GLUCOCORTICOIDS, VITAMIN D AND BONE. A PATHOPHYSIOLOGIC AND THERAPEUTIC STUDY OF GLUCOCORTICOID-INDUCED BONE DISEASE.

GLUCOCORTICOIDS, VITAMIN D AND BONE.

A PATHOPHYSIOLOGIC AND THERAPEUTIC STUDY OF GLUCOCORTICOID-INDUCED BONE DISEASE.

Glucocorticoiden, vitamine D en botweefsel.
Pathophysiologische en therapeutische aspecten van glucocorticoid-osteoporose.

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. M. W. VAN HOF
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 23 MEI 1984 TE 15.45 UUR.

DOOR

Jacob Johannes Braun
geboren te Dordrecht

Promotor: Prof. Dr. J. C. Birkenhäger

Promotiecommissie: Prof. Dr. R. O. van der Heul
Prof. Dr. W. C. Hülsmann
Prof. J. H. P. Wilson

The support from Leo, Pharmaceutical Products, Holland for the publication of this manuscript is gratefully acknowledged.

Aan Birgit
Annemarie, Saskia en Hanneke
Aan mijn moeder
ter herinnering aan mijn vader

CONTENTS

LIST OF ABBREVIATIONS

BBMV: brush border membrane vesicles
BMU: basic multicellular unit
cAMP: cyclic adenosine 3^1, 5^1-monophosphate
CT: calcitonine
GC: glucocorticoid(s)
HP: hypoparathyroidism
(i)PTH: (immunoreactive) parathyroid hormone
PHP: primary hyperparathyroidism
TA: triamcinolone-acetonide
25-(OH)D: 25-hydroxyvitamin D
1,25-$(OH)_2$D: 1,25-dihydroxyvitamin D
1α-$(OH)D_3$: 1-alpha-hydroxyvitamin D_3
24,25-$(OH)_2$D: 24,25-dihydroxyvitamin D

Definitions in relation to intestinal calcium absorption studies:
Ca-efflux: unidirectional movement of calcium from the intestinal lumen to tissue.
Ca-influx: unidirectional movement of calcium from tissue into the intestinal lumen.

CHAPTER I

Introduction and objectives

Mooser's report (1921) of a patient with osteoporosis and obesity is one of the first descriptions of endogenous hypercortisolism. Later Cushing (1932) described the syndrome as a clinical entity and Albright et al. (1941) reported 9 patients who at autopsy all appeared to have sustained a severe degree of cortical and trabecular bone loss. In 1949 glucocorticoids (GC) became available as therapeutic agents. In addition to their favourable antiinflammatory action deleterious side effects (e.g. on bone mass) have become apparent (Kjellstrand, 1975).

A diminished bone mass, measured as trabecular bone volume of iliac crest biopsies (Birkenhäger et al., 1967) or by total body Ca with neutron activation analysis (Aloia et al., 1974), is a typical hallmark in patients with *endogenous hypercortisolism*. The impact of *exogenous GC excess* on bone mass is more difficult to evaluate. In the quantification of the amount of bone in such patients by several techniques one has to take into account not only the physiological age related bone loss (see Chapter II), but also the underlying disease for which GC are prescribed. This is illustrated by Stresemann & Krokowski (1967) who found signs of a decreased bone mineral content on X-rays of the vertebral column (using a quantitative measurement technique) in 32% of patients treated with varying doses of GC for chronic obstructive lung disease, but similar signs were observed in an equal percentage of patients who never had used steroids. However, Adinof & Hollister (1983) described a significant loss of trabecular bone mass at the metaphysis of the radius with single photonabsorptiometry only in asthmatic subjects on long-term daily steroids. Measurement of total body Ca with neutron activation analysis showed lower values in patients with rheumatoid arthritis treated with non steroidal antiinflammatory drugs than controls, but even lower values in rheumatic patients treated with steroids (Reid et al., 1982). Similar findings were reported by Hahn et al. (1973) with photonabsortiometry of the metaphyseal mass of the forearm. The decreased mineral content was correlated with the duration of GC therapy.

Trabecular bone is specially vulnerable for the deleterious effects of GC. Using single photonabsorptiometry of the proximal part of the radius alone (Gluck et al., 1981) or combined with dual photoabsorptiometry of the lumbar spine (Seeman et al., 1982), patients with exogenous or endogenous hypercortisolism have been found to have low density values at these sites. In a retrospective evaluation of iliac crest biopsies Bressot et al. (1979) found trabecular bone volumes of less than 11% of total bone volume in 42 of 62 patients chronically treated with GC.

Extensive studies of physiologic and pathologic loss of bone mass have failed to provide a unifying concept for the pathogenesis of the common forms of osteoporosis. The pathophysiology of the osteoporosis which developes during GC excess is also not clear. We therefore decided to study the relationships between GC, factors thought to be involved in bone mineral homeostasis and the target organs of such factors. We investigated the effects of GC on:

1. vitamin D activation and metabolism,
2. the secretion and activity of parathyroid hormone (PTH),
3. the mechanism by which GC decrease intestinal Ca absorption and
4. the possible interrelation between these processes.

Active vitamin D metabolites have recently become available for clinical administration. The findings of this study therefore could have practical implications for the prophylaxis of GC induced osteoporosis.

We looked at the influence of short-term GC excess on vitamin D metabolism in man at several levels of parathyroid activity (Chapter V). The mutually antagonistic effects of GC and vitamin D on intestinal Ca absorption has been studied on a subcellular level in rat intestinal brush border membrane vesicles (BBMV) and in the in situ ligated gut loop (Chapters VI and VII). Finally, in line with a concept of the pathophysiology of GC induced bone disease as outlined in Chapter IV, a double-blind placebo-controlled trial with 1α-$(OH)D_3$ is described, which we performed in patients on long-term corticosteroid treatment. The patients were matched for age, sex, dose of GC and underlying disease (Chapter VIII). In Chapters II and III the physiology and pathology of the skeletal mass in relation to Ca homeostasis is discussed. Special emphasis is given to the regulation of vitamin D activation and the effects of vitamin D on gastrointestinal Ca absorption.

CHAPTER II

Physiology and pathology of skeletal mass.

2.1 *Physiological changes*

Skeletal weight increases during human life in both sexes with equal velocity up till the pubertal growth spurt. During this period of accelerated growth boys gain more bone, in concert with lean body mass, than girls (Garn, 1981). Beyond the age of 35 years the weight of the skeleton decreases gradually; this physiologic process starts somewhat earlier and progresses faster in females than in males. In 1960 Trotter et al., studying postmortal skeletons, described in a cross-sectional study an age related loss of bone density. Besides this aging process the influence of racial factors, socio-economic status, food composition and physical activity on the weight and density of the skeleton is probably important, but not always easy to assess. Some aspects will be discussed later in this Chapter in relation to the pathophysiology of primary osteoporosis.

2.2 *Non-invasive measurements of bone (mineral) mass.*

Age related changes in total body Ca, 99% of which is located in the skeleton, can be measured in vivo by means of *neutron activation* analysis (Cohn et al., 1976), but this technique is not widely available. As a substitute for the measurement of skeletal mass (that is the total amount of bone mineral) *radiographic morphometry*, measuring for example cortical area, has been used in large cross-sectional population surveys (Garn et al., 1967). With this and other techniques such as *single photonabsorptiometry* of the radius the sex difference and the bone loss with age have been documented (Smith et al., 1975) for the amount of cortical bone in those locations. In normal subjects the amount of cortical bone in general correlates quite well with the total skeletal (mineral) mass (Manzke et al., 1975; Horsman et al., 1983). However, in osteoporotics changes in these parameters do not need to correlate (Aloia et al., 1975). *Dual photonabsorptiometry* and *computer assisted*

tomography can provide data with regard to the amount of mineral in the vertebrae (usually the lumbar ones), which largely consist of trabecular bone. Also with these techniques age related bone loss is apparent on cross-sectional surveys (Riggs et al., 1981; Cann & Genant, 1982) and in a longitudinal study Krølner & Nielsen (1982) found evidence for an accelerated loss of lumbar bone mineral content in postmenopausal women.

2.3 *Histomorphometry, the concept of the basic multicellular unit (BMU).*

Krølners findings are in agreement with the increased rate of trabecular bone loss in women during five years following the menopause, as derived from histomorphometric analysis of bone from iliac crest biopsies (Meunier et al., 1973). Histomorphometry of bone obtained from the iliac crest, as part of the central skeleton, may not only provide data on trabecular and cortical bone volume, but also on static and dynamic (tetracycline labelling) parameters of bone formation and resorption (Bordier & Tun Chot, 1972). After skeletal growth is completed, the mass and shape of the normal adult skeleton is determined by a process of balanced bone resorption and formation called remodeling. According to the theory of Frost, as recently reviewed by Jaworski (1981), bone formation and resorption are coupled in the form of so-called BMU's activated at the three bone envelopes: periosteal, Haversian and endosteal. Osteoclastic bone resorption at one site is, via a reversal phase, systematically followed by recruitment of osteoblasts and deposition of bone matrix. Mineralization of osteoid completes the remodeling cycle leaving osteocytes embedded in the calcified bone, while the bone surface is covered with (resting) lining cells. The activation frequency of BMU's in bone determines the turnover of the total skeletal mass. The balance between the amount of bone resorbed and formed at each of the BMU's determines whether locally, skeletal mass is gained or lost (remodeling balance). The change in the total amount of bone for the whole skeleton is determined by the sum of remodeling balances of the BMU's at the three bone envelopes, while the rate of change of bone mass is determined by the activation frequency of BMU's. On theoretical grounds a net loss of bone mass, as occurs during aging, can be the result of increased resorption, decreased formation or a combination of both. At the cellular level quantitative or qualitative changes of bone resorbing and bone forming cells can be responsible. An increase in bone turnover rate is initiated by stimulation of bone resorption, for instance by one of the regulators of bone mineral homeostasis as discussed later in Chapter III. In the initial phase of certain metabolic bone diseases, when increased bone resorption is not (yet) accompanied by increased bone formation, this increased "remodeling space" results in a temporary loss of bone mass (primary hyperparathyroidism, hyperthyroidism).

2.4 *Osteopenia and osteoporosis*

When the quantity of bone is too small for age, sex and body size, while the composition of its constituents is grossly normal (in the sense that there is no mineralization defect), the term osteopenia has been applied. Newton-John &

Morgan (1970) defined osteopenia arbitrarely as a bone quantity less than 2 standard deviations below the mean value reached at maturaty. With this definition one does not take into account the physiologic bone loss with increasing age, or the fact that certain parts of the skeleton are more liable to lose bone than others, resulting in vertebral crush fractures and fractures of the ribs, forearm and proximal part of the femur. When, as a result of decreased resistance to stress, these fractures occur without a so-called adequate trauma (pathologic fractures), the term osteoporosis is generally used. According to Courpron et al. (1976) patients with pathologic vertebral fractures had histomorphometrically iliac crest trabecular bone volume percentage values below 11% (vertebral fracture threshold).

In Table 2.1 some clinical situations are listed that may be associated with less than the normal amount of bone. Whether patients with so-called primary osteoporosis represent the lower part of a normal distribution curve, as suggested by Newton-John et al. (1970), or have lost and will lose bone more rapidly than normal people of the same age and sex (Doyle, 1972), is still a matter of debate.

Table 2.1

CAUSES OF OSTEOPOROSIS

I SECONDARY	d. gastrointestinal tract diseases - stomach resection - malabsorption syndrome - chronic liver disease
a. congenital, hereditary - osteogenesis imperfecta - homocystinuria - Marfan syndrome, Ehlers-Danlos disease	e. immobilization
b. endocrine - hyperparathyroidism* - hyperthyroidism* - glucocorticoid excess (endogenous or exogenous) - hypogonadism - diabetes mellitus	f. medicinal - heparin
c. nutritional - protein malnutrition - deficiency of Ca, vit. D**, vit. C - ethanol excess	g. hematologic diseases - myelomatosis
	II PRIMARY - juvenile (idiopathic) - postmenopausal - osteoporosis of elderly people

* Refers to the bone tissue status after successful treatment of the endocrine disease

** Usually with admixture of osteomalacia

CHAPTER III

Regulators of Ca homeostasis and skeletal mass.

About 99% of total body Ca (1300 g) and 85% of total body P (700 g) are located in the skeleton (Nordin, 1976). *Short-term* regulation of the homeostasis of both minerals depends upon their free-exchangeable skeletal pools, while the extraskeletal soft tissue also is an important source of P. In the *long-term* regulation of Ca and P homeostasis changes in activation frequency of BMU's, their activity and life-span and the mineralization process are involved. In this Chapter PTH and calcitonin (CT), as two regulators of Ca homeostasis, as well as other hormones and factors with a more indirect influence on bone cells and bone mineral metabolism will be discussed. With regard to the investigations described in this thesis, the activation of vitamin D and the function of vitamin D in gastrointestinal Ca absorption are dealt with in more detail.

3.1 *Parathyroid hormone (for review see Habener & Jacobs, 1982).*

A decrease of plasma Ca concentration within the range of 2.62 - 2.25 mmol/l is one of the main stimuli for the secretion of PTH. It is the ionized form of Ca that regulates the PTH secretion. Other factors involved are magnesium, adrenergic agents and histamine. The secretion of PTH is inhibited by an increase in plasma Ca concentration. Some in vivo (perfusion studies) and in vitro (parathyroid tissue culture) studies point to inhibition of PTH secretion by 1,25-$(OH)_2$D, 25-(OH)D or 24,25-$(OH)_2$D (Canterbury et al., 1978; Dietel et al., 1979). The evidence for such a short-loop regulation of PTH secretion is, however, not conclusive (Rothstein et al., 1983). Glucocorticoids may also have a role in the regulation of PTH secretion (see Chapter V). PTH certainly has effects on osteoblast-like cells and stimulation of bone formation by PTH or one of its fragments has been reported (Hermann-Erlee et al., 1976; Reeve et al., 1980[a]). The most striking effect of PTH is stimulation of the number and activity of osteoclasts, leading to resorption of bone tissue and mobilization of bone mineral. Osteocytic osteolysis is possibly involved in short-term Ca homeostasis. PTH lowers reabsorption of P in the proximal renal tubule. The Ca reabsorption in the distal tubule is influenced in the opposite direction. PTH is an important stimulator of mitochondrial 1α-hydroxylation of 25-(OH)D in the proximal renal tubular cells. Both in the secretory process and in some of the actions of PTH on target organs, the generation of cAMP is involved.

Secondary hyperparathyroidism (elevated PTH levels and characteristic bone histology) may play a role in the pathogenesis of primary osteoporosis in a subgroup of patients (Teitelbaum et al., 1976). In many patients with primary osteoporosis normal PTH levels and PTH secretory reserve (Bouillon et al., 1979) or even low basal PTH concentrations (Gallagher et al., 1979) have been reported.

3.2 *Calcitonin (for review see Austin & Heath III, 1981).*

Hypercalcaemia is probably the most important stimulus for the secretion of CT by the parafollicular C-cells of the thyroid gland. Several gastrointestinal hormones and biogenic amines may also be involved in its secretion. CT inhibits bone resorption by decreasing the number and activity of osteoclasts and this results in a decreased efflux of bone minerals. The renal clearence of Ca and P is increased by CT and a short-term water diuresis with increased excretion of Na and K has been described. There is no agreement with regard to the effects of CT on the 1α-hydroxylation of 25-(OH)D and on intestinal absorption of Ca. CT may be regarded as a "bone mass sparing" hormone with elevated plasma levels during states of increased demands for Ca (pregnancy, lactation). Repeatedly, lower basal and stimulated CT levels have been found in women as compared to men of the same age group (Deftos et al., 1980).

In both sexes the secretory reserve for CT has been reported to decrease with age (Deftos et al., 1980, Shamonki et al., 1980). With the aid of a sensitive assay for CT monomers, Body & Heath III (1983) could confirm the above mentioned sex difference in basal CT and its secretion reserve, but not the age related difference. This could be relevant for the development of postmenopausal osteoporosis. Bone mass was found to be decreased in thyroidectomized patients on adequate dosage of thyroxine (MacDermott et al., 1983).

3.3 *Vitamin D (for review see Norman et al., 1982; Kanis et al., 1982; Mawer, 1982).*

3.3.1 Production

The third regulatory factor in bone mineral and bone mass homeostasis is activated vitamin D. At least one of its derivatives (1,25-$(OH)_2$D) has been shown to act as a hormone in physiological concentrations. After photochemical conversion of 7-dehydrocholesterol in the skin to previtamin D and isomerization to vitamin D_3 or cholecalciferol it is transported to the liver and hydroxylated at the C_{25} position. Bound to the same plasma protein it is subsequently delivered to the kidney, where it is further hydroxylated at C_1 or C_{24} to 1,25-$(OH)_2$D or 24,25-$(OH)_2$D respectively.

3.3.2 Regulation

Several factors are involved in the regulation of these renal hydroxylases. *PTH* and low plasma levels of *Ca* and *P stimulate* the renal 1α-hydroxylase. There is no consensus as to whether the presence of the parathyroid glands is a prerequisite for

the stimulation of 1α-hydroxylase by low Ca levels (Tanaka & DeLuca, 1973; Trechsel et al., 1980). In patients with hypoparathyroidism low levels of 1,25-$(OH)_2D$ have been found in the presence of hypocalcaemia and normal serum P levels (Lund et al., 1980). High normal concentrations of *Ca, P* and *1,25-$(OH)_2D$* in plasma *inhibit* the formation of 1,25-$(OH)_2D$ and *stimulate* the 24-hydroxylase activity. In kidney tissue from birds the formation of 1,25-$(OH)_2D$ is found to be increased by *prolactin* (Spanos et al., 1976) and *estrogens* (Castillo et al., 1977). During short-term administration of pharmacologic dosis of *growth hormone* in growth hormone deficient children, the plasma levels of 1,25-$(OH)_2D$ increase (Burnstein et al., 1983). In man during growth, pregnancy and lactation, high levels of 1,25-$(OH)_2D$ have been reported (Aksnes & Aarskog, 1982; Brown et al., 1979), but data on 1,25-$(OH)_2D$ levels in patients with acromegaly or hyperprolactinaemia are inconsistent (Lund et al., 1981; Adams et al., 1979). In patients with *hyperthyroidism* decreased serum 1,25-$(OH)_2D$ and increased 24,25-$(OH)_2D$ concentrations have been found (MacFarlane et al., 1982; Jastrup et al., 1982), probably secondary to an increased efflux of bone mineral to the plasma. Recently, similar changes have been reported in patients with *type I diabetes mellitus* (Frazer et al., 1981) or streptozotocin induced diabetes mellitus in rats (Hough et al., 1983). A role for *CT* in the regulation of vitamin D metabolism is at least doubtful and the effect of *glucocorticoids* is reviewed in Chapter IV.

A seasonal variation for 25-(OH)D and 24,25-$(OH)_2D$ with peak levels in late summer is now widely accepted. We also reported a similar seasonal variation for 1,25-$(OH)_2D$ in normal individuals studied longitudinally (Juttmann et al., 1981[b]). With increasing age 25-(OH)D, 24,25-$(OH)_2D$ (Weisman et al., 1981) and 1,25-$(OH)_2D$ (Gallagher et al., 1979) concentrations decrease.

Further hydroxylation of the dihydroxy-metabolites to inactive compounds occurs in the kidney and the intestine. Recently 1α- and/or 24-hydroxylase activities have been discovered in bone, placenta, mammary gland and intestine.

3.3.3 Functions

In view of the wide variety of tissues with cytoplasmic and nuclear receptors for 1,25-$(OH)_2D$, vitamin D metabolites may have a large number of biological functions, many of which are unknown. In addition to the intestine, kidney and bone, these receptors are present in parathyroids, pituitary, placenta, gonads, mammary glands, skin and mononuclear leucocytes.

Bone

In physiological concentrations, vitamin D (1,25-$(OH)_2D$, but possibly also 24,25-$(OH)_2D$) is necessary for adequate mineralization of bone matrix (Kanis et al., 1978). In vitro 1,25-$(OH)_2D$ stimulates bone resorption and inhibits bone matrix synthesis (Holtrop et al., 1981; Raisz et al., 1978). This bone mineral mobilizing action is important during vitamin D intoxication (Streck et al., 1979).

Kidney

Changes in phosphaturia and calciuria, ascribed primarily to the action of 25-(OH)D or 1,25-$(OH)_2D$, have been reported in the literature, but conflicting data

may result from concomittant changes in serum levels of Ca, P or PTH. Administration of pharmacologic dosis of 25-(OH)D to thyroparathyroidectomized (TPTX) dogs during volume expansion raised tubular reabsorption of both Ca and P (Puschett et al., 1972), and 1,25-$(OH)_2$D lowered phosphaturia and stimulated tubular reabsorption of Ca in D deficient TPTX rats (Constanzo et al., 1974; Puschett & Kuhrman, 1978).

Intestine

Intestinal Ca absorption and the stimulatory activity of physiological concentrations of vitamin D (especially 1,25-$(OH)_2$D) on this process have been studied extensively and will be reviewed in connection with the investigations described in Chapters VI and VII (for reviews see Bikle et al., 1981 and Nemere & Norman, 1982).

Intestinal absorption of Ca can occur against a concentration gradient by an energy dependent process (Schachter & Rosen, 1959). The results of absorption studies in the small intestine of the rat and man, using varying Ca concentrations, are compatible with a saturable carrier mediated transport process in combination with a passive diffusion component (Walling & Rothman, 1969; Ewe, 1972; Wilkinson, 1976; Pansu et al., 1983). The first mechanism is only demonstrable in the more proximal parts of the small intestine, it is vitamin D dependent and related to the high transport capacity for Ca in the duodenum.

Kinetic data of this carrier mediated transport process, studied in the rat in vivo (Pansu et al., 1983), differ from those of in vitro studies with everted gut sacs, Ussing chambers or in vitro perfusion of intestinal segments (Walling & Rothman, 1969 and 1973; Ewe et al., 1972). Pansu et al. (1983) reported an apparent half-saturation constant for the saturable component of net Ca absorption (K_t) in rat duodenum of about 20 mM, while in in vitro experiments with rat gut K_t's in the range of 1-2 mM have been found (Walling & Rothman, 1973; Ewe, 1972). With triple lumen perfusion studies of the proximal jejunum in human volunteers an apparent half saturation constant of 1-5 mM for saturable Ca absorption has been reported by Ireland & Fordtran (1973). In the rat the net absorption of Ca in vivo correlates, in the more distal parts of the small intestine, with the absorption of fluid (Behar & Kernstein, 1976). This suggests Ca absorption by passive diffusion via a mechanism called "solvent drag".

Net Ca absorption is determined by kinetics of Ca-efflux (lumen to tissue) and Ca-influx (tissue to lumen). In the rat ileum, studied in vitro, Ca-influx occurs probably exclusively via the paracellular pathway (Nellans & Kimberg, 1978). The distribution between cellular and paracellular components of Ca-efflux is not exactly known, but, in the rat, at low luminal Ca concentrations, the cellular pathway is probably predominant, especially in the proximal part of the small intestine (Nellans & Kimberg, 1979).

The relative contribution of several parts of the total intestinal tract to the net Ca balance must also be taken into account. For instance in the rat the cecum and colon seem to be important sites of Ca absorption (Nellans & Goldsmith, 1981; Favus et al., 1981).

Patient factors as age, pregnancy, intestinal transit time and food composition

modify the net Ca absorption in the gastro intestinal tract and this occurs partly via influences on the activation of vitamin D.

At a normal dietary intake of Ca the net intestinal Ca absorption, composed of digestive juice Ca (3 mg/kg.day) and true intestinal Ca absorption (7 mg/kg.day), amounts to 4 mg/kg.day. This corresponds with 25-30 percent of the dietary intake (Wilkinson, 1976).

Vitamin D deficiency causes a lowering of intestinal Ca absorption, that can be reserved by administration of vitamin D (Nicolaysen, 1937) or one of its active metabolites. A few hours after the parenteral repletion of vitamin D deficient animals with 1,25-$(OH)_2D_3$ an increase of mucosal Ca uptake and transepithelial Ca transport is demonstrable (Morrissey et al., 1978; Spencer et al., 1978). This correlates with the appearance of several intracellular proteins: calcium binding protein (CaBP) (Wasserman & Taylor, 1966), intestinal membrane calcium-binding complex (IMCal) (Kowarski & Schachter, 1980) or increased enzyme activities: alkaline phosphatase (Norman et al., 1970). These effects of vitamin D are probably receptor-mediated. There is no agreement on the time sequence of the events after vitamin D repletion nor on the rate limiting step in the transepithelial Ca transport. One of the earliest changes is seen at the level of the luminal membrane: an increased Ca uptake in BBMV, prepared from vitamin D deficient chicks, is found within 2 hours after the in vivo administration of 1,25-$(OH)_2D_3$. This rise is not prevented by pretreating the animals with an inhibitor of protein synthesis (cycloheximide) (Rasmussen et al., 1979). These data are in agreement with those of Bikle et al. (1978), who found that the early increase in transepithelial Ca transport in the duodenum of vitamin D depleted rats after repletion with 1,25-$(OH)_2D$, was not inhibited by actinomycin D or cycloheximide. Changes in lipid composition and luminal membrane fluidity have been described and appear to be correlated with the early action of vitamin D on transepithelial Ca transport (Matsumoto et al., 1981). It is not known whether this effect is specific for vitamin D. Ca uptake by other membranes from intestinal cells (for instance: Golgi, mitochondrial) is also considerably changed by vitamin D. Golgi membrane vesicles have a 10 fold higher Ca accumulation rate and a 3 fold higher binding capacity for Ca compared to BBMV (Freedman et al., 1981). Their role in transcellular Ca transport is not exactly known. Extrusion of Ca at the serosal side of the intestinal epithelial cell is an energy dependent process, in which a Ca-Na exchange and a Ca-ATPase are involved (Ghijssen & v.Os, 1982).

3.3.4 Vitamin D and osteoporosis

Both normal and low serum levels of 25-(OH)D and 1,25-$(OH)_2D$ and a smaller rise of serum 1,25-$(OH)_2D$ after injection of exogenous PTH have been reported in elderly patients with osteoporosis (Slovik et al., 1981; Sørensen et al., 1982; von Knorring et al., 1982). The same is true for the intestinal Ca absorption, which was found to be decreased in patients with osteoporosis of the aged (Caniggia et al., 1963; Spencer et al., 1964). However, these changes occur also during the normal aging process (Bullamore et al., 1970; Gallagher et al., 1979), so that their role in the pathophysiology (via secondary hyperparathyroidism) of primary osteoporosis has not yet been established.

3.4 *Other hormones that directly or indirectly may influence bone mass, but that are not primarily involved in bone mineral homeostasis are: (for review see Wallach, 1979).*

Thyroid hormone: Bone turnover is decreased or increased respectively in hypo- and hyperthyroidism in man and this last condition can, via an increased "remodeling space", lead to a reversible loss of bone mass (Krølner et al., 1983). In vitro high concentrations of thyroxine are able to stimulate bone resorption (Raisz & Kream, 1983).
Growth hormone (GH): This is the only hormone known to be able to increase the amount of bone in the adult. An increased cellular activity is seen in this condition in combination with an increased cortical thickness and a decreased number of trabeculae (with increased diameter). Like the effects of GH on other tissues those on bone tissue are probably mediated by somatomedins.
Gonadal hormones: Both androgen and estrogen deficiency are associated with a rapid loss of bone mass and this may, at least for the cortical bone, be prevented by timely and adequate substitution (Meema et al., 1975; Lindsay et al., 1980). The effects of these hormones on bone tissue are probably indirect, as no androgen or estrogen receptors are demonstrable in bone cells. In vitro, estrogens can reduce the bone resorbing action of PTH (Atkins & Peacock, 1975).

Schlechte et al. (1983) recently described a decreased bone density in the distal part of the radius in patients with *hyperprolactinaemia*. The underlying pathogenetic mechanism is not established, but may be related to hypo-estrogenism.

The association of long standing *diabetes mellitus* with osteoporosis is well-known. In this connection the in vitro demonstrated stimulation of bone collagen synthesis by insulin (Raisz & Kream, 1983) may be important. The role of *glucocorticoids* in the regulation of bone mineral homeostasis and bone cell turnover will be discussed in Chapter IV.

Finally, an increasing number of agents affecting bone cells locally have been described in in vitro studies: *prostaglandins, lymfokines* and *local growth factors* (Canalis, 1983[a]). Their role in the regulation of bone cell turnover is yet to be explored.

From epidemiological surveys it appears that primary osteoporosis (post-menopausal or of elderly people) may be associated with changes in concentrations of regulators of bone mineral homeostasis and bone cell turnover. Although some of these factors will be causal, others are secondary. As is evident from this short survey, the pathogenesis of primary osteoporosis is multifactorial and in individual patients risk factors may be identified. Glucocorticoid induced bone disease is more uniform in its pathophysiology. The influence of GC excess on hormonal and other factors regulating bone mineral homeostasis and on their target organs is reviewed in more detail in Chapter IV.

CHAPTER IV

Interaction between glucocorticoids, calcium homeostasis and bone.

The serum calcium lowering effects of high doses GC in hypercalcaemia due to vitamin D intoxication (Streck et al., 1979), sarcoidosis (Bell et al., 1979), malignancies, especially hematologic ones (Bentzel et al., 1964), hyperthyroidism (Parfitt & Dent, 1970) and in idiopathic hypercalcaemia of infancy (Morgan et al., 1956) indicate an interaction between GC and Ca homeostasis. In the preceding Chapter is outlined that regulators of bone mineral metabolism influence bone mass. Studying the pathogenesis of GC induced bone disease, one should look for GC associated changes in these factors as well as for direct actions of GC on bone cells.

4.1 *Parathyroid hormone.*

4.1.1 Interaction with physiologic levels of GC.

With regard to Ca homeostasis, PTH and the glucocorticoid hormones are antagonists, GC having a hypocalcaemic action. In rats (Williams et al., 1974; Feigal & Messer, 1981) and mice (Meyer & Brinkley, 1972) the fall in serum Ca that occurs after (thyro)parathyroidectomy is diminished, when it is combined with adrenalectomy. This effect is demonstrable in the presence as well as in the absence of the thyroid gland. Adequate suppletion with GC leads to a further decrease of serum Ca, which is probably not due to increased renal loss (Feigel & Messer, 1981). Another example of opposing actions of PTH and GC in Ca homeostasis is the hypercalcaemia that is frequently seen in patients with adrenal insufficiency (Walser et al., 1963; Walker & Davies, 1981). The hypercalcaemic action of exogenous PTH in mice is diminished by previous adrenalectomy; adrenalectomy does not affect the phosphaturic action of exogenous PTH in these animals (Meyer & Brinkley, 1972). These findings fit in the concept of a permissive role of GC in the normal activity of PTH in bone mineral mobilization.

4.1.2 Interaction of PTH with supraphysiologic levels of GC (short-term).
Variable effects of GC excess on the parathyroid tissue have been reported. This variability probably depends upon the experimental conditions. Au (1976) found a dose related stimulatory effect on the PTH release from rat parathyroid glands cultured for 48 hrs in the presence of cortisol (10^{-6}-10^{-8}M). Chertov et al. (1977), however, were unable to stimulate the PTH release from bovine parathyroid glands cultured with 10^{-5}M cortisol for 36 hrs. Intravenous administration of hydrocortisone (200 mg/4 hrs) raised serum PTH levels in man without detectable changes in serum Ca concentrations (Fucik et al., 1975). Williams et al. (1974) and Kukreja et al. (1976) found increased levels of PTH in rats treated with high doses of GC for 1 and 2 weeks, respectively. Hahn et al. (1980) and Zerwekh et al. (1980) observed no significant changes in serum PTH levels when normal volunteers were given 20 mg or 50 mg prednisone daily for 15 or 7 days respectively, but when similar doses of GC were continued for 30 days, a significant increase was found (Gennari et al., 1981).

4.1.3 Interaction with supraphysiologic levels of GC (long-term).
In patients with chronic endogenous or exogenous hypercortisolism serum PTH levels are often found to be elevated (Fucik et al., 1975; Lukert & Adams, 1976; Bressot et al., 1979; Findling et al., 1982 and Suzuki et al., 1983) when compared with matched "eucorticoid" patients. These findings are compatible with the increased numbers of osteoclasts, as described by Bressot et al. (1979) and Hahn et al. (1979[b]), in trabecular bone from patients with hypercortisolism.

4.2 *Calcitonin*

4.2.1 Interaction with physiologic levels of GC.
Cortisol probably does not have a permissive role in the action of CT on bone mineral metabolism, as adrenalectomy does not influence the hypocalcaemic and hypophosphataemic effect of exogenous CT in mice (Meyer & Brinkley, 1972). The adrenals are, however, indispensable for the induction of increased renal secretion of water, Na and K by CT, as demonstrated by Aldred et al. (1971) in the rat.

4.2.2 Interaction with supraphysiologic levels of GC.
The hypocalcaemic effect, that is seen after injection of a standard dose of CT in rats, is diminished by high doses of GC (Thompson et al., 1968). There is not much information on levels of serum CT during hypercortisolism. Lo Cascio et al. (1982) reported low serum CT levels in 7 patients treated for 2-8 months with high doses of GC. Thompson & Urist (1973) observed faster bone loss during exogenous hypercortisolism in thyroidectomized rabbits (T4 suppleted) as compared to intact (not CT deficient) controls. His hypothesis of an antagonism between GC and CT is supported by the fact, that a lesser degree of osteoporosis was induced by GC in these animals when they were also treated with CT (Thompson et al., 1972).

4.3 *Vitamin D metabolism.*

Besides the therapeutic effect of GC in patients presenting with vitamin D intoxication, another example of antagonism between these two hormones is given by Farrell et al. (1976), who described a patient with idiopathic hypoparathyroidism developing, during treatment with vitamin D, a severe hypercalcaemia when an adrenal insufficiency supervened. Of course such an apparent antagonism can be indirect, resulting from a GC induced increased loss of Ca in the faeces or in the urine.

4.3.1 Interaction with supraphysiologic levels of GC (short-term).
In 1968 Avioli et al. found a decreased plasma half life of ^{3}H-labelled vitamin D_3, with the appearance of increased levels of biologically inactive metabolites in the plasma of volunteers, treated with prednisone for 10 days. In other experiments with vitamin D deficient rats, treated with supraphysiologic doses of GC, less 1,25-$(OH)_2D_3$ appeared to be formed from labelled precursors, while there was a rapid accumulation of more polar inactive metabolites (Favus et al., 1973; Carré et al., 1974; Edelstein et al., 1977). However, when determinations of the vitamin D metabolites became available, no changes in serum levels of 1,25-$(OH)_2$D were found during short-term administration of supraphysiologic doses of GC in men (Zerwekh et al., 1980; Keck et al., 1982; Gennari et al., 1982[b]). Hahn et al. (1981) even reported an increased concentration of 1,25-$(OH)_2$D after 14 days of prednisone administration (in a daily dosage of 20 mg) to 12 volunteers. This was accompanied by a decrease of the fractional intestinal absorption of ^{47}Ca. Spanos et al. (1977) found a slight stimulation of 1α-hydroxylase activity in kidney tubules, following treatment of chickens with cortisol.

4.3.2 Interaction with supraphysiologic levels of GC (long-term).
During chronic hypercortisolism most patients have normal serum levels of 25-(OH)D (Aloia et al., 1974; Hahn et al., 1977; Bressot et al., 1979; Findling et al., 1982). For the subgroup in which decreased concentrations of this metabolite have been found (Klein et al., 1977; Slovik et al., 1980), one has to take into account the influence of the underlying disease for which GC have been prescribed, sunlight exposure, dietary factors, seasonal variation of vitamin D metabolites etc. In a few studies low levels of 1,25-$(OH)_2$D have been observed in children with renal disease and adolescents with lupus erythematodes, both treated with GC (Chesney et al., 1978; O'Regan et al., 1979). These findings are in contradiction with the normal levels, production rates and metabolic clearance rates of 1,25-$(OH)_2$D reported by Seeman et al. (1980), studying patients before and during treatment with high doses of GC, or before and after succesfull treatment of endogenous hypercortisolism. Nothing is known about the levels of 24,25-$(OH)_2$D in man during chronic GC excess.

