CALCIUM HOMEOSTASIS IN THE ELDERLY

by

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DEDICATED TO

MY PARENTS AND MY BROTHER ERROL

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SYNOPSIS

The initial aims of this investigation were to develop a reliable assay system for measuring serum 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] concentrations and to establish a normal range in young healthy adults. Compared with young healthy individuals, the elderly population are indeed vitamin D deficient.

Vitamin D deficiency was also demonstrated in a group of elderly osteomalacic patients. Slight improvements in osteomalacia was achieved by one month of treatment with vitamin D_3, or alpha-calcidiol with or without calcium supplements. The improvements were small and occured slowly. A significant increase in the strength of bone seems unlikely to occur in the short term. On the present evidence the combination of alphacalcidiol and calcium supplements seems no better than vitamin D_3 or alphacalcidiol alone although it may require closer monitoring to avoid hypercalcaemia.

In a group of elderly patients with osteoporosis and femoral neck fracture (FNF), serum osteocalcin concentrations rose significantly in the first week after fracture fixation. The change in osteocalcin correlated well ($p < 0.001$) with the change in serum $1,25(OH)_2D$ concentration. Histomorphometric measurements of the extent of osteoid correlated better with osteocalcin than alkaline phosphatase.

Serum concentrations of $1,25(OH)_2D$ were also reduced in elderly patients with FNF irrespective of the presence of osteomalacia and therefore cannot be used as a screening test for osteomalacia in this patient group. Reduction of $1,25(OH)_2D$ was not due to a reduction in vitamin D binding protein. It is suggested that the low rate of bone turnover in these elderly patients reduces the requirement of vitamin D.

Of the ten elderly patients who had underwent laryngo-pharyngeal surgery all developed hypocalcaemia. This immediate

post-operative decrease, due to a rapid reduction in circulating PTH concentrations, lead to an overall increase in urinary calcium excretion. Serum concentration of $1,25(OH)_2D$ also fell post-operatively thus potentiating the hypocalcaemic state in these patients. Thus, it is important to give parenteral feeding supplemented with calcium and vitamin D, preferably alphacalcidiol. If delayed then profound as well as prolonged hypocalcaemia can occur.

The human osteosarcoma cell 2OS metabolised $25(OH)D_3$ in a substrate concentration and time dependent manner to produce products which were secreted into the extracellular medium. These products eluted from HPLC with a retention time coincident with $24,25(OH)_2D_3$ and exhibited an UV absorption spectrum characteristic of a vitamin D sterol. Mass spectroscopy analysis indicated at least two products were synthesised by the cells. One was identical to $24,25(OH)_2D_3$; the other appeared to be an unsaturated tri-hydroxylated derivative of vitamin D_3.

ACKNOWLEDGEMENTS

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And finally, I must not forget all my family and friends who were very caring and supportive when thieves broke into the boot of my car and stole the bulk of this thesis. I am forever grateful for getting me through that ordeal.

ABBREVIATIONS USED:

D	Vitamin D_2 and D_3
$PreD_3$	Previtamin D_3
7-DHC	7-dehydrocholesterol
25(OH)D	25-hydroxyvitamin D_3 25-hydroxycholecalciferol
$1,25(OH)_2D_2$	1α,25-dihydroxyvitamin D_2 1α,25-dihydroxyergocalciferol
$1,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3 1α,25-dihydroxycholecalciferol
$1,25(OH)_2D$	Both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$
$24,25(OH)_2D_3$	24R,25-dihydroxyvitamin D_3
$25,26(OH)_2D_3$	25,26-dihydroxyvitamin D_3
$1\alpha,24,25(OH)_3D_3$	1α,24,25-trihydroxyvitamin D_3
PTH	Parathyroid Hormone
Pre Pro-PTH	Preproparathyroid Hormone
Pro-PTH	Proparathyroid Hormone
iPTH	Immunoreactive Parathyroid Hormone
bPTH	Highly purified bovine Parathyroid Hormone
ATP	Adenosine triphosphate
ADP	Adenosine 5' diphosphate
cAMP	Adenosine-3',5'-cyclic monophosphate
dbcAMP	Dibutyryl cyclic AMP
PPi	Inorganic pyrophosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
NADP	Nicotinamide adenine dinucleotide

ABBREVIATIONS USED: continued

Ca^{2+}	Calcium ion
CaBP	Vitamin D induced Calcium binding protein
DBP	Vitamin D binding protein
BSA	Bovine serum albumin
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane sulphonate
PBS	Phosphate-buffered saline
ER	Endoplasmic reticulum
^{47}Ca	^{47}Calcium
^{85}Sr	^{85}Strontium
MW	Molecular weight
Da	Dalton
(m)M	(milli)Molar
min	minute(s)
sec	second(s)
h	hour(s)
$t_{\frac{1}{2}}$	turnover rate
rpm	revolutions per minute
dpm	disintigrations per minute
cpm	counts per minute
ml	millilitre (10^{-3}litre)
ul	microlitre (10^{-6}litre)
mg	milligram (10^{-3}gram)
ug	microgram (10-6gram)

ABBREVIATIONS USED: continued

ng	nanogram (10^{-9}gram)
pg	picogram (10^{-12}gram)
vol	volumes
v/v	volume to volume
w/v	weight to volume

CHAPTER 1

GENERAL INTRODUCTION

1.1 CALCIUM HOMEOSTASIS

Calcium plays an important role in a variety of cellular and organ functions, in particular muscle contraction, blood clotting, hormone release and action, cell motility and cell growth and division. Calcium constitutes approximately 1% of the body weight of an adult man. A substantial amount of total body calcium (99%) is immobilised in bone and teeth, in the form of hydroxyapatite, $[Ca_{10}(PO_4)_6(OH)_2]$, and since calcium has little or no ability to form covalent compounds the body is able to sustain a small amount in the intra- and extracellular compartments. Bone also contains a considerable amount of non-crystalline calcium phosphate and carbonate, as well as small amounts of other salts and it is the movement of calcium from this source which maintains intra- and extracellular calcium concentrations. Calcium concentrations differ greatly in these compartments; extracellular calcium is around 2.2mM, whereas calcium in the intracellular fluid is less than 1uM.

Calcium is present in plasma in 3 forms: ionised calcium accounts for around 50% of total extracellular calcium, the remainder is either bound to plasma proteins (eg. albumin) or associated with the anionic groups of a variety of organic acids, such as calcium citrate. These forms assist in the maintenance of extracellular calcium concentration within the normal range of 2.2-2.5mM, thus ensuring that there is always a constant source of calcium available to the cells.

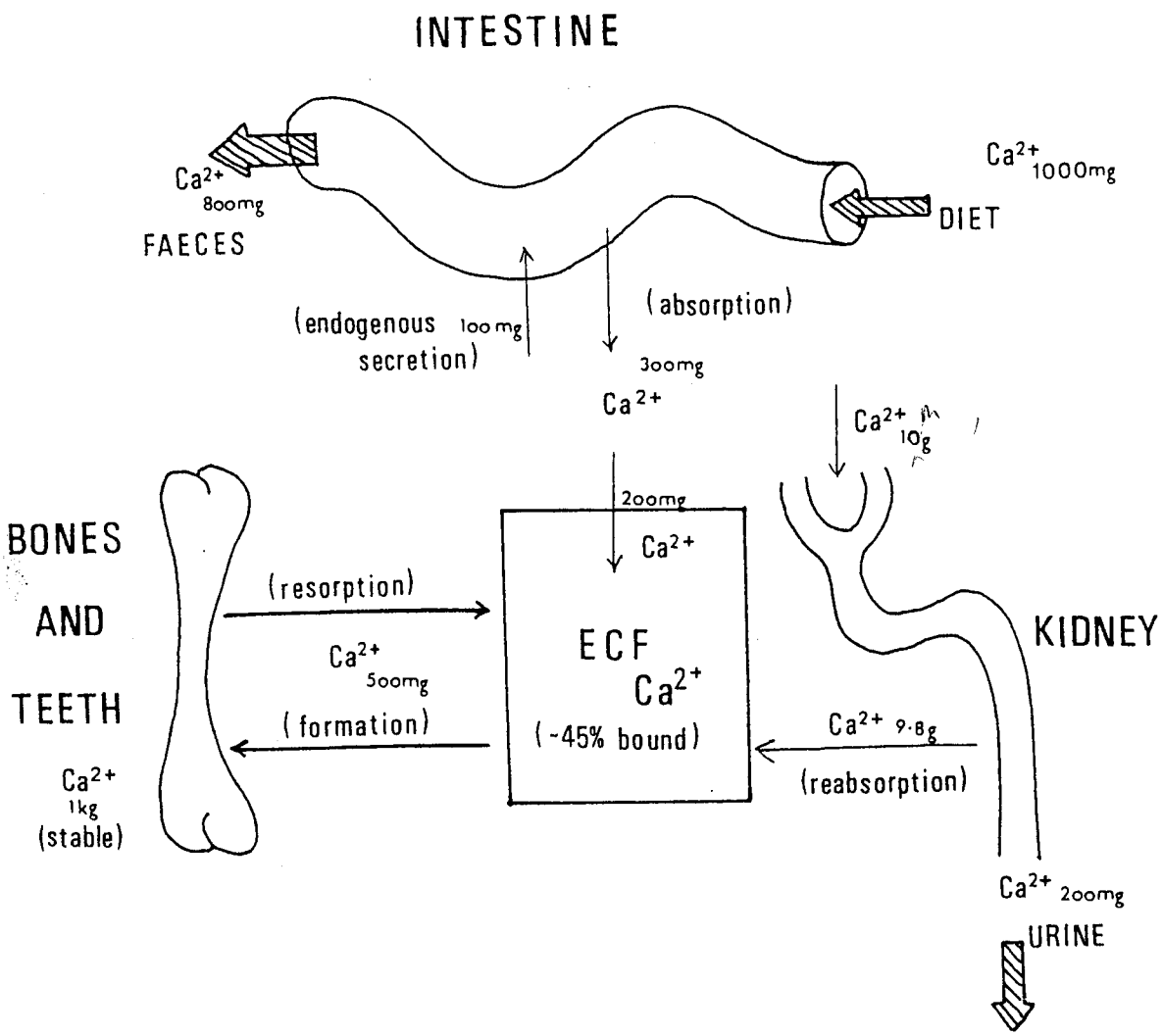
Figure 1.1 illustrates an example of normal calcium balance in adult man. On an average dietary intake of 1000mg, around 30-35% is absorbed in the small intestine. There is no net gain or loss of calcium from bone because of the two processes of bone formation and bone resorption which involve the movement of calcium into and out of bone, respectively. In a young adult, these two processes are balanced, i.e. skeletal homeostasis is maintained. An equivalent amount of calcium must therefore be excreted daily in the urine to maintain overall calcium balance. Only when there is an imbalance between these two systems does bone serve as a calcium reservoir. The magnitude of this calcium exchange is difficult to estimate, but it becomes significant when the calcium imbalance, whether in the hyper- or hypocalcaemic state, persists for a long period and metabolic bone disorders develop.

One additional excretory route of calcium, not shown in figure 1.1, is through the skin. Calcium ions are a normal component of sweat, and around 30-120mg of calcium are excreted via this route each day.

Calcium has two major functional roles in human physiology. Firstly, it has a structural role as exemplified by the strength and rigidity that it, in the form of calcium salts, contributes to the human skeleton. Its second role is that of a "second messenger" which mediates cellular responses to a wide range of stimuli in a manner analogous to the regulatory actions of the cyclic nucleotides. Calcium has the ability to associate reversibly with anionic structures present on intra- and extracellular proteins and on phospholipid bilayers of membranes, and it is this ability which allows regulated calcium fluxes in and out of cells.

FIGURE 1.1. An example of normal calcium homeostasis in an individual with a dietary intake of 1000mg of elemental calcium. There is no net movement of calcium between the skeleton and the extracellular fluid (ECF), thus urinary excretion of calcium is 200mg to maintain zero external calcium balance.

(from: Brown et al, 1987. Recent Prog. Horm. Res. 43, pp337-96).



The calcium binding proteins found intracellularly differ to those found in the extracellular space. Intracellular calcium binding proteins recognise and bind to the low concentrations of messenger calcium that appear in the cell. They are small proteins in which several oxygen-containing anionic groups are arranged in a conformation that bind tightly to calcium at micromolar concentrations. One such example is calmodulin (Means and Dedman, 1980), which is found in every nucleated cell type examined. It binds calcium ions and by doing so modulates the activities of a variety of enzymes, in particular those involved in cyclic nucleotide metabolism, protein phosphorylation, muscle contraction, microtubule assembly and calcium flux. This contrasts with the calcium receptor on the cell surface which possess the dicarboxylic acid gamma-carboxyglutamic acid, which is able to form a complex with the higher (millimolar) calcium concentrations found in the extracellular fluid. These distinctions reflect the very large differences in calcium concentrations in these two regions of the body.

The control mechanism responsible for maintaining normal calcium homeostasis involves the interplay between two hormones, namely parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], which act either directly or indirectly on three target tissues: bone, kidney and the small intestine. It is worth noting at this point that another peptide hormone, calcitonin, is rapidly released by the parafollicular or "C" cells of the thyroid gland in local response to small increases in plasma calcium concentration (White et al, 1984). However, in disorders where calcitonin is absent or in excess patients do not become permanently hypercalcaemic or hypocalcaemic, respectively. This would suggest that its overall contribution to calcium homeostasis is minor compared with that of PTH or the vitamin D metabolite. At pharmacological

doses calcitonin is able to reduce plasma calcium and phosphate concentrations by effectively inhibiting bone resorption and increasing renal calcium and phosphate clearance. In view of the uncertainty in its physiological role no further mention will be made of calcitonin.

The purpose of this chapter is to give the reader a general insight into the mechanism of action of both PTH and $1,25(OH)_2D$ in the maintenance of calcium homeostasis.

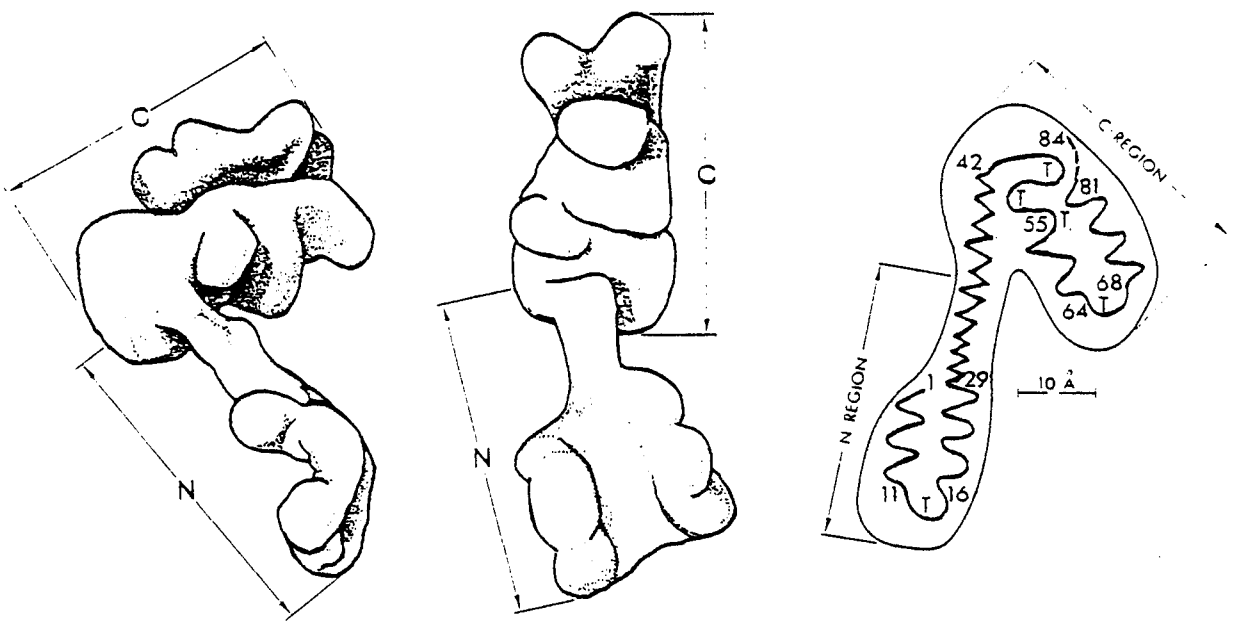
1.2. PARATHYROID HORMONE

Parathyroid hormone (parathormone, PTH) is synthesised within the chief cells of the parathyroid gland. It comprises of 84 amino acid residues in a single peptide chain, and contain no disulphide bonds, no carbohydrate moeities or any other covalently bound structure (Cohn and Elting, 1986). The amino acid sequence of bovine, porcine and human PTH have been established, although the conformational structure is still uncertain. The differences among them prevent complete immunologic cross-reactivity. This accounts for the difficulties in developing radioimmunoassays for the measurement of human PTH using antisera directed against the more readily available bovine or porcine peptides.

Indirect techniques, other than crystallography, have suggested that the molecule is folded in such a manner to produce a two domain structure linked by a linear region (figure 1.2). Biological activity of the hormone resides in the first third of the molecule (1-34) and this occupies one domain. Removal of residues 1 and 2 renders this amino-terminal region biologically inert. The inactive carboxy-terminal portion (41-84) produces the second domain and to date no biological activity has been assigned to this

FIGURE 1.2. The proposed tertiary and secondary structures of bovine PTH. Left and centre: The 3D distribution of mass as visualised by dark-field electron microscopy. The view of the molecule pictured in the centre is rotated 90° clockwise relative to the view on the left. Right: The predicted distribution of α-helix (sine waves), β-sheet (saw-tooth), β-turn (T), and random coil (---) in bovine PTH. Structural predictions illustrated in the view on the right suggest that the region marked N represents the biologically active amino-terminal portion of the molecule and that marked C the inactive carboxyl-terminal region.

(from: Cohn and Elting, 1983. Recent Prog. Horm. Res. 39, pp181-203).



region. Linking both domains is the connecting 'stalk' (amino acid residues 35-40) which have been shown to be the region which enzymes cleave PTH with ease.

1.2.1. BIOSYNTHESIS AND SECRETION

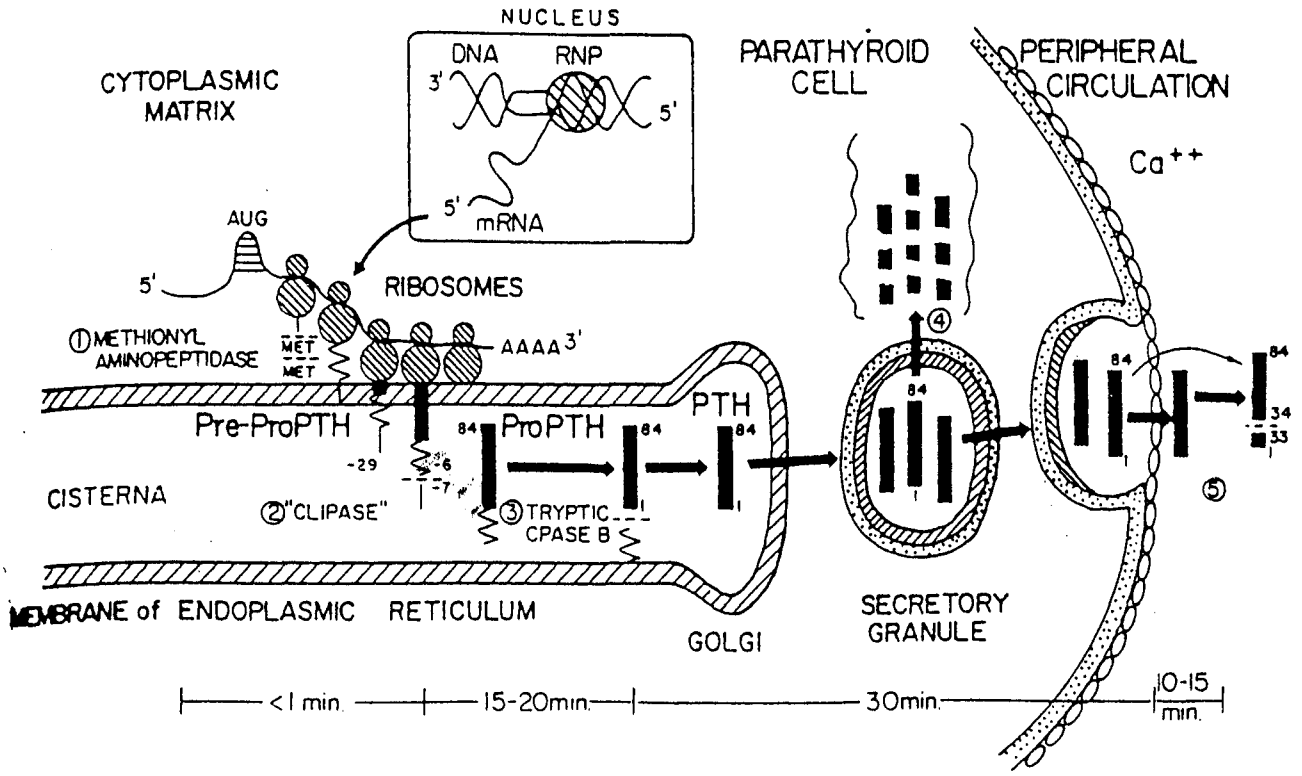
The synthesis of PTH, a 9500dalton molecular weight peptide (Habener and Kronenberg, 1978) is depicted in figure 1.3. Like many other peptide hormones PTH is synthesised on polyribosomes as a larger inactive precursor molecule (115 amino acids) termed pre-proparathyroid hormone (pre-proPTH). As the growing peptide chain emerges from the ribosome, the hydrophobic 25-amino-acid terminal sequence associates with the endoplasmic reticulum (ER) membrane, in accordance with the signal hypothesis, allowing the pre-proPTH to enter the cisternal space of the ER during translation. This N-terminal leader sequence is removed, either during or immediately after completion of synthesis, thus liberating an intermediate molecule of 90 amino acid residues, the pro-parathyroid hormone, pro-PTH. During translocation to the Golgi area the pro-PTH molecule undergoes post-translational cleavage of 6 amino acid sequences, yielding the native 84 amino acid PTH molecule. Enclosed in secretory vesicles the newly synthesised PTH has 4 fates:

(1) Immediate secretion- PTH passes to the liver where it undergoes post-secretory proteolysis, leaving a 33 amino acid peptide which represents the biological and immunologically active fragment. It is this fragment which is responsible for the peripheral actions of PTH.

(2) Degradation in the cell by late post-translational proteolysis between amino acids 33 and 34.

FIGURE 1.3. Schema depicting the proposed intracellular pathway of the biosynthesis of PTH. Pre-ProPTH, the initial product of synthesis on the ribosomes, is converted into ProPTH by removal of 1) the NH_2-terminal methionyl residues and 2) the NH_2-terminal sequence (-29 to -7) of 23 amino-acids during and within seconds after synthesis, respectively. By 20min after synthesis, ProPTH reaches the Golgi region and is converted into PTH by 3) removal of the NH_2-terminal hexapeptide. PTH is stored presumably in the secretory granule and is either partially degraded within the cell 4) when secretion of the hormone is supressed by high serum calcium concentrations or 5) is released into the circulation in response to a fall in serum calcium. The time needed for these events to take place is given below the schema.

(from: Habener and Kronenberg, 1978. Federation Proc. 37, pp2561-66).



(3) Transportation into storage pool although relatively little hormone is stored in the gland, or

(4) Complete degradation in the parathyroid gland.

There is no evidence to suggest that either of the PTH precursor molecules or the "pre" or "pro" peptide sequences are secreted into the circulation (Habener and Kronenberg, 1978).

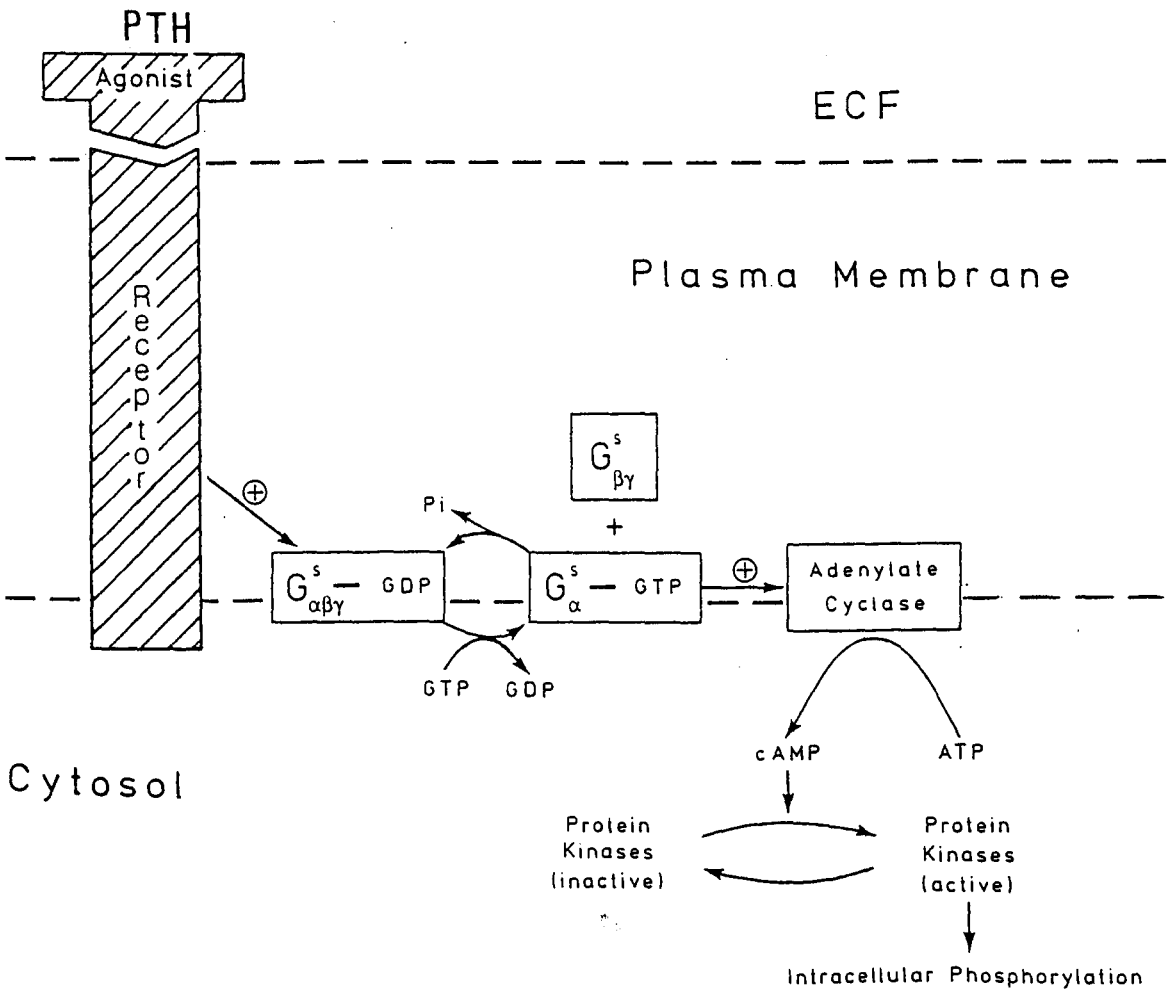
The major physiological regulator for PTH synthesis and secretion is extracellular calcium concentration. Although a rise in plasma calcium supresses PTH secretion complete inhibition is not seen. There is evidence to suggest that intracellular stores of PTH may be regulated by a degradative pathway that is stimulated by high or inhibited by low extracellular calcium concentrations (Habener et al, 1975). It is not known which enzymes within the parathyroid gland are responsible for the degradation of PTH but certain lysosomal enzymes, in particular the cathepsins, have been implicated (MacGregor et al, 1979). Fragments of the hormone produced during intracellular degradation have been detected in the circulation and some of these have been shown to have immunoreactive properties (Potts et al, 1982).

1.2.2. MECHANISM OF ACTION

Like many other peptide hormones, PTH acts by binding to a specific cell surface receptor and this interaction activates the intracellular adenylate cyclase system and hence the production of cyclic adenosine monophosphate (cAMP) (Stryer and Bourne, 1986). The binding of PTH to the receptor molecule (figure 1.4) initiates the activation of the membrane-bound G proteins (formally known as the N proteins).

FIGURE 1.4. The activation of the adenylate cyclase system by PTH.

Refer to text for description of steps.

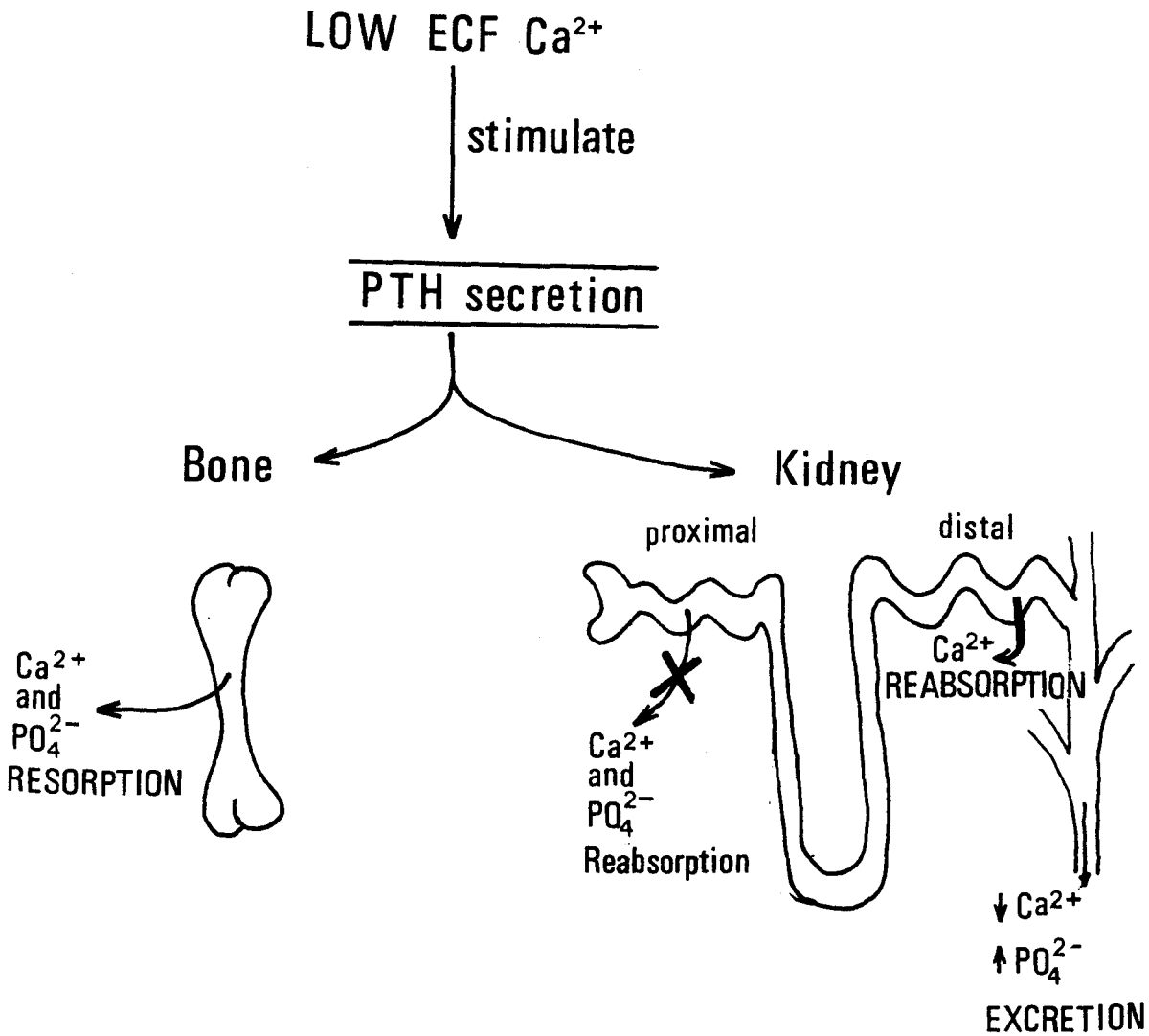


Before activation, the G proteins are in a quiescent state with all three polypeptide subunits, α, β and γ, associating with the GDP molecule, denoted GDP-$G_{\alpha\beta\gamma}$ (Stryer and Bourne, 1986). Upon activation by the hormone-receptor complex, GTP binds to the α chain which in turn dissociates from the β and γ chains to form G_{α}-GTP and $G_{\beta\gamma}$ respectively with the loss of GDP. The G_{α}-GTP then stimulates the activity of adenylate cyclase resulting in the accelerated production of cAMP from adenosine triphosphate (ATP). Raised cAMP induces specific metabolic events, one of which is to increase intracellular calcium. The role, therefore, of the $G_{\beta\gamma}$ molecule is to present G_{α}-GTP to the receptor-hormone complex. Once stimulation of adenylate cyclase is achieved, GTP is hydrolysed to GDP thus terminating activation and the GDP-$G_{\alpha\beta\gamma}$ molecule reforms.

The major sites for the receptor-mediated actions of PTH are in bone and kidney. In the hypocalcaemic state (Figure 1.5) PTH exerts its major effect on the distal nephrons of the kidney by producing an increase in renal tubular reabsorption of calcium whilst inhibiting calcium reabsorption in the proximal convoluted tubule. The combination of the two leads to a decrease in calcium clearance. However, PTH also inhibits phosphate reabsorption in the proximal tubule thus producing an increase in renal phosphate clearance, phosphaturia. The adaptive value of the latter requires further explanation.

PTH also exerts its effect on bone (figure 1.5) by releasing calcium, in conjunction with phosphate, in to the extracellular fluid. If phosphate concentration is allowed to increase, further movement of calcium from bone will be retarded; but in addition to this effect on bone, PTH also decreases tubular reabsorption of phosphate, thus permitting the excess phosphate to be eliminated in the urine.

FIGURE 1.5. The action of PTH on bone and kidney in response to low extracellular fluid (ECF) calcium.



Therefore, calcium can be mobilised from bone without a major disturbance in phosphate concentrations.

Another function of PTH in the kidney is to stimulate the synthesis of the vitamin D metabolite, $1,25(OH)_2D$, by activation of the 25(OH)-1 alpha-hydroxylase. The mechanism of this activation is unclear but may be related to a fall in intracellular phosphate.

1.3 VITAMIN D

In the early 1900s rickets was one of the most common diseases of infancy and childhood. Investigations by Mellanby in 1919 led to the discovery that this disease could be cured and prevented by the administration of cod liver oil (Mellanby, 1919). The beneficial effect of sunlight on healing rickets was also demonstrated in the early 1900s. Since cod liver oil was known to contain vitamin A, Mellanby (1919) suggested that this was the antirachitic substance. However, sceptical of this claim, McCollum was able to show that this organic factor was not vitamin A, but a group of anti-rachitic substances which were detected in several natural oils and designated the collective name vitamin D (McCollum, 1922). Collaborative work by Windaus and his collegues in the 1930s led to the structural identification of these substances (figure 1.6) (Windaus et al, 1936).

The term vitamin D refers to a group of ten secosteroids with anti- rachitic properties, all of which are derivatives of the 3(ß)hydroxy 9,10 seco 5,7,10(19)triene structure and differ only in the C_{17} side chain (figure 1.7). Of the ten, only vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are of any significance, both nutritionally and

FIGURE 1.6. Numbering system for the vitamin D carbons.

Vitamin D_2: (5Z,7E,22E)-9,10-seco-5,7,10(19)22-ergostatriene-3β-ol.

Vitamin D_3: (5Z,7E)-9,10-seco-5,7,10(19)-cholestatrien-3β-ol.

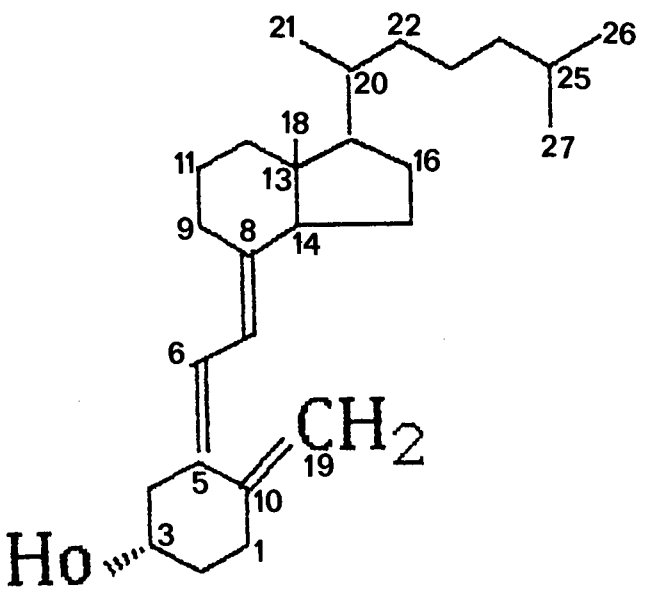
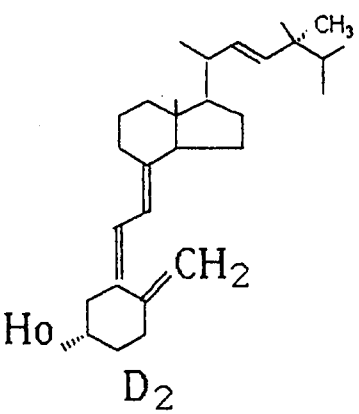
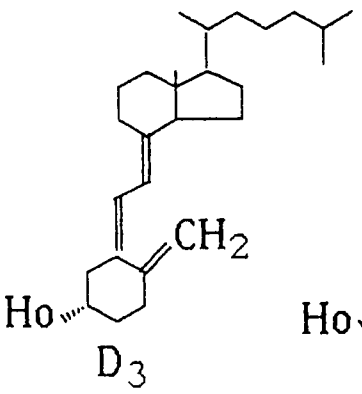


FIGURE 1.7. The D vitamins

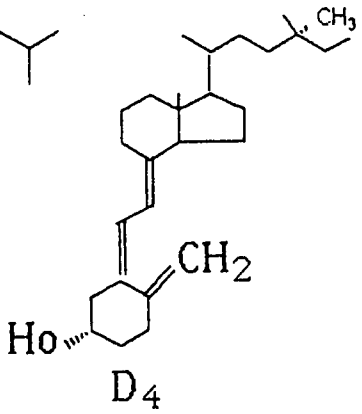
Not all 10 D vitamins are illustrated. Vitamin D_2 (ergocalciferol) and Vitamin D_3 (cholecalciferol) are the only two commonly known D vitamins. The others are known from the literature as biochemical "curiosities".



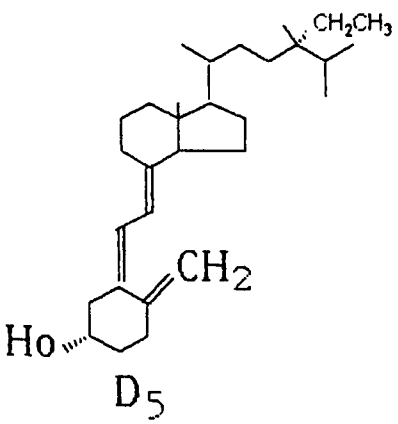
D_2



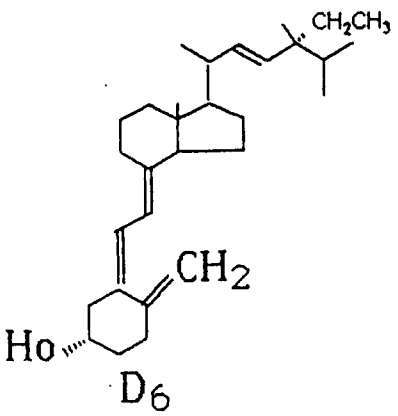
D_3



D_4



D_5



D_6

biologically, to man and, therefore, little or no reference will be made to the other eight.

Vitamin D (in the form of D_2 and D_3) plays an important role in calcium homeostasis, as attested by the fact that its deficiency results in poorly calcified bone. Unlike vitamin D_2, vitamin D_3 can be produced in the skin in the presence of sunlight. Only when adequate sunlight is not available are people absolutely dependent on dietary intake. Approximately 100IU of vitamin D are required per day by a healthy adult and only during childhood, pregnancy and times of stress does this value increase to as much as *400IU per day (Recommended Dietary Allowances, 1974).

Few natural foods contain appreciable amounts of vitamin D_3 and for many people endogenous production, by the action of sunlight on the skin, is the most important source of the vitamin. Natural dietary sources are fatty fish, such as herrings and mackerel, eggs and butter, but the highest vitamin D content is present in cod liver oil, approximately 2ug/g. Vitamin D_2 is generally the commercially favoured source of vitamin D used in the fortification of food products such as margarine, infant foods, some yoghurts and cereal products because of the ease with which it can be synthesised. However, in man, concentrations of vitamin D_3 are normally considered higher than those of vitamin D_2 unless there is a high intake of vitamin pills containing D_2. Nevertheless, the molecular form in which vitamin D is ingested or formed in the skin is relatively inactive in maintaining calcium homeostasis and must undergo hydroxylation in the liver and kidney to form the active hormonal metabolite, $1,25(OH)_2D$.

*nb. 1μg ≡ 1000IU.

1.3.1. THE PHOTOCHEMICAL FORMATION OF VITAMIN D_3

In 1979 Holick and co-workers established that the compound 7-dehydrocholesterol (7-DHC), an intermediate in cholesterol biosynthesis present in skin, is not immediately converted by ultraviolet light to the expected vitamin D_3 (D_3) but to a precursor form termed previtamin D_3 (pre D_3) (Holick et al, 1979). This compound, a double-bonded isomer of D_3, is quickly converted to D_3 (figure 1.8). Further work by this group (Holick et al, 1980) led to the identification of the site for the production of this precursor form as the epidermis nearest the capillary bed, just beneath the epidermal-dermal junction.

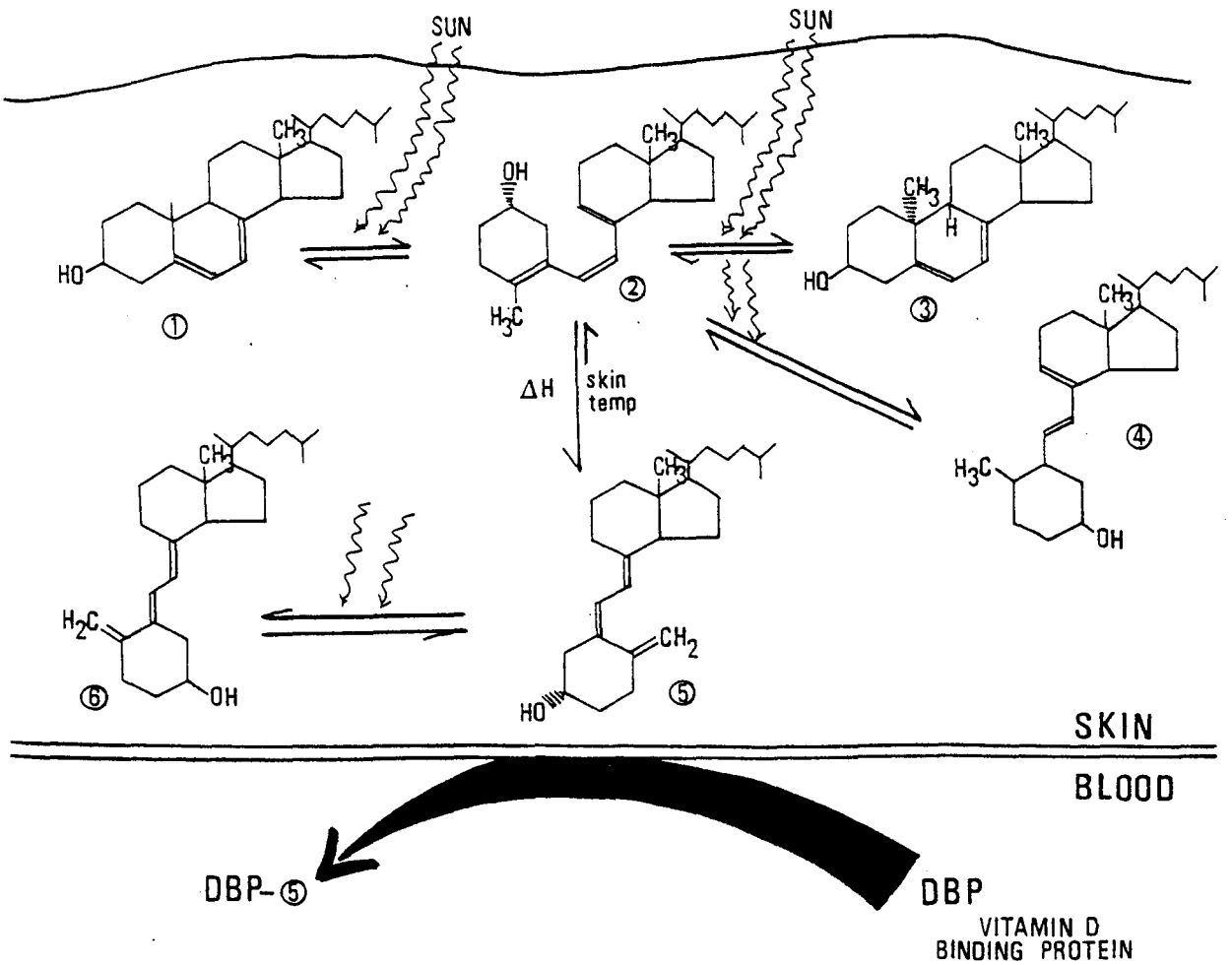
Once preD_3 is formed (figure 1.8) from 7-DHC, which involves the breakage of the B-ring structure, it is thermally converted to D_3 by a non-enzymatic reaction which appears to be much slower than the initial step (Holick et al, 1980). The greater rate of thermal conversion is due to the rapid removal by the circulation of D_3 and this drives the equilibrium to preD_3 conversion. Longer exposure to sunlight produces other photoproducts from preD_3, the biologically inactive compounds tachysterol and lumisterol, thus avoiding any danger of endogenous vitamin D_3 intoxication. Neither tachysterol nor lumisterol is transported out of the skin and, consequently, both are sloughed off with dead skin. The reaction of preD_3 to lumisterol is reversible under the influence of sunlight so that if preD_3 were to be depleted by the thermal reaction, more could be produced from lumisterol.

Once vitamin D_3 is formed it is selectively removed by way of the dermal capillaries by the vitamin D binding protein (DBP), which has a high affinity for the vitamin. This protein was originally termed 'group-specific component' or 'Gc protein'. Because DBP has a low affinity

FIGURE 1.8. The photosynthesis of $PreD_3$ in human skin and its photoisomerisation.

(1) 7-dehydrocholesterol (2) Previtamin D_3 (3) Lumisterol
(4) Tachysterol (5) Vitamin D_3 (6) 5,6-Trans-vitamin D_3

(from: Holick et al, 1981. Science 211, pp590-93).



for preD_3, this metabolite remains in the dermis for eventual conversion to vitamin D_3. The majority of D_3 is transported on DBP to the liver. Only a small amount of vitamin D is transported on serum albumin, and even a smaller amount on lipoproteins (chylomicron remnants) (Dueland et al, 1983). In the liver the newly synthesised vitamin will enter a common pool with any vitamin D (D_2 and/or D_3) from the diet and either undergo hydroxylation if needed by the body or will be stored in fat deposits. Vitamin D_2 is a plant sterol and cannot be produced in the skin of animals. It is prepared by the photolysis of the sterol ergosterol.

VITAMIN D BINDING PROTEIN

Vitamin D binding protein (DBP), previously known as Gc protein, is a glycoprotein of molecular weight of around 55,000 daltons (Bouillon and Van Baelen, 1981). It is synthesised by the liver as a single polypeptide chain. Each DBP molecule has a single binding site for all the known vitamin D metabolites; the order of affinity being 25(OH)D-lactone> 25(OH)D> 24,25$(OH)_2$D> 1,25$(OH)_2$D> Vitamin D. The affinity for 1,25$(OH)_2$D is approximately $10^7 M^{-1}$ whereas vitamin D itself binds only with a 100-fold lower affinity (Bouillon and Van Baelen, 1982). Two functions have been described for this binding protein:

(1) To facilitate in the skin production of vitamin D_3 by rapidly removing the end-product.

(2) To act as a transport protein for parent vitamin D and its metabolites.

A third possible role for DBP has been suggested. In vitro studies have shown that DBP will form tight 1:1 complexes with globular actin and is thereby able to depolymerise fibrous actin (Van Baelen et al, 1980). The

formation of extracellular actin-DBP complexes may occur in vivo after cell rupture but the exact function is still unknown.

1.3.2. THE METABOLISM OF VITAMIN D

Vitamin D, either in the D_2 or D_3 form, are metabolised in the same manner and are of equal biologic potency, as too are their metabolites.

(i) CONVERSION OF VITAMIN D TO 25(OH)D

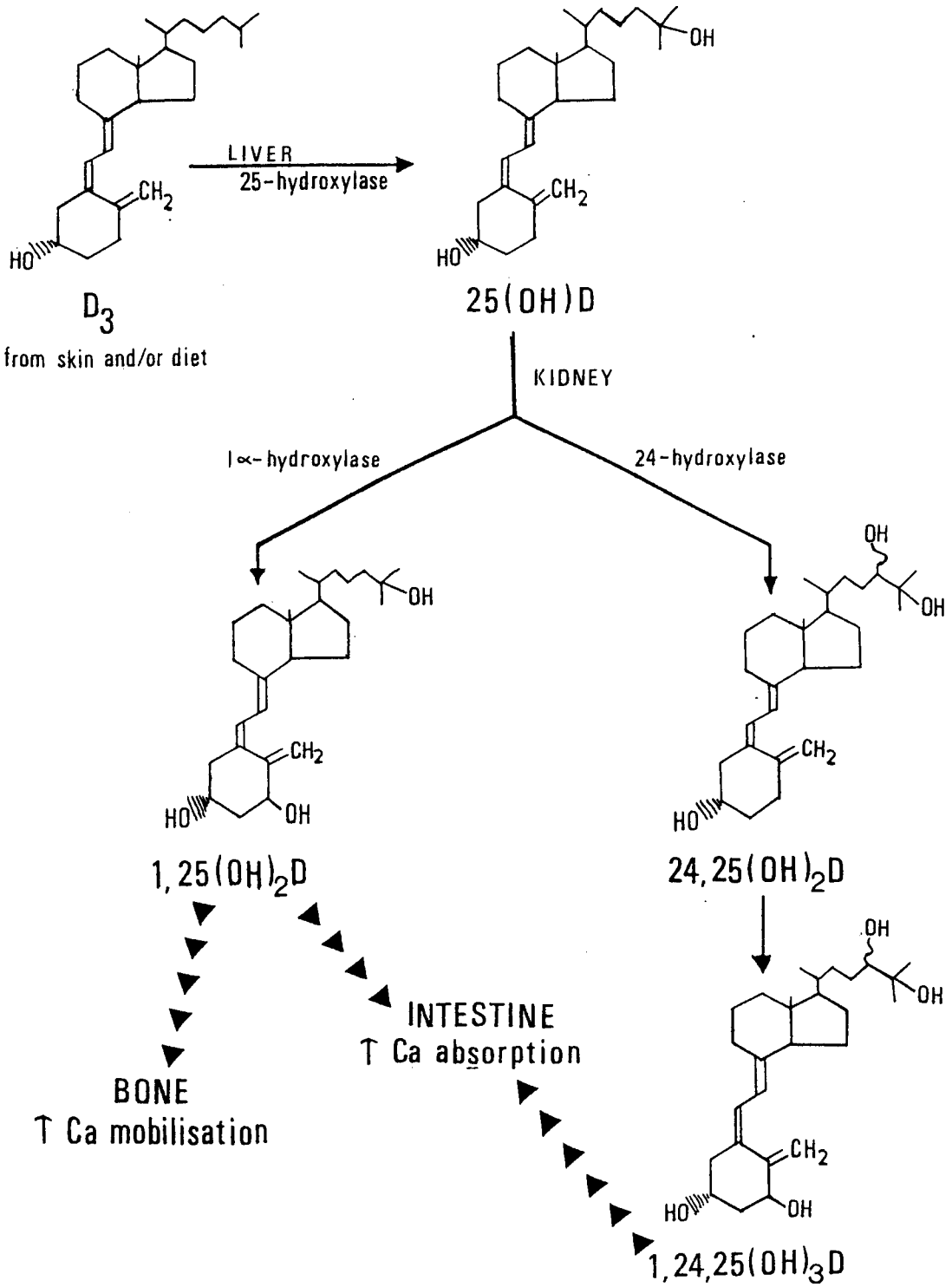
Once in the liver, vitamin D may be retained by the tissue (Silver and Berry, 1982) or it may be hydroxylated to 25-hydroxyvitamin D [25(OH)D], by a mixed function oxidase enzyme, located principally in the liver micosomes (figure 1.9). This vitamin D-25-hydroxylase enzyme requires reduced NADP, magnesium ions and molecular oxygen (Bhattacharyya and DeLuca, 1974): ie. it is dependent on a cytochrome P450 system. 25-hydroxylase is regulated by a feedback inhibition mechanism so that the product, 25(OH)D, is maintained at a relatively low concentration in the serum (20ng/ml). 25(OH)D is the major ciculating form of vitamin D and also the major storage form. Accordingly, circulating concentrations of 25(OH)D are considered to be a reflection of the availability of vitamin D and are thought to be the best indicator of vitamin D levels. Like parent vitamin D, it is unable to stimulate intestinal calcium transport or bone calcium mobilisation at physiological concentrations (Lawson, 1974).

(ii) CONVERSION OF 25(OH)D TO $1,25(OH)_2D$

The kidney is the major site of 25(OH)D metabolism, the most important reaction being the conversion of 25(OH)D to

FIGURE 1.9. Schematic summary of the origin of Vitamin D and its various biologically active metabolites.

(from: DeLuca and Schnoes, 1983. Ann. Rev. Biochem. 52, pp411-39).



the active hormone $1,25(OH)_2D$ by the renal mitochondrial 25(OH)D-1 alpha-hydroxylase (DeLuca and Schnoes, 1983). This enzyme requires internally generated NADPH, molecular oxygen and magnesium ions and, like the liver 25-hydroxylase, it is a mixed functional oxidase dependent on a P450 system. The hydroxylation of 25(OH)D to $1,25(OH)_2D$ (figure 1.9) by the tubular cells of the renal cortex, is tightly regulated by a variety of factors, most important of which are PTH, extracellular inorganic phosphorus and $1,25(OH)_2D$ itself. It is the only naturally occuring form of vitamin D that in physiological amounts is capable of maintaining plasma calcium concentrations. In fact, $1,25(OH)_2D$ has a biologic activity 500-1000 fold higher than that of the precursor 25(OH)D.

The kidney also possesses another hydroxylase enzyme, 25(OH)D-24-hydroxylase which catalyses the conversion of 25(OH)D to the 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) metabolite (Tanaka et al, 1979). This enzyme is known to be activated by $1,25(OH)_2D$ under conditions where $1,25(OH)_2D$ concentrations are adequate. In this manner $1,25(OH)_2D$ has a negative feedback on its own production. Both $1,25(OH)_2D$ and $24,25(OH)_2D$ may undergo an additional hydroxylation to form 1,24,25-trihydroxyvitamin D (Holick et al, 1973). Upon oxidative cleavage of the side chain, $1,25(OH)_2D$ forms a C-23 carboxylic acid, calcitroic acid , which is biologically inactive (Esvelt et al, 1979). Production of this acid in target tissues may be an important mechanism for the inactivation of the biologically potent $1,25(OH)_2D$.

26-Hydroxylation of 25(OH)D producing the 25,26-dihydroxyvitamin D metabolite can also occur (Suda et al, 1970) but the function of this metabolite remains unknown. It has only a slight action on intestinal calcium transport and little activity on bone mineralisation and mobilisation (Lam et al, 1975).

Table 1.1 illustrates the different vitamin D metabolites present in blood and their respective concentrations.

(iii) MINOR METABOLITES OF 25(OH)D

Other metabolites of 25(OH)D have been isolated and identified and these are summarised in figure 1.10. These metabolites were isolated either from *in vitro* incubations or from animals given large doses of vitamin D *in vivo* and, consequently, the metabolic significance of these metabolites are unknown.

(iv) METABOLIC EXCRETION OF VITAMIN D

Little is known about the excretory pathway of vitamin D and its metabolites. Although bile represents the major excretory route (DeLuca and Schnoes, 1983), only small amounts of 25(OH)D, $1,25(OH)_2D$ and two dehydration products of $1,25(OH)_2D$ have been positively identified (Onisko *et al*, 1980).

1.3.3. REGULATION OF VITAMIN D METABOLISM

The synthesis of $1,25(OH)_2D$ is under stringent control so that the renal output of $1,25(OH)_2D$ is related to the body's need for calcium. PTH is the principal regulator of the renal synthesis of $1,25(OH)_2D$. The stimulation of 1-alpha-hydroxylase activity by PTH is enhanced under conditions of decreased extracellular calcium (figure 1.11a) although the exact mechanism is still uncertain. This increase in $1,25(OH)_2D$ production restores plasma calcium concentration by stimulating calcium absorption in the intestine, notably in the regions of the duodenum and upper jejunum, and also by mobilising calcium from bone. Once plasma calcium concentration is restored PTH secretion is

TABLE 1.1. Some characteristics of the major Vitamin D_3 metabolites in plasma from normal individuals.

(from: Kanis, 1982. J. Bone Joint Surg. 64, pp542-60)

	Plasma Concentration (ug/L)	Turnover rate ($t_{\frac{1}{2}}$) (days)	Production rate (estimated) (ug/day)
Vitamin D_3	4-30	1-2	-
$25(OH)D_3$	5-50	5-20	10
$1,25(OH)_2D_3$	0.02-0.04	1-3	0.2
$24,25(OH)_2D_3$	1-5	12-31	1
$25,26(OH)_2D_3$	0.2-1.2	3-6	1.5
$1,24,25(OH)_3D_3$	0.01	unknown	uncertain

FIGURE 1.10. Minor metabolites of Vitamin D_3.

(from: DeLuca and Schnoes, 1983. Ann. Rev. Biochem. 52, pp411-39).

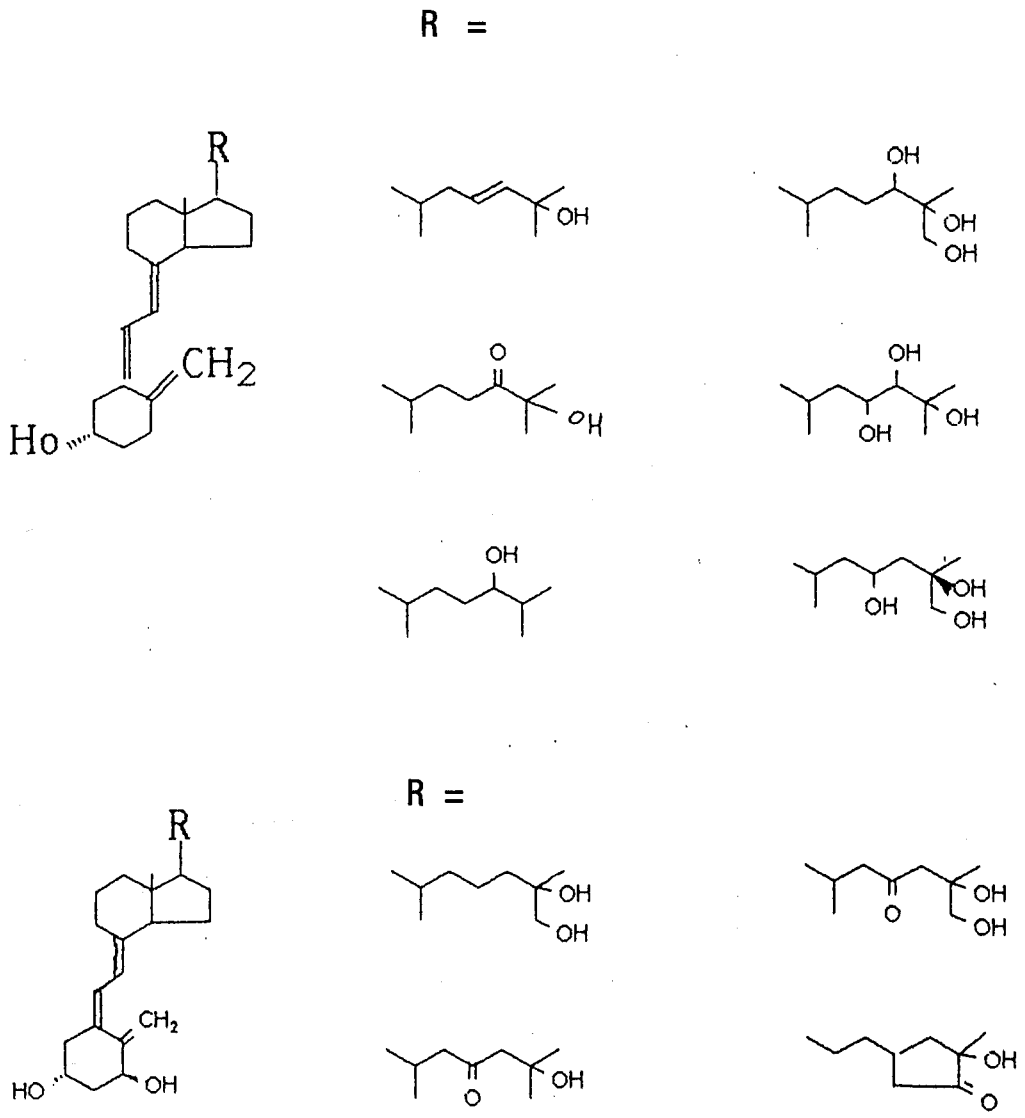
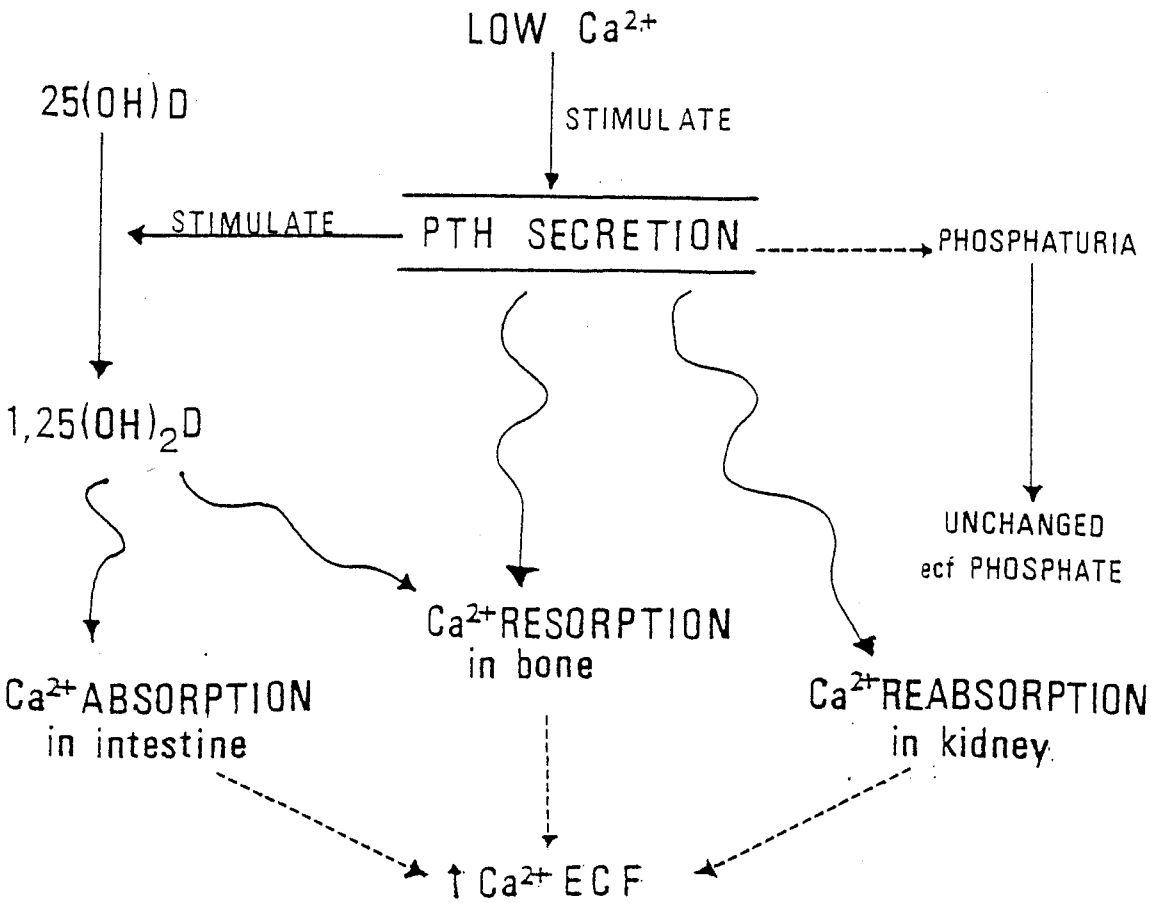


FIGURE 1.11a. Sequence of events following low plasma calcium concentration. The net effect is to elevate plasma calcium concentration without affecting plasma phosphate concentration.



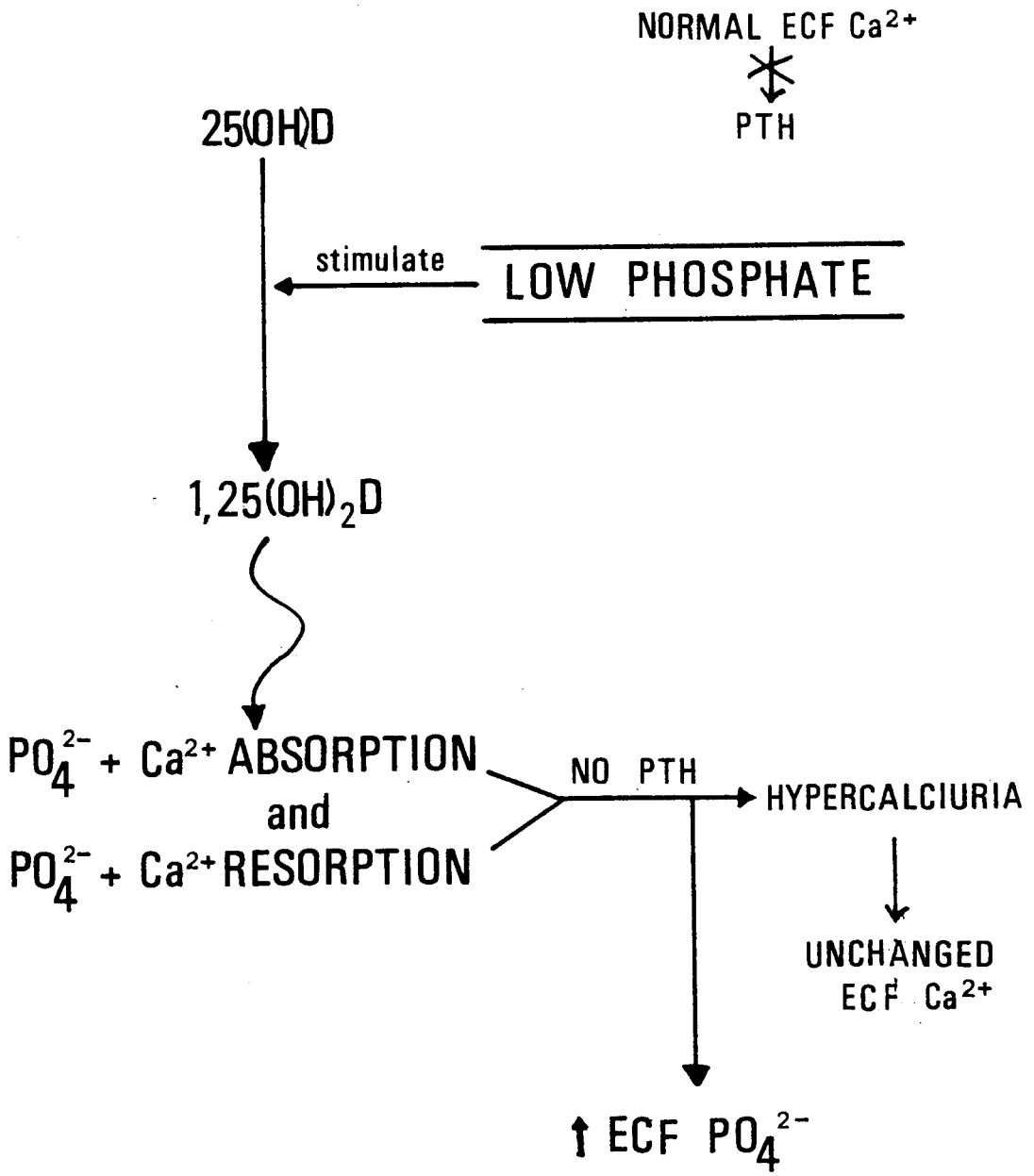
supressed thus leading to the decreased synthesis of $1,25(OH)_2D$. Elevated $1,25(OH)_2D$ concentrations also inhibit the synthesis of PTH by the parathyroid glands by interacting with the preproPTH gene (Russell et al, 1986).

Another regulator of $1,25(OH)_2D$ synthesis is $1,25(OH)_2D$ itself which inhibits the 1 alpha-hydroxylase. This feedback inhibition stimulates the renal 24-hydroxylase enzyme to produce $24,25(OH)_2D$ which is biologically less potent than $1,25(OH)_2D$ (Tanaka et al, 1979).

Other factors which seem to influence $1,25(OH)_2D$ production include plasma phosphate concentration (Tanaka and DeLuca, 1973), sex hormones, and possibly calcitonin and magnesium ions (Rude et al, 1985). In situations where there is low plasma phosphate, but normal calcium concentration, the production of $1,25(OH)_2D$ is enhanced, without the secretion of PTH (figure 1.11b). $1,25(OH)_2D$ will, therefore, stimulate intestinal absorption of phosphate (as well as calcium) and mobilise phosphate and also calcium from bone. In the absence of PTH, hypercalciuria is potentiated and the kidney reabsorbs all filtered phosphate. This sequence of events results in an increase in serum phosphate without appreciable elevation of plasma calcium.

Whether extracellular calcium directly regulates $1,25(OH)_2D$ production is still uncertain. It is firmly established that increased calcium down-regulates the secretion of PTH and that PTH can directly stimulate production of this metabolite. Thus, any changes in calcium that alter PTH secretion can indirectly affect $1,25(OH)_2D$ synthesis. Consequently, direct effects of calcium on its production are difficult to assess. However, in vivo results from Bushinsky et al (1985) suggest that serum calcium can regulate $1,25(OH)_2D$ independently of PTH and phosphorus. This conclusion was drawn from data obtained from rats

FIGURE 1.11b. Sequence of events following low plasma phosphate concentration. The net effect is to elevate plasma phosphate concentration without affecting plasma calcium concentration.



infused with high levels of PTH. Whether calcium has a strong regulatory effect when PTH is normal, rather than high, and how calcium actually affects plasma $1,25(OH)_2D$ are questions which still need to be answered.

1.3.4. MECHANISM OF ACTION OF $1.25(OH)_2D$ ON TARGET TISSUES

$1,25(OH)_2D$ is the most potent vitamin D metabolite in stimulating calcium transport in target tissues, namely bone and intestine. Evidence to date imply that these two organs are not the only sites for $1,25(OH)_2D$ action. In general, it is possible to classify target tissues with $1,25(OH)_2D$ receptors into three broad groups: Table 1.2. Walters et al, 1983; Haussler et al, 1985).

(a) Tissues where $1,25(OH)_2D$ receptors may regulate calcium and phosphate translocation across epithelial barriers. These include intestine (Zile et al, 1978), bone (Walters et al, 1982), kidney (Christakos and Norman, 1979), placenta (Zerwekh and Breslau, 1986), mammary and egg shell glands (Bell and Freeman, 1971).

(b) Tissues concerned with the regulation of the vitamin D system. These include kidney, skin (Strumpf et al, 1981), parathyroid (Wecksler et al, 1980) and possibly the pituitary (Haussler et al, 1980).

(c) Tissues where the role is currently unknown e.g. pancreas (Christakos and Norman, 1979), uterus (Walters et al, 1983), thymus (Reinhardt et al, 1982), fibroblasts (Pike and Haussler, 1983) and activated lymphocytes (Provvedini et al, 1983).

The mechanism of action of $1,25(OH)_2D$ complies with that of the classical steroid hormone-receptor model. The majority of unoccupied $1,25(OH)_2D$ receptor is apparently in

TABLE 1.2. Relevant tissues and cells that possess the $1,25(OH)_2D$ receptor.

(from: Haussler et al, 1985. Vitamin D: A Chemical, Biochemical and Clinical update)

Category	Tissue	Specific cell types	Established cell lines
Calcium Control	Intestine	Absorptive epithelial	Intestine-407 (H)
	Bone	Osteoblast	ROS 17/2.8 (R)
	Kidney	Distal/Proximal epithelial	LLC-MK_2 (M)
			LLC-PK_1 (P)
Endocrine	Parathyroid	Chief	---
	Pancreas	ß-cells	---
	Pituitary	Somatomammotroph	GH_3 Tumour (R)
	Ovary	?	Chinese hamster ovary
Other	Breast	Epithelial	MCF-7 carcinoma (H)
	Skin	Epidermal	3T6 fibroblast (M)
	Bone		
	Marrow	Monocyte	HL-60 leukaemia (H)
	Thymus	Reticular/T-lymphocyte	HSB-2 lymphoblast (H)
	Brain	Specific neurons	---

(H) Human (M) Mouse
(P) Porcine (R) Rat

the cytosol. $1,25(OH)_2D$ enters the cell and associates noncovalently but stereospecifically with the unoccupied receptor (figure 1.12). This association strengthens the binding capacity of the receptor and the receptor-hormone complex moves into the nucleus where it locates and binds to high affinity upstream activating sequences of DNA that control vitamin D regulated genes. Altered DNA transcription then results in enhanced or repressed levels of various mRNAs, examples of which include an increase in the mRNA which codes for the vitamin D-dependent calcium binding protein (Wasserman and Taylor, 1966) and a decrease in collagen mRNA.

A list of all the genes that are known to be regulated by $1,25(OH)_2D$ are shown in Table 1.3. These induced proteins, either singularly or in combination, are thought to perform the diverse biological functions of the hormone in the various target tissues. Thus, some target cells (i.e. the kidney) may primarily inactivate $1,25(OH)_2D$ and commence trans-epithelial calcium transport. Bone cells respond by inducing a range of proteins which are required for the complex process of bone mineralisation and remodelling. This will be discusssed in section 1.4. Finally, cells of the haematopoietic system and transformed cells appear to respond to $1,25(OH)_2D$ in a variety of ways involving differentiation and proliferation.

RECEPTOR FOR $1,25(OH)_2D$

This monomeric receptor has been conserved throughout evolution with immunocrossreactivity between fish, avian and mammalian $1,25(OH)_2D$ receptors (Pike *et al*, 1983). The entire sequence of the human intestinal receptor to $1,25(OH)_2D$ has been established (Baker *et al*, 1988) and the DNA-binding domain of this molecule shows strong homologies

FIGURE 1.12. Proposed mechanism of action of $1,25(OH)_2D$ in stimulating intestinal calcium transport: calcium is either packaged near the terminal web into vesicles or is taken up by mitochondria. Calcium is then extruded by a sodium-dependent process.

(from: DeLuca and Schones, 1983. Ann. Rev. Biochem. 52, pp411-39).

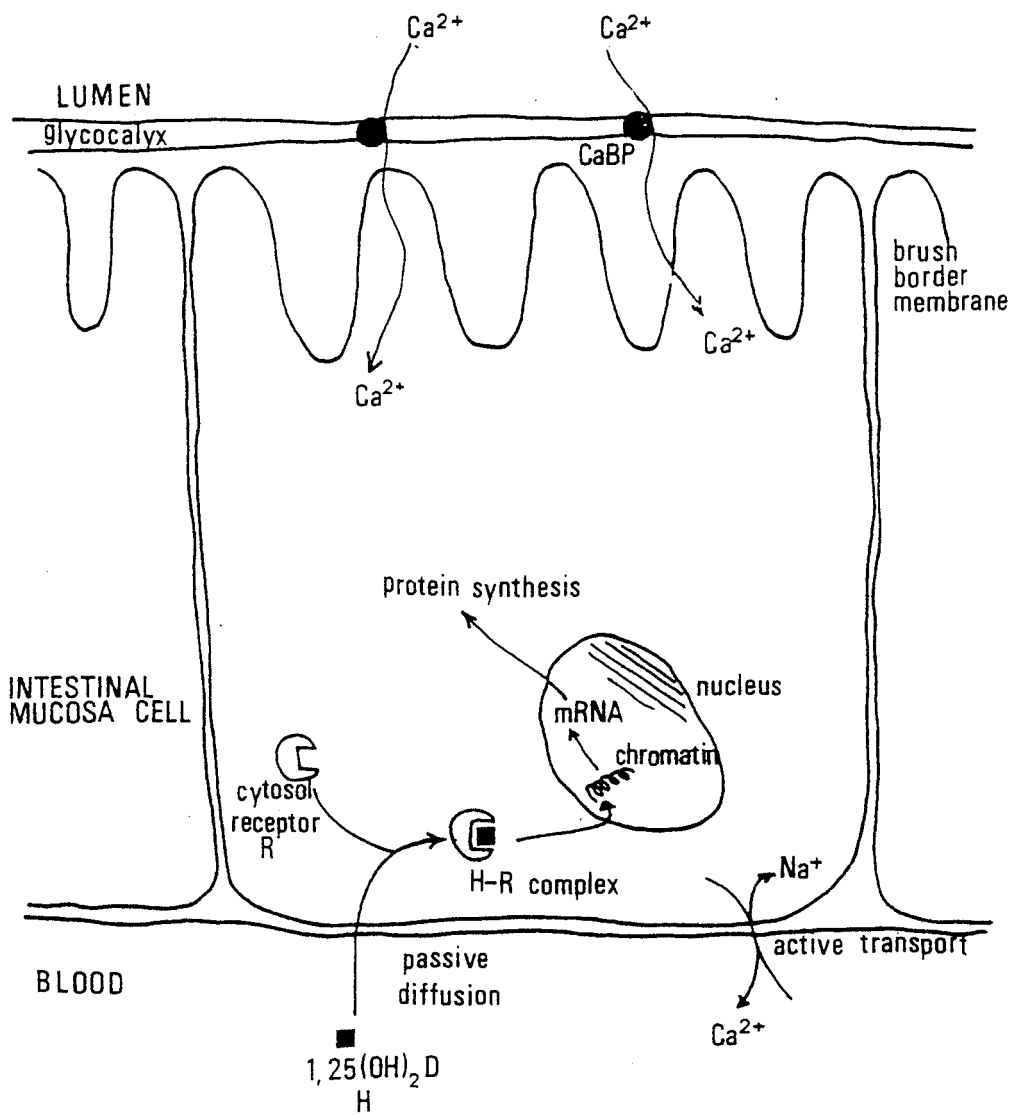


TABLE 1.3. Genes regulated by $1,25(OH)_2D$ at the level of mRNA accumulation.

