EFFECTS OF GROWTH AND OOPHORECTOMY ON CALCIUM BALANCE

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ABSTRACT

This thesis assesses calcium balance and its components, measured by metabolic balance studies, in ovary-intact (sham) and oophorectomised (oophx) young (1.5-4 month) and adult (7-14 month) rats.

In the young rat, calcium balance diminished with age as rats approached full size. The major factor leading to reduced balance with age was reduced true calcium absorption. The age-related reduction in calcium balance was more rapid in oophx rats, due mainly to a transient rise in intestinal calcium secretion and partly to a fall in true calcium absorption.

Oophorectomy in adult rats led to reduced calcium balance resulting from transient rises in intestinal calcium secretion and urine calcium excretion in the short term and in the long term reduced calcium balance was sustained by reduced intestinal calcium absorption. Although calcium balance and intestinal calcium absorption were lower in oophx rats, the magnitude of adaptation in balance and absorption to dietary calcium restriction (0.02% Ca) was unaffected by oophorectomy. Oophorectomy did not affect the slopes for the relationships between calcium consumption with calcium balance and intestinal calcium absorption, but the intercepts for both relationships were lower in oophx rats. However, circulating 1,25 dihydroxyvitamin D was not reduced in oophx rats. Administered 1,25 dihydroxyvitamin D stimulated intestinal calcium absorption in both sham and oophx rats but did not significantly increase calcium balance, whereas oestradiol stimulated intestinal calcium absorption in oophx rats (without affecting circulating 1,25 dihydroxyvitamin D) and increased calcium balance.

It is concluded from the findings of the study that oophorectomy reduces calcium balance regardless of age by transient rises in calcium excretion and prolonged reduction in intestinal calcium absorption. In adult rats the impairment to intestinal calcium absorption is not the result of reduced circulating 1,25 dihydroxyvitamin D or reduced intestinal responsiveness to 1,25 dihydroxyvitamin D. Oestradiol stimulates intestinal calcium absorption probably by a direct effect on the intestine in addition to its effects on bone.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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CHAPTER 1

CALCIUM HOMEOSTASIS AND CALCIUM BALANCE

1.1 INTRODUCTION

Calcium balance is the difference between the amount of calcium consumed in the diet and the total amount of calcium excreted. Thus, positive calcium balance indicates retention of calcium, whereas negative calcium balance indicates depletion of the body's calcium stores. Dietary calcium absorbed by the intestine, reaches the extracellular fluid (ECF) from where it is either incorporated into bone or excreted via the urine, faeces, sweat or other body secretions.

Calcium balance varies as mammals pass through different stages of life with highly positive balance during growth, neutral balance during the stable years of early adulthood and negative balance during senescence. The skeleton stores 99% of the whole body calcium reserve in mammals and thus, calcium balance is synonymous with bone balance. Apart from calcium storage the major function of the skeleton is to provide mechanical support for the body. Maintenance of the quantity and integrity of calcium stored in bone is essential to avoid failure of the skeleton in the form of fractures. The control of calcium balance is highly complex and the intricate interrelationship between endocrine, paracrine and environmental factors is yet to be fully clarified. The accumulation, maintenance or loss of whole-body calcium is determined by the complex combination of bidirectional calcium

fluxes in the gastrointestinal tract, kidney and secretory cells of the skin, mucous membranes and the lungs.

Calcium is involved as a link in many physiological pathways. The normal function of these physiological events requires maintenance of ECF ionised calcium levels within critical limits by a group of hormones known as the calciotropic hormones. ECF ionised calcium is maintained on a minute-to-minute basis by modulation of renal calcium excretion, then less rapidly by movement of calcium in and out of bone and over a longer term by modulation of intestinal absorption of calcium. These events occur even under conditions in which the ECF ionised calcium can only be maintained at the expense of calcium stored in the skeleton.

It has long been recognised that fracture rates increase as bone density falls below a 'fracture threshold' (Newton-John & Morgan 1968). The mean bone density in groups of fracture patients is generally lower than age-matched controls regardless of the site of bone density measurement in relation to the site of fracture (Nordin et al 1993). The onset of menopause in women causes a relatively acute change in calcium balance (Heaney et al 1978b), reflecting a loss of skeletal calcium. Regardless of the loss of whole body calcium stores there is no change in the physiologically relevant ionised fraction of calcium (Nordin et al 1989, Nordin et al 1990a).

Osteoporosis is described as a reduction in the amount of bony tissue in a given volume of bone, or a reduction in bone density in a given area, without change to the composition of the remaining bone (Albright & Reifenstein 1948). Postmenopausal osteoporosis is a

disorder of multifactorial origin, characterised by low bone density (Nordin et al 1993) which results in skeletal fragility (Heaney 1993) and consequently, fracture without major trauma. The major factor leading to bone fragility is reduced bone mineral density (Dalen et al 1976, Hansson et al 1980), which is the result of a combination of genetic factors (Smith et al 1973, Dequeker et al 1987, Krall & Dawson-Hughes 1993) and environmental/risk factors such as menopausal status, age, diet, alcohol and caffeine consumption, smoking habits, physical activity, immobilisation, thyroid disease and corticosteroid therapy (Nordin et al 1993). Bone loss results from a negative calcium balance, where calcium excretion is greater than intestinal calcium absorption. Thus, factors that reduce intestinal calcium absorption or increase excretion of calcium can be included in the list of risk factors for osteoporosis. The exact mechanism by which ovarian hormone deficiency leads to bone loss remains controversial (Gallagher et al 1979, Gennari et al 1990, Morris et al 1991, Nordin et al 1991).

The multifactorial nature of osteoporosis makes it difficult to identify the contribution of each of the risk factors by clinical studies. The use of an appropriate animal model for this purpose is essential. The oophorectomised rat has been used extensively as a model for postoophorectomy bone loss and a number of aspects of the model have been characterised in recent times (Kalu et al 1989, Yamazaki & Yamaguchi 1989, Frost & Jee 1992). There are, however, many characteristics of the physiology of calcium metabolism in the rat, including, the role of the ovarian hormones that remain to be identified.

1.2 CALCIUM

1.2.1 Physiology

Calcium forms approximately 2% of total body weight and is the most common mineral in vertebrates (Nordin 1976a). It is the most important structural mineral, combining with phosphate in bone and teeth and with phospholipids and proteins in cell membranes where it plays an important role in the maintenance of membrane integrity and in controlling the permeability of the membrane to many ions including calcium itself (Robertson 1988). It participates in blood coagulation, cellular adhesiveness, transmission of nerve impulses, maintenance of cell membranes (Avioli 1980) and muscle contraction (Kirschberger 1990). Calcium also acts as an important second messenger in intracellular signal transduction. In response to the detection of a signal on the plasma membrane of a cell, intracellular ionised Ca concentration can be raised from approximately 10^{-7} M to approximately 10^{-6} M, enabling its interaction with bioactive proteins such as calmodulin. This interaction induces a conformational change in calmodulin, which triggers a number of appropriate intracellular responses, such as glycogen metabolism and cell division (Robertson 1988).

1.2.2 Maintenance of circulating calcium

Calcium in serum can be divided into essentially three fractions: ionised calcium (approximately 45%), protein-bound calcium (approximately 45%) and complexed calcium (approximately 10%) (Marshall 1976). It is the ionised fraction of calcium that is physiologically important (Nordin 1976b) and it is the principal factor regulating PTH release in vivo (Brown et al 1990).

Physiologically the most important parameter in terms of calcium regulation is the serum ionised calcium, which is maintained within narrow limits (Nordin 1976b). Calcium is continually being lost in the urine even under fasting conditions (Suki & Rouse 1991), at which times serum ionised calcium must be maintained at the expense of bone. Tight regulation of circulating calcium is necessary for the proper function of nerve, muscle and bone. Serum calcium is regulated primarily through the actions of 1,25 dihydroxyvitamin D, the biologically active metabolite of vitamin D, and parathyroid hormone (PTH) on the intestine, kidney and bone.

Approximately 99% of the calcium in the body is in the skeleton in the form of hydroxylapatite and 1% of the skeletal content of calcium is freely exchangeable with the extracellular fluids and soft tissues. Although small as a percentage of the skeletal content, this exchangeable pool is approximately equal to the content of calcium in the extracellular fluid and soft tissues, and serves as an important buffer of calcium (Broadus 1993).

1.2.3 Nutritional requirement for calcium

The calcium requirement varies throughout life and is determined by the relative activities of bone formation and bone resorption. During periods of growth, calcium balance must be positive to enable accumulation of calcium into bone. Increased calcium intake during adolescence and early adulthood results in increased bone mass. Even after the epiphyses have closed and linear growth has stopped, bone mass continues to increase until about 30 years of age (Peck et al 1988). The requirement for dietary calcium is, therefore, higher during these periods than it is during the period when growth has stopped, bone mass has peaked and bone density is merely being maintained. Notwithstanding that 80% of an

individual's bone mass is controlled genetically (Dequeker et al 1987, Pocock et al 1987, Slemenda et al 1991), high calcium intake is associated with a large bone mass, protects against osteoporosis and reduces the incidence of fractures (Matkovic et al 1979). It has been argued that the requirement for calcium rises again during the period of bone deterioration that occurs as a result of the menopause and increasing age (Heaney 1992). With advancing age gastrointestinal calcium absorption is reduced, therefore older persons need to increase calcium intake to maintain the level of dietary calcium absorption that is required to maintain normal bone (Peck et al 1988).

In laboratory rats dietary calcium intake is a determinant of peak bone mass. Low dietary calcium intake during adolescence reduces peak bone mass, whereas high dietary calcium intake promotes higher peak bone mass, providing potential protection against age-related bone loss (Peterson et al 1995).

1.3 BONE TISSUE

Bone is a dynamic tissue with properties of growth and renewal similar to most other tissues. Among its many functions are the provision of support and weight bearing for the body and a repository for calcium stores. Calcium is stored in bone in the form of hydroxylapatite crystals, which are deposited onto a collagen matrix. Once formed, bone is continuously remodelled (ie resorbed and reformed) throughout life (Frost 1969) in order to maintain the viability of the cells (especially the entrapped osteocytes), biomechanical integrity (Melsen & Mosekilde 1988) and to provide calcium to the extracellular fluid during fasting periods.

1.3.1 Bone Remodelling

Bone remodelling takes place at localised sites in the cortical and trabecular bone. Bone resorption begins after the retraction of bone lining cells, at which time osteoclast precursor cells are activated and attracted to the bone surface (Frost 1969). It is probable that cytokines produced by surface cells or adjoining lymphocytes attract cells of the osteoclast lineage to the bone surface (Melsen & Mosekilde 1988). Following the resorption of a quantum of bone by resorptive cells, the resultant pit is refilled with concentric layers of lamellar bone by osteoblasts (Melsen & Mosekilde 1988). Each individual site on a trabecular surface undergoes remodelling approximately every 2 years under normal conditions (Eriksen et al 1986). It has been proposed that during remodelling, a complete coupling must exist between bone resorption and bone formation (Parfitt 1982). The coupling ensures the integrity of the bone mass in spite of the disruptive process of remodelling.

In spite of the coupling that exists between bone resorption and bone formation, changes in bone mass can occur due to a number of different mechanisms (Parfitt 1976, Parfitt 1984). A negative balance between the amount of bone resorbed and the amount later formed will create an irreversible bone loss. This process occurs in osteoporosis (Eriksen 1986).

1.4 CALCIUM BALANCE AND ITS COMPONENTS

As 99% of total body calcium is found in the bone (Nordin 1976a), alterations to any of the fluxes of calcium that constitute whole body calcium balance will lead to alterations in bone

calcium balance. The major components of calcium balance are intestinal calcium absorption, bone formation, bone resorption, urine calcium excretion, faecal calcium excretion and insensible losses via sweat and other body secretions.

1.4.1 Intestinal absorption

Calcium is absorbed from the lumen of the gastrointestinal tract by both saturable and non-saturable mechanisms. Saturable calcium absorption occurs via a transcellular route and is mediated by the action of 1,25 dihydroxyvitamin D on the enterocytes in the proximal small intestine. Calcium is also absorbed by a non-saturable, paracellular route along a concentration gradient, and its rate is therefore, determined by the concentration of available calcium in the gastric contents. Paracellular absorption of calcium predominates when the luminal calcium concentration is high, whereas transcellular (active) transport is needed for efficient absorption when dietary calcium is restricted (Nellans 1990). The regulation of paracellular calcium absorption is poorly understood and data concerning the effects of 1,25 dihydroxyvitamin D are conflicting (Nellans 1990, Karbach 1992).

1.4.1.1. Transcellular calcium transport

Transcellular calcium transport involves three separate steps: 1. Entry into the enterocyte through the brush-border membrane down an electrochemical gradient; 2. Translocation of calcium through the cell; and 3. extrusion of calcium at the basolateral membrane surface against an electrochemical gradient.

Calcium entry into the enterocyte

Intracellular ionised calcium concentration in the enterocyte is approximately 100 nM, compared to available calcium in the luminal fluid of approximately 1-10mM. In addition, the electrical potential within the enterocyte is about -55mV relative to the outside of the cell. Thus, both chemical gradient and electrical forces favour entry of calcium into the cell, requiring no metabolic energy. Brush-border membrane vesicle studies have confirmed that calcium entry into the enterocyte is not energy dependent (Ghishan et al 1989, Schedl et al 1990, Kaune et al 1992). Calcium uptake into brush-border vesicles prepared from vitamin D-sufficient animals is comprised of saturable and non-saturable components and consists of both binding to the membrane and transport into the vesicular space (Schedl et al 1990). The initial rate of uptake is greater in vesicles prepared from vitamin D-replete animals or in animals treated with 1,25 dihydroxyvitamin D than in vesicles from animals on a vitamin D-deficient diet (Schedl et al 1990, Kaune et al 1992). The observation of a saturable component of calcium entry into vesicles is consistent with the involvement of calcium transporters or channels, but direct proof of their existence is lacking. The vitamin D status does not affect vesicle uptake at longer times, indicating that the hormone alters the rate, but not the capacity of calcium entry (Sitrin & Brasitus 1994).

Intracellular calcium translocation

Inside the enterocyte, calcium must move from the brush-border membrane to the basolateral membrane where it can be extruded into the circulation. The rate of calcium flux across the enterocyte by simple diffusion has been estimated from the diffusion constant of calcium in water, the cell size and the estimated transcellular concentration gradient. The

calculated rate of diffusion is nearly two orders of magnitude slower than the experimentally verified rate of transcellular movement (Bronner et al 1986).

In mammals, the intestinal calcium binding protein, calbindin-D is a 9 kilodalton protein, which is conventionally referred to as calbindin-D9k. Its synthesis in the enterocyte is induced by 1,25 dihydroxyvitamin D (Christakos et al 1992). Many studies using a variety of experimental preparations have demonstrated a linear relationship between intestinal calbindin-D9k content and the rate of calcium absorption (Bronner 1990). Additionally, it has been shown that calbindin-D9k can act as an intracellular carrier to facilitate movement of calcium through the enterocyte by greatly increasing the transcellular calcium gradient (Feher et al 1989, Stein 1992). The actual route of calcium during transcellular transport is unknown. A commonly held hypothesis on the possible role of calbindin-D9k in facilitating calcium diffusion assumes that calcium complexed with calbindin-D9k at the brush border traverses the cytosolic compartment to the basolateral membrane. It has also been suggested, however, that calcium traverses the cell within mitochondria (Bikle et al 1980), microsomes (Rubinoff & Nellans 1985), golgi (Freedman et al 1977) or within special lysosomes (Nemere et al 1986). It has recently been suggested that 1,25 dihydroxyvitamin D-stimulated intestinal calcium absorption can occur via a vesicular pathway, involving sequestration of absorbed calcium in endocytic vesicles, fusion of these endosomes with lysosomes, movement of vesicles and lysosomes along microtubules to the basolateral membrane surface and exocytosis from the enterocyte (Nemere & Norman 1990, Nemere & Norman 1991b, Nemere 1992). The importance of this mechanism has not been established.

Basolateral membrane calcium extrusion

The extrusion of calcium at the basolateral membrane of the enterocyte, against a steep gradient, is an energy dependent step. The plasma membrane calcium pump is found in all eukaryotic cells (Carafoli 1991). Its primary role is to maintain free intracellular Ca^{++} within the range of 10^{-7} to 10^{-8} M in order to prevent the potential toxic effects of high concentrations of intracellular Ca^{++} and to prevent interference in the intracellular processes that utilise Ca^{++} as a second messenger. In the enterocyte these properties of the ATP-dependent plasma membrane calcium pump ensure that absorbed Ca^{++} is extruded at the basolateral membrane (Wasserman et al 1994). Wasserman and co-workers (1992a) calculated that the calcium pumping rate is 2-7 times greater than the calcium absorption rate in vitamin-D replete animals, but in vitamin-D deficiency, pump activity could potentially be rate limiting.

The calcium binding affinity of the basolateral membrane calcium pump is approximately 2.5 times that of calbindin-D9K, thus, providing a gradient of binding affinities from the brush border to the basolateral membrane that promotes one-way transport of calcium (Sitrin & Brasitus 1994). Experimental evidence suggests that the calbindins can serve as activators of the calcium pump by a direct, calmodulin-like manner or by the so-called "EGTA effect" (Wasserman 1992a). The concept of the EGTA effect was derived from studies with the erythrocyte calcium pump (Carafoli 1991) and the theory for this effect is based on the shielding of the calcium pump's Ca^{++} binding site by positive charges. Ca^{++} complexed to EGTA is presumed to be more accessible to the Ca^{++}-binding site.

1.4.1.2 Paracellular calcium transport

Paracellular absorption of calcium from the lumen of the gastrointestinal tract to the serosal side of the enterocytes occurs down an electrochemical gradient. Luminal calcium concentration is usually higher than the extracellular fluid ionised calcium concentration of approximately 1.2 mmol/l (Bronner 1992). The paracellular pathway consists of three sequential segments: 1) the tight junction, 2) the intermediate junction and 3) the much wider basolateral space (Trier 1968). It has been calculated that the rate of diffusion of calcium through the region of the tight junction is much slower than expected for a simple diffusion process (Bronner et al 1986). Hyperosmolar solutions, regardless of their chemical nature, can cause the rate of passive calcium transport to double or triple (Pansu et al 1976). It has been suggested that this occurs because the water moving into the hyperosmolar space causes the tissue to expand and the tight junctions to widen (Bronner 1992). Wasserman and co-workers (1956) demonstrated that L-lysine in the diet caused an increase in intestinal calcium absorption. More recently it has been shown that a number of amino acids cause contraction of the cytoskeleton of the cells lining the junctions, thus causing increased calcium flow (Madara & Pappenheimer 1987, Pappenheimer 1987, Pappenheimer & Reiss 1987).

1.4.2. Excretion of calcium

There are two major pathways by which calcium is excreted from the body: excretion in the urine and excretion via the gastrointestinal tract. In the human, of these two routes the minor route is via the gastrointestinal tract (Peacock 1988). The rat, on the other hand excretes approximately 10 times more calcium via the gastrointestinal tract than it does via the kidney (Aubert et al 1964). There are also insensible losses of calcium via sweat,

desquamated cells, hair and intermittent secretions such as menstrual fluid. In adult humans, dermal calcium loss correlates with total serum calcium (Charles et al 1983) and accounts for approximately 13% to 18% of the total excretion of calcium in urine, faeces and skin combined (Taagehøj Jensen et al 1983, Weaver 1994). The loss of calcium in desquamated cells, hair and intermittent secretions is very small (Peacock 1991). Excretion of calcium in the gut, sweat, and secretions are all generally thought to be passive and determined by the concentration of calcium in the extracellular fluid (Peacock 1988), although there is a weak correlation between dermal calcium loss and body surface area (Charles et al 1983).

1.4.2.1 Urine calcium excretion

The calcium excreted in the urine must be derived from either the diet or from body stores. The amount derived from the diet will be determined by the dietary content of calcium and the efficiency of intestinal calcium absorption, while the amount derived from body stores will be determined by the relative rates of bone formation and bone resorption.

Ultrafilterable calcium, forming less than 60% of total plasma calcium is the fraction of plasma calcium which is filtered at the glomerulus (Suki & Rouse 1991). By definition, ultrafilterable calcium is the fraction of plasma calcium made up of free calcium ions and calcium complexed to anions such as phosphate, citrate and bicarbonate, which is filterable through a semi-permeable membrane (Marshall 1976).

Calcium that is filtered through the glomerulus is partially reabsorbed, mainly in the later part of the proximal convoluted tubule, to a lesser degree in the distal convoluted tubule and less again in the ascending limb of the loop of Henle (Suki & Rouse 1991). Although

the majority of calcium reabsorption occurs in the proximal tubule, regulation of the amount finally excreted occurs in the distal convoluted tubule (Peacock 1988). For humans the total filtered load of calcium is about 8.0 g/d. In order to maintain neutral calcium balance, 98 to 99% of this filtered load must be reabsorbed by the renal tubules (Suki & Rouse 1991). At reduced levels of filtered load, calcium never completely disappears from the urine and thus the percentage reabsorbed is reduced (Peacock 1988).

Reabsorption of calcium from the glomerular filtrate in the proximal convoluted tubule is thought to follow a largely paracellular passive route (Suki & Rouse 1991). However, in the rat it has been shown that tubular fluid calcium concentrations in the proximal convoluted tubule could fall below the calcium concentration in the plasma, suggesting that an active component may exist (Lassiter et al 1963). This, however, remains controversial.

The thin descending and ascending portions of the loop of Henle are not involved in the reabsorption of calcium from the glomerular filtrate. On the other hand the thick ascending limb of the loop of Henle does play an important role in calcium handling by the nephron, but again the mechanism of calcium reabsorption is controversial with evidence for both mainly passive absorption and also for mainly active absorption (Suki & Rouse 1991).

Calcium transport in the distal convoluted tubule occurs against an electrochemical gradient, indicating that it is active and most likely transcellular. The kidney has been identified as a target tissue for 1,25 dihydroxyvitamin D (Chandler et al 1979) and has been reported to have high levels of vitamin D-dependent calcium binding proteins (Thomasset et al 1982). Calbindin-D28k is present in the principal cells of the distal tubule in which the

selective reabsorption of calcium occurs. This protein is found in the cytosol and nucleus and it is suggested that it is involved in the translocation of calcium from the luminal membrane to the basolateral membrane (Christakos et al 1989). The role of the calbindins in renal tubular calcium transport is discussed further in section 1.5.1.1.

The calcium in the final urine is less than that observed in the distal convoluted tubule (Lassiter et al 1963), suggesting that further reabsorption of calcium from the glomerular filtrate occurs in the collecting ducts. Vitamin D-dependent calbindin has also been identified in this segment of the kidney (Taylor et al 1982) and it is thought that the mechanism for calcium reabsorption from this segment is likely to be active (Suki & Rouse 1991)

Parathyroidectomy leads to a rise in urine calcium in rats (Talmage & Kraintz 1954), dogs (Kleeman et al 1960) and hamsters (Biddulph 1972). Parathyroid hormone-sensitive adenylate cyclase activity has been identified in the proximal convoluted tubule, proximal straight tubule and in the cortical thick ascending limb of the loop of Henle in the rabbit. The presence of parathyroid hormone-sensitive adenylate cyclase correlates well with the observed actions of this hormone on calcium reabsorption in these segments (Suki & Rouse 1991). Parathyroid hormone has also been shown to enhance calcium reabsorption along the distal convoluted tubule (Shareghi & Stoner 1978, Costanzo & Windhager 1980).

The interrelationship between vitamin D and PTH in renal tubular reabsorption of calcium is not entirely clear. Administration of vitamin D enhances calcium reabsorption in the

parathyroid-intact rat (Costanzo et al 1974), but not in the thyroparathyroidectomised vitamin D replete rat (Hugi et al 1977).

The rat may not be a good animal model for the study of renal handling of calcium. The amount of calcium excreted via the urine in the rat is very small, less than 1% of dietary calcium compared to about 20% in humans (Kerstetter & Allen 1994). In addition, it has been reported that ovarian hormone deficiency has no effect on urine calcium excretion in the rat (Morris et al 1992), unlike the effects observed in women. However, this is currently controversial, with reports of an inconsistent effect (Yamazaki & Yamaguchi 1989) and increased urine calcium excretion (Morris et al 1995) suggesting that renal calcium handling in the oophorectomised rat may not be entirely different from that observed in humans.

1.4.2.2 Faecal calcium excretion

Although calcium excretion in the faeces is commonly thought to be passive and determined by the concentration of calcium in the extracellular fluid, there is evidence to suggest that the transport of calcium into the gastrointestinal tract at a number of sites is determined by other factors. Calcium is excreted in the digestive tract via digestive secretions from the salivary glands, stomach, small intestine, pancreas and liver, and factors that influence the excretion of calcium via these excretions have been identified (Layer & Goebel 1989, Rege et al. 1990, Yu 1990, Karbach 1991). Of particular interest to the study of the effect of oestrogen deficiency on calcium homeostasis is the control of calcium excretion in the bile. The concentration of calcium in the bile is similar to total plasma calcium concentration (Bronner 1964) and is determined by bile flow and composition (Cummings & Hoffman 1984, Loria et al. 1989). The composition and rate of production of bile are influenced by

the sex steroids in a number of species, including the rat (Collado et al. 1989, LeBlanc & Waxman 1990), and alterations to hepatobiliary function following menopause in women have been identified (Maurer et al. 1990).

Excretion of calcium via the gastrointestinal tract has been identified as a determinant of trabecular bone loss in postmenopausal women. Tellez and co-workers (1995) identified a positive correlation between trabecular bone loss and the level of endogenous calcium excretion in calcium balance studies.

1.5 FACTORS AFFECTING CALCIUM BALANCE AND ITS COMPONENTS

While calcium balance can be affected by changes in any of its components there are a variety of mechanisms, particularly those controlled by the calciotropic hormones, which are capable of compensating one or more of the opposing components to ultimately achieve a level of calcium balance that is appropriate for the stage of growth of the individual.

Because calcium balance and bone balance are synonymous, factors that directly affect bone balance also affect calcium balance. For example, factors that shift the balance between bone formation and bone resorption towards resorption lead to a net release of calcium from bone into the ECF. In order to maintain the ECF calcium concentration within critical limits there must be a compensatory reduction in intestinal calcium absorption or and increase in calcium excretion or a combination of both. The net result is a reduction in calcium balance, which may be either a less positive calcium balance or a more negative calcium balance depending on the stage of maturity of the subject.

Many studies have described the effect of various factors on histological or densitometric measurements of bone at specific sites within the skeleton. The findings of these studies are often extrapolated to suggest that the site-specific changes reflect changes that occur throughout the skeleton. Calcium balance evidence in the literature usually supports these extrapolations, but this type of evidence is not available to support all studies that have identified the effects of various factors bone.

This section discusses the hormonal, dietary, environmental and genetic factors that have been shown to affect calcium balance and the components of calcium balance. Where calcium balance studies have not been conducted, the published evidence suggestive of effects on calcium balance is presented. The physiology and mechanisms of action of the major factors affecting calcium balance are also discussed.

1.5.1 The calciotropic hormones

1.5.1.1 Vitamin D

Vitamin D is a fat soluble steroid hormone synthesised in the skin when 7 dehydrocholesterol is exposed to UV light. Its initial classification as a vitamin resulted from early observations that vitamin D, a fat soluble nutrient present in cod liver oil, was capable of preventing and curing rickets (Mellanby 1918, McCollum et al 1922). It is now generally accepted that most of the biological responses produced in target tissues by vitamin D, particularly 1,25 dihydroxyvitamin D in intestine and bone, are mediated via pathways analogous to those used by other steroid hormones (Henry & Norman 1984). After its synthesis in the skin, vitamin D bound to the specific vitamin D binding protein (DBP)

circulates through the liver where it is converted to 25 hydroxyvitamin D. This metabolite of vitamin D, although biologically inactive, is the major circulating metabolite and is the substrate from which the biologically active metabolite, 1,25 dihydroxyvitamin D is produced. The rate of conversion of 25 hydroxyvitamin D to 1,25 dihydroxyvitamin D is determined by the activity of the 1-hydroxylase enzyme located in the distal tubule of the kidney. The activity of the enzyme is in turn determined by the circulating levels of PTH and 1,25 dihydroxyvitamin D (DeLuca HF 1988).

The classic action of 1,25 dihydroxyvitamin D is to elevate plasma calcium and plasma phosphorus to levels required for normal skeletal and neuromuscular function. 1,25 dihydroxyvitamin D's role in maintenance of plasma calcium is achieved primarily by stimulating the intestinal enterocyte to transport calcium against an electrochemical gradient from the lumen of the intestine to the plasma (DeLuca 1988). Another site of 1,25 dihydroxyvitamin D mediation of calcium homeostasis is the bone, where 1,25 dihydroxyvitamin D stimulates the release of calcium to the circulation. This process requires the presence of PTH (Garabedian et al 1974). The third site for 1,25 dihydroxyvitamin D mediation of calcium homeostasis is the distal renal tubule where PTH and 1,25 dihydroxyvitamin D are required to facilitate the renal reabsorption of the last 1% of the filtered load of calcium (Yamamoto et al 1984).

Vitamin D has both genomically and non-genomically mediated actions on a wide variety of target tissues. The genomically mediated actions of vitamin D involve complex and highly integrated signalling pathways modulating the expression of genes that include, but are not restricted to, the control of calcium homeostasis. It has become clear in recent years that the

transduction of the vitamin D signal at its target tissues is complex in nature and thus variance in factors other than vitamin D and its receptor may play an important role in calcium homeostasis.

The vitamin D receptor (VDR) is the central element in the signal transduction pathway leading to the genomically mediated actions of this hormone. It belongs to the superfamily of intracellular nuclear receptors that includes thyroid, steroid and retinoid receptors (Pike 1991). High affinity interaction of VDR with its specific DNA response element requires the formation of a heterodimer between VDR and a member of the retinoid X receptor (RXR) subfamily (Yu et al 1991, Kliewer et al 1992). It has been suggested that VDR exists as a homodimer located in the nucleus and the addition of 1,25 dihydroxyvitamin D causes its dissociation to enable the formation of a heterodimer with RXR, followed by interaction with specific VDRE's (Freedman et al 1994). The exact nature of homodimerisation of VDR and heterodimerisation of VDR with RXR and the physiological significance of these events have not been determined.

VDR is phosphorylated at several sites, each by different kinases (McDonnell et al 1989, Hsieh et al 1993, Jurutka et al 1993a, Jurutka et al 1993b) and it has been demonstrated that the degree of phosphorylation at one of these sites is modulated by 1,25 dihydroxyvitamin D (McDonnell et al 1989). It has been suggested that these events regulate the nuclear functions of 1,25 dihydroxyvitamin D and provide a mechanism for modulation of vitamin D mediated actions within target cells by hormones/growth factors and the state of cell differentiation (Haussler et al 1994).

Perhaps the most widely accepted role for vitamin D in the intestine is the stimulation of the production of the calcium binding protein, calbindin (Christakos et al 1992). The mammalian intestinal calbindin-D9k was first identified by Wasserman & Feher (1977) and has subsequently been shown to be under the strict control of 1,25 dihydroxyvitamin D (Darwish et al 1991). The distribution of calbindin-D9k within the intestine is consistent with the relative absorption rates, in that high levels of calcium absorption occur in the proximal intestine where calbindin content of the enterocytes is high and low levels of calcium absorption occur in the lower intestine where calbindin content of enterocytes is low. There is a linear relationship between intestinal calbindin content and the rate of calcium absorption (Bronner 1990), but calbindin alone does not account for the transport process, because temporal studies have shown this protein to be present when calcium transport has subsided (Harmeyer & DeLuca 1969, Spencer et al 1976).

Other gene products of the enterocyte are also controlled by 1,25 dihydroxyvitamin D. It has been shown to increase brush-border-associated calmodulin, and calmodulin antagonists inhibit vitamin D mediated brush-border membrane vesicle calcium uptake (Bikle et al 1984). Alkaline phosphatase at the brush border appears to be under the control of vitamin D (Pileggi et al 1955), although its role in the calcium transport process remains unknown. Administration of 1,25 dihydroxyvitamin D to vitamin D deficient animals is followed by a significant alteration in membrane lipids (O'Doherty 1978, Matsumoto et al 1981). For instance, phospholipase A2 activity and lysophosphatidyl choline acetyltransferase activity in rat intestinal cells were increased 2-3 fold within 3 hours of 1,25 dihydroxyvitamin D administration (O'Doherty 1978). Cai and co-workers (1993) recently demonstrated that the

transcript of the chicken intestinal plasma membrane calcium pump (PMCA) was elevated following 1,25 dihydroxyvitamin D administration to vitamin D deficient chicks.

The non-genomically mediated actions (usually referred to as the "rapid" effects) were first observed in 1979 by Bachelet et al (1979), who reported stimulation of brush border alkaline phosphatase activity after only 30 min ex vivo perfusion of rat intestine with 1,25 dihydroxyvitamin D. Subsequently, stimulation of alkaline phosphatase activity has been reported after just 10 min (Ben Nasr 1988). These mechanisms appear to be independent of de novo protein synthesis and it has been proposed that the increase in alkaline phosphatase may reflect a segment of the proposed vesicular calcium transport pathway (Nemere & Norman 1991a). Evidence against the involvement of alkaline phosphatase in calcium entry, however, is the observation of Pansu et al (1989) that theophylline inhibited alkaline phosphatase activity, but did not change calcium uptake by brush border vesicles.

In the intestine, the relative importance of each of the identified actions of vitamin D on intestinal calcium transport remains controversial. The mechanisms of vitamin D mediated transcellular calcium transport are discussed in more detail in section 1.4.1.

In bone 1,25 dihydroxyvitamin D stimulates the release of calcium to the circulation. 1,25 dihydroxyvitamin D added to cultures of promyelocytes stimulates their differentiation to monocytes (Abe et al 1981, Tanaka et al 1982) which become macrophages, the precursors of giant osteoclasts (Suda et al 1992). Receptors for 1,25 dihydroxyvitamin D have been identified in osteoblasts, but not in osteoclasts, indicating that the major target cells for 1,25 dihydroxyvitamin D in the bone are the osteoblasts (Suda et al 1992).

Several protein products of osteoblasts or osteoblast-like osteosarcoma cell lines are under the control of 1,25 dihydroxyvitamin D. The promoter regions of the rat (Demay et al 1990) and human (Ozono et al 1990) osteocalcin genes contain vitamin D responsive elements (VDRE), and it has been suggested that osteocalcin stimulates osteoclast development by chemotaxis of osteoclast progenitors (Lian et al 1984). The promoter region of the human osteopontin gene also contains a VDRE (Noda et al 1990). The synthesis of matrix GLA protein (MGP) has also been reported to be stimulated by 1,25 dihydroxyvitamin D in an osteosarcoma cell line, UMR 106 (Fraser et al 1988). It is suggested that either these products or other as yet unidentified products of osteoblasts act as osteoclast differentiation-inducing factor(s) in response to 1,25 dihydroxyvitamin D (Suda et al 1992).

Renal vitamin D receptors have been identified by immunolocalisation in the cells of the distal convoluted tubule (DCT), the collecting duct, the proximal tubule (PCT) and in the parietal epithelial cells of the glomerulus (Kumar et al 1994). Renal 25 hydroxyvitamin D-1-hydroxylase and 25 hydroxyvitamin D-24-hydroxylase enzymes, located mainly in the PCT (Brunette et al 1978, Kawashima et al 1981), are regulated reciprocally (Kawashima & Kurokawa 1986, DeLuca HF 1988) by genomically mediated events which involve the regulation of vitamin D receptor number in tubular cells (Iida et al 1995).

Two vitamin-D dependent proteins involved in Ca transport, calbindin-D9k and calbindin-D28k, have been identified in the mammalian kidney (Christakos et al 1989). Vitamin D deplete rabbits have a marked reduction in ATP-dependent Ca transport in both luminal and basolateral membrane vesicles prepared from the distal tubule (Bouhtiauy et al 1993). More recently, a combined role for calbindin-D9k and calbindin-D28k in vitamin D mediated

transcellular calcium transport in the distal nephron has been proposed (Bouhtiauy et al 1994a, Bouhtiauy et al 1994b).

A deficiency of vitamin D leads to the condition which is usually referred to as rickets in children or osteomalacia when it occurs in adults. This metabolic bone disorder can also be caused by end-organ resistance to vitamin D and less commonly by hypophosphataemia (Peacock 1993). Osteomalacia, which has been reported to be associated with negative calcium balance (Hioco et al 1967), is a bone disorder characterised by delayed initiation of mineralisation of bone matrix as it is laid down. In children the disease affects skeletal growth, modelling and remodelling, whereas only bone remodelling is affected in adults (Peacock 1993).

1.5.1.2 Parathyroid Hormone

The parathyroid glands are usually embedded in the four poles of the thyroid gland, although they may also be found elsewhere in the neck or in the mediastinum. Parathyroid hormone (PTH), after synthesis as a 115 amino acid prepro-peptide in the chief cells of the parathyroid gland, undergoes successive cleavages in the endoplasmic reticulum and the golgi apparatus prior to storage in secretory granules as an 84 amino acid polypeptide. Only a small amount of the hormone is stored in the chief cells and the response to a hypocalcaemic stimulus is both to secrete and synthesise more hormone (Horowitz & Nordin 1993). The effect of parathyroid hormone on ECF-Ca^{++} is rapid and the biologically active hormone is cleared quickly from the circulation (Aurbach et al 1981). PTH maintains the ECF-Ca^{++} at approximately 1.20 mmol/l with a high index of individuality (Morris et al 1990).

Extracellular fluid Ca^{++} interacts with a guanine nucleotide regulatory protein (G-protein) coupled Ca^{++} receptor on the chief cells of the parathyroid gland to regulate intracellular Ca^{++}. The intracellular Ca^{++} in-turn inhibits the secretion of parathyroid hormone from these cells (Brown et al 1993). Although Ca^{++} is the major regulator of PTH secretion, both 1,25 dihydroxyvitamin D (Cantley et al 1985) and Mg^{++} (Rude et al 1978) are also involved in the regulation of its secretion.

The biological activity of PTH is mediated by specific receptors located on the plasma membrane of the target cells and the signal is transduced by a number of effector systems. Apart from adenylate cyclase, the effector systems include cyclic GMP, phosphodiesterase, phospholipase C and ion channels (St John 1995).

The major target tissue for PTH is the kidney. PTH increases the fractional reabsorption of calcium from the glomerular filtrate in the thick ascending limb of the loop of Henle, the distal convoluted tubule and the collecting duct. In the kidney, the PTH signal can be transduced by either a cyclic AMP mediated pathway or by a phospholipase C mediated pathway (Hruska et al 1987). The relative importance of the two pathways is unclear.