From these data an absolute deficiency of active vitamin D does not seem likely to be of predominant pathophysiologic importance in GC induced bone disease. In agreement with these findings osteomalacia is not a hallmark of the histology of glucocorticoid bone disease. In view of the negative bone mineral balance, result-

ing from renal and gastrointestinal losses, a compensatory increase of vitamin D activity might be expected. As this has not been found, a relative deficiency of vitamin D may still be involved.

To obtain further information on the interactions between GC and vitamin D, we studied the effect of short-term administration of GC on serum concentrations of vitamin D metabolites in patients with different levels of parathyroid gland function (Chapter V).

4.4 *Influence of GC excess on other hormones.*

4.4.1 Thyroid hormones.

Supraphysiologic doses of GC, administered for 5 days to volunteers, induce a fall in serum T4 and T3 and a rise of rT3 (Gamstedt, 1981). It appears that the secretion of T4 as well as the peripheral conversion of T4 to T3 are impaired. Basal TSH levels in substituted hypothyroid patients fell during treatment with prednisone (20 mg daily for 9 days), while plasma levels of T4, T3 and TBG remained unchanged (Jensen et al., 1978). The possible impact of these changes in thyroid hormone secretion and -metabolism on GC induced bone disease is not known.

4.4.2 Gonadal hormones.

In high doses GC have a depressive effect on the levels of gonadal and gonadotrophic hormones in serum. Many data are from studies in hyperandrogenic premenopausal women (Boehm et al., 1979; Rodriguez-Rigau et al., 1979) in whom even low doses of GC have a suppressive effect on androgen production both from ovaries and adrenals (Kirschner et al., 1976). Addition of dexamethasone to cultures of testicular tissue from hypophysectomized rats (Welsh et al., 1982) or rat ovarial granulosa cells (Hsueh & Erickson, 1978), induces a local inhibitory effect on androgen release and the aromatisation of androgens to estrogens respectively. In postmenopausal women circulating plasma estrogen levels are mainly determined by peripheral conversion of adrenal precursors. No consistent data are available as to possible differences in serum levels of androgens or estrogens between postmenopausal osteoporotic women and non-osteoporotic controls (Riggs et al., 1973; Manolagas et al., 1979[a]; Crilly et al., 1979), but there are indications that treatment with GC under these circumstances lowers the level of oestron, adrostenedione and testosterone (Crilly et al., 1979).

4.4.3 Growth hormone.

The stunted growth in children as well as the loss of bone mass in adults treated chronically with high doses of GC, are frequently ascribed to changes in growth hormone (GH) secretion or depletion of its tissue mediators. Both normal and subnormal levels of plasma GH or responses to provocative tests have been reported in patients with exogenous or endogenous hypercortisolism (Hartog et al., 1964; Frantz et al., 1964). Normal levels of serum somatomedins, determined with radioreceptor- or radioimmunoassay have been found in patients with Cushing's syndrome or exogenous hypercortisolism (Thoren et al., 1981; Gour-

melen et al., 1982). However, somatomedin activity measured in a bioassay was found to be decreased (Gourmelen et al., 1982). The inhibition of growth by GC in children could not be overcome by the simultaneous long-term treatment with human GH (Morris et al., 1968).

4.4.4 Insulin

GC excess is associated with an insulin resistant state, characterized by hyperinsulinaemia and hyperglycaemia (Olefsky & Kimmerling, 1976; Nosadini et al., 1983). It is not known whether this state of insulin resistance also influences the stimulatory effect of insulin on bone collagen synthesis, which has been demonstrated in vitro by Raisz & Kream (1983).

4.5 *Gastrointestinal calcium absorption and GC excess.*

4.5.1 Measurements in vivo.

Though it is generally accepted that high doses of GC decrease the net intestinal Ca absorption, there are only a few well documented *balance studies* in man that demonstrate an increase in faecal Ca loss (Bunim et al., 1958; Lichtwitz et al., 1961). In man most studies on the effects of GC on intestinal Ca absorption have been performed with *radioactive Ca* (e.g. comparing oral with parenteral administration). When high doses (more than 40 mg prednisone-equivalent daily) are used for at least one week, a decreased fractional intestinal absorption of radiocalcium is generally found (Klein et al., 1977; Hahn et al., 1980; Gennari et al., 1982[a]). When administered in a lower dosage or for shorter periods of time, GC have no or only a small influence on intestinal radiocalcium absorption (Lekkerkerker et al., 1970; Zerwekh et al., 1980). The disappearance of radiocalcium from *ligated* chicken *duodenal loops* in situ is inhibited by the prior administration of GC in supraphysiologic doses. This decrease of Ca-efflux can be reversed by feeding the chickens a low Ca or low P diet (Fox et al., 1978), but not by a single oral dose of 1,25-$(OH)_2D_3$, given 20 hours previously (Feher & Wasserman, 1979). The same authors reported a concomittant decrease of mucosal Calcium Binding Protein and alkaline phosphatase activity.

4.5.2 Measurements in vitro.

Early studies of the interference of GC with transepithelial Ca transfer were performed with *everted gut sacs,* as described by Schachter et al. (1959). The inside-outside (I/O) ratio's for Ca, measured after one hour or longer, were considered representative for active transepithelial ion transport. They were found decreased in sacs prepared from segments of small intestine from rats that were treated with high doses GC for about one week (Harrison & Harrison, 1960; Kimberg et al., 1971). This effect was most clearly demonstrable in the proximal part of the small intestine and was of the same order of magnitude as the one obtained by vitamin D depletion. High doses of vitamin D or its active metabolite 1,25-$(OH)_2D_3$ corrected the decrease of the I/O ratio to that of non-vitamin D deficient animals (Williams et al., 1961; Krawitt, 1972; Carré et al., 1974). GC

pretreatment reduced the mucosa to serosa Ca fluxes in rat duodenum, mounted in *Ussing chambers* (Kimberg et al., 1971; Favus et al., 1973). This, combined with a slightly increased serosa to mucosa flux, led to a markedly depressed net Ca absorption.

4.5.3 Mechanism of action

GC have been reported to decrease the small intestinal villous height in the rat depending on the dose administered, the duration of treatment and the segment of intestine studied (Batt & Peters, 1976; Batt et al., 1978 Ananna et al., 1979;). A diminution of absorptive surface could explain the reported reduction in net Ca absorption during GC excess. It is also possible that intestinal Ca absorption is decreased by GC at *subcellular* level. As reviewed (4.3), the involvement of a primarily decreased availability of active vitamin D metabolites during GC excess may be considered improbable. Downward regulation of intestinal 1,25-$(OH)_2D$ receptors by GC is found in the mouse but not in the rat (Hirst & Feldman, 1982) and finally, GC could change the action of activated vitamin D at a postreceptor level.

Even in the absence of vitamin D,GC excess can diminish intestinal mucosal transfer of Ca from lumen to serosa, as shown by Favus et al. (1973) in duodenal mucosa of vitamin D depleted rats, mounted in an Ussing chamber. Corradino (1979) found that ^{45}Ca accumulation in cultured embryonic rat duodena, where the vitamin D dependent Ca transport system is not yet developed, was decreased in a 48h culture with hydrocortisone (10^{-5}—10^{-6}M). Addition of vitamin D prevented this decreased uptake completely. In the rat supraphysiologic doses of GC also reduce the gastrointestinal absorption of P (Ferraro et al., 1976) and cations such as Sr and Fe (Kimberg et al., 1971), while the absorption of Na, water and some hexoses is increased (Charney et al., 1975; Batt & Peters, 1976) in association with an increased Na,K -ATPase activity (Charney et al., 1975).

We studied the effects of GC excess and vitamin D on Ca uptake and glucose transport, at the subcellar level, in BBMV from rat small intestinal epithelial cells and provide some preliminary data of the influence of GC on Ca absorption, measured in in situ ligated small intestinal loops of rats (Chapters VI and VII).

4.6 *Renal handling of calcium and phosphorus during GC excess.*

When supraphysiologic doses of GC are administered to patients or volunteers, a rise of urinary Ca is observed after 1-2 weeks (Lichtwitz et al., 1961; Wajchenberg et al., 1969; Hahn et al., 1980). Patients chronically treated with supraphysiologic doses of GC usually have a urinary Ca excretion in the normal range (Miravet et al., 1977; Bressot et al., 1979; Hahn et al., 1979[b]), but this excretion may be elevated when compared with that of a simultaneously studied control group (Adams et al., 1981; Suzuki et al., 1983). In patients with Cushing's syndrome urinary Ca is also frequently found to be elevated (Molinatti et al., 1960; Kleemann et al., 1975). Mobilization of Ca from the skeleton could, by raising the filtered load and inhibiting PTH secretion, result in the hypercalciuria observed in hypercortisolism. Also a decrease of fractional renal Ca reabsorption in 7 of 16

patients, studied for 1-2 weeks after the initiation of prednisone administration, has been described by Laake (1960). During short-term (hours) administration of high doses of GC in dogs (Massry et al., 1967) or men (Lemann et al., 1970) renal Ca handling is not changed. In contrast, phosphaturia increases under these circumstances in men (Rosental et al., 1982). In rats Frick et al. (1981) found a decrease of the maximal tubular transport of P over the glomerular filtration rate (TmP/GFR) to be responsible for this phenomenon, that also has been observed after parathyroidectomy. Turner et al. (1982) described a decreased Na dependent P uptake in BBMV, prepared from renal cortical tubular cells from GC treated rats. Increased urinary P excretion and low serum P levels are frequently found in patients with Cushing's syndrome (Camanni et al., 1967) or on GC treatment (Rosental et al., 1982). Little is known about the direct impact of such changes in P metabolism on bone mass.

4.7 *Influence of GC excess on skeletal tissue*

4.7.1 Histology and histomorphometry.

The rat is not a very suitable animal model for the study of GC induced bone disease. Increased loss of bone only occurs with diets unbalanced for Ca and P (Storey, 1960). The rabbit seems to be more appropriate and administration of supraphysiologic doses of GC for some weeks results in a marked trabecular and cortical porosity at both central and appendicular skeletal sites, measured with histomorphometry and microradiography (Epker, 1970; Duncan et al., 1973; Jee et al., 1981). Generally, an increased number of osteoclasts, a decreased number of osteoblasts or a combination of both is described. Tam et al. (1979), using double labelling with tetracycline, found a significantly reduced bone mineral appositional rate in rabbits, treated with hydrocortisone (2 mg/kg for 1 month). By histological and histomorphometrical examination of trabecular bone from iliac crest biopsies, an increased number of osteoclasts has been observed in many patients with hypercortisolism (Birkenhäger et al., 1967; Bressot et al., 1979; Hahn et al., 1979[b]). In men indirect evidence for the induction of a reduced bone formation rate in hypercortisolism has been obtained in the form of a low mean wall thickness per basic multicellular unit (Birkenhäger-Frenkel et al., unpublished results). According to Dempster et al. (1983) this is the result of a decrease in the active formation period of trabecular bone packets. Bone mineralization rate (measured by tetracycline double labelling) has also been shown to be lowered under these circumstances (Bressot et al., 1979).

4.7.2 Bone cultures in vitro.

It has to be stressed that the data reviewed here concern the bone matrix formation by osteoblasts rather than true bone formation in vitro. Osteoblast collagen synthesis, measured by means of the incorporation of ^{14}C-proline (as ^{14}C OH-proline) into the collagen of bone tissue culture, is found to be decreased by excess GC (Peck et al., 1967; Blumenkrantz & Asboe-Hansen, 1976; Choe et al., 1978). This effect is probably not specific, as the incorporation of amino acids into other proteins is also inhibited (Peck et al., 1967; Blumenkrantz & Asboe-Hansen,

1976). Similar findings have been reported with regard to the incorporation of ^{35}S in cartilagenous ground substance (Murota et al., 1967; Tessler & Salmon, 1975). Dietrich et al. (1979) described an increased incorporation of ^{3}H-proline into collagen and to a lesser extent into non-collagenous proteins in foetal rat calvaria, cultured in the presence of high concentrations of cortisol for the first 24 hours. Incorporation of ^{3}H-thymidine into DNA and of ^{3}H-uridine into RNA were unchanged. In 96 hours cultures cortisol decreased all of these activities. Recently, the same workers found in foetal rat calvaria cultures that the early stimulation of collagen synthesis by GC could only be detected in the central part, after separation of the central from the periosteal part of the bone (Raisz & Kream, 1983). Their hypothesis that GC have a stimulatory effect on the activity of the mature osteoblasts, while the differentiation of periosteal precursors to osteoblasts is inhibited, is attractive. A similar dual effect in time, as Dietrich et al. found, is described for the transient stimulation by GC of alkaline phosphatase activity in cultured foetal rat calvaria (Canalis, 1983[b]) or mouse cartilage (Silberman et al., 1981).

In contrast to the histological signs of increased bone resorption in bone from patients, exposed to high doses of GC, these substances in vitro appear to have an inhibitory action on the ^{45}Ca release from prelabeled bone material. This has been described for the release as stimulated by PTH, prostaglandin E_2, vitamin A, 25-(OH)D, arachidonic acid and epidermal growth factor (Raisz et al., 1972; Eilon & Raisz, 1978; Tashjian & Levine, 1978; Sandberg et al., 1982). However, the resorbing action of rat peritoneal macrophages in an in vitro system, using devitalized bone particles, is enhanced by GC (Teitelbaum et al., 1981).

4.7.3 Interactions between GC and vitamin D and PTH at the (sub)cellular level. In fetal rat calvaria the existence of cytoplasmatic GC receptors has been demonstrated by Feldman et al. (1975). Conflicting results have been reported with regard to the effects of GC on the number or stability of 1,25-$(OH)_2D$ receptors in several species (Manolagas et al., 1979; Chen et al., 1983).

A number of biochemical responses of bone tissue or bone cells in culture to PTH is changed in the presence of GC. It has repeatedly been observed that the cAMP release in response to PTH in cultured rat bones is augmented by dexamethasone or high levels of cortisol (Chen & Feldman, 1978; Hahn & Halstead, 1979; Ng et al., 1979). Another example of GC *altering the sensitivity* of the target cell (the osteoblast) to other hormones was demonstrated by the finding that the PTH and 1,25-$(OH)_2D_3$ induced inhibition of the decarboxylation of citrate is more pronounced when osteoblast-like bone cells from mouse calvaria are cultured in the presence of GC (Wong, 1979; Wong et al., 1980). The discrepancy between the increased number of osteoclasts seen in the trabecular bone from patients with hypercortisolism and the in vitro observed inhibition by GC of the bone mineral mobilizing action of PTH and other factors at first sight seems difficult to explain.

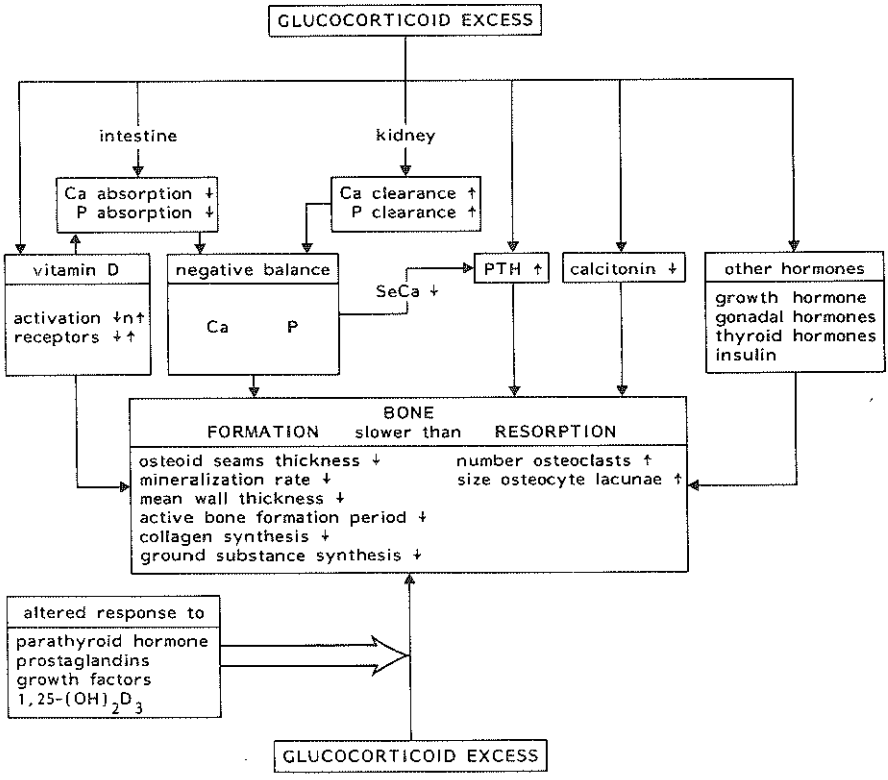

Figure 4.1 Influence of glucocorticoid excess on factors involved in the regulation of bone mass. The data are accumulated from literature studies in man and animal, both in vivo and in vitro. How the various factors may influence bone cell activity is not indicated.

4.8 *The pathogenesis of glucocorticoid induced bone disease. A concept from the literature.*

The factors that may play a role in the pathogenesis of GC induced bone disease are summarized in Figure 4.1.

There is evidence that a state of secondary hyperparathyroidism as a consequence of a decreased intestinal Ca absorption and an increased renal loss of bone mineral is an important factor. An increased level of parathyroid gland activity is indicated by the elevated serum levels of PTH that have frequently been observed in patients or animals during chronic exposure to supraphysiologic levels of GC. It is also reflected by an increased number of osteoclasts on histologic examination of bone biopsies. As proposed in the preceding section the induction of an altered

sensitivity of bone cells to bone resorbing agents like PTH and 1,25-$(OH)_2D$ may also be operative in the development of GC induced bone disease. Furthermore there are probably indirect effects of supraphysiologic doses of GC on bone mediated by their influence on the production of other hormonal and local regulators of bone cell activity. These other factors may comprise growth hormone, calcitonin, local growth factors, prostaglandins etc.. By this last mechanism and/or by the direct depressive effect of GC on protein synthesis, a decreased activity (per cell) of both the bone resorbing and the bone forming cells results.

In theory, 1α-hydroxylated derivatives of vitamin D could have favourable actions on GC induced bone disease. Administration of activated vitamin D results in an ample influx of Ca and P into the extracellular fluid by increasing intestinal absorption of both minerals. The state of secondary hyperparathyroidism, with increased bone resorption, will be counteracted directly by the vitamin D metabolites or indirectly by elevation of plasma Ca levels. It is speculative whether bone formation will be stimulated under these circumstances, for example by an increased availability of Ca and P or by an "anabolic" action of one or more of the vitamin D metabolites. To further investigate these possibilities a double-blind, placebo-controlled trial with 1α-$(OH)D_3$ was performed in patients, chronically treated with supraphysiologic doses of GC (Chapter VIII).

CHAPTER V

Short-term effect of prednisone or hydrocortisone on serum 1,25-dihydroxyvitamin D in normal individuals and patients with hyper- and hypoparathyroidism.

The content of this Chapter has been published in somewhat different form: J.J. Braun, J.R. Juttmann, T.J. Visser and J.C. Birkenhäger (1982). *Clinical Endocrinology 17,* 21-28.

SUMMARY

Oral administration of prednisone (30 mg/day for 9 days) to six normal individuals induced a significant rise in the concentration of serum 1,25-dihydroxyvitamin D (1,25-$(OH)_2D$) within 2 days. In eight patients with primary hyperparathyroidism a larger increase of 1,25-$(OH)_2D$ was observed within 3 days. In these patients the 1,25-$(OH)_2D$ concentration remained elevated during the whole period of prednisone administration (8-10 days), whereas in the control group it had returned to basal levels or below after 9 days of prednisone administration. This response appeared to be dependent upon the presence of parathyroid hormone (PTH) as we found no change in the (basally low) 1,25-$(OH)_2D$ concentrations in five patients with hypoparathyroidism during 3-4 days of prednisone administration (30 mg/day). In these patients vitamin D medication had been interrupted 3-5 days before the administration of prednisone, whereafter serum calcium was kept between 2.10 and 2.30 mmol/l by means of calcium infusion. The response of 1,25-$(OH)_2D$ to prednisone is probably not due to a stimulatory action of the glucocorticoids on PTH secretion, but rather to an increased sensitivity of the renal tubular cells to PTH. Evidence was obtained in a hypoparathyroid patient in whom we studied the response of the serum 1,25-$(OH)_2D$ level to exogenous PTH. This response was markedly increased when PTH was injected during intravenous administration of hydrocortisone in a dosage scheme that was able to raise serum 1,25-$(OH)_2D$ significantly in 8 hours in a group of six normal individuals.

INTRODUCTION

In contrast to the well-known inhibition of intestinal Ca absorption by high dose GC (Lichtwitz et al., 1961; Klein et al., 1977) conflicting data with regard to the serum levels of vitamin D metabolites have been reported in hypercortisolism.

A lowered concentration of 25-(OH)D has been observed by Klein et al. (1977) in patients on prednisone therapy, while Aloia et al. (1974) and Hahn et al. (1977) reported normal levels in patients with Cushing's syndrome or on chronic corticosteroid therapy. Under similar circumstances subnormal concentrations of serum 1,25-$(OH)_2$D have been reported by Chesney et al. (1978) and O'Regan et al. (1979); the earlier study concerning children with kidney disease. Altered metabolism of vitamin D, involving either decreased production of active vitamin D or increased turnover, has been described by Avioli et al. (1968) in man and Carré et al. (1974) in rats. Seeman et al. (1980) reported normal serum concentrations, production and metabolic clearance rates of 1,25-$(OH)_2$D in patients with endogenous and exogenous hypercortisolism. Recently Zerwekh et al. (1980) and Hahn et al. (1981) found no significant change of 1,25-$(OH)_2$D concentrations in healthy volunteers taking supraphysiologic doses GC for 7-8 days. Continuing the administration of prednisone for 14 days Hahn found a small but significant rise of serum 1,25-$(OH)_2$D together with a fall in fractional intestinal absorption of ^{47}Ca.

To elucidate some of these conflicting data we studied the short-term effect of prednisone on the serum levels of vitamin D metabolites in healthy volunteers, patients with primary hyperparathyroidism (PHP) and post-operative or idiopathic hypoparathyroidism (HP). We furthermore studied in two hypoparathyroid patients the effect of exogenous PTH alone or combined with hydrocortisone on 1,25-$(OH)_2$D in serum and on the urinary excretion of inorganic phosphate and cAMP.

PATIENTS AND METHODS

Group I consisted of six healthy volunteers (two women and four men, ages 23-40 years, numbered 1-6). Prednisone was given orally, in a dose of 30 mg/day (in three divided doses) for 9 days.

Group II comprised 8 patients with PHP (patients 1-8). The clinical and biochemical data together with the adenoma weights found at operation are presented in Table 5.1. Preoperatively, 30 mg of prednisone was given per day (in three divided doses) for 8-10 days.

Group IIIA consisted of four patients with postoperative HP (two women and two men, 16-67 years, patients 1-4) and one women with idiopathic HP (aged 35 years, patient 5).

Group IIIB is formed by two women, aged 27 and 51 years, with postoperative HP.

In all hypoparathyroid patients vitamin D medication (1α-$(OH)D_3$ or dihydrotachysterol) was interrupted. After 2-5 days, when the serum Ca level had dropped to 2.0 mmol/l or less, an intravenous infusion of Ca (as calcium glucobionate in dextrose 5%) was started. After stabilization of the serum Ca level between 2.10 and 2.30 mmol/l for 24 h the infusion of Ca was continued and in patients of group IIIA prednisone was administered orally in a dose of 30 mg/day (three divided doses) for 3-4 days. During this period the serum Ca was checked regularly and the infusion rate adapted when necessary to maintain the level withing the range indicated. Before the administration of prednisone, the Ca dose needed varied

TABLE 5.1

PREOPERATIVE DATA AND WEIGHTS OF PARATHYROID ADENOMAS IN 8 PATIENTS WITH PRIMARY HYPERPARATHYROIDISM

patient	age	sex	serum				
no*	yrs	M/F	Ca	P	iPTH**	creatinine clearance	adenoma weight
			mmol/l	mmol/l	µg/l	ml/min	mg
1	48	F	2.64	0.72	1.1	138	330
2	52	F	2.75	0.71	0.7	65	1030
3	45	M	2.75	0.77	0.2	125	250
4	68	F	3.04	0.75	2.1	84	1800
5	62	F	2.89	0.75	0.9	86	300
6	64	F	2.71	0.90	0.6	116	380
7	53	F	2.98	0.80	1.3	106	175
8	49	M	2.71	0.86	0.1	135	160
normal range			2.25-2.65 mmol/l	0.95-1.45 mmol/l	<0.2 µg/l	100-140 ml/min	

* The patient numbers correspond to those in Fig. 5.2
** As assayed by Dr. Bouillon (see: Methods section)

from 275-444 mg/24h, during prednisone from 355-675 mg/24h (not significantly different, paired t-test). In the two patients of group IIIB the effect of intravenous administration of 400 mg hydrocortisone (100 mg bolus followed by 300 mg in four hours) alone or in combination with parathyroid extract (PTE: i.v. bolus 200 USP U, Lilly) was studied. To keep the serum P level within the normal range aluminium hydroxide (1.5-3.0 g/day) was administered to patient no. 5. This resulted in a mean P $\pm$ 1 SD of 1,31 $\pm$ 0.13 mmol/l. In patients 2 and 3 serum P was at the upper limit of normal (mean P $\pm$ 1 SD of 1.41 $\pm$ 0.12 and 1.35 $\pm$ 0.22 mmol/l, respectively), while it was moderately elevated in patients 1 and 4 (mean $\pm$ 1 SD of 2.00 $\pm$ 0.09 and 1.59 $\pm$ 0.13 mmol/l, respectively. Both patients of group IIIB received 3.0 grams aluminium hydroxide daily and their serum P levels did not exceed 1.59 mmol/l during the whole period of the study. All patients with post operative HP required thyroxine substitution. Their biochemical parameters of thyroid function were normal. When not indicated otherwise, blood samples were taken after overnight fasting.

Serum and urinary Ca, P and creatinine were determined by Technicon auto-analyzer. In the two patients of group IIIB hourly urinary excretion of cAMP was determined by a competitive protein binding assay as described by Brown et al. (1971) and the urinary P excretion during the same period was used to calculate the

maximal tubular P reabsorption per unit glomerular filtrate (TmP/GFR) according to Bijvoet (1972). Serum 25-(OH)D and 24,25$(OH)_2$D were determined by competitive protein binding assay after chromatography on Sephadex LH20, for 24,25-$(OH)_2$D followed by high pressure liquid chromatography (HPLC). Serum 1,25-$(OH)_2$D was estimated by means of a radioimmunoassay (RIA) also after Sephadex LH20 chromatography and HPLC (Juttmann et al., 1981[a]). The antiserum we used (kindly donated by Dr. R. Bouillon, Louvain) recognizes 1,25-$(OH)_2D_3$ but not the vitamin D_2 derivative. All samples obtained from one individual were measured in the same assay. The intra- and interassay variation is 20 and 13% respectively. Normal values ± 1 SD throughout the year for 25-(OH)D, 24,25-$(OH)_2$D and 1,25-$(OH)_2$D are 56 ± 20 nmol/l (n = 94), 5.0 ± 2.1 nmol/l (n = 94) and 113 ± 30 pmol/l (n = 20), respectively. In group I the serum iPTH concentrations were determined by W. Hackeng (Municipal Hospital "Bergweg", Rotterdam) with a RIA, as described recently (Lips et al., 1983). In this assay the antiserum recognizes the mid-region of the molecule. Serum iPTH levels in the 8 PHP patients were determined by means of a RIA that recognizes mainly the C-terminal fragment of the molecule (Bouillon & de Moor, 1974). In 4 of the patients with PHP the course of vitamin D-binding protein concentrations in serum during prednisone administration was measured by R. Bouillon (Louvain) by means of a single radial immunodiffusion technique (Bouillon et al., 1977).

For statistical analysis of data within one group or between groups Wilcoxon's matched-pairs signed-ranks test and Mann-Whitney U test were used respectively. The Spearman test was employed for the detection of correlations between parameters. This study was approved by the Medical Ethical Committee of the University Hospital "Dijkzigt" and informed consent was obtained from all participants.

RESULTS

In all individuals of the control group (group I) a significant rise of the serum 1,25-$(OH)_2$D concentration was observed within 2 days after starting the administration of prednisone. Despite continued administration of prednisone the concentration of 1,25-$(OH)_2$D then declined to reach the initial values by 9 days (Fig. 5.1). There were no consistent changes in the concentrations of serum Ca, P, 25-(OH)D, 24,25-$(OH)_2$D or PTH in any of the control individuals.

In the eight patients with PHP (group II) the level of 1,25-$(OH)_2$D showed also an increase within 3 days after the initiation of the administration of prednisone (Fig 5.2). The average maximal increament (Δ) was larger than observed in the controls 88.8 ± 35.5 and 49.1 ± 18.3 pmol/l, respectively (Δ ± 1 SD; MWU-test p 0.05). In 7 of the 8 patients the 1,25-$(OH)_2$D concentration remained elevated during the whole period of prednisone administration. As in the control group no significant change in the concentrations of Ca or the other vitamin D metabolites was noted. The serum iPTH and the ratio of Ca to creatinine clearance had significantly risen after 8-10 days of prednisone administration, while serum P

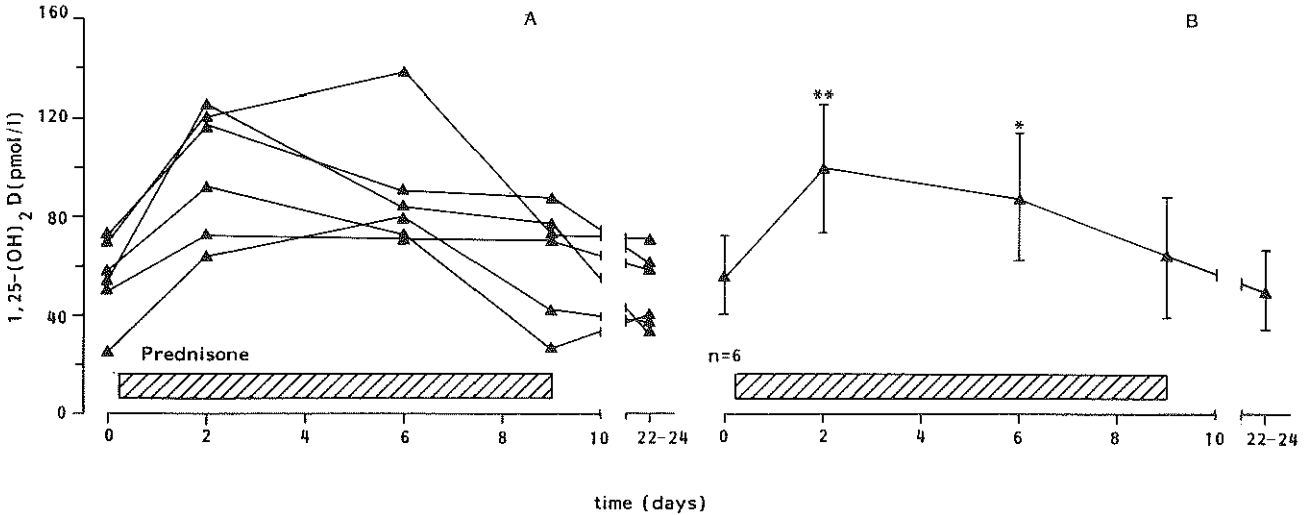

Fig. 5.1 Serum 1,25-$(OH)_2$D concentrations in six healthy volunteers (group I) taking 30 mg prednisone/day for 9 days (hatched bar). A, individual curves; B, mean values ± 1 SD. Significance of the difference from baseline value: ** $p < 0.01$; * $p < 0.05$. Average maximal △ ± 1 SD for 1,25-$(OH)_2$D: 49.1 ± 18.3 pmol/l.

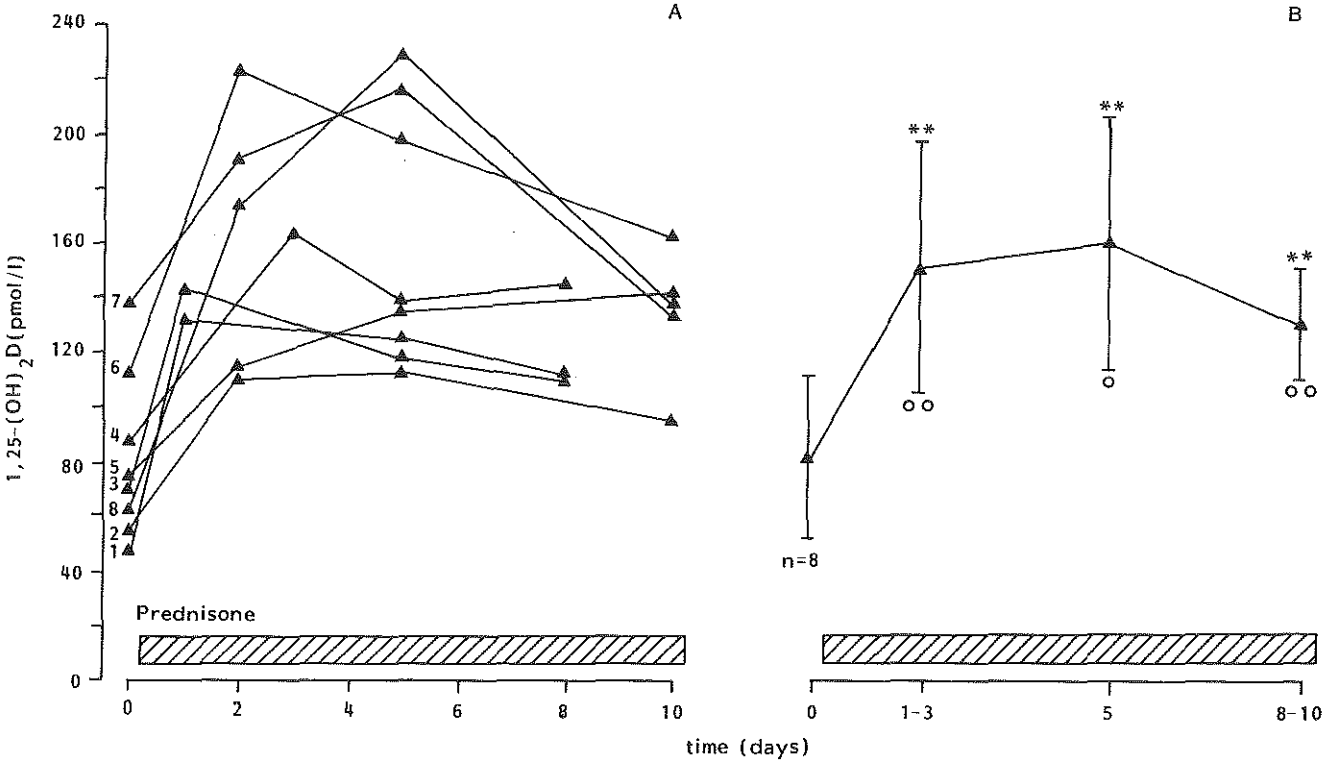

Fig. 5.2 Serum 1,25-$(OH)_2D$ concentrations in eight patients with PHP (group II) during preoperative administration of 30 mg prednisone/day for 8-10 days (hatched bar). A, individual curves; B, mean values ± 1 SD. Significance of the difference from baseline value: ** $p < 0.01$. Significance of the difference from group I at corresponding time points in the course of treatment: ^{00} $p < 0.01$; ^{0} $p < 0.05$; Average maximal △ ± 1 SD for 1,25-$(OH)_2D$: 88.8 ± 35.5 pmol/l.

