A STUDY ON THE REGULATION OF RENAL MITOCHONDRIAL VITAMIN D HYDROXYLASES

by

Daniel Elias Hay Afar

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Department of Pharmacology & Therapeutics,
McGill University, Montreal.

November, 1987

ABSTRACT

The regulation of vitamin D hydroxylases in renal mitochondria has been investigated.

Rats raised on a normal diet had measurable 25-hydroxycholecalciferol 24-hydroxylase (24-OHase) and non-detectable 25-hydroxycholechalciferol 1-hydroxylase (1-OHase) activities in their renal mitochondria. Adult 8 weeks old rats had a 2 fold higher 24-OHase activity than 2-4 weeks old pre-pubertal rats. The enzyme activity was stimulated 2-3 fold by fasting for 24 hours in the young and old rats. The mechanism of this induction was examined and did not appear to involve parathyroid hormone (PTH), pituitary hormones or 1,25-dihydroxycholecalciferol.

Female rats weaned on a vitamin D-deficient, low calcium diet exhibited only 1-OHase activity and no 24-OHase activity in their renal mitochondria. Female adult (8-30 weeks old) rats had a similar 1-OHase activity compared to female 5-week old pubertal rats, although adult rats were more hypocalcaemic, hypophosphataemic and hyperparathyroid than pubertal rats. During lactation 1-OHase activity was stimulated 3 fold over non-lactating adult 1-OHase activity levels. The hormonal involvement in this stimulation is unknown. PTH does not appear to be involved in this stimulation, because lactating and non-lactating rats were both hyperparathyroid and PTH sensitive.

It can be concluded that renal vitamin D hydroxylase activities can be induced by dietary and hormonal manipulations. An increase in

the enzyme content in renal mitochondria was not demonstrated and awaits molecular quantitation of the enzymes.

RÉSUMÉ

Lors de ce travail, nous nous sommes interessés a l'étude de la régulation des Vitamines D hydroxylases a partir de préparations de mitochondries rénales de rats.

Les mitochondries rénales de rats soumis a un régime normal montrent une activité de la 25-hydroxycholecalciférol-24-hydroxylase (24-OHase) tandis que l'activité de la 25-hydroxycholecalciférol-1-hydroxylase (1-OHase) n'est pas détectable. Des rats adultes de 8 semaines présentent une activité de la 24-OHase deux fois supérieure a celle de rats pre-pubertaires de 2 semaines. Cette activité peut etre stimulée de 2 a 4 fois, autant chez les jeunes rats que chez les rats âgés, après un jeune de 24 h. Le mécanisme de cette induction a été étudié et il ne semble pas faire intervenir l'hormone parathyroidienne (PTH), les hormones pituitaires ou la 1,25-dihydroxycholecalciférol.

Sur des mitochondries rénales de rates, soumises durant leur sevrage a un régime déficient en vitamine D et à faible teneur en calcium, seule l'activité de la 1-OHase est décelable. Par ailleurs, l'activité de cet enzyme est similaire chez des rates adultes (8 - 30 semaines) et des rates pubères de 5 semaines bien que les rates adultes soient plus hypocalcémiques, hypophosphatémiques et hyperparathyroidiénnes que les rates pubères. Chez les femelles lactantes, l'activité de la 1-OHase est augmentée de 3 fois par rapport a des femelles adultes non lactantes. Les mécanismes hormonaux impliques dans ce phénomène ne sont pas connus, a l'heure actuelle, mais la PTH

ne semble pas intervenir dans cette stimulation car les femelles lactantes et non lactantes sont, a la fois, hyperparathyroidiennes et sensibles a la PTH.

En conclusion, nos études semblent indiquer que des variations hormonales et de régime augmentent les activités rénales des vitamines D hydroxylases. Aucune étude sur l'augmentation du contenu enzymatique des mitochondries rénales n'a été réalisé et une quantification par des techniques de biologie moléculaire semble approprier pour répondre à cette question.

DEDICATED TO MY MOTHER AND FATHER

The limitations of our biological equipment may condemn us to the role of Peeping Toms at the key-hole of eternity, but at least let us take the stuffing out of the key-hole which blocks even our limited view.....

Arthur Koestler

This thesis is divided into two chapters that are based on the following manuscripts and which will be referred to by their Roman numerals:

CHAPTER I. Afar DEH, Tenenhouse A, Warner M. Regulation of renal 25-hydroxy-cholecalciferol 24-hydroxylase in vitamin D-replete rats. Manuscript submitted to J. Steroid Biochem.

CHAPTER II. Afar DEH, Warner M. Induction of renal mitochondrial 25-hydroxycholecalciferol 1-hydroxylase during lactation. Manuscript, not submitted for publication.

TABLE OF CONTENTS

ORIGINAL CONTRIBUTION TO SCIENTIFIC KNOWLEDGE

This thesis provides novel information concerning the regulation of vitamin D metabolism. It was demonstrated that renal mitochondrial 24-OHase activity is regulated as a function of age and food intake in normal rats. The significance of this finding is unknown, but it implies some functional role for 24,25-dihydroxycholecalciferol.

It has been previously shown that during lactation 1,25-dihydroxycholecalciferol production is elevated. Here, it is reported that 1-OHase activity is induced several fold in renal mitochondrial preparations from lactating rats, which confirms the previous in vivo findings.

LIST OF TABLES

CHAPTER I

CHAPTER II

LIST OF FIGURES

CHAPTER I

CHAPTER II

LIST OF ABBREVIATIONS

BMAR	linear rate of bone mineral apposition
CaBP	vitamin D-dependent calcium binding protein
cAMP	cyclic adenosine 3'5'monophosphate
DNA	deoxyribonucleic acid
E_2	estradiol
GH	growth hormone
G_1	first generation vitamin D-deficient
G_2	second generation vitamin D-deficient
hGH	human growth hormone
IGFs	insulin like growth factors
mRNA	messenger ribonucleic acid
PRL	prolactin
PTX	parathyroidectomized
P450	cytochrome P450
TPTX	thyroparathyroidectomized
T_3	L-3,5,3'triiodothyronine
T_4	L-3,5,3',5'-tetraiodothyronine (thyroxine)
1-OHase	25-hydroxycholecalciferol 1-hydroxylase
24-OHase	25-hydroxycholecalciferol 24-hydroxylase
25-OHase	cholecalciferol 25-hydroxylase
16α-OHase	testosterone 16α-hydroxylase

7-DHC	7-dehydrocholecalciferol
1-HCC	1-hydroxycholecalciferol
25-HCC	25-hydroxycholecalciferol
1,25-DHCC	1,25-dihydroxycholecalciferol
24,25-DHCC	24,25-dihydroxycholecalciferol
1,24,25-THCC	1,24,25-trihydroxycholecalciferol

INTRODUCTION

Historical Background

Vitamin D_3 (cholecalciferol) is one of the most important regulators of calcium and phosphorus homeostasis in higher animals [39,40,63,97,125]. Vitamin D_3 was discovered in the early 1920s and was identified as a fat soluble nutritional factor necessary for proper growth and health [39]. A bone disorder, rickets, was attributed to a deficiency in this fat soluble factor . McCollum showed that the anti rachitic substance found in butter fat and cod liver oil was not identical to vitamin A, the only known fat soluble vitamin at the time [85]. This work led to the isolation and purification of vitamin D_3 by Askew et al. [6].

A discovery, which turned out to be critical in understanding the vitamin D_3 system, was the finding that ultraviolet (UV) rays cured rickets in rachitic infants [65,71]. This suggested that UV light converted an endogenous substance into vitamin D_3. Subsequent studies determined that vitamin D_3 is a seco-steroid that is produced from 7-dehydrocholesterol (7-DHC) in the skin by UV irradiation [39,40,97]. Only much later did it become apparent that vitamin D_3 has to undergo several metabolic steps in order to be active [39].

Metabolic Activation of Vitamin D_3

Vitamin D_3 is synthesized in the skin by a nonenzymatic photolysis reaction [97]. 7-DHC is converted to previtamin D_3 when it

absorbs light at wavelengths in the UV range. Previtamin D_3 exists in a simple thermoequilibrium with vitamin D_3. The equilibrium ratio of vitamin D_3 to previtamin D_3 depends on temperature and at 37°C the ratio is 89:11 [97]. Vitamin D_3 then enters the circulation where it is bound by the vitamin D-binding protein (DBP) [16].

Vitamin D_3 accumulates in the liver, where it is hydroxylated in the 25 position to produce 25-hydroxycholecalciferol (25-HCC) [39,40,63,97]. This reaction is catalyzed by a microsomal [82] and a mitochondrial enzyme system [13]. The microsomal 25-hydroxylase (25-OHase) is composed of a cytochrome P450 and a flavoprotein which require NADPH, molecular oxygen for activity [40,82]. The kinetics of this enzyme have been shown to be altered by phenobarbital administration to the animal [41]. The mitochondrial enzyme is a three component mixed function oxidase consisting of a cytochrome P450, an iron sulfur protein and a flavoprotein [14]. As will be discussed later, some studies demonstrate convincing evidence, that the mitochondrial 25-OHase is more important in the 25-hydroxylation of vitamin D_3.

25-HCC enters the circulation bound to DBP [16]. The kidney is the major site of metabolism of 25-HCC [39,40,63,97,125]. The most important metabolic reaction in the vitamin D_3 system is the 1-hydroxylation of 25-HCC in renal mitochondria [39,40,63,97,125]. The product of this reaction, 1,25-dihydroxycholecalciferol (1,25-DHCC), is the active hormone with respect to calcium absorption from the gut [18] and calcium mobilization from bone [68]. The 1-hydroxylation of 25-HCC is also the most tightly regulated step in vitamin D_3 metabolism

and will be discussed below in greater detail.

The most abundant renal metabolite of 25-HCC is 24,25-dihydroxycholecalciferol (24,25-DHCC) [40]. The 24-hydroxylation reaction of 25-HCC is also catalyzed by a renal mitochondrial enzyme. Both, the 1-hydroxylase (1-OHase) and the 24-hydroxylase (24-OHase) are cytochrome P450s [22,56,66,139] and belong to the same P450 family as the mitochondrial 25-OHase in the liver and the adrenal mitochondrial cytochrome P450-side chain cleavage [142]. Although the serum concentration of 24,25-DHCC in normal animals is 40-100 times higher than 1,25-DHCC [16,50], it is controversial whether 24,25-DHCC exhibits any specific hormonal actions [40,97]. Other renal metabolites of 25-HCC have been found, but they are quantitatively unimportant and do not exhibit any biological activity [40,97].

Regulation of Vitamin D_3 Metabolism

(a) 25-OHase

The 25-hydroxylation reaction is the first metabolic step in the activation of vitamin D_3. Andersson and Jörnvall [3] reported that the microsomal 25-OHase is identical to the 16α-hydroxylase (16α-OHase) of testosterone, a well characterized male specific P450. The 16α-OHase is regulated by the growth hormone (GH) secretion pattern of the animal [91,92]. Female rats exhibit a different GH secretion pattern from males [46] and show very little 16α-OHase activity [3,91,92] or

vitamin D_3 25-OHase activity in their hepatic microsomes [3]. Even though female rats do not exhibit microsomal 25-OHase activity, their serum 25-HCC levels are comparable to those in males [38]. Dahlbäck and Wickwall [38] demonstrated that serum 25-HCC concentrations correlated with mitochondrial 25-OHase activity, but not with microsomal 25-OHase activity. It is still not known what factors are involved in the regulation of the mitochondrial 25-OHase.

(b) 1-OHase

The critical reaction in vitamin D_3 metabolism is the 1-hydroxylation of 25-HCC. This reaction occurs primarily in the kidney [51], however bone cells [70] and placenta [133,146] have also been shown to exhibit 1-OHase activity. The regulation of bone cell 1-OHase is not known and the placental 1-OHase is under a different regulation than the renal enzyme [149]. This short review on the regulation of 1-OHase activity will concentrate on the renal 1-OHase.

The renal 1-hydroxylation reaction is tightly regulated by a number of endocrine factors. The main regulators of 1-OHase activity are hypocalcaemia, hypophosphataemia, parathyroid hormone (PTH), GH, prolactin (PRL), estrogen and 1,25-DHCC itself.

Hypocalcaemia stimulates 1-OHase activity. This stimulation is thought to be mediated primarily by PTH [52], although some studies have shown that hypocalcaemia can induce 1-OHase activity independent of PTH [21,136]. A decrease in serum calcium stimulates PTH secretion, which then acts on the kidney to increase 1-OHase activity

[39,40,63,74,125]. The result is a rise in serum 1,25-DHCC concentration, which promotes an increase in calcium absorption from the gut and calcium mobilization from bone (see later section). The resulting increase in serum calcium then feeds back to the parathyroids and shuts off PTH secretion [39,40,63,97,125], thereby closing the loop.

PTH is a polypeptide hormone. In the kidney it binds to plasma membrane receptors, which are coupled to an adenylate cyclase [124]. PTH stimulates intracellular cAMP production which correlates with the PTH mediated increase in urinary phosphorus excretion [80,124]. Urinary cAMP and phosphorus excretion are, therefore, indices of PTH action on the kidney [80].

In contrast to the immediate increase in cAMP production, the effect of PTH on 1-OHase activity takes several hours to occur [4,5,64,67]. Infusion of bovine PTH into thyroparathyroidectomized (TPTX) vitamin D-deficient rats stimulated 1-OHase activity within 6 hours [67]. This effect was mimicked by infusion of cAMP or dibutyryl cAMP instead of bovine PTH [67]. Forskolin, a direct activator of adenylate cyclase, can stimulate 1-OHase activity within 4 hours in chick kidney cell culture [64] as well as in rat renal slices in vitro [4]. The effect of forskolin was additive with PTH in chick kidney cell culture depending on the concentrations used [64]. In rat renal slices, however, forskolin and PTH were not additive [4]. PTH has also been shown to stimulate 1-OHase activity in isolated mitochondria from guinea pig kidney [75]. This effect was rapid and peaked after 15 minutes of incubation [75]. The mechanism of this in vitro stimulation

of 1-OHase activity by PTH was, however, not determined.

The product of the 1-hydroxylation reaction, 1,25-DHCC, can act on the kidney to decrease its own synthesis [98,130,134]. This feedback regulation of 1,25-DHCC synthesis is not due to a 1,25-DHCC mediated suppression of PTH secretion (see later section), since it directly antagonizes the effect of PTH on 1-OHase activity in TPTX rats [98]. Furthermore, in rat renal slices 1,25-DHCC blocked the forskolin mediated induction of 1-OHase activity without blocking the effect of PTH and forskolin on cAMP production and protein kinase activity [4,5]. Therefore, 1,25-DHCC acts directly on the kidney to suppress 1-OHase activity at a site distal to cAMP generation and protein kinase activity. It is still unknown how cAMP activates the 1-OHase. However, it has been shown that other mitochondrial P450s are also regulated cAMP [142].

Hypophosphataemia, induced by dietary phosphorus deprivation, stimulates 1-OHase activity independent of PTH [129]. The mechanism of this stimulation has been shown to require a pituitary factor [57], which was identified as GH [58,59]. Other studies have also shown that hypophysectomy decreases the constitutive level of 1-OHase activity in rats [120,147]. In doses that do not affect PTH secretion, human GH (hGH) could increase serum levels of 1,25-DHCC back to normal in hypophysectomized rats [120]. However, hGH has more lactogenic properties than somatogenic properties in the rat [106]. Wongsurawat et al. [147] demonstrated an effect of hypophysectomy in rats on in vitro renal slice 1-OHase activity, which is reversed by administration

of rat GH, a specific somatogen in the rat [108], over a period of 10 days. Bickle et al. [12] failed to see an effect of GH on 1-OHase activity. However, they added GH directly to renal tubules and slices and incubations were 20 minutes long [12]. Since GH often requires the production of insulin-like growth factors (IGFs) for action [103], 20 minutes may not be enough time for GH to exert any effects on 1-OHase activity.

Insulin has been shown to affect 1-OHase activity in a similar manner as GH during phosphate deprivation of rats. Matsumoto et al. [84] demonstrated that streptozotocin induced diabetic rats had lower constitutive 1-OHase activity and a diminished response to dietary phosphorus deprivation. Treatment with insulin reversed the effects of the diabetic state. The kidney is one of the sites of IGF production [42]. Therefore, it is possible that 1-OHase activity during phosphorus deprivation is regulated by an IGF mediated mechanism, that can also be activated by insulin. To date, no such mechanism has been demonstrated[1].

In mammals pregnancy and lactation are physiologic states in which the calcium homeostatic system is stressed. In pregnancy calcium is needed for fetal bone development and during lactation calcium is required for milk production. These states are also characterized by exhibiting a 2-fold increase in serum 1,25-DHCC concentrations and enhanced intestinal calcium transport [15,103]. A simple explanation for these observations is that a decrease in serum calcium stimulates PTH secretion, which then induces 1-OHase activity. The rise in serum

1,25-DHCC is then responsible for the increased calcium absorption in the gut. However, parathyroidectomy of lactating rats does not completely abolish the increase in serum 1,25-DHCC [103]. This suggests that PTH is not the only factor invoived in regulating 1-OHase activity during lactation. Several reports have since then shown that PRL can stimulate 1,25-DHCC production in vivo as well as in vitro [12,110,121,122]. During pregnancy and lactation PRL levels are increased 30-60 fold [113]. In the rat administration of bromocryptine, a dopaminergic agonist that inhibits PRL secretion [27], blocks the rise in serum 1,25-DHCC and significantly reduces the enhanced intestinal calcium absorption observed during lactation [110]. Simultaneous PRL administration of bromocryptine treated rats prevents the changes seen with bromocryptine alone [110]. Although these results are very convincing, Matsumoto et al. [83] did not observe a change in the in vivo metabolism of 25-HCC by constant infusion of PRL. The doses of PRL used in both studies were approximately the same. However, the method of administration of the hormone was different. Matsumoto et al. [83] used a constant infusion protocol, while Robinson et al. [110] treated the rats with 2-3 bolus injections of PRL per day. Since PRL is normally secreted in a pulsatile fashion, the protocol used by Robinson et al. [110] may be more physiological. It still remains unknown by what mechanism PRL activates the 1-OHase.

