1,25-Dihydroxyvitamin D and the Free Hormone Hypothesis

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1,25-Dihydroxyvitamin D and the free hormone hypothesis

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CHAPTER 1

General introduction

1.1 HISTORICAL BACKGROUND

The history of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) probably started about 500 million years ago, when the molecule was produced in ocean-dwelling plankton [Holick 1989]. Although its function is not yet clear, it may have acted as a photon sink to protect the DNA from damaging UV radiation, since the UV absorption spectrum of vitamin D overlaps that of DNA, RNA and proteins [Holick 1973]. It also seems to have been extremely important during the evolution of animals with ossified endoskeletons [Holick 1989]. The more recent history starts with the industrial revolution. At that time, many people moved to the cities where they lived in crowded and dark streets and many children developed rickets. At the end of the nineteenth century, 90% of the children in Leiden (The Netherlands) suffered from this disease. Already in 1822 Sniadecki observed that children living in Warsaw developed more rickets than children living in rural areas and concluded that sunlight must be the determining factor [Sniadecki 1840]. Palm in the 1890s performed an epidemiologic survey of the British Empire and the Orient and also concluded that lack of sunlight must be the key factor in the development of rickets [Palm 1919]. However, since it was inconceivable at that time that such a simple remedy for such a serious illness would suffice, this was not accepted by the medical establishment. It was not until 1919 that Huldschinsky was capable of proving unequivocally that ultraviolet radiation had a beneficial effect on children suffering from rickets [Huldschinsky 1919]. Hess hereafter found that the ultraviolet component of normal sunlight is enough to cause the same effects [Hess & Unger 1921]. Not only exposition to radiation proved effective, but also the intake of food, radiated beforehand with UV light, turned out to improve rickets [Goldblatt & Soames 1923, Steenbock & Black 1924]. This seemed to indicate two different mechanisms for a cure of rickets. Then, Steenbock and Hess showed that the substance generated by radiation of food could also be generated by exposing different organs to UV radiation, including the human skin, and thus the two different mechanisms turned out to be one and the same [Steenbock & Black 1924, Hess & Weinstock 1924].

Meanwhile, in 1919, Mellanby showed that dogs could be cured of rickets with a special diet [Mellanby 1919] and McCollum showed that this food contained a fat soluble substance responsible for this effect [McCollum *et al.* 1922]. First it was mistaken for vitamin A, but soon it turned out to be a new vitamin. Vitamins (vital amines) by definition are essential micronutrients that are necessary for a healthy life, but cannot be synthesized in the body. At the time, three such substances were known, namely vitamins A, B, and C. The new substance, which was able to cure rickets, was therefore considered the fourth vitamin, and named vitamin D.

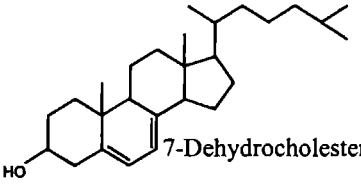
When it became clear that the substance formed in the skin upon radiation was the same as the one McCollum had found, it became obvious that this new molecule thus was a hormone and not a vitamin at all, as it can be synthesized by the body. Nevertheless, it is the name vitamin D that is still generally used[1].

In 1926, it was Windaus who discovered that the compound from which the rickets curing hormone was formed after UV irradiation was similar to ergosterol [Windaus & Hess 1926]. This molecule had been discovered in 1890 by Tanret in a fungus growing on grain called ergot (*Claviceps purpurea*). Now the molecule had been identified, and this soon lead to the use of ergosterol as a medicine for rickets, although it would still take a long time before the exact mechanism by which it exerted its effects was solved. In 1928, Windaus received the Nobel prize in chemistry "for the services rendered through his research into the constitution of sterols and their connection with the vitamins". There appeared to be several different analogues of vitamin D, which were numbered in order of discovery (D_1, D_2, etc.) Later, in 1932 and 1936, Windaus was able to solve the structural formulas of vitamin D_2 and D_3 respectively [Windaus *et al.* 1932, Windaus *et al.* 1936]. Vitamin D_1 had turned out to be a mixture of vitamin D_2 and vitamin D_3 [Hess & Windaus 1927], whose structures are very similar and only differ in the side chain: vitamin D_2 contains an extra methyl group on carbon 24 and a double bond between carbons 22 and 23 (figure 1.1). The name vitamin D_1 is therefore no longer used. The sources of vitamin D_3 are formation in the skin as well as uptake from food, while vitamin D_2 can only be obtained from vegetable dietary sources. Strictly speaking, this actually means that vitamin D_3 must be considered a hormone and D_2 a vitamin. However, since the metabolism and functions in humans for both analogues are identical, the term *vitamin D* is used to refer to both.

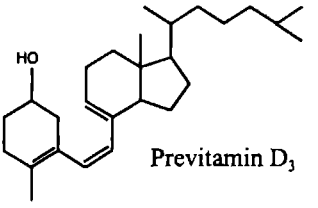
Further research was limited for a long time due to the very small concentrations involved.

After the introduction of radioactive labels however, Norman was able to synthesize tritiated-vitamin D in 1963 [Norman & DeLuca 1963]. This gave an enormous boost to the vitamin D research, since now the small concentrations of metabolites could be traced. Already in 1964, his group found that more polair metabolites were formed [Norman *et al.* 1964] and Haussler showed in 1967 that some of those metabolites bind to the nucleus of intestinal cells [Haussler & Norman 1967]. This latter finding was the first indication of the mechanism behind the direct involvement of vitamin D in the calcium metabolism, since this was a strong hint of its involvement in intestinal calcium uptake. In 1968 Blunt found one of

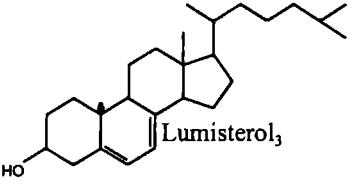
[1]The systematic name of vitamin D_2 is (5Z,7E,22E)-(3S)-9,10-seco-5,7,10(19),22-ergostatetraen-3-ol, while the IUPAC recommended trivial name is either ercalciol or ergocalciferol
The systematic name of vitamin D_3 is (5Z,7E)-(3S)-9,10-seco-5,7,10(19)-cholestatrien-3-ol, while the IUPAC recommended trivial name is either calciol or cholecalciferol [IUPAC-IUB 1982].



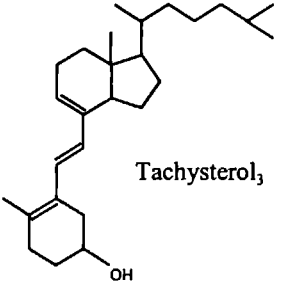
7-Dehydrocholesterol



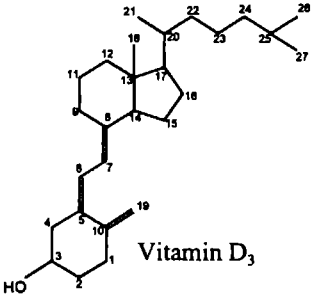
Previtamin D_3



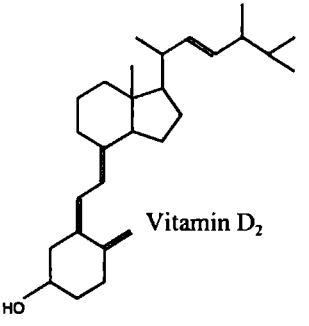
Lumisterol$_3$



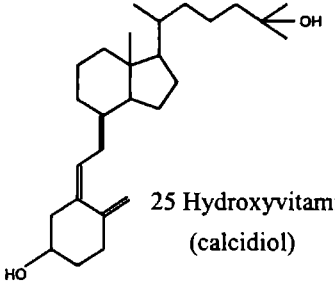
Tachysterol$_3$



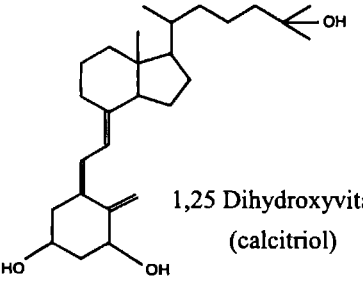
Vitamin D_3



Vitamin D_2



25 Hydroxyvitamin D_3
(calcidiol)



1,25 Dihydroxyvitamin D_3
(calcitriol)

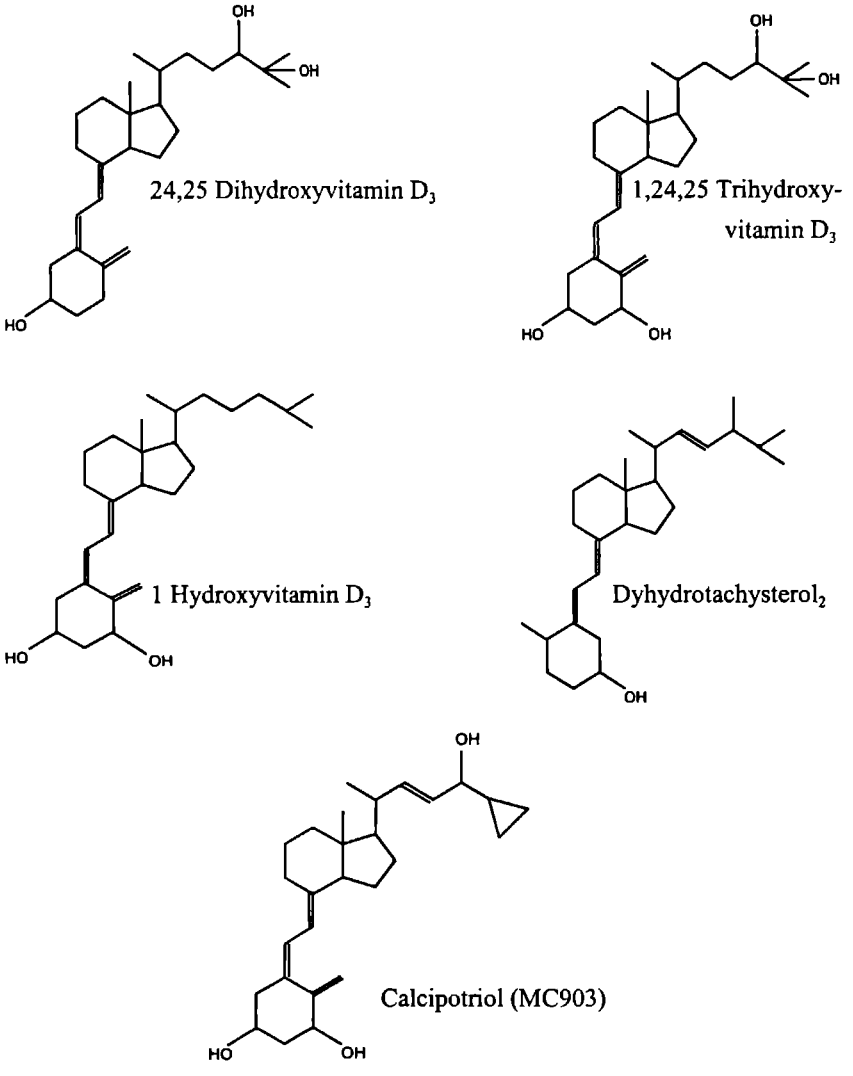


Figure 1.1: Structural formulas of vitamin D_3 and vitamin D related compounds.

