STUDIES ON FACTORS WHICH INFLUENCE

CYCLIC 3',5'- ADENOSINE MONOPHOSPHATE

PRODUCTION IN-VIVO AND IN-VITRO

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LIST OF ABBREVIATIONS USED IN THIS THESIS

Adenosine 3',5'-cyclic monophosphate	cAMP
Adenosine 5'-phosphate	AMP
Adenosine 5'-diphosphate	ADP
Adenosine 5'-triphosphate	ATP
Adrenocorticotrophic hormone	ACTH
Amino terminal	N terminal
Analysis of variance	ANOVA
Antibody	Ab
Antigen	Ag
Arginine hydrochloride	Arg HCl
Bovine parathyroid hormone	bPTH
Calcium	Ca
Calcium adjusted for albumin concentration	Adj Ca
Carboxy terminal	C terminal
Cholesterol side chain cleavage cytochrome P450	$P450_{scc}$
Coefficient of variation	CV
Correlation coefficient	r
Curies	Ci
Day	d
Degrees centigrade	C
Deoxyribonucleic acid	DNA
Enzyme immunoassay	EIA
Enzyme linked immunosorbent assay	ELISA
Enzyme multiplied immunoassay technique	EMIT
Ethylene diamine tetra-acetic acid	EDTA
Ethylene glycol tetra-acetic acid	EGTA

Femtomole	fmol
Follicle stimulating hormone	FSH
Free thyroxine	FT_4
Free tri-iodothyronine	FT_3
Glomerular filtrate	GF
Gram	g
Growth hormone	GH
Growth hormone releasing hormone	GHRH
Guanosine nucleotide binding proteins	G proteins
Guanosine 3', 5'-cyclic monophosphate	cGMP
Guanosine 5'-phosphate	GMP
Guanosine 5'-diphosphate	GDP
Guanosine 5'-triphosphate	GTP
Haemoglobin	Hb
Half-life	t1/2
High performance liquid chromatography	HPLC
Hour	h
Human parathyroid hormone	hPTH
Humoral hypercalcaemia of malignancy	HHM
Hydrogen ion activity	pH
Hypercalcaemia associated with malignancy	HCM
Immunoradiometric assay	IRMA
Immunoreactive PTH	iPTH
Intact parathyroid hormone	PTH (1-84)
International unit	IU
Intravenous	IV
Isobuthylmethylxanthine	IBMX
Kilogram	kg

Lithium	Li
Litre	L
Luteinizing hormone	LH
Magnesium	Mg
Messenger ribonucleic acid	mRNA
Metre	m
Michaelis constant	K_m
Micromole	μmol
Millilitre	mL
Millimolar	mmol/L
Milliunit	mU
Minute	min
Molar	mol/L
Mole	mol
Molecular weight	MW
Nanogram	ng
Nanomole	nmol
Nephrogenous adenosine 3',5'-cyclic monophosphate	NcAMP
Not significant	NS
Number in sample	n
Parathyroid hormone	PTH
Parathyroid hormone related peptide	PTHrP
Per cent	%
Phosphate	PO_4
Phosphodiesterase	PDE
Picogram	pg
Picomole	pmol
Plasma adenosine 3',5'-cyclic monophosphate	PcAMP

Primary hyperparathyroidism	1°HPT
Probability of an event being due to chance alone	p
Prolactin	PRL
Radioimmunoassay	RIA
Ribonucleic acid	RNA
Second	s
Sodium fluoride	NaF
Standard deviation	SD
Standard error of mean	SEM
Technetium	^{99}Tcm
Threshold for renal tubular reabsorption of phosphate	$TmPO_4$
Thyroid stimulating hormone	TSH
Total thyroxine	TT_4
Total tri-iodothyronine	TT_3
Unit	U
Urinary adenosine 3',5'-cyclic monophosphate	UcAMP
Urinary calcium creatinine ratio	Ca/Cr
Versus	v
1,25 dihydroxy vitamin D	$1,25 (OH)_2 D3$
3-amino-1-hydroxypropylidene-1,1-bisphosphonate (Pamidronate)	APD

DECLARATION

I performed the work described in this thesis in the Institute of Biochemistry, Glasgow and the University Department of Clinical Chemistry, Royal Liverpool University Hospital, between 1987 and 1993. I developed the assay for urinary and acetylated cyclic adenosine monophosphate and personally performed all the plasma, urinary and acetylated cAMP measurements. I was involved in the design, performance and analysis of results of all the work presented in this thesis. The contribution made by my colleagues who collaborated with me is acknowledged in the appropriate sections of the thesis. I worked in collaboration with several members of staff in each of the above departments and particularly acknowledge the assistance given in the planning and performance of the 24 hour studies. I wrote the thesis and the opinions expressed herein are mine.

William Duncan Fraser

PUBLICATIONS

Some of the studies in this thesis have already been published or are about to be published in peer-review journals. Parts of some of the studies have contributed to publications in peer-review journals. They include:-

Logue FC, Fraser WD, O'Reilly DStJ, Beastall GH. The circadian rhythm of intact parathyroid hormone (1-84) and nephrogenous cyclic adenosine monophosphate in normal men. J Endocrinol 1989; 12: R1-R3.

Ralston SH, Gallacher SJ, Patel U, Dryburgh FJ, Fraser WD, Cowan RA, Boyle IT. Comparison of three intravenous bisphosphonates in cancer-associated hypercalcaemia. Lancet 1989; ii: 1180-2.

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men. J Clin Endocrinol Metab 1990; 71: 1556-60.

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Fraser WD, Logue FC, MacRitchie K, Wilson RM, Gray HW, Beastall GH, O'Reilly DStJ. Intact parathyroid hormone concentration and cyclic AMP metabolism in thyroid disease. Acta Endocrinol 1991; 124: 652-7.

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Fraser WD, Logue FC, Christie JP, Cameron DA, O'Reilly DStJ, Beastall GH. Alteration of the circadian rhythm of intact parathyroid hormone following a 96-hour fast. Clin Endocrinol 1994; 40: 523-8.

Submitted for Publication

Fraser WD, Williams BC, O'Reilly DStJ. Cyclic AMP production *in-vitro* studied using superfusion of rat renal tubules. J Endocrinol.

Fraser WD, Robinson J, Durham B, Gallacher SJ, Boyle IT, Beastall GH, Logue FC, O'Reilly DStJ. PTH (1-84), PTHrP and cAMP metabolism in primary hyperparathyroidism. J Clin Endocrinol Metab.

Fraser WD, Logue FC, Christie JP, Gallacher SJ, Boyle IT, Beastall GH, O'Reilly DStJ. Variation in the circadian rhythm of cAMP in hyperparathyroid patients before and after parathyroidectomy. Clin Endocrinol.

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SUMMARY

Daily variations in plasma (PcAMP), urine (UcAMP), nephrogenous cAMP (NcAMP) and hormones known to affect cAMP metabolism were studied. Circadian variations in PTH (1-84) and NcAMP were observed. Both increased significantly overnight and early morning. PcAMP and glucagon decreased overnight and following meals. PcAMP had greater fluctuations over 24h than glucagon indicating several hormones have transient effects on PcAMP production.

A 7h shift of the sleep-wake cycle moved sleep-associated prolactin secretion but caused minimal alteration in PTH (1-84). NcAMP secretion overnight decreased, suggesting PTH (1-84) end organ responses may be modified by other hormones and effectors when sleep is disturbed. PcAMP decreased overnight reaching a nadir corresponding to the lowest glucagon concentration. PcAMP fluctuations were greater when compared to normal 24h profiles.

Following a 96h fast serum phosphate, PTH (1-84) and NcAMP circadian rhythms were attenuated. Mean serum calcium and NcAMP production were significantly decreased. Fasting increased variability in PTH (1-84) secretion which may be an important signalling mechanism inducing bone resorption. Serum phosphate fluctuations may play an important role in the genesis of PTH (1-84) and NcAMP circadian rhythms. Mean glucagon concentration was increased with attenuation of the circadian rhythm and mean PcAMP concentration increased. Dissociation of bone and kidney effects of PTH (1-84) in fasting may be due to acute acidosis.

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In 1°HPT the concerted overnight increase in PTH (1-84) and NcAMP was absent. Parathyroid adenoma resection restored phosphate, PTH (1-84) and NcAMP circadian rhythms. The phosphate increase may stimulate PTH (1-84) secretion which then influences the phosphate rhythm. Mean PcAMP was increased in 1°HPT but 24h PcAMP profiles differed little pre and post surgery.

24h studies indicate control of the circadian rhythms of PTH (1-84), NcAMP and PcAMP is complex. Several hormones act in concert to regulate cAMP generation and end organ responses.

Thyrotoxic patients had mean PTH (1-84) and NcAMP lower than normals, hypothyroid and treated hypothyroid patients. Increased serum calcium decreases PTH (1-84) and subsequently NcAMP in thyrotoxicosis. In hypothyroid patients PTH (1-84) and NcAMP are dissociated with mean PTH (1-84) increased compared to thyrotoxic patients but NcAMP remained similar to euthyroid subjects indicating resistance to PTH (1-84). Thyroxine treated patients have mean PTH (1-84) concentrations lower than euthyroid controls with a relatively increased NcAMP. PcAMP is increased in thyrotoxic and thyroxine treated patients and decreased in hypothyroid patients.

Synacthen increased PcAMP with the pattern similar to the cortisol profile. This was either due to simultaneous

release of cAMP and cortisol following adrenal stimulation or was a direct effect of cortisol. Dexamethasone decreased NcAMP and UcAMP with a variable effect on PcAMP. Variability in the PcAMP response is probably due to combined effects of dexamethasone and cortisol whereas decreased UcAMP and NcAMP production may represent altered kidney cell sensitivity to PTH (1-84) caused by decreased cortisol.

In 1°HPT patients a significant correlation between PTH (1-84) and NcAMP exists (r=0.66, p<0.001). A subgroup of 1°HPT patients have NcAMP inappropriately high for the measured PTH (1-84). Two patients had increased PTHrP but in the majority PTHrP was low and the discrepancy was unexplained. In three patients PcAMP concentration was lower than expected. Twelve 1°HPT patients, divided equally, received atenolol or placebo and were studied prospectively for 6 months. No significant effect of atenolol on any parameter was observed.

In patients with Paget's disease treated with bisphosphonate (APD) calcium decreased, PTH (1-84) and NcAMP increased significantly. Patients with HCM prior to therapy with APD had a suppressed or low PTH (1-84). A high percentage had elevated NcAMP indicating the presence of a circulating humoral factor, probably PTHrP, causing hypercalcaemia. APD therapy in HCM patients resulted in a significant reduction in serum calcium. No patient

developed significant hypocalcaemia. PTH (1-84) secretion was stimulated as serum calcium decreased and PTH (1-84) was detectable or elevated whilst some patients remained hypercalcaemic. The absolute concentration rather than rate of decrease of serum calcium was more important in regulating PTH (1-84) secretion. NcAMP correlated poorly with PTH (1-84) in treated HCM patients. An increase then decrease in NcAMP was observed associated with a decrease then increase in $TmPO_4$ indicating PTHrP may increase transiently following APD therapy.

PTHrP measurement confirmed the hormone's role in the aetiology of HCM and a strong correlation of PTHrP with NcAMP. In HCM patients with breast, lung and kidney malignancy PTHrP secretion commonly results in elevated NcAMP. Patients with HCM and haematological, gastrointestinal and ear-nose-throat malignancies have low circulating PTHrP and normal or low NcAMP. Squamous cell carcinomas commonly secrete PTHrP. 78% of normals had detectable PTHrP which may reflect sample collection into protease inhibitor tubes, age distribution of the normal population, assay sensitivity and properties of the assay antibodies. Alternatively the assay may be subject to non-specific binding effects or matrix effects. A subgroup of patients was detected with low PTHrP and low PTH (1-84) with inappropriate or elevated NcAMP. These patients may be producing a factor which stimulates NcAMP production. This factor(s) may be a fragment of PTHrP or PTH not

recognised by the IRMAs or may be a new molecule causing hypercalcaemia mediated via cAMP.

Superfusion of rat renal tubules was used to study cAMP production in response to PTH stimulation. Optimal conditions producing a consistent, reproducible cAMP response were identified. Tubules were equilibrated on columns for 90 min, prior to stimulation with 2.5 units bovine PTH (1-84) at 10 min intervals. A significant decrease in cAMP production in response to PTH was observed when tubules were perfused with buffer acidic (pH 7.1) relative to normal physiological pH (pH 7.4). Perfusion with buffer relatively alkalotic (pH 7.65) had no significant effect on PTH stimulated cAMP production. 7mM arginine hydrochloride in the perfusate significantly decreased cAMP production. Superfusion of rat renal tubules was a reliable, reproducible method for studying the action of PTH.

Chapter 1

Introduction and Literature Review

1.1 Cellular Control of Cyclic Adenosine 3′,5′ Monophosphate Production: Historical Perspective to Current Belief

Cyclic adenosine 3′,5′ monophosphate (cyclic AMP, cAMP) was first identified in biological systems by Sutherland and Rall (1958) (1) whilst studying hormone dependent glycogenolysis in liver cells. Sutherland and co-workers (1962) (2) subsequently discovered the enzyme responsible for the formation of cAMP from adenosine triphosphate (ATP), adenylate cyclase (EC. 4.6.1.1) and proposed the concept that extracellular hormones were "first messengers" and cAMP was a "second messenger" in the information transfer process of some endocrine pathways. This "second messenger" was generated intracellularly and mediated the effects of hormones, which did not themselves enter the cell, by activation of intracellular protein kinases (3,4).

The specificity of the response resides firstly in the presence of a particular hormone receptor on the cell surface and secondly on the presence of a specific protein kinase substrate, usually an enzyme, within the cell (5).

Cell surface receptors are coupled to the adenylate cyclase catalytic component through two classes of guanine-nucleotide binding proteins (G proteins). Gs stimulates adenylate cyclase in response to PTH, TSH, gonadotrophins, ACTH, GHRH, β-adrenergic agonists and many other hormones or neurotransmitters (Table 1). Gi mediates inhibition of

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adenylate cyclase in response to α-adrenergic agonists, muscarinic agonists, somatostatin and opiate or opiate like molecules (Table 1). The G proteins are heterotrimers, consisting of an α-subunit and a tightly coupled $\beta\gamma$-dimer. A model for G-protein action has been developed which revolves around the unique functional characteristics of each G-protein. Binding of an agonist to a stimulatory receptor facilitates the exchange of guanine triphosphate (GTP) for guanine diphosphate (GDP) in the guanine-nucleotide-binding site of Gsα. This results in the release of Gsα from the receptor and its dissociation into free Gsα-GTP and free Bγ-dimer. Free Gsα-GTP stimulates adenylate cyclase activity and thereby increases the synthesis of the intracellular second-messenger cAMP. Hydrolysis of GTP to GDP by the intrinsic GTPase of Gsα enables reassociation of Gsα-GDP with the $\beta\gamma$-dimer, thus restoring Gs to its inactive state. Gs can now re-associate with the receptor and participate in another cycle. GTPase activity functions as a molecular switch which regulates the duration of adenylate cyclase activity resulting from the formation of the Gsa-GTP complex. After a preset time, determined by the kinetics of the GTPase activity, Gsα is turned off by the hydrolysis of GTP to GDP. This summary of the current theory of G-protein function is the summation of many years of postulation and investigation (6,7,8,9,10,11,12,13).

	Adenylate Cyclase Stimulatory	Adenylate Cyclase Inhibitory
G Protein	Gs	Gi
	ACTH	A_1-Adenosine
	A_2-Adenosine	α_2-Adrenergic
	β-Adrenergic	D_2-Dopamine
	Calcitonin	Enkephalin (M,K,E)
	CRH	Muscarinic (M_2M_3)
	D_1-Dopamine	Somatostatin
	FSH	
	GHRH	
	Glucagon	
	H_2-Histamine	
	LH	
	MSH	
	Prostacyclin	
	PTH	
	Secretin	
	S_2-Serotonin	
	TSH	
	VIP	

Table 1   **Receptors coupled to adenylate cyclase mediated through G proteins**

The catalytic activity of adenylate cyclase is influenced in intact cells, via the G-proteins, by hormones and antibodies (14,15). Broken cell preparations have also been used to study adenylate cyclase activity and several activators and effectors have been identified. Such studies of adenylate cyclase activity only partially reflect the properties of the enzyme observed from studies of intact cells. Only ATP and magnesium (Mg^{2+}) are required for the expression of basal enzyme activity (16). A divalent cation is essential for activity and under normal circumstances this is Mg^{2+} (17,18,19,20). Monovalent cations have minimal effects on activity with the exception of Lithium (Li^+) which can inhibit adenylate cyclase activity (21,22,23). Sodium fluoride (NaF) stimulates activity (2,24) although this is not observed to any extent in whole cell preparations (25). Forskolin significantly stimulates adenylate cyclase activity by interacting with the catalytic subunit at the internal face of the cell membrane structure. Forskolin activation of adenylate cyclase has been shown to occur in a significant number of endocrine cell types and is a standard means of investigating cAMP dependent pathways (26).

Phosphodiesterase (EC. 3.1.4.17) present in most tissues, rapidly hydrolyses cAMP to an inactive metabolite adenosine 5' monophosphate (5'AMP) and phosphoprotein phosphatases inactivate protein substrates by dephosphorylation thus ensuring the transient nature of the cAMP signal within the

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cell. Phosphodiesterase (PDE) enzymes are found in both the cytosol and membrane fractions of the cell and are usually classified in terms of their differing affinities for the hydrolysis of cAMP (27,28,29). The Km for PDEI to cAMP is in the range of 10^{-5} M, whereas PDEII, which is thought to be predominantly membrane bound, has a Km value of 10^{-6} M (25). These enzymes are also Mg^{2+} dependent and can be inhibited by divalent cation binders such as ethylene-diamine-tetracetic acid (EDTA) and ethylene-glycol-tetracetic acid (EGTA) (30). Methylxanthine-derivatives are a group of important inhibitors of PDE activity and this property is seen medically and socially following the ingestion of theophylline and caffeine respectively. A very potent methylxanthine-derivative is isobuthylmethyl-xanthine (IBMX) which is widely used as a PDE-inhibitor under experimental conditions especially when studying cellular responses to hormones *in-vitro*. IBMX is particularly useful because unlike several other methylxanthines it has very little inhibitory activity on adenylate cyclase. Intracellular calcium and calmodulin are known to activate PDE (31,32,33) and there is some evidence that they can also activate adenylate cyclase (34) which suggests that co-ordinate regulation of cAMP production can occur through these two effectors.

Once cAMP is generated it exerts its regulatory effects through activation of intracellular protein kinase enzymes (3,4). These in turn phosphorylate a number of

intracellular substrates and the specificity of the cAMP signal depends on the substrate availability within the cell (5). The phosphorylation may result in activation of an enzyme important in a metabolic pathway and may also inhibit another enzyme which would have opposite metabolic effects, eg glycogen phosphorylase and glycogen synthase in liver cells. Termination of such signals is accomplished by dephosphorylation of proteins by phosphoprotein-phosphatase (4,35,36,37). This enzyme is itself regulated by cAMP as has been shown in skeletal muscle where activation of cAMP-dependent protein kinase phosphorylates a phosphatase inhibitory protein (37). This protein binds to and temporarily inhibits the activity of the phosphoprotein phosphatase which otherwise would immediately dephosphorylate and reverse the phosphorylation reactions stimulated by cAMP. There is thus a cycle of phosphorylation/dephosphorylation which appears to be a widespread intracellular regulatory system and ensures exquisite control over the cAMP signal.

An ever increasing number of hormones have been shown to use cAMP as their "second messenger" (Table 1). The majority of these hormones exert a net catabolic effect on their target tissues. Other characteristics are that they activate cells very rapidly, within seconds or minutes, with a dose-response relationship and have been termed immediate hormones in contrast to permissive hormones whose cellular effects are only fully expressed about one hour or

more after the hormones reach their target cells (38). The amount of cAMP generated within a target cell is a reflection of hormone receptor interaction and is dependent on the concentration of hormone present in the extracellular fluid and the number of cell surface receptors. Steady state concentrations of cAMP give minimal information about hormone action but serial measurement after hormonal stimulation can give important information on the initial steps of hormone action. Early studies measuring cAMP in isolated cells, cell membranes or extracellular fluids resulted in valuable information on the control of cAMP production, action, release and pointed the way to the clinical usefulness of cAMP measurements. Although many hormones act via an increase in intracellular cAMP a certain basal formation of cAMP occurs in most cell systems, independent of the level of immediate hormones in the local environment. Alteration of this basal formation of cAMP can be observed in growth phases (decrease) and when contact inhibition of cell growth occurs (increase) (39). These observations highlight the fact that under such *in-vitro* conditions all synthesis of cAMP is not necessarily mediated through hormonal stimulation.

The concentration of cAMP in the intracellular fluid is far greater than that in the extracellular fluid (40). The efflux of cAMP from some cells is enhanced by hormone receptor interaction. In 1971 Exton *et al* (40) were the first to demonstrate in the isolated perfused liver that

glucagon was required in the perfusate for the release of cAMP into the effluent. Increasing concentrations of glucagon resulted in a dose dependent increase in cAMP and glucose production with the extracellular cAMP rising before measurable elevations of intracellular cAMP. This led to the theory of two pools of cAMP with one tightly bound and inactive, the other being freely diffusible and open to hormonal regulation (40). Changes in the extracellular concentration of cAMP are therefore deemed to reflect the net effects of hormonal stimulation on tissue cAMP production.

1.2 Physiology and Early Clinical Studies

In clinical practice the extracellular fluids that are most readily available are plasma and urine. The relatively high concentration of cAMP in urine and the ease with which individual or serial specimens can be obtained has made urine a useful fluid for clinical studies. Measurement of cAMP in human urine was first made by Butcher and Sutherland (1962) (41) and early work confirmed that under basal conditions cAMP was present in urine at micromolar concentrations (42,43,44). Physiological studies in the late 1960s and 1970s, largely pioneered by Broadus and his colleagues, demonstrated that the mechanism of renal plasma clearance of cAMP was glomerular filtration but that *de novo* production of cAMP also occurred in the kidney. Detailed studies with radioactive cAMP tracers (45) established the basis for the currently accepted mechanism

of total urinary cAMP (UcAMP) production (46) which is summarised in Figure 1. The total amount of cAMP excreted in the urine is the sum of that derived from two sources: a) the nucleotide filtered at the glomerulus and b) the nucleotide produced by the renal tubule. The term nephrogenous cAMP (NcAMP) was introduced to describe the latter component. Under basal conditions each component accounts for approximately 50% of the total quantity of cAMP excreted in the urine of normal individuals. Thus factors which affect both the plasma concentration and the renal production of cAMP can contribute to UcAMP and renal function will also influence plasma cAMP (PcAMP) and UcAMP concentrations.

In normal subjects virtually every cell in the body contributes to the PcAMP concentration with no single tissue known to be of particular importance at rest. A variety of tissues contribute to metabolism of PcAMP and only one-fifth of the metabolic clearance of PcAMP is accounted for by excretion in the urine (47). Cyclic AMP distributes into a space several fold larger than the extracellular volume and turns over rapidly, with a half life of approximately 25 min and a metabolic clearance rate of about 650 ml/min. It is recognised that physiological variations in PcAMP may not be reflected in total UcAMP but pharmacological doses of certain hormones and hormonal concentrations in disease states can increase PcAMP with a resultant increase in total UcAMP.

TISSUE
SOURCES

```
┌─────────────────────────────────┐
│   Extracellular Cyclic AMP      │
└─────────────────────────────────┘
```

CIRCULATING

```
        ┌──────────────────┐
        │     Plasma       │
        │   Cyclic AMP     │
        └──────────────────┘
```

KIDNEY

```
    ┌──────────────────────────────┐
    │                              │
    │         Filtered             │
    │        Cyclic AMP            │
    │            +                 │
    │        Nephrogenous          │
    │        Cyclic AMP            │
    │                              │
    │                              │
    └──────────────────────────────┘
```

OUTPUT

```
        ┌──────────────────┐
        │  Total Urinary   │
        │   Cyclic AMP     │
        └──────────────────┘
```
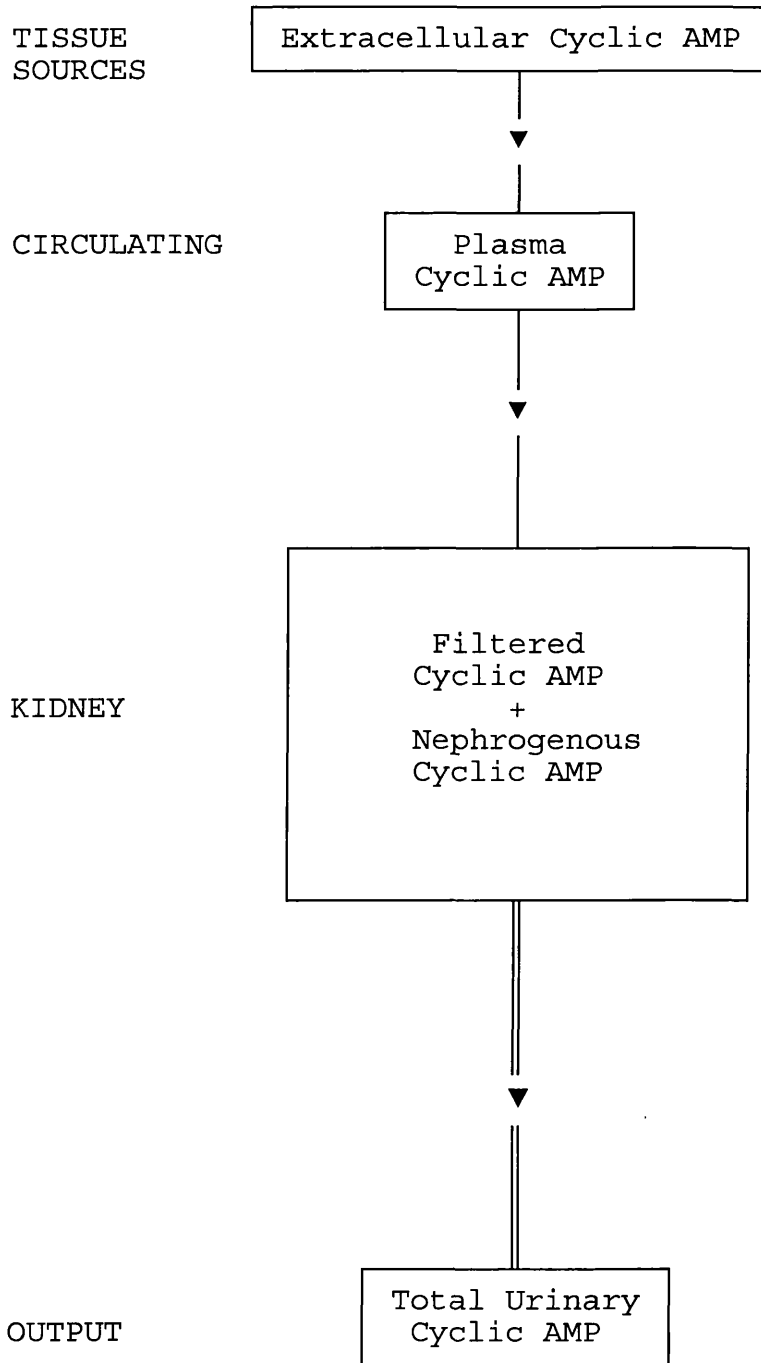
Figure 1   The production of urinary cAMP

The hormones that have the greatest effects on PcAMP in pharmacological doses are adrenaline (48,49,50), glucagon (49,51,52,53), parathyroid hormone (PTH) (43,54,55) and calcitonin (49,56,57). The increases in UcAMP were significant but relatively small in comparison to the rise in PcAMP following calcitonin, glucagon and adrenaline but a pronounced increase in UcAMP was observed following PTH administration.

Studies using renal cortical preparations demonstrated that PTH can stimulate the release of cAMP from renal cortical tissue (58). The absence of an increase in PcAMP after PTH administration in anephric patients (54) confirmed the kidney as the source of the increase in both PcAMP and UcAMP in response to PTH. Early reports suggested that calcitonin (59,60) and arginine vasopressin (AVP) (42,61,62) were able to increase NcAMP. However, it has been shown that the effects of calcitonin on NcAMP are mediated by secondary rises in PTH (46,63) and the action of AVP on NcAMP has not been confirmed (47,49,64). The experimental evidence available confirms the importance of PTH in the generation of UcAMP with PTH accounting for 90-95% of NcAMP and some 45-50% of total UcAMP in normal man.

1.3 Urinary/Nephrogenous cyclic AMP and Parathyroid Function

Measurement of UcAMP is useful in investigating disorders of calcium metabolism and provides an assessment of

parathyroid function (46). Several studies have reported that UcAMP is elevated in patients with primary hyperparathyroidism and lowered in patients with hypoparathyroidism (44,49,61,63,65). However, considerable overlap between normals and hyperparathyroid patients existed especially in mild hyperparathyroidism (66). Improved discrimination between the groups can be achieved by expressing UcAMP as a function of glomerular filtrate (GF), as given by :

$$\frac{\text{Urinary cyclic AMP}}{\text{Urinary creatinine}} \text{ x Serum creatinine}$$

which provides a parametric index of UcAMP and overcomes the problems associated with mild to moderate renal impairment, changes in creatinine excretion in prolonged illness and differences in creatinine excretion in males and females (46). The best discrimination between normals and hyperparathyroidism is achieved when both plasma and UcAMP are measured which enables NcAMP, expressed per litre of glomerular filtrate, to be calculated as:

$$\frac{\text{Urinary cyclic AMP}}{\text{Urinary creatinine}} \text{ x Serum creatinine - Plasma cyclic AMP}$$

(46,67,68).

The relationship between NcAMP and parathyroid function has been elegantly reviewed by Broadus (47). NcAMP values range from near zero to more than 150 nmol/L GF. Typical values obtained in hypoparathyroid (2.9 $\pm$ 1.6), hyperparathyroid (44.8 $\pm$ 17.0) and euparathyroid subjects (3.4-27.0 nmol/L GF) clearly demonstrate that NcAMP

measurements enable the confident assessment of parathyroid activity. Confirmation of the work of Broadus has been obtained by other authors (69,70) and in general an overlap of NcAMP with normals is obtained in 7-9% of hyperparathyroid patients and 3-10% of hypoparathyroid patients.

1.4 Urinary/Nephrogenous cyclic AMP in Malignant Disease

Elevation of UcAMP is not specific for hyperparathyroidism and interest has centred on the elevation of UcAMP in patients with malignant disease. Since patients with hypercalcaemia associated with malignancy (HCM) characteristically have suppressed PTH concentrations UcAMP and NcAMP would be expected to be low in HCM. A number of authors have reported elevations of UcAMP and/or NcAMP in patients with malignant disease and this finding is most frequently observed in patients with hypercalcaemia associated with malignancy (47,71,72,73,74). In these studies it was observed that patients with squamous cell carcinoma of the lung and uro-genital carcinoma were more likely to have an increased UcAMP or NcAMP while patients with carcinoma of the breast or haematological malignancies tended to have normal or low UcAMP and or NcAMP excretion (74,75). It was postulated (74) that patients with hypercalcaemia associated with malignancy (HCM) could be subdivided into two groups depending on whether their NcAMP values were increased or decreased. Those with increased NcAMP had few or no bone metastases and those with low or

decreased NcAMP invariably had widespread skeletal invasion by tumour. These results suggested that in patients with HCM measurements of NcAMP can be used to detect humoral hypercalcaemia of malignancy (HHM) when NcAMP values were high and that low NcAMP values were associated with local osteolytic hypercalcaemia. In patients with low or undetectable plasma PTH and elevated UcAMP or NcAMP it was postulated that a tumour derived humoral factor was produced with actions similar to PTH (74,76,77,78). Subsequently a peptide has been isolated from tumours which has remarkable sequence homology at its amino terminal with PTH (79,80,81). As a result of similarities in structure and function with PTH it has been named parathyroid hormone related protein (PTHrP). Measurements of UcAMP and or NcAMP with plasma PTH can be used to indicate, indirectly, the presence of PTHrP. Differential diagnosis of hypercalcaemia based on UcAMP or NcAMP excretion alone is not possible, however, since considerable overlap of results exists between hyperparathyroidism and HCM (71,82).

1.5 Cyclic AMP in Pseudohypoparathyroidism

In 1942 Albright and colleagues described a syndrome characterised by mental deficiency, fits, tetany, dwarfism, shortened metacarpals, soft tissue calcification with hypocalcaemia and hyperphosphataemia (83). The cause of this syndrome is a combination of separate defects in the target-cell response to PTH, end organ resistance. Early observations were based on the failure of these patients to

respond to infusions of PTH extract by increasing the fractional excretion of phosphate (84) but this test remained unreliable even when purified preparations of PTH became available. It was demonstrated that the urine excretion of cAMP in response to intravenous PTH was markedly decreased in patients with pseudo-hypoparathyroidism (44) and the test was further modified by Tomlinson *et al* in 1976 who showed that the PcAMP response to PTH is also impaired (85). Measurement of the urinary phosphate excretion response to PTH is still of diagnostic value. Two types of pseudohypoparathyroidism are now recognised. Type 1 is characterised by an absence of both the increase in UcAMP and phosphate in response to PTH and in Type 2 patients have a normal cAMP response to PTH but a subnormal phosphaturic response (86).

1.6 Measurement of cyclic AMP

It is clear from the preceding review that measurement of UcAMP is mainly useful in metabolic studies but it can supply information of clinical value in well defined conditions. Several assays exist for the measurement of cAMP and in selecting particular methods for use in the following experimental work it has been necessary to consider the problems presented by each method, the expertise and the facilities available to perform the method.

The earliest assays were based on enzymatic methods and were time consuming, insensitive, highly dependent on enzyme purity and prone to interference (25,87,88,89). Competitive protein binding assays were developed based on the binding of unlabelled and radioactive cAMP to cAMP dependent protein kinase obtained from muscle or adrenal cortex (90,91). These methods have a far greater sensitivity than enzyme methods and are able to measure cAMP in the picomolar range. There is some debate, however, about interferences in these assay systems. Feinglos *et al* (92) in a review of cAMP methodologies concluded that cAMP binding proteins present in plasma interfered and that proteins present in ileal secretions also interfered in the assay. Other authors have not found such interferences with various fluids, including plasma and urine (93,94).

A major breakthrough in cAMP assays occurred when Steiner *et al* (95) raised antibodies in guinea pigs towards succinylated cAMP conjugated to human serum albumin. Native cAMP exhibits sufficient cross reactivity with the antibodies to succinylated cAMP that the antibodies could be used in a radioimmunoassay for cAMP. This method was sensitive enough to measure cAMP in the picomolar range. The sensitivity of this assay can be increased by acetylation or succinylation of the cAMP in the sample prior to analysis (96). Acetylation yields a more stable product than succinylation, is easy to perform and results

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in an assay capable of measuring femtomole amounts of cAMP. Such sensitivity is not usually required when measuring urine and plasma but is often necessary when measuring tissue extracts, cell culture supernatants or tissue perfusates.

RIAs for cAMP have been developed that are sensitive, precise, accurate and convenient to use. Assays can also be modified in-house so that they are targeted to measure cAMP at the concentration of interest in a particular sample type. As a result of these assay characteristics the vast majority of current research involving measurement of cAMP is conducted using RIAs.

Chapter 2

Technical Methods

2.1   **Biochemical Methods**

2.1.1 <u>Cyclic Adenosine Monophosphate</u>

cAMP was measured in urine, which was collected in sterile containers using no preservatives, using an in-house radioimmunoassay (RIA) which was developed at the Institute of Biochemistry and utilises rabbit antibody to cAMP kindly donated by Dr B Williams, Department of Medicine, Western General Infirmary, Edinburgh. A full evaluation of this assay, in thesis format, was submitted to the Royal College of Pathologists as part of the final examination for membership of the College (97). The assay has a detection limit of 2.6 nmol/L (22% CV as defined by Ekins (98)) and between-assay CV <10% over the working range of the assay (10-150 nmol/L). The rabbit anti-cAMP antiserum was raised against an O-2'-succinyl cAMP-albumin conjugate. The % cross reactivities, based on the concentration causing 50% displacement, are: cyclic guanosine 3':6'-monophosphate 0.25%, adenosine <0.025%, guanosine <0.025%, 5'-adenosine monophosphate <0.025%, 5'-guanosine monophosphate <0.025%, adenosine diphosphonate <0.025% and adenosine triphosphate <0.025%. Separation was performed by using donkey anti-rabbit antibody (Scottish Antibody Production Unit, Law Hospital, Carluke) coupled to Sepharose (99). The sensitivity of this assay can be increased 1000 fold by acetylation of samples. This is performed by adding 15 μL of triethylamine and acetic anhydride mixture (ratio 2:1) to 500 μL of sample and vortexing immediately prior to

assay.

cAMP was measured in plasma after appropriate dilution using a commercial RIA supplied by Amersham International (Aylesbury, Bucks). Blood is collected into EDTA anti-coagulant, separated within 30 min and stored frozen (-70°C) prior to analysis.

2.1.2 Parathyroid Hormone

In the majority of studies the intact PTH (PTH (1-84)) molecule was measured in serum using an in-house immunoradiometric (IRMA) assay developed at the Institute of Biochemistry, Glasgow. The assay utilises two mouse monoclonal antibodies to PTH directed at the N and C terminal ends of the PTH molecule. A full evaluation of the assay has been published (100). The assay has a sensitivity of 0.5 pmol/L (22% CV) (98) and a CV <10% across the range 1.5-250 pmol/L. Quantitative recovery of PTH (1-84) standard is obtained (mean recovery 107%) and the assay is unaffected by a 200 molar excess of C-terminal fragments (PTH (53-84)). A reference range of 1.0-5.0 pmol/L has been established for this assay.

PTH (1-84) was measured in one study (Chapter 8) using the Nichols Institute (San Juan Capistrano) Allegro PTH (1-84) IRMA. This assay has a claimed sensitivity of 0.1 pmol/L, based on precision in counting small amounts of bound radioactivity and an inter assay CV from 5.6% to 6.1% for

replicate measurements of control sera (control A 3.0-5.3, mean 4.2 and control B 23.6-39.3, mean 31.5 pmol/L). Mean recovery of PTH (1-84) is 100% and the assay is unaffected by 300 molar excess of the synthetic peptides PTH (1-34), PTH (39-68), PTH (53-84), PTH (44-68) and PTH (39-84) (101).

2.1.3 Parathyroid Hormone Related Protein

In two studies (Chapters 6,8) PTHrP was measured in plasma using a commercial IRMA supplied by the Nichols Institute (San Juan Capistrano). A full evaluation of this assay was performed as a component of this thesis (Chapter 8) and has recently been published (102). Blood samples for this assay have to be collected into tubes containing protease inhibitors (aprotinin, leupeptin, pepstatin and EDTA). Sensitivity of the assay is 0.7 pmol/L (22% CV) (98) and the CV <10% over the range 1.5-50 pmol/L. The assay is highly specific and cross reactivity was <0.1% with human PTH (1-34) and PTH (1-84) at 10, 100 and 1000 pmol/L of PTH peptide.

2.1.4 Thyroid Hormones and Thyroid Stimulating Hormone

Total thyroxine (TT_4) was measured in serum by an in-house RIA utilising antibody supplied by the Scottish Antibody Production Unit (SAPU, Law Hospital, Carluke ML8 5ES). The assay sensitivity is 20 nmol/L (22% CV) (98) and the CV of the assay is <10% across the range 40-250 nmol/L.

Total tri-iodothyronine (TT_3) was measured in serum by an in-house RIA utilising antibody supplied by SAPU (Law Hospital, Carluke ML8 5ES). The assay sensitivity is 0.3 nmol/L (22% CV) (98) and the CV <10% across the range 0.5-5.0 nmol/L.

Free thyroxine was measured in serum using an in-house RIA which uses magnetic antibody-containing microcapsules. Anti-thyroxine antibody for encapsulation was obtained from Dr T Merrit, Rast Allergy Unit, Benedin Chest Hospital, Kent. This assay has a detection limit of 3 pmol/L (22% CV) (98) and a CV of <10% over the range 8-75 pmol/L (103).

Thyroid stimulating hormone (TSH) was measured in serum by an immunoflurometric assay (IFMA) (Delfia, Pharmacia Ltd., Milton Keynes). This assay has a detection limit of 0.05 mIU/L (22% CV) (98) and a CV of <10% across the range 0.01-50 mIU/L (104).

2.1.5 <u>Cortisol</u>

Cortisol was measured in extracted urine and serum using a direct solid-phase RIA employing ^{125}I iodohistamine linked to the carboxyl derivative, 3-(o-carboxymethyl) oxime of cortisol (105). The between batch CV over the range 100-1000 nmol/L was 11% and the within batch CV 7% over the range 300-1500 nmol/L. Sensitivity of this assay was 20 nmol/L as determined by the 22% CV obtained from cumulative precision profile data (98).

2.1.6 Glucagon

Measurement of glucagon was performed using a commercial RIA supplied by Cambridge Medical Technology (Billerica USA) that has a detection limit of 70 ng/L (22% CV) (98) and a working range of 100-4000 ng/L (<10% CV) as defined by the precision profile for the assay. Samples for glucagon assay were taken into EDTA tubes separated immediately and stored at -20°C prior to assay.

2.1.7 Prolactin

Prolactin was measured using an IRMA method (NETRIA, St. Bartholomew's Hospital, London, UK) that has a detection limit of 30 mU/L (22% CV) (98) and a working range of 60-3500 mU/L (CV <10%).

2.1.8 Routine Biochemical Analysis

Serum calcium, phosphate, creatinine, and albumin were measured using standard automated methods on either a Technicon SMAC II (Tarrytown USA) or Hitachi 704 (Boehringer Mannheim, Lewes, UK). Calcium was adjusted for albumin as described by Gardner et al (106). Urine calcium, phosphate and creatinine were measured by standard automated methods on a Hitachi 704 (Boehringer Mannheim, Lewes, UK).

2.1.9 Renal Tubular Reabsorption of Phosphate

The notional threshold for renal tubular reabsorption of phosphate ($TmPO_4$) was determined from fasting serum and

urine measurements using a nomogram (107). The reference range is 0.80-1.40 mmol/L of glomerular filtrate.

2.1.10 Urinary Calcium Excretion

Urinary excretion of calcium was expressed as a molar ratio relative to urine creatinine (Ca/Cr; mmol/mmol; reference range less than 0.70). In the fasting state Ca/Cr ratio is thought to be an index of net bone resorption (108).

2.2 Imaging Techniques

2.2.1 Thyroid Hormone Uptake Scan

Thyroid scans were performed using standard techniques following injection of 80 megabequerels of Technetium (^{99}Tcm).

2.2.2 Radionuclide Bone Scan

Radionuclide bone scans were performed using standard techniques three hours after the intravenous injection of 530 megabequerels of ^{99}Tcm labelled methylene diphosphonate.

2.2.3 Technetium/Thallium Subtraction Scan

Parathyroid localisation studies were performed using a scanning technique where images were acquired in 1 min segments for 20 min after the intravenous administration of 80 megabequerels of ^{201}Tl then 10 min after 180 megabequerels of ^{99}Tcm. Images were stored in a computer interfaced to the gamma camera and a "subtraction" scan was then obtained

which highlighted the parathyroid adenoma (109).