In mammals the effects of steroids on vitamin D_3 metabolism is unresolved. Clinical data on human subjects has shown that a marked increase in serum testosterone levels during male puberty has no ef-

fect on serum concentrations of vitamin D_3 metabolites [74]. In women, endogenous estrogen levels fluctuate during the menstrual cycle. While some investigators report a correlation between high serum estrogen levels and an increased production of 1,25-DHCC [20,60], others have shown that no correlation exists [10]. An effect of estrogen on the metabolism of vitamin D_3 in the mammal, particularly the human, could be of great clinical importance, since osteoporosis, a bone disorder which primarily affects postmenopausal women is also characterized by a loss in endogenous estrogen production [109].

(c) 24-OHase

The 24-hydroxylation reaction occurs predominantly in renal mitochondria. However, nephrectomy does not prevent the formation of 24,25-DHCC in the body, although the plasma concentration of 24,25-DHCC in nephrectomized rats is lower than in sham operated rats [54]. It was determined that rat intestine [78], human placenta [144], rat bone cells [70], rat cartilage and cultured chondrocytes [53] also exhibit 24-OHase activity.

There exists a reciprocal relationship between renal 24-OHase activity and 1-OHase activity. In effect, when 1-OHase activity is high, such as during hypocalcaemic conditions (induced by a low calcium diet or a vitamin D-deficient diet), then 24-OHase activity is low [17,99]. In normocalcaemic or hypercalcaemic animals 1,25-DHCC production is low and 24,25-DHCC is the predominant metabolite of 25-HCC

[17,99]. Treatment of vitamin D-deficient rats with 1,25-DHCC over a 5 day period, reduced the in vivo production of 1,25-DHCC to non-detectable levels, while stimulating 24,25-DHCC production [130]. The 1,25-DHCC mediated increase in 24-25-DHCC production proved to be sensitive to administration of actinomycin D or cycloheximide to the animal, if given within 24 hours of 1,25-DHCC treatment [134]. This suggests that transcriptional and translational events are involved in the induction of 24-OHase activity by 1,25-DHCC.

PTH, an inducer of the 1-OHase, decreases constitutive levels of 24-OHase activity and also antagonizes the 1,25-DHCC mediated stimulation of 24,25-DHCC production [98]. Thyroparathyroidectomy of vitamin D replete rats with measurable in vitro renal 24-OHase activity, induced the enzyme activity about 2.5 fold over sham operated rats [131]. The mechanism of action of PTH in regulating urinary phosphate excretion and 1-OHase activity involves the second messenger cAMP. Infusion of PTH or cAMP, or a submaximal dose of PTH plus theophylline (a cyclic nucleotide phosphodiesterase inhibitor) into TPTX rats, all inhibited 24-OHase activity [115].

It is not known at what level PTH and 1,25-DHCC interact to regulate 24-OHase activity, but it is possible that the 24-OHase is regulated by a balance between PTH and 1,25-DHCC action. In other words, during hypocalcaemia PTH secretion is increased, PTH then acts on the kidney to induce 1-OHase activity and suppress 24-OHase activity. The result is an elevation in serum 1,25-DHCC concentration, which leads to enhanced calcium transport in the gut. As serum calcium

levels increase PTH secretion is suppressed. The removal of an inhibitory influence (PTH) and the presence of a stimulator (1,25-DHCC) results in an increased 24-OHase activity.

It is not known whether other factors are involved in regulating 24-OHase. Hypophysectomy increases 24,25-DHCC production, while GH administration to hypophysectomized rats reverses the effects of hypophysectomy [48,147]. However, hypophysectomy also decreases parathyroid gland function, which returns upon GH treatment [79,120]. Therefore, the effects of hypophysectomy and GH on 24-OHase activity are probably secondary to effects on the parathyroid glands.

Chronic treatment of vitamin D-replete rats with thyroxine (T_4) increased plasma levels of 24,25-DHCC and decreased plasma levels of 1,25-DHCC [143]. Consistent with this report Miller and Ghazarian [89] demonstrated that daily injections of T_4 or triiodothyronine (T_3) for 6 days induced renal 24-OHase activity and repressed renal 1-OHase activity. The authors conclude from the study that thyroid hormones affect vitamin D_3 metabolism either directly or by their ability to simulate bone resorption. A stimulation of bone resorption would have the effect of elevating serum calcium concentration, which would depress PTH secretion, resulting in decreased 1,25-DHCC and increased 24,25-DHCC synthesis. This sequence of events has not been confirmed, and the possibility still exists that thyroid hormones affect renal vitamin D metabolism directly.

The 24-hydroxylation reaction has been regarded as a deactivation pathway for 1,25-DHCC [78]. The product of the 24-hydroxylation

of 1,25-DHCC, 1,24,25-trihydroxycholecalciferol (1,24,25-THCC), is biologically less active than 1,25-DHCC [69]. A high 24-OHase activity would work in two ways to inactivate the vitamin D system: (a) by 24-hydroxylating 1,25-DHCC to produce the less active metabolite; (b) by 24-hydroxylating 25-HCC to produce a compound that, according to many investigators, is biologically inactive (reviewed by Brommage and DeLuca, ref.19). This hypothesis is in agreement with the data showing a major involvement of 1,25-DHCC in regulating its own degradation by inducing 24-OHase activity. However, 1,25-DHCC is mainly excreted in the bile and as a side-chain oxidation product [77]. These excretory processes are non-saturable and are not regulated by serum calcium, phosphorus, or 1,25-DHCC levels. Although 24-hydroxylase activity is regulated by 1,25-DHCC, it is unlikely that a change in 24-hydroxylase activity will cause a change in the excretion of 1,25-DHCC.

The presence of the 24-OHase in renal mitochondria suggests that it is an enzyme that produces a biologically important hormone, because other mitochondrial P450s are usually involved in hormone biosynthesis, rather than their degradation [142].

(d) The Effect of Estrogen on Vitamin D Hydroxylases in Birds

In birds the egg-laying cycle represents a physiological calcium stress. The production of the egg shell can require as much as one gram of calcium in one hour every 24 hours during the egg laying cycle [28]. Consequently an increase in intestinal calcium transport and deposition of medullary bone occurs in preparation for the egg

production process [28]. Kenny [73] showed that following ovulation in the egg-laying Japanese Quail 1,25-DHCC production is enhanced, even at a time before any calcification of the egg shell begins. If ovulation does not happen after oviposition takes place, then 1,25-DHCC production rapidly declines [73]. In female birds estrogen secretion increases as the bird reaches maturity and it was propcsed that estrogen is responsible for the changes in calcium metabolism during reproduction [28]. A number of studies have reported that estradiol (E_2) treatment can stimulate 1-OHase activity in female birds [7,8,9,28,128] and in male birds when given together with testosterone [128]. The effect of E_2 is larger in magnitude than that of PTH, and the effects of PTH and E_2 given together are additive, suggesting that they act via different mechanisms [9,28]. Pretreatment with tamoxifen citrate, an anti-estrogen, prevented the E_2 mediated increase in 1,25-DHCC production, but did not affect the inducibility of 1-OHase activity by PTH [9]. This is further evidence supporting the notion that E_2 and PTH act via different mechanisms. The induction of 1-OHase activity by E_2 happens only in the intact bird, because E_2 treatment of cultured chick kidney cells had no effect on 1-OHase activity [119]. This data implies that E_2 does not induce the 1-OHase directly, but acts via an indirect mechanism. E_2 is known to stimulate the synthesis and secretion of PRL and GH [2,30,117]. It is, therefore, possible that E_2 increases 1,25-DHCC production via a pathway that involves PRL and/or GH. This has not been determined yet.

E_2 treatment of immature females had no effect on 24-OHase ac-

tivity, but in immature males 24-OHase activity was markedly decreased [7]. On the other hand, tamoxifen citrate stimulated 24-OHase activity in female birds, which was partially inhibited by E_2 and completely inhibited by PTH [9]. It is difficult to assess what these data mean in the context of the normal hormonal regulation of 24-OHase activity. It has not been determined yet whether E_2 has an effect on synthesis or activation of the 24-OHase , or whether the effects are mediated via PTH or some other intermediary factor.

Physiological Actions of Vitamin D_3

By definition a hormone is a substance that is secreted by a specific tissue into the blood stream and is transported to other specific tissues, upon which a physiological effect is exerted. Hormones are effective in minute quantities and exert their effects by binding to specific high affinity receptors. All these criteria considered, 1,25-DHCC is an endocrine hormone, with the kidney as its endocrine gland for synthesis and secretion. The intestine, bone and parathyroid glands are then the major target organs with specific receptors for 1,25-DHCC.

(a) Action on Intestine

The most thoroughly investigated effect of 1,25-DHCC is its role in increasing calcium absorption from the intestine [18]. 1,25-DHCC has also been shown to increase intestinal phosphorus absorption [31], but

most studies have concentrated on the molecular mechanisms of the 1,25-DHCC mediated increase in calcium transport. Autoradiographic studies on target tissues for 1,25-DHCC demonstrated evidence for a nuclear concentration and retention of 1,25-DHCC in the intestine [126,150] as well as other tissues [126]. Using chick embryonic intestinal organ cultures it was then demonstrated that the intestinal calcium transport in response to 1,25-DHCC is blocked by RNA and protein synthesis inhibitors [49]. These studies provided strong evidence for a nuclear mechanism of 1,25-DHCC action, similar to classical steroid hormone action on gene expression. In other words, 1,25-DHCC acts on specific genes that code for proteins involved in calcium transport in the intestine. A cytosolic receptor for 1,25-DHCC has been found in the intestine, which upon binding of 1,25-DHCC translocates to the nucleus [137]. The avian intestinal 1,25-DHCC receptor has been well characterized and cloned [86].

An important discovery in the vitamin D field was the identification of a vitamin D-dependent calcium binding protein in the gut (CaBP) [141]. Since then a number of vitamin D-dependent CaBPs have been found in the intestine and various other tissues [97]. Initially it was thought that the intestinal CaBP is involved in the calcium transport process. Corradino et al. [34] showed that adding CaBP to intestinal mucosa, deficient in CaBP, increased calcium transport in these cells. Taylor [135] demonstrated CaBP specific immunofluorescence over absorptive cell cytoplasm. However, other investigators demonstrated that 1,25-DHCC mediated intestinal calcium transport occurs at least 2

hours before CaBP can be detected immunologically and at least 1 hour before protein synthesis occurs on the polyribosomes [123]. Also, rachitic chicks require the presence of dietary calcium for a maximal stimulation of CaBP production by 1,25-DHCC [123]. These data suggested that CaBP is an intracellular protein whose synthesis is increased as a consequence of raised intracellular calcium content. Therefore, Spencer et al. [123] proposed that CaBP acts as a buffer for intracellular calcium, permitting a continued 1,25-DHCC mediated calcium absorption. CaBP has been cloned [43] and an increase in its mRNA by 1,25-DHCC administration has been observed [46]. Further studies will be required to determine what the exact function of CaBP is.

(b) Action on Bone

Vitamin D_3 has two major actions on bone: (a) mineralization of bone, which is characterized morphologically as a decrease in the amount of osteoid, when vitamin D_3 is administered to rachitic animals; (b) bone resorption, which is manifested as a decrease in bone mineral and matrix content [125]. Vitamin D deficiency leads to bone disorders such as rickets during growth and osteomalacia in the adult [39,125]. This is due to the hypocalcaemia and decreased bone mineralization that accompanies vitamin D deficiency [125]. Ingestion of high doses of vitamin D can result in vitamin D toxicity, one of its characteristics being excessive bone resorption, without a corresponding increase in bone mineralization [125].

Bone is a highly complex tissue that consists of a number of different cell types. The osteoblasts are the bone forming cells and have been shown to exhibit receptors for 1,25-DHCC [32] and PTH [116]. Although 1,25-DHCC is known to have a variety of effects on osteoblasts, the mechanisms of 1,25-DHCC mediated bone mineralization is still poorly understood [95].

Bone resorption is mediated by another cell type, the osteoclast. PTH and 1,25-DHCC are the major bone resorbing hormones [95,125]. In fact they require the presence of one another in order to be active [26,55]. Osteoclasts, however, exhibit neither 1,25-DHCC [93] nor PTH receptors [116]. Isolated mature osteoclasts also do not respond to 1,25-DHCC [29]. One hypothesis that has been put forward is that PTH and 1,25-DHCC mediate osteoclastic bone resorption via another cell type, possibly the osteoblast [111]. Recently, it has been shown that PTH mediated osteoclastic bone resorption in vitro requires the presence of osteoblastic cells [87]. Alone, neither osteoclasts nor osteoblasts are able to respond to PTH with bone resorption [87].

Another line of research has also shown that mononuclear phagocytes, the postulated precursor cells that differentiate into pre-osteoclasts [95], respond to 1,25-DHCC by enhancement of cell maturation and phagocytic activity [11]. Therefore, the exact mechanism of 1,25-DHCC mediated bone resorption is not completely understood.

(c) Action on Other Tissues

The parathyroid glands have for a long time been suspected of being a target organ for 1,25-DHCC. Since PTH is a stimulator of 1,25-DHCC production, it seems plausible that 1,25-DHCC regulates PTH secretion by a feedback mechanism. In fact, 1,25-DHCC can suppress the secretion of PTH in intact animals [23,25] as well as in bovine parathyroid cells in culture [24]. This study also showed that PTH secretion closely parallels levels of pre-pro PTH mRNA. It was then determined that 1,25-DHCC acts on the parathyroids by suppressing transcription of the pre-pro PTH gene [112].

The hepatic metabolism of vitamin D_3 has been determined to be affected by 1,25-DHCC [61]. Dietary supplementation of vitamin D-deficient rats with 1,25-DHCC resulted in a decrease in circulating levels of vitamin D_3 and 25-HCC and an increase in more polar metabolites [61]. This suggested that 1,25-DHCC accelerates the in vivo metabolism of vitamin D_3 and 25-HCC.

The kidney has also been shown to be a target organ for 1,25-DHCC. Besides inducing renal 24-OHase activity, 1,25-DHCC has also been implicated in increasing renal calcium [62] and phosphate reabsorption [107]. There is no doubt that other target organs for 1,25-DHCC will be found, especially in light of finding vitamin D-dependent CaBPs in a variety of tissues [97] and having specific binding and retention of radioactive 1,25-DHCC in different organs as seen by autoradiography [126].

(d) Evidence for a Biological Role of 24,25-DHCC

The 24-OHase catalizes the formation of 24,25-DHCC. Although this metabolite of 25-HCC is quantitatively more important in normal animals than 1,25-DHCC, its role as a hormone is not as clear as for 1,25-DHCC. Tanaka et al. [132] used 24,24-difluoro 25-HCC, a compound that can be hydroxylated in the 1-position but not in the 24-position, to test whether the 24-hydroxylation is important for vitamin D_3 action. The results showed that there was no significant difference between the action of the difluoro compound and 25-HCC in vitamin D-deficient rats with respect to intestinal calcium transport, calcium mobilization from bone, healing and mineralization of rachitic bone, elevating serum phosphorus and preventing rachitogenesis [132]. They concluded that the 24-hydroxylation of 25-HCC is not important for any of the effects attributed to vitamin D.

On the other hand, studies have shown that 24,25-DHCC, in physiological doses, can effectively suppress PTH secretion in the dog [23], in the goat [25], and in bovine parathyroid glands in vitro [33]. Although 1,25-DHCC is more effective in suppressing PTH secretion in bovine parathyroid glands in vitro [33], it was concluded by all these studies that 24,25-DHCC, by virtue of being the predominant metabolite in the normo- and hypercalcaemic state, plays an important role in the inhibition of PTH secretion [23,25,33]. In contrast, Dietel et al.[44] found no effect of 24,25-DHCC on PTH secretion in porcine parathyroid glands and in human parathyroid adenomas in organ cul-

ture.

Corvol et al.[35] presented biochemical evidence for an action of 24,25-DHCC on chondrocytes. In physiological concentrations 24,25-DHCC increased the incorporation of radioactive sulfate into proteoglycans [35]. Subsequent studies, using the same model, demonstrated increased DNA polymerase activity in response to 24,25-DHCC [36] and a specific nuclear uptake of [^{3}H] 24,25-DHCC in chondrocytes [37]. In both studies 1,25-DHCC had similar effects in the same dose range. The authors concluded that 24,25-DHCC can act in the same manner as 1,25-DHCC and other steroid hormones.

Effects of 24,25-DHCC on bone were also reported. 24,25-DHCC was observed to prevent bone resorption in nephrectomized and nephrectomized parathyroidectomized hypocalcaemic rats [101]. A direct action on osteoclasts was excluded as the mechanism of action [101]. Rather, 24,25-DHCC was postulated to act via osteocytes, or by inhibiting cell activity [101]. Ornoy et al.[100] and Endo et al.[47] have demonstrated that 24,25-DHCC is required for proper bone formation in chicks. Both studies showed that although 1,25-DHCC is the important hormone for increasing serum calcium and phosphorus levels, a combination treatment of 1,25-DHCC or 1-HCC with 24,25-DHCC was most effective in curing rachitic lesions and causing bone mineralization [47,100]. Recent evidence for a role of 24,25-DHCC in the regulation of bone formation and mineralization stems from the finding that administration to vitamin D-deficient rats of 25-HCC or 24,25-DHCC, but not 1,25-DHCC, restored the linear rate of bone mineral apposition

BMAR) to normal [127]. Thyroparathyroidectomy abolished the effect of 24,25-DHCC, but simultaneous administration of PTH and 24,25-DHCC to vitamin D-deficient TPTX rats restored the BMAR to normal [127]. These results demonstrated that PTH and 24,25-DHCC can act in concert to regulate bone formation and mineralization.