the polair metabolites to be 25 hydroxyvitamin D [Blunt *et al.* 1968], which is produced in the liver. Finally in 1971, the true active metabolite turned out to be $1,25(OH)_2D$ [Norman 1971].This activating 1-hydroxylation step takes place in the kidney. After this discovery, the rough outlines of the calcium metabolism soon became clear.

1.2 SYNTHESIS AND METABOLISM OF VITAMIN D

Today, the metabolism of vitamin D is fairly well established (figure 1.2). Until now, 37 different metabolites have been discovered [Bouillon *et al.* 1995], only one of which seems to be biologically active, namely $1,25(OH)_2D$, which binds to the vitamin D receptor. The synthesis of these metabolites starts with the formation of squalene by the papillary reticulum [Brooks *et al.* 1963], which in turn is converted into 7-dehydrocholesterol (also known as provitamin D) by cells of the sebaceous glands [Gaylor & Sault 1964]. This 7-dehydrocholesterol then is converted in the Malpighian layer of the skin to previtamin D under UV exposure (290-315 nm) [Holick *et al.* 1980]. The thermolabile previtamin D isomerizes during 1½ to 2 days into vitamin D under influence of bodyheat [Holick *et al.* 1980]. In cases of prolonged exposure to UV radiation, still only 10-15% of the 7-dehydrocholesterol is converted into previtamin D. This is made possible by isomerization of previtamin D into two other metabolites, namely tachysterol and lumisterol [Holick *et al.* 1981]. Although tachysterol has a vitamin D-like effect when administered directly into the bloodstream, these metabolites are believed to remain in the skin and function as a buffer storage to isomerize into previtamin D when UV radiation is limited. No other biological functions of these two molecules are known at this time.

The vitamin D thus formed enters the bloodstream. The exact mechanism underlying this process is not known yet, but the vitamin D binding protein (DBP) may play an important role [Haddad *et al.* 1993]. In the blood, the vitamin D binding protein bound vitamin D is transported to the liver where 25 hydroxylation takes place in the endoplasmatic reticulum of the hepatic cells [Ponchon & DeLuca 1969]. The 25-hydroxylation occurs without feedback regulation and the concentration of 25(OH)D is directly correlated with the concentration of vitamin D in blood.

The following step in the vitamin D metabolism takes place in the kidney. Here, two hydroxylases in the mitochondria are capable of further hydroxylating 25(OH)D. The 24-hydroxylase converts 25(OH)D into $24,25(OH)_2D$ [Holick *et al.* 1972]. This is a more polar metabolite and is thought to be the first product in the catabolism of vitamin D. It probably has no biological functions, although some discussion about this subject still exists. There is an unconfirmed report of a $24,25(OH)_2D$ receptor in the chick tibial membrane,

playing a role in egg laying [Norman *et al* 1983] The 1-hydroxylase converts 25(OH)D into $1,25(OH)_2D$, which is the main active metabolite of vitamin D [Fraser & Kodicek 1970, Norman 1971]

This hydroxylation step is strictly regulated by the concentrations of calcium [Suda *et al* 1973, Bushinsky *et al* 1985], phosphate [Tanaka & DeLuca 1973], PTH [Fraser & Kodicek 1973], and $1,25(OH)_2D$ [Henry 1979] in blood Further regulating agents that can influence the 1-hydroxylation either directly or indirectly include calcitonin [Kawashima *et al* 1981], estrogen [Castillo *et al* 1977], prolactin [Spanos *et al* 1976], cortisol [Lukert *et al* 1973], insulin [Schneider *et al* 1977], growth hormone [Pahuja & DeLuca 1981], and androgens [Tanaka *et al* 1978], although the

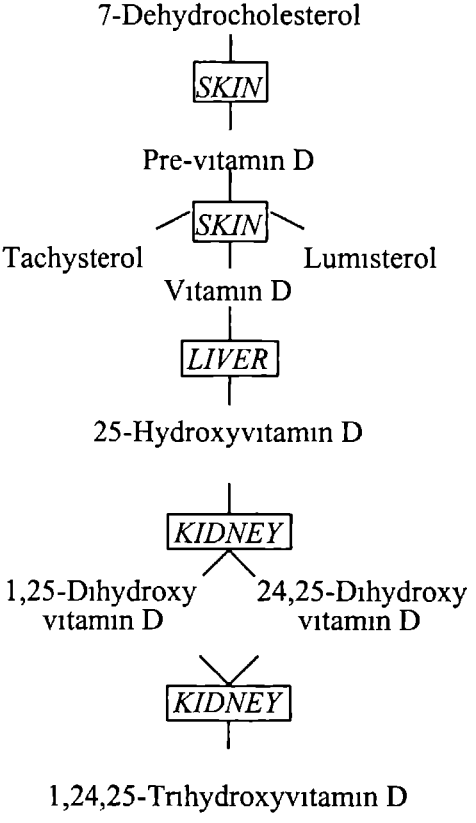


Figure 1 2 Metabolism of vitamin D

biological importance of these substances in the vitamin D regulation is not yet fully elucidated [Holick 1991] (figure 1.3).

The kidney hydroxylases are mitochondrial mixed function oxidases [Ghazarian *et al.* 1974, Henry & Norman 1974]. The electrons needed for the reduction of molecular oxygen to H_2O and OH are ultimately derived from NADPH. They are passed on by ferredoxin reductase and ferredoxin to a cytochrome P450, which specifically catalyses the hydroxylation of 25(OH)D. It is not clear yet which, if any, components are shared between the 1- and 24-hydroxylase. Ghazarian suggested that the P450 can function both in 1- or 24-hydroxylation, whereby the hydroxylation activity is switched by allosteric changes [Ghazarian 1990]. This view is consistent with the reciprocal relationship between $1,25(OH)_2D$ and $24,25(OH)_2D$ synthesis. However, solubilization of a separate 24 hydroxylase has been reported [Burgos-Trinidad *et al.* 1986] and lately Postlind described to have separated the 1- and 24- hydroxylases [Postlind 1990]. These findings strongly indicate that two different hydroxylases are responsible for the two hydroxylation processes of 25(OH)D.

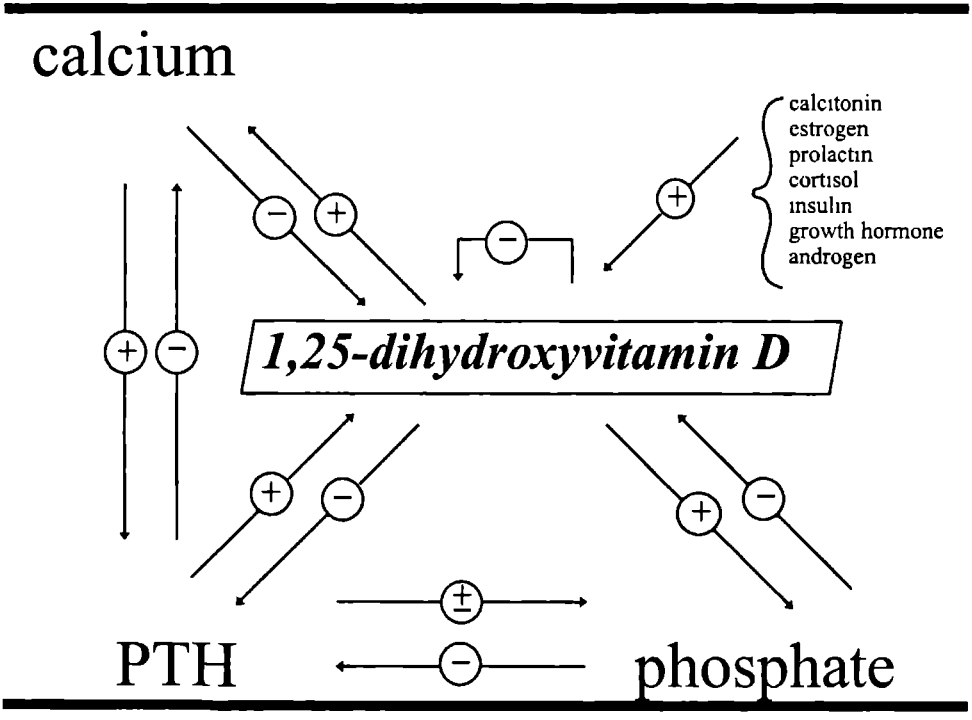


Figure 1.3: Regulatory influences on the synthesis of $1,25(OH)_2D$.

Deactivation of $1,25(OH)_2D$ is achieved by hydroxylation to $1,24,25(OH)_3D$ or $1,23,25(OH)_3D$ and further catabolism to more polar metabolites until the end products 1,(OH)-24,25,26,27-tetranor-23-COOH-D and $1,25(OH)_2$-26,23-lactone-D are excreted [Norman *et al.* 1982]. It has been found that the placenta [Weisman *et al.* 1979] as well as activated macrophages [Adams *et al.* 1983] are also capable of hydroxylating 25(OH)D to $1,25(OH)_2D$. Although the kidney is the principal source of $1,25(OH)_2D$, this extra-renal production may indicate an important function in both pregnancy and infections.