In addition to its calcium conserving role in the kidney, PTH also determines the activity of the 25 hydroxyvitamin D-1-α-hydroxylase (1-hydroxylase) enzyme, located in the proximal tubule. This enzyme is responsible for the conversion of 25 hydroxyvitamin D to its active metabolite, 1,25 dihydroxyvitamin D (Kawashima et al 1981). The indirect effect of PTH on the gastrointestinal tract to stimulate intestinal calcium absorption is mediated by 1,25 dihydroxyvitamin D.

The other important target tissue for PTH action is bone. Receptors for PTH have been identified on cells of the osteoblast lineage (Aubin et al 1995), which mediate the signal for osteoclast formation (Takahashi et al 1988) and hence bone resorption.

Many other local factors can stimulate osteoclastic development including the interleukins and tumour necrosis factor (TNF) and some of these factors are influenced by systemic hormones such as PTH (St John 1995).

Primary hyperparathyroidism, that is, the uncontrolled excessive secretion of PTH is a metabolic disorder characterised by negative calcium balance (Gallagher & Wilkinson 1973). This form of hyperparathyroidism is one of the most common endocrine disorders and is usually the result of a single parathyroid adenoma and less commonly the result of hyperplasia of all parathyroid glands (Horowitz & Nordin 1993). The disease is associated with reduced bone density in both the forearm and the spine (Wishart et al 1990).

1.5.1.3 Calcitonin

Calcitonin is synthesised by the "C" cells of the thyroid gland as a large precursor polypeptide (136 amino acids in the rat), which is cleaved into 3 peptides, the mid-region peptide being calcitonin (Aurbach 1988).

The secretion of calcitonin is stimulated by calcium and cyclic AMP. When ECF-Ca^{++} is elevated above normal levels, calcitonin production and secretion are stimulated and there is evidence in the rat that plasma calcitonin levels are increased after feeding (referred to in

Talmage et al 1980). A number of agonists capable of activating the adenylate cyclase-cyclic AMP system have been identified. These include glucagon, cholecystokinin, gastrin and cerulein (Aurbach 1988).

Calcitonin acutely reduces the activity of the osteoclasts by a direct effect on the cells (Melsen & Mosekilde 1988). It also inhibits the direct action of PTH on the release of calcium from bone in vitro. Although receptors for calcitonin have been identified in the medullary ascending thick limb of the nephron, the activity of calcitonin in vivo to produce hypocalcaemia reflects predominantly inhibition of calcium resorption from bone (Aurbach 1988).

The effects of both calcitonin excess and calcitonin deficiency on bone metabolism are controversial. Early bone histomorphometry studies suggested that in patients suffering from medullary carcinoma of the thyroid, chronic elevation of calcitonin is associated with a generalised decrease in bone turnover, but later studies identified increased bone turnover (referred to in Heath 1993). Clinically, there is no elevation of bone mineral density in either the spine or the forearm in patients suffering from hypercalcitoninaemia (Hurley et al 1987).

There is some evidence for a mild resistance to hypercalcaemic stress in calcitonin deficiency (Anast & Guthrie 1971) but this defect cannot be of vital importance to calcium homeostasis because plasma calcium concentrations remain normal (Heath 1993). Patients suffering from calcitonin deficiency following thyroidectomy have no reduction in bone mass in the spine or the forearm (Hurley et al 1987). It has been suggested that endogenous calcitonin may have a suppressive effect on bone resorption, perhaps only under certain

conditions, but that loss of calcitonin secretion causes no major disturbance of bone metabolism or calcium homeostasis (McDermott & Kidd 1987).

1.5.1.4 Oestrogen as a Calciotropic Hormone

Oestrogens are not generally referred to as calciotropic hormones because their influence on calcium homeostasis (ie maintenance of extracellular fluid ionised calcium concentration) is controversial (Young & Nordin 1967, Gallagher et al 1980, Sokoll & Dawson-Hughes1989, Adami et al 1992). Oestrogen receptors have recently been identified in a non-transformed rat enterocyte cell line (Thomas et al 1993) and 17β-oestradiol has been shown to stimulate in vitro ^{45}Ca uptake in isolated rat enterocytes (Arjmandi et al 1993). In addition, 17β-oestradiol stimulates in vivo intestinal calcium absorption without increasing circulating PTH or 1,25 dihydroxyvitamin D in ovary-intact rats demonstrated by calcium balance studies (Arjmandi et al 1994). More recently a negative calcium response element type 2, an element which is involved in the downregulation of PTH transcription in response to raised extracellular calcium concentrations (Okazaki et al 1991), has also been identified on the oestrogen receptor gene (McHaffie & Ralston 1995). This is consistent with the notion that oestrogens play a role in overall calcium homeostasis rather than just bone calcium balance.

The body of evidence that indicates a role for oestrogens in maintenance of whole body calcium levels is very strong for both women and the rat. Much of the evidence for a calciotropic hormone role for oestrogens comes from observed effects of oestrogen deficiency on calcium metabolism and bone metabolism in women and rats, which are discussed in some detail in sections 1.5.6 and 1.6.

1.5.1.5 Other Calcium Regulating Hormones

In addition to the major calciotropic hormones, there are a number of other hormones that have important influence on calcium metabolism. During normal growth and development in man and animals, the bones increase in size, change in shape (modelling) and undergo changes in their internal structure and chemical composition. In immature animals, the growth of the skeleton is accompanied by progressive whole-body retention of calcium until skeletal size reaches its peak which occurs at approximately 6 months in the rat (Kalu et al 1989). These changes are brought about by the activity of osteoblasts and osteoclasts, which are in turn governed by a variety of internal and external factors.

Among the internal factors, growth hormone, adrenal glucocorticoids, thyroid hormone and the sex steroids are necessary in addition to the calciotropic hormones for the normal growth and development of the skeleton and maintenance of bone balance. The precise mechanisms of their actions are not fully understood.

There is abundant evidence from clinical and experimental studies that indicates that glucocorticoid excess causes loss of cortical and trabecular bone and in immature animals (including humans), delayed growth and maturation of the skeleton. Patients suffering from Cushing's disease (overproduction of endogenous cortisol) and patients receiving long-term glucocorticoid therapy are predisposed to osteoporosis and bone fracture (Adams 1988).

The mechanisms underlying the accelerated loss of bone induced by glucocorticoids are complex, but there is general agreement that reduced bone formation as a result of impaired osteoblastic activity is particularly important (Sissons 1960, Riggs et al 1966, Jowsey &

Riggs 1970, Bressot 1979). The effect of glucocorticoids on osteoclastic activity is controversial. Sissons (1960) noted a marked depletion of osteoclasts in Cushing's disease, whereas Jowsey & Riggs (1970) found increased bone resorption as assessed by quantitative microradiography.

Glucocorticoids reduce transport of calcium across the duodenum (Kimberg et al 1971), without reducing the circulating levels of 1,25 dihydroxyvitamin D (Hahn et al 1981). Renal excretion of calcium is elevated in humans with Cushing's disease (Findling et al 1982). Glucocorticoids have been shown to stimulate the secretion of PTH in organ culture (Au 1976).

Growth hormone is essential for normal skeletal development, and deficiency during growth leads to dwarfism. An anabolic effect of growth hormone has been demonstrated on bone in humans (Rudman et al 1990). Fleet and co-workers (1994) demonstrated in the rat that growth hormone stimulates intestinal calcium absorption by increasing duodenal calbindin levels without increasing circulating 1,25 dihydroxyvitamin D. Cell culture experiments using transformed enterocytes, Caco-2, in the same study suggested that the growth hormone effect on calcium absorption may be mediated by insulin-like growth factor-1 (IGF-1). In addition, insulin appears to be necessary for the stimulatory effect of PTH on the 1-hydroxylase enzyme in cultured kidney cells (Henry 1981) and diabetes mellitus is associated with osteopaenia (McNair et al 1978).

The metabolic activity of bone cells varies directly with thyroid function (Jowsey & Detenbeck 1969) and hyperthyroidism is associated with bone loss (Fraser et al 1971). The

male sex steroids are also required for normal skeletal growth and maintenance. Treatment with testosterone causes retention of calcium, phosphorus and nitrogen (Albright & Reifenstein 1948) and castrated male rats develop osteoporosis with loss of both cortical and trabecular bone (Winks & Felts 1980).

Specific receptors for progesterone have been identified in human osteoclasts (Pensler et al 1990). Progesterone also competes with dexamethasone for glucocorticoid receptors and it has been proposed that progesterone could modulate the effects of corticosteroids on bone (referred to in Aurbach 1988). Bone densities in the femur and lumbar spines of progesterone-treated oophorectomised rats are similar to data obtained for intact controls (Barbagallo et al 1989) and it has been demonstrated by single photon absorptiometry that norethistrone increases radial mineral density in postmenopausal women (Abdalla et al 1985). The role of progesterone in the bone loss that follows oophorectomy or menopause has not yet been determined.

1.5.2 Non hormonal factors affecting calcium balance and absorption

For calcium to be absorbed in the gastrointestinal tract it must be in a soluble, ionised form (Pansu et al 1993). The proportion of dietary calcium that is in a soluble form in the small intestine is dependent on the pH, presence of phosphate, fatty acids and other ions forming insoluble salts of calcium (Allen 1982). Thus, under certain conditions, the amount of calcium absorbed by a segment of intestine may depend more on luminal factors which determine calcium solubility, or on the transit rate of the luminal contents, and less on the rate and capacity of saturable transport mechanisms.

A number of dietary factors other than dietary calcium play a role in calcium balance. As early as 1920 it was recognised that dietary protein affected calcium balance, when Sherman (1920) described an increase in urine calcium excretion in humans that were fed an all-meat diet and high dietary protein intake has been shown to lead to lower calcium balance as a result of increased urine calcium excretion (Anand & Linkswiler 1974). It has subsequently been suggested that in the human this is the result of an increase in the glomerular filtration rate that occurs on higher dietary protein intakes (Hegsted et al 1981). The mechanism in the rat appears to be different. Increased protein intake does increase urine calcium excretion, but this is achieved by an increase in intestinal calcium absorption and a shift in the route of calcium excretion from the faeces to the urine (Bell et al 1975). In humans, high dietary phosphorus is associated with decreased urine calcium excretion accompanied by increased intestinal calcium secretion such that there is no net effect on calcium balance (Heaney & Recker 1982). Other observations of dietary modulation of calcium absorption include, modulation of the efficiency of intestinal calcium absorption by lactose (Greenwald & Gross 1929, Wasserman 1964, Favus & Angeid-Backman 1984), starch (Schulz et al 1993), protein content (Kerstetter & Allen 1994), dietary fibre (Harmuth-Hoene & Schelenz 1980) and other factors affecting calcium bioavailability (Amman et al 1986, Pansu et al 1993).

1.5.3 Genetic factors affecting calcium balance

Twin studies suggest that genetic factors account for up to 80% of the variance in bone mineral density in young adults (Dequeker et al 1987, Pocock et al 1987, Slemenda et al 1991). The demonstration of a genetic influence on serum osteocalcin (Kelly et al 1991) and type-1-collagen propeptide (Tokita et al 1994), markers of bone formation, and the rate of

bone gain (Kelly et al 1993) suggest that the genetic control of peak bone mass is mediated through genetic effects on bone turnover. A correlation between vitamin D receptor (VDR) gene polymorphisms and serum osteocalcin levels has been identified (Morrison et al 1992) suggesting a relationship with bone turnover, and it has subsequently been reported that these polymorphisms may account for up to 75% of the total genetic influence on peak bone mass (Morrison et al 1994). However, other studies have failed to identify a relationship between VDR gene polymorphisms and bone density (Hustmeyer et al 1994, Spector et al 1995). Thus, the exact nature of the influence of polymorphisms of the VDR gene on bone density remains controversial and their effect on other vitamin D target tissues has not been elucidated.

1.5.4 The effect of ageing on calcium balance

All species of vertebrates examined so far, from laboratory rodents to non-human species living in the wild are subject to loss of bone with advancing age. Although the pattern of bone loss differs among species and no animal model has been identified in which the pattern of bone loss exactly simulates that in humans, studies on age-related bone loss in animals have yielded valuable information on the causes of bone loss in human subjects, particularly about the effects of physical activity and nutrition (Draper HH 1994).

In humans there is no effect of age on total serum calcium (Insogna et al 1981, Fujisawa et al 1984, Tsai et al 1984), but a slight reduction in ionised calcium has been identified in humans with advancing age (Wiske et al 1979, Yendt et al 1986). However, intestinal calcium absorption declines with age (Avioli et al 1965, Bullamore et al 1970, Alevisaki et al 1973). The age-related reduction in intestinal calcium absorption in women has been

attributed to an age-related intestinal resistance to 1,25 dihydroxyvitamin D, which was caused by reduced duodenal vitamin D receptor (Ebeling et al 1992). There is also a fall in 1,25 dihydroxyvitamin D production with advancing age due to renal dysfunction (Halloran et al 1990).

The rat demonstrates many of the age-related changes in calcium absorption that have been observed in the human. Similar to the human, there is no change in total serum calcium with age in the rat (Armbrecht et al 1980, Gray & Gambert 1982, Kalu et al 1984b Armbrecht et al 1988b). An age-related decrease in calcium absorption in the rat has been demonstrated using everted gut sacs (Horst et al 1978, Armbrecht et al 1979, Armbrecht 1986), perfused duodenal loops (Wood et al 1988) and in isolated duodenal cells (Liang et al 1988).

Active intestinal calcium absorption declines most rapidly during the growth period in the rat until about 6 months of age (Horst et al 1978, Armbrecht et al 1979), consistent with the age at which skeletal growth ceases (Kalu et al 1989). During this period of diminishing growth the levels of intestinal calbindin-D9k also declined in a similar manner (Armbrecht et al 1979, Armbrecht et al 1989). A reduction in calcium absorption after 6 months of age has been demonstrated using everted gut sacs (Horst et al 1978) and isolated duodenal cells (Liang et al 1988). However, Armbrecht and co-workers (1979) were unable to demonstrate a reduction in calcium transport in everted gut sacs after about 6 months of age, but did show that the magnitude of adaptation to low dietary calcium continued to decline after 6 months of age. Calbindin-D9k levels in the duodenum continue to decline after 6 months but plateau after 13 months (Armbrecht et al 1989).

The reduction in absorption when young (2-3 month) rats were compared to adult (12-14 month) rats was most pronounced in the duodenum, less pronounced in the jejunum and there is no age-related change in the ileum (Armbrecht 1986). Thus the major age-related change occurs in the region that exhibits a large active absorption component, while there is no change in the region where calcium absorption is primarily passive. ATP-dependent calcium transport by basolateral membrane vesicle preparations of duodenal enterocytes was also reduced in young (2-3 month) rats compared to adult (12-14 month) rats (Armbrecht et al 1988a).

Circulating levels of 1,25 dihydroxyvitamin D peak at 28 days of age, after which they decline by approximately 80% of the peak value over the next 60 days (Clark et al 1986) and similarly a fall of 78% has been reported in adult rats (>16 month) compared to young rats (1 month) (Horst et al 1990). There is also a reduction in enterocyte vitamin D receptor number in adult rats (>16 months of age) compared to young rats (1 month of age) (Horst et al 1990) and reduced responsiveness to 1,25 dihydroxyvitamin D has been demonstrated in old (23 month) compared to young (3 month) rats using a perfused duodenal loop technique (Wood et al 1988). In addition, a decrease in growth hormone levels has also been demonstrated in aged rats (Millard et al 1986) and as mentioned previously growth hormone has been shown to stimulate intestinal calcium absorption by increasing duodenal calbindin-D9k levels without increasing circulating 1,25 dihydroxyvitamin D (Fleet et al 1994).

An age-related reduction in renal tubular calbindin-D28k which parallels the age-related changes in intestinal calbindin-D9k has also been demonstrated (Armbrecht et al 1989). The

calbindin-D28k response to 1,25 dihydroxyvitamin D administration to primary cultures of rat renal tubule cells is actually greater with advancing age (Chen et al 1992). These data suggest that the reduction in calbindin-D28k, which has been proposed to have a role in transcellular calcium transport in the distal nephron (Bouhtiauy et al 1994a) is the result of reduced circulating 1,25 dihydroxyvitamin D.

The effect of age on calcium balance studies has not been established in humans, but an age-related reduction in bone mass has been reported (Hui et al 1988). In rats a decreased ability to maintain a positive calcium balance in old (18 month) compared to very young rats (1.5 month) has been reported (Armbrecht et al. 1981) and an age-related reduction in bone mass has also been demonstrated (McDonald et al 1986, Kiebzak 1988a). The age-related loss of bone mineral in the rat is mainly from trabecular rather than cortical bone (Kiebzak et al 1988b).

1.5.5 The effect of dietary calcium restriction on calcium balance

Restriction of dietary calcium intake leads to a reduction in calcium balance in both the human (Nordin 1976a) and the rat (Armbrecht et al 1981). It is well recognised, however, that restriction of dietary calcium intake leads to adaptation by calcium homeostatic mechanisms (Nicolaysen et al 1953, Schacter et al 1960, Kimberg et al 1961). The term adaptation can be defined in several ways, and so without clarification the term can be easily misconstrued. For the purpose of this treatise, adaptation refers to the ability of the organism to modify the efficiency of calcium homeostatic mechanisms to accomplish homeostasis while maintaining calcium balance at a level that is appropriate for the organism and for its stage of maturity (ie growth, maintenance or senescence). The ability to

increase the efficiency of calcium absorption and to decrease the amount of calcium lost in the excretions is essential for the maintenance of whole body calcium when the supply of dietary calcium is restricted. It is also essential that when calcium intake is high there are mechanisms in place that prevent excessive accumulation. Young animals have a great capacity to adapt to low dietary calcium intakes (Henry & Kon 1953) and "under these conditions urinary calcium can drop to near zero and retention of dietary calcium can be nearly 100%" (Hegsted 1994). Since the work of Nicolaysen et al (1953) it has been clear that periods of increased skeletal calcium requirements, such as during growth, pregnancy, or lactation or when the diet is low in calcium, are associated with increased efficiency of intestinal calcium absorption (Nicolaysen et al 1953, Schacter et al 1960, Kimberg et al 1961). It is also known that vitamin D is required for intestinal regulation or adaptation of calcium absorption to occur (Nicolaysen et al 1953, Dowdle et al 1960, Kimberg et al 1961). Boyle et al (1971) demonstrated that the production of 1,25 dihydroxyvitamin D could be modulated by dietary calcium content in rats. Dietary calcium restriction stimulates the activity of the 25 hydroxyvitamin D-1-hydroxylase enzyme in the kidney (Omdahl et al 1972) which leads to increased circulating levels of 1,25 dihydroxyvitamin D (Rader et al 1979).

Dietary calcium restriction is also followed by an increase in active intestinal calcium transport which is paralleled by changes in calbindin-D9k in rats up to at least 12 months of age. However, the magnitude of adaptation in intestinal calcium transport declined with age up to 12 months (Armbrecht et al 1979). In the adult rat (over 12 months of age), however, there was a slight rise in circulating 1,25 dihydroxyvitamin D, but no induction of calbindin-D9k and no increase in calcium absorption by the in vitro everted gut sac technique

(Armbrecht et al 1984). It has also been shown in chickens that dietary calcium restriction stimulated a vitamin D-dependent increase in the plasma membrane calcium pump (Wasserman et al 1992b) by a genomically mediated mechanism (Cai et al 1993). Kalu and co-workers (1989) compared intestinal calcium absorption using a balance technique in sham operated and oophorectomised adult rats at both normal and low dietary calcium levels. These workers were unable to clearly demonstrate a reduced intestinal calcium absorption in the oophorectomised rats when fed a normal dietary calcium. When dietary calcium was restricted, however, the oophorectomised rats exhibited a clearly demonstrable reduction in intestinal calcium absorption, suggesting that the oophorectomised rats were unable to adapt to dietary calcium restriction as efficiently as the sham operated rats.

1.5.6 The effect of the menopause on calcium balance

The menopause has a substantial effect on calcium balance with significantly lower balance in postmenopausal women compared to premenopausal women, the difference being due approximately equally to decreased intestinal calcium absorption and to increased urine calcium excretion. The impairment of calcium balance can be corrected by the administration of oestrogen (Heaney et al 1978b).

After the menopause there is a rise in plasma calcium, which may be due to the high level of bone resorption that occurs at this time (Nordin & Polley 1987, Nordin et al 1989, Adami et al 1992). The rise in plasma calcium does not occur in the physiologically relevant ionised fraction, but in the complexed fraction of plasma calcium (Nordin et al 1989, Nordin et al 1990a), which may be the result of a rise in plasma bicarbonate also identified in the postmenopausal woman (Nordin et al 1989).

At the glomerulus, both the ionised and complexed fractions are filtered from the plasma and enter the glomerular filtrate. The majority of the filtered calcium is reabsorbed in the renal tubules and collecting ducts, but the increase in the amount of calcium that is filtered at the glomerulus, resulting from the rise in the complexed fraction contributes to a higher obligatory loss of calcium in the urine (Heaney et al 1978b, Nordin & Polley 1987, Kelly et al 1989, Adami et al 1992). It has also been shown by comparing the calcium excretion in pre- and post-menopausal women matched for filtered calcium loads, that an increase in urine calcium excretion occurs independently of the increased filtered load (Nordin et al 1991). As a result of the strong evidence for an increase in urine calcium at the menopause it has been proposed that the urine 'calcium leak' is a major factor contributing to the continued loss of bone that occurs following the menopause (Nordin & Polley 1987, Nordin et al 1991, Nordin et al 1993). In addition to the excretion of calcium via the kidney, excretion of calcium via the gastrointestinal tract has also been identified as a positive determinant of trabecular bone loss in postmenopausal women (Tellez et al 1995).

Intestinal calcium absorption has been shown by two longitudinal studies to decrease as women pass through menopause regardless of whether the menopause was natural or the result of surgical oophorectomy (Heaney et al 1989, Gennari et al 1990). When corrected for the effect of age on calcium absorption, the withdrawal of oestrogen at the menopause reduces intestinal calcium absorption by approximately 9% (Heaney et al 1989). Calcium malabsorption in postmenopausal osteoporotic women has been attributed variously to reduced circulating 1,25 dihydroxyvitamin D levels (Gallagher et al 1979) and to gastrointestinal resistance to the action of 1,25 dihydroxyvitamin D (Gennari et al 1990, Morris et al 1991). Gennari and co-workers demonstrated that the rise in intestinal calcium

absorption following administration of 1,25 dihydroxyvitamin D was smaller in women after bilateral oophorectomy, but full responsiveness to administered 1,25 dihydroxyvitamin D was restored by oestrogen replacement (Gennari et al 1990). Dietary calcium restriction evokes a similar increase in circulating 1,25 dihydroxyvitamin D in pre- and postmenopausal women suggesting no effect of oestrogen deficiency on the capacity for 1,25 dihydroxyvitamin D production (Prince et al 1990). Although 1,25 dihydroxyvitamin D is regarded as the major regulator of calcium absorption in the intestine (Gallagher et al 1979, Sheikh et al 1988), this hormone only accounts for 33% of the variance in radiocalcium absorption in postmenopausal women (Morris et al 1991).

Recent evidence suggests that oestrogen may also have a direct effect on intestinal calcium absorption. Oestrogen receptors have been identified in a non-transformed rat enterocyte cell line (Thomas et al 1993) and oestradiol stimulates calcium uptake in isolated rat enterocytes (Arjmandi et al 1993) and in vivo calcium absorption in rats (Arjmandi et al 1994).

Calcium imbalance following the menopause leads to a rapid loss of bone mineral density (Nordin & Polley 1987), which is independent of the effect of age on bone mineral density (Nordin et al 1990b). The initial rapid loss of bone is exponential and self limiting (Nordin et al 1990b) and appears to occur mainly in trabecular bone (Riggs & Melton 1983, Nordin et al 1992). After the first five years of rapid bone loss a slower rate of bone loss continues over the following 20 years (Nordin et al 1990b), which appears to result mainly in a reduction in cortical bone (Nordin et al 1993).

Bone loss in postmenopausal women is associated with an increase in bone turnover (Heaney et al 1978a, Christiansen et al 1982, Nordin & Polley 1987). In elderly postmenopausal women high rates of bone turnover are associated with low bone mass (Garnero et al 1996). Heaney and co workers also demonstrated that the resorptive component of remodelling rises significantly more than the formation component of bone turnover, creating an imbalance that results in bone loss (Heaney et al 1978a). The imbalance has been attributed to an increase in the life span of the osteoclast (Parfitt 1979), resorption of deeper pits by osteoclasts (Parfitt et al 1983) and impaired osteoblast function (Parfitt 1979, Parfitt et al 1983, Kelly et al 1989). The amount of bone which has been removed by the osteoclasts and not yet reformed by the osteoblasts during the remodelling sequence is referred to as the remodelling space (Eriksen 1986). The total remodelling space within the skeleton depends on the number of ongoing remodelling cycles (activation frequency), the duration of the remodelling periods and the final resorption depth. Steiniche and co-workers (1989) demonstrated that a combined oestrogen/gestagen treatment did not affect the remodelling periods or the final resorption depth, but reduced the activation frequency by 50% in trabecular bone, consistent with the theory that oestrogen deficiency leads to reduced bone density by increasing the activation frequency of bone resorptive units.

Bone strength depends not only on bone mass but also to a large extent on the integrity of the trabecular lattice (Mosekilde et al 1987). During normal remodelling the trabecular structures are perforated when deep resorption pits hit thinner trabeculae (Parfitt 1984). The risk of trabecular perforations, which disrupt the three-dimensional trabecular network, depends on the activation frequency, the final resorption depth and the trabecular thickness

(Eriksen 1986). Oestrogens do not affect trabecular bone balance, which affects trabecular thickness, or the final resorption depth. Increased activation frequency in the absence of oestrogens increases the risk of perforations (Steiniche et al 1989).

1.5.6.1 Osteoporosis

In many women the ultimate consequence of the increased bone loss that occurs following menopause is osteoporosis. Osteoporosis is a condition in which there is a deficiency of bony tissue in any given volume of anatomical bone (Albright & Reifenstein 1948), but the composition of the remaining tissue is normal (Nordin et al 1993). The increased fracture risk associated with osteoporosis is the result of reduced bone density (Nordin et al 1987). Clinically, osteoporosis can be defined as a reduction in bone density of 2 standard deviations or more below the young normal mean bone density (Nordin 1987). By this definition, not all postmenopausal women become osteoporotic. The degree of reduction in bone density is the result of a combination of the genetic factors that determine peak bone mass (Smith et al 1973, Dequeker et al 1987, Krall & Dawson-Hughes 1993) and factors that have a detrimental effect on bone density such as the menopause, advanced age, low dietary calcium, alcohol and caffeine consumption, smoking, reduced physical activity or immobilisation, thyroid disease and corticosteroid therapy (Nordin et al 1993).

Osteoporosis also occurs in men (Mazess 1982, Riggs & Melton 1986, Nordin et al 1993), young adults (Nordin & Roper 1955, Griffith et al 1965, Gutteridge et al 1986) and in children (Nordin et al 1993). Although calcium balance data has not been collected for the osteoporoses other than postmenopausal osteoporosis, their classification as osteoporoses is based on fracture rates, bone densitometry and histomorphometric data, which indicate a

reduction in bone density (Nordin et al 1993) and therefore suggest negative calcium balance.

1.6 THE OOPHORECTOMISED RAT AS A MODEL FOR POSTMENOPAUSAL BONE LOSS

The oophorectomised rat has been described as a suitable model for the loss of bone that occurs following cessation of ovarian function in women (Kalu 1991, Frost & Jee 1992). Removal of the ovaries from mature rats results in a loss of mainly trabecular bone (Wronski et al 1985, Kalu et al 1989, Wronski et al 1989, Yamazaki & Yamaguchi 1989) with rapid osteoclastic perforation of trabecular plates, but no generalised thinning of the plates, such that there is a decrease in trabecular number without a change in trabecular thickness (Dempster et al 1995).

A study of the effect of oophorectomy on whole body calcium using neutron activation analysis has suggested an impairment to calcium balance following oophorectomy (Beall 1984). The effect of oophorectomy on metabolic calcium balance studies in the rat has not yet been established.

The ultimate result of oestrogen deficiency is an imbalance between the rates of bone formation and bone resorption which results in a net outflow of calcium from the bone. The bone loss that occurs following oophorectomy in the rat is associated with increased bone turnover (Wronski et al 1988a, Morris et al 1992) and it has been demonstrated that the action of oestradiol in bone is to inhibit bone resorption (Wronski et al 1988b, Yamamoto

& Rodan 1990, Kalu et al 1991) and variously to inhibit (Wronski et al 1988b, Kalu et al 1991) and stimulate bone formation (Yamamoto & Rodan 1990, Chow et al 1992). Two recent reports demonstrated that following oophorectomy bone resorption is increased within 5 to 6 days, whereas bone formation is not increased until 9 to 12 days post-operation with bone loss occurring between 12 and 15 days (Dempster et al 1995, Sims et al 1996). An earlier study, however demonstrated that although both osteoclast and osteoblast surface as a percentage of total trabecular surface were increased within 14 days, the rate of bone formation was not maximally stimulated until 35 days post-oophorectomy (Wronski and co-workers 1988a).

Studies utilising a calcium balance technique in the oophorectomised rat have identified an impairment to net intestinal calcium absorption at low dietary calcium intakes, without a reduction in 1,25 dihydroxyvitamin D (Kalu et al 1989). More recently, the administration of oestradiol to ovary-intact rats has been shown to stimulate net intestinal calcium absorption without increasing circulating PTH or 1,25 dihydroxyvitamin D levels (Arjmandi et al 1994). However, studies utilising isolated duodenal loops (Thomas & Ibarra 1987, Thomas et al 1988, Miller et al 1991) and everted gut sacs (Lindgren & DeLuca 1982) have failed to show a decrease in calcium absorption following oophorectomy. In fact, several of the isolated duodenal loop studies (Thomas & Ibarra 1987, Thomas et al 1988) actually reported an increase in active calcium transport.

The measurement of net intestinal calcium absorption by the balance technique does not separate endogenous faecal calcium from true intestinal calcium absorption. Therefore, the fall in net calcium absorption observed by Kalu and co-workers (1989) may actually reflect

a rise in secretion of calcium into the gut, or a combination of reduced intestinal calcium absorption and increased secretion of calcium into the gut. This is a likely route for the excretion of the extra calcium resorbed from bone following oophorectomy in the rat, because the rat excretes approximately 10 times more calcium via the gastrointestinal tract than it does via the kidney (Aubert et al 1964), compared to the human, which excretes more calcium via the kidney (Peacock 1988).

The effect of oophorectomy on urine calcium excretion is currently controversial with no effect (Morris et al 1992), an inconsistent effect (Yamazaki & Yamaguchi 1989) and a small increase in urine calcium excretion (Morris et al 1995) having been reported. These conflicting data may also be explained by the fact that renal calcium excretion is a relatively minor calcium excretory pathway in the rat, thus an effect of oophorectomy on urine calcium, if there is one, may be small and difficult to detect.

1.7 SUMMARY, AIMS AND HYPOTHESES

The principal aim of this thesis is to further characterise the oophorectomised rat model for postmenopausal bone loss by determining the effect of oophorectomy on calcium balance and the components of calcium balance in young and adult rats. The study utilises the metabolic calcium balance technique for this purpose. Many of the characteristics of this animal model have been well described, particularly in terms of the effects of oophorectomy on bone histomorphometry, and metabolic markers of bone turnover. The evidence for an effect of oophorectomy on intestinal calcium absorption and/or urine calcium excretion remains controversial and there has been very little data published on the effects of oophorectomy on metabolic calcium balance studies and the components of calcium balance.

1.7.1 Calcium balance during growth and the effect of oophorectomy

Calcium balance must be highly positive during periods of rapid growth and as growing rats approach full skeletal size the requirement for accumulation of calcium diminishes. Through this period of growth, intestinal calcium absorption decreases markedly. Longitudinal studies of metabolic calcium balance through the growth period have not previously been published. The aim of this study was to characterise the changes in calcium balance and its components through the growth period.

Hypothesis 1: Calcium balance in young growing rats decreases rapidly in parallel with the diminution in growth rate and approaches neutral calcium balance as rats approach full size. The decrease in balance is mainly the result of a decrease in intestinal calcium absorption.

1.7.2 The effect of oophorectomy on calcium balance in the adult rat.

Rapid bone loss after the menopause is associated with an imbalance between the rate of bone formation and the rate of bone resorption. There is also decreased intestinal calcium absorption and increased urine calcium excretion. Oophorectomy in the rat has been shown to reduce bone at specific sites within the skeleton and is associated with an imbalance between the rate of bone formation and the rate of bone resorption, resulting in bone loss within a very short time frame. The effect of oophorectomy on intestinal calcium absorption and urine calcium excretion is currently controversial. The effect of oophorectomy on intestinal calcium secretion (the major route of calcium excretion in the rat) has not been established.

Hypothesis 2: Site-specific reductions in bone following oophorectomy in young growing rats and adult rats, compared to ovary-intact control rats reflect a reduction of calcium throughout the entire skeleton rather than a redistribution of calcium within the skeleton. Thus, oophorectomy leads to a reduced calcium balance, which may be a less positive or a more negative balance. The major component leading to the reduction in calcium balance is decreased intestinal calcium absorption. The extra calcium resorbed from bone following oophorectomy is excreted into the gastrointestinal tract.

1.7.3 The effect of dietary calcium intake on calcium balance in sham and oophorectomised rats.

Restriction of dietary calcium is associated with increased efficiencies of intestinal calcium absorption and renal tubular calcium reabsorption, nevertheless low dietary calcium intake still leads to reduced calcium balance in humans and rats. Oophorectomy, which has been

shown to reduce the efficiencies of intestinal calcium absorption and renal tubular reabsorption in humans, also reduces calcium balance. The daily requirement for dietary calcium is determined by the stage of growth and the efficiencies of intestinal calcium absorption and the reabsorption of secreted calcium.

Hypothesis 3: Calcium balance is related to dietary calcium intake in both ovary-intact and oophorectomised rats. Oophorectomy leads to reduced calcium balance at all levels of dietary calcium intake. Consequently, oophorectomy increases the level of dietary calcium required to maintain neutral balance compared to ovary-intact rats. Adaptation to dietary calcium restriction is achieved by increased intestinal calcium absorption and decreased excretion of calcium in the urine and these adaptations are associated with increased circulating levels of parathyroid hormone and 1,25 dihydroxyvitamin D. The major adaptive response will be a rise in the efficiency of intestinal calcium absorption, as urine calcium excretion is low in the rat. Oophorectomy reduces the efficiency of the adaptive mechanisms such that oestrogen deficient rats are unable to increase intestinal calcium absorption or decrease calcium excretion as efficiently as ovary-intact rats.

1.7.4 The effect of treatment with 1,25 dihydroxyvitamin D and oestradiol on calcium balance.

A major contributor to calcium imbalance after the menopause is reduced intestinal calcium absorption and data obtained from the oophorectomised rat support a similar contribution from decreased intestinal calcium absorption, although this finding is controversial. Administration of 1,25 dihydroxyvitamin D to oestrogen deficient women stimulates intestinal calcium absorption, although the response is diminished compared to oestrogen

replete women. In ovary-intact rats 17β-oestradiol stimulates intestinal calcium absorption without increasing circulating PTH or 1,25 dihydroxyvitamin D levels.

Hypothesis 4 : Administration of 1,25 dihydroxyvitamin D stimulates intestinal calcium absorption and increases calcium balance in the rat. The increase in calcium absorption after 1,25 dihydroxyvitamin D administration to oophorectomised rats is less than that achieved in ovary-intact rats. Oestrogen replacement to oophorectomised rats stimulates intestinal calcium absorption without increasing circulating PTH or 1,25 dihydroxyvitamin D levels and restores calcium balance to a level equivalent to ovary-intact rats.

CHAPTER 2

MATERIALS AND METHODS

2.1 BACKGROUND

Calcium balance has been studied extensively in humans using a variety of protocols (Nordin et al 1976, Heaney et al 1977, Hesp et al 1979, Hegsted et al 1981, Taagehϕj Jensen et al 1983, Eastell et al 1989). Many of these protocols are capable of simultaneous estimation of endogenous faecal calcium and hence true calcium absorption (Nordin et al 1976, Heaney et al 1977, Hesp et al 1979, Taagehϕj Jensen et al 1983, Eastell et al 1989) and some even estimate the dermal losses of calcium (Taagehϕj Jensen et al 1983). Thus in the human, calcium balance studies are capable of determining not only calcium balance and net calcium absorption, but also true absorption of calcium in the intestine and excretion of calcium in the urine, faeces and sweat.

There are also a variety of protocols for the measurement of calcium balance in the rat (Cohn et al 1968, Goulding & Malthus 1971, Armbrecht et al 1981, Goulding & Campbell 1984, Adler et al 1985, Goulding & McIntosh 1986, Schulz et al 1992, Pansu et al 1993), but they are most commonly applied to the simple measurement of calcium balance and net intestinal calcium absorption and are rarely used to obtain the broad spectrum of information that has been obtained from calcium balance studies in humans. These rat protocols vary mainly in terms of duration of balance study, ranging from 24 hours (Cohn et al 1968) to 13 days (Goulding & Campbell 1984), and the use of a period of acclimatisation

(Goulding & Campbell 1984) prior to the performance of the calcium balance. Essentially the common aim of the various techniques is to accurately determine the calcium intake and the calcium content of the excreta that are specifically related to the intake during a given period of time. Endogenous faecal calcium is measured in only one of the listed calcium balance studies in the rat (Goulding & Campbell 1983).

In humans the excretion of calcium occurs mainly via the kidney, with less calcium being excreted via the gastrointestinal tract (Peacock 1988). Urine calcium excretion, which has been described as the major contributor to postmenopausal bone loss (Nordin & Polley 1987) is the major route of calcium excretion in humans. However, the rat, as previously mentioned, excretes approximately 10 times more calcium via the gastrointestinal tract than it does via the kidney (Aubert et al 1964). Thus without measuring the secretion of endogenous calcium into the faeces it would not be possible to determine the relative contributions of each of the components of calcium balance to the reduction in calcium balance that is predicted to occur following oophorectomy in the rat. It is therefore essential, when using the rat as a model for human postmenopausal bone loss to assess the effects of oophorectomy on endogenous faecal calcium.

Intestinal calcium absorption in the rat is commonly studied by invasive techniques such as isolated perfused duodenal loops (Thomas & Ibarra 1987, Miller et al 1991) and everted gut sacs (Lindgren & DeLuca 1982), which do not permit longitudinal studies and are not able to determine the proportional contribution of altered intestinal calcium absorption to altered calcium balance. The present study was designed as a longitudinal study and sought to identify the relative contributions of the various components of calcium balance to the

predicted oophorectomy-induced changes in calcium balance. It was therefore, considered necessary to determine true calcium absorption by a metabolic calcium balance technique.