To date, conclusive evidence for a 24,25-DHCC receptor is lacking. 24,25-DHCC has been observed to accumulate in fetal rat bones [96] and in chick callus tissue, after experimental fracture, at a time coinciding with cartilage synthesis [81]. Furthermore, long bones of new born rats exhibited specific high affinity binding sites for 24,25-DHCC [118]. Long bones from new born rats consist predominantly of cartilage. It was, therefore, suggested that these binding sites are located in the chondrocytes and that 24,25-DHCC is involved in the regulation of cartilage synthesis [118], as has been suggested by other studies [35,37,81].

In summary, evidence for a specific biological action for 24,25-DHCC is controversial. Although the results obtained with 24,24-difluoro 25-HCC are convincing data against a biological role for 24,25-DHCC, other studies have equally convincing data supporting a role for 24,25-DHCC in suppressing PTH secretion and in the synthesis of cartilage and bone. Conclusive demonstration of a 24,25-DHCC receptor that interacts with DNA, or some form of second messenger system, is needed to prove that 24,25-DHCC is an important hormone.

Another criteria for 24,25-DHCC being a hormone is that its synthesis is tightly regulated. So far, the regulation of the 24-OHase is

shown to be secondary to that of the 1-OHase. In effect, the production of 1,25-DHCC is responsible for inducing 24-OHase activity.

Objective of Study

The molecular mechanisms involved in the induction of 1-OHase and 24-OHase activity are unknown. It is not clear whether the increased enzyme activities are due to de novo enzyme synthesis or due to the activation of pre-existing enzyme. The induction of several hepatic microsomal P450s by phenobarbital, pregnenolone 16-carbonitrile and 3-methylcholanthrene involves an increase in total hepatic microsomal P450 and an accumulation of the respective mRNAs [102,114,138]. The renal mitochondrial P450 content does not change when rats are made vitamin D-deficient, despite large changes in renal vitamin D hydroxylase activities [139]. However, it is not known what proportion of renal mitochondrial P450 is made up of 1-OHase or of 24-OHase enzyme.

The production of molecular probes, such as specific polyclonal or monoclonal antibodies, against the renal vitamin D hydroxylases would allow us to quantify the individual enzymes. These molecular tools would also allow us to determine whether transcriptional, translational or post-translational events are involved in the induction of enzyme activities by various treatments.

The objective of this study is to identify conditions under which 1-OHase activity and 24-OHase activity are consistently high in an in

vitro assay system. The purpose of this is to obtain mitochondria with either high 1-OHase or high 24-OHase activity, from which the enzymes can be isolated and purified. Specific antibodies to the 1-OHase and 24-OHase can then be produced, in order to study the regulation of vitamin D metabolism at the molecular level.

RESULTS and DISCUSSION

Age Related Changes in Renal Vitamin D Metabolism

Renal vitamin D hydroxylase activities can be changed by dietary manipulations. Rats raised on a diet with adequate calcium, phosphorus and vitamin D content have measurable 24-OHase activity and non-detectable 1-OHase activity in their renal mitochondria. Rats that are weaned on a vitamin D-deficient, low calcium diet have measurable 1-OHase activity and non-detectable 24-OHase activity in their renal mitochondria.

Normal male rats exhibited an age related increase in 24-OHase activity [Ch.I]. The enzyme activity was lower in 2-4 week old rats than in 8 week old adult rats[2]. It was not determined at what time point adult levels of 24-OHase activity were reached, but it must have occurred after 30 days of age. The mechanism of this induction is not known, but age related changes in other forms of hepatic and renal microsomal P450s do occur [94]. The significance of the change in enzyme activity with age can at this point not be evaluated, since it is still debatable whether 24,25-DHCC has a biological function.

First generation vitamin D-deficient (G_1) female rats did not exhibit an age related change in renal mitochondrial 1-OHase activity [Ch.II]. Young (5 weeks old) rats had measurable 1-OHase activity and no 24-OHase activity. Adult rats (8-30 weeks old) had a highly variable 1-OHase activity, which on average was not significantly different from young rats. The mechanism of induction of 1-OHase activity is

likely to involve PTH. While the adult rats were hypocalcaemic, hypophosphataemic and hyperparathyroid, the young rats had normal serum concentrations of calcium and phosphorus, but serum PTH levels were slightly elevated [Ch.II]. Serum PTH levels were determined using the mid-molecule radioimmunoassay (RIA) for PTH. This assay has its limitations, since it does not measure biologically active hormone. The fact that the young rats exhibited measurable 1-OHase activity and increased urinary excretion of cAMP, indicated that they were effectively hyperparathyroid.

Second generation vitamin D-deficient rats (G_2) exhibit an induction of 1-OHase activity at the time of puberty [140]. The enzyme activity becomes non-detectable after 65 days of age, which correlates with the loss of PTH sensitivity [90,140]. At no time did any of the G_1 rats display PTH resistance, as measured by their urinary cAMP excretion [Ch.II]. Therefore, the initial development of hyperparathyroidism was probably responsible for the appearance of 1-OHase activity in G_1 rats.

Effects of Fasting on Vitamin D Metabolism

In an attempt to determine whether vitamin D metabolism is altered on a day to day basis, according to the bodies' need for calcium and phosphorus, normal rats were deprived of food for 24 hours and renal mitochondrial vitamin D hydroxylase activities were measured [Ch.I]. At no time was any 1-OHase activity detectable in normal rats. Fasting for 24 hours induced 24-OHase activity 2-3 fold in adult as

well as young rats. This finding was unexpected in light of what is known about vitamin D metabolism. Food deprivation in effect is equivalent to the removal of dietary calcium and phosphorus. In order to satisfy the animals' requirement for calcium and phosphorus, bone resorption must take place. Therefore, fasting should increase PTH secretion and possibly 1,25-DHCC production, since both are potent bone resorbing hormones. However, neither serum PTH nor serum 1,25-DHCC concentrations significantly change with fasting [Ch.I].

There are 3 possibilities that could explain the observed phenomena: (1) The effective levels of PTH were higher than determined by the mid-molecule RIA (in effect, bone resorption takes place with small changes in serum PTH levels, that cannot be detected by the used methodology); (2) The induced 24-OHase activity increases serum levels of 24,25-DHCC, which then acts as a calcium regulating hormone; (3) There is some unknown mechanism at work. In man 24,25-DHCC has been shown to increase intestinal calcium absorption [72]. However, fasted rats had no dietary calcium intake. Bone resorption has been shown to be inhibited by 24,25-DHCC [101]. Therefore, scenario (2) is unlikely to occur. It remains a distinct possibility that the effective PTH levels are increased (1). In order to test this hypothesis further, a PTH assay that detects biologically active hormone would have to be used in these experiments, and bone resorption would have to be measured in fasting rats.

Mechanism of 24-OHase Induction

Hormones that have been shown to influence 24-OHase activity include thyroid hormones [89,143], PTH [98,134], pituitary hormones [48,147] and 1,25-DHCC [98,130,134]. Thyroid hormones are unlikely to be involved in the stimulation of 24-OHase activity by fasting, since their serum concentrations are reduced in starvation [105]. A removal of an inhibitory influence on 24-OHase activity by PTH can also be ruled out, since parathyroidectomy did not prevent the response to fasting and fed PTX rats exhibited an enzyme activity not significantly different from intact fed rats [Ch.I].

Similarly, pituitary hormones can also be eliminated as possible mediators of the induction of 24-OHase activity by fasting, since an increase in enzyme activity was observed in hypophysectomized rats [Ch.I].

A high dose (2.5 nmol) of 1,25-DHCC stimulated 24-OHase activity within 10 hours in normal rats [Ch.I]. If 1,25-DHCC is involved in the effect of fasting on 24-OHase activity, then serum levels of 1,25-DHCC should rise within 10 hours of food removal. However, serum 1,25-DHCC did not significantly change within that time period [Ch.I], suggesting that the induction of 24-OHase activity by fasting is independent of 1,25-DHCC.

Serum 1,25-DHCC [88] and 24,25-DHCC [148] concentrations follow a diurnal rhythm. Both the light/dark cycle and the time of feeding can act as synchronizers of a diurnal rhythm [148]. It is not known whether a connection exists between the diurnal rhythm of 24,25-DHCC

production and the induction of 24-OHase activity by food deprivation.

The biological significance of this enzyme regulation by food intake is not understood, mainly because the exact biological rcle of 24,25-DHCC is not known. The finding that anorectic girls also exhibit elevated serum levels of 24,25-DHCC [1] confirms that starvation can induce a change in vitamin D metabolism.

Induction of 1-OHase Activity During Lactation

In the present investigation it was shown that renal mitochondrial 1-OHase activity in G_1 adult female rats is induced by lactation [Ch.II]. The enzyme activity in lactating rats is consistently high, regardless of the age of the rat or the length of the lactational period. The 1-OHase activity in non-lactating G_1 females was highly variable. Although some non-lactating females exhibited activities equivalent to values obtained for lactating rats, on average lactating rats had a 3-4 fold higher 1-OHase activity.

Lactating rats have been shown to exhibit elevated serum levels of 1,25-DHCC [15,104]. Therefore, the present findings were not unexpected. The mechanism of 1-OHase induction, however, is not clear. Lactating and non-lactating rats were hypocalcaemic, hypophosphataemic, hyperparathyroid and PTH sensitive, as determined by their increased urinary excretion of cAMP [Ch.II]. It is, therefore, unlikely that PTH, hypocalcaemia and hypophosphataemia alone mediate the stimulation of 1-OHase activity during lactation. Some other factor present during lactation may be responsible for the observed enzyme

induction. PRL is known to stimulate 1,25-DHCC production in vivo [110,121] as well as in vitro [12,121]. In this investigation, 24 hours after litter removal, a procedure known to decrease serum PRL levels, 1-OHase activity was still elevated [Ch.II]. It was not determined whether a longer period after litter removal is necessary for the 1-OHase activity to decrease, since the enzyme half-life is not known. Therefore, it could not be demonstrated whether a lactational factor plays a role in the induction of renal mitochondrial 1-OHase activity during lactation.

General Summary

Normal adult (8 weeks old) rats exhibited 24-OHase activity in their renal mitochondria which was higher than the activity displayed by pre-pubertal (2-4 weeks old) rats. The 24-OHase activity was induced by food deprivation for 24 hours, regardless of the age of the animal. The mechanism of this induction is not clear, but it is independent of PTH, pituitary hormones and 1,25-DHCC.

Adult (8-30 weeks old) G_1 females had variable 1-OHase activity in their renal mitochondria, which on average was not significantly different from the activity seen in young 5 week old rats. The 1-OHase activity was stimulated during lactation. The mediators of this induction could not be identified, but it is suspected that some factor other than PTH, hypocalcaemia and hypophosphataemia is responsible.

This investigation has demonstrated conditions under which renal

mitochondrial vitamin D hydroxylases are easily and rapidly stimulated. It is not known whether the described phenomena are due to a post-translational activation of the enzymes or whether translational and/or transcriptional events are involved. The information presented here can be used to prepare renal mitochondria with induced 1-OHase or 24-OHase activities, from which the enzymes can be purified and studied at the molecular level.

FOOTNOTES

While writing this thesis, new data has been published that is relevant to this thesis. Footnote (1) refers to an article that presents evidence implicating somatomedin C in the stimulation of 1-OHase activity during hypophosphataemia. Footnote (2) refers to an article demonstrating an effect of age on 24-OHase and 1-OHase activity. Their results show an increase in 24-OHase activity and a decrease in 1-OHase activity with an increase in age. The hydroxylase activity measurements were taken starting with 2 month old rats and ending with 24 month old rats.

(1) Gray RW (1987) Evidence that somatomedins mediate the effect of hypophosphataemia to increase serum 1,25-dihydroxyvitamin D_3 levels in rats. Endocrinol. 121: 504-512.

(2) Ishida M, Bulos B, Takamoto S, Sacktor B (1987) Hydroxylation of 25-hydroxyvitamin D_3 by renal mitochondria from rats of different ages. Endocrinol. 121: 443-448.

ACKNOWLEDGMENTS

I would like to express my gratitude and appreciation to all those people who have contributed to this thesis and who have made life enjoyable in this department. In particular I would like to thank:

My supervisor, Dr. Margaret Warner, who has been an incredible scientific inspiration to me and who is largely responsible for my current views on science. I consider her departure from McGill a great loss to this department.

Dr. Brian Collier, professor at the Department of Pharmacology & Therapeutics, for his encouragement and support in the sciertific field, and for his advice on many other matters. He will always be remembered as someone who has an absolutely incredible imagination. People who know him understand what this means.

Dr. Jan-Åke Gustafsson, professor at the Department of Medical Nutrition, Huddinge, Sweden, for permitting me to spend one very exciting year at his department. The experience I gained in Sweden has contributed a great deal to my development as a scientist and person.

My fellow slaves who have been there when times where rough, at Huddinge: Dr. Gunnar Johansson, Lauryl van Neste and Maria Sundin, and at McGill: Helena Koa, Robyn Schecter, Ligia Stern, Dr. Rogelio Mosqueda, Dr. Louben Tchakarov, and Dr. Serge Mykita.

I have reserved this special line for a dear "colleague" who has not only been a good friend who has helped me a great deal over the

years, but who never said no to a Pilsner when we really needed it. This friend is none other than Dr. Rafáel deJesus Cabeza.

I am also indepted to Drs. Bernard Robaire and Alan Tenenhouse, professors at the Department of Pharmacology & Therapeutics, for their help in designing my experiments, their advice and encouragement especially in my last year.

Finally I wish to thank the Fonds de la Recherche en Santé du Québéc for funding me throughout the years.

REFERENCES

1. Aarskog D, Aksnes L, Markestad T (1986) Plasma concentrations of vitamin D metabolites in pubertal girls with anorexia nervosa. Acta Endocrinol. Suppl. 279 (113): 458-467.

2. Amara JF, Van Italie C, Dannies PS (1987) Regulation of prolactin production and cell growth by estradiol: difference in sensitivity to estradiol occurs at the level of messenger ribonucleic acid accumulation. Endocrinol. 120: 264-271.

3. Andersson S, Jörnvall H (1986) Sex differences in cytochrome P-450-dependant 25-hydroxylation of C_{27}-steroids and vitamin D_3 in rat liver microsomes. J. Biol. Chem. 253: 842-849.

4. Armbrecht HJ, Forte LR, Wongsurawat N, Zenser TV, Davis BB (1984) Forskolin increases 1,25-dihydroxyvitamin D_3 production by rat renal slices in vitro. Endocrinol. 114: 644-649.

5. Armbrecht HJ, Wongsurawat N, Zenser TV, Davis BB (1984) Effect of PTH and $1,25(OH)_2D_3$ on renal $25(OH)D_3$ metabolism, adenylate cyclase, and protein kinase. Am. J. Physiol. 246: E102-107.

6. Askew FA, Bourdillon RB, Bruce HM, Callow RK StL, Philpot J,

Webster TA (1932) Crystalline vitamin D. Proc. R. Soc. London (Biol) 109: 488-506.

7. Baksi SN, Kenny AD (1977) Vitamin D_3 metabolism in immature Japanese Quail: effects of ovarian hormones. Endocrinol. 101: 1216-1220.

8. Baksi SN, Kenny AD (1978) Acute effect of estradiol on the renal vitamin D hydroxylases in Japanese Quail. Biochem. Pharmacol. 27: 2765-2768.

9. Baksi SN, Kenny AD (1979) Parathyroid hormone stimulation of 1,25-dihydroxyvitamin D_3 production in antiestrogen-treated Japanese Quail. Mol. Pharmacol. 16: 932-940.

10. Baran DT, Whyte MP, Haussler MR, Deftor LJ, Slatopolski E, Avioli LV (1980) Effect of the menstrual cycle on calcium-regulating hormones in the normal young woman. J. Clin. Endocrinol. Metab. 50: 377-379.

11. Bar-Shavit Z, Teitelbaum SL, Reitsma P, Hall A, Pegg LE, Trial JA, Kahn AJ (1983) Induction of monocyte differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. Proc. Natl. Acad. Sci.80: 5907-5911.

12. Bickle DD, Spencer EM, Burke WH, Rost CR (1980) Prolactin but not growth hormone stimulates 1,25-dihydroxyvitamin D_3 production by chick renal preparations in vitro. Endocrinol. 107: 81-84.

13. Björkhem I, Holmberg I (1978) Assay and properties of a mitochondrial 25-hydroxylase active on vitamin D_3. J. Biol. Chem. 253: 842-849.

14. Björkhem I, Holmberg I, Oftebro H, Pedersen J (1980) Properties of a reconstituted vitamin D_3 25-hydroxylase from rat liver mitochondria. J. Biol. Chem. 255: 5244-5249.

15. Boass A, Toverud JW, McCain TA, Pike JW, Haussler MR (1977) Elevated serum levels of 1,25-dihydroxycholecalciferol in lactating rats. Nature 267: 630-632.

16. Bouillon R, Van Baelen H (1981) Transport of vitamin D: significance of free and total concentrtations of the vitamin D metabolites. Calc. Tissue Int. 33: 451-453.

17. Boyle IT, Gray RW, DeLuca HF (1971) Regulation by calcium of in vivo synthesis of 1,25-dihydroxycholecalciferol and 21,25-dihydroxycholecalciferol. Proc. Natl. Acad. Sci. 68: 2131-2134.

18. Boyle IT, Miravet L, Gray RW, Holick MF, DeLuca HF (1972) The

response of intestinal calcium transport to 25-hydroxy and 1,25-dihydroxyvitamin D in nephrectomized rats. Endocrinol. 90: 605-608.