Gene	Tissue	Direction of Regulation
PreproPTH	Rat parathyroid glands	↓
Calcitonin	Rat thyroid glands	↓
Type I collagen	Rat foetal calvaria	↓
Fibronectin	Human fibroblast line	↑
Osteocalcin	Rat osteosarcoma cells	↑
Interleukin-2	Activated human lymphocytes	↓
Interferon-γ	Activated human lymphocytes	↓
c-myc c-fos oncogenes c-fms	Human HL-60-myeloid leukaemia	↓ ↑ ↑
$1,25(OH)_2D$ receptor	Mouse fibroblasts	↑
Calbindin-D_{9K}	Rat intestinal mucosa	↑
Calbindin-D_{28K}	Chick intestinal mucosa	↑

An effect of $1,25(OH)_2D$ on transcription has thus far been demonstrated only for the genes encoding preproPTH, calcitonin, c-myc and the calcium-binding proteins calbindins D_{9K} and D_{28K}. $1,25(OH)_2D$ modifies granulocyte-macrophage colony-stimulating factor mRNA post-transcriptionally and also has post-transcriptional effects on the mRNA expression of both calbindins. For the other genes, it is not known whether $1,25(OH)_2D$ modulates gene transcription or induces the synthesis of other factors that alter the accumulation of mRNA after transcription.

(from: Reichel et al, 1989. N. Engl. J. Med. 320, pp980-91)

with that of the DNA-binding domains of other steroid hormone receptors, particularly within the DNA-binding domain. This suggests that the $1,25(OH)_2D$ receptor may belong to the same supergene family as all the other classic steroid-hormone receptor proteins.

The $1,25(OH)_2D$ receptor is a cytoplasmic protein of molecular weight between 50,000-60,000daltons and has a sedimentation coefficient around 3.1-3.7S (Pike and Haussler, 1979). It binds specifically, and with high affinity to the $1,25(OH)_2D$ metabolite and mediates its actions in the nucleus of the target cells (DeLuca and Schones, 1983). The hormone binding site is separate from the DNA binding domain (Pike, 1984) however, both contain reactive sulphydryl groups in or near their binding domains and the DNA binding site is highly sensitive to proteolysis (Allegretto and Pike, 1985).

MECHANISM OF ACTION:

(1) Actions of $1,25(OH)_2D$ on the intestine

The mechanism whereby $1,25(OH)_2D$ stimulates intestinal calcium absorption is well established. The stimulation of calcium binding proteins such as calbindin-D, by $1,25(OH)_2D$ facilitates the passive diffusion of calcium from the intestine and with the assistance of the calcium pump, calcium passes into the blood (figure 1.12). Induction of these binding proteins is known to be strictly dependent upon the regulated production of $1,25(OH)_2D$ (Christakos et al, 1981) although synthesis of these proteins is not optimal until after maximum stimulation of calcium transport by $1,25(OH)_2D$ (Spencer et al, 1976).

There is significant transport of calcium into the intestinal mucosa cell before the detection of any calcium binding proteins. Work by Bikle et al (1978) showed that inhibitors of protein and RNA synthesis did not block the ability of $1,25(OH)_2D$ to stimulate calcium movement by the chick intestinal mucosa which suggest that the stimulation of calcium transport in situ and in vitro are not totally dependent on the synthesis of new proteins and that other factors must be responsible.

Another calcium binding protein, calmodulin, is also found in the intestinal epithelium and may play a major role in calcium absorption. Although total calmodulin concentrations are not increased in the intestine (Thomasset et al, 1981), there is a redistribution of calmodulin to the brush border membrane when the tissue is stimulated with $1,25(OH)_2D$ (Bikle et al, 1984). This effect is coupled to an increase in calcium transport and is blocked by specific calmodulin antagonists.

An alternative mechanism for the action of $1,25(OH)_2D$ on intestinal calcium absorption has been proposed by Matsumoto et al (1981) and involves the alteration of the membrane lipid composition by the hormone. O'Doherty (1979) first demonstrated that $1,25(OH)_2D$ stimulated the enzymes involved in the phosphatidylcholine deacylation-reacylation cycle in duodenal villus cells of the rat intestine. Matsumoto et al showed that $1,25(OH)_2D$ acts specifically on the brush border membranes by causing increases in

(a) the total phosphatidylcholine content,
(b) the rate of incorporation of arachidonate into phosphatidyl-choline and also
(c) the amount of linoleic acid in the phospholipid fraction.

These observations were consistent with findings of O'Doherty (1979). This led subsequently to a proposal for the alternative mechanism of action of $1,25(OH)_2D$ on the intestine which involved the retailoring of the fatty acid composition of the villus cell membrane. This would modify general properties of the membrane, such as fluidity and permeability and perhaps these modifications would enable the passive diffusion of calcium into the cell.

Although the conventional classical hormone-receptor model is more favoured, it is possibile that the three mechanisms described above may interplay in the actions of $1,25(OH)_2D$ on the intestine.

(2) Actions of $1,25(OH)_2D$ on bone

The cellular basis for the direct effect of $1,25(OH)_2D$ on bone has been obtained from in vitro studies because of the difficulty in demonstrating bone remodelling in vivo. This metabolite is well characterised as an essential hormone for the regular mineralisation of new bone and as a potent bone-resorptive agent. This will be dicussed in section 1.4.2.

(3) Actions of $1,25(OH)_2D$ on cell growth and differentiation

There is growing evidence to support the idea that $1,25(OH)_2D$ has additional activities in tissues not primarily related to mineral homeostasis. Receptors to $1,25(OH)_2D$ have been demonstrated in a variety of tissues but of particular interest cells of the monocyte/macrophage series and in activated, proliferating B and T lymphocytes; quiescent lymphocytes do not seem to possess such receptors (Manolagas, 1985). In vitro experiments suggest that

$1,25(OH)_2D$ promotes the differentiation of monocytes towards the macrophage phenotype (Olsson et al, 1983) and also enhances the phagocytic activity of macrophages in culture. Activated lymphocytes have also been shown to be affected by $1,25(OH)_2D$. This hormone is a potent inhibitor of interleukin-2 and interferon production by these cells (Manolagas, 1985). These discoveries indicate that $1,25(OH)_2D$ interacts with the immune system and therefore might play an important role in the regulation of the immune response.

1.4 BONE

The ability of bone to serve a dual function, the first acting mechanically as a scaffold for the organs of the body and the second playing a role in the maintenance of mineral homeostasis, is achieved by its capacity to be constantly remodelled throughout life and yet still retain its strength and durability. As a living tissue, bone is unique in that it is not only rigid but it is also light enough to be moved by the coordinated action of muscles. This is due to the presence of two major types of bone which are strategically positioned (figure 1.13). Cortical (or compact) bone, composed of densely packed mineralised collagen laid down in layers, provides rigidity and is found in a majority of tubular bones. It has the familiar structure of Haversian systems or osteons. Trabecular (or cancellous) bone is spongy in appearance and is made up of a series of interconnecting plates of bone, each perforated by holes rather than bars. This bone provides strength and elasticity and constitutes the major portion of the axial skeleton (Revell, 1986).

In the normal healthy adult, the process of remodelling, where old bone is replaced by new bone, takes place primarily on cancellous bone surfaces, and to a lesser extent along the walls of the Haversian canals. It occurs in discrete units called Basic Multicellular Units (BMU's) each of which has a co-ordinated sequence involving activation of bone resorbing cells (osteoclasts), resorption of bone matrix and mineral, activation of bone formation cells (osteoblasts), and formation of new bone and its subsequent mineralisation (figure 1.14). Each remodelling unit replaces approximately 0.05-0.1mm^3 of bone and requires around 3 months to complete its task. The duration of the activation phase is a few days; of the resorption phase, 3 to 4 weeks,

FIGURE 1.13. Longitudinal section of the human femur to show dense cortical bone, the compacta; spongiosa showing fine trabeculae; and marrow cavities between both the trabeculae and within the cortical bone.

(from: Vaughan, 1975. The physiology of bone, Oxford University Press, London).

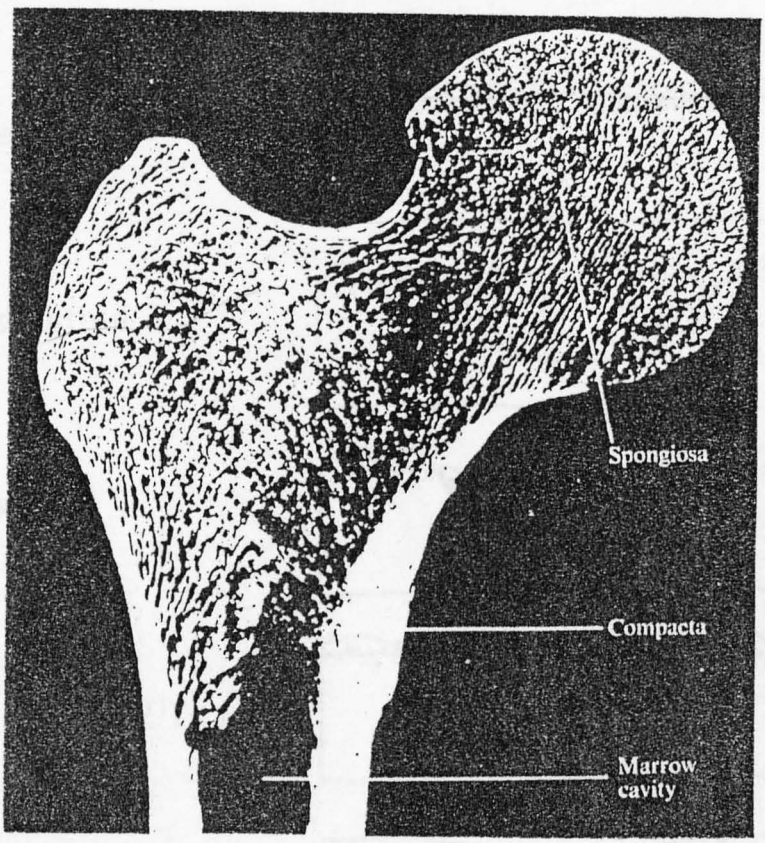
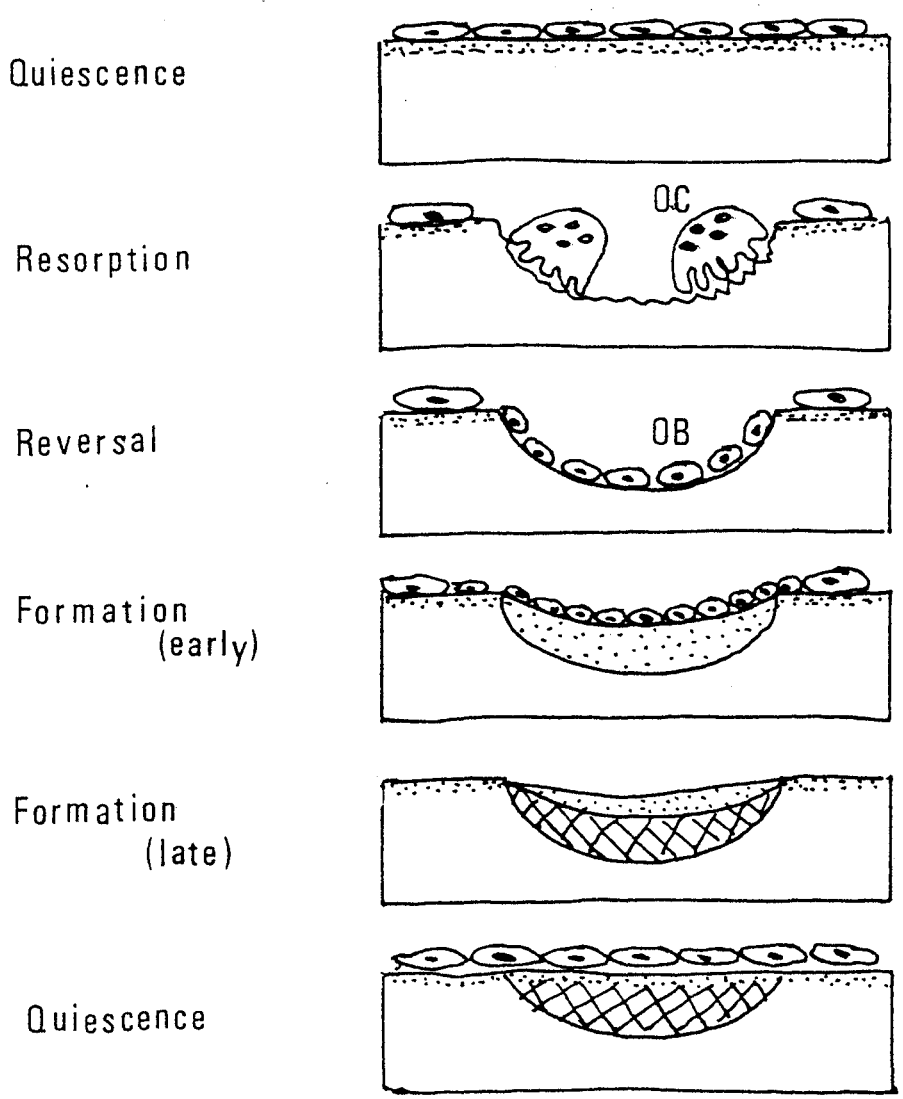


FIGURE 1.14. Bone remodelling sequence. The quiescent surface is covered by resting osteoblasts. The resorption surface has osteoclasts (OC). In the reversal phase the osteoblasts (OB) move in and deposit osteoid. In late formation, mineralisation has occured to complete the cycle.

☐ Old bone; ⊠ New bone; ⁘ Osteoid.

(from: Martin et al, 1988. Bailliere's Clin. Endocrinol. Metab. 2, pp1-9).



and of the formation phase, 3 to 4 months (Bordier, 1976). However, this time lengthens to 4-6 months in older adults and in some patients with bone disease it has been found that this time period could be as long as 30 months.

Surfaces of osseous tissue on which there is active deposition of bone show a narrow zone of osteoid tissue which differs from bone matrix in that it is not calcified. As the osteoblasts deposit the uncalcified matrix the lacuna gradually fills thus producing a wide osteoid seam. The interface between the calcifed bone and the osteoid is termed 'the calcification front'. Mineralisation commences, osteoblasts produce less matrix and the osteoid seam, initially wide narrows and eventually disappears. Thus, there is neither a gain nor loss of bone.

This process of bone resorption followed by equal bone formation is known as coupling and each BMU is excavated and filled to a precise pattern. However, very little is known about how the osteoclast cells are activated and how the bone volume excavated is determined. Even less is known about the mechanism which triggers the osteoblasts to appear at the excavated site and to lay down exactly the same amount of bone that has been resorbed by the osteoclasts.

Bone remodelling is an active process which is regulated by several hormones as well as local factors generated within bone and bone marrow. Most if not all metabolic bone diseases occur because there is an imbalance between bone formation and resorption.

1.4.1. THE CELLS OF BONE

The two main cells responsible for the turnover of bone tissue are derived from two separate cell systems. The

osteoblast, the cell responsible for bone formation, is a product of the osteogenic cell line, one of several lines of the stromal system of bone and marrow (figure 1.15), whereas the osteoclast, the cell mainly responsible for bone resorption, is derived from mononuclear phagocytic cell line, a product of the haemopoietic system. A third bone cell, the osteocyte, is considered to be an inactive osteoblast cell which has become embedded in the bone matrix and whose primary function is to 'communicate' with other osteocytes as well as osteoblasts on the surface. The relationship of all three cell types to the bone surface are shown diagramatically in figure 1.16.

1.4.1a THE OSTEOBLAST CELL

Active osteoblasts occur as a continuous layer of cells which are cuboidal and contain intracellular organelles typical of cells vigorously engaged in protein synthesis- i.e. abundant ER, a prominent Golgi apparatus and eccentric nuclei rich in RNA. They form functional gap junctions with neighbouring osteoblasts and with subjacent osteocytes processes and they contain numerous microtubles and bundles of actin filaments along the inner surface of the plasma membrane (Revell, 1986).

The primary function of the active osteoblast is to synthesise the extracellular bone matrix components. Figure 1.17 illustrates the major components synthesised by this cell type. Type I collagen (possessing two α_1 and one α_2 chains) is the major protein synthesised and is almost the exclusive collagen of the bone matrix. It represents at least 90% of the total organic matrix of bone. Type III and V collagens are generally associated with blood vessels found within the bone.

FIGURE 1.15. The origin of bone cells.

(from: Revell, 1986. Pathology of bone, eds. Springer-Verlag).

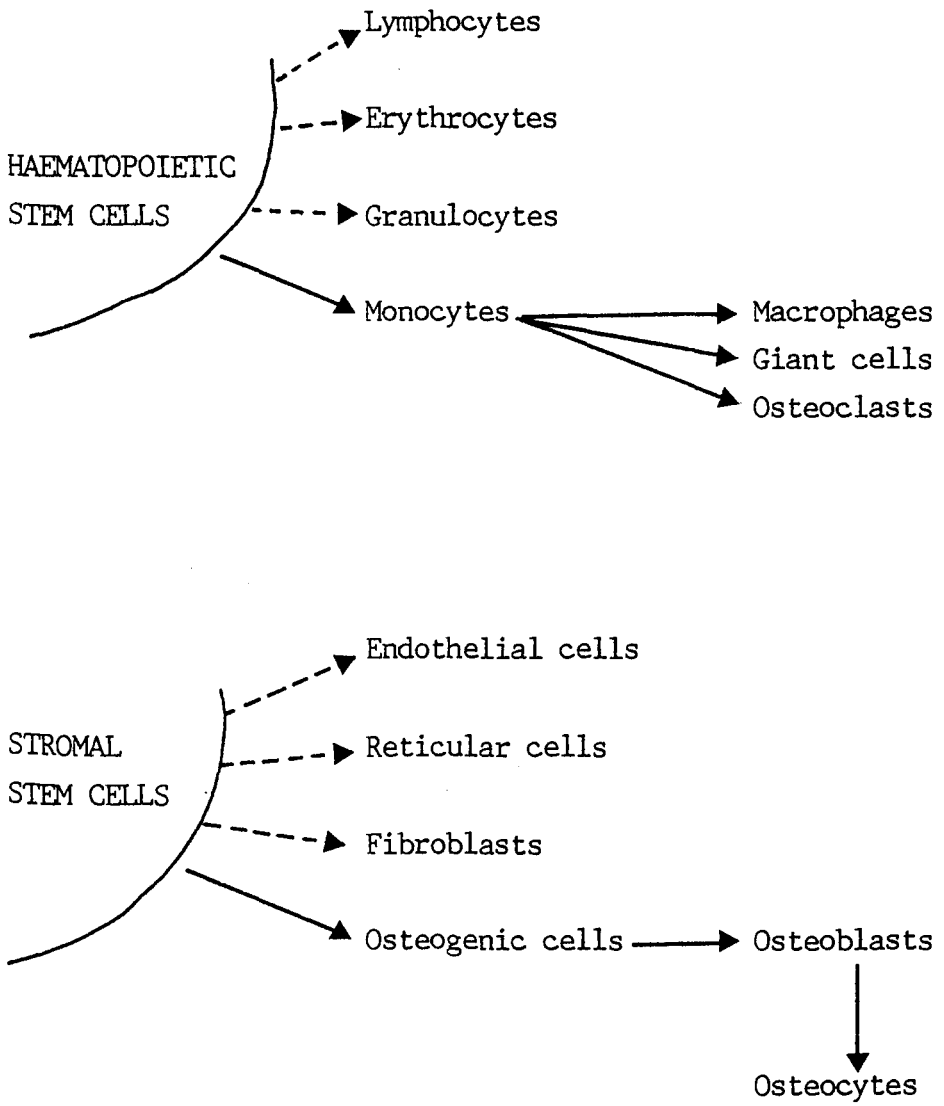
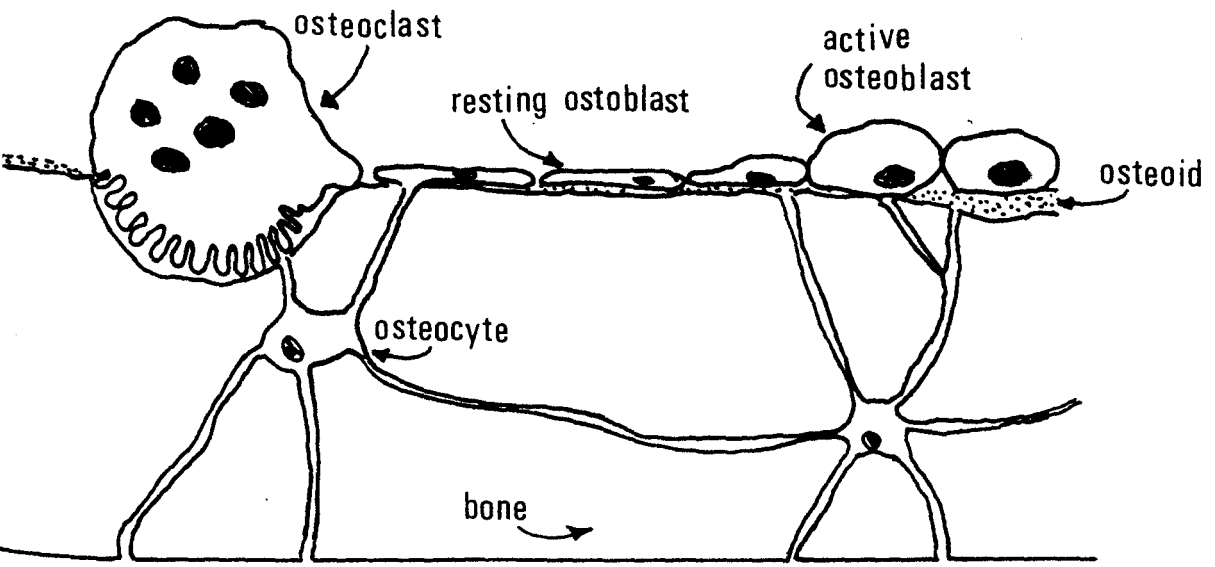


FIGURE 1.16. Schematic representation of bone cell types.

(from: Martin et al, 1988. Bailliere's Clin. Endocrinol. Metab. 2, pp1-29).



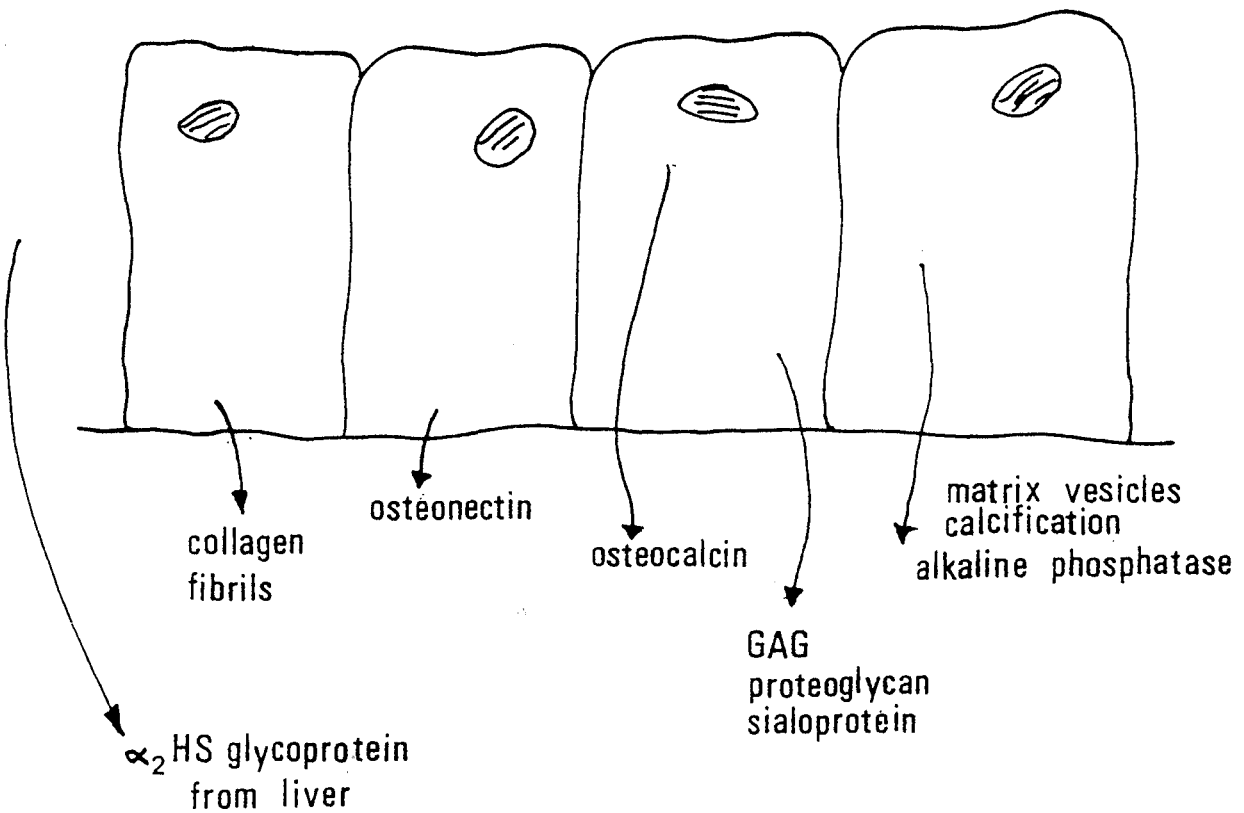
The osteoblast cell is rich in alkaline phosphatase and it is this enzyme which is the hallmark of the active cell. However, the function of this enzyme still remains unclear but it may be involved in maintaining low extracellular concentrations of inorganic pyrophosphate (PPi) since PPi is an inhibitor of calcification. Osteoblasts release small vesicles from their plasma membranes and these become embedded in the extracellular matrix. The enzyme is located on the outer membrane of these vesicles (Posner, 1985).

In addition to collagen molecules, osteoblasts synthesise and secrete other protein components of the bone matrix (figure 1.17). These are referred to as the non-collagenous proteins, of which osteocalcin is the most abundant and the best characterised (Lian and Gundberg, 1987). Also known as bone Gla protein and γ-carboxyglutamate protein, osteocalcin contains 49 amino acids (5,800 Da) of which three are the vitamin K dependent amino acid γ-carboxyglutamic acid (Gla). In the presence of calcium these three residues allow specific conformational changes in the osteocalcin molecule, changes which promote osteocalcin to bind to hydroxyapatite and subsequently accumulate in the bone matrix (Lian and Gundberg, 1987). Only a small amount of osteocalcin, which can be measured by the conventional radioimmunoassay method, is found circulating in blood. The precise biological function of osteocalcin is uncertain but it may play a role in regulating mineral deposition and crystal growth.

Another noncollagenous protein synthesised by the osteoblasts is osteonectin, a phosphoprotein which is analogous to fibronectin because it binds to both calcium and collagen fibres. This protein, therefore, forms the potential link between these two major constituents of bone (Russell et al, 1983) and there is strong evidence for its

FIGURE 1.17. Products of osteoblasts.

(from: Russell et al, 1983. Osteoporosis: a multi-disciplinary problem. Academic press, London).



involvement in the mineralisation of the bone extracellular matrix (Termine et al, 1981b). Other phosphoproteins have been identified but are not yet fully characterised. They are known as the 75,000 Da, 62,000 Da and 24,000 Da bone phosphoproteins. All are relatively acidic proteins but the latter differs from the other two in that it has a high hydroxyproline content which is unusual for a noncollagenous protein. It may well be a fragment of a collagenous protein rather than a distinct low molecular weight bone cell product. The function of these three phosphoproteins are unknown (Termine et al, 1981a).

Proteoglycans, glycosaminoglygans and sialoproteins have also been shown to be produced by the osteoblast but their respective functions are still uncertain.

Not all proteins found in the bone matrix are synthesised by osteoblasts. Albumin, α_2HS-glycoprotein and transferrin are derived from blood and have been reported to be deposited in bone at the time of mineralisation. It is presumed that these proteins bind to hydroxyapatite crystals and subsequently become trapped in the matrix as mineralisation is completed. However, the functions of these proteins, if any, are unknown (Russell et al, 1983).

Each osteoblast carries out a cycle of matrix synthesis, afterwhich it becomes inactive and either becomes buried in the bone tissue as an osteocyte or it remains on the surface as an inactive osteoblast.

1.4.1b. THE OSTEOCYTE CELL

The osteocyte, derived from the osteoblast, lies within the mineralised bone and is in contact with other osteocytes and also with osteoblasts on the bone surface by extensive

projections, or canaliculi. The osteocyte may be involved in the control of mineral exchange between bone and plasma.

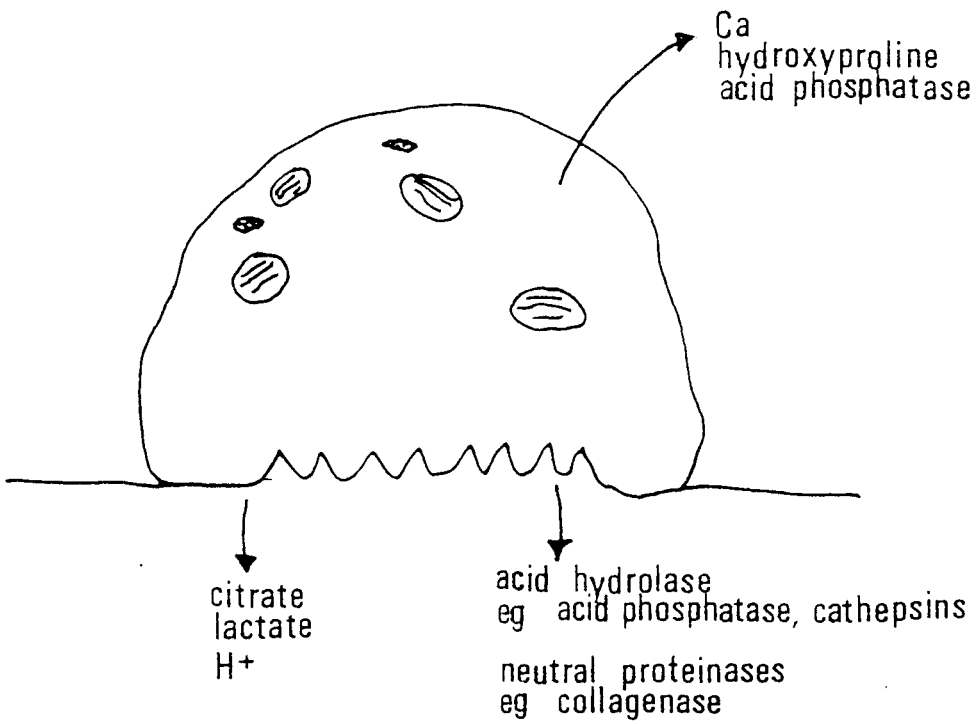
1.4.1c. THE OSTEOCLAST CELL

Osteoclasts are multinucleated cells which are rich in lysosomal enzymes, such as acid phosphatase and cathepsins but, in contrast to osteoblasts, their rough ER is much less well developed (Martin et al, 1988). They occur either singularly or in a small group on the inner surface of resorbing trabecular and compact bones. When in contact with the bone surface, the plasma membrane of the osteoclast which is immediately adjacent to the bone matrix shows deep invaginations with numerous microvilli. This ruffled border forms a tight physical bond with the bone and is considered to be the area of active resorption. Adjacent to this region is the 'clear zone', a region which has moderately dense granular cytoplasm and lacks any organelles. This is in contrast to the ruffled region which is dense in mitochondria and lysosomes and contains smooth and coated vesicles, phagosomes and residual bodies.

Osteoclasts synthesise and release various substances which are capable of resorbing the bone matrix (Revell, 1986). These are illustrated in figure 1.18. Possible mechanisms involved in bone resorption include the local production of acid (as H^+ as a result of carbonic anhydrase activity or as lactate) and of calcium chelating organic anions such as citrate. These assist in the ability of the osteoclast cell to dissolve the bone mineral phase. The osteoclasts also produce lysosomal acid hydrolases and proteinases such as collagenase and it is thought that these are involved in the degradation of the matrix components. However, much still needs to be learnt about how these enzymes function.

FIGURE 1.18. Products of osteoclasts.

(from: Russell et al, 1983. Osteoporosis: a multi-disciplinary problem. Academic press, London).



There is now strong evidence to show that osteoblasts participate more directly in the removal of bone matrix, rather than the osteoclasts. Work by Chambers and Fuller (1985) clearly shows that osteoclasts can only resorb bone and not the bone matrix. If the osteoid layer is removed by preincubating with collagenase then osteoclasts can proceed to resorb bone. Extensive studies have demonstrated that osteoblasts release latent collagenase as well as a proteinase called tissue plasminogen activator (tPA), into the matrix (Sakamoto and Sakamoto, 1986; Hamilton et al, 1985; Martin et al, 1985). tPA is able to generate plasmin from plasminogen (Hamilton et al, 1985) which in turn is capable of activating latent collagenase to degrade the bone matrix.

1.4.2. FACTORS REGULATING BONE REMODELLING

The majority, if not all, of the information on the control of bone remodelling has been obtained from studying the effect of various hormones and other factors on either the formation and resorption of foetal or newborn rodent bone in culture or the effects on cultured osteoblast-like cells. It is very difficult to obtain a homogeneous layer of osteoclasts because of the difficulty in removing fibroblast-like cells.

The humoral control of bone remodelling results from the combined interaction of many factors. These can be divided into four main groups. Although the effects of some of these factors have been well established there are a few in which a specific role is uncertain (Russell et al, 1983; Martin et al, 1988b).

(i) THE CALCIUM REGULATING HORMONES

At the cellular level both PTH and $1,25(OH)_2D$ have similar effects on bone remodelling. Their major effect on

bone is to increase bone resorption and to have a direct inhibitory effect on formation. Calcitonin, however, is a specific hormonal inhibitor of bone resorption.

PTH:

PTH has been shown to be a potent stimulator of bone resorption (Raisz, 1965) but the presence of receptors for PTH on the osteoclast cell have not been demonstrated. Although there is no convincing evidence to show that it acts directly on the osteoclasts or their precursors, studies using organ cultures of bone have noted an increase in both the activity and number of osteoclasts in the presence of this hormone (Raisz, 1965; Holtrop et al, 1974). The well documented in vitro effects of PTH includes (a) stimulating carbonic anhydrase activity (Minkin and Jennings, 1972) and citric acid production (Vaes and Nichols, 1971) thereby decreasing the pH of the resorbing environment and (b) encouraging lysosomal enzyme release from the osteoclasts. It now seems likely that PTH acts on the osteoclast indirectly via the osteoblast lineage.

Unlike the osteoclasts, osteoblasts possess high-affinity plasma membrane receptors for PTH (Partridge et al, 1981). Studies have shown that after several minutes of exposure to PTH these cells increase their adenylate cyclase activity, cAMP accumulation and intracellular calcium uptake. At the same concentration PTH also inhibits both matrix synthesis, including bone collagen synthesis, and alkaline phosphatase activity. However, the intracellular messenger(s) for PTH action is still unknown although studies have shown that the cyclic AMP analogues 8-bromo cyclic AMP and dibutyryl cyclic AMP and the phosphodiesterase inhibitor isobutylmethylxanthine mimic these effects of PTH.

Recently, a PTH-like peptide has been described in extracts of tumours and in culture medium from tumour cell lines obtained from patients presenting with humoral hypercalcaemia of malignancy (Suva et al, 1987). This factor has great homology with PTH, in particular within the N-terminal region and, like PTH, will stimulate adenylate cyclase activity in both renal membranes and osteoblast-like cells. Although immunochemically and chromatographically distinct from PTH, this PTH-like peptide of 141-amino acids might nevertheless interact with the PTH receptor. If this is the case it would explain the potent resorbing activity of this peptide.

1,25-DIHYDROXYVITAMIN D:

$1,25(OH)_2D$ has an important role in normal bone growth and mineralisation. Like PTH it is a potent stimulator of bone resorption, increasing the number and activity of osteoclasts in organ cultures and yet the presence of $1,25(OH)_2D$ receptors in the osteoclast or its precursor has not been demonstrated. It is generally thought that $1,25(OH)_2D$, like PTH, induces bone resorption indirectly by stimulating the osteoblast to release a soluble factor which increases osteoclastic bone resorption (McSheeny and Chambers, 1987).

Although receptors to $1,25(OH)_2D$ has been demonstrated in osteoblasts its exact role in bone formation is still controversial. In bone organ culture and in osteoblastic cell cultures $1,25(OH)_2D$ stimulates osteocalcin synthesis (Price and Baukol, 1980) and decreases collagen synthesis but can either increase or decrease alkaline phosphatase activity depending on the dose administered and the stage of growth of the cultures used. The interrelationship between osteocalcin and $1,25(OH)_2D$ in bone formation is still unclear and the physiological significance of reduced collagen synthesis in the presence of $1,25(OH)_2D$ is

interesting since the same hormone promotes normal growth and development *in vivo*.

Several studies have suggested that another vitamin D metabolite, such as 25(OH)D or $24,25(OH)_2D$, may stimulate bone growth and development. 25(OH)D is the most abundant vitamin D metabolite in bone and is associated with areas of active mineralisation (Wezeman, 1976).

CALCITONIN:

Calcitonin directly inhibits bone resorption by inhibiting osteoclastic activity but does not affect osteoblasts directly.

(ii) LOCAL FACTORS

PROSTAGLANDINS

Prostaglandins (PGs), particularly of the E series, are synthesised by the osteoblast and are potent bone-resorbing agents (Klein and Raisz, 1970). *In vitro* studies have shown that synthesis is stimulated by epidermal growth factor and platelet-derived growth factor. PGs are direct stimulators of bone formation as well as resorption and therefore could play an important role in the coupling mechanism. However, their exact physiological role is still not established.

CYTOKINES

A variety of monokines and lymphokines have been shown to act directly on bone to promote resorption and inhibit bone collagen synthesis *in vitro* (Raisz *et al*, 1975). The term OAF (osteoclast activating factor) was introduced to describe the substance which was present in the supernatant of activated proliferative lymphocytes and which had powerful bone resorbing activity (Horton *et al*, 1972). It is now inappropriate to use this term since more than one

factor was present in the supernatant. The cytokines, as they are now known, which are capable of stimulating bone resorption include interleukin-1 (IL-1) and tumour necrosis factor alpha and beta, and like PTH and $1,25(OH)_2D$, they do not have a direct effect on osteoclasts but their actions are mediated by osteoblasts (Martin et al, 1988). Their synthesis has been linked with the production of PGs by the osteoblasts but their physiological importance, if any, is still unclear.

(iii) GROWTH FACTORS

Growth factors may play an important role in bone formation by stimulating the growth and development of the osteoblast precursor cells. Cultured rat osteoblast-like cells possess receptors to epidermal growth factor (EGF) and in the presence of EGF, DNA and prostaglandin E_2 synthesis are increased whilst synthesis of type I collagen and alkaline phosphatase activity are both inhibited.

Transforming growth factors alpha and beta (TGFα and β), platelet-derived growth factor (PDGF) and EGF are powerful stimulators of bone resorption (Raisz et al, 1980; Tashjian et al, 1985). Although the effects of EGF, TGFα and β on mouse calvaria appear to be dependent on endogenous production of prostaglandins further studies using foetal rat long bones have shown them to be independent of prostaglandin synthesis. It is difficult to explain these actions since the physiological role(s) for these factors are unknown.

There is a possibility that PDGF may have a role in the healing of fractures since this factor is secreted by platelets and has been shown to stimulate both DNA and protein synthesis in cultured rat calvaria (Canalis, 1981).

1.4.3 BONE DISEASE IN THE ELDERLY

The loss of bone mineral (osteopenia) is a normal feature in the ageing process and occurs when the two active processes of bone formation and resorption become dissociated in favour of net resorption. The amount of trabecular bone in the vertebrae begins to decline in the third decade of life whereas loss of cortical bone occurs much later (Revell, 1986). This loss of mineral from bone is proportionately greater and begins a decade earlier in women than in men (Woolf and St John Dixon, 1988). This could be related to the period of accelerated bone loss accompanying menopause which superimposes upon the age-related bone loss. The ultimate result is that by the age of 90years women have lost around one fifth of their original peak cortical bone mass and 40-50% of their peak trabecular bone mass. Contrast this with the loss of less than 5% of peak cortical bone mass and 10-25% of peak trabecular bone mass in men. As a direct consequence, bone strength decreases and fracture risk increases with age (Newton-John and Morgan, 1970).

There is a negative calcium balance in the elderly, especially in elderly women (Heaney et al, 1982). Intestinal absorption of calcium declines with advancing age (Bullamore et al, 1970; Gallagher et al, 1979) and may be attributed to either a fall in circulating concentrations of $1,25(OH)_2D$ or to an imbalance between bone formation and resorption. In the former hypothesis the decreased secretion of $1,25(OH)_2D$ is brought on by age-related decreases in renal function. Malabsorption of calcium follows and this stimulates the secretion of PTH which in turn increases renal secretion of $1,25(OH)_2D$. Although concentrations of the active vitamin D metabolite is maintained the elevated PTH concentrations cause an increase in bone resorption.

In the latter hypothesis, however, the imbalance between formation and resorption is due either to reduced physical activity, age, oestrogen deficiency or some unknown factor (Heaney et al, 1982). PTH secretion is supressed because calcium released from the skeleton exceeds that which is taken up by the skeleton, and, as a consequence of this $1,25(OH)_2D$ synthesis is supressed. Calcium malabsorption therefore develops.

Although the reason for the deterioration in intestinal calcium absorption is not known with certainty it appears that vitamin D may play an important role. Vitamin D deficiency in the elderly has been noted (Baker et al, 1980; Lips et al, 1982) although not all investigators have confirmed this finding (Meller et al, 1985). It is quite clear that a healthy individual cannot obtain enough vitamin D through dietary sources alone and, therefore, exposure to sunlight or dietary supplementation is necessary to ensure adequate vitamin D status (Parfitt et al, 1982). These factors therefore have a great influence on vitamin D status in the elderly:

(i) Diminished exposure to sunlight
(ii) Reduced dietary intake of vitamin D, and
(iii) Intestinal malabsorption of fat-soluble vitamin D.

Because not many foods are fortified with vitamin D the body store of vitamin D is more dependent on the cutaneous synthesis of D_3.

Skin thickness decreases linearly with age after 20 years (Tan et al, 1982). The age-related changes take place principally in the dermis, namely the shrinking and sagging of the elastic fibres in the papillary dermis (Montagna et al, 1979), the marked reduction in 7-dehydrocholesterol (the precursor of vitamin D_3) and the decreased ability of the skin to synthesise $PreD_3$ (MacLaughlin and Holick, 1985). In fact, skin obtained from elderly subjects demonstrated only

half the ability to synthesise $PreD_3$ compared with skin from young individuals.

The decreasing ability of the skin to synthesise D_3 with age coupled with a low dietary intake of calcium and of vitamin D these factors bring about the observed calcium deficiency in this age group. This deficiency, if persistent, will eventually lead to bone disease as the body tries to compensate for the imbalance by removing calcium from bone. Two such bone diseases which are common in the elderly are osteoporosis and osteomalacia.

(i) OSTEOPOROSIS:

Osteoporosis is becoming one of the major bone diseases in the United Kingdom as well as in other western countries. It is now considered to be a "heterogeneous disorder" thus making it unlikely that any single element is the causative factor. However, risk factors are known and these are summarised in Table 1.4. The two major risk factors for osteoporosis are loss of ovarian function and peak adult bone mass (Lindsay, 1988).

Females are at a greater risk of developing osteoporosis than their male counterparts, especially slender women. Although tobacco smoking is a risk factor the mechanism is still uncertain. Smoking disturbs pulmonary function, causes some degree of acidosis and, in general, induces an earlier menopause. Excessive alcohol consumption and sedentary lifestyle are also factors which may induce osteoporosis. The final risk factor is the onset of an early menopause. If these five factors are combined in a woman she is surely predisposed to become osteoporotic (Lindsay, 1988).

TABLE 1.4. Factors thought to be associated with increased risk for osteoporosis.

(from: Lindsay, 1988. Bailliere's Clin. Endocrinol. Metab. 2, pp103-24).

Female sex
Caucasian or Asiatic ethnicity
Positive family history
Low dietary calcium intake (lifelong)
Early menopause (or Oophorectomy)
Slim body build
Sedentary lifestyle
Alcohol abuse
Cigarette smoking
High caffeine intake
High protein intake
High phosphate intake
Secondary causes of bone loss (steroids, hyperthyroidism)

Roughly arranged in descending order of importance

The transition from osteopenia to osteoporosis commences when the degree of bone loss is such that the skeletal components no longer ensure their weight bearing function. Thus, the underlying defect is an uncoupling between bone formation and resorption, favouring an increase in resorption. Thus, the net result is loss of trabecular and cortical bone mass which leaves behind normal but thin cortical bone and normal but sparse trabecular bone. Eventually a fracture threshold is reached as trabecular bone volume diminishes in thickness and it is at this point that spinal fractures can occur.

Detecting the thinning of long bone cortical tissue due to osteoporosis, is easy and in advanced cases is visible to the naked eye on inspection of good hand or limb radiographs. Unfortunately, it is on the vertebrae, where detection is most difficult, that bone ageing is the most severe. To prevent osteoporosis means to maintain the peak bone mass, i.e. the maximum bone mass present at maturity, thus preventing repeated spinal compression fractures that may lead to loss of stature, a bent back and even kyphosis.

Osteoporosis is normally classified into three catergories (Riggs, 1985):
Type I osteoporosis (or postmenopausal osteoporosis)
Type II osteoporosis (age-related or senile osteoporosis)
Type III osteoporosis (with secondary hyperparathyroidism).

Type I osteoporosis (Table 1.5), characterised by rarefaction of trabecular bone, affects exclusively women aged from 50-65years, in the period following the menopause and has been linked with oestrogen deficiency and impaired calcium absorption. Such women have normal circulating concentrations of 25(OH)D but reduced concentrations of $1,25(OH)_2D$ (Gallagher et al, 1979; Riggs et al, 1981)

TABLE 1.5. Abnormalities of vitamin D metabolism associated with various types of involutional osteoporosis.

(from: Riggs, 1985. Vitamin D: A Chemical, Biochemical and Clinical update, pp976-85).

TYPE	DEFINITION	MECHANISM
I	Postmenopausal osteoporosis	Seconary decrease in 25(OH)D 1α-hydroxylase activity
IIa	Senile osteoporosis (SO)	Primary defect (mild) in 25(OH)D 1α-hydroxylase
IIb	SO with nutritional vit D deficiency	Insufficient substrate
III	Associated with secondary hyper-parathyroidism	Primary defect (moderate) of 25(OH)D 1α-hydroxylase activity

implying a defect in the 25(OH)D-1 alpha-hydroxylase activity.

The majority of patients with type I osteoporosis have normal or low circulating concentrations of serum PTH. However, a small subgroup have been shown to have elevated values and these patients are said to have type III osteoporosis.

Type II osteoporosis (senile osteoporosis) occurs in individuals over 65years and is due to the loss of both cortical and trabecular bone. The major risk factor for developing this type is the initial bone mass (Riggs, 1985). Bone mass is greater in men than in women and in blacks compared to whites. Thus, osteoporosis is correspondingly three to five times more frequent in women than in men and is much less frequent in blacks than in whites (Stevenson, 1988).

The first clinical indication of senile osteoporosis is usually a complication, the fracture. This occurs because the bone mass has diminished sufficiently to produce a bone density value below the fracture threshold. Elderly patients with the lowest bone mineral density values will therefore have the greatest risk of fracture and fractures may occur on minimal and trivial trauma. Crushed fractures of the vertabrae are very common in this disorder and it is now recognised that osteoporosis is not only the principle cause of this spontaneous vertebral compression but is also a major contributor to most of the fractures seen in the elderly (fractures in the neck and intertrochanteric regions of the proximal femur and distal radius or Colles' fractures). All these regions have a high proportion of trabecular bone.

(ii) OSTEOMALACIA:

In osteomalacia (and in the juvenile form, rickets) the metabolic abnormality results in the failure to mineralise newly formed organic matrix. Consequently, trabecular bone surfaces have a greater percentage of osteoid and usually an increase in the width of the osteoid seams. Such bones become weak and are prone to fracture. The classical description of osteomalacia includes bone pain, which is worse on weight bearing or pressure, limb weakness, waddling gait and the presence of pseudofractures or Looser's zones on bone radiographs. Biochemically, serum alkaline phosphatase is usually raised as is urinary excretion of hydroxyproline. Serum calcium and phosphate may be low or normal.

The development of osteomalacia (and rickets) can be due to a number of factors (Table 1.6) but the most common cause in the elderly is vitamin D deficiency (Revell, 1986). Hodkinson et al (1973) showed that the elderly, especially those who are housebound, generally have a low dietary intake of vitamin D and that this deficiency can be aggravated by not exposing themselves to sunlight. Although malabsorption of calcium also occurs in osteomalacia, an occurrence related to the reduced concentrations of vitamin D, the exact patho-physiology is still uncertain.

The presence of osteomalacia in the elderly is very often in a subclinical form. It is therefore important to be able to diagnose these patients before the disease develops to a severe form to cause the possibility of fractures.

TABLE 1.6. Causes of osteomalacia and rickets.

(from: Revell, 1986. in: Pathology of bone, eds. Springer-Verlag).

A Vitamin D deficiency:
  1. Dietary lack
  2. Low sunlight exposure
  3. Neonatal rickets

B Vitamin D malabsorption:
  1. Coeliac disease
  2. Postgastrectomy
  3. Small bowel operations

C Impaired Vitamin D metabolism:
  1. Impaired 25-hydroxylation in the liver
     a) Neonatal hepatitis
     b) Cirrhosis and chronic hepatitis
  2. Impaired 1 alpha-hydroxylation in the kidney
     a) Enzyme defect
     b) Chronic renal failure
     c) PTH deficiency/resistence

D Increased catabolism:
  Induction of microsomal enzymes by drugs (e.g. barbiturates)

E Inhibition of mineralisation:
  1. Diphosphonates
  2. Sodium fluoride
  3. Aluminium

F Phosphate depletion and hypophosphataemia:
  1. Negative phosphate balance due to malabsorption, haemodialysis, antacids
  2. Excess PTH or lack of Vitamin D
  3. Primary (X-linked) or Secondary (tumour related) hypophosphataemia

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(iii) FRACTURE OF THE FEMORAL NECK:

When standing about one third of the body's weight is supported by each hip and the neck of femur is said to be loaded normally. Between the ages of 20 and 90 years bone mineral density at the femoral neck falls an estimated 58% in women and 39% in men (Riggs et al, 1982). This age-related decline in femoral neck bone mass is correlated with decreased bone strength in both sexes although less in women than in men.

As age-related bone mass progresses, especially around the femur, an increasing number of elderly men and women will have bone density values below the fracture threshold; those with the lowest values will have the greatest risk of fracture. The majority of femoral fractures occurs when the patient falls on the affected hip. What, therefore, is the cause of reduced bone strength?

While it is generally thought that osteoporosis is the main cause of reduced bone strength and therefore femoral fractures, there are a few cases in which the underlying defect is due to osteomalacia, which is usually in the subclinical form. However, the prevalence of femoral neck fracture with osteomalacia is only about 2% (Wilton et al, 1987a). There is no clear biochemical marker to distinguish femoral neck fracture patients with osteomalacia from those with osteoporosis (Table 1.7). The only means is to biopsy every femoral fracture case.

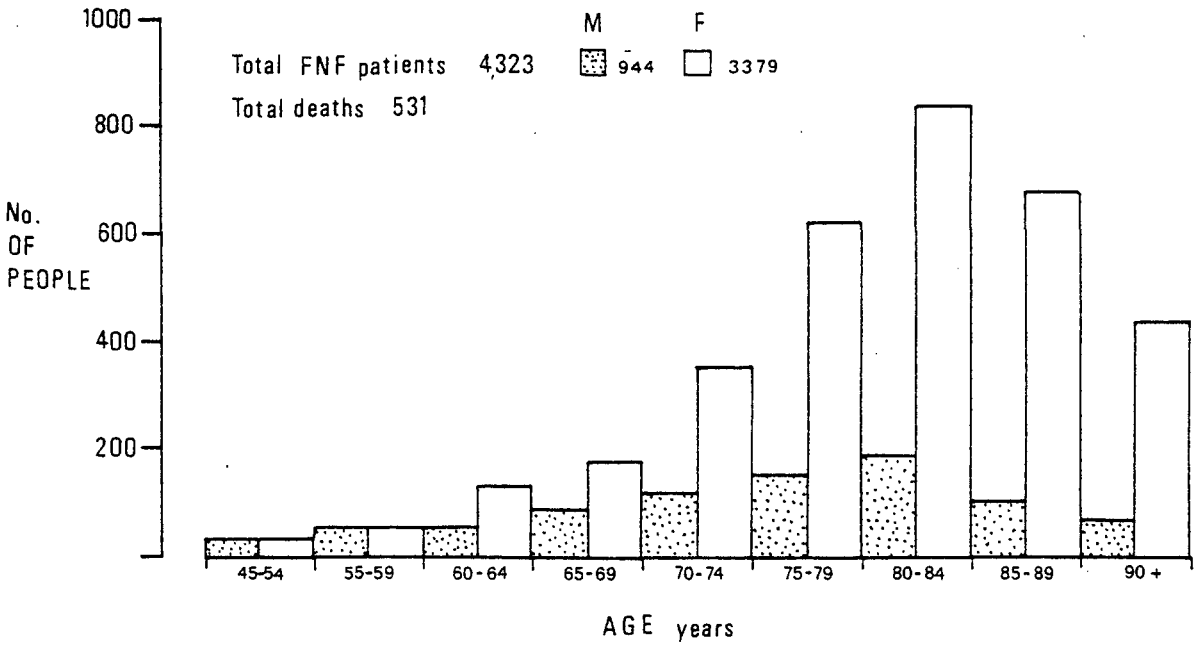
It is easy to evaluate the incidence of femoral neck fractures (FNF) since all patients are hospitalised. In the Hospital in-patient enquiry of 1985 over 60% of new cases of FNF were over 65 years old (Figure 1.19) and within this group the male:female ratio was 1:4. With the increase in life expectancy, which is related to the decrease in infant

Table 1.7 Clinical-pathologic differences between osteoporosis and osteomalacia.

	Osteoporosis	Osteomalacia
Definition	Decreased bone mass	Demineralised bone
Symptoms	Localised to fracture sites	Generalised musculo-skeletal
Radiographic features	Osteopenia, frank fractures	Pseudofractures with Looser lines, frank fractures
Pathology	Thin and discontinuous bone trabeculae	Increased osteoid (the non-mineralised part of bone)
Calcium	Normal	Low or normal
Phosphate	Normal	Low or normal
Alkaline Phosphatase	Normal or mild increase	High
PTH	Normal or mild increase	High or normal
Calcification rate	Normal	Decreased

FIGURE 1.19. The number of femoral neck fracture (FNF) cases in England and Wales in 1985.

(from: The Hospital in-patient enquiry, 1985).



mortality and also to a decrease in the mortality rate after 55 years, FNF will have a major impact on the cost of health care.

It should be pointed out just how serious these fractures are. As stated previously, the incidence increases in patients over 65 years and in many cases other diseases often co-exist with the fracture, thereby worsening the prognosis. Complications are frequent because of the age of the patient and may affect at least 40% of the operated patients. The main complications are related to infection, to the urinary tract or cardiovascular system. Unfortunately, mortality is high, around 17% (Ions and Stevens, 1987) and those patients who survive rarely recover their independence. In fact, hip fracture is often the event that precipitates institutionalisation and as many as 8% of all nursing home residents have had a FNF. The economic burden of age-related fractures is hugh because of the large number of people affected and because of the expense and the protracted care that is often required.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS:

2.1.1. CHEMICALS

SIGMA (London) CHEMICALS CO. Ltd., Poole, Dorset, U.K.
Sephadex G-25 and G100, Rabbit anti-Bovine Whole Serum, DL-dithiothreitol (Cleland's Reagent, DTT), Monothioglycerol, Dowex-50W (dry mesh 100-200), N^6,2'-O-butryladenosine 3'5'-cyclic monophosphate (Sodium salt), Adenosine 3'5'-cyclic monophosphate, 2-mercaptoethanol, Agarose (type I- low EEO), Theophylline, Adenine, Adenosine, Adenosine 5'-triphosphate, Adenosine 5'-diphosphate, Imidazole, Gentamycin, Bovine serum albumin, Ovalbumin.

THE RADIOCHEMICAL CENTRE, Amersham International, Little Chalfont, Bucks., U.K.
25-hydroxy[26,27-methyl-^{3}H]cholecalciferol (176Ci/mmol), 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol (180Ci/mmol), [8-^{3}H]Adenosine 3'5'-cyclic phosphate, ammonium salt (26.5Ci/mmol), [8-^{3}H]Adenine (22Ci/mmol), [^{14}C]Adenosine 3'5'-cyclic phosphate (268mCi/mmol).

NEW ENGLAND NUCLEAR, Harants, Hants., U.K.
24R,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol (160Ci/mmol).

FISONS SCIENTIFIC APPARATUS, Loughborough, Leics, U.K.
HPLC-grade Hexane, HPLC-grade Methanol, HPLC-grade Propan-2-ol, Sodium azide, 50 x 9/10mm soda glass tubes.

RATHBURN CHEMICALS (Walkerburn) Ltd., Walkerburn.
Peebleshire, Scotland, UK.
HPLC-grade Ethanol, HPLC-grade Acetonitrile.

BDH CHEMICAL Ltd., Poole, Dorset, U.K.
Folin and Ciocalteau's phenol reagent, Aluminum oxide, Barbitone sodium, Activated charcoal, Dextran T_{70}, Amido black 10.

CIS (UK) Ltd., High Wycombe, Bucks., U.K.
Osteocalcin radioimmunoassay kit.

IMPERIAL LABORATORIES, Sailsbury, U.K.
Newborn calf serum, Minimum essential medium- Eagles α-modification with 2g/L $NaHCO_3$, Hank's balanced salt solution, 0.25% Trypsin/0.02% EDTA solution in Dulbecco's phosphate bufered saline, Trypan blue.

WATERS ASSOCIATES (Inst.) Ltd., Cheshire, U.K.
Sep-Pak^{TM}(C_{18}) and Sep-Pak^{TM}(silica) cartridges.

THE FOLLOWING CHEMICALS WERE KINDLY DONATED BY:

ROCHE PRODUCTS Ltd., Welwyn Garden City, Hert., U.K.
Crystalline vitamin D_2 and D_3, 25-hydroxyvitamin D_3, 24R,25-dihydroxy-vitamin D_3, 1α,25-dihydroxyvitamin D_2 and D_3, 25,26-dihydroxyvitamin D_3, 1,24R,25-trihydroxyvitamin D_3.

All vitamin D metabolites were dissolved in HPLC-grade ethanol and the concentrations determined by UV spectroscopy using an extinction coefficient (ϵ_{264}) of 19,400 M^{-1} cm^{-1} for the vitamin D_2 metabolites and (ϵ_{264}) of 18,300 M^{-1} cm^{-1} for the vitamin D_3 metabolites.

NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL, Hampstead, U.K.
Bovine parathyroid hormone (Stock 77/533)

All other chemicals were of AR- grade and obtained from commercial suppliers.

2.1.2. EQUIPMENT:

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

HPLC was performed using a Beckman HPLC pump fitted to a Zorbax-Sil column (4.6mm x 25cm) and a silica guard-pak precolumn module (Waters Associates). Two Zorbax-Sil columns were used in these procedures. The first was used solely for clinical studies and purification of radiolabelled vitamin D metabolites and the second for all experimental cell culture work.

Batches of ^{3}H-labelled vitamin D metabolites ranged from 89-95% purity. Because of the oxidative decomposition of radioactivity with time, each batch of radiolabelled metabolite was purified by HPLC every 4 months prior to use. To minimise decomposition, the metabolites were stored in HPLC-grade ethanol, under nitrogen and below -20°C in the dark.

CENTRIFUGATION:

High speed centrifugation was carried out in either the MSE Prepspin 75, the Centrikon T-2070 ultracentrifuge or the Europa 24M centrifuge with 10 x 10ml, 8 x 14ml swing out or 8 x 50ml fixed angle rotors. The MSE Mistral 3000 bench centrifuge and the MSE Microcentaur were used for low speed centrifugation.

SONICATION:

The MSE Sonicator was used for all breakage of cell.

MEASUREMENT OF RADIOACTIVITY:

Radioactivity in samples was determined using either a Packard Tri-carb CD 460 liquid scintillation spectrometer or gamma counting efficiency in the region of 40%.

SPECTROSCOPY:

Absorbance measurements were made using a Spectroflow 773 absorbance detector.

MASS SPECTROSCOPY:

Mass spectrometry was performed on a AEI MS902 mass spectrometry (modified by VG Analytical Ltd.) in combination with a VG DS2000 data system. MS conditions were as follows:
Source temperature = 200°C
Scan rate = 7s/decade (approx. 14s total scan time)
Probe heated at a rate of 2°C/s

2.1.3. CELL LINES:

The human osteosarcoma cell line 20S was kindly provided by Dr. J. Embleton, Cancer Research Laboratories, University of Nottingham. For comparative studies, two rat osteosarcoma cell lines, 791T and T278, as well as the transformed human macrophage cell line U937 were also used. The macrophage cell line was a gift from Dr. G. Leslie, Department of Immunology, Queens Medical Centre, Nottingham.

2.2. METHODS 1: ASSAY PROCEDURES

2.2.1. EXTRACTION AND PURIFICATION OF THE VITAMIN D METABOLITES IN HUMAN SERUM

Biological samples contain a great number of compounds which can interfere with the analysis of the different vitamin D metabolites, in particular various fatty acids and steroids. As 25(OH)D is the most abundant form of vitamin D in circulation it is important to separate this metabolite from other vitamin D metabolites which appear in smaller quantities in serum. Therefore, in order to separate successfully the metabolites in question, a purification step is required, after extraction and before the quantitation of the vitamin D metabolites by competitive protein binding assays. This step involves the use of high performance liquid chromatography, or HPLC for short.

(i) EXTRACTION PROCEDURE:

All glassware was washed thoroughly with detergent and distilled water followed by HPLC-grade ethanol and HPLC-grade hexane to minimise non-specific binding of the vitamin D metabolites, in particular the $1,25(OH)_2D$ metabolite. HPLC-grade solvents were also used throughout the following procedure.

Recovery tracer for each vitamin D metabolite, approximately 3,500dpm (<4pg) of ^3H-$25(OH)D_3$, ^3H-$24,25(OH)_2D_3$ and ^3H-$1,25(OH)_2D_3$ in a total volume of 45ul ethanol, was added to test tubes containing serum or 0·9% saline (1-3ml). An aliquot of each tracer was dispensed into separate scintillation vials to monitor recovery. These vials were stored at 4°C until required. Assay tubes were

vortex-mixed briefly and incubated in the dark at room temperature for 30min to allow recovery tracers to equilibrate. An equal volume of acetonitrile was added to each tube, to precipitate serum proteins, and the tubes were vortexed for 20sec before finally centrifuging at 2,500rpm for 15min. The supernatant fractions were decanted into glass tubes containing 0·5vol 0·4M K_2HPO_4 solutions (pH 10·6) and the pellets discarded. The addition of this solution to the acetonitrile extracts before column chromatography results in the additional removal of lipids during the chromatography step (Reinhardt et al, 1984).

Column chromatography was accomplished using C_{18} cartridges attached to 10ml Lueur-tipped glass syringes which acted as solvent reservoirs. Each cartridge has an octadecylsilane bonded phase packing system which is designed for reverse phase chromatography using small volumes of solvents. This system has been shown to be a convenient and reproducible method of separating the polar vitamin D metabolites from low polarity compounds which may interfere in their final analysis.

Each cartridge was prewashed with 5ml methanol, followed by 5ml distilled water. This washing procedure was used between sample applications. Samples were decanted individually into the solvent reservoirs and slowly aspirated through the cartridges. The eluates were discarded. It was unnecessary to wash the sample tubes after application since greater than 85% recovery was obtained off the columns. Low polarity compounds were eliminated by washing each cartridge with 5ml distilled water, followed by 3ml 70% (v/v) methanol in water. These washes remove excess salts and water soluble lipids, respectively. The addition of 4ml acetonitrile, a more polar solvent, to the cartridges eluted all the vitamin D metabolites. Approximately 87-99% recovery of each metabolite was achieved by this method. The

acetonitrile fractions, collected in glass conical tubes, were dried under a stream of nitrogen before storing at -20°C in ethanol under nitrogen, until required for the HPLC stage. Each C_{18} cartridge was used four times, being rinsed with 5ml methanol and 5ml distilled water before each sample application.

(ii) HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC was performed on a Waters HPLC apparatus fitted with a Zorbax-Sil column (4.6mm x 25cm) and a silica guard column. The use of HPLC allows rapid separation of the vitamin D metabolites under moderately high pressures. Zorbax-Sil is a small-particle silica column packing that has strong affinity for the hydroxyl group(s) of vitamin D and its metabolites. Thus, an increase in the number of hydroxyl groups on the vitamin D molecule increases the interaction with the silica absorbant and therefore the retention time for the metabolite.

The eluting solvent used for this system was hexane: isopropanol: methanol (92:4:4 by vol). Using this straight phase solvent system and a flow rate of 2ml/min, all the vital vitamin D metabolites were sufficiently resolved. A typical elution profile is depicted in figure 2.1. The resolution of both the $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ metabolites can be observed, although only partial separation was achieved. In general, the D_2 analogues elute before the complementary D_3 analogues.

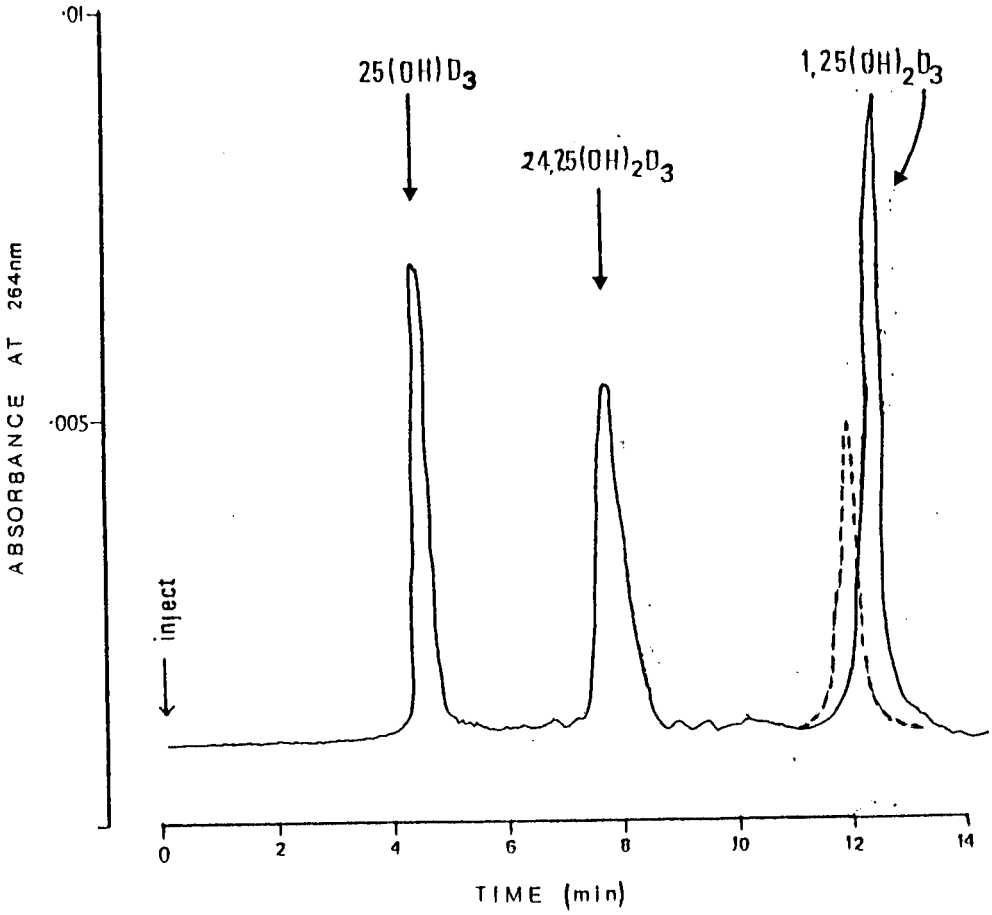


FIGURE 2.1. Typical elution profile of the authentic vitamin D_3 metabolites.

Vitamin D metabolites were separated on a Zorbax-Sil straight phase HPLC column eluted with hexane:methanol:isopropanol (92:4:4 by volume). Arrows mark the retention times of each metabolite determined by U.V. absorption at 264nm.

Dashed peak represents $1,25(OH)_2D_2$ elution profile.

Establishment of elution profile for the vitamin D metabolites

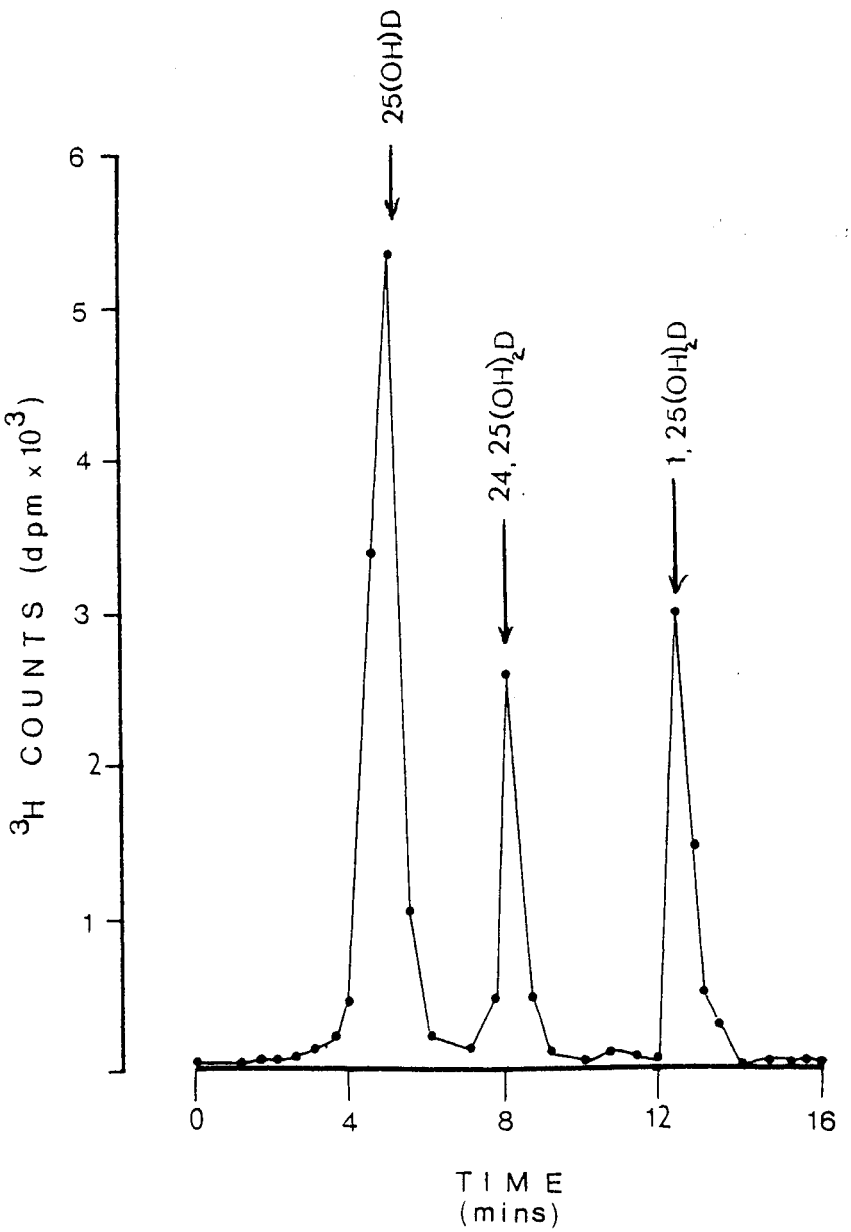
Before a batch of sample ~~run~~ application, the retention time of all three vitamin D metabolites was established using known amounts of tritiated samples. Initially, the elution profile was generated by injecting small quantities of unlabelled standards (approximately 0.1ug/metabolite) on to the column and monitoring the retention time by UV detection at 264nm. This procedure however was abandoned due to the interference of these metabolites with final serum sample values. Extensive washing of the column with hexane:isopropanol (75:25 v/v), once the elution profile was obtained, still produced excessively high values. Therefore, a more suitable technique of applying known amounts of labelled metabolites was employed.

Approximately 5,000dpm of each vitamin D metabolite was dispensed into a glass tube and individual scintillation vials to monitor recovery. After evaporating to dryness, the metabolites were redissolved in 1ml running buffer and applied to the column. A stop clock was started simultaneously with the injection of the sample and 1ml aliquots (corresponding to 30sec intervals) were collected into clean scintillation vials. These vials, along with the recovery vials, were monitored for radioactivity and an elution profile of the metabolites was obtained. The elution profile for the tritiated metabolites, shown in figure 2.2., was identical to that of the synthetic vitamin D metabolites and because of the minute quantities applied to the column (<6pg per metabolite) there was no interference with final serum values.

The above method, therefore, was performed for each batch of sample runs. Once the profile was established, the column was washed for 10min with hexane:isopropanol (75:25

FIGURE 2.2. Typical elution profile of the tritiated vitamin D_3 metabolites.

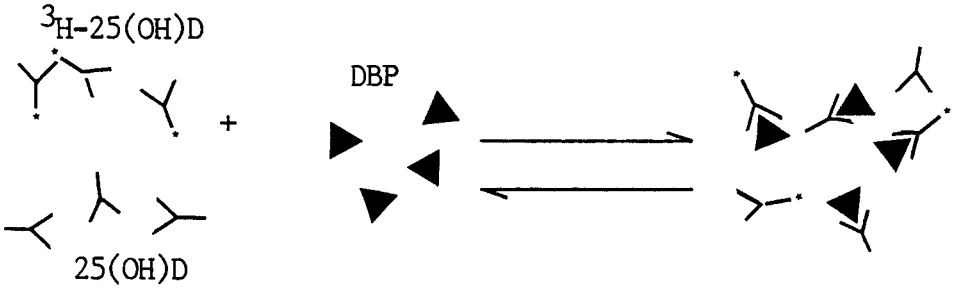
A sample of the tritiated vitamin D metabolites was separated on a Zorbax-Sil straight phase HPLC column with hexane: methanol: isopropanol (92:4:4 by volume) as the mobile phase. See text for procedure.



v/v) and the column re-equilibrated with running solvent for 20min before the first sample application. Each sample from the C_{18} columns was evaporated to dryness and redissolved in 1ml running solvent before applying to the HPLC column. Fractions corresponding to each metabolite were collected into glass conical tubes and evaporated to a small volume before storing under nitrogen at -20°C until required for assay. The column washing procedure was repeated for every three sample applications.

2.2.2. PRINCIPLE OF THE BINDING ASSAYS

The techniques of radioimmunoassay are similar to that of the radioreceptor assays and to all competitive protein binding assays. In brief, the binding assay is based on competition between a known fixed amount of radioactively labelled ligand and unlabelled ligand for the binding sites on the given protein. For example, consider the 25(OH)D binding assay. The labelled ligand, ^{3}H-25(OH)D, will compete with unlabelled 25(OH)D for the binding domains on the serum vitamin D binding protein (DBP), as illustrated below:



Assuming that there is no difference in the affinity of both labelled and unlabelled ligand binding to the protein, then the percentage radioactivity bound will decrease as the concentration of unlabelled ligand increases. When the reaction reaches equilibrium any labelled ligand not bound to the protein is removed by a phase separation reagent, either a second antibody (eg in the osteocalcin assay) or by the much used dextran-coated charcoal which adsorbs any free ligand. The protein/ligand complex in the supernatant is decanted and radioactivity determined by liquid scintillation counting. By using known concentrations of the unlabelled compound a calibration curve can be constructed and used to evaluate the amount of ligand present in unknown samples.

