

ALTERED BONE METABOLISM  
IN THE PROTEIN DEPRIVED RAT

by

DEREK LE ROITH

A Thesis submitted for the degree, doctor of Philosophy to  
the Department of Medicine, University of Cape Town.

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To Galia and Tania.

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### INTRODUCTION

The twentieth century has seen outstanding advances in science, communication and space travel. Remarkable achievements such as modern heart surgery and hormonal biosynthesis have revolutionised the practice of medicine. Despite these achievements, malnutrition remains one of the most serious problems facing the world and the medical profession alike. It has been estimated that 60% of the world's preschool population suffer from some degree of protein-calorie malnutrition<sup>(1)</sup>.

In South Africa today the problem of malnutrition amongst the poorer socio-economic groups is in many ways similar to that seen in the indigenous populations of the rest of Africa. It is therefore not surprising that considerable interest and research has been directed towards this problem<sup>(2)</sup>, both at the Medical School of the University of Cape Town and in other centres throughout the world.

Protein-calorie malnutrition embraces a wide spectrum of nutritional deficiency. Kwashiorkor and marasmus can be regarded as two extremes of this spectrum, with the intermediate syndrome by far the commonest form. The major etiological factor responsible for the syndrome is a dietary lack of protein which may be associated with a concomitant lack of calories<sup>(3)</sup>.

Since adequate dietary protein is essential for normal body function and growth<sup>(4)</sup>, it is not unexpected therefore that retardation of growth is the earliest and commonest manifestation of protein-calorie malnutrition. Arrested growth due to inadequate protein intake manifests as a reduction in body length and weight for age.

It is this reduction in body length which is produced by retardation of skeletal growth<sup>(5,6)</sup>. It has been estimated that one third by weight and three quarters by volume, of the total body skeleton is comprised of protein in the form of collagen<sup>(7)</sup>. It is therefore conceivable that normal skeletal growth and turnover may be severely affected in protein deprivational states.

In contrast to the vast amount of active research and available knowledge on many aspects of protein-calorie malnutrition, surprisingly little attention has been focused on the skeletal effects of this syndrome. As early as the 1920's it was realised that the skeletal metabolism in protein-calorie malnutrition was deranged. Notwithstanding this, there is still to date inadequate understanding of the mechanisms by which these skeletal changes are brought about. This is partly due to the fact that the research workers were "victims of their times" in that the techniques utilised gave limited information and it has only been in the past decade or two that more sophisticated techniques have become available. (Evidence for this will be borne out in the following chapter where the literature is reviewed). It was with this in mind that the work presented in this thesis was undertaken.

Part I of the thesis consists of a review of the literature, followed by a dissertation on the normal physiological concepts of calcium metabolism as well as a description of the calcium pools used in kinetic models.

Part II includes all the chapters on the various experiments performed. Included in each chapter are the methods used, results obtained as well as a discourse relevant to that particular study, with special reference to similar experiments performed by other authors.

Part III encompasses the integrated summary of the results and a discussion theorising the mechanisms involved in those changes.

Finally, an appendix which deals with the statistical methods used for analyses, as well as details of those techniques which were used, but were established by other authors.

PART I

## CHAPTER ONE

REVIEW OF THE LITERATURE

The earliest workers to notice abnormalities of the skeleton in protein-calorie malnutrition were HARRIS<sup>(8)</sup> and ELIOT et al<sup>(9)</sup>. They observed radiological changes in the growing ends of the long bones in children suffering from protein deprivational states. These changes took the form of "transverse trabeculations", or what were later termed growth arrest lines. For the following two decades studies were limited to post mortem histology, radiological findings and measurements of the chemical composition of the serum and tissues.

In 1954, a detailed and erudite review of the histological appearances was presented by Higginson<sup>(10)</sup>, who performed postmortem microscopic evaluations of the bones of Bantu children dying of kwashiorkor. Reduced thickness of the epiphyseal cartilage plate in all the bones was the most striking feature. He also found the metaphyseal trabeculation to be abnormal with the formation of transverse bars. These bars corresponded closely to the transverse trabeculations seen radiologically. Osteoblastic and osteoclastic cells were reduced in number. In addition to the transverse trabeculations skeletal immaturity and retarded growth of the long bones was found<sup>(11)</sup>.

Subsequently more detailed radiological observations were reported including cortical deficiency of the long bones<sup>(12)</sup>, and trabecular bone loss<sup>(13)</sup>. Some authors<sup>(14)</sup> maintained that only a small proportion of their cases of kwashiorkor showed radiological changes, whilst others<sup>(15)</sup> suggested that many of these children also

had varying degrees of rickets which probably complicated the radiological assessment.

Dreizen et al<sup>(16)</sup> in reviewing the causes of transverse trabeculation, also noted that children suffering from malnutrition commonly exhibited this radiological sign. He maintained however, that during normal development healthy children may likewise show these trabeculations transiently. Children suffering from either systemic infections, vitamin or mineral deficiencies were also found to exhibit these radiographic changes.

Normal skeletal development maybe influenced by a deficiency of calories, protein or vitamins in starvation. All of these may produce nonspecific bone changes<sup>(17)</sup>. The likely coexistence of so many inter-related and causative factors in the pathogenesis of human protein-calorie malnutrition makes the interpretation of data somewhat difficult. For this reason many workers turned to the laboratory animal in an attempt to produce a pure model of protein-calorie malnutrition.

A protein deprivational state was established and studied in pigs<sup>(18)</sup>. Similar changes to those found in malnourished children were seen. Transverse trabeculation, generalised rarefaction (osteoporosis) and epiphyseal thinning were found radiologically together with normal mineral content. Growth in length of the long bones was more markedly reduced than lateral growth. Models utilising rats<sup>(19)</sup> and monkeys<sup>(20)</sup> have likewise been studied with similar findings.

Studies on the biochemical composition of the bones revealed that although bone growth was retarded by protein deprivation, the mineral content was normal<sup>(18,20,21,22,23)</sup>.

More recently dynamic studies have been performed on animal models. Ultraviolet light fluorescence of tetracycline deposited in bones has been used as a measurement of the rate of bone formation. These studies in protein depleted monkeys showed a marked reduction in appositional bone growth<sup>(20)</sup>. Radiocalcium has been used as a tracer in kinetic studies designed to measure calcium accretion. In protein deprived rats measurements of accretion rates were found to be reduced<sup>(23)</sup>.

No comprehensive study of disordered bone metabolism is complete without studies on collagen turnover<sup>(24)</sup>. Collagen, which comprises 95% of bone matrix, is a unique protein and almost certainly is affected in protein privational states. Studies were performed using urinary hydroxyproline excretion as a measure of the collagen turnover<sup>(25)</sup>. The excretion was found to be low in both human kwashiorkor<sup>(26,27)</sup> and animal models<sup>(28,29)</sup> of protein calorie malnutrition. The significance of this reduced excretion is doubtful since urinary hydroxyproline also reflects skin collagen turnover which is even more severely affected in protein-calorie malnutrition<sup>(22,30,31)</sup>.

#### SUMMARY

Outlined in this chapter is the earlier work described on the skeletal effects of protein deprivation. Further details of the data presented by these authors will be discussed, more fully, under the relevant chapters.

The skeletal changes due solely to protein depletion are masked in the individual case by the fact that Kwashiorkor is commonly associated with multiple deficiencies. Notwithstanding this, however,

it seems that the animal models studied show similar morphological features to those described in human kwashiorkor. What remains to be elucidated are the mechanisms involved in these changes.

Bone formation proceeds in an orderly manner. Firstly matrix deposition occurs followed closely by calcification of this protein matrix. Platt and Stewart<sup>(18)</sup> suggested that inadequate protein supply during the critical growth period may affect chondroblastic activity with resultant matrix deficiency. McCance and Widdowson<sup>(32)</sup> however suggested that protein is vital for intestinal calcium absorption. A diet lacking in protein may then cause reduced calcium absorption and thereby interfere with bone formation.

It was the object of this thesis to study and elucidate the mechanisms involved in the skeletal changes of protein deprivation. For this purpose a laboratory animal model has been chosen and various dynamic techniques are included in the studies performed.

## CHAPTER TWO

CALCIUM PHYSIOLOGYINTRODUCTION

A brief outline of calcium physiology and the compartmentalisation of calcium into various physiological pools is presented in this chapter. Many of the dynamic studies utilised in this thesis are based on the understanding of these concepts.

PHYSIOLOGY OF CALCIUM IN THE INTACT ORGANISM

Calcium is one of the most important divalent ions in living organisms. There are many physiological functions of calcium unrelated to bone metabolism. These include membrane permeability, neuromuscular transmission, coagulation and hormonal secretion.

Calcium exists in the serum in three fractions:

- |                  |   |                        |
|------------------|---|------------------------|
| 1) Ionised       | } | diffusible ... 60%     |
| 2) Non-ionised   |   |                        |
| 3) Albumin bound |   | non-diffusible ... 40% |

Calcium in bone exists either in the form of hydroxyapatite  $\left[ \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \right]$  the stable bone mineral crystal, or in the ionised form. The ionised calcium is present in the superficial hydration shell which comprises the extra-cellular fluid surrounding the bone cells and which is in equilibrium with other tissue fluids. In contrast to the high concentration of calcium in extracellular fluids, the concentration of intracellular calcium is very small and usually insignificant.

Dietary calcium is absorbed from the gastro-intestinal tract and rapidly enters the serum, from where it diffuses through a number of larger calcium pools. The extracellular fluid compartment includes the "hydration shell" of the bone (Figure 1).

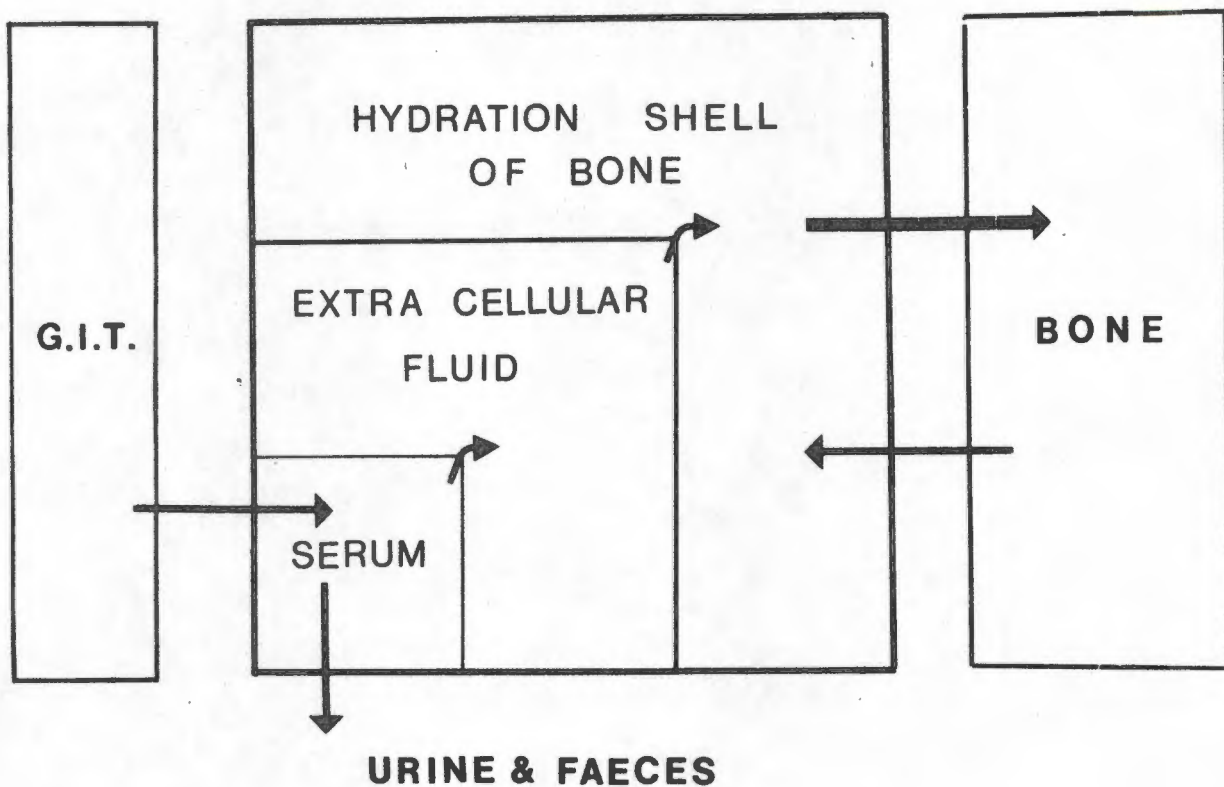
Bone formation is achieved by the laying down of collagen (matrix) followed by the nucleation of hydroxyapatite (calcium-phosphate crystals). This calcium is derived from the extrasosseous pools of calcium and the rate of laying down of calcium into this stable hydroxyapatite is the rate limiting process known as the accretion rate of calcium into bone. Rapid exchanges of calcium occur continuously between the various pools, but it is felt that this accretion of calcium into bone is unidirectional<sup>(33)</sup>.

Resorption of bone involves the simultaneous dissolution of the hydroxyapatite crystal and break down of the collagen matrix with resultant return of bone calcium into the extra-osseous calcium pools<sup>(34)</sup>.

Calcium excretion from the whole body occurs continuously via urine and faeces, the intestinal loss being described as endogenous faecal calcium. Faecal calcium is thus comprised of endogenous faecal calcium and unabsorbed dietary calcium.

#### A SIMPLIFIED POOL CONCEPT

Early studies of derangements in bone metabolism have utilised balance studies, but since the introduction of tracer studies it has become possible to study the dynamic interchange of calcium between the various pools of the body<sup>(35)</sup>. This requires the delineation of various pools.



**FIGURE 1**

The physiological compartmentalisation of calcium. The diffusion between the serum, extracellular fluid and hydration shell is rapid. In contrast, the accretion and resorption of calcium are rate limiting, unidirectional processes.

Many models have been constructed in attempting to define the pools and though it is well recognised that many pools exist, it is more practical to confine any particular study to a simplified model<sup>(36)</sup>.

The model shown in figure 2 is a simplification of that from figure 1, where all the extraosseous exchangeable calcium pools are incorporated, conceptually, into one single compartment called the "exchangeable calcium pool". Since the various anatomical pools making up this larger compartment are in rapid equilibrium with each other it becomes feasible to consider this exchangeable calcium pool as a single physiological unit. Obviously this precludes the measurements of the rates of interchange between the different pools. However, the computation required to analyse the results derived from these multicompartmental kinetic models is not practical. It is currently believed that there is little to choose between the various published compartmental models, since they all involve the same basic assumptions and give similar results<sup>(36)</sup>.

## COMPARTMENTAL POOLS

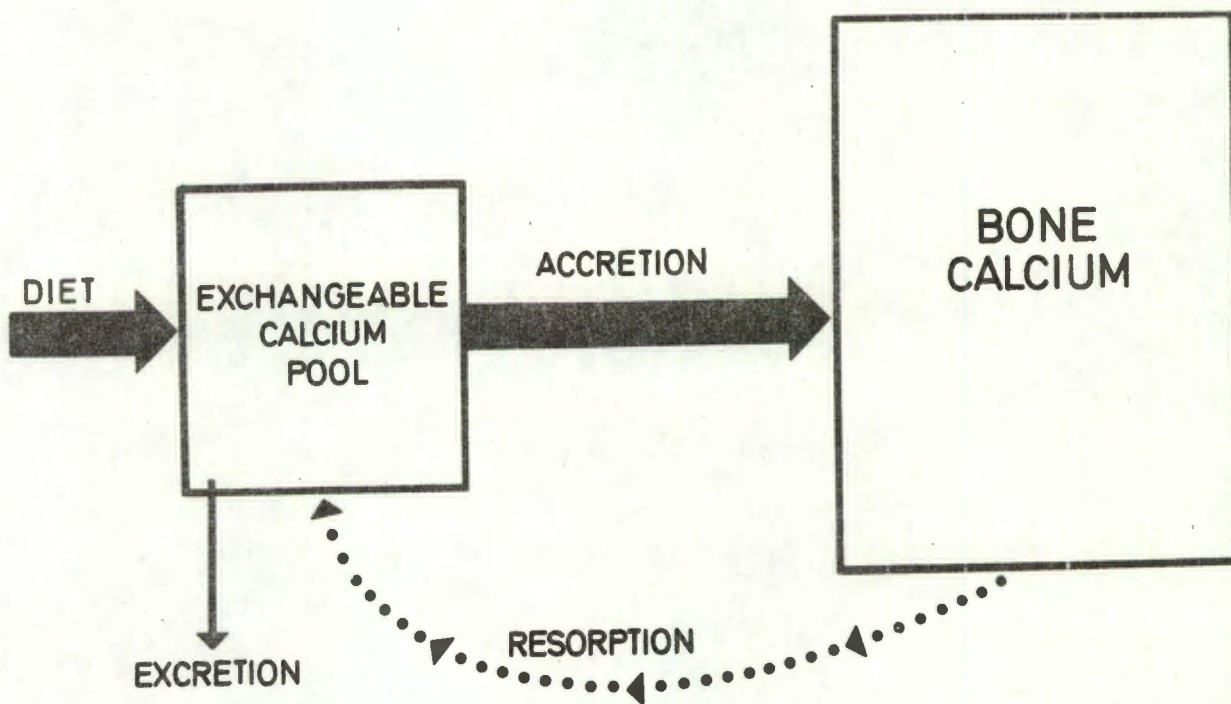


FIGURE 2

The single compartmental model, in which all the extraosseous calcium pools are incorporated, conceptually, into one single compartment, the exchangeable calcium pool.

## CHAPTER THREE

EXPERIMENTAL MODEL OF PROTEIN MALNUTRITIONINTRODUCTION

Isolated deficiencies of protein, calorie, vitamins or minerals are rare in the clinical syndromes of malnutrition. By far and away the most common is a multiple deficiency state of any of the above, which may be compounded by the presence of infection<sup>(37)</sup>. Since the purpose of this project was to study the specific effects of pure protein depletion on bone metabolism, it was imperative to utilise a model of protein deprivation which was unaffected by other deficiencies. In this regard the experimental laboratory animal is ideal.

In a research project where adequate numbers of animals are a prerequisite, the rat is a most convenient species. The rapidity at which this species procreate and the ease with which they are housed and maintained, outweighs the disadvantages inherent in a species which is unrelated to humans, when extrapolation of experimental data is desired.

Protein deficient states have been produced in the rat<sup>(38,39,40,41,42)</sup>. Essential to the production of a pure protein depleted state is the intake of a low protein diet. Reduced protein intake, however, results in a certain degree of inanition with resultant calorie deficiency<sup>(43,44)</sup>. Rats fed on a low protein diet will rapidly reduce their food intake as an adaptation to this low protein diet<sup>(45)</sup>.

In choosing the appropriate model, four alternatives were examined, three of which were techniques designed to overcome the problem of reduced food intake resulting from the low protein diet.

- (a) Adult rats fed a low protein diet ad lib<sup>(40)</sup>.
- (b) Intragastric "force feeding" a low protein diet<sup>(39)</sup>.
- (c) Early weaned rats fed a low protein diet ad lib<sup>(42)</sup>.
- (d) Interval feeding of a low protein diet to early post weaned rats<sup>(42)</sup>.

Reduced total food intake prevented the use of technique (a); whereas technique (b) produced an acute depletion state which resulted in an unacceptably high mortality rate. Technique (c) resulted in a protein depleted rat which was too small for adequate examination of the state of calcium metabolism, by the techniques described in the following chapters. Technique (d) "interval" or "meal feeding"<sup>(44,46,47)</sup> was adapted<sup>(42)</sup> to reduce the anorexia normally associated with amino acid imbalance<sup>(48)</sup>. This feeding technique resulted in the maintenance of calorie intake equal to the control rats, even in the face of a dietary protein deficiency. Thus adequate calorie intake, together with protein depletion resulted in a rat model in which the effects of pure protein depletion could be studied.

## METHODS

### (a) FEEDING REGIME

Male rats of the WISTAR strain were weaned and fed a standard laboratory cube diet until they weighed 70 - 90 g. They were then placed in groups of 4 - 5 rats in stainless steel wire-bottom cages. The room housing the animals was air conditioned and maintained at 22°C, with artificial light for 10 hours daily.

Each group was trained to consume its daily ration of food within a regular two hourly period (8.00 a.m. - 10.00 a.m.) after which they were allowed only water for the 22 hours remaining. 20% casein diets (Table I) were utilised for this one week training period. They were then divided into two groups of cages. One group (henceforth labelled "experimental") was fed a low protein diet (4% casein - Table I). The other group (age matched controls) continued on the normal protein (20% casein) diet. The diets were isocaloric and contained equal vitamin and mineral supplements. Methionine was added to the 4% diet to obviate any deficiency changes due specifically to methionine deficiency, which is the first limiting amino acid in casein<sup>(49)</sup>.

The rats were maintained on this feeding regime for 35 days. Daily food consumption was measured by weighing the food containers before and after the two hour meal, as well as accounting for any food spilt during the meal. Body weights were measured twice weekly prior to the meal.

At the end of the thirty-five day feeding period the rats were subjected to various experiments as described in the following chapters.

In order to substantiate the efficacy of this feeding regime in producing a model of pure protein depletion, numbers of groups were anaesthetised under light ether anaesthesia and sacrificed. Blood was withdrawn from the abdominal aorta and both tibia were cleaned, fixed in 10% formalin, decalcified and mounted in paraffin wax. Livers were subjected to frozen section examination. Serum albumin was measured by the buiret method<sup>(50)</sup>. Histological sections of the liver and epiphyseal plates of bones were cut and stained. Haematoxylin and eosin stains were performed on the bone and sudan staining for fat was performed on the liver sections.

TABLE I

COMPOSITION OF THE DIETS (g/100g)

<u>Ingredient</u>	<u>4% protein</u>	<u>20% protein</u>
Casein	4.0	20.0
Dextrin	85.4	69.7
Mineral Mixture	4.0	4.0
Vitamin Mixture	1.0	1.0
Maize oil	4.0	4.0
Cod-liver oil	1.0	1.0
Choline chloride	0.3	0.3
DL-Methionine	0.3	-

Both diets contained an equal number of joules/g the only difference relating to the proportion of protein. They were equally supplemented with vitamin D (1% w/v cod liver oil), calcium (4.9 g/kg food) and phosphorus (3.05 g/kg).

(b) ADDITIONAL CONTROL GROUPS OF RATS

Three additional groups of rats were included in the project as controls.

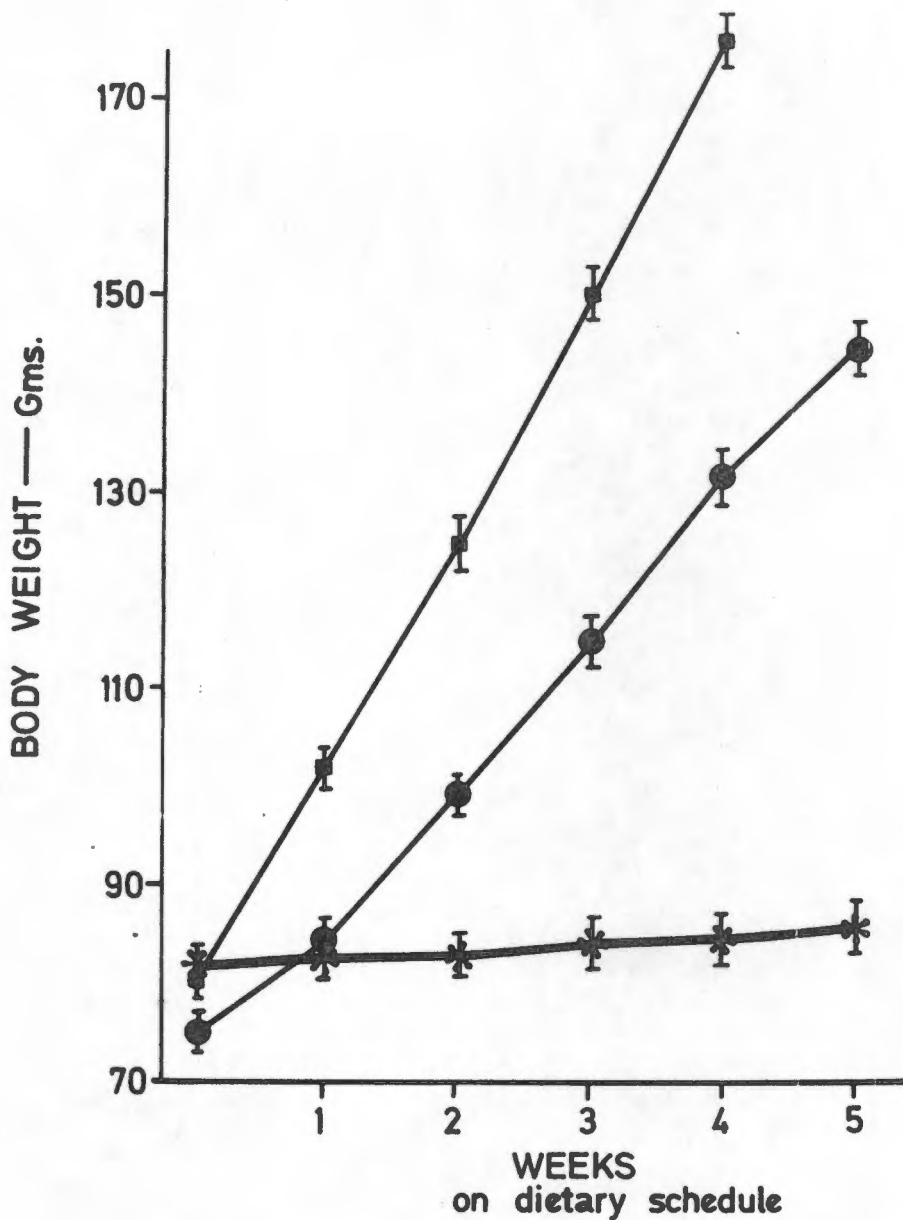
(i) Weight matched controls. Since the experimental rats had weights markedly less than the 20% controls it was necessary to include a control group of rats whose weights were matched to the experimental group. These baseline control rats were studied two weeks after weaning when their body weights were comparable to the protein deprived group.

