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Effects of vitamin D

A comparison of 1 αOHD$_2$ and 1 αOHD$_3$ in rats

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NOMENCLATURE

Vitamin D_2	ergocalciferol
Vitamin D_3	cholecalciferol
$1\alpha OHD_2$	1-alphahydroxyvitamin D_2
$1\alpha OHD_3$	1-alphahydroxyvitamin D_3
$25OHD_2$	25-hydroxyergocalciferol
$25OHD_3$	25-hydroxycholecalciferol
$1,25(OH)_2D_2$	1,25-dihydroxyergocalciferol
$1,25(OH)_2D_3$	1,25-dihydroxycholecalciferol
25-hydroxylase	Vitamin D-25-hydroxylase
1-hydroxylase	25-hydroxyvitamin D-1-alphahydroxylase
DBP	vitamin D Binding Protein
CaBP	Calcium Binding Protein
PTH	ParaThyroid Hormone
HPLC	High Pressure Liquid Chromatography
IU	International Unit (1 µg of vitamin D = 40 IU)

Note: Vitamin D without subscript denotes both vitamin D_2 and vitamin D_3 when not considered separately.

The picture on page 4 reproduces the title page of the third edition of Francis Glisson's: "De rachitide sive morbo puerli, qui vulgo the rickets dicitur", Leyden 1671.

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This thesis is based upon the following studies, which will be referred to by their roman numerals:

I ANTIRACHITIC ACTIVITY OF 1α-HYDROXYERGOCALCIFEROL AND 1α-HYDROXYCHOLECALCIFEROL IN RATS.
Sjödén, G., Lindgren, U., DeLuca, H.F.
Journal of Nutrition 114:2043-2046, 1984.

II 1α-HYDROXYVITAMIN D_2 IS LESS TOXIC THAN 1α-HYDROXYVITAMIN D_3 IN THE RAT.
Sjödén, G., Smith, C., Lindgren, J.U., DeLuca, H.F.
Proceedings of the Society for Experimental Biology and Medicine 178:432-436, 1985.

III THE EFFECT OF 1α-HYDROXYVITAMIN D_2 ON CALCIUM METABOLISM IN GLUCOCORTICOID-TREATED RATS.
Sjödén, G., Lindgren, J.U., DeLuca, H.F.
Bone. In press (1985).

IV EFFECTS OF 1αOHD_2 ON BONE TISSUE. Studies of 1αOHD_2 and 1αOHD_3 in normal rats and in rats treated with prednisolone.
Sjödén, G., Johnell, O., DeLuca, H.F., Lindgren, J.U.
Acta Endocrinologica Scandinavica 106:564-568, 1984.

V THE EFFECT OF PREDNISOLONE ON KIDNEY CALCIFICATION IN VITAMIN D TREATED RATS
Sjödén, G. & Lindgren, J.U.
Calcified Tissue International, In press, 1985.


GLISSONIUS
De
RACHITIDE
G.W. sculp
LUGD. BATAV. Ex Officina FELICIS LOPEX de HARO et CORNELII DRIEHUYSEN, 1671

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INTRODUCTION

In the early 1920's cod liver oil was found to cure rickets. The term Vitamin D was given to the anti-rachitic constituent of the oil. During the last 15 years, it has become evident that vitamin D in itself is physiologically inactive. Instead at least one metabolite, namely 1,25-dihydroxyvitamin D, is responsible for the biological activity. This metabolite acts in a hormonal fashion and its formation is feedback-controlled. Ergocalciferol (vitamin D_2) is the alimentary form of the vitamin. It is derived from irradiated ergosterol. Cholecalciferol (vitamin D_3) is the endogenous form and it is derived from 7-dehydrocholesterol.

Vitamin D_2 was introduced into the drug market first; vitamin D_3 and its metabolites came later. Today, mainly the various vitamin D_3 metabolites are in therapeutic use.

$1\alpha OHD_2$ has been synthesized only recently in adequate amounts, so its effects are less well known than those of related vitamin D_3 compounds.

The present study was undertaken in order to study $1\alpha OHD_2$ in direct comparison with $1\alpha OHD_3$. Its influence on calcium and phosphate metabolism in rats was studied in different situations, namely:

- in the healing of rickets
- in the stimulation of intestinal calcium uptake
- in the effect on bone
- in the prevention of osteopenia during prednisolone treatment
- in causing nephrocalcinosis, weight loss and death following toxic doses

HISTORICAL NOTES

The discovery of vitamin D is related to the treatment of rickets. Whistler (in 1645) and Glisson (in 1650) described extensively this disease of children with swelling of the wrists, ankles, and abdomen and deformity of the chest,

producing a prominent sternum and knotty junctions of the ribs.

At the beginning of the 20th century rickets was endemic in North America and northwestern Europe. The ailment was more common in urban areas (Owen, 1889). An estimated 80% of children under the age of two coming from poor circumstances in Boston suffered from the disease (Morse, 1900).

Fresh air, sunlight and cod liver oil were used early (in folk medicine) in the treatment of rickets. As early as 1919, Huldschinsky demonstrated that ultraviolet light heals rickets. The first clinical trial for treating rickets with Bergen cod liver oil was made in 1924 (Schütte).

The true etiology of rickets was not unraveled until the concept of vitamins was established. For many decades during the 1800's scientists had considered the possibility of raising animals on chemically defined diets, based on analytical values for protein, carbohydrates, lipids, and salts. It was found impossible to support growth and reproduction with these artificial diets. This led to the opinion that something vital was missing in these chemically purified diets. The term "vital amines" was coined to designate the substances needed in minute amounts to support life (Funk, 1911).

The demonstration of curing scurvy with lime juice was the first evidence of a vitamin (Holst & Frölich, 1903). In 1916 McCollum could demonstrate that a fat-soluble substance was needed to support health in rats. He named this substance vitamin A.

It was in this context that Mellanby in 1919 conducted his famous nutritional experiments using puppies. By keeping the dogs out of sunlight and feeding them a diet of milk, porridge and bread, Mellanby could induce a disease in the dogs that closely resembled human rickets. Mellanby further showed that cod liver oil cured rickets. Subsequently the antirachitic factor in the fish liver oil was identified as

vitamin D (McCollum et al., 1922). For some time the situation was puzzling, since both cod liver oil and ultraviolet light had been found to cure rickets.

Then Goldblatt and Soames (1923) were able to cure rachitic rats by feeding them extracts of livers from rats subjected to ultraviolet irradiation. Steenbock and Black (1924) showed that irradiating various foods such as milk, wheat flour, and vegetable oils induced antirachitic properties. Once the connection between ultraviolet light and antirachitic activity had been made, the work to isolate and characterize the reponsible factor was initiated. Investigation of plant-derived material led to the identification of vitamin D_2 by Askew (1931) and Windaus (1932). (What was called vitamin D_1 was an artifactual mixture of vitamin D_2 and a contaminating sterol, lumisterol.)

It was noted that birds did not respond as well to treatment with vitamin D_2 as to cod liver oil, and Waddel (1934) suggested the existence of another vitamin D derived from cholesterol. The isolation and chemical identification of vitamin D_3 from irradiated cholesterol was carried out by Windaus (1936). The role of vitamin D in maintaining normal calcium concentrations in plasma by improving the intestinal absorption was established by Nicolaysen in 1937.

In the search for the role of vitamin D in the mineralization process, the other aspect of bone metabolism, bone resorption, was over-looked. Carlsson (1952), using isotopes, showed that vitamin D is important for the mobilization of calcium from bone to support plasma calcium.

The method of preparing radiolabelled vitamin D with high specific activity, using ^3H, led to a breakthrough in modern vitamin D research. In 1968 DeLuca and associates discovered

that vitamin D is hydroxylated in the liver to 25-hydroxyvitamin D (Blunt et al., 1968, 1969). This discovery was followed by the finding by Fraser and Kodicek (1970) that the kidney produces the biologically most active form of vitamin D, eventually identified as 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$).

The complicated and expensive synthesis of $1,25(OH)_2D_3$ was described in 1972 (Semunter et al.). During this work, synthesis of $1\alpha OHD_3$ was also accomplished, and this procedure was easier and less expensive (Holick et al., 1973). The liver converts the $1\alpha OHD_3$ into $1,25(OH)_2D_3$ (Holick et al. 1976). Synthesis of $1\alpha OHD_2$ was carried out by Lam et al. in 1974.

PHYLOGENETICS

Vitamin D is found in some green plants. A $1,25(OH)_2D_3$ derivative has been found in Solanum malacoxylon in South America and Trisetum flavescens (oat grass) in Germany (Wasserman, 1975). Recently Horst et al. (1984) demonstrated that Medicago sativa (alfalfa) contains large amounts of vitamin D_2. Under normal conditions, 1 kg of alfalfa contains 1920 IU of vitamin D_2 but only 25 IU of vitamin D_3.

Provitamin D_2, ergocalciferol, is found in many lower organisms such as fungi and yeasts. Very large amounts of ergocalciferol are found on rye when ergot is present. Algae, sea anemones, mussels, oysters, and worms also contain ergocalciferol. Clover hay that has dried in sunshine acquires antirachitic activity due to formation of vitamin D_2 (Norman, 1979).

Vitamin D_3 is found in large amounts in the livers of fish, even in species that do not have a calcified skeleton such as sharks (DeLuca, 1978). The source of this vitamin is unknown. The cod, for example, is a deep-water fish not subjected to irradiation by ultraviolet light. The vitamin could emanate from a nonphotochemical production process (Blondin, 1967), although this is doubtful, since vitamin D

seems required in the diet of some fish (Lovell & Li, 1978). The source could well be plankton and other small organisms. Renal 1-hydroxylase has been documented in both fresh-water and salt-water fish (Henry & Norman, 1975)

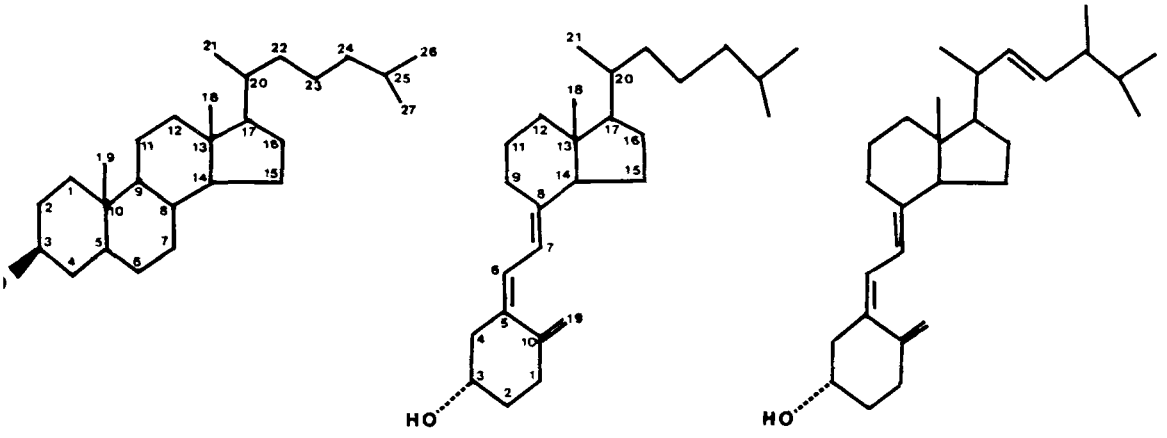
In birds, vitamin D_2 is about ten times less potent than vitamin D_3 (Chen & Bosmann, 1964). This has been considered to be due to a more rapid degradation of vitamin D_2 by the liver (Imrie et al., 1967).

In mammals other than new world monkeys vitamin D_2 and vitamin D_3 have earlier been considered equipotent (Hunt et al., 1967). Recent reports have identified species differences. The rat, pig and dairy calf metabolize vitamin D_2 and vitamin D_3 differently (Horst et al., 1982; Sommerfeldt et al., 1983). This implicates that the effects of vitamin D_2 and vitamin D_3 on mammals in general differ quantitatively and maybe also qualitatively.

BACKGROUND

THE D VITAMINS

The vitamin D molecule is termed a secosteroid - a steroid in which one ring has opened. Carbon numbering and configurational designation of the precursor (7-dehydrocholesterol) is retained in the secosteroid (Fig. 1).



7-dehydrocholesterol **Vitamin D_3** **Vitamin D_2**

Fig. 1. Structure and carbon numbering of 7-dehydrocholesterol vitamin D_3 and vitamin D_2

The two major forms of vitamin D are vitamin D_2 and vitamin D_3. They share a common steroid nucleus but have different side chains. Vitamin D_2 has a methyl group in position 24 and a double bond between carbon atoms 22 and 23. Vitamin D_2 (ergocalciferol) is produced from irradiation of ergosterol with 250 to 310 nm ultraviolet light (Velluz & Amiard, 1949). Ergosterol is present in higher plants and yeasts, and its abundance in brewers yeast (Saccharomyces cerevi-

siae) has made this the most common source for vitamin D_2 (Norman, 1979).

Vitamin D_3 (cholecalciferol) is the endogenous form of vitamin D. Naturally occurring dietary sources of vitamin D_3 include egg yolk, fatty fish, fish liver oil, and butter. Vitamin D_3 is produced in mammalian skin after ultraviolet irradiation of 7-dehydrocholesterol.

Since the natural content of vitamin D in food is low, vitamin D_3 has been added to milk and margarine in Sweden in order to ensure an estimated adequate intake of vitamin D in the order of 2.5 µg/day to 10 µg/day (100-400 IU/day) in healthy adults.

In Table 1, the normal plasma levels of vitamin D and the major metabolites are given together with the estimated half-lives.

TABLE 1 NORMAL PLASMA LEVELS AND ESTIMATED HALF LIFE IN MAN OF VITAMIN D AND ITS MAJOR METABOLITES

	Plasma concentration	Half life
Vitamin D_3	1-6 ng/ml (Shepard, 1979)	20-24 hr (Avioli, 1967)
25-OH-D_3	15-30 ng/ml (Haddad, 1974)	12 days (Haddad, 1976)
1,25$(OH)_2D_3$	30-50 pg/ml (Haussler, 1976)	10 hr (Gray, 1978)

VITAMIN D METABOLISM

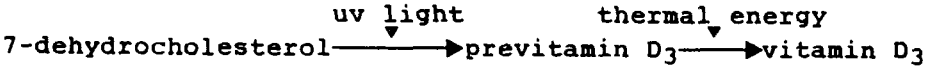
An outline of vitamin D metabolism is shown in fig. 2.