2.3   Statistical Techniques

2.3.1 Cosinor Rhythmometry

Statistical analysis of circadian rhythm parameters was performed using the Cosinor technique (110). In this procedure, a cosine curve with a period of 24h was 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 parameters calculated are: the mesor (rhythm adjusted mean), the amplitude (half the total extent of the predictable change) and the acrophase (crest time of the best fit cosine function, in relation to local midnight).

2.3.2 Mann-Whitney U Test

In studies where the data collected is not normally distributed and the sample sizes result in different variances a nonparametric statistical method of analysis has been applied (111).

2.3.3 Analysis of Variance (ANOVA)

In studies where repeated t-tests or Mann-Whitney analysis would be inappropriate ANOVA was performed. In this analysis the data sets are usually greater than two and the distribution may or may not be normal. This analysis is applied in situations where multisample hypotheses are

being tested (112).

2.3.4 Students t-Test

Comparisons of paired data within group or within subject where the data is normally distributed have been performed using the Students t-test. In all cases where this test has been applied the two-tailed hypotheses have been tested (112,113).

2.3.5 Wilcoxon Signed Rank Test

Comparisons of paired data where the values are not from a normal distribution have been performed using the Wilcoxon signed rank test (114).

2.3.6 Linear Regression

The functional dependence of two variables on one another has been analysed by simple regression analysis. Data suitable for this analysis consists of a dependent variable that is a random effect factor and an independent variable that is either a fixed effect or a random effect factor. In this analysis values are assumed to be normally distributed, the variances are equal, errors in X are minimal and Y are additive (115).

Experimental Work

Chapter 3

Variation in Cyclic Adenosine Monophosphate
in Plasma and Urine During a 24 hour Period

3.1   Variation in cAMP in Normal Male Subjects

3.1.1 Introduction

The importance of fluctuations in the secretion of hormones throughout a 24h time period has been increasingly recognised during the past decade.   The variations in plasma hormone concentrations have physiological importance and the absence or alteration of normal rhythms can be pathological and may be diagnostic of certain disease states.   Both urine and plasma cAMP concentrations reflect an integrated effect of several hormones on their receptors, as well as kidney function, making interpretation of the cause of cyclical variations in cAMP difficult.   Several authors have demonstrated the existence of a circadian rhythm in UcAMP (63,116,117,118,119) with a remarkable consistency in timing of the excretion trough (0300-0900h).   In contrast there has been little agreement on the timing of the peak excretion of UcAMP with 1300-1500h (63), 0900-1200h (116), 1800-2100h (118), 0900-1200h (119) and a bimodal population with both 0400-0800h and 1200-1600h (117) proposed as the period of peak UcAMP excretion.

A circadian rhythm for cAMP in plasma has been described (117,120,121,122) and again whilst there is good agreement on the timing of the PcAMP trough (0200-0400h) major differences are found in the timing of the PcAMP peak (0800, 0600, 1200, 1400, 1600 and 2400h).

There has been little investigation into the factors controlling or affecting the daily variation in cAMP. In addition to PTH, adrenaline and noradrenaline have been proposed as the main hormones affecting both plasma and UcAMP rhythms (118,122,123). UcAMP in females is maximal around the time of ovulation and minimal during menses (124). There is also a significantly higher UcAMP excretion in women with the pre-menstrual tension syndrome (124). These effects are thought to be mediated by LH and FSH since amenorrhoea and the use of a contraceptive pill significantly reduced the mid cycle UcAMP excretion whilst decreasing UcAMP output (124). Indirect effects have been proposed with exercise, caffeine and smoking all implicated in the generation of PcAMP variations (125,126,127). There is strong evidence that the circadian rhythm in PcAMP is the result of postural variations and orthostatism and that the rhythm is absent in subjects confined to bed for 32h (128). Few studies have analysed data on both urine and plasma cAMP concentrations simultaneously and surprisingly there has been no attempt to study the role of the major cAMP controlling hormones PTH and glucagon in generating cAMP 24h rhythms. This probably reflects the previous difficulties experienced in measuring accurately both these hormones.

Improvements in immunoassay techniques in recent years have led to the development of immunometric assays for the intact molecule of PTH, PTH (1-84), which are specific and

sensitive and are able to measure PTH (1-84) in all normal subjects with a concentration well above the detection limit of the assay (100,101,129). These assays overcome the problems of circulating fragments of PTH interfering in the measurement of PTH (1-84) and enable physiological studies to be performed.

An understanding of the factors responsible for glucagon degradation and improved antibody technology targeted at the correct portion of the glucagon molecule has meant that glucagon measurements can now be made in normal subjects and physiological studies performed with confidence.

3.1.2 <u>Subjects and Methods</u>

In all the 24h studies described in this chapter a standard blood and urine sampling technique was adopted. Subjects were admitted to a hospital ward between the hours 1000-1200. A venflon cannula was placed in a vein in the median cubital fossa for blood sampling and urine was collected into a volumetric flask at 4h intervals during the day and one sample obtained immediately on rising from sleep corresponding to an overnight sample. The studies were performed over the period 1400-1330h and the maximum number of subjects sampled at one time was 6 with the usual number being 3. All subjects ate at the same times when studied and a standard diet was consumed during each study period, although no run in period of standard diet was given. All subjects were ambulant but avoided exercise and lay down to

sleep and arose from bed at the same time.

In the initial study six healthy male volunteers (age 29-
40; mean 33 years) were studied over a 24h period. None of
these males were suffering from any illness or taking
medication known to interfere with calcium metabolism. The
subjects were ambulant but avoided exercise during the
study period.   They lay down to sleep at 0100h, were
awakened at 0700h arising at 0800h.

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

The sample type for each analysis has been described in
Chapter 2. Each serum or plasma was obtained within 30 min
of venesection and immediately frozen and stored at -70°C
or -20°C, depending on the analyte being measured, prior to
analysis.   In this study PTH (1-84), UcAMP, NcAMP,
glucagon, calcium, phosphate, creatinine and albumin were
measured. All measurements were performed as specified in
Chapter 2.

3.1.3 <u>Results</u>

The mean 24h profiles of PTH (1-84), NcAMP, glucagon and
PcAMP in the six subjects are shown in Figure 2. PTH (1-
84) rises from 0130h in a broad peak through the night and
early morning. The concentrations from 0200 to 0600h were
significantly elevated (p<0.001) compared to baseline
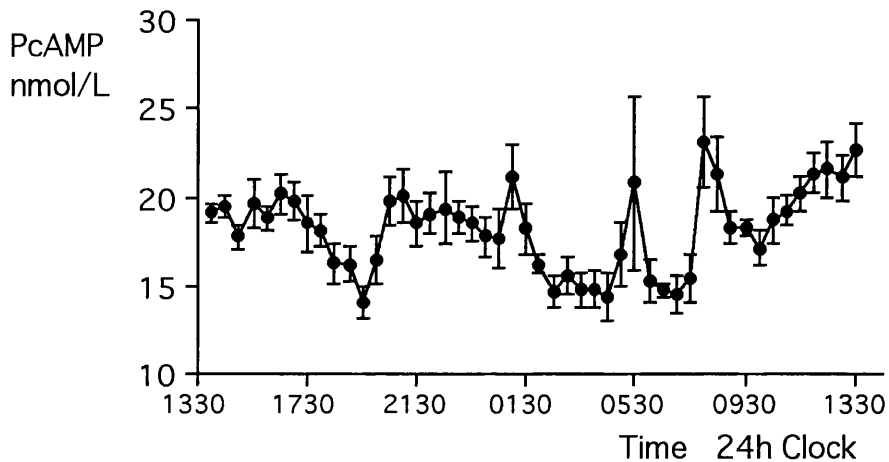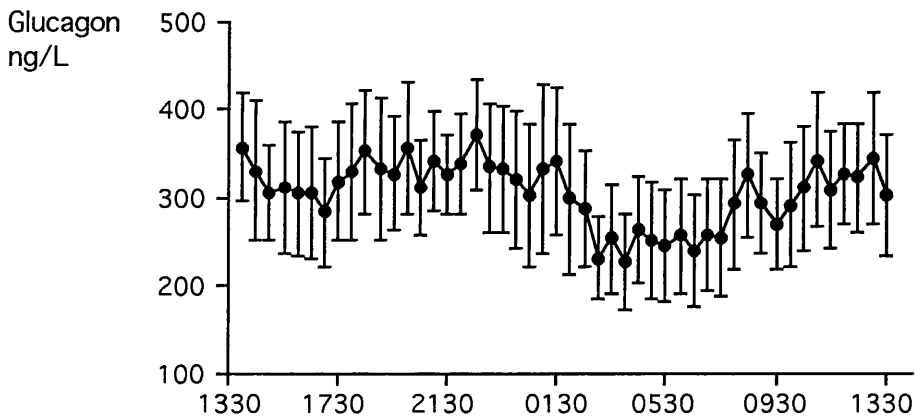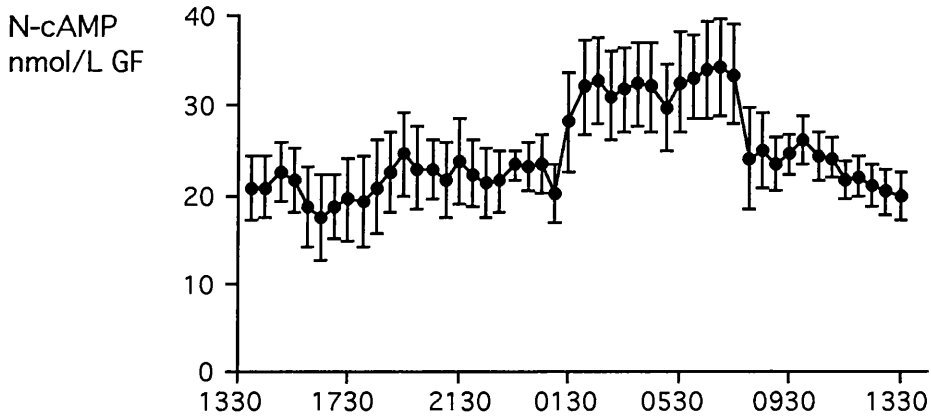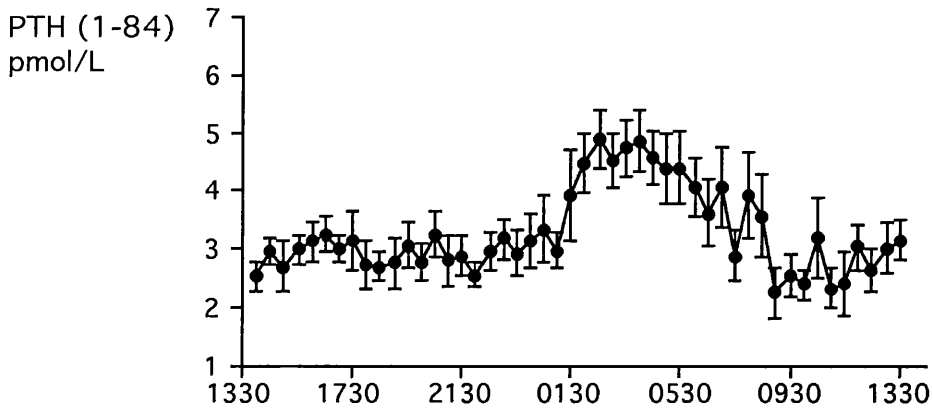
Figure 2   24 h profiles of PTH(1-84), NcAMP, Glucagon and
            PcAMP in 6 normal males.   Results are mean ± SEM.

(1400-1800h). The PTH (1-84) concentrations in all subjects returned to baseline values by 1000h although the rate of fall varied markedly between individuals. The mean intra-individual CV between 0630 and 1000h was 30% (range 12.9-42.5) compared to 17% (12.6-23.6) in the basal period.

Mean NcAMP concentrations rose throughout the night and early morning in parallel to the PTH(1-84). The mean NcAMP concentrations at eight of the eleven time points during the period 0200 to 0700h were significantly elevated ($p < 0.01$) compared to the baseline means (1400-1800h).

Plasma cAMP fluctuates considerably during the 24h period and there is wide individual variation. A significant ($p < 0.01$) concerted decrease in PcAMP occurs during the night and early morning with eleven of the twelve time points from 0200-0700h lower than baseline (1400-1800 h). A significant decrease ($p < 0.01$) in PcAMP was observed in the post-prandial period from 1800-2100h. The most significant increase in PcAMP was noted when all subjects rose from their beds in the morning peaking 1h after moving from recumbency to standing. PcAMP decreased following breakfast from the peak at 0800h to a nadir at 1000h then gradually increased over the remaining study period. PcAMP decreased significantly following each meal time (1800, 2300, 0800 and 1300h) ($p < 0.05$).

Glucagon decreased significantly (p<0.05) during the night and early morning in parallel to PcAMP with all twelve time points from 0200-0700h significantly lower than baseline (1400-1800). The changes in glucagon showed less fluctuation than PcAMP but similar decreases in glucagon were observed following each meal time.

Cosinor analysis of the data was performed Table 2 and indicated a tight phase and frequency synchronisation in the PTH(1-84) and glucagon concentrations of the six subjects over the 24h period. The results confirm a significant circadian rhythm in PTH (1-84) and glucagon but although there is phase synchrony in the NcAMP concentrations variability in amplitude is such that the mean amplitude of 5.9 from the mesor concentration of 25 nmol/L GF is not statistically significant at the 5% level. Variability in the PcAMP data is such that no statistically significant circadian rhythm is demonstrated.

3.1.4 <u>Discussion</u>

Both serum PTH (1-84) and NcAMP concentrations increase significantly during the night and early morning, with mean values above the day-time reference ranges for these analytes. The cosinor analysis confirms significant circadian rhythm in PTH (1-84), with an acrophase at 0405h. This is consistent with the night-time rise in immunoreactive PTH previously reported using C-terminal specific assays (130,131) and also with the hypothesis that

	Acrophase 24 h clock	Mesor	Amplitude	Significance
PTH 1-84	0405	3.3 pmol/L	0.7 pmol/L	P<0.05
NcAMP	0524	25.0 nmol/L GF	5.9 nmol/L GF	NS
Glucagon	1759	305.0 ng/L	36.8 ng/L	P<0.01
PcAMP	1443	18.2 nmol/L	1.2 nmol/L	NS

NS - Not Significant

Table 2   Cosinor analysis of 24 h profiles of normal males

the rise may be sleep related (132).

These results show that the circadian variations in PTH (1-84) are accompanied by parallel variations in NcAMP levels. This supports the conclusion that the PTH (1-84) released through the night is physiologically active at the kidney. The kidney has not been exposed to an increased filtered load of cAMP as PcAMP fell overnight and so the results explain why measurements of total urinary cAMP failed to correlate with proposed PTH effects and circadian rhythm in the past. The close correlation between NcAMP and PTH which is independent of PcAMP supports the use of NcAMP as a marker of end organ PTH activity.

Although the NcAMP concentrations increased significantly, the cosinor analysis failed to detect a significant phase and frequency synchronised rhythm for the six subjects. This is probably related to the number of subjects studied and the inherent variability in this measurement which is calculated from four separate analyses.

NcAMP concentration remained elevated (0600-0700h) after the main peak of PTH (1-84). This is partly due to the study design as urine was collected on rising in the morning after sleep and so the NcAMP calculations overnight will be a reflection of the changes in PcAMP at each time point over an integrated urine collection for each subject. However, studies using catheterised animals or human

subjects (46) have indicated that total UcAMP decreases by a minimal amount then remains constant overnight and so the calculated NcAMP will be a good estimate of true NcAMP production at each time point.

Plasma glucagon and cAMP decrease significantly overnight and glucagon demonstrates a significant circadian rhythm. The overall pattern of glucagon and cAMP secretion over 24h is very similar and a strong correlation exists between the two analytes over the 24h period. It is known that glucagon stimulates cAMP production from liver and from this current data glucagon would appear to be making a major contribution to the circulating PcAMP. A 50% decrease in glucagon overnight is accompanied by a 50% decrease in PcAMP and the role of glucagon in the generation of PcAMP is further supported by the transient falls in both analytes following mealtimes. These data would indicate that PcAMP in the physiological situation is influenced more by glucagon than PTH. Although PcAMP in pharmacological studies can be used to rapidly monitor PTH action (55,85) in physiological and pathophysiological studies NcAMP is a more reliable index of PTH activity.

Significant fluctuations exist in PcAMP on top of the inherent circadian rhythm which make the cosinor analysis difficult to apply and inappropriate for this data. PcAMP reflects the integrated effect of several hormones and it is likely that other hormones, particularly the

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catecholamines, are affecting the values obtained. A transient fall in catecholamines occurs on recumbency and sleep with a significant increase on rising early in the morning and these hormone changes will also contribute to the overall appearance of the PcAMP profile. Several other hormones act through cAMP and in disease states, or in altered physiology, the effects of one or other of these hormones may predominate altering cAMP metabolism.

The existence of a circadian rhythm in PTH (1-84) which is physiologically active raises a number of questions related to the control of PTH (1-84) release and its effects on cAMP metabolism. In subsequent chapters studies have been performed to analyse cAMP metabolism during health, under physiological manipulation and in disease during 24h periods and at selected single time points.

3.2 Sleep Shift Effects on cAMP Metabolism, PTH(1-84), Glucagon and Prolactin in Normal Male Subjects

3.2.1 Introduction

The mechanism controlling the nighttime rise in PTH (1-84) and NcAMP is not yet known. It has been concluded from a number of studies (130,132,133,134,135,136,137) that the PTH (1-84) rhythm cannot be accounted for solely by changes in serum calcium or phosphate concentrations and therefore the central nervous system may play a role either directly or indirectly in modulating normal PTH (1-84) secretion. Prolactin (PRL) secretion follows a similar pattern to PTH (1-84) over a 24h period in normal male subjects with an early evening rise and a major night-time elevation occurring shortly after the onset of sleep (138,139). Cross-correlation analysis of 24h profiles confirmed a strong temporal correlation between PTH (1-84) and PRL secretion in normal male subjects with the changes in PRL secretion occurring approximately 2h after those in PTH (1-84) (136). It has been postulated therefore that there may be neuroendocrine control common to PTH (1-84) and PRL secretion which would subsequently affect cAMP production.

The overall circadian characteristics (excluding food intake or dietary effects) of a 24h hormonal profile generally represent an integration of 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 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 7-12h, any masking effects of sleep on circadian inputs are removed and the effects of sleep at an abnormal circadian time can be revealed. In this study a sleep-shift experiment was performed in which the time of sleep was delayed by 7h in six normal male subjects.

3.2.2 Subjects and Methods

The six normal healthy male subjects from the previous study were sampled exactly one year following the original study thus avoiding the possible effects of seasonal changes in calcitropic hormones. Study A (circadian) was described in Chapter 3.1. In study B (sleep shift) the subjects were awake from 1400-0800h and slept from 0800-1400h. All subjects ate identical meals in both studies (at 1800, 2300, 0800 and 1300h in study A and 1800, 2300, 0730 and 1430 in study B). Timed urine samples were obtained 4 hourly when awake and one sample obtained after awaking from sleep.

All analytes studied in Chapter 3.1 were measured and in addition serum prolactin (PRL) was measured in this study. Methods of analysis were as outlined in Chapter 2 and NcAMP

was calculated by the method of Broadus *et al* (46). Statistical analysis was performed using a paired t-test to compare results within an individual and the Mann-Whitney U test between studies.

3.2.3 <u>Results</u>

The mean (± SEM) PTH (1-84), NcAMP are shown Figure 3, PcAMP, glucagon Figure 4 and PRL Figure 5 in each of the studies. Overall mean 24h concentrations, the range of values and the coefficient of variation for PTH (1-84), NcAMP, PcAMP, PRL, glucagon and adjusted calcium in each of the six subjects for the circadian and sleep-shift studies are shown in Tables 3a and 3b.

A comparison of the profiles has been made Figure 6 (PTH (1-84), NcAMP, PRL and adjusted Ca) and Figure 7 (P-cAMP and glucagon) which highlights the trends during the hours of sleep by smoothing the profiles using a three-sample moving average technique to minimise the perturbations due to either pulsatile secretion or assay noise and the error bars omitted for clarity.

Comparison of the profiles of each analyte indicates that sleep shift has altered the pattern of both secretion of the hormones studied and cAMP. The nocturnal rise in PTH (1-84) is present in both studies despite the shift in the sleep-wake cycle. However, the start of the sleep wake cycle is attenuated by sleep delay with the PTH
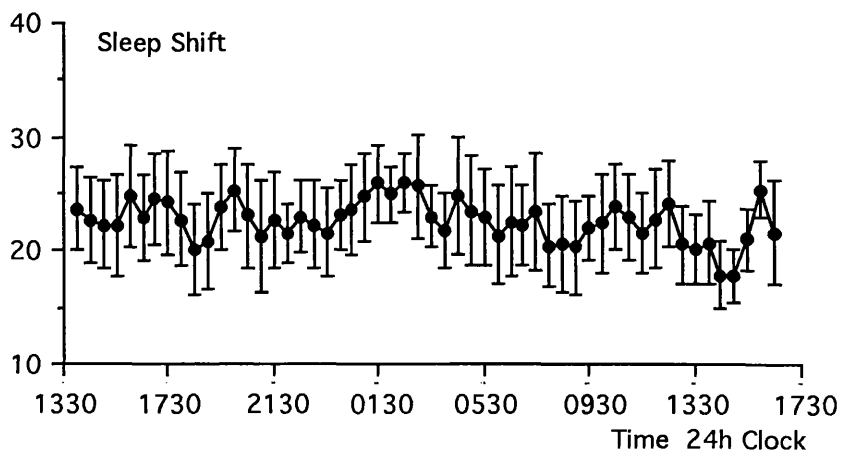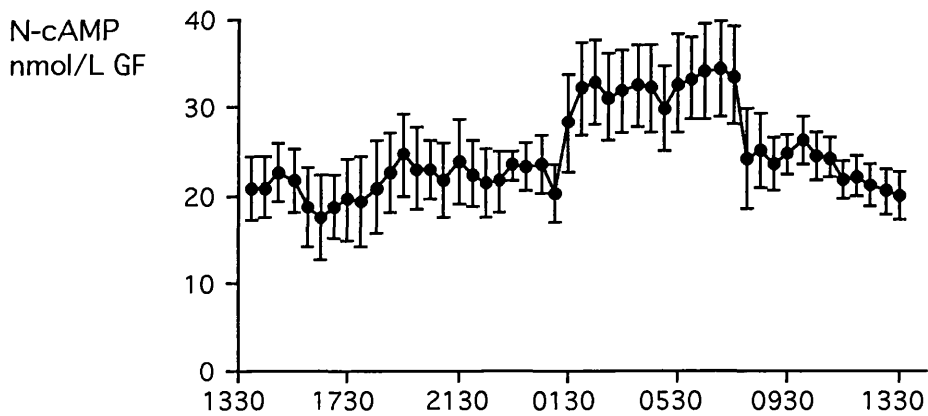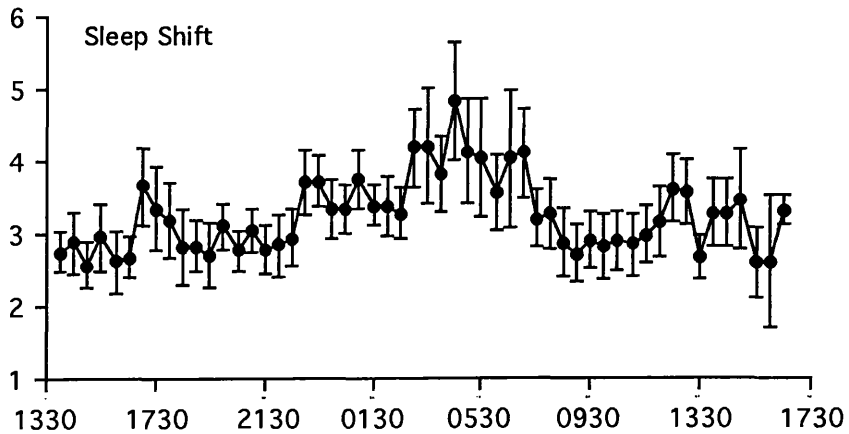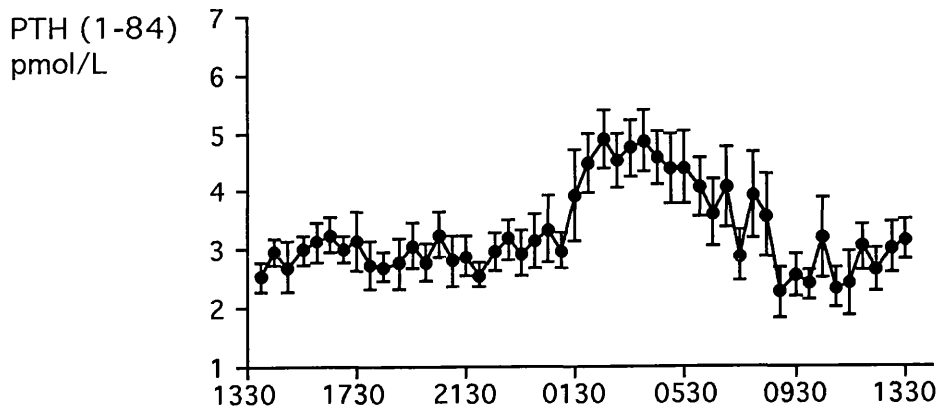
Figure 3 Sleep shift effects on PTH(1-84) and NcAMP
profiles in 6 normal males.
Results are mean ± SEM.
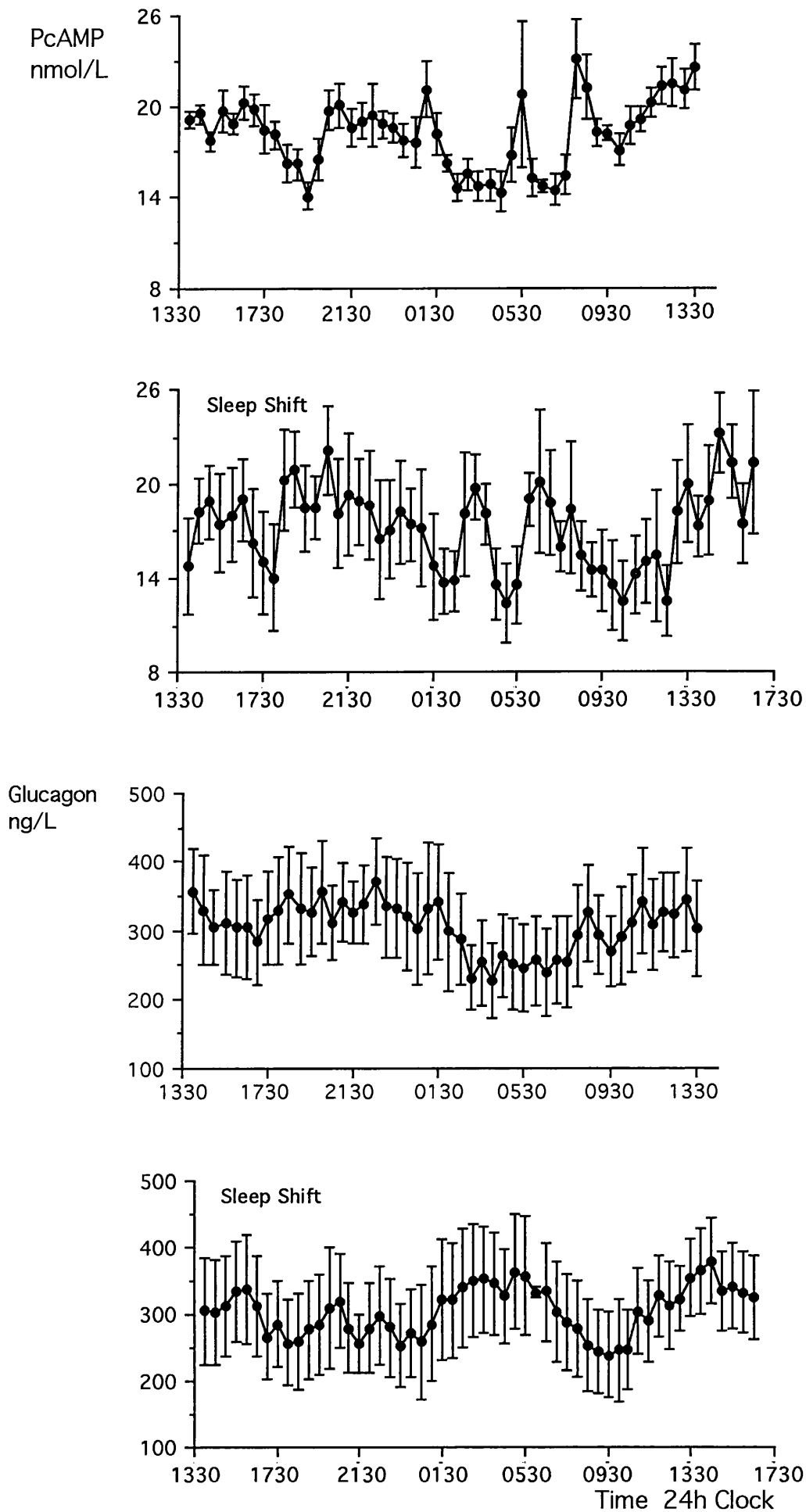
Figure 4 Sleep Shift effects on PcAMP and Glucagon 24 hr profiles in 6 normal male subjects. Results are mean ± SEM
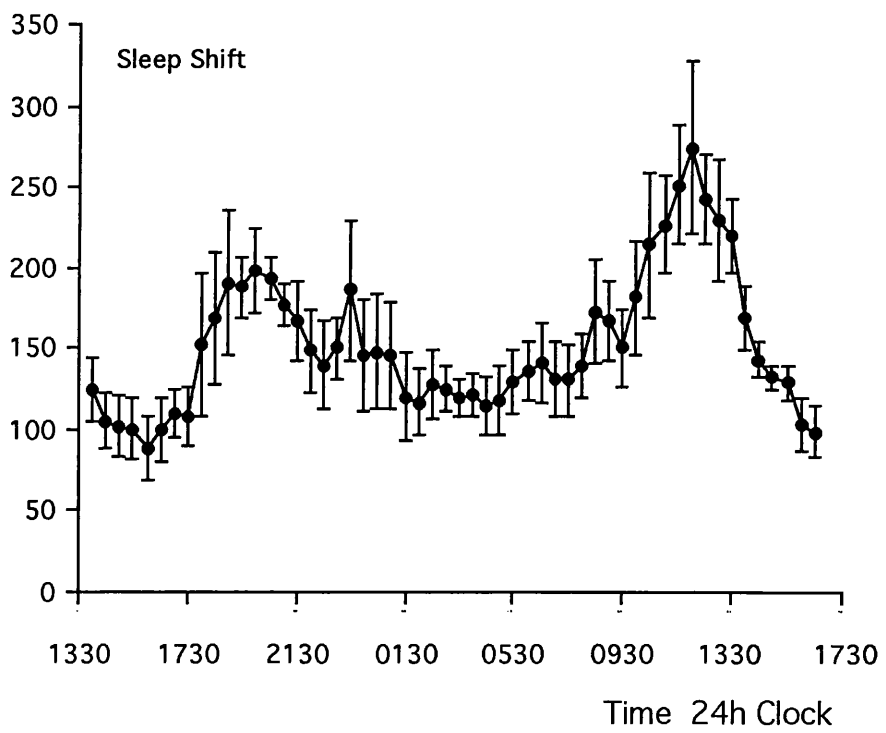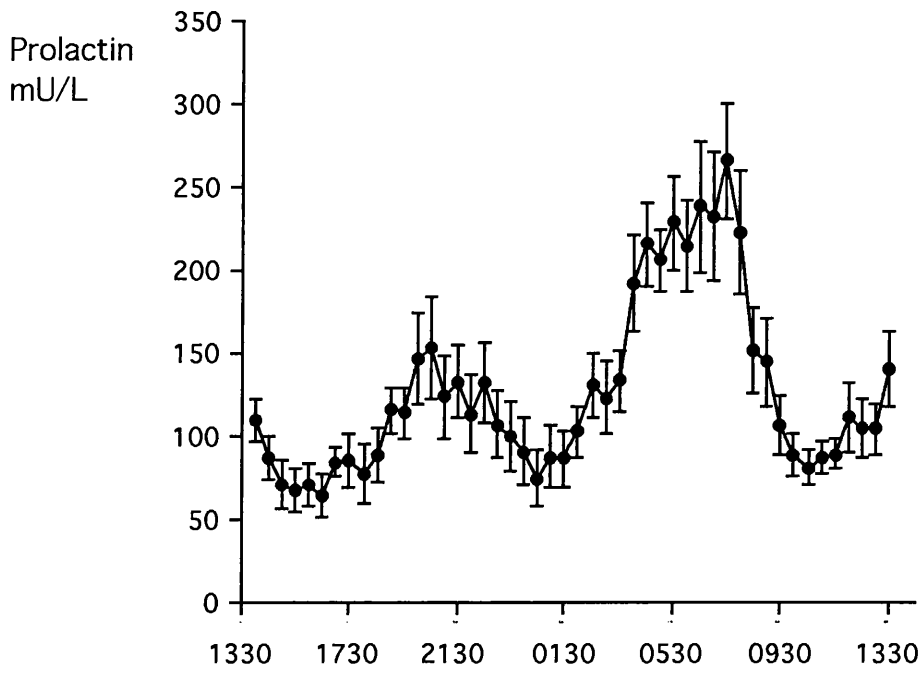
Figure 5   Sleep shift effects on Prolactin 24 h profile in
6 normal male subjects.   Results are mean ± SEM.

		Circadian: Study A			Sleep shift: Study B		
		24 h Mean	Range	CV	24 h Mean	Range	CV
PTH (1-84) (pmol/L) Ref Range (1-5)	1	2.3	1.1-4.5	36.7	1.8	0.6-3.2	32.1
	2	3.9	1.6-6.5	25.1	3.3	1.7-4.9	22.4
	3	4.6	2.1-7.6	32.2	4.4	2.0-8.6	32.6
	4	3.2	1.4-5.6	35.2	2.8	1.2-4.5	23.1
	5	2.6	0.9-4.2	30.6	3.5	1.7-8.5	30.1
	6	3.2	2.0-4.2	14.8	3.7	1.5-5.6	22.1
NcAMP (nmol/L GF) Ref Range (8-28)	1	23.0	4.9-36.9	37.5	15.2	10.2-23.1	17.6
	2	19.4	6.7-32.3	32.0	17.8	2.6-34.5	45.8
	3	33.4	14.2-56.7	38.8	31.8	21.8-48.1	15.6
	4	27.0	10.6-40.6	31.4	28.2	18.8-42.6	19.7
	5	13.9	4.5-22.7	33.4	16.1	3.9-32.8	50.5
	6	31.5	20.6-39.7	13.3	26.0	17.4-37.0	16.7
PRL (mU/L) Ref Range (60-360)	1	144	32-310	55.6	194	111-384	56.5
	2	96	10-351	80.0	124	31-313	48.1
	3	168	55-319	40.8	203	111-411	32.3
	4	156	58-389	48.8	92	34-266	59.7
	5	93	46-171	32.7	139	93-307	32.4
	6	103	69-207	33.9	168	50-532	49.3
Adjusted calcium (mmol/L) Ref Range (2.20-2.60)	1	2.45	2.38-2.49	1.0	2.38	2.31-2.45	1.5
	2	2.33	2.13-2.48	4.3	2.46	2.40-2.53	1.2
	3	2.39	2.34-2.45	1.2	2.40	2.33-2.48	1.6
	4	2.40	2.27-2.52	1.9	2.46	2.38-2.56	1.6
	5	2.48	2.42-2.54	1.0	2.45	2.37-2.51	1.4
	6	2.47	2.30-2.54	1.6	2.43	2.32-2.50	2.0

Table 3a   PTH (1-84), NcAMP, PRL and adjusted calcium results for individual subjects during the circadian and sleep shift studies

		Circadian: Study A			Sleep Shift: Study B		
		24h Mean	Range	CV	24h Mean	Range	CV
PcAMP (nmol/L)	1	17.8	9.6 - 25.2	22.3	17.4	9.0 - 29.1	26.2
Ref Range (15-30)	2	18.8	12.2 - 30.0	18.2	17.7	4.6 - 28.6	30.0
	3	20.3	11.0 - 29.2	21.6	20.1	10.5 - 24.9	26.1
	4	16.9	11.5 - 23.1	15.4	17.8	6.8 - 31.8	35.3
	5	18.5	13.1 - 28.7	17.4	23.9	4.8 - 41.3	36.3
	6	17.1	11.3 - 44.3	28.3	15.9	8.0 - 28.5	28.1
Glucagon (ng/L)	1	583	358 - 747	15.5	593	428 - 745	12.9
Ref Range (70-350)	2	334	267 - 508	14.2	370	265 - 469	12.7
	3	116	53 - 206	37.5	163	63 - 259	24.3
	4	193	116 - 321	24.7	228	164 - 312	15.1
	5	305	186 - 575	20.2	324	222 - 404	15.9
	6	295	210 - 426	17.6	285	245 - 475	14.7

Table 3b PcAMP and glucagon results for individual subjects
during the circadian and sleep shift studies
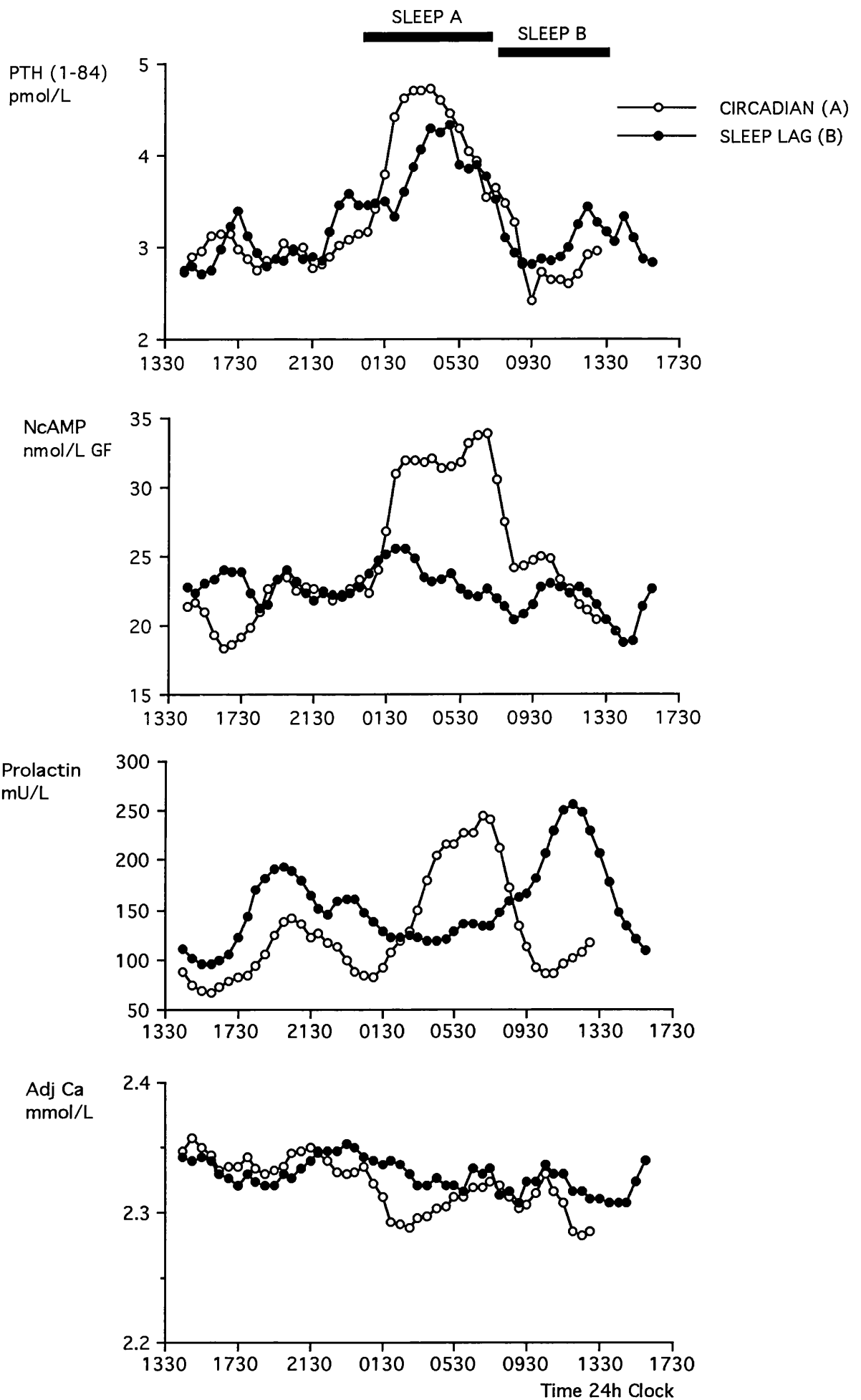
Figure 6 Smoothed (3 point moving average) mean 24h profiles for PTH (1-84), NcAMP, PRL and adjusted calcium under normal (circadian) and sleep shift conditions in 6 normal males.
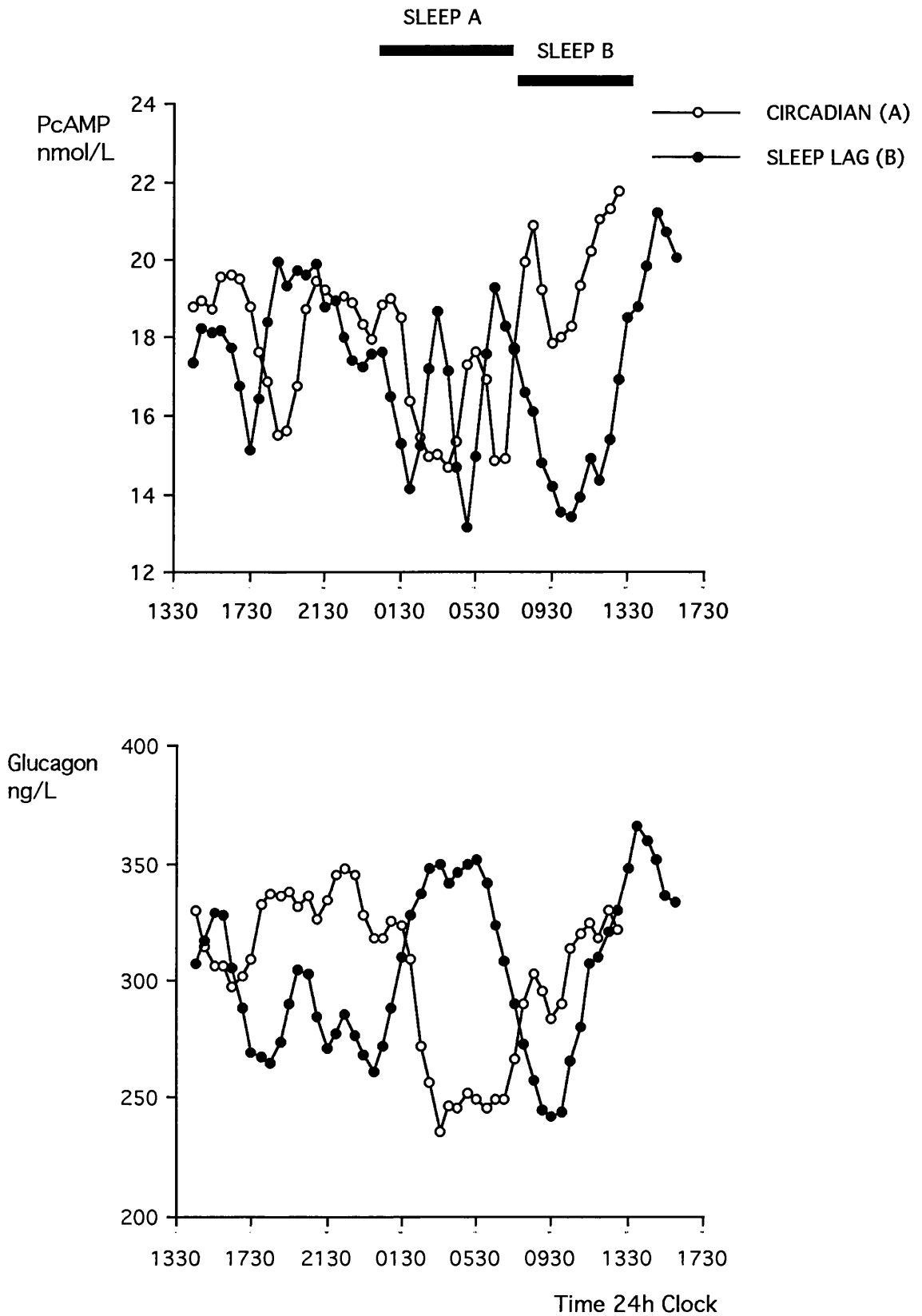
Figure 7   Smoothed (3 point moving average) mean 24h profiles for PcAMP and glucagon under normal (circadian) and sleep shift conditions in 6 normal males.

concentrations in each individual significantly lower in the sleep-shift study from 0200-0300h ($p < 0.05$). NcAMP rises overnight in both studies but the rise during the sleep-shift experiment is markedly reduced with mean NcAMP from 0130-0700h calculated to be 23.8 nmol/L GF compared to 31.9 ($p < 0.01$) in the circadian study. In the circadian study an early morning peak in PRL is seen which is absent in the sleep-shift study. However, an increase in PRL occurs coincident with the time of delayed sleep in the sleep-shift study. Initial comparisons of the PcAMP profiles are very similar and a decrease in PcAMP from 0130-0230h is seen in both studies. After 0230h the two profiles differ markedly with peaks and troughs occurring at different times in each study and a concerted decrease in PcAMP coincident with the time of delayed sleep in the sleep-shift experiment. The profile of glucagon excretion is also very similar in the early part of the studies. A significant difference in excretion exists at two time periods coincident with sleep in both studies with glucagon significantly lower ($p < 0.01$) when asleep compared to basal secretion in the same study or the equivalent time period between the studies ($p < 0.05$).