19. Brommage R, DeLuca HF (1985) Evidence that 1,25-dihydroxyvitamin D_3 is the physiologically active metabolite of vitamin D_3. Endocrine Rev. 6(4): 491-511.

20. Buchanan JR, Santen R, Cauffman S, Cavaliere A, Greer RB, Demers LM (1986) The effect of endogenous estrogen fluctuation on metabolism of 25-hydroxyvitamin D. Calc. Tissue Int. 39: 139-144.

21. Bushinsky DA, Rieva GS, Favus MJ, Coe FL (1985) Evidence that blood ionized calcium can regulate serum $1,25(OH)_2D_3$ independantly of parathyroid hormone and phosphorus in the rat. J. Clin. Invest. 76: 1599-1604.

22. Burgos-Trinidad M, Brown AJ, DeLuca HF (1986) Solubilization and reconstitution of chick renal mitochondrial 25-hydroxyvitamin D_3 24-hydroxylase. Biochemistry 25: 2692-2696.

23. Canterbury JM, Lerman S, Claylin AJ, Henry H, Norman A, Russ E (1977) Inhibition of parathyroid hormone secretion by 25-hydroxycholecalciferol and 24,25 dihydroxycholecalciferol in the

dog. J. Clin. Invest. 61: 1375- 1383.

24. Cantley LK, Russel J, Lettieri D, Sherwood LM (1985) 1,25-dihydroxyvitamin D_3 suppresses parathyroid hormone secretion from bovine parathyroid cells in tissue culture. Endocrinol. 117: 2114-2119.

25. Care AD, Bates RFL, Pickard DW, Peakock M, Tomlinson S, O'Riordan JLH, Mawer EB, Taylor CM, DeLuca HF, Norman AW (1976) The effects of vitamin D metabolites and their analogues on the secretion of parathyroid hormone. Calcif. Tissue Res. 21: 142-146.

26. Carlsson A, Lindqvist M, Magnusson T (1954) The cause of hypophosphataemia and hypocalcaemia in vitamin D deficiency. Acta Physiol. Scand. 31: 308-311.

27. Caron MG, Beaulieu M, Raymond V, Gagne B, Drouin J, Lefkowitz RJ, Labrie F (1978) Dopaminergic receptors in the anterior pituitary gland. J. Biol. Chem. 253: 2244-2253.

28. Castillo L, Tanaka Y, DeLuca HF (1977) The stimulation of 25-hydroxyvitamin D_3-1-hydroxylase by estrogen. Arch. Biochem. Biophys. 179: 211-217.

29. Chambers TJ, Mc Sheehy PMJ, Thompson BM, Fuller K (1985) The ef-

fect of calcium regulating hormones and prostaglandins on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. Endocrinol. 116: 234-239.

30. Chen CL, Meites J (1970) Effects of estrogen and progesterone on serum and pituitary prolactin levels in ovariectomized rats. Endocrinol. 86: 503-505.

31. Chen TC, Castillo L, Korycka-Dahl M, DeLuca HF (1974). Role of vitamin D metabolites in phosphate transport of rat intestine. J. Nutr. 104. 1056-1060.

32. Chen TL, Hirst MA, Feldman D (1979) A receptor-like binding macromolecule for 1,25-dihydroxycholecalciferol in cultured mouse bone cells. J. Biol. Chem. 254: 7491-7494.

33. Chertow BS, Baker GR, Henry H, Norman AW (1980). The Effects of vitamin D metabolites on bovine parathyroid hormone release in vitro. Am. J. Physiol. 238: 384- 388.

34. Corradino RA, Fullmer CS, Wasserman RH (1976) Embrionic chick intestine in organ culture: stimulation of calcium transport by exogenous vitamin D-induced calcium-binding protein. Arch. Biochem. Biophys. 174: 738.

35. Corvol M, Dumontier MF, Garabedian M, Rappaport R (1978) Vitamin D and cartilage. II. Biological activity of 25-hydroxycholecalciferol and 24,25- and 1,25-dihydroxycholecalciferol on cultured growth plate chondrocytes. Endocrinol. 102: 1269.

36. Corvol M, Dumontier MF, Ulmann A, Garabedian M, Witmer G (1979) Nuclear binding of 24,25-$(OH)_2D_3$ and its effects on DNA polymerase activity in cultured chondrocytes. In Vitamin D: Basic Research and its Clinical Application. Edited by A.W. Norman, K. Schaefer, D. von Herrath, H.G. Grigoleit, J.W. Coburn, H.F. DeLuca, E.B. Mawer and T. Suda. Walter de Gruyter, Berlin. pp.419.

37. Corvol M, Ulmann A, Garabedian M (1980). Specific nuclear uptake of 24,25-dihydroxycholecalciferol a vitamin D_3 metabolite biologically active in cartilage. FEBS Lett. 116: 273- 276.

38. Dahlbäck H, Wikwall K (1986) 25-hydroxylation of vitamin D_3 in rat liver: roles of mitochondrial and microsomal cytochrome P-450. Biochem. Biophys. Res. Commun. 142: 999-1005.

39. DeLuca HF (1981) The vitamin D system: a view from basic science to the clinic. Clin. Biochem. 14: 213-222.

40. DeLuca HF, Schnoes HJ (1983) Vitamin D Recent Advances. Ann. Rev. Biochem. 52: 411-439.

41. Delvin EE, Arabian A, Glorieux FH (1980) Kinetic alterations in rat liver microsomal cholecalciferol 25-hydroxylase associated with phenobarbital administration. Biochem. Pharmacol. 29: 441-445.

42. D'Ércole AJ, Stiles AD, Underwood LE (1984) Tissue concentration of somatomedin C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. Proc. Natl. Acad. Sci. 81: 935-939.

43. Désplan C, Thomasset M, Moukhtar M (1983) Synthesis, molecular cloning and restriction analysis of DNA complementary to vitamin D-dependant calcium-binding protein mRNA from rat duodenum. J. Biol. Chem. 258: 2762-2769.

44. Dietel M, Dorn G, Montz R, Altenahr E (1979) Influence of vitamin D_3, 1,25 dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 on parathyroid hormone secretion, adenosine 3',5'-monophosphate release, and ultrastructure of parathyroid glands in organ culture. Endocrinol. 105: 237- 245.

45. Dupret JM, Brun P, Thomasset M (1986) In vivo effects of transcriptional and translational inhibitors on duodenal vitamin D-dependant calcium-binding protein messenger ribonucleic acid stimulation by 1,25-dihydroxycholecalciferol. Endocrinol. 119: 2476-

2483.

46. Eden S (1979) Age and sex related differences in episodic growth hormone secretion in the rat. Endocrinol. 105: 555-560.

47. Endo H, Kiyoki M, Kawashima K, Naguchi T, Hashimoto Y (1980). Vitamin D_3 Metabolites and PTH synergistically stimulate bone formation. Nature 286: 262- 264.

48. Fontaine O, Pavlovitch H, Balsan S (1978) 25-hydroxycholecalciferol metabolism in hypophysectomized rats. Endocrinol. 102: 1822-1826.

49. Franceschi RT, DeLuca HF (1981) Characterization of 1,25-dihydroxyvitamin D_3-dependant calcium uptake in cultured embryonic chick duodenum. J. Biol. Chem. 256: 3840-3847.

50. Fraser DR (1980) Regulation of the metabolism of vitamin D. Physiol. Rev. 60: 551-613.

51. Fraser DR, Kodicek E (1970) Unique biosynthesis by kidney of a biologically active vitamin D metabolite. Nature 228: 764-766.

52. Fraser DR, Kodicek E (1973) Regulation of 25-hydroxycholecalciferol-1-hydroxylase activity in kidney by

parathyroid hormone. Nature New Biol. 241: 163-165.

53. Garabedian M, Bailly DuBois M, Corvol MT, Pezant E, Balsan S (1978) Vitamin D and cartilage. I. In vitro metabolism of 25-hydroxycholecalciferol by cartilage. Endocrinol. 102: 1262-1269.

54. Garabedian M, Pavlovitch H, Fellot C, Balsan S (1974) Metabolism of 25-hydroxyvitamin D_3 in anephric rats: a new active metabolite. Proc. Natl. Acad. Sci. 71: 554-557.

55. Garabedian M, Tanaka Y, Holick MF, DeLuca HF (1974) Response of intestinal calcium transport and bone calcium mobilization to 1,25-dihydroxyvitamin D_3 in thyroparathyroidectomized rats. Endocrinol. 94: 1022-1027.

56. Ghazarian JG, Jefcoate CR, Knutson JC, Orme-Johnson WH, DeLuca HF (1974) Mitochondrial cytochrome P_{450}. J. Biol. Chem.249: 3026-3033.

57. Gray RW (1981) Control of plasma 1,25-$(OH)_2$-vitamin D concentrations by calcium and phosphorus in the rat: effects of hypophysectomy. Calcif. Tissue Int. 33: 485-488.

58. Gray RW, Garthwaite TL (1985) Activation of renal 1,25-dihydroxyvitamin D_3 synthesis by phosphate deprivation: evidence

for a role for growth hormone. Endocrinol.116: 189-193.

59. Gray RW, Garthwaite TL, Phillips LS (1983) Growth hormone and triiodothyronine permit an increase in plasma $1,25(OH)_2D$ concentrations in response to dietary phosphate deprivation in hypophysectomized rats. Calcif. Tissue Int. 35: 100-106.

60. Gray TK, McAdoo T, Hatley L, Lester GE, Thierry M (1982) Fluctuation of serum concentration of 1,25-dihydroxyvitamin D_3 during the menstrual cycle. Am. J. Obstet. Gynecol. 144: 880-884.

61. Haddad P, Gascon-Barré M, Brault G, Plourde V (1986) Influence of calcium or 1,25-dihydroxyvitamin D_3 supplementation on the hepatic microsomal and in vivo metabolism of vitamin D_3 in vitamin D-depleted rats. J. Clin. Invest. 78: 1529-1537.

62. Harris CA, Sutton RAL, Seeley JF (1976) Effect of $1,25(OH)_2$ vitamin D_3 on renal electrolyte handling in the vitamin D deficient rat: dissociation of calcium and sodium excretion. Clin. Res. 24: 685A.

63. Haussler MR, McCain TA (1977). Basic and clinical concepts related to vitamin D metabolism and action. New Engl. J. Med.297: 974-983.

64. Henry HL (1985) Parathyroid hormone modulation of 25-hydroxyvitamin D_3 metabolism by cultured chick kidney cells is mimicked and enhanced by forskolin. Endocrinol. 116: 503–510.

65. Hess AF, Gutman MB (1922) Cure of infantile rickets by sunlight. JAMA 78:29.

66. Hiwatashi A, Nishii Y, Ichikawa Y (1982) Purification of cytochrome $P\text{-}450_{D1}$ (25-hydroxyvitamin D_3-1 -hydroxylase) of bovine kidney mitochondria. Biochem. Biophys. Res. Commun. 105: 320-327.

67. Horiuchi N, Suda T, Takahashi H, Shimazawa E, Ogata E (1977) In vivo evidence for the intermediary role of 3'5'-cyclic AMP in parathyroid hormone-induced stimulation of 1,25-dihydroxyvitamin D_3 synthesis in rats. Endocrinol.101: 969-974.

68. Holick MF, Garabedian M,DeLuca HF (1972). 1,25-Dihydroxycholecalciferol: metabolite of vitamin D_3 active on bone in a nephric rat. Science 176: 1146-1147.

69. Holick MF, Kleiner-Borsaller A, Schnoes HK, Kasten PM, Boyle IT, Deluca HF (1973) 1,24,25-trihydroxyvitamin D_3 a metabolite of vitamin D_3 effective on intestine. J. Biol. Chem.248: 6691-6696.

70. Howard GA, Turner RT, Sherrard DJ, Baylink DJ (1981) Human bone cells in culture metabolize 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3. J. Biol. Chem. 256: 7738-7740.

71. Huldschinsky K (1919) Heilung von Rachitis durch künstliche Höhensonne. Dtsch. Med. Wochenschr. 45: 712.

72. Kanis JA, Cundy T, Bartlett M, Smith R, Heynen G, Warner GT, Russell RGG (1978) Is 24,25-dihydroxycholecalciferol a calcium regulating hormone in man. Brit. Med. J. 1. 1382-1386.

73. Kenny AD (1976) Vitamin D metabolism: physiological regulation in egg-laying Japanese Quail. Am. J. Physiol. 230(6): 1609-1615.

74. Krabbe S, Hummer L, Christiansen C (1986) Serum levels of vitamin D metabolites and testosterone in male puberty. J. Clin. Endocrinol. Metab. 62: 503-507.

75. Kremer R, Goltzman D (1982) Parathyroid hormone stimulates mammalian renal 25-hydroxyvitamin D_3-1-hydroxylase in vitro. Endocrinol. 110: 294-296.

76. Kulkowski JA, Chan T, Martinez J, Ghararian JG (1979). Modulation of 25-hydroxyvitamin D_3 -24-hydroxylase by aminophylline: A

cytochrome P450 monooxygenase system. Biochem. Biophys. Res. Comm. 90: 50-57.

77. Kumar R (1984) Metabolism of 1,25-dihydroxyvitamin D_3. Physiol. Rev. 64(2): 478-504.

78. Kumar R, Schnoes HK, DeLuca HF (1978) Rat intestinal 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3-24-hydroxylase. J. Biol. Chem. 253: 3804-3809.

79. Lancer SR, Bowser EN, Hargis GK, Williams GA (1976) The effect of growth hormone on parathyroid function in rats. Endocrinol. 98: 1289-1293.

80. Lewin IG, Papapoulos SE, Hendy GN, Tomlinson S, O'Riordan JLH (1982) Reversible resistance to the renal action of parathyroid hormone in human vitamin D deficiency. Clin. Sci. 62, 381- 387.

81. Lidor C, Dekel S, Edelstein S (1987) The metabolism of vitamin D_3 during fracture healing in chicks. Endocrinol. 120: 389-393.

82. Madhok TC, Deluca HF (1979) Characteristics of the rat liver microsomal enzyme system converting cholecalciferol into 25-hydroxycholecalciferol: evidence for the participation of cytochrome P-450. Biochem. J. 184: 491-499.

83. Matsumoto T, Horiuchi N, Suda T, Takahashi H, Shimazawa E, Ogata E (1979) Failure to demonstrate stimulatory effect of prolactin on vitamin D metabolism in vitamin D-deficient rats. Metabol. 28: 925-927.

84. Matsumoto T, Kawanobe Y, Ezawa I, Shibuya N, Hata K, Ogata E (1986) Role of insulin in the increase in serum 1,25-dihydroxyvitamin D concentrations in response to phosphorus deprivation in streptozotocin-induced diabetic rats. Endocrinol. 118: 1440-1444.

85. McCollum EV, Simmonds N, Becker JE, Shipley PG (1922) Studies on experimental rickets.XXI. An experimental demonstration of the existence of a vitamin which promotes calcium deposition. J. Biol. Chem. 53: 293-312.

86. McDonnell DP, Mangelsdorf DJ, Pike JW, Haussler MR, O'Malley BW (1987) Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. Science 235: 1214-1217.

87. McSheehy PMJ, Chambers TJ (1986) Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. Endocrinol. 118: 824-828.

88. Miller B, Norman AW (1982) Evidence for circadian rhythms in the serum levels of the vitamin D-dependant calcium-binding protein and in the activity of the 25-hydroxycholecalciferol-1-hydroxylase in the chick. FEBS Lett. 141: 242-244.

89. Miller ML, Ghazarian JG (1981) Differential response of kidney mitochondrial calcium-regulating mixed function oxidases to thyrotoxicosis. J. Biol. Chem. 256: 5643-5645.

90. Mitchell J, Warner M, Tenenhouse A, Goltzman D (1985) Widespread reduction in guanyl nucleotides-stimulated renal adenylate cyclase activity in hypocalcaemic vitamin D-deficient rats. Am. Soc. Bone and Mineral Res. 397.

91. Morgan ET, MacGeoch C, Gustafsson J-Å (1985) Sexual differentiation of cytochrome P-450 in rat liver: evidence for a constitutive isozyme as the male-specific 16α-hydroxylase. Mol. Pharmacol. 27: 471-479.

92. Morgan ET, MacGeoch C, Gustafsson J-Å (1985) Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16α-hydroxylase cytochrome P-450 apoprotein in the rat. J. Biol. Chem. 260: 11895-11898.

93. Narbaitz R, Stumpf WE, Sar M, Huang S, DeLuca HF (1983)

Autoradiographic localization of target cells for 1,25-dihydroxyvitamin D_3 in bones from fetal rats. Calcif. Tissue Int. 35: 177-182.

94. Neims AH, Warner M, Loughnan P, Aranda JV (1976) Developmental aspects of the hepatic cytochrome P-450 monooxygenase system. Ann. Rev. Pharmacol. 16: 427-445.

95. Nijweide PJ, Burger EH, Feyen JHM (1986) Cells of bone: proliferation, differentiation, and hormonal regulation. Physiol. Rev. 66(4): 855-886.

96. Noff D, Edelstein S (1978) Vitamin D and its hydroxylated metabolites in the rat. Hormone Res. 9: 292-300.

97. Norman AW, Roth J, Orci L (1982) The vitamin D endocrine system: steroid metabolism, hormone receptors, and biological response (calcium binding proteins). Endocrine Rev. 3: 331-366.

98. Omdahl JL, (1978) Interaction of the parathyroid and 1,25-dihydroxyvitamin D_3 in the control of renal 25-hydroxyvitamin D_3 metabolism. J. of Biol. Chem. 253: 8474-8478.

99. Omdahl JL, Gray RW, Boyle IT, Knutson J, DeLuca HF (1972). Regulation of metabolism of 25-hydroxycholecalciferol by kidney

tissue in vitro by dietary calcium. Nature New Biol. 237: 63-64.