The following groups of tubes were prepared for each binding assay:

- T_c group for the determination of total activity
- C_b group for the calculation of non-specific binding
- O group for the determination of maximum binding ability of the protein
- Standard group for the determination of the standard curve
- S_x group for the samples to be assayed
- C_{bx} group for the calculation of non-specific binding of the samples

A standard curve was constructed by plotting the percentage of B/B_o against the log of the amount of ligand added to each tube, where:

B = (total counts bound by standards - total C_b counts) ÷ T_c

and

B_o = (total counts bound by zero standards - total Cb counts) ÷ T_c

2.2.3. 25-HYDROXYVITAMIN D PROTEIN BINDING ASSAY

MATERIALS:

STOCK BUFFER (500ml) ..... 7.87g sodium barbitone
4.86g sodium acetate
0.5g sodium azide

WORKING BUFFER (1L) ..... 50ml stock buffer in 850ml 0.9% saline, pH 8.6. Made up to 1L with distilled water

GELATINE BUFFER (1g/L) ..... 100mg gelatine in 100ml working buffer

PROTEIN BINDING SOLUTION ..... Human serum diluted 1 in 10,000 fold with gelatine buffer (see figure 2.3 for serial dilution)

DEXTRAN-COATED CHARCOAL* ..... 750mg charcoal + 75mg Dextran T_{70} in 20ml stock buffer at 4°C

*This is prepared a day before the assay. Dextran is preswollen in 10ml stock buffer and added, after 15min, to charcoal suspended in 10ml stock buffer.

^{3}H-25(OH)D..... Approx 45,000dpm (in 20ul ethanol) per assay tube

25(OH)D STANDARDS..... Ranging from 0.03-8 ng/20ul (in ethanol)

ASSAY PROCEDURE: Protocol 2.1

All assay tubes were performed in triplicate. 10mm x 75mm glass tubes were labelled according to the protocol. Aliquots of approximately 45,000dpm ^{3}H-25(OH)D were dispensed into all labelled tubes. Standard curve tubes received various concentrations of standard 25(OH)D in 20ul ethanol. HPLC-purified sample fractions containing 25(OH)D were evaporated under nitrogen and reconstituted in 300ul ethanol. Aliquots (50ul) from each sample were transferred to glass counting vials, to assess final recovery, and 20ul aliquots were dispensed into labelled assay tubes. Protein binding solution (1ml) was added to all except tubes labelled T_c and C_b. To these tubes 1ml gelatine buffer was added. All tubes were vortex-mixed and incubated at 4°C for 30min. Tubes were transferred to an ice bath and, to the appropriate tubes, 250ul charcoal suspension added (stirring continuously throughtout the additions). Tubes were vortex-mixed and incubated at 4°C, and after 20min the tubes were centrifuged at 2,500rpm for a further 20min at 4°C. Aliquots (800ul) of the supernatant fractions were transferred to scintillation vials, 10ml of scintillation fluid added and the radioactivity in each vial counted for 5min.

ASSAY SYSTEM CHARACTERISTICS: For a typical standard curve

The standard curve was constructed by plotting the percentage of B/B_0 against the log of the amount of 25(OH)D added to each tube. A typical standard curve is shown in figure 2.4. Total counts bound in the supernatant of the unknowns were corrected for non-specific binding.

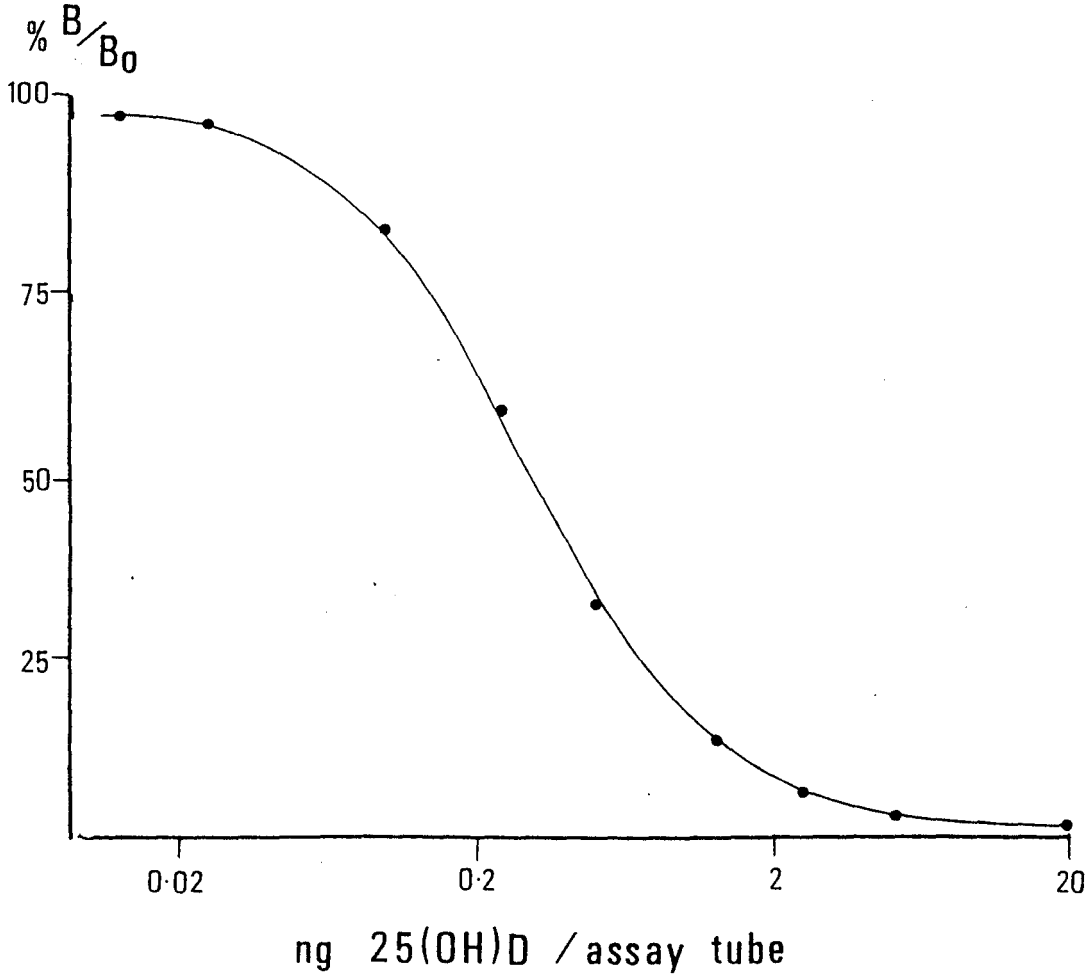
Sensitivity: Defined as the amount of 25(OH)D required to reduce the zero dose binding by two standard deviations ..... 15 pg/assay tube (i.e. 11.8 pg/ml).

PROTOCOL 2.1. Protein binding assay for $25(OH)D_3$

TUBES	STANDARD $25(OH)D_3$ (ng/20ul)	^3H-$25(OH)D_3$	PROTEIN BINDING SOLUTION (1ml)	BUFFER (1ml)		CHARCOAL SUSPENSION (250ul)	
0	-	+	+	-		+	
T_c	-	+	-	+ 2ml		-	
C_b	-	+	-	+ 2ml	Mix	+	Mix
1	0.01	+	+	+	Incubate	+	Incubate 20min
2	0.02	+	+	+		+	@
3	0.1	+	+	+	for	+	4°C
4	0.2	+	+	+		+	
5	0.4	+	+	+	30min	+	
6	1.0	+	+	+		+	Centrifuge
7	2.5	+	+	+	@	+	20min
8	10.0	+	+	+		+	@
9	20.0	+	+	+	4°C	+	4°C
Unknowns	-	+	+	+		+	

FIGURE 2.4. An example of a typical standard curve for 25(OH)D assay.

Each point represents the average of triplicate determinations.



Mid-point: Defined as the amount of 25(OH)D required to reduce the bound tracer by 50% ..... 0.36 ng/assay tube (i.e. 0.28 ng/ml).

Specific binding: Defined as the proportion of tracer specifically bound to the binding protein in the absence of unlabelled 25(OH)D ..... 78% (B_0 tubes − C_b tubes).

Non-specific binding: Defined as the proportion of tracer bound to the binding protein in the presence of 500ng of unlabelled 25(OH)D 1.7%

SEASONAL CHANGES IN PLASMA 25(OH)D MEASUREMENTS

Measurements of plasma 25(OH)D concentrations provide a precise index of the vitamin D nutritional status in man (Stamp and Round, 1974). However, concentrations seem to show a marked seasonal variation as demonstrated in Table 2.1 (taken from Stamp and Round, 1974). Values for autumn and summer produce similar reference ranges whereas those obtained during the spring season were much lower. In view of this the normal 25(OH)D range was established as follows:

Measurements of plasma 25(OH)D concentrations were obtained from 36 young healthy individuals (mean age 36; range 23-53) throughout the year and these values were divided into two groups according to their sampling date (Table 2.2): Group A -sampled between May and October and Group B -sampled between November and April. In some instances 25(OH)D values for groups A and B were obtained from the same individual and did show statistically significant differences.

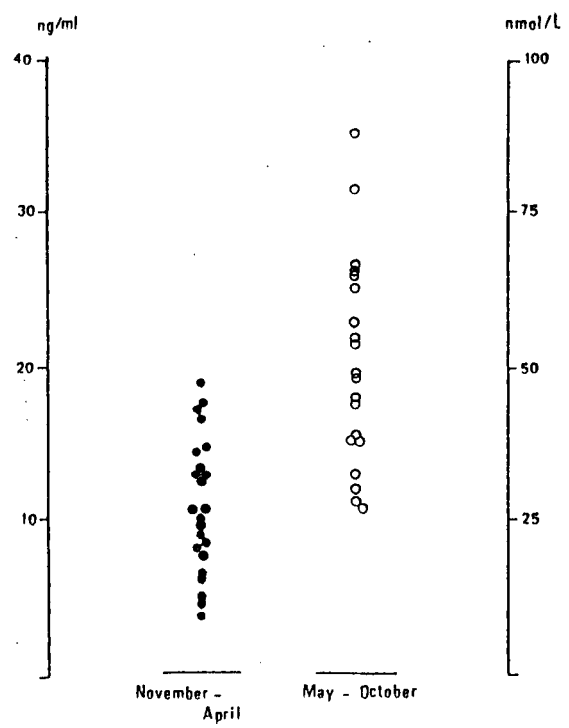
TABLE 2.1. Plasma 25(OH)D concentrations in healthy white British subjects of both sexes sampled over a period of 22.5 months and grouped according to season.

(from: Stamp and Round, 1974. Nature 247, pp563-65)

Population data			Season	Plasma 25(OH)D (ng/ml)	
Age range	Sex	No.		Mean	Range
18 to 37	Both	27	October - early November	21.3	10.8 - 41.8
18 to 37	Both	19	Late March	12.9	6.6 - 25.2
19 to 36	Both	20	Early August	20.4	9.7 - 42.8

TABLE 2.2. Mean plasma 25(OH)D concentrations from young healthy individuals sampled throughout the year.

Group	No.	Sampling Date	Plasma 25(OH)D ng/ml mean	range (± 2SD)
A	20	May - October	19.5	16.8 - 22.2
B	22	November - April	10.5	7.6 - 13.5



2.2.4. 24,25-DIHYDROXYVITAMIN D PROTEIN BINDING ASSAY

MATERIALS:

STOCK BUFFER	see 2.2.3 materials
WORKING BUFFER	" "
GELATINE BUFFER	" "
PROTEIN BINDING SOLUTION	Human serum diluted 1 in 75,000 fold with gelatine buffer
DEXTRAN-COATED CHARCOAL	see 2.3.1 materials
$24,25(OH)_2D$ STANDARDS	Ranging from 1.33-680 pg/20ul (in ethanol)
^3H-$24,25(OH)_2D$ OR* ^3H-25(OH)D	approx 3,500dpm in 20ul ethanol per assay tube

*Initially, labelled $24,25(OH)_2D$ was used for competitive binding, but due to the withdrawal of this radioactive compound from the market the assay was slightly modified by introducing labelled 25(OH)D as the competitor. There was no difference in the ability of the unlabelled $24,25(OH)_2D$ to bind to the protein (see figure 2.5).

METHOD: Protocol 2.2.

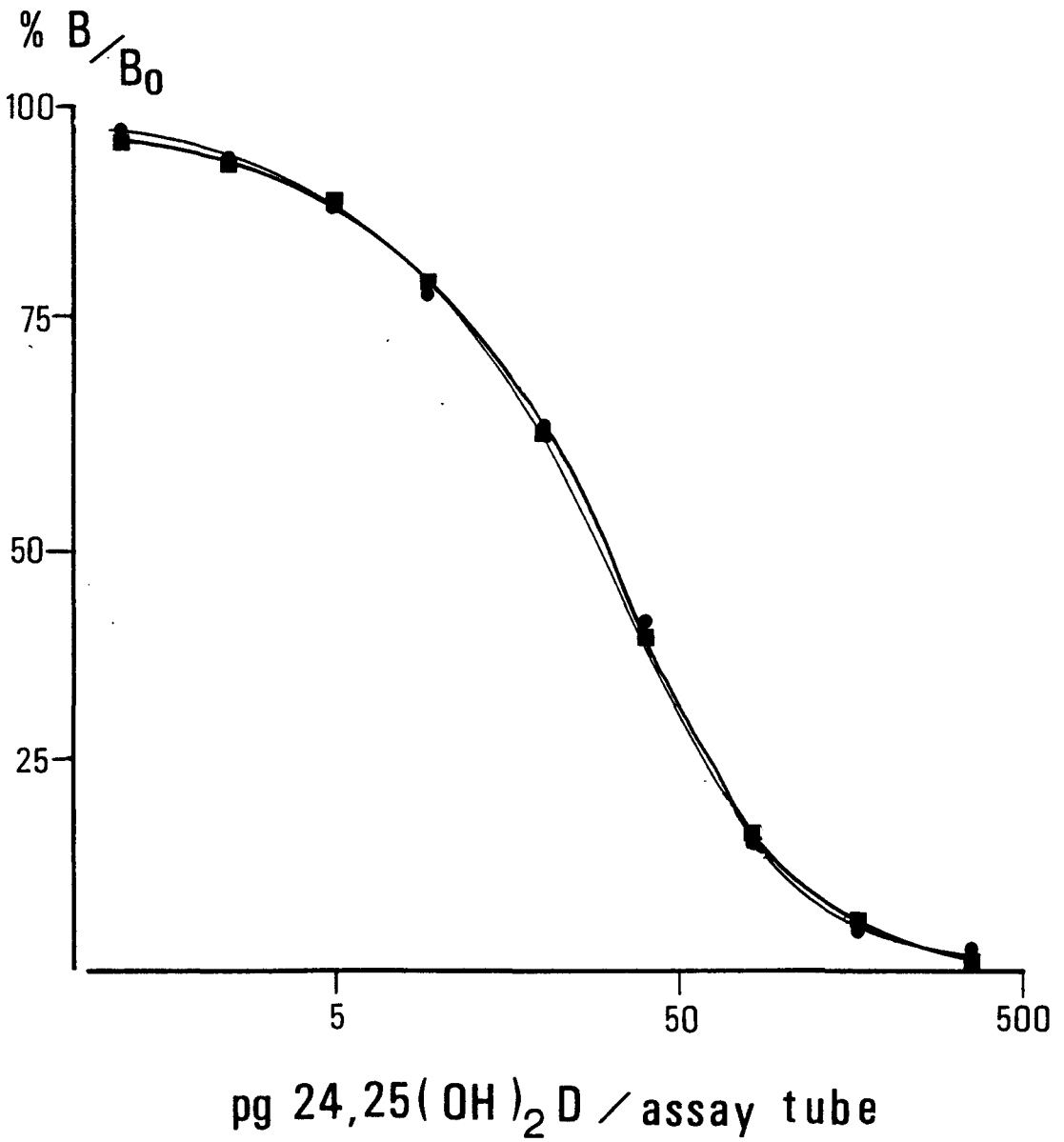
The 25(OH)D assay procedure was employed using $24,25(OH)_2D$ standards to construct a standard curve with approximately 3,500dpm (<4pg) of tracer. The unknown samples

PROTOCOL 2.2. Protein binding assay for $24,25(OH)_2D_3$:
Using 1 in 75,000-fold dilution of human serum.

TUBES	STANDARD $24,25(OH)_2D_3$ (pg/20ul)	^3H-$25(OH)D_3$	PROTEIN BINDING SOLUTION (1ml)	BUFFER (1ml)		CHARCOAL SUSPENSION (250ul)	
0	-	+	+	-		+	
T_c	-	+	-	+2ml		-	
C_b	-	+	-	+2ml	Mix	+	Mix
							Incubate
1	1.33	+	+	+	Incubate	+	20min
2	2.66	+	+	+		+	@
3	5.30	+	+	+	for	+	4°C
4	10.60	+	+	+		+	
5	21.25	+	+	+	30min	+	
6	42.50	+	+	+		+	Centrifuge
7	85.00	+	+	+	@	+	20min
8	170.00	+	+	+		+	@
9	340.00	+	+	+	4°C	+	4°C
Unknowns	-	+	+	+		+	

FIGURE 2.5. Standard curve for $24,25(OH)_2D$ using 75,000-fold dilution of human serum as binding protein and:
(●) ^3H-$24,25(OH)_2D_3$ and (■) ^3H-$25(OH)D_3$

Each point represents the average of triplicate determinations.



were reconstituted in 100ul ethanol and after removing 20ul aliquots from each sample, to monitor final recovery, three 20ul aliquots were used for the assay.

ASSAY SYSTEM CHARACTERISTICS: For a typical standard curve

Sensitivity: 9.6 pg/assay tube (i.e. 7.6 pg/ml).

Mid-point: 39 pg/assay tube (i.e. 30.7 pg/ml).

Specific binding: 49%

Non-specific binding: 2.2%

2.2.5. 1,25-DIHYDROXYVITAMIN D RADIORECEPTOR ASSAY

MATERIALS:

HOMOGENISING BUFFER*..... 500mM KCl, 50mM Tris-HCl, 10mM $Na_2MoO_4.2H_2O$, 5mM DTT, 1.5mM EDTA pH 7.5

*This buffer was prepared without DTT and stored at 4°C until assay. DTT was added prior to use.

THYMIC CYTOSOL SUSPENSION ..... Each pellet was resuspended in 5ml homogenising buffer and then further diluted to give a B/B_t 35% (see figure 3.1; chapter 3)

BORIC ACID BUFFER ..... 0.1M Boric acid + 0.05% BSA pH 8.6

CHARCOAL SUSPENSION* ..... 1.2g Charcoal + 0.12g Dextran T_{70} suspended in 100ml boric acid buffer stirred over night at 4°C then centrifuged at 15,000 rpm for 20min. The pellet was resuspended in 100ml boric acid buffer.

*This charcoal suspension was stable for at least 4 weeks at 4°C.

$1,25(OH)_2D$ STANDARDS ..... Ranging from 1.25-800 pg/20ul (in ethanol)

^3H-$1,25(OH)_2D$ ..... Approx 3,600dpm (in 20ul ethanol) per assay tube

ASSAY PROCEDURE: Protocol 2.3.

Eppendorf tubes were labelled according to the protocol and placed on ice. Standard solutions of $1,25(OH)_2D$, ranging from 1.25-800 pg/20ul, were dispensed into appropriate tubes. HPLC-purified samples were evaporated under nitrogen and reconstituted in 100ul ethanol. Three 20ul aliquots were pipetted into labelled tubes and a further 20ul dispensed into scintillation vials to monitor final recovery.

Thymic cytosol suspension was placed on ice and, whilst stirring, 450ul aliquots were pipetted into all except the T_c and C_b tubes. After vortexing, tubes were incubated in a 25°C water bath for 45min with gentle shaking. After this initial incubation period all tubes were placed on ice for 5min where they received approximately 3,600dpm ^3H-$1,25(OH)_2D$ tracer and, after vortex-mixing, they were incubated for a further 15min in a 25°C shaking water bath. The tubes were again cooled for 5min on ice and 200ul charcoal suspension added to appropriate tubes. The tubes were vortex-mixed and left on ice. The tubes were further vortexed after 10min, and after a total of 20min charcoal treatment, were centrifuged at 6,000rpm for 45sec and aliquots (400ul) of each supernatant fraction were decanted into scintillation vials. With the addition of 10ml volumes of scintillation fluid, each vial was vortex-mixed and the radioactivity in each vial counted for 10min.

ASSAY SYSTEM CHARACTERISTICS: Refer to chapter 3 for typical standard curve and assay characteristics.

PROTOCOL 2.3. Radioreceptor assay for $1,25(OH)_2D_3$.

TUBES	STANDARD $1,25(OH)_2D_3$ (pg/20ul)	THYMIC CYTOSOL SUSPENSION (450ul)	BUFFER (450ul)		^3H-$1,25(OH)_2D_3$		CHARCOAL SUSPENSION (200ul)	
0	-	+	-		+		+	
T_c	-	-	+		+		-	
C_b	-	-	+	Mix	+	Mix	+	Mix
				Incubate		Incubate		Incubate
1	1.25	+	-		+	for	+	20min
2	2.50	+	-		+	15min	+	on ice
3	5.00	+	-	for	+	@	+	
4	10.00	+	-		+	25°C	+	Mix after
5	20.00	+	-	45min	+		+	10min
6	50.00	+	-		+		+	
7	100.00	+	-	@	+	Cool	+	Centrifuge
8	200.00	+	-		+	on	+	45sec
9	800.00	+	-	25°C	+	ice	+	@
								4°C
Unknowns	-	+	-		+		+	

2.2.6. ESTIMATION OF OSTEOCALCIN CONCENTRATIONS IN HUMAN SERUM

MATERIALS:

OSTEOCALCIN RADIOIMMUNOASSAY KIT ..... which supplied the following reagents:

^{125}I Osteocalcin- approximately 74kBq (2 Ci) highly purified bovine osteocalcin.

Anti Osteocalcin antiserum- raised in rabbit by injection of bovine osteocalcin. Using the specified reconstitution volume the antiserum binds 50-60% of the added radiolabelled osteocalcin.

Osteocalcin standards- ranging from 0.5-27 ng/ml bovine osteocalcin.

Human control serum

Immunoprecipitating (PR) reagent- contains polyethylene glycol, sodium azide, an insoluble complex of sheep anti-rabbit gamma globulins and non immunized rabbit gammaglobulins

METHOD:

All reagents (except PR reagent) were raised to room temperature (18-25°C) prior to use. Each sample was performed in duplicate. Protocol 2.4. was followed for this assay. Briefly, aliquots (100ul) of standards, control serum or unknown samples were dispensed into appropriate tubes. Aliquots (200ul) of radioactive osteocalcin (tracer) were dispensed into all tubes. Standard and sample tubes received

PROTOCOL 2.4. Osteocalcin Assay

TUBES	STANDARDS (ul)	SAMPLES (ul)	^{125}I OSTEOCALCIN (ul)	ANTISERUM (ul)		PR REAGENT (ml)		
								Centrifuge
T_c	-	-	200	-		-		Discard
C_b	100	-	200	-	Mix	1	Mix	Supernatant
0	100	-	200	100	Incubate	1	Incubate	
Standards	100	-	200	100	20-24 h	1	15 min	Count
Unknowns	-	100	200	100	@	1	@	Pellet
Control	-	100	200	100	4°C	1	4°C	

aliquots of 100ul of anti-osteocalcin antiserum and all tubes were vortex-mixed and incubated for 24h at 4°C.

1ml PR reagent was added to all tubes, except those of the T_c group, and after vortexing, the tubes were incubated for a further 15min at 4°C. The tubes were then centrifuged at 3,500rpm for 15min at 4°C. The supernatant fractions were discarded by inverting the tubes over a sink suitable for collecting radioactivity and left inverted on absorbing paper for 10min. Each tube was counted for 100sec for ^{125}I. A typical standard curve is depicted in figure 2.6a.

ASSAY SYSTEM CHARACTERISTICS: For a typical standard curve

Sensitivity: 0.35 ng/ml

Mid-point: 4.9 ng/ml

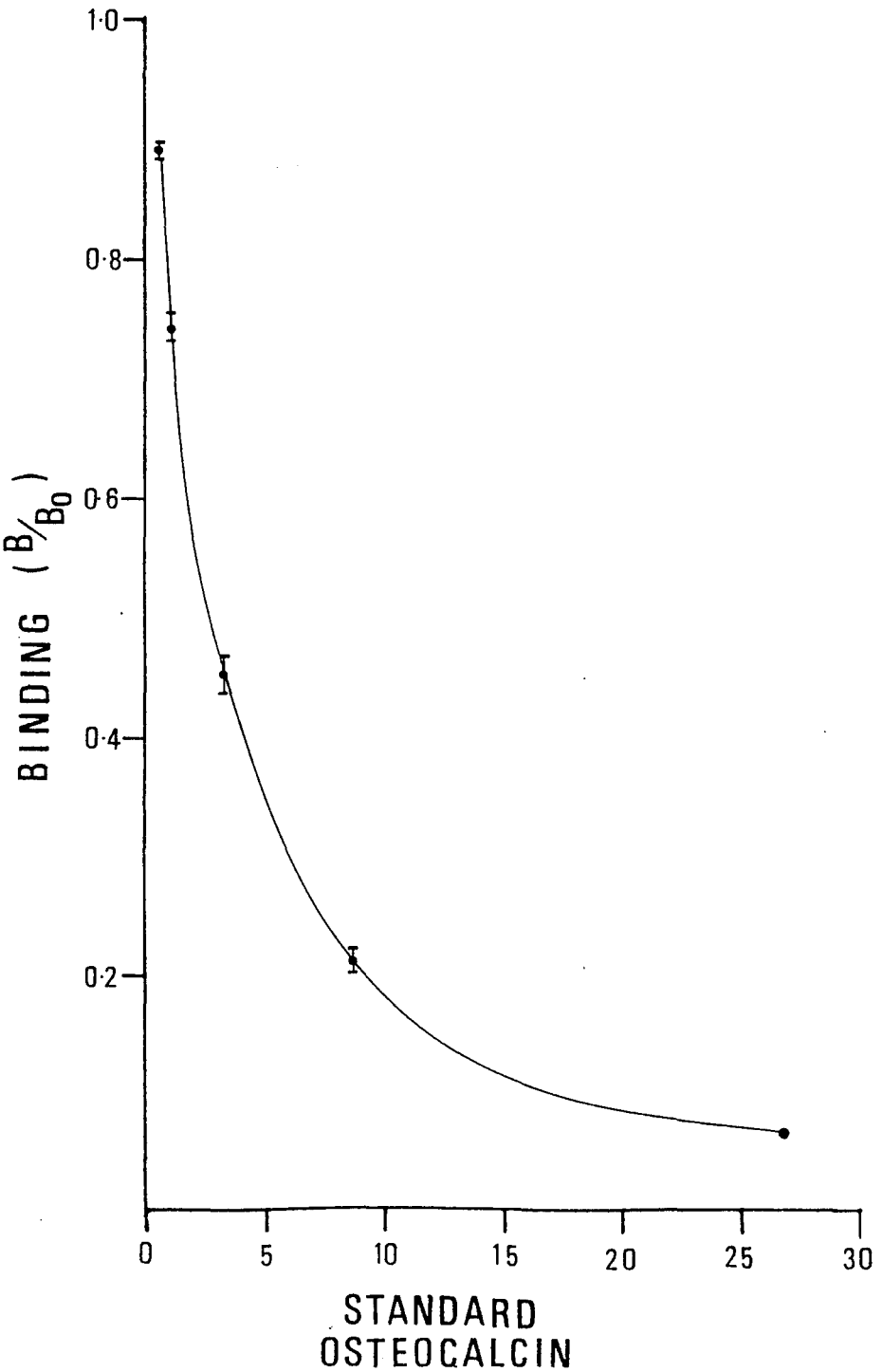
Specific binding: 54%

Non-specific binding: 3.8%

Serum samples, including those from young healthy individuals, which were stored for more than one year at -20°C demonstrated low osteocalcin concentrations; values around 0.5 ng/ml were frequently observed. These low values could be explained if the serum samples were frozen and thawed a few times or if the original blood samples were collected in EDTA bottles. Since the bonding between calcium and the GLA residues of the osteocalcin molecule is influencing the predicted alpha helical structure, the presence of EDTA will lead to the severe loss of immunoreactivity.

FIGURE 2.6a An example of a typical standard curve for the determination of osteocalcin concentrations.

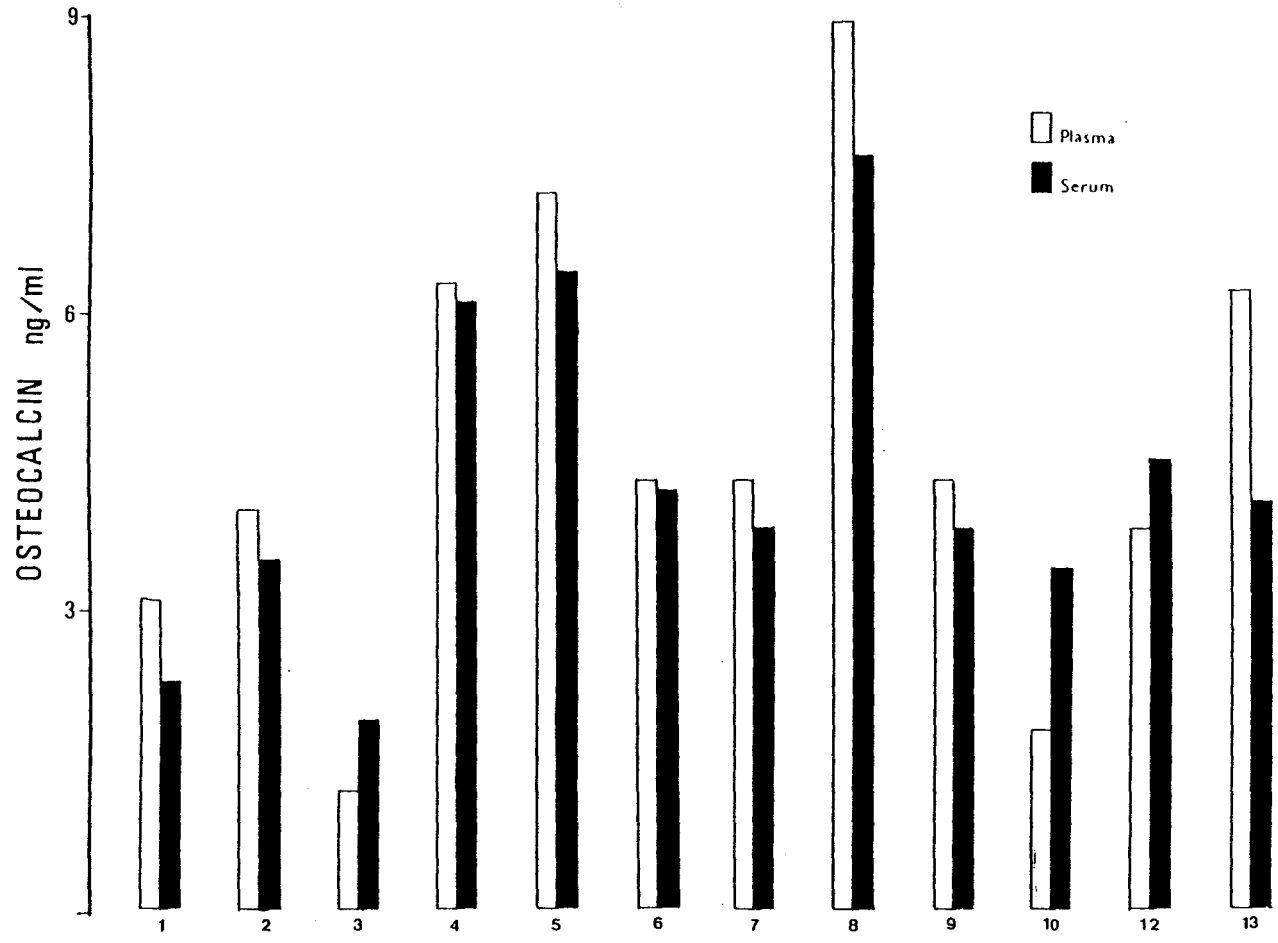
Each point represents the average of duplicate determinations.



It is recommended by the manufacturers to perform the assay on serum samples. To investigate whether plasma specimens would produce similar results to serum samples from the same individual the following experiment was performed.

Blood from 13 healthy individuals were sampled in to lithium heparin bottles as well as plain bottles and the osteocalcin concentration for each determined. There were no significant differences between the two set of results (figure 2.6b). Therefore, either serum or plasma samples can be used to estimate osteocalcin concentrations.

FIGURE 2.6b Differences between serum and plasma osteocalcin measurements from 13 healthy individuals.



2.2.7. ESTIMATION OF HUMAN VITAMIN D BINDING PROTEIN IN SERUM (Mancini method)

MATERIALS:

VERONAL BUFFER (1L) ..... 9.0g barbital nitrate
0.5g sodium azide
6.5ml 1M HCL pH 8.6

1% AGAROSE GEL ..... made up in veronal buffer and stored at 4°C in 10ml aliquots

RABBIT ANTI-HUMAN DBP ANTIBODY ... kindly provided by Dr. B. Mawer, University of Manchester.

0.1% AMIDO BLACK STAIN ..... 1g Amido black (Napthol blue black)
450ml 1M acetic acid
450ml 0.1M sodium acetate
100ml distilled water

10% ACETIC ACID

0.9% SALINE

METHOD:

1. Preparation of gel:

Two 10mm x 10mm glass plates were clipped together with a 1-2mm plastic spacer between them in a manner to allow one plate to be slightly offset. This enables easy pouring of the agarose between the two plates. 10ml agarose gel was heated until fluid and then placed in a 45°C water bath. After the addition of the antibody, the agarose was mixed and then rapidly poured between the two plates until there

was only approximately 5mm from the top of the edge. After leaving at room temperature for 15min, in order to set, the upper glass plate and plastic spacer were removed. The agarose plate was transferred to a moist chamber (a 'lunch-box' containing a small amount of adsorbant paper soaked in veronal buffer). This prevents the gel from drying out.

2. Plating the samples:

With the aid of a 3mm hole punch and a 25-hole stencil, 25 holes were punctured in the gel. The loose agarose were carefully removed from the wells with a wide pasteur pipette attached to a vacuum line. Care was taken at this stage not to tear the wells. The gel was then transferred to its chamber.

Serum samples were defrosted and mixed before application to the gel. After placing the gel on a white background, 5ul of each sample were pipetted into the first 21 wells. Calibrated standard serum (400·25ug vitamin D binding protein/ml) was diluted to 25%, 50%, 75% and 100% with veronal buffer and 5ul of each dilution pipetted into the remaining 4 wells. The plate was again transferred to its chamber and left for 48h at room temperature.

3. Staining the gel:

After 48h the antigen-antibody complexes were visible as precipitated rings. The gel was soaked for 12h in 0.9% saline. This eliminated all non-precipitated proteins from the gel. The gel was carefully removed from saline and rinsed with distilled water. After ensuring that the wells did not contain any air bubbles, a damp square of filter paper was placed on top of the gel and a further 8-10 layers of adsorbant paper on top of that. With the addition of a large glass plate a 500g weight was positioned on top of the 'sandwich'. After 20min the gel was removed and dried with a hairdryer. At this stage the gel became transparent.

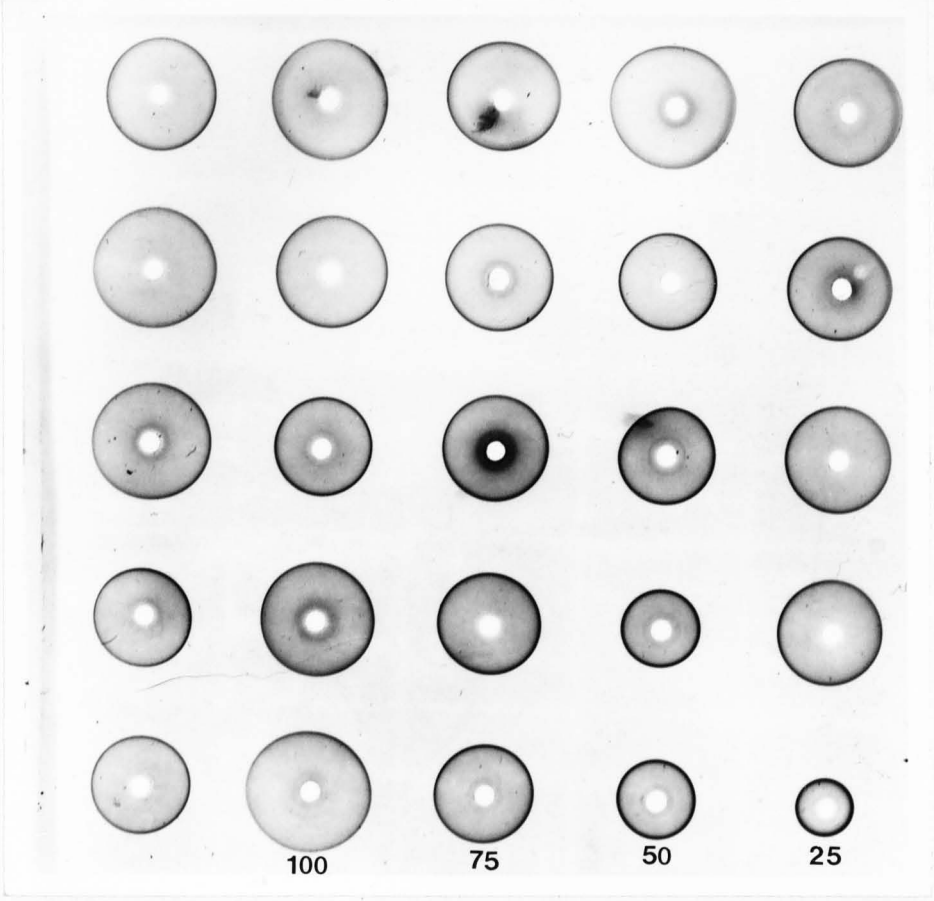
Once cooled, the gel was immersed for 2-3min in 0.1% amido black and then washed for 5-10min in distilled water. After a further 5min in 10% acetic acid solution, the gel was dried. This procedure stains the precipitated rings blue thus enabling the rings to be clearly visible (see figure 2.7a).

4. Calculation of samples:

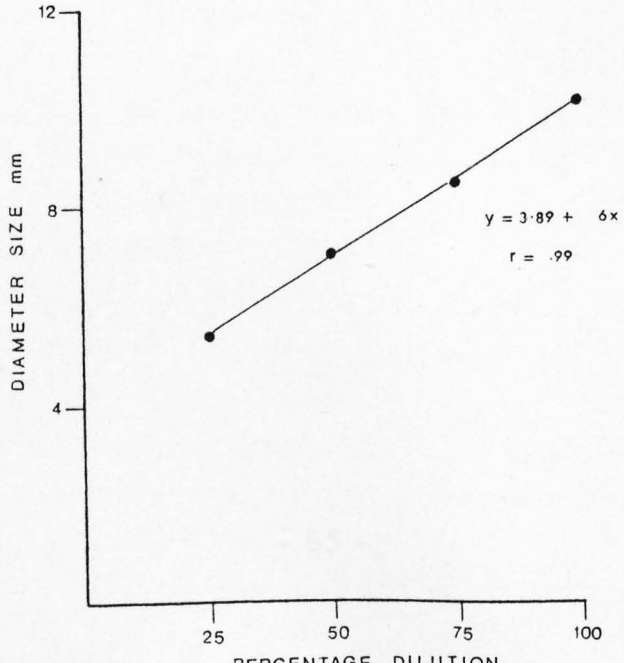
In order to calculate the diameter of each ring, the gel was placed on top of a sheet of graph paper and a magnifying apparatus placed in position over the gel. The diameter was measured twice- at right angles to compensate for any rings which had become askewed during the staining procedure. The precipitated rings of antibody-antigen complex have a diameter proportional to the concentration of vitamin D binding protein in the sample. A standard curve was obtained from the mean diameters of the known standards (figure 2.7b) and from this the unknowns were calculated.

FIGURE 2.7. **(a)** Typical diagram of an agarose gel depicting precipitated rings of the calibrated standards and patients samples. (b) By measuring the diameter of each calibrant a standard curve can be plotted and the unknowns calculated.

A



B



2.2.8. ESTIMATION OF PARATHYROID HORMONE CONCENTRATIONS IN HUMAN SERUM

Parathyroid hormone (PTH) estimations were kindly measured by the Department of Chemical Pathology and Medicine, Derbyshire Royal Infirmary, Derby, UK., using a recently available 'C-terminal' PTH radioimmunoassay kit purchased from Dac-Cel, Wellcome Diagnostics, Temple Hill, Dartford DA1 5AH, UK (Ingle et al, 1987).

The PTH assay kit comprised of the following reagents:

BOVINE PTH STANDARD in human serum
ANTI-BOVINE PTH ANTISERUM (raised in guinea-pig)
^{125}I-BOVINE PTH
DONKEY ANTI-GUINEA-PIG ANTIBODY SUSPENSION (linked to cellulose)

2.2.9. DOUBLE-ISOTOPE CALCIUM ABSORPTION TEST (WOOTON AND REEVE, 1979)

PROTOCOL

(i) Patients received a normal diet on the day before the test but water from midnight on the day of the test.

(ii) At 08.15 an intravenous cannula was inserted into one arm, for blood sampling, and infused with 0.9% saline.

(iii) A fasting 20ml blood sample was taken, half of which acted as the isotope 'blank' and the remainder used for measuring the vitamin D metabolites.

(iv) Patient name and the weights of the pre-weighed isotope syringes were carefully noted.

(v) At 08.30 15uCi of ^{47}Calcium (^{47}Ca) and 2.5mmol of calcium chloride BP in 100ml of distilled water were given by mouth, whilst 2uCi ^{85}Strontium (^{85}Sr) was injected intravenously as the patient drank. The time of injection was carefully noted.

(vi) Both syringes were re-weighed to ascertain the exact weight of isotope given.

(vii) 10ml blood samples were taken at:
T + 5, 10, 35, 50, 75, 100, 140, 180, 240, 300 and 360min.

(viii) The patient was allowed a standard meal 300min after administration of the isotopes.

(ix) Blood samples were spun at 2400rpm for 6min and 4ml aliquots of plasma counted for gamma emissions.

The test employs the use of an intravenous dose of ^{85}Sr in conjunction with an oral dose of ^{47}Ca. In order to calculate accurately the percentage of the oral dose absorbed by the intestine, allowance must be made for the removal of ^{47}Ca into soft tissue and bone. Since ^{85}Sr administered intravenously is distributed in the same manner as the oral dose of ^{47}Ca, the ratio of activity of the two isotopes in plasma is used to calculate the percentage absorption (figure 2.8).

Using a computer program designed for this study, the following data were inserted:

(i) Plasma radioactivity data from gamma-counting of the serial plasma samples- simultaneous counts for ^{47}Ca and ^{85}Sr.

(ii) Background counts for both ^{47}Ca and ^{85}Sr.

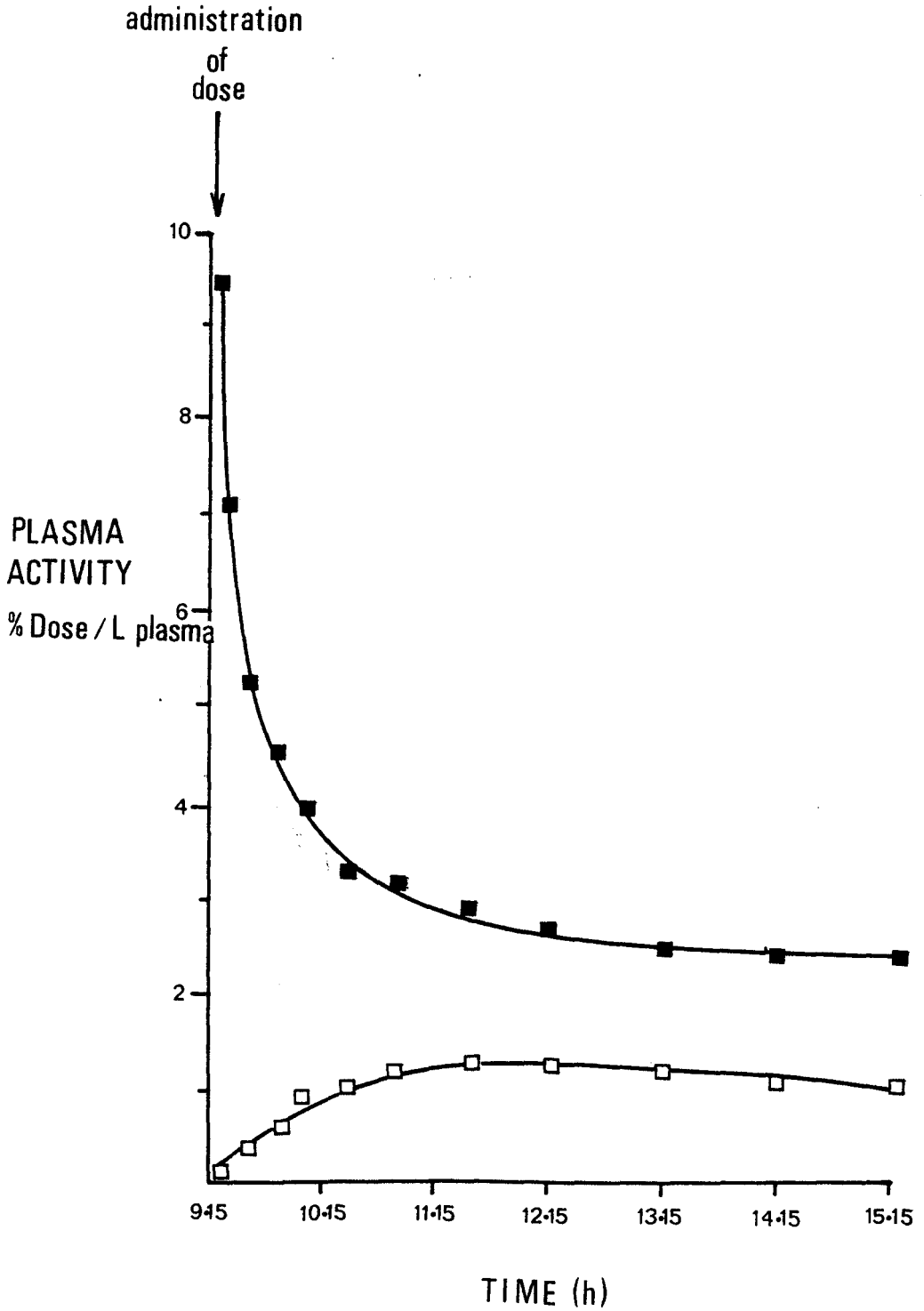
(iii) Counts for standard ^{47}Ca and ^{85}Sr administered.

(iv) Weights of standards used and their dilutions.

(v) Weight of each isotope administered to subject.

The percentage absorption was calculated as a function of time. By differentiating this graph, the rate of calcium absorption can be calculated for each hour. It should be noted that the effect of recirculation of orally administered tracer before its clearance into the skeleton and excreta is eliminated by a process known as deconvolution, so that only oral tracer absorbed during the course of the preceding circulation is taken into account. This process was included in the computer program.

FIGURE 2.8. Changes in plasma activity over a 6h time period following administration of oral ^{47}Ca (□) and I.V. ^{85}Sr (■).



2.3. METHODS 2: EXPERIMENTS ON 20S CELLS — See chapter 9

The human osteosarcoma cell line 20S was grown in α-modified minimum essential medium (MEM) supplemented with 10% (v/v) heat-inactivated newborn calf serum (NCS) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.3.1. SUBCULTURE AND PASSAGING OF CELLS

Cells were grown at a cell density of 8 x 10^6 cells/90mm culture dish until confluent. Growth medium was discarded and a few drops of Trypsin/EDTA solution added to the cell monolayer. Cells were trypsinised for approximately 5min, carefully agitating the culture dish to dislodge cells from the surface. Approximately 0.5ml fresh growth medium was added and the cell suspension transferred to a sterile universal bottle. After centrifuging for 10min at 2,000rpm,, the pellet was resuspended in fresh growth medium and the cells seeded out at 2 x 10^6 cells/90mm dish. Growth medium was changed every 2-3 days of cell growth until confluent when the procedure was repeated. When grown past confluence the majority of the cells floated off the surface and died.

2.3.2. MEASUREMENT OF CELL VIABILITY:
Trypan blue exclusion (Seglen, 1976)

Trypan blue (0.4% v/v solution in saline) was mixed with equal volume of cell suspension and left for 5min. A drop of this mixture was placed on a haemocytometer and a cover slip placed on top. The cells were viewed under a light microscope. Any damaged cells will have their nuclei stained blue because they were unable to prevent the uptake of the dye. Viable cells, which are not permeable to the dye appear normal colour. Therefore the number of viable and non-viable

(blue) cells were counted and the viable cells expressed as a percentage of total number of cells recorded. This was always greater than 95%

2.3.3. STORAGE AND PROPAGATION OF CELLS

Cells were harvested, as above, and the resulting pellet of cells was resuspended in 1ml of 5% (v/v) dimethyl-sulphoxide (5% DMSO) in NCS for every 90mm dish of cells. After transferring the suspension to sterile cryo-tubes, the tubes were placed in a -80°C freezer for 2 days after which they were placed in a -196°C liquid nitrogen bank for storage.

For the thawing procedure, each cryo-tube was warmed rapidly in a 37°C water bath and the contents transferred to a sterile universal bottle. An equal volume of growth medium was slowly added dropwise whilst shaking the universal. Cells were then pelleted and resuspended in 1ml of fresh growth medium prior to transfer to a culture dish.

2.3.4. EXTRACTION AND PARTIAL PURIFICATION OF LIPIDS FROM CELL MONOLAYER

Medium was removed from the cell monolayer and the cells treated with 4ml of hexane:isopropanol (3:2 v/v) for 20min. This solvent mixture was sucessful in removing lipids from cells. The solvent layer was transferred to a glass conical tube and the monolayer washed with a further 1ml of hexane:isopropanol (3:2 v/v) for 5min. The extracts were pooled and evaporated to dryness under a stream of nitrogen before resuspending in 1ml of 0.9% saline solution. An equal volume of acetonitrile was added to the extract in order to precipitate any protein and after vortex-mixing, the tube

was centrifuged at 1,200rpm for 10min. The supernatant fraction was applied directly to a prewashed C_{18} Sep pak column (refer to section 2.2.1). The column was washed, as described previously, and the acetonitrile fraction evaporated down to a small volume (approximately 500ul) and stored under nitrogen at -20°C.

2.3.5. EXTRACTION AND PARTIAL PURIFICATION OF LIPIDS FROM MEDIA

A mixture of chloroform and methanol was added to a known volume of medium such that the final ratio of chloroform: methanol: water was 1:1:0.9 (by vol). The lower organic phase was removed and evaporated under vacuum using a rotary evaporator. The residual lipid extract was resuspended by sonication in 0.9% saline solution and the above extraction procedure followed.

2.3.6. HPLC PURIFICATION OF LIPID EXTRACT FROM CELLS OR MEDIA

Purification of lipid extract from initial column chromatography was achieved by high performance liquid chromatography (section 2.2.1). A UV detector and chart recorder was attached to the system to monitor the various vitamin D metabolites at 264nm.

The retention times for the standard vitamin D metabolites were determined by injecting approximately 0.1ug of each metabolite on to the column and monitoring the retention time by UV detection. Once this was achieved satisfactorily, the column was washed thoroughly with isopropanol in hexane (25%, v/v), followed by re-equilibration with eluting solvent. The lipid extract was

then injected on to the column and fractions corresponding to the $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ retention times were collected separately, evaporated to small volumes and stored at -20°C under nitrogen.

2.3.7. PROTEIN DETERMINATION: Burgi et al (1967)

Each cell monolayer from a 90mm culture dish was scraped in 4ml of phosphate-buffered saline (PBS: 0.14M NaCl, 7.37mM Na_2HPO_4, 2.68mM KCl, 1.47mM KH_2PO_4, 0.49mM $MgCl_2$) and transferred to a conical tube. After centrifuging at 3,600rpm for 10min, the pellet was resuspended in 4ml of 0.1M NaOH and tranferred to a 37°C water bath to dissolve the protein. Aliquots (200ul) were assayed for protein by the Lowry Folin microassay method .

Alkaline reagent: 5ml 2% Na_2CO_3, 0.5ml 1% $CuSO_4$ and 0.5ml 2% NaK Tartrate
-Made up just prior to use.

Folin's reagent: 0.5ml Folin-Ciocalteau's reagent
4.5ml 0.1M NaOH

Samples for protein determination were pipetted in duplicate into a 24-well microassay plate and to these 50ul aliquots of alkaline reagent were added. After 5min at room temperature 50ul of Folin's reagent was added to each sample well and the samples left for a further 20min at room temperature. The absorbance at 620nm was read against a reagent blank, consisting of 200ul 0.1M NaOH, 50ul of alkaline reagent and 50ul of Folin's reagent.

A calibration curve was constructed using 1mg/ml BSA (in 0.1M NaOH) as standard protein. A suitable range of 10-150ug/well was used. Volumes were made up to 200ul/well with 0.1M NaOH.

2.3.8. PREPARATION OF CYTOSOL FROM OSTEOSARCOMA CELLS

2.5 $x10^9$ cells were grown to near-confluence in growth medium. After preincubation for 24h in MEM without NCS (to remove the serum vitamin D binding protein from the incubate) cells were washed twice with PBS and trypsinised to dislodge them from the plates. The cell suspension was centrifuged at 2,000rpm for 10min at 4°C and the resulting pellet was placed on ice.

The remainder of the procedure was carried out at 4°C. The pellet was washed five times with ice-cold isotonic buffer (0.25M Sucrose; 50mM Tris-HCl, pH 7.4; 25mM KCl; 12mM Thioglycerol; 5mM $MgCl_2$ and 1mM EDTA) and then finally suspended in hypertonic buffer (0.3M KCl; 10mM Tris-HCL, pH 7.4; 1.5mM EDTA and 0.5mM DTT). After 10min in hypertonic buffer the cell suspension was subjected to five rounds of freezing and thawing using liquid nitrogen. An aliquot (10ul) of the resulting suspension was viewed under low power magnification to confirm cell disruption.

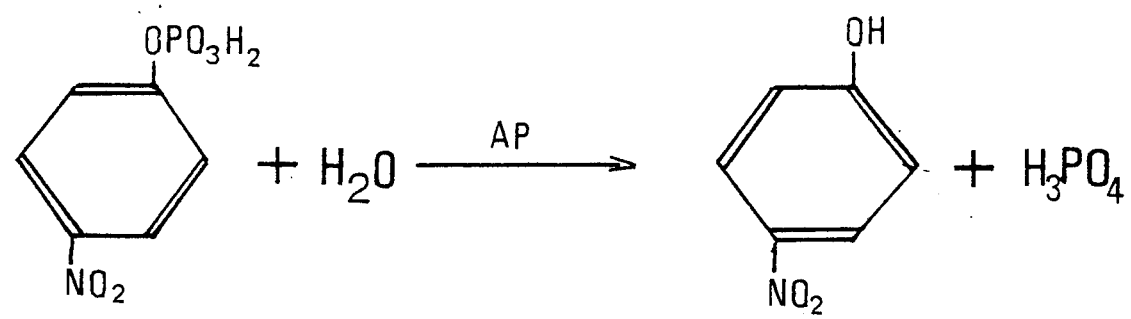
The broken cell suspension was centrifuged at 3,000rpm for 10min to remove cell debris and the supernatant fraction centrifuged at 25,000rpm for 1h to yield a cytosolic fraction. This supernatant fraction was stored in aliquots (2ml) at -20°C until required for analysis. A similar procedure was followed to obtain a cytosolic fraction from U937 cells. A calf thymic cytosol fraction was prepared as described by Reinhardt *et al* (1984).

2.3.9. ALKALINE PHOSPHATASE ACTIVITY IN 2OS CELL

Cytosol fraction from 2OS cells was prepared as described previously. For comparative purposes a cytosolic fraction from U937 cells was prepared in the same manner.

content was measured by the method of Burgi et al (see section 2.3.7). The protein content of the U937 cytosol was lower than that of the 20S cytosol (0.78 mg/ml compared to 2.5 mg/ml) and this was taken into account during the remaining procedures. An aliquot of each cytosolic fraction (with equal protein content) was dispensed into clean glass tubes. Alkaline phosphatase activity was measured by incubating each cytosolic fraction with p-nitro-phenyl phosphate buffer (50mM glycine, pH 10.5; 5.5mM p-nitrophenyl phosphate, sodium salt; 0.5mM $MgCl_2$) for 30min at 37°C. As control the phosphate buffer was incubated with hypertonic buffer (see section 2.3.8). In the presence of alkaline phosphatase the colourless substrate is converted to the yellow p-nitro phenol (figure 2.9).

FIGURE 2.9. Hydrolysis of p-nitrophenyl phosphate (colourless in alkaline medium) to p-nitrophenol (yellow in alkaline medium) by alkaline phosphatase (AP).



Alkaline phosphatase activity was also measured in the culture medium. Because NCS contains alkaline phosphatase, 20S cells were grown to confluence in growth medium and then transferred to serum-free medium. Small aliquots of medium was removed every 6h and alkaline phosphatase activity determined.

2.4. EXPERIMENTATION

2.4.1. CELLULAR RESPONSE OF 2OS CELLS TO PTH

1. Incubation medium: a) MEM + 10% NCS + 1uCi 3H-adenine
   b) MEM + 2% NCS + 0.2mM Theophylline
   c) MEM + 2% NCS + 6nM PTH

2. Hank's balanced salt solution (Ca and Mg free)

3. Carrier solution- containing 1mM of the following
   Adenine, Adenosine, ADP, ATP and cAMP

4. Recovery tracer- ^{14}C-cAMP

Cells were plated out at a cell density of 10 x 10^3 cells/35mm culture dish in 3ml growth medium. Medium was changed every 2 days until the time of experiment. Cells were incubated with 2ml of incubation medium (a) for 2h, after which the medium was discarded and the cell washed twice with 2ml Hank's balanced salt solution before incubating for a further 10min with the following:

Control dishes..... 0.5ml incubation medium (b) +
0.5ml (MEM + 2% NCS)

3mM PTH dishes..... 0.5ml incubation medium (b) +
0.5ml incubation medium (c)

1.5mM PTH dishes.... 0.5ml incubation medium (b) +
0.25ml incubation medium (c) +
0.25ml (MEM + 2% NCS)

Reactions were stopped by transferring reaction media to labelled conical tubes and adding 1.2M TCA (1ml) to the cell

monolayer. After a few minutes the TCA solution was removed and added to the appropriate tubes containing the reaction media. The cells were discarded at this atage. Approximately 6,000dpm of recovery tracer was added to all tubes, and into a scintillation vial to monitor final recovery. After vortexing, tubes were centrifuged at 2,000rpm for 10min and the supernatant fractions dispensed into clean tubes. All fractions were neutralised with 300ul of 4M KOH solution before the addition of 50ul of carrier solution. Tubes were then stored at -20°C until chromatography.

Stage 1: Using Dowex-50W (strongly acid cation exchange resin) constituted in 1M HCl and poured into glass pipettes plugged with cotton wool to make 5mm x 150cm columns. All columns were washed with distilled deionised water until the pH of the elutes were within the pH 6.0 region. 0.8ml of distilled deionised water was added to all tubes and after vortex-mixing, the contents of each tube were decanted onto the Dowex columns. The eluate from this, and two succesive washes with 1ml water were discarded. Water (3ml) was then added to each column and the eluates collected into clean labelled scintillation vials.

Stage 2: Using neutral alumina (aluminium oxide). 0.2ml of imidizole-HCl (pH 7.5) was added to each of the 3ml fractions collected from the Dowex columns which raised the pH 7.5. The contents in each vial were vortex-mixed before decanting onto a column containing 0.6g neutral alumina that had been washed previously with 8ml of 0.1M imidizole-HCl pH 7.5. Each eluate was collected directly into a clean scintillation vial. An additional 1ml of 0.1M imidizole-HCL pH 7.5 was added to the columns and collected into the vials. The contents of each vial were freeze-dried before the addition of 10ml of scintillation fluid and counting for both ^{3}H and ^{14}C.

2.4.2. BINDING STUDIES WITH 20S CYTOSOLIC FRACTION

Incubation with ^{3}H-vitamin D metabolites:

Aliquots (500ul) of 20S cytosol were incubated with either ^{3}H-25(OH)D_3 or ^{3}H-1,25(OH)$_2D_3$ (approx 80,000dpm in 10ul ethanol/incubation tube) at 25°C for 30min. Equivalent volumes of hypertonic buffer and human serum, diluted 1 in 10,000 fold with gelatine buffer (refer to section 2.2.2), were used as controls. All samples were cooled on an ice-bath and treated for 20min with 200ul of dextran-coated charcoal suspension (see section 2.2.2) to remove any ^{3}H-vitamin D metabolites not bound to the protein. After centrifugation at 2,000rpm for 20min the resultant supernatant fractions were counted for radioactivity.

Since these initial studies showed high ^{3}H counts in the tubes containing cytosol and ^{3}H-25(OH)D_3 the experiment was repeated but using cytosol which had previously undergone an overnight incubation at 4°C with an equal volume of rabbit-antibovine whole serum. Incubation with this antibody will form precipitated complexes with any serum proteins which may be present in the cytosolic preparation. After pelleting the precipitate by centrifugation at 6,000rpm for 3min, the supernatant fraction was incubated with ^{3}H-25(OH)D_3 for 30min then the sample was applied on to a G-25 sephadex column.

Preparation of Sephadex™ G-25 and G-100 columns:

Two dextran gels were employed; G-25 which was used to separate radiolabelled vitamin D metabolites bound to the cytosolic protein from the unbound (free) radioactivity, and G-100 for molecular weight determination of the radio-labelled bound protein.

Sephadex bead gels were pre-swollen as instructed by the manufacturer, in column buffer (100mM Tris-HCl, pH 7.4 containing 0.01% (w/v) sodium azide). A Sephadex G-25 column (25cm x 1cm) and a Sephadex G-100 column (85cm x 1.4cm) were prepared and allowed to equilibrate in column buffer before determination of their exclusion volumes. The G-100 column was then calibrated with proteins of known molecular weight.

Two mixtures of proteins were prepared in column buffer:

Mixture A contained 200ul of the following proteins:

Cytochrome c (10mg/ml)	MW 17,000
BSA (10mg/ml)	MW 66,000
Immunoglobulin G (10mg/ml)	MW 250,000

Mixture B contained 200ul of the following proteins:

Ovalbumin (10mg/ml)	MW 45,000
Aldolase (10mg/ml)	MW 160,000
Cytidine (1mg/ml)	MW 243,000

Mixture A was applied onto the G-100 column and 1ml fractions collected immediately after application. Each fraction was monitored at 280nm wavelength using quartz spectrophotometer cells. Collection was stopped when three absorption peaks were recorded. The column was washed with 20ml of column buffer and mixture B applied as before.

20S cytosol was incubated with ^{3}H-25(OH)D_3, as previously described. At the end of the incubation period the reaction mixture was applied to the G-25 column and 1ml fractions collected. Aliquots (100ul) of each fraction were counted for radioactivity. The fractions corresponding to the exclusion volume were pooled and applied to the G-100 column. Again 1ml fractions were collected and analysed for radioactivity.

For comparative purposes this procedure was repeated using (a) cytosolic fractions from U937 cells, (b) thymic cytosol (refer to section 3.1.1 for preparation) and (c) human serum, diluted 1 in 1,000 times in gelatine buffer (section 2.2.5).

Analysis of cytosolic protein by Sucrose density gradient centrifugation:

Two solutions of sucrose, at 4 and 24% (w/v) in 0.3M KCl were used to create continuous sucrose density gradients in six 16.5ml centrifuge tubes (7cm x 1.25cm). The following samples (500ul total volume) were applied to the top of each gradient:

Tube 1 A mixture of 300ul BSA (10mg/ml) and 200ul hypertonic buffer

Tube 2 A mixture of 300ul Ribonuclease (10mg/ml) and 200ul hypertonic buffer

Tube 3 A mixture of 300ul Ovalbumin (10mg/ml) and 200ul hypertonic buffer

Tube 4 500ul of 20S cytosol, previously incubated with ^{3}H-25(OH)D_3 and applied onto a G-25 column

Tube 5 500ul of U937 cytosol- as for tube 4

Tube 6 500ul of human serum (diluted 1 in 1,000 fold with hypertonic buffer- as for tube 4

The tubes were spun simultaneously at 4°C for 56h at 30,000rpm. Tubes were unloaded from the bottom using a peristaltic pump and 200ul fractions collected. For tubes 1 to 3, fractions were collected in plastic disposable tubes. Fractions obtained from these tubes were subjected to the Lowry-Folin assay (refer to section 2.3.7), to identify the position of each protein in the gradient. Because of the non-specific binding of the vitamin D metabolites to

plastic, fractions from tubes 4 to 6 were collected into glass tubes and 100ul aliquots from these fractions were monitored for radioactivity.

2.4.3. IMMUNODIFFUSION ANALYSIS OF 20S CYTOSOLIC PROTEINS

The following double immunodiffusion analysis was performed to examine the possibility that there may be some contamination of serum proteins in the cytosolic preparation and therefore some radioactively bound $25(OH)D_3$ might be associated with these proteins.

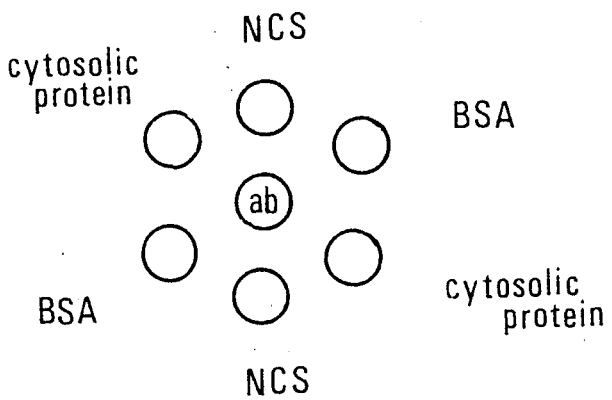
Immunodiffusion was carried out on a 10mm x 10mm glass plate using 1% agarose gel (Axelsen et al, 1979). Rabbit antibovine whole serum (2ul) was placed in the centre well (figure 2.10) and the following samples (2ul aliquots) were positioned in the remaining wells:

20S cytosolic protein
Newborn calf serum (NCS) ..... 1 in 1,000-fold dilution of stock.
Bovine serum albumin (BSA) ..... 0.25 mg/ml

After 48h at 4°C in a moist chamber antigen-antibody complexes were visible as precipitated lines. The procedure for staining the gel is similar to that outlined in section 2.2.7. The gel was dried and stained for 10min in Coomassie Brilliant Blue R (0.1% w/v) in acetic acid/methanol/water (1:5:5 by volume) and destained in acetic acid (7% w/v) and methanol (5% w/v) to remove non-cross reactive proteins. The gel was dried and analysed.

FIGURE 2.10. Diagram showing the position of each of the proteins on the immunodiffusion plate.

ab represents rabbit antibovine whole serum, NCS newborn calf serum and BSA bovine serum albumin.



2.4.4. INVESTIGATION INTO THE METABOLISM OF $25(OH)D_3$ BY 20S CELLS

Cells were maintained in culture in 90mm culture dishes at a cell density of approximately 2 x 10^6 cells/dish in growth medium. Once cells were near confluent, medium was removed and the cells washed well with MEM before incubating for 18h in serum-free medium. The time and concentration dependence of $25(OH)D_3$ metabolism was measured after this initial serum-free incubation using ^3H-$25(OH)D_3$ (170Ci/mmol) as radioactive precursor.

After the initial incubation, cells were again washed in serum-free medium before incubating for 3h in serum-free medium containing 50nM ^3H-$25(OH)D_3$ (in ethanol) or vehicle (ethanol alone). The reaction was stopped by cooling the culture dish and removing the media. Lipids were extracted from cells and media, as described in sections 2.3.4 to 2.3.6, and the vitamin D metabolites isolated using prewashed C_{18} Sep-Pak cartridges. Separation of the vitamin D metabolites was performed by HPLC.

Time and concentration experiments were performed under similar conditions. Cells were exposed to 50nM ^3H-$25(OH)D_3$ for different time periods (ranging from 10min to 3h) whilst for the concentration studies cells were incubated for 3h with concentrations of ^3H-$25(OH)D_3$ up to 0.5uM.

The effect of dibutyryl cyclic AMP (dbcAMP) on the metabolism of $25(OH)D_3$ was also investigated. Near-confluent cells were washed with serum-free medium before incubating overnight with either serum-free medium containing 1mM dbcAMP or in serum-free medium alone. The following day cells were incubated for 3h in serum-free medium (with or without dbcAMP) containing various concentrations of ^3H-$25(OH)D_3$.

Identification of unknown vitamin D metabolite by mass spectroscopy

The identity of the product which eluted with $24,25(OH)_2D_3$ was identified by mass spectroscopy. Large scale incubations involving eighty culture dishes (140mm diameter) were carried out to isolate the unknown metabolite. Approximately 10uM of $25(OH)D_3$ was used for each culture dish. The fraction corresponding to the $24,25(OH)_2D_3$ from the HPLC column was rechromatographed a further three times. After the fourth step fractions corresponding to the unknown metabolite were pooled, evaporated to dryness and redissolved in ethanol for UV and mass spectroscopy. An aliquot (15ul) was placed into the probe of a MS902 mass spectometer and this was evaporated to dryness before introducing into the system. The probe was heated at a rate of 2°C/sec to the point where a good signal was produced. The spectrum was examined on the mass spectrometer oscilloscope and its spectrum compared with those produced from the authentic vitamin D metabolites, $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$.

CHAPTER 3

DEVELOPMENT OF AN ASSAY SYSTEM FOR THE MEASUREMENT OF SERUM $1,25(OH)_2D$

3.1 INTRODUCTION:

The development of an assay system which measures serum concentrations of $1,25(OH)_2D$ has its share of problems. Concentrations of this metabolite in serum are extremely low; normal values are within the range of 20-50pg/ml. The presence of other vitamin D metabolites at concentrations two or three orders of magnitude greater than $1,25(OH)_2D$, as well as non-specific factors, possibly lipids, tend to interfere with its measurement. It is therefore essential to develop an assay system which will be able to overcome these problems. One of the first assay procedures to be reported, by Brumbaugh et al (1974), utilised the chick intestinal cytosol-chromatin receptor system to create a competitive radioreceptor assay for $1,25(OH)_2D$. Although modified by Eisman et al in 1976, the assay was laborious requiring a lengthy hormone purification scheme. Due to the instability of the receptor protein, this had to be prepared frequently which meant a regular supply of rachitic chicks. Therefore, other methods which would overcome these problems were sought.

By the early 1980s methods such as radioimmunoassays (e.g. Clemens et al, 1978), several radioreceptor assays (e.g. Eisman et al, 1976, Reinhardt et al, 1984), a bioassay (Stern et al, 1978) and an isotope-dilution-mass fragmentographic method (Bjorkhem et al, 1979) were reported. Due to the expensive and time-consuming techniques of mass fragmentography and the bioassay, these methods have

been abandoned and the most favoured methods for routine determination of serum $1,25(OH)_2D$ concentrations have been the radioimmunoassays and radioreceptor assays, both of which utilising chromatographic techniques, in particular high-performance liquid chromatography (HPLC) for extraction and purification of the vitamin D metabolite before quantification.

The aims of this present study were to compare the efficiency of an antibody to $1,25(OH)_2D$ with a cytosolic receptor derived from calf thymus, in the assay of serum concentrations of $1,25(OH)_2D$ and to establish a normal range in a group of healthy adults.

3.2 METHODS:

In order to reduce non-specific binding, all glassware was treated as described in section 2.2.1.

3.2.1 PRODUCTION OF ANTISERA:

Antisera were raised in sheep against $1,25(OH)_2D_3$-3-hemisuccinate coupled to bovine serum albumin (BSA), as described by Clemens et al (1978) for the 25-hemisuccinate derivative.

3.2.2 PREPARATION OF THYMIC CYTOSOL:

Thymus gland cytosol was prepared as described by Reinhardt et al (1985) modified as follows.

Thymus gland was removed from a 6-week old calf and washed in 0.9% ice-cold saline. The glands were cut into

small cubes, frozen in liquid nitrogen and stored at -80°C until required. Under these conditions there was no loss in receptor activity for at least one year.

The following steps were carried out at 4°C. Thymus glands were homogenised (25% w/v) in homogenising buffer (500mM KCl; 50mM Tris-HCl, pH 7.5; 10mM $Na_2MoO_4 2H_2O$; 5mM DTT; 1.5mM EDTA). The homogenate was centrifuged for 1h at 100,000rpm and the cytosol (minus pellet and floating lipid layer) was removed and fractionated by the slow addition of solid ammonium sulphate to 35% saturation (ie. 19.4g $(NH_4)_2SO_4$/100ml cytosol solution). After stirring slowly for 30-60min, aliquots of cytosol (10ml) were centrifuged at 15,000rpm for 20min. The supernatant fractions were discarded and the pellets stored under nitrogen at -80°C. The pellets were resuspended in 5ml homogenising buffer prior to use in binding studies.

3.2.3 EXTRACTION AND PURIFICATION OF SERUM $1,25(OH)_2D$:

See method section 2.2.1

3.2.4 RADIOIMMUNOASSAY PROCEDURE:

-as described by Gray (1983).

Triplicate glass tubes (10mm x 75mm), were labelled according to protocol 3.1. Stock antiserum was diluted 200,000-fold using gelatine buffer (50mg gelatine in 500ml of 0.1mmol/L K_2PO_4, pH 7.4) and this was kept on ice until required. Standard $1,25(OH)_2D_3$ in ethanol, ranging from 1.25-800 pg/20ul ethanol, were dispensed into appropriate tubes. Tubes labelled 0, T_c and C_b (refer to section 2.3) received the ethanol vehicle. The chromatographed serum extracts were reconstituted in 100ul ethanol. An aliquot (20ul) from each extract was dispensed into clean

PROTOCOL 3.1. Radioimmunoassay for $1,25(OH)_2D_3$

TUBES	STANDARD $1,25(OH)_2D_3$ (pg/20ul)	^3H-$1,25(OH)_2D_3$	ANTISERUM (250ul)	BUFFER (250ul)		CHARCOAL SUSPENSION (250ul)	
0	-	+	+	+		+	
T_c	-	+	-	++		-	
C_b	-	+	-	++	Mix	+	Mix Incubate
1	1.25	+	+	+	Incubate	+	15min
2	2.50	+	+	+		+	@
3	5	+	+	+	for	+	4°C
4	10	+	+	+		+	
5	20	+	+	+	4h	+	
6	40	+	+	+		+	Centrifuge
7	80	+	+	+	@	+	20min
8	160	+	+	+		+	@
9	320	+	+	+	4°C	+	4°C
Unknowns	-	+	+	+		+	

scintillation vials to monitor recovery and three 20ul aliquots pipetted into appropriately labelled assay tubes. Approximately 3,500dpm ^{3}H-1,25$(OH)_2D_3$ (in 20ul of ethanol) was added to all tubes and a clean scintillation vial. All tubes except those labelled T_c and C_b received 250ul of diluted antiserum; tubes T_c and C_b received buffer without antibody. A further 250ul gelatin buffer was added to all tubes and the tubes vortex-mixed.

After a 4h incubation at 4°C, aliquots (250ul) of dextran-coated charcoal suspension were added to all tubes except those of the T_c group. The suspension was made by mixing 1g activated charcoal and 0.1g dextran T_{70} with 200ml of 0.1mol/L potassium phosphate buffer, pH 7.4. After brief vortexing, the tubes were further incubated for 15min at 4°C and then centrifuged at 3,000rpm for 20min. An aliquot (500ul) from each of the supernatant fractions was dispensed into scintillation vials containing 10ml liquid scintillator and the vials monitored for tritium counts.

3.2.5 RADIORECEPTOR ASSAY PROCEDURE:

See method section 2.2.1.

3.3 RESULTS:

Before the antisera or cytosol can be used for the development of protein binding assays they must be characterised carefully. The three important properties are Titre, Sensitivity and Specificity.

3.3.1 TITRE:

Titre was established by measuring the ability of varying dilutions of protein to bind ^{3}H-1,25$(OH)_2D_3$ in the

absence of $1,25(OH)_2D_3$. Figure 3.1 shows the titre for the antiserum and thymic cytosol. In general, the dilution of antisera or cytosol which binds 30-50% of radioactivity was used for the radioassay since these conditions optimise the ratio of sensitivity to precision. Complete binding of ^3H-$1,25(OH)_2D_3$ to the antisera was obtained when the antiserum was diluted 200,000 fold and this dilution was used to construct a calibration curve; a typical curve is depicted in figure 3.2. Dilution of the cytosolic suspension by 5-fold produced similar radioactive binding (figure 3.1b) but this dilution varied slightly for each batch of cytosolic preparation. A much wider variation was observed between thymuses of different ages (figure 3.3). The correct dilution was therefore found empirically for each preparation batch and a calibration curve constructed using the estimated dilution factor (figure 3.2).

