The influence of
dietary calcium and phosphorus
on bone metabolism

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
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des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

Drukkerij Giethoorn - Meppel

Dit proefschrift verschijnt ook als Verslag V219 van het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede

Stellingen

1. Een suboptimale vitamine-D-voorziening kan bijdragen tot het ontstaan van osteoporose.
   Dit proefschrift.

2. De Ca/P-verhouding, als maat voor de kwaliteit van de voeding, heeft slechts betekenis indien de absolute hoeveelheid van een der betrokken elementen bekend is.
   Dit proefschrift.

3. De gunstige werking die het innemen van kalktabletten heeft tegen het optreden van botontkalking bij vrouwen na de menopauze, kan worden verklaard door de remmende invloed van calcium op de absorptie van fosfaat in de darm.
   Recker, R. R. et al., Effect of estrogens and calcium carbonate on bone loss in postmenopausal women. Ann. Int. Med. 87 (1977) 649-655.

4. Het calciumgehalte van het bloed is niet of weinig geschikt als graadmeter voor de vitamine-D-status en de produktie van bijschildklierhormoon.

5. Het is zeer aannemelijk dat de aanwezigheid van lactose in melk bijdraagt tot de anti-rachitische werking van dit voedingsmiddel.
   Pansu, D. et al., Effect of lactose on duodenal calcium-binding protein and calcium absorption. J. Nutr. 109 (1979) 508-512.

6. De uitdrukking „homeostasis of inorganic phosphate" komt voort uit een onjuiste woordkeuze.
   Fleisch, H. et al., Homeostasis of inorganic phosphate: an introductory review. Calcif. Tiss. Res. 21 (1976) Suppl. 327-331.

7. Elke commissie van de Voedingsraad die een opdracht heeft op het terrein van de samenstelling van de voeding, dient, alvorens adviezen aan de Raad uit te brengen, in overleg te treden met de Commissie Voedingsnormen.

8. Het reageren op de vaak verwarrende en deels onjuiste berichten die nieuwsmedia over voeding verspreiden, kan worden gecoördineerd door het Voorlichtingsbureau voor de Voeding te Den Haag.

9. De uitspraak van de voedingskundige E. H. Groot: „Voeding is geen wiskunde", heeft nog onvoldoende ingang gevonden bij de gebruikers van de formules van Keys.

10. Het zou de eerlijkheid en de oprechtheid van de zuivelindustrie benadrukken indien ze voor het produkt dat wordt verkregen door het microbiologisch aanzuren van zoete ondermelk een andere naam bedacht dan „karnemelk".

11. Een lichtpunt in de achteruitgang van de economie in de ontwikkelde landen is dat men mag verwachten dat de voedingsgewoonten erdoor zullen verbeteren.

12. De instelling van medezeggenschapsraden in het onderwijs, naar analogie van de ondernemingsraad in het bedrijfsleven, is overbodig waar oudercommissies en schoolraden goed functioneren.

13. In tegenstelling tot wat de epitheta suggereren, zijn gewone, buitengewone en bijzondere hoogleraren meestal even gewoon.

14. Indien het praktisch uitvoerbaar zou zijn, kon een verhoging van de ziektekostenpremies voor rokers van sigaretten worden bekostigd uit een verlaging van de pensioenpremies voor deze mensen.

15. Als de bal niet rond was, leek voetbal nóg meer op rugby.

16. Bermtoerisme wordt bevorderd door het aanleggen van grote verkeerswegen.

17. Het moet dubieus worden geacht of het euvel van spookrijders op zowel autowegen als rijwielpaden kan worden verholpen door de algemene invoering van linkshoudend verkeer, of door deze categorie weggebruikers te verplichten bij duisternis een rood voorlicht te voeren.

Proefschrift G. Schaafsma
The influence of dietary calcium and phosphorus on bone metabolism
Wageningen, 12 juni 1981

Aan Marijke,
Daan, Else, Tamara,
mijn ouders en schoonouders

Voorwoord

Hoewel het voorwoord doorgaans weinig bijdraagt tot de wetenschappelijke waarde van de inhoud van een proefschrift, is het er een wezenlijk onderdeel van. Het biedt immers de schrijver de gelegenheid om degenen die hem met raad en daad ter zijde hebben gestaan te bedanken, op een voor de lezer in het oog lopende wijze.

Als eersten wil ik in dit verband noemen mijn promotoren, Prof. Dr. J. G. A. J. Hautvast en Prof. Dr. S. A. Duursma. Reeds tijdens mijn studie aan de Landbouwhogeschool heb ik mogen ervaren hoe Prof. Hautvast bij zijn studenten belangstelling weet te wekken voor het vak voedingsleer, met name voor de fysio-pathologische aspecten ervan. Ik ben hem veel dank verschuldigd voor zijn terechte en kritische opmerkingen over het manuscript. De discussies met Prof. Duursma zijn voor mij eveneens van bijzondere betekenis en van grote waarde geweest, mede door zijn speciale kennis van de botstofwisseling en zijn opmerkelijke belangstelling voor de voeding.

Het bestuur en de directie van het NIZO ben ik erkentelijk voor het feit dat zij mij in de gelegenheid hebben gesteld om deze promotie te realiseren.

Niet alleen doordat Huug de Waard een belangrijk aandeel heeft gehad in het onderzoek bij de bejaarden, ook door zijn enthousiasme voor en kennis van voedingsleer, alsmede door zijn collegialiteit, is hij voor mij een belangrijke steun geweest. Ik dank hem daarvoor graag.

Walter Visser van de Afdeling Botstofwisselingsziekten van het Academisch Ziekenhuis te Utrecht ben ik dank verschuldigd voor het vele werk dat hij heeft verzet bij het verrichten van de sectie op de proefdieren en bij het histologisch onderzoek van de botjes. Ook dank ik hem voor de kennis die hij mij op deze gebieden heeft bijgebracht.

Grote waardering heb ik voor de wijze waarop Gos Stel het manuscript op taalkundige wijze heeft gestileerd en het heeft gereedgemaakt voor de drukkerij. Zijn spitsvondige woordspelingen en opmerkelijk gevoel voor humor maakten dat de bezoeken die ik aan hem bracht een aangename onderbreking vormden van het schrijven van dit boekje.

Niet onvermeld mag blijven wat mijn collega's van de afdeling Voedingsfysiologie, Renske van Beresteyn, Rob Dekker, Rinus van Schaik en Romana Visser, hebben bijgedragen aan de totstandkoming van deze dissertatie. In dit verband moeten ook worden genoemd Elly Buurma - Rethans en Ina Haze - de Ruiter, die thans elders werkzaam zijn. Hen allen bedank ik hierbij zeer.

Het waren Jo Haas en Gerrit van Tintelen van het Centrum Kleine Proefdieren van de Landbouwhogeschool die op buitengewoon kundige en plezierige wijze de dierexperimenten hebben begeleid. Voor deze medewerking ben ik hen zeer erkentelijk.

De dames van het secretariaat van het NIZO zeg ik hierbij dank voor alle

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Voorts wil ik Henk van Brakel, Jacques Maessen en Joop Mondria bedanken voor het vervaardigen van de figuren in dit proefschrift. Ook gaat mijn dank uit naar alle anderen die mij op een of andere wijze bij de totstandkoming van deze dissertatie behulpzaam zijn geweest.

Ten slotte, maar zeker niet het minst nadrukkelijk, dank ik mijn vrouw en kinderen voor de morele steun die ik van hen heb mogen ontvangen tijdens het schrijven van dit proefschrift. Ik ben hen er ook erkentelijk voor dat zij het hebben geaccepteerd dat mijn gedachten in die periode soms ver weg waren van de huiselijke aangelegenheden van elke dag.

Contents

Chapter 1

Introduction and objectives

Osteoporosis can be described as a disorder or group of disorders in which the density of the bone is reduced. In recent years a distinction is made between osteopenia and osteoporosis. Osteopenia is considered to be a non-pathological condition, which results from the normal physiological loss of bone which starts in the fifth decade of life. Osteoporosis is a metabolic bone disease, so a pathological state, which involves an increased risk of bone fracture and which results from an imbalance between bone resorption and bone formation. Osteoporosis may occur either in women after the menopause (post-menopausal osteoporosis), in old men and women (senile osteoporosis), in young adults (idiopathic osteoporosis) or in adolescent children (juvenile osteoporosis). Osteoporosis is, with good reason, a matter of great concern to public health authorities. It has been estimated that almost 25% of the female population in the USA of over 60 years have osteoporotic bones. An exponential increase in the collum fracture rate with advancing age, in males as well as in females, has been documented in the Netherlands and other countries.

Several factors have been put forward in the etiology of osteoporosis. Among them are hormonal, physical and nutritional factors. Although it is well known that dietary calcium deficiency causes osteoporosis in laboratory animals, an association between dietary calcium and osteoporosis in man is not clear. Actually, there is no general agreement on the human calcium requirement. Experiments with aging rodents have indicated that diets containing an excess of phosphorus cause increased resorption of calcium from the bones and produce osteoporosis after mild but chronic stimulation of the parathyroid glands.

According to several investigators, the calcium-to-phosphorus ratio in the average diet in the USA, which is substantially lower than that recommended in animal feeding practice, might contribute to the high prevalence of osteoporosis. Hence, restriction of dietary phosphorus has been advocated as a prevention of this disease.

The possible relation between the amounts of calcium and phosphorus in the diet and human osteoporosis has given rise to the following questions.

1. Are there any relations between dietary calcium and phosphorus on the one hand and bone mass and bone metabolism on the other in normal, healthy, elderly people, who subsist on their own customary diets?
2. What influence have dietary calcium and phosphorus on bone mass and bone metabolism under controlled conditions in rats?

In the first part of this thesis the literature is reviewed with regard to the regulation of bone mineral metabolism and to the effect of diets on bone and bone mineral homeostasis. In the second part experiments are described which we conducted in order to provide an answer to the questions mentioned above.

These experiments comprised a cross-sectional study among 89 free-living elderly people in the community of Ede and three series of trials with rats. In short, the cross-sectional study consisted of a dietary survey, measurement of the bone mineral content by photon absorptiometry, and determination of biochemical parameters in serum and urine. It was investigated whether the estimated daily intake of calcium and phosphorus was related to bone mineral mass or to biochemical data. In the trials with rats the effect of varying dietary levels of calcium and phosphorus on bone, calcium and phosphorus metabolism was investigated by means of balance studies, determination of biochemical parameters in serum and urine, and chemical analysis and histological investigation of bone samples.

Chapter 2

Bone, bone metabolism and homeostasis of bone minerals

2.1 Introduction

This chapter deals with the composition, structure and metabolism of bone tissue. It is not intended, however, as an extensive review of all aspects concerned with these subjects, because such a review would be outside the scope of this thesis. Instead of this, attention is drawn only to the essentials required for a general understanding of the effects of endogenous and exogenous factors on bone metabolism.

2.2 Chemical composition of the bone

Like other supporting tissues, bone is derived from mesenchyme or, embryonically, from mesoderm. The bone tissue (amounting to 9 kg at a body weight of 65 kg) provides elasticity and strength to the body. It consists of an organic matrix and a bone mineral phase. On a fresh-weight basis, about 28% is organic matrix, 47% is mineral and 25% is water (Draper & Chalmers, 1971).

About 90% of the organic matrix is collagen and roughly 10% is ground substance (Herring, 1970). The collagen is a unique type of protein with regard to its high content of hydroxyproline (13% of the weight). The ground substance of the bone matrix is made up of amino-polysaccharides, non-collagen protein and a small quantity of lipids. The amino-polysaccharides, mainly chondroitin-4-sulphate, chondroitin-6-sulphate and hyaluronic acid, are associated with protein. Because of their acid sulphate and carboxyl groups, the amino-polysaccharides have water- and ion-binding properties. The lipid in the ground substance consists of triglycerides, phospholipids, cholesterol and cholesterol esters (Herring, 1968).

The bone mineral phase is present mainly in the form of hydroxy-apatite crystals, $Ca_{10}(PO_4)_6(OH)_2$, varying quantities of amorphous calcium phosphate, and small but variable quantities of carbonate, citrate, sodium, potassium, magnesium and fluoride (Irving, 1973).

2.3 Bone structure

Macroscopically, two main structural forms of bone tissue can be differentiated: compact bone and cancellous bone. Both forms are deposited in lamellae which are characterized by the orientation pattern of the collagen fibres.

Compact bone is present in the shaft of long bones (cortical bone). This type of bone is made up of units, called Haversian systems or osteons, which are rod-shaped and provided with a central canal surrounded by concentric lamellae. The osteons branch each with another and interweave. The central canal contains vessels and nerve fibres. The direction of the long axes of the osteons runs concurrently with the lines of stress that have to be resisted.

Cancellous or spongy bone is present in irregular and flat bones, such as the vertebrae, the pelvic bone and in the ends of long bones. Spongy bone is also

called trabecular bone, as the lamellae are arranged in the form of trabeculae. These are laid down in such a pattern that optimum resistance to stress is obtained.

2.4 Bone cells

On morphological and functional grounds four types of bone cell can be identified: the undifferentiated mesenchyme cell (osteoprogenitor cell), the osteoblast, the osteoclast and the osteocyte.

Osteoblasts orginate from the osteoprogenitor cells. They are involved in the synthesis of bone matrix, which is called the osteoid as long as it has not yet mineralized. The active cuboidal cells with basophilic cytoplasma and large nuclei cover the growing bone surfaces in a continuous layer. After the bone has formed, they have the appearance of flat spindles and are called lining cells. From this resting phase they can resume their osteogenic function.

During bone formation some osteoblasts are trapped in new bone tissue, where they are lying as spindle-shaped cells in lacunae. These cells, called osteocytes, are conneced with tissue fluids at the bone surface by fine protoplasma offsets or canaliculi, which also interconnect these cells. The osteocytes are involved in the withdrawal and deposition of bone mineral, according to the biochemical needs of the body.

Osteoclasts, originating presumably from the bone marrow, are involved in the resorption of bone mineral and in the removal of bone matrix. They are large cells with several nuclei and lie against the bone at sites of resorption. This resorption causes the so-called Howship's lacunae. Electron micrographs have shown that active osteoclasts have a ruffled border where they lie against the bone.

2.5 Bone formation

*Two types of osteogenesis are recognized: endochondral ossification and intra*membranous bone formation. The first involves calcification of previously generated cartilage. Soon after birth, endochondral ossification is restricted to epiphysial cartilage plates, where it enables the long bones to grow in length, and to the articular surface of bone, where it contributes to the size of the epiphyses. Intramembranous bone formation proceeds under the influence of osteoblasts. It occurs beneath the periosteum, the membrane that covers the bone in places where no cartilage is present. Osteoblastic bone formation is responsible for the increase in bone diameter during the growing period. The periosteum is composed of two layers, an inner one consisting of osteoblasts and an outer one made up of fibrous tissue. The outer layer provides attachment for muscles and ligaments and carries part of the blood supply of the bone.

The osteoblasts synthesize and secrete the osteoid. Procollagen molecules, which are formed within the osteoblasts are made up of three separate polypeptide chains which are spindled around a central axis. Each polypeptide chain contains about 1000 amino acids, mainly glycine, proline and hydroxyproline. After incorporation of proline molecules in the peptide chains, a proportion of these amino acids is hydroxylated to hydroxyproline (Kivirikko & Prockop, 1967). The procollagen is incorporated in the bone matrix, but part of it is lost in the blood stream, metabolized by the body or excreted in the urine as breakdown products (Laitinen, 1967; Weiss & Klein, 1969). The urinary hydroxyproline excretion is used for the study of collagen metabolism. Procollagen molecules arrange and assemble to form microfibrils from which the collagen is made up.

Mineralization takes place in spaces between the microfibrils along the collagen axis in an ordered fashion. It is not known why accumulation of minerals is specific for the collagen of bone.

2.6 Bone mineralization

The mechanism of bone mineralization has been the subject of many speculative theories (Irving, 1973) and is as yet not elucidated. An interesting theory is that of the process called epitaxy (Neuman & Neuman, 1958). This theory assumes the existence of a specific ionic configuration or nucleation centre similar to that of bone crystals in the organic matrix. In this centre, which is formed by components of the osteoid, either phosphate or calcium are bound, after which crystal formation can commence. Crystal growth can proceed since the serum is supersaturated with respect to bone salts. Glycolysis appears to be a necessary accompaniment of bone salt formation, which indicates that the process is energy-dependent. The question why mineralization is restricted to bone matrix and does not occur elsewhere in the body where collagen is present, has been explained by the existence of a crystal poison at the sites of crystal seeding. Inorganic pyrophosphate might be such a poison; it is split by alkaline phosphatase, an enzyme which is present in active osteoblasts (Fleisch, 1968).

Another theory of bone mineralization, put forward by Bonucci, Anderson and others (see Jowsey, 1977), is that of the matrix vesicles. These vesicles have been demonstrated, by means of electron microscopy in zones of mineralization. They contain needle-like structures, which are probably calcium phosphate crystals since they are removed by EDTA. It has been found recently that electron-dense granules are present within the osteoblasts in areas of rapidly mineralizing bone. These granules, which are 20 to 80 nm in size, appeared to consist of agglomerates of calcium phosphate molecules in sac-like envelopes. It has been suggested that these particles are extruded from the osteoblast down the caniculi to the mineralization front.

Surface ions of the bone crystal bind water. This water forms an hydratation shell, which is an integral part of the hydroxy-apatite. This shell provides the possibility of ionic exchange taking place between bone salts and body fluids. Crystal growth is associated with a relative decrease in the amount of absorbed water, and with maturation of the bone its water content decreases.

2.7 Bone resorption

Two mechanisms of bone resorption are recognized: resorption by osteoclasts and resorption by osteocytes: the latter is called osteocytic osteolysis. Osteoclastic bone resorption occurs particularly at the endosteal surfaces of cortical and trabecular bone and also at the surfaces within the Haversian systems. On the inside of the shafts of long bones it causes widening of the cavity of the growing bone. One osteoclast can break down an amount of bone similar to that formed by 100 to 1000 osteoblasts in the same time (Frost, 1963). The osteoclasts are known to contain proteolytic enzymes and to produce organic acids such as citric acid and lactic acid.

Osteolysis by osteocytes involves removal of mineral and matrix matter from around the lacunae. It has been suggested that osteolysis is an important factor in calcium homeostasis, in that it accounts for the acute release of calcium from bone due to increased secretion of parathyroid hormone (Belanger, 1969; Jowsey, 1968).

2.8 Bone remodelling and bone turnover

Bone is a dynamic tissue that is formed and broken down continuously by the integrated action of osteoblasts and osteoclasts. This process is called bone remodelling. During growth, bone formation dominates bone resorption. In people over the age of about 45 years, bone resorption is no longer compensated by bone formation, which may lead to decalcification of the bones (Albanese, 1977). Compared with cortical bone, trabecular bone has a high surface-to-volume ratio and, as bone remodelling is a surface phenomenon, trabecular bone is considered to be more responsive to bone loss than is cortical bone (Jowsey, 1976).

Kinetic studies with radio active tracer calcium have shown that about 500 mg calcium is exchanged for bone tissue each day (see Morgan, 1973); this value can be taken as a measure of the daily bone turnover rate.

2.9 Calcium homeostasis

The adult human body contains about 1200 g calcium, 99% of which is present in the skeleton. The remaining 1% is found in the circulation and in soft tissues. A small proportion (approx. 1%) of skeletal calcium is rapidly exchangeable for circulating calcium. The plasma calcium concentration is homeostatically controlled and kept within narrow limits, normally between 2.20 and 2.60 mmol/l.

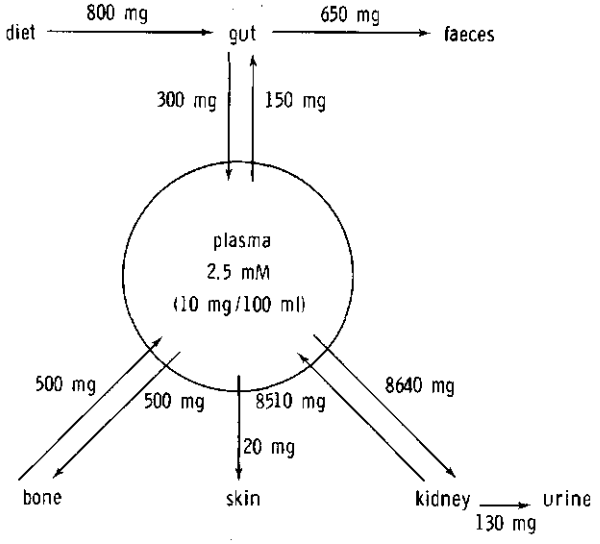

Fig. 1. Outline of calcium metabolism under balance conditions in adults (input = output). If the diet contains 800 mg of calcium per day, about 300 mg is absorbed from the intestinal tract to meet the daily endogenous losses: 150 mg in the faeces (from digestive juices and mucosal cells), 20 mg through the skin, 130 mg in the urine. Bone turnover is 500 mg per day. If the glomerular filtration is 100 ml/min and 60% of the plasma calcium is ultrafiltrable, the primary urine flow corresponds to 8510 mg/24 h, almost 99% of which is reabsorbed by the tubular cells, leaving a urinary excretion of 130 mg. Data were taken from Marshall et al. (1976), Heany et al. (1975), Lutwak et al. (1974), and Morgan (1973).

Calcium in plasma occurs in three different forms: (1) ionic or free calcium (± 47%), (2) calcium bound to protein, particularly to albumin, (± 39.5%), and (3) calcium complexed with citrate, phosphate, bicarbonate, lactate and sulphate (± 13.5%) (Moore, 1970). Protein-bound calcium is non-diffusable. The normal plasma calcium concentration is maintained by complex and integrated hormonal regulation of intestinal absorption, urinary excretion and deposition into or resorption from the bone. Figure 1 shows an outline of the calcium metabolism as it takes place in adults.

2.10 Phosphate metabolism

The total amount of body phosphorus is about 600 to 900 g, more than 80% of which is present in the skeleton. The rest can be found in soft tissues and blood, either as organic phosphate (phospholipids and phosphate esters) or as inorganic phosphate. In blood plasma the normal amount of organic (phospholipid-)phosphorus ranges from 1.6 to 3.0 mmol/l and that of inorganic phosphate (P_i) from 0.8 to 1.6 mmol/l (Henry et al., 1974).

It has been reported that about 53% of P_i in plasma is present as free ions, mainly as HPO_4^{2-} (43%) and $H_2PO_4^-$ (10%), the rest being bound to proteins (12%) or to other ions, especially sodium (29%), calcium (3%) and magnesium (3%) (Walser, 1961; see Van Paassen, 1979), Micropuncture studies of the kidney indicate that 90 to 95% of plasma P_i is ultrafiltrable (Fleisch et al., 1976). The kidneys play a major role in P_i metabolism by varying the tubular reabsorption. This reabsorption strongly depends on the amount of phosphate ingested with the diet. Under balance conditions the urinary P_i excretion equals the net intestinal phosphate absorption which amounts to more than 60% of the total intake (Spencer et al., 1965).

2.11 Summary

Bone is a dynamic tissue which is continuously formed by osteoblasts and broken down by osteoclasts. Osteocytes are involved in both processes. Together with the intestinal absorption and renal handling of calcium and phosphate, the deposition of these minerals into the bone and their resorption from it (bone turnover) are important parameters in the maintenance of calcium homeostasis and in phosphate metabolism.

Chapter 3

Hormonal control of bone mineral homeostasis

3.1 Introduction

Several hormones are involved in the complex mechanism of bone mineral homeostasis. The following will be discussed: the parathyroid hormone (PTH), calcitonin (CT), vitamin D, thyroxine, glucocorticoids, growth hormone, somatomedin, oestrogens and prolactine.

3.2 Parathyroid hormone

PTH is synthesized by four small glands which are situated beside the thyroid gland. It is a single-chain peptide, consisting of 84 amino acids. The hormone is secreted into the circulation after six amino acids have been detached from the N-terminal part of a pro-hormone (Hamilton et al., 1973). The biological activity of the native 1-84 molecule is located almost exclusively in the 1-34 N-terminal fragment.

The native hormone is degraded (rapidly) by liver and kidney, some of its fragments remaining behind in the circulation. By means of a radio-immuno-assay, the native hormone, its degradation products, or both, can be determined, depending on the type of antiserum used. Determination of the entire molecule or N-terminal fragments is useful if information is required about acute PTH-secretion; the fact is that these peptides have a rapid turnover and are short-lived (minutes). Determination of carboxy-terminal fragments provides information about chronic PTH-secretion, because these fragments have prolonged turnover rates (hours) (Kenny & Dacke, 1975; Root & Harrison, 1976).

The secretion of PTH and probably also its synthesis are directly stimulated by a low plasma-ionized calcium concentration, or inhibited by an elevated one. In vitro, PTH release appears to be enhanced by low serum magnesium levels, but severe hypomagnesemia inhibits the secretion of the hormone (Root & Harrison, 1976). The main function of PTH is to maintain the plasma calcium concentration within narrow limits. The hormone acts upon bone, kidney and intestine.

The well known effect that PTH has on bone is mobilization of calcium by: (1) increase in the number of osteoclasts, (2) rise in the metabolic activity of the osteoclasts and (3) increase in osteocytic osteolysis. It is generally agreed that PTH also acts upon the osteoblasts, initially inhibitory and subsequently stimulatory (Kenny & Dacke, 1975), but resorption dominates apposition.

The effect of PTH on the kidney is that: (1) in the proximal tubule the reabsorption of phosphate and calcium is decreased, and (2) that in the distal tubule calcium reabsorption is enhanced. The results are that the renal phosphate clearance increases and the calcium clearance decreases. In addition, PTH stimulates the urinary excretion of sodium, potassium and bicarbonate by lowering the tubular reabsorption of these ions, and reduces the urinary excretion of hydrogen and magnesium ions by increasing the tubular reabsorption (Kenny &

Dacke, 1975; Massry et al., 1973; Root & Harrison, 1976). PTH activates the renal enzyme 25-hydroxycholecalciferol-1-alpha-hydroxylase, which stimulates the synthesis of 1,25-dihydroxycholecalciferol, a potent vitamin D metabolite (see Section 'Vitamin D').

The effect of PTH upon the intestine is probably indirect and is exerted through the medium of vitamin D.

Regarding the effect of PTH on a cellular level, many observations are in line with a second messenger concept, as has been put forward by Borle and by Rasmussen & co-workers (see Root & Harrison, 1976). According to this concept, PTH directly or indirectly influences the concentration of cyclic AMP, cytosol-ionized calcium and monohydrogen phosphate, three intracellular second messengers which act in an interrelated manner and stimulate the cell to carry out characteristic functions.

After PTH has been bound at the receptor site of the cell, membrane adenyl cyclase is activated. This results in the formation of 3,5-cyclic AMP from ATP, and in a change in the permeability of the cell membrane to ionized calcium, so that these ions enter the cell from the interstitial fluid, thereby increasing the cytosol calcium concentration. In bone cells these changes bring about stimulation of enzymes involved with resorption of bone, inhibition of osteoblast activity, and acceleration of osteoclast production from precursor cells. In renal cells they stimulate a mechanism that inhibits phosphate reabsorption. It is postulated that monohydrogen phosphate inhibits the metabolic activity of PTH by lowering the cytosol calcium concentration upon increasing deposition of calcium in mitochondria. In contrast, cyclic AMP is claimed to accelerate the transfer of calcium from mitochondria into cytosol, by which the cytosol calcium concentration is increased. This might inhibit the activity of adenyl cyclase, so that cyclic AMP production is partly autoregulatory. This theory explains why there is no responsiveness of bone cells to PTH in vitamin D deficiency, as has been reported by Au & Raisz (1967), who made experiments with rats. They state that, since in the vitamin-D-deficient animal intra- and extracellular calcium and phosphate concentrations are low, PTH is unable to increase cytosol-ionized calcium.

3.3 Calcitonin

Calcitonin is a linear peptide consisting of 32 amino acids, the entire structure being necessary for biological activity. The hormone is produced in the thyroid gland by the parafollicular cells, and its secretion is directly proportional to that of plasma calcium above the level of about 2.25 mmol/l; below this concentration the hormone is not detectable (Catt, 1970).

Calcitonin has a hypocalcaemic action, mainly because it inhibits bone resorption; it activates adenyl cyclase, as a result of which cellular levels of cAMP are increased, and stimulates active efflux of calcium from the cytosol to the interstitial fluid, thus antagonizing PTH (Root & Harrison, 1976).

In the kidney, calcitonin is known to inhibit the tubular reabsorption of calcium, phosphate and sodium (Massry et al., 1973). No clear-cut direct effect of calcitonin on the intestine has been demonstrated (Root & Harrison, 1976).

Human calcitonin has a low biological activity and is rapidly cleared from the circulation, probably by the kidneys. Its role in human calcium homeostasis has been questioned, since plasma calcium is essentially normal in patients with medullary carcinoma of the thyroid. These subjects have high circulating levels of the hormone. Moreover, administered calcitonin does not produce hypocalcae-

mia in the normal adult to any marked extent, and thyroidectomy is not associated with a rise in plasma-calcium levels (Kalu & Foster, 1976). However, as postulated by Stevenson (1980), calcitonin might be of some importance in antagonizing the bone-resorptive action of 1,25-DHCC,, the active vitamin D metabolite, and of PTH, during periods of calcium stress such as pregnancy and lactation.

3.4 Vitamin D

3.4.1 Introduction

Vitamin D is known mainly in two forms, one from the animal and one from the plant kingdom. Provitamin D_3 (7-dehydrocholesterol) is formed in the intestinal mucosa from cholesterol and is converted in the skin by ultraviolet irradiation (230-313 nm) into vitamin D_3 (cholecalciferol); provitamin D_2 (ergosterol) in foodstuffs of plant origin is similarly converted upon ultraviolet irradiation into vitamin D_2 (ergocalciferol) in vitro. In both conversions the B-ring of the steroid molecule is opened. The difference between vitamin D_2 is in the side chain (see Fig. 2). The primary action of the calciferols is to prevent rickets in the young and

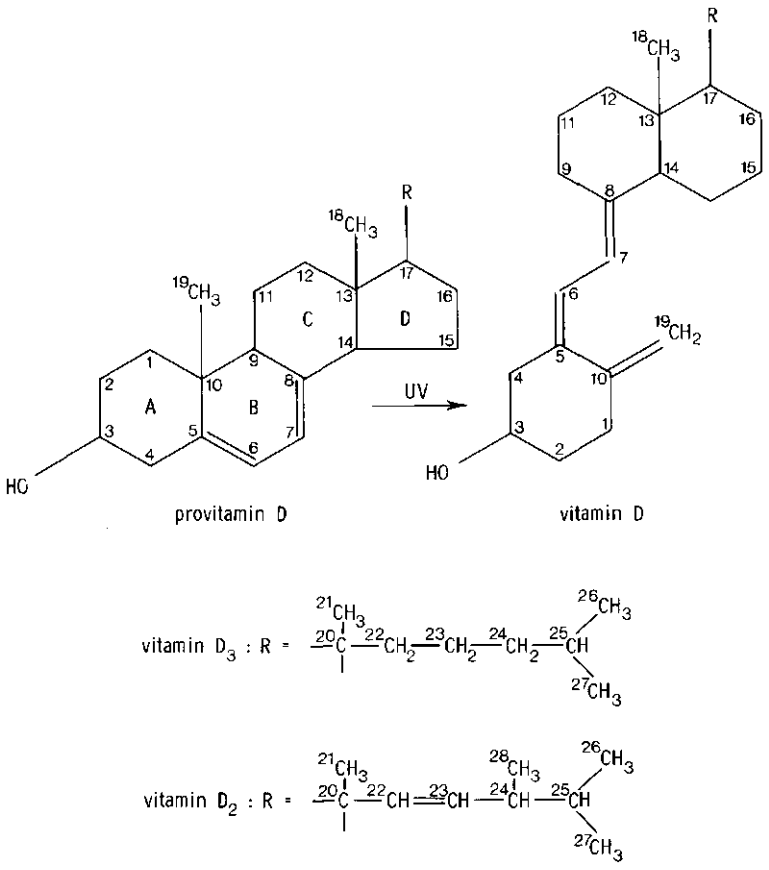

Fig. 2. Formation of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) from their precursors by UV-irradiation.

osteomalacia in the adult. The physiological response to vitamin D_2 and D_3 is similar in rat and man, but in birds as well as in the green monkey vitamin D_2 is much less active than vitamin D_3 (Harrison, 1975).

3.4.2 Metabolism of vitamin D

It is now generally recognized, mainly on account of the work of DeLuca and co-workers (Ponchon et al., 1969) and of Fraser & Kodicek (1970), that vitamin D must be hydroxylated in liver and kidney before it obtains its full biological activity. After transport by an alpha-globulin protein from skin to liver, vitamin D_3 is hydroxylated in liver cells by a microsomal 25-hydroxylase. The metabolite, 25-hydroxycholecalciferol (25-HCC), is further hydroxylated to 1,25-dihydroxycholecalciferol (1,25-DHCC) by a mitochondrial 25-hydroxy-1-alpha-hydroxylase in the renal cortex. This 1,25-DHCC, the hormonal form of vitamin D, is known to be most active in the stimulation of intestinal calcium absorption and bone calcium mobilization (see Fraser, 1975). The reported positive effect of 1,25-DHCC on renal calcium reabsorption is not specific and is probably related to the phosphataemic action of the hormone (Hugi et al., 1979).

Plasma values of 25-hydroxy-vitamin D which depend on the vitamin D content of the diet and the degree to which the skin is exposed to the sun, serve as an excellent index of the vitamin D status; the normal concentration is about 30 ng/ml, more than 80% of which has been reported to be 25-HCC (Edelstein et al., 1974; Haussler & McCain, 1977). Plasma 25-HCC levels are subject to seasonal variation, because they depend on the degree to which the skin is exposed to the sun. Values are highest in August and lowest in February (Postkitt et al., 1979). The plasma concentration of 1,25-DHCC is less than 1/500th that of 25-HCC. Other circulating vitamin D metabolites are 24,25-DHCC and 25,26-DHCC, which are renal products of 25-HCC. Their plasma concentration is about 1/10th to 1/20th that of 25-HCC, but their function has not been completely defined yet (Root & Harrison, 1976; Haussler & McCain, 1977; Care et al., 1979). It has been suggested that 24,25-DHCC has a positive action on skeletal mineralization (Kanis et al., 1979), possibly by occupying bone cell receptor sites for 1,25-DHCC.

3.4.3 Regulation of the vitamin D metabolism

The activity of renal 25-HCC-1-alpha-hydroxylase is known to be increased by PTH (Fraser & Kodicek, 1973; Garabedian et al., 1972; Rasmussen et al., 1972; Boyle & DeLuca, 1971), and this leads to an enhanced synthesis of 1,25-DHCC. PTH is not essential to the synthesis of this metabolite; in parathyroidectomized rats 1-alpha-hydroxylase is stimulated by a low-phosphorus diet (Tanaka & DeLuca, 1973). According to DeLuca (1973) 1-alpha-hydroxylase is stimulated if the inorganic phosphorus concentration in the renal parenchyma is below 400 μg/g tissue. It is now well established that either a low-calcium diet (via decreased plasma calcium and increased PTH secretion) or a low-phosphorus diet (independently of PTH via hypophosphataemia) increases circulating levels of 1,25-DHCC (Henry et al., 1974; Baxter & DeLuca, 1976; Hughes et al., 1975; Rader et al., 1979). On the other hand, another group of investigators (MacIntyre et al., 1979) believe, mainly on the strength of results of experiments with renal cell culture, that, in addition to PTH, calcium itself can regulate the activity of 1-alpha-hydroxylase; they found that elevation of calcium in the culture medium inhibited the activity of this enzyme and enhanced that of 24-hydroxylase.

The PTH-induced stimulation of renal 1-alpha-hydroxylase operates through

the medium of cAMP, both in vivo (Horiuchi et al., 1977) and in vitro (Rasmussen et al., 1972). The common denominator in both the PTH- and hypophosphataemia-stimulated 1-alpha-hydroxylation of 25-HCC may be a low intracellular, possibly mitochondrial, phosphorus content of the renal cortex. Rasmussen et al., (1975) (see Root & Harrison, 1976) believe that PTH has a major direct effect upon 1-alpha-hydroxylase in that it increases the renal cell uptake of calcium and the cytosol concentration of ionized calcium. In the rat, the renal synthesis of 1,25-DHCC declines and the synthesis of 24,25-DHCC increases when serum calcium rises above 2.25 mmol/l and serum phosphorus above 2.60 mmol/l (Tanaka & DeLuca, 1973). Renal 1-alpha-hydroxylase is inhibited by 1,25-DHCC, whereas renal 24-hydroxylase is stimulated by it (Tanaka & DeLuca, 1974; Omdahl, 1976).

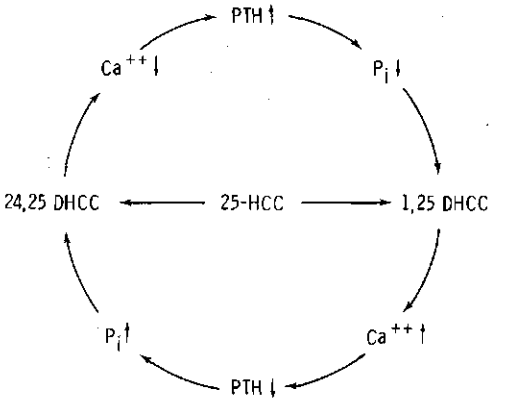

Fig. 3. Regulation of the conversion of 25-HCC by the intracellular phosphate concentration (P_i) in the renal cortex; Ca^{++} and PTH are plasma concentrations of these regulators.

PTH suppresses renal production of 25,26-DHCC (Tanaka et al., 1978). It is not known whether calcitonin influences the renal metabolism of 25-HCC; Rasmussen et al. (1972) reported an inhibitory effect of this hormone on the production of 1,25-DHCC, but this was not confirmed by others (Lorenc et al., 1977).