3.2.4 Discussion

Early studies using this type of experimental design demonstrated that the secretion of some hormones, such as cortisol (140) and melatonin (141), is primarily modulated by circadian rhythmicity, whereas others such as PRL

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(138,142) and growth hormone (143) are strictly sleep dependent. The shift in the timing of the PRL peak seen in this acute sleep-shift experiment is consistent with early studies on PRL secretion which had shown that daytime naps are associated with increases in PRL (142) and that acute reversal or delay of the sleep wake cycle is accompanied by an increase in PRL during daytime sleep (138). However, more recent studies on repeated sleep shifts as part of a jet lag study have shown a splitting of the PRL peak revealing an endogenous circadian component to PRL secretion in addition to the sleep-related component (139). 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.

In this acute sleep-shift experiment the overall timing of the main nocturnal PTH (1-84) rise did not alter substantially from the normal circadian pattern. Thus the data on PTH (1-84) secretion from this experiment is consistent with a primary circadian rhythm, i.e. intrinsically linked to the time of day. However, nocturnal PTH concentrations have been shown previously to be temporally correlated with cycles of stage 3 and 4 sleep (132) and this may be reflected in this study by the early part of the peak being blunted with a significant decrease in PTH (1-84) from 0200-0300h. These data are consistent

with the postulate that the control of PTH (1-84) secretion is primarily circadian but 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 (144).

Comparison of the NcAMP profiles between the two studies indicates a markedly attenuated nocturnal NcAMP output in the sleep-shift experiment. These results suggest that the end organ response, the nocturnal production of NcAMP at the kidney, may not solely be determined by PTH (1-84) but is modified by other factor(s). For example cortisol (145, 146 and Chapter 5), T_4 (147, 148 and Chapter 4), T_3 (149) and insulin (150) can modify the cAMP response evoked to PTH in cell systems and in humans. In particular cortisol and thyroid hormones can alter the threshold concentration at which PTH can produce a cAMP response (145,146,148,149). Although such effects have largely been shown to occur at supraphysiological concentrations and *in- vitro*, it is conceivable that under normal circumstances the increase in NcAMP during the night is likely to be the result of a concerted effect of a number of hormones acting to facilitate PTH stimulation of cAMP. This phenomenon may be disrupted by an acute sleep shift.

It can be seen that a significant change in the profile of PcAMP has taken place which only in part reflects the

alteration in glucagon secretion. The nadir of secretion corresponds to sleep and recumbency in both studies at the time when glucagon concentrations are at their lowest. In the sleep-shift experiment, however, a decrease in PcAMP is observed at a time when mean glucagon concentrations are rising and considerable fluctuations are observed in PcAMP over the period 0130-0700h which probably reflect the disturbed balance between hormones that are entrained to the biological clock and those that are entrained to the time of day.

There is evidence to link PRL to PTH and calcium metabolism (151,152,153). The recent finding that PRL can stimulate PTHrP release into breast milk and influence PTHrP messenger RNA expression adds weight to the association of PRL and calcium metabolism (154). It is interesting to speculate whether the night-time rise in NcAMP during the normal sleep wake cycle has a PRL-dependant, and therefore sleep-dependent, component perhaps related to the production of PTHrP rather than PTH (1-84).

Thus, although the results of this acute sleep-shift experiment argue against a direct neuroendocrine link between PTH (1-84) and PRL secretion, the circadian studies, performed to date, demonstrate that these hormones are maintained by the circadian clock in a distinct phase relationship throughout a 24h period in normal subjects. The changes observed in cAMP metabolism, particularly

NcAMP, in this study reflect the alteration of activity of several hormones and it may be that a high degree of temporal organisation allows concerted metabolic effects between PTH and other hormones leading to optimised end organ responses.

3.3   The Effect of a 96 Hour Fast on cAMP Metabolism, PTH

(1-84) and Glucagon in Normal Male Subjects

3.3.1 Introduction

PTH (1-84) secretion and NcAMP production in normal males

follows a defined circadian rhythm (137,155, Chapter 3.1).

Evidence for neuroendocrine control arises from the

observation of a strong temporal correlation between PTH

(1-84) and PRL (136). The sleep shift experiment (Chapter

3.2) argues against a direct neuroendocrine link between

PRL and PTH (1-84) indicating that the PTH (1-84) and NcAMP

rhythms are mainly circadian in nature, that is

intrinsically linked to the time of day with the

possibility of modification by the presence of sleep.

Throughout the studies on the circadian secretion of PTH

(1-84) the most striking and consistent relationship found

has been between PTH (1-84) and serum PO_4. From evidence in

the literature it has been concluded that this relationship

reflects the influence of PTH on the renal handling of

phosphate with this action mediated via cAMP. The

strongest evidence against a direct role for phosphate in

the control of the circadian rhythm of PTH secretion is

that of Jubiz *et al* (130) who reported that following a

fast the PTH circadian rhythm persisted while that of

phosphate was abolished.

It should be noted that this key study on the inter-
relationship between phosphate and PTH secretion was
performed some twenty years ago. Since that time the
highly specific two-site immunometric assays have been
developed which allow the measurement of subtle changes in
PTH (1-84) concentrations and have permitted improved
definition of the physiology and pathophysiology of PTH (1-
84) secretion (156).

In the following study the effects of fasting on the 24h
rhythms of PTH (1-84), NcAMP, PcAMP, glucagon, serum
adjusted Ca and serum PO_4 have been investigated.

3.3.2 Subjects and Methods

Six normal male volunteers (age 28-40, mean 32 years) were
studied on two separate occasions. During one study
(Control) on the day of sampling the subjects ate identical
meals at defined times (1800, 2230, 0800 and 1200h), were
ambulant but avoided exercise and lay down to sleep at an
identical time (0100-0700h). For the other study (Fasting)
the subjects consumed no food but were allowed water *ad
libitum* for 96h and were sampled during the final 24h of
the fast. The subjects followed an exercise and sleep
regime identical to that of the control study. Three
subjects were randomised to the control study followed by
the fasting study whilst the other three subjects fasted
first then subsequently took part in the control study. A
minimum of three months separated each study period. Blood

and urine samples, for the measurements described in Chapter 3.1, were obtained, stored and assayed as described Chapter 2.

Statistical analysis was performed using the paired difference t-test to analyse significant changes within 24h profiles. Mann Whitney U comparisons were used to analyse differences between study periods. Cosinor techniques were used to analyse circadian rhythm parameters.

3.3.3 Results

The 24h profiles for serum PO_4, adjusted calcium, PTH (1-84) and NcAMP are shown in Figures 8 and 9. Comparisons of the profiles of PcAMP and glucagon are given Figure 10. Results from the control study are shown above the corresponding results from the fasting study figures. The results of the cosinor analysis of the data contained in the figures are summarised in Table 4. As anticipated, the process of fasting for 96h effected a dramatic change in the 24h pattern of serum PO_4. In the control study there was a pronounced circadian rhythm of PO_4 which is highly significant when analysed by cosinor analysis. In the fasting study the nocturnal peak of phosphate was almost completely abolished and cosinor analysis confirms the absence of any significant circadian rhythm. It is worthy of note that for most of the 24h period serum PO_4 concentrations were higher in the control study (mean 1.20 mmol/L) than in the fasting study (mean 1.13 mmol/L,
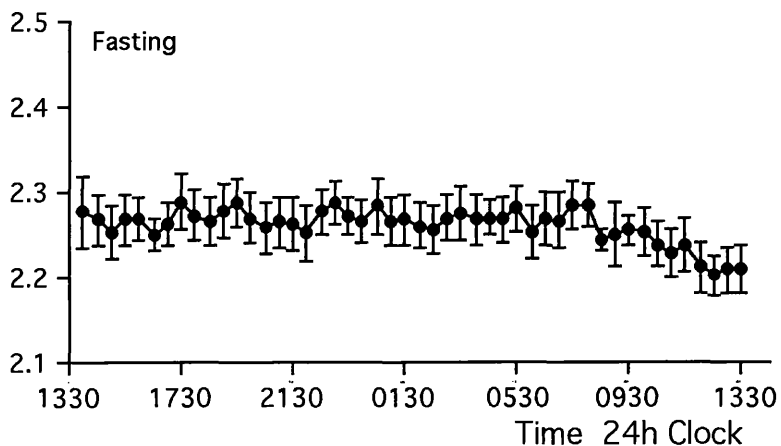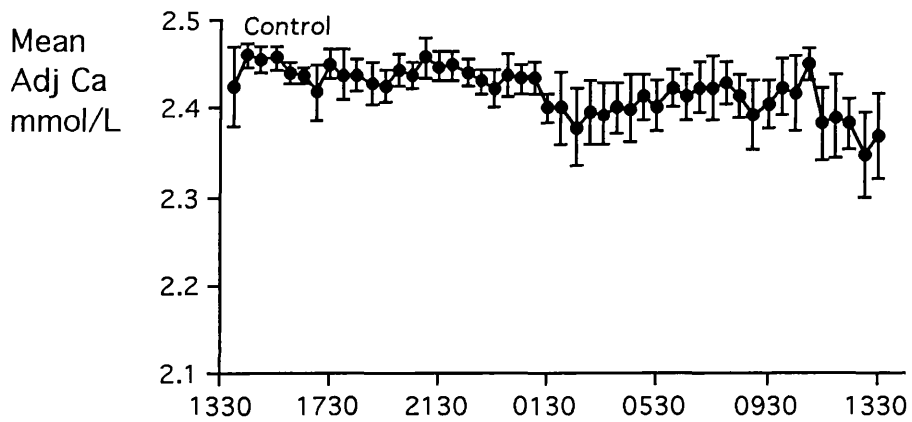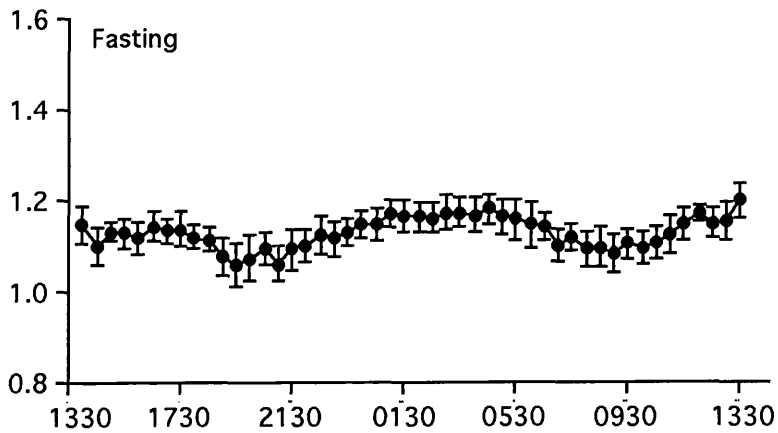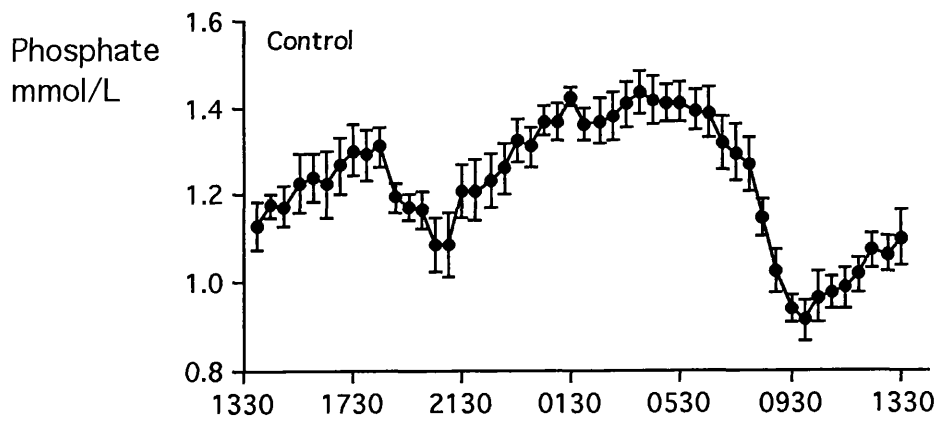
Figure 8   24 h profiles of serum PO_4 and adjusted Ca during normal diet and after a 96 h fast in 6 normal male subjects.   Results are mean ± SEM.
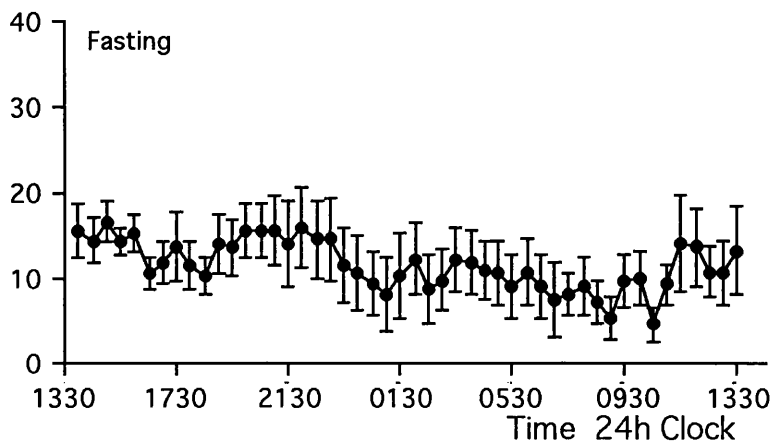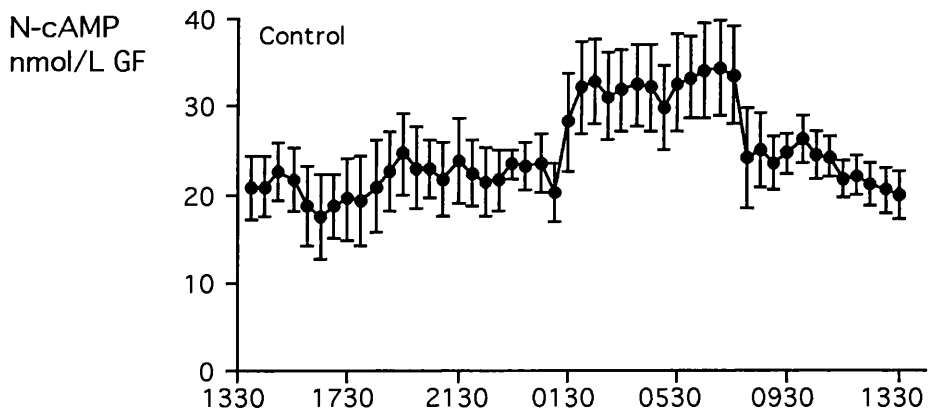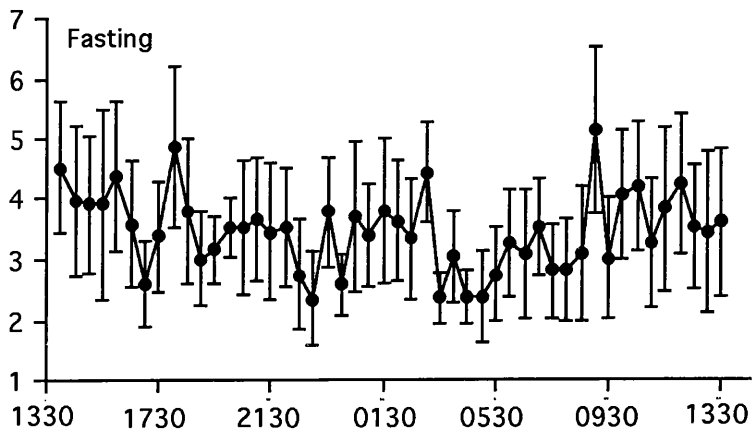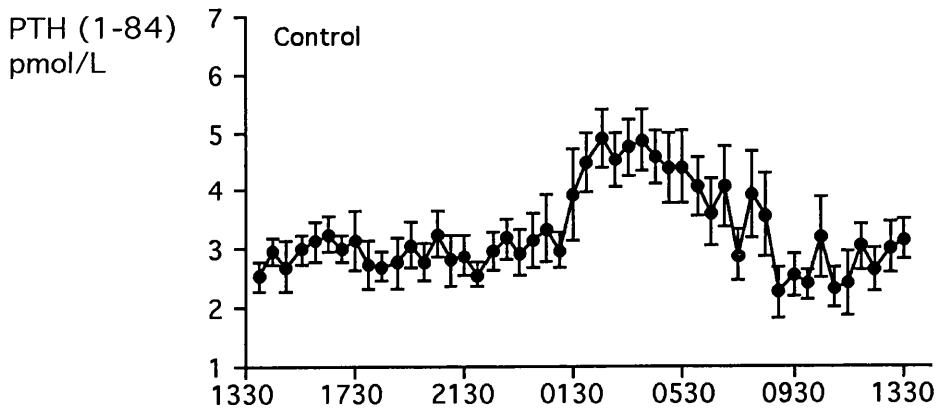
Figure 9 24 h profiles of PTH(1-84) and NcAMP during normal diet and after a 96 h fast in 6 normal male subjects. Results are mean ± SEM.
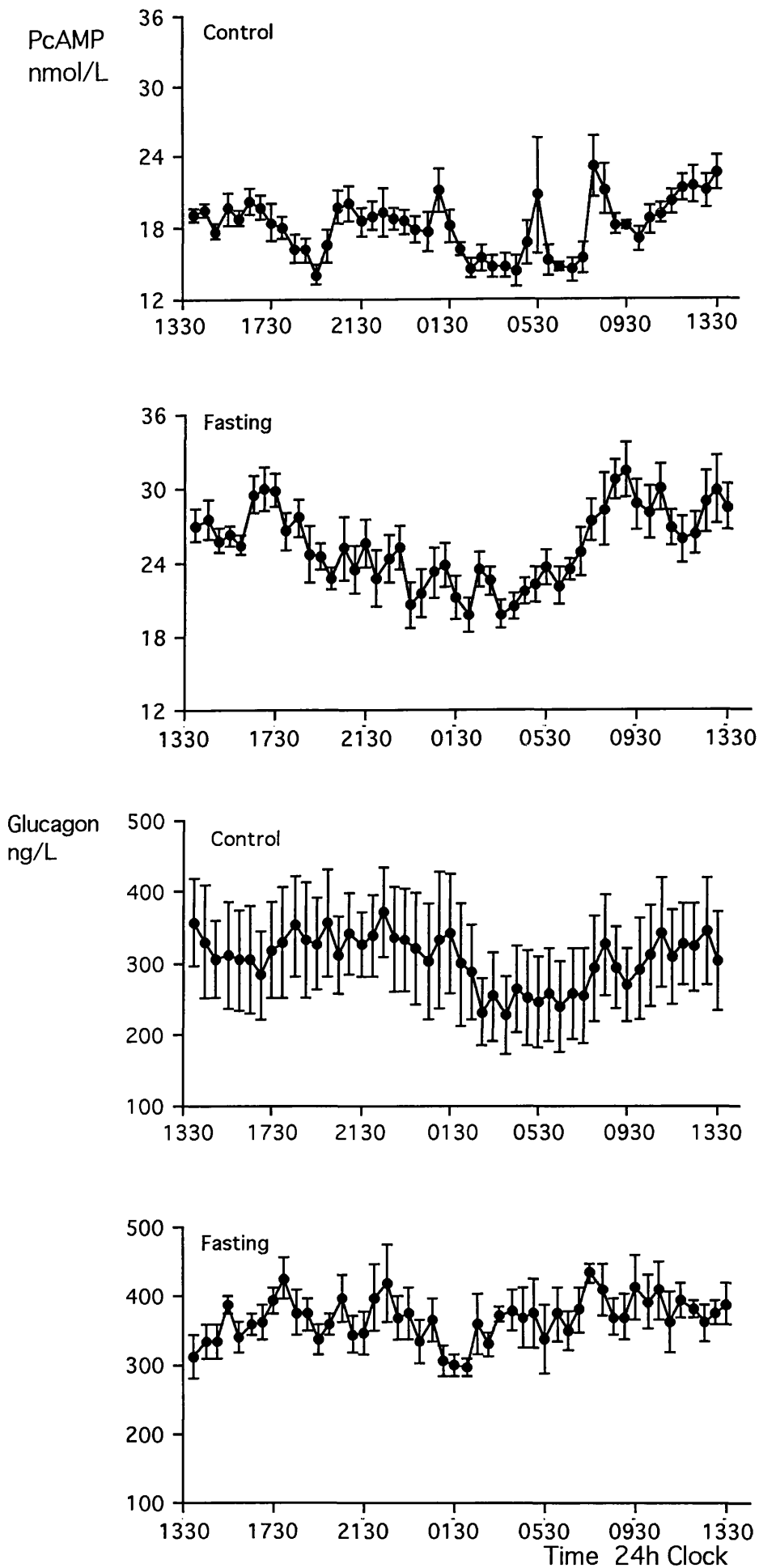
Figure 10   24 h profiles of PcAMP and Glucagon during normal diet and after a 96 h fast in 6 normal male subjects.   Results are mean ± SEM.

	Acrophase 24h Clock	Mesor	Amplitude	P
Phosphate				
Control	0155	1.20	0.14	<0.01
Fasting	NS	1.13	NS	NS
		(mmol/L)	(mmol/L)	
Adjusted calcium				
Control	NS	2.40	NS	NS
Fasting	NS	2.26	NS	NS
		(mmol/L)	(mmol/L)	
PTH (1-84)				
Control	0405	3.3	0.7	<0.05
Fasting	NS	3.5	NS	NS
		(pmol/L)	(pmol/L)	
NcAMP				
Control	0524	25.0	5.6	0.08 (NS)
Fasting	NS	11.6	NS	NS
		(nmol/L GF)	(nmol/L GF)	
PcAMP				
Control	NS	18.2	1.2	NS
Fasting	1300	25.1	3.3	<0.02
		(nmol/L)	(nmol/L)	
Glucagon				
Control	1759	305.0	36.8	<0.01
Fasting	NS	354.5	11.9	NS
		(ng/L)	(ng/L)	

NS - Not Significant

Table 4 Cosinor analysis of 24h profiles of serum PO_4, adjusted Ca, NcAMP, PTH (1-84), PcAMP and glucagon whilst consuming normal diet and following a 96h fast

p<0.01).

The 24h mean of serum adjusted Ca concentration was also higher in the control study (2.40 mmol/L) than in the fasting study (2.26 mmol/L, p<0.01) but the pattern throughout the day did not differ markedly between studies. Cosinor analysis did not detect a synchronised circadian rhythm in serum adjusted calcium concentration in either study. Using the group mean concentrations over the initial period of the study as baseline (1400-1800h) a statistically significant transient decrease in adjusted calcium was observed in the control study during the sleep period (2.44 to 2.40 mmol/L at 0130h; p<0.05). No corresponding decrease in adjusted calcium was detected in the fasting study. The mean adjusted calcium was lower at the end of both studies than at the beginning and may in part reflect daily variation. This was also a consequence of greater individual variability in the latter part of the study.

Figure 9 reveals a marked alteration in the pattern of PTH (1-84) concentrations over the 24h period as a result of fasting, despite similar 24h mean concentrations (control 3.3 pmol/L; fasting 3.5 pmol/L). The nocturnal rise seen in the control study is absent in the fasting profile with PTH (1-84) concentrations decreasing during the night and early morning when fasting. In the fasting study the mean PTH (1-84) concentrations were significantly lower during

the time of sleep (fasting 2.8 pmol/L; control 3.5 pmol/L; p<0.01) but higher during waking hours (fasting 3.1 pmol/L; control 2.4 pmol/L; p<0.001). Figure 9 also reveals that the between subject variability was greater in the fasting study than in the control study. Cosinor analysis of the PTH (1-84) profiles indicates a significant circadian rhythm in the control study but fails to detect a synchronised rhythm in the fasting study.

NcAMP results are very similar in pattern to those of PTH (1-84). Thus the nocturnal peak of NcAMP coincident with that of PTH (1-84) in the control study was absent following fasting. Of major importance was the significant decrease in mean NcAMP following fasting (control 25.0 nmol/L GF; fasting 11.6 nmol/L GF; p<0.001). This difference is in part due to the absence of the nocturnal rise in NcAMP but is also due to a significant decrease in mean NcAMP during the waking hours 0800-0100 (control 22.1 nmol/L GF; fasting 12.1 nmol/L GF p<0.001). Although an increased variability in PTH (1-84) is observed when fasting the variability in NcAMP production is reduced.

PcAMP is significantly higher (p<0.01) following fasting when compared to the control and the profile over 24h altered so that a gradual decrease in PcAMP was noted from 2100-0600h with a less pulsatile pattern of secretion observed when compared to control. Glucagon concentrations are also significantly higher (p<0.01) after fasting with

little variation in concentration over the 24h and in particular the post prandial decreases noted in the control study are absent during fasting and the sleep/orthostatism related decrease is not so obvious during fasting. These alterations in glucagon and PcAMP secretion abolish the glucagon circadian rhythm but result in a significant PcAMP rhythm following the fast.

3.3.4 Discussion

The 96h fast incorporated into this study resulted in the loss of the 24h rhythm of serum PO_4 from a group of normal adult male subjects. This result, together with the accompanying reduction in the mean 24h serum adjusted calcium concentration, is consistent with the findings of other fasting studies (157,158). Of major significance, however, is that unlike the one previous study in which it was examined (130) the current study has demonstrated that a 96h fast results in attenuation of the normal circadian rhythm of both PTH (1-84) and NcAMP with a significant reduction in NcAMP production following this fast.

There are four main possible explanations for the difference between the results of the present study and that of Jubiz and his colleagues in 1972 (130). Firstly, there were more subjects in the current study (six versus two). Secondly, the current study employed an increased sampling frequency (30 min versus 2h). Thirdly, the period of fast was longer (96h versus 48h). Finally, improvements

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in immunoassay in the current study meant that only the intact PTH molecule was measured without interference from fragments of the PTH molecule, with a sensitivity and precision capable of distinguishing small changes in PTH (1-84) concentration within the reference range for normal subjects (100,156). This combination of improved study design and methodology contribute to the differences in outcome but the possibility also exists that there is a gradual loss in the nocturnal surge in PTH (1-84) with an increasing length of fast.

The present study provides insights into our understanding of the chemical modulation of the circadian rhythm of PTH (1-84) and NcAMP. Although the higher setting of serum PTH (1-84) during the waking hours of the fasting study is probably a consequence of the reduction in the mean adjusted calcium concentration, the relative fall in PTH (1-84) during the period of sleep when fasting cannot be explained by changes in calcium status. These results are in accordance with the view that factors other than calcium contribute to the circadian rhythm of PTH (1-84) in normal subjects (130,132,133,159, Chapters 3.1, 3.2 and 3.4). Data from the previous key study (130) sustained the view that the PO_4 rhythm must be a consequence of the PTH (1-84) rhythm. The present study results suggest that the converse argument is at least as valid as the serum PO_4 rise occurs before the nocturnal PTH rise. Thus the role of phosphate as a PTH secretagogue in normal physiology must

be considered.

While the circadian rhythm of PTH (1-84) is lost on fasting the between and within subject variability of PTH (1-84) concentrations is increased. It is not possible with the sampling frequency used in this study (30 min intervals) to analyse directly the pulsatility of PTH (1-84) secretion since PTH (1-84) has a half life of 2-3 min. However, it is possible that the increase in variability observed represents alterations in the pulsatile nature of PTH (1-84) secretion. The increased variability in secretion of PTH (1-84) and the loss of the nocturnal rise may represent an adaptation to the fasting state which facilitates the mobilisation of calcium from bone. It has been calculated that approximately half the quantities of calcium and phosphate excreted in urine during a prolonged fast have been mobilised from bone (158).

The changes in signal mode for PTH (1-84) observed in this current study may offer a means of mediating bone resorption in normal and pathological states. It is known that trabecular bone formation may be stimulated by transient rises in serum PTH following daily injection (160) but that continuous infusions of PTH are ineffective or counterproductive (161). This knowledge has led to the postulate that the circadian rhythm of PTH (1-84), particularly the nocturnal rise then fall, is an essential element in the process of bone formation. Conversely,

abnormalities in the rhythm of PTH (1-84) secretion as seen in this current study and in hyperparathyroidism (Chapter 3.4) may lead to an alteration in the normal pattern of bone turnover and may contribute to the aetiology of bone disease including osteoporosis.

NcAMP production has been significantly altered following fasting and probably reflects the combined effects of loss of the PTH (1-84) circadian rhythm and the increased variability of PTH (1-84) secretion. Added to these changes are alterations in the mean circulating calcium, phosphate and possibly vitamin D which will have effects on the PTH receptor at the kidney. The overall result is that the kidney response to PTH (1-84) in terms of cAMP production, is significantly decreased following a fast.

As expected, mean glucagon concentrations following the fast are significantly elevated with no significant concerted decrease overnight observed although a transient decrease is observed between 2130 and 0230. The overall increase in glucagon reflects the alterations in glucagon metabolism that take place following a fast. It has been demonstrated that fasting for 3 days results in a significant increase in plasma glucagon as a result of decreased catabolism by the kidney rather than increased secretion by the pancreas (162). PcAMP is also increased over the 24h period with the development of a significant circadian rhythm and the retention of the nocturnal

decrease in PcAMP. Part of the increase in PcAMP will be due to the rise in glucagon but the overall increase in PcAMP will also be due to contributions of other hormone effects not measured in this study. Prolonged fasting will result in increases of GH, PRL and cortisol which will all contribute to the increased PcAMP. It is also possible that PTH (1-84) effects are different on bone and kidney resulting in increased contribution to PcAMP from bone cells but a decreased contribution from the kidney. These effects may be due to the acute ketosis that exists following a fast as altered PTH responses in acute acidosis have been observed in the kidney and bone. An increase in cAMP production by isolated perfused dog bone has been reported in acidosis (163) whilst decreased UcAMP excretion has been found in rats or dogs made acidotic (164,165).

This study has shown that a prolonged fast can markedly alter phosphate, calcium, PTH (1-84), NcAMP and glucagon rhythms. The results suggest that phosphate may play an important role in the genesis of the PTH (1-84) rhythm and subsequent NcAMP rhythm. Further work is required to investigate such mechanisms and the role other circadian hormones may play, such as GH and cortisol, in regulating calcium and phosphate homeostasis and the action of PTH (1-84) in generating cAMP.

3.4 Variation in The Circadian Rhythm of cAMP in Hyperparathyroid Patients Before and After Parathyroidectomy

3.4.1 Introduction

The demonstration of the significant elevation of NcAMP rising in parallel with PTH (1-84) in normal males (Chapter 3.1) and the ability to modify these profiles by manipulating sleep (Chapter 3.2) and diet (Chapter 3.3) raises questions about the role of these rhythms, if any, in health and disease. Patients with primary hyperparathyroidism (1°HPT) have inappropriately detectable or high serum concentrations of PTH (1-84) in the presence of hypercalcaemia (101,166,167) and an elevated NcAMP in the majority of cases (46,69,70). Some of the available evidence indicates that in 1°HPT 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. Thus we would expect that the circadian rhythm observed in normal subjects could be lost in 1°HPT. Studies measuring N and C terminal PTH have shown either the existence (131,159) or absence (168,130) of a circadian rhythm for PTH in primary hyperparathyroidism. If the circadian rhythms for PTH (1-84) and NcAMP are lost in 1°HPT then following removal of the adenoma there may be restoration or alteration of these circadian rhythms. In this study the data obtained from normal volunteers

(Chapter 3.1) and 2 additional subjects has been compared to that obtained in patients with 1°HPT before and after surgical removal of a parathyroid adenoma.

3.4.2 <u>Subjects and Methods</u>

An additional two normal male volunteers (aged 38 and 43) were sampled at the same time as the hyperparathyroid patients giving a total of eight normal healthy males (six of these as described in Chapter 3.1) and eight patients with 1°HPT studied. The patients with 1°HPT (age 32-79, mean 52 years, 4 male, 4 female) were all hypercalcaemic (serum adjusted Ca >2.6 mmol/L) with detectable or elevated PTH(1-84) (4.8-68.0 pmol/L) on more than one occasion and parathyroid adenomas were detected by technetium/thallium subtraction scan in all cases. Six 1°HPT patients had parathyroid adenomas (histological confirmation) removed surgically after neck exploration. Following operation all six patients became normocalcaemic and PTH(1-84) concentrations normalised. Patients with 1°HPT were sampled over a 24h period on two occasions, 1) at least 2 months prior to surgery and, 2) at least 6 months following surgery. During the study periods the patients were hospitalised, they ate hospital diet at identical times, were ambulant but avoided exercise and all lay down to sleep at an identical time (2300h). The standard sampling protocol was followed as described previously.

PTH (1-84), NcAMP and PcAMP values showed a wide scatter in patients with 1°HPT so, for clarity, the results were calculated for each subject as percentage difference from their 24h mean value. Statistical analysis was performed using paired difference t-test to analyse significant changes within 24h profiles. Mann-Whitney U-test was used to analyse differences between groups. Circadian rhythm parameters were calculated using the cosinor technique.

3.4.3 Results

Figures 11, 12, 13, 14 and 15 show the 24h profiles for PTH (1-84), NcAMP, (each expressed as percentage of the individual 24h mean), PcAMP, adjusted serum Ca and serum PO_4 for normal subjects, 1°HPT patients pre and post surgery respectively. The PTH (1-84) and NcAMP values throughout the 24h periods in the three groups are summarised in Table 5. Cosinor analysis of the data for the five measurements in each group studied are shown Table 6.

In comparison to the normal subjects the circadian rhythm in both PTH (1-84) and NcAMP is lost completely in patients with 1°HPT with no synchronised rise in PTH (1-84) or NcAMP observed at any time during the 24h in these patients.

In normal subjects the greatest variability in PTH (1-84) occurs between 0630-1000h. In patients with 1°HPT, concentrations of PTH (1-84) occasionally fell within the reference range during the 24h period and these results coincided with normal values for adjusted Ca. In two

Figure 11   PTH (1-84) in normals, and 1°HPT patients pre
            and post parathyroidectomy. Results are
            expressed as % of 24 h mean ± SEM of each value.
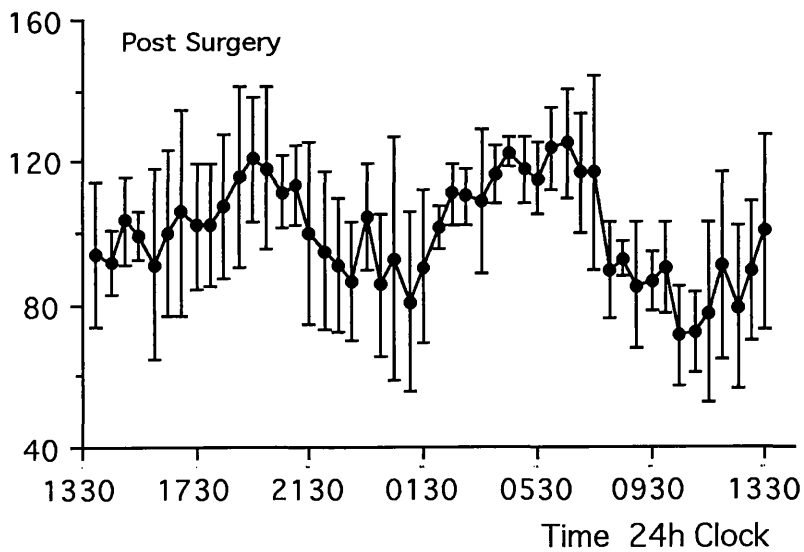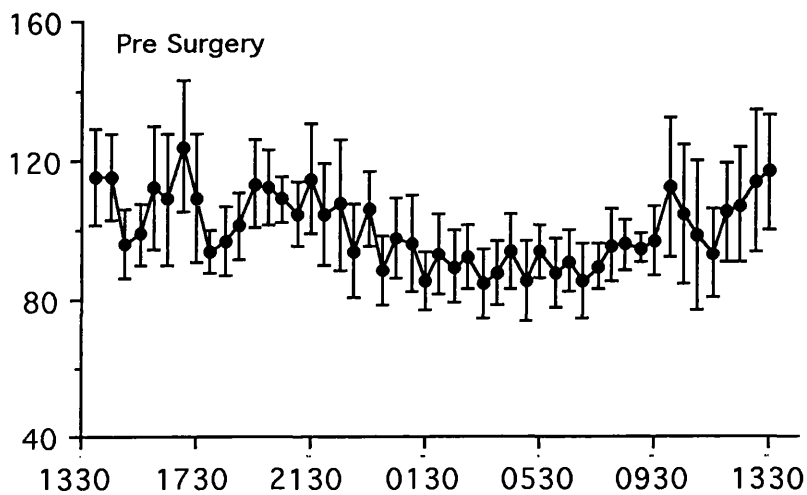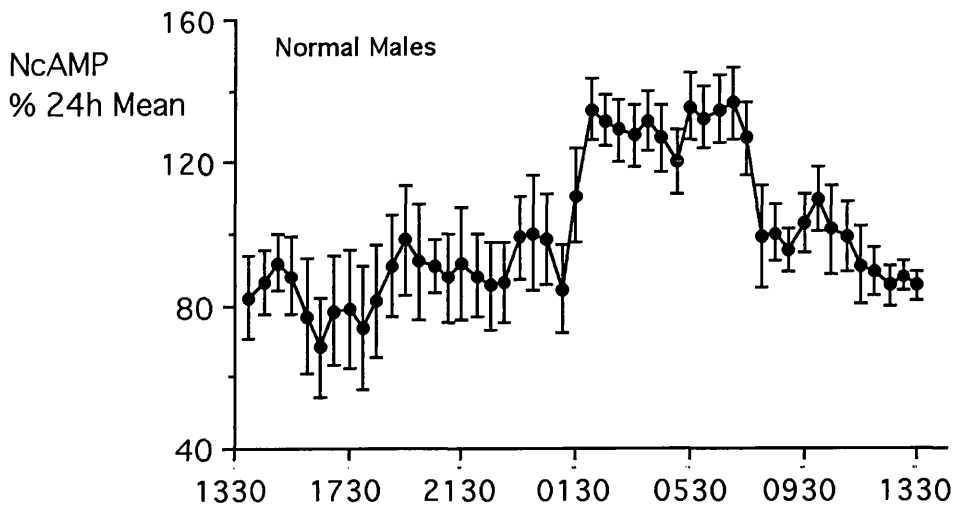
Figure 12   NcAMP in normals, and 1°HPT patients pre and
post parathyroidectomy. Results are expressed
as % of 24 h mean ± SEM of each value.

		Normal males 24h Mean	Range	CV		1°HPT Pre Surgery 24h Mean	Range	CV		1°HPT Post Surgery 24h Mean	Range	CV
PTH (1-84)	A	2.3	1.1-4.5	36.7	1	13.8	11.2-17.2	11.1	1	2.5	1.4-3.6	21.2
(1-5 pmol/L)	B	3.9	1.6-6.5	25.1	2	7.2	4.7-11.9	20.4	2	2.5	1.0-3.7	28.9
	C	4.6	2.1-7.6	32.2	3	20.8	12.6-25.8	10.9	3	3.9	2.1-6.0	25.0
	D	3.2	1.4-5.6	35.2	4	24.7	16.0-33.0	14.4	4	3.1	1.1-5.2	31.8
	E	2.6	0.9-4.2	30.6	5	13.1	4.0-20.1	19.2	5	3.0	1.8-4.3	20.2
	F	3.2	2.0-4.2	14.8	6	37.3	22.2-68.4	29.1	6	5.6	2.5-8.1	24.7
	G	2.7	1.6-3.8	23.4	7	19.5	16.7-26.0	10.8				
	H	3.8	1.8-5.1	21.6	8	20.3	15.4-27.4	13.6				
NcAMP	A	23.0	4.9-36.9	37.5	1	41.6	17.3-72.9	39.0	1	9.9	2.8-21.0	40.6
(8-28 nmol/L)	B	19.4	6.7-32.3	32.0	2	37.7	13.8-74.4	43.8	2	20.3	5.3-31.4	25.2
	C	33.4	14.2-56.7	38.9	3	99.9	62.4-126.3	13.4	3	17.6	10.5-26.0	23.9
	D	27.0	10.6-40.6	31.4	4	73.9	42.6-101.4	17.6	4	22.8	6.6-44.2	33.9
	E	13.9	4.5-22.7	32.9	5	37.6	18.2-96.6	39.7	5	15.2	8.4-21.3	21.2
	F	31.5	20.6-39.7	13.3	6	90.0	42.2-169.6	37.3	6	17.5	2.8-35.2	37.3
	G	36.7	18.8-55.9	25.5	7	62.8	47.9-78.6	11.7				
	H	21.2	10.0-35.1	31.3	8	111.8	74.4-172.8	17.9				

Table 5   PTH (1-84) and NcAMP individual results in normal males and 1°HPT patients pre and post parathyroidectomy

patients with 1°HPT values for NcAMP within the reference range were obtained intermittently throughout the 24h. PcAMP was significantly higher in 1°HPT patients throughout the 24h (p<0.001). There was no synchronised rhythm present in 1°HPT and no prolonged trough in PcAMP overnight although a decrease in PcAMP was observed on recumbency and an increase on rising from sleep in the morning. Following surgery PcAMP varied in a similar manner to the normal subjects with the lowest PcAMP concentrations observed early morning. Variability in PcAMP was greater in patients with 1°HPT. Analysis of the data in 1°HPT patients indicates that there may be 6 regular peaks of PcAMP in a 24h period if a simple peak counting analysis technique is applied to the data. A similar but less obvious set of peaks can also be recognised in the normal subjects. It is not immediately obvious from this data what is stimulating this pattern of secretion and why these peaks are bigger in 1°HPT.