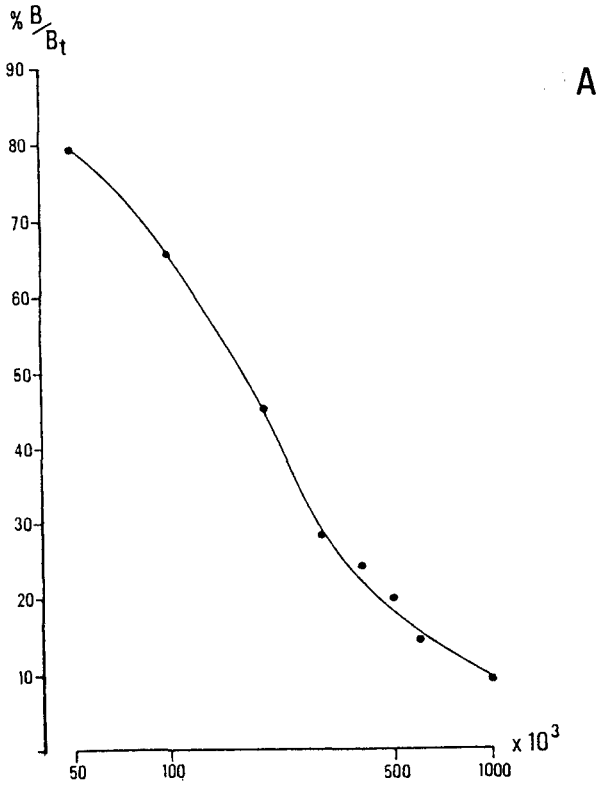
The performance characteristics of each binding assay are outlined in Table 3.1. Both standard curves were reproducible from assay to assay with little change in either the detection limits (see Table 3.1) or the shape of the curves. Maximum binding for both assays were similar but non-specific binding, defined as the proportion of ^3H-$1,25(OH)_2D_3$ which will bind to the protein in the presence of a 100-fold excess of the unlabelled hormone, was over three and a half times greater in the radioimmunoassay. This was also reflected in the mid-range of each binding curve.

3.3.2 SENSITIVITY:

Sensitivity, defined as the least amount of hormone that can be distinguished from no hormone by the antisera or receptor, was estimated as 2 standard deviations (2SD) below the maximal binding, or B_o value. The receptor assay

FIGURE 3.1. $1,25(OH)_2D_3$ binding dilution curves for:
(A) Antisera raised against the $1,25(OH)_2D_3$-hemisuccinate coupled to BSA, and
(B) The calf thymus receptor.

Each point represents the average of triplicate determinations. B_t represents total added radiolabelled $1,25(OH)_2D_3$.



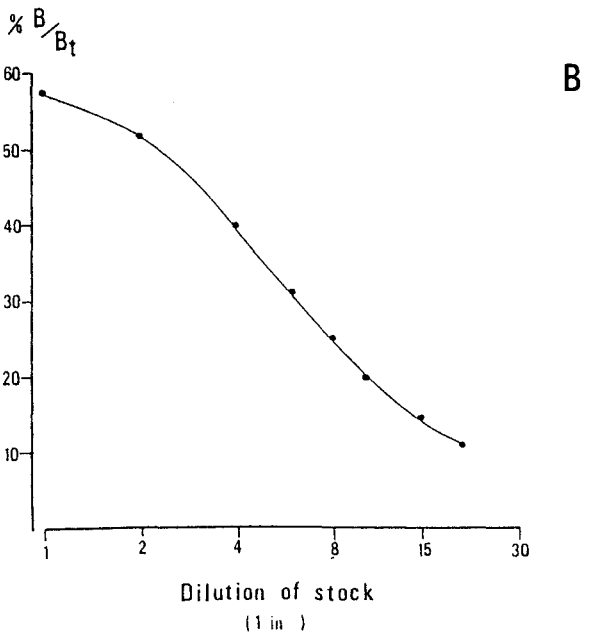


FIGURE 3.2. Standard curves for $1,25(OH)_2D_3$ using:
(A) Antiserum diluted 200,000-fold, and
(B) Thymus $1,25(OH)_2D$ cytosolic receptor.

Each point represents the average of triplicate determinations.

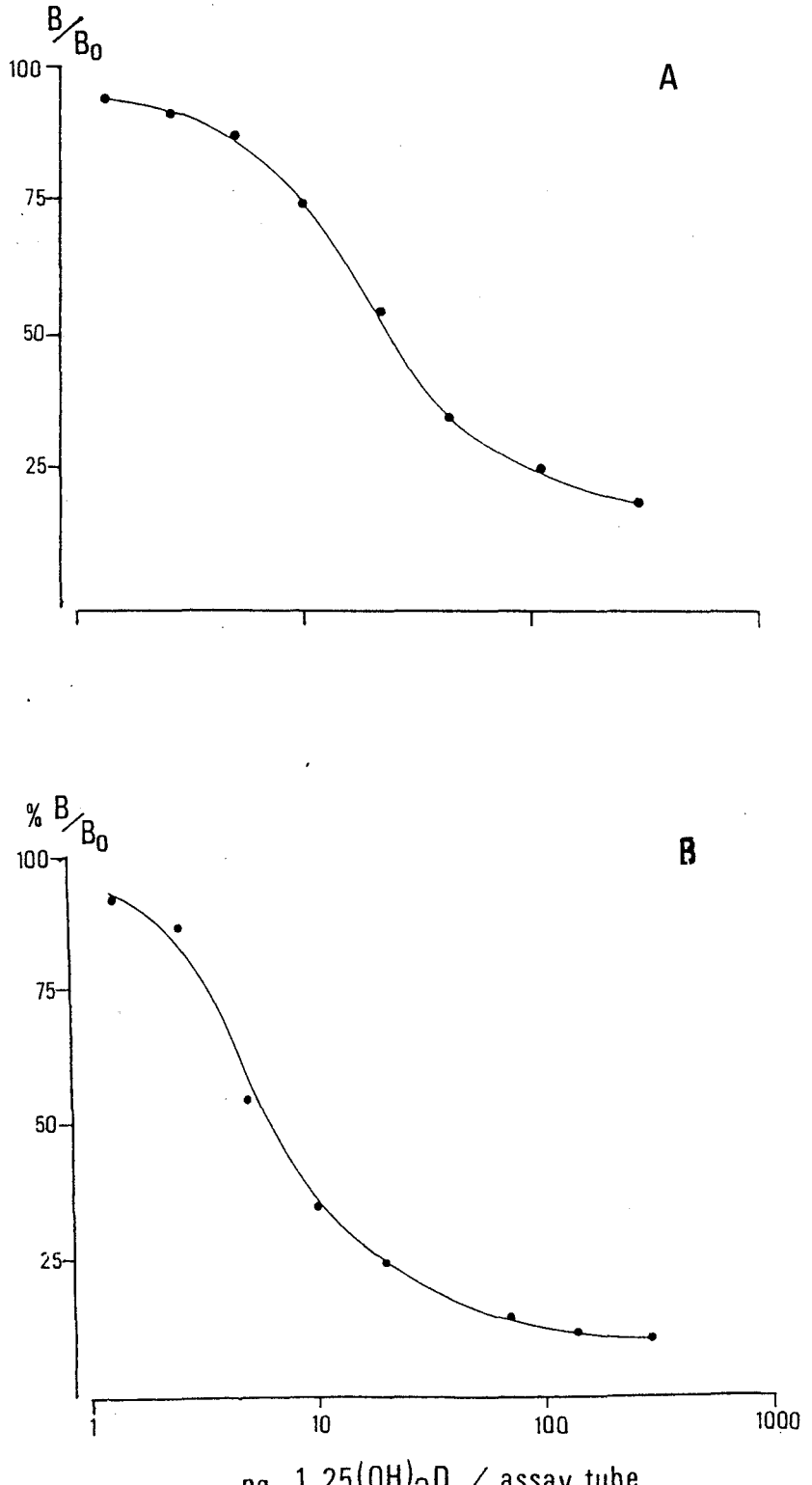
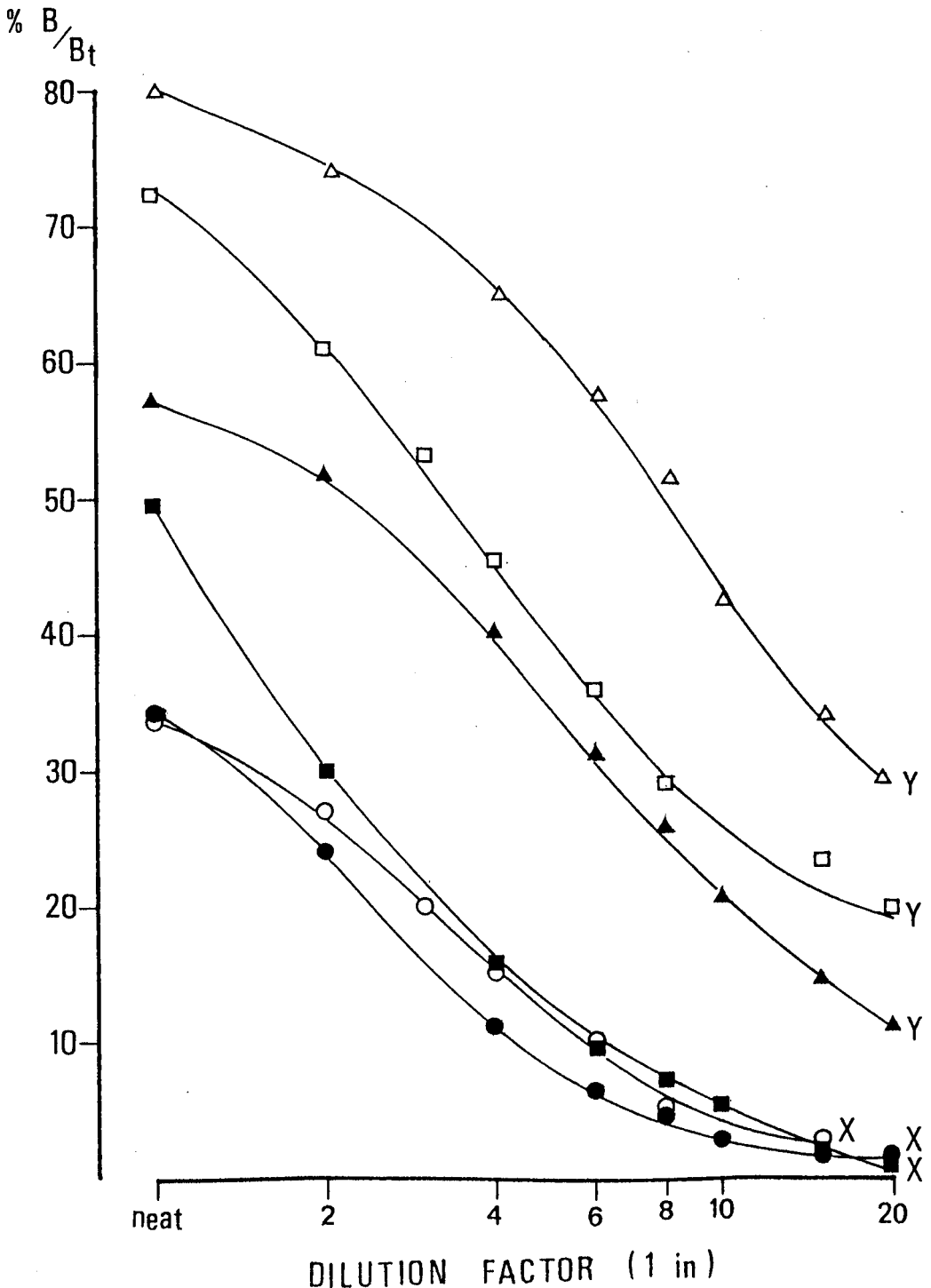


FIGURE 3.3. Variation in binding ability of thymuses from calves of different ages.

Y represents thymuses from calves < 16 weeks old; X represents > 16 weeks old. ▲ represents thymus frozen for 2 years prior to cytosolic preparation. Each point represents the average of triplicate determinations.



demonstrated a sensitivity of 1.27 pg/tube in comparison with 5 pg/tube for the immunoassay.

TABLE 3.1 PERFORMANCE CHARACTERISTICS OF THE CALF THYMUS RECEPTOR ASSAY AND THE RADIOIMMUNOASSAY

	Thymus receptor assay n = 10	Radioimmuno-assay n = 10
Maximum binding (%)	93.00	95.00
Non-specific binding (%)	1.36	4.70
Mid-range $0.5B_o$ (pg/tube)	6.50	24.0
Sensitivity 2SD B_o (pg/tube)	1.27	5.0
Detection range (pg/tube)	1.5 - 40	5 - 100

values determined from 10 standard curves and shown as mean

3.3.3 SPECIFICITY:

Specificity, defined as the freedom from interference by substances other than the hormone, is the characteristic which ultimately determines the usefulness of the antiserum or receptor. The cross-reactivity of various vitamin D analogues other than $1,25(OH)_2D_3$ was examined by comparing the ability of the unlabelled metabolites to displace ^3H-

$1,25(OH)_2D_3$ from the antibody and the thymus receptor (figure 3.4a and 3.4b, respectively). Although both receptor and antibody cross-react with other vitamin D compounds they do so with low affinity and they still retain their specificity towards the $1,25(OH)_2D_3$ metabolite. Table 3.2 compares the displacement of both systems; the amount of $1,25(OH)_2D_3$ which result in 50% displacement of the maximal binding (B_o) is defined as 1. Results indicate that with respect to the antibody although specific for $1,25(OH)_2D_3$ it will cross-react with vitamin D compounds which lack the 1-alpha hydroxyl group- such as $25(OH)D_3$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ if presented at a higher concentration than the $1,25(OH)_2D_3$ metabolite. However, the thymic receptor showed little or no specificity towards these analogues. In fact, the receptor displayed specificity towards vitamin D metabolites which possessed the hydroxyl function at C-1 on the A ring and so has equal affinity for both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ (figure 3.4b) reacting poorly with metabolites hydroxylated only on the side chain of the vitamin D molecule.

3.3.4 · AFFINITY OF THE BINDING PROTEINS:

Affinity of the binding protein is the energy required for the protein to bind the hormone and is denoted as K_a, the affinity constant. The affinity of each binding protein, determined by means of the Scatchard plot (Scatchard, 1949; Walker, 1977), and outlined in appendix 3, was 1.7×10^{10} L/mol for the antiserum and 4.75×10^{10} L/mol for the cytosol protein. The Scatchard analysis of total bound ligand showed non-linear plots for both proteins, indicating the presence of more than one class of binding sites in both instances.

FIGURE 3.4. Displacement of ^{3}H-$1,25(OH)_2D_3$ from:
(A) Antisera diluted 200,000-fold and
(B) Thymic $1,25(OH)_2D_3$ cytosolic receptor
by various vitamin D metabolites.
(1) $1,25(OH)_2D_3$ (2) $1,25(OH)_2D_2$ (3) $1,24,25(OH)_3D_3$
(4) $25(OH)D_3$ (5) $24,25(OH)_2D_3$ (6) $25,26(OH)_2D_3$ and
(7) D_2 or D_3.

Each point represents the average of triplicate determinations.

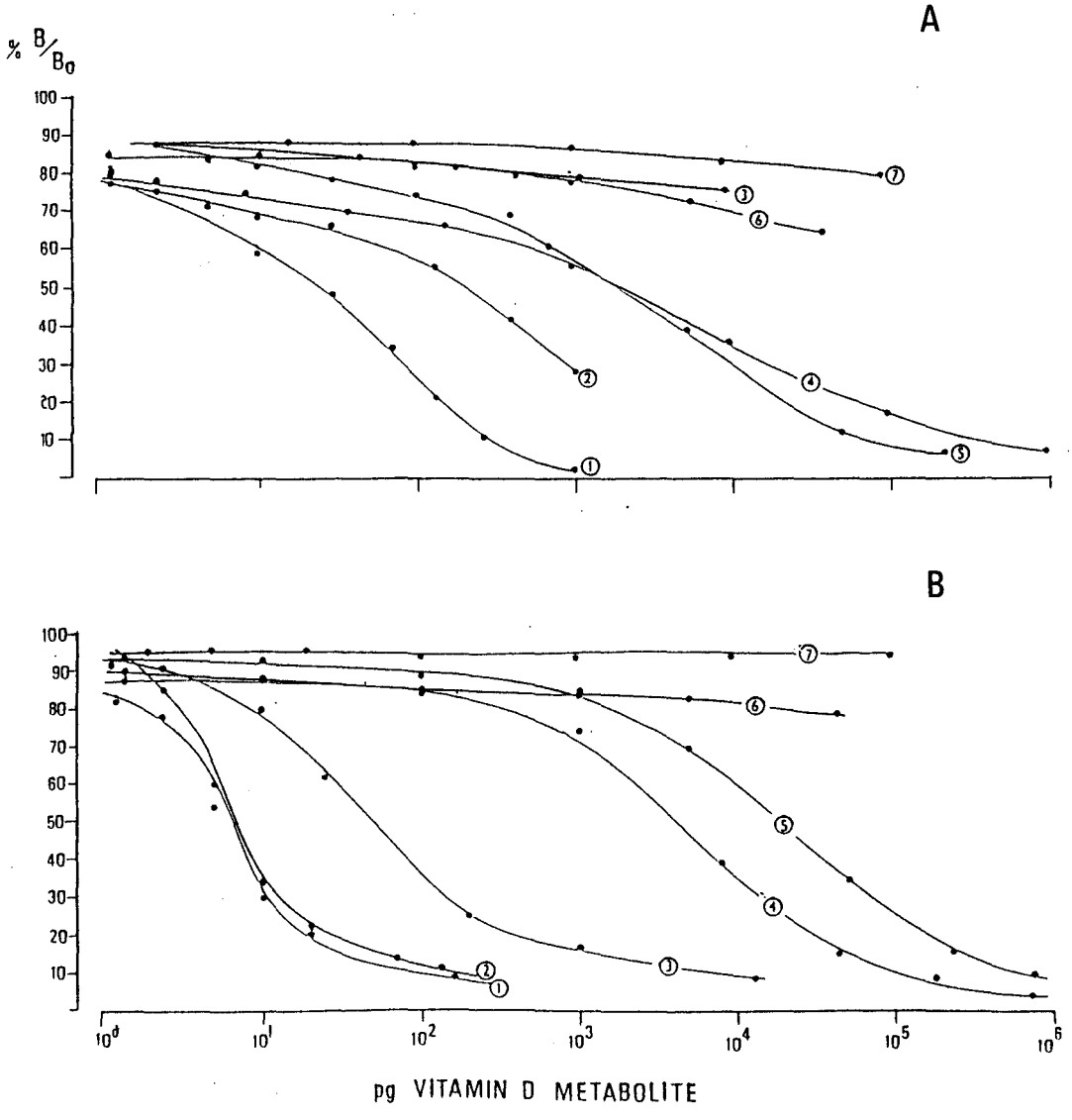


TABLE 3.2. Specificity of the radioimmunoassay and the Thymus receptor assay.

Vitamin D metabolite	Calf thymus receptor	Antibody
$1,25(OH)_2D_3$	1.0	1.0
$1,25(OH)_2D_2$	1.06	13.1
Vitamin D_3	>15,000	>15,000
Vitamin D_2	>15,000	>15,000
$25(OH)D_3$	430	285
$24,25(OH)_2D_3$	1,4000	570
$25,26(OH)_2D_3$	>3000	>4300
$1,24,25(OH)_3D_3$	7.58	>700

The amount of $1,25(OH)_2D_3$ which result in 50% displacement of the maximal binding is defined as 1.0

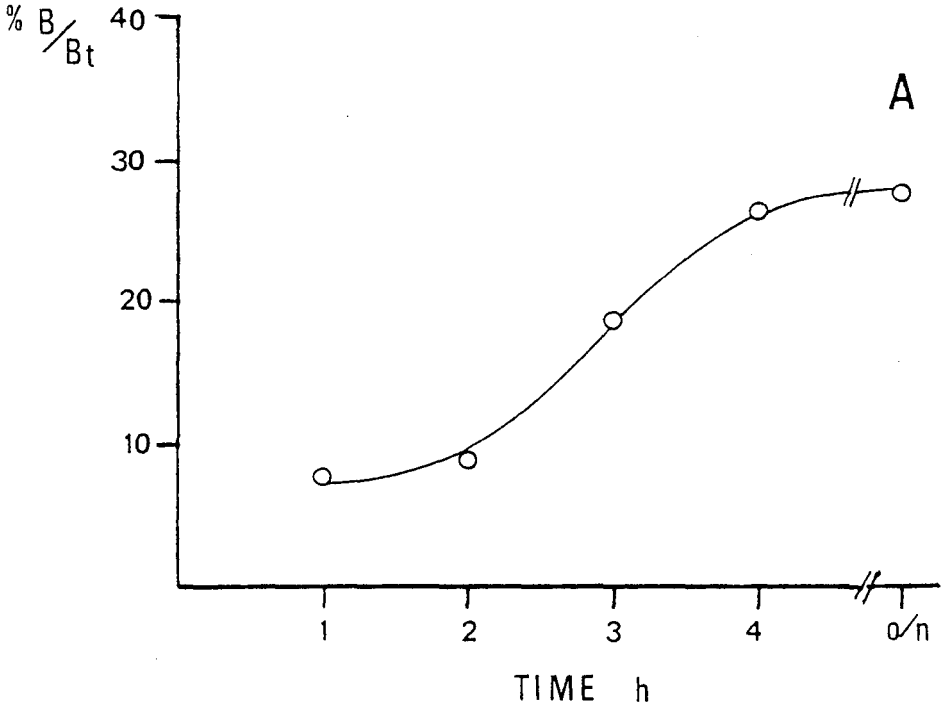
Investigation into the binding behaviour of the antiserum with different incubation times and temperatures revealed little or no binding during the first three hours but a marked increase in binding at 4h (figure 3.5a). It should be noted that an overnight incubation did not significantly increase the binding ability of the antiserum for the hormone. Different incubation temperatures produced different binding curves (figure 3.5b). At 25°C the binding afffinity of the antibody was lower than that observed at an incubation temperature of 4°C.

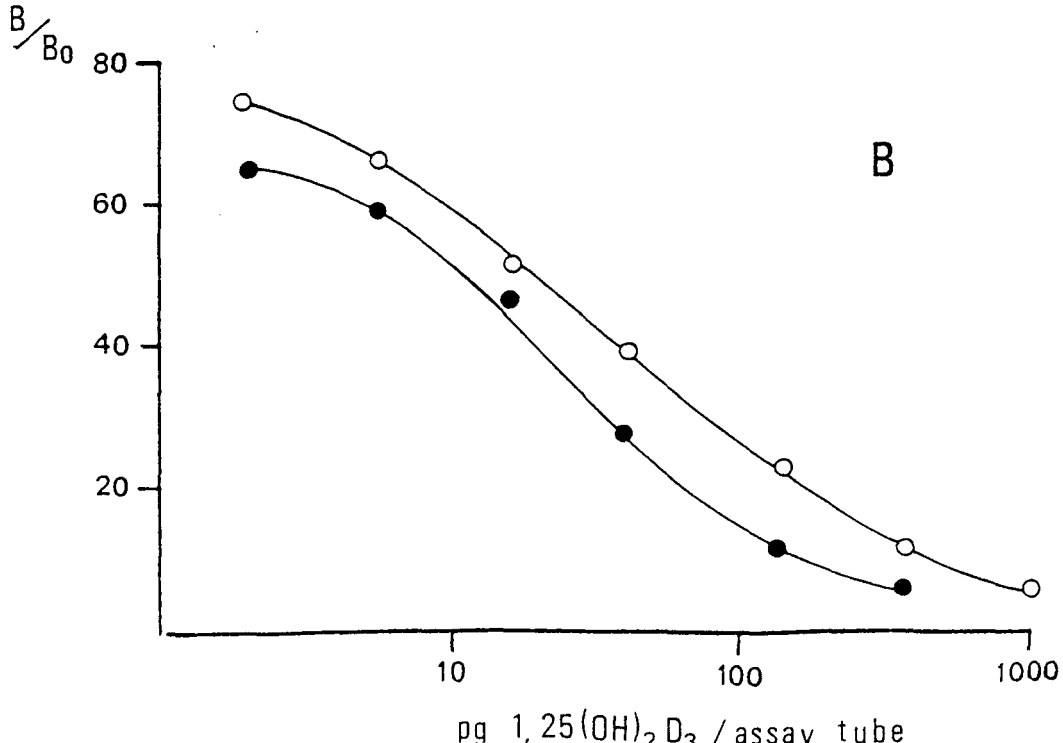
3.3.5 EFFICIENCY OF THE EXTRACTION AND PURIFICATION PROCEDURE:

Known amounts of radioactive vitamin D_3 metabolites, including the ^3H-1,25$(OH)_2D_3$ metabolite, were added to plasma and the procedure for the extraction and purification of the metabolites was followed (section 2.2.1). Removal of serum proteins by precipitation with acetonitrile was rapid and efficient with no loss of the radiolabelled metabolites. After the removal of salts and polar lipids using a C_{18} cartridge the vitamin D metabolites were eluted with 4ml acetonitrile. This volume produced the highest recovery value for all metabolites; at this stage in the procedure recovery of each radiolabelled metabolite was greater than 95% (n = 4).

The use of á silica Sep-Pak cartridge, rather than the HPLC step, to separate the different vitamin D metabolites, was investigated (Reinhardt et al, 1984). The eluate from the C_{18} cartridge was evaporated to dryness, redissolved in 1ml hexane/isopropanol (96:4 vol/vol) and applied to a silica Sep-Pak cartridge which had been washed previously by sequential additions of 5ml methanol, 5ml hexane and 5ml hexane/iso-propanol (96:4 vol/vol). The cartridge was washed

FIGURE 3.5 (A) Binding ability of the antiserum (diluted 200,000-fold) at different incubation times. (B) Standard curve for $1,25(OH)_2D_3$ using antiserum (diluted 200,000-fold) at 4°C (○) and 25°C (●) incubation temperatures.





with 11ml hexane/isopropanol (96:4) to elute the 25(OH)D metabolite and then with 8ml hexane/isopropanol (94:6) to elute the $24,25(OH)_2D$ metabolite. Finally, the cartridge was washed with 10ml hexane/isopropanol (85:15) which removed the $1,25(OH)_2D$ metabolite. The three fractions were evaporated to dryness and monitored for radioactivity. The results from four separate applications showed that the mean recovery of ^3H-$25(OH)D_3$, ^3H-$24,25(OH)_2D_3$ and ^3H-$1,25(OH)_2D_3$ were 89.3%, 72.6% and 118.5%, respectively. These results suggest that there may be some overlap between ^3H-$24,25(OH)_2D_3$ and ^3H-$1,25(OH)_2D$. Thus, the HPLC step was used for purification and separation of the vitamin D metabolites.

The radiolabelled vitamin D metabolites showed an identical elution profile to that of the unlabelled metabolites (figure 2.2) and therefore this profile was used to determine the retention time for each metabolite. Direct application of the radiolabelled metabolites to the HPLC column produced recovery values which were always greater than 87%. Samples from the C_{18} cartridges were therefore subjected to straight phase HPLC. Fractions corresponding to the 25(OH)D (3-6½min), $24,25(OH)_2D$ (7-9½min) and $1,25(OH)_2D$ (11-15min) were collected and evaporated to a small volume (approx. 200ul) before storing under N_2 at -20°C until required for quantitative determination.

The overall recovery (i.e. from plasma extraction to quantitative determination) for ^3H-$25(OH)D_3$, ^3H-$24,25(OH)_2D_3$ and ^3H-$1,25(OH)_2D_3$ were 84 $\pm$ 5.6 % (mean $\pm$ SD, n = 30), 79 $\pm$ 4.9 % (n = 30) and 75 $\pm$ 6.1 % (n = 30), respectively. Analytical recovery of unlabelled $1,25(OH)_2D$ was assessed at two levels; when 22pg and 69pg of unlabelled $1,25(OH)_2D$ were added to 1ml aliquots of either control serum or 0.9% (w/v) saline solutions. After extraction and purification the $1,25(OH)_2D$ extracts were determined either by the

radioimmunoassay or by the receptor assay method. Results are shown in Table 3.3.

No detectable levels of $1,25(OH)_2D$ were seen when blank saline samples were subjected to the thymic receptor assay. This was in contrast with the radioimmunoassay which demonstrated values within the region of 50pg/ml. It was impossible to reduce this value.

To determine the accuracy of the radioreceptor assay pooled serum was divided into 10 x 1ml aliquots. The average ($\pm$ 2SD) of $1,25(OH)_2D$ concentration was 37.7 $\pm$ 7.8pg/ml. The intra-assay coefficient of variation, calculated as (SD ÷ mean) x 100%, was 10.3%. Assay of the same pooled serum in 20 successive assays yielded an inter-assay coefficient of variation of 13.2%.

Figure 3.6 shows values for $1,25(OH)_2D$ in human serum determined by the radioreceptor assay. In a group of 50 normal subjects (mean age 36 years; range 19 - 53 years) concentrations of $1,25(OH)_2D$ ranged from 20-65 pg/ml with a mean ($\pm$ 2SD) concentration of 35.9 $\pm$ 15.6 pg/ml. No sex-related difference was found (p= NS) nor was there any seasonal variation in $1,25(OH)_2D$ concentrations. These reference intervals are comparable to those found by other investigators, especially with those from European countries.

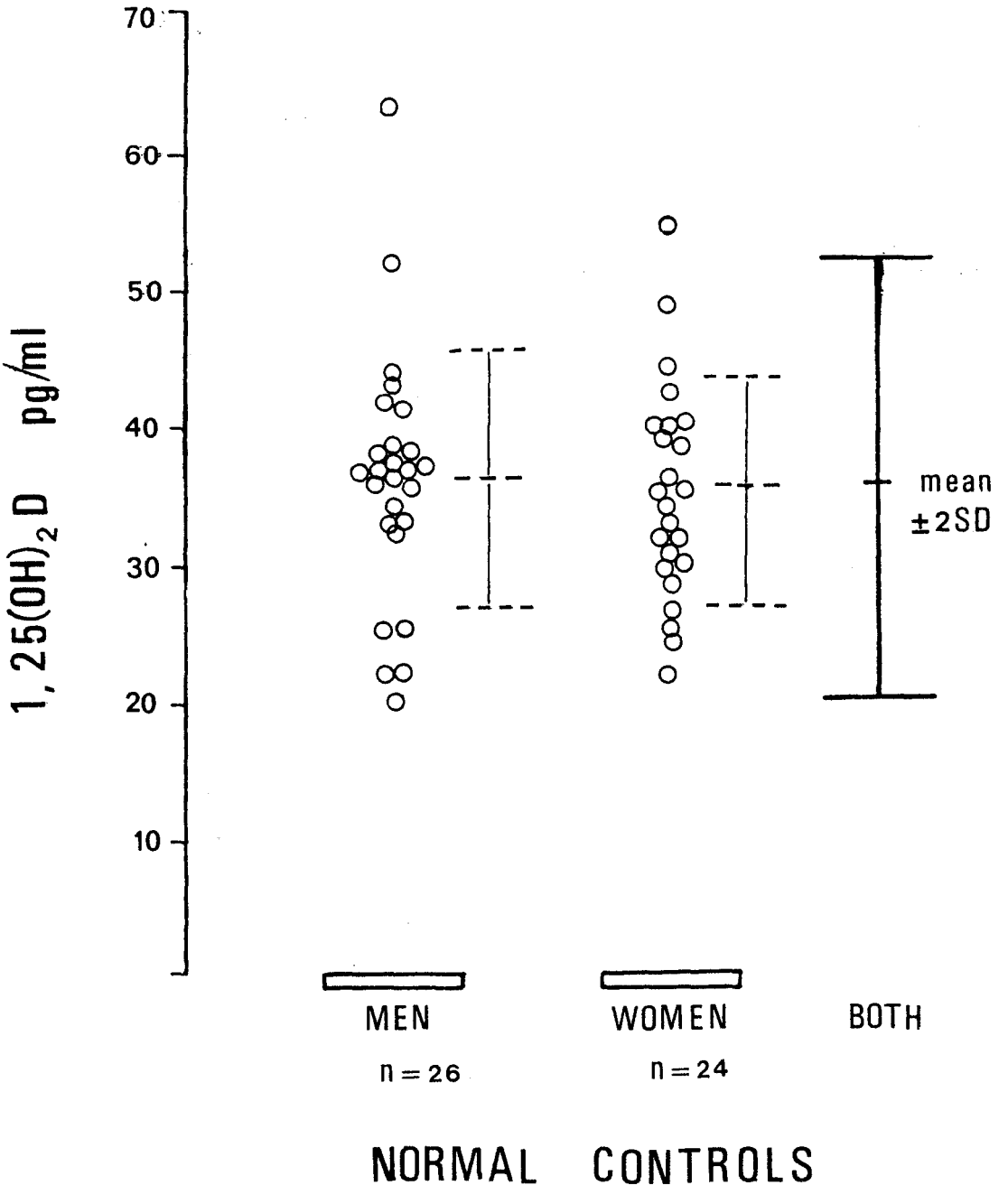
TABLE 3.3. Analytical recovery of $1,25(OH)_2D$ from either 1ml serum or 0.9% saline

A represents actual values. **B** represents estimated values.

		0.9% SALINE			SERUM		
		$1,25(OH)_2D$			$1,25(OH)_2D$		
	n	A pg/ml	B pg/ml	Recovery (%)	A pg/ml	B pg/ml	Recovery (%)
Thymus Receptor Assay	4	0	0	-	0	34.3	-
	4	22	17.9	81.4	22	59.7	115.5
	4	69	60.5	87.7	69	89.2	79.6
Radio-immuno assay	4	0	52.5	-	0	172.5	-
	4	22	117.5	-	22	213.6	-
	4	69	121.8	-	69	344.7	-

FIGURE 3.6 Serum $1,25(OH)_2D$ concentrations from 50 normal healthy adults

Bold horizontal lines represent the mean $\pm$ 2SD for all values.



3.4 DISCUSSION:

Since the vitamin D metabolite $1,25(OH)_2D$ is important in calcium homeostasis, it is essential to establish a sensitive and reliable assay system for the measurement of serum $1,25(OH)_2D$ in patients with a large variety of mineral and skeletal disorders. However, assays have been limited in part because of the difficulty in measuring this metabolite in serum, or plasma, with any accuracy. This has been attributed largely to non-specific factors, such as lipids and other vitamin D metabolites, which seem to interfere in its assay.

Over the past decade several assay systems have been developed which have overcome these problems by introducing extensive extraction and purification schemes prior to quantification. The time-consuming schemes involving the use of Sephadex LH-20 columns have now been abandoned and instead HPLC-based methods are used sucessfully in purifying $1,25(OH)_2D$ from other vitamin D metabolites before its estimation by competitive binding radioassay, either by radioimmunoassay or radioreceptor assay. The work presented here was designed to compare the efficiency of these two assay systems in the measurement of serum concentrations of $1,25(OH)_2D$ after its extraction and purification by HPLC.

Radioimmunoassay has proved to be a very sucessful method for measuring serum concentrations of $1,25(OH)_2D$. It offers the advantage of producing large quantities of stable gamma-globulin which, when stored under appropriate conditions, can be used for several years without loss of binding ability. This is in contrast to assay systems which utilise a cytosol receptor as the binding protein. Because of its limited stability, continual preparation of the cytosol receptor is necessary. However, this was not seen when the thymus receptor was used as binding protein. Once

prepared the thymic cytosol protein was stable for up to six months at -80°C. Reinhardt et al (1984) showed that intact thymus glands stored at -80°C still retained their binding ability. This was confirmed in the present study. Cytosol, prepared from a gland which had been frozen for two years, still demonstrated consistent binding properties (figure 3.3). The occasional need for new supplies of thymus emphasised clearly the importance of the age at which the calf must be slaughtered. Insufficient binding activities were observed with thymuses from calves older than sixteen weeks old (figure 3.3). It is difficult to interpret this result because of the uncertainty as to why the thymus should possess receptors for $1,25(OH)_2D$.

A critical step in the development of an assay system is the specificity of the binding protein for the hormone. Both assays demonstrated similar specificity towards the $1,25(OH)_2D_3$ metabolite. The antiserum, raised against the $1,25(OH)_2D$-3-hemisuccinate coupled to bovine serum albumin, showed cross-reactivity with several vitamin D metabolites which appeared in 100-fold excess to the radiolabelled hormone, in particular analogues which lack the 1 alpha-hydroxyl group. The relative order of the displacement of bound radioligand by several vitamin D metabolites was $1,25(OH)_2D_3$ > $1,25(OH)_2D_2$ > $24,25(OH)_2D_3$ > 25(OH)D > $25,26(OH)_2D_3$ > $1,24,25(OH)_3D_3$. However, parent vitamin D_2 and D_3 did not displace the bound radioligand when added to assay tubes in quantities greater than 100ng per tube.

In contrast, the thymus receptor displayed specificity towards vitamin D metabolites which contain an hydroxyl function at the C-1 position on the A ring, and therefore demonstrated identical affinity for both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ (figure 3.2) while reacting poorly with metabolites hydroxylated only on the side chain. The relative order of displacement for the thymus receptor was

$1,25(OH)_2D_3$ and $1,25(OH)_2D_2$ > $1,24,25(OH)_3D_3$ > $25(OH)D_3$ > $24,25(OH)_2D_3$ > $25,26(OH)_2D_3$ > vitamin D_2 or D_3.

Under normal circumstances vitamin D_2 metabolites constitute around 5% of the total vitamin D activity in serum (Hughes et al, 1976). However, when considering patients on vitamin D supplements, especially when the parent vitamin D administered is in the D_2 form, then the biologically active form will be represented by both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$. Because the affinity of the antiserum for $1,25(OH)_2D_2$ is less than for $1,25(OH)_2D_3$, the radioimmunoassay will underestimate total $1,25(OH)_2D$. The radioreceptor assay, however, has the ability of measuring both forms equally and simultaneously and, therefore, will not underestimate the true value.

Investigation into the sensitivity of each assay system revealed that the radioreceptor was far more sensitive than the radioimmunoassay. The receptor assay was capable of detecting as little as 1.5pg of $1,25(OH)_2D$ per assay tube with a useful range of measurement of 1.5-40pg/tube compared with 5pg of $1,25(OH)_2D$ per tube for the immunoassay and a range of 5-100pg/tube. These detection limits imply that for the estimation of serum concentrations of $1,25(OH)_2D$ the receptor assay will require less sample volume. Thus, to work within the useful ranges, approximate serum volumes used were 1ml for the radioreceptor assay and 3ml for the radioimmunoassay. The large serum volumes, as in the case for the immunoassay, increases the chance of interference of other serum vitamin D metabolites with the assay.

Although identical extraction and purification techniques were performed for both assay systems, interference was still prominent in the immunoassay (Table 3.3). By no means could this interference be removed and, because of this, the radioimmunoassay was abandoned. The

receptor assay, however, proved very sucessful, showing no detectable levels of $1,25(OH)_2D$ with blank saline samples and an average value of 34.3pg/ml of $1,25(OH)_2D$ with pooled serum samples obtained from normal individuals.

The reproducibility of the receptor assay was confirmed by showing low intra- and interassay coefficients of variations. In addition, analytical recovery was estimated using two amounts- when 22pg and 69pg of standard $1,25(OH)_2D$ were added to 1ml aliquots of either saline or pooled serum samples (Table 3.3). Recovery was found to be greater than 75% for both samples.

The mean value for serum $1,25(OH)_2D$ in normal subjects was similar to those values obtained previously with other methods. In fact, the normal mean value of 35.9 (SD 7.8) pg/ml does not differ markedly from those of Bouillon et al (1980) and Hughes et al (1976), both of which utilise a radioimmunoassay for the measurement of $1,25(OH)_2D$, nor with Reinhardt et al (1984) who employed the same calf thymus receptor protein. Methods differ, however, in that they utilised the silica Sep-Pak cartridge rather than the HPLC methodology for the final purification step. When this was attempted unsatisfactory results were obtained. Recovery values for radiolabelled $1,25(OH)_2D$ from the cartridge always produced values greater than 100% which implied that the radiolabelled $24,25(OH)_2D$ was coeluting with the $1,25(OH)_2D$. This was confirmed when the $1,25(OH)_2D$ fraction was quantitated by the receptor assay. Normal pooled serum produced values far greater than 34pg/ml.

When serum volumes greater than 1ml were extracted and purified by the two Sep-Pak method values were again overestimated. This could only be attributed to the lack of complete separation of the two vitamin D metabolites. However, Reinhardt et al did report that values obtained

from normal serum samples spiked with 10ng of $24,25(OH)_2D$ gave values identical to those of unspiked samples. Other factors must, therefore, be interfering with the assay and in order to eliminate these unknown factors the HPLC method was used.

From the results presented here it is clear that for the measurement of serum concentrations of $1,25(OH)_2D$ the thymus cytosol radioreceptor assay is acceptable; demonstrating high precision, reproducibility and specificity. However, extensive purification of the metabolite is necessary because serum contains factors, including other vitamin D metabolites, which will interfere with the assay. When employing this method for clinical situations it was vital to perform this assay under strict quality control. This was achieved by including reference sera and blank samples (saline) in each assay.

CHAPTER 4

INTESTINAL CALCIUM ABSORPTION AND THE VITAMIN D METABOLITES

4.1 INTRODUCTION:

The two important determinants of effective calcium absorption are (a) the amount of calcium in the diet and (b) the efficiency of its absorption (Heaney et al, 1982). It is clearly recognised that $1,25(OH)_2D$, the hormonally active vitamin D metabolite, stimulates intestinal calcium absorption. It could be argued, therefore, that if this metabolite is the sole initiator of calcium absorption in the gut then the rate of calcium uptake will depend largely upon the plasma concentration of $1,25(OH)_2D$. Would low $1,25(OH)_2D$ concentration suggest some form of calcium malabsorption?

Methods for the in vivo measurement of intestinal calcium absorption have employed the use of radiolabelled calcium isotopes, ^{45}Ca and/or ^{47}Ca. These techniques are based on comparing the disappearance of calcium from plasma after intravenous and oral administration. The single tracer technique uses ^{47}Ca in monitoring intestinal calcium absorption (Bhandarkar et al, 1961) whereas the double tracer method uses both ^{47}Ca and ^{45}Ca isotopes (Reeve et al, 1976).

The curves of the disappearance and appearence of the two tracers in the blood can be analysed by a process known as the deconvolution method to provide, in addition, the distributive function of absorptive rates with respect to time. There is no doubt that the deconvolution method

provides the most information about the absorption process. However, this method of using ^{45}Ca and ^{47}Ca as the two isotopes was inconvenient in practise because of the six weeks delay in calculating the results. The delay is unavoidable. To determine the activity of ^{45}Ca allowance must be made for the ^{47}Ca to decay because of its interference in the measurement of ^{45}Ca. This takes around 6-8 weeks. Therefore another radiolabelled isotope, ^{85}Sr, having almost identical plasma kinetics over the first 12h after injection (Hart and Spencer, 1967) was used in place of ^{45}Ca.

The double tracer deconvolution calcium absorption test, employing the use of ^{47}Ca and ^{85}Sr has been shown to be a precise method in determining in vivo calcium absorption (Reeve et al, 1975). This method was therefore employed to measure intestinal calcium absorption in a selected group of patients with various metabolic disorders. Plasma concentrations of the vitamin D metabolites were also measured to determine whether 25(OH)D or $1,25(OH)_2D$ had any relationship with intestinal calcium absorption.

4.2 PATIENTS AND METHODS:

All studies were carried out on a metabolic out-patient unit with the informed consent of the 39 patients in this investigation. Those patients who were on medication did not take any on the day of the test.

All patients were fasted overnight and on the day of the test blood was removed for measurement of the vitamin D metabolites (sections 2.2.3 and 2.2.5). The double-isotope method was used to assess intestinal calcium absorption (section 2.2.9). After the administration of an oral dose of ^{45}Ca and an intravenous dose of ^{85}Sr, blood samples were taken at specified intervals into heparinised tubes and the

plasma separated. Patients were not allowed lunch until after the four hour sample had been taken. Aliquots (4ml) of each plasma sample were counted for both radio-isotopes.

Data from each patient was then submitted to a computer programme kindly provided by Dr. JR Greenwell, Department of Physiological Sciences, University of Newcastle upon Tyne. This programme initially calculates for each isotope the percentage of the dose absorbed at a given time during the 6 hour study. It then calculates the true absorption of ^{47}Ca into the intestinal tract by a method known as deconvolution. The calculation uses a mathematical spline curve-fitting procedure to enhance the change in the plasma concentration of the oral dose to correct for the clearance of the isotopes from plasma using the changing concentration of the intravenous isotope. By differentiating this graph the rate of calcium absorption can be determined.

4.3 RESULTS:

The procedures employed to calculate the initial fractional rate (λ) and the total percentage calcium absorbed after 6h are outlined in appendix 4. Table 4.1 shows individual patient responses to the calcium absorption test and their measured vitamin D metabolites whereas results for the different patient groups are summarised in Table 4.2.

The patients with renal stones (cases 1 to 4), Bartter's syndrome (case 5), sarcoidosis (case 6) and myeloma (case 7) all represented control patients.

Four patients with osteoporosis were studied (cases 8 to 11). All had similarly reduced values for both initial fractional absorption and total calcium absorbed. These

TABLE 4.1. Individual responses to the calcium absorption test and their corresponding vitamin D concentrations.

Case No.	Diagnosis	25(OH)D (ng/ml)	$1,25(OH)_2D$ (pg/ml)	Initial fract. rate (%)	Maximal Absorption (%)
1	Renal stones	19.3	38.4	31.0	78.2
2	"	14.1	36.1	37.5	69.4
3	"	7.1	20.8	6.7	50.9
4	"	2.9	22.0	16.5	30.6
5	Bartter's syndrome	19.5	40.1	42.2	78.0
6	Sarcoidosis	9.3	37.5	37.8	104.2
7	Myeloma	11.3	4.2	18.3	53.2
8	Osteoporosis	7.7	11.2	13.3	39.7
9	"	33.4	14.6	1.9	34.7
10	"	3.5	17.3	23.5	43.1
11	"	6.3	25.7	20.2	58.4
12	Osteomalacia: controls	4.1	22.5	19.4	32.1
13	"	9.75	10.9	22.1	51.4
14	"	3.1	27.3	6.5	25.9
15	"	3.6	33.4	25.4	55.3
16	Osteomalacia: on D_3	10.2	58.1	53.0	69.9
17	"	25.5	69.2	40.6	68.9
18	"	21.7	37.9	25.1	63.9
19	"	16.5	41.5	33.3	52.6
20	"	4.3	19.8	19.8	52.6
21	"	41.1	53.9	31.1	40.4
22	"	29.7	37.6	15.0	39.7
23	"	26.2	68.9	67.1	84.6
24	Osteomalacia: on 1	6.8	50.2	47.9	83.0
25	" alpha	9.1	65.7	26.2	46.9
26	"	2.8	28.0	21.4	52.5
27	"	3.6	37.8	40.5	72.2
28	"	3.2	33.6	25.4	55.3
29	Osteomalacia: on	18.6	34.4	17.6	19.0
30	" 1 alpha + Ca	2.9	36.2	51.0	91.4
31	"	11.7	77.0	24.9	78.1
32	"	9.1	65.7	26.2	54.3
33	"	3.0	27.3	6.5	25.9
34	Hypoparathyroid	9.7	5.3	23.8	48.9
35	"	16.9	27.4	15.9	48.3
36	"	6.0	1.8	19.7	45.4
37	"	4.1	14.0	22.1	54.9
38	"	9.6	21.3	32.5	94.2
39	"	17.0	12.1	14.7	49.5

TABLE 4.2. Patient group response to the calcium absorption test -summary of data from Table 4.1. ± SD.

Diagnosis	Case No.	25(OH)D ng/ml MEAN ± SD.	$1,25(OH)_2D$ pg/ml	Initial frac rate %	Maximal Ab %
Renal stones	1 - 4	10.9 ± 9.2	29.3 ± 9.2	25.4 ± 10.6	57.3 ± 21.1
Bartter's syndrome	5	19.5	40.1	42.2	78.0
Sarcoidosis	6	9.3	37.5	37.8	104.2
Myeloma	7	11.3	4.14	18.3	53.2
Osteoporotics	8 - 11	10.1 ± 8.8	17.2 ± 6.2	14.7 ± 9.6	44.0 ± 10.2
Osteomalacic on no treatment	12 - 15	5.2 ± 3.1	23.5 ± 9.5	18.4 ± 8.3	41.2 ± 14.4
Osteomalacic on D3 treatment	16 - 23	21.9 ± 11.6	48.1 ± 17.2	35.6 ± 17.4	64.3 ± 19.5
Osteomalacic on 1 alpha	24 - 28	5.1 ± 2.7	36.0 ± 8.7	34.7 ± 11.0	62.0 ± 15.1
Osteomalacic on 1 alpha + Ca	29 - 33	9.1 ± 6.6	48.1 ± 21.8	25.3 ± 16.4	53.7 ± 31.6
Hypoparathyroid	34 - 39	10.6 ± 5.4	13.7 ± 9.6	21.5 ± 6.4	56.9 ± 18.6

results agreed with those from the four untreated osteomalacic patients (cases 12 to 15). However, there were variations in the concentrations of the vitamin D metabolites. The osteomalacic patients had low concentrations of 25(OH)D and this was reflected in the corresponding low concentrations of $1,25(OH)_2D$. Concentrations of 25(OH)D in the osteoporotic patients were varied but $1,25(OH)_2D$ concentrations were below the normal reference range in all four patients.

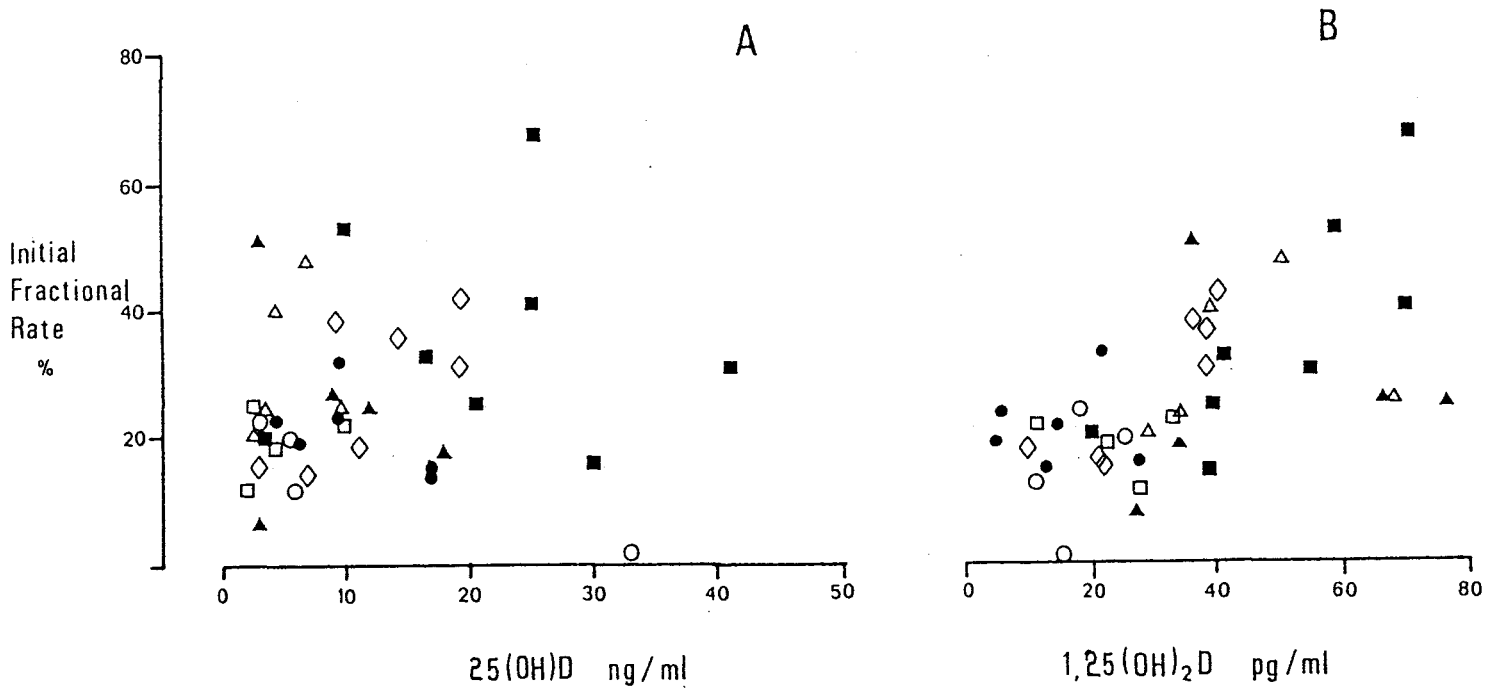
The eighteen osteomalacic patients on various vitamin D treatments prior to study (cases 16 to 33), produced statistically significant increases in their absorption rates and percentage absorption compared to their untreated counterparts. Values for the different vitamin D metabolites were varied especially between the osteomalacic patients on the alphacalcidiol treatment, either with or without calcium supplements. It must be emphasised that all medication was stopped on the day of the test and so therefore the last dose was the previous morning.

The six hypoparathyroid patients (cases 34 to 39) all had low concentrations of $1,25(OH)_2D$ although their 25(OH)D values were within the normal seasonal ranges. Initial fractional rates were also low but the maximal percentage of calcium absorbed after the six hours were within the normal range.

Values for total 25(OH)D and $1,25(OH)_2D$ concentrations were plotted against initial fractional absorption (figures 4.1a and 4.1b) and total percentage calcium absorption after 6h (figures 4.2a and 4.2b). There was a highly significant correlation between the initial absorption rate and plasma concentrations of the $1,25(OH)_2D$ metabolite [$r = 0.60$; $p<0.001$]. Total calcium absorbed also showed a significant correlation with this metabolite [$r = 0.56$; $p<0.01$]. The

FIGURE 4.1. The relationship between the initial rate of calcium absorbed and (A) 25(OH)D and (B) $1,25(OH)_2D$ concentrations in all groups.

□ - osteomalacic patients on no treatment; ○ - osteoporotic patients
■ - " " " D_3 " ; ● - patients with hypoparathyroidism
△ - " " " 1 alpha ; ◇ - others
▲ - " " " 1 alpha + Ca.



25(OH)D metabolite showed no correlation with either of the two absorption parameters. A closer relationship between $1,25(OH)_2D$ and initial fractional absorption [$r = 0.75$; $p<0.001$] was observed when patients on the alphacalcidiol medication were excluded (figure 4.3a). No such relationship was observed between the fractional absorption and the 25(OH)D metabolite when these patients were removed (figure 4.3b).

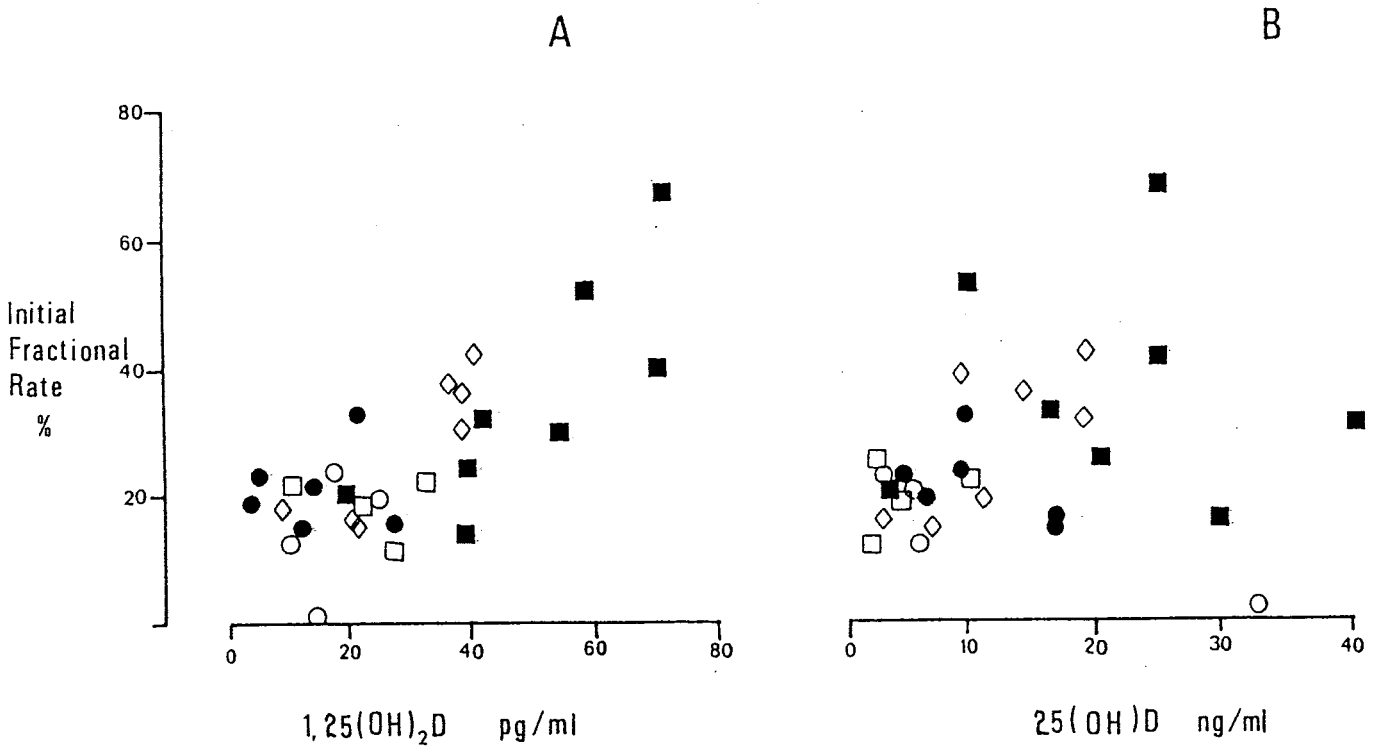
4.4 DISCUSSION:

The present finding that plasma concentrations of $1,25(OH)_2D$ correlate positively with the initial fractional calcium absorption rates in a random group of individuals is consistent with the strong belief that this vitamin D metabolite is an important physiological regulator of intestinal calcium absorption. This was further supported by the lack of correlation between either of the measured parameters of calcium absorption and the 25(OH)D metabolite. Previous studies have demonstrated a similar relationship, however, data was somewhat restricted to either normal individuals (Gallagher et al, 1979) or patients with primary hyperparathyroidism (Kaplan et al, 1977). This study is the first to demonstrate such a significant correlation within a wide range of patients, both with and without disorders relating to the vitamin D endocrine system.

The relationship between initial calcium absorption and circulating concentrations of $1,25(OH)_2D$ was partially masked when data from osteomalacic patients on the alphacalcidiol treatment were included. The $1,25(OH)_2D$ analogue, alphacalcidiol, has a half life ($t_{\frac{1}{2}}$) of approximately 3 hours, although it varies from patient to patient. Since all medication was stopped on the day of the test, the effect of the last dose (the previous day's) would

FIGURE 4.3. The relationship between the initial rate of calcium absorbed and (A) $1,25(OH)_2D$ and (B) 25(OH)D concentrations -omitting patients on 1 alpha medication.

□ - osteomalacic patients on no treatment; ○ - osteoporotic patients
■ - " " " D_3 " ; ● - patients with hypoparathyroidism
◇ - others



be receding at the time of the study. This was clearly demonstrated by the wide diversity of $1,25(OH)_2D$ values measured in these patients. However, it is difficult to specify on the time period to which the $1,25(OH)_2D$ will stimulate intestinal calcium absorption; whether it would be a matter of minutes or hours after the alphacalcidiol dose. Clearly the increased ability of the intestine to absorb calcium in these patients must be due to the medication since osteomalacic patients on no treatment demonstrated notably reduced absorption values. In fact, these values were similar to those observed for the three osteoporotic patients.

Although only four patients in this study had osteoporosis they all demonstrated low intestinal calcium absorption. Previous reports have shown that malabsorption of calcium is a risk factor for the development of osteoporosis (Gallagher et al, 1979; Nordin et al, 1985). It has been suggested that this could be due to one of two factors. Either that circulating $1,25(OH)_2D$ concentrations were low in osteoporotic patients and therefore contributed to the malabsorption of calcium or that concentrations of $1,25(OH)_2D$ were normal but the primary problem was an absorptive defect in the gut. The former would comply with this present study. In these patients osteoporosis was due to a primary deficiency in the $1,25(OH)_2D$ metabolite which, in turn, caused diminished calcium absorption and eventually the negative calcium balance so often seen in this disorder.

There was a definite tendency for reduced initial absorption rates in the six hypoparathyroid patients. However, in each case, this retarded start was compensated for by an increased rate of absorption later. The reduced initial rates corresponded to the low $1,25(OH)_2D$ concentrations in these patients and would suggest, therefore, that the hypoparathyroid state somehow adjusts to

the low values of $1,25(OH)_2D$ and by doing so alters the absorptive pattern for calcium uptake in the gut. Previous studies by Haussler et al (1976) also demonstrated reduced calcium absorption rates in hypoparathyroid patients.

The remaining patients, those with renal stones, sarcoidosis, myeloma and Bartter's syndrome were chosen as control subjects. The stone formers as well as the Bartter's patient were selected because their diseases did not relate to the vitamin D endocrine system.

The patient with sarcoidosis represented the hypercalcaemic state. Hypercalcaemia in this case was due to the excessive extrarenal production of $1,25(OH)_2D$, the source of the which was unknown. This patient was on prednisone therapy before the calcium absorption test was performed. Prednisone is known to lower plasma $1,25(OH)_2D$ concentrations and therefore corrects hypercalcaemia (Bell et al, 1979). This would explain the normal value attained for $1,25(OH)_2D$. Thus, it is possible that the regression of hypercalcaemia during prednisone therapy in this patient may have occured because of a reduction in plasma $1,25(OH)_2D$ concentration as well as a decrease in intestinal calcium absorption.

Finally, the myeloma patient again represented the hypercalcaemic state but unlike the sarcoid patient, hypercalcaemia was due to factors, such as PTH or PTH-like factors, which caused total bone breakdown. The extremly low $1,25(OH)_2D$ value in this patient would suggest that PTH-like factors, rather than PTH itself, were the sole instigators of the raised calcium.

In conclusion, this study has demonstrated that the double isotope procedure for measuring intestinal calcium absorption is suitable for most clinical studies, in

particular when required to investigate malabsorption in individuals. With the use of this technique the study has clearly demonstrated a close relationship between intestinal calcium absorption and the $1,25(OH)_2D$ metabolite.

CHAPTER 5

TREATMENT OF SUBCLINICAL OSTEOMALACIA IN THE ELDERLY

5.1 INTRODUCTION:

Osteomalacia is defined as the defective mineralisation of the organic bone matrix. This is histologically identified in uncalcified bone as an excess of osteoid. The increase in osteoid will undoubtedly lead to an increase in both the amount of osteoid covering trabecular surfaces and the seam width of the osteoid. Osteomalacia is attributed to several factors including a fall in dietary intake of vitamin D, a reduction in the exposure to sunlight, malabsorption and the impairment of $1,25(OH)_2D$ synthesis as a result in the decline in renal function. All these predisposing factors are often seen in the elderly. The latter factor would suggest that hydroxyl metabolites or analogues would be more useful than the parent vitamin D_3 in correcting this vitamin D disturbance and therefore osteomalacia. There are a few synthetic vitamin D analogues which have been manufactured to treat various metabolic bone diseases. One such analogue is 1 alpha-hydroxyvitamin D, or alphacalcidiol, which by-passes the need for renal hydroxylation. However, Zerwekh et al (1974) demonstrated in rachitic chicks that alphacalcidiol must undergo 25-hydroxylation in the liver before exerting its biological effects.

Alphacalcidiol has been demonstrated to be similar to $1,25(OH)_2D$ in potency and clinical effectiveness, but is slightly slower-acting and has a longer duration of action in humans (Brickman et al, 1976). While its therapeutic use

in treating hypoparathyroidism and renal osteodystrophy has been documented (Russell et al, 1974; Davie et al, 1976), its benefit in the treatment of osteomalacia is unclear. The present study therefore investigates the use of this $1,25(OH)_2D$ analogue in the correction of osteomalacia in a group of elderly patients. It also compares these patients with control patients and patients on parent vitamin D3 in order to evaluate which treatment would be the more advantageous routine in healing subclinical osteomalacia.

5.2 PATIENTS AND METHODS:

The study was based on a group of 38 patients (mean age 78.8; range 46-91) who presented with subclinical osteomalacia over a period of 4 years. These patients, with histological evidence to confirm osteomalacia, were randomly allocated to one of four treatment programmes. The first group, consisting of nine patients, received 3000IU vitamin D_3 daily in the form of cod liver oil capsules. The second and third groups, both consisting of eleven patients, received daily dosage of 1ug alphacalcidol and alphacalcidiol plus calcium supplements (20mmol Ca^{2+}/day: Ossopan), respectively. The final group (7 patients) received placebo. Patients with cases of severe osteomalacia were randomised separately between groups 1-3. Blood was sampled before starting treatment and after one month for measurement of plasma calcium, albumin, phosphate, creatinine, alkaline phosphatase (refer to appendix 2) and vitamin D metabolites (see sections 2.2.1 and 2.2.2).

An iliac crest bone biopsy was performed after treatment for one month in order to assess the degree of healing of their osteomalacia (refer to appendix 1). The effect of treatment in improving intestinal calcium absorption was also investigated. A small number of patients from each

study group therefore consented to the in vivo calcium absorption test (section 2.2.9).

5.3 RESULTS:

Individual responses to treatment are outlined in appendix 5 and are shown in figures 5.1 and 5.2. The overall responses are summarised in Table 5.1.

During the one month study period none of the patients developed hypercalcaemia (figure 5.1), nor was there a deterioration of renal function. However, alkaline phosphatase activity remained elevated (figure 5.1) and in the control group values markedly increased after a month under investigation [$0.01 < p < 0.05$].

Patient compliance with treatment was satisfactory, as shown by the significant increases in circulating concentrations of $25(OH)D_3$ (for patients on vitamin D_3) and $1,25(OH)_2D$ (for patients on alphacalcidiol and alpha-calcidiol plus calcium). Only one patient was subsequently excluded from the study for not taking the prescribed tablets (see figure 5.2). Pretreatment values of 25(OH)D concentrations were below the normal age-matched reference range (refer to section 2.2.3) and did not show seasonal variation (figure 5.3). Those patients on vitamin D_3 treatment demonstrated significant increases in their 25(OH)D values [$p<0.001$] after a month of treatment. In contrast to the low pretreatment concentrations of 25(OH)D, basal $1,25(OH)_2D$ concentrations were varied, for all groups under investigation, but generally were within the lower end of the reference range (figure 5.2). $1,25(OH)_2D$ concentrations also increased significantly in patients on treatment. Values for the vitamin D metabolites were unchanged in the control group.

TABLE 5.1. Summary of biochemical and histomorphometric responses to treatment.

Variable	Treatment	Mean at Baseline (±SD)	Mean at 1 month (±SD)	Change	95% Confidence Interval	Significance P
Calcium (mmol/L)	Control	2.02 (0.19)	2.09 (0.15)	+0.07	-0.07 to +0.22	
	D_3	2.13 (0.27)	2.26 (0.08)	+0.13	-0.09 to +0.35	
	1 alpha	2.06 (0.25)	2.18 (0.13)	+0.12	-0.02 to +0.26	
	1 alpha + Ca	2.12 (0.23)	2.23 (0.16)	+0.11	-0.01 to +0.20	< 0.05
Creatinine (umol/L)	Control	147 (47)	144 (30)	-3	-47.9 to +41.9	
	D_3	103 (30)	111 (27)	+8	-6.5 to +22.7	
	1 alpha	124 (41)	122 (45)	-2	-18.1 to +13.4	
	1 alpha + Ca	93 (36)	97 (30)	+4	-12.8 to +21.0	
Alkaline Phosphatase (IU/L)	Control	423 (167)	722 (371)	+299	-309 to +906	
	D_3	538 (384)	713 (602)	+174	-81 to +430	
	1 alpha	751	970	+219	-405 to +843	
	1 alpha + Ca	449 (246)	440 (168)	-9	-149 to +132	
$25(OH)D_3$ (ng/ml)	Control	14.1 (8.7)	8.6 (4.3)	-5.5	-9.1 to +1.7	
	D_3	4.7 (1.6)	22.4 (8.0)	+17.7	+9.2 to +26.2	< 0.005
	1 alpha	4.6 (2.1)	7.4 (4.7)	+2.8	-3.6 to +9.2	
	1 alpha + Ca	3.6 (2.2)	5.8 (3.4)	+2.2	-1.8 to +6.3	

TABLE 5.1. Summary of biochemical and histomorphometric responses to treatment contd.

Variable	Treatment	Mean at Baseline (± SD)	Mean at 1 month (± SD)	Change	95% Confidence Interval	Significance P
$1,25(OH)_2D_3$ (pg/ml)	Control	14.3 (3.9)	8.6 (5.1)	-5.7	-12.2 to +3.4	
	D_3	18.2 (6.5)	50.9 (12.5)	+32.7	+16.1 to +49.4	< 0.005
	1 alpha	16.9 (5.8)	36.5 (7.5)	+19.6	+16.8 to +22.4	< 0.001
	1 alpha + Ca	12.0 (6.9)	46.3 (12.7)	+34.3	+16.6 to +51.9	< 0.005
Osteoid Area (OA)	Control	31.2 (18.9)	25.0 (18.1)	-6.3	+0.1 to -12.7	
	D_3	25.3 (16.2)	18.3 (10.0)	-7.0	+0.2 to -14.2	
	1 alpha	24.6 (16.9)	20.0 (17.1)	-4.6	+0.5 to -9.8	
	1 alpha + Ca	26.5 (18.6)	17.3 (11.8)	-9.2	-0.6 to -17.9	< 0.05
Osteoid Surface (OS)	Control	73.1 (29.1)	60.6 (37.2)	-12.5	+26.2 to -51.2	
	D_3	63.0 (27.9)	59.5 (23.9)	-3.5	+6.9 to -13.9	
	1 alpha	62.9 (28.9)	61.3 (27.9)	-1.6	+12.6 to -15.8	
	1 alpha + Ca	68.2 (32.0)	54.9 (27.4)	-13.3	-6.1 to -20.5	< 0.005
Osteoid Index (OI)	Control	0.41 (0.18)	0.38 (0.14)	-0.03	+0.10 to -0.07	
	D_3	0.38 (0.11)	0.29 (0.09)	-0.09	-0.01 to -0.16	< 0.05
	1 alpha	0.38 (0.12)	0.29 (0.14)	-0.09	-0.02 to -0.15	< 0.05
	1 alpha + Ca	0.36 (0.15)	0.30 (0.09)	-0.06	+0.02 to -0.16	

p values relate to significance on paired t-test

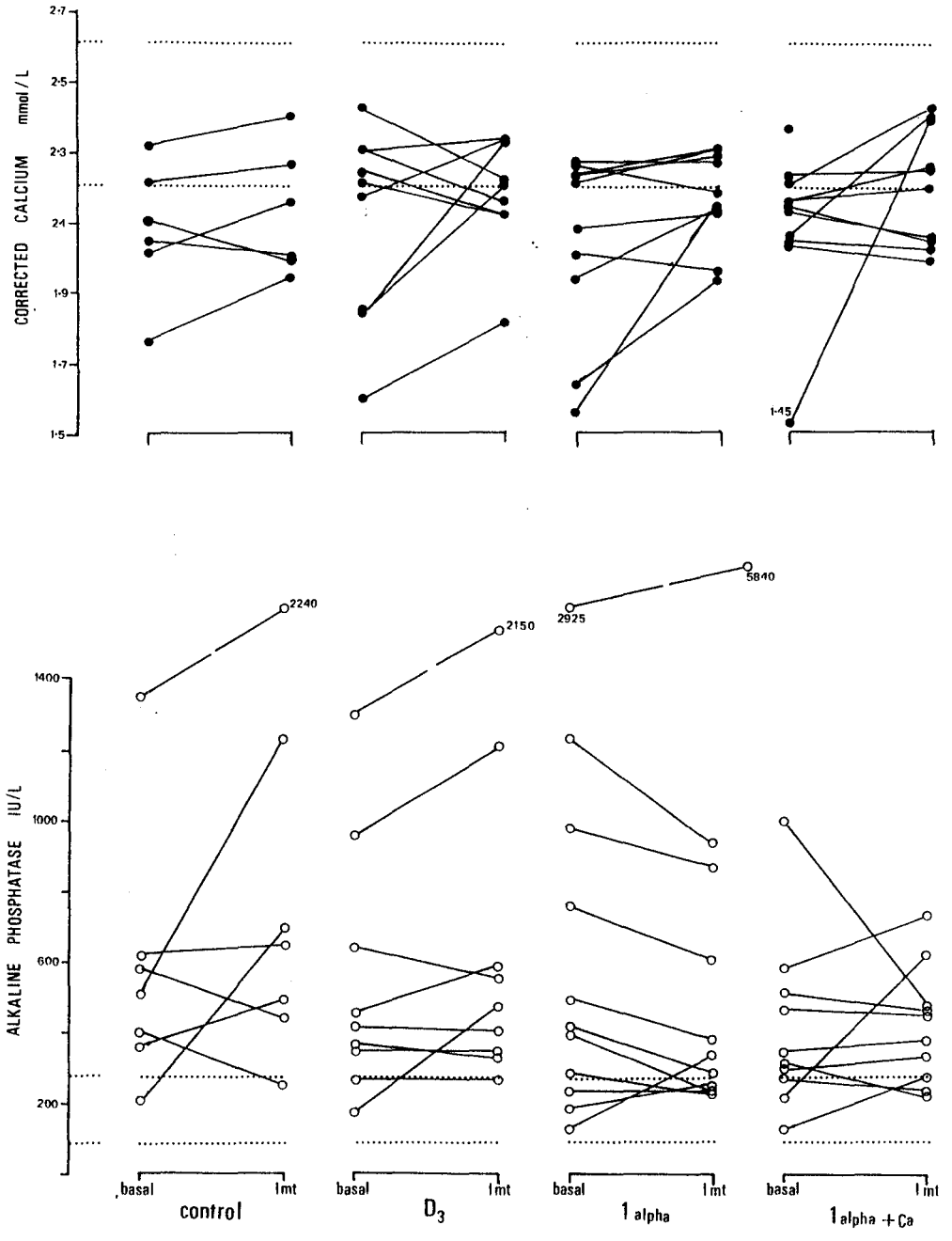


FIGURE 5.1. Change in plasma calcium concentrations and alkaline phosphatase activity after one month's treatment.

Dotted horizontal lines represent the normal range.