This chapter describes the development of a method for the determination of calcium balance, which is capable of concomitant determination of endogenous faecal calcium to enable estimation of intestinal calcium excretion and hence true calcium absorption in rats. The performance of various aspects of calcium balance technique were assessed. It also describes the surgical procedures and standard clinical laboratory techniques utilised in this study for the determination of parathyroid hormone (PTH), 1,25 dihydroxyvitamin D (125D) and oestradiol (E2).

2.2 MATERIALS

Unless otherwise stated, basic chemicals used were analytical grade reagents purchased from BDH (Kilsyth, Australia).

2.3 SURGICAL PROCEDURES

Weighed rats were anaesthetised individually in an anaesthetic box filled with 2% halothane (Zeneca Limited, Macclesfield, UK) in O_2:N_2O (2:1). The anaesthetised rat was laid supine on a table with its limbs held extended by adhesive tape and 2% halothane was delivered continuously by a face mask. Surgery was not commenced until the rat was sufficiently anaesthetised. The rat was considered to be sufficiently anaesthetised when there was no reflex response to pinching its foot.

After clipping the hair from the abdomen a 1cm incision was made along the lower abdomen, taking care not to lacerate the underlying muscle layers. The exposed muscle layers and the peritoneum were incised along the mid-line using the smallest incision possible to still allow the removal of the ovaries. The use of such small incisions prevented problems occurring when rats occasionally chewed out their stitches between twice daily post-operative inspections.

The fat pad from either the left or the right side of the ventral pubic region was grasped with forceps and pulled out through the incision until the oviduct was exposed. The ovary was located by pulling the oviduct out through the incision and artery forceps were used to clamp the oviduct below the ovary. The oviduct was tied off with silk and cut through with a scalpel between the silk tie and the ovary. The procedure was repeated for the ovary on the other side. In the case of sham operated rats, once the ovary was located it was handled for a similar period of time and replaced into the abdomen. The muscle layer incision was stitched with a dissolving polyglactin suture (Vicryl, Johnson & Johnson Medical, North Ryde, Australia) and the skin incision was stitched with a silk suture (Johnson & Johnson Medical, North Ryde, Australia). The sutures were checked twice a day for the three days immediately following the operations and restitched when necessary. The rats were allowed a minimum of 7 days to recover from the operation before any further procedures, such as metabolic balance studies, were undertaken.

2.4 SERUM HORMONE ANALYSES

2.4.1 1,25 dihydroxyvitamin D

1,25 dihydroxyvitamin D was determined by a modification of the method of Taylor et al (1980). A 1ml sample of plasma or serum was extracted into 2 × 5ml washes of cyclohexane:ethyl acetate (1:1), evaporated to dryness and reconstituted in chloroform:hexane (65:35). The extract was cleaned and partially purified by LH-20 chromatography, poured in 5ml glass columns with chloroform:hexane (65:35) as a mobile phase. The vitamin D metabolite fraction, which eluted between 7 and 17ml, was evaporated to dryness and reconstituted in hexane:isopropanol (91:9). 1,25 dihydroxyvitamin D was separated from the other vitamin D metabolites by HPLC (ICI model LC1500, ICI Australia, Dandenong, Australia) using a Zorbax Sil column (Du Pont, Wilmington, DE) 4.6mm ×15mm and a hexane:isopropanol (91:9) mobile phase pumped at 2ml/min. Fractions of 1ml were collected on a Biorad model 2110 fraction collector (Biorad, Richmond, CA). 1,25 dihydroxyvitamin D eluted between 11 and 13ml. The 1,25 dihydroxyvitamin D fractions were combined, evaporated to dryness and reconstituted in ethanol. 1,25 dihydroxyvitamin D concentration of the purified extract was determined by radioimmunoassay which utilised an antibody donated by M Peacock (Leeds University, Leeds, UK) and tritiated 1,25 dihydroxyvitamin D (Amersham, Little Chalfont, U.K.) in a phosphate buffer pH 6.1. Following a 48h incubation period, the free fraction was removed from solution by charcoal separation and centrifugation and the bound fraction was counted in the supernatant by scintillation counting. The assay had a calibration range between 5 and 625 pmol/l.

2.4.2 Total serum oestradiol

Oestradiol was measured in unextracted serum samples by a chemiluminescent immunoassay on an ACS:180 immunoassay analyser (CIBA-Corning Diagnostics Corporation, Medfield, MA). The manufacturers instructions as supplied with the kit indicated that this assay had a coefficient of variation of 11.7% at 267pmol/l and an assay range between 3.7pmol/l and 11,000pmol/l (CIBA-Corning 1994).

2.4.3 Intact Parathyroid hormone

Serum levels of intact parathyroid hormone were determined by a two-site immunoradiometric assay for intact rat PTH (Nichols Institute Diagnostics, San Juan Capistrano, CA). The manufacturers instructions as supplied with the kit indicated that this assay had a coefficient of variation of 4.0% at 5.3 pmol/l and standard values ranged from 0 to 200 pmol/l. (Nichols Institute Diagnostics 1993).

2.5 CALCIUM BALANCE METHOD DEVELOPMENT

2.5.1 Animals

Virgin female Sprague-Dawley rats used for all experiments described in this thesis were purchased from Gilles Plains Animal Research Centre (Adelaide, South Australia). For the development of the calcium balance method rats aged 16 - 22 weeks were used. Stress in rats was indicated by the appearance of chromodacryorrhea (bloody tears) (Harkness & Ridgway 1980). All animal procedures, including housing, dietary manipulation, balance and surgical procedures were approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science.

2.5.2 Housing

On arrival in the animal house the rats were transferred from communal cages to individual cages measuring 41×28×14 cm. The animal house environment was maintained at 22 ±2°C, with a 12:12hr light:dark cycle.

2.5.3 Diet

The animals were allowed 24hr access to water and were fed either standard laboratory chow (Milling Industries, Adelaide, South Australia) (Table 2.1) or one of two semi-synthetic diets: AIN-76A (American Institute of Nutrition 1977, American Institute of Nutrition 1980) (Table 2.2) or AIN-76A-starch (Table 2.3), in which the sucrose content of the diet was replaced by cornstarch. Ad libitum feeding was compared with restricted feeding during the balance period. For balances that employed restricted feeding the mean daily food consumption of ovary-intact rats was determined during the period of acclimatisation, immediately prior to the balance measurement period and then both ovary-intact and oophorectomised rats were given a daily allowance of 90% of that mean for the duration of the balance period.

2.5.4 Diet preparation

2.5.4.1 Standard laboratory chow

Standard laboratory rat chow was powdered in a Waring blender (Waring Products Co., Winsted, Conn, USA) before being fed to the rats in metabolic cages. The chow used in the experiment to determine the effects of oophorectomy on calcium balance in the young rat was prepared as a single batch to avoid the possibility of inter batch variation.

TABLE 2.1 Composition of standard rat chow.

Ingredient	Amount (g/100g diet)
Whole wheat	45.95
Whole oats	15.0
Soyabean meal	12.37
Fish meal	7.5
Lucerne meal	5.5
Peas	5.0
Meat meal	2.5
Tallow	2.0
Vitamin-mineral mix[1]	0.55
Sodium chloride	0.5
Choline chloride	0.08
Lysine	0.05
Sunflower oil	2.5
Blood meal	0.5

[1]Composition of the vitamin-mineral mix was as follows (mg/kg diet): all-*trans* retinol, 75; cholecalciferol, 0.05; *RRR*-α-tocopherol, 50; menadione, 8.3; cyanocobalamin, 120; thiamine, 58; riboflavin, 10; pyridoxine, 12; calcium pantothenate, 20; niacin, 18; folic acid, 2; biotin, 110; zinc, 61; copper, 5; manganese, 102; molybdenum, 1; iodine, 0.6; cobalt, 420; butylated hydroxyanisole, 100.

TABLE 2.2 Composition of AIN-76A[1] semi-synthetic diet.

Ingredient	Amount (g/100g diet)
Casein	20
Corn starch	15
Sucrose	50
Cellulose	5
Corn oil	5
DL-methionine	0.3
Choline bitartrate	0.2
Modified AIN 76 mineral mix[2]	3.5
AIN 76A vitamin mix[3]	1

[1]American Institute of Nutrition 1977

[2]Composition of AIN 76 mineral mix (modified to give 0.4% calcium) was as follows (mg/kg diet): Calcium phosphate (dibasic), 17500; NaCl, 2590; Potassium citrate monohydtrate, 7700; Potassium sulphate, 1820; Magnesium oxide, 840; Manganous carbonate, 123; Ferric citrate, 210; Zinc carbonate, 56; Cupric carbonate, 11; Potassium iodate, 0.4; Sodium selenite, 0.4; Chromium potassium sulphate, 19; Sucrose, 4130.

[3]Composition of AIN 76A vitamin mix (ICN Biomedicals Australasia, Seven Hills, Australia) was as follows (mg/kg diet): Thiamine hydrochloride, 6; Riboflavin, 6; Pyridoxine hydrochloride, 7; Nicotinic acid, 30; D-calcium pantothenate, 16; Folic acid, 2; D-biotin, 0.2; Cyanocobalamin, 0.01; Retinyl palmitate, 16; DL-α-tocopherol acetate, 200; Cholecalciferol, 2.5; Menadione sodium bisulphite complex, 0.5; Finely powdered sucrose, 9729.

TABLE 2.3 Composition of AIN-76A[1]-starch semi-synthetic diet (0.02-0.4% calcium).

Ingredient	Amount (g/100g diet)
Casein	20
Corn starch	65
Cellulose	5
Corn oil	5
DL-methionine	0.3
Choline bitartrate	0.2
Calcium deplete AIN 76 mineral mix[2]	3.5
AIN 76A vitamin mix[3]	1
Calcium carbonate (0.02-0.4%)	0.5-10

[1]American Institute of Nutrition 1977

[2]Composition of calcium deplete AIN 76 mineral mix was as follows (mg/kg diet):Sodium di-hydrogen phosphate ($2H_2O$), 6895; Potassium di-hydrogen phosphate, 9625; Potassium sulphate, 1820; Magnesium oxide, 840; Manganous carbonate, 123; Ferric citrate, 210; Zinc carbonate, 56; Cupric carbonate, 11; Potassium iodate, 0.4; Sodium selenite, 0.4; Chromium potassium sulphate, 19; Finely powdered sucrose, 15400.

[3]Composition of AIN 76A vitamin mix (ICN Biomedicals Australasia, Seven Hills, Australia) was as follows (mg/kg diet): Thiamine hydrochloride, 6; Riboflavin, 6; Pyridoxine hydrochloride, 7; Nicotinic acid, 30; D-calcium pantothenate, 16; Folic acid, 2; D-biotin, 0.2; Cyanocobalamin, 0.01; Retinyl palmitate, 16; DL-α-tocopherol acetate, 200; Cholecalciferol, 2.5; Menadione sodium bisulphite complex, 0.5; Finely powdered sucrose, 9729.

2.5.4.2 Semi-synthetic diets

The semi synthetic diets were prepared in the laboratory to the standard formulae described in Tables 2.2 and 2.3. The components of the mineral mix were weighed, crushed to a fine powder with a mortar and pestle, placed in a sealable plastic container and mixed by manual shaking for 5 minutes. Cornstarch, casein and cellulose were mixed for 30 minutes in a pizza dough mixer (OEM, VE201, Bozzolo(MN) Italy). The trace components, DL-methionine (Sigma, St Louis, USA), choline bitartrate (Sigma, St Louis, USA), mineral mix, vitamin mix (ICN, Aurora, USA) and appropriate amount of $CaCO_3$ (BDH, Kilsyth, Australia) were combined in a sealable plastic container and mixed by manual shaking for 5 minutes. The mixture of the trace components was then added gradually to the mixture of cornstarch, casein and cellulose while the dough mixer was in motion and mixing was allowed to continue for a further 30 minutes. Finally the corn oil was added to the mixture while the dough mixer was in motion and mixing was allowed to continue for a further 30 minutes or until the semi-dry powder was smooth and consistent to the touch, indicating complete blending of the oil. The semi-synthetic diet was used in this form for feeding during periods in the metabolic cages. Powdered semi-synthetic diets were stored at room temperature for not more than 2 weeks. For feeding during periods outside the metabolic cages the diet was further processed by adding sufficient water to form a thick slurry, which was poured into trays and allowed to partially dry before being cut into conveniently sized cakes and frozen at -20°C. Frozen semi synthetic diets were kept for periods up to 6 weeks.

2.5.5 Non-absorbable dietary markers

Balance data obtained with the inclusion of one of three non-absorbable dietary markers were compared to data obtained without the use of a non-absorbable marker. The markers

assessed in this study were carmine red (BDH, Kilsyth, Australia), polyethylene glycol 4000 (PEG) (BDH, Kilsyth, Australia) and ^{51}chromium chloride (Amersham, Little Chalfont, UK). PEG was added to powdered rat chow at 1g PEG/100g chow and mixed thoroughly in a sealable plastic container capable of holding 3kg of powdered diet. At the time that the PEG experiment was conducted, the calcium balance protocol included a 4 day acclimatisation period followed by a 3 day balance period with ad libitum feeding of rat chow. ^{51}Chromium and carmine red were assessed later in the method development when the AIN-76A diet was being used. Carmine red (a visible faecal marker) was added until the diet was sufficiently coloured (approximately 1g/kg diet) and ^{51}Cr was added at 2MBq/kg diet ($^{51}CrCl_2$: 10 GBq/mg Cr). In both cases the marker was added to the trace component mixture prior to combination with the mixture of cornstarch, casein and cellulose. Carmine red was used with a balance protocol that included 4 days acclimatisation followed by a 3 day balance period and ad libitum feeding of the AIN-76A diet. The balance protocol used with ^{51}Cr included a 5 day acclimatisation followed by a 6 day balance period, with 90% restricted feeding during days 5-11 as described in 2.5.3. For balances that included either PEG or ^{51}Cr as a non-absorbable marker the calculations of calcium balance and its components were corrected by calculating calcium consumption from the amount of marker recovered and the ratio of calcium to marker in the diet, determined by assay.

2.5.6 Estimation of endogenous faecal calcium

Radioactive ^{45}Ca was administered by either intramuscular (IM) or intraperitoneal (IP) injection to monitor endogenous faecal calcium. A solution containing ^{45}Ca 20×10^3MBq/l (CaCl2, 83.6 GBq/l; Amersham, Little Chalfont, UK) and NaCl 150mmol/l was sterilised by

filtration through a 0.2μm syringe-mounted filter. 100μl of the sterilised solution, containing 2MBq $^{45}CaCl_2$, was injected either intraperitonealy or intramuscularly (thigh).

The rate of reduction in urine ^{45}Ca following IM administration of ^{45}Ca 20×10^3MBq/l was determined by collection of 24h urine samples for 8 days immediately after the dose. To determine the effect on the variance in endogenous faecal calcium of the length of the lag period between administration of IM ^{45}Ca and commencement of the balance period, IM ^{45}Ca was administered either 16 hours, 3 days or 6 days prior to the commencement of calcium balance.

2.5.7 The effect of duration of balance period on variance

The length of time over which calcium balance studies were performed was assessed by performing a balance study with 4 day acclimatisation period followed by a 9-day balance period, during which faeces and urine collections were made at the end of day 3, at the end of day 6 and at the end of day 9. ^{45}Calcium was administered by IM injection at 1700h on the day prior to commencement of the 9-day balance period and was not readminstered during this period. From these data it was possible to calculate 3 × 3-day balances for each rat, a 6-day balance was calculated from day 4 to day 9 inclusive and the data from all 3 collections were combined to calculate a 9 day balance. The effect of a lag time between administration of IM ^{45}Ca and the commencement of the balance was also assessed.

2.5.8 Performance of calcium balance studies

Calcium balances were performed in individual metabolic cages (Techniplast, Buguggiate, Italy). Each balance study included an **acclimatisation period** of 4 or 5 days followed by a **balance period** of either 3, 6 or 9 days.

The rats were weighed and placed in metabolic cages on day 1 and given a known amount of powdered diet. On the morning of day 2 the remaining food was weighed and the food consumption for day 1 calculated and recorded. The process was repeated for days 2-4. Radioactive ^{45}Ca ($CaCl_2$, 83.6 GBq/L; Amersham, Little Chalfont, U.K.) was administered on day 4 by either intraperitoneal or intramuscular injection to monitor the secretion of endogenous calcium into the faeces (endogenous faecal calcium). Those balances that included a four day acclimatisation period, the balance period commenced on day 5 and the rats were fed ad libitum. For those that included a 5 day acclimatisation period, the daily consumptions for days 1-4 for the ovary-intact rats were meaned and the food allowance for day 5 and for the subsequent balance period, was restricted to 90% of the mean acclimatisation period consumption of the ovary intact animals.

On the morning of the first day of the balance period the collecting funnels, urine and faeces containers and the feed containers were rinsed of fur, urine and faeces. 2ml of concentrated HCl was added to the urine container to prevent precipitation of calcium salts that can result from the rise in pH which occurs when urine is kept at room temperature. The daily food allowance for the balance period was added to the food containers at 0900 each day and the actual consumption was determined by weighing the food remaining in the container before discarding. On the final day of the balance period the feed containers were removed from

the metabolic cages at 0900 and the urine and faeces samples were collected into separate 250ml containers, weighed, and the rats were returned to their normal cages.

2.5.9 Preparation of samples for analysis

2.5.9.1 Faeces

For all calcium balance studies using rat chow and the AIN-76A diets, faecal samples were diluted 1:4 (w/v) with water and homogenised in a Sorvall Omni Mixer (Sorvall, Newtown, CT). Two aliquots, approximately 1g each, were placed into numbered 20ml porcelain crucibles and the exact weights recorded. The samples were then charred on a ceramic hotplate for approximately 30 minutes, porcelain lids were placed on the crucibles and the charred material was ashed at 600°C for 16h in a muffle furnace. The ash was reconstituted in exactly 2ml 5N HCl. The samples were centrifuged at 5000×g for 10 minutes to remove particulate matter. The remaining non-ashed homogenate was saved for PEG analysis in the studies for which it was used as a non-absorbable marker.

For calcium balance studies in rats fed the AIN-76A-starch diet, the entire faecal sample was placed into a numbered 70ml porcelain crucible and covered with a porcelain lid. The crucibles were then placed into a muffle furnace and charred by heating gradually to 400°C for 4h and then ashed at 800°C for at least 16h. The ash was dissolved in 4ml 5N HCl and heated to 75°C for 5 minutes, then transferred to a 10ml volumetric flask. A further 4ml 5N HCl was used to rinse the crucible, then transferred to the same volumetric flask. The volume was made up to exactly 10ml with 5N HCl. These samples were transferred to 10ml plastic tubes and centrifuged at 5000×g for 10 minutes to remove particulate matter.

Prior to calcium analysis the faecal extracts were prediluted to bring them within the standard range of the atomic absorption calcium assay used. For example faecal extracts from animals on a 0.02% Ca diet were prediluted 1/41 prior to analysis and faecal extracts from animals on a 0.4% Ca diet were prediluted 1/401 prior to analysis.

2.5.9.2 Diet

For experiments in which standard laboratory chow or AIN-76A were used, triplicate diet samples were diluted 1:4 (w/v) with water and homogenised in a Sorvall Omni Mixer (Sorvall, Newtown, CT). Duplicate aliquots, approximately 1g each, were placed into numbered 20ml porcelain crucibles and the exact weights were recorded. The samples were then charred on a ceramic hotplate for approximately 30 minutes, porcelain lids were placed on the crucibles and the charred material was ashed at 600°C for 16h in a muffle furnace. The ash was reconstituted in exactly 2ml 5N HCl. The samples were centrifuged at 5000×g for 10 minutes to remove particulate matter.

For the calcium balance studies in which AIN-76A-starch was used, triplicate 10g samples of the diet were placed in separate crucibles and covered with porcelain lids. The samples were charred, ashed, reconstituted and centrifuged in the same way in which the faecal samples were prepared for rats fed the AIN-76A-starch diet. The dilutions used to bring the diet extracts within the standard range of the calcium assay were lower than those required for faecal samples. For example diet extracts from the 0.02% diet were not prediluted and diet extracts from the 0.4% diets were prediluted 1/101 prior to analysis.

2.5.9.3 Urine

The pH of each urine sample was checked to ensure that it was below pH 2 and adjusted with concentrated HCl when necessary. The samples were centrifuged at 5000×g for 10 minutes to remove any particulate matter. Urine samples did not require predilution prior to calcium analysis.

2.5.10 Analysis of urine, faeces and diet

2.5.10.1 ^{40}Calcium

Calcium analysis of faecal extracts, diet extracts and urine samples was performed by atomic absorption spectrometry on a Perkin-Elmer model 3030 (Perkin-Elmer, Norwalk, CT). The instrument was set up with a wavelength of 422.7nm, slit width 0.7nm, lamp current 15mA with a fuel rich flame (air 45, acetylene 25[flow units]). Calibrators, 0, 1.5, 2.0, 2.5, 2.75 and 3.0 mmol/l were prepared according to the method of Cali et al (1973). The imprecision of the assay was 1.2% at 2.45 mmol/l.

2.5.10.2 ^{45}Calcium

The ^{45}Ca content of faecal extracts and urine samples was determined by scintillation counting. Duplicate aliquots of the urine (1ml) and faecal extracts (200µl) were transferred to 20ml scintillation vials and dissolved in 20ml scintillation fluid (Optiphase Hisafe III, Wallac Oy, Turku, Finland) for faecal extracts and 10ml scintillation fluid for acidified urine samples. Each vial was counted for 20 minutes in a Packard Minaxi, Tri-carb 4000 (Packard, Downers Grove, IL).

2.5.10.3 Polyethylene glycol 4000

Polyethylene glycol 4000 was determined in faeces by the method of Wilkinson (1971). 1g of faecal homogenate was weighed into a plastic tube and diluted with 10ml water. 1ml of PEG standards (0 - 10 g/l) were diluted with 10ml water. To the standards, controls and unknowns 1.0ml of BaCl2 100g/l and 2ml 0.3N BaOH was added and all tubes were mixed. Then 2.0ml ZnSO4 50g/l was added to all tubes which were then allowed to stand for 10 min. All tubes were filtered through individual Whatman 542 filter papers. 1ml of filtrate was measured into another tube and 4ml of a solution of trichloracetic acid 300g/l and BaCl2 60g/l was added and mixed. The tubes were incubated at room temperature for 60 - 90 min and the turbidity was determined by measuring optical density at 650nm in a 1cm glass cuvette.

2.5.10.4 ^{51}Chromium

^{51}Chromium was determined in diet extracts by the same method used to determine ^{45}Ca in the faecal extracts. ^{45}Calcium and ^{51}Cr were determined simultaneously in faecal extracts. After samples were prepared in the same manner used for faecal ^{45}Ca counting, the samples were counted 4 times with a time lag between each count (days 0, 12, 38 and 48). The portion of the total count on day zero that was attributed to each ^{45}Ca and ^{51}Cr was calculated by a simultaneous equation method using either the 12, 38 or 48 day count as the second count. The calculations using days 1 to 12, days 1 to 38 and days 1 to 48 were compared with each other to determine the best time lag between counts 1 and 2. The equations used to calculate ^{45}Ca and ^{51}Cr for day one were:

$$TC_1 = {}^{45}Ca_1 + {}^{51}Cr_1$$

$$TC_2 = \alpha_1 {}^{45}Ca_1 + \alpha_2 {}^{51}Cr_1$$

$${}^{45}Ca_1 = ((TC_2/\alpha_2)-TC_1)/((\alpha_1/\alpha_2)-1)$$

$${}^{51}Cr_1 = TC_1 - {}^{45}Ca_1$$

Where: TC_1 = total count day 1

TC_2 = total count day 2

${}^{45}Ca_1$ = ${}^{45}Ca$ day 1

${}^{51}Cr_1$ = ${}^{51}Cr$ day 1

α_1 = calculated remaining fraction of day 1 activity of ${}^{45}Ca$ ($t_{½}$ ${}^{45}Ca$ = 163d)

α_2 = calculated remaining fraction of day 1 activity of ${}^{51}Cr$ ($t_{½}$ ${}^{51}Cr$ = 27.7d)

2.5.11 Calculation of calcium balance and components of balance

Calcium balance and its components, endogenous faecal calcium, intestinal calcium secretion, net calcium absorption, and true calcium absorption were calculated by the following equations adapted from Nordin et al (1976):

Ca consumption = average food consumption × food ${}^{40}Ca$ (mmol/d)

or Ca consumption = faecal non-absorbable marker × (diet Ca/ diet non-absorbable marker) (mmol/d)

Ca balance = Ca consumption - (faecal ${}^{40}Ca$ + urinary ${}^{40}Ca$) (mmol/d)

Endogenous faecal Ca = urinary ${}^{40}Ca$ × (faecal ${}^{45}Ca$/urinary ${}^{45}Ca$) (mmol/d)

Unabsorbed dietary Ca = faecal ${}^{40}Ca$ - endogenous faecal Ca (mmol/d)

Net Ca absorption = Ca consumption - total faecal Ca

Fractional calcium absorption = (Ca consumption - unabsorbed dietary Ca) Ca consumption (mmol/d)

True calcium absorption = Ca consumption - unabsorbed dietary calcium

(mmol/d)

or True calcium absorption % = fractional calcium absorption × 100

Intestinal Ca secretion = endogenous faecal Ca/(1- fractional Ca absorption)

(mmol/d)

The components of calcium balance were calculated by these formulae using the Microsoft, Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA). Each parameter was initially calculated as a six-day balance (mmol/6d) then divided by 6 in order to express them as mmol/d. An example of an extract from a calcium balance spreadsheet is shown in Table 2.4.

2.5.12 Statistical analyses

For the development of the calcium balance method, the mean, standard deviation and interindividual coefficient of variation were determined for each calcium balance performed on groups of 6 rats for rat chow and AIN-76A experiments and 10 rats for AIN-76A-starch experiments. The calcium balance technique was optimised by adopting the techniques that provided the lowest interindividual coefficients of variation for calcium balance, net calcium absorption, endogenous faecal calcium and true calcium absorption.

Table 2.4 Example of the Microsoft Exel spreadsheet used for the calculation of metabolic calcium balance and its components

										DietCa Level =	0.015	mmol/g								
Rat ID	UCa45/ml	UCa40/L	U vol	UCa45/6d	UCa40/6d	FCa45/200ul	FCa45/6d	FCa40/L	FCa40/6d	fdwt	CaCon/6d	CaBal /6d	NtAbs/6d	EndFCa/6d	UnabCa/6d	TruAbsFrac/6d	TruAbs/6d	TruAbs %	IntCaSec/6d	NtAbs%
65	627	0.25	34.5	21631.5	0.008625	5863	293150	84	0.84	46.3	0.6945	-0.154	-0.146	0.117	0.723	-0.041	-0.029	-4.120	0.112	-20.950
66	1204	0.4	66.8	80186.4	0.02664	6117	305850	121	1.21	76.4	1.148	-0.091	-0.064	0.102	1.108	0.033	0.038	3.282	0.105	-5.585
67	942	0.83	91.9	86669.8	0.076277	8208	410400	145	1.45	84.2	1.263	-0.263	-0.187	0.362	1.088	0.138	0.175	13.825	0.420	-14.806
68	1929	0.58	41.8	80246.4	0.024128	4968	248300	86	0.86	79.2	1.188	0.304	0.328	0.075	0.785	0.339	0.403	33.894	0.113	27.609
69	996	0.4	52.5	52290	0.021	7320	366000	143	1.43	85	1.275	-0.176	-0.155	0.147	1.283	-0.006	-0.008	-0.628	0.146	-12.157
70	1223	0.73	77.4	94660.2	0.056502	3938	196900	109	1.09	84.7	1.2705	0.124	0.181	0.118	0.972	0.235	0.298	23.458	0.154	14.207
71	1344	0.49	60.1	80774.4	0.029449	7026	351300	121	1.21	84.9	1.2735	0.034	0.064	0.128	1.082	0.150	0.192	15.043	0.161	4.986
72	1224	0.41	46.8	57038.4	0.019106	6967	348350	132	1.32	84.8	1.272	-0.067	-0.048	0.117	1.203	0.054	0.069	5.400	0.123	-3.774
73	1334	0.57	50.9	67900.6	0.029013	5908	295400	138	1.38	84.3	1.2645	-0.145	-0.116	0.126	1.254	0.008	0.011	0.848	0.127	-9.134
74	2335	0.88	53.7	125389.5	0.047256	5087	254350	106	1.06	82.1	1.2315	0.124	0.172	0.098	0.964	0.217	0.267	21.710	0.122	13.926
	UCa40/d	FCa40/d	fdwt/d	CaCon/d	CaBal/d	NtAbs/d	NtAbs %	EndFCa/d	TruAbs frac/d	TruAbs/d	TruAbs %	IntCaSec/d	operation							
65	0.0014	0.140	7.717	0.116	-0.026	-0.024	-20.950	0.019	-0.007	-0.005	-4.120	0.019	2							
66	0.0044	0.202	12.733	0.191	-0.015	-0.011	-5.585	0.017	0.005	0.006	3.282	0.018	1							
67	0.0127	0.242	14.033	0.211	-0.044	-0.031	-14.806	0.060	0.023	0.029	13.825	0.070	2							
68	0.0040	0.143	13.200	0.198	0.051	0.055	27.609	0.012	0.056	0.067	33.894	0.019	1							
69	0.0035	0.238	14.167	0.213	-0.029	-0.026	-12.157	0.024	-0.001	-0.001	-0.628	0.024	2							
70	0.0094	0.182	14.117	0.212	0.021	0.030	14.207	0.020	0.039	0.050	23.458	0.026	1							
71	0.0049	0.202	14.150	0.212	0.006	0.011	4.986	0.021	0.025	0.032	15.043	0.025	2							
72	0.0032	0.220	14.133	0.212	-0.011	-0.008	-3.774	0.019	0.009	0.011	5.400	0.021	1							
73	0.0048	0.230	14.050	0.211	-0.024	-0.019	-9.134	0.021	0.001	0.002	0.848	0.021	2							
74	0.0079	0.177	13.683	0.205	0.021	0.029	13.926	0.016	0.036	0.045	21.710	0.020	1							
means				0.196	-0.008					0.024	11.271	0.026								
sh mean	0.006	0.185	13.573	0.204	0.013	0.019	9.277	0.017	0.029	0.036	17.549	0.021								
sd	0.003	0.029	0.605	0.009	0.027	0.028	13.903	0.003	0.022	0.026	12.947	0.003								
sem	0.001	0.013	0.271	0.004	0.012	0.012	6.218	0.001	0.010	0.012	5.790	0.001								
ox mean	0.005	0.210	12.823	0.192	-0.023	-0.018	-10.412	0.029	0.008	0.011	4.993	0.032								
sd	0.004	0.042	2.855	0.043	0.018	0.017	9.646	0.017	0.015	0.018	8.815	0.021								
sem	0.002	0.019	1.277	0.019	0.008	0.007	4.314	0.008	0.007	0.008	3.942	0.010								

Legend: UCa45 = urine ^{45}Ca; UCa40 = urine ^{40}Ca; FCa45 = faecal ^{45}Ca; FCa40 = faecal ^{40}Ca; fdwt = food consumed; CaCon = Ca consumed; CaBal = Ca balance; NtAbs = net absorbed Ca; EndFCa = endogenous faecal Ca; UnAbCa = unabsorbed dietary Ca; TruAbsFrac = true absorbed fraction; TruAbs = true absorbed concentration; TruAbs% = true absorbed percentage; IntCaSec = intestinal calcium secretion; NetAbs % = net absorbed percentage, sh = sham; ox = oophorectomy.

2.5.13 RESULTS

When the rats were allowed unrestricted access to AIN-76A diet, the correlation coefficient for the relationship between the weight of faeces (fcwt) produced and the weight of food consumed (fdwt) was very low, but when they were allowed unrestricted access to standard rat chow the correlation coefficient improved markedly (AIN-76A; fcwt = 0.79 + 0.094 × fdwt r^2 = 0.27, ns, chow; fcwt = -3.17 + 0.433 × fdwt r^2 = 0.74, P<0.01). These data represent the data collected from all the balances performed on the AIN-76A diet (n=37) and all the balances performed under the same conditions on rat chow (n=17). For rats fed the AIN-76A diet the range of food intakes was 3.8 - 13.5 g/d ([mean,CV%]8.4g/d, 26.6%) and the range of faeces production was 0.35 - 2.14 g/d (1.01g/d, 37.7%), whereas for rats fed rat chow the range of food intakes was 9.2 -16.3 g/d (12.1g/d, 17.7%) and the faeces production 2.2 - 6.3 g/d (4.2g/d, 25.6%). Both AIN-76A and rat chow data were collected from 3-day balances. When the AIN-76A-starch diet was introduced, the range of food intakes was 11.1-13.8 g/d (13.1g/d, 6.3%) and the faeces production ranged from 1.2-1.9 g/d (1.6g/d, 12.2%). These data were collected from 6-day balances and the daily allowance of food was restricted to 90% of the mean ovary-intact consumption for the acclimatisation period. Because food allowance was restricted it was not possible to assess the relationship between fcwt and fdwt on this diet.

The variance in the estimate of calcium balance was improved considerably by restricting food intake during the balance period to 90% of the mean intake consumed during the acclimatisation period ([mean, CV%]unrestricted; 0.63 mmol/d, 32%, restricted, 0.53 mmol/d, 19.2%). When food was restricted, there was no longer a relationship between fcwt and fdwt.

The calcium content of the standard rat chow, received in batches from the supplier, varied considerably. In three consecutive batches of chow analysed, the calcium content was 0.79%, 0.92% and 0.72%.

The variance in the estimate of endogenous faecal calcium was higher when the radioactive ^{45}Ca was administered by intraperitoneal injection compared to intramuscular injection (IP; 0.25mmol/d, 123%, IM; 0.06mmol/d, 39%) (Table 2.5). The reduction in variance was even more impressive in molar terms, with standard deviations of 0.26mmol/d for IP versus 0.02mmol/d for IM. Radioactivity in the urine fell exponentially after the administration of IM ^{45}Ca from 26.3×10^3 (3.6×10^3) cpm/d after 24h to 4.6×10^3 (0.6×10^3) cpm/d after 8 days (fig 2.1). Longer lag times between IM administration of ^{45}Ca and commencement of the balance also reduced the variance in the estimate of endogenous faecal calcium (16 hour lag; 0.29mmol/d, 22.7%, 3 day lag; 0.25mmol/d, 6.0%, 6 day lag; 0.29mmol/d, 7.6%).

Increasing the duration of the balance study above 3 days reduced the variance in almost every component of the calcium balance (Table 2.6). Both 6 and 9 day balances had reduced variance in the estimates of calcium consumed, faecal calcium, calcium balance, net calcium absorption, endogenous faecal calcium and true calcium absorption, with the most marked improvement in the estimates of endogenous faecal calcium. Increasing the duration of the balance period did not improve the variance in the estimate of urine calcium excretion.

TABLE 2.5 Intraperitoneal Vs intramuscular administration of ^{45}Ca for the determination of endogenous faecal calcium.

	Endogenous faecal calcium mmol/d	SD	CV%
Intraperitoneal	0.21	0.26	123
Intramuscular	0.058	0.023	39

n=6

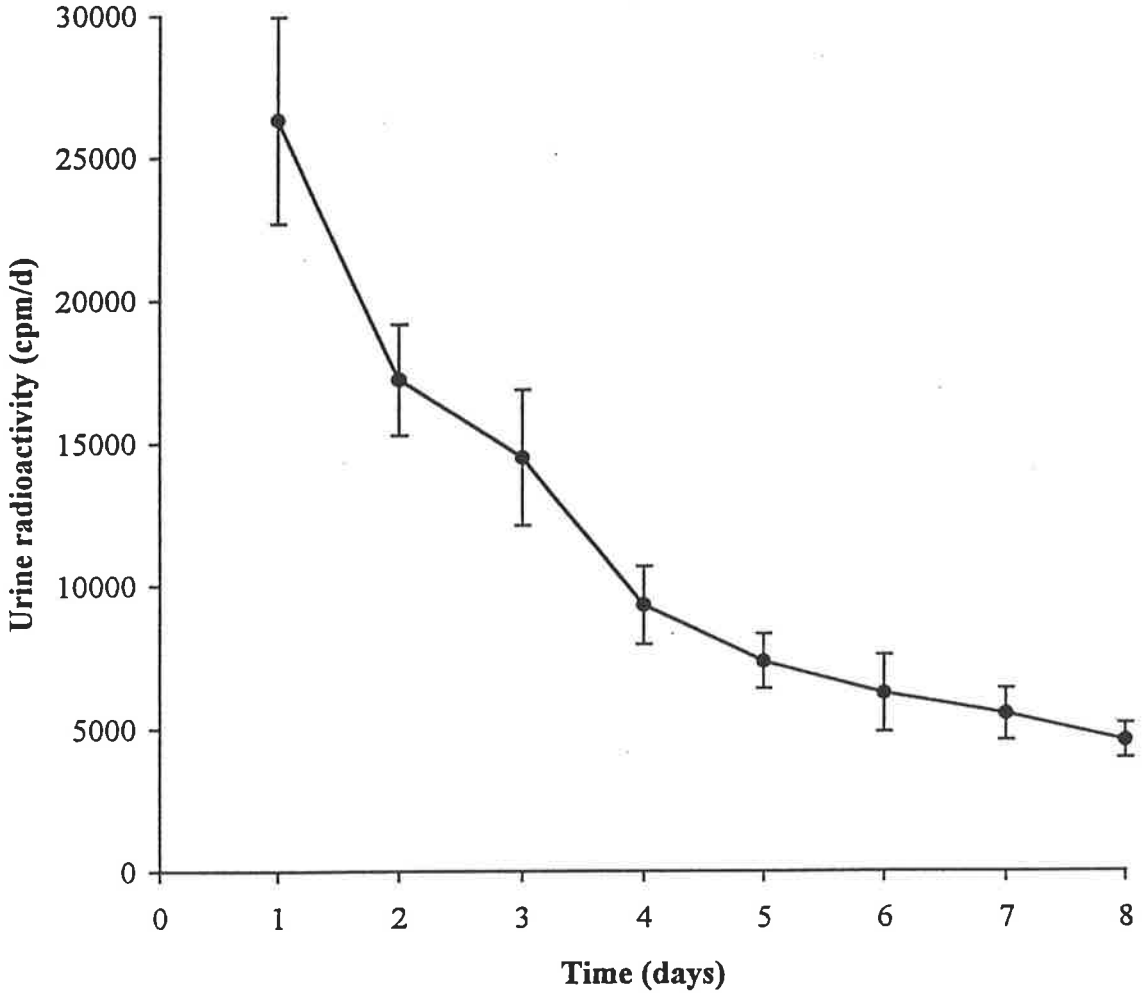

Figure 2.1 The effect of time on urine radioactivity following intramuscular administration of ^{45}Ca 20 × 10^3 MBq ($CaCl_2$, 83.6 GBq/l) to female Sprague-Dawley rats.