Adjusted calcium varied very little during the 24h in both groups. A transient, statistically significant decrease (p<0.05) in mean adjusted calcium from 2.44 to 2.40 mmol/L at 0130h in normals, and from 2.94 to 2.84 mmol/L at midnight in patients with 1°HPT was observed when compared with the group mean concentration between 1400-1800h. The adjusted calcium mean concentration in the patient group was predictably higher pre treatment (p<0.001) and was also significantly lower post treatment (p<0.001) than in the
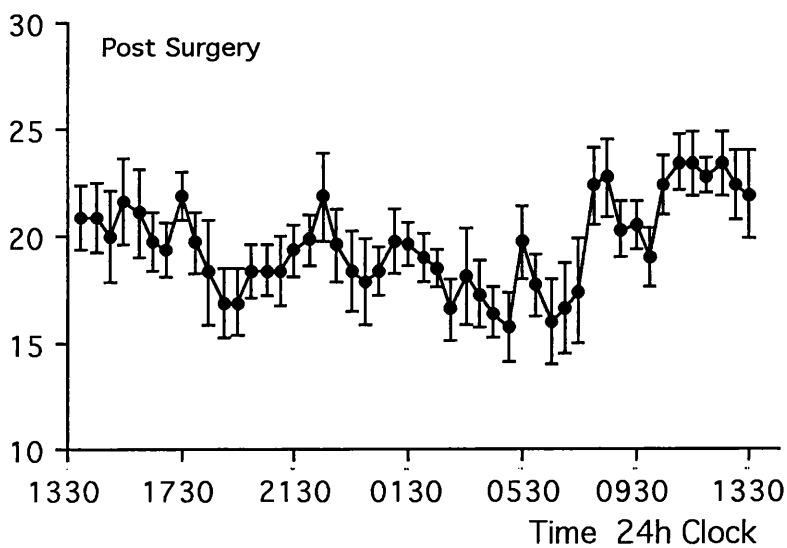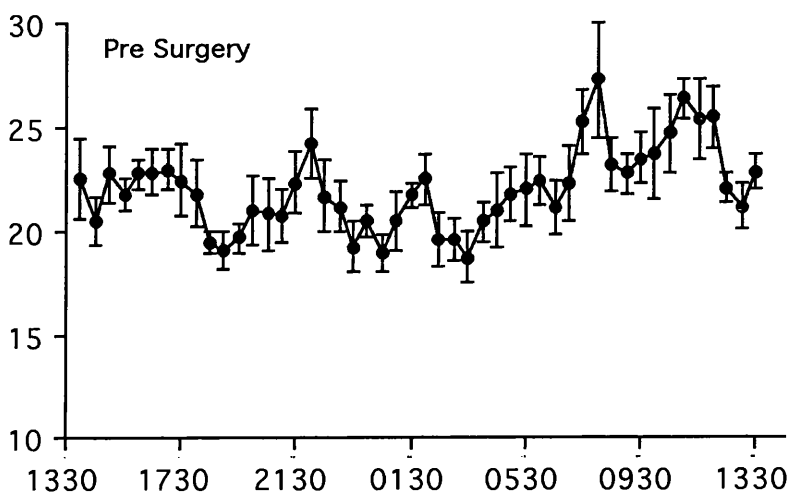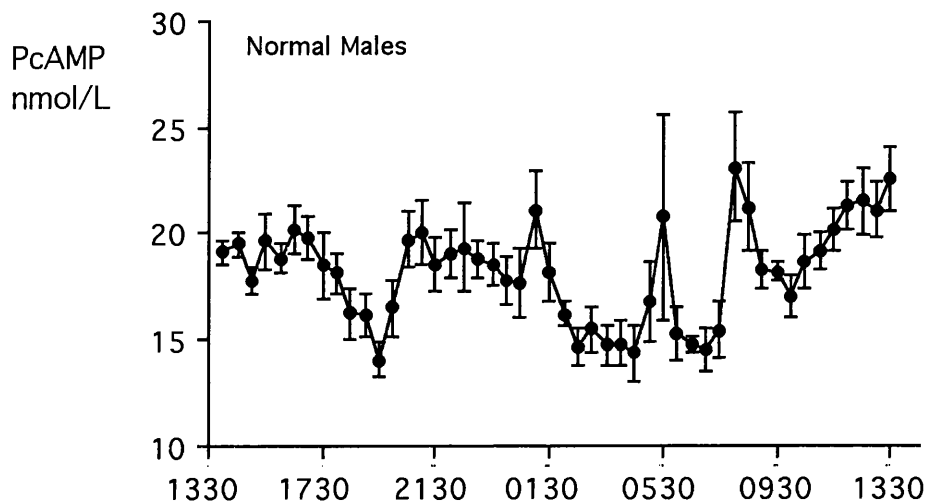
Figure 13   PcAMP in normals, and 1°HPT patients pre and
             post parathyroidectomy. Results are expressed
             as mean ± SEM.
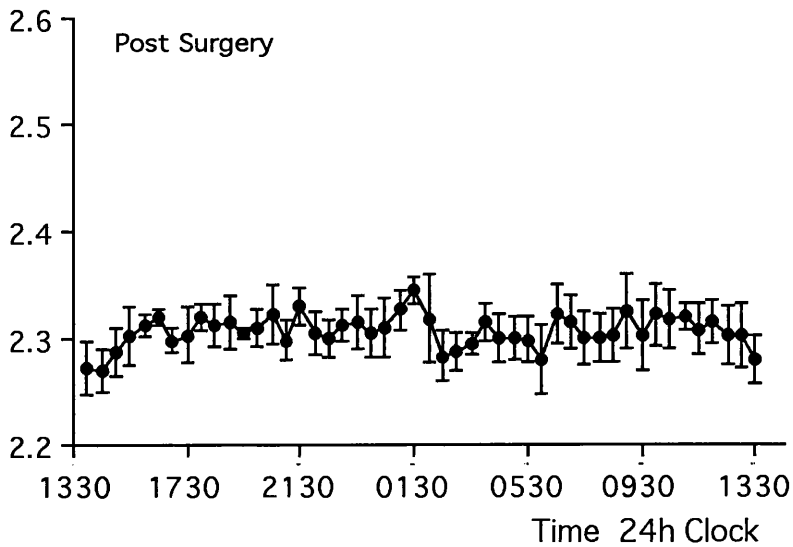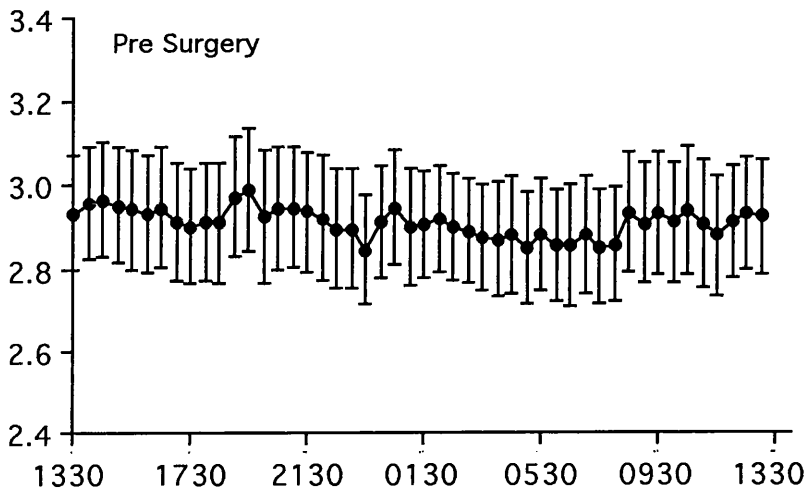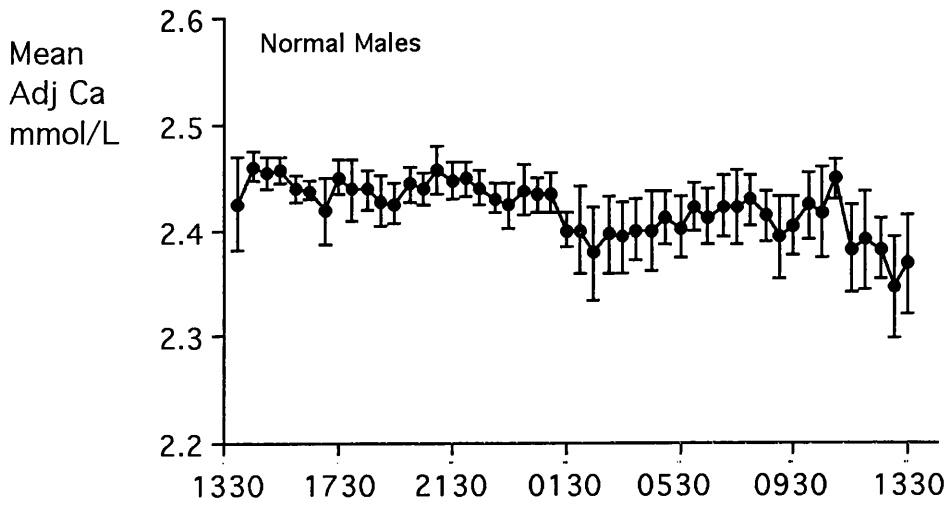
Figure 14   Adjusted calcium in normals, and 1°HPT patients
            pre and post parathyroidectomy.   Results are
            expressed as mean ± SEM.

normal subject group throughout the 24h period.

Serum PO_4 was significantly lower in the 1°HPT patients pre treatment throughout the 24h (p<0.001). A marked synchronized rhythm in phosphate was observed in all groups with a transient decrease following the evening meal, which was the main meal of the day, a significant nocturnal rise and early morning fall being seen in all groups. The percentage change in phosphate during the 24h was greater in normal subjects and post treatment than in those with 1°HPT pre treatment.

3.4.4 Discussion

This study has shown that the circadian rhythm for PTH (1-84) and NcAMP which is present in normal males is not present in patients with 1°HPT but can be restored by surgical removal of the parathyroid adenoma. Although the profile of PTH (1-84) and NcAMP secretion is different in patients post parathyroidectomy compared to normal males a statistically significant circadian rhythm is restored following operation. The subtle differences in the shape of the profiles may reflect age and sex differences between the groups not just the presence or absence of a single pathology. Biological activity of PTH (1-84) has also been restored to normal post operation as judged by NcAMP production and PcAMP profiles although examination of individual profiles detects two patients whose biological response remains blunted. In these two patients cosinor
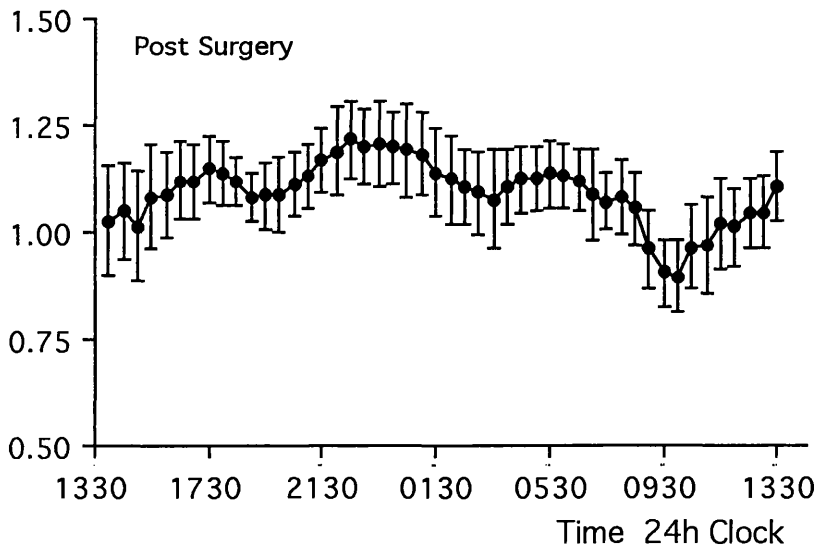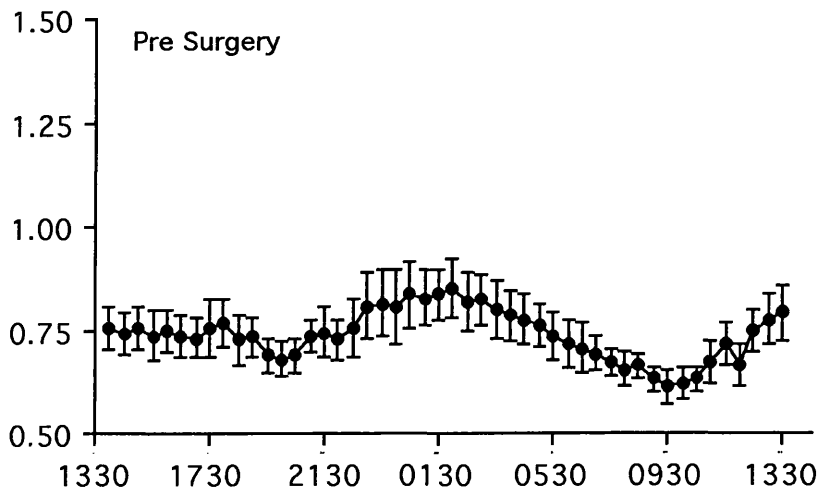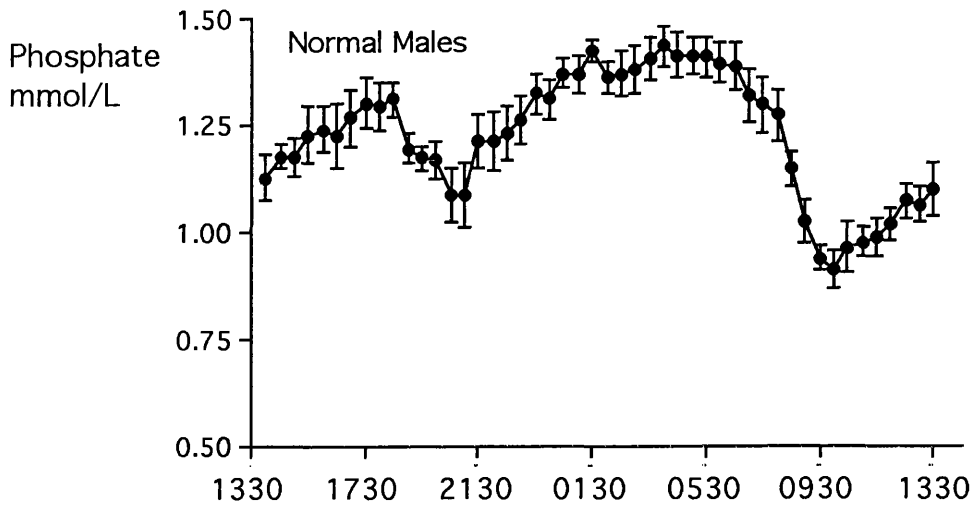
Figure 15   Serum phosphate in normals, and 1°HPT patients
            pre and post parathyroidectomy. Results are
            expressed as mean ± SEM.

	Acrophase 24h Clock	Mesor	Amplitude	Significance
Normal Subjects				
PTH (1-84)	0412	3.2 pmol/L	0.7 pmol/L	P<0.02
NcAMP	0514	25.7 nmol/L GF	6.1 nmol/L GF	P<0.05
Adj Ca	2010	2.4 mmol/L	0.02 mmol/L	NS
PO_4	0156	1.4 mmol/L	0.16 mmol/L	P<0.001
1°HPT Pre surgery				
PTH (1-84)	2339	14.2 pmol/L	0.80 pmol/L	NS
NcAMP	1804	58.0 nmol/L GF	5.6 nmol/L GF	NS
Adj Ca	1613	2.9 mmol/L	0.03 mmol/L	NS
PO_4	0116	0.8 mmol/L	0.07 mmol/L	NS
1°HPT Post surgery				
PTH (1-84)	0326	3.1 pmol/L	0.7 pmol/L	P<0.01
NcAMP	0359	17.9 nmol/L GF	1.9 nmol/L GF	NS
Adj Ca	0225	2.3 mmol/L	0.00 mmol/L	NS
PO_4	2357	1.1 mmol/L	0.09 mmol/L	P<0.01

NS - Not Significant

Table 6 Cosinor analysis of 24 h profiles of PTH (1-84), NcAMP, adjusted Ca and serum PO_4 in normal subjects and 1°HPT patients pre and post parathyroidectomy

analysis of individual rhythms indicates a low mesor and non significant amplitude for both PTH (1-84) and NcAMP. It is possible that this is a refection of both the length of time these patients were hyperparathyroid and the time from operative removal of the adenoma.

These observations raise important questions for the control of PTH (1-84) secretion in normal and abnormal states and the subsequent action of PTH (1-84) at its receptor. Previous studies have demonstrated a fall in ionised calcium during the night in both normal subjects and patients with 1°HPT (159). In this study we have also shown a transient but significant lowering of adjusted Ca in all groups between midnight and 0200h. The aetiology of such a transient fall in calcium remains unclear. A reduction in ionised calcium might be expected to trigger a compensatory surge in PTH (1-84) secretion and therefore provide the mechanism of the nocturnal rise in PTH (1-84) in normal subjects and the subsequent increase in NcAMP. However, this explanation appears to be an over simplification, 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 (130). Predictably, infusion of calcium at higher doses leads to decreased nocturnal PTH secretion (130,131). Methodological problems also exist with the measurement of ionised calcium that make interpretation of previous data difficult as protein

effects may be contributing to changes in measured ionised calcium (169). Clearly, therefore, factors other than changes in ionised calcium concentration are important in generating 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 (170,171). It has been suggested that the basis of 1°HPT is an alteration in the calcium-PTH set point rather than autonomous secretion of PTH by the parathyroid gland (172,173,174). In this study the absence of a synchronised nocturnal increase in PTH (1-84) in hyperparathyroid patients pre-op has been shown, despite the detection of a transient lowering in adjusted calcium consistent with the previous data on ionised calcium (159). Although the precise incremental fall in serum calcium required to produce a response in hyperparathyroidism has not been defined in these patients, this is further evidence against the fall in calcium being the sole 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 and 1°HPT post parathyroidectomy. 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 small decrease is followed by a much larger increase in phosphate prior to the nocturnal surge of PTH (1-84). This increase in phosphate could be regarded as the stimulus for PTH release, which in turn would cause phosphaturia and the observed subsequent fall in serum phosphate. Abolition of the phosphate rise by fasting, until recently (Chapter 3.3), was believed to have no effect on PTH secretion with claims that the nocturnal surge persists after a short fast (130). Furthermore, infusion of calcium in an appropriate dose results in suppression of PTH secretion and an exaggerated phosphate circadian rhythm (130,131). Thus it would appear that phosphate has little or no influence on secretion of PTH throughout the night, but that the PTH status of the individual may well influence the extent of the phosphate circadian rhythm. Further support for this conclusion is obtained from the phosphate results in the hyperparathyroid patients in this current 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. Thus, using PTH (1-84) as the discriminant, the optimal time for sampling is 1000-1600h when the variability within and between normal subjects is at its minimum. Similarly, this

data suggests that the optimal time for sampling using NcAMP as the discriminant is between 1100-1500h and not early in the morning as recommended by previous authors (46,175).

The profile of PcAMP excretion is remarkably consistent between the three groups studied. 1°HPT patients have a significantly increased mean PcAMP throughout the 24h period presumably reflecting increased PTH (1-84) concentrations. It would appear that hormones making the major contribution to the daily variations in PcAMP, and hence the pattern of PcAMP secretion, are relatively unaffected by the development of 1°HPT.

Further study is required to establish whether the loss of the circadian rhythm of PTH (1-84) and NcAMP in 1°HPT is an early event in the natural history of the disease or whether it is a secondary consequence of increases in the plasma calcium beyond critical threshold concentrations. It will also be important to discover whether or not restoring the circadian rhythms of PTH (1-84) and NcAMP in patients post parathyroidectomy has any significance for the metabolic bone disease observed in patients with 1°HPT.

Combining all the currently 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) and parallel changes in

NcAMP. A component of neuroendocrine control may exist and concerted effects of hormones are probably required to control the end organ responses and cAMP generation.

Chapter 4

The Effects of Thyroid Status on cAMP Metabolism

4.1.1 <u>Introduction</u>

Alterations in calcium and skeletal metabolism are well recognised in patients with thyroid disease. Prolonged hyperthyroidism is associated with negative calcium balance, increased bone turnover, reduced bone density, decalcification and fractures (176,177,178,179,180, 181). Hypothyroidism is characterised by decreased rates of skeletal turnover (177,182,183) and treatment of hypothyroidism results in a rapid decrease in bone density in some patients (184). The precise mechanisms for these changes are still poorly understood and conflicting evidence exists on the concentrations of immunoreactive parathyroid hormone (iPTH) present and cAMP metabolism in thyroid disease. In hyperthyroidism some studies have demonstrated suppressed iPTH (180,185,186,187), others have observed no change in iPTH concentration (181,188,189) and more recently no change in intact PTH (190). Several groups have reported an increased excretion of UcAMP in hyperthyroidism (28,191,192) and an elevated PcAMP (28,189,193). An elevated UcAMP contrasts with suppressed iPTH since UcAMP is mainly dependent on PTH activity (45,54,61). However, as discussed earlier, it is more correct to determine NcAMP as an index of PTH action at the kidney (67,46) and a study measuring NcAMP has found lower concentrations in patients with hyperthyroidism (189).

Some authors have argued that alterations in thyroid status modify the end organ responses to PTH with claims that

excess thyroid hormone sensitizes and deficient thyroid hormone blunts the responsiveness of bone to PTH (187) and that hypothyroidism impairs the renal response to PTH (147).

Comparison of previous studies is complicated by the fact that the immunoassays used to measure PTH have been relatively insensitive and of variable specificity. The recent development of immunoradiometric assays (100,101,129) measuring intact PTH (PTH 1-84) has improved clinical discrimination in diseases of calcium metabolism (101,156) and has enabled small changes in PTH (1-84) concentration to be demonstrated even within the reference interval (Chapter 3, 134). In this chapter the relationship between serum PTH (1-84) concentrations and indices of PTH activity in thyrotoxic, hypothyroid, treated hypothyroid patients and euthyroid controls has been examined.

4.1.2 <u>Patients and Methods</u>

All patients were attending a thyroid outpatient clinic. In total 35 newly diagnosed hyperthyroid, 20 newly diagnosed hypothyroid, 35 treated hypothyroid patients and 30 euthyroid volunteers were studied. Hyperthyroidism, hypothyroidism or euthyroidism was diagnosed following clinical examination, estimation of total thyroxine (TT_4), total triiodothyronine (TT_3), thyroid stimulating hormone (TSH), thyroid antibodies and, where appropriate, thyroid

scan. Hypothyroid patients all received treatment with thyroxine following diagnosis. All treated hypothyroid patients had received thyroxine replacement therapy for a minimum of 3 months and were considered to be clinically euthyroid with thyroid function tests within the reference ranges for a patient on thyroxine (194). In this study measurements of TT_4, TT_3, TSH, PTH(1-84), PcAMP, UcAMP, serum albumin, serum and urine calcium, phosphate and creatinine were performed on appropriate samples as outlined Chapter 2. NcAMP was calculated by the method of Broadus *et al* (46) and serum calcium adjusted for albumin as described previously (106). Statistical analysis was performed using a Kruskal Wallis (ANOVA) test to detect significance for each measurement for the four study groups prior to applying the Mann-Whitney U-test to compare between group significance.

4.1.3 Results

The thyroid function test results for each category of subject studied are given in Table 7. These results confirm biochemical hyperthyroidism, hypothyroidism and euthyroidism. The expected increased mean TT_4 and decreased TSH is observed in patients receiving thyroxine replacement therapy (194).

PTH (1-84) concentrations and NcAMP are shown for comparison in the scattergrams (Figure 16). As can be seen PTH (1-84) was undetectable (<0.5 pmol/L) in 10/35 patients

(Reference range)	Euthyroid N = 30	Thyrotoxic N = 35	Hypothyroid N = 20	T$_4$-treated N = 35
Total T$_4$ (55-144) nmol/L	92.5(16.3)	236.4(52.8)	41.3(12.6)	140.6(28.4)
Free T$_4$ (8-18) pmol/L	12.8(1.2)	36.8(5.3)	5.4(1.8)	18.1(3.7)
Total T$_3$ (0.9-2.8) nmol/L	1.91(0.23)	5.30(2.21)	1.40(0.42)	1.94(0.24)
TSH (0.35-5.0) mU/L	1.90(0.76)	<0.10	41.80(28.30)	0.95(1.30)

Figures are mean (SD)

Table 7   Thyroid function tests in euthyroid, thyrotoxic, hypothyroid and thyroxine treated hypothyroid subjects
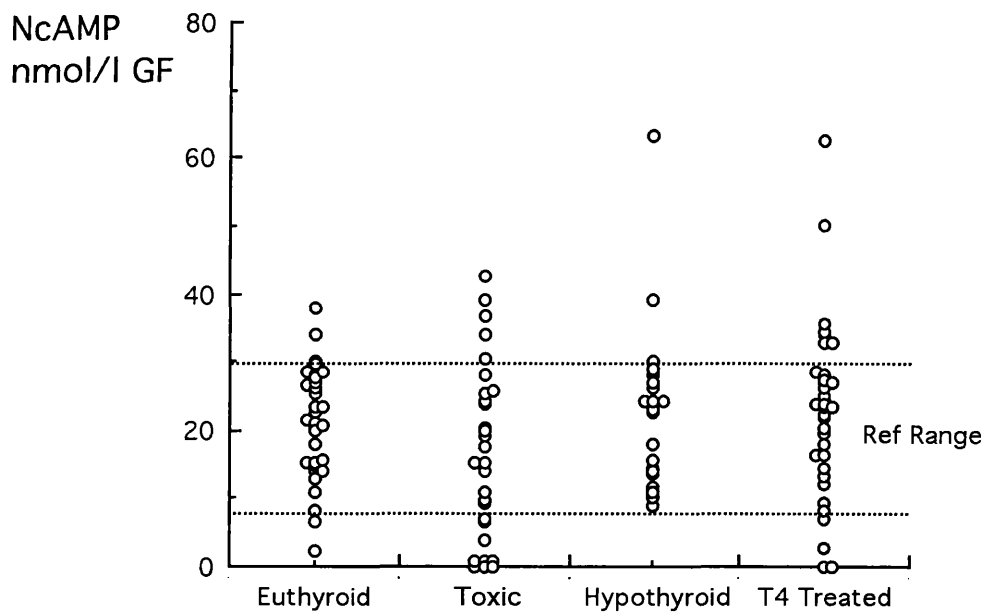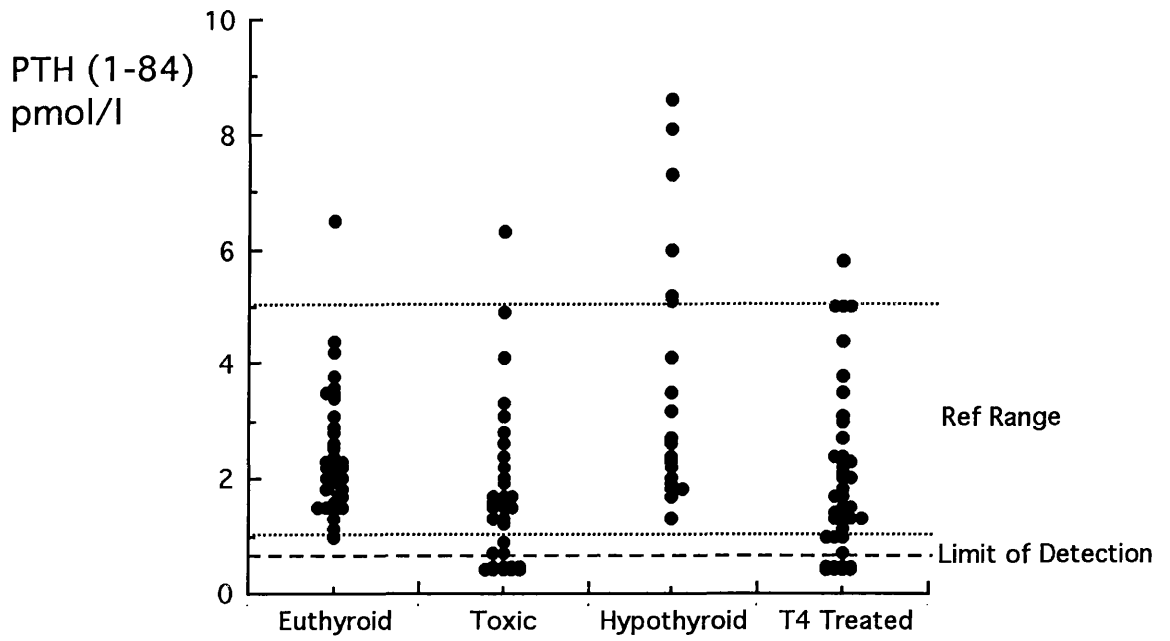
Figure 16   PTH (1-84) and NcAMP in euthyroid, thyrotoxic,
            hypothyroid and thyroxine treated hypothyroid
            subjects.

with hyperthyroidism and in 8/30 treated hypothyroid patients for the purposes of statistical comparison patients with undetectable PTH (1-84) have been assigned a value of 0.5 pmol/L. PTH (1-84) is elevated in 6/20 patients with hypothyroidism. In contrast NcAMP is undetectable in only 6/35 hyperthyroid patients and in only 2/35 treated hypothyroid patients. In hypothyroid patients NcAMP is lower than would be predicted by the PTH (1-84) concentrations with only 2/20 patients having elevated NcAMP.

Table 8 shows the values obtained for adjusted Ca, calcium/creatinine ratio, PcAMP and UcAMP in the subjects studied. Significant differences for these measurements are given for comparison between thyrotoxic, hypothyroid, treated hypothyroid patients or euthyroid controls. Hypercalcaemia was present in 8 patients with thyrotoxicosis. PTH (1-84) was undetectable in these 8 patients. All treated hypothyroid patients were normocalcaemic.

4.1.4 <u>Discussion</u>

Modern assays for serum PTH (1-84) overcome the sensitivity problems experienced in earlier studies of PTH in thyroid disease. It is clear from these results that mean intact PTH concentrations are decreased in thyrotoxicosis and increased in hypothyroidism. In thyrotoxic patients the relative decrease in PTH (1-84) would appear to be a

(Reference ranges)	Euthyroid N = 30	Thyrotoxic N = 35	Hypothyroid N = 20	T_4-treated N = 35	Significant differences (Mann-Whitney comparison)
Adjusted Ca (2.2-2.6) mmol/L	2.40 (0.09)	2.50 (0.20)	2.39 (0.07)	2.41 (0.08)	$P<0.05$ Thyrotoxic vs euthyroid Thyrotoxic vs T_4-treated
Ca/Cr (<0.5)	0.40 (0.16)	0.62 (0.37)	0.32 (0.12)	0.52 (0.34)	$P<0.01$ Hypothyroid vs T_4-treated $P<0.001$ Thyrotoxic vs hypothyroid
PO$_4$ (0.8-1.4) mmol/L	1.05 (0.12)	1.06 (0.26)	1.02 (0.14)	1.10 (0.18)	NS
TmPO$_4$ (0.8-1.35) mmol/L GF	0.95 (0.27)	1.10 (0.34)	0.92 (0.31)	1.06 (0.28)	NS

Plasma cAMP
(15-30)
nmol/L
	19.0 (5.5)	28.7 (8.3)	16.8 (3.9)	23.2 (4.8)	$P<0.05$ Euthyroid vs T_4-treated
					         Thyrotoxic vs euthyroid
					$P<0.001$ Thyrotoxic vs hypothyroid
					         Thyrotoxic vs T_4-treated
					         Hypothyroid vs T_4-treated

Urinary cAMP (26-66) nmol/L GF	43.8 (11.2)	48.4 (14.7)	42.6 (13.6)	48.8 (14.8)	NS

Values are mean (SD)

NS - No significance

Table 8   <u>Adjusted Ca, urine Ca/Cr ratio, serum PO$_4$, TmPO$_4$, PcAMP and UcAMP in euthyroid,
thyrotoxic, hypothyroid and thyroxine treated hypothyroid subjects</u>

physiological response to an increase in mean serum Adj Ca. 8 thyrotoxic patients with PTH (1-84) values below the detection limit were hypercalcaemic (Adj Ca >2.6 mmol/L) and the mean Adj Ca level was increased relative to controls in thyrotoxicosis. This increase in serum calcium is probably due to Ca release from bone as a result of direct effects of endogenous thyroid hormones on skeletal metabolism. PTH (1-84) is the active circulating form of PTH and in thyrotoxic patients indices of PTH activity are consistent with decreased serum PTH (1-84) concentrations: NcAMP is decreased, $TmPO_4$/GFR is elevated and Ca/Cr ratio is elevated.

In hypothyroid and treated hypothyroid patients the correlation between PTH (1-84) and indices of PTH activity is weaker. Hypothyroid patients have relatively higher PTH (1-84) levels than euthyroid controls and this is associated with normocalcaemia. NcAMP is lower than expected for the observed PTH concentrations, $TmPO_4$ is higher and Ca/Cr ratios lower than normal. These indicate that there is relative end organ resistance to PTH (1-84) in hypothyroid patients.

Impaired responses to exogenous PTH have been demonstrated in hypothyroid patients (187,147) and the reasons for this may be due to the effects of thyroid hormone status on adenyl cyclase (149,195) and cAMP phosphodiesterase activity (148,149,196). Hypothyroid animals have decreased

adenyl cyclase with either increased or normal phosphodiesterase activity which results in decreased cAMP production. It is likely that cAMP production is affected similarly in all cells and this would explain the increased PTH (1-84) observed in hypothyroid patients but absence of hyperparathyroid symptoms.

Hypothyroid patients on thyroxine have a mean PTH (1-84) concentration that is lower than normals. This is associated with a relatively increased NcAMP, a lower $TmPO_4$, a non statistically significant higher mean Ca/Cr ratio and normocalcaemia. Thyroxine therapy has been associated with reduced bone density (184,197) and it has been suggested that this may be due to either increased sensitivity to PTH or a direct effect of exogenous thyroid hormones (198). These results would indicate that at the kidney, although PTH (1-84) is lower than normal, cAMP production is increased. This may be due to an increase in adenylate cyclase activity and a decrease in cAMP phosphodiesterase activity, as has been observed when hypothyroid rats were treated with thyroxine (148). The cAMP responses observed are therefore likely to be secondary to thyroxine therapy and the decreased PTH (1-84) concentrations a consequence of thyroxine action on skeletal metabolism. The combination of relative PTH suppression and thyroxine induced bone resorption resulting in osteopenia.

These observations have demonstrated the significant changes in intact PTH concentrations that occur in thyroid disease and when taken in conjunction with indices of PTH activity help to explain the mechanisms involved in calcium metabolism in these patients. Treated hypothyroid patients have subtle differences from euthyroid controls that may begin to explain why these patients have a relatively reduced bone density. Whether these differences are the result of over replacement with thyroid hormones or simply a consequence of the differences between endogenous thyroid hormone production and exogenous thyroxine therapy will require further investigation.

Chapter 5

Adrenocorticotrophic and Glucocorticoid

Effects on Plasma and Urine cAMP

5.1   Adrenocorticotrophin Effects on Plasma cAMP

5.1.1 Introduction

The list of hormones whose effects are mediated through cAMP or can modify cAMP mediated events continues to increase. Most of the data on cAMP production has been obtained by studying *in-vitro* systems and the knowledge on *in-vivo* effects of many hormones is minimal. This is particularly true of the effects of glucocorticoids and those hormones involved in the hypothalamic-pituitary-adrenal axis.

The synthesis of steroid hormones involves up to five different steroid hydroxylases belonging to the cytochrome P450 superfamily (199) and the rate limiting reaction is catalysed by an enzyme complex comprising adrenodoxin reductase, adrenodoxin and cholesterol side-chain cleavage cytochrome P450 ($P450_{scc}$). Synthesis of $P450_{scc}$ is induced by adrenocorticotropin (ACTH) through the mediation of cAMP (200). Evidence for the role of cAMP in steroidogenesis has accumulated over many years. Early observations on the effects of exogenous cAMP (201,202), then ACTH action on adrenal quarters (203) and perfusion of adrenal cells (204) all suggest an important role for cAMP in the control of adrenal steroid production. Most recent data suggests that cAMP activates cAMP-dependent protein kinases and these are capable of activating pathways linked to the stimulation of steroidogenesis (205,206,207,208).

Bovine adrenocortical cells in primary culture respond to ACTH and adrenaline stimulation by increasing intracellular cAMP and releasing cAMP into their culture medium (209). The rate of release of cAMP from ACTH stimulated adrenal cells is slower (204) than that observed from PTH stimulated renal cells (55) which suggests that a time delay in cAMP production may exist in adrenal cells or that the rate of release via the cell membrane is slower in such cells. It is conceivable that circulating cAMP in plasma may be affected by ACTH secretion and that supra physiological doses, such as those used during a synacthen test, may cause an increase in circulating cAMP. Initially in this chapter cAMP production in normal subjects in response to ACTH administration has been studied.

5.1.2 Subjects and Methods

23 healthy volunteers (age 18-59, mean 32, 14 male, 9 female) were studied after an overnight fast. A venflon cannula 18G was inserted in a vein in the ante cubital fossa at 0900h. All subjects remained seated throughout the test and consumed no food or drink during the test. At 1000h a baseline blood sample (10 ml) was taken prior to intra-venous injection of 0.25 mg Tetracosactrin (Synacthen) in 5 ml sterile water via the cannula. Further blood samples were obtained at 2.5, 5, 10, 15, 30, 60 min following synacthen injection. 8 of the above volunteers were sampled on a second occasion after insertion of a venflon cannula in the median cubital fossa with the

cannula flushed with saline after each sample. Plasma
cortisol, cAMP and PTH (1-84) were measured as described
previously. Statistical analysis was performed using
paired t-test within each study and Mann-Whitney U test
between studies.

5.1.3 Results

Figures 17 and 18 show the cortisol, PcAMP and PTH (1-84)
response in the subjects studied during the control period
and following administration of synacthen (mean ± SD). The
time profile for the increase in both plasma cortisol and
cAMP are similar for Synacthen with a significant elevation
of these analytes occurring 5 min after Synacthen
injection. Cortisol and PcAMP continue to increase over
the 60 min following Synacthen injection. The magnitude of
the responses are different with the plasma cortisol
doubling after 30 min and PcAMP increasing by approximately
30% over the same period of time. There is no significant
change in cortisol or PcAMP in the control study and no
significant differences in PTH (1-84) in either study
period. Individual cortisol and PcAMP responses following
Synacthen are shown Figure 19 which demonstrate the
consistency in response in all the subjects studied.