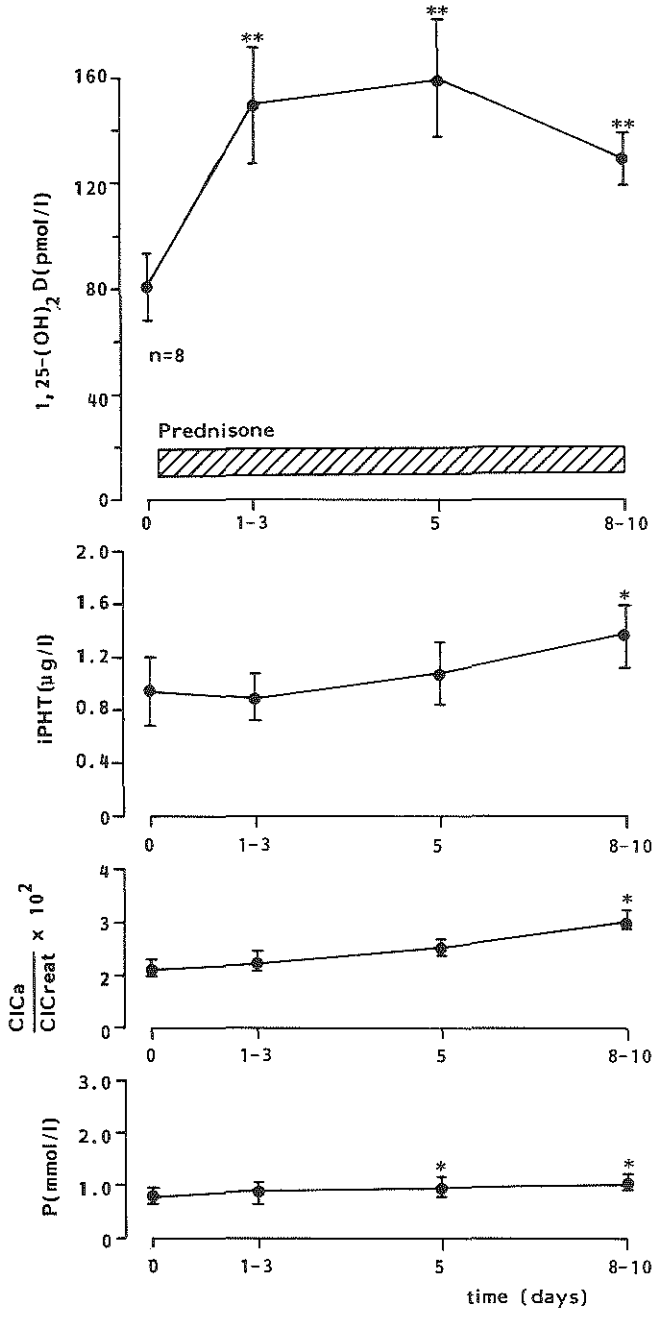

Fig. 5.3 Mean values ± 1 SD for serum 1,25-$(OH)_2$D, iPTH, the ratio of Ca over creatinine clearance and serum P in eight patients with PHP (group II), during administration of prednisone. Significance of the difference from basal value: ** $p < 0.01$; * $p < 0.05$.

increased above basal values from 5 days onwards (Wilcoxon $p < 0.05$) (Fig. 5.3). Serum vitamin D binding protein did not change during the whole period of prednisone administration in the 4 patients with PHP, studied in this respect. No correlation between the serum levels of iPTH, Ca and P on the one hand and serum 1,25-$(OH)_2$D concentrations or maximal Δ 1,25-$(OH)_2$D during prednisone on the other hand was demonstrable in the two groups. In the patients with PHP adenoma weights did neither correlate with basal levels of 1,25-$(OH)_2$D nor with the maximal Δ 1,25-$(OH)_2$D. Mean basal levels $\pm$ 1 SD of 1,25-$(OH)_2$D did not differ significantly between group I and II; 55.6 $\pm$ 16.7 and 81.6 $\pm$ 30.3 pmol/l, respectively. Serum 1,25-$(OH)_2$D concentrations in the hypoparathyroid patients decreased rapidly after vitamin D medication was discontinued.

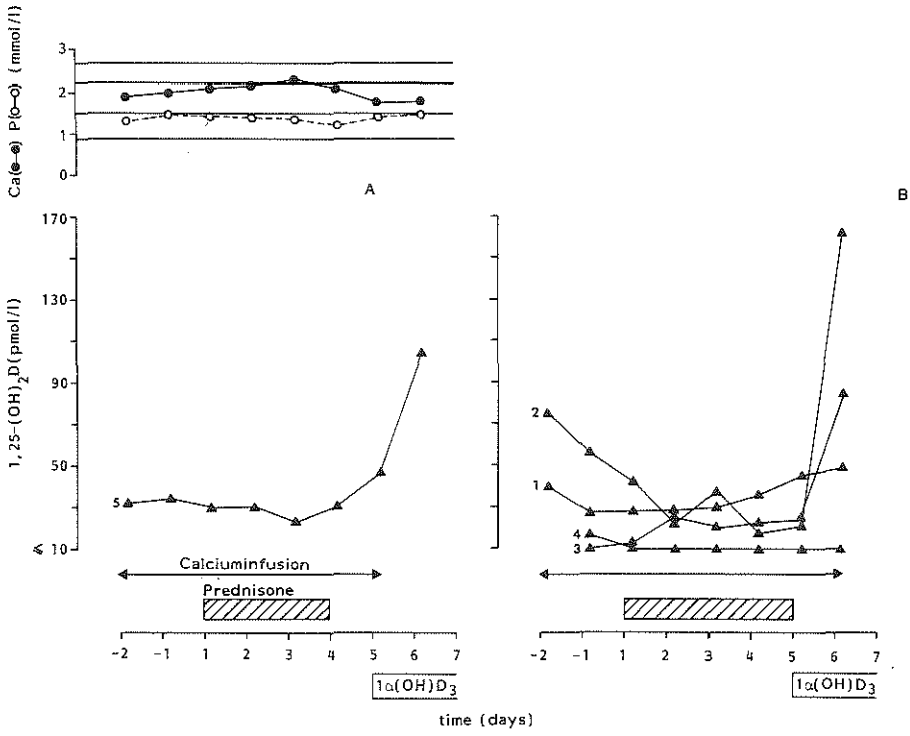

Fig. 5.4 A, serum Ca, P and 1,25-$(OH)_2$D concentrations in a patient with idiopathic HP (patient 5) during the period of infusion of Ca, before and during the administration of 30 mg prednisone/day for 3 days and subsequently treated with 1α-$(OH)_3$D (1-2 μg daily); 1,25-$(OH)_2$D 10$\leq$ pmol/l means at the lower limit of detection or undetectable.
B, individual course of the serum 1,25-$(OH)_2$D concentrations in the other four hypoparathyroid patients (postoperative HP) during infusion of Ca, before and during prednisone administration (30 mg/day for 4 days) and during subsequent reinstitution of treatment with 1α-$(OH)_3$D (1-3 μg daily).

No significant rise of 1,25-$(OH)_2D$ was seen during the administration of prednisone to patients with HP of group IIIA (Fig. 5.4). Their mean basal level $\pm$ 1 SD (24.4 $\pm$ 14.3 pmol/l was significantly lower than in group I and II (MWU-test $p < 0.05$). Fig. 5.5[A] shows a marginal increase of serum 1,25-$(OH)_2D$ after the i.v. injection of 200 USP U of PTE on two consecutive days in one of the hypoparathyroid patients of group IIIB. The third injection, given 15 h after the initiation of a hydrocortisone infusion, did not elicit a larger response of 1,25-$(OH)_2D$, although the level of the whole curve had risen. After changing the time-table somewhat for the other patient with HP, in such a way that the third injection of PTE was given during the hydrocortisone infusion, an augmented response of 1,25-$(OH)_2D$ (and of urinary cAMP as well) was seen, as compared to the effect of the two previous injections (Fig. 5.5[B]). The decrease of TmP/GFR appeared also to be larger after the third injection. Hydrocortisone alone, administered several months later under similar conditions, scarcely elicited an increase of serum 1,25-$(OH)_2D$, but lowered TmP/GFR somewhat.

In six normal individuals infusion of hydrocortisone with the same dosage scheme caused a gradual rise of serum 1,25-$(OH)_2D$, which was significant 8 h after starting the hydrocortisone administration (data not shown).

DISCUSSION

The early rise in serum 1,25-$(OH)_2D$ concentration during prednisone administration found by us in normal individuals differs from the findings of Zerwekh et al. (1980) and Hahn et al. (1981). After 7 days they observed no significant increase of the 1,25-$(OH)_2D$ concentration, while Hahn et al. saw a late rise (after 14 days of GC). In our hands 1,25-$(OH)_2D$ would already have fallen to normal after 9 days. One has of course to consider the possibility of a secondary increase after 9 days and the fact that Hahn et al. did not use a divided dose scheme for prednisone administration. Lukert et al. (1973), using silicic acid chromotography, found an augmented peak corresponding to 1,25-$(OH)_2D$, in serum extracts of rats that were treated with 5 mg prednisone/day for 7 days. Cortisol may induce a moderate increase of chicken kidney tubule 1α-hydroxylase in vitro as well as in vivo (Spanos et al., 1977).

The extensive and more sustained elevation of 1,25-$(OH)_2D$ that we found in the eight patients with PHP and the absence of an increase of the 1,25-$(OH)_2D$ level in the five patients with HP during short-term prednisone administration indicate, that the observed response of 1,25-$(OH)_2D$ to GC administration is mediated by PTH.

Breslau et al. (1982), using a higher dose of prednisone (50 mg daily) and a chicken intestine radioreceptorassay for 1,25-$(OH)_2D$, could not find consistent changes in serum 1,25-$(OH)_2D$ concentrations. These authors compared in 7 patients with PHP over 8 days the mean values of 4 control days and those of the last 4 days of prednisone treatment of each patient.

With regard to the absence of a response of 1,25-$(OH)_2D$ in HP we feel it

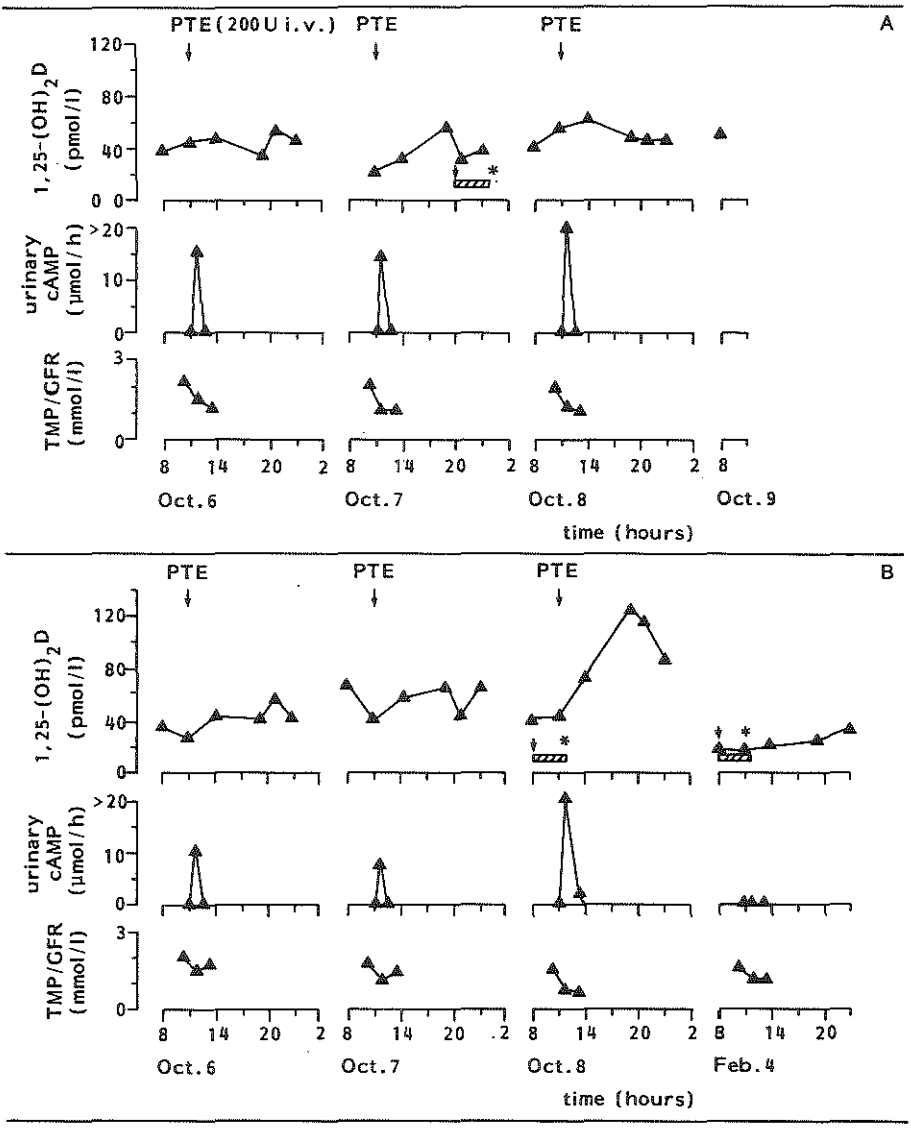

Fig. 5.5 Serum 1,25-$(OH)_2D$ concentration, hourly urinary cAMP excretion and TmP/GFR in response to i.v. administration of PTE (200 USP U) and hydrocortisone* (bolus of 100 mg followed by 300 mg in 4 h) in two patients with postoperative HP. A, hydrocortisone administered before the third PTE injection. B, PTE injected during the hydrocortisone infusion.

unlikely, that this is due to a suppresive effect of serum P on the production of $1,25\text{-}(OH)_2D$, as in three of the five patients serum P was not elevated during the study. As to the kinetics of the changes observed in the concentration of $1,25\text{-}(OH)_2D$, one has to note that our measurements during prednisone administration were performed from 1 to 2 days onwards. Parenteral administration of hydrocortisone in normal individuals induced an even earlier response. We have as yet no explanation for the much slower secondary decline of the $1,25\text{-}(OH)_2D$ levels in PHP. This phenomenon indicates the need for studies of longer duration.

The basal $1,25\text{-}(OH)_2D$ levels in the control and PHP groups, being below the normal range mentioned in the methods section, deserve comment. In the relatively small group of individuals from which our normal values were derived ($n = 20$; ten women, ten men) the average age was 31 years (range 22-43), while in eight patients with PHP the average age was considerably higher at 55 years (range 45-68). Besides group size and age, other factors such as sex, nutrition and season (Juttmann et al., 1981[b]) may have been responsible for this apparent discrepancy.

An effect of GC on vitamin D binding protein could not be demonstrated in four patients with PHP and if one assumes that the early rise of serum $1,25\text{-}(OH)_2D$ concentrations, induced by GC, reflects a change in its renal production, PTH could be operative in one of the following ways:

1. direct stimulation of PTH secretion with subsequent activation of 1α-hydroxylase by PTH;
2. indirect stimulation of PTH secretion by GC through increased intestinal or renal loss of Ca;
3. increase in the sensitivity of the PTH target organ(s) by GC;
4. a permissive action of PTH, involved in a direct effect of GC on 1α-hydroxylase.

Although in vivo Fucik et al. (1975) and in vitro Au (1976) have obtained some evidence for mechanism 1, we observed no change in serum iPTH levels, that would support this concept. The rise of iPTH in group II after 8-10 days prednisone treatment is in agreement with results of Breslau et al. (1982) in patients with PHP and Hahn et al. (1979[a]) in normal individuals. It probably reflects a reaction to an increased loss of Ca (intestinal as well as renal) in hypercortisolism, as has been amply documented (Wajchenberg et al., 1969; Hahn et al., 1980). It has to be stressed, that different RIA's were used for group I and II. This makes the interpretation of the absence of a rise of iPTH in group I difficult. No changes in serum Ca could be found during prednisone administration in group I or II. The fact, however, that in our hypoparathyroid patients the amount of Ca administered intravenously needed to maintain a serum Ca level of 2.10 to 2.30 mmol/l tended to increase during prednisone treatment (although not significantly so), is in agreement with the early induction of a negative Ca balance by GC excess.

A direct stimulatory effect of GC on the 1α-hydroxylase (without involvement of PTH) is not supported by the findings in the hypoparathyroid patients in group IIIA, while the augmented response in patients with PHP advocates against the role of PTH purely as a permissive factor (mechanism 4).

Induction by GC of an increased sensitivity of the renal 1α-hydroxylase activity to the stimulatory action of PTH might very well explain the early rise in serum

1,25-$(OH)_2D$ levels. This mechanism is illustrated by the results obtained in the patients with HP, who received a PTE injection concomitantly with a hydrocortisone infusion. GC appear to potentiate the PTH induced release of cAMP from bone culture (Chen & Feldman, 1978) and (in patients) from the kidney (Gennari et al., 1981).

The transient character of the stimulation may be caused by counter-regulatory effects, such as the inhibition of 1α-hydroxylase by 1,25-$(OH)_2D$ itself, changes in Ca transport from and to the (renal tubular) cell or the induction of increased metabolism of 1,25-$(OH)_2$)D.

The (patho)physiological significance of this relatively rapid and short-lived stimulation of 1,25-$(OH)_2D$ production by GC is not clear. With regard to the pathogenesis of bone disease in chronic hypercortisolism it may reflect an (inadequate) adaptation to a negative bone mineral balance which, after a short course of prednisone at the dose level used, is known to be present.

For references see the common reference list.

CHAPTER VI

Calcium and glucose uptake in rat small intestinal brush border membrane vesicles: modulation by exogenous hypercortisolism and 1,25-dihydroxyvitamin D_3.

The content of this Chapter has been accepted for publication in *Biochem. Biophys. Acta*:
Hans J. Braun, Jan C. Birkenhäger and Hugo R. de Jonge.

SUMMARY

The effect of exogenous hypercortisolism and 1,25-dihydroxyvitamin D_3 on small intestinal calcium and glucose transport in the rat was studied at the level of brush border membrane vesicles generated from isolated villous cells by a freeze-thaw procedure. At 5.10^{-5}M extravesicular calcium initial uptake rates in vesicles prepared from triamcinolone treated adult rats were decreased by 30% after 5 days. Since calcium ionophore A23178 virtually abolished the difference in calcium uptake, triamcinolone appeared to affect calcium channel density or activity rather than intravesicular binding capacity. Kinetic analysis showed that a decrease in V_{max} of a saturable calcium transport system could entirely account for the diminished rate of vesicular calcium uptake. Calcium transport rates could be partially restored by in vivo administration of 1,25-dihydroxyvitamin D_3 at a dosage which did not affect vesicular calcium uptake in control animals. Conversely, sodium-driven glucose accumulation in brush border vesicles from triamcinolone treated rats was stimulated by 50-70% after 36h and appeared insensitive to vitamin D. A specific triamcinolone action on the glucose carrier itself rather than on the driving force of the sodium gradient was indicated by (i) a similar stimulation of glucose transport under equilibrium exchange conditions and (ii) an opposite effect of triamcinolone on sodium-driven alanine transport. The triamcinolone induced changes in calcium and glucose uptake were not accompanied by a gross alteration of membrane integrity in vitro or by major alterations in vesicular protein composition, intra-vesicular glucose space and sucrase or alkaline phosphatase activity. The modification of vesicular transport properties is discussed in relation to the vitamin D antagonized inhibition of intestinal calcium uptake and the stimulation of glucose absorption in response to supraphysiologic amounts of glucocorticoids observed in intact epithelium.

INTRODUCTION

Reduction of net Ca absorption in the proximal small intestine in response to exogenous hypercortisolism has been documented in animals in vitro using everted gut sacs or Ussing chambers (Harrison & Harrison, 1960; Kimberg et al., 1971; Krawitt, 1972). Studies, monitoring fractional intestinal uptake of radioactive calcium in man, showed a diminished absorption during treatment with supraphysiologic doses of GC (Klein et al., 1977; Hahn et al., 1979[b]). Malabsorption of Ca may contribute to the pathogenesis of GC induced bone disease, presumably by induction of a state of secondary hyperparathyroidism (Hahn et al., 1979[b]).

A major regulatory site in transepithelial Ca transport is the passive entry of luminal Ca across vitamin D sensitive channels in the brush border membrane (Rasmussen et al., 1979 and 1982). According to the "liponomic control" hypothesis the active vitamin D metabolite 1,25-dihydroxyvitamin D (1,25-$(OH)_2D$) would unmask cryptic Ca channels in the luminal membrane by changing the lipid annulus surrounding the channel. In these studies duodenal brush border membrane vesicles (BBMV) have been succesfully applied as in vitro tools to discriminate vitamin D action at the luminal membrane from its potential effects on other epithelial components, e.g. the Ca binding protein (CaBP) in the cytosol (Wasserman & Taylor, 1966), the basolateral Ca pump (Ghijsen & v.Os, 1982) and Na-Ca exchanger (Hildman et al., 1982) or the Ca counter-transport system in Golgi membranes (Freedman et al., 1981).

In view of the apparent antagonism between 1,25-$(OH)_2D$ and GC with regard to transepithelial Ca absorption the possibility was considered, that GC might likewise interfere with Ca transport across the luminal membrane. In the present study the BBMV model was chosen to monitor functional changes at the brush border level in adult rats treated with supraphysiologic doses of GC. Effects of exogenous 1,25-$(OH)_2D_3$ on this model were also examined in order to clarify the mechanism underlying the beneficial action of vitamin D or its metabolites on intestinal Ca uptake in GC treated animals or patients (Krawitt, 1972; Klein et al., 1977; Braun et al., 1983). Na-glucose cotransport served as a marker confined exclusively to the luminal membrane (Hopfer et al., 1973) and as a sensitive indicator of the integrity of the vesicle membrane.

MATERIALS AND METHODS

Materials: Triamcinolone (Kenacort® A 40) was obtained from Squibb, 1,25-$(OH)_2D_3$ for parenteral administration was kindly provided by Hoffmann La Roche, phlorizin was obtained from Fluka, D-[1-^{3}H]-glucose from NEN and other radioactive isotopes were obtained from the Radiochemical Centre, Amersham. The Ca ionophore (A23178) was obtained from Boehringer. Other chemicals were analytical grade. Nitrocellulose filters were obtained from Sartorius, The Netherlands.

Animals: Adult male Wistar rats weighing 300-350 gram and fed normal laboratory chow were injected subcutaneously (s.c.) with a suspension of triamcinolone acetonide (TA), sustained release, at a dosage of 6 mg/kg (0.05 ml), five days prior to vesicle preparation (Group II). In Group III this treatment was combined with s.c. administration of 1,25-$(OH)_2D_3$, 300 ng/kg (0.05 ml), 36 and 12 hours prior to vesicle preparation. Group IV was pretreated with 1,25-$(OH)_2D_3$ alone and Group I, the control group, was injected with equivolumes 0.9% NaCl. In combined experiments vesicle preparations obtained from different groups were studied simultaneously. The existence of a state of hypercortisolism after 5 days was confirmed by the finding of suppressed endogenous corticosterone levels (not shown). For the time-effect study TA, 6 mg/kg, or 0.9% NaCl (both 0.05 ml) was administered s.c. 10, 36 and 120 hours prior to vesicle preparation.

Preparation of vesicles: BBMV were prepared from the proximal half of the rat small intestine. Intestines from 2 rats were pooled for a single vesicle preparation. Under light aether anaesthesia the small intestine was removed and rinsed 3 times with 20 ml icecold 0.9% NaCl. All further steps were performed at 0-4°C. First intestinal villous cells were released by mechanical vibration as described earlier (Hülsmann et al., 1974). Cells were collected by centrifugation (160 g, 5 min) and the cell pellet derived from 2 rats was washed 3 times with 40 ml buffer containing 10 mM maleate, 300 mM mannitol and 0.02% NaN_3 brought at pH 7.2 with Tris (buffer A). The method used for vesiculization of the brush border membrane and purification of BBMV will be published in full detail elsewhere. In short, the cell pellet was resuspended in 10 ml buffer A and quickly frozen in liquid nitrogen. After 10 min the frozen suspension was thawed slowly in ice water for 60-90 minutes. Vesiculization by mechanical homogenization (Kessler et al., 1978[b]; Hauser et al., 1980) was completely avoided. Released brush border membranes were separated from other membrane fragments by differential precipitation with 10 mM $MgSO_4$(Hauser et al., 1980) and centrifugation (3000 g, 15 min). BBMV were isolated from the supernatant by a second centrifugation step (27000 g, 30 min). The crude vesicle pellet was resuspended in 20 ml buffer (pH 7.2) containing 10 mM Tris-maleate, 100 mM mannitol and 0.02% NaN_3 (buffer B). The suspension was homogenized in a Potter-Elvehjem homogenizer and the differential centrifugation procedure was repeated once. The final pellet was suspended in Buffer B prior to use. The enrichment factor of the brush border marker enzyme sucrase in BBMV as compared to mucusal scrapings was 32 ± 5 ($n = 10$).

Vesicular transport: Vesicular Ca uptake was determined by preincubating 50 μl of a BBMV suspension (25-50 μg protein) in buffer B for 1 minute at 25°C, followed by addition of an equivolume of 0.1 mM $^{45}CaCl_2$ (specific activity 170-200 dpm/pmol) in the same buffer. Transport was stopped by addition of 2 ml icecold buffer B, followed by rapid filtration through nitrocellulose filters (pore size 0.45 μ). After washing 4 times with 2 ml icecold buffer B the filter was dissolved in 5 ml scintillation fluid (Instagel, Packard) and ^{45}Ca determined in a Packard 2650 Tricarb scintil lation counter. Data were corrected for blank values obtained by omitting BBMV from the incubation mixture. In kinetic studies of Ca uptake, 2 mM EGTA was included in the stopbuffer in order to reduce Ca binding to the

vesicle exterior, which became significant at Ca levels exceeding 0.05 mM. Initial Ca influx rates were calculated from the difference in vesicular Ca uptake at two initial time points (15 and 60 sec) when uptake was virtually linear in time at all Ca concentrations tested (0.015-0.9 mM). Following preloading of the BBMV during 3 min in the presence of 50 μM $^{45}CaCl_2$ in some experiments ^{45}Ca efflux was studied by diluting the incubation mixture 40-fold with buffer B containing 2 mM EGTA plus or minus 10 μM Ca ionophore followed within 0-2 min by filtration and washing.

Na-dependent glucose and alanine transport into BBMV were determined following preincubation of the vesicle suspension (50-100 μg protein) at 25°C for 1 min or at 37°C for 30 min followed by 1 min 25°C. Transport was started by rapidly mixing a droplet of 50 μl BBMV suspension with an equivolume of 0.2 M NaSCN in buffer B containing 2.6 μM D-[1-^{3}H]-glucose (specific activity 3 x 10^4 dpm/pmol) or 1.0 μM L-[2,3-^{3}H]-alanine (specific activity 8 x 10^4 dpm/pmol). Since the dissipation rate of the transmembranal Na gradient strongly affects the driving force for intravesicular glucose accumulation, the activity of the glucose carrier was also measured under isotope equilibrium conditions, as described by Hopfer & Groseclose (1980). In this way gradient related driving forces are completely avoided and changes in labeled glucose transport reflect alterations in glucose carrier activity. In these experiments BBMV were preincubated in the presence of 0.1 mM glucose and 0.1 M NaCl in buffer B for 30 min at 25°C. Isotope uptake was started by mixing 50 μl BBMV suspension with an equivolume of the preincubation medium containing 0.1 mM D-[1-^{3}H]-glucose (specific activity 2 x 10^3 dpm/pmol). In case of sodium dependent glucose uptake, transport was stopped at 0.1 min and in case of equilibrium exchange at 10, 20, 40 and 120 sec with 2 ml icecold buffer B containing 0.1 M NaCl and 0.5 mM phlorizin. Mixing and stopping were performed by means of a semiautomatic apparatus constructed according to Kessler et al. (1978[a]). Following filtration on nitrocellulose filters and repeated washings (4 times with 2 ml icecold stopbuffer) the filters were processed for liquid scintillation counting. Equilibrium values for ^{3}H-glucose uptake were obtained after 60 min of incubation. In the isotope equilibrium exchange experiments the half-time for maximal uptake of labeled glucose ($t_{1/2}$) was estimated by interpolation on plots of ln (1- $uptake_t/uptake_\infty$) versus time, whereby $uptake_t$ = tracer uptake at the indicated time points and $uptake_\infty$ = uptake at equilibrium (cf. Hopfer & Groseclose, 1980).

Miscellaneous: Protein concentrations were determined according to Lowry et al. (1951). Alkaline phosphatase activity was assayed at 37°C, as described by Iemhoff et al. (1970). Sucrase activity measured in 0.1% Triton X_{100} was determined according to Forstner et al. (1968). Brush border proteins were analyzed by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) in principle according to Laemmli (1970).

Statistical evaluation: Results from animal Groups I-IV obtained in one experiment were statistically compared with the outcome of separate experiments by a two way analysis-of-variance test, utilising Tukey multiple confidence intervals. In experiments were only 2 groups were compared the (unpaired) Student's t-test was applied.

RESULTS

Characterization of vesicular Ca and glucose uptake: Some of the basic features of the Ca uptake process in BBMV prepared by the freeze-thaw technique are demonstrated in Fig. 6.1[A] and can be summarized as follows:

1. Binding of Ca to the vesicle exterior following the filter washing procedure was apparently insignificant at the low Ca concentration (0.05 mM) in the medium (Ca_{out}) selected, since (i) 1 mM EGTA or 0.01 mM $LaCl_3$ added to the washing buffer did not affect Ca uptake values (results not shown) and (ii) excess EGTA added to the vesicle exterior at 25°C started a slow release of Ca from ^{45}Ca preloaded vesicles, which could be accelerated substantially by addition of the Ca ionophore A23178 (see Fig. 6.1[A], efflux experiments);

2. Based on an osmotically active intravesicular glucose space of 1.46 μl/mg of protein (Table 6.1), equilibrium uptake of Ca at 0.05 mM Ca_{out} should be reached following transport of 0.07 nmol Ca/mg protein, provided that binding to internal Ca binding sites and the Donnan potential (Liedtke & Hopfer, 1982) could be neglected. However, according to Fig. 6.1[A] uptake of Ca reached a 15 fold higher

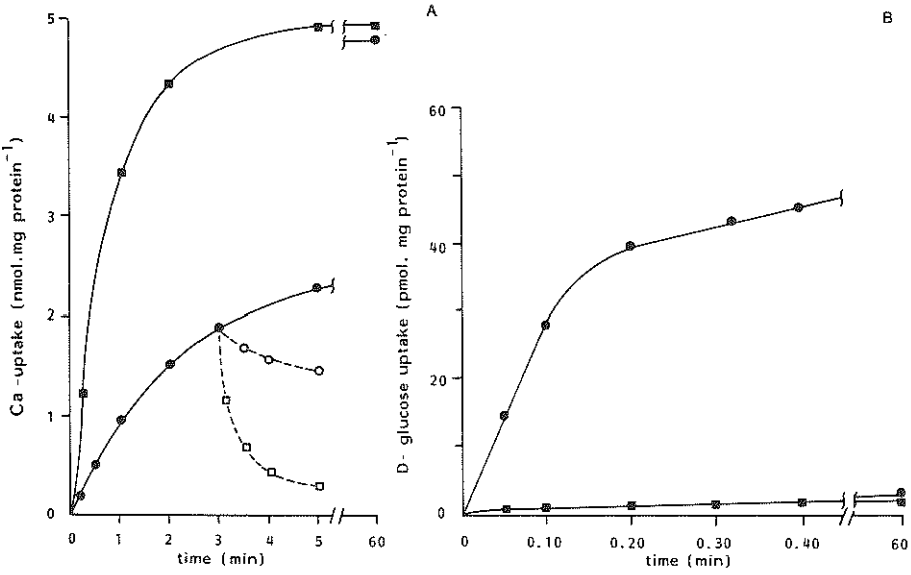

Fig 6.1 A. Time course of Ca uptake and Ca efflux, at 50 μM Ca_{out} by BBMV in absence (●—●, O—O) and presence (■—■, □—□) of Ca ionophore A23178. Release of ^{45}Ca (O—O, □—□) was started by diluting samples of the incubation mixture 40-fold in the same buffer containing EGTA (2 mM). For experimental details see Methods.
B. Time course of glucose uptake by BBMV in the presence (●—●) or absence (■—■) of a NaSCN gradient (100 mM outside, 0 mM inside at t = 0). Equilibrium uptake was reached after 30 min of incubation. Curve (■—■): BBMV were preincubated in the presence of 100 mM NaSCN for 30 min prior to ^{3}H-glucose addition.

value already at 1 min and a 30 fold higher value at 5 min, implying that virtually all Ca uptake reflects intravesicular binding;

3. Preincubation of BBMV in the presence of the Ca ionophore caused a 4-6 fold increase in initial rate of vesicular ^{45}Ca uptake (Fig. 6.1^{A}). This level of stimulation was persistently seen within a broad concentration range of Ca_{out} (0.015-0.9 mM; data not shown). Equilibrium uptake was reached already after 5 min, about 1 h earlier as found for BBMV in the absence of ionophore (Fig. 6.1^{A}). The finding that equilibrium values of Ca uptake in the absence or presence of ionophore were not significantly different, suggests that the ionophore acts on the same class of vesicles responsible for basal Ca transport. Since the ionophore merely increases the rate of Ca influx across the vesicle membrane, without affecting Ca binding sites, Ca entry into the vesicle interior apparently functions as a rate limiting step in the Ca uptake process, at least during the first few minutes. Therefore initial rates of vesicular ^{45}Ca uptake will primarily reflect the kinetic properties of Ca channels embedded in the microvillous membrane.

Na-driven glucose accumulation increased linearly with time for about 6 sec (Fig. 6.1^{B}). At this point the "overshoot" above the equilibrium values was 15-18 fold, a value similar to or better than reported for other BBMV preparations (Hopfer et al., 1973; Kessler et al., 1978^{b}). Glucose overshoot was completely abolished if ionic gradients were dissipated by preincubating BBMV with NaSCN (0.1 M) for 30 min prior to ^{3}H-glucose addition (Fig. 6.1^{B}). The glucose "overshoot", reflecting the activity of the Na glucose carrier, is also a sensitive indicator of the integrity of the vesicle membrane. Monitoring vesicle stability was considered essential, because in initial experiments using BBMV prepared according to Kessler et al. (1978^{b}), we noticed a variable loss of glucose overshoot during in vitro incubation parallelled by increased ^{45}Ca uptake and proteolysis of intravesicular proteins, e.g. actin (data not shown). These in vitro artifacts were rarely seen in vesicles prepared from TA treated rats. By using the "freeze-thaw" procedure for vesicle isolation however, such artifacts were completely avoided. This is illustrated by the persistently high levels of glucose overshoot in vesicles from group I and II upon preincubation for 30 min at 37°C (Table 6.1).

Changes in vesicular Ca and glucose uptake induced by GC and 1,25-$(OH)_2D_3$: Five days after injection of TA we found a significant fall in the initial Ca uptake rates (average 30%) in BBMV isolated from proximal small intestine compared to the same preparation from saline-injected rats (c.f. group II versus group I in Table 6.1). Addition of Ca ionophore A23178 virtually eliminated any difference in Ca uptake (measured at t = 5 min) between all four groups (Table 6.1). Since at least 95% of total Ca uptake at 5 min reflects binding to intravesicular receptor sites, the TA effect is unlikely to be the consequence of a change in intravesicular free Ca space. Moreover the osmotically active intravesicular glucose space was unaltered by TA treatment (Table 6.1). This table additionally shows that treatment of rats with 1,25-$(OH)_2D_3$ alone (Group IV) had no influence on vesicular Ca uptake rate compared to controls (group I). In contrast administration of the vitamin D_3 derivative to TA treated rats partially counteracted the TA induced depression of vesicular Ca transport (c.f. Group III versus II).