TABLE 2.6 The effect of duration of balance period on the variance in calcium balance and components of calcium balance.

	All 3-day balances	**6-day balance**	**9-day balance**
Calcium consumption (mmol/d)	3.04(2.9)	3.09(1.8)	3.04(1.2)
Urine calcium (mmol/d)	0.06(56.2)	0.06(56.5)	0.06(57.5)
Faecal calcium (mmol/d)	2.46(8.9)	2.51(5.7)	2.46(5.2)
Calcium balance (mmol/d)	0.52(37.8)	0.52(27.7)	0.52(24.0)
Net calcium absorption (mmol/d)	0.58(34.1)	0.58(24.5)	0.58(21.8)
Endogenous faecal calcium (mmol/d)	0.29(22.7)	0.25(6.0)	0.29(7.6)
True calcium absorption (mmol/d)	0.87(22.9)	0.83(16.0)	0.87(13.6)
n	18	6	6

Values are mean(CV%).

There was no decrease in the variance of the estimates of calcium balance or net calcium absorption when these parameters were expressed as a ratio of body weight for each individual animal (Table 2.7).

The inclusion of either ^{51}Cr or carmine red as non-absorbable markers in the diets consumed during the balances did not reduce the variance of the estimates of calcium balance or its components. Carmine red, a visible marker indicated the faeces that represented the beginning of food intake during a balance period by a distinct red colouration of the faecal pellets, but the disappearance of red stained faeces following withdrawal of dietary carmine red was gradual, persisting for several days. The effect of the use of ^{51}chromium as a non-absorbable dietary marker on calcium balance and its components is described in Table 2.8. When calcium consumption was corrected for faecal ^{51}Cr, regardless of the time lag between counts 1 and 2, the variance in Ca consumption was approximately double the variance for the uncorrected Ca consumption. The variance in both calcium balance and net intestinal absorption were not reduced by correction for faecal ^{51}Cr and when the lag between counts 1 and 2 was extended to 48d the variance increased by almost three fold. The means for ^{51}Cr-corrected values for calcium consumption, calcium balance and net calcium absorption varied considerably depending on the lag between counts 1 and 2, with a threefold range in both calcium balance and net intestinal absorption.

Calcium consumption corrected for polyethyleneglycol recovered in the faeces had a coefficient of variation similar to that determined without correction for PEG recovered (Table 2.9). PEG recovery was 109% of the mean food consumption determined by weight. The mean estimates of calcium balance and net intestinal calcium absorption were markedly

TABLE 2.7 The effect of correcting for body weight on variance in calcium balance and its components.

	Calcium balance	**Net calcium absorption**
Not corrected for rat weight mmol/d	0.63(32.3)	0.65(30.7)
Corrected for body weight mmol/100g/d	0.21(31.6)	0.22(29.9)

Values are mean(CV%) for 6 rats.

TABLE 2.8 The effect of the use of ^{51}chromium as a non-absorbable dietary marker on variance in calcium balance.

	Uncorrected	**Corrected for faecal ^{51}Cr**		
Lag time between count 1 and count 2	-	12 days	38 days	48 days
Calcium consumption (mmol/d)	1.52 (6.5)	1.66 (12.1)	1.73 (15.6)	1.48 (14.6)
Calcium balance (mmol/d)	0.143 (53.1)	0.283 (55.1)	0.356 (69.9)	0.104 (204.8)
Net intestinal calcium absorption (mmol/d)	0.151 (49.0)	0.291 (52.9)	0.364 (67.8)	0.122 (188.4)
Estimated day 1 faecal ^{45}Ca (cpm/d)	-	51028 (8.1)	58118 (9.9)	52341 (8.8)
Estimated day 1 faecal ^{51}Cr (cpm/d)	-	58606 (14.5)	73443 (15.8)	52293 (17.5)

Values are mean(CV%) for 10 rats.

TABLE 2.9 The effect of the use of polyethyleneglycol (PEG) as a non-absorbable dietary marker on variance in calcium balance.

	Uncorrected	**Corrected for faecal polyethyleneglycol**
Calcium consumption (mmol/d)	1.0(7.7)	1.09(8.8)
Calcium balance (mmol/d)	0.142(45.1)	0.234(20.5)
Net intestinal calcium absorption (mmol/d)	0.164(37.8)	0.255(18.8)
Faecal Calcium (mmol/d)	0.84(8.7)	0.84(8.7)
Faecal PEG (mmol/d)	-	0.098(8.2)

The calcium content of the diet was 11.2 mmol/g PEG. Values are mean(CV%) for 6 rats.

raised when PEG corrected calcium consumption was used in the calculation of these parameters (balance: +65%, absorption: +55% Vs uncorrected). The coefficients of variation for both calcium balance and net intestinal calcium absorption were approximately half those observed for uncorrected calcium balance and net intestinal calcium absorption.

2.5.14 DISCUSSION

2.5.14.1 Housing

The incidence of chromodacryorrhea during balance studies was reduced in rats that were permanently adapted to individual housing compared to animals that were housed in communal cages between balance studies. Chromodacryorrhea, an indicator of stress, is caused by the secretion of "bloody tears" from the harderian gland around the eye (Harkness & Ridgway 1980). This condition leads to an obvious red/rust coloured stain of the fur of the muzzle and around the ears. The periods of stress were also associated with reduced food intake, which in the worst cases ceased altogether for periods of several days. In order to avoid marked variance in food intake during metabolic balance measurement, it was essential to reduce stress to a minimum.

The reduction of stress to minimum is of particular importance in calcium balance studies. Glucocorticoids, the secretion of which are stimulated under conditions of stress (Gill 1990) have been shown to reduce transport of calcium across the duodenum (Kimberg et al 1971), without reducing the circulating levels of 1,25 dihydroxyvitamin D (Hahn et al 1981) and hypersecretion of glucocorticoids in humans with Cushing's disease is associated with elevated renal excretion of calcium (Findling et al 1982). In order to study the factors

regulating calcium balance it is essential to minimise variations in any of the other factors that may also affect calcium balance.

2.5.14.2 Diet

Pelleted standard laboratory chow was unsuitable for use in metabolic cages. When served in pelleted form, the food was generally carried by the rats into the main body of the metabolic cage before it was consumed. As the pellets were broken up, the smaller pieces fell through the wire floor into the faecal collection containers. To prevent contamination of faecal samples, rat chow had to be powdered in a Waring blender and semi-synthetic diets were only served to the rats in a powdered form.

The rationale for restricting the food consumption on the last acclimatisation day to the same amount allowed for the balance period was to ensure that sudden changes in food intake at the commencement of restricted feeding occurred prior to faecal sample collection. Faeces collected on any given day will be affected to some extent by food consumed on the previous day. If the amount of food ingested immediately prior to food restriction was large by comparison to the restricted allowance the faeces collected on the first day of restricted allowance would be biased by the remains of the previous days ingested food. By introducing food restriction 24 hours before the balance period began, the possibility of a marked change in consumption affecting the faeces collected during the balance period was avoided, provided gastric transit time was less than 24 hours. Gastric transit time in the rat varies according to the composition of the diet, and is particularly affected by the fibre type. The fastest transit from stomach to excretion at the anus is approximately 550 minutes for a diet containing wheat bran and the slowest is approximately 950 minutes for a fibre-free

diet. Diets which contain cellulose as the only form of fibre have a transit time of approximately 900 minutes (Lupton & Meacher 1988). Thus, the slowest transit time that could be expected on the diets used in this study would be approximately 900 minutes for the semi-synthetic diets.

For the balances using AIN-76A diet, the variance in the estimate of calcium balance was high and not considered suitable for the assessment of the effect of oophorectomy on calcium balance. These balances were characterised by a poor relationship between the amount of food consumed and the amount of faeces produced. This finding suggests that the collected faeces was either an incomplete collection of the faeces that arose from the food consumed during the balance period or that it was contaminated with faeces which did not arise from the food consumed during the balance period . It is an essential aspect of the performance of metabolic balance studies that the excretions analysed from a balance period arise from the food that was consumed during that balance period. The diet was fed ad libitum during these balance studies and food intakes varied over a 4-fold range, so the poor relationship between consumption and excretion was not due to uniform consumption by all rats within groups. The low end of the range of faecal outputs of 0.35g/d on the AIN-76A diet represents an average production of about 2 to 3 faecal pellets per day. Such low levels of faecal production make it difficult to determine the appropriate portion of the faeces produced that represents the food consumed during the balance period. Not only is it difficult to determine the appropriate portion of the faeces to collect, but when faecal production is so low, the inclusion in a faecal collection of one pellet too many or one pellet too few will consequently lead to large errors in the estimation of calcium balance and its

components. It is likely that this type of error was a factor contributing to the poor relationship between food consumption and faeces production.

There was a higher incidence of chromodacryorrhea, indicating a high level of stress, when the rats were fed the AIN-76A diet compared to any of the other dietary preparations. Diets high in sucrose, such as AIN-76A, have been shown to cause hepatic lesions (Hamm et al 1982, Medinsky et al 1982, Bacon et al 1984), intranephronic calculosis (Nguyen & Woodward 1980) and increased numbers of gut microflora and changes in type (Winitz et al 1970). It is hypothesised that the AIN-76A diet affected gastric transit time, possibly mediated by changes in gut microflora or the induction of stress or a combination of the two, in such a way as to lead to the poor relationship between food consumption and faeces production that was observed in this study.

When rats were fed standard rat chow there was a much stronger relationship between food consumption and faeces production. The levels of food intake were higher, but the level of faeces production was markedly higher. In fact, on rat chow the slope of the relationship between faeces production and food consumption was 4 times higher compared to the same relationship on the AIN-76A diet.

Balance studies conducted using standard rat chow were characterised by lower variance than those which used AIN-76A diet, but diets such as rat chow are made from whole foods and are thus subject to seasonal variations that affect the compositions of whole foods. In this study, although the rat chow used was nominally 0.7% Ca, the 3 batches of chow analysed varied from 0.72 to 0.92%. In addition to dietary calcium levels, other dietary

components such as lactose (Greenwald & Gross 1929, Wasserman 1964, Favus & Angeid-Backman 1984), starch (Schulz et al 1993), protein content (Kerstetter & Allen 1994), sucrose and fat (Zernicke et al 1995), dietary fibre (Harmuth-Hoene & Schelenz 1980) and other factors affecting calcium availability (Amman et al 1986, Pansu et al 1993), have been shown to affect calcium metabolism. The relative levels of these components are not controlled in whole food diets, thus introducing further variables that may complicate the interpretation of calcium balance data. Consequently, for the experiments in which rat chow was used, such as those conducted in young rats, a single batch of chow was set aside for the entire experiment, both during and between balance studies. Synthetic or semi-synthetic diets are generally used to avoid the composition variation that affects diets prepared from whole foods.

The AIN-76A-starch diet was introduced as an alternative semi-synthetic diet to the AIN-76A diet to ensure that all experiments were performed on a standardised, consistent diet. Hamm and co-workers (1982) demonstrated that substitution of starch for sucrose in the AIN-76A diet eliminated the severe periportal lipidosis observed in those rats fed the AIN-76A diet with normal sucrose levels, without affecting body weight. More recently the American Institute of Nutrition's ad hoc committee on the reformulation of the AIN-76A rodent diet recommended that the sucrose content of the diet be replaced by either cornstarch or glucose to avoid the adverse effects of a high sucrose diet (Reeves et al 1993).

The AIN-76A-starch diet was well tolerated by the rats, with no apparent increase in chromodacryorrhea when compared to rats fed the standard rat chow. While the level of

faeces production in rats fed the AIN-76A-starch diet was reduced compared to standard rat chow, there was very low variance in food consumption and low variance in faeces production. The lack of variance in food consumption was due to the introduction of restricted feeding for the balances using the AIN-76A-starch diet and thus it was not possible to determine the effect of this diet on the relationship between food consumption and faeces production.

2.5.14.3 Non-absorbable dietary markers

In the present study the use of non-absorbable dietary markers, was not advantageous. While methods which include the use of a non-absorbable dietary marker for metabolic balance studies in the rat have been published (Dunstan et al 1988, Arjmandi et al 1994, Donkoh et al 1994), this practise is relatively uncommon.

Carmine red

Because the disappearance of carmine red stain from the faeces was very gradual following the cessation of dietary carmine red, it was not possible to use this method to identify the last faecal pellet associated with the food consumed during a balance period. The use of this marker may be improved by feeding carmine red diet only on the first day of a balance period to colour the faecal pellets associated with the beginning of the balance period and then feeding carmine red-free diet for the remainder of the balance period to allow its gradual disappearance. The carmine red diet could then be fed again on the first day following the balance period to identify the first faecal pellet to be discarded after the completion of the balance study. It is possible that these precautions may still not overcome the unpredictable fate of faecal pellets in the large bowel, where pellets can overtake other

pellets associated with earlier dietary intake. Studies in humans have demonstrated that there is extensive mixing of the stools in the colon such that the remains of a given meal can be passed in this region by the remains of subsequent meals (Elliott & Barclay-Smith 1904).

^{51}Chromium

It has been demonstrated that ^{51}Cr is a good non-absorbable dietary marker for metabolic balance studies (Eastell et al 1989). However, ^{51}Cr was not found to be a suitable non-absorbable dietary marker for the present study. It was relatively complex to measure ^{45}Ca, used to monitor endogenous faecal calcium, and ^{51}Cr simultaneously in faecal samples, because the beta emission spectra for ^{45}Ca and ^{51}Cr are not sufficiently different to enable dual discrete counting of both radionuclides in a single sample. Theoretically it is possible to count each sample twice with a suitable time lag between the two counts and then apply simultaneous equations that utilise the difference in the half-lives for the two radionuclides to calculate the proportion of the first count that could be attributed to each of the radionuclides. Correcting the calcium intake for the amount of ^{51}Cr recovered in the faeces, however, actually led to an increase in the variance in the estimates of calcium intake, calcium balance and net intestinal calcium absorption. In addition to the increase in the variance when these parameters were corrected for ^{51}Cr recovery, the accuracy was dependent on the time lag between counts 1 and 2. These findings suggest that this method for determining the proportion of beta counts in the faeces that can be attributed to ^{45}Ca and ^{51}Cr is neither precise nor accurate enough to be suitable for correcting the calcium consumption for the amount of ^{51}Cr recovered.

Polyethylene glycol

Although calcium balance and intestinal calcium absorption corrected for PEG recovery had lower coefficients of variation, the accuracy of the means for each of these parameters was questionable. The estimate of calcium consumption was 9% higher when determined by PEG recovery compared to uncorrected calcium consumption and this led to marked increases in the estimates of calcium balance and net intestinal calcium absorption (+65% and +55% respectively). The uncorrected calcium consumption was determined by simply measuring the reduction in weight of food (of known calcium content) in the food containers at the end of each 24h period during the balance. Food that was occasionally spilled into the collecting funnel and/or faecal collection containers was retrieved and weighed to prevent overestimation of food consumption. Underestimation of food consumption, on the other hand, is most unlikely as this could only occur if the weight of food remaining in the food containers at the end of each 24h period was overestimated. As the content of PEG in the diet was verified by PEG analysis, the high value for PEG-corrected calcium consumption suggests the estimated recovery of PEG in the faecal samples was falsely elevated, leading to an overestimate of consumption, which has in turn led to markedly elevated estimates for calcium balance and net calcium absorption. This occurred even though a blank tube was set up for each sample to allow for non-specific turbidity in the faecal samples. Previous studies have also reported poor performance of PEG as a non-absorbable dietary marker (Corbett et al 1958, Eastell et al 1989).

In addition to the difficulties in interpretation of the accuracy of PEG-corrected calcium consumption figures, the homogenisation of faecal samples, which was necessary to allow analysis of faecal PEG was difficult because the samples commonly contained swallowed

fur. After homogenisation, the essentially liquid slurries were difficult to sample, because the fur blocked even the largest opening made in the sampling syringe, acting as a filter through which particulate matter did not pass. Consequently, it was difficult to determine whether the sampled aliquot was a true representation of the faecal homogenate. Because aliquots of samples processed in this way were also analysed for calcium after ashing, the accuracy of the estimates of faecal calcium were also questionable. PEG-corrected calcium balance and intestinal calcium absorption had lower variance than uncorrected values possibly because the PEG recovery corrected somewhat for the inefficiency of homogenate sampling. This specimen handling technique was only necessary when faecal specimens were to be analysed for PEG. For specimens that did not require PEG analysis the entire sample could be ashed to avoid this problem.

2.5.14.4 Duration of balance period

The length of time over which a calcium balance was performed was an important factor in maintaining a low variance in the estimates of calcium balance and its components. As Table 2.6. shows, increasing the duration of the balance period from 3 to 6 days led to a reduction of almost 30% in the variance in calcium balance and net calcium absorption. This was brought about by reductions in the variances in both calcium consumption and faecal calcium. Net calcium absorption, the difference between consumption and faecal excretion and calcium balance, the difference between consumption and both faecal and urine excretion, are both relatively small values and thus more susceptible to the variations that occur in calcium consumption and faecal excretion. Urine calcium excretion was the smallest component of calcium balance and there was no change in its variance with increased duration of the balance period. The reduction in the estimate of endogenous faecal

calcium in the 6-day compared to the 3-day balance period was almost 75%. This marked improvement when duration of the balance period was extended to 6 days may also be partly due to the fact that these were calculated from day 4 to day 9 inclusive and, therefore included a 3 day lag after the administration of ^{45}Ca, the advantages of which are discussed in more detail in section 2.5.14.6.

There was a further reduction in variance in calcium balance and its components when the duration of the balance period was increased to 9 days compared to 6 days, but this further improvement was relatively modest. Including the acclimatisation period, a 9-day balance would take 14 days to perform. The improvement in performance offered by the 9-day period protocol was not considered to be sufficient to warrant the extra metabolic cage time required. There were a total of 10 metabolic cages available for the performance of the present study, making metabolic cage-time precious. Consequently a 6-day balance period (11 days including the acclimatisation period) was considered to be the optimal compromise between low variance and economy of time.

2.5.14.5 Prebalance acclimatisation period

A period of acclimatisation at the beginning of each balance study was considered important to allow the rats to settle into the metabolic cage environment. It was noted that on many occasions the food consumption of some individual rats fell to zero in the first day or two of being transferred into metabolic cages, but gradually rose to levels similar to those of the rest of the group. As this phenomonon was almost always associated with chromodacryorrhea, it was considered to be a stress related reduction in food consumption. The use of a period of acclimatisation in the metabolic cage before commencement of the

balance period prevented the stress related reduction in food consumption from impinging on the balance measurement. The incidence of the stress related reduction in food consumption was also reduced by housing the rats in individual cages between each balance study. Thus, by acclimatising the rats to individual caging, the periods spent in metabolic cages were less foreign than they would have been if the animals were accustomed to communal caging.

2.5.14.6 Endogenous faecal calcium

The administration of ^{45}Ca by intramuscular injection reduced the variance in the estimate of endogenous faecal calcium to approximately one third of that observed following intraperitoneal administration of ^{45}Ca. Because there was also a lower mean endogenous faecal calcium in the IM group the reduction in variance was even more impressive in molar terms, with nearly a 20 fold reduction in the IM group compared to IP. The high imprecision following IP administration may be the result of variable equilibration of IP ^{45}Ca directly with the contents of the intestinal lumen. The composition of the lumenal contents in immediate contact with the injected ^{45}Ca may be a major determinant of the rate of diffusion of ^{45}Ca into the lumen directly from the intraperitoneal space prior to its even distribution within the extracellular fluid. IM administration on the other hand would not be affected by direct equilibration into the intestinal lumen.

The variance in the estimate of endogenous faecal calcium was also reduced by increasing the lag period between IM administration of ^{45}Ca and commencement of the balance period. The disappearance of ^{45}Ca from the extracellular fluid is exponential with the most rapid reductions in the first 24 hours and the rate of reduction diminishes with time after

administration. It was also noted throughout the study that food consumption was reduced in some animals in the 24 hour period immediately following the administration of IM ^{45}Ca. This may have also contributed to the high variance when the lag period was only 16 hours. Although lag periods of 3 and 6 days were shown to reduce the variance in endogenous faecal calcium in this experiment, it was subsequently found that a lag period of 2 days was suitable, convenient and avoided disruption of the rats in the middle of the acclimatisation period when food consumption was being monitored.

2.5.14.7 Other considerations

The availability of metabolic cages for the present study was limited to 10 individual cages, thus restricting the number of rats that could be studied concurrently. Because of the variance associated with the performance of calcium balance studies even after exhaustive optimisation it was decided to conduct all experiments in 2 groups of 5 rats each (5 × sham and 5 × oophorectomy). An additional rest period between balance studies was also introduced on the advice of the institutional animal ethics committee. The committee suggested that the clear perspex cages with wire grid floors would induce stress due to a sensation of exposure and consequently advised that rats be allowed a minimum of a one week rest period between each 11 day balance study to recover. In the majority of experiments conducted in the present study it was important to be able to assess calcium balance as frequently as possible following oophorectomy and, so balance studies were performed on a three week cycle; 11 days in the metabolic cages followed by 1 weeks rest outside the metabolic cages. Therefore, to conduct one preoperative balance study followed by 3 or 4 post-operative balance studies on 5 sham and 5 oophorectomised rats would take 12 to 15 weeks merely to collect the samples. Apart from the slow rate of data production,

the process is laborious, requiring considerable time for the manufacture of synthetic diets, sample processing and regular washing of metabolic cages.

2.6 ADOPTED PROTOCOL FOR CALCIUM BALANCE STUDIES

Calcium balance studies were performed in individual metabolic cages. The balances were divided into 2 parts: **acclimatisation period**, from days 1-5, immediately followed by a **6-day balance period**, from days 6 -11 inclusive.

The rats were weighed and placed into metabolic cages on day 1 and given a known amount of powdered diet. On the morning of day 2 the remaining food was weighed and the food consumption for day 1 calculated and recorded. The process was repeated for days 2-4. Radioactive ^{45}Ca ($CaCl_2$, 83.6 GBq/L; Amersham, Little Chalfont, U.K.) was administered on day 4 by intramuscular injection to monitor the secretion of endogenous calcium into the faeces. On the morning of day 5 the consumptions for days 1-4 were summed and meaned. The food allowance for day 5 and for the balance period, was restricted to 90% of the mean acclimatisation period consumption of the ovary intact animals.

On the morning of day 6 the collecting funnels, urine and faeces containers and the feed containers were rinsed of fur, urine and faeces. 2ml of concentrated HCl was added to the urine container to prevent precipitation of calcium salts. The daily food allowance for the balance period was added to the food containers at 0900 each day and the actual consumption was determined by weighing the container the following morning before adding the next day's allowance. On the final day of the balance period the feed containers

were removed from the metabolic cages at 0900 and the urine and faeces samples were collected into separate 250ml containers, weighed, and the rats were returned to their normal cages. The faeces and urine samples were processed and analysed as described in 2.5.9 and 2.5.10.

CHAPTER 3

THE EFFECT OF OOPHORECTOMY ON CALCIUM BALANCE IN YOUNG, GROWING RATS

3.1 BACKGROUND

Calcium accretion is essential for skeletal growth during development in all vertebrate species. However, as animals approach full skeletal size the requirement for calcium retention by the body as a whole subsides and calcium balance might be expected to become less positive. In the growing laboratory rat, in the absence of intervention, dietary calcium intake remains essentially constant regardless of a reduced calcium requirement, calcium balance must reduce by means of either reduced calcium absorption efficiency or increased calcium excretion or a combination of the two.

Intestinal calcium absorption occurs via both passive (paracellular) and active (transcellular) processes. The age-related reduction in calcium absorption through the growth period in the rat, which results from a reduction in the active component of calcium absorption (Pansu et al. 1983b), has been demonstrated by both in vitro (Horst et al 1978, Armbrecht et al 1979, Armbrecht 1986 Liang 1988) and in vivo techniques (Pansu et al. 1983a). The decline is most rapid during the growth period until about 6 months of age (Horst et al 1978, Armbrecht et al 1979), consistent with the age at which skeletal growth ceases (Kalu et al 1989). As there is no change in total serum calcium with age (Armbrecht et al 1980, Gray &

Gambert 1982, Kalu et al 1984b, Armbrecht et al 1988b) the changes in calcium absorption and skeletal growth described must be closely linked and suggest that calcium balance measured by metabolic balance studies reduces with age.

The major route of calcium excretion in the rat is via the secretions of the gastrointestinal tract, with calcium also being excreted in the urine and other body secretions. There is no clear evidence for an age-related change in either intestinal calcium secretion or urine calcium excretion.

The role of ovarian hormones in the metabolism of calcium is not well understood, but several studies have indicated that intact ovaries are essential for the maintenance of normal bone density and normal gastrointestinal handling of calcium in women and rats. Oophorectomy in adult rats leads to decreased bone density at a number of skeletal sites (Wronski et al. 1988a, Kalu et al. 1989, Yamazaki & Yamaguchi 1989, Frost & Jee 1992), and is accompanied by impaired net intestinal calcium absorption (Kalu et al 1989). Furthermore, a role for oestrogens in intestinal calcium absorption is supported by the recent identification of oestrogen receptors in a non-transformed rat enterocyte cell line (Thomas et al 1993) and enhanced calcium uptake by isolated duodenal cells in the presence of oestrogen (Arjmandi et al. 1993). Furthermore, in vivo stimulation of calcium absorption by oestradiol has also been demonstrated (Arjmandi et al. 1994). In young rats oophorectomy also causes reduced femoral bone density, but there is an increase in overall femur length, such that there is little effect on total femur calcium content (Yamazaki &

Yamaguchi 1989). The effect of oophorectomy on urine calcium excretion in rats is controversial (Yamazaki & Yamaguchi 1989, Morris et al 1992, Morris et al 1995).

While a reduced calcium balance in old (18mth) compared to young (1.5mth) rats has previously been reported (Armbrecht et al 1981), the overall changes that take place through the growth period have not, nor has the relative importance of changes in each of the components of calcium balance been determined through this period. There is considerable evidence for a role for ovarian hormones in calcium metabolism, but their role in the developing skeleton is not clear. It has not been determined whether the oophorectomy-induced reduction in bone density at specific sites in the rat results from a net reduction in whole body calcium or merely a redistribution of calcium within the skeleton. Therefore, in the present study metabolic calcium balance studies have been performed and the components of calcium balance including net calcium absorption, true calcium absorption, urine calcium excretion and intestinal calcium secretion have been assessed in young intact and oophorectomised rats.

3.2 PROTOCOL

3.2.1 Animals

On arrival in the laboratory, ten 4 week old female rats were separated into individual cages and allowed unrestricted access to standard rat chow. Following one week of acclimatisation to individual cages, animals underwent a 6-day calcium balance study as described in 2.6, (balance 1). At 6 weeks of age the animals were randomly allocated to

either sham or oophorectomy operation as described in 2.3, with 5 animals in each group. Calcium balance studies were repeated at 9,12 and 15 weeks of age (balances 2, 3 and 4). Throughout the present study rats were fed standard rat chow (Table 2.1). Between balances, the rats were pair fed to prevent hyperphagia in the oophorectomised animals.

3.2.2 Statistical analyses

The data are expressed as the mean ± sem or mean change ± sem. The effect of oophorectomy and age were analysed by two-way analysis of variance and Tukey's posthoc test (Pagano 1986) to identify specific differences between groups. One-tailed t-test was used to test the difference between operational groups for the change in intestinal calcium absorption at 15 weeks of age. All other comparisons were assessed by one-way analysis of variance. Differences of $P<0.05$ were considered significant.

3.3 RESULTS

3.3.1 Body weight

Body weight increased rapidly with age in both the sham operated and oophorectomised groups (fig. 3.1). Despite matched food intake the weight gain in the oophorectomised group was significantly greater than in the sham group. In both groups there was a significantly greater increase in weight between 6 and 9 weeks of age compared to the increase between 12 and 15 weeks of age (Table 3.1) .

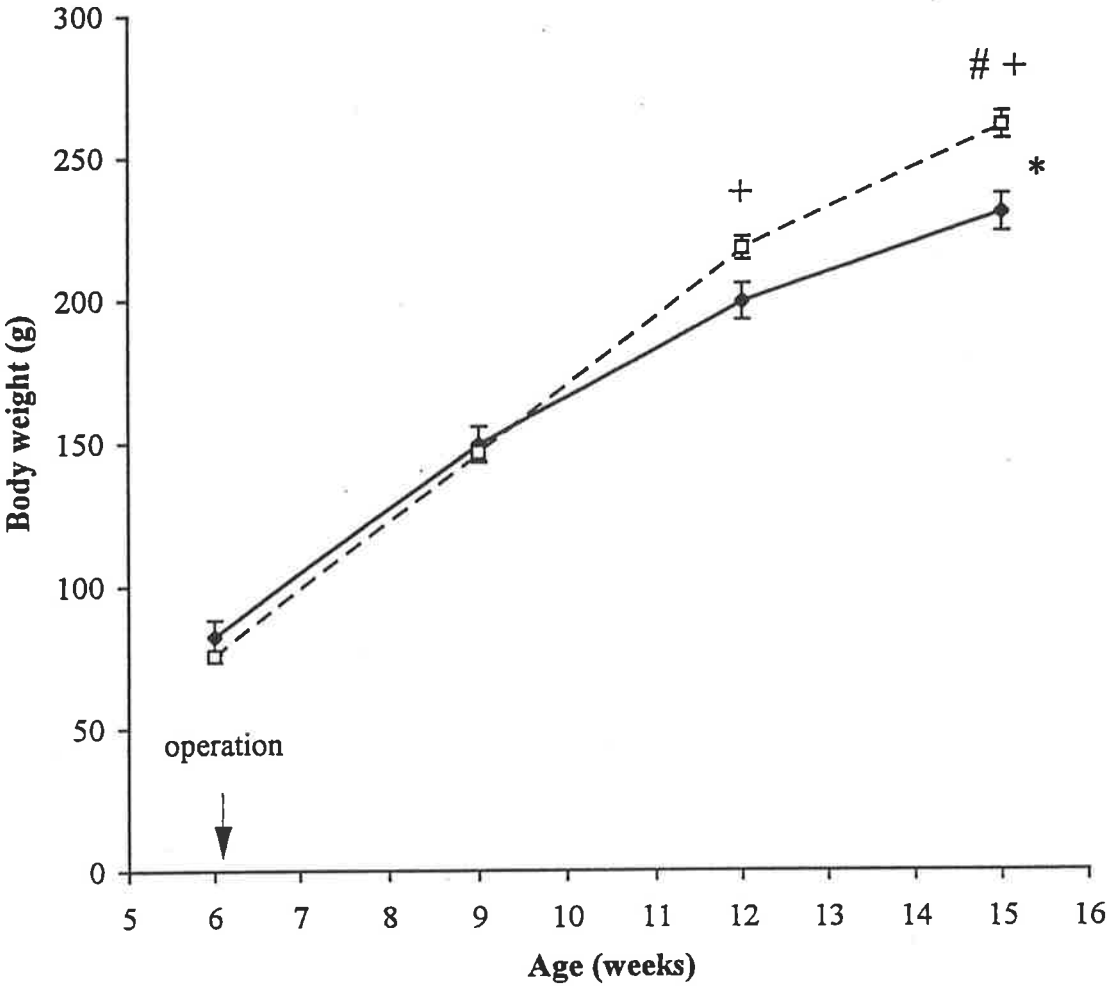

Figure 3.1 The effect of age on body weight for sham-operated control —♦— and oophorectomised – – □ – – rats. The data are expressed as the mean ± sem for 5 rats. *P <0.0001 effect of age for both oophx and sham groups, #P<0.02 effect of oophx compared to sham group, +P<0.01 effect of oophx compared with sham for same age.

TABLE 3.1 Diminution of the effect of age on body weight, true calcium absorption and calcium balance.

		Change between 6 and 9 weeks	Change between 12 and 15 weeks	P
Body weight (g)	sham	67.2(1.3)	30.8(1.1)	<0.001
	oophx	71.4(1.6)	42.8(1.6)	<0.001
Calcium balance (mmol/d)	sham	-2.1(0.027)	-0.9(0.3)	<0.025
	oophx	-2.1(0.12)	-1.0(0.1)	<0.001
True calcium absorption (%)	sham	-15.6(1.4)	-5.8(1.0)	<0.001
	oophx	-16.4(1.4)	-3.7(2.0)	<0.001

Values are the mean change (sem) for 5 rats.

3.3.2 Calcium balance

Calcium balance decreased rapidly with age in both groups (fig. 3.2) from 0.86(0.02)mmol/d at 6 weeks of age to 0.27(0.04)mmol/d at 15 weeks of age ($P<0.0001$) in sham rats and 0.91(0.03)mmol/d at 6 weeks of age to 0.22(0.03)mmol/d at 15 weeks of age ($P<0.0001$) in oophorectomised rats. There was a significantly greater decrease in balance between 6 and 9 weeks of age compared to the decrease between 12 and 15 weeks of age, regardless of operation type (Table 3.1) . Comparison of the difference between the pre-operative balance and each subsequent balance revealed a greater reduction in calcium balance in the oophx group compared to the sham group, which was significant from 6 weeks following the operation (12 weeks of age).

3.3.3 Calcium absorption

Net calcium absorption also decreased with age in both groups from 0.91(0.02)mmol/d at 6 weeks of age to 0.27(0.03)mmol/d at 15 weeks of age ($P<0.0001$) in sham rats and 0.94(0.03)mmol/d at 6 weeks of age to 0.21(0.03)mmol/d at 15 weeks of age ($P<0.0001$) in oophorectomised rats. Comparison of the difference between the pre-operative net absorption and net absorption in each subsequent balance revealed a greater reduction in net absorption in the oophx group compared to the sham group, which was significant from 6 weeks following the operation (12 weeks of age) (fig. 3.3). When net absorption was corrected for endogenous faecal calcium, true calcium absorption expressed as a percentage of calcium consumption also decreased with age in both groups from 46.3(1.5)% at 6 weeks of age to 22.6(1.1)% at 15 weeks of age ($P<0.0001$) in sham rats and from 48.2(1.4)% at 6 weeks of age to 21.2(1.2)% at 15 weeks of age ($P<0.0001$) in

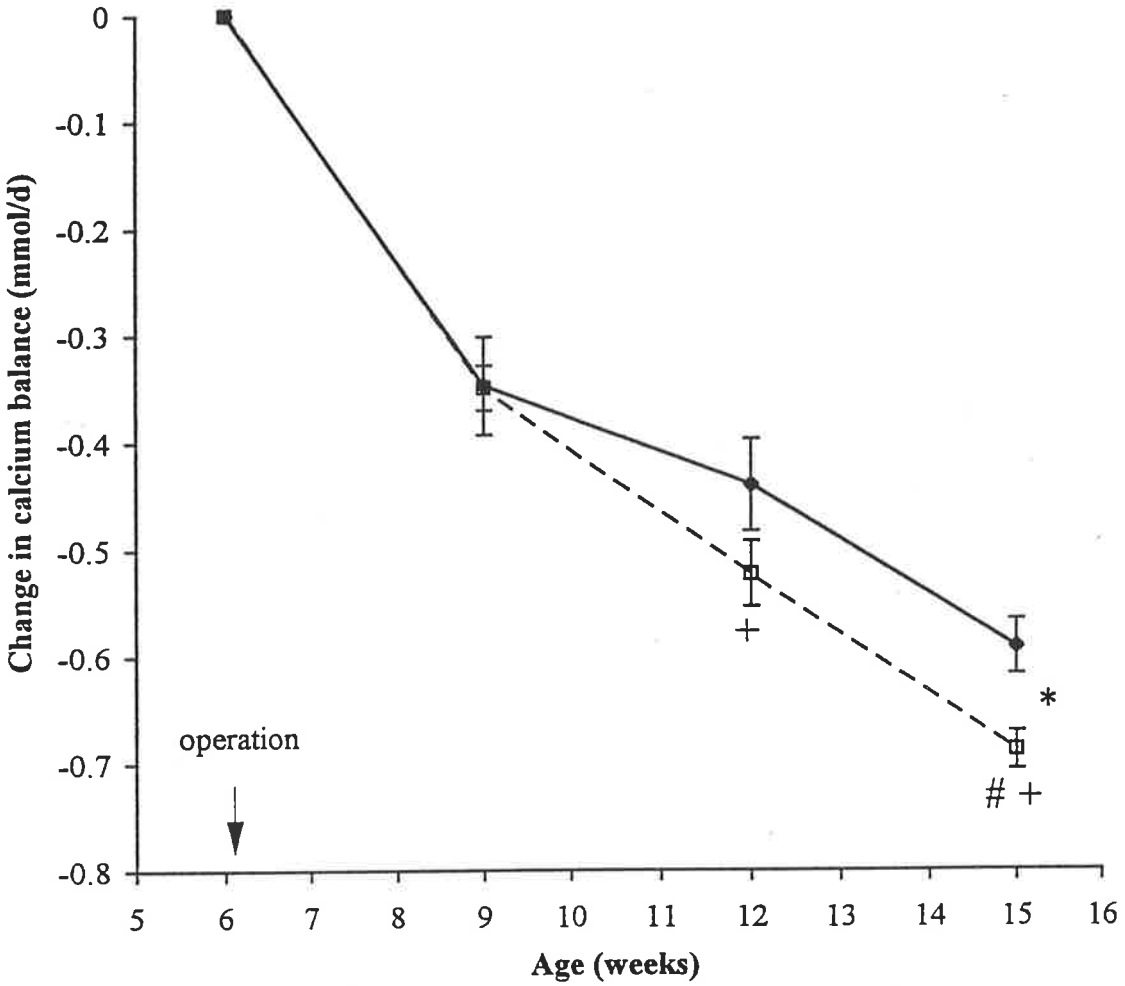

Figure 3.2 The change in calcium balance with age for sham-operated control —♦— and oophorectomised – – □ – – rats. The difference between preoperative balance and the subsequent postoperative balances for each individual rat were determined and the data expressed as the mean ± sem change in calcium balance for 5 rats. *P <0.0001 effect of age for both oophx and sham groups, #P<0.05 effect of oophx compared to sham group, +P<0.01 effect of oophx compared with sham for same age.
The raw data are presented in appendix 1.