1.3 MODE OF ACTION OF $1,25(OH)_2D$

1.3.1 BIOAVAILABILITY OF $1,25(OH)_2D$

Many substances circulate in blood bound to specific carrier proteins. Several functions for this phenomenon have been proposed: *i*) increasing the solubility of the material in aqueous solutions such as blood, *ii*) providing a buffer amount to be available when there is a sudden rise in demand, *iii*) protecting a substrate against degradation, or *iv*) regulation of the cellular uptake by specific receptors for the binding protein-ligand complex [Bouillon & van Baelen 1981a]. Protein-ligand complex uptake can be found for instance with vitamin A [Smith & Goodman 1979] and vitamin B_{12} [Moestrup *et al.* 1996]. However, vitamin D seems to behave like other steroid and also thyroid hormones, for which no such receptor has been found. It has been shown that vitamin D binds to a specific binding protein [Haddad 1979], to albumin, and perhaps to lipoproteins [Silver & Fainaru 1979]. The specific vitamin D binding protein (DBP) is produced in the liver and circulates in blood [Prunier *et al.* 1964, Imawari *et al.* 1982]. This protein (also known as Gc protein) consists of a single chain with a molecular weight between 50,000 and 60,000 and circulates in blood at a concentration of 5-10 μM [Bouillon *et al.* 1976]. One vitamin D binding site is available per molecule. After binding its ligand a conformational change takes place [Nilsson *et al.* 1972]. Whether this change has any biological function is still subject to debate. It could mean that a receptor exists which can only bind the complex, but until now this receptor has not been identified for the $1,25(OH)_2D$-DBP complex. An interesting recent discovery indicates however, that there may be a receptor for the 25(OH)D-DBP complex [Christensen *et al.* 1997]. It was found that megalin, a protein in the renal proximal tubule, is a receptor for 25(OH)D-DBP and enables reabsorption of the complex. This would mean that the conversion of 25(OH)D to $1,25(OH)_2D$ is dependent on megalin and that two separate functions of DBP, e.g. transport of 25(OH)D to the kidney and of $1,25(OH)_2D$ to its target organs, are regulated by two different processes. Moreover, it has

lately been found that megalin expression is regulated by $1,25(OH)_2D$ and this could indeed mean that this molecule has an important role in the function of $1,25(OH)_2D$ [Liu *et al.* 1998].
DBP is capable of binding vitamin D as well as all its major metabolites, albeit with different association constants (table 1.1). Because of its high concentration in relation to those of the vitamin D metabolites, most binding sites are still unoccupied and the percentage free hormone is very low (< 1%). It has been found that polyunsaturated fatty acids bind to DBP [Williams *et al.* 1988, Ena *et al.* 1989] and that this binding can decrease the binding affinity of DBP for $1,25(OH)_2D$ [Calvo & Ena 1989, Bouillon *et al.* 1992]. Moreover, Cooke and Haddad showed that in situations with an excessive amount of 25(OH)D present, the availability of unoccupied binding sites on DBP for $1,25(OH)_2D$ may be reduced [Cooke & Haddad 1989]. DBP is also capable of binding actin and an additional function of DBP may be the capturing of free actin which appears in the blood after cell lysis [van Baelen *et al.* 1980]. The binding of actin however does not seem to influence the vitamin D binding capabilities of DBP.
Since no receptors have been found to bind the $1,25(OH)_2D$-DBP complex, while there is a cytoplasmatic receptor for $1,25(OH)_2D$, this indicates that most probably free $1,25(OH)_2D$ must enter the cell to bind to the receptor and thus exerts its effect.
This suggests that the free hormone hypothesis must also apply to $1,25(OH)_2D$. The free hormone hypothesis states that the free concentration of a hormone best reflects the biological activity of that hormone. This hypothesis predicts that, in case homeostasis is maintained, mere modification of binding protein capacity or affinity does not affect the free hormone concentration [Robbins & Rall 1957, Mendel 1989, Ekins 1990]. Higher DBP levels are capable of binding more $1,25(OH)_2D$, thus decreasing the free concentration. This will lead to a diminished disposal rate. When the synthesis rate remains normal, a positive balance results until the free concentration is normal again. This is the essence of the free hormone hypothesis. From this it can be concluded that when binding protein properties change, the resulting changes in the total concentration actually reflect homeostasis.
A rapid dynamic equilibrium exists between the free and bound fractions and to replace the lost fraction after the free hormone enters the cells, there is a fast dissociation from the binding protein [Mendel 1989]. Although some discussion still exists about the general validity of this view and the precise mechanism behind it, it is now widely accepted as a working hypothesis.
This theoretical issue can play an important role, as in practice there is a large variability in the concentrations of binding proteins. The concentrations of binding proteins as steroid hormone binding globulin (SHBG), corticosteroid binding globulin (CBG), thyroid-hormone binding globulin (TBG) and DBP rise under the influence of estrogens and will therefore be

elevated during pregnancy and oral contraceptive use. Lower values can be expected for example in nephrotic syndrome or liver cirrhosis.

Because the free concentration of $1,25(OH)_2D$ is very low and difficult to assess, most authors use the free index as an indication of the free concentration [Bouillon *et al.* 1981b]. This index is the molar ratio of DBP and total $1,25(OH)_2D$.

However, it can be shown that the free index is not unambiguously correlated with the true free concentration. According to the law of mass action, the dissociation constant K is given by:

$$\frac{[H][P]}{[HP]}=K \qquad \text{Formula 1.1}$$

Where [H] = concentration free hormone; [P] = concentration free binding protein; [HP] = concentration hormone-binding protein complex.

This can be rewritten for a specific binding protein i as:

$$\frac{F(P_i-B_i)}{B_i}=K_i \qquad \text{Formula 1.2}$$

which after rearrangement gives:

$$B_i=\frac{P_i F}{K_i+F} \qquad \text{Formula 1.3}$$

Where B_i = concentration hormone bound to protein i; P_i = total concentration binding protein i; F = concentration free hormone; K_i = dissociation constant for binding protein i.

When there are more binding proteins, the total hormone concentration can be written as the sum of all the bound fractions and the free hormone. In the case of $1,25(OH)_2D$, there are two binding proteins, namely DBP and albumin. When it is further assumed that there is no competition by 25(OH)D, the total concentration can then be written as:

$$H=F\left(\frac{DBP}{K_{DBP}+F}+\frac{ALB}{K_{ALB}+F}+1\right) \qquad \text{Formula 1.4}$$

Where H = total $1,25(OH)_2D$ concentration; F = free $1,25(OH)_2D$ concentration; DBP = total DBP concentration; ALB = total albumin concentration; K_{DBP} = dissociation constant of DBP for $1,25(OH)_2D$; K_{ALB} = dissociation constant of albumin for $1,25(OH)_2D$.

The free index can be expressed as the total hormone concentration divided by the DBP

concentration. When we assume that the free concentration is very low compared to the Ks and that the last term is so small that it can be ignored, the formula then becomes:

$$\frac{H}{DBP} = index = F\left(\frac{1}{K_{DBP}} + \frac{ALB}{K_{ALB}\,DBP}\right) \qquad \text{Formula 1.5}$$

It can be seen from formula 1.5 that the relationship between the free index and the true free concentration is not constant and depends on the ratio of ALB/DBP. This ratio is known to differ per individual, and hence the free indexes of a group of persons will not always be linear with the free concentrations.

Further it has to be considered that the apparent K_{DBP} in part depends on the 25(OH)D concentration. It has been reported that DBP has only one binding site for the vitamin D metabolites and that DBP binds both 25(OH)D and 24,25$(OH)_2$D more strongly than 1,25$(OH)_2$D [Haddad & Walgate 1976a, Kawakami *et al.* 1979].

Because the concentration of 25(OH)D is much larger than that of 24,25$(OH)_2$D, the potential disturbing factor will be the concentration of 25(OH)D. This means that formula 1.4 should be written as [Feldman 1972]:

$$H = F_{1,25}\left(\frac{DBP}{K'_{1,25} + F_{25}\frac{K_{1,25}}{K_{25}} + F_{1,25}} + \frac{ALB}{K_{ALB}\,F_{1,25}} + 1\right) \qquad \text{Formula 1.6}$$

In which $K'_{1,25}$ is the true dissociation constant of DBP for 1,25$(OH)_2$D; K'_{25} is the true dissociation constant of DBP for 25(OH)D; $F_{1,25}$ is the free concentration 1,25$(OH)_2$D; F_{25} is the free concentration 25(OH)D. The reported values of the $K'_{1,25}$ and K'_{25} are in the order of 10^{-7} and 10^{-8} M respectively [Bouillon *et al.* 1981b, Bikle *et al.* 1986]. If we assume that an effect of 10% can be detected, this means that the free 1,25$(OH)_2$D concentration will start to deviate when free 25(OH)D levels reach concentrations in the order of 10^{-9} M. The free 25(OH)D fraction is thought to be around 0.03% [Bikle *et al.* 1986], which means that total 25(OH)D concentrations of µM will start to result in elevated free 1,25$(OH)_2$D levels. Indeed, exactly this phenomenon has been reported in several patients who had accidentally ingested large amounts of vitamin D concentrate [Pettifor *et al.* 1995]. Moreover, the aforementioned effects of free fatty acids on the binding affinity of DBP for 1,25$(OH)_2$D are also not taken into account when using the free index.

Although it is rather probable that no problems will occur when using the free index as an indication of the free concentration in health, it is very well likely that the free index might result in spurious results in disease. It should therefore cause no surprise to find conflicting

reports with regard to the significance of free $1,25(OH)_2D$ in the literature.
Thus far only one technique for the measurement of free $1,25(OH)_2D$ has been described, *e.g.* a centrifugal ultrafiltration technique [Bikle *et al.* 1984]. However, because this method is known to be rather prone to disturbances, the results obtained with this technique should be considered with caution.