Intestinal absorption

Intestinal absorption of vitamin D occurs mainly in the jejunum (Schachter et al., 1964). About 80% of the ingested amount is normally absorbed (Parfitt & Kleerekoper, 1980) by simple passive diffusion into the intestinal mucosa (Hollander, 1981) and enters the circulation via the portal system and the thoracic duct (Sitrin et al., 1982). Micellar incorporation of vitamin D and chylomicron transport are enhanced in the presence of bile salts (Davies et al., 1980).
The absorption of 25OHD is less dependent on bile salts and this form is transported predominantly via portal blood into the circulation (Davies et al., 1980).
Since the administration of radioactive vitamin D leads to excretion of radioactive material in the bile (Bell & Kodicek, 1969) it has been suggested that there is an enterohepatic circulation conserving vitamin D similar to that for bile salts (Arnaud et al., 1975). However, this recycling concerns probably the vitamin D catabolites and may be of small significance for vitamin D status (Fraser, 1983).

Dermal formation

7-Dehydrocholesterol is present in large amounts in the stratum germinativum of the epidermis (Reinertson & Wheatley, 1959). Ultraviolet light of wavelengths 250-310 nm penetrates the epidermis and is absorbed by 7-dehydrocholesterol which is converted to previtamin D_3 by chemical photolysis (Holick et al., 1979). This compound undergoes thermal isomerization to vitamin D_3, requiring about 36 hr at body temperature to equilibrate (Holick et al., 1977):



7-Dehydrocholesterol may, on further photoactivation, be transformed to tachysterol and lumisterol as side reaction products (Havinga, 1973). Production of vitamin D by skin depends on geographic location (latitude) (Holick et al., 1981), season of the year, altitude and atmospheric condi-

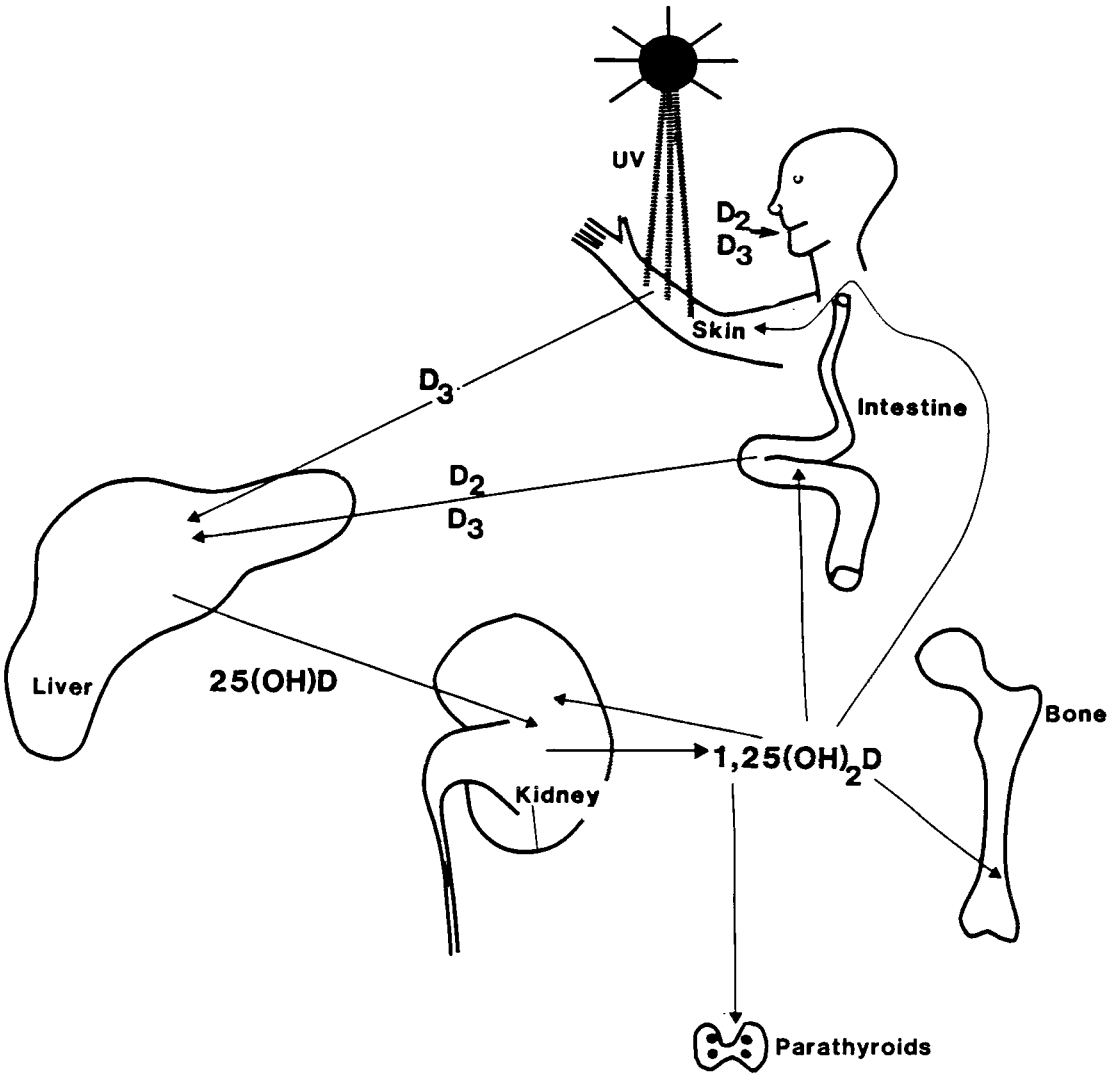


Fig. 2
Vitamin D is made available to the organism by synthesis in the skin after UV irradiation or by intestinal absorption. The vitamin D must then be hydroxylated first in the liver to 25(OH)D and then in the kidney to $1,25(OH)_2D$ before being biologically active. $1,25(OH)_2D$ acts on target tissue to maintain Ca and P homeostasis.

tions, clothing, and skin pigmentation (Loomis, 1967; Clemens et al., 1982).

Following formation in the skin, vitamin D_3 is bound to vitamin D binding protein in plasma and transported to the liver (Holick & Clark, 1978), where hydroxylation to $25OHD_3$ takes place. The importance of the skin in maintaining the vitamin D status is demonstrated by the seasonal variations in $25OHD_3$ levels, where late summer levels are twice as high as late winter concentrations (Chesney et al., 1981).

The continuous release of vitamin D_3 from skin into the circulation have greater long-term effects on vitamin D metabolism than discontinuous ingestion of vitamin D. The absence of toxic manifestations even after prolonged intense exposure to ultraviolet irradiation is noteworthy. This implicate a control in the release of vitamin D_3 from the skin (Stanbury, 1980).

Storage

Depletion of vitamin D is a slow process. Vitamin D is stored primarily in the fat depots (Rosenstreich et al., 1971) and depletion in man or animals may take months. The storage form appears to be mainly the free vitamin D sterol. The existence of a vitamin-binding protein in muscle (Haddad, 1982) could have physiological significance.

Hepatic conversion

Vitamin D circulating in blood is taken up by the liver and rapidly metabolized to 25OHD (Ponchon & DeLuca, 1969). The liver is the major site for 25-hydroxylation (Olsson et al., 1976). The enzymes involved - the vitamin D 25-hydroxylases - have been found in microsomal (Bhattarachyya & DeLuca, 1974) and mitochondrial fractions (Björkhem & Holmberg, 1978). Vitamin D 25-hydroxylase is a mixed-function monooxy-

genase requiring NADPH, oxygen and magnesium (Bhattarachyya & DeLuca, 1974; Madhok et al., 1978).

The substrate cholecalciferol suppresses the 25-hydroxylase (Bhattarachyya & DeLuca, 1973) but large amounts of substrate may overcome the block, resulting in very high circulating levels of 25OHD (Haddad & Stamp, 1974). 25OHD is not accumulated in the liver but transported on the vitamin D-binding protein (DBP) in serum. At present it is believed that the same 25-hydroxylase acts on vitamin D_2 and D_3 (Jones et al., 1976b).

It is possible that under physiological conditions, the microsomal enzyme is operative, while the mitochondrial enzyme operates when vitamin D levels are elevated (Fukushima et al., 1978; Björkhem et al., 1980). The microsomal and mitochondrial 25-hydroxylases from rat liver preparations hydroxylates vitamin D_2 more slowly than vitamin D_3 (Andersson et al., 1983; Holmberg, 1984).

There are 25-hydroxylases present in the liver acting on other metabolites such as dihydrotachysterol (Bhattarachyya & DeLuca, 1973) and $1\alpha OHD_3$ (Fukushima et al., 1978; Holick et al., 1976). 25-hydroxylation of $1\alpha OHD_3$ is not under metabolic control (Fukushima et al., 1978).

Renal activation

The second step in vitamin D metabolism occurs in the kidney. 25OHD is the precursor for at least two metabolites: $1,25(OH)_2D$ and $24,25(OH)_2D$. The 1-alphahydroxylation takes place in the mitochondria of renal cortical cells (Fraser & Kodicek, 1970).

1-hydroxylase activity is conditioned by a number of hormones (Table 2). The renal intracellular concentration of phosphorus also influences the activity of 1-hydroxylase (Tanaka & DeLuca, 1973). The principal modulator of 1-hydroxylase activity appears to be PTH and serum calcium concentration (DeLuca, 1979).

TABLE 2 Hormones that enhance the activity of 1 - hydroxylase

PTH	(Garabedian et al., 1972)
Estrogen	(Castillo et al., 1977)
Prolactin	(Spanos et al., 1976)
Insuline	(Schneider et al., 1977)
Growth hormone	(Spanos et al., 1978)

Under certain circumstances extrarenal production of $1,25(OH)_2D$ can occur, such as in placental cells (Whitsett et al., 1981) and in sarcoid tissue (Barbour et al., 1981), but normally the exclusive source of $1,25(OH)_2D$ production is the kidney (DeLuca, 1979).

When the supply of calcium and phosphorus is adequate, $1,25(OH)_2D$ formation decreases and the formation of the other metabolite $24,25(OH)_2D$ increases. 25-hydroxylation occurs primarily in the kidney (Horst et al., 1979) and the activity of the 24-hydroxylase is induced by the presence of $1,25(OH)_2D$ (Tanaka & DeLuca, 1974). 24-hydroxylation appears essential for the inactivation of the vitamin D molecule (Holick et al., 1976), while other roles for $24,25(OH)_2D$ are doubtful (Jarnagin et al., 1983; Brommage et al., 1983).

Plasma transport

Vitamin D, 25OHD, $24,25(OH)_2D$ and $1,25(OH)_2D$ are transported in plasma bound to vitamin D binding protein (DBP) with the highest binding affinity for 25OHD and $24,25(OH)_2D$ (Bouillon et al., 1976; Haddad & Walgate, 1976). DBP is synthesized by the liver (Prunier et al., 1964; Bouillon et al., 1976) and circulates in large excess over all the vitamin D compounds. Consequently more than 99% of all metabolites are bound to DBP (Bouillon & Van Baelen, 1981).

Inactivation and excretion

The further metabolism of $1,25(OH)_2D_3$ includes hydroxylation in the kidney and intestine to 1,24,25-trihydroxyvitamin D_3, a metabolite one tenth as active as $1,25(OH)_2D_3$ (Holick et al., 1973; Kumar et al., 1978). In the liver and intestine $1,25(OH)_2D_3$ is oxidized to a 23-carbon acid called calcitroic acid. Calcitroic acid is relatively inactive and is rapidly cleared from circulation (Kumar et al., 1976; Esvelt & DeLuca, 1981). In the intestine there are enzymes to degrade $1,25(OH)_2D_3$ into 1,25,26-dihydroxy-23-oxy-D_3 (Ohnuma & Norman, 1982).

Some metabolites are in the form of glucuronides, others are neutral compounds, sulfates and bisulfates (Weisner et al., 1980). Approximately 4% of administered vitamin D is excreted in the urine (Avioli et al., 1967), and the major route of exretion is via the bile and feces.

PHYSIOLOGIC ACTION OF VITAMIN D

The receptor concept

Vitamin D is most properly considered a pro-hormone. Steroid hormones act on a variety of target cells by influencing gene expression. The functioning of the steroid hormones depends on their interaction in the cells with protein molecules termed receptors. Each steroid hormone affects only the cells that contain the appropriate receptor. The complex formed by the hormone and the receptor controls protein synthesis by acting on DNA in the nucleus (Grudy et al., 1982).

Cellular action

Within half an hour after administration, radiolabeled $1,25(OH)_2D_3$ is localized specifically in the nuclei of villus cells and crypt cells (Stumpt et al., 1979). As with other steroid hormones, the presence of the active vitamin D

metabolite in the nuclei indicate that there exists a specific binding protein (receptor molecule) for $1,25(OH)_2D_3$ within these cells. A receptor molecule with a MW around 60.000 has been demonstrated in the small intestine (Wecksler et al., 1979).

Lately it was reported that the synthesis of the polyamine putrescine was induced via induction of duodenal spermidine N^1-Acetyltransferas half an hour after the injection of $1,25(OH)_2D_3$ (Shinki et al., 1985). Putrescine and spermidine are found in normal and tumor cells, and the concentration of these polyamines is related to the rate of cell differentiation and growth.

Intestine

It is well known that vitamin D stimulates the intestinal absorption of calcium, and also that vitamin D is necessary for adaptation of calcium absorption to the needs of the organism (Nicolaysen et al., 1953) and it was recognized early that vitamin D also influences phosphate transport (Harrison & Harrison, 1961).

Calcium is transported against an electrochemical potential gradient in the small intestine. The most rapid rate of transport occurs in the duodenum (Schachter & Rosen, 1959). How Ca traverses the epithelial cell is not known. Possibly it is bound to mitochondria (Borle, 1974) and is later released at the basement membrane surface, a process which requires sodium ions (Martin & DeLuca, 1969).

Suckling rat pups are insensitive to the stimulation of $1,25(OH)_2D_3$ on intestinal calcium uptake until they begin eating solid food (Halloran & DeLuca, 1980). Since rat pups lack the receptor for $1,25(OH)_2D_3$ in the small intestine until they approach weaning (Halloran & DeLuca, 1981b), it appears that interaction of $1,25(OH)_2D_3$ with the receptor plays an important role in the initiation of intestinal calcium transport.

In embryonic chick intestinal organ culture, inhibitors of protein and RNA synthesis, such as actinomycin D and cycloheximide, block $1,25(OH)_2D_3$-induced calcium transport. This indicates the existence of a nucleus-mediated mechanism for $1,25(OH)_2D_3$ action on calcium uptake (Francesci & DeLuca, 1981).

The nature of the 4-5 Ca transport proteins now known to be formed in response to $1,25(OH)_2D_3$ (DeLuca, 1985) is uncharacterized except for one: this is the calcium-binding protein (CaBP) of about 10.000 daltons that binds two atoms of Ca to each molecule (Wasserman & Feher, 1977).
$1,25(OH)_2D_3$ could also promote calcium uptake by a non-nucleus-mediated mechanism. Changes in lipid composition of brush border membranes (Goodman et al., 1972) and the appearence of vesicles at these membranes increasing the permeability to calcium (Rasmussen et al., 1979), have been reported following $1,25(OH)_2D_3$ dosing.