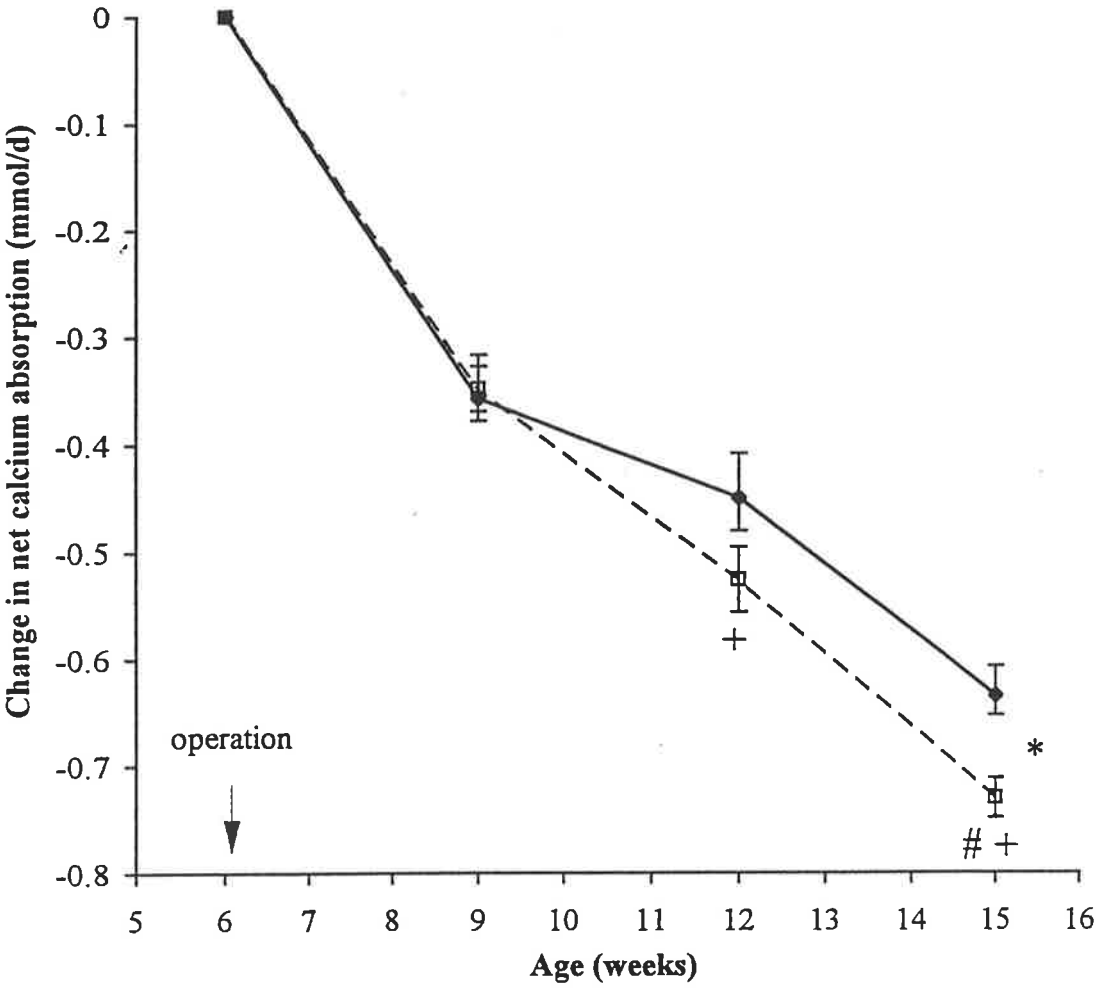

Figure 3.3 The change in net calcium absorption with age for sham-operated control —♦— and oophorectomised – – □ – – rats. The difference between preoperative net calcium absorption and the subsequent postoperative net calcium absorption values for each individual rat were determined and the data expressed as the mean ± sem change in net calcium absorption for 5 rats. *P <0.0001 effect of age for both oophx and sham groups, #P<0.05 effect of oophx compared to sham group, +P<0.01 effect of oophx compared with sham for same age. The raw data are presented in appendix 1.

oophorectomised rats. There was a significantly greater decrease in true calcium absorption between 6 and 9 weeks of age compared to the decrease between 12 and 15 weeks of age regardless of operation type (Table 3.1). The difference between pre-operative true calcium absorption and true calcium absorption at 9 weeks following operation (15 weeks of age) was significantly greater by one-tailed t-test in the oophx rats compared to sham rats (fig. 3.4).

3.3.4 Body weight as a determinant of calcium balance and true calcium absorption

Both calcium balance and true calcium absorption were strongly, inversely related to body weight. The relationships are described by the equations: calcium balance = +6.77 - 0.0216 x body weight (r^2=0.89, P<0.0001) and true calcium absorption = +55.9 - 0.142 x body weight (r^2=0.83, P<0.0001).

3.3.5 Calcium excretion

Endogenous faecal calcium was significantly higher in the oophx group compared to sham (fig. 3.5), with the maximum differential between the groups at 12 weeks of age (Table 3.2). Total faecal ^{45}Ca was higher in the oophx group at 12 weeks of age although it did not reach statistical significance due to lower urine specific activity of ^{45}Ca in the oophx group at the same time. Intestinal calcium secretion was significantly greater in the oophx compared to sham (fig. 3.6), achieving maximum difference between operational groups at 12 weeks of age (intestinal calcium secretion at 12 wk; sham: 0.23(0.03), oophx: 0.33(0.02) mmol/d, p<0.01). Urine calcium did not change with age and there was no difference between the sham and oophx groups at any of the monitored ages (fig. 3.7).

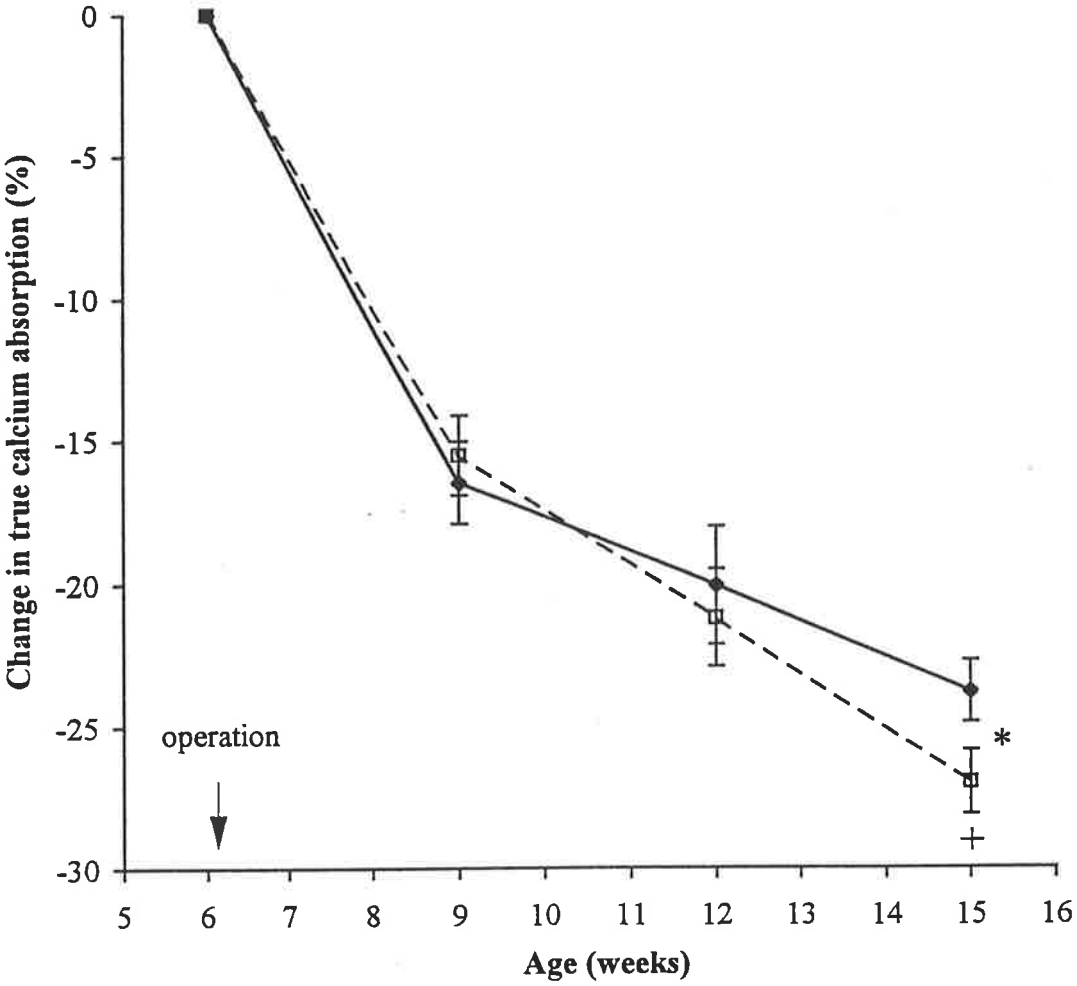

Figure 3.4 The change in true calcium absorption with age for sham-operated control —♦— and oophorectomised – – □ – – rats. The difference between preoperative true calcium absorption and the subsequent postoperative values for true calcium absorption for each individual rat were determined and the data expressed as the mean ± sem change in true calcium absorption for 5 rats. *P <0.0001 effect of age for both oophx and sham groups, +P<0.05 effect of oophx compared with sham at 15 weeks of age. The raw data are presented in appendix 1.

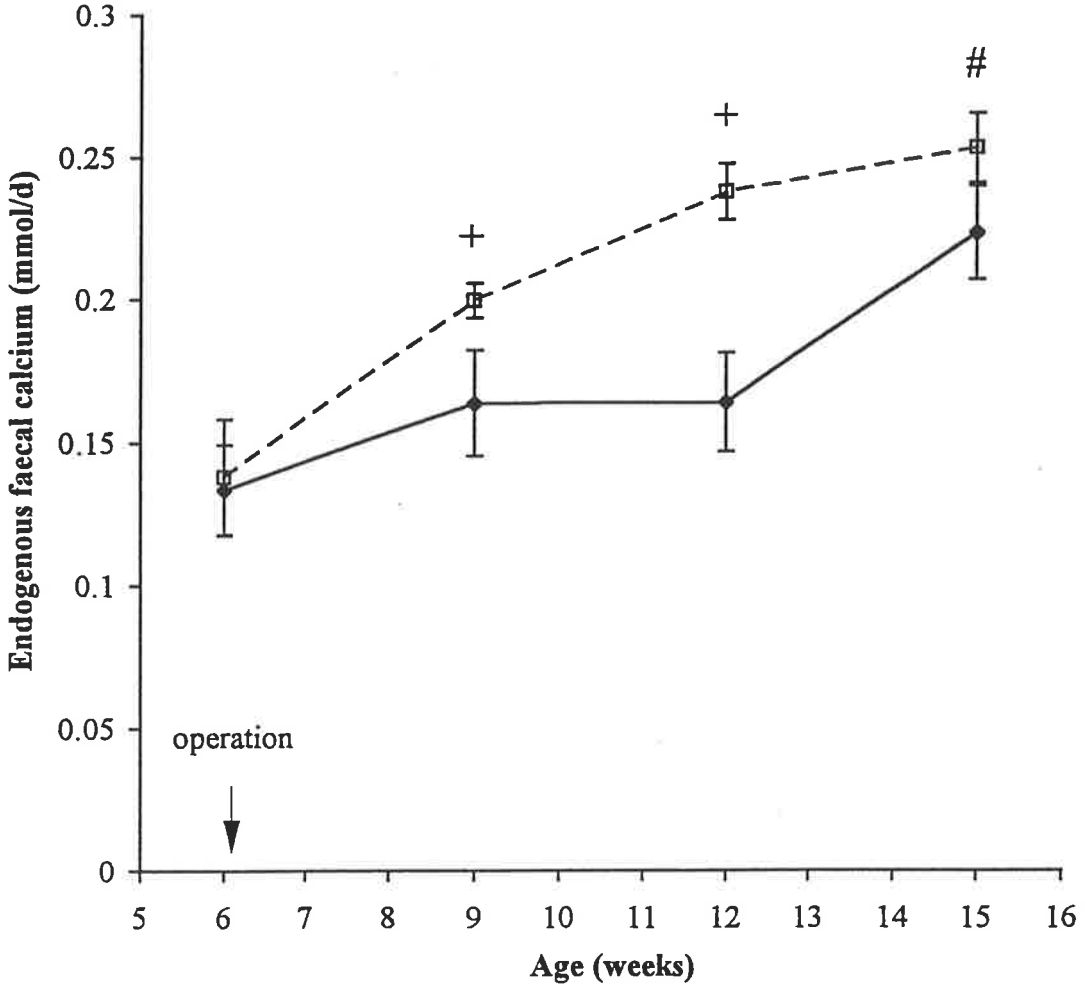

Figure 3.5 The effect of age on endogenous faecal calcium for sham-operated control —♦— and oophorectomised – – □ – – rats. The data are expressed as the mean ± sem for 5 rats. #P<0.05 effect of oophx compared to sham group, +P<0.01 effect of oophx compared with sham for same age.

TABLE 3.2 The effect of age and oophorectomy on endogenous faecal calcium and its components in youngrats.

Age	Operation	Faecal 45Ca kBq/d	Urinary ^{45}Ca specific radioactivity kBq/mmol	Endogenous faecal calcium mmol/d
6 weeks	sham	4.2(0.08)	32.6(2.5)	0.134(0.015)
	oophx	4.1(0.21)	32.2(4.0)	0.138(0.02)
9 weeks	sham	12.8(1.26)	82.7(11.7)	0.164(0.019)
	oophx	12.3(0.45)	62.1(3.0)	0.199(0.006)
12 weeks	sham	25.7(1.69)	162.6(15.5)	0.164(0.017)
	oophx	28.5(2.53)	119.9(8.0)*	0.237(0.01)*
15 weeks	sham	24.4(0.19)	112.7(5.9)	0.219(0.013)
	oophx	25.7(0.73)	102.3(2.6)	0.253(0.012)

Values are mean(sem) for 5 rats; *P<0.05 Vs sham for same age group.

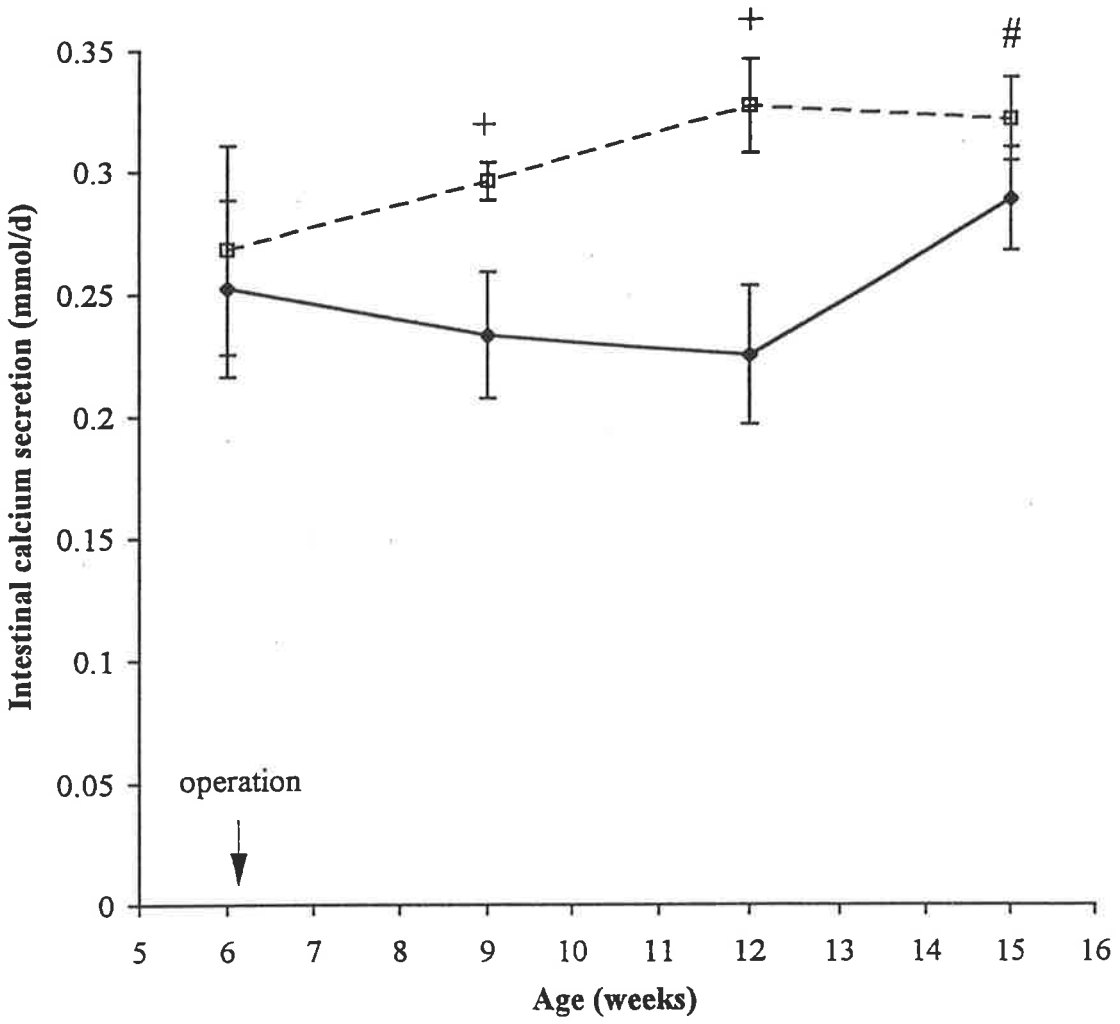

Figure 3.6 The effect of age on intestinal calcium secretion for sham-operated control —♦— and oophorectomised – – □ – – rats. The data are expressed as the mean ± sem for 5 rats. #P<0.05 effect of oophx compared to sham group, +P<0.01 effect of oophx compared with sham for same age.

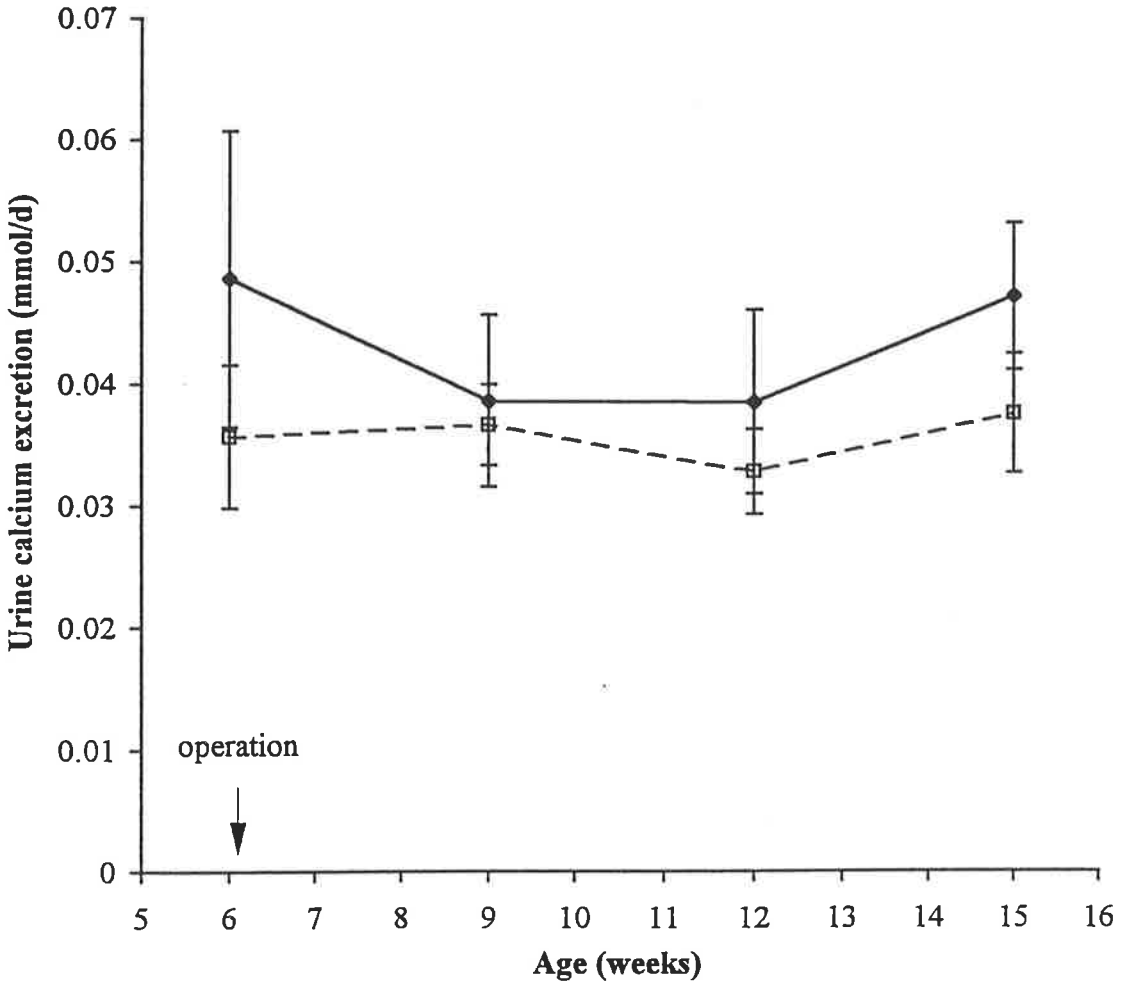

Figure 3.7 The effect of age on urine calcium excretion for sham-operated control —♦— and oophorectomised – – □ – – rats. The data are expressed as the mean ± sem for 5 rats.

3.4 DISCUSSION

We have identified a rapid reduction in calcium balance in female rats between the ages of 6 and 15 weeks, which was accompanied by similar reductions in both net and true calcium absorption, but there was no age-related change in either urine calcium excretion or intestinal calcium secretion. These findings indicate that the diminution in calcium accumulation as growing rats approach full size is due entirely to a decreased efficiency of dietary calcium absorption. A decreased ability to maintain a positive calcium balance in 18 month old animals compared to 6 week old animals has previously been reported (Armbrecht et al. 1981), and a reduction in intestinal calcium absorption through the growth period has been demonstrated using everted gut sacs (Horst et al 1978, Armbrecht et al 1979) and perfused duodenal loops (Pansu et al 1983a). The reduction in calcium absorption is most pronounced in the duodenum, the region of the intestine in which active absorption primarily occurs (Armbrecht 1986). The present study is the first to measure calcium balance and its components by metabolic balance studies through the growth period to identify the relative contribution of reduced intestinal calcium absorption to reduced calcium balance as growth rate diminishes. The longitudinal data presented clarify the overall changes that occur in whole body calcium balance in both the intact and oophorectomised young rat during this period.

The exact nature of the mechanism for the age-related decline in intestinal calcium absorption through the growth period has not been established, but circulating levels of 1,25 dihydroxyvitamin D decline from a peak of 272 pg/ml at 28 days of age to 51 pg/ml at 90

days of age (Clark et al 1986) and similarly a fall of 78% in circulating levels of 1,25 dihydroxyvitamin D in adult rats (>16 month) compared to young rats (1 month) has also been reported (Horst et al 1990). The fall in 1,25 dihydroxyvitamin D alone may account for the reported age-related reductions in intestinal calbindin-D9k (Armbrecht et al 1989), enterocyte vitamin D receptor number (Horst et al 1990) and ATP-dependent calcium transport by basolateral membrane vesicle (Armbrecht et al 1988a). The effects of age on other mechanisms of intestinal calcium absorption, such as passive paracellular absorption or the putative vesicular mechanism (Nemere et al 1986) have not been studied.

There was no change with age in the excretion of calcium via either the gastrointestinal tract or the kidney. It is interesting to note, however that a marked reduction in renal tubular calbindin-D28k between the ages of 2 and 6 months has been reported (Armbrecht et al 1989) and a combined role for calbindin-D9k and calbindin-D28k in vitamin D mediated transcellular calcium transport in the distal nephron has been proposed (Bouhtiauy et al 1994a, Bouhtiauy et al 1994b). The decline in renal tubular calbindin-D28k does not appear to be the result of impaired renal tissue function, because the response of calbindin-D28k to 1,25 dihydroxyvitamin D administration in primary cultures of rat renal tubule cells does not decline with age (Chen et al 1992). The age-related reduction in calbindin-D28k may be the result of reduced circulating 1,25 dihydroxyvitamin D. The interrelationship between vitamin D and PTH in renal tubular reabsorption of calcium is not entirely clear. Administration of vitamin D enhances calcium reabsorption in the parathyroid-intact rat (Costanzo et al 1974), but not in the thyroparathyroidectomised vitamin D replete rat (Hugi et al 1977). The excretion of calcium in the urine is affected by others factors such

as sodium and phosphate excretion (Morris et al 1995), so it is difficult to interpret the meaning of an effect or even a lack of effect of a factor such as age. However, in the present study the data do not support an effect of the reported age-related reduction in renal tubular calbindin-D28k on the excretion of calcium between the ages of 6 and 15 weeks.

Since 99% of whole body calcium is stored in bone, a positive calcium balance largely represents the total accretion of calcium into bone. Therefore, the rapid reduction in calcium balance that occurs between the ages of 6 and 15 weeks represents a rapid reduction in bone mineral accumulation. The rate of change in calcium balance was greater between 6 and 9 weeks of age than between 12 and 15 weeks of age indicating an attenuation in the rate of bone mineral accumulation, consistent with the report that femur length and volume increase more rapidly in 4 week old rats than in 10 week old rats (Yamazaki & Yamaguchi 1989). The strong inverse relationships between body weight and calcium balance and the coincident diminution in the rate of change of both of these variables between the ages of 6 and 15 weeks indicate that the attenuation of bone mineral accumulation is associated with attenuation of whole body growth rate. There was also a strong inverse relationship between body weight and true calcium absorption, which indicates that the rate of diminution in the body's requirement for calcium corresponds with the rate of diminution of the ability of the intestine to extract calcium from the diet. Previous studies in male rats have identified similar diminution in calcium absorption with age (Horst et al 1978, Armbrecht et al 1979, Pansu et al. 1983b, Armbrecht 1986). Although passive absorption is particularly important in the first week or two after birth it

diminishes to a modest level, which remains constant from 4 weeks of age. Active calcium absorption declines from a peak at 4 weeks of age down to a level which remains steady from 15 weeks of age until at least 21 weeks of age (Pansu et al. 1983b).

It is important that the effect of age on calcium absorption be studied in the female rat regardless of studies that have been conducted on the male rat. Extrapolation of the findings of calcium absorption studies in the male rats to predict effects on calcium absorption in female rats may not be valid. The identification of oestrogen receptors in a non-transformed enterocyte cell line (Thomas et al 1993) and demonstration of 17β-oestradiol stimulated calcium absorption in vivo without altered circulating levels of PTH or 1,25 dihydroxyvitamin D (Arjmandi et al 1994), suggests that the control of intestinal calcium absorption may be influenced through the growth period by oestrogen. However, the findings in the present study of diminishing intestinal calcium absorption as female rats approach full skeletal size is consistent with the observations for age-related diminution of calcium absorption that has been reported for the male rat.

Assessment of the effect of oophorectomy on calcium metabolism in the young rat is confounded by the rapid changes in growth rate and consequent effects on calcium balance and absorption. In addition, it has previously been reported that calcium balance studies are characterised by a large coefficient of variation due to small differences between variables involving large amounts of calcium, even though there are only small errors in individual measurements from which the calcium balance is calculated (Taagehϕj Jensen et al 1983). Consequently, in this study the data were paired by calculating the difference

between the pre-operative balance and each subsequent balance, which revealed that the oophorectomised rats had a significantly greater reduction in calcium balance compared to the sham operated rats. These data indicate that oophorectomy in the young growing rat results in a net reduction in calcium retention and, hence a reduction in the accretion of calcium to bone. Our observation indicates that the report of a relative reduction in femur density in oophorectomised rats at 10 weeks of age (Yamazaki & Yamaguchi 1989) is not merely the result of a redistribution of calcium within the skeleton. The increased femur length observed following oophorectomy in young rats (Yamazaki & Yamaguchi 1989) may be the result of a direct effect of oestrogens on the growth plate, an hypothesis which is supported by the observation that the rate of reduction in the growth plate was attenuated following oophorectomy in young rats (Baldock et al 1996)

At 15 weeks of age the calcium balance for the oophx group was 0.05mmol/d lower than the calcium balance for the sham group. Given that total body calcium is approximately 50mmol for a 250g rat (Staub et al. 1988), such a deficit will produce a 10% reduction in total body calcium in oophx animals compared to sham controls in 14 weeks. As the majority of total body calcium is stored in bone it represents a 10% reduction in bone mineral over a period of time equivalent to approximately 10% of the animal's life span.

The lower specific activity of ^{45}Ca in the urine of the oophorectomised animals at 12 weeks of age was probably the result of the increased bone turnover that occurs following oophorectomy (Wronski et al 1988a, Morris et al 1992). In a state of high bone turnover, both ^{40}Ca and ^{45}Ca will be deposited into bone at each bone mineralisation unit (BMU),

while the calcium released from newly formed BMUs is likely to be predominantly ^{40}Ca. Thus in a high bone turnover environment the specific activity of ^{45}Ca in the ECF probably falls more rapidly than it does when bone turnover is lower. This is presumably the cause of the lower specific activity of ^{45}Ca in the urine which has occurred within six weeks of oophorectomy in the present study.

There was a greater reduction with age in net calcium absorption in the oophorectomised rats compared to sham operated rats and there was also a trend for a greater reduction in intestinal calcium absorption in the oophx group which became significant at 12 weeks of age. The data indicate that impaired intestinal calcium absorption may play only a partial role in post-oophorectomy bone loss in young rats. Impaired net calcium absorption following oophorectomy has been reported in animals fed a diet low in calcium (Kalu et al. 1989) and oestradiol has been shown to stimulate calcium absorption in vivo without altered circulating levels of PTH or 1,25 dihydroxyvitamin D (Arjmandi et al 1994). Our data suggest that these effects may be less important in the growing rat.

The major component of the greater reduction in calcium balance in the oophorectomised group was a transient increase in intestinal calcium secretion that was observed at 3 and 6 weeks post-operation, but was not different from the sham group at 9 weeks post-operation. In the rat, alteration of faecal calcium excretion will have considerably greater effect on whole body calcium stores than alteration of urine calcium excretion, as the ratio of calcium excretion via these pathways is approximately 10:1 in favour of intestinal calcium secretion (Aubert et al. 1964). At 12 weeks of age (6 week post-oophorectomy),

intestinal calcium secretion reaches a maximum 43% higher than the sham operated group at the same time, while no other component of calcium balance is significantly affected. Six weeks post-oophorectomy is also the time when the greater reduction in calcium balance in the oophorectomised group was first detected. An increase in intestinal calcium secretion following cessation of ovarian function in women has not been reported, but endogenous faecal calcium has been identified as a significant determinant of trabecular bone loss in osteoporotic women (Tellez et al. 1992, Tellez et al 1995).

There has been criticism of the use of radioactive isotopes of calcium for the estimation of intestinal calcium secretion (Sheikh et al. 1990). Essentially, the issue is whether this method measures the physiological process of calcium excretion via the gastrointestinal tract or simply represents the equilibration of the radioactive isotope from the extracellular fluid to the contents of the intestinal lumen. The differential effect of oophorectomy on intestinal calcium secretion compared to the sham animals demonstrated in this study strongly suggests that the method is measuring a physiological process. Isotope equilibration may account for a portion of the estimates of intestinal calcium secretion, but it is unlikely that oophorectomy is responsible for enhanced equilibration of radioactive calcium between the extracellular fluid and the faeces.

Calcium is excreted in the gastrointestinal tract via digestive secretions from the salivary glands, stomach, small intestine, pancreas and liver and factors that influence the excretion of calcium via these secretions have been identified (Layer & Goebel 1989, Rege et al. 1990, Yu 1990, Karbach 1991). Of particular interest is the control of calcium excretion in

the bile. The concentration of calcium in bile is similar to total plasma calcium concentration (Bronner 1964) and is determined by bile flow and composition (Cummings & Hofmann 1984, Loria et al 1989). The composition and rate of production of bile are influenced by the sex steroids in a number of species including the rat (Collado et al 1989, LeBlanc & Waxman 1990) and alterations to hepatobiliary function following menopause in women have been identified (Maurer et al. 1990). It is reasonable to suspect, therefore, that oestrogen deprivation may modify the total calcium excreted via the bile.

The conclusion from the data presented in this chapter is that oophorectomy in the young rat leads to impaired calcium balance, indicating that observed histomorphometric changes in the long bones of oophorectomised young rats (Yamazaki & Yamaguchi 1989) are not merely the result of redistribution of calcium within the skeleton. Impaired intestinal calcium absorption makes only a minor contribution to the impaired calcium balance when compared to the contribution made by increased excretion of calcium via the gastrointestinal tract. Further work is required to determine whether the increased gastrointestinal loss of calcium, resulting from both increased secretion and reduced absorption, is the cause or the result of loss of bone mineral.

CHAPTER 4

SHORT TERM EFFECTS OF OOPHORECTOMY ON CALCIUM BALANCE IN THE ADULT RAT

4.1 BACKGROUND

Bone loss that follows the cessation of ovarian function in women is biphasic. The rapid loss of bone mineral density that follows the menopause (Nordin & Polley 1987), independent of the effect of age on bone mineral density (Nordin et al 1990b), is exponential and self limiting (Nordin et al 1990b) and appears to occur mainly in trabecular bone (Riggs & Melton 1983, Nordin et al 1992). The rapid rate of bone loss continues for approximately five years following menopause after which a slower rate of bone loss continues over the following 20 years (Nordin et al 1990b). The more gradual loss of bone is more closely related to chronological age than menopausal age and appears to result mainly in a reduction in cortical bone (Nordin et al 1993). The mechanism by which ovarian hormone deficiency modulates bone mass is not fully understood, but a number of risk factors have been implicated. Two longitudinal studies have demonstrated a decrease in intestinal calcium absorption as women pass through menopause regardless of whether the menopause was natural or the result of surgical oophorectomy (Heaney et al 1989, Gennari et al 1990). In addition to reduced intestinal calcium absorption, there is evidence that excretion of calcium via both the kidney and the intestine contributes to postmenopausal bone loss. Increased urine calcium excretion has been identified as a major factor

contributing to the continued loss of bone that occurs following the menopause (Nordin & Polley 1987, Nordin et al 1991, Nordin et al 1993). Excretion of calcium via the gastrointestinal tract has also been identified as a positive determinant of trabecular bone loss in postmenopausal women (Tellez et al 1995).

The pattern of bone loss that occurs in the oophorectomised rat is very similar to that which has been observed in postmenopausal women, in that the loss is mainly from trabecular bone (Wronski et al 1985, Kalu et al 1989, Wronski et al 1989, Yamazaki & Yamaguchi 1989). The rate of bone loss in the rat is also attenuated after an initial rapid rate of loss (Wronski et al 1988a, Dempster et al 1995). In both young and adult rats, oophorectomy causes a reduction in bone density at a number of skeletal sites (Wronski et al 1988a, Kalu et al 1989, Yamazaki & Yamaguchi 1989), but a loss of whole body calcium has not been demonstrated. The effect of oophorectomy on urine calcium excretion in rats is controversial (Yamazaki & Yamaguchi 1989, Morris et al 1992, Morris et al 1995), but net calcium absorption has been shown to be impaired at low dietary calcium (Kalu et al, 1989). Net absorption, however, is not corrected for intestinal calcium secretion, so decreased net absorption can result from either decreased true calcium absorption or increased intestinal calcium secretion.

It has clearly been difficult to determine the relative importance of changes in the components of calcium balance to the presumed oestrogen deficiency-induced loss of whole body calcium stores. The present study assessed the effects of oophorectomy on calcium balance and its components (intestinal calcium absorption, intestinal calcium secretion and urine calcium excretion) within the first 9 weeks of operation in adult rats.

4.2 PROTOCOL

4.2.1 Animals

On arrival in the laboratory, 20 adult (28 weeks of age) female Sprague-Dawley rats were separated into individual cages and allowed unrestricted access to AIN-76A-starch diet containing 0.4% Ca (Table 2.3). After 2 weeks acclimatisation (30 weeks of age) to individual housing, calcium balance and its components, intestinal calcium absorption, intestinal calcium secretion and urine calcium excretion were determined as described in 2.6. Immediately following the initial balance, rats were randomly allocated to either sham or oophorectomy operations as described in 2.3, and calcium balance studies were repeated at 33, 36 and 39 weeks of age. Throughout the experiment rats were fed the 0.4% Ca AIN-76A-starch diet.

4.2.2 Statistical analyses

The data were expressed as the mean ± sem or mean change ± sem. The effects of oophorectomy and time were analysed by two-way ANOVA. Differences were considered significant at $P<0.05$.

4.3 RESULTS

4.3.1 Body weight.

There was a significant positive relationship between body weight and age in both sham and oophorectomised rats. The relationships are described by the following equations: sham, body weight = +230 + 3.42 × age; oophorectomy, body weight = +120 + 7.39 × age. The increase in weight between 30 and 39 weeks of age was greater in the oophorectomised rats than in the sham rats ([mean(sem)]sham; 30wk 337(4.8) to 39wk 366(7.5)g, oophx; 30wk 345(12.3) to 39wk 407(11.2)g, $t=4.04$, $P<0.001$).

4.3.2 Calcium balance and calcium absorption.

The data for calcium balance studies in 30 week old intact female rats are shown in Table 4.1. When the calcium balance data were paired by calculating the difference between the preoperative balance and each subsequent balance the oophorectomised rats had a significantly more negative change in calcium balance compared to sham rats (fig. 4.1). Similarly there was a significantly more negative change in net calcium absorption in the oophorectomised rats compared to sham rats (fig. 4.2). Neither true calcium absorption nor the change in true calcium absorption (fig. 4.3) were significantly affected by oophorectomy. There was no effect of time on calcium balance, net calcium absorption or true calcium absorption.

TABLE 4.1 Pre-operative calcium balance and the components of calcium balance for adult rats..