3.4.4 Action of vitamin D metabolites

The main target tissues of vitamin D metabolites are intestine, bone and kidney. It is known that the presence of vitamin D in the body is essential to the regulation of the intestinal calcium absorption. Active calcium absorption occurs in the upper portion of the small intestine against an electrochemical gradient; it is a vitamin-D-dependent process which requires energy from the oxidative metabolism of mucosal cells (Schachter & Rosen, 1959). These cells contain cytoplasmic and nuclear receptor proteins which bind the 1,25-DHCC-hormone. After 1,25-DHCC has been bound to the nucleus, it induces the formation of a messenger RNA, which brings about the synthesis of an intracellular calcium-binding protein (CaBP). This protein, which was isolated for the first time by Wasserman et al. from chick intestine in 1966 (Wasserman & Taylor, 1968; Root & Harrison, 1976), may increase the permeability of the intestinal cell to calcium. The calcium-bin-

ding activity of CaBP has been shown to correlate well with the efficiency of calcium absorption (Swaminathan et al., 1977). Vitamin D also increases the activity of intestinal alkaline phosphatase, a membrane-bound enzyme that exhibits calcium-stimulated ATP-ase activity (Haussler et al., 1970). This enzyme may be involved in calcium transport, but its precise function remains to be clarified. Active intestinal calcium transport is not stimulated by 25-HCC in anephric animals, which indicates that renal 1-alpha-hydroxylation is required for this effect (Boyle et al., 1972). Across the wall of the entire length of the intestine, vitamin-D-facilitated diffusion of calcium has been established (Harrison, 1960). This transport is independent of the oxidative metabolism and is believed to be solely related to calcium intake (Heany et al., 1975).

In normal individuals intestinal calcium absorption is positively correlated with serum levels of 1,25-DHCC (Gallagher et al., 1979; Wilz et al., 1979). It has been reported that 25-HCC and 1,25-DHCC also augment intestinal phosphate transport, independently of the presence of calcium (Chen et al, 1974; Walling & Kimberg, 1975). Brickman et al. (1977) reported that when patients suffering from advanced renal failure and normal individuals were treated with 1,25-DHCC or with its synthetic analogue, 1-alpha-HCC, intestinal absorption of phosphate was augmented. However, Wilz et al. (1979) noted that there was no significant correlation between plasma levels of 1,25-DHCC and intestinal absorption of phosphate or magnesium in vitamin-D-replete humans. Any direct action of 24,25-DHCC or 25,26-DHCC upon the intestine has not been reported.

Regarding the action of vitamin D metabolites on bone, it is recognized that 1,25-DHCC is most potent in stimulating bone resorption, and that this action requires the presence of PTH in vivo (Garabedian et al., 1974). PTH is not required for 1,25-DHCC-stimulated bone resorption in hypophosphataemic rats (Castillo et al., 1975). Although any action of 24,25-DHCC and 25,26-DHCC on bone has not been clearly defined, Queille et al. (1978) reported that both metabolites mineralized the osteoid in vitamin-D-deficient rats.

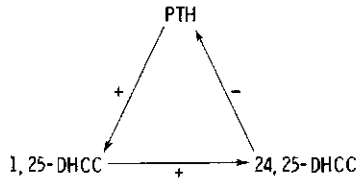

Fig. 4. Autoregulation of PTH secretion, as proposed by Canterbury et al. (1978).

In the kidney, both 25-HCC and 1,25-DHCC decrease the excretion of filtered phosphate, sodium and calcium by increasing proximal tubular reabsorption of these ions (Puschett et al., 1972a and 1972b).

Evidence is accumulating that vitamin D metabolites also act on the parathyroid glands. It has been demonstrated that 1,25-DHCC localizes selectively in the parathyroids (Henry & Norman, 1975) and that cytoplasmic and nuclear binding sites are present (Brumbaugh et al., 1975). Canterbury et al. (1978) observed that in an in-vivo canine model 25-HCC and 24,25-DHCC, in a near physiological concentration, completely suppressed the secretion of PTH. They further demonstrated that pharmacological, but not physiological doses of 1,25-DHCC sti-

mulated PTH secretion. Care et al. (1979) reported that 24,25-DHCC and 25,26-DHCC depressed PTH secretion and stimulated calcitonin secretion from in situ perfused goat parathyroid glands; 1,25-DHCC had an opposite action in this respect.

3.5 Thyroxine

Thyroxine increases the bone turnover rate, and this is particularly evident in thyrotoxicosis. In this condition serum levels of calcium and phosphorus are elevated, and there is a marked loss of bone minerals in urine consequent on an increased filtered load; since the increase in bone apposition does not fully compensate for the increase in bone resorption, thyrotoxicosis may lead to osteoporosis (Irving, 1973).

3.6 Glucocorticoids

Long-term administration or an increased synthesis of glucocorticoids (Cushing's syndrome) may cause loss of bone substance, resulting from a negative nitrogen balance. This loss of bone is associated with an increased loss of bone minerals in the urine (Massry et al., 1973). Glucocorticoids antagonize the effect of vitamin D on the intestinal calcium absorption (Root & Harrison, 1976).

3.7 Growth hormone and somatomedin

Growth hormone stimulates endochondral bone formation and increases skeletal mass by accelerating subperiostal bone apposition. Hypersecretion may lead to acromegaly with overgrowth of hands, feet and face, and to gigantism, whereas hyposecretion may give rise to dwarfism. The effect of growth hormone on bone is not a direct one, but is controlled by somatomedin, the synthesis of which is assumed to take place in the liver under the influence of growth hormone (Wiedemann & Schwarz, 1972). The effect of somatomedin on bone is dependent on the presence of thyroxine. Regarding the renal handling of calcium and phosphate, growth hormone has an effect opposite to that of PTH (Massry et al., 1973) and this probably explains the higher levels of serum phosphate during growth. Spencer et al. (1979) reported that growth hormone, injected into hypophysectomized rats, stimulated the renal conversion of 25-HCC into 1,25-DHCC. They proposed that this effect could be of importance to the long-term stimulation of renal hydroxylase during growth.

3.8 Oestrogens and prolactine

There is convincing evidence that oestrogens inhibit bone resorption; administration of these hormones to postmenopausal women decreases bone loss (Riggs et al., 1969 and 1972; Meema & Meema, 1976; Lindsday et al., 1976; Recker et al., 1977) and this effect appears to be associated with a decrease in the urinary excretion of calcium and hydroxyproline and in serum calcium, phosphorus and alkaline phosphatase. According to Heany (1965), sex hormones in women alter the responsiveness of bone to the action of PTH. Oestrogen deficiency, such as occurs in natural and artificial menopause, would increase the sensitivity of bone to PTH without the sensitivity of other PTH-target organs being changed. Indeed, Atkins et al. (1972) found that oestrogen inhibited the calcium-mobilizing action of PTH in an organ system of mouse calvaria. According to Van Paassen et al. (1978), however, the oestrogen concentration used in this experiment might have caused cell damage. The latter investigators were unable to demonstrate the

presence of a specific oestrogen receptor in the cell cytosol of the bone of rabbits, rats and humans, which would favour an indirect effect of oestrogens on bone. In his thesis, Van Paassen (1979) postulates that the effect of oestrogen administration to postmenopausal females might result from an inhibitory effect of oestrogens on somatomedin synthesis in the liver (Wiedemann & Schwarz, 1972).

There is evidence from in-vitro studies with chick renal tubules that prolactine directly stimulates the activity of renal 1-alpha-hydroxylase, but this might be specific to birds (Spencer et al., 1979; MacIntyre et al., 1979).

3.9 Summary

Calcium homeostasis is directed to the maintenance of the serum calcium concentration within narrow limits, mainly by the complex and integrated action of PTH and 1,25-DHCC, the active vitamin D metabolite, on three target organs: intestine, kidney and bone.

Along the proximal part of the intestinal tract, active transport of calcium takes place against a concentration gradient. This process is dependent on the presence of 1,25-DHCC. More distally, passive calcium transport, depending on the concentration gradient, has been established.

In the kidneys diffusable calcium is filtered from the plasma into the primary urine and subsequently largely reabsorbed from the tubuli. This tubular reabsorption is influenced positively by PTH and 1,25-DHCC.

The bone can be considered to be a closed system in which the intercellular calcium concentration is low (approx. 1.6 mmol/l) as compared with that in the blood (approx. 2.5 mmol/l). This gradient causes a diffusion transport of calcium from plasma to bone. On the other hand, transport of calcium from bone to plasma is an active process, which is influenced positively by PTH and 1,25-DHCC.

The secretion of PTH, the main regulator of calcium homeostasis, is triggered by a low serum-ionized calcium concentration. PTH stimulates the renal synthesis of 1,25-DHCC from 25-HCC. The calcaemic action of PTH and 1,25-DHCC upon bone, kidney and intestine is accompanied by a phosphaturic action of PTH to prevent hyperphosphataemia. The regulation of phosphate metabolism can be considered to be subservient to calcium homeostasis. 1,25-DHCC inhibits renal 1-alpha-hydroxylase and stimulates 24-hydroxylase. This gives rise to the formation of 24,25-DHCC, which metabolite inhibits the secretion of PTH.

Chapter 4

Effects of non-nutritional factors on bone and bone mineral metabolism

4.1 Introduction

In this chapter the effects of a number of non-nutritional, more or less physiological factors on bone and bone mineral metabolism are discussed. It is felt that for a proper understanding or for an evaluation of the results of studies dealing with this subject, especially with reference to osteoporosis, the factors discussed below cannot be overlooked. Although merely referring to a pathological rather than to a physiological status, renal failure is given attention. The hyperparathyroidism and osteodystrophia associated with this disease resemble, at least to a certain extent, nutritional hyperparathyroidism, which subject will be discussed in Chapter 5.

4.2 Age and sex

As was already mentioned in Chapter 1, an important feature of aging is the associated loss of bone tissue, which may lead to osteoporosis. Cross-sectional studies in different populations have shown that bone loss starts in the fifth decade of life and proceeds about twice as fast in females as in males. In the former the rate of loss is about 12% of the bone mass per decade, as indicated by radiogrammatric measurements of the cortical thickness of the metacarpal bone (Garn et al., 1967). Newton-John and Morgan (1970) have collected considerable data from the literature on the loss of bone tissue with age; the process is approximately linear in both sexes; the inter-individual variation in the amount of bone does not increase with age, and within various age groups the amount of bone appears to be distributed normally. On the basis of their observations these investigators postulated that nearly all persons lose bone with age and that there is no large subgroup of persons who lose more bone than others. This interpretation suggests that those who develop osteoporosis have not lost bone at a more rapid rate but just had a smaller amount of bone all the time. However, it should be recognized that results of cross-sectional studies, on which the above interpretation is based, may give a false picture of reality since the effects of selective survival or differential mortality are not taken into account. Moreover, as stressed by Smith et al. (1975), models exist that allow inter-individual variation in the rate of loss without a change in the inter-individual variation in the amount of bone. The few longitudinal population studies that have been carried out as yet have indeed shown that inter-individual variation in the rate of bone loss exists (Garn et al., 1967; Adams et al., 1970; Dequeker, 1972). According to Garn et al., (1967), a small stature, resection of the stomach and early artificial menopause would favour the loss of minerals from the skeleton. Cohn et al. (1976) have analysed the amount of total body calcium in 40 women by means of neutron activation. These investigators reported that loss of calcium from the body can be characterized by two components, one with a rate of 0.37% per year and the other, a more rapid one

which starts postmenopausally, of 1.08% per year. The fast component was superimposed on the slow component and the latter continued throughout life.

A second age-associated phenomenon is the reduced intestinal absorption of calcium in elderly people. It has been demonstrated by several groups of investigators that calcium absorption gradually decreases with age, in males as well as in females (Gallagher et al., 1979; Avioli et al., 1965; Bullamore et al., 1970; Nordin et al., 1976). Calcium absorption was found to be significantly lower than normal in postmenopausal osteoporotic patients when either age or dietary calcium was used as a co-variable (Gallagher et al., 1979). A decrease in calcium absorption with age, predominantly affecting the active transport component, has also been established in rats (Wilkinson, 1971; Armbrecht et al., 1979). Horst et al. (1978) reported decreased calcium absorption, low levels of 1,25-DHCC in serum and a decline in activity of renal 25-HCC-1-alpha-hydroxylase in aged rats. Gallagher et al. (1979) found that osteoporotic patients and elderly healthy subjects had normal serum levels of 25-HCC but lower levels of 1,25-DHCC. In addition to the observations made by Nordin et al. (1976) that malabsorption of calcium in elderly people can be corrected with pharmacological doses of vitamin D_2, the above findings suggest that the decline in calcium absorption with age is the consequence of reduced renal synthesis of the active vitamin D metabolite. This phenomenon may be caused by decreases of PTH secretion in the absence of oestrogens or by diminished renal functioning, as has been postulated by Nordin et al. (1976).

Other features of aging are a decrease in the urinary excretion of calcium, phosphorus and hydroxyproline, as has been reported by Dequeker (1973) on the basis of cross-sectional data on 59 normal females between 20 and 90 years. The decrease in mineral excretion was attributed to reduced intestinal absorption, and the decrease in hydroxyproline excretion to loss of bone mass. In females there is an increase in serum concentration of calcium and phosphorus and in the activity of alkaline phosphatase after the menopause. Values of these parameters return to pre-menopausal levels upon administration of oestrogens (Riggs et al., 1969 and 1972; Van Paassen, 1979).

During bone growth, when bone turnover is rapid, serum alkaline phosphatase activity, and the levels of serum phosphorus and urinary hydroxyproline are higher than during adulthood, when bone turnover has slowed down.

It has been reported that women have lower plasma calcitonin levels than men, that during pregnancy and lactation plasma calcitonin levels are increased, and that women taking oestrogen/progesterone oral contraceptives show a similar increase in calcitonin levels (Stevenson, 1980). The physiological implications of these findings may be that calcitonin protects the bone against the resorptive action of PTH and 1,25-DHCC during periods of increased calcium requirements and that it provides the protective effect of oestrogens against bone resorption. Furthermore, the difference in plasma levels of calcitonin between sexes may be involved in the corresponding difference in rate of bone loss.

4.3 Race

There is convincing evidence that the prevalence of fracture and osteoporosis is much lower among people of African than among those of Caucasian background, that black males and females have a greater skeletal mass and that they lose less bone with age than their white counterparts (Moore et al., 1975; Cohn et al., 1977). The greater skeletal mass in black than in white women is associated

with a larger muscle mass (Cohn et al., 1977). It seems unlikely that these differences are due to factors other than genetic background. On the other hand, the earlier start and greater intensity of bone loss in Alaskan Eskimos, by 'white' standards, might be related to nutritional factors, such as low calcium, high protein and high phosphorus intake levels (Mazess & Mather, 1974).

4.4 Pregnancy and lactation

For the calcification of the human foetus 35 g calcium are required, most of which is delivered during the last trimester of pregnancy. As the amount of calcium in human milk ranges from 25 to 35 mg/100 ml and the daily output of breast milk is about 850 ml, up to about 50 g of calcium are required from the maternal body during a six-month lactation period. Plasma 1,25-DHCC levels are raised in early pregnancy, continue to increase throughout gestation, and remain elevated during lactation (Kumar et al., 1979). Probably as a consequence, 25-HCC and 24,25-DHCC concentrations in plasma decrease during gestation (Reiter et al., 1979). It has also been reported that in late gestation plasma PTH levels tend to go up (Cushard et al., 1972). These changes in plasma levels of vitamin D metabolites and PTH undoubtedly reflect a physiological response to higher calcium requirements and explain why intestinal calcium absorption is increased in human pregnancy, as has been reported by Heany & Skillman (1971). Calcium balance may be negative during periods of high milk production in mammals as well as in the human species, in spite of large calcium intakes; it seems that during lactation bone calcium is more readily available than dietary calcium; the calcium balance again becomes positive when milk production declines and the maternal calcium stores are replenished (Irving, 1973).

4.5 Physical activity

It is wel known that bone adapts itself to environmental conditions. Stress and strain imposed upon the bone improve its trabecular structure, cortical thickness and density. Stress produces a piezo-electric response in bone, which stimulates osteoblastic activity. This effect, which may be an important factor with respect to increase of bone loss with age, is well illustrated by the observation that trabeculae subjected to the greatest stress and strain are the last to disappear as osteoporosis develops (Jowsey, 1977), and by the establishment that postmenopausal bone loss can be prevented, at least on a one-year basis, by exercise programmes (Aloia et al., 1978).

Resting in bed definitely increases the urinary excretion of calcium and phosphorus within a few days and this leads to a net loss of these minerals from the skeleton. Immobilization or denervation of a motor nerve of a limb is a commonly used technique for inducing disuse osteoporosis in laboratory animals (Kenny & Dacke, 1975).

Aloia et al. (1978) observed that marathon runners had an 11% larger skeletal mass than that of more or less sedentary subjects. Furthermore it is known that manual labourers have more bone mass than sedentary workers (Jowsey, 1977). Emiola & O'shea (1978) found that young adults who were classified as highly active, on the basis of a questionnaire and a handgrip strength test, had denser finger bones than either moderately or lowly active groups. All these findings demonstrate the adaptive response of bone to muscular stress and strain.

4.6 Renal function

Because the kidneys play an important role in the maintenance of calcium and phosphate homeostasis, it can be no matter of surprise that renal failure will upset this homeostasis. Indeed a complex disorder is known, called renal osteodystrophy, which occurs particularly in uremic patients suffering from end-stage renal failure, when the tubular function is less than about 30% of normal (glomerular filtration rate 40 ml/min) (Kumar, 1979). The bone lesions are mainly osteitis fibrosa and osteomalacia, the latter being more common in areas where vitamin D intake and/or exposure to sunshine are minimal; less frequently osteosclerosis is observed; osteoporosis is uncommon but is seen with increasing frequency among patients undergoing haemodialysis (David, 1975). It has been shown, for example by Juttman et al. (1979), that uremic patients lose bone and that this loss, as measured in the distal radius by ^{125}I-photon absorptiometry, is roughly proportional to the decrease in renal function (glomerular filtration rate between 5 and 45 ml/min).

The most important factor that contributes to the development of renal osteodystrophy is secondary hyperparathyroidism. It is the first detectable abnormality in very early renal failure (glomerular filtration rate 70 to 80 ml/min). According to the 'Bricker hypothesis' (Bricker et al., 1969), a decline in the glomerular filtration rate leads to a small increase in plasma phosphate, as phosphate excretion is temporarily decreased. The rise in plasma phosphate depresses plasma-ionized calcium and it is this that triggers secretion of PTH, which in its turn decreases tubular reabsorption of phosphate and promotes a higher rate of phosphaturia per remaining nephron. In this way a new state of phosphate balance is obtained, but at the cost of a higher rate of PTH-secretion. In later stages of renal failure (glomerular filtration rate 30 ml/min) the body can no longer cope with the intake of phosphate, and hyperphosphataemia develops.

Slatopolsky & Bricker (1973) have indeed found, from experiments with a uremic dog model, that phosphate homeostasis is maintained at the cost of a higher rate of PTH secretion. They also showed that dietary phosphate restriction, in proportion to the reduction in glomerular filtration, could prevent the rise in hormone secretion.

Patients with advanced renal failure have decreased circulating levels of 1,25-DHCC (Eisman et al., 1976), which explains the decreased intestinal absorption of calcium, the low responsiveness of bone to PTH, the defective bone mineralization and the hyperparathyroidism, which have been observed in these patients (David, 1975; Duursma & Dorhout Mees, 1973).

It is well known, from cross-sectional studies as well as from a longitudinal study, that the normal aging process is associated with a continuing linear decline in renal function; as measured by the creatinine clearance technique this decline amounts to about 30% in persons from 30 to 80 years (Rowe et al., 1976). Whether this loss of renal function has any consequences for the regulation of calcium and phosphate homeostasis remains to be established.

4.7 Summary

Several factors, directly or indirectly related to the metabolism of bone and bone minerals, appear to be associated with the normal aging process. These factors are: loss of bone mass, which may lead to osteoporosis, decrease in intestinal calcium absorption, decline in renal function and, probably, decrease in renal

synthesis of dihydroxylated vitamin D metabolites. It is not precisely known to what extent these features are interrelated.

It is undoubtedly the hormonal status (oestrogen deficiency) in postmenopausal females which makes them more predisposed to bone loss than males of the same age. Differences in race or genetic background are of significance in the etiology of osteoporosis as is illustrated by the fact that in black people this disease is relatively rare and bone loss with age proceeds at a lower rate than in whites.

Physical activity is necessary for the maintenance of skeletal integrity. A decreased physical activity probably contributes to bone loss through age.

The decrease in renal function with advancing age might be of importance from a nutritional point of view, since it has been shown that the adaptive response of the body to early renal failure is hyperparathyroidism. This disease can, as is apparent from an experimental study with dogs, be prevented by restriction of phosphorus intake levels.

Chapter 5

Effects of nutritional factors on bone and bone mineral metabolism

5.1 Introduction

This chapter deals with the effect of nutritional factors on calcium and phosphate metabolism. Special attention is given to the influence of different amounts of calcium and phosphate in the diet, but first some remarks will be made on other dietary components which have indirect effects in this respect.

5.2 Fat

Dietary fat is a vehicle for vitamin D and facilitates its intestinal absorption, which in patients with fat malabsorption (steatorrhoea) is insufficient. The intestinal calcium absorption is depressed by the formation of insoluble calcium soaps in the more distal parts of the intestine (ileum) where conditions are more alkaline. The formation of these calcium soaps is increased as the digestibility of the dietary fat is lower. Depending on the sequence of the fatty acids in the glycerol molecule, their chain length and degree of saturation, triglycerides may differ in digestibility. In general the intestinal absorption of long-chain fatty acids ($> C_{18}$) is lower than that of short-chain, medium-chain and unsaturated fatty acids (Laval-Jeantet & Laval-Jeantet, 1976). It has been shown that a triglyceride containing a long-chain fatty acid in the terminal position is more readily absorbed than a fat in which such a fatty acid is situated centrally (Barltrop, 1974).

5.3 Lactose

5.3.1 In laboratory animals

It is well known that lactose (milk sugar) stimulates the intestinal absorption and retention of calcium, but the mechanism underlying this effect remains unresolved as yet (Ali & Evans, 1973). According to Vaughan & Filer (1960), lactose shares this property with other carbohydrates. These also have a low digestibility, with the result that they reach the ileum, where the effect referred to is exerted. Armbrecht & Wasserman (1976) obtained evidence that lactose interacts with the absorptive cells, which leads to an increase in the permeability to calcium. Pansu et al. (1979) observed that feeding of lactose to rats (30% in the diet) was associated with a lower concentration of calcium-binding protein in the mucosa. It is therefore realistic to suggest that lactose depresses the synthesis of 1,25-DHCC.

5.3.2 In man

The positive effect of lactose on calcium utilization has been confirmed by means of experiments with humans (Mills et al., 1940; Kobayashi et al., 1975; Condon et al., 1970). However, this effect has been found to be absent in lactase-deficient (lactose intolerant) people, who showed a diminished calcium absorption after lactose ingestion (Condon et al., 1970; Kocian et al., 1973). On the other hand,

Pansu & Chapuy (1970) reported calcium absorption to be enhanced by lactose in lactase deficient subjects.

Flatz & Rotthauwe (1973) have postulated that the lactose tolerance (sufficient lactase activity in the intestine) in northern and western Europeans is a selective advantage in countries where vitamin D intake and exposure to sunshine are low and a tendency to develop vitamin D deficiency exists. The beneficial effect of lactose on calcium absorption would be of importance in the prevention of rickets and osteomalacia under these conditions.

A high prevalence of lactase deficiency (47%) was observed by Birge et al. (1967) among patiens with osteoporosis. In patients who had undergone partial gastrectomy, Kocian et al. (1973) found significantly more thinning of the clavicle cortex in those who were lactase deficient than in those with normal lactase activity. More recently, Newcomer et al. (1978) reported in a well-controlled study that among 30 women with postmenopausal osteoporosis 8 were lactase deficient, whereas among 31 control subjects without metabolic bone disease only one subject showed lactose intolerance. In this study, intakes of lactose and calcium were significantly lower in subjects with lactase deficiency than in those with normal lactase activity, because the former left dairy products out of their diets, even while they were not aware of their lactose intolerance. So it has been suggested that a low grade of calcium deficiency associated with lactose intolerance contributes to the pathogenesis of osteoporosis (Anonymous, 1979).

5.4 Proteins

5.4.1 In laboratory animals

As early as 1956, Wasserman et al. reported that the presence of certain amino acids, particularly lysine and arginine, in the intestinal tract improved calcium absorption. Engstrom & DeLuca (1963) investigated the effects of increasing amounts (from 18 to 36%) of three types of protein in the diet (egg-white, blood fibrin and casein) on calcium metabolism in growing rats fed a low-phosphorus diet (0.02%). In relation to egg-white, an increase in the net intestinal absorption and urinary excretion of calcium and a decrease in the femur-ash content were observed. Blood fibrin induced similar changes but had no effect on the femur-ash content, and casein increased the intestinal absorption of calcium and the femur ash content but had no effect on urinary calcium. The specific characteristic of egg-white, its reducing effect on the femur-ash content, disappeared when conalbumin, ovomucoid and lysozyme were removed from it. Denis et al. (1973) reported that a rise in the protein content (blood fibrin) of a low-calcium, low-phosphorus diet of young growing rats from 3.3 to either 11 or 33%, increased the epiphyseal plates. These effects were found to be independent of PTH and calcitonin, as they also occurred in thyro-parathyroid- and parathyroid-ectomized rats. Bell et al. (1975) demonstrated, by means of experiments with adult rats fed adequate amounts of dietary calcium and phosphorus, that an increase in the prctein content of the diet from 10 to either 20 or 40% (by adding equal parts of lactalbumin and gelatin to a 10% casein diet) was associated with a shift in the route of endogenous calcium excretion from faeces to urine, and with an increase in intestinal calcium absorption. It was found to have no effect on the rate of bone resorption.

More recently, Allen & Hall (1978), who worked with growing rats, reported that a high-protein diet (36% casein), as compared with a control diet (18% casein), resulted in a temporary increase (for the first two weeks) in urinary calcium

excretion. Other parameters of calcium metabolism (body calcium retention, intestinal absorption and intestinal calcium-binding protein content, bone turnover, bone development and plasma concentration) remained unchanged. Endogenous faecal calcium excretion, however, was reduced in animals fed the high-protein diet for 14 and 28 days, although this effect was not mentioned explicitly. A recent paper by Whiting & Draper (1980) shows that the degree of hypercalciuria seen with high-protein feeding in the rat depends on the duration of the diet period and on the type and level of protein used. A peak excretion occurs two days after feeding of the high-protein diet has started. The paper demonstrates the existence of positive correlations between the urinary excretion of calcium, that of sulphate and the content of sulphur-amino acids (methionine and cystine) of the diet.

5.4.2 In man

There is increasing evidence that the amount of dietary protein affects calcium metabolism. As early as in 1920, Sherman reported that a man who maintained calcium balance when given a diet supplying 390 mg calcium daily, lost body calcium when meat was added to his diet. McCance et al. (1942) observed that a raise in the protein content of the diet of three healthy subjects resulted in increased urinary excretion and intestinal absorption of calcium and magnesium. It was suggested that the primary action of dietary protein was that it increased the intestinal calcium absorption. More recent studies, carried out mainly by the group of Linkswiler and co-workers (Johnson et al., 1970; Walker & Linkswiler, 1972; Anand & Linkswiler, 1974; but see Linkswiler et al., 1974), have confirmed the hypercalciuric effect of dietary protein and have shown that this effect is not secondary to an increased intestinal absorption. These investigators studied the effect of three levels of dietary protein (47, 95 and 142 g/day) in healthy young male adults during 15-day experimental periods at three levels of calcium intake (500, 800 and 1400 mg/day). Regardless of the level of calcium intake, urinary calcium increased with the protein content of the diet. If calcium intake was low (500 mg/day), the intestinal absorption of calcium was unaffected by the protein content of the diet, and calcium retention became negative if the protein content of the diet was raised from 47 to either 95 or 142 g/day. At higher levels of calcium intake an increase in the protein content of the diet was associated with an increase in the intestinal calcium absorption, the maximal effect being reached at the 95 g protein level. Almost all balances were negative at the highest level of protein intake. Kim & Linkswiler (1979) have shown that the calciuric effect of dietary protein is caused by a change in the renal handling of calcium: in a ten-day experimental study with six healthy adults, a 142 g protein diet, as compared with a control diet containing 47 g protein, resulted in an acceleration of the glomerular filtration, an increase in the filtered calcium load, and a reduction of the tubular reabsorption of calcium. These effects were associated with a slight, though not significant increase in the urinary excretion of hydroxyproline and were not related to parathyroid function. The calciuric effect of an increase in the amount of dietary protein from 47 to 112 g/day has recently been demonstrated by this group in older men and women (Schuette et al., 1980). In these people, who consumed about 800 mg calcium per day, the calcium balance became more negative whereas the circulating levels of 1,25-DHCC and PTH and the urinary excretion of cAMP remained unchanged.

The findings described above were obtained in experiments with humans who

were given purified proteins, care being taken that other dietary components known to affect calcium metabolism were kept constant. This probably explains the somewhat different results obtained by Spencer et al. (1978 a). They found that an increase in the protein intake from 1 to 2 g/kg body weight by the ingestion of meat had no effect on urinary calcium when the calcium intake was either 200 or 800 mg/day, and only had a temporary effect in this respect when calcium intake was 1100 or 2000 mg/day. It was assumed that the calciuric effect of protein was counteracted by a concomitant increase in the intake of phosphate (see Section 5.10.2).

Licata et al. (1979) have stressed that the calciuric effect of dietary protein has to be taken into account with respect to evaluation and treatment of patients with hypercalciuria and urolithiasis. It has been hypothesized on the basis of a retrospective clinical and dietary survey that consumption of animal protein is a factor in the incidence of urinary calculi (Anonymous, 1980).

5.5 Acid-base balance, dietary acidity

When adult rats were subjected to acid stress by the addition of 1.5 or 2% NH_4Cl to their drinking water for 6 to 11 months, femur bone loss occurred independently of the level of dietary calcium (Barzel & Jowsey, 1969; Barzel, 1969). However, Newell & Beachene (1975) could not demonstrate any loss of bone mineral from the tibias in 13- and 25-month old rats which were fed diets containing 2% NH_4Cl with either 0.1 or 0.5% dietary calcium for nine months. But the acid-stressed animals, particularly those fed the low-calcium diet, had a decreased urinary pH, excreted more titratable acid with the urine and had an increased urinary excretion of calcium, phosphorus and ammonia. In addition, the renal phosphate-dependent glutaminase activity had increased in these animals and their serum calcium and phosphate levels tended to be depressed. These observations indicate that rats may adapt themselves to an acid load and maintain acid-base balance without loss of minerals from the skeleton. This view is supported by the results of a study made by Bell et al. (1976), who demonstrated that rats fed acid diets excreted amounts of labelled calcium resorbed from the bones that were very similar to the amounts excreted by rats fed neutral diets. In the first mentioned animals the route of endogenous calcium excretion was found to have shifted from faeces to urine. Human subjects on acid diets showed increased urinary excretion of calcium, which appeared to be due to decreased calcium reabsorption from renal tubules (Lemann et al., 1979). According to these investigators, this phenomenon is not related to PTH or 1,25-DHCC. On the contrary, Wachman & Burnstein (1970) reported that NH_4Cl-acidosis in five healthy subjects resulted in a higher serum PTH concentration associated with an increased excretion of calcium, phosphorus, magnesium and hydroxyproline in the urine, and negative calcium balances. The same investigators (Wachman & Burnstein, 1968) have framed the hypothesis that the acid load associated with the consumption of high-protein omnivorous diets in western communities is a contributing factor in the etiology of osteoporosis.

5.6 Vitamin D

The importance of vitamin D in the regulation of the calcium metabolism has been discussed in Chapter 3. Vitamin D deficiency causes rickets in the child and osteomalacia in the adult, with the well known classical symptoms: the histological picture of the bone is characterized by failure of mineralization as it undergoes

remodelling, leaving wide zones of unmineralized osteoid at the junction of mineralized bone and the layer of osteoblasts; serum calcium is normal or low, and serum phosphorus is reduced; alkaline phosphatase activity is increased and secondary hyperparathyroidism occurs. Hypervitaminosis D, a toxic condition resulting from excessive intake (more than 2000 units daily) over several weeks, is associated with hyperabsorption of calcium, hypercalciuria, renal calcinosis, progressive renal insufficiency and hypercalcaemia. The hypercalcaemia causes anorexia, nausea, obstipation, thirst and polyuria (Harrison, 1975).

5.7 Dietary fibre, phytate and oxalate

Dietary fibre (cellulose, lignin and non-cellulose polysaccharides) impairs the intestinal absorption of minerals, particularly calcium, magnesium, iron and zinc. Thus it is known that a high proportion of whole-wheat products in the diet may cause a negative calcium balance in man, despite calcium intakes as high as 1300 mg/day (Cummings, 1978). Traditionally this effect is attributed to the calcium-binding properties of phytic acid, a cell-wall constituent of cereals. However, it has been shown by James et al. (1978) that uronic acids also have strong binding properties in this respect. These investigators estimate that a typical western diet, which contains on average 12.3 mmol uronic acids, can bind about 3.8 mmol calcium (= 152 mg). In tropical communities with a much higher intake of dietary fibre, the calcium binding capacity of uronic acids may exceed the calcium intake. However, microbial digestion of uronic acids in the colon will liberate the calcium, so that colonic absorption of calcium may occur, particularly since the transit time of food residues in the colon is about 20 times that in the small intestine. Experiments with rats suggest that the large intestine may play a role in calcium homeostasis by colonic calcium absorption, particularly when the calcium intake is restricted (Petith et al., 1976).

Oxalate, present in foodstuffs such as rhubarb, spinach and purslane, binds calcium irreversibly in the intestinal tract. The calcium-oxalate complex formed is unavailable for absorption.

5.8 Fluoride

Fluoride is a 'bone-seeking' mineral: in rats 6% of a dose of radio-fluoride can be detected in the skeleton within two hours after administration. The incorporation of fluoride in bone and teeth results in a more stable mineral phase, the fluorohydroxyapatite. Experiments showed that, when the fluoride content of the diet fed to weanling rats was increased from 0.2 to 200 mg/kg for a period of four weeks, the fluoride content of the bone was 200 times, that of plasma and liver 3 times, and that of the kidneys 8 times as high as the corresponding levels in control animals (Deshmuth et al., 1970). Administration of fluoride has been reported to result in stimulation of osteoblasts and in formation of poorly mineralized osteoid; this leads to secondary hyperparathyroidism with increased bone resorption. By increasing the calcium and vitamin D content of the diet, the osteoid can be mineralized and hyperparathyroidism prevented (Jowsey et al., 1972).

Excessive dietary fluoride causes fluorosis, the symptoms of which are mottled enamel and white patches on the teeth, and osteosclerosis, characterized by thickened and densely calcified bones and bony outgrowths (exostoses). It has been tested whether fluoride could prevent periodontal disease and osteoporosis in dogs which were fed diets with a low calcium and a high phosphorus content; the results were negative (Henrikson et al., 1970).

People living in areas where the fluoride content of the drinking water is high show a decreased incidence of both caries and osteoporosis; in the Netherlands Melman et al. (1973) found that in almost all age groups, people living in the town of Tiel, who received fluoride in their drinking water (1 mg/l), had a higher radial bone density than people living in the control town of Culemborg. Fluoride and vitamin D, used in the treatment of osteoporosis, were found to increase the amount of bone (Van Kesteren, 1978).

5.9 Calcium

5.9.1 In laboratory animals

5.9.1.1 Calcium metabolism

It is known that intestinal calcium absorption, expressed as a fraction of the calcium intake, decreases when calcium is added to the diet (Hansard & Plumlee, 1954; Whittemore et al., 1973; Clark, 1969) or when body stores of calcium are filled (Nicolayson et al., 1953). It is now generally accepted that this adaptive response is regulated by the active intestinal transport system which is under the control of vitamin D metabolism (see Chapter 3). Hansard & Plumlee (1954) have shown that in the rat the endogenous faecal route of calcium is used as an excretory pathway when the dietary calcium content is varied. The cause of this phenomenon, which does not appear to occur in man to any significant extent, is that in the rat the urinary calcium excretion is very low (usually less than 1 mg/day or less than 1% of the amount ingested). This remarkable species difference has been demonstrated by Wanner et al. (1956) by injecting ^{45}Ca intravenously and by determining the ratio of faecal to urinary ^{45}Ca in excreta collected for five days after injection of the tracer. In rat and dog this ratio was 22/1 and 10/1 respectively, and in man and monkey it was 1/2. There is no information available as to whether this endogenous-faecal calcium excretion in the rat is under hormonal control.

5.9.1.2 Phosphorus metabolism

Hansard & Plumlee (1954) have demonstrated that an increase in the calcium content, from 0.013 to 0.5%, of the diet of rats stimulates the faecal excretion of injected ^{32}P and diminishes the urinary excretion of this isotope. This effect may be attributed to depression of PTH secretion. Clark (1969) has shown that the intestinal absorption of phosphate is directly proportional to its intake, but inversely proportional to calcium intake. He also demonstrated that low dietary calcium increases the intestinal absorption of magnesium.

5.9.1.3 Osteoporosis

There is a lot of evidence showing that feeding rats calcium-free or calcium-deficient diets (approx. 0.02% calcium) results in osteoporosis, irrespective of the age of the animals; in young rats bone formation is reduced and in older animals bone removal predominates (Volpin & Salomon, 1978). Similar observations have been made in chicks, rats, pigs and dogs (Sevastikoglou et al., 1977). Bone loss is accelerated in aging female mice after fertility and in adult ovariectomized rats, when they are given a low-calcium diet (Ambrus et al., 1978; Blanusa et al., 1978). The osteoporotic changes induced by dietary calcium deficiency are extreme in lactating rats: Rasmussen (1977 a and b) reported hypocalcaemia, slightly elevated serum phosphorus and hydroxyproline, and reduction in the femur-ash content of the trabecular bone at the proximal and distal parts and, to a lesser

extent, of the cortical bone of the diaphyseal shaft. Histologically, there was reduced apposition of bone at the periosteal surface and increased resorption of bone at the endosteal surface of the diaphysis. Calcium deficiency is associated with increased circulating levels of PTH and 1,25-DHCC (Rader er al., 1979). Stauffer et al. (1973) reported that calcium deficient rats with hypocalcaemia develop a mineralization defect similar to that seen in hypocalcaemic vitamin D-deficient animals, but bone resorption is much higher in the former than in the latter. Whether the osteoporotic changes induced by dietary calcium deficiency are completely reversible remains a matter of debate; reversibility seems to depend on the age of onset and on the length of the period during which the animal is subjected to calcium deficiency (Sevastikoglou et al., 1977).