The biphasic nature of intestinal Ca transport response to a single injection of $1,25(OH)_2D_3$ suggests that there are two mechanisms of Ca transport (Halloran & DeLuca, 1981a). After a lag of 2 hours, the rate of intestinal Ca transport rises to a peak at 6 hours, then drops back to pre-injection levels at 12 hours. A late response occurs at 24 hr and continues for several days. The initial response is thought to reflect an interaction of $1,25(OH)_2D_3$ with existing villus cells, while the late response could involve intestinal crypt cells that mature and migrate along the villus membrane surface. The dual reponse could also reflect an initial direct effect on brush border membrane permeability, while the late response could require protein synthesis (Halloran & DeLuca, 1981; Wasserman et al., 1982).

Phosphate is also transported against an electrochemical gradient in response to $1,25(OH)_2D_3$ (Walling, 1977). Maximal transport takes place in the jejunum (Harrison & Harrison, 1961).

The same type of dual response to a single injection of $1,25(OH)_2D_3$ is reported for P transport (Kabakoff et al., 1982).

Bone

The healing of rachitic lesions and the normalization of bone mineralization by vitamin D were the basis for the discovery of vitamin D. The action of vitamin D on bone mineralization appears to be indirect via providing sufficient amounts of Ca and P to the chondroblasts and osteoblasts in newly formed matrix (Howland & Kramer, 1921). Vitamin D is not necessary for mineralization to occur at a normal rate as long as concentrations of Ca and P in the plasma are kept normal, either by dietary manipulations (Howard & Baylink, 1980) or by continuous infusion of Ca and P (Underwood et al., 1984; Weinstein et al., 1984).
$1,25(OH)_2D_3$ stimulates the activity of osteoclasts directly and increases their number (Holtrop et al., 1981).
$1,25(OH)_2D_3$ is the most potent vitamin D metabolite in inducing bone resorption (Reynolds et al., 1973). Bone cells have a high affinity for $1,25(OH)_2D_3$ (Kream et al., 1977), and there is evidence for a nuclear localization of the metabolite prior to bone cell response (Weher et al., 1971). The presence of PTH is necessary in vivo to induce bone Ca mobilization (Garabedian et al., 1974).

Kidney

The effects of vitamin D on renal handling of Ca and P is difficult to evaluate. Vitamin D also changes Ca and P in serum, and affects PTH secretion. All these factors influences the renal homeostasis. The physiological significance of a possible effect on renal mineral handling is also unestablished.

Ninety-nine per cent of all filtered Ca is reabsorbed even in the absence of either vitamin D or PTH (Kleeman et al., 1961). Since man filters around 10 g of Ca per day, the remaining appears a significant amount (Nordin & Smith,

1965). Vitamin D acutely improves the reabsorption of Ca, probably by acting on the distal tubules (Sutton et al., 1975). The effect of vitamin D on the tubules seems dual, i.e. to facilitate tubular Ca reabsorption and to enhance the responsiveness of the tubules to PTH (Yamamoto et al., 1984).

When used clinically vitamin D leads to hypercalciuria even without concomitant hypercalcemia (Litvak et al., 1958). The increase in Ca excretion is likely to be due to the suppression of PTH release secondary to an increase in intestinal Ca absorption.

It was suggested early that vitamin D might conserve P by reducing renal excretion of P (Harrison & Harrison, 1941). The response of the kidney to stimulation by $1,25(OH)_2D_3$ seems, however, dependent on body stores of P. When $1,25(OH)_2D_3$ was given to rats that were slightly hyperphosphatemic, the results was phosphaturia (Bonjour et al., 1977); but if the rats were hypophosphatemic, administration led to a conservation of P by the kidney (Constanzo et al., 1974). The tubular action that leads to these divergent responses to vitamin D administration remains unknown.

Muscle

Muscle weakness and lack of muscle tone are prominant findings in rachitic patients. The symptoms are readily ameliorated by the administration of $1,25(OH)_2D_3$ (Russel et al., 1974). Ultrastructural changes of muscle in vitamin D deficient animals reverted to normal after $1,25(OH)_2D_3$ administration (Sjöström et al., 1978). However, muscle does not contain receptors for $1,25(OH)_2D_3$ (DeLuca, 1981).

Parathyroids

The parathyroid glands contain specific receptors for $1,25(OH)_2D_3$ (Brumbaugh et al., 1975) and nuclear localization of tritiated $1,25(OH)_2D_3$ has been demonstrated on autoradiography (Stumpf et al., 1979). It is not established

whether $1,25(OH)_2D_3$ influences directly PTH secretion (Chertow et al., 1975, Kugai et al., 1981) or acts by increasing the responsiveness of the glands to changes in serum Ca levels (Oldham et al., 1979).

Other tissue

The number of target organs with receptors for $1,25(OH)_2D_3$ are numerous. Notable exceptions are skeletal and intestinal muscle, liver, spleen and bone marrow cells (DeLuca, 1981). In Table 3 the tissues were receptors for $1,25(OH)_2D_3$ have been found are listed.

Vitamin D induces the redifferentiation of myeloid leukemia cells into macrophages (Miyaura et al., 1981) and have antitumor effects (Sato et al., 1982).

Table 3. Tissue with evidence of receptors for $1,25(OH)_2D_3$

Intestine	(Wecksler et al., 1979)
bone	(Kream et al., 1977)
kidney	(Christakos & Norman, 1979)
parathyroid gland	(Brumbaugh et al., 1975)
pituary gland	(Stumpf et al., 1979)
skin	(Feldman et al., 1980)
brain and spinal cord	(Stumpf et al., 1982)
mammary gland	(Colston et al., 1980)
placenta	(Pike et al., 1980)
pancreas	(Christakos & Norman, 1979)
thymus	(Reinhardt et al., 1982)

VITAMIN D THERAPY

With the introduction of new active metabolites and analogs of vitamin D in the past few years, the management of mineral homeostasis derangements and diseases of bone is changing.

The half-life of vitamin D in plasma is approximately one day (Avioli et al., 1967), but since large amounts of vitamin D are stored in fat, withdrawal of vitamin D in patients with intoxication still leaves hypervitaminosis persisting for months. When active vitamin D metabolites are used, hypervitaminosis is faster corrected upon withdrawal of medication.

The drugs most commonly used at present are listed in Table 4, together with their commonly employed dose regiments. The persistence of hypervitaminosis upon withdrawal of the drug is also stated.

Table 4 Vitamin D preparations used in treatment of hypocalcemia and renal osteodystrophy, their dosing and their persistence of toxicity after withdrawal in cases of hypervitaminosis (After Haussler & Cordy, 1982)

Drug	Human daily dose	Persistence of toxicity (days)
Vitamin D	1-10 mg	17-60
$25OHD_3$	0,05-0,5 mg	7-30
Dihydrotachysterol	0,2 - 1,2 mg	3-14
$1\alpha OHD_3$	1 - 2 µg	5-10
$1,25(OH)_2D_3$	0,5 - 1 µg	2-10

Rickets and osteomalacia

Rickets and osteomalacia are the juvenile and adult forms of vitamin D deficiency. Both diseases are characterized by the failure of newly formed matrix to mineralize (Mankin, 1974). With vitamin D supplementation of dairy products, rickets has largely been eliminated in the western world. However, in a world-wide perspective, rickets still represent a major health problem (Stephens et al., 1982; Groen et al., 1965). Osteomalacia, on the other hand, is not uncommon

in the aged population in industrialized western countries. The major predisposing factors are to little sunlight, low dietary intake of vitamin D, and intestinal malabsorption of Ca (Parfitt et al., 1982).

Osteomalacia may occur in adults with a dietary intake of less than 70 IU/day of vitamin D (Dent & Smith, 1969), which implies that strict vegetarians are at risk. Diets high in phytic acid found in unleavened bread, Indian chepatte (Wills et al., 1972) and Bedouin raghif (Berlyne et al., 1973), have complex effects on mineral metabolism, inducing osteomalacia. Interference of phytate with vitamin D metabolism has been suggested as one factor (Hill, 1972). The formation of amorphous calcium phytate in the intestine could add to the hypocalcemic state (Mellanby, 1949).

Vitamin D depletion occurs after various disorders accompanied by intestinal malabsorption and steatorrhea, such as biliary obstruction and hepatic parenchymal disease (Long et al., 1976), partial gastrectomy (Gertner et al., 1977) and intestinal by-pass surgery (Mosekilde et al., 1982). The causes of vitamin D depletion are multiple: decreased absorption, altered enterohepatic circulation, decreased DBP production, and changing hydroxylation capacity of the liver (Kumar, 1983).

Patients given total parenteral nutrition for more than three months may develop osteomalacia, due to low levels of circulating $1,25(OH)_2D_3$ (Klein et al., 1982). Chronic treatment with antiepileptic drugs such as phenobarbital or diphenylhydantoin increases the incidence of osteomalacia and reduces plasma levels of 25OHD (Christiansen et al., 1974). Low plasma levels of 25OHD in the nephrotic syndrome could result from urinary losses of DBP together with vitamin D (Barragry et al., 1977). These vitamin D deficiency states are successfully treated with vitamin D or any active metabolite.

Vitamin D dependant rickets is an extremely rare disease characterized by the inability to convert $25OHD_3$ to

$1,25(OH)_2D_3$: Inherited as an autosomal recessive disease, it is probably due to a dificiency of 1-hydroxylase in the kidney (Fraser et al., 1973) - A type II form has been reported, where hypocalcemia is present despite increased amounts of $1,25(OH)_2D_3$ and PTH. This defect is due to end-organ resistence (Silver et al., 1985). Vitamin D dependent rickets reacts favourably to $1,25(OH)_2D_3$ (Fraser et al., 1973) or $1\alpha OHD_3$ (Balsan et al., 1977).

Vitamin D resistant hypophosphatemic rickets displays an X-linked dominant inheritance pattern. The level of $1,25(OH)_2D_3$ probably is normal (Haussler & McCain, 1977). The disease is characterized by severe hypophosphatemia, and there may be a defect in the PTH-sensitive phosphate transport site in the proximal renal tubuli (Scriver, 1974). Treatment with a combination of phosphate and $1\alpha OHD_3$ is the most successful therapy (Rasmussen et al., 1981).

Osteoporosis

Osteoporosis is defined as a reduction in bone mass leading to fractures of the spine, hip, or distal forearm caused by bone mineral resorption exceeding new bone formation. **Postmenopausal osteoporosis** is associated with reduced intestinal calcium absorption (Gallagher et al., 1979). The Ca balance is negative and reduced levels of $1,25(OH)_2D_3$ have been reported in these patients (Gallagher et al., 1979). The efficacy of long-term treatment with $1,25(OH)_2D_3$ to prevent spinal fractures in patients with postmenopausal osteoporosis has recently been reported (Gallagher, 1985).

Hypercortisonism results in loss of trabecular bone, with decreased bone formation and increased bone resorption (Hahn, 1978). Intestinal Ca absorption is decreased and hyperparathyroidism results as a consequence of lower serum Ca. Serum $1,25(OH)_2D_3$ has been found normal (Seeman et al., 1980) or reduced (Chesney et al., 1978). Administration of $25OHD_3$ (Hahn et al., 1979), $1\alpha OHD_3$ (Lindgren et al., 1978), or $1,25(OH)_2D_3$ (Klein et al., 1977) to patients receiving

glucocorticoids led to increase of intestinal Ca absorption. However, the administration of $1,25(OH)_2D_3$ did not increase bone mass or reduce fracture rate, even though it suppressed PTH secretion (Dykman et al., 1984).

Renal osteodystrophy

About 50 per cent of patients with chronic renal failure exhibit pathologic changes in their skeleton when beginning dialysis (Malluche et al., 1976). These bone lesions may take the form of osteomalacia, osteitis fibrosa cystica with or without osteosclerosis, or osteopenia (Felsenfeld & Llach, 1982).

Patients with chronic renal failure were the first to be treated with $1\alpha OHD_3$ (Brickman et al., 1972) and they are still the largest group of patients to receive this compound. Effective treatment of renal osteodystrophy has been reported using vitamin D_3 in large doses (Lumb et al., 1971), dihydrotachhysterol (Cordy & MIlls, 1981), or $25OHD_3$ (Witmer et al., 1976). Treatment with $1\alpha OHD_3$ or $1,25(OH)_2D_3$ is more effective in reducing PTH secretion (Balsan et al., 1982; Varghese et al., 1979); therefore these latter compounds are preferred. The use of $24,25(OH)_2D_3$ seems of no therapeutic value, not even in combination with $1,25(OH)_2D_3$ (Muirhead et al., 1982).

Hypoparathyroidism

Hypoparathyroid patients lack the ability to secrete PTH in response to hypocalcemia. The lack of PTH leads to low circulating levels of $1,25(OH)_2D_3$. Hypoparathyroidism leads to hyperphosphatemia, which also suppresses $1,25(OH)_2D_3$ production. These patients may be treated with $1,25(OH)_2D_3$ or $1\alpha OHD_3$ plus dietary Ca supplementation (Neer et al., 1975).

Pseudohypopharathyroid patients secrete adequate amounts of PTH but the kidney fails to respond, and the patients become hypocalcemic due to low levels of circulating $1,25(OH)_2D_3$ (Drezner et al., 1976). These patients may benefit from treatment with $1,25(OH)_2D_3$ or $1\alpha OHD_3$ (Kooh et al., 1975).

VITAMIN D TOXICITY

Vitamin D is one of two vitamins that are toxic at high doses the other being vitamin A. Symptoms of vitamin D intoxication include muscle weakness, polyuria, dehydration, vomiting, obstipation, nausea, demineralization of bone, elevated serum Ca and P, and most seriously ectopic calcification in lungs, heart, and vessels, and nephrocalcinosis (Howard & Mayer, 1948; Anning et al., 1948; Danowski, 1962).

The report of vitamin D intoxication in an anephric patient (Counts et al., 1975) and the finding that $1,25(OH)_2D$ levels were low or moderately elevated in intoxicated patients while 25OHD levels were exceedingly high point to 25OHD as the metabolite causing intoxication (Hughes et al., 1976; Mawer et al., 1984). 25OHD is known to increase intestinal Ca absorption when present in excessive amounts, and 25OHD may also mobilize Ca from bone (DeLuca, 1978).

The hepatic 25-hydroxylases do not seem as tightly regulated as the renal 1-hydroxylase, and levels of 25OHD in 40-fold excess have been reported (Haddad & Stumpf, 1974). Whether or not normocalcemic hypercalciuria can be harmful to kidney function is uncertain, although this seems possible after prolonged vitamin D treatment (Parfitt, 1976; Christiansen et al., 1981).