TABLE 6.1 TRANSPORT RATES OF CALCIUM, GLUCOSE AND ALANINE AND ACTIVITIES OF BRUSH BORDER ENZYMES IN BBMV FROM TA AND 1,25-$(OH)_2D_3$ TREATED RATS

Animal groups	I	II	III	IV
triamcinolone treated	-	+	+	-
1,25-$(OH)_2D_3$ treated	-	-	+	+
		$nmol.min^{-1}.mg\ protein^{-1}$		
Ca uptake (initial rate)[a]	0.97 ± 0.25	0.68 ± 0.20*	0.88 ± 0.16°	0.93 ± 0.25
Ca uptake (5 min)[a]		$nmol.mg\ protein^{-1}$		
- ionophore	2.44 ± 0.44	1.74 ± 0.38*	2.17 ± 0.33°	2.53 ± 0.39
+ ionophore	4.87 ± 0.62	3.92 ± 0.79	4.03 ± 0.96	4.89 ± 0.48
glucose transport (NaSCN gradient)[a]		$pmol.0.1min^{-1}.mg\ protein^{-1}$		
preincubation 1 min, 25°C	27.2 ± 8.8	45.9 ± 15.7 *	55.4 ± 11.8 *	34.8 ± 7.8
preincubation 30 min, 37°C	29.0 ± 14.0	42.8 ± 16.0 *	53.4 ± 16.6 *	38.4 ± 8.1
glucose transport[b]		sec		
($t_{\frac{1}{2}}$ under isotope equilibrium conditions)	7.8 ± 2.1	3.1 ± 1.0 ▪	n.d.	n.d.
		$pmol.0.1min^{-1}.mg\ protein^{-1}$		
alanine transport (NaSCN gradient)[b]	5.61 ± 1.27	3.26 ± 0.75▪	n.d.	n.d.
		$\mu l.mg\ protein^{-1}$		
intravesicular glucose space[b]	1.46 ± 0.15	1.48 ± 0.07	1.40 ± 0.11	1.47 ± 0.09
		$U.mg\ protein^{-1}$		
sucrase activity[c]	2.40 ± 0.51	2.65 ± 0.65	2.70 ± 0.49	2.44 ± 0.55
		$mU.mg\ protein^{-1}$		
alkaline phosphatase activity[a]	3.74 ± 1.27	3.27 ± 1.52	4.53 ± 1.74	5.85 ± 2.04*

a: mean values ± SD (n=6); b: mean values ± SD (n=3); c: mean values ± SD (n=4); n.d.: not determined
significance of the difference (two way analysis of variance): *from Group I ($p<0.05$); °from Group II ($p<0.05$)
significance of the difference (Student's t-test) : ▪from Group I ($p<0.05$)

Table 6.1 also shows a 1.7 fold increase of Na-dependent glucose uptake in BBMV from TA treated animals. Interestingly, this stimulatory effect of TA on glucose transport was not counteracted by 1,25-$(OH)_2D_3$, but even showed a tendency to increase further (Group III versus II). An opposite effect of TA was found on the half filling time ($t^1/_2$) for vesicular glucose uptake, measured under isotope equilibrium exchange conditions (Table 6.1), indicating that the TA induced increase in glucose uptake was the consequence of a change in glucose carrier activity rather than of a fall in Na permeability of the vesicle membrane. Moreover, Na-driven alanine accumulation was significantly depressed in BBMV from TA treated rats as compared to control vesicles (Table 6.1).

Changes in vesicular Ca and Na-driven glucose transport were also studied at several time points following a single injection of saline or TA (Fig. 6.2). Significant changes in Na-driven glucose transport rates in TA rats were detectable as early as 36h, whereas a depression of Ca uptake became significant only at 120h after injection of TA.

Effects of TA and 1,25-$(OH)_2D_3$ on other properties of BBMV: Table 6.1 additionally shows that the specific activity of the brush border membrane marker enzyme sucrase was not significantly different in BBMV isolated from animal Groups I-IV. The activity of alkaline phosphatase was also unaffected in TA treated rats. As expected (Norman et al., 1970), 1,25-$(OH)_2D_3$ significantly increased the activity of this enzyme (Group IV). As a trivial explanation for the TA effects on Ca and glucose transport it was also considered possible that TA treatment could lead to the generation or selection of a different class of vesicles no longer representative of BBMV. Therefore, we analyzed the protein composition of

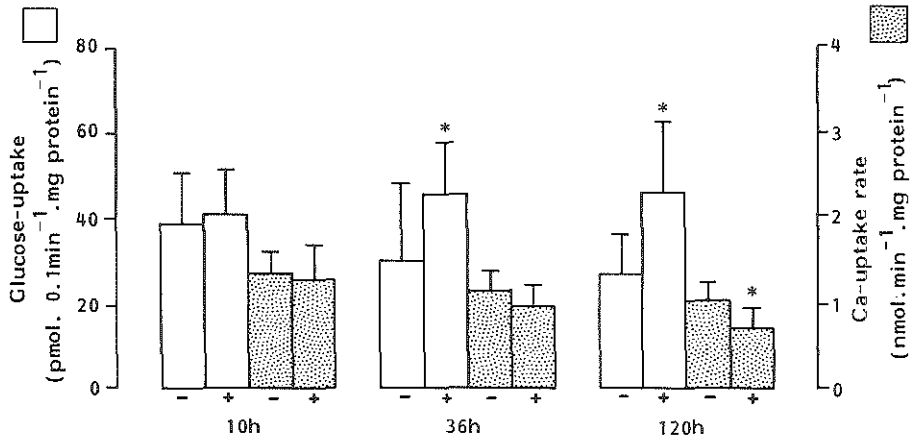

Fig. 6.2 Effect of TA injection on glucose and Ca transport in BBMV. Na-dependent glucose transport was measured at t = 0.1 min as indicated in Fig. 6.1[B]. Initial Ca uptake rates were measured at 50 μM Ca_{out} as described in Methods. Rats were killed at 10, 36 and 120h following injection of TA. Mean values ± 1 SD (n = 6) are presented. The difference from controls was significant for glucose transport at t = 36 and 120 h: * $p < 0.05$ and for Ca transport at t = 120 h: * $p < 0.05$ (Student's t-test).

vesicles obtained from all groups (I-IV) by SDS-PAGE. Although changes in minor protein components cannot be detected accurately by this technique, the overall protein composition appeared very similar (results not shown).

Effects of TA on kinetic properties of vesicular Ca and glucose transport: The decrease of initial Ca uptake rate in BBMV from rats treated with TA for 5 days was persistently found within a broad range of extravesicular Ca concentrations (0.15-0.9 mM; Fig. 6.3). Analysis of the data of 4 consecutive experiments in an Eadie-Hofstee plot (Fig. 6.3, inset) indicated that the decrease of Ca uptake in BBMV resulted from a sharp decrease in the apparent V_{max} (mean ± SEM: 4.7 ± 0.6 for control versus 2.3 ± 0.05 nmol.min^{-1}.mg protein^{-1} for TA), predominating the effects of a decrease in the K_m value of the transport system (mean ± SEM: 138 ± 4.8 for control versus 78 ± 4.8 μM for TA).

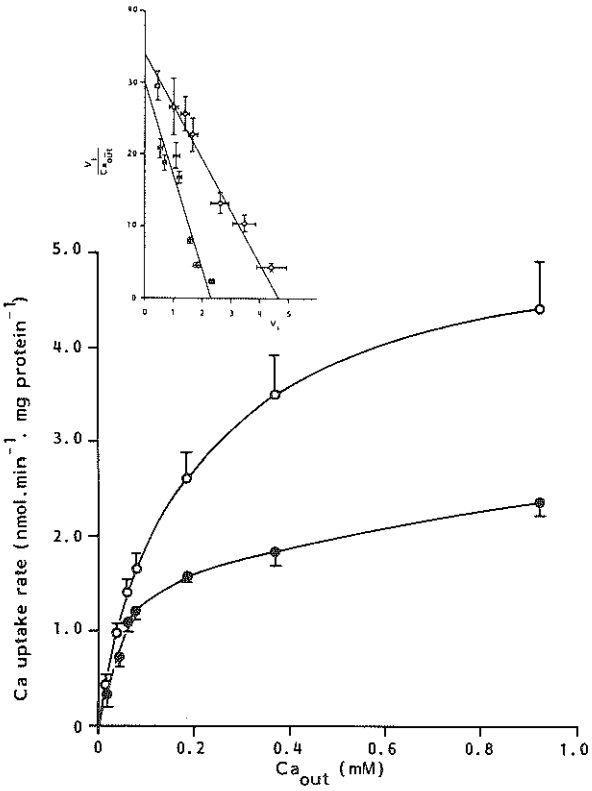

Fig. 6.3 Initial Ca uptake rates (V_i) as a function of extravesicular Ca concentration (Ca_{out}) in BBMV from control (O—O) and TA pretreated (●—●) rats. Mean values ± SEM are shown (n = 4). The difference from controls was significant over the whole range of Ca_{out} measured ($p < 0.05$, Students t-test). An Eadie-Hofstee plot of these data is shown in the inset. The r value for control and TA vesicles amounted to 0.99 and 0.93 respectively (Linear regression analysis).

Measurements of Na-driven ^{3}H-glucose accumulation at 6 sec as a function of extravesicular glucose concentration and analysis of the data in a Lineweaver-Birk plot suggested, that TA treatment shifted the apparent V_{max} of glucose transport from 1.1 to 1.7 nmol. 0.01 min^{-1}.mg $protein^{-1}$ without affecting the K_m (approximately 80 μM; data not shown). A similar K_m value has been reported for the Na-glucose symport in renal BBMV (Aronson & Sacktor, 1975).

DISCUSSION

The 30% decrease in Ca uptake rate found in BBMV from rats treated with GC could be due to a change in Ca channel properties, an alteration of the number or affinity of intravesicular Ca binding sites or both. The finding that the Ca ionophore A23178, which increases the Ca permeability of the vesicle membrane but does not affect intravesicular Ca buffering systems, eliminated the difference in Ca uptake between control and TA vesicles, is strongly in favour of a TA effect on transmembrane Ca transport rather than on intravesicular Ca binding. Kinetic analysis of the Ca transport data showed that the TA induced depression of vesicular Ca uptake could be ascribed completely to a fall in apparent V_{max} of the Ca transport system, which is only partially compensated by a concomittant decrease in K_m. The effect is unlikely to result from a general membrane stabilizing action of GC preventing in vitro breakdown of the membrane barrier (Weissman & Thomas, 1963), since the BBMV prepared by the freeze-thaw method were extremely stable as appeared from the persistence of the glucose "overshoot" phenomenon upon prolonged incubation at 37°C (Table 6.1). The lower level of vesicular Ca transport found in TA treated rats corresponds to reported effects of GC on intact intestinal segments in vitro (Harrison & Harrison, 1960; Kimberg et al., 1971). A partial restoration of GC suppressed Ca transport by concomittant treatment with vitamin D or 1α-hydroxylated vitamin D has also been observed with intact intestinal epithelium both in vitro (Krawitt, 1972) and in vivo (Fox et al., 1978). Our finding of a 1,25-$(OH)_2D_3$ correctable depression of vesicular Ca transport is not in contradiction with recent data from Schultz et al. (1982), who found a normal increase of Ca uptake in BBMV from vitamin D depleted chicks treated with GC upon repletion with 1,25-$(OH)_2D_3$. Unfortunately, these authors did not present data of GC effects on BBMV from untreated normal chicks and they confined the period of treatment to 48h.

Although the results of the BBMV experiments offer a plausible explanation for the apparent decrease in segmental intestinal Ca absorption induced by hypercortisolism and for the partial reversal of this decrease by vitamin D, they do not rule out additional effects of GC on other parts of the transmucosal Ca transport system, e.g. on Ca binding to cytosolic proteins, Ca-pump activity or Ca-Na exchange at the basolateral membrane or on paracellular Ca transport.

The molecular mechanism involved in GC action on the Ca transport system in the intestinal brush border is not established by the present study. Depression of vitamin D activation by TA or stimulation of its catabolism are unlikely in view of

the normal or even increased 1,25-$(OH)_2D$ serum levels found in rat (Lukert et al., 1973) and men (Braun et al., 1982) during short-term hypercortisolism. However, those findings do not exclude changes in intracellular 1,25-$(OH)_2D$ receptor concentrations (Hirst & Feldman, 1982) or post-receptor events such as the production of cytosolic CaBP (Feher & Wasserman, 1979). A local effect of GC in the villous cells, mediated by binding to steroid receptors and affecting either a "liponomic control" mechanism of the Ca channel (cf. Rasmussen et al., 1982) or the synthesis and incorporation of Ca channels into the brush border membrane, appears also unlikely in view of the rather slow response of the Ca transport system to exogenous hypercortisolism, significant only after 5 days. In contrast such a local mechanism could certainly play a role in the stimulation of vesicular glucose transport measured at 36h following TA injection. Moreover, if GC would exert their action exclusively on intestinal crypt cells, implying a lag phase in the effects on villous cell membranes, one should expect a maximal effect on microvillar Ca transport not earlier than 2-3 days after TA injection, corresponding with the transit time of enterocytes from crypt to villous tip (Hughes et al., 1958). We therefore suggest that the inhibitory action of TA on Ca transport either originates in the intestinal crypt cell or is secondary to effects of TA on non-intestinal tissues.

The stimulation of glucose transport in response to GC at the level of BBMV has not been reported earlier, but seems to correspond in time with the GC induced stimulation of glucose absorption reported for intact intestinal segments (Charney et al., 1975). Charney et al. have suggested a positive correlation between the activation of Na,K-ATPase in the basolateral membrane of the enterocyte and Na, water and glucose absorption by the intact epithelium. Since BBMV are devoid of this enzyme and depleted of endogenous ATP (unpublished results), such a mechanism cannot play a role in the stimulation of glucose transport at the vesicle level. On the other hand it is more plausible to explain the increased transmucosal glucose transport, observed in intact epithelium, by a GC mediated increase of glucose carrier activity in the brush border membrane, since this system seems to function as the rate-limiting step in overall-glucose transport (Sellin & Field, 1981).

In conclusion, the results of our vesicle studies indicate that exogenous hypercortisolism exerts a stimulatory effect on the Na-symport carrier for glucose in the brush border membrane at 36h, followed later (120h) by an inhibitory effect on the Ca permeability of the membrane. The active vitamin D metabolite 1,25-$(OH)_2D_3$ was found capable to counteract the GC effect on Ca transport but not on glucose transport. The specific effects of GC on intestinal brush border components not only form an interesting basis for further studies regarding the molecular mechanism underlying GC action, but are also of clinical importance.

For references see the common reference list.

CHAPTER VII
(Addendum to Chapter VI)

Influence of triamcinolone on calcium and water absorption in rat small intestinal loops in situ.

INTRODUCTION

Most studies demonstrating depression of net mucosal to serosal intestinal Ca transport in the rat by GC have been performed with isolated everted sacs of the proximal intestine (cf. Schachter & Rosen, 1959) or with unstripped mucosa mounted in Ussing chambers (cf. Walling & Rothman, 1969). In vivo however, Clark et al. (1959) found no changes in combined gastrointestinal and faecal recovery or oral ^{45}Ca loadings in rats treated with high doses of GC and Ferretti et al. (1978) even observed an increased net Ca absorption, as measured by Ca balance, in rats with doses of cortisol roughly equivalent to those used in our experiments; increasing the doses of GC caused a gradual elevation of endogenous Ca secretion until at extremely high doses ($\geqslant$32 mg cortisol/kg/day) the net intestinal Ca balance became negative.

It seemed important to compare the results of the vesicle experiments reported in Chapter VI with Ca transport measurements in intact intestinal segments of rats subjected to the same GC regimen, using an in situ loop technique. Thus the contribution of paracellular pathways to overall Ca transport and the effect of the so-called unstirred layer on transport rates can be taken into account. Furthermore artifacts induced by in vitro conditions (e.g. tissue hypoxia) are herewith avoided. Some preliminary data are reported.

MATERIALS AND METHODS

$^{45}CaCl_2$ (10-40 mCi/mg Ca) was obtained from Amersham and [1,2-^{3}H] polyethyleneglycol (PEG, MW 4000; 0.5-2 mCi/g) was obtained from NEN.

Male Wistar rats of similar age and weight as used in the vesicle experiments received a s.c. injection of triamcinolone-acetonide (TA), long-acting suspension,

6 mg/kg, or saline 5 days before the transport study. Absorption of fluid, ^{40}Ca and ^{45}Ca was assessed in 6 rats from each group (non-fasted state) by means of a modification of the in situ loop technique described by Zornitzer and Bronner (1971). Under light aether anaesthesia the peritoneal cavity was opened and the whole small intestine was flushed 2 times with 20 ml isotonic saline kept at 37°C. Residual saline was largely removed by gently stripping the intestine between fingertips. Subsequently 3 segments of about 15 cm each were ligated: a proximal jejunal loop just distal to the ligament of Treitz, a distal jejunal loop from 30 cm distal to the ligament of Treitz and the terminal 15 cm of the ileum. A control and TA treated rat were always studied simultaneously. One ml of transport medium, prewarmed to 37°, was injected by means of a syringe with a 18 gauge needle in each ligated segment. The transport medium contained 164 mM NaCl, 0.88 mM $^{45}CaCl_2$ (610 -680 dpm/nmol) and [1,2-^{3}H] polyethyleneglycol (720 dpm/nmol). Following an equilibration period of 5 min a 10 μl sample from the luminal content was taken by means of a transmural puncture, enabling us to calculate the initial intra-luminal solvent volume and ^{45}Ca content. The sample was dissolved in 5 ml scintillation fluid (Instagel, Packard) and ^{45}Ca and ^3H were determined in a Packard 2650 Tricarb scintillation counter. After 30 min the loop was removed, its length measured and a 10 μl sample was again taken from the luminal content for determination of ^{45}Ca and ^3H radioactivity. Also the ^{40}Ca concentration in the luminal fluid was determined by means of EGTA titration of a fluorescent Ca-calceine complex, a modification of the method described by Wallach & Steck (1963). Finally, the excised loop was flushed with 20 ml icecold saline and dissolved during 48 hrs in 3 ml soluene (Packard) at 60° C. The extract was diluted 12.5 fold and a 1 ml sample was counted for ^{45}Ca and ^3H. For determination of the ^{45}Ca uptake into the intestinal wall a correction for the amount of ^{45}Ca associated with the "^{3}H PEG-space" was applied. The following absorption parameters were determined and calculated according to Wasserman et al. (1961).

1. *Solvent absorption* ($A_{solv.}$: μl fluid per cm intestine per 30 min) defined as the total amount of fluid absorbed from the intestinal lumen into the tissue

$$A_{solv.} = \frac{V_i - V_f}{L} = \frac{V_i - PR \times V_i}{L}, \text{ whereby}$$

V_i (initial volume in μl) refers to the injected solvent volume corrected for dilution by residual endogenous luminal fluid at t_o on the basis of the change in PEG concentration in the previous 5 minutes.

V_f (final volume in μl) refers to solvent volume left at the end of the absorption period (t_{30}). This parameter is calculated as the product of PR and V_i.

PR (PEG ratio) refers to the ratio of PEG concentration at t_o versus t_{30}.

L (length in cm) refers to the length of the ligated intestinal loop.

2. *Ca-efflux* (nmol Ca per cm intestine per 30 min) is defined as the unidirectional movement of calcium from the intestinal lumen to tissue (mean value over the period studied).

$$\text{Ca-efflux} = \frac{V_i\,(^{45}Ca_i) - V_f\,(^{45}Ca_f)}{\frac{SA_i + SA_f}{2}\,L}, \text{ whereby}$$

$^{45}Ca_i$ and $^{45}Ca_f$ (dpm per μl intraluminal fluid) refer to the initial and final ^{45}Ca concentration at t_o and t_{30}, respectively.

SA_i and SA_f (dpm per nmol Ca) refer to specific activity of Ca at t_o and t_{30}, respectively.

3. *Net Ca absorption* (Net Ca abs.: nmol Ca per cm intestine per 30 min) defined as the total amount of Ca absorbed from the intestinal lumen.

$$\text{Net Ca abs.} = 880 - {}^{40}Ca_f V_f, \text{ whereby}$$

880 refers to the total amount of ^{40}Ca (nmoles) administered at t_o.

$^{40}Ca_f$ (nmol per μl intraluminal fluid) refers to the final ^{40}Ca concentration at t_{30}.

4. *Ca-influx* (nmol Ca per cm intestine per 30 min) defined as unidirectional movement of calcium that enters the intestinal lumen from the tissue.

$$\text{Ca-influx} = \text{Ca-efflux} - \text{Net Ca abs.}$$

In these calculations it is assumed that backflux of ^{45}Ca from mucosa into the lumen is negligible. For statistical analysis Student's t-test (unpaired) and linear regression analysis were employed.

RESULTS

The treatment with TA induced a significant increase of the solvent absorp tion in each segment (Fig. 7.1). As illustrated in Fig. 7.2 this solvent uptake was correlated positively with Ca-efflux at the level of the proximal jejunum in the control as well as in the TA treated animals ($r = 0.91$ and 0.96, respectively). In the distal jejunum and the terminal ileum this applied only to the TA treated animals ($r = 0.95$ and 0.82, respectively; data not shown). Although the Ca-efflux was higher at the level of the proximal and distal jejunum of TA treated rats as compared to controls, the net absorption of Ca was decreased along the whole length of the small intestine as a consequence of an increased Ca secretion (Ca-influx), which was most pronounced in the distal jejunal and ileal segments (Fig. 7.3). The net gain of radioactive Ca in the intestinal tissue (corrected for ^{45}Ca in the adhering fluid as quantified by ^{3}H-PEG measurements) was not influenced by TA treatment (data not shown). This means that the TA induced efflux of ^{45}Ca from the lumen into the gut wall is exactly compensated by an enhanced flux of the tracer from the intestinal wall into the body.

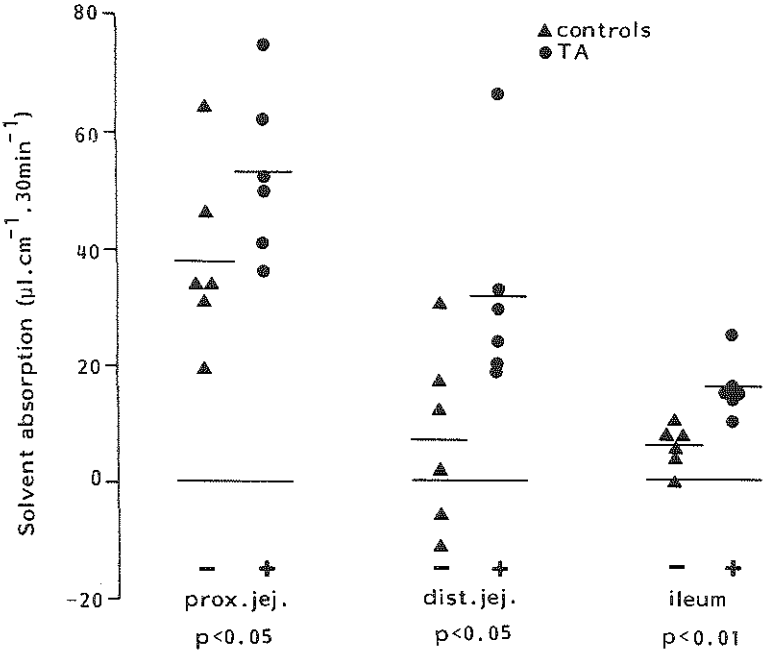

Figure 7.1 Influence of TA treatment (studied 5 days after s.c. injection of 2 mg TA) on the solvent absorption in rat proximal jejunum, distal jejunum and terminal ileum. The mean value for each group is presented by a horizontal bar.

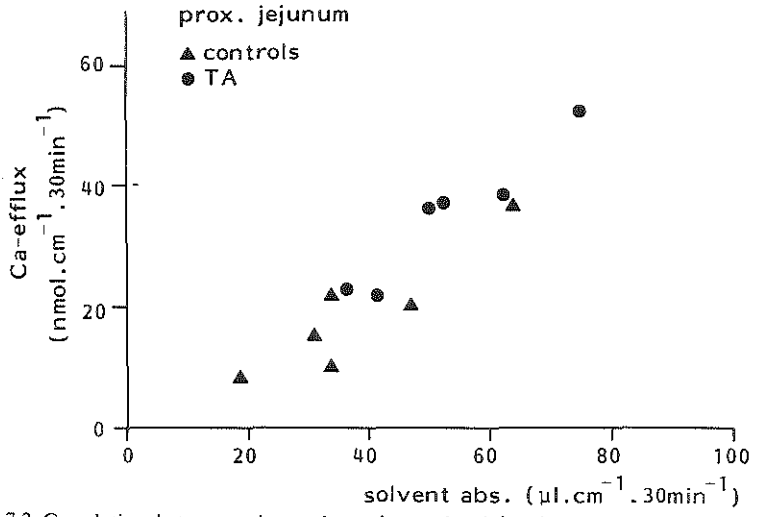

Figure 7.2 Correlation between solvent absorption and unidirectional Ca-efflux from proximal jejunum in control and TA treated rats. Linear regression: for control group: $y = 1.3\,x\text{-}13$ ($r = 0.91$; $p < 0.05$) and for TA group: $y = 1.2\,x\text{-}10$ ($r = 0.96$; $p < 0.01$).

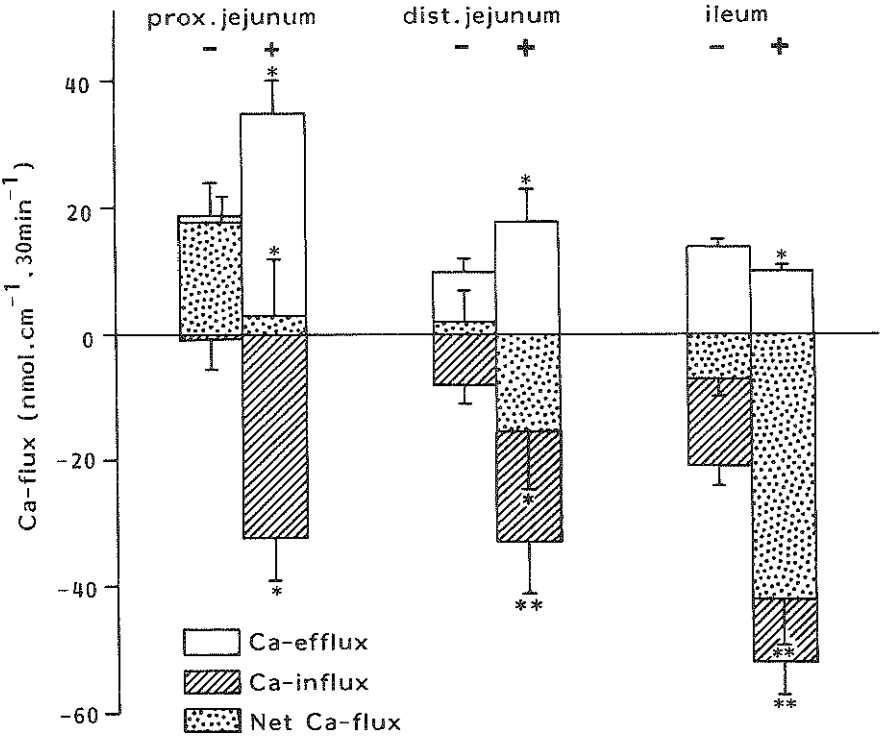

Figure 7.3 Influence of TA treatment (studied 5 days after s.c. injection of 2 mg TA) on unidirectional Ca-efflux and net Ca absorption in rat proximal jejunum, distal jejunum and terminal ileum. Mean values ± 1 SD are presented. Differences between control and TA treated animals: * $p < 0.05$; ** $p < 0.01$.

DISCUSSION

Based on the 30% fall in Ca permeability of the brush border membrane observed in BBMV isolated from the proximal half of the rat small intestine, following a similar pretreatment with TA (see Chapter VI), a decrease of Ca-efflux (lumen to tissue) was also expected in the Ca transport experiments with in situ ligated loops. Paradoxically, the in situ experiments showed a significant increase of lumen to tissue Ca-efflux. At least two explanations for this apparent discrepancy seem plausible: (1) GC may exert a different effect on Ca extrusion across the basolateral membrane of the enterocyte, e.g. by stimulating the Ca pump. This will result in a decrease of the steady-state level of intracellular Ca and a rise in Ca entry into the enterocyte across the brush border (due to a steeper Ca gradient), which may amply compensate for the apparent decrease in Ca-channel density measured in BBMV; (2) in the in situ experiments increased "solvent drag" of Ca through a

paracellular route may mask a decrease of Ca-absorption through the microvillous membrane. The positive correlation found between Ca-efflux and solvent absorption provides strong evidence for this second hypothesis. Transepithelial transport of cations by such a mechanism seems especially important in the more distal parts of the small intestine (Behar & Kernstein, 1976). More data on the in vivo contributions of cellular and paracellular pathways in the Ca transport across the intestinal wall are needed. It is probable, that the transcellular route predominates in the duodenum where vitamin D exerts its main action.

Using in situ loop techniques similar to ours, Feher & Wasserman (1979) found a marked decrease of duodenal ^{45}Ca absorption in growing chicks after two days treatment with cortisol (2 mg daily i.m.) and Fox et al. (1978) reported similar results in chickens after oral administration of betamethasone (25 μg/kg/day) for 14 days. These two studies differ from our experimental set-up in several respects. (1) Both investigations were carried out with duodenum only, which amplifies possible effects of GC on vitamin D dependent Ca transport. (2) The chicken is far more sensitive to vitamin D deprivation than the rat. (3) Fox et al. used a much lower dose of GC than we used in our experiments.

The decrease in net Ca absorption by GC found in our in situ loop experiments results predominantly from an increased Ca secretion. The question arises as to the nature of the driving force involved in this process. High doses of GC stimulate the transepithelial movement of Na by enhancement of Na,K-ATPase (Charney et al., 1975) and/or brush border Na permeability (Sellin & Field, 1981). The increased pumping of Na leads to hyperpolarisation of the intestinal mucosa (the potential difference between mucosa and serosa becomes more negative). The magnitude of this effect increases from the proximal to the distal part of the small intestine (Charney et al., 1975). Thus a driving force for the secretion of cations such as Ca is created, which increases from duodenum to ileum.

In the calculation of Ca absorption from in situ ligated small intestinal loops possible changes in the specific activity resulting from ^{40}Ca secretion were neglected by Ewe (1972). He found a decreased ^{45}Ca absorption from in situ ligated proximal jejunal loops of adult rats treated with high doses of GC (2 mg methylprednisolone daily) for 6 days, i.e. with doses considerably higher than used in our study.

The increased small intestinal Ca secretion provoked by GC may provide an important contribution to the total intestinal Ca balance. However, the effect of GC on Ca absorption in the large intestine also has to be taken into account. In Ussing chamber experiments the rat coecum and colon appeared to be effective sites of Ca absorption (Nellans et al., 1981; Favus et al., 1981). With the same technique Lee (1983) found an increased net Ca-flux in the distal colon of rats pretreated with GC for 4 days.

The effect of GC and active vitamin D metabolites, alone or in combination, on the absorption of Ca have yet to be studied in situ along the whole length of the intestine. In these studies the contribution of cellular and paracellular fluxes should be determined separately, in order to elucidate the mechanism underlying the beneficial effect of active vitamin D metabolites on intestinal Ca absorption in patients treated with GC (Chapters VIII and IX).

CONCLUSIONS

In in situ ligated small intestinal loops of rats pretreated with GC we have found a decreased net Ca absorption. This was the result of a marked stimulation of the Ca secretion (along the whole intestinal length tested) which was greater than the stimulation of the Ca-efflux from the proximal and distal jejunum. The relative contribution of the trans- and paracellular pathway in this process is unknown, but it seems plausible that effects of GC on the paracellular route play an important role in the net transepithelial Ca absorption in vivo. The use of BBMV is therefore an important tool to examen GC and vitamin D effects on Ca transport across the microvillus without interference of possible effects on the paracellular route (Chapter VI).

CHAPTER VIII

Influence of 1α-(OH)D_3 administration on bone and bone mineral metabolism in patients on chronic glucocorticoid treatment; a double-blind controlled study.

The contents of this Chapter have been published:
J.J. Braun, D.H. Birkenhäger-Frenkel, A.H. Rietveld, J.R. Juttmann, T.J. Visser and J.C. Birkenhäger (1983) *Clin. Endocrinol.* 18, 265-273. The figures 8.1-8.4 have been added.

SUMMARY

We have performed a double-blind placebo controlled study of 1α-hydroxyvitamin D_3 (1α-(OH)D_3) 2 μg daily for 6 months, in 14 patients receiving long-term glucocorticoid treatment. Patients were matched for age, sex, underlying disease and dose of glucocorticoid.

Initial values for serum calcium, phosphorus and alkaline phosphatase were in the normal range. Two of the 14 patients showed an increased serum immunoreactive parathyroid hormone (iPTH) concentration. Serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D) were normal but the average 24,25-dihydroxyvitamin D (24,25-$(OH)_2$D) was low.

The histomorphometrically determined trabecular bone volume of an iliac crest biopsy appeared to be low in 6 patients. The average active bone resorption and osteoid seams were increased, while the average osteoblast seams were within the normal range. Treatment with 1α-(OH)D_3 raised ^{47}Ca intestinal absorption and 24h urinary Ca excretion significantly at 3 and 6 months and at 6 months serum iPTH concentration and 24h urinary hydroxyproline excretion had fallen significantly in the treated group. During treatment with 1α-(OH)D_3 the serum 1,25-$(OH)_2$D and 24,25-$(OH)_2$D levels increased significantly.

In all cases of the 1α-(OH)D_3 group, the second bone biopsy, taken at the end of the treatment period, showed a decrease of active resorption and a more positive course of trabecular bone volume than the biopsy of the placebo treated counterpart: trabecular bone volume remained constant or even increased in the 1α-(OH)D_3 group. In both groups osteoid seams and osteoblast seams did not change significantly. We conclude that treatment with 1α-(OH)D_3 during 6 months inhibits the increased bone resorption seen in glucocorticoid induced bone disease, while bone formation is not suppressed by this therapy.

INTRODUCTION

The pathophysiology of osteoporosis associated with GC excess is complex. The well-known decrease of intestinal absorption of Ca (Gallagher et al., 1973) and increase of renal loss of Ca (Wajchenberg et al., 1969) may both stimulate PTH secretion. In short-term experiments a direct stimulatory effect of GC on the secretion of PTH has also been observed (Fucik et al., 1975; Au, 1976). Thus secondary hyperparathyroidism may contribute to the loss of bone in patients treated chronically with pharmacological doses of GC. The significance of changes in vitamin D status for the pathogenesis of glucocorticoid bone disease remains controversial. Both normal and decreased serum levels of 25-(OH)D (Klein et al., 1977) and 1,25-$(OH)_2$D (Chesney et al., 1978; O'Regan et al., 1979; Seeman et al., 1980) have been reported. Intending to counteract the effects of GC excess on intestinal absorption of Ca, and to inhibit PTH secretion and bone resorption, we have conducted a double-blind placebo-controlled trial with 1α-$(OH)D_3$ in patients on chronic GC treatment. We report here the biochemical and histological data obtained during this trial.

PATIENTS AND METHODS

Fourteen patients treated chronically (range 6-192 months) with a pharmacological dose of GC were matched in pairs for sex, age, dose of GC and underlying disease (Table 8.1). After obtaining informed consent individuals from each pair entered the study within a 3 month period to minimize seasonal bias. No medication with known influence on bone metabolism was used. All patients with chronic obstructive lung disease used a β-adrenergic drug and a xanthine-derivative. Dietary intake of Ca was at least 500 mg daily. Patients were not immobilized. All women were premenopausal. Renal disease was excluded and the two patients with liver disease were in a stable phase with normal values for serum albumin and coagulation factors. One patient from each pair was treated with a daily dose of 2 μg 1α-$(OH)D_3$ (Etalpha®, Leo Pharmaceutical Products, Holland) for 6 months, while the other patient received a placebo (in double-blind fashion). In retrospect the duration of previous GC treatment appeared to be somewhat longer in the 1α-$(OH)D_3$ treated group than in the placebo group (mean 81 versus 57 months).