Table 1.1
Ranges of association constants of DBP for different vitamin D metabolites.

vitamin D	$3x10^5$ - $2.3x10^6$	Imawari *et al.* 1976 Haddad & Walgate 1976b
25 hydroxyvitamin D	$1x10^8$ - $1.2x10^{10}$	Haddad & Walgate 1976a Bouillon *et al.* 1976
24,25 dihydroxyvitamin D	$1x10^8$	Haddad *et al.*1977
1,25 dihydroxyvitamin D	$2.9x10^6$ - $1.5x10^7$	Haddad & Walgate 1976a Kawakami *et al.* 1979

1.3.2 RECEPTOR(S) FOR $1,25(OH)_2D$

The wide range of effects that is induced by $1,25(OH)_2D$ can only be explained by postulating two different pathways. The first of these pathways is a fairly well established genomic route, in which a new protein is synthesized and the response time to $1,25(OH)_2D$ in this case is typically several hours. Second, there is a non-genomic route with responses within seconds or minutes [Toffolon *et al.* 1975]. The first route is accomplished via a cytosolic vitamin D receptor [Tsai & Norman 1973]. This receptor is a protein of 50,000-60,000 daltons [Brumbaugh & Haussler 1975, Wecksler *et al.* 1980] and belongs to the superfamily of steroid and thyroid hormone receptors. It most closely resembles the thyroid hormone receptor [Laudet *et al.* 1992]. Vitamin-K dependent γ-carboxylation of some of the glutamic acid residues present in the receptor, probably modulates its binding to DNA [Sergeev & Norman 1992].
The active form of the receptor consists either of a homodimer or of a heterodimer with the 9-*cis*-retinoic receptor [Schräder *et al.* 1993]. Although the affinity constant for $1,25(OH)_2D$ hasn't yet been precisely established, the K_a is probably about $1\text{-}50*10^{11}$ M [Norman *et al.* 1982]. Upon binding $1,25(OH)_2D$, the receptor-hormone complex migrates to the nucleus where it binds to specific sites on the chromatin and then induces mRNA production and

subsequent protein synthesis [Haussler *et al.* 1981].

Recently it has been found that vitamin D receptor alleles can be used to predict most of the total genetic effect on bone density [Morrison *et al.* 1994], although some controversy does exist about this finding [Lim *et al.* 1995, Mocharla *et al.* 1997]. Until now, five gene polymorphic restriction sites have been reported, namely *Bsm* [Morrison *et al.* 1992], *Apa* [Hustmeyer *et al.* 1993], *Taq* [Hustmyer *et al.* 1993], *Eco*RV [Morrison *et al.* 1992], and *Fok* [Gross *et al.* 1996]. A combination of the first three, increases the correlation between bone mineral density and the polymorphism, indicating that such a relation indeed does exist [Uitterlinden *et al.* 1996]. Further, Barger-Lux *et al.* found a correlation between the vitamin D receptor polymorphism and body size, suggesting that this may be the basis of the correlation between the polymorphism and bone density [Barger-Lux *et al.* 1995]. Another hypothesis states that the different alleles may lead to various receptors with differences in the receptor-receptor or receptor-DNA binding, thus leading to the observed relationship [Dawson-Hughes *et al.* 1995]. Although any direct link between these polymorphisms and receptor gene-expression or function is still unclear, the evidence seems to point in the direction of a connection and this matter is being further investigated [Pols *et al.* 1998].

The non-genomic actions are rather difficult to explain, since no mechanism behind these processes is known yet. Until now, two theories have been put forward to explain this wide range of actions. One proposes a direct insertion of the $1,25(OH)_2D$ into the lipid bilayer of the membrane where it should exert its effect [Norman & Ross 1979]. The other theory proposes a second, membrane-bound, receptor which upon binding $1,25(OH)_2D$ would mediate the opening of calcium channels [Nemere & Szego 1981]. Studies with analogues of $1,25(OH)_2D$ which can produce either the genomic or the non-genomic effect indicate that this latter hypothesis may be closest to the truth. Some analogues with virtually no capacity to bind to the cytosolic vitamin D receptor are very well capable of blocking the non-genomic effects of $1,25(OH)_2D$ [Bouillon *et al.* 1995]. A reported finding by Nemere *et al.* of a membrane located $1,25(OH)_2D$ binding protein however, hasn't been confirmed yet [Nemere *et al.* 1994]. Effects induced in this second, non-genomic route are reported in many tissue types [Nemere *et al.* 1993], including intestine [Nemere & Szego 1981], bone [Civitelli *et al.* 1990], muscle [Fernandez *et al.* 1990], hepatocytes [Baran *et al.* 1988], kidney [Dick *et al.* 1990], and keratinocytes [Tang *et al.* 1987].

1.4 FUNCTION OF $1,25(OH)_2D$

1.4.1 EFFECTS OF $1,25(OH)_2D$

The nucleus of intestinal cells was the place where $1,25(OH)_2D$ was first demonstrated in 1968 [Haussler *et al.* 1968]. Because of the extremely low concentrations involved, it was only after radiolabeling of $1,25(OH)_2D$ became possible and these small quantities could be measured, that the study of the metabolites and the major functions of the hormone could really start (table 1.2) [Norman & DeLuca 1963].

A feedback system was found between $1,25(OH)_2D$ and PTH, as $1,25(OH)_2D$ inhibits PTH synthesis [Chertow *et al.* 1975], while PTH increases the 1-hydroxylation of 25(OH)D [Fraser & Kodicek 1973]. A second feedback system exists as elevated $1,25(OH)_2D$ inhibits its own formation (figure 1.3)[Henry 1979].

Table 1.2

Effects of $1,25(OH)_2D$.

Organ	Effect	Reference
intestine	increase of calcium absorption	Nicolaysen 1937
parathyroid	inhibition PTH release	Chertow *et al.* 1975
kidney	inhibition $1,25(OH)_2D$ synthesis	Larkins *et al.* 1974
	stimulation $24,25(OH)_2D$ synthesis	Tanaka & DeLuca 1974
	stimulation of calcium resorption	Bindels *et al.* 1991
bone	stimulation osteoclast formation	Holtrop *et al.* 1981
bone marrow	differentiation of mast cells	Yetgin & Ozsoyly 1982
immune system	stimulation activated T lymphocytes	Lemire *et al.* 1985
	stimulation B lymphocytes	Iho *et al.* 1986
muscle	differentiation of myoblasts into microtubules	Giuliani & Bolland 1984
skin	differentiation of keratinocytes	Regnier & Darmon 1991
malignancies	differentiation of breast cancer cells	Eisman *et al.* 1979
	differentiation of myeloid leukaemia cells	Abe *et al.* 1981
	differentiation of colon cancer cells	Cross *et al.* 1991
	differentiation of prostate cancer cells	Skrowonski *et al.* 1993

The synthesis of many proteins is induced by $1,25(OH)_2D$ after binding of the hormone-receptor complex to DNA. Among others these are calbindins, both in intestine

[Thomasset *et al.* 1981] en kidney [Bindels *et al.* 1991], osteocalcin [Zerwekh *et al.* 1985, Kerner *et al.* 1989], osteopontin [Oldberg *et al.* 1986], c-myc [Reitsma *et al.* 1983], c-fos [Brelvi & Studzinski 1986], and c-jun [Candeliere *et al.* 1991].

Further, $1,25(OH)_2D$ stimulates the synthesis of $24,25(OH)_2D$ [Tanaka & DeLuca 1974], which is thought to have little or no biological function and merely to be a first step in the catabolism of vitamin D, although lately there have been some indications that there may be a role for $24,25(OH)_2D$ in the egg-laying of hens [Elaroussi *et al.* 1993]. The intestine, parathyroid, kidney, and bone, are the four classic $1,25(OH)_2D$ target organs and are involved in the calcium metabolism. The net effect of the actions of $1,25(OH)_2D$ is a rapid increase in blood calcium levels by increased absorption through the intestine and a reduced secretion of calcium through the kidneys. On a longer timescale, an increase in osteoclast activity occurs [Reichel *et al.* 1989].

Table 1.3

Tissues containing $1,25(OH)_2D$ receptors [Hannah & Norman 1994].

Adipose	Parathyroid
Adrenal	Pancreas
Bone Marrow	Parotid
Brain	Pituitary
Breast	Placenta
Cartilage	Prostate
Colon	Retina
Eggshell Gland	Skin
Epididymis	Stomach
Intestine	Testis
Kidney	Thymus
Liver (fetal)	Thyroid
Lung	Uterus
Muscle	Yolk Sack
Osteoblast	Cancer Cells (many)
Ovary	

More recently, a remarkable discovery has been made [Stumpf *et al.* 1979]. He showed that the $1,25(OH)_2D$ receptor is present in many more tissues (table 1.3) than the classic target organs. This discovery lead to the finding of more possible functions of $1,25(OH)_2D$ (table

1.2). Activation of lymphocytes indicate a role of $1,25(OH)_2D$ in the regulation of the immune system. It has been reported that vitamin D deficient patients have a decreased inflammatory response and patients with rickets have been reported to have recurrent infections [Ströder & Franzen 1975]. These findings could be related to the vitamin D status, since after treatment with $1,25(OH)_2D$ these symptoms disappear. However, patients with a vitamin D receptor defect do not seem to suffer more from infections. This may either mean that the role of $1,25(OH)_2D$ on the immune system is rather limited or that another, until now unknown, receptor is involved in the effect $1,25(OH)_2D$ has on the immune system.Further it has been reported that myoblasts differentiate into fused microtubules after stimulation with $1,25(OH)_2D$ [Giuliani & Bolland 1984], but again patients suffering from a receptor defect do not suffer from extensive muscle weakness.