5.9.2 In man

5.9.2.1 Calcium metabolism

Heany et al. (1975) have confirmed the inverse relationship between intake and fractional absorption of calcium in man. They gave a mathematical equation for the absorption (y) versus intake (x), which consists of an exponential term, representing a controlled active process, and a linear component, representing a passive process solely related to the level of intake:

$$y = 0.3127\, e^{-1.0539\,x} + 0.1541\, x \text{ (x and y in g/day).}$$

Thus an enlargement of the calcium intake leads to increased net calcium absorption, which within 24 hours causes a rise in the filtered load of calcium and a drop in the circulation levels of PTH, and within 24 to 36 hours a decrease in serum levels of 1,25-DHCC. As can be expected from the equation described above, urinary calcium excretion depends slightly on calcium intake; at a given variation of calcium intake the urinary calcium changes by an average of only 6% (Lemann et al., 1979). Excretion of endogenous calcium in faeces is known to be largely independent of calcium intake, and amounts to about 150 mg/day. However, Spencer et al. (1964) found that endogenous-faecal calcium excretion in three osteoporotic subjects was 60 to 96 mg/ day during periods of a low calcium intake (75 to 177 mg/day) and 75 to 144 mg/day during periods of a high calcium intake (880 to 1505 mg/day).

5.9.2.2 Phosphorus metabolism

Dietary calcium increases the faecal excretion of phosphorus and decreases its urinary excretion. According to Spencer et al. (1964, 1965 and 1978b), this shift is caused by the formation of insoluble calcium phosphates in the intestinal tract.

5.9.2.3 Calcium requirement and allowance

There is no general consensus of the calcium requirement of adult humans, which is illustrated by the fact that calcium allowances may differ substantially in various countries. For example, the FAO/WHO allowance for adults is 400 to 500 mg/day, whereas in the USA the Food and Nutrition Board recommends 800 mg/day for this group. The FAO/WHO committee on calcium requirements is of opinion that calcium intake may be low, since there are no clear-cut signs of calcium deficiency in countries where calcium intake is supposed to be as low as 300 mg/day, and since man is able to adapt himself to low levels of calcium intake, as has been shown by Hegsted et al. (1952) and by Malm (1958). Proponents of a high level of calcium intake, however, point out that adaptation to low levels is not a desirable

situation. One of their arguments is that Malm (1958) has shown that some people, particularly older ones, may find difficulties in such an adaptation or do not adapt at all, and another that cultural differences in dietary pattern (high intake of protein and phosphate) may increase calcium requirements. Anyhow, the results of calcium balance studies raise doubt of the general applicability of the FAO/WHO allowance. Thus Malm (1958) found that the average calcium requirement of 25 prisoners in Norway, who were fully adapted to a low calcium intake, was 440 mg per day (range 337 to 617 mg). The calcium allowance for such people should largely exceed 400 to 500 mg/day. Marshall et al. (1976), in a recent review of 212 calcium balances in 84 subjects, found that most balances were negative at intakes below 600 mg calcium per day. These workers point out that the recommended intake that would preserve calcium balance in most of the population may be as high as 900 mg/day.

Heany et al. (1977) found a weak but significant correlation between calcium intake and calcium balance in 130 perimenopausal women who had a mean calcium intake of 661 mg/day. On an average, the calcium balance in these women was slightly negative. On the basis of these findings, which indicate that the actual calcium intake was suboptimal, the investigators computed the calcium requirement (to maintain zero balance for the group as a whole) at 1.24 g/day. It should be recognized that balance studies are liable to large errors. The main reasons for this are the difficulties associated with the accurate measurement of intake and output, and the statistical fact that the small difference between intake and output cannot be determined with a high degree of precision.

5.9.2.4 Calcium deficiency and osteoporosis

Calcium deficiency in man is not well recognized. There are two case reports (Waltz et al., 1970; Kooh et al., 1977) in which the existence of dietary calcium deficiency in two infants is mentioned. The disease was characterized by rickets-like symptoms, such as rachitic rosary, enlargement of the epiphyseal regions of the long bones, low serum levels of calcium and phosphorus, increased alkaline phosphatase and aminoaciduria. Only in the report by Kooh et al. were vitamin D deficiency and resistance excluded. Recently Pettifor et al. (1979) described high incidence of hypocalcaemia, increased alkaline phosphatase and low urinary excretion of calcium in black schoolchildren living in a rural community in South Africa. These abnormalities were attributed to a low calcium intake (125 mg/day) as they did not occur in children who consumed some milk (calcium intake 337 mg/day), and they disappeared after calcium supplementation.

Whether osteoporosis in man is, in part, the result of dietary calcium deficiency is still a matter of discussion. Several groups of investigators have claimed to possess evidence, on the basis of retrospective studies, that a low calcium intake is more prevalent in osteoporotic patients than in control subjects, but other groups have reported negative results (Exton-Smith, 1972). In many studies in which calcium consumption is related to osteoporosis or to bone mass, the lack of a control group matched with the experimental group as regards sex, age, height and weight, ethnic background and geographic area is evident. Furthermore, a reliable estimate of the subject's individual habitual calcium intake is difficult to obtain. It is for these reasons that results of retrospective studies have to be interpreted with care. In this respect Reshef et al. (1971) found a negative correlation between calcium intake and severity of osteoporosis. This correlation, however, was age-dependent, since calcium intake decreased with age and

osteoporosis was more common in the elderly than in the young. Nordin (1960), who revived the calcium deficiency theory, reported that 71 osteoporotic patients had a calcium intake of 13.4 mg/kg/day, whereas 69 control subjects consumed 17.9 mg/kg/day. However, the control subjects differed markedly from the osteoporotic patients as their average body weight exceeded that of the latter by 14 kg.

There are other lines of evidence which indicate that a low calcium intake predisposes to osteoporosis. The high incidence of this disease in patients suffering from lactose intolerance has already been stressed (see Section 5.3.). The administration of either calcium supplements alone (Lutwak, 1974; Recker et al., 1977) or calcium supplements with vitamin D (Albanese et al., 1973; Riggs et al., 1976) has proved to be effective in inhibiting bone loss with age. Cohn et al. (1968) have demonstrated in a study with seven osteoporotic patiens that calcium supplements inhibit bone resorption.

Matkovic et al. (1979), in a cross-sectional study among two rural populations in two Yugoslavian districts, found that in both men and women the relative cortical area of the second metacarpal bone was larger and the femur fracture rate was lower in a cattle raising district where dairy products were consumed than in a purely agricultural district where the consumption of these products was low. The difference in the amount of cortical bone which was already present at adult age, could not be attributed to genetic or geographic factors, tended to decrease with age, and was probably the consequence of a large difference in calcium consumption between the two population groups (one group twice as much as the other).

5.10 Phosphorus and the calcium-to-phosphorus ratio

5.10.1 In laboratory animals

5.10.1.1 Calcium and phosphorus metabolism

Feeding rats phosphorus-deficient diets (approx. 0.03% P) leads to phosphorus depletion, cessation of growth and characteristic biochemical changes including hypercalcaemia, hypophosphataemia, hypercalciuria and hypophosphaturia (Lee et al., 1979). In addition, as demonstrated by Rader et al. (1979), circulating levels of 1,25-DHCC are increased and serum levels of PTH are decreased in rats fed 0.04% dietary phosphorus instead of 0.6%.

In recent years a great deal of attention has been given to the effect on bone metabolism of diets with either a high phosphorus content or with a low Ca/P-ratio; according to Krook et al. (1975), such diets with their relative excess of dietary phosphorus slightly depress the serum-ionized calcium concentration, which stimulates the parathyroid glands and leads to nutritional hyperparathyroidism. Scott & Greaves (1961) induced a generalized osteitis fibrosa in young cats which were fed beef heart for six to eight weeks. As a result of this all-meat diet, with a Ca/P-ratio of 1/20, the parathyroids of the animals were enlarged, hypertrophic and hyperplastic. Addition of calcium carbonate, in amounts sufficient to obtain a Ca/P-ratio of 1/2, only delayed the osteopathy, whereas addition of calcium carbonate and gluconate until a Ca/P-ratio of 1/1 was reached, prevented the skeletal lesions. More recently Clark & Brashear (1976) induced osteitis fibrosa cystica and nephrocalcinosis in adult rats by feeding them a diet with a normal calcium content (0.4%) and a very high phosphorus content (3.2%) for two months. Very interesting in this respect is the extensive work, mainly on aging rats and mice, carried out by the group of Draper and co-workers. Initially

they showed (Shah et al., 1967) that the femur-ash content of aged mice was significantly influenced by feeding them diets with different Ca/P-ratios for 14 months. A ratio of 2/1 always resulted in higher bone-ash contents than did a ratio of 1/1 at levels of dietary calcium that varied between 0.1 and 1.2%. A further study of mice which were fed diets high in calcium (1.2%) (Krishnaro & Draper, 1972) showed that in animals of 11 months and over a reduction of dietary phosphorus from 1.2 to 0.6% lowered bone loss during the following seven months. Draper and co-workers, in a series of experiments with adult rats, investigated the effect of dietary phosphorus upon the mobilisation of calcium from bone by monitoring the excretion of ^{45}Ca that had been injected one month before the start of the dietary studies (Anderson & Draper, 1972; Draper et al., 1972; Sie et al., 1974). It was demonstrated that high-phosphorus diets (1.2%) with a normal (0.6%) or a high (1.2%) calcium content stimulated bone resorption, that this effect could be eliminated by parathyroidectomy and that the high-phoshorus diets stimulated PTH-synthesis and reduced urinary excretion of calcium. Meal-fed rats on a high-phosphorus diet exhibited depressed serum-calcium concentrations in postprandial as well as in fasting blood samples. The bone mass of these rats was reduced, but the chemical composition of the bones was found to be normal. Even a diet with 0.6% dietary phosphorus and 0.6% calcium gave, during a six-month period, a 16% higher excretion of ^{45}Ca than did a similar diet with 0.3% dietary phosphorus. A 1.8% phosphorus diet (0.6% calcium) produced calcification of soft tissues (kidneys and heart). Although the technique of monitoring the excretion of stable ^{45}Ca from the skeleton is very sensitive as regards the resorption of calcium from bone, it cannot be used for differentiating between net loss of bone and increased bone turnover.

The observations made by the group of Draper regarding aging bone loss in rats rely on determinations of bone ash, since differences in the retention of calcium could not be demonstrated.

Another group of investigators, Jowsey and co-workers, studied the effects of dietary phosphate supplements in rabbits (Jowsey & Balasubramanian, 1972) and in adult dogs (Laflamme & Jowsey, 1972). In rabbits a decrease in the Ca/P-ratio from either 1.7 to 0.55 or from 1.1 to 0.42 by the addition of phosphate resulted in increased porosity of the bones, as shown by microradiography, and in calcification of soft tissues (kidneys and thoracic aorta). In dogs, long-term dietary phosphate supplementation (for ten months) led to higher bone resorption, loss of bone tissue, increased bone porosity and raised circulating levels of PTH. In addition, calcification of soft tissues (kidneys, thoracic aorta, tendon and eye-lens) was observed in animals fed the high-phosphorus diets. The feeding of these diets was further associated with a post-prandial decrease in serum-ionized calcium and an increase in serum phosphorus. In fasting serum samples, however, calcium was normal and phosphorus slightly depressed. Serum phosphorus was found to correlate negatively with the bone resorption rate. Increased PTH-activity in serum was reflected by a 40 to 60% decrease in urinary calcium excretion. Urinary phosphorus increased by a factor of 2 to 3, which illustrates the lack of a barrier against intestinal phosphate transport. The phosphate-supplemented dogs had a phosphorus intake of about 0.22 g/kg/day, roughly three times the amount fed to the control animals.

In contrast to the findings described above, Anderson et al. (1977) reported that diets with a low dietary Ca/P-ratio (0.30% Ca and 1.20% P, or 0,95% Ca and 2.0% P) had no significant effects on either the skeleton or plasma calcium, phospho-

rus and alkaline phosphatase in young growing and maturing Cebus monkeys. This observation might indicate that a species difference exists with respect to the response to changes in dietary calcium and phosphorus content, or that this response depends on the age of the animal.

5.10.1.2 Nephrocalcinosis

Of particular interest is the dietary induction of nephrocalcinosis. It is clear that this condition can be induced by means of diets with a high phosphorus content, particularly if these diets are semi-purified with highly available inorganic phosphates (Woodard, 1971; Goulding & Malthus, 1969; Van Beek et al., 1974). In female rats fed a semi-purified diet even 0.5% dietary phosphorus appeared to be sufficient to induce nephrocalcinosis (Hitchman et al., 1979). The significance of the calcium content of the diet in this respect remains indistinct. According to Woodard (1971), increasing dietary calcium worsens the lesions, but Hitchman et al. (1979) reported decreased severity of renal calcification in rats on an 0.5% phosphorus diet when the dietary calcium content was raised from 0.5 to 1.0%. Among rats of different strains the susceptibility to nephrocalcinosis varies, and female rats are more susceptible than male animals (Du Bruyn, 1970).

5.10.2 In man

5.10.2.1 Phosphorus requirement

Phosphorus nutrition in man has never received considerable attention, because nutritionists are generally of opinion that phosphorus is no problem as long as calcium intake is satisfactory. Phosphates are so ubiquitous in foods that the occurrence of a state of deficiency is very unlikely. An estimate of the average level of phosphorus intake, based on Dutch market figures, is 1520 mg/day, but in a sub-population of farmers the intake averaged about 2200 mg/day (El-Shaarawy, 1971). On the basis of balance studies, collected from the literature, Marshall et al. (1976) have reported that the intake is very close to the output of phosphorus down to levels of 400 mg/day. These investigators commented that balance studies below this level will be needed to define a phosphorus requirement. In the USA 800 mg is the recommended daily allowance for adults, and this seems a generous amount.

5.10.2.2 Calcium metabolism

According to Irving (1973), it might be concluded on the strength of evidence that in healthy subjects large increases in phosphorus ingestion, either as phosphoric acid or as neutral phosphate, do not affect the calcium balance or the absorption of calcium to any significant extent. This conclusion is based on the results of calcium balance studies, such as those made by Malm (1953) and Spencer et al. (1965). More recent studies by Spencer et al. (1975 and 1978b) have confirmed this conclusion.

On the other hand, dietary phosphorus affects several parameters of calcium metabolism. Thus Reiss et al. (1970) reported that an oral dose of phosphate (1 g P) to five healthy adult subjects resulted in a 60 tot 125% increase in serum PTH, initiated by a small decrease in total and ionized serum calcium. This observation is consistent with the well known phenomenon that an increase in phosphorus intake diminishes urinary calcium excretion (Malm, 1953; Spencer et al., 1965, 1975, 1978a, 1978b; Bell et al., 1977a). Bell et al. (1977a) studied the physiological response in eight students to foods containing phosphate additives. Commer-

cially available foods containing phosphate additives were substituted for natural foods. As a result of this substitution, phosphorus intake was increased from 979 mg/day during the four-week control period to 2124 mg/day during the four-week experimental period, whereas calcium intake only changed from 677 to 715 mg/day in these eight weeks. The high-phosphorus diets significantly depressed the urinary excretion of calcium (by 37% on average), and increased the urinary phosphorus excretion (by 112% on average). Also the urinary hydroxyproline excretion increased, whereas six subjects out of eight showed a larger urinary cAMP excretion. Serum calcium was found to be depressed up to three hours after the high-phosphorus evening meal, whereas no such depression was observed after the control evening meal. The depression of serum calcium was associated with an increase in the post-prandial serum-phosphorus concentration. These observations are consistent with the view, based on studies with laboratory animals, that high-phosphorus diets stimulate PTH-secretion and bone resorption.

With regard to the effect of low-phosphorus diets on calcium metabolism, it has been shown by Dominguez et al. (1976), in an investigation concerning eleven healthy adults, that phosphate depletion (phosphorus intake about 90 mg/day) is associated with an increase in urinary calcium, and in intestinal calcium absorption, and with negative calcium balances (particularly in females), hypophosphataemia (in females but not in males), depressed serum PTH-levels and increased turnover of the serum 25-HCC-pool, probably as a consequence of enhanced renal synthesis of 1,25-DHCC. In addition, urinary phosphate excretion was reduced to less than 3 mg/day.

5.10.2.3 Relation to osteoporosis

Although there is no doubt that phosphorus intake is generally far above the amount required to maintain phosphorus balance, there is no conclusive or direct evidence that a high phosphorus intake or a low dietary calcium-to-phosphorus ratio contributes to the high incidence of osteoporosis in western communities. However, in addition to the observations made by Bell et al. (1977a) and Reiss et al. (1970) (see Section 5.10.2.2), which strongly support the evidence from studies with laboratory animals that diets with either a low calcium-to-phosphorus ratio or a high phosphorus content lead to nutritional secondary hyperparathyroidism, there are several indications which link a high phosphorus intake or a low dietary Ca/P-ratio with osteoporosis in man. Thus a study of bone density in 25 elderly ovo-lacto-vegetarians, reported by Ellis & Ellis (1972), revealed that these people had significantly denser bones and lost less bone with age than did sex- and age-matched omnivorous control subjects. These differences could be due to a more favourable Ca/P-ratio in the diets of the vegetarians. Mazess & Mather (1974) measured the bone mineral content in Alaskan Eskimos and found a greater bone loss with age and an earlier start of this loss in these mainly meat-eating people, as compared with standard values in white humans. Again a low dietary Ca/P-ratio (in the Eskimo diet) may be implicated. Jowsey et al. (1972) observed in eleven osteoporotic patients a significant correlation between previous phosphorus intake and the rate of bone resorption.

The discrepancy between the fact that studies in both man and laboratory animals failed to show that high-phosphorus diets affect calcium retention and the hypothesis that dietary phosphorus is a factor in osteoporosis, can be explained by taking into consideration that the result of phosphate-stimulated bone

turnover depends on the physiological status. If a positive calcium balance exists (during growth), increased bone turnover will tend to accelerate this positive balance. On the other hand, if there is a negative calcium balance (during the aging process), increased bone turnover will also tend to accelerate this negative balance. A second factor which might explain the discrepancy mentioned above is that the degree to which bone turnover is stimulated by dietary phosphorus depends on the renal ability to handle the phosphorus load, and this ability may have a connection with age. The normal aging process in the human kidney leads to a decrease in the glomerular filtration rate and this will be associated with a decrease in the tubular reabsorption of phosphate per remaining nephron, possibly controlled by increased circulating levels of PTH. It has been found that serum PTH-levels increase with age (Riggs et al., 1978; Berlyn et al., 1974).

5.11 Conclusion

Calcium, phosphorus, vitamin D and protein are nutrients which have an important influence on the calcium and phosphate metabolism. It is well established that the influence of the first three dietary components is exerted to a large extent through the medium of parathyroid hormone (PTH). This hormone plays a major role in calcium homeostasis. As far as dietary protein is concerned, its relation with PTH is not very clear. On the basis of the available literature it is possible to compose a model which gives an outline of the pathogenesis of secondary hyperparathyroidism. In this model (see Figure 5) dietary calcium, phosphorus, the vitamin D status and the renal function are the variables which influence the secretion of PTH. In normal conditions, an equilibrium exists between serum-

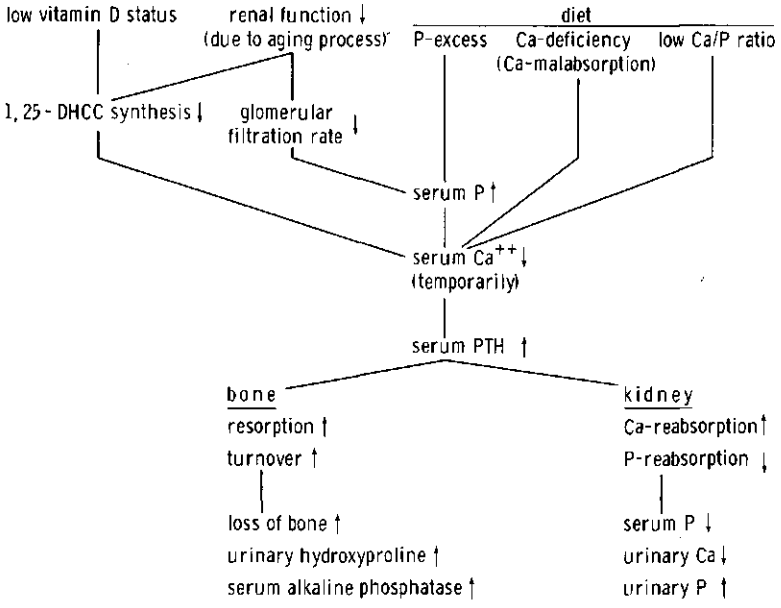

Fig. 5. Outline of the pathogenesis of secondary hyperparathyroidism.

ionized calcium and PTH. Either a low vitamin D status, diminished renal function, excess of dietary phosphorus, dietary calcium deficiency (or calcium malabsorption) or a low dietary CA/P-ratio depresses (temporarily) the serum-ionized calcium concentration. This results in the secretion of PTH being stimulated. The parathyroid hormone acts on bone and kidney, in order to restore the serum-ionized calcium concentration. In that new situation, again an equilibrium between a normal level of serum-ionized calcium and an increased amount of PTH is reached. The action of PTH on bone may proceed at the expense of skeletal calcium, so that osteoporosis develops.

5.12 Summary

Many dietary components influence the calcium and phosphate metabolism, the most important being calcium, phosphorus, vitamin D and protein.

Though adaptation to low-calcium diets is, in the presence of vitamin D, quite normal, results of epidemiological and clinical studies suggest that, at least in western societies, an ample intake of calcium (800 mg/day or more) is desirable with a view to prevention and treatment of osteoporosis.

From studies witx laboratory animals it appears that feeding high-phosphorus diets results in secondary hyperparathyroidism, calcification of soft tissues (nephrocalcinosis), and stimulation of bone resorption. The amount of phosphorus normally consumed by man largely exceeds the amount required to maintain the phosphorus balance. It is not precisely known whether a high phosphorus intake is without risks. Restriction of dietary phosphorus might be valuable in the prevention and treatment of osteoporosis.

Dietary protein increases the urinary excretion of calcium, which may lead to negative calcium balances, especially when protein intake is high and calcium intake is low.

In the evaluation of effects of dietary components on calcium metabolism the phenomenon of adaptation has to be accounted for, and in this respect a difference must be made between short-term and long-term studies. Short-term studies (duration less than a few weeks) are useful only for studying mechanisms of calcium homeostasis. Long-term studies (duration more than four weeks) are needed if information is required about the influence of dietary components on the genesis of osteoporosis.

On the basis of the available literature a model was composed which gives an outline of the pathogenesis of secondary hyperparathyroidism. According to this model, either a calcium-deficient diet (or calcium malabsorption) or a high-phosphorus diet or a diet with a low calcium-to-phosphorus ratio stimulates the secretion of parathyroid hormone (PTH), especially when the renal function has decreased and/or the vitamin D status is low. By its action on bone, PTH may cause osteoporosis.

Chapter 6

Cross-sectional study in elderly people

6.1 Introduction

On a long-term basis a low calcium intake, a high phosphorus intake or a low dietary Ca/P-ratio might cause osteoporosis in man via a mechanism which involves secondary hyperparathyroidism. This mechanism might particularly occur when renal function has declined (diminished glomerular filtration rate and diminished synthesis of 1,25-DHCC). A schematic presentation of causes and consequences of this so-called nutritional secondary hyperparathyroidism is shown in Figure 5 (Section 5.11). Given the fact that the human calcium requirement is subject to much debate and that the level of phosphorus intake in western countries is considered to be excessive, we found it necessary to investigate whether such a mechanism as described above is operative in man on his ususal diet. Since the prevalence of osteoporosis is high among the aged and since renal function is known to decrease with advancing age, we planned a study in a group of normal healthy elderly people. The aim of this study was to investigate whether the existence of relationships between dietary calcium and phosphorus on the one hand and the development of hyperparathyroidism on the other could be demonstrated.

6.2 Methods

The study was carried out between March and December 1976 in the community of Ede. Four hundred addresses of elderly people living in their own homes were kindly provided by a service centre in Ede (director Mr. S. J. H. Hartsema). Contact persons from this centre visited the elderly people at home and invited them to participate in the study on a voluntary basis. In a brochure the aim of the study and the study procedure were explained. From a response population of 25%, 89 normal healthy elderly people (53 women and 36 men) were selected according to criteria discussed below (see under 'Medical investigation'). The study procedure consisted of three parts: (1) dietary (history) survey by a dietitian; (2) medical investigation by a physician, and (3) laboratory investigation of serum and urine.

6.2.1 Dietary survey

Food consumption was estimated by a trained dietitian who used the cross-check method during a visit at home. By this method habitual rather than actual food intake is estimated. The cross-check method has been recommended as the method of choice for the estimation of individual food consumption in scientific studies in which nutrient intake levels will be correlated with physical and physiological parameters (Loo-Bunnik & Van Staveren, 1973). After the dietitian has interviewed the participant about the daily food consumption pattern, including possible variation during the weekend, and has listed the varieties and quantities of food items, the list is checked with a table of commonly consumed food

products to make sure that one or more items are not forgotten. The estimated quantities are compared with the contents of cups, spoons etc. which are used in that particular household. Finally as a cross-check the list is compared with the weekly food purchases. In spite of all checks the cross-check method has the essential limitation that it cannot be completely free of subjectivity and, since it is merely based on interview, only a rough estimate of the habitual food intake is obtained. Furthermore there is no guarantee as to whether the dietary habit is life-long or has been adopted fairly recently.

The recorded food consumption pattern was translated into nutrient intake with the aid of a computer programme, which is based on figures of the Dutch Food Composition Table (Hautvast, 1975). It was found by El-Shaarawy (1971) that figures in this table of the calcium and phosphorus content of food products correlate well with results of chemical analyses and can be used for the estimation of calcium and phosphorus intake in Dutch diets. In addition we have tested the suitability of the Dutch Food Composition Table for this purpose by the determination of calcium and phosphorus contents of 15 food items obtained from local stores. The items were selected as representative of dairy products, meat, vegetables and luxury foods. After homogenization in a mortar or a Waring Blendor a sample of 1 gram was wet-ashed according to the method of Clarkson (1967). After proper dilution with distilled water, calcium and phosphorus were estimated according to the methods of Ripoll (1976) and Griswold et al. (1951) respectively. Table 1 shows the results of these estimations as well as the corresponding figures taken from the Dutch Food Composition Table. The figures compare

Table 1. Calcium and phosphorus content (mg/100 g) of 15 food items by analysis and by the Dutch Food Composition Table[1]).

food item	analysis		DFCT	
	Ca	P	Ca	P
milk (full cream)	121	97	125	90
Gouda cheese (young)	803	510	600	350
processed cheese (full cream)	541	767	450	800
pork (lean)	45	212	10	200
beef (lean)	51	175	10	200
minced meat (beef)	24	152	6	125
liver (cooked)	17	449	10	225
ham	12	308	10	300
potato	10	38	10	60
onion	47	36	30	40
endive	90	44	20	70
chicory	20	27	20	20
cola drink	13	17	5	20
salted peanuts	58	364	60	350
salted potato crisps	51	103	25	150

[1]) The Spearman rank-order correlation coefficients (rho) between values obtained from analyses and those obtained from the Dutch Food Composition Table are 0.82 for calcium and 0.98 for phosphorus.

favourably. The Spearman rank-order correlation coefficient was found to be good for calcium (rho = 0.82) and excellent for phosphorus (rho = 0.98).

Physical activity was estimated by interview and classified according to the five-point scale as defined in the Dutch Food Composition Table (Edition 1978).

6.2.2 Medical investigation

All participants visited the physician, to judge whether they could be regarded as 'apparently healthy'. Special attention was given to medical history, prescription of medicines and to complaints related to health. The occasional use of medicines and/or vitamin supplements was considered to be normal. Persons who used blood pressure-lowering drugs or diuretics were not excluded from participation. Blood pressure (systolic and diastolic) was measured in the sitting position, body height (to the nearest mm) in upright position and body weight (to the nearest 100 g) with indoor clothing but without shoes. The ratio W/H^2 (Quetelet index) was used as an index of obesity: W is the measured body weight (in kg) reduced by 2 and 3 kg for women and men respectively (to account for clothes) and H is the standing height (in metres).

For the measurement of the skeletal mass the non-invasive sensitive technique of photon-absorptiometry was chosen. With the Norland-Cameron Bone Mineral Analyzer (BMA type 178) the bone mineral content (BMC) and bone width (BW) at the distal third of the radius were measured. With regard to the radiation dose, a set of 4 scans is computed to be 1/100 of the dose delivered to a patient for a radiograph of the forearm if radiation area and exposure time are taken into account (communications from Norland Instruments). The principle of this method is as follows. A low-energy gamma-beam from a collimated 200 mCi ^{125}I source and a NaI-detector are moved by an electromotor across the arm with a speed of 2 mm/sec. The translated energy, not absorbed by the radius, is detected and a built-in computer module computes the BMC (in g/cm) and the BW (in cm). Both values are read out digitally. During the measurement the lower arm is immobilized by a limb-holder and, at the site of the scan, surrounded by a tissue bag equivalent (a rubber bag filled with distilled water). This bag has similar characteristics to those of soft tissue with respect to photon-absorption and makes sure that the bone is equally surrounded by 'soft tissue' so that a straight base line is obtained. The BMA is calibrated before each series of measurements against an aluminium phantom with a mineral content similar to that of an average radius. The radius was measured as the second bone (after the ulna), which has the advantage that the base line is set between ulna and radius where only a small amount of adipose tissue is present. Adipose tissue has a lower photon-absorption coefficient than lean soft tissue and may disturb the measurement. The scan site was at the point 1/3 the distance from the distal end of the radius (the distal third). Here the region is quite uniform and repositioning has little effect on the measurement. Each participant was measured 4 times in one session and the mean value was computed. The precision (reproducibility) of the method has been reported to be between 2 and 3% and the accuracy is about 5% (Mazess et al., 1964; Smith et al., 1972). In our hands the variation coefficient between measurements in one session was well below 2% for both BMC and BW. In normal subjects BMC-values of the radius at the distal third correlate well ($r = 0.973$) with values of total body calcium as measured by neutron activation analysis. In osteoporotic patients this correlation is somewhat lower ($r = 0.826$; Cohn et al., 1974). The ratio BMC/BW has been used in many studies to correct BMC-values

for differences in skeletal size. Normal values for BMC, BW and BMC/BW depend on sex, age and race. A BMC-value for females less than 0.68 g/cm is suspect of clinical osteoporosis*).

6.2.3 Laboratory investigation

Haemoglobin, haematocrit, mean cell haemoglobulin concentration (MCHC), pH and bicarbonate in blood, and urea, creatinine, cholesterol, uric acid, alkaline phosphatase, acid phosphatase and proteins in serum were estimated as a check of the general status of health. Inorganic phosphorus, ionized calcium, bone alkaline phosphatase, PTH and 25-HCC in serum and calcium, phosphorus and total hydroxyproline in urine were estimated as indicators of the calcium and bone metabolism.

Serum was prepared within one to two hours after venapuncture (blood was drawn during the medical investigation) by centrifuging the clot at room temperature for 20 minutes at 2000 g. Serum was frozen (-18°C) or refrigerated (4°C, less than 4 days), until analyzed. The phosphatases, urea, creatinine and uric acid were estimated in fresh or refrigerated samples.

Participants were instructed in detail (verbal and on paper) to collect a complete 24-hour urine sample including the morning urine of the day on which the blood was drawn. The procedure for this collection was according to Henry et al. (1974). Urine volume was measured and the urine was checked for presence of glucose, protein, nitrite, ketones, bilirubin, blood and urobilinogen by the Combur-8-test (Boehringer, Mannheim). Urine was frozen (-18°C) until analyzed.

Blood pH, bicarbonate (Astrup-apparatus) and haematocrit were estimated by standard procedures (Henry et al., 1974). The determination of haemoglobin was according to the cyan methaemiglobin method (reagent test combination, Boehringer, Mannheim). MCHC values were computed from the ratio between blood haemoglobin and haematocrit.

Serum proteins (albumin, alpha 1, alpha 2, beta and gamma globulins were separated by cellulose acetate electrophoresis (Helena Laboratories, Beaumont, Texas). Bands were stained with Ponceau-S dye and measured densitometrically in transmission with a Shimadzu CS-900 dual wavelength densitometer at 525 nm. The reference beam was set at 635 nm. The scans were inspected visually for abnormalities. The serum albumin concentration was computed by multiplying the relative area of the albumin peak by the serum total-protein concentration. The inter-assay variation coefficient of the relative area of the albumin peak in an electropherogram of proteins in a control serum was found to be 5.1%.

Total cholesterol, uric acid and urea in serum were estimated enzymatically, creatinine in serum and urine with the Jaffé reaction (in serum after precipitation of the proteins with 2N trichloroacetic acid), acid and alkaline phosphatase with para-nitrophenol-phosphate as a substrate and serum total protein with the Biuret reaction. All these methods were carried out using commercial reagent kits (Boehringer, Mannheim).

Alkaline phosphatase isoenzymes (liver, bone and intestine) were separated by polyacrylamide gel electrophoresis (Ames, division Miles, Indiana). Bands were visualized by incubation of gels in a solution containing 5-bromo-4-chloro-3-indolyl-phosphate, a substrate which is specific for alkaline phosphatase. Bands

*) Smith & Cameron, Interpretation of Fracture Index Charts, University of Wisconsin, Bone Mineral Laboratory Communication from Norland Instruments.

were measured densitometrically in transmission with the sample beam set at 620 nm and the reference beam at 550 nm. The activity of the bone isoenzyme was computed by multiplying the relative area of the bone isoenzyme peak by the total alkaline phosphatase activity in the serum. The inter-assay variation coefficient of the relative area of the bone isoenzyme peak of a control serum was 4.5%

Calcium in serum and urine was determined by atomic absorption. Serum-ionized calcium was computed using a modification of the formula of Zeissler as proposed by Pottgen & Davis (1976). This formula accounts for serum concentrations of albumin and globulin. Phosphorus in serum and urine was estimated by a molybdenum blue method according to Griswold et al. (1951). Urinary total hydroxyproline was estimated according to the method of Kivirikko et al. (1967).

Within each of the above-mentioned series of determinations a control serum or urine sample was run and the inter-assay variation coefficients were found to be below 6%.

PTH in serum was measured by radio-immunoassay by Dr. R. Bosch (University of Utrecht). Results are expressed in μEq/l PTH-135, a bovine standard. The antiserum used is specific to carboxyterminal PTH-fragments and was developed by Dr. R. Bouillon (University of Leuven, Belgium). The inter-assay variation coefficient of this assay is 8%. 25-hydroxycholecalciferol (25-HCC) was measured by a competitive protein-binding assay by Dr. J. H. Schade (CIVO-TNO, Zeist) according to a modified method of Edelstein et al. (1974). The inter-assay variation coefficient of this assay is 10%.

As, in spite of detailed instructions, errors in collection time of urine could be expected, urinary calcium, phosphorus and hydroxyproline were expressed as their ratios to creatinine which are much less dependent on the accuracy of the collection period.

6.2.4 Statistics

To investigate correlations between biochemical, physical and dietary parameters, data were classified according to nutrient intake levels, age groups and bone mineral content values. Although this method has the disadvantage that some information is lost because individual data are pooled, it was given preference over simple linear regression as a primary evaluation since grouping of data provides readable tables. Students t-test was used to test the significance of differences between group means. If variances differed significantly ($P \leqslant 0.05$), as shown by the F-test, the modified t-test of Cochran & Cox was used (Downie & Heath, 1965). As a secondary evaluation of data correlational techniques (multiple-linear or simple-linear regression analysis) were applied (Snedecor & Cochran, 1973). A Hewlett Packard model 9830A table computer and a Hewlett Packard model 9866A printer were used for the processing of the data. Three levels of significance were applied: $P \leqslant 0.01$; $P \leqslant 0.05$; $P \leqslant 0.10$, one-tailed, unless otherwise indicated. The latter level was used as an indicator of a trend effect.

6.3 Results

6.3.1 Basic data in males and females (Tables 2 to 5)

The medical investigation revealed complaints related to old age (sleeplessness and obstipation), histories of gastric resection (1 woman), hysterectomy (5 women), ovariectomy (1 woman) and of mastectomy (1 woman). Moreover 1 woman had achlorhydria and 2 women and 1 man were non-insulin-dependent diabetics. Diuretics were used by 13 women and 2 men. No significant abnormalities were

found in urine by the Combur-8-test. These data together with those of Tables 2 to 5 indicate that we deal with a rather healthy group of elderly people.

Table 2 shows data on age and somatometry and reference values (normative data) of U.S. whites, reported by Mazess & Cameron (1973). The mean value for the Quetelet index for females was high. In 23 women the index exceeded the value 27.0 which indicates 20% overweight or more for women with a normal frame (James et al., 1976). Mean values for BMC, BW and BMC/BW and their standard deviations compare with the U.S. normative data. Our values for BW are slightly higher and those for BMC/BW slightly lower than these normative values. In the study of Van Paassen (1979) the mean values for BMC, BW and BMC/BW of 44 postmenopausal women in Utrecht (mean age 54 years) were 0.87 g/cm, 1.31 cm and 0.65 g/cm² respectively. If the line is taken that postmenopausal bone loss proceeds with an annual rate of 1%, than these values compare favourably with those of the present study. An osteopenic BMC-value (BMC $<$ 0.68 g/cm) was found in 9 women (18%).

Table 2. Age and somatometric data.

		females (n = 53)			males (n = 36)			reference values (± sd)	
		mean	sd	range	mean	sd	range	♀	♂
age	(years)	69.9	5.5	57 – 89	70.4	4.7	64 – 82	60 – 70	60 –
weight	(kg)	70.2	10.4	51 – 107	75.6	10.9	56 – 101	62.8 ± 11.3	75.9 ± 1
height	(cm)	159.6	6.3	146 – 173	171.8	5.7	162 – 184	159.1 ± 6.9	175.2 ±
Quetelet index	(kg/m²)	26.7	3.3	19.6 – 38.9	24.6	3.2	16.6 – 31.9	24.8	24.7
BMC	(g/cm)²)	0.78	0.12	0.53 – 1.06	1.19	0.15	0.88 – 1.50	0.77 ± 0.14	1.23 ± 0
BW	(cm)²)	1.39	0.14	1.02 – 1.71	1.63	0.14	1.26 – 1.92	1.26 ± 0.13	1.57 ± 0
BMC/BW	(g/cm²)²)	0.56	0.07	0.39 – 0.69	0.73	0.08	0.55 – 0.85	0.61 ± 0.09	0.79 ± 0

[1]) Reference values are normative data of U.S. whites, reported by Mazess & Cameron (1973); reference values for U.S. whites in the age group of 70 - 80 years are very similar, with the exceptio of BMC- and BMC/BW-values for females, which are 0.72 ± 0.13 g/cm and 0.58 ± 0.09 g/cm² respectively.