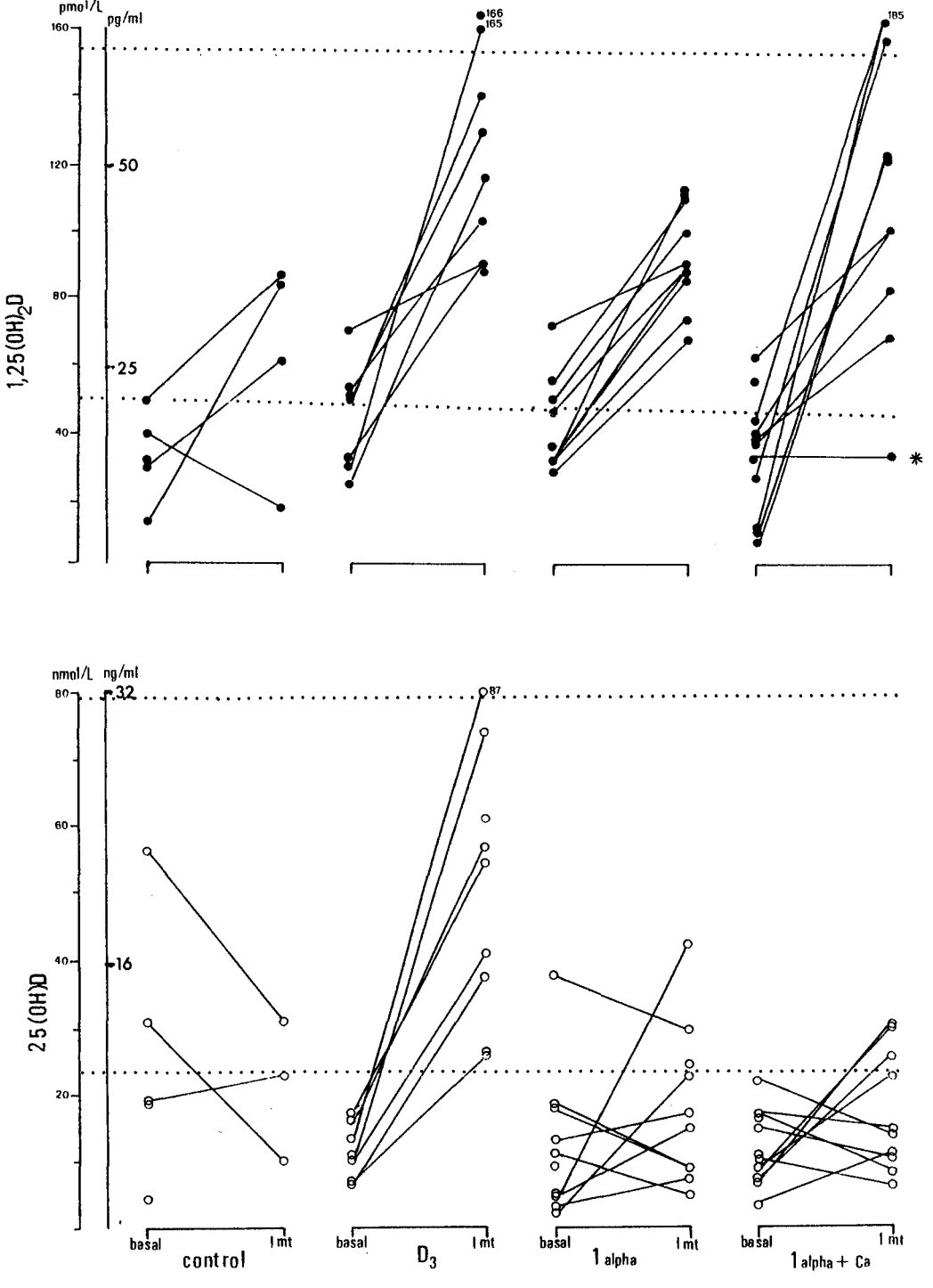
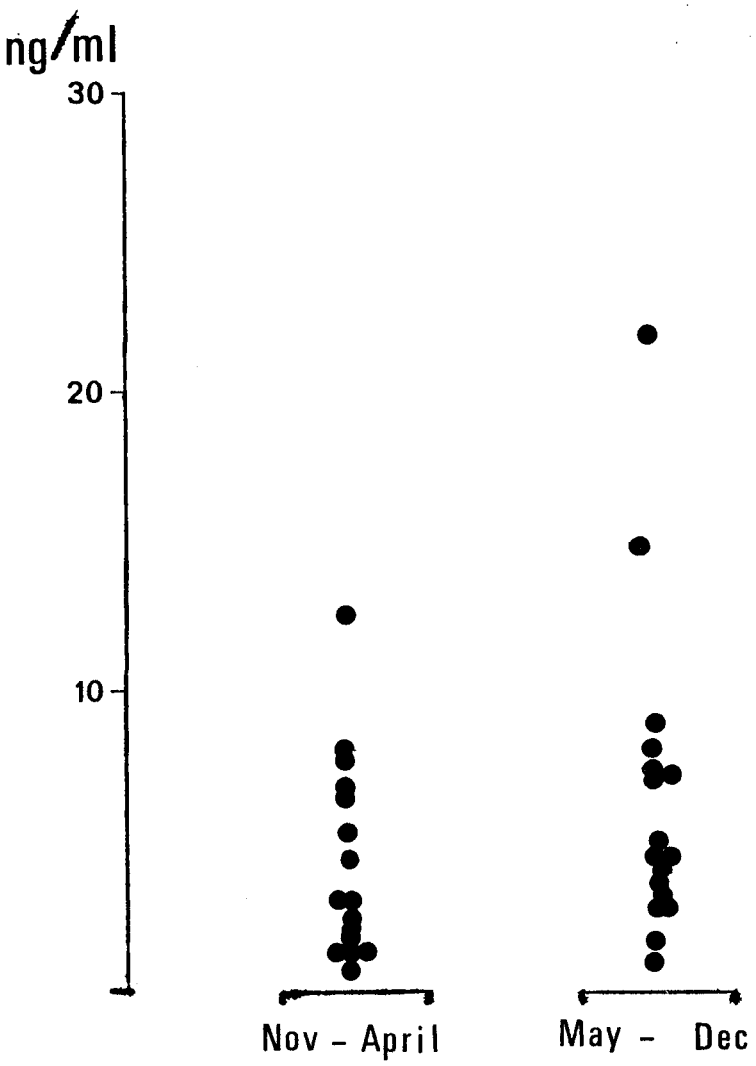


FIGURE 5.2. Change in 25(OH)D and $1,25(OH)_2D$ concentrations after one month's treatment.

Dotted horizontal lines represent the normal range.
*Patient omitted for not complying to treatment.

FIGURE 5.3. Plasma 25(OH)D concentrations from elderly osteomalacic patients sampled throughout the year.

Pretreatment values show no seasonal variation.



There were no statistically significant differences between the three treatment regimes with respect to changes in histomorphometric indices (figure 5.4 and Table 5.1). Osteoid area showed a decrease with all three treatments but this was statistically insignificant in each case. Only those patients treated with the alphacalcidiol plus calcium combination showed a significant decrease in surface extent of osteoid [$p<0.01$]. In contrast, those treated with either vitamin D_3 or alphacalcidiol alone showed a significant decrease in osteoid seam thickness [$p<0.05$ and 0.01, respectively]. There were no significant changes in the bone histology of the control patients during the one month study period (Table 5.1).

Individual responses to the in vivo calcium absorption test are shown in figure 5.5. The absolute values for calcium absorption in the pre-treatment patients were substantially lower compared with data obtained from patients on medication. However, all three treatment programmes showed statistically significant increases in absorption rates as well as similar trends in total percentage calcium absorption.

FIGURE 5.4. Changes in histomorphometric indices over 1 month.

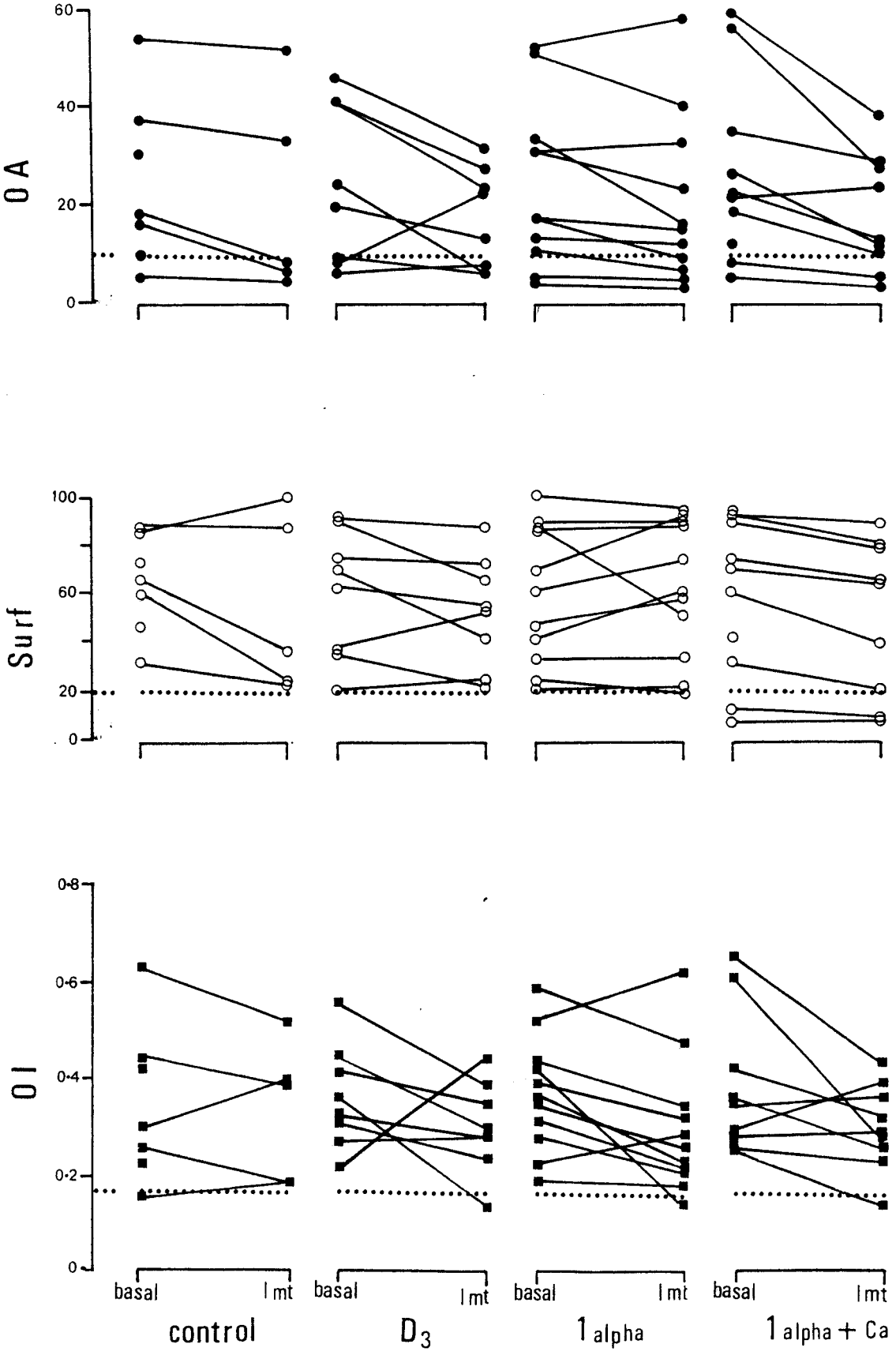
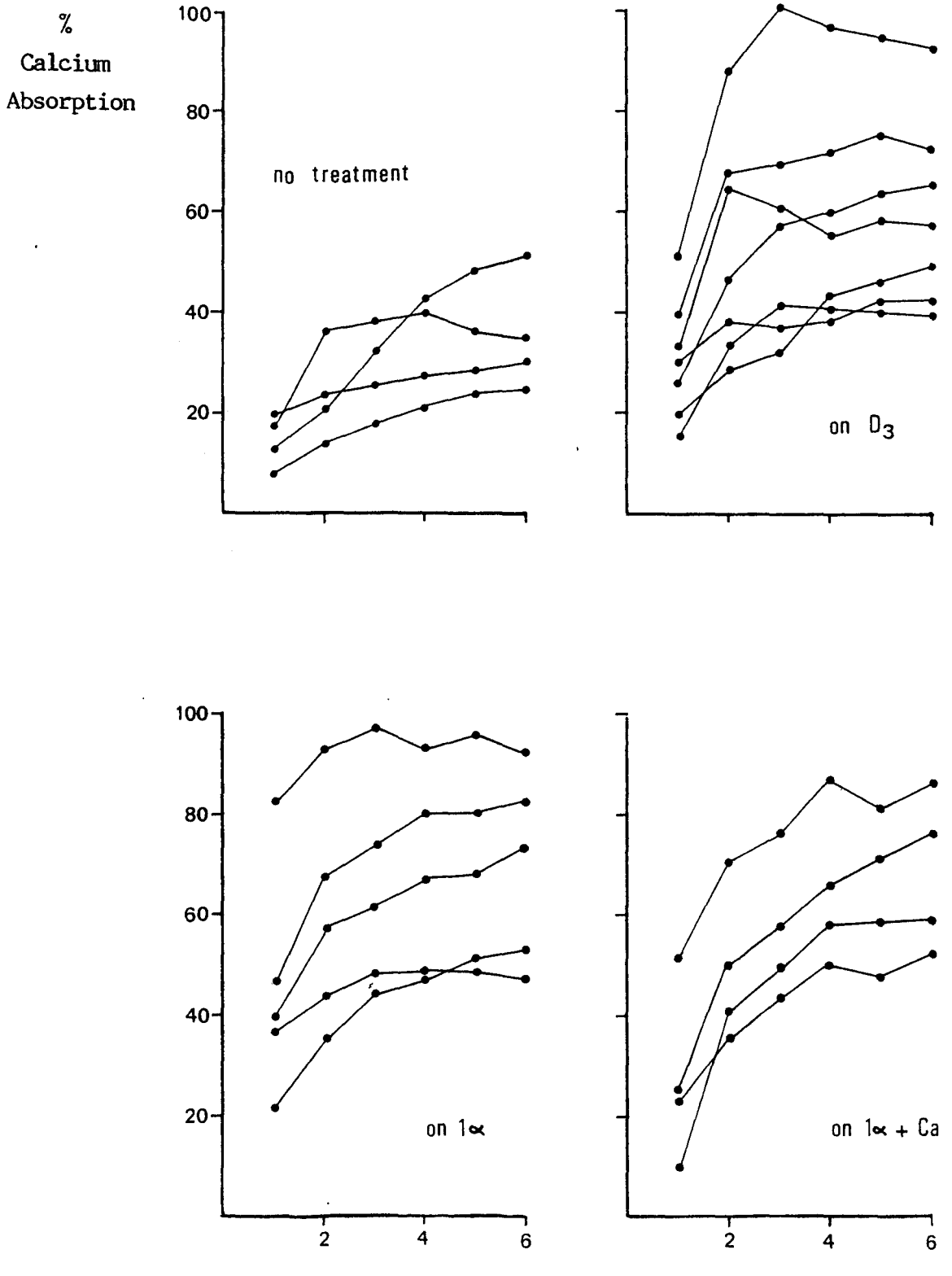


FIGURE 5.5. Individual patient response to the in vivo calcium absorption test.



5.4 DISCUSSION:

The high incidence of osteomalacia in the elderly has been documented in many studies (Hodkinson et al, 1973; Aaron et al, 1974) but many cases probably go unrecognised and untreated because the disease is often subclinical. The major aetiological factor associated with the high incidence of osteomalacia in the elderly is inadequate exposure to sunlight, possibly due to immobility in a population whose dietary intake of vitamin D is low. Many of these patients are either hospitalised for long-term geriatric care or are housebound due to a combination of chronic diseases. As well as these two factors which play a role in the development of osteomalacia others include intestinal malabsorption as well as a decline in renal function. Renal problems will undoubtedly impair the conversion of 25(OH)D to the active hormonal vitamin D metabolite $1,25(OH)_2D$. Once osteomalacia is identified treatment must be administered carefully and effectively.

The present study was devised to investigate the use of the synthetic derivative of vitamin D_3, alphacalcidiol, in the effective treatment of subclinical osteomalacia. Further, since calcium supplementation in the elderly has produced some degree of slowing of age-related bone loss, the use of this derivative with calcium supplements was also investigated. These actions were compared with those of parent vitamin D_3 which is conventionally used in the treatment of osteomalacia.

The study has confirmed that all three treatment regimes under investigation were safe to administer. No patient developed hypercalcaemia during the one month clinical trials and those on parent vitamin D_3 medication showed no indication of vitamin D intoxication. Hypercalcaemia is the primary hazard during therapy with any of the vitamin D

derivatives and may be associated with potentially serious complications, particularly in old age. It is well known that prolonged excessive intake of vitamin D can also result in deterioration in renal function as well as extra-osseous calcification, the latter appearing in many soft tissues including kidney, heart, lung and arterial walls (Parfitt et al, 1982). The mechanism by which this occurs is still not clear although it has been suggested that it is related to the enhanced intestinal calcium absorption and bone resorption which accompanies hypervitaminosis D. None of these symptoms were encountered during the term of the present investigation. However, had hypercalcaemia been induced by alphacalcidiol this would have easily be reversed by simply withdrawing the medication. Because of its short half life ($t_{\frac{1}{2}}$ = 3.4 $\pm$ 0.4 days) the toxic effect would have resolved relatively quickly. This does not hold true for the parent vitamin. Loss of toxic effects, and therefore hypercalcaemia, would exist for appreciably longer, probably between 17 to 60 days because of its slow release from storage tissues such as adipose and liver.

Basal alkaline phosphatase activities were invaribly raised in all patients indicating that osteomalacia was accompanied by high bone turnover. However, none of the treatment regimes was able to reduce these values significantly possibly due to the short period of investigation. In some of the patients on treatment there was an initial paradoxical increase in alkaline phosphatase activity after one month. This is not necessarily surprising since healing of bone may take several months to complete and will depend on the initial severity. Approximately 30% of patients were still hypocalcaemic after treatment for one month although it was evident in most of these patients that if treatment had been continued beyond one month they would have probably achieved the normocalcaemic state.

The plasma concentration of 25(OH)D is a valid index of vitamin D status in the body (Preece et al, 1975). The present study showed quite clearly that, compared with healthy adults, patients with osteomalacia were indeed vitamin D deficient. This statement holds true when sampling throughout the year. Basal $1,25(OH)_2D$ values were usually reduced in the osteomalacic patients or remained within the lower end of the normal reference range. That these values increased substantially after vitamin D therapy, either with alphacalcidiol or parent vitamin D itself, reinforces the general idea that the cause of osteomalacia was indeed a deficiency in the parent vitamin rather than a defect in renal hydroxylation.

The reduced vitamin D status in these patients also suggested a decreased absorption of calcium in the intestine. Calcium absorptive efficiency has been noted to decline with advancing age and has been linked with the fall in vitamin D status (Parfitt et al, 1982). Large fractions of the middle-aged and elderly population have low concentrations of the essential vitamin D metabolites (both 25(OH)D and $1,25(OH)_2D$). Thus, any further decline in these concentrations will result in a decline in absorptive efficiency. This has clearly been demonstrated here. Untreated control patients who consented to the in vivo calcium absorption test demonstrated reduced calcium absorption in the gut compared with their treated counterparts. Treatment with either of the vitamin D medications obviously led to a positive calcium balance as demonstrated by increases in intestinal calcium absorption at the time when plasma $1,25(OH)_2D$ concentrations were elevated. However, there were no substantial differences in the percentage calcium absorbed between patients on either of the vitamin D treatment regimes. The present observations therefore confirm that the vitamin D analogue is as

effective as parent vitamin D_3 in the stimulation of intestinal calcium absorption.

There was no evidence to suggest that any of the treatment regimes accelerates the rate of healing. Changes in bone histology showed a similar trend in all three treatments. There was considerable improvement in most patients with one exception, the patient on the alphacalcidiol treatment who did not take the medication. It was interesting to find that alphacalcidiol with the addition of calcium supplements showed no advantage over the analogue alone in the healing process. Previous studies (Hosking et al, 1983) demonstrated slight improvement in bone histology after three months of treatment with alphacalcidiol compared with vitamin D_3.

To conclude, in the treatment of osteomalacia both sterols (vitamin D_3 and alphacalcidiol) performed successfully, if not effectively, in bringing about the healing process. Both were equally capable of restoring calcium absorptive efficiency in the intestine after one month's treatment and thus the choice of vitamin D would appear arbitary. But, since parent vitamin D is considerably less expensive than alphacalcidiol it would seem reasonabe to suggest that this should be the treatment of choice. However, in cases where renal impairment is present it would be advisable to prescribe the $1,25(OH)_2D$ analogue. Whichever sterol is used it is advisable to monitor plasma calcium concentrations frequently and, if possible, the vitamin D metabolites at regular intervals.

CHAPTER 6

CHANGES IN OSTEOCALCIN AND VITAMIN D METABOLITES AFTER FRACTURE OF THE FEMORAL NECK

6.1 INTRODUCTION:

The presence of osteomalacia complicating femoral neck fractures is now less frequently seen. In a recent large survey of 422 femoral neck fracture cases only 2% had underlying osteomalacia (Wilton et al, 1987a). However, it is still an important criterion to identify because of the effect it has on fracture fixation and patient mobilisation. To date there is no biochemical marker which is able to distinguish osteomalacia from the almost inevitable osteoporosis in this fracture group and, thus, the only means of successfully identifying every case of osteomalacia, however mild, is to perform an iliac crest bone biopsy on all femoral neck fracture patients. Wilton et al (1987b) recently suggested a means of detecting all the severe cases of osteomalacia by employing the combination of hypocalcaemia and a raised alkaline phosphatase. However, this combination has a high false positive rate since alkaline phosphatase, the conventional marker used in determining bone turnover, originates not only from bone but also from other sources such as the liver. Furthermore, alkaline phosphatase is often raised in non-osteomalacic elderly patients both with and without fracture (Wilton et al, 1987b, Campbell et al, 1984). Thus, this marker gives a less specific estimation of bone formation.

Osteocalcin (BGP, bone GLA protein), the major non-collagenous protein of bone matrix, is produced solely by osteoblasts and has been shown to reflect directly the level

of new bone formation (Lian and Gundberg, 1988). The synthesis of this vitamim K-dependent bone protein, found circulating in plasma, is subject to stimulation by $1,25(OH)_2D$ (Price, 1980). Although its precise biological function in bone is still unclear, it has been found to be a sensitive indicator of bone turnover in patients with a variety of metabolic bone diseases.

The recent availability of radioimmunoassays for osteocalcin raises the possibility that it may be a more specific screening test for osteomalacia than measuring serum alkaline phosphatase activity. However, the main advantage of measuring serum alkaline phosphatase in this instance is that its activity changes little during the first week after a fracture (Hosking, 1978) and therefore reflects the 'pre-fracture' state. No such information about changes in concentration during the immediate post fracture period is available for osteocalcin. This chapter describes changes in osteocalcin concentrations in the first week following a femoral neck fracture and its fixation. It also examines its relationship with alkaline phosphatase, the vitamin D metabolites and bone histomorphometry. Indeed, this is a necessary step before attempting to assess its value, if any, in the screening of subclinical osteomalacia complicating femoral neck fracture.

6.2 PATIENTS AND METHODS:

The study comprised 30 patients (3 males and 27 females; mean age 78yrs; range 58-92 yrs) who had sustained a traumatic fracture of the femoral neck. The criterion for entry into this study was that perioperative investigations had excluded osteomalacia. All had osteoporosis. None were taking calcium or vitamin D supplements before or during the study. Fasting blood samples were taken preoperatively from

all patients (within 24h of the fracture), and repeated one week later, by which time all fractures had been treated by internal fixation. All blood samples were collected into lithium heparin and the plasma stored at -20°C until assay.

Plasma calcium, phosphate, creatinine, albumin concentrations and alkaline phosphatase activity were measured by standard laboratory techniques and calcium concentrations corrected to a plasma albumin level of 40g/L (appendix 2). Plasma osteocalcin and PTH concentrations were measured by radioimmunoassay (sections 2.2.6. and 2.2.8., respectively) and the vitamin D metabolites by competitive protein binding assays (see appropriate sections). DBP concentrations were measured by radial immunodiffusion (section 2.3.5). Free 25(OH)D and $1,25(OH)_2D$ indices were calculated as the molar ratio 25(OH)D/DBP x 10^3 and the molar ratio $1,25(OH)_2D$/DBP x 10^5, respectively. These molar ratios give good estimations of free vitamin D concentrations (Lips et al, 1985). Iliac crest bone biopsies were performed perioperatively on all patients to exclude the presence of metabolic bone diseases other than osteoporosis. Measured histomorphometric indices were as described in appendix 1.

For comparative purposes, an additional 9 patients (1 male and 8 females; mean age 81yrs, range 74-89) who fulfilled the criterion for entry into this study, were included. However, these patients had raised alkaline phosphatase activity on admission and were only studied once; within 24 hours of their fracture.

Reference values for osteocalcin, vitamin D metabolites and binding protein were derived from a group of age- and sex-matched normal individuals (8 males and 13 females; mean age 70yrs; range 60-81). Both patients and control subjects were studied throughout the year.

6.3 RESULTS:

Individual values of plasma biochemistry and histomorphometric indices are outlined in appendix 6 and summerised in Table 6.1. Changes in plasma calcium, phosphate, alkaline phosphatase and creatinine in the first week after a femoral fracture are shown in figure 6.1. Only four patients had raised alkaline phosphatase activity on admission, and in three cases other liver function tests were abnormal (gamma GT = 135, 224 and 294 IU/L: normal range 11-51 IU/L). Alkaline phosphatase activity in the majority of patients increased during the first week after the fracture but this difference did not reach a statistical significance [p = 0.097]. While forty-five percent of patients were hypocalcaemic at some stage of this study, concentrations of phosphate and creatinine generally remained within the age-matched reference range (figure 6.1).

Figure 6.2a shows individual changes in osteocalcin, DBP and PTH concentrations, whilst those for free and total 25(OH)D and $1,25(OH)_2D$ are depicted in figure 6.2b. PTH concentrations were not measured in all patients, but in cases where they were, values remained either undetectable or at the lower end of the reference range throughout this study. Over half the patients had admission values of total and free 25(OH)D and DBP concentrations below the reference ranges, although both free and total $1,25(OH)_2D$ were less commonly abnormal. All the initial concentrations of osteocalcin lay below the upper limit of the reference range, even in those selected because of a raised alkaline phosphatase (refer to table in appendix 6). Moreover, the increments in osteocalcin which occured within the first week after the fracture also lay below this limit with only one exception. In contrast, of the 22 patients with a normal

TABLE 6.1. Changes in plasma biochemistry after fracture of the femoral neck.

PARAMETER	FEMORAL NECK FRACTURE (n = 30) Basal	1 week	REFERENCE RANGE
	mean + 2SD		
Calcium (mmol/L)	2.24 (± 0.14)	2.27 (± 0.14)	2.2-2.6
Phosphate (mmol/L)	1.21 (± 0.32)	1.11 (± 0.18)	0.8-1.4
Alkaline Phosphatase (IU/L)	208.5 (± 143)	269.5 (± 184)	98-280
Creatinine (umol/L)	96.9 (± 19.4)	104.8 (± 45)	60-120
Osteocalcin (ng/ml)	3.07 (± 1.8)	3.74 (± 1.9)*	3.2-8.1
Vitamin DBP (mg/L)	295 (± 65.3)	347 (± 77)*	284-373
Total 25(OH)D (ng/ml)	6.1 (± 5.5)	4.3 (± 2.6)	6.0-17.8
Total $1,25(OH)_2D$ (pg/ml)	16.5 (± 12.9)	18.3 (± 8.8)	11.2-30.1
Free 25(OH)D index ($\times 10^3$)	2.32 (± 1.37)	2.02 (± 1.5)	2.57-9.45
Free $1,25(OH)_2D$ index ($\times 10^5$)	0.78 (± 0.4)	0.77 (± 0.46)	0.44-1.28

Test of significance between basal and one week values

* $p < 0.01$

FIGURE 6.1. Changes in plasma calcium, phosphate, creatinine and alkaline phosphatase in the first week after femoral neck fracture and its fixation.

Normal ranges (mean ± 2SD) are shown within the dashed lines.

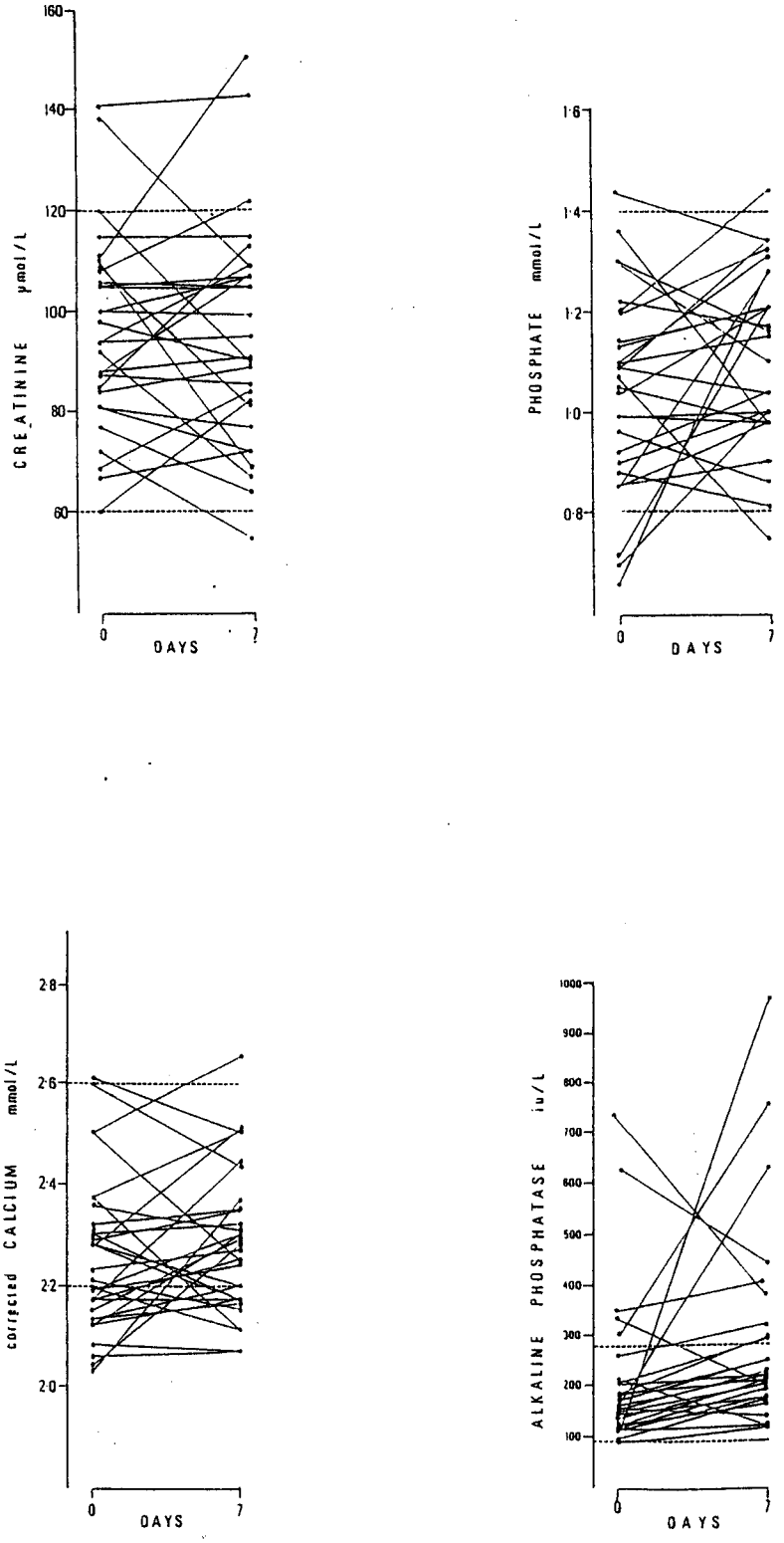


FIGURE 6.2a. Changes in osteocalcin, DBP and PTH concentrations in the first week after femoral neck fracture and its fixation.

Normal ranges (mean ± 2SD) for osteocalcin and DBP are shown within the dashed lines. PTH concentrations were only measured in 14 patients, of which half had values below the sensitivity of the assay (dashed line).

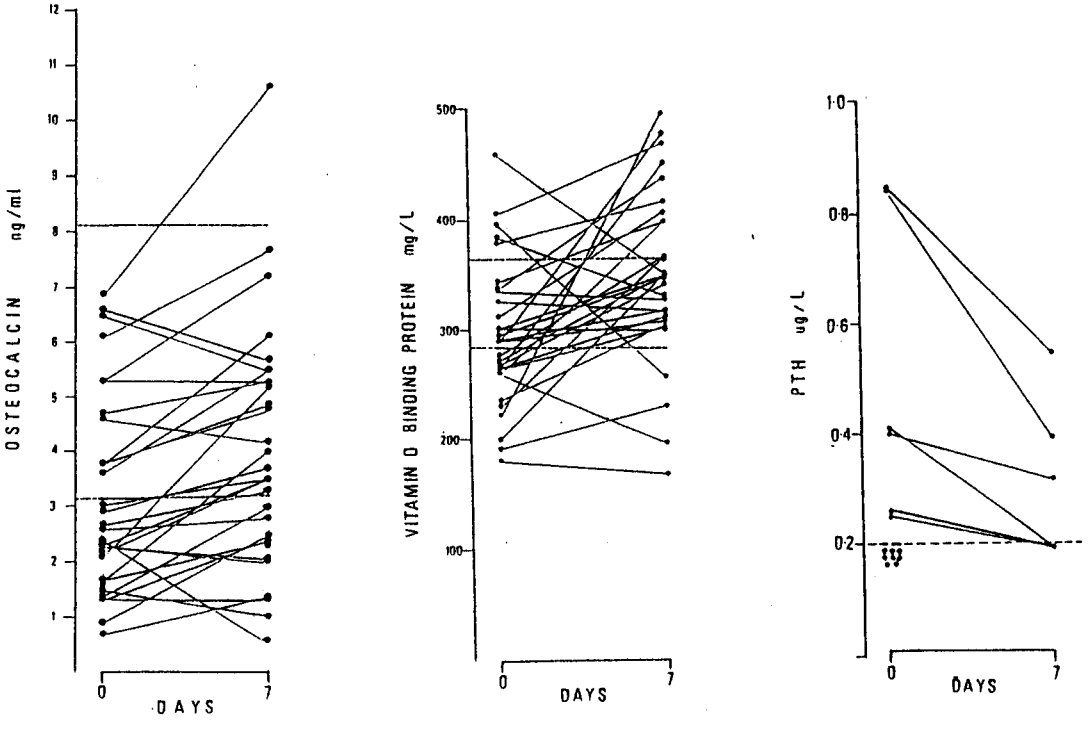
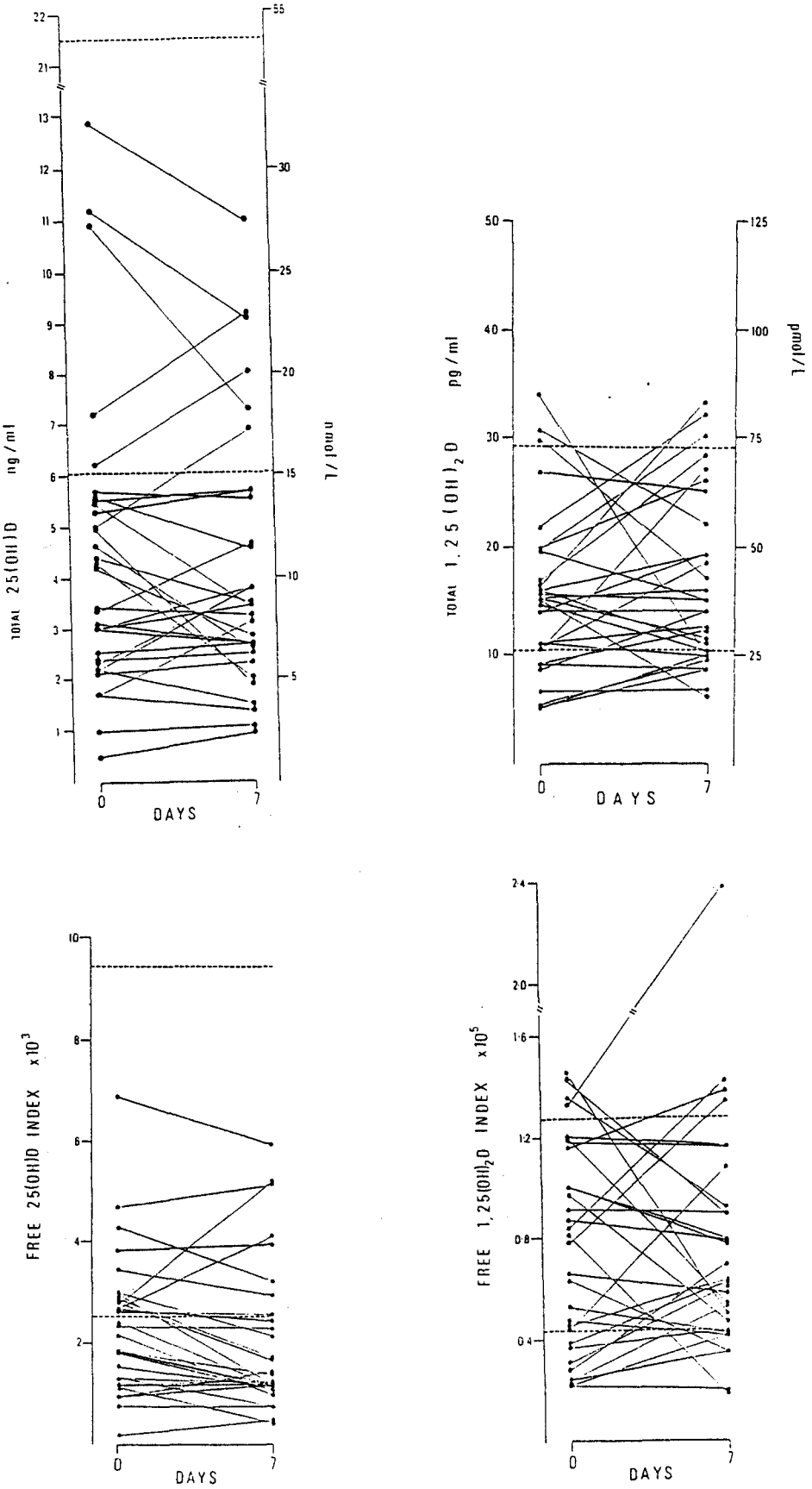


FIGURE 6.2b. Changes in both total and free vitamin D metabolites in the first week after femoral neck fracture and its fixation.

Normal ranges (mean ± 2SD) are shown within the dashed lines.



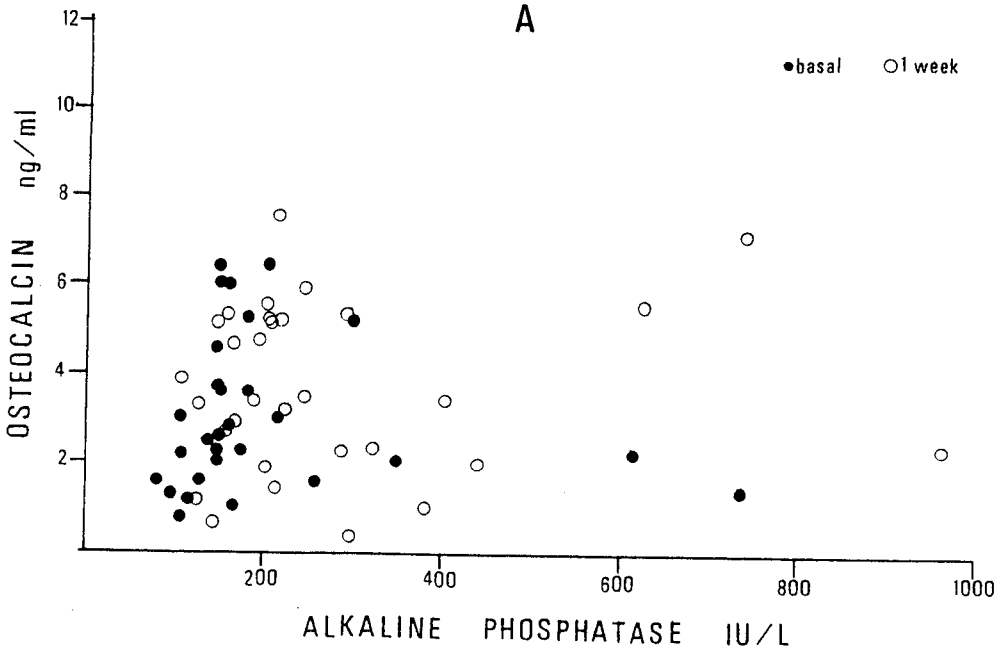
alkaline phosphatase activity on admission, five rose to a level above the upper limit of normal after one week.

Of all parameters measured only the concentrations of osteocalcin and DBP increased significantly within the week following fracture and its fixation [$p = 0.0039$ and 0.0022, respectively]. There were no significant changes in the vitamin D metabolite concentrations, neither free nor total.

Basal values of osteocalcin concentrations did not show a significant correlation with alkaline phosphatase (figure 6.3a), DBP or with total or free values of the vitamin D metabolites. Figure 6.3b illustrates the lack of relationship between osteocalcin and total $1,25(OH)_2D$. However, there were strong correlations [$p<0.001$] between the changes in osteocalcin concentration and those of total and free $1,25(OH)_2D$ (figure 6.4). No such relationship was observed with respect to 25(OH)D (figure 6.5), DBP or alkaline phosphatase activity. Neither was there a relationship between alkaline phosphatase and $1,25(OH)_2D$.

There was no statisticallly significant correlation between the admission concentrations of osteocalcin or alkaline phosphatase and the surface extent of osteoid, an index of bone formation (figure 6.6) or with osteoid area or osteoid seam thickness. This is also true when values from the nine osteoporotics with elevated alkaline phosphatase activity were included. This seemed to be due to some patients with apparently adequate bone turnover, as judged by the extent of osteoid surfaces, having low values of osteocalcin (figure 6.6). However, when these patients were stratified according to the prevailing level of total $1,25(OH)_2D$, the low osteocalcin concentrations appeared to be associated with very low concentrations of this vitamin D metabolite (figure 6.7).

FIGURE 6.3. Relationship between osteocalcin concentrations and (a) alkaline phosphatase activity and (b) total $1,25(OH)_2D$ concentrations.



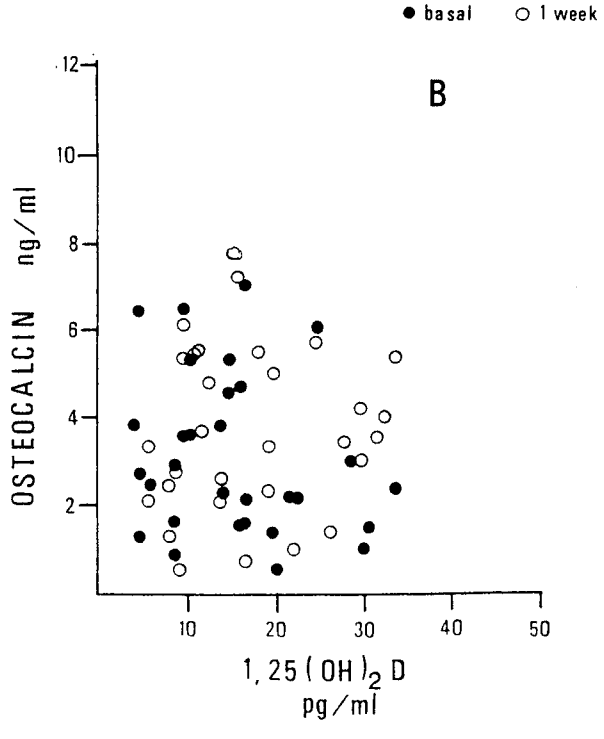


FIGURE 6.4. Correlation between the changes in osteocalcin and the changes in (a) total and (b) free $1,25(OH)_2D$ concentrations in the first week after femoral neck fracture.

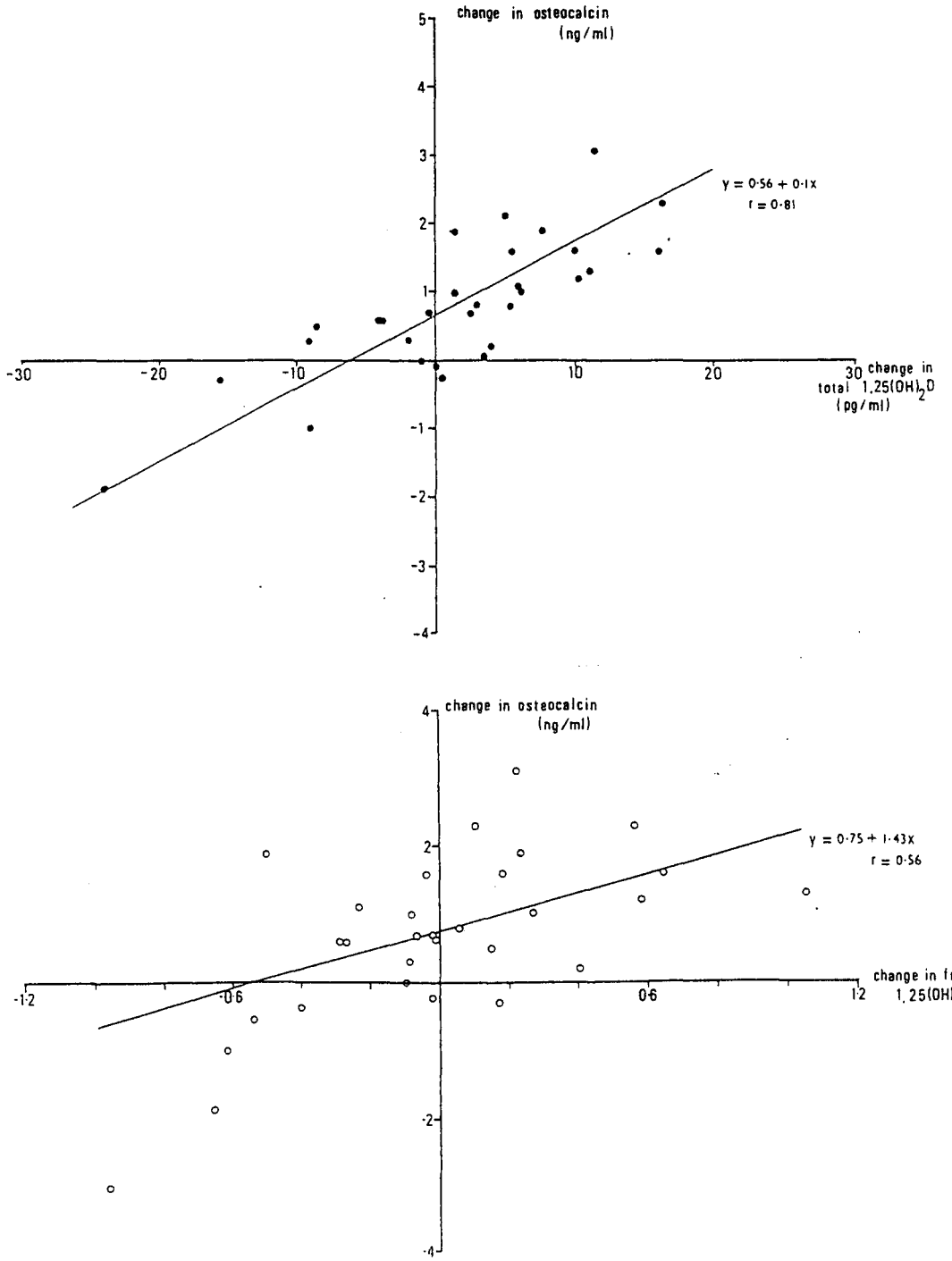


FIGURE 6.5. Relationship between the changes in osteocalcin and total 25(OH)D concentrations in the first week after femoral neck fracture.

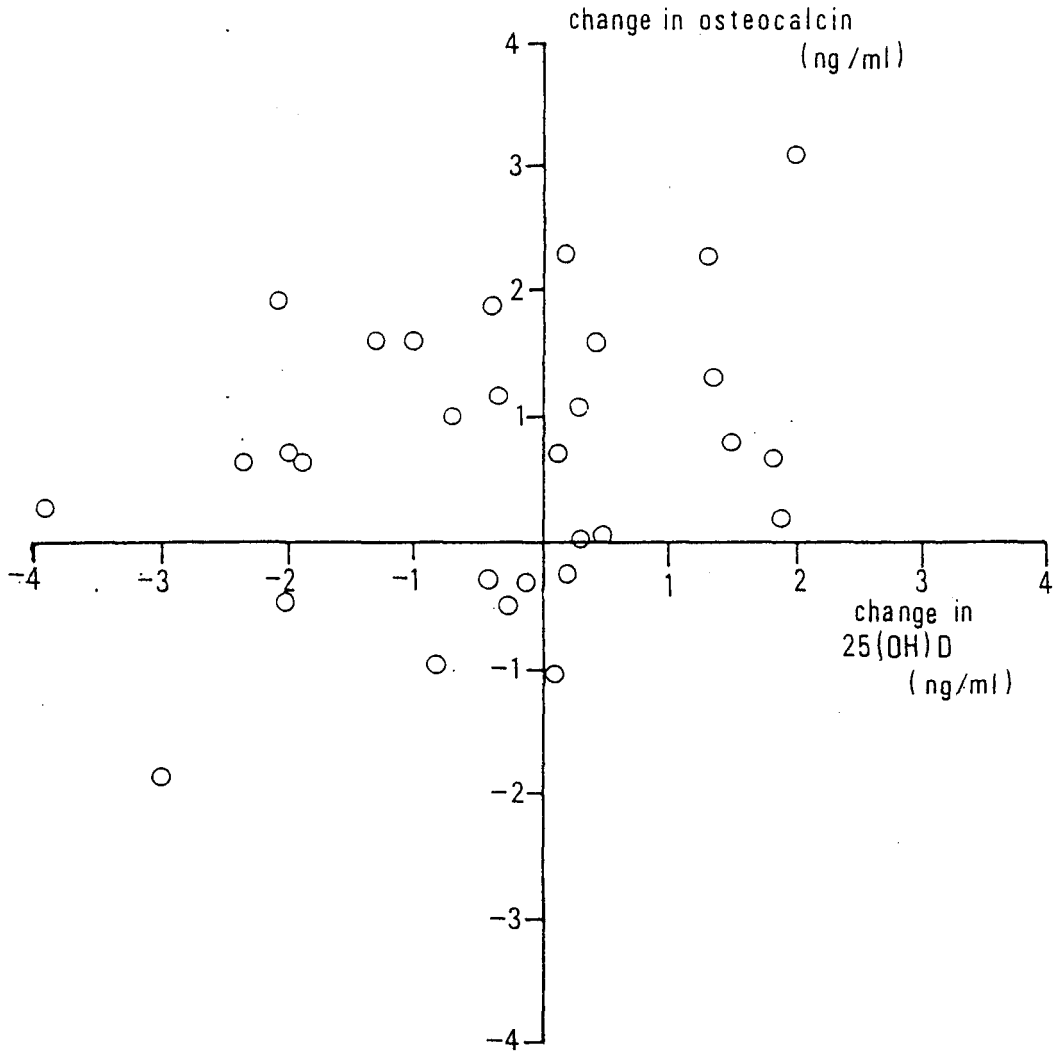


FIGURE 6.6. Plasma alkaline phosphatase activity and osteocalcin concentrations in relation to surface extent of osteoid in osteoporotic patients with femoral neck fractures.

The horizontal dotted lines indicate the references ranges for alkaline phosphatase and osteocalcin. □ represents the nine osteoporotics with elevated alkaline phosphatase activity.

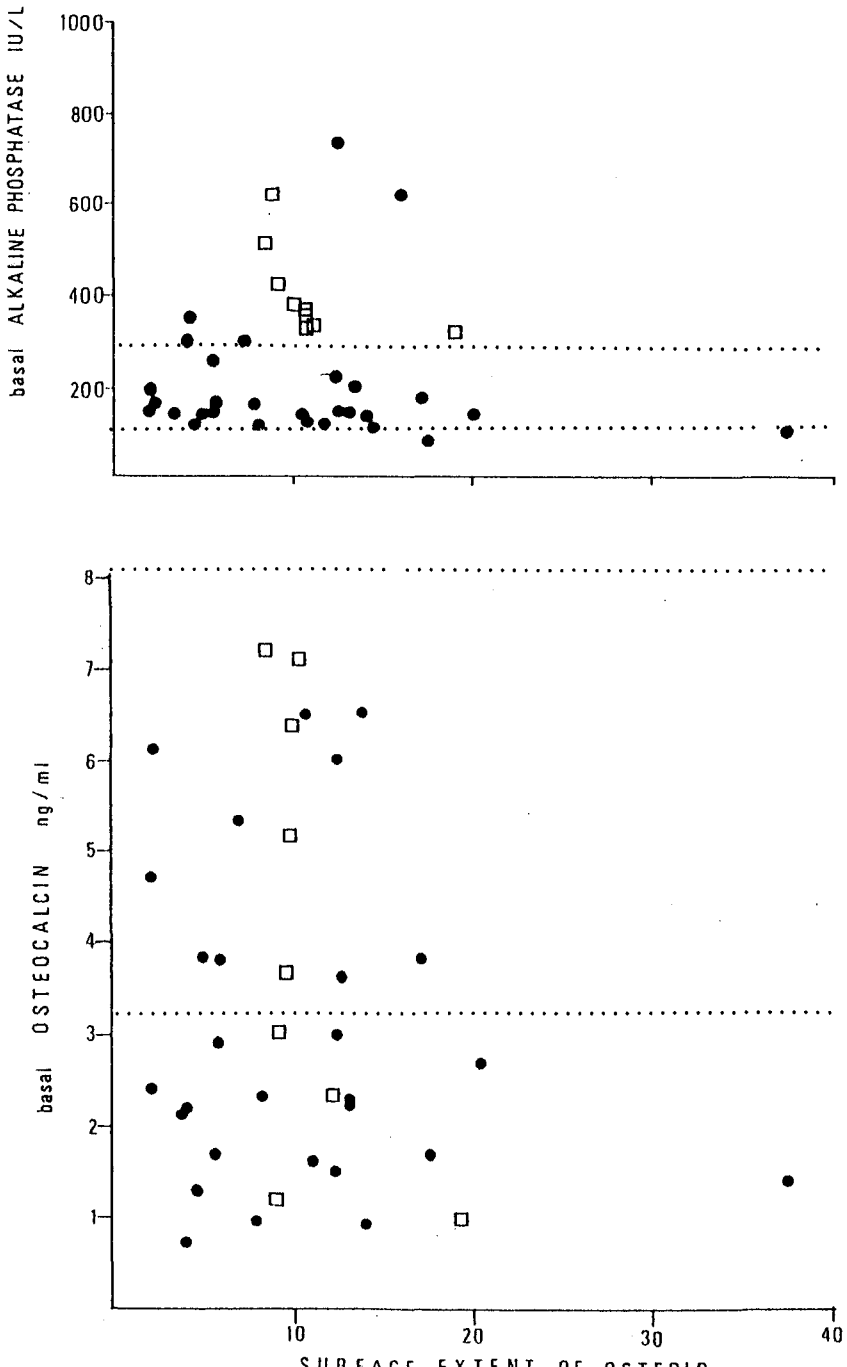
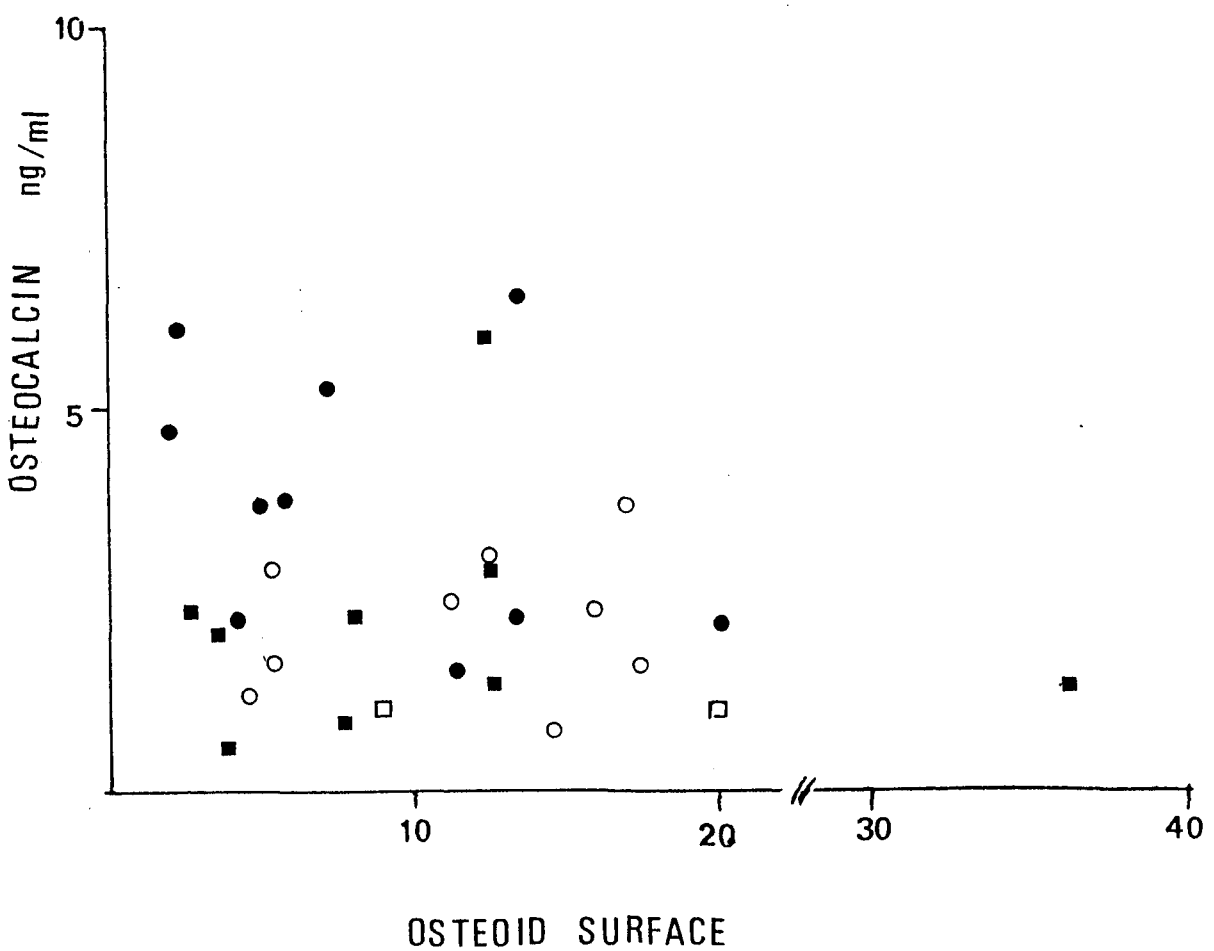


FIGURE 6.7. Plasma osteocalcin concentrations in relation to surface extent of osteoid in osteoporotic patients with with FNF.

$1,25(OH)_2D$: ○ represent values < 10pg/ml; ● 10-20pg/ml; ■ values > 20pg/ml.

□ represent values from osteoporotic patients with elevated alkaline phosphatase activity. Only two patients had their $1,25(OH)_2D$ concentrations measured and both were < 10pg/ml.



6.4 DISCUSSION

Osteocalcin, the vitamin K-dependent protein of bone, is synthesised by osteoblasts (Price et al, 1981). Previous studies have shown it to be a noninvasive specific marker of bone formation in various metabolic bone diseases (Lian and Gundberg, 1988). It may be a more specific marker than alkaline phosphatase because the latter originates not only from bone, but also from liver, kidney and placenta. There is a tendancy to find raised alkaline phosphatase values in the elderly, particularly in those with no evidence of bone disease. This is probably attributable to mild liver dysfunction.

In the present study of very elderly patients with femoral neck fractures neither osteocalcin concentrations nor alkaline phosphatase activity correlated with measured histomorphometric parameters of bone formation. Previous studies of younger women with osteoporosis (Price et al, 1980b; Brown et al, 1984) demonstrated a correlation between osteocalcin and bone formation. Our patients were much older than those in previous studies but since osteocalcin concentration tends to rise with age (Delmas et al, 1983a) this cannot be an explanation for these findings. The problem seemed to be that a significant proportion of these elderly patients with bone turnover towards the upper part of the reference range had low concentrations of osteocalcin. A number of possible explanations for this observations need considering.

Plasma osteocalcin has a short half life (Price et al, 1981) and may be depressed by the stress associated with a fracture. A similar mechanism has been proposed for the reversible depression of DBP (Lips et al, 1985), which was also demonstrated in this present study. However, the lack of a correlation between these two variables in the week

after the fracture argues against a common mechanism. It is possible that stress might depress osteocalcin through adrenal stimulation since such an effect has been reported with the use of glucocorticoids (Lukert et al, 1986). Unfortunately plasma cortisol was not measured in these patients, but is likely to be increased.

Although the precise biological function of osteocalcin is still unclear it may play a role in bone mineral deposition (Lian and Gundgerg, 1988). No function has been assigned to osteocalcin in the circulation and therefore, any increase in plasma concentration probably reflects the portion of newly synthesised protein not bound to the mineral phase of bone (Price and Nishimoto, 1980). With the significant rise in osteocalcin concentrations during the one week study period one could speculate that this rise is a direct effect of the fracture. Even though osteocalcin values increased they remained within the lower end of the reference range. This is in contrast to the report by Slovik et al (1984) who found elevated values in a group of hip fracture patients who had undergone similar operative treatment. However, the timing of blood sampling was not given and it may be that the elevated osteocalcin concentrations reflected a later phase of callus formation. Another report, from Borsalino et al (1985), also demonstrated contrasting data. They showed that osteocalcin concentrations reduced significantly in the first five days after orthopaedic surgical operations but again sampling time after the fracture was not specified.

The concentrations of osteocalcin tended to be associated with very low concentrations of $1,25(OH)_2D$ (<10pg/ml). That $1,25(OH)_2D$ may be an important factor is supported by the further observations that although there was no correlation between the absolute concentrations of osteocalcin and $1,25(OH)_2D$, there was a relationship between

the changes in these variables in the first week after the fracture. This is consistent with the observation that while osteoblast production of osteocalcin is stimulated by $1,25(OH)_2D$ in cell culture (Price and Baukol, 1980; Silve et al, 1986), it does not appear to be vitamin D dependent.

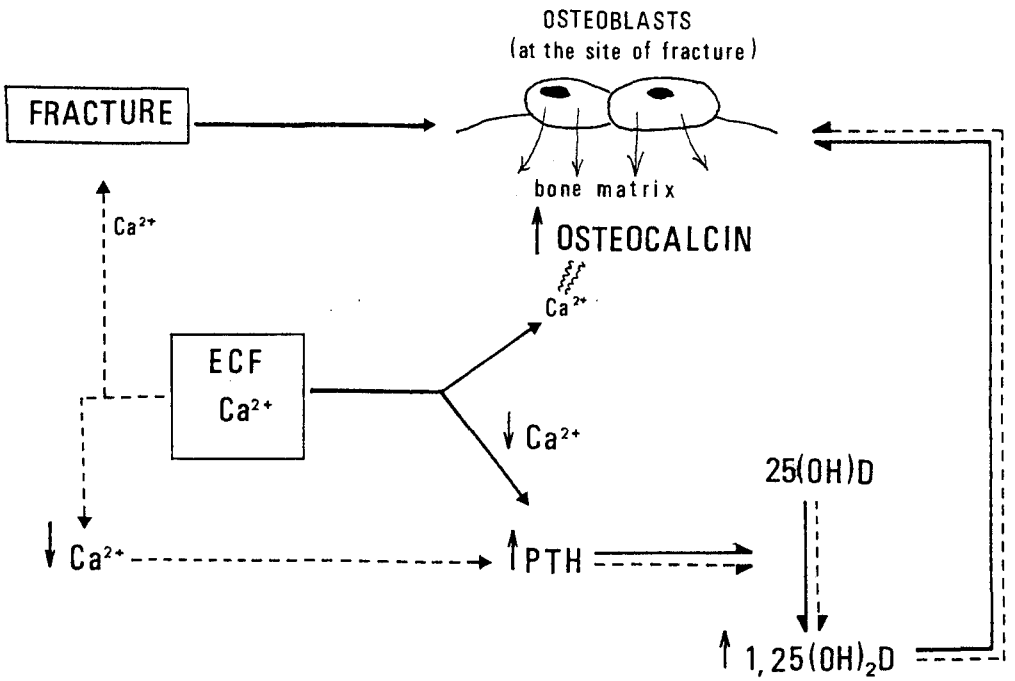
There are several possible explanations for the observed relationship between osteocalcin and $1,25(OH)_2D$, as outlined in figure 6.8. It may be that as the fracture heals calcification of callus results in the slight fall in serum calcium (refer to route a in figure 6.8), leading to stimulation of PTH secretion and augmentation of $1,25(OH)_2D$ production with further enhancement of osteocalcin production. Alternatively, the production of the fracture callus, by stimulating osteoblastic production of osteocalcin, leads to the movement of calcium into bone with secondary effects on $1,25(OH)_2D$ synthesis mediated via hypocalcaemia (route b of figure 6.8). A direct relationship between $1,25(OH)_2D$ and osteocalcin seems most likely since in vitro studies on human osteoblast-like cells (Lian et al, 1985) and cultures of foetal rat bone (Skjodt et al, 1985) have confirmed that $1,25(OH)_2D$ has a stimulatory effect on osteocalcin synthesis.

The necessary increases in PTH and $1,25(OH)_2D$ were not seen in the present study but this may reflect either the insensitivity of the assays used to small changes or those changes may have diminished when sampled a week after the fracture. In the case of the vitamin D metabolite, absolute concentrations and sequential changes showed considerable individual variation and perhaps for this reason the paired values for the study, as a whole, did not show a statistically significant increase.

In conclusion, the determination of the precise role of osteocalcin in bone, and its physiological role in fracture

FIGURE 6.8. Two possible theories for the observed relationship between osteocalcin and $1,25(OH)_2D$ during fracture healing. Refer to discussion section for explanation.

Bold arrows indicate route A; dashed arrows indicate route B.



healing, require further study. It remains to be seen whether osteocalcin is useful in screening very elderly patients with femoral neck fractures with osteomalacia. Experience with the use of osteocalcin for this purpose is surprisingly limited. The generation of diagnostically useful raised values may depend critically on the opposing effects of $1,25(OH)_2D$ deficiency and the ability of the very elderly to increase both PTH secretion and bone formation. The timing of sampling is also critical. The present study shows that osteocalcin concentrations remain within the reference range within the first week after a fracture. Other studies where the timing of samples were not stated, found elevated values, further complicating, and diminishing the diagnostic usefulness of this measurement.

CHAPTER 7

VITAMIN D DEFICIENCY AND FEMORAL NECK FRACTURE

7.1 INTRODUCTION:

Despite earlier reports to the contrary (Aaron et al, 1974; Jenkins et al, 1973), a recent large survey has shown that osteomalacia is now an uncommon accompaniment of femoral neck fractures in the elderly (Wilton et al, 1987a). Moreover, in another population potentially at risk, the acutely ill elderly, the prevalence of subclinical osteomalacia also appears to be low (Campbell et al, 1986).

It is uncertain whether the decreased frequence of osteomalacia reflects improved nutrition of the elderly or whether there is some other explanation. The former seems unlikely since the limited data which is available suggests that circulating concentrations of 25(OH)D are low in the elderly both with and without femoral neck fracture (Baker et al, 1979; Brown et al, 1976; Weisman et al, 1978;). The situation is particularly confused with respect to $1,25(OH)_2D$ where concentrations have been reported to be either low, normal or high (Francis et al, 1984; Hordon and Peacock, 1987; Lips et al, 1987; Meller et al, 1985). This apparent paradox between the decreasing incidence of osteomalacia despite vitamin D deficiency and the currently high incidence of osteoporotic femoral neck fractures seems to be at variance with the accepted role of vitamin D in the maintenance of calcium and skeletal homeostasis.

In the present study serum concentrations of the hydroxylated vitamin D metabolites were measured in elderly

patients at the time of presentation with femoral neck fractures. Comparisons between those with and without osteomalacia have been made in an attempt to assess the pathogenetic significance of vitamin D deficiency in this age group.

7.2 PATIENTS AND METHODS:

The study was based on a group of patients obtained from a 4 year screening programme, designed to detect sub-clinical osteomalacia in elderly patients presenting with a femoral neck fracture. All patients in this present study underwent an iliac crest bone biopsy at the time of their operation. Osteomalacia was defined as a relative osteoid area greater than 5% of the total trabecular area with osteoid covering more than 25% of the total trabecular surface (Wilton et al, 1987a).

A total of three groups were studied. The first group consisted of 41 patients with a femoral neck fracture without osteomalacia (mean age 79 years; range 59-94 years). The second consisted of 26 patients (mean age 82 years; range 57-95 years) who had osteomalacia at the time of their femoral neck fracture. The third group comprised of 12 young osteomalacic patients without fractures (mean age 48 years; range 43-58 years) who had been referred to the metabolic unit over the same period.

Fasting blood samples were taken from all patients within 24h of admission for the measurement of serum calcium, phosphate, creatinine, alkaline phosphatase and albumin (refer to appendix 2). Samples for the measurement of vitamin D binding protein and vitamin D metabolites were taken on a separate occasion but generally within the first

few days after admission. For further timing of these samples refer to appendix 7.

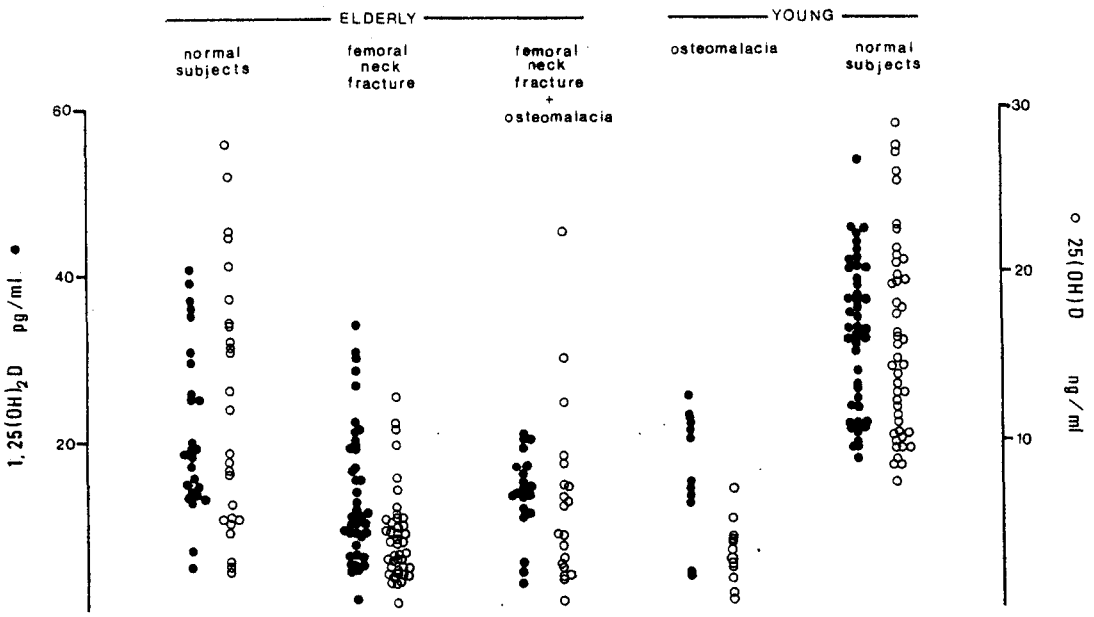
Reference values for vitamin D metabolites and binding protein were obtained from normal elderly subjects (mean age 70 years; range 60-82 years), selected from relatives of patients attending a metabolic out-patient clinic. These patients appeared well and none was known to suffer from renal, gastrointestinal or musculoskeletal diseases. Reference data was also derived from 45 young normal subjects working in the hospital laboratories (mean age 34 years; range 18-54 years).

7.3 RESULTS:

Individual plasma biochemistry and histomorphometric indices are outlined in appendix 7.

Serum concentrations of 25(OH)D and $1,25(OH)_2D$ for all the groups under investigation are shown in figure 7.1. The mean (±SD) serum 25(OH)D and $1,25(OH)_2D$ concentrations of the 26 normal elderly subjects were 12.8 (±7.2) ng/ml and 21.6 (±9.1) pg/ml respectively while the corresponding values for the 42 young normal subjects were 19.4 (±8.4) ng/ml and 34.6 (±9.3) pg/ml. These differences between age groups were significant: [25(OH)D $p<0.02$; $1,25(OH)_2D$ $p<0.01$]. Vitamin D concentrations were predictably low in both young and elderly patients with osteomalacia. However, a considerable number of the elderly controls (who did not have a bone biopsy) and the elderly fracture patients without osteomalacia also had equally low concentrations of 25(OH)D and $1,25(OH)_2D$. There was no statistically significant difference between the measurements of the vitamin D metabolites in any of the elderly groups.

FIGURE 7.1. Serum 25(OH)D and $1,25(OH)_2D$ concentrations in the elderly patients with fractures, control subjects and young patients with osteomalacia.



The severity of the osteomalacia, as judged by the osteoid area (O.A.) showed no significant correlation with either of the vitamin D metabolites [25(OH)D v O.A. r = -0.14 p NS; $1,25(OH)_2D$ v O.A. r=0.05 p NS]. The relationship between $1,25(OH)_2D$ and osteoid surface for the fracture patients without osteomalacia is shown in figure 7.2. Many of those with low concentrations of $1,25(OH)_2D$ also had low rate of bone turnover, as assessed from the surface extent of osteoid and this tended to be associated with hypocalcaemia. Under these circumstances, osteoid seam thickness is difficult to measure with any degree of accuracy but where this could be done, in patients with osteoid greater than 12.5%, a high proportion of the osteoporotic patients with low $1,25(OH)_2D$ concentrations had abnormally thick osteoid seams (figure 7.3). This suggests that there was a defect in the mineralisation of newly formed osteoid, even though osteoid had not accumulated to a sufficient extent to fulfil the criteria for a diagnosis of osteomalacia.

Serum concentrations of the vitamin D metabolites and DBP have the potential for more rapid change than any of the histomorphometric parameters. Therefore, in order to assess whether the interval between hospital admission and blood sampling was an important determinant for the low concentrations of $1,25(OH)_2D$, both DBP and $1,25(OH)_2D$ are shown in relation to the time of blood sampling (figure 7.4). In neither case was time an important variable, even though measurements of DBP were only available from 30 of the 41 patients.

Another important factor to consider is whether the stress of a fracture lowers the concentration of DBP which might account for the low $1,25(OH)_2D$ concentrations found in these elderly fracture patients. Measurements of DBP were only available from 56/80 patients and 19/26 elderly

FIGURE 7.2. Relationship between serum $1,25(OH)_2D$ and bone turnover (extent of osteoid surface).

□ - osteoporotic patients with FNF

■ - osteomalacic patients with FNF

FIGURE 7.3. Relationship between serum $1,25(OH)_2D$ and osteoid index.

× - osteoporotics with osteoid surface < 12.5

□ - " " " " > 12.5

■ - osteomalacic patients.

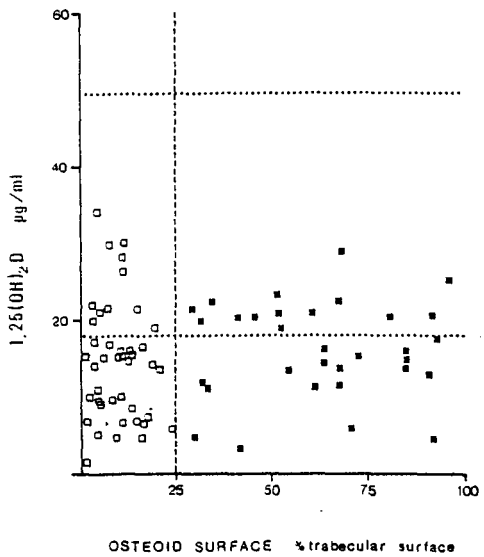


FIGURE 7.2.

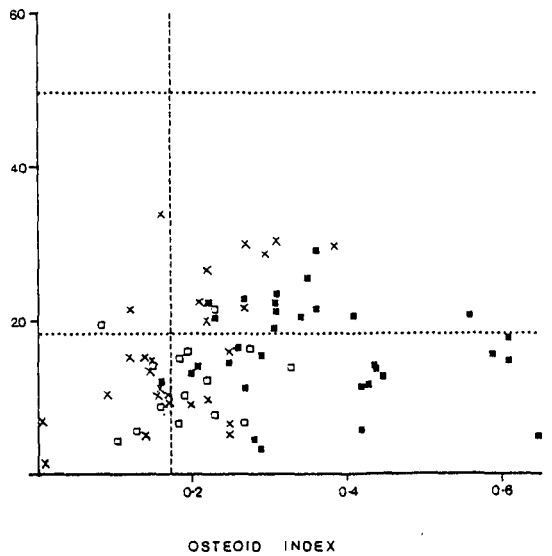
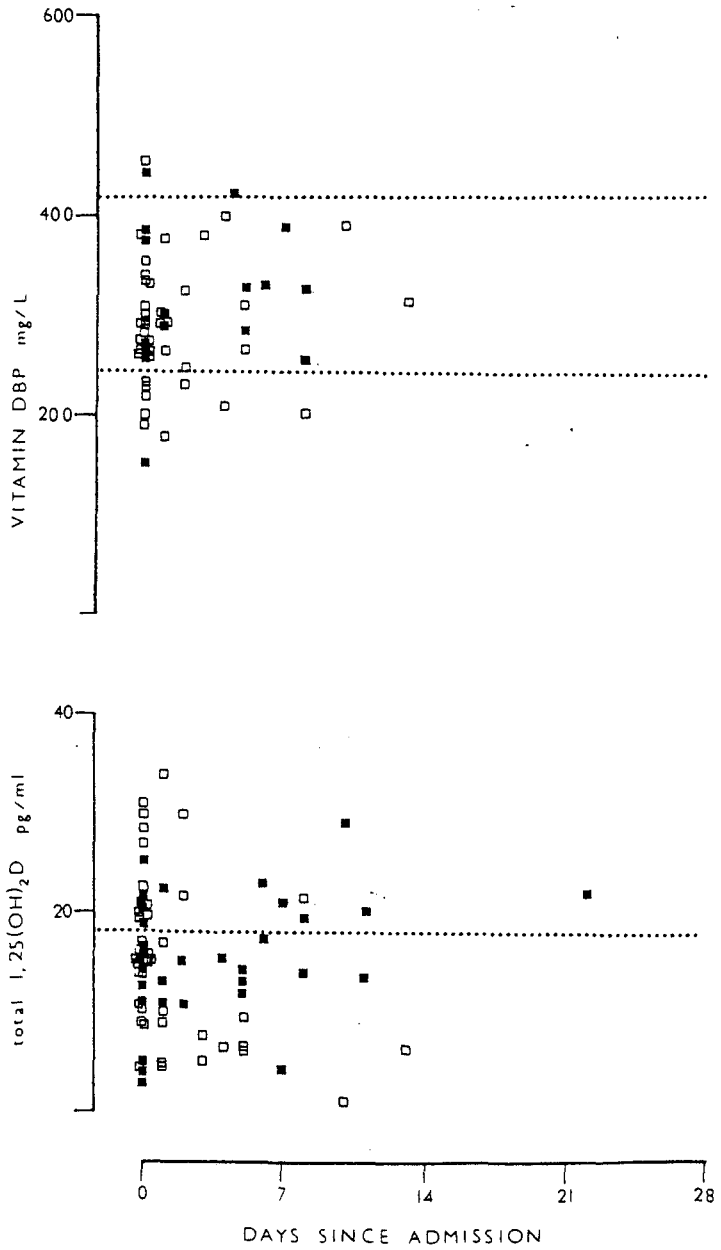


FIGURE 7.3.