Apart from the differentiation of many cell types, epidemiologic studies also indicate a role for $1,25(OH)_2D$ in the prevention of breast cancer [Garland *et al.* 1990], colorectal cancer [Garland *et al.* 1985], and prostate cancer [Hanchette & Schwartz 1992]. These findings have already lead to the use of $1,25(OH)_2D$ as treatment of psoriasis [Morimoto *et al.* 1986] and studies are underway to investigate the suitability of $1,25(OH)_2D$ for cancer treatment. Due to the calcemic effects of $1,25(OH)_2D$ however, the dose that can be applied is relatively low. To solve this problem, many analogues have been developed to find one which does possess the differentiating capabilities of $1,25(OH)_2D$, but has either none or a diminished effect on the calcium metabolism [Bouillon *et al.* 1995]. The importance of the effects of $1,25(OH)_2D$ *in vivo* on cell growth and differentiation still needs further elucidation, but these effects may prove to have a great impact on medical practice in the near future.

1.4.2 $1,25(OH)_2D$ AND OSTEOPOROSIS

Osteoporosis is internationally defined as "a progressive systemic skeletal disease, characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture". With age, both men and women lose bone, which may result in osteoporosis. In women around menopause, there is some extra, rapid, bone loss, so that they are more at risk than men [Riggs *et al.* 1981]. Since $1,25(OH)_2D$ is responsible for regulation of calcium levels, its deficiency may be one of the causes of osteoporosis. Decreased $1,25(OH)_2D$ levels would result in decreased calcium uptake through the intestines and increased excretion through the kidneys, and thus to lower bone mass. Moreover, low calcium levels will also result in higher PTH levels, in turn leading to increased bone resorption.

However, reports on the levels of $1,25(OH)_2D$ in ageing are conflicting. Some studies report

decreases [Tsai *et al.* 1984, Bouillon *et al.* 1987], whereas others find no change with age [Orwol & Meier 1986, Sherman *et al.* 1990]. There are several possible factors that might be responsible for a decrease in $1,25(OH)_2D$ concentrations with age. These are changes in DBP concentration, decreased renal 1-hydroxylase activity, increased renal $1,25(OH)_2D$ clearance, or 25(OH) substrate deficiency. None of these factors however, seem to be of major importance in healthy people. The levels of DBP are reported not to decline with age [Aksnes *et al.* 1989]. Renal 1-hydroxylase activity in ageing has only be studied in rats and conflicting results are reported [Armbrecht *et al.* 1984, Ishida *et al.* 1987]. Clearance of $1,25(OH)_2D$ is reported to rather decrease than increase with age [Eastell *et al.* 1991]. Indeed, it has been shown that there is a decrease of plasma 25(OH)D with age [Aksnes *et al.* 1989]. However, in that study it was also reported that in healthy people $1,25(OH)_2D$ levels were normal nonetheless. In institutionalised people however, both 25(OH)D and $1,25(OH)_2D$ levels were lowered and substitution of 25(OH)D did result in an increase in both hormones [Aksnes *et al.* 1989]. It is therefore far from certain whether there is an age-dependent decrease in $1,25(OH)_2D$ levels in healthy people and its importance in the development of osteoporosis is not at all clear. On the other hand however, it has been shown that $1,25(OH)_2D$ supplementation in osteoporotic women did lead to a decrease in the occurrence of vertebral fractures [Gallagher *et al.* 1989, Tilyard *et al.* 1992], indicating that there indeed may exist a state of relative hypovitaminosis in osteoporosis.

1.4.3 $1,25(OH)_2D$ AND ESTROGENS

The complex interrelationship between $1,25(OH)_2D$ and estrogens that arises from literature data deserves some extra attention. It is now known that these hormones play a role in both breast cancer and osteoporosis. Insights in their mutual influence could be of great importance for the treatment of either disease.

It has been shown that $1,25(OH)_2D$ has effects on the aromatase enzyme and thus the estrogen production in different cell types. In bone cells $1,25(OH)_2D$ increases the aromatase activity [Nawata *et al.* 1995]. In human placental cells however, no change in activity was found [Evans *et al.* 1994]. The direct effect of $1,25(OH)_2D$ on the production of estrogen *in vivo* seems rather limited. A larger effect can be found at the level of expression of the estrogen receptor. In bone marrow derived stromal cells, $1,25(OH)_2D$ has been shown to increase the amount of estrogen receptor [Bellido *et al.* 1993]. In mammary tumours however, $1,25(OH)_2D$ decreases the estrogen receptor amount in the tumour [Noguchi *et al.* 1989].

Estrogen has also been reported to have effects on the $1,25(OH)_2D$ concentration [Kumar *et al.* 1979]. It has been shown that oral estrogens increase the $1,25(OH)_2D$ concentration in

blood. However, it is unclear whether this increase is due to a direct stimulation of the renal 1-hydroxylase [Pike *et al.* 1978], an indirect stimulation of this enzyme [Henry 1981], or just an increase in bound hormone [Bouillon & van Baelen 1981a] as result of the increase in the hepatic DBP production [Haddad & Walgate 1976b]. Assuming that the free hormone hypothesis applies to $1,25(OH)_2D$, this latter mechanism would mean that no rise in biological activity occurs.

Apart from the change in $1,25(OH)_2D$ levels, there are also reports of effects on the regulation of the vitamin D receptor (VDR) induced by estrogens. Estrogens are reported to increase the VDR concentration in the liver [Duncan *et al.* 1991], uterus [Walters *et al.* 1983], and osteoblast like cells [Liel *et al.* 1992]. An estrogen induced decrease of the VDR is reported in the kidney [Duncan *et al.* 1991], while no effect could be found in the intestine [Duncan *et al.* 1991], even though an intestine resistance to $1,25(OH)_2D$ is suspected in hypoestrogen conditions.

Furthermore, estrogen and $1,25(OH)_2D$ antagonize each other in an indirect way. The resorption of bone is increased by $1,25(OH)_2D$ [Holtrop *et al.* 1981] through stimulation of osteoclasts, whereas estrogen suppresses these cells [Most *et al.* 1995]. Estrogen also increases the formation of bone through differentiation of osteoblast like cells [Scheven *et al.* 1992].

Additionally, there are reports of a direct stimulation of calcitonin [Agnusdei *et al.* 1990] and PTH secretion [Greenberg *et al.* 1987] by estrogens, although controversy exists about the latter [Prince 1994]. These two hormones are also known to stimulate the $1,25(OH)_2D$ synthesis [Kawashima *et al.* 1981, Fraser & Kodicek 1973], thus further complicating the relationship between $1,25(OH)_2D$ and estrogens.

In conclusion, the two hormones have antagonizing effects on bone, since $1,25(OH)_2D$ stimulates bone resorption, whereas estrogens inhibit it. Opposing effects can also be found in mammary carcinomas, whose growth is induced by estrogens [Korenman & Dukes 1970], but differentiate under the influence of $1,25(OH)_2D$ [Eisman *et al.* 1979]. The effect of estrogens on the $1,25(OH)_2D$ synthesis is probably indirect through PTH, calcitonin, calcium, phosphorus, or a combination of these substances. However, the exact extent of this effect and the physiological meaning have not yet been clearly established.

1.5 ASSAYS FOR $1,25(OH)_2D$

1.5.1 ASSAY APPROACHES

After the discovery of the active metabolite of vitamin D, $1,25(OH)_2D$, many researchers set

out to obtain a suitable assay to measure $1,25(OH)_2D$ concentrations in plasma. The first assay described was a two step assay in which $1,25(OH)_2D$ was bound to the cytosolic receptor from the intestine of rachitic chickens [Brumbaugh *et al.* 1974]. The hormone-receptor complex was then bound to the purified chromatin, after which the whole complex was separated. An important improvement came when instead of complexation with the chromatin, polyethylene glycol was used to separate bound and free $1,25(OH)_2$ D [Eisman *et al.* 1976]. Later, radioimmunoassays were developed by Clemens *et al.* [Clemens *et al.* 1979] and Bouillon *et al.* [Bouillon *et al.* 1980], but the characteristics of these assays were not much better than those of the radioreceptorassay. The raising of antibodies in rabbits proved to be a rather difficult procedure, since most rabbits died before high enough titers of antibodies in blood were obtained.

Because the chick intestine is rather small, the yield of receptor per chicken was not very large and another source for a $1,25(OH)_2D$ receptor was sought for. Duncan used the intestinal receptor of rachitic rabbits [Duncan *et al.* 1986], but the real improvement came when Reinhardt found that the calf thymus contains large amounts of easily obtainable receptor [Reinhardt & Hollis 1986]. Moreover, this source obviates the necessity of a special diet for the animal [Reinhardt *et al.* 1982]. At this time, this radioreceptorassay is the most widely $1,25(OH)_2D$ assay used, despite several new techniques that have been introduced during the years. These new assays include a scintillationproximityassay [Wildermuth *et al.* 1993], a gas chromatographic mass fragmentographic method [Poon *et al.* 1993], and a method using thermospray liquid chromatography / mass spectrometry [Watson *et al.* 1991]. A new development is the finding of even larger amounts of receptor in bovine mammary glands [Watanabe *et al.* 1994], which should result in even more easily available receptor. However, the exact characteristics of this receptor still have to be studied.

The introduction of a ^{125}I label [Hollis *et al.* 1996] as well as a fluorescent label [Shimizu *et al.* 1997] may be of even more importance for the development of assays for $1,25(OH)_2D$, routinely used around the world.

1.5.2 SAMPLE PURIFICATION

The radioreceptorassay now widely used for the assessment of $1,25(OH)_2D$ cannot be performed without a separation of the major vitamin D metabolites. The binding of the receptor to 25(OH)D is a thousand times weaker than the binding to $1,25(OH)_2D$, but the concentration of the former is also a thousand times higher [Feldman *et al.* 1979]. This means that major disturbances will occur without separation prior to the assay. The strength of the binding to $24,25(OH)_2D$ is of the same order as the binding to 25(OH)D, but because the

concentration of $24,25(OH)_2D$ normally is ten times lower, usually no major interference is found. In hypervitaminosis however, serious interference may be expected and separation of $1,25(OH)_2D$ from $24,25(OH)_2D$ will be preferable.