(ii) In order to exclude rickets as being responsible for the bony changes in protein deprived rats, age matched rachitic rats were studied after being housed in dark cages and fed a vitamin D deficient diet, rich in calcium and low in phosphorus<sup>(51,52)</sup>. Histological sections were made of the epiphyseal ends of the long bones in these rachitic rats.

(iii) Finally a further group of rats were fed 20% casein diet ad lib for a thirty-five day period in order to compare the effect of meal feeding with ad lib feeding.

RESULTS

During the first week, interval-feeding a 20% casein diet caused an initial fall in body weight. This was due to a reduction in food intake, but once the rats became well-trained, they acquired a normal intake and attained their initial starting weights at the end of that week<sup>(53)</sup>.



**Figure 3.** The contrast in weight gain between groups of twenty-four rats fed ad lib (■), 20% casein by meal-feeding (●), and 4% casein by meal-feeding (✱).

The mean  $\pm$  SEM is shown.

TABLE II

FOOD CONSUMPTIONProtein deprived experimental (4%)

Weeks on diet	1	2	3	4	5	6
g/day	7.9	7.7	7.9	7.6	8.1	8.0
g/100g body weight	9.6	9.1	9.3	8.8	9.5	9.2

Age-match controls (20%)

Weeks on diet	1	2	3	4	5	6
g/day	6.3	8.0	9.3	10.6	12.2	12.9
g/100g body weight	8.8	10.0	9.9	8.6	9.4	8.8

Mean daily food consumption of twenty-four protein deprived and twenty-four age-matched control rats, showing no reduction in the proportional food intake of the protein deprived group.

Protein deprivation is characterised by growth retardation, a low serum albumin, periportal fatty infiltration of the liver and inactivity of the growth plates at the epiphyseal ends of the long bones<sup>(54)</sup>.

Growth retardation in the experimental group is illustrated in figure 3 where weekly body weights of each group are shown. Age matched control rats gained weight at a rate almost equal to the growth rate of ad lib fed rats of the same strain. The experimental group showed no appreciable weight gain, over the thirty-five day period.

In order to provide a means of comparison between the smaller experimental group of rats and the larger age matched controls, food intake was calculated as grammes consumed per 100 g. body weight.

As shown in table II, the experimental group of rats did not reduce their food consumption below that of the control group when food consumption was expressed as g. eaten per 100 g. body weight.

Serum albumins were significantly reduced in the experimental group (Table III).

Fatty infiltration of the liver in varying degrees was demonstrated in the livers of experimental rats whereas this change was not seen in control rats (figure 4).

Experimental rats had narrower epiphyseal cartilage plates and did not exhibit excess osteoid when compared to age matched controls. Rats on the vitamin D deficient diet had widened epiphyseal cartilage plates and excessive osteoid (figure 5).

TABLE III

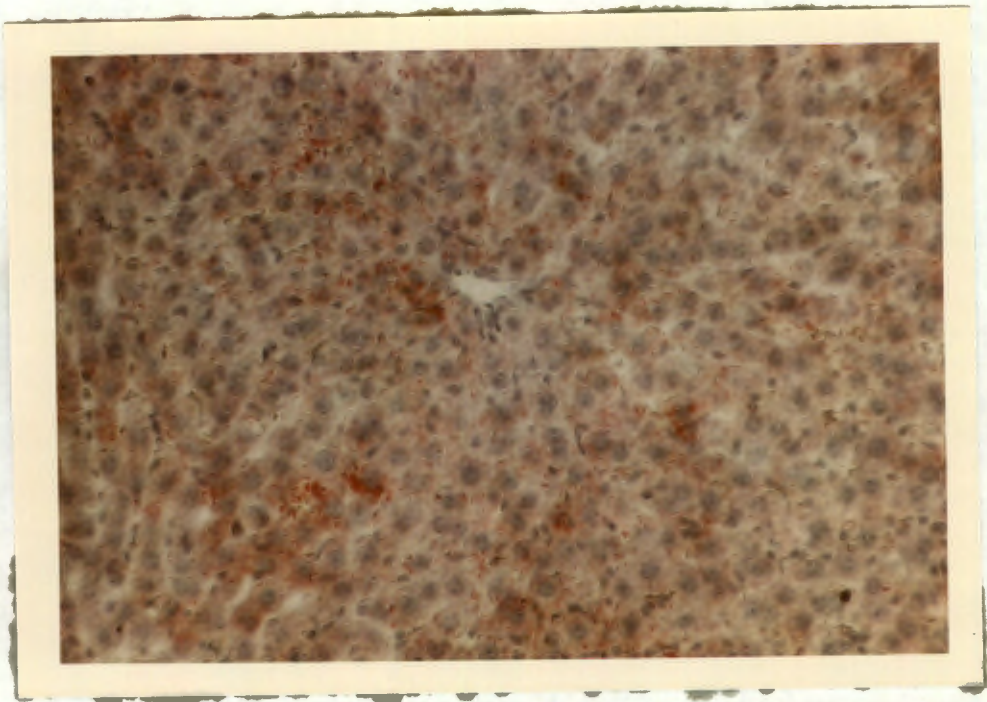
SERUM ALBUMIN (g/100 ml)

<u>Groups</u>	<u>No. of rats</u>	<u>Mean values (SEM)</u>
Protein deprived experimental (4%)	20	2.16 (0.05)
Age-matched controls (20%)	15	3.20 (0.09)

Significant reduction of the serum albumin concentration, in the experimental group (4%) was demonstrated by the t test.

(t = 10.704,  $p < 0.001$ )

(a)



(b)

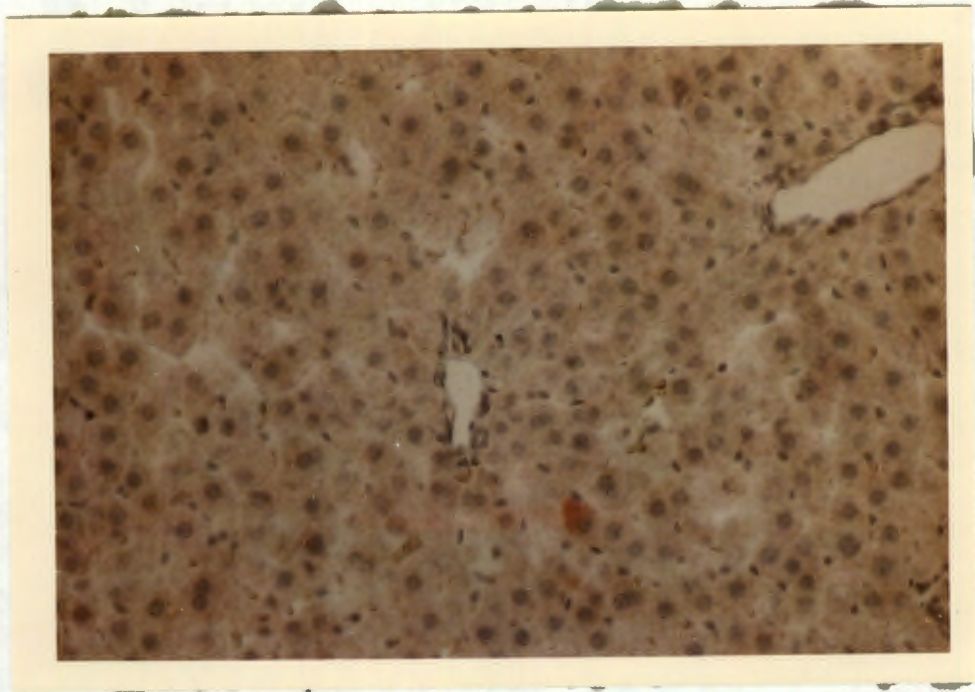


Figure 4 (a and b). Sudan staining for fat, in frozen sections of the liver from

(a) protein deprived (4%) rat, and

(b) age-matched control rat (20%).

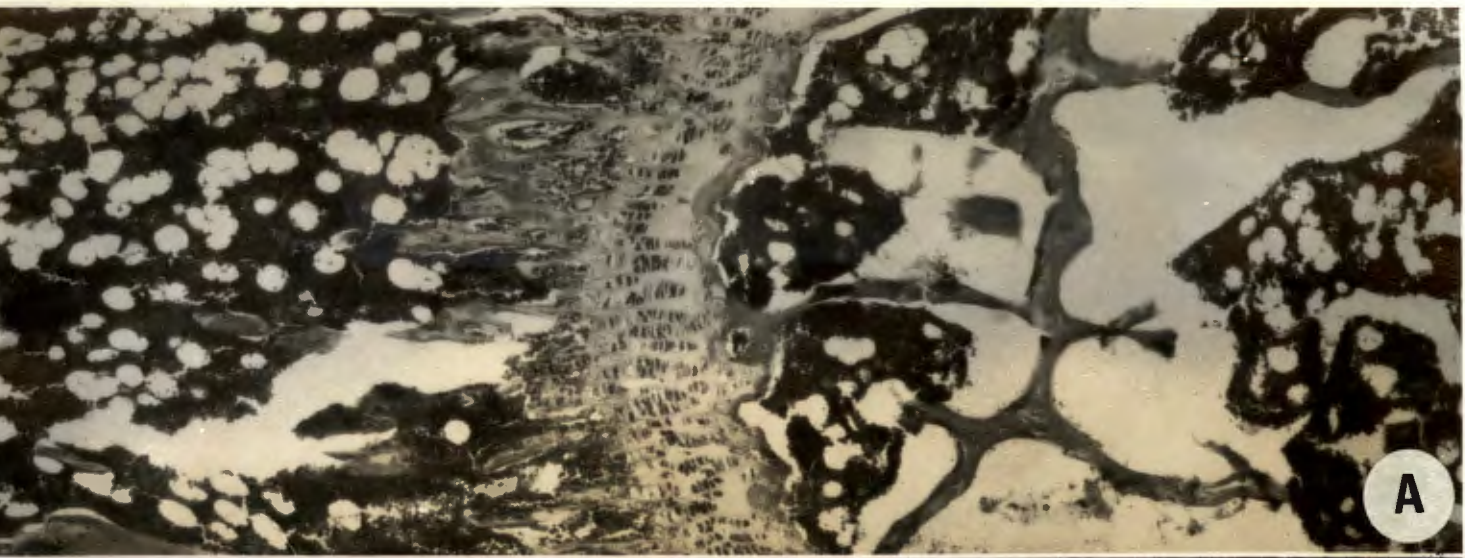
Fatty infiltration of the liver was marked in the experimental (4%) group.

Figure 5

Haematoxylin and eosin stains of decalcified sections of the epiphyseal ends of the long bones from

- (a) a protein deprived rat (4%),
- (b) age-matched control (20%), and
- (c) an age-matched rachitic rat.

Protein deprived rats showed narrower epiphyseal plates and no increase in the amount of osteoid when compared to age-matched controls. Rachitic rats demonstrated widened epiphyseal cartilage plates and excessive osteoid. (Photograph overleaf).



A



B



C

CRIP

## DISCUSSION

Protein deprived, post weaned rats as an experimental model for human protein malnutrition has previously been validated<sup>(42)</sup>. It was necessary however to evaluate this model and test its reproducibility.

In addition to the meal-fed experimental and the control groups of rats, three groups of controls were included. The weight matched controls were studied as a baseline group since they matched the weight of the smaller experimental rats and were therefore only two weeks post weaning. Age matched rachitic rats were included to demonstrate that the metabolic changes in the skeleton were not due to vitamin D deficiency. The inclusion of rats fed a 20% casein diet ad lib demonstrated that the meal fed control group had only a small reduction in calorie intake.

The characteristics of protein calorie malnutrition include growth retardation, a lowered serum albumin, fatty infiltration of the liver and thinning of the epiphyseal cartilage all of which were produced in the experimental groups. Despite adequate food consumption, when expressed as g. eaten per 100 g. body weight, the experimental group failed to gain weight. This may therefore be concluded as being due to pure protein deprivation since the diets were isocaloric. In order to exclude the effects of mineral or vitamin deficiencies on skeletal metabolism, adequate and equal supplementation of all vitamins and minerals required were added to the diets (Table I). 4.9 g. of calcium<sup>(55)</sup> and 3.0 g. of phosphorus were present in each Kg. of the diet. The ratio of 3 : 2 calcium to phosphorus being ideal for normal skeletal growth development<sup>(51,56)</sup>.

Adequate vitamin D was ensured by the addition of cod liver oil (1.0%) which supplied 200 international units per 100 g. diet. Magnesium was present in the concentration of 0.8 g./kg. diet.

Further confirmation was afforded by the demonstration that rachitic changes could only be produced in a similar strain of rat by housing in a dark cage, and feeding the rats a diet completely lacking in vitamin D with a high calcium and low phosphorus content (figure 5).

#### SUMMARY

The use of the protein deprived post weaned rats as an experimental model was tested and found to be valid in that the growth retardation, lowered serum albumin, fatty infiltration of the liver and thinned epiphyseal plates simulated other models of protein malnutrition<sup>(44,54)</sup>.

PART . I I

## CHAPTER FOUR

SERUM CALCIUM, PHOSPHORUS, MAGNESIUM AND ALKALINE PHOSPHATASEINTRODUCTION

Appropriate concentrations of calcium, magnesium and phosphate ions are essential for the proper working of many of the more delicate cellular mechanisms throughout the body.

In relation to skeletal metabolism, calcium and phosphorus make up the bulk of the mineral component of the bone. Magnesium is present in the hydration shell of the bone, and is the fourth most abundant cation in the skeleton. About half the body magnesium is present in the skeleton<sup>(57)</sup>. The most important function of magnesium is probably its activation of a large number of enzymes, some of which are especially important in the skeleton, such as alkaline phosphatase<sup>(58)</sup>.

The concentration of calcium, phosphorus and magnesium should remain constant in plasma and in extracellular and intra-cellular fluids. Both physico-chemical and biological factors are involved in the control of mineral homeostasis<sup>(59)</sup>.

The level of alkaline phosphatase in the serum is usually indicative of the activity of the osteoblasts (bone forming cells) in the bone<sup>(60)</sup> provided that liver function is normal.

METHODSa) Analytical Procedures

Full details of these are presented in Appendix One.

b) Experimental Groups

Serum calcium was measured in thirteen experimental and eleven control rats, magnesium in seven experimental and seven controls.

Since duplicate estimations of inorganic phosphorus and alkaline phosphatase required large quantities of serum, pooled blood from four rats from the same cage was used. A total of fourteen pooled experimental and twelve control bloods were measured.

### RESULTS

Table IV illustrates the significant reduction in serum calcium, magnesium and inorganic phosphorus in the protein deprived rats as compared to controls. Alkaline phosphatase activity remained unchanged.

### DISCUSSION

Measurements of the serum concentrations of calcium, phosphorus and magnesium are important adjuncts in studies of skeletal metabolism. The actual amounts of these ions in the serum are, however, small relative to the total body content. About 1% of the bodies total magnesium<sup>(61)</sup> and calcium content<sup>(62)</sup> are present in the extracellular fluid compartment. Thus a significant change in total body content or an alteration in skeletal turnover, may not necessarily be reflected in the serum levels of these ions. Serum phosphorus is further limited by the fact that its level varies with dietary intake and time of day<sup>(63)</sup>.

The levels of serum calcium, phosphorus and magnesium were significantly reduced in the protein deprived experimental rats. Similar alterations have been noted by other authors<sup>(64)</sup>. Children with advanced protein calorie malnutrition showed reduced total serum calcium<sup>(64)</sup>. On recovery the ultrafiltrable calcium levels rose by about 10%, whereas the albumin bound calcium rose by about 30% which paralleled the rise in serum albumin. Stewart<sup>(17)</sup> emphasised that the reduced serum albumin lowered the total serum calcium in protein malnutrition.

TABLE IV

SERUM CHEMISTRY ESTIMATIONS

Compound and concentration	Protein deprived (4%) experimental rats		Age matched (20%) controls		t test values
	No of rats	Mean (SEM)	No of rats	Mean (SEM)	
Calcium (mg/100 ml)	13	6.82 (0.40)	11	10.01 (0.57)	4.674 < 0.001
Inorganic Phosphorus (mg/100 ml)	14	6.98 (0.20)	12	8.68 (0.55)	3.065 < 0.01
Magnesium (mEq/litre)	7	1.40 (0.05)	7	1.85 (0.06)	5.201 < 0.001
Alkaline Phosphatase (Sinowara units/litre)	14	32.37 (1.92)	12	32.29 (2.05)	1.038 < 0.4

Using the t test, significant reductions in calcium, inorganic phosphorus and magnesium were noted in the experimental (4%) group as compared to control rats. Alkaline phosphatase activity remained unchanged.

Surprisingly, Jha et al<sup>(20)</sup> in their studies on experimental protein deficiency in the rhesus monkey found no reduction in serum calcium, despite a significantly reduced serum albumin level. Elmaraghi et al<sup>(19)</sup> described varying serum calcium and albumin concentrations in protein malnourished and control rats. Unfortunately they did not correlate the calcium and albumin levels and thus no comparisons can be made from their data. The calcium binding capacity of serum albumin in the rat, is not known. It is possible that the 30% reduction in serum albumin described in the previous chapter may account for the total reduction of serum calcium in the protein deprived rats. On the other hand serum ionised calcium may also have accounted for a significant proportion of this fall. Unfortunately serum ionised calcium levels were not estimated and thus the role of the ionised calcium in the fall in serum calcium must remain speculative.

The existence of hypomagnesaemia in children suffering from protein calorie malnutrition is well documented<sup>(65,66)</sup>. The exact mechanism has not yet been elucidated, though it could possibly represent the consequence of poor magnesium intake<sup>(65)</sup>. Significant hypomagnesaemia was found in the protein deprived rats in the present study, despite adequate supplementation of the diets with magnesium (Table I). This suggests either poor absorption of magnesium from the gastro-intestinal tract or excessive loss via the kidney. Hypocalcaemia and hypomagnesaemia are commonly associated<sup>(67)</sup> and it has been suggested that magnesium deficiency interferes with calcium release from bone making the bone resistant to parathyroid hormone<sup>(68)</sup>. Cyclic 3'5' - adenosine monophosphate (3'5'-AMP) is a key biochemical intermediate in the action of parathyroid hormone<sup>(69)</sup>. Parathyroid hormone binds to the cell membrane, activates adenylyl cyclase which

causes an intracellular rise in the concentration of cyclic 3'5' - AMP<sup>(70)</sup>. This in turn causes the release of calcium and bone resorption. Magnesium ions are required for the conversion of adenosine triphosphate (ATP) to cyclic 3'5' - AMP<sup>(71)</sup> and magnesium deficiency would then prevent the formation of cyclic 3'5' - AMP and thus reduce the effectiveness of parathyroid hormone.

The exact relationship of calcium and magnesium ions is still under review, but the homeostasis of both ions is interdependent. They compete actively for intracellular sites in the renal tubules and gastro-intestinal mucosa<sup>(72)</sup>. Parathyroid hormone secretion is related to the magnesium concentration in the serum<sup>(73)</sup>. In contrast to most other species magnesium deficiency in rats usually causes hypercalcaemia presumed to be mediated by the secondary hyperparathyroidism<sup>(67)</sup>. The secondary hyperparathyroidism causes an increase in calcium absorption, which can easily be achieved in the rat. In the protein deprived rats studied here the hypomagnesaemia did not cause hypercalcaemia, in fact hypocalcaemia was present. This probably reflects the poor absorption of calcium demonstrated in the following chapter. A further contribution to the hypocalcaemia is the relative insensitivity of the skeleton to parathyroid hormone in the presence of hypomagnesaemia<sup>(68)</sup>. The presumed secondary hyperparathyroidism will thus be ineffective in increasing bone resorption in an attempt to raise the serum calcium levels.

The protein deprived experimental rats in this study showed a significantly reduced serum inorganic phosphorus level. Similar findings were noted in children suffering from protein calorie malnutrition,<sup>(64)</sup> but not in experimental protein depleted monkeys<sup>(20)</sup>.

The cause for the lowered inorganic phosphorus is not apparent from the above study, but might represent the result of secondary hyperparathyroidism if the lowered serum calcium is due partly to a decrease in the ionised fraction. It may however reflect poor absorption of dietary phosphorus, since serum phosphorus levels are easily affected by dietary phosphorus<sup>(63)</sup>. Further estimations of urinary phosphorus and serum parathyroid levels, which would certainly answer these questions, were not done.

Alkaline phosphatase activity was normal in the protein deprived rats, as has previously been observed in the rhesus monkey<sup>(20)</sup> rendered protein malnourished. Without alkaline phosphatase isoenzyme studies, the relative contributions of bone and liver to this normal value cannot be determined. Raised liver alkaline phosphatase levels were obtained in children with kwashiorkor<sup>(74)</sup> which later fell on refeeding. Swartz<sup>(75)</sup> postulated that bone alkaline phosphatase rose on recovery from kwashiorkor signifying that bone development had restarted. It is possible that the normal serum alkaline phosphatase activity in the protein deprived rats studied, reflects reduced bone osteoblastic activity together with increased liver isoenzyme levels. Isoenzyme estimations are necessary to establish this point. Magnesium is a cofactor for the alkaline phosphatases<sup>(76)</sup>. Rats rendered magnesium deficient demonstrated reduced serum alkaline phosphatase activity<sup>(77)</sup>. Thus the hypomagnesaemia found in the protein deprived rats may well be the cause of reduced bone alkaline phosphatase activity. The postulated elevation in the liver isoenzyme is possible in view of the fatty infiltration of the liver seen (chapter three). This however is surprising in view of the hypomagnesaemia, which should inhibit all alkaline phosphatase activity.

SUMMARY

Serum levels of calcium, inorganic phosphorus and magnesium were significantly reduced in the protein deprived rats when compared to controls, whereas alkaline phosphatase activity was unchanged. Postulates as to the mechanisms producing these changes are discussed. Without knowledge of the serum parathyroid activity, alkaline phosphatase isoenzyme and serum ionised calcium levels, such assumptions must remain theoretical.

## CHAPTER FIVE

CALCIUM ABSORPTION IN THE PROTEIN DEPRIVED RATINTRODUCTION

Until the introduction of radio-isotopes, calcium absorption was studied using metabolic balance techniques<sup>(78)</sup>. Most of these techniques are tedious and difficult to perform<sup>(79)</sup>. They require accurate collection of excreta which is especially difficult in small laboratory animals like the rat<sup>(80)</sup>.

Radio isotope methods have afforded more practical means for measuring calcium absorption. There are numerous techniques described, but unfortunately no accurate and simple method has yet been developed or sufficiently proved<sup>(79)</sup>.

Bhandarkar et al<sup>(81)</sup> suggested that the plasma radio-activity level, two hours after the oral administration of the isotope is a good measure of intestinal calcium absorption. Although this technique is the least demanding in terms of time, it is the most liable to factors other than absorption<sup>(78)</sup>. The size of the exchangeable calcium pool into which the isotope enters will also determine the plasma level.

Radioactive counting of the isotope in isolated limbs<sup>(82)</sup> is impractical in small laboratory rats. The double isotope technique described by Degrazia et al<sup>(83)</sup> requires the measurement of radio-activity of both <sup>45</sup>Ca and <sup>47</sup>Ca in the urine, the former having been administered orally and the latter intravenously. Faecal measurements of the unabsorbed isotopic calcium given orally indicates the extent of calcium absorption but errors in collection can invalidate the results unless nonabsorbable markers are used<sup>(84)</sup>.

To some extent these problems have been overcome by using whole body retention counting.  $^{47}\text{Ca}$ , a  $\gamma$  emitting isotope is used as the tracer and is introduced orally, followed by whole body counting of the animal. Once all the unabsorbed radio-calcium is excreted from the bowel a second count is made. This provides a direct measure of the percentage retention of the isotope (78, 85). The technique was originally described for use in humans, but has subsequently been adapted for use in small animals (86). The percentage retention thus calculated is taken to represent calcium absorption.

#### METHOD

##### a) Pilot Study

In order to determine the time by which all the unabsorbed isotope had been cleared from the intestine, two rats were studied, one experimental protein deprived and the other an age matched control rat. Whole body retention counts were obtained twice daily for five days after the oral administration of the  $^{47}\text{Ca}$  dose.

##### b) Practical Aspects

After an overnight fast 5  $\mu$  Ci of  $^{47}\text{Ca}$  was introduced into the stomach of the rat using plastic tubing, 1 mm in diameter. The rat was then placed in a small plastic container and counted in a small whole body gamma counting system to obtain the count of the initial dose (Appendix Two). The rats were then maintained for three days, after which all the unabsorbed isotope had been excreted. They were then recounted in the small whole body counter using the same counting geometry. Values were corrected for decay of the isotope by including a standard dose of  $^{47}\text{Ca}$ .

$$\% \text{ Retention} = \frac{\text{whole body count rate after three days}}{\text{initial whole body count rate}} \times 100$$

c) Experimental Groups

Three sets of experiments were performed on both the protein deprived and age matched control rats.

- (i) 5  $\mu$  Ci  $^{47}\text{Ca}$  was administered alone.
- (ii) 5  $\mu$  Ci  $^{47}\text{Ca}$  was given premixed with a homogenised meal appropriate to that particular group of rats and introduced intragastrically.

- (iii) 5  $\mu$  Ci  $^{47}\text{Ca}$  was premixed with homogenised food taken from the diet of the opposite group of rats in order to assess whether the protein content of the meal influenced the absorption of  $^{47}\text{Ca}$ .