In sarcoidosis there is a greater risk of intoxication with vitamin D as the control of 1-hydroxylation is defective (Chesney et al., 1981).

Treatment of vitamin D intoxication consists of reducing Ca intake and administering glucocorticoids (Verner et al., 1958). The mechanims of glucocorticoid action is unclear, but involves decreased bone resorption (Streck et al., 1979), reduced intestinal Ca absorption and reduced levels of $1,25(OH)_2D_3$ by reducing 1-hydroxylase activity (Blahos et al., 1983). Urinary Ca excretion is also enhanced by glucocorticoids (Gallagher et al., 1973).

PRESENT STUDY

INTRODUCTION

The potential toxicity of vitamin D presents a major problem in clinical use of the vitamin. Even when using active metabolites or analogs such as $1\alpha OHD_3$ or $1,25(OH)_2D_3$ the result of therapy can be severe metabolic disturbances.

$1,25(OH)_2D_2$ was prepared in 1975 and found to have an antirachitic activity equal to that of $1,25(OH)_2D_3$ in rats, while $1,25(OH)_2D_2$ was 1/10 as active as $1,25(OH)_2D_3$ in chicks (Jones et al., 1975, 1976a).

$1\alpha OHD_2$ was first synthesized in 1974 (Lam et al.) but was not studied further until 1977 when Reeve et al. showed that $1\alpha OHD_2$ in comparison with $25OH_3$ was equally active in stimulating intestinal calcium transport, but induced less bone resorption.

$1\alpha OHD_2$ probably requires 25-hydroxylation before being active. There are recent reports indicating that the liver microsomal and mitochondrial 25-hydroxylase of the rat metabolize vitamin D_2 at a slower rate than vitamin D_3 (Andersson et al., 1983; Holmberg, 1984). Therefore, because of the need for vitamin D therapy and the promise of the $1\alpha OHD_2$ compound, we felt it was important to carry out a comparative study of $1\alpha OHD_2$ and $1\alpha OHD_3$.

MATERIALS AND METHODS

Animals

Rats have been used throughout the study because
a) the calcium metabolism is in several aspects similar to that of man (Bronner & LeMaire, 1969; Nicolaysen et al., 1953),
b) the rat has been used in studies of rickets since the early days of vitamin D research (McCollum et al., 1922), and vitamin D metabolism has been studied extensively in this animal, and

c) the rat has been used in experimental models of clinically important conditions such as glucocorticoid-induced osteoporosis (Storey, 1969; Lindgren et al., 1982).

Male rats were used in these series of experiments to exclude the influence of oestrogens on vitamin D metabolism.

English Wistar rats were obtained from the National Food Administration, Uppsala, Sweden (I). Sprague-Dawley rats were obtained from Holtzman Co, Madison, WI (II) or Anticimex, Sollentuna, Sweden (III, IV, V). The age of the animals ranged from 3 weeks to 4 months. The rats were kept in individual wire-topped plastic cages approximately 60 cm by 30 cm by 30 cm. The rats were housed in a climate-controlled room with 55% humidity and a temperature of 20°C. Light/dark cycles were 12 hours, running from 6 a.m. to 6 p.m. (Exp I, III, IV, V). Individual hanging wire cages approximately 40 cm by 30 cm by 30 cm were used in the toxicologic experiment (II). Experiments involving rachitic rats were performed under UV-free light.

Experimental models

Rickets was induced by feeding vitamin D-deficient 21 day old rats a diet very low in phosphate (Steenbock & Gerting, 1955). A four week depletion period is necessary to reach low levels in serum of $1,25(OH)_2D_3$ (Mallon et al., 1981). We did not have access to vitamin D-deficient rat pups from vitamin D-deficient mothers. In these rats, rachitic leasions can be induced without the use of diets low in phosphate (Halloran et al., 1981c), but the model used in experiment I is the traditional method of inducing rickets in rats (U.S. Pharmacopoea, 1955).
As a model of a clinical condition, osteoporosis was induced by the administration of 1.5 mg/day of prednisolone to rats given a diet somewhat restricted in Ca intake (0.3% Ca). This regimen induces changes in bone tissue of the rat similar to osteoporosis (Storey, 1960), and was thought relevant to the clinical situation of hypercortisonism in Cushings disease or in the situation of high-dose therapy with glucocorticoids.

Diet

Pelleted food was used in all studies except (II), where the food was administered as powder. The Ca and P content was different in different experiments (Table 5).

The rats were fed every other day. The rations were 15 g/day for 250-g rats and 5 g/day for 90-g rats. When prednisolone was added to the diet, the rations were adjusted so that as a rule all the food was consumed. Deionized water was provided *ad libitum*.

The sources of the diets are listed in Table 5.

Table 5. Diet composition with regard to Ca and P in the different experiments, and the source of the diets

Experiment	Ca (%)	P (%)	Obtained from
I	1.6	0.04	National Food Administration, P.O.Box 622, S-751 26 UPPSALA, Sweden
II	1.2	1.0	Wayne Lab Blox, Allied Mills, Chicago, Ill., USA
II	0.02	0.3	Department of Biochemistry, University of Wisconsin, Madison, WI 53706 , USA (Diet 11-Ca)
II	1.2	0.1	Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA (Diet 24)
III, IV, V	0.3	0.5	Ewos, P.O.Box 618, S-151 27, Södertälje, Sweden (R 219)
V	0.02	0.5	Ewos, Södertälje, Sweden (R 223)

Pharmaceutics

$1\alpha OHD_2$ was kindly provided by H. Paaren, Department of Biochemistry, Madison, WI, USA. It was obtained as a crystalline powder, which was eventually dissolved in absolute alcohol and then kept in darkness at -70°C until use. $1\alpha OHD_3$ was obtained from Løven Co., Industriparken 55, DK-2750 Ballerup, Denmark, and treated in the same manner as $1^{\alpha}OHD_2$.

Prior to use the concentrated solution containing $1\alpha OHD_2$ or $1\alpha OHD_3$ was diluted with absolute alcohol. The concentration of the solution was determined by the ultraviolet absorption spectrum. This shows a characteristic pattern. In Fig. 3 the absorption curve of $1\alpha OHD_2$ is shown, with the typical vitamin D absorption maximum at 265 nm and a minimum at 228 nm (Koshy, 1981).

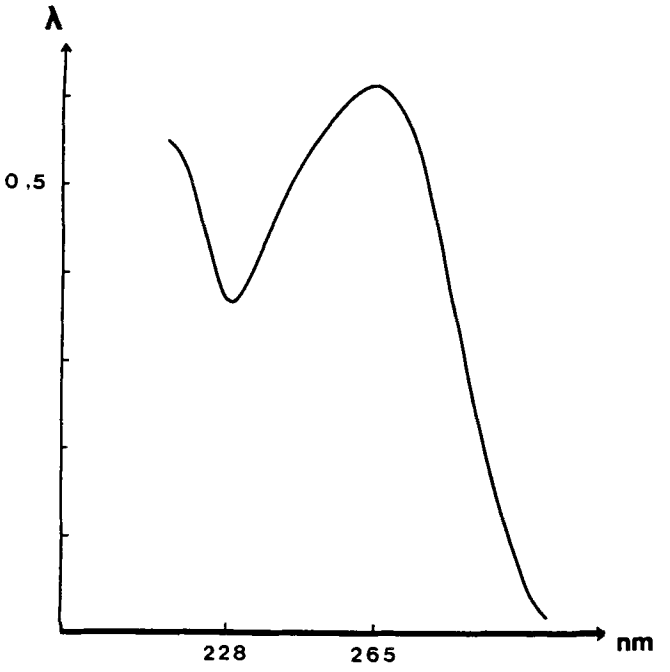


Fig. 3. The absorption curve of $1\alpha OHD_2$

The concentration is determined by the following equation:

$$\frac{M.W. \times \lambda \text{ max}}{18.3} = \text{conc } (\mu g/ml)$$

λ = absorption

MW (molecular weight) for $1\alpha OHD_2$ = 412.3

MW for $1\alpha OHD_3$ = 400.3

18.3 = molar extinction coefficient

Before use, the vitamin D solutions were mixed with Wesson oil or with propylene glycol to the appropriate concentration. The preparations were at all times shielded from light. They were stored at +8°C.

The dose of $1\alpha OHD_2$ and $1\alpha OHD_3$ was varied in the different experiments. The doses and the duration of treatment is listed in Table 6.

Table 6. Daily doses of $1\alpha OHD_2$ and $1\alpha OHD_3$ and the duration of treatment in the various experiments

Experiment	$1\alpha OHD_2$ (dose/kg/day)	$1\alpha OHD_3$ (dose/kg/day)	duration (days)
I	27.5, 275 or 2750 ng	27.5, 275 or 2750 ng	7
II	3.5, 6.5 or 10 mg*	0.1, 0.2, 0.5, 1.0 or 1.5 mg*	10
	8, 20, 30, 60, 120 or 300 µg	-	
	2.5, 5.0 or 20 µg	2.5, 5.0 or 20 µg	28
	25, 125 or 250 ng	25, 125 or 250 ng	7
III, IV	1.5 µg	1.5 µg	42
V	1.5, or 6.0 µg	1.5 or 6.0 µg	42

* Given as a single dose

Oral dosing was accomplished by a syringe fitted with a blunt needle ending in a short plastic tube. The tube was introduced into the hypopharynx and the dose (0.1-0.2 ml) was slowly administered.
Intraperitoneal dosing was carried out by injections of 0.2 ml in the lower right quadrant of the abdomen.

Prednisolone was dissolved in absolute alcohol to a concentration of 10 mg/ml. With a syringe, prednisolone was dropped evenly over the pelleted food rations and the alcohol was allowed to evaporate before the food was presented to the rats.

Ethical aspects

All experiments were performed in accordance with ethical recommendations of the National Board of Agriculture (S-551 83 Jönköping, Sweden). The experiments were classed as grade 2 on a 5-grade scale; i.e. the experiments were not painful for the rats.

Technical procedures

a) Analysis of serum

Serum was obtained by tail vein sampling or by aortic exsanguination. The samples were collected between 9 a.m. and noon. Blood was collected in plastic tubes, allowed to clot at room temperature for 2 h, and then centrifuged at 2000 rpm in a Sorvall GLC-2 centrifuge. Serum was pipetted off and stored at -20°C. Serum Ca was measured after dilution with 0.4% $SrCl_2$ or with 0.1% $LaCl_3$ on a Pye-Unicam atomic absorption spectrometer (model 2900). Serum P was determined by a photometric method (Kallner, 1975): 20 µg of serum (or 1:20 diluted urine) was added to 1 ml of 5 mmol/l of urea solution. Four milliliters of malachite green reagent was added and mixed well. After 20 min the absorbance was read at 630 nm on a Beckman model 20 spectrophotometer. In experiment II, serum P was determined by the method of Chen et al., (1956).

Serum $1,25(OH)_2D$ was determined using a modification of the technique described by Eisman et al. (1976). 0.9-4.2 ml of serum was diluted to 5 ml with distilled water. To each sample 20 µl ethanol containing 3000 c.p.m. of ^3H-$1,25(OH)_2D_3$ was added. The lipids were extracted with three volumes of methylene chloride and reextracted with three volumes of methanol/methylene chloride (2:1 v/v) and two separate volumes of methylene chloride. The combined volumes of methylene chloride were evaporated under reduced pressure. The lipid residue was solubilized in chloroform/hexane (1:1 v/v), and the procedure was repeated twice. The sample was dried under nitrogen, then solubilized in 0.5 ml hexane/chloroform/ethanol (9:1:1 by vol) and applied to a column (0.7 cm by 12 cm) of Sephadex LH-20 (Pharmacia, S-751 82

Uppsala, Sweden) in hexane/chloroform/ethanol. Another two rinses of 0.5 ml of the lipid extract were applied to the column, then 9.8 ml of solvent was added to the column and this eluate was discarded. Another 16.5 ml was added and this fraction was collected and dried under nitrogen. The residue was redissolved in 40 µl of isopropanol/hexane (90:10 v/v). Final purification was carried out by HPLC on a Zorbax-Sil column (4.6 mm by 25 cm) with a flow rate of 2.3 ml/min. HPLC was done on a LC 204 chromatograph fitted with a model 6000A pumping system, a U6K injection valve and a model 440 UV fixed wavelength (254 nm) detector (all from Waters Associates, Milford, MA., USA). The $1,25(OH)_2D$ was eluted at 13-20 ml, as determined with $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$ standards. The metabolite was assayed by a slight modification of the method of Shephard et al. (1979) so that samples were incubated on ice with chick intestinal cytosol binding protein for 18 h. Scintillation counting was performed at room temperature in an LS-100C liquid scintillation system (Beckman, Palo Alto, Calif., USA).

Serum creatinine was measured using a photometric method involving picrate and detergent (Bergman & Öhman, 1980). The samples were automatically analyzed on a Vitatron AKES reaction rate analyzer (Vitatron Scientific BW, Dieren, Netherlands).

b) Analysis of urine

Urine was collected from the rats in metabolic cages (Fig. 4). The rats were kept in the cages for 1-3 days, and during this period they were given free access to deionized water and to their regular diet.

After addition of 0.5 ml 2N HCl, the urine was stored in plastic bottles at -20°C. Urinary Ca and P were analyzed in the same manner as serum Ca and P.