To separate all three metabolites, Brumbaugh described a HPLC method [Brumbaugh *et al.* 1974]. To save costs as well as time, Reinhardt described a two step method [Reinhardt *et al.* 1984]. The first step consisted of a separation of salts and polair lipids on a C-18 cartridge, the second step was a separation of the three vitamin D metabolites on a silica column. Hollis described an even faster and cheaper method using phase-switching on a single C-18 column, but this method does not separate $1,25(OH)_2$ D and $24,25(OH)_2D$ [Hollis 1986]. This limitation is of no consequence when in the subsequent assay no cross-reactivity occurs between these two metabolites, otherwise, as for example in the RIA of Clemens *et al.* [Clemens *et al.* 1979], this method fails.

To separate $1,25(OH)_2D_2$ from $1,25(OH)_2D_3$, Masuda *et al.* applied an eluate from a Bond Elut C-18OH column to HPLC, using a Zorbax SIL column. [Masuda *et al.* 1993]. Because normally this separation step is unnecessary, the method is hardly ever used. A recent development has been the introduction of immunoaffinity chromatography, using polyclonal antibodies to selectively capture the $1,25(OH)_2D$ from plasma prior to the radioreceptorassay [Kobayashi *et al.* 1997].

1.6 SCOPE OF THIS THESIS

Osteoporosis is a serious disease that strikes millions of people every year, mostly the elderly. Presently, the treatment of choice to halt bone degradation in postmenopausal women is estrogen replacement therapy. However, despite its wide use, the mechanism by which estrogens exert this effect is still not known. There have been indications that at least part of the effects of estrogens on bone is mediated through $1,25(OH)_2D$. It is hypothesised that postmenopausal estrogen treatment results in a rise in biologically active $1,25(OH)_2D$. According to the free hormone hypothesis, this biologically active fraction is best represented by the non-protein bound or free $1,25(OH)_2D$. With assessment of total $1,25(OH)_2D$ the true hormonal status may be obscured by unrelated or only indirectly related factors pertaining to its transport. These factors are the concentration and affinity of binding proteins as well as the presence of inhibitors of binding. In view of the fact that estrogens influence the hormone binding in various ways, most prominently by increasing vitamin D binding protein levels, the total $1,25(OH)_2D$ concentration cannot be used as a reliable marker for the free concentration. Therefore, the free $1,25(OH)_2D$ must be measured to study any effects of estrogens..

First, it was therefore necessary to develop an accurate and reliable assay for total plasma

$1,25(OH)_2D$ concentrations, since plasma $1,25(OH)_2D$ levels are quite low and prevailing methods are either rather complex or expensive. Moreover, the only previously published method to assess the free $1,25(OH)_2D$ concentration has several major drawbacks. It was therefore also necessary to develop a better assay for the measurement of the free $1,25(OH)_2D$ concentration in plasma.

It was then investigated whether the free hormone hypothesis also applies to $1,25(OH)_2D$. For this purpose, both total and free $1,25(OH)_2D$ concentrations were studied in conditions expectedly associated with altered binding, *i.e.* use of oral contraceptives, pregnancy, and nephrotic syndrome.

Finally, the effects of postmenopausal hormone replacement therapy on the levels of both total and free $1,25(OH)_2D$ were investigated, in an attempt to clarify the possible role of $1,25(OH)_2D$ in the beneficial effect of estrogens on postmenopausal osteoporosis.

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CHAPTER 2

Advantages of paper chromatography as a preparative step in the assay of 1,25-dihydroxyvitamin D

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2.1 ABSTRACT

Ether extraction and paper chromatography were used to separate the main metabolites of vitamin D in plasma [(25(OH), 24,25$(OH)_2$ and 1,25$(OH)_2$ vitamin D] prior to radioreceptorassay. The overall procedural loss of the 1,25$(OH)_2$ vitamin D was 58%±5% (n=40), corrected for by tracer addition. The sensitivity of the assay was 0.5 fmol/tube, corresponding to 4 pmol/l and the intra- and interassay coefficients of variation were 10.5 and 11.5 %, respectively. The range of values measured in healthy controls was 80-200 pmol/l (n=60), which is in agreement with findings reported in the literature. A comparison of the results of the present procedure with those obtained with a procedure employing C-18 purification, disclosed a correlation coefficient of 0.92 ($p \leq 0.0001$), a slope of 0.89 ($p \leq 0.0001$) and a small non-significant intercept of 5.0 pmol/l (n=53).

2.2 INTRODUCTION

Vitamin D is hydroxylated in the liver to 25(OH) vitamin D (25(OH)D), the main circulating metabolite of the hormone in human blood [Blunt *et al.* 1968]. Hydroxylation to 1,25$(OH)_2$ vitamin D (1,25$(OH)_2$D) and 24,25$(OH)_2$ vitamin D (24,25$(OH)_2$D) mainly takes place in the kidney [Fraser & Kodicek 1970, Holick *et al.* 1972). The 1,25$(OH)_2$D is the biologically active form and is involved in many regulating processes, whereas 24,25$(OH)_2$D has little, if any, biological activity [DeLuca 1975], and 25(OH)D, as far as known, has none. The main role of 1,25$(OH)_2$D is the regulation of calcium levels in blood [Reichel *et al.* 1989]. There is growing evidence that it is also involved in insulin synthesis, sex hormone synthesis, cell differentiation and cell growth [Cade & Norman 1987, Meggouh *et al.* 1991, Pols *et al.* 1990, Frampton *et al.* 1983].

The most recent and most accurate method for assessing 1,25$(OH)_2$D is by making use of high-performance liquid chromatography/mass spectrography (HPLC/MS) [Watson *et al.* 1991]. The currently most widely used method is assessment by a radioreceptorassay, using calf thymus tissue for the preparation of the receptor. This receptor, though, not only binds with 1,25$(OH)_2$D, but also the other metabolites, only weakly so with 25(OH)D, but which circulates at a concentration about 1000 times higher than 1,25$(OH)_2$D [Reinhardt *et al.* 1982]. Consequently, assaying of 1,25$(OH)_2$D is only feasible after separating it from the other metabolites. Such purification can be effectively achieved by HPLC [Watson *et al.* 1991, Eisman *et al.* 1976] as well as by a dual- or single-column solid phase extraction [Reinhardt *et al.* 1984, Hollis 1986], the latter however, not separating 24,25$(OH)_2$D from 1,25$(OH)_2$D.

Because in our laboratory liquid-liquid extraction followed by paper chromatography has

proven to be an effective and a convenient purification technique for various other steroid hormones, requiring little time and instrumentation of limited cost, we have investigated its efficacy in the purification of $1,25(OH)_2D$ preceding a radioreceptorassay.

2.3 MATERIALS AND METHODS

2.3.1 SAMPLES

Human plasma was obtained (1) from the local blood bank and pooled for control experiments, (2) from 60 healthy individuals (34 men, 26 women) in order to establish a normal range of $1,25(OH)_2D$ concentrations, and (3) from 53 patients, selected for the purpose of obtaining a wide range of $1,25(OH)_2D$ concentrations (determined at TNO Food and Nutrition Research laboratories, Zeist, the Netherlands, by making use of solid phase chromatography conform a previously described method [Mudde *et al.* 1987]).

2.3.2 REAGENTS AND CHEMICALS

Radioactive materials were purchased from Dupont (NEN Products, Dreieich, Germany). Nonlabeled $1,25(OH)_2D$ was kindly donated by Dr. U. Fisher (F. Hoffmann-La Roche & Co, Basel, Switzerland).
All other chemicals used were of analytical-reagent grade.
C-18 and silica columns were purchased from Waters (Millipore, Milford, Ma, USA).

2.3.3 EXTRACTION AND PAPER CHROMATOGRAPHY

To 1.0 ml of plasma, 25 µl (10,000 dpm) ^3H $1,25(OH)_2D_3$ (166.4 Ci/mmol, 26,27-^3H) in ethanol was added. The samples were incubated for 15 min at room temperature and extracted with 15 ml of diethylether. The solvent was removed by drying under a stream of dry nitrogen. The residue was dissolved in an aliquot of diethylether and applied to a sheet of paper (Whatman Nº 1) divided into 8 lanes (2.5 x 42 cm), which was prewashed with ethanol in order to reduce the reagent blank and was chromatographed for 3 h in a descending system [Bush 1961] (petroleumether 80-110 : toluene : methanol : water = 333:167:400:100, v/v). The tracer label was located by radioscanning with a model 7201 radiochromatogram scanner (Packard Instruments Co). The appropriate area of paper was cut out and the $1,25(OH)_2D$ was eluted with 2 ml of ethanol for 1 h at room temperature. The ethanol was then evaporated under a stream of

dry nitrogen and the residue was dissolved in 200 µl of 25% ethanol in phosphate buffer (0.04 M Na_2HPO_4 , 0.01 M NaH_2PO_4 , 0.10 mM MTG)(pH = 7.4) in order to concentrate the sample.

2.3.4 C-18/SILICA AND C-18 PURIFICATION

To purify samples by means of solid-phase columns, to obtain processed water blanks and recovery data, two different procedures were employed. The first was a two-column purification employing C-18 and silica columns as described by Reinhardt *et al.* [Reinhardt *et al.* 1984], the second was a single-column purification employing only a C-18 column, as described by Hollis [Hollis 1986].

2.3.5 PREPARATION OF CALF THYMUS RECEPTOR

Calf thymus was obtained from the local slaughterhouse and stored until use at -80°C. To make a receptor preparation, 20 g of calf thymus was cryopulverized in a motor-driven mortar. The thymus, vat and piston were all cooled with liquid nitrogen. A 45 ml volume of 0.05 M phosphate buffer was added, containing 0.4 M KCl, 1.5 mM EDTA, 10 Mm Na_2MoO_4, 0.1 mM monothioglycerol and 0.1 mM bacitracin and allowed to stand for 30 min at 4°C. The thymus tissue was then further suspended with an Ultra Turrax for about 5 min. The suspension was centrifuged at 100,000 x g for 60 min at 4°C and the supernatant was stored in 1 ml aliquots at -80°C until use. Prior to assay the solution was diluted 1:30 with 0.05 M phosphate buffer containing 0.1 M KCl, 1.5 mM EDTA 10 mM Na_2MoO_4, 0.1 mM monothioglycerol and 0.1 mM bacitracin, to achieve optimal initial binding. No significant loss of binding capacity of the receptor preparation was detected even after 1 year of storage.