[2]) Measured on 51 females and 35 males.

Table 3 shows the data on blood pressure and biochemical parameters in blood (plasma). No participants had a diastolic blood pressure exceeding 110 mm Hg. In 5 women and 1 man systolic blood pressure exceeded 170 mm Hg. Mean values for both systolic and diastolic blood pressure are quite acceptable for this age group (Kitchin & Julian, 1971). Anaemia did not occur in women whereas only 3 men had haemoglobin values below 14 g/100 ml. In 2 of them haematocrit was below 40% but their MCHC was normal. In 6 women and 2 men plasma bicarbonate was below 21 mEq/l, but their blood pH was normal. In 1 woman and 2 men blood pH was slightly below 7.35 but their plasma bicarbonate was normal.

Table 4 shows the biochemical parameters in serum and urine. Mean values for females and males are well within the range of normality, although for several parameters the observed ranges are broader. As far as calcium and bone metabolism are concerned, the following comments can be made. The bone fraction of serum alkaline phosphatase (APB) was moderately increased (activity $>$ 24 U/l) in 5 women and 1 man. Mean serum calcium values are rather low. For

instance, van Paassen (1979) reported a mean value of 10.3 mg/100 ml in postmenopausal women. The cause of this difference might be of methodological origin. The mean value of serum phosphorus is significantly lower ($P < 0.01$) in males than in females; 6 men, but no women, had values below 2.5 mg/100 ml. In the study by van Paassen a lower mean value (3.16 $\pm$ 0.47 mg/100 ml) for serum phosphorus was found in postmenopausal women who were on average 15 years younger. These observations are consistent with the sex difference in serum phosphorus and with an increase in serum phosphorus with advancing age (Section 4.2). Serum 25-HCC was subnormal ($<$12 ng/ml) in 5 women and 1 man. In 16 women (31%) and 8 men (22%) PTH exceeded the upper limit of normality, and this is consistent with an increase in serum PTH with age (Section 4.2). Hypercalciuria occurred in 2 women. No excessive values were found for the urinary excretion of hydroxyproline. Regarding renal function, mean values for serum creatinine were at the upper limit of normality and in 17 subjects (20%) serum urea values exceeded this limit. These findings are consistent with a slightly diminished renal function in elderly people.

Table 5 shows data on dietary parameters and physical activity. If a comparison is made with recommendations by the Dutch Nutrition Board (see the Dutch Food Composition Table, edition 1978), the following comments can be made. Energy intake is in conformance with the recommendation. The intake level of protein exceeds the amounts recommended for women and men with 13 and 15 g/24 h respectively. Fat intake is within the recommended range but the contribution of the saturated fat to the total fat intake is relatively high. Calcium intake largely exceeds the recommendation of 0.8 g/24 h. Nevertheless 13 women (25%) and 6 men (17%) had an intake below that level. In spite of an ample intake of phosphate (25% of the women and men had an intake exceeding 1.6 and 2.0 g phosphorus daily respectively) the dietary Ca/P-ratio was more favourable than that of the general Dutch population (0.76 and 0.71 versus 0.58, Bosman & Kosten-Zoethout, 1978). The mean intake of vitamin D is well above the level of 100 IU/24 h below which osteomalacia has been reported to occur in moderate climates (Dent & Smith, 1968). Physical activity of the women was low and this might relate to the high frequency of obesity among them.

6.3.2 Evaluation of the data in females (Tables 6 to 12)

6.3.2.1 Factors associated with the bone mineral content

Table 6 shows the results of grouping data according to different BMC-values. With the exception of the value of 0.68 g/cm (osteopenic BMC) these values were chosen arbitrarily. If the means of somatometric, biochemical and nutritional parameters in group I are compared with those in group III, significant differences can be observed with respect to age, height, weight, the Quetelet index, BW, BMC/BW, serum phosphorus, serum 25-HCC and dietary protein and phosphorus. The positive correlations between BMC on the one hand and BW, BMC/BW, height and weight on the other are related to either differences in skeletal size or to the loss of bone and height with advancing age. The latter is indicated by the high mean age in subgroup I (osteopenic BMC). Since the oestrogen oestrone is produced in adipose tissue (Vermeulen & Verdonck, 1978), the high-value of the Quetelet index in the subgroup with the highest BMC-value might reflect the protective effect of oestrogen against postmenopausal bone loss. The high serum phosphorus concentration in subgroup I (low BMC-value) might reflect a loss of renal function as a consequence of the aging process. The positive correlation

Table 3. Blood pressure and biochemical parameters in blood.

		females (n = 53)			males (n = 36)			normal range[2])	
		mean	sd	range	mean	sd	range	♀	♂
blood pressure[1])	(mm Hg)								
systolic		150	16	100 – 185	147	20	113 – 200	< 170	< 170
diastolic		88	10	70 – 108	86	9	68 – 110	< 110	< 110
haemoglobin	(g/100 ml)	14.2	1.0	12.6 – 17.7	15.7	1.5	11.6 – 18.5	12 – 16	14 – 18
haematocrit	(%)	41.5	2.6	36 – 47	45.6	3.7	33 – 52	36 – 47	40 – 54
MCHC	(g/100 ml)	34.4	2.5	30.2 – 43.2	34.8	3.1	31.5 – 46.8	30 – 36	30 – 36
bicarbonate	(mEq/l)	23.6	2.4	15.8 – 27.6	23.5	2.0	17.4 – 27.5	21 – 25	21 – 25
PH		7.38	0.03	7.31 – 7.44	7.38	0.03	7.31 – 7.44	7.35 – 7.45	7.35 – 7.45

[1]) Measured on 51 females and 32 males.
[2]) Derived from Henry et al. (1974) and from Passmore & Robson (1971).

Table 4. Biochemical parameters in serum and urine.

		females (n = 53)			males (n = 36)			normal range[4])	
		mean	sd	range	mean	sd	range	♀	♂
serum:[1])									
AcP	(U/l)	8.6	2.4	4.1 – 15.2	8.6	2.3	2.4 – 14.0	< 11	< 11
AP	(U/l)	38.4	12.8	12.2 – 81.4	38.6	10.1	22.8 – 70.0	20 – 48	20 – 48
APB	(U/l)	14.8	5.8	4.0 – 28.2	13.9	5.2	7.7 – 30.1	[5])	[5])
Ca	(mg/100 ml)	9.1	0.7	7.3 – 10.4	9.1	0.7	7.1 – 10.6	9.2 – 11	9.2 – 11
Ca^{++}	(mg/100 ml)	4.48	0.43	3.7 – 5.6	4.42[2])	0.36	3.4 – 5.0	4.84 – 5.56	4.84 – 5.56
TP	(g/l)	69.8	5.3	61.0 – 83.0	69.5	3.8	61.1 – 78.0	66 – 87	66 – 87
Alb	(g/l)	45.1	4.8	34.1 – 58.5	46.3	4.6	39.0 – 51.9	[6])	[6])
P	(mg/100 ml)	3.46	0.46	2.5 – 4.4	2.99	0.55	1.8 – 4.1	2.5 – 4.8	2.5 – 4.8
creat	(mg/100 ml)	1.02	0.17	0.71 – 1.53	1.13	0.14	0.85 – 1.44	0.5 – 0.9	0.6 – 1.1
urea	(mg/100 ml)	39.7	10.3	19.4 – 60.0	43.8	13.5	21.6 – 90.0	10 – 50	10 – 50
uric acid	(mg/100 ml)	5.0	1.2	2.5 – 8.7	5.5	1.0	3.5 – 7.7	2.4 – 5.7	3.4 – 7.0
chol	(mg/100 ml)	244	41	151 – 350	218	37	155 – 290	177 – 340	177 – 340
PTH	(μ Eq/l)	0.36[3])	0.18	0.02 – 1.12	0.33	0.14	0.02 – 0.66	0.02 – 0.40	0.02 – 0.40
25-HCC	(ng/ml)	21.6	7.7	7.0 – 38.4	23.1	6.1	6.6 – 35.0	12 – 40	12 – 40
urine:[1])									
Ca/cr	(mg/g)	141	80	18 – 445	118	56	25 – 295	30 – 280	30 – 280

[2]) n = 35, one value was not available.
[3]) n = 52, one value was not available.
[4]) Derived from Henry et al. (1974) or from Boehringer, Mannheim specifications.
[5]) Normal activity = less than 67% of AP-activity (Ames, Indiana).
[6]) Normal concentration = 56.8 - 76.4% of TP-concentration (Henry et al. 1974).
[7]) Depends on dietary phosphorus.
[8]) Normal range not available.

Table 5. Dietary parameters and physical activity.

		females (n = 53)			males (n = 35)			recommended[2])	
		mean	sd	range	mean	sd	range	♀	♂
energy	(MJ/24 h)	7.9	1.7	3.8 — 11.5	11.0	2.5	6.7 — 14.4	7.6	10.5
protein	(g/24 h)	68	17	37 — 117	85	19	56 — 140	55	70
	energy %)	14.6			12.9				
carbohydrates	(g/24 h)	211	60	99 — 351	306	81	180 — 509		
	(energy %)	45.0			56.6				
fat	(g/24 h)	80	22	36 — 127	110	35	35 — 215		
	(energy %)	38.3			37.5			30 — 40	30 — 40
saturated	(%)	46.3			42.2			33	33
mono-unsaturated	(%)	35.8			34.0			33	33
poly-unsaturated	(%)	17.9			17.5			33	33
Ca	(g/24 h)	1.04	0.33	0.52 — 1.97	1.22	0.40	0.58 — 2.22	0.8	0.8
P	(g/24 h)	1.37	0.37	0.77 — 2.49	1.70	0.38	1.10 — 2.71		
Ca/P		0.76	0.13	0.49 — 1.06	0.71	0.15	0.45 — 1.12		
vit. D	(IU/24 h)	169[1])	81	22 — 473	243	138	22 — 729		
physical activity[3])		1.5	0.9	0 — 3	2.7	1.1	1 — 5		

[1]) one woman who used vit. D supplements (1200 IU/24 h) was excluded.
[2]) Derived from the Dutch Food Composition Table, edition 1978.
[3]) Physical activity was graded as follows: predominantly sitting or indoor walking:0 — 1;
strenuous work for one to two hours a day: 2 — 3;
strenuous work for more than two hours a day: 4 — 5.

Table 6. BMC in relation to age and somatometric, biochemal and nutritional parameters females.

		BMC (g/cm)			t-tes I-III
		I	II	III	P-val
		< 0.68	0.68-0.85	> 0.85	
mean		0.61 (n = 9)	0.77 (n = 27)	0.91 (n = 15)	
somatometry:					
age	(years)	74.6 ± 6.1	69.5 ± 5.3	67.7 ± 4.5	< 0.
height	(cm)	155.6 ± 7.8	158.7 ± 5.3	163.0 ± 5.7	< 0.
weight	(kg)	65.6 ± 9.7	66.3 ± 6.9	77.6 ± 8.1	< 0.
Quetelet index	(kg/m²)	26.2 ± 3.1	25.6 ± 2.6	28.4 ± 2.3	< 0.
BW	(cm)	1.22 ± 0.12	1.42 ± 0.12	1.44 ± 0.11	< 0.
BMC/BW	(g/cm²)	0.51 ± 0.06	0.54 ± 0.05	0.64 ± 0.03	< 0.
serum:[5])					
Ca^{++}	(mg/100 ml)	4.42 ± 0.39	4.49 ± 0.40	4.45 ± 0.50	r
P	(mg/100 ml)	3.82 ± 0.21	3.39 ± 0.52	3.36 ± 0.38	< 0.
25-HCC	(ng/ml)	18.9 ± 7.5	21.3 ± 8.3	24.1 ± 7.1	= 0.
PTH	(μ Eq/l)	0.35 ± 0.18	0.32 ± 0.12[1])	0.36 ± 0.15[2])	r
creat	(mg/100 ml)	1.06 ± 0.16	1.01 ± 0.18	0.99 ± 0.13	r
APB	(U/l)	12.9 ± 4.3	16.1 ± 6.1	13.8 ± 6.0	r
urine:[5])					
Ca/cr	(mg/g)	130 ± 76[3])	132 ± 63[3])	127 ± 57	r
P/cr	(mg/g)	708 ± 188	764 ± 322	691 ± 201	r
hypro/cr	(mg/g)	31.7 ± 11.2	26.5 ± 9.8	28.5 ± 8.9	r
diet:					
energy	(MJ/24 h)	6.8 ± 1.7	8.5 ± 1.7	7.4 ± 1.5	r
protein	(g/24 h)	58 ± 11	70 ± 18	70 ± 12	< 0.
	(energy %)	14.3	13.8	15.9	
Ca	(g/24 h)	0.91 ± 0.13	1.04 ± 0.35	1.07 ± 0.38	r
	(g/MJ)	0.13	0.12	0.15	
P	(g/24 h)	1.16 ± 0.14	1.40 ± 0.40	1.41 ± 0.36	< 0.
	(g/MJ)	0.17	0.17	0.19	
Ca/P		0.79 ± 0.17	0.74 ± 0.12	0.75 ± 0.12	r
vit. D	(IU/24 h)	121 ± 71	198 ± 85[4])	150 ± 64	r
physical activity[6])		1.0 ± 0.9	1.9 ± 1.0	1.3 ± 0.6	r

[1]) One value not available
[2]) With exclusion of one abnormal value (1.12 μEq/l).
[3]) With exclusion of one (hypercalciuric) value.
[4]) With exclusion of one woman who used vit. D supplements.
[5]) For abbreviations see subscript to Table 4.
[6]) See subscript to Table 5.

between serum 25-HCC and BMC indicates that the vitamin D status is a factor associated with BMC. The lower intake of protein and phosphorus in subgroup I can be attributed mainly to a lower intake of food.

6.3.2.2 Factors associated with age

Since age is an important variable with regard to bone mass and calcium metabolism (Section 4.2), differences in age have to be taken into account if the effect of diet on calcium metabolism and bone mass is studied. Table 7 shows the results of grouping data according to different age categories. From this table it can be seen that body height, BMC, BMC/BW, and the urinary excretion of calcium decrease with age. Correlation and regression coefficients are shown in Table 8. The

average loss of height with age of 0.35 cm per annum exceeds that of 0.2 cm reported by Mazess & Cameron (1973) for US females after the thirties. Our value for the loss of height with age may be an overestimation since it does not take into account a possible secular trend. The loss of bone with age is 0,8% per annum as computed from the ratio between the regression of BMC/BM on age and the BMC/BM value of Table 2. This rate of loss harmonizes with the literature on the rate of postmenopausal bone loss (Smith et al., 1976). The decrease in the urinary excretion of calcium with age (about 3.5% per annum) occurred without any decrease in calcium intake and may therefore be attributed to a decrease in the intestinal calcium absorption. There was a positive correlation between serum-ionized calcium and age. This is in harmony with observations by Dequeker (1973), who reported an increase in serum calcium in females after the age of about 50 years. This increase might be attributed to an increase in the responsiveness of bone to the calcium-mobilizing action of PTH as a consequence of oestrogen deficiency. The increase in the dietary Ca/P-ratio with age is of interest since it might obscure a positive effect of this ratio on bone.

6.3.2.3 Effect of calcium and phosphorus intake on bone parameters

From Table 6 it cannot be concluded that BMC-values are related to dietary parameters. However, since Tables 6, 7 and 8 indicate that age and skeletal size (height and weight) correlate with bone parameters and that the dietary Ca/P-ratio increases with age, it was found necessary to account for differences in age and skeletal size in the possible relation between diet and bone parameters. To account for differences in skeletal size the parameter body height $\times$ body weight $^{1}/^{2}$ was used. It appeared from linear regression analysis that this parameter gave the same correlation coefficients with the bone parameters as did linear combinations of height and weight.

Table 9 shows a matrix of correlation coefficients between age, somatometric and nutritional parameters. It can be seen that $H.W^{1}/^{2}$ correlates significantly positive with BMC, BW and BMC/BW. The latter correlation shows that the ratio BMC/BW does not completely correct BMC-values for differences in skeletal size. The negative correlation between $H.W^{1}/^{2}$ and the Ca/P-ratio is probably related to the observation that the older females, who have a smaller stature, have a higher dietary Ca/P-ratio. The positive correlation between dietary phosphorus and BW as well as the negative correlation between dietary Ca/P and this bone parameter will be referred to below. The correlations between dietary calcium, phosphorus and the Ca/P-ratio are explained by the fact that dairy products, the main source of dietary calcium, contain much phosphorus and have a high dietary Ca/P-ratio.

Table 10 shows partial regression coefficients of multiple linear regression of bone parameters (dependent variables) on age, $H.W^{1}/^{2}$, Ca, P and Ca/P as independent variables. It can be seen that the inclusion of either Ca, P or both or the inclusion of Ca/P in the regression of BMC on age and $H.W^{1}/^{2}$ does not improve the multiple correlation coefficient. In addition, partial regression coefficients (b_3, b_4 and b_5) are not significant. The same procedure applied to BW resulted in a significant partial regression coefficient for dietary P and in an improvement of the multiple correlation coefficient. With reference to BMC/BW there was an increase in the multiple correlation coefficient if both Ca and P were included in the regression equation, whereas partial regression coefficients (b_3 and b_4) for Ca and P were significantly positive and negative respectively. These

Table 7. Age in relation to somatometric, biochemical and nutritional parameters in females

		age				t-test
		I	II	III	IV	I-
		< 66 yr	66-71 yr	71-76 yr	> 76 yr	III + IV
		62.6 (n = 11)	68.0 (n = 18)	73.5 (n = 18)	78.7 (n = 6)	P-value
Somatometry:						
height	(cm)	161.3 ± 4.9	161.1 ± 4.9	157.3 ± 7.6	156.7 ± 8.0	< 0.1
BMC	(g/cm)	0.88 ± 0.10	0.80 ± 0.09[1])	0.72 ± 0.11[1])	0.73 ± 0.10	< 0.0
BW	(cm)	1.42 ± 0.12	1.44 ± 0.13[1])	1.34 ± 0.14[1])	1.34 ± 0.17	< 0.1
BMC/BW	(g/cm²)	0.62 ± 0.05	0.56 ± 0.08[1])	0.54 ± 0.07[1])	0.54 ± 0.04	< 0.0
serum:[5])						
Ca^{++}	(mg/100 ml)	4.26 ± 0.29	4.50 ± 0.50	4.52 ± 0.43	4.73 ± 0.27	< 0.0
P	(mg/100 ml)	3.43 ± 0.42	3.38 ± 0.48	3.47 ± 0.56	3.55 ± 0.33	n
25-HCC	(ng/ml)	21.7 ± 5.8	25.9 ± 7.2	19.6 ± 7.8	14.7 ± 5.5	n
PTH	(μEq/l)	0.38 ± 0.16	0.29 ± 0.11[2])	0.34 ± 0.17	0.44 ± 0.11	n
creat	(mg/100 ml)	1.00 ± 0.15	1.02 ± 0.15	1.02 ± 0.21	1.07 ± 0.20	n
APB	(U/l)	16.9 ± 6.5	14.6 ± 6.0	13.5 ± 5.5	15.1 ± 2.1[3])	n
urine:[5])						
Ca/cr	(mg/g)	161 ± 69	140 ± 60	116 ± 57[3])	85 ± 43[4])	< 0.0
P/cr	(mg/g)	715 ± 258	757 ± 294	701 ± 274	742 ± 237	n
hypro/cr	(mg/g)	27.8 ± 7.5	26.6 ± 10.2	29.0 ± 11.0	25.3 ± 9.3	n
diet:						
energy	(MJ/24 h)	7.1 ± 1.8	8.3 ± 1.6	7.7 ± 1.9	8.3 ± 1.0	n
protein	(g/24 h)	70 ± 17	71 ± 14	64 ± 18	68 ± 18	n
Ca	(g/24 h)	0.93 ± 0.30	1.08 ± 0.39	1.06 ± 0.31	1.08 ± 0.31	n
P	(g/24 h)	1.32 ± 0.35	1.44 ± 0.36	1.33 ± 0.37	1.35 ± 0.43	n
Ca/P		0.70 ± 0.11	0.74 ± 0.14	0.81 ± 0.12	0.81 ± 0.06	< 0.0
vit. D	(IU/24 h)	170 ± 56	177 ± 63	152 ± 79	185 ± 81	n
Physical activity[6]		1.4 ± 0.7	1.6 ± 0.9	1.4 ± 1.0	2.0 ± 0.9	n

[1]) n = 17.
[2]) with exclusion of one abnormal value (1.12 μEq/l).
[3]) n = 4.
[4]) With exclusion of one (hypercalciuric) value.
[5]) For abbreviations see subscript to Table 4.
[6]) See subscript to Table 5.

Table 8. Linear regression of somatometric, biochemical and nutritional parameters with age ($y = b_0 + b_1 x$) in females (n = 51).

y		b_0	b_1	r	P (t-test)
BMC	(g/cm)	1.54	− 0.0110	− 0.53	< 0.01
BMC/BW	(g/cm²)	0.86	− 0.0043	− 0.35	< 0.01
height	(cm)	184	− 0.350	− 0.31	< 0.01
serum Ca	(mg/100 ml)	3.19	0.0186	0.24	< 0.05
urinary Ca/cr	(mg/g)	444	− 4.49	− 0.40	< 0.01
dietary	Ca/P	0.23	0.0076	0.33	< 0.01

Table 9. Matrix of correlation coefficients between age, somatometric and nutritional parameters in females (n = 51).

	age	*H.W.$^{1/2}$*	dietary *Ca*	dietary P	dietary *Ca/P*
age	1.00				
H.W.$^{1/2}$	− 0.23**	1.00			
dietary Ca	0.13	0.01	1.00		
dietary P	− 0.01	0.16	0.88***	1.00	
dietary Ca/P	0.34***	− 0.32**	0.49***	0.03	1.00
BMC	− 0.53***	0.60***	0.06	0.15	− 0.20
BW	− 0.33***	0.50***	0.06	0.24**	− 0.28**
BMC/BW	− 0.34***	0.32**	0.03	0.00	− 0.03

** = $P \leqslant 0.05$; *** = $P \leqslant 0.01$.

Table 10. Partial regression coefficients (± SEM) of multiple linear regression ($y = b_0 + b_1x_1 + b_2x_2 + \ldots b_nx_n$) of bone parameters (y) on age (x_1), H.W.$^{1/2}$(x_2), Ca (x_3), P(x_4) and Ca/P (x_5) in females (n = 51).

y	regression coefficients b_0	b_1 (age)	b_2 (H.W.$^{1/2}$)	b_3 (Ca)	b_4 (P)	b_5 (Ca/P)	multiple r	P
BMC (mg/cm)	765	− 8.4*** ± 2.1	45.2*** ± 9.3	−	−	−	0.72	< 0.01
	753	− 8.7*** ± 2.1	44.8*** ± 9.3	38.6 ± 34.6	−	−	0.73	< 0.01
	751	− 8.4*** ± 2.1	44.2*** ± 9.5	−	21.4 ± 32.0	−	0.72	< 0.01
	771	− 9.0*** ± 2.2	46.7*** ± 9.7	88.3 ± 77.6	− 51.2 ± 71.4	−	0.73	< 0.01
	691	− 9.0*** ± 2.2	48.1*** ± 9.6	−	−	111 ± 101	0.73	< 0.01
BW (cm 10^{-2})	115	− 0.57** ± 0.31	4.8*** ± 1.4	−	−	−	0.55	< 0.01
	113	− 0.60** ± 0.31	4.8*** ± 1.4	3.7 ± 5.0	−	−	0.55	< 0.01
	110	− 0.58** ± 0.30	4.5*** ± 1.3	−	6.6* ± 4.6	−	0.57	< 0.01
	107	− 0.48** ± 0.31	4.1*** ± 1.4	− 13.6 ± 11.0	17.8** ± 10.1	−	0.59	< 0.01
	120	− 0.52** ± 0.33	4.6*** ± 1.4	−	−	8.2 ± 14.7	0.55	< 0.01
BMC/BW (mg/cm^2)	631	− 3.5*** ± 1.7	13.5** ± 7.3	−	−	−	0.42	<0.01
	626	− 3.6*** ± 1.7	13.4** ± 7.4	13.2 ± 27.6	−	−	0.42	< 0.01
	636	− 3.5*** ± 1.7	14.0** ± 7.5	−	− 8.9 ± 25.4	−	0.42	< 0.01
	660	− 4.3*** ± 1.7	17.0*** ± 7.5	108.7** ± 60.2	−98.2** ± 55.3	−	0.48	< 0.01
	565	− 4.1*** ± 1.7	16.1*** ± 7.6	−	−	98.2 ± 79.2	0.45	< 0.01

* = $P \leqslant 0.10$; ** = $P \leqslant 0.05$; *** = $P \leqslant 0.01$.

observations indicate that dietary calcium has a positive effect on BMC/BW if differences in age, skeletal size and P-intake are taken into account and that dietary phosphorus has a negative effect on BMC/BW if differences in age, skeletal size and calcium intake are taken into account.

The dietary Ca/P-ratio did not give a significant partial correlation with any of the bone parameters. This might be explained by the fact that information is not fully utilized when, instead of calcium and phosphorus separately, the ratio between them is taken.

6.3.2.4 Effect of calcium and phosphorus intake on biochemical parameters

Table 11 shows the results of classification of biochemical data into groups according to intake levels of Ca, P and Ca/P. These levels (low, medium and high) were chosen arbitrarily. Comparison of group means (low-high) shows that there are no significant relations between diet and serum parameters. Serum P tends to increase with the intake of Ca and with the Ca/P-ratio and this trend is associated with a significant increase in urinary P. These effects may be a reflection of high availability of dietary P derived from dairy products being rich in calcium. Serum-ionized calcium (not mentioned in Table 11) was not related to dietary parameters. The positive trend between Ca intake and urinary Ca is in harmony with literature data (Section 5.9). A positive trend between the dietary Ca/P-ratio and urinary Ca is obscured by the age-associated decrease in urinary Ca and increase in dietary Ca/P. Regarding P-intake, the positive relation with urinary P indicates that the use of the cross-check method and that of the Dutch Food Composition Table for the estimation of P-intake have their value. The positive relation between P-intake and urinary Ca has no physiological basis and is probably the consequence of the fact that diets rich in P contain much calcium and protein, which nutrients increase urinary Ca. The increase in the excretion of hydroxyproline with P-intake might indicate an increase in bone turnover. However, bone alkaline phosphatase (APB) and PTH were not significantly influenced by P-intake.

6.3.2.5 Correlations between biochemical parameters and BMC/BW

Since correlations between biochemical parameters and bone might provide information about the possible existence of nutritional secondary hyperparathyroidism, a correlation matrix was computed between biochemical parameters and BMC/BW (Table 12). For this purpose we had to exclude women whose data set was not complete ($n = 6$), women with hypercalciuria ($Ca/cr \geqslant 280$ mg/g, $n = 2$) and one woman who had an abnormal urinary P-excretion ($P/cr = 1620$ mg/g). Table 12 shows a positive correlation ($r = 042$; $P < 0.01$) between P and hydroxyproline in urine and a negative one ($r = -0.32$; $P < 0.05$) between urinary P and BMC/BW. These correlations, which are in striking harmony with the positive relation between P-intake and urinary hydroxyproline (Table 9) and with the negative partial correlation between P-intake and BMC/BW (Table 10), support the concept of nutritional secondary hyperparathyroidism. This concept is also supported by the negative correlation ($r = -0.32$; $P < 0.05$) between serum ionized Ca and urinary hydroxyproline. We have no satisfactory explanation for the negative correlation ($r = -0.40$; $P < 0.01$, two tailed) between bone alkaline phosphatase in serum and urinary hydroxyproline. Serum P showed a weak negative correlation with serum PTH ($r = 0.20$; $P = 0.10$) and BMC/BW ($r = 0.20$; $P = 0.10$) and a positive correlation with serum creatinine ($r = 0.32$; $P < 0.05$). These correlations might reflect a slight loss of renal function with loss of

Table 11. Intake level of dietary variables in relation to biochemical parameters in females.

		intake level of dietary variable			t-test low-high P-value
		low	medium	high	
			calcium		
mean Ca-intake	(g/24 h)	0.66 (n = 13)	0.98 (n = 26)	1.49 (n = 14)	
range		< 0.8	0.8 – 1.2	> 1.2	
serum:[5])					
P	(mg/100 ml)	3.34 ± 0.52	3.48 ± 0.45	3.61 ± 0.46	< 0.10
PTH	(μEq/l)	0.33 ± 0.18	0.34 ± 0.14	0.38 ± 0.12[1])	n s
APB	(U/l)	15.8 ± 6.1	15.8 ± 6.6	14.0 ± 4.9	n s
urine:[5])					
Ca/cr	(mg/g)	118 ± 57	127 ± 65[2])	149 ± 62	< 0.10
P/cr	(mg/g)	615 ± 109[3])	678 ± 197	867 ± 318	< 0.05
hypro/cr	(mg/g)	26.7 ± 10.1	28.1 ± 9.4	30.1 ± 10.6	n s
			phosphorus		
mean P-intake	(g/24 h)	1.02 (n = 19)	1.37 (n = 21)	1.87 (n = 13)	
range		<1.2	1.2 – 1.6	>1.6	
serum:					
P	(mg/100 ml)	3.41 ± 0.52	3.51 ± 0.44	3.55 ± 0.48	n s
PTH	(μEq/l)	0.36 ± 0.17	0.32 ± 0.13	0.37 ± 0.14[1])	n s
APB	(U/l)	16.0 ± 4.8	14.3 ± 6.7	15.2 ± 5.7	n s
urine:					
Ca/cr	(mg/g)	111 ± 63	132 ± 58[2])	151 ± 68	< 0.05
P/cr	(mg/g)	639 ± 154	665 ± 192[3])	882 ± 320	< 0.01
hypro/cr	(mg/g)	25.3 ± 7.3	28.8 ± 11.8	31.6 ± 9.0	< 0.05
			calcium to phosphorus		
mean Ca/P		0.61 (n = 16)	0.76 (n = 15)	0.88 (n = 22)	
range		< 0.7	0.7 – 0.8	> 0.8	
serum:					
P	(mg/100 ml)	3.34 ± 0.51	3.45 ± 0.49	3.56 ± 0.39	< 0.10
PTH	(μEq/l)	0.30 ± 0.18	0.38 ± 0.13	0.35 ± 0.13[1])	n s
APB	(U/l)	15.3 ± 6.9	15.6 ± 5.7	14.3 ± 5.0	n s
urine:					
Ca/cr	(mg/g)	138 ± 57	106 ± 48[4])	142 ± 72[4])	n s
P/cr	(mg/g)	616 ± 108[3])	674 ± 215	799 ± 289	< 0.05
hypro/cr	(mg/g)	27.5 ± 8.4	27.4 ± 9.9	30.4 ± 11.9	n s

[1]) One value not available, one abnormal value excluded (1.12 μEq/l).
[2]) Two hypercalciuric values excluded.
[3]) One abnormal value excluded (1620 mg/g).
[4]) One hypercalciuric value excluded.
[5]) For abbreviations see subscript to Table 4.

Table 12. Matrix of correlation coefficients between biochemical parameters and BMC/BW in females (n = 44).

	1	2	3	4	5	6	7	8	9	10
1. Ca/cr[1])	1.00									
2. P/cr	0.35***	1.00								
3. hypro/cr	0.13	0.42***	1.00							
4. Ca^{++}	0.02	0.01	−0.32**	1.00						
5. P	0.12	0.19	0.03	0.12	1.00					
6. PTH	−0.04	−0.02	−0.04	0.10	−0.20*	1.00				
7. APB	0.17	−0.02	−0.40***	0.01	0.03	0.05	1.00			
8. 25-HCC	0.01	−0.10	0.07	−0.06	−0.18	−0.14	−0.17	1.00		
9. creat	−0.03	−0.12	−0.01	0.11	0.32**	0.09	−0.05	−0.04	1.00	
10. BMC/BW	−0.14	−0.32**	−0.09	−0.07	−0.20*	0.12	0.04	0.27**	−0.03	1.00

* = $P \leq 0.10$; ** = $P \leq 0.05$; *** = $P \leq 0.01$. [1]) For abbreviations see subscript to Table 4.

bone as a consequence. The positive correlation ($r = 0.27$; $P < 0.05$) between serum 25-HCC and BMC/BW which was also found with BMC (Table 6) probably indicates a suboptimal vitamin D status.

6.3.3 Evaluation of the data in males (Tables 13 to 20)
For reasons of systematics and simplicity, evaluation of the data in males was done according to the procedures applied to the data of females.

6.3.3.1 Factors associated with the bone mineral content
Table 13 shows that BMC values correlate positively with height and weight, BW, BMC/BW, and thus with skeletal size. The positive correlation between BMC and

Table 13. BMC in relation to age and somatometric, biochemical and nutritional parameters in males.

		BMC (g/cm)			t-test I-III
		I	II	III	
mean		< 1.10 0.99 (n = 8)	1.10-1.25 1.17 (n = 17)	> 1.25 1.37 (n = 10)	P-value
somatometry:					
age	(years)	70.4 ± 4.7	70.3 ± 4.7	69.6 ± 3.7	n s
height	(cm)	166.4 ± 3.0	172.4 ± 5.1	175.4 ± 5.6	< 0.01
weight	(kg)	69.5 ± 7.2	76.0 ± 11.9	80.5 ± 10.1	< 0.01
Quetelet index	(kg/m²)	24.0 ± 2.5	24.5 ± 3.5	25.3 ± 3.4	n s
BW	(cm)	1.56 ± 0.16	1.61 ± 0.14	1.74 ± 0.08	< 0.01
BMC/BW	(g/cm²)	0.64 ± 0.06	0.74 ± 0.06	0.79 ± 0.04	< 0.01
serum:[4])					
Ca^{++}	(mg/100 ml)	4.45 ± 0.29[1])	4.37 ± 0.44	4.54 ± 0.19	n s
P	(mg/100 ml)	3.01 ± 0.55	2.96 ± 0.58	3.06 ± 0.58	n s
25-HCC	(ng/ml)	21.5 ± 5.6	23.8 ± 7.2[2])	23.5 ± 5.2	n s
PTH	(µEq/l)	0.36 ± 0.23	0.36 ± 0.12	0.26 ± 0.07	n s
creat	(mg/100 ml)	1.04 ± 0.10	1.14 ± 0.14	1.20 ± 0.14	< 0.01
APB	(U/l)	17.0 ± 4.8	13.8 ± 5.7	11.8 ± 3.8	< 0.01
urine:[4])					
Ca/cr	(mg/g)	123 ± 51	122 ± 63	121 ± 48	n s
P/cr	(mg/g)	535 ± 142	652 ± 179[3])	583 ± 159	n s
hypro/cr	(mg/g)	20.9 ± 5.0	22.5 ± 7.1	21.2 ± 5.4	n s
diet:					
energy	(MJ/24 h)	10.7 ± 3.0	11.0 ± 2.8[2])	11.9 ± 2.8	n s
protein	(g/24 h)	70 ± 10	91 ± 21[2])	86 ± 26	< 0.10
	(energy %)	11.0	13.9	12.1	
Ca	(g/24 h)	1.03 ± 0.33	1.31 ± 0.46	1.20 ± 0.29	n s
	(g/MJ)	0.10	0.12	0.10	
P	(g/24 h)	1.49 ± 0.28	1.79 ± 0.44[2])	1.71 ± 0.33	< 0.10
	(g/MJ)	0.14	0.16	0.14	
Ca/P		0.68 ± 0.17	0.73 ± 0.17[2])	0.70 ± 0.10	n s
vit.D		237 ± 152	246 ± 168[2])	254 ± 76	n s
physical activity[5])		2.0 ± 0.8	2.8 ± 1.3[2])	3.2 ± 0.9	< 0.0

[1]) n = 7, one value not available.
[2]) n = 16, one value not available.
[3]) n = 16, one extreme value (1752 mg/g) excluded.
[4]) For abbreviations see subscript to Table 4.
[5]) See subscript to Table 5.

serum creatinine and that between BMC and physical activity probably reflect the association between skeletal and muscle mass. The relatively low consumption of protein and phosphorus in the subgroup with the low BMC-values (subgroup 1) can be attributed to a lower intake of food. The absence of a negative correlation between BMC and age is attributable to the lower rate of bone loss with aging in males than in females (Section 4.2) as well as to the rather small age range of the males (Table 2). The negative correlation between bone alkaline phosphatase activity in serum and BMC will be referred to below.

6.3.3.2 Factors associated with age

Table 14 shows a remarkable decrease of height with age. On average this height loss amounted to 0.45 cm per annum (Table 15). It undoubtedly represents bone loss from the vertebrae (trabecular bone) but it could be in part the consequence of a secular trend. Between 1920 and 1930 the average height of conscripts in the Netherlands increased by 1.5 cm (den Hartog, 1972). This trend is insufficient to account for the observed height loss in the present study. The decrease in the urinary phosphorus excretion with age parallels a decrease in food intake. The decrease in the urinary excretion of hydroxyproline with age is attributable to a decrease in either protein intake, bone collagen metabolism or bone mass.

6.3.3.3 Effect of calcium and phosphorus intake on bone parameters

Table 16 shows significant positive correlations between skeletal size ($H.W^{1/2}$) and bone parameters. A positive correlation between calcium intake and skeletal size can also be seen in this table. This correlation was investigated further using body height as a bone parameter (see Table 18). The positive correlations between calcium and phosphorus intake and dietary Ca/P are attributable to the consumption of dairy products. The positive trend correlation between BW on the one hand and dietary calcium and Ca/P on the other appeared insignificant if differences in skeletal size and age were taken into account by multiple linear regression (Table 17).

From Table 17 it can be concluded that nutritional parameters did not give any partial correlation with bone parameters. However, if body height was used as a bone parameter, which seems justified because of the remarkable loss of height with age, the inclusion of calcium intake as an independent variable in the regression of height on age improved the correlation coefficient from 0.37 (Table 15) to 0.49 and the partial regression coefficient for calcium intake was significant ($P < 0.05$). The partial correlation between height and calcium intake was superior to that between either height and phosphorus intake or height and dietary Ca/P (Table 18). This suggests that the positive relation between calcium intake and body height is specific.

6.3.3.4 Effect of calcium and phosphorus intake on biochemical parameters

Table 19 shows there are no significant correlations between serum parameters (P, PTH and APB) and dietary variables (Ca, P and Ca/P).

