J. S. HERON LTD
5 QUEENS CRESCENT
ST. GEORGES CROSS
GLASGOW 041 332 1883

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

IN VITRO STUDIES WITH BONE

IN

METABOLIC BONE DISEASE

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Medicine.

Patricia McLoughlin, BSc. Honours,
University Department of Medicine,
University of Glasgow and the
Royal Infirmary, Glasgow G4 OSF.

AUGUST, 1982.

C O N T E N T S

TABLES AND ILLUSTRATIONS

TABLE 2G. ^{45}Ca - release and ^{47}Ca - uptake in the presence of serum from Paget's disease of bone.

TABLE 2H. ^{45}Ca - release and ^{47}Ca - uptake in the presence of primary hyperparathyroid serum.

TABLE 2I. ^{45}Ca - release and ^{47}Ca - uptake in the presence of malignant hypercalcaemic serum.

TABLE 2J. ^{45}Ca - release and ^{47}Ca - uptake in the presence of normal calcium serum from patients with carcinoma.

TABLE 2K. ^{45}Ca - release and ^{47}Ca - uptake in the presence of hypercalcaemic serum.

TABLE 2L. ^{45}Ca - release and ^{47}Ca - uptake in the presence of serum from patients with hypervitaminosis D.

TABLE 2M. ^{45}Ca - release and ^{47}Ca - uptake in the presence of osteoporotic serum.

TABLE 2N. ^{45}Ca - release and ^{47}Ca - uptake in the presence of osteogenesis imperfecta serum.

TABLE 2O. ^{45}Ca - release and ^{47}Ca - uptake in the presence of hypophosphataemic serum.

TABLE 2P. ^{45}Ca - release and ^{47}Ca - uptake in the presence of ricketic/osteomalacic serum.

TABLE 2Q. ^{45}Ca - release and ^{47}Ca - uptake in the presence of 1,25 $(OH)_2D_3$.

TABLE 2R. ^{45}Ca - release and ^{47}Ca - uptake in the presence of 25 $(OH)D_3$.

TABLE 2S. ^{45}Ca - release and ^{47}Ca - uptake in the presence of parathyroid hormone.

TABLE 2T. ^{45}Ca - release and ^{47}Ca - uptake in the presence of calcitonin.

TABLE 2U. ^{45}Ca - release and ^{47}Ca - uptake in the presence of oestrogen.

FIGURE 7B. The effect of killing mouse calvaria on the release of alkaline phosphatase into the culture medium.

TABLE 7E. Looking at the effect of pH on the release
FIGURE 7C. of alkaline phosphatase by calvaria in the presence of aluminium.

TABLE 7F. Ca^{2+} levels in culture medium supporting
FIGURE 7D. neonatal mouse calvaria, in the presence of aluminium chloride.

TABLE 7G. The relationship between alkaline phosphatase
FIGURE 7E. and Ca^{2+} levels in culture medium supporting neonatal mouse calvaria, in the presence of aluminium chloride.

TABLE 7H. ^{45}Ca-release and ^{47}Ca-uptake in the presence of aluminium chloride.

FIGURE 7F. ^{45}Ca-release in the presence of aluminium.

FIGURE 7G. ^{47}Ca-uptake in the presence of aluminium.

TABLE 7I. Alkaline phosphatase levels within mouse
FIGURE 7H. calvaria in the presence of aluminium.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr I T Boyle, for allowing me to work in the field of metabolic bone disease and for his advice and encouragement in the course of this project.

I would also like to thank Professor A C Kennedy for the use of the laboratory facilities in the University Department of Medicine and Tenovus Scotland for providing the money which made the research possible.

To J McKenna, B Boyce, J Byers, F Spiers and W Waterhouse, whose technical advice, supervision and assistance have proved invaluable, I would like to express my grateful thanks.

I would like to thank Dr J J Reynolds and the staff of the Strangeways Research Laboratories in Cambridge for making my stay so enjoyable and fruitful.

Special thanks must be given to my parents, whose encouragement and enthusiasm over the past 24 years have made this work possible, and to my husband, Mick, who has made it worthwhile.

SUMMARY

Improvements in living standards and health care have caused a great increase in the number of elderly individuals in our community. The problems associated with age are many, but one in particular is of interest in this thesis: bone loss and the problems of metabolic bone disease, leading to various studies concerned with their effects on bone and bone metabolism.

Organ culture preparations of neonatal mouse calvaria were used to look at several aspects of bone metabolism. By preparing standard cultures of neonatal mouse calvaria, but with the bone prelabelled with ^{45}Ca and the culture medium labelled with ^{47}Ca, it was possible to look at the effects of human serum from patients suffering from metabolic bone disease on bone turnover in vitro. The results of this study proved interesting, suggesting that such sera act on bone to produce particular patterns of formation and resorption, reminiscent, in general, of the symptoms of the bone disease in vivo. Although there are other and better ways of diagnosing metabolic bone disease, such as serum biochemistry and bone histology, this method could be useful in assessing the development of any disorder. Human serum is abundant and easy to obtain, unlike human bone, and the cultures are easy to prepare; the effects of any drug therapy on bone turnover

can be seen quickly, by monitoring ^{45}Ca and ^{47}Ca in the culture medium, and by constant monitoring of serum biochemistry, it may be possible to understand how particular drug therapies affect bone.

The enzyme alkaline phosphatase stimulated a lot of interest. First of all the relationship between serum alkaline phosphatase and the level of the enzyme within bone itself was studied, showing a strong correlation between the two parameters and supporting the study of serum alkaline phosphatase levels as a tool in the diagnosis of metabolic bone disease. Dialysis osteomalacia is thought to be caused by the inhibition of alkaline phosphatase, through the build-up of aluminium. By studying the effect of aluminium in vitro, it was found that it does inhibit alkaline phosphatase, the inhibition of which is accompanied by high levels of free calcium in the culture medium, showing a strong inverse relationship between alkaline phosphatase and calcium and supporting the role of the enzyme in mineralisation. This relationship was also supported when alkaline phosphatase and free calcium levels were compared in cultures containing foetal calf serum and with human serum.

Other studies concerned with metabolic bone disease involved the assay of two different and unrelated enzymes: collagen prolyl hydroxylase and creatine kinase.

Collagen is the main structural component of bone and collagen prolyl hydroxylase is the enzyme responsible for the hydroxylation of proline, without which the classic triple helix structure cannot be formed. By assaying homogenates of bone obtained by biopsy of the iliac crest, it was hoped to discern any differences in collagen metabolism between the different metabolic bone diseases. Unfortunately, for a variety of reasons, it was not possible to pursue this line of study as much as was desired.

The assay of creatine kinase was more successful. Creatine kinase is the enzyme involved in the breakdown of the high energy molecule creatine phosphate to form ATP and is particularly abundant in skeletal muscle. Normal serum has little or no creatine kinase content, but the level has been found to change with different disorders, particularly those related to muscle weakness. As many metabolic bone diseases are severely debilitating, it was decided, looking at homogenates of striated muscle, to see if there is any pattern of creatine kinase activity in different metabolic bone diseases. Results showed no difference in creatine kinase levels in striated muscle in different metabolic bone diseases, but showed higher levels in the muscle of males than in females and increasingly high levels of the enzyme, for both sexes, with increasing age. Previous work, looking at serum

creatine kinase levels, related the sex-difference to the inhibition of the enzyme by oestrogen in pre-menopausal women.

The culture of human bone fragments is possible, but not desirable as long as uniform samples, containing equivalent amounts of cells and bone, of a suitable size are difficult to obtain. Human bone cell cultures are more desirable as they would be easier to reproduce and control. Although unsuccessful here, new technology will aid the culture of human bone cells and these cultures will play an important role in the study of metabolic bone diseases.

ABBREVIATIONS:

Ca	= calcium
Ca^{2+}	= free ionised calcium
PTH	= parathyroid hormone
AP	= alkaline phosphatase
CT	= calcitonin
C.P.M.	= counts per minute
C.M.R.	= cell-mediated resorption
EXPTAL.	= experimental
1^{o} hyperpara	= primary hyperparathyroidism
$25(OH)D_3$	= 25-hydroxycholecalciferol
$1,25(OH)_2D_3$	= 1,25-dihydroxycholecalciferol
$24,25(OH)_2D_3$	= 24,25-dihydroxycholecalciferol
$1(OH)D_3$	= 1-hydroxycholecalciferol

GENERAL INTRODUCTION

The recent advances made in the field of modern medicine have produced many important improvements in the lives and life expectancy of countless thousands of people; however these advances themselves may lead to new or previously unconsidered problems, for example, those related to metabolic bone diseases. As individuals age they lose bone, becoming evident in men over 50 and women over 40, and once bone loss has begun it proceeds linearly with age. Until recently, in this country, the life expectancy of the average individual was only 40 or 50 years and bone loss with increasing age posed few problems, but with today's aging population: a 14% increase in the number of women over 80 (1971-1977, Scotland) (Boyle, 1981), this is no longer the case and we have many elderly people suffering from the consequences of prolonged bone loss. The increasing significance of metabolic bone diseases makes them a very relevant topic of study, because of both social and medical aspects. This thesis is concerned with studies of bone metabolism in metabolic bone diseases, using various biological, biochemical, chemical and histological means in an attempt to understand the basis of these clinical problems.

Bone is a complex organ, well known for its supportive role in providing the skeleton, but also very important in maintaining mineral homeostasis within the body through its constant formation and resorption (Vaughan, 1965; Jackson, 1967; Rasmussen, et al. 1974). Frost (1981) has hypothesised that bone is made up of bone modelling units (B.M.U.), whereby specific areas of bone are undergoing constant turnover in an attempt to maintain the strength of the skeleton, as well as answering the need for minerals throughout the body. Changes through the process of bone remodelling, where formation follows resorption and takes place at sites of previous resorption, are fundamental to most metabolic bone diseases and can often result in serious dysfunction of the bone.

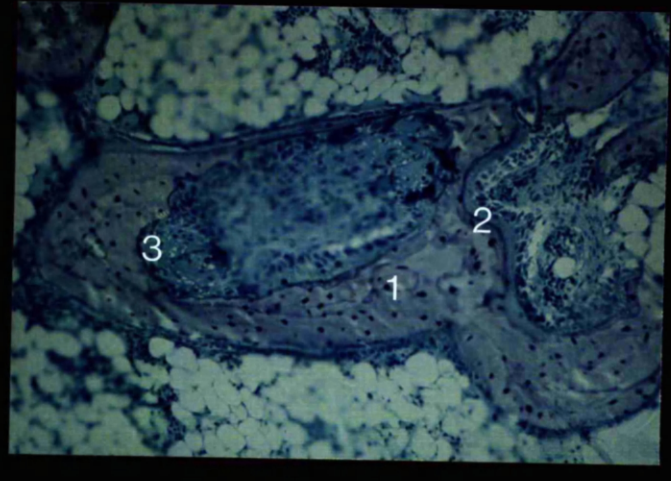
The basis of all bone function relates to the complex arrangements of cells and matrix within the bone, with each type of bone cell: osteoclasts, osteoblasts and osteocytes, performing specific tasks in the maintenance of bone, bone matrix and ground substance. The spacial arrangements of these cells, as well as their differing shapes and sites, can be seen in FIGURE A.

Osteoclasts are the largest cells found in bone and can be easily recognised as they may contain up to 20 nuclei; vacuolated cytoplasm contains those enzymes, chiefly acid phosphatase, so important in their role as bone-resorbing cells (Smith, 1979). Circulating monocytes have been found

FIGURE A

Histological section showing osteocytes in bone lacunae, or chambers, osteoblasts at the bone-forming surface and multinucleate osteoclasts in Howship's lacunae.

a



1:osteocyte

2:osteoblasts

3:osteoclast

to be attracted to sites of bone resorption, as well as being capable of resorbing bone, leading Mundy, et al. (1977, 1978) to suggest that these cells may be the origin of osteoclasts found in bone.

Biochemical changes produced by osteoclasts during bone resorption have been studied by Wong, et al. (1977), who noted increased levels of hyaluronate, a matrix component, and increased acid phosphatase activity; acid phosphatase is involved in the breakdown of bone matrix (Marks, 1974). Osteoclasts, as well as monocytes, phagocytose any bone fragments produced during bone resorption (Kahn, et al. 1978). Chambers (1980) describes the origin and role of osteoclasts, with evidence supporting their origin from circulating monocytes and their production of lysosomal enzymes during bone resorption.

Bone resorption occurs in the general maintenance of bone structrue, but also occurs in response to biochemical stimuli from throughout the body, such as parathyroid hormone, the vitamin D metabolite $1,25(OH)_2D_3$ (Wong, et al. 1977) and prostaglandins (Bookman, 1980). It would seem that parathyroid hormone acts directly on the osteoclasts to promote bone resorption, increasing their numbers (Rowe, et al. 1977), while prostaglandins act by stimulating β-lymphocytes, which in turn produce an osteoclast-activating factor (O.A.F.) (Bookman, 1980). Vitamin D metabolites, it would seem, act generally to stimulate bone turnover (Mittleman, et al.1968;

Raisz et al. 1972; Boyle, 1974; Stern, et al. 1976; Peacock, et al. 1976; Wong, et al. 1977). Interestingly, recent work by Howard, et al. (1981) would seem to suggest that osteoclasts may be involved in the extra-renal synthesis of vitamin D metabolites in patients suffering from chronic renal failure, where the patient cannot synthesise these metabolites in the normal way and the number of osteoclasts is found to be increased.

Osteoblasts are the bone-forming cells: small cells with single nuclei, rich in the enzyme alkaline phosphatase, which is believed to increase local phosphate concentrations for the formation of calcium phosphate and the deposition of bone mineral (Jackson, 1967; Scott, et al. 1980); these cells are also important in bone matrix formation. There are several different views as to the origin of osteoblasts. Owen (1977) holds the opinion that, while osteoclasts are derived from circulating monocytes, osteoblasts are derived, separately, from reticular cells of the reticulo-endothelial system; Rasmussen, et al. (1973,1974) believe that they are derived from osteoclasts. Osteoblasts form a single layer on the surface of bone, lying parallel to the underlying collagen fibres, which form the basis of the bone structure.

Osteocytes are osteoblasts which have become encapsulated within bone during its formation and can be found within small chambers, or lacunae (Jackson, 1967).

From within these lacunae osteocytes send out cytoplasmic processes of up to 100 μm in length, connecting with similar processes from other osteocytes and with the circulatory system (Rasmussen, et al. 1974), allowing them to respond to chemical changes throughout the body and forming an organised network between the cells of bone (Parfitt, 1976). When osteocytes die and contact between bone cells and their environment is lost, bone can no longer function effectively (Aaron, 1976).

Various groups have studied the relationship between age and bone cell function. The famous American endocrinologist Albright (Albright, et al. 1948) provided what is now regarded as the classic view on bone loss in elderly women, concentrating on osteoporosis, the most prevalent disorder in aging bone. He believed, like Rasmussen, et al. (1973, 1974), that osteoblasts are derived from osteoclasts and that the increasing loss of bone with increasing age is due to a decrease in the number of bone-forming cells. Through a lack of progression from osteoclasts to osteoblasts, or because the number of cells which could derive from osteoclasts became decreased, osteoclasts in osteoporotic bone averaging only 2.5 nuclei per cell as opposed to 4 nuclei per cell in normal age-matched bone, the ability to form bone decreases with age; at the same time, though, the number of osteoclasts and the degree of bone resorption remains unchanged, leading to net bone loss.

Two other, independent, studies of bone histology by Mert, et al. (1969) and Bordier, et al. (1972) note an increased accumulation of bone resorption surface, while the rate of active bone resorption remains the same; the number of osteoblasts, also in accordance with Albright, et al. (1948), was found to decrease. It would seem, therefore, that bone loss with age occurs through decreased bone formation, with no compensatory change in bone resorption, leading to progressive loss of bone.

Collagen is the major extracellular protein found in the body and more than 50% of it is found in bone, forming the basis of the extracellular bone matrix (Grant, et al. 1972). Osteoblasts, the bone-forming cells, produce procollagen precursors, α-chains, which are extruded into the extracellular matrix and formed into collagen bundles, triple helices (Rasmussen, et al. 1974; Smith, 1979). In a study of the role of collagen in mineralisation, Strates, et al. (1957) successfully induced calcification in collagen from tissues which do not normally calcify, while Bachra, et al. (1959) found that the lamellar organisation of collagen is important as a 'local factor' in initiating mineralisation. Rasmussen, et al. (1974) supported the idea of some cellular involvement in the mineralisation of bone, with Münzenberg, et al. (1975) believing that the calcium phosphate crystals and collagen

matrix in bone form a specific spacial interaction by which calcification can take place and Vaughan (1975) claiming that the mucopolysaccharides in the matrix also have some role to play. It would seem then that collagen could be important, perhaps along with other factors, in bone mineralisation. In this thesis collagen is studied with respect to the enzyme collagen prolyl hydroxylase, involved in the hydroxylation of α- for the formation of the triple helices; this enzyme is discussed in greater detail in a later chapter.

Abnormalities in bone metabolism can give rise to several clinical pictures: osteoporosis, rickets or osteomalacia, hyperparathyroidism and, perhaps, Paget's disease of bone, among many others. Patients suffering from these disorders have formed the basis of this study.

The most widespread disorder of bone affecting the elderly, particularly women, is osteoporosis. This disorder is characterised by a continuous loss of bone with age, although the biochemistry and pathology of the bone present is absolutely normal (Albright, et al. 1948). Albright and Reifenstein, as mentioned above, found that this continuous bone loss resulted mainly from an inability to form sufficient bone in reply to normal bone resorption. The clinical consequences of osteoporosis are characterised by femur and fore-arm fractures, as well as crush fractures of the vertebrae (Smith, 1979).

Coupron (1972) has suggested that those persons suffering most from the effects of osteoporosis had, in general, less bone than normal to begin with. However, Chalmers, et al. (1970), in their comparative study of the incidence of vertebral crush fractures in Oriental and European women, found that the former, with their smaller skeletons, showed no greater tendency towards fractures. Doyle (1972) has suggested that Oriental women perform more physical labour, which has been shown to increase bone mass and calcification (Harell, et al. 1977), thus counter-acting normal bone loss with age (Geiser, et al. 1958). A study by Jensen, et al. (1980) has shown that 44% of all Danish women suffer from fractures.

Possible clinical cures for osteoporosis have been studied. Reeve, et al. (1976) found that parathyroid hormone (PTH), administered to post-menopausal women, stimulated bone turnover, with more formation than resorption. Calcitonin, a strong inhibitor of bone resorption, was studied by Sha monki, et al. (1980); they found that post-menopausal women show progressively lower levels of serum calcitonin with increasing age. Calcitonin acts by inhibiting the proliferation of osteoclasts and fibroblasts and inhibits the loss of bone matrix (Raisz, et al. 1968) and so we can see how its progressive loss would enhance progressive bone loss. In addition to these treatments, Marx (1980) supports some role for the vitamin D metabolite

1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) in the management of osteoporosis. All of these factors are discussed in more detail later in this introduction.

Oestrogen is thought to hold the key to the prevention or cure of osteoporosis, as the condition is predominant among post-menopausal females whose oestrogen levels have become drastically reduced. Atkins, et al. (1972) showed that oestrogen inhibits the bone-resorptive activities of parathyroid hormone, suggesting that the loss of oestrogen with the menopause causes a decrease in the control of bone resorption. Wink, et al. (1980) showed the induction of osteoporosis in castrated rats, again suggesting the involvement of the sex-hormones. Robin, et al. (1980) found that a conjugated natural oestrogen preparation, Premarin prevented heparin-induced accelerated bone loss, but Coope, et al. (1980) found that a synthetic steroid produced no change in the osteoporotic condition. It would seem then that oestrogen could play an important role in the prevention or cure of osteoporosis, but this treatment can have unpleasant side-effects, such as cancer of the uterine endometrium or the inconvenience of an elderly woman continuing her menstrual cycle. Orloff, et al. (1980) have described the preventive or curative agent required in the treatment of osteoporosis: any agent which influences the balance between bone deposition and resorption, which can control the time-dependent evolution of trabecular bone loss.

Osteomalacia, along with its juvenile form of rickets, is another common metabolic disorder of bone (Smith, 1979). This disorder is characterised mainly by a lack of calcification in the organic osteoid bone matrix, leading to distortion of bone, pseudofractures and severe bone pain. There are several well-known causes of this disorder which, unlike osteoporosis, is not an age-related complaint.

The problems of osteomalacia and rickets have largely been eradicated in the native population of this country, but it has been found with increasing regularity among Scotland's Asian immigrant population. In 1962 the Scottish Medical Journal published a report by Dunnigan, et al. (1962) concerning the incidence of osteomalacia and rickets in Glasgow's Asians, followed by studies from Ford, et al. (1972) and Denman (1979). Felton, et al. (1966) noted its occurrence during pregnancy, leading to its appearance in newly-born Asian children (Ford, et al. 1973).

The high occurrence of osteomalacia and rickets among the Asian population was thought to be associated with high gluten and phytate content of the traditional Asian bread. Studies by Moss, et al. (1965), Van de Berg, et al. (1972) and Holmes, et al. (1973) showed that gluten, phytate and hydrolysates of phytate all inhibit resorption of calcium in the gut by means of the formation of insoluble calcium salts, but interestingly, orally-

administered phytate can be readily digested in the gut. Wills, et al. (1972) however claim some success in curing rickets by reducing dietary phytate. The role played by phytates and gluten in the incidence of osteomalacia is therefore inconclusive, leading to the study of other factors.

Dietary or environmental lack of vitamin D, through inadequate supplies in food or insufficient exposure to sunlight, have also been implicated in the occurrence of osteomalacia. Various studies have shown that the vitamin D metabolites: $25(OH)D_3$, the main circulating form, and $1,25(OH)_2D_3$, the active form, are important in the prevention and cure of osteomalacia (De Luca, 1977; Kanis, et al. 1977; Frame, et al. 1978; Ahmed, et al. 1979; Offerman, et al. 1979). The mechanisms of these metabolites will be discussed in more detail later in the introduction.

It could be that osteomalacia and rickets occur purely through a lack of vitamin D. Those Asians in Scotland suffering from the effects of osteomalacia and rickets did not suffer to the same extent in their native countries, where they had the same diet and a lot more exposure to sunlight. Patients on long-term anti-convulsant therapy show a high incidence of osteomalacia (Hoikka, et al. 1980), but they are usually institutionalised and lack exposure to sunlight, as well as a healthy diet; children of mothers on anti-convulsant therapy and suffering from osteomalacia

show no increased tendency towards the disorder themselves (Christiansen, et al. 1980). The importance of diet, sunlight and vitamin D in the prevention and cure of osteomalacia and rickets are also emphasised by Parfitt, et al. (1979).

An abnormal serum calcium level is the main disorder resulting from hyperparathyroidism and is the result of increased calcium absorption from the gut (Avioli, et al. 1965), increased bone turnover, particularly resorption (Harris, et al. 1969), and increased tubular resorption of calcium from the kidneys. Clinical bone disease is not common in this disorder, but the bone is nearly always histologically abnormal (Byers, et al. 1971), with excessive osteoclastic resorption, marrow fibrosis and increased bone formation, accompanied by a woven collagen matrix (Smith, 1979). Apart from bone disease, clinical problems manifest themselves in increased cardiac problems (Hargreaves, et al. 1980), increased tumour development (Wajngot, et al. 1980), muscle weakness, malaise and severe dementia (Heath, et al. 1980b).

Like most other metabolic bone diseases, hyperparathyroidism tends to increase in incidence with age (Heath, 1980a, Mannix, et al. 1980), although Jason, et al. (1981) report a case of this disease in an 18 month-old child. Similar to hypophosphatasia, mentioned later, hyperparathyroidism may occasionally have a familial incidence, often

associated with other endocrine disorders, manifesting themselves in adulthood (Orwall, et al. 1981), although Sandler, et al. (1980) discuss the occurrence of the disease in a mother and her 12-year-old son.

Hyperparathyroidism takes two main forms. The usual stimulus to parathyroid overactivity is low serum calcium, found particularly with chronic renal failure or intestinal malabsorption, with prolonged overactivity of this type classed as secondary (2^{o}) hyperparathyroidism (McCarron, et al. 1981). Primary (1^{o}) hyperparathyroidism occurs through overproduction of the hormone, parathyroid hormone, through the presence of an adenoma in one or more of the parathyroid glands (O'Riordan, et al. 1972). Cure or prevention of hyperparathyroidism has, understandably, been under study for many years now, with surgery providing a common means of removing the adenoma which cause 1^{o} hyperparathyroidism (Heath, et al. 1980b; Sandler, et al. 1980; McCarron, et al. 1981). Various drugs have been used to combat the disorder, with a lot of attention recently focusing on Cimetidine. This drug is capable of blocking the synthesis or release of the excess parathyroid hormone causing the disease (Sherwood, et al. 1980a), without affecting serum levels of calcium, phosphorus or magnesium (Jacob, et al. 1980).

The enzyme alkaline phosphatase, found in great abundance in osteoblasts, is thought to be very important in bone mineralisation, or calcification, and dramatically high or low levels of the enzyme have been found to result in equally dramatic changes in bone structure and composition. Hypophosphatasia is an inherited recessive genetic disorder affecting alkaline phosphatase and bone (Smith, 1979); a condition of hyperphosphatasia was thought to exist, but is now regarded as a form of Paget's disease of bone, discussed later (Eyring, et al. 1968). Hypophosphatasia is characterised by very low levels of bone-related alkaline phosphatase in the serum, with any enzyme present originating from its other major sources: the liver and the gut (Warshaw, et al. 1971; Inglis, et al. 1972). This disorder manifest itself in defectively-mineralised bone, similar to that found in osteomalacia, with the age of onset determining the severity of the effects, decreasing with age (Fraser, 1957). O'Duffy (1970) also found the disorder to be related to cartilage calcification. The lack of mineralisation found with the absence or very low levels of alkaline phosphatase in this disorder emphasises the importance of the enzyme in calcification, discussed in a later chapter.

Hypophosphatasia is a very rare disorder, but deserves mention here as it became possible to include two hypophosphataemic patients in this study.

Although those bone diseases already described may be regarded as the results of changes in metabolic processes, metabolic bone disease may appear as a result of non-biological factors. Renal bone disease, or renal osteodystrophy, appears as one or all of several bone diseases: osteitis fibrosa, osteomalacia and osteosclerosis (Ellis, et al. 1973), with one condition tending to predominate. Bone disorders accompanying chronic renal failure tend to show themselves in increased bone formation, increased osteoid and increased mineralised bone, compared to age-matched controls, although bone problems accompanying chronic renal failure are not inevitable (Malheche, et al. 1976). One of the main factors thought to enhance renal osteodystrophy is aluminium (Ward, et al. 1978).

Ellis, et al. (1979) found that patients suffering from chronic renal failure, and receiving regular dialysis treatment, had abnormally high levels of aluminium in their bones, which could be associated with osteomalacia and dialysis encephalopathy, a mental disorder. Changes in bone histology associated with osteomalacia, dialysis and high levels of aluminium have been found to be common (Stanbury, 1972; Malluche, et al. 1976; Alfrey, et al. 1976; Elliot, et al. 1978; Wing, et al. 1980; Boyce, et al. 1981). Experimental studies with laboratory animals, along with some of my own studies, have shown that aluminium

is readily taken up by bone (Berlyne, et al. 1970) and that prolonged administration leads to decreased calcification and increased osteomalacia (Ellis, et al. 1979).

The origin of the aluminium involved is probably the dialysate water used in renal dialysis (Platts, et al. 1977; Drueke, 1980; Boyce, et al. 1981), with increased incidence of renal osteomalacia in areas where the level of aluminium in the local water supply is high. The effects of aluminium on bone mineralisation is of particular interest in this study and is discussed in greater detail in a later chapter.

Albright, et al. (1948) described Paget's disease of bone as a local bone disease, not a metabolic bone disease, characterised by very high levels of serum alkaline phosphatase and urinary hydroxyproline, indicative of high rates of bone turnover, noted by Nagant de Deuxchaines, et al. (1964). This bone disease is fairly common, with 3% of all people over 40 suffering from it (Smith, 1979) and, although it is not a metabolic bone disease as such, it has been used in several comparative studies within this thesis and so requires description. Paget's disease of bone is characterised by gross macroscopic and microscopic changes in bone (Singer, 1977), with enlargement and deformation of the skull and long bones following the deposition of woven, disorganised bone, as opposed to normal, organised lamellar bone (Smith, 1979; Krane, 1980), through increased bone turnover.

The increased levels of serum alkaline phosphatase characteristic of this disorder are known to originate from the bone (Woodward, et al. 1969), suggesting increased osteoblastic activity. Cheung, et al. (1980) noted that, in addition to increased alkaline phosphatase production, pagetic osteoblasts also synthesised more collagen and protein than other osteoblasts, while pagetic osteoclasts are found to be bigger and more numerous, indicative of increased bone resorption (Rowe, et al. 1977) and, as discussed before, providing the source of more osteoblasts. All of this increased bone turnover leads to the formation of dense, highly disorganised and deformed bone (Khairi, et al. 1973; Franck, et al. 1974).

The incidence of Paget's disease of bone has been found to follow interesting geographical patterns. Smith (1979) noted that it never occurs in Scandinavia, Japan or the Tropics, but is common in Australia and Western Europe, leading to a general assumption that it is a disorder of Anglo-Saxon populations. A particularly interesting study by Barker, et al. (1980) has shown that certain communities can exhibit unusually high incidences of Paget's disease of bone, particularly in Lancashire. Rebel, et al. (1980) and Singer (1980) have noticed a measles-like viral inclusion in the genetic material contained within the nuclei of osteoclasts from Paget's disease of bone and such antigens

could, it seems, explain the course of the disease: the lack of an immune response, indicative of a slow virus infection, and the incidence of the disease within families and communities.

Various drugs have been used in the treatment of Paget's disease of bone. Mithramycin, a cytotoxic antibiotic which has been shown to inhibit the bone-resorbing activities of parathyroid hormone (Engracio, et al. 1972; Minkin, 1973), has produced effective results. On administration to patients, Mithramycin relieved all types of pain, more effectively than any synthetic drug, and decreased all types of bone cellular activity (Condon, et al. 1971; Hadjipavlou, et al. 1977). Diphosphonates, thought to be naturally-occurring inhibitors of bone turn-over in their natural form of pyrophosphate, discussed in a later chapter, were also studied in association with Paget's disease of bone. Bijvoet, et al. (1980) used dichloro-methylidene diphosphonate and (3-amino-1-hydroxypropylidene)-1, 1-diphosphonate and found that they produced a total biochemical and histological cure of the disease within 6 months. Calcitonin has proved less successful, initially decreasing bone turnover, but becoming ineffective after only 4 months (Gattereau, et al. 1980).

Metabolic bone diseases can, therefore, take several forms, each associated with the dysfunction of one or more

of the systems involved in the formation and maintenance of normal calcified bone. There are a few hormones and metabolites of particular importance in the normal functioning of bone, some of which have already been mentioned, which bear further discussion.

Parathyroid hormone (PTH) has been widely researched over the years: normal physiological levels of the hormone producing no net gain or loss of bone (Malluche, et al. 1981), while severe variations result in those bone disorders characteristic of hyperparathyroidism, already discussed, and hypoparathyroidism, which has opposite effects. The main effects of PTH are known to result in increased bone resorption (Rosenbach, et al. 1967; Vaes, 1968; Shubina, et al. 1969; Chu, et al. 1971; Messer, et al. 1973a; Minkin, 1973; Peck, et al. 1976; Reeve, et al. 1976; Langeland, 1977; Wong et al, 1977; Luben, et al.1977; Maria, et al. 1977; Ibbotson, et al. 1978; Krieger, et al. 1980; Malluche, et al. 1981), with the mechanisms of this action under investigation.

Under normal conditions PTH production is stimulated by low serum calcium levels, with high serum calcium levels resulting in a decrease in the available PTH (Birkenhager, et al. 1973; Pitkin, et al. 1980; Weinstein, et al. 1980; Blum, et al. 1981). Krieger, et al. (1980) maintains the existence of a Na-Ca exchange mechanism for this regulation

of serum calcium levels under the influence of PTH, fuelled by glucose (Fenton, et al. 1978; Zamboni, et al. 1980). It has been shown that PTH effects the release of calcium from bone by stimulating the production of acids and enzymes, mainly lactate and citric acids (Nisbet, 1968; Heersch, et al. 1969; Stern, et al. 1970; Messer, et al. 1973b; Luben, et al. 1976), resulting in decreased calcification and the breakdown of bone matrix; increased bone resorption is also accompanied by increased levels of cAMP within osteoblasts (Peck, et al. 1973; McPartlin, et al. 1976; Wong, et al. 1977; Peck, et al. 1977; Marcus, et al. 1977). The role of osteoclasts in this PTH-stimulated bone resorption has been supported by the discovery that calcitonin (CT) inhibits PTH-stimulated bone resorption by inhibiting osteoclastic activity (Feldman, et al. 1980), while other studies have shown that PTH acts through inhibiting collagen formation (Dietrich, et al. 1976; Kream, et al. 1980), increasing serum calcium by inhibiting bone formation.

One interesting point, related to the severe bone loss experienced by many post-menopausal women, is the relationship between PTH and the steroid sex hormones: these hormones decline with the menopause and recent studies have shown that they are effective in inhibiting PTH-stimulated bone resorption (Stern, 1969; McPartlin, et al. 1976; Reeve, et al. 1976; Langeland, 1977).

Calcitonin has the opposite effect to PTH, inhibiting bone resorption (Raisz, et al. 1967; Reynolds, et al. 1968; Raisz, et al. 1968; Nisbet, et al. 1968; Mittleman, et al. 1968; Binderman, et al. 1972; Gozariu et al. 1973; Geusens, et al. 1980; Maier, et al. 1980; Stevenson, et al. 1981). Many studies have shown that although CT produces results contrary to PTH, it does not work directly against PTH itself, only against its effects: it does not inhibit PTH-inhibition of collagen formation (Dietrich, et al. 1976), it has been found to act on the cells of the periosteum, where PTH works on sub-periosteal cells (Peck, et al. 1977); it does not inhibit PTH-induced inhibition of citrate metabolism, although it does inhibit hyaluronate synthesis and demineralisation (Luben, et al. 1976). CT acts by inhibiting the proliferation of osteoclasts and fibroblasts and inhibits the loss of bone matrix (Raisz, et al. 1968), effects which are enhanced by the presence of fluoride (Messer, et al. 1973b).

The important association between steroid hormones, CT and post-menopausal bone loss has been followed in recent years. It has been shown that the purpose of CT would seem to be the prevention of excessive bone loss, rather than the formation of new bone, as it acts only to reverse any bone resorption which may have already occurred (Mittleman, et al. 1968). In post-menopausal women we find serum CT levels

drastically different than those in younger women, decreasing with age (Shamonki, etal. 1980; Padilla, et al. 1980). Oestrogens have been found to stimulate CT secretion (Woloszczuk, et al. 1980; Stevenson, et al. 1980; Morimoto, et al. 1980; Cressent, et al. 1980; Stevenson, et al. 1981), suggesting that lack of oestrogens, through the menopause, would lead to loss of control over bone resorption and accumulated bone loss over the years (Geusens, et al. 1980). This situation would suggest that a suitable treatment for post-menopausal osteoporosis would be the administration of CT; CT is already used to control bone turnover in Paget's disease of bone (Singer, et al. 1980; MacIntyre, et al. 1980), but it has been found that patients soon become resistant to the effects of the drug and it becomes ineffective.

Vitamin D_3 is involved in bone mineralisation and resorption, promoting both (Mittleman, et al. 1968; Raisz, et al. 1972; Boyle, 1974; Stern, et al. 1976; Peacock, et al. 1976; Wong, et al. 1977) through the action of its metabolites, mainly 25-hydroxycholecalciferol ($25(OH)D_3$) and 1,25-dihydroxycholecalciferol ($25(OH)_2D_3$). Cholecalciferol, or vitamin D_3, can be synthesised in the skin from 7-dehydrocholesterol or can be ingested from food in the small intestine (Smith, 1979). $25(OH)D_3$, the main circulating form, is synthesised from vitamin D_3 in the

liver microsomes and subsequently converted to $1,25(OH)_2D_3$, the active form, in the kidneys (Boyle, 1974; DeLuca, et al. 1978). Two specific cases resulting from defects in this procedure occur, in one case, with chronic renal failure, where $1,25(OH)_2D_3$ cannot be synthesised (Boyle, 1974; Weinstein, et al. 1980), and in elderly people where reduced stores of 7-dehydrocholesterol mean decreased ability to produce vitamin D_3 (Holick, et al. 1981); these ailments contrast with osteomalacia and rickets, where lack of exposure to vitamin D in the environment or the diet is the problem.

Metabolites of vitamin D_3 are widely used in the treatment of metabolic bone diseases, treating osteoporosis (DeLuca, 1977; Wandless, et al. 1980; Cohen, et al. 1980) and osteomalacia, as discussed earlier, promoting bone strength and decreasing pain. One novel point to note is the recent work by Howard, et al. (1981), which suggests that osteoclasts may be involved in the extra-renal synthesis of vitamin D metabolites, their numbers increasing during chronic renal failure.

Fluoride has been the subject of a lot of controversy in recent years, with evidence suggesting that it is effective in reducing the incidence of dental caries when added to drinking water (Jackson, 1967), through its action in promoting bone matrix/tooth formation (Smith, 1979) and

inhibiting bone resorption (Messer, et al. 1973). However excesses of fluoride would appear to have severe effects on bone, not differing greatly from those found with osteomalacia (Arnala, et al. 1980; Christie, 1980) and osteoporosis (Alhava, et al. 1980; Robin, et al. 1980; Laitenen, et al. 1980), meaning that the use of this element must be strictly controlled.

We can see that bone is a very complex organ, subject to the influences of many drugs, hormones and environmental factors and not solely responsible for providing mechanical skeletal support. The purpose of this study is to investigate further aspects of bone metabolism to try to aid our understanding of metabolic bone diseases, with particular interest being placed on alkaline phosphatase and calcification, the effects of aluminium on alkaline phosphatase and calcification, changes in muscle creatine kinase and collagen prolyl hydroxylase activities in different metabolic bone diseases and the effects of human serum on bone turnover in vitro. Each particular subject has been described in greater detail within each chapter and the results would seem to suggest that we still have a lot to learn about metabolic bone diseases.

CHAPTER ONE: PRELIMINARY STUDIES IN THE ORGAN CULTURE OF BONE

The in vitro culture of foetal and neonatal mouse calvaria has, for many years, been important in the study of the mechanisms of bone metabolism, particularly bone resorption. These studies, some of which will be described here, serve to illustrate the most valuable aspect of in vitro cultures: they allow the effects and interactions on bone of hormones, drugs and other metabolites to be seen directly, independent of other, complex, systemic factors.

General studies in bone resorption have been performed by Reynolds, et al. (1970) and Messer, et al. (1972). who looked at PTH, which increased bone resorption, CT and fluoride, which inhibited bone resorption, in in vitro cultures with mouse calvaria. Susi, et al. (1966); Nisbet (1968) and Nisbet, et al. (1970) found using such cultures that PTH-stimulated bone resorption is accompanied by intense acid phosphatase activity and the accumulation of lactate and citric acids. Minkin (1972) used this bone-resorbing activity of PTH to study the effects of the antibiotic Mithramycin on in vitro bone cultures, finding that it inhibited bone resorption, while Maria,et al. (1977) studied the effects of Verapamil, a drug involved in calcium homeostasis, on the same system. Many studies have been performed using in vitro bone culture, looking at various steroid hormones (Nisbet, et al. 1969; Messer, 1977), vitamin D metabolites (Herrmann-Erlee, et al. 1978), hyperoxia

(Goldhaber, 1958) and collagenase (Stern, et al. 1970), for example. Although these examples serve to illustrate the wide use of this system, it must always be remembered that it is only a model system and not an absolute replica of in vivo bone systems (Reynolds, 1975):

> "One of the most important experimental advantages of organ cultures of bone and cartilage is the ability to isolate tissue under study from systemic effects, and to be able to test the actions of substances under controlled conditions. However, this can also be one of the disadvantages of the method unless care is taken to integrate the organ culture results with in vivo findings."

One important example which illustrates the differing effects a substance may exhibit in vivo and in vitro is that concerning vitamin D. DeLuca (1978) demonstrated that vitamin D is metabolised in the liver to $25(OH)D_3$ and then in the kidney to $1,25(OH)_2D_3$, before it has any effect on bone, increasing calcification. When vitamin D is added to bone culture medium in vitro it can undergo no such transformations and is therefore unable to reflect the effects of vitamin D in vivo.

Of particular interest in this study is the relationship between the enzyme alkaline phosphatase (AP) and the level of free ionised calcium (Ca^{2+}) found within the culture medium supporting mouse calvaria in vitro. The possible

involvement of AP in bone mineralisation is discussed in greater detail later in this thesis, however the basic studies will be outlined here. Robison (1923) noted that AP was readily found in localised areas where calcification was occurring, or would soon occur, as did Bevelander, et al. (1950) and Morse, et al. (1951). The suggestion resulting from these discoveries was that AP was present to facilitate the precipitation of calcium phosphate, by causing a local supersaturation of phosphate esters, the precipitation of the calcium phosphate would in turn cause a decrease in the Ca^{2+} present. By looking at the relationship between AP and Ca^{2+} in the culture medium supporting the mouse calvaria, it is hoped here to find any possible correlation between these two parameters.

The means of mouse calvaria culture employed in this study, as in many others, was that developed by John Reynolds in the Strangeways Laboratory in Cambridge. This method of bone culture can be found in Reynolds and Dingle (1970), Reynolds (1972) and Reynolds (1975) and generally involves the very careful dissection of calvaria from 6-7 day old mice and their culture under optimum conditions. In this study mouse calvaria have been cultured and quantified for viability, both histologically and biochemically, as a preliminary to organ culture of human bone samples and the culture of mouse calvaria with human serum. It is hoped to discern any relationship between AP and Ca^{2+} using all of those culture systems mentioned.

FIGURE (i)

Organ culture dish with central well for culture medium. The stainless steel grid sits over the well, supported by its walls. Each dish is approximately 5 cm in diameter.

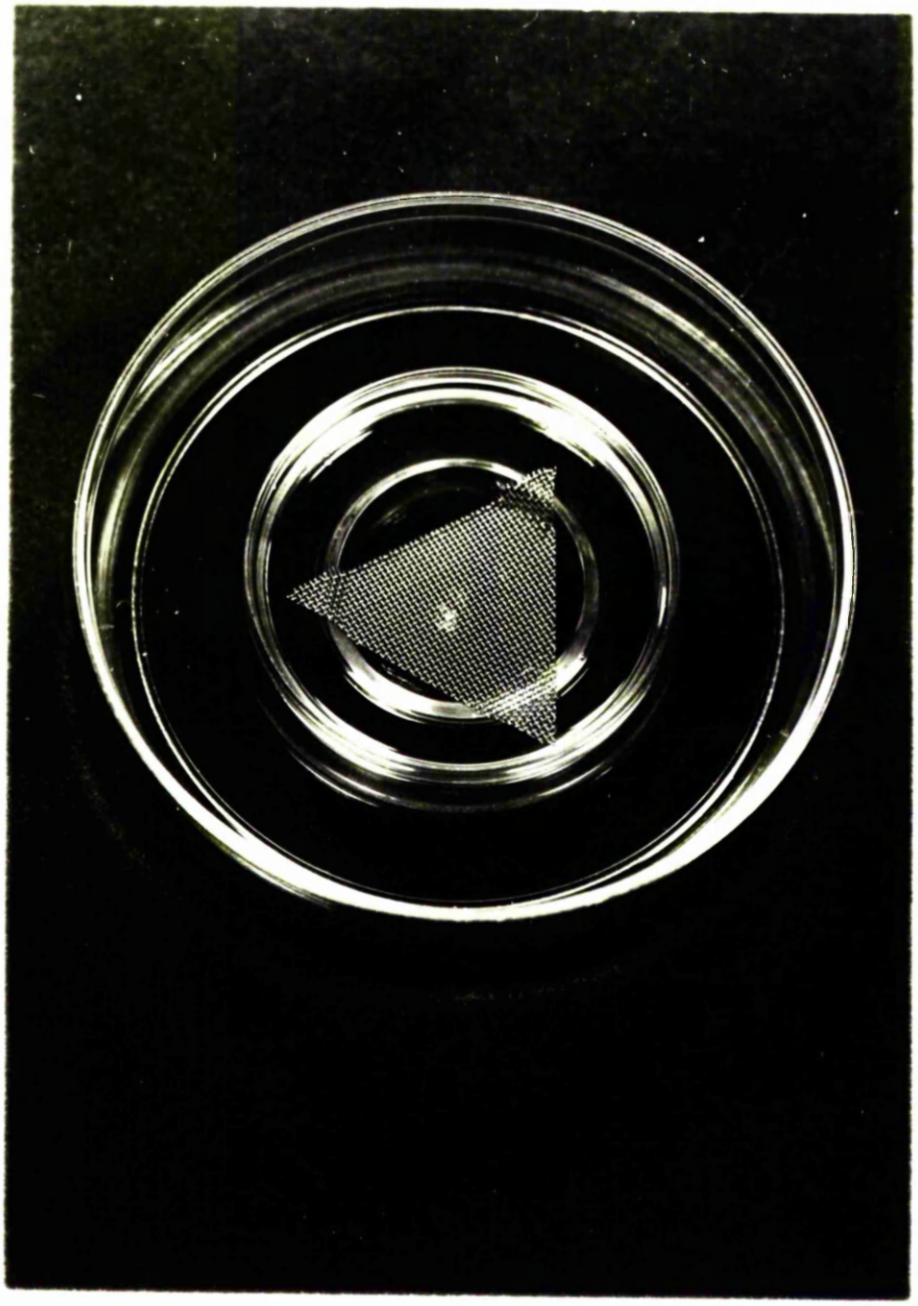
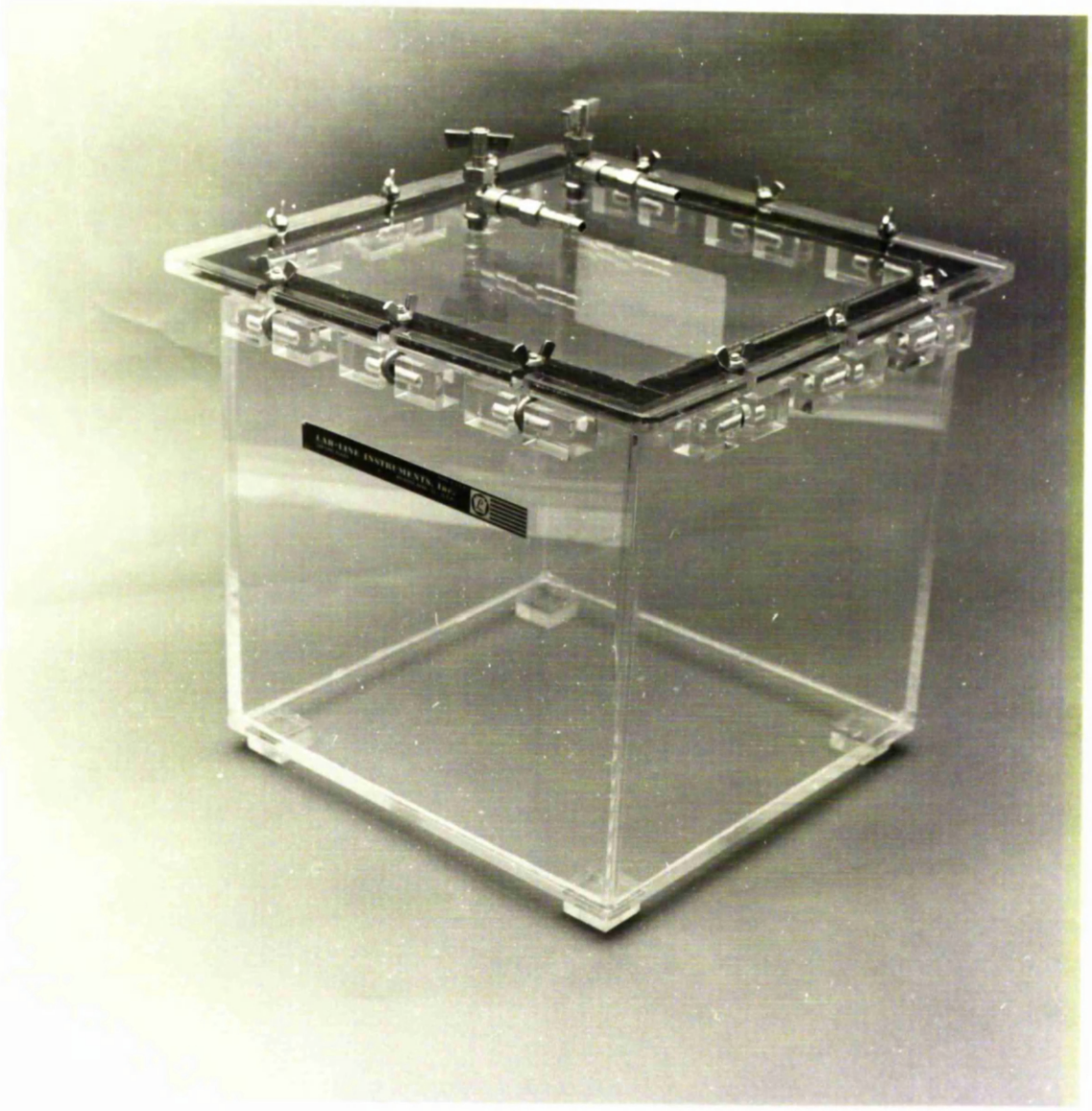


FIGURE (ii)

McIntosh-Fildes jar, which can hold up to 24 culture dishes at any time. This is a glass jar, commonly known as an aneorbic jar, which can be made air-tight by means of a tightly-fitting, rubber-lined lid, with the inlet and outlet taps allowing gassing with 5% CO_2 in air.