RESULTS

a) Pilot Study

In both the protein deprived and age matched control rats, there was no further reduction in whole body retention value after the third day indicating that all the unabsorbed calcium had been excreted by the third day. (Table V).

b) Experimental Groups

Significant impairment of calcium absorption in the protein deprived group is noted in Table VI, when compared to control groups. Both groups of animals showed a further proportionate reduction in calcium absorption when the  $^{47}\text{Ca}$  was introduced premixed with the appropriate meal. Administration of the isotope with a meal from the opposite group of animals did not alter calcium absorption further.

DISCUSSION

The study of calcium absorption by calcium balance techniques is inaccurate especially when absorption is poor. Small losses in the collection of faeces can invalidate the results<sup>(78)</sup>. Techniques requiring the collection of faeces or urine are inconvenient in

TABLE V

PILOT STUDY

(To determine when all the unabsorbed  $^{47}\text{Ca}$  had been excreted)

Hours after oral administration of $^{47}\text{Ca}$	<u>% Retention</u>	
	Protein Deprived 4%	Age matched control 20%
0	100.00	100.00
11	66.90	85.95
24	53.28	86.28
33	49.07	80.02
48	46.62	78.33
59	43.29	76.94
73	44.90	78.54
85	42.87	78.43
96	42.06	76.22
105	43.53	79.76
120	43.89	78.54

TABLE VI

CALCIUM ABSORPTION% RETENTION OF INTRAGASTRIC <sup>47</sup>Ca

Expt. (i)	No. of rats studied	Protein deprived rats (4%) Mean (SEM)	No. of rats studied	Age-matched controls (20%) Mean (SEM)	t test	
					t	p
Expt. (i)	11	52.04 (3.29)	10	75.26 (3.45)	4.865	< 0.001
Expt. (ii)	17	37.40 (2.83)	19	62.51 (2.92)	6.144	< 0.001
Expt. (iii)	11	38.32 (2.91)	11	66.41 (3.32)	6.359	< 0.001

Calcium absorption in protein deprived and age-matched control rats, as measured by the percentage whole body retention of intragastric <sup>47</sup>Ca after three days. In experiment (i) both groups received <sup>47</sup>Ca only; in experiment (ii) they received the isotope with a homogenised meal consisting of their appropriate experimental diet; in experiment (iii) the diets with which the <sup>47</sup>Ca was administered were of the opposite group. In all three experiments a significantly lowered calcium retention was found in the protein deprived group using the t test.

small laboratory animals. The technique of whole body retention obviates all these difficulties. Since all the unabsorbed radio-calcium is excreted in the faeces, whole body retention values are indicative of the degree of intestinal absorption.

The pilot study (Table V) illustrated that in both the protein deprived and age matched control rats all unabsorbed calcium had been excreted by the third day after oral administration of the isotope. All whole body count rates were therefore measured on the third day.

It is possible that the reduced whole body retention values obtained in the protein deprived rats do not in fact reflect poor calcium absorption. As shown in the next chapter, protein deprived rats demonstrated significant reductions in calcium accretion into bone. In normal young animals 80% - 90% of the calcium absorbed within the first twenty-four hours reaches the skeleton<sup>(87)</sup>. Reduced calcium accretion would then result in a larger proportion of the absorbed isotope remaining in the exchangeable calcium pool. Increased excretion of this radio-calcium would then be possible. Thus the whole body retention value, after three days in the protein deprived rats, may therefore merely represent the poor calcium accretion into bone. However Hansard et al<sup>(88)</sup> showed that less than 2% of the isotope absorbed from the intestine is resecreted into the gut as endogenous faecal calcium. Secondly, the excretion of calcium in the protein deprived rats was only increased by 50% (Chapter Six) when compared to age matched controls, and this increase is insufficient to affect the whole body retention values by any significant degree. Thus any reduction in whole body retention was due almost entirely to impaired calcium absorption.

As illustrated in Table VI, intestinal calcium absorption was significantly reduced in the protein depleted group. The addition of a homogenised meal further reduced the absorption. This could possibly be due to an inhibiting substance in the food, though more likely it was due to a dilutional effect on the isotope, thus lowering the concentration of the isotope presented to the intestinal mucosa for absorption. The reduction in calcium absorption in the protein deprived rats was not merely related to the amount of protein in the food in which calcium is being administered, as a single meal containing adequate protein did not improve the absorption of the radio-calcium in the chronically deprived animals.

Many animal experiments have indirectly illustrated the possible relationship between the protein content of the diet and calcium absorption from the intestinal tract<sup>(19,89,90,91)</sup>. Greatly improved calcium balance was reported in patients, when changed from periods of low to high protein intake and these authors<sup>(32)</sup> concluded that little calcium would be absorbed if the diet contained no protein or amino-acids. The results of experiment (iii) are at variance with this concept. The age matched control rats were able to absorb <sup>47</sup>Ca normally in the presence of a low protein diet, and the introduction of a high protein diet to the protein deprived rats did not increase calcium absorption. This suggests that the protein content of the meal given with the <sup>47</sup>calcium is not important for the absorption of calcium.

It has recently been shown that active calcium absorption in the gut requires the synthesis of a specific calcium binding protein<sup>(92)</sup>, probably under the control of 1,25 dihydroxycalciferol<sup>(93)</sup>. In fact, others<sup>(94)</sup> have shown, in protein depleted rats that the activity of

calcium binding protein in the rat mucosa is significantly reduced. Thus the synthesis of this important carrier protein is impaired in chronic protein deprivation and this impairment results in reduced calcium absorption from the intestine.

Impaired synthesis of calcium binding protein in the protein depleted rats was probably not due to deficient Vitamin D, since their diets contained adequate supplements of Vitamin D (Table I).

#### SUMMARY

Calcium absorption was reduced in the protein deprived rats. This was not due to the absence of protein in the test diet, but due to chronic protein deprivation probably resulting in reduced synthesis of an important carrier protein necessary for active intestinal absorption of calcium.

## CHAPTER SIX

CALCIUM POOL, BONE ACCRETION AND CALCIUM EXCRETIONINTRODUCTION

Kinetic analysis of calcium metabolism is a well established technique used in investigating metabolic derangements of the skeleton<sup>(95)</sup>. These tracer kinetic studies allow accurate measurements of the movement of calcium between the various physiological compartments in the intact organism. They have the added advantage of not interfering with the state of the various compartments and thus not altering the steady state of the organism during the study.

Since the first descriptions of techniques for the study of calcium metabolism based on kinetic models<sup>(96, 97, 98)</sup> many adaptations have been reported. No existing technique, however, seemed practical for the examination of calcium kinetics in the small laboratory rat used in these experiments. The technique described by Bauer et al<sup>(96)</sup> requires the direct measurement of the radioactive tracer content of extirpated bone from the experimental animals which have to be sacrificed to obtain each point of the kinetic curve. The consequent need for numerous animals for adequate statistical evaluation of the results is a great disadvantage.

The technique of Heaney et al<sup>(98)</sup> requires the accurate collection of urine and faeces. This was found to be a great source of error and inconvenience especially as the smaller rats used in these studies required frequent handling out of the metabolic cage, which leads to urine and faecal loss.

It became necessary therefore to establish a technique based on the model of Heaney et al<sup>(98)</sup> but avoiding the necessity of urine and faecal collections.

#### THEORETICAL CONSIDERATION OF THE MODEL

##### a) Introduction to tracer kinetics.

In order to understand the adaptations made, a brief description of the concept of tracer kinetics is presented.

Tracer amounts of the radio-isotope are introduced into a pool which is undisturbed by the tracer. The total amount of tracer becomes instantaneously and uniformly distributed throughout the pool. Its concentration is described as specific activity (millicuries of the isotope per milligram of element).

As calcium leaves the pool, the total amount of tracer diminishes (Figure 6). The amount of tracer leaving is proportional to the stable calcium and therefore the concentration or specific activity remains unchanged. Since a steady state is assumed to exist in the pool the amount of calcium leaving must be replaced by an equivalent amount entering. The calcium which enters is as yet unlabelled by tracer and will thus cause the specific activity to fall, although it does not affect the total amount of tracer still present.

While the total amount of isotopic tracer present at any moment is determined by the amount of calcium leaving, the specific activity is in fact determined by both the exit and entry of calcium. With the passage of time the specific activity will decline and the rate of this decline can be used as a measure of the total turnover of stable calcium (exit and entry) during that period.

The rate of fall-off of the isotopic concentration will remain constant in the steady state situation and maybe described by one rate

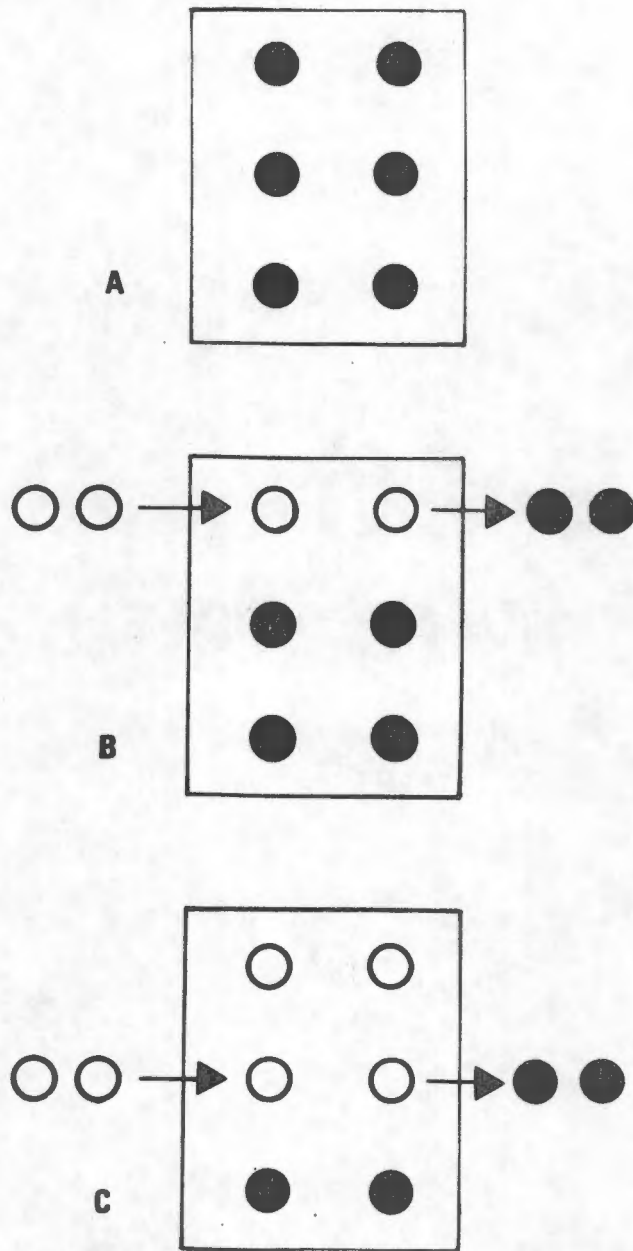


FIGURE 6

In a steady state, the number of isotope-labelled molecules (●) leaving the pool, are simultaneously replaced by an equal number of unlabelled molecules (○). Thus the S.A. declines with time and is dependant on both the entry into and the exit from the pool. The rate of fall off of the S.A. is a measure of the total turnover of stable calcium.

constant. Where there are more routes of entry and exit from a single pool, many rate constants must be accounted for.

b) Mathematical Theory

Several attempts have been made to describe calcium kinetics in terms of multicompartmental models. Most investigators have considered it worthwhile to divide the exchangeable calcium pool (Figure 2) into two or more compartments as indicated by the exponential decay curves obtained from plasma samples. Heaney<sup>(99)</sup> employed a two compartmental model. Cohn<sup>(100)</sup> described a two compartmental open-ended system based on the computer programme designed by Berman et al<sup>(101)</sup>. Aubert et al<sup>(97)</sup> went a stage further and utilised a four-compartmental model.

Because of the complexity of multicompartmental analysis it is currently held that simplified approaches using single compartmental systems are more practical<sup>(102)</sup> and since they involve the same basic assumptions, give similar results<sup>(36)</sup>.

The single compartmental model (Figure 2) is based on the measurement of the rate at which a radio-active calcium tracer disappears from the rapidly exchangeable calcium pool after its introduction into that pool. The level at which the tracer label reaches equilibrium depends on the size of the pool and therefore its dilution effect. This fact is used to calculate the magnitude of the exchangeable calcium pool. The "disappearance" of tracer is due to accretion of calcium into bone and excretion into urine and faeces. The rate (p) at which the isotope disappears from the exchangeable pool is obtained by sampling the serum at regular intervals and calculating the specific activity (S.A.). The rate of excretion (q) is measured from the urine and faecal collections.

Since  $p = q + a$  .....(1)  
 ("a" being the rate of accretion of calcium into bone), the latter is readily obtained by simple substitution of the now measured values for  $p$  and  $q$ .

To avoid the collection of urine and faeces in order to calculate  $q$ , two isotopes are introduced into the exchangeable pool by intravenous injection. The first,  $^{47}\text{Ca}$ , a  $\gamma$  emitting isotope, is then measured in the whole animal by external whole body counting. This will estimate the retention of the isotope by the body and will drop as a result of excretion of the tracer into the urine and faeces.  $^{45}\text{Ca}$ , a  $\beta$  emitting isotope, injected simultaneously, is measured serially in serum samples from which can be calculated the rate of disappearance of this tracer from the exchangeable calcium pool. The fall off of the serum S.A. of  $^{45}\text{Ca}$  must be due, as already indicated, both to accretion of calcium into bone and its excretion into urine and faeces. It thus follows that any difference between the disappearance of the one isotope from the blood and the disappearance of the simultaneously administered second isotope from the whole body, must be a measure of bone accretion.

Let  $E$  be the amount of  $^{45}\text{Ca}$  in the serum pool at any time instant. Assuming that  $E$  is a function  $f(t)$  of time  $t$  only, then

$$E = f(t) \dots\dots\dots(2)$$

Let  $E_0$  be the initial amount of  $^{45}\text{Ca}$  introduced into the serum pool at time  $t_0$ . The amount of tracer flowing out of the pool is assumed to be a constant fraction of the amount left in the pool, expressed mathematically as

$$\frac{dE}{dt} = -pE \dots\dots\dots(3)$$

where  $p$  is the rate constant.

Solving this as a differential equation we get

$$E = E_0 \cdot e^{-pt} \dots\dots\dots(4)$$

the fall off of E being exponential.

Similarly the rate of excretion is assumed to be a constant fraction of the amount left in the pool. Let this rate constant = q, and the amount excreted from the whole body can be obtained from two consecutive whole body counts - say  $b_1$  and  $b_2$  at times  $t_1$  and  $t_2$  respectively. Then using integral calculus, the total amount of excretion ( $b_1 - b_2$ ) between  $t_1$  and  $t_2$  is given by (5).

$$b_1 - b_2 = \int_{t_1}^{t_2} q \cdot E \cdot dt \dots\dots\dots(5)$$

From equation (2) and (4)  $E = f(t) = E_0 \cdot e^{-pt}$  and by substituting in (5) the total amount excreted

$$\begin{aligned} (b_1 - b_2) &= \int_{t_1}^{t_2} q \cdot E_0 \cdot e^{-pt} \cdot dt \\ &= q \cdot \frac{E_0}{p} \left[ e^{-pt_1} - e^{-pt_2} \right] \dots\dots\dots(6) \end{aligned}$$

The amount excreted is calculated from the whole body retention and thus q can be obtained by rearranging (6) as follows:-

$$q = (b_1 - b_2) \cdot \frac{p}{E_0 \left[ e^{-pt_1} - e^{-pt_2} \right]} \dots\dots\dots(7)$$

where p = rate of loss of tracer from the pool derived from the serum S.A. curve and expressed as a fraction of the amount in the pool.

This model in its present form is obviously an idealised representation of an in vivo process. The model assumes that minute to minute variations due to ongoing physiological processes play an insignificant role.

c) Practical Considerations

A similar approach to the problem was recently attempted in dogs<sup>(103)</sup>. Only one isotope  $^{47}\text{Ca}$  was used for both serum specific activity and whole body retention counting. The total intravascular volume is very small in the rats used for these experiments and since repeated blood sampling is necessary, only small quantities are used to avoid interference with fluid balance. The use of  $^{47}\text{Ca}$  for the measurement of the fall off of isotope from the serum is associated with very low counts especially during the crucial period, when the total specific activity of the pool is only 0.1% of the original dose and each blood sample represents less than  $1/100$ th of the serum pool (Appendix Three). It was therefore necessary to use, as already mentioned, the  $\beta$  emitting isotope,  $^{45}\text{Ca}$ , which can be measured with enhanced counting efficiency by liquid scintillation.

Pool size is calculated by dividing the dose of isotope by the S.A. at the time of equilibrium and expressed as mg. of stable calcium. Calcium accretion and excretion are expressed as fractional rates (% of pool per day) and absolute rates (mg. stable calcium per day).

d) Methods.

i) Analytic Methods

The rats were housed in separate wire bottom cages.  $10 \mu\text{Ci}$  of  $^{47}\text{Ca}$  and  $35 \mu\text{Ci}$  of  $^{45}\text{Ca}$  were simultaneously introduced into the exchangeable calcium pool by injection into the tail vein of each rat. (Appendix three).

$^{47}\text{Ca}$  Whole body counting. Immediately after the injection, each rat was counted in the small whole body counter (Appendix Two) to obtain the initial whole body retention value. Thereafter serial measurements were obtained over a thirty-six hour period.

$^{45}\text{Ca}$  Serum counting. At the time the animals were measured in the whole body counter, a 50  $\mu\text{l}$  sample of rat serum was obtained for  $^{45}\text{Ca}$  measurement by bleeding from the tail vein. The serum was processed (Appendix Three) by the technique of Humphreys<sup>(104)</sup>.

ii) Experimental groups

a) Pilot Study (A)

To test the use of the model which is based essentially on first order reaction kinetics, a preliminary experiment was performed on two normal 150 g. rats in which whole body retention and serum S.A. were measured  $1\frac{1}{2}$  hourly over the 36 hour period following introduction of the isotopes. The  $1\frac{1}{2}$  hour periods between samples was selected on the basis of finding a reasonably practical number of data points to attempt a straight line fit, in conformity with the theory, and to minimize handling of the animals.

b) Pilot Study (B)

As total body retention (exchangeable calcium pool + bone) is always less than measured retention (pool + bone + intestinal content), the proportion of the isotope retained in the intestinal content during the period of whole body counting was measured. During a 30 hour period after the injection of both isotopes urinary and faecal  $^{45}\text{Ca}$  were counted in approximately 4 hour periods in addition to measurements of whole body retention and serum S.A.

c) Studies on experimental protein deprived and control rats.

The kinetic studies were performed on seventeen protein deprived, fourteen age-matched and seven weight-matched control rats.

RESULTS

a) Pilot Study (A)

Figure 7 illustrates the serum  $^{45}\text{Ca}$  S.A. curve which can best be fitted by two straight line exponential segments which intersect about 4 hours after commencement of the experiment. During the first 4 hours the serum curve reflects both disappearance of  $^{45}\text{Ca}$  due to accretion and excretion, as well as mixing in the exchangeable calcium pool. The serum disappearance curve from 4 to 24 hours is the result of bone accretion and excretion only, and is the slope used for the calculation utilising the model as, at 24 hours, serum levels are elevated by the return of tracer from the skeleton.

Figure 8 illustrates the whole body retention values. By contrast with figure 7, this curve is unaffected by equilibration and is valid from the first measurement. However this curve does not portray the situation in the exchangeable calcium pool which is of direct concern and some extrapolation is necessary to satisfactorily determine the starting point at which the model can be assumed to be suitable. As already indicated, the rate of calcium excretion ( $q$ ) is obtained from the whole body retention and serum S.A. curve.  $E_0$ , which is the theoretical serum tracer S.A. at zero time, is derived from backward extrapolation of the serum S.A. curve after mixing is complete, since the actual readings in this period are falsely high. By contrast, the whole body retention values are not, and falsely elevated excretion rates ( $q$ ) may be obtained if the calculations are derived from this part of the retention curve. To test whether differences in whole body retention measured prior to equilibration markedly influence the

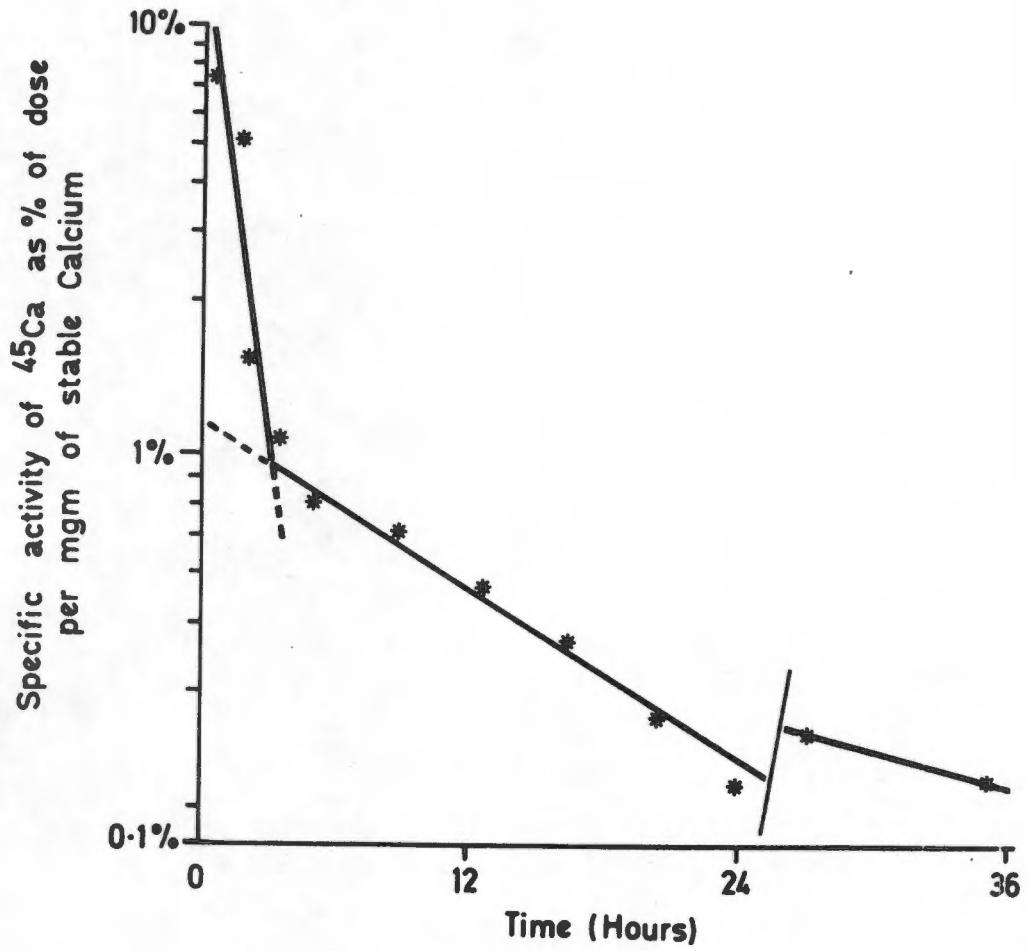


Figure 7. Disappearance of Serum  $^{45}\text{Ca}$  specific activity after the intravenous injection in a normal 150 g. rat.

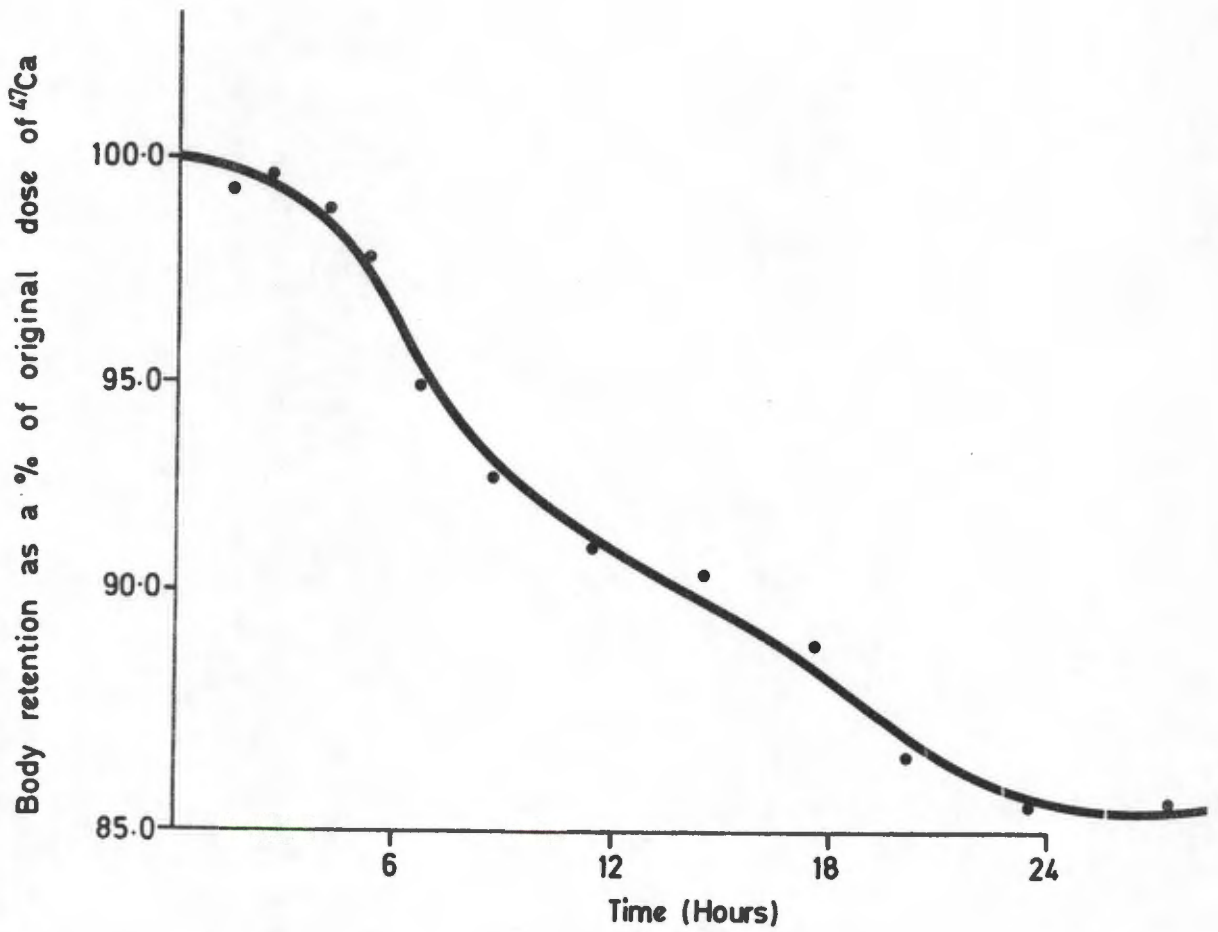


Figure 8.  $^{47}\text{Ca}$  whole body retention values after intravenous injection in a normal 150 g. rat.