5.1.4 Discussion

A highly consistent response to Synacthen is observed in
all subjects studied with a remarkable similarity in the
timing of the profiles for both cortisol and PcAMP. It
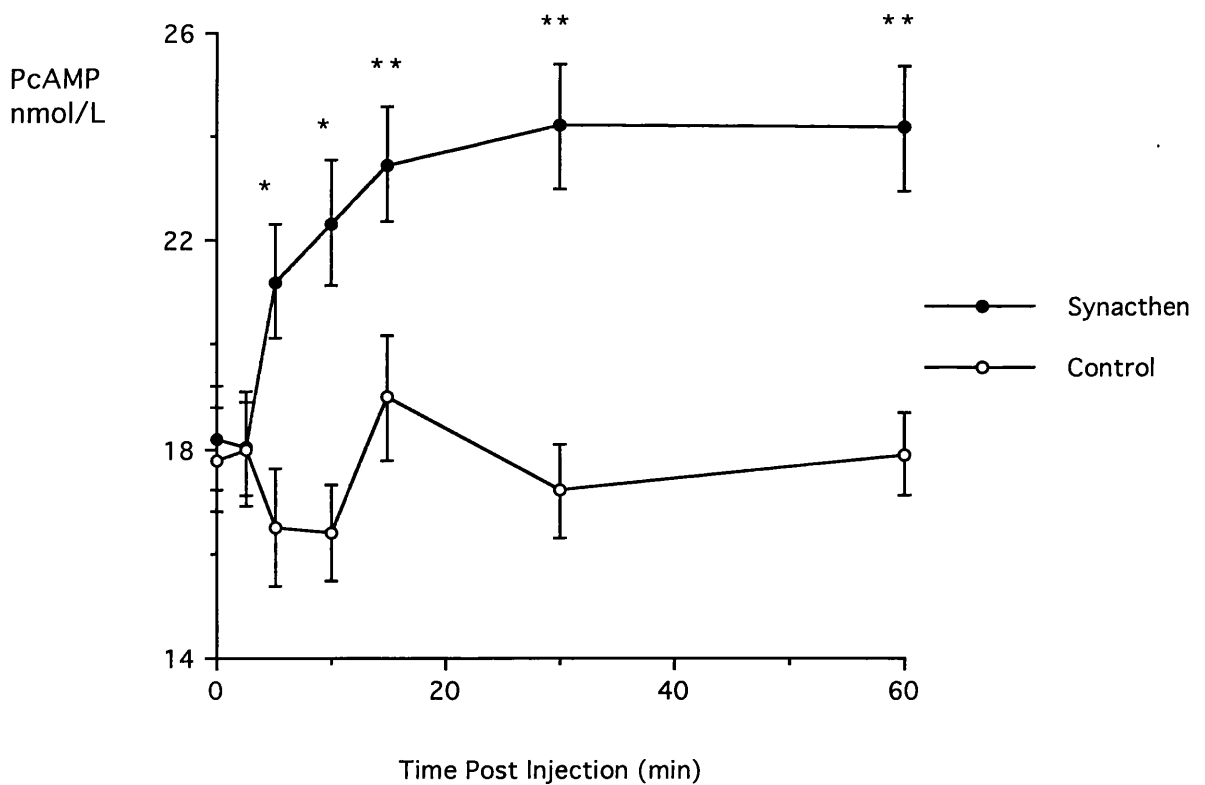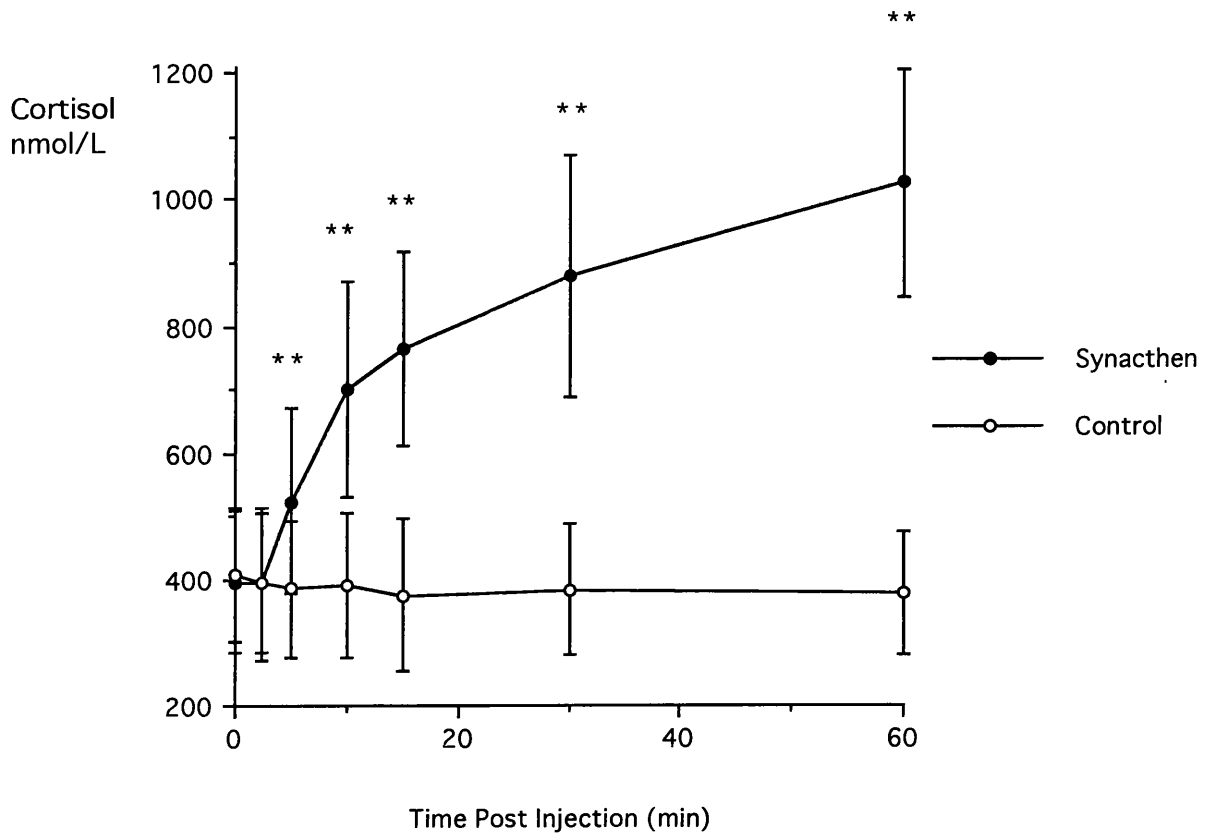
Figure 17   Cortisol and PcAMP response to 0.25 mg synacthen
             in normal subjects.
             Significance compared to baseline   * p < 0.01,
             ** p < 0.001.
             Results are expressed as mean ± SEM.
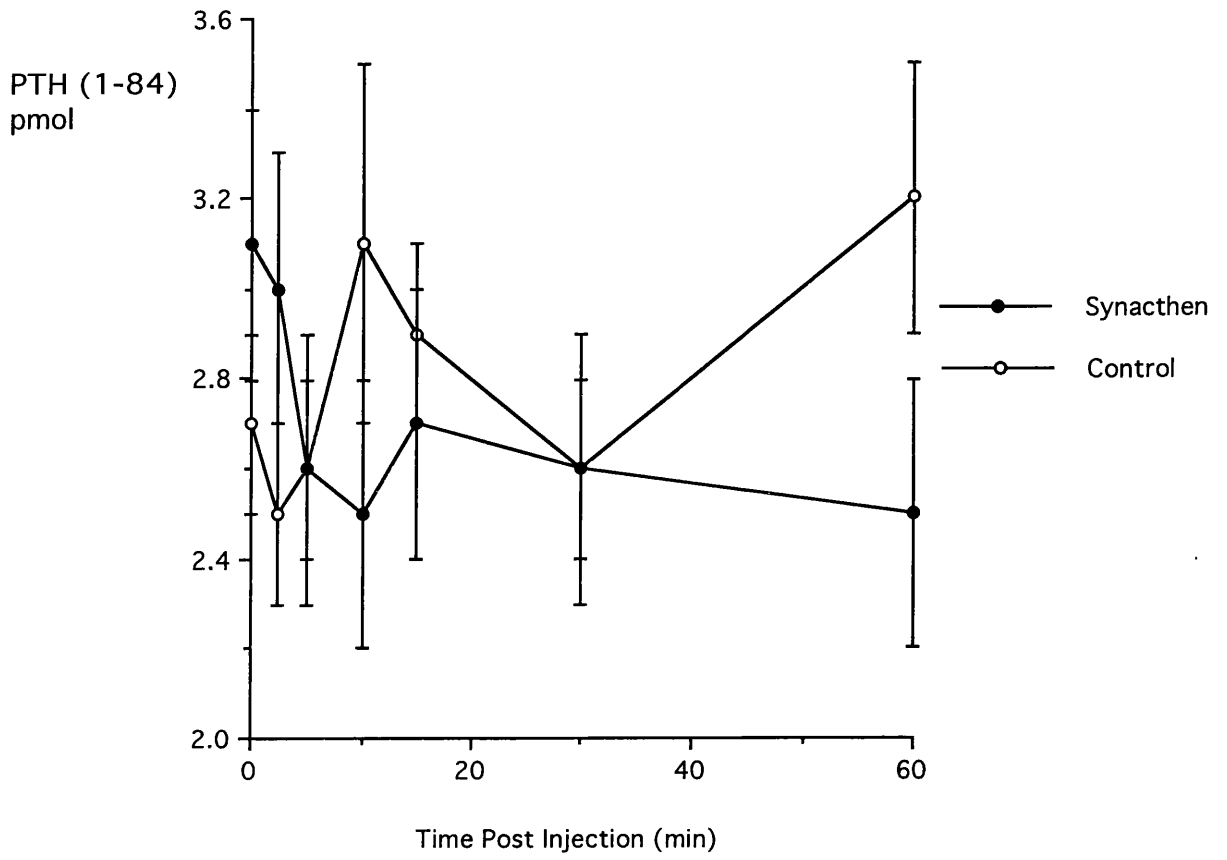
Figure 18   PTH (1-84) response to 0.25 mg synacthen in
            normal subjects.
            Results are expressed as mean ± SEM.
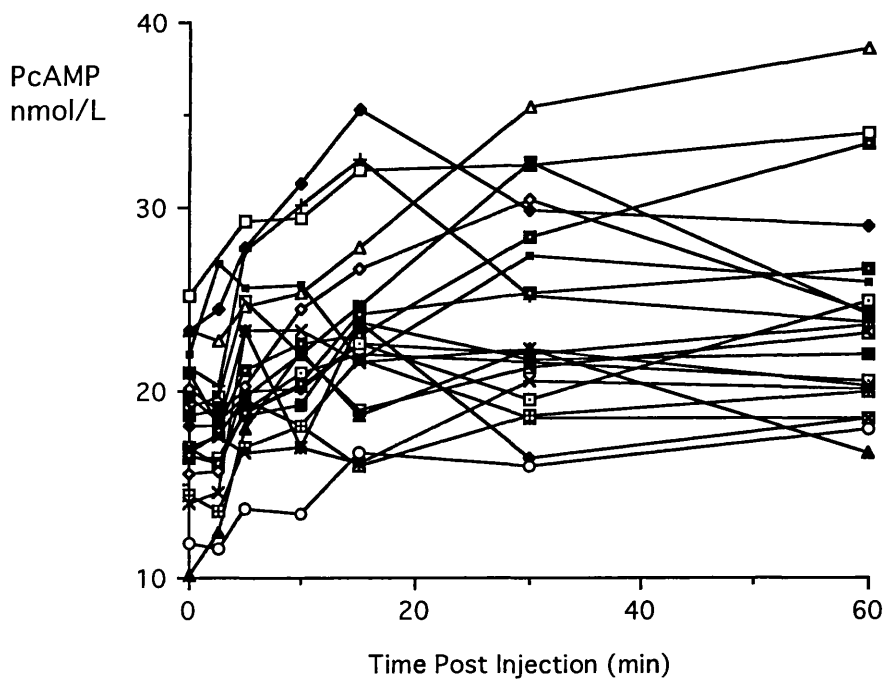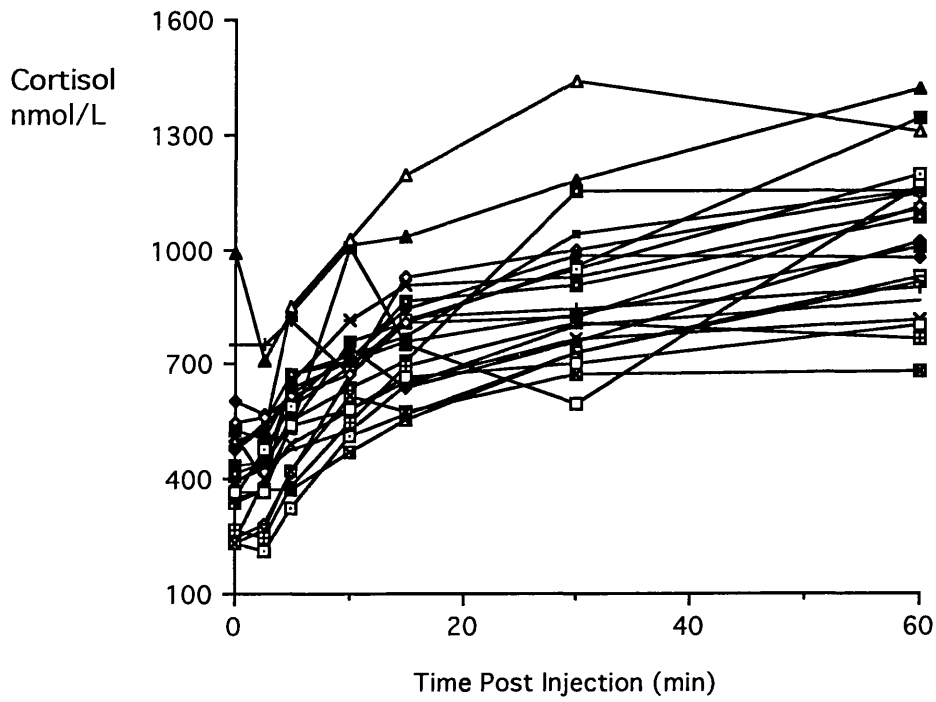
Figure 19   Individual cortisol and PcAMP responses to
0.25 mg synacthen in normal subjects.

would be expected that if Synacthen was the major stimulus to PcAMP release the rise in PcAMP would have occurred earlier than the rise in cortisol and would be much shorter in duration than the cortisol rise. The rise in PcAMP after PTH infusion is immediate and the peak cAMP response is observed within 5-10 min, reflecting the short half life of PTH and the transient receptor mediated generation of cAMP (55). In contrast the PcAMP response to Synacthen is delayed until cortisol rises at 5 min, parallels the rise in cortisol and remains elevated at 60 min.

PTH (1-84) secretion remains unaffected by either Synacthen injection or cortisol secretion in this study and so the changes in PcAMP observed are not due to PTH mediated cAMP generation. From the data available it would appear that the increase in PcAMP that occurs following synacthen injection is due to cortisol production and a general systemic effect rather than the action of Synacthen on the adrenal. Glucocorticoids are known to enhance cAMP accumulation in cell cultures (210) which may be due to increased receptor number (211,212,213) or reduced phosphodiesterase activity (145,214,215,216,217), although most of these effects require prolonged exposure to the steroid for a period greater than 12h.

The short term effects of glucocorticoids resulting in secondary hyperparathyroidism reported by Fucik *et al* (218) have not been confirmed by this current experiment and this

may be a reflection of the relatively lower level of endogenous cortisol produced following Synacthen injection compared to the rise in glucocorticoid observed following administration of exogenous glucocorticoid. Chronic exposure to glucocorticoids in man also results in increased secretion of PTH (219) and so the available data would appear to indicate that in order to stimulate PTH secretion glucocorticoids need to be administered at relatively high concentrations or for a long period of time.

5.2   Glucocorticoid Effects on cAMP Metabolism

5.2.1 Introduction

Enhanced cAMP accumulation in response to hormone stimulation has been observed in several cell types when glucocorticoids are added to culture media (210). The mechanism of this effect varies amongst cells of different tissues. In astrocytoma, neutrophil and human lung cells glucocorticoids increase the number of β-adrenergic receptors (211,212,213). In fibroblast, hepatoma, lymphocyte and a variety of bone derived cell types there is a reduction in phosphodiesterase activity in response to glucocorticoids (145,214,215,216,217). In the osteosarcoma cell line dexamethasone increases adenylate cyclase activity (220,221), increases hormone receptors and the abundance of the regulatory G_s protein (222).

Most of the observed effects of glucocorticoids on cAMP production by cultured cells require exposure to the steroid for a minimum of 12h and the continued presence of steroid during hormonal stimulation. Some authors have reported increased cAMP generation in eukaryotic cells (223,224,225) and erythrocytes (226) when exposed for short time periods (5-60 min) to pharmacological concentrations of glucocorticoids.

Little is known about the *in-vivo* effects of glucocorticoids on cAMP production, although this has

obvious importance for patients receiving glucocorticoid therapy. One study has failed to demonstrate any effect of acute or chronic treatment with dexamethasone on the PTH-induced rise of cAMP by rat kidney (227) but it has been reported that acute and chronic glucocorticoid treatment in man results in secondary hyperparathyroidism (218,219) and this would be expected to increase cAMP production.

In this section of Chapter 5 the acute effects of dexamethasone, given as part of a standard overnight dexamethasone suppression test, on PTH (1-84), plasma, urinary and nephrogenous cAMP have been investigated.

5.2.2 Subjects and Methods

21 healthy volunteers were studied (age 17-61 years, mean 31, 14 male, 7 female) on two consecutive mornings pre and post an overnight Dexamethasone suppression test (2 mg dexamethasone orally at 2300h). Each subject acted as his/her own control and emptied their bladder at 2300h then collected all urine voided until 0900h the next morning. A blood sample was obtained by venesection at 0900-0930h. All biochemical measurements were performed as described previously. Statistical comparisons were performed using the paired t-test.

5.2.3 Results

Table 9 compares the results obtained in the control period and post dexamethasone. Dexamethasone caused a significant

	Pre Dose	Post Dexamethasone	Significance
Urine Cortisol (nmol/L)	94.8 ± 34.6	<40	P<0.0001
PcAMP (nmol/L)	26.3 ± 7.4	27.6 ± 5.4	NS
UcAMP (nmol/L GF)	46.8 ± 18.6	35.2 ± 8.5	P<0.01
NcAMP (nmol/L GF)	19.3 ± 1.6	7.7 ± 0.8	P<0.001
PTH (1-84) (pmol/L)	2.9 ± 0.7	2.7 ± 0.6	NS

Table 9   Urine cortisol, UcAMP, PcAMP, NcAMP and PTH (1-84) before and after an overnight dexamethasone suppression test

decrease in urine cortisol, total UcAMP and NcAMP but had no effect on PTH (1-84) or PcAMP obtained at 0900h in the morning following dexamethasone. The individual responses to dexamethasone for cortisol, NcAMP, UcAMP and PcAMP are given in Figures 20 and 21 which confirm that all subjects studied responded to dexamethasone by suppressing urinary cortisol and 18 out of 21 decreased NcAMP production. PcAMP showed the greatest variability with an increase in PcAMP observed in 12 and a decrease in 9 subjects.

5.2.4 <u>Discussion</u>

Dexamethasone resulted in the well recognised suppression of urinary cortisol in all subjects studied (228,229). Associated with this effect was a significant decrease in total UcAMP and NcAMP. Although a variable response in PcAMP was observed, overall there was no significant difference in PcAMP following dexamethasone. The main contributor to the decrease in total UcAMP was the nephrogenous component. The significant decrease in NcAMP may be due to a number of factors. NcAMP production will decrease in response to a decrease in a) plasma PTH concentration b) the number of PTH receptors on the renal tubular cell surface c) PTH receptor recycling and d) adenylate cyclase activity. Cyclic AMP breakdown may be enhanced by an increase in phosphodiesterase activity or by alterations in the renal handling of cAMP.
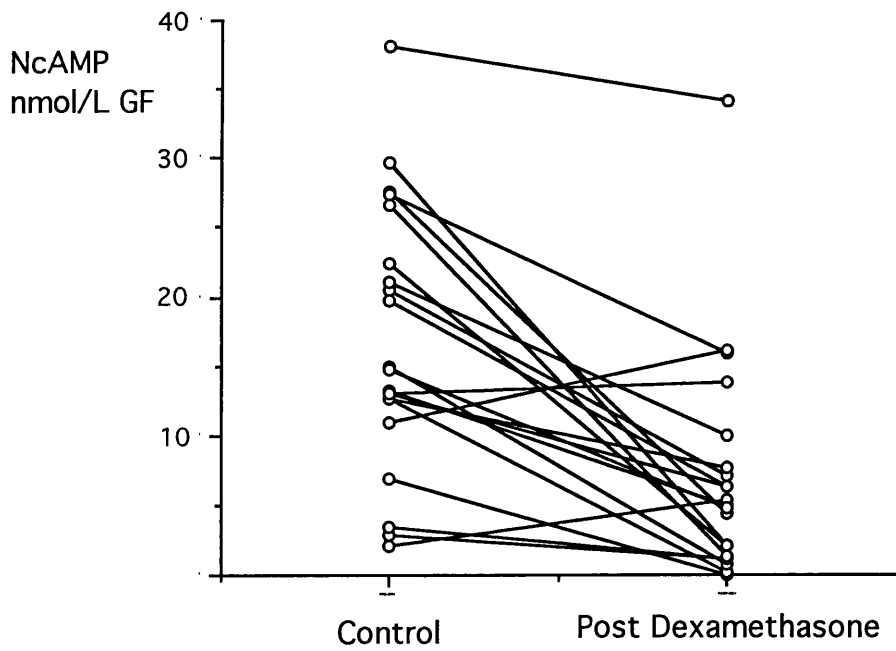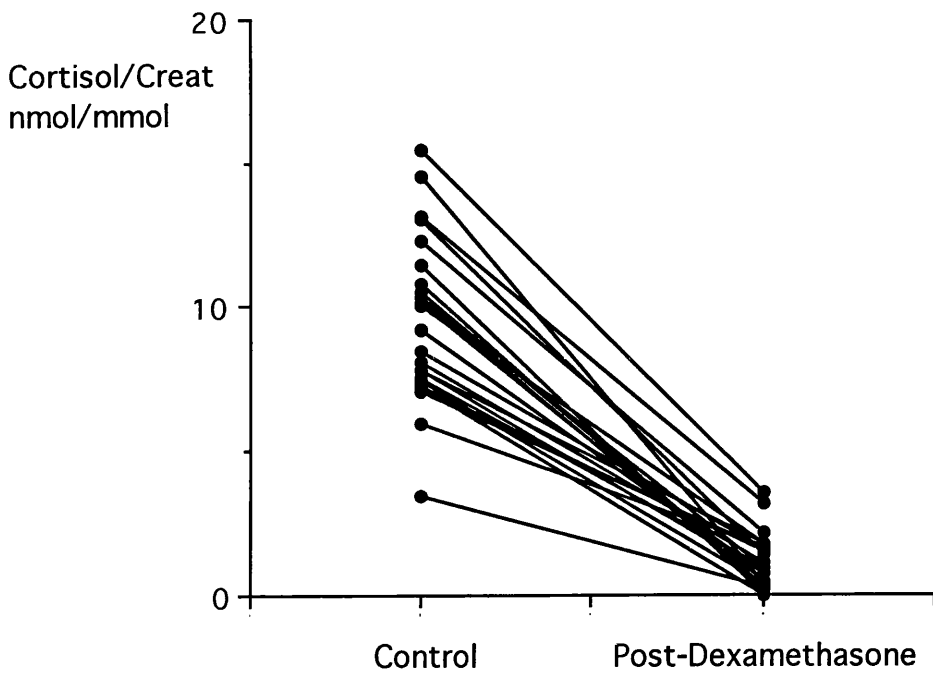
Figure 20   Cortisol and NcAMP responses to an overnight
dexamethasone suppression test in normal
subjects.
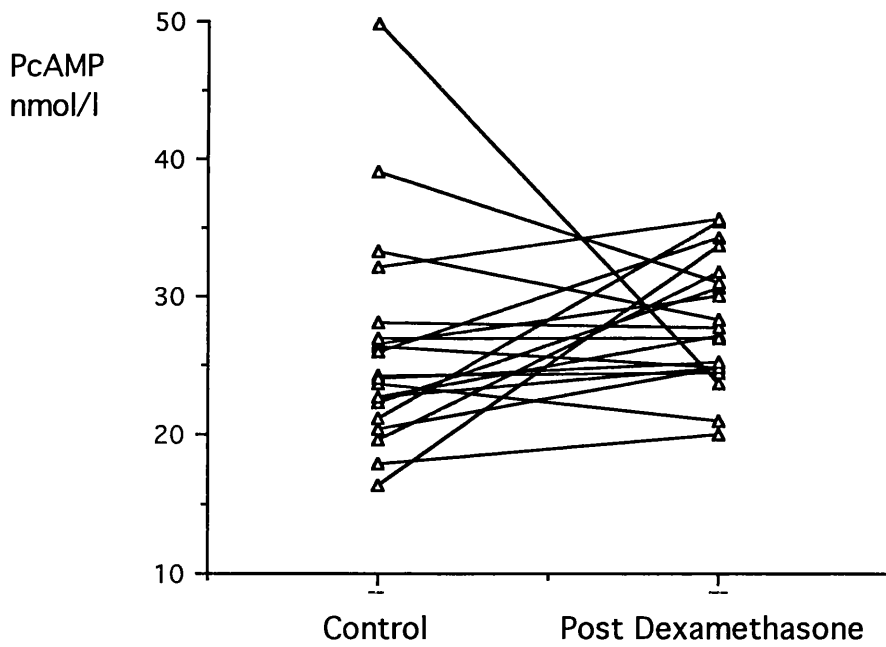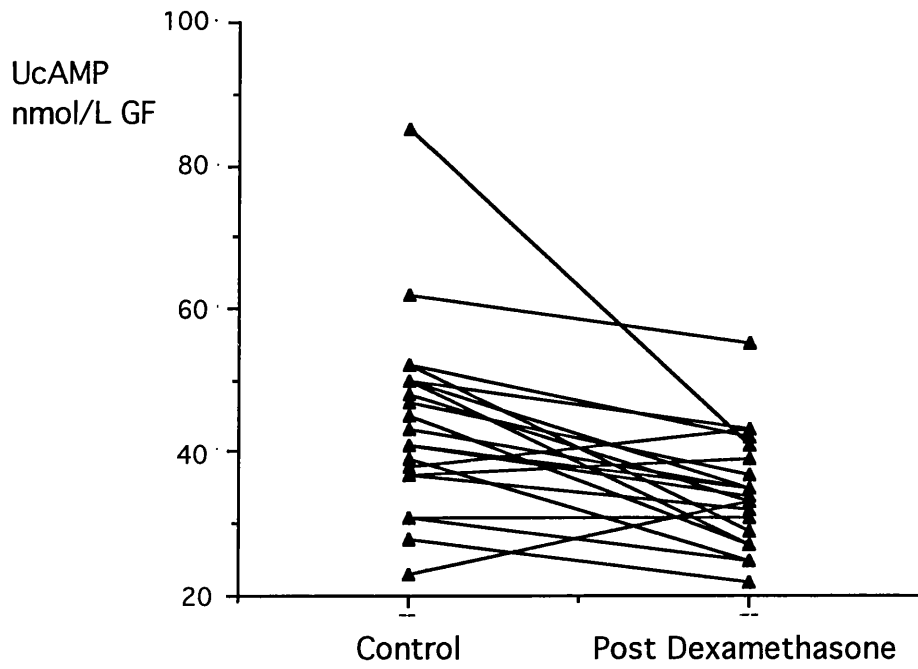
Figure 21 UcAMP and PcAMP responses to an overnight dexamethasone suppression test in normal subjects.

In this study the significant effects on cAMP metabolism may be caused directly by dexamethasone or indirectly through the resultant decrease in cortisol. Studying the effects of these hormones on cAMP production *in-vitro* and in animal models has given conflicting results. In cell culture systems dexamethasone enhances cAMP production (222) whilst in rat kidneys dexamethasone has little effect on the PTH mediated generation of cAMP (227). Hydrocortisone and dexamethasone has been shown to enhance the response of ROS 17/2.8 cells in culture to PTH resulting in a dose-dependent increase in cAMP production (220,221). The current study suggest that the decrease in circulating cortisol in response to dexamethasone may have caused the decrease in NcAMP possibly by altering the kidney cell response to PTH.

At the time this study was being performed the detailed knowledge on the circadian rhythm of PTH (1-84) was not known and so the possibility exists that the dexamethasone test acutely suppresses PTH (1-84) secretion and interferes with the nocturnal rise in PTH (1-84) resulting in the decrease in NcAMP production. However, the failure of the increase in cortisol in response to Synacthen to influence PTH secretion acutely (Chapter 5.1) would suggest that this is unlikely. PTH (1-84) measurements were not significantly different at 0900-0930h but this does not exclude the possibility of overnight effects and so further study is required to fully explain these findings.

In view of the apparent failure of hydrocortisone to suppress the action of PTH in patients with 1°HPT (Hydrocortisone or Steroid Suppression Test, 230,231,232,233) together with the results of this study and the ACTH stimulation study it would appear that dexamethasone and cortisol effects on cAMP metabolism in humans merit further study.

Chapter 6

PTH(1-84), PTHrP and cAMP in

Primary Hyperparathyroidism

6.1.1 Introduction

Total urinary cAMP (UcAMP) is a combination of filtered cAMP derived from the circulation and cAMP produced by the kidney, nephrogenous cAMP (NcAMP). Between 40-45% of the UcAMP and 90-95% of the NcAMP is produced as a consequence of PTH action on the kidneys (46,67,68). NcAMP has been proposed as an accurate indicator of PTH function but published positive correlations between immunoreactive PTH and either UcAMP or NcAMP, although always significant, vary from strong to weak (r = 0.76 to r = 0.31) (46,67,69,71,93). There are a number of possible reasons for the variability and low correlations observed. Immunoassays for PTH used in the 1970s and early 1980s were subject to several technical and biological problems, including the heterologous nature of the assays and the heterogeneity of circulating forms of PTH. This meant that the assays often measured inactive fragments of PTH which may be present in the circulation especially in patients with renal impairment. Inaccuracy in the measurement of PTH is likely to be an important factor in the variable correlations with NcAMP.

Hyperparathyroidism with persistent elevations of PTH results in a down regulation of PTH receptors with a reduced cAMP and phosphaturic response to PTH stimulation (234,235,236,237,238,239). The precise length of time required to develop this down regulation and its effect on basal NcAMP production in 1°HPT is unknown but infusion of

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bovine PTH for 2h at a rate of 500 units/h results in impairment of the cAMP response to PTH injection which lasts for 12-18h (234,236).

There are several problems inherent in NcAMP estimations as outlined in chapters 1,2 and 3. Inaccuracy may be introduced due to marked PcAMP variability and unrecognised spikes in PcAMP concentrations (Chapter 3). Consistency in duration and timing of both urine and plasma sampling is required to eliminate variability. Measurement of cAMP has to be performed with as precise and specific an assay as possible with sample dilutions targeted at the part of the immunoassay curve which results in the best assay precision.

Calcitropic hormones other than PTH may also play a role in NcAMP production in 1°HPT. Immunoassays for parathyroid hormone related protein (PTHrP) have recently been developed and some authors have detected elevated levels of PTHrP in a high percentage of patients with 1°HPT (240). Parathyroid adenomas have DNA and mRNA for PTHrP present in significant quantities as well as expressing PTHrP (241,242,243). There is also evidence to suggest that fetal parathyroids express PTHrP in preference to PTH and that PTHrP is the major fetal calcitropic hormone in some species (244,245). It is therefore possible that PTHrP may be secreted by the parathyroid gland in 1°HPT and this would affect PTH correlations with NcAMP.

Recent advances in assay technology has led to the development of immunometric assays which measure the intact molecule of PTH (PTH (1-84)) without interference from PTH fragments. The sensitivities and specificity of these assays allows PTH (1-84) to be measured in the low picomolar range and has improved the discrimination between normals and patient groups such as 1°HPT, HCM, hypoparathyroid and secondary hyperparathyroidism (100,101,129). Immunoradiometric assays for PTHrP have also recently become available (Chapter 9) with excellent specificity and sensitivity and so it is now possible to evaluate the relationship between PTH (1-84), PTHrP and cAMP metabolism in patients with 1°HPT.

6.1.2 Patients and Methods

Over a two year period 62 patients with 1°HPT were prospectively included in the study. There were 24 males and 38 females (29 - 82 years, mean 56). All patients were hypercalcaemic on more than one occasion (calcium adjusted for albumin greater than 2.65 mmol/L) with a PTH (1-84) concentration on the same specimen greater than 3.0 pmol/L. In 46 patients an adenoma or hyperplastic parathyroid glands have been removed surgically, in 48 patients an adenoma was detected either by ultrasound or by technetium/thallium subtraction scan, in 16 patients the diagnosis is currently based on the clinical, radiological and biochemical findings since these patients have negative technetium/thallium scans and are unwilling or unfit for

operation. In 4 of 16 patients a previous selective venous catheterisation of the neck veins indicated an increase in iPTH on the left side of the neck.

Blood samples were obtained by standard venesection in the middle of a timed urine collection obtained between 0900-1400h. All patients were asked to void urine prior to collection of the timed sample (4h) which was obtained by spontaneous voiding. Patients remained recumbent for at least 15 min prior to blood sampling and were allowed to be ambulant but avoided exercise throughout the time of the urine sample collection. Blood was collected into tubes containing lithium heparin, EDTA, EDTA with protease inhibitors and no anticoagulant. Plasma was separated within 30 min and stored frozen -70°C prior to analysis. Serum was obtained after 30-45 min and stored either frozen (-70°C) or refrigerated (4°C) prior to analysis. All urine voided during the timed collection period was collected in one sterile container containing no preservatives, volumed and an aliquot stored frozen (-20°C) prior to analysis.

PTH (1-84), plasma and urine cAMP, PTHrP and routine chemistries were all measured as described in Chapter 2. NcAMP was calculated as described by Broadus *et al* (46). Statistical differences between groups was evaluated using the Mann-Whitney test and correlation coefficients were determined as described Chapter 2.

A subgroup of 12 patients with proven 1°HPT were followed for a period of 6 months. Patients were selected prospectively and randomised into a double blind placebo controlled study to receive either placebo or 100 mg atenolol daily for 6 months. Patients were sampled monthly as described previously and within group comparison of data performed using Student's paired t-test and between groups comparison by Mann-Whitney test.

6.1.3 Results

The baseline values for Adj Ca, PO_4, PTH(1-84), PTHrP, PcAMP, UcAMP and NcAMP are given in Table 10. Adj Ca, PTH (1-84), PcAMP, UcAMP and NcAMP were higher than the reference range in 100%, 87%, 24%, 81% and 90% respectively. PO_4 was lower than the reference range in 16% of patients. PTHrP was increased above the reference range (2.7 pmol/L) in only 2 patients (3%), was above the assay detection limit (0.7 pmol/L) in another 5 (8%) and was below the assay detection limit in the remaining 55 patients (89%).

The correlation of PTH (1-84) with NcAMP is shown in Figure 22a. It is notable that a subset of patients exist (outlined in Figure 22a) whose NcAMP values are such that they appear discordant with the measured PTH (1-84). In all cases the level of NcAMP is far higher than expected for the measured PTH (1-84). If this group of patients are removed from the analysis the r value for the correlation

(Ref Range)	Adj Ca mmol/L (2.20-2.60)	Serum PO$_4$ mmol/L (0.7-1.4)	PTH (1-84) pmol/L (1.0-5.0)	PTHrP pmol/L (<0.7-2.6)	PcAMP nmol/L (15-30)	UcAMP nmol/LGF (22-66)	NcAMP nmol/LGF (8-28)
Mean	3.09	0.92	22.1	0.9	22.5	75.5	69.5
SD	0.39	0.18	14.0	1.2	6.9	36.6	37.2
Range	2.66-4.10	0.48-1.41	3.1-59.8	0.3-4.9	10.4-38.6	44.6-190.4	23.0-158.3

Table 10   Baseline Biochemical Measurements in 62 Patients with 1°HPT
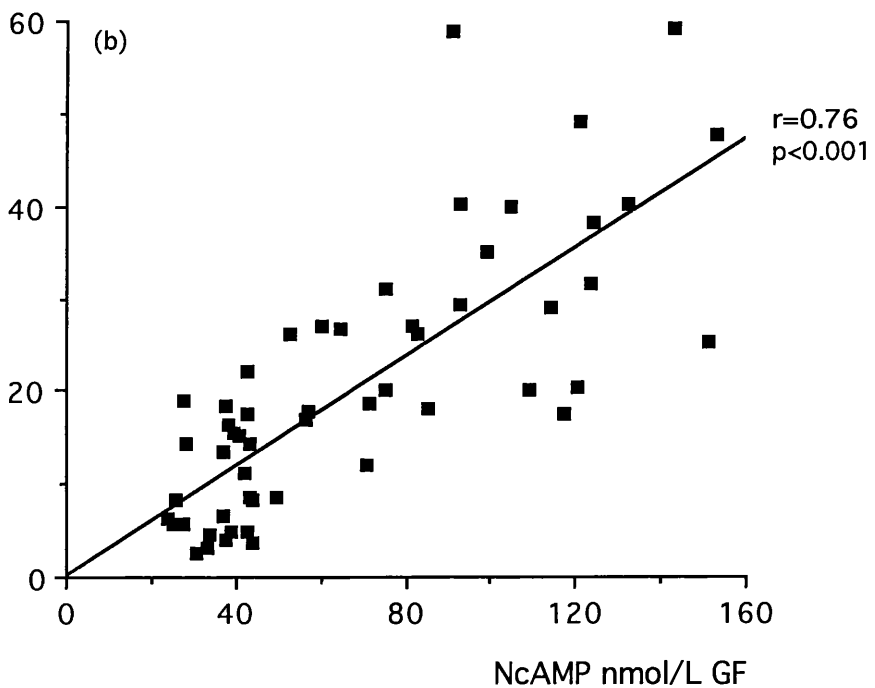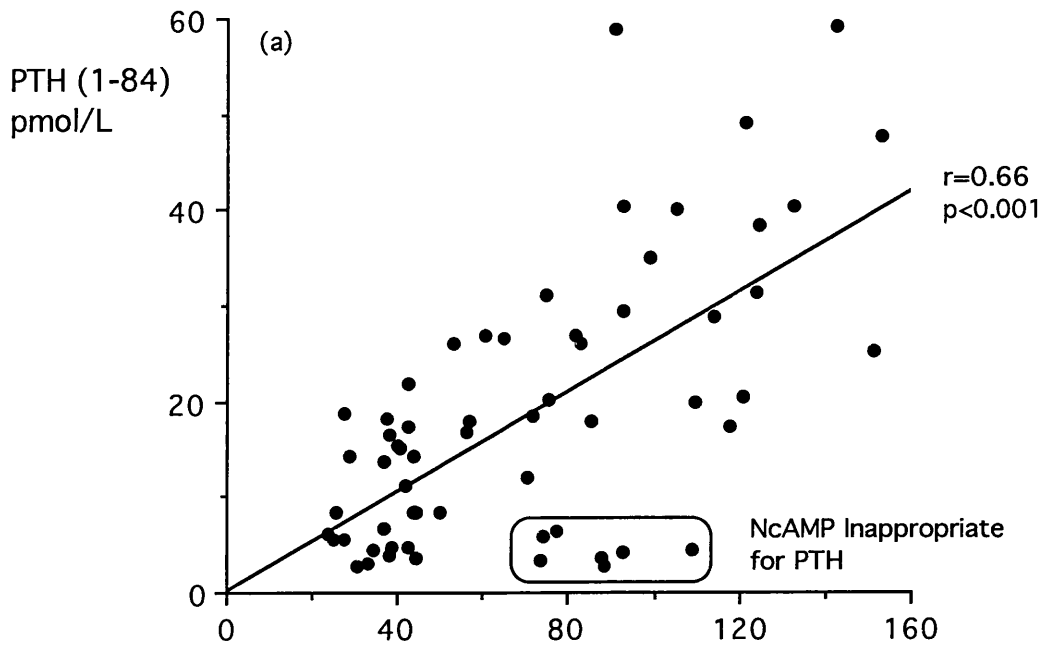
Figure 22   Linear regression of PTH (1-84) and NcAMP in
            1°HPT patients a) including all results
            obtained   b) excluding results were NcAMP is
            deemed inappropriate for PTH (1-84).