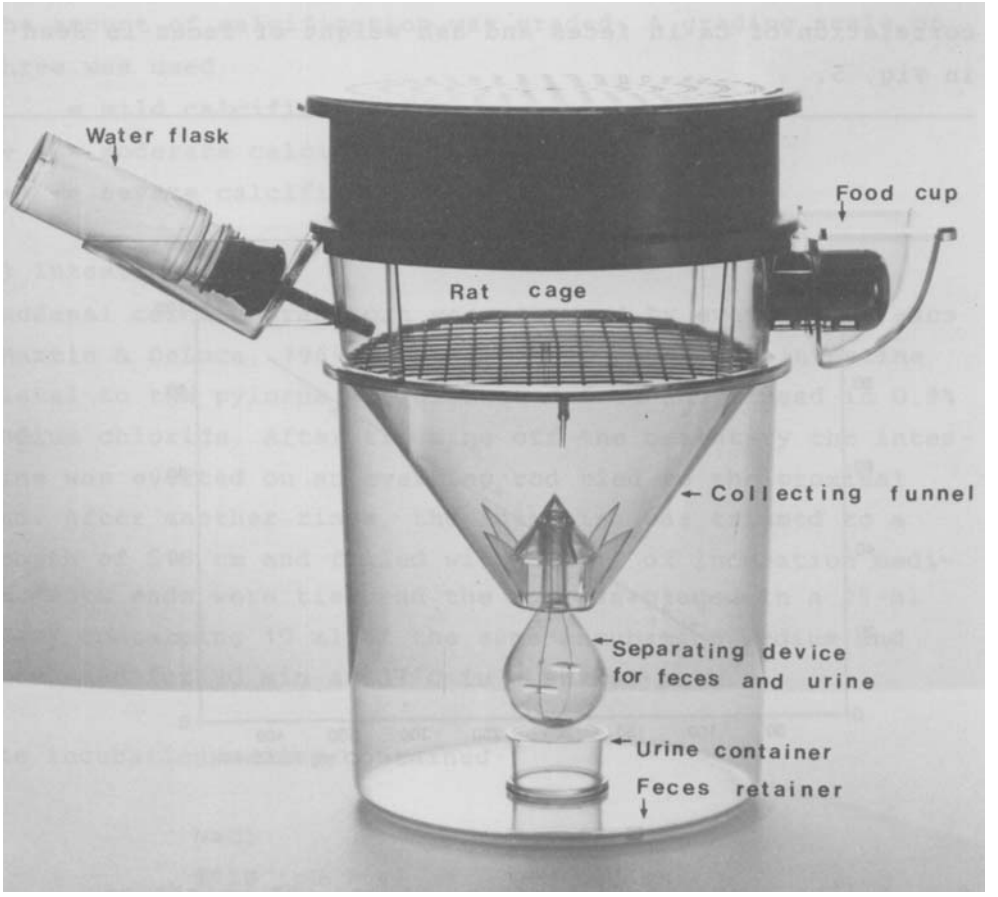


Fig. 4. Metabolic cage for the collection of urine and feces

c) Analysis of feces

Feces were collected during three-day periods when the rats were kept in metabolic cages. The feces were dried at 60°C for 12 h and then ashed at 700°C for 24 h and weighed.

The ash weight is closely correlated to the Ca content in feces. This was established in eighteen samples by first recording the ash weight and then dissolving the ash in 10 ml of 6N HCl. After proper dilution with distilled water, Ca was analyzed after adding 0.4% $SrCl_2$ using a Pye-Unicam atomic absorption spectrophotometer (model 2900). The close

correlation of Ca in feces and ash weight of feces is seen in Fig. 5.

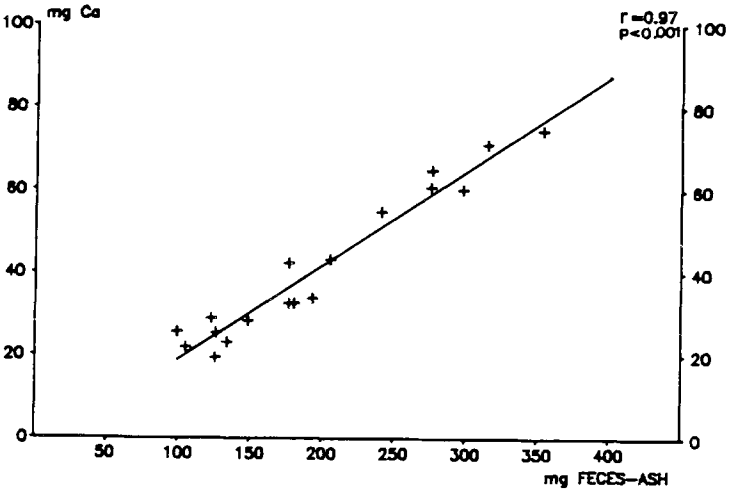


Fig. 5. The correlation of fecal Ca and ash weight of feces. After ashing and weighing, the ash was analyzed for Ca by atomic absorption spectrophotometry.
n = 18

d) Analysis of kidneys

At sacrifice the left kidney was removed. After drying at 60°C for 12 h, the kidneys were ashed in a muffle furnace at 700°C for 24 h. The ash was dissolved in 2 ml 6N HCl, then diluted with distilled water and 0.4% $SrCl_2$ or 1% $LaCl_3$. Ca was measured as for serum Ca analysis.

For histologic examination the right kidney was removed and put in 10% buffered formaline at pH 7.0. After paraffin embedding, 5 µm thick sections were stained for calcium-phosphate using von Kossa method (McMannus & Mowry, 1960).

The amount of calcification was graded. A grading scale of three was used:

+ = mild calcification

++ = moderate calcification, and

+++ = severe calcification

e) Intestinal tests

Duodenal calcium transport was measured by everted gut sacs (Martin & DeLuca, 1969). The first 10 cm of the intestine distal to the pylorus was dissected free and rinsed in 0.9% sodium chloride. After trimming off the mesentery the intestine was everted on an everting rod tied to the proximal end. After another rinse, the intestine was trimmed to a length of 5.5 cm and filled with 0.5 ml of incubation medium. Both ends were tied and the sac was placed in a 25-ml flask containing 10 ml of the same incubation medium and incubated for 90 min at 37°C in a shaker bath.

The incubation medium contained:

NaCl	125 mM
TRIS (pH 7.4)	30 mM
Fructose	10 mM
$CaCl_2.2H_2O$	0.25 mM

Oxygen was continuously bubbled through the flasks and at the end of incubation, the sacs were removed and blotted, then opened and drained. The serosal medium (inside the sac) and the mucosal medium (in the flask) were analyzed for Ca by atomic absorption spectrophotometry.
Data were expressed as the ratio of Ca inside the sac to Ca outside the sac (= serosa/mucosa).

Phosphate uptake was studied in jejunal discs by the method described by Kabakoff et al. (1982). The first 15 cm of the small intestine was excised and the distal end was opened lengthwise. With a cork borer two discs were cut from the jejunum and put into separate 25-ml flasks containing 9 ml of incubation solution of the following composition:

HEPES (pH 7.4)	10 mM
NaCl	150 mM
$CaCl_2.2H_2O$	2.5 mM
Fructose	22.2 mM (O_2 flask only)

After pre-incubating the two discs, one under an O_2 atmosphere and the other under an N_2 atmosphere, for 5 min, 1 ml of tracer solution containing ^{32}P was added:

HEPES (pH 7.4)	10 mM
NaCl	150 mM
KCl	60 mM
Na_2HPO_4	10 mM +^{32}P

The incubation with phosphate was allowed to proceed for 15 min and then it was quenched by washing the discs in two rinses of ice-cold solutions containing an excess of non-radioactive phosphate:

Na_2HPO_4	5 mM
HEPES (pH 7.4)	10 mM
NaCl	150 mM

The discs were blotted on tissue paper and put into glass scintillation vials containing 0.4 ml of tissuse solubilizer Soluene 100 (Packard Instruments, Downers Grove, Ill., USA) and incubated at 37°C for 12 h. The solubilized tissue was neutralized in concentrated acedic acid and 10 ml of Aquasol was added (New England Nuclear, Boston, Ma., USA). The radioactivity was counted on a liquid scintillation counter (Intertechnique SL 30).

The results were expressed as nmol/cm^2/15 min and represented the value obtained when passive uptake (that under nitrogen atmosphere and without metabolizable sugar) was subtracted from the uptake in tissue under oxygen atmosphere.

f) Bone analysis

Endochondral calcification (= line test)

In rachitic rats, radii were dissected free from adherent tissue. The right radius was rinsed in 0.9 % NaCl, and the distal end was split longitudinally. The two pieces were put in 2% $AgNO_3$ solution for 1 min. Both sections were then rinsed in distilled water and exposed, in water, to daylight for 1 h. The calcified areas in the endochondral growth plate developed a clearly defined stain. The degree of staining was scored under a dissecting microscope on a scale of 0 to 5 (U.S. Pharmacopoeia, 1955).

Quantitative histology

Histological studies were performed on the proximal left tibia (Fig. 6). After dehydration in successive changes of alcohol/chloroform (1:1, 2:1, then 1:0) the specimens were embedded in methyl methacrylate. Undecalcified sections 5 µ thick cut in the frontal plane were obtained and stained according to Goldner (1939). No cartilage was included in the area of measurement. Osteoid surface and total bone area were counted with a point-wave template according to the method of Merz & Schenk (1970). The osteoclasts were counted at a magnification of x 400 and identified as multinuclear cells in contact with bone.

Physical parameters

The femurs, tibias or radii were immersed in distilled water for 12 h. After drying the exterior of the bone with tissue paper, the weight was recorded on a Sartorius 2004 MP balance (Sartorius, Göttingen, W Germany). Then a fine thread was attached to the bone and it was again immersed in water and weighed. The bone volume was determined according to Archimedes' principle.

The density was calculated as wet weight per volume and expressed as g/cm^3. The bones were then defatted in six changes of acetone over a period of three days. The dry weight was determined after the bones had been kept in a drying oven at 60°C for 24 h. The bones were subsequently

ashed in a furnace at 700°C for 24 h and the ash weight was recorded under constant conditions.

In experiment III, the tibiae were sectioned 10 mm distal to the proximal end. The two parts of each bone were weighed and ashed separately.
Fig. 6 illustrates the bone analysis of experiment III.

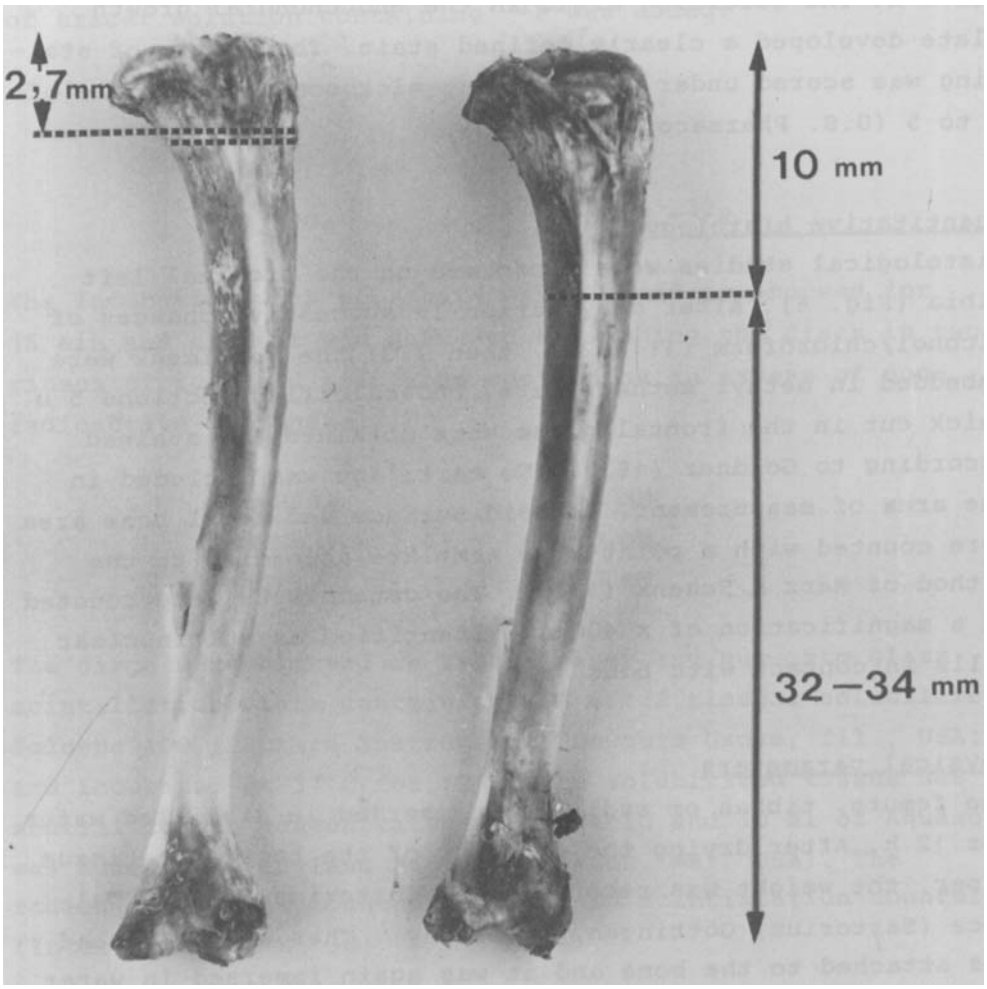


Fig. 6. Schematic illustration of bone analysis in experiment III. Histological examination was done on nine fields, each 0.06 mm^2 and usually 2.7 mm from the epiphysis of the tibiae. The contralateral tibiae were divided 10 mm from the proximal end and the physical parameters were recorded.

Statistical methods

In experiment I the statistical significance of obtained differences was proved by using Dunnett's procedure (Kirk, 1968). In the other experiments (II, III; IV and V), the hypothesis of no difference between groups was tested using Student's t-test. The analyses were performed on a WANG 2200 computer (WANG Laboratories, Tewksbury, Mass., USA). The differences between groups were considered significant when $p < 0.05$.

Data are presented as means ± S.D. or ±S.E.M. as indicated in the text.

RESULTS

Rickets (I)

Beside the data presented in experiment I (see page 3) oral dosing with 12.5 ng/day were given to rachitic rats for 7 days. Neither dosing with $1\alpha OHD_2$ or $1\alpha OHD_3$ affected growth of the animals (Table I:I), but oral dosing resulted in the same increase in skeletal mineralization for both compounds (Table I:II). Calcium transport across the intestinal wall increased significantly with both compounds, and serum Ca was concomitantly raised with no consistent change in serum P.

Serum $1,25(OH)_2D$ was measurable in vitamin D-deficient controls. It rose to very high levels after dosing, indicating a similar absorption rate for the two compounds.

Table I:I Body weight of rachitic rats treated for seven days with daily oral administration of 12.5 ng of $1\alpha OHD_2$ or $1\alpha OHD_3$
Values expressed as mean ± S.E.
n = 9

Treatment	Body weight (g) Initial	Final
$1\alpha OHD_2$	86.1 ± 2.0	83.9 ± 2.3
$1\alpha OHD_3$	85.0 ± 1.9	85.0 ± 2.0
Control	82.8 ± 1.9	83.9 ± 3.3

Table I:II Values of various parameters in rachitic rats after seven days of oral dosing of 12.5 ng of $1\alpha OHD_2$ or $1\alpha OHD_3$
Values are given as mean ± S.E.
n = numbers within paranthesis.

	Treatment		
Measurement	$1\alpha OHD_2$	$1\alpha OHD_3$	Control
serum Ca (mmol/l)	2.78±0.11** (9)	2.97±0.11*** (8)	2.19±0.11 (9)
serum phosphate (mmol/l)	1.21±0.06 (9)	1.05±0.07 (9)	1.12±0.20 (9)
Ca transport serosa/mucosa	3.65±0.047*** (9)	3.60±0.46*** (9)	1.45±0.08 (8)
Line test score	2.9±0.4	3.3±0.5	0.3±0.2
Serum $1,25(OH)_2D$ (pg/ml)	225[a] 1[b]	189[a] 2[b]	34[a] 2[b]

[a] = plasma pooled from 2 or 3 rats
[b] = sample numbers

** = different from control at $p < 0.01$
*** = different from control at $p<0.001$

In experiment I when $1\alpha OHD_2$ and $1\alpha OHD_3$ was given intraperitoneally, the rats lost weight. Serum Ca increased in a largely dose-dependent manner with both analogs, while the increase in serum P was less consistent.
The healing of rickets was evident by increased endochondral calcification and increased bone mineral.