100. Ornoy AD, Goodwin D, Noff D, Edelstein S (1978). 24,25-Dihydroxyvitamin D_3 is a metabolite of vitamin D essential for bone formation. Nature 276: 517- 519.

101. Pavlovitch JH, Cournot-Witmer G, Bourdeau A, Balsan S, Fischer JA, Heynen G (1981) Suppressive effects of 24,25-dihydroxycholecalciferol on bone resorption induced by acute bilateral nephrectomy in rats. J. Clin. Invest. 68: 803-810.

102. Phillips IR, Shephard EA, Mitani F, Rabin BR (1981) Induction by phenobarbital of the mRNA for a specific variant of rat liver microsomal cytochrome P-450. Biochem. J. 196: 839-851.

103. Phillips LS, Vassilopoulo- Sellin R (1980). Somatomedins. New Engl. J. Med. 302: 371-380.

104. Pike JW, Parker JB, Haussler MR, Boass A, Toverud SU (1979) Dynamic changes in circulating 1,25-dihydroxyvitamin D during reproduction in rats. Science 204: 1427-1429.

105. Portnay GI, O'Brian JT, Bush J, Vagenakis AG, Azizi F, Arky RA, Ingbar SH, Braverman LE (1974). The effect of starvation on the concentration and binding of thyroxine and triiodothyronine in

serum and on the response to TRH. J. Clin. Endocrinol. Metab. 39: 191-194.

106. Postel-Visnay MC (1976) Binding of human groth hormone to rat liver membranes: lactogenic and somatotropic sites. FEBS Lett. 69: 137-142.

107. Pushett JB, Moranz J, Kurnick WS (1972) Evidence for a direct action of cholecalciferol and 25-hydroxycholecalciferol on the renal transport of phosphate, sodium and calcium. J. Clin. Invest. 51: 373-385.

108. Ranke MB, Stanley CA, Tenore A, Rodbard D, Bongiovanni AM, Parks JS (1976) Characterization of somatogenic and lactogenic binding sites in isolated rat hepatocytes. Endocrinol. 99: 1033-1045.

109. Richelson LS, Wahner HW, Melton LJ, Riggs BL (1984) Relative contributions of aging and estrogen deficiency to postmenopausal bone loss. New Engl. J. Med. 311: 1273-1275.

110. Robinson CJ, Spanos E, James MF, Pike JW, Haussler MR, Makeen AM, Hillyard CJ, McIntyre I (1982). Role of prolactin in vitamin D metabolism and calcium absorption during lactation in the rat. J. Endocrinol.94: 443-453.

111. Rodan GA, Martin TJ (1981) Role of osteoclasts in hormonal control of bone resorption- a hypothesis. Calcif. Tissue Int. 33: 349-351.

112. Russel J, Lettieri D, Sherwood LM (1986) Suppression by $1,25(OH)_2D_3$ of transcription of the pre-proparathyroid hormone gene. Endocrinol.119: 2864-2866.

113. Saunders A, Terry LC, Audet J, Brazeau P, Martin JB (1976) Dynamic studies of growth hormone and prolactin secretion in the female rat. Neuroendocrinol. 21: 193-203.

114. Schuetz EG, Wrighton SA, Safe SH, Guzelian PS (1986) Regulation of cytochrome P-450p by phenobarbital and phenobarbital-like inducers in adult rat hepatocytes in primary monolayer culture and in vivo. Biochemistry 25: 1124-1133.

115. Shigematsu T, Horiuchi N, Ogura Y, Miyahara T, Suda T (1986) Human parathyroid hormone inhibits renal 24-hydroxylase activity of 25-hydroxyvitamin D_3 by a mechanism involving adenosine 3',5'-monophosphate in rats. Endocrinol. 118: 1583-1589.

116. Silve CM, Hradek GT, Jones AL, Arnaud CD (1982) Parathyroid hormone receptors in intact embryonic chicken bone: characterization and cellular localization. J. Biol. Chem. 94: 379-386.

117. Simard J, Hubert J-F, Hosseinzadeh T, Labrie F (1986). Stimulation of growth hormone release and synthesis by estrogens in rat anterior pituitary cells in culture. Endocrinol. 119: 2004-2011.

118. Sömjen D, Sömjen GJ, Weisman Y, Binderman I (1982). Evidence for 24,25-dihydroxycholecalciferol receptors in long bones of newborn rats. Biochem. J. 204: 31- 36.

119. Spanos E, Barret D, Chong KT, MacIntyre I (1978) Effect of oestrogen and 1,25-dihydroxycholecalciferol on 25-hydroxycholecalciferol metabolism in primary chick kidney-cell cultures. Biochem. J. 174: 231-236.

120. Spanos E, Barrett D, MacIntyre I, Pike JW, Safilian EF, Haussler MR (1978) Effect of growth hormone on vitamin D metabolism. Nature 273: 246-247.

121. Spanos E, Brown DJ, Stevenson JC, MacIntyre I (1981) Stimulation of 1,25-dihydroxycholecalciferol production by prolactin and related peptides in intact renal cell preparations in vitro. Biochim. Biophys. Acta 672: 7-15.

122. Spanos E, Pike JW, Haussler MR, Colston KW, Evans IMA, Goldner AM, McCain TA, MacIntyre I (1976) Circulating 1,25-dihydroxyvitamin D in the chicken: enhancement by injection of prolactin and

during egg laying. Life Sci. 19: 1751-1756.

123. Spencer R, Charman M, Wilson PW, Lawson DEM (1978) The relationship between vitamin D-stimulated calcium transport and intestinal calcium-binding protein in the chicken. Biochem. J. 170: 93-102.

124. Spiegel AM, Marx SJ (1983) Chapter 12- Parathyroid hormone and vitamin D receptors. In: Clnics in Endocrinology and Metabolism- Vol.12, No.1, pp.221-241.

125. Stern P (1980) The D vitamins and bone. Pharmacol. Rev. 32: 47-80.

126. Stumpf WE, Sar M, Reid FA, Tanaka Y, DeLuca HF (1979) Target cells for 1,25-dihydroxyvitamin D_3 in intestinal tract, stomach, kidney, skin, pituitary, and parathyroid. Science 206: 1188-1190.

127. Tam CS, Heersche JNM, Jones G, Murray TM, Rasmussen H (1986). The effect of vitamin D on bone in vivo. Endocrinol. 118: 2217-2224.

128. Tanaka Y, Castillo L, DeLuca HF (1976) Control of renal vitamin D hydroxylases in birds by sex hormones. Proc. Natl. Acad. Sci. 73: 2701-2705.

129. Tanaka Y, DeLuca HF (1973). The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. Arch. Biochem. Biophys. 154: 566-574.

130. Tanaka Y, DeLuca HF (1974). Stimulation of 24,25-dihydroxyvitamin D_3 production by 1,25-dihydroxyvitamin D_3. Science 183: 1198-1200.

131. Tanaka Y, DeLuca HF (1981) Measurement of mammalian 25-hydroxyvitamin D_3-24R- and 1-hydroxylase. Proc. Natl. Acad. Sci. 78: 196-199.

132. Tanaka Y, DeLuca HF, Kobayashi Y, Taguchi T, Ikekawa N, Morisaki M (1979) Biological activity of 24,24-difluoro- 25-hydroxyvitamin D_3: effect of blocking 24-hydroxylation on the functions of vitamin D. J. Biol. Chem. 254: 7163-7167.

133. Tanaka Y, Halloran B, Schnoes HK, DeLuca HF (1979) In vitro production of 1,25-dihydroxyvitamin D_3 by rat placental tissue. Proc. Natl. Acad. Sci. 76: 5033-5035.

134. Tanaka Y, Lorenc RS, DeLuca HF (1975). The role of 1,25-dihydroxyvitamin D_3 and parathyroid hormone in the regulation of chick renal 25-hydroxyvitamin D_3-24-hydroxylase. Arch. Biochem.

Biophys. 171: 521-526.

135. Taylor AN (1981) Immunocytochemical localization of the vitamin D-induced calcium-binding protein: relocation of antigen during frozen section processing. J. Histochem. Cytochem. 29: 65-73.

136. Trechsel U, Eisman JA, Bonjour JP, Fleisch H (1979) Evidence for a calcium dependant, PTH-independant regulation of plasma 1,25-dihydroxyvitamin D in rats. In Vitamin D: Basic Research and its Clinical Application. Edited by A.W. Norman, K. Schaefer, D. von Herrath, H.G. Grigoleit, J.W. Coburn, H.F. DeLuca, E.B. Mawer and T. Suda. Walter de Gruyter, Berlin. pp.511-513.

137. Tsai HC, Wong RC, Norman AW (1973) Studies on calciferol metabolism. VIII. Evidence for a cytoplasmic receptor for 1,25-dihydroxyvitamin D_3 in the intestinal mucosa. J. Biol. Chem. 248: 5967-5975.

138. Tukey RH, Hannah RR, Negishi M, Nebert DW, Eisen HJ (1982) The Ah locus: correlation of intranuclear appearance of inducer-receptor complex with induction of cytochrome P_1-450 mRNA. Cell 31: 275-284.

139. Warner M (1982) Catalytic activity of partially purified renal 25-hydroxyvitamin D hydroxylases from vitamin D-deficient and

vitamin D-replete rats. J. Biol. Chem. 257: 12995-13000.

140. Warner M, Tenenhouse T (1985). Regulation of renal vitamin D hydroxylase activity in vitamin D deficient rats. Can. J. Physiol. Pharmacol. 63, 978-982.

141. Wasserman RH, Taylor AN (1966) Vitamin D_3-induced calcium binding protein in chick intestinal mucosa. Science 152: 791.

142. Waterman MR, Simpson ER (1985) Regulation of the biosynthesis of cytochrome P-450 involved in steroid hormone synthesis. Mol. Cell. Endocrinol. 39: 81-89.

143. Weisman Y, Eisenberg Z, Lubelski R, Spirer Z, Edelstein S, Harell A (1981). Decreased 1,25-dihydroxycholecalciferol and increased 25-hydroxy- and 24,25-dihydroxycholecalciferol in tissues of rats treated with thyroxine. Calc. Tissue Int. 33: 445-447.

144. Weisman Y, Harell A, Edelstein S, David M, Spirer Z, Golander A (1979) 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in vitro synthesis by human decidua and placenta. Nature 281: 29-31.

145. Weisman Y, Vargas A, Duckett G, Reiter E, Root AW (1978) Synthesis of 1,25-dihydroxyvitamin D_3 in the nephrectomized pregnant rat. Endocrinol. 103: 1992-1996.

146. Whitsett JA, Ho M, Tsang RC, Norman EJ, Adams KG (1981) Synthesis of 1,25-dihydroxyvitamin D_3 by human placenta in vitro. J. Clin. Endocrinol. Metab. 53: 484-488.

147. Wongsurawat N, Armbrecht HJ, Zenser TV, Forte LR, Davis BB (1984). Effects of hypophysectomy and growth hormone treatment on renal hydroxylation of 25-hydroxycholecalciferol in rats. J. Endocrinol. 101: 333-338.

148. Wrôbel J, Ghazarian JG (1982) Diurnal rhythmicity in plasma 24,25-dihydroxycholecalciferol: correlation with intestinal calcium transport and plasma minerals. Biochem. Biophys. Res. Commun. 106: 1233-1239.

149. Zerwekh JE, Breslan NA (1986) Human placental production of 1,25-dihydroxyvitamin D_3: biochemical characterization and production in normal subjects and patients with pseudohypoparathyroidism. J. Clin. Endocrinol. Metab. 62: 192-196.

150. Zile M, Bunge EC, Barsness L, Yamada S, Schnoes HK, DeLuca HF (1978) Localization of 1,25-dihydroxyvitamin D_3 in intestinal nuclei in vivo. Arch. Biochem. Biophys. 186: 15-24.

Regulation of renal 25-hydroxycholecalciferol- 24 hydroxylase in vitamin D replete rats

Daniel E.H. Afar, Alan Tenenhouse and Margaret Warner

Department of Pharmacology and Therapeutics, McGill University, 3655 Drummond str., Montreal, P.Q., Canada H3G 1Y6

This research was supported by Grant MA # 6421 from the Medical Research Council of Canada.

SUMMARY

Changes in renal mitochondrial 25-hydroxycholecalciferol 24-hydroxylase (24-OHase) activity as a function of age and during fasting and refeeding was examined.

In adult male rats (8 weeks old) the level of 24-OHase activity in renal mitochondria expressed as pmol 24,25-dihydroxycholecalciferol (24,25-DHCC) produced/g kidney/30 minutes was 103 ± 9.5. Between two and four weeks of age the activity was 40-50% of the adult control activity.

Fasting of 8 week old rats for 24 hours increased the 24-OHase activity 3 fold to 268 ± 29 pmol 24,25-DHCC/g kidney/30 min. These elevated levels of enzyme activity were maintained throughout a fasting period of up to 32 hours. Within 10 hours of refeeding of rats, which had been starved for 24 hours, the 24-OHase activity returned to normal. Fasting of 3 week-old rats also resulted in a 3 fold increase in 24-OHase activity.

Neither parathyroid hormone (PTH) nor pituitary hormones appear to mediate the increase in enzyme activity in response to fasting, since the increase in enzyme activity during fasting was not abolished by parathyroidectomy or hypophysectomy. Furthermore, no changes in serum concentrations of calcium, phosphorus and PTH were detected during starvation.

Within 10 hours of administering 1,25 dihydroxycholecalciferol (1,25 DHCC) 24-OHase activity is induced 10 fold. Food deprivation for

3 and 10 hours, however, did not change serum 1,25 DHCC concentrations.

We conclude that the 24-OHase is regulated in vitamin D replete rats as a function of age and in response to food intake. The mechanism of this regulation is unknown, but these changes in 24-hydroxylase activity suggests some physiological role for 24,25-DHCC.

INTRODUCTION

Two major metabolites of 25-hydroxycholecalciferol (25-HCC) are produced in renal mitochondria. These are 1,25-dihydroxycholecalciferol (1,25-DHCC) and 24,25-dihydroxycholecalciferol (24,25-DHCC) [10,20]. In normal rats with adequate calcium, phosphorus and vitamin D intake 24,25-DHCC is the predominant renal metabolite. During periods of calcium, phosphorus or vitamin D deprivation 1,25-DHCC predominates [2,28]. The hormonal actions of 1,25-DHCC in facilitating calcium and phosphorus absorption from the gut [3,7] and osteoclastic bone resorption [13] are well documented. The biological role of 24,25-DHCC is less clear. Its effects on bone formation [12,19,21,22,27] and PTH secretion [5,6,8,11,31] are still controversial. Putative receptors for 24,25-DHCC have been found in chondrocytes [9], fetal bone and limb bud mesenchymal cells [26], and in parathyroid glands [17].

If 24,25-DHCC has endocrine functions, its synthesis should be regulated in response to the body's need for its action. So far the only hormones known to stimulate the 24-OHase are 1,25-DHCC [29] and thyroid hormone [16]. We now report evidence that regulation of the 24-OHase activity changes with age in rats and that activity also changes as a function of food intake.

EXPERIMENTAL

Animals

Intact and parathyroidectomized male Sprague Dawley rats (14-30 days old and 8 weeks old) were purchased from Charles River (Canada) and maintained on a normal diet of Purina Rat Chow. For starvation experiments the food was removed from the rats at 10 am, while water was still available ad libitum. The rats were anaesthesized with ether and killed by exsanguination at 10 am or at 6 pm the following day. For treatment with 1,25-DHCC rats were injected with 2.5 nmoles of the the hormone at midnight and were sacrificed at 10 am the following morning.

Materials

25-hydroxy (26,27-[^{3}H]) cholecalciferol (20.6 Ci/mmol), 1,25-dihydroxy (26,27-[^{3}H]) cholecalciferol (130-180 Ci/mmol), and 24,25-dihydroxy(26,27-[^{3}H])cholecalciferol (130-180 Ci/mmol) were purchased from Amersham and were purified by high performance liquid chromatography (HPLC) prior to use. Nonradioactive 25-HCC was a gift from Hoffman- La Roche and nonradioactive 1,25-DHCC was a gift from Duphar B.V., Amsterdam, Holland.

Renal Mitochondrial 24-OHase Assay

Kidneys were homogenized in 10 volumes (w/v) of ice cold 0.25 M sucrose using a Brinkman Polytron PT10-35, fitted with a PT20 probe, for 5 seconds at speed setting No 5. Crude mitochondria were prepared by differential centrifugation. The nuclear fraction was

removed by centrifugation at 1000 x g for 10 minutes. The mitochondrial fraction was obtained by centrifuging the postnuclear fraction at 10000 x g for 10 minutes. The mitochondrial pellet was resuspended in 20 mM Hepes buffer, pH 7.4, containing 125 mM KCl, 2 mM $MgCl_2$, 10 mM succinate, 0.25 mM EDTA and 1.0 mM DTT,at a concentration of 0.5 g of original kidney weight/ml of buffer. Mitochondrial 24-OHase activity was measured according to Vieth and Fraser [32]. One ml of homogenate was incubated in the presence of 500 nM [^{3}H] 25-HCC (400-800 cpm/pmol). After incubation at 37°C for 30 minutes the reaction was stopped by addition of 5 ml of chloroform-methanol (2:1). In the original method by Vieth and Fraser [32] the incubation was performed at a lower temperature and for a shorter time period. We have determined that similar values were obtained using either incubation method. The mixture was then vortexed and the phases separated by centrifugation. The organic phase was removed and the extraction repeated.