2.3.6 RADIORECEPTORASSAY

The radioreceptorassay was performed as described by Hollis [Hollis 1986] with a few modifications. A standard curve (0-1200 pmol/l) was set up in duplicate. To 50 µl of standard, control or unknown, 50 µl of ^{3}H-1,25$(OH)_2D_3$ (10,000 dpm) in ethanol:phosphate buffer (1:3, v/v) and 500 µl receptor solution were added in a glass tube. After 3 h incubation at room temperature, bound and unbound 1,25$(OH)_2$D were separated using dextran coated charcoal to adsorb the unbound fraction. This separation went as follows. An aliquot of 150 µl 1% charcoal suspension in 0.05 M phosphate buffer containing 0.1 M KCl and 0.1 % Dextran T70 was pipetted into the cavity of a plastic test tube cap, where, due to adhesion, the slurry stayed in

position until shaken out. The caps thus filled were put on the tubes and then the tubes were shaken simultaneously to mix the suspension with the contents of the tubes. After 3 min the samples were centrifuged at 2000 x g for 10 min at room temperature. The supernatants were decanted simultaneously into counting vials using the device described by Vecsei and Gless [Vecsei & Gless 1975]. After adding of 4 ml scintillation liquid, the radioactivity was determined.

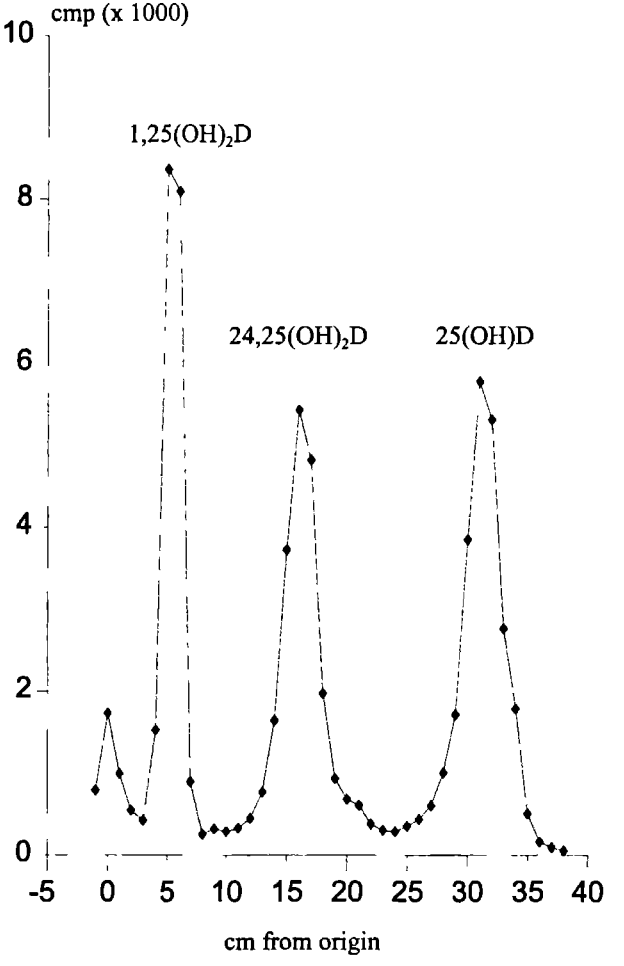

Figure 2.1: Separation of 1,25(OH)$_2$D, 24,25(OH)$_2$D, and 25(OH)D after paper chromatography using a descending system [Bush 1961].

2.3.7 CALCULATIONS

Measurement results were corrected for procedural losses (recovery), reagent blank, and the

effects caused by the use of recovery tracer itself. The standard curve was fitted to a four-parameter model as described by Healey [Healey 1972], using a non-linear fit algorithm according to Marquardt [Marquardt 1963]. The concentration of hormone present in the sample eluate was read from the standard curve after correction of the total counts for the contribution of the radioactivity by the recovery tracer. The concentration was then corrected for mass-contribution of recovery tracer and reagent blank. Finally, a correction for procedural losses was performed.

Student's t-test and Spearmann correlations were performed with the SAS program on a VAX/VMS computer.

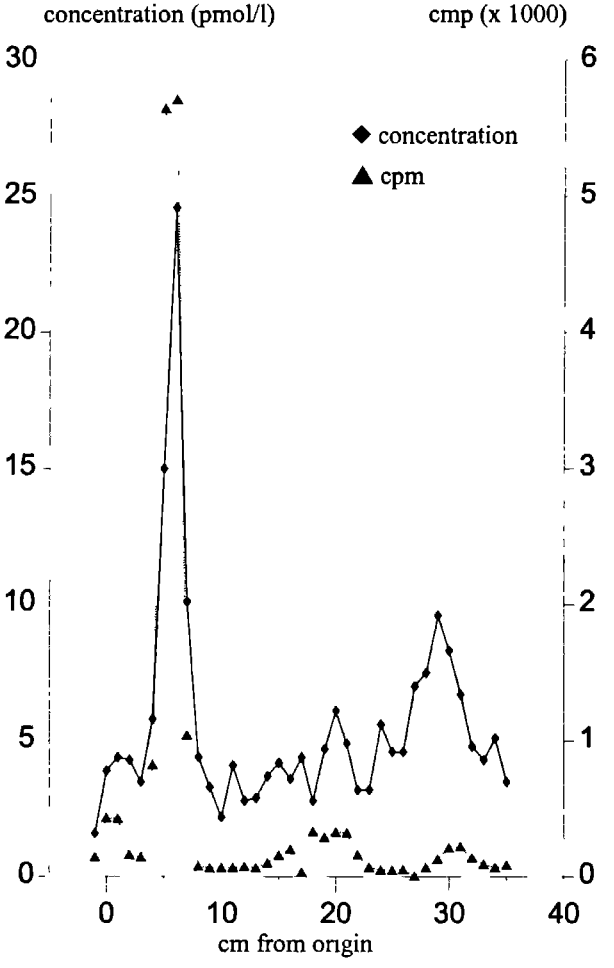

Figure 2.2: Radioactivity (cpm) of ^{3}H-1,25(OH)$_2$D along the paper chromatogram and concentration of 1,25(OH)$_2$D measured in plasma after chromatography. Radiolabeled 1,25(OH)$_2$D comigrates with nonlabeled 1,25(OH)$_2$D.

2.4 RESULTS

The $1,25(OH)_2D$ tracer was extracted from plasma by diethylether with an efficiency of 89 ± 4%. After paper chromatography and elution of the central area of the peak, the overall recovery of $1,25(OH)_2D$ was 42±5% (n=40).

The three main metabolites of vitamin D in plasma, 25(OH)D, $24,25(OH)_2D$ and $1,25(OH)_2D$ were efficiently separated by paper chromatography as shown in figure 2.1.

Radiolabeled $1,25(OH)_2D$ comigrated with nonlabeled $1,25(OH)_2D$, as was demonstrated in an experiment in which 10,000 dpm tracer was added to 1 ml of plasma. After extraction and chromatography, the chromatogram was scanned for radioactivity and then cut into pieces 1 cm long. Each piece of paper was eluted and the eluate was assayed for $1,25(OH)_2D$. Both the radioactivity and the concentration along the entire chromatogram are shown in figure 2.2. The peak in concentration observed at about 30 cm from the origin corresponds with the location of 25(OH)D.

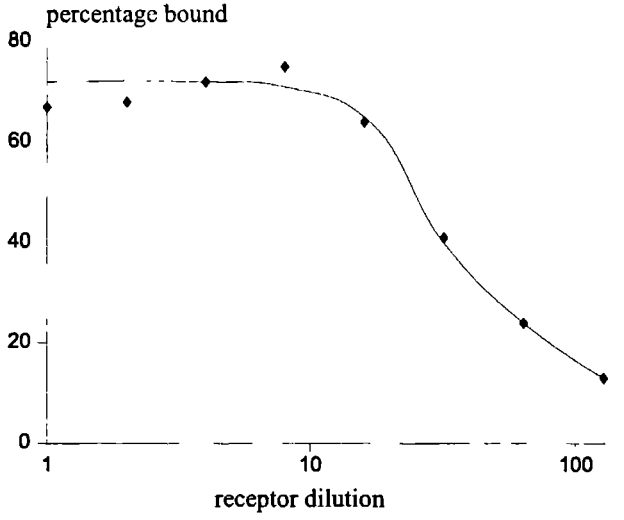

Figure 2.3: Percentage binding of ^3H-$1,25(OH)_2D$ after incubation for 3h at room temperature with different dilutions of calf thymus receptor preparation. The preparation was used in a 1:30 dilution.

In order to establish the appropriate dilution of the receptor preparation for the $1,25(OH)_2D$ assay, serial dilutions of the calf thymus receptor preparation were made. Figure 2.3 shows the binding of ^3H-$1,25(OH)_2D$ at different dilutions of receptor preparation. To obtain an initial binding of approximately 40%, a 1:30 (v/v) dilution was used for the assay.

Figure 2.4 shows a typical standard curve for the radioreceptorassay using the appropriate 1:30 dilution of the receptor preparation. The sensitivity was calculated as three times the standard deviation from the data on the zero sample. The detection limit was found to be 0.5 fmol/tube, corresponding to 4 pmol/l when 1 ml of plasma was assayed, recovery being taken into account.