Lab-line tissue culture box, which can hold up to 64 cultures at a time. This is a perspex box, which can be made air-tight by means of a tightly-fitting, rubber-lined lid, again with inlet and outlet taps for controlling the inner atmosphere.



Materials and Methods:

1. All organ cultures of bone were maintained in organ culture dishes (Falcon Plastics, 3037. Becton, Dickinson and Co., Cockeysville, M.D. USA). These dishes (FIGURE (i)) are approximately 5 centimetres in diameter and have a central well, to which bone culture medium is added, and an outer moat, which may contain a sterile filter paper ring soaked in sterile distilled water, to stop or reduce evaporation of the culture medium. In those cases where no paper ring was used, a sterile tissue soaked in sterile distilled water and wrapped in foil would be placed in the tissue culture jar or box.
2. In initial studies, McIntosh-Fildes anaerobic jars (Anaerobic culture jar. A. Gallenkamp and Co. Ltd., London) were used for the incubation of cultures; each jar held up to 24 cultures and, here, filter paper rings were used to provide humidity. Latterly these jars were replaced by tissue culture boxes (Lab-line Instruments, Inc.; Melrose Park, Illinois, USA) which hold up to 64 cultures each; sodden tissue paper provides a humid environment here. Both of these vessels can be seen in FIGURE (ii).

   It is important that such air-tight containers are used to house the bone organ cultures, as the cultures must be maintained in an atmosphere of 5%

CO_2 in air, which is added through inlet tubes each time the boxes have been opened for changing of culture medium or when the cultures are set up.

3. Sterile disposable pipettes (Falcon Plastics; Becton, Dickinson and Co., Cockeysville, M.D. USA) were used in the transfer of all culture media and other sterile solutions throughout each experiment.

4. Various types of culture media were used: Medium 199 with Hanks salts, Medium 199 with Earle's salts, BGJb Original medium and BGJb Modified medium, with the Fitton-Jackson modification (Gibco Bio-cult Ltd., Paisley); P_4 medium, another modification, was supplied by J. Reynolds (Strangeways Laboratory, Cambridge). Each type of culture medium, except in particular cases which will be mentioned, was supplemented with 15% foetal calf serum (Gibco Bio-cult Ltd., Paisley), or by 5% rabbit serum, in the case of the P_4 medium. To reduce any incidence of contamination, penicillin-streptomycin solution (Gibco Bio-cult Ltd., Paisley) was added to the culture medium: 1 ml of solution containing 5000 units of penicillin and 5000 mcg. of streptomycin was added to each 100 ml of culture medium. Glutamine is an essential factor in bone culture medium and was added to those culture media which did not already contain it: BGJb Original and Modified; 1 ml of glutamine (Gibco Bio-cult Ltd., Paisley), containing 29.2 mg, was added to each 100 ml of culture medium.

5. Before and during dissection, bone was supported in Hanks BSS (Gibco Bio-Cult Ltd., Paisley) with added penicillin-streptomycin or Medium 199 (Gibco Bio-cult Ltd., Paisley) with added penicillin-streptomycin. The latter maintains a steady pH over a longer period of time and was used mainly in the transport of human bone biopsies, which may take as long as one hour.
6. Bone in culture was supported over the central well of the tissue culture dish, allowing uniform access between bone and culture medium, while holding the bone out of the culture medium and allowing culture medium to be changed without disturbing the bone. Various means of support were tested in an attempt to find the best way of holding the bone over the culture medium: stainless steel mesh grids (Falcon Plastics; Becton, Dickinson and Co., Cockeysville, M.D. USA), nylon mesh grids (supplied by Professor A Curtis, Department of Cell Biology, University of Glasgow), and filter paper rafts (Reynolds, 1972). Both the nylon mesh grids and the filter paper rafts failed to support the bone, each becoming waterlogged and collapsing into the culture medium. Stainless steel grids, however, provided strong support while still allowing diffusion and passage of nutrients and waste to occur between bone and culture medium as well as being easily sterilised. From this point all organ cultures of bone were prepared using stainless steel grids.

7. Organ culture dishes containing a stainless steel grid and the appropriate culture medium were always prepared before bone was made ready.

8. For organ cultures of mouse calvaria, mice were killed instantly by decapitation and the head placed in sterile Hanks BSS with added penicillin-streptomycin. Using sterile scissors and forceps, and in a sterile petri dish (Falcon Plastics; Becton, Dickinson and Co., Cockeysville, MD. USA) containing sterile Hanks solution, calvaria were carefully dissected from each mouse, being particularly careful not to damage the bone. Following the lines of the frontal and parietal bones, these bones were dissected-out, cleansed of all tissue and placed in a fresh dish of Hanks solution. Again, being very careful, the two parietal bones, or calvaria, were cut out and each was placed, separately, in prepared culture dishes, concave side down.

It was also suggested that single mouse vertebrae could be used in organ cultures of bone, since they are intact pieces of bone of a uniform size, suitable for organ culture. However, after several unsuccessful attempts, it proved impossible to remove all of the surrounding muscle and tendons from these bones sufficiently quickly to keep the bone alive for culture.

9. Human bone organ cultures were prepared from biopsies of the iliac crest. When an iliac crest bone biopsy .... was taken from the patient it was placed immediately in Medium 199 solution, which is specially buffered

to maintain a more constant pH, contained in a sterile universal container (Falcon Plastics; Becton, Dickinson and Co., Cockeysville, MD. USA) on ice. As soon as possible, usually within 45 minutes after the removal of the biopsy from the patient, the biopsy was placed in a sterile petri dish and all muscle removed, the bone was then placed in fresh Medium 199 solution to remove any blood.

Using sharp scissors and forceps, under sterile conditions, the bone biopsy was cut horizontally into two pieces. Generally the bone biopsy would be a cylinder of bone, mainly trabecular bone, but with an end-plate of cortical bone at the top and bottom; by cutting the bone horizontally it became possible to gain access to the trabecular bone. Using sharp scissors, it was quite a simple matter to pare away at the trabecular bone and obtain small fragments suitable for culture. These small fragments of trabecular bone were placed in organ culture in the same way as the mouse calvaria.

10. All bone cultures, as described, were placed in McIntosh-Fildes jars or tissue culture boxes and gassed with 5% CO_2 in air.
11. Culture medium was removed and replaced as required, usually daily, but always within 3 days.

12. In those experiments where the effects of serum from patients suffering from metabolic bone disease were studied, 15% human serum replaced 15% foetal calf serum in the standard culture medium. Human serum was sterilised using multipore filters (Swinnex-25, Millipore S.A., France).

13. In some cases, to obtain control bone samples, sample calvaria were killed by thrice freezing and thawing and subsequently treated as live cultures.

14. Alkaline phosphatase assays on the culture medium were performed by means of kits (Boehringer Diagnostics, London) for total alkaline phosphatase in serum, but proved suitable for use here. This assay for alkaline phosphatase incorporates paranitrophenol phosphate as the substrate.

15. Ionised calcium (Ca^{2+}) measurements in the culture medium were performed by means of a Nova 2 ionised calcium analyser (American Hospital Supply (U.K.) Ltd., Oxon).

Initially, to allow the viability of the calvaria in culture to be assessed, sample calvaria were removed from culture and fixed for histological studies. For comparison, calvaria of the same age as the bone in culture were removed from mice and assessed in the same manner.

16. All bone studied was fixed in the following buffered formalin solution:

formalin	10 ml
acid sodium phosphate monohydrate	0.4g
anhydrous disodium phosphate	0.65g
distilled water	to 100 ml

Samples were fixed in buffered formalin solution for at least 48 hours. Later, when it was realised that the fine calvaria were difficult to discern during embedding and sectioning procedures, red acid fuchsin dye was added to the formalin, making the calvaria red; the dye was readily removed from the calvaria during the staining procedures.

17. Decalcification of the calvaria became necessary as it was found that, although quite thin and delicate, the mouse calvaria did not become completely embedded in the paraffin wax used and parted company with the wax during sectioning. When bones had been fixed in buffered formalin for at least 48 hours, they were then placed in 10% formic acid overnight, this decalcified the bone, and then into a solution of 50% saturated lithium carbide solution and 50% tap water, to neutralise the acid.

18. Embedding of samples was by the standard paraffin-embedding technique.

19. Three main histological stains were used on the 6 μm sections of calvaria produced:

A. Standard haemalum and eosin. This stain highlights

the nucleus and cytoplasm of each cell: the nucleus is blue and the cytoplasm pink.

B. Masson's acid fuchsin aniline blue trichrome. This stain shows the nuclei to be black, cytoplasm a range of reds, muscle fibres bright red and collagen an intense blue, mucin is also blue. This stain was chosen to highlight the presence of collagen in the calvaria sections, the major component of the organic phase of bone.

C. Schmorl's method for compact bone. This stain shows the cells to be blue to black, while the bone matrix is light blue. As with the previous stain, this stain was chosen to highlight the matrix component of the mouse calvaria sections.

20. All photographs were taken on Ektachrome 50 film using a tungsten lamp.

21. Human serum from a variety of metabolic bone disorders were used in this study.

Results:

Stained sections of both cultured and non-cultured calvaria have allowed us to see whether the bone in culture is being maintained, using the criterion that living nuclei are large and round, while dead nuclei are small and dilated, pyknotic. The various staining methods were employed to let us see if the sections of cultured calvaria compared favourably with sections of calvaria taken directly from mice.

FIGURE 1A shows a section of a 6-day-old mouse calvaria which has been in culture for 2 days, the section is stained using haemalum and eosin. In this photograph we can see the large nuclei of the cells, the cell cytoplasm and the bone matrix stain pink, indicating that cells are alive. FIGURE 1B shows a section of 6-day-old mouse calvaria, which has been cultured for 2 days, stained using Masson's trichrome stain; here again we see the large dark-stained nuclei and healthy collagen matrix, indicating that the bone is both alive and maintaining its structure.

Later, looking at sections of calvaria kept in culture for 4 days (FIGURES 1C and 1D) we can see that the nuclei are still large and densely-stained; the staining methods used here are haemalum and eosin and Masson's trichrome, respectively. Even after 6 days in culture (FIGURES 1E and 1F) we still see large dark nuclei, with these sections comparing favourably with sections of

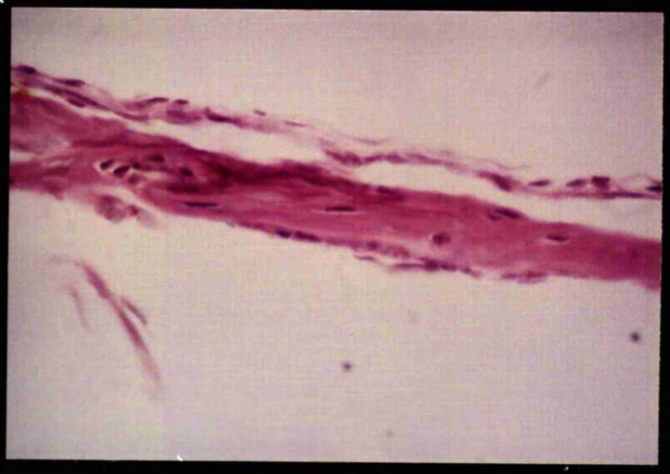
FIGURE 1A

A histological section of 6 day-old mouse calvaria which has been cultured for two days. This section is stained with haemalum and eosin.

FIGURE 1B

A histological section of 6 day-old mouse calvaria which has been cultured for two days. This section is stained with Masson's trichrome stain.

a



b

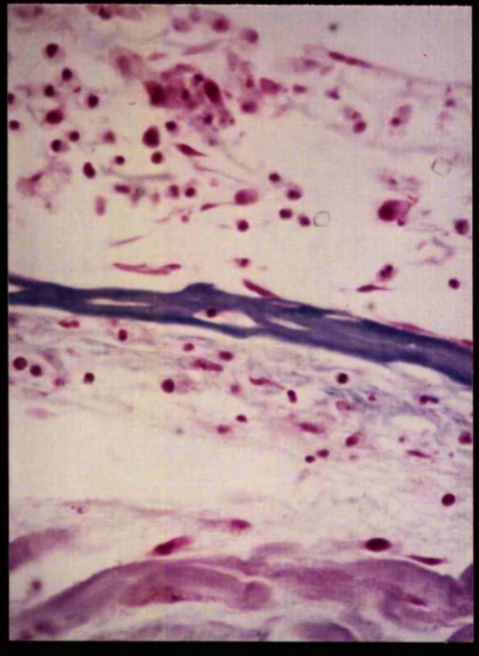


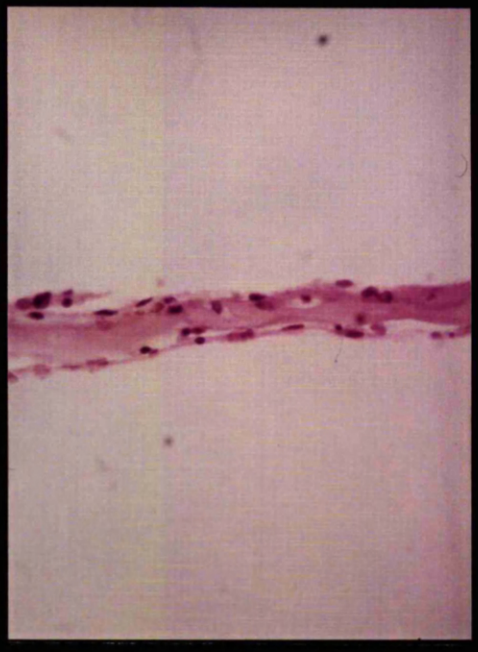
FIGURE 1C.

A histological section of 6 day-old mouse calvaria which has been cultured for 4 days. This section has been stained with haemalum and eosin.

FIGURE 1D

A histological section of 6 day-old mouse calvaria which has been cultured for 4 days. This section has been stained with Masson's trichrome stain.

c



d

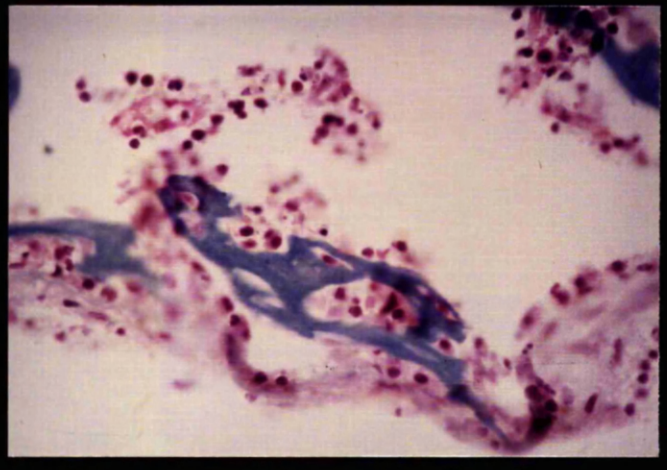


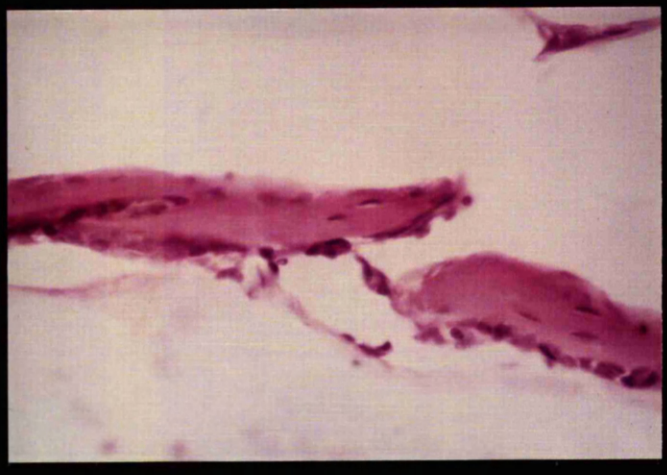
FIGURE 1E

A histological section of 6-day-old mouse calvaria which has been cultured for 6 days. This section has been stained with haemalum and eosin.

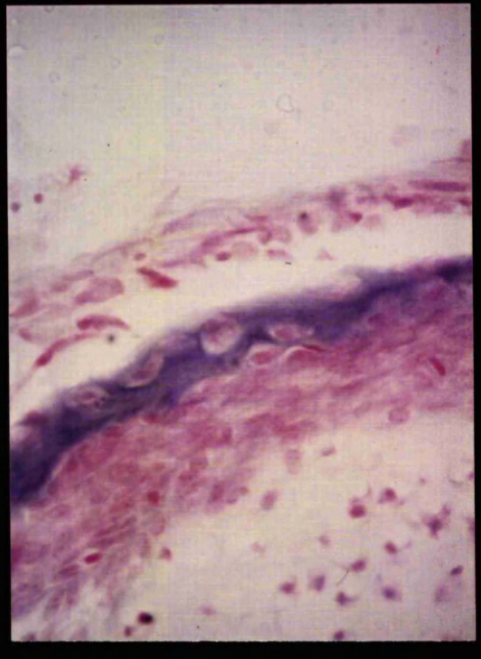
FIGURE 1F

A histological section of 6-day-old mouse calvaria which has been cultured for 6 days. This section has been stained with Masson's trichrome stain.

e



f



calvaria taken from a 13-day old mouse (FIGURE 1G). After 9 days in culture the bone is still alive and maintaining its structure (FIGURE 1H).

Haemalum and eosin staining and Masson's trichrome stain produced the clearest results, although for ease and simplicity haema um and eosin staining was preferred. Schmorl's stain for compact bone produced poor and indefinite results. This histological study of mouse calvaria in culture showed that bone can be successfully maintained by the culture regime described, with all culture media tested producing healthy calvaria. From this point, however, only BGJb Original medium was used, allowing accurate comparisons to be made between cultures.

Alkaline phosphatase and Ca^{2+} assays were performed daily on the culture medium supporting the mouse bones, noting first of all that both methods of assay chosen: the Nova 2 ionised calcium analyser and the Boehringer-Mannheim kit for total AP, gave reproducible results, with the appropriate quality controls. Fresh culture medium contains no AP but has a 1.1 mM concentration of Ca^{2+}; the results produced in culture medium when bone is cultured are shown in FIGURE 1I. These results show that, initially, the AP level in culture medium rises and the Ca^{2+} level falls, only to decrease and increase, respectively, as the cultures proceed. The general trends for AP and Ca^{2+} levels would seem to suggest an inverse relationship between the two

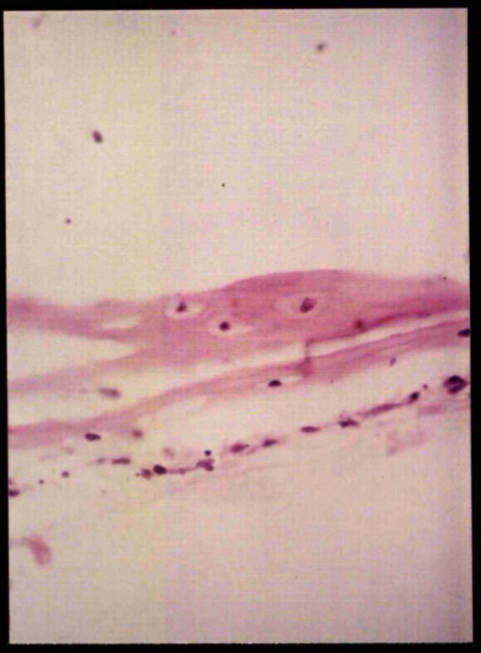
FIGURE 1G

A histological section of a 13 day old mouse calvaria, taken directly from a 13-day-old mouse and not cultured. This section has been stained with haemalum and eosin.

FIGURE 1H

A histological section of a 6-day-old mouse calvaria which has been cultured for 9 days. This section has been stained with haemalum and eosin.

g



h

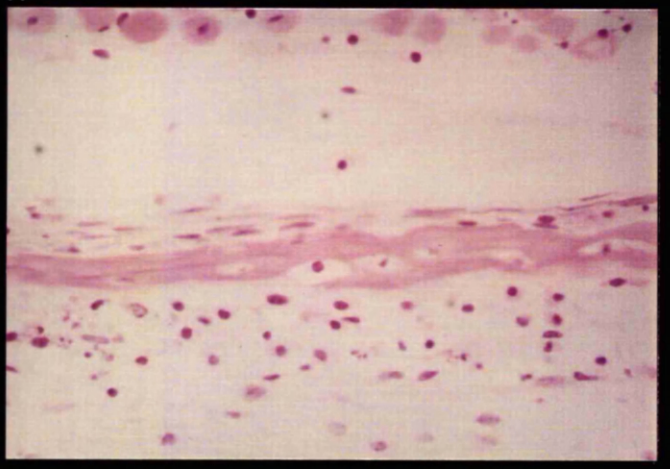
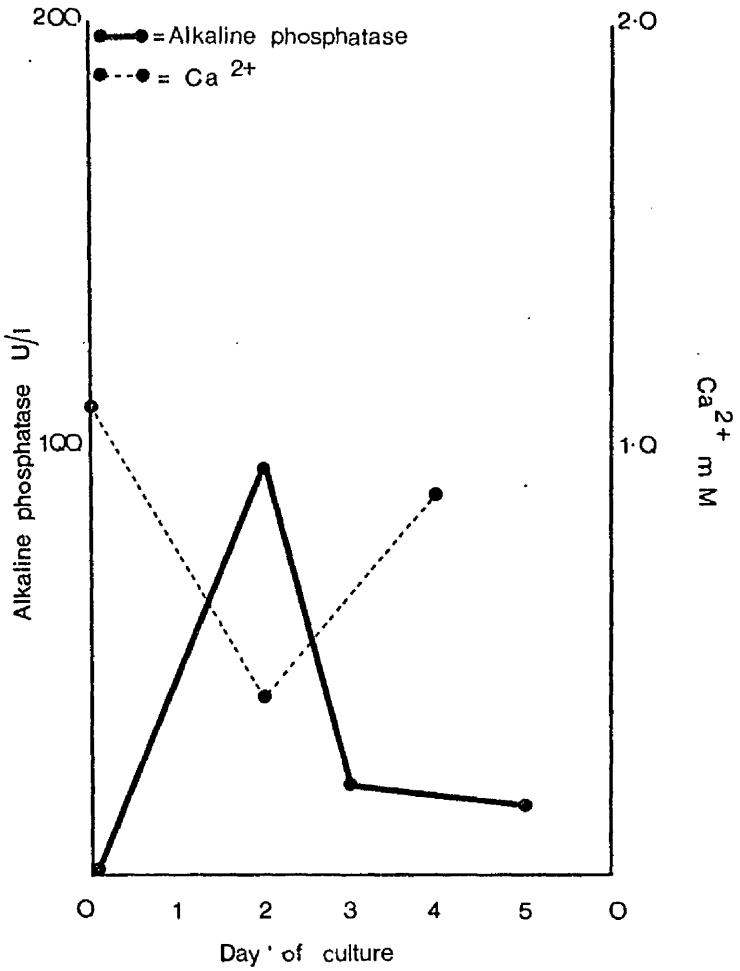


FIGURE 1I

Levels of alkaline phosphatase and Ca^{2+} in culture medium supporting 6-day-old mouse calvaria in culture, on each day of culture.

FIGURE 11


200
100
0
2·0
1·0
0
= Alkaline phosphatase
= Ca 2+
Alkaline phosphatase U/l
Ca2+ m M
0 1 2 3 4 5
Day ' of culture

parameters, which will be studied in more detail later.

Biochemical tests equivalent to those performed on mouse calvaria culture medium were carried out on culture medium supporting small fragments of human bone. Results of AP assays and Ca^{2+} assays performed are shown in FIGURES 1J and 1K, respectively. These results would suggest that the amount of AP released by calvaria in culture and the Ca^{2+} levels in the culture medium, again, show some kind of inverse relationship. The results, however, do not show any particular trends for any of the bone disorders studied, except that bone from a patient with Paget's disease of bone releases more AP than the others, reflecting serum AP levels in vivo.

Looking at the results for AP and Ca^{2+} levels in culture medium supporting human bone fragments, and taking some other factors into consideration, it was decided not to continue preparing cultures of human bone. It is very difficult to produce fragments of human bone of a uniform size and content, in terms of cortical and trabecular bone and cellular distribution, making accurate comparisons very difficult. The complex structure of bone also makes it difficult to get a uniform distribution of nutrients throughout the bone, as well as a uniform release of enzymes and metabolites, again making accurate comparisons difficult.

However, mouse calvaria cultures were prepared using serum from patients suffering from metabolic bone disease,

FIGURE 1J

Results of alkaline phosphatase assays performed on culture medium supporting fragments of bone from patients suffering from metabolic bone diseases.

FIGURE 1J

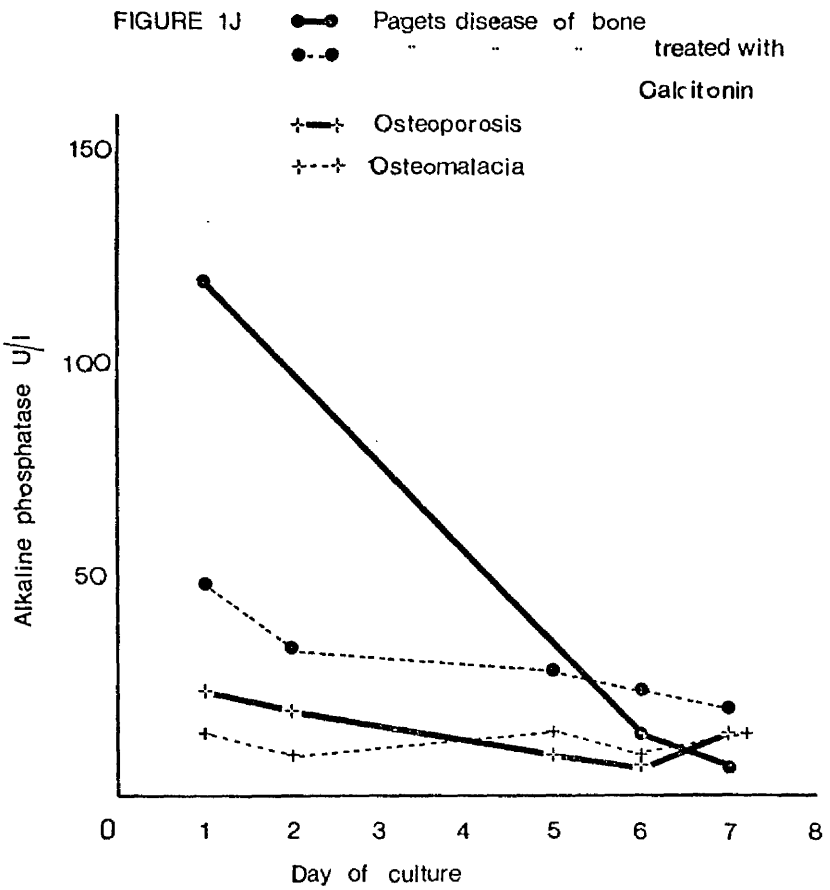
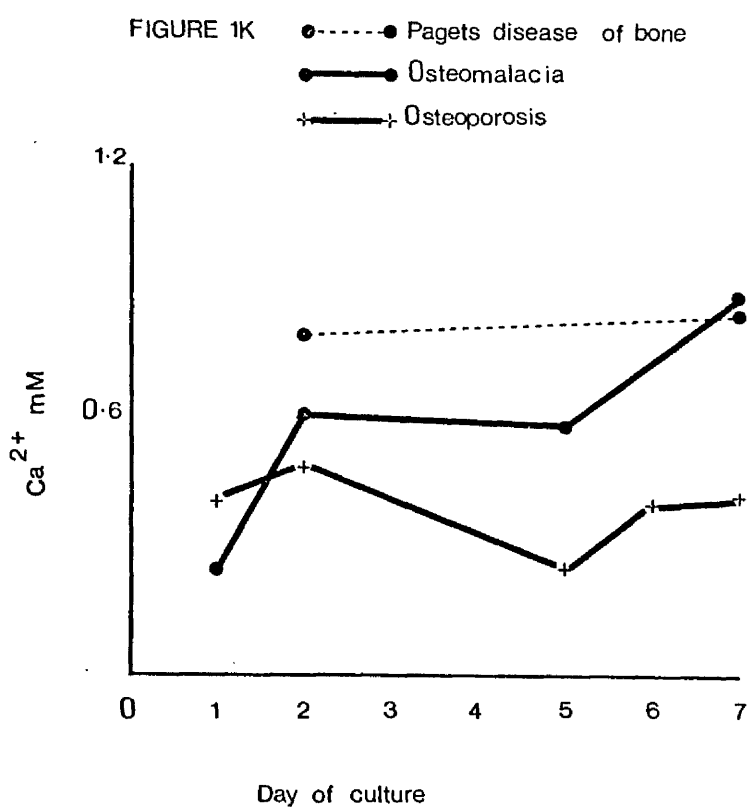

Pagets disease of bone
" " " treated with Calcitonin
Osteoporosis
Osteomalacia
150
100
50
Alkaline phosphatase U/l
0
1
2
3
4
5
6
7
8
Day of culture

FIGURE 1K

Results of Ca^{2+} assays performed on culture medium supporting fragments of bone from patients suffering from metabolic bone diseases.

FIGURE 1K


Pagets disease of bone
Osteomalacia
Osteoporosis
1·2
0·6
Ca^{2+} mM
0
1
2
3
4
5
6
7
Day of culture

substituting 15% human serum for 15% foetal calf serum. Alkaline phosphatase and Ca^{2+} assays were performed on the culture medium each day and the results are shown in TABLES 1A(i),(ii).

TABLE 1A (i)

	ALKALINE PHOSPHATASE LEVEL (U/L) + DAY OF CULTURE					
	DAY 1 $\bar{x} \pm S$	DAY 2 $\bar{x} \pm S$	DAY 3 $\bar{x} \pm S$	DAY 4 $\bar{x} \pm S$	DAY 5 $\bar{x} \pm S$	DAY 6 $\bar{x} \pm S$
Foetal calf serum	136 ± 55	43 ± 15	42 ± 27	48 ± 25	69 ± 34	23 ± 4
Osteoporosis	131 ± 38	47 ± 18	18 ± 2	8 ± 2	67 ± 31	20 ± 7
Primary Hyperpara	101 ± 26	48 ± 20	12 ± 3	23 ± 7	47 ± 27	32 ± 12
Paget's Disease of Bone	90 ± 20	30 ± 6	5 ± 0	0	0	0
Pseudohypopara	11 ± 2	11 ± 3		11 ± 1		
Hypochondroplasia	137 ± 54	3 ± 0	0	0	0	0
Normal human	107 ± 49	48 ± 33	29 ± 10	44 ± 6		
Malignant Hypercalcaemia	90 ± 6	29 ± 4	25 ± 10	31 ± 13		
Osteogenesis Imperfecta	65 ± 43	20 ± 5	4 ± 1	3 ± 3	4 ± 0	

Results of AP assays performed on culture medium containing 15% human serum, from patients suffering from metabolic bone disease, and supporting 6-day-old mouse calvaria in culture, along with control results for medium containing 15% foetal calf serum. The average levels for each day are given, along with the standard deviation.

TABLE 1A (ii)

	Ca^{2+} LEVEL (mM) + DAY OF CULTURE					
	DAY 1 $\bar{x} \pm S$	DAY 2 $\bar{x} \pm S$	DAY 3 $\bar{x} \pm S$	DAY 5 $\bar{x} \pm S$	DAY 6 $\bar{x} \pm S$	DAY 7 $\bar{x} \pm S$
Foetal calf serum	1.9±0.04	2.0 ±0.4		1.97±0.02	0.89±0.06	
Osteoporosis	1.62±0.14	1.7 ±0.1		1.53±0.34	1.69±0.22	
Normal human serum	1.02±0.03	1.02±0.15		0.71±0.03	0.83±0.02	
Paget's Disease of Bone	1.37±0.14	1.48±0.01		1.07±0.21	1.19±0.18	
Hypercalcaemia	1.39±0.09	1.39±0.04		1.09±0.11	1.44±0.07	
Malignant Hypercalcaemia	1.49±0.06	1.6 ±0.32		1.04±0.07	1.39±0.07	
Hypophosphatasia	1.56±0.1	1.62±0.09		1.05±0.13	1.5 ±0.06	
Chronic Renal Failure			1.12±0.14	1.29±0.16	1.444 ±0.14	1.333±0.1

Results of Ca^{2+} assays performed on culture medium containing 15% human serum, from patients suffering from metabolic bone disease, and supporting 6-day-old mouse calvaria in culture, along with control results for medium containing 15% foetal calf serum. The average levels for each day are given, along with the standard deviation.

By rearranging the results shown in TABLES 1A, to show the levels on the first day calculated as 100%, we can gauge the changes in AP and Ca^{2+} concentrations over the culture period. The results of this exercise are shown in TABLES 1B.

TABLE 1B(i)	PERCENTAGE ALKALINE PHOSPHATASE LEVEL + DAY OF CULTURE					
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
Foetal calf serum	100	32	31	35	51	17
Osteoporosis	100	36	14	6	51	15
Primary Hyperparathyroidism	100	48	12	23	46	32
Paget's Disease of Bone	100	33	5	0	0	0
Pseudohypoparathyroidism	100	100		100		
Hypochondroplasia	100	2	0	0	0	0
Normal human serum	100	45	27	41		
Malignant Hypercalcaemia	100	32	28	34		
Osteogenesis Imperfecta	100	30	6	5	6	

TABLE 1B(ii)	PERCENTAGE Ca^{2+} LEVEL + DAY OF CULTURE					
	DAY 1	DAY 2	DAY 3	DAY 5	DAY 6	DAY 7
Foetal calf serum	100	105		104	47	
Osteoporosis	100	105		94	104	
Normal human serum	100	100		70	81	
Paget's Disease of Bone	100	108		78	87	
Hypercalcaemia	100	100		78	104	
Malignant Hypercalcaemia	100	107		70	93	
Hypophosphatasia	100	104		67	96	
Chronic Renal Failure			100	115	129	119

Discussion:

From the results of histological studies made concerning the maintenance of mouse calvaria in culture, we can see that the culture regime which has been developed successfully supports the calvaria in culture, as well as maintaining their structural integrity. This method of culture may now be used with confidence in other studies.

Results of biochemical studies also appear to be promising, with the Ca^{2+} and AP parameters easily measurable in the culture medium. Standard culture medium, containing foetal calf serum, contains no AP but a 1.1 mM Ca^{2+} concentration. When the medium is placed in culture with bone we see a dramatic increase in the level of AP and changes in the Ca^{2+} level, which can increase or decrease, but the trends would seem to suggest an inverse relationship between Ca^{2+} and AP.

Robison (1923), Bevelander, et al. (1950) and Morse, et al. (1951) suggest that AP is present in areas preparing for calcification and, as such, one might expect to find some sort of relationship between Ca^{2+} and AP. As AP stimulates or engenders mineralisation through the precipitation of calcium phosphate, one would expect the levels of free Ca^{2+} to decrease, increasing again when levels of AP decrease. Further studies in this thesis will attempt to clarify this relationship.

Mouse calvaria cultures are very simple to replicate: 6-day-old mice from a standard litter are of similar size, making their calvaria of similar size and allow accurate comparisons to be made between different calvaria in culture. Cultures of human bone are a different matter: Meunier trephine biopsies of the iliac crest are cylinders of bone, 1 cm in diameter and 2.5 cm in length, but the amount of bone contained in each biopsy may vary a great deal, depending on the condition of the patient. Osteomalacic bone is soft and poorly calcified, osteoporotic bone is fine and fragments easily, while bone from a patient with Paget's disease of bone is dense and strongly calcified. This comparison shows that a 2 mm^3 piece of bone from each type of patient would vary a great deal, as it also can within disorders, where severity may vary. It is fairly clear then that many factors may combine to make comparisons between results produced by human bone fragments in culture very suspect and inaccurate. From this point no further human bone cultures were prepared.

However, human serum from patients suffering from a variety of metabolic bone disorders was used in culture with mouse calvaria: 15% human serum replacing the standard 15% foetal calf serum in the medium. The results of Ca^{2+} and AP assays performed on the culture medium of such cultures are shown in TABLES 1A(i),(ii) and TABLES 1B(i),(ii).

These results show no particular pattern of progressive increase or decrease in AP or Ca^{2+} over the culture period, with the AP and Ca^{2+} levels inherent in the patient's serum interfering with those produced by the effects of the serum on the bone itself, making comments on the effects of the serum difficult.

What has been learned from the work carried out in this chapter is that the culture system used is keeping the calvaria alive and healthy in culture and that AP and Ca^{2+}, of particular interest in this thesis, are easily measurable in the culture medium, with the suggestion of some kind of inverse relationship between the two parameters. Such results allow further studies with calvaria in culture to be carried out with confidence.

CHAPTER TWO: STUDIES IN BONE RESORPTION USING ^{45}Ca AND ^{47}Ca WITH HUMAN SERUM

Over the past 20 years calcium radioisotopes have been used in many studies concerned with the effects of various drugs, hormones or metabolites on bone resorption and, it is generally agreed, the release of ^{45}Ca from prelabelled bones in culture is an accurate means of assessing bone resorption. These various substances have been tested, either individually or in combination, allowing the effects of the drugs on in vitro bone resorption to be assessed.

One of the main bone resorbing agents to be studied has been parathyroid hormone, which has been shown, after causing an initial uptake of calcium by bone, to promote bone resorption and serum calcium levels (Parsons, et al. 1971). Changes in bone resorption on the addition of other substances may also be assessed. Engracio, et al. (1972) and Minkin (1973) studied the effects of the antibiotic Mithramycin on parathyroid-hormone-induced release of ^{45}Ca from prelabelled bone in culture, finding that the drug inhibits the action of the hormone. Stern (1969) had followed a similar line of enquiry, looking at the effects of corticosteroids on parathyroid hormone-induced resorption of bone, also finding that the hormone was inhibited. Calcitonin is an effective inhibitor of bone resorption (MacIntyre, et al. 1971) and also inhibits the in vitro resorption of bone by parathyroid hormone (Raisz, et al. 1967).

Raisz, et al. (1969) also studied the effects of phosphate, calcium, magnesium, parathyroid hormone and calcitonin, in combination, on bone resorption in culture, providing some interesting results. Phosphate inhibits the release of ^{45}Ca from bone, particularly in the presence of parathyroid hormone, with the effects of phosphate and calcitonin appearing to be additive. Increasing the concentration of magnesium ions has no effect on bone resorption, but decreasing the concentration results in decreased bone resorption, particularly in the presence of parathyroid hormone. Calcium concentration has apparently no effect on bone resorption in vitro. These studies, among many others, serve to indicate the wide acceptance of this technique as a means of assessing bone resorption.

Experimental bones may be labelled for study by one of several methods. Some workers prelabel young bones in utero, injecting up to 500 μCi of $^{45}CaCl_2$ into a pregnant rat or mouse on the 18th day of gestation, using foetal bones in culture a day or two later (Raisz, et al. 1967; Vaes, 1968; Raisz, et al. 1968; Stern, 1969; Raisz, et al, 1969; Engracio, et al. 1972; Stern, et al, 1976; Rowe, et al. 1977; Holtrop, et al. 1978; Horton, et al. 1978). Other researchers prefer to inject 2 day old mice subcutaneously, with doses of between 10 μCi and 12.5 μCi of $^{45}CaCl_2$ each, using their labelled calvaria in culture 4 days later (Reynolds, et al. 1970; Fang, et al. 1971; Minkin, et al.

1972; Minkin, 1973; Gozariu, et al. 1973; Marks, 1974). The prelabelled bones are dissected out from the freshly-killed young animals and placed in sterile culture with either control medium or experimental medium, the latter containing some test substance. The effects of the test substance are gauged by intermittent monitoring of the ^{45}Ca levels in the culture medium.

As in the previous chapter, thrice frozen and thawed dead bone cultures were sometimes used as experimental controls. Reynolds, et al. (1970) have shown that the degree of actual cell-mediated resorption in bone, as opposed to diffusion or active transport, of calcium may be calculated by subtracting the level of ^{45}Ca released by dead bones from that released by living bones. In addition to the ^{45}Ca released by bone in culture, changes in the ^{45}Ca content of the prelabelled bones themselves may also be assessed: Vaes (1968) dissolved labelled calvaria in 1 ml of 1 Normal hydrochloric acid, while Fang, et al. (1971) used 0.5 ml of concentrated formic acid, assessing the ^{45}Ca content of the dissolved bone on β-scintillation counter.

In this study, after various preliminary experiments, the effects of various substances were examined. Many studies have been made concerning the effects of para-thyroid hormone, calcitonin, oestrogen and vitamin D metabolites, separately or in combination, on bone in culture and so it was decided to use these as trial

substances for the experimental system used here, using the doses of these substances tested in previous studies (Nisbet, et al. 1969; Heersche, 1969; Messer, et al. 1973; Peck, et al. 1976; Jones, et al. 1976; Reynolds, 1977; Messer, 1977; Jones, et al. 1977; Herrmann-Erlee, et al. 1977; Hermann-Erlee, et al. 1978).

The main point of this study, however, was to look at the effects of human serum, taken from patients suffering from a variety of metabolic bone diseases, on bone turnover in vitro. Mice were prelabelled by subcutaneous injection of $^{45}CaCl_2$ at 2 days old and their calvaria placed in culture at 6 days old, but on each occasion, in addition to prelabelling the bone with ^{45}Ca, the culture medium was prelabelled using $^{47}CaCl_2$. These isotopes of calcium have distinct half-lives, which allow them to be used together, while still allowing them to be assessed separately. Once test serum has been added to the culture medium, samples of medium are removed daily and assessed, initially for ^{45}Ca and ^{47}Ca content and after four weeks for ^{45}Ca content alone. ^{47}Ca has a half-life of only 4.7 days, decaying within 4 weeks, with the ^{45}Ca, with a half-life of 160 days, remaining. It was hoped that by using both isotopes of calcium, bone turnover, and not just bone resorption, in the presence of the different sera could be assessed.

Materials and Methods:

1. Preparation of cultures. Two-day-old BKW mice were injected subcutaneously with 10 μCi of $^{45}CaCl_2$, in initial studies, or 0.5 μCi of $^{45}CaCl_2$ in all subsequent studies with patient serum. At six-days-old the mice were killed by decapitation and their calvaria placed in culture, as described in the previous chapter. In those experiments where it is specified, 0.25 μCi of $^{47}CaCl_2$ were added to the culture medium. Each day of culture the medium was removed and replaced with fresh medium of the same type and, occasionally, bone was removed from culture and dissolved in 1 ml of concentrated formic acid, to allow the isotope content of the bone to be assessed.

2. Radio-active assay of samples. All samples, after initial trial experiments, were counted for 5 minutes on a β-scintillation counter (Packard TRI-CARB, Model 3375), each vial containing 5 ml of instagel, a scintillation cocktail, and 25 μl of the sample.

The culture medium used, as in the previous chapter, contained phenol red indicator and it was thought that this may produce a colour-quenching effect on the activity recorded, reducing the counts per minute (CPM) detected. To test this effect, some samples were treated with 0.2 mls of hydrogen peroxide and incubated at 40^{o}C for ½ hour; samples without bleach were also treated in this way. The results showed

that the phenol red produces no colour-quenching effect and bleaching of samples is unnecessary.

3. To look at the effects of parathyroid hormone (PTH) and calcitonin (CT) on the release of ^{45}Ca from prelabelled bone in culture, cultures were prepared as normal, but with either 0.5 U/ml of PTH or 50 mU/ml of CT (Sigma, London) added to the experimental cultures; control cultures contained no test substance. The PTH and CT were not added to the experimental cultures until day 2, allowing the calvaria to 'acclimatise' themselves in culture. The release of ^{45}Ca in the presence of each substance was assessed by β-scintillation. Dead control cultures were also prepared. Results are shown in TABLE 2A. (FCS = Foetal Calf Serum Control).

TABLE 2A

TEST → SUBSTANCE ADDED NOW	DAY OF CULTURE	CULTURES COMPARED		p
	1	PTH (860± 350)	+ FCS (1120±160)	0
		CT (1570± 90)	+ FCS (1120±160)	0
		PTH (860± 350)	+ CT (1570± 90)	0
		FCS (1120±160)	+ FCS Dead (940±180)	0
	2	PTH (1190±400)	+ FCS (2680±120)	0.01
		CT (2540±940)	+ FCS (2680±120)	0
		PTH (1190±400)	+ CT (2540±100)	0
		FCS (2680±120)	+ FCS Dead (1420±100)	0.001
	3	PTH (450±77)	+ FCS (1700±60)	0.001
		CT (1640±40)	+ FCS (1700±60)	0
		PTH (450±77)	+ CT (1640±40)	0.001
		FCS (1700±60)	+ FCS Dead (960±240)	0
	4	PTH (380±92)	+ FCS (1160±40)	0.001
		CT (760±320)	+ FCS (1160±40)	0
		PTH (380±92)	+ CT (760±320)	0
		FCS (1160±40)	+ FCS Dead (700±60)	0.02

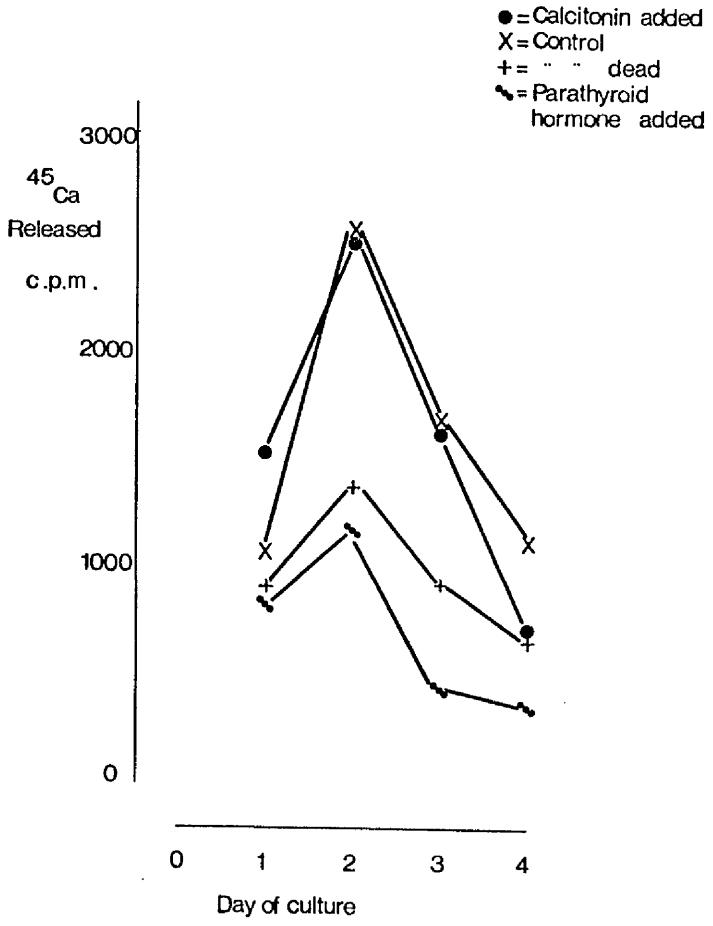
The results demonstrate that PTH strongly inhibits bone resorption in vitro, while CT only slightly inhibits bone resorption. Dead bone in culture releases less ^{45}Ca than corresponding living control cultures. FIGURE 2A demonstrates the general trends.