between PTH (1-84) and NcAMP increases (Figure 22b). In one of the patients in this subgroup PTHrP was elevated and if the PTHrP and PTH (1-84) values for that patient are added together (assuming PTHrP and PTH (1-84) to be biologically equipotent *in-vivo*) the correlation with NcAMP for that patient is much stronger. A plot of PTHrP added to PTH (1-84) for all patients where the measurements are available is shown Figure 23. The correlation obtained in this comparison is little different from that obtained when PTH (1-84) and NcAMP are compared for all patients studied. The variation in PTH (1-84), NcAMP (Figure 24), UcAMP and PcAMP (Figure 25) for the 12 patients studied over a 6 month period are shown for placebo and atenolol treated patients. Although the variability in PTH (1-84) was greater than that in NcAMP, reflecting the greater error of estimation for NcAMP, there was remarkably little difference in the values obtained in the majority of the patients over the study period. No significant difference in any measurement was observed between the two groups at any time point. Correlations between PTH (1-84) and NcAMP for this group of 12 patients remained remarkably consistent throughout the period of study in both placebo (P1-P6) and atenolol (P7-P12) treated patients (Figure 26). In particular 2 patients who had NcAMP that was identified as inappropriate for the PTH (1-84) maintained the same correlations over the period of study whether receiving placebo or atenolol (Figure 26).
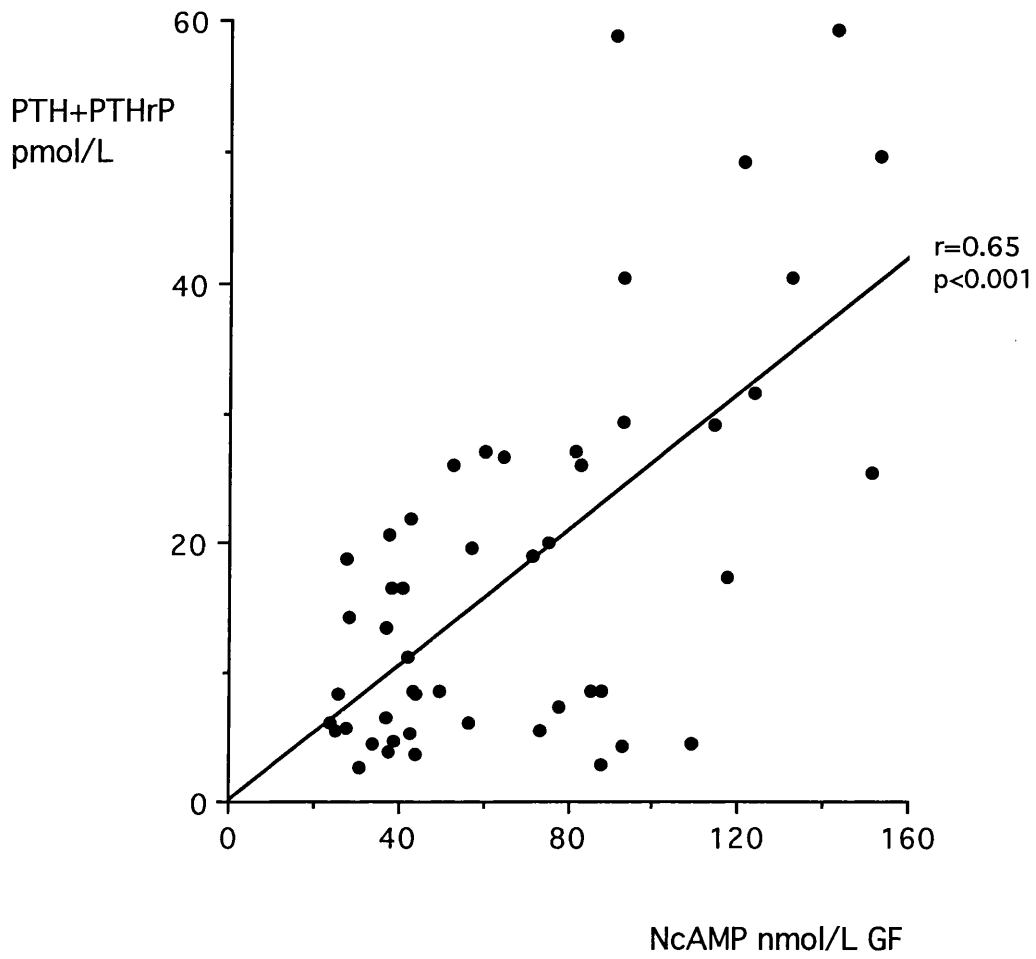
Figure 23   Linear regression of PTH + PTHrP and NcAMP in
            1°HPT patients.
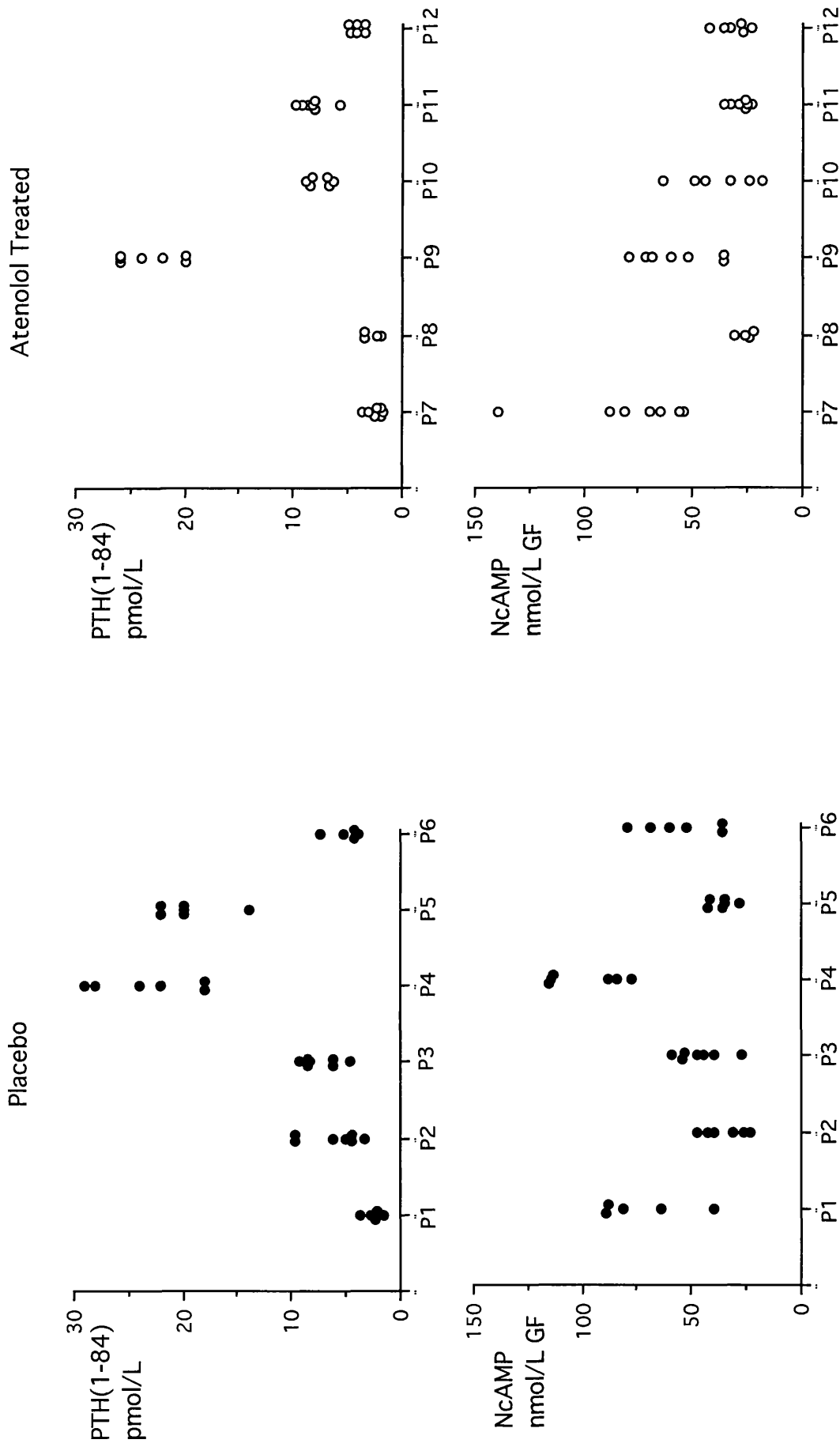
Figure 24 Scatter plots of PTH (1-84) and NcAMP in 12 patients (P_1-P_{12}) with 1°HPT sampled monthly for 6 months. Each subject was sampled prior to the 6 month study period. P_1-P_6 were placebo controls. P_7-P_{12} received 100 mg atenolol daily for 6 months.
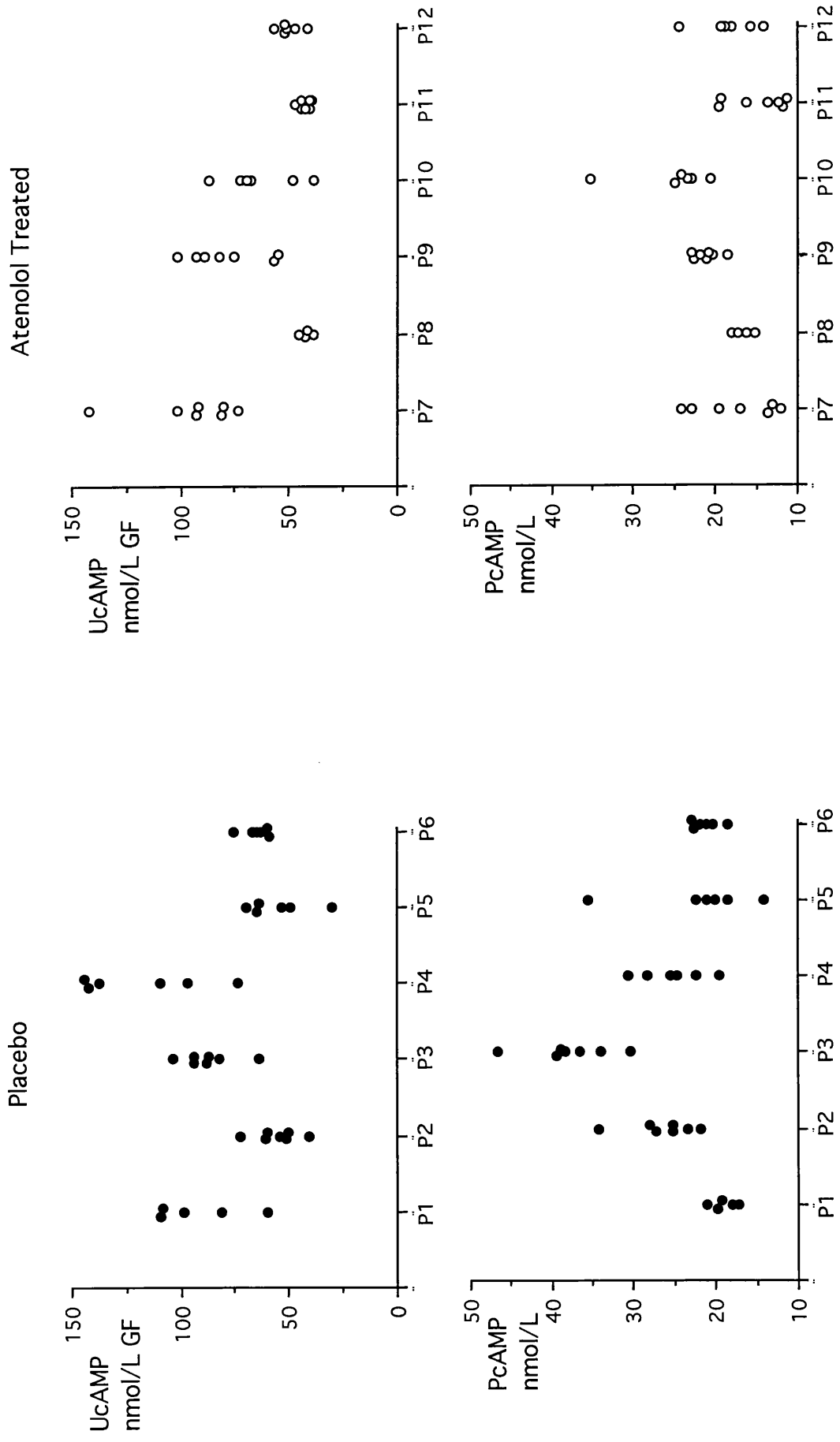
Figure 25   Scatter plots of UcAMP and PcAMP in 12 patients (P_1-P_{12}) with 1°HPT sampled monthly for 6 months. Each subject was sampled prior to the 6 month study period. P_1-P_6 were placebo controls. P_7-P_{12} received 100 mg atenolol daily for 6 months.
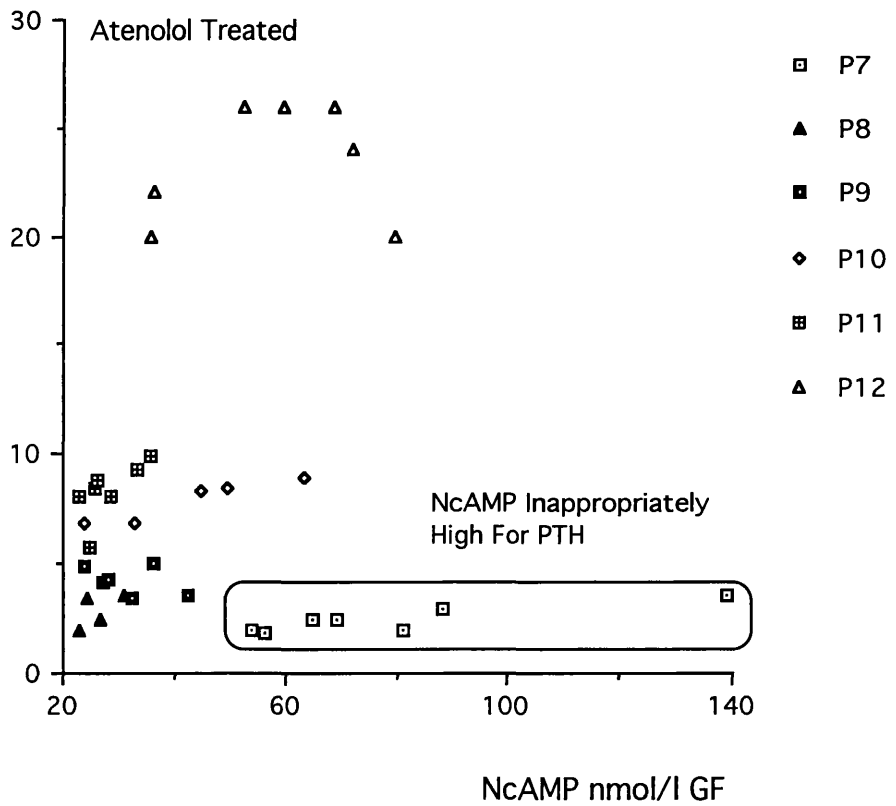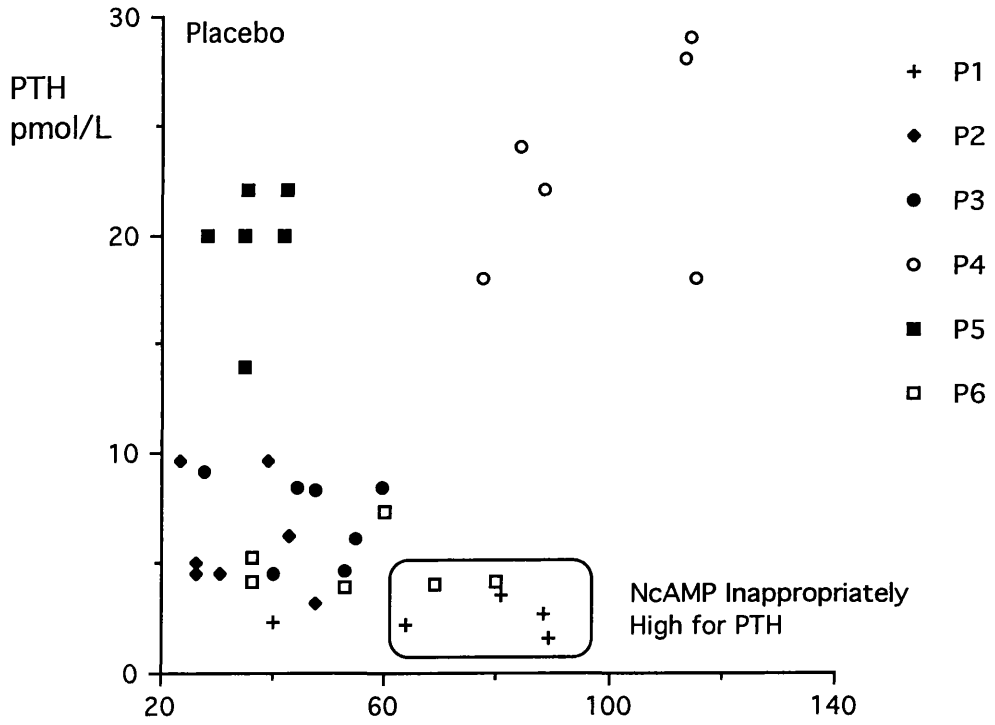
Figure 26   Correlation of individual values for PTH (1-84) and NcAMP in 12 patients (P_1-P_{12}) with 1°HPT sampled monthly for 6 months.

6.1.4 Discussion

Correlations between PTH and NcAMP have varied markedly in published studies although they have always been positive and significant (46,67,69,71,93). In this study one of the strongest and highly significant correlations between the two measurements has been obtained and this is due to a number of factors.

Improvement in the measurement of PTH with estimation of the major circulating biologically active molecule PTH (1-84) is a major factor. The current generation of PTH (1-84) IRMAs are able to measure the intact PTH molecule with excellent specificity and accuracy without interference from fragments of PTH (100,101,129) and should therefore correlate better with the *in-vivo* biological estimate of activity NcAMP. It is possible that PTH (1-34) could contribute to biological effects but in studies performed on humans using infused PTH (1-34) the half life of this molecule was found to be relatively short (t 1/2 = 2.5 min) and in all subjects measured to date, including patients with 1°HPT, PTH (1-34) circulating concentrations were < 0.5 pmol/L (246). It would appear that in most studies the molecule of major biological significance *in-vivo* is PTH (1-84).

The current generation of RIAs for cAMP have excellent performance characteristics and will contribute to the improved correlation between PTH (1-84) and NcAMP.

Variability in NcAMP is greater than that in PTH (1-84), with repeated estimates, which is not surprising since NcAMP is a derived value calculated from 4 measurements. In those patients who were studied over 6 months there was little change in either the measurement of PTH (1-84) or estimate of NcAMP and the correlation between the two remained remarkably constant. This may in part be due to our use of urine collections over a relatively short time period under close supervision rather than 24h urine samples with the associated difficulty of ensuring accurate collections.

It is known that the presence of hypercalcaemia, Vitamin D abnormalities and 1°HPT can alter the cAMP response to PTH and down regulation of receptors will occur in patients with 1°HPT (234,235,236,237,238,239). In 12 patients studied for 6 months, however, there was little change in either PTH (1-84) or NcAMP in the majority of patients and this confirms that any changes that take place in 1°HPT probably occur very slowly over a long period of time.

An early study of PTHrP measurements in patients with 1°HPT indicated 30% of 1°HPT patients were producing PTHrP simultaneously with PTH (1-84) (240). In subsequent studies with more specific and sensitive assays for PTHrP these initial findings have not been confirmed and taken overall only a small percentage of 1°HPT patients have circulating PTHrP levels of any significance (247,248,249,

Chapter 8).   In this current study only one patient had a PTHrP level that would be considered as significantly detectable and this was in one of the patients in whom NcAMP was inappropriate for the level of PTH (1-84). Assuming equal biological potency in humans for equivalent concentrations of PTHrP and PTH (1-84), then by addition of these two calcitropic hormones the NcAMP production was exactly as expected by the correlation statistics. However, PTHrP was low or undetectable in the majority of 1°HPT patients in this study who had inappropriate NcAMP for their PTH (1-84) concentrations and in 2 of these patients the NcAMP production and PTH (1-84) concentrations remained relatively constant over a 6 month period.   It is unlikely, therefore, that secretion of PTHrP by parathyroid adenomas plays a significant part in the abnormalities of calcium homeostasis that are observed in the majority of patients with 1°HPT.   There was no obvious reason, in the remaining patients of this group, for the discordance between PTH (1-84) and NcAMP.   It is possible that biologically active fragments of either PTH or PTHrP are produced by these patients which are not recognised by the IRMAs used in this study due to the epitopes that the antibodies employed in the assay detect.   The only other significant difference observed was a lower PcAMP concentration in these patients (P_1, P_6, P_7) when compared to the rest of the 1°HPT patients.   This may indicate that the renal production of cAMP selectively appears mainly at the tubular luminal side of the renal cells or that other

substances are circulating in some 1°HPT that can have predominant effects on renal cAMP production, perhaps by enhancing PTH (1-84) activity, whilst having little effect on PcAMP.

An increased prevalence of hyperparathyroidism in hypertensive patients has been reported (250) and also of hypertension in patients with 1°HPT (251,252,253). The precise mechanism of this hypertension is open to debate as the evidence for the role of hypercalcaemia (254,255,256), renal function (250,251,256), the renin-angiotensin system (255,257,258), the sympathetic nervous system (259) and a parathyroid hypertensive factor (PHF) (260) is conflicting. Treatment of 1°HPT patients with parathyroidectomy has variable effects on blood pressure when studies are compared. Some show improvement or cure (251,255,261) while others have found no significant change in blood pressure following surgery (258,262,263,264,265). Alterations in indices of calcium metabolism have been reported in hypertensive patients treated with either propranolol, bendroflumethiazide or verapamil (266). Hvarfner reported an increase in ionised calcium and phosphate and decreased PTH (by RIA), free fatty acids and glycerol in hypertensive patients treated for 6 months with propranolol. This previous publication raised the possibility that β-blockers might modify PTH secretion and calcium metabolism in patients 1°HPT. In this current study there was no significant effect of atenolol on any measurement related

to calcium homeostasis, although a small number of subjects were studied over a relatively short period of time. Despite this it is obvious that atenolol has no important or dramatic effects on calcium homeostasis in the short term.

In this study a significant correlation between NcAMP and PTH (1-84) has been demonstrated that persists unchanged for a significant period of time in a selected group of 1°HPT patients. A subgroup of patients exists where NcAMP production is apparently inappropriately high for the measured PTH (1-84) and in a very small percentage of 1°HPT patients this may be due to the simultaneous production of PTHrP. The main factor influencing the amount of NcAMP produced is the amount of circulating PTH (1-84) in patients with 1°HPT. The factors that can further influence the NcAMP produced for a consistent PTH (1-84) level are not immediately obvious and further study will be required to investigate these current findings in the future.

Chapter 7

cAMP Response to Bisphosphonate Treatment

7.1 Comparison of the Daily Variation in cAMP Following Pamidronate in Patients with Hypercalcaemia Associated with Malignancy and Paget's Disease of Bone

7.1.1 Introduction

The bisphosphonates are a group of pyrophosphate analogues that are potent inhibitors of osteoclast-mediated bone resorption. They are used in the treatment of bone diseases associated with increased bone resorption such as Paget's disease (267,268,269,270,271), hypercalcaemia associated with malignancy (HCM) (272,273,274,275,276) and involutional osteoporosis (277). Pamidronate (3-amino-1-hydroxypropylidene-1,1-bisphosphonate, APD) is one of the more effective bisphosphonates in the treatment of Paget's disease and HCM and the effects of treatment on calcium and phosphate homeostasis have been well documented (272,273,274,276,278). There is no unifying theory that explains all the alterations in calcium and phosphate metabolism that occur following pamidronate treatment, and various mediators of the ionic changes have been postulated (278,279,280).

To date there have been no studies on the response of cAMP to pamidronate therapy and studies on PTH have been limited by assay technology with increases in plasma PTH only observed when patients became hypocalcaemic (279).

7.1.2 Patients and Methods

12 patients with Paget's disease (4 male, 8 female, aged 51-63 years) were studied. All had their bone disease confirmed by standard biochemical, radiological (X-ray, radionuclide bone scan) methods and, where appropriate, by histomorphometry. All were studied prior to and following a single intravenous dose of 45 mg pamidronate, which was given intravenously over 4h in 0.5 L of 0.9% NaCl.

12 patients with HCM (7 male, 8 female, aged 48-78 years, Adj calcium > 2.7 mmol/L) 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). 8 of the 12 patients had metastatic bone disease confirmed by radionuclide bone scan and the tumour load was estimated as being heavy in 2 and light in 6 patients. All patients were rehydrated over a 24h period with a minimum of 2 L of intravenous 0.9% NaCl prior to the administration of a single intravenous dose of 30 mg pamidronate given over 4h in 0.5 L of 0.9% NaCl. Serum creatinine concentrations were within the reference range (60-130 μmol/L) in 10 patients and elevated in 2 (150 and 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 treatment (day 0) and daily thereafter for 7 days. All blood specimens were

collected between 1000 and 1100h, and a timed 2-4h fasting urine was obtained after discarding the first morning urine specimen (as discussed Chapter 3). Serum, K-EDTA plasma and urine were stored at -20°C prior to analysis.

All measurements were performed as described previously (Chapter 2). Each series of samples for an individual patient was analysed within a single batch.

Statistical analysis was performed using a Kruskal Wallis (ANOVA) test to detect significance for each measurement for each day prior to applying a paired difference t-test to compare each sample point with the appropriate day 0 result for the patient.

7.1.3 Results

The mean ($\pm$ SEM) results for each measurement in the patients with Paget's disease are shown Figures 27 and 28. Prior to therapy, the mean concentration of all analytes was within the appropriate reference range. Following pamidronate therapy, there was a small significant decrease 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 NcAMP on day 4 ($p < 0.01$), PcAMP on day 5 ($p < 0.05$) and UcAMP on day 3 ($p < 0.05$). PTH (1-84) increased significantly from day 2 ($p < 0.05$) and continued to increase throughout the study reaching a final mean value more than twice the upper limit
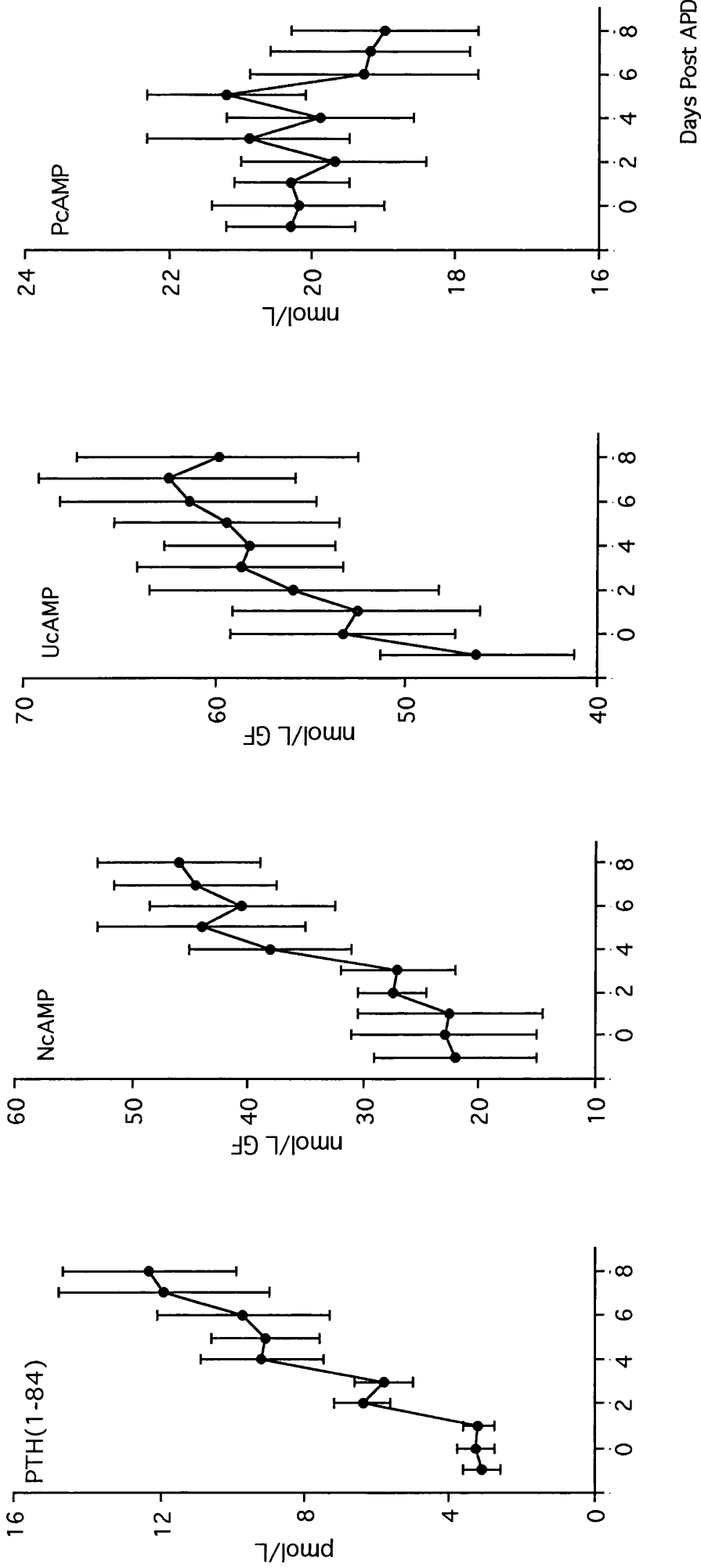
Figure 27 Daily changes in PTH (1-84), NcAMP, UcAMP and PcAMP in patients with Paget's disease of bone following intravenous infusion of APD. Results are mean ± SEM.
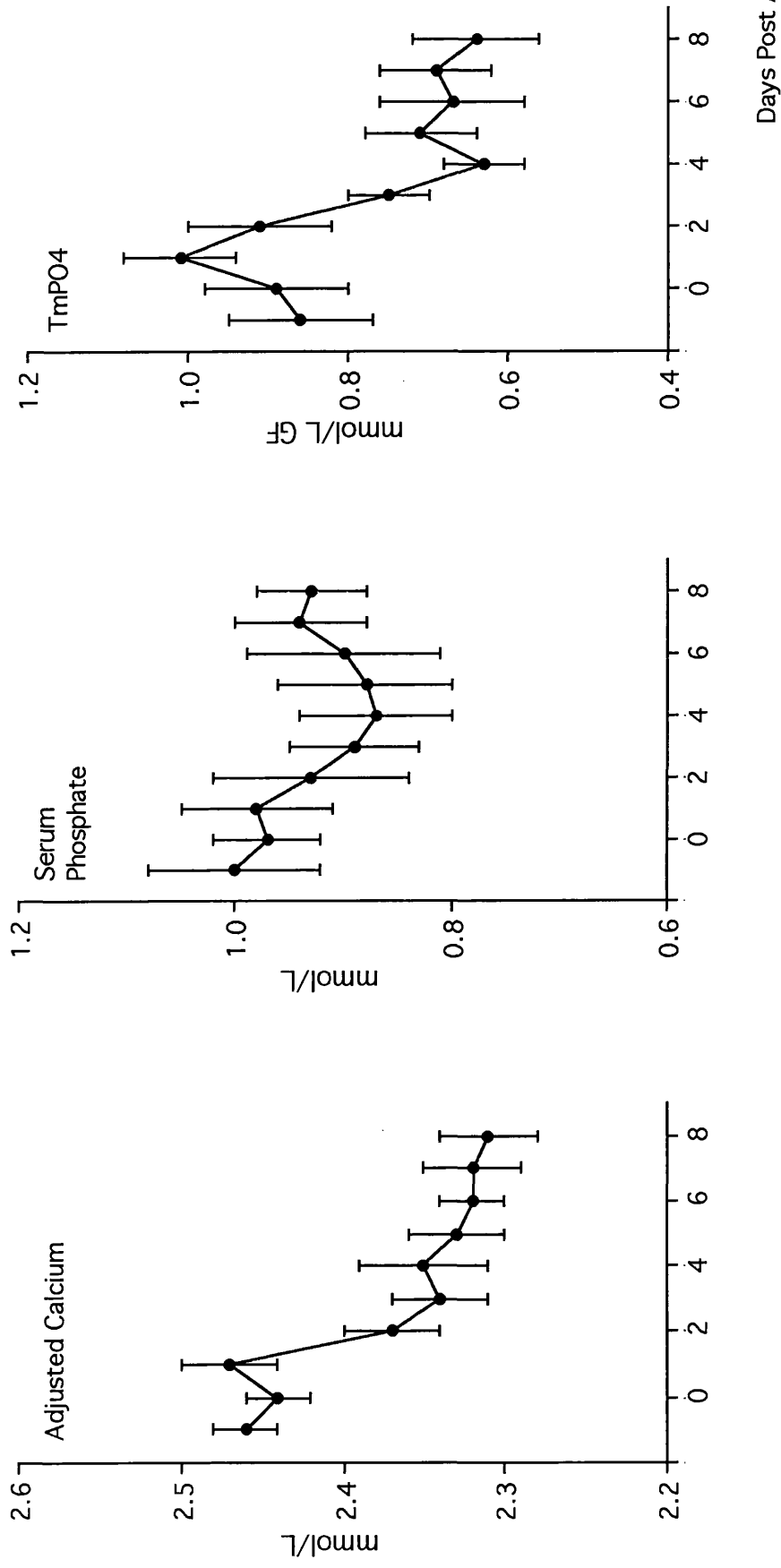
Figure 28 Daily changes in adjusted calcium, serum phosphate and threshold for renal tubular reabsorption of phosphate ($TmPO_4$) in patients with Paget's disease of bone following intravenous infusion of APD. Results are mean ± SEM.

of the reference interval. $TmPO_4/GFR$ decreased gradually to a trough on day 4 ($p<0.001$) and remained at this low value thereafter.

The mean ($\pm$ SEM) results for the analytes measured in the patients with HCM are shown Figures 29 and 30. Following rehydration, but prior to pamidronate therapy, all patients had elevated concentrations of serum adjusted calcium (range 2.85-3.95 mmol/L). 9 patients had PTH (1-84) concentrations below the assay detection limit (0.5 pmol/L), whilst the other 3 patients had concentrations at the low end of the reference range (0.9-1.1 pmol/L). Despite this suppression of PTH (1-84), the mean NcAMP concentration was significantly increased and was "inappropriate" for the PTH (1-84) in 8 patients. The mean $TmPO_4/GFR$ was also significantly decreased and was "inappropriate" for the PTH (1-84) in 7 patients.

Following pamidronate therapy, there was a rapid, consistent and continuing decrease in the serum adjusted calcium in all patients, with the mean concentration reaching the upper limit of the reference range on day 5. PTH (1-84) concentrations remained suppressed for days 1-3 but increased significantly on day 4 ($p<0.05$) and continued to increase, such that the mean concentration was above the reference interval at the end of the study. The NcAMP, PcAMP, UcAMP and $TmPO_4/GFR$ responses to pamidronate therapy were complex. The initially subnormal $TmPO_4/GFR$ decreased
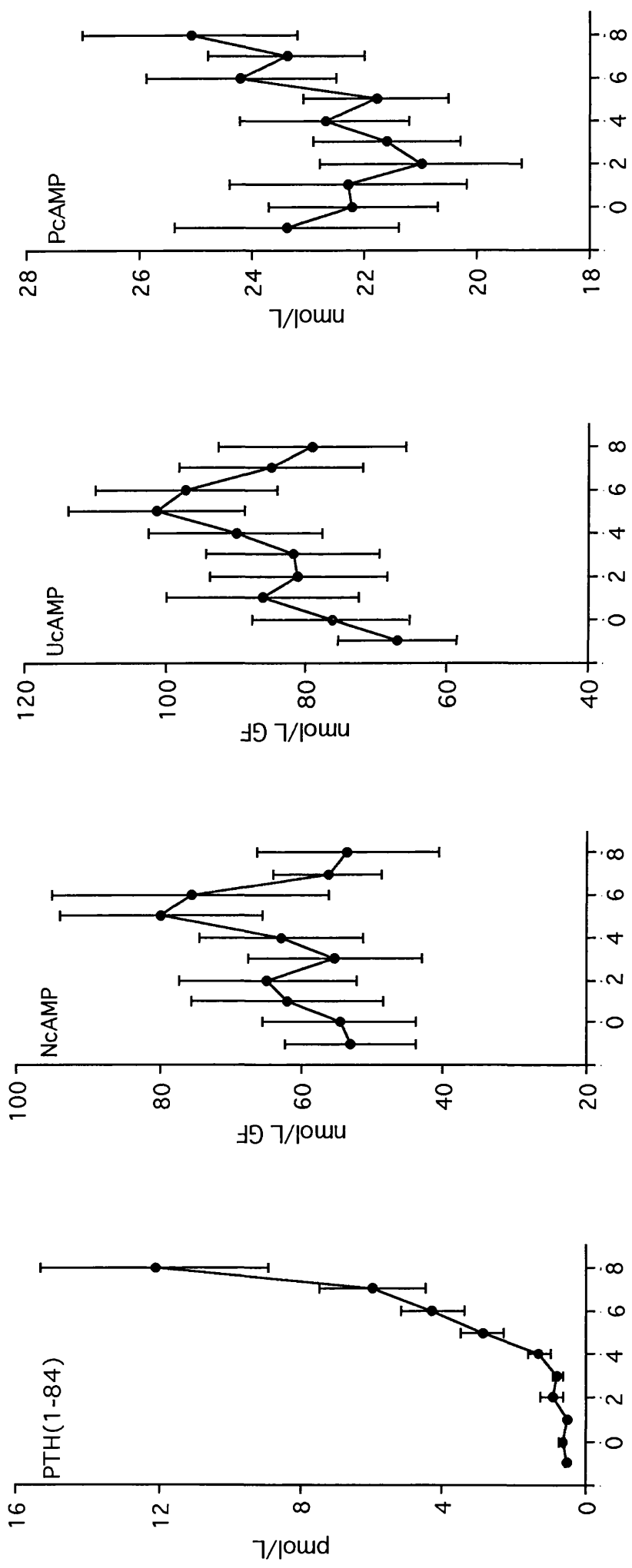
Figure 29   Daily changes in PTH (1-84), NcAMP, UcAMP and PcAMP in patients with HCM following intravenous infusion of APD. Results are mean ± SEM.
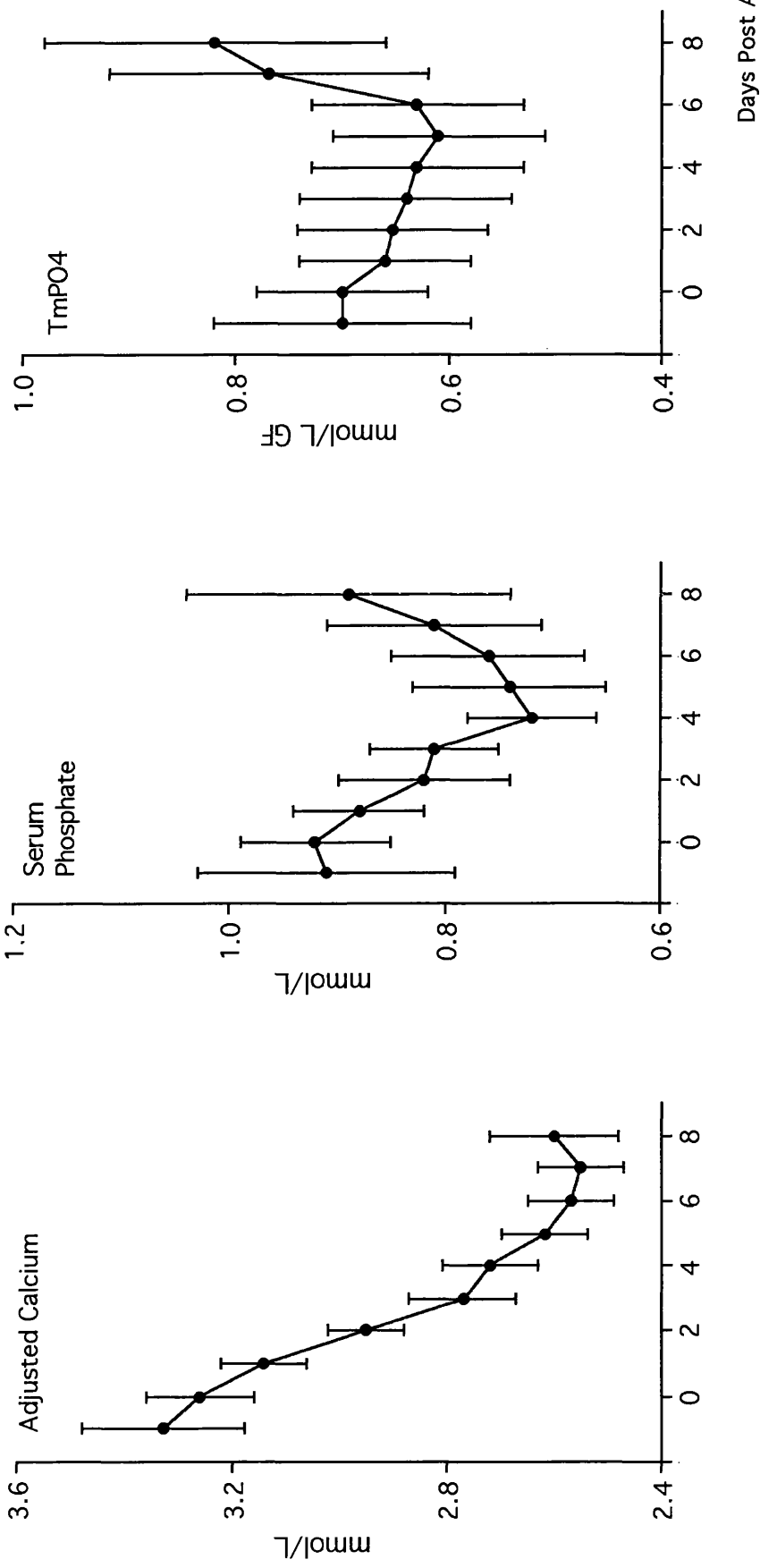
Figure 30 Daily changes in adjusted calcium, serum phosphate and threshold for renal tubular reabsorption of phosphate ($TmPO_4$) in patients with HCM following infusion of APD. Results are mean ± SEM.

significantly, reaching a nadir on day 5 (p<0.001) before increasing significantly on the final day of the study (p<0.01). NcAMP measurement showed a significant increase from the elevated basal concentration to a peak on day 5 (p<0.01) and then decreased significantly from this value by day 7 (p<0.05). UcAMP had a similar profile to NcAMP increasing significantly on day 5 (p<0.01) and decreasing significantly from this value by day 7 (p<0.05). PcAMP decreased significantly on day 3 (p<0.05) and increased significantly on day 6 compared to day 0 (p<0.02).

No patient from either group became hypocalcaemic during the study (all adjusted calcium results ≥2.20 mmol/L). Serum phosphate concentrations decreased following pamidronate from 1.05 (± 0.04) to 0.76 (± 0.05) mmol/L in Paget's disease and from 1.34 (± 0.06) to 0.62 (± 0.08) mmol/l in HCM (day 0 v day 7).

The concentrations of PTH (1-84) corresponding to adjusted calcium values obtained for each patient following pamidronate therapy are shown in Figure 31. The majority of patients with Paget's disease had detectable PTH (1-84); in only one sample (adjusted calcium 2.55 mmol/L) was PTH (1-84) undetectable. In patients with HCM having adjusted calcium concentrations greater than 2.85 mmol/L, only 9.8% of samples had detectable PTH (1-84) concentrations (1.0-2.4 pmol/L); between 2.6 and 2.85 mmol/L, 75.9% of samples had detectable PTH (1-84); below 2.6 mmol/L, 93.6% of samples had detectable PTH (1-84) (0.8-19.0 pmol/L).
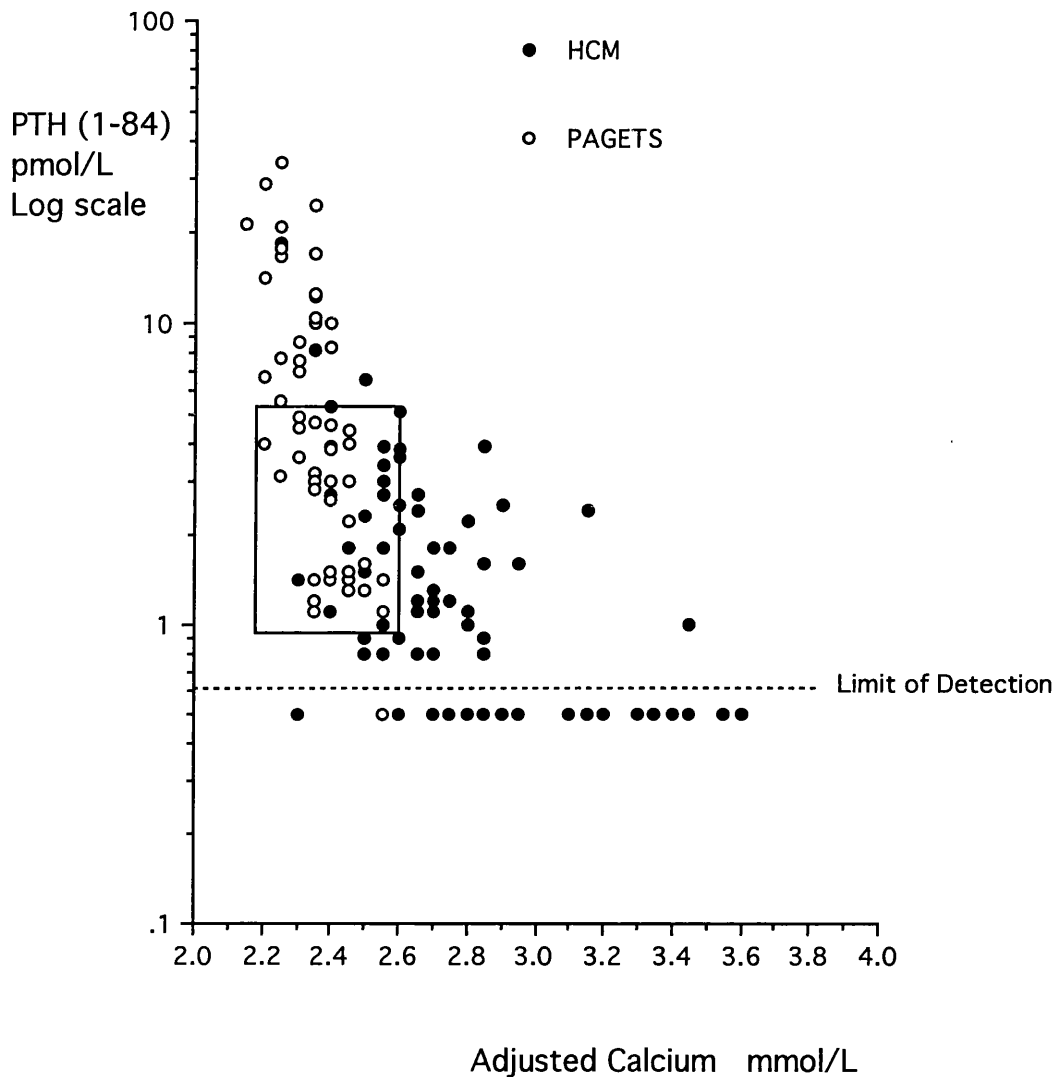
Figure 31   PTH (1-84) and corresponding adjusted calcium in
            patients with HCM (●) and Paget's disease of
            bone (○) before and after APD. All results
            obtained in the study for each patient are
            plotted. The rectangle includes all
            observations within the reference range for both
            PTH (1-84) and adjusted calcium.

The mean rate of fall in adjusted Ca throughout the study varied between patients from 0.06 to 0.27 mmol/day (mean 0.14). There was no relationship between this rate of fall and the time after pamidronate at which serum PTH (1-84) became detectable.

7.1.4 Discussion

The patients with Paget's disease initially exhibited the expected pattern of calcium homeostasis. Therapy with pamidronate induced a rapid but small decrease in serum calcium which was sustained throughout the study. This effect is consistent with previous reports of pamidronate therapy in Paget's disease and results from reduced osteoclastic resorption of calcium from bone (267,268,269,270,271). Using the immunoradiometric assay for PTH (1-84), it was possible to demonstrate a significant increase in secretion coincident with the fall in serum calcium. The concentration of PTH (1-84) rose progressively during pamidronate 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.

This increased secretion of PTH (1-84) following pamidronate therapy of Paget's disease could also be detected by measurement of indirect parameters of PTH action. PTH acts via a renal tubular receptor and cAMP second messenger to reduce the maximal tubular reabsorption

of phosphate (TmPO$_4$). Thus, the findings of decreased serum phosphate, decreased TmPO$_4$/GFR and increased NcAMP following pamidronate therapy are consistent with the known biological effects of increased PTH (1-84) secretion (46,281).

Patients with HCM showed the expected combination of hypercalcaemia and suppressed PTH (1-84) prior to pamidronate therapy. However, the pretreatment concentrations of TmPO$_4$/GFR and NcAMP were decreased and increased, respectively. Such observations are consistent with the recognised excretion in a proportion of these patients of PTHrP, a molecule that is structurally distinct from PTH but which contains a striking homology in the N-terminal amino acid sequence and so binds to the renal tubular PTH receptor (77,78,79).