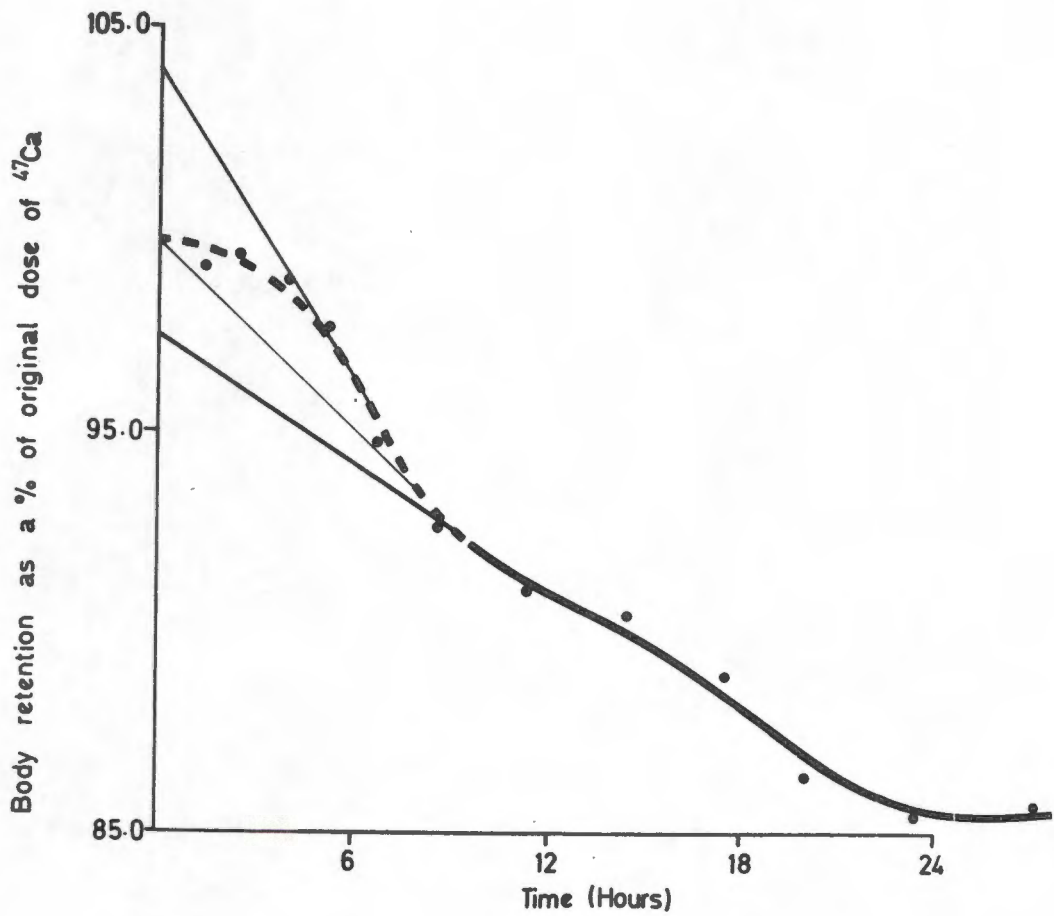


Figure 9. Three hypothetical backward extrapolations of the whole body retention values shown in Figure 8, taken from the time of equilibration of the administered isotope in the exchangeable calcium pool.

final calculation of calcium excretion, three different hypothetical extrapolations backwards from the time of equilibration (4 hours) have been made on the whole body retention curve of one of the rats studied from the pilot study group (A) (Figure 9).

For this, the calculated mean fractional  $q$  values, utilising the three extrapolated curves were 8.33%, 7.34%, 6.65% for curves 1, 2 and 3, respectively with an overall mean of 7.44% and a maximum deviation of 10%.

b) Pilot Study (B)

Table VII illustrates the urine and faecal  $^{45}\text{Ca}$  collections during the 30 hour period. Urinary  $^{45}\text{Ca}$  appears immediately and then declines. In no period does it form more than 15% of the total calcium excretion. Faecal  $^{45}\text{Ca}$  by contrast appears maximally 5 hours after intravenous administration and then declines.

Table VIII shows faecal  $^{45}\text{Ca}$  expressed as a percentage of the whole body radio-calcium retention during the different periods studied. In view of the delay in faecal excretion of  $^{45}\text{Ca}$  as shown by Table VII the values were calculated using the whole body retention values of the preceding period. During no period does the intestinal content of  $^{45}\text{Ca}$  represent more than 5% of the whole body retention.

c) Pool size, accretion and excretion rates of calcium in groups of protein deprived, age-matched and weight-matched controls.

The results of pool size, accretion and excretion rates of calcium are shown in Table IX.

There was a striking and significant reduction in the size of the calcium pool in the protein deprived rats as compared to both age and weight-matched control groups. The weight-matched controls who were considerably younger than the other two groups show the largest calcium

TABLE VII

URINE AND FAECAL <sup>45</sup>Ca

expressed as percentage of initial dose, during timed periods after i.v. administration of the isotope to two normal 150 g. rats.....

---

Hours after i.v. <sup>45</sup> Ca injection	RAT I		RAT II	
	Urine	Faeces	Urine	Faeces
0 - 5	0.59	1.26	0.27	0.32
5 - 9	0.26	3.23	0.14	3.84
9 - 14	0.19	1.57	0.06	2.38
14 - 18	0.16	1.19	0.03	1.29
18 - 23	0.03	0.76	0.05	0.98
23 - 29	0.04	0.70	0.02	0.94

TABLE VIII

FAECAL  $^{45}\text{Ca}$ 

expressed as a percentage of whole body radiocalcium retention during different periods in the 30 hours after the i.v. administration of the isotope to two normal 150 g. rats.

---

<u>Hours after i.v. <math>^{45}\text{Ca}</math></u>	<u>RAT I</u>	<u>RAT II</u>
0 - 5	4.63	4.38
5 - 9	3.43	4.67
9 - 14	4.32	4.17
14 - 18	2.44	2.84
18 - 23	1.88	2.80

TABLE IX

Exchangeable calcium pool, calcium accretion and excretion in protein deprived, age and weight-  
matched control rats.

The Means (S.E.M.) are shown.

	No. of Exchangeable rats	Calcium pool (mg.)	Calcium Accretion		Calcium Excretion	
			Fractional % of pool	Absolute (mg./day)	Fractional % of pool	Absolute (mg./day)
Protein deprived (4%)	17	32.9 (1.96)	65.95 (2.65)	20.70 (0.94)	11.40 (0.81)	3.88 (0.44)
Age matched control group	14	59.81 (3.23)	73.42 (3.84)	45.49 (2.05)	6.21 (0.57)	3.68 (0.33)
Weight matched control group	7	147.93 (15.52)	68.35 (4.30)	98.96 (8.61)	6.49 (0.78)	9.11 (0.89)

Using the WILCOXON test, significant differences between the protein deprived and age matched control rats are shown in the exchangeable pool, absolute accretion and fractional excretion ( $p \leq 0.05$ ). Significant differences between the protein deprived and weight matched control groups are shown in exchangeable pool, absolute calcium accretion and both fractional and absolute excretion ( $p \leq 0.05$ ). All three groups showed similar fractional accretion rates.

pool. The fractional calcium accretion rate was equal in all three groups but because of the difference in calcium pool size the total daily accretion of calcium was lowest in the protein deprived rats and highest in the younger weight-matched controls.

A striking finding was a large increase in the fractional rate of calcium excretion in the protein deprived rats when compared to both other groups. As a result of this, in spite of a substantially smaller pool of calcium, the protein deprived rats excreted the same amount of calcium per day as did the age-matched controls. Because of a very large calcium pool and an equal fractional excretion rate, the young weight-matched control rats excreted a large amount of calcium daily compared to the age-matched group.

#### DISCUSSION

There are inherent problems in most of the techniques used to measure calcium kinetics. Heaney<sup>(99)</sup> maintains that although bone formation removes mineral from the exchangeable calcium pool, tracer movements into bone do not necessarily equate with bone formation. He maintains that there are two exchange processes involved, a rapid and a slow one. Both exchange processes are reflected in the serum S.A. disappearance curve but the rapid exchange between the pool and the bone mineral does not really form part of the slow "accretion" of calcium into bone. Thus bone formation is not synonymous with the non-excretory removal of calcium from the exchangeable calcium pool, and these measurements will over estimate the calcium accretion rates. However, while calcium kinetic studies are of little diagnostic value they are extremely useful when utilised for investigatory purposes<sup>(99)</sup>.

The model of Bauer et al<sup>(96)</sup> requires animals to be killed at intervals after the initial injection of isotopes to obtain individual points on the curve derived from counts of plasma and excised bones. Biological variation in individual animals would thus necessitate large numbers of rats to be used for the desired statistics. The technique of Heaney and Whedon<sup>(98)</sup> requires accurate urine and faecal collections which was found to be difficult especially since frequent tail bleeding and handling of rats often initiates defecation and micturition at times when animals are not in their metabolic cages. Any unmeasured loss of excreta would result in a low (q) value and a corresponding falsely elevated accretion rate. Difficulties with such collections have previously been stressed<sup>(80, 105)</sup>.

The use of the double isotope technique where urine and faeces are not collected has a further advantage. The rats were housed in wired bottom stainless steel cages and the faecal "droppings" are thus lost from the cage. This precludes any coprophagia which most rat species practice especially when the rats are subjected to starvation or irregular feeding schedules, as was done to the protein deprived and control rats. The absence of coprophagia thus prevents any untoward recycling of excreted isotope which may re-enter the pools via the oral route. If the faecal excretion of isotope is large then this recycling could theoretically interfere to a significant degree.

The tracer kinetic model used in the above experiment is based on the accepted calcium kinetic theory and therefore also has some of the inherent shortcomings outlined by Heaney<sup>(99)</sup>. This model uses a first order reaction to describe the observed results and since samples were spaced at more than hourly intervals any reaction with time constants around 30 minutes or less are not accounted for.

To extend the results to a higher order reaction model as suggested by Heaney (99) and Robertson et al (106) would require the computation of at least two rate constants and several rate coefficients and would mean more frequent bleeding of the experimental animal to obtain a greater number of data points. The complexity involved in additional bleeding did not seem necessary in view of the current belief that there is little to choose between the various published compartmental models as they all involve the same basic assumptions and give similar results (36).

The results obtained in the first pilot study (Figure 7) are in accord with those found by other workers (107) in which the equilibrium of the tracer has been shown to occur in the exchangeable calcium pool at between three and five hours after injection of the isotope. The recycling of tracer out of bone occurs between 24 - 36 hours after injection as can be seen by a peak in the serum S.A. curve (Figure 7). Theoretically, the use of the results during the first five hours of the whole body retention curve may be erroneous, however testing of the three hypothetical slopes during the initial part of the whole body curve show a 10% variation in subsequent  $q$  values. This suggests that variations of the initial slope of the whole body retention curve did not alter the calculated rates of excretion to any marked degree, probably because in the young rapidly growing rats, the calcium accretion was much greater than calcium excretion. Moreover, further confirmation of this was obtained in the second pilot study where it was shown that the measurement of whole body retention of radio-calcium was not significantly contributed to by the intestinal content of radio-calcium in as yet unexcreted faeces. Urinary calcium

excretion did not contribute significantly to total calcium excretion which is in accord with other studies in young rats (80,108).

As illustrated in Table IX the fractional accretion rates are the same in all three groups of rats. This reflects the constant relationship between absolute accretion rate and pool size in both large and small rats. Similar results were shown in sheep<sup>(109)</sup>. The smaller more actively growing animals have a larger turnover of calcium and a correspondingly greater pool and increased accretion rates. During ageing and maturation rats reduce their turnover of calcium and therefore have smaller pools and accretion rates<sup>(110,111)</sup>. This was borne out by the findings in Table IX where the smaller, younger weight-matched controls had larger pool size and accretion rates than the older and larger age-matched controls. The experimental rats however showed an even further reduction in pool size and accretion rates, which is in agreement with the findings of Shenolikar<sup>(23)</sup> in their protein depleted rat experiments.

Excretion results were rather surprising. Compared to the age-matched controls the protein deprived rats showed a fractional increase in the calcium excretion which resulted in equal excretion of absolute calcium per day. This relative increase in excretion was despite the marked reduction in exchangeable calcium pool size in the protein deprived rats. Recently calcium binding protein, the protein responsible for calcium absorption from the intestinal tract has been isolated in the kidney<sup>(112)</sup>. It was postulated in Chapter five that in protein malnutrition inadequate protein synthesis resulted in a reduction in the activity of calcium binding protein, which in turn caused the reduction in calcium absorption from the gut. It is

possible that chronic protein deprivation could result in a depletion of calcium binding protein which is currently thought to be responsible for tubular reabsorption of calcium in the kidney<sup>(112)</sup>. This would then allow calcium to "leak" into the urine, despite a contracted exchangeable calcium pool. On the other hand endogenous faecal calcium was increased in protein depleted rats<sup>(23)</sup>, and since young rapidly growing rats usually have insignificant calcium excretion<sup>(108)</sup>, it seems more likely that the loss was via the endogenous faecal calcium route. More direct measurements are therefore required to answer this question. Because the new kinetic model used in these experiments was designed to eliminate the problem of urine and faecal collection in these small rats, the elucidation of this problem was not possible.

#### SUMMARY

The calcium kinetic model presented in this chapter is one derived from established models and adapted for use in small, young rats. It precludes the necessity for urine and faecal collections.

The theoretical computation is borne out by the results obtained in the pilot studies. Calcium accretion paralleled the size of the exchangeable calcium pool in all the groups studied. Protein deprived rats demonstrated reduced calcium pool size and lowered calcium accretion rates. Despite this the total excretion of calcium was not reduced in these rats compared to the age-matched controls.

## CHAPTER SEVEN

HYDROXYPROLINE SYNTHESIS

Normal bone is composed of three distinct elements, the cells, organic matrix and the mineral fraction. The organic matrix constitutes about three-quarters of the volume, but only one-third of the weight of normal bone<sup>(7)</sup>.

In recent years, most investigators of bone metabolism have used dynamic techniques involving the mineral fraction of bone. These methods for studying calcium accretion into bone are generally accepted as indicating total bone formation rates. In normal, healthy bone, the rate of formation of the organic matrix parallels the deposition of mineral<sup>(113,114)</sup>. However, under pathological conditions, matrix and mineral turnover rates are not necessarily identical. It is imperative therefore, to measure the relative turnover rates of both these fractions, when studying bone metabolism in pathological states<sup>(24)</sup>.

The organic matrix is made up primarily of collagen. It is a unique protein in its amino acid composition, being especially rich in glycine and proline. Two of its amino acids, hydroxylysine and hydroxyproline are found exclusively in connective tissue<sup>(115)</sup>. It is currently believed that collagen itself is one of the major catalysts involved in the nucleation of the calcium and phosphate ions, thereby initiating the formation of the hydroxyapatite crystals of bone<sup>(116, 117, 118, 119, 120)</sup>.

Half the body collagen is present in bone<sup>(30)</sup>. Urinary hydroxyproline, which is derived solely from collagen degradation,<sup>(25, 121)</sup> has therefore been used to estimate the rate of turnover of bone collagen.

It was found to be reduced in both human kwashiorkor<sup>(26,27)</sup> and experimental protein depleted animals<sup>(28,29)</sup>. This reduction is however, due to an alteration of both skin and bone collagen, the former comprising a quarter of the body collagen content<sup>(30)</sup>. Thus urinary hydroxyproline is only an indirect and approximate estimate of skeletal collagen turnover, especially in states affecting total body collagen as does protein malnutrition<sup>(22,30,31)</sup>.

A more satisfactory parameter for the study of quantitative alterations of collagen, is the estimation of the rate of incorporation of precursors into the collagen molecule.

#### METHODS

##### a) Theory

The hydroxyproline in the collagen molecule is derived solely from the proline residues in the protocollagen precursors<sup>(25)</sup> (figure 10) and not from dietary hydroxyproline<sup>(122)</sup>. The specific hydroxylation of free proline into hydroxyproline can thus be used as a direct measure of the collagen formation rate.

<sup>14</sup>C-proline is injected into the rats and the rate of incorporation into hydroxyproline is measured by extracting the organic material from the bone. Proline and hydroxyproline fractions are separated and the specific activity of the <sup>14</sup>C hydroxyproline is measured by the method described by Firschein<sup>(123)</sup>. (Appendix four).

##### b) Practical Aspects

50  $\mu$  Ci of <sup>14</sup>C-proline was injected intraperitoneally into the rats which were then sacrificed at both three and six hours after the injection. The tibiae were removed, cleaned, demineralised and hydrolysed. Hydroxyproline was separated from proline using an exchange

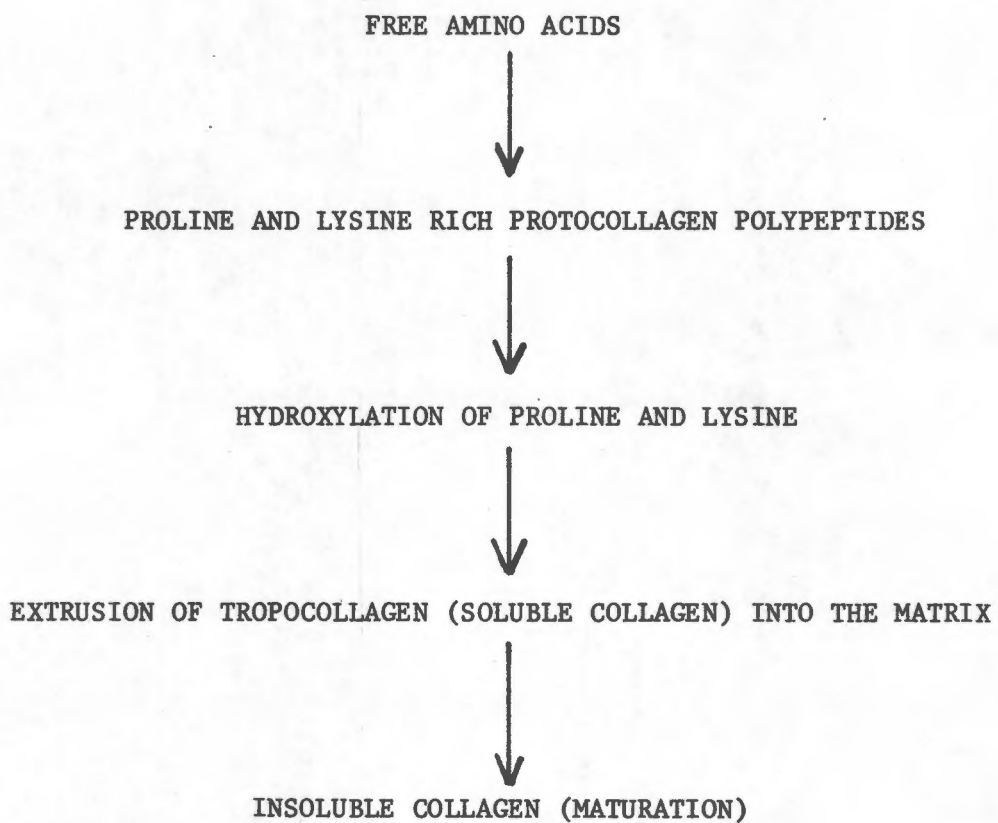


FIGURE 10

General scheme for the biosynthesis of collagen<sup>(25)</sup>.

resin technique. Aliquots of hydroxyproline were taken for stable hydroxyproline estimations as well as radioactive assay. (Appendix four).

c) Experimental Groups

These studies were performed on seven protein deprived and seven age-matched controls.

RESULTS

As demonstrated in figure 11 there was a significant reduction in the incorporation of proline into hydroxyproline in the experimental group after six hours.

DISCUSSION

The direct measurement of collagen synthesis by incorporation of  $^{14}\text{C}$  proline into hydroxyproline has been well documented (24, 124), Firschein (123) showed that the incorporation of the isotope in the long bones of rapidly growing rats increased to a maximum at about ten hours after the introduction of  $^{14}\text{C}$ -proline. After this the specific activity begins to fall, due to the remodelling of the newly formed matrix and dilution of the labelled matrix by non-radioactive proline incorporation into hydroxyproline. Thus, provided measurements are made within the first ten hours, hydroxyproline synthesis rates are obtained.

A significant reduction in hydroxyproline synthesis was found in the protein deprived group of rats after six hours. When taken in conjunction with the reduced rates of calcium accretion (Chapter Six) it probably implies reduction of total bone formation. The absence of any significant difference at the three hour measurements possibly reflects inadequate sampling numbers.

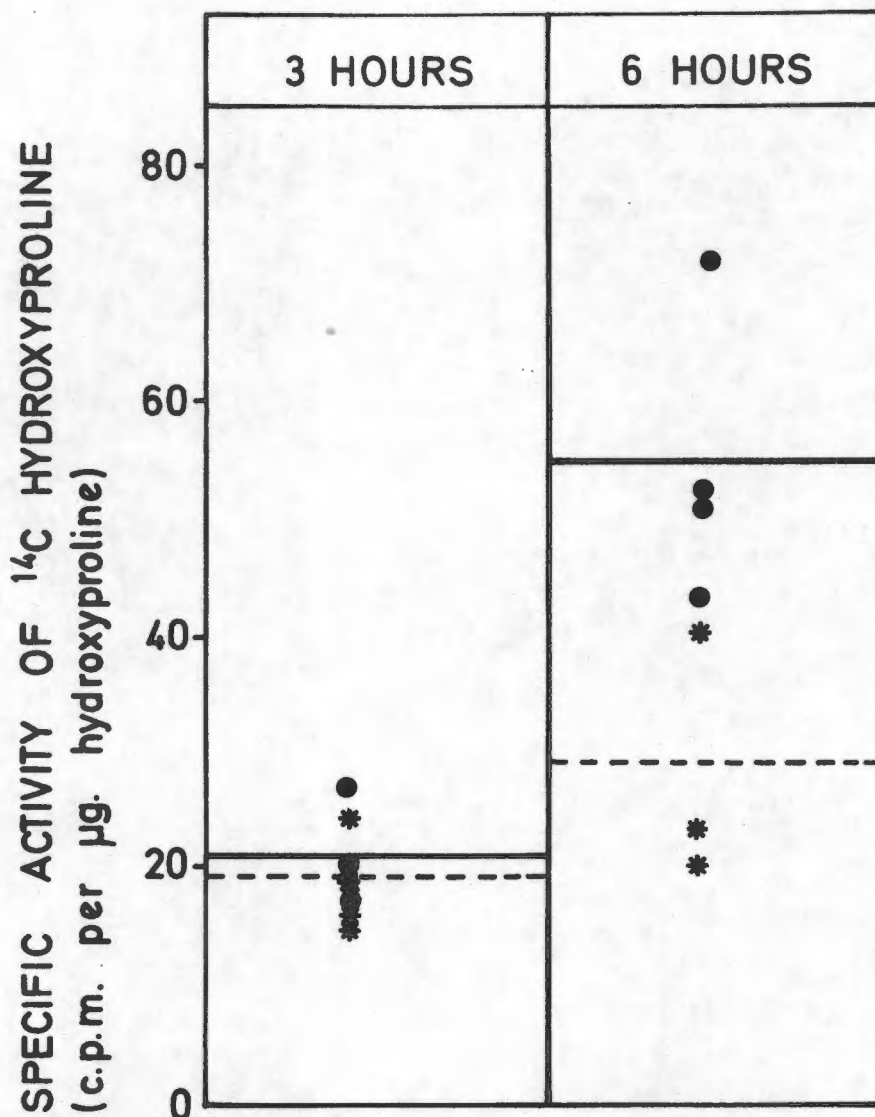


FIGURE 11

Incorporation of <sup>14</sup>C proline into tibial hydroxyproline three and six hours after intra-peritoneal administration of the isotope in protein deprived ( \* ) and age matched controls ( ● ). Using the t test a significant decrease in incorporation is seen in the protein deprived rats after six hours. (t = 3.612, p < 0.02).

As relative sizes of the proline and hydroxyproline pools are not known in the two groups of rats, it is conceivable that the reduced incorporation of proline into hydroxyproline in protein deprivation, reflects dilution of the isotope in a larger pool. This however, seems unlikely in a state of severe protein deprivation, where reduced availability of amino acids would lead to a contracted pool. Further measurements of pool sizes are required to elucidate this point.