4. ^{45}Ca and ^{47}Ca in Culture Medium. On each day that fresh medium was placed in culture, 0.25 μCi $^{47}CaCl_2$ were added to the medium. It was hoped that by using two distinct calcium isotopes that the passage of calcium into and out of bone, throughout the duration of each culture could be studied: looking at the release of ^{45}Ca by bone and the uptake of ^{47}Ca by bone. Results in TABLE 2B, looking at ^{45}Ca and ^{47}Ca levels in the culture medium on each day of culture show that the culture medium in each case contains both calcium isotopes, where it had originally contained only ^{47}Ca. The level of ^{45}Ca in the medium seems to remain fairly constant, although it falls towards the end of the given culture period, the level of ^{47}Ca rises. The values of ^{47}Ca have been corrected, taking into account the short half-life and the rapid decay of the isotope. At the end of the given culture period the bone, on dissolution with concentrated formic acid, can be seen to contain a mixture of both ^{45}Ca and ^{47}Ca, where it had originally contained only ^{45}Ca.

FIGURE 2 A

Results of comparisons between ^{45}Ca levels in culture medium in the presence of parathyroid hormone and calcitonin, as well as in control medium. These results demonstrate that, generally, less ^{45}Ca is released in the presence of parathyroid hormone or calcitonin than with control medium.

FIGURE 2A


●=Calcitonin added
X=Control
+= " " dead
= Parathyroid hormone added
3000
45Ca Released c.p.m.
2000
1000
0
0
1
2
3
4
Day of culture

It is possible to use two isotopes of calcium together in such a manner, and detect them separately, as they have distinct half-lives. In the initial count made on the culture medium, and dissolved bone samples, we obtain a total count for all ^{45}Ca and ^{47}Ca present, however ^{47}Ca has a half-life of 4.7 days and all ^{47}Ca decays to a stable form within 4 weeks; assessment of the culture medium after 4 weeks will show only the ^{45}Ca present, ^{45}Ca having a half-life of 160 days. The activity of ^{47}Ca in the samples was obtained by subtracting the bone ^{47}Ca level from the combined ^{45}Ca and ^{47}Ca level obtained 4 weeks previously.

TABLE 2B

DAY OF CULTURE	^{45}Ca (CPM) $\bar{x} \pm S$	^{47}Ca (CPM) $\bar{x} \pm S$
1	1304 ± 544	5887 ± 1214
2	1494 ± 637	7638 ± 1448
3	1401 ± 597	8723 ± 1899
4	1489 ± 665	11877 ± 1137
5	1421 ± 565	13520 ± 3572
6	1344 ± 463	14421 ± 3403
7	1141 ± 380	15610 ± 2804
BONE ON DAY 7	8726 ± 4815	43745 ± 7833

Results of ^{45}Ca and ^{47}Ca activities present in bone culture medium on each day of culture and in acid-dissolved bone at the end of the culture period. These results show that both bone and culture medium contain a mixture of the 2 calcium isotopes, where each had previously contained only one.

To give a better impression of how ^{45}Ca and ^{47}Ca levels in bone are affected on each day of culture, sample calvaria were removed from culture on each day and dissolved in concentrated formic acid. As controls, cultures were set up using dead calvaria, killed by thrice freezing and thawing, to let us see whether the exchange of calcium isotopes between bone and culture medium is active or passive. The results of this study are shown in TABLE 2C and FIGURE 2B, showing us that as cultures progress the amount of ^{47}Ca in the bone itself rises then falls again, suggesting that ^{47}Ca is lost from bone. ^{45}Ca levels also seem to show a lot of variation, probably due to differing uptake at the time of injection. The ^{45}Ca and ^{47}Ca levels in both living and dead bones seem to fluctuate, with no great differences in the levels of isotopes on each day.

TABLE 2C. (C.P.M. = Counts per minute).

DAY BONE REMOVED FROM CULTURE	CONTENT OF LIVING BONE		CONTENT OF DEAD BONE	
	^{45}Ca (C.P.M.)	^{47}Ca	^{45}Ca (C.P.M.)	^{47}Ca
1	4263	5491	4704	3626
2	3654	9118	2929	7021
3	2892	12990	2886	12540
4	2409	20599	2337	19921
5	3578	23740	4246	14390
6	3751	19019	4930	18181
7	4237	19805	3370	35488

Results of ^{45}Ca and ^{47}Ca activities in bone removed from culture each day and dissolved in concentrated formic acid, showing that the ^{45}Ca and ^{47}Ca in both living and dead bone fluctuates greatly.

FIGURE 2B

Results of ^{45}Ca and ^{47}Ca assays performed daily on pre-labelled calvaria. The calvaria were removed from culture on each day, dissolved in concentrated Formic acid and assayed for ^{45}Ca and ^{47}Ca content.

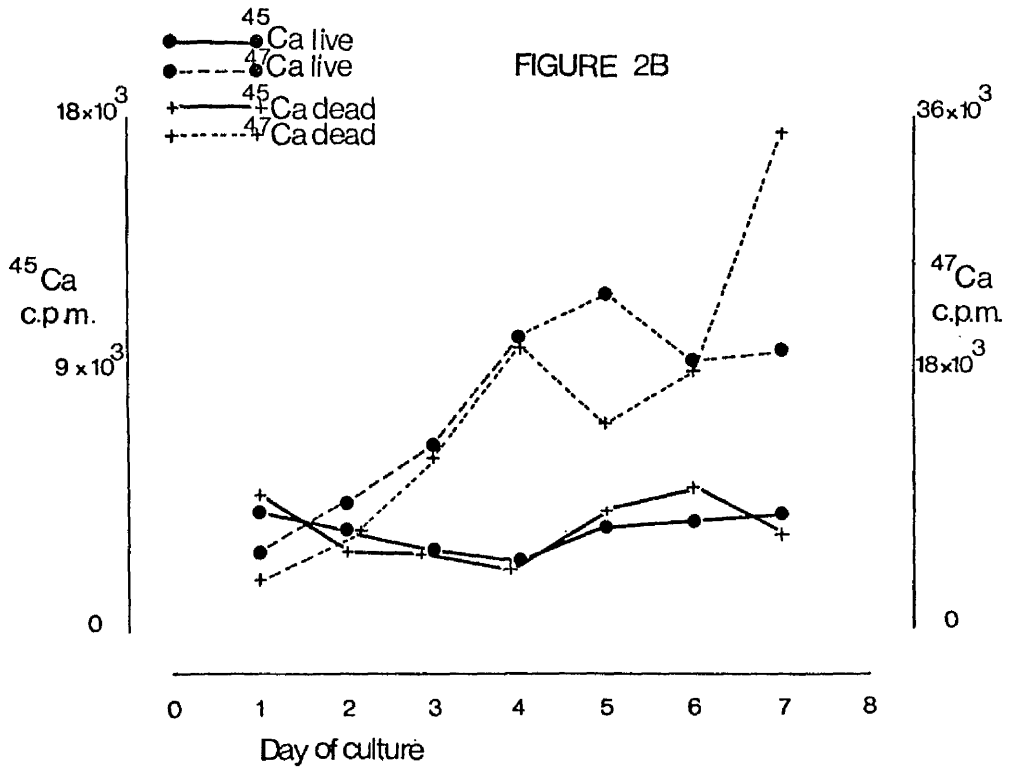

45Ca live
47Ca live
45Ca dead
47Ca dead
18x10^3
45Ca c.p.m.
9 x10^3
0
36x10^3
47Ca c.p.m.
18x10^3
0
0 1 2 3 4 5 6 7 8
Day of culture

FIGURE 2B

Looking at the levels of ^{45}Ca and ^{47}Ca released by living and frozen dead control bones into the culture medium, TABLE 2D FIGURE 2C, we see that dead bones tend to release less ^{45}Ca into the culture medium than living bones. The uptake of ^{47}Ca by living and dead bone following a similar pattern, decreasing then increasing, but with living bone tending to take up slightly less ^{47}Ca.

TABLE 2D

(i)	DAY OF CULTURE	^{45}Ca (C.P.M.) $\bar{x} \pm S$	^{47}Ca (C.P.M.) $\bar{x} \pm S$
LIVING BONE CULTURE MEDIUM	1	734 ± 207	6114 ± 941
	2	859 ± 226	8720 ± 1338
	3	864 ± 311	9967 ± 1260
	4	863 ± 219	12157 ± 1618
	5	1001 ± 294	14732 ± 2988
	6	1055 ± 273	12925 ± 1820
	7	677 ± 16	11285 ± 358

(ii)	DAY OF CULTURE	^{45}Ca (C.P.M.) $\bar{x} \pm S$	^{47}Ca (C.P.M.) $\bar{x} \pm S$
DEAD BONE CULTURE MEDIUM	1	596 ± 128	6805 ± 2254
	2	596 ± 83	7638 ± 1540
	3	593 ± 73	9496 ± 995
	4	644 ± 77	10763 ± 1561
	5	640 ± 62	12874 ± 1548
	6	598 ± 395	10019 ± 1740
	7	650 ± 7	12068 ± 985

Results of ^{45}Ca and ^{47}Ca activities in culture medium supporting living and dead calvaria on each day of culture. These results show that dead bone releases less ^{45}Ca than living bone and the release of ^{45}Ca from dead bone is at an almost constant rate, suggesting a passive process. The results also show that living bone tends to take up less ^{47}Ca than dead bone, but the amount taken up fluctuates.

FIGURE 2C

Results of ^{45}Ca and ^{47}Ca assays performed daily on bone culture medium supporting living and dead calvaria; medium is labelled with ^{45}Ca.

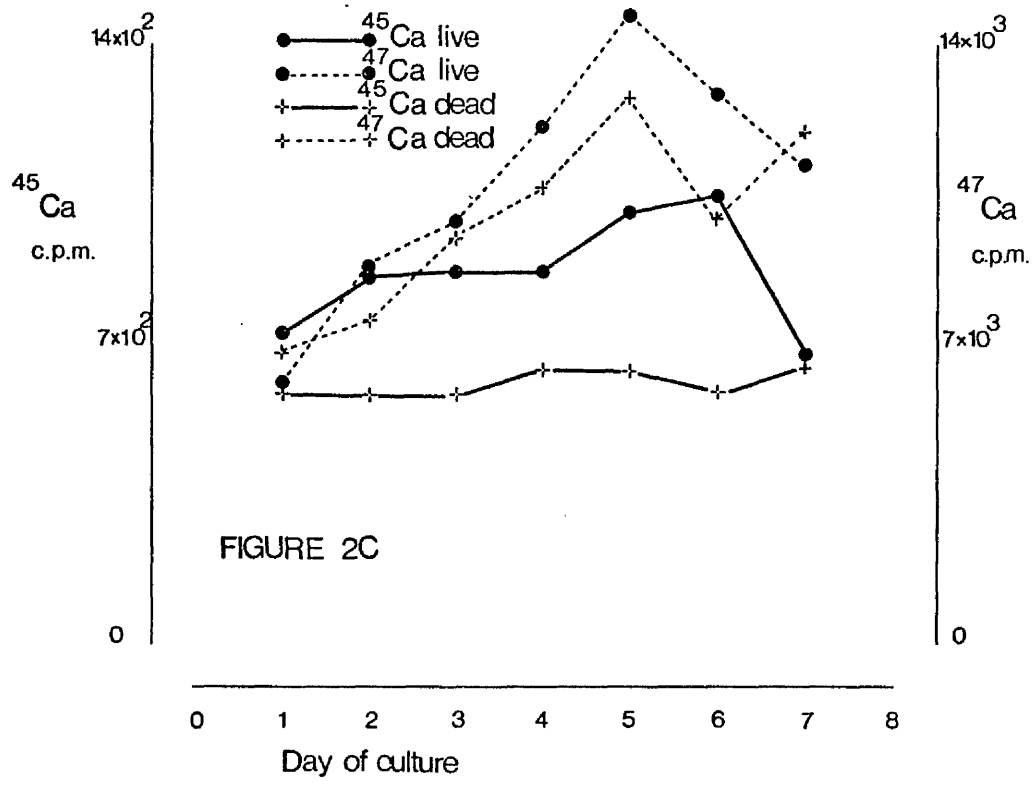

45Ca live
47Ca live
45Ca dead
47Ca dead
14x10^2
7x10^2
0
45Ca c.p.m.
14x10^3
7x10^3
0
47Ca c.p.m.
0 1 2 3 4 5 6 7 8
Day of culture

FIGURE 2C

Reynolds (1970) used the release of ^{45}Ca from prelabelled living and dead calvaria to give a measure of the actual cell-mediated resorption (C.M.R.), by subtracting the ^{45}Ca released by dead calvaria from the ^{45}Ca released by living calvaria, the release of ^{45}Ca from dead bone being caused by diffusion. However, results from cultures containing both ^{45}Ca and ^{47}Ca suggest that ^{47}Ca, originally taken up by bone in culture, may be released by bone towards the end of the culture period, replacing ^{45}Ca which would normally be released. This trend suggests that when both calcium isotopes are used we cannot get a true measure of the C.M.R., making the use of dead calvaria controls unnecessary.

From this point in the study of bone turnover in culture, the levels of the ^{45}Ca and ^{47}Ca isotopes in the culture medium will be used in comparative studies. The short half-life of ^{47}Ca makes it unsuitable for long-term cultures and so all cultures will be maintained for only 4 days, with the ^{47}Ca levels on each day being corrected for decay.

The main point of this investigation is the study of the effects of serum, from patients suffering from metabolic bone disorders, on bone turnover in vitro. We have already seen that many groups have studied the effects of bone-related substances, such as parathyroid hormone, on bone resorption in culture, using ^{45}Ca alone; these substances: parathyroid hormone, oestrogen, calcitonin and vitamin D

metabolites have been studied in relation to ^{45}Ca and ^{47}Ca bone turnover in vitro. By looking at the effects of these metabolites in culture with this experimental system, we can look at the usefulness of this system compared with studies in bone turnover using ^{45}Ca alone.

Cultures were prepared containing 15% human serum, from patients or normal individuals, replacing 15% foetal calf serum; the normal serum is pooled. For those cultures where PTH or some other substance is studied, the test substance, in the concentration given, is added to the standard culture medium, containing 15% foetal calf serum, at the beginning of the culture period. Control cultures, containing standard culture medium with 15% foetal calf serum, were prepared with each set of experimental cultures. Between 3 and 16 cultures of each type were prepared, depending on the availability of the serum or metabolite. On each day of culture, the culture medium was changed, being replaced by fresh medium of the same type, as before. The culture medium removed was assayed by β-scintillation for ^{45}Ca and ^{47}Ca content, by the method described; the average ^{45}Ca and ^{47}Ca levels in each set of control and experimental cultures are given in TABLES 2E-2U.

Initially, the ^{45}Ca and ^{47}Ca levels found in culture medium in the presence of any test substance, whether serum or metabolite, are compared with those levels found

in control medium, control medium acting as the standard, producing a ratio: $\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$. By comparing these ratios between different sets of experimental cultures, we can see how ^{45}Ca release and ^{47}Ca uptake compare in the presence of each test substamce. Results for days 3 and 4 only, where bone has 'acclimatised' in culture, are given.

Results:

TABLE 2E. Pooled normal human serum

TYPE OF CULTURE		DAY 3		DAY 4	
		^{45}Ca (C.P.M.)	^{47}Ca	^{45}Ca (C.P.M)	^{47}Ca
EXPERIMENTAL	$\bar{x}$	79	485	85	454
	S	14	192	8	152
CONTROL	$\bar{x}$	87	547	82	665
	S	13	119	17	222
$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.91	0.89	1.04	0.68

TABLE 2F. Serum from hypoparathyroid patients.

	TYPE OF CULTURE		DAY 2 ^{45}Ca(C.P.M)	DAY 2 ^{47}Ca	DAY 4 ^{45}Ca(C.P.M)	DAY 4 ^{47}Ca
(i)	EXPERIMENTAL	$\bar{x}$	489	16662		
		S	24	3305		
	CONTROL	$\bar{x}$	596	24312		
		S	17	4529		
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		0.82	0.69		
(ii)	EXPERIMENTAL	$\bar{x}$	161	4308	210	4769
		S	86	354	135	490
	CONTROL	$\bar{x}$	178	3025	144	3311
		S	35	730	70	513
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		0.9	1.42	1.46	1.44
(iii)	EXPERIMENTAL	$\bar{x}$	211	4034	280	4345
		S	111	600	83	341
	CONTROL	$\bar{x}$	178	3025	144	3311
		S	35	730	70	513
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.18	1.33	1.94	1.31
(iv)	EXPERIMENTAL	$\bar{x}$	481	9072	526	10167
		S	45	686	73	863
	CONTROL	$\bar{x}$	506	5525	630	6199
		S	44	572	63	825
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		0.95	1.64	0.84	1.64

(i) Female, age 37. Serum Ca 1.65; serum AP 246.
(ii) Female, age 37. Serum Ca 1.95; serum AP 127. On 3 µg $1(OH)D_3$ per day.
(iii) Female, age 46. Serum Ca 2.4 ; serum AP 205; serum PTH 280. On 1.25 µg calciferol per day.
(iv) Female, age 67. Serum Ca 1.95; serum AP 211.

TABLE 2G. Serum from patients with Paget's Disease of Bone

	TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M)	DAY 4 ^{47}Ca
(i)	EXPERIMENTAL	$\bar{x}$	906	24863	1195	31054
		S	58	2571	0	0
	CONTROL	$\bar{x}$	997	23985	964	26494
		S	92	924	0	0
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		0.91	1.03	1.24	1.18
(ii)	EXPERIMENTAL	$\bar{x}$	291	2547		
		S	55	215		
	CONTROL	$\bar{x}$	269	1688		
		S	28	299		
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.08	1.51		
(iii)	EXPERIMENTAL	$\bar{x}$			580	13243
		S			81	1550
	CONTROL	$\bar{x}$			628	10955
		S			29	1460
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$				0.92	1.21
(iv)	EXPERIMENTAL	$\bar{x}$	849	8590	730	10377
		S	23	1520	89	1045
	CONTROL	$\bar{x}$	509	6821	546	9021
		S	13	706	99	466
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.67	1.26	1.34	1.15

TABLE 2G (continued) ...

	TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M.)	DAY 4 ^{47}Ca
(v)	EXPERIMENTAL	$\bar{x}$	427	16432	498	18086
		S	29	194	24	2311
	CONTROL	$\bar{x}$	346	18121	410	21538
		S	44	1424	26	1067
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.23	0.91	1.21	0.84
(vi)	EXPERIMENTAL	$\bar{x}$	410	7954	432	10187
		S	68	1296	101	3637
	CONTROL	$\bar{x}$	486	5474	453	8580
		S	104	1077	92	167
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		0.84	1.45	0.95	1.19

(i) Male, age 59. Serum Ca 2.25; serum AP 353.

(ii) Female, age 55. Serum Ca 2.3; serum AP 828.

(iii) Female, age 71.

(iv) Male, age 51. Serum Ca 2.3; serum AP 265.

(v) Male, age 72. Serum Ca 2.5; serum AP 142.

(vi) Male, age 78. Serum Ca 2.35; serum AP 1542. On calcitonin, last injection 98 hours previously.

TABLE 2H. Serum from patients with primary hyperparathyroidism.

	TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M.)	DAY 4 ^{47}Ca
(i)	EXPERIMENTAL	$\bar{x}$	477	10465	393	18128
		S	0	0	0	0
	CONTROL	$\bar{x}$	587	6680	606	9906
		S	0	0	79	1131
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.81	1.56	0.65	1.83
(ii)	EXPERIMENTAL	$\bar{x}$	262	4730	342	5090
		S	176	553	35	401
	CONTROL	$\bar{x}$	178	3025	144	3311
		S	35	730	70	513
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		1.47	1.56	2.37	1.53
(iii)	EXPERIMENTAL	$\bar{x}$	198	4190	369	4228
		S	163	927	74	535
	CONTROL	$\bar{x}$	178	3025	144	3311
		S	35	730	70	513
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		1.11	1.38	2.56	1.27
(iv)	EXPERIMENTAL	$\bar{x}$	378	440	232	538
		S	0	7	14	89
	CONTROL	$\bar{x}$	662	347	476	431
		S	0	0	34	147
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.57	1.27	0.49	1.24

(i) Male. (ii) Male, age 79

(iii) Female, age 66. (iv) Male.

TABLE 2I. Serum from patients with malignant hypercalcaemia

	TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M)	DAY 4 ^{47}Ca	
(i)	EXPERIMENTAL	$\bar{x}$	132	390	150	449	Male. Serum AP 1040 (mostly liver iso-enzyme)
		S	30	115	10	129	
	CONTROL	$\bar{x}$	72	321	75	300	
		S	12	81	4	178	
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.83	1.21	2.0	1.5	
(ii)	EXPERIMENTAL	$\bar{x}$	150	457	129	174	Male. Serum AP 274.
		S	14	163	16	70	
	CONTROL	$\bar{x}$	75	321	75	300	
		S	12	81	4	178	
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		2.0	1.42	1.72	0.58	
(iii)	EXPERIMENTAL	$\bar{x}$	119	415	116	196	Male.
		S	13	70	7	87	
	CONTROL	$\bar{x}$	75	321	75	300	
		S	12	81	4	178	
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.59	1.29	1.55	0.65	
(iv)	EXPERIMENTAL	$\bar{x}$	170	439	118	293	Male. Serum AP 2200 (mostly liver iso-enzyme)
		S	38	113	34	124	
	CONTROL	$\bar{x}$	75	321	75	300	
		S	12	81	4	178	
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		2.27	1.37	1.57	0.98	
(v)	EXPERIMENTAL	$\bar{x}$	712	3613	585	4554	Female.
		S	66	747	116	273	
	CONTROL	$\bar{x}$	671	3692	740	5197	
		S	412	904	48	790	
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.06	0.98	0.79	0.88	

TABLE 2J. Serum from patients suffering from carcinoma, but with a normal serum calcium.

	TYPE OF CULTURE		DAY 3		DAY 4	
			^{45}Ca(C.P.M)	^{47}Ca	^{45}Ca(C.P.M)	^{47}Ca
(i)	EXPERIMENTAL	$\bar{x}$	345	1838		
		S	70	534		
	CONTROL	$\bar{x}$	269	1688		
		S	28	299		
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.28	1.09		
(ii)	EXPERIMENTAL	$\bar{x}$	356	2223		
		S	45	297		
	CONTROL	$\bar{x}$	269	1688		
		S	28	299		
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.32	1.32		
(iii)	EXPERIMENTAL	$\bar{x}$	185	150	170	343
		S	56	105	67	138
	CONTROL	$\bar{x}$	75	321	75	300
		S	12	81	4	178
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		2.47	0.47	2.27	1.14
(iv)	EXPERIMENTAL	$\bar{x}$	103	425	103	250
		S	9	125	9	28
	CONTROL	$\bar{x}$	75	321	75	300
		S	12	81	4	178
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		1.37	1.32	1.37	0.83

(i) Male. Serum PTH <300. (ii) Male.
(iii) Male. (iv) Male.

TABLE 2K. Serum from patients suffering from hypercalcaemia

	TYPE OF CULTURE	DAY 3 ^{45}Ca(C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca(C.P.M)	DAY 4 ^{47}Ca
(i)	EXPERIMENTAL $\bar{x}$	300	2547		
	S	29	591		
	CONTROL $\bar{x}$	269	1688		
	S	28	299		
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$	1.11	1.51		
(ii)	EXPERIMENTAL $\bar{x}$	206	2148	176	2564
	S	15	182	9	212
	CONTROL $\bar{x}$	167	3947	183	3826
	S	19	216	17	14
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$	1.23	0.54	0.96	0.70
(iii)	EXPERIMENTAL $\bar{x}$	726	17663	657	20663
	S	53	2307	44	1054
	CONTROL $\bar{x}$	748	15255	348	19890
	S	99	904	337	1082
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$	0.97	1.16	1.89	1.04

(i) Female, age 72. Serum Ca 2.65; Serum AP 296

(ii) Female, age 66. On diabetic drugs.

(iii) Male, age 63.

TABLE 2L. Serum from patients suffering from hypervitaminosis D.

	TYPE OF CULTURE		DAY 3		DAY 4	
			^{45}Ca(C.P.M)	^{47}Ca	^{45}Ca(C.P M)	^{47}Ca
(i)	EXPERIMENTAL	$\bar{x}$	520	4208	698	4468
		S	130	421	489	1055
	CONTROL	$\bar{x}$	671	3692	740	5197
		S	412	904	48	790
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		0.77	1.14	0.94	0.86
(ii)	EXPERIMENTAL	$\bar{x}$	492	3940	579	4927
		S	231	544	41	1250
	CONTROL	$\bar{x}$	671	3692	740	5197
		S	412	904	48	790
	EXPERIMENTAL $\bar{x}$ / CONTROL $\bar{x}$		0.73	1.07	0.78	0.95

(i) Female. Serum 25(OH)D_3 1940 nm/litre

(ii) Male. Serum 25(OH)D_3 1760 nm/litre.

TABLE 2M. Serum from patients suffering from osteoporosis.

	TYPE OF CULTURE		DAY 3 ^{45}Ca(C.P.M)^{47}Ca		DAY 4 ^{45}Ca(C.P.M)^{47}Ca	
(i)	EXPERIMENTAL	$\bar{x}$	142	1290	263	1179
		S	99	72	70	0
	CONTROL	$\bar{x}$	349	774	304	890
		S	76	63	61	0
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.41	1.67	0.87	1.32
(ii)	EXPERIMENTAL	$\bar{x}$	266	4206	291	4311
		S	87	451	87	675
	CONTROL	$\bar{x}$	178	3025	144	3311
		S	35	730	70	513
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		1.49	1.39	2.02	1.30
(iii)	EXPERIMENTAL	$\bar{x}$	366	3360	290	5043
		S	131	595	56	559
	CONTROL	$\bar{x}$	236	3092	410	4020
		S	67	493	99	429
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		1.55	1.09	0.71	1.25
(iv)	EXPERIMENTAL	$\bar{x}$	552	4207	459	5446
		S	45	667	100	1242
	CONTROL	$\bar{x}$	671	3692	740	5197
		S	412	904	48	790
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.82	1.14	0.62	1.05

(i) Female.
(ii) Female, age 51. Serum Ca 2.3; Serum AP 171.
(iii) Female, age 58.
(iv) Female, on oestrogen tablets for 3 weeks.

TABLE 2N. Serum from a patient suffering from osteogenesis imperfecta

TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M)	DAY 4 ^{47}Ca
EXPERIMENTAL	$\bar{x}$	493	4225	794	4810
	S	71	228	48	560
CONTROL	$\bar{x}$	671	3692	740	5197
	S	412	904	48	790
$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.73	1.14	1.07	0.94

Male, age 31. Serum Ca 2.5; Serum AP 436.

TABLE 2O. Serum from a patient suffering from hypophosphatasia

TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M)	DAY 4 ^{47}Ca
EXPERIMENTAL	$\bar{x}$	254	2507		
	S	58	400		
CONTROL	$\bar{x}$	269	1688		
	S	28	299		
$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.94	1.49		

Female, age 21. Serum Ca 2.8; no AP.

TABLE 2P. Serum from patients suffering from rickets/osteomalacia

	TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M)	DAY 4 ^{47}Ca
(i)	EXPERIMENTAL	$\bar{x}$	263	4436	181	5150
		S	118	289	85	416
	CONTROL	$\bar{x}$	178	3025	144	3311
		S	35	730	70	513
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		1.48	1.47	1.26	1.56
(ii)	EXPERIMENTAL	$\bar{x}$	436	15518	466	15127
		S	34	2883	20	3470
	CONTROL	$\bar{x}$	402	16655	407	18123
		S	139	4619	51	2077
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		1.08	0.93	1.14	0.83
(iii)	EXPERIMENTAL	$\bar{x}$	355	3805	294	4671
		S	63	1091	84	786
	CONTROL	$\bar{x}$	178	3025	144	3311
		S	35	730	70	513
	$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		1.99	1.26	2.04	1.41

(i) Male. Nutritional rickets

(ii) Male, age 79. Serum Ca 1.3; Serum AP 619

(iii) Female, age 18. Serum Ca 2.25; Serum AP 302; Serum PTH 230. Phosphaturic rickets.

TABLE 2Q. 1 ng/ml $1,25(OH)_2D_3$

TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M)	DAY 4 ^{47}Ca
EXPERIMENTAL	$\bar{x}$	129	1633	155	2987
	S	24	273	38	796
CONTROL	$\bar{x}$	175	1186	200	3068
	S	29	335	39	493
$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.74	0.71	0.78	0.97

TABLE 2R. 100 ng/ml $25(OH)D_3$

TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M)	DAY 4 ^{47}Ca
EXPERIMENTAL	$\bar{x}$	120	1432	132	1422
	S	18	245	19	408
CONTROL	$\bar{x}$	175	2286	200	3068
	S	29	335	39	493
$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.69	0.63	0.66	0.46

TABLE 2S. 0.5 U/ml parathyroid hormone

TYPE OF CULTURE		DAY 3 ^{45}Ca (C.P.M)	DAY 3 ^{47}Ca	DAY 4 ^{45}Ca (C.P.M)	DAY 4 ^{47}Ca
EXPERIMENTAL	$\bar{x}$	62	128	62	129
	S	11	54	10	74
CONTROL	$\bar{x}$	87	547	82	665
	S	13	119	17	222
$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.71	0.23	0.76	0.19

TABLE 2T. 50 mU/ml calcitonin

TYPE OF CULTURE		DAY 3		DAY 4	
		^{45}Ca (C.P.M)	^{47}Ca	^{45}Ca (C.P.M)	^{47}Ca
EXPERIMENTAL	$\bar{x}$	61	379	74	418
	S	10	79	13	62
CONTROL	$\bar{x}$	87	547	82	665
	S	13	119	17	222
$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.70	0.69	0.90	0.63

TABLE 2U. 100 µg/ml oestrogen

TYPE OF CULTURE		DAY 3		DAY 4	
		^{45}Ca (C.P.M)	^{47}Ca	^{45}Ca (C.P.M)	^{47}Ca
EXPERIMENTAL	$\bar{x}$	62	118	60	131
	S	7	43	7	45
CONTROL	$\bar{x}$	87	547	82	665
	S	13	119	17	222
$\frac{\text{EXPERIMENTAL } \bar{x}}{\text{CONTROL } \bar{x}}$		0.71	0.22	0.73	0.20

FIGURE 2D (i), (ii), (iii)

Results of $\frac{\text{EXPERIMENTAL } ^{45}Ca}{\text{CONTROL } ^{45}Ca}$ calculations made for control medium, containing foetal calf serum, and experimental medium, containing either 15% human serum, from a patient with a metabolic bone disorder, or some other bone-related test substance.

FIGURE 2D1.

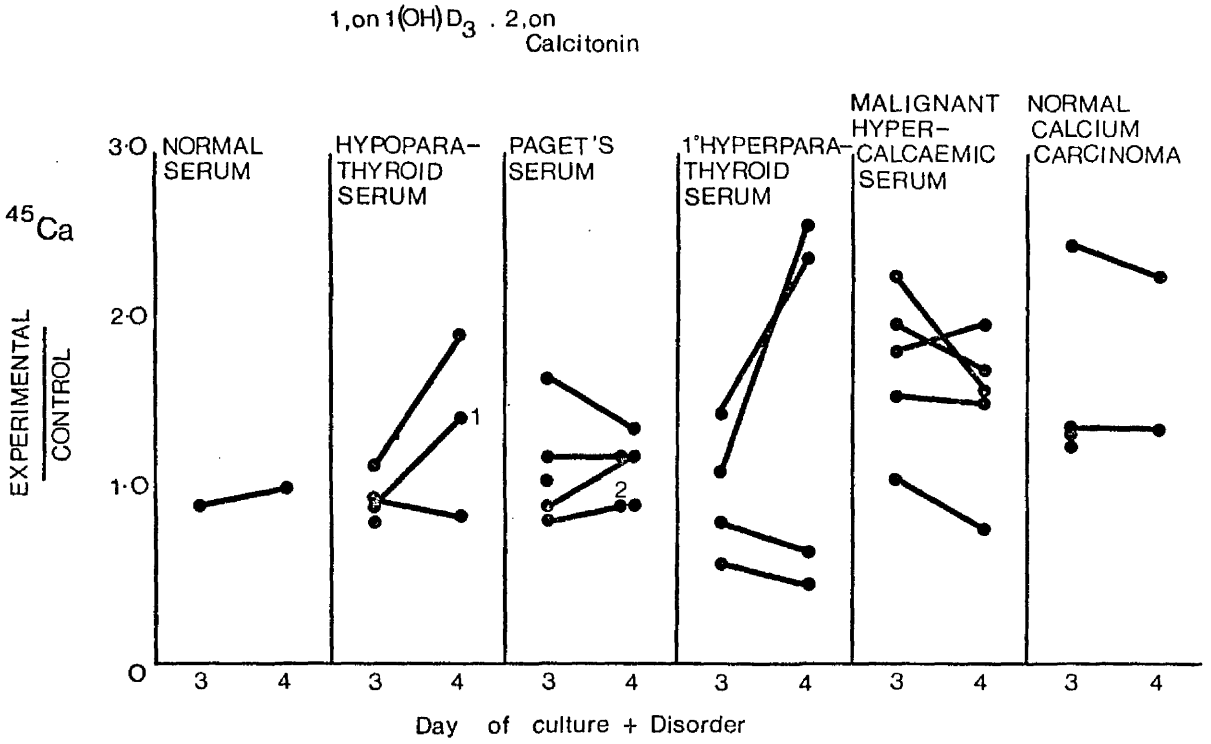
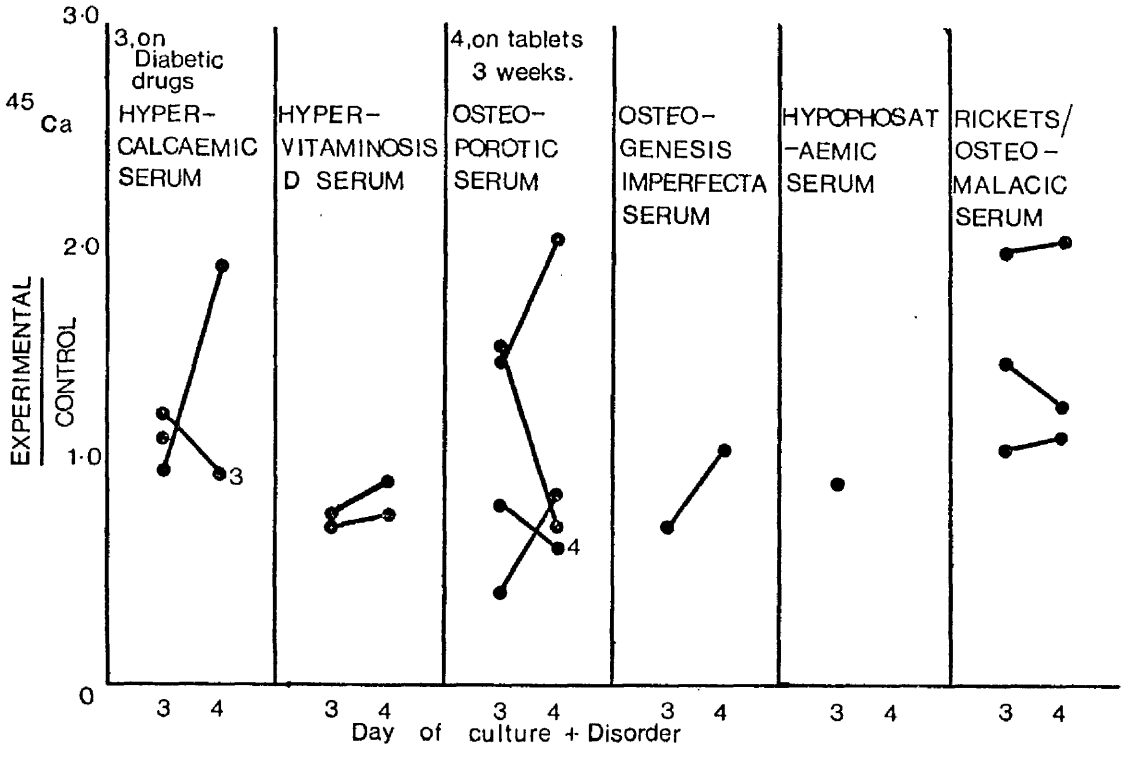

1,on 1(OH)D3 . 2,on Calcitonin
NORMAL SERUM
HYPOPARA-THYROID SERUM
PAGET'S SERUM
1°HYPERPARA-THYROID SERUM
MALIGNANT HYPER-CALCAEMIC SERUM
NORMAL CALCIUM CARCINOMA
45Ca
EXPERIMENTAL / CONTROL
3·0
2·0
1·0
0
1
2
3
4
Day of culture + Disorder

FIGURE 2D2


3·0
2·0
1·0
0
45 Ca
EXPERIMENTAL / CONTROL
3,on Diabetic drugs
HYPER-CALCAEMIC SERUM
HYPER-VITAMINOSIS D SERUM
4,on tablets 3 weeks.
OSTEO-POROTIC SERUM
OSTEO-GENESIS IMPERFECTA SERUM
HYPOPHOSAT-AEMIC SERUM
RICKETS/ OSTEO-MALACIC SERUM
3
4
3 4 3 4 3 4 3 4 3 4 3 4
Day of culture + Disorder

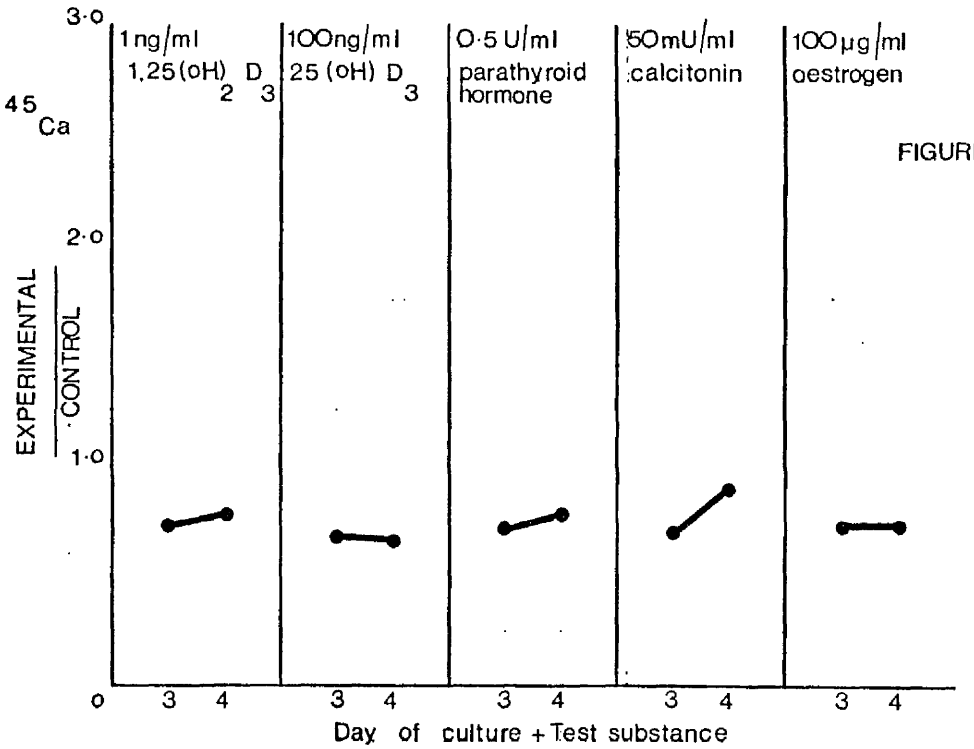

3·0
2·0
1·0
0
45Ca
EXPERIMENTAL / CONTROL
1 ng/ml 1,25(OH)2 D3
100ng/ml 25 (OH) D3
0·5 U/ml parathyroid hormone
50mU/ml calcitonin
100µg/ml oestrogen
3 4
3 4
3 4
3 4
3 4
Day of culture + Test substance

FIGURE 2D3

FIGURE 2E (i), (ii), (iii)

Results of $\frac{\text{EXPERIMENTAL } {}^{47}Ca}{\text{CONTROL } {}^{47}Ca}$ calculations made for control medium, containing foetal calf serum, and experimental medium, containing either 15% human serum, from a patient with a metabolic bone disorder, or some other bone-related test substance.

FIGUR 2E1

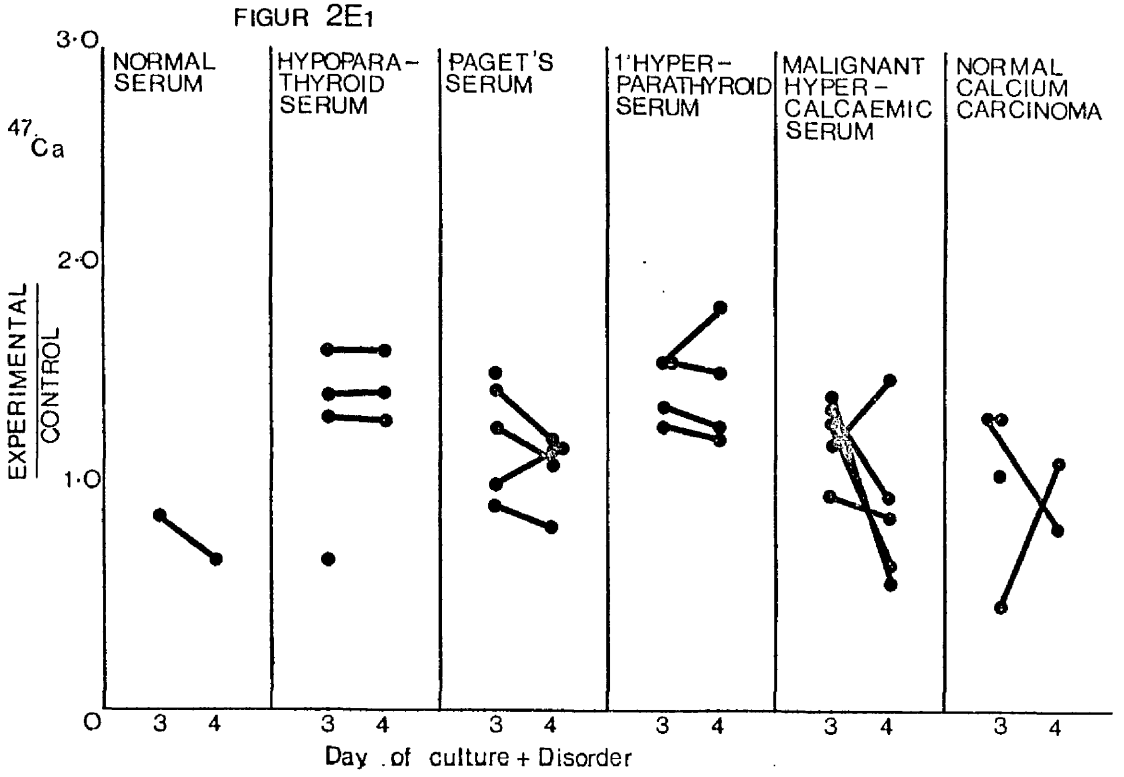
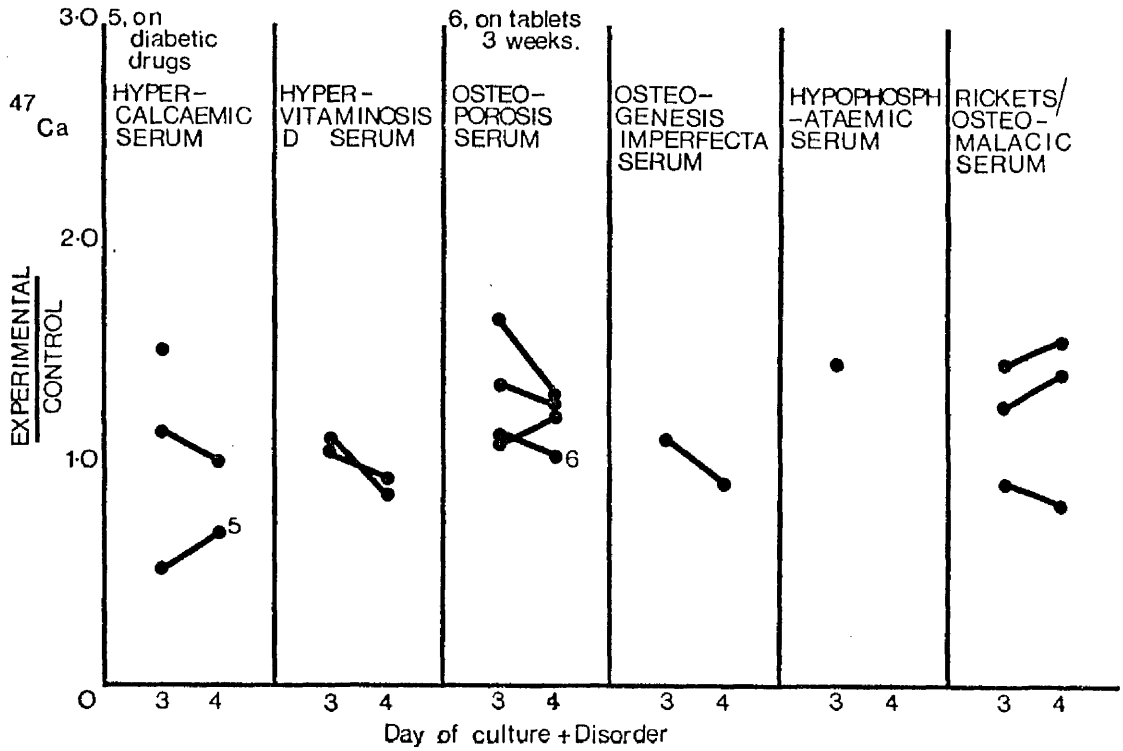

NORMAL SERUM
HYPOPARA-THYROID SERUM
PAGET'S SERUM
1°HYPER-PARATHYROID SERUM
MALIGNANT HYPER-CALCAEMIC SERUM
NORMAL CALCIUM CARCINOMA
47Ca
EXPERIMENTAL / CONTROL
3·0
2·0
1·0
0
3 4 3 4 3 4 3 4 3 4 3 4
Day of culture + Disorder

FIGURE 2E2


3·0
2·0
1·0
0
47 Ca
EXPERIMENTAL / CONTROL
5, on diabetic drugs
6, on tablets 3 weeks.
HYPER-CALCAEMIC SERUM
HYPER-VITAMINOSIS D SERUM
OSTEO-POROSIS SERUM
OSTEO-GENESIS IMPERFECTA SERUM
HYPOPHOSPH-ATAEMIC SERUM
RICKETS/ OSTEO-MALACIC SERUM
5
6
3 4 3 4 3 4 3 4 3 4 3 4
Day of culture + Disorder

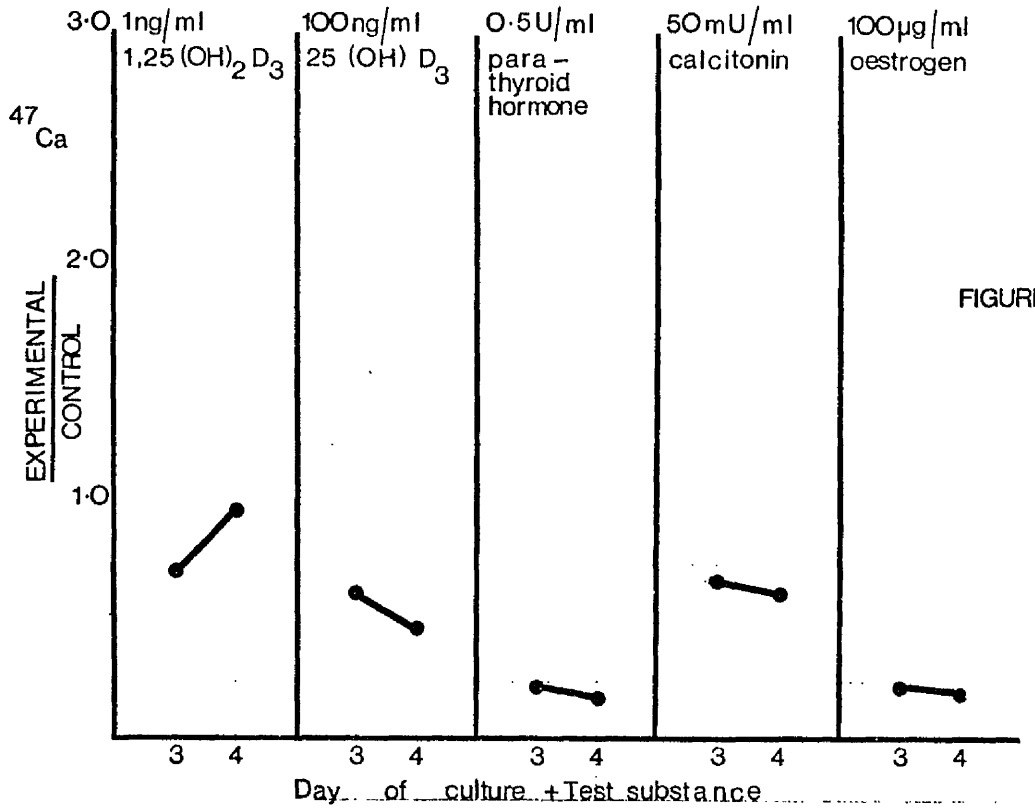

3·0
2·0
1·0
47Ca
EXPERIMENTAL / CONTROL
1ng/ml
1,25 (OH)2 D3
100ng/ml
25 (OH) D3
0·5U/ml
para – thyroid hormone
50mU/ml
calcitonin
100µg/ml
oestrogen
3 4
3 4
3 4
3 4
3 4
Day of culture + Test substance

FIGURE 2E3

Discussion:

As was described in the introduction to this chapter, bone pre-labelled with ^{45}Ca has been very useful, in in vitro culture, in the study of the effects of various metabolites or other test substances on bone resorption. However, as well as knowing the effects a substance may have on resorption, it is sometimes of interest to know the effect of the same substance on bone formation, involving the use of a ^{47}Ca label in the culture medium. In this chapter, the effects of various standard test substances: PTH, CT, oestrogen and the vitamin D metabolites $25(OH)D_3$ and $1,25(OH)_2D_3$, and of various human sera on bone resorption and formation have been studied. By comparing ^{45}Ca and ^{47}Ca levels in the presence of control culture medium, containing 15% foetal calf serum, with the levels in experimental medium, containing added test substance, we can see any effect the substance may have on bone turnover. The effects of medium containing 15% serum from patients suffering from metabolic bone disease are compared in the same way, with control medium, and then compared with the effect of medium containing normal human serum.