Mean serum PTH tended to be higher in the low-calcium than in the high-calcium intake subgroup but this correlation was not consistent. Serum Ca (not tabulated) was unaffected by dietary variables. With respect to the urinary parameters, an increase in either Ca or P intake was associated with an increase in both the excretion of P and that of hydroxyproline. These observations in males are more or less similar to those in females.

Table 14. Age in relation to somatometric, biochemical and nutritional parameters in males

mean		age I < 68 years 65.8 (n = 12)	II 68-73 years 69.8 (n = 14)	III > 73 years 76.9 (n = 10)	t-test I - III P-valu
somatometry:					
height	(cm)	174.4 ± 6.1	170.9 ± 6.2	169.7 ± 3.4	< 0.(
BMC	(g/cm)	1.17 ± 0.17	1.22 ± 0.16	1.15 ± 0.11[1])	n
BW	(cm)	1.63 ± 0.18	1.62 ± 0.15	1.65 ± 0.10[1])	n
BMC/BW	(g/cm²)	0.72 ± 0.08	0.75 ± 0.08	0.70 ± 0.08[1])	n
serum:[5])					
Ca^{++}	(mg/100 ml)	4.42 ± 0.45[2])	4.47 ± 0.28	4.36 ± 0.41	n
P	(mg/100 ml)	2.86 ± 0.62	3.05 ± 0.60	3.14 ± 0.41	n
25-HCC	(ng/ml)	22.4 ± 7.0	25.0 ± 6.3[3])	21.3 ± 5.2	n
PTH	(μEq/l)	0.32 ± 0.10	0.33 ± 0.15	0.35 ± 0.18	n
creat	(mg/100 ml)	1.14 ± 0.14	1.10 ± 0.17	1.16 ± 0.10	n
APB	(U/l)	15.5 ± 6.4	13.4 ± 3.1	13.5 ± 5.6	n
urine:[5])					
Ca/cr	(mg/g)	133 ± 68	102 ± 36	124 ± 64	n
P/cr	(mg/g)	638 ± 177	610 ± 182[4])	535 ± 139	< 0.1
hypro/cr	(mg/g)	23.8 ± 5.3	21.2 ± 7.4	18.9 ± 3.8	< 0.(
diet:					
energy	(MJ/24 h)	12.4 ± 3.2	10.7 ± 1.6	9.7 ± 2.0[1])	< 0.(
protein	(g/24 h)	95 ± 21	84 ± 17	75 ± 14[1])	< 0.(
Ca	(g/24 h)	1.24 ± 0.31	1.26 ± 0.40	1.15 ± 0.52[1])	n
P	(g/24 h)	1.85 ± 0.40	1.70 ± 0.35	1.52 ± 0.36[1])	< 0.(
Ca/P		0.67 ± 0.13	0.74 ± 0.15	0.73 ± 0.17[1])	n
vit.D	(IU/24 h)	292 ± 208	222 ± 70	212 ± 89[1])	n
physical activity[6])		2.9 ± 1.6	2.9 ± 1.2	2.1 ± 0.8[1])	n

[1]) n = 9, one value not available.
[2]) n = 11, one value not available.
[3]) n = 13, one value not available.
[4]) n = 13, one extreme value (1752 mg/g) excluded.
[5]) For abbreviations see subscript to Table 4.
[6]) See subscript to Table 5.

Table 15. Linear regression of somatometric, biochemical and nutritional parameters on age ($y = b_0 + b_1x$) in males.

y		b_0	b_1	r	P-value (t-test)
height	(cm)[1])	204	− 0.45	−0.37	< 0.05
urinary hypro/cr	(mg/g)[1])	51.8	− 0.43	−0.34	< 0.05
diet:					
energy	(MJ/24 h)[2])	26.4	− 0.22	−0.41	< 0.01
protein	(g/24 h)[2])	194	− 1.54	−0.38	< 0.05
phosphorus	(g/24 h)[2])	3.31	−0.023	−0.28	< 0.05

[1]) n = 36.
[2]) n = 35, one value not available.

Table 16. Matrix of correlation coefficients between age, somatometric, and nutritional parameters in males (n = 34).

	age	H.W.½	dietary Ca	dietary P	dietary Ca/P
age	1.00				
H.W.½	−0.09	1.00			
dietary Ca	−0.14	0.41***	1.00		
dietary P	−0.38**	0.23	0.74***	1.00	
dietary Ca/P	0.14	0.36**	0.77***	0.32**	1.00
BMC	−0.15	0.54***	0.20	0.23	0.08
BW	−0.06	0.50***	0.25*	0.13	0.24*
BMC/BW	−0.14	0.24*	0.04	0.15	−0.06

* = $P \leq 0.10$; ** = $P \leq 0.05$; *** = $P \leq 0.01$.

Table 17. Partial regression coefficients (± SEM) of multiple linear regression ($y = b_0 + b_1x_1 + b_2x_2 + \ldots b_nx_n$) of bone parameters (y) on age (x_1) H.W.½(x_2), Ca (x_3), P (x_4) and Ca/P (x_5) in males (n = 35)

	regression coefficients						multiple	P
y	b_0	b_1 (age)	b_2 (H.W.½)	b_3 (Ca)	b_4 (P)	b_5 (Ca/P)	r	
BMC (mg/cm)	591	− 3.7 ± 5.3	57.2*** ± 16.2	—	—	—	0.55	< 0.01
	593	− 3.8 ± 5.3	58.9*** ± 17.9	− 15.6 ± 64.0	—	—	0.55	< 0.01
	495	− 2.7 ± 5.8	55.6*** ± 16.8	—	30.4 ± 66.3	—	0.56	< 0.01
	310	1.4 ± 6.1	61.3*** ± 18.1	− 82.6 ± 95.4	93.8 ± 99.0	—	0.57	< 0.01
	558	− 3.0 ± 5.4	61.6*** ± 17.6	—	—	− 112 ± 167	0.56	< 0.01
BW ($cm\ 10^{-2}$)	90	− 0.05 ± 0.53	5.1*** ± 1.6	—	—	—	0.50	< 0.01
	90	− 0.03 ± 0.53	4.9*** ± 1.8	2.1 ± 6.3	—	—	0.50	< 0.01
	89	− 0.03 ± 0.51	5.1*** ± 1.7	—	0.48 ± 6.58	—	0.50	< 0.01
	97	− 0.09 ± 0.57	4.8*** ± 1.8	3.9 ± 9.5	− 2.5 ± 9.8	—	0.51	< 0.01
	92	− 0.09 ± 0.55	4.8*** ± 1.7	—	—	6.7 ± 16.5	0.51	< 0.01
BMC/BW (mg/cm^2)	634	− 2.0 ± 3.0	12.7* ± 9.5	—	—	—	0.27	< 0.01
	687	− 2.2 ± 3.2	14.6* ± 10.4	− 17.1 ± 37.3	—	—	0.28	< 0.10
	644	− 1.6 ± 3.3	12.0 ± 9.8	—	12.6 ± 39.0	—	0.27	< 0.10
	514	− 0.7 ± 3.5	16.0* ± 10.5	− 57.8 ± 55.6	57.0 ± 57.8	—	0.33	< 0.05
	661	− 1.6 ± 3.2	15.8* ± 10.2	—	—	− 79.1 ± 97.8	0.30	< 0.05

* = $P \leq 0.10$; *** = $P \leq 0.01$.

Table 18. Partial regression coefficients (± SEM) of multiple linear regression of body height (y) on age (x_1), dietary Ca (x_2), P (x_3) and Ca/P (x_4) in males (n = 35).

	regression coefficients						
y	b_0	b_1	b_2	b_5	b_8	multiple r	P-value
height (cm)	195.5	− 0.42** ± 0.22	5.07** ± 2.35	—	—	0.49	< 0.01
	194.8	− 0.42** ± 0.24	4.90* ± 3.60	0.24 ± 3.99	—	0.49	< 0.01
	188.4	− 0.34* ± 0.24	—	4.30* ± 2.66	—	0.44	< 0.01
	202.3	−0.53** ± 0.22	—	—	9.62* ± 6.50	0.43	< 0.01

* = $P \leq 0.10$; ** = $P \leq 0.05$.

Table 19. Intake level of dietary variables in relation to biochemical parameters in males.

		intake level of dietary variable			t-test low-hig
		low	medium	high	P-value
			calcium		
mean Ca-intake	(g/24 h)	0.76 (n = g)	1.16 (n = 15)	1.68 (n = 11)	
range		< 1.0	1.0 — 1.4	> 1.4	
serum:[1])					
P	(mg/100 ml)	3.07 ± 0.45	2.93 ± 0.58	3.04 ± 0.64	n
PTH	(µEq/l)	0.42 ± 0.18	0.26 ± 0.12	0.33 ± 0.09	< 0.1
APB	(U/l)	13.7 ± 6.1	13.9 ± 5.5	15.5 ± 3.4	n
urine:					
Ca/cr	(mg/g	94 ± 32	134 ± 72	118 ± 46	= 0.1
P/cr	(mg/g)	517 ± 93	608 ± 170[2])	657 ± 207	< 0.0
hypro/cr	(mg/g)	18.5 ± 4.6	22.2 ± 5.8	23.7 ± 7.0	< 0.0
			phosphorus		
mean P-intake	(g/24 h)	1.31 (n = 12)	1.67 (n = 11)	2.13 (n = 12)	
range		< 1.5	1.5 — 1.9	> 1.9	
serum:					
P	(mg/100 ml)	2.97 ± 0.47	2.82 ± 0.57	3.10 ± 0.61	n
PTH	(µEq/l)	0.36 ± 0.20	0.29 ± 0.09	0.32 ± 0.11	n
APB	(U/l)	13.7 ± 5.1	14.3 ± 6.5	14.5 ± 3.9	n
urine:					
Ca/cr	(mg/g)	117 ± 46	125 ± 68	115 ± 61	n
P/cr	(mg/g)	539 ± 147	611 ± 134[2])	651 ± 212	< 0.1
hypro/cr	(mg/g	19.1 ± 5.4	20.9 ± 6.0	25.0 ± 5.8	< 0.0
			calcium to phosphorus		
mean Ca/P		0.55 (n = g)	0.67 (n = 12)	0.86 (n = 14)	
range		< 0.6	0.6 — 0.75	> 0.75	
serum:					
P	(mg/100 ml)	3.01 ± 0.35	3.05 ± 0.58	3.04 ± 0.58	n
PTH	(µEq/l)	0.37 ± 0.18	0.32 ± 0.14	0.30 ± 0.12	n
APB	(U/l)	13.5 ± 4.3	15.4 ± 7.9	13.6 ± 3.4	n
urine:					
Ca/cr	(mg/g)	124 ± 71	102 ± 52	130 ± 52	n
P/cr	(mg/g)	551 ± 157[3])	637 ± 152	595 ± 197	n
hypro/cr	(mg/g)	22.1 ± 4.1	21.7 ± 6.2	21.4 ± 7.2	n

[1]) For abbreviations see subscript to Table 4.
[2]) n = 14, one abnormal value (1752 mg/g) is excluded.
[3]) n = 8, see footnote 1.

6.3.3.5 Correlations between biochemical parameters and BMC/BW

Table 20 shows a correlation matrix based on data of 33 males. Because of incomplete data sets (2 cases) and hyperphoshaturia (1 case; 1753 mg/g creatinine), 3 males were excluded from this matrix. There was a significant correlation ($r = -0.34$; $P < 0.05$) between urinary Ca and serum PTH and this is consistent with the positive effect of the hormone on renal tubular reabsorption of calcium.

Table 20. Matrix of correlation coefficients between biochemical parameters and BMC/BW in males (n = 33).

	1	2	3	4	5	6	7	8	9	10
1. Ca/cr[1])	1.00									
2. P/cr	0.22	1.00								
3. hypro/cr	0.07	0.27*	1.00							
4. Ca**	−0.12	0.21	−0.01	1.00						
5. P	−0.05	−0.39**	0.13	0.07	1.00					
6. PTH	−0.34**	−0.08	0.19	−0.02	−0.03	1.00				
7. APB	−0.12	0.04	0.21	−0.05	0.01	0.09	1.00			
8. 25-HCC	−0.21	−0.05	−0.07	0.05	0.07	0.07	−0.15	1.00		
9. creat	−0.06	−0.34**	−0.05	−0.04	0.27*	−0.03	−0.01	0.03	1.00	
10. BMC/BW	−0.09	0.12	0.04	0.06	0.03	0.09	−0.36**	0.42***	0.13	1.00

* = $P \leq 0.10$;
** = $P \leq 0.05$;
*** = $P \leq 0.01$.
[1]) For abbreviations see subscript to Table 4.

The positive correlation ($r = 0.27$; $P < 0.10$) between urinary P and hydroxyproline is consistent with the concept that excess dietary phoshorus stimulates PTH secretion and bone turnover. The negative correlations between urinary P and serum P ($r = -0.39$; $P < 0.05$) and between urinary P and serum creatinine ($r = 0.34$; $P < 0.05$) as well as the positive correlation between serum creatinine and serum P ($r = 0.27$; $P < 0.10$) point to a slightly diminished renal function. The positive correlation ($r = 0.42$; $P < 0.01$) between serum 25-HCC and BMC/BW may indicate that the vitamin D status of the group as a whole is not optimal. This is supported by the negative correlation ($r = -0.36$, $P < 0.05$) between bone alkaline phosphatase (APB) in serum and BMC/BW.

6.3.4 Abstract of results

Calcium intake largely exceeded the Dutch recommendation of 0.8 g/24 hours for adults and averaged 1.04 g/24 hours in the women and 1.22 g/24 hours in the men. In spite of an ample phosphorus intake (25 % of the men and the women had an intake exceeding 1.6 and 2.0 g phosphorus daily respectively), the dietary Ca/P-ratio (0.76 in the women and 0.71 in the men) was more favourable than that of the general Dutch population (0.58).

In the women BMC/BW-values clearly decreased with advancing age (0.8% per year) and this bone loss was associated with loss of height (0.35 cm per year). The BMC/BW-ratio showed a significant positive partial correlation with calcium intake and a negative one with phosphorus intake (both $P < 0.05$). An increase in phosphorus intake was associated with an increase in the urinary excretion of

phosphorus and that of hydroxyproline, and the urinary phosphorus excretion showed a significant negative correlation ($P < 0.05$) with the BMC/BW-ratio. The urinary excretion of calcium slightly but significantly decreased with advancing age ($P < 0.01$), probably as a consequence of a decrease in the intestinal absorption.

In the men there was no loss of radial bone with advancing age. However, a marked loss of height was established (0.45 cm per year). This decrease of body height with age probably reflects, at least in part, loss of trabecular bone from the vertebrae. BMC-values as well as BMC/BW-values increased with the degree of physical activity but did not show any relation with either calcium or phosphorus intake. In contrast body height showed a significant positive partial correlation with calcium intake ($P < 0.05$). As in the women, an increase in phosphorus intake was associated with an increase in the urinary excretion of phosphorus and that of hydroxyproline. A significant negative correlation was observed between the urinary excretion of phosphorus and the serum phosphorus concentration ($P < 0.05$).

In both the men and the women serum concentrations of PTH and creatinine were at the upper limit of normality, and this is in line with a slightly diminished renal function. In both sexes positive correlations ($P < 0.05$) were found between BMC/BW values and the serum concentrations of 25-HCC. These correlations existed despite serum concentrations of this parameter of the vitamin D status were within normal limits.

6.3.5 Discussion of results

For several reasons the interpretation of the results has to be done with great care. In the first place the study was carried out among a rather small and selected group of elderly people. So extrapolation of results to the population of elderly people in Ede or in The Netherlands as a whole is not permissible. In the second place it should be recognized that in an observational study like this it is not possible to have all the interfering variables sufficiently under control. In the third place it should be recognized that in an observational study the change of one variable is mostly associated with that of many others. These two latter considerations imply that the study cannot provide conclusive evidence. At best the results can support an hypothesis or give rise to further study.

Although significant correlations were found, they were generally low and, as a consequence explained only a small part of the variation. In this respect the marked inter-individual variation of biological parameters which normally occurs should be stressed. It should also be stressed that the cross-check method yields somewhat rough estimations of habitual rather than actual nutrient intake. Furthermore there is no guarantee as to whether the constancy of food habits on which the cross-check method is based really occurred in all participants. For these reasons the correlation between nutrient intakes and biochemical parameters in a single urine or serum sample cannot be high.

One complication of a cross-sectional study among aged subjects is that effects of selective survival or differential mortality might give a false picture of observed relationships. A significant point in this study is that observed differences in bone mass may be the consequence of either differences in bone mass already present at maturity or to differences in the rate of bone loss after maturity or to both.

The results will be discussed further within the scope of the pathogenesis of

hyperparathyroidism as outlined in Figure 5 (Section 5.11). Both in women and men there was evidence of a slightly diminished renal function and this might have been expected in subjects of this age group (Rowe et al., 1976). The positive correlation, found in both sexes, between creatinine and phosphorus in serum also points to early loss of renal function, and the negative correlation in women between serum phosphorus and BMC or BMC/BW is consistent with the view that loss of renal function is a factor in bone loss with aging.

Regarding the effect of diet on bone in women, the positive correlation between calcium intake and BMC/BW and the negative correlation between phosphorus intake and this bone parameter are consistent with the hypothesis of nutritional hyperparathyroidism. However, since these correlations were only significant if differences in age and skeletal size were taken into account and since they could not be demonstrated in men, the chance of an artefact should not be overlooked. In men bone loss with advancing age was not detected but there was a marked loss of height with age, probably reflecting a loss of trabecular bone. It is therefore possible that the photon-absorptiometric measurement of the BMC of the radius at the distal third (almost completely cortical bone) is not representative of the bone status of the entire body. In this respect differences in manual labour affecting the muscle and bone mass of the right (often dominating) forearm might have interfered. It is also possible that, particularly in men, loss of trabecular bone dominates that of cortical bone. In this sense the positive correlation in men between calcium intake and body height is remarkable.

Regarding the effect of diet on biochemical parameters in both women and men, an increase in P intake was found to be associated with an increase in the urinary excretion of phosphorus and hydroxyproline. In addition, these latter parameters were correlated positively with each other. Since diets which are rich in phosphorus usually contain much protein, possibly including collagen-protein and hydroxyproline, these correlations might be of dietary rather than of physiological origin. It should be remembered, however, that urinary phosphorus, which is mainly determined by the amount of phosphorus consumed, correlated negatively with BMC/BW in women. This correlation is consistent with the partial negative correlation between dietary phosphorus and this bone parameter. In men urinary phosphorus correlated negatively with serum phosphorus, suggesting hyperparathyroidism. This latter phenomenon was not observed in the women. The possible cause of this sex difference will be referred to in the General Discussion (Chapter 9). The observed correlations between either dietary or urinary phosphorus and hydroxyproline excretion and serum phosphorus in the men and hydroxyproline excretion and BMC/BW in the women, are in line with the concept that high-phosphorus diets stimulate PTH secretion and bone turnover (Figure 5, Section 5.11).

An important observation was that in the elderly people, serum 25-HCC correlated positively with BMC/BW despite the fact that the concentrations of this metabolite in serum were normal. This correlation probably reflects a diminished conversion of 25-HCC to 1,25-DHCC or 24,25-DHCC consequent to renal aging (Section 4.6), an increased need for vitamin D and a sub-optimal vitamin D status of the elderly. A diminished synthesis of 1,25-DHCC consequent to renal aging was also outlined in Figure 5 (Section 5.11). This corresponds with the decline in the urinary calcium excretion with age which was observed in the women, and with the negative correlation between bone alkaline phosphatase and BMC/BW in the men.

It can be concluded that the observed relations, discussed above, fit into the outline of the pathogenesis of hyperparathyroidism (Figure 5, Section 5.11). This suggests that in elderly people either a low-calcium or a high-phosphorus diet contributes to the development of hyperparathyroidism, which may cause osteoporosis.

Chapter 7

Nutritional interrelationships between calcium, phosphorus and lactose in rats

7.1 Introduction

This Chapter, in which an experiment with rats is reported, deals with the effect of dietary calcium, phosphorus and lactose on calcium and bone metabolism. It was published in the 'Journal of Nutrition' in 1980 and is reproduced in its entirety on the following pages.

Although it was not intended that this thesis should deal with lactose as a dietary factor which influences calcium metabolism (Section 5.3), the observations made in this experiment, as far as the effect of dietary calcium and phosphorus on calcium and bone metabolism is concerned, are relevant to the subject of this thesis. The study at issue preceded the experiments described in Chapter 8.

Before the reader loses himself in the article, some remarks have to be made. First, it should be mentioned that rat plasma alkaline phosphatase, in contrast to human plasma alkaline phosphatase, could not be separated by polyacrylamide gel electrophoresis into its isoenzymes in a satisfactory way. It appeared that the bone isoenzyme did not separate from the intestinal isoenzyme. For this reason, only total alkaline phosphatase activity has been reported in the experiments with rats. Secondly, attention is drawn to a few errors in the text:

page 64 Abstract, line 11: 'excretion' should read: 'exception'.
Abstract, line 21: 'in line' should read: 'are in line'.

page 65 first column, line 15: 'calcium phosphorus ratio' should read: 'calcium-to-phosphorus ratio'.

Nutritional Interrelationships Between Calcium, Phosphorus and Lactose in Rats

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ABSTRACT From the age of 3 months, six groups of rats consisting of six animals each were fed one of three types of diet with either 15% lactose or dextrose. The diets used were a control diet containing 0.6% calcium and 0.45% phosphorus and two high-phosphorus (1.3%) diets with either a normal (0.6%) or a low (0.2%) calcium content. Whereas calcium retention and femur mass appeared to be unaffected, feeding the high-phosphorus diets resulted in significant ($P \leq 0.05$ or $P \leq 0.01$) biochemical changes including: decreased plasma phosphorus almost throughout the whole of the study; increased plasma alkaline phosphatase and urinary total hydroxyproline after 17 and 42 weeks; nephrocalcinosis; depressed urinary calcium, and reduced femur density. With the excretion of nephrocalcinosis and depressed urinary calcium, these changes were more marked if the calcium content of the diet was low than when it was normal, and this difference was associated with a significant ($P < 0.01$) inhibiting effect of dietary calcium on the intestinal absorption and urinary excretion of phosphorus. Although lactose, as compared with dextrose, significantly ($P < 0.01$) improved the intestinal absorption and retention of calcium during body weight gain, which was reflected by an increased femur mass after 42 weeks, this sugar did not reduce the detrimental effects on bone and soft tissue resulting from feeding the high-phosphorus diets. The results of the study in line with the induction of nutritional secondary hyperparathyroidism and increased bone turnover in rats fed high-phosphorus diets and indicate that lactose-stimulated calcium absorption will not prevent or diminish the biochemical changes associated with this disease; the results also stress the significance of the calcium content of the diet as a factor that may protect the body against excessive dietary phosphorus. J. Nutr. *110*: 1101–1111, 1980.

INDEXING KEY WORDS calcium · phosphorus · lactose · calcium balance · bone parameters

It is well known (1) that diets containing either a phosphorus excess or a low calcium:phosphorus ratio induce accelerated bone resorption in experimental animals. This bone resorption may, depending on age of onset, animal species and dietary manipulation, result in osteoporosis (2), osteitis fibrosa (3) and periodontal disease (4). These bone lesions are sometimes associated with soft tissue calcification, especially of kidneys. According to Krook et al. (5) the underlying mechanism is secondary hyperparathyroidism, initiated by a depression of the plasma calcium concentration following an increased phosphate flow through the blood. Osteoporosis (diminished amount of bone) has been reported by Draper and co-workers (2, 6) in aging

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TABLE 1

Composition of the diets (g/100 g)

	Diets		
	CC	CH	LH
Basal diet[1,2]	93.24	93.24	93.24
$CaCO_3$[2]	1.50	1.50	0.50
KH_2PO_4[2]	1.32	5.28	5.28
Washed sea sand	3.94	—	0.98
Total	100	100	100

[1] Ingredients (g/kg total diet): casein, 195; DL-methionine, 5; palm oil, 149; wheat starch, 298; cellulose, 100; lactose or dextrose, 150; KCl, 10; NaCl, 15; $MgSO_4 \cdot 7H_2O$, 6.2; $FeSO_4 \cdot 7H_2O$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.1; $MnSO_4 \cdot 4H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.1, and KIO_3, 0.001. Vitamins per kilogram of total diet: thiamin-HCl, 3 mg; riboflavin, 4 mg; pyridoxine, 3 mg; niacin, 25 mg; pantothenic acid, 20 mg; biotin, 0.12 mg; cyanolcabalamin, 0.04 mg; folic acid, 0.84 mg; myo-inositol, 84 mg; choline chloride, 1.5 g; dextrose, 360 mg; retinol, $12.5.10^3$ IU; cholecalciferol, 2.9×10^3 IU; alpha tocopherol, 51 mg; phyllochinone, 2 mg, and soy oil, 1 g. [2] Dietary mineral composition and the supplementation with $CaCO_3$ and KH_2PO_4 adapted from Whittemore et al. (9).

rats fed diets containing 1.2 or 1.8% versus 0.3 or 0.6% phosphorus with 0.6% calcium, as well as in rats fed 1.2 versus 0.6% dietary phosphorus with 1.2% calcium. Although in both studies an increased loss of labeled calcium from the bones of rats fed the high-phosphorus diets was observed, neither intestinal absorption nor retention of calcium was affected, which is difficult to reconcile with the development of osteoporosis. Increasing the dietary calcium content did not prevent accelerated bone resorption induced by feeding high-phosphorus diets, and it was therefore postulated that the dietary calcium phosphorus ratio loses significance at high phosphorus intake levels (6).

However, as it has been shown that dietary calcium has a marked inhibiting effect on intestinal phosphate absorption, (2, 6, 7) one might expect that lowering the calcium content of the diet would aggravate the detrimental effects of a high phosphorus load. It might also be expected that the effects of excess dietary phosphorus would be counterbalanced if the stimulus to parathyroid stimulation were reduced by an increased intestinal calcium absorption. A factor that has long been known to increase intestinal absorption and retention of calcium is the presence of lactose in the diet (8). The aim of the subsequent study was therefore to investigate the effects of high-phosphorus diets with or without lactose on calcium metabolism and bone and the effects of different levels of calcium in high-phosphorus diets on phosphate metabolism and bone.

METHODS

Thirty-six male lean Zucker rats (Rattus norvegicus) 3 months old and weighing about 270 g were divided into six groups of six which were fed one of three types of diet with either 15% lactose or dextrose. The diets used were a control diet (diet CC) containing 0.6% calcium and 0.45% phosphorus and two high-phosphorus (1.3%) diets with either a normal (0.6%) or a low (0.2%) calcium content (diets CH and LH, respectively). The diets of which the composition is shown in table 1 were fed for a period of 42 weeks. The animals were housed individually under conditions of controlled lighting, temperature and humidity, and deionized water and food were provided ad libitum. Food consumption was recorded daily and body weights were measured biweekly. During weeks 4, 8, 30 and 42, 96-hour collections of feces and urine were made. Fasting blood samples were drawn under light ether anesthesia (orbita puncture) after these collection periods and also after 17 weeks.

After 42 weeks of feeding, the rats were killed by N_2O, and heart, kidneys and right femur were removed from their bodies. Feces, dry fat-free femurs, heart and kidneys were dry ashed in a muffle furnace for 16 hours at 600°. The ash was dissolved in 1 ml 4 N HCl and diluted with distilled water for the determination of calcium and phosphorus. Femurs were cleaned of adhering tissue, weighed under water for determination of bone volume, defatted for 48 hours in ethanol (95%) and for 48 hours in petroleum ether (b.p. 30–60°), dried for 16 hours at 105° and weighed. Calcium was estimated by a methylthymol blue method

(10) and phosphorus by a molybdenum blue method (11). Urinary total hydroxyproline was assayed according to the method of Kivirikko et al. (12). Plasma alkaline phosphatase activity was estimated using nitrophenylphosphate as a substrate (commercial reagent kit, Boehringer, Mannheim, W. Germany).

Two-way analysis of variance was applied to test the significance of main and interaction effects. If F-values reached statistical significance ($P \leqslant 0.05$) or indicated a trend effect ($P \leqslant 0.10$), differences between group means of rats fed the same type of diet (lactose versus dextrose) were further investigated using Student's *t*-test (one-sided). Differences due to the type of diet and/or carbohydrate × diet interaction effects were further investigated using Sheffe's test to prevent loss of confidence due to multiple comparison (13). If inhomogeneity of variances was apparent, the parameter-free Kruskal-Wallis test was used. Three levels of significance were applied: $P \leqslant 0.01$, $P \leqslant 0.05$ and $P \leqslant 0.10$.

RESULTS

Body weight gain and food consumption (table 2). Body weight gain was almost linear during the first 12 weeks and slowed down thereafter in all groups. Rats fed the dextrose diets with the normal calcium content gained faster than did rats fed these diets with lactose ($P < 0.05$), whereas food consumption was not significantly different between these animals. Body weight gain as well as food consumption was higher in rats fed the control diets than in rats fed the high-phosphorus diets ($P < 0.01$).

TABLE 2

Body weight gain and food consumption[1]

		Body wt gain	Food consumption
		g/42 weeks	*g/week*
Diets			
Lactose	CC[2]	129	116
	CH	88	105
	LH	118	113
Dextrose	CC	162[b]	117
	CH	126[b]	110
	LH	111	110
SEM[4]		13	2
ANOV[5]	CHO	$P < 0.10$	N.S.
	Ca/P	$P < 0.01$	$P < 0.01$
	CHO × Ca/P	N.S.	N.S.
Main[6] contrast	CC − CH	38.5±13.4[3,c]	9.0±2.4[f]
	CC − LH	31.0±13.4[c]	5.0±2.4
	CC − ½(CH + LH)	34.7±11.6[f]	7.0±2.1[f]
	CH − LH	−7.5±13.4	−4.0±2.4

[1] Values shown are means of six rats. [2] CC, CH and LH indicate control, normal-calcium, high-phosphorus and low-calcium, high-phosphorus diets, respectively. [3] Indicates significance of difference from value in rats fed the corresponding diet with lactose: [a] $P \leqslant 0.10$; [b] $P \leqslant 0.05$; [c] $P \leqslant 0.01$, by Students *t*-test. [4] SEM derived from pooled error variance. [5] Analysis of variance: CHO, Ca/P and CHO × Ca/P indicate effects of dietary carbohydrate, calcium/phosphorus ratio and carbohydrate × calcium-phosphorus ratio interaction effect, respectively. [6] Values of lactose-fed and dextrose-fed rats combined. [7] Mean ± SEM; [d] $P \leqslant 0.10$, [e] $P \leqslant 0.05$, [f] $P \leqslant 0.01$ by Scheffe's test.

Plasma parameters and urinary total hydroxyproline (fig. 1)

Calcium. There were no significant main effects of dietary variables on plasma calcium, which was within the normal range in all groups. After 42 weeks, values tended to be lower in rats fed the normal calcium high-phosphorus diets than in rats fed the control diets (mean difference ± SEM: 0.30 ±0.14 mg/100 ml; $P < 0.10$).

Phosphorus. In all groups plasma phosphorus decreased with advancing age. The type of dietary carbohydrate did not influence the concentration in four of the five occasions that it was measured; after 30 weeks levels were slightly higher in rats fed lactose: mean differences ± SEM were 0.55±0.33 in rats fed the control diets, and 0.48±0.33 and 0.50±0.33 mg/100 ml (all $P \leqslant 0.10$) in those fed the high-phosphorus diets with the normal and low calcium content respectively. Throughout the study rats fed the high-phosphorus diets had lower concentrations than did those fed the control diets; the lowest values were observed on the low-calcium, high-phosphorus diets. These values differed significantly from those of rats fed the control diets for the first 30 weeks: mean differences ± SEM were 1.09 ± 0.37, 1.39 ± 0.32, 0.69±0.23 and 0.84 ±0.23 mg/100 ml (all $P \leqslant 0.01$) after 4, 8, 17 and 30 weeks, respectively. After 42 weeks the difference was smaller (mean value 0.48±0.23 mg/100 ml; $P < 0.10$). Consistent intermediate values which were not significantly different from those in rats fed either type of other diet were

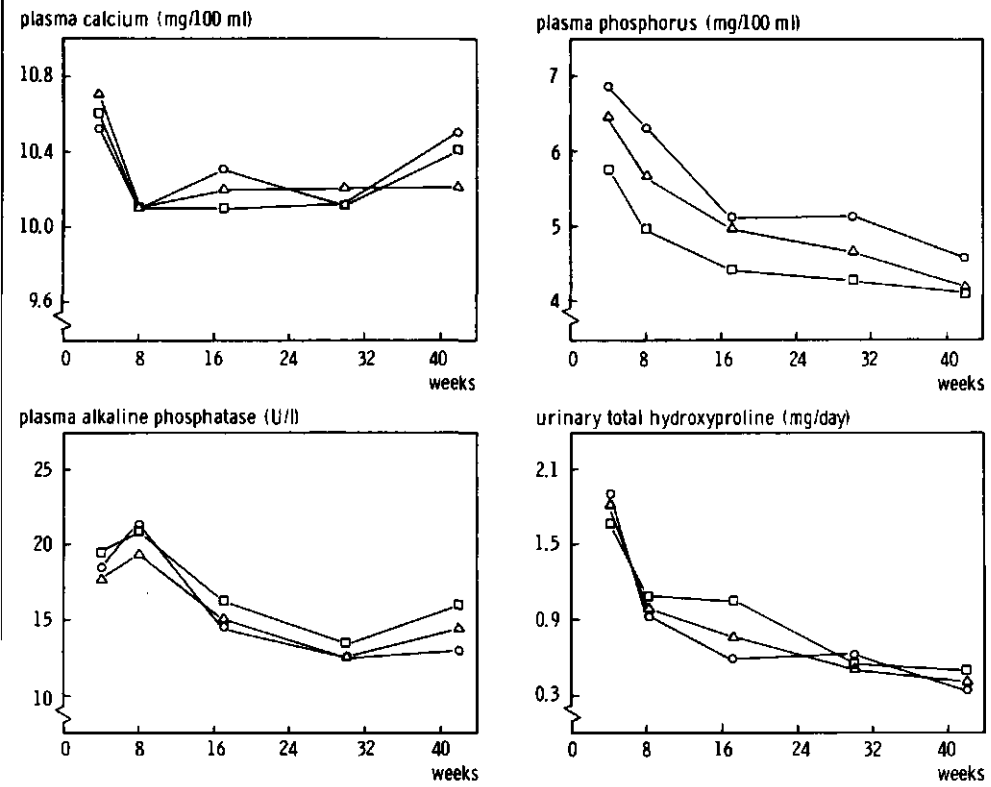

Fig. 1 Plasma calcium, phosphorus and alkaline phosphatase and urinary total hydroxyproline. Lactose-fed and dextrose-fed rats combined: ○——○ = control groups (CC diets); △——△ normal-calcium, high-phosphorus groups (CH diets); □——□ low-calcium, high-phosphorus groups (LH diets).

observed in rats fed the normal-calcium, high-phosphorus diets.

Alkaline phosphatase. Enzyme activity was not significantly influenced by the type of dietary carbohydrate throughout the study. After 17, 30 and 42 weeks the activity was higher in rats fed the low-calcium, high-phosphorus diets than in those fed the control diets. (mean difference ± SEM: 2.01 ±0.79, $P = 0.05$; 0.81±0.55, $P < 0.10$, and 2.99 ± 0.66, $P < 0.01$, respectively). After 17 and 42 weeks intermediate values were measured in rats fed the normal-calcium, high-phosphorus diets, which were not significantly different from values in rats fed either type of other diet.

Hydroxyproline. No significant carbohydrate effect was detected throughout the study. After 17 weeks rats fed the control diets had lower values than rats fed either the normal-calcium, high-phosphorus diets (mean difference ± SEM: 0.20±0.08 mg/24 hours, $P < 0.05$) or the low-calcium, high-phosphorus diets (mean difference ± SEM: 0.49±0.08 mg/24 hours $P < 0.01$). Of the rats fed these high-phosphorus diets, those fed low dietary calcium had higher values than those fed normal dietary calcium (mean difference ± SEM: 0.30±0.08 mg/100 ml, $P < 0.01$). These differences were not observed after 30 weeks but reappeared, though to a smaller extent, after 42 weeks. At that time rats fed the low-calcium, high-phosphorus diets excreted more than rats fed either the control diets or the normal-calcium, high-phosphorus diets (mean differences ± SEM: 0.14±0.04, $P < 0.01$, and 0.11±0.04, $P < 0.05$, respectively).

Calcium and phosphorus balance (tables 3 and 4). Data on balances obtained after 3 and 7 weeks, when body weight gain was almost linear (designated as period 1) and after 29 and 41 weeks when body weights were almost constant (designated as period 2) were pooled, since the results obtained during each of these periods were similar. Results obtained during period 1 are shown in tables 3 and 4, and those achieved during period 2 appear in the text as far as they differed from those achieved during period 1.

Urinary calcium. Effects of dietary treatment on urinary calcium were identical during periods 1 and 2. Rats fed lactose excreted more calcium in urine than did rats fed dextrose ($P < 0.01$), this effect being greater in rats fed the control diets than in those fed either type of the high-phosphorus diet ($P < 0.01$). Rats fed the control diets excreted more than did rats fed the high-phosphorus diets ($P < 0.01$), and in rats fed these latter diets excretion was slightly higher when the calcium content of the diet was low than when it was normal ($P < 0.05$).

Calcium retention. Calcium retention in all groups was positive during period 1. Lactose improved retention during this period in rats fed either the control diets ($P < 0.01$) or the low-calcium, high-phosphorus diets ($P < 0.05$). This positive effect was not observed during period 2. There was no significant effect of the dietary Ca/P ratio on retention during period 1. When body weights were almost constant (period 2), a positive calcium retention was only observed in rats fed the normal-calcium, high-phosphorus diets, which amounted to 4.4±1.8 mg/24 hours for both lactose-fed and dextrose-fed rats, whereas in the remaining groups retention was not significantly different from zero −0.05± 0.91 mg/24 hours).