No difference in response was found when comparing $1\alpha OHD_2$ and $1\alpha OHD_3$ in rachitic rats. The antirachitic activity of both compounds is equal to $25OHD_3$ or to 100-200 IU/µg (Reeve et al., 1978).

Toxicity (II)

Toxicity was studied after both chronic and acute overdosage with $1\alpha OHD_2$ and $1\alpha OHD_3$. Oral dosing with high doses of $1\alpha OHD_2$ and $1\alpha OHD_3$ for four weeks resulted in decreased growth (Fig. 4). The doses ranged from 0.5 µg/day (=2.5 µg/kg/day) to 4 µg/day (=20 µg/kg/day).

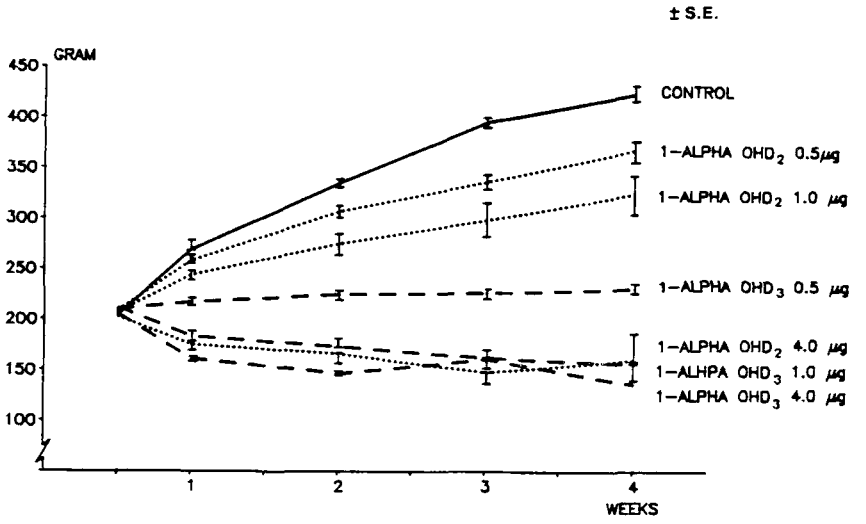


Fig. II:I Weight record of rats on graded doses of $1\alpha OHD_2$ or $1\alpha OHD_3$ given orally by daily injections into the hypopharynx. 0.5 µg/day and 1.0 µg/day of $1\alpha OHD_2$ differ significantly ($p < 0.001$) from equal doses of $1\alpha OHD_3$.

The serum Ca level was raised in animals chronically dosed with hydroxylated vitamin D compounds. This increase was dose-correlated. When followed over the course of the experiment, serum Ca had a tendency to decrease slightly in rats given the highest doses of $1\alpha OHD_2$ and $1\alpha OHD_3$ (Fig. II:II a-c).

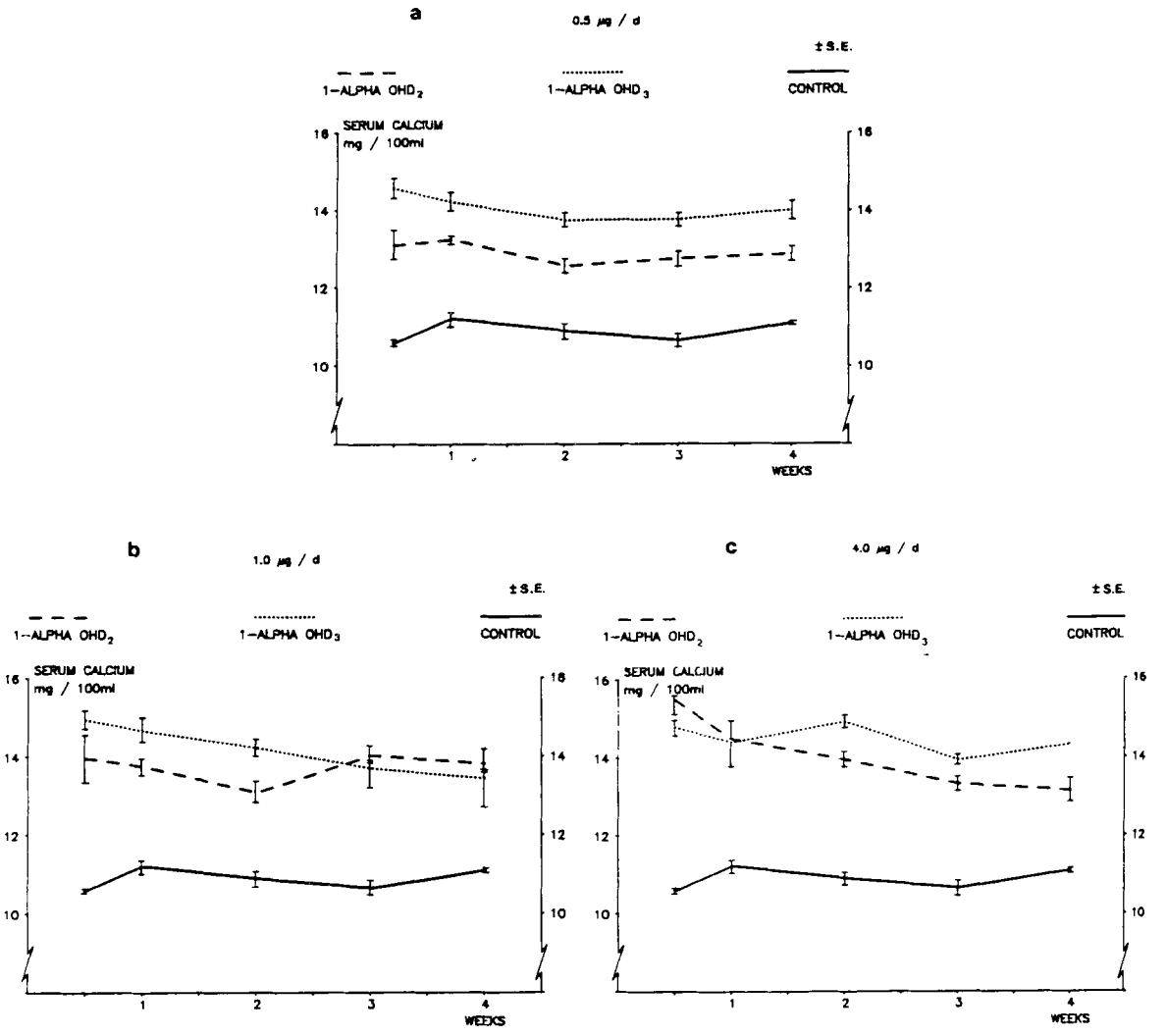


Fig. II:IIa,b,c Serum Ca of rats given graded doses of 1-hydroxylated vitamin D compounds. All values of dosed groups were significantly higher than controls ($p < 0.001$).

During chronic administration, five of the six rats that received 4 µg/day of $1\alpha OHD_3$ and two rats given 1.0 µg/day of the same compound died. In contrast, only two rats given the highest dose of $1\alpha OHD_2$ (4 µg/d) died during the experimental period. The mortality rate was correlated with the amount of renal calcification. It is likely that the massive calcification that occurred after the induction of hypervitaminosis lead to uremia and death (Fig. II:III). In fact some kidneys remained as a skeleton of calcified tubuli and calices after ashing.

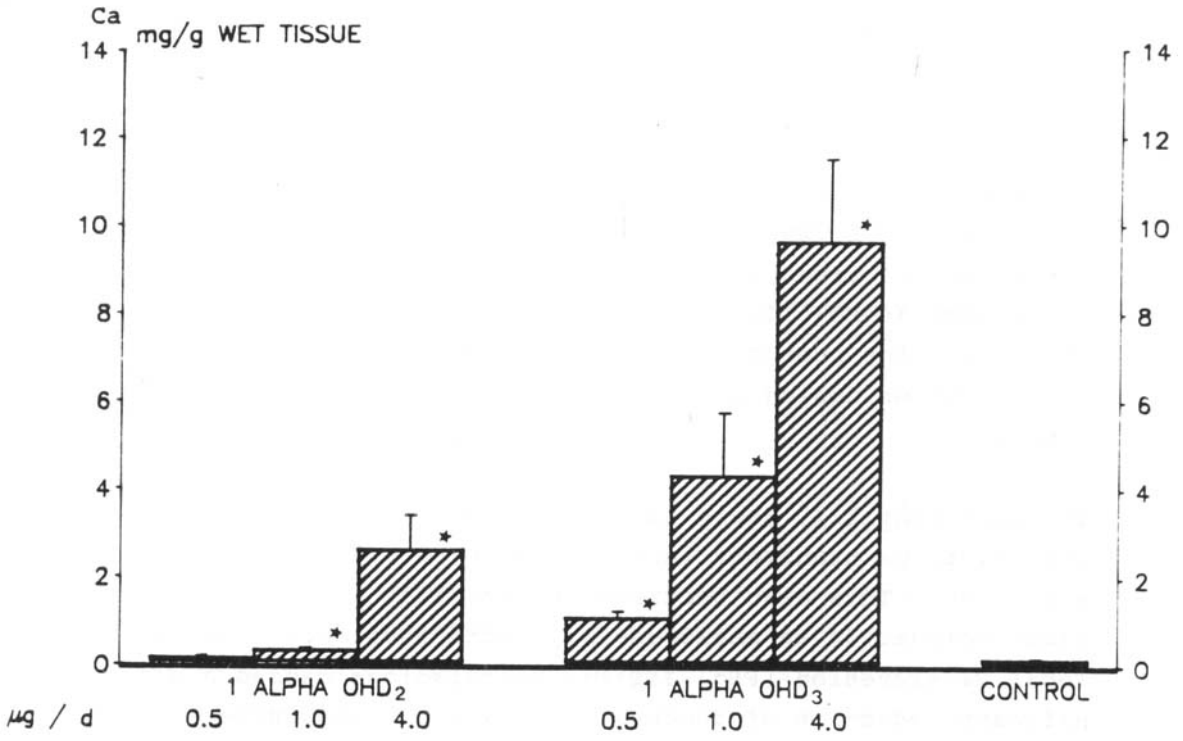


Fig. II:III Amount of Ca in the right kidney of rats on four weeks oral medication with graded doses of $1\alpha OHD2$ or $1\alpha OHD3$.
n = 6 Vertical bars = S.E.M.
* = different from control at $p < 0.001$

Calcium is deposited in renal tissue when filtered Ca is increased and the tubular reabsorption mechanism is satura-

ted (Nordin, 1973). The amount of renal Ca reflects the cumulative effect of vitamin D compounds on serum Ca, leading to increases in Ca excretion. Renal calcification was determined by the dose of $1\alpha OHD_2$ and $1\alpha OHD_3$. $1\alpha OHD_3$ induced more renal calcifications and it appeared four to five times more toxic than $1\alpha OHD_2$ when administered chronically. The mortality rate was related to the amount of renal Ca.

For determination of LD_{50}, a single oral dose of the $1\alpha OHD$ compound was given. The deaths were recorded, and the results calculated on the basis of dose per kg of body weight required to cause death of 50% of the animals. The LD_{50} for $1\alpha OHD_2$ appeared to be 15 times greater than that for $1\alpha OHD_3$.

Intestinal action (I, III)

In experiment I, where young rachitic rats were used, the intestinal Ca transport increased as a result of dosing with $1\alpha OHD_2$ and $1\alpha OHD_3$. The system seemed saturated at a dose of 25 ng/day. The phosphate uptake in jejunal segments also increased after dosing with either of the 1-hydroxylated compounds.

In experiment III, where older rats (3 months) were used the intestinal Ca transport increased after dosing with $1\alpha OHD_2$ and $1\alpha OHD_3$. This also occurred in rats that were given prednisolone. In an analogous way, both substances reduced fecal Ca excretion (Fig. III:I). Prednisolone caused a significant reduction of duodenal Ca transport and increased fecal Ca loss.

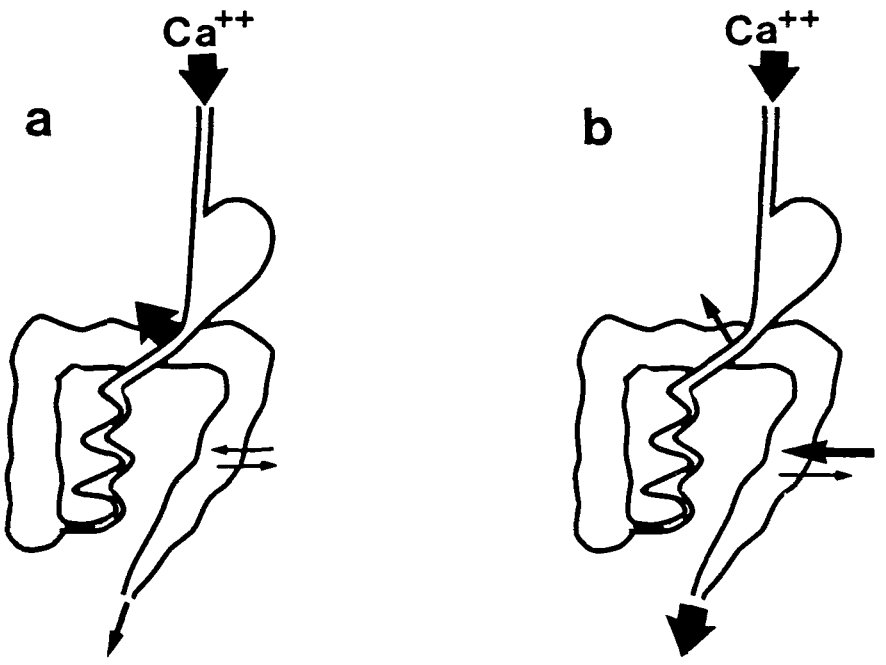


Fig. III:I The effect of vitamin D and prednisolone on the intestinal uptake of Ca and the fecal excretion of Ca.

a) Administration of vitamin D increases Ca uptake and reduces fecal loss of Ca

b) Prednisolone decreases Ca uptake in duodenum and increases secretion of Ca in colon, thereby increasing fecal Ca.

Although $1\alpha OHD_3$ appeared slightly more effective than $1\alpha OHD_2$ in promoting intestinal Ca transport, no statistically significant difference between the compounds could be established. When intestinal Ca transport was reduced by prednisolone treatment, both 1-hydroxylated compounds enhanced the transport mechanism to the same extent.