There are two basic ways by which the results produced in this study can be assessed. Firstly, the effects of the standard test substance: PTH, CT, oestrogen and the vitamin D metabolites, on ^{45}Ca and ^{47}Ca bone turnover

in vitro can be compared with the effects found in previous studies, of the same substances on ^{45}Ca bone resorption in vitro. Secondly, the effects of the different human sera on bone turnover in vitro can be studied, with the effects of any in vivo drug therapy, such as calcitonin treatment for Paget's disease of bone, on bone turnover being assessed.

As has already been mentioned, PTH is a powerful stimulator of bone resorption. In the condition of hyperparathyroidism, with a high serum PTH (Harris, et al. 1969), we find increased bone turnover, particularly resorption, through increased osteoblastic activity (Rosenbach, et al. 1967; Vaes, 1968; Shubina, et al. 1969; Byers, et al. 1971; Chu, et al. 1971; Messer, et al. 1973a; Minkin, 1973; Peck, et al. 1976; Reeve, et al. 1976; Langeland, 1977; Wong, et al. 1977; Luben, et al. 1977; Maria, et al. 1977; Ibbotson, et al. 1978; Krieger, et al. 1980, Malluche, et al. 1981), although accompanied by increased bone formation (Smith, 1979). Using pre-labelled bone to study bone resorption, it was found that PTH acts on bone in vitro by inhibiting bone resorption, for the first few days of culture, followed by a stimulation of bone resorption (Raisz, et al. 1967, 1969; Stern, 1969; Parsons, et al. 1971; Engracio, et al. 1971; Minkin, 1973).

With ^{45}Ca-prelabelled bone and ^{47}Ca-prelabelled culture medium it was found that, over the 4 day culture period, PTH decreased bone resorption, as compared to control medium with no added PTH, but increased calcium uptake

from the labelled culture medium. The inhibition of bone resorption in these cultures compares favourably with these results found with ^{45}Ca-labelled bone alone, with the increased uptake of ^{47}Ca from the prelabelled medium supporting the role of PTH in increasing bone formation, although to a lesser extent than promoting resorption. These trends are also promising in that the comparable results would seem to suggest that the uptake and re-release of ^{47}Ca from bone, mentioned earlier, may not significantly interfere with the experiment.

Calcitonin inhibits bone resorption (Raisz, et al. 1967; Reynolds, et al. 1968; Raisz, et al. 1968; Nisbet, et al. 1968; Mittleman, et al. 1968; Binderman, et al. 1972; Gozariu, et al. 1973; Luben, et al. 1976; Geusens, et al. 1980; Maier, et al. 1980; Stevenson, et al. 1981),by inhibiting the loss of bone matrix and mineral. Looking at the effect of CT on PTH-induced bone resorption, and on bone in general, it has been found that CT acts on prelabelled bone in culture to inhibit bone resorption (Raisz, et al. 1967, 1969; MacIntyre, et al. 1971). In this study, using pre-labelled bone and pre-labelled culture medium, it was found that CT does indeed inhibit bone resorption, also increasing calcium uptake. This role of CT in the control of bone turnover is of particular interest in the study of post-menopausal osteoporosis, a condition of accumulated bone loss accompanied by normal bone formation, as serum CT levels fall drastically with the menopause (Shamonki, et al. 1980; Padilla, et al. 1980). As CT falls one

would expect the effects of bone resorption to increase, with no effect on bone resorption; with this experiment it has been found that high levels of CT in the culture medium result in decreased bone resorption, a result which compares favourably with that found in vivo and already researched in vitro.

The most common metabolic bone disease affecting post-menopausal women is osteoporosis and with the onset of the menopause comes the disappearance of oestrogen. Oestrogen has been shown to inhibit PTH-stimulated bone resorption (Atkins, et al. 1972; Robin, et al. 1980; Wink, et al. 1980), as well as being able to stimulate the production of CT (Woloszczuk, et al. 1980; Stevenson, et al. 1980; Morimoto, et al. 1980; Cressent, et al. 1980; Stevenson, et al. 1981) which, as we have seen, also inhibits bone resorption, the loss of both oestrogen and CT leading to accumulated loss of bone (Geusens, et al. 1980). The inhibition of bone resorption by oestrogen has been demonstrated, looking at ^{45}Ca-labelled bone (Stern, 1969). Using ^{45}Ca-labelled bone and ^{47}Ca-labelled culture medium, it was found that oestrogen decreased bone resorption in vitro, while stimulating the uptake of calcium. Again, we have results which compare favourably with those found in previous studies, with added information concerning the effect of oestrogen on calcification.

Metabolites of vitamin D have, in general, been found to promote both bone resorption and formation (Mittleman, et al. 1968; Raisz, et al. 1974; Boyle, 1974, Stern, et al. 1976; Peacock, et al. 1976; DeLuca, 1977; Wong, et al. 1977; Cohen, et al. 1980), promoting bone strength. Looking at the effects of the metabolites $25(OH)D_3$ and $1,25(OH)_2D_3$ on bone turnover, using ^{45}Ca-labelled bone and ^{47}Ca-labelled culture medium, it was found that both inhibited bone resorption, with no apparent effect on calcification.

Those experiments relating to the effects of PTH, CT, oestrogen and the vitamin D metabolites on bone turnover in vitro, using ^{45}Ca-labelled bone and ^{47}Ca-labelled medium, have proved promising, generally reflecting those trends found using ^{45}Ca-labelled bone alone, but providing additional information concerning bone formation under the same conditions. Just as important, however, is that these results show that any re-release of ^{47}Ca taken up by bone in the early stages of culture has no detrimental effect on the results. It will now be interesting to look at the effects of the various human sera on bone turnover in vitro.

Looking at the effect of serum from osteoporotic patients on bone turnover in vitro, there are indications of a general trend towards increasing resorption and decreasing formation, as compared with normal human serum.

One patient, being treated with oestrogen, shows decreased resorption and decreased uptake of calcium, as compared to other osteoporotic patients. Osteoporosis is the most widespread bone disorder affecting the elderly and is characterised by an accumulated loss of bone, although the biochemistry and pathology of the remaining bone is normal (Albright, et al. 1948). As the biochemistry and pathology of the bone are normal, one may expect to find that serum from osteoporotic patients and normal individuals would produce the same effects, concerning bone turnover, on bone in vitro, however we find that osteoporotic serum promotes bone resorption over formation, reflecting the osteoporotic condition. The 'normalising' effect of the administration of oestrogen to an osteoporotic patient, reducing bone resorption, is interesting to note, with oestrogen being seen as a potential cure for osteoporosis.

Serum from patients suffering from osteomalacia/rickets would seem to enhance resorption of bone, accompanied by decreased calcification. The osteomalacic/ricketic condition is characterised by soft bones, with a lot of uncalcified bone matrix (Smith, 1979). The effects of serum from such patients would seem to reflect the situation in vivo, with resorption and lack of mineralisation being the rule.

Parathyroid hormone, as we have already seen, acts in vitro by inhibiting bone resorption, initially, but promoting it as cultures get older (Raisz, et al. 1967, 1969; Stern, 1969; Parsons, et al. 1971; Engracio, et al. 1972;

Minkin, 1973). Serum from patients suffering from 1^{o} hyperparathyroidism, where there is an abnormally high serum PTH level, acts on ^{45}Ca-labelled bone and ^{47}Ca-labelled medium by decreasing uptake of calcium and, in general, stimulating bone resorption. These results differ from those found using standard PTH, in that here calcium uptake is decreased and bone resorption is stimulated more quickly. This result suggests that, similar to vitamin D, PTH may act slightly differently in vivo and in vitro.

Hypoparathyroidism, as the name would suggest, is a condition where PTH is absent and bone resorption is severely decreased. Looking at the effects of hypoparathyroid serum on bone turnover in vitro, we see that the effects, as compared with normal human serum, suggest decreased calcification, through decreased uptake of ^{47}Ca from the culture medium. Results concerning bone resorption are unconvincing. It could be that decreased resorption, in vivo, is counteracted to some extent by decreased mineralisation, in an attempt to keep the amount of bone present as normal as possible. The vitamin D metabolite $1\alpha(OH)D_3$ has, as far as this study is concerned, little effect on bone turnover.

Although not a metabolic bone disease, Paget's disease of bone is used here in a comparative role. Serum from patients suffering from P.D. would seem to increase bone

resorption, while showing decreased mineralisation compared to normal human serum. Treatment with CT, as seen with one patient, is effective in decreasing bone resorption, as well as 'normalising' calcification. As P.D. is characterised by increased bone turnover, leading to the deposition of disorganised woven bone (Smith, 1979; Krane, 1980), the increased bone resorption in vitro reflects that found in vivo, however increased mineralisation may have been expected. The effect of CT in reducing bone resorption and 'normalising' calcium uptake indicates its importance in treating this disorder. When the standard test CT was added to ^{45}Ca-labelled bone and ^{47}Ca-labelled medium we saw that it increased the uptake of calcium to a more normal level, suggesting a normalising effect in vivo.

A group of patients suffering from malignant hypercalcaemia provided a special interest in this study, such patients having very high serum Ca levels which were thought to originate from bone; their serum was placed in culture to test this theory. From the results of ^{45}Ca-resorption and ^{47}Ca-uptake, we can see that serum from patients with malignant hypercalcaemia promotes bone resorption and decreases calcification, suggesting that some factor in the serum indeed promotes bone resorption. However, when we look at the effects of serum from patients suffering from carcinoma but with no elevated serum Ca, we see a very

similar pattern of calcium uptake and release. More, specific, investigations would be necessary to explain this action of serum from malignant patients.

Two patients suffering from hypervitaminosis D, who had each taken a massive overdose of $25(OH)D_3$, also provided particular interest in this study. Despite the very high $25(OH)D_3$ levels in their serum, the Ca and AP levels in their serum remained normal. When placed in culture with ^{45}Ca-labelled bone and ^{47}Ca-labelled culture medium, their serum caused decreased bone resorption, as compared with normal human serum, in much the same way as the two test vitamin D metabolites. Mineralisation, or ^{47}Ca-uptake, was slightly less than that found with normal human serum and with the test vitamin D metabolites.

Hypophosphatasia is a rare, inherited, disorder affecting AP and bone (Smith, 1979), where there is no, or very little, bone AP. As this disorder is so rare, it was fortunate that serum from a hypophosphataemic patient became available for this study. In culture with labelled bone and medium, hypophosphataemic serum caused decreased calcification, with no effect on bone resorption, as compared with normal human serum. This result may support the positive relationship between AP and mineralisation, discussed elsewhere in this thesis.

Serum from patients suffering from hypercalcaemia, apart from the malignant hypercalcaemic patients, produced increased bone resorption in culture, with no effect on calcification. This result suggests that the increased serum

calcium originates from bone. Osteogenesis imperfecta is characterised by decreased bone mass, accompanied by multiple fractures (Teitelbaum, 1981), with serum from such a patient producing decreased calcification, with no effect on resorption, in culture. As with osteomalacia, this decreased calcification and softening of the bone would lead to the accumulation of pseudofractures, as have been found in vivo.

From these results we can see that this method of observing bone turnover in vitro has certain attractions. With previous studies it was possible to look at the effects of substances on bone resorption in vitro, but now it is also possible to look at the effects of substances on bone formation in vitro. Previous studies using ^{45}Ca-labelled bone have shown how various standard test substances: PTH, CT, oestrogen, $25(OH)D_3$ and $1,25(OH)_2D_3$ affect bone resorption, effects which have been repeated here, using ^{45}Ca-labelled bone and ^{47}Ca-labelled culture medium; any effects of such substances on bone formation in vitro can be accepted confidently in view of the duplicated resorption effects. Additional information concerning bone formation in vitro gained from this study of CT, PTH, oestrogen and the vitamin D metabolites is that, as in 1^{o} hyperparathyroidism, PTH stimulates calcium uptake in vitro, CT normalises calcification while oestrogen promotes calcification. It would have been interesting to look at the effects of

fluoride, thought to promote strong bones and teeth (Jackson, 1967; Messer, et al. 1973; Smith, 1979) on bone turnover in vitro, however time did not allow.

Although ^{47}Ca has been used here to demonstrate the uptake of calcium by bone in the presence of different test substances, the uptake of labelled calcium from the gut has previously been used to demonstrate bone mineralisation (Spencer, et al. 1978). The advantage of using this in vitro system as opposed to any in vivo system is that this system is much less complicated, with the many systemic effects found in experimental animals being removed. Another advantage of this in vitro system is that it allows investigation of the effects of various human sera on bone turnover in vitro; the administration of human serum to an experimental animal would be complicated by its immune response.

From the limited number of human sera studied here, it is still possible to discern particular patterns of bone formation and resorption accompanying particular bone diseases, as well as their treatment. Human serum is, of course, a very complex mixture of enzymes, metabolites, nutrients and other factors and the main problem in using serum in any study is that any effect cannot, without further analysis, be linked to any particular factor. With those sera used here, for example, no link between serum AP or Ca levels and bone resorption or formation in vitro can be found. As far as the study of the effects of human sera on bone turnover in vitro is concerned, this system could be important,

first of all, in indicating the severity of bone loss or formation in a disorder, and, secondly, in determining the effects of any drug therapy on the patient's condition, the latter being the most important aspect of this system.

NOTE: The work contained within this chapter was presented, in poster form, at the joint meeting of the Scottish Society for Experimental Medicine and the Medical Research Society in Edinburgh on 9th, 10th July, 1982.
Abstract: McGowan, P., Boyle, I.T. (1982) In vitro Bone Turnover in Neonatal Mouse Calvaria: The Influences of Serum from patients with a Variety of Metabolic Problems. Clinical Science, 63, 24p.

CHAPTER THREE: HOW SERUM ALKALINE PHOSPHATASE LEVELS RELATE TO BONE ALKALINE PHOSPHATASE LEVELS IN METABOLIC BONE DISEASE.

The enzyme alkaline phosphatase (AP) has, for many years, been of great interest in the study of the mechanisms of bone mineralisation and many groups have looked at various aspects of its distribution and activity, in attempts to understand its association with calcification. The purpose of this study is to look at the relationship between serum AP levels and AP measurements in bone from patients suffering from metabolic bone disease.

Early work on the role of AP in calcification was performed by Robison (1923,1932), who looked at AP activity in rachitic cartilage. Robison found that the enzyme is detectable in the cytoplasm of hypertrophic cells located in areas of imminent calcification, leading him to postulate that AP is responsible for the localised increase in phosphate esters at these sites, which eventually react within calcium to precipitate as calcium phosphate. Bevelander, et al. (1950) and Morse, et al. (1951) followed the same line of histological study and suggested that AP dissipates following this localised build-up of phosphate esters and before mineralisation occurs, playing no part in mineralisation itself. When they studied the mineralisation of bone in relation to the underlying collagen fibres, Bachra, et al. (1959) also found evidence that AP acts on inorganic

phosphates as a 'local' factor, but suggested that it had some role in calcification itself. All of these results suggest a specialised build-up of AP for mineralisation, although there seems to be some controversy about the part played by the enzyme.

In an attempt to understand the changes produced by AP and, therefore, the part it plays in the calcification process, the relationship between the enzyme and the inorganic phosphate, pyrophosphate, was studied. Fleisch, et al. (1962) proposed that AP removes a layer of pyrophosphate, covering inactive bone surfaces, allowing mineralisation to proceed, showing that pyrophosphate in normal physiological concentrations stabilises both bone formation and resorption (Fleisch, et al. 1966; Nordin, et al. 1969; Fast, et al. 1977). These results lead to the conclusion that pyrophosphate, in a layer covering inactive bone surfaces, is a naturally-occurring regulator of bone turnover, preventing unnecessary formation and resorption and that, acting locally, AP removes the layer and allows bone turnover to proceed (Fleisch, et al. 1970; Russell, et al. 1970; Thomas, et al. 1978; Smith, et al. 1979). This conclusion would seem to support the localised activity of AP without any involvement in the mineralisation process itself, as suggested by Robison (1923, 1932), Bevelander, et al (1950) and Morse, et al. (1951).

The role of AP in relation to pyrophosphate and mineralisation is therefore fairly well understood, but

where does the enzyme originate? Osteoblasts, the bone-forming cells are known to be rich in AP (Jackson, 1967; Smith, 1979) and are the most likely source of the AP involved in mineralisation. Khairi, et al. (1973) and Franck, et al. (1974) found that they could relate increased AP activity with increased osteoblast activity and the level of calcification in bone from patients suffering from Paget's disease of bone. Burks, et al. (1978) also found a visible rise in AP during bone cell proliferation, looking at cells in culture. It would seem then that the AP involved in the localised calcification of bone arises from increased osteoblast activity, as would be expected: increased bone formation requiring increased mineralisation under normal circumstances. One point to note, however, is that the osteoblasts which produce AP are often separated from the mineralisation front by a layer of osteoid tissue; one might expect then that the osteoid nearest to the osteoblasts would calcify first, but this does not happen: the AP would seem only to interact with the surface of mineralised bone; could this be a measure to prevent unwanted calcification?

The level of AP in human serum has been used as a diagnostic index in several metabolic bone diseases, varying between different disorders (Rasmussen, et al. 1974; Smith, 1979). An increase in serum AP is often to be found related to osteomalacia and rickets, renal osteo-dystrophy, Paget's disease of bone and hyperparathyroidism,

although it may remain normal with each of these disorders, as it does with osteoporosis and osteomalacia as a result of renal tubule disorders. The level of serum AP is always very low in hypophosphatasia, a rare disease where there is a congenital lack of AP in most tissues, resulting in gross defects in mineralisation and, therefore, providing support for the importance of AP in the calcification process.

Although the level of serum AP is used in the diagnosis of metabolic bone disease, not all circulating AP originates from bone: it derives from gastro-intestinal mucosa, placenta, bone and liver, with the last two providing the bulk of the enzyme (Smith, 1979); the level of liver derived AP in the serum remains constant, while the level of bone-derived enzyme tends to fluctuate. Pritchard (1952) found AP located in 'soft' tissue, which does not normally calcify, and in mesenchymal cells, which earlier workers found difficult to reconcile with the role of AP in mineralisation but, as discussed earlier, the lack of mineralisation in these tissues may be due to the lack of a calcification front.

The diverse origins of serum AP must, therefore, place some doubt as to whether the level of serum AP genuinely reflects the level of AP and therefore calcifcation, in bone (Rasmussen, et al. 1974). We will see from the study of creatine kinase in muscle, that enzyme levels in serum may not always reflect enzyme levels in the organ of origin itself; in this study, therefore, the levels of alkaline phosphatase in various bone samples have been assessed and the relationship to serum AP determined.

Materials and Methods:

1. Transiliac Meunier trephine biopsies were frozen immediately after removal from the patient and stored deep-frozen until required. Shortly before the assay was due to be performed, the bone was cut on a Cryostat, a microtome in a -20^{o}C refrigerated cabinet, to slices of 12 µm thickness. These sections were stored deep-frozen until required.
2. To prepare bone homogenates, 0.01g of bone flakes were added to 1.0 ml of distilled water and homogenised at setting 5 on a TRI-R homogeniser (Model 563C. TRI-R Instruments, Rockville Centre, New York, USA) for 5 minutes, on ice. The homogenates were then left, on ice, to settle and the supernatant was assayed for total alkaline phosphatase.
3. Alkaline phosphatase assay was by means of a Boehringer-Mannheim Diagnostic Kit for total alkaline phosphatase, substituting 100 µl of bone homogenate for the 100 µl of serum required by the assay. Four assays were carried out on each sample, producing a measure of alkaline phosphatase in U/L.
4. Serum alkaline phosphatase levels were determined in the hospital laboratories.

Results:

1. TABLE 3A shows several bone disorders and the levels of serum AP and bone AP found for each patient suffering from each disorder.

BONE DISORDER + PATIENT		SERUM AP U/L	BONE AP U/L
OSTEOPOROSIS	H.W.	184	18.6
	I.K.	1609	81.4
	A.K.		5.8
	E.R.	258	25.6
	E.O.	200	25.5
	C.R.	289	11.5
	A.B.	211	6.0
1^{O} HYPERPARA	M.T.	598	158.9
	M.D.	322	16.4
HYPOPHOSPHAT.	D.R.	15	3.8
OSTEOMALACIA	J.S.	327	54.4
	E.I.	470	85.0
C.R.F.	M.R.	202	24.4
	G.R.	110	16.4
	M.H.	264	24.8
	J.B.	281	26.0
	J.B.	270	7.0
	R.K.		15.0

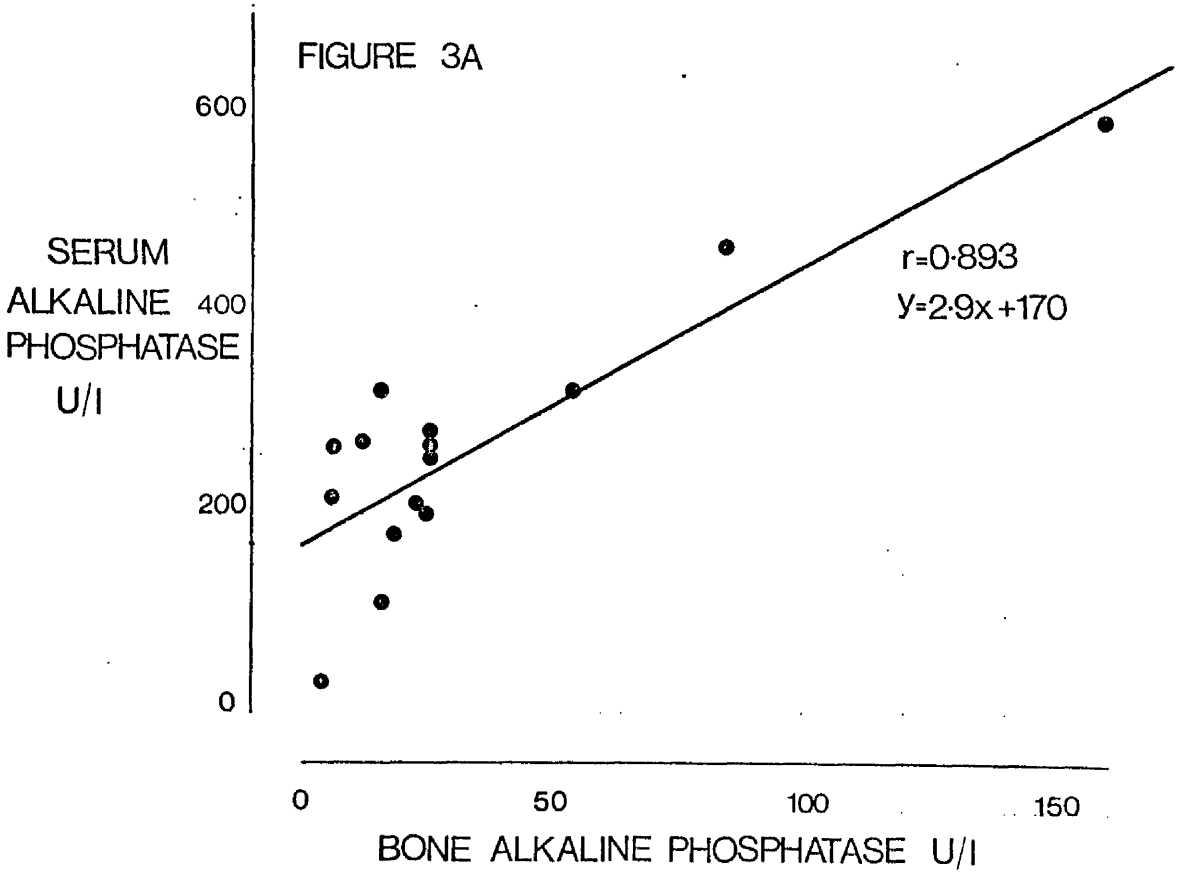
A.P. = Alkaline Phosphatase
1^{O} HYPERPARA = Primary Hyperparathyroidism
HYPOPHOSPHAT. = Hypophosphatasia
C.R.F. = Chronic Renal failure

FIGURE 3A

Results of serum alkaline phosphatase versus bone homogenate alkaline phosphatase, with line of regression $y = 2.9x + 170$.

Correlation co-efficient 0.893.

FIGURE 3A


600
400
200
0
SERUM
ALKALINE
PHOSPHATASE
U/l
r=0·893
y=2·9x+170
0
50
100
150
BONE ALKALINE PHOSPHATASE U/l

2. Using all of the paired data for serum AP levels and bone AP levels, the co-efficient of correlation (r) between these two parameters was found to be 0.893, showing a strong correlation between the level of AP in the serum and the AP level in bone.

Discussion:

It is generally assumed that changes in the level of serum AP reflect both pathological and physiological changes in those organs rich in the enzyme, namely liver and bone (Rasmussen, et al. 1974; Smith, 1979). Serum AP levels are used in the diagnosis of metabolic bone disease, but as the enzyme originates from other organs, apart from bone, it cannot accurately be assumed that changes in serum AP levels reflect those in bone (Rasmussen, et al. 1974). In addition to this, it has been found, in the case of creatine kinase discussed in the next chapter, that the circulating level of an enzyme may not reflect the level of enzyme in the organ of origin, the muscle, as in that particular case the levels of enzyme in the organ were so high that changes were difficult to detect. The purpose of this study was to clarify the relationship between serum AP levels and bone AP levels, to let us see whether or not serum AP accurately reflects metabolic bone disease.

Assays for AP were performed on human bone homogenates, using longitudinal slices of Meunier trephine biopsies, incorporating both trabecular and cortical bone; a measure of the enzyme present in each bone homogenate was obtained and compared with the level of AP in the corresponding serum. This comparison showed a correlation co-efficient of 0.893, indicating that even a measure of total serum AP clearly reflects the level of enzyme activity in bone and proving

that a measure of serum AP is a good indicator of the level of active calcification in bone. Alkaline phosphatase, it would seem, is produced by bone only when required, it is not continuously abundant, as is creatine kinase, which suggests a specialised role, probably related to calcification.

CHAPTER FOUR. COLLAGEN PROLYL HYDROXYLASE ACTIVITY IN HUMAN BONE

In the past the biosynthesis of collagen has been studied with particular reference to the occurrence of a proline-rich precursor, which is converted by hydroxylation to collagen; the enzyme involved in this hydroxylation is collagen prolyl hydroxylase. This enzyme was first demonstrated in chick embryo microsomal fractions (Peterkopsky and Udenfriend, 1962), with activity also being detected in foetal rat skin, adult rat liver and guinea pig granuloma (Stone and Meister, 1962; Hutton, et al. 1966). A rapid assay for collagen prolyl hydroxylase activity was developed by Hutton, et al (1966) based on the measurement of tritiated water formed when 3,4-^{3}H-L-proline {^{3}H = tritium} in specially prepared substrate is converted to 3-^{3}H-L-hydroxyproline. This assay has been found to be suitable for crude enzyme preparations, as will be used here.

Proline constitutes 20% of all amino acids in collagen and any failure in proline hydroxylation leads to the failure to form the triple helix structure which is so characteristic of collagen. Steps involved in the hydroxylation of proline to hydroxyproline are illustrated in FIGURES 4A, 4B. Various groups, particularly Prockop, et al. (1963, 1964), Juva and Prockop (1964, 1966) and Meister, et al. (1964) have shown that this reaction involves the loss of only one tritium atom, the release of this atom and its assessment giving

FIGURE 4A

The proposed mechanism for the coupled oxygenation of hydroxyproline and α-ketoglutanate by collagen prolyl hydroxylase.

FIGURE 4A

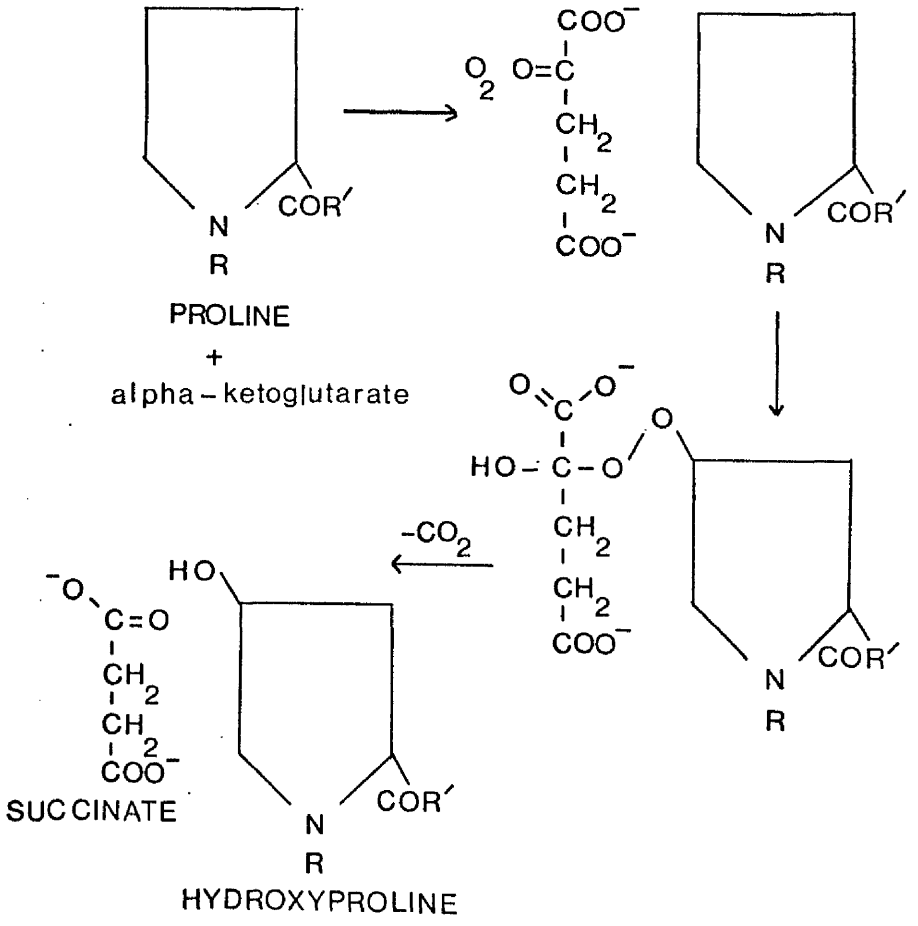
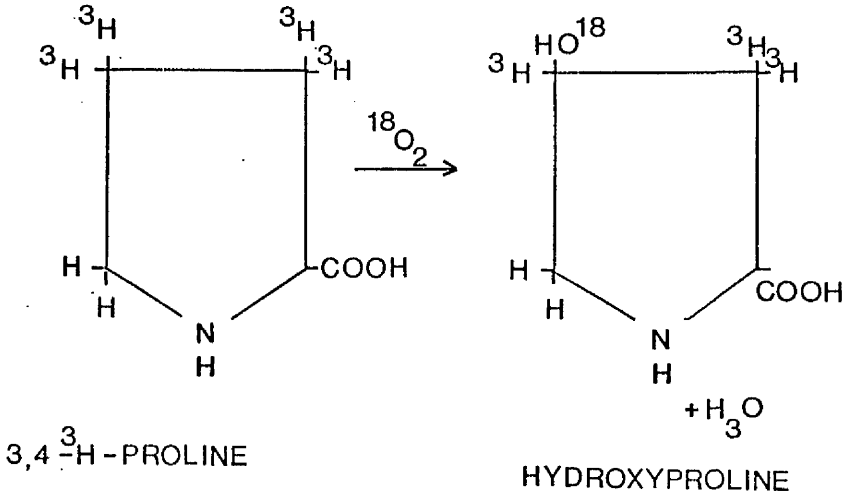


FIGURE 4B

The hydroxylation of tritium-labelled proline to hydroxyproline, with the liberation of tritiated water. This also incorporates the inclusion of molecular oxygen as the reaction proceeds.

FIGURE 4B



3,4 ^{3}H - PROLINE

HYDROXYPROLINE

an accurate measure of enzyme activity, while Fujimoto and Tamiya (1962), using molecular $^{18}O_2$, have demonstrated that the reaction cannot occur in anaerobic conditions.

Hutton, et al. (1966) have shown that α-ketoglutarate is an essential co-factor, but its function as a reactant or as a catalyst in the hydroxylation of proline is not clear, since 50% of it disappears during the reaction (Hutton, et al. 1967). Work by Flanagan and Nichols (1962) had already suggested that intermediates in glycolysis may play some part in proline hydroxylation. Ascorbic acid and iron, in the Fe^{2+} ionic form, are also essential to the reaction: ascorbic acid acting as a reducing agent, which can be replaced by tetrahydrop teridine (Peterkofsky and Udenfriend, 1965), Fe^{2+} is unique in its role as an electron-transferring agent (Hutton, et al. 1967), although it can be chelated by α, α'-dipyridyl (Hurych and Chvapil, 1965). All of these important factors and co-factors reflect the complexity of the enzyme reaction involved in the hydroxylation of proline and our understanding is reflected in the accuracy of the assay itself.

The incorporation of labelled proline and the formation of labelled hydroxyproline are found to be unaffected in the presence of unlabelled hydroxyproline. Granuloma minces and cell-free supernatants from chick embryo homogenates are unable to use free hydroxyproline for incorporation into collagen (Hutton, et al. 1966), although Mitamo, et al (1959) claimed some success in incorporating hydroxyproline

directly into collagen. The former, however, is generally held to be the case and the presence of labelled hydroxyproline indicates collagen synthesis.

Collagen is one of the main structural components of bone and as such any changes in bone metabolism, such as are found in osteoporosis, osteomalacia, or renal osteodystrophy, for example, may be reflected in changes in collagen metabolism. In this study, using a method for assay of collagen prolyl hydroxylase activity based on that developed by Hutton, et al. (1966), human bone homogenates have been examined to assess any changes in collagen metabolism with different metabolic bone diseases.

Materials and Method:

1. Bone from Meunier trephine biopsies of the iliac crest was frozen immediately after removal from the patient and stored deep-frozen until required. Shortly before the assay was due to be performed, bone was cut on a Cryostat, a microtome contained in a -20°C refrigerated cabinet, to slices 12 µm in thickness. These sections were stored deep-frozen until required for homogenisation.
2. To prepare the substrate, 14 dozen six-day-old chick embryos were decapitated and the bodies placed in buffer, with constant gassing with 5% CO_2 in air. Hydroxylation of proline depends on molecular oxygen and is prevented by imposing anaerobic conditions (Hutton, et al. 1967), preventing spontaneous formation

of hydroxyproline in the substrate. The bodies were washed three times, using 200 ml of buffer each time, and once the buffer was clear it was decanted-off. The tissue was then divided into 5g aliquots and added to 100 ml beakers along with 5 ml of buffer and 0.1 ml of 0.1 Molar α, α'-dipyridyl in absolute alcohol, to inhibit collagen formation by inhibiting Fe^{2+} (Hurych and Chvapil, 1965). This mixture was incubated at $37^{o}C$, with constant gassing with 5% CO_2 in air and constant shaking, for 20 minutes. Following incubation ½ mCi H_30 was added to each of the beakers, which were then incubated under the same conditions as before for a further two hours. This two hour incubation was followed by spinning at 15,000 r.p.m. on a Superspeed 50 centrifuge for 20 minutes, after which the supernatant was removed and discarded. Each pellet formed was extracted with 10 ml of 0.5 Molar CH_3COOH overnight, forming extract I, which was further centrifuged at 15,000 r.p.m. for another 20 minutes. The supernatant obtained from this last centrifugation was placed in a dialysis bag and dialysed against 5 litres of chilled distilled water for approximately 3 hours, then two changes of 1 litre of 0.01 Molar tris buffer, pH 7.5 . The pellet produced by this extraction was also dialysed with 10 ml of 0.5 Molar CH_3 COOH, forming extract 2; extract I was further dialysed against tris buffer, with both extracts forming the enzyme substrate.

3. Buffer for substrate preparation:

0.3 Molar KCl	10 ml
1.2 Molar $MgSO_4$ $7H_2O$	1 ml
1.22 Molar NaCl	100 ml
1.3 Molar $CaCl_2$ $6H_2O$	1 ml
0.4 Molar KH_2PO_4	1 ml
0.25 Molar $NaHCO_3$	100 ml

D-glucose to 0.01 Molar
Distilled water up to 1 litre.

4. Buffer X for preparation of bone homogenates:

0.5 Molar tris buffer, pH7.2	10 ml
10^{-3}Molar E.D.T.A.	1 ml

0.25 Molar Sucrose up to 90 ml.
Before use add 1/10 volume of 10^{-2}Molar dithiothreitol.

5. Bone homogenates were prepared from 0.3 g of frozen bone slices, added to 5 ml of Buffer X plus 1/10 volume of Dithiothreitol, and homogenised at setting 5 on a TRI-R STIR-R homogeniser {Model S63C. TRI-R Instruments, Rockville Centre, New York, USA} for 5 minutes. This homogenate, kept on ice throughout its preparation, was allowed to settle before use.

6. The reaction mix for each assay tube was made as follows:

0.5 Molar Tris buffer, pH 7.2.	0.1 ml
10^{-2}Molar Ferrous Ammonium Sulphate (ANALAR GRADE)	0.1 ml
50 mMolar Na Ascorbate	0.1 ml
1% Bovine Serum Albumin	0.2 ml
Catalase	0.02 ml
10^{-2}Molar α-ketoglutaric acid	0.1 ml
10^{-2}Molar Dithiothreitol	0.01 ml
Distilled water	0.07 ml.

The total volume of this reaction mix is 0.7 ml and the amount required is obtained by multiplying all constituents by the number of samples used in the assay.

7. The contents of each reaction tube are as follows:

Reaction mix	0.7 ml
Substrate	0.05 ml
Homogenate	as specified.

Distilled water making homogenate up to 300 µl, with 300 µl of distilled water, only, forming the experimental blank.

8. All reaction tubes were sealed and incubated at 30°C for 30 minutes, after which the reaction was stopped by the addition of 0.1 ml of 50% Tricarboxylic Acid, to precipitate the protein.

9. The tritium-labelled water produced by the reaction was was obtained by standard distillation methods.

10. Assessment of the tritium-content of the water was by means of adding 0.8 ml of the distilled water to 10 ml of Aqua Luma scintillation cocktail and counting on a β-scintillation counter. By subtracting the counts per minute (C.P.M) produced by the experimental blank from that produced in each experimental tube containing bone homogenate, a measure of the collagen Prolyl hydroxylase activity in each sample was obtained.

Results:

A (i) Results obtained when homogenates of bone from patients suffering from osteoporosis were assayed for collagen prolyl hydroxylase activity. In each case the volume of bone homogenate assayed is given and the C.P.M./ml produced.

TABLE 4A (i)

PATIENT	VOL. OF BONE HOMOGENATE IN ASSAY (µl)	C.P.M./ml
A.K.	300	620
	150	1360
E.R.	300	1023
	150	2753
J.M.	300	863
	150	1513
	100	1986
	50	10820
E.D.	300	896
	150	2440
	100	2550
	50	6920
T.W.	300	763
	100	710
	50	1580

A (ii) Mean values and standard deviations, for the levels of collagen prolyl hydroxylase activity produced by homogenates of bone from patients with osteoporosis, and the volume of homogenate present in each assay.

TABLE 4A (ii)

VOL. OF BONE HOMOGENATE IN ASSAY (µl)	$\bar{x} \pm S$ (C.P.M)
300	833 ± 151
150	2016 ± 685
100	1748 ± 943
50	6440 ± 4639

B (i). Results obtained when homogenates of bone from patients suffering from renal osteodystrophy were assayed for collagen prolyl hydroxylase activity. In each case the volume of bone homogenate assayed is given and the C.P.M./ml produced.

TABLE 4B (i)

PATIENT	VOL. OF BONE HOMOGENATE IN ASSAY (µl)	C.P.M/ml
M.H.	300	1836
	150	2666
	100	6710
	50	6620
M.R.	300	3393
	150	1246
	100	3610
	50	-
G.R.	300	1120
	150	3327
	100	5240
	50	8360
J.B.	300	210
	150	366
	100	420
	50	1900

B (ii) Mean values and standard deviations, for the level of collagen prolyl hydroxylase activity produced by homogenates of bone from patients with renal osteodystrophy, and the volume of homogenate present in each assay.

TABLE 4B (ii)

VOL. OF BONE HOMOGENATE IN ASSAY (µl)	$\bar{x} \pm S$ (C.P.M)
300	1640 ± 1345
150	1901 ± 1342
100	3995 ± 2699
50	5627 ± 3343

C. Results obtained when homogenates of bone from a patient suffering from osteomalacia were assayed for collagen prolyl hydroxylase activity. In each case the volume of bone homogenate assayed is given and the C.P.M./ml produced.

TABLE 4C

PATIENT	VOL OF BONE HOMOGENATE IN ASSAY (µl)	C.P.M./ml
J.S.	300	1200
	150	1633

D. Results obtained when homogenates of bone from a patient suffering from primary hyperparathyroidism were assayed for collagen prolyl hydroxylase activity. In each case the volume of bone homogenate assayed is given and the C.P.M./ml produced.

TABLE 4D

PATIENT	VOL. OF BONE HOMOGENATE IN ASSAY (µl)	C.P.M./ml
M.D.	300	413
	150	626
	100	1380
	50	2800

E. Results obtained when the C.P.M./ml for all patients suffering from metabolic bone disease studied were averaged; the volume of bone homogenate in each case is also given.

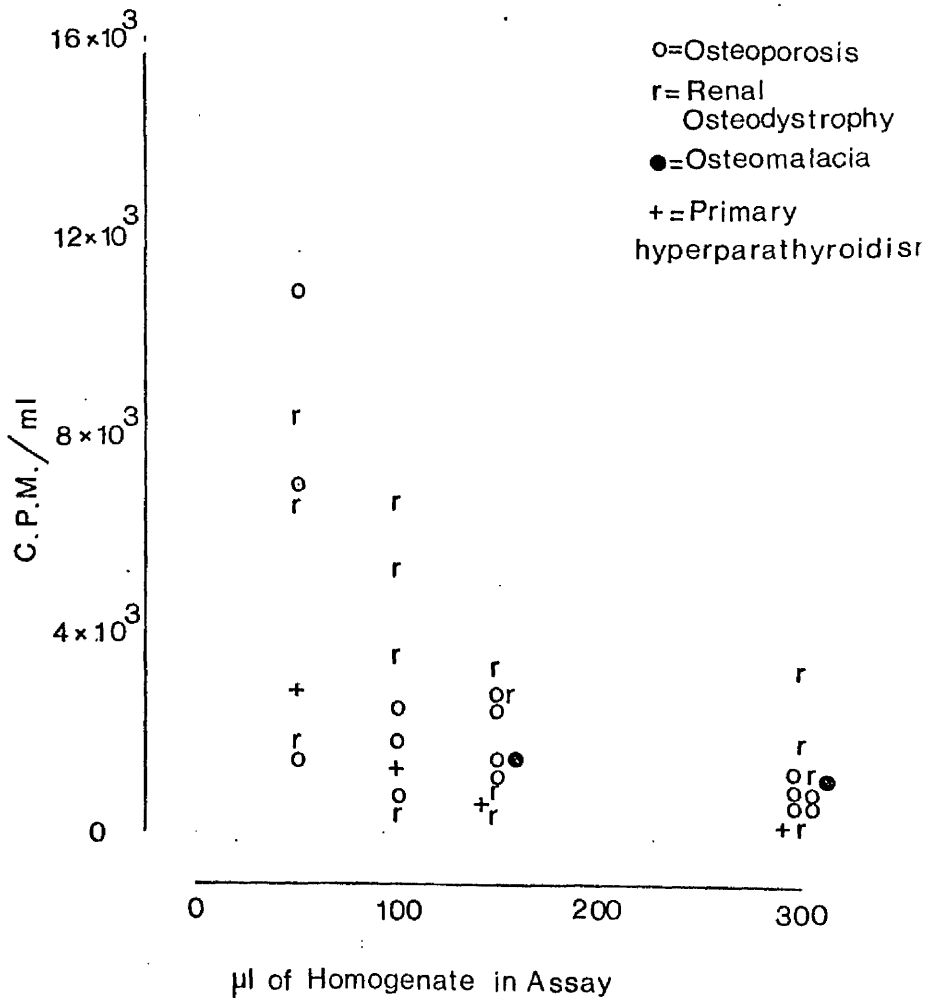
TABLE 4E

VOL. OF BONE HOMOGENATE IN ASSAY (µl)	$\bar{x} \pm S$ (C.P.M)
300	1121 ± 867
150	1793 ± 969
100	2826 ± 2225
50	5371 ± 3543

FIGURE 4C

Results illustrating the level of collagen prolyl hydroxylase activity, in C.P.M./ml, found with each bone disorder, using different volumes of bone homogenate. The level of activity would seem to increase with increasing dilution of homogenate.