Percentage net intestinal calcium absorption. During period 1 lactose improved absorption in rats fed either the control diets

TABLE 3

Parameters on calcium balance and percentage net intestinal calcium absorption[1]

		Intake	Urine	Retained	Absorption
			mg/24 hours		*%*
Diets					
Lactose	CC	89.0	2.1	9.7	13.1
	CH	80.3	1.1	8.8	12.3
	LH	31.8	1.3	8.5	30.8
Dextrose	CC	85.3	1.3[c]	2.7[c]	4.8[c]
	CH	81.8	0.8[b]	7.5	10.1
	LH	27.2	1.0[b]	4.1[b]	18.8[c]
SEM		2.1	0.1	1.3	1.6
ANOV	CHO	—	$P < 0.01$	$P < 0.01$	$P < 0.01$
	Ca/P	—	$P < 0.01$	N.S.	$P < 0.01$
	CHO × Ca/P	—	$P < 0.01$	$P < 0.10$	$P < 0.01$
Main contrast	CC − CH	—	0.8±0.1[f]	—	− 2.1±1.6
	CC − LH	—	0.6±0.1[f]	—	−15.9±1.6
	CC − ½(CH + LH)	—	0.7±0.1[f]	—	− 9.1±1.4[f]
	CH − LH	—	0.2±0.1[e]	—	−13.6±1.6[f]
Interaction contrast[2]	CC − CH	—	0.6±0.2[f]	5.6±2.6	6.2±3.2
	CC − LH	—	0.5±0.2[f]	2.6±2.6	− 3.8±3.2
	CC − ½(CH + LH)	—	0.6±0.1[f]	4.1±2.2	1.2±2.8
	CH − LH	—	0.1±0.2	−3.1±2.6	− 9.9±3.2[f]

[1] Average of mean values of six rats during weeks 3 and 7. See footnotes to table 2. [2] Difference between contrast on lactose and contrast on dextrose diets.

TABLE 4

Parameters on phosphorus balance and percentage net intestinal phosphorus absorption[1]

		Intake	Urine	Retained	Absorption
			mg/24 hours		%
Diets					
Lactose	CC	68.9	32.1	4.7	53.2
	CH	174.1	113.8	7.9	69.8
	LH	188.0	159.1	6.2	87.9
Dextrose	CC	66.5	30.9	−1.1	44.8[c]
	CH	177.5	124.5	−2.7[b]	68.5
	LH	168.2	145.9	−53.[b]	83.6[c]
SEM		4.6	5.5	4.1	0.9
ANOV	CHO	—	N.S.	$P < 0.01$	$P < 0.01$
	Ca/P	—	$P < 0.01$	N.S.	$P < 0.01$
	CHO × Ca/P	—	N.S.	N.S.	$P < 0.01$
Main contrast	CC − CH	—	− 87.6±5.5[f]	—	−20.2±0.9[f]
	CC − LH	—	−121.0±5.5[f]	—	−36.8±0.9[f]
	CC − ½(CH + LH)	—	−104.3±4.8[f]	—	−28.5±0.8[f]
	CH − LH	—	− 33.4±5.5[f]	—	−16.6±0.8[f]
Interaction contrast[2]	CC − CH	—	—	—	7.1±1.8[f]
	CC − LH	—	—	—	4.1±1.8
	CC − ½(CH + LH)	—	—	—	5.6±1.4[f]
	CH − LH	—	—	—	− 3.0±1.8

[1] Average of mean values of six rats during weeks 3 and 7. See footnotes to table 2. [2] Difference between contrast on lactose and contrast on dextrose diets.

($P < 0.01$) or the low-calcium, high-phosphorus diets ($P < 0.01$), and in rats fed the high-phosphorus diets the positive effect of lactose was significantly greater when the calcium content of the diet was low than when it was normal ($P < 0.01$). The positive effect of lactose was not observed during period 2. Rats fed the low-calcium, high-phosphorus diets had higher values than rats fed either type of the other diet during period 1 ($P < 0.01$) but not during period 2. During this latter period absorption tended to be higher in rats fed the normal-calcium, high-phosphorus diets than in those fed the other diets (7.2±1.5 versus 3.0±1.0%, $P < 0.10$).

Urinary phosphorus. During periods 1 and 2 there was no significant carbohydrate effect, but rats fed the high-phosphorus diets excreted much more phosphorus in urine than did rats fed the control diets ($P < 0.01$). In rats fed the high-phosphorus diets excretion was markedly higher when the calcium content of the diet was low than when it was normal ($P < 0.01$).

Phosphorus retention. During period 1, but not during period 2, rats fed lactose retained more phosphorus than did rats fed dextrose. This effect was significant on either type of the high phosphorus diet ($P < 0.05$). Retention was not significantly influenced by the dietary Ca/P ratio during period 1, but during period 2 positive retention occurred in rats fed the normal-calcium, high-phosphorus diets (12.0±3.1 mg/24 hours), whereas in rats fed the other diets retention was not significantly different from zero (1.4±2.2 mg/24 hours).

Percentage net intestinal phosphorus absorption. During period 1 but not during period 2, rats fed lactose had higher absorption values than did rats fed dextrose. This effect was significant in rats fed the control diets ($P < 0.01$) and in those fed the low-calcium, high-phosphorus diet and was greater in rats fed the control diets

than in rats fed the high-phosphorus diets ($P < 0.01$). Absorption was markedly influenced by the dietary Ca/P ratio: during both periods values were higher in rats fed the high-phosphorus diets than in those fed the control diets ($P < 0.01$); and in rats fed the former diets absorption was higher when the calcium content of the diet was low than when it was normal ($P < 0.01$).

Bone parameters (tables 5 and 6)

Bone volume. Rats fed lactose tended to have higher values than did rats fed dextrose ($P < 0.10$), the difference being significant only in rats fed the control diets ($P < 0.05$). Rats fed the high-phosphorus diets had higher values than rats fed the control diets ($P < 0.01$).

Dry fat-free weight. Rats fed lactose had higher values than did rats fed dextrose ($P < 0.01$), the difference being significant in rats fed the control diets ($P < 0.01$) and the normal-calcium, high-phosphorus diets ($P < 0.05$). Of the rats fed the high-phosphorus diets, those fed low dietary calcium tended to have lower values than those fed normal dietary calcium ($P < 0.10$).

Total ash. Regarding the effect of lactose versus dextrose, results were similar to those for dry fat-free weight. Rats fed the low-calcium, high-phosphorus diets had significantly lower values than rats fed either type of other diet ($P < 0.01$). The effect tended to be more marked in lactose-fed animals than in dextrose-fed ones ($P < 0.10$).

Derived bone parameters. Dry fat-free weight/volume, total ash/volume and total ash/(dry fat-free weight—total ash) were similar in lactose-fed rats and in dextrose-fed rats. Values for these parameters were lower in rats fed either type of the high-phosphorus diet than in rats fed the control diets ($P < 0.01$), the difference being most marked between rats fed the control and those fed the low-calcium, high-phosphorus diets.

TABLE 5

Bone quantity parameters (right femur)[1]

		Volume	Dry fat-free weight	Total ash
		cm³/kg BW	*g/kg BW*	*g/kg BW*
Diets				
Lactose	CC	1.38	1.59	1.04
	CH	1.52	1.59	1.01
	LH	1.43	1.49	0.93
Dextrose	CC	1.27[b]	1.42[c]	0.92[c]
	CH	1.44[a]	1.50[b]	0.96[b]
	LH	1.43	1.45	0.91
SEM		0.04	0.03	0.02
ANOV	CHO	$P < 0.10$	$P < 0.01$	$P < 0.01$
	Ca/P	$P < 0.01$	$P < 0.10$	$P < 0.01$
	CHO × Ca/P	N.S.	N.S.	$P < 0.10$
Main contrast	CC − CH	−0.16±0.04[f]	−0.04±0.03	−0.01±0.02
	CC − LH	−0.11±0.04[f]	0.07±0.03	0.06±0.02[f]
	CC − ½(CH + LH)	−0.13±0.04[f]	−0.01±0.03	0.03±0.02
	CH − LH	0.05±0.04	0.08±0.03[d]	0.07±0.02[f]
Interaction contrast[2]	CC − CH	—	—	0.07±0.04
	CC − LH	—	—	0.10±0.04[d]
	CC − ½(CH + LH)	—	—	0.09±0.03[d]
	CH − LH	—	—	0.03±0.04

[1] See footnotes to table 2. [2] Difference between contrast on lactose and contrast on dextrose diets.

TABLE 6

Bone quality parameters (right femur)[1]

		Dry fat-free weight/volume	Total ash/volume	Total ash/fat-free organic matter
		g/cm³	*g/cm³*	
Diets				
Lactose	CC	1.15	0.75	1.89
	CH	1.05	0.67	1.76
	LH	1.05	0.65	1.70
Dextrose	CC	1.12	0.73	1.89
	CH	1.05	0.67	1.76
	LH	1.02	0.64	1.70
SEM		0.02	0.02	0.03
ANOV	CHO	N.S.	N.S.	N.S.
	Ca/P	$P < 0.01$	$P < 0.01$	$P < 0.01$
	CHO × Ca/P	N.S.	N.S.	N.S.
Main contrast	CC − CH	0.09±0.02[f]	0.07±0.02[f]	0.13±0.03[f]
	CC − LH	0.10±0.02[f]	0.10±0.02[f]	0.19±0.03[f]
	CC − ½(CH + LH)	0.09±0.02[f]	0.08±0.02[f]	0.16±0.03[f]
	CH − LH	0.02±0.02	0.03±0.02	0.06±0.03

[1] See footnotes to table 2.

Bone ash Ca/P ratio. The Ca/P ratio in the bone ash of rats fed either the control or the normal-calcium, high-phosphorus diets was 2.03±0.02 and was not influenced by the dietary treatments. In rats fed the low-calcium, high-phosphorus diets there was a wide variation in the bone ash Ca/P ratio: mean values were 1.98 (range: 1.69–2.54) and 1.88 (range: 1.46–2.17) for rats fed lactose and those fed dextrose, respectively.

Calcium content of soft tissues (table 7). There was a marked calcification of renal tissue in rats fed the high-phosphorus diets, although with a variable response. These rats also showed a slightly but not significantly increased calcium content of the heart. Soft-tissue calcification was not significantly influenced by the type of dietary carbohydrate or by the dietary calcium content.

DISCUSSION

Body weight gain and food consumption. The lower body weight gain and food consumption in rats on the high-phosphorus diets possibly indicate a low degree of toxicity of these diets. That the body weight gain in lactose-fed rats was lower than that in dextrose-fed rats must be due to a higher fecal loss of dietary energy in the former, since food intake was equal in both groups of rats. An increased loss of protein and fat in post-weanling lactose-fed rats, the consequence of low intestinal lactase levels, has been reported by Leichter and Tolensky (14).

Effects of high-phosphorus diets. As might be expected from the literature, feeding high-phosphorus diets to rats would induce nutritional secondary hyperparathyroidism (NSH). Depending on experimental conditions, inconsistent and variable results have been reported in this disease with respect to plasma calcium, phosphorus and alkaline phosphatase (5). Henrikson (4) stressed the importance of appreciation of compensatory mechanisms in the evaluation of single determinations. The present experiment shows that in rats fed the high-phosphorus diets plasma phosphorus was lower than in those fed the control diets. Laflamme and Jowsey (15) also observed depressed plasma phosphorus concentrations in dogs that received oral acid potassium phosphate supplements, and these

TABLE 7

Calcium content of kidneys and heart[1]

		Kidneys (range)	Heart
		mg/kg dry tissue	
Diets			
Lactose	CC	0.33 (0.23–0.46)	0.41
	CH	0.47 (0.45–0.62)	0.43
	LH	0.87 (0.41–1.64)	0.46
Dextrose	CC	0.36 (0.26–0.44)	0.42
	CH	1.50 (0.45–4.17)	0.46
	LH	0.60 (0.51–0.85)	0.46
SEM		—	0.02
Kruskal-Wallis test	CHO	N.S.	—
	Ca/P	$P < 0.01$	—
ANOV	CHO	—	N.S.
	Ca/P	—	$P < 0.10$
	CHO × Ca/P	—	N.S.
Main contrast	CC − CH	−0.64[a]	−0.03±0.02
	CC − LH	−0.39	−0.05±0.02
	CC − ½(CH + LH)	−0.52	−0.04±0.02
	CH − LH	0.25	−0.02±0.02

[1] See footnotes to table 2. [a] $P \leqslant 0.01$ by Kruskal-Wallis test.

concentrations were negatively correlated with the bone resorption rate and with the plasma parathyroid hormone (PTH) level.

We did not measure PTH levels but using the creatinine clearance which amounts to about 2.6 ml/minute in this strain of rats [1] as a measure of glomerular filtration, it can be estimated that tubular reabsorption of phosphate, which is known to be inhibited by PTH (16), was about 81 and 12% in rats fed the control and high-phosphorus diets, respectively. Consistent with NSH is also the decreased urinary calcium excretion (6, 16), the slightly increased activity of plasma alkaline phosphatase after 17, 30 and 42 weeks, the increased urinary excretion of hydroxyproline after 17 and 42 weeks, the calcification of renal tissue and the maintenance of normal plasma calcium concentrations in rats fed the high-phosphorus diets. The observed increase in plasma alkaline phosphatase and urinary hydroxyproline indicates PTH mediated accelerated bone remodeling (17).

Like the results of other studies concerned with rats (2, 7) as well as with man (18, 19), calcium retention as measured by the balance technique was not negatively influenced by feeding the high-phosphorus diets. This harmonizes with the quantity of bone ash and dry fat-free weight of femurs that did not differ between rats fed the control and those fed the high-phosphorus diets. However, in rats fed these latter diets bone quality parameters (amount of ash and dry fat-free weight per unit of bone volume and amount of ash per unit of fat-free organic matter) were significantly reduced. In rat studies carried out by Draper and co-workers (2, 6) just the reverse was observed, but our results are in line with a rabbit study conducted by Jowsey and Balasubramanian (20), who reported increased porosity of bones associated with increased bone turnover in phosphate-supplemented animals without any effect on the bone calcium content. Differences in experimental conditions (age and strain of the rats, length of feeding period and dietary composition) might explain the discrepancy between our results

[1] Schaafsma, G. (1978) Unpublished results.

and those of the group of Draper. In one study (2) they used 8-month-old rats. An average urinary excretion of hydroxyproline of 0.15 mg/24 hours, which is far below the values we observed, was reported and no significant differences in hydroxyproline excretion between rats fed either 0.3, 0.6 or 1.2% dietary phosphorus were observed. Since hydroxyproline is a measure of collagen breakdown and bone turnover, it is attractive to postulate that quantitative loss of bone induced by feeding high-phosphorus diets will occur only in older animals in which bone turnover is low and stimulated bone resorption is not easily balanced by increased bone formation.

To increase the phosphorus content of the diets, acid potassium phosphate was used as a supplement and no attempts were made to adjust potassium levels in the control diets. This is why the observed effects cannot theoretically be attributed to phosphate alone. However, we have no information available as to whether differences in the dietary potassium content might explain any of the observed effects.

Effects of dietary calcium. Since the effects of high-phosphorus diets on bone and soft tissue are attributed to the high phosphate flow through the plasma, the intestinal absorption of phosphate is a critical parameter in NSH. Intestinal absorption and urinary excretion of phosphate were significantly higher when the calcium content of the diet was low than when it was normal. An inverse relationship in rats between phosphate absorption and the dietary calcium content has been reported by Clark (7) and was also apparent from studies of Draper and co-workers (2, 6). The inhibiting effect of dietary calcium in this regard is probably related to the formation of insoluble calcium phosphates in the intestinal tract. In the present study the observed effects of feeding high-phosphorus diets were—with the exception of renal calcification and depressed urinary calcium—more marked when the calcium content of the diet was low than when it was normal, stressing the importance of the dietary Ca/P ratio at high phosphorus intake levels. In addition to an inhibiting effect on phosphate absorption, dietary calcium also inhibits intestinal magnesium absorption (7) This might explain why high levels of dietary calcium do not fully counteract the effects of high-phosphorus diets on bone (2). Low levels of dietary magnesium not only predispose to renal stone formation (21) but probably also reduce bone formation, as Lai et al. (22) reported a decreased activity of bone alkaline phosphatase in magnesium deprived rats.

Effects of lactose versus dextrose. With the exception of slightly higher plasma phosphorus values in rats fed lactose after 30 weeks, for which we have no satisfactory explanation, there were no significant effects of the type of dietary carbohydrate on plasma parameters and on the urinary hydroxyproline excretion. During body weight gain, but not thereafter, rats fed lactose absorbed and retained more calcium and phosphorus, and this anabolic effect was reflected by higher bone values for dry fat-free weight and amount of ash. Throughout the study the urinary excretion of calcium was higher in lactose-fed rats. This probably reflects a continuing positive action of lactose on calcium absorption, which could not be detected by the balance method that does not allow the measurement of endogenous fecal calcium. The endogenous fecal route is used by the rat as a regulatory way of calcium excretion (9). The precise mechanism by which lactose stimulates intestinal calcium absorption is not understood as yet. Recent evidence (23) suggests that lactose and several other carbohydrates are able to increase the activity of an intestinal brush border alkaline phosphatase, which is associated with calcium transport, by a facilitated diffusion mechanism. The enzyme stimulation occurs predominantly in the ileum where, according to classical observations (24), the effect of lactose on calcium absorption is most marked.

The failure of lactose to diminish the change in bone quality and plasma parameters, in urinary hydroxyproline excretion and renal calcification produced by feeding the high-phosphorus diets, indicates that enhancement of calcium absorption does not interfere with the detrimental effects of high-phosphorus diets. This observation

is in harmony with a study conducted by Bell et al. (25) who showed that high-protein diets promoted calcium absorption but did not prevent an increased loss of calcium from the bones of rats fed high-phosphorus diets.

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Chapter 8

Effect of dietary calcium and phosphorus on calcium and phosphorus metabolism, bone parameters and renal function in rats

8.1 Introduction

Several investigators have pointed to a low dietary Ca/P-ratio as a factor in the pathogenesis of hyperparathyroidism and osteoporosis (Section 5.10). However, it is not clear whether in this respect similar ratios at different (absolute) levels of the elements have the same physiological significance. Therefore it was found necessary to investigate the effect of the dietary Ca/P-ratio on calcium and phosphorus metabolism and on bone parameters at different levels of both elements. This was the first objective of the study.

The development of nephrocalcinosis in rats fed high-phosphorus diets is considered to be one of the effects of hyperparathyroidism on the kidney. Nephrocalcinosis may impair renal function and further aggravate the hyperparathyroidism. In the previous study (Chapter 7) nephrocalcinosis was found in male lean Zucker rats which were fed, from the age of three months onwards, high-phosphorus diets for 42 weeks, but renal function was not measured. No information is available as to whether the length of the experimental period has any influence on the development of nephrocalcinosis in rats fed high-phosphorus diets. Furthermore, results of studies on the effect of dietary calcium on this phosphate-induced nephrocalcinosis are inconsistent (Section 5.10) Therefore, it was found necessary to investigate the effects of dietary calcium and the length of the experimental period on nephrocalcinosis and renal function in rats fed high-phosphorus diets. This was the second objective of the study.

In two experiments, which only differed in the duration of the experimental period, it was tried to find answers to the two objectives of the study. A short-term experiment (experiment A) was designed particularly to obtain information about the effects of the diet on calcium and phosphorus metabolism, which can be obtained fairly quickly. A long-term experiment (experiment B) was designed particularly to obtain information about the effects of the diet on bone parameters, which requires more time to establish. In both the long-term and short-term experiment the effect of the diet on nephrocalcinosis and renal function was studied. The difference between the results of these experiments would provide information about the effect of the duration of the feeding period in this respect. In order to be able to compare the results of this study with those of the previous one (Chapter 7), rats of the same strain, sex and age were used. From weaning until the time that the rats were used for the experiments, they were fed a commercial diet (RMH-B, Hope Farms N.V., Woerden). This diet contained 1.03% calcium and 0.78% phosphorus.

8.2 Methods

8.2.1 Experiment A (short-term)

Thirty-two male lean Zucker rats, three months old and weighing about 220 g,

were divided into eight groups of four. The animals were given either low-phosphorus control diets (0.15% P; L-diets) or high-phosphorus diets (1.20% P; H-diets). These diets had Ca/P-ratios of either 0.25, 0.50, 1.00 or 2.00. The composition of these diets was similar to that reported in Chapter 7, except that wheat starch and lactose were substituted for dextrose, that $CaCO_3$ and KH_2PO_4 were added to obtain Ca/P-ratios as described above and that Cr_2O_3 was added at a level of 0.5% as a non-absorbable faecal marker. The diets were fed for a period of four weeks during which the animals were kept individually in metabolic cages under the conditions described in Chapter 7. In order to reduce differences in the amount of food consumed, each rat was provided with 15 g food daily. Food consumption was recorded daily and body weights were measured bi-weekly. After 6 and 24 days, 72-hour collections of faeces and urine were made and in the morning of days 10 and 28, after an overnight fast, blood was drawn by orbita puncture under light ether anaesthesia. After the last bleeding the animals were sacrificed and femora, tibiae and kidneys were removed from the bodies. Total ash, dry fat-free weight and volume of the left femur and calcium, phosphorus, plasma alkaline phosphatase and urinary hydroxyproline were estimated as described in Chapter 7. The left kidneys and samples of faeces and food were wet-ashed (with nitric acid and perchloric acid). After wet-ashing, the chromium content of faeces and food was estimated according to the method of Clarkson (1967). For the purpose of balance studies, food intake was estimated by using the following formula:

$$\text{food intake (g/24 hours)} = \frac{\text{conc. } Cr_2O_3 \text{ in faeces}}{\text{conc. } Cr_2O_3 \text{ in food}} \times \text{faeces output (g/24 h)}$$

In plasma and urine, urea was estimated enzymatically and creatinine was estimated by the Jaffé-reaction according to the method designed by the Dutch Normalization Institute (Design NEN 2416, 1972). PTH in plasma was estimated by Dr. R. Bosch, as described in Section 6.2.3.

For bone histology, tibiae (right side) were removed from the bodies, and cleaned of adhering tissue. Cross-sections were made by using a thin-bladed diamond saw which was cooled with physiological water. The bone pieces were fixed in a solution according to Burkhardt. This solution consists of a mixture of formalin, methanol and a glucosephosphate-buffer (pH 7.4). After fixation and dehydration, the proximal parts of the tibiae were embedded in methyl metacrylate. With a heavy Jung-K microtome undecalcified 3-6 μm thick sections were made and stained according to Olah (pentachrome). Osteoblasts, osteoclasts, osteoid and mineralized bone could be distinguished most satisfactorily in this way.

8.2.2 Experiment B (long-term)

With the exception of the following modifications, the design of experiment B was similar to that of experiment A. The diets were fed for a period of 16 weeks. The rats were housed in stainless steel cages with wired bottoms. No collections of urine and faeces were made. Blood was drawn after 6, 44 and 111 days of feeding the experimental diets.

8.2.3 Statistics

Analysis of variance and/or of covariance was applied to test the significance of main and interaction effects. The statistical procedures were those described by

Snedecor & Cochran (1973). Differences between group means were further investigated using Student's t-test (one-sided, unless otherwise indicated). Three levels of significance were used: $P \leq 0.01$, $P \leq 0.05$ and $P \leq 0.10$. This last level was used as indicative of a statistical trend. If variances differed significantly, as indicated by an F-test, logarithmic transformation was applied before subjecting the data to further statistical treatment.

8.3 Results

8.3.1 Experiment A

8.3.1.1 Body weight gain and food consumption (Table 21)

In the previous study (Chapter 7) it was observed that rats fed high-phosphorus diets consumed slightly less food than those fed the control diet. In the present study we fed to all rats a restricted amount of food. It was expected that this amount would be consumed completely. However, it was observed that three groups of rats fed the high-phosphorus diets (H-diets) yet consumed slightly less,

Table 21. Body weight gain (g/4 weeks) and average food consumption (g/24 hours) in experiment A.

	body weight gain[1])		food consumption[1])	
	L-diets[2])	H-diets[2])	L-diets	H-diets
Ca/P				
0.25	41.5	38.5	14.1	14.2
0.50	49.0	30.5	14.4	13.6
1.00	46.2	21.2	14.3	13.6
2.00	44.4	16.5	14.4	13.1
SEM[3])	5.1		–	
ANOV[4])				
diets (F1,24)	22.68***		–	
Ca/P (F3,24)	1.48		–	
diets × Ca/P (F3,24)	2.06		–	

[1]) Values are the means for four rats.
[2]) L-diets: 0.15% phosphorus; H-diets: 1.20% phosphorus.
[3]) SEM derived from pooled error variance (df = 24).
[4]) Analysis of variance; F-test values are given
* = $P \leq 0.10$; ** = $P \leq 0.05$, *** = $P \leq 0.01$.

because they spilled more food. A change-over to paired-feeding was considered but this idea was abandoned, since the spillage appeared to be independent of the amount of food provided. The reduced consumption in rats fed H-diets was closely associated with a lower body weight gain. The mean difference (± SEM) in body weight gain between rats fed the low- and those fed the high-phosphorus diets was 17.1 ± 3.6 g after the four weeks of duration of this experiment ($P < 0.01$).

8.3.1.2 Plasma parameters and urinary hydroxyproline

Calcium (Table 22). There were no significant main effects of dietary variables on the plasma calcium concentration. After six days a significant ($P < 0.05$) diets × Ca/P-interaction was observed: with an increase in the dietary Ca/P-ratio, plasma calcium tended to increase in rats fed the low-phosphorus control diets

Table 22. Plasma calcium (mg/100 ml) in experiment A[1]).

	after 6 days		after 28 days	
	L-diets	H-diets	L-diets	H-diets
Ca/P				
0.25	9.5	9.9	10.3	10.5
0.50	9.4	9.7	10.5	10.4
1.00	9.6	9.6	10.5	10.6
2.00	9.8	9.3	10.5	10.6
SEM	0.18		0.10	
ANOV				
diets (F1,24)	0.20		1.49	
Ca/P (F3,24)	0.39		1.05	
diets × Ca/P (F3,24)	3.18**		0.53	

[1]) See footnotes to Table 21.

Table 23. Plasma phosphorus (mg/100 ml) in experiment A[1]).

	after 6 days		after 28 days	
	L-diets	H-diets	L-diets	H-diets
Ca/P				
0.25	9.0	7.5	8.5	7.2
0.50	8.8	6.8	8.5	6.3
1.00	7.9	7.1	8.4	6.8
2.00	8.6	7.3	8.4	7.1
SEM	0.37		0.36	
ANOV				
diets (F1,24)	29.04***		39.59***	
Ca/P (F3,24)	1.41		0.63	
diets × Ca/P (F3,24)	0.87		0.73	

[1]) See footnotes to Table 21.

Table 24. Tubular reabsorption of phosphate as a ratio to the glomerular filtration rate (TRP/GFR; μmol/ml)[1]) after 28 days in experiment A[2]).

	L-diets	H-diets
Ca/P		
0.25	2.68	1.58
0.50	2.68	1.58
1.00	2.68	1.67
2.00	2.68	2.05
SEM	0.15[3])	
ANOV		
diets (F1,3)	78.97***	
Ca/P (F3,3)	1.14	

[1]) $\text{TRP/GFR} = \frac{\text{creatinine clearance (ml/min)} \times \text{plasma P (μmol/ml)} - \text{urinary P (μmol/min)}}{\text{creatinine clearance (ml/min)}}$

[2]) Values are group means obtained from analyses in samples pooled per group.

[3]) df = 3

*** = $P \leqslant 0.01$

(L-diets) whereas it tended to decrease in rats fed the H-diets. After 28 days plasma calcium values were consistently higher than those after 6 days (mean difference: 0.89 mg/100 ml). The cause of this difference is probably of methodological origin, since it was not observed in the long-term experiment (see results of experiment B).

Total protein. No significant differences were observed between the groups in the plasma total protein concentration after 28 days. The overall mean value (± SEM) was 61.9 ± 0.3 g/l. No values can be given after 6 days, since insufficient plasma was available for this assay. The absence of effects of dietary variables on the concentration of total protein in plasma indicates that it is not necessary to correct plasma calcium values for differences in plasma total protein.

Phosphorus (Table 23). Both after 6 and 28 days the plasma phosphorus concentrations were consistently lower in rats fed H-diets than in those fed L-diets (mean differences ± SEM: 1.40 ± 0.26 and 1.59 ± 0.25 mg/100 ml respectively; both $P < 0.01$).

Parathyroid hormone. The plasma concentration of PTH was estimated in samples, drawn after 28 days. Unfortunately in most samples there was no clear cross-reaction and the assay was lacking the specificity to estimate PTH in Zucker rats. To estimate parathyroid activity, the tubular reabsorption of phosphate, expressed as a ratio to the glomerular filtration rate (TRP/GFR), was computed. The results are shown in Table 24. TRP/GFR was invariably 2.68 μmol/ml in rats fed L-diets. In rats fed H-diets TRP/GFR increased from 1.58 to 2.05 μmol/ml if the Ca/P ratio was raised from 0.25 to 2.00. These data indicate that PTH activity was higher in rats fed H-diets than in those fed L-diets, and that in rats on the former diets this activity decreased with an increase of the Ca/P-ratio.

Alkaline phosphatase (Table 25). Both after 6 and 28 days, but particularly after 6 days, the activity of alkaline phosphatase in plasma was significantly lower in rats fed H-diets than in those fed L-diets. The mean difference (± SEM) after 6 days was 7.5 ± 1.8 U/l ($P < 0.01$, two-tailed) and after 28 days it was 5.4 ± 2.2 U/l ($P < 0.05$, two-tailed). After 6 days, the activity of alkaline phosphatase on either diet was the lowest if the dietary Ca/P-ratio was 0.5. The significance of this observation is obscure since enzyme activity did not change consistently with the dietary Ca/P-ratio. The observations indicate that bone turnover in rats fed H-diets was lower than that in rats fed L-diets. This difference in bone turnover is not attributable to the high phosphorus content of the H-diets but must be related to the higher average calcium content of these latter diets. This conclusion can be derived from a comparison of alkaline phosphatase activity between rats fed diet $L_{2.00}$ and those fed diet $H_{0.25}$, which differ only in their phosphorus content (calcium content 0.3%). Enzyme activities between these groups are not significantly different.

Urinary hydroxyproline (Table 26). Both after 6 and 28 days, the urinary excretion of hydroxyproline which, like plasma alkaline phosphatase, is an indicator of the bone turnover rate, was lower in rats fed H-diets than in those fed L-diets (mean differences ± SEM: 0.11 ± 0.06 mg/24 h; $P < 0.10$, two-tailed and 0.13 ± 0.04 mg/24 h; $P < 0.01$, two-tailed, after 6 and 28 days respectively). Excretion decreased in rats fed H-diets with an increase in the dietary Ca/P-ratio after 6 days. This decrease was observed on both L- and H-diets after 28 days and at that time the mean difference (± SEM) between rats fed the lowest and those fed the highest Ca/P-ratio was 0.14 ± 0.08 mg/24 h on L-diets ($P < 0.05$) and 0.18 ± 0.08 mg/24 h ($P < 0.05$) on H-diets.

Table 25. Plasma alkaline phosphatase (U/l) in experiment A[1]).

	after 6 days		after 28 days	
	L-diets	H-diets	L-diets	H-diets
Ca/P				
0.25	50.0	48.6	47.1	43.2
0.50	42.6	37.7	47.1	34.5
1.00	53.2	41.9	45.1	38.8
2.00	54.8	42.4	42.0	43.4
SEM	2.6		3.1	
ANOV				
diets (F1,24)	16.90***		6.10***	
Ca/P (F3,24)	5.36***		0.72	
diets × Ca/P (F3,24)	2.07		1.80	

[1]) See footnotes to Table 21.

Table 26. Urinary hydroxyproline in experiment A[1]).

	hydroxyproline (mg/24 hours)				hydroxyproline/creatinine (mg/g)[2]) after 28 days	
	after 6 days		after 28 days			
	L-diets	H-diets	L-diets	H-diets	L-diets	H-diets
Ca/P						
0.25	0.83	1.00	0.75	0.69	52.2	52.7
0.50	1.02	0.74	0.75	0.59	52.0	46.8
1.00	0.90	0.76	0.69	0.47	47.7	41.0
2.00	0.88	0.70	0.61	0.51	42.6	41.8
SEM	0.08		0.05		2.4[3])	
ANOV						
diets (F1,24)	3.43*		11.70***		F1,3 = 3.13	
Ca/P (F3,24)	0.90		3.63**		F3,3 = 7.35*	
diets × Ca/P (F3.24)	2.88*		0.80		—	

[1]) See footnotes to Table 21.
[2]) Values of hydroxyproline/creatinine are group means obtained from analyses in samples pooled per group.
[3]) df = 3.

Table 27. Plasma urea (mg/100 ml) in experiment A[1]).

	after 6 days		after 28 days	
	L-diets	H-diets	L-diets	H-diets
Ca/P				
0.25	31.8	37.0	30.2	37.0
0.50	32.0	36.4	29.5	36.8
1.00	27.4	40.0	28.3	41.5
2.00	31.3	35.2	29.6	37.8
SEM	2.3		2.0	
ANOV				
diets (F1,24)	15.41***		39.15***	
Ca/P (F3,24)	0.09		0.28	
diets × Ca/P (F3,24)	1.52		1.07	

[1]) See footnotes to Table 21.

To account for differences in body weight, hydroxyproline excretion was computed as the ratio to creatinine excretion. Values of this ratio decreased with an increase of the dietary Ca/P-ratio. Rats fed diet $L_{2.00}$ had a significantly lower urinary hydroxyproline/creatinine-ratio than did rats fed diet $H_{0.25}$ (mean difference ± SEM: 10.1 ± 3.45 mg/g; $P < 0.05$). Since the calcium content of these diets was equal (0.3%), this difference can be attributed to dietary phosphorus (0.15 versus 1.20%).

8.3.1.3 Parameters on renal function

Plasma urea (Table 27). Both after 6 and 28 days the plasma concentrations of urea were significantly higher in rats fed H-diets than in those fed L-diets and this difference tended to increase with the duration of the experiment (mean differences ± SEM after 6 and 28 days: 6.5 ± 1.6 and 8.9 ± 1.4 mg/100 ml respectively; both $P < 0.01$). Since similar differences were observed between rats fed diet $L_{2.00}$ and those fed diet $H_{0.25}$ (the Ca content of these diets is equal) the difference in plasma urea values is attributable to the phosphorus content of the diets. No significant main effect of the Ca/P-ratio was observed.

Table 28. Creatinine and urea clearance (ml/min) after 28 days in experiment A[1]).

	creatinine clearance[2])		urea clearance	
	L-diets	H-diets	L-diets	H-diets
Ca/P				
0.25	2.85	2.55	2.12	1.74
0.50	2.90	2.77	2.49	1.61
1.00	2.87	2.43	2.53	1.58
2.00	3.09	2.63	2.44	1.63
SEM	0.11[3])		0.14	
ANOV				
diets	$F(1,3) = 18.99^{***}$		$F(1,24) = 55.43^{***}$	
Ca/P	$F(3,3) = 1.79$		$F(3,24) = 0.34$	
diets × Ca/P	–		$F(3,24) = 1.62$	

[1]) See footnotes to Table 21.
[2]) Values of creatinine clearance are group means obtained from analyses in plasma and urine samples pooled per group.
[3]) df = 3.

Urea and creatinine clearances (Table 28). Clearances of urea and creatinine were only estimated after 28 days. They were found to be significantly lower in rats fed the H- than in those fed the L-diets (mean differences ± SEM: 0.33 ± 0.16 ml/min; $P < 0.05$ and 0.76 ± 0.10 ml/min; $P < 0.01$, for the creatinine and urea clearance respectively). No significant main effect of the dietary Ca/P-ratio was observed: however, the lowest clearance values (and the highest plasma urea values) were those in rats fed diet $H_{1.00}$.

8.3.1.4 Calcium metabolism (Table 29)

In rats fed L-diets, retention and faecal excretion of calcium increased with the dietary Ca/P-ratio (and thus with Ca-intake) during both week 2 and week 4 and this was associated with an increase in urinary calcium, particularly during week

Table 29. Parameters of calcium metabolism (mg/24 hours) during week 2 and week 4 in experiment A[1]).

	L-diets				H-diets			
	intake	faeces	urine	retention	intake	faeces	urine	retention
				week 2				
Ca/P								
0.25	5.9	3.6	0.38	2.0	33	24	0.47	8.2
0.50	10.3	5.3	0.41	4.7	58	50	0.46	7.9
1.00	17.4	9.8	0.56	7.0	113	103	0.53	9.2
2.00	34.8	23.2	0.50	11.1	233	218	0.73	14.8
SEM[2])	—	1.2	0.03	0.6	—	9	0.03	2.2
				week 4				
Ca/P								
0.25	4.2	0.8	0.31	3.1	32	21	0.38	10.6
0.50	9.7	1.9	0.36	7.5	68	52	0.37	15.1
1.00	18.8	7.3	0.45	11.2	136	124	0.40	11.2
2.00	35.7	24.1	0.52	11.1	248	233	0.85	13.9
SEM[2])	—	0.6	0.30	0.8	—	7	0.05	1.3

[1]) Values are the means for four rats.
[2]) SEM derived from pooled error of analysis of variance of data on L-diets and H-diets separately.

Table 30. Parameters of phosphorus metabolism (mg/24 hours) during week 2 and week 4 in experiment A[1]).

	L-diets				H-diets			
	intake	faeces	urine	retention	intake	faeces	urine	retention
				week 2				
Ca/P								
0.25	19.3	6.3	16.1	— 3.1	134	26	158	— 51
0.50	17.9	5.2	13.5	— 0.8	128	37	118	— 27
1.00	18.1	6.0	11.3	0.8	122	53	92	— 23
2.00	17.6	7.3	5.8	4.1	124	60	61	2
SEM	—	0.7	0.8	1.0	—	3	10	7
				week 4				
Ca/P								
0.25	14.0	3.0	7.0	4.0	130	25	78	26
0.50	16.8	3.7	5.0	8.1	149	38	49	61
1.00	19.7	4.9	5.5	9.3	146	73	46	27
2.00	18.1	7.7	2.8	7.6	130	82	21	26
SEM	—	0.4	0.6	1.2	—	4	4	5

[1]) See footnote to Table 29.

4. At that time, but not yet during week 2, the increase in calcium retention with calcium intake flattened to a dietary Ca/P-ratio over 1. The phenomenon that calcium retention on the dietary Ca/P-ratios below 1 was lower during week 2 than that during week 4, shows that the adaptive response to diets low in calcium was not yet complete during week 2.

In rats fed H-diets, faecal calcium increased with the dietary Ca/P-ratio. On this diet, urinary calcium increased with the dietary Ca/P-ratio only if this ratio exceeded the value 0.5. Calcium retention increased very slightly with the dietary Ca/P-ratio during week 2 but this increase was no longer observed during week 4.