	Mean(sem)
Calcium balance (mmol/d)	-0.0047(0.028)
Net calcium absorption (mmol/d)	0.0053(0.027)
True calcium absorption (%)	2.42(1.8)
Calcium consumption (mmol/d)	1.433(0.033)
Faecal calcium excretion (mmol/d)	1.428(0.043)
Intestinal calcium secretion (mmol/d)	0.032(0.007)
Urine calcium excretion (mmol/d)	0.01(0.002)

n=20

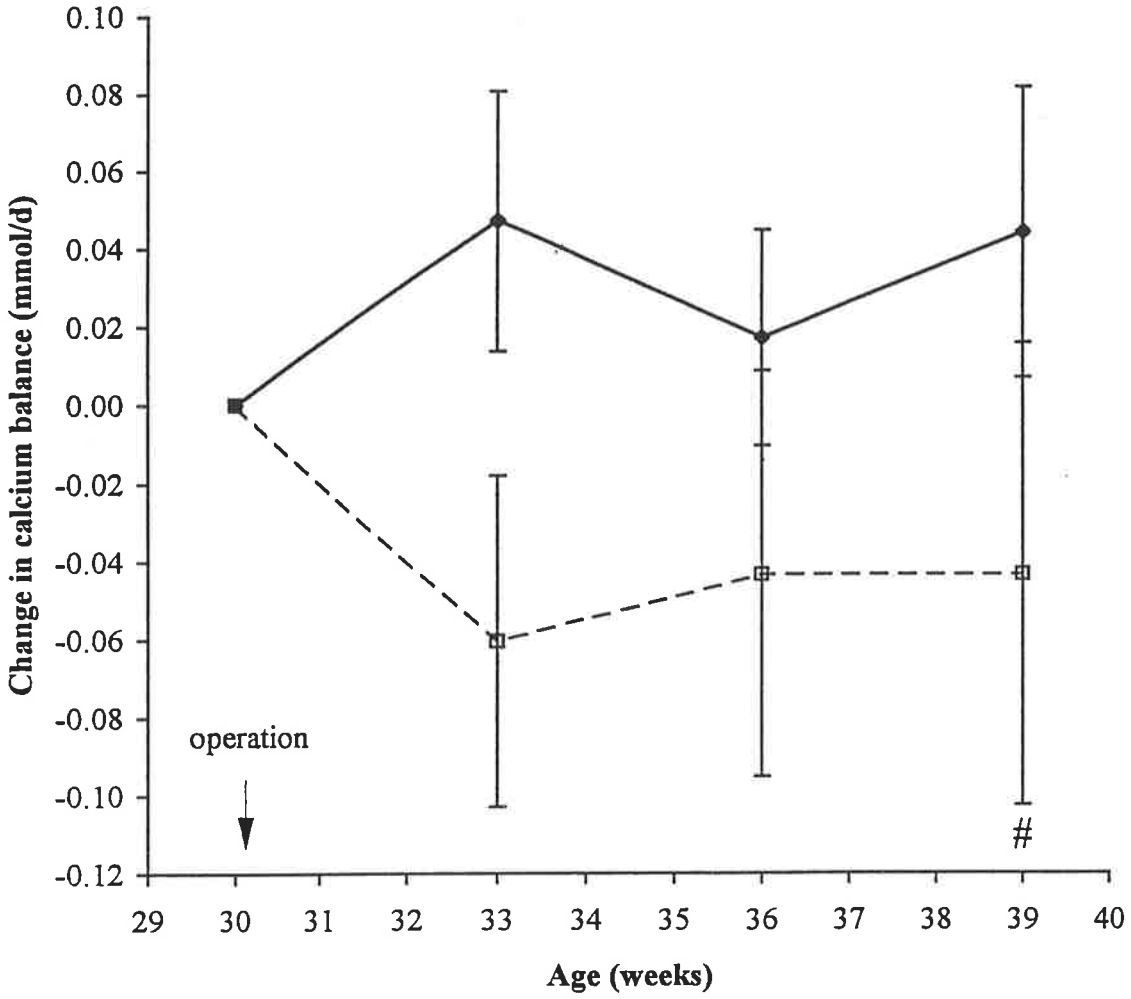

Figure 4.1 The effect of oophorectomy on the change in calcium balance within 9 weeks of operation for adult sham-operated control —♦— and oophorectomised – – □ – – rats. The differences between preoperative balance and the subsequent postoperative balances for each individual rat were determined and the data expressed as the mean ± sem change in calcium balance for 10 rats. #P<0.05 effect of oophx compared to sham group.

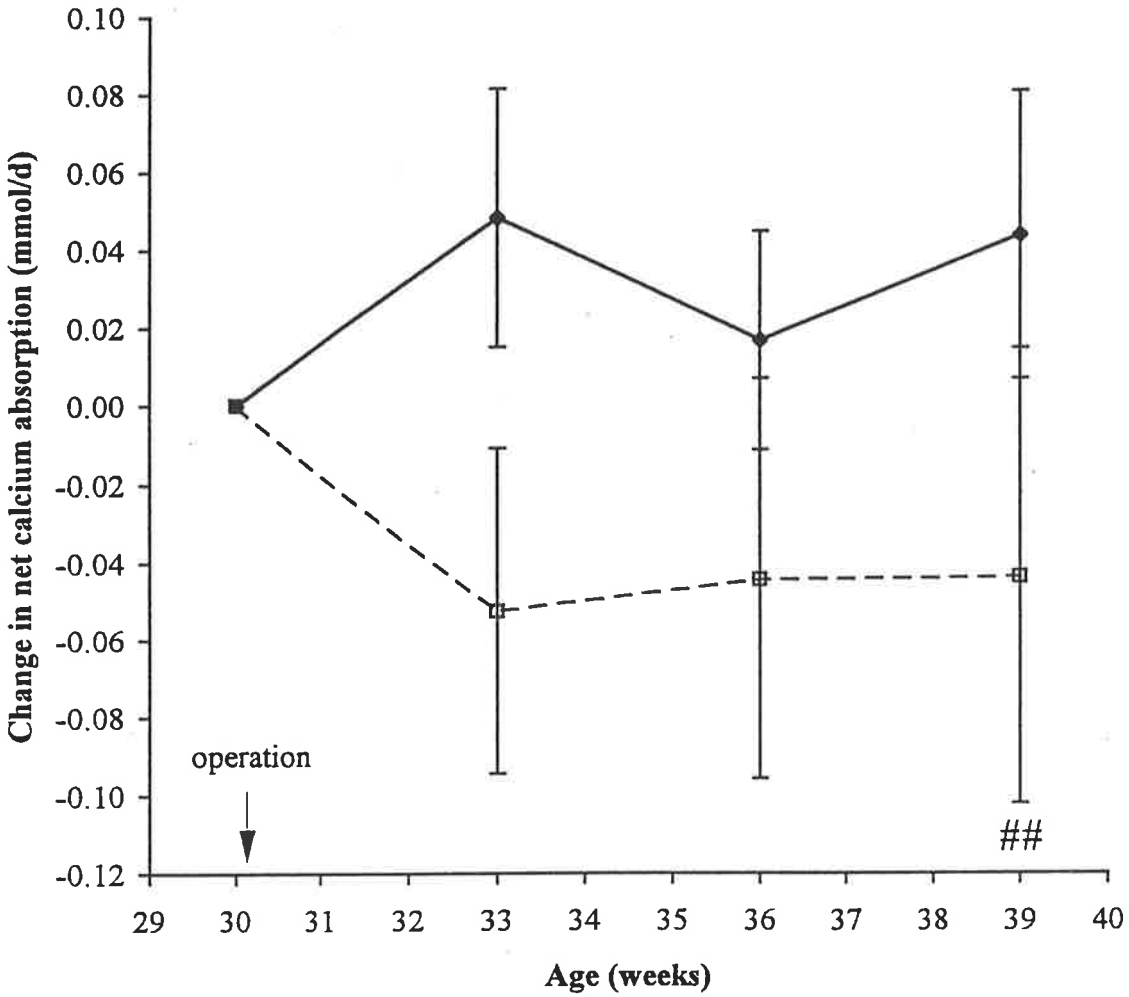

Figure 4.2 The effect of oophorectomy on the change in net calcium absorption within 9 weeks of operation for adult sham-operated control —♦— and oophorectomised – – □ – – rats. The differences between preoperative net calcium absorption and the subsequent postoperative net calcium absorption values for each individual rat were determined and the data expressed as the mean ± sem change in net calcium absorption for 10 rats. ##P<0.02 effect of oophx compared to sham group.

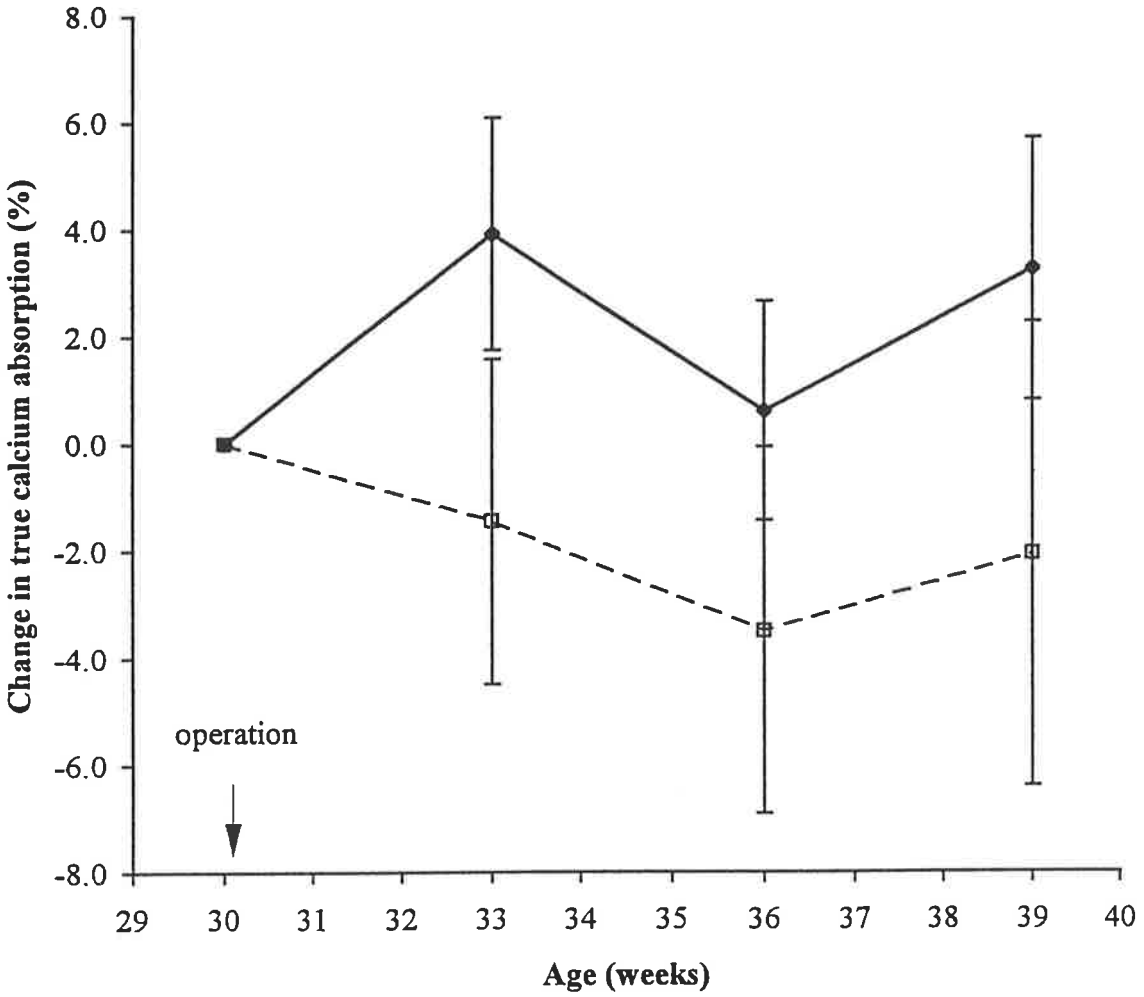

Figure 4.3 The effect of oophorectomy on the change in true calcium absorption within 9 weeks of operation for adult sham-operated control —♦— and oophorectomised – – □ – – rats. The differences between preoperative true calcium absorption and the subsequent postoperative values for true calcium absorption for each individual rat were determined and the data expressed as the mean ± sem change in true calcium absorption for 10 rats. There was no significant effect of oophorectomy on the change in true calcium absorption.

4.3.3 Calcium excretion

There was a significant rise in urine calcium excretion in oophorectomised rats compared to sham rats, but there was no effect of time following operation on urine calcium excretion (fig. 4.4). The effect of oophorectomy on urine calcium excretion was most distinct at 3 weeks post-oophorectomy. By two-way analysis of variance there was a significant rise in endogenous faecal calcium (Table 4.2, fig. 4.5) and a strong trend for a rise in intestinal calcium secretion ($P = 0.08$) (fig. 4.6) in the oophorectomised group compared with the sham-operated group. As for urine calcium excretion the effect of oophorectomy on intestinal calcium secretion was most distinct at 3 weeks post-oophorectomy.

4.4 DISCUSSION

At 30 weeks of age, calcium balance and net calcium absorption were not different from zero, indicating that skeletal growth had ceased in these rats. However, the rats did gain weight between 30 and 39 weeks, which probably reflects growth of non-skeletal tissues only. Kalu and co-workers (1989) demonstrated in the rat that femur density and femur calcium did not change after 6 months of age, regardless of an increase in body weight which continued to at least 24 months. In the present study, the increase in rat weight after operation was greater in the oophorectomised rats than in sham, consistent with previous reports indicating that oophorectomy leads to increased body weight even when oophorectomised rats are pair fed with ovary-intact animals (Kalu et al 1984a).

The demonstration of physiologically meaningful changes in calcium balance and intestinal calcium absorption is difficult due to high coefficients of variation in their measurement.

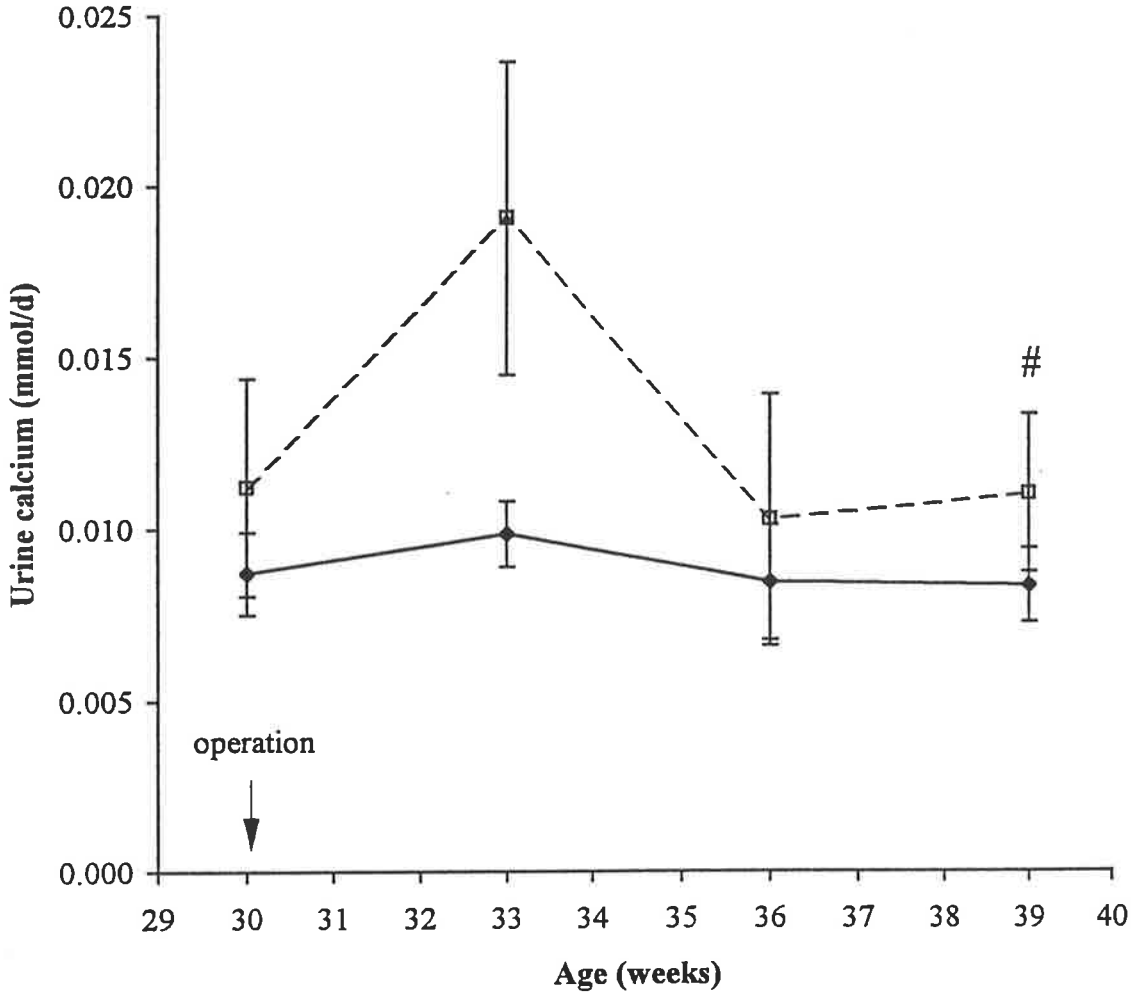

Figure 4.4 The effect of oophorectomy on urine calcium excretion within 9 weeks of operation for adult sham-operated control —♦— and oophorectomised – – □ – – rats. The data are expressed as the mean ± sem for 10 rats. #P<0.05 effect of oophx compared to sham group.

TABLE 4.2 The effect of oophorectomy on endogenous faecal calcium and its components within 9 weeks of operation in adult rats.

Age	Operation	Faecal ^{45}Ca kBq/d	Urinary ^{45}Ca specific radioactivity kBq/mmol	Endogenous faecal calcium mmol/d
30 weeks	sham	18.8(6.1)	615(161)	0.026(0.007)
	oophx	18.8(5.2)	728(221)	0.034(0.012)
33 weeks	sham	14.9(4.8)	409(94)	0.028(0.005)
	oophx	12.5(3.5)	273(59)	0.061(0.016)*
36 weeks	sham	6.8(1.8)	374(57)	0.015(0.003)
	oophx	5.9(1.3)	326(48)	0.019(0.004)
39 weeks	sham	12.5(2.8)	524(89)	0.023(0.006)
	oophx	13.1(2.6)	445(69)	0.041(0.012)

Values are mean(sem) for 10 rats; *P<0.05 Vs sham for same time point.

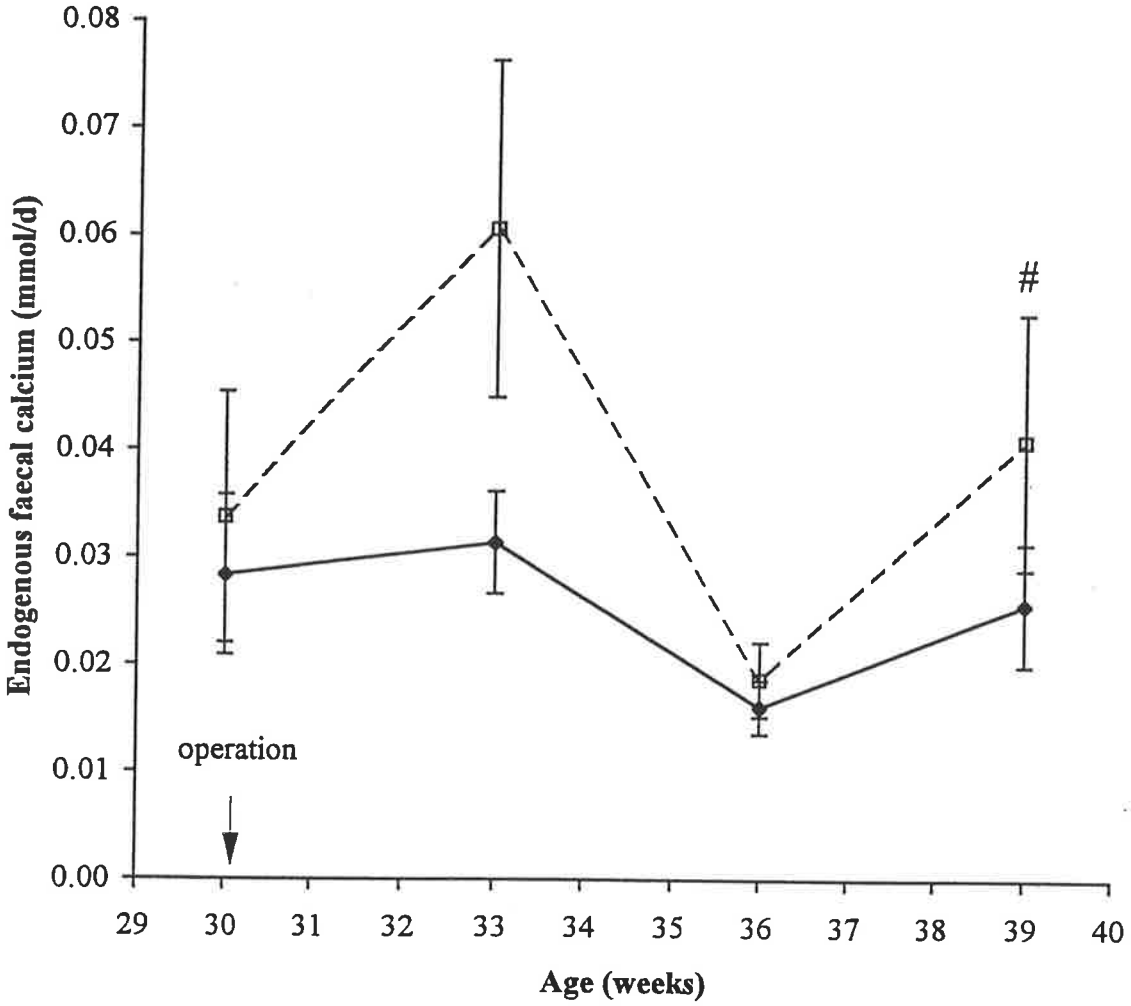

Figure 4.5 The effect of oophorectomy on endogenous faecal calcium within 9 weeks of operation for adult sham-operated control —♦— and oophorectomised – – □ – – rats. The data are expressed as the mean ± sem for 10 rats. #P<0.05 effect of oophx compared to sham group.

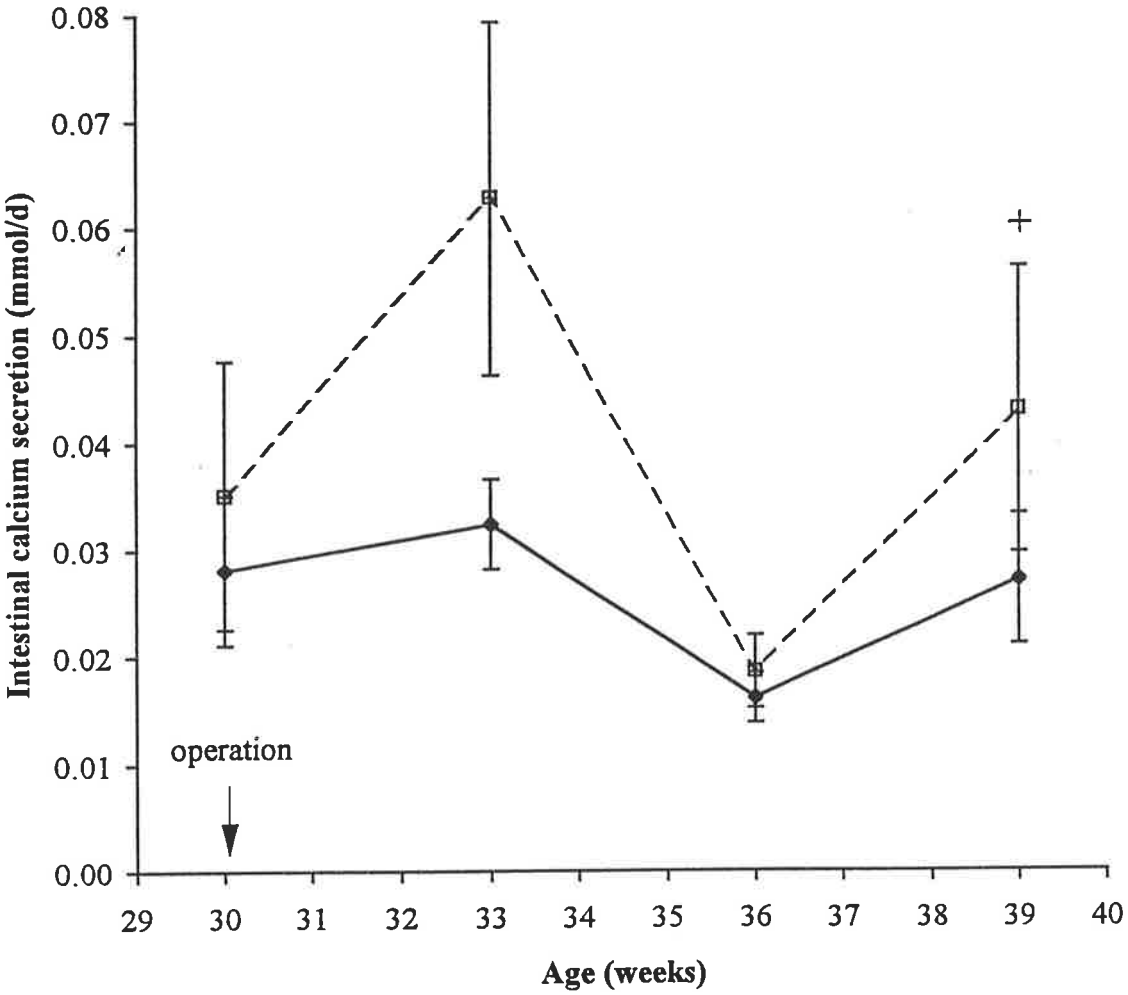

Figure 4.6 The effect of oophorectomy on intestinal calcium secretion within 9 weeks of operation for adult sham-operated control —♦— and oophorectomised – – □ – – rats. The data are expressed as the mean ± sem for 10 rats. +P=0.08 effect of oophx compared to sham group.

The coefficient of variation of calcium balance is high because it is determined as a small difference between calcium intake and calcium excretion, both of which are large numbers. This problem with the measurement of calcium balance has been described in detail in balance studies in humans (Taagehøj Jensen et al 1983). In the present study calcium balance and net calcium absorption data were paired by calculating the difference between the values obtained from preoperative balance studies and the values obtained from each subsequent post-operative balance study. Thus, the data are presented as mean changes in calcium balance and net calcium absorption compared to the values obtained pre-operatively at 30 weeks of age.

The more negative change in calcium balance in the oophorectomised rats compared to sham operated controls indicated a net loss of whole body calcium stores in the adult rat. Further, the effect of oophorectomy on the change in calcium balance was most distinct at 3 weeks post-operation. This finding is consistent with previous reports of bone loss within 14 days of oophorectomy in the rat (Wronski et al 1988a, Dempster et al 1995). Consistent with the finding of greater reduction in calcium balance following oophorectomy in young rats (section 3.3.2), these data indicate that reports of oophorectomy-induced bone loss in adult rats at specific sites within the skeleton (Wronski et al 1985, Kalu et al 1989, Yamazaki & Yamaguchi 1989) are not merely the result of a redistribution of calcium within the skeleton. The reports of site-specific reductions in bone following oophorectomy do not unequivocally identify a loss of whole body calcium after oophorectomy.

Similar to the findings for calcium balance, there was also a more negative change in net calcium absorption in oophorectomised rats compared to sham rats. This confirms the

finding of reduced net calcium absorption after oophorectomy in adult rats reported by Kalu and co-workers (1989). Their study demonstrated reduced net calcium absorption in oophorectomised rats fed a restricted calcium diet (0.1%) 5 months after oophorectomy. The present study goes a step further by demonstrating reduced net calcium absorption on a diet containing the level of calcium (0.4%) recommended by the American Institute of Nutrition (1977) for laboratory rodents. Further, the present study also demonstrates that the reduction in net calcium absorption is most distinct at 3 weeks after oophorectomy.

The present study was unable to clearly demonstrate an effect of oophorectomy on true calcium absorption in adult rats fed a normal calcium diet within 9 weeks of oophorectomy. Kalu and co workers (1989) demonstrated reduced net calcium absorption well beyond the brief period of increased excretion of calcium into the intestine that is demonstrated in the present study, suggesting that their finding is the result of reduced true calcium absorption rather than increased intestinal calcium secretion. In the present study, pre-operative true calcium absorption was only 2.42±1.8% (Table 4.1), making it difficult to identify a further reduction due to oophorectomy. It has previously been demonstrated that dietary calcium restriction stimulates active calcium transport in the rat (Armbrecht et al 1979). Thus repeating the present study at lower dietary calcium levels to increase the percentage of consumed calcium that is absorbed may improve the discrimination between oophorectomy and sham groups for true calcium absorption.

There was a positive change in calcium balance and net calcium absorption values obtained for sham rats after 33 weeks of age compared to the preoperative values obtained at 30 weeks of age for the same rats (figs. 4.1 & 4.2). This finding may be the result of

adaptation to the AIN-76-starch 0.4% Ca diet, which had been fed to the rats for only 5 weeks when the 33-weeks-of-age calcium balance study was conducted. The calcium content of standard rat chow, which the rats were fed until arrival in the laboratory, is nominally 0.7%, although this varies considerably (section 2.5.13). Dietary calcium restriction in the rat increases active calcium transport (Armbrecht et al 1979) by increasing circulating levels of 1,25 dihydroxyvitamin D (Rader et al 1979). A sample of the standard rat chow fed to the rats prior to the commencement of the AIN-76-starch diet was not available for calcium analysis to determine if there was a marked change in calcium intake at this time. In addition to the higher nominal calcium content of rat chow, seasonal variations in other components known to affect calcium absorption such as lactose (Greenwald & Gross 1929, Wasserman 1964, Favus & Angeid-Backman 1984), starch (Schulz et al 1993), protein content (Kerstetter & Allen 1994), sucrose and fat (Zernicke et al 1995), dietary fibre (Harmuth-Hoene & Schelenz 1980) and other factors affecting calcium availability (Amman et al 1986, Pansu et al 1993) may also lead to enhanced calcium absorption, which would effectively suppress the active component of calcium absorption. Thus, although the exact content of the diet could not be determined it is possible that switching from chow to AIN-76-starch 0.4% Ca induced a period of adaptation in intestinal calcium absorption that may be sufficient to explain the positive changes between 30 and 33 weeks of age in both net calcium absorption and calcium balance in the sham operated rats. Moreover, if adaptation to dietary calcium restriction accounts for the positive changes in calcium balance and net calcium absorption in sham rats, then the oophorectomised rats would also have been subject to the same degree of dietary calcium restriction. Thus, the findings of negative changes in calcium balance and net intestinal calcium absorption are strong evidence for the effect of oophorectomy on

these variables in the rat. The length of time over which adaptation to dietary calcium restriction occurs for net calcium absorption and calcium balance is discussed in more detail in section 5.4.

In addition to the reduction in net calcium absorption, there was also a significant increase in urine calcium excretion which was most distinct at 3 weeks post-oophorectomy. After 3 weeks post-operation urine calcium excretion was similar in both sham and oophorectomy rats. The effect of oophorectomy on urine calcium excretion is currently controversial. Yamazaki and Yamaguchi (1989) reported an effect of oophorectomy on fasting urine calcium excretion in 10 week old rats but not in either 4 or 52 week old rats. Morris and co-workers found no effect of oophorectomy on non-fasting urine Ca excretion in rats fed a diet with 0.7% Ca (1992), but subsequently demonstrated an oophorectomy-induced increase in urine calcium excretion in fasted rats fed a similar diet (1995). Thus, it would appear that an effect of oophorectomy on renal excretion of calcium can only be demonstrated when urine specimens are collected under fasting conditions that prevent the effects of variations in dietary calcium intake on the variance in urine calcium excretion. Urine samples in the present study were collected over a 6 day period in rats that were given a restricted food allowance, which would prevent the effects of variation in dietary intake contributing to the variance in urine calcium excretion. This should be taken into consideration when designing future studies to assess the effect of oophorectomy on renal calcium excretion.

Endogenous faecal calcium was significantly higher in oophorectomised rats compared to sham operated controls and there was a strong trend for a rise in intestinal calcium

absorption in the oophorectomised group. Intestinal calcium secretion is calculated by correcting endogenous faecal calcium for that portion of calcium from endogenous sources that is reabsorbed in the intestine along with dietary calcium, according to the calculation described in section 2.5.11. Because true calcium absorption is very low in these adult rats, intestinal calcium secretion is essentially the same as endogenous faecal calcium. However, the calculation of intestinal calcium secretion combines the variances from endogenous faecal calcium and true calcium absorption, making it more difficult to identify an effect of oophorectomy on intestinal calcium secretion than it is to identify the effect on endogenous faecal calcium. Thus, although intestinal calcium secretion did not reach statistical significance, these data combined indicate an increase in the excretion of calcium into the gastrointestinal tract after oophorectomy. Further, the increased excretion of calcium into the gastrointestinal tract was distinct at 3 weeks post-operation, but subsequently, endogenous faecal calcium and intestinal calcium absorption were similar in sham and oophorectomised rats. This finding is consistent with the demonstration of increased intestinal calcium secretion following oophorectomy in the young rat (section 3.3.5), which also appeared to be transient, although in the young rats it persisted until 6 weeks after oophorectomy. Endogenous faecal calcium has been identified as a positive determinant of trabecular bone loss in osteoporotic women (Tellez et al. 1992, Tellez et al 1995).

The ratio of intestinal calcium secretion to urine calcium excretion was approximately 3:1, both pre-operatively (Table 4.1) and at 3 weeks post-operation (figs. 4.4 & 4.6). Therefore in the present study 75% of the total measured excretion of calcium is accounted for by intestinal calcium secretion and 25% by urine calcium excretion. This is consistent with the

previous report of Aubert and co-workers' (1964) of higher calcium excretion via the kidney than the gastrointestinal tract.

As the excretion of calcium by both the gastrointestinal tract and the kidney rose transiently in the same manner and the relative portion of calcium excretion attributable to each was not affected by operation, it is probable that they arise from a common mechanism, rather than separate mechanisms for each occurring simultaneously. It has been shown that bone is lost from both the tibia and femur within 10 days of oophorectomy in the rat (Dempster et al 1995) and thus it is likely that the increased excretion of calcium at 3 weeks post-oophorectomy observed in the present study is the result of increased resorption of calcium from bone. Increasing the load of calcium filtered at the glomerulus, by increasing plasma calcium, leads to increased urine calcium excretion (Suki & Rouse 1991). An effect of increasing plasma calcium on the excretion of calcium via the gastrointestinal tract has not been described, but is possible that raised plasma calcium would increase the amount of calcium that enters the secretions of the salivary glands, stomach, small intestine, pancreas and liver, all of which contribute to the calcium that is excreted by the gastrointestinal tract. The plasma calcium fractions were not measured in the present study, but for the postulated mechanism of increased calcium excretion following oophorectomy described here to hold true there must be a rise in ionised calcium and/or complexed calcium, the fractions of plasma calcium that are filtered at the glomerulus and it is also likely that these fractions also enter the secretions of the gastrointestinal tract.

The data reported in the present study are consistent with the notion of a rapid phase of bone loss within 3 weeks of oophorectomy followed by a slower rate of bone loss after 3 weeks. Recently, it has been demonstrated that within 5 days of oophorectomy in adult rats there is an increase in osteoclast surface area in secondary spongiosa, followed at 10 days by a reduction in tibial trabecular bone volume and a reduction in femur bone density. After an initial rapid loss of bone to 40 days post-oophorectomy there was an attenuated rate of bone loss (Dempster et al 1995). Wronski and co-workers (1988a) have reported post-oophorectomy trabecular bone loss in the rat, which is most rapid to 14 days post-operation and gradually attenuates until 100 days when trabecular bone mass appears to stabilise. The rapid loss of bone within 14 days coincided with maximal increases in both osteoclast and osteoblast surface as percentages of total trabecular surface, but the rate of bone formation was not maximally stimulated until 35 days post-oophorectomy. Similarly, percent acid phosphatase surface and urine hydroxyproline excretion, markers of bone resorption have been reported to rise before the mineral appositional rate rises following oophorectomy in the rat (Sims et al 1996). This temporal relationship between resorption and formation is consistent with a rapid increase in bone resorption leading to the increased excretion of calcium demonstrated at 3 weeks post-oophorectomy in the present study. Furthermore, the subsequent increase in bone formation probably prevents on-going elevated calcium excretion.

The findings of increased excretion of calcium via both the kidney and the gastrointestinal tract after oophorectomy in the adult rat differ from the findings in the young rat of a rise in excretion of calcium by the gastrointestinal tract, but not by the kidney. It may be that the mechanism of the loss of whole body calcium differs in the young oophorectomised rat

compared to the adult oophorectomised rat. Alternatively the different patterns of calcium excretion may be the result of the differences in diet, with the young rats having been fed standard rat chow (0.7%), while the adult rats were fed AIN-76-starch (0.4% Ca).

The mechanism of the rapid bone loss that occurs within a finite period after the cessation of ovarian function in the human has not been elucidated. Postmenopausal bone loss may not be uniform at all sites within the skeleton. In postmenopausal women there is a reduction in both cortical thickness and trabecular bone density, but the reduction in density of trabecular bone is more pronounced (Ettinger et al 1987). It has been suggested that postmenopausal bone loss is characterised by an initial rapid loss of trabecular bone due to the menopause, followed by a slower rate of cortical bone loss that is age-related (Nordin et al 1993). Thus reduced bone mineral is likely to be evident in trabecular-rich bones in the early stages of postmenopausal bone loss, whereas cortical-rich bones are likely to lose mineral at a later stage. The findings of calcium balance studies are therefore valuable to understand the effects of oestrogen on whole body calcium handling. The menopause has a substantial effect on calcium balance with significantly lower balance in postmenopausal women compared to premenopausal women, the difference being due approximately equally to decreased intestinal calcium absorption and to increased urine calcium excretion (Heaney et al 1978b). However, the time course of the development of oestrogen deficiency-induced reduction in calcium balance has not been elucidated and can only be inferred from studies on the effect of menopause on bone density, usually measured at specific sites within the skeleton. The present study has used the oophorectomised rat model for postmenopausal bone loss to assess the effect of oophorectomy on calcium balance in the adult rat.

At the glomerulus, both the ionised and complexed fractions are filtered from the plasma and enter the glomerular filtrate. Although the majority of the filtered calcium is reabsorbed in the renal tubules and collecting ducts, an increase in the amount of calcium that is filtered at the glomerulus, resulting from a rise in the complexed fraction of plasma calcium contributes to a higher loss of calcium in the urine (Heaney et al 1978b, Nordin & Polley 1987, Kelly et al 1989, Adami et al 1992). An increase in urine calcium excretion, independent of the increased filtered load has also been demonstrated by comparing the calcium excretion in pre- and post-menopausal women matched for filtered calcium loads (Nordin et al 1991). It has been proposed that the urine 'calcium leak' is a major factor contributing to the continued loss of bone that occurs following the menopause (Nordin & Polley 1987, Nordin et al 1991, Nordin et al 1993).

It can be concluded from the data presented that oophorectomy in adult rats causes a reduction in calcium balance, which is most distinct at 3 weeks post-operation. The temporal pattern of changes in calcium balance after oophorectomy correspond very closely with changes in net calcium absorption. The distinct effect of oophorectomy on calcium balance at 3 weeks after operation is the result of transient increases in both renal and gastrointestinal excretion of calcium, which are probably the result of increased bone resorption at this time when bone formation has not reach maximal activity. It cannot be determined from the data presented if oophorectomy impairs true calcium absorption, but repeating the present study on rats fed lower dietary calcium levels may improve the discrimination between oophorectomy and sham groups for true calcium absorption.

CHAPTER 5

EFFECT OF DIETARY CALCIUM RESTRICTION, 1,25 DIHYDROXYVITAMIN D ADMINISTRATION AND OESTRADIOL REPLACEMENT ON CALCIUM BALANCE IN OVARY-INTACT AND OOPHORECTOMISED ADULT RATS.