FIGURE 4C


16×10³
12×10³
8×10³
4×10³
0
C.P.M./ml
o=Osteoporosis
r=Renal Osteodystrophy
●=Osteomalacia
+=Primary hyperparathyroidism
0
100
200
300
µl of Homogenate in Assay

Discussion:

In this study of the enzyme collagen prolyl hydroxylase it was hoped that the level of activity of the enzyme could be assessed in homogenates of bone from patients suffering from metabolic bone disease. However, due to circumstances beyond the control of the author, it was not possible to continue using this assay beyond initial exploratory experiments. Those results which were obtained serve only to show that collagen prolyl hydroxylase activity can be detected in human bone homogenates, but it was not possible to proceed with enzyme kinetic studies to find the maximum activity (V_{max}) for the enzyme for different patients. The one interesting point to note is that, from those results obtained here, the activity of the enzyme seems to increase as the bone homogenate becomes more dilute. Hutton, et al. (1966) found, using pure enzyme preparations, that the activity produced, in C.P.M. increased as the amount of enzyme increased; it could be that there is some kind of non-competitive inhibitor of the enzyme present in the bone homogenate which is gradually diluted as the homogenate becomes more dilute, but no conclusions can be made without further experiment.

CHAPTER FIVE. CREATINE KINASE IN MUSCLE FROM PATIENTS SUFFERING FROM METABOLIC BONE DISEASE

All forms of human muscle: smooth muscle, cardiac muscle and striated muscle, contain abundant stores of the high-energy phosphate compound creatine phosphate (Conn and Stumpf, 1967; Yudkin and Offord,1971; Wood, 1975). This compound plays an important role in muscle contraction, during which it is degraded to creatine and inorganic phosphate, providing energy for the formation of another high-energy compound: adenosine triphosphate (ATP). The enzyme which catalyses the breakdown of creatine phosphate to form ATP is creatine kinase.

Creatine kinase is found in decreasing order of activity in the following human organs: skeletal muscle, cardiac muscle, cerebral cortex, smooth muscle, thyroid gland, kidney and liver (Forster, et al. 1974). It is plentiful in tissues which require a lot of energy for their efficient functioning. Normal human serum has no, or very little, creatine kinase activity (Wilson, 1965), however the serum level can rise with such conditions as hypothyroidism, severe physical exercise, muscular trauma, epileptic fits and primary hyperparathyroidism. Forster (1967) found that a rise in serum creatine kinase also occurred with progressive muscular dystrophy and coma, where muscle is inactive and muscle weakness is a prominent feature.

As it was seen that tissue creatine kinase levels are low in tissues which require less energy and serum creatine kinase levels rise under similar conditions, creatine kinase levels in tissue from metabolic bone diseases were studied. Many metabolic bone diseases cause such pain and disfigurement that movement becomes difficult and patients become less active or even totally immobile.

As Goto (1974) has indicated that creatine kinase increases in serum originate from skeletal muscle, it was decided to compare levels of creatine kinase in muscle homogenates, prepared according to Oliver (1955), of skeletal muscle of patients suffering from metabolic bone disease. As well as studying muscle creatine kinase levels in relation to metabolic bone disease, sex-differences were also studied. Wiesmann, et al. (1966) have previously shown that a higher serum creatine kinase level can be found in male serum than in female serum. Further studies, by Smith, et al. (1979) and Thomson and Smith (1980), have shown that pregnancy is accompanied by an increase in female serum creatine kinase levels, suggesting a strong link, possibly inhibition of creatine kinase, by oestrogen, which falls during pregnancy. Variations of muscle creatine kinase levels with age were also studied.

Materials and Methods:

1. Some striated muscle fragments from the vastus lateralis were found attached to through and through Meunier trephine biopsies of the anterior iliac crest. These fragments were frozen immediately after removal from the patient. Shortly before each assay was to be performed, the frozen muscle was homogenised in 6 volumes of 0.1 molar potassium chloride solution (KCl) at 6000 r.p.m. on a TRI-R STIR-R homogeniser (Model S63C. TRI-R Instruments, Rockville Centre, New York) for 5 minutes on ice.

2. Lowry protein estimations were made by means of the standard method laid down by Lowry, et al. (1951). A standard graph was prepared using varying concentrations of bovine serum albumin (BSA) (FIGURE 5A), from which the protein concentration of the muscle homogenates could be assessed. For every muscle sample studied, the following dilutions of muscle homogenate in distilled water were prepared.

   25×10^3 p.p.m. in 1 ml.
   50×10^3 p.p.m. in 1 ml.
   100×10^3 p.p.m. in 1 ml.
   200×10^3 p.p.m. in 1 ml.
   1 ml. of distilled water. (p.p.m = parts per million).

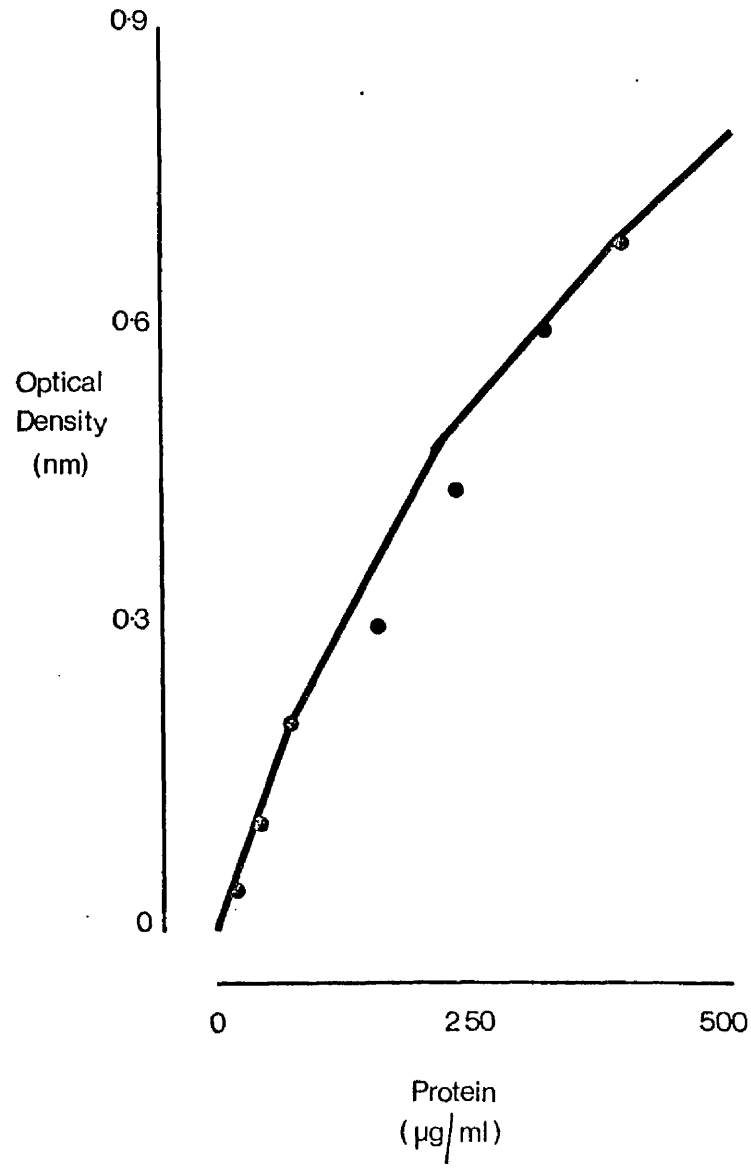
   In the final analysis, only one concentration of muscle homogenate was used, that of 25×10^3 p.p.m.

3. Creatine kinase estimation. The following dilutions of muscle homogenate were prepared from each muscle biopsy, using distilled water:

FIGURE 5A

Standard curve of protein concentration versus optical density, used in the calculation of protein concentration following the Lowry test.

FIGURE 5A



2.5×10^3 p.p.m. in 1 ml.

5.0×10^3 p.p.m. in 1 ml.

10.0×10^3 p.p.m. in 1 ml.

20.0×10^3 p.p.m. in 1 ml.

1 ml of distilled water.

Smaller concentrations of muscle homogenate were used in the creatine kinase analysis, as initial studies showed concentrations similar to those used in the Lowry protein estimation were too high to allow the level of creatine kinase to be calculated accurately.

The reaction mechanism for creatine kinase analysis is outlined in FIGURE 5B. The method for assay is based on that developed by Oliver (1955) and ultimately results in the measurement of NADPH, another high-energy molecule, but is performed using a Spinchem kit on an LKB autoanalyser. The Spinchem kit uses N-acetyl cysteine (NAC) as an activator, a modification of Oliver's method, which used glutathione as an activator. This method was developed for measuring serum creatine kinase, but has been found to be successful in measuring creatine kinase levels in muscle homogenates.

Each creatine kinase result was determined empirically and did not involve the calculation of a standard curve. All appropriate quality control methods were used. Only one concentration of muscle homogenate was used in the final analysis, that of 2.5×10^3 p.p.m.

FIGURE 5B

The mechanism involved in the assay of creatine kinase.

(a) To enable this reaction to be measured on the LKB 8600 auto-analyser, it is linked to two further reactions. The ATP formed in this reaction reacts with glucose.

(b) ATP formed in the previous reaction used in the conversion of glucose to glucose-6-phosphate by hexokinase.

(c) The glucose formed in the previous reaction acts as a substrate for glucose-6-dehydrogenase.

FIGURE 5B

(A)

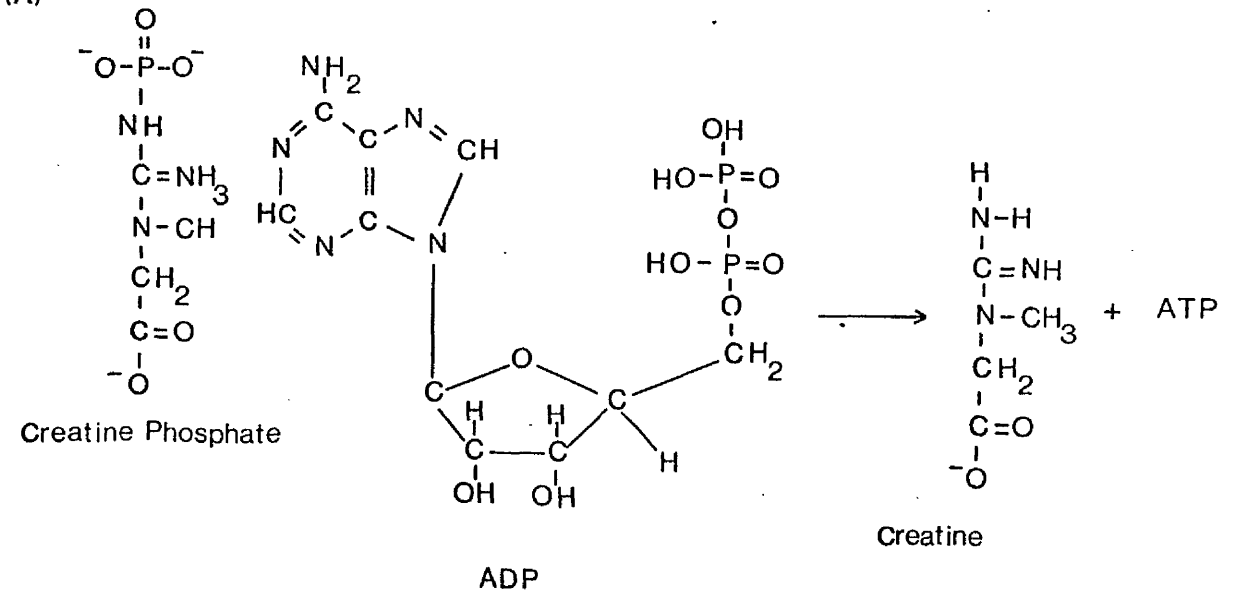


FIGURE 5B

(B)

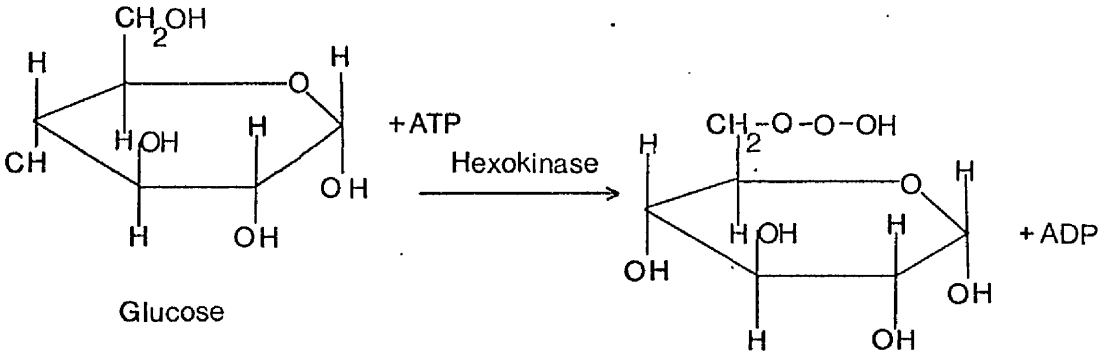
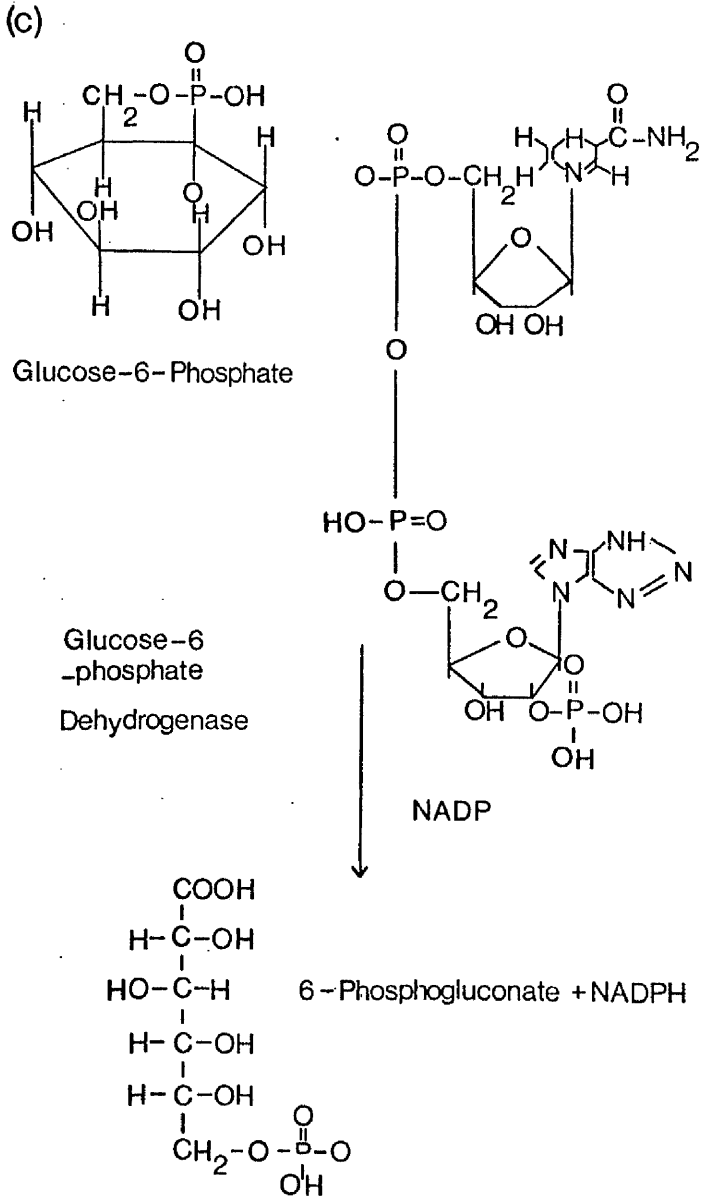

CH2OH
H
O
H
H OH
H
CH
OH
H
OH
Glucose
+ATP
Hexokinase
CH2-O-O-OH
H
O
H
H OH
H
OH
OH
H
OH
+ADP
Glucose-6-phosphate

FIGURE 5B


(c)
Glucose-6-Phosphate
Glucose-6
-phosphate
Dehydrogenase
NADP
6-Phosphogluconate +NADPH

Results:

1. As a result of the Lowry protein estimations it was possible to obtain a measure of the protein present in each muscle homogenate in µg protein per ml of homogenate. From the creatine kinase assays, a result in ng creatine kinase per litre of homogenate was obtained. From these two results a measure of the amount of creatine kinase, in ng. per mg of protein was calculated and all results are shown in TABLE 5A.

N.B. C.K = Creatine kinase.
1^{O} HYPERPARA. = Primary hyperparathyroidism
R.O. = Renal osteodystrophy.

TABLE 5A

PATIENT, DISORDER, AGE AND SEX		µg PROTEIN/ml ($\times 10^5$)	ng CK/L ($\times 10^4$)	ng CK/mg PROTEIN
OSTEOPOROSIS	M.J.(F)(62)	2.6	7.9	0.3
	M.M.(F)(53)	1.5	7.8	0.52
	T.W.(M)(51)	1.9	13.4	0.68
	E.M.(F)(58)	2.3	5.8	0.25
	E.R.(F)	1.9	6.5	0.34
	E.D.(F)(64)	3.7	3.4	0.34
	I.E.(F)	1.0	6.0	0.16
	C.B.(F)(58)	1.9	1.2	0.06
	A.B.(F)(59)	1.5	2.6	0.17
	M.B.(F)(66)	2.9	3.7	0.13
	A.M.(F)(50)	4.0	3.6	0.09
	E.L.(F)(53)	0.8	5.7	0.08
	J.M.(M)(52)	0.9	3.0	0.35
	J.M.(F)(74)	1.1	6.8	0.62
	L.M.(F)	1.6	12.9	0.81
	M.H.(F)(81)	5.9	35.6	0.61
1^{o} HYPERPARA	M.D.(F)(66)	3.0	13.7	0.47
	J.R.(F)(72)	1.2	3.6	0.3
	J.C.(F)(65)	0.2	0.5	0.25
	A.A.(F)(55)	1.5	29.4	0.2
	M.J.(F)(69)	0.9	3.3	0.36
OSTEOMALACIA	I.K.(F)(48)	1.4	5.2	0.37
	J.D.(F)(30)	2.1	0.8	0.04
	M.G.(F)(19)	2.1	13.1	0.62
	J.B.(M)(54)	2.0	17.5	0.85
R.O.	E.M.(F)	2.0	8.8	0.44
	J.B.(M)(64)	2.7	11.8	0.44
	B.M.(M)	1.4	5.7	0.41
	R.K.(M)	2.5	2.8	0.11
	G.R.(M)(53)	3.5	29.9	0.86

2. Students t-tests comparing the muscle levels of ngCK/mg Protein in each disorder.

TABLE 5B.

DISORDERS COMPARED				P
OSTEOPOROSIS	0.34±0.24	1^{o} HYPERPARA-THYROIDISM	0.32±0.1	NSD
OSTEOPOROSIS	0.34±0.24	OSTEOMALACIA	0.47±0.35	NSD
OSTEOPOROSIS	0.34±0.24	C.R.F.	0.45±0.27	NSD
1^{o} HYPERPARA-THYROIDISM	0.32±0.1	OSTEOMALACIA	0.47±0.35	NSD
1^{o} HYPERPARA-THYROIDISM	0.32±0.1	C.R.F.	0.45±0.27	NSD
OSTEOMALACIA	0.47±0.35	C.R.F.	0.45±0.27	NSD

Results here show no significant difference in the levels of ng creatine kinase/mg protein in muscle for each of the different metabolic bone diseases studied.

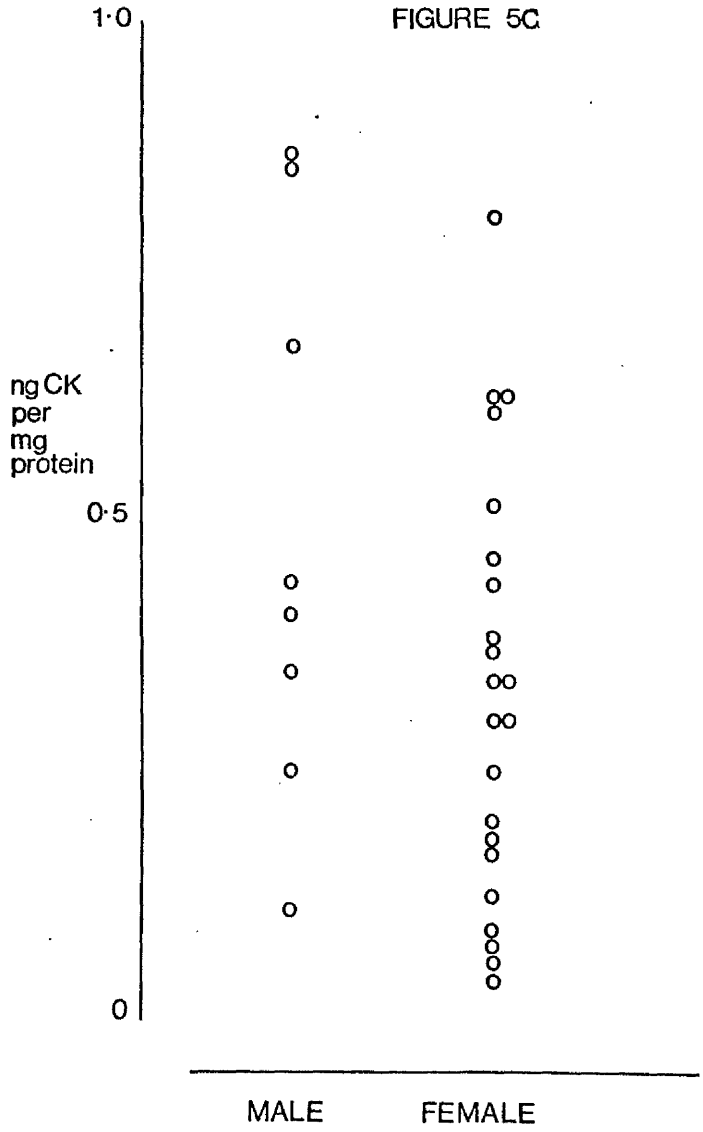
3. Comparison of levels of ng creatine kinase/mg protein in muscle from males and females.

The level of creatine kinase in striated muscle from male patients showed an average of 0.5 ± 0.275 ng creatine kinase/mg protein, while in female patients the level was 0.33 ± 0.21 ng creatine kinase/mg protein. FIGURE 5C shows the results grouped according to sex and the means are significantly different at $p < 0.05$.

FIGURE 5C

Results in ng creatine kinase per mg of muscle protein for each male and female patient studied.

FIGURE 5C



4. Correlation between the level of creatine kinase per mg of protein and the age of the patient studied.

For females, the correlation co-efficient (r) between levels of creatine kinase in striated muscle homogenates and age is 0.81, which is significant in the range -1 to +1 for all correlation co-efficients.

For males, the correlation co-efficient (r) between the levels of creatine kinase in striated muscle homogenates and age is 0.7, which is also significant in the range -1 to +1 for all correlation co-efficients.

Discussion:

Most of the creatine kinase in striated muscle is contained within the cell cytoplasm, with some in the mitochondria, therefore complete homogenisation of muscle is necessary to obtain a measure of the total creatine kinase present. The whole homogenate was used in this study, rather than the supernatant, but Oliver (1955) showed that either can be used to produce accurate results. Methodology is important in the determination of creatine kinase. Potassium chloride solution has been shown to be effective (Oliver, 1955) in retarding any inactivation of the enzyme through the action of an inhibitor, as demonstrated by Von Rotthauwe (1967).

Students t-tests performed between the four groups of patients in this study, each group suffering from a different metabolic bone disease, showed no significant difference in the levels of creatine kinase in homogenates of their striated muscle (TABLE 5B). Previous studies, looking at serum creatine kinase levels in different disorders, showed that they became raised with hypothyroidism, primary hyperparathyroidism (Wilson, 1965) and muscular dystrophy (Forster, 1967; Smith, 1979). However changes in creatine kinase levels are easier to detect in human serum, where there is normally little present (Wilson, 1965), than in human striated muscle, which is abundant in the enzyme. A large change in serum creatine kinase

would result from only a very small change in the level in the muscle, meaning that a result of no significant difference between levels of creatine kinase in muscle from any disorder would be expected.

However, comparisons made between the levels of creatine kinase in male and female patients showed that males have a higher striated muscle creatine kinase level than female; the difference is significant at $p < 0.05$. Wiesmann, et al. (1966) showed that adult males have a higher serum creatine kinase level than adult females, with Smith, et al. (1979) and Thomson and Smith (1980) finding that low levels of serum creatine kinase are linked with high levels of blood oestrogen. The comparison of creatine kinase levels in male and female muscle would seem to support these results and show that lower levels of serum creatine kinase in females reflects lower levels in the muscle itself.

Looking at any possible correlation between the level of muscle creatine kinase and age, it was found that a strong relationship existed with both males and females. The level of muscle creatine kinase would seem to increase with age, as one might expect through increased inactivity, as found with coma and muscular dystrophy (Forster, 1967). For female patients the correlation between age and muscle creatine kinase levels is stronger than that for males, which may reflect the dependence of serum creatine kinase levels on oestrogen levels, as oestrogen decreases with

age and becomes greatly reduced at the menopause and thereafter.

From this study of creatine kinase in striated muscle homogenates, we can see that the levels in muscle from males and females reflect those already found in their serum, while no relationship can be found between muscle creatine kinase levels and different metabolic bone diseases.

CHAPTER SIX. BONE CELL CULTURE

The various types of cells which can be found in bone have already been described in the introductory chapter, showing us the different role each plays in the formation and maintenance of normal bone. As with mouse calvaria providing a model system for looking at the effects of substances on bone as a whole, isolated bone cells in culture also provide the opportunity to test the effects of such substances on the different cells themselves. In 1964 Peck, et al. developed a method by which collagenase, an enzyme capable of degrading bone matrix, was used to isolate bone cells and that method has been used in many studies (Yu, et al. 1976; McPartlin et al. 1976; Peck, et al. 1976; Fast, et al. 1977; Felix, et al. 1978), although others have preferred to use the proteolytic enzyme trypsin (Binderman, et al. 1974; Harell, et al. 1976), making it fairly easy to isolate bone cells to study the many aspects of bone metabolism.

To show, initially, that the cells in culture are bone cells, various studies concerning calcification, matrix formation and the appearance of bone-related enzymes have been pursued. Collagen synthesis by osteoblast-like cells in culture has been demonstrated by Binderman, et al. (1974) and Scott, et al. (1980), while the formation of other bone matrix components has been noted by Martin, et al. (1971), among others. The most important sign of bone

formation is, of course, mineralisation and work by Rose, et al. (1964), Binderman, et al. (1974) Harell, et al. (1976) and Williams, et al. (1980) has shown that osteoblast-like cells, isolated in tissue culture, are very capable of forming bone.

The effects of various hormones and metabolites on bone metabolism have been studied looking at these isolated cells in culture (Peck, et al. 1964; Rosenbusch, et al. 1967; Park, et al. 1967; Binderman, et al. 1972; Peck, et al. 1973; Peck, et al. 1974; Peck, et al. 1976; Yu, et al. 1976; Miller, et al. 1976; Wong, et al. 1977; Luben, et al. 1977; Harell, et al. 1977; Fast, 1977; Burns, et al. 1978; Goldring, 1978; Smith, 1979; Wong, 1980), showing that bone cells in culture, like bone itself, respond to calcitonin, parathyroid hormone, vitamin D metabolites, oestrogen and prostaglandins, among others. Some interesting work by Howard, et al. (1981) has also suggested that osteoclasts in culture are capable of producing $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, in much the same way, they suspect, as osteoclasts of patients with chronic renal failure synthesise these metabolites.

In this particular study it is hoped that, by using bone cells isolated from mouse calvaria and human bone biopsies, using the method of Peck, et al. (1964), the effects of various bone-related substances, such as oestrogen, PTH, CT and fluoride, as well as human serum from patients with metabolic bone disease, on alkaline

phosphatase and Ca^{2+} levels in the culture medium and alkaline phosphatase within the cells themselves may be studied. In addition to this biochemical study it is also hoped to study any bone formation which may take place under the influence of these substances.

Materials and Methods:

1. Sterile $25cm^2$ tissue culture flasks (Falcon Plastics, Becton, Dickinson and Co. Cockeysville, M.D. USA) were prepared by coating the culture surface with a thin layer of sterile 0.1% gelatin solution. These flasks were then stored at $4^{o}C$.
2. Culture medium was prepared by the same means as described in previous chapters, using either Medium 199 or BGJb Original medium, with 15% foetal calf serum or 15% human serum, as specified.
3. Mouse cell cultures were prepared using calvaria removed from mice aged 5-21 days and human bone biopsies of the iliac crest provided the material for human cell culture. All bones used were washed carefully in Medium 199 solution immediately after removal to clean off any blood, then placed in sterile enzyme solution.

4. Enzyme solution was made according to Peck, et al. (1964), with collagenase as the main active component. For 10 calvaria or one iliac crest Meunier trephine biopsy:

   4.0 ml Tris-buffered saline (pH 7.4)

   Glucose to 5 μmoles/ml

   Penicillin-Streptomycin solution to 50 units/ml (Gibco Bio-Cult Ltd., Paisley)

   Crude collagenase to 0.1-6.0 mg/ml (Sigma).

5. The enzyme solution plus bone was then shaken 90 times per minute for 1.5 hours at 37°C on a rotary shaker (Luckham Ltd., Sussex), by which time there was virtual disappearance of calvaria and disintegration of the biopsies, forming a suspension.

6. The resultant cell suspension was then spun at 3000 r.p.m. for 5 minutes on a bench centrifuge, forming a pellet.

7. 1 ml of the supernatant formed was added to flasks each containing 9 ml of fresh culture medium.

8. In order to release more free cells from the pelleted digest, the pellet was resuspended in 5 ml of fresh culture medium and spun at 5000 r.p.m. for 5 minutes on a bench centrifuge. The supernatant was removed and the pellet resuspended; the supernatant was made into 1 ml aliquots and placed in culture as before.

9. The cell pellet was suspended and washed in fresh culture medium a total of three times, the final suspension being divided into 0.2 ml aliquots and added to prepared tissue culture flasks, each containing 9.8 ml of fresh culture medium.
10. All flasks were then gassed with 5% CO_2 in air and incubated at 37°C on a rotating tray (Luckham Ltd., Sussex), rotating 5 times per minute.
11. Cultures were observed at intervals under a Leitz-Diavert inverted objective phase contrast microscope.
12. Culture medium was changed at intervals of about one week, removing old medium by means of a sterile pipette and replacing with fresh medium. Old medium was assayed for alkaline phosphatase and Ca^{2+}.
13. Staining cells:

To fix cells for staining within the tissue culture flasks themselves, culture medium was removed and replaced by 80% alcohol. To fix cells for staining on histological slides, the cells were removed from the tissue culture flasks by trypsinisation and placed in specially-prepared petri dishes; these petri dishes contained gelatinised sterile coverslips and culture medium, incubation of the cells in these dishes would allow the cells to attach to the coverslips, which were then attached to histological slides, after fixation in the dishes with 80% alcohol. After fixation, all cells were dehydrated in two changes of absolute alcohol, two hours each, and cleared in toluene for 12-18 hours.

A. Cells were stained for alkaline phosphatase activity using Gomori's cobalt method, sites of activity were indicated by black cobalt deposits.

B. Standard haemalum and eosin staining was used to show cell nuclei and cytoplasm, which stained blue and pink, respectively.

C. Giemsa stain was used to show erythrocytes, eosinophilic granules, basophilic granules, neutrophilic granules cytoplasm and thrombocytes, pale red, brown, blue, violet, blue and blue, respectively.

14. Trypsinisation:

A sterile trypsin solution was made and stored at $4^{o}C$:

Trypsin	0.025%
EDTA Na_2	1 mM
Chick serum in PBS (Ca^{2+} and Mg^{2+} free)	1% $^{v}/u.$

To remove all attached cells, remove culture medium and replace with 5 ml of phosphate-buffered saline (PBS) wash over the cells and remove. Add 0.3 ml of trypsin solution to the flask and wash over the adherent cells, shaking the flask as you do so, removing any attached cells; this proceeds more quickly at $37^{o}C$ and should be observed under a microscope, in this case a Leitz-Diavert inverted objective phase contrast microscope.

When all cells are detached, add 10 ml of culture medium and resuspend.

15. Alkaline phosphatase assays on culture medium were performed using Boehringer-Mannheim diagnostic kits for total alkaline phosphatase.

16. Ca^{2+} was measured using a Nova 2 ionised calcium analyser (American Hospital Supplies Ltd.).

Results:

In initial mouse cell cultures, it was noted that after only six days the cells became strongly adherent to the tissue culture flasks, assuming elipsoid form, with large round nuclei. Changing culture medium produced no change in the condition of the cells, remaining adherent throughout; throughout the culture period the medium removed was assayed for alkaline phosphatase and Ca^{2+} (FIGURE 6A). After nine weeks of culture, two of the original flasks now contained a confluent monolayer of cells, originating from about 15 cells which had been seeded to the flasks; the density of cells in the flasks was similar to that in FIGURE 6B, similar to those described by Burks, et al. 1978).

FIGURE 6A

Results of alkaline phosphatase and Ca^{2+} assays performed on the culture medium supporting cells in culture.

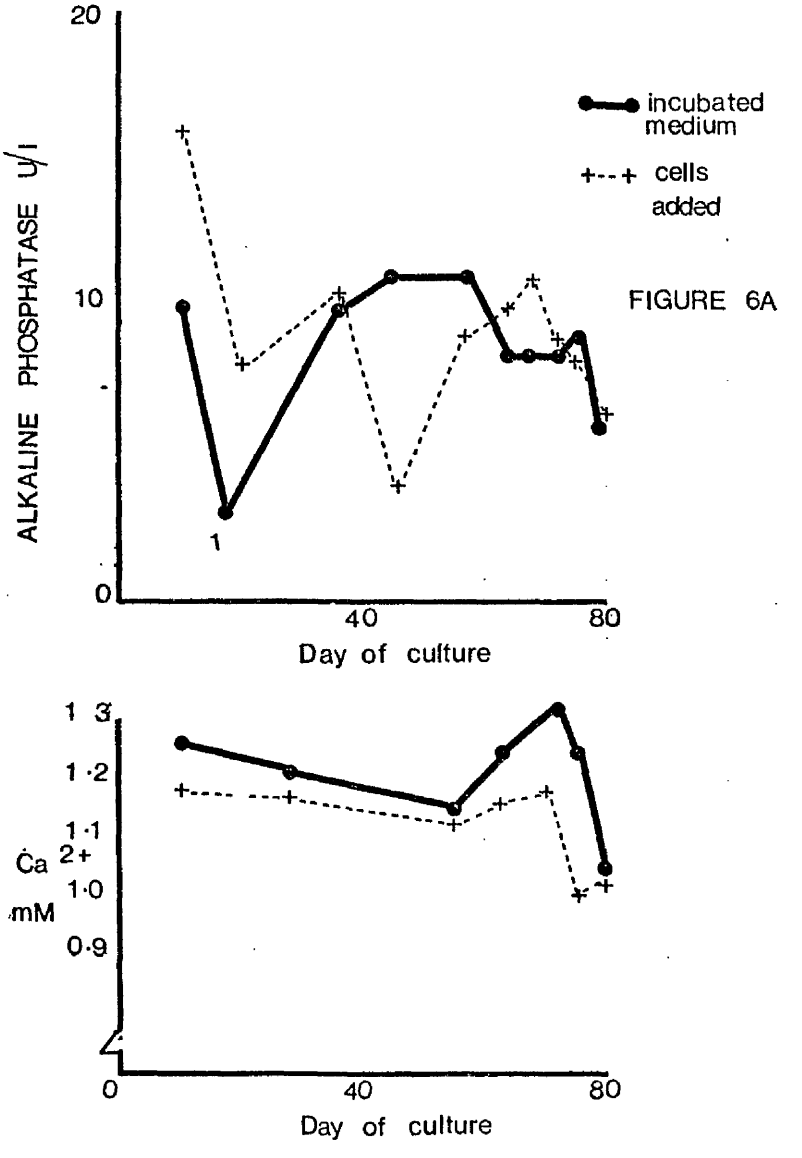

20
10
0
ALKALINE PHOSPHATASE U/l
incubated medium
+--+ cells added
1
40
80
Day of culture
1·3
1·2
1·1
1·0
0·9
Ca 2+ mM
0
40
80
Day of culture

FIGURE 6A

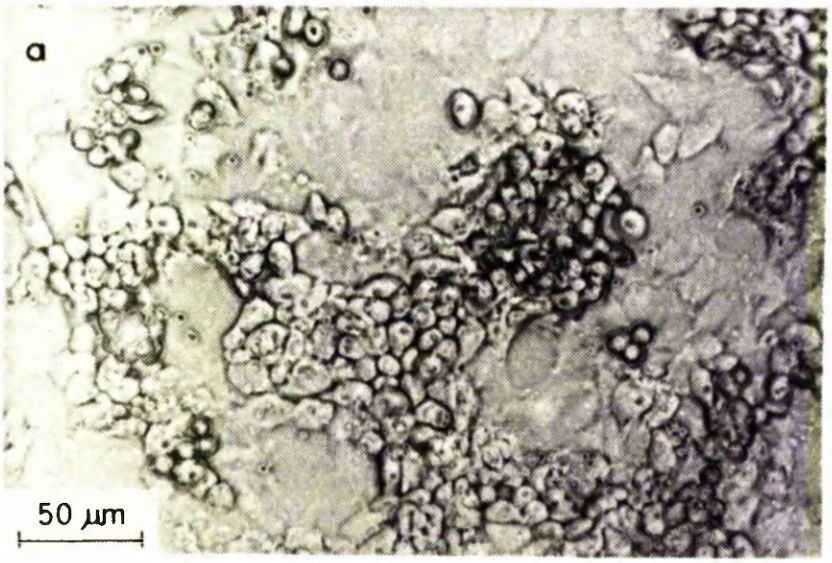
FIGURE 6B

Embryonic rat cells grown to confluence in vitro. (From Science, 199 (4328), 542-544, Burks J K, Peck W A. Permission has been given to use photograph.

FIGURE 6C

Embryonic rat calvaria cells grown in culture and stained for alkaline phosphatase activity, using Gomori's cobalt method. Sites of alkaline phosphatase activity are indicated by black cobalt deposits. (From Science, 199 (4328), 542-4. Burks J K, Peck W A). Permission has been given to use photograph.

Footnote: It was not possible to use the author's own photographs of alkaline phosphatase activity in neonatal mouse cells in culture as the negatives were lost, beyond the author's control.


a
50 µm


b
1 µm
10 µm

Sub-culturing, or passaging, was attempted on the confluent cell monolayers after the cells had been in culture for 11 weeks, using trypsinisation. It was found that cells successfully detached and entered suspension with fresh culture medium; new cell cultures were prepared by aliquoting this cell suspension into prepared tissue culture flasks, as before.

After 12 weeks in culture all cell cultures were fixed and stained for alkaline phosphatase, showing that some, but not all, cells in culture contained traces of the enzyme (FIGURE 6C). Results of alkaline phosphatase and Ca^{2+} assays performed on the culture medium supporting the cells are shown in FIGURE 6A, along with results for control culture medium which was incubated without cells. These results suggest that the cells in culture took up Ca^{2+} from the medium, as the level in medium incubated with cells lower than in that incubated without cells; alkaline phosphatase levels suggest no patterns, only a lot of fluctuation.

Further attempts at mouse cell culture were successful in as much as cells were obtained, which replicated very slowly, but they tended to die before confluence was obtained. These cells were stained for alkaline phosphatase activity, using Gomori's cobalt method, and it was generally found that about 30% of the cells exhibited alkaline phosphatase activity in their cytoplasm, perhaps indicating the presence of osteoblasts.

Initial attempts at preparing bone cell cultures from samples of human bone proved to be quite interesting. Bone obtained through the course of operations in the hospital's orthopaedic theatre was treated in the same way as mouse calvaria: placed in collagenase solution, incubated and shaken (Peck, et al. 1964), resulting in tissue culture flasks containing many spherical bodies of uniform site, 80% of which adhered within 48 hours. Fixation and staining of these bodies with haemalum and eosin showed that they were spheres of cytoplasm with no apparent nuclei. Further attempts at human bone cell culture produced similar results, but with, occasionally, adherent elongate cells with nuclei, which proved to stain positive for alkaline phosphatase.

A few subsequent cultures of human cells terminated by day 2, with all cells dying, prompting me to change the medium after day 1 to remove any possible lingering effects of enzyme solution. At the same time, the effects of shaking the bone in the enzyme solution for 0.5 hours, as opposed to 1.5 hours, were compared and showed that fewer cells were obtained on the shorter exposure to collagenase and that longer incubation with the enzyme solution does not affect the numbers or viability of the cells.

It became increasingly apparent that the spherical bodies of cytoplasm isolated in culture were probably erythrocytes. Previously there had been some doubt as to the nature of the bodies as they seemed to increase in

number while in culture, not a feature of erythrocytes; it is now thought that the other, elongate, cells were responsible for the increase in numbers and caused confusion by becoming detached and rounded during cell division. Attempts were made to stain the spherical bodies for the characteristic erythrocyte colour produced with Giemsa stain, but this proved ineffective as this stain was developed for blood smears and not erythrocytes alone. As an alternative the cells were suspended in culture medium and spun down on a centrifuge, producing a red pellet, indicative of erythrocytes.

Subsequent attempts at human bone cell culture involved repeated washing and re-spinning of the cell suspension to remove any erythrocytes, but few cells were isolated.

Discussion:

As a result of the inability to isolate and culture large numbers of bone cells, studies of bone cell by-products in culture medium were not possible; the results in FIGURE 6A represent assays performed on culture medium supporting relatively few cells, only reaching confluence after 9 weeks, of mixed character. Had the cell cultures produced greater cell numbers it may have been possible to separate all cell types: osteoblasts, osteoclasts and osteocytes, for study of their individual biochemical characteristics.

Human bone cell cultures also produced too few bone cells, but an abundance of erythrocytes. Erythrocytes in culture did not pose a problem as far as mouse cell cultures were concerned, as the blood supply to the wafer-thin calvaria was minimal, compared to the rich blood supply to human trabecular bone, but proved to be a regular hazard in the preparation of human cell cultures. Another problem in trying to isolate bone cells from human bone is related to the density of the bone: most bone supplied from the orthopaedic theatre was in the form of chips of cortical bone, too dense to allow easy access to any collagenase solution, making the isolation of cells through the breakdown of bone very difficult. It should be realised that there is a thin line between the concentration of an enzyme solution which will digest bone matrix and an enzyme solution which will also digest bone cells, this

happy medium was not, unfortunately, found in this study and culture of bone cells was unsuccessful.

If it had been possible to successfully culture large amounts of human bone cells, it was hoped that bone may have formed in 3-dimensional culture, using Cytodex tissue culture beads. These beads would have provided an array similar to that found within bone itself, where cells are separated by matrix and sit within small cavities or lacunae, or on the outside layers of the 'trabecular' bone, involved in bone turnover. This, however, was not possible, but it would have allowed us to see how various substances related to bone metabolism, as well as serum from patients with various metabolic bone diseases, have their effects on bone.

CHAPTER SEVEN. A STUDY OF THE EFFECTS OF ALUMINIUM ON ALKALINE PHOSPHATASE AND IONISED CALCIUM LEVELS IN CULTURE MEDIUM SUPPORTING NEONATAL MOUSE CALVARIA

Studies by several other groups have suggested that aluminium may play some role in influencing calcium and phosphorus metabolism. Initial and more recent studies (Fauley, et al. 1941; Freeman, et al. 1941; Kirsner, et al. 1943; Lotz, et al. 1968; Spencer, et al. 1978) have noted that excessive or prolonged use of aluminium-containing antacids in the management of peptic ulcers resulted in the formation of insoluble phosphate complexes within the intestine, resulting in inhibition of phosphate absorption within the gut, decreased urinary phosphate and increased urinary calcium. Spencer, et al. (1978) showed that, while the administration of aluminium-containing antacids increased urinary and faecal calcium, intestinal absorption of calcium, as determined by an oral ^{47}Ca method, remained unchanged. This, along with results from Baylink, et al. (1971), suggested that calcium loss during the phosphate depletion would be through increased bone resorption.

More recently, attention has again been focused on aluminium, but with particular reference to the high levels found in the brain and bone of patients on regular haemo-dialysis therapy for chronic renal failure. These high aluminium concentrations may be associated with dialysis dementia, a mental disorder and dialysis osteomalacia

(Bloom, et al. 1960; Berlyne, et al. 1970; Stanbury, 1972; Platts, et al. 1973; Alfrey, et al. 1976; Malluche, et al. 1976; Flendrig, et al. 1976; Platts, et al, 1977; Elliot, et al. 1978; McDermott, et al. 1978; Rozas, et al. 1978; Dunea, et al. 1978; Ellis, et al. 1979; Parkinson, et al. 1979; Wing, et al. 1980; Shore, et al. 1980; Boyce, et al. 1981; Prior, et al. 1982). The source of the increased aluminium levels found in these patients is thought to be the dialysate water used in artificial kidney machines, with the incidence of osteomalacia and dialysis dementia increasing in those areas where the local water has a higher than average aluminium content (Platts, et al. 1977; Drueke, 1980; Boyce, et al. 1981); oral phosphate-binding gels have also been implicated (Alfrey, et al. 1978; Boyce, et al. 1981).

Ellis, et al. (1979) studied the relationship between aluminium and osteomalacia. Taking samples of bone from the iliac crests of patients suffering from chronic renal failure, they studied their aluminium content and compared them with levels found in equivalent bone samples from normal patients. Results showed that while on regular dialysis the aluminium content of the bone had significantly increased. In a further animal study, they administered intraperitoneal injections of aluminium chloride to rats for periods of up to 3 months, finding that osteomalacia developed with increasing concentrations of aluminium, disappearing eventually after treatment had ceased, which

reflected results found in the patient studies. Le Fevre, et al (1980) also found evidence that aluminium became incorporated into the bone.

Boyce, et al. (1981) found that once the concentration of aluminium within bone rises above a certain level it inhibits mineralisation, but not bone matrix formation, i.e. osteomalacia develops where calcification is inhibited. It is thought likely that aluminium acts by inhibiting one or more of the enzymes concerned with mineralisation, probably involving alkaline phosphatase. This latest work has formed the basis for this study. Already within this thesis attempts have been made to try to elucidate the relationship between alkaline phosphatase and bone calcification and by studying the effect of aluminium on this system it is hoped to clarify the relationship further.