During week 2, calcium retention on diet $L_{2.00}$ was superior to that on the diets $H_{0.25}$, $H_{0.50}$ and $H_{1.00}$, in spite of a similar or higher calcium content of these latter diets. This difference in calcium retention was not observed during week 4. At that time the urinary calcium excretion on the H-diets was lower than that on diet $L_{2.00}$.

The results with respect to calcium metabolism indicate that dietary phosphorus excess did not adversely influence either intestinal absorption or retention of calcium to a significant extent. They also indicate that calcium retention in rats fed L-diets is significantly decreased if the dietary Ca/P-ratio is 0.25 or 0.50. Moreover, the results indicate that the urinary calcium excretion is decreased in rats fed H-diets.

8.3.1.5 Phosphorus metabolism (Table 30)

On both L- and H-diets, the urinary excretion of phosphorus decreased and the faecal excretion of phosphorus increased when the dietary Ca/P-ratio was raised. As a consequence, net intestinal phosphorus absorption decreased with an increase in this ratio. For instance, during week 4 the absorption, expressed as a percentage of phosphorus intake, was 91% on diet $H_{0.25}$ whereas it was only 37% on diet $H_{2.00}$.

During week 2, phosphorus retention on both L- and H-diets increased with the dietary Ca/P-ratio as did calcium retention. However, at that time phosphorus retention was found to be markedly negative in rats fed the H-diets with Ca/P-ratios between 0.25 and 1.00. The cause of this negative retention is probably an artefact. Since urinary phosphorus excretion on these diets is high, small inaccuracies in the collection time of urine have substantial effects on the phosphorus balance. Anyhow, retention values were clearly positive during week 4.

8.3.1.6 Bone parameters and kidney calcium content

Results on bone parameters and kidney calcium content will be reported in conjunction with those of experiment B.

8.3.2 Experiment B

8.3.2.1 Body weight gain and food consumption (Table 31)

Body weight gain and food consumption were slightly lower in rats fed H-diets than in those fed L-diets. The mean difference in body weight gain (± SEM) between these rats was 13.6 ± 5.3 g/16 weeks. These results compare favourably with those of experiment A, although the differences in body weight gain and food consumption were smaller in experiment B.

8.3.2.2 Plasma parameters

Calcium (Table 32). There were no significant differences in the plasma calcium

Table 31. Body weight gain (g/16 weeks) and average food consumption (g/24 hours) in experiment B[1]).

	body weight gain		food consumption	
	L-diets	H-diets	L-diets	H-diets
Ca/P				
0.25	80.8	74.0	14.7	14.4
0.50	100.8	78.3	14.8	14.6
1.00	80.5	74.8	14.5	14.3
2.00	78.8	59.3	14.2	14.1
SEM	7.5		—	
ANOV				
diets (F1,24)	6.54***		—	
Ca/P (F3,24)	2.50		—	
diets × Ca/P (F3,24)	0.65		—	

[1]) See footnotes to Table 21.

Table 32. Plasma calcium (mg/100 ml) in experiment B[1]).

	after 6 days		after 44 days		after 111 days	
	L-diets	H-diets	L-diets	H-diets	L-diets	H-diets
Ca/P						
0.25	10.5	10.5	10.5	10.2	10.1	10.0
0.50	10.1	10.3	10.0	9.9	10.0	9.8
1.00	10.4	10.6	10.1	10.5	10.2	10.1
2.00	10.3	10.6	10.7	10.5	9.8	10.0
SEM	0.12		0.27		0.12	
ANOV						
diets (F1,24)	3.70*		0.02		0.09	
Ca/P (F3,24)	3.30**		1.89		1.96	
diets × Ca/P (F3,24)	0.30		0.66		1.15	

[1]) See footnotes to Table 21.

Table 33. Plasma phosphorus (mg/100 ml) in experiment B[1]).

	after 6 days		after 44 days		after 111 days	
	L-diets	H-diets	L-diets	H-diets	L-diets	H-diets
Ca/P						
0.25	7.7	7.0	7.5	6.0	6.6	4.6
0.50	7.6	7.2	7.0	5.4	6.0	5.1
1.00	7.3	6.7	7.0	6.2	6.3	5.4
2.00	7.5	7.6	7.3	6.3	6.0	5.7
SEM	0.24		0.32		0.28	
ANOV						
diets (F1,24)	4.83**		26.31***		25.96***	
Ca/P (F3,24)	1.83		1,40		0.61	
diets × Ca/P (F3,24)	0.96		1.43		3.16**	

[1]) See footnotes to Table 21.

concentration between groups after 44 and 111 days. After 6 days, values in rats fed L-diets tended to be lower than those in rats fed H-diets (mean difference ± SEM: 0.16 ± 0.08 mg/100 ml; $P < 0.10$, two-tailed). Although analysis of variance indicated a statistically significant main effect of the dietary Ca/P-ratio, no systematic changes in plasma calcium could be observed in this respect. Since results on plasma calcium after 6 days in experiment A were different, it seems unlikely that differences in plasma calcium between groups are attributable to diet.

Total protein. No significant differences in the plasma total protein concentration were observed after 44 and 111 days. The overall mean values (± SEM) were 62.4 ± 0.7 and 63.8 ± 1.5 g/l respectively. No values can be given after 6 days since insufficient plasma was available for the estimation.

Phosphorus (Table 33). During the whole of the study the concentration of phosphorus in plasma was significantly lower in rats fed H-diets than in those fed L-diets. Mean differences (± SEM) after 6, 44 and 111 days were 0.38 ± 0.17 ($P < 0.05$), 1.23 ± 0.23 ($P < 0.01$) and 1.03 ± 0.20 mg/100 ml ($P < 0.05$) respectively. After 111 days, but not at earlier times, plasma phosphorus showed a slight but clear-cut increase with the dietary Ca/P-ratio in rats fed H-diets. In this respect the mean difference (± SEM) between rats fed the $H_{2.00}$- and those fed the $H_{0.25}$-diet was 1.08 ± 0.40 mg/100 ml ($P < 0.01$).

Alkaline phosphatase (Table 34). The activity of alkaline phosphatase in plasma was found to be slightly lower in rats fed H-diets than in those fed L-diets. After 6, 44 and 111 days, the differences (± SEM) were 6.3 ± 2.0 ($P < 0.01$, two-tailed), 4.0 ± 1.08 ($P < 0.01$, two-tailed) and 2.8 ± 1.5 U/l ($P < 0.10$, two-tailed) respectively. These differences thus tended to fade away with advancing age. On both L- and H-diets, alkaline phosphatase showed a slight decrease with an increase of the dietary Ca/P-ratio after 6 and 44 days. This effect was no longer observed after 111 days.

As in experiment A, these results indicate that the bone turnover, as measured by alkaline phosphatase activity in plasma, was slightly lower in rats fed H-diets than in those fed L-diets. This difference is attributable to the higher average calcium content of the H-diets. This conclusion can be drawn from a comparison of alkaline phosphatase activity between rats fed the $L_{2.00}$-diet and those fed the $H_{0.25}$-diet. Enzyme activities on these diets, which differ only in their phosphorus content, are not significantly different.

Urea (Table 35). Both after 44 and 111 days the plasma urea concentration was significantly lower in rats fed L-diets than in those fed H-diets. Mean differences (± SEM) were 5.2 ± 1.3 and 9.7 ± 1.8 mg/100 ml (both $P < 0.01$). The highest plasma urea concentration was observed in rats fed the $H_{1.00}$-diet. After 111 days, this concentration (46.3 mg/100 ml) was significantly higher than that in rats fed the other diets. In rats fed the H-diets the plasma concentration of urea increased with the duration of the experiment.

8.3.2.3 Kidney calcium content (experiments A and B, Table 36)

There was a wide variation in the kidney calcium content of rats fed H-diets in both experiments A and B. Values ranged from 120 to 1520 μg/g fresh tissue. The kidney calcium content in rats fed L-diets was lower and much less variable (range: 70 to 180 μg/g). Because of the high variability on H-diets, values were transformed into their ^{10}log-derivations for statistical treatment. In Table 36 it can be observed that the transformed values were significantly higher in rats fed H-

Table 34. Plasma alkaline phosphatase (U/l) in experiment B[1]).

	after 6 days		after 44 days		after 111 days	
	L-diets	H-diets	L-diets	H-diets	L-diets	H-diets
Ca/P						
0.25	66.9	61.1	31.0	26.6	33.4	29.6
0.50	62.0	58.3	29.0	25.0	33.5	27.6
1.00	59.8	49.4	26.7	22.9	32.4	34.2
2.00	58.6	53.0	27.3	23.6	32.9	30.0
SEM	2.8		1.5		2.1	
ANOV						
diets (F1,24)	10.02***		13.56***		3.67*	
Ca/P (F3,24)	4.55**		2.73*		0.61	
diets × Ca/P (F3,24)	0.50		0.02		1.28	

[1]) See footnotes to Table 21.

Table 35. Plasma urea (mg/100 ml) in experiment B[1]).

	after 44 days		after 111 days	
	L-diets	H-diets	L-diets	H-diets
Ca/P				
0.25	30.1	30.7	31.3	34.0
0.50	28.4	34.3	31.6	38.3
1.00	27.7	36.9	27.0	46.3
2.00	28.9	34.1	27.8	38.1
SEM	1.9		2.6	
ANOV				
diets (F1,24)	15.70***		26.73***	
Ca/P (F3,24)	0.34		1.05	
diets × Ca/P (F3,24)	1.83		3.78**	

[1]) See footnotes to Table 21.

Table 36. Kidney calcium content in experiments A and B ([10]log-derivations of μg/g fresh tissue[1]).

	experiment A		experiment B	
	L-diets	H-diets	L-diets	H-diets
Ca/P				
0.25	2.13	2.70	2.03	2.26
0.50	2.09	2.56	1.98	2.79
1.00	2.14	2.98	1.97	3.00
2.00	2.15	2.24	1.98	2.53
SEM[2])	0.09			
ANOV				
diets (F1,48)	18.28***			
Ca/P (F3,48)	9.24***			
experiments (F1,48)	1.69			
Ca/P × diets (F3,48)	10.69***			
experiments × diets (F1,48)	3.55*			
experiments × Ca/P (F3,48)	3.35**			
experiments × diets × Ca/P (F3,48)	4.31***			

[1]) See footnotes to Table 21.
[2]) SEM derived from pooled error variance (df = 48).

than in those fed L-diets in both experiments A and B. The mean difference (± SEM) was 0.58 ± 0.04 μg/g ($P < 0.01$, experiments A and B combined). The dietary Ca/P-ratio significantly, but not systematically, influenced the kidney calcium content: as with plasma urea, peak values were observed in rats fed the $H_{1.00}$-diet. In rats fed L-diets, the kidney calcium content was significantly lower in experiment B than in experiment A (mean difference of ^{10}log-values ± SEM: 0.14 ± 0.06 μg/g; $P < 0.05$, two-tailed). This phenomenon was not observed in rats fed H-diets (mean difference ± SEM: −0.02 ± 0.06 μg/g; n.s.). From a comparison of values in rats fed the $L_{2.00}$-diet with those in rats fed the $H_{0.25}$-diet, it must be concluded that the higher values on the latter diet can be attributed to dietary phoshorus, since the calcium content ot both diets was equal (0.3%).

The higher kidney calcium content in rats fed H-diets was associated with an increased relative weight of this organ. In experiment A the weight of the left kidney, expressed as a percentage of the body weight (± SEM) was 0.396 ± 0.09% on L-diets and 0.452 ± 0.09% on H-diets. In experiment B these values were 0.391 ± 0.08 and 0.449 ± 0.07% respectively. The differences in relative kidney weight between rats fed the L- and those fed the H-diets were statistically significant ($P < 0.01$).

The kidney calcium content in rats fed H-diets was significantly correlated with the phosphorus content of this organ. In experiment A the correlation between these variables (x = Ca in mg/g fresh tissue; y = P in mg/g fresh tissue) could be described as: $y = 0.42 x + 2.56$ ($r = 0.83$; $P < 0.01$) and in experiment B as: $y = 0.44 x + 2.34$ ($r = 0.92$; $P < 0.01$). The slopes of these lines are very close to the weight ratio between P and Ca in apatite ($Ca_{10}(PO_4)_6 \cdot (OH)_2$) which is 0.46.

There was a significant positive correlation ($r = 0.74$; $P < 0.01$) between ^{10}log-values of the kidney calcium content and the plasma urea concentration in rats fed H-diets in experiment B. In experiment A this correlation was not statistically significant ($r = 0.33$; $P < 0.10$). In rats on L-diets in both experiments A and B, plasma urea varied independently of the kidney calcium content.

8.3.2.4 Bone parameters (experiments A and B)

Bone quantity (left femur, Table 37). Since the bone mass is related to the body weight and since differences between groups in body weight gain occurred, bone quantity parameters were adjusted to the overall final mean body weight in experiment A and that in experiment B. This was done by regression of body weight on these bone parameters. The regression coefficients (the bs) were obtained from analyses of covariance. For the adjustments the following formula was used:

$$y_{adj} = y_{obs} - b(x_{obs} - \bar{x})$$

with y = bone parameter and x = body weight.

In rats fed L-diets, values on dry fat-free weight and total ash increased markedly with the Ca/P ratio in both experiments. This increase also occurred in rats fed H-diets, though to a lesser extent. For instance in experiment A and in experiment B, the differences (± SEM) in femur ash between rats fed the $L_{0.25}$- and those fed the $L_{2.00}$-diet were 34 ± 8 and 72 ± 8 mg respectively (both $P < 0.01$) and between rats fed the $H_{0.25}$- and those fed the $H_{2.00}$-diet, these respective differences were 10 ± 8 ($P < 0.10$) and 21 ± 8 mg ($P < 0.01$).

In both experiments A and B, values on dry fat-free bone weight and total bone ash were higher in rats fed H-diets than those in rats fed L-diets. These differences

were mainly caused by the low values in rats fed the $L_{0.25}$- and $L_{0.50}$-diets. The marked increase in total ash and dry fat-free weight of the femur with the dietary Ca/P-ratio in rats fed L-diets as well as the low values of these parameters in rats fed the $L_{0.25}$- and $L_{0.50}$-diets, indicate that these latter animals suffered from calcium deficiency. As is indicated in Table 37, values on total ash and dry fat-free weight were slightly higher in rats fed the $L_{2.00}$-diet than those in rats fed the $H_{0.25}$-diet, in both experiments A and B. These differences were not statistically significant. Since the calcium content of these latter diets was similar (0.3%), this observation indicates that dietary phosphorus excess did not adversely influence the bone mass to a significant extent.

Bone quality (left femur, Table 38). Bone quality parameters increased with the dietary Ca/P-ratio in rats fed the L-diets. In rats fed the H-diets this increase was

Table 37. Bone quantity parameters (mg, left femur) in experiments A and B[1] [2]).

	dry fat-free weight	total ash	fat-free org. matter
experiment A			
L-diets, Ca/P			
0.25	345	215	130
0.50	351	224	127
1.00	376	245	131
2.00	388	249	139
H-diets, Ca/P			
0.25	381	245	137
0.50	381	246	135
1.00	394	258	136
2.00	395	255	139
experiment B			
L-diets, Ca/P			
0.25	406	258	148
0.50	447	289	158
1.00	474	312	162
2.00	496	330	166
H-diets, Ca/P			
0.25	484	320	164
0.50	494	327	168
1.00	507	336	172
2.00	508	341	167
SEM[3])	8.2	5.6	3.3
ANOV			
diets (F1,46)	52.81***	74.50***	12.23***
Ca/P (F3,46)	20.33***	26.94***	5.98***
experiments (F1,46)	44.49***	49.92***	21.23***
Ca/P × diets (F3,46)	3.93**	4.53***	3.23**
experiments × diets (F1,46)	0.00	0.17	0.23
experiments × Ca/P (F3,46)	1.99	2.03	2.05
experiments × diets × Ca/P (F3,46)	3.11**	2.97**	6.14***

[1]) See footnotes to Table 21.

[2]) Values are adjusted to the overall mean body weight of 242.5 g in experiment A and of 280.7 g in experiment B. Regression coefficients (± SEM) for the respective bone parameters are 0.58 ± 0.25, 0.29 ± 0.16 and 0.28 ± 0.11 in experiment A (all $P < 0.05$), and 1.71 ± 0.20, 1.22 ± 0.14 and 0,50 ± 0.08 in experiment B (all $P < 0.01$).

[3]) SEM derived from pooled error variance (df = 46).

only slight. The overall mean values of bone quality parameters in rats fed the H-diets were superior to those in rats fed the L-diets in both experiments A and B. This superiority is attributable to low values in rats fed the $L_{0.25}$- and $L_{0.50}$-diets. These rats suffered from calcium deficiency. Bone quality parameters in rats fed the $L_{1.00}$- or $L_{2.00}$-diet did not differ significantly from those in rats fed the H-diets, neither in experiment A nor in experiment B. This observation indicates that dietary phosphorus excess did not adversely influence the bone quality parameters to a significant extent.

In general, values for bone quality parameters in experiment B were higher than those in experiment A. This reflects the maturation of the bone tissue. For instance the mean value (± SEM) for the ratio total ash/fat-free organic matter in rats fed L-diets increased from 1.76 ± 0.02 in experiment A to 1.85 ± 0.02 in experiment B.

Table 38. Bone quality (left femur) in experiments A and B[1]).

	dry fat-free weight	total ash	total ash
	volume	volume	fat-free organic matter
experiment A			
L-diets, Ca/P			
0.25	0.91 g/cm³	571 g/cm³	1.67
0.50	0.96	611	1.74
1.00	1.00	650	1.86
2.00	1.01	653	1.78
H-diets, Ca/P			
0.25	0.94	641	1.79
0.50	1.04	655	1.83
1.00	1.03	684	1.91
2.00	1.06	668	1.87
experiment B			
L-diets, Ca/P			
0.25	1.00	602	1.77
0.50	1.02	677	1.88
1.00	1.04	683	1.94
2.00	1.03	693	1.88
H-diets, Ca/P			
0.25	1.03	682	1.94
0.50	1.06	701	1.94
1.00	1.03	678	1.95
2.00	1.07	717	2.01
SEM	0.015	12	0.031
ANOV			
diets (F1,48)	28.02***	34.41***	32.29***
Ca/P (F3,48)	18.51***	17.62***	11.13***
experiments (F1,48)	24.95***	36.95***	49.54***
Ca/P × diets (F3,48)	6.05***	4.93***	2.39
experiments × diets (F1,48)	1.32	0.59	0.12
experiments × Ca/P (F3,48)	2.36	2.15	0.99
experiments × diets × Ca/P (F3, 48)	0.86	0.95	0.58

[1]) See footnotes to Table 21.

Bone histology (right tibia, Table 39). Sections from tibiae from all rats fed diets $L_{0.25}$, $L_{2.00}$, $H_{0.25}$ and $H_{2.00}$ were judged by Dr. W. Visser, who is an experienced investigator in bone histology, and by the writer of this thesis. The area of interest was uniformly that beneath the epiphyseal line in the metaphyseal trabecular bone. Semi-quantitative estimations were made of the number and size of the trabeculae, the bone turnover rate (lines of osteoclastic activity along the trabeculae) and of the amount of osteoid. The procedure was as follows. Dr. Visser selected one of the sections that showed a histologic picture that was not different from that of a normal rat and this section was used as a reference. All sections were studied without knowledge of the experimental situation. When the investigators had come to agreement about a particular section, it was judged in terms of signs. These were (in decreasing order) + +, + ±, +, ±, −, ± −, and −− (± = normal)

In experiment A the amount of trabecular bone was considered to be below normal in all groups. It tended to be higher in rats fed a Ca/P-ratio of 2.00 than in those fed a ratio of 0.25. Bone turnover was found to be normal in rats fed L-diets, but in rats fed H-diets bone turnover was slightly increased.

In experiment B the amount of trabecular bone was found to be slightly below normal in all groups except in the group fed diet $H_{2.00}$ where it was normal. Bone turnover and amount of osteoid were slightly elevated in rats fed diet $L_{0.25}$. In the other groups these parameters were normal.

In addition to the observations with respect to the bone mass and bone density, it can be concluded from these histological results that rats fed the low-phosphorus control diets developed osteoporosis as a consequence of calcium deficiency if the dietary Ca/P-ratio was low (either 0.25 or 0.50).

Table 39. Bone histological parameters (right tibia) in experiments A and B.

	Ca/P	rat No.	experiment A			rat No.	experiment B		
			trabeculae	turnover	osteoid		trabeculae	turnover	osteoid
L-diets	0.25	102	− −	±	±	360	−	+	±
		130	− −	±	±	376	−	+ +	+
		140	± −	±	±	380	−	±	±
		037	±	±	+	092	± −	± +	± +
		mean	−	±	±	mean	−	+	± +
	2.00	123	−	±	±	377	−	±	±
		132	±	±	±	385	− −	±	±
		009	−	±	−	372	± −	±	±
		112	±	±	±	373	±	±	±
		mean	± −	±	±	mean	−	±	±
H-diets	0.25	103	− −	+	−	345	− −	± +	±
		131	− −	−	−	002	−	−	−
		042	−	+	±	086	±	±	±
		051	− −	+	±	095	±	±	±
		mean	− −	+	± −	mean	−	±	±
	2.00	124	± −	± +	±	378	±	±	±
		143	−	±	−	397	±	+	±
		080	− −	+	−	087	±	±	±
		085	±	±	±	094	± −	±	±
		mean	−	± +	± −	mean	±	±	±

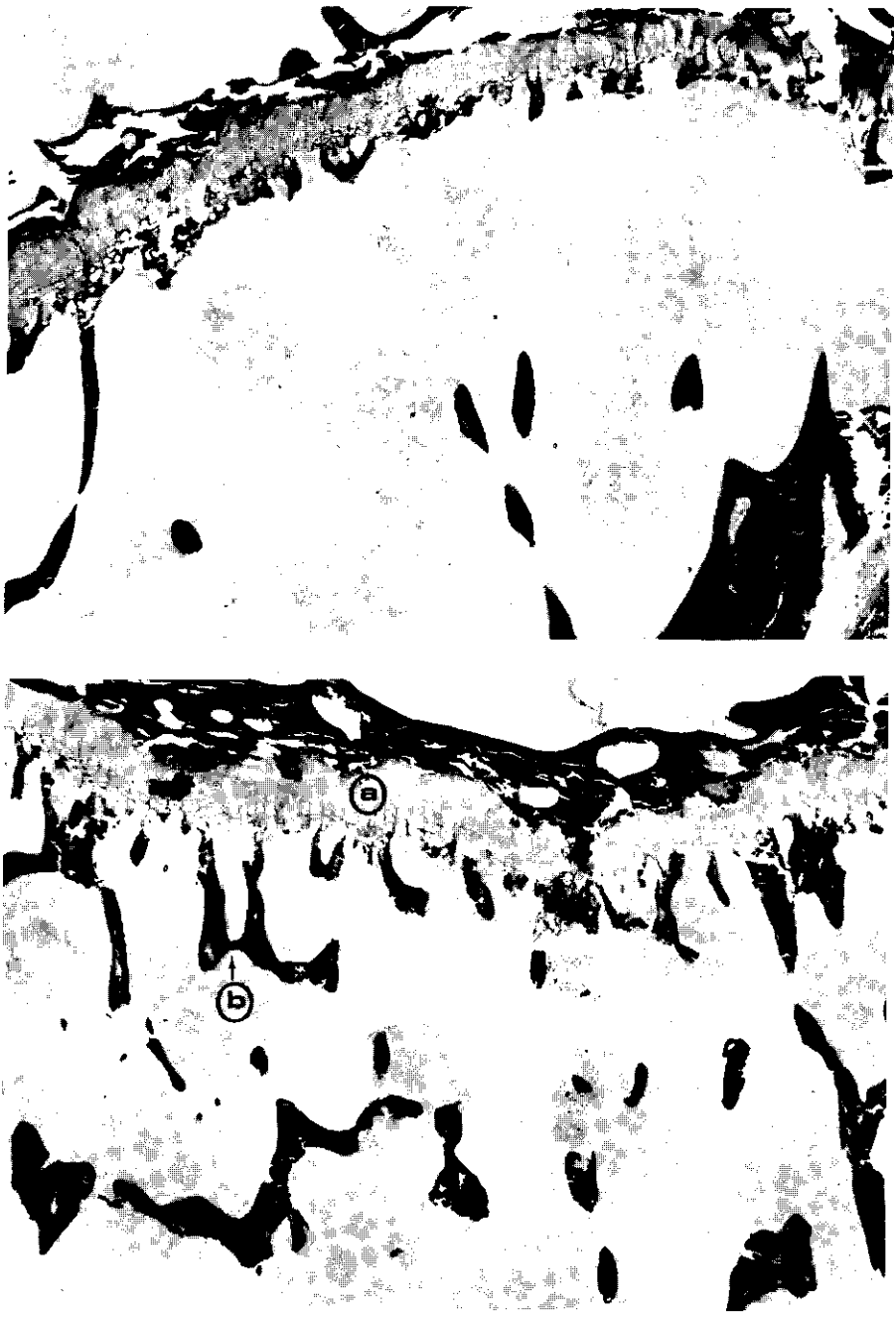

Fig. 6. Cross-sections of the proximal part of the tibia, magnified 320 times. The upper photograph (rat No. 345) shows a reduced amount of trabecular bone below the epiphyseal line. The lower photograph (rat No. 397) shows a normal number of trabeculae; the epiphyseal line and a trabecula are indicated by (a) and (b) respectively.

8.3.3 Abstract of results

8.3.3.1 Body weight gain and food consumption

In both the short- and long-term experiment, body weight gain was significantly lower ($P < 0.01$) in rats fed the high-phosphorus diets (H) than in those fed the low-phosphorus control diets (L). This difference in body weight gain was attributable to a reduced food intake.

8.3.3.2 Biochemical parameters

Plasma concentrations of calcium and total protein were not systematically or significantly influenced by dietary variables. Plasma phosphorus was significantly ($P < 0.01$ or $P < 0.05$) decreased in rats fed the H-diets during the whole of the experiments. At the end of the long-term experiment, the severity of this hypophoshataemia decreased with an increase of the dietary Ca/P-ratio. In the short-term experiment, PTH activity, as measured by the tubular reabsorption of phosphate per ml glomerular filtrate (TRP/GFR), was higher in rats fed the H-diets than in those fed the L-diets. On these former diets, PTH activity decreased with an increase of the dietary Ca/P-ratio. The activity of alkaline phosphatase in plasma was significantly ($P < 0.01$ or $P < 0.05$) lower in rats fed the H-diets than in those fed the L-diets in both experiments. This difference in alkaline phosphatase activity was attributable to the lower calcium content of the latter diets. A slight decrease in enzyme activity with an increase of the dietary Ca/P-ratio was observed on both diets during the first part of the long-term study. The observed differences in alkaline phosphatase activity decreased with the duration of the long-term experiment. In the short-term experiment, the urinary hydroxyproline excretion was lower ($P < 0.10$ or $P < 0.01$) in rats fed the H-diets than in those fed the L-diets. This difference was attributable to the lower calcium content of these latter diets. On either diet the excretion of hydroxyproline decreased with an increase of the dietary Ca/P-ratio. The hydroxyproline/creatinine ratio in urine was significantly ($P < 0.05$) higher in rats fed the H-diets than in those fed the L-diets if the calcium content of these diets was similar (0.3%).

8.3.3.3 Calcium and phosphorus metabolism

An increase in the Ca/P-ratio of either the H-diets or the L-diets inhibited the intestinal absorption and decreased the urinary excretion of phosphorus. The urinary calcium excretion was found to be depressed in rats fed the H-diets. With an increase of the dietary Ca/P-ratio, intestinal absorption and retention of calcium markedly increased in rats fed the L-diets. In rats fed the H-diets, this increase in absorption and retention of calcium was only slight. Dietary phosphorus excess did not adversely influence either calcium absorption or calcium retention to a significant extent.

8.3.3.4 Bone parameters

Parameters of bone quantity as well as those of bone quality increased clearly with the dietary Ca/P-ratio in rats fed the L-diets.

In rats fed the H-diets, this increase was only slight. In rats on the L-diets, osteoporosis developed as a consequence of calcium deficiency if the Ca/P-ratio of the diet was low (either 0.25 or 0.50). It was found that dietary phosphorus excess did not adversely influence either the quality or the quantity of the bone to a significant extent. Histologically, only small differences between groups were observed. In the short-term experiment, bone turnover was slightly increased in

rats fed the H-diets. On either diet, the amount of trabecular bone tended to be higher if the dietary Ca/P-ratio was 2.00 than when it was 0.25. In the long-term experiment, bone turnover and amount of osteoid were slightly elevated in rats fed the L-diet with a Ca/P-ratio of 0.25.

8.3.3.5 Renal function
In both experiments, feeding the H-diets resulted in a rapid (within one week) but moderate loss of renal function, as measured by the clearance rates of urea and creatinine and/or the plasma urea concentration. The loss of renal function which increased slowly with the duration of the studies was attributable to the deposition of calcium apatite in the kidneys (nephrocalcinosis). The extent of renal calcification varied with the calcium content of the diet. It increased if the calcium content was raised from 0.3 to 1.2% (Ca/P from 0.25 to 1.00), but decreased when the calcium content of the diet was raised to 2.4% (Ca/P to 2.00).

8.3.4 Discussion of results
According to the outline in Figure 5 (Section 5.11), either a low-calcium diet, a high-phosphorus diet or a diet with a low Ca/P-ratio decreases the plasma-ionized calcium concentration (temporarily) so that the parathyroid glands are stimulated to secrete parathyroid hormone (PTH). This hormone acts on bone and kidney in order to restore the plasma-ionized calcium concentration and this may proceed at the expense of skeletal calcium so that osteoporosis develops. It was explained (Figure 5) why the stimulation of PTH secretion by the diet occurs particularly when renal function is decreased. In the present experiments rats were given high-phosphorus diets (H) and low-phosphorus diets (L). These diets were provided with four different Ca/P-ratios (0.25, 0.50, 1.00 and 2.00) during either a long- or short-term experiment. By these experiments we were able to investigate the effect of dietary phosphorus excess and that of the dietary Ca/P-ratio (at different levels of both elements) on the metabolism of calcium, phosphorus and bone. We were also able to investigate the effect of dietary calcium on nephrocalcinosis and subsequent loss of renal function, induced by feeding high-phosphorus diets.

8.3.4.1 Dietary phosphorus
As could have been expected, the plasma calcium concentration was not clearly influenced by dietary phosphorus excess. On the other hand, it was observed that in rats fed the H-diets the tubular reabsorption of phosphate (TRP/GFR), the plasma phosphorus concentration and the urinary calcium excretion were decreased. Moreover, in these rats nephrocalcinosis developed. These biochemical changes in rats fed the H-diets reflect the effect of hyperparathyroidism on the kidney. The urinary excretion of hydroxyproline (expressed as a ratio to creatinine) was significantly higher in rats fed the H-diet with 0.3% calcium (Ca/P = 0.25) than in those fed the L-diet with the same calcium content (Ca/P = 2.00). Histologically, bone turnover was found to be slightly higher in rats fed the H-diets than in those fed the L-diets. These observations reflect the effect of hyperparathyroidism on bone. The intestinal calcium absorption, calcium retention and parameters of bone quantity and bone quality were not adversely influenced by dietary phosphorus excess to any significant extent. However, these bone parameters were slightly (but not significantly) lower in rats fed the H-diet with 0.3% calcium (Ca/P = 0.25) than in those fed the L-diet with the same calcium content

(Ca/P = 2.00). This observation, which is consistent with the calcium retention values in the short-term experiment, may indicate a small negative effect of dietary phosphorus excess on calcium utilization.

8.3.4.2 Dietary Ca/P-ratio

On both the L- and H-diets, the urinary excretion of hydroxyproline decreased with an increase of the dietary Ca/P-ratio. The activity of alkaline phoshatase in plasma and the urinary excretion of hydroxyproline were lower in rats fed the H-diets than in those fed the L-diets. These effects on the parameters of the bone turnover were attributable to differences in the calcium content of the diets. The secretion of PTH and the bone turnover rate are known to be inhibited by dietary calcium. The activity of alkaline phosphatase in plasma did not decrease so clearly with the amount of calcium in the diets as did the excretion of hydroxyproline in urine.

This may indicate that the activity of alkaline phosphatase in plasma is not such a sensitive indicator of the bone turnover rate as is the urinary excretion of hydroxyproline. Plasma alkaline phosphatase activity declined with the duration of the long-term experiment as did the difference in this activity between rats fed the L-diets and those fed the H-diets. This probably reflects a decrease in the bone turnover rate and calcium need with further maturation when body growth diminishes.

The observation, at the end of the long-term study, in rats fed the H-diets, that the severity of the hypophosphataemia decreased with an increase of the dietary Ca/P-ratio, probably reflects the inhibiting effect of dietary calcium on PTH secretion. This explanation is consistent with the observation that PTH activity, as measured by the tubular reabsorption of phosphate, decreased with an increase of the dietary Ca/P-ratio in rats fed the H-diets in the short-term experiment.

The inhibition of the intestinal absorption and decrease of the urinary excretion of phosphorus, which were observed if the dietary Ca/P-ratio of either the L- or H-diets was raised, can be explained in two ways:

1. dietary calcium inhibits the intestinal phosphorus absorption by the formation of insoluble calcium phosphates in the intestinal tract and
2. dietary calcium inhibits PTH secretion, which is followed by a reduction in the urinary phosphorus excretion. Further research is needed to establish the relative importance of these mechanisms.

The positive relation between the dietary Ca/P-ratio and calcium retention in rats fed the L-diets was reflected by the bone parameters. If the Ca/P-ratio of the L-diets was low (either 0.25 or 0.50), osteoporosis developed: bone mass and bone density were decreased, particularly in the long-term experiment; histologically, the amount of trabecular bone was found to be reduced, whereas bone turnover and amount of osteoid were normal (short-term experiment) or slightly increased (long-term experiment). In rats fed the H-diets, a positive trend between the dietary Ca/P-ratio and calcium retention was also reflected by the bone parameters. In these rats osteoporosis did not develop if the dietary Ca/P-ratio was low.

8.3.4.3 Renal function

In the long-term as well as in the short-term experiment, renal function, as measured by the plasma urea concentration and/or the renal clearances of urea and creatinine, was decreased in rats fed the H-diets. This decrease in renal function

was the consequence of nephrocalcinosis as indicated by the significant correlation between plasma urea values and extent of renal calcification. Since plasma urea was already increased after 6 days of feeding the H-diets and slightly increased further with the duration of the experiments, it can be concluded that loss of renal function by dietary phosphate-induced renal calcification is caused rapidly and progresses slowly. This calcification is known to comprise the deposition of von Kossa-positive particles (probably calcium apatite) in the interstitial tissue of the outer zone of the medulla; this deposition is associated with tubular cell damage involving the distal portions of the proximal convoluted tubules; these renal lesions have been shown to be irreversible (McKay & Oliver, 1935; Hitchman et al., 1979).

On the low-phosphorus diets renal calcium and phosphorus contents were on average 137 and 2640 μg/g fresh tissue in experiment A and 99 and 2468 μg/g fresh tissue in experiment B respectively. These contents compare favourably with those reported by Goulding & Malthus (1969) for the normal rat kidney (75 μg calcium and 3250 μg phosphorus per gram wet tissue). If compared with values of the renal calcium content on the low-phosphorus diet, values were increased eight-fold in rats fed the high-phosphorus diet if the dietary Ca/P-ratio was 1.0 and four-fold if the Ca/P ratio was 0.25 in experiment A. These figures were fourteenfold and two-fold respectively in experiment B. These increases are rather small if compared to those reported by Hitchman et al. (1979). These investigators observed a fifty-fold increase in the renal calcium content (from 67 to 3250 μg/g wet tissue) in the female rat if the phosphorus content of a semi-synthetic diet which contained 1% calcium was raised from 0.2 to 1.0% during a feeding period of 6 weeks.

The cause of the observed nephrocalcinosis is undoubtedly hyperparathyroidism. Anderson & Draper (1972) have found that nephrocalcinosis in rats fed 1.2% dietary phosphorus can be prevented by removal of the parathyroid glands. Moreover, the decreased plasma phosphorus values and the decreased tubular reabsorption of phosphate in rats fed the H-diets also point to hyperparathyroidism. Although this was not reported explicitly in their publication, Hitchman et al. (1979) observed significantly lower plasma phosphorus values in female rats fed a semi-synthetic diet which contained 1% calcium and 1% phosphorus than in rats fed a commercial diet which contained 1% calcium and 0.72% phosphorus. Rats on the former diet developed severe nephrocalcinosis whereas those on the latter diet did not.

In rats fed the L-diets, the kidney calcium content and the renal function were not influenced by the dietary Ca/P-ratio. On the other hand, in rats fed the H-diets the extent of nephrocalcinosis and loss of renal function was most severe if the calcium content of the diet was 1.2% (Ca/P = 1.0). Either an increase of the dietary calcium content to 2.4% (Ca/P = 2.0) or a decrease to 0.3% (Ca/P = 0.25) was associated with a marked reduction in the extent of renal calcification and in the loss of renal function. Since the intestinal phosphorus absorption decreased with an increase of the calcium content (and thus of the Ca/P-ratio) of the diets, these observations indicate that the extent of nephrocalcinosis is not simply a function of the availability of dietary phosphorus as has been suggested by van Beek et al. (1974) and Hitchman et al. (1979). In fact in the present study the lowest degree of nephrocalcinosis on the H-diets was observed in the group where the availability of dietary phosphorus was the highest. Thus other factors also play a role. In this respect the inhibiting effect of dietary calcium on the

intestinal absorption of magnesium may be recalled. Low levels of dietary magnesium have been shown to predispose the rat to nephrocalcinosis (Goulding & Malthus, 1969).

Whether the diminished renal function in rats fed the H-diets has had any influence on parameters of the bone metabolism is difficult to evaluate. In the short-term experiment the creatinine clearance was decreased by about 17% in rats that had the highest plasma urea concentration. It is not likely that such a decrease in the glomerular filtration rate causes significant disturbance. The decreased plasma phosphorus concentration in rats fed the H-diets might be a reflection of a diminished tubular reabsorption caused by tubular cell damage, but this would be in contrast with the observation that at the end of the long-term experiment plasma phosphorus was the lowest in rats that had the lowest degree of nephrocalcinosis and the lowest urea concentration. Therefore it is more logical to attribute the decrease in plasma phosphorus to hyperparathyroidism.