Reduction in collagen synthesis in the protein depleted rats was most probably secondary to inadequate dietary protein with resultant reduction in amino acid pool available for protein synthesis. Tissues with high protein turnover are the most severely and earliest to be affected. Examples of this are seen in the liver, intestines and pancreas<sup>(125)</sup>. It is therefore understandable that protein deprivation should affect collagen synthesis. Furthermore, it has recently been shown that protein deprived weanling rats had impairment of the intra- and inter-molecular crosslinkage of the collagen molecules<sup>(126)</sup> which suggests that protein deprivation may affect collagen maturation as well as synthesis.

#### SUMMARY

The rate of incorporation of  $^{14}\text{C}$  proline into hydroxyproline was used as a measure of collagen synthesis. Protein deprived rats showed a reduction in this rate. However it must be emphasised that because of the lack of data on the pool sizes, the interpretation of these results must be made cautiously.

## CHAPTER EIGHT

BONE RESORPTIONINTRODUCTION

The final structure and composition of bone is dependant on both formation and removal. Whereas bone formation is achieved by the mineralisation of organic matrix, bone resorption of both elements occurs simultaneously<sup>(34)</sup>. This has recently been confirmed using isotopic tracer studies of mineral and collagen resorption from the bones of young rats<sup>(114)</sup>. Thus the measurement of removal of either component from bone is considered sufficient when studying total bone resorption<sup>(24)</sup>.

METHODa) Theory

The method used was adapted from that described by Firschein et al<sup>(114)</sup>. <sup>45</sup>Ca is injected intravenously into the rats which are then sacrificed at 5 day intervals over a 20 day period. The tibiae are removed, cleaned and ashed. Equal samples from each rat are analysed for <sup>45</sup>Ca activity. (Appendix Three). As tracer-labelled bone is remodelled and calcium is removed, the amount of the isotope in the bone will fall. The rate of fall off over the 20 day period will therefore reflect the rate of bone resorption.

b) Adaptation.

The total activity of <sup>45</sup>Ca in a standard aliquot of ashed bone will depend on the dose given and the degree of resorption. The major portion of the injected isotope exists in the exchangeable calcium pool for the first few hours. During this time urine and faecal

excretion may contain a high concentration of the isotope. "High excretors"<sup>(78)</sup> are especially liable to loss of tracer during the first few hours when the serum concentration of the isotope is high (figure 7). This would considerably lower the amount of tracer entering the bone. Reduced amounts of the isotope in the bone would then erroneously be considered to represent greater resorption of bone.

To overcome this problem an internal marker has been added to the technique of Firschein et al<sup>(114)</sup>.  $^{47}\text{Ca}$ , the  $\gamma$  emitting isotope, was used for this purpose.

c). Practical Aspects.

$^{47}\text{Ca}$  and  $^{45}\text{Ca}$  were simultaneously administered intravenously. Percentage retention of the  $^{47}\text{Ca}$  was obtained by immediate whole body counting (Appendix Two) and repeating the measurement after twenty-four hours. This retention value then indicated the degree of loss of isotope by excretion and was used to readjust the value of the dose. This readjusted dose value therefore represented the effective injected dose.

The values of  $^{45}\text{Ca}$  activity in the various ashed samples over the 20 day period were expressed as a percentage of the corrected dose. Maximum retention of  $^{45}\text{Ca}$  was found to be present at day 5, which was then taken to represent 100%. All subsequent values were expressed as a percentage of this 5 day value.

d) Experimental Groups

Twenty five protein deprived and twenty five age-matched control rats were studied. Five rats from each group were sacrificed every fifth day.

## RESULTS

Figure 12 demonstrates that there was no significant reduction in bone resorption in the protein deprived rats when compared to age-matched controls.

## DISCUSSION

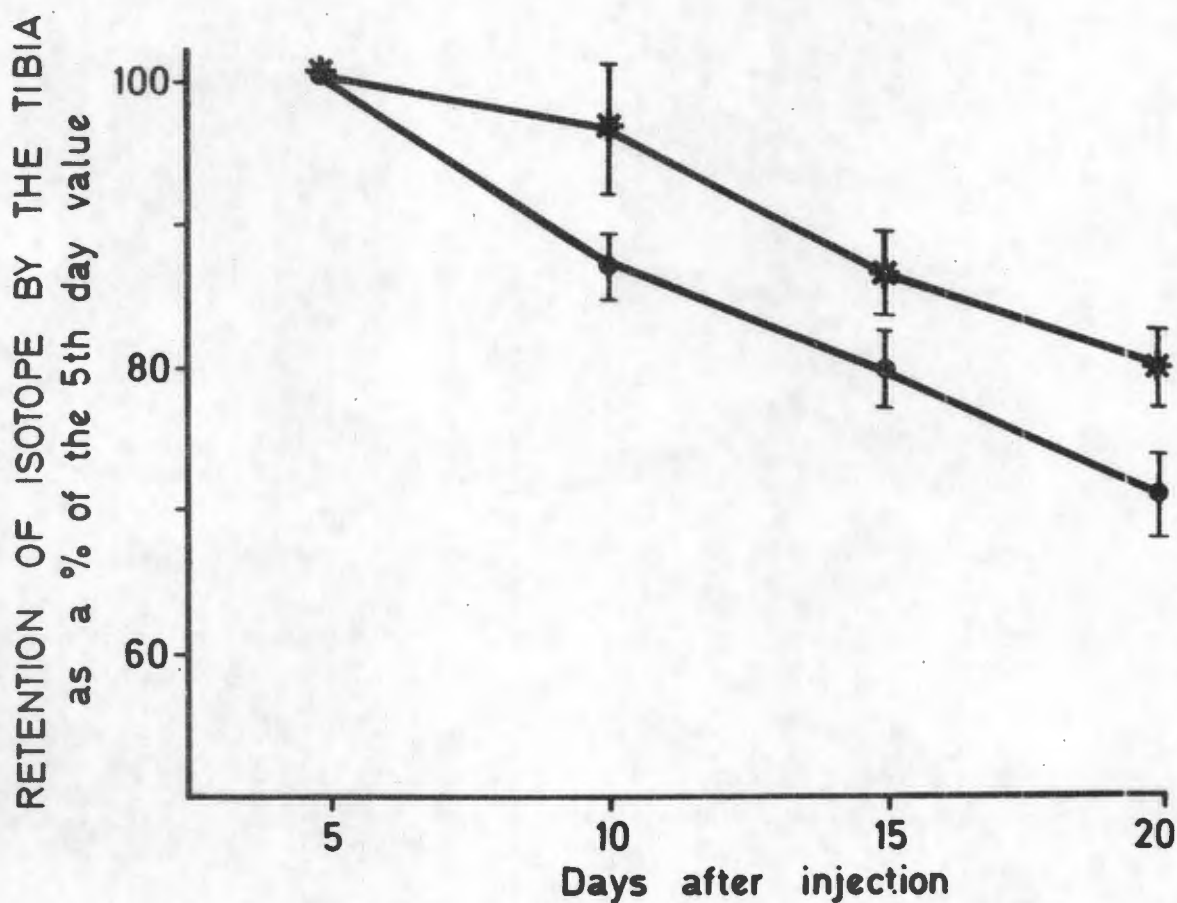
By adapting the technique of Firschein et al<sup>(114)</sup> with the addition of an internal marker (<sup>47</sup>Ca) the problem of early total body tracer loss by excretion was overcome. Any loss will then be accounted for by the twenty-four hour whole body retention value, which was then used as the corrected dose.

The rate of removal of calcium from bone generally reflects total bone remodelling<sup>(34,114)</sup>. Bone formation is composed of two inter-related though temporally separate, processes. In contrast the removal of both matrix and mineral elements generally occurs simultaneously even under pathological conditions<sup>(34)</sup>.

The lack of significant change in bone resorption should be viewed in the light of the marked reduction in calcium accretion (Chapter Six). In young rapidly growing animals the rate of resorption is small compared to accretion<sup>(127)</sup>, however the lack of change in resorption despite the markedly lowered calcium accretion resulted in reduction in total bone.

## SUMMARY

The technique of Firschein et al<sup>(114)</sup> was used to measure calcium resorption. There was no change in the resorption rate in experimental protein depleted rats when compared to age matched controls.



**FIGURE 12**

Calcium resorption in protein deprived ( \* ) and age matched control rats ( ● ) as measured by percentage retention of  $^{45}\text{Ca}$  in extirpated tibiae at five day intervals after intravenous administration of the isotope. Each point represents the mean  $\pm$  S.E.M. of five observations. Using the least squares regression line, no significant difference was seen between the two groups  $0.1 < p < 0.2$

## CHAPTER NINE

DENSITOMETRY

Extirpated bone measurements, as presented in the next chapter, showed detectable differences in the length of the bones between protein deprived and control rats. Information regarding the composition of the bones is only semiquantitative when naked eye comparisons of radiographs are used. Visual detection of bone porosity can only be made once 30 - 50% of bone mass has been lost<sup>(128,129)</sup>.

The need for a more sensitive method for the earlier detection of minor changes in mineral content and bone mass resulted in the introduction of quantitative radiological techniques, such as the direct measurement of cortical bone thickness<sup>(130,131)</sup>. However significantly more information is yielded by densitometric scanning of bone radiographs<sup>(132)</sup>.

The technique employed in this experiment was adapted for rat work from that of Albanese et al<sup>(133)</sup>.

METHODSa) Theory

Conventional radiographs of the long bones of the rats were made. An aluminium step wedge which was included with each radiograph (figure 13), minimised the possible error arising from variations in exposure and processing of the plates, thus serving as an internal reference standard<sup>(134, 135)</sup>. That part of the radiograph displaying the hind limbs was then positioned on a scanning densitometer and a tracing made across the midshaft of both femurs of each rat after appropriate adjustment of the sensitivity of the scanner, using the aluminium wedge as the standard. The midshaft of the femur was



FIGURE 13

Radiograph of the hind legs of a rat, showing the aluminium step-wedge used as an internal standard.

studied since it is the most symmetrical and cylindrical part of the bone. The femur is regarded as representative of the total skeleton<sup>(136)</sup>.

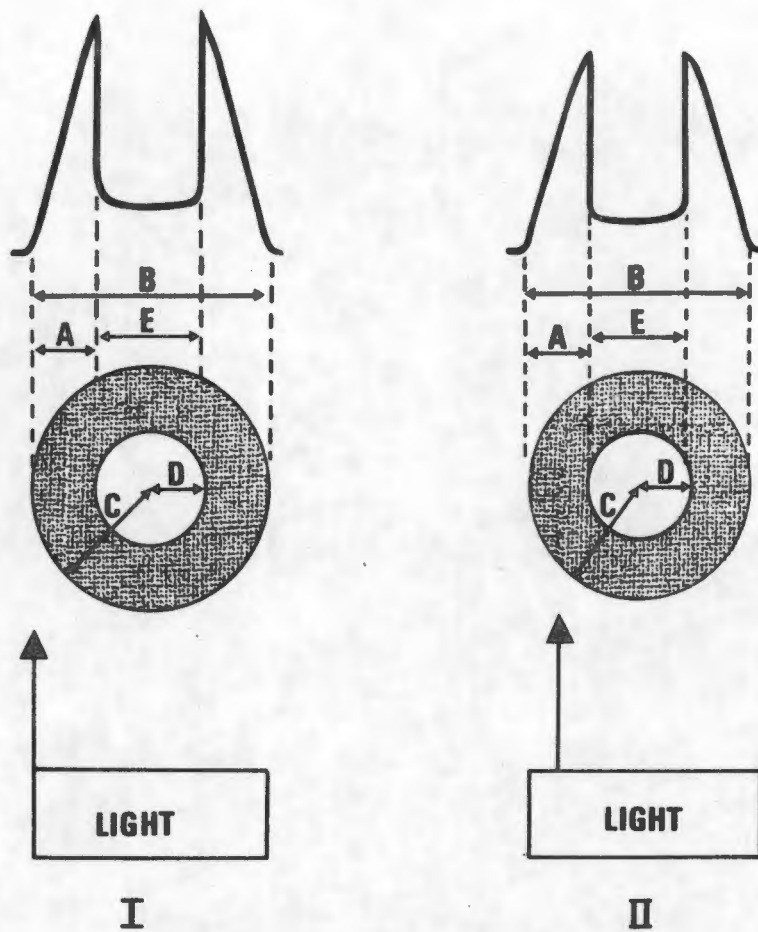
From the tracings the following measurements were made (figure 14).

1) Outer bone width (B)

2) Medullary cavity width (B - 2A), which is measured as inter-peak distance, since the peaks correspond to the inner edge of the cortical bone<sup>(137)</sup>.

3) Area under the graph, expressed as  $\text{mm}^2$ , was measured using a haff planimeter. This represents the "total bone density" at the femoral midshaft.

Since the deflection of the densitometric recording needle is dependant on the optical density of the radiograph, the height of the deflection is a measure of both the mineral content of that bone and the actual amount of cortical bone. Thus the planimetric area obtained (total bone density) is influenced by both factors. In order to selectively assess the mineral content of the bone by this technique, the total bone density has to be evaluated in relationship to the quantity of bone, measured as cortical surface area. This latter estimation is determined by measuring the radius of the inner and outer circle of cortical bone (D and C figure 14), calculating the circular areas subtended by the radii ( $\pi r^2$ ) and subtracting the smaller from the larger area. By dividing the total bone density by the cortical surface area, a factor "f" was obtained which expresses the relationship of these two factors. Any change in this relationship is likely to be due to a difference made by the bone mineral content.



**FIGURE 14**

Schematic representation of bone densitometry tracings of the femoral midshaft from control I and protein deprived II rats. The light source moves from left to right across the bone radiograph and the density is continuously recorded. A = cortical thickness; B = total bone width; C = the outer radius, and D = the inner radius. Cortical bone surface area is calculated by subtracting the circular area subtended by D from that subtended by C. The total area under the density curve is measured by planimetry and represents the "total bone density".

Note that the bone density is less in the femoral midshaft of protein deprived rats compared with that in the control animals in spite of nearly similar cortical bone thickness in both groups. The difference in two groups can be accounted for by differences in the cortical surface areas.

b) Practical Aspects

The technical aspects are outlined in Appendix Five.

c) Experimental Groups

Radiographs of eight protein deprived and eight age-matched control rats were taken. The femora of each rat was examined as described.

RESULTS

The results obtained are represented in Table X and represented schematically in figure 14.

Total bone density (the total planimetric area under the bone density graph) is significantly reduced in the protein deprived rats. Similarly, a significant reduction in outer and inner cortical radii is noted in the same group, the diminution of the outer cortical radius being a little more marked.

Cortical bone surface area, calculated from the cortical radii, shows a 25% drop in the protein deprived rats. This accounts for almost all the reduction in the total bone density, as factor "f" is not significantly different in either groups of rats.

DISCUSSION

Densitometric measurements allow the accurate assessment of bone mineral density and cortical thickness<sup>(128)</sup>. The aluminium wedge is included as an internal standard as it automatically corrects for any variation in the radiographic plates and the processing thereof<sup>(134,135)</sup>. The densitometric evaluation of bone radiology enables one to obtain quantitative data on bone density combined with an indication of its mineral content. Radiographic measurements of cortical bone thickness are of limited value when cortical porosity is suspected, as loss of bone substance may not necessarily be accompanied by alteration in

TABLE X

BONE DENSITOMETRIC MEASUREMENTSTaken at the femoral midshaft of protein-deprived and age-matched control rats

Rats	No. of measurements	Total bone density (Planimetric area sq.mm.)	2 x Outer radius (mm)	2 x Inner radius (mm)	Cortical bone surface area (sq. mm.)	Planimetric area/cortical bone area (factor "f")
I Age-matched controls (20% Casein)	16	35.73 (2.83)	2.99 (0.05)	1.94 (0.04)	4.11 (0.22)	8.64 (0.41)
II Protein deprived (4% Casein)	16	23.60 (1.53)	2.63 (0.03)	1.72 (0.04)	3.07 (0.09)	7.68 (0.47)

Using the Wilcoxon's test, the protein-deprived group showed significantly lower parameters with the exception of factor "f" (at a level of  $p < 0.005$ .)

cortical thickness.

Significant loss of total bone density in protein deprived rats, compared to age-matched controls was found. A proportionate reduction of cortical bone surface area was noted in the protein deprived animals. Thus the loss of bone density seemed to be a consequence of quantitative reduction of bone rather than a change in mineral content.

Cortical thickness (A - figure 14) obtained by subtraction of the radii alone, does not equate with total cortical surface area, the former can be identical in two rats with different surface areas (as shown in figure 14). Thus measurements of cortical bone thickness maybe misleading and the total bone density is the more reliable measurement.

The findings in the above experiment of reduced cortical bone mass with normal mineral composition is similar to that found by other authors<sup>(12,15,18)</sup>. Garn et al<sup>(12)</sup> demonstrated cortical bone loss in kwashiorkor as did Adams et al<sup>(15)</sup>. The rarefaction of bone in experimental protein deficiency in the pig corresponded to this cortical bone loss, although those authors judged the rarefaction subjectively and used arbitrary gradings<sup>(18)</sup>.

#### SUMMARY

The technique of Albanese et al<sup>(133)</sup> was adapted for densitometric study of the radiographs of the long bones in the rat. The protein deprived group of rats demonstrated a significant loss of total bone density when compared to controls. Cortical bone surface area was reduced by a similar degree and therefore this loss of bone density was due almost entirely to reduction of bone mass and not to any change in mineral content.

## CHAPTER TEN

EXTIRPATED BONE MEASUREMENTSINTRODUCTION

Accurate measurements of the composition of bone are valuable adjuncts to kinetic studies. They describe the chemical status of the bone at the time of measurement. The effects of altered skeletal metabolism on the morphology of the bone may therefore be confirmed by these measurements.

METHODSa) Radiographs

Conventional radiographs of the tibiae and femora were made after sacrificing the rats and removing these long bones intact (figure 15). The aluminium wedge was included as an internal standard (appendix six). Total bone and epiphyseal lengths were accurately measured on the radiographs using Vernier calipers.

b) Dry Bone Weight.

The bones were then dried in an oven at 125°C for twenty four hours after which they were carefully weighed using a Sartorius balance

c) Mineral Content

After drying, the bones were divided into three sections. The proximal, midshaft and distal fractions were then ashed for thirty-six hours in an oven at 600°C, and the ash weighed. Ash weights were expressed as a percentage of the dry weight of bone. Ash weights represent the mineral content, the matrix having been combusted.

d) Experimental Groups

Eight experimental and eight age-matched control rats were studied as well as four baseline weight-matched control rats.

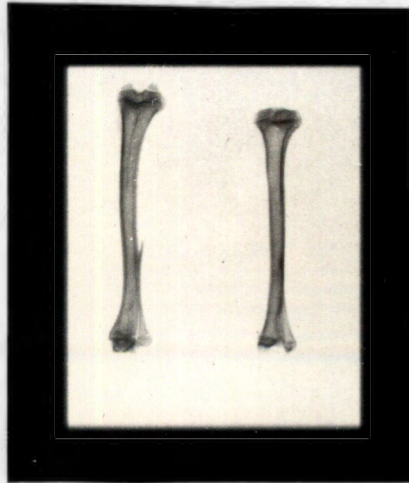


FIGURE 15

Comparative radio-autographs of tibiae of the control rats (left) and protein deprived rats (right). The bone from the protein deprived rat is smaller than the control.

## RESULTS

The error of the radiological measurements using the Vernier calipers was 4.3% and the weighing error on the balance was 0.1%

### a) Radiological assessment of total bone and epiphyseal lengths

Table XI compares the total length of tibia and femur in the protein deprived, age and weight-matched control groups. In addition, the epiphyseal length has been measured separately in the three groups and its proportion of tibial length separately calculated. Bone length appears greatest in the age-matched control group and least in the younger weight-matched group. The protein deprived rats show values intermediate between the two groups and significantly different from both. The weight-matched control rats show the largest proportional epiphyseal length, and this declines with age as shown in the older, age-matched group. In spite of attaining bone growth intermediate between the two control groups, the protein deprived rats show the thinnest tibial epiphyses, whether expressed in absolute terms or as a percentage of the total tibial length.

A typical example of the contrast between the bones of the protein deficient and age-matched rats is shown in figure 15.

### b) Dry bone weight

Figure 16 compares tibial weight with body weight in the protein deprived, age and weight-matched control groups. In both control groups a close correlation can be seen between dry bone weight and total body weight. The trend being for tibial weight to increase as body weight increases. However, the protein deprived rats, although

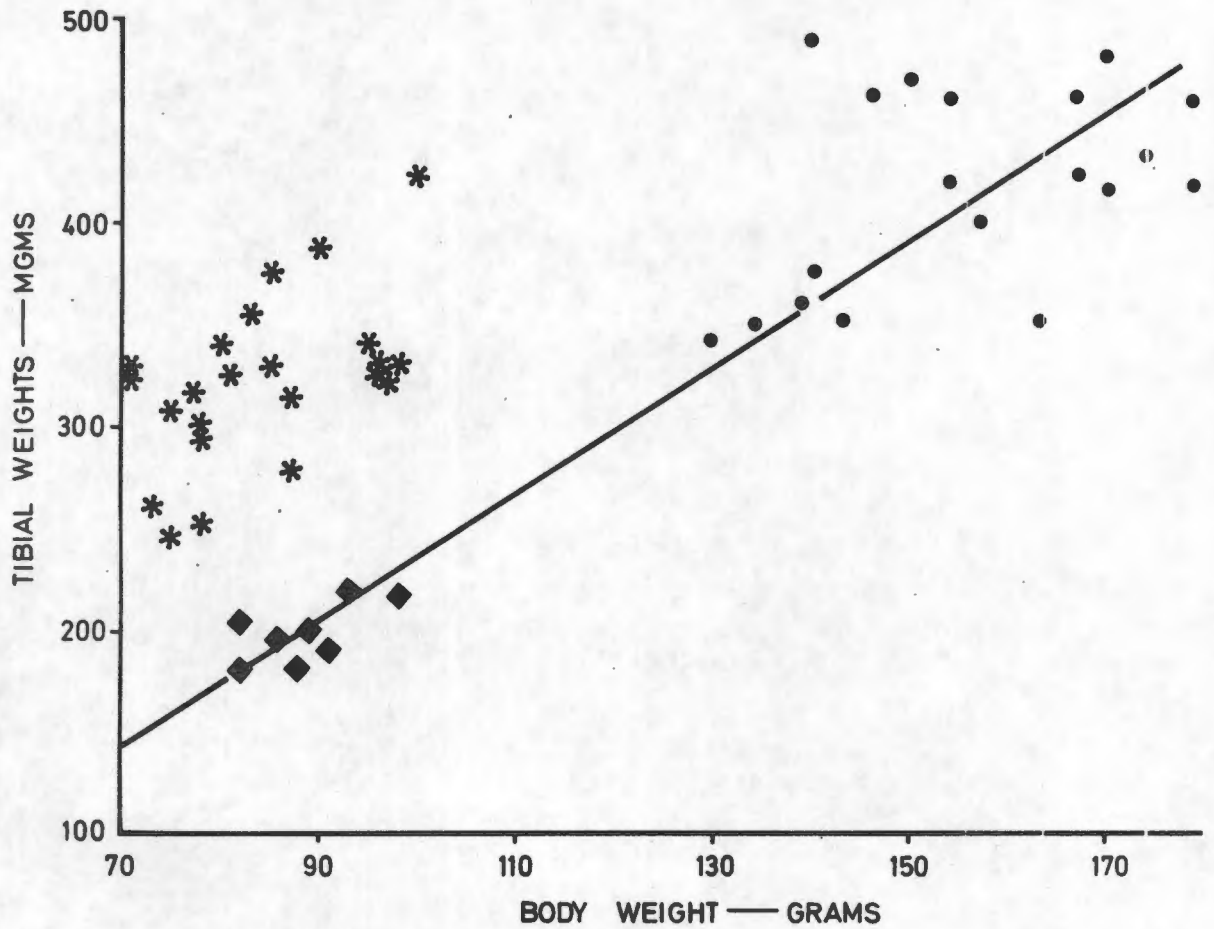
TABLE XI

Total Bone and Epiphyseal lengths in protein deprived, age and weight-matched control rats.

The means (S.E.M.) are shown

	No. of rats	The means (S.E.M.) are shown		
		Tibia (mm)	Tibial Epiphysis (mm)	Epiphyses as % of tibia
Protein deprived group (4%)	16	2.84 (0.02)	0.13 (0.01)	4.71 (0.23)
Age-matched controls 20%	16	3.03 (0.03)	0.21 (0.01)	6.85 (0.31)
Weight-matched controls	8	2.46 (0.04)	0.20 (0.02)	8.26 (0.68)
				Femur (mm)
				2.68 (0.02)
				2.89 (0.02)
				2.27 (0.02)

Using the t test, the protein deprived rats showed a significant reduction in femoral and tibial length, when compared to the age-matched controls ( $t = 6.493$ ,  $p < 0.001$ ;  $t = 7.397$ ,  $p < 0.001$  respectively). The epiphyses as % of tibial length was significantly reduced in protein deprivation compared to both control groups ( $t = 4.019$ ,  $p < 0.001$ ;  $t = 5.782$ ,  $p < 0.001$ ; for age and weight-matched control groups).



**FIGURE 16**

A correlation between body and tibial weights in protein-deprived rats ( \* ) weight matched ( ■ ) and age-matched controls ( ● ). Bone has grown in the protein deprived rats, in spite of cessation of weight gain; however bone weight remains below that of the age matched controls.

achieving a tibial weight less than expected for age, nevertheless show a substantial increase in spite of almost total failure to gain body weight.

c) Mineral Content

Table XII contrasts the bone ash of protein deprived and age-matched controls in the proximal, mid-shaft and distal tibia and femur. Reduced mineral content (bone ash expressed as a percentage of dry bone weight) is found only at the proximal and distal ends of the tibia in the protein deprived rats.