Materials and Methods:

1. Neonatal mouse calvaria cultures were prepared in the same manner as described in previous chapters, using Medium 199 supplemented with 15% foetal calf serum and glutamine.
2. Aluminium chloride solution, sterilised using an 0.22 μm filter (Falcon Plastics; Becton, Dickinson and Co.), was added to various cultures to concentrations ranging from 0.031 to 7.44 mMoles. The volume of aluminium chloride solution added was kept constant at 0.1 ml, making a total volume of 0.6 ml in each well of each tissue culture dish. Control cultures were also prepared, with no added aluminium in the culture medium.
3. Culture medium was removed and replaced with fresh medium of the same type on days 1, 3, 5 and 7.
4. On each occasion when the culture medium was removed it was assayed either for alkaline phosphatase content, using a Boehringer-Mannheim diagnostic kit for total alkaline phosphatase (Boehringer Diagnostics), or for Ca^{2+}, using a Nova 2 ionised calcium analyser (American Hospital Supplies), as specified.
5. In addition to control cultures containing no added aluminium chloride in their culture medium, other frozen-dead control calvaria were placed in culture and treated as normal.

6. As in a previous chapter, mice were prelabelled with ^{45}Ca by subcutaneous injection at 2 days old and placed in culture with medium supplemented with ^{47}Ca. In addition to the ^{45}Ca and ^{47}Ca, in this case, aluminium chloride solution was added to the culture medium at a concentration of 0.372 mM, to test the effect of aluminium on calcium exchange by bone in culture. Control cultures, where no aluminium was added to the culture medium, were also prepared. Medium was replenished each day, with the medium removed from culture being assessed for ^{45}Ca and ^{47}Ca activity in the usual way.

Results:

1. Alkaline phosphatase levels in culture medium supporting neonatal mouse calvaria, in the presence of aluminium chloride.

TABLE 7A

mM Aluminium in culture			U/L Alkaline Phosphatase DAY 1	DAY 3	DAY 5	DAY 7
0.000	LIVE	n	4	4	4	4
		$\bar{x}$	113	60	37	13
		S	25	25	11	6
	DEAD	n	2	2	2	2
		$\bar{x}$	247	135	49	7
		S	140	42	11	6
0.031	LIVE	n	4	3	3	3
		$\bar{x}$	92	26	18	10
		S	71	9	5	1
	DEAD	n	3	3	3	3
		$\bar{x}$	330	108	25	16
		S	87	63	18	15
0.062	LIVE	n	4	4	4	4
		$\bar{x}$	78	39	10	12
		S	20	18	4	5
	DEAD	n	3	3	3	3
		$\bar{x}$	350	153	38	9
		S	135	77	30	0

TABLE 7A (continued) ...

mM Aluminium in culture			U/L Alkaline Phosphatase DAY 1	DAY 3	DAY 5	DAY 7
0.093	LIVE	n	4	4	4	4
		$\bar{x}$	52	50	8	15
		S	10	20	2	8
	DEAD	n	3	3	3	3
		$\bar{x}$	280	81	11	20
		S	13	49	5	1
0.186	LIVE	n	4	4	4	4
		$\bar{x}$	98	46	23	6
		S	6	16	2	1
	DEAD	n	3	3	3	3
		$\bar{x}$	327	97	35	10
		S	48	47	10	2
0.310	LIVE	n	4	4	4	4
		$\bar{x}$	51	23	11	14
		S	11	5	4	2
	DEAD	n	3	3	3	3
		$\bar{x}$	303	51	17	16
		S	102	28	7	2
0.372	LIVE	n	4	4	4	4
		$\bar{x}$	54	18	20	10
		S	22	4	12	3
	DEAD	n	3	3	3	3
		$\bar{x}$	307	85	36	7
		S	78	58	4	4

TABLE 7A (continued) ...

mM Aluminium in culture			U/L Alkaline Phosphatase DAY 1	DAY 3	DAY 5	DAY 7
0.558	LIVE	n	3	4	4	4
		$\bar{x}$	33	26	22	9
		S	8	10	8	8
	DEAD	n	3	3	3	3
		$\bar{x}$	292	84	27	6
		S	41	42	6	1
0.620	LIVE	n	4	4	4	4
		$\bar{x}$	48	13	5	13
		S	5	5	3	1
	DEAD	n	3	3	3	3
		$\bar{x}$	328	129	25	20
		S	56	78	21	7
0.930	LIVE	n	4	4	4	4
		$\bar{x}$	48	26	8	9
		S	7	19	4	3
	DEAD	n	3	3	3	3
		$\bar{x}$	284	125	19	21
		S	128	36	6	7
1.240	LIVE	n	4	4	4	4
		$\bar{x}$	71	15	16	12
		S	23	11	6	4
	DEAD	n	3	3	3	3
		$\bar{x}$	382	76	22	5
		S	54	48	9	2

TABLE 7A (continued) ...

mM Aluminium in culture			U/L Alkaline Phosphatase DAY 1	DAY 3	DAY 5	DAY 7
1.860	LIVE	n	3	4	4	4
		$\bar{x}$	88	28	22	11
		S	6	12	5	6
	DEAD	n	3	3	3	3
		$\bar{x}$	421	127	40	12
		S	85	50	17	7
3.720	LIVE	n	4	4	4	4
		$\bar{x}$	82	42	25	10
		S	57	14	10	3
	DEAD	n	3	3	3	3
		$\bar{x}$	315	91	38	6
		S	91	39	14	5
5.580						
7.440			NO RESULTS			

Standard culture medium contains no alkaline phosphatase.

FIGURE 7A

Levels of alkaline phosphatase found when mouse calvaria are cultured in the presence of various concentrations of aluminium chloride.

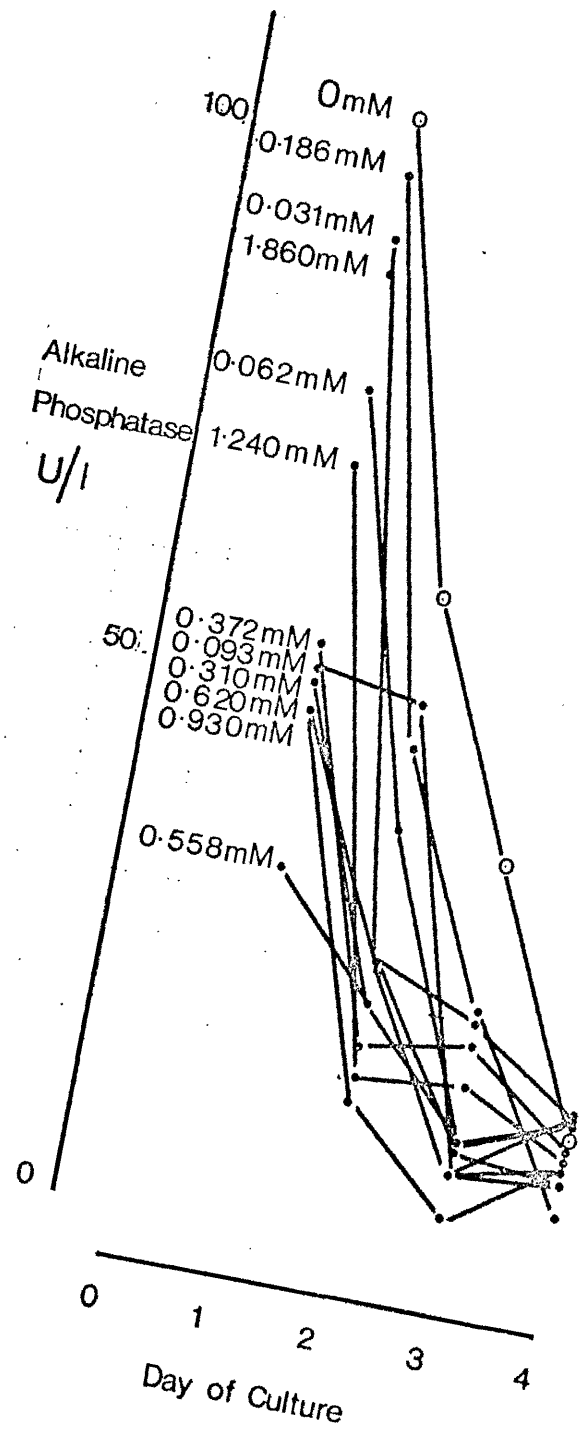

100
50
0
Alkaline
Phosphatase
U/l
0mM
0·186mM
0·031mM
1·860mM
0·062mM
1·240mM
0·372mM
0·093mM
0·310mM
0·620mM
0·930mM
0·558mM
0
1
2
3
4
Day of Culture

FIGURE 7A

2. Students t-tests comparing alkaline phosphatase levels in culture medium to which aluminium has been added to levels in culture medium with no added aluminium.

TABLE 7B

mM concentrations of aluminium compared			p
DAY 1	None (113 ± 25)	+ 0.031 (92 ± 71)	NSD
		+ 0.062 (78 ± 20)	NSD
		+ 0.093 (52 ± 10)	0.01
		+ 0.186 (98 ± 6)	NSD
		+ 0.310 (51 ± 11)	0.01
		+ 0.372 (54 ± 22)	0.02
		+ 0.558 (33 ± 8)	0.001
		+ 0.620 (48 ± 5)	0.01
		+ 0.930 (48 ± 7)	0.01
		+ 1.240 (71 ± 23)	0.01
		+ 1.860 (88 ± 3)	NSD
		+ 3.720 (82 ± 57)	NSD
DAY 3	None (60 ± 25)	+ 0.031 (26 ± 9)	NSD
		+ 0.062 (39 ± 18)	NSD
		+ 0.093 (50 ± 20)	NSD
		+ 0.186 (46 ± 16)	NSD
		+ 0.310 (23 ± 5)	0.05
		+ 0.372 (18 ± 4)	0.02
		+ 0.558 (26 ± 10)	0.05
		+ 0.620 (13 ± 5)	0.02
		+ 0.930 (26 ± 19)	NSD
		+ 1.240 (15 ± 11)	0.02
		+ 1.860 (28 ± 12)	NSD
		+ 3.720 (42 ± 14)	NSD

TABLE 7B (continued) ...

	mM concentrations of aluminium compared		p
DAY 5	None (37 ± 11)	+ 0.031 (18 ± 5)	0.05
		+ 0.062 (10 ± 4)	0.01
		+ 0.093 (8 ± 4)	0.01
		+ 0.186 (23 ± 2)	0.05
		+ 0.310 (11 ± 4)	0.01
		+ 0.372 (20 ± 12)	NSD
		+ 0.558 (22 ± 8)	NSD
		+ 0.620 (5 ± 3)	0.002
		+ 0.930 (8 ± 4)	0.01
		+ 1.240 (16 ± 6)	0.02
		+ 1.860 (22 ± 5)	0.05
		+ 3.720 (25 ± 10)	NSD
DAY 7	None (13 ± 6)	+ 0.031 (10 ± 1)	NSD
		+ 0.062 (12 ± 5)	NSD
		+ 0.093 (15 ± 8)	NSD
		+ 0.186 (6 ± 1)	0.01
		+ 0.310 (14 ± 2)	NSD
		+ 0.372 (10 ± 3)	NSD
		+ 0.558 (9 ± 8)	NSD
		+ 0.620 (13 ± 1)	NSD
		+ 0.930 (9 ± 3)	NSD
		+ 1.240 (12 ± 4)	NSD
		+ 1.860 (11 ± 6)	NSD
		+ 3.720 (10 ± 3)	NSD

3. Students t-tests comparing alkaline phosphatase levels in culture medium supporting living and frozen dead calvaria in the presence of the same concentration of aluminium.

TABLE 7C

	Cultures Compared			p
DAY 1	0mM living	(113±25) +	dead (247±140)	NSD
	0.031mM living	(92±71) +	dead (330±87)	0.01
	0.062mM living	(78±20) +	dead (350±135)	0.01
	0.093mM living	(52±10) +	dead (280±13)	0.001
	0.186mM living	(98±6) +	dead (327±48)	0.001
	0.310mM living	(51±11) +	dead (303±102)	0.01
	0.372mM living	(54±22) +	dead (307±78)	0.002
	0.558mM living	(33±8) +	dead (292±41)	0.001
	0.620mM living	(48±5) +	dead (328±56)	0.001
	0.930mM living	(48±7) +	dead (284±128)	0.02
	1.240mM living	(71±23) +	dead (382 ±54)	0.001
	1.860mM living	(88±6) +	dead (421±85)	0.001
	3.720mM living	(82±57) +	dead (315±91)	0.01
DAY 2	0mM living	(60±25) +	dead (135±42)	0.05
	0.031mM living	(26±9) +	dead (108±63)	NSD
	0.062mM living	(39±18) +	dead (153±77)	0.05
	0.093mM living	(50±20) +	dead (81±49)	NSD
	0.186mM living	(46±16) +	dead (97±47)	NSD
	0.310mM living	(23±5) +	dead (51±28)	NSD
	0.372mM living	(18± 4) +	dead (85±58)	NSD
	0.558mM living	(26±10) +	dead (84±42)	NSD
	0.620mM living	(13±5) +	dead (129±78)	0.05
	0.930mM living	(26±19) +	dead (125±36)	0.01
	1.240mM living	(15±11) +	dead (76±48)	NSD
	1.860mM living	(28±12) +	dead (127±50)	0.02
	3.720mM living	(42±14) +	dead (91±39)	NSD

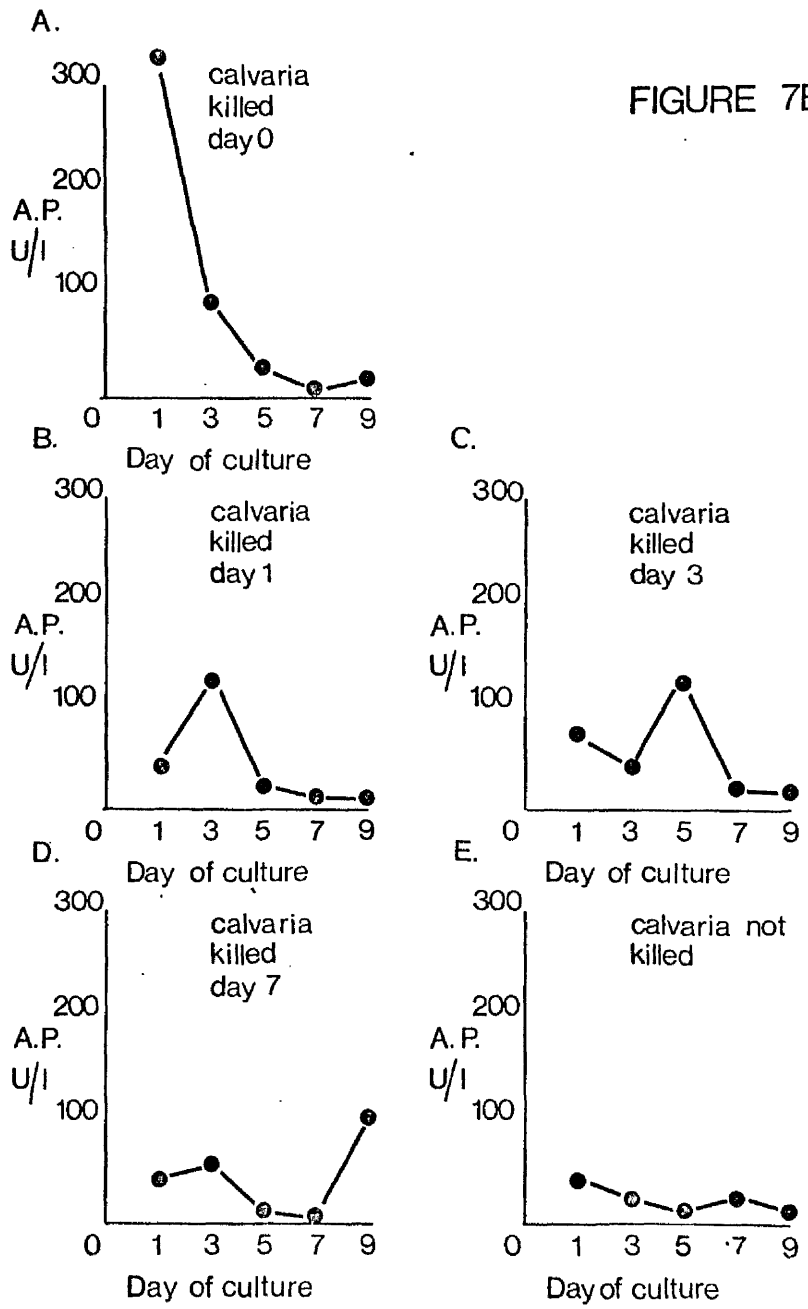
TABLE 7C (continued) ...

	Cultures Compared		p
DAY 5	0mM living (37±11)	+ dead (49±11)	NSD
	0.031mM living (18±5)	+ dead (25±18)	NSD
	0.062mM living (10±4)	+ dead (38±30)	NSD
	0.093mM living (8±2)	+ dead (11±5)	NSD
	0.186mM living (23±2)	+ dead (35±10)	NSD
	0.310mM living (11±4)	+ dead (17 ±7)	NSD
	0.372mM living (20±12)	+ dead (36±4)	NSD
	0.558mM living (22±8)	+ dead (27±6)	NSD
	0.620mM living (5±3)	+ dead (25±21)	NSD
	0.930mM living (8±4)	+ dead (19±6)	0.05
	1.240mM living (16±6)	+ dead (22±9)	NSD
	1.860mM living (22±5)	+ dead (40±17)	NSD
	3.720mM living (25±10)	+ dead (38±14)	NSD
DAY 7	0mM living (13±6)	+ dead (7±6)	NSD
	0.031mM living (10±1)	+ dead (16±15)	NSD
	0.062mM living (12±5)	+ dead (9±0)	-
	0.093mM living (15±8)	+ dead (20±1)	NSD
	0.186mM living (6±1)	+ dead (10±2)	0.05
	0.310mM living (14±2)	+ dead (16±2)	NSD
	0.372mM living (10±3)	+ dead (7±4)	NSD
	0.558mM living (9±8)	+ dead (6±1)	NSD
	0.620mM living (13±1)	+ dead (20±7)	NSD
	0.930mM living (9±3)	+ dead (21±7)	0.02
	1.240mM living (12±4)	+ dead (5±2)	NSD
	1.860mM living (11±6)	+ dead (12±7)	NSD
	3.720mM living (10±3)	+ dead (6±5)	0.05

FIGURE 7B

The effects of killing mouse calvaria by freezing and thawing on the release of alkaline phosphatase into the calvaria culture medium. No aluminium chloride has been added to any of the cultures studied here.

FIGURE 7B


A.
300
200
A.P.
U/l
100
calvaria
killed
day 0
0 1 3 5 7 9
Day of culture
B.
calvaria
killed
day 1
C.
calvaria
killed
day 3
D.
calvaria
killed
day 7
E.
calvaria not
killed

The first and most noticeable feature of this experiment was the effect of the 5.58mM and the 7.44mM concentrations of aluminium on the mouse calvaria in culture. When added to the culture medium, within 24 hours these solutions had resulted in fragmenting of the calvaria in culture, with small particles of bone floating freely in the culture medium; these cultures were subsequently abandoned.

Results here, in TABLE 7A and FIGURE 7A, show that any concentration of aluminium in culture medium causes a decrease in the level of alkaline phosphatase in the culture medium. The effect of aluminium on the alkaline phosphatase levels does not seem to be dose-dependent or related to concentration, although there is a lot of variation in the amount of inhibition exhibited. Alkaline phosphatase levels in all of the cultures, with or without added aluminium, fall as the cultures proceed and eventually reach the same low level. It is important to remember that fresh standard culture medium, as used here, has no alkaline phosphatase present before it is placed in culture with the calvaria and any alkaline phosphatase present at any time during the culture period comes from the bone itself.

An interesting point to note is that dead calvaria in culture release more alkaline phosphatase into the culture medium than the live calvaria. This phenomenon probably reflects the breakdown of the bone as a result of the death and damage due to freezing and thawing, explaining perhaps the initially high levels of alkaline phosphatase produced when live calvaria have just been dissected-out and placed in culture.

4. To see whether we could detect calvaria dying in the course of culture by a similar rise in alkaline phosphatase level in the culture medium, sample calvaria were killed at intervals throughout the culture period and replaced in culture, the alkaline phosphatase levels in their culture medium measured as normal.

TABLE 7D

DAY CULTURE KILLED		ALKALINE PHOSPHATASE LEVEL (U/L)				
		DAY 1	DAY 3	DAY 5	DAY 7	DAY 9
0	n	4	4	4	4	4
	$\bar{x}$	331	96	23	7	15
	S	62	41	15	3	5
1	n	2	2	2	2	2
	$\bar{x}$	41	124	22	10	11
	S	24	40	12	8	1
3	n	3	3	3	3	3
	$\bar{x}$	73	44	111	22	16
	S	20	30	48	8	8
7	n	3	3	3	3	3
	$\bar{x}$	45	53	14	4	98
	S	33	9	2	4	10
-	n	3	3	3	3	3
	$\bar{x}$	40	25	15	29	10
	S	26	4	13	12	1

The results in this table (TABLE 7D) show that following the death of any calvaria by freezing is followed by a steep rise in the level of alkaline phosphatase in the culture medium on the following day, complementing results by Lenaers-Claeys, et al. (1976) who found that freezing and thawing to produce dead explants always resulted in a rise

in the alkaline phosphatase level in their culture medium. As well as confirming that it is always the case that dead explants of bone release more alkaline phosphatase than live explants, not a feature of adding aluminium, we also have a competent yardstick by which we can assess the inability of those calvaria which we hope to maintain alive.

5. Alkaline phosphatase, as the name suggests, works generally in alkaline conditions of up to pH 9.8; the culture medium used here is neutral, as indicated by phenol red, but became more acidic on addition of the aluminium chloride solution. It was thought that, perhaps, the decreased level of alkaline phosphatase found in the presence of aluminium chloride may be an effect of the pH rather than the aluminium itself. To test whether the decreased levels of the enzyme reflected a pH or aluminium effect, the pH of the culture medium was raised using 0.1M NaOH, 1 drop in each well producing an alkaline pH, as indicated by the phenol red. More accurate determinations of the pH of the culture medium seemed inappropriate as after a short period of incubation the pH would change again. Results of this experiment are shown in TABLE 7E and FIGURE 7C, where the alkaline phosphatase levels produced in the new, higher, pH are compared with those at the lower pH, in the presence of the same concentration of aluminium.

TABLE 7E

mM Aluminium present		U/L Alkaline Phosphatase DAY 1	DAY 2	DAY 3	DAY 4
0.000	n	3	3	3	3
	$\bar{x}$	232	79	25	19
	S	25	22	8	6
0.031	n	4	4	4	4
	$\bar{x}$	248	75	21	13
	S	25	9	6	3
0.062	n	4	4	4	4
	$\bar{x}$	162	39	21	14
	S	23	2	8	1
0.093	n	3	4	3	3
	$\bar{x}$	165	71	17	10
	S	51	31	5	1
0.186	n	4	4	4	4
	$\bar{x}$	122	52	16	10
	S	26	7	2	0
0.310	n	4	4	4	4
	$\bar{x}$	131	50	16	10
	S	11	7	3	2
0.372	n	4	4	4	4
	$\bar{x}$	143	47	13	13
	S	17	2	1	2

Disregarding the artificially high level of alkaline phosphatase recorded for each type of culture on day 1, the result of damage during the dissection procedure, we can

FIGURE 7C

Comparing the effects of an alkaline or an acid pH on the release of alkaline phosphatase from calvaria, in the presence of aluminium chloride.

FIGURE 7C(1)

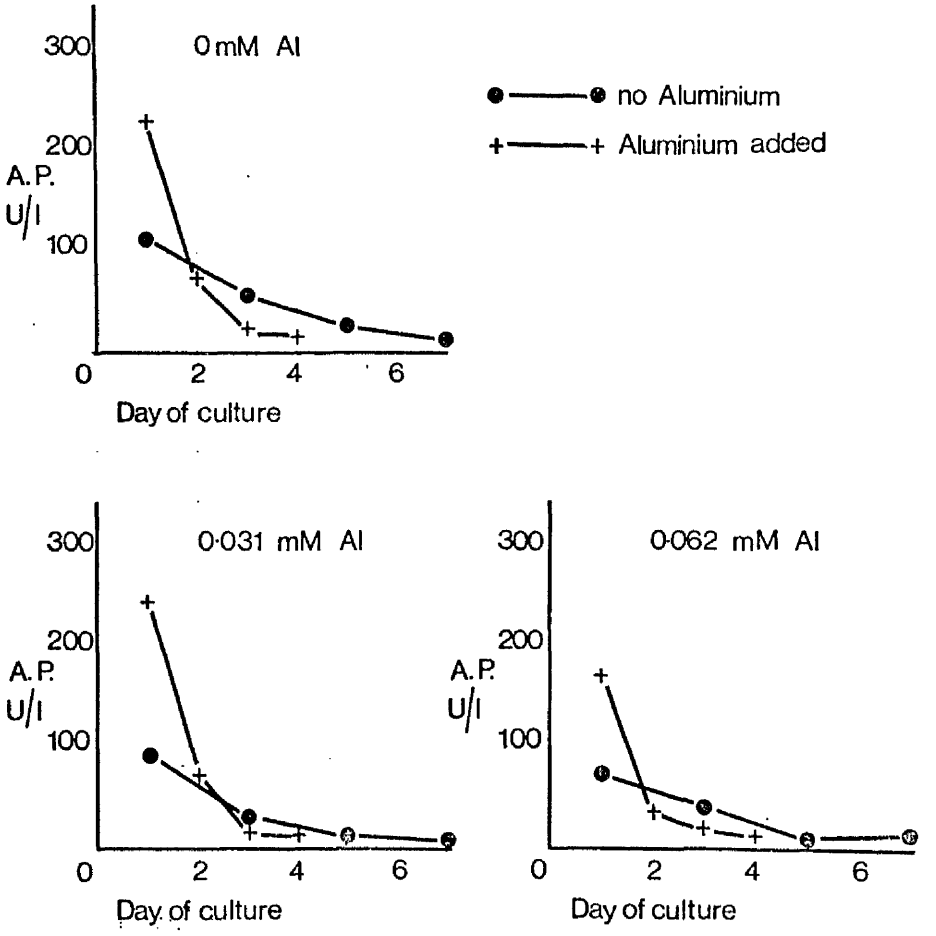
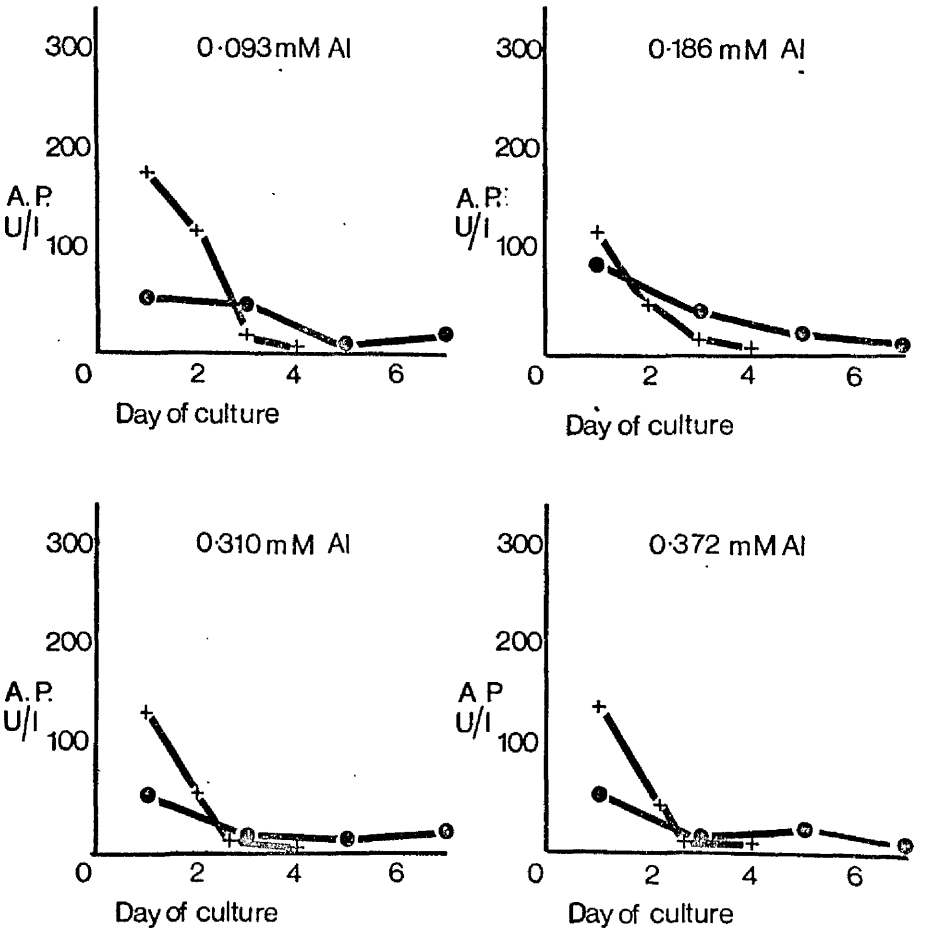

0 mM Al
300
200
100
A.P.
U/l
0
2
4
6
Day of culture
no Aluminium
Aluminium added
0·031 mM Al
0·062 mM Al

FIGURE 7C (2)


0·093 mM Al
0·186 mM Al
0·310 mM Al
0·372 mM Al
A.P. U/l
300
200
100
0
2
4
6
Day of culture

see that increasing the pH decreases the level of alkaline phosphatase recorded. From these results we can see that the aluminium, therefore, decreases the alkaline phosphatase levels slightly beyond those which we already found, but the trends are the same, with no significant differences.

6. Ionised calcium (Ca^{2+}) levels in culture medium supporting neonatal mouse calvaria, in the presence of aluminium chloride.

TABLE 7E.

mM Aluminium in culture			mM Ca^{2+} DAY 1	DAY 3	DAY 5	DAY 7
0.000	LIVE	n	3	3	3	
		$\bar{x}$	0.370	0.597	0.569	
		S	0.172	0.074	0.071	
	DEAD	n	3	3	3	
		$\bar{x}$	1.254	1.412	1.180	
		S	0.051	0.012	0.058	
0.031	LIVE	n	3	3	3	3
		$\bar{x}$	1.374	1.781	1.95	1.839
		S	0.120	0.212	0.151	0.102
	DEAD	n	3	3	3	3
		$\bar{x}$	1.196	1.658	1.485	1.563
		S	0.017	0.184	0.086	0.353
0.062	LIVE	n	3	3	3	3
		$\bar{x}$	1.444	2.117	2.244	2.043
		S	0.073	0.346	0.028	0.138
	DEAD	n	3	3	3	3
		$\bar{x}$	1.045	1.323	1.271	1.151
		S	0.127	0.543	0.065	0.474
0.093	LIVE	n	3	3	3	
		$\bar{x}$	1.51	1.834	1.853	
		S	0.199	0.297	0.329	
0.186	LIVE	n	3	3	3	
		$\bar{x}$	1.45	1.854	2.05	
		S	0.038	0.214	0.225	

FIGURE 7D

Levels of Ca^{2+} found in culture medium supporting mouse calvaria, in the presence of various concentrations of aluminium chloride.

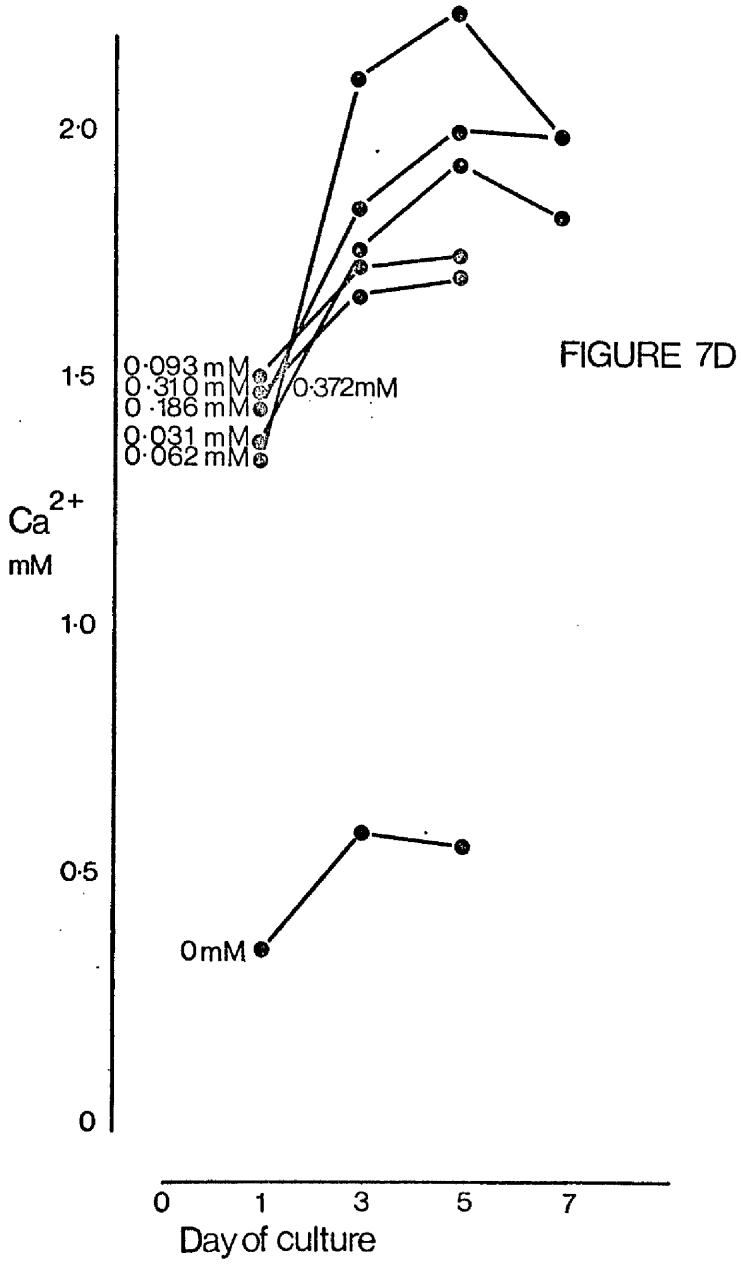

2·0
1·5
1·0
0·5
0
Ca2+
mM
0·093 mM
0·310 mM
0·186 mM
0·031 mM
0·062 mM
0·372mM
0mM
0
1
3
5
7
Day of culture

FIGURE 7D

mM Aluminium in culture			mM Ca^{2+} DAY 1	DAY 2	DAY 3	DAY 4
0.310	LIVE	n	3	3	3	
		$\bar{x}$	1.48	2.042	2.103	
		S	0.020	0.062	0.066	
0.372	LIVE	n	3	3	3	
		$\bar{x}$	1.48	1.684	1.716	
		S	0.051	0.294	0.538	

Standard culture medium has a 1.1 mM concentration of Ca^{2+}.

Students t-tests, comparing the levels of Ca^{2+} in culture medium supporting mouse calvaria in the presence of aluminium with those where no aluminium is present, show that levels in the presence of aluminium are significantly greater (from $p < 0.01$ to $p < 0.001$). T-tests comparing the levels of Ca^{2+} in culture medium supporting living and dead calvaria, in the presence of aluminium show that, in general, as time proceeds, the level of Ca^{2+} in the presence of dead calvaria is significantly lower than that in the presence of live calvaria.

7. The main purpose of this study of the effect of aluminium on the levels of alkaline phosphatase and Ca^{2+} in mouse calvaria culture medium was to see if we could learn more about the possible relationship between the two parameters. The following table lists the alkaline phosphatase (A.P) and Ca^{2+} levels in equivalent cultures on each day of culture and in the presence of different concentrations of aluminium.

TABLE 7G

U/L

mM Aluminium present	Average A.P. and Ca^{2+} (mM) on each day DAY 1		DAY 3		DAY 5	
	A.P.	Ca^{2+}	A.P.	Ca^{2+}	A.P.	Ca^{2+}
0	113	0.370	60	0.598	37	0.569
0.031	92	1.374	50	1.781	20	1.950
0.062	78	1.444	39	2.117	10	2.244
0.093	52	1.510	50	1.834	8	1.853
0.186	98	1.450	46	1.854	23	2.050
0.310	51	1.480	23	2.042	11	2.103
0.372	54	1.480	18	1.684	20	1.716

Taking all Ca^{2+} amd A.P. pairs in the above table we find a strong correlation between the two parameters ($r = -0.835$), showing that as alkaline phosphatase levels in culture decrease the ionised calcium levels increase.

FIGURE 7E

The relationship between Ca^{2+} and alkaline phosphatase levels found in culture medium in the presence of aluminium chloride; each pair of results was found with the same concentration of aluminium chloride. A correlation co-efficient of -0.835 was found to exist between Ca^{2+} and alkaline phosphatase levels in culture, with a line of regression: $y = 0.0076 x + 2.1$.

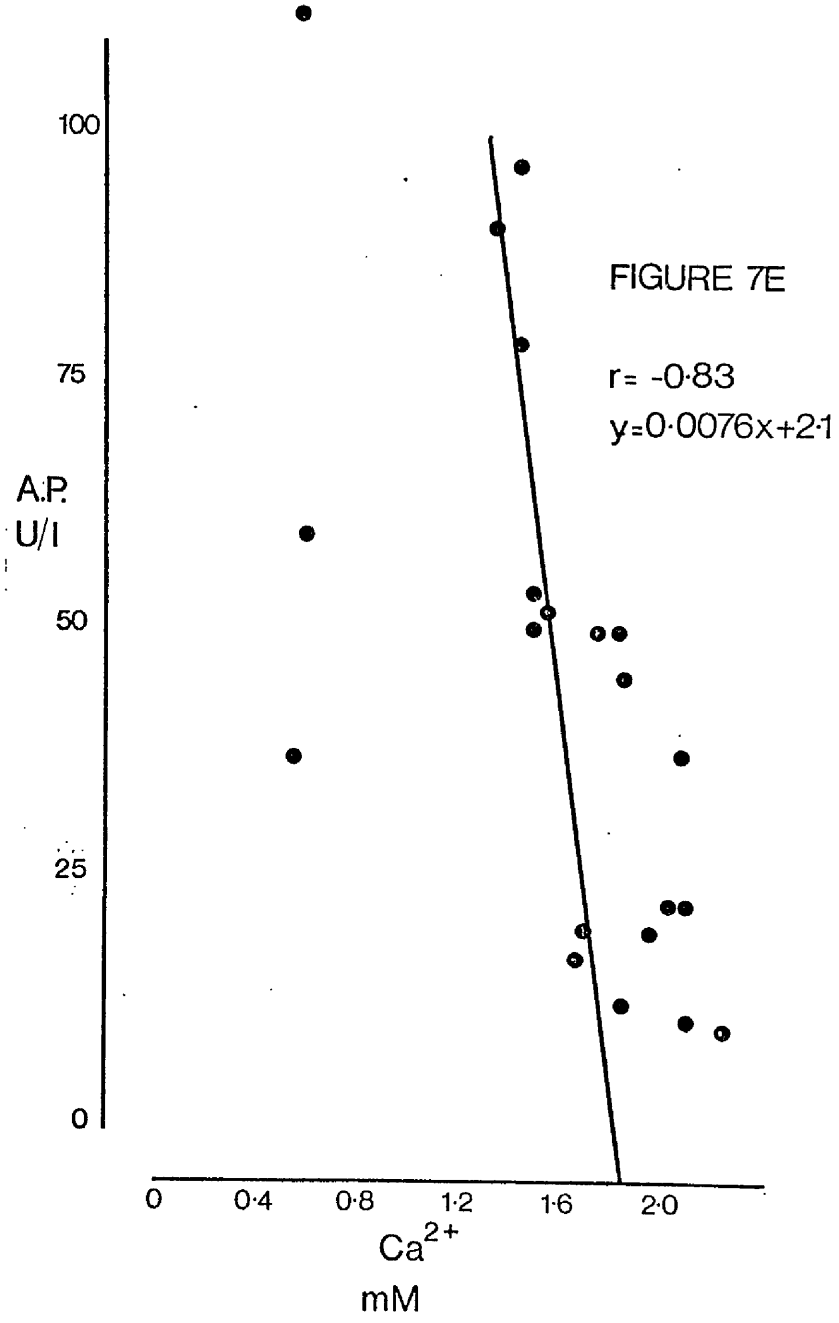


FIGURE 7E

r= -0·83

y=0·0076x+2·1

8. To look at the effect of aluminium on calcium exchange by bone in culture, ^{45}Ca and ^{47}Ca - labelled cultures were prepared as described, with experimental cultures containing culture medium with an 0.372 mM concentration of aluminium. Control culture medium had no added aluminium.

TABLE 7H

c.p.m. ^{45}Ca ^{47}Ca

TYPE OF CULTURE		DAY 1		DAY 2		DAY 3		DAY 4		DAY 5	
		^{45}Ca	^{47}Ca	^{45}Ca	^{47}Ca	^{45}Ca	^{47}Ca	^{45}Ca	^{47}Ca	^{45}Ca	^{47}Ca
EXPERIMENTAL	n	3	3	3	3	3	3	3	3	3	3
	$\bar{x}$	300	374	296	613	261	970	303	1234	222	1264
	S	28	33	50	40	27	183	46	0	47	0
CONTROL	n	3	3	3	3	3	3	3	3	3	3
	$\bar{x}$	304	207	206	740	349	757	304	890	335	754
	S	47	118	118	127	76	62	61	0	98	0
S.D.		NSD	NSD (0.1)	NSD	NSD	NSD	NSD	NSD	-	NSD	-

These results, along with FIGURE 7F, demonstrate that the level of ^{45}Ca in the absence of and presence of aluminium show no significant difference and, although results here show no significant difference, ^{47}Ca levels in culture medium indicate that less calcium is taken up in the presence of aluminium, suggesting decreased calcification, as may be expected.

FIGURE 7F

Levels of ^{45}Ca in culture medium in the presence of aluminium chloride. The release of ^{45}Ca from the pre-labelled bone indicates the level of bone resorption.

FIGURE 7F

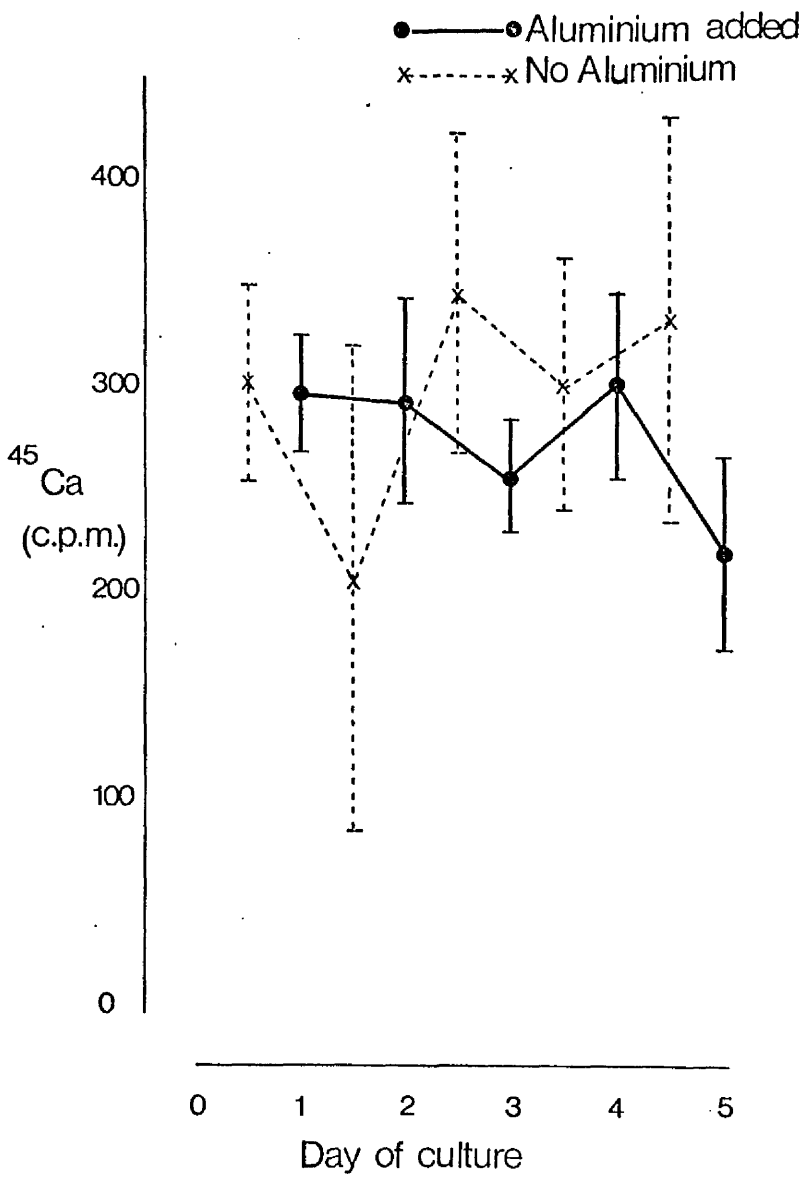
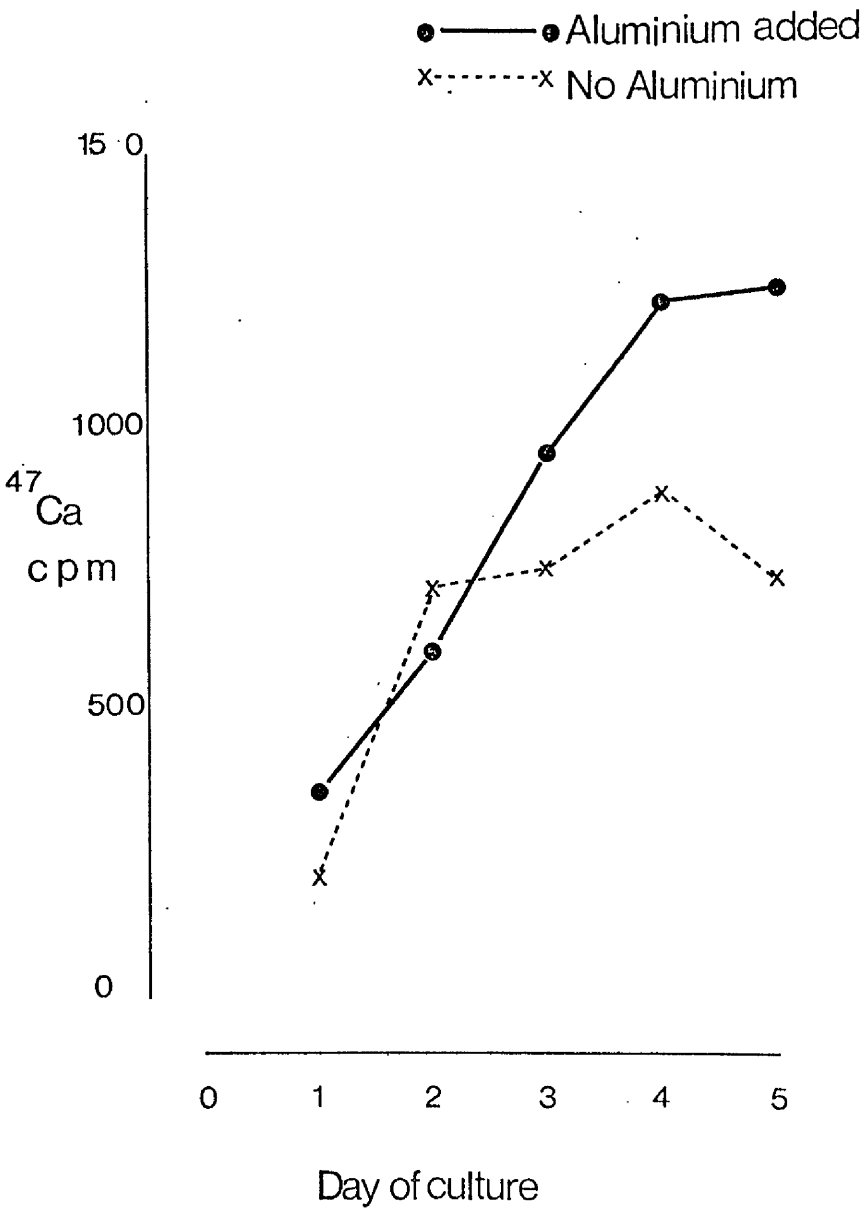

Aluminium added
No Aluminium
400
300
45Ca
(c.p.m.)
200
100
0
0
1
2
3
4
5
Day of culture

FIGURE 7G

Levels of ^{47}Ca in culture medium in the presence of aluminium chloride. The uptake of ^{47}Ca from the pre-labelled culture medium indicates the level of calcification.