One patient (no 2 of the 1α-$(OH)D_3$ treated group) had a crush fracture of one thoracic vertebra. Rib fractures had occurred in patient no 1 of the placebo group. During the period of treatment no additional fractures occurred. In 7 other patients radiological density of the spine appeared to be decreased. Four of these were in the placebo group. Bone mineral content was measured by photon absorptiometry at the distal third of the right radius (Norland-Cameron absorptiometer). This measurement was carried out before treatment and subsequently at intervals of 3 months. The initial values reported in Table 8.1 were corrected for bone width (BMC/BW, g/cm^2). In our laboratory mean values ($\pm$1SD) for

TABLE 8.1

CLINICAL CHARACTERISTICS AND BONE MASS PARAMETERS AT THE START OF THE TRIAL

	PLACEBO						1α-$(OH)D_3$					
pair	sex F/M	age yrs	diagnosis	average daily dose of prednisone during trial mg	bone mineral content* g/cm^2	trabecular bone volume[B] %	sex F/M	age yrs	diagnosis	average daily dose of prednisone during trial mg	bone mineral content g/cm^2	trabecular bone volume %
1	F	30	COLD	22	0.67	11.1	F	21	COLD	16.5	0.69	16.6
2	M	60	COLD	15	0.69	15.4	M	54	arthritis	15	0.66	15.3
3	F	42	COLD	10	0.68	21.2	F	36	COLD	3 x 0.5 dexa	0.58	17.3
4	F	24	CALD	13.5	0.46	15.9	F	21	CALD	10	0.61	17.2
5	F	35	SLE	15	0.69	23.6	F	28	SLE	20	0.63	19.4
6	F	37	SLE	9.5	0.68	20.3	F	27	SLE	10	0.65	14.5
7	F	45	COLD	10	0.68	11.1	F	51	COLD	10	0.58	22.0

COLD: Chronic Obstructive Lung Disease; CALD: Chronic Active Liver Disease
* Photonabsorptiometry of the radius. For normal range see Methods
[B] Histomorphometry of iliac crest bone. For normal values see Methods

normal women (age 20-60 years, (n = 59) and for normal men (age 20-60 years, n = 36) are: 0.68 ± 0.06 and 0.77 ± 0.06 g/cm^2, respectively.

Before treatment, after 3 and 6 months of treatment and 3 months after the conclusion of the treatment period, serum Ca, P, creatinine, total protein and alkaline phosphatase were determined by means of a Technicon-Autoanalyzer in a blood sample obtained after an overnight fast. At the same time, iPTH was determined by radioimmuoassay (RIA) with a sheep antiserum reacting with the mid-region (44-68) of the PTH molecule (kindly assayed by Dr. W.H.L. Hackeng, Municipal Hospital Bergweg, Rotterdam). Levels of serum vitamin D metabolites were determined as previously described (Juttmann et al., 1981[a]). In Table 8.2 normal values are given as means over the year (Juttmann et al., 1981[b]). Intestinal absorption of ^{47}Ca was studied by means of an external counting method (Armac, right forearm) (Juttmann et al., 1978) together with the 24h urinary excretion of Ca. Before and after 6 months of treatment urinary hydroxyproline was determined in 2-4 successive 24 h collections. A collagen poor diet was used for 3 days before and during the collection period. Hydroxyproline was determined after resin uptake and hydrolysis (Hypronosticon, Organon Teknika).

At the beginning and at the end of the treatment period bone biopsies were taken from the iliac crest 3 cm dorsal to the anterior superior iliac spine on contralateral sites successively, using a trephine with an internal diameter of 6 mm. Undecalcified specimens were embedded in methyl metacrylate and cut into sections of 5 μm. From each biopsy 6 sections were measured at distances of at least 100 μm. For each section this involved an area of 13.7 mm^2. Staining was done with a modified Von Kossa procedure and histomorphometry was performed by means of x-y tabloid connected to a PDP-11 computer using a specially developed program (Birkenhäger-Frenkel et al., 1980). Parameters for bone density (trabecular bone volume as a percentage of the section area measured), bone formation (osteoid and osteoblast seams length as percentage of the total trabecular perimeter) and bone resorption, (number of osteoclasts per section area and active resorption i.e. interface of osteoclasts and mineralized bone surface as a percentage of total trabecular perimeter) were measured. For the age groups involved the means of the normal trabecular bone volume vary from 21.6 to 23.5% (Meunier et al., 1973). The normal values of active resorption have been calculated from the data of Bordier & Tun Chot (1972) and Merz & Schenk (1970). For the comparison of the changes of parameters as observed in the individuals of matched pairs, Wilcoxon's matched-pairs signed-ranks test was used. For correlations Spearman's rank correlation test was applied.

RESULTS

Initial investigations

Biochemistry: The average initial values for most biochemical parameters fell within the normal range, one exception being the level of 24,25-$(OH)_2$D (Table 8.2). Initial serum iPTH level was above the normal range in only 1 patient of each

TABLE 8.2

BIOCHEMICAL PARAMETERS AT THE START OF THE STUDY, AFTER 3 AND 6 MONTHS TREATMENT WITH 1α-(OH)D_3(1α) OR PLACEBO (PI) AND 3 MONTHS AFTER DISCONTINUING MEDICATION

	reference range or mean ± 1 SD	initial mean ± 1 SD		3 months mean Δ		6 months mean Δ		3 months off treatment mean Δ	
serum		PI	1α	PI	1α	PI	1α	PI	1α
Ca	2.25 - 2.75 mmol/l	2.36 ± 0.09	2.35 ± 0.13	+0.03	+0.06	-0.03	+0.07	-0.02	+0.01
iPTH	2 - 12 pmol/l	7.2 ± 4.4	7.4 ± 4.8	-0.8	-1.7	+2.6	-3.1*	+3.0	+4.1
25-(OH)D	54.3 ± 22.1 nmol/l	50.6 ± 24.5	51.1 ± 36.2	+4.3	-6.0	-0.4	-5.5	+3.8	-11.8
24,25-$(OH)_2$ D	4.6 ± 2.2 nmol/l	2.1 ± 1.1	2.1 ± 1.0	-0.1	+1.1*	+0.9	+1.8	+0.4	+0.4
1,25-$(OH)_2$ D	160 ± 54 pmol/l	149.0 ± 35.1	110.8 ± 34.0	+2.7	+50.2*	-19.9	+38.8	-14.0	+7.1
urine 24 h									
Ca	< 275 mg	189 ± 81	178 ± 86	+18	+242*	+30	+178*	+27	-18
hydroxyproline[■]	15 - 43 mg	30 ± 3	37 ± 13			-6	-18*		
intestinal absorption of ^{47}Ca	28.5 - 55.0 %	42.9 ± 8.0	48.4 ± 8.2	+3.4	+21.0*	+4.0	+22.1*	+4.1	+1.0

Δ: difference between the mean and the mean initial value
* $p<0.05$: Wilcoxon's matched-pairs signed-ranks test
(comparison of Δ values obtained for the individuals of each pair)
■ 6 pairs

group. Only 1 patient of the placebo group showed a subnormal fractional intestinal absorption of ^{47}Ca and in 3 patients (2 of the placebo group) the 24h urinary excretion of Ca exceeded 275 mg.

Bone histomorphometry: In 7 of the 14 patients the trabecular bone volume (%) was low (Table 8.1 and Fig. 8.1). The mean active resorption (%) (Table 8.3) as well as the osteoid seams (%) turned out to be high, while osteoblast seams (%) were normal.

Bone mineral content. All the initial values except one were in the normal range (Table 8.1).

TABLE 8.3

DIFFERENCES IN MORPHOMETRIC RESULTS BETWEEN FIRST AND SECOND ILIAC CREST BIOPSIES OF MATCHED PATIENT PAIRS

	PLACEBO		1α-(OH)D_3		Wilcoxon's matched-pairs signed-ranks test	
parameters	pre-treatment mean	6 months mean	pre-treatment mean	6 months mean	p value	normal values
trabecular bone volume (%)	16.9	14.5	17.5	19.9	< 0.05	22.0*
active resorption (%)	1.10	1.24	0.92	0.34	< 0.05	0.01 - 0.30#

* Meunier et al. (1973)
# Calculated from Bordier & Tun Chot (1972) and Merz & Schenk (1970)

Double-blind placebo-controlled trial with 1α-(OH)D_3

Biochemistry: Comparing the paired individuals we found no changes in serum Ca (corrected for total protein), P, alkaline phosphatase and 25-(OH)D concentrations during the observation period. Hypercalcaemia did not occur in any of the patients treated with 1α-(OH)D_3. ^{47}Ca absorption and 24h urinary excretion of Ca appeared to have increased significantly after 3 and 6 months of treatment with 1α-(OH)D_3 as compared to the course of the values in the placebo group, while iPTH showed a decrease which was significant after 6 months treatment, followed by a significant increase 3 months after the conclusion of the trial (as compared to the value after 6 months treatment). As expected, the serum level of 1,25-$(OH)_2$D rose during treatment with 1α-(OH)D_3 (after 3 months of treatment significantly). Simultaneously the concentration of 24,25-$(OH)_2$D had also increased significantly (Table 8.2). After 6 months treatment a significant decrease of 24h urinary hydroxyproline excretion was found in the 1α-(OH)D_3 treated patients. Three months after terminating the treatment, none of the parameters studied differed from the values found at the beginning of the trial in both groups.

Bone histomorphometry: In Table 8.3 the changes of the values of trabecular bone volume (%) and active resorption (%) of the individuals treated with 1α-(OH)D_3

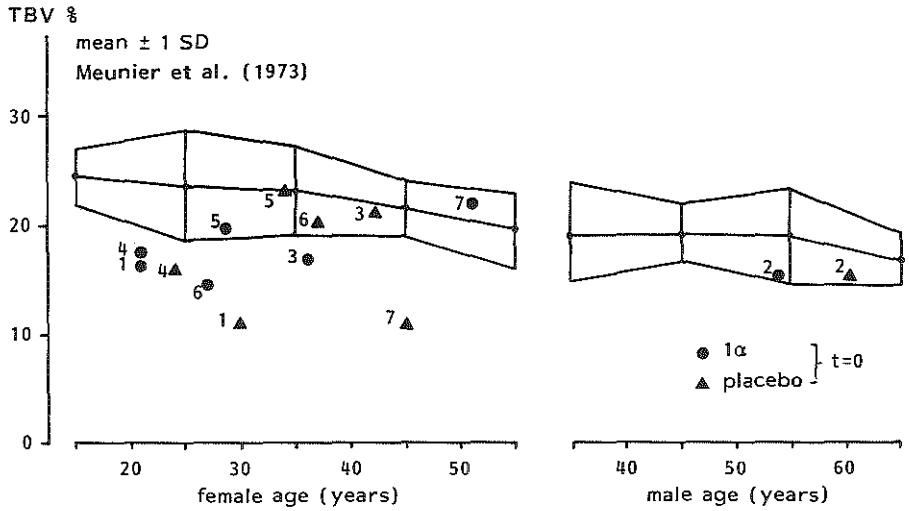

Figure 8.1 Initial trabecular bone volume percentage (TBV%) of the patients compared to age related normal values after Meunier et al. (1973). The numbers correspond to the patient pairs as listed in Table 8.1.

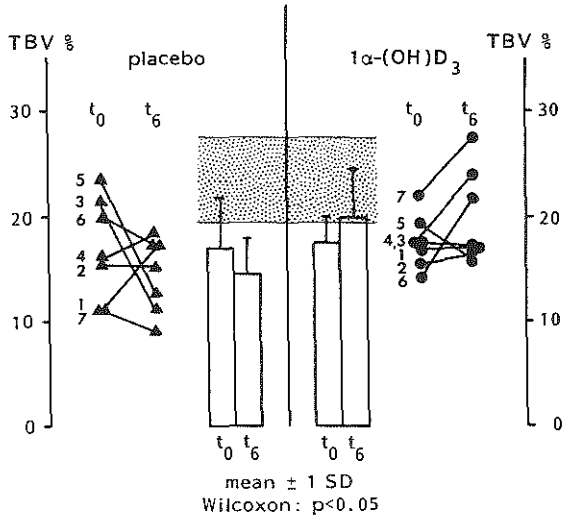

Figure 8.2 Individual and mean ± 1 SD values for trabecular bone volume percentage (TBV%) of the patients treated with placebo or 1α-(OH)D_3 at the start (t_0) and 6 months later at the end of the treatment period (t_6). The shaded area indicates the normal values (mean ± 1 SD) for females in the third decade after Meunier et al. (1973).

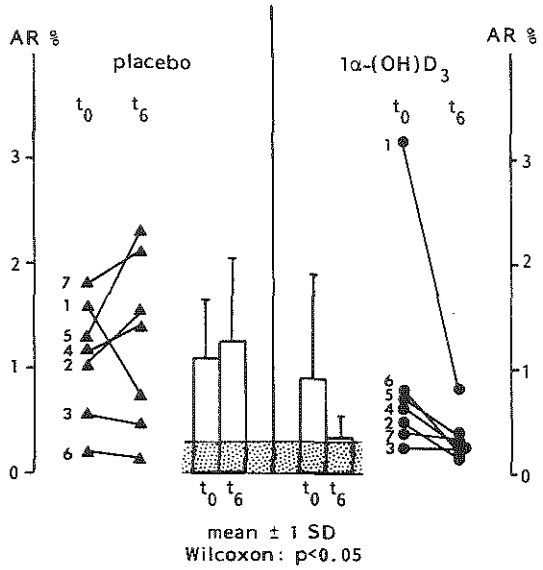

Figure 8.3 Individual and mean ± 1 SD values for active resorption percentage of trabecular bone surface (AR%) in the patients treated with placebo or 1α-(OH)D_3 at the start (t_0) and the end (t_6) of the treatment period. The shaded area indicates the normal range as calculated from data of Bordier & Tun Chot (1972) and Merz & Schenk (1970).

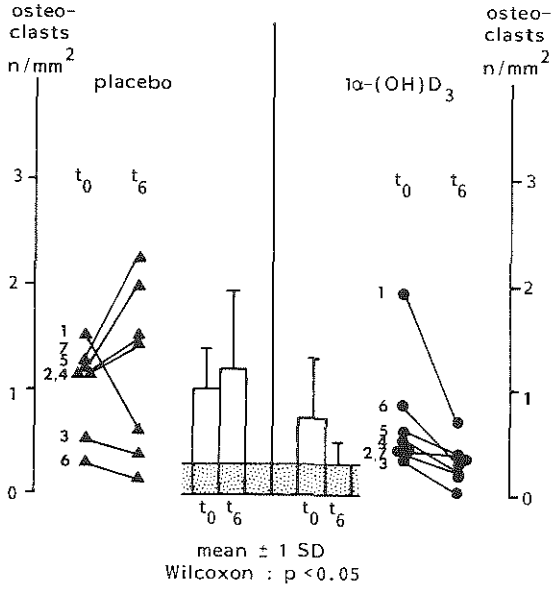

Figure 8.4 Individual and mean ± 1 SD values for the number of osteoclasts per surface unit section area of trabecular bone in the patients treated with placebo or 1α-(OH)D_3 at the start (t_0) and the end (t_6) of the treatment period. The shaded area indicates the normal range after Bordier & Tun Chot (1972).

are compared with those of his/her placebo-treated counterpart. Comparing the changes of trabecular bone volume observed in each pair of patients, the 1α-$(OH)D_3$ treated individuals showed a larger increase or smaller decrease (Fig. 8.2). During treatment with 1α-$(OH)D_3$ active resorption and the number of osteoclasts per section area (Fig. 8.3 and 8.4) decreased in all patients. Whenever a decrease was found in a patient of the placebo-group, it was smaller than in the matched 1α-$(OH)D_3$ treated patient. Bone formation parameters (osteoid seams (%), osteoblast seams (%)) did not change significantly within the pairs of patients. *Bone mineral content* did not change significantly in either group during the treatment period.

DISCUSSION

In general the initial concentration in serum of Ca, iPTH and the three vitamin D metabolites studied was normal (Table 8.2). The only exception is the subnormal average level of 24,25-$(OH)_2D$. Although it has been suggested that this metabolite of vitamin D might be important for the mineralization of bone matrix by a direct action on bone (Kanis et al., 1978), its biological activity or role has not yet been established (Papapoulus et al., 1980). Although increased levels of iPTH in serum in chronic GC excess have been reported (Bressot et al., 1979; Hahn et al., 1979[b]), we found a supranormal concentration in only two patients. Mean urinary Ca and hydroxyproline excretion were also normal. Initial values of 1,25-$(OH)_2D$ levels were not low, in contrast to what has been reported by some authors (Chesney et al., 1978; O'Regan et al., 1979) and in agreement with the normal average intestinal absorption of ^{47}Ca we observed. Initial ^{47}Ca absorption, urinary Ca and levels of the three metabolites of vitamin D studied did not correlate with each other. However, initial serum iPTH levels were positively correlated with the 1,25-$(OH)_2D$ concentrations ($r_s = 0.53$, $p < 0.05$).

During treatment with 1α-$(OH)D_3$, a significant rise of ^{47}Ca absorption and urinary Ca excretion was seen after 3 and 6 months (Table 8.2). Serum iPTH levels were significantly lowered after 6 months. Urinary hydroxyproline (measured in 6 pairs) after 6 months treatment also fell significantly (Table 8.2). The decrease of these two parameters did not correlate. The serum level of 2 of the 3 vitamin D metabolites studied, 1,25-$(OH)_2D$ and 24,25-$(OH)_2D$, were found to have risen after 3 months treatment with 1α-$(OH)D_3$. The formation of 24,25-$(OH)_2D$ from 25-(OH)D in the kidney is known to be stimulated by 1,25-$(OH)_2D$ (Tanaka & DeLuca, 1974). We have as yet no explanation for the fact that after 6 months treatment with 1α-$(OH)D_3$ the levels of these 2 metabolites were no longer significantly increased in comparison with the initial values. The significance of the correction by 1α-$(OH)D_3$ of the initially low 24,25-$(OH)_2D$ concentrations for the changes in bone histology remains to be assessed. Three months after the conclusion of the trial serum iPTH, 1,25- and 24,25-$(OH)_2D$, urinary Ca and ^{47}Ca absorption all had returned to the initial value.

The fact that no change in the bone mineral content of the radius occurred in both groups of patients is not surprising, as at this site it is almost exclusively cortical bone which is measured.

The initial histomorphometrically determined trabecular bone volume (%) was negatively correlated with active resorption (%) ($r_s = 0.54$, $P < 0.025$). It is therefore not surprising that the 6 patients who had a subnormal trabecular bone volume, also showed the greatest degree of bone resorption. No relationship was found between trabecular bone volume and active resorption on one hand and iPTH, 1,25-$(OH)_2D$ and ^{47}Ca absorption on the other.

The raised active bone resorption, that was observed in the majority of our patients, might be due to an enhanced sensitivity of the bone to PTH. During short-term administration of pharmacological doses of GC, responses of bone and kidney cells to PTH have been shown to be increased with regard to the production of cAMP and 1,25-$(OH)_2D$ (Chen & Feldman, 1978; Gennari et al., 1981; Braun et al., 1982).

After 6 months of treatment with 1α-$(OH)D_3$, active bone resorption had diminished in all 7 patients and this parameter became normal in 6 of them. The same applied to the total number of osteoclasts. Bone resorption and bone formation are normally rather tightly coupled processes (Frost, 1963), which implies that bone formation is dependent upon immediately preceding bone resorption in the same area. It may therefore be of importance that in our patients treated for 6 months with rather a high dose of 1α-$(OH)D_3$ bone resorption was not completely inhibited. Trabecular bone volume either had increased or had decreased to a lesser extent than in the placebo treated counterparts. It appears that the enhanced activity of bone resorbing cells[(+)] had been counteracted by the administration of 1α-$(OH)D_3$ by inhibition of the PTH secretion, either directly or by the stimulation of intestinal Ca absorption. No correlation was found between the extent of the decrease of active bone resorption or the increment of trabecular bone volume and the decrease of the serum iPTH level, the increase of 1,25-$(OH)_2D$ concentration or the increase of intestinal Ca absorption. On the other hand, the degree of inhibition of active bone resorption appeared to be reflected in the extent of the fall in urinary hydroxyproline ($r_s = 0.71$, $0.10 > P > 0.05$, $n = 6$). The somewhat surprising fact that the degree of inhibition of bone resorption seems to correlate with the change of hydroxyproline excretion, but not with the decrease of the iPTH level may be due to a lack of sensitivity of the radio-immunological technique or to the involvement of other bone resorption-determining factors.

Hahn et al. (1979[b]), who treated patients with GC osteoporosis with 25-hydroxyvitamin D, similarly found a rise of ^{47}Ca absorption and of urinary Ca and suppression of iPTH values. The only change in bone histomorphometry they found after 12 months (comparing seven 25-$(OH)D_3$-treated patients with seven unmatched control patients) was a decreased number of osteoclasts in the cortical part of the biopsies. The more positive result we found, after 6 months treatment with 1α-$(OH)D_3$, must be due partly to the different design of our study, allowing us to apply Wilcoxon's matched-pairs signed-ranks test in which even a less negative course of a parameter within a pair contributes to a significant result of

[(+)] It might be better to use here the term enhanced recruitment of bone resorbing cells.

the test. Sørensen et al., (1977) administered 2 μg of 1α-(OH)D_3 for 3-4 months to 3 patients with prednisone induced bone loss and 7 patients with senile osteoporosis: fractional ^{47}Ca absorption and urinary Ca rose, while the authors claimed that histomorphometry in both types of patients showed changes comparable to those reported here. Several authors (Marshall & Nordin, 1977; Gallagher et al., 1979) have found that the low intestinal absorption of Ca in postmenopausal women is caused by a low production of 1,25-$(OH)_2D_3$. Evidently, the condition can be corrected by administration of 1,25-$(OH)_2D_3$, leading to a more positive balance of Ca skeletal mass. One should not, however, see too much of an analogy between GC induced bone loss and postmenopausal osteoporosis. In GC excess the negative skeletal balance is probably caused by two mechanisms. Apart from an increased resorption of bone that can be counteracted by raising 1,25-$(OH)_2D$ levels and intestinal Ca absorption, excess of GC also causes inhibition of protein synthesis by the bone forming cells (Raisz, 1980). It is not clear yet, whether administration of 1α-(OH)D_3 can counteract this inhibition to any degree. From the work reported here we conclude that there is a need for a similar study over a longer period, using a lower dosage of 1α-(OH)D_3 in which a parameter of bone formation rate is measured.

For references see the common reference list.

CHAPTER IX
(Addendum to Chapter VIII)

Comparison of intestinal radiocalcium absorption and intestinal calcium balance in patients treated with glucocorticoids with and without 1α-(OH)D_3.

INTRODUCTION

Complementary to the study of fractional intestinal ^{47}Ca absorption in GC treated patients, this Chapter contains Ca balance data obtained in 5 of the 7 patients pairs from the study, described in Chapter VIII. Initial data and data obtained after 6 months treatment with placebo or 1α-(OH)D_3 have been compared.

METHODS

During the Ca balance studies the patients were admitted to the metabolic ward. Each balance study started with an equilibrium period of 3 days, followed by 2 collection periods of 6 days. Using the diet history of the patient (calories, Ca and P), the balance diet was prepared from stock for the whole study and the meals were stored frozen until use. The diet was analyzed for Ca in duplicate. Polyethyleneglycol (PEG,MW 4000) was administered in a dosage of 400 mg 3 times daily as nonabsorbable marker to correct faecal collection. The start and end of a collection period were marked by faecal appearence of orally administered carmine-red. In the diet (1 day collection) and faeces (6 day collection period) Ca content was determined after dilution with distilled deionized water, homogenization and destruction with HNO_3 (65% v/v) and $HClO_4$ (70% v/v) by means of EGTA titration of a fluorescent Ca-calceïne complex (a modification of the method described by Wallach & Steck (1963)). The faecal PEG concentration was determined by a turbidimetric method (Boulter & McMichael, 1970). The faecal recovery of PEG was always between 80 and 90%. Net intestinal Ca absorption was obtained by substracting daily faecal loss of Ca from daily oral Ca intake. Net intestinal Ca absorption is composed of true intestinal absorption minus endogenous Ca excretion. From Wilkinson's study (1976) of the metabolic Ca balances in

more than 200 individuals, we derived a mean ± 2 SD value for the fractional net Ca absorption of 28 (± 12)% (at an average daily Ca intake of 1100 mg).

The fractional intestinal ^{47}Ca absorption, determined by an external counting method as described by Juttmann et al. (1978), was always performed at the end of the balance study, while the patient used his normal diet. Our normal range is 28,5 - 55,0% (n=19).

For statistical analysis Student's t-test and Spearman's correlation test were employed.

RESULTS

Both dietary fractional Ca absorption and fractional ^{47}Ca absorption were measured in 5 of the 7 patient pairs described in Chapter VIII (no. 1, 3, 4, 5 and 7), at the start of the study (t_o) and after 6 months (t_6). At t_o the fractional intestinal absorption measured by radiocalcium was below the normal range in only one

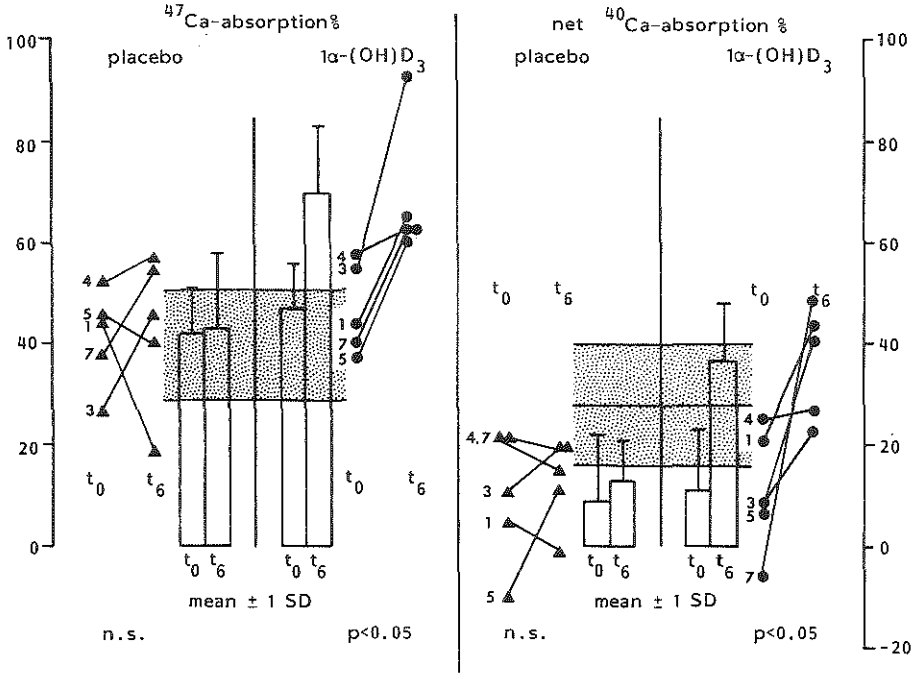

Figure 9.1 Individual and mean values ± 2 SD for fractional intestinal absorption of ^{47}Ca and net intestinal ^{40}Ca absorption in patients on chronic GC at the start of the study (t_o) and after treatment with placebo or 1α-$(OH)D_3$ for 6 months (t_6). The shaded areas indicate the normal range of ^{47}Ca absorption (Juttmann et al., 1978) and the mean value ± 2 SD of net ^{40}Ca absorption (Wilkinson, 1976). The numbers correspond to the patient pairs described in Chapter VIII.

patient (patient number 3 from the placebo-group). However, the net Ca absorption derived from the balance was lower than -2 SD of the mean value from Wilkinson et al. in 6 of the 10 patients at the start of the study (Fig. 9.1). In patient number 5 from the placebo- and patient number 7 from the 1α-group a net intestinal Ca secretion was found. In all 5 patients in the 1α-group a significant increase of the intestinal Ca absorption was observed with both methods, after treatment with 1α-(OH)D_3 (2 μg daily for 6 months). There was no correlation between the results of both measurements in these 5 patient pairs at t_o and t_6 (Spearman's correlation test).

DISCUSSION

Reeve et al. (1980[b]) compared fractional net intestinal Ca absorption, calculated from balances, with radiocalcium absorption studies and found a rather weak correlation with maximum and mean rate of absorption of the tracer. Comparing the two methods for the determination of intestinal Ca absorption, one has to realize that the processes measured are not identical.

Radiocalcium absorption is studied by us with 200 mg of Ca as carrier in an easily absorbable, largely ionized form in an individual after an overnight fast. These circumstances enhance Ca absorption. In the radiocalcium absorption test one measures ^{47}Ca uptake into a large body pool of ^{40}Ca over 5 hours. Assuming that within this period no substantial amount of ^{47}Ca is secreted into the intestinal lumen, one measures only unidirectional Ca flux from lumen to tissue, mainly in the proximal part of the small intestine. When fractional Ca absorption is calculated from the difference between dietary intake and faecal excretion, one obtains the net result of Ca-efflux and Ca-influx (from and to the intestinal lumen) along the entire length of the gastrointestinal tract. The fact, that in normal people a higher mean value (40%) has been found for unidirectional Ca-efflux (expressed as fraction radiocalcium absorbed) than for fractional net Ca absorption calculated from the Ca balance (30%), is probably the result of Ca secretion (Ca-influx). The 10% difference between the results of these two methods agrees quite well with the reported endogenous Ca secretion of 100-200 mg per day at an intake of 1000 mg Ca (Nordin et al., 1976).

The discrepancy between the values obtained by the two methods is accentuated in patients treated with a moderately high dose of GC. This is in agreement with an enhancement of intestinal Ca secretion by excess GC as reported for dogs (Collins et al., 1962), rats (Ferretti et al., 1978) and by us in rat small intestinal loops (Chapter VII).

During treatment of patients on GC with 1α-(OH)D_3 the intestinal Ca absorption increased mainly by stimulation of the unidirectional Ca-efflux, represented by ^{47}Ca fractional absorption, to supranormal levels. As a result the net intestinal absorption of Ca was normalized.

Studies in man on the intestinal secretion of parenterally administered radiocalcium during GC excess have to be done to confirm this hypothesis.

CONCLUSIONS

The intestinal fractional absorption of ^{47}Ca, as measured over 5 hours after an oral load by an external counting method, is normal in patients treated chronically with a moderately high dose of GC (cf. Lekkerkerker et al., 1970; Klein et al., 1977). Net Ca absorption expressed as fraction of dietary Ca, however, is subnormal in a considerable number of these patients, probably as a result of an increased endogenous Ca secretion. Treatment with 1α-$(OH)D_3$ can reverse this low net Ca absorption by increasing the true Ca absorption (via stimulation of the unidirectional Ca-efflux) to supranormal levels.

CHAPTER X

General discussion

In this Chapter we will try to fit the results of the clinical trial and the other experimental investigations into a concept of the pathogenesis of GC induced bone disease.

An *increased sensitivity* of the renal 1α-hydroxylase to the stimulatory action of PTH might be responsible for the rise of serum 1,25-$(OH)_2$D during short-term GC administration in men (Chapter V). Teleologically, this rise is a beneficial reaction that, by stimulation of the intestinal absorption of Ca and P, could compensate for the primary induction of intestinal and renal loss of both minerals induced by high dose GC. The rapidity of the rise of the serum 1,25-$(OH)_2$D level during infusion with hydrocortisone (see Chapter V) makes it improbable that it is the result of the inhibition of intestinal Ca absorption, leading in turn to stimulation of 1α-hydroxylase by a decrease of serum ionized Ca. It is well-known that the negative influence of excess GC on intestinal Ca absorption and the stimulatory effect on renal Ca secretion become apparent after days. In the hypoparathyroid patients we found an increase of urinary P and in normal individuals a decrease of serum P during hydrocortisone infusion within hours. The possibility that this stimulates 1α-hydroxylase is not excluded. However experimentally, serum P levels have to be very low to demonstrate the enhancing effect on 1α-hydroxylase activity (Tanaka & DeLuca 1973). Furthermore, in our study of the normal and hypoparathyroid individuals, using a prednisone dosage that admittedly was much lower than that of the experiments with hydrocortisone, we saw no change of the serum P level during the first 5 days.

The high level of 1,25-$(OH)_2$D is not sustained, however, when the administration of prednisone is continued in normal individuals for more than 5 days, in contrast to patients with PHP. Possible explanations for the transient character of this response are given in Chapter V. The diminished intestinal Ca absorption and the activation of vitamin D by GC excess are probably different, not causally related responses.

The loss of Ca, caused by decreased intestinal absorption and increased renal clearance, leads to secondary hyperparathyroidism and this is reflected by an increased number of osteoclasts on histological examination of trabecular bone. However, in our patients chronically treated with GC serum PTH levels have predominantly been found in the normal range. The combination of the characteristics of bone histology and the generally normal PTH concentrations point to an *increased sensitivity* of the bone to the resorbing action of PTH caused by the GC excess. An alternative explanation would be the involvement of an unknown bone-resorbing agent. In vitro the release of bone mineral is diminished after treatment with high doses of GC, indicating that osteoclasts are depressed in their activity. Probably an increased recruitment of osteoclasts in combination with a decrease in bone (matrix) formation results in a net loss of bone.The amount of bone formed and resorbed per unit time at the level of the BMU's is schematically illustrated for the normal situation and during GC excess in Fig. 10.1^{A} and 10.1^{B}.

Enhancement or permissive effects of GC on the action of other hormones (e.g. glucagon and epinephrine) have frequently been reported in the literature (for review see Baxter & Forsham, 1972; Granner, 1979). At the cellular level this type of effect could be obtained through the influence of GC on *second messengers as cAMP and cGMP* [(+)]. The amplification of hormonal effects by GC probably lies beyond the generation of cAMP (Lamberts et al., 1975; Granner, 1979), but data are not conclusive. The activity of cAMP-phosphodiesterase is reported to be inhibited by GC in several tissues (Manganiello & Vaughan, 1972; Schmidtke et al., 1976). The *"Ca-generating system"* is another pathway through which hormones can transduce their effect on target cells. GC can induce, probably via gene activation, the synthesis of an antiphospholipase A_2 protein (macrocortin or lipomodulin), as recently described in guinea pig lung tissue and rat peritoneal leucocytes (Flower & Blackwell, 1979; Blackwell et al., 1980). Changes in composition and conformation of cellular membranes, altered turnover of phospholipids, decreased release and production of arachidonic acid and prostaglandins could result. Via such mechanisms not only the availability of membrane hormone-receptors can change, but above all cellular Ca-influx can be limited by a decreased production of substances with a "Ca-gating" action (arachidonic acid, certain prostaglandins). In theory, GC can modulate the effects of other hormones via such changes of intracellular Ca as second messenger.

The decreased influx of Ca in BBMV prepared from small intestine of rats pretreated with triamcinolone is probably an example of decreased "Ca-gating" in the cell membrane (Chapter VI). The active vitamin D metabolite 1,25-$(OH)_2D_3$ was capable to antagonize this GC effect. Active vitamin D metabolites are thought to enhance Ca entry across the intestinal brush border membrane by changes in the phospholipid metabolism (Matsumoto et al., 1981) resulting in an increased phosphatidylcholine/phosphatidylaethanolamine ratio. One might speculate whether a similar interaction between GC and 1,25-$(OH)_2D$ applies to other target tissues of both hormones, such as bone and kidney cells. A decreased

[(+)] cyclic guanosine $3^{1},5^{1}$-monophosphate

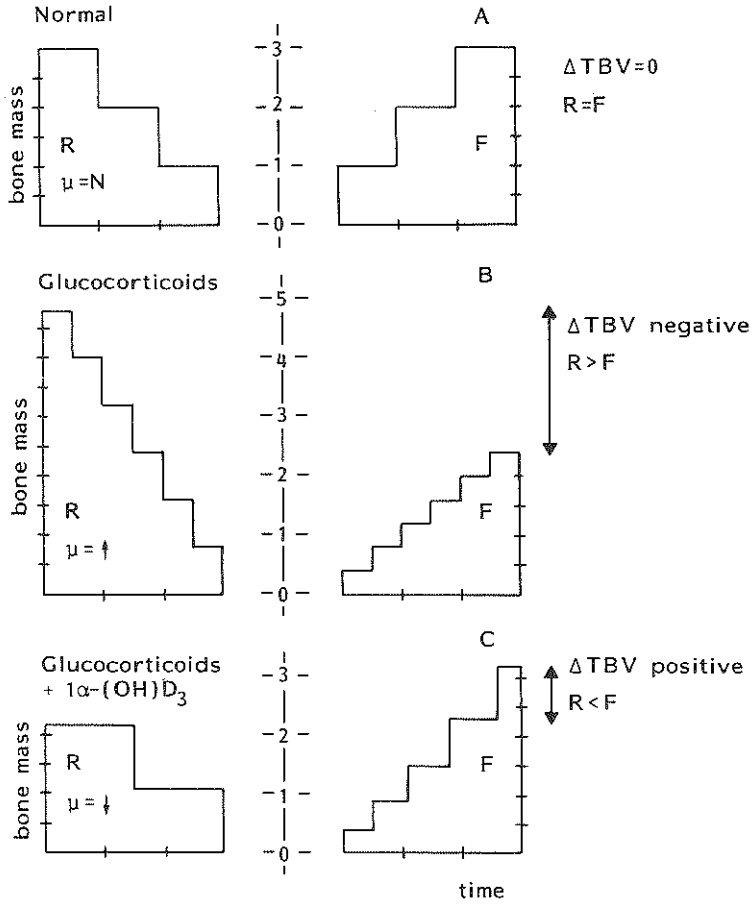

Figure 10.1 In this figure the activation frequency (μ) of basic multicellular units (BMU) per unit time is illustrated schematically. The amount of bone resorbed (R) or formed (F) within a given time period is presented by means of "stair-cases". The height of each step represents the amount of bone resorbed or formed by one BMU during the whole period of its existence. Net gain or loss of trabecular bone volume (TBV) is obtained by the difference between R and F.