Dotted horizontal lines represent the normal reference range for $1,25(OH)_2D$. The vertical lines indicate the upper limit of normal for the surface extent of osteoid and the osteoid index.

FIGURE 7.4. Relationships between serum vitamin D binding protein (DBP) and $1,25(OH)_2D$ and the delay between admission and sampling.

□- osteoporotics with FNF; ■- osteomalacic with and without FNF.



Dotted horizontal lines represent the normal reference range for DBP and the lower end of the reference range for $1,25(OH)_2D$.

controls. Table 7.1 shows the mean concentration of DBP in the elderly patient groups and the corresponding free $1,25(OH)_2D$ indices. Only a fifth of the patients had free $1,25(OH)_2D$ values below the normal range and this could not explain the higher incidence of low $1,25(OH)_2D$ values. No correlation was seen between total $1,25(OH)_2D$ and DBP. There were no statistically significant differences between DBP and free $1,25(OH)_2D$ index in any of the elderly sub groups, or between the elderly patients and the younger subjects with non fracture osteomalacia. However, there was a good correlation [$r=0.91$, $p<0.001$] between total and free $1,25(OH)_2D$ (figure 7.5) which suggest that DBP is not an important variable with respect to the low concentrations of $1,25(OH)_2D$.

7.4 DISCUSSION:

Many of the patients with femoral neck fractures and osteoporosis in this study had low serum 25(OH)D and $1,25(OH)_2D$ concentrations. Moreover, low concentrations of $1,25(OH)_2D$ do not seem to be inevitably associated with the development of osteomalacia. These findings are consistent with previous reports of low 25(OH)D (Brown et al, 1976; Baker et al,1979; Meller et al, 1985) and $1,25(OH)_2D$ (Lips et al, 1982; Francis et al, 1984) concentrations in patients with femoral neck fractures. However, although Meller et al (1985) found low concentrations of 25(OH)D in their fracture group, the $1,25(OH)_2D$ concentrations were higher than those of their normal elderly controls.

This study shows quite clearly that, compared with young healthy controls, the elderly population with or without femoral neck fractures, are vitamin D deficient. It was surprising to find that many elderly subjects in apparent good health also had low concentrations of 25(OH)D and

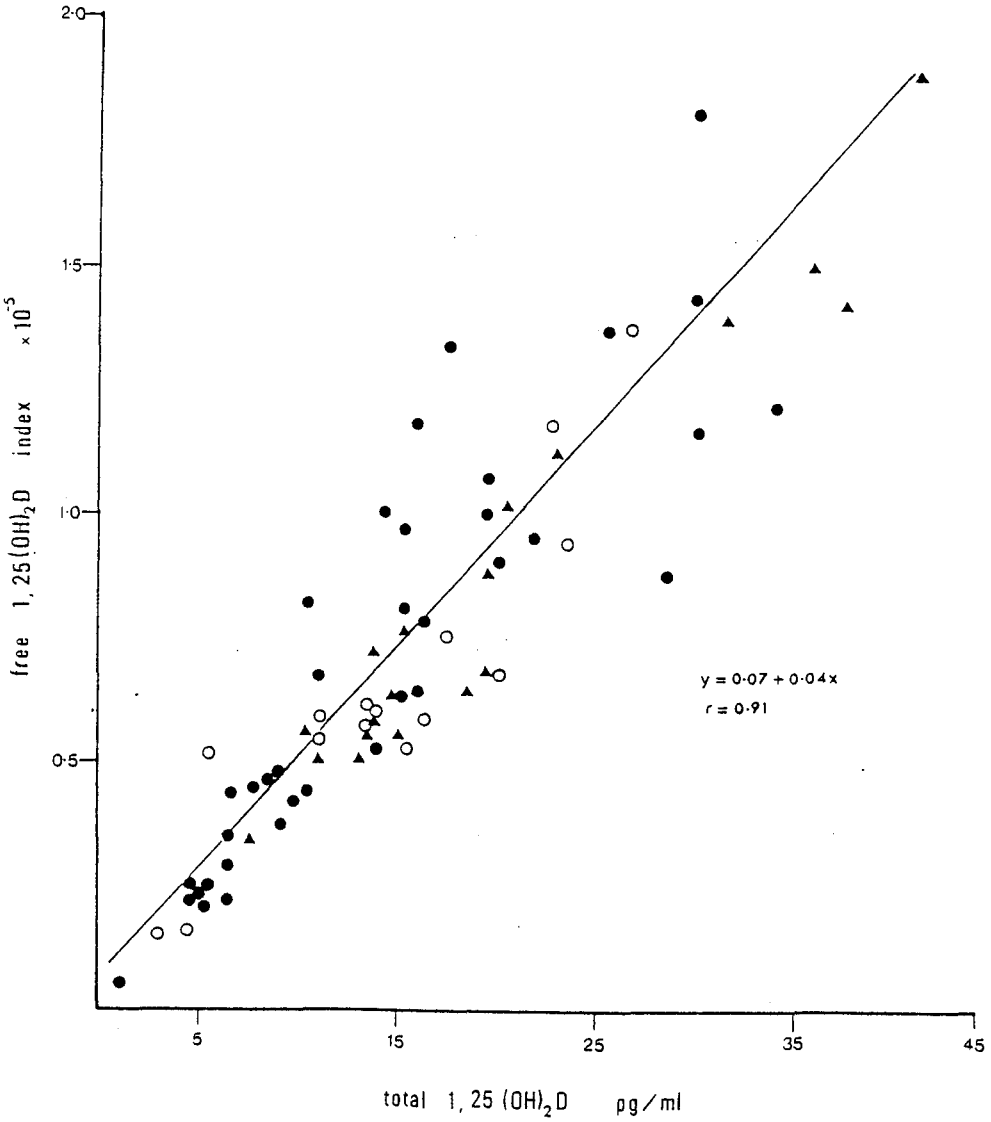
PATIENT GROUP	n	VITAMIN D BINDING PROTEIN mg/L	FREE $1,25(OH)_2D$ INDEX x 10^{-5}
Elderly normal subjects	19	326 (± 45)	0.85 (± 0.42)
FNF + osteoporosis	41	293 (± 65)	0.78 (± 0.41)
FNF + osteomalacia	11	316 (± 76)	0.50 (± 0.20)
Non fracture osteomalacia	5	346 (± 75)	0.94 (± 0.32)

TABLE 7.1. Vitamin D binding protein and free $1,25(OH)_2D$ indices in the elderly patient groups.

Values represent the mean ± SD

FIGURE 7.5. Linear relationship between total and free $1,25(OH)_2D$.

▲- elderly controls; ●- osteoporotic patients with FNF;
○- osteomalacic patients with and without FNF.



$1,25(OH)_2D$. This is in agreement with other studies which have not found any differences in the vitamin D metabolite concentrations between fracture patients and controls (Hordon and Peacock, 1987; Wootton et al, 1982). It has been suggested that the vitamin D deficient state may be characteristic of old age in general, rather than confined to the elderly at risk group. However, the data is at variance with the findings of Lips et al (1982) who found much lower concentrations of the vitamin D metabolites in a similar group of hip fracture patients compared to controls. Their observations were attributed to low concentrations of DBP, which was probably due to the traumatic effect of the fracture (Lips et al, 1985). In this study DBP concentrations were normal but this could be attributed to the inclusion of patients sampled several days after the fracture.

There are a number of possible explanations why low concentrations of $1,25(OH)_2D$ may not invariably be associated with osteomalacia. DBP concentrations may be low after a femoral neck fracture (refer to chapter 6; Lips et al, 1982) although they generally recover within the subsequent week (refer to chapter 6). However, most of the patients in the present study had normal concentrations of DBP and it is unlikely that the low concentrations of $1,25(OH)_2D$ are falsely low because of the reduced binding protein. Although DBP values were not available on every subject, the correlation between total $1,25(OH)_2D$ concentration and the free $1,25(OH)_2D$ index was so good that it makes it unlikely that reliance upon total $1,25(OH)_2D$ concentrations incorporates a systematic error.

Another possible explanation is that the low $1,25(OH)_2D$ concentration reflects the presence of Type II osteoporosis, which may be an almost universal phenomenon in the very elderly and commonly presents with proximal femoral

fractures. This is characterised by decreased renal 25(OH)D-1 alpha-hydroxylase activity leading to impaired $1,25(OH)_2D$ production and reduced osteoblastic activity (Riggs and Melton, 1983). In this respect the low values for the surface extent of trabecular osteoid found in the present study would be consistent with decreased osteoblast function. It may be that in the presence of a low rate of bone formation less $1,25(OH)_2D$ than normal is required for adequate mineralisation.

Bone turnover is known to be low in the elderly (Meunier, 1983) and it seems that this may have a 'protective' effect in reducing the requirement for vitamin D. Although many of the patients in the present study had low rates of bone formation, as assessed by the surface extent of osteoid, several also had thick osteoid seams. This implies that there may be some form of mineralisation defect but because the rate of activation of new forming surfaces is so low vitamin D deficiency can exist for a prolonged period of time before osteoid has accumulated to such an extent as to satisfy the diagnostic measurements for osteomalacia.

There are a number of practical implications associated with these findings. Firstly, measurement of plasma 25(OH)D and $1,25(OH)_2D$ concentrations are of no value in screening for subclinical osteomalacia complicating femoral neck fractures. Reliability must therefore be placed on the conventional combination of low plasma calcium concentration and raised alkaline phosphatase activity despite the high false positive rate associated with this combination (Wilton et al, 1987b). Secondly, in the very old the rate of activation of new bone remodelling units may be so low that vitamin D deficiency may need to be present for a long period before osteoid accumulates to such an extent that it fulfils the generally accepted criteria for osteomalacia.

Moreover, low turnover may be associated with a very slow response of vitamin D therapy to heal osteomalacia.

Supplementation of the diet of the elderly with vitamin D either on the suspicion of a low vitamin D intake or because of low plasma concentrations of vitamin D metabolites does not seem likely to improve bone structure unless there is overt osteomalacia. Whether long term vitamin D supplementation starting at an earlier age may improve bone mass, by offsetting the age-related decline in intestinal calcium absorption which is seen in the elderly (Francis et al, 1984; Peacock and Nordin, 1980), is uncertain at present. Identification of patients with low serum concentrations of 25(OH)D and $1,25(OH)_2D$ cannot, by itself, be equated with the presence of osteomalacia. Such measurements do not help to resolve the problem of eliminating the high false positive rate associated with biochemical screening of fracture patients for the presence of osteomalacia (Wilton et al, 1987b).

CHAPTER 8

CHANGES IN CALCIUM HOMEOSTASIS AFTER TOTAL THYROPARATHYROIDECTOMY IN MAN

8.1 INTRODUCTION:

Surgical excision of squamous cell carcinoma of the larynx, hypopharynx and upper oesophagus often includes partial or total removal of the thyroid gland (ie. thyroidectomy). Both pairs of the parathyroid glands are normally situated on or beneath the posterior surface of the thyroid lobes, located near the junction of the middle thyroid artery and the recurrent laryngeal nerve. Only rarely are they embedded within the thyroid. Either way, during thyroidectomy some if not all of the parathyroid glands are removed because identification at thyroidectomy is often difficult. On many occasions positive search of the glands, with a view to their preservation, is not undertaken because of the high risk of damage to their blood supply in the process. Nevertheless, it has been shown that three of the four parathyroid glands can be resected without necessarily inducing hypoparathyroidism (Wade, 1972).

In 1983 up to 46% of all new cases of laryngeal carcinoma in both England and Wales (1689 cases in total) underwent laryngectomy (National Cancer Registration, 1987). Hypopharyngeal carcinomas are less common; 383 new cases were reported in 1983 and from this group only 5% underwent surgical excision (unpublished data). Thus, the number of patients undergoing extensive laryngo-pharyngeal surgery is significant and may give rise to a number of metabolic problems.

The induction of hypocalcaemia in man following this type of surgery has been shown to be within the region of 11-15% (Harrison, 1973; Isaacson and Snow, 1978). However, this value could be an underestimate as most studies have relied on the appearance of signs and symptoms of hypocalcaemia before proceeding with any biochemical measurements. There have been no reports to suggest that radiotherapy for laryngo-pharyngeal squamous cell carcinoma causes hypocalcaemia. The cause of either the temporary or permanent hypocalcaemia occurring after thyroidectomy may result from hypoparathyroidism either following damage to the parathyroid glands at operation or their excision with the thyroid. Excision of calcitonin-secreting tissues is inevitable at thyroidectomy but would not cause hypocalcaemia since its overall contribution to calcium homeostasis is minor compared with that of parathyroid hormone.

The aims of this study was to examine the effect of laryngectomy and laryngo-pharyngo-oesophagectomy (LPO) with thyroparathyroidectomy on the calcium homeostatic system in man and to determine the efficacy of the standard post-operative treatment regimes.

8.2 PATIENTS AND METHODS:

The study was carried out in 10 patients (4 males and 6 females: mean age 61; range 41-82) with histologically proven squamous cell carcinoma of the larynx, hypopharynx or upper oesophagus. All had normal pre-operative levels of serum calcium, magnesium, creatinine and thyroid hormones. Clinical details of the individuals are summarised in Table 8.1. Of the ten patients, seven underwent laryngo-pharyngo-oesophagectomy (which involved total thyroparathyroidectomy) with pharyngo-gastric anastomosis (LPO). From the first

Case No.	Sex	Age	Diagnosis	pre-op Ca^{2+}	pre-op Mg	Oper-ation	Post-op treatment commenced (days) Calcium IV	Calcium Oral	T_3 IV	T_4 Oral	1,25 Oral
1	F	82	Post cricoid carcinoma	2.44	0.75	LPO	1	11	-	9	11
2	M	48	Post cricoid carcinoma	2.44	0.93	LPO	1	12	1	12	12
3	F	62	Post cricoid carcinoma	2.46	0.73	LPO	1	*	9	*	9
4	F	72	Post cricoid carcinoma	2.42	0.83	LPO	1	35	9	35	35
5	F	75	Post cricoid carcinoma	2.41	0.82	LPO	1	17	1	19	19
6	F	68	Post cricoid carcinoma	2.45	0.93	LPO	1	17	1	17	17
7	M	53	Post cricoid carcinoma	2.43	0.90	LPO	1	12	9	12	12
8	M	57	Larynx carcinoma	2.56	0.85	LTT	-	3	-	4	4
9	M	52	Larynx carcinoma	2.50	0.75	LTT	-	2	-	7	3
10	F	41	Larynx carcinoma	2.27	0.90	LPT	-	6	-	12	6

LPO	Laryngopharyngo-oesophagectomy	T_3	Triiodothyronine
LTT	Total laryngectomy with total thyroidectomy	T_4	Thyroxine
LPT	Total laryngectomy with partial thyroidectomy	1,25	$1,25(OH)_2D$

* Died after 17 days

TABLE 8.1. Clinical details on the ten patients in the study.

post-operative day total parenteral nutrition (TPN) from a 2 litre pre-prepared bag was infused via a central venous line. The composition of the feeding together with vitamin D and mineral supplementation is summmarised in Table 8.2. In addition all patients received intravenous triiodothyronine (10μg three times daily) throughout the study.

Two patients underwent total laryngectomy and total thyroidectomy (LTT) whilst one other had a total laryngectomy with partial-thyroidectomy (LPT). Post-operative nutrition for these patients, maintained using a naso-gastric tube, was supplemented with calcium, $1,25(OH)_2D_3$ (calcitriol) and thyroxine (150ug/day) -see Tables 8.1 and 8.2.

Fasting blood samples were taken from each patient for daily measurements of calcium, phosphate, albumin, magnesium, creatinine, electrolytes and the vitamin D metabolites. All serum calcium concentrations were corrected to a reference albumin concentration of 40g/L (see appendix 1). Measurements of vitamin D binding protein, osteocalcin and parathyroid hormone concentrations were measured in a selected group of patients. Free vitamin D indices were calculated as in section 6.1.

Fasting urine samples were taken for measurement of sodium, calcium, creatinine and phosphate. Urinary excretion of calcium (CaE) and sodium (NaE) were expressed as umol/L glomerular filtrate:

$$\frac{(\text{urinary Ca or Na [mmol/L]}) \times (\text{serum Creatinine [mol/L]})}{(\text{urinary Creatinine [mmol/L]})}$$

TABLE 8.2. Calcium, magnesium and sodium replacement after thyroparathyroidectomy.

ROUTE	PREPARATION	CALCIUM (mmol)	MAGNESIUM (mmol)	SODIUM (mmol)
INTRAVENOUS	20% Intralipid (500ml)	-	-	-
	Vamin 14 (1000ml)	5.0	8.0	100
	50% Dextrose (500ml)	-	-	-
	Addamel (10ml)	5.0	1.5	-
	10% Calcium gluconate (10ml)	2.25	-	-
	50% Magnesium sulphate (2ml)	-	4.0	-
ORAL	Calcitriol (1mg) or Alphacalcidiol (1mg)	-	-	-
	Microcrystalline hydroxyapatite	70.0	-	-
	Calcium lactate gluconate (1.6g)	40.0	-	24
	Magnesium hydroxide (10ml)	-	14.0	-
	Magnesium amino chelate (200mg)	-	8.2	-
	Osmolite (250ml)	4.5	-	-

The notional setting of renal calcium reabsorption measured as the maximal tubular reabsorption of calcium related to glomerular filtration rate (TmCa/GFR) was calculated using a nomogram (see appendix 8) and compared to that of normal subjects.

Statistics

Analysis of the results were performed by standard paired t-tests. The relationship between TmCa/GFR and sodium excretion (NaE) for normal subjects was determined by standard linear regression fitted by the least squares method, and the 95% confidence limits expressed.

8.3 RESULTS

Parenteral feeding in the laryngopharyngo-oesophagectomy group (LPO) was continued for a mean of 17.3 days (range 11-35). This was replaced by an oral diet with calcium, calcitriol and thyroxine supplements, once all alimentary anastomoses had healed. The laryngectomy group (LPT and LTT) were fed by means of a naso-gastric tube for a mean of 10.2 days (range 7-14 days), thereafter resuming a normal diet with added calcium, calcitriol and thyroxine (Table 8.1).

Table 8.3 summarises the mean serum calcium, albumin, phosphate, magnesium, sodium, potassium, creatinine, 25(OH)D and $1,25(OH)_2D$ concentrations before and 24 hours after surgery. Individual responses are given in appendix 8.

The individual daily calcium concentrations following surgery and immediate infusion of calcium are summarised in figure 8.1a and 8.1b. Mean serum calcium concentrations fell significantly from a pre-operative value of 2.44 [$\pm$ 0.03] to 2.02 [$\pm$ 0.04] mmol/L after 24 hours [$p<0.01$] and to a nadir of 1.83 [$\pm$0.06] mmol/L three to thirteen days post-

TABLE 8.3. Changes in plasma biochemistry after TPTx.

Values represent the mean from the 10 patients ± SD.

PARAMETER	BASAL (± SD)	24h AFTER SURGERY (± SD)
Calcium (mmol/L)	2.44 (0.03)	2.02 (0.04)
Albumin (g/L)	36.5 (1.9)	28.7 (0.5)
Phosphate (mmol/L)	1.10 (0.05)	1.16 (0.09)
Magnesium (mmol/L)	0.87 (0.13)	0.76 (0.10)
Sodium (mmol/L)	139 (2.6)	138 (1.8)
Potassium (mmol/L)	3.88 (0.98)	3.37 (0.88)
Creatinine (umol/L)	86 (16)	87 (24)
25(OH)D (ng/ml)	6.61 (4.6)	3.65 (2.3)
$1,25(OH)_2D$ (pg/ml)	36.3 (6.8)	21.1 (11.9)

FIGURE 8.1a. Daily serum albumin and corrected calcium concentrations following surgery (TPTx) and immediate calcium infusion.

- for patients 1-5.

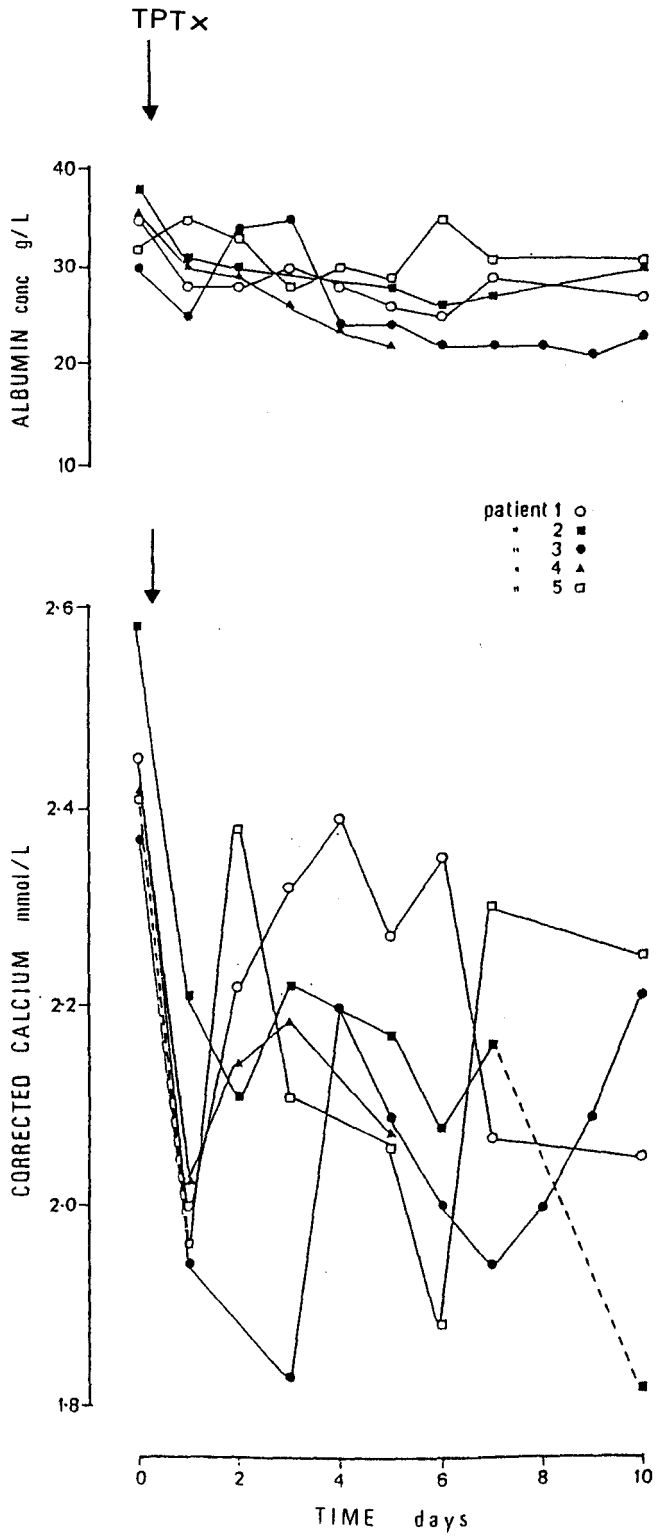
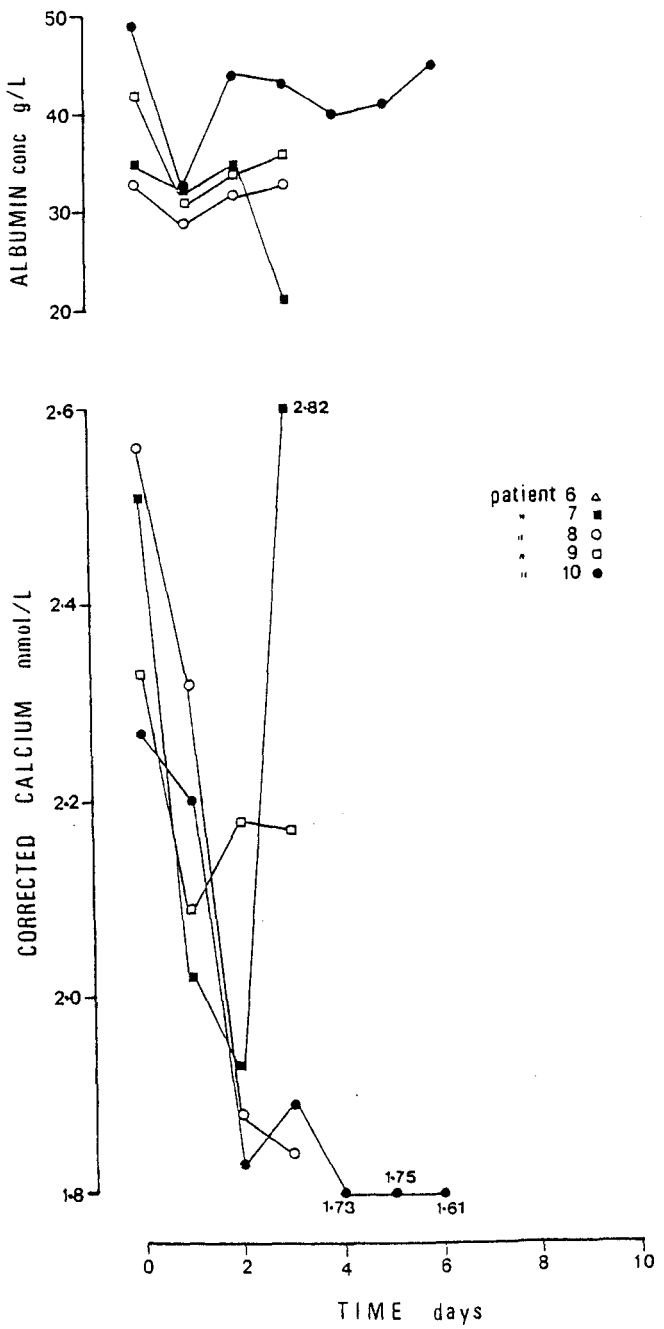


FIGURE 8.1b. Daily serum albumin and corrected calcium concentrations following surgery (TPTx) and immediate calcium infusion.

- for patients 6-10.



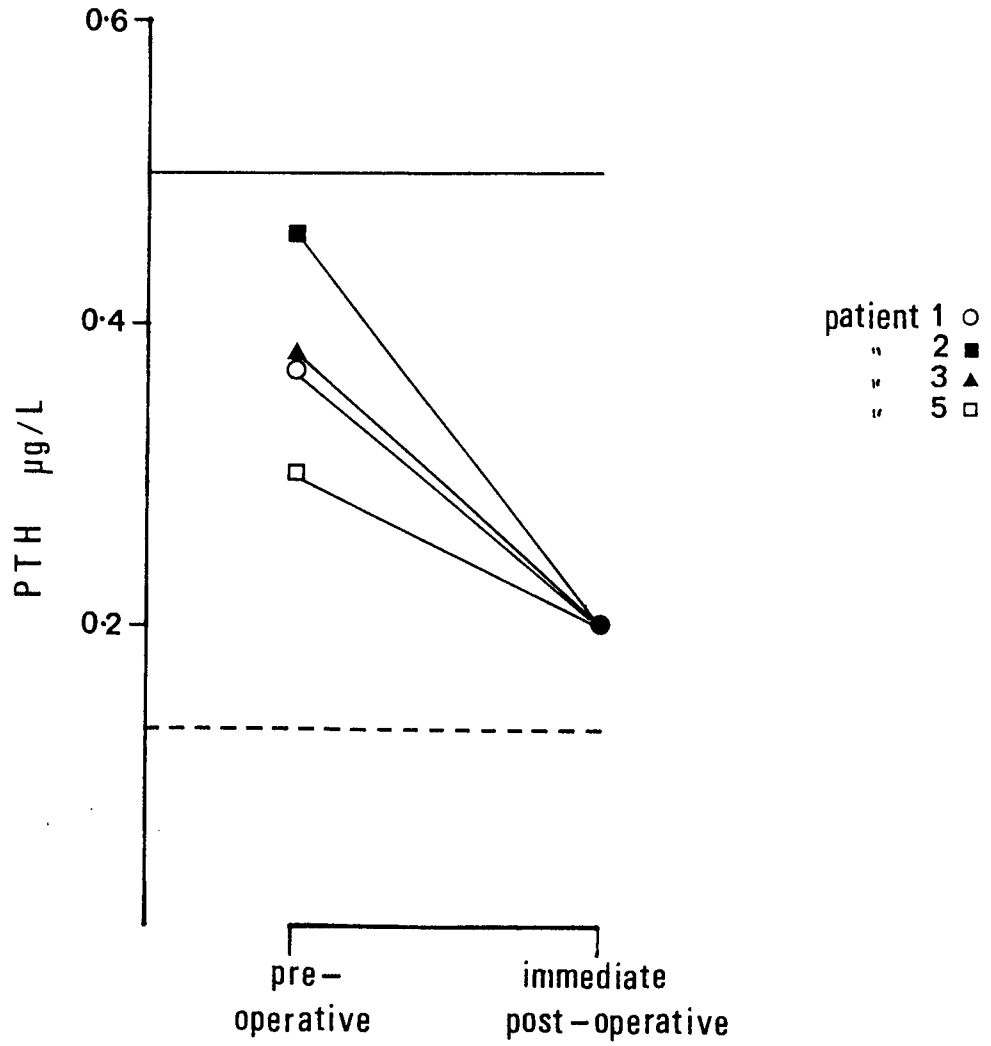
operatively [$p<0.01$]. Serum albumin also fell (figures 8.1a and b) from a mean pre-treatment value of 36.5 [$\pm$ 1.9] g/L to 28.7 [$\pm$ 0.5] g/L post-operatively [$p<0.01$]. There was a delay in nasogastric feeding in two of the three laryngectomy patients. These two patients became profoundly hypocalcaemic and this was not corrected until calcium and calcitriol had been given for five to six days (not shown). There was only one episode of tetany during this period which required treatment with a bolus injection of 10% calcium gluconate (2.25mmols).

Serum magnesium concentrations remained within the normal range throughout. Phosphate concentrations, however, increased from a mean pre-operative value of 1.10 [$\pm$ 0.05] to 1.16 [$\pm$ 0.09] mmol/L on the first post-operative day and peaked at 1.79 [$\pm$ 0.11] mmol/L seven to nine days after the operation [$p<0.001$]. There was no significant change in the mean phosphate concentration 24 hours after surgery.

Circulating serum concentrations of PTH were monitored in four patients (patients 1, 2, 3 and 5; refer to Table 8.1). Mean PTH concentrations decreased rapidly from a pre-operative value of 0.38 [$\pm$ 0.07] ug/L to below 0.2 ug/L. Unfortunately exact values could not be determined because of the insensitivity of the assay used (figure 8.2). PTH values remained at the lower end of the reference range throughout the study.

Measurements of total vitamin D metabolites were performed in all patients (figure 8.3a and 8.3b). The mean serum concentration of 25(OH)D before surgery was in the lower end of the reference range, taking into account any seasonal variation. However, both $1,25(OH)_2D$ and $24,25(OH)_2D$ concentrations were within the normal reference ranges. In general concentrations of the vitamin D metabolites decreased after surgery but only $1,25(OH)_2D$ fell

FIGURE 8.2. Serum PTH concentrations in four patients (1, 2, 3 and 5) before TPTx and post-operative.



Solid line indicates upper limit of normal; dotted line shows assay sensitivity.

FIGURE 8.3a. Concentrations of the vitamin D metabolites-pre-operatively and after surgery (TPTx) for patients 1-5.

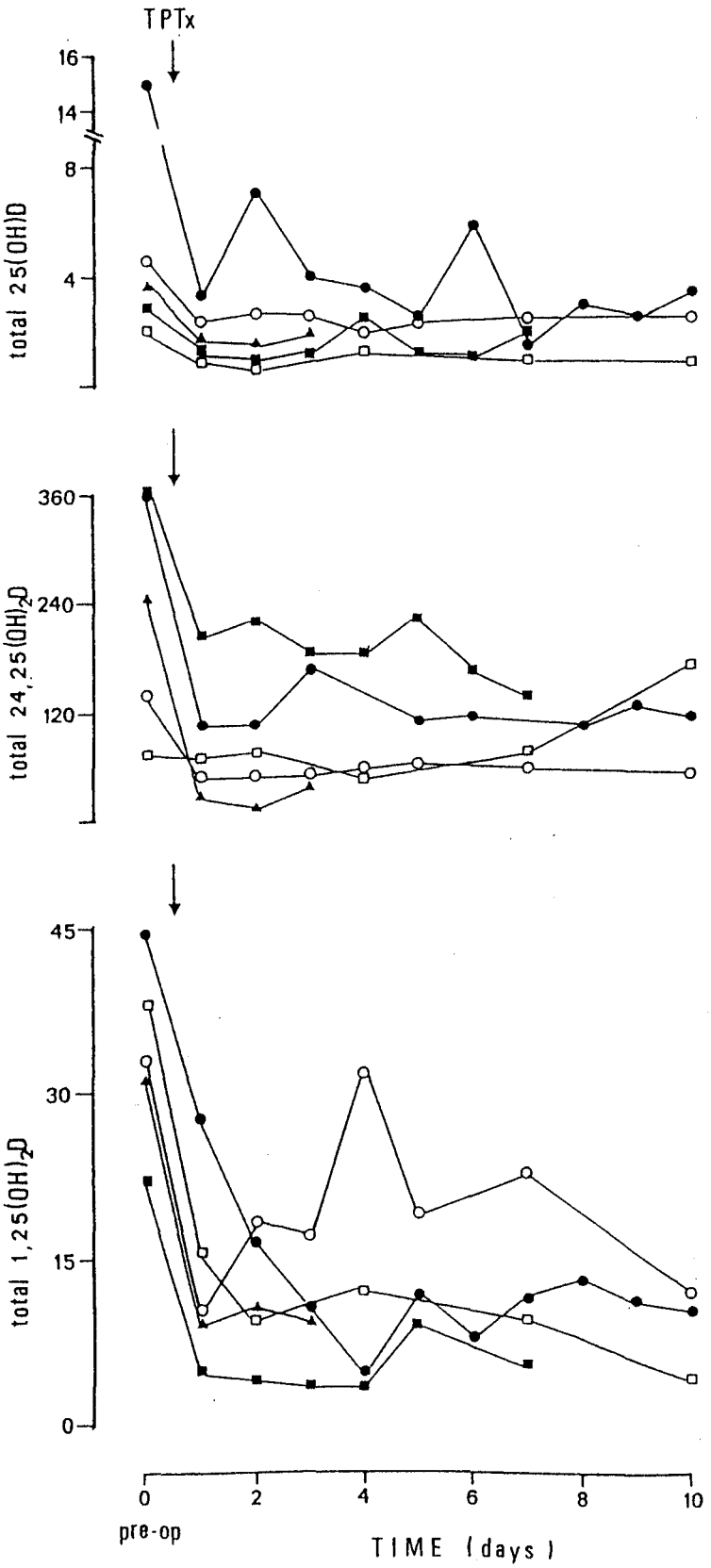
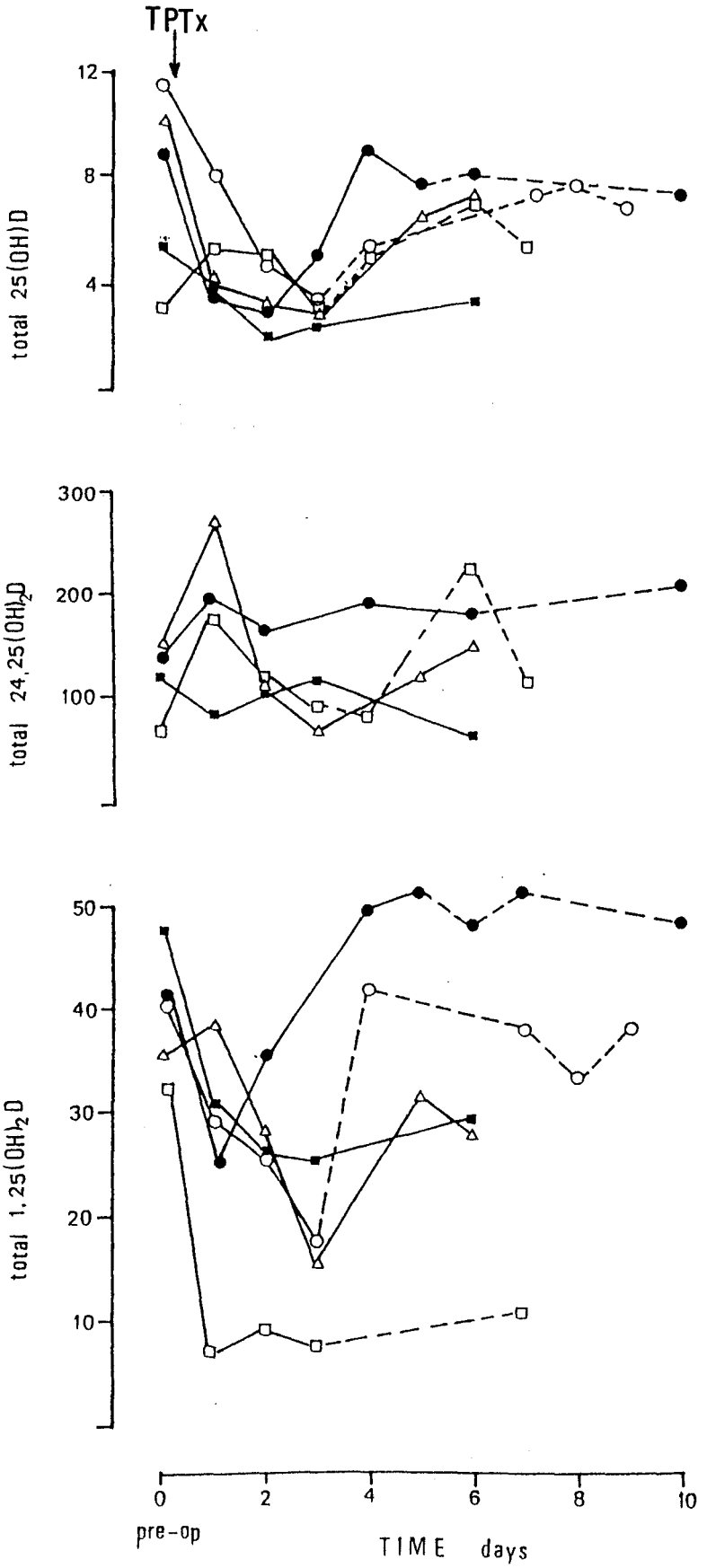


FIGURE 8.3b. Concentrations of the vitamin D metabolites pre-operatively and after surgery (TPTx) for patients 6-10.



significantly within 24 hours [$p<0.001$]. These values remained below the lower normal range and did not change significantly until the administration of calcitriol [$p<0.001$].

There were also no significant correlations between serum $1,25(OH)_2D$ and that of the serum calcium, phosphate, magnesium or albumin concentrations.

Concentrations of DBP were monitored for seven to ten days post-operatively in all but patient 4. The individual results are depicted in figures 8.4a and 8.4b. The mean pre-operative value of 374 [$\pm$ 80] mg/L decreased significantly to 241 [$\pm$ 99] mg/L 24 hours after surgery [$p<0.01$]. However, after 7 to 10 days there was a significant increase from this mean value to 315 [$\pm$ 72] mg/L, demonstrating that DBP values were able to revert back to normality. This is not true, however, for the total vitamin D metabolite values. There was no correlation between total serum $1,25(OH)_2D$ and DBP concentrations either before surgery or after therapy. No correlation was seen between DBP and either of the other vitamin D metabolites. Nor was there a relationship between DBP and serum albumin concentrations (not shown). There was no correlation between the changes seen in any of the vitamin D metabolites and the changes in DBP concentrations.

To determine whether the low DBP concentrations could account for the low $1,25(OH)_2D$ concentrations free $1,25(OH)_2D_3$ indices were calculated as described in section 6.1. Figure 8.4 shows the free $1,25(OH)_2D_3$ indices for the nine patients. There were no statistically significant changes throughout this period.

Osteocalcin concentrations were measured in patients 6, 7, 8 and 10, before and after treatment (figure 8.5). Although concentrations were within the normal age-matched

FIGURE 8.4a. Concentrations of vitamin D binding protein (DBP) and free $1,25(OH)_2D$ indices after TPTx (↓) - in patients 1, 2, 3 and 5.

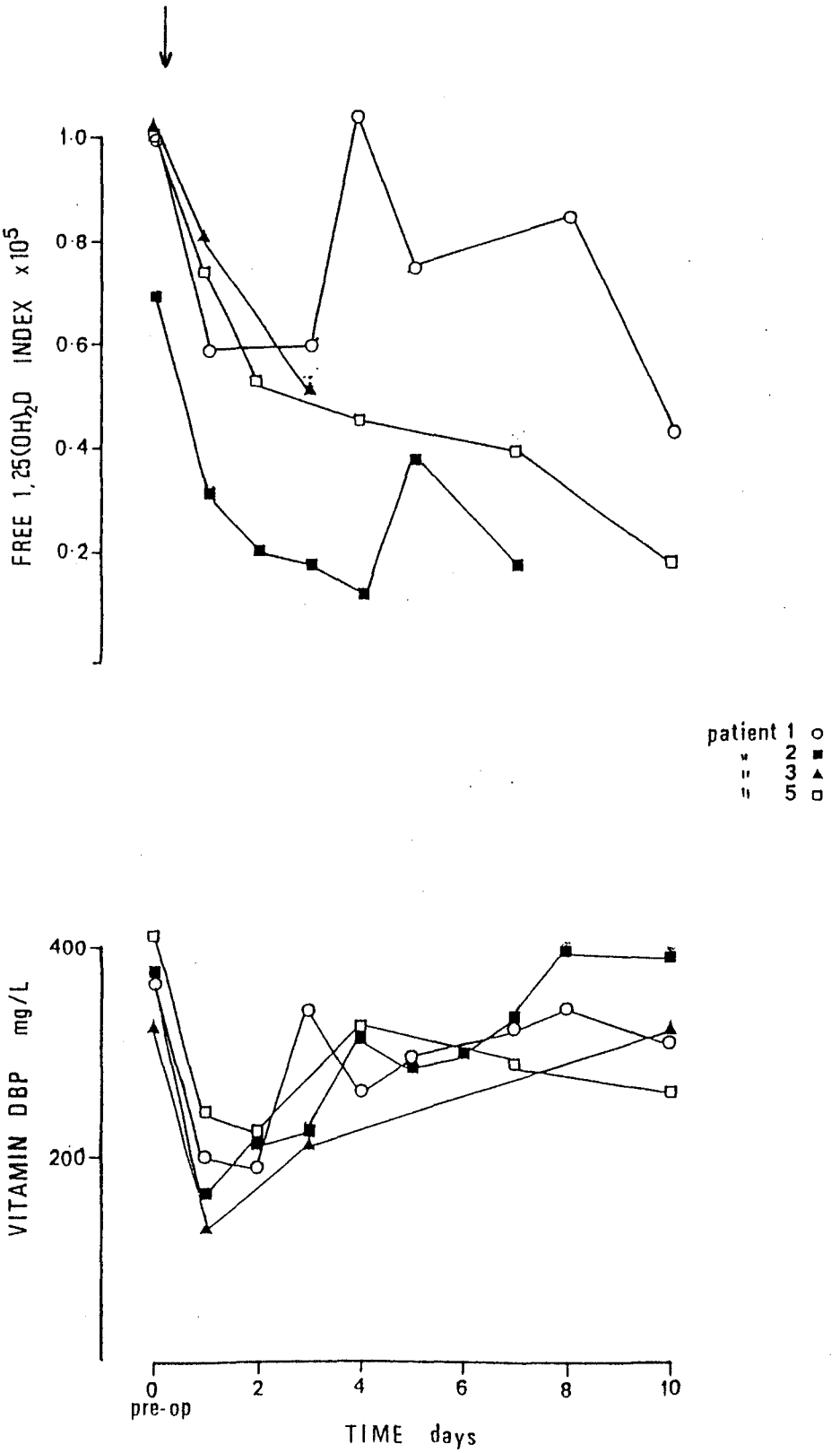


FIGURE 8.4b. Concentrations of vitamin D binding protein (DBP) and free $1,25(OH)_2D$ indices after TPTx (↓) - in patients 6-10.

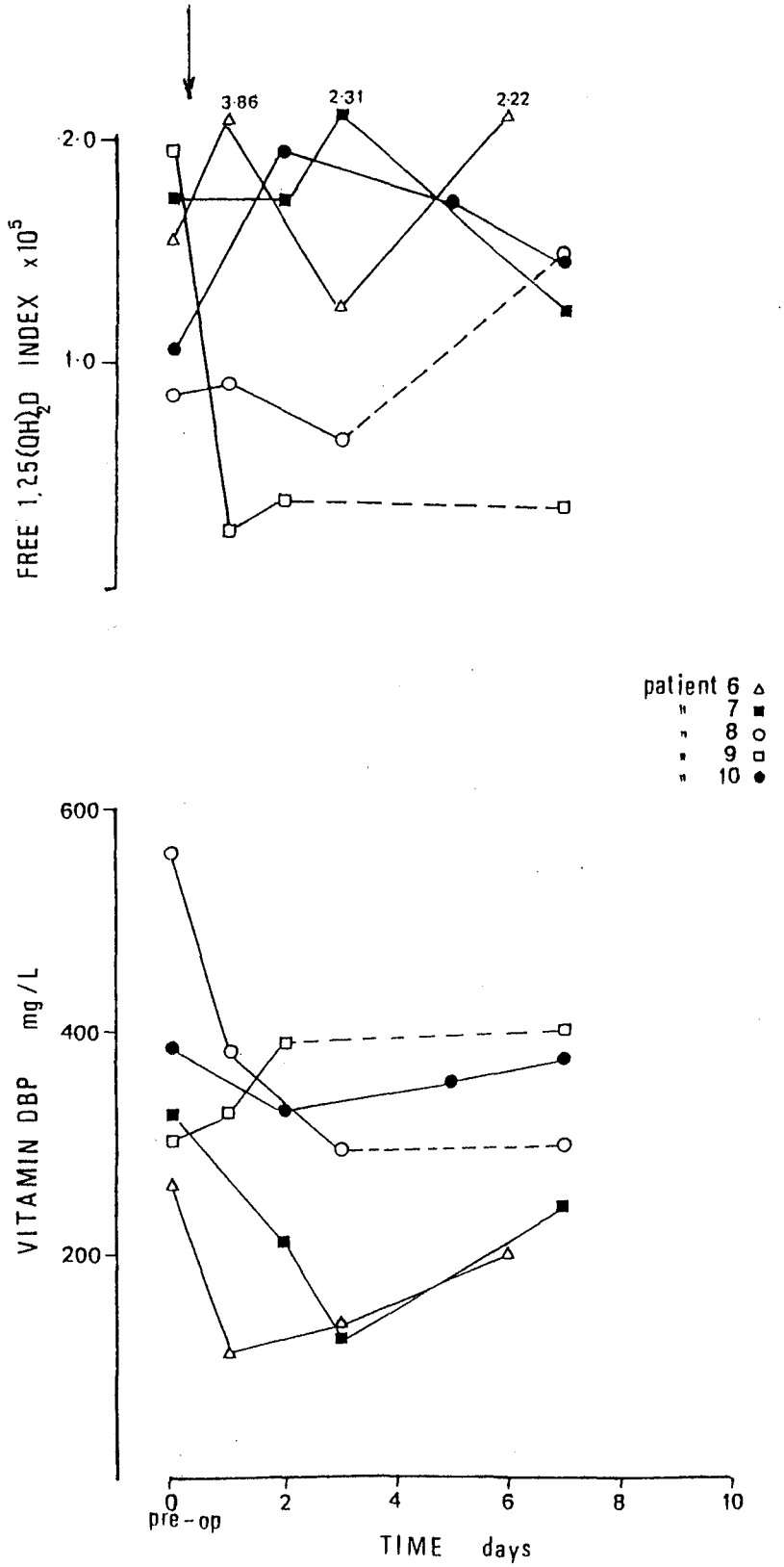
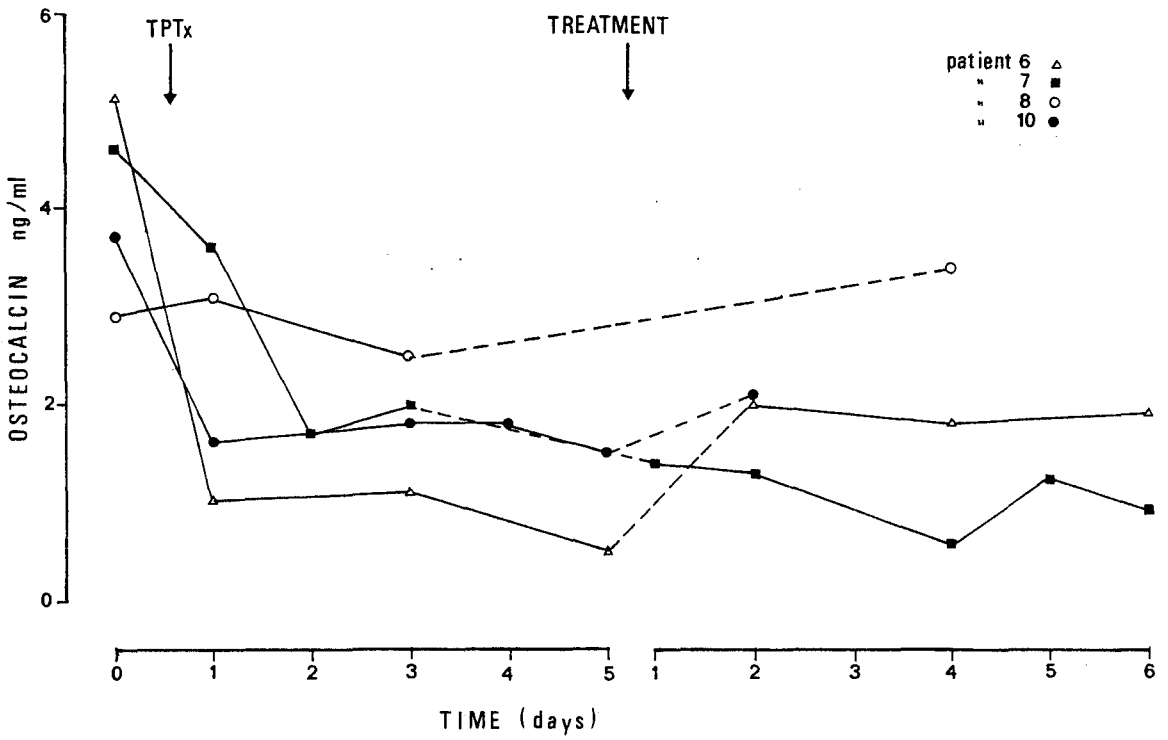


FIGURE 8.5. Osteocalcin concentrations in four patients before and after surgery (TPTx) and during treatment.



reference range before surgery (refer to Table 6.1 for range), values decreased significantly within 48 hours after surgery [$p<0.001$] and did not revert back to pre-operative values even when treatment with calcitriol had commenced.

Urinary calcium excretion (CaE) was compared with the prevailing concentrations of serum calcium (figure 8.6) in all patients. Results show that the infusion of calcium in these patients caused an inappropriately high excretion of calcium implying that they had a reduced renal threshold for calcium reabsorption. This is consistent with loss of the effect of PTH on the distal nephron of the kidney to increase renal tubular calcium reabsorption. The relationship between serum and urinary calcium was similar for both oral and intravenous routes of calcium administration.

Renal handling of calcium and that of sodium are known to be interdependent. Because of this association the assessment of the influence of any factor on urinary calcium excretion must take into account any simultaneous change in urinary sodium excretion. The relationship between the setting of calcium reabsorption (TmCa/GFR) and the rate of sodium excretion is shown in figure 8.7. TmCa/GFR represents the overall effect of both the proximal and the distal (PTH-dependent) renal tubular handling of calcium. Although the normocalcaemic state was maintained by increasing calcium output (either from enhanced intestinal absorption or by calcium infusion) the TmCa/GFR fell post-operatively. In the immediate post-operative period, patients were deliberately kept hypovolaemic, with very low sodium excretion rates, and at this time the setting of TmCa/GFR was within the normal range. As volume re-expansion was permitted to occur, sodium excretion increased while TmCa/GFR decreased. The setting of TmCa/GFR (figure 8.7b) tended to be lower when the patients had been persistently hypocalcaemic (ie $Ca<2.0$ mmol/L for

FIGURE 8.6. Renal handling of calcium following TPTx.

Shaded area represents the range for normal individuals during calcium infusion.

□ - laryngopharygo-oesophagectomy patients

■ - total laryngectomy with total/partial thyroidectomy.

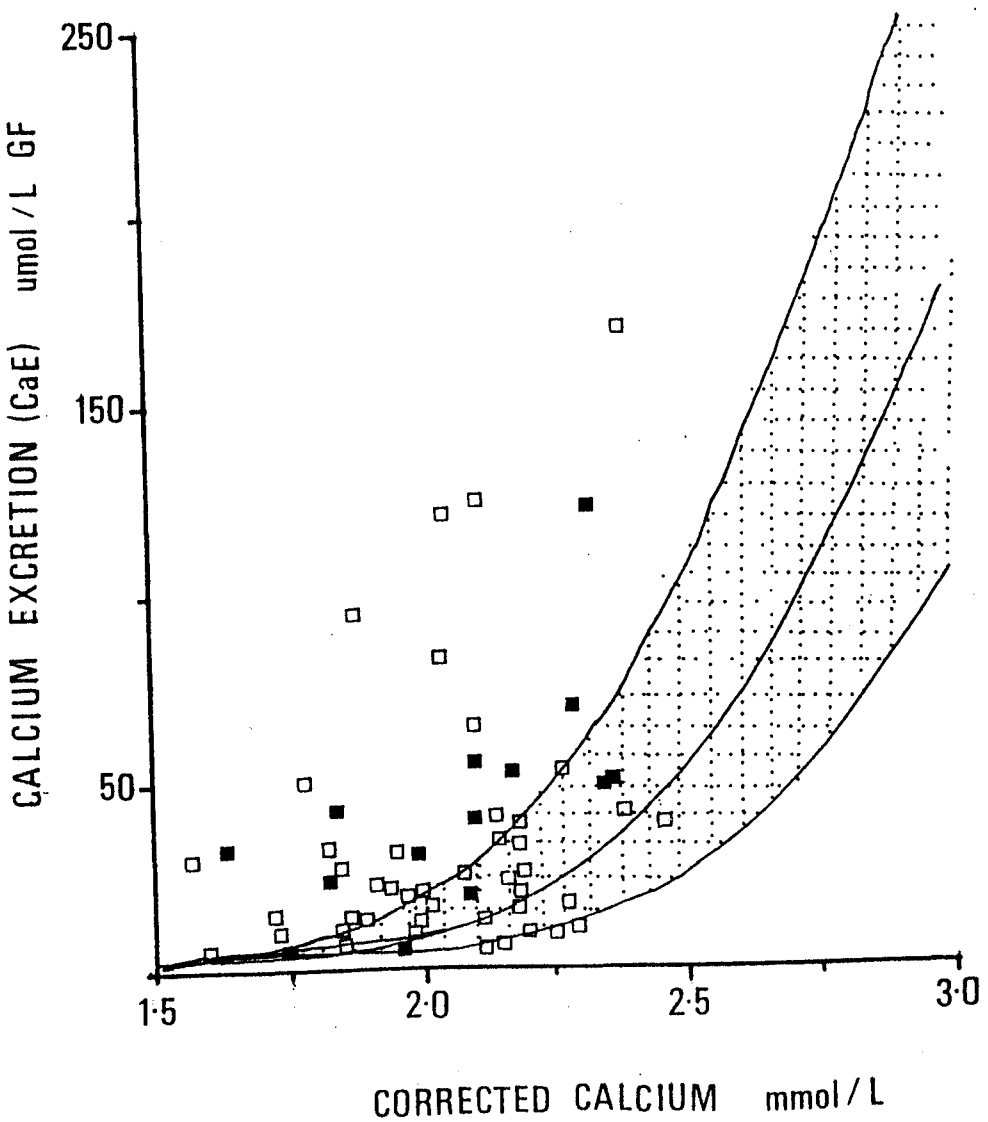
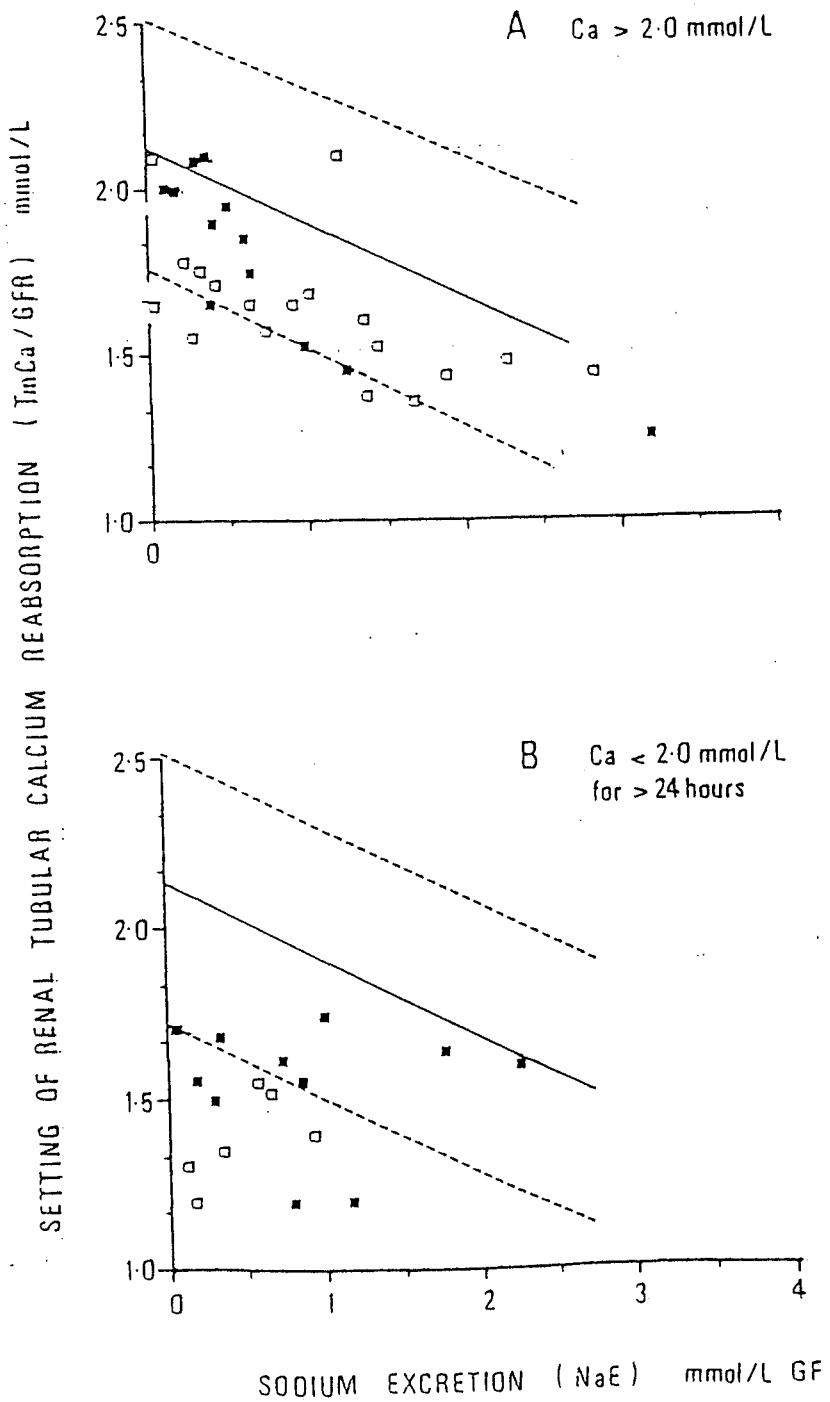


FIGURE 8.7. Relationship between the setting of calcium reabsorption and the rate of sodium excretion in (a) patients with corrected serum calcium >2.0 mmol/L and (b) patients with corrected serum calcium <2.0 mmol/L for greater than 24h.

□- laryngopharygo-oesophagectomy patients

■- total laryngectomy with total/partial thyroidectomy



greater than 24 hours). However, thyroid hormone replacement had little influence on the setting of renal tubular calcium reabsorption.

8.4 DISCUSSION:

In the present study hypocalcaemia was an invariable consequence of laryngo-pharyngeal surgery where the thyroid gland is partially or completely excised. The overall incidence of permanent hypoparathyroidism following this type of surgery has been reported to be in the region of 5-15% (Thomson and Harness, 1970) however, transient hypocalcaemia is more common (Smith and Quiney, 1987). Thus, patients undergoing extensive neck surgery are immediately infused with calcium in order to anticipate the post-operative hypocalcaemia. The pathogenesis of this temporary hypocalcaemia is not clearly understood. One concept which has been suggested implicates the release of calcitonin (the "calcium-lowering" hormone) during the manipulation of the thyroid gland at surgery (Watson et al, 1981). However, this suggestion has been questioned and the most commonly accepted reason suggests that the removal or damage of the parathyroid gland during surgery results in hypo-parathyroidism and therefore hypocalcaemia.

There was no evidence of parathyroid gland function in these patients after surgery, as assessed by the presence of undetectable concentrations of the circulating hormone. However, Buchanan et al (1975) demonstrated a delayed response in the gland 200 days after total thyroidectomy. It is therefore suggested that the rapid reduction in circulating PTH was the major cause for the observed hypocalcaemia in this group of patients. With this evidence the next stage was to get an insight into the effects of

laryngo-pharyngeal surgery on the calcium homeostatic system with particular attention to the vitamin D metabolites.

After laryngo-pharyngeal surgery serum calcium concentrations decreased in asociation with temporary hypoalbuminaemia. Low albumin concentration is commonly seen after any type of surgery and is associated with the stress of the surgery. However, the reason for continued hypocalcaemia in these patients cannot be reduced albumin concentrations because when albumin concentrations returned to near-normal serum calcium concentrations still remained low. There was no relationship between serum albumin and calcium concentrations.

Post-thyroidectomy hypocalcaemia can also be due to either magnesium deficiency (Rude and Singer, 1981) or the rapid entry of calcium into depleted bone as concentrations of the thyroid hormone fall. Both of these ideas have been eliminated in this study. All patients had normal magnesium and thyroid hormone concentrations which were sustained by intravenous or oral supplementations.

With the immediate post-operative reduction of PTH concentrations it was important to investigate the concentrations of the vitamin D metabolites throughout the study period. The study demonstrates that before surgery all patients had serum $1,25(OH)_2D$ concentrations within the normal reference range but values fell significantly immediately after surgery. Potential explanations for this rapid reduction in this metabolite include a decrease in $1,25(OH)_2D$ synthesis, an increase in $1,25(OH)_2D$ catabolism and a reduction in the serum binding of $1,25(OH)_2D$. It is highly unlikely that the reduced $1,25(OH)_2D$ is related to the 25(OH)D concentrations although low concentrations of 25(OH)D were reported in 30% of the patients. However, there

was no correlation, either before or after surgery, between the serum concentrations of 25(OH)D and $1,25(OH)_2D$.

Less than 1% of total $1,25(OH)_2D$ concentration in the circulation is free and presumably biologically active; the remainder is transported by DBP to the target sites. Thus, the low concentration of this binding protein could account for the observed decrease in total $1,25(OH)_2D$ concentrations. Although total $1,25(OH)_2D$ concentrations after surgery were low, free $1,25(OH)_2D$ indices were normal and did not vary significantly during the post-operative study period. Mean concentration of DBP, measured in nine patients, was low immediately after surgery, but returned to normal a few days later. The reduction of DBP corresponded to the low concentrations of serum albumin and is therefore probably a consequence of surgical stress.

The lack of correlation between DBP and $1,25(OH)_2D$ and the failure of $1,25(OH)_2D$ to rise with DBP suggest that the concentrations of DBP were not responsible for the continually low concentrations of $1,25(OH)_2D$. This therefore implicates a defect in the synthesis of $1,25(OH)_2D$ and this deficiency has a profound affect on the synthesis of osteocalcin. The close interrelationship between circulating $1,25(OH)_2D$ and osteocalcin, as outlined in the chapter 6, has been reinforced in this study.

Although osteocalcin measurements were obtained from a small number of patients, results clearly demonstrate a similar reduction in circulating concentrations immediately post-operatively and remained low throughout the investigation. The administration of calcitriol, however, was unable to raise osteocalcin concentrations to pre-operative values after six days of treatment.

The two important physiological regulators of $1,25(OH)_2D$ synthesis and secretion are serum phosphate and PTH concentrations. In the present study the mean serum phosphate concentration rose after surgery but no correlation was found between the rise in serum phosphate concentrations and the change in serum $1,25(OH)_2D$. The rise was most probably related to the mobilisation of phosphate from bone to compensate for the prolonged reduction in extracellular calcium.

PTH maintains serum calcium concentrations within the normal range is by its action on the 1 alpha-hydroxylase activity in the kidney. Reduction or even removal of this hormone will therefore upset the synthesis of $1,25(OH)_2D$. This has been clearly demonstrated by the rapid reduction of the $1,25(OH)_2D$ metabolite immediately after surgery. There were no similar reductions in either the 25(OH)D or the $24,25(OH)_2D$ metabolites. Neither synthesis of which are independent of the action of PTH. Serum concentrations of $1,25(OH)_2D$ remained in the lower end of the normal reference range until the commencement of dietary calcitriol.

The reduction of circulating concentrations of PTH also had a profound effect on the renal reabsorption of calcium in these patients. The effect of PTH is to stimulate net calcium transport across renal tubular cells. Over 70% of calcium reabsorption occurs in the proximal convoluted tubules, and is tightly coupled to sodium reabsorption. Factors, therefore, which will enhance sodium reabsorption in the proximal tubules (e.g. volume depletion) will also enhance calcium reabsorption. Conversely, factors which diminish proximal tubular sodium reabsorption (e.g. saline infusion) will similarly reduce calcium reabsorption (Stewart and Broadus, 1981).

Only a small fraction of the filtered calcium load remains at the distal convoluted tubules. Calcium reabsorption in this segment is not associated with sodium reabsorption. It is here where PTH acts on calcium reabsorption by enhancing distal reabsorption. The absence or reduction of circulating PTH, as seen in these post-thyroidectomy patients, results in an overall increase in urinary calcium excretion, consistent with the loss of the effect of PTH on the distal nephrons.

With respect to treatment of laryngo-pharyngeal patients after surgery it is important to give parenteral feeding supplemented to give 10mmol/day of calcium in order to produce satisfactory concentrations (around 2.0 mmol/L) of serum calcium. Additionally, calcium given through a peripheral vein may result in a higher level of serum calcium but experience shows that this causes problems with venous thrombosis. Calcium supplementation with 10mmols/day allows a wide range of safety while protecting against tetany. It also avoids the risk of hypercalcaemia during the change over to oral supplements if the latter is commenced whilst the patient is still receiving intravenous calcium. Such an overlap of treatment is recommended in order to avoid hypocalcaemia which, once established, may take several days to correct. This was well demonstrated in the two patients who became severely hypocalcaemic.

There are two approches to the prevention and treatment of hypocalcaemia in patients receiving oral replacement therapy. Firstly, the efficiency of intestinal calcium absorption can be increased by the use of large doses of vitamin D, to achieve maximal calcium absorption with a normal diet. One disadvantage, however, is the potential toxicity of vitamin D. The other alternative is to use calcium supplements with smaller doses of vitamin D, preferably in the form of $1,25(OH)_2D$. This allows adjustment

in calcium intake to regulate the concentration of serum calcium. Whichever approach is adopted it is important to be aware that the setting of renal tubular calcium reabsorption may rise if the patient becomes salt depleted and also increase after restoration of normocalcaemia. This present study has demonstrated that persistant hypocalcaemia lowers the setting of TmCa/GFR, resulting in an increased renal leak of calcium with a tendency to worsening hypocalcaemia.

Hypothyroidism (Bouillon and DeMoor, 1974) does not seem to be a major factor in regulating calcium reabsorption in these patients since the three patients who had become profoundly hypocalcaemic after surgery, either due to interruption or a delay in the initiation of replacement therapy, all had impaired renal tubular reabsorption of calcium. In these three cases the additional contributing factor was likely to be the loss of the regulatory function of parathyroid hormone in controlling skeletal calcium influx and efflux.

Protection from hypocalcaemia is thought to occur in two distinct stages (Parfitt, 1976). The immediate but limited response is the release of stored calcium from a labile pool. This is then followed by a more prolonged (and quantitatively greater) increase in osteoclastic bone resorption. In the presence of inadequate post-operative treatment with calcium and vitamin D supplements the labile pool may become depleted which together with reduced osteoclastic recruitment, due to parathyroid hormone deficiency, will contribute to the development of a profound hypocalcaemia.

Thus, supplementation for patients following laryngo-pharyngectomy surgery is necessary and also similar irrespective of the type of operation performed. Although replacement therapy is straightforward, if the

administration of appropriate therapy is delayed profound as well as prolonged hypocalcaemia can occur.

CHAPTER 9

METABOLISM OF 25-HYDROXYVITMAN D IN OSTEOSARCOMA CELL LINE 20S

9.1 INTRODUCTION:

The physiological actions of vitamin D require activation by hydroxylations in both the liver and kidney to produce $1,25(OH)_2D$. This form of the vitamin is the active metabolite which is known to play an important role in normal bone homeostasis, both in growth and mineralisation. However, the major vitamin D metabolite in blood (Bouillon, 1983) and bone itself (Haussler & Rasmussen, 1972) is the 25-hydroxy derivative. In bone, 25(OH)D appears to be associated with areas of active mineralisation (Wezeman, 1976) and it has been suggested that this metabolite may play a role in stimulating bone formation.

Although synthesis of $1,25(OH)_2D$ occurs predominantly in the kidney, extrarenal production has been shown in a variety of tissues including placenta (Weisman et al, 1979; Whitsett et al, 1981), human pulmonary alveolar macrophages (Barbour et al, 1981), human keratinocytes (Bikle et al, 1986), transformed lymphocyte (Reichel et al, 1987b) and chick and human bone cells (Turner et al, 1980; Howard et al, 1981; Puzas et al, 1987). In vivo evidence has been demonstrated by Rosenthal et al (1985) who found elevated $1,25(OH)_2D$ concentrations in some patients with non-Hodgkins lymphoma. Since these hypercalcaemic patients had renal impairment, suppressed PTH and nephrogenous cAMP it is possible that the $1,25(OH)_2D$ originated from the lymphoma rather than the kidney, a view which is supported by the

decline in $1,25(OH)_2D$ concentrations when the tumour was removed surgically or treated medically.

24-hydroxylation of 25(OH)D, like 1 alpha-hydroxylation, occurs predominantly in the kidney and is induced by raised concentrations of $1,25(OH)_2D$. It is suggested that renal 24-hydroxylation may either limit production of $1,25(OH)_2D$ by competing for 25(OH)D, or inactivate $1,25(OH)_2D$ by conversion to the trihydroxy metabolite (DeLuca and Schnoes, 1983). Extrarenal 24-hydroxylation has also been reported in a variety of tissues including bone cells (Lohnes and Jones, 1987), calvarial cell (Pusaz et al, 1987), virus-transformed lymphocytes (Reichel et al, 1987) and human decidua and placenta (Weisman et al, 1979). However, the physiological role of $24,25(OH)_2D$ in these cells remains uncertain but studies have demonstrated a possible role for this vitamin D metabolite in bone mineralisation (Ornoy et al, 1978) and in normal fracture repair (Meller et al, 1984).

An intrinsic function for $24,25(OH)_2D$ in bone formation has been proposed (Endo et al, 1980; Lohnes and Jones, 1987) and Tam et al (1986) suggest that, in conjunction with PTH, $24,25(OH)_2D$ may play a role in the regulation of bone formation and/or bone mineralisation. Indirect evidence to support this comes from Muirhead et al (1980) who observed that long-term treatment with $24,25(OH)_2D$ alone in chronic renal failure increases plasma alkaline phosphatase activity without increasing plasma hydroxyproline. Plasma alkaline phosphatase is considered to be a valid index of bone formation whereas hydroxyproline an index of bone resorption.

Since knowledge about the production of vitamin D metabolites by bone cells and its regulation may be essential for understanding the effect of vitamin D on the skeleton the present study investigates the metabolism of

25(OH)D by the human osteoblast-like osteosarcoma cell line 20S. This cell line still retains many of the native osteoblast phenotype, such as increased alkaline phosphatase activity, PTH sensitive adenylate cyclase activity and the ability to synthesise osteocalcin and, therefore, is an ideal system to study. With this line of investigation and by searching for possible receptors for 25(OH)D, $24,25(OH)_2D$ and $1,25(OH)_2D$ in this cell line it is proposed that one would gain some insight into the active form or forms of vitamin D in bone.

9.2 METHODS:

For all experimental procedures refer to section 2.3.

9.3 RESULTS:

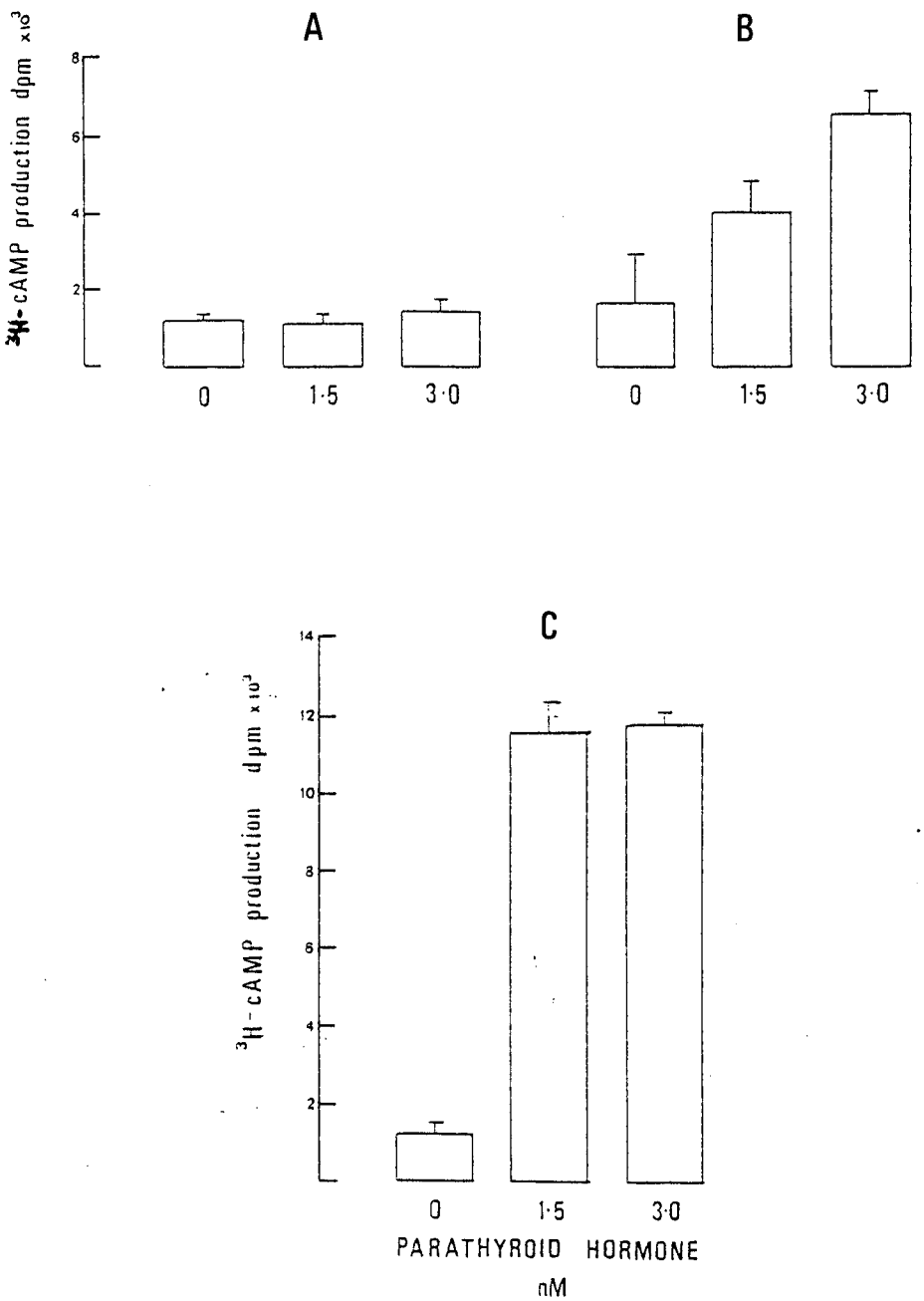
9.3.1 CHARACTERISATION OF THE 20S CELL LINE

(i) Cellular response of 20S to Parathyroid hormone

Three osteosarcoma cell lines were kindly provided by Dr. J Embleton (cell lines 791T, T278 and 20S) however, only cell line 20S produced a satisfactory response to PTH stimulation as illustrated in figure 9.1. Treatment with different concentrations of human 3-34 PTH produced a dose-dependent increase in the production of cAMP in both 20S and 791T but the extent of stimulation of adenylate cyclase by PTH in the 20S cell line was far greater (figure 9.1). Significant production of cAMP was observed at a dose of 1.5nM PTH whereas in cell line 791T the dose was 3nM. In contrast, cell line T278 totally lacked PTH responsiveness; doses of up to 3nM PTH failed to stimulate the production of

FIGURE 9.1. Dose response effect for hPTH (3-34) stimulation of cAMP in (A) T278, (B) 791T and (C) 20S cells, as described in section 2.4.1.