Bone action (III, IV)

Mineralization of the femur increased in rats given $1\alpha OHD_2$ as expressed by significantly increased amounts of bone ash, and higher dry weight (III). The rats given $1\alpha OHD_3$ also showed increased mineralization, but this increase was not statistically significant.

The tibia in experiment IV were further analyzed by dividing the bone into two parts, the proximal (mainly trabecular bone) and the distal part (mainly cortical bone). The two parts reacted differently to treatment with vitamin D In trabecular bone, $1\alpha OHD_2$ significantly increased the amount of mineralized bone (Table III:II). $1\alpha OHD_3$ appeared to cause resorption of cortical bone. Growth was unaffected by either compound.

Histological examination of the proximal part of the right tibia demonstrated that total bone area was slightly increased in rats dosed with $1\alpha OHD_2$, but this increase was not statistically significant (Table III:II). Osteoid area did not change, but osteoclast number was significantly reduced in rats given $1\alpha OHD_2$. In contrast, rats receiving $1\alpha OHD_3$ had slightly increased osteoclast count.

TABLE III:II TIBIAL PARAMETERS IN RATS GIVEN 0.3% Ca DIET AND 500 ng $1\alpha OHD_2$ or $1\alpha OHD_3$ ORALLY FOR SIX WEEKS (IV) Values are means ± S.D. n = 12 Values in paranthesis = cortical part

Measurement	Treatment 1-alphaOHD$_2$	1-alphaOHD$_3$	Control
Whole tibial length mm	44.17±0.53	44.02±0.51	44.18±0.38
Whole tibial density g/cm^3	1.54±0.02	1.53±0.02	1.54±0.02
Dry weight, g			
trabecular	0.18±0.01*	0.17±0.01	0.17±0.01
cortical	(0.36±0.01)	(0.35±0.03)*	(0.37±0.02)
Ash weight, g			
trabecular	0.11±0.00*	0.10±0.01	0.10±0.00
cortical	(0.24±0.01)	(0.23±0.02)	(0.24±0.01)
Bone area, %	26.53±0.05	24.14±0.04	24.16±0.06
Osteoid, %	0.05±0.14	0.00±0.00	0.59±0.61
Osteoclast no/field	0.33±0.04**	0.49±0.09	0.46±0.11

* = different from control $p < 0.05$
** = different from control $p < 0.01$

Glucocorticoid-induced osteopenia (III, IV)

Prednisolone at a dose of 1.5 mg/day for 6 weeks induced osteopenia in the rats. As seen in Fig. IV:I, both ash weight and density of the femur and tibia were reduced in rats given prednisolone. The reduced mineralization was partly, but not completely, counteracted by the administration of $1\alpha OHD_2$ and $1_\alpha OHD_3$. Furthermore, $1_\alpha OHD_2$ increased trabecular bone area while decreasing the number of osteoclasts (Table IV:II).

Table IV:II Osteoclast number, osteoid area and bone area in the tibiae of rats given 1.5 mg/day prednisolone and 0.5 µg/day $1\alpha OHD_2$ or $1_\alpha OHD_3$ for six weeks (IV).
Data presented as mean ± S.D. n = 12

	$1\alpha OHD_2$ + prednisolone	$1\alpha OHD_3$ + prednisolone	Prednisolone
Osteoclasts (n:o/field)	0.39 ± 0.11	0.42 ± 0.11	0.41 ± 0.07
Osteoid (%)	0.0 ± 0.0	0.3 ± 0.4	0.0 ± 0.5
Bone area (%)	36.1 ± 4.2	33.9 ± 5.2 — c —	27.4 ± 5.4
	b		

^{b} = significantly different $p < 0.01$
^{c} = significantly different $p < 0.05$

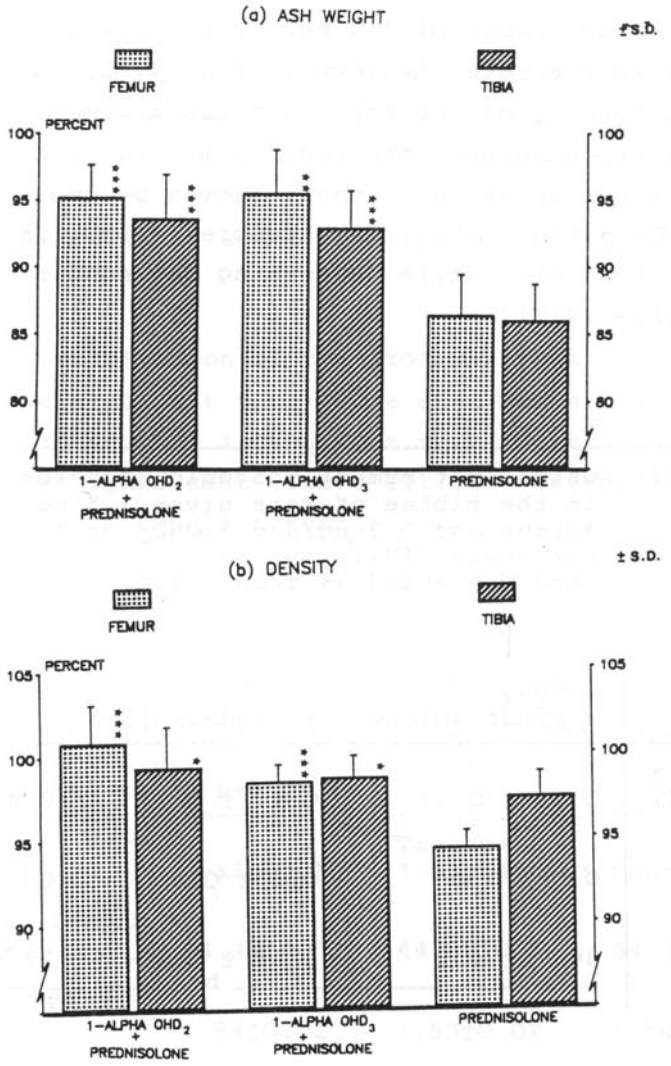


Fig. IV:I Femoral and tibial ash and density in rats given 1.5 mg of prednisolone and 0.5 μg of $1\alpha OHD_2$ or $1\alpha OHD_3$ daily for six weeks.
100% = value for rats given the same diet without prednisolone or vitamin D compounds (III, IV).
* = significantly different from prednisolone treated rats at $p < 0.05$
** = significantly different at $p < 0.01$
*** = significantly different at $p < 0.001$

Renal effects (V)

The effect of 1.5 mg/day of prednisolone on the amount of Ca deposited in the kidney of rats dosed with $1\alpha OHD_2$ or $1\alpha OHD_3$ was studied. The dose of the 1-hydroxylated compound was 0.5 µg/day or 2.0 µg/day and the diet contained either 0.3% Ca or 0.02% Ca and 0.5% P.

Histologic examination of the kidneys of rats dosed with prednisolone and 0.5 µg/day of 1-hydroxylated vitamin D compounds and given the 0.3% Ca diet showed an increased amount of Ca deposited predominantly peritubularly and in the calices (Fig. V:I).

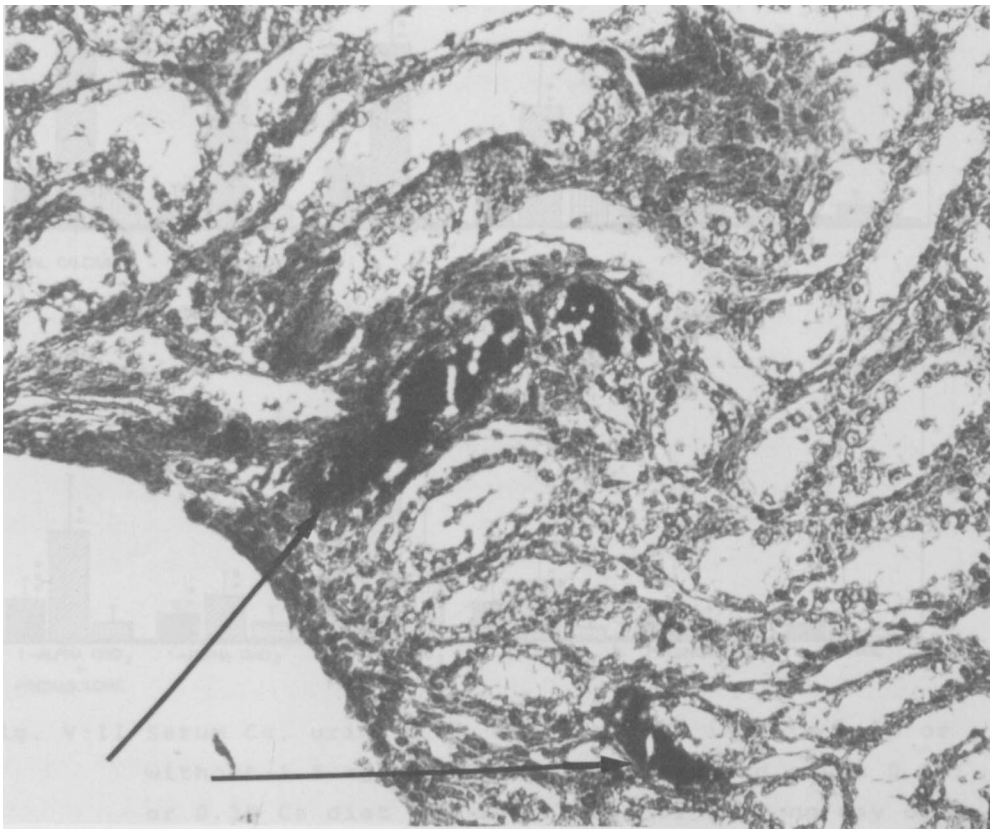


Fig. V:I Von Kossa stained kidney in rat given $1\alpha OHD_3$ and prednisolone. x 50, showing calcium deposition in renal tubule (arrows)

Rats dosed with active vitamin D analogs were hypercalcemic and hypercalciuric. When the rats also were dosed with prednisolone, they still had hypercalciuria in the absence of hypercalcemia (Fig. V:II). The renal content of calcium was substantially increased in rats given vitamin D analogs and Ca in the kidney was increased further by the simultaneous administration of prednisolone (Fig. V:II).Serum creatinine being dependent on muscle mass as well as renal functioning was not changed by administration of vitamin D analogs or prednisolone.

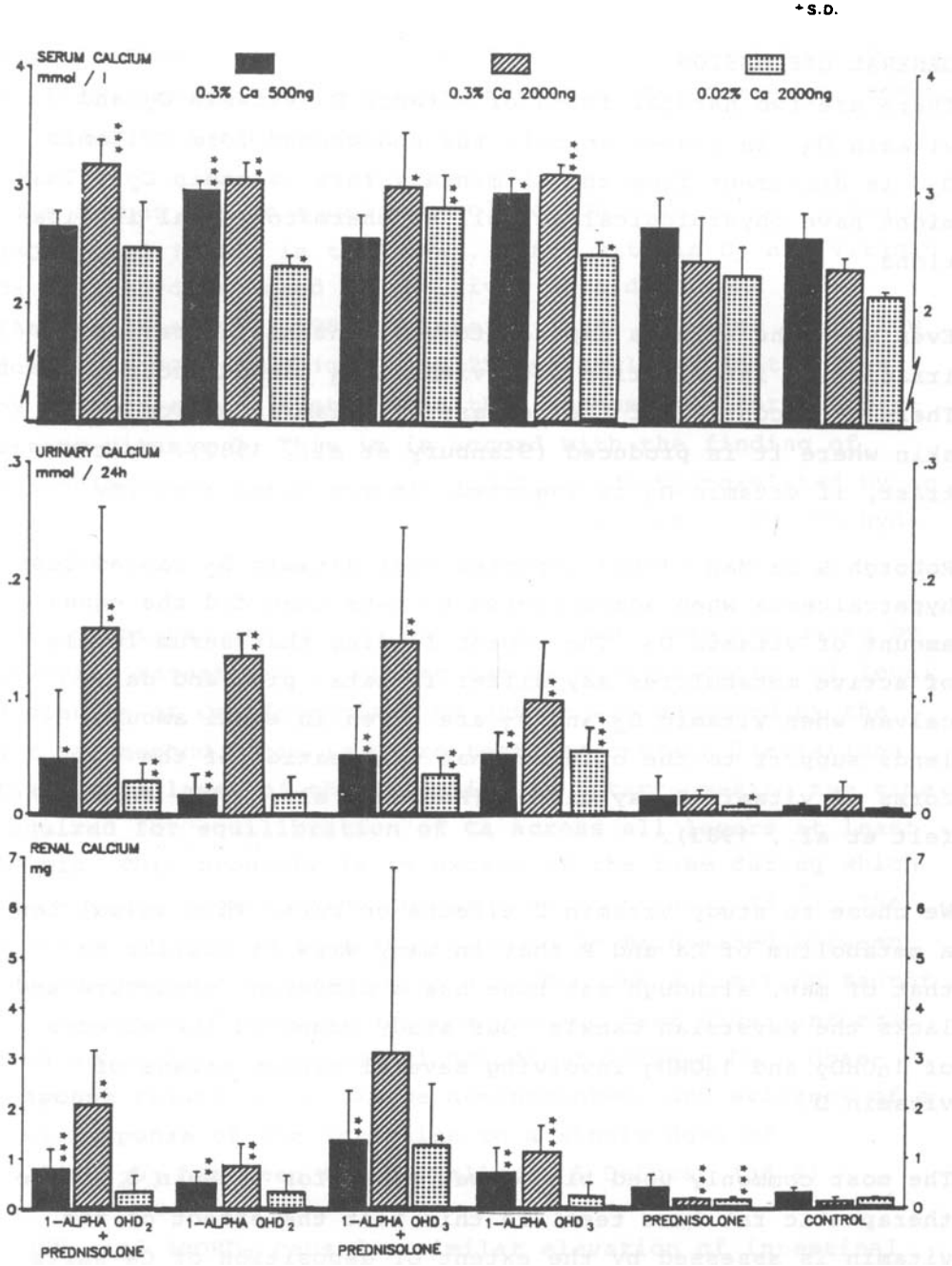


Fig. V:II Serum Ca, urinary Ca and renal Ca in rats with or without 1.5 mg/day of prednisolone and given 0.02% or 0.3% Ca diet and 500 ng/day or 2000 ng/day of $1\alpha OHD_3$ or $1\alpha OHD_2$ for six weeks.

* = different from control $p < 0.05$

** = different from control $p < 0.01$

*** = different from control $p < 0.001$

Statistical evaluation only between randomized groups

GENERAL DISCUSSION

There are two natural forms of vitamin D, vitamin D_2 and vitamin D_3. In higher animals the endogenous form (vitamin D_3) is different from the alimentary form (vitamin D_2). This might have physiological as well as pharmacological implications.