A. In the *normal* situation R and F are in equilibrium or R exceeds F slightly.

B. We postulate that during *hypercortisolism* an increased recruitment of BMU's ($\mu\nearrow$) is accompanied by a decreased bone forming and bone resorbing activity per BMU.

C. When 1α-hydroxylated derivatives of vitamin D are given to patients chronically treated with GC, the activation frequency of bone resorbing units is depressed to subnormal ($\mu\searrow$) The amount of bone resorbed per BMU may be increased. Bone formation is determined by a progressively decreasing activation frequency of bone forming units (resulting from persistent coupling of bone formation and resorption) and a gradually increasing amount of bone formed per newly activated BMU when 1α-(OH)D_3, directly or indirectly, has growth factor-like activity.

availability of intracellular Ca, induced by GC excess in renal tubular cells and precursors of bone cells, could act as extra stimulus for the renal 1α-hydroxylase enzyme and the recruitment of (pre)osteoclasts by PTH. However, one should be careful not to see too much of an analogy between BBMV from rat small intestinal villous cells, specialized in Ca transport and membranes of other cells. Furthermore, when Ca-influx is inhibited, one would generally expect a diminished biological response to hormones for which Ca probably also acts as a second messenger.

The beneficial effect of treatment with 1α-$(OH)D_3$ on the progression of GC induced bone disease is demonstrated by the results of the clinical trial, described in Chapter VIII. There are several loci where active vitamin D metabolites interfere in the pathogenesis of GC osteoporosis. This is schematically illustrated in Fig. 10.2 and concerns the following main points:

I. Fractional intestinal absorption of ^{47}Ca and circulating levels of 1,25-$(OH)_2D$ generally were within the normal range in the patients chronically treated with GC. For the net intestinal ^{40}Ca absorption we found values below the normal range in a large number of these patients (Chapter IX). Extrapolation of the data on the influence of high doses of GC on Ca fluxes in the experiments with rat ligated small intestinal loops in situ suggests, that GC have an important effect on intestinal Ca absorption by increasing the Ca secretory flux. Treatment with 1α-$(OH)D_3$ (being very rapidly converted to 1,25-$(OH)_2D_3$) will compensate for this intestinal Ca loss by stimulating the unidirectional intestinal Ca absorption in the proximal part of the small intestine to a supranormal level. A hypothesis on the effects of GC and/or 1,25-$(OH)_2D_3$ on cellular and paracellular fluxes of Ca in the rat small intestine, derived from our data on in situ experiments with loops (Chapter VII) and studies with BBMV (Chapter VI), is schematically illustrated in Fig. 10.3.

II. Active vitamin D derivatives inhibit the increased secretion of PTH and/or the enhanced recruitment of pre(osteoclasts) by the stimulation of intestinal Ca absorption and possibly also directly. In the histomorphometric study of bone from our patients treated for 6 months with 1α-$(OH)D_3$, this was reflected by the striking decrease of the parameters for bone resorption. In the 1α-$(OH)D_3$ treated patients the positive course of trabecular bone volume, as compared to the placebo treated ones and the lack of change of the parameters for bone formation, are evidence at least for an unhampered bone formation.

III. A stimulatory effect of the treatment with 1α-$(OH)D_3$ on bone formation cannot be excluded. Factors reported to have a beneficial effect on bone formation and/or mineralization are Ca and P (Bingham et al., 1974; Harris et al., 1976), 1,25-$(OH)_2D$ (Larsson et al., 1977) and 24,25-$(OH)_2D$ (Kanis et al., 1978). We found low serum levels of 24,25-$(OH)_2D$ in about half of the patients treated with medium dose of GC, which levels rose during the administration of 1α-$(OH)D_3$.

The increment of intestinal Ca absorption in the patients treated with 1α-$(OH)D_3$ was largely excreted in the urine. In some patients this led to an unacceptable rise in urinary Ca excretion. The data on the Ca balances, expressed as percentage of dietary Ca intake, in 5 patient pairs before and after 6 months treatment with 1α-$(OH)D_3$ or placebo, are presented in Fig. 10.4. After 6 months

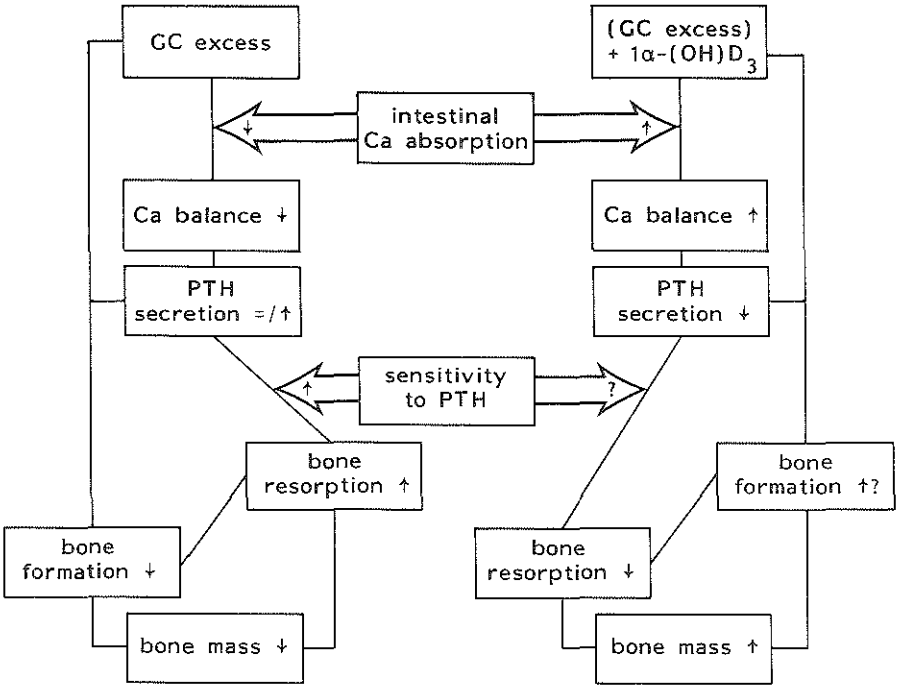

Figure 10.2 Main features in the pathogenesis of glucocorticoid induced bone disease and the influence of treatment with 1α-$(OH)D_3$ on its progress in a model derived from the results of our studies.

I An increased intestinal Ca secretion, induced by GC excess, is counteracted by 1α-$(OH)D_3$ via stimulation of the intestinal Ca-efflux (lumen to tissue).

II An increased bone resorption, resulting from an increased PTH secretion or sensitivity to PTH, is suppressed by the active vitamin D derivative.

III Bone formation is unhampered, may be even stimulated, during treatment (6 months) with 1α-$(OH)D_3$.

treatment with the vitamin D derivative the Ca balance showed a tendency to be less negative than in the untreated state and was also less negative than in the placebo-group, although not significantly, due to rather large inter- and intra-individual variations. This finding in combination with the positive course of trabecular bone mass and the decreased number of osteoclasts as found on bone histomorphometry, is strong evidence against a bone mobilizing action of the $1,25\text{-}(OH)_2D_3$, formed from 1α-$(OH)D_3$, in the dosage used. This is in contrast to the negative Ca balances induced by the administration of $1,25\text{-}(OH)_2D_3$ (3 μg daily) to normal volunteers (Maierhofer et al., 1983). In that study biochemical evidence of increased bone resorption was demonstrable in association with supraphysiologic serum levels of $1,25\text{-}(OH)_2D$. This may reflect an important difference in reactivity of normal bone and bone of hypercortisolistic patients.

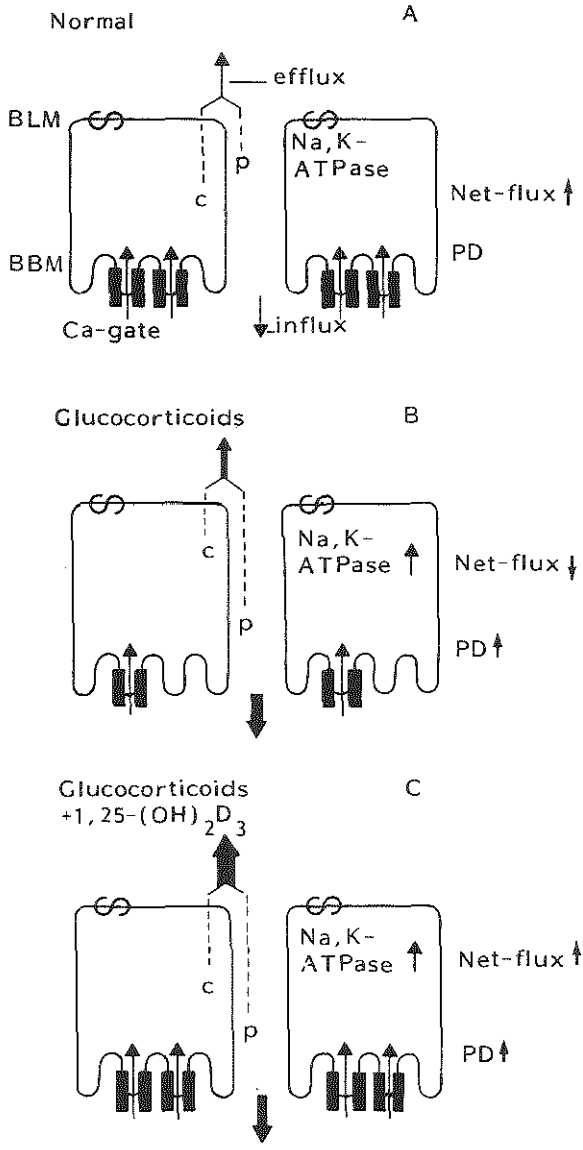

Figure 10.3 Schematic illustration of a hypothetical model for transepithelial Ca fluxes in the small intestine. Ca-efflux is composed of cellular (c) and paracellular (p) components. Ca-influx is exclusively paracellular (p). During hypercortisolism Ca-efflux is probably increased by "solvent drag" via the paracellular pathway, while the number of "Ca-gates" in the brush border membrane (BBM) is diminished. The Ca-influx, however, is stimulated to a larger extent, driven by the more negative (lumen to serosa) transmural potential difference (PD) resulting from a stimulated Na,K-ATPase at the basolateral membrane (BLM). Treatment with the active vitamin D metabolite 1,25-$(OH)_2D_3$, during GC excess, restores the number of "Ca-gates" in the BBM and the still augmented Ca-influx is overruled by stimulation of the transcellular Ca-efflux.

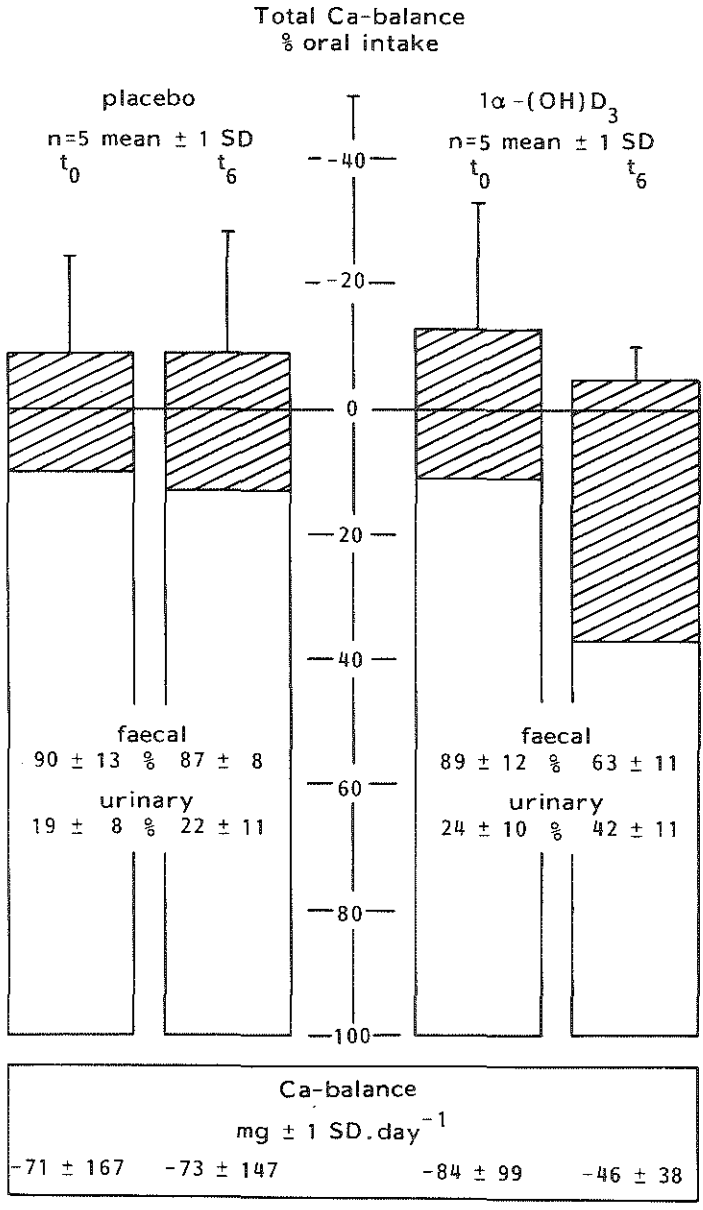

Figure 10.4 Components of Ca balance presented as percentage of food intake in 5 patients pairs who were chronically treated with GC, before (t_o) and after 6 months (t_6) administration of 1α-(OH)D_3 (2 μg daily) or placebo. For methods see Chapter IX. The Ca intake is plotted downwards from the base line as 100 percent. Faecal and urinary Ca are represented by open or hatched bars, respectively. At the bottom mean values ± 1 SD for the Ca balance are given in mg/day.

Whether the beneficial effect of 1α-$(OH)D_3$ in GC induced bone disease is the result of suppression of secondary hyperparathyroidism only, or is (also) achieved by stimulation of bone formation, is yet to be established.

To assess the exact value of 1α-hydroxylated vitamin D derivatives for the prevention or treatment of GC induced bone disease, in our opinion, a second study with these compounds has to be performed, using a lower dosage for a longer observation period and in a larger group of patients with the use of non-invasive bone mass measurements (dual photonabsorptiometry and/or computed tomography). One argument to carry out such a study is the possibility that the beneficial effect on bone mass observed after 6 months treatment, would subsequently be arrested. Bone formation being then depressed to a similar degree as bone resorption has been previously. This would be the result of persistence of the well-known tight coupling of bone formation to bone resorption. Seen in this light intermittent treatment with active vitamin D metabolites would be an attractive alternative, as has been reported by Rasmussen et al. (1980), treating postmenopausal patients with calcitonin. When, however, one or more vitamin D metabolites, c.q. 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, also act(s) as bone "growth factor(s)", the balance between bone formation and bone resorption will remain positive beyond 6 months treatment. At the level of the BMU's this may be visualized as in Fig. 10.1[C].

With regard to the treatment of postmenopausal and other age related forms of osteoporosis with 1α-hydroxylated vitamin D derivatives, both positive (Gallagher et al., 1982) and negative (Christiansen et al., 1981; Finn et al., 1982) results have been reported. The negative results have been obtained with the rather low dosage of 0,25-0,50 μg 1,25-$(OH)_2D_3$ daily. One should realize however, that the pathogenesis of GC induced osteoporosis, albeit complex, probably is more uniform than that of age related osteoporosis and that increased intestinal loss of Ca is an important factor in the negative bone and Ca balances of GC induced bone disease.

SUMMARY

Chronic exposure to supraphysiologic levels of glucocorticoids is associated with the loss of mainly trabecular bone. The result is osteopenia, defined as a subnormal bone mass for age, sex, race and body size, while bone mineralization is normal. The clinical term osteoporosis is generally used when pathologic fractures, especially compression fractures of the vertebrae, occur due to insufficient supportive strength. In this sense osteoporosis is a frequent complication in patients with endogenous or exogenous hypercortisolism. However, one has to take into account that the underlying disease for which glucocorticoids are prescribed may in itself also cause abnormal bone loss (Chapters I and II).

An important problem in the diagnosis of osteopenia is that, with the available non-invasive radiological and isotope techniques, one only obtains quantitative or semiquantitative information about the local bone mass, usually in a peripheral (appendicular) part of the skeleton with a preponderance of cortical bone. Histomorphometrical measurements in biopsy material from the iliac crest provide information on the amount of trabecular bone at that site and also quantitative data on bone resorption and bone formation, reflecting the dynamic process of bone remodeling (Chapter II).

By their influence on bone turnover, regulators of Ca homeostasis may play an important role in the pathogenesis of several forms of osteoporosis. In this context the most important regulators of Ca metabolism are parathyroid hormone, calcitonin and vitamin D, which are discussed in Chapter III. The most frequent form of osteoporosis (primary) is probably multifactorial in its pathogenesis and one should realize that changes reported in circulating concentrations of parathyroid hormone and other factors involved in Ca homeostasis and bone cell activity may not be related to the cause of the process. The regulation of vitamin D metabolism and the mode of action of the most active metabolite 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D) on its target organs are discussed in Chapter III. Clinically, the serum level of Ca, P and parathyroid hormone are the main regulators of the formation of 1,25-$(OH)_2$D in the kidney. The action of 1,25-$(OH)_2$D is to stimulate the flux of Ca (and P) into the extracellular fluid from intestine, bone and kidney.

The mode of action of 1,25-$(OH)_2$D has the characteristics of that of a steroid hormone: cytoplasmatic and nuclear receptors have been demonstated and de novo protein synthesis is induced by gene activation.

In Chapter IV a review is given of the literature on the effect of glucocorticoids, at physiological and supraphysiological levels, on regulators of Ca homeostasis and bone cell activity. A negative Ca and P balance, resulting from a decreased intestinal Ca and P absorption and an increased renal clearance of both minerals, appears to be of crucial importance. In this respect the (slightly) elevated levels of circulating parathyroid hormone, as frequently reported in patients with hypercortisolism, represent probably a state of secondary hyperparathyroidism. It is also possible that the secretion of parathyroid hormone is directly stimulated by glucocorticoids. Histomorphometric analysis of bone biopsy material from patients with hypercortisolism often reveals features of

increased *bone resorption* together with decreased bone formation. The increased number and/or activity of osteoclasts is not always associated with supranormal serum levels of parathyroid hormone. This could be explained by an augmented response of (pre)osteoclasts to parathyroid hormone induced by glucocorticoids. Glucocorticoids interfere with *bone formation* by direct inhibition of the synthesis of bone matrix by osteoblasts and by inhibition of the production of local growth factors or of hormones indirectly involved in the process of bone formation.

Glucocorticoid excess affects the target organs of vitamin D (bone, gut, kidney). The possible influence of glucocorticoids on the metabolism (activation) of vitamin D itself has therefore been studied rather extensively. Definite conclusions, however, cannot be drawn from the data derived from these studies.

In Chapter V we describe the effect of short-term hypercortisolism on the metabolism of vitamin D in man, as studied by us, at different levels of parathyroid gland activity. During the administration of 30 mg of prednisone daily we found within 1-3 days a rise of the concentration of serum 1,25-$(OH)_2$D in 6 normal individuals and 8 patients with primary hyperparathyroidism. This rise turned out to be dependent on the presence of parathyroid gland activity, as in 5 patients with hypoparathyroidism no rise of serum 1,25-$(OH)_2$D could be detected when the same dose of prednisone was administered for 3-4 days. In normal individuals the intravenous infusion of hydrocortisone induced within 8 hours a similar increase of the serum 1,25-$(OH)_2$D level. An increased sensitivity of the renal 1α-hydroxylase for parathyroid hormone could explain the observed reaction. No changes in the concentrations of parathyroid hormone, Ca and P in the serum were found, capable of stimulating the production of 1,25-$(OH)_2$D. Furthermore the increase of serum 1,25-$(OH)_2$D after injection of parathyroid extract in a patient with hypoparathyroidism was distincly enhanced, when the extract was administered in combination with hydrocortisone. In normal individuals the rise of serum 1,25-$(OH)_2$D during prednisone administration was transient, the concentration of 1,25-$(OH)_2$D returning to basal levels after 9 days of prednisone administration. From these data it seems highly unlikely that in man changes in the metabolism of vitamin D are responsible for the decrease of the intestinal absorption of Ca, observed during administration of similar doses of glucocorticoids for 1-2 weeks. It is, on the other hand, also unlikely that the early and transient rise of serum 1,25-$(OH)_2$D is a reaction to the inhibition of intestinal Ca absorption by glucocorticoids.

We studied the mechanism by which glucocorticoid excess lowers intestinal Ca absorption, at subcellular level, using brush border membrane vesicles prepared from epithelial cells from the proximal half of rat small intestine (Chapter VI). Five days after the injection of a long-acting suspension of triamcinolone we found a decrease of the initial rate of Ca uptake in the vesicles, presumably resulting from a decrease of the number or activity of Ca carriers in the brush border membrane. This decrease of the initial Ca uptake, that probably is associated with a fall in the transcellular Ca transport, could be antagoni-

zed by the administration of 1,25-$(OH)_2D_3$.

Although the fall in the Ca uptake at the level of the brush border membrane induced by glucocorticoid excess, is in agreement with the effect reported by others in experiments with intestinal segments in vitro, it is probably not the transcellular Ca flux that determines the lowered net Ca absorption under these conditions. We studied Ca absorption in isolated rat small intestinal loops in situ after treatment with triamcinolone. Treatment with triamcinolone increased the unidirectional Ca-efflux (from lumen to tissue) in the proximal part of the small intestine and this flux correlated positively with the net absorption of solvent. However, Ca-influx (secretion) into the small intestinal lumen was stimulated to an even greater extent, resulting in a decreased net Ca absorption along the whole length of the small intestine (Chapter VII).

The fraction of Ca absorbed from the diet turned out to be subnormal in 6 of 10 patients on long-term treatment with a medium dose of glucocorticoids (Chapter IX). In only one of these 6 patients the fraction of orally administered ^{47}Ca (with 200 mg ^{40}Ca as carrier) absorbed in 5 hours was also subnormal. Assuming that the fractional intestinal absorption of radioactive calcium is representative for unidirectional Ca-efflux, the discrepancy between the two methods can be explained by glucocorticoids stimulating the endogenous secretion of Ca. This is in agreement with the results of our in situ experiments with rat small intestinal segments (Chapter VII). After treatment with 1α-$(OH)D_3$ (2 μg daily) for 6 months the fraction of Ca absorbed from the diet was normalized in patients on glucocorticoids, while the fractional absorption of ^{47}Ca was increased to a supranormal level.

The effect of treatment with 1α-$(OH)D_3$ (2 μg daily) on the bone disease, induced by long-term glucocorticoid treatment, was studied in a double-blind placebo-controlled trial of 6-9 months with 7 patient pairs matched for sex, age, underlying disease and dose of glucocorticoids (Chapter VIII). In general the biochemical parameters for Ca- and bone-metabolism (serum Ca, P, alkaline phosphatase, parathyroid hormone, vitamin D metabolites and urinary excretion of Ca and hydroxyproline) were all in the normal range at the beginning of the study. One exception was formed by the low levels of 24,25-dihydroxyvitamin D (24,25-$(OH)_2D$) in the serum present in more than 50 percent of the participants. Although serum parathyroid hormone was found to be elevated at the start of the study in only one patient, histomorphometry of trabecular bone from the iliac crest revealed an increased number of osteoclasts in nearly every patient, often in combination with a subnormal trabecular bone volume.

During treatment with 1α-$(OH)D_3$ the fractional intestinal absorption of ^{47}Ca and urinary Ca excretion increased significantly in comparison to the placebo treated control patient. After 6 months treatment with 1α-$(OH)D_3$ the concentrations of serum parathyroid hormone and the urinary excretion of hydroxyproline had fallen, while in the treated group the concentrations of both 1,25-$(OH)_2D$ and 24,25-$(OH)_2D$ in the serum had risen. Histomorphometric comparison of trabecular bone from a second iliac crest biopsy, taken after 6 months treatment with 1α-$(OH)D_3$ or placebo, with material from the first

biopsy, showed a decrease of bone resorption parameters in all patients treated with 1α-(OH)D_3, while the course of trabecular bone volume was always more positive in the patients treated with 1α-(OH)D_3 than in the placebo-treated controls. Changes in the parameters for bone formation (osteoblast seams, osteoid seams) occurring during treatment, did not differ significantly between the treatment groups (Wilcoxon's matched-pairs signed-ranks test).

On the basis of the results of the studies described in this thesis a concept of the pathogenesis of glucocorticoid induced bone disease and of the possibilities for its treatment or prevention with active vitamin D derivates is outlined in Chapter X. An enhancement of the sensitivity of the renal tubular cells and of (pre)osteoclasts for the action of parathyroid hormone by glucocorticoid excess is postulated. An antagonism between glucocorticoids and vitamin D expresses itself not only at the level of Ca uptake in brush border membrane vesicles, but also in an improvement of the intestinal Ca absorption and in a beneficial effect on trabecular bone mass when patients, who chronically use glucocorticoids, are treated with a 1α-hydroxylated derivative of vitamin D. It is not known whether the improvement in trabecular bone mass results from the suppression of parathyroid hormone secretion or is (also) due to a growth factor-like activity of the active vitamin D metabolite.

SAMENVATTING

Langdurige blootstelling aan suprafysiologische doses glucocorticoiden gaat gepaard met een snel verlies van vooral trabeculair bot. Het resultaat is osteopenie: een verminderde botmassa voor leeftijd, geslacht, ras en lichaamsbouw, terwijl de mineralizatie van het bot nagenoeg normaal is. Wanneer hierbij als gevolg van onvoldoende steunfunctie van het skelet pathologische fracturen, met name van de wervellichamen optreden, spreken we van osteoporose. Osteoporose is dan ook een frequent voorkomende complicatie bij het syndroom van Cushing en bij het gebruik van hoge doses glucocorticoiden als therapeuticum. Ook de ziekten waarvoor glucocorticoiden worden voorgeschreven kunnen zelf aanleiding zijn tot een abnormaal snel verlies van botmassa. Bij de bestudering van het effect van glucocorticoiden op het bot van deze patienten dient hiermee rekening te worden gehouden (Hoofdstukken I en II).

Een belangrijk probleem bij het vaststellen van osteopenie is het feit dat men met de thans gangbare, niet-invasieve radiologische en isotoop-technieken kwantitatieve of semikwantitatieve gegevens krijgt van de lokale botmassa, als regel in een perifeer deel van het skelet waar corticaal bot overheerst. Histomorfometrie van botbiopsiemateriaal b.v. uit de cristailiaca biedt naast de informatie over de hoeveelheid (trabeculair) bot ter plaatse ook kwantitatieve gegevens over de botafbraak en de daaraan gekoppelde botaanmaak als een momentopname in het dynamische proces van botombouw (Hoofdstuk II).

Regulatoren van de Ca homeostase kunnen door hun invloed op botafbraak en botaanmaak een belangrijke rol spelen in de pathogenese van de diverse vormen van osteoporose. De belangrijkste hormonale regulatoren van de Ca huishouding: parathyroidhormoon, calcitonine en vitamine D en hun mogelijke betekenis voor de pathogenese van osteoporose worden behandeld in Hoofdstuk III. Primaire osteoporose is een multifactorieel bepaalde toestand, waarbij veranderingen in spiegels van bijschildklierhormoon of andere factoren betrokken bij Ca homeostase en botcel activiteit, niet causaal behoeven te zijn. De regulatie van het vitamine D metabolisme en het werkingsmechanisme van het meest active vitamine D metaboliet 1,25-dihydroxyvitamine D (1,25-$(OH)_2$D) op zijn doelwitorganen worden eveneens in Hoofdstuk III besproken. In klinische zin zijn voor de regulatie van de vorming van 1,25-$(OH)_2$D in de nier de concentratie van Ca, P en bijschildklierhormoon in het serum de belangrijkste factoren. De werking van 1,25-$(OH)_2$D is er vooral op gericht de influx van Ca (en P) in de extracellulaire ruimte te stimuleren vanuit het maagdarmkanaal, het skelet en de nier.

Het werkingsmechanisme van 1,25-$(OH)_2$D heeft vele kenmerken van dat van een steroid hormoon: er zijn receptoren in het cytoplasma en op de kern en er vindt de novo eiwitsynthese plaats via gen-activatie door het 1,25-$(OH)_2$D.

In Hoofdstuk IV wordt een literatuuroverzicht gegeven van het effect van fysiologische en suprafysiologische spiegels glucocorticoiden op regulatoren van de Ca homeostase en de activiteit van botcellen. Een negatieve Ca en P balans als gevolg van een verminderde intestinale Ca en P absorptie en een

toegenomen renale klaring van beide mineralen lijkt van beslissende betekenis voor de pathogenese van glucocorticoid osteoporose. In het licht hiervan is de regelmatig verhoogd gevonden bijschildklierhormoon spiegel in het serum van deze patienten te beschouwen als een uiting van secundaire hyperparathyreoidie. Daarbij kan de bijschildklierhormoon secretie ook direct door glucocorticoiden worden gestimuleerd. Bij histomorfometrisch onderzoek van botbiopten van hypercortisolistische patienten worden veelal kenmerken gevonden van een verhoogde *botafbraak* en een verminderde botaanmaak. Aangezien deze histologisch manifest verhoogde botafbraak lang niet altijd gepaard gaat met een verhoogde concentratie bijschildklierhormoon in het serum, lijkt het erop dat de "response" van het botweefsel op parathyreoidhormoon versterkt kan zijn onder invloed van glucocorticoiden. Glucocorticoiden interferen met de *botaanmaak* door direkte remming van de synthese van botmatrix door de osteoblasten en door remming van de produktie van lokale groeifactoren of van hormonen die op indirekte wijze betrokken zijn bij de botvorming.

Teneinde de effecten van een overmaat glucocorticoiden op de doelwitorganen van vitamine D (bot, darm, nier) te kunnen verklaren is de mogelijke invloed van glucocorticoiden op het metabolisme (de activatie) van vitamine D zelf vrij uitgebreid onderzocht. Eensluidende conclusies kunnen uit deze onderzoekingen echter niet worden getrokken.

Wij onderzochten het effect van kortdurend hypercortisolisme op de activatie van vitamine D bij de mens (Hoofdstuk V). Tijdens toediening van 30 mg prednison per dag vonden wij bij 6 gezonde proefpersonen en 8 patienten met primaire hyperparathyreoidie binnen 1-3 dagen een stijging van de 1,25-$(OH)_2$D concentratie in het serum. Deze stijging bleek afhankelijk te zijn van de aanwezigheid van de bijschildklieren, aangezien bij 5 patienten met hypoparathyreoidie tijdens toediening van 30 mg prednison per dag gedurende 3-4 dagen door ons geen stijging van het 1,25-$(OH)_2$D gehalte van het serum werd waargenomen. Bij normale mensen bleek infusie van hydrocortison reeds in 8 uur een dergelijke verhoging van de 1,25-$(OH)_2$D spiegel te veroorzaken. Een verhoging van de gevoeligheid van het renale 1α-hydroxylase voor parathyreoidhormoon zou dit reactiepatroon kunnen verklaren, o.m. omdat geen veranderingen werden waargenomen van de concentraties van Ca, P en bijschildklierhormoon in het serum. Bovendien werd bij een patiente met hypoparathyreoidie een veel sterkere stijging van het serum 1,25-$(OH)_2$D na injectie van parathyreoid extract gezien als dit tesamen met hydrocortison werd toegediend. Bij de normale proefpersonen was de stijging van de serum 1,25-$(OH)_2$D concentratie tijdens prednison toediening van voorbijgaande aard met terugkeren van de spiegel tot het uitgangsniveau na 9 dagen toediening van prednison. Op grond van deze gegevens lijkt het uitgesloten dat bij de mens een afname van de intestinale Ca absorptie, zoals regelmatig waargenomen tijdens behandeling met vergelijkbare dosering glucocorticoiden gedurende 1-2 weken, zou kunnen berusten op een stoornis in het metabolisme van vitamine D. Anderzijds zou ook een compensatoire toename van de 1,25-$(OH)_2$D spiegel in het serum, als gevolg van de remming van de absorptie van Ca in de darm door glucocorticoiden, niet goed

verantwoordelijk kunnen zijn voor de waargenomen snelle en voorbijgaande stijging van de 1,25-$(OH)_2D$ spiegel.

De wijze waarop glucocorticoiden in overmaat de intestinale absorptie van Ca verlagen werd door ons, op subcellulair niveau, onderzocht door gebruik te maken van zgn. "brush border membrane vesicles" bereid uit epitheelcellen van de proximale helft van de dunne darm van de rat (Hoofdstuk VI). Vijf dagen na injectie van een triamcinolon-depot-preparaat vonden wij een verminderde initiele snelheid van opname van Ca in de vesicles, vermoedelijk als gevolg van een afname van het aantal of de activiteit van de Ca carriers in de membraan. Deze afname, die waarschijnlijk gepaard gaat met een vermindering van het transcellulaire Ca transport, bleek te kunnen worden tegengegaan door gelijktijdige toediening van 1,25-$(OH)_2D_3$. In onze handen kon op het niveau van de "brush border" door een overmaat glucocorticoiden een afname van de Ca opname worden geinduceerd, die gelijk gericht is aan hetgeen door anderen gevonden is in experimenten met dunne darmlissen in vitro. Wij constateerden echter, bij onderzoek met in situ geisoleerde dunne darmlissen van de rat, dat onder deze omstandigheden een verlaagde netto Ca absorptie door andere mechanismen wordt bepaald. In het proximale deel van de dunne darm bleek bij de met triamcinolon behandelde ratten de unidirectionele Ca-efflux (van lumen naar weefsel) gestimuleerd te zijn, positief gecorreleerd met de netto absorptie van water. Daarbij was een nog sterkere toename van de Ca-influx (secretie) o.i.v. triamcinolon verantwoordelijk voor de lagere netto absorptie van Ca (Hoofdstuk VII).

Bij 6 van 10 patienten, die langdurig middelmatig hoge dosis glucocorticoiden gebruikten, bleek de fractionele intestinale absorptie van ^{40}Ca uit de voeding subnormaal (Hoofdstuk IX). Bij slechts 1 van deze 6 patienten was de in 5 uur geabsorbeerde fractie van oraal (met 200 mg ^{40}Ca als carrier) toegediend ^{47}Ca ook verlaagd. Wanneer deze laatste meting representatief mag worden geacht voor de unidirektionele intestinale Ca-efflux, kan alleen een toegenomen endogene secretie van Ca verantwoordelijk zijn voor deze discrepantie. Dit is in overeenstemming is met de uitkomsten van onze darmlis experimenten bij de rat (Hoofdstuk VII). Onder behandeling met 1α-$(OH)D_3$ (2 μg dd.) gedurende 6 maanden kwam de geabsorbeerde fractie van Ca uit de voeding weer op normaal niveau bij de patienten die met glucocorticoiden werden behandeld, onder toename van de fractionele ^{47}Ca absorptie tot supranormaal.