DISCUSSION

The techniques utilised in these experiments on bone morphology are of limited value, in that they merely indicate the final composition of the bones. They are incapable of measuring alterations in the dynamic processes which maybe affected by protein deprivation. Their usefulness, however, lies in the fact that they correlate the predicted effects of the interrelated kinetic studies, on bone morphology.

Naked eye, radiographic comparison of bone morphology is inaccurate and to minimise the error in processing, an aluminium wedge was included as the internal standard. The error of parallax was minimised by the large distance between the bones and the x-ray source, which is kept constant (Appendix Six).

The results of both age and weight-matched control rats (Table XI) bears out the knowledge that there is normally a parallel between the rate of bone growth and the epiphyseal cartilage plate thickness<sup>(138)</sup>. As the rats grow in size, so the rate of growth diminishes and the epiphyseal plate becomes thinner. The thinnest epiphyseal plate was however found in the protein deprived groups indicating a significant reduction in the rate of linear growth as compared to both control groups.

TABLE XII

BONE ASH WEIGHT

expressed as percentage dry bone weight, in the tibiae and femora of protein deprived and age matched control rats

The means (S.E.M.) are shown.

TIBIA

	<u>No. rats</u>	<u>TIBIA</u>			<u>FEMUR</u>		
		<u>Proximal</u>	<u>Midshaft</u>	<u>Distal</u>	<u>Proximal</u>	<u>Midshaft</u>	<u>Distal</u>
Protein deprived group (4%)	8	48.88 (0.96)	62.73 (1.0)	48.23 (0.86)	50.55 (1.13)	61.29 (0.78)	48.42 (0.78)
Age Matched Controls	8	52.54 (0.68)	63.26 (0.71)	54.98 (0.85)	50.88 (1.38)	62.70 (0.68)	49.97 (1.02)

Using the t test a significant reduction in bone ash is found only in the proximal and distal portions of the tibiae of the protein deprived group ( $t = 3.941$ ,  $p < 0.005$ ;  $t = 5.562$ ,  $p < 0.001$  respectively), but the magnitude of change is small.

Similar findings of narrowed epiphyseal cartilage plates have been described (17,18). Moreover the thinner epiphyseal plates confirms the histological appearances described in Chapter three. The end result was shorter bones in the protein deprived rats (Table XI) which is in keeping with the findings in children suffering from kwashiorkor (15) and experimental protein depleted pigs (18).

Under normal circumstances tibial weight bears a fixed relationship to body weight, (136) but in the protein deprived rats bone growth continued despite the almost total failure to gain body weight. This ability to maintain bone growth in the face of failure to gain body weight has been noted in protein malnutrition (17,39) as well as in starvation (138,139).

Measurements of the mineral content of the bone in protein depleted states have been performed (18,19,20,21,22,23). Protein deprived monkeys had normal matrix and mineral composition when compared to controls (20). Although total ash content was reduced in protein depleted rats, (18) the mineral to matrix ratio in the shafts of the long bones was normal. These authors concluded that the protein content of the diet did not affect the mineral content of the bones, nor did further addition of calcium to the diet. Similar observations were described by others (21,22,23). In an attempt to distinguish the effects of protein from calcium deprivation, Elmaraghi (19) described protein lack as causing "matrix-osteoporosis", whereas calcium deficiency resulted in "mineral-osteoporosis".

The experiments performed in this thesis, although not identical, showed similar results. Both percentage ash and the ratio of ash to matrix, reflect the relative proportions of the mineral and matrix content. The femoral and tibial mid-shafts had normal mineral

content in the protein deprived rats as compared to controls. This confirms the densitometric findings in the femoral midshaft described in the previous chapter. The proximal and distal ends of the tibia in the protein deprived rats showed, however, a significant reduction in mineral content (Table XII). This reduction, though statistically significant, was small and probably not of any biological significance, since no rachitic changes were seen either histologically (figure 5) or radiologically (figure 15).

The differences in mineral content between tibial ends and the midshaft do, however, indicate that, where possible, densitometric and morphological studies on bone should be performed at all sites.

#### SUMMARY

Extirpated bone measurements revealed reduced linear growth in the protein deprived group of rats when compared to age-matched groups. Tibial bone weight was similarly reduced despite total failure in body weight gain.

Mineral content expressed as percentage ash was normal in the midshafts, confirming the densitometric findings of the previous chapter. Reduced mineral content of proximal and distal tibial ends although significant was considered as not being of any biological significance.

PART III

## CHAPTER ELEVEN

CORRELATION OF RESULTS, HYPOTHESES AND CONCLUSIONSINTRODUCTION

Each preceding chapter has included relevant discussion of the techniques and results of the individual studies. In this concluding section an attempt will be made to correlate these results, to crystallise the discussions and to put forward tentative hypotheses linking them.

SUMMARY OF RESULTS

THE MODEL: After a period of thirty-five days, the rats were depleted of protein using a 4% protein diet administered by the technique of interval feeding. Protein deficiency was confirmed by lowered serum albumin concentrations, fatty infiltration of the liver, retarded growth and thinning of the epiphyseal cartilage plate. This regime resulted in the production of pure protein deprivation in the rat, in which calorie deficiency was minimised. Histological and radiological evidence excluded vitamin D deficiency.

EXPERIMENTAL DATA: The absorption of dietary calcium was markedly reduced in the protein depleted rats. The addition of protein to the test meal did not increase absorption, suggesting that changes in protein in the meal had no relevance. Chronic protein deprivation by contrast presumably effected these changes by lowering the calcium binding protein (CaBP) which is responsible for calcium absorption. The kinetic studies showed a large reduction in exchangeable calcium pool, indicating reduced calcium turnover. Bone formation, measured as calcium accretion into bone and the incorporation of proline into

hydroxyproline was also low. However, bone resorption did not change significantly (Figure 17). The excretion of calcium in the experimental rats was high relative to the reduced exchangeable calcium pool.

The changes in these dynamic processes caused a reduction of bone growth and a lowering of the total amount of bone in the protein deprived rats. This was however unaccompanied by any significant change in mineral content as shown by ash weight and densitometric testing. Serum levels of calcium, phosphorus and magnesium were significantly reduced whereas alkaline phosphatase activity remained unchanged.

### DISCUSSION

There are numerous factors involved in the control of normal skeletal metabolism. Bone formation requires the synthesis of matrix, followed by a precise electro-chemical process of calcification<sup>(59)</sup>. Remodelling of bone may be affected by stresses exerted on the bone, as well as by nutritional and hormonal factors.

Protein, vitamins and minerals are the major nutritional factors necessary for normal bone metabolism. Since the prime object of this thesis was to study the effect of protein deprivation on the bone adequate supplementation of all the vital minerals and vitamins were included in the experimental diets. No clinical, radiological or biochemical evidence of rickets or scurvy was noted in the bones of the protein deprived rats. It is therefore reasonable to assume that the disordered bone metabolism described in this thesis was not primarily due to deficiencies of the bone related vitamins or minerals (although impairment of their biological effects cannot be excluded).

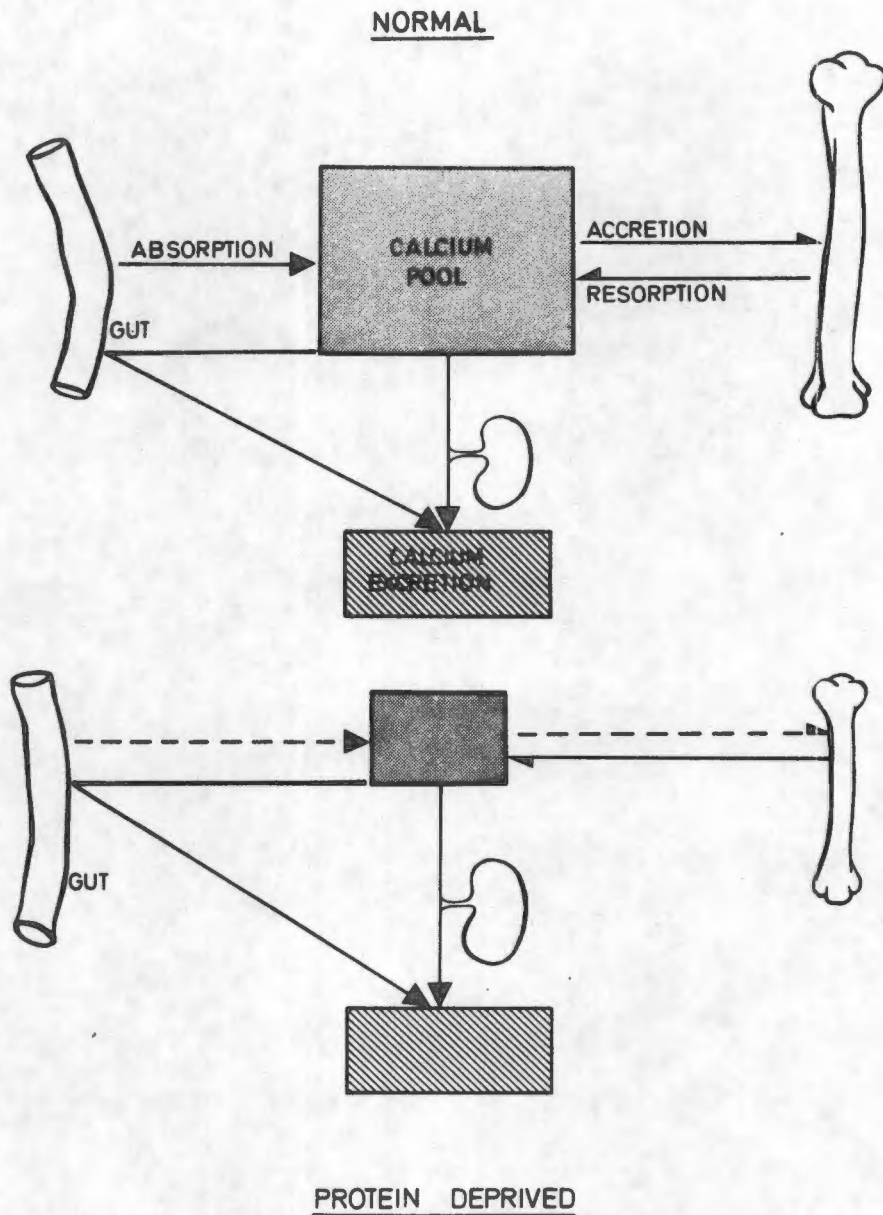


FIGURE 17

Schematic representation of the results described in this thesis. The absorption of calcium, exchangeable calcium pool and accretion of calcium into bone are reduced in the protein deprived rat as compared to age matched controls. Bone resorption remained unchanged and thus the result was reduced total bone with normal mineral content. Despite the marked reduction in exchangeable calcium pool, fractional calcium excretion increased.

Protein is an essential constituent of all cells. Bone cell proteins have numerous and varied functions, including matrix formulation, enzymatic activity required for bone remodelling, the maintenance of membrane permeability and transport of ions across cell membranes. Similarly, the intestinal mucosal cell has a rapid turnover and therefore a high protein requirement. It is therefore conceivable that protein deprivation may affect both these sites simultaneously.

Dietary calcium absorption requires the presence of CaBP<sup>(92)</sup>, a carrier protein in the mucosal cell of the intestine. Although the synthesis of this protein is stimulated by vitamin D and its metabolites, it requires an adequate protein supply. CaBP has recently been shown to be low in protein deprived rats<sup>(94)</sup> which would result in reduced calcium supply to the body despite adequate dietary supplementation with calcium.

Deprivation of calcium will result in less calcium being made available for bone accretion. The mitochondria of the bone cells are directly responsible for the process of calcification<sup>(140)</sup> and require an adequate supply of calcium ions. Calcium deprivation would then hinder this process and prevent adequate hydroxyapatite formation.

Indirect attempts to measure collagen synthesis were made by studying the rate of incorporation of proline into hydroxyproline. Significantly reduced rates of incorporation were seen in the experimental rats which probably reflected a lowered synthesis of all the body proteins in chronic protein deprivation. This reduction in matrix formation and the adequate supplementation of vitamin D in the diets possible explains the absence of any radiological or histological features of rickets in the experimental rats.

The fractional excretion rate of calcium did not fall in the protein deprived rats despite markedly reduced turnover of calcium. Whether this excretion was via faecal or urinary route could not be examined by the techniques used in the present study. Endogenous faecal calcium is normally the major route of excretion in young growing rats and in fact was noted to be increased in protein depleted rats<sup>(23)</sup>. It is possible that additional amounts are lost via the kidneys. CaBP, recently demonstrated in the kidney may be responsible for renal tubular calcium binding and hence tubular reabsorption. Since gut CaBP is low in malnutrition it is conceivable that renal CaBP may be likewise affected, increasing urinary calcium loss.

#### HYPOTHESIS

It is probable that a primary effect of protein deprivation is a reduction in CaBP in the gut resulting in lowered calcium absorption. The consequent reduction in calcium pool turnover causes a reduced accretion of calcium into bone.

The impaired synthesis of hydroxyproline (an independent phenomenon) together with the reduced calcium accretion, results in lowered bone formation. Despite this reduction, bone resorption continues at a normal rate, so that the end result is a reduction in total bone.

The increased fractional excretion rate of calcium despite the reduced calcium pool turnover is due either to increased endogenous faecal calcium, lowered CaBP in the kidney or a combination of both these factors. The role of parathyroid hormone, calcitonin and vitamin D must remain conjectural until direct measurements of these hormones are made.

APPENDIX ONEESTIMATIONS OF SERUM ALBUMIN, CALCIUM, PHOSPHORUS,  
MAGNESIUM AND ALKALINE PHOSPHATASEA) SERUM ALBUMIN ESTIMATIONBiuret Method<sup>(50)</sup>.REAGENTS

1) 0.9% (w/v) Sodium chloride

2) Hydrochloric acid - ethanol

To 600 ml of "denatured" ethanol add 1.0 ml. concentrated hydrochloric acid. (Denatured ethanol is a mixture of 95% absolute ethanol and 5% methanol). Store at 4°C.

3) 0.2 M Sodium acetate-ethanol

Dissolve 2.7218 mg. sodium acetate in 5.0 ml. methanol. Add 95.0 ml. absolute alcohol. Store at 4°C.

(Note: Sodium acetate is soluble in methanol but not in absolute alcohol. Solution must therefore be achieved in methanol prior to the addition of ethanol.)

4) 3% (w/v) Sodium hydroxide

5) Biuret Reagent.

Dissolve (i) 17.3 g. copper sulphate pentahydrate in 100 ml. hot distilled water, (ii) 173 g. sodium citrate and 100 g. anhydrous sodium carbonate in 800 ml. distilled water while heating. When cool, pour (ii) into (i) with rapid stirring. Dilute to one litre with distilled water.

6) Protein Standard.

30 mg./ml. aqueous solution of crystalline bovine albumin.

METHOD

- a) To 0.2 ml. of serum add 0.8 ml of normal saline.
- b) Add 9.0 ml. HCl-ethanol dropwise with continuous shaking.
- c) Incubate for thirty minutes at 37°C.
- d) Centrifuge.
- e) Transfer 5.0 ml of the albumin rich supernatant fluid to another centrifuge tube.
- f) Add 0.5 ml. of 0.2 M Sodium acetate-ethanol dropwise with continuous shaking.
- g) Stopper and allow to stand at room temperature for ten minutes.
- h) Centrifuge. Tip off the supernatant. Invert tubes on filter paper to ensure all the supernatant fluid is removed.
- i) Dissolve the precipitate in 5.0 ml. 3% sodium hydroxide, add 1.0 ml. biuret reagent, and mix by inversion.
- j) Read intensity of colour produced after thirty minutes at 545 m $\mu$ , using a Klett Summerson photo-electric colorimeter.

A reagent blank consists of 5.0 ml. 3% sodium hydroxide plus 1.0 ml. biuret reagent. The known protein standard is made to consist of 4.9 ml. 3% sodium hydroxide, 0.1 ml. of a 3 g.% bovine albumin solution, and 1.0 ml. biuret reagent.

CALCULATION

$$\frac{\text{Sample - blank}}{\text{Standard - blank}} \times \frac{3}{1} = \text{g. of albumin per 100 ml.}$$

All samples were estimated in duplicate with an error of 6.32%.

**B) TOTAL SERUM CALCIUM ESTIMATION**

By the method of Baron and Bell<sup>(141)</sup>

**REAGENTS****1) Glycine buffer ph 12.66.**

To 20 ml. of 0.1 M - glycine + 0.1 M Sodium chloride add  
80 ml. of 0.1 M Sodium hydroxide.

**2) 40% (w/v) Sodium hydroxide.****3) E.D.T.A. Solution.**

Dissolve 0.93 g. disodium ethylene diamine tetra-acetate in  
distilled water and make up to 1 litre.

**4) Calcein-thymol-phthalein indicator.**

Grind together 0.2 g. calcein, 0.12 g. thymol-phthalein and  
20 g. potassium chloride, to a fine powder.

**5) Stock calcium solution (100 mg/100 ml.).**

Add 2.5 g. calcium carbonate (previously dried at 105°C for  
24 hours) to 200 ml. distilled water. Add 50 ml. N-hydrochloric  
acid. Allow to stand overnight. Dilute up to 1 litre.

**6) Calcium standard (10 mg/100 ml.).**

Dilute 10 ml. of the stock calcium solution, up to 100 ml.

**METHOD**

a) To 1.0 ml. of serum add 5.0 ml. glycine buffer in a white porcelain  
crucible.

b) Add 40% (w/v) sodium hydroxide, dropwise, until the ph of the  
solution is 12.

c) 0.5 - 1.0 mg. of the calcein-thymol-phthalein indicator is added  
until the solution is uniformly orange green in colour.

d) Add the E.D.T.A. slowly, titrating, until the colour of the  
solution changes from green to mauve.

e) Repeat all samples twice.

A reagent blank consists of 1.0 ml. distilled water plus 5.0 ml. glycine buffer. The known calcium standard is made to consist of 1.0 ml. of a 10 mg/100 ml. calcium standard solution plus 5.0 ml. glycine buffer.

CALCULATION

$$\frac{\text{Sample - Blank}}{\text{Standard - Blank}} \times \frac{10}{1} = \text{mg. of calcium per 100 ml.}$$

All samples were estimated in duplicate with an error of 3.56%.

C) SERUM MAGNESIUM ESTIMATION

Using the technique of Neill et al<sup>(142)</sup>.

REAGENTS

- 1) 2/3 N. Sulphuric Acid.
- 2) 10% Sodium Tungstate.
- 3) 4 N. Sodium hydroxide.
- 4) 0.05% Titan yellow.

Dissolve 50 mg. of titan yellow in distilled water. Make up to 100 ml. Store in an amber coloured bottle in a dark cupboard.

- 5) 0.05% Polyvinyl alcohol.

Disperse 50 mg. polyvinyl alcohol in 50 ml. of distilled water. Dissolve by heating in a 65°C water bath. Dilute to 100 ml. with water and filter. Store in the refrigerator.

- 6) Stock magnesium solution. (50 mg./100 ml).

Dissolve 50 mg. magnesium metal turnings in 5 ml. 1N hydrochloric acid in a 100 ml. volumetric flask. Make up to 100 ml. using distilled water.

- 7) Working magnesium standard. (0.5 mg./100 ml.).

Dilute 1 ml. of the stock magnesium solution to 100 ml. using distilled water.

METHOD

- a) To 0.5 ml. of serum add 2.5 ml. distilled water.
- b) Then add 1.0 ml. 10% sodium tungstate, followed by 1.0 ml. 2/3 N. sulphuric acid.
- c) Centrifuge and transfer 2.5 ml. to another tube. Add 1.0 ml. polyvinyl alcohol, and 0.5 ml. titan yellow. Mix well.

- d) 1.0 ml. 4 N. Sodium hydroxide is then added slowly with gentle mixing.
- e) The reagent blank consists of 3.0 ml. distilled water processed as for the serum (b, c and d). 2.0 ml. working magnesium standard (10  $\mu$ g) is added to 1.0 ml. distilled water and processed in a similar fashion.
- g) Read the absorbance of the Standard and unknown versus the blank at 540 m $\mu$  on a Beckman DB Spectro-photometer.

CALCULATION

$$\frac{\text{Unknown}}{\text{Standard}} \times 1.64 = \text{meq. of magnesium per litre.}$$

All samples were estimated in duplicate with an error of 5.20%.

D) SERUM INORGANIC PHOSPHORUS ESTIMATION

By the technique of Dryer et al<sup>(143)</sup>

REAGENTS

- 1) 30% Trichloroacetic acid.
- 2) 0.0202 M. Ammonium Molybdate.

Dissolve 25 g. ammonium molybdate in 700 ml. of distilled water. Slowly add 84 ml. concentrated sulphuric acid. Allow to cool and dilute to 1 litre with distilled water. Store in a polyethylene bottle.

- 3) p - semidine reagent. (N - phenyl - p - phenylenediamine hydrochloride).

Place 50 mg. of p - semidine in a flask. Wet the salt with a few drops of 95% ethanol and add 100 ml. 1% sodium hydrogen sulphite with shaking. Filter off the insoluble residue. Store in the refrigerator.

- 4) Stock phosphorus standard (1 mg./ml.).

Place 439 mg. potassium dihydrogen phosphate in a 100 ml. volumetric flask and add distilled water to volume. Add a few drops of chloroform as preservative.

- 5) Working phosphorus standard (10 $\mu$  g/ml.).

Transfer 1.0 ml. of the stock phosphorus standard to a 100 ml. volumetric flask. Add 16 ml. 30% trichloroacetic acid and then distilled water up to 100 ml.

METHODS

- a) To 0.5 ml. serum add 4.5 ml. distilled water.
- b) Add 1.0 ml. 30% trichloroacetic acid, mix well and let stand for 5 minutes. Centrifuge.

- c) Transfer 2.0 ml. of the supernatant to another tube and add 0.4 ml. molybdate reagent. Mix well then add 4.0 ml. p - semidine reagent.
- d) Allow to stand for 10 minutes and then read the intensity of the colour produced at 770 m $\mu$  using a Klett Summerson photo-electric colorimeter.

A reagent blank consists of 0.33 ml. 30% trichloroacetic acid, 1.67 ml. distilled water, 0.4 ml. molybdate reagent and 4.0 ml. p-semidine. The known phosphorus standard is made up of 2.0 ml. of the working standard (20  $\mu$ g), 0.4 ml. molybdate agent and 4.0 ml. p-semidine.

#### CALCULATION

$$\frac{\text{Unknown - Blank}}{\text{Standard - Blank}} \times 12 = \text{mg. inorganic phosphate per 100 ml.}$$

All samples were estimated in duplicate with an error of 4.52%.

E) SERUM ALKALINE PHOSPHATASE ESTIMATION

By the method of Shinowara et al<sup>(144)</sup>.

REAGENTS

## 1) Buffered substrate, stock solution.

Measure 3 ml. petroleum ether and about 200 ml. distilled water into a 250 ml. volumetric flask. Add 2.5 g. sodium -  $\beta$  - glycerophosphate and 4.25 g. sodium diethyl barbiturate. Dissolve and dilute with distilled water to bring aqueous level to 250 ml. Mix well and store in refrigerator.

## 2) Working alkaline phosphatase substrate.

To a 100 ml. volumetric flask, add 3 ml. petroleum ether, 50 ml. stock substrate and 2.9 ml. 0.10 N sodium hydroxide. Dilute with distilled water to the 100 ml. level and mix well. Check the ph and correct using 0.10 N sodium hydroxide or 0.10 N hydrochloric acid to maintain the ph at 10.8.

## 3) All the reagents employed in the determination of inorganic phosphate (section D).

METHODS

- a) Place 4.5 ml. of the working alkaline phosphatase substrate in a test tube and place in a water bath at 37°C for 5 minutes.
- b) Add 0.5 ml. serum and mix well.
- c) Replace in the water bath for one hour.
- d) Remove the tube from the water bath and add 1.0 ml. 30% trichloroacetic acid, mix well and let stand for five minutes.
- e) Centrifuge. Transfer 2.0 ml. of the supernatant to another test tube.

- f) Add 0.4 ml. molybdate reagent, mix well and then add 4.0 ml. p - semidine reagent.
- g) Read absorbance at 770 m $\mu$  using a Klett - Summerson photo-electric colorimeter.

A substrate blank consists of 4.5 ml. of the working substrate and 0.5 ml. distilled water processed from d to g. The reagent blank (consisting of 0.33 ml. 30% trichloroacetic acid and 1.67 ml. of distilled water) and a standard phosphate (20 $\mu$ g) are processed from f to g.

Inorganic phosphate determinations on the same serum samples are made as in section D.

#### CALCULATION

$$\frac{\text{Unknown - Substrate Blank.}}{\text{Standard - Reagent Blank}} \times 12 = \text{mg. of inorganic phosphate per 100 ml.}$$

From this result subtract the concentration of inorganic phosphate determined on the same sample of serum in section D.

The result is expressed as SHINOWARA UNITS.

All the pooled samples were estimated in duplicate with an error of 9.04%.

APPENDIX TWO<sup>47</sup>CALCIUM WHOLE BODY COUNTINGA) INTRAGASTRIC INTRODUCTION OF THE <sup>47</sup>CALCIUM

Figure 18 illustrates the method of introducing the dose of <sup>47</sup>Calcium. Plastic tubing with an inner diameter of 1 mm. is attached to a needle and syringe. The tubing is passed into the stomach of the rat and the <sup>47</sup>Ca injected, slowly. Since the dose is calculated from the initial whole body retention value, flushing of the plastic tubing to ensure complete introduction of the isotope is unnecessary.