Pamidronate therapy to the patients with HCM produced the expected sharp and prolonged decrease in serum adjusted Ca (272,273,274,275,276). At the end of the study, the mean calcium was within the reference interval with the range of patient values from 2.25-2.80 mmol/L. Therefore, this study confirms the effectiveness of pamidronate 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 a continuing increase to a mean concentration above the reference interval. The initial increase in PTH (1-84) occurred at a time when the mean

serum calcium was above the reference interval, and the highest PTH (1-84) concentrations above the reference range occurred when the mean serum calcium was within the reference interval with no subject hypocalcaemic. This current data contrasts with a previous report that PTH secretion only occurs as patients become hypocalcaemic (279). The data strongly suggest a resetting of the 'trigger point' for PTH (1-84) 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 (282). Previous studies using acute changes in calcium have shown both rate of change and direction of change to be important determinants of the PTH response in normal subjects and patients with secondary hyperparathyroidism (283,284). 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 major clinical implication for these results is related to the interpretation of PTH (1-84) concentrations in patients receiving pamidronate therapy. Confusion between the two major causes of hypercalcaemia (1°HPT and malignancy) will occur if a sample is obtained after treatment of hypercalcaemia, as in some cases of HCM modest lowering of Ca results in PTH (1-84) becoming detectable. It is clear that a valid diagnosis will only be made on a sample taken prior to any treatment being given.

The observed changes in $TmPO_4$/GFR and NcAMP following treatment of HCM with pamidronate require careful interpretation. If PTHrP is responsible for the abnormal pretreatment concentrations of these parameters in some patients, then the exacerbation of the abnormality (decreasing $TmPO_4$/GFR; increasing NcAMP) from days 2-5 after pamidronate may suggest increased secretion of PTHrP at this time, either as a direct consequence of the therapy or as a manifestation of the natural history of the disease. The paradoxical increase in $TmPO_4$/GFR and decrease in NcAMP on day 7 is especially interesting, as it occurs at a time of increasing PTH (1-84) secretion and is suggestive of decreased stimulation of the renal tubular PTH receptor.

This study was performed prior to the availability of validated assays for measurement of PTHrP (Chapter 8) and so the explanation of the results for NcAMP and $TmPO_4$ cannot be as comprehensive as one would wish. In recent studies, where PTHrP has been measured following pamidronate therapy, there has been little or no change in PTHrP concentrations in the week following treatment (285,286,287). These results may reflect differences in patient selection since the most recent studies have in the main analysed data on patients who had significantly elevated PTHrP levels and hypercalcaemia. Further investigation will be required to explain the NcAMP and $TmPO_4$/GFR results obtained in this current study.

Chapter 8

Parathyroid Hormone Related Protein
and Cyclic Adenosine Monophosphate

8.1.1 Introduction

Hypercalcaemia is a common finding in clinical practice and has many possible causes (75). The important differential diagnosis is between primary hyperparathyroidism (1°HPT) and hypercalcaemia associated with malignancy (HCM). Modern immunometric assays for PTH have the sensitivity and specificity to distinguish 1°HPT from non parathyroid causes of hypercalcaemia (100,101,129). It has been known for some time that certain malignancies are associated with the production of a humoral factor that has very similar properties and actions to PTH. This factor can cause hypercalcaemia, hypophosphataemia, increased nephrogenous cyclic adenosine monophosphate (NcAMP), renal conservation of calcium, phosphaturia and stimulate osteoclast activation (73,78,288). The term humoral hypercalcaemia of malignancy (HHM) was adopted to describe the syndrome of HCM in association with the cardinal features of an elevated NcAMP and a low or undetectable PTH (289). In the late 1980s the humoral factor responsible for HHM was cloned, sequenced and purified and because of the remarkable N-terminal sequence homology with PTH was named Parathyroid Hormone-Related Protein (PTHrP) (79,80). Immunoassays have recently been developed to measure PTHrP and several in-house methods have been described which provide direct evidence for the role of PTHrP in HHM (247,249,290). These assays also offer the potential value of diagnosing malignancy in those hypercalcaemic patients where the PTH (1-84) measurement is low or undetectable.

In this chapter the clinical value of first commercial assay available for the measurement of PTHrP the Nichols Institute (Allegro) PTHrP assay has been evaluated and the relationship of PTHrP with NcAMP investigated.

8.1.2 Subjects and Methods

Four subject groups were studied, (i) 31 normal volunteers, all fit, healthy, ambulant subjects, normocalcaemic not taking any medication known to affect calcium metabolism (mean age 32, range 19-60, female 11, male 20) (ii) 29 fit ambulant elderly subjects (mean age 76 years, range 60-88, female 17, male 12) (iii) 20 patients with 1°HPT (mean age 58, range 38-75, female 12, male 8) (iv) 95 patients with HCM (mean age 61, range 42-85, female 39, male 46). All patients with hypercalcaemia had an elevated adjusted serum calcium (>2.7 mmol/L) on more than one occasion. 1°HPT was diagnosed by the presence of hypercalcaemia and a serum PTH (1-84) concentration greater than 3.0 pmol/L in all cases, surgical removal of a parathyroid adenoma in 10 cases and evidence of an adenoma on technetium/thallium subtraction scan in 9 cases. In 88 patients with malignant disease the primary tumour was identified by a combination of radiology, surgery and histology; in 7 patients the primary tumour was not identified. Bone metastases were diagnosed by the presence of characteristic lesions that showed increased tracer uptake in radionuclide bone scans; however, not all patients had bone scans performed.

All subjects studied had venepuncture performed between 1000 and 1200h. Serum was obtained within 30 min for PTH (1-84) assay, Li Heparin plasma for calcium, phosphate, creatinine, albumin measurement, EDTA plasma for cAMP measurement and all samples for PTHrP estimation were collected into vacutainer tubes supplied by Nichols Institute containing aprotinin (500 kallikrein units/L), leupeptin (5 mg/L), Pepstatin (5 mg/L) and EDTA (100 mmol/L). Serum and plasma were stored at -70°C prior to hormone and cAMP assay. A 2-3h timed urine (0900-1300) was obtained on all normal volunteers, all 1°HPT patients and 61 patients with malignancy (urine was not available on all patients with malignancy due to the severity of illness in some patients).

PTH (1-84) was measured using an IRMA kit (Nichols Institute, San Juan Capistrano, CA), PTHrP using an IRMA kit (Nichols Institute, San Juan Capistrano, CA), plasma cAMP using a commercial RIA kit (Amersham International, Bucks), urine cAMP by an in-house RIA (Chapter 2) and NcAMP calculated by the method described by Broadus et al (46). Plasma calcium, phosphate, creatinine, albumin and urine creatinine were measured by standard automated methods (Technicon Tarrytown). Calcium was adjusted for albumin as described by Gardner et al (106). PTHrP and PTH peptides used in interference and recovery experiments were obtained from Peninsula (Peninsula Laboratories Europe Ltd, St Helens, Merseyside, UK).

8.1.3 <u>Results</u>

Specificity of the PTHrP IRMA was assessed by the addition of human PTH (1-34) and PTH (1-84) in 10, 100 and 1,000 pmol/L concentration. No cross reactivity or assay interference was observed. The assay does not recognise human PTHrP (1-34) fragments.

Parallelism of the assay was assessed by dilutions of 3 day and 7 day human keratinocyte culture medium, known high patient PTHrP samples and a spiked (PTHrP (1-86)) low plasma pool. The results are shown in Table 11.

Replicate analysis of the zero standard defines the sensitivity of this assay as 0.3 pmol/L. A precision profile obtained using 6 assays is given for comparison (Figure 32). Using the 22% CV as an estimate of assay sensitivity (98) a value of 0.7 pmol/L is obtained. The degradation of PTHrP observed in plasma when no protease inhibitors were included in the collection tubes is shown Table 12.

Figure 33 shows the PTHrP and PTH (1-84) results obtained in all subjects studied comparing normals against patients with 1°HPT and HCM. PTHrP was greater than the detection limit of 0.7 pmol/L in 78% of normals and a reference range of <0.7-2.6 pmol/L has been established by calculating the 95% reference interval for the values obtained in these subjects. All ambulant elderly patients had detectable

Sample	Dilution	Observed (pmol/L)	PTHrP Concentration Corrected for Dilution (pmol/L)
Plasma	Undil	8.4	8.4
	1:2	4.2	8.4
	1:4	2.0	8.0
	1:8	1.1	8.8
	1:16	0.6	9.6
Plasma	Undil	13.3	13.3
	1:2	6.4	12.8
	1:4	3.4	13.6
	1:8	1.4	11.2
	1:16	0.9	14.4
Keratinocyte Fluid (3 day)	Undil	>44.0	>44.0
	1:5	26.0	130.0
	1:10	14.0	140.0
	1:20	6.1	122.0
Keratinocyte Fluid (7 day)	Undil	6.7	6.7
	1:5	1.4	7.0
	1:10	0.6	6.0
	1:20	<0.3	-
Spiked plasma (PTHrP (1-86))	Undil	50.0	50.0
	1:2	26.0	52.0
	1:4	13.4	53.6
	1:8	7.6	60.8
	1:16	3.5	56.0
	1:32	1.6	51.2

Table 11   Parallelism of PTHrP Assay
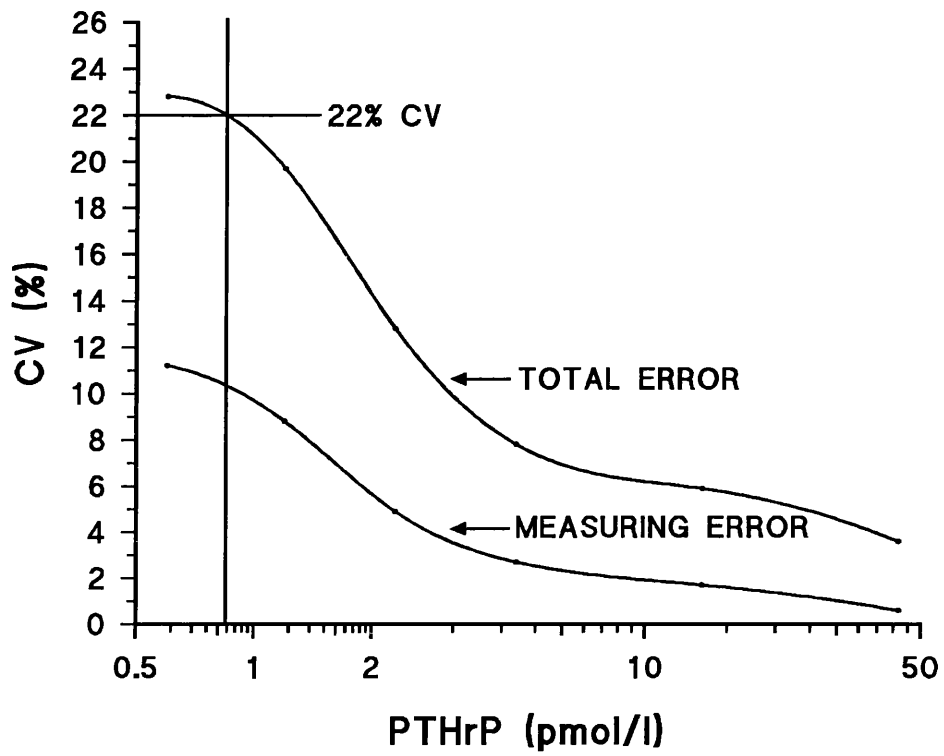
Figure 32 Precision profile of Nichols Institute PTHrP assay

PTHrP concentrations (pmol/L) obtained using different collection tubes

Samples	EDTA with Protease Inhibitors	EDTA Alone
1	7.9	3.4
2	3.7	1.9
3	3.4	3.3
4	21.4	17.7
5	3.3	2.4

PTHrP concentrations (pmol/L) obtained after rapid or delayed separation of plasma

Samples	Separated within 30 min of collection into EDTA with Protease Inhibitors	Plasma separated after 3h at Room Temperature in EDTA with Protease Inhibitors
6	9.6	7.7
7	5.1	4.1
8	2.8	2.1
9	2.5	1.9
10	2.5	1.7

Table 12   Degradation of PTHrP in plasma in the absence of protease inhibitors or following a prolonged delay before separation

PTHrP values (range 0.7-2.8 pmol/L, mean 1.7 pmol/L), but these were not significantly different from values for the "young normal" subjects (range <0.7-2.5 pmol/L).

The distribution of PTHrP results in patients with HCM subdivided into tumour type is shown in Figure 33. Table 13 gives the histological diagnosis in each patient.
The relation between PTHrP concentrations (values above the detection limit) and NcAMP or adjusted Ca is shown in Figure 34. A significant correlation exists between PTHrP and NcAMP (r=0.63, p<0.01) and a weak correlation between PTHrP and adjusted calcium (r=0.46, p=0.01). The correlation was poor between PTHrP and serum phosphate (r=0.28, p=0.10), total alkaline phosphatase (r=0.35, p=0.05), serum creatinine (r=-0.19, p=0.28), and creatinine clearance (r=0.21, p=0.14).

NcAMP was measured in 61 patients with HCM. 12 had low or undetectable NcAMP (<10 nmol/L GF), leaving 49 patients classified as having HHM. Of these 49, 24 had above-normal PTHrP with low or undetectable PTH (1-84), 13 had detectable PTHrP with low or undetectable PTH (1-84), 4 had above normal PTHrP with above-normal PTH (1-84) and 8 had low or undetectable PTHrP and PTH (1-84). A sub group of patients is shown in Table 14 in whom the NcAMP value is inappropriate for the calcitropic hormones PTHrP and PTH (1-84). The histological diagnosis is given for each patient and it can be seen that a high percentage of these
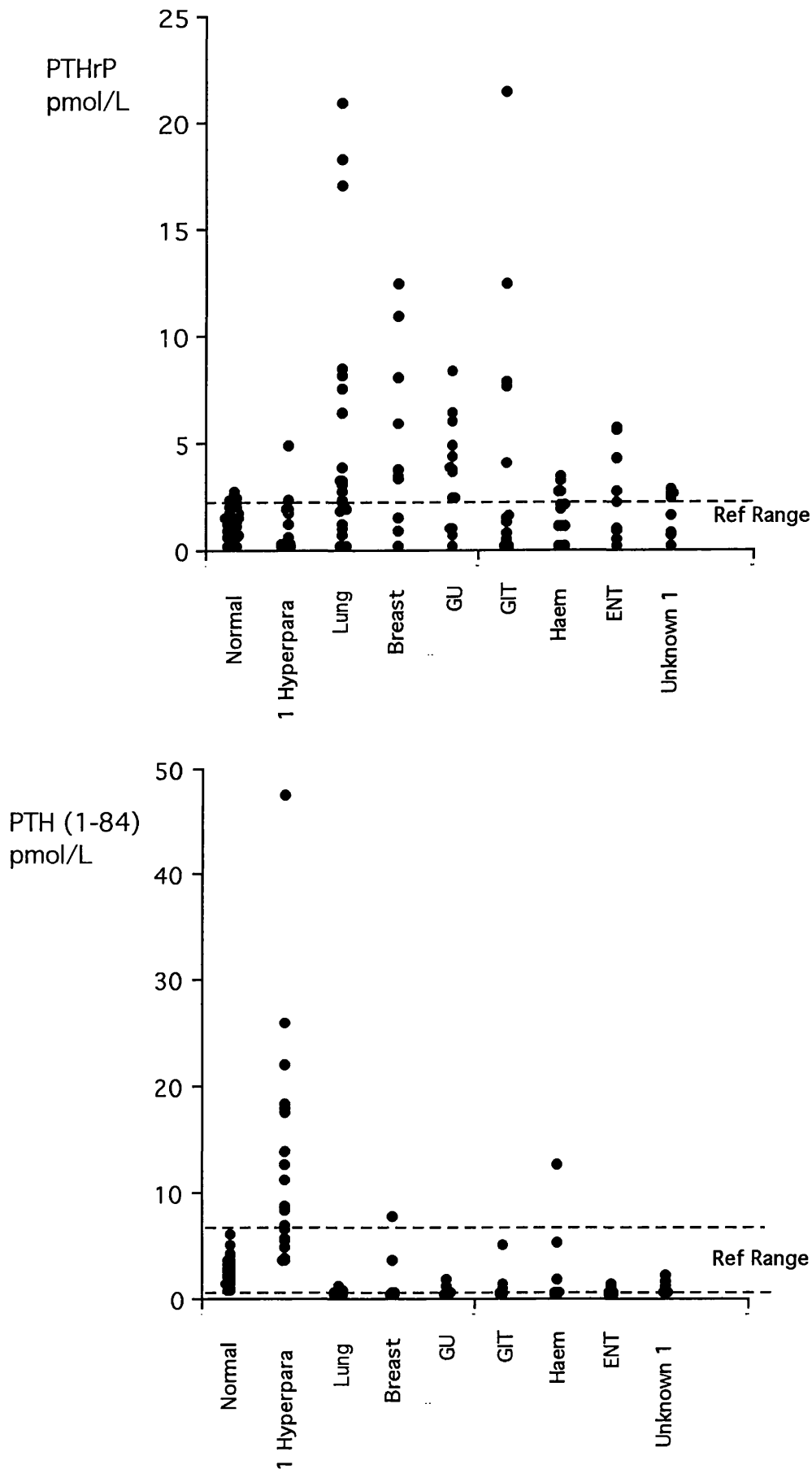
Figure 33   PTHrP and PTH (1-84) in normal subjects, patients with 1°HPT and HCM. The HCM patients have been divided into broad groups based on the primary source of tumour.

Tumour Type	Histological diagnosis	Elevated PTHrP	
Lung (n = 26)		12/26	46%
	Squamous Carcinoma	9/14	64%
	Small Cell Carcinoma	0/7	0%
	Adenocarcinoma	1/2	50%
	Unknown	2/3	67%
Breast (n=11)		8/11	73%
	Ductal Carcinoma	3/4	75%
	Lobular Carcinoma	5/6	83%
	Papillary Carcinoma	0/1	0%
Genito Urinary (n=14)		9/14	64%
	Carcinoma Cervix	1/1	100%
	Carcinoma Ovary	0/1	0%
	Carcinoma Kidney	1/3	33%
	Transitional Cell Carcinoma Kidney	0/1	0%
	Hypernephroma	4/4	100%
	Transitional Cell Carcinoma Bladder	1/2	50%
	Carcinoma Prostate	2/2	100%
Gastrointestinal (n=15)		5/15	33%
	Adenocarcinoma Colon	0/5	0%
	Adenocarcinoma Stomach	0/1	0%
	Carcinoma Stomach	2/2	100%
	Carcinoma Oesophagus	2/3	67%
	Poorly Differentiated Carcinoma Oesophagus	0/1	0%
	Somatostatinoma Pancreas	1/1	100%
	Carcinoma Rectum	0/2	0%
Haematological (n=14)		4/14	29%
	Myeloma	4/9	44%
	Lymphoma	0/3	0%
	Leukaemia	0/2	0%
Ear Nose Throat (n=7)		3/7	43%
	Squamous Carcinoma Larynx	3/3	100%
	Anaplastic Carcinoma Larynx	0/2	0%
	Squamous Carcinoma Tongue	0/1	0%
	Squamous Carcinoma Hypopharynx	0/1	0%
Others, unknown (n=8)		3/8	38%
	Melanoma	0/1	0%
	Adenocarcinoma	0/3	0%
	Poorly differentiated Carcinoma	0/1	0%
	Squamous Carcinoma	3/3	100%

Table 13   Tumour analysis in Patients with HCM
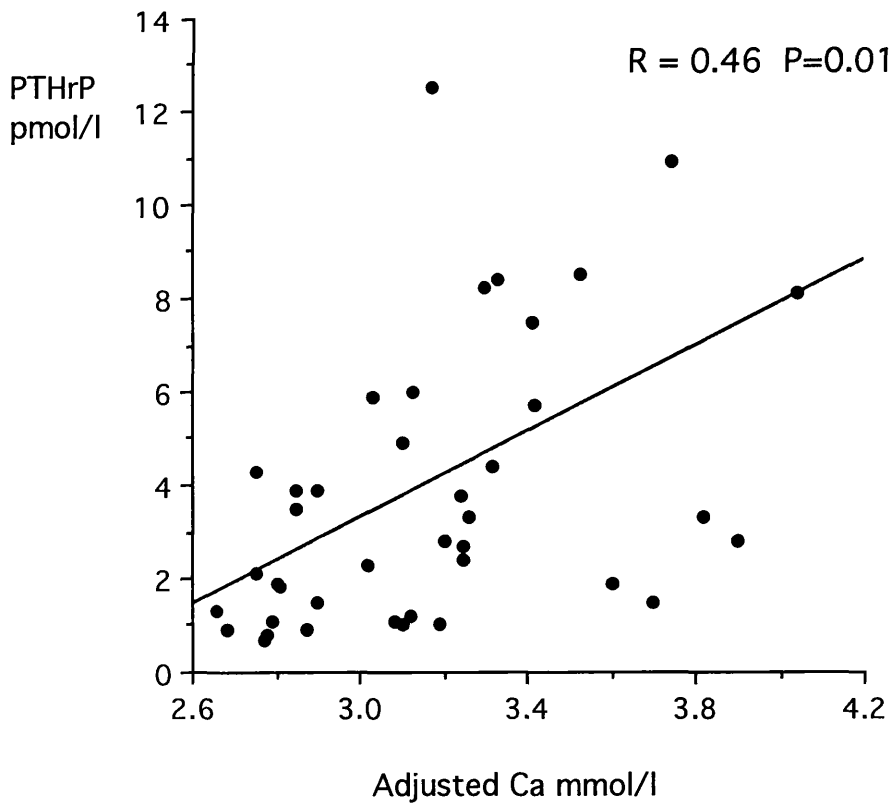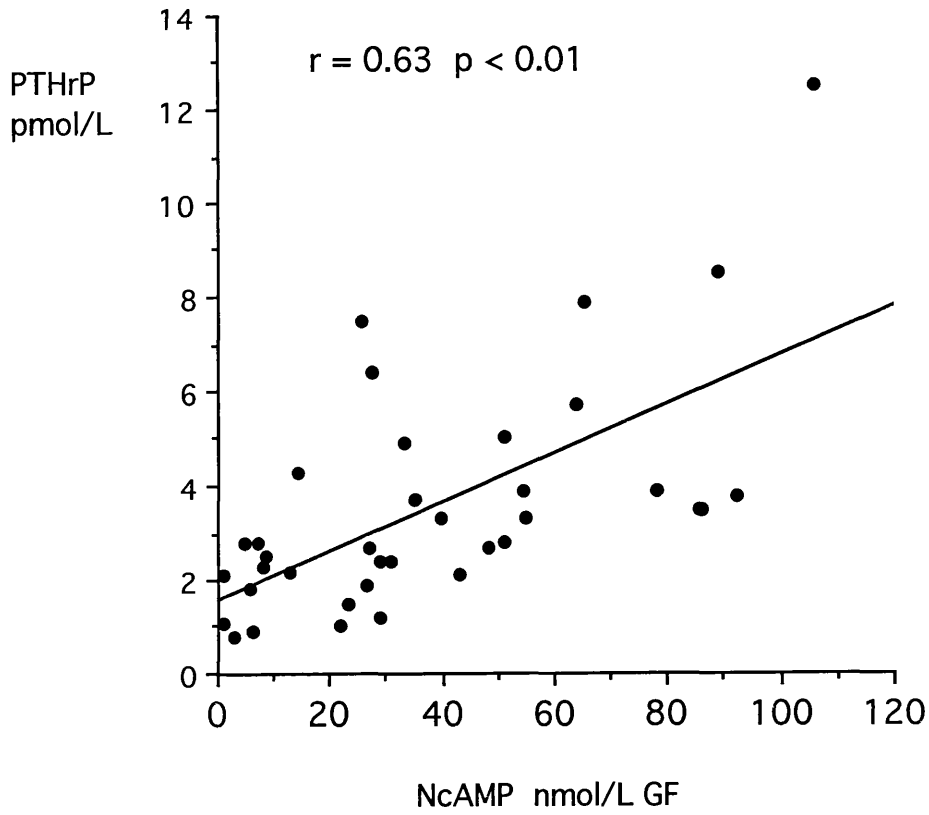
Figure 34   Linear regression of PTHrP and NcAMP and PTHrP
and adjusted calcium in patients with HCM.

Tumour type	PTHrP pmol/L	PTH (1-84) pmol/L	NcAMP nmol/L of GF
Small cell carcinoma, lung	<0.3	<0.7	12.6
Small cell carcinoma, lung	<0.3	<0.7	17.8
Small cell carcinoma, lung	<0.3	<0.7	19.0
Small cell carcinoma, lung	<0.3	<0.7	80.4
Small cell carcinoma, lung	<0.3	0.7	145.0
Carcinoma, larynx	<0.3	<0.7	14.5
Adenocarcinoma, stomach	<0.3	0.8	14.4
Poorly differentiated carcinoma, oesophagus	<0.3	<0.7	17.6

Table 14 Tumour type in patients with HCM whose NcAMP is inappropriate

for an undetectable or low PTHrP and PTH (1-84)

patients (62.5%) have small cell carcinoma of lung.

8.1.4 <u>Discussion</u>

PTHrP was elevated in a high percentage of patients with solid tumours and in those patients with tumours associated with HHM. These results add further weight to rapidly accumulating data on the role of PTHrP as a mediator of HHM. In patients with squamous cell carcinomas 68% had elevated PTHrP, in keeping with recent findings (247,248,249) and in patients with breast carcinoma 73% had elevated PTHrP as was predicted using indirect measurements of PTHrP action (291) and has been shown in more recent studies using PTHrP measurements (292). Malignancies of the genito-urinary tract gave elevated PTHrP if the kidney or prostate was the primary tumour source and we rarely found elevated PTHrP in gastrointestinal malignancies or haematological malignancies. The differences in the percentage elevation found between reported studies will undoubtedly reflect the patient population studied and the tumour histology. Comparison of this HCM population studied with that of previous publications (249,290) indicates that this study had a greater percentage of gastrointestinal, haematological, and head and neck malignancies but a lower percentage of breast and gynaecological malignancies. The percentage of squamous carcinomas in this study population was also lower than in the previous studies. These results show elevations of PTHrP in a lower percentage of patients than has been

reported in some published assays and may reflect a number of important problems, apart from patient selection, in the measurement of PTHrP.

An important observation is that PTHrP was detectable in some 78% of normals sampled. This normal population reflected a broader spectrum of subjects than previous studies and contained a more representative percentage of normal elderly subjects who are likely to be the age group who have malignant disease. These data and that of Pandian and his colleagues (249) demonstrate that PTHrP is unstable in plasma and must be collected into tubes with protease inhibitors. When samples are properly collected PTHrP is measurable in a high percentage of healthy subjects. These findings indicate that PTHrP is circulating in normal individuals and may have a role in calcium and phosphate homeostasis.

It is also possible that the assay for PTHrP is being affected by non specificity and matrix effects which has resulted in an increased detection of PTHrP in normals. Such effects can often cause positive bias and interference in immunoassays and are more often observed close to the detection limit of the assay. Against this possibility is the evidence that PTHrP has been detected and measured in other assays in normals and the average concentration observed is close to 1.0 pmol/L. However further work is required to fully investigate the possibility of matrix

effects possibly using affinity chromatography of samples, charcoal extraction and antibody blocking tubes.

An important component of the validation of any PTHrP assay is the association between NcAMP production and PTHrP concentration. NcAMP has been used as one of the main indirect indicators of PTH (1-84) and PTHrP activity. Classification of patients with HCM based on NcAMP has been advocated in the past (73,288); thus it would be expected that PTHrP should correlate with PTHrP. The cross correlation analysis of PTHrP above the reference range and NcAMP was excellent, further validating the value of this assay and confirming the role of PTHrP in the syndrome of HHM. In the patients in whom we measured all three variables, an elevated PTHrP and a low or undetectable PTH (1-84) were associated with an inappropriate or elevated concentration of NcAMP, ranging from 14.1 to 105.6 nmol/L GF.

A subgroup of patients with low PTHrP and low or undetectable PTH (1-84) with inappropriate or elevated NcAMP was detected. In these patients a factor which is not detected as PTHrP or PTH (1-84) which is capable of stimulating NcAMP production and causing hypercalcaemia may be produced. Alternatively these patients may be producing an active fragment of either PTHrP or PTH which is not detected by the IRMA assays due to the size of the fragments. A less likely possibility is that another new

molecule is being produced that has a hypercalcaemic action and can stimulate NcAMP production. It is interesting that small cell carcinoma of lung is very prevalent in this group of patients as a recent publication (293) has suggested that all major lung cancer cell types can produce PTHrP and that small cell lung cancer cell lines produce PTHrP whose molecular weight is 3,500. A PTHrP molecule of this size would not be measured in this assay and it is possible that other patients studied have tumours which may, by a mechanism of post translational processing, produce PTHrP of varying sizes. Such results also emphasise the continuing importance of NcAMP measurements when studying this group of patients and evaluating PTHrP assays.

At present, due to its poor specificity, PTHrP has no role in detecting patients with malignant disease prior to developing hypercalcaemia. In those patients with proven malignancy who were normocalcaemic only 15% had elevated plasma PTHrP. This is in keeping with previous reports of elevated NcAMP in groups of normocalcaemic patients with lung neoplasms and mixed neoplasms (288).

PTHrP was elevated in 1 patient with 1°HPT who to date has no evidence of having a malignancy. This result is consistent with the findings of other groups and suggests that although PTHrP DNA and mRNA may be present in parathyroid adenomas (241,243) the production of PTHrP by

this tissue is insufficient to elevate circulating PTHrP in most patients with 1°HPT. The incidence of simultaneous 1°HPT and malignancy in this study is higher than had previously been reported or expected (294,295) and is in keeping with recent findings (247,248,249). Such an association is of clinical importance as treatment of both the condition and the hypercalcaemia would be influenced by the diagnosis. This data also emphasises the importance of obtaining samples for PTH (1-84) estimation prior to treatment of hypercalcaemia as lowering the calcium can result in an increase of PTH (1-84) prior to restoring normocalcaemia which could result in diagnostic confusion (Chapter 7).

These results indicate the value and limitations of this new PTHrP assay in investigating patients with hypercalcaemia. The assay will confirm the presence of PTHrP-producing tumours in a high percentage of patients with hypercalcaemia. An increase of PTHrP may help indicate the likely type of tumour present and the possible primary source of tumour. Treatment of hypercalcaemia may be influenced by the finding of an increased PTHrP since bisphosphonate treatment, whilst the best therapy available (296), is now known to be less effective in patients with HCM and PTHrP secretion (297). The combination of antibody specificities and assay technology means that circulating forms of PTHrP smaller than PTHrP (1-72) will not be detected, so that the percentage of PTHrP-producing tumours

may be underestimated. NcAMP remains an important measurement in such patients and will continue to provide information on the factors responsible for HHM. PTHrP results obtained by this assay suggest that PTHrP may be degraded in plasma by proteases; therefore, inhibiting these enzymes may allow detection of PTHrP in normal subjects. This possibility is of obvious importance and requires further research and validation using alternative assays before the true significance of the finding will be known.

Chapter 9

Cyclic AMP Production *in-vitro* Studied

Using Superfusion of Rat Renal Tubules

9.1.1 Introduction

Superfusion of dispersed cells is a technique that has been used to study the production and direct action of many hormones at the cellular level (204,298,299,300). Such a technique overcomes several of the problems of static systems and results in improved sensitivity, reduces the possibility of accumulation of products or metabolites and will minimise possible unwanted effects such as negative feedback of end organ response or degradation of the molecule of interest. The methods also allow assessment of dose response effects and the efficacy of inhibition using the same tissue preparation over a short period which will minimise biological variation. A modification of the method established by Lowry and McMartin (204) has been developed to study dispersed rat adrenal cells using a column superfusion technique (301) which is readily adaptable to other cell types.

The biological activity of PTH can be quantified in terms of cAMP production by kidney cells either *in-vivo* or *in-vitro* (43,46,54,55,67). Stimulation of the kidney or renal cells by PTH has been investigated for many years and cAMP production by the kidney forms the basis of an important diagnostic test for pseudohypoparathyroidism (43,55). Renal tubular cells would therefore appear to be suitable candidates for studying the effects of PTH on cAMP generation by superfusion techniques.

Opinion has varied concerning the effect that acidosis can have on PTH action at both kidney and bone. Some authors claim that PTH action, assessed by UcAMP excretion or tubular reabsorption of phosphate or urinary calcium excretion, is inhibited in acute and chronic metabolic acidosis in rats or dogs (164,165) and that this contributes to the metabolic bone disease that occurs in renal failure. Others have failed to confirm this conclusion and have argued that prolonged metabolic acidosis has little or no effect on bone or renal responsiveness to PTH in man (302) and the actions of PTH are not impaired during chronic metabolic acidosis in dogs (303). In some studies acute metabolic acidosis has enhanced PTH action with increased cAMP production by isolated perfused dog bone (163) and increased NcAMP production in rats made acutely acidotic (304).

Alkalosis has little effect on the biological actions of PTH investigated to date although chronic metabolic alkalosis in rats results in an increased circulating PTH but no change in total serum calcium and a decrease in ionised calcium (305). Inducing acute alkalosis in rats has no effect on the tubular reabsorption of phosphate in response to PTH (164) nor any effect on the change in serum calcium concentrations following PTH injection (306).

In human studies that have been performed it has been demonstrated that arginine hydrochloride can block the

action of PTH on the renal tubule resulting in a significant decrease in UcAMP production (307). This effect if confirmed may be important for patients receiving parenteral nutrition where arginine can form a major component of the infusion. In patients on long term parenteral nutrition arginine infusion may result in inhibition of PTH action at the kidney and contribute to the metabolic bone disease observed in these patients. Since the superfusion technique is an ideal system for studying the effects of changing an individual component of the perfusate on biological activity this technique was adapted to study factors that may influence the production of cAMP by rat renal tubules in response to PTH stimulation.

9.1.2 Materials and Methods

Each superfusion experiment was performed using the kidneys obtained from 2 adult male Wistar rats (200-250 g) killed by cervical dislocation. Renal cortical tissue was dissected from the kidney, after removal of the capsule, and then minced using a scalpel and scissors. This tissue was then digested with 10 ml of enzyme solution (0.05% collagenase) (Worthington Biochemical Corporation, Freehold, NJ, U.S.A.) and 0.1% hyaluronidase (Sigma Chemical Company Ltd, Poole, Dorset, U.K.) in a Hepes-based collagenase buffer (Table 15) for 60 min in total at 37°C. The supernatant was removed after 30 min and the remaining cortical tissue mechanically dispersed with a pipette into

Collagenase Buffer Concentrate

NaCl	4.0 g
KCl	0.5 g
$CaCl_2$ $2H_2O$	0.7 g
HEPES	24.0 g
1M NaOH	66 ml
H_2O	100 ml
Collagenase	0.5 g

5 ml aliquots were stored at -50°C.   Each aliquot was diluted 1/10 prior to use in distilled H_2O with 2% BSA.

Superfusion/Suspension Buffer

Three separate concentrated buffers were combined together when required composed of 5 ml A, 5 ml B and 10 ml C made up to 100 ml with distilled H_2O containing 0.2% BSA with 4% glucose.

A   Calcium/Magnesium Concentrate

$MgCl_2$ $6H_2O$	0.52 g
$CaCl_2$ $2H_2O$	0.72 g
H_2O	200 ml

5 ml aliquots were stored at -50°C.

B   Calcium Concentrate

$CaCl_2$ $2H_2O$	1.8 g
H_2O	200 ml

5 ml aliquots were stored at -50°C.

C   Suspension Buffer Concentrate

NaCl	16.0 g
KCl	1.6 g
KH_2PO_4	0.6 g
Na_2SO_4	0.4 g
HEPES	28.8 g
TES	27.6 g
Tricine	26.0 g
NaOH	8.4 g
H_2O	400 ml

10 ml aliquots were stored at -50°C.

Table 15   Composition of buffers used in preparing renal tubules and in superfusion experiments

10 ml fresh enzyme solution for a further 30 min digestion at 37°C. The resultant suspension of isolated cortical tubules was filtered through nylon mesh (100 μm) and centrifuged at 300 g for 10 min. Pellets obtained were resuspended in 10 ml Hepes-based superfusion buffer (Table 15) and the washing procedure repeated twice. The final tubule pellet was resuspended in 2-3 ml superfusion buffer and a tubule count performed using a Buchner Haemocytometer. Viability of the tubules was assessed using the Typan Blue exclusion technique. An identical volume of tubule preparation was introduced onto the top of especially designed Teflon columns (301). 4 columns were run simultaneously. The tubules were retained on top of Sephadex G50 coarse gel (Pharmacia, Milton Keynes, UK) which was retained by 30 μm nylon gauze. A reservoir of Hepes-based superfusion buffer, all tubing (including a debubbler) and the 4 teflon columns were maintained at 37°C in a water bath. Fluid was pumped from the reservoir through the columns from top to bottom by means of a Technicon continuous flow pump (Technicon Tarrytown USA) via continuous flow tubing. The flow rate was controlled by altering the tubing diameter so that a rate of 600 μL/min was obtained. Effluent from the bottom of the columns was collected at 2 min intervals and stored at -20°C before analysis. The volume of effluent obtained was measured accurately by weighing. A line diagram of the perfusion apparatus outlining the components of the system is shown in Figure 35.
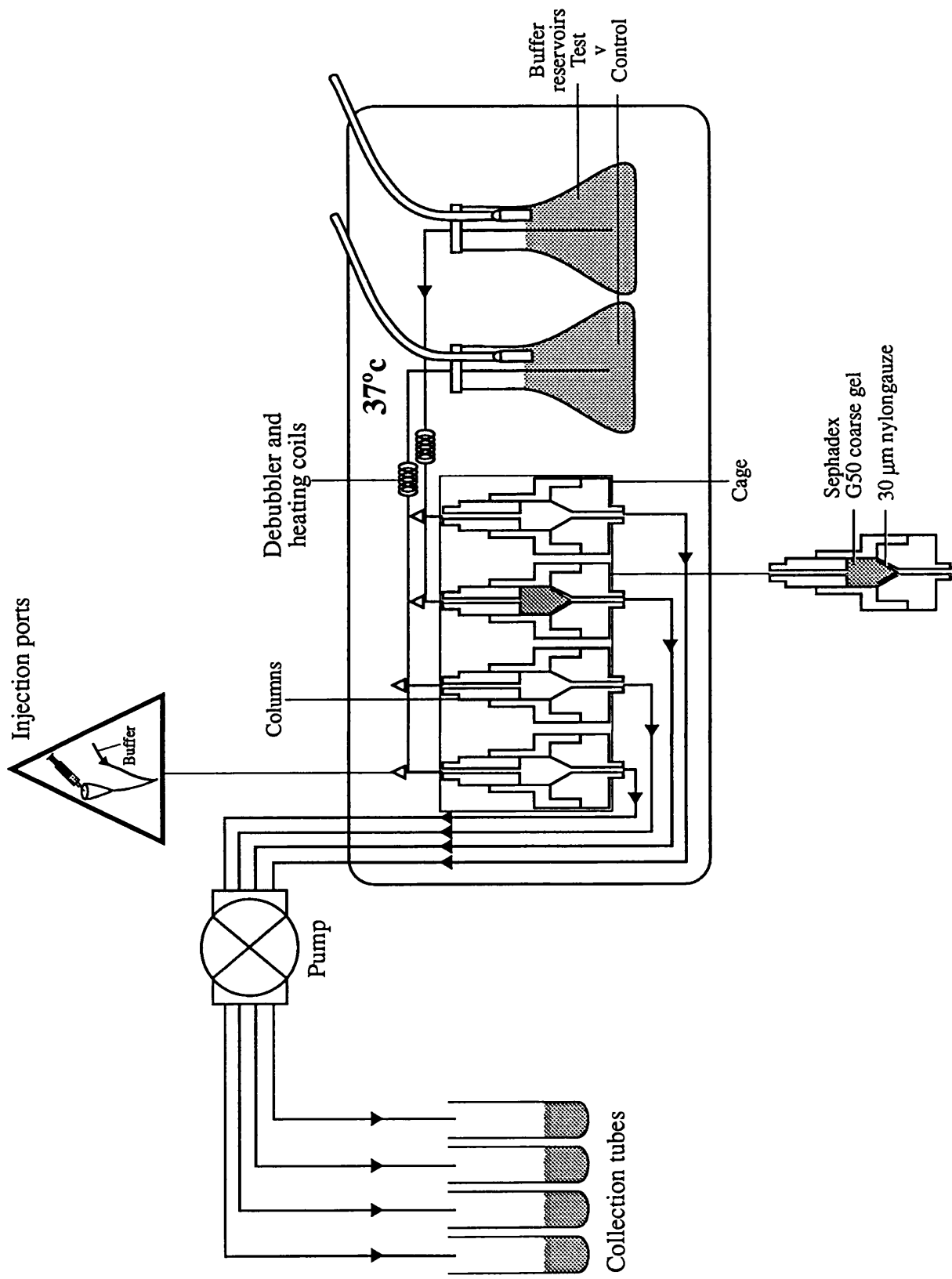
Figure 35. Column Superfusion Apparatus

In initial experiments to establish optimum conditions for stimulation all 4 columns were perfused with standard Hepes superfusate buffer. Subsequently 2 columns were perfused with standard buffer and 2 with test perfusates. The test perfusates contained standard Hepes based buffer superfusates with the addition of a) HCl (BDH, Poole, Dorset, UK) to produce a pH of 7.1 b) NaOH (BDH) to produce a pH of 7.65 and c) arginine hydrochloride (Sigma Chemical Company Ltd) at a concentration of 7 mmol/L. Experiments were performed in the absence and presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) (Sigma Chemical Company Ltd). IBMX was added in an attempt to optimise cAMP concentration by preventing phosphodiesterase degradation of cAMP in the eluent.

Stimulation was performed using PTH (1-84) (National Institute for Biological Standards, South Mimms, Potters Bar U.K.) or synthetic bovine PTH (1-34) (Sigma Chemical Company Ltd). PTH was dissolved in 0.1% acetate buffer pH 4.0 and stored at -50°C in 100 μL aliquots containing 250 IU per 100 μL. An aliquot was thawed and diluted in the appropriate perfusate buffer just prior to use.

Cyclic AMP was measured in an in-house radioimmunoassay (97) following acetylation of 500 μL of sample with 5 μL of an acetylation mixture containing 2:1 (vol/vol) triethylamine/acetic anhydride (BDH). This assay has a sensitivity of 30 pmol/L and a between batch CV of <12%

across the range 45-400 pmol/L. The characteristics of the antibody have been described Chapter 2.

Cyclic AMP in the comparative experiments (pH and Arg HCl) was expressed as total output, which was estimated from the volume of effluent and concentration of cAMP obtained, per 10^6 tubules per PTH pulse. Comparisons were made of the mean of 3 separate experiments involving four separate periods of stimulation with PTH. The columns were rotated clockwise following each experiment so that control and test columns varied. Statistical differences were evaluated using Mann-Whitney U statistics for each experiment.