5.1 BACKGROUND

The mechanism by which ovarian hormones modulate bone mass is not fully understood, but the menopause is associated with increased renal excretion of calcium (Nordin et al 1991) and decreased intestinal calcium absorption (Heaney et al 1989). The reduced calcium absorption has been attributed variously to reduced circulating 1,25 dihydroxyvitamin D levels (Gallagher et al 1979) and to gastrointestinal resistance to the action of 1,25 dihydroxyvitamin D (Gennari et al 1990, Morris et al 1991). In addition, the role of ovarian hormones in adaptation to dietary calcium restriction is unclear, although it has been shown in humans that oestrogens modulate the end organ effect of 1,25 dihydroxyvitamin D on intestinal calcium absorption (Gennari et al 1990).

Oophorectomy in the rat produces a reduction in bone density at a number of skeletal sites (Wronski et al 1988a, Kalu et al 1989, Yamazaki & Yamaguchi 1989). Net intestinal calcium absorption in adult rats fed a low calcium diet (0.1%) is impaired by oophorectomy (Kalu et al 1989). The study of the short term effects of oophorectomy on calcium balance in adult rats (chapter 4) demonstrated reduced net calcium absorption at higher dietary calcium levels (0.4%), but the oophorectomy-induced reduction in true calcium absorption

did not reach statistical significance. A decrease in intestinal 1,25 dihydroxyvitamin D receptor number (Chan et al 1984) following oophorectomy may contribute to reduced intestinal calcium absorption. Given that oophorectomy leads to reduced calcium absorption in the rat, the mechanism for this effect could include either reduced circulating levels of 1,25 dihydroxyvitamin D, impaired intestinal response to 1,25 dihydroxyvitamin D or reduction in a vitamin D independent mechanism for calcium absorption.

The present study reports the long-term effects of oophorectomy on calcium balance and its components in adult rats. In particular, this study assesses adaptation to dietary calcium restriction in oophorectomised and sham-operated control rats, determines the relationships between calcium consumption and calcium balance and its components, including true intestinal calcium absorption and intestinal calcium secretion in oophorectomised and sham operated control. In addition, the effect of oophorectomy on circulating levels of 1,25 dihydroxyvitamin D and the response to treatment with 1,25 dihydroxyvitamin D or oestradiol are presented.

5.2 PROTOCOL

5.2.1 Animals

Female Sprague-Dawley rats were randomly allocated to either oophorectomy or sham operation (section 2.3) at 7 months of age. Calcium balance studies (section 2.6) were conducted when animals were between 10 and 14 months of age. Dietary calcium was varied within the range of 0.02% and 0.4% by the addition of calcium carbonate to an otherwise calcium free AIN-76A-starch diet (Table 2.3). Calcium balance and net intestinal

calcium absorption was determined at all levels of dietary calcium and intestinal calcium secretion and true calcium absorption were estimated in rats fed between 0.05% and 0.4% dietary calcium.

5.2.2 Adaptation to dietary calcium restriction

Adaptation to dietary calcium restriction was assessed in 5 sham and 5 oophx rats. The animals were maintained on a 0.4% calcium diet from 2 weeks prior to operation until 15 weeks after operation (11.5 months of age). At this time a calcium balance study was performed with rats fed a 0.02% Ca diet from the beginning of the acclimatisation period such that the calcium balance period commenced after 5 days of adaptation to the 0.02% Ca diet. The rats were maintained on the 0.02% Ca diet until the end of the experiment with 2 further calcium balance studies being performed such that the balance periods commenced at 26 and 47 days after commencement of the 0.02% Ca diet. In experiments 2 and 3, described below, a minimum period of 8wk of adaptation to altered dietary calcium before measurement of calcium balance was adopted on the basis of the data obtained from experiment 1.

5.2.3 Calcium balance at varying dietary calcium levels

Following operation, 40 female Sprague-Dawley rats were separated into groups of 5 sham and 5 oophx and placed on one of the following dietary calcium levels; 0.02%, 0.05%, 0.1%, 0.15%, 0.2% or 0.4%. The rats were maintained on the allocated diet for at least 10 weeks to allow for adaptation to the new dietary Ca level before measurement of calcium balance and its components. On completion of a balance measurement, the rats were reallocated to another dietary calcium level for another period of at least 10 weeks and

balance measured again, until calcium balance was performed on at least 5 rats in each group at each dietary calcium level.

5.2.4 1,25 dihydroxyvitamin D administration and oestradiol replacement

20 female Sprague-Dawley rats were randomly allocated to either oophorectomy or sham operation (section 2.3) at 7mth of age. The rats were fed the 0.05% Ca diet from 8wk before the operation until the end of the experiment. At 11wk post operation calcium balance was assessed in all animals, following which 10 of the animals (5 sham and 5 oophx) were killed under halothane anaesthesia by cervical dislocation. Blood was collected from the tail vein and by cardiac puncture for intact parathyroid hormone (section 2.4.3) and 1,25 dihydroxyvitamin D (section 2.4.1) analyses respectively.

5.2.4.1 1,25 dihydroxyvitamin D administration

1,25 dihydroxyvitamin D was administered at 20ng/kg/d to the remaining 5 sham and 5 oophx rats by a subcutaneous 14-day mini-osmotic pump (Alzet 2002, Alza Corporation, Palo Alto, CA) from 8 days prior to the commencement of the balance period (14wk post-op) until the end of the balance period. On completion of the balance, blood was collected from the tail vein for intact parathyroid hormone (section 2.4.3) and 1,25 dihydroxyvitamin D (section 2.4.1) analyses.

5.2.4.2 Oestradiol replacement to oophorectomised rats

Calcium balance was measured again at 19wk post-op as a baseline prior to oestradiol replacement to ensure that calcium balance and its components had returned to pre-1,25 dihydroxyvitamin D treated levels. 17β-oestradiol (20μg/kg/d) was administration via

subcutaneous 14d mini-osmotic pumps inserted into oophorectomised rats at 13d and 1d prior to the commencement of the balance period. The oestradiol replacement balance was performed at 23wk post-op. Immediately following the balance the rats were killed under halothane anaesthesia by cervical dislocation. Blood was collected from the tail vein for intact parathyroid hormone (section 2.4.3) and by cardiac puncture for total oestradiol (section 2.4.2) and 1,25 dihydroxyvitamin D (section 2.4.1).

5.2.5 Statistical analyses

The data are expressed as the mean ± sem. The effect of oophorectomy and time were analysed by two-way ANOVA. To determine the function that best described the relationship between calcium consumption and faecal calcium, the consumption values were standardised for each mathematical function by subtracting the mean from fitted values and a diagnostic plot of residuals was used to eliminate the functions with systematic lack-of-fit (Draper & Smith 1966). A similar method was used to determine the relationships between calcium consumption and calcium balance and its components. The two-tailed *t*-test was used to assess differences between operation groups and two-tailed paired *t*-test was used to assess the effects of treatment with 1,25 dihydroxyvitamin D or oestradiol. Differences were considered significant at $P < 0.05$.

5.3 RESULTS

5.3.1 Adaptation to dietary calcium restriction

There was a significant increase in calcium balance in both sham and oophx groups between 5 and 47 days after commencement of dietary calcium restriction (fig 5.1) ($P<0.0001$),

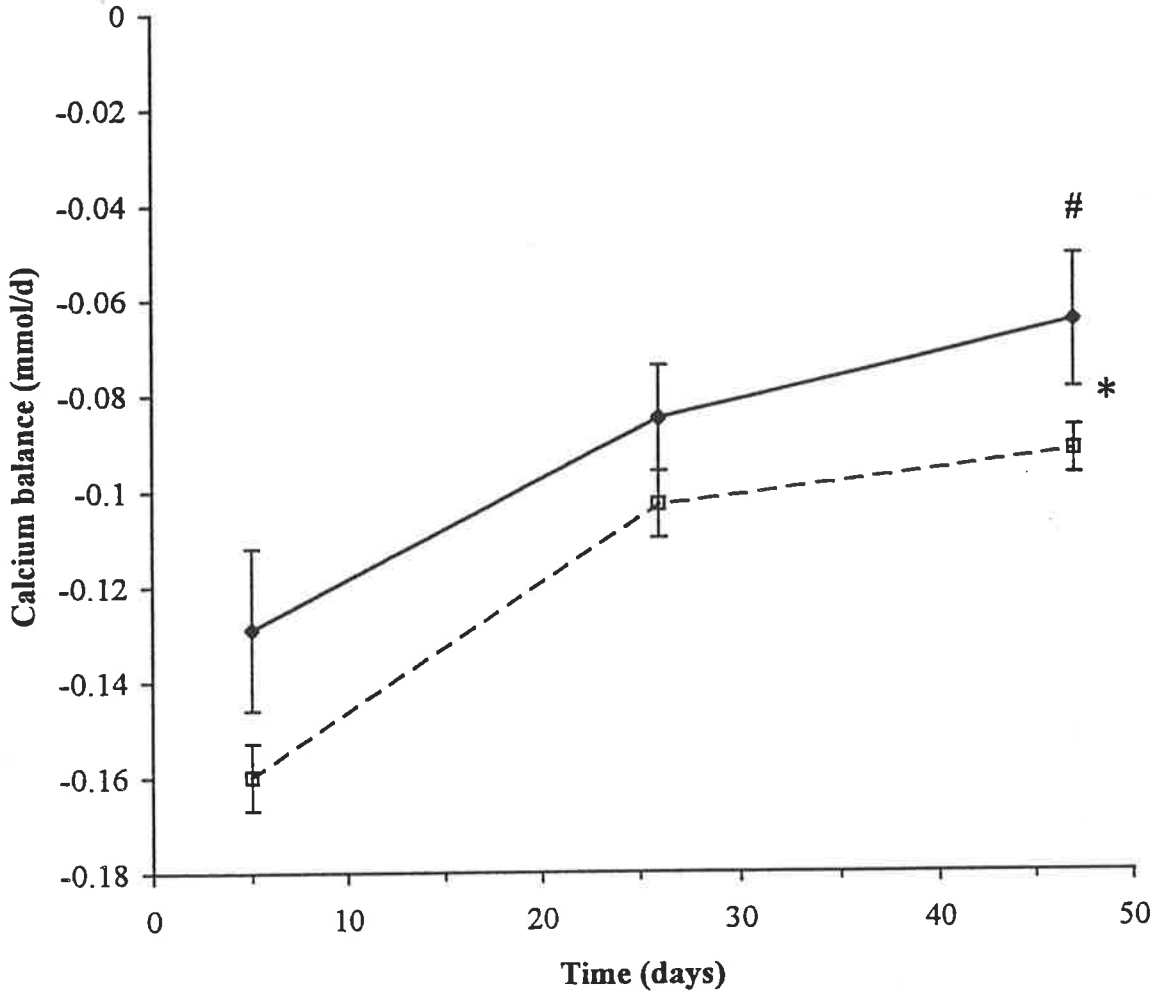

Figure 5.1 Adaptation in calcium balance to dietary calcium restriction in sham operated control —♦— and oophorectomised – – □ – – rats for 47 days after commencement of 0.02% Ca diet. The data are expressed as the mean ± sem for 5 rats. *P<0.0001 effect of time after commencement of dietary calcium restriction, #P<0.02 effect of oophx compared to sham group.

although the oophx group was significantly lower than the sham group at each time point throughout this period ($P<0.02$). Net calcium absorption also increased significantly in both groups between 5 and 47 days following commencement of dietary calcium restriction (fig 5.2) ($P< 0.0001$), but, as for calcium balance, the oophx group remained lower than the sham group for each time point ($P<0.02$). Urine calcium excretion decreased in both the sham group and oophx groups from day 5 to day 47, but there was no difference between the two operation groups (fig 5.3). There was no significant change in intestinal calcium secretion between days 5 and 47 following commencement of dietary calcium restriction

Serum 1,25 dihydroxyvitamin D levels after 12 weeks on 0.02% Ca diet were significantly higher in the oophx group compared to the sham group and both groups were higher than data obtained for rats fed the same modified AIN-76A-starch diet containing 0.4% Ca (Mason and Morris, submitted for publication) (Table 5.1). PTH levels after 12 weeks on the 0.02% Ca diet were not affected by oophorectomy, but were higher than those obtained for rats fed 0.4% calcium (Table 5.1).

5.3.2 Calcium balance at varying dietary calcium levels

The relationship between calcium consumption and faecal calcium was best described by a quadratic polynomial function. The relationships between standardised consumption and faecal calcium for both the sham and oophorectomised groups were highly statistically significant (sham $r^2 = 0.99$, oophx $r^2 = 0.99$). When these relationships were compared by general linear modelling, the difference in the intercepts was statistically significant (intercepts: sham +0.48(0.013), oophx +0.53(0.015), $F = 4.6$, $P < 0.05$), but there was no difference between the slopes or the quadratic terms for the two groups. From the

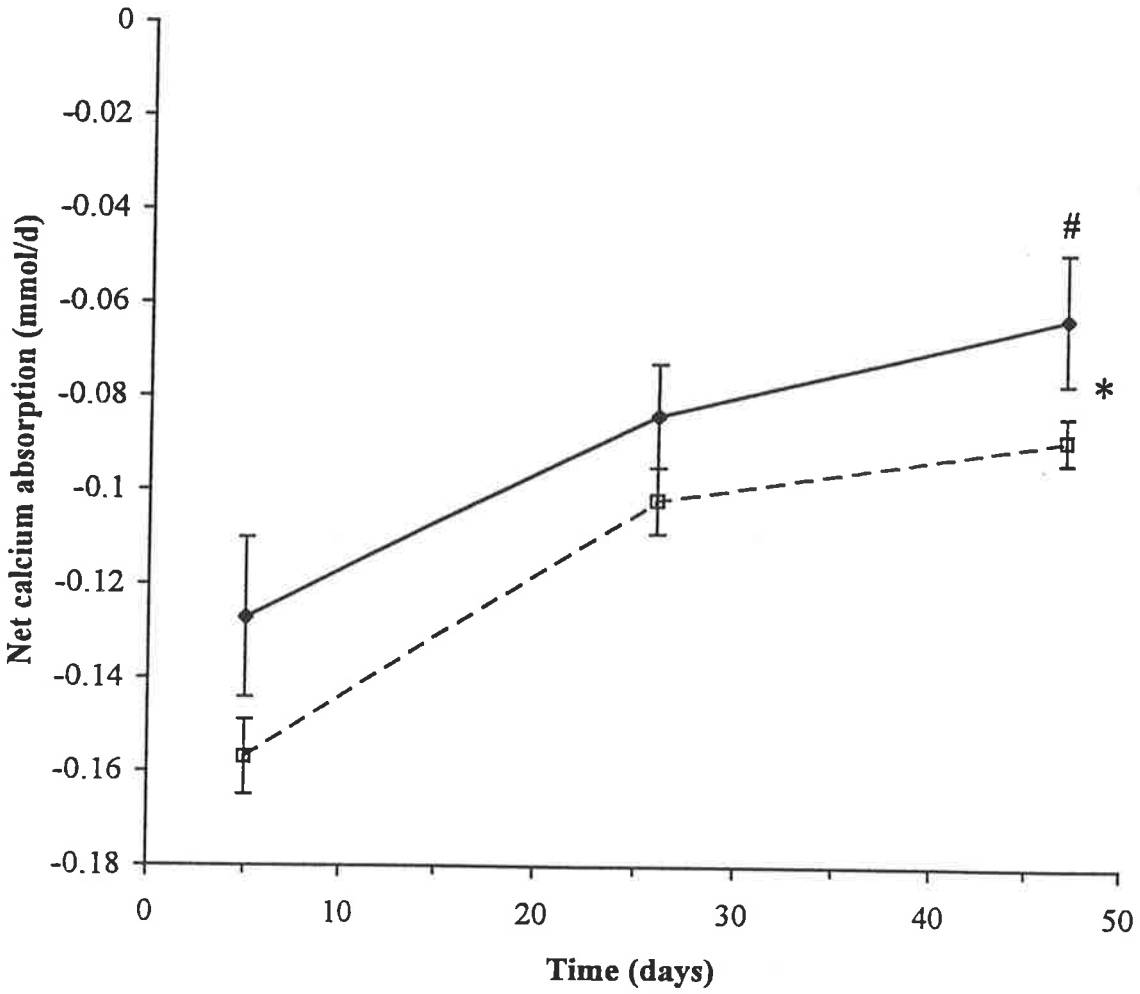

Figure 5.2 Adaptation in net intestinal calcium absorption to dietary calcium restriction in sham operated control —♦— and oophorectomised – – □ – – rats for 47 days after commencement of 0.02% Ca diet. The data are expressed as the mean ± sem for 5 rats. *P<0.0001 effect of time after commencement of dietary calcium restriction, #P<0.02 effect of oophx compared to sham group.

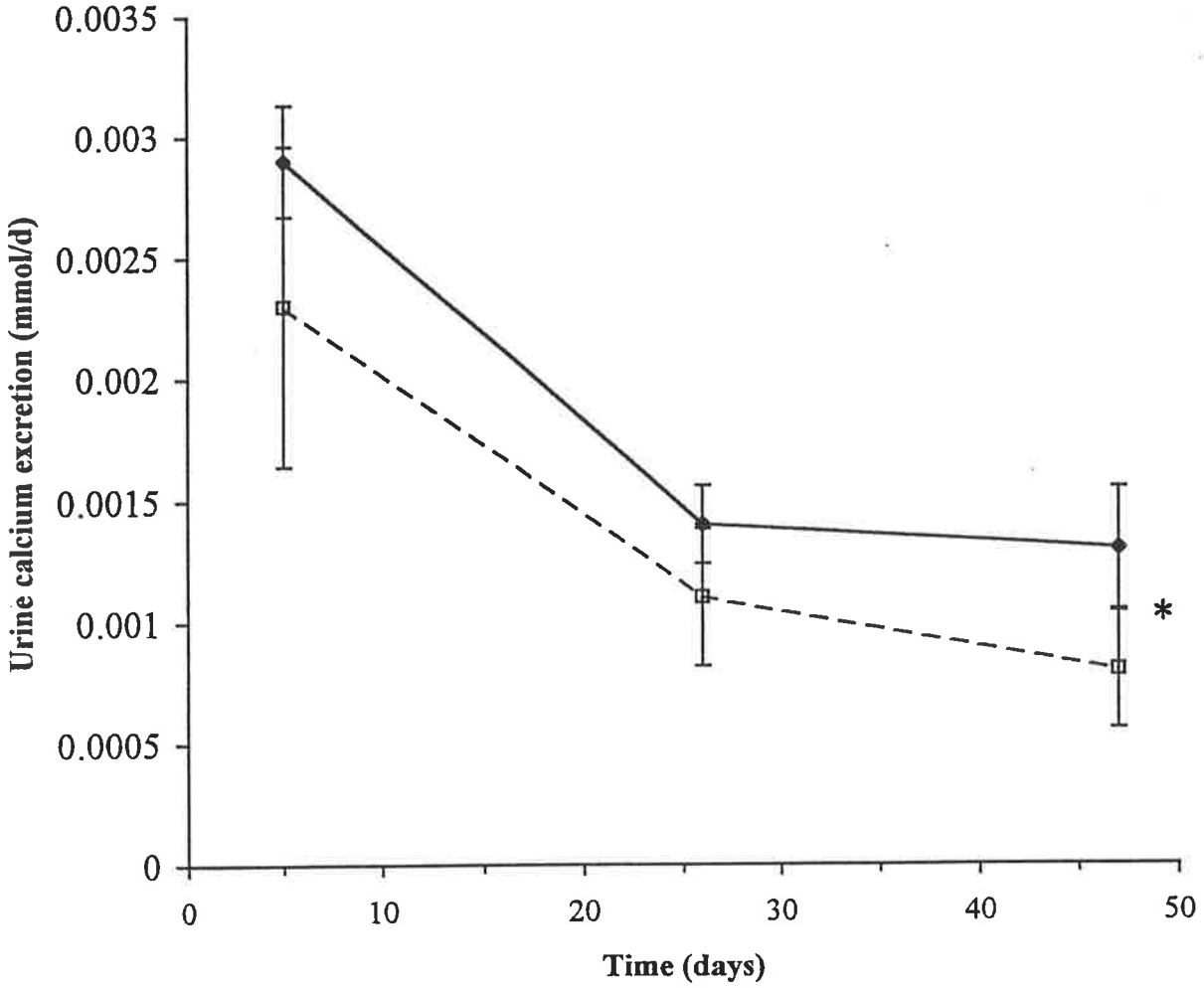

Figure 5.3 Adaptation in urine calcium excretion to dietary calcium restriction in sham operated control —♦— and oophorectomised – – □ – – rats for 47 days after commencement of 0.02% Ca diet. The data are expressed as the mean ± sem for 5 rats. *P<0.001 effect of time after commencement of dietary calcium restriction.

TABLE 5.1 Serum 1,25 dihydroxyvitamin D and PTH before and after dietary calcium restriction or hormone treatment.

		1,25 dihydroxyvitamin D (pmol/l)		PTH (pmol/l)	
	Dietary Ca level	sham	oophx	sham	oophx
Before dietary Ca restriction	0.4%	83 (19.6)	104 (22.9)	6.2 (0.2)	3.4‡‡ (0.6)
After 12weeks on 0.02% Ca	0.02%	140* (7.1)	198*‡ (25.6)	13.9** (1.8)	12.1** (2.6)
Before hormone treatment	0.05%	150 (9.2)	156 (7.0)	13.0 (1.2)	11.5 (1.7)
Post 1,25 dihydroxyvitamin D	0.05%	229# (22)	211# (21)	13.3 (1.6)	11.7 (2.2)
Post oestradiol	0.05%	-	168 (10)	-	25.2## (2.8)

Values are mean(sem) for 5 rats, except 1 data from Mason and Morris (1996), mean(sem) for 4 to 8 rats. *P<0.05 compared with 0.4% dietary calcium, **P<0.01 compared with 0.4% dietary calcium level, #P<0.05 compared with the same dietary calcium level before hormone treatment, ##P<0.01 compared with the same dietary calcium level before hormone treatment, ‡P<0.05 compared with sham for the same dietary calcium level. ‡‡P<0.01 compared with sham for the same dietary calcium level.

relationships for the two operational groups for dietary calcium levels between 0.02% and 0.2% the intercept for the relationship in the oophorectomised rats was 20% higher than for the sham operated rats and highly significant (intercepts: sham +0.25(0.009), oophx +0.3(0.009), $F = 12.8$, $P < 0.001$). There was no effect of oophorectomy on either the slope or the quadratic term

There was a logarithmic relationship between calcium balance and calcium consumption in both the sham and oophorectomised rats (fig 5.4). General linear modelling analysis indicated that the intercept for the oophx group was significantly lower than for the sham group ($P<0.005$). There was no statistically significant difference between the slopes of these equations for the two operation groups. The relationship between net intestinal calcium absorption and calcium consumption was also described by a logarithmic function in both the sham and oophorectomised rats (fig 5.5) with the intercept for the oophx group again significantly lower than for the sham group ($P<0.005$). There was no statistically significant difference between the slopes of these equations for the two operation groups.

The relationship between intestinal calcium secretion and calcium consumption was best described by a logarithmic function and was unaffected by oophorectomy (intestinal calcium secretion = $0.039 + 0.024 \times \log$(calcium consumption), $r^2 = 0.65$. The relationship between urinary excretion of calcium and calcium consumption was also best described by a logarithmic function and was unaffected by oophorectomy (urine calcium excretion = $0.008 + 0.004 \times \log$(calcium consumption), $r^2 = 0.27$)

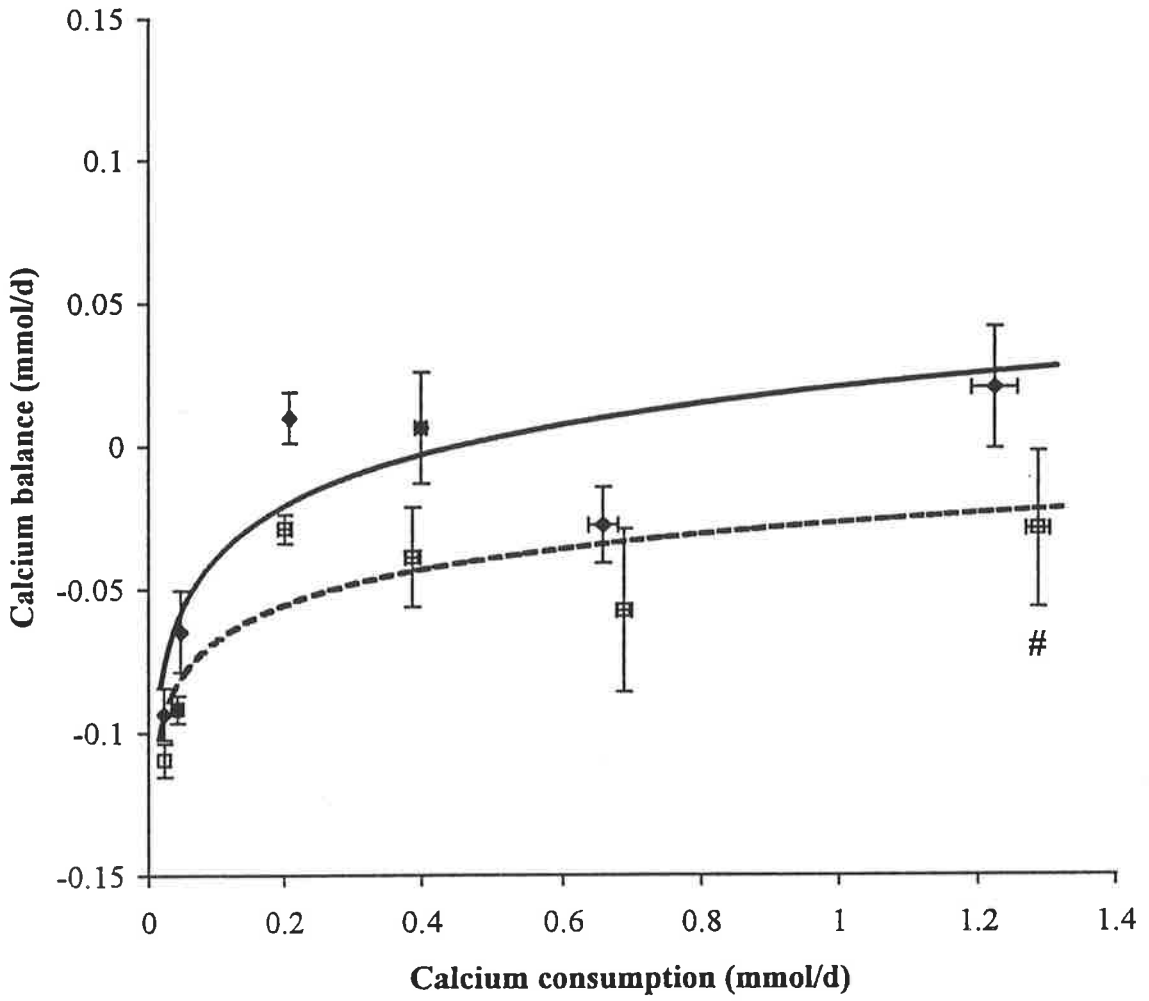

Figure 5.4 The relationship between calcium consumption and calcium balance for sham operated control —♦— and oophorectomised – – □ – – rats fed diets containing between 0.02% and 0.4% Ca. The relationships were described by the following equations:

sham, calcium balance = +0.0204 + 0.059 × log[calcium consumption], $r^2 = 0.319$.

oophx, calcium balance = -0.0269 + 0.0407 × log[calcium consumption], $r^2 = 0.181$.

The data are expressed as the mean ± sem for 5 to 10 rats. #P<0.005 effect of oophx on intercept for the relationship.

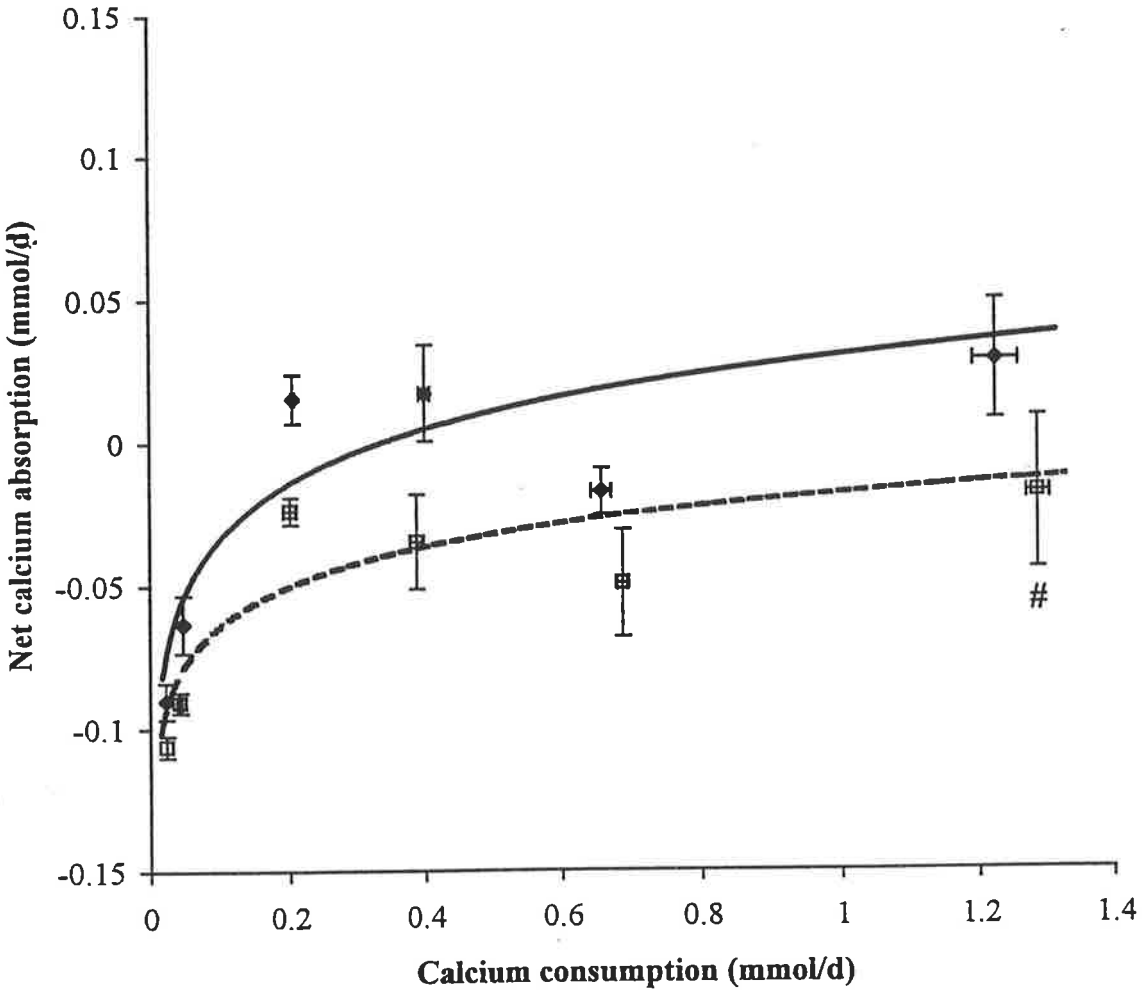

Figure 5.5 The relationship between calcium consumption and net intestinal calcium absorption for sham operated control —♦— and oophorectomised – – □ – – rats fed diets containing between 0.02% and 0.4% Ca. The relationships were described by the following equations:

sham, net calcium absorption = +0.03 + 0.0633 × log[calcium consumption],
$r^2 = 0.371$

oophx, net calcium absorption = -0.0184 + 0.0453 × log[calcium consumption],
$r^2 = 0.219$.

The data are expressed as the mean ± sem for 5 to 10 rats. #P<0.005 effect of oophx on intercept for the relationship.

5.3.3 1,25 dihydroxyvitamin D administration and oestradiol replacement

5.3.3.1 1,25 dihydroxyvitamin D administration

Administration of 1,25 dihydroxyvitamin D significantly increased circulating 1,25 dihydroxyvitamin D levels in sham and oophx rats fed 0.05% dietary calcium, but there was no change in PTH levels (Table 5.1).

True absorbed calcium increased by an equivalent amount in the sham and oophorectomised rats when treated with 1,25 dihydroxyvitamin D and the rise achieved statistical significance in the oophx group (Table 5.2). There was also an increase in urine calcium excretion in both the sham and oophorectomised groups, a significant rise in intestinal calcium secretion in the sham group and a very strong trend for a rise in intestinal calcium secretion in the oophorectomised group (Table 5.2). Consequently, the rise in calcium balance following administration of 1,25 dihydroxyvitamin D did not achieve statistical significance in either sham or oophorectomised rats.

5.2.3.2 Oestradiol replacement

Circulating oestradiol levels in the oophorectomised group following oestradiol replacement were significantly increased compared to untreated sham rats (sham 361(67) pmol/l; oophx+E2 927(211) pmol/l, $t = 2.56$, $P<0.05$). Oestradiol replacement in the oophorectomised group elicited a highly significant rise in true intestinal calcium absorption and calcium balance (Table 5.3). Increases were also observed for intestinal calcium secretion and urine calcium excretion, but these were not statistically significant. Circulating levels of 1,25 dihydroxyvitamin D were unaffected by oestradiol administration in oophorectomised rats when compared to untreated oophorectomised rats or untreated sham

TABLE 5.2 The response of calcium balance and its components to administered 1,25 dihydroxyvitamin D (125D) in sham and oophorectomised rats.

	Sham			Oophorectomy		
	Baseline mean(sem)	+ 125D mean(sem)	Paired t (P)	Baseline mean(sem)	+ 125 D mean(sem)	Paired t (P)
Balance (mmol/d)	0.025 (0.02)	0.051 (0.008)	1.14 ns	-0.03 (0.012)	-0.003 (0.002)	2.07 ns
Net absorption (mmol/d)	0.029 (0.026)	0.06 (0.009)	1.68 ns	-0.026 (0.011)	0.007 (0.003)	1.93 ns
Intestinal calcium secretion (mmol/d)	0.016 (0.0005)	0.031 (0.002)	8.1 (0.0013)	0.02 (0.005)	0.032 (0.001)	2.48 (0.068)
True absorption (mmol/d)	0.042 (0.019)	0.079 (0.008)	1.91 ns	-0.005 (0.009)	0.03 (0.003)	3.18 (0.034)
True absorption (%)	19.1 (8.5)	38.6 (3.3)	2.30 (0.083)	-4.4 (6.84)	18.6 (2.5)	2.83 (0.047)
Urine calcium (mmol/d)	0.0038 (0.0006)	0.010 (0.002)	3.23 (0.032)	0.005 (0.001)	0.01 (0.001)	5.77 (0.005)
Faecal calcium (mmol/d)	0.191 (0.02)	0.144 (0.005)	2.49 (0.068)	0.215 (0.01)	0.18 (0.02)	3.93 (0.017)

n = 5. The raw data not shown are presented in appendix 2

TABLE 5.3 The response of calcium balance and its components to oestradiol (E2) replacement in oophorectomised rats.

	Baseline mean(sem)	+ E2 mean(sem)	Paired t (P)
Balance (mmol/d)	-0.029 (0.011)	0.053 (0.01)	6.89 (0.0023)
Net absorption (mmol/d)	-0.025 (0.011)	0.061 (0.009)	13.9 (0.0002)
Intestinal calcium secretion (mmol/d)	0.015 (0.005)	0.031 (0.013)	1.78 ns
True absorption (mmol/d)	-0.01 (0.014)	0.08 (0.008)	8.25 (0.0012)
True absorption (%)	-5.0 (7.0)	45.8 (4.4)	13.9 (0.0002)
Urine calcium (mmol/d)	0.004 (0.001)	0.008 (0.003)	2.39 (0.075)
Faecal calcium (mmol/d)	0.21 (0.015)	0.11 (0.01)	12.6 (0.0002)

n=5. The raw data not shown are presented in appendix 3.

rats on the same diet (Table 5.1). However, oestradiol stimulated a twofold rise in PTH levels.

5.4 DISCUSSION

Both sham and oophorectomised rats exhibited a significant improvement in calcium balance between 5 and 47 days after the commencement of the 0.02% calcium diet. An increase in net intestinal calcium absorption contributed approximately 97% to this adaptation. Although urine calcium excretion reduced by more than 50% over 47 days on the 0.02% Ca diet, this adaptation contributed only about 2% to the overall improvement in the conservation of whole body calcium observed during this period. The reduction in urine calcium excretion is probably the result of enhanced renal tubular calcium reabsorption resulting from the elevated circulating levels of 1,25 dihydroxyvitamin D and PTH that were observed in rats fed the 0.02% Ca diet. Although both sham and oophorectomised rats were able to adapt to dietary calcium restriction, the oophorectomised rats were unable to achieve the same calcium balance or net absorption as the sham rats at any given time point. The increments of adaptation for calcium balance and net absorption following dietary calcium restriction were equivalent in both sham and oophorectomised rats as were the rises in circulating levels of 1,25 dihydroxyvitamin D and PTH. This finding suggests that the mechanism of adaptation to dietary calcium restriction is not impaired by oophorectomy. However the increased levels of 1,25 dihydroxyvitamin D in the oophorectomised rats were unable to stimulate intestinal calcium absorption to the levels of the sham rats and consequently restore calcium balance to that of the controls.

In young rats, dietary calcium restriction increases circulating PTH, stimulating renal production and increasing circulating levels of 1,25 dihydroxyvitamin D (Rader et al 1979). Dietary calcium restriction also increases active calcium transport which is paralleled by changes in calbindin-D9k in rats up to at least 12 months of age (Armbrecht et al 1979). In the present experiment, adaptation studies were conducted on rats between 11.5 and 13.5 months of age. Our finding of adaptation of intestinal calcium absorption is in contrast to that of Armbrecht et al (1984), who found that rats over 12 months of age increased circulating 1,25 dihydroxyvitamin D, but did not increase calcium absorption in response to a low calcium diet. The difference in the two observations may be explained by methodological differences, with the present study utilising the in vivo calcium balance method, whereas the Armbrecht group (1984) determined calcium absorption in vitro using the everted gut sac technique.