FIGURE 18

## B) WHOLE BODY GAMMA COUNTING SYSTEM

The whole body counting system was specially designed for human faecal counting and adapted for small animal studies (figure 19). It consisted of a box with two inch lead shielding, in which were two opposing 5 inch diameter plastic scintillators with photomultiplier tubes connected to a gamma counting system (Nuclear Enterprises).

The opposing photomultiplier tubes enabled reproducible whole body retention values to be obtained independant, both of the position and movement of the rat. Any effect due to redistribution of the isotope within the body was also minimised by the opposing position of the photo-multiplier tubes. Pulse height analysis was used to exclude counts from the decay product  $^{47}\text{Sc}$ .

Each rat was counted in a plastic container (figure 19) which is first counted without the rat to provide the background count. Background counts are subtracted from each estimation of whole body retention counts.

An equivalent dose (standard) of  $^{47}\text{Ca}$  was counted in a plastic container of similar geometry to the rat, thus obviating the need to include calculations for isotopic decay and to minimise the day to day variations which maybe introduced by the machine.

Counting efficiency for  $^{47}\text{Ca}$  was 0.8% and the error was 1%.

C) The  $\gamma$  emitting isotope  $^{47}\text{Ca}$  was obtained from the Radio-chemical Centre, Amersham, which supplied 150  $\mu\text{Ci}$  in 3.0 ml. of sterile calcium chloride solution.

S.A. = 150  $\mu\text{Ci}/\text{mgm Ca}$ .

At the reference date there was less than 2.5%  $^{45}\text{Ca}$  in the solution. The half life of  $^{47}\text{Ca}$  is 4.53 days and it has an energy of 1.30 mev.

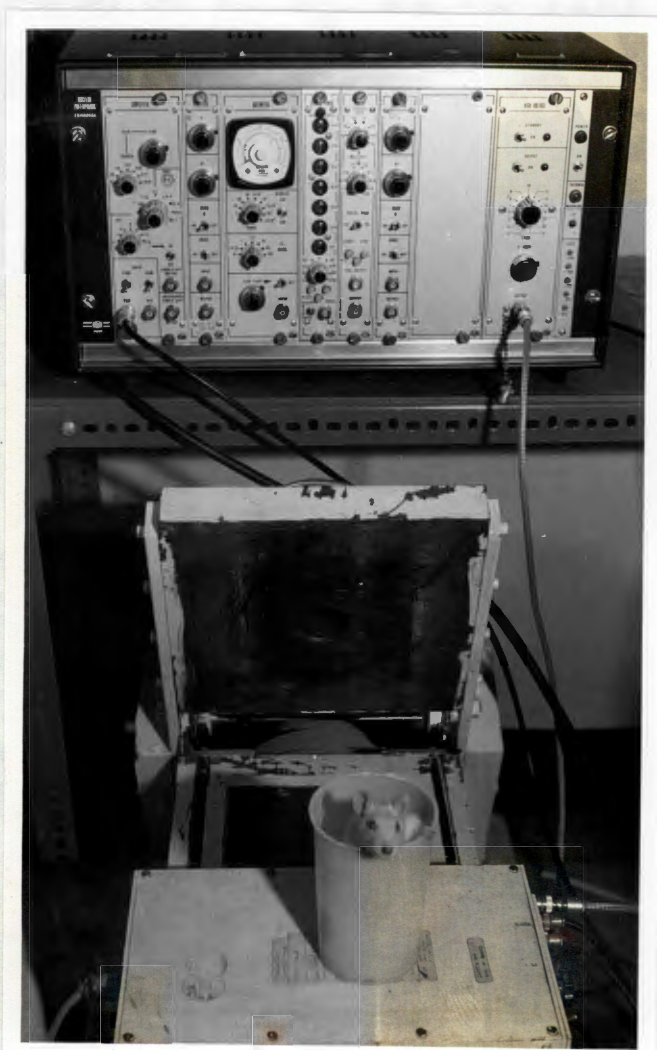


FIGURE 19

Illustrates the whole body gamma counting system. The rat is placed in a plastic container and counted in the lead box which is connected to a gamma counting system.

APPENDIX THREESERUM <sup>45</sup>Ca CALCIUM S.A. ASSAYA) INTRAVENOUS INJECTION OF THE RADIO-ISOTOPES

Figure 20 illustrates the intravenous injection of the radio-isotopes. To calculate the dose given, the syringe is weighed before and after the injection. The difference in weights being the amount of radio-isotope solution injection. 1 g. is assumed to equal 1 ml. of the solution.

The standard is obtained by weighing out a known amount of the radio-isotope solution. <sup>45</sup>Ca the  $\beta$  emitting isotope which has a half life of 159 days with an energy of 0.258 Mev was obtained from the Radio-Chemical Centre, Amersham, S.A.=1 m Ci/ml. with a concentration of 25  $\mu$ g Ca/ml.

B) SERUM SAMPLES

Serial serum samples are obtained by bleeding the tail vein of the rat, at the tip. Each sample of blood is collected in a 0.1 ml. pipette, passed into a small centrifuge tube and centrifuged.

50  $\mu$ l of the serum is then removed. The actual amount of serum used is determined by weighing the pipette before and after dispensing of the serum and adjusting the results proportionately in order to obtain uniformity in all the serum samples.



FIGURE 20

Intravenous injection of the radiocalcium isotopes into the tail vein of the rat.

C. PROCESSING OF THE SERUM SAMPLE (104)

PRINCIPLE

Perchloric acid incineration, of the precipitated calcium oxalate, eliminates all oxidizable material and allows the preparation of calcium perchlorate which is free from all interfering substances.

Since calcium perchlorate is insoluble in toluene, a secondary solvent (tri-n-butyl phosphate) is essential.

REAGENTS

1) Buffered oxalate.

82 g. sodium acetate is dissolved in 57 ml. glacial acetic acid and made up to two litres with 4% ammonium oxalate.

2) (PPO) 2.5 - diphenyloxazole.

3g. is dissolved in 1 litre of toluene.

3) Standard Calcium Solution (10 mg/100 ml.).

4) Tri-n-butyl phosphate(TBP)

5) 60% perchloric acid.

METHODS Radioactive counting.

a) To 50  $\mu$ l of the serum sample add 1 ml. of the standard calcium solution as a carrier.

b) 5 ml. buffered oxalate solution is added.

c) Allow the solution to stand for one hour. Centrifuge and decant the supernatant.

d) Add 0.5 ml. 60% perchloric acid, followed by heating to 180°C until dry.

e) The residue is then dissolved in 2 ml. TBP and 4 ml. PPO in Toluene with gentle warming.

f) Transfer the mixture to the counting vial followed by 6 ml. PPO in toluene as washings.

- g) Standards containing known amount of activity and approximately the same amount of carrier calcium are run at the same time.
- h) Counting is performed in a Beckman liquid scintillation counter with a counting efficiency of 53.4% and an error of less than 5%.

D) CALCULATION THE S.A. OF <sup>45</sup>CA AS % OF DOSE

The following estimations are made.

- a. The radio activity of <sup>45</sup>Ca expressed as c.p.m. in each 50 μl sample of serum.
- b. total serum calcium (Appendix one) expressed as mg. of calcium per 100 ml. serum.

Calculation

There are X mg. of calcium in 100 ml. of serum, therefore 1 mg. of calcium is present in  $\frac{1}{X} \times 100$  ml. .... (1)

If there are Y c.p.m. in a 50 μl sample, then there will be

$$Y \times \frac{1000}{50} \text{ c.p.m. in one ml. .... (2)}$$

For 1 mg. of calcium there are

$$Y \times \frac{1000}{50} \times \frac{1}{X} \times 100 \text{ c.p.m. .... (3)}$$

Expressed as percentage of original dose

$$\frac{Y \times \frac{1000}{50} \times \frac{1}{X} \times 100}{Z} \times 100 \text{ .... (4)}$$

where Z is the dose in c.p.m.

E) THE USE OF  $^{45}\text{Ca}$  FOR SERUM SAMPLING IN THE DOUBLE ISOTOPE KINETIC STUDY

The total dose of radiocalcium is rapidly distributed in the large exchangeable calcium pool. Assuming 100  $\mu\text{Ci}$  of radiocalcium is administered, and 1  $\mu\text{Ci} = 2.2 \times 10^6$  c.p.m. (100% efficiency)

$$\therefore 100 \mu\text{Ci} = 2.2 \times 10^8 \text{ c.p.m.}$$

If there are 100 mgm in the calcium pool, then the S.A. at the time of equilibrium will be  $2.2 \times 10^6$  c.p.m./per mg. of calcium.

Since there are approximately 10 mg. of stable calcium per 100 ml. and only 50  $\mu\text{l}$  serum samples are tested, therefore only

$$\begin{aligned} \frac{0.05}{100} \times 10 &= 0.005 \text{ mg. are counted which equals} \\ 2.2 \times 10^6 \times 0.005 & \\ = 1.1 \times 10^4 \text{ c.p.m.} \end{aligned}$$

At a point half way along the serum S.A. curve, the counting will only be 5,500 c.p.m. To minimise the error in counting a minimum of 10,000 c.p.m. is required.

Thus  $^{47}\text{Ca}$  with its poor counting efficiency cannot be economically used for the serum sampling, whereas very much less  $^{45}\text{Ca}$  can be utilised because of enhanced counting, achieved by liquid scintillation.

F) PROCESSING THE TIBIAE FOR S.A. OF  $^{45}\text{Ca}$  CALCIUM CONTENT. (104)

PRINCIPLE

Ashing of the bones is necessary prior to processing the samples as described for the serum samples.

REAGENTS

- 1) All the reagents required for processing the serum samples.
- 2) 4% Ammonium oxalate.
- 3) 2 N. hydrochloric acid.

METHODS

- a) Accurately weighed amounts of dried tibia are ashed at  $600^{\circ}\text{C}$ .
- b) Dissolve the ash in 3 ml. 2 N hydrochloric acid.
- c) To 0.5 ml. of the solution (which contains less than 50 mg. calcium) add 5 ml. 4% ammonium oxalate followed by sufficient ammonia to make the solution alkaline.
- d) Allow to stand for one hour, centrifuge and decant the supernatant.
- e) To the residue add 1.0 ml. 60% perchloric acid, heat at  $180^{\circ}$  until dry and dissolve in 10 ml. of distilled water.
- f) Continue processing as for serum samples b - f.

G) CALCULATION OF S.A.  $^{45}\text{Ca}$  IN BONE

1. The dose is calculated by weighing the syringe before and after injection.
2. Samples of clean, dried tibiae are accurately weighed. The bones are ashed, dissolved and tested for radioactivity.
3. The radioactivity is expressed as c.p.m. per 100 mgm dried bone weight and related to the dose, i.e. c.p.m./100 mgm. bone as a % of dose.

APPENDIX FOURRATE OF INCORPORATION OF  $^{14}\text{C}$  PROLINE INTO HYDROXYPROLINE

Using the method described by Firschein<sup>(123)</sup>

PRINCIPLE

The tibiae are demineralised and the organic matrix is hydrolysed. Proline is separated off from the hydroxyproline using an ion exchange resin and the specific activity of the  $^{14}\text{C}$  hydroxyproline is measured.

REAGENTS

- 1) Hydrochloric acid.

0.33N, 1N, 3N and 6N.

- 2) 0.1M citrate buffer (ph 6.0).

58.8 g. sodium citrate is dissolved in two litres of distilled water. 19.2 g. citric acid is dissolved in one litre of distilled water. Add 150 ml. of the citric acid solution to the two litres of sodium citrate solution and adjust to ph 6.0 by addition of small amounts of either solution.

- 3) Bray's Solution<sup>(145)</sup>

Naphthalene	60 g.
2,5 - diphenyloxazole (PPO)	4 g.
1,4 - Bis - $\left[ 2(5\text{-phenyloxazoly}) \right]$ -benzene (POPOP)	0.2 g.
methanol	100 ml.
ethylene glycol	20 ml.
p-Dioxane	to make up to 1 litre

4) Isopropyl alcohol.

5) Oxidising Reagent.

a) 3.5 g. chloramine T is dissolved in 50 ml. distilled water.

b) buffered solution ph 6.0.

57 g. sodium acetate, 37.5 g. sodium citrate, 5.5 g. citric acid and 385 ml. isopropyl alcohol are placed in a 1 litre flask and diluted to volume with distilled water. Shortly before use 4 parts buffer and 1 part chloramine T solution are mixed.

6) Ehrlichs Reagent.

20 g. p - dimethylamino-benzaldehyde is dissolved in 22 ml. concentrated hydrochloric acid by warming on a steam bath. 128 ml. of isopropyl alcohol is then added and the solution allowed to cool.

#### METHOD

a) Remove the tibiae and clean off all soft tissue. Soak them in 0.33 N hydrochloric acid for three days with daily changes of the acid and washings with distilled water.

b) The organic residue is then refluxed with 5 ml distilled water using a sand bath and air condenser for twenty four hours.

c) The dissolved organic fraction is then hydrolysed in 6N Hydrochloric acid for eighteen hours at 125°C in an autoclave.

#### Separation Procedure

d) Filter the hydrolysate through a scintered glass funnel and evaporate to 2 ml.

e) Pass the hydrolysate through a 0.8 x 20 cm. column of Dowex 50 W - x 8, 200 - 400 mesh, previously equilibrated, with 1N hydrochloric acid.

- f) Wash the sample into the column with small amounts of 1N hydrochloric acid and elute with the same reagent.
- g) Collect 5 ml. fractions. The fourth and fifth fraction contains the hydroxyproline. Eighth and ninth fractions contain the proline.
- h) Evaporate the hydroxyproline sample to 2 ml. and pass through a second column of Dowex 50 W - x 8, equilibrated with 0.1 m. citrate buffer.
- i) Elute with the citrate buffer. Hydroxyproline is present in the fourth and fifth of 5 ml. fractions.

#### Assay Procedure

- j) Add 0.2 ml. 3 N hydrochloric acid and 10 ml. Bray's solution to 2 ml. of the separated hydroxyproline in citrate buffer.
- k) Count in a Beckman liquid scintillation counter. Quenching is determined for each sample by the addition of a known amount of radioactive standard, after each sample has been counted. The samples are then recounted. The difference between the actual counts obtained and the expected counts is the degree of quenching. Each individual sample is corrected for its own particular degree of quenching. Counting efficiency was 13.6% with an error of 8.2%.
- l) A known amount of standard is counted in the identical manner.

#### Total Hydroxyproline Determination <sup>(146)</sup>

- m) Place 3 ml. of the hydroxyproline sample in citrate buffer, in a 20 x 200 mm glass-stoppered test tube.
- n) Add 4 ml. isopropyl alcohol and 1 ml. oxidising reagent.
- o) Place the sample in an ice bath, and add 10 ml. of Ehrlich's reagent.
- p) Heat the samples in a boiling water bath for two minutes and cool in ice.

q) Stand at room temperature for 90 minutes and then read on a Klett Summerson photo-electric spectrometer at  $575 \text{ m}\mu$ . Standard hydroxyproline solution (0.05 g. per litre) is processed in an identical manner, and all samples are read against a blank (citrate buffer). Error of estimation 6.32%.

The S.A. results are expressed as counts per minute per  $\mu\text{g.}$  of hydroxyproline. Each result must be corrected for the various doses of  $^{14}\text{C}$  given to each animal. The dose is calculated by weighing the syringe before and after injection and assuming that 1 g. is the weight of 1 ml. of the dose solution.

$^{14}\text{C}$  labelled proline was obtained from the Radiochemical Centre, Amersham in 500  $\mu\text{Ci}$ , freeze dried solid batches

S.A. = 10 mCi/m.mole

87  $\mu\text{Ci/mg.}$

APPENDIX FIVEDENSITOMETRY

- 1) Roentgenograms of the femurs of anaesthetized rats were simultaneously exposed with the aluminium step wedge. The x-ray unit was a Philips Polytome operated at 35 KV, 19 mas with 0.03 second exposure. The distance between the rat and the x-ray tube was 1.22 metres. Cronex film was used; 90 second automatic processing of the film was achieved with a Kodak Rapid Processor.
- 2) Step wedge. The wedge consisting of five steps with 0.5 mm increments was machined from aluminium alloy.
- 3) The densitometric apparatus consists of a Hilgert and Watts H 451 micro densitometer, with a Hilgert and Watts L454 motor. Scanning speed was 0.25 mm/minute. The densitometer was coupled to a Hitachi 2 PD.54 recorder, the response being Standardised to 1.5 milli volts.

APPENDIX SIXRADIOGRAPHS OF EXTIRPATED BONES

Extirpated tibiae and femora were cleaned and radiographs performed using a Philips Polytome at 35 Kv, 19 Mas with 0.03 second exposures.

The distance between the bones and x-ray tube was 1.22 metres thus minimising the error due to parallax.

The Step wedge was included which acted as an internal standard for the automatic processing of the Cronex film.

Vernier Calipers were used to measure accurately total bone and epiphyseal lengths of the tibiae and femora on the radiographs.

Variations in the measurements were minimised to 3.1%.

APPENDIX SEVENSTATISTICAL METHODS (147,148)

$$1) \text{ MEAN } \bar{x} = \frac{\sum x}{n}$$

where  $x$  = the observations

(sigma)  $\Sigma$  = the sum of

$n$  = the number of observations.

2) STANDARD ERROR OF THE MEAN (S.E.M.)

$$\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n(n-1)}}$$

$$3) \text{ VARIANCE } = \frac{\sum (x - \bar{x})^2}{n-1}$$

5) STUDENT T TEST

The following criteria are used to decide whether or not to use the t test.

a) Given two samples, the variances of each sample should not be significantly different.

This test of homogeneity is performed using the F test.

$$F = \frac{S_1^2}{S_2^2}$$

where  $S_1^2$  is the larger of the two variances and  $S_2^2$  the smaller.

One determines its significance by entering in the appropriate table (upper significance limits of the F distribution) with  $n_1 - 1$  degrees of freedom for the numerator and  $n_2 - 1$  degrees of freedom for the denominator. F values that are smaller than the table value, at a level of  $P = 0.05$ , show no significant difference in the sizes of the two variances.

b) Underlying distributions must be normal. Provided there was nothing to suggest any possibility of a skew distribution of the data, normality was assumed (the t Test is robust to any slight deviation from normality in the data).

4) LEAST SQUARE REGRESSION LINE

A line of the form  $y = a + bx$

is fitted to each of two sets of data, each set being of the form

$(x_1, y_1)$   $(x_2, y_2)$  .....  $(x_n, y_n)$

It can be shown that the constant

$$a = \frac{(\Sigma y) (\Sigma x^2) - (\Sigma x) (\Sigma xy)}{n \Sigma x^2 - (\Sigma x)^2}$$

and the constant

$$b = \frac{n \Sigma xy - (\Sigma x) (\Sigma y)}{n \Sigma x^2 - (\Sigma x)^2}$$

where  $b$  is referred to as the regression coefficient and represents the rate of change in  $y$  for a unit change in  $x$ . The coefficients  $b$  for each line, together with their respective variances were calculated. The  $t$  distribution was used to determine whether there was any significant difference between the two coefficients.

## 6. WILCOXON'S NON-PARAMETRIC RANK TEST

In the situation where the F test was applied and the variances were significantly different, the Wilcoxon test was used.

Computation: Ranking by magnitude.

Given are two samples 1 and 2 with  $X_1$  values and  $X_2$  values.

They are ranked in ascending order

e.g.  $X_1 = 1,06, 1,53, 1,68, 1,69$

$X_2 = 1,30, 1,55, 1,88, 1,90$

when ranked

$X_1$	=	1,06	1,53,	1,68,	1,69				
$X_2$	=	1.30	1.55			1.88	1.90		
		1	2	3	4	5	6	7	8

Rank numbers  $O_1$  of the  $X_1$  values are 1, 3, 5, 6

and their sum  $T_1 = \sum O_1 = 15$

The significant limits for  $T_1$  are given in the appropriate table, where  $T_1$  refers to the smaller of the two samples. If  $T_1$  lies outside of the values of the limits given in the table then a significant difference exists between the two sets of data, at that level of significance.

## 7) SIGNIFICANCE LEVELS

In all the experiments, differences at a level of  $p < 0.05$  have been taken as being significant.

8) ERROR IN ESTIMATIONS

All blood sample estimations were performed in duplicate (Appendix one). The error in estimation described was the variation of the duplicate samples from their mean. This error was calculated for each pair of duplicate samples and the maximum variation was called the error in estimation for that particular study.

REFERENCES

1. SCRIMSHAW, N.S. and BEHAR, M. (1961)  
Protein Malnutrition in Children.  
Science, 133, 2039 - 2047.
2. BROCK, J.F. (1966)  
Dietary Protein Deficiency; Its influence on body  
structure and function.  
Annals of Internal Medicine, 65, 877 - 899
3. HANSEN, J.D.L. (1968)  
Protein-Calorie Malnutrition.  
Medicine Today, 2, 25 - 35.
4. PARKS, J.R. (1970)  
Growth curves and the physiology of growth.  
111. Effects of dietary protein.  
American Journal of Physiology, 219, 840 - 843.
5. DOWNS, E.F. (1964)  
Nutritional Dwarfing; A syndrome of early protein-  
calorie malnutrition.  
The American Journal of Clinical Nutrition, 15, 275 - 281.
6. DUGDALE, A.E., CHEN, S.T. and HEWITT, G. (1970)  
Patterns of Growth and Nutrition in Childhood.  
The American Journal of Clinical Nutrition, 23, 1280 - 1287.
7. LAITINEN, O. (1967)  
The metabolism of collagen and its hormonal control in  
the rat.  
Acta Endocrinologica. Supplementum 120, Chapter V, pp.44 - 51.
8. HARRIS, H.A. (1927)  
Cessation of growth in the long bones in health and disease.  
Proceedings of the Staff meetings of the Mayo Clinic,  
2, 228 - 229.
9. ELIOT, M.M., SOUTHER, S.P. and PARK, E.A. (1927)  
Transverse lines in the X-ray plates of the long bones  
in children.  
Bulletin of the Johns Hopkins Hospital, 41, 364 - 338.
10. HIGGINSON, J. (1954)  
Studies on Human bone from South African Bantu Subjects.  
II. Histopathological changes in the ribs of South African  
Bantu Infants.  
Metabolism, 3, 392 - 399.

11. JONES, P.R.M. and DEAN, R.F.A. (1956).  
The Effects of Kwashiorkor on the Development of the  
Bones of the Hand.  
The Journal of Tropical Pediatrics, 2, 51 - 68.
12. GARN, S.M., ROHMANN, C.G., BEHAR, M., VITERI, F. and  
GUZMAN, M.A. (1964)  
Compact Bone Deficiency in Protein-Calorie Malnutrition.  
Science, 145, 1444- 1445.
13. WHAYBURNE, S. (1968)  
Malnutrition in Johannesburg.  
In: Calorie Deficiencies and Protein Deficiencies.  
(Editors R.A. McCance and E.M. Widdowson), pp 7 - 22,  
Churchill, London.
14. REICHMAN, P. and STEIN, H. (1968)  
Radiological features noted on plain radiographs in  
malnutrition in African children.  
British Journal of Radiology, 41, 296 - 299.
15. ADAMS, P. and BERRIDGE, F.R. (1969)  
Effects of Kwashiorkor on Cortical and Trabecular Bone.  
Archives of Disease in Childhood, 44, 705 - 709.
16. DREIZEN, S., CURRIE, C., GILLEY, E.J. and SPIES, T.D. (1956)  
Observations on the association between nutritive failure,  
skeletal maturation rate and radio-opaque transverse lines  
in the distal end of the radius in children.  
The American Journal of Roentgenology, 76, 482 - 487.
17. STEWART, R.J.C. (1965)  
Bone Pathology in experimental malnutrition.  
In: World Review of Nutrition and Dietetics,  
5, 275 - 337. (Ed. Bourne, G.H. and Wilson, E.M.H.)  
S. Karger. Basel and New York.
18. PLATT, B.S. and STEWART, R.J.C. (1962)  
Transverse Trabeculae and Osteoporosis in bones in  
experimental protein-calorie deficiency.  
British Journal of Nutrition, 16, 483 - 495.
19. EL-MARAGHI, N.R.H., PLATT, B.S. and STEWART, R.J.C. (1965)  
The effect of the interaction of dietary protein and  
calcium on the growth and maintenance of the bones of young,  
adult and aged rats.  
British Journal of Nutrition, 19, 491 - 509.
20. JHA, G.J., DEO, M.G. and RAMALINGASWAMI, V. (1968)  
Bone Growth in Protein Deficiency.  
American Journal of Pathology, 53, 1111 - 1123.

21. ESTREMER, H.R., and ARMSTRONG, W.D. (1948)  
Effect of protein intake on the bones of mature rats.  
The Journal of Nutrition, 35, 611 - 618.
22. CABAK, V., DICKERSON, J.W.T. and WIDDOWSON, E.M. (1963)  
Response of young rats to deprivation of protein or  
of calories.  
British Journal of Nutrition, 17, 601 - 616
23. SHENOLIKAR, I.S. and NARASINGA RAO, B.S. (1968)  
Influence of dietary protein on calcium metabolism in  
young rats.  
The Indian Journal of Medical Research, 56, 1412 - 1422.
24. FIRSCHEIN, H.E. (1967)  
Collagen turnover in Calcified Tissues.  
Archives of Biochemistry and Biophysics, 119, 119 - 123.
25. PROCKOP, D.J. and KIVIRIKKO, K.I. (1967)  
Relationship of hydroxyproline excretion in the urine to  
collagen metabolism.  
Annals of Internal Medicine, 66, 1243 - 1266
26. PICOU, D., ALLEYNE, G.A.O. and SEAKINS, A. (1965)  
Hydroxyproline and creatinine excretion in infantile  
protein malnutrition.  
Clinical Science, 29, 517 - 523.
27. ANASUYA, A. and NARASINGA RAO, B.S. (1966)  
Urinary excretion of hydroxyproline in Kwashiorkor.  
The Indian Journal of Medical Research, 54, 849 - 857.
28. WIDDOWSON, E.M. and WHITEHEAD, R.G. (1966)  
Plasma aminoacid ratios and urinary hydroxyproline  
excretions in rats deficient in protein and calories.  
Nature, 212, 683 - 686.
29. ANASUYA, A. and NARASINGA RAO, B.S. (1970)  
Relationship between body collagen and urinary  
hydroxyproline excretion in young rats fed on a  
low-protein or low-calorie diet.  
British Journal of Nutrition 24, 97 - 107.
30. BOLLET, A.J. (1970)  
Effect of protein depletion on skin and bone collagen.  
The Mount Sinai Journal of Medicine, 37, 445 - 449.
31. VASANTHA, L. (1970)  
Biochemical changes in skin in experimental protein  
and calorie deficiency.  
The American Journal of Clinical Nutrition, 23, 99 - 104.