Each point represented by triplicate determinations ± SD.



cAMP. Because of its retained ability to respond to PTH cell line 20S was used for the remainder of experimental work.

(ii) Presence of alkaline phosphatase activity

Alkaline phosphatase activity in cell line 20S was assessed as described in section 2.3.9. The production of p-nitro phenol, indicated by the yellow appearance of the incubation mixture, clearly established the presence of alkaline phosphatase activity in 20S cells. No yellow colour appeared when, as controls, the monocytic cell line U937 and buffer were incubated with the substrate.

(iii) Production of osteocalcin by 20S cells

In order to establish that this human osteosarcoma cell line was synthesising and secreting osteocalcin, a 12,000 molecular weight bone-specific γ-carboxyglutamic acid-containing protein, a RIA which can detect nanograms of human osteocalcin (see section 2.6) was used to examine a 24h (or 48h) sample of serum-free media harvested from confluent cultures. The presence of 20ng/ml of osteocalcin in the medium suggest that these cells do synthesise osteocalcin. However, no further experiments were carried out to verify that this protein, which cross-reacted with the anti-osteocalcin antiserum, was indeed osteocalcin. Analysis of the harvested serum-free medium on SDS-polyacrlamide gel electrophoresis showed the presence of a protein band which migrated with an apparent molecular weight of 12,000 daltons on 15% SDS-polyacrylamide gel (Salhi, A., personal communication). This 12,000 dalton product may represent osteocalcin synthesised and released from the 20S cell line.

From these preliminary studies the human osteosarcoma cell line 20S appears to maintain its differentiated function in culture as indicated by production of alkaline phosphatase and osteocalcin, and by its responsiveness to parathyroid hormone.

9.3.2. THE METABOLISM OF 25(OH)D BY THE OSTEOSARCOMA CELL LINE 20S

The metabolism of physiological concentrations of 25(OH)D was studied initially. When 20S cells were incubated for 3h with 50nM ^{3}H-25(OH)D a second peak of radioactivity was seen on HPLC analysis of the lipid extracts from the cells. The radioactive profile of the eluate is shown in figure 9.2. The major peak at 3.5min represents the substrate 25(OH)D_3. The unknown metabolite had a retention time (7.5-9.5min) identical to that of authentic 24,25$(OH)_2$D. This metabolite was not produced in medium that did not contain cells nor was it produced when a similar quantity of radioactive 25(OH)D was applied directly onto the HPLC column. When this second peak was re-chromatographed on the same HPLC system the radioactivity again co-migrated with 24,25$(OH)_2$D.

Since separation of this unknown polar vitamin D_3 metabolite (denoted metabolite X) from 25(OH)D and 1,25$(OH)_2$D was successful (figure 9.2) one can postulate that this cannot be the hormonally active vitamin D_3 metabolite. These observations prompted more detailed investigation of the 25(OH)D metabolising system of these cells. The results of time course study is illustrated in figure 9.3. The synthesis of this metabolite was linear with time for 8h. Little synthesis occured after this time and only a small amount was detected in the incubation medium. There was no detection of any metabolite more polar than

FIGURE 9.2. Analysis by HPLC of radioactive lipid extracts of 20S cells incubated with ^{3}H-25(OH)D_3.

Lipid extracts were separated on a Zorbax-Sil straight phase HPLC column eluted with hexane:methanol:isopropanol (92:4:4 by volume). Fractions (30sec) were collected and assayed for radioactivity. Arrows mark the retention times of authentic vitamin D_3 metabolites determined by U.V. absorption.

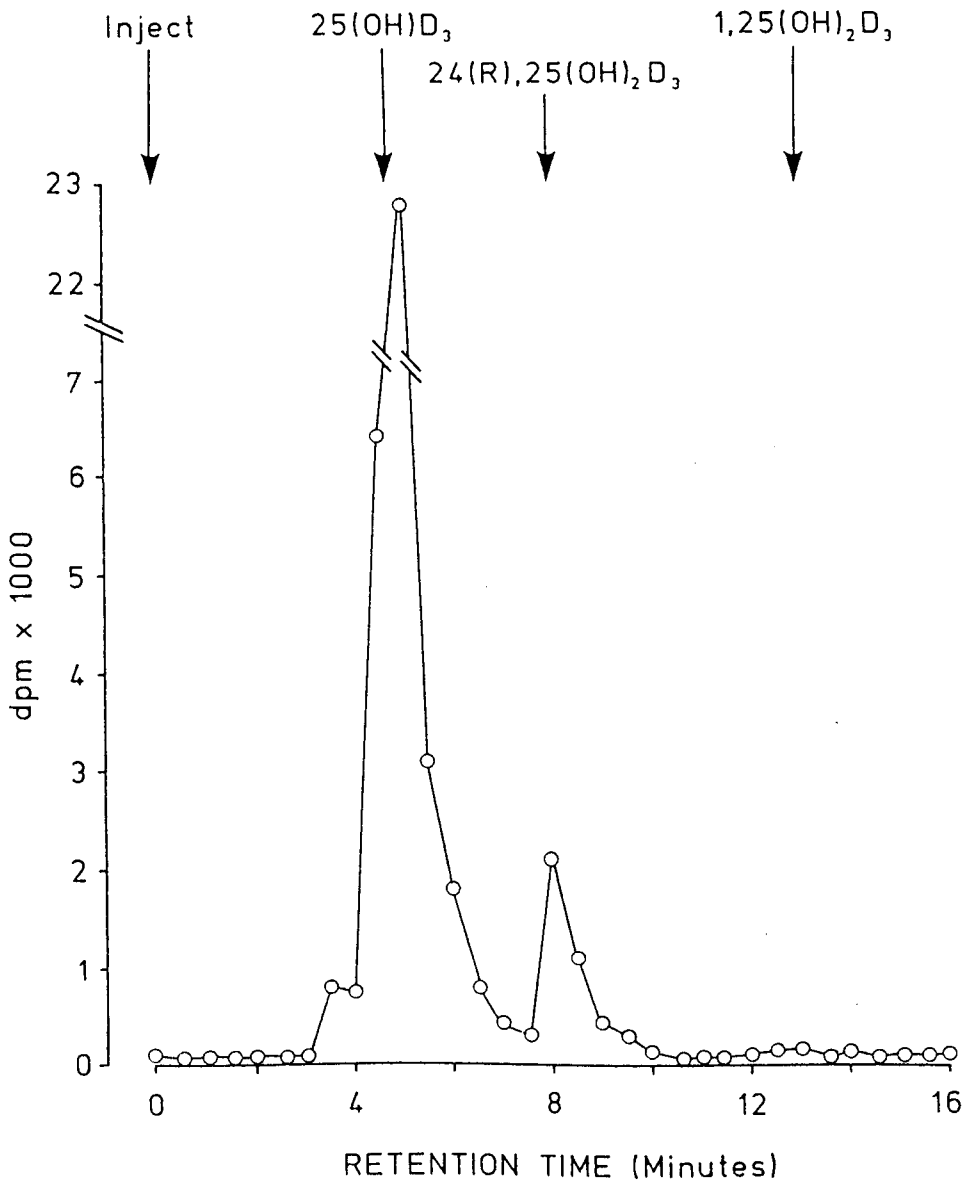
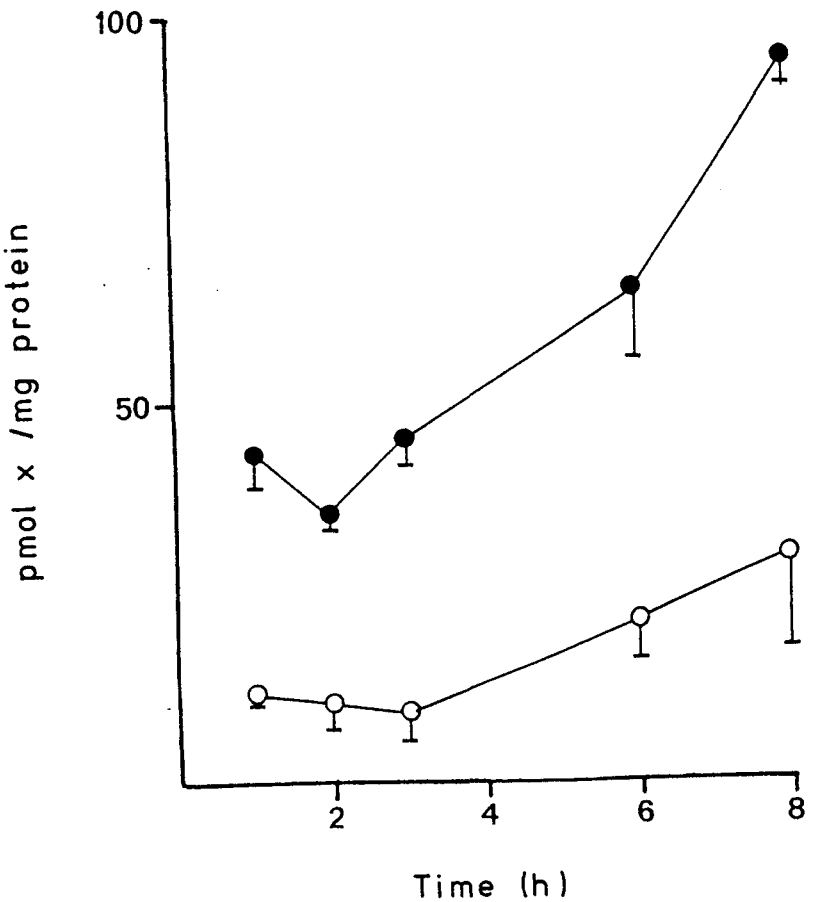


FIGURE 9.3. Time course of the synthesis and secretion of $25(OH)D_3$ metabolites by 20S cells.

Cells were grown to near-confluence and transferred to serum-free medium for 18h. They were incubated with 0.25nM $25(OH)D_3$ containing 0.25uCi ^3H-$25(OH)D_3$ per dish. Lipids were extracted from cells and incubation medium and separated by HPLC as described in methods section. Each point represents mean $\pm$ SD of four determinations.

● cells ○ medium



$24,25(OH)_2D$ during these incubation times and neither was there a redistribution of substrate and product into media since the metabolite was recovered almost exclusively from cells (figure 9.3).

Synthesis was also dependent on substrate concentrations, producing a sigmoidal response with concentrations up to 0.5uM ^{3}H-25(OH)D (figure 9.4).

In further experiments, 20S cells were pre-incubated for 14h with 1mM dibutyryl cAMP (dbcAMP) before repeating the substrate concentration experiments in the presence of 1mM dbcAMP. Exposure of these cells to this cAMP analogue produced an unexplainable decrease in the progressive synthesis of this polar vitamin D_3 metabolite as substrate concentrations increased (figure 9.5). This became significantly different at 25(OH)D concentrations greater than 0.5uM.

The identification of the product which elutes with $24,25(OH)_2D$ was then investigated by mass spectrometric analysis. Large scale incubations, involving eighty culture dishes (140mm diameter), were used to generate sufficient quantity of the unknown metabolite for analysis. Fractions corresponding to the metabolite (retention time 7.5-9.5min) (figure 9.6) were combined from the Zorbax-Sil column and applied to the silica sep pak column, as described in the method section. The corresponding $24,25(OH)_2D$ fractions were collected and re-chromatographed a further three times on the same HPLC system. The absorbance at 263nm was measured in fractions eluting from the fourth chromatographic step (figure 9.7). Unfortunately, the efficiency of the extraction and purification procedures could not be monitored due to the unavailability of a suitable recovery standard. A strongly absorbing peak eluted from the column after 7.5min and corresponded to that of $24,25(OH)_2D$. The

FIGURE 9.4. Substrate concentration dependence of the formation of $25(OH)D_3$ metabolites in 20S cells with increasing concentration of $25(OH)D_3$ containing 0.1uCi/dish.

Vitamin D metabolites were extracted and separated on HPLC. Values represent the mean of triplicate determinations.

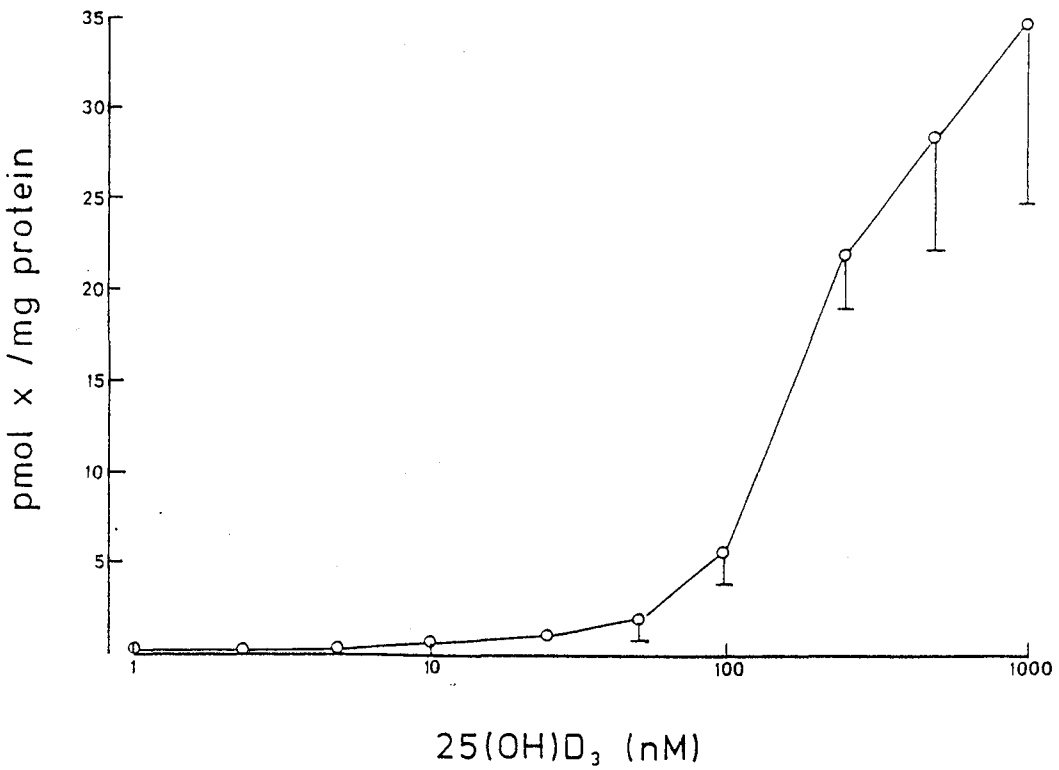


FIGURE 9.5. The synthesis of the unknown vitamin D metabolite with increasing concentration of $25(OH)D_3$ containing 0.1uCi/dish: in the presence ▨ or absence □ of 1mM dibutyryl cAMP (dbcAMP).

(A) 0.05uM $25(OH)D_3$ (B) 0.1uM $25(OH)D_3$
(C) 0.5uM $25(OH)D_3$ (D) 1.0uM $25(OH)D_3$

Each point represents the mean ± SD of four determinations.
* $0.02<p<0.05$ ** $0.01<p<0.001$

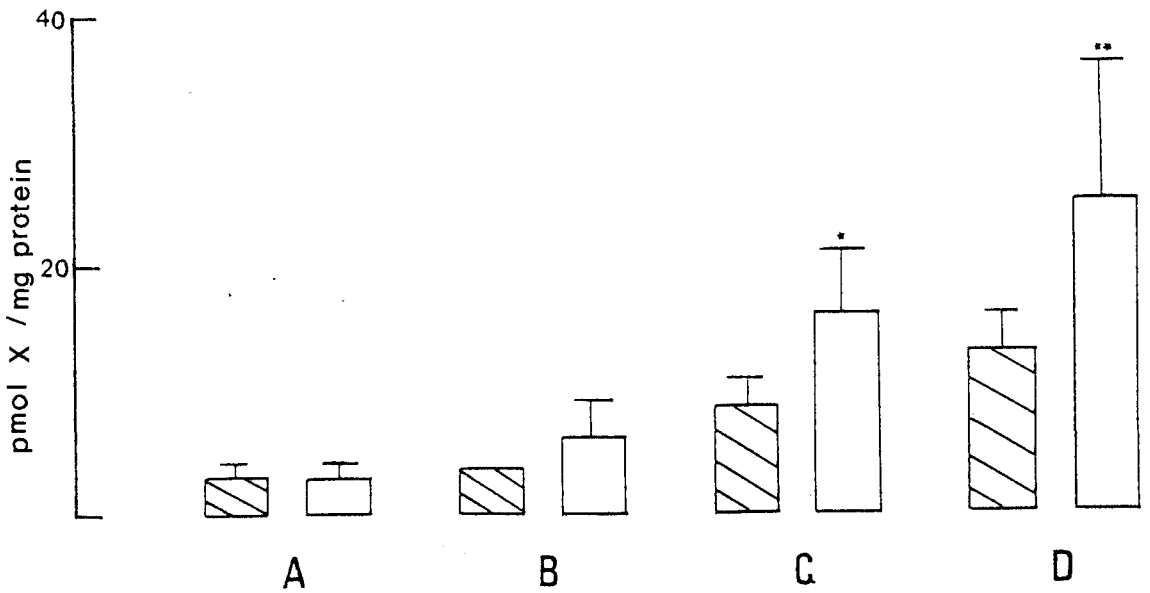


FIGURE 9.6. Initial HPLC chromatogram of lipid extracts from media and cells.

Arrows mark the retention times for the authentic vitamin D_3 metabolites.

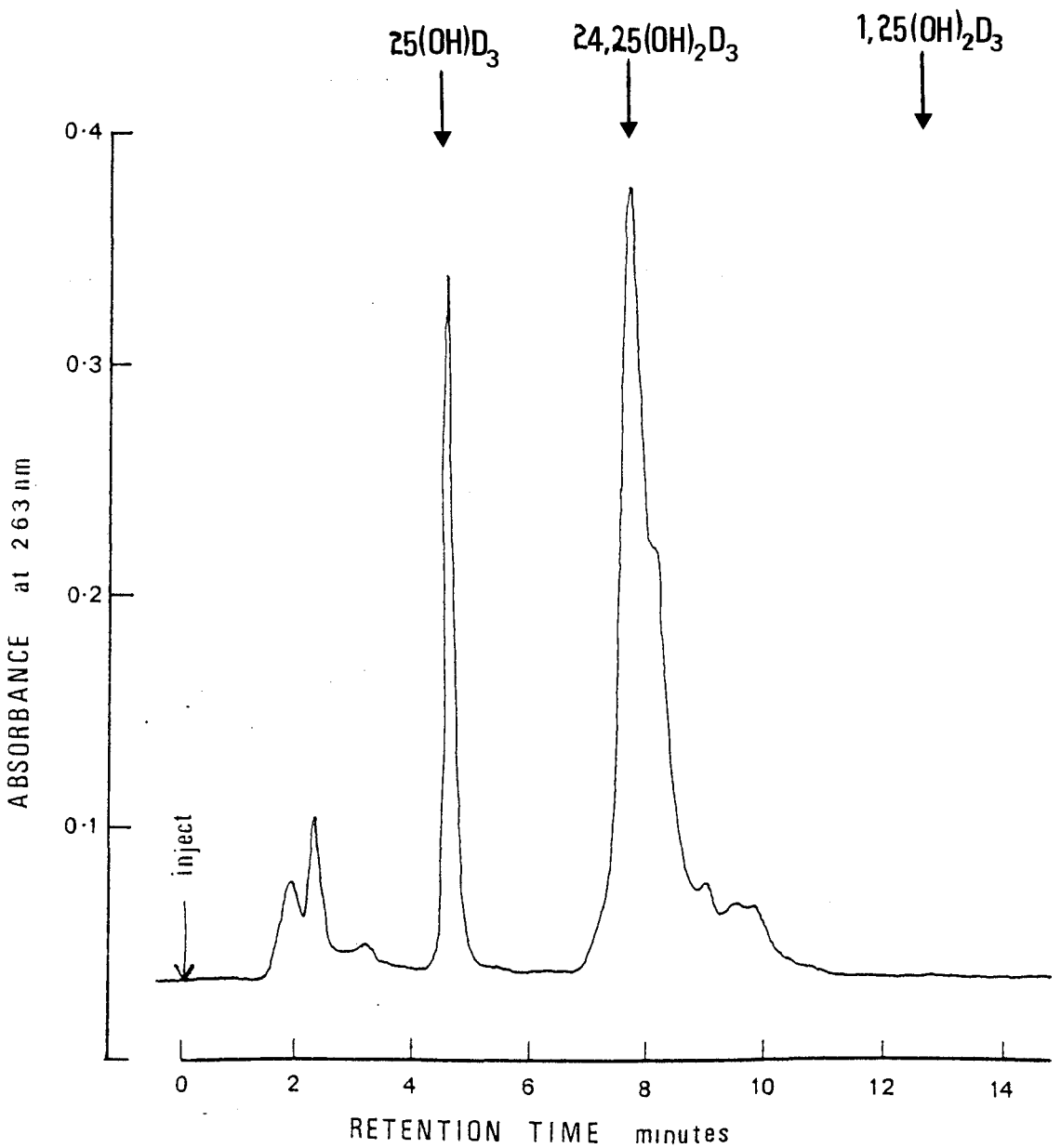
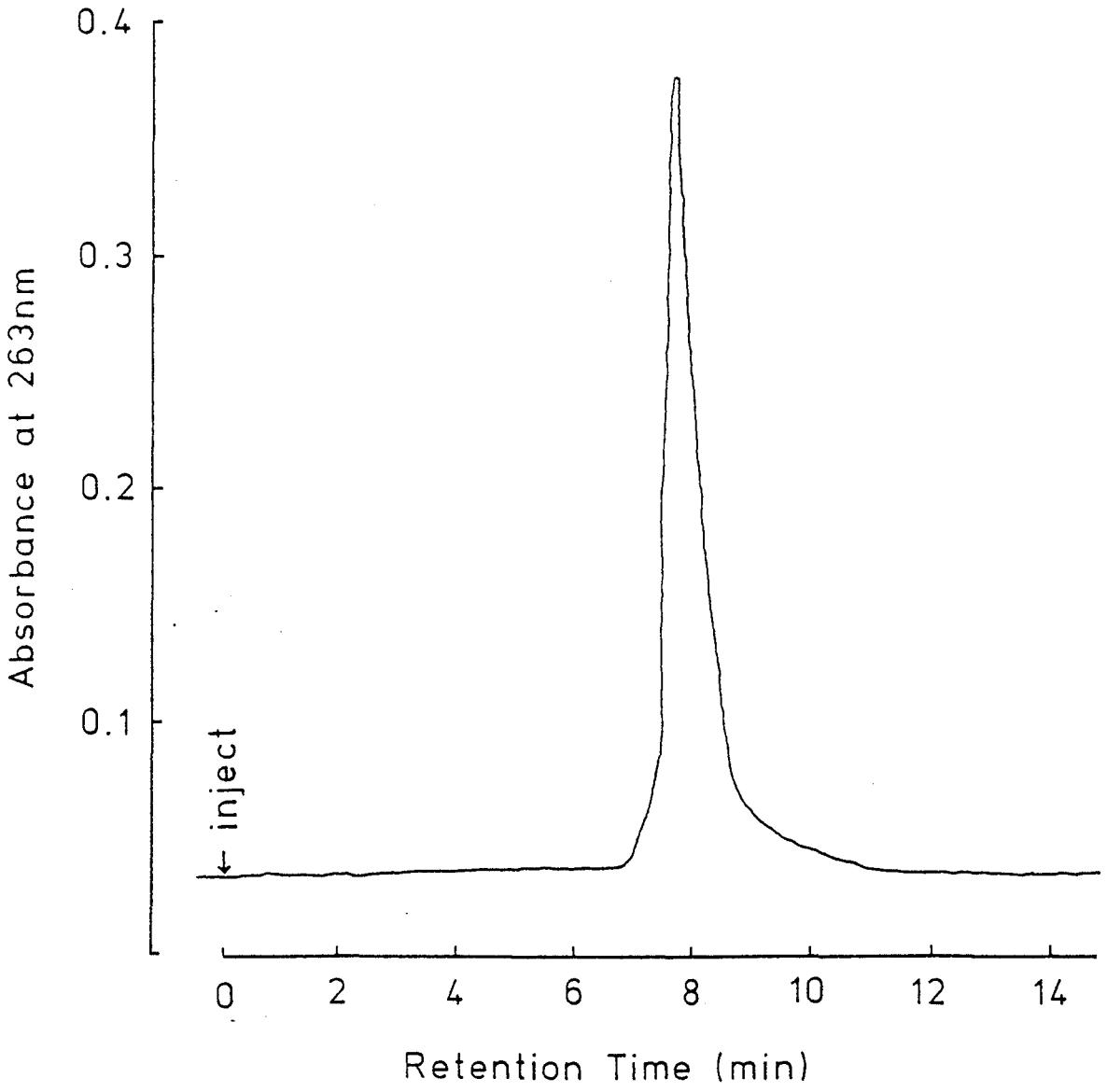


FIGURE 9.7. The final HPLC chromatogram of the unknown metabolite.



retention time of the 25(OH)D substrate, which also absorbed at this wavelength was 3.5-6min.

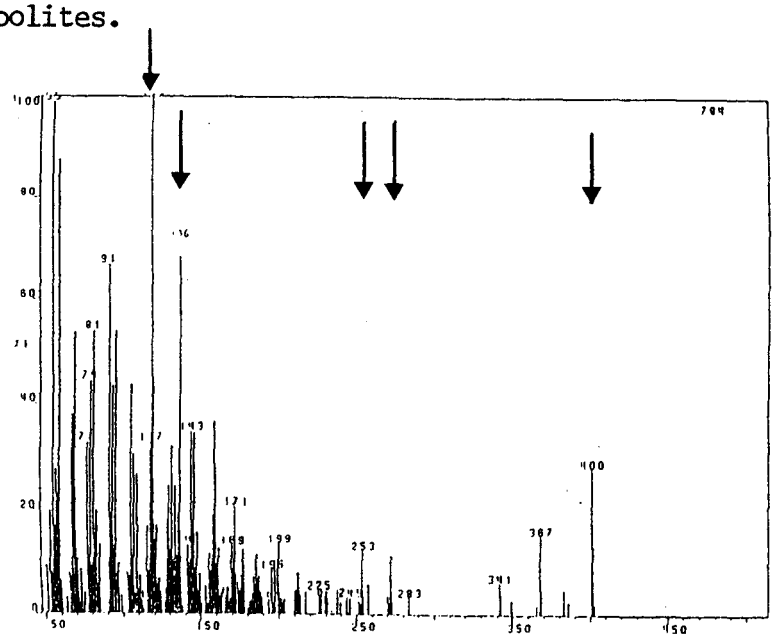
Fractions eluting from the column between 7.5min and 9min were combined, evaporated to dryness under N_2 and subjected initially to UV analysis followed by mass spectroscopy analysis. The mass spectrum obtained from this unknown (figure 9.9) was compared with those of the authentic vitamin D_3 metabolites: $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ (figure 9.8). The 25(OH)D substrate extracted and purified by similar methods was also analysed by mass spectroscopy (figure 9.10).

Ultraviolet (UV) spectral analysis of the unknown had a UV absorption maximum (λ_{max}) at 264nm and an absorption minimum (λ_{min}) of 228nm, which was typically seen when authentic vitamin D_3 metabolites were analysed by UV absorption (figure 9.11).

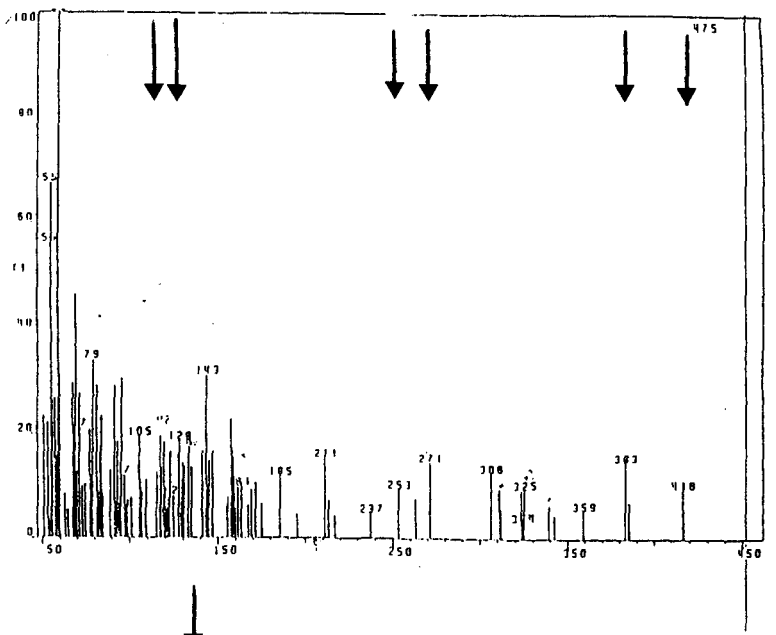
Spectral analysis confirmed the HPLC evidence. The mass spectrum of the $24,25(OH)_2D$ sample was obtained using a temperature probe of 300°C. (figure 9.9a). Despite the presence of some impurities the spectrum obtained for the sample clearly shows the presence of $24,25(OH)_2D$; particularly noteworthy is the occurance of the molecular ion (M^+) at m/z 416 and the characteristic fragmentation at m/z 383 (M^+ minus H_2O and CH_3). The following smaller fragmentation ions are also common to both spectra to further support the assignment:

m/z	271	..... loss of side chain
	253	..... 271 minus H_2O
	136	..... cis-triene cleavage
	118	..... 136 minus H_2O

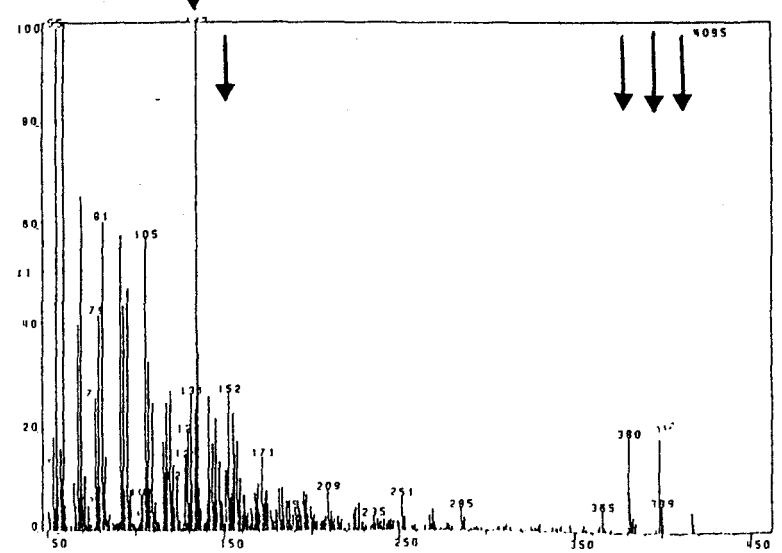
FIGURE 9.8. Mass spectral analysis of the authentic vitamin D_3 metabolites.



$25(OH)D_3$



$24,25(OH)_2D_3$



$1,25(OH)_2D_3$

FIGURE 9.9. Mass spectra of metabolites extracted from incubation of 20S cells with $25(OH)D_3$.

Mass spectra were produced using a temperature probe of (a) 400°C and (b) 250°C as outlined in method section 1.4.4.

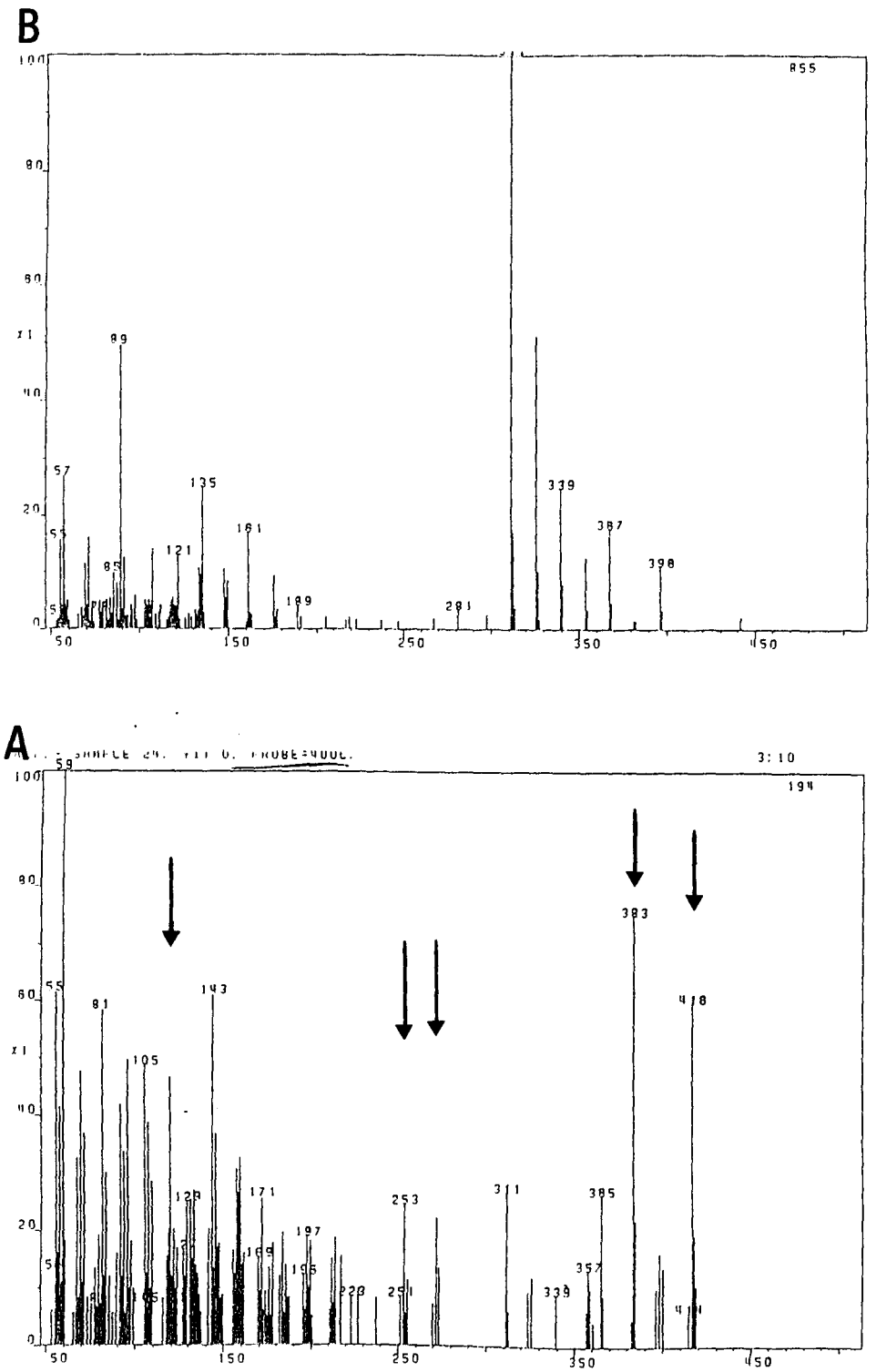
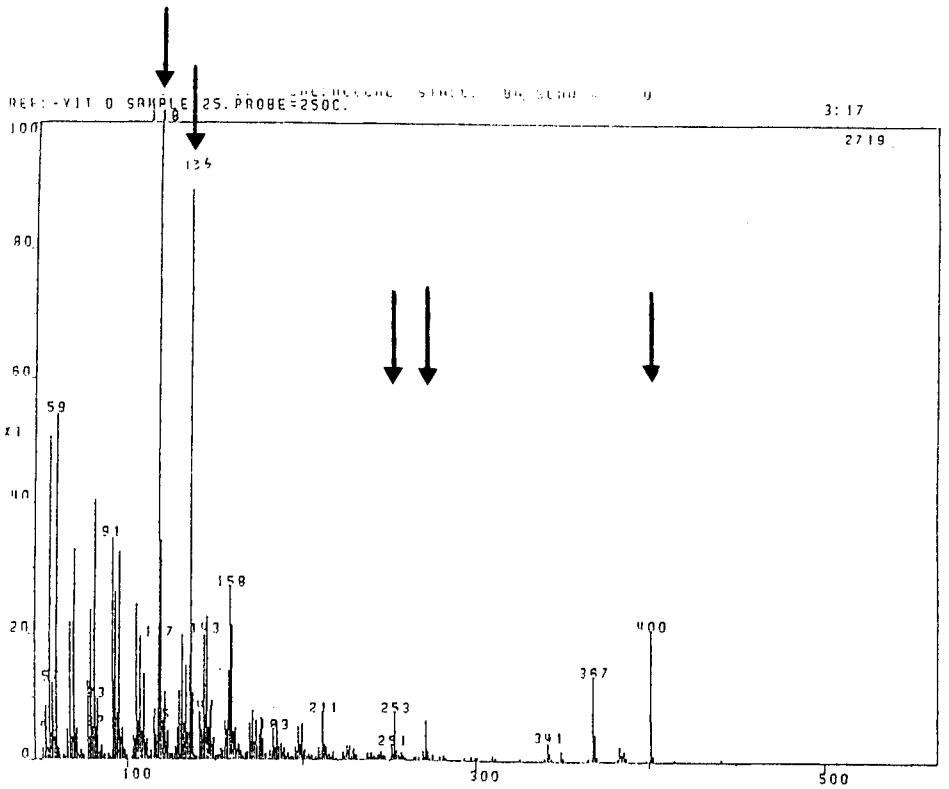
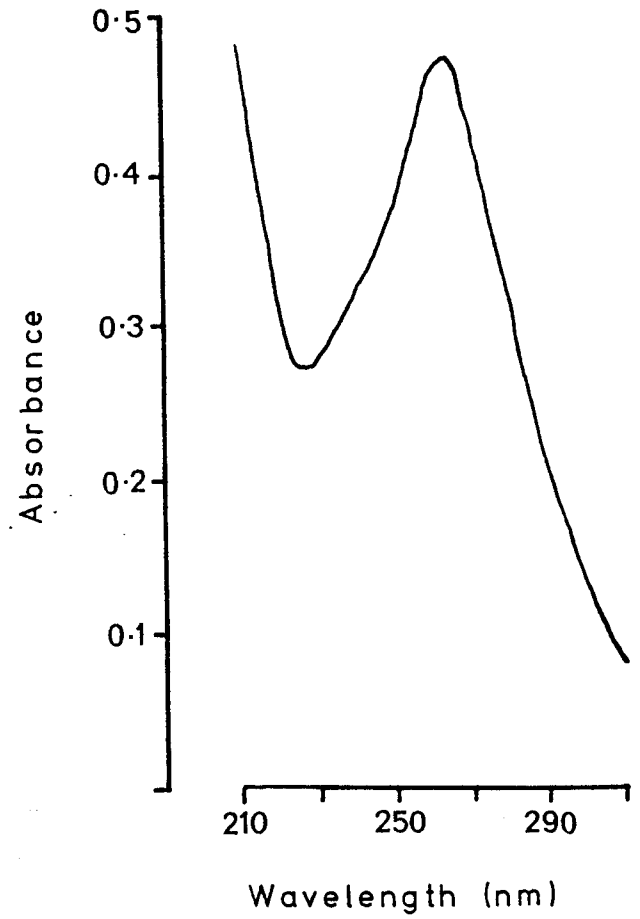


FIGURE 9.10. Mass spectral analysis of the 25(OH)D_3 substrate extracted and purified from 20S cells as detailed in methods section.



Vertical arrows in figures 9.8., 9.9. and 9.10. highlights the characteristic m/z fragments produced when vitamin D_3 metabolites are subjected to mass spectroscopy.

FIGURE 9.11. Ultraviolet absorption spectrum of the unknown metabolite eluting between 7.5min and 9.5min from the final HPLC separation.



The spectrum showed no resemblance to that produced by authentic 25(OH)D indicating that the chromatography steps were efficient in separating the two vitamin D metabolites completely.

On analysis of the purified 25(OH)D fraction by mass spectroscopy a similar, if not identical spectrum to authentic $25(OH)D_3$ was produced with a characteristic molecular ion at m/z 400 (figure 9.10). The major fragment ions, also characteristically seen in both spectra, are m/z 271, 253, 136 and 118.

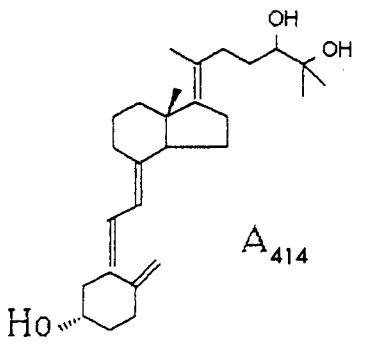
At the lower probe temperature of 250°C an unexpected second metabolite was detected which exhibited the spectrum shown in figure 9.9b, and which was obtained prior to that of the $24,25(OH)_2D$ spectrum. The fact that this second metabolite co-eluted from the HPLC column with $24,25(OH)_2D$ strongly suggests that it is also a tri-hydroxylated species. On the basis of this spectrum it is tentatively suggested that this unknown metabolite has the C_{17}-C_{20} unsaturated structure A (refer to figure 9.12).

The base peak at m/z 311 could be due to the relatively stable allylic cation (structure B in figure 9.12). Loss of water from the molecular ion (which was not observed) would account for the m/z 396 while dehydration could give rise to the ketone structure C. Fragmentation of this would lead to both m/z 325 (structure D) and m/z 353 (structure E) and the corresponding protonated species, m/z 354.

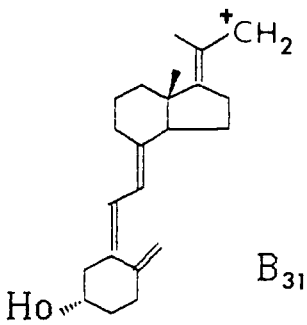
The ion m/z 367 seen in the spectrum could be due to dehydration of the secondary alcohol function followed by the loss of both methyl groups; m/z 339 would then arise by decarbonylation of this fragment. A number of other related pathways could also explain these fragments and it is stressed that the above is only a tentative assignment.

FIGURE 9.12. Structures deduced from the mass spectrum produced at 250°C.

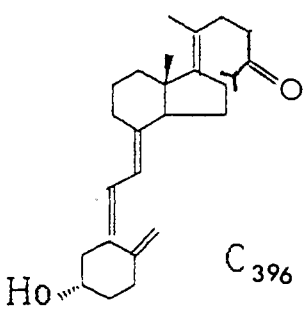
Refer to results section for explanation.



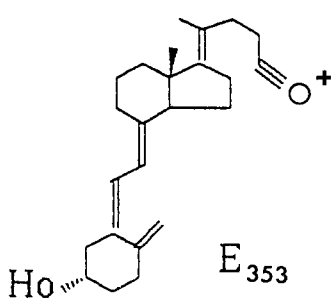
A_{414}



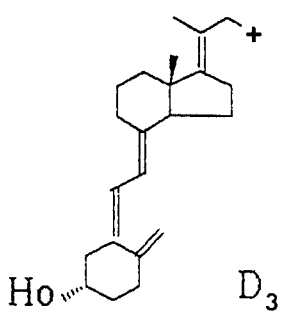
B_{311}



C_{396}



E_{353}



D_{325}

9.3.3 BINDING OF 25(OH)D_3 TO 20S CYTOSOLIC PROTEINS

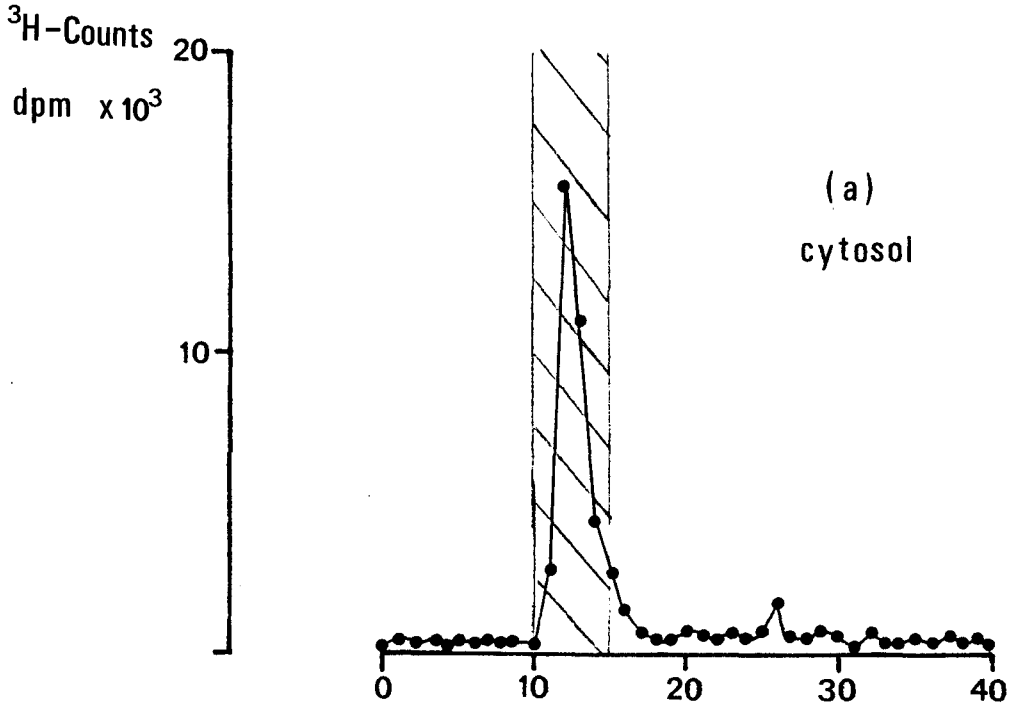
(i) Initial incubation with ^{3}H-vitamin D metabolites

Incubation of 20S cytosolic proteins with ^{3}H-25(OH)D produced a single peak of radioactivity when the sample was applied on to a G-25 column. This peak was totally displaced by unlabelled 25(OH)D present at 100-fold molar excess. A similar peak was also observed when 20S cytosol was treated with rabbit antibovine whole serum prior to incubation with ^{3}H-25(OH)D. There was little or no difference between the amount of radioactivity present in these two peaks (figure 9.13). No radioactive peak was seen when cytosol was incubated with an equivalent amount of ^{3}H-1,25$(OH)_2$D or ^{3}H-24,25$(OH)_2$D.

Results from the double immunodiffusion analysis (figure 9.14) showed no line of precipitation between the antibody and the cytosolic protein therefore demonstrating that the cytosolic preparation was free from contamination by serum proteins. As controls NCS (at 1,000-fold dilution) and BSA (0.25mg/ml) were used, both of which formed lines of precipation with the antibody.

A single peak of radioactivity was seen on Sephadex G-100 gel filtration of 20S cytosolic proteins incubated with ^{3}H-25(OH)D (figure 9.15). The positions of the reference proteins are also shown and suggest that the 25(OH)D ligand binds to a protein(s) of molecular weight approximately 75,000Da. This molecular weight was confirmed by analysis of the cytosolic proteins by sucrose density gradient centrifugation (figure 9.16), producing a sedimentation coefficient (S) of 4.7. The binding activity of normal serum (MW 56,000Da) is quite separate from that of the cytosolic binding protein (refer to figures 9.15 and 9.16) although a shoulder on the latter suggests some serum binding protein is carried through during the cellular fractionation

FIGURE 9.13. Sephadex G-25 chromatography of 20S cytosolic proteins previously incubated with (a) ^{3}H-25(OH)D_3 and (b) rabbit antibovine whole serum (A/B) followed by ^{3}H-25(OH)D_3.



500ul fractions were collected and the fractions corresponding to the shaded area were pooled and assessed on Sephadex G-100.

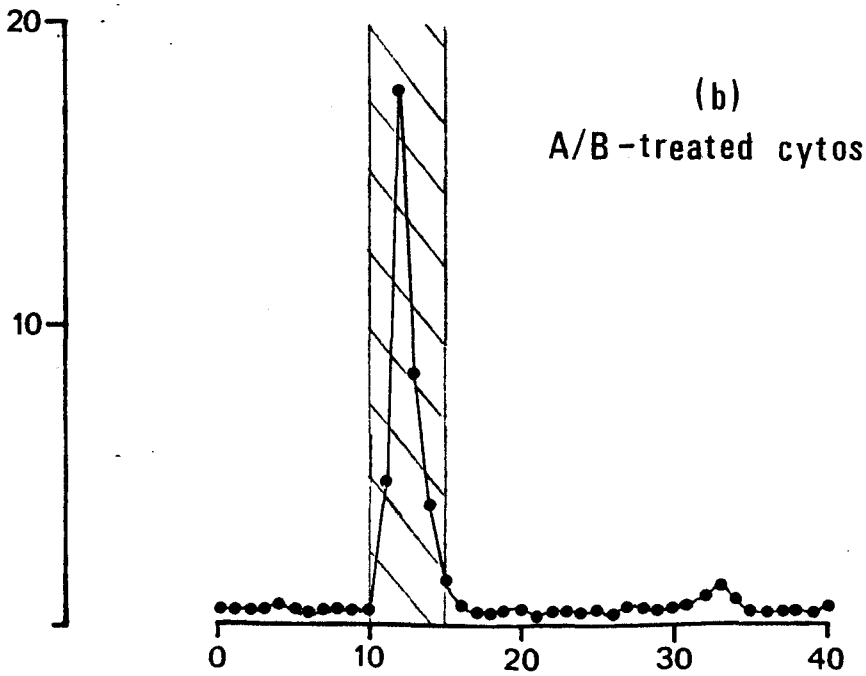


FIGURE 9.14. Double immumodiffusion analysis of 20S cytosolic proteins.

No line of precipitation between the antiserum and the cytosolic proteins (c) was observed but both BSA (a) and NCS (b) demonstrated antibody-antigen reaction with the antiserum.

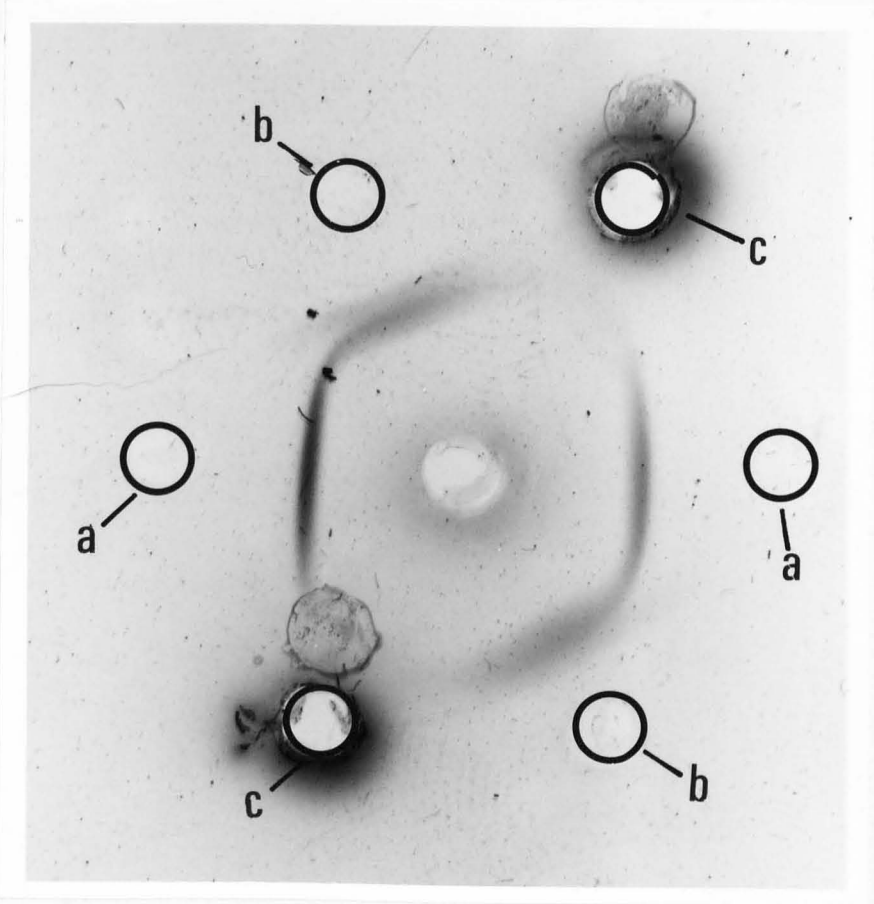


FIGURE 9.15. Sephadex G-100 chromatography of 20S cytosolic proteins and human serum previously incubated with ^{3}H-25(OH)D_3.

^{3}H-25(OH)D_3 (80,000dpm) was incubated at 4°C overnight with the preparations and applied to a G-25 column before finally applying on to the G-100 column. Fractions (1ml) were collected and assayed for radioactivity.

b - bovine serum albumin, M.W. 66,000; o - ovalbumin, M.W. 45,000; c - cytochrome c, M.W. 17,000.

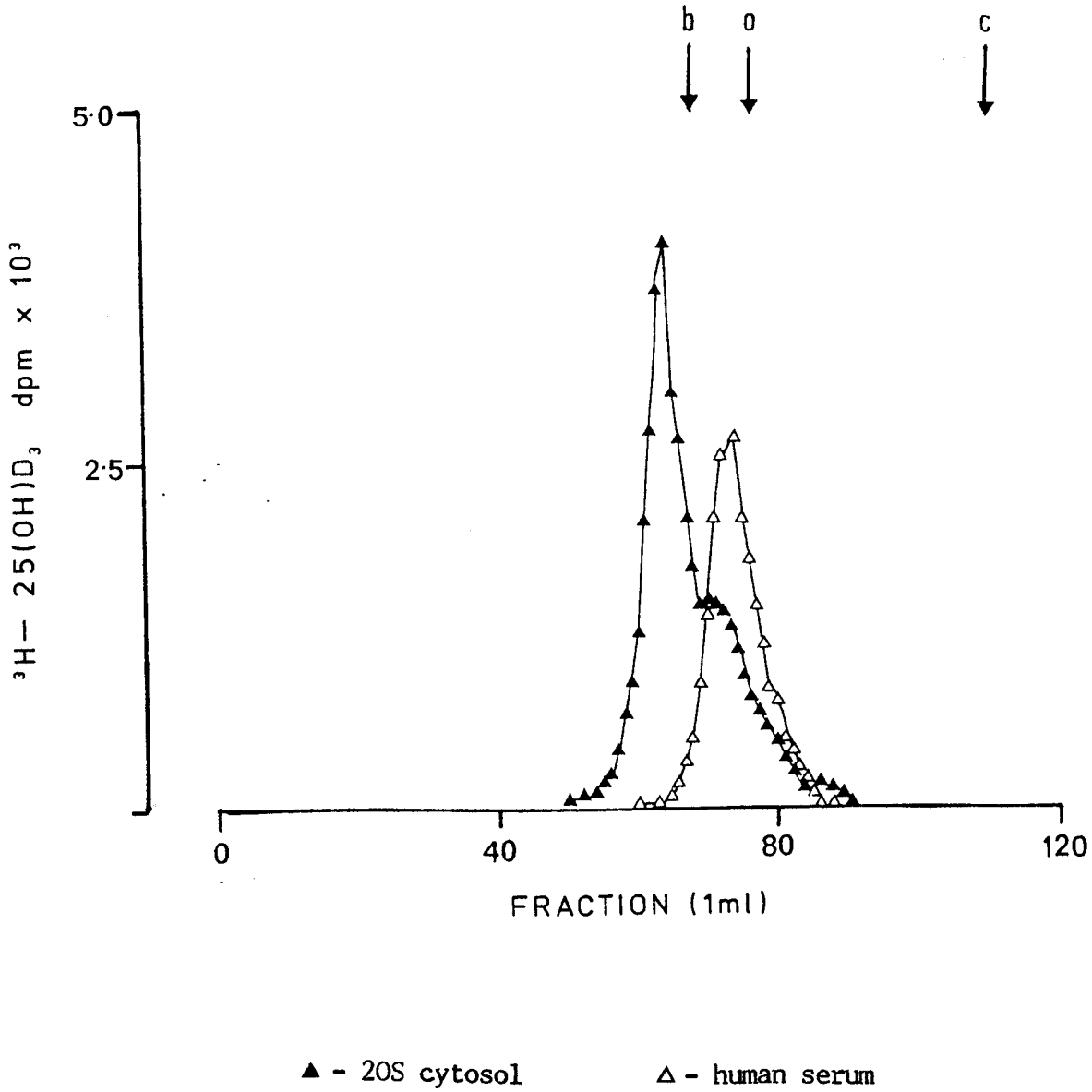
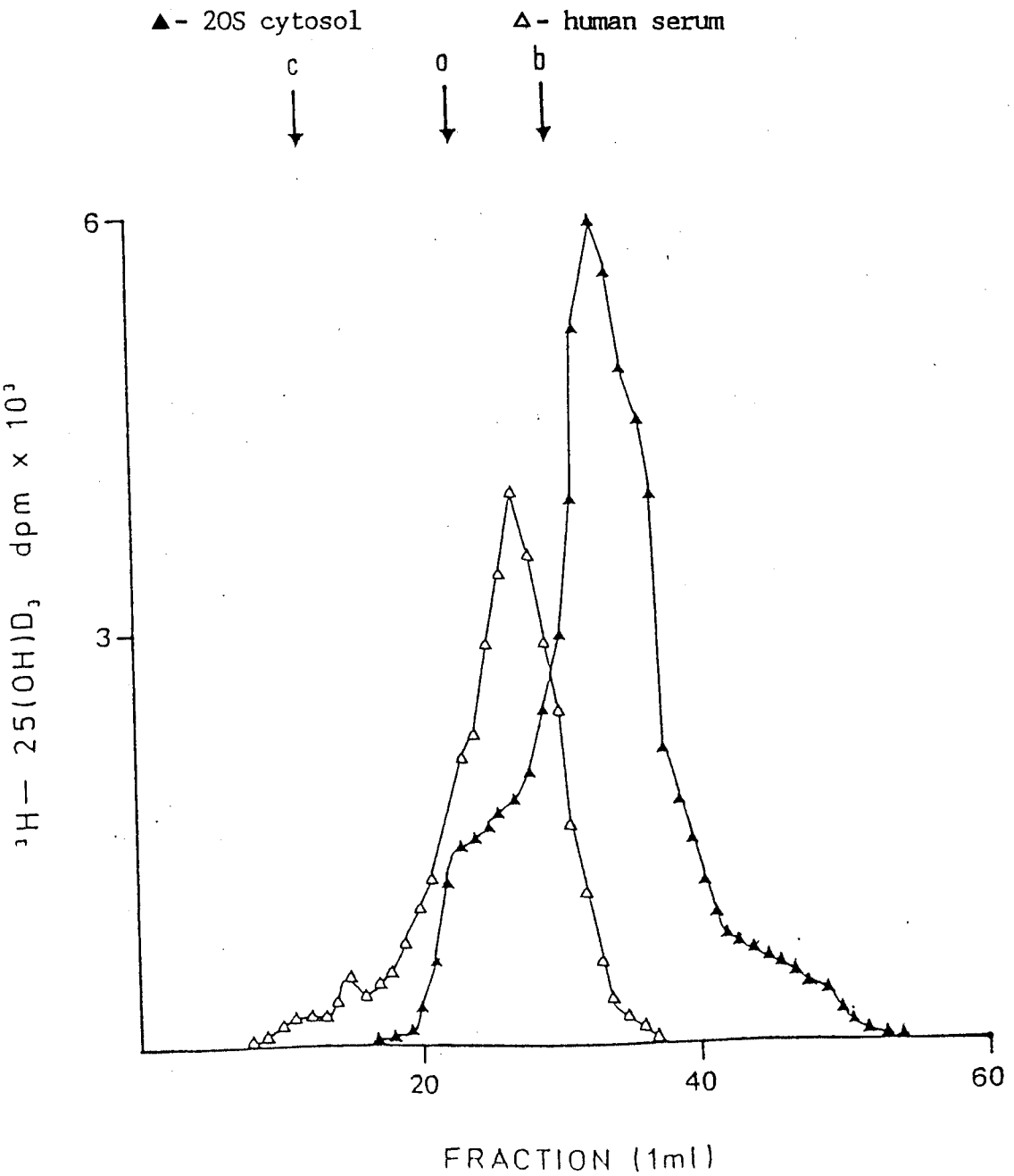


FIGURE 9.16. Sucrose density sedimentation analysis of 20S cytosolic proteins and human serum previously incubated with ^{3}H-25(OH)D_3.

Samples were applied to a continuous sucrose density gradient (4-24% in 0.3M KCl) in 16.5ml centrifuge tubes (7 x 1.25 cm). After centrifugation at 30,000rpm for 56h at 4°C, fractions (200ul) were collected and assayed for radioactivity.

b - bovine serum albumin, M.W. 66,000; o - ovalbumin, M.W. 45,000; c - cytochrome c, M.W. 17,000.



procedure. No peak of 25(OH)D binding activity was seen by either gel filtration or density gradient centrifugation of cytosols of U937 or calf thymus cytosol which had been incubated with ^{3}H-25(OH)D. While calf thymus cytosol displayed binding activity towards $1,25(OH)_2D$, as reported by Reinhardt et al (1984) no such activity was present in 20S cytosolic proteins.

9.4 DISCUSSION

The human osteosarcoma cell line 20S was chosen as an appropriate bone model system for the following reasons. Firstly, unlike the two previous osteosarcoma cell lines 791T and T278, this cell line still retained its osteoblastic phenotype, as illustrated by its responsiveness to PTH and its ability to produce alkaline phosphatase and osteocalcin. The expression of these characteristics in vitro rendered it amenable to experimental investigation. Secondly, it offered the advantage of being a cell line which could be maintained in culture after unlimited propagation.

This study was unable to demonstrate the presence of $1,25(OH)_2D_3$ receptors in the human osteosarcoma cell line 20S. Other studies, however, have been able to characterise the receptor in rat osteogenic sarcoma cells (Manolagas et al, 1980) as well as in cultured osteoblast-like cells from mouse calvaria (Chen et al, 1979), however the latter with some difficulty. Problems arose because the concentration of $1,25(OH)_2D_3$ receptors varied directly with the rate of DNA synthesis in the mouse model (Chen and Feldman, 1981). If a relationship between cell growth and receptor concentration does exist for this human osteosarcoma cell line, i.e. if receptor levels are high at log growth phase and low at

confluence, then perhaps the difficulty in demonstrating receptors may be due to preparing cytosolic fractions from near-confluent cells, thus working at a point in the culture cycle near the nadir of the receptor concentration.

This study, however, revealed the presence of a cytosolic binding protein which specifically bound $25(OH)D_3$. $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ had little or no binding to this protein. This novel binding protein, of molecular weight around 75,000Da, sedimented at 4.7S over sucrose density gradients and was shown, by immunodiffusion analysis, to be clearly distinguishable from serum DBP. Because human serum contains a high affinity $25(OH)D_3$ binding protein (MW 56,000Da) it was important to remove this activity from the cells as far as possible. This was achieved as follows. The cells were grown in medium containing 10% serum and prior to preparation of the cytosolic fractions they were washed extensively at confluence and cultured in serum-free medium for 48h. The $25(OH)D_3$ binding activity of these fractions separated from the serum activity on gel exclusion chromatography and sucrose density gradient.

There is evidence from studies using tissue homogenates to demonstrate the presence of a 5.8-6.0S binding protein which binds both $25(OH)D_3$ and $24,25(OH)_2D_3$ with high affinity (Van Baelen et al, 1980). However, this is not an in vivo phenomenon but is apparently formed after homogenisation. In fact, it occurs when serum DBP complexes with a cytosolic component, which has been identified as actin (Van Baelen et al, 1980; Manolagas and Deftos, 1981). This 20S cytosolic binding protein is presumably that which binds $25(OH)D_3$ for subsequent metabolism in the cells and is not an actin complex of the sreum binding protein.

The evidence presented here demonstrates that the major metabolite of $25(OH)D_3$ produced by the human osteosarcoma cell line 2OS is $24,25(OH)_2D_3$. This is the first report to demonstrate a cell line which preferentially synthesises $24,25(OH)_2D_3$ rather than $1,25(OH)_2D_3$. The structural conformation of the metabolite as $24,25(OH)_2D_3$ was supported by three lines of evidence:

(1) the anomalous chromatographic behaviour of the metabolite was similar to that of the $24,25(OH)_2D_3$ metabolite when applied onto two separation systems.

(2) The UV absorption spectrum displayed a typical vitamin D_3 cis-triene chromophore with λ_{max} = 264nm and λ_{min} = 228nm.

(3) The mass spectrum of the metabolite gave a parent molecular ion of 416 and fragments m/z of 396, 271 (loss of the side chain), 253 (271 minus H_2O), 136 (cis-triene cleavage of m/z 253) and 118 (136 minus H_2O). The fragments m/z 271 and 253 indicate that the secosteroid nucleus of vitamin D_3 has remained unchanged and that all the metabolic alterations have taken place on the side chain. Additional fragmentation patterns were indistinguishable from the authentic $24,25(OH)_2D_3$. Consequently, the structural assignment as $24,25(OH)_2D_3$ is unambiguous.

Mass spectral analysis of this fraction suggested that it contained two metabolites, one of which, obtained at a probe temperature of 300°C was clearly shown to be $24,25(OH)_2D_3$. At the lower probe temperature of 250°C a second spectrum, quite different from $24,25(OH)_2D_3$ was obtained. That this second metabolite co-eluted with $24,25(OH)_23$ from the HPLC column is suggestive of another tri-hydroxylated species. On the basis of this spectrum and from helpful comments from Dr M.R. Uskokovic (Hoffman- La

Roche, Nutley, New Jersey, USA) regarding the assignment of the spectrum it is tentitively suggested that it is the C_{17}-C_{20} unsaturated structure A.

The discovery of a new vitamin D metabolite, metabolite "A," has raised a fundemental question as to why these bone cells should produce such a metabolite and for what purpose. This compound is chromatographically indistinct from $24,25(OH)_2D_3$ and their relative proportions in the fraction which eluted from the HPLC column is unknown. The intensity of the two mass spectra suggest that they are present in approximately equal amounts. Production of either or both metabolites appears to be inhibited by the presence of the cAMP analogue dbcAMP when the substrate concentration exceeds 0.5uM.

It is suggested that this new molecule may represent some form of catabolic process which eventually leads to reduced biological activity of the $24,25(OH)_2D_3$ and therefore its excretion from the cell. It should be noted that pharmacological doses of $25(OH)D_3$ were required to generate this polar metabolite to sufficient quantities for analysis. It could be argued, therefore, that this process is only instigated under these conditions, i.e. under hypervitamin D. This could therefore represent a pathway whereby in circumstances of large amounts of $25(OH)D_3$ bone cells can induce the 24-hydroxylase to synthesise $24,25(OH)_2D_3$ which in turn is metabolically modified to this unknown metabolite.

Experiments using mammalian kidney cells have shown that $25(OH)D_3$ may be metabolised through an oxidation pathway to yield numerous inactive metabolites which have extensive metabolic modification particularly in the side chain. These enzymes, which cleave the C_{23}-C_{24} bond as well as other regions of the vitamin D sterol, are only induced by

supraphysiological concentrations of $1,25(OH)_2D_3$. A similar pathway has been demonstrated in the rat osteoblast-like cell UMR-106 but again this pathway was only detectable when the cells were exposed to $1,25(OH)_2D_3$. This is in contrast with the present study. Production of the new metabolite A did not require $1,25(OH)_2D_3$ and its mass spectrum did not resemble any of the inactivated compounds in the literature.

As stated in the introduction the extrarenal production of $24,25(OH)_2D_3$ has been reported in a number of tissues but apart from its possible role in the regulation of bone formation and/or mineralisation its function is unknown. This is the first report of a cell system which preferentially synthesises $24,25(OH)_2D_3$ and a second metabolite A. Unfortunately, the metabolic role of metabolite A and its <u>in vivo</u> actions need further investigation.

APPENDIX 1

QUANTIFICATION OF ILIAC CREST BONE BIOPSY

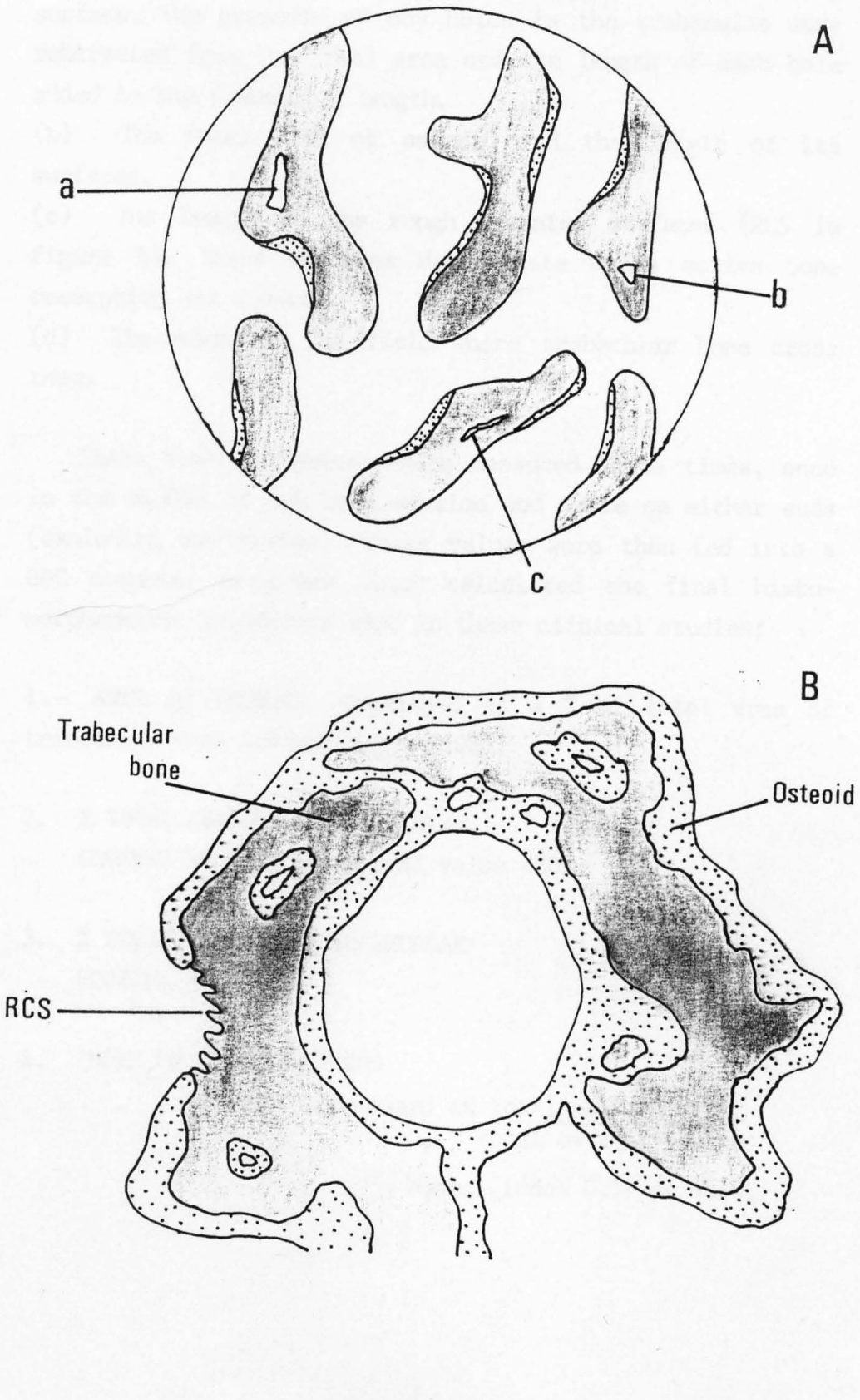
Resin sections from the bone biopsy was stained by the Goldner trichrome method in order to demonstrate trabecular bone and osteoid regions. Bone quantification was performed with the aid of a Kontron computer linked to an electronic drawing-board (graphic tablet).

With the aid of a low powered microscope, the image of the bone section was superimposed onto the tablet, enabling the outlining of features seen in the microscope field. Figure A illustrates a typical field of view under low power; the mineralised bone shown shaded and the osteoid stippled. The following parameters were measured using the scanning pen which fed the information to a computer:

(1) The **area** of the field to be estimated. This is represented by the bold line in figure A and represents most of the biopsy. This area was chosen because it excluded cortical bone and the crushed edges of the biopsy.
(2) The **total area of trabecular bone,** including the osteoid.
(3) The presences of any holes in the trabeculae (eg. a, b and c in figure A). These were caused by tearing when cutting the sections. These values, therefore were subtracted from the area.

The final result gave the area of bone as a percentage of the total area viewed. The section was then viewed under high power in order to obtain further parameter readings. A characteristic high power vision is depicted in figure B.

Figure



The following parameters were obtained:

(1) The **total trabecular area** and **trabecular length** of the surface. The presence of any holes in the trabeculae were subtracted from the total area and the length of each hole added to the trabecular length.
(b) The **total area of osteoid** and the length of its surfaces.
(c) The **length of any rough crenated surfaces** (RCS in figure B). These surfaces demonstrate where active bone resorption has occured.
(d) The edges of the field where trabecular bone cross them.

These final parameters were measured three times, once in the middle of the bone section and twice on either ends (excluding any cortex). These values were then fed into a BBC computer programme which calculated the final histomorphometric parameters used in these clinical studies:

1. **AREA OF OSTEOID** -expressed as a % of total area of trabecular bone (normal value <10%).

2. **% TOTAL SURFACE OF TRABECULAE COVERED BY OSTEOID** -normal value <20%

3. **% TOTAL SURFACE OF TRABECULAE COVERED BY RCS**

4. **INDEX OF SEAM THICKNESS**

-expressed as $\frac{\text{total osteoid area}}{\text{total osteoid length}}$

normal index 0.17 or less.

APPENDIX 2

SERUM BIOCHEMISTRY MEASUREMENTS

Routine serum biochemistry was measured in the Department of Clinical Chemistry, Queen's Medical Centre, Nottingham.

Measurement	Instrument	Technique employed	Normal Plasma Range
*Plasma and Urinary Calcium	Clinicon Hitachi 712 Automatic Analyser	Ca^{2+} forms a violet complex with O-cresolphythalein in alkaline conditions, detected at 600-660nm wavelength at 37°C	2.2 - 2.6 mmol/L
Plasma and Urinary inorganic Phosphate	Multi-channel Technicon Auto-Analyser	Phosphate reacts with ammonium molybdate. Stannous ions (stannous chloride) reduces molybdiphosphate to heteropoly molybdenum blue, detected at 660nm	0.8 - 1.4 mmol/L
Plasma and Urinary Creatinine	Technicon Auto-Analyser II	Creatinine produces a red colour in saturated alkaline solution of picric acid, detected at 505nm at room temperature	60 - 120 mol/L
Plasma Albumin	Technicon SMA Multi-channel biochemical Analyser	Albumin reacts with bromo-cresyl green dye at pH 4.2 (+ 0.1) to form a coloured complex, detected at 630nm wavelength	31 - 51 g/L
Plasma Alkaline Phosphatase	Clinicon Hitachi 712 Automatic Analyser	Alkaline phosphatase converts p-nitro phenylphosphate to phosphate and p-nitro phenol (yellow in alkaline conditions) detected at 405nm at 37°C	98 - 280 IU/L

*Measured plasma calcium values must be corrected for albumin levels as illustrated on the following page. Unless otherwise stated, plasma calcium concentrations quoted in the result sections were corected for albumin.

THE CORRECTION OF PLASMA CALCIUM
(Editorial, 1977)

There are three definable fractions of calcium in plasma: ionised, protein- bound and complexed fractions. The total plasma calcium is the sum of these three fractions. The ionised fraction is the physiologically relevant fraction but laboratory analysis of plasma measures total calcium. These results may be misleading especially when there is a change in plasma protein concentration or pH. To overcome this total plasma concentration is corrected albumin concentrations. Plasma pH is generally constant and has negligable effect on protein binding.

Correction of plasma calcium is then simply corrected for an albumin concentration (Alb) of 40g/L using the following formula:

$$Ca_c = Ca_m \pm 0.02(40 - Alb)$$

where:

Ca_c = corrected plasma calcium concentration (mmol/L)

Ca_m = measured plasma calcium concentration (mmol/L)

and Alb = measured plasma albumin concentration (g/L)

APPENDIX 3

SCATCHARD ANALYSIS IN IMMUNOMETRIC ASSAY

(Taken from Walker, 1977)

In radioimmunoassay, and indeed radioreceptor assay, the response is measured in terms of labelled ligand total counts, L*, and labelled ligand bound counts, B*.

Information on:
(i) the heterogeneity of the binding sites,
(ii) the binding capacity of either the antibody or binding protein (Ab), and
(iii) the affinity constant of ligand-Ab interaction (K_a)
are conveniently obtained by using the linear Scatchard equation in the form:

$$yL = Ab - K_a^{-1}y \div (1-y)$$

where B = bound ligand concentration, both labelled and unlabelled, L = total ligand concentration, and y = B/L.

If there is no protein discrimination between labelled and unlabelled ligand, y = B*/L*. With axes y/(1-y) and yL, a linear relationship results, with slope $-K_a$ and intercept Ab on the yL axis.