Even when the skin is exposed to very intense ultraviolet irradiation intoxication with vitamin D_3 cannot occur. There is a control of the release of vitamin D_3 from the skin where it is produced (Stanbury et al., 1980). By contrast, if vitamin D_3 is ingested, it may cause toxicity.

Roborgh & De Man (1960) reported that vitamin D_2 caused less hypercalcemia when administered to rats than did the equal amount of vitamin D_3. The recent finding that serum levels of active metabolites may differ in rats, pigs and dairy calves when vitamin D_2 and D_3 are given in equal amounts lends support to the opinion that utilization of the two forms of vitamin D may differ (Horst et al., 1982; Sommerfelt et al., 1983).

We chose to study vitamin D effects on rats. This animal has a metabolism of Ca and P that in many ways is similar to that of man, although rat bone has a different structure and lacks the Haversian canals. Our study compared the effects of $1\alpha OHD_2$ and $1\alpha OHD_3$ involving several target organs of vitamin D.

The most commonly used biological assay for vitamin D is the therapeutic rat line test. In this test the effect of the vitamin is assessed by the extent of deposition of Ca salts in osteoid tissue of long bones. Rickets is induced in rats by vitamin D depletion for three to four weeks and by feeding the animals a low-phosphorous diet. When vitamin D is given small increments in Ca deposition are noted by the line test as mineralization begins at the metaphysis. The ashing of long bones and the recording of the ash weight is also a sensitive test of increased mineralization. By both

methods $1\alpha OHD_2$ and $1\alpha OHD_3$ were found equally biopotent in healing rickets. The two substances are therefore equipotent on an equimolar basis (I).

This argues for an equal metabolization rate of low doses of $1\alpha OHD_2$ and $1\alpha OHD_3$ in the liver. Since vitamin D_2 and vitamin D_3 is 25-hydroxylated in the liver at a different speed (Andersson et al., 1983; Holmberg, 1984) it seems as if low doses of $1\alpha OHD_2$ and $1\alpha OHD_3$ is 25-hydroxylated in the liver by another enzyme system than that responsible for hydroxylating vitamin D. This is in accord with the finding of Fukushima et al. (1978) that $1\alpha OHD_3$ is 25-hydroxylated by an enzyme without metabolic control in contrast to the 25-hydroxylase for vitamin D_3.

The intestinal action of $1\alpha OHD_2$ and $1\alpha OHD_3$ was evaluated by studying intestinal Ca uptake and by quantification of fecal excretion of Ca. Intestinal Ca uptake was measured by the gut sat method. This in vitro test has certain limitations. The muscle layer of the intestine is intact, making the time required for equilibration of Ca across all layers at least 90 min. This probably is in excess of the time during which the villus layer remains intact (Levine et al., 1970). The maximum ionized Ca gradient that can be maintained between the serosal and mucosal layer also becomes a limiting factor in the assay. Nevertheless, the gut sac test gives information about the physiological action of vitamin D. A dose-response relationship can be demonstrated, and evidence of a dual response of the intestine to a single dose of $1,25(OH)_2D_3$ has been found (Halloran & DeLuca, 1981a).

$1\alpha OHD_2$ and $1\alpha OHD_3$ caused a similar elevation of intestinal Ca transport (I, III). This finding is physiologically relevant as demonstrated by the fact that when Ca uptake was enhanced, the exretion of Ca in feces diminished (III).

Intestinal uptake of phosphate was studied in rachitic rats (I). The method used has recently been developed. The technique of incubating jejunal discs of known area under nitrogen or oxygen atmosphere and measuring the uptake of ^{32}P makes it possible to estimate the active uptake of P. Uptake of ^{32}P without oxygen or metabolizable sugar represents passive diffusion, and this value is subtracted from that obtained in tissue with oxygen and sugar present. The active uptake of P can then be calculated which has been difficult earlier due to interference with inorganic P released from tissue. The finding that $1\alpha OHD_2$ and $1\alpha OHD_3$ stimulate P uptake to the same extent in rachitic rats in experiment I is consistent with the demonstrated rise of serum P following the administration of 1-hydroxylated compounds in rachitic rats in experiment II. The rise in serum phosphorus is directly related to the healing of rickets (Tanaka & DeLuca, 1974).

$1\alpha OHD_2$ and $1\alpha OHD_3$ were equally well absorbed, as serum levels of $1.25(OH)_2D$ was similarly elevated in rachitic rats given oral doses of either compound. The reason that the vitamin D-deficient rats after three weeks still had measurable amounts of $1,25(OH)_2D_3$ in serum is that at least four weeks of depletion is necessary to reach very low levels (Mallon et al., 1981). In study I, a depletion period of four weeks was used, but in the study with oral dosing of rachitic rats (Table I:I and Table I:II) a three week depletion period was employed. Serum levels of $1,25(OH)_2D$ were lower in normal rats given $1\alpha OHD_2$ than in rats given $1\alpha OHD_3$. Prednisolone treatment reduced serum $1,25(OH)_2D$; this reduction was counteracted by dosing with $1\alpha OHD_3$ or with $1\alpha OHD_2$ (Experiment III). This is in accordance with the finding by Lindgren et al. (1982), that lowered serum $1,25(OH)_2D_3$ levels were normalized after $1,25(OH)_2D_3$ administration to prednisolone treated rats.

The estimation of serum $1,25(OH)_2D$ levels could be influenced by different binding affinities of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ to the chick cytosol receptor. Eisman et al. (1976) did not report any difference in receptor affinity of

$1,25(OH)_2D_2$ or $1,25(OH)_2D_3$, but Jones et al. (1980) found a 30% reduction of displacement potency of $1,25(OH)_2D_2$ in his receptor assay. A difference in the metabolic turnover of vitamin D_2 and D_3 could also influence the serum-levels of $1,25(OH)_2D$. In consequence, the reduced level of $1,25(OH)_2D$ in rats given $1\alpha OHD_2$ in experiment IV could be the result of technical failure to measure correctly $1,25(OH)_2D_2$, or it could be due to slower rate of 25-hydroxylation of $1\alpha OHD_2$ compared with that of $1\alpha OHD_3$, when the two compounds were given in large doses.

The effect of $1\alpha OHD_2$ and $1\alpha OHD_3$ on bone was studied by previously used methods with known high reproducibility (Lindgren & Lindholm, 1979; Johnell et al., 1977). In normal rats, treatment with $1\alpha OHD_3$ increased the number of osteoclasts. This is in accordance with previous studies, where the administration of $1,25(OH)_2D_3$ increased the number of osteoclasts (Holtrop et al., 1981). In contrast, rats dosed with $1\alpha OHD_2$ had a significant reduction of osteoclast number (Experiment IV). Furthermore $1\alpha OHD_2$ led to increased bone mineral, while $1\alpha OHD_3$ slightly reduced the mineral content (Experiment IV). This difference in the effect on bone tissue could be due to a relatively more pronounced depression of the parathyroid glands following stimulation with $1,25(OH)_2D_2$ as compared with $1,25(OH)_2D_3$. The effect could also result from different peak concentrations of $1,25(OH)_2D$. It was previously shown that the administration of $1,25(OH)_2D_3$ could not reverse osteopenia in rats if given parenterally (Lindgren et al., 1982), but if the compound was given orally osteopenia was prevented (Lindgren & DeLuca, 1983). The resulting osteoclasia might be caused by high peaks of active vitamin D metabolites. By a different rate of hepatic 25-hydroxylation, resulting in lower peaks of active vitamin D, the D_2 analog could have fewer negative effects on bone tissue.

By studying the action of the two vitamin D analogs in prednisolone-induced osteopenia, the compounds were tested in a model simulating a clinical condition, i.e. hypercorti-

sonism. In order to induce osteopenia in rats, high doses of glucocorticoids must be given. Such doses lead to a catabolic state in the animals, including a decrease in protein synthesis and a decrease in bone matrix formation (Peck et al., 1967,1969). Prednisolone administration also reduced intestinal Ca transport and increased fecal excretion of Ca. Both $1\alpha OHD_2$ and $1\alpha OHD_3$ were antagonistic to prednisolone in the last respect (Experiments III, IV). Even in extreme conditions of glucocorticoid excess, administration of 1-hydroxylated compounds reduced osteopenia, implicating the potency of both analogs on bone mineral homeostasis. Therefore, both $1\alpha OHD_2$ and $1\alpha OHD_3$ are active in preventing prednisolone-induced osteopenia (Experiments III, IV); and $1\alpha OHD_2$ reduces osteoclast number in contrast to $1\alpha OHD_3$, which increases the osteoclast number.

In order to study vitamin D toxicity and the renal effects of $1\alpha OHD_2$ and $1\alpha OHD_3$, histologic examination of kidneys and analysis of renal calcium content were performed. Both $1\alpha OHD_2$ and $1\alpha OHD_3$ induced hypercalciuria and increased calcification of renal tissue. The effects of $1\alpha OHD_3$ were more pronounced than those of $1\alpha OHD_2$. Clinically, renal calcification and deterioration of kidney function is recognized as the major risk factor in vitamin D pharmacotherapy. Treatment with very high doses of $1\alpha OHD_2$ and $1\alpha OHD_3$ were lethal (Experiment II). The mortality rate closely paralleled the amount of Ca present in renal tissue. $1\alpha OHD_3$ was five to fifteen times more toxic than $1\alpha OHD_2$ (Experiment II). No change in renal function appeared when studying serum creatinine (Experiment V), although renal calcifications are considered a threat to normal functioning of the kidneys.

When vitamin D compounds are used clinically, the urinary excretion of Ca has to be followed as nefrocalcinosis can appear even in the absence of hypercalcemia, especially in hypercortisonism (Experiment V).

What then are the differences between $1\alpha OHD_2$ and $1\alpha OHD_3$? The two compounds differ in situations where pharmacological doses are employed. The efficacy of healing rickets was

the same for the two analogs. In non-rachitic rats, 1αOHD$_2$ reduced the osteoclast number and increased the bone mineral content. Prednisolone-induced osteopenia was counteracted by either compound. Intestinal Ca transport and P uptake were almost equally enhanced by either compound. 1αOHD$_3$ induced more hypercalciuria than 1αOHD$_2$, and 1αOHD$_2$ appeared much less toxic than 1αOHD$_3$.

One possible explanation for the apparent difference in effect and toxicity is that different cells have receptors for 1,25(OH)$_2$D that discriminate between active vitamin D$_2$ and D$_3$. In the chick intestinal receptor D$_2$ and D$_3$ bind differently (Jones et al., 1980). The possibility of the receptor discriminating between 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$ is well argued for by the fact that the synthetic analog 24-epi-1,25(OH)$_2$D$_2$ has different effects on bone and intestine. While 24-epi-1,25(OH)$_2$D$_2$ stimulates intestinal calcium transport, it does not induce bone resorption in contrast to 1,25(OH)$_2$D$_2$ (DeLuca, 1985). This indicates that the side chain configuration is important for receptor activity. Since vitamin D$_2$ differs in the side chain from vitamin D$_3$, the receptor affinity could change. It is unlikely that the absorption of 1αOHD$_2$ and 1αOHD$_3$ should differ because the same levels were found in rachitic rats when measuring serum 1,25(OH)$_2$D after dosing with 1αOHD$_2$ or 1αOHD$_3$. Vitamin D is absorbed from the intestine mainly by passive diffusion (Hollander, 1981). The more polar metabolite 25(OH)D$_3$ was found to be absorbed more efficiently than vitamin D$_3$ when gall production was insufficient (Davies et al., 1980), but the slight difference in polarity between 1αOHD$_2$ and 1αOHD$_3$ is unlikely to lead to different intestinal uptake of the two compounds.

Another possibility that could cause dissimilar properties of the two 1-hydroxylated analogs is a difference in hepatic metabolization rate. The existence of a microsomal and a mitochondrial 25-hydroxylase that discriminate against vitamin D$_2$ and prefers vitamin D$_3$ has been demonstrated in rat liver (Andersson et al., 1983; Holmberg, 1984). This would

lead to lower peak levels of $1,25(OH)_2D_2$ than of the corresponding D_3 metabolite. Whether the same enzyme acts on vitamin D as on $1\alpha OHD_2$ or $1\alpha OHD_3$ is however unlikely (Fukushima et al., 1978).

As it is now possible to measure effects and plasma levels of vitamin D_2 and D_3 metabolites in humans, studies in man seem to be warranted in order to establish the effects of vitamin D_2 compounds in pharmacotherapy. It was recently demonstrated in man on antiepileptic drugs that vitamin D_2 and vitamin D_3 given daily in equal doses led to different serum levels of 25(OH)D, and also to dissimilar effects on bone metabolism such that vitamin D_2 therapy led to an increase in bone mass while vitamin D_3 dosing led to unchanged bone mass (Tjellesen et al., 1985 a, b).

The present study suggests that $1\alpha OHD_2$ would constitute a valuable tool in medicine.

Oral vitamin D_2 analogs may be safer than currently used vitamin D_3 preparations. The endogenous form of vitamin D is not toxic when emanating from the natural forming-sites, but sudden peaks of D_3 metabolites in pharmacotherapy is difficult for the organism to handle. In contrast, the alimentary form of vitamin D, (vitamin D_2) might be recognized by the enzymes as foreign and handled differently, with less risk for toxic manifestations.

SUMMARY AND CONCLUSIONS

The effect of $1\alpha OHD_2$ and $1\alpha OHD_3$ have been studied in rachitic, normal, and prednisolone-treated male rats. The healing of rickets, the stimulation of intestinal Ca and P transport, the effect on bone mineral, and the induction of renal calcifications have been examined.

1 The two 1-hydroxylated compounds are equally potent in healing rickets, and by comparison with previous data are equivalent to 100-200 IU/ug.

2 $1\alpha OHD_2$ and $1\alpha OHD_3$ stimulated intestinal Ca and P transport to the same extent and were antagonistic to prednisolone in this respect.

3 $1\alpha OHD_2$ reduced the number of osteoclasts and increased bone mineral in normal rats in contrast to $1\alpha OHD_3$, which increased osteoclasts and slightly reduced bone mineral. Prednisolone-induced osteopenia was more effectively counteracted by $1\alpha OHD_2$ than by $1\alpha OHD_3$.

4 Mortality rate was higher in rats intoxicated with $1\alpha OHD_3$ than for rats given $1\alpha OHD_2$. LD_{50} was estimated to be five to fifteen times higher for $1\alpha OHD_2$.

5 Renal calcification were more pronounced after dosing with $1\alpha OHD_3$ than after treatment with $1\alpha OHD_2$. When prednisolone was given together with $1\alpha OHD_2$ or $1\alpha OHD_3$, renal calcifications were further increased.

These observations demonstrate physiological dissimilarities between vitamin D_2 and vitamin D_3 in rats which are in accordance with a different metabolism of the two vitamins. The findings, in particular that $1\alpha OHD_2$ is less toxic than $1\alpha OHD_3$, are of potential clinical importance.

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