32. McCANCE, R.A., WIDDOWSON, E.M. and LEHMANN, H. (1942)  
The effect of protein intake on the absorption of calcium and magnesium.  
The Biochemical Journal, 36, 686 - 691.
33. VAUGHAN, J.M. (1970)  
The movement of ions in and out of the skeleton.  
In: The Physiology of Bone. Chapter 8, pp 113 - 149.  
Clarendon Press. Oxford.
34. URIST, M.R. (1969)  
Rarefying diseases of bone.  
In: The Biological Basis of Medicine. Volume 3.  
Chapter 13, pp 425 - 452.  
(Editors E. Bittar and N. Bittar), Academic Press,  
London and New York.
35. BAUER, G.C.H., CARLSSON, A. and LINDQUIST, B. (1955)  
Some properties of the exchangeable bone calcium.  
Acta Physiologica Scandinavia, 35, 67 - 72.
36. REEVE, J. and VEALL, N. (1972)  
Radioisotope studies of calcium metabolism and their interpretation:  
Calcium metabolism and bone disease.  
In: Clinics in Endocrinology and Metabolism. pp 185 - 196.  
(Editor I. MacIntyre,) W.B. Saunders, London, Philadelphia  
and Toronto.
37. STEWART, R.J.C. (1970)  
Experimental protein calorie deficiency.  
The Proceedings of the Nutrition Society, 29, 206 - 213.
38. EDOZIEN, J.C. (1968)  
Experimental Kwashiorkor and Marasmus.  
Nature, 220, 917 - 919.
39. SIDRANSKY, H. and VERNEY, E. (1970)  
Skeletal muscle protein metabolism changes in rats force-fed  
a diet inducing an experimental Kwashiorkor-like model.  
The American Journal of Clinical Nutrition, 23, 1154 - 1159.
40. KIRSCH, R.E., BROCK, J.R. and SAUNDERS, S.J. (1968)  
Experimental protein-calorie malnutrition.  
The American Journal of Clinical Nutrition, 21, 820 - 826.
41. ENWONWU, C.O. and SREEBNEY, L.M. (1970)  
Experimental protein calorie malnutrition in rats:  
Biochemical and ultra-structural studies.  
Experimental and Molecular Pathology, 12, 332 - 353.

42. STEAD, R.H. and BROCK, J.F. (1972)  
Experimental protein calorie malnutrition: Rapid  
induction of protein depletion signs in early-weaned rats.  
The Journal of Nutrition, 102, 1357 - 1366.
43. FRANSEN, A.M., NELSON, M.M., SULON, E., BECKS, H. and  
EVANS, H.M. (1954)  
The effects of various levels of dietary protein on  
skeletal growth and endochondral ossification in rats.  
The Anatomical Record, 119, 247 - 266.
44. PADWALDESAI, S.R., NINJOOR, V., RAMAKRISHNAN, T.V.,  
SAWANT, P.L., KUMTA, U.S. and SREENIVASAN, A. (1969)  
Protein deficiency in spaced-fed rats.  
British Journal of Nutrition, 23, 745 - 753.
45. HARPER, A.E. (1959)  
Amino acid balance and imbalance. Dietary level of  
protein and amino acid imbalance.  
The Journal of Nutrition, 68, 405 - 418.
46. FINLAYSON, J.S. and BAUMANN, C.A. (1956)  
Responses of rats to urea and related substances:  
The use of a spaced-feeding technique.  
The Journal of Nutrition, 59, 211 - 221
47. HARPER, A.E. and SPIVEY, H.E. (1958)  
Relationship between food intake and osmotic effect of  
dietary carbohydrate.  
American Journal of Physiology, 193, 483 - 487.
48. LEUNG, P.M.B., ROGERS, Q.R. and HARPER, A.E. (1968)  
Effect of amino acid imbalance in rats fed ad libitum,  
interval fed or force-fed.  
The Journal of Nutrition, 95, 474 - 482.
49. HARPER, A.E. (1959)  
Sequence in which the amino acids of casein become  
limiting for the growth of the rat.  
The Journal of Nutrition, 67, 109 - 122.
50. FERNANDEZ, A., SOBEL, C. and GOLDENBERG, H. (1966).  
An improved method for determination of serum albumin  
and globulin.  
Clinical Chemistry, 12, 194 - 205.
51. BETHKE, R.M., KICK, C.H. and WILDER, W. (1932)  
The effect of the calcium phosphorus relationship on  
growth, calcification and blood composition of the rat.  
The Journal of Biological Chemistry, 98, 389 - 403.
52. McCOY, R.H. (1942)  
Dietary requirements of the rat.  
In: The rat in laboratory investigation.  
(Editors: J.Q. Griffith and E.J. Farris), Chapter 5, pp 67 - 101.  
J.B. Lippincott Co., Philadelphia, London, Montreal.

53. HOLLIFIELD, G. and PARSONS, W. (1962)  
Metabolic Adaptations to a "Stuff and Starve" feeding programme.  
I. Studies of adipose tissue and liver glycogen in rats limited to a short daily feeding period.  
Journal of Clinical Investigation, 41, 245 - 249.
54. DEO, M.G., SOOD, S.K. and RAMALINGASWAMI, V. (1965)  
Experimental protein deficiency: Pathological features in the rhesus monkey.  
Archives Pathology, 80, 14 - 23.
55. VAN DYNE, F.O., LANFORD, C.S., TOEPFER, E.W. and SHERMAN, H.C. (1941)  
Life time experiments upon the problem of optimal calcium intake.  
The Journal of Nutrition, 21, 221 - 224.
56. GREENFIELD, H. and BRIGGS, G.M. (1971).  
Nutritional Methodology in metabolic research with rats.  
In: Annual Review of Biochemistry, 40, 549 - 572.
57. WACKER, W.E.C. and PARISI, A.F. (1968)  
Magnesium Metabolism.  
The New England Journal of Medicine, 278, 658 - 776.
58. JENNER, H.D. and KAY, H.D. (1931)  
The phosphatases of mammalian tissues.  
III. Magnesium and the phosphatase system.  
The Journal of Biological Chemistry, 93, 733 - 748.
59. VAUGHAN, J.M. (1970)  
Bone as a tissue  
In: The Physiology of Bone, Chapter 1, pp 1 - 22.  
Clarendon Press. Oxford.
60. VAUGHAN, J.M. (1970)  
Enzymes of Bone and Cartilage  
In: The Physiology of Bone, Chapter 13, pp 246 - 253.  
Clarendon Press. Oxford.
61. JACKSON, W.P.U. (1967)  
Physiology of Calcium and Phosphorus  
In: Calcium Metabolism and Bone disease, Chapter 1, pp 1 - 9,  
Arnold Publishers. London.
62. RASMUSSEN, H. (1968)  
The Parathyroids.  
In: Textbook of Endocrinology, Chapter II, pp 847 - 966.  
(Ed. R.H. Williams)  
W.B. Saunders, Co. Philadelphia, London, Toronto.
63. FOURMAN, P. (1960)  
Supply and transport of calcium and phosphorus.  
In: Calcium Metabolism and the Bone, Chapter 2, pp 33 - 47  
Blackwell Scientific Publications, Oxford.

64. FRENK, S. (1971).  
Calcium and Phosphorus in third degree malnutrition:  
XIII International Congress of Pediatrics.  
Vol III Nutrition and Gastroenterology, pp 239 - 242.
65. CADDELL, J.L. and GODDARD, D.R. (1967)  
Studies in protein calorie malnutrition  
I. Chemical Evidence for Magnesium Deficiency.  
The New England Journal of Medicine, 276, 533 - 535
66. ROSEN, E.U., CAMPBELL, P.G. and MOOSA, G.M. (1970)  
Hypomagnesaemia and magnesium therapy in protein  
calorie malnutrition.  
The Journal of Paediatrics, 77, 709 - 714.
67. MacMANUS, J. and HEATON, F.W. (1969)  
The effect of magnesium deficiency on calcium homeostasis  
in the rat.  
Clinical Science, 36, 297 - 306.
68. MacMANUS, J., HEATON, F.W. and LUCAS, P.W. (1971)  
A decreased response to parathyroid hormone in magnesium  
deficiency.  
The Journal of Endocrinology, 49, 253 - 258.
69. CHASE, L.R. and AURBACH, G.D. (1970)  
The effect of parathyroid hormone on the concentration  
of adenosine 3', 5' - monophosphate in skeletal tissue  
in vitro.  
Journal of Biological Chemistry, 245, 1520 - 1526
70. AURBACH, G.D., KEUTMANN, H.T., NIALL, H.D., TREGGAR, G.W.,  
O'RIORDAN, J.L.H., MARCUS, R., MARX, S.J. and POTTS, J.T. (1972)  
Structure, Synthesis and Mechanism of action of parathyroid  
hormone.  
In: Recent Progress in Hormone Research, 28, 353 - 398.  
Academic Press. New York and London.
71. RALL, T.W. and SUTHERLAND, E.W. (1962)  
Adenyl cyclase II: The enzymatically catalysed formation  
of adenosine 3', 5' - phosphate and inorganic pyrophosphate  
from adenosine triphosphate.  
Journal of Biological Chemistry. 237, 1228 - 1232.
72. ZIMMET, P., BREIDAHL, H.D. and NAYLER, W.G. (1968).  
Plasma ionised calcium in hypomagnesaemia.  
British Medical Journal, 1, 622 - 623.
73. MacINTYRE, I., BOSS, S. and TROUGHTON, V.A. (1963).  
Parathyroid hormone and magnesium homeostasis.  
Nature, 198, 1058 - 1060.

74. MUKHERJEE, K.L. and SARKAR, N.K. (1958).  
Liver Enzymes in human under-nutrition.  
British Journal of Nutrition, 12, 1 - 7.
75. SWARTZ, R. (1956).  
Alkaline phosphatase activity of the serum in Kwashiorkor.  
Journal of Clinical Pathology, 9, 333 - 340.
76. KAY, H.D. (1930)  
Plasma Phosphatase.  
I, Method of determination: Some properties of the Enzyme.  
The Journal of Biological Chemistry, 89, 235 - 247.
77. PIMSTONE, B., EISENBERG, E. and STALLONE, W. (1966)  
Decrease in serum alkaline phosphatase activity produced by  
magnesium depletion in rats.  
Proceeding of the Society for Experimental Biology and  
Medicine, 123, 201 - 203.
78. AGNEW, J.E., KEHAYOGLOU, A.K. and HOLDSWORTH, C.D. (1969).  
Comparison of three isotopic methods for the study of  
calcium absorptions.  
Gut, 10, 590 - 597.
79. MAUTALEN, C.A., CABREJAS, M.L. and SOTO, R.J. (1969).  
Isotopic determination of intestinal calcium absorption  
in normal subjects.  
Metabolism, 18, 395 - 405.
80. COHN, S.H., TERE, J.M. and GUSMANO, E.A. (1968)  
Effect of varying calcium intake on the parameters  
of calcium metabolism in the rat.  
Journal of Nutrition, 94, 261 - 267.
81. BHANDARKAR, S.D., BLUHM, M.M., MacGREGOR, J. and NORDIN, B.E.C.  
(1961)  
An isotope test of calcium absorption.  
British Medical Journal, 2 1539 - 1541.
82. LUTWAK, L. (1969)  
Tracer studies of intestinal calcium absorption in man.  
The American Journal of Clinical Nutrition, 22, 771 - 785.
83. DE GRAZIA, J.A., IVANOVICH, P., FELLOWS, H. and RICH, C. (1965)  
A double isotope method for measurement of intestinal  
absorption of calcium in man.  
Journal of Laboratory and Clinical Medicine, 66, 822 - 829
84. ROSE, G.A. (1964)  
Experiences with the use of interrupted carmine red and  
continuous chromium sesquioxide marking of human faeces  
with reference to calcium phosphorus and magnesium.  
Gut, 5, 274 - 279.

85. CAMERON, J.S., BUTTERFIELD, W.J.H., VEAL, N., REES, R.J. and PARSON, V. (1962)  
Studies on intestinal calcium absorption in man using  $^{47}\text{Ca}$ .  
In: Medical uses of  $^{47}\text{Ca}$ , pp 29 - 34.  
International Atomic Energy Agency (Vienna).
86. TADAYYON, B. and LUTWAK, L. (1969)  
Effects of dietary triolein, tripalmitin and L-phenylalanine on calcium absorption in the rat.  
Proceedings of the Society for Experimental Biology and Medicine, 130, 978 - 979.
87. HARRISON, H.E., and HARRISON, H.C. (1951)  
Studies with radiocalcium: The intestinal absorption of calcium.  
The Journal of Biological Chemistry, 188, 83 - 90.
88. HANSARD, S.L. and CROWDER, H.M. (1957)  
The physiological behaviour of calcium in the rat.  
The Journal of Nutrition, 62, 325 - 339.
89. KAO, H.C., CONNER, R.T. and SHERMAN, H.C. (1941)  
Influence of protein intake upon growth, reproduction and longevity studied at different calcium levels.  
The Journal of Nutrition, 22, 327 - 331.
90. ADOLPH, Wm.H. and CHEN, S.C. (1932)  
The utilisation of calcium in soya bean diets.  
The Journal of Nutrition, 5, 379 - 385.
91. CONNER, R.T. and Sherman, H.C. (1936)  
Some aspects of protein intake in relation to growth and rate of calcification.  
The Journal of Biological Chemistry, 115, 695 - 706.
92. WASSERMAN, R.H. and TAYLOR, A.N. (1966)  
Vitamin  $\text{D}_3$  - induced calcium binding protein in chick intestinal mucosa.  
Science, 152, 791 - 793.
93. NORMAN, A.W., MYRTLE, J.F., MIDGETT, R.J., NOWICK, H.G. and POPJAK, V.W.G. (1971)  
1.25 - dihydroxycholecalciferol identification of the proposed form of vitamin  $\text{D}_3$  in the intestine.  
Science, 173, 51 - 54.
94. BANDARU, S.R. (1972)  
Effect of protein malnutrition on calcium transport system in rats.  
Federation Proceedings, Volume 31, Abstract 2665.

95. McLEAN, F.C. and URIST, M.R. (1968)  
Mineral Metabolism  
In: Bone, Chapter II, pp 147 - 172.  
The University of Chicago Press. Chicago and London.
96. BAUER, G.C.H., CARLSSON, A. and LINDQUIST, B. (1955)  
Evaluation of accretion, resorption and exchange reactions  
in the skeleton.  
Kungliga Fysiografiska Sällskapet I.  
Lund Förhandlingar 25, 1 - 16.
97. AUBERT, J.P. and MILHAUD, G. (1960)  
Méthode des mesure des principales voies du métabolisme  
calcifique chez l'homme.  
Biochimica et Biophysica Acta, 39, 122 - 139.
98. HEANEY, R.P. and WHEDON, G.D. (1958)  
Radiocalcium studies of bone formation rate in human  
metabolic bone disease.  
The Journal of Clinical Endocrinology and Metabolism,  
18, 1246 - 1267.
99. HEANEY, R.P. (1963)  
Evaluation and interpretation of calcium kinetic data in man.  
Clinical Orthopaedics and Related Research, 31, 153 - 183.
100. COHN, S.H., BOSSO, S.R., JESSEPH, J.E., CONSTANTINIDES, C.,  
HUENE, D.R. and GUZANO, E.A. (1965)  
Formulation and testing of a compartmental model for  
calcium metabolism in man.  
Radiation Research, 26, 319 - 333.
101. BERMAN, M., SHAHN, E. and WEISS, M.F. (1962)  
A routine fitting of kinetic data to models: A mathematical  
formalism for digital computers.  
Biophysical Journal, 2, 275 - 287.
102. GONICK, H.C. and BROWN, M. (1970)  
Critique of multi-compartmental analysis of calcium kinetics  
in man based on 27 cases.  
Metabolism, 19, 919 - 933.
103. KALLFELZ, F.A. and WENTWORTH, R.A. (1969)  
Evaluation of bone calcium accretion rate as a function of  
age in Beagle dogs.  
The Journal of Nutrition, 99, 459 - 464.
104. HUMPHREYS, E.R. (1965)  
An improved method for determining calcium - 45 in biological  
materials.  
International Journal of Applied Radiation and Isotopes.  
16, 345 - 348.

105. WALLACE, W.M. (1959)  
Nitrogen content of the body and its relation to retention and loss of nitrogen.  
Federation Proceedings, 18, 1125 - 1130.
106. ROBERTSON, J.S., TOSTESON, D.C. and GAMBLE, J.L. (1957).  
The determination of exchange rates in three compartment steady state closed systems through the use of tracers.  
Journal of Laboratory and Clinical Medicine, 49, 497 - 503.
107. AUBERT, J.P., MILHAUD, G., MANOUSSOS, G. and DORMAND, Y. (1958)  
Mesure de l'exchange chimique et du renouvellement physiologique du calcium osseux chez l'homme et de Rat.  
Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences, 246, 2817 - 2820.
108. CARLSSON, A. (1951)  
Metabolism of radiocalcium in relation to calcium intake in young rats.  
Berlingska Boktryckeriet. Lund.
109. BRAITHWAITE, G.D. and RIAZUDDIN, SH. (1971)  
The effect of age and level of dietary calcium intake on calcium metabolism in sheep.  
British Journal of Nutrition, 26, 215 - 225.
110. BRONNER, F. and LEMAIRE, R. (1969)  
Comparison of calcium kinetics in man and the rat.  
Calcified Tissue Research, 3, 238 - 248.
111. HARRISON, M. and FRASER, R. (1960)  
Bone metabolism in rats studied with stable strontium.  
The Journal of Endocrinology, 21, 191 - 196.
112. WASSERMAN, R.H., CORRADINO, R.A. and TAYLOR, A.N. (1968)  
Vitamin D. dependant calcium binding protein: Purification and some properties.  
The Journal of Biological Chemistry, 243, 3978 - 3986.
113. SHTACHER, G. and FIRSCHEIN, H.E. (1967)  
Collagen and mineral kinetics in bone after fracture.  
American Journal of Physiology, 213, 863 - 866.
114. FIRSCHEIN, H.E. and ALCOCK, N.W. (1969)  
Rate of removal of collagen and mineral from bone and cartilage.  
Metabolism, 18, 115 - 119.
115. VAUGHAN, J.M. (1970)  
Chemistry of bone matrix,  
In: The Physiology of Bone, Chapter 3, pp 61 - 76.  
Oxford University Press. London.

116. BACHRA, B.N. (1967)  
Some molecular aspects of tissue calcification.  
Clinical Orthopaedics and related research, 51, 199 - 222.
117. JETHI, R.K., INLOW, C.W. and WADKINS, C.L. (1970)  
Studies of the mechanism of Biological Calcification.  
Calcified Tissue Research, 6, 81 - 92.
118. BACHRA, B.N. (1972)  
Calcification in vitro of demineralised bone matrix:  
Electron microscopic and chemical aspects.  
Calcified Tissue Research, 8, 287 - 303
119. CAMERON, D.A. (1963)  
The fine structure of Bone and Calcified Cartilage.  
Clinical Orthopaedics and Related Research, 26, 199 - 228.
120. UITTO, J. and LAITINEN, O. (1968)  
Relationship of collagen metabolism to calcium metabolism in  
the bone.  
Acta Chemica Scandinavia, 22, 1039 - 1040.
121. LINDSTEDT, S. and PROCKOP, D.J. (1961)  
Isotopic studies on urinary hydroxyproline as evidence  
for rapidly catabolised forms of collagen in the young rat.  
The Journal of Biological Chemistry, 236, 1399 - 1403.
122. STETTEN, M.R. (1949)  
Some aspects of the metabolism of hydroxyproline studied  
with the aid of isotopic nitrogen.  
The Journal of Biological Chemistry, 181, 31 - 37.
123. FIRSCHEIN, H.E. (1969)  
Collagen and mineral dynamics in bone.  
Clinical Orthopaedics and Related Research, 66, 212 - 225.
124. HAHN, T.J. and AVIOLI, L.V. (1970)  
Effect of chronic uraemia on Collagen Metabolism in  
skin and bone.  
Archives of Internal Medicine, 126, 882 - 886.
125. RAMALINGASWAMI, V. (1969)  
Interface of Protein Nutrition and Medicine in the Tropics.  
The Lancet, 2, 733 - 736.
126. UPENDRA PRASAD, C. and BOSE, S.M. (1972)  
Effect of dietary protein level on the formation of  
cross-links in collagen.  
The Indian Journal of Nutrition and Dietetics, 9, 153 - 156.

127. BAUER, G.C.H., CARLSSON, A. and LINDQUIST, B. (1961)  
Metabolism and Homeostatic Function of Bone  
In: Mineral Metabolism: an advanced treatise.  
(Editors C.L. Comar and F. Bronner), Chapter 14, pp 609 - 677,  
Volume 1 B.  
Academic Press. New York and London.
128. HURXTHAL, L.M. and VOSE, G.P. (1969)  
The relationship of dietary calcium intake to radiographic  
bone density in normal and osteoporotic persons.  
Calcified Tissue Research 4, 245 - 256.
129. LACHMAN, E. (1955)  
Osteoporosis: the potentialities and limitations of its  
roentgenologic diagnosis.  
The American Journal of Roentgenology, 74, 712 - 715.
130. ROSE, G.A. (1967)  
A Critique of modern methods of diagnosis and treatment  
of osteoporosis.  
Clinical Orthopaedics and Related Research, 55, 17 - 41.
131. SAVILLE, P.D. (1967)  
A quantitative approach to single radiographic diagnosis  
of osteoporosis. Its application to osteoporosis  
of rheumatoid arthritis.  
Arthritis and Rheumatism, 10, 416 - 422.
132. MEEMA, H.E., HARRIS, C.K. and PORRET, B.A. (1964)  
A method for determination of Bone - Salt content  
of Cortical Bone.  
Radiology, 82, 986 - 997.
133. ALBANESE, A.A., EDELSON, A.H., LORENZE, E.J. and WEIN, E.H.  
(1969)  
Qualitative radiographic survey technique for detection  
of bone loss.  
American Geriatrics Society Journal, 17, 142 - 154.
134. VIRTAMA, P. and HELETA, T. (1969)  
Radiographic measurements of cortical bone.  
Acta Radiologica Supplementum, 293.
135. MACK, P.B., BROWN, W.N. and TRAPP, H.D. (1949)  
The quantitative evaluation of bone density.  
The American Journal of Roentgenology, 61, 808 - 825.
136. WALLACH, S., CHAUSMER, A., MITTLEMAN, R. and DIMICH, A. (1967)  
In vivo inhibition of bone resorption by thyrocalcitonin.  
Endocrinology, 80, 61 - 64.

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137. WEST, R.R. and REED, G.W. (1970)  
The measurement of bone mineral in vivo by photon beam scanning.  
The British Journal of Radiology, 43, 886 - 893.
138. SISSONS, H.A. (1956)  
The growth of bone  
In: The Biochemistry and Physiology of Bone.  
(Editor G.H. Bourne), Chapter 15, pp 443 - 474  
Academic Press, New York.
139. SMITH, H.A. (1931)  
The phenomena of retarded growth.  
The Journal of Nutrition, 4, 427 - 442.
140. SHAPIRO, I.M. and GREENSPAN, J.S. (1969)  
Are Mitochondria directly involved in Biological Mineralisation?  
Calcified Tissue Research, 3, 100 - 102.
141. BARON, D.N. and BELL, J.L. (1959)  
Compleximetric determination of calcium in pathological and  
Physiological specimens.  
Journal of Clinical Pathology, 12, 143 - 148.
142. NEILL, D.W. and NEELY, R.A. (1956)  
The estimation of magnesium in serum using Titan Yellow.  
Journal of Clinical Pathology, 9, 162 - 163.
143. DRYER, R.L., TAMMES, A.R. and ROUTH, J.I. (1957)  
The determination of phosphorus and phosphates with  
N-phenyl-p-phenylenediamine.  
Journal of Biological Chemistry, 225, 177 - 183.
144. SHINOWARA, G.Y., JONES, L.M. and REINHART, H.L. (1942)  
The estimation of serum inorganic phosphate and acid, and  
alkaline phosphatase activity.  
Journal of Biological Chemistry, 142, 921 - 933.
145. BRAY, G.A. (1960)  
A simple efficient liquid scintillator for counting  
aqueous solutions in a liquid scintillation counter.  
Analytical Biochemistry 1, 279 - 285.
146. FIRSCHEIN, H.E. and SHILL, J.P. (1966)  
The determination of total hydroxyproline in urine and  
bone extracts.  
Analytical Biochemistry, 14, 296 - 304.
147. Statistical Methods in: Scientific Tables, pp. 145 - 198.  
(Editors K. Diem and C. Lentner), Seventh Edition (1970).  
Ciba-Geigy, Basle, Switzerland.
148. SPEIGEL, M.R. (1961)  
Theory and problems of statistics.  
Schaum Publishing Co. New York.