Het effect van behandeling met 1α-$(OH)D_3$ (2 μg dd.) op de botafwijkingen bij langdurig gebruik van glucocorticoiden, werd nagegaan in een dubbelblind zg. "placebo-controlled" onderzoek van 6-9 maanden bij 14 patienten onderling paargewijs overeenkomend in geslacht, leeftijd, onderliggende ziekte en dosis glucocorticoiden (Hoofdstuk VIII). Voor de biochemische parameters van de Ca huishouding: serum Ca, P, alkalische fosfatase, vitamine D metabolieten en uitscheiding van Ca en hydroxyproline in de urine, werden aan het begin van het onderzoek over het algemeen normale waarden gevonden. Een uitzondering werd gevormd door de lage concentraties 24,25-$(OH)_2D$ in het serum, die bij meer dan de helft van de onderzochte patienten werden gevonden. Hoewel de concentratie bijschildklierhormoon in het serum bij slechts 1 van de

14 patienten verhoogd was, werd bij histomorfometrisch onderzoek van trabeculair bot uit de crista iliaca als regel een verhoogd aantal osteoclasten gevonden, veelal in combinatie met een te laag trabeculair botvolume. Onder behandeling met 1α-$(OH)D_3$ stegen de fractionele intestinale ^{47}Ca absorptie en de calciurie significant in vergelijking tot bij de met placebo behandelde patient(e), "gematched" als bovengenoemd. Na 6 maanden behandeling bleken de bijschildklierhormoon concentratie in het serum en de uitscheiding van hydroxyproline in de urine te zijn verlaagd, terwijl de concentraties van 1,25-$(OH)_2D$ en 24,25-$(OH)_2D$ in het serum stegen tijdens behandeling met 1α-$(OH)D_3$. Histomorfometrisch onderzoek van trabeculair bot van een tweede biopsie, genomen na 6 maanden behandeling met 1α-$(OH)D_3$ dan wel placebo, toonde een afname van de botresorptie parameters bij alle met 1α-$(OH)D_3$ behandelde patienten. Het verloop van het trabeculaire botvolume was steeds meer positief bij de met 1α-$(OH)D_3$ behandelde patient in vergelijking tot de met placebo behandelde "partner". Er werden geen verschillen in verloop van de parameters voor botvorming (osteoblastenzomen en osteoidzomen) gevonden bij de paarsgewijze vergelijking van de patienten.

Naar aanleiding van de onderzoeksresultaten beschreven in dit proefschrift, wordt in Hoofdstuk X een concept gegeven van de pathogenese en van de mogelijkheden tot behandeling van de door glucocorticoiden teweeggebrach te botaandoening met actieve vitamine D derivaten of -metabolieten. Hierbij wordt gepostuleerd dat onder invloed van een overmaat glucocorticoiden de gevoeligheid van de niertubuli en de (pre)osteoclasten voor de werking van bijschildklierhormoon toeneemt.

Een antagonisme tussen glucocorticoiden en vitamine D komt niet alleen tot uiting op het niveau van de Ca opname in "brush border membrane vesicles", maar ook in een verbeterde netto Ca absorptie in de darm en een anabool effect op de trabeculaire botmassa bij behandeling van patienten, die langdurig glucocorticoiden gebruiken, met een 1α-gehydroxyleerd derivaat van vitamine D. Het blijft onduidelijk of het positieve effect op de botmassa een gevolg is van de suppressie van de bijschildklierhormoon secretie of dat het actieve vitamine D metaboliet ook als "groeifactor" functioneert.

REFERENCES

Adams, N.D., Garthwaite, T.L., Gray, R.W., Hagen, T.C. and Lemann, J. jr. (1979). The interrelations among prolactin, 1,25-dihydroxyvitamin D and parathyroid hormone in humans. J. Clin. Endocrinol. Metab. 49, 628-630.

Adams, J.S., Wahl, T.O. and Lukert, B.P. (1981). Effects of hydrochlorothiazide and dietary sodium restriction on calcium metabolism in corticosteroid treated patients. Metabolism. 30, 217-221.

Adinoff, A.D. and Hollister, J.R. (1983). Steroid-induced fractures and bone loss in patients with asthma. N. Engl. J. Med. 309, 265-268.

Albright, F., Parson, W. and Bloomberg, E. (1941). Cushing's syndrome interpreted as hyperadrenocorticism leading to hypergluconeogenesis: result of treatment with testosterone propionate. J. Clin. Endocrinol. 1, 375.

Aldred, J.P., Kleszynski, R.R., Stubbs, R.K. and Bastian, J.W. (1971). Requirement of the adrenal for certain urine electrolyte effects of salmon calcitonin in rats. Proc. Soc. Exp. Biol. Med. 137, 1145-1151.

Aloia, J.F., Roginsky, M., Ellis, K., Shukla, K. and Cohn, S. (1974). Skeletal metabolism and body composition in Cushing's syndrome. J. Clin. Endocrinol. Metab. 39, 981-985.

Aloia, J.F., Ellis, K., Zanzi, I. and Cohn, S.H. (1975). Photon absorptiometry and skeletal mass in the treatment of osteoporosis. J. Nucl. Med. 16, 196-199.

Ananna, A., Eloy, R., Bouchet, P., Clendinnen, G. and Grenier, J.F. (1979). Effects of oral and parenteral corticosteroids on intestinal villous morphology and brush border enzymes in the rat. Lab. Invest. 41, 83-88.

Aronson, P.S. and Sacktor, B. (1975). The Na^+ gradient-dependent transport of D-glucose in renal brush border membranes. J. Biol. Chem. 250, 6032-6039.

Asknes, L. and Aarskog, D. (1982). Plasma concentrations of vitamin D metabolites in puberty: effect of sexual maturation and implications for growth. J. Clin. Endocrinol. Metab. 55, 94-101.

Atkins, D. and Peacock, M. (1975). A comparison of the effects of the calcitonins, steroid hormones and thyroid hormones on the response of bone to parathyroid hormone in tissue culture. J. Endocrinol. 64, 573-583.

Au, W.Y.W. (1976). Cortisol stimulation of parathyroid hormone secretion by rat parathyroid glands in organ culture. Science. 19, 1015-1017.

Austin, L.A. and Heath III, H. (1981). Calcitonin, physiology and pathophysiology. N. Engl. J. Med. 304, 269-278.

Avioli, L.V., Birge, S.J. and Lee, S.W. (1968). Effects of prednisone on vitamin D metabolism in man. J. Clin. Endocrinol. 28, 1341-1346.

Batt, R.M. and Peters, T.J. (1976). Effects of prednisolone on the small intestinal mucosa of the rat. Clin. Sc. Mol. Med. 50, 511-523.

Batt, R.M., Wells, G. and Peters, T.J. (1978). The effects of prednisolone on the rat enterocyte at a subcellular level. Clin. Sc. Mol. Med. 55, 435-443.

Baxter, J.D. and Forsham, P.H. (1972). Tissue effects of glucocorticoids. Am. J. Med. 53, 573-589.

Behar, J. and Kernstein, M.D. (1976). Intestinal calcium absorption: differences in transport between duodenum and ileum. Am. J. Physiol. 230, 1255-1260.

Bell, N.H., Stern, P.H., Pantzer, E., Sinha, T.K. and Deluca, H.F. (1979). Evidence that increased circulating 1,25-dihydroxyvitamin D is the probable cause for abnormal calcium metabolism in sarcoidosis. J. Clin. Invest. 64, 218-225.

Bentzel, C.J., Carbone, P. and Rosenberg, L. (1964). The effect of prednisone on calcium metabolism and Ca^{47} kinetics in patients with multiple myeloma and hypercalcemia. J. Clin. Invest. 43, 2132-2145.

Bijvoet, O.L.M. (1972). Renal phosphate excretion in man. Folia Med. Neerl. 15, 84-93.

Bikle, D.D., Zolock, D.T., Morrissey, R.L. and Herman, R.H. (1978). Independence of 1,25-dihydroxyvitamin D_3-mediated calcium transport from de novo RNA and protein synthesis. J. Biol. Chem. 25, 484-488.

Bikle, D.D., Morrissey, R.L., Zolock, D.T. and Rasmussen, H. (1981). The intestinal response to vitamin D. Rev. Physiol. Biochem. Pharmacol. 89, 63-142.

Bingham, P.J. and Raisz, L.G. (1974). Bone growth in organ culture: effects of phosphate and other nutrients on bone and cartilage. Calcif. Tissue Res. 14, 31-48.
Birkenhäger, J.C., v.d. Heul, R.O., Smeenk, D., v.d. Sluys Veer, J. and v. Seters, A.P. (1967). Bone changes associated with glucocorticoid excess. Proc. R. Soc. Med. 60, 1134-1136.
Birkenhäger-Frenkel, D.H., Clermonts, E.C.G.M. and Richter, H. (1980). Histomorphometry by means of an x-y tabloid. Design of a computer programme; disposition of equipment. Metab. Bone Dis. Relat. Res. 25, 453-457.
Blackwell, G.J., Carnuccio, R., DiRosa, M., Flower, R.J., Parente, L. and Persico, P. (1980). Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. Nature 287, 147-149.
Blumenkrantz, N and Asboe-Hansen, G. (1976). Cortisol effect on collagen biosynthesis in embryonic explants and in vitro hydroxylation of protocollagen. Acta Endocrinol. 83, 665-672.
Body, J.J. and Heath III, H. (1983). Estimates of circulating monomeric calcitonin: physiological studies in normal and thyroidectomized man. J. Clin. Endocrinol. Metab. 57, 897-903.
Böhm, P., Cöllü, H, Pitzel, L and König, A. (1979). Effect of dexamethasone on plasma concentrations of LH, FSH and testosterone in women with hirsutism. Endokrinologie. 73, 301-306.
Bordier, Ph.J. and Tun Chot, S. (1972). Quantitative histology of metabolic bone disease. Clinics Endocrinol. Metab. 1, 197-215.
Bouillon, R. and de Moor, P. (1974). Parathyroid function in patients with hyper- or hypoparathyroidism. J. Clin. Endocrinol. 38, 999-1004.
Bouillon, R., van Baelen, H., de Moor, P. (1977). The measurement of the vitamin D-binding protein in human serum. J. Clin. Endocrinol. Metab. 45, 225-231.
Bouillon, R., Geusens, P., DeQueker, J. and de Moor, P. (1979). Parathyroid function in primary osteoporosis. Clin. Sci. 57, 167-171.
Boulter, J.M. and McMichael, H.B. (1970). Modification of polyethylene glycol estimation suitable for use with small mammals. Gut. 11, 268-270.
Braun, J.J., Juttmann, J.R., Visser, T.J. and Birkenhäger, J.C. (1982). Short-term effect of prednisone on serum 1,25-dihydroxyvitamin D in normal individuals and in hyper- and hypoparathyroidism. Clin. Endocrinol. 17, 21-28.
Braun, J.J., Birkenhäger-Frenkel, D.H., Rietveld, A.H., Juttmann, J.R., Visser, T.J. and Birkenhäger, J.C. (1983). Influence of 1α-$(OH)D_3$ administration on bone and bone mineral metabolism in patients on chronic glucocorticoid treatment; a double-blind controlled study. Clin. Endocrinol. 18, 265-273.
Breslau, N.A., Zerwekh, J.E., Nicar, M.J. and Pak, C.Y.C. (1982). Effects of short-term glucocorticoid administration in primary hyperparathyroidism: comparison to sarcoidosis. J. Clin. Endocrinol. Metab. 54, 824-830.
Bressot, C., Meunier, P.J., Chapuy, M.C., Lejeune, E., Edouard, C. and Darby, A.J. (1979). Histomorphometric profile, pathophysiology and reversibility of corticosteroid-induced osteoporosis.
Brown, B.L., Albano, J.D.M., Ekins, R.P., Sgherzi, A.M. and Tampion, W. (1971). A simple and sensitive saturation assay method for the measurement of adenosine $3^1,5^1$-cyclic monophosphate. Biochem. J. 121, 561-562.
Brown, D.J., Spanos, E., Raptis, P. and MacIntyre, I. (1979). Effect of pregnancy, acromegaly, primary hyperparathyroidism and prolactinoma on 1,25-dihydroxyvitamin D in man. In: Vitamin D, basic research and its clinical application (Norman, A.W., Schaefer, K., v. Herrath, D., Grigoleit, H.G., Coburn, J.W., DeLuca, H.F., Mawer, E.B. and Suda, T. eds.), pp. 625-628. Walter de Gruyter & Co, Berlin, New York.
Bullamore, J.R., Wilkinson, R., Gallagher, J.C., Nordin, B.E.C. and Marshall, D.H. (1970). Effect of age on calcium absorption. Lancet ii, 535-537.
Bunim, J.J., Black, R.L., Lutwak, L., Peterson, R.E. and Whedon, G.D. (1958). Studies on dexamethasone, a new synthetic steroid in rheumatoid arthritis - a preliminary report. Arthritis Rheum. 1, 313-331.
Burstein, S., Chen, I. and Tsang, R.C. (1983). Effects of growth hormone replacement therapy on 1,25-dihydroxyvitamin D and calcium metabolism. J. Clin. Endocrinol. Metab. 56, 1246-1251.
Camanni, F., Losana, O, Massara, F. and Molinatti, G.M. (1967). Increased renal phosphate excretion in Cushing's syndrome. Acta Endocrinol. 56, 85-92.
Canalis, E. (1983[a]). The hormonal and local regulation of bone formation. Endocr. Rev. 4, 62-77.

Canalis, E. (1983[b]). Effect of glucocorticoids on type I collagen synthesis, alkaline phosphatase activity and deoxyribonucleic acid content in cultured rat calvariae. Endocrinology. 112, 931-939.
Caniggia, A., Gennari, C., Bianchi, V. and Guideri, R. (1963). Intestinal absorption of ^{45}Ca in senile osteoporosis. Acta Med. Scand. 173, 613-617.
Cann, C.E. and Genant, H.K. (1982). Cross-sectional studies of vertebral mineral using quantitative computed tomography. J. Comput. Assist. Tomogr. 6, 216-217.
Canterbury, J.M., Lerman, S., Claflin, A.J., Henry, H., Norman, A. and Reiss, E. (1978). Inhibition of parathyroid hormone secretion by 25-hydroxycholecalciferol and 24,25-dihydroxycholecalciferol in the dog. J. Clin. Invest. 61, 1375-1383.
Carré, M., Ayigbedé, O., Miravet, L. and Rasmussen, H. (1974). The effect of predisolone upon the metabolism and action of 25-hydroxy and 1,25-dihydroxyvitamin D_3. Proc. Nat. Acad. Sci. USA. 71, 2996-3000.
Castillo, L., Tanaka, Y., DeLuca, H.F. and Sunde, M.L. (1977). The stimulation of 25-hydroxyvitamin D_3-1α-hydroxylase by estrogen. Arch. Biochem. Biophys. 179, 211-217.
Charney, A.N., Kinsey, M.D., Myers, L., Giannella, R.A. and Gots, R.E. (1975). Na^+-K^+-activated adenosine triphosphatase and intestinal electrolyte transport. Effect of adrenal steroids. J. Clin. Invest. 56, 653-660.
Chen, T.L. and Feldman, D. (1978). Glucocorticoid potentiation of the adenosine $3^1,5^1$-monophosphate response to parathyroid hormone in cultured rat bone cells. Endocrinology. 102, 589-596.
Chen, T.L., Cone, C.M., Morey-Holton, E. and Feldman, D. (1983). 1,25-dihydroxyvitamin D_3, receptors in cultured rat osteoblast-like cells. J. Biol. Chem. 258,4350-4355.
Chertov, B.S., Williams, G.A., Norris, R.M., Baker, G.R. and Hargis, G.K. (1977). Vitamin A stimulation of parathyroid hormone: interactions with calcium, hydrocortisone and vitamin E in bovine parathyroid tissues and effects of vitamin A in man. Eur. J. Clin. Invest. 7, 307-314.
Chesney, R.W., Hamstra, A.J., Mazess, R.B., DeLuca, H.F. and O'Reagan, S. (1978). Reduction of serum 1,25 dihydroxyvitamin D_3 in children receiving glucocorticoids. Lancet ii, 1123-1125.
Choe, J., Stern, P. and Feldman, D. (1978). Receptor mediated glucocorticoid inhibition of protein synthesis in isolated bone cells. J. Steroid Biochem. 9, 265-271.
Christiansen, C., Christensen, M.S., Rødbro, P., Hagen, C. and Transbøl, I. (1981). Effect of 1,25-dihydroxy-vitamin D_3 in itself or combined with hormone treatment in preventing postmenopausal osteoporosis. Eur. J. Clin. Invest. II, 305-309.
Clark, I., Geoffroy, R.F. and Bowers, W. (1959). Effects of adrenal cortical steroids on calcium metabolism. Endocrinology. 64, 849-856.
Cohn, S.H., Vaswani, A., Zanzi, I., Aloia, J.F., Roginsky, M.S. and Ellis, K.J. (1976). Changes in bone chemical composition with age measured by total-body neutron activation. Metabolism. 25, 85-95.
Collins, E.J., Garrett, E.R. and Johnston, R.L. (1962). Effect of adrenal steroids on radio-calcium metabolism in dogs. Metabolism. 11, 716-726.
Constanzo, L.S., Sheehe, P.R. and Weiner, I.M. (1974). Renal actions of vitamin D in D-deficient rats. Am. J. Physiol. 226, 1490-1495.
Corradino, R.A. (1979). Embryonic Chick intestine in organ culture: hydrocortisone and vitamin D-mediated processes. Arch. Biochem. Biophys. 192, 302-310.
Courpron, P., Meunier, P., Bressot, C. and Giroux, J.M. (1976). Amount of bone in iliac crest biopsy. Significance of the trabecular bone volume. Its values in normal and pathological conditions. In: Bone histomorphometry (Meunier, P.J. ed.), pp. 39-53. Société de la Nouvelle Imprimerie Fournié, Toulouse, France.
Crilly, R.G., Marshall, D.H. and Nordin, B.E.C. (1979). Metabolic effects of corticosteroid therapy in postmenopausal women. J. Steroid Biochem. 11, 429-433.
Cushing, H. (1932). The basophil adenomas of the pituitary body and their clinical manifestations (pituitary basophilism). Bull. Johns Hopkins Hosp. 50, 137-195.
Deftos, L.J., Weisman, M.H., Williams, G.W., Karpf, D.B., Frumar, A.M., Davidson, B.J., Parthemore, J.G. and Judd, H.L (1980). Influence of age and sex on plasma calcitonin in human beings. N. Engl. J. Med. 302, 1351-1353.
Dempster, D.W., Arlot, M.A. and Meunier, P.J. (1983). Mean wall thickness and formation periods of trabecular bone packets in corticosteroid-induced osteoporosis. Calcif. Tissue. Int. 35, 410-417.
Dietel, M., Dorn, G., Montz, R. and Altenähr, E. (1979). Influence of vitamin D_3, 1,25-dihydroxy-

vitamin D_3, and 24,25-dihydroxyvitamin D_3 on parathyroid hormone secretion, adenosine $3_1,5_1$-monophosphate release and ultrastructure of parathyroid glands in organ culture. Endocrinology. 105, 237-245.
Dietrich, J.W., Canalis, E.M., Maima, D.M. and Raisz, L.G. (1979). Effects of glucocorticoids on fetal rat bone collagen synthesis in vitro. Endocrinology. 104, 715-721.
Doyle, F. (1972). Involutional osteoporosis. Clinics Endocrinol. Metab. 1(1). 143-167.
Duncan, H., Hanson, C.A. and Curtiss, A. (1973). The different effects of soluble and crystalline hydrocortisone on bone. Calcif. Tissue Res. 12, 159-168.
Edelstein, S, Noff, D., Matitiahu, A., Sapir, R. and Harell, A. (1977). The functional metabolism of vitamin D in rats treated with cortisol. Febs. Lett. 82, 115-117.
Eilon, G. and Raisz, L.G. (1978). Comparison of the effects of stimulators and inhibitors of resorption on the release of lysosomal enzymes and radioactive calcium from fetal bone in organ culture. Endocrinology. 103, 1969-1975.
Epker, B.N. (1970). Studies on bone turnover and balance in the rabbit. Effects of hydrocortisone. Clin. Orthop. 72, 315-326.
Ewe, K. (1972). Calcium transport in rat small intestine in vitro and in vivo. Naunyn Schmiedebergs Arch. Pharmacol. 273, 352-365.
Farrell, P.M., Rikkers, H. and Moel, D. (1976). Cortisol-dihydrotachysterol antagonism in a patient with hypoparathyroidism and adrenal insufficiency: apparent inhibition of bone resorption. J. Clin. Endocrinol. Metab. 42, 953-957.
Favus, M.J., Kimberg, D.V., Millar, G.N. and Gershon, E. (1973). Effects of cortisone administration on the metabolism and localization of 25-hydroxycholecalciferol in the rat. J. Clin. Invest. 52, 1328-1335.
Favus, M.J., Kathpalia, S.C. and Coe, F.L. (1981). Kinetic characteristics of calcium absorption and secretion by rat colon. Am. J. Physiol. 240, G350-354.
Feher, J.J. and Wasserman, R.H. (1979). Intestinal calcium-binding protein and calcium absorption in cortisol-treated chicks: effects of vitamin D_3 and 1,25-dihydroxyvitamin D_3. Endocrinology. 104, 547-551.
Feigal, R.J. and Messer, H.H. (1981). Comparison of the hypocalcaemic actions of the thyroid and adrenal glands in parathyroidectomized rats. IRCS Med. Sci. 9, 317-318.
Feldman, D, Dziak, R, Koehler, R and Stern, P. (1975). Cytoplasma glucocorticoid binding proteins in bone cells. Endocrinology. 96, 29-36. Ferraro, C., Ladizesky, M., Cabrejas, M., Montoreano, R. and Mautalen, C. (1976). Intestinal absorption of phosphate: action of protein synthesis inhibitors and glucocorticoids in the rat. J. Nutr. 106, 1752-1756.
Ferretti, J.L., Bazan, J.L., Alloatti, D. and Puche, R.C. (1978). The intestinal handling of calcium by the rat in vivo, as affected by cortisol. Effect of dietary calcium supplements. Calcif. Tissue Res. 25, 1-6.
Findling, J.W., Adams, N.D., Lemann, J. jr., Gray, R.W., Thomas, C.J. and Tyrrell, J.B. (1982). Vitamin D metabolites and parathyroid hormone in Cushing's syndrome: Relationship to calcium and phosphorus homeostasis. J. Clin. Endocrinol. Metab. 54, 1039-1044.
Finn Jensen, G., Christiansen, C. and Transbøl (1982). Treatment of postmenopausal osteoporosis. A controlled therapeutic trial comparing oestrogen/gestagen, 1,25-dihydroxyvitamin D_3 and calcium. Clin. Endocrinology. 16, 515-524.
Flower, R.J. and Blackwell, G.J. (1979). Anti-inflammatory steroids induce biosynthesis of a phospholipase A_2 inhibitor which prevents prostaglandin generation. Nature. 278, 456-459.
Forstner, G.G., Sabesin, S.M. and Isselbacher, K.J. (1968). Rat intestinal microvillous membranes. Purification and biochemical characterization. Biochem. J. 106,381-390.
Fox, J., Care, A.D. and Blahos, J. (1978). Effects of low calcium and low phosphorus diets on the duodenal absorption of calcium in betamethasone-treated chicks. J. Endocrinol. 78, 255-260.
Frantz, A.G. and Mitchell, T.R. (1964). Human growth hormone. Clinical measurement, response to hypoglycemia and suppression by corticosteroids. N. Engl. J. Med. 271, 1375-1381.
Frazer, T.E., White, N.H., Hough, S., Santiago, J.V., McGee, B.R., Bryce, G., Mallon, J. and Avioli, L.V. (1981). Alternations in circulating vitamin D metabolites in the young insulin-dependent diabetic. J. Clin. Endocrinol. Metab. 53, 1154-1159.
Freedman, R.A., MacLaughlin, J.A. and Weiser, M.M. (1981). Properties of Ca^{2+} uptake and release by Golgi membrane vesicles from rat intestine. Arch. Biochem. Biophys. 206, 233-241.

Frick, A., Durasin, I. and Neuweg, M. (1981). Phosphaturic response of hydrocortisone in the presence and the absence of parathyroid hormone. Pflügers Arch. 392, 99-105.
Frost, H.M. (1963). In: Bone remodeling dynamics. Thomas, C.C., Springfield, USA.
Fucik, R.F., Kukreja, S.C., Hargis, G.K., Bowser, E.N., Henderson, W.J. and Williams, G.A. (1975). Effect of glucocorticoids on function of the parathyroid glands in man. J. Clin. Endocrinol. Metab. 40, 152-155.
Gallagher, J.C., Aaron, J., Horsman, A., Wilkinson, R. and Nordin, B.E.C. (1973). Corticosteroid osteoporosis. Clin. Endocrinol. Metab. 2, 355-368.
Gallagher, J.C., Riggs, B.L., Eisman, J., Hamstra, A., Arnaud, S.B., and DeLuca, H.F. (1979). Intestinal calcium absorption and serum vitamin D metabolites in normal subjects and osteoporotic patients. Effect of age and dietary calcium. J. Clin. Invest. 64, 729-736.
Gallagher, J.C. (1982). In: Vitamin D, chemical, biochemical and clinical endocrinology of calcium metabolism. (Norman, A.W., Schaefer, K., v. Herrath, D. and Grigoleit, H.G. eds.), pp. 909-913. Walter de Gruyter & Co., Berlin, New York.
Gamstedt, A., Järnerot, G. and Kogedal, B. (1981). Dose related effects of betamethasone on iodothyronines and thyroid hormone-binding proteins in serum. Acta Endocrinol. 96, 484-490.
Garn, S.M. (1981). The phenomenon of bone formation and bone loss. In: Osteoporosis: recent advances in pathogenesis and treatment (DeLuca, H.F., Frost, H.M., Jee, W.S.S., Johnston, C.C. and Parfitt, A.M., eds.), pp. 3-16. University Park Press, Baltimore.
Garn, S.M., Rohmann, C.G. and Wagner, B. (1967). Bone loss as a general phenomenon in man. Fed. Proc. 26, 1729-1736.
Gennari, C., Francini, G., Maioli, E., Civitelli, R. and Gonnelli, S. (1981). Acute effects of human PTH 1-34 infusion on plasma and urinary cAMP in subjects undergoing short and long-term treatment with glucocorticoids. Calcif. Tissue Int. 33 (suppl.), 198 (abstract).
Gennari, C., Bernini, M., Nardi, P., Fusi, L. and Avioli, L.V. (1982[a]). Glucocorticoids and intestinal absorption of calcium and phosphate in man. In: Vitamin D, chemical, biochemical and clinical endocrinology of calcium metabolism. (Norman, A.W., Schaefer, K., v.Herrath, D. and Grigoleit, H.G. eds.), pp. 257-259. Walter de Gruyter & Co., Berlin, New York.
Gennari, C., Scala, C. and Montagnani (1982[b]). Glucocorticoids and parathyroid hormone activity in man. Calcif. Tissue Int. 34, 577 (Abstract).
Ghijsen, W.E.J.M. and v.Os, C.H. (1982). 1,25-dihydroxyvitamin D_3 regulates ATP-dependent calcium transport in basolateral plasma membranes of rat enterocytes. Biochim. Biophys. Acta. 689, 170-172.
Gluck, O.S., Murphy, W.A., Hahn, T.J. and Hahn, B. (1981). Bone loss in adults receiving alternate day glucocorticoid therapy. A comparison with daily therapy. Arthritis Rheum. 24, 892-898.
Gourmelen, M., Girard, F. and Binoux (1982). Serum sometomedin/insulin -like growth factor (IGF) and IGF carrier levels in patients with Cushing's syndrome or receiving glucocorticoid therapy. J. Clin. Endocrinol. Metab. 54, 885-892.
Granner, D.K. (1979). The role of glucocorticoid hormones as biological amplifiers. In: Monogr. Endocrinol. (Baxter J.D. and Rousseay G.G., eds.), pp. 593-611. Springer, Berlin.
Habener, J.F. and Jacobs, J.W. (1982). Biosynthesis and control of secretion of the calcium-regulating peptides. In: Endocrinology of calcium metabolism (Parsons, J.A. ed.). Comprehensive Endocrinology Series (Martini, L. ed.), pp. 143-166. Raven Press, New York.
Hahn, T.J., Boisseau, V.C. and Avioli, L.V. (1973). Effect of chronic corticosteroid administration on diaphyseal and metaphyseal bone mass. J. Clin. Endocrinol. Metab. 39, 274-282.
Hahn, T.J., Halstead, L.R. and Haddad jr., J.G. (1977). Serum 25-hydroxyvitamin D concentrations in patients receiving chronic corticosteroid therapy. J. Lab. Clin. Med. 90, 399-404.
Hahn, T.J. and Halstead, L.R. (1979[a]). Cortisol enhancement of PTH-stimulated cyclic AMP accumulation in cultured fetal rat bone rudiments. Calcif. Tissue Int. 29, 173-175.
Hahn, T.J., Halstead, L.R., Teitelbaum, S.L. and Hahn, B.H. (1979[b]). Altered mineral metabolism in glucocorticoid-induced osteopenia. Effect of 25-hydroxyvitamin D administration. J. Clin. Invest. 64, 655-665.
Hahn, T.J., Halstead, L.R., Strates, B., Imbimbo, B. and Baran, D.T. (1980). Comparison of subacute effects of oxazacort and prednisone on mineral metabolism in man. Calcif. Tissue Int. 31, 109-115.
Hahn, T.J., Halstead, L.R. and Baran, D.T. (1981). Effects of short-term glucocorticoid administration on intestinal calcium absorption and circulating vitamin D metabolite concentrations in man. J. Clin. Endocrinol. Metab. 52, 111-115.

Harris, W.H., Heaney, R.P., Davis, L.A., Weinberg, E.H., Coutts, R.D. and Schiller, A.L. (1976). Stimulation of bone formation in vivo by phosphate supplementation. Calcif. Tissue Res. 22, 85-98.
Harrison, H.E. and Harrison, H.C. (1960). Transfer of Ca^{45} across intestinal wall in vitro in relation to action of vitamin D and cortisol. Am. J. Physiol. 199, 265-271.
Hartog, M., Gaafar, M.A. and Fraser, R. (1964). Effect of corticosteroids on serum growth hormone. Lancet ii, 376-378.
Hauser, H., Howell, K., Dawson, R.M.C. and Bowyer, D.E. (1980). Rabbit small intestinal brush border membrane preparation and lipid composition. Biochim. Biophys. Acta. 602, 567-577.
Herrmann-Erlee, M.P.M., Heersche, J.N.M., Hekkelman, J.W., Gaillard, J., Tregear, G.W., Parsons, J.A. and Potts, J.T. jr. (1976). Effects on bone in vitro of bovine parathyroid hormone and synthetic fragments representing residues 1-34, 2-34 and 3-34. Endocrinol. Res. Commun. 3, 21-35.
Hildman, B., Schmidt, A. and Murer, H. (1982). Ca^{2+}-transport across basal-lateral plasma membranes from rat small intestinal epithelial cells. J. Membr. Biol. 65, 55-62.
Hirst, M. and Feldman, D. (1982). Glucocorticoid regulation of 1,25-$(OH)_2$ vitamin D_3 receptors: divergent effects on mouse and rat intestine. Endocrinology. 111, 1400-1402.
Holtrop, M.E., Cox, K.A., Clark, M.B., Holick, M.F. and Anast, C.S. (1981). 1,25-dihydroxycholecalciferol stimulates osteoclasts in rat bones in the absence of parathyroid hormone. Endocrinology. 108, 2293-2301.
Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973). Glucose transport in isolated brush border membrane from rat small intestine. J. Biol. Chem. 245, 25-32.
Hopfer, U. and Groseclose, R. (1980). The mechanism of Na^+-dependent D-glucose transport. J. Biol. Chem. 255, 4453-4462.
Horsman, A., Burkinshaw, L., Pearson, D., Oxby, C.B. and Milner, R.M. (1983). Estimating total body calcium from peripheral bone measurements. Calcif. Tissue Int. 35, 135-144.
Hough, S., Faurto, A., Sonn, Y., Dong Jo, O.K., Birge, S.J. and Avioli, L.V. (1983). Vitamin D metabolism in the chronic streptozotocin-induced diabetic rat. Endocrinology 113, 790-796.
Hsueh, A.J.W. and Erickson, G.F. (1978). Glucocorticoid inhibition of FSH-induced oestrogen production in cultured rat granulosa cells. Steroids. 32, 639-648.
Hughes, W.L., Bond, V.P., Brecher, G., Cronkite, E.P., Painter, R.B., Quastler, H. and Sherman (1958). Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine. Proc. Natl. Ac. Sci. 44, 476-483.
Hülsmann, W.C., v.d. Berg, J.W.O. and de Jonge, H.R. (1974). Isolation of intestinal mucosa cells. In: Methods in enzymology. (Fleischer, S., Packer L. eds.), 32, pp. 665-673. Academic Press, New York.
Iemhoff, W.G.J., v.d. Berg, J.W.O., de Pijper, A.M. and Hülsmann, W.C. (1970). Metabolic aspects of isolated cells from rat small intestinal epithelium. Biochim. Biophys. Acta. 215, 229-241.
Ireland, P. and Fordtran (1973). Effect of dietary calcium and age on jejunal calcium absorption in humans studied by intestinal perfusion. J. Clin. Invest. 52, 2672-2681.
Jastrup, B., Mosekilde, L., Melsen, F., Lund, Bi., Lund, Bj. and Sørensen, O.H. (1982). Serum levels of vitamin D metabolites and bone remodelling in hyperthyroidism. Metabolism. 31, 126-132.
Jaworski, Z.F.G. (1981). Physiology and pathology of bone remodelling. Orthop. Clinics North America. 12(3), 485-512.
Jee, W.S.S., Black, H.E. and Gotcher, J.E. (1981). Effect of dichloromethane diphosphonate on cortisol-induced bone loss in young adult rabbits. Clinical Orthop. 156, 39-51.
Jensen, J., Nolan, G. and Jubiz, W. (1978). The effect of prednisone on serum thyrotropin, thyroxine and triiodothyronine concentrations in hypothyroid patients. J. Endocrinol. Invest. 2, 171-173.
Juttmann, J.R., Ruis, A.M., Hagenouw-Taal, J.C.W., Lockefeer, J.H.M. and Birkenhäger, J.C. (1978). A comparative study of two methods of measurement of fractional calcium absorption: results in normal individuals and patients with various disturbances of calcium metabolism. Eur. J. Clin. Invest. 8, 137-142.
Juttmann, J.R., Buurman, C.J., de Kam, E., Visser, T.J. and Birkenhäger, J.C. (1981[a]). Serum concentrations of metabolites of vitamin D in patients with chronic renal failure (CRF). Consequences for the treatment with 1α-hydroxyderivatives. Clin. Endocrinol. 14., 225-236.
Juttmann, J.R., Visser, T.J., Buurman, C., de Kam, E. and Birkenhäger, J.C. (1981[b]). Seasonal fluctuations in serum concentrations of vitamin D metabolites in normal subjects. Br. Med. J. 282, 1349-1352.

Kanis, J.A., Cundy, T., Bartlett, M., Smith, R., Heynen, G., Warner, G.T. and Russell, R.G.G. (1978). Is 24,25-dihydroxycholecalciferol a calcium-regulating hormone in man? Br. Med. J. 6124, 1382-1386.

Kanis, J.A., Guillard-Cumming, D.F. and Russell, R.G.G. (1982). Comparative physiology and pharmacology of the metabolites and analogues of vitamin D. In: Endocrinology of calcium metabolism (Parsons, J.A. ed.). Series: Comprehensive Endocrinology (Martini, L. ed.), pp. 321-362. Raven Press, New York.

Keck, E., Peerenboom, H., Ernst, A., West, T.B., Starke, A., v.Lilienfeld-Toal, H. and Krüskemper, H.L. (1982). Influence of exogenous and endogenous glucocorticoid excess on vitamin D metabolites in humans. In: Vitamin D, chemical, biochemical and clinical endocrinology of calcium metabolism. (Norman, A.W., Schaefer, K., v.Herrath, D. and Grigoleit, H.G. eds.), pp. 689-691. Walter de Gruyter & Co, Berlin, New York.