Identification of 24,25-DHCC

The pooled organic extracts from the incubations were dried under nitrogen and vitamin D metabolites were separated by HPLC on a Zorbax Sil column (Dupont Co.), by elution with hexane-isopropanol (9:1) or on an Ultrasphere- ODS column (Beckman Instruments, Inc.), eluted with methanol- water (4:1). 24,25-DHCC and 25-HCC were identified by their co-elution with radiolabelled standards. Since the resolution between 25-HCC and 24,25-DHCC was not ideal using the Zorbax Sil sys-

tem, the identity of 24,25-DHCC eluted from this column was further confirmed using the Ultrasphere- ODS system and by sodium metaperiodate cleavage performed according to Tanaka, Lorenc and DeLuca [30]. Vitamin D metabolites were quantitated by liquid scintillation counting.

Measurement of serum calcium and phosphorus

Blood was collected from rats anaesthesized with ether. The blood samples were left to clot for 2 hours at room temperature and then centrifuged at 5000 x g for 20 minutes. Calcium and phosphorus concentrations were determined using specific diagnostic kits from the Lancer division of Sherwood Medical, St.Louis, Mo.

Serum PTH Levels

Serum PTH was determined using the midmolecule RIA kit from Immuno Nuclear Corporation, Stillwater, MN.

Serum 1,25-DHCC Concentrations

Food was removed from 8 week old rats at 6 pm and at midnight. Blood was collected 3 hours and 10 hours later respectively. Serum 1,25-DHCC concentrations were determined using a radioreceptor assay from Immuno Nuclear Corporation- Star, Stillwater, MN.

Statistical Analyses

Statistical comparisons were performed using Dunnett's test. The

results are expressed as means ± standard errors.

RESULTS

Effect of food deprivation on 24-OHase activity

Renal mitochondrial 24-OHase activity was measured in adult rats maintained on normal rat chow. The enzyme activity in fed animals was 103 ± 9.5 pmol 24,25-DHCC/g kidney/30 min. Starvation of these rats for 24 hours increased the 24-OHase activity to 268 ± 29 pmol 24,25-DHCC/g kidney/30 min. Figure 1 is an example of an HPLC elution profile of the chloroform-methanol extract after incubating [^{3}H] 25-HCC with renal mitochondria from a fed and a starved rat. The main metabolite, 24,25-DHCC, was identified by its co- elution with radioactive standard 24,25-DHCC on two HPLC systems (Fig.1a, Fig.1b). Sodium periodate cleavage resulted in the disappearance of 100% of the 24,25-DHCC peak chromatographed on an Ultrasphere-ODS column, eluted with methanol-water (4:1).

The 24-OHase activity remained high for at least 32 hours of starvation. Ten hours after refeeding fasted rats the 24-OHase activity returned to near normal levels (Table 1).

Effect of parathyroidectomy on the induction of the 24-OHase

Parathyroidectomized starved adult rats exhibited a 2-3 fold higher 24-OHase activity compared to fed parathyroidectomized rats (Figure 2). The enzyme activities were comparable to the activities seen in intact animals. Similarly, hypophysectomized rats also showed

an increase in 24-OHase activity, when fasted, which was equivalent to the induced activity seen in starved intact rats (Figure 2).

Age dependency of 24-OHase Activity

Prepubertal rats ranging in age from 2-4 weeks and receiving a normal diet had constitutively lower 24-OHase activity than adult rats (Table 2). Food deprivation for 24 hours of 22 day day old rats increased the enzyme activity to 151 ± 23 pmol 24,25-DHCC/g kidney/30 min. Although this represented a 3 fold increase in 24-OHase activity over fed prepubertal rats, it was nevertheless lower than the activity seen in fasted adult rats.

Other effects of Starvation

Starvation for 24 hours had no significant effect on serum calcium, phosphorus and PTH concentrations (Table 3).

Ten hours after treating fed adult rats with 1,25-DHCC, 24-OHase activity is induced 10 fold (Table 1). This would suggest that if an increase in serum 1,25-DHCC occurs within 10 hours of fasting, it could be responsible for the change in 24-OHase activity seen with food deprivation. However, no change in serum 1,25-DHCC is observed after 3 and 10 hours of fasting (Table 4).

DISCUSSION

Vitamin D 24-hydroxylase is a renal mitochondrial cytochrome P450 [4,15,23] whose activity is stimulated by 1,25-DHCC [29,30] and by thyroid hormone [16]. Although the physiological roles of 24,25-DHCC are still debated [18], the presence of the 24-hydroxylase in mitochondria suggests a hormonal function of this metabolite, since mitochondrial P450s are usually involved in hormone biosynthesis rather than their degradation.

The present study provides evidence for changes in the level of 24-hydroxylase activity with age and for its induction upon fasting. Elevated levels of 24,25-DHCC have been found in anorectic girls [1] and in children with type-1 diabetes [25]. These observations suggest that hormonal and/or dietary changes may be involved in the abnormal pattern of vitamin D metabolites.

We have ruled out the involvement of PTH and pituitary hormones as mediators of the response to starvation in rats, since an increase in 24-hydroxylase activity was observed in both parathyroidectomized and hypophysectomized rats upon food withdrawal.

Of the two hormones known to stimulate 24-hydroxylase activity, thyroid hormone is unlikely to be involved, since its levels are decreased during starvation of rats [24]. In the case of 1,25-DHCC, no change in the serum levels of this hormone was detected during the starvation period. Serum 1,25-DHCC measurements were only taken at

two time points. Therefore, it is possible that a temporary increase in the serum concentration of 1,25-DHCC was missed. However, if fasting is a stimulus for 1,25-DHCC production, then 1-hydroxylase activity should be measurable throughout the period of food withdrawal. At no time was any 1-hydroxylase activity measurable in the renal mitochondria of these rats.

The increase in 24-hydroxylase activity upon fasting could not be predicted on the basis of what is understood about the role of vitamin D metabolites in calcium homeostasis [10]. Withdrawal of food for 10-32 hours should have caused an elevation of PTH, osteoclastic bone resorption and an increase in 1-hydroxylase activity, in order to satisfy the calcium needs of the body. In the starved rats there was no elevation in serum levels of PTH. The midmolecule immunoassay used in these studies does not measure biologically active PTH. Therefore, it remains possible that the effective levels of PTH were elevated during starvation, but not enough to cause a detectable increase in serum 1,25-DHCC concentrations.

The biological significance of the elevated 24-OHase activity during food deprivation and the lower enzyme activity during the prepubertal period is not clear. One important function attributed to the 24-OHase is to hydroxylate 1,25-DHCC to produce 1,24,25-trihydroxycholecalciferol (1,24,25-THCC), a less active metabolite of 1,25-DHCC [14]. In effect, 1,25-DHCC is able to induce its own inactivation by stimulating 24-hydroxylase activity. The evidence presented in this study demonstrates that 24-OHase activity can be induced

without a change in serum 1,25-DHCC concentrations. The regulation of 24-OHase activity by dietary manipulations suggests that 24,25-DHCC has a biological role and is not merely a by-product of an excretory pathway for 1,25-DHCC.

FIGURE LEGENDS

Figure 1:

HPLC elution profiles of extracts of renal mitochondria from a fed and a starved adult rat, incubated with [^{3}H] 25-HCC for 30 min at 37 C. The extract was chromatographed using: (A) a Zorbax Sil column, equilibrated with hexane-isopropanol (9:1) at a flow rate of 1ml/min; (B) an Ultrasphere-ODS column equilibrated in water-methanol (4:1) at a flow rate of 1ml/min. The fractions were analyzed for radioactivity by liquid scintillation counting. The retention time of standard vitamin D metabolites is indicated at the top of the figures.

Figure 2.

Renal mitochondrial 24-OHase activity in parathyroidectomized, hypophysectomized and intact adult rats. There were 3-17 rats per group. The results were expressed as % activity of fasted intact adult rats. * $P < 0.05$ compared to fasted intact adult rats.

HYPOX= hypophysectomized.

PTX= parathyroidectomized.

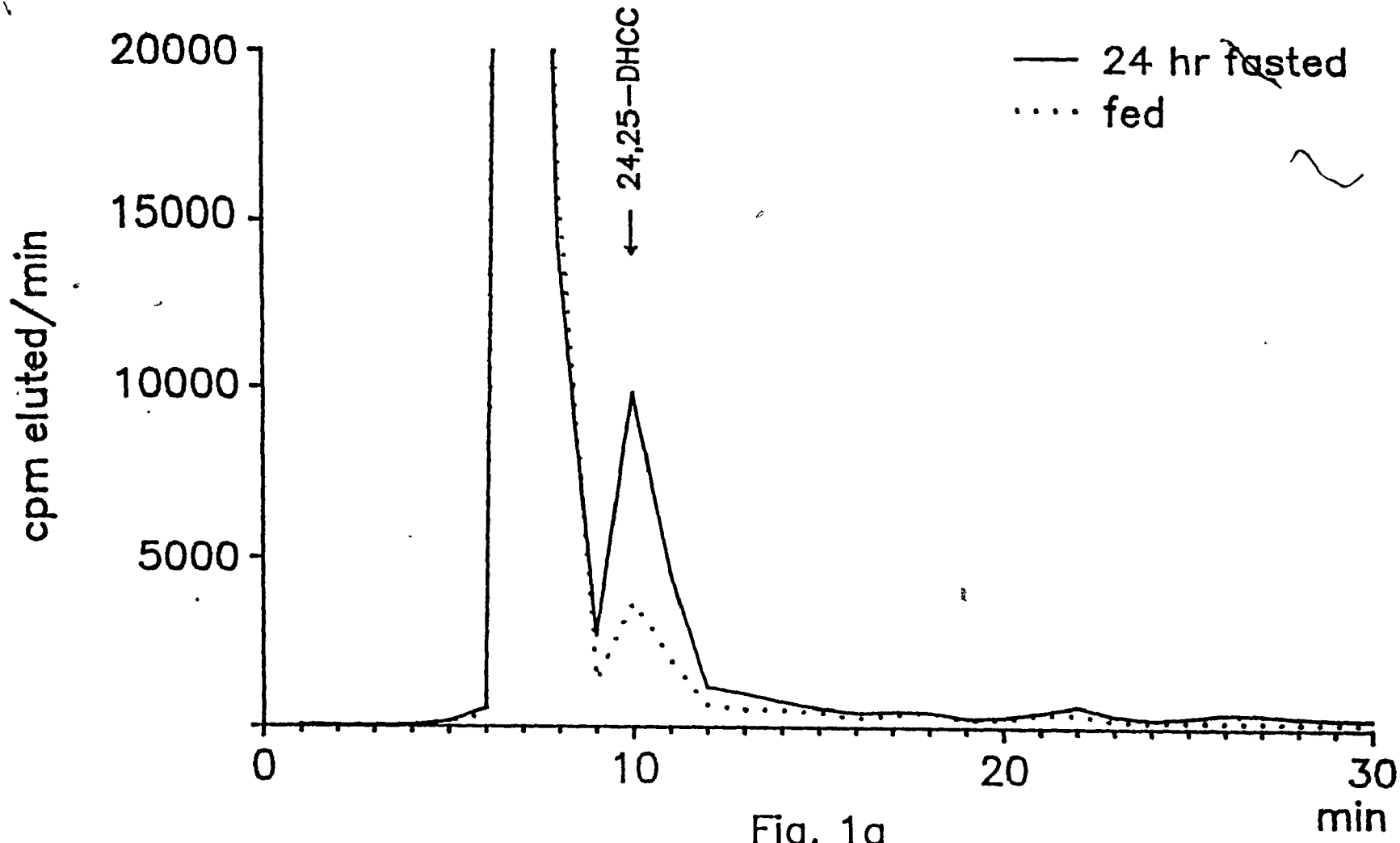
20000
15000
10000
5000
cpm eluted/min
24,25-DHCC
24 hr fasted
fed
0
10
20
30
min

Fig. 1a

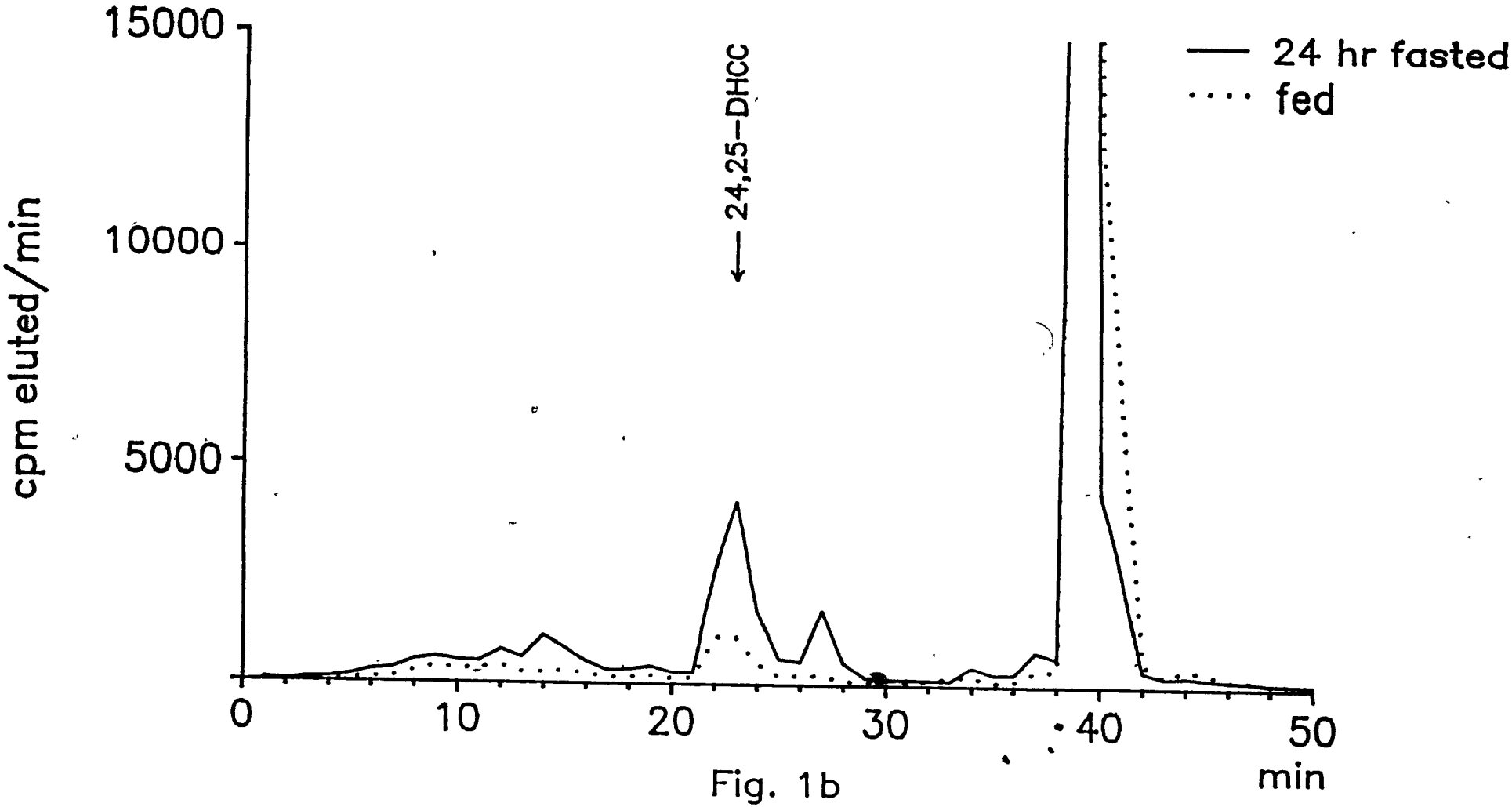
15000
10000
5000
cpm eluted/min
24,25-DHCC
24 hr fasted
fed
0
10
20
30
40
50
min

Fig. 1b

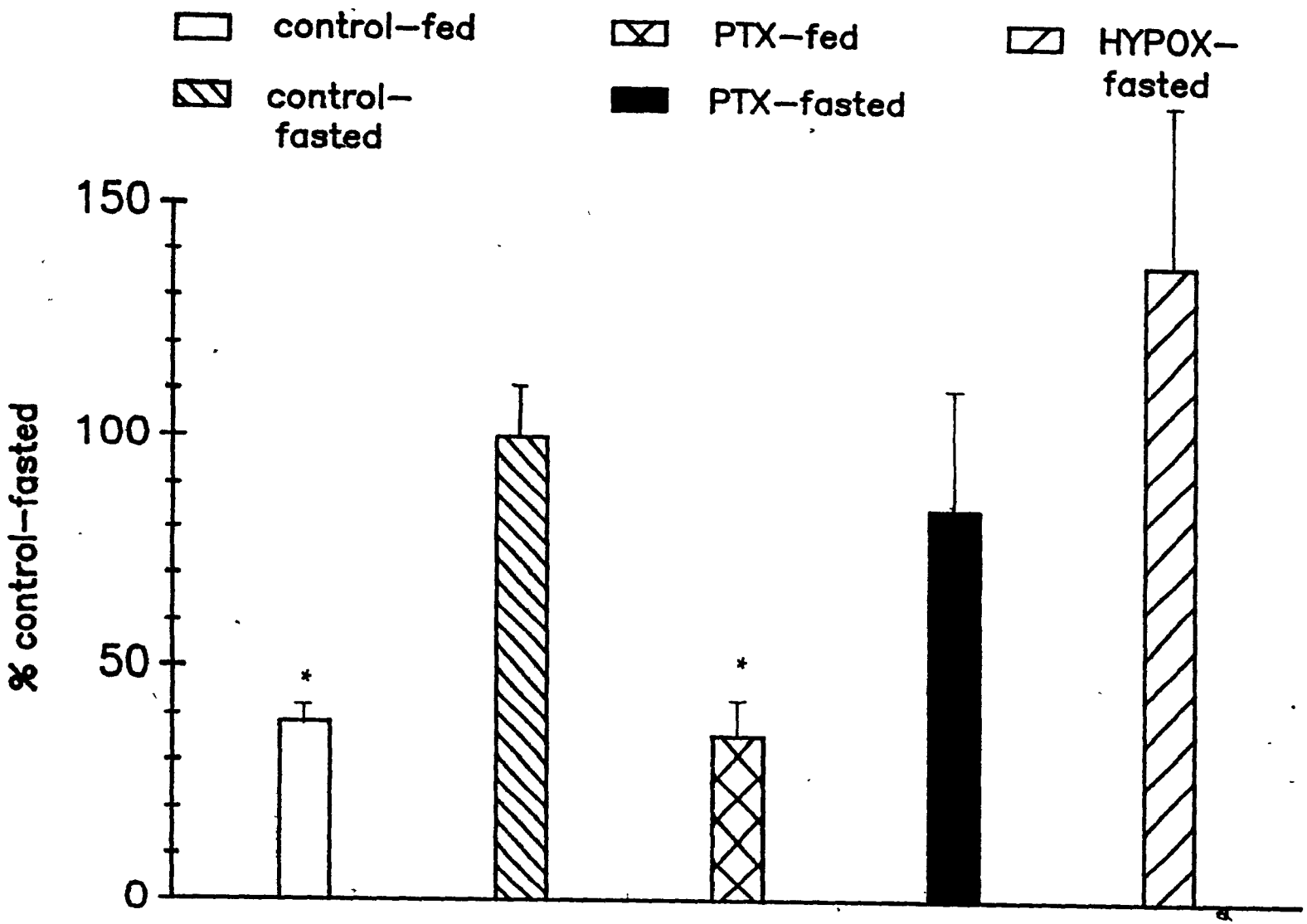

Figure 2: Effect of hypophysectomy and parathyroidectomy on the induction of 24–hydroxylase activity by fasting.