Oophorectomy reduced calcium balance at all levels of dietary calcium. For any given level of dietary calcium the oophorectomised rats excrete more calcium in the faeces compared to ovary-intact rats, a consequence of reduced intestinal calcium absorption. The intercept for the relationship between faecal calcium and dietary calcium consumption was 20% higher in the oophorectomised rats compared to their sham operated controls, but oophorectomy did not affect the slope or the quadratic term for this relationship. This difference between the intercepts was more clearly demonstrated when only the data for balances performed on rats fed diets containing from 0.02% to 0.2% Ca were included in the analysis. Taagehϕj Jensen and co-workers (1983) reported in humans a large coefficient of variation in calcium balance due to small differences between variables involving large amounts of calcium, even though the error on individual measurements from which the calcium balance was calculated

was quite small. Thus, at the highest levels of calcium consumption with a concomitant increase in total faecal calcium, the effect of oophorectomy on this relationship is masked due to higher variance in the estimate of calcium balance. Furthermore, the effect of oophorectomy on calcium balance is likely to be more distinct at lower dietary calcium levels. It is for this reason that the effects of 1,25 dihydroxyvitamin D and oestradiol were determined in rats fed a 0.05% Ca diet.

With 99% of whole body calcium stored in bone, calcium balance is analogous to bone balance. It has been well recognised that dietary calcium restriction decreases bone density in animals (Jowsey & Raisz 1968) and more recently, Shen and co-workers (1995), demonstrated an additive effect on bone loss when calcium deficiency and oestrogen deficiency were combined. They found that calcium deficiency caused bone loss from both cortical and trabecular-rich sites, while oestrogen deficiency caused bone loss from trabecular-rich bone sites only. From the equations describing the relationship between calcium consumption and calcium balance the minimum daily consumption of calcium required to maintain a neutral calcium balance on the AIN-76 starch diet for sham rats is 0.45 mmol/d rising by tenfold for oophorectomised rats to 4.58 mmol/d. It is difficult to extrapolate these findings to other studies using diets of different composition, because components, such as lactose (Greenwald & Gross 1929, Wasserman 1964, Favus & Angeid-Backman 1984), starch (Schulz et al 1993), protein content (Kerstetter & Allen 1994), dietary fibre (Harmuth-Hoene & Schelenz 1980) and other factors affecting calcium bioavailability (Amman et al 1986, Pansu et al 1993), modulate the efficiency of intestinal calcium absorption.

The major factor leading to the more negative calcium balance in the oophorectomised rats was an impairment to net calcium absorption. This is consistent with a previous report that oophorectomy in the rat leads to a reduced net intestinal calcium absorption when dietary calcium is restricted (Kalu et al 1989), although not detected at normal dietary calcium intakes. The present data suggest that calcium absorption is impaired regardless of the level of dietary calcium. However previous studies which have assessed calcium absorption in isolated intestinal segments utilising isolated duodenal loops (Thomas & Ibarra 1987, Thomas et al 1988, Miller et al 1991) and everted gut sacs (Lindgren & DeLuca 1982) have not detected a decrease in calcium absorption following oophorectomy. Several of the isolated duodenal loop studies (Thomas & Ibarra 1987, Thomas et al 1988) actually reported an increase in active calcium transport. A possible explanation for the discrepancy between the balance studies and the isolated duodenal studies may be that the contribution of passive absorption is reduced in the isolated duodenal studies compared to the balance studies where both passive and active calcium absorption are assessed along the entire length of the gut.

Urine calcium excretion was not affected by oophorectomy in the present study, consistent with findings of other studies (Yamazaki & Yamaguchi 1989, Morris et al 1992) and with the finding of only a brief rise in urine calcium excretion, which normalised within 6 weeks of oophorectomy in adult rats (chapter 4, fig 4.4). However, a small but significant rise in the fasting urine calcium excretion for 130 days following oophorectomy in the adult rats has recently been reported (Morris et al 1995). It is of considerable importance to note that the relationship between urine calcium excretion and calcium consumption in the present study was unaffected by oophorectomy even though the efficiency of intestinal calcium

absorption was impaired by oophorectomy. These data indicate that urine calcium excretion by oophorectomised rats was high relative to the amount of calcium absorbed from the diet.

There was no effect of oophorectomy on intestinal calcium secretion in the present study. Calcium balance was not measured until at least 10 wk post-oophorectomy, thus the data remain consistent with the notion of a transient rise in intestinal calcium secretion following oophorectomy as suggested by the findings discussed in sections 3.4 and 4.4.

Our data confirm the previous report that oophorectomy does not reduce circulating 1,25 dihydroxyvitamin D levels (Kalu et al 1989). The impairment to true intestinal calcium absorption that follows oophorectomy is, therefore, not mediated by reduced production of 1,25 dihydroxyvitamin D. In fact after 12wk on the 0.02% Ca diet, 1,25 dihydroxyvitamin D was significantly higher in the oophx group compared to the sham group indicating stimulation of 1,25 dihydroxyvitamin D production. This finding is in sharp contrast with the notion of calcium malabsorption induced by reduced circulating 1,25 dihydroxyvitamin D that has been proposed for postmenopausal women (Gallagher et al 1979). Therefore the impairment to intestinal calcium absorption as a result of oophorectomy despite elevated 1,25 dihydroxyvitamin D levels may have three possible explanations: **1**. that there is an impaired intestinal response to 1,25 dihydroxyvitamin D, **2**. that 1,25 dihydroxyvitamin D active transport is saturated and cannot be further increased or **3**. that the impairment to intestinal calcium absorption that follows oophorectomy is not an impairment to vitamin D-mediated calcium absorption.

In the present study 1,25 dihydroxyvitamin D administration to rats fed a 0.05% Ca diet produced an equivalent increase in intestinal calcium absorption in both sham and oophorectomised groups, indicating no impairment of the response to 1,25 dihydroxyvitamin D at the level of the gut. This finding is inconsistent with the proposal that the oophorectomy-induced impairment to intestinal calcium absorption is the result of down-regulation of vitamin D receptors in the enterocyte (Chan et al 1984, Chen & Kalu 1995). Moreover, this finding indicates that the vitamin D mediated intestinal calcium absorption is not saturated following oophorectomy. Our data suggest that the oophorectomy-induced impairment to intestinal calcium absorption is due to an impairment to a non vitamin D-dependent mechanism of calcium absorption.

In humans the rise in intestinal calcium absorption following 1,25 dihydroxyvitamin D administration was increased in oestradiol replaced oophorectomised women compared with placebo control oophorectomised women, suggesting that oestradiol does influence vitamin D mediated calcium absorption (Gennari et al 1990). In postmenopausal osteoporotic women the relationship between 1,25 dihydroxyvitamin D and fractional calcium absorption had an equivalent slope but lower intercept than the relationship for normal postmenopausal women (Morris et al 1991), indicating that the osteoporotic group had a constant negative bias for intestinal calcium absorption at all levels of circulating 1,25 dihydroxyvitamin D. Thus, for an equivalent incremental rise in circulating 1,25 dihydroxyvitamin D in both normal and osteoporotic women there is an equivalent rise in fractional calcium absorption. These data also suggest that osteoporotic patients demonstrate an impairment to a mechanism of calcium absorption that is not vitamin D dependent. An age related decrease in intestinal responsiveness to 1,25 dihydroxyvitamin D

due to decreased intestinal vitamin D receptor has been reported in normal women (Ebeling et al 1992). Because there was also an age-related rise in 1,25 dihydroxyvitamin D there was no decrease in intestinal calcium absorption.

Although intestinal calcium absorption remained equally responsive to 1,25 dihydroxyvitamin D administration in the oophorectomised rats compared to sham rats, there was not a significant improvement in calcium balance in 1,25 dihydroxyvitamin D treated oophorectomised rats due to the concomitant rises in the excretion of calcium at both the gut and the kidney. This study was performed on a restricted calcium diet and it is possible that significant changes in calcium balance would be achieved with higher dietary calcium.

The administration of oestradiol to oophorectomised rats reversed the impairment in intestinal calcium absorption. Since the rise in intestinal calcium absorption was not brought about by a rise in circulating 1,25 dihydroxyvitamin D and the intestinal response to 1,25 dihydroxyvitamin D is unaffected by oestrogen deficiency, oestradiol appears to be acting directly on the gut to stimulate calcium absorption. This hypothesis is supported by the identification of oestrogen receptors in a non-transformed rat enterocyte cell line (Thomas et al 1993) and 17β-oestradiol has been shown to stimulate ^{45}Ca uptake in isolated rat enterocytes (Arjmandi et al 1993) and in vivo intestinal calcium absorption in rats demonstrated by calcium balance studies (Arjmandi et al 1994).

Unlike the findings with 1,25 dihydroxyvitamin D administration, oestradiol administration caused a marked improvement in calcium balance, with neither intestinal calcium secretion

nor urine calcium excretion rising significantly. This positive calcium balance indicates that the extra calcium absorbed from the diet is mainly accreted to bone, further suggesting that oestradiol also has a direct affect on bone cells shifting the balance between bone formation and bone resorption to achieve net accumulation of mineral. The elevated circulating PTH at this time suggests that serum ionised calcium was reduced concomitant with increased calcium balance. Previous studies have indicated that oestradiol administration of similar regimen to that used in the present study causes a rise in serum calcium (Kalu et al 1991, Arjmandi et al 1994), but there is no change in circulating PTH even though intestinal calcium absorption was stimulated (Arjmandi et al 1994). However, the previous studies were performed on rats fed a calcium sufficient diet (0.4%), whereas the present study used a diet low in calcium (0.05%). The discrepancy between the studies suggests that when dietary calcium is restricted, although calcium absorption efficiency is directly stimulated by oestradiol, the level of calcium absorbed is insufficient for the stimulated rate of accretion of calcium to bone, which results in a fall in serum calcium and a rise in PTH. Further, when dietary calcium is sufficient or in excess oestradiol administration stimulates intestinal calcium absorption above that required for the stimulated rate of calcium accretion to bone and consequently causes a rise in serum calcium.

The present study has demonstrated that oophorectomy in the adult rat decreases calcium balance by decreasing intestinal calcium absorption, however the magnitude of the adaptive response to dietary calcium restriction is unaffected by oophorectomy. Decreased intestinal calcium absorption in the oophorectomised rat is not the result of decreased circulating 1,25 dihydroxyvitamin D or a decreased intestinal response to 1,25 dihydroxyvitamin D, but was

corrected by oestradiol replacement. These findings suggest that oestradiol stimulates a non-vitamin D mediated mechanism of intestinal calcium absorption.

CHAPTER 6

CONCLUSIONS

6.1 Relationship between calcium balance and intestinal calcium absorption

There is a close relationship between calcium balance and intestinal calcium absorption in the rat that is clearly demonstrable through the growth period and continues after skeletal growth ceases (chapters 3 and 5). The nutritional requirement for calcium varies according to the relative activities of bone formation and bone resorption. During periods of growth, calcium balance must be positive to enable accumulation of calcium to bone.

The data presented in this thesis are consistent with a model in which calcium balance and intestinal calcium absorption are controlled in unison, such that variation in the demand for calcium accretion to bone is associated with the efficiency of intestinal calcium absorption. Figure 6.1 shows the very strong relationship, which exists between calcium balance and true calcium absorption in ovary-intact growing young rats (adapted from chapter 3) and in adult rats (adapted from chapter 5). Although the intercepts for the relationships are different, the slopes are similar, such that for a given increase in either calcium balance or absorption there is a similar increase in the other regardless of the age of the rat.

The higher intercept in the young rats for the relationship between calcium balance and true calcium absorption was due to higher levels of calcium excretion via both the gastrointestinal tract and the kidney when compared to adult rats (Table 6.1). However,

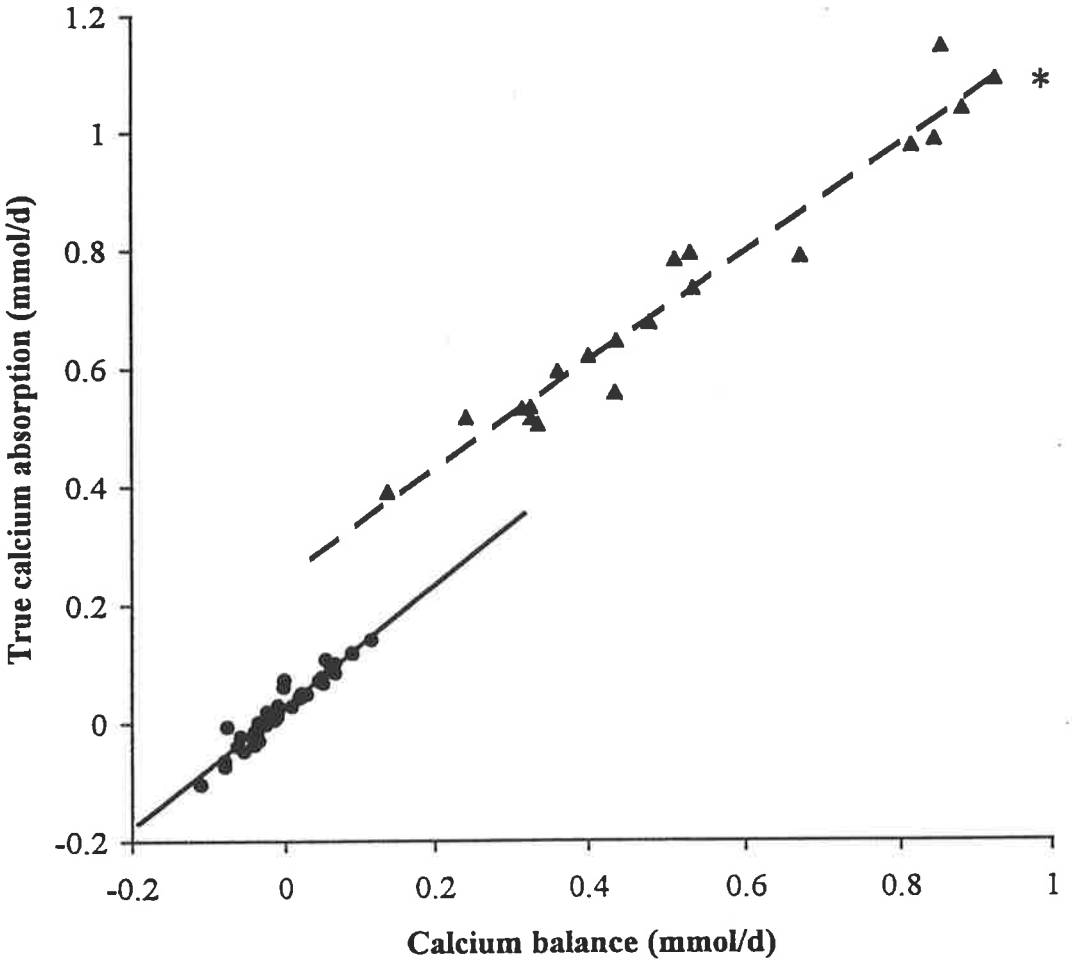

Figure 6.1 The relationship between calcium balance and true calcium absorption for ovary-intact young – –▲– – and adult rats —●—. The relationships were described by the following equations:

young rats fed 0.72% Ca diet, true calcium absorption = + 0.244 + 0.919[calcium balance], $r^2 = 0.95$

adult rats fed 0.05% to 0.4% Ca diet, true calcium absorption = + 0.028 + 1.02[calcium balance], $r^2 = 0.92$

*P<0.0001 young Vs adult rats for the intercept for the relationship.

TABLE 6.1 Pre-operative calcium excretion in young rats fed standard rat chow (table 2.1) and adult rats fed AIN-76A-starch (table 2.3).

	Urine Ca (mmol/d)	Intestinal calcium secretion (mmol/d)
Young rats	0.049(0.012)	0.25(0.036)
Adult rats	0.009(0.0012)	0.028(0.007)

Values are mean(sem) for 5 to 10 rats

there was no indication of an age-related change in calcium excretion between 6 and 15 weeks of age (chapter 3). Increased excretion of calcium in the young rats compared to adult rats may be due to differences in the diets used in the 2 experiments, with young rats fed the non-defined standard rat chow (Table 2.1), whereas the adult rats were fed the AIN-76A-starch diet (Table 2.3). Dietary factors known to cause variation in urine calcium include dietary protein (Bell et al 1975) and phosphorus content (Heaney & Recker 1982) and factors affecting the availability of dietary calcium in the lumen of the intestine probably affect the rate of excretion of calcium of endogenous origin along the intestine by modifying the concentration gradient from the serosal to mucosal sides of the intestinal wall. As the exact composition of the rat chow was not determined in the present study, it was not possible to identify specific differences in the components of the two diets which may have accounted for the differences in calcium excretion.

The factor/s which control calcium absorption and calcium balance in unison have not been fully elucidated. The calciotropic hormones are intricately linked with both processes and are probable candidates for the coupled control of calcium absorption and calcium balance, but other hormones such as oestradiol also affect both calcium absorption (Arjmandi et al 1994) and oophorectomy affects bone balance at specific sites (Wronski et al 1985, Kalu et al 1989, Wronski et al 1989, Yamazaki & Yamaguchi 1989).

The age-related reduction in calcium balance as growth diminishes is associated with the age-related reduction in intestinal calcium absorption, in support of hypothesis 1 (section 1.7). Furthermore, there is no age-related change in the excretion of calcium via the gastrointestinal tract or the kidney in young growing rats (chapter 3). It is not surprising

that during the growth period the supply of calcium from the diet is very tightly coupled with the rate of accretion of calcium to bone. Thus, as growth diminishes so does intestinal calcium absorption. It has previously been demonstrated that 18 month old animals have a decreased ability to maintain a positive calcium balance compared to 6 week old animals (Armbrecht et al. 1981), and a reduction in intestinal calcium absorption through the growth period has been demonstrated using everted gut sacs (Horst et al 1978, Armbrecht et al 1979) and perfused duodenal loops (Pansu et al 1983a).

The present study is the first to measure calcium balance and its components by metabolic balance studies through the growth period to identify the relative contribution of reduced intestinal calcium absorption to reduced calcium balance as growth rate diminishes. Furthermore, calcium balance studies measure total calcium absorption, that is, both saturable (active) and non-saturable (passive) calcium absorption along the entire length of the intestine, in contrast to the commonly used techniques for measuring absorption such as perfused duodenal loops (Wood et al 1988) and everted gut sacs (Horst et al 1978), which assess calcium absorption in an isolated segment of the intestine and do not mimic the conditions of absorption of dietary calcium.

Circulating levels of 1,25 dihydroxyvitamin D were not measured in young rats in the present study, but have been demonstrated to decline in young rats between 4 and 13 weeks of age (Clark et al 1986) in much the same way that calcium balance and true calcium absorption both declined between 6 and 15 weeks of age in the present study (chapter 3). 1,25 dihydroxyvitamin D is regarded as the major regulator of intestinal calcium absorption (Sheikh et al 1988). During this period of diminishing growth the levels of the intestinal

vitamin D-dependent protein, calbindin-D9k also decline in a similar manner (Armbrecht et al 1979, Armbrecht et al 1989). Enterocyte vitamin D receptor number is also reduced in old rats (>16 months of age) compared to young rats (1 month of age) (Horst et al 1990), but no change in receptor number through the period of rapid growth has been reported. Thus it appears that the major cause of reduced calcium absorption during this period of rapid, but constantly diminishing growth is the decline in circulating 1,25 dihydroxyvitamin D.

Two possible mechanisms can be proposed for a 1,25 dihydroxyvitamin D mediated coupling of calcium balance and intestinal calcium absorption, consistent with the age-related falls observed in each of these variables: **1**, simply, the age related fall in 1,25 dihydroxyvitamin D per se reduces calcium absorption efficiency, thus limiting bone growth, or **2**, bone growth diminishes due to other factors such as the age-related reduction in growth hormone in rats (Millard et al 1986), so that as the flux of extracellular fluid calcium into bone decreases, the production of 1,25 dihydroxyvitamin D is either directly or indirectly inhibited, ultimately leading to reduced intestinal calcium absorption. There is no change in circulating PTH levels between the ages of 1.5 and 6 months (Kalu et al 1984b). Regardless of the mechanism, 1,25 dihydroxyvitamin D appears to be an important factor in the coupling of calcium balance and intestinal calcium absorption in growing rats.

Urine calcium excretion did not change with age in the present study, suggesting that reported age-related changes in 1,25 dihydroxyvitamin D (Clark et al 1986) did not affect renal tubular handling of calcium in young rats. The control of renal tubular reabsorption of calcium is complex and the interrelationship between 1,25 dihydroxyvitamin D and PTH in

controlling this reabsorption is not fully understood. PTH has been shown to be a potent stimulator of calcium reabsorption along the distal convoluted tubule (Shareghi & Stoner 1978, Costanzo & Windhager 1980). It has also been demonstrated, that vitamin D depletion is associated with increased renal clearance of calcium in rats (Costanzo et al 1974) but the association does not exist in thyroparathyroidectomised, vitamin D replete rats (Hugi et al 1977). More recently it has been demonstrated that vitamin D depletion in rabbits reduces calcium uptake in distal tubular basolateral and luminal membrane vesicles (Bouhtiauy et al 1993). The finding of constant urine calcium excretion through the growth period in the present study suggests that there is no relationship between circulating 1,25 dihydroxyvitamin D and renal tubular reabsorption of calcium. As well, the lack of an age-related change in urine calcium excretion is consistent with the notion that circulating PTH, which does not change between the ages of 1.5 and 6 months (Kalu et al 1984b) is the major determinant of renal tubular calcium reabsorption.

Observations in adult rats are also consistent with a tight coupling between intestinal calcium absorption and calcium balance in which circulating levels of 1,25 dihydroxyvitamin D play an important role. Both calcium balance and net calcium absorption were related to calcium consumption in a similar manner. Dietary calcium restriction in the adult rat led to reduced calcium balance, but over a period of 47 days, as shown in the present study, a process of adaptation which increased the efficiency of intestinal calcium absorption, also enhanced calcium balance. Dietary calcium restriction was also associated with higher circulating levels of PTH and 1,25 dihydroxyvitamin D (chapter 5), consistent with previous demonstrations in young rats that dietary calcium restriction increases circulating PTH, stimulating renal production and increasing circulating levels of 1,25

dihydroxyvitamin D (Rader et al 1979), which stimulates enterocyte levels of calbindin-D9k in rats up to at least 12 months of age (Armbrecht et al 1979).

Further evidence for a close coupling between intestinal calcium absorption and calcium balance come from studies of the effects of dietary calcium intake on bone mass. In humans, high calcium intake is associated with a large bone mass (Matkovic et al 1979) even though approximately 80% of an individuals bone mass is controlled genetically (Dequeker et al 1987, Pocock et al 1987, Slemenda et al 1991). Further, Peck and co-workers (1988) suggested that because gastrointestinal calcium absorption is reduced with age, older persons need to increase calcium intake to maintain the level of calcium absorption required to maintain normal bone.

Dietary calcium intake has also been identified as a determinant of peak bone mass in laboratory rats. Low dietary calcium intake during adolescence reduces peak bone mass, whereas high dietary calcium intake promotes higher peak bone mass, providing potential protection against age-related bone loss (Peterson et al 1995).

6.2 The effect of oophorectomy on calcium balance and its components

Oophorectomy led to a reduction in calcium balance in both young and adult rats, in support of hypothesis 2 (section 1.7), indicating that the site-specific reductions in bone following oophorectomy reflect a reduction in calcium throughout the entire skeleton in both young and old rats. In young rats, oophorectomy led to less positive calcium balance, whereas oophorectomy in adult rats led to a more negative calcium balance. The major

cause of reduced calcium balance in the short term following oophorectomy was a transient increase in calcium excretion following oophorectomy in young and old rats until 6 weeks and 3 weeks respectively. In the young rat, increased excretion was only observed in the gastrointestinal tract (chapter 3), while in the adult rat excretion of calcium was increased via both the kidney and the gastrointestinal tract (chapter 4). The transient rise in excretion of calcium may reflect the time following oophorectomy when bone is being lost at the maximum rate. Reduced calcium balance was observed in adult rats within the shortest time frame assessed in the present study (21 days). In young rats the effect of oophorectomy on calcium balance was not detected until 42 days post-oophorectomy. However, in the young growing rats calcium balance was constantly changing as they approached full size, and therefore it was more difficult to assess the effects of oophorectomy on calcium balance. It is possible that changes in calcium balance earlier than 42 days post-oophorectomy may not have been detected.

Oophorectomy also reduced intestinal calcium absorption in both young and adult rats, which became more evident at later times following oophorectomy when the transient rises in calcium excretion returned to levels similar to those observed in sham rats (chapters 3 and 5). Thus, apart from the brief periods of raised calcium excretion following oophorectomy in adult rats the reduction in calcium balance was associated with reduced intestinal calcium absorption (chapter 5).

There was a strong relationship between calcium consumption and calcium balance in both sham and oophorectomised rats. The relationships differed in that oophorectomised rats had a significantly lower intercept compared to sham rats, supporting hypothesis 3 (section 1.7),

which proposed that oophorectomy reduces calcium balance at all levels of dietary calcium intake. Thus the level of dietary calcium required to maintain neutral calcium balance increases from 0.45 mmol/d for sham rats to 4.58 mmol/d for oophorectomised rats.

As discussed in 6.1, dietary calcium restriction led to an adaptive process over a period of 47 days in which intestinal calcium absorption was increased and urine calcium excretion was reduced with a consequent increase in calcium balance (chapter 5). However, intestinal calcium absorption and calcium balance always remained lower in oophorectomised rats compared to sham rats. The mechanism of adaptation involved higher circulating levels of PTH and 1,25 dihydroxyvitamin D, supporting hypothesis 3 (section 1.7). The circulating levels of these hormones after 12 weeks of dietary calcium restriction was not lower in oophorectomised rats compared to sham rats and the magnitude of adaptation in intestinal calcium absorption, urine calcium excretion and calcium balance was equivalent in both operational groups contrary to the suggestion in hypothesis 3 (section 1.7) that oophorectomy reduces the adaptive response to dietary calcium restriction.. These findings indicate that oophorectomy did not affect the calciotropic hormone response to dietary calcium restriction, nor did it impair the renal response to the calciotropic hormones or the magnitude of the intestinal response to 1,25 dihydroxyvitamin D.

Oophorectomy did not affect the slope of the relationship between calcium balance and intestinal calcium absorption (fig 6.2). In young rats, oophorectomy caused an upward shift in the intercept for the relationship at 3 and 6 weeks post-operation, when calcium excretion was raised compared to sham rats. The shift in the intercept was relatively small, because the rise in intestinal calcium secretion was less than 0.5-fold above sham operated controls

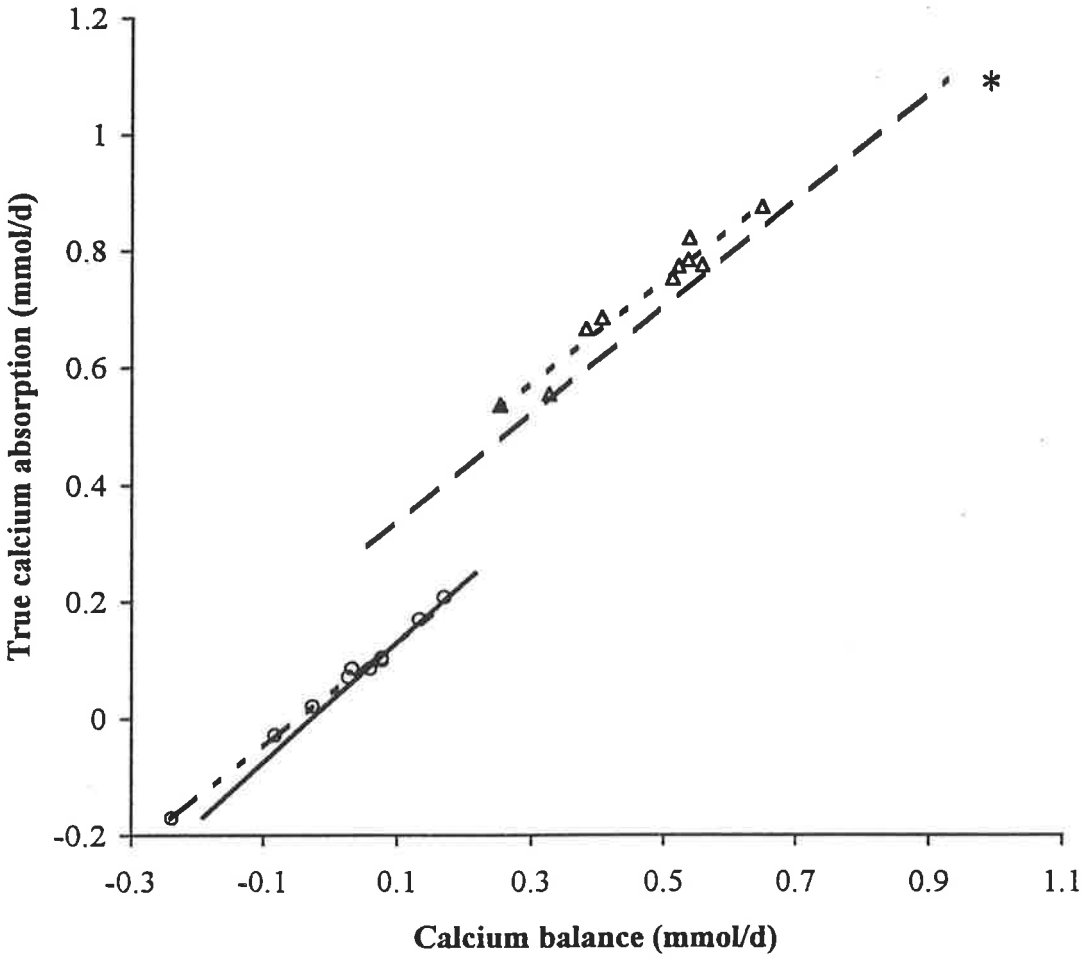

Figure 6.2 Comparison of the relationship between calcium balance and true calcium absorption for ovary-intact young – – – – and adult rats ——— with the relationships between calcium balance and true calcium absorption during the periods of raised levels of calcium excretion for young oophorectomised rats fed 0.72% Ca diet at 3 and 6 weeks post-oophorectomy - - Δ - - and adult oophorectomised rats fed 0.4% Ca diet at 3 weeks post-oophorectomy – - - -O- - - -. The relationships were described by the following equations:

young rats, true calcium absorption = + 0.302 + 0.9[calcium balance], $r^2 = 0.96$

adult rats, true calcium absorption = + 0.044 + 0.9[calcium balance], $r^2 = 0.99$.

*P<0.0001 young Vs adult rats for the intercept for the relationship.

and there was no increase in urine calcium excretion. In adult rats, an upward shift in the intercept was not observed in the oophorectomised group at 3 weeks post-operation when calcium excretion was raised compared to sham rats, but the data for intestinal calcium secretion and urine calcium excretion, although significantly higher at this time, were quite variable. To put these observations into perspective, the significant upward shift in the intercept for the relationship between intestinal calcium absorption and calcium balance in young compared to adult rats (fig 6.1) was due to a 5-fold higher urine calcium excretion and a 9-fold higher intestinal calcium secretion (Table 6.1).

When the transient oophorectomy-induced rises in calcium excretion had normalised in young and adult rats, the relationship between calcium absorption and calcium balance was not different from that observed in ovary-intact rats. Thus, the long-term reduction in intestinal calcium absorption following oophorectomy reduced calcium balance in a manner consistent with the relationship observed between these variables in ovary-intact rats. This finding suggests that oophorectomy causes a quantum reduction in calcium absorption and calcium balance that is not mediated by the calciotropic hormones. In this model calcium balance can be increased only if calcium absorption is stimulated without a concomitant increase in calcium excretion.

The time course for the development of reduced calcium absorption following oophorectomy is not clear from the data obtained in the present study. An effect of oophorectomy on intestinal calcium absorption was demonstrated in young rats fed 0.72% calcium at 9 weeks after operation (fig 3.4). The oophorectomised adult rats fed 0.4% calcium appeared to have a more negative change in calcium absorption within 3 weeks of

oophorectomy (figs 4.2 and 4.3), but did not reach statistical significance at this particular time point. However, the relationship between net calcium absorption and calcium consumption in adult rats indicated reduced absorption in oophorectomised adult rats at least 10 weeks after operation (fig 5.5). Calcium absorption, like calcium balance is calculated as a small difference between variables involving large amounts of calcium and is thus subject to a high interindividual variation (Taagehϕj Jensen et al 1983), particularly at higher levels of dietary calcium intake. The assessment of the relationships between calcium consumption and intestinal calcium absorption (fig 5.5) demonstrated that there was a better discrimination between sham and oophorectomised rats when calcium consumption was approximately 0.4mmol/d (0.15% Ca diet) or less. Although an effect of oophorectomy on intestinal calcium absorption was not demonstrated until 9 weeks or 10 weeks after oophorectomy in the young and old rats respectively, it is possible that intestinal calcium absorption was reduced before this time. It would be valuable to reassess the short term effects of oophorectomy on calcium absorption in young and old rats with rats fed lower dietary calcium levels to clarify the time of onset of oestrogen-deficiency induced calcium malabsorption.

Since the major contributor to reduced calcium balance in the long term in adult rats was reduced intestinal calcium absorption, it suggests that direct stimulation of calcium absorption would lead to increased calcium balance. However, administration of 1,25 dihydroxyvitamin D to oophorectomised rats while stimulating intestinal calcium absorption also resulted in a concomitant increase in urine calcium excretion with a trend for increased intestinal calcium secretion, such that the rise in calcium balance was not statistically significant. There was no effect of 1,25 dihydroxyvitamin D administration on circulating

levels of PTH. It is therefore important to note that stimulation of intestinal calcium absorption by 1,25 dihydroxyvitamin D treatment was insufficient to overcome the impairment to the oestradiol deficiency-induced reduction in calcium balance. These findings do not support hypothesis 4 (section 1.7), which proposed that although the response to administered 1,25 dihydroxyvitamin D would be impaired in oophorectomised rats compared to sham rats, intestinal calcium absorption would be stimulated, leading to increased calcium balance.

Oestradiol replacement to oophorectomised rats was a potent stimulus for increased intestinal calcium absorption and led to a marked increase in calcium balance, in support of hypothesis 4 (section 1.7). There was also a marked rise in circulating levels of PTH, which probably stimulated renal tubular reabsorption of calcium and thus prevented a marked increase in urine calcium excretion. As discussed in chapter 5 the elevated circulating PTH at this time suggests that serum ionised calcium was reduced concomitant with increased calcium balance and that the oestradiol-stimulated rate of accretion of calcium to bone during this short-term oestradiol replacement dominated the normally tight regulation of ECF ionised calcium by the calciotropic hormones. Previous studies have demonstrated that oestradiol administration to rats fed 0.4% Ca diet increased serum calcium (Kalu et al 1991, Arjmandi et al 1994). The restricted calcium diet (0.05%) used in the present study appears to have limited the rate of intestinal calcium absorption such that the induction of calcium accretion to bone produced a fall in ionised calcium, with the observed elevation of circulating PTH.

With oestradiol administration to oophorectomised rats in the present study there was no rise in circulating 1,25 dihydroxyvitamin D, although intestinal calcium absorption was markedly stimulated, suggesting that oestradiol stimulates intestinal calcium absorption directly. These data are consistent with the previous report in ovary-intact rats that 17β-oestradiol stimulated net intestinal calcium absorption without increasing circulating 1,25 dihydroxyvitamin D (Arjmandi et al 1994).

The data presented in this thesis, while consistently demonstrating and effect of oestradiol on intestinal calcium absorption do not identify the mechanism by which this occurs. Oestradiol receptors have, however, been identified in a non-transformed rat enterocyte cell line (Thomas et al 1993) and oestradiol-stimulated ^{45}Ca uptake in isolated rat enterocytes suggests that the mechanism involves transcellular calcium absorption (Arjmandi et al 1993). Although oestradiol stimulated calcium absorption without causing a rise in circulating 1,25 dihydroxyvitamin D, this does not eliminate the possibility of an interaction of oestradiol with vitamin D mediated calcium absorption at the level of the enterocyte, such as enhancing the calbindin-D9k response to 1,25 dihydroxyvitamin D. Alternatively, oestradiol may stimulate calbindin-D9k directly. It is, however, also possible that oestradiol stimulated passive calcium absorption. Previous studies have demonstrated an increase in duodenal weight per unit length following oophorectomy, which may alter the characteristics of passive, paracellular calcium absorption (Miller et al 1991). Future studies in this laboratory to determine the mechanism of action of oestradiol on calcium absorption will assess the effect of oophorectomy and oestradiol replacement to oophorectomised rats on calbindin-D9k protein and mRNA and vitamin D receptor protein and mRNA in duodenal enterocytes.

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Appendix 1 The components of calcium balance between 6 and 15 weeks of age in sham and oophorectomised rats.

Age (weeks)		6	9	12	15
Calcium consumption (mmol/d)	sham	2.25(0.012)	2.39(0.019)	2.37(0.021)	2.35(0.001)
	oophx	2.24(0.016)	2.42(0.002)	2.42(0.003)	2.35(0.001)
Urine calcium excretion (mmol/d)	sham	0.049(0.012)	0.038(0.007)	0.038(0.007)	0.047(0.006)
	oophx	0.036(0.006)	0.037(0.004)	0.033(0.003)	0.037(0.004)
Faecal calcium excretion (mmol/d)	sham	1.34(0.03)	1.84(0.05)	1.91(0.03)	2.08(0.04)
	oophx	1.3(0.04)	1.83(0.02)	2.0(0.05)	2.14(0.03)
Net calcium absorption (mmol/d)	sham	0.91(0.022)	0.55(0.047)	0.46(0.034)	0.28(0.034)
	oophx	0.94(0.026)	0.59(0.024)	0.41(0.048)	0.21(0.028)
True calcium absorption (%)	sham	46.4(1.5)	29.9(1.5)	26.3(1.8)	22.6(1.1)
	oophx	48.1(1.4)	32.6(0.9)	26.9(2.1)	21.2(1.2)
Calcium balance (mmol/d)	sham	0.86(0.019)	0.51(0.05)	0.42(0.03)	0.27(0.036)
	oophx	0.90(0.027)	0.55(0.024)	0.38(0.047)	0.22(0.029)

n = 5

Appendix 2 The components of calcium balance before and after 1,25 dihydroxyvitamin D (125D) in sham and oophorectomised rats.

	Sham		Oophorectomy	
	Baseline mean(sem)	+ 125D mean(sem)	Baseline mean(sem)	+ 125 D mean(sem)
Ca consumption (mmol/d)	0.22 (0.001)	0.20 (0.004)	0.19 (0.02)	0.19 (0.018)
Urine ^{45}Ca (KBq/d)	13.5 (1.91)	17.8 (3.2)	10.6 (1.24)	16.4 (2.8)
Faecal ^{45}Ca (KBq/d)	48.5 (5.72)	36.7 (3.1)	47.5 (6.13)	43.8 (3.5)

n = 5.

Appendix 3 The components of calcium balance before and after oestradiol (E2) replacement in oophorectomised rats.

	Baseline mean(sem)	+ E2 mean(sem)
Ca consumption (mmol/d)	0.184 (0.009)	0.168 (0.014)
Urine ^{45}Ca (KBq/d)	9.5 (0.84)	15.3 (1.48)
Faecal ^{45}Ca (KBq/d)	38.5 (1.51)	30.3 (1.32)

n=5.