FIGURE 7G


Aluminium added
No Aluminium
15 0
1000
47Ca
cpm
500
0
0
1
2
3
4
5
Day of culture

9. As the aluminium has any effect inside the bone itself, it was suggested that it may have been more relevant to study the alkaline phosphatase content of the bone in culture, not the alkaline phosphatase in the medium. By setting up two sets of cultures, one set with 0.093 mM aluminium solution and one without and removing two sample calvaria from each set of culture each day, homogenising them in 5 ml of culture medium and assaying their homogenate for alkaline phosphatase, it was possible to test if decreases in alkaline phosphatase culture medium containing aluminium reflected changes within the bone itself. The results obtained are shown in TABLE 7I and FIGURE 7H.

TABLE 7I

		ALKALINE PHOSPHATASE (U/L) + DAY OF CULTURE			
		DAY 1	DAY 2	DAY 5	DAY 6
CONTROL	$\bar{x}$	38	24	22	24
	S	8.5	2.8	2.2	5
ALUMINIUM	$\bar{x}$	33	10	7	7
ADDED	S	1.4	0	0	0
S.D.		0	0.01	0.01	0.001

These results show that as well as reducing the alkaline phosphatase levels in bone culture medium, aluminium significantly lowers the level of alkaline phosphatase in the bone itself.

FIGURE 7H

Looking at the levels of alkaline phosphatase within the bone itself in the presence of aluminium chloride solution, as well as in the presence of control medium, which contains no aluminium.

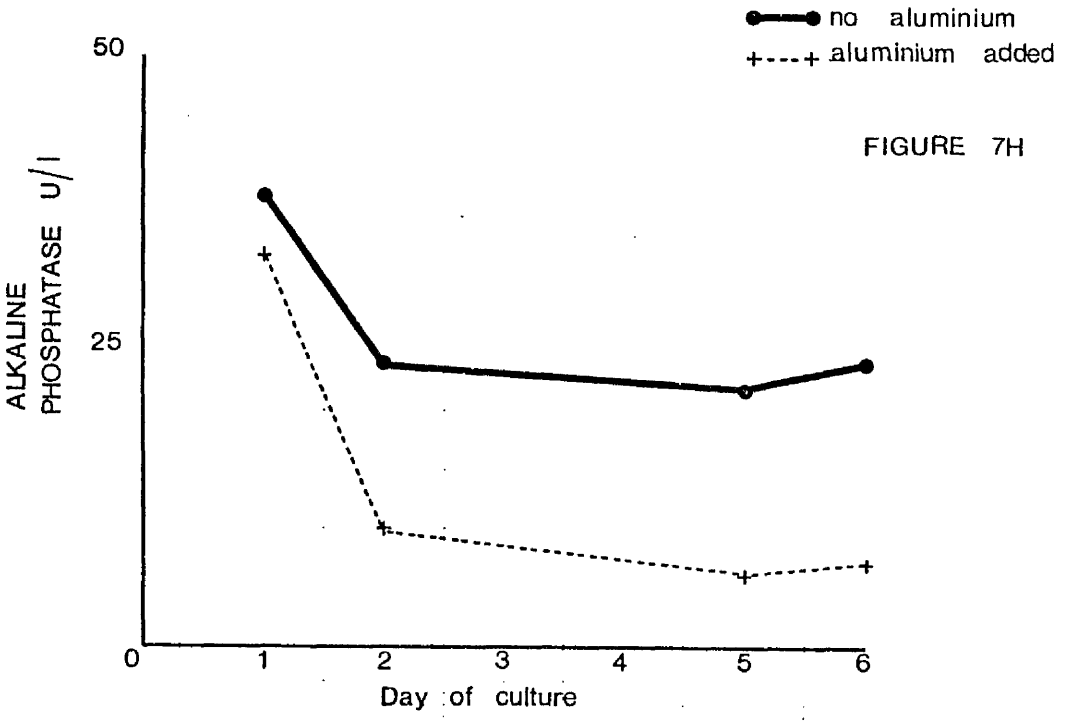

no aluminium
aluminium added
50
25
0
ALKALINE PHOSPHATASE U/l
1
2
3
4
5
6
Day of culture

FIGURE 7H

DISCUSSION:

Ellis, et al. (1979) and Boyce, et al. (1981) found that the continued administration of aluminium in vivo leads to osteomalacia, through the inhibition of calcification as a result of, perhaps, as Boyce, et al. (1981) suggests, the inhibition of enzymes involved in calcification, such as A.P. Both of these groups, along with Le Fevre, et al. (1980), have shown that aluminium is readily incorporated into bone in vivo. The basis of this study was the effect of aluminium on bone mineralisation in vitro, looking specifically at A.P. and Ca^{2+}, with a view, in general, of looking at the relationship between these two parameters as well as their reaction to aluminium.

Firstly, the effects of aluminium on A.P. release by bone in vitro was studied, adding aluminium in varying concentrations to the culture medium and assessing the effect on the enzyme. Note that fresh culture medium contains no A.P. and any enzyme activity present can only have originated from the bone itself. Results in TABLE 7A, 7B and FIGURE 7A suggest that aluminium has some effect on decreasing the amount of A.P. released by bone; results in TABLE 7I and FIGURE 7H suggest that this may be through decreasing enzyme activity within the bone itself and not by inhibiting its release from bone in culture. Comparisons made between the levels of A.P. in culture medium supporting living and dead calvaria, in the presence of aluminium (TABLE 7C), show that A.P. is released in abundance from

bones, but that the amount available for release is less in the presence of aluminium than when none is present; metabolic controls which are influencing A.P. become ineffective when the bone dies and the enzyme is freely released (TABLE 7D, FIGURE 7B).

If, as is suggested, aluminium inhibits calcification one would expect that the presence of the element in culture medium supporting mouse calvaria would either prevent or reduce the uptake of calcium from the culture medium, which normally has a 1.1 mM concentration of Ca^{2+}. As before, aluminium was added to culture medium supporting neonatal mouse calvaria, in varying concentrations, and the effect on the Ca^{2+} levels in the culture medium assessed. Looking at results in TABLE 7F, FIGURE 7D, we can see quite clearly that while the Ca^{2+} level in culture medium with no added aluminium is quite low compared to the original concentration of 1.1 mM, probably as a result of calcium uptake for mineralisation, the levels of Ca^{2+} in the presence of aluminium are all higher than 1.1 mM, probably as a result of resorption releasing calcium, with no uptake for mineralisation. These results would strongly suggest that aluminium acts by inhibiting calcification.

As was stated earlier, one of the main quests of this thesis is to find out more about the relationship between A.P. activity and calcification. It has been seen that aluminium causes decreased A.P. activity, with an accompanying increase in the Ca^{2+} levels in the culture

medium. Looking at levels of A.P. and Ca^{2+} in the presence of the same concentration of aluminium (TABLE 7G. FIGURE 7E) we can see the strong relationship between the two parameters, producing a correlation co-efficient of -0.835, and suggesting that the enzyme A.P. is, beyond doubt, involved in mineralisation.

Adding strength to the conclusion that aluminium acts on A.P. and decreases the uptake of calcium for mineralisation are results in TABLE 7H and FIGURES 7F, 7G, using ^{45}Ca and ^{47}Ca labels, that uptake of calcium in the presence of aluminium is noticeably decreased.

These results would suggest, then, that aluminium inhibits the activities of the enzyme A.P. in relation to the calcification of bone. From these results it cannot be concluded at which concentration, within the range used, aluminium has its effect, that would require further and more specific studies, but its action on calcification would seem to be convincing.

GENERAL DISCUSSION AND CONCLUSIONS

Metabolic bone diseases are a widespread and complicated group of disorders affecting bone, with various problems related to bone pain and fractures, as well as the less obvious problems concerned with hyper and hypocalcaemia and bone loss. As a group they are probably among the most widespread and least appreciated of diseases, having none of the more usual and distressing symptoms normally associated with disease, with their characteristics generally being accepted as those accompanying old age, but their almost universal occurrence makes their control and treatment important with today's continually increasing population. The control and treatment of these disorders cannot however be successfully attained without a full or, at least, comprehensive understanding of how these disorders occur and how they develop. All of the studies contained within this thesis have been performed in an attempt to understand more about metabolic bone diseases.

Organ culture preparations of neonatal mouse calvaria have been used many times in studies associated with bone metabolism (Goldhaber, 1958; Susi, et al. 1966; Nisbet, 1968; Nisbet, et al. 1969; Reynolds, et al. 1970; Nisbet, et al(197 Stern, et al. 1970; Messer, et al. 1973; Minkin, 1973; Maria, et al. 1977; Messer, 1977; Herrmann-Erlee, et al. 1978) and proved invaluable in this study. However it was also hoped to study human bone in organ culture, the 3-dimensional integrity of human bone being nearer to the

true structure of bone than the wafer-thin layer of cartilage in calvaria, also allowing the in vitro study of bone from patients suffering from metabolic bone disease. It was the complex structure of human bone which proved to be the stumbling block as far as its in vitro culture was concerned. Where mouse calvaria cultures are easily reproducible, human bone cultures are not. Calvaria from an average litter of six days old are of a fairly uniform size, but human bone fragments, of mature calcified bone, may often appear to be of the same size, but they can vary greatly in the degree of calcification, the number of cells and even the amount of bone present; the severity of bone disease may also vary between bones within the same individual. As all studies involving bone cultures were comparative, results produced from human bone in culture could not be compared with confidence and such cultures were not pursued. Attempts at preparing cultures of human bone cells also proved difficult, but with continued attempts it should be possible to culture all kinds of bone cells successfully, as has been found with other cell types.

Human serum was studied in culture with mouse calvaria and proved more fruitful than studies of human bone in culture. By preparing standard cultures of mouse calvaria, with bone prelabelled with ^{45}Ca and culture medium containing ^{47}Ca, it became possible to look at the effects of human serum, from a variety of metabolic problems, on bone turnover in vitro. Many groups have studied bone resorption

in culture, looking at the release of ^{45}Ca from prelabelled bone, under the influence of specific bone-related test substances. Studies have helped to show that PTH stimulates bone resorption in vitro, after initially inhibiting resorption, (Raisz, et al. 1967, 1969; Stern, 1969; Parsons, et al. 1971; MacIntyre, et al. 1971; Engracio, et al. 1972; Minkin, 1973), CT inhibits bone resorption (Raisz, et al. 1967; Reynolds, et al. 1968; Raisz, et al. 1968; Nisbet, et al. 1968; Mittleman, et al. 1968; Binderman, et al. 1972; Gozariu, et al. 1973; Luben, et al. 1976; Geusens, et al. 1980; Maier, et al. 1980; Stevenson, et al. 1981), oestrogen inhibits bone resorption (Atkins, et al. 1972; Robin, et al. 1980; Wink, et al. 1980) and vitamin D metabolites promote bone strength (Mittleman, et al. 1968; Raisz, et al. 1974; Boyle, 1974; Stern, etal. 1976; Peacock, et al. 1976; DeLuca, 1977; Wong, et al, 1977; Cohen, et al. 1980). As the pattern of bone resorption under the influence of these substances is so well known, they were placed in culture with the ^{45}Ca labelled bone, to measure resorption, and the ^{47}Ca labelled culture medium, as a measure of calcification, to see if bone resorption occurred as normal, without interference from the ^{47}Ca, and to see what effect these substances may have on bone formation.

Results obtained showed that as in previous studies, over the 4 day culture period, PTH inhibits bone resorption before stimulating it, CT inhibits bone resorption, oestrogen decreases bone resorption, while the vitamin D metabolites inhibited bone resorption where they were expected to stimulate it, this however would still have the effect of

increasing bone strength. The trends suggested that the uptake of ^{47}Ca from the culture medium, and its possible re-release by the bone in culture, did not seem to interfere with the assay of bone resorption and could possibly be providing a fairly accurate representation of the effects of these same test substances on bone formation. The effects of PTH, CT, oestrogen and the vitamin D metabolites on bone formation would seem to be that PTH stimulates bone formation, as in vivo; CT normalises bone formation, which is interesting when compared to previous studies which have only suggested its involvement in bone resorption; oestrogen stimulates the uptake of calcium, which may help to explain why the loss of oestrogen with the menopause causes such devastating bone loss; while the vitamin D metabolites, according to this study, have no effect on calcification, where previous results have shown that they promote bone turnover. This last result is interesting, but no definite conclusions may be drawn without repeating the experiment several times to check the result, which time did not allow for here.

The duplication of results concerning bone resorption found in previous studies by this system meant that the effects of new substances, which have not previously been assessed, could be studied with a degree of confidence, involving the investigation of the effects of sera from patients suffering from a variety of metabolic disorders. In general, the results have suggested that serum from patients suffering from these metabolic bone disorders acts

on bone in vitro to produce similar patterns of bone turnover to those found in vivo.

Osteoporotic serum acts to increase bone resorption and decrease calcification, as compared with normal human serum, which mirrors the accumulated bone loss found with osteoporosis (Albright, et al. 1948). The treatment of one osteoporotic patient with oestrogen had the effect of reducing bone resorption and increasing calcium uptake, bringing bone turnover nearer to that found in normal individuals and suggesting that oestrogen is effective in the control of osteoporosis.

Osteomalacic/ricketic serum acts on bone in vitro to enhance bone resorption and decrease mineralisation, which would lead to the osteomalacic/ricketic condition of soft, mainly osteoid bone (Smith, 1979). These conditions, one the adult form and one the juvenile form, are often treated using $25(OH)D_3$ and $1,25(OH)_2D_3$ (DeLuca, 1977; Kanis, et al. 1977; Frame, et al. 1978; Ahmed, et al. 1979; Offerman, et al. 1979) and these metabolites, in culture with ^{45}Ca and ^{47}Ca, have been shown to decrease bone resorption, which would indeed help the osteomalacic condition, while their effects on mineralisation are unclear.

The condition of 1^{o} hyperparathyroidism is recognised by increased bone turnover, particularly resorption, through the action of PTH (Rosenbach, et al. 1967; Vaes, 1968; Harris, et al. 1969; Shubina, et al. 1969; Byers, et al. 1971; Chu, et al. 1971; Messer, et al. 1973a; Minkin, 1973;

Peck, et al. 1976; Reeve, et al. 1976; Langeland, 1977; Wong, et al. 1977; Luben, et al. 1977; Maria, et al. 1977; Ibbotson, et al. 1978; Krieger, et al. 1980; Malluche, et al. 1981). accompanied by increased bone formation. Hyperparathyroid serum acts on bone in vitro to increase bone resorption, as compared to normal human serum, but decreases the uptake of calcium, which is not indicative of bone formation.

Hypoparathyroidism, where patients have no, or very little serum PTH, exhibits decreased bone resorption through the lack of available PTH. Serum from such patients acts on bone in vitro to decrease calcium uptake from the culture medium, while results concerning bone resorption are inconclusive; it could be that the decreased formation of bone is to counteract the decreased bone resorption and keep the level of bone as normal as possible. One hypoparathyroid patient studied was treated with 1α $(OH)D_3$ which, as far as this study is concerned, seems to have little effect on bone turnover.

Paget's disease of bone is recognised by increased bone turnover, which leads to the deposition of disorganised woven bone, as opposed to normal lamellar bone (Nagant de Deuxchaines, et al. 1964; Singer, 1977) and the deforming of skull and limb bones (Smith, 1979; Krane, 1980). It is thought by some to be caused by a viral inclusion found within the nucleus of osteoclasts of such patients (Rebel, et al. 1980; Singer, 1980) and so it was interesting to

note the effects of the serum from P.D. patients. Serum from patients suffering from P.D. of bone caused increased bone resorption in vitro, with decreased mineralisation, as compared to normal human serum. The latter, lack of calcium uptake, indicative of a lack of bone formation, was unexpected as P.D. is characterised by both increased formation and resorption. Treatment of P.D. with C.T, as in the case of one patient studied, acted to decrease bone resorption and 'normalise' calcification, showing that it can be effective in treating P.D. of bone.

Sample sera from patients suffering from malignant hypercalcaemia acted on bone in vitro to increase bone resorption and decrease calcium uptake, suggesting that the excess serum calcium came from the bone. As a comparison, though, serum from patients with carcinoma but with no elevated serum calcium produced the same effect, which may suggest the presence of some factor in the malignant patient's serum which causes bone resorption in vitro but not in vivo, further study would be required.

A family, all of whom had taken a massive overdose of $25(OH)D_3$, provided two samples for the study of the effects of serum from patients suffering from hypervitaminosis D on bone turnover in vitro. This serum caused decreased bone resorption, in the same way as the two test vitamin D metabolites, although calcium uptake was less than expected, as compared to normal human serum and the two metabolites. As the serum Ca and AP in both cases was normal, we see serum acting differently in vivo and in vitro,

stimulating bone resorption only in the latter.

Hypophosphataemic serum, from patients suffering from the rare disorder where the individuals have little or no serum AP, was studied in culture with ^{45}Ca-labelled bone and ^{47}Ca-labelled culture medium, causing decreased mineralisation, with no effect on resorption. As AP is thought to be important in mineralisation, this result is important in supporting this supposition, with no AP leading to decreased mineralisation. A test substance such as this, where bone formation is affected more than bone resorption, shows the value of this system in showing aspects of bone turnover.

On the whole, this study of bone turnover in vitro, under the influence of different sera has been interesting, showing how both bone resorption and formation can be affected. Although there are other and more accurate ways of diagnosing metabolic bone diseases, this method of studying bone turnover could be useful in assessing the development of any disorder. Many metabolic bone diseases are diagnosed by means of histological study of sections of iliac crest bone biopsies, which are useful in as much as the condition of the bone can be seen, but inaccurate in that the severity of a disorder may vary between bones in the same patient, there cannot be repeated sampling from the same site, as well as taking into account the fact that changes occur slowly in bone, different disorders taking years to manifest or treat. Using serum from such patients, serum which can be sampled repeatedly

without affecting the result, unlike bone, and which is more dynamic than bone, adapting to changes more quickly, changes in a patient's condition can be assessed. The effects of drug therapy may be seen more quickly, as has already been seen in those cases where the serum used came from patients who had already received some kind of drug therapy. The latter is probably the most important aspect of this study; by constant monitoring of serum biochemistry and bone turnover in vitro, it may also be possible to understand the effects of particular drug therapies in more detail.

The enzyme alkaline phosphatase (AP) has stimulated a lot of interest over the years concerning its role in the mineralisation of bone. In the first half of this century Robison (1923, 1932) pioneered the study of this enzyme, finding that AP was always to be found in areas where calcification would soon occur and leading him to suggest that it was responsible for a localised increase in phosphate esters, which would precipitate with calcium, forming calcium phosphate and mineralising bone. Further studies by Bachra, et al. (1959) also provided evidence that AP acts locally, but suggested that the enzyme itself was involved in mineralisation. Khairi, et al. (1973) and Franck, et al. (1974) related increased AP activity with increased osteoblastic activity and increased mineralisation, when studying Paget's disease of bone.

The level of AP in human serum is used as a diagnostic index of the degree of mineralisation in several metabolic

bone diseases, the level varying with different disorders (Rasmussen, et al. 1974; Smith, 1979), although no studies have been performed to show that the level of serum AP relates directly to the level of AP in bone itself. Alkaline phosphatase is not exclusive to bone, it is also found in gastro-intestinal mucosa, placenta and liver (Smith, 1979), although the bulk of the enzyme derives from bone and liver. It was important, then, that a study should be made to see if serum AP can be associated with bone AP, as an index of mineralisation and bone disease.

Using sample iliac crest bone biopsies from patients suffering from several metabolic bone disorders, homogenates of bone were prepared and assayed for AP content; the serum AP level in the serum of each patient, as near to the day of biopsy as possible, was also noted. By looking at bone and serum AP levels, it was found that a strong correlation exists between bone AP and serum AP levels, with a correlation co-efficient of 0.893, indicating that serum AP levels closely reflect AP levels within bone. This result was encouraging, with the correlation being stronger than expected, the AP from other sources not interfering significantly with the result.

Many studies were carried out for this thesis using in vitro cultures of mouse calvaria, assaying the culture medium for changes in AP and Ca^{2+}, which are thought to reflect levels in bone itself. One particular study involved the investigation of the effects of aluminium on AP and mineralisation of bone. Many patients suffering

from chronic renal failure (C.R.F) have been found to have high levels of aluminium concentrated in both their brain and bone tissue, which are thought to be associated with the conditions of dialysis dementia and dialysis osteomalacia, which often accompany long-term kidney dialysis (Bloom, et al. 1960; Berlyne, et al. 1970; Stanbury, 1972; Platts, et al. 1973; Alfrey, et al. 1976; Malluche, et al. 1976; Flendrig, et al. 1976; Platts, et al, 1977; Elliot, et al. 1978; McDermott, et al. 1978; Rozas, et al. 1978; Dunea, et al. 1978; Ellis, et al. 1979; Parkinson, et al. 1979; Wing, et al. 1980; Shore, et al. 1980; Boyce, et al. 1981; Prior, et al. 1982) and are thought to be associated in turn, with levels of aluminium in dialysate water or oral phosphate-binding gels (Platts, et al. 1977; Alfrey, et al. 1978; Drueke, 1980; Boyce, et al. 1981).

Ellis, et al. (1979) and Le Fevre, et al. (1980) found that aluminium is easily incorporated into bone, with osteomalacia developing as the aluminium concentration within bone increased. Increasing concentrations of aluminium were also found to be linked with the inhibition of mineralisation, probably by inhibiting AP (Boyce, et al, 1981). This finding formed the basis of this particular study into the effects of aluminium, looking at its effects on AP and Ca^{2+} levels in vitro when placed in culture with neonatal mouse calvaria.

When compared with cultures where no aluminium has

been added, it can be seen that aluminium does decrease the amount of AP released into culture medium by bone in culture (TABLES 7A, 7B, FIGURE 7A), which was found to reflect decreased levels of AP within the bone itself, found by homogenising bone which had been exposed to aluminium and assaying for AP (TABLE 7I, FIGURE 7H). These results supported the idea that aluminium causes dialysis osteomalacia by inhibiting AP activity within the bone; they also suggest that, as with serum AP and bone AP levels, levels of AP in culture medium reflect those in cultured bone.

If, as is suspected, AP is involved in mineralisation, it was also of interest to look at the effects of aluminium on Ca^{2+} levels in culture medium, which are normally high, around 1.1mM. From the results (TABLE 7F, FIGURE 7D) obtained when Ca^{2+} assays were carried out in culture medium in the presence of aluminium, it is clear that the presence of aluminium, enhancing decreased AP levels, causes Ca^{2+} levels in the culture medium to remain high, where they fall if no aluminium is present. This result supports the strong link between high levels of aluminium, low levels of AP, decreased mineralisation and the occurrence of dialysis osteomalacia.

As one of the main interests of this thesis was to find out more about the relationship between bone AP and mineralisation, the relationship between AP and Ca^{2+} levels in the face of aluminium inhibition was studied (TABLE 7G, FIGURE 7E), showing a correlation co-efficient of -0.835.

This result shows the strong relationship between AP and mineralisation, calcium levels remaining high while AP levels are low and vice versa. Using ^{45}Ca-labelled bone and ^{47}Ca-labelled culture medium, the relationship between AP, Ca^{2+} and aluminium was again exhibited, when aluminium caused the uptake of calcium from the culture medium to be decreased (TABLE 7H, FIGURES 7F, 7G).

It would seem then that aluminium does inhibit mineralisation through inhibiting AP, which in turn has a strong influence on Ca^{2+} levels in culture, high levels of the enzyme causing Ca^{2+} levels to fall as mineralisation occurs, although from this study it is not possible to tell whether AP facilitates mineralisation or takes part in it itself.

As with the bone turnover studies, it was hoped to study the effects of human sera, from a variety of metabolic bone diseases, on AP and Ca^{2+} levels in culture medium, but this proved to be difficult. Human serum has its own inherent AP and Ca^{2+} content, making it difficult to use these parameters as a measure of the effects of the serum on mineralisation, the ^{45}Ca, ^{47}Ca system proved more successful. Preliminary studies, however, using foetal calf serum gave indications of a strong relationship between AP and Ca^{2+} levels in culture, which was later confirmed by the aluminium studies.

Other studies related to metabolic bone disease involved the assay of two different and unrelated enzymes:

collagen prolyl hydroxylase and creatine kinase.

Collagen is the main structural component of bone and as such any changes in bone metabolism, such as in the various metabolic bone diseases, may be reflected in changes in collagen metabolism. In this case collagen metabolism was to be studied by looking at the activity of the enzyme collagen prolyl hydroxylase in different disorders. Proline represents 20% of all of the amino acids in collagen and any failure in its hydroxylation prevents the formation of the characteristic triple helix shape found in collagen. The assay for this enzyme was developed by Hutton, et al. (1966), where activity is measured by means of assay of the $^{3}H_{2}0$ formed at the end of the experiment. Various co-factors and conditions essential to this assay have already been described (Mitamo, et al. 1959; Fujimoto and Tamiya, 1962; Flanagan and Nichols, 1962; Prockop, et al. 1963, 1964, Juva and Prockop, 1964, 1966; Meister, et al. 1964, Peterkofsky and Udenfriend, 1965; Hurych and Chvapil, 1965; Hutton, et al. 1966, 1967).

Here the assay was performed using homogenates prepared from slivers of bone cut from biopsies of the iliac crest, but the assay did not pass the developmental stage of seeing if the assay was working, caused by many technical hitches beyond the control of the author. No comparative studies could be made, although what results were obtained did suggest that increasing the dilution of

the bone homogenate increased the enzyme activity detected, perhaps suggesting the presence of an inhibitor. It is unfortunate that this assay could not be developed further as it showed promise in the study of metabolic bone disease.

The assay of muscle creatine kinase was more successful. Creatine kinase, the enzyme involved in the breakdown of the high energy molecule creatine phosphate to form ATP, is found in many human tissues, but is most abundant in skeletal muscle (Forster, et al. 1974), a tissue which requires a lot of energy for normal functioning. Normal human serum has little or no creatine kinase activity (Wilson, 1965), but this can rise with such disorders as hypothyroidism, severe physical exercise, muscular trauma, epileptic fits and primary hyperparathyroidism, as well as in coma and progressive muscular dystrophy (Forster, 1967). As many metabolic bone disorders can affect movement, through increased bone pain and disfigurement, it was decided to see if creatine kinase levels vary with different metabolic bone diseases.

The assay for creatine kinase used here is based on that determined by Oliver (1955), using homogenates prepared from fragments of the vastus lateralis, the muscle found attached to Meunier trephine biopsies of the iliac crest. Most of the creatine kinase in striated

muscle is found within the cell cytoplasm and so complete homogenisation is necessary to obtain a true measure of the enzyme present. Lowry protein assays were also performed to allow a measure of creatine kinase in ng creatine kinase/mg protein to be obtained.

Results of these assays showed little difference between creatine kinase levels in muscle from patients suffering from different metabolic bone disease and, in retrospect, it would probably have been of interest to compare the levels of the enzyme found in these patients with that found in muscle from a normal individual. Changes in serum creatine kinase levels, as found in previous studies, are easier to detect as normal serum contains no creatine kinase; homogenates of skeletal muscle, however, contain an abundance of creatine kinase, making subtle changes difficult to detect. Comparisons made between levels of creatine kinase in muscle from male and female patients showed that males have a higher content in skeletal muscle than females, reflecting results for serum creatine kinase levels found by Wiesmann, et al (1966). Work by Smith, etal. (1979) and Thomson and Smith (1980) linked low levels of serum creatine kinase in females to the inhibition of the enzyme by oestrogen; the levels rise during pregnancy, as oestrogen falls. Looking at the effect of age on muscle creatine kinase levels, the levels rise in both male and female

subjects, but most noticeably in females, probably related to the decline of oestrogen with the menopause.

From these studies concerning bone metabolism in metabolic bone disease, various conclusions can be drawn, with indications of lines for further study.

Organ cultures of neonatal mouse calvaria continue to provide invaluable assistance in the study of bone metabolism. The use of pre-labelled calvaria and pre-labelled culture medium in the study of the effects of substances on bone turnover shows promise in many further studies, concerned with drug therapies and other treatments for metabolic bone diseases. Results concerning the effects of human sera on bone turnover proved interesting, with more studies, using larger numbers of samples, being required to discern any particular effects of different sera.

The importance of the enzyme alkaline phosphatase in mineralisation has been further emphasised by these studies, perhaps suggesting that an alkaline phosphatase 'substitute' could be used in the treatment of bone loss. The relationship between serum alkaline phosphatase and bone alkaline phosphatase has also been determined, meaning that serum alkaline phosphatase,unless additional information suggests to the contrary, is a good index of bone metabolism.

Creatine kinase levels in skeletal muscle bear no relation to metabolic bone disease and further study is not required, unless it is to look at serum creatine kinase levels in the different disorders. The study of collagen

prolyl hydroxylase levels in human bone continues to be of interest, as does the culture of human bone cells, with further studies doubtless producing interesting results in the study of metabolic bone diseases.

Aaron J E (1976). Histology and Microanatomy of Bone. In: Calcium, Phosphate and Magnesium Metabolism. Ed. Nordin B E C. 298-356. London: Churchill Livingstone.

Ahmed K Y, Varghese Z, Moorhead J F, Wills M R. (1979). The Response to 1,25$(OH)_2D_3$ and to Dihydrotachysterol in Adult-Onset Hypophosphataemic Osteomalacia. Clinica Chimica Acta, 97, 33-37.

Alfrey A C, Le Gendre G R, Kaehny W D. (1976). The Dialysis Encephalopathy Syndrome: Possible Aluminium Intoxication. New England Journal of Medicine, 294, 184-188.

Alhava E M, Olkkonen H, Kauranen P, Kari T (1980). The Effect of Drinking Water Fluoridation on the Fluoride Content, Strengh and Mineral Density of Human Bone. Acta. Orthop. Scand., 51(3). 413-420.

Arnala I, Kauranen P, Alhava E M (1980). The Effect of Drinking Water Fluoride Concentration on Spongy Bone. Calcified Tissues International, 31, 41.

Avioli L V, McDonald J E, Singer R A, Henneman P H (1965). A New Oral Isotopic Test of Calcium Absorption. Journal of Clinical Investigation, 44, 128-139.

Bachra B N, Sobel A E, Stanford J N (1959). Calcification XXIV. Mineralisation of Collagen and Other Fibres. Archives of Biochemistry and Biophysics, 84, 79-95.

Barker D J P, Chamberlain A T, Guyer P B, Gardner M J (1980). Paget's Disease of Bone: The Lancashire Focus. British Medical Journal, 280 (6222), 1105-1107.

Baylink D, Wergedal J, Stauffer M (1971). Formation, Mineralisation and Resorption of Bone in Hypophosphataemic Rats. Journal of Clinical Investigation, 50, 2519-2530

Berlyne G M, Ben-Ari J, Pest D., et al. (1970). Hyper-aluminaemia from Aluminium Resins in Renal Failure. Lancet, 11, 494-496.

Bevelander G, Johnson R L (1950). Histochemical Study of Development of Membrane Bone. Anatomical Research, 108, 1.

Bijvoet O L M, Reitsma P H, Frijlink W B (1980). Diphosphonates and Paget's Disease. Lancet, 1 (8183), 1416-1417.

Binderman I, Duksin D, Sachs L, Harell A (1972). Thyrocalcitonin and Accumulation of Calcium by Cultured Bone Cells. Abstract of the 4th International Congress of Endocrinology, 256, 181.

Binderman I, Duksin D, Harell A, Katzir E, Sachs L (1974). Formation of Bone Tissue in Culture from Isolated Bone Cells. The Journal of Cell Biology, 61, 427-439.

Birkenhager S C, Seldenrath H S, Hackeng W H L., et al. (1973). Calcium and Phosphorus Metabolism , PTH, CT and Bone Histology in Pseudohypoparathyroidism. European Journal of Clinical Investigation, 3, 27-34.

Bloom W L, Flinchum D (1960). Osteomalacia with Pseudofractures Caused by the Injection of Aluminium Hydroxide. J.A.M.A. 174, 1327-1330.

Blum J W, Kunz P, Rodriguez S M., et al. (1981). Parathyroid Hormone Response to Hypocalcaemia Following Hypercalcaemia. Acta. Endocrinol, 96(1), 75-80.

Bookman R (1980). Prostaglandin Dependent Osteoclast - Activating Factor Production by β-lymphocytes. Clinical Research, 28(2), 386A.

Boyce B F, Elder H Y, Fell G S., et al. (1981). Quantitation and Localisation of Aluminium in Human Cancellous Bone in Renal Osteodystrophy. In: Scanning Electron Microscopy 1981/111. Ed. O'Hare A M F, Chicago, S E M. Inc.

Boyle I T (1974). Vitamin D - An Old Hormone Rediscovered. Scottish Medical Journal, 19 (6), Editorial.

Boyle I T (1981). Osteoporosis. Scottish Medical Journal, 26, 1.

Burks J K, Peck W A (1978). Bone Cells: A serum-free Medium Supports Proliferation in Primary Cultures. Science, 199 (4328) 542-544.

Byers P D, Smith R (1971). Quantitative Histology of Bone in Hyperparathyroidism. Quarterly Journal of Medicine 40, 471-486.

Chalmers J, Ho K C (1970). Geographical Variations in Senile Osteoporosis. The Assocation with Physical Activity. Journal of Bone and Joint Surgery, 52B, 667-675.

Chambers T J (1980). The Cellular Basis of Bone Resorption. Clinical Orthopaedics and Related Research, 151, 283-293.

Cheung H S, Singer F R, Mills B, Nimni M E (1980). IN VITRO Synthesis of Normal Bone (Type I) Collagen by Bones of Paget's Disease Patients. Proceedings of the Society for Experimental Biology and Medicine, 163(4), 547-552.

Christiansen C, Brandt N J, Ebbensen F, Sandemann H, Trolle D (1980). Do Newborn of Epileptics on Anticonvulsants Develop Biochemical Signs of Osteomalacia? A Controlled Prospective Study. Acta Neurol Scand. 62(3), 158-164.

Christie D P (1980). The Spectrum of Radiographic Bone Changes in Children with Fluorosis. Radiology, 136(1), 85-90.

Chu L L, Macgregor R R, Hamilton J W, et al. (1971). A Bioassay for PTH on Hormonal Inhibition of CO_2 Production Iron Citrate in Mouse Calvaria. Endocrinology 89(6), 1425-1431.

Cohen H N, Farrah D, Fogelman I, et al. (1980). A Low Dose Regime of 1 αhydroxyvitamin D_3 in the Management of Senile Osteoporosis: A Pilot Study. Clinical Endocrinology 12(6), 537-542.

Condon J R, Reith S B M, Nassim J R, Millard F J C, Hilb A, Stainthorpe E M (1971). Treatment of Paget's Disease of Bone with Mithramycin. British Medical Journal, 1. 421-423.

Coope J, Lindsay R, McKay Hart D (1980). Trial of Synthetic Steroid for Preventing Post-menopausal Osteoporosis. British Medical Journal, 281(6237), 456-457.

Cressent M, Pidoux E, Tabaulet J, et al. (1980). Ovariectomy and Calcitonin Levels in Adult Female Rats. Calcified Tissues International, 31 (Suppl), 51.

DeLuca H F (1977). Vitamin D Metabolism. In: The Clinical View of 1 α-hydroxyvitamin D_3. Ed. Munro Peacock, 15-175.

DeLuca H F, Avioli L V (1978). Mechanisms of Action of Vitamin D Metabolism. In: Endocrinology of Calcium Metabolism. Ed. Copp, D H. Talmage D V. 157-158. Amsterdam: Excerpta Medica.

Denman A M (1979). Joint Complaints in Some Immigrant Groups. Medicine, 13(2), 670.

Dent C E. Sutor D J (1971). Presence or Absence of Inhibitor of Calcium Oxalate Crystal Growth in Urine of Normals and Stone-Formers. Lancet, 2, 775-778.

Dietrich J W, Canalis E M, Maina D M, et al. (1976). Hormonal Control of Bone Collagen Synthesis IN VITRO: Effects of Parathyroid Hormone and Calcitonin, Endocrinology, 98(4), 943-949.

Doyle F H (1972). Involutional Osteoporosis. In: Calcium Metabolism and Bone Disease. Clinics in Endocrinology. Ed. MacIntyre I. Vol 1. 143-167. London: W B Saunders Co. Ltd.

Drueke T (1980). Dialysis Osteomalacia and Aluminium Intoxication. Nephron, 26, 207-210.

Dunea G, Mahurkar S D, Marmolani B, Smith E G (1978). The Role of Aluminium in Dialysis Dementia. Annals of Internal Medicine, 88, 502-504.

Dunnigan M G, Paton J P S, Haase S, et al. (1962). Late Rickets and Osteomalacia in the Pakistani Community in Glasgow. Scottish Medical Journal, 7, 159.

Dunnigan M G, Robertson J. (1980). Residence in Britain as a Risk Factor for Asian Rickets and Osteomalacia. Lancet 1 (8171), 770.

Elliot H L, Dryburgh F, Fell G S, Sabet S, MacDougall A I (1978). Aluminium Toxicity During Regular Haemodialysis. British Medical Journal 1, 1101-1103.

Ellis H A, McCarthy J H, Herrington J (1979). Bone Aluminium in Haemodialysed Patients and in Rats Injected with Aluminium Chloride: Relationship to Impaired Bone Mineralisation. Journal of Clinical Pathology, 32, 832-844.

Ellis H A, Peart K M (1973). Azotaemic Renal Osteodystrophy. A Quantitative Study on Iliac Bone. Journal of Clinical Pathology, 16, 89-101.

Engracio E P, Holland J F, Moskowitz R, Depoli E (1972). Effect of Mithramycin on Bone Resorption IN VITRO. Cancer Research, 32, 74-76.

Eyring E J, Eisenberg E (1968). Congenital Hypophosphatasia. A Clinical, Pathological and Biochemical Study of Two Cases. Journal of Bone and Joint Surgery, 50A, 1099-1117.

Fang V S, Minkin C, Goldhaber P (1971). 2-thiophene-carboxylic Acid: Inhibitor of Bone Resorption in Tissue Culture. Science, 172 (979), 163-165.

Fast D K, Felix R, Neuman W F, Sallis J, Fleisch H (1977). The Effects of Diphosphonates on Cells in Culture. Calcified Tissue Research Supplement, 22, 449-451.

Fauley G B, Freeman S, Ivy A C., et al. (1941). Aluminium Phosphate in the Therapy of Peptic Ulcer. Effect of Aluminium Hydroxide on Phosphate Absorption. Arch. Int. Med., 67, 563-578.

Feldman R S, Krieger N S, Tashjian A H (1980). Effects of Parathyroid Hormone and Calcitonin on Osteoclast Formation IN VITRO. Endocrinology, 107 (4), 1137-1143.

Felix R, Neuman W F, Fleisch H (1978). Aerobic Glycolysis of Bone: Lactic Acid Production by Rat Calvaria Cells in Culture. American Journal of Physiology, 234 (1), C51-55.

Felton D J C, Stone W D (1966). Osteomalacia in Asian Immigrants During Pregnancy. British Medical Journal, 1, 1521-1522.

Fenton S, Heath D A (1978). A Quantitative Cytochemical Assay for Human P T H. Clinical Science and Molecular Medicine, 55 (3) 7p.

Flanagan B, Nichols G (1962). Metabolic Studies of Bone IN VITRO IV: Collagen Biosynthesis by Surviving Bone Fragments IN VITRO. Journal of Biochemistry and Chemistry, 237, 3688.

Fleisch H, Bisaz S (1962). Mechanisms of Calcification: Inhibitory Role of Pyrophosphate. Nature (London), 195, 911.

Fleisch H, Bonjour J P, Morgan D B, et al. (1972). Diphosphonates. In: Endocrinology. Ed. Taylor S.

Fleisch H, Russell R G G (1970). Pyrophosphate and Polyphosphate. International Encyclopaedia of Pharmacology and Therapeutics, 1 (51), 61-100.

Fleisch H, Russell, R G G, Straumann F (1966). Effect of Pyrophosphate on Hydroxyapatite and its Implications in Calcium Homeostasis. Nature, 212, 901-903.

Flendrig J A, Kruis H, Das H (1976). Aluminium Dialysis Dementia. Lancet, 1, 1235.

Ford J A, Colhoun E M, McIntosh W B, Dunnigan M G (1972). Rickets and Osteomalacia in the Glasgow Pakistani Community, 1961-1971. British Medical Journal, 2, 677.

Ford J A, Davidson D C, McIntosh W B, Fyfe W M, Dunnigan M C (1973). Neonatal Rickets in Asian Immigrant Populations. British Medical Journal, 3, 211-212.

Forster G (1967). Diagnostic Method for Serum Creatine Kinase. Praxis, 52, 1177-1183.

Forster G, Bernt E, Bergmeyer H U (1974). Creatine Kinase. In: Methods of Enzymatic Analysis. 2nd Edition. Vol 2. Ed. Bergmeyer H U. New York and London: Academic Press.

Frame B, Parfitt A M (1978). Osteomalacia: Current Concepts (Review). Annals of Internal Medicine, 59, 966-982.

Franck W A, Bress N M, Singer F R, Krane S M (1974). Rheumatic Manifestations of Paget's Disease of Bone. American Journal of Medicine, 56, 592-603.

Fraser D (1957). Hypophosphatasia. American Journal of Medicine, 22, 730-746.

Freeman S, Freeman W M C (1941). Phosphorus Retention in Children with Chronic Renal Insufficiency. Am. J. Dis. Child, 61, 981-1002.

Frost H M (1981). Mechanical Microdamage, Bone Remodelling and Osteoporosis: A Review. In: Osteoporosis: Recent Advances in Pathogenesis and Treatment. Ed. DeLuca H F. Frost H M, Jee W S S, Johnston C C, Parfitt A M. 185-190. Baltimore: University Park Press.

Fujimoto D, Tamiya N (1962). Incorporation of ^{18}O from Air into Hydroxyproline by Chick Embryo. Biochemical Journal, 84, 333.

Gaillard P J, Thesingh C W (1980). Bone Culture Studies with Thyrocalcitonin. In: Calcitonin: Proceedings of the Symposium on Thyrocalcitonin and the C-cells. 238-246.

Gattereau A, Bielman P, Durivage J, et al. (1980). Effect of Acute and Chronic Administration of Calcitonin on Serum Glucose in Patients with Paget's Disease of Bone. Clinical Research 28(2), 393A.

Geiser M, Truetta J (1958). Muscle Action, Bone Rarefaction and Bone Formation. Journal of Bone and Joint Surgery, 40B, 282-311.

Geusens P, Dequeker J (1980). Calcitonin-Test in Osteoporosis. Calcified Tissues International, 31 (Suppl) 60.

Goldhaber P (1958). The Effect of Hyperoxia on Bone Resorption in Tissue Culture. Archives of Pathology, 52, 57.

Goldring S R (1978). Response to Hormones of Cells Cultured from Human Giant Cell Tumours of Bone. The Journal of Clinical Endocrinology and Metabolism, 46(3), 425-433.

Goto I (1974). Serum Creatine Phosphokinase in Hypothyroidism, Convulsion, Myocardial Infarction and Other Diseases. Clin. Chim. Acta, 52, 27.

Gozariu L, Bellminkel S, Ziegler R (1973). Effects of Serum on the Calcitonin-Dependent Bone Resorption IN VITRO. Endocrinology Experimentalis (Brazil), 7(2), 129-133.

Grant M E, Prockop D J (1972). The Biosynthesis of Collagen. New England Medical Journal, 286, 194-199.

Hadjipavlou A G, Tsoukas G M, Siller T N, Danais S, Greenwood F (1977). Combination Drug Therapy in Paget's Disease of Bone. Journal of Bone and Joint Surgery, 59A, 1045-1051.

Harell A, Binderman I, Guez M (1976). Tissue Culture of Bone Cells: Mineral Transport, Calcification and Hormonal Effects. Israel Journal of Medical Science, 12(2), 115-123.

Harell A, Dekel S, Binderman I (1977). Biochemical Effects of Mechanical Stress on Cultured Bone Cells. Calcified Tissue Research Supplement, 22, 202-207.

Harris W H, Heaney R P (1969). Skeletal Renewal and Metabolic Bone Disease. New England Medical Journal, 280, 193-202.

Heath H (1980A). Primary Hyperparathyroidism. Lancet, 2(8187), 204.

Heath D A, Wright A D, Barnes A D, Oates G D, Derricott N J (1980). Surgical Treatment of Primary Hyperparathyroidism in the Elderly. British Medical Journal, 280 (6229), 1406-1408.

Heersche J N M (1969). The Effects of Thyrocalcitonin and Parathyroid Hormone on Bone Metabolism in Tissue Culture. Metabolism of Bone in Tissue Culture: The Effects of Parathyroid Hormone and Thyrocalcitonin on the Process of Bone Demineralisation. Proc. K. Ned Akadwet (Biology and Medicine). 72(5), 594-606.

Herrmann-Erlee M P M, Gaillard P J (1977). The Effect of Verapamil on the Action of Parathyroid Hormone on Embryonic Bone in vitro. European Journal of Pharmacology, 46(11), 51-58.

Herrmann-Erlee M P M, Gaillard P J (1978). The Effects of $1,25(OH)_2D_3$ on Embryonic Bone IN VITRO: A Biochemical and Histological Study. Calcified Tissue Research, 25(2), 111-118.

Hoikka V, Savolainen K, Alhava E M, Sivenius J, Karjalainen P (1980). Osteomalacia in Institutionalised Epileptic Patients with Chronic Anticonvulsant Therapy. Calcified Tissue Research, 31 (Suppl), 68.

Holick M F, MacLoughlin J A (1981). Aging significantly Decreases the Capacity of Human Epidermis to Produce Vitamin D_3. Clinical Research, 29(2): 408A.

Holmes A M, Enoch B A, Taylor J L, Jones M E (1973). Occult Rickets and Osteomalacia Amongst the Asian Immigrant Population. Quarterly Journal of Medicine. New Series, XLII (165), 125-149.

Holtrop M E, King G J, Raisz L G (1978). Factors influencing Osteoclast Activity as Measured by Ultrastructural Morphometry. Endocrinology of Calcium Metabolism, 91-96.

Horton J E, Wezeman F H, Kuettwer K E (1978). Resorption IN VITRO by a Cartilage-Derived Anti-Collagenase Factor. Science, 199 (4335), 1342-1345.

Howard G A, Baylink D J (1980). IN VITRO Evidence for a Coupling Factor in Bone Metabolism. Clinical Research, 28(2), 395A.

Howard G A, Turner R T, Sherrard D J, et al (1981). Human Bone Cells Produced $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$. Clinical Research, 29(1), 54A.

Hurych J, Chvapil M (1965). Influences of Chelating Agents on the Biosynthesis of Collagen. Biochemistry and Biophysics. Acta, 97, 3688.

Hutton J J, Tappel A L, Udenfriend S (1966A). A Rapid Assay for Collagen Prolyl Hydroxylase. Analytical Biochemistry, 16, 384.

Hutton J J, Tappel A L, Udenfriend S (1967). Co-factor and Substrate Requirements of Collagen Proline Hydroxylase. Archives of Biochemistry and Biophysics, 118, 231.