TABLE 1

Effect of starvation on 24-OHase activity in renal mitochondria of 8 week old rats

	pmol 24,25 DHCC produced/g kidney/30 min	
control fed	103 ± 9.5	(17)
starved 24 hr.	268 ± 29	(12) *
starved 32 hr.	287 ± 36	(5) *
10 hr. post refeeding of 24 hr. starved	92 ± 18	(5)
10 hr. post injection of 1,25 DHCC (2.5 nmoles)	978 ± 46	(4) *

* $P < 0.05$ compared to control fed rats.

TABLE 2

Effect of age and fasting on renal mitochondrial 24-OHase activity in rats

	pmol 24,25-DHCC produced/g kidney/30 min	
	fed	24 hr. fasted
14 day old	42 ± 5.0 (4)	ND
22 day old	46 ± 9.3 (11)	151 ± 23 (5)*
30 day old	50 ± 15 (5)	ND
56 day old	103 ± 9.5 (17)	268 ± 29 (12)*

* $P < 0.05$ compared to 22 day old fed rats.

ND= not determined.

TABLE 3:

Effects of starvation on plasma levels of calcium, phosphorus and PTH in 8 week old rats

	control fed	starved 24 hr	
Ca(mg/dl)	9.07 ± 0.19 (18)	9.09 ± 0.22 (9)	NS
P_I(mg/dl)	8.10 ± 0.22 (18)	8.27 ± 0.23 (10)	NS
PTH(pmol/l)	48 ± 6.5 (4)	53 ± 5.0 (4)	NS

NS = not significantly different from control fed.

TABLE 4

Effect of fasting on serum 1,25 DHCC concentration in 8 week old rats

	pg 1,25-DHCC/ml	
control fed	122 ± 6.9 (5)	
3 hr. fasted	111 ± 7.2 (5)	NS
10 hr. fasted	131 ± 15 (5)	NS

NS= not significantly different from control fed.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Majid Gharemani for doing the serum 1,25-DHCC extractions and assisting in the serum 1,25-DHCC assay.

REFERENCES

1. Aarskog D, Aksnes L, Markestad T (1986) Plasma concentrations of vitamin D metabolites in pubertal girls with anorexia nervosa. Acta Endocrinol. Suppl. 279 (113). 458-467.

2. Boyle IT, Gray RW, DeLuca HF (1971). Regulation by calcium of In vivo synthesis of 1,25 - dihydroxycholecalciferol and 21,25-dihydroxycholecalciferol. Proc. Nat. Acad Sci.(USA) 68 2131 - 2134.

3. Boyle IT, Miravet L, Gray RW, Holick MF, De Luca HF (1972) The response of intestinal calcium transport to 25-hydroxy and 1,25 - dihydroxyvitamin D in nephrectomized rats Endocrinol. 90: 605-608.

4. Burgos-Trinidad M, Brown AJ, De Luca HF (1986) Solubilization and reconstitution of chick renal mitochondrial 25-hydroxyvitamin D_3-24-hydroxylase. Biochem. 25: 2692-2696.

5. Canterbury JM, Lerman S, Claylin AJ, Henry H, Norman A, Russ E (1977) Inhibition of parathyroid hormone secretion by 25-hydroxycholecalciferol and 24,25 dihydroxy cholecalciferol in the dog. J. Clin. Invest. 61: 1375- 1383.

6. Care AD, Bates RFL, Pickard DW, Peakock M, Tomlinson S, O'Riordan JLH, Mawer EB, Taylor CM, DeLuca HF, Norman AW (1976).The effects of vitamin D metabolites and their analogues on the secretion of parathyroid hormone. Calcif. Tissue Res. 21: 142- 146.

7. Chen TC, Castillo L, Korycka-Dahl M, DeLuca HF (1974). Role of vitamin D metabolites in phosphate transport of rat intestine. J. Nutr. 104. 1056-1060.

8. Chertow BS, Baker GR, Henry H, Norman AW (1980). The Effects of vitamin D metabolites on bovine parathyroid hormone release in vitro. Am. J. Physiol. 238: 384- 388.

9. Corvol M, Ulmann A, Garabedian M (1980). Specific nuclear uptake of 24,25-dihydroxycholecalciferol a vitamin D_3 metabolite biologically active in cartilage. FEBS Lett. 116: 273- 276.

10. DeLuca HF, Schnoes HJ (1983). Vitamin D Recent Advances. Ann. Rev. Biochem. 52: 411-439.

11. Dietel M, Dorn G, Montz R, Altenahr E (1979). Influence of vitamin D_3, 1,25 dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 on parathyroid hormone secretion, adenosine 3',5'-monophosphate release, and ultrastructure of parathyroid glands in organ cul-

ture. Endocrinol.. 105: 237- 245.

12. Endo H, Kiyoki M, Kawashima K, Naguchi T, Hashimoto Y (1980). Vitamin D_3 Metabolites and PTH synergistically stimulate bone formation. Nature 286: 262- 264.

13. Holick MF, Garabedian M,DeLuca HF (1973). 1,25-Dihydroxycholecalciferol: metabolite of vitamin D_3 active on bone in a nephric rat. Science 176: 1146-1147.

14. Holick MF, Kleiner-Borsaller A, Schnoes HK, Kasten PM, Boyle IT, Deluca HF (1973) 1,24,25-trihydroxyvitamin D_3 a metabolite of vitamin D_3 effective on intestine. J. Biol. Chem.248: 6691-6696.

15. Kulkowski JA, Chan T, Martinez J, Ghararian JG (1979). Modulation of 25-hydroxyvitamin D_3 -24-hydroxylase by aminophylline: A cytochrome P450 monooxygenase system. Biochem. Biophys. Res. Comm. 90: 50-57.

16. Miller ML, Ghazarian JG (1981) Differential response of kidney mitochondrial calcium-regulating mixed function oxidases to thyrotoxicosis. J. Biol. Chem. 256: 5643-5645.

17. Merke J, Norman AW (1981). Studies on the mode of action of calciferol. Evidence for a 24R,25$(OH)_2$-vitamin D_3 receptor in the

parathyroid gland of the rachitic chick. Biochem. Biophys. Res. Comm. 100: 551- 558.

18. Norman AW, Roth J, Orci L (1982) The vitamin D endocrine system: steroid metabolism, hormone receptors, and biological response (calcium binding proteins). Endocrine Rev. 3: 331-366.

19. Okamota SY, Tanaka HJ, DeLuca S, Yamada S, Takayama H (1981). 24,24-Difluoro-25 dihydroxyvitamin D enhanced mineralization in rats compared with 24-hydroxyvitamin D_3 and vitamin D_3. Arch. Biochem. Biophys. 206: 8- 14.

20. Omdahl JL, Gray RW, Boyle IT, Knutson J, DeLuca HF (1972). Regulation of metabolism of 25-hydroxycholecalciferol by kidney tissue in vitro by dietary calcium. Nature New Biology 237: 63-64.

21. Ornoy AD, Goodwin D, Noff D, Edelstein S (1978). 24,25-Dihydroxyvitamin D_3 is a metabolite of vitamin D essential for bone formation. Nature 276: 517- 519.

22. Parfitt AM, Mathews CH, Brommage R, Jarnagin K, DeLuca HF (1984). Calcitriol but no other metabolite of vitamin D is essential for normal bone growth and development in the rat. J. Clin. Invest. 73: 576- 586.

23. Pedersen JI, Shobaki HH, Holmberg I, Bergseth S, Björkhem I (1983) 25-Hydroxyvitamin D_3-24-hydroxylase in rat kidney mitochondria. J. Biol. Chem. 258: 742-746.

24. Portnay GI, O'Brian JT, Bush J, Vagenakis AG, Azizi F, Arky RA, Ingbar SH, Braverman LE (1974). The effect of starvation on the concentration and binding of thyroxine and triiodothyronine in serum and on the response to TRH. J. Clin. Endocrinol. Metab. 39: 191-194.

25. Rodland O, Markestad T, Aksnes L, Aarskog D (1985) Plasma concentrations of vitamin D during puberty of diabetic children. Diabetologica 28: 663-666.

26. Sömjen D, Sömjen GJ, Weisman Y, Binderman I (1982). Evidence for 24,25-dihydroxycholecalciferol receptors in long bones of newborn rats. Biochem. J. 204: 31- 36.

27. Tam CS, Heersche JNM, Jones G, Murray TM, Rasmussen H (1986). The effect of vitamin D on bone in vivo. Endocrinol. 118: 2217-2224.

28. Tanaka Y, DeLuca HF (1973). The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. Arch. Biochem. Biophys. 154:

566-574.

29. Tanaka Y, DeLuca HF (1974). Stimulation of 24,25-dihydroxyvitamin D_3 production by 1,25-dihydroxyvitamin D_3. Science 183: 1198-1200.

30. Tanaka Y, Lorenc RS, DeLuca HF (1975). The role of 1,25-dihydroxyvitamin D_3 and parathyroid hormone in the regulation of chick renal 25-hydroxyvitamin D_3-24-hydroxylase. Arch. Biochem. Biophys. 171: 521-526.

31. Tanaka Y, DeLuca HF, Ghazarian JG, Hargis GK, Williams GA (1979), Effect of vitamin D and its metabolites on serum parathyroid hormone levels in the rat. Mineral Electrol. Metab. 2: 20.

32. Vieth R, Fraser D (1979) Kinetic behaviour of 25-hydroxyvitamin D-1-hydroxylase and 24-hydroxylase in rat kidney mitochondria. J. Biol. Chem. 254: 12455-12460.

7

Induction of renal mitochondrial 25-hydroxycholecalciferol 1-hydroxylase during lactation

Daniel E.H. Afar and Margaret Warner

Department of Pharmacology & Therapeutics, McGill University, 3655 Drummond str., Montreal, P.Q., Canada H3G 1Y6

This research was supported by Grant MA # 6421 from the Medical Research Council of Canada.

SUMMARY

Female rats maintained from weaning on a vitamin D-deficient diet have measurable levels of 25-hydroxycholecalciferol 1-hydroxylase (1-OHase) in their renal mitochondria (71 ± 9 pmol 1,25-dihydroxycholecalciferol produced [1,25-DHCC]/g kidney/30 min.) and no detectable 24-hydroxylase activity at 5 weeks of age. In the adult females (8-30 weeks old) the 1-OHase activity is variable with a mean of 121 ± 29 pmol 1,25-DHCC/g kidney/30 min. Lactating females exhibited consistently high 1-OHase activity (355 ± 37 pmol/g kidney/30 min). Twenty-four hours after the removal of the litters 1-OHase activity was still elevated (340 ± 59 pmol/g kidney/30 min).

Serum PTH levels were 3-15 fold elevated in all the adult vitamin D-deficient rats, regardless of lactational status. Five-week old females only exhibited a slight elevation in serum PTH levels. Urinary cAMP excretion was increased in all the vitamin D-deficient rats. This indicates that all the vitamin D-deficient rats were PTH sensitive.

We conclude that 1-OHase activity becomes measurable in vitamin D-deficiency due to hyperparathyroidism and renal PTH responsiveness. Lactation induces a high level of 1-OHase activity. It could not be determined whether the induced 1-OHase activity in lactating females was due to PTH alone, or the combined effect of PTH and some other factor present during lactation.

INTRODUCTION

One of the major functions of 1,25-dihydroxycholecalciferol (1,25-DHCC) is the maintenance of calcium homeostasis through regulation of intestinal calcium absorption [3,8] and calcium mobilization from bone [15]. The enzyme involved in the formation of 1,25-DHCC, the 25-hydroxycholecalciferol 1-hydroxylase (1-OHase) is a renal mitochondrial cytochrome P450 [5]. The 1-OHase activity is stimulated by hypocalcaemia [12], hypophosphataemia [7,17], parathyroid hormone (PTH) [4], prolactin (PRL) [13,15], growth hormone (GH) [14] and estrogen [2]. Hypocalcaemia can induce 1-OHase activity either via PTH or in the absence of PTH [18]. The effects of PTH on 1-OHase activity can be mimicked by cAMP [9] and forskolin [1], a direct activator of adenylate cyclase. The induction of the enzyme activity by hypophosphataemia has been shown to require GH [6]. Although the mechanisms by which these factors increase 1-OHase activity are unknown, there are likely to be several levels of regulation and several mediators of the action of the different hormones.

It has been shown that PTH resistance develops in older second generation vitamin D-deficient male rats, and that this is largely responsible for the disappearance of the 1-OHase activity after puberty [20]. In this study we report on the PTH sensitivity, as measured by urinary cAMP excretion, and induction of 1-OHase activity in first generation vitamin D-deficient female rats as a function of age and lactation.

EXPERIMENTAL

Animals

Female Sprague Dawley rats (Charles River, Canada) were weaned on a vitamin D-deficient diet , containing 0.4% calcium and 0.6% phosphorus. The rats were anaesthesized with ether and killed by exsanguination.

Materials

25-hydroxy (26,27-[^{3}H]) cholecalciferol (20.6 Ci/mmol), 1,25-dihydroxy (26,27-[^{3}H]) cholecalciferol (130-180 Ci/mmol), and 24,25-dihydroxy (26,27-[^{3}H]) cholecalciferol (130-180 Ci/mmol) were purchased from Amersham and were purified by high performance liquid chromatography (HPLC) prior to use. Nonradioactive 25-hydroxycholecalciferol was a gift from Hoffman-La Roche.

Renal mitochondrial 1-OHase assay

Kidneys were homogenized in 10 volumes (w/v) of ice cold 0.25 M sucrose using a Brinkman Polytron PT10-35, fitted with a PT-20 probe, for 5 seconds at speed setting No 5. Crude mitochondria were prepared by differential centrifugation. The nuclear fraction was removed by centrifugation at 1000 x g for 10 minutes. The mitochondrial fraction was obtained by centrifuging the postnuclear

fraction at 10000 x g for 10 minutes. The mitochondrial pellet was resuspended in 20 mM HEPES buffer, pH 7.4, containing 125 mM KCL, 2 mM $MgCl_2$, 10 mM succinate, 0.25 mM EDTA and 0.4 mM DTT. Mitochondrial 1-OHase activity was measured by a modification of the method of Vieth and Fraser [21]. Five ml of buffer, containing 0.5-0.75 mg mitochondrial protein, were incubated in the presence of 20 nM [^{3}H] 25-HCC (400-800 cpm/pmol). We have found that this modification of the assay renders it more sensitive for the measurement of 1-OHase activity. After incubation at 37° C for 30 minutes the reaction was stopped by addition of 5 ml of chloroform-methanol (2:1). The mixture was vortexed and the phases separated by centrifugation. The organic phase was removed and the extraction repeated.

Identification of 1,25-DHCC

The pooled organic extracts from the incubations were dried under nitrogen and vitamin D metabolites were separated by HPLC on a Zorbax Sil column (Dupont Co.), by elution with Hexane-isopropanol (9:1). 1,25-DHCC and 25-HCC were identified by their co-elution with radiolabelled standards. Vitamin D metabolites were quantitated by liquid scintillation counting.

Determination of serum concentrations of calcium, phosphorus and PTH and urinary excretion of cAMP

Urinary excretion of cAMP was measured in urine collected from rats anaesthesized with ether. The flanged end of a polyethylene

catheter was tied into the urinary bladder such that residual volume was minimal. Urine was collected in the catheter over a period of 30 minutes for the analysis of cAMP and creatinine. At the end of the experiment blood was collected for the determination of serum calcium, phosphorus and PTH. Cyclic AMP excretion is expressed as nmoles/mg creatinine. Calcium, phosphorus and creatinine concentrations were determined using specific diagnostic kits from the Lancer division of Sherwood Medical, St. Louis, Mo. Urinary cAMP was measured using a radioimmunoassay (RIA) kit (Amersham). Serum PTH was determined using the midmolecule RIA kit from Immuno Nuclear Corporation, Stillwater, MN.

Statistical analyses

Statistical comparisons were performed using Dunnett's test. The results are expressed as means ± standard errors.