APPENDIX 4

DOUBLE-ISOTOPE CALCIUM ABSORPTION TEST

The test employs the use of an intravenous dose of ^{85}Sr in conjunction with an oral dose of ^{47}Ca. To calculate the percentage of the oral dose absorbed by the intestine accurately, allowance must be made for the removal of ^{47}Ca into soft tissue and bone. Since ^{85}Sr administered intravenously is distributed in the same manner as the oral dose of ^{47}Ca, the ratio of activity of the two in plasma is used to calculate the percentage absorption.

Using a computer program designed for this study by Dr. J.R. Greenwell, University of Newcastle, the following data were inserted:

WORKED EXAMPLE: Patient E.S.

^{47}Ca wt given to patient = **5.0485**mg wt standard = **0.977**g
^{85}Sr wt given to patient = **5.1200**mg wt standard = **0.990**g

	^{47}Ca (cpm)	^{85}Sr (cpm)
Standard counts	a) 5877 b) 5271	a) 3082 b) 3216
^{47}Ca std in ^{85}Sr channel		a) 2824 b) 2573
^{85}Sr std in ^{47}Ca channel	a) 5 b) 8	
Background counts	a) 7 b) 7	a) 14 b) 15

Imputing the patient values:

TIME (min)	VOL OF SERUM (ml)	^{47}Ca (cpm)	^{85}Sr (cpm)
0	4	7	14
5	4	7	760
10	4	16	392
20	4	39	343
35	4	67	292
50	4	91	273
75	4	129	272
100	4	126	258
140	4	159	257
180	4	169	243
240	4	165	231
300	4	159	218
360	4	163	219

From the above data the computer programme is able to (a) calculate the percentage of ^{47}Ca absorbed at a given time, and

(b) plot a graph of the percentage of calcium absorbed against time. By differentiating this graph the initial fractional rate and the total percentage calcium absorbed after 6h can be estimated.

NB. Initial fractional rate = amount of Calcium absorbed at 1hr.

APPENDIX 5

TREATMENT OF SUBCLINICAL OSTEOMALACIA IN THE ELDERLY:

Tables showing individual biochemical and histomorphormetric responses after one month's treatment.

1 - 7	Control patients
8 - 16	Patients on Vitamin D_3
17 - 27	" " 1 Alpha
28 - 38	" " 1 Alpha + Calcium supplements (Ca)

			BASAL						1 MONTH					
CASE	AGE	SEX	Cac mmol/L	P mmol/L	AP IU/L	Cr umol/L	t25 ng/ml	t1,25 pg/ml	Cac mmol/L	P mmol/L	AP IU/L	Cr umol/L	t25 ng/ml	t1,25 pg/ml
1	75	F	2.31	0.71	413	129	1.8	12.0	2.39	1.04	264	144	-	25.5
2	85	F	2.21	0.74	212	148	12.4	16.2	2.26	1.15	709	131	4.0	7.1
3	80	F			628	131	22.5	20.8	1.84	1.08	648	142	12.5	36.6
4	73	F	2.10	0.81	1352	71	7.5	5.8	1.98	0.71	2240	73	9.2	35.0
5	85	M	2.04	0.66	518	153	-	-	2.00	0.81	1232	124	-	-
6	70	F	2.07	0.91	588	90	-	-	2.16	0.74	444	126	-	-
7	89	M	1.76	1.10	374	197	7.2	13.5	1.94	1.03	505	195	-	-
8	87	F	1.60	0.70	277	114	6.7	29.3	2.22	1.82	275	143	21.7	37.9
9	86	F	2.42	0.68	464	75	6.2	11.1	2.22	0.85	597	75	22.6	49.1
10	46	F	1.84	0.67	355	104	2.6	12.8	2.20	1.10	359	105	15.0	68.9
11	95	F	2.30	0.93	187	104	4.4	14.1	2.33	1.09	484	109	29.7	37.6
12	71	M	2.21	0.81	1303	70	2.7	20.6	2.12	1.42	2150	76	10.2	58.1
13	53	F	2.24	0.68	427	153	4.1	22.5	2.12	0.81	417	132	16.5	43.3
14	57	F	2.17	1.11	373	104	5.4	21.2	2.32	1.45	346	135	34.8	53.9
15	80	M	2.30	0.92	960	147	-	-	2.16	1.09	1211	136	24.5	69.2
16	80	F	1.84	1.08	648	142	-	-	2.32	1.45	546	135	12.5	36.6
17	83	F	2.27	1.13	295	119	15.0	21.4	2.27	0.85	238	132	11.6	41.3
18	90	F	1.64	1.36	980	203	5.2	13.3	1.94	1.21	877	203	6.8	50.2
19	80	M	2.21	0.68	195	103	1.3	12.5	2.30	1.12	251	91	2.8	28.0
20	77	F	2.08	0.87	2925	112	0.8	13.4	2.12	0.87	5840	64	9.1	30.5
21	81	F	2.23	1.04	241	141	7.4	30.0	2.28	1.19	264	109	3.5	37.8.
22	79	F	2.23	0.86	130	90	4.5	13.4	2.28	0.78	349	90	1.9	35.2
23	89	M	1.94	1.03	505	195	7.2	13.5	2.13	1.11	394	201	3.5	35.7
24	78	F	2.26	1.53	421	79	1.8	19.2	2.18	0.95	294	91	5.9	36.7
25	76	M	2.25	1.04	427	109	2.1	23.4	2.29	1.01	237	112	16.8	48.1
26	85	M	2.01	0.81	1232	124	-	-	1.96	0.73	941	118	9.8	47.5
27	90	F	1.56	1.34	759	164	3.6	15.4	2.14	1.37	616	141	-	-
28	90	F	2.16	1.31	357	121	3.1	11.3	2.20	1.48	388	98	9.1	65.7
29	86	F	2.16	0.83	321	74	6.8	4.5	2.26	1.38	218	97	2.9	77.1
30	43	F	2.03	0.64	473	53	3.9	4.4	1.99	0.83	455	49	2.4	51.0
31	52	F	1.45	0.98	131	66	8.7	15.4	2.38	1.23	280	70	5.4	28.3
32	83	F	2.04	0.77	1000	79	3.7	17.7	2.03	0.94	481	74	11.8	77.0
33	81	M	2.15	0.76	587	104	2.7	15.2	2.04	0.60	744	129	10.2	34.3
34	85	F	2.23	1.09	277	57	6.6	16.6	2.25	1.17	242	84	5.9	41.9
35	88	M	2.13	0.42	227	111	5.8	25.7	2.06	0.53	629	93	3.9	41.5
36	87	F	2.06	1.05	527	107	1.3	14.0	2.39	1.19	458	114	4.4	13.9
37	78	F	2.36	0.64	314	91	2.6	3.3	-	-	-	-	12.1	50.4
38	86	F	2.21	0.92	307	170	4.0	22.9	2.42	1.04	342	152	-	-

		BASAL			1 MONTH		
CASE No.	TREATMENT	OA	Surf	OI	OA	Surf	OI
1	Control	4.9	31.6	0.16	4.1	22.0	0.19
2	"	16.8	64.4	0.26	6.6	34.5	0.19
3	"	10.7	45.7	0.23	-	-	-
4	"	30.3	71.3	0.42	-	-	-
5	"	53.4	84.9	0.63	51.7	100	0.52
6	"	17.9	57.9	0.31	8.8	22.1	0.40
7	"	37.3	85.3	0.44	33.0	85.8	0.39
8	Vitamin D_3	24.5	68.0	0.36	5.9	42.2	0.14
9	"	9.0	33.7	0.27	6.2	22.2	0.28
10	"	41.4	91.3	0.45	27.3	89.7	0.30
11	"	6.6	20.0	0.33	7.3	26.1	0.28
12	"	45.7	81.2	0.56	31.3	80.0	0.39
13	"	8.2	37.5	0.22	22.7	52.2	0.44
14	"	19.1	61.8	0.31	13.1	54.4	0.24
15	"	40.8	94.6	0.41	22.8	65.8	0.35
16	"	1.0	-	-	-	-	-
17	1 Alpha	10.7	29.4	0.36	6.6	28.5	0.23
18	"	30.6	88.1	0.35	23.0	90.1	0.26
19	"	5.2	23.6	0.22	5.02	18.0	0.28
20	"	30.1	68.7	0.44	31.9	93.0	0.34
21	"	3.8	20.2	0.19	3.8	21.0	0.18
22	"	16.8	59.5	0.28	14.9	75.2	0.20
23	"	33.0	85.8	0.39	15.8	49.2	0.32
24	"	13.3	43.6	0.31	12.8	58.3	0.22
25	"	17.3	41.2	0.42	8.5	60.5	0.14
26	"	51.7	100.0	0.52	58.4	93.7	0.62
27	"	51.8	87.4	0.59	39.7	85.2	0.47
28	1 Alpha	25.6	61.2	0.42	12.0	38.1	0.32
29	+ Ca	8.4	30.2	0.28	5.7	20.0	0.29
30	"	59.9	92.2	0.65	37.6	87.9	0.43
31	"	5.4	14.8	0.36	2.7	10.4	0.26
32	"	56.2	92.8	0.61	19.8	70.9	0.28
33	"	20.9	73.7	0.29	24.9	64.3	0.39
34	"	21.5	8.8	0.25	1.24	10.0	0.12
35	"	33.5	96.2	0.35	28.4	78.8	0.36
36	"	13.2	64.5	0.21	11.6	52.2	0.22
37	"	12.0	41.6	0.29	-	-	-
38	"	18.5	68.1	0.27	14.7	63.0	0.23

APPENDIX 6

CHANGES IN OSTEOCALCIN AND VITAMIN D METABOLITES AFTER FRACTURE OF THE FEMORAL NECK:

Table showing individual biochemical and histomorphormetric values at the time of the fracture and one week later. Note, two patients histomorphometric data were mislaid.

CASE No.	AGE	SEX	SAMPLE	Cac mmol/L	P mmol/L	AP IU/L	Cr umol/L	GGT IU/L	Bili umol/L	ALT IU/L	t25 ng/ml	t1,25 pg/ml	DBP mg/L	fi,25 index x10^{5}	OCal ng/ml	OA	Surf	OI
1	92	F	b	2.20	1.30	260	115	39	7	19	2.4	9.2	267	0.48	1.7	0.95	5.46	0.17
			1 wk	2.11	1.16	327	115				2.6	8.8	305	0.41	2.4			
2	65	F	b	2.30	0.92	106	100	22	13	32	3.0	19.8	231	1.21	1.4	5.60	37.7	0.15
			1 wk	2.32	1.04	174	100				3.4	30.2	366	1.16	3.0			
3	78	F	b	2.13	0.90	123	105	26	7	18	3.0	21.8	328	0.84	2.3	2.17	7.96	0.27
			1 wk	2.20	1.01	194	107				2.7	32.0	317	1.42	3.5			
4	86	F	b	2.59	0.88	356	94	38	10	14	3.4	17.1	180	1.34	2.2	0.66	4.26	0.16
			1 wk	2.43	0.81	411	95				4.7	28.3	167	2.39	3.5			
5	68	F	b	2.21	1.07	178	60	40	7	9	5.0	34.0	395	1.21	2.4	0.37	2.38	0.16
			1 wk	2.15	0.75	295	82				1.9	9.8	255	0.54	0.6			
6	79	F	b	2.33	1.13	619	143	294	9	14	2.1	6.4	404	0.22	2.4	3.08	16.08	0.19
			1 wk	2.17	1.21	447	143				2.4	6.7	466	0.20	2.1			
7	83	F	b	2.13	1.05	118	88	17	9	14	0.5	5.0	302	0.23	1.3	1.11	4.51	0.25
			1 wk	2.16	0.98	127	91				1.0	8.6	299	0.41	1.4			
8	72	F	b	2.12	0.99	112	69	224	12	34	5.6	8.7	265	0.46	0.9	2.33	14.4	0.16
			1 wk	2.30	0.98	970	84				4.6	14.1	313	0.63	2.5			
9	62	F	b	2.04	1.09	84	92	33	10	26	5.5	16.4	294	0.79	1.7	4.56	17.43	0.26
			1 wk	2.28	1.04	116	67				5.7	33.1	346	1.35	4.0			
10	71	F	b	2.08	0.96	138	100	12	1	6	5.0	5.4	261	0.29	2.6	no bone biopsy		
			1 wk	2.07	0.86	167	107				6.9	9.4	193	0.69	2.8			
11	63	M	b	2.07	1.70	126	87	10	10	40	11.2	10.5	379	0.39	3.0	2.41	12.74	0.19
			1 wk	2.07	0.86	167	107				9.1	18.3	414	0.62	5.5			
12	92	F	b	2.28	1.22	148	110	12	12	14	4.7	5.3	290	0.26	6.5	1.24	10.49	0.12
			1 wk	2.20	1.17	131	68				4.8	11.5	306	0.53	5.5			
13	67	M	b	2.17	1.10	170	77	22	20	17	2.3	9.0	343	0.37	2.9	1.15	5.75	0.20
			1 wk	2.25	1.15	251	55				3.8	12.1	394	0.43	3.7			
14	65	F	b	2.17	0.85	182	67	30	6	4	5.3	10.9	290	0.53	5.3	no bone biopsy		
			1 wk	2.17	0.98	211	72				5.7	9.8	326	0.42	5.3			
15	89	F	b	2.12	1.36	183	109	4	9	12	1.7	4.7	261	0.25	3.8	1.43	17.05	0.08
			1 wk	2.17	0.98	252	81				3.1	9.9	394	0.35	6.1			

CASE No.	AGE	SEX	SAMPLE	Cac mmol/L	P mmol/L	AP IU/L	Cr umol/L	GGT IU/L	Bili umol/L	ALT IU/L	t25 ng/ml	t1,25 pg/ml	DBP mg/L	f1,25 index x10^{-5}	OCal ng/ml	OA	Surf	OI
16	70	F	b	2.19	0.66	208	77	20	38	13	4.4	15.2	264	0.81	6.5	2.50	13.51	0.19
			1 wk	2.27	1.28	293	64				3.6	6.0	448	0.19	5.5			
17	86	F	b	2.24	1.44	152	106	26	14	24	3.2	14.1	384	0.3	2.3	2.03	13.3	0.15
			1 wk	2.41	1.34	212	105				2.7	14.1	331	0.61	2.0			
18	58	F	b	2.25	1.01	102	69	-	-	-	5.7	30.0	289	1.46	1.0	2.11	7.91	0.27
			1 wk	2.66	1.44	144	71				5.6	16.9	474	0.50	0.7			
19	84	F	b	2.32	0.86	302	120	19	9	9	1.7	15.2	221	0.97	5.3	1.17	6.91	0.17
			1 wk	2.35	0.90	757	90				1.4	16.4	492	0.47	7.2			
20	86	F	b	2.15	0.90	147	81	17	20	6	2.5	14.2	200	1.00	3.8	0.72	4.85	0.15
			1 wk	2.29	0.95	203	77				2.8	19.1	348	0.77	4.9			
21	82	F	b	2.03	0.69	151	108	17	17	13	2.2	11.2	236	0.67	3.8	0.91	5.73	0.16
			1 wk	2.37	1.02	170	122				1.5	12.6	302	0.59	4.8			
22	84	F	b	2.23	1.20	157	84	35	17	74	12.9	19.6	269	0.91	2.7	1.75	20.79	0.08
			1 wk	2.27	1.33	231	89				11.0	12.6	341	0.62	3.3			
23	90	F	b	2.30	1.97	221	87	25	31	13	10.9	28.5	458	0.88	3.0	3.80	12.5	0.30
			1 wk	2.18	1.33	134	85				7.0	19.5	350	0.78	3.3			
24	79	M	b	2.28	1.16	130	138	8	8	9	6.2	16.2	191	1.19	1.6	2.80	11.35	0.25
			1 wk	2.46	1.20	291	110				8.0	18.9	228	1.17	2.3			
25	80	F	b	2.19	1.08	159	85	21	15	15	4.3	15.2	339	0.63	4.7	0.27	1.87	0.15
			1 wk	2.30	1.33	217	113				2.0	11.1	435	0.36	5.3			
26	80	F	b	2.17	0.72	201	94	45	12	9	4.2	10.6	336	0.44	6.1	0.21	2.27	0.09
			1 wk	2.44	1.21	222	109				2.9	27.1	352	1.08	7.7			
27	82	F	b	2.50	1.94	304	114	32	15	21	4.6	20.2	313	0.91	0.7	0.86	3.97	0.22
			1 wk	2.26	0.96	215	114				2.6	25.9	406	0.90	1.4			
28	78	F	b	2.36	0.84	150	111	37	7	27	7.2	22.5	272	1.16	2.1	0.79	3.75	0.21
			1 wk	2.32	1.27	165	150				9.2	33.7	343	1.38	5.2			
29	85	F	b	2.37	1.31	159	98	25	10	27	5.5	26.8	276	1.37	6.6	2.69	12.46	0.22
			1 wk	2.03	1.10	201	115				3.4	25.0	366	0.96	5.7			
30	85	F	b	2.61	1.09	742	108	135	31	13	3.2	30.6	300	1.44	1.5	3.78	12.36	0.31
			1 wk	2.50	1.21	388	281				3.0	22.0	346	0.90	1.0			

APPENDIX 7

VITAMIN D DEFICIENCY AND FEMORAL NECK FRACTURE

Table showing individual biochemical and histomorphormetric values for the three groups studied:

Table A : Osteoporotic patients who had sustained a fracture of the femoral neck.

Table B : Osteomalacic patients who had sustained a fracture of the femoral neck.

Table C : Osteomalacic patients without fractures.

CASE No.	AGE	SEX	N	Ca mmol/L	P mmol/L	AP IU/L	Cr umol/L	t25 ng/ml	t1,25 pg/ml	DBP mg/L	f1,25 index x 10^{-5}	OA	SurF	OI
1	84	F	5	2.42	0.84	605	98	8.1	9.4	312	0.42	2.0	9.1	0.22
2	63	F	1	2.30	1.13	202	180	9.8	4.7	297	0.22	1.5	10.4	0.14
3	90	M	13	2.13	0.46	175	105	2.6	6.5	316	0.29	0	0	0
4	73	F	4	1.80	-	-	-	3.6	6.5	211	0.43	2.81	11.3	0.25
5	79	F	8	2.20	0.90	123	105	1.9	21.5	201	1.50	0.74	6.4	0.12
6	78	F	0	2.13	0.90	123	105	3.0	21.9	328	0.84	2.17	7.96	0.27
7	86	M	0	2.59	0.88	356	94	3.4	17.1	180	1.33	0.66	4.26	0.16
8	68	F	1	2.21	1.07	178	60	5.0	34.0	395	1.21	0.37	2.38	0.16
9	92	F	0	2.20	1.30	260	115	2.4	9.2	267	0.48	0.95	5.46	0.17
10	83	F	0	2.13	1.05	118	88	0.5	5.0	302	0.23	1.11	4.51	0.25
11	92	F	0	2.24	1.22	148	110	4.8	15.3	290	0.26	1.24	10.5	0.12
12	67	M	0	2.17	1.10	170	77	2.3	9.0	343	0.53	1.15	5.75	0.20
13	58	F	0	2.66	-	-	-	5.7	30.0	289	1.47	2.11	7.91	0.27
14	90	F	0	2.30	1.97	221	87	10.9	20.2	458	0.88	3.80	12.5	0.30
15	81	F	0	2.28	1.16	130	138	6.2	16.2	191	1.19	2.80	11.4	0.25
16	80	F	0	2.19	1.08	159	85	4.3	15.2	339	0.63	0.27	1.87	0.15
17	86	F	0	2.15	0.90	147	81	2.5	14.2	200	1.00	0.72	4.85	0.15
18	84	F	0	2.32	0.90	302	120	1.7	15.2	221	0.97	1.17	6.91	0.17
19	82	F	0	2.03	0.69	151	108	2.2	11.2	236	0.67	0.91	5.73	0.16
20	80	F	0	2.17	0.72	201	94	4.3	10.6	336	0.45	0.21	2.27	0.09
21	82	F	0	2.50	1.94	304	114	4.6	20.3	313	0.91	0.86	3.97	0.22
22	78	F	0	2.36	0.84	150	111	7.2	22.5	272	1.17	0.79	3.75	0.21
23	85	F	0	2.61	-	742	108	3.3	30.6	300	1.44	3.78	12.4	0.31
24	85	F	0	2.31	1.3	159	98	5.5	26.8	276	1.37	2.69	12.5	0.22
25	80	F	0	2.23	1.09	277	57	6.5	16.6	-	-	2.10	8.9	0.25
26	70	M	0	2.32	1.98	220	195	3.8	14.6	254	1.03	3.63	10.9	0.33
27	81	F	3	2.36	0.98	169	135	3.5	5.3	381	0.20	3.30	24.9	0.13
28	79	F	0	2.33	1.13	619	143	2.1	6.4	404	0.22	3.08	16.1	0.19
29	72	F	0	2.12	0.99	112	69	5.6	8.8	265	0.46	2.33	14.4	0.16
30	71	M	0	2.07	1.70	126	87	11.2	10.5	379	0.39	2.41	12.7	0.19
31	85	M	4	2.13	0.80	125	76	1.6	21.7	-	-	3.56	15.7	0.23
32	89	F	0	2.12	1.36	183	109	1.7	4.7	261	0.25	1.43	17.1	0.08
33	70	F	0	2.19	0.66	208	77	4.4	15.2	264	0.81	2.50	13.5	0.19
34	86	F	0	2.24	1.44	152	106	3.2	14.1	384	0.52	2.03	13.3	0.15
35	84	F	0	2.23	1.20	157	84	12.9	19.6	269	1.02	1.75	20.8	0.08
36	90	F	10	2.30	1.34	276	INS	10.9	28.5	392	0.88	3.80	12.5	0.30
37	94	F	0	2.30	1.07	139	89	2.4	16.3	355	0.83	3.90	14.3	0.28
38	82	F	5	2.23	1.04	241	141	7.4	30.0	-	-	3.80	20.2	0.19

PATIENTS WITH OSTEOPOROSIS + FEMORAL NECK FRACTURE

B

CASE No.	AGE	SEX	N	Ca mmol/L	P mmol/L	AP IU/L	Cr umol/L	t25 ng/ml	t1,25 pg/ml	DBP mg/L	f1,25 index x 10^5	OA	Surf	OI
39	78	F	0	2.26	1.53	431	ins	1.9	19.5	-	-	13.3	43.6	0.31
40	87	F	0	2.06	1.05	527	107	1.3	14.3	258	0.77	13.2	64.5	0.21
41	90	F	0	2.35	1.46	352	289	5.5	11.7	272	0.61	28.2	68.1	0.43
42	92	F	0	2.03	0.98	917	77	0.8	21.0	447	0.65	37.4	92.0	0.41
43	80	F	11	2.03	1.52	715	165	22.5	20.8	-	-	10.7	45.7	0.23
44	81	M	2	2.15	0.76	587	104	2.7	15.3	-	-	20.9	43.3	0.29
45	87	M	10	1.60	0.72	428	114	6.7	29.3	-	-	24.5	68.0	0.36
46	86	F	6	2.21	0.92	307	170	2.5	23.0	271	1.18	18.5	68.1	0.27
47	90	F	2	2.16	1.31	357	121	3.1	11.3	-	-	25.6	61.2	0.42
48	78	F	0	2.36	0.64	314	ins	2.6	3.0	278	0.15	12.0	41.6	0.29
49	78	F	1	2.23	0.86	130	90	4.5	13.5	307	0.61	10.9	55.4	0.20
50	83	F	6	2.04	0.77	1000	79	3.7	17.8	328	0.76	56.2	92.8	0.61
51	80	M	8	2.17	1.02	328	ins	2.5	19.5	254	1.08	5.9	31.1	0.19
52	77	F	5	2.08	0.87	2925	88	0.8	13.5	328	0.58	30.1	68.7	0.44
53	75	F	0	2.13	0.81	1352	71	9.2	5.7	151	0.53	30.3	71.3	0.42
54	83	F	0	2.30	0.92	206	100	3.0	20.1	231	1.16	5.6	37.7	0.15
55	76	M	22	2.26	0.81	289	109	23.0	21.3	-	-	17.3	41.2	0.42
56	75	F	5	2.31	0.71	413	129	1.8	12.0	283	0.59	4.9	31.6	0.16
57	86	F	1	2.42	0.86	464	75	6.2	11.1	292	0.57	9.0	33.7	0.27
58	86	F	7	2.16	0.83	321	89	6.8	4.5	392	0.16	8.4	30.2	0.28
59	83	F	7	2.27	1.13	295	119	15.0	21.4	-	-	10.7	29.4	0.36
60	89	M	11	1.76	1.10	374	197	7.2	13.5	-	-	33.0	85.8	0.39
61	85	F	0	2.21	0.74	212	148	12.4	16.4	392	0.59	16.8	64.4	0.26
62	87	F	5	2.03	0.81	252	49	2.4	14.7	-	-	15.7	63.2	0.25
63	76	M	2	2.21	0.68	195	103	1.1	12.1	-	-	5.2	23.6	0.22

PATIENTS WITH OSTEOMALACIA + A FEMORAL NECK FRACTURE

C

CASE No.	AGE	SEX	N	Ca mmol/L	P mmol/L	AP IU/L	Cr umol/L	t25 ng/ml	t1,25 pg/ml	DBP mg/L	f1,25 index x 10^5	OA	Surf	OI
64	43	F	0	2.02	0.64	473	53	3.9	4.6	-	-	59.9	92.2	0.65
65	46	F	0	1.84	0.67	355	104	2.6	13.0	-	-	41.4	91.3	0.45
66	90	F	0	1.56	1.34	759	164	3.7	16.0	-	-	51.8	87.4	0.59
67	81	M	0	2.21	0.81	1303	70	2.7	21.0	-	-	45.7	81.2	0.56
68	71	F	0	1.87	1.17	1058	43	0.7	14.8	-	-	51.9	84.8	0.61
69	70	F	0	1.17	0.98	817	104	1.0	23.9	351	0.94	16.2	52.0	0.31
70	52	F	2	1.45	0.98	131	60	8.7	16.0	426	0.52	5.38	14.8	0.36
71	53	M	1	2.24	0.68	427	153	2.5	22.9	-	-	8.2	37.5	0.22
72	29	F	0	2.26	1.33	276	ins	3.9	47.1	-	-	15.1	53.4	0.28
73	57	F	0	2.17	1.11	373	104	5.4	21.5	-	-	19.1	61.3	0.31
74	88	M	0	2.13	0.42	227	111	4.1	26.1	386	0.31	33.5	96.2	0.35

NON FRACTURE OSTEOMALACIA

APPENDIX 8

CHANGES IN CALCIUM HOMEOSTASIS AFTER TOTAL THYROPARATHYROIDECTOMY IN MAN

Tables showing individual biochemical values for the 10 patients studied:

NAME Patient 1

HOSPITAL NUMBER N 54 37 92

AGE 82

PLASMA

Date	21/2	23/2	24/2		25/2	26/2	27/2	28/2	1/3	2/3	3/3	4/3	5/3	6/3	7/3	8/3	9/3	10/3
Time				Po/op	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Creatinine	70	78		81	97	83	73	80	57	73	64	86	33	67	78	61	70	71
Urea	3.9	3.5		4.1	5.1	4.0	4.6	5.6	6.8	8.2	8.1	8.4	9.2	8.0	5.2	5.2	4.0	2.9
Sodium	132	138		138	140	138	142	139	138	140	140	139	135	132	126	130	128	129
Potassium		4.4		3.4	3.8	3.5	2.8	3.1	4.1	4.5	3.4	4.0	6.6	3.8	3.3	3.9	3.9	4.1
Bicarb	26	28		18	17		24			23	22	17	18	21			18	
Calcium	2.42	2.35	2.24	1.99	1.76	1.98	2.12	2.15	1.99	2.05	1.85	1.90	1.84	1.79	1.91	2.12	2.21	2.18
Corr Ca	2.48	2.45	2.40	2.19	2.00	2.22	2.32	2.39	2.27	2.35	2.07	2.14	2.10	2.05	2.27	2.44	2.47	2.46
Phosphate	1.32	1.26	1.20	1.65	0.79	0.71	0.47	0.63	0.87	1.33	1.64	1.60	1.38	1.49	1.48	1.54	1.59	1.42
Magnesium		0.75			0.73	0.91	0.48		0.87	0.97	0.90	0.95	0.93	0.88			0.71	
Alk Phos	220	180	163	147	113	133	139	143	159	167	195	203	219	212	228	173	269	147
Albumin	37	35	32	30	28	28	30	28	26	25	29	28	27	27	24	24	27	26
Globulin																		
GGT	25	25	23			13	14	14	31	41	44	67	78	81	66		55	
Bilirubin	5	9	9			16	21	12	13	14	15	15	15	8	10		9	
ALT	9	20	9			19	18	18	14	11	12	5	2	4	1		4	
Glucose																		

FASTING URINE

Creatinine	2.5	3.0						2.70		9.8		9.3	9.7	2.1	2.9			
Calcium	1.45	2.5		2.82		2.14		5.70		6.76		3.75	4.69	3.81	5.40			
Phosphate	6.30	7.6		20.3		0.66		0.60		0.06		35.83	49.4	6.66	17.02			
Sodium	30	10				124		96		46		48	32	100	40			
CaE	47	65				296		169		50.3		34.4	40.0	121	53			
NaE	840	260		1492		19153		2844		343		906	274	3190	395			
TmCa/GFR	1.90	1.78		1.35		1.10		1.43		1.75		1.65	1.55	1.25	1.65			
TMP/GFR	1.20	1.08						0.48		>1.4				1.34				

24 HR URINE

Volume	1680	821	373	1105	2653	2469	2556	1345	Lost	1509	1094	864	Lost	1281	Lost	977		
Creatinine	5.1	5.4	1.9	6.6	6.4	6.2	5.6	5.4										
Calcium	4.2	4.4	3.2	3.9	5.4	5.2	10.0	7.7										
Phosphate	7.7	8.3	9.0	14.0	4.08													
Sodium	43	17	35	87														

NAME Patient 2

HOSPITAL NUMBER N 54 37 89

AGE 48

PLASMA																		
Date	5/2	6/2	11/2		12/2	13/2	14/2	15/2	16/2	17/2	18/2	19/2	20/2	21/2	22/2	23/2	27/2	3/3
Time				Po/op	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Creatinine	63	63		97	67	72	56	52	52	49	44	65	67	68		51		71
Urea																		
Sodium																		
Potassium				4.4	3.7	3.3	2.9	3.2	3.2	3.8	4.1	4.0	3.9	4.1		4.6		4.1
Bicarb																		
Calcium	2.33		2.54	2.15	2.03	1.91	2.00		1.93	1.80	1.90	1.94	1.96	1.64		1.49	1.53	1.87
Corr Ca	2.37	2.37	2.58	2.47	2.21	2.11	2.22		2.17	2.08	2.16	2.18	1.98	1.82		1.71	1.69	1.93
Phosphate	1.27		1.51	1.43	1.06	0.76			1.62	1.67	1.94	1.74	1.84	1.77		1.67	1.96	1.84
Magnesium	0.93				0.66	0.73		0.57	0.66	0.61	0.80	0.66	0.69			0.81		0.70
Alk Phos	152		170	102	73	98			108	111	122	172	184	278		156	221	233
Albumin	38		38	24	31	30	29		28	26	24	22	29	31		28	32	33
Globulin					14				26									
GGT	26				18				16	18		52				48		41
Bilirubin	6				24				17	11		13				8		8
ALT	17				38				19	15		13				18		45
Glucose																		

FASTING URINE																		
Creatinine		4.6	10.2		12.2		4.6	0.9	7.1	4.3	2.3	3.7	7.0					
Calcium		3.5	10.0		0.20	1.4	0.75	0.55	0.45	1.4	2.7	3.4						
Phosphate		0.85	57.4		27.5	45.4	0.48	0.62	29.1	21.5	15.7	18.0						
Sodium		33	41		66		170	102	67	182	156	115	73					
CaE		31.7	61.7		1.09		4.1		3.29	15.9	51.6	59.7					25	
NaE		174	253	560	362		2069		490	2074	2984	2020					135	
TmCa/GFR		1.90	1.85	2.10	>2.5	1.85	2.05		2.35	1.75	1.55	1.55					1.32	
TMP/GFR			1.16		0.94				1.48	1.46	1.68	1.44						

24 HR URINE																		
Volume	2230		165	740	2665	1796	759	2405	1312	2818	2380	906	641	850	1067	580	641	
Creatinine	8.0		1.68	8.14	7.46	7.72	1.44	5.0	7.08	6.76	5.71	5.3		4.6	6.7	4.0	2.1	
Calcium			1.65	1.70	3.78	1.49	0.83	1.6	0.32	4.9	5.14	0.52	0.94			0.74		
Phosphate			9.5	16.2	23.5	1.40	3.37	2.5	25.6	40.3	38.9	19.6				3.3		
Sodium	85		68	47	309	276	108	360	147	397	192			17	5	64	4	

NAME Patient 3

HOSPITAL NUMBER N 55 10 23

AGE 62

PLASMA																		
Date	24/4	27/4	28/4		29/4	30/4	1/5	2/5	3/5	4/5	5/5	6/5	7/5	8/5	9/5	10/5	11/5	12/5
Time				Po/op	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Creatinine	64		63	71	107	88	77	61	61	62	59	58	50	56	54	60	47	62
Urea	2.6		3.6	5.3	5.3	6.5	6.4	6.5	5.3	6.4	6.1	4.8	5.5	5.1	6.1	5.5	6.9	6.0
Sodium	136		142	139	139	141	140	140	136	137	136	136	133	133	136	134	132	134
Potassium	3.5		3.7	4.1	4.1	3.9	4.3	3.9	4.2	4.2	3.8	4.1	3 9	4.1	3.8	4.1	4.2	3.9
Bicarb							24	23			25	26	27		25		22	25
Calcium	2.43		2.20	2.31	1.80	1.92	1.90		1.72		1.51	1.60	1.63	1.57	1.58	1.72	1.62	1.63
Corr Ca	2.51		2.41	2.54	2.00	2.14	2.18		2.08		1.85	1.94	1.97	1.91	1.94	2.00	2.00	1.95
Phosphate	1.20		1.12	1.33	1.04	0.74	0.88		1.34		1.24	1.23	1.49	1.65	1.46	1.59	1.65	1.71
Magnesium	0.78		1.10	0.77	0.72	0.94			0.74		0.73			0.84	0.82			0.68
Alk Phos	333		155	254	112	62	125	124	144		139	212	184	229	228	252	262	347
Albumin	36		23	35	30	29	26	24	22		24	23	23	23	22	26	22	24
Globulin																		
GGT	15		24		30	8	14	14	18		18	34					65	
Bilirubin	10		5		10	16	12	10	11		11	11					21	
ALT	4		8		43	27	25	7	7		7	9					2	
Glucose												14			<9			

FASTING URINE																		
Creatinine			5.6	5.6	16.4	14.0	9.7		4.9		4.7			6.3	5.8		5.7	
Calcium			4.8	3.3	0.2	6.5	4.8		2.03		2.19			2.6	1.64		2.5	
Phosphate			6.1	8.3	0.12	1.55	24.3		7.9		20.4			23.7	19.1		24.8	
Sodium			24	40		2	4		51		151			96	122		118	
CaE			54	42	1.3	41	38		25		27			23			20.6	
NaE			270	507	1450	12.6	32		635		1895			853			973	
TmCa/GFR			1.88	1.83	2.43	1.60	1.65		1.65		1.43			1.55			1.53	
TMP?GFR			1.28	1.41		1.06	0.67		1.46		0.99			1.52				

24 HR URINE																		
Volume					1387													
Creatinine					7.6													
Calcium					3.3													
Phosphate					1.3													
Sodium					103													

NAME Patient 4

HOSPITAL NUMBER N 55 22 65

AGE 72

PLASMA																		
Date	11/6		15/6	16/6		17/6	18/6	19/6	20/6	21/6	22/6	23/6	24/6	25/6	26/6	27/6	28/6	29/6
Time					Po/op	1	2	3	4	5	6	7	8	9	10	11	12	13
Creatinine	85			101	101	95	105	102	61	57	70	73	68	69	78	69	56	73
Urea	4.4			5.5	5.5	8.5	9.7	10.5	9.4	6.9	7.9	10.4	9.2	7.3	8.4	9.1	10.0	11.8
Sodium	136			144	144	139	140	141	143	141	142	139	139	138	139	141	137	141
Potassium	3.9			3.6	3.6	3.0	3.2	3.6	3.4	3.4	3.5	3.8	3.9	3.7	3.8	3.6	3.6	3.3
Bicarb						25	24	23	25			24		21	20	21		22
Calcium			2.29	2.32	2.07	1.82		1.73		1.86	1.75	1.64	1.56	1.64	1.67	1.61	1.55	1.64
Corr Ca			2.47	2.37	2.52	1.94		1.83		2.20	2.09	2.00	1.94	2.00	1.85	1.95	1.89	1.94
Phosphate			1.23	1.61	1.11	1.50		1.47		1.50	1.75	1.96	1.78	1.74	2.09	2.21	2.17	2.07
Magnesium			0.81	0.85			0.50	0.66		0.56	0.57	0.54	0.71	0.63	0.64			0.70
Alk Phos	172		167	171	132	107		126		237	237	277	312	386	384	405	433	550
Albumin	31		31	30	25	34		35		24	24	22	22	22	21	23	23	25
Globulin								20			29			29				38
GGT	25		23					13			79			100				150
Bilirubin	10		7					10			12			15				18
ALT	5		5					14			13			11				7
Glucose														19				

FASTING URINE																		
Creatinine				8.5		12.5	11.0	6.6		1.6		9.4	7.1	6.2	6.8	6.1	8.4	
Calcium			1.42	1.21	0.94	2.84		2.11		2.24		0.10	0.34	0.39	0.6	0.57	0.37	
Phosphate			10.2	15.5	23.8	7.62		12.36		1.74		36.8	36.2	23.2	40.5	24.1	48.3	
Sodium				140		78	37	60		127	57	23	85	114	56	69	41	
CaE				14.4		21.6		32.6		9.8		<1	3.2	4.3	6.9	6.4	2.5	
NaE						593		927				179	814	1269	642	780	273	
TmCa/GFR				2.05		1.55		1.40		1.50					1.75	1.90	2.10	
TMP/GFR																		

24 HR URINE																		
Volume																		1730
Creatinine																		7-8
Calcium																		
Phosphate																		
Sodium																		144

NAME Patient 5

HOSPITAL NUMBER

AGE 75

PLASMA																		
Date	2/2	3/2	4/2		5/2	6/2	7/2	8/2	9/2	10/2	11/2	12/2	13/2	14/2	15/2	16/2	17/2	18/2
Time		Po/op	1		2	3	4	5	6	7	8	9	10	11	12	13	14	15
Creatinine		64	51		88	72	58	50	57	69	65	62	64	67	76	53	62	63
Urea		6.8	6.8		7.0	8.2	9.7	9.3	9.5	10.7	10.9	10.8	12.2	12.2	11.0	11.5	10.3	8.4
Sodium		141	139		139	135	136	137	139	138	142	137	139	131	134	131	131	132
Potassium		3.3	3.3		3.2	3.8	3.6	3.2	3.6	3.9	4.0	4.3	4.7	4.2	4.3	4.4	3.9	3.9
Bicarb		27					15		26	23	22	24	17	22		21	16	
Calcium		1.85	1.86		2.24	1.87		1.84	1.78	2.13		2.02	2.04			1.90		
Corr Ca			1.96			2.11		2.06	1.88	2.30		2.20	2.35			2.00		
Phosphate			1.20		1.35	1.56		1.64	1.68	1.48		1.63	1.80			1.64		
Magnesium			0.70		0.51		0.52		0.58				0.92			0.71		
Alk Phos			122		140	145	146	218	344	419	390	470	545			556		
Albumin			35		33	28	30	29	35	31	32	31	31			27		
Globulin			22		21	21										35		
GGT			15		16	12	13	37	83	123	100					151		
Bilirubin			32		33	20	19	18	24	29	10					21		
ALT			17		13	18	9	6	18	9	11					15		
Glucose																		

FASTING URINE																		
Creatinine			16.3		11.4		7.6		10.5		4.3	6.6						
Calcium			1.86		5.46		0.54		0.60		0.6	1.11	0.4					
Phosphate			1.38		2.10		26.6		18.12		38.9	2.82	45.6					
Sodium			81		81		16		10		12	13						
CaE			5.8		42				3.2		9.1	10.4						
NaE			253		625				54		181	122						
TmCa/GFR			1.92		1.80				2.00			2.0						
TMP/GFR																		

24 HR URINE																		
Volume	1025				1510	1085	1636	1457	1664		2149	1891	847	619				467
Creatinine	6.5				6.8	6.0	6.4	5.9	4.3		6.0	4.7	4.6	3.1				2.8
Calcium	2.2				4.3			2.1	2.6		2.7	1.6	0.4	0.3				0.8
Phosphate	0.7				1.0			11.1	18.2		8.7	21.1	22.5	21.9				6.0
Sodium	118				177	61	136	122	116		135	161	39	25				9.0

NAME Patient 6

HOSPITAL NUMBER

AGE 68

PLASMA																	
Date	15/1	16/1	18/1	19/1	20/1	21/1	22/1	23/1	24/1	25/1	26/1	27/1	28/1	29/1	30/1	31/1	1/2
Time					1	2	3	4	5	6	7	8	9	10	11	12	13
Creatinine	83	87	84	82	96	75	84	67	55	63	69	62	64	61	63	64	65
Urea	3.8	4.8	4.7	3.7	5.6	5.8	7.6	8.5	7.8	7.5	2.2	8.2	8.1	8.8	8.9	8.5	9.5
Sodium	137	139	136	138	136	136	140	141	144	142	136	137	140	137	136	133	134
Potassium	4.0	4.4	3.9	3.2	3.2	3.3	5.0	4.3	4.1	4.1	3.7	3.3	3.5	3.5	4.1	3.9	3.9
Bicarb		29	30	20	22	20	28	24		24		20	22	23			26
Calcium	2.33	2.44	2.42	2.33	1.82	1.97	1.97	2.05		2.10	1.85	1.91	1.72	1.75	1.73	1.92	2.05
Corr Ca	2.39	2.50	2.50	2.39	1.98	2.21	2.29	2.47		2.28	2.15	2.27	2.12	2.15	2.11	2.30	2.43
Phosphate	1.40	1.47	1.51	1.27	1.05	0.81	0.59	0.79	1.08	1.38	1.33	1.32	1.51	1.37	1.59	1.41	1.40
Magnesium		0.94								0.84			0.72				0.80
Alk Phos	194	96	125	101	80	104	143	122		136	115	120	140	291	327	691	618
Albumin	37	37	36	37	32	28	24	19	26	31	25	22	20	20	21	21	21
Globulin						19											41
GGT		42	36			30				60			49				207
Bilirubin		8	7			13				25			9				14
ALT		14	12							231			32				19
Glucose																	

FASTING URINE																	
Creatinine	4.0	6.8	4.4	8.0	11.0	1.8	13.5	5.2	3.6	5.0	11.3	7.9	12.0	12.1	8.1	12.0	9.7
Calcium	3.7	3.1	1.4	2.8	1.21	3.6	1.6			0.88		2.1	1.12	1.2	1.73	0.66	
Phosphate	6.2	15.9	15.0	16.0	24.0	6.5	47.1			10.0		40.3	12.3	81.0	49.0	78	
Sodium	86	50	26	26	7	88	6	13	107	96	40	64	68	60	77	33	33
CaE	77	39.7	26.7	28.7	10		10			11		16.5	6.0	6.0	13.4	3.5	
NaE	1784	640	496	266	61		37.3	167		1210	244	502	362	302	599	176	221
TmCa/GFR	1.65	1.94	2.01	1.90	1.79		2.10			2.10		1.95	2.10	2.08	1.85		
TMP/GFR																	

24 HR URINE																	
Volume			1288							974	882					1459	
Creatinine			8.1							7.3	5.0					5.7	
Calcium			3.0							0.70	1.30					2.7	
Phosphate			33.1							19.9	29.5					26.3	
Sodium			95							77	59					143	

NAME Patient 7

HOSPITAL NUMBER

AGE 53

PLASMA																		
Date	29/10	2/11	3/11	Po/op	4/11	5/11	6/11	7/11	8/11	9/11	10/11	11/11	12/11	13/11	14/11	15/11	16/11	17/11
Time																		
Creatinine	95	106		137	99	125	120		125	130	106	89	87	97	100	90	93	
Urea	2.9	3.1		5.7	5.3	5.0	4.5		5.1	7.0	7.7	7.3	5.4	4.6	4.1	4.1	4.5	
Sodium	137	139		138	135	141	143		141	148	147	148	146	145	143	138	142	
Potassium	4.0	4.1		4.3	3.2	3.6	3.5		4.8	4.9	4.4	3.8	3.5	4.8	4.5	3.9	5.0	
Bicarb	25			23	23	27				23	22	26		25				
Calcium	2.27	2.53	2.41	1.84	1.79		2.27	2.38	2.44	2.44	2.28	2.14	2.07	2.29		2.26	2.29	2.31
Corr Ca	2.47	2.63	2.51	2.02	1.93		2.39	2.68	2.82	2.82	2.68	2.60	2.55	2.73		2.72	2.71	2.71
Phosphate	0.82	1.39	1.18	1.58	1.28		1.11	0.84	0.84	0.84	1.63	1.39	1.47	1.71		1.58	1.69	1.75
Magnesium	0.84					0.80				0.84			0.84	0.93			0.95	
Alk Phos	184	178	166	44	80		114	163	147	147	149	155	140	222		228	199	200
Albumin	30	35	35	31	33		34	25	21	21	20	17	16	18		17	19	20
Globulin					16								33					
GGT	111	120			34		30			45			67	137			164	161
Bilirubin	14	13			38		20			34			25	25			89	17
ALT	26	41			26		34			25			28	20			31	25
Glucose																		

FASTING URINE																		
Creatinine	9.3	10.3			8.8		21.5	15.5		14.9	11.3			13.5	17.0	15.0	17.3	
Calcium	4.3	3.7	6.1				0.4	1.9		9.2	8.3			1.2	5.1	0.9	17.7	
Phosphate	15.3	21.1	38.6				1.1	1.7		1.9	5.9			23.2	35.9	38.8	23.7	
Sodium	138	45	5		17		2	3	9	3	13			126	135	14	77	
CaE	43.9	38.1	18.9				2.3	15.1		80.3	77.9			8.7	30	5.4		
NaE	1410	463					11.2			26.2	122			905	794	84	414	
TmCa/GFR																		
TMP/GFR																		

24 HR URINE																		
Volume	990	493	1456															
Creatinine	7.0	11.0	15.3															
Calcium	3.8	4.2																
Phosphate	8.0	27.3																
Sodium	69	14	28															

NAME Patient 8

HOSPITAL NUMBER S 68 82 03

AGE 57

PLASMA											
Date	12/10	13/10	14/10	15/10	16/10	17/10	18/10	19/10	20/10	21/10	22/10
Time		Po/op									
Creatinine	65	71	77	82	76	77		73	85	76	88
Urea	3.5	4.3	5.5	6.6	5.0	3.6		4.1	3.9	3.1	3.7
Sodium	139	134	136	138	139	138		139	138	138	138
Potassium	4.8	4.4	4.1	4.0	4.1	4.1		4.8	4.5	4.7	4.5
Bicarb	31	28	30	30	28	27		28	27	29	28
Calcium	2.42	2.10	1.72	1.70	1.5	1.75	1.87	1.74	1.71	2.28	2.06
Corr Ca	2.56	2.32	1.88	1.86	1.78	1.85	1.95	1.84	1.81	2.34	2.16
Phosphate	1.15	1.00	0.68	0.67	0.59			2.01	1.75	1.79	1.45
Magnesium	0.85	0.65		0.70					0.75		
Alk Phos	389	269		259	234			235			
Albumin	33	29	32	33	26	35	36	35	35	37	35
Globulin											
GGT											
Bilirubin											
ALT											
Glucose											

FASTING URINE											
Creatinine	8.6	7.1	11.2	13.2	9.8		9.3				14.1
Calcium	6.2	12.3	13.7	6.7	6.5		3.9				3.9
Phosphate	2.7	5.3	2.2	2.2	0.7		14.3				17.1
Sodium	51	102	23	56	129		84				28
CaE	46.9	123	96.2	41.6	50.4		31.0				24.3
NaE	355.5	1020	158	347	127		659				174.8
TmCa/GFR											
TMP/GFR											

24 HR URINE																		
Volume	1.70	1.64	1.40	1.44	1.38		1.53											
Creatinine																		
Calcium																		
Phosphate																		
Sodium																		

NAME Patient 9

HOSPITAL NUMBER N 67 07 20

AGE 52

PLASMA																			
Date	27/4	28/4	28/4	29/4	30/4	1/5	2/5	3/5	4/5										
Time			Po/op																
Creatinine	72	82	104	84	80	71	80	102	79										
Urea	5.7	5.0	6.5	6.2	3.8	6.7		4.9	6.2										
Sodium	140	136	138	135	131	133		131	137										
Potassium	5.0	6.6	4.7	6.1	3.4	4.0		6.4	6.5										
Bicarb	28	31	26	29	26	28		25	30										
Calcium	2.43	2.37	2.34	1.91	2.06	2.09	2.12	2.27	2.34										
Corr Ca	2.39	2.33	2.5	2.09	2.18	2.17	2.18	2.29	2.34										
Phosphate	0.93	1.06	1.05	1.02	1.05	0.55	0.93	0.84	1.2										
Magnesium	0.75																		
Alk Phos	292	278	195	201	227		273												
Albumin	42	42	32	31	34	36	37	39	40										
Globulin	75				59														
GGT	61				40														
Bilirubin	6				11														
ALT	18				14														
Glucose																			

FASTING URINE																			
Creatinine	22.9	15.5	11.1	17.2	6.1	9.9	14.1	10.2	21.6										
Calcium	11.3	5.2	6.3	4.1	1.4	7.4	5.9	7.0	13.8										
Phosphate	22.5	19.6	9.0	16.3	4.9	4.6	3.0	4.0	9.6										
Sodium	76	75	80	54	23	102	182	136	113										
CaE	35.5	27.5	59	20	18	53	33	70	50										
NaE		397	750	264	301	731	1032	1360	413										
TmCa/GFR	1.85	1.85	1.80	1.70	1.84	1.57	1.68	1.60	1.71										
TMP/GFR																			

24 HR URINE																			
Volume		0.92		0.9	1.79	2.0	1.07		1.35										
Creatinine		14.7		14.4															
Calcium				5.4															
Phosphate				13.8															
Sodium																			

NAME Patient 10

HOSPITAL NUMBER 30 31 53

AGE 41

PLASMA																		
Date	18/9	18/9	19/9	20/9	21/9	22/9	23/9	24/9	28/9	29/9	30/9	1/10	5/10					
Time		Po/op																
Creatinine	83	85	74	78	68	62	68	63	71	64	86	68	72					
Urea																		
Sodium	142	139	135	139	141	139	139	139	135	135	138	139	139					
Potassium	4.5		3.8	2.8	3.1	3.3	3.2	3.7	3.6	4.2	4.2	4.3	4.5					
Bicarb	32		29	28	34	33	30	30	30	32	31	30	30					
Calcium	2.45	2.06	1.91	1.95	1.73	1.77	1.70	1.61	1.71	1.97	2.04	2.15	2.28					
Corr Ca	2.27	2.20	1.83	1.89	1.73	1.75	1.60	1.57	1.87	2.09	2.20	2.27	2.32					
Phosphate	0.95	1.02	0.87	1.37	0.52	0.86	1.36	1.48		1.12	0.82		1.45					
Magnesium	0.90	0.65	0.90	0.75	0.75	0.85												
Alk Phos					177				176	172		179	150					
Albumin	49	33	44	43	40	41	45	42	32	34	32	34	38					
Globulin																		
GGT						29					33		25					
Bilirubin						15					5		6					
ALT						15					18		13					
Glucose																		

FASTING URINE																		
Creatinine	13.4		9.1	5.5		16	9.7	3.4	12.2	7.5	8.5							
Calcium	3.7		3.0	1.0		1.4	0.9	1.6	7.6	9.1	7.5							
Phosphate	20.1		8.1	4.6		2.3	3.5	2.8	21.5	16.1	20.2							
Sodium	49		90	24		16	43	43	34	30	35							
CaE	22.9	29.9	24.4	14.2	9.9	5.4	6.3	29.6	44.2	77.6	75.8							
NaE	303	777	732	340	184	62	301	797	191	256	354							
TmCa/GFR	1.87	1.82	1.45	1.60	1.55	1.70	1.50	1.20	1.35	1.43	1.54							
TMP/GFR	0.87	1.04	0.93	1.68	0.68	1.24	1.12	1.86										

24 HR URINE																		
Volume																		
Creatinine																		
Calcium																		
Phosphate																		
Sodium																		

PATIENT 1

	Pre op	1	2	3	4	5	6	7	8
PTH (ug/L)	0.37	<0.25	<0.25					<0.25	<0.25
$1,25(OH)_2D$ (pg/ml)	33.0	10.1	18.7	17.7	32.6	19.6		23.6	11.4
$24,25(OH)_2D$ (pg/ml)	150	47	40	48	61	76		60	54
25(OH)D (ng/ml)	4.6	2.2	2.5	2.3	1.9	2.4		2.7	2.6
Osteocalcin (ng/ml)									
DBP (mg/L)	383	200	197	340	260	294		324	309
Free $1,25(OH)_2D$ index x 10^5	1.0	0.59	1.11	0.60	1.45	0.77		0.85	0.43

PATIENT 2

	Pre op	1	2	3	4	5	6	7	9	10
PTH (ug/L)	<0.25	<0.25	<0.25			<0.25		<0.25	<0.25	
$1,25(OH)_2D$ (pg/ml)	22.2	4.5	3.8	3.3	3.4	9.4		5.1		
$24,25(OH)_2D$ (pg/ml)	360	220	250	190	190	250	160	120		
25(OH)D (ng/ml)	2.8	1.6	1.0	1.4	2.5	1.5	1.3	1.9		
Osteocalcin (ng/ml)										
DBP (mg/L)	406	164	211	216	311	289	290	330	399	393
Free $1,25(OH)_2D$ index x 10^5	0.69	0.32	0.21	0.18	0.13	0.38		0.18		

PATIENT 3

	Pre op	1	2	3	4	5	6	7	8
PTH (ug/L)									
$1,25(OH)_2D$ (pg/ml)	31.2	9.1	10.2	9.3					
$24,25(OH)_2D$ (pg/ml)	250	32	11	50					
25(OH)D (ng/ml)	3.6	1.4	1.2	1.8					
Osteocalcin (ng/ml)									
DBP (mg/L)	327	131		214					391
Free $1,25(OH)_2D$ index x 10^5	1.11	0.81		0.51					

PATIENT 4

	pre-op		1	2	3	4	5	6	7	8
PTH (ug/L)										
$1,25(OH)_2D$ (pg/ml)	44.9	27.1	16.4	10.2	4.45	11.9	7.5	11.9	13.0	11.6
$24,25(OH)_2D$ (pg/ml)	360	100	107	160		93	120		116	147
25(OH)D (ng/ml)	14.8	3.7	7.22	3.94	3.65	2.6	6.13	1.4	2.91	2.13
Osteocalcin (ng/ml)										
DBP (mg/L)										
Free $1,25(OH)_2D$ index x 10^5										

PATIENT 5

	Pre op	1	2	3	4	5	6	7	8
PTH (ug/L)	<0.25	<0.25	<0.25		<0.25				
$1,25(OH)_2D$ (pg/ml)	38.1	15.4	9.5		12.2			9.7	3.7
$24,25(OH)_2D$ (pg/ml)	73.0	72.0	77.0		55.0			82.0	180
25(OH)D (ng/ml)	1.9	0.93	0.93		0.41			1.16	1.05
Osteocalcin (ng/ml)									
DBP (mg/L)	410	241	214		318			286	260
Free $1,25(OH)_2D$ index x 10^5	1.08	0.74	0.52		0.45			0.39	0.17

PATIENT 6

	Pre op	1	2	3	4	5	6	7	8	9	11	12
PTH (ug/L)												
$1,25(OH)_2D$ (pg/ml)	35.5	37.3	27.9	15.0	31.5	27.7	39.6		22.8	17.9	15.2	27.2
$24,25(OH)_2D$ (pg/ml)	0.15	0.26	0.11	0.07	0.12	0.15	0.14	109		120	140	105
25(OH)D (ng/ml)	10.2	4.22	2.96	275		6.52	7.0	2.96	2.73	2.56	1.88	2.84
Osteocalcin (ng/ml)	5.1	1.0		1.1		0.5		2.0	1.8		1.8	
DBP (mg/L)	267	112		138			207					
Free $1,25(OH)_2D$ index x 10^5	1.54	3.86		1.26			2.22					

PATIENT 7

	Pre op	1	2	3	4	5	6	7	8	9
PTH (ug/L)										
$1,25(OH)_2D$ (pg/ml)	43.9	47.8	31.2	26.1	25.0	29.7		23.7	29.1	48.3
$24,25(OH)_2D$ (pg/ml)	140	120	80	100	110	70	50	100	150	60
25(OH)D (ng/ml)		5.38	4.0	1.95	2.0	3.17	3.62	3.5	4.6	4.17
Osteocalcin (ng/ml)	3.6	4.6	3.6	1.7	2.0	1.4	1.3		0.6	1.2
DBP (mg/L)	383	323	210	131	240					
Free $1,25(OH)_2D$ index x 10^5	1.33	1.72	1.72	2.31	1.21					

PATIENT 8

	Pre op	1	2	3	4	5	6	7	8	9
PTH (ug/L)										
$1,25(OH)_2D$ (pg/ml)	40.9	29.6	26.6	16.4	42.0	38.2	33.3	38.4	27.7	
$24,25(OH)_2D$ (pg/ml)										
25(OH)D (ng/ml)	11.7	8.0	4.58	3.61	5.75	7.17	7.81	6.92		
Osteocalcin (ng/ml)	2.9	3.1		2.5		3.4				
DBP (mg/L)	560	386		293		298				
Free $1,25(OH)_2D$ index x 10^5	1.03	1.08		0.65		1.49				

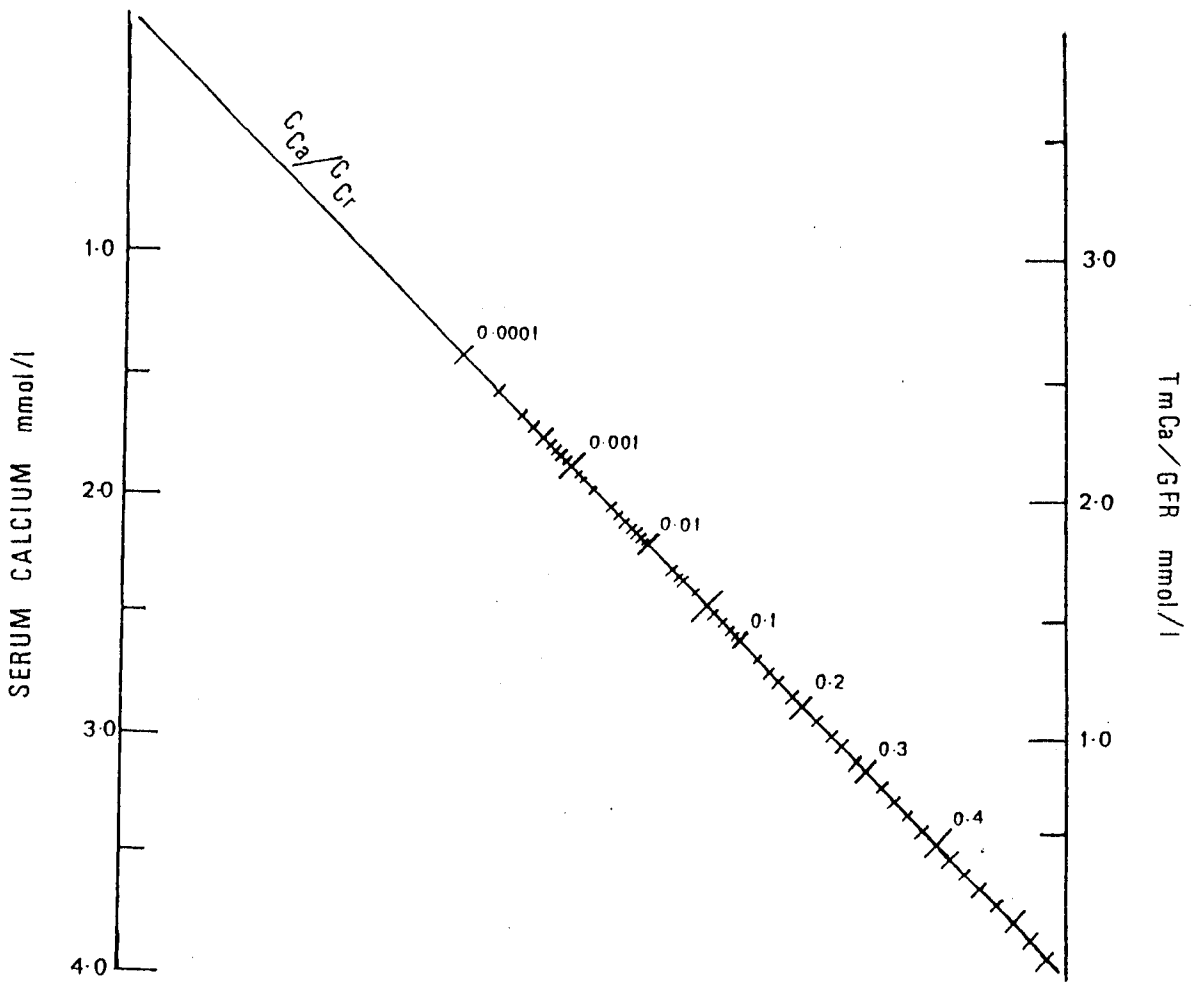
PATIENT 9

	Pre op	1	2	3	4	5	6	7	8
PTH (ug/L)									
$1,25(OH)_2D$ (pg/ml)	32.3	5.3	8.6	5.3				10	
$24,25(OH)_2D$ (pg/ml)	60	170	110	80	73		220	100	
25(OH)D (ng/ml)	3.1	5.6	5.0	2.5	5.2		6.9	5.1	
Osteocalcin (ng/ml)									
DBP (mg/L)	304	329	387					399	
Free $1,25(OH)_2D$ index x 10^3	2.07	0.23	0.37					0.35	

PATIENT 10

	Pre op	1	2	3	4	5	6	7	8	11
PTH (ug/L)										
$1,25(OH)_2D$ (pg/ml)	41.1	24.9	34.5		52.6	51.4	48.2	56.2	48.7	47.0
$24,25(OH)_2D$ (pg/ml)	130	200	160			190		180		210
25(OH)D (ng/ml)	8.6	3.6	3.5	5.4	8.8	8.1	7.3	7.6	7.6	7.3
Osteocalcin (ng/ml)	3.7		1.6		1.8	1.8	1.5		2.1	
DBP (mg/L)	383	339			355		375			
Free $1,25(OH)_2D$ index x 10^3	1.04	2.09			1.72		1.49			

Nomogram to estimate TmCa/GFR (mmol/L)



- FINIS CORONAT OPUS -

(The end crowns the work)

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Changes in osteocalcin after femoral neck fracture

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SUMMARY. In a group of elderly patients with osteoporosis and femoral neck fracture, osteocalcin concentrations rose significantly in the first week after fracture fixation. The changes in osteocalcin correlated well with the changes in plasma 1,25-dihydroxycholecalciferol ($P < 0.001$).

Osteomalacia is an important[1,2] but uncommon[3] treatable accompaniment of femoral neck fracture. Current biochemical screening tests which rely on measurements of serum calcium and alkaline phosphatase have a high false positive rate.[4] This is largely because alkaline phosphate arises from both bone and liver: hepatic dysfunction is common in the elderly fracture population.[5]

The recent availability of radioimmunoassays for osteocalcin, a bone specific protein that is released during osteoblastic activity[6,7] and is stimulated by 1,25-dihydroxyvitamin D (1,25-$(OH)_2D$)[8,9] raises the possibility that it may be a more specific test for osteomalacia than serum alkaline phosphatase. The problem is that there is no information about changes in osteocalcin concentration during the immediate post-fracture period when it might be used for screening.

In the present study we describe the changes in osteocalcin concentration which occur in the first week after a femoral neck fracture and its fixation. We have also examined its relationship to alkaline phosphatase, 1,25-$(OH)_2D$ and bone histomorphometry.

Patients and methods

The study was based on 26 consecutive patients (3 male, 23 female; mean age 78·2 years, range 58–92 years) who had sustained a traumatic fracture of the femoral neck. Perioperative iliac crest bone biopsies had excluded osteomalacia in all cases: osteoid area < 3·5%; surface extent of osteoid < 25%, osteoid seam thickness index < 0·14, < 4 osteoid lamellae/seam.[10,11] No patient took calcium or vitamin D supplements either before or during the study.

Fasting blood samples were taken pre-operatively, within 24 h of the fracture in all patients, and were repeated one week later by which time all fractures had been treated by internal fixation.

Blood samples were collected into lithium heparin tubes and the plasma stored at −20°C until assay. Plasma calcium, phosphate, creatinine, albumin concentrations and alkaline phosphatase were measured by standard autoanalyser techniques.[10] Calcium concentrations were adjusted to a plasma albumin of 40 g/L.[12]

Plasma osteocalcin concentrations were measured by radioimmunoassay (CIS(UK) Ltd, London, UK) using a rabbit antibody raised against bovine osteocalcin. 1,25-$(OH)_2D$ was extracted from plasma and purified by straight phase high performance liquid chromatography on a Zorbax-Sil column.[13] The 1,25-$(OH)_2D$ peak was collected and quantitated by a competitive protein binding assay using calf thymus cytosol.[14] Vitamin D binding protein (DBP) concentrations were measured by radial immunodiffusion.[15,16] The free 1,25-$(OH)_2D$ index was calculated as the molar ratio of 1,25-$(OH)_2D$/DBP × 10^5. This gives a good estimate of the free 1,25-$(OH)_2D$ concentration.[16] Parathyroid hormone (PTH) concentrations were measured using a C-terminal assay.[17]

Reference values for osteocalcin, 1,25-$(OH)_2D$ and DBP were derived from a group of healthy elderly normal subjects (8 male, 13 female; mean age 69·6 years, range 60–81 years). Both patients and control subjects were studied throughout the year. Iliac crest bone biopsies were quantitated as described previously.[3,10]

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TABLE 1. Biochemical changes after femoral neck fracture

	Femoral neck fracture ($n=26$) (mean ± SD)		Reference range
	Basal	1 Week	
Calcium (mmol/L)	2·26 ± 0·15	2·28 ± 0·14	2·2–2·6
Phosphate (mmol/L)	1·15 ± 0·34	1·11 ± 0·18	0·8–1·4
Alkaline phosphatase (IU/L)	221 ± 150	281 ± 196‡	98–280
Creatinine (mmol/L)	98·0 ± 19·9	101·4 ± 54·6	60–120
Osteocalcin (ng/mL)	3·12 ± 1·8	3·73 ± 2·0*	3·2–8·1
Vitamin DBP (mg/L)	299 ± 69	354 ± 76**	284–373
Total 25-(OH)D (nmol/L)	11·6 ± 7·6	8·9 ± 6·5	15·1–53·5
Total 1,25-$(OH)_2$D (pmol/L)	39·6 ± 20·3	43·7 ± 20·0	26·5–72·3
Free 25-(OH) D index ($\times 10^3$)	2·32 ± 1·37	2·02 ± 1·5	2·57–9·45
Free 1,25-$(OH)_2$D index ($\times 10^5$)	0·78 ± 0·4	0·77 ± 0·46	0·44–1·28

Test of significance between basal and one week values: * $P<0·02$ ** $P<0·01$ ‡ $P<0·001$.

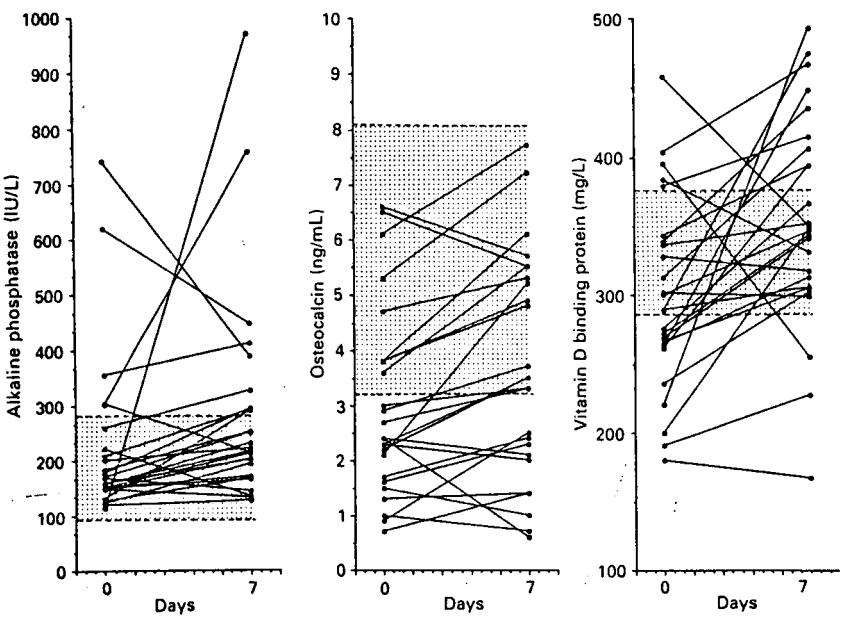

FIG. 1. Changes in serum alkaline phosphatase, osteocalcin and vitamin D binding protein in the first week after a femoral neck fracture and its fixation. Reference range for each variable shown hatched.

Statistical analysis

Differences between and within group were assessed using Student's *t*-test, the paired *t*-test or the Wilcoxon signed-ranks test as appropriate.

Results

The changes in the measured biochemical variables within the first week after femoral neck fracture are summarised in Table 1. There were significant increases in osteocalcin ($P<0.02$), DBP ($P<0.01$) and alkaline phosphatase ($P<0.001$: Wilcoxon signed-ranks test). All the initial values of osteocalcin lay below the upper limit of the reference range, even in those selected because of a raised alkaline phosphatase (Fig. 1). Moreover, the increments in osteocalcin which occurred within the first week after the fracture also lay below this limit. Of the 21 patients with a normal alkaline phosphatase on admission only two rose to a level above the upper limit of normal after one week.

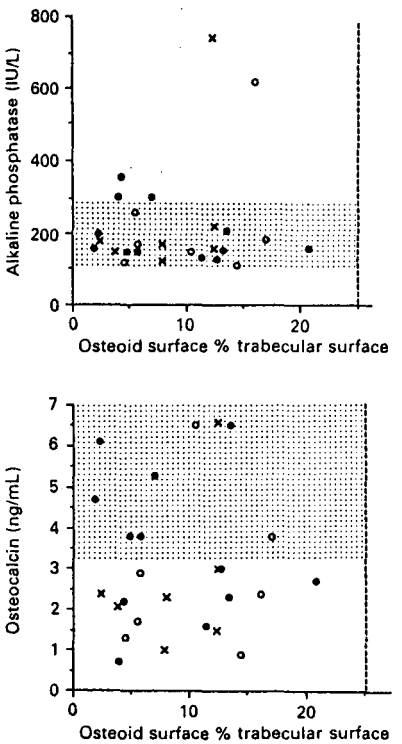

FIG. 2. Plasma osteocalcin and alkaline phosphatase concentrations in relation to surface extent of osteoid in osteoporotic patients with femoral neck fractures. The vertical line indicates the upper limit of normal for the surface extent of osteoid. The hatched area indicates the reference ranges for osteocalcin and alkaline phosphatase. 1,25-$(OH)_2$D: o, 10–25 pmol/L; •, 25–50 pmol/L, ×, > 50 pmol/L.

There was no statistically significant relationship between the admission concentrations of osteocalcin or alkaline phosphastase and the surface extent of osteoid, an index of bone formation (Fig. 2) nor with osteoid area or osteoid seam thickness. This seemed to be because some patients with apparently adequate bone turnover as judged by the extent of osteoid surfaces had low values of osteocalcin. However, when these patients were stratified according the prevailing level of free 1,25-$(OH)_2$D the low osteocalcin values appeared to be associated with very low levels of vitamin D.

There was no correlation between the absolute concentrations of osteocalcin and alkaline phosphatase, DBP, or free and total 1,25-$(OH)_2$D. There was however a strong correlation between the changes in osteocalcin and the changes in free ($r=0.56$; $P<0.001$) or total ($r=0.81$; $P<0.001$) 1,25-$(OH)_2$D (Fig. 3). There was no correlation between the changes in osteocalcin and DBP nor was there any relationship between alkaline phosphatase and 1,25-$(OH)_2$D.

Serum PTH concentrations were either undetectable or at the lower end of the reference range throughout the study.

Neither the type of fracture, its degree of comminution or displacement nor the type of operative fixation appeared to influence the magnitude of the change in osteocalcin over the course of the study.

Discussion

Evaluation of the significance of osteocalcin concentrations after femoral neck fracture requires an appreciation of the factors influencing both the initial post-fracture value and its subsequent change as healing occurs. Since osteocalcin originates only from bone it is potentially a more specific marker of osteoblastic activity than alkaline phosphatase.[18, 19] In the present study patients without osteomalacia but with normal or low bone turnover, assessed from the surface extent of osteoid, had admission values of osteocalcin which lay below the upper limit of the reference range. In contrast alkaline phosphatase was elevated in several of the same patients.

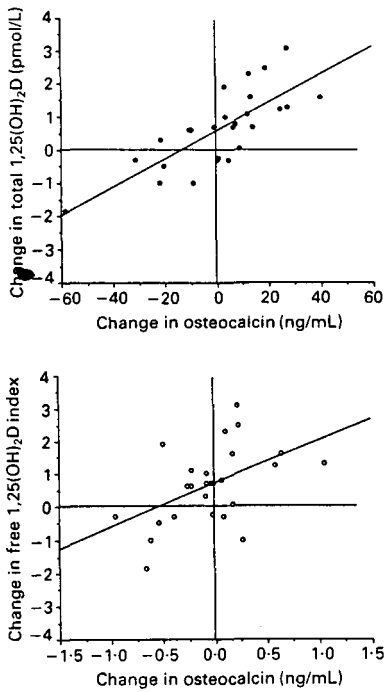

FIG. 3. Correlation between the changes in osteocalcin and the changes in total and free 1,25-$(OH)_2$D concentrations in the first week after femoral neck fracture.

Unlike previous studies of younger women with osteoporosis[18-20] we were unable to find a correlation between osteocalcin and bone formation (surface extent of osteoid). The problem seemed to be that a significant proportion of the patients with osteoid surface values towards the upper part of the reference range had low levels of osteocalcin. A number of possible explanations for this observation need to be considered.

Plasma osteocalcin has a short half life[21] and may be depressed by the stress associated with the fracture. A similar mechanism has been proposed for the reversible depression of vitamin D binding protein,[16] which was also seen in the present study. However the lack of a correlation between these two variables in the week after the fracture argues against a common mechanism. However, stress might depress osteocalcin through adrenal stimulation since such an effect has been reported with the use of glucocorticoids.[22] Unfortunately plasma cortisol was not measured in the present study. Our patients were much older than those in previous studies, but since osteocalcin tends to rise with age[23] this canot be the explanation for our findings.

The disproportionately low levels of osteocalcin tended to be associated with very low (<25 pmol/L) levels of 1,25-$(OH)_2$D. That 1,25-$(OH)_2$D may be an important factor is supported by the further observation that although there was no correlation between the absolute concentrations of osteocalcin and 1,25-$(OH)_2$D, there was a relationship between the changes in these variables in the first week after the fracture. This is consistent with the observation that while osteoblast production of osteocalcin is stimulated by 1,25-$(OH)_2$D, both in cell culture[7, 8] and in experimental animals,[24] it does not appear to be vitamin D dependent.

The role of osteocalcin in bone mineralisation remains uncertain and plasma concentrations probably reflect the portion of newly synthesised protein not bound to the mineral phase of bone.[24-26] It may be that as the fracture heals calcification of callus results in a slight fall in serum calcium, stimulation of parathyroid hormone secretion and augmentation of 1,25-$(OH)_2$D production with further enhancement of osteocalcin production. The necessary increases in PTH and 1,25-$(OH)_2$D were not seen in the present study but this may be because of the insensitivity of our assays to small changes or because those changes had diminished when sampled a week after the fracture.

It remains to be seen whether osteocalcin is useful in screening very elderly patients with femoral neck fractures for osteomalacia. Experience with the use of osteocalcin for this purpose is surprisingly limited. The generation of diagnostically useful raised values may depend critically on the opposing effects of 1,25-$(OH)_2$D deficiency and the ability of the very elderly to increase both parathyroid hormone secretion and bone formation. The timing of samples is also critical. The present study shows that osteocalcin concentrations remain within the reference range within the first week after a fracture. Other studies where the timing of samples was not stated, found elevated values[26] further complicating, and diminishing, the diagnostic usefulness of this measurement.

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PAPERS AWAITING PUBLICATION

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Changes in osteocalcin after femoral neck fracture.

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Short term response to treatment of osteomalacia.
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Thompson S.P., Hosking D.J., Knight D. and White D.A.
Metabolism of $25(OH)D_3$ by the human osteosarcoma cell line.
Awaiting publication.