Ibbotson K J, Atkins D, Martin T J (1978). Stimulation of Bone Resorption by Various Prostglandins in Tissue Culture. Clinical Science and Molecular Medicine, 55 (3), 9p.

Inglis N R, Fishman L, Stolbach L L, Warshaw J B, Fishman W H (1977). A Comparison of Chyle Isoenzymes of Alkaline Phosphatase in Chyle and Hypophosphataemic Sera. Clin. Chim. Acta, 38, 67-73.

Jacob A I, Lanier D, Canterbury J, Bourgoignie J J (1980). Reduction by Cimetidine of Serum Parathyroid Hormone Levels in Uraemic Patients. New England Journal of Medicine, 302 (12), 671-674.

Jason C, Arnaud S B, Harrison M, Styne D M, Kaplan S L (1981). Primary Hyperparathyroidism in Infancy. Clinical Research, 29(1), 55A.

Jenkins M V, Harris M, Wills M R (1973). The Effects of Anticonvulsant Drugs IN VITRO on Bone Calcium Metabolism by Parathyroid Hormone. Clinical Science, 45(1), 1p-2p.

Jensen G F, Boesen J, Hegedus V, Transbol I, Christiansen C (1980). Osteoporosis in 70-year-old Women in Denmark - An Epidemiological Investigation. Calcified Tissues International 31 (Suppl), 56.

Jones S J, Boyde A (1976). Morphological Changes of Osteoblasts IN VITRO. Cell Tissue Research, 166, 101-107.

Jones S J. Boyde A (1977). Some Morphological Observations on Osteoclasts. Cell Tissue Research, 185 (3), 387-397.

Jones S J, Boyde A (1978). Scanning Electron Microscopy of Bone Cells in Culture. Endocrinology of Calcium Metabolism. Ex. 89(421), 97-104.

Jones S J, Ness A R (1977). A Study of the Arrangement of Osteoblasts of Rat Calvarium Cultured in Medium with, or without, Added Parathyroid Extract. Journal of Cell Science, 25, 247-263.

Juva K, Prockop D J (1964). Puromycin Inhibition of Collagen Synthesis as Evidence for a Ribosomal or Post-ribosomal Site for the Hydroxylation of Proline. Biochemistry and Biophysics Acta, 91, 174.

Juva K, Prockop D J (1966). An Effect of Puromycin on the Synthesis of Collagen by Embryonic Cartilage in vitro. Journal of Biological Chemistry, 241, 4419.

Kalm A J, Stewart C C, Teitelbaum S L (1978). Contact-Mediated Bone Resorption by Human Monocytes IN VIVO. Science, 199 (4332), 988-990.

Kanis J (1977). Correlation of Clinical, Biochemical and Skeletal Responses to $1\alpha(OH)D_3$ in Renal Bone Disease. Clinical Endocrinology, 7.

Khairi M R A, Wellman H N, Robb J A, Johnston C C (1973). Paget's Disease of Bone (Osteitis Deformans): Symptomatic Lesions and Bone Scan. Annals of Internal Medicine, 79, 348-351.

Kirsner J B (1943). Effect of Calcium Carbonate, Aluminium Phosphate and Aluminium Hydroxide on Mineral Excretion in Man. Journal of Clinical Investigation, 22, 47-52.

Krane S M (1980). Skeletal Metabolism in Paget's Disease of Bone. Arthritis and Rheumatology, 23 (10), 1007-1094.

Kream B E, Rowe D W, Gworek S C, et al. (1980). PTH Alters Collagen Synthesis and Procollagen mRNA Levels in Foetal Rat Calvaria. Proceedings of the Society of the National Academy of Sciences (USA, Biol. Sci.), 77(10), 5654-5658.

Krieger N S, Tashjian A H (1980). Parathyroid Hormone Stimulates Bone Resorption via a Na-Ca Exchange Mechanism. Nature, 287 (5785), 843-845.

Laitenen O, Simonen O, Knekt P (1980). Fluoride Consumption and Osteoporosis. Calcified Tissues International, 31 (Suppl), 78.

Langeland N. (1977). The IN VITRO Effect of Oestradiol on Collagen Metabolism in Metaphyseal Rat Bone. Acta. Orthop. Scand. 48(3), 266-272.

Leneārs-Claeys G, Vaes G (1976). Conditions Governing the Release of Collagenase and Procollagenase by Bone Explants in Culture: Effect of Heparin. In: Société Belge de Biochemie, Reunion de Lille. pp. 634-636.

Lotz M, Zisman E, Bartter F C (1968). Evidence of a Phosphorus Depletion Syndrome in Man. New England Journal of Medicine, 278, 409-415.

Lowry O H, Rosenbrough N J, Farr A L, Randall R (1951). Protein Measurement with Folin Phenol Reagent. Journal of Biological Chemistry, 265-275.

Luben R A, Cohn D V (1976). Effects of Parathyroid Hormone and Calcitonin on Citrate and Hyaluronate Metabolism in Cultured Bone. Endocrinology, 98(2), 413-419.

Luben R A, Wong G L, Cohn D V (1977). Parathyroid Hormone-Stimulated Resorption of Devitalised Bone by Cultured Osteoblast-type Bone Cells. Nature, 265 (5596), 629-630.

MacIntyre I, Byfield P G H, Galante L, Matthews E W, Mosely J M, Woodhouse N J Y (1971). Studies of the Effects of Human Synthetic Calcitonin in Experimental Animals and Man. In: Endocrinology. Proceedings of the Third International Symposium. 21-26. London: William Heinemann Medical Books Ltd.

MacIntyre I, Evans I M A, Hebitz H H G, et al (1980). Chemistry, Physiology and Therapeutics of Calcitonin. Arthritis and Rheumatology, 23(10), 1139-1147.

Maier R, Wilhelmi G (1980). Effect of Calcitonin on Senile Skeletal Changes in Mice. Calcified Tissues International, 31 (Suppl), 90.

Malheche J J, Rite E, Lange H P, et al. (1976). Bone Histology in Incipient and Advanced Renal Failure. Kidney International, 9 (4) 355-362.

Malluche H H, Rite E, Lange H P, Schoeppe W (1976). Changes in Bone Histology During Maintenance Haemodialysis at Various Levels of Dialysate Calcium Concentration. Clinical Nephrology, 6(4), 440-447.

Malluche H H, Sherman D, Healy M, et al. (1981). Steady State Effects of Physiologic Concentrations of Parathyroid Hormone on Bone. Clinical Research, 29(1), 96A.

Mannix H, Pyrtek L J, Crombie H D, Canalis E (1980). Hyperparathyroidism in the Elderly. American Journal of Surgery, 139(4), 581-585.

Marcus R, Orner E B (1977). Cyclic AMP Production in Rat Calvaria IN VITRO: Interaction of Prostaglandins with PTH. Endocrinology, 101 (5), 1570-1578.

Maria P M, Herrmann-Erlee M P M, Gaillard P J, Hekkelman J W, Nijweide P J (1977). The Effect of Verapamil on the Action of Parathyroid Hormone on Embryonic Bone IN VITRO. European Journal of Pharmacology, 46(1), 51-58.

Marks S C (1974). A Discrepancy Between Measurements of Bone Resorption IN VIVO and IN VITRO in Newborn Osteopetrotic Rats. American Journal of Anatomy, 141(3), 329-339.

Martin T J, Harris G S, Melick R A (1971). Glycosaminoglycan Secretion by Fetal Cartilage Cells IN VITRO. Israel Journal of Medical Science, 7 (3), 356-357.

Marx J L (1980). Osteoporosis: New Help for Thinning Bones. Science, 207 (4431), 628-630.

McCarron D A, Lenfesty B, Vetto M (1981). Parathyroid Hyperplasia: The Anatomical Distribution. Clinical Research, 29(1), 57A.

McDermott J R, Smith A I, Ward M K, etal (1978). Brain-Aluminium Concentrations in Dialysis Encephalopathy. Lancet, 1, 901-904.

McPartlin J, Powell D (1976). A Bioassay for Parathyroid Hormone and Other Substances Affecting Bone Metabolism. Irish Journal of Medical Science, 145(10), 319-329.

Meister A, Stone N, Manning J M (1964). In: Advances in Chemical Sciences. Ed. Gould, R F, Stekol, J A. Vol 44, 67. American Chemical Society.

Messer H H (1977). Hormonal Responses of Bone in a Continuous Flow Cultural System. Journal of Dental Research, 56(8), 971-975.

Messer H H, Armstrong W D, Singer L (1973a). Fluoride, Parathyroid Hormone and Calcitonin: Inter-relationships in Bone Calcium Homeostasis. Calcified Tissue Research, 13, 217-225.

Messer H H, Armstrong W D, Singer L (1973b). Fluoride, Parathyroid Hormone and Calcitonin: Effects on Metabolic Processes Involved in Bone Resorption. Calcified Tissue Research, 13, 227-233.

Miller S S, Wolf A M, Arnaud C D (1976). Bone Cells in Culture: Morphologic Transormation by Hormones. Science, 192(4246), 1340-1343.

Minkin C (1973). Inhibition of Parathyroid Hormone-Stimulated Bone Resorption IN VITRO by the Antibiotic Mithramycin. Calcified Tissue Research, 13, 249-257.

Minkin C, Jennings J M (1972). Carbonic Anhydrase and Bone Re-modelling: Sulphonamide Inhibition of Bone Resorption in Organ Culture. Science, 176 (38), 1031-1033.

Mitamo C, Smith T E, Friedberg F, Rayford C (1959). Incorporation of Hydroxyproline into Tissue Proteins by Chick Embryos. Journal of Biological Chemistry, 234, 78.

Mittleman R, Wallach S, Chausmer A, Bellavia J (1968). Interaction of Thyrocalcitonin and Vitamin D on Bone. In: Calcitonin: Proceedings of the Symposium on Thyrocalcitonin and the C-cells. 276-281.

Morimoto S, Tsuji M, Okado Y, et al. (1980). The Effect of Oestrogens on Human Calcitonin Secretion After Calcium Infusion in Elderly Female Subjects. Clinical Endocrinology, 13 (2), 135-143

Morse A, Greep R O (1951). Effect of Abnormal Metabolic States Upon Histochemical Distribution of Alkaline Phosphatase in the Tibia of the Albino Rat. Anatomical Research, 111, 193.

Moss A J, Waterhouse C, Terry R (1965). Gluten-sensitive Enteropathy with Osteomalacia but Without Steatthorea. New England Journal of Medicine, 272, 825-830.

Mundy G R, Altman A J, Gondek M D, Bandelin J G (1977). Direct Resorption of Bone by Human Monocytes. Science, 196(4294), 1109-1111.

Mundy G R, Varani J, Orr W, Gonder M, et al. (1978). Resorbing Bone is Chemotactic for Monicytes. Nature, 275 (5676), 132-135.

Münzenberg K J, Dennert R D (1975). The Cell-Independent Influence on Calcification in Living Bone and IN VITRO. Archives of Orthopaedics. UNI. ALLCHIR. 82(2), 157-168.

Nagant de Deuxchaines C, Krane S M (1964). Paget's Disease of Bone: Clinical and Metabolic Observation. Medicine, 43, 253-266.

Nisbet J, Nordin B E C (1968). Thyrocalcitonin Inhibition of Bone Resorption in Tissue Culture. In: Calcitonin: Proceedings of the Symposium on Thyrocalcitonin and the C-cells. 230-238.

Nisbet J, Nordin B E C (1969). Some Hormonal Effects on Bone in Tissue Culture. Proceedings of the Soy. Soc. Medicine, 62, 239-240.

Nisbet J A, Helliwell S, Nordin B E C (1970). Relationship of Lactic Acid and Citric Acid Metabolism to Bone Resorption in Tissue Culture. Clinical Orthopaedics, 70, 220-230.

Nordin B E C, Pautard F G (1969). Calcified Tissues. Science, 164 (878), 466-469.

O'Duffy J D (1970). Hypophosphatasia Associated with Calcium Phosphate Dihydrate Deposits in Cartilage. Arthritis and Rheumatology, 13, 381-387.

Offerman G, Manhold C (1979). Immigrant Osteomalacia: Occurence in Turkish Guest Workers in Germany. In: Vitamin D Basic Research and its Clinical Applications. Proceedings of the Fourth Workshop on Vitamin D. Berlin. Ed. Norman A W. Berlin: W de Gruyter.

Oliver I T (1955). A Spectrophotometric Method for the Determination of Creatine Phosphokinase and Myokinase. Biochemical Journal, 61, 116-122.

O'Riordan J L H, Watson L, Woodhead J J (1972). Secretion of Parathyroid Hormone in Primary Hyperparathyroidism. Clinical Endocrinology, 1, 149-155.

Orloff S, Manicourt D (1980). Osteoporosis: A Cautionary Note. Science, 208(4441), 240.

Orwall E S, McClung M R (1981). Familial Hyperparathyroidism: A Varied and Relatively Common Disorder. Clinical Research, 29(1), 58A.

Owen M (1977). Precursors of Osteogenic Cells. 13th European Symposium on Calcified Tissues. Calcified Tissue Research (Supplement), 24, R19.

Padilla A J, Deftos L J (1980). Influence of Age on Calcitonin. New England Journal of Medicine, 303(14),821-822,

Parfitt A M (1976). The Actions of Parathyroid Hormone on Bone: Relation to Bone Remodelling and Turnover, Calcium Homeostasis and Metabolic Bone Disease. Metabolism, 25, 909-955.

Park H Z, Talmage R V (1967). Relation of Endogenous Parathyroid Secretion to ^{3}H-cytidine Incorporation into Bone Cells. Endocrinology, 80(4), 552-560.

Parkinson I S, Feest T G, Ward M K, et al (1979). Fracturing Dialysis Osteodystrophy and Dialysis Encephalopathy. An Epidemiological Study. Lancet, 1, 406-409.

Parsons J A, Robinson C J (1971). Further Evidence that the Initial Calcium Shift into Bone is a Primary Response to Parathyroid Hormone. In: Endocrinology. Proceedings of the Third International Symposium. 358-362. London: William Heinemann Medical Books Ltd.

Peacock M, Taylor G A, Redel J (1976). The Action of Two Metabolits of Vitamin D_3; 25,26-dihydroxy-cholecalciferol {25, 26$(OH)_2D_3$} and 24, 25-dihydroxy-cholecalciferol {24, 25$(OH)_2D_3$} on Bone Resorption. FEBS Lett, 62(3), 248-250.

Peck W A, Birge S J, Fedak S A (1964). Bone Cells: Biochemical and Biological Studies After Enzyme Isolation. Science, 146, 1476-1477.

Peck W A, Burks J K, Wilkins J, Rodan S B, Rodan G A (1977). Evidence for Preferential Effects of Parathyroid Hormone, Calcitonin, and Adenosine on Periosteum and Bone. Endocrinology, 100(5), 1357-1364.

Peck W A, Carpenter J, Messinger K, etal. (1973). Cyclic 3', 5' - adenosine monophosphate in Isolated Bone Cells. Response to Low Concentrations of Parathyroid Hormone. Endocrinology, 92(3), 692-697.

Peck W A, Carpenter J, Messinger K (1974). Cyclic 3', 5' - adenosine Monophosphate in Isolated Bone Cells II. Responses to Adenosine and PTH. Endocrinology, 94(1), 148-154.

Peck W A, Dowling I (1976). Failure of 1,25$(OH)_2D_3$ to Modify Cyclic AMP Levels in Parathyroid Hormone-treated and Untreated Bone Cells. Endocrine Research Communications, 3(2), 157-166.

Peterkofsky B, Udenfriend S (1961). Conversion of {14_C} Proline to Peptide-Bound {14_C} Hydroxyproline in a Cell-free System from Chick Embryos. Biochem. Biophys. Res. Comm, 6, 184-190.

Peterkofsky B, Udenfriend S (1965). Enzymatic Hydroxylation of Proline in Microsomal Polypeptides Leading to Formation of Collagen. Proceedings of the Society of the National Academy of Sciences, 53, 335-342.

Pitkin R M, Reynolds W A, Williams G A, Kawahara W, Baumann A F, Hargis G K (1980). Maternal and Foetal Parathyroid Hormone Responsiveness in Pregnant Primates. Journal of Clinical Endcrinology and Metabolism, 51(5), 1044-1047.

Platts M M, Goode G G, Hislop J S (1977). Composition of Domestic Water Supply and the Incidence of Fractures and Encephalopathy on Home Dialysis. British Medical Journal, 2, 657-660.

Platts M M, Grech P, McManners T, Cochran M (1973). Skeletal Changes in Patients Treated by Regular Haemodialysis in the Sheffield Area. British Journal of Radiology, 46, 585-593.

Poskitt E M E, Cole J J, Lawson D E M (1979). Diet, Sunlight and 25-hydroxyvitamin D in Healthy Children and Adults. British Medical Journal, 1, 221-223.

Prior J C, Cameron E C, Knickerbocker W J, et al. (1982). Dialysis Encephalopathy and Osteomalacic Bone Disease. A Case-Controlled Study. The American Journal of Medicine, 72 (1), 33-43.

Pritchard J J (1952). Cytological and Histochemical Study of Bone Cartilage Formation in Rat. Journal of Anatomy, 86, 259.

Prockop D J, Ebert P S, Shapiro B M (1964). Studies with Proline-3,4-H^3 on the Hydroxylation of Proline During Collagen Synthesis. Archives of Biochemistry and Biophysics, 106, 112.

Prockop D J, Kaplan A, Udenfriend S (1963). Oxygen-18 Studies in the Conversion of Proline to Hydroxyproline. Archives of Biochemistry and Biophysics, 101, 499.

Raisz L G. Au W Y W, Friedman J, Niemann I (1969). Thyrocalcitonin and Bone Resorption. Studies Employing a Tissue Culture Bioassay. American Journal of Medicine, 13, 684-689.

Raisz L G, Au W Y W, Friedman J, Niemann I (1968). Inhibition of Bone Resorption in Tissue Culture by Thyrocalcitonin. In: Calcitonin: Proceedings of the Symposium on Thyrocalcitonin and the C-cells. 215-223.

Raisz L G, Niemann I (1969). Effect of Phosphate, Calcium and Magnesium on Bone Resorption and Hormonal Responses in Tissue Culture. Endocrinology, 85, 446-452.

Raisz L G, Trummel C L, Holick M F, DeLuca H F (1972). $1,25(OH)_2D_3$: A Patent Stimulator of Bone Resorption in Tissue Culture. Science, 175, 768-770.

Rasmussen H, Bordier P (1973). The Cellular Basis of Metabolic Bone Diseases. New England Medical Journal, 289, 25-32.

Rebel A, Basle M, Paupard A, Kauyoumoljan S, Filman R, Lepatezaur A (1980). Viral Antigens in Osteoclasts from Paget's Disease of Bone. Lancet, 2(8190), 244-246.

Reeve J, Hesp R, Williams D, Hulme P, Klenerman L (1976). Anabolic Effects of Low Doses of a Fragment of Parathyroid Hormone on the Skeleton in Post-Menopausal Osteoporosis. Lancet, 1 (7968), 1035-1038.

Reynolds J J (1972). Skeletal Tissue in Culture. In: The Biochemistry and Physiology of Bone. 2nd Edition. Ed. Bourne G H. Vol 1. 69-126. New York and London: Academic Press.

Reynolds J J (1975). Studies on the Physiology and Pathology of Resorption. In: Organ Culture in Biomedical Research (British Society for Cell Biology, Symposium I). Eds. Balls and Monnickendam. 355-366. London, New York and Melbourne: University Press.

Reynolds J J, Dingle J T, Gudmundsson T V, MacIntyre I (1968). Bone Resorption IN VITRO and its Inhibition by Calcitonin. In: Calcitonin: Proceedings of the Symposium on Thyrocalcitonin and the C-cells. 223-230.

Reynolds J J, Dingle J T (1970). A sensitive IN VITRO Method for Studying the Induction and Inhibition of Bone Resorption. Calcified Tissue Research, 4(4), 339-349.

Robin J C, Schepart B, Calkins H, Lo Sasso G, Thomas C C, Ambrus J L (1980). Studies in Osteoporosis: II. Effects of Oestrogen and Fluoride. Journal of Medicine, 11(1), 1-14.

Robison R (1923). The Possible Significance of Hexosephosphoric Esters on Ossification. Biochemical Journal, 17, 286.

Rose G G, Shindler T O (1964). The Mineralisation of Emigrating Bone Cells in Tissue Culture. Tex. Rep. Biol. Med. 22, 174-88.

Rosen V, Laverty G, Clark N B (1980). Stimulation of Calcium Release from Cultured Embryonic Chick Bones by Parathyroid Gland of the Freshwater Turtle. Gen. Comp. Endocrinol. 41(2), 150-155.

Rosenbusch J P, Nichols G (1967). Parathyroid Hormone Effects on Amino Acid Transport into Bone Cells. Endocrinology, 81(3), 553-557.

Rowe D J, Hausmann E (1977). Quantitative Analyses of Osteoclast Changes in Resorbing Bone Organ Cultures. Calcified Tissue Research, 23, 283-289.

Rozas V V, Port F K, Rutt W M (1978). Progressive Dialysis Encephalopathy from Dialysate Aluminium. Archives of Internal Medicine, 138, 1375-1377.

Russell R G G, Bisaz S, Donath A, Morgan D B, Fleisch H (1971). Inorganic Phosphate in Plasma in Normal Persons and in Patients with Hypophosphatasia, Osteogenesis Imperfecta and Other Disorders of Bone. Journal of Clinical Investigation, 50, 961-969.

Russell R G G, Fleisch H (1970). Inorganic Phosphate and Pyrophosphates in Calcification and Calcium Homeostasis. Clinical Orthopaedics, 69, 101-117.

Sandler L M, Moncrieff M W (1980). Familial Hyperparathyroidism. Archives Dis. Child, 55 (2), 146-147.

Scott D M, Kent N, Cohn D V (1980). Collagen Synthesis in Cultured Osteoblast-like Cells. Archives of Biochemistry and Biophysics, 55 (2), 146-147.

Shamonki I M, Frumar A M, Takamya I V, et al. (1980). Age-Related Changes of Calcitonin Secretion in Females. Journal of Clinical Endocrinology and Metabolism, 50 (3), 437-439.

Sherwood J K, Ackroyd F W, Garcia M (1980A). Effect of Cimetidine on Circulating Parathyroid Hormone in Primary Hyperparathyroidism. Lancet, 1 (8169), 616-620.

Sherwood J K, Ackroyd F W, Garcia M (1980B). Cimetidine in Hyperparathyroidism. Lancet (8181), 1298.

Shore D, King S W, Kaye W, et al. (1980). Serum and Cerebrospinal Fluid Aluminium and Circulating Parathyroid Hormone in Primary Degenerative (Senile) Dementia. Neurotoxicology, 1 (4), 55-63.

Shubina L I, Vendt V P (1969). Effect of Vitamin D_2 and Parathyroid Hormone on Calcification of Bone Tissue in Experiments IN VITRO. Ukrainian Biochemical Journal, 41 (6), 715-718.

Singer F R (1980). Paget's Disease of Bone: A Slow Virus Infection? Calcified Tissue Research, 31 (3), 185-187.

Singer F R, Fredericks R S, Minkin C (1980). Salmon Calcitonin Therapy for Paget's Disease of Bone. The Problems of Acquired Intestinal Resistance. Arthritis and Rheumatology, 23 (10), 1148-1154.

Smith I, Elton R A, Thomson W H S (1979). Muscular Dystrophy: Serum Creatine Phosphokinase Values in Premenarchal, Menstruating, Post-menopausal and Pregnant Normal Women. Clin. Chim. Acta, 98, 207-216.

Spencer H, Norris C, Gatza C, et al. (1978). Aluminium-induced Changes in Mineral and Fluoride Metabolism in Man. Clinical Research, 26, 586A.

Stanbury S W (1972). Azotaemic Renal Osteodystrophy. Clinics in Endocrinology and Metabolism, 1, 267-304.

Stern P (1969). ^{45}Inhibition by Steroids of Parathyroid Hormone Induced ^{45}Ca Release from Embryonic Rat Bone IN VITRO. The Journal of Pharmacology and Experimental Therapeutics, 168, 211-217.

Stern P, Golub L, Goldhaber P (1970). Effects of Demineralisation and Parathyroid Hormone on the Availability of Bone Collagen to Degradation by Collagenase. Journal of Periodontal Research, 5, 116-121.

Stern P, Mavereas T T (1976). Bone Resorbing Activity of Analogues of 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol: Effects of Side-Chain Modifications and Stereo-Isomerisation on Responses of Foetal Rat Bones IN VITRO. Molecular Pharmacology, 12(6), 879-886.

Stone N, Meister A (1962). Function of Ascorbic Acid in the Conversion of Proline to Collagen Hydroxyproline. Nature, 194, 555.

Strates B S, Neuman W F, Levinskas G J (1957). The Solubility of Bone Mineral. II. Precipitation of Near-Neutral Solutions of Calcium and Phosphate. Journal of Physical Chemistry, 61, 279-282.

Stevenson J C, Abeyasekera G, Hillyard C J, et al. (1981) Calcitonin and the Calcium-Regulating Hormones in Post-Menopausal Women: Effects of Oestrogen. Lancet, 1 (8222), 693-695.

Stevenson J C, Hillyard C J, Spanos E, et al. (1980). Effect of Oestrogen on Calcitonin Secretion in Post-Menopausal Women. Journal of Endocrinology, 87 (2), 18P-19P.

Susi F R, Goldhaber P, Jennings J M (1966). Histochemical and Biochemical Study of Acid Phosphatase in Resorbing Bone in Culture. American Journal of Physiology, 211(4), 959-962.

Teitelbaum S L (1981). Osteogenesis Imperfecta: A Heterogeneous Disorder of Collagen. In: Osteoporosis: Recent Advances in Pathogenesis and Treatment. Ed. De Luca H F, Frost H M, Jee W S S, Johnston C C, Parfitt A M, Baltimore: University Park Press.

Thomas M L, Ramp W K (1978). Increased ATPaseActivity and Decreased Alkaline Phosphatase Activities by Parathyroid Hormone and Cultured Chick Embryo Tibiae. Proceedings of the Royal Society for Experimental Biology and Medicine, 157 (3), 358-362.

Thomas W H S, Smith I (1980). Effects of Oestrogens on Erythrocyte Enzyme Efflux in Normal Men and Women. Clin Chim. Acta, 103, 203-208.

Treatise on Collagen, Vol 2A. Biology of Collagen. Ed. Gould B S. 165-181. London, New York: Academic Press.

Vaes G (1968). On the Mechanisms of Bone Resorption. Journal of Cell Biology, 39 (3), 676-679.

Van de Berg C J, Hill L F, Stanbury S W (1972). Inositol Phosphate and Phytic Acid as Inhibitors of Biological Calcification in the Rat. Clinical Science, 43, 377-383.

Von Rotthowe H W, Kowalewski S (1967). Creatine Kinase. Zschr. Klin. Chem, 5, 254-260.

Wandless I, Jarvis S, Grimley Evans J, Aird E G A, Stevens J (1980). Vitamin D_3 in Osteoporosis. British Medical Journal, 280 (6227), 1320.

Ward M K, Feest T G, Ellis H A, Parkinson I S, et al. (1978). Osteomalacic Dialysis Encephalopathy: Evidence for a Water-Borne Aetiological Agent, Probably Aluminium. Lancet, 1, 841-845.

Warshaw J B, Littlefield J W, Fishman W H, Inglis N R, Stolbach L L (1971). Serum Alkaline Phosphatase in Hypophosphatasia. Journal of Clinical Investigation, 50, 2137-2142.

Weinstein R S, Hudson J B (1980). Parathyroid Hormone and 25(OH)D_3 Levels in Hypercalcaemia of Acute Renal Failure. Archives of Internal Medicine, 140 (3), 410-411.

Wiesmann U, Colombo J P, Adam A (1966). Determination of Cysteine Activated Creatine Kinase in Serum. Enzymal Biol. Clin. 7, 266-284.

Williams D C, Boder G B, Toomey R E, et al. (1980). Mineralisation and Metabolic Response to Serially Passaged Adult Rat Bone Cells. Calcified Tissues International, 30 (3), 233-246.

Wills M R, Phillips J B, Day R C, Bateman E C (1972). Phytic Acid and Nutritional Rickets in Immigrants. Lancet, 1, 771-773.

Wilson T G G (1965). Clinical Research, 13, 223.

Wing A J, Brunner F P, Brynger H, et al. (1980). Dialysis Dementia in Europe. Lancet, 11, 190-192

Wink C S, Felts W J L (1980). Effects of Castration on the Bone Structure of Male Rats: A Model for Osteoporosis. Calcified Tissues International, 32(1), 77-82.

Woloszczuk W, Kovarik J, Linkesch W, et al. (1980). Calcitonin and Parathyroid Hormone During Pregnancy and in Newborn Babies. Calcified Tissues International, 31 (Suppl), 142.

Wong G L (1980). Bone Cells Cultures on an Experimental Model. Arthritis and Rheumatology, 23 (10), 1081-1086.

Wong G L, Luben R A, Cohn R V (1977). 1,25(OH)$_2$D$_3$ and Parathyroid Hormone: Effects on Isolated Osteoclast-like and Osteoblast-like Cells. Science, 197 (4304), 663-665.

Woodward H Q, Marcove R C (1969). A Comparison of the Chemistry of Blood from Bone and Peripheral Veins. Clinical Orthopaedics, 66, 254-264.

Yu J H, Wells H, Ryan W J, Lloyd W S (1976). Effects of Prostaglandins and Other Drugs on the Cyclic AMP Content of Cultured Bone Cells. Prostaglandins, 12 (4), 501-513.

Zamboni G, Albertini A, Zoppi G, et al. (1980). Parathyroid Hormone and Calcitonin in Glucose Regulation. European Journal of Pediatrics, 135 (2), 195-198.

Bibliography

Albright F, Reifenstein E C (1948). The Parathyroid Glands and Metabolic Bone Disease. Baltimore: Williams and Wilkins.

Conn E, Stumpf P K (1967). Outlines of Biochemistry. 2nd Edition. New York, London and Sydney: John Wiley and Sons, Inc.

Courpron P (1972). Dounees Histologiques Quantitatives sur le Viellissement Osseux Humain. Thesis of Medicine, University of Lyon, France.

Jackson W P U (1967). Calcium Metabolism and Bone Disease. London: Edward Arnold (Publishers) Ltd.

Nisbet J (1968). Metabolic Aspects of Bone Resorption in Tissue Culture. Ph.D. Thesis. University of Leeds.

Rasmussen H, Bordier P (1974). The Physiological and Cellular Basis of Metabolic Bone Disease. Baltimore: Williams and Wilkins Co.

Robison R (1932). The Significance of Phosphoric Esters in Metabolism. New York, University Press.

Singer F R (1977). Paget's Disease of Bone. New York and London: Plenum Medical Book Company.

Smith R (1979). Biochemical Disorders of the Skeleton. London, Boston: Butterworths.

Vaughan J (1975). The Physiology of Bone. 2nd Edition. Oxford: Clarendon Press.

Wood D (1975). Principles of Animal Physiology. 2nd Edition. London: Edward Arnold.

Yudkin M, Offard R (1971). A Guidebook to Biochemistry. Cambridge University Press.

CHAPTER EIGHT. APPENDIX: ADDITIONAL REVIEW OF RECENT LITERATURE

In the main text of the thesis, the origin, development and activities of the main bone cell types were discussed. Osteoclasts, the bone-resorbing cells, were found to originate from circulating monocytes (Mundy, et. al. 1977, 1978) and more recent studies by Bonnuci (1981) would seem to support this, with monocytes becoming macrophages and giant multinucleate cells, as are osteoclasts, in vitro (Sutton, et. al. 1972). Biochemical changes produced in the cells under the influence of various bone-resorbing agents, such as PTH, 1, 25$(OH)_2$ D_3, prostaglandins and osteoclast-activating factor, produced by lymphocytes, have been found to include increases in levels of hyaluronate and acid phosphatase activity (Luben, et. al. 1976; Wong, et. al. 1977; Raisz, et. al. 1981; Simmons, et. al. 1982). Physical changes include increased activity in the ruffled border of the osteoclasts, which accommodates enzyme-rich vacuoles (Rifkin, et. al. 1980; Bonnuci, 1981), and the discharge of enzymes into the extra-cellular space. The ruffling effect produces pseudopodia, which indeed make the cells motile in vitro (Chambers, et. al. 1982), the production of which is halted by calcitonin (CT), a potent inhibitor of bone resorption, apparently via a reduction in serum calcium, perhaps suggesting a role for pseudopodia in the mechanism of bone resorption. Osteoclasts are also found to undergo a continuous loss and incorporation of nuclei in the maintenance of their activity (Young, 1962).

It seems likely that osteoclasts do not have receptors for PTH, which stimulates bone resorption, but it is probable that they have CT receptors (Chambers, et. al. 1982). Parathyroid hormone

may influence osteoclastic bone via its effect on osteoblasts (Rodan, et. al. 1981) and the adenylate cyclase system (Luben, et.al. 1976; Braidman, et. al. 1981).

Osteoblasts, as well as osteoclasts, are influenced by PTH, prostaglandins and $1,25(OH)_2D_3$ (Rodan, et. al. 1981; Walters, et. al. 1982). In their studies of the role of osteoblasts in bone resorption, Rodan, et. al. (1981) have found that PTH, generally a bone-resorbing agent, affects osteoblasts, bone-forming cells, in several ways: increasing adenylate cyclase activity to increase cyclic AMP (cAMP), the activation of cAMP-dependent protein kinase, the inhibition of collagen synthesis, inhibition of AP activity, stimulation of Ca-uptake, production of cell-shape changes resulting in a more relaxed arrangement of osteoblasts. It would seem that these changes are communicated chemotactically to osteoclasts, which are attracted to the site and proceed to breakdown the bone matrix using their projections and the enzymes contained inside. Ramp, et. al. (1982) have found that PTH generally causes an increase in the level of cAMP within the skeleton and suggest a role for it as the secondary messenger involved in bone resorption.

Peck, et. al. (1981) showed that the cAMP increase associated with the stimulation of osteoblasts by PTH is directly linked to increases in extracellular Ca^{2+} concentrations, which in turn increase the activity of PTH. The results suggest that Ca^{2+} is also involved in communicating the influence of PTH, with Ca^{2+} in effect controlling the cAMP response to PTH stimulation of osteoblasts and therefore the activity of adenylate cyclase, which acts on ATP to produce cAMP, or by inhibiting the use of cAMP.

The more general effects of PTH, CT and $1,25(OH)_2D_3$ involve the control of serum calcium levels. Ramp, et. al. (1982) looked at the action of PTH on bone and found that it affects both bone formation and resorption, with high levels stimulating bone resorption and then bone formation, and lower levels influencing mainly bone formation; two sides to PTH activity, as found in ^{45}Ca, ^{47}Ca studies in the main text. The release of calcium from bone via osteoclastic bone resorption is, however, slow and PTH has been found to be responsible for speedy increases in the level of serum calcium, the suggestion now being that it acts on bone extracellular fluid, which somehow compels the prompt release of calcium from bone.

Various studies have shown that CT increases bone formation, although it has no direct effect on collagen formation or mineralisation CRaisz, et. al. 1981), but Weiss, et. al. (1981) have found that the administration of CT following a period of bone formation actually suppresses any further bone formation, meaning that CT also has a dual effect on bone metabolism. Where PTH stimulates the release of calcium into bone extracellular fluid, CT acts to inhibit that release (Ramp, et. al. 1982), which would seem to be important in the control of hypercalcaemia during feeding.

Vitamin D and its metabolites are known to have great influence on bone metabolism and calcium homeostasis, allowing the release of calcium from bone (Putkey, et. al. 1982; Ramp, et. al. 1982), with $1,25(OH)_2D_3$, for example, stimulating osteoclastic bone resorption and inhibiting bone collagen synthesis. In cases of severe vitamin D deficiency, bone fails to mineralise normally and there is a decrease in osteoclastic activity, however the administration of vitamin D_3 to such patients manifests itself in

the resumption of calcification, while no detectable change in serum calcium levels are found. The lack of change in serum calcium levels on the application of vitamin D_3 suggests that it too, like PTH and CT, somehow acts directly on bone in times of calcium crisis (Putkey, et. al. 1982). Interestingly, although PTH has been shown to act directly on bone, it cannot do so in vitamin D-deficient animals (Crowell, et. al. 1981).

It has been suggested that a lack of exposure to vitamin D in the environment, either from the sun or the diet, may be responsible for the expression of rickets and osteomalacia in certain communities, as discussed in the main text of the thesis. However, Sasson, et. al. (1982) maintain that phytate contained in the unleavened bread of Bedouins is responsible for their mineral defects; the argument will, doubtless, continue.

Many of the studies concerning the effects of various factors on bone metabolism have used in vitro cell and organ cultures as their experimental model, the importance of which have already been discussed earlier in the thesis. More recently, several groups have concentrated on the characterisation of the cells grown in culture and the isolation of specific cell types.

Parry, et. al. (1981) isolated cells from a human iliac crest bone biopsy of a 12 year old girl. Once established in culture the cells produced alkaline phosphatase levels 10x those found in the medium of comparable skin cells, collagen of the type associated with bone-forming cells was also detected; when cultured in chambers grown inside mammalian peritoneal cavities, the cells were found to have produced an amorphous calcium deposit. These results compare well with results following the isolation of living cells, successfully achieved by Bard, et. al. in 1972. It

is more common to isolate cells from calvaria of rodents, as attempted and described in the main text. Aubin, et. al. (1982) obtained many clones of cells on digestion of foetal rat calvaria and found cells responsive to PTH and prostaglandin E_2, the clones also produced type I (bone) and type III collagen as well as matrix components in the form of glycosaminoglycans.

Simmons, et. al. (1982) also cultured bone cells in the peritoneal cavities of mice, finding that osteoblast-like cells formed mineralised bone in proportion to AP activity, detected in the environs, and in association with type I collagen. Wong (1982) has been working on the separation of osteoblast-like (OB) and osteoclast-like (OC) cells using sedimentation techniques, producing cells of different sizes in 4 fractions, which were further characterised, following 6 days in culture, using biochemical criteria. These results indicate promise in the growth of bone from cells in vitro and more intricate experimentation concerning bone metabolism in vitro in the future.

The growth of bone from bone rudiments has been attempted for many years, with Rajan, et. al. (1970) placing the intact digits of 12-16 week human embryos in culture and growing the cartilagenous rod in culture until it had increased significantly in length, within 6 days. Recent attempts by Tenenbaum et. al. (1982), using periostea from 17-day-old emryonic chick calvaria, folded and grown in culture on plasma clots, produced osteoid tissue between the folds, which subsequently mineralised in the presence of the AP substrate beta-glycerophosphate.

Perhaps the formation of bone in vitro could be assessed using the new staining method developed by Kimmel, et. al. (1981), which stains bone and cartilage specifically and gives results within a few days. This stain could also be used, perhaps, to judge

the effects of metabolites on in vitro bone cultures, as it has been developed for use particularly with young bone.

Diphosphonates were mentioned in chapter seven and are generally thought to be inhibitors of bone turnover. Further studies looking at the possible uses of both naturally-occurring and synthetic diphasphonate compounds have produced promising results. The diphosphonates disodium ethylidine-1-hydroxy-1,1-diphosphonate (EHDP) and dichloromethylene diphosphonate (CI_2MDP), have been shown to inhibit soft tissue calcification and bone resorption, but McGuire, et. al. (1982) suggest a possible role for it in preventing inflammation which produces arthritis. However, a study by Boyce, et. al. (1982) has shown that while EHDP does reduce mineralisation, but at the doses required to reduce the increased bone resorption found in Paget's Disease, the decrease in mineralisation is so dramatic as to result in osteomalacia, therefore strict control of the drug dosage is required. A study of the activity of EHDP has also shown that it affects all bone cell types and not one specifically (Felix, et. al. 1981).

The main interest in this thesis concerned osteoporosis, a bone disorder found most commonly in post-menopausal women. A common factor thought to be the cause of the decrease in bone mass found in these individuals is the notable decrease in serum oestrogen levels, consequently oestrogen has been used in the treatment of this post-menopausal bone loss. Nevertheless, a recent survey by Boyle (1982) shows that many general practitioners prescribe vitamin D, on its own or in association with oestrogen, as treatment, suggesting that vitamin D deficiency may also play a part in this disorder. The use of eostrogen in the treatment of bone loss would also seem to be inadvisable following the work by

Raisz, et. al. (1981) which shows that oestrogen actually decreases bone growth and that formation of bone at the menopause is actually maintained, even increased. They found that there are no oestrogen receptors in bone and that it has no particular effect on collagen synthesis or bone resorption. Results did suggest that oestrogen may act on vitamin D, indicating perhaps that the G.P.'s are correct in their treatment, that it may increase muscle mass and so increase bone mass by mechanical stress, but that resorption of bone at the menopause probably increases as a result of some osteoblastic defect.

An interesting paper by Mazess (1982) shows that there is no history of sustained loss of trabecular bone following the menopause, that minimal changes in the mineral content of bone, alone, may in effect greatly alter the strength of bone, making it liable to pseudofractures, or decreases in the circulation may reduce the amount of energy available to sustain the bone, leading to a reduction in mechanical strength. Mazess (1982) also shows that men also lose bone as they get older, although less than women, and that the loss is slow and progressive, but accelerated in women at the menopause. Oestrogen does inhibit the loss of bone at the menopause, but its effectiveness as a preventive agent decreases again, perhaps indicating that, after a while, the loss of oestrogen is no longer a factor in ageing bone loss.

Another possible 'cure' or treatment of osteoporosis, which, although it may not be caused solely by the loss of oestrogen, is still a dominant feature in the elderly, has been studied by Dent, et. al. (1980). They looked at a microcrystalline calcium hydroxy-apatite compound (MCHC), a bone mineral compound, in association with dihydrotachysterol, and found that the treatment improved the calcium balance in patients with osteogenesis inperfecta, although

it produced no obvious changes in the bone itself, and they hope that it may be used for osteoporotic patients. Clearly, though, a greater understanding of the causes and effects of bone disorders and disorders of calcium metabolism is needed before any great steps can be made in their eradication.

References to Appendix

Aubin J E, Heersche J N M, Merrilees M J, Sadek J (1982). Isolation of Bone Cell Clones with Differences in Growth, Hormonal Responses and Extracellular Matrix Production. Journal of Cell Biology, 92(2), 452-61.

Bard D R, Dickens M J, Smith A U, Zarek J M (1972). Isolation of Living Cells from Mature Mammalian Bone. Nature, 236, 314-315.

Bonnuci E (1981). New Knowledge on the Origin, Function and Fate of Osteoclasts. Clinical Orthopaedics, 158, 256-269.

Boyce B F, Smith L, Fogelman I, Ralston S, Boylde I T (1982). Diphosphonates and Inhibition of Bone Mineralisation. Lancet, 1(8278), 964-965.

Boyle I T (1982). Treatments for Postmenopausal Osteoporosis. Lancet, 2(8234), 1376.

Braidman I P, Anderson D C (1981). Studies on Separated Foetal Rat Bone Cell Populations - A New View of PTH-induced Bone Resorption. Abstracts of the Bone and Tooth Society, London.

Chambers T J, Magnus C J (1982). Calcitonin Alters Behaviour of Isolated Osteoblasts. Journal of Pathology, 136(1), 27-39.

Crowell J A, Cooper C W, Toverud S U, Boass A (1981). Influence of Vitamin D on Parathyroid Hormone-Induced Adenosine $3^{-},5^{-}$-Monophosphate Production by Bone Cells Isolated from Rat Calvaria. Endocrinology, 109(5), 1715-1722.

Dent C E, Dayies I J T (1980). Calcium Metabolism in Bone Disease. Journal of the Royal Society of Medicine, 73, 780-785.

Felix R, Fleisch H (1981). The Effect of Diphosphonates on Periosteal and Bone Cells in Culture. Experimentia, 37(8), 817-819.

Kimmel C A, Trammel C (1981). A Rapid Procedure for Routine Double Staining of Cartilage and Bone in Foetal and Adult Animals. Stain Technology, 56(5), 271-273.

Luben R A, Wong G L, Cohn D V (1976). Biochemical Characterisation with Parathyroid Hormone and Calcitonin of Isolated Bone Cells: Provisional Identification of Osteoclasts and Osteoblasts. Endocrinology, 99(2), 526-34.

Mazess R B (1982). On Ageing Bone Loss. Clinical Orthopaedics, 165, 239-252.

McGuire M K B, Russell R G G, Murphy G, Reynolds J J (1981). Diphosphonates Inhibit Production of Prostaglandins and Collagenase by Human Cells in vitro. Annals of Rheumatic Disorders, 40(5), 575.

Parry R, Green J R, Harvey J J, Nicholls A C, Reeve J, Tyrell D A J (1981). A Method for the Cultivation and Selection of Human Bone Cells. Abstracts of the Bone and Tooth Society, London.

Peck W A, Kohler G, Barr J (1981). Calcium-mediated Enhancement of the Cyclic AMP Response in Cultured Bone Cells. Calcified Tissues International, 33(4), 409-416.

Putkey J, Norman A W (1982). Hormonal Regulation of Calcium Homeostasis I: Vitamin D. In: The Role of Calcium in Biological Systems, Volume II. Eds. Anghileri L J, Tuffet-Anghileri A M. CRC Press, Inc. Boca Raton, Florida, 171-202.

Raisz L G, Kream B E (1981). Hormonal Control of Skeletal Growth. Annual Reviews in Physiology, 43, 225-238.

Rajan K J, Hopkins A M (1970). Human Digits in Organ Culture. Nature, 227, 621-622.

Ramp W R, Waite L C (1982). Hormonal Regulation of Calcium Homeostasis II: Parathyroid Hormone, Calcitonin and Summary. In: The Role of Calcium in Biological Systems, Volume II. Eds. Anghileri L J. Tuffet-Anghileri A M. CRC Press, Inc. Boca Raton, Florida, 203-215.

Rifkin B R, Brand J S, Cushing J E, Coleman S J, Sanovi F (1980). Fine Structure of Foetal Rat Calvarium; Provisional Identification of Preosteoclasts. Calcified Tissues International, 31(1), 21-28.

Rodan G A, Martin T J (1981). Role of Osteoblasts in Hormonal Control of Bone Resorption - A Hypothesis. Calcified Tissues International, 33(4), 349-351.

Sasson A, Etzion Z, Shany S, Berlyne G M, Yagil R (1982). Growth and Bone Mineralisation Affected by Dietary Calcium, Phytic Acid and Vitamin D. Comparative Biochemistry and Physiology, 72A(1), 43-48.

Simmons D J, Kent G N, Jilka R L, Scott D M, Fallon M, Cohn D V (1982). Formation of Bone by Isolated, Cultured Osteoblasts in Millipore Diffusion Chambers. Calcified Tissues International, 34(3), 291-294.

Sutton G G, Ghash L, Dorfman H B (1972). Ultrastructure of Giant Cell Tumours of Bone. Human Pathology, 3, 569.

Tenenbaum H C, Heersche J N M (1982). Differentiation of Osteoblasts and Formation of Mineralised Bone in vitro. Calcified Tissues International, 34(1), 76-79.

Walters M R, Rosen D M, Norman A W, Luben R A (1982). $1,25(OH)_2D_3$ Receptors in an Established Bone Cell Line. Journal of Biological Chemistry, 257, 7481-7484.

Weiss R E, Singer F R, Gorn A H, Hofer D P (1981). Calcitonin Stimulates Bone Formation When Administered Prior to Initiation of Osteogenesis. Journal of Clinical Investigation, 68, 815-818.

Wong G L (1982). Characterisation of Subpopulations of OC and OB Bone Cells Obtained by Sedimentation at Unit Gravity. Calcified Tissues International, 34(1), 67-75.

Young R Y (1962). Cell Proliferation and Specialisation During Endochondral Osteogenesis in Young Rats. Journal of Cell Biology, 14, 357.