Kessler, M., Tannenbaum, V. and Tannenbaum, C. (1978[a]). A simple apparatus for performing short-time (1-2 seconds) uptake measurements in small volumes; its application to D-glucose transport studies in brush border vesicles from rabbit jejunum and ileum. Biochim. Biophys. Acta. 509, 348-359.

Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M. and Sememza, G. (1978[b]). A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems. Biochim. Biophys. Acta. 506, 136-154.

Kimberg, D.V., Baerg, R.D., Gershon, E. and Graudusius, R.T. (1971). Effect of cortisone treatment on the active transport of calcium by the small intestine. J. Clin. Invest. 50, 1309-1321.

Kirschner, M.A., Zucker, I.R. and Jespersen, D. (1976). Idiopathic hirsutism, an ovarian abnormality. N. Engl. J. Med. 294, 637-640.

Kjellstrand, C.M. (1975). Side effects of steroids and their treatment. Transplant. Proc. VII, 123-129.

Kleeman, C.R., Levi, J. and Better, O. (1975). Kidney and adrenocortical hormones. Nephron. 15, 261-278.

Klein, R.G., Arnaud, S.B., Gallagher, J.C., DeLuca, H.F. and Riggs, B.L. (1977). Intestinal calcium absorption in exogenous hypercortisolism. Role of 25-hydroxyvitamin D and corticosteroid dose. J. Clin. Invest. 60, 253-259.

v.Knorring, J., Slätis, P., Weber, T.H. and Helenius, T. (1982). Serum levels of 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D and parathyroid hormone in patients with femoral neck fracture in southern Finland. Clin. Endocrinol. 17, 189-194.

Kowarski, S. and Schachter, D. (1980). Intestinal membrane calcium-binding protein. J. Biol. Chem. 25, 10834-10840.

Krawitt, E.L. (1972). The role of intestinal transport proteins in cortisone- mediated suppression of Ca^{2+} absorption. Biochim. Biophys. Acta, 179-188.

Krølner, B. and Nielsen, S.P. (1982). Bone mineral content of the lumber spine in normal and osteoporotic women: cross-sectional and longitudinal studies. Clin. Sci. 62, 329-336.

Krølner, B., Jørgensen, J.V. and Nielsen, S.P. (1983). Spinal bone mineral content in myxoedema and thyrotoxicosis. Effects of thyroid hormone(s) and antithyroid treatment. Clin. Endocrinol. 18, 439-446.

Kukreja, S.C., Bowser, E.N., Hargis, G.K., Henderson, W.J. and Williams, G.A. (1976). Mechanisms of glucocorticoid-induced osteopenia: role of parathyroid glands. Proc. Soc. Exp. Biol. Med. 152, 358-361.

Laake, H. (1960). The action of corticosteroids on the renal reabsorption of calcium. Acta Endocrinol. 34, 60-64.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature. 227, 680-685.

Lamberts, S.W.J., Timmermans, H.A.T., Kramer-Blankestijn, M. and Birkenhäger, J.C. (1975). The mechanism of the potentiating effect of glucocorticoids on catecholamine-induced lipolysis. Metabolism. 24, 681-689.

Larsson, S.E., Lorentzon, R. and Boquist, L. (1977). Low doses of 1,25-dihydroxycholecalciferol increase mature bone mass in adult normal rats. Clinical Orthop. 127, 228-235.

Lee, D.B.N. (1983). Unanticipated stimulatory action of glucocorticoids on epithelial calcium absorption. Effect of dexamethasone on rat distal colon. J. Clin. Invest. 71, 322-328.

Lekkerkerker, J.F.F., v.Woudenberg, F. en Doorenbos, H. (1970). De invloed van prednison op de calciumresorptie uit de darm. Ned. Tijdschr. Geneeskd. 114, 987-988.
Lemann, L. jr., Piering, W.F. and Lennon, E.J. (1970). Studies of the acute effects of aldosterone and cortisol on the interrelationship between renal sodium, calcium and magnesium excretion in normal man. Nephron. 7, 117-130.
Lichtwitz, A., de Sèze, S., Hicco, D., Parlier, R., Lanham, C. et Sfikakis, P. (1961). Etude biochimique des decalcifications cortisoniques. Les corticoides et le métabolisme phosphocalcique. Sem. Hop. Paris. 37, 682-697.
Liedtke, C.M. and Hopfer, U. (1982). Mechanism of Cl^- translocation across small intestinal brush-border membrane. I. Absence of Na^+-Cl^- cotransport. Am. J. Physiol. 242, G263-G271.
Lindsay, R., Hart, D.M., Forrest, C. and Baird, C. (1980). Prevention of spinal osteoporosis in oophorectomised women. Lancet ii, 1151-1153.
Lips, P., Hackeng, W.H.L., Jongen, M.J.M., v.Ginkel, F.C. and Netelenbos, J.C. (1983). Seasonal variation in serum concentrations of parathyroid hormone in elderly people. J. Clin. Endocrinol. Metab. 57, 204-206.
Lo Cascio, V., Adami, S., Avioli, L.V., Cominacini, L., Galvanini, G., Gennari, C., Imbimbo, B. and Scuro, L.A. (1982). Suppressive effect of chronic glucocorticoid treatment on circulating calcitonin in man. Calcif. Tissue Int. 34, 309-310.
Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.
Lukert, B.P., Stanbury, S.W. and Mawer, E.B. (1973). Vitamin D and intestinal transport of calcium: effects of prednisolone. Endocrinology. 93, 718-722.
Lukert, B.P. and Adams, J.S. (1976). Calcium and Phosphorus homeostasis in man. Effect of corticosteroids. Arch. Intern. Med. 136, 1249-1253.
Lund, Bj, Sørensen, O.H., Lund, Bi., Bishop, J.E. and Norman, A.W. (1980) Vitamin D metabolism in hypoparathyroidism. J. Clin. Endocrinol. Metab. 51, 606-610.
Lund, B., Eskildsen, P.C., Lund, B., Norman, A.W. and Sørensen, O.H. (1981). Calcium and vitamin D metabolism in acromegaly. Acta Endocrinol. 96, 444-450.
MacFarlane, I.A., Mawer, E.B., Berry, J. and Hahn, J. (1982). Vitamin D metabolism in hyperthyroidism. Clin. Endocrinol. 17, 51-59.
Maierhofer, W.J., Gray, R.W., Cheung, H.S. and Lemann, J. (1983). Bone resorption stimulated by elevated serum 1,25-$(OH)_2$ vitamin D concentrations in healthy men. Kidney Int. 24, 555-560.
Manganiello, V. and Vaughan, M. (1972). An effect of dexamethasone on adenosine $3^1,5^1$-monophosphate content and adenosine $3^1,5^1$-monophosphate phosphodiesterase activity of cultured hepatoma cells. J. Clin. Invest. 51, 2763-2766.
Manolagas, S.C., Anderson, D.C. and Lindsay (1979[a]). Adrenal steroids and the development of osteoporosis in oophorectomised women. Lancet ii, 597-600.
Manolagas, S.C., Andersen, D.C. and Lumb, G.A. (1979[b]). Glucocorticoids regulate the concentration of 1,25-dihydroxycholecalciferol receptors in bone. Nature. 277, 314-315.
Manzke, E., Chesnut III, C.H., Wergedal, J.E., Baylink, D.J. and Nelp, W.B. (1975). Relationship between local and total bone mass in osteoporosis. Metabolism. 24, 605-615.
Marshall, D.H. and Nordin, B.E.C. (1977). The effect of 1α-hydroxyvitamin D_3 with and without oestrogens on calcium balance in postmenopausal women. Clin. Endocrinol. 75, 1595-1685.
Massry, S.G., Coburn, J.W., Chapman, L.W. and Kleeman, C.R. (1967). The acute effect of adrenal steroids on the interrelationship between the renal excretion of sodium, calcium and magnesium. J. Lab. Clin. Med. 70, 563-570.
Matsumoto, T., Fontaine, O. and Rasmussen, H. (1981). Effect of 1,25-dihydroxyvitamin D_3 on phospholipid metabolism in chick duodenal mucosal cell. J. Biol. Chem. 256, 3354-3360.
Mawer, E.B. (1982). Functional control over the metabolic activation of calciferol. In: Endocrinology of calcium metabolism (Parsons, J.A. ed.). Series: Comprehensive Endocrinology (Martini, L. ed.), pp. 271-294. Raven Press, New York.
Mc Dermott, M.T., Kidd, G.S., Blue, P., Ghaed, V. and Hofeldt, F.D. (1983). Reduced bone mineral content in totally thyroidectomized patients: possible effect of calcitonin deficiency. J. Clin. Endocrinol. Metab. 56, 936-939.
Meema, S., Bunker, M.L. and Meema, H.E. (1975). Preventive effect of estrogen on postmenopausal bone loss. Arch. Intern. Med. 135, 1436-1440.

Merz, W.A. and Schenk, R.K. (1970). A quantitative structural analysis of human cancellous bone. Acta Anat. 75, 54-66.
Meunier, P., Courpron, P., Edouard, C., Bernard, J., Bringuier, J. and Vignon (1973). Physiological senile involution and pathological rarefaction of bone. Quantitative and comparative histological data. Clin. Endocrinol. Metab. 2, 239-256.
Meyer, R.A. and Brinkley, H.J. (1972). Effects of adrenalectomy on the response of mice to parathyroid hormone and thyrocalcitonin. J. Pharmacol. Exp. Ther. 181, 171-175.
Miravet, L., Gueris, J., Rousselet, F. and Ryckewaert, A. (1977). Effect des cortisoniques sur le metabolism calcique. Nouv. Press. Med. 6, 1847-1851.
Molinatti, G.M., Camanni, F. and Olivetti, M. (1960). A study on the metabolism of calcium in the hyperadrenocortical syndrome. Acta Endocrinol. 34, 323-334.
Mooser, H. (1921). Ein Fall von endogener Fettsucht mit hochgradiger Osteoporose. Virchows Arch. Pathol. Anat. 229, 247-.
Morgan, H.G., Mitchell, R.G., Stowers, J.M. and Thomson, J. (1956). Metabolic studies on two infants with idiopathic hypercalcaemia. Lancet i, 925-934.
Morris, H.G., Jorgensen, J.R., Elrick, H. and Goldsmith, R.E. (1968). Metabolic effects of human growth hormone in corticosteroid-treated children. J. Clin. Invest. 47, 436-451.
Morrissey, R.L., Zolock, D.I., Bikle, D.D., Empson, R.N. and Bucci, T.J. (1978). Intestinal response to 1,25-dihydroxycalciferol. Biochim. Biophys. Acta. 538, 23-33.
Murota, S., Endo, H. and Tamaoki, B. (1967). Identification of metabolites of cortisol in cultured bone and their effects upon bone formation. Biochim. Biophys. Acta. 136, 379-385.
Nellans, H.N. and Kimberg, D.V. (1978). Cellular and paracellular calcium transport in rat ileum: effects of dietary calcium. Am. J. Physiol. 235, E726-737.
Nellans, H.N. and Kimberg, D.V. (1979). Anomalous calcium secretion in rat ileum: role of paracellular pathway. Am. J. Physiol. 236, E473-481.
Nellans, H.N. and Goldsmith, R.S. (1981). Transepithelial calcium transport by rat cecum: high-efficiency absorptive site. Am. J. Physiol. 240, G424-431.
Nemere, I. and Norman, A.W. (1982). Vitamin D and intestinal cell membranes. Biochim. Biophys. Acta. 694, 307-327.
Newton-John, H.F. and Morgan, D.B. (1970). The loss of bone with age, osteoporosis and fractures. Clinical Orthop. 71, 229-252.
Ng, B., Hekkelman, J.W. and Heersche, J.N.M. (1979). The effect of cortisol on the adenosine $3^1,5^1$-monophosphate response to parathyroid hormone of bone in vitro. Endocrinology. 104, 1130-1135.
Nicolaysen, R. (1937). Studies upon the mode of action of vitamin D. Biochem. J. 31, 107-129.
Nordin, B.E.C. (1976). In: Calcium, phosphate and magnesium metabolism. (Nordin, B.E.C. ed.), p. 3, Churchill Livingstone Edinburgh, London and New York.
Norman, A.W., Mircheff, A.K., Adams, T.H. and Spielvogel, A. (1970). Studies on the mechanism of action of calciferol. III Vitamin D-mediated increase of intestinal brush border alkaline phophatase activity. Biochim. Biophys. Acta. 215, 348-359.
Norman, A.W., Roth, J. and Orci, L. (1982). The vitamin D endocrine system: steroid metabolism, hormone receptors and biological response (Calcium binding proteins). Endocr. Rev. 3, 331-366.
Nosadini, R., Del Prato, S., Tiengo, A., Valerio, A., Muggeo, M., O Pocher, G., Mantero, F., Duner, E., Marescotti, C., Mollo, F. and Belloni, F. (1983). Insulin resistance in Cushing's Syndrome. J. Clin. Endocrinol. Metab. 57, 529-535.
Olefsky, J.M. and Kimmerling, G. (1976). Effects of glucorticoids on carbohydrate metabolism. Am. J. Med. Sci. 271, 202-210.
Pansu, D., Bellaton, C., Roche, C. and Bronner, F. (1983). Duodenal and ileal calcium absorption in the rat and effects of vitamin D. Am. J. Physiol. 244, G695-700.
Papapoulus, S.E., Clemens, T.L., Fraher, L.J., Gleed, J. and O'Riordan, J.L.H. (1980). Metabolites of vitamin D in human vitamin D-deficiency: effect of vitamin D_3 or 1,25-dihydroxyholecalciferol. Lancet ii, 612-615.
Parfitt, A.M. and Dent, C.E. (1970). Hyperthyroidism and hypercalcaemia. Q. J. Med. 154, 171-187.
Peck, W.A., Brandt, J. and Miller, I. (1967). Hypercortisone-induced inhibition of protein synthesis and uridine incorporation in isolated bone cells in vitro. Proc. Natl. Acad. Sci. USA.57, 1599-1606.

Puschett, J.B., Moranz, J. and Kurnick, W.S. (1972). Evidence for a direct action of cholecalciferol and 25-hydroxycholecalciferol on renal transport of phosphate, sodium and calcium. J. Clin. Invest. 51, 373-385.

Puschett, J.B. and Kuhrman, M.S. (1978). Renal tubular effects of 1,25-dihydroxyvitamin D_3: interactions with vasopressin and parathyroid hormone in the vitamin D-depleted rat. J. Lab. Clin. Med. 92, 895-903.

Raisz, L.G., Trummel, C.L., Wener, J.A. and Simmons, H. (1972). Effect of glucocorticoids on bone resorption in tissue culture. Endocrinology. 90, 961-967.

Raisz, L.G., Maina, D.M., Gworek, S.C., Dietrich, J.W. and Canalis, E.M. (1978). Hormonal control of bone collagen synthesis in vitro: inhibitory effect of 1-hydroxylated vitamin D metabolites. Endocrinology. 102, 731-735.

Raisz, L.G. (1980). Effect of corticosteroids on calcium metabolism. Prog. Biochem. Pharmacol. 17, 212-219.

Raisz, L.G. and Kream, B.E. (1983). Regulation of bone formation. N. Engl. J. Med. 309, 29-35 and 83-89.

Rasmussen, H., Fontaine, O., Max, E.E. and Goodman, D.B.P. (1979). The effect of 1α-hydroxyvitamin D_3 administration on calcium transport in chick intestine brush border membrane vesicles. J. Biol. Chem. 254, 2993-2999.

Rasmussen, H., Bordier, P., Marie, L., Auquier, L., Eisinger, J.B., Kuntz, D., Caulin, F., Argemi, B., Gueris, J. and Julien, A. (1980). Effect of combined therapy with phosphate and calcitonin on bone volume in osteoporosis. Metab. Bone Dis. Relat. Res. 2, 107-111.

Rasmussen, H., Matsumoto, T., Fontaine, O. and Goodman, D.B.P. (1982). Role of changes in membrane lipid structure in the action of 1,25-dyhydroxyvitamin D_3. Fed. Proc. 41, 72-77.

Reeve, J., Meunier, P.J., Parsons, J.A., Bernat, M., Bijvoet, O.L.M., Courpron, P., Edouard, C., Klenerman, L., Neer, R.M., Renier, J.C., Slovik, D., Vismans, F.J.F.E. and Potts, J.T. jr. (1980[a]). Anabolic effects of human parathyroid hormone fragment on trabecular bone in involutional osteoporosis: a multicenter trial. Br. Med. J. 6228, 1340-1344.

Reeve, J., Bijvoet, O.L.M., Neer, R.M., Slovik, D., Tellez, M., Vismans, F.J.F.E. and Zanelli, G.D. (1980[b]). A comparison between the balance method and radiotracer methods for measuring calcium absorption in treated and untreated patients with osteoporosis. Metab. Bone Dis. Relat. Research 2, 233-238.

O'Regan, S., Chesney, R.W., Hamstra, A., Eisman, J.A., O'Gorman, A.M. and DeLuca, H.F. (1979). Reduced serum 1,25-$(OH)_2$vitamin D_3 levels in prednisone-treated adolescents with systemic lupus erythematosus. Acta Paediatr. Scand. 68, 109-111.

Reid, D.M., Kennedy, N.S.J., Smith, M.A., Tothill, P. and Nuti, G. (1982). Total body calcium in rheumatoid arthritis: effects of disease activity and corticosteroid treatment. Br. Med. J. 285, 330-332.

Riggs, B.L., Ryan, R.J., Wahner, H.W., Jiang, N. and Matton, V.R. (1973). Serum concentrations of estrogen, testosteron, and gonadotropins in osteoporotic and nonosteoporotic postmenopausal women. J. Clin. Endocr. Metab. 36, 1097-1099.

Riggs, B.L., Wahner, H.W., Dunn, W.L., Mazess, R.B., Offord, K.P. and Melton 111, L.J. (1981). Differential changes in bone mineral density of appendicular and axial skeleton with aging. Relationship to spinal osteoporosis. J. Clin. Invest. 67, 328-335.

Rodriguez-Rigau, L.J., Smith, K.D., Tcholakian, R.K. and Steinberger, E. (1979). Effect of prednisone on plasma testosterone levels and on duration of phases of the menstrual cycle in hyperandrogenic women. Fertil. Steril. 32, 408-413.

Rosental, R., Babarykin, D., Fomina, O., Smelters, G., Valiniece, M. und Baumann, V. (1982). Hypophosphatämie nach erfolgter Transplantation der Niere. Klinisch-experimentelle Untersuchung. Z. Urol. Nephrol. 75, 393-399.

Rothstein, M., Olgaard, K., Arbelaez, M., Finco, D., Klahr, S. and Slatopolsky, E. (1983). Lack of influence of 24,25-dihydroxyvitamin D_3 on parathyroid hormone secretion from normal or hyperplastic glands. Calcif. Tissue Int. 35, 449-454.

Sandberg, A.L., Raisz, L.G., Wahl, L.M. and Simmons, H.A. (1982). Enhancement of complement-mediated prostaglandin synthesis and bone resorption by arachidonic acid and inhibition by cortisol. Prostaglandins Leukotriennes Med. 8, 419-427.

Schachter, D. and Rosen, S.M. (1959). Active transport of ^{45}Ca by the small intestine and its dependence on vitamin D. Am. J. Physiol. 196, 357-362.
Schlechte, J.A., Sherman, B. and Martin, R. (1983). Bone density in amenorrheic women with and without hyperprolactinemia. J. Clin. Endocrinol. Metab. 56, 1120-1123.
Schultz, T.D., Ballman, S. and Kumar, R. (1982). Decreased intestinal calcium absorption in vivo and normal brush border membrane vesicle calcium uptake in cortisol-treated chickens: Evidence for dissociation of calcium absorption from brush border vesicle uptake. Proc. Natl. Acad. Sci. USA. 79, 3542-3546.
Schmidtke, J., Wienker, Th., Flügel, M. and Engel, W. (1976). In vitro inhibition of cyclic AMP phosphodiesterase by cortisol. Nature. 262, 593-594.
Seeman, E., Kumar, R., Hunder, G.G., Scott, M., Heath III, H. and Riggs, B.L. (1980). Production, degradation and circulating levels of 1,25-dihydroxyvitamin D in health and in chronic glucocorticoid excess. J. Clin. Invest. 66, 664-669.
Seeman, E., Wahner, H.W., Offord, K.P., Kumar, R., Johnson, W.J. and Riggs, B.L. (1982). Differential effects of endocrine dysfunction on the axial and the appendicular skeleton. J. Clin. Invest. 69, 1302-1309.
Sellin, J.H. and Field, M. (1981). Physiologic and pharmacologic effects of glucocorticoids on ion transport across rabbit ileal mucosa in vitro. J. Clin. Invest. 67, 770-778.
Shamonki, I.M., Frumar, A.M., Tataryn, I.V., Meldrum, D.R., Davidson, B.H., Parthemore, J.G., Judd, H.L. and Deftos, L.J. (1980). Age-related changes of calcitonin secretion in females. J. Clin. Endocrinol. Metab. 50, 437-439.
Silbermann, M., Toister, Z. and Lewinson, D. (1981). Glucocorticoid-induced changes in the activity of cartilage alkaline phosphatase. Metab. Bone Dis. Relat. Res. 3, 67-75.
Slovik, D.M., Neer, R.M., Ohman, J.L., Lowell, F.C., Clark, M.B., Segre, G.V. and Potts jr. J.T. (1980). Parathyroid hormone and 25-hydroxyvitamin D levels in glucocorticoid-treated patients. Clin. Endocrinol. 12, 243-248.
Slovik, D.M., Adams, J.S., Neer, R.M., Holick, M.F. and Potts, J.T. (1981). Deficient production of 1,25-dihydroxyvitamin D in elderly osteoporotic patients. N. Engl. J. Med. 305, 372-374.
Smith, D.M., Khairi, M.R.A. and Johnston, C.C. (1975). The loss of bone mineral with aging and its relationship to risk of fracture. J. Clin. Invest. 56, 311-318.
Sørensen, O.H., Lund, B., Friis, Th., Hjorth, L., Reimann, L., Kjaer, I. and Andersen, R.B. (1977). Effect of 1α-hydroxycholecalciferol in senile osteoporosis and in bone loss following prednisone treatment. Iss. J. Med. Sci. 13, 253-258.
Sørensen, O.H., Lumholtz, B., Lund, Bi., Lund, Bj., Hjelmstrand, I.L., Mosekilde, L., Melsen, F., Bishop, J.E. and Norman, A.W. (1982). Acute effects of parathyroid hormone on vitamin D metabolism in patients with the bone loss of aging. J. Clin. Endocrinol. Metab. 54, 1258-1261.
Spanos, E., Colston, K.W., Evans, I.M.S., Galante, L.S., Macauley, S.J. and Macintyre, I. (1976). Effect of prolactin on vitamin D metabolism. Mol. Cell. Endocrinol. 5, 163-167.
Spanos, E., Colston, K.W. and Macintyre, I. (1977). Effect of glucocorticoids on vitamin D metabolism. Febs Lett. 75, 73-76.
Spencer, H., Menczel, J., Lewin, I. and Samachson, J., (1964). Absorption of calcium in osteoporosis. Am. J. Med. 37, 223-234.
Spencer, R., Charman, M., Wilson, P.W, and Lawson, D.E.M. (1978). The relationship between vitamin D-stimulated calcium transport and intestinal calcium-binding protein in the chicken. Biochem. J. 170, 93-101.
Storey, E. (1960). Bone changes associated with cortisone administration in the rat. Br. J. Exp. Pathol. 41, 207-213.
Streck, W.F., Waterhouse, C. and Haddad, J.G. (1979). Glucocorticoid effects in vitamin D intoxication. Arch. Intern. Med. 139, 974-977.
Stresemann, E. and Krokowski, E. (1967). Der Mineralisationsgrad der Wirbelsäule nach langfristiger Corticosteroidbehandlung der chronischen Bronktialasthmas. Klin. Wochenschr. 45, 564-573.
Suzuki, Y., Ichikawa, Y., Saito, E. and Homma, M. (1983). Importance of increased urinary calcium excretion in the development of secondary hyperparathyroidism of patients under glucocorticoid therapy. Metabolism. 32, 151-156.
Tam, C.S., Cruickshank, B., Swinson, D.R., Anderson, W. and Little, H.A. (1979). The response of bone apposition rate to some non-physiologic conditions. Metabolism.28, 751-755.

Tanaka, Y. and DeLuca, H.F. (1973). The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. Arch. Biochem. Biophys. 154, 566-574.
Tanaka, Y. and DeLuca, H.F. (1974). Stimulation of 24,25-dihydroxyvitamin D_3 production by 1,25-dihydroxyvitamin D_3. Science. 183, 1198-1200.
Tashjian, A.H. jr. and Levine, L. (1978). Epidermal growth factor stimulates prostaglandin production and bone resorption in cultured mouse calvaria. Biochem. Biophys. Res. Commun. 85, 966-975.
Teitelbaum, S.L., Rosenberg, E.M., Richardson, C.A. and Avioli, L.V. (1976). Histological studies of bone from normocalcemic postmenopausal osteoporotic patients with increased circulating parathyroid hormone. J. Clin. Endocrinol. Metab. 42, 537-543.
Teitelbaum, S.L., Malone, J.D. and Kahn, A.J. (1981). Glucocorticoid enhancement of bone resorption by rat peritoneal macrophages in vitro. Endocrinology. 108, 795-799.
Tessler, R.H. and Salmon, jr., W.D. (1975). Glucocorticoid inhibition of sulfate incorporation by cartilage of normal rats. Endocrinology. 96, 898-902.
Thompson, J.S., Palmieri, G.M.A., Eliel, L.P. and Cutler, G.A. (1968). Effects of cortisone and adrenalectomy on the response to thyrocalcitonin. Endocrinology. 83, 470-474.
Thompson, J.S., Palmieri, G.M.A., Eliel, L.P. and Crawford, R.L. (1972). The effect of porcine calcitonin on osteoporosis induced by adrenal cortical steroids. J. Bone Joint Surg. 54A, 1490-1500.
Thompson, J.S. and Urist, M.R. (1973). Effects of cortisone on bone metabolism in intact and thyroidectomized rabbits. Calcif. Tissue Res. 13, 197-215.
Thoren, M., Hall, K. and Rähn, T. (1981). Somatomedin A levels in patients with Cushing's disease. Acta Endocrinol. 97, 12-17.
Trechsel, U., Eisman, J.A., Fischer, J.A., Bonjour, J.P. and Fleisch, H. (1980). Calcium-dependent, parathyroid hormone-independent regulation of 1,25-dihydroxyvitamin D. Am. J. Physiol. 239, E119-124.
Trotter, M., Broman, G.E. and Peterson, R.R. (1960). Densities of bones of white and negro skeletons. J. Bone Joint Surg. 42A, 50-58.
Turner, S.T., Kiebzak, G.M. and Dousa, T.P. (1982). Mechanism of glucocorticoid effect on renal transport of phosphate. Am. J. Physiol. 243, C227-236.
Wajchenberg, B.L., Pereira, V.G., Kieffer, J. and Ursic, S. (1969). Effect of dexamethasone on calcium metabolism and ^{47}Ca kinetics in normal subjects. Acta Endocrinol. 61, 173-192.
Walker, D.A. and Davies, M. (1981). Addison's disease presenting as a hypercalcaemic crisis in a patient with idiopathic hypoparathyroidism. Clin. Endocrinol. 14, 419-423.
Wallach, D.F.H. and Steck, T.L. (1963). Fluorescence techniques in microdetermination of metals in biological materials. II: An improved method for direct complexometric titration of calcium in small serum samples. Anal. Biochem. 6, 176-180.
Wallach, S. (1979). Hormonal factors in osteoporosis. Clinical Orthop. 144, 284-292.
Walling, M.W. and Rothman, S.S. (1969). Phosphate-independent, carrier-mediated active transport of calcium by rat intestine. Am. J. Physiol. 217, 1144-1148.
Walling, M.W. and Rothman, S.S. (1973). Adaptive uptake of calcium at the duodenal brush border. Am. J. Physiol. 225, 618-623.
Walser, M., Robinson, B.H.B. and Duckett, J.W. (1963). The hypercalcemia of adrenal insufficiency. J. Clin. Invest. 42, 456-465.
Wasserman, R.H., Kallfelz, F.A. and Comar, C.L. (1961). Active transport of calcium by rat duodenum in vivo. Science. 133, 883-884.
Wasserman, R.H. and Taylor, A.N. (1966). Vitamin D_3-induced calcium-binding protein in chick intestinal mucosa. Science. 152, 791-793.
Weisman, Y., Schen, R.J., Eisenberg, Z., Edelstein, S. and Harell, A. (1981). Inadequate status and impaired metabolism of vitamin D in the elderly. Isr. J. Med. Sci. 17, 19-21.
Weissmann, G. and Thomas, L. (1963). Studies on Lysosomes. II The effect of cortisone on the release of acid hydrolases from a large granule fraction of rabbit liver induced by an excess of vitamin A. J. Clin. Invest. 42, 661-669.
Welsh, T.H. jr., Bambino, T.H. and Hsueh, A.J. (1982). Mechanism of glucocorticoid-induced suppression of testicular androgen biosynthesis in vitro. Biol. Reprod. 27, 1138-1146.
Wilkinson, R. (1976). In: Calcium, phosphate and magnesium metabolism (Nordin, B.E.C. ed.), pp. 36-72. Churchill Livingstone, Edinburgh, London and New York.

Williams, G.A., Bowser, E.N., Henderson, W.J. and Uzgiries, V. (1961). Effects of vitamin D and cortisone on intestinal absorption of calcium in the rat. Proc. Soc. Exp. Biol. Med. 106, 664-666.
Williams, G.A., Peterson, W.C., Bowser, E.N., Henderson, W.J., Hargis, G.K. and Martinez, N.J. (1974). Interrelationship of parathyroid and adrenocortical function in calcium homeostasis in the rat. Endocrinology. 95, 707-712.
Wong, G.L. (1979). Basal activities and hormone responsiveness of osteoclast-like and osteoblast-like bone cells are regulated by glucocorticoids. J. Biol. Chem. 254, 6337-6340.
Wong, G.L., Lukert, B.P. and Adams, J.S. (1980). Glucocorticoids increase osteoblast-like bone cell response to 1,25-$(OH)_2D_3$. Nature. 285, 254-257.
Zornitzer, A.E. and Bronner, F. (1971). In situ studies of calcium absorption in rats. Am. J. Physiol. 220, 1261-1266.
Zerwekh, J.E., Pak, C.Y.C., Kaplan, R.A., McGuire, J.L., Upchurch, K., Breslau, N. and Johnston, R. jr. (1980). Pathogenetic role of 1,25-dihydroxyvitamin D in sarcoidosis and absorptive hypercalciuria: different response to prednisolone therapy. J. Clin. Endocrinol. Metab. 51, 381-386.

VERANTWOORDING

Dit proefschrift kwam tot stand op de afdelingen Inwendige Geneeskunde III, Pathologische Anatomie I en Biochemie I.

Het was niet in ieder opzicht gemakkelijk als internist op de praktische ingestelde afdeling Heelkunde werkzaam te zijn en tegelijkertijd een onderzoek met preklinische aspecten te voltooien. Toch had ik dit niet willen en kunnen missen en ik dank met name Eveline Ong voor de gelegenheid die ik hiervoor heb kunnen nemen.

Alle anderen die op enigerlei wijze behulpzaam waren bij het tot stand komen van dit proefschrift wil ik bedanken, ook degenen die ik hier niet zal noemen.

Reeds in een vroeg stadium van mijn opleiding tot internist wist Prof. Birkenhäger mijn belangstelling te wekken voor botstofwisseling en wel speciaal voor een aspect dat hem zelf reeds langer bezighield: corticosteroidosteoporose. Zijn nooit aflatend enthousiasme, aansluitend bij mijn vasthoudendheid (c.q. "drammen"), was voor mij een belangrijke steun en stimulans. Dit resulteerde in een samenwerking waarvan ik heel veel heb geleerd.

Dorie Birkenhäger "voerde" mij, met grote kennis van zaken, in in de wereld van de bothistomorfometrie en zij mat met veel gevoel voor detail en geduld, geholpen door Ad Rietveld, de botbiopten van het patienten onderzoek.

Veel dank ben ik verschuldigd aan Hugo de Jonge voor het onderricht in de eigenschappen van zijn "borstelzoomblaasjes" en voor zijn inzet bij mijn begeleiding. Hierdoor kon mijn inzicht in de invloed van hormonen op calciumtransport in de darm zich ontwikkelen.

Cootje van Aller, Cok Buurman, Desirée Peul, Ed de Kam, Marcel van Edixhoven en Ronald Lammers hebben op voortreffelijke wijze de diverse laboratoriumonderzoeken uitgevoerd. Theo Visser stond altijd klaar voor het oplossen van problemen bij de wispelturige bepaling van vitamine D metabolieten. Ook de laboranten van het Centraal Klinisch Chemisch Laboratorium (hoofd Dr. B.G. Blijenberg) past een woord van dank. Zij waren in staat en bereid ook in de "kleine uurtjes" serum calciumbepalingen te verrichten.

Ik dank Wil Hackeng en Roger Bouillon voor de bepalingen van bijschildklierhormoon en vitamine D bindend eiwit die in hun laboratoria werden verricht.

De botdensitometrie en de absorptietesten met radioactief calcium werden nauwgezet uitgevoerd door Marcel van de Pluym op de afdeling Nucleaire Geneeskunde (Drs. W. Bakker).

Het personeel van de balansafdeling, Joke van Vure, Cootje van Aller, Marijke Hengeveld en Henny van der Stel-van de Berg, is op onnavolgbare wijze een steun geweest voor de patienten die aan het onderzoek deelnamen, zowel in menselijk opzicht als bij het praktisch uitvoeren van diverse proeven.

Het personeel van de interne afdelingen 4 Zuid en 4 Noord was behulpzaam bij het verzamelen van patientengegevens, waarvoor ik hen zeer erkentelijk ben.

Secretariële hulp bij het verwerken van de publicaties en het uittypen van de definitieve tekst van het proefschrift werd verleend door Dieneke van Wessem, Ankie Bos-Voogd en Yolande te Giffel.

Paul Schmitz (afdeling Biostatistica) gaf advies bij het statistisch bewerken van de diverse resultaten.

De figuren en tekeningen in het proefschrift zijn uitgevoerd door de Audiovisuele Dienst. Kees de Vries gaf de omslag zijn definitieve vorm.

De firma Leo Pharmaceutische Producten Nederland leverde de 1α-hydroxycholecalciferol en placebo tabletten voor de klinische trial. Met name Otto Bauermann heeft ervoor gezorgd dat het proefschrift in zijn huidige vorm kan verschijnen.

Speciaal wil ik de patienten bedanken die bereid waren deel te nemen aan de diverse onderdelen van het onderzoek. Hun loyale medewerking was zeker niet alleen ingegeven door eigenbelang.

Mijn gezin, Birgit, Annemarie en Saskia, is misschien wel het meest van allen betrokken geweest bij het tot stand komen van dit proefschrift, maar gelukkig werd deze "schepping" overvleugeld door de geboorte van onze jongste dochter, Hanneke.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 24 oktober 1949 te Dordrecht geboren. Na het behalen van het eindexamen Gymnasium β aan het Gemeentelijk Lyceum te Dordrecht in 1968, studeerde hij geneeskunde aan de Medische Faculteit te Rotterdam. Op 13 december 1974 legde hij het artsexamen af. Daarna volgde hij gedurende 1 jaar de beroepsopleiding tot huisarts en vervulde vervolgens zijn militaire dienstplicht bij de Koninklijke Landmacht. In 1976 ging hij in opleiding tot internist op de afdeling Inwendige Geneeskunde III tevens Klinische Endocrinologie van het Academisch Ziekenhuis Dijkzigt te Rotterdam (hoofd: Prof. Dr. J.C. Birkenhäger). Op 1 april 1982 volgde inschrijving in het specialistenregister. Thans is hij werkzaam als internist op de afdeling Algemene Heelkunde van het Dijkzigt Ziekenhuis.