9.1.3 <u>Results</u>

The viability of the tubular cells as measured by the Typan Blue exclusion technique varied between 95-98%. Cyclic AMP production obtained when the tubules were allowed to equilibrate on the columns for 60, 90 and 180 min before stimulation with PTH (1-84) is shown Figure 36. An initial dose-response study was performed which indicated that 2.5 units of bovine PTH (1-84) would result in an accurately measured cAMP response from the tubules. An erratic production of cAMP was observed when tubules were equilibrated for 60-90 min, following 90 min equilibration a stable reproducible response was obtained. After 180 min equilibration cAMP output became inconsistent when stimulation was attempted beyond 196 min. In all

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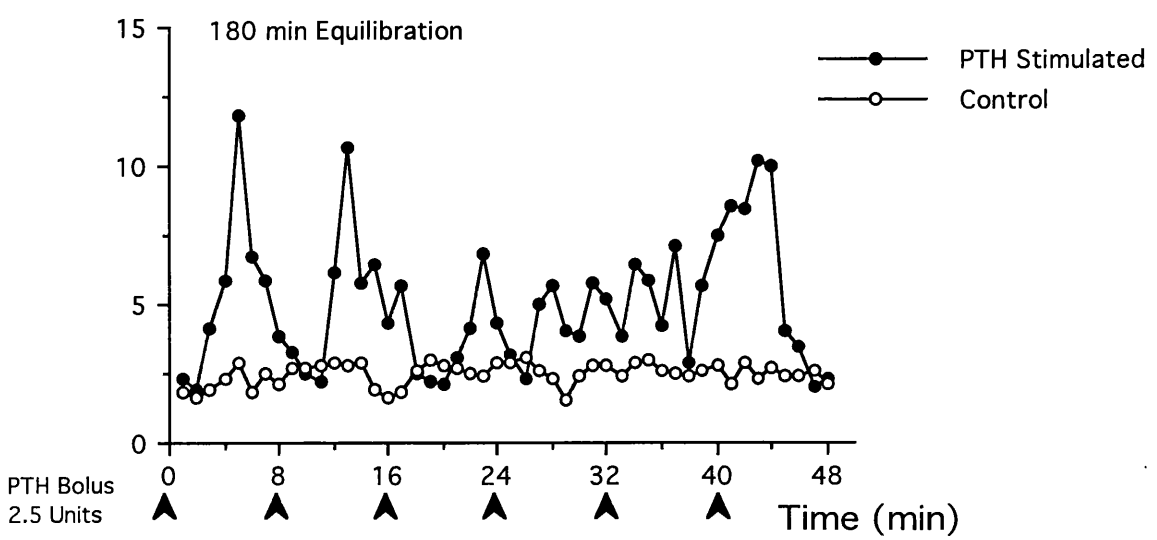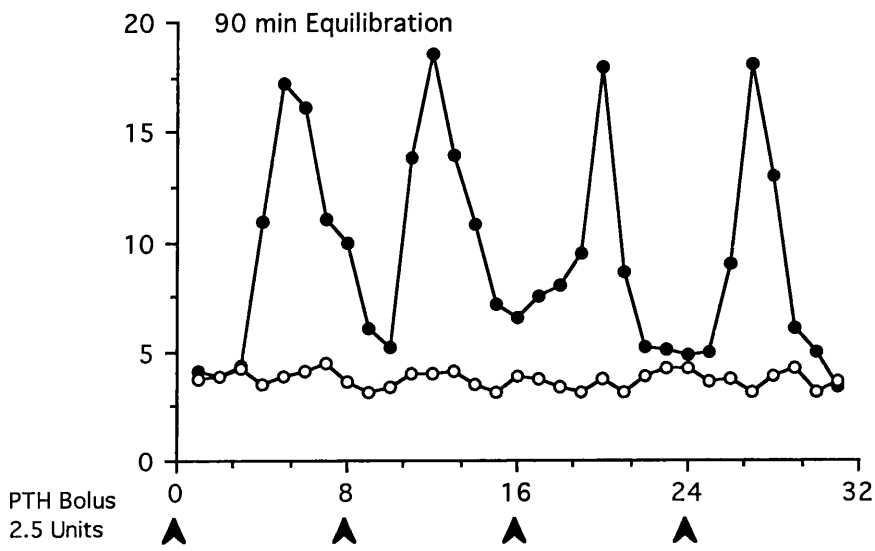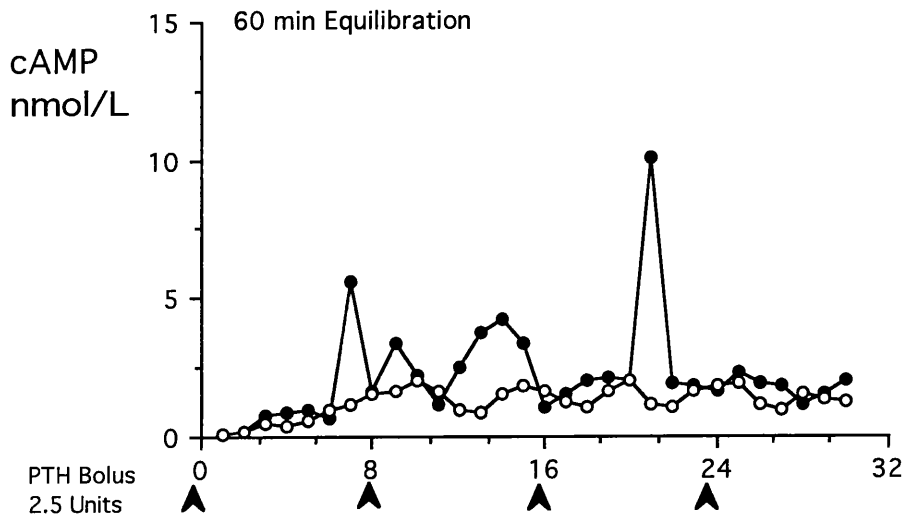
Figure 36   Renal tubules stimulated with bovine PTH (1-84) after 60, 90 and 180 min equilibration on the superfusion columns. Each line represents the results from a single column. PTH was introduced as a bolus on top of the columns at times indicated by the arrows.

subsequent experiments the tubules were allowed to equilibrate on the columns for 90 min before stimulation experiments were performed. Figure 37 shows the cAMP produced by the tubules when stimulated by bovine PTH (1-84) in the presence and absence of 1 mM of the phosphodiesterase inhibitor iso-butyl-methylxanthine (IBMX) and when unstimulated with IBMX present. For all subsequent experiments 1 mM IBMX was added to the superfusion buffer. The responses to repeated stimulation for 75 min after 90 min equilibration on the columns are shown Figure 38. Comparison of the tubule responses from 2 columns stimulated in an identical manner are shown Figure 38a for control v stimulated and 38b for 2 columns stimulated with PTH.

Figure 39 shows the dose response curves obtained for tubule stimulation using bovine PTH (1-84) and synthetic bovine sequence PTH (1-34). An equivalent response to both molecules is observed over the range of concentrations studied and the response is consistent and reproducible over three experiments.

Figures 40, 41 and 42 show the tubule responses obtained when perfusion was performed with buffer at pH 7.4, pH 7.1 (Figure 40), pH 7.4, pH 7.65 (Figure 41) and 7 mM arginine hydrochloride compared to control (Figure 42). Table 16 gives the mean values obtained and the statistical comparisons between each group of experiments. There is a
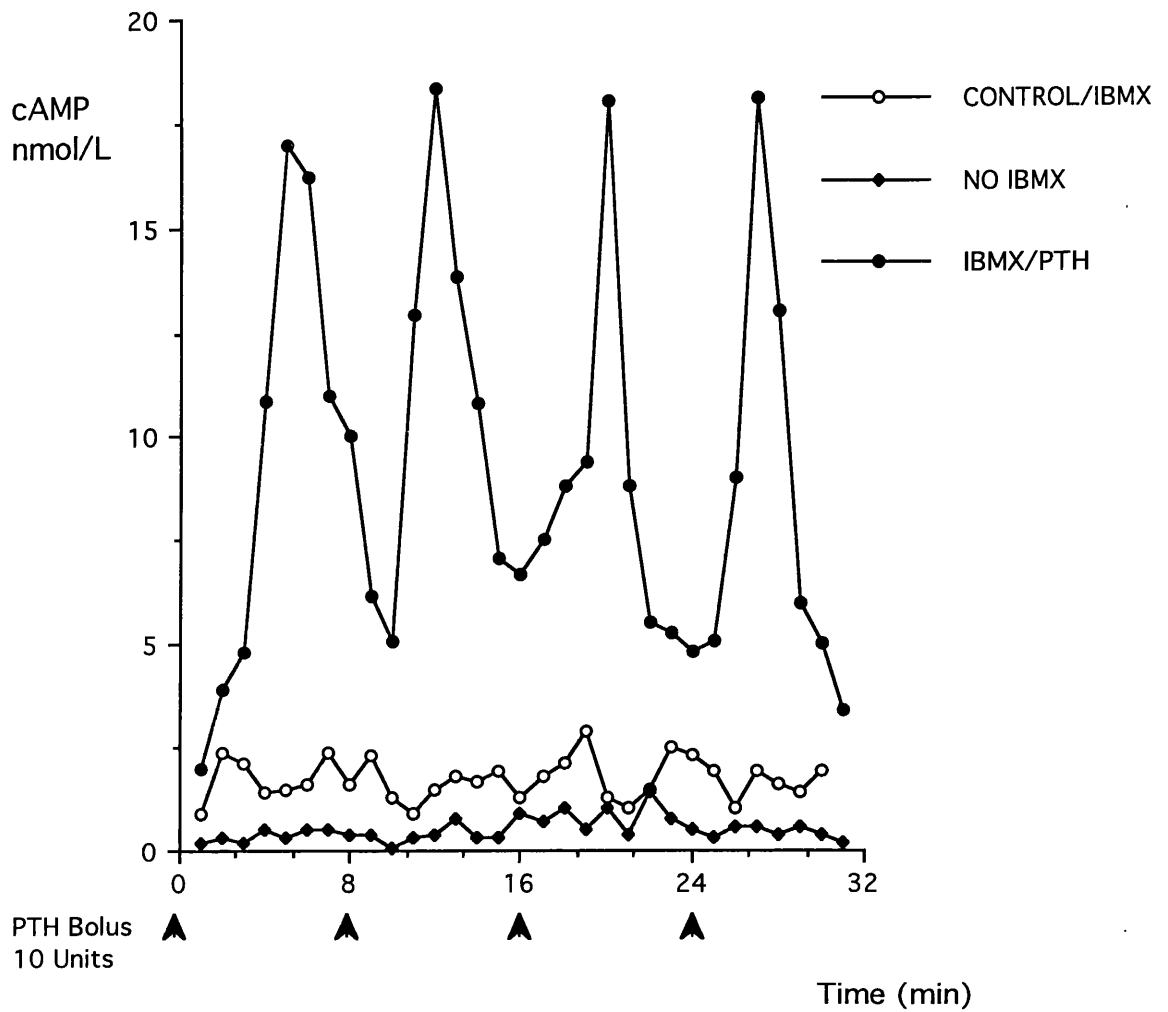
Figure 37 Renal tubules stimulated with bovine PTH (1-84) in the presence and absence of IBMX. Each line represents the results from a single column. PTH was introduced as a bolus on top of the columns at times as indicated by the arrows.
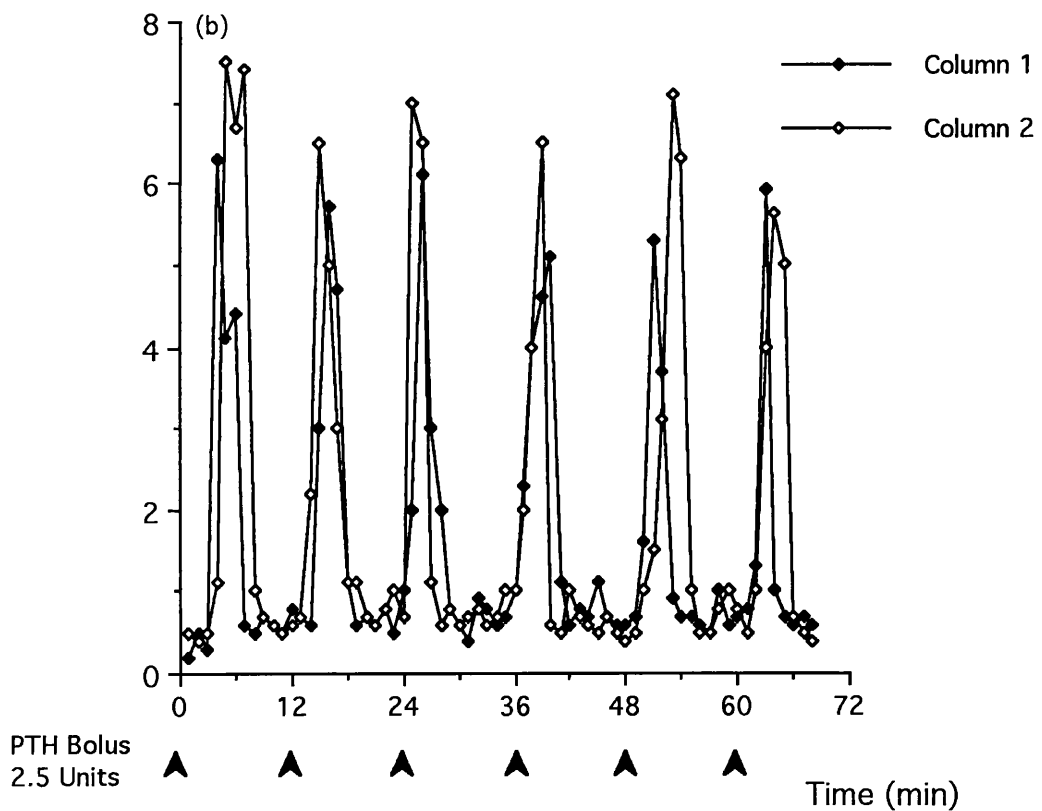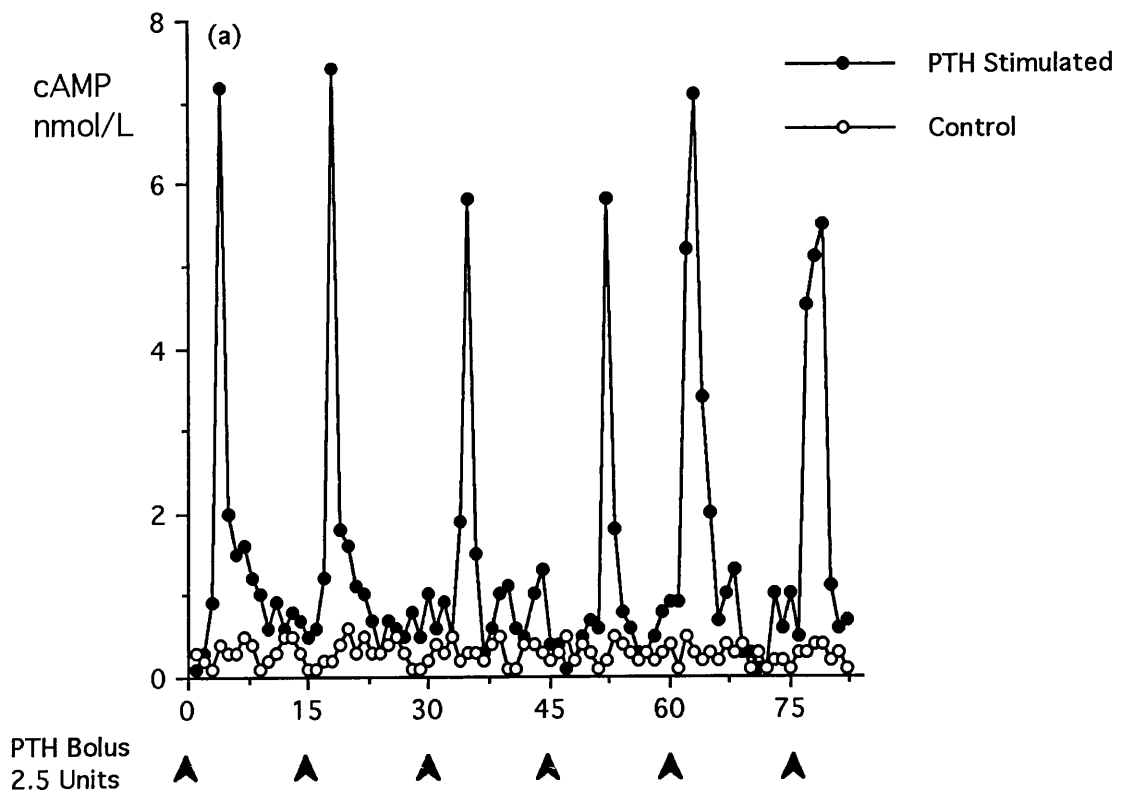
Figure 38   Renal tubules repeatedly stimulated with bovine
PTH (1-84) after 90 min equilibration on the
superfusion columns   a) stimulated column and
unstimulated control   b) two columns stimulated.
Each line represents the results from a single
column.   PTH was introduced as a bolus on top of
the columns at times indicated by the arrows.
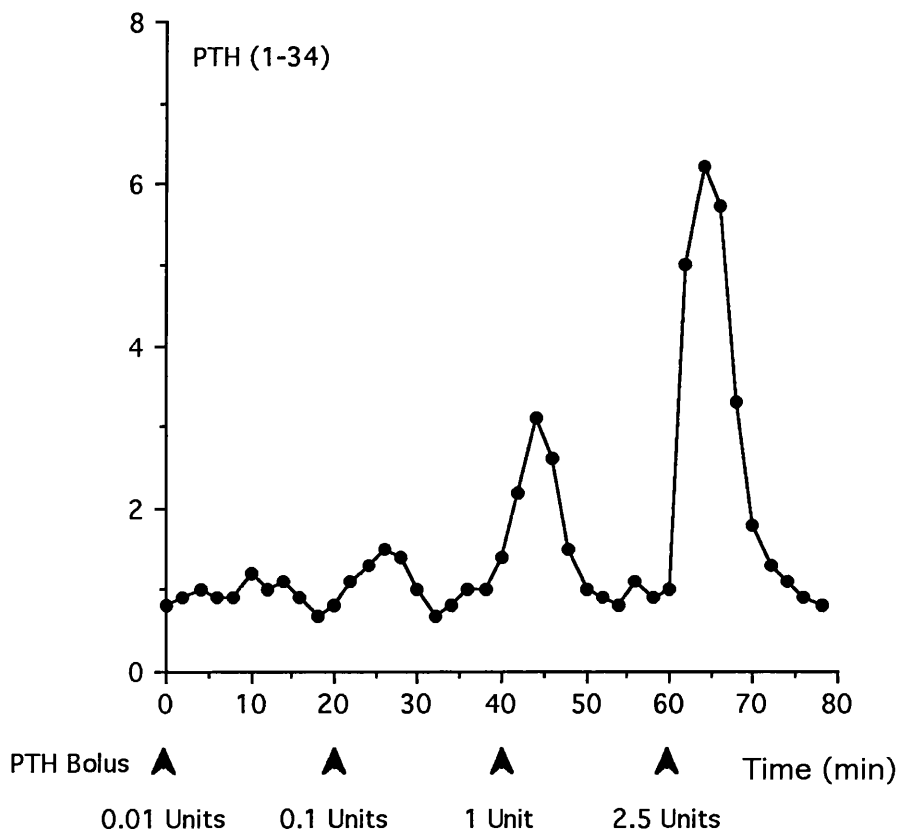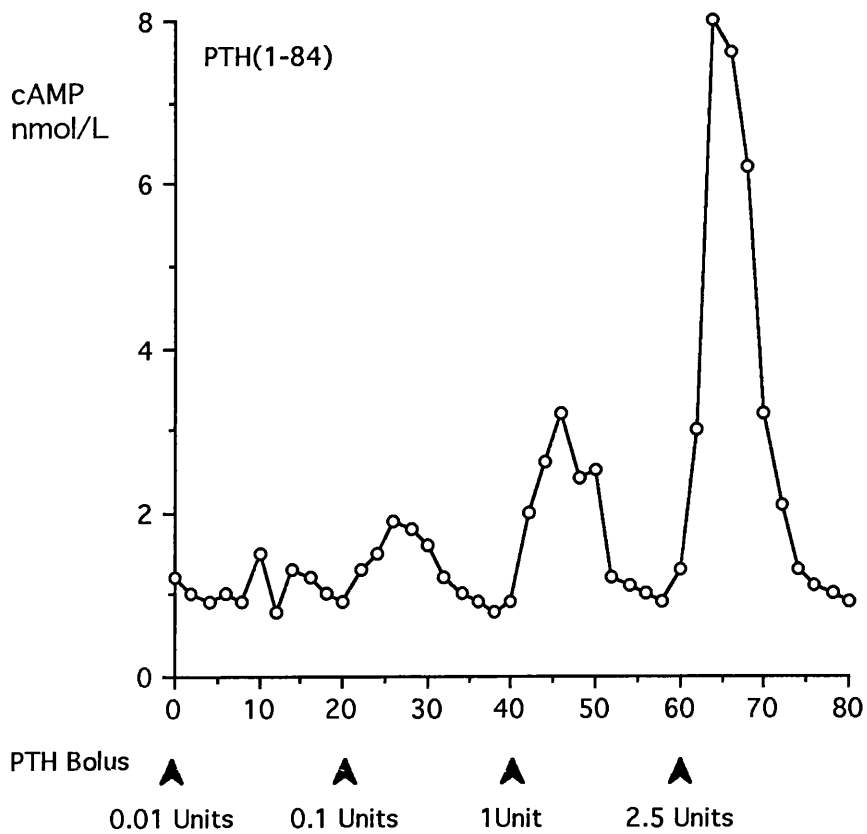
Figure 39   Dose responses to bovine PTH (1-84) and
            synthetic human PTH (1-34). Each line
            represents the results from a single column.
            PTH was introduced as a bolus on top of the
            columns at times indicated by the arrows.
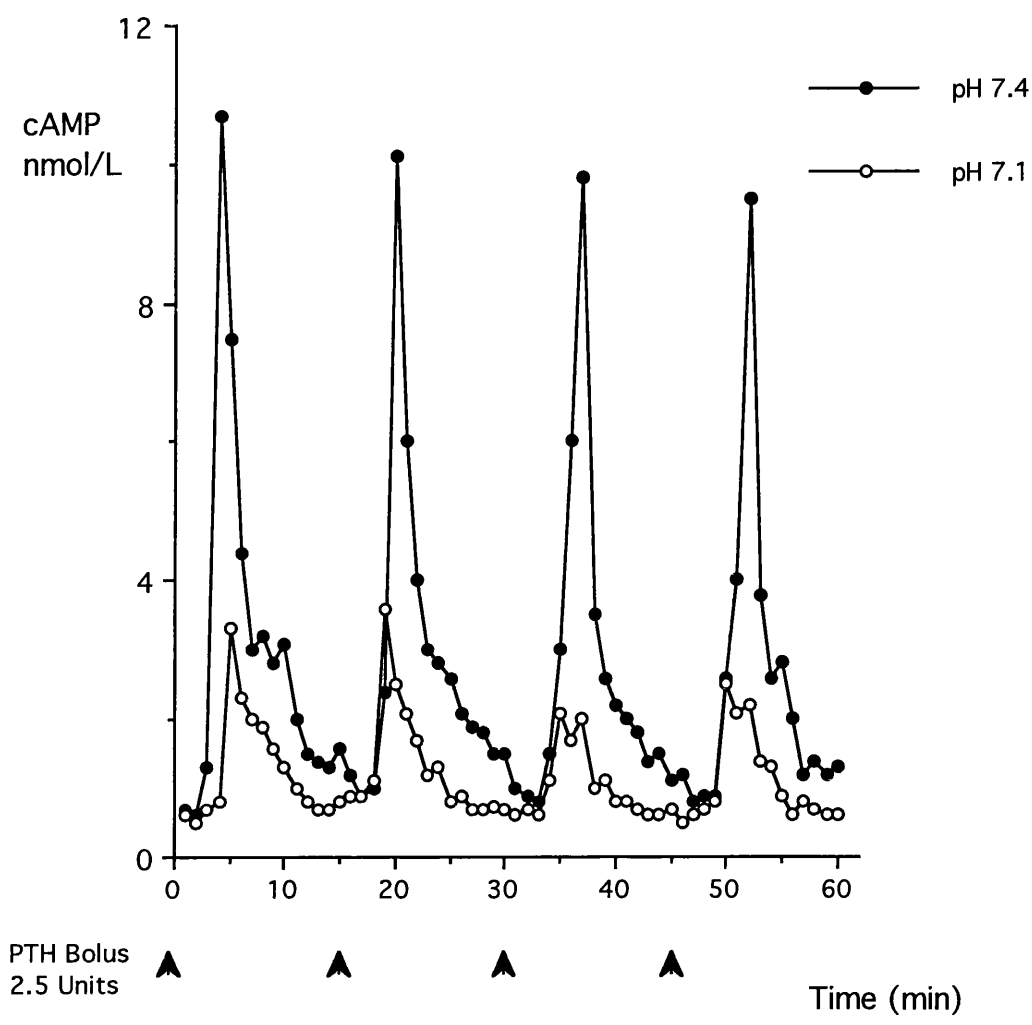
Figure 40   Comparison of cAMP production by renal tubules
            in response to stimulation with bovine PTH
            (1-84) when perfused at pH 7.4 or pH 7.1. Each
            line represents the results from a single
            column. PTH was introduced as a bolus on top of
            the columns at times indicated by the arrows.
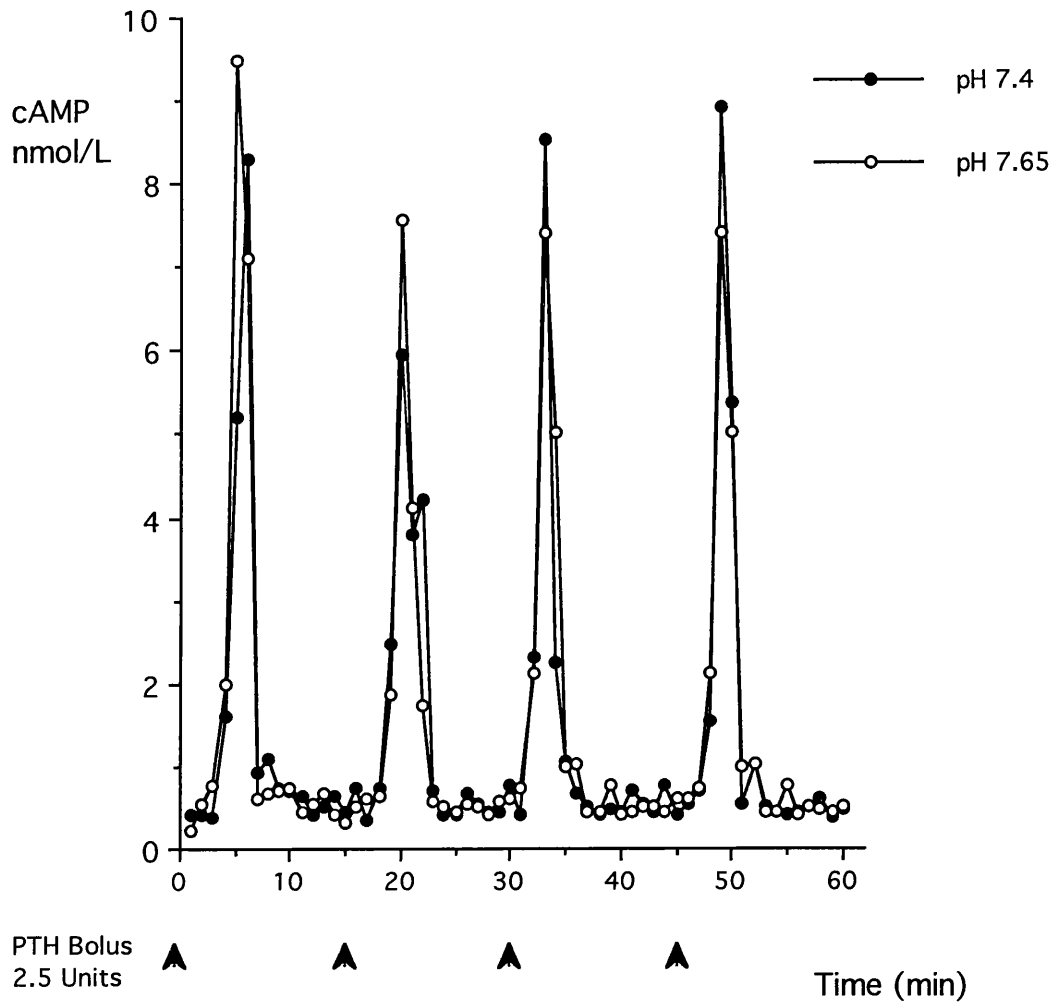
Figure 41   Comparison of cAMP production by renal tubules
in response to stimulation with bovine PTH
(1-84) when perfused at pH 7.4 or pH 7.65. Each
line represents the results from a single
column. PTH was introduced as a bolus on top of
the columns at times indicated by the arrows.
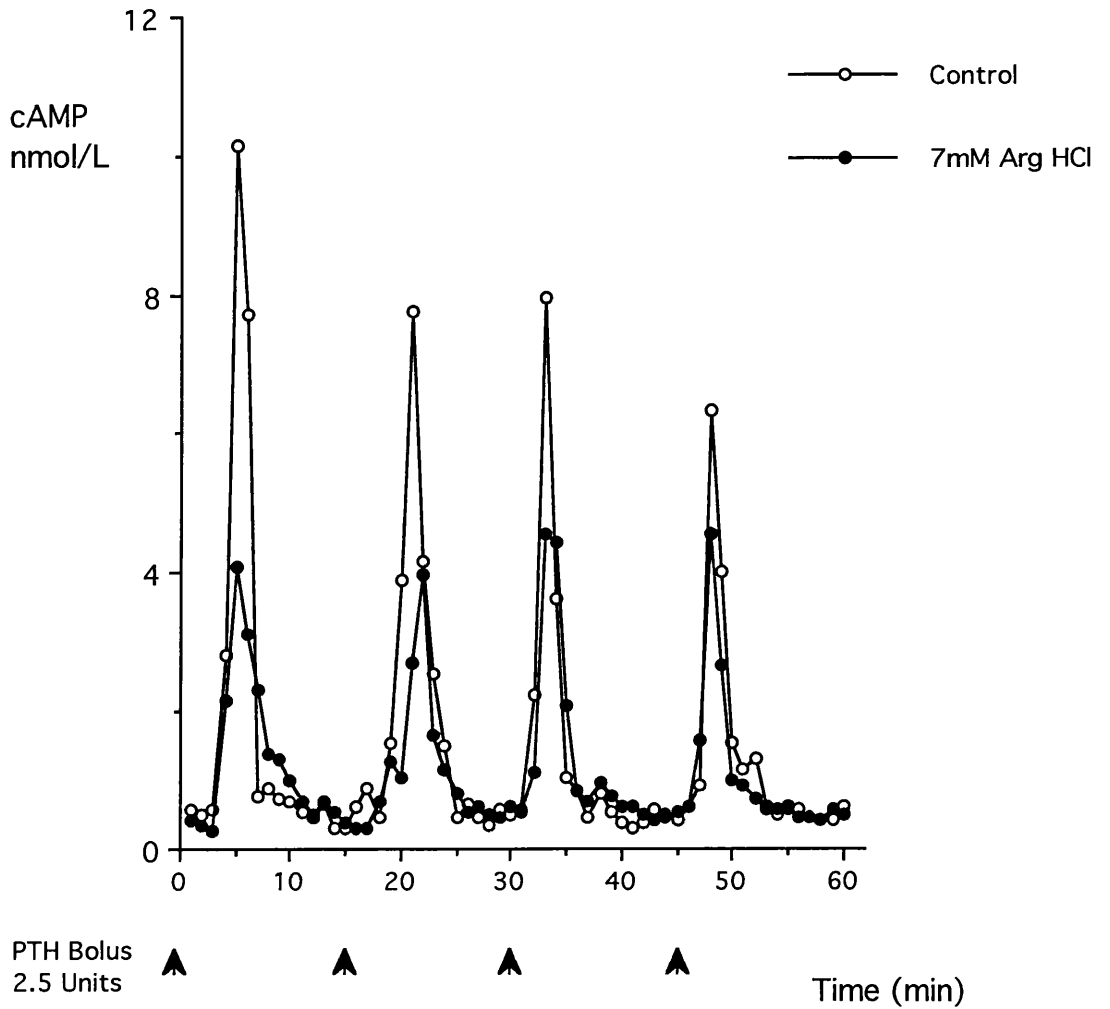
Figure 42   Comparison of cAMP production by renal tubules
            in response to stimulation with bovine PTH
            (1-84) when perfused in the presence or absence
            of 7mM Arg HCl. Each line represents the
            results from a single column. PTH was
            introduced as a bolus on top of the columns at
            times indicated by the arrows.

Conditions for Perfusion	Mean cAMP output fmol/10^6 renal tubules/pulse	Significant Difference
Control (1) pH 7.4	55.2 ± (14.9)	
pH 7.1	36.9 ± (13.1)	$p < 0.01$
Control (2) pH 7.4	51.2 ± (10.2)	
pH 7.65	49.9 ± (10.3)	NS
Control (3) pH 7.4	53.6 ± (8.8)	
7 mM Arg HCl present	45.1 ± (9.3)	$p < 0.05$

Mean ± SD

Table 16   **Comparison of cAMP Production by Superfused Renal Tubules pulsed with PTH whilst perfused at pH 7.4, pH 7.1, pH 7.65 and in the presence or absence of 7mM Arg HCl**

statistically significant decrease in cAMP output in response to bPTH (1-84) when the tubules are exposed to the acidic perfusate but not the alkaline perfusate. A statistically significant decrease in cAMP output in response to bPTH (1-84) is also noted when the tubules were perfused with arginine hydrochloride.

9.1.4 <u>Discussion</u>

In these experiments it has been demonstrated that superfusion of rat renal cortical tubules can be used to study the factors affecting cAMP production by renal tubules in response to bPTH (1-84) stimulation. This system has the advantage that the biological activity of PTH can be assessed in a consistent reproducible manner, after 90 min equilibration of the tubules on the columns, as demonstrated by both bPTH (1-84) and hPTH (1-34) fragment stimulation. By running control experiments in parallel with the test columns the effects of changing individual factors in the perfusate are readily observed and any unexpected effects or perturbations of the experimental system are immediately obvious.

Although the superfusion technique is not a physiological system it has many advantages over other *in-vitro* bioassay systems most of which will also require the use of supraphysiological doses of hormone. The superfusion system responses to increasing doses of both bPTH (1-84) and hPTH (1-34) were consistent, linearly increased over

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the concentration ranges studied and are reproducible. There was an indication that under current conditions the tubule response diminished with the length of time the cells were on the columns which may reflect a number of factors. Obvious explanations may be that the cell receptors may down regulate on repeated stimulation, receptor cycling may slow down, precursors for cAMP generation may be gradually diminished, there may be an insufficient energy supply for the cells and the number of functioning tubules may decrease with time.

A significant decrease in cAMP production by the tubules in response to bPTH (1-84) was noted when they were perfused with acidic buffer (pH 7.1). This effect has occurred acutely and persisted throughout the period of perfusion of the tubules. This down regulation of response to PTH may be due to acidosis affecting the tubular cells such that receptor function changes or receptor cycling is slowed down or possibly due to effects of the acidic environment on the PTH molecule itself. Conflicting evidence exists in the literature on the effects of changes in acid-base status on PTH action at both bone and kidney and reflects the fact that different animal species have been studied, different cell systems have been used and different methods have been adopted to induce acidosis or alkalosis. Experiments using rats have detected a decrease in UcAMP production, decreased adenylate cyclase activity and decreased PO_4 excretion in animals made acutely acidotic

(164). The response to PTH injection was also reduced with decreased UcAMP production, reduced phosphaturic effect and increased urinary Ca excretion (164,306). However, after a period of prolonged acidosis in man and dogs there has been no impairment observed in PTH effects as assessed by urinary calcium, phosphate, bicarbonate excretion and by changes in circulating ionised calcium (302,303). In dogs made acutely acidotic cAMP production in response to PTH stimulation increased from perfused canine tibia (163). It would appear that in the intact animal changes may occur with time in chronic acidosis to overcome the reduced response to PTH we have observed in tubules made acutely acidotic. One way of overcoming such effects would be by increasing PTH secretion from the parathyroid gland and so chronic acidosis may result in secondary hyperparathyroidism.

While compensatory effects are occurring the acute effects of acidosis observed in these experiments may have important consequences for calcium homeostasis at the kidney and may contribute to the pathogenesis of metabolic bone disease observed in acidotic patients.

Perfusion of the tubules with alkalotic buffer had no measurable effect on the production of cAMP by renal tubules. This result is in keeping with previous data where intact animals have been made acutely alkalotic (164,306). Chronic metabolic alkalosis in rats results in

several changes in calcium metabolism. A decrease in ionised calcium and an increase in PTH are the most obvious effects. An appropriate response to this increased PTH is observed in chronic alkalosis with increased $1,25 (OH)_2D3$, phosphaturia and hypocalciuria (305). Taken together with the current data these results indicate that neither acute nor chronic alkalosis interferes with PTH action at the tubule.

Arginine hydrochloride has been used in clinical medicine to treat severe metabolic alkalosis, diagnose growth hormone deficiency and investigate insulin release. Infusion of arginine hydrochloride into healthy male volunteers results in a diuresis, a fall in urine osmolality, a decrease in free water clearance, a decrease in renal phosphate clearance and a decrease in UcAMP output (307). The response to 230 i.u. of PTH intravenously is also significantly reduced during the infusion of arginine hydrochloride (307). In superfused tubules there is a significant decrease in cAMP output in response to PTH stimulation in the presence of arginine hydrochloride. A minimal decrease in pH of the perfusate is observed with the concentration of arginine hydrochloride used in this experiment and so the effect on PTH action is due to arginine hydrochloride. There are a number of possible explanations for the effect of arginine hydrochloride on cAMP production. Arginine is actively transported into renal tubular cells and the high intracellular

concentration of arginine could inhibit the endocytosis of receptor-ligand complexes or receptor recycling in these cells. Arginine may block the action of PTH at its receptor by occupying the receptor binding site. Biological activity of PTH resides in the first 1-34 amino acids of the polypeptide, a sequence which contains three lysine and two arginine residues. The minimum polypeptide required for biological activity is the 2-25 N-terminal sequence which contains two arginine and one lysine residues. PTHrP also has a similar number of arginine and lysine residues in both the 1-34 (5) and 1-84 (14) peptides. Cyclohexanedione modification of arginine residues blocks receptor mediated uptake of low density lipoprotein by several cells. The cumulated evidence would indicate that arginine plays an important role in hormone-receptor interaction and it is possible that exogenous arginine may interfere with PTH activity. This may have implications for patients receiving total parenteral nutrition (TPN). Many patients receiving TPN have hypercalciuria and long term may develop calcium and phosphate imbalance and metabolic bone disease (308). Amino acid infusions used in such therapy have relatively high concentrations of arginine and this may produce the effect that we have seen in the tubule superfusion experiments resulting in a decreased response to PTH and abnormal calcium/phosphate homeostasis.

The superfusion technique is a reliable, reproducible method for studying the direct action of PTH in terms of cAMP production from renal tubules in isolation. Superfusion has many similarities with the normal physiology of the tubules, where PTH is delivered to the target cell via the arterial supply and cellular products are removed via the venous drainage and/or urine. In these experiments this technique has shown its value in studying PTH activity *in-vitro* and the method described will be of value in investigating the many factors that can alter cAMP metabolism in renal tubular cells.

Chapter 10

Current and Future Research in Relation
to the Work of This Thesis

The importance of the circadian rhythms of the hormones controlling cAMP production and the alteration of cAMP metabolism throughout a 24h period has given new insight into mechanisms that could control calcium homeostasis and bone turnover. Further understanding of the physiological and pathological modification of the circadian rhythms of PTH (1-84) and cAMP should lead to a better understanding of normal bone remodelling and metabolic bone disease. Studies have been initiated and have produced interesting data on differences in the circadian rhythms of PTH (1-84) and NcAMP in pre-menopausal, normal post-menopausal and osteoporotic post-menopausal women (309). The circadian rhythms of PTH (1-84) and NcAMP are present in pre-menopausal women, attenuated in post-menopausal women without osteoporosis and absent in post-menopausal osteoporotic women. These findings have resulted in the initiation of a programme of investigation into manipulation of the PTH (1-84) and cAMP response using timed ingestion of calcium and phosphate (310). The studies in this thesis formed the basis of a theory that phosphate was important in the genesis of the PTH (1-84) circadian rhythm and by increasing ingestion of phosphate at 22.00 the PTH (1-84) and cAMP rhythms would be augmented resulting in beneficial effects on bone metabolism. Measurement of overnight NcAMP production, first voided morning urine, as a ratio against second voided urine gives an accurate indication of PTH (1-84) secretion overnight and enables a rapid assessment of the effects of different

therapeutic manoeuvres (311).

Neuroendocrine control of the circadian rhythm of PTH (1-84) and subsequent cAMP production is being investigated using the LHRH agonist Buserelin to modify the pituitary production of gonadotrophins and prolactin (312). Long term studies on cAMP metabolism in the "chronic fasting state" anorexia nervosa are planned in the light of results obtained from the sleep shift and fasting studies.

It will be important to investigate whether intradian variation (minute to minute) in PTH (1-84) will result in modification of the cAMP responses thus altering calcium and phosphate homeostasis and whether manipulation of calcium and phosphate concentrations can change PTH (1-84) and cAMP metabolism with beneficial effects on bone. Studies have commenced looking at the pulsatility of PTH (1-84) and cAMP generation in normal subjects before and after EDTA infusion (313), hyperparathyroid patients (314), fasting subjects (315) and reports of alteration in PTH (1-84) pulsatility in osteoporotic patients have recently been published (316,317). The cAMP production by cells in response to hormonal stimulation and subsequent cell function is likely to alter depending on the combination of intradian and circadian stimuli.

Studies in this thesis have demonstrated that cAMP metabolism may be affected by a number of circulating

hormones and so interpretation of cAMP concentrations *in-vivo* requires detailed knowledge of the status of several hormones. There have been few attempts to study simultaneously hormones which affect cAMP production even in the short term and circadian studies on the combined effect of hormones on cAMP metabolism are non existent. Future research will attempt to isolate separate hormonal, particularly thyroid, cortisol and catecholamine effects *in-vivo* and *in-vitro*.

Development of assays for PTHrP open up major avenues of scientific and clinical research. Measurement of PTHrP in hypercalcaemia can help resolve difficult diagnostic problems and help in the management of patients. Recent data obtained using the assay evaluated in this thesis has indicated that elevated PTHrP in patients with HCM is associated with decreased survival, increased metastatic tumour load and increased bone turnover (318). Responses to bisphosphonate therapy are poorer in patients with elevated PTHrP (297) and treatment regimens and therapeutic approaches in patients with HCM may be modified as more knowledge is obtained on the physiology and pathophysiology of PTHrP. Studies performed during and after pregnancy have demonstrated that PTHrP increases gradually during pregnancy and can become markedly elevated at the time of lactation (319, 320). In this situation PTHrP may be produced by the placento/uterine and breast tissue and may stimulate increased bone turnover that is observed in

pregnancy. Evidence for the role of local PTHrP production by osteoblasts has recently been obtained and indicates that an autocrine/paracrine function of PTHrP may be very important in maintaining normal bone and in metabolic bone disease (321, 322, 323).

The superfusion system successfully allowed the study of individual factors that can affect the production of cAMP by renal tubular cells. Modification of this system will be required to make this technique less laborious and time consuming with alteration of the perfusion flow rate possible, collection of fewer samples following stimulation, integrating cAMP production after each stimulus and supplementing the perfusate buffer to allow prolonged survival of the tubular cells. Commercial equipment has recently become available that has automated several of the processes employed in superfusion experiments which confers a greater flexibility on the system and would allow quicker optimisation of the experimental technique.

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