Plasma urea values in rats fed the high-phosphorus diets slowly increased with the duration of the experiments and this reflects a gradual loss of renal function. It is therefore clear that experimental periods exceeding those of the present study in this respect might cause problems in the maintenance of the bone mineral metabolism.

8.3.4.5 Conclusions

From the results of the experiments, the following conclusions can be drawn:

1. Similar dietary Ca/P-ratios at different levels of the elements do not have the same physiological significance. Low ratios (either 0.25 or 0.50) in low-phosphorus diets produce osteoporosis whereas the same low ratios in high-phosphorus diets do not.
2. High-phosphorus diets produce hyperparathyroidism, the effect of which on the kidney is more marked than that on bone. In the kidney the hyperparathyroidism reduces the tubular reabsorption of phosphate, increases that of calcium and induces nephrocalcinosis. In the bone, the hyperparathyroidism slightly accelerates the bone turnover but it does not adversely influence to a significant extent either the bone mass or the bone density. With an increase of the dietary Ca/P-ratio the hyperparathyroidism is reduced.
3. Nephrocalcinosis, induced by excess of dietary phosphorus, results in a rapid loss of renal function which increases slowly with the duration of feeding the high-phosphorus diet. With a rise of the dietary calcium content to a Ca/P-ratio of 1.0 the renal lesion increases, while with a further rise of the dietary calcium content to a Ca/P-ratio of 2.0 the lesion decreases.

Chapter 9

General discussion

9.1 Literature review

It was deduced from the literature that the diet might contribute to the pathogenesis of osteoporosis according to a mechanism in which secondary hyperparathyroidism plays a leading role. A calcium-deficient diet, a phosphate-rich diet or a diet with a low Ca/P-ratio depresses the concentration of ionized calcium in plasma, which in its turn leads to stimulation of the secretion of PTH. This hormone acts on bone and kidney in order to restore the plasma calcium concentration, which may proceed at the expense of skeletal calcium.

A model was composed (Figure 5, Section 5.11) according to which hyperparathyroidism by the diet occurs particularly under conditions of a diminished renal function (decreased GFR and synthesis of 1,25-DHCC) and/or a low vitamin D status. By measuring the plasma PTH concentration, a good impression of the activity of the parathyroid glands can be obtained. This can also be accomplished indirectly by estimating biochemical parameters in plasma and urine, which reflect the action of the hormone on the bone (stimulation of bone turnover) and on the kidney (stimulation of tubular reabsorption of calcium, inhibition of tubular

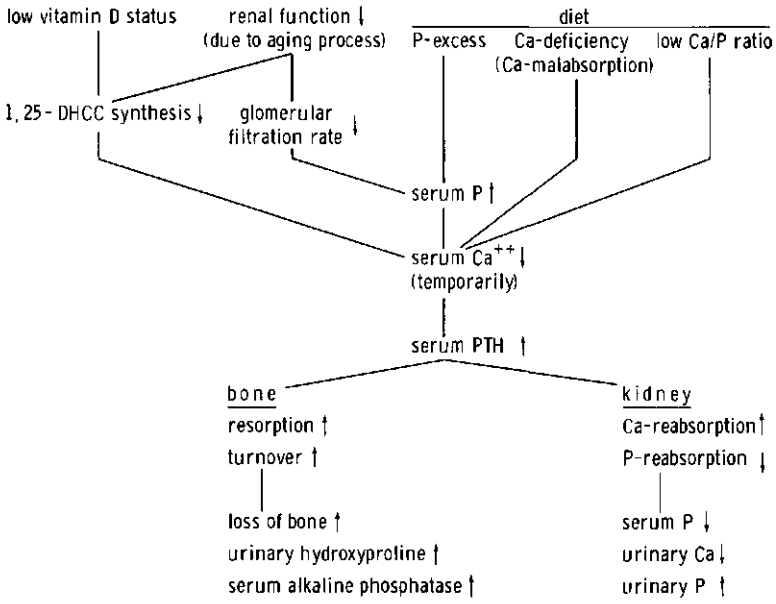

Fig. 5. Outline of the pathogenesis of secondary hyperparathyroidism.

reabsorption of phosphate, and stimulation of 1,25-DHCC synthesis). In this respect the urinary excretion of hydroxyproline can be used as an indicator of the bone turnover rate, and the tubular reabsorption of phosphate (TRP) as an indicator of PTH activity on the kidney. With this model as a guide, the possible relationships between dietary calcium and phosphorus on the one hand and bone mass and bone metabolism on the other were investigated in an observational cross-sectional study in elderly people and in three experimental studies with rats.

9.2 Cross-sectional study in elderly people

The many limitations which are inherent in the design of this study are such that the results cannot be explained as a conclusive confirmation of the hypothesis that secondary hyperparathyroidism contributes to the pathogenesis of osteoporosis. Nevertheless, several observations were indeed in line with this hypothesis and add to its probability.

They may be compared with the relationships outlined in Figure 5, Section 5.11.

As regards the women, the following results suggest that dietary phosphorus stimulates the secretion of PTH, accelerates the bone turnover rate and brings about a loss of cortical bone:

- a negative correlation between dietary phosphorus and bone mineral content, corrected for bone width (BMC/BW), if any effects of other parameters (age, body size, calcium intake) on the bone mineral content are taken into account;
- a negative correlation between the BMC/BW-ratio and the urinary excretion of phosphorus (the latter parameter reflects the phosphorus intake);
- a positive correlation between dietary phosphorus and the urinary excretion of hydroxyproline (the latter parameter is considered to reflect the bone turnover rate);
- a positive correlation between urinary phosphorus and hydroxyproline;
- the absence of any significant correlation between plasma calcium and either dietary or urinary phosphorus.

As regards the men, the following results suggest that dietary phosphorus stimulates the secretion of PTH and accelerates the bone turnover rate, but does not bring about any loss of cortical bone:

- no significant correlation between dietary phosphorus and the bone mineral content, corrected for bone width (BMC/BW);
- no significant correlation between the BMC/BW-ratio and the urinary excretion of phosphorus;
- a positive correlation between dietary phosphorus and the urinary excretion of hydroxyproline;
- a positive correlation between urinary phosphorus and hydroxyproline;
- a negative correlation between urinary phosphorus and serum phosphorus (this correlation reflects a decrease of the tubular reabsorption of phosphorus in the kidney with an increase of phosphorus intake, as a result of a raised production of PTH);
- the absence of any significant correlation between plasma calcium and either dietary or urinary phosphorus.

It should be recognized that these results in men and women were not supported by significant positive correlations between either dietary or urinary phosphorus and the serum concentrations of PTH and alkaline phosphatase (bone fraction). These correlations might have been expected to occur, according to Figure 5, Section 5.11. However, it is plausible that the large inter-indivi-

dual variation in the serum concentrations of PTH and alkaline phosphatase, which, at least in part, is the consequence of the rather large variation coefficient in the biochemical determination of these parameters, obscure relations within the range of normal values. Moreover, the activity of alkaline phosphatase in serum might not be such a sensitive indicator of the bone turnover rate as is the urinary secretion of hydroxyproline. As was to be expected in a population of elderly people, serum values of PTH as well as those of creatinine (renal function) were at the upper limit of normality.

In both the men and the women, the serum 25-HCC concentration, which reflects the vitamin D status, correlated positively with the BMC/BW-ratio, despite the fact that the serum concentrations of this vitamin D metabolite were within normal limits. This positive correlation might reflect a decreased production of 1,25-DHCC in elderly people consequent on either a low vitamin D intake, a low degree of skin exposure to sunshine, or both. A decline in the production of the active vitamin D metabolite is compatible with the observation that in the women the urinary calcium excretion decreased with age, probably reflecting a decline of the intestinal calcium absorption and/or an increase of the tubular reabsorption in the kidney, the latter being brought about by a raised production of PTH. The observation that in the men the BMC/BW-ratio correlated negatively with the serum alkaline phosphatase activity is also compatible with a decreased production of 1,25-DHCC, because it has been shown that the activity of this enzyme is increased in vitamin D-deficiency. In our opinion these observations indicate that elderly people may need a larger quantity of vitamin D.

In spite of the observation that the mean calcium intake exceeded the Dutch recommended allowance for adults, a positive correlation between calcium intake and the BMC/BW-ratio was found in the women, if allowance was made for differences in age, body size and phosphorus intake. In the men, but not in the women, a positive correlation was found between body height and calcium intake. Since loss of body height, which is a sign of trabecular bone loss, occurred particularly in the men, these correlations in women and men might reflect a protective action of dietary calcium against aging bone loss.

9.3 Studies with rats

The results of the three experiments with rats are consistent with the induction of hyperparathyroidism by feeding high-phosphorus diets. This appeared from biochemical changes in serum and urine (hypophosphataemia, decreased tubular reabsorption of phosphate and urinary calcium excretion, increased urinary hydroxyproline excretion) and from the occurrence of nephrocalcinosis. Feeding rats high-phosphorus diets resulted in decreased bone density after a period of forty-two weeks, but did not do so after shorter periods (either four or sixteen weeks). This difference in the response of bone might be related to differences between the experiments in the level of dietary phosphorus used and the amount of food that the rats consumed. In the experiment that lasted 42 weeks, the dietary phosphorus content was 1.3% and the diets were fed ad libitum. In the experiments that lasted either four or sixteen weeks, the dietary phosphorus content was slightly lower (1.2%) and the diets were fed in restricted amounts. However, the response of bone to hyperparathyroidism is more likely to depend on the duration of the intake of high-phosphorus diets, since it is to be expected that quite some time must elapse before the effect of moderate hyperparathyroidism on bone becomes observable.

Dietary calcium tended to reduce not only the biochemical changes in serum and urine induced by high-phosphorus diets, but also the adverse influence of these diets on bone density. These 'protective' effects of dietary calcium were associated with an inhibition of the net intestinal phosphorus absorption. It should be recognized, however, that addition of calcium to the high-phosphorus diets up to a level of a Ca/P-ratio of 1.0, increased the severity of nephrocalcinosis.

It was observed that maturing rats fed a low-phosphorus diet with Ca/P-ratios of 0.25 or 0.50 developed osteoporosis as a consequence of calcium deficiency, whereas rats fed a high-phosphorus diet with similar Ca/P-ratios did not. This is an indication of the limited effect that the dietary Ca/P-ratio has on bone metabolism.

The decrease of renal function which was associated with the induction of nephrocalcinosis continued slowly during the course of the studies. Although no effect of this process on bone metabolism could be demonstrated, it should be recognized that loss of renal function in the long run will contribute to hyperparathyroidism.

9.4 Final view, a new hypothesis

With respect to the influence of dietary phosphorus on bone metabolism, some remarkable parallels can be observed between the results of the cross-sectional study of elderly people and those of the studies with rats. These parallels have given rise to the construction of a theory which accounts for the difference in physiological response to dietary phosphorus between the postmenopausal women on the one hand and the elderly men and the male rat on the other. In Table 40 it is shown that an increase of phosphorus intake is accompanied with an

Table 40. Outline of changes of parameters regarding bone metabolism and bone mass, associated with an increase of phosphorus intake.

	elderly women	elderly men	male rats
urinary phosphorus	+	+	+
urinary hydroxyproline	+	+	+
BMC/BW or femur mass	−	±	±
serum phosphorus	±	−	−

increase in the urinary excretion of phosphorus and hydroxyproline in the postmenopausal women, the elderly men and the male rats. The increase in the excretion of hydroxyproline reflects an increase in the bone turnover rate, brought about by PTH. The secretion of this hormone, which is triggered by a depressed plasma-ionized calcium concentration consequent on an increased flux of phosphate through the blood, acts on bone and kidney in order to restore the plasma calcium concentration. As was expected, in all groups plasma calcium concentration did not change with either the phosphorus content of the diet or with the urinary phosphorus excretion. In the postmenopausal women the skeleton is liable to the action of PTH because of oestrogen deficiency, which action results in bone loss and thus in a net release of phosphorus into the circulation. By the phosphaturic action of PTH on the kidney in these women, the plasma phosphorus concentration will not change to any significant extent. In the elderly man as well as in the male rat, on the other hand, the net result of the action of PTH

on bone is that no phosphorus is liberated into the circulation, since the stimulated bone resorption is balanced by bone formation, so that bone loss does not occur. The phosphaturic action of PTH on the kidney leads to a decrease of the plasma phosphorus concentration.

9.5 Conclusions

To the questions which were posed in Chapter 1, the following answers have been formulated:

Question 1. Are there any relations between dietary calcium and phosphorus on the one hand and bone mass and bone metabolism on the other in normal, healthy, elderly people, who subsist on their own customary diets?

a. In elderly women, a positive correlation between dietary calcium and bone mass and a negative one between dietary phosphorus and bone mass was observed. These correlations, which were significant ($P < 0.05$), could be demonstrated only if the effects of other factors which influence the bone mass (age, body size and dietary calcium or phosphorus) were taken into account.
b. In elderly men, no significant correlation between either calcium or phosphorus intake and bone mass was detected, but body height and calcium intake showed a significant positive correlation ($P < 0.05$).
c. In elderly women as well as in men, correlations between dietary phosphorus and biochemical parameters of the bone metabolism were found, which supports the hypothesis that high-phosphorus diets cause hyperparathyroidism.

Question 2. What influence have dietary calcium and phosphorus on bone mass and bone metabolism under controlled conditions in rats?

a. Excess of dietary phosphorus caused hyperparathyroidism, as shown by a reduced tubular reabsorption of phosphate in the kidney, hypophosphataemia, increased urinary hydroxyproline excretion and nephrocalcinosis.
b. The hyperparathyroidism, induced by dietary phosphorus excess, did not significantly influence either the bone mass or the bone quality in the maturing animal fed the high-phosphorus diet for either four or sixteen weeks, but it reduced the bone density if this diet was fed for forty-two weeks.
c. The hyperparathyroidism, induced by dietary phosphorus excess, and its effect on bone, were reduced if the dietary calcium content was raised.
d. In rats fed a high-phosphorus diet, the extent of nephrocalcinosis increased if the calcium content of the diet was raised to a Ca/P-ratio of 1.0.
e. The nephrocalcinosis, induced by dietary phosphorus excess, was associated with a loss of renal function which continued slowly with the duration of feeding the phosphorus excess.
f. In maturing animals, fed diets with a low Ca/P-ratio, osteoporosis developed if the calcium content of the diet was insufficient but not if the calcium content of the diet was adequate.

9.6 Dietary prevention of osteoporosis

With regard to the high prevalence of osteoporosis, particularly among postmenopausal women, it is absolutely necessary to utilize all possibilities which might contribute to the prevention of this disease. It is very likely that the diet plays a role in the etiology of osteoporosis. Four main factors emerge: calcium, phosphorus, vitamin D and protein. As yet, the available data do not allow us to make scientifically based, exact recommendations of the optimum levels of these nutrients in the diet. Nevertheless, current knowledge points to dietary measures

which may be considered as useful for the maintenance of bone health in elderly people living in western communities. These measures are:
- ensure a proper vitamin D status;
- restrict intake of phosphorus;
- take ample calcium;
- restrict intake of protein.

Whether supplementary dietary vitamin D is necessary, depends on the extent to which the skin is exposed to sunshine. In our moderate climate the endogenous synthesis of vitamin D is probably limited, particularly in people who have a pigmented skin, in night-workers and in those who are house-bound (elderly people, sick persons). For these people, vitamin D supplementation (200 – 400 IU per day = 5 – 10 μg) is necessary.

The average western diet contains an abundance of (animal) protein and is rich in phosphorus. A 20%-reduction in the intake of these nutrients would be completely without risk in terms of nutrient deficiency. For instance, in the Netherlands such a reduction would bring down the protein intake to about 70 g per head per day and that of phosphorus to about 1.3 g per head per day. These levels are still in excess of the USA recommended daily allowances for male adults (70 kg body weight) with regard to these nutrients (56 and 0.8 g respectively).

Table 41. Estimated amount (g per head per day) of calcium, phosphorus and protein in Dutch diets[1]).

	period		
	1950 – 1960	1970	1975
Ca	1.00	0.93	0.94
P	1.48	1.54	1.63
Ca/P	0.68	0.60	0.58
protein	79.3	82.1	87.9
vegetable	35.7	28.0	28.7
animal	43.6	54.1	59.2

[1]) Derived from market data of foods available for human consumption, as reported by Bosman (1979), Bosman & Kosten-Zoethout (1978), and Mulder (1962).

Table 41 shows that a slight decrease in the consumption of calcium and an increase in that of phosphorus and protein have taken place in the Netherlands between the fifties and the seventies. The dietary Ca/P-ratio has declined from 0.68 to 0.58 in that period. The increase in the consumption of phosphorus and protein is almost completely attributable to a higher consumption of meat and poultry. It should be remembered that the hypercalciuric action of dietary protein is related particularly to the sulphur-containing amino-acids which are present in the protein (Section 5.4). Since the content of these amino-acids in animal protein generally exceeds that in vegetable protein, it is not only the rise in the intake of protein but also the shift from vegetable to animal protein that has to be taken into consideration. In fact, the ratio of vegetable to animal protein decreased from 0.82 in the fifties to about 0.50 in the seventies. The increase in the consumption of phosphorus, mentioned above, is probably an underestimation, as the use of phosphate and polyphosphate additives by the food-processing industry has been left out of account. These compounds are used for such purposes as

increasing the water-binding capacity, fat emulsification, acidification, protein stabilization and others. It has been estimated that in the USA the contribution of these additives to the total phosphorus intake is more than 25% (Sie et al., 1974). The sodium salts of ortho-, pyro- and tripoly-phosphates as well as those of the cyclic trimeta- and linear hexameta-phosphate are of particular commercial significance. Unfortunately, only few data are available about the current use of phoshate additives by the Dutch food-processing industry.

In order to decrease the intake of animal protein and phosphorus, a reduction in the consumption of dairy products and meat and poultry may be suggested for consideration. These food groups together account for more than 50% of the intake of phosphorus and for more than 90% of the intake of animal protein (see Table 42). However, a reduction in the consumption of dairy products would be

Table 42. Relative contribution (%) of dairy products, meat and poultry to the intake of calcium, phosphorus and animal protein in Dutch diets in 1975[1]).

	dairy products[2])	meat and poultry
Ca	75	2
P	31	23
animal protein	36	55

[1]) Derived from market data of foods available for human consumption, as reported by Bosman (1979).
[2]) Milk and milk products, excluding butter.

unwise, since these foods are by far the most important sources of dietary calcium. Moreover, the Ca/P-ratio of dairy products (between 1.0 and 1.5) is favourable and exceeds that of almost all other foods. On the other hand, an excessive consumption of dairy products should also be avoided, because of their high content of animal protein and phosphorus. In addition to the fact that excess of protein raises the calcium requirement due to its hypercalciuric action, it is also suspect by reason of the high frequency of nephrocalcinosis in western societies (Section 5.4). Furthermore, our results indicate that dietary calcium may aggravate nephrocalcinosis in rats fed high-phosphorus diets. Although it is not permissible to extrapolate this effect to man without observing the greatest reservation, it can be used as a warning to those who think that a high phosphorus intake does not do harm as long as the dietary Ca/P-ratio is kept within acceptable limits. If, for therapeutical reasons, more than a normal amount of calcium in the diet is required, calcium tablet supplementation (for instance as calcium lactate or as calcium carbonate) is indicated.

Considering the matters discussed above, a reduction in the intake of phosphorus and animal protein should be brought about by a decrease in the consumption of meat, poultry and meat products. These foods do not contribute to any significant extent to the intake of calcium, and their Ca/P-ratio is extremely low (1/20). A further reduction in the intake of phosphorus can be achieved by limiting the consumption of processed foods to which phosphates have been added. A list of these products with their content of added phosphates should be made available for nutritionists, dietitians and others. It is also recommended that the use of phosphate additives should be limited as much as possible.

Chapter 10

Summary

By means of this study it was attempted to obtain a better insight into the possible influence of the diet on the development of human osteoporosis. This disease, which is a consequence of decalcification of the bones, occurs frequently in elderly people, particularly in postmenopausal women.

On the basis of data from the literature a model was composed describing a mechanism according to which either a low-calcium diet, a high-phosphorus diet or a diet with a low calcium-to-phosphorus ratio (temporarily) decreases the plasma-ionized calcium concentration, so that the parathyroid glands are stimulated to secrete parathyroid hormone (PTH). This hormone acts on bone and kidney in order to restore the plasma-ionized calcium concentration, which may proceed at the expense of skeletal calcium, so that osteoporosis develops. The model explains why the dietary stimulation of PTH secretion occurs especially when the renal function has decreased and/or when a suboptimal vitamin D status is prevalent. In an observational cross-sectional study in elderly people and in three experimental studies with three month old male rats, the influence of dietary calcium and phosphorus on bone metabolism was investigated.

The cross-sectional study was carried out among 89 healthy eldery people (53 women and 36 men), whose age ranged from 57 to 89 years (mean: 70 years). It was investigated whether relationships could be demonstrated which fitted in the proposed model. For this purpose the calcium and phosphorus intake was estimated by the cross-check method. The bone mineral content (BMC) and the bone width (BW) were measured on the distal third of the radius (cortical bone) by means of ^{125}I-photon absorptiometry. In addition, parameters of the bone metabolism in serum and urine were estimated. Because of the observational nature of the study, which lacked an experimental basis, it was stressed that the results had to be interpreted with caution. The mean daily calcium intake of the women was 1.04 g and that of the men 1.22 g. These amounts largely exceeded the current Dutch recommended allowance for adults of 0.8 g per day. Phosphorus intake was considered to be high: 25% of the men and women had an intake of more than 1.6 and 2.0 g per day respectively. In the men as well as in the women the serum concentrations of PTH and creatinine were at the upper limit of normality. The level of this latter parameter pointed to a slightly decreased renal function such as could be expected in people of this age group.

In the women the amount of cortical bone, corrected for bone width (BMC/BW-ratio) showed a significant negative correlation with age, and the regression corresponded with an annual bone loss of about 0.8%. This loss of bone was associated with a decrease of body height of about 0.35 cm per year, which was considered to be, at least in part, the consequence of vertebral collapse. The BMC/BW-ratio correlated positively with calcium intake and negatively with phosphorus intake, if differences in age, body size and either calcium or

phosphorus intake were taken into account. The excretion of phosphorus with the urine also showed a negative correlation with the BMC/BW-ratio, and dietary phosphorus correlated positively with the urinary excretion of phosphorus as well as with that of hydroxyproline. The possibility was discussed that these correlations are in line with the concept that high-phosphorus diets contribute to bone loss in that they stimulate the secretion of PTH.

In the men, loss of cortical bone with advancing age was not observed, and no relations were found between dietary calcium or phosphorus and the amount of this bone. However, a marked loss of height with age (0.45 cm per year) was established and a positive correlation between dietary calcium and body height was found. It was considered that this correlation might reflect a 'protective' action of dietary calcium against loss of trabecular bone from the vertebrae. The urinary excretion of phosphorus showed a negative correlation with the serum phosphorus concentration, and dietary phosphorus correlated positively with the urinary excretion of phosphorus as well as with that of hydroxyproline. The possibility was discussed that these correlations are in line with a concept according to which high-phosphorus diets stimulate the secretion of PTH and increase the bone turnover rate, but do not bring about loss of cortical bone.

In the men as well as in the women, the serum concentration of 25-hydroxy-vitamin D (25-HCC) correlated positively with the BMC/BW-ratio, despite the fact that serum values of this metabolite (the serum 25-HCC concentration is used as an indicator of the vitamin D status) were within normal limits. The possibility was discussed that this positive correlation might indicate that elderly people require larger amounts of vitamin D as a consequence of diminished renal synthesis of the active vitamin D metabolite.

The experiments with rats, which lasted either four, sixteen or forty-two weeks, showed that high-phosphorus (1.2 or 1.3%) diets did not significantly influence calcium retention and/or bone mass, as opposed to diets with either a low (0.15%) or a normal (0.45%) phosphorus content. From histological and chemical analyses of bone samples, it was concluded that the quality of the bone was not obviously affected by the phosphorus content (0.15 versus 1.20%) of the diet in experiments which lasted four or sixteen weeks. However, in the experiment which lasted forty-two weeks, bone density was found to have been reduced in rats fed 1.3% dietary phosphorus, as opposed to those that had been fed 0.45% dietary phosphorus. It was suggested that the effect of high-phosphorus diets on bone depends either on age or on the duration of feeding these diets. There were consistent and strong indications that the function of the parathyroids and the bone turnover rate were stimulated in rats fed high-phosphorus diets. In addition to the diminished bone density after forty-two weeks, these indications were: decreased renal tubular reabsorption of phosphate, hypophosphataemia, decreased urinary calcium excretion, increased urinary phosphorus excretion, nephrocalcinosis and increased urinary excretion of hydroxyproline. With the exception of nephrocalcinosis and decreased urinary calcium, these biochemical changes were reduced if the calcium content of the diet was elevated. This was associated with an inhibiting effect of dietary calcium on the intestinal phosphorus absorption. In the experiments which lasted four or sixteen weeks, the cause and consequence of nephrocalcinosis were studied in more detail. It was found that nephrocalcinosis was attributable to the deposition of calcium apatite and that it resulted in a clear but moderate loss of renal function. This loss appeared to increase slowly during the course of the experiments. The extent of nephrocalci-

nosis and loss of renal function depended on the calcium content of the diet. When the diet contained 1.2% phosphorus, the extent of renal calcification and loss of renal function increased if the calcium content of the diet was raised from 0.3 to 1.2%, but these parameters decreased if the calcium content of the diet was further raised to 2.4%. There were no indications as to whether the loss of renal function had any influence on the composition or metabolism of the bone. In rats fed the low-phosphorus control diets, but not in those fed the high-phosphorus diets, osteoporosis developed when the dietary Ca/P-ratio was low (0.25 or 0.50). It was concluded that this stresses the importance of indicating absolute levels of calcium and phosphorus in the diet and that reporting only the ratio between these nutrients should be avoided.

The results of the cross-sectional study of elderly people and those of the experiments with rats gave rise to the framing of a hypothesis. This hypothesis explains why hyperparathyroidism, induced by excess of dietary phosphorus, brings about a decrease in the plasma phosphorus concentration in the elderly man and in the rat, whereas no such effect is seen in the postmenopausal woman.

Finally, recommendations were made regarding dietary measures which are considered to be useful in the prevention of osteoporosis in western societies. These measures are: (1) ensure a proper vitamin D status, (2) restrict the intake of phosphorus, (3) take ample calcium, and (4) restrict the intake of protein. In order to reduce the intake of phosphorus and protein, it was suggested that the consumption of meat and poultry should be decreased and the consumption of products which contain added phosphates should be limited. A further discussion was concerned with a regular consumption of dairy products which is necessary to ensure a sufficient intake of calcium.

Samenvatting

Door middel van het hier beschreven onderzoek werd getracht een beter inzicht te verkrijgen in de mogelijke invloed die de voeding heeft op het ontstaan van osteoporose bij de mens. Deze ziekte, die het gevolg is van een verlies van kalk uit het skelet, komt bij de oudere mens veelvuldig voor, vooral bij de vrouw na de menopauze.

Aan de hand van literatuuronderzoek werd een model opgesteld dat een mechanisme beschrijft volgens hetwelk een voeding die arm is aan calcium, of veel fosfaat bevat of een lage Ca/P-verhouding heeft, het gehalte aan geïoniseerd calcium in het bloedplasma (tijdelijk) verlaagt, zodat de bijschildklieren worden gestimuleerd tot het uitscheiden van parathormoon (PTH). Dit hormoon werkt in op botten en nieren, met als doel de plasmaconcentratie aan geïoniseerd calcium weer te verhogen; de hiervoor benodigde kalk kan aan het skelet worden onttrokken, zodat osteoporose ontstaat. Het model verklaart waarom de afscheiding van PTH die door de voeding is geïnduceerd, vooral wordt gestimuleerd wanneer er sprake is van een verminderde nierfunctie en/of van een suboptimale vitamine-D-status. In een observationeel transversaal onderzoek bij bejaarden en in drie experimenten met drie-maanden-oude mannelijke ratten werd de invloed onderzocht die het calciumgehalte en het fosfaatgehalte van de voeding op de botstofwisseling hebben.

Het transversale onderzoek werd verricht bij 89 gezonde bejaarden (53 vrouwen en 36 mannen), die in leeftijd varieerden van 57 tot 89 jaar (gemiddeld 70 jaar). Er werd onderzocht of er relaties konden worden aangetoond die pasten in het voorgestelde model. Hiertoe werd de opneming van calcium en fosfaat bepaald door middel van de kruis-vraagmethode. Het botmineraalgehalte (BMC) en de botbreedte (BW) werden gemeten aan het distale derde deel van de radius (corticaal bot) door middel van ^{125}I-fotonabsorptiometrie. Daarnaast werden parameters van de botstofwisseling bepaald in bloedserum en urine. Er werd met nadruk gesteld dat de interpretatie van de resultaten met voorzichtigheid moet geschieden, omdat het onderzoek observationeel van aard was zonder dat er een experimentele basis aan ten grondslag lag. De gemiddelde dagelijkse calciumopneming bedroeg 1,04 g bij de vrouwen en 1,22 g bij de mannen. Deze hoeveelheden overschrijden ruimschoots de huidige in Nederland aanbevolen hoeveelheid voor volwassenen van 0,8 g per dag. De opneming van fosfor werd hoog bevonden: 25% van de mannen en vrouwen hadden een dagelijkse opneming van meer dan 1,6, respectievelijk 2,0 g. Zowel bij de mannen als bij de vrouwen waren het gehalte aan PTH en aan creatinine in het bloedserum hoog-normaal. Het niveau van deze laatste parameter wees op een licht verminderde nierfunctie, zoals overigens verwacht mocht worden bij mensen uit deze leeftijdscategorie.

Bij de vrouwen vertoonde de hoeveelheid corticaal botmineraal, gecorrigeerd voor de botbreedte (de BMC/BW-verhouding), een significant negatieve corre-

latie met de leeftijd en de regressie kwam overeen met een botontkalking van ongeveer 0,8% per jaar. Deze botontkalking ging samen met een jaarlijkse vermindering van de lichaamslengte van ca. 0,35 cm. Deze vermindering in lengte werd, althans ten dele, toegeschreven aan het inzakken van de rugwervels. De BMC/BW-verhouding correleerde positief met de calciumopneming en negatief met de fosfaatopneming, indien verschillen in leeftijd, lichaamsafmetingen en calcium- of fosfaatopneming in aanmerking werden genomen. De uitscheiding van fosfaat in de urine vertoonde eveneens een negatieve correlatie met de BMC/BW-verhouding, en de fosfaatopneming met de voeding correleerde positief met de uitscheiding van fosfaat in de urine evenals met die van hydroxyproline. De mogelijkheid werd besproken dat deze correlaties in overeenstemming zijn met het concept dat een fosfaatrijke voeding botontkalking bevordert doordat deze voeding de afscheiding van PTH stimuleert.

Bij de mannen werd met het klimmen der jaren geen ontkalking van corticaal bot waargenomen, en bij hen bestond ook geen verband tussen de hoeveelheid calcium en fosfaat in de voeding en de hoeveelheid van dit bot. Er werd echter wel een duidelijke vermindering van de lichaamslengte waargenomen (0,45 cm per jaar), en de lichaamslengte had een positieve correlatie met de calciumopneming. Er werd verondersteld dat deze correlatie een afspiegeling zou kunnen zijn van een beschermende werking die calcium in de voeding uitoefent tegen verlies van trabeculair bot uit de rugwervels. De uitscheiding van fosfaat in de urine vertoonde een negatieve correlatie met de serum-fosfaatconcentratie, en de hoeveelheid fosfaat in de voeding correleerde positief met zowel de uitscheiding van fosfaat in de urine als met die van hydroxyproline. De mogelijkheid werd besproken dat deze correlaties in overeenstemming zijn met een concept volgens hetwelk een voeding met veel fosfaat de afscheiding van PTH stimuleert en de botstofwisseling versnelt zonder ontkalking van corticaal bot te bewerkstelligen.

Bij zowel de mannen als de vrouwen was er een positieve correlatie tussen de BMC/BW-verhouding en de concentratie aan 25-hydroxy-vitamine D (25-HCC) in bloedserum ondanks het feit dat de serumwaarden van deze metaboliet — de 25-HCC-concentratie in serum wordt gebruikt als een indicator van de vitamine-D-status — in het normale gebied lagen. De mogelijkheid werd besproken dat deze positieve correlatie erop zou kunnen wijzen dat de oudere mens een hogere behoefte aan vitamine D heeft als gevolg van een verminderde synthese van de actieve vitamine-D-metaboliet in de nier.

Uit de resultaten van proeven met ratten, die vier, zestien of tweeënveertig weken duurden, bleek dat fosfaatrijke voeders (1,2 of 1,3% P) geen significante invloed hadden op de calciumretentie en/of op de hoeveelheid bot, ten opzichte van controlevoeders die een lage (0,15% P) dan wel een normale (0,45% P) hoeveelheid fosfaat bevatten. Het histologisch en chemisch onderzoek van botmonsters toonde aan dat de kwaliteit ervan niet duidelijk door het fosfaatgehalte van het voer was beïnvloed (0,15 t.o.v. 1,20% P), althans niet bij de proeven die vier of zestien weken duurden. Na afloop van het experiment dat tweeënveertig weken duurde, werd bij ratten die 1,3% P in het voer hadden een geringere botdichtheid vastgesteld dan bij die welke voer kregen met 0,45% P. Er werd verondersteld dat de invloed die voeders met veel fosfaat uitoefenen op het bot afhankelijk kan zijn van de leeftijd van de rat of van de tijd gedurende welke deze voeders worden verstrekt. Er waren consistente en sterke aanwijzingen dat de bijschildklierfunctie en de botstofwisseling werden gestimuleerd door de voeders met veel fosfaat. Naast een verminderde botdichtheid na tweeënveertig weken

waren deze aanwijzingen: een vermindering van de tubulaire reabsorptie van fosfaat, hypofosfatemie, een verminderde calciumuitscheiding met de urine, een toegenomen uitscheiding van fosfaat in de urine, nierverkalking en een verhoogde uitscheiding van hydroxyproline in de urine. Met uitzondering van de nierverkalking en de verminderde calciumuitscheiding in de urine, werden deze biochemische veranderingen tegengegaan indien het calciumgehalte van het voer werd verhoogd; dit ging samen met een remmend effect dat het calcium in het voer op de absorptie van fosfaat in de darm bleek te hebben. Bij de proeven die vier tot zestien weken duurden, werden de oorzaak en het gevolg van de nierverkalking meer gedetailleerd onderzocht. Er werd waargenomen dat het verkalken van de nieren was toe te schrijven aan de afzetting van calciumapatiet, en dat deze afzetting een zekere vermindering van de nierfunctie ten gevolge had. De mate van de nierverkalking en van de verminderde nierfunctie bleken afhankelijk te zijn van het calciumgehalte van het voer. Indien het voer 1,2% P bevatte, namen de mate van nierverkalking en de vermindering in nierfunctie toe als het calciumgehalte van het voer van 0,3 tot 1,2% werd verhoogd, maar deze parameters namen af als het calciumgehalte van het voer verder werd verhoogd tot 2,4%. Er werden geen aanwijzingen gevonden dat de vermindering in nierfunctie enige invloed zou hebben op de botstofwisseling. Bij ratten die het controlevoer kregen met een laag fosfaatgehalte ontstond osteoporose wanneer de Ca/P-verhouding van het voer laag was (0,25 of 0,50) maar dit gebeurde niet bij ratten die voer kregen met een hoog fosfaatgehalte. Hieruit werd geconcludeerd dat het van groot belang is de absolute gehalten aan calcium en fosfaat van de voeding te vermelden, en dat men zich niet moet beperken tot het aangeven van de verhouding tussen deze nutriënten.

De resultaten van het transversale onderzoek bij de bejaarden en die van de proeven met de ratten gaven ons aanleiding een hypothese op te stellen. Deze hypothese verklaart waarom hyperparathyreoïdie, die is geïnduceerd door een teveel aan fosfaat in de voeding, een daling van de plasmafosfaatconcentratie kan bewerkstelligen bij de oudere man en bij de rat, terwijl dit effect bij de vrouw na de menopauze niet wordt waargenomen.

Ten slotte werden adviezen gegeven over voedingsmaatregelen die als nuttig kunnen worden beschouwd om het optreden van osteoporose in westerse samenlevingen te voorkomen. Deze maatregelen zijn: (1) zorg voor een behoorlijke vitamine-D-status, (2) beperk de opneming van fosfaat, (3) neem een ruime hoeveelheid calcium, en (4) beperk de opneming van eiwit. Ten einde de opneming van fosfaat en eiwit te beperken, werd in overweging gegeven de consumptie van vlees en gevogelte te verminderen en de consumptie van produkten waaraan fosfaten zijn toegevoegd te beperken. Ook werd erop gewezen dat een regelmatige consumptie van melkprodukten noodzakelijk is om verzekerd te zijn van een voldoende opneming van calcium.

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Curriculum vitae

Gertjan Schaafsma werd op 28 maart 1947 in Overveen (gemeente Bloemendaal) geboren. Na het behalen van het diploma HBS-B aan het toenmalige Karel van Manderlyceum te Haarlem in 1967, vervulde hij tot juni 1969 zijn militaire dienstplicht bij de Luchtdoelartillerie in Ede.

In datzelfde jaar begon hij zijn studie aan de Landbouwhogeschool en in januari 1975 behaalde hij het doctoraal examen met als hoofdvak Voedingsleer (verzwaard) en als bijvak Fysiologie. Gedurende deze studie werkte hij een half jaar in het klinisch-chemisch laboratorium van het St. Jozef-ziekenhuis te Doetinchem onder leiding van Dr. A. P. M. van Oudheusden, en was hij enige tijd werkzaam aan de Landbouwhogeschool als student-assistent bij de Vakgroep Organische Chemie en als technisch assistent bij de Vakgroep Humane Voeding.

In maart 1975 trad hij als wetenschappelijk medewerker in dienst van het Nederlands Instituut voor Zuivelonderzoek (NIZO) bij de afdeling Voedingsfysiologie (hoofd: Dr. H. de Waard). Hier was hij ruim twee jaar betrokken bij het onderzoek naar de invloed van de voeding op de cholesterolstofwisseling van de genetisch vetzuchtige rat. Daarna werd het in dit proefschrift beschreven onderzoek verricht.

Hij is lid van de Commissie Voedingsnormen van de Voedingsraad.