RESULTS

Effect of age and lactation on 1-OHase activity

Renal mitochondrial 1-OHase activity was determined in female rats that were weaned on a vitamin D-deficient, low calcium diet. Within 2 weeks on the diet 1-OHase activity became measurable and was high compared to normal rats (fig.1). Adult females, that have been on the diet for 8-30 weeks, had a highly variable 1-OHase activity,

with a range from 25.0-465 pmol 1,25-DHCC produced/g kidney/30 minutes. After 8 weeks on the diet there was no correlation between the age of the animal and 1-OHase activity. In the same age group, lactating rats showed consistently high 1-OHase activity, with a range from 190-830 pmol 1,25-DHCC produced/g kidney/30 min. In lactating rats, there was no correlation between 1-OHase activity and age of the animal or the length of the period of lactation. Twenty-four hours after the removal of the litters of rats that have been lactating for one week, 1-OHase activity was still elevated (fig.1).

Serum levels of calcium, phosphorus and PTH

The young vitamin D-deficient females where normocalcaemic, normophosphataemic and had serum levels of PTH comparable to vitamin D-replete rats (table 1). The adult rats, whether lactating or non-lactating were all hypocalcaemic, hypophosphataemic and hyperparathyroid. Twenty-four hours after litter removal the serum PTH levels of the mothers increased 3-5 fold over values obtained for nursing mothers (table 1). The cause of this increase in serum PTH is unknown.

Urinary excretion of cAMP

Urinary cAMP excretion is an index of PTH action on the kidney [10]. Urinary cAMP excretion was elevated in all the rats, regardless of the age and whether or not they were lactating (fig.2). This indicates that all the vitamin D-deficient rats studied were PTH respon-

sive.

DISCUSSION

We have demonstrated that in first generation vitamin D-deficient (G_1) females 1-OHase activity is is induced within 2 weeks after weaning. Second generation vitamin D-deficient (G_2) rats of the same age exhibit 3-4 fold higher 1-OHase activity [20]. In contrast to the hypocalcaemic, hyperparathyroid G_2 rats, the pubertal G_1 rats were normocalcaemic and had only slightly elevated levels of PTH in the blood. Since the midmolecule RIA used in this study does not measure biologically active PTH, it is likely that the effective levels of PTH were significantly elevated after 2 weeks on the diet, especially in light of the increased urinary cAMP excretion in these animals.

During lactation the level of 1-OHase activity is increased in renal mitochondria from G_1 female rats. Although 1-OHase activity can also be high in adult non-lactating females, the enzyme activity is highly variable and in some cases it can be close to non-detectable. Lactating rats had a consistently higher level of 1-OHase activity and neither serum PTH nor urinary excretion of cAMP was significantly different between lactating and non-lactating rats. If these PTH levels are indicative of active hormone, then this is suggestive evidence of some other factor in lactation being responsible for the induction of

the enzyme activity. Removal of suckling pups, which would decrease milk production, might eliminate such a lactational factor. Within 24 hours after litter removal from lactating females 1-OHase activity was still elevated. However, it is possible that the 1-OHase activity declines only slowly after a stimulant is removed and that 24 hours is not enough time for the enzyme activity to decrease. Therefore, the cause of 1-OHase induction during lactation remains unresolved.

It has been shown previously that G_2 rats, at an age of 9 weeks and older, develop PTH resistance as measured by the urinary excretion of cAMP [20] and by changes in the regulatory subunit of renal adenylate cyclase [11]. In G_1 rats PTH resistance did not develop in any of the rats studied. These G_2 rats are, therefore, the ideal tool to determine other factors can stimulate 1-OHase activity independently of PTH, or whether other factors can influence 1-OHase activity by altering PTH resistance.

It is still unknown by what molecular mechanisms 1-OHase activity is induced during vitamin D-deficiency and lactation. The renal mitochondrial cytochrome P450 content does not change when rats are made vitamin D-deficient [19]. It is, however, unknown what proportion of renal mitochondrial P450 is 1-OHase enzyme. To date, an increase in the amount of 1-OHase has not been demonstrated yet and awaits immunological quantitation of the enzyme.

We conclude that 1-OHase activity in G_1 females is induced by vitamin D-deficiency and increases as a function of lactation. The induction of 1-OHase activity in vitamin D-deficient rats is due to the

development of hyperparathyroidism. The cause of the elevated levels of 1-OHase activity during lactation, however, was not determined.

FIGURE LEGENDS

Figure 1:

Renal mitochondrial 1-OHase activity in female vitamin D-deficient rats. There were 4-20 rats per group. The young rats were 5 weeks old. The adult rats (non-lactating, lactating and post-litter removal) were 8-30 weeks old. Vitamin D-replete rats had no detectable 1-OHase activity.

Figure 2:

Urinary excretion of cAMP in control and vitamin D-deficient rats. The control rats were 8 week old males that were weaned on normal rat chow. There were 3-6 rats per group. * $P < 0.05$ compared to control rats.

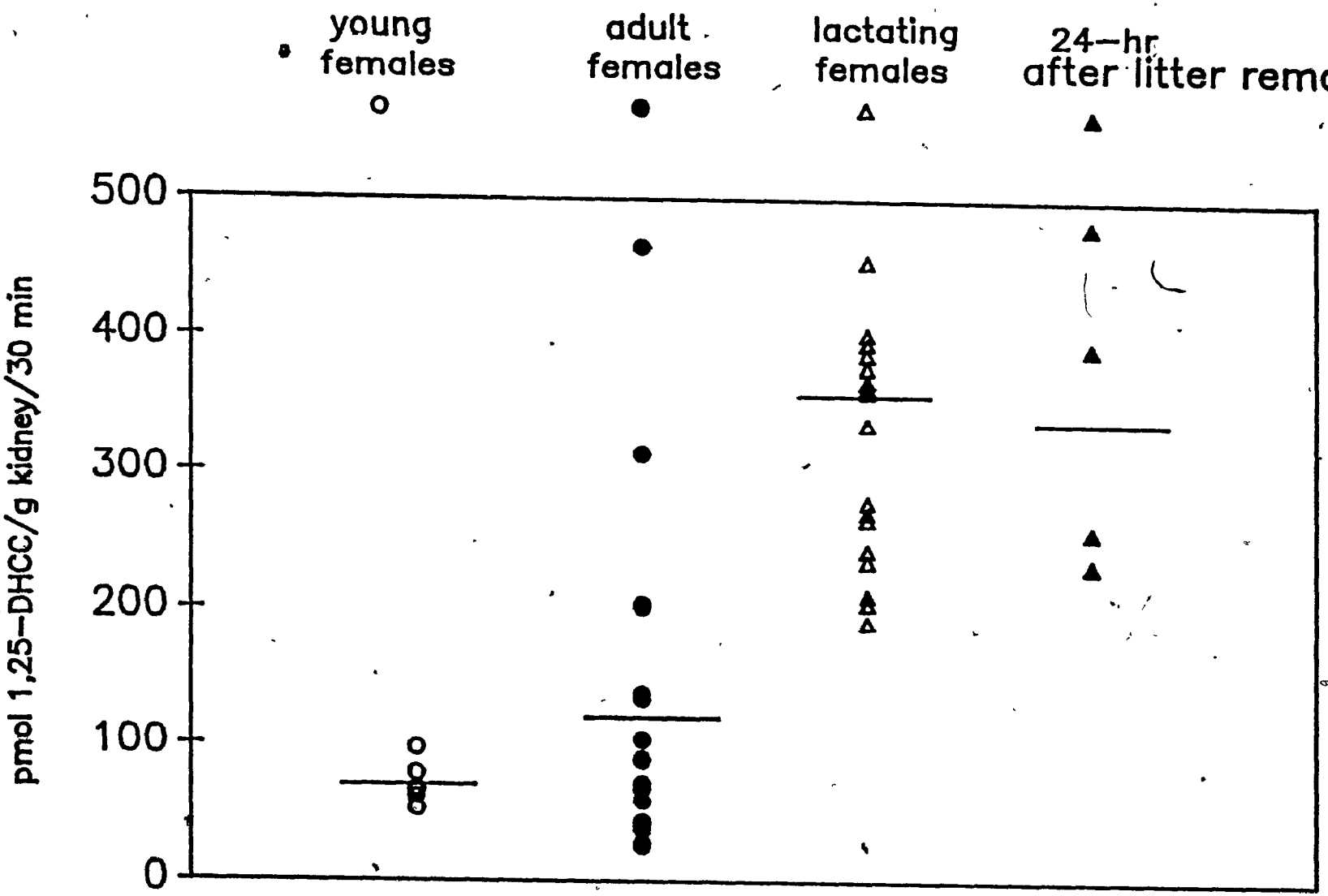

Figure 1: Renal mitochondrial 1–hydroxylase activity in female vitamin D–deficient rats.

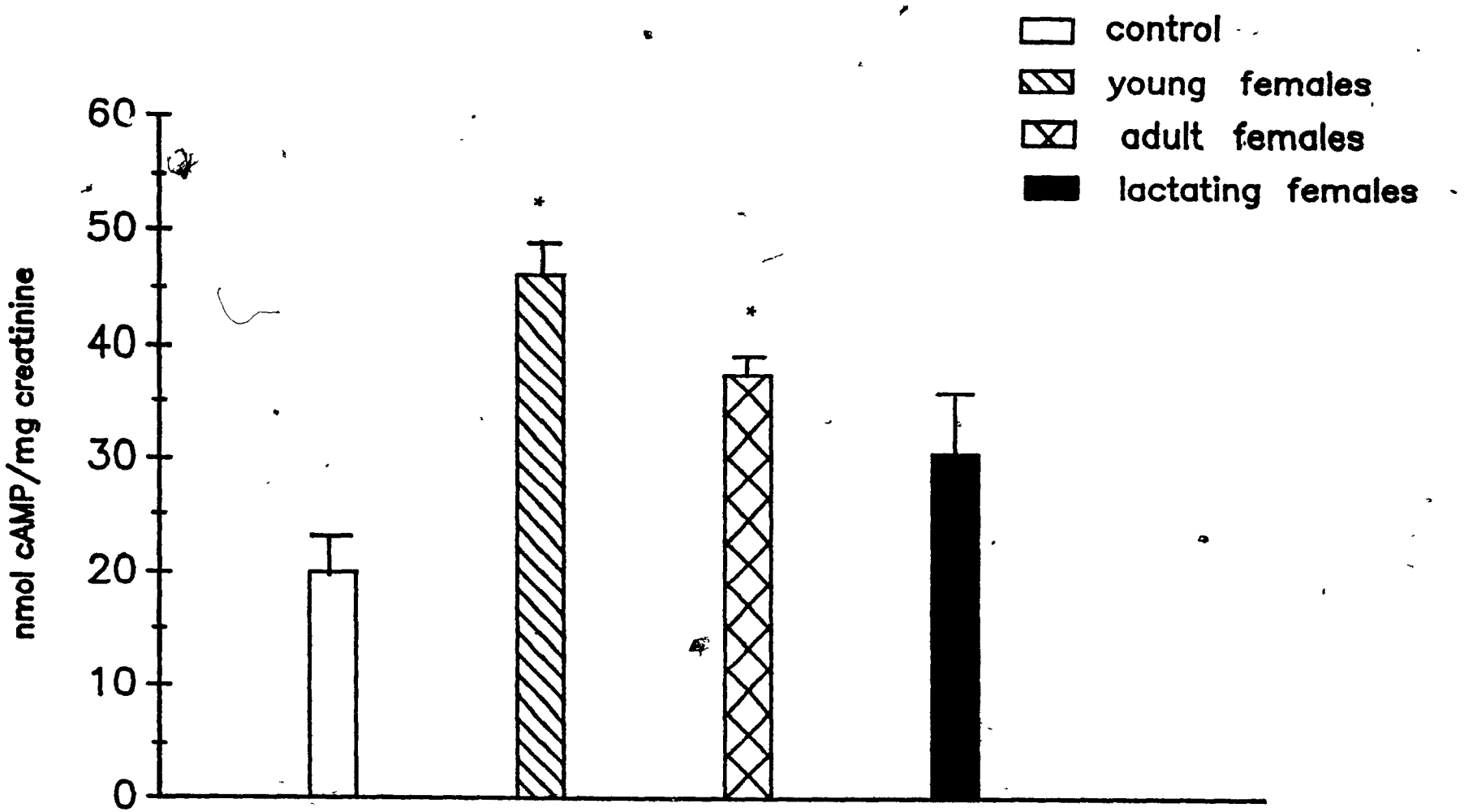

Figure 2: Urinary excretion of cAMP in vitamin D–deficient and control rats.

TABLE 1

Serum concentrations of calcium, phosphorus and PTH in vitamin D-deficient (D^-) female rats

	Ca(mg/dl)	P_i(mg/dl)	PTH(pmol/l)
control	9.07 ± 0.19	9.09 ± 0.22	48 ± 6.5
young D^-	9.50 ± 0.23	9.57 ± 0.43	61 ± 6.6
adult D^-	7.02 ± 0.41 *	4.68 ± 0.34 *	179 ± 25 *
lactating D^-	5.84 ± 0.42 *	6.71 ± 0.48 *	216 ± 19 *
lactating D^- -24hr ppr***	ND	ND	677 ± 119 **

There were 4-18 rats per group. ND= not determined. * $P < 0.05$ compared to control rats. ** $P < 0.05$ compared to lactating D^- rats. *** ppr= post-pup removal.

REFERENCES

1. Armbrecht HJ, Forte LR, Wongsurawat N, Zenser TV, Davis BB (1984) Forskolin increases 1,25-dihydroxyvitamin D_3 production by rat renal slices in vitro. Endocrinol. 114: 644-649.

2. Baksi SN, Kenny AD (1977) Vitamin D_3 metabolism in immature Japanese Quail: effects of ovarian hormones. Endocrinol. 101 1216-1220.

3. DeLuca HF, Schnoes HJ (1983). Vitamin D Recent Advances. Ann. Rev. Biochem. 52, 411-439.

4. Fraser DR, Kodicek E (1973) Regulation of 25-hydroxycholecalciferol-1-hydroxylase activity in kidney by parathyroid hormone. Nature New Biol. 241: 163-166.

5. Ghazarian JG, Jefcoate CR, Knutson JC, Orme-Johnson WH, DeLuca HF (1974) Mitochondrial cytochrome P450. J. Biol. Chem.249 3026-3033.

6. Gray RW, Garthwaite TL (1985) Activation of renal 1,25-dihydroxyvitamin D_3 synthesis by phosphate deprivation: evidence for a role for growth hormone. Endocrinol.116: 189-193.

7. Gray RW, Nappli JL (1983) Dietary phosphate deprivation increases 1,25-dihydroxyvitamin D_3 synthesis in rat kidney in vitro. J. Biol. Chem.258: 1152-1155.

8. Haussler MR, McCain TA (1977). Basic and clinical concepts related to vitamin D metabolism and action. New Engl. J. Med.297: 974-983.

9. Horiuchi N, Suda T, Takahashi H, Shimazawa E, Ogata E (1977) In vivo evidence for the intermediary role of 3'5-cyclic AMP in parathyroid hormone-induced stimulation of 1,25-dihydroxyvitamin D_3 synthesis in rats. Endocrinol.101: 969-974.

10. Lewin IG, Papapoulos SE, Hendy GN, Tomlinson S, O'Riordan JLH (1982) Reversible resistance to the renal action of parathyroid hormone in human vitamin D deficiency. Clin. Sci. 62, 381- 387.

11. Mitchell J, Warner M, Tenenhouse A, Goltzman D (1985) Widespread reduction in guanyl nucleotides-stimulated renal adenylate cyclase activity in hypocalcaemic vitamin D-deficient rats. Am. Soc. Bone and Mineral Res. 397.

12. Omdahl JL, Gray RW, Boyle IT, Knutson J, DeLuca HF (1972). Regulation of metabolism of 25-hydroxycholecalciferol by kidney

tissue in vitro by dietary calcium. Nature New Biology 237: 63-64.

13. Robinson CJ, Spanos E, James MF, Pike JW, Haussler MR, Makeen AM, Hillyard CJ, McIntyre I (1982). Role of prolactin in vitamin D metabolism and calcium absorption during lactation in the rat. J. Endocrinol.94: 443-453.

14. Spanos E, Barrett D, MacIntyre I, Pike JW, Safilian EF, Haussler MR (1978) Effect of growth hormone on vitamin D metabolism. Nature 273: 246-247.

15. Spanos E, Colston KW, Evans IMS, Galante LS, Macauly SJ, MacIntyre I (1976) Effect of prolactin on vitamin D metabolism. Mol. Cell. Endocrinol. 5: 163-167.

16. Stern P (1980) The D vitamins and bone. Pharmacol. Rev. 32:47-80.

17. Tanaka Y, DeLuca HF (1973). The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. Arch. Biochem. Biophys. 154, 566-574.

18. Trechsel U, Eisman JA, Bonjour JP, Fleisch H (1979) Evidence for a calcium dependant, PTH-independant regulation of plasma 1,25-dihydroxyvitamin D in rats. In Vitamin D: Basic Research and its

Clinical Application. Edited by A.W. Norman, K. Schaefer, D. von Herrath, H.G. Grigoleit, J.W. Coburn, H.F. DeLuca, E.B. Mawer and T. Suda. Walter de Gruyter, Berlin. pp.511-513.

19. Warner M (1982) Catalytic activity of partially purified renal 25-hydroxyvitamin D hydroxylases from vitamin D-deficient and vitamin D-replete rats. J. Biol. Chem. 257: 12995-13000.

20. Warner M, Tenenhouse T (1985). Regulation of renal vitamin D hydroxylase activity in vitamin D deficient rats. Can. J. Physiol. Pharmacol. 63, 978-982.

21. Vieth R, Fraser D (1979) Kinetic behaviour of 25-hydroxyvitamin D-1-hydroxylase and 24-hydroxylase in rat kidney mitochondria. J. Biol. Chem. 254, 12455-12460.