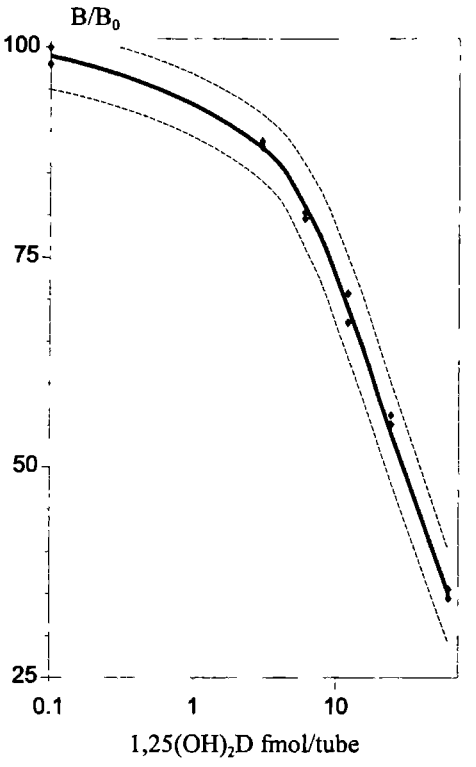

Figure 2.4: Typical standard curve for 1,25(OH)$_2$D assay. The sensitivity was 0.5 fmol/tube.

The intra- and interassay coefficient of variation were 10.5% (n=15 at 93.4 pmol/l) and 11.5% (n=8 at 103.3 pmol/l), respectively. Of one and the same plasma sample 4, 2, 1 and 0.5 ml were assayed to determine parallelism (n=5). The values (± SD) measured were 99 (±2), 95 (±12), 95 (±11) and 96 (±18) pmol/l, respectively. To investigate the efficacy of the recovery corrections, different aliquots of standard 1,25(OH)$_2$D were added to a plasma sample. The recovery of this standard was 107% ± 5% (100 pmol/l added)(n=9) and 115% ± 8% (200 pmol/l added)(n=8). Normal levels of 1,25(OH)$_2$D were obtained from plasma of 34 healthy men and 26 healthy women with ages ranging from 22-79 yr (men 50 ± 16 ; women 42 ± 13). The normal range for men was 81-195 pmol/l, the normal range for women was 83-200 pmol/l. There was no significant difference in range related to sex. Nor was there any significant difference in

concentration related to age, neither in men nor in women.

The present assay was compared with a $1,25(OH)_2D$ assay using C-18 purification. 53 samples were assayed with both methods. The results are shown in figure 2.5. The coefficient of correlation was 0.92 ($p \leq 0.0001$), the slope was 0.89 ($p \leq 0.0001$) and the intercept was 5.0 pmol/l (N.S.).

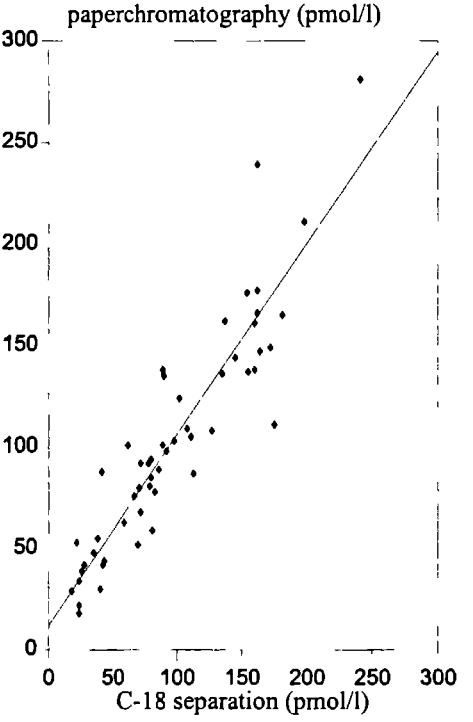

Figure 2.5: Correlation of measured values after solid-phase C-18 chromatography and paper chromatography. (n=53, r=0.92, p<0.0001).

The processed water blanks were obtained by assaying 1 ml of water instead of plasma. The water blank obtained after the C-18 purification (0.60 ± 0.28 fmol/tube) (n=20) was slightly lower than that obtained after paper chromatography (0.95 ± 0.33 fmol/tube) (n=20) ($p \leq 0.05$). The processed water blank after C-18/silica purification was significantly higher than those of the other two (1.95 ± 1.25 fmol/tube) (n=16) ($p \leq 0.01$).

The water blank after extraction and chromatography was found to be mainly due to the chromatographic step. The water blank resulting from ether extraction was about 2.5 times lower than that obtained from the paper chromatography and did not significantly contribute to the latter.

2.5 DISCUSSION

Liquid-liquid extraction and paper chromatography were successfully used to separate $1,25(OH)_2D$ from 25(OH)D and $1,24(OH)_2D$. Presently this separation is generally achieved by making use of HPLC [Watson *et al.* 1991, Eisman *et al.* 1976] or C-18/silica columns. However, HPLC and the C-18/silica method of Reinhardt *et al.* [Reinhardt *et al.* 1984] are rather expensive and time-consuming. The single-column method of Hollis [Hollis 1986] takes less time but does not separate $1,25(OH)_2D$ from $24,25(OH)_2D$. This limitation is of no consequence when in the assay no cross-reactivity occurs between these two metabolites, as is the case in the present RRA. Otherwise, as for example in the RIA of Clemens *et al.* [Clemens *et al.* 1978], Hollis' method fails.

The recovery of $1,25(OH)_2D$ after paper chromatography (42%±5%) (n=40) was not significantly different from the recovery after C-18 chromatography (38%±10%) (n=12). The processed water blank after paper chromatography (0.95 ± 0.33 fmol/tube) was slightly higher than that after C-18 chromatography (0.60 ± 0.28 fmol/tube). The intra- and interassay coefficients of variation (10.5%, 11.5%) of the present assay are of magnitudes similar to those reported by Hollis [Hollis 1986].

The sensitivity of our assay was 0.5 fmol/tube, calculated as three times the standard deviation of the counts of the zero sample. Reinhardt *et al.*, using two times the standard deviation of the zero sample, reported a sensitivity of 3.6 fmol/tube [Reinhardt *et al.* 1984], while Hollis, also using two times the standard deviation, reported a sensitivity of 1.7 fmol/tube [Hollis 1986]. A possible explanation for these differences may be found in different qualities of the receptorpreparations used, since the decline of the standard curve, and thus the sensitivity, is dependent on the quality of the receptor. The intra- and interassay CVs at normal range values were about 10% in all three assays, which means that even though we found a lower detection limit, the values within the normal range have a similar degree of accuracy.

Although the range of $1,25(OH)_2D$ concentrations in healthy people differs between laboratories, the range of our assay (80-200 pmol/l) is comparable with those found in the literature. (e.g. Reinhardt *et al.* [Reinhardt *et al.* 1984] 24-192 pmol/l ; Bouillon *et al.* [Bouillon *et al.* 1987] 92-168 pmol/l)

In accordance with Sherman *et al.* [Sherman *et al.* 1990] and Orwoll and Meier [Orwol & Meier 1986], we found no correlation between age and concentration of $1,25(OH)_2D$ in plasma. This is in contrast with the results of Bouillon *et al.* [Bouillon *et al.* 1987] and Tsai *et al.* [Tsai *et al.* 1984], who found concentrations and age inversely related. The latter two studies however, were not confined to healthy volunteers, but also comprised diseased subjects, so there is no point in comparing our results with theirs.

Because the production of $1,25(OH)_2D$ is an enzymatic process, low production in healthy persons must result either from low availability of 25(OH)D or from an age related decrease in enzymatic activity. Since we exclusively assessed plasma of healthy subjects with no history of any disease related to bone metabolism or vitamin D uptake, abnormally low 25(OH)D levels were not to be expected. Neither was there, in view of results published by other authors, any reason to expect a correlation between enzyme activity and age [Sherman *et al.* 1990, Orwoll & Meier 1986, Bouillon *et al.* 1987]. So the fact that we did not find such a correlation did not come as a surprise.

Interestingly, Bouillon *et al.* [Bouillon *et al.* 1987], in common with Sherman *et al.* [Sherman *et al.* 1990] found seasonal differences in $1,25(OH)_2D$ concentrations. Such fluctuations might influence the range of normal values.

We measured 53 samples using both paper chromatography and C-18 chromatography to purify the samples. The results were very similar as appears from the slope of 0.89 and a correlation of 0.92.

To conclude, the application of the old technique of paper chromatography has proven to be equally effective and reliable for the purification of $1,25(OH)_2D$ as state of the art techniques, except HPLC/MS. The latter however, requires costly instrumentation and, like solid-phase and HPLC purification, produces considerably more chemical waste per sample than paper chromatography. Solid-phase extraction and HPLC also require far more time per sample than paper chromatography: in our laboratory, 64 samples can be purified simultaneously with paper chromatography, as compared to 1 sample with HPLC and 10 samples with solid-phase extraction.

An additional advantage is that paper chromatography separates all three main metabolites of vitamin D in human plasma, in contradistinction to solid-phase extraction.

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CHAPTER 2

ADDENDUM

Effects of dihydrotachysterol and 1α(OH)D in the radioreceptorassay for $1,25(OH)_2D$

2A.1 INTRODUCTION

Administration of $1,25(OH)_2D$ has shown to be rather dangerous in the treatment of hypocalcemic disorders due the fact that an overdosage easily occurs, which leads to hypercalcemia. To circumvent this problem, both 1α-hydroxyvitamin D (1(OH)D) or dihydrotachysterol (DHT_2) are frequently used in the treatment of hypocalcemic disorders.
When both the 25- (liver) and 1-hydroxylases (kidney) are still functioning, vitamin D can be administered to increase the $1,25(OH)_2D$ concentration during hypoparathyroidism. In cases of renal failure however, administration of vitamin D or 25 hydroxyvitamin D (25(OH)D) is no solution, since there is no active renal 1-hydroxylase to produce $1,25(OH)_2D$. Because the hepatic 25-hydroxylase is still working in this case, 1(OH)D will be metabolized into $1,25(OH)_2D$, thus circumventing the diseased kidney [Saarem & Pedersen 1985]. However, since the 25-hydroxylase is not feedback regulated, caution must be taken when administering this substance. There are some reports of a direct action of 1(OH)D, but these actions are related only to the differentiation of cells and do not interfere with the calcium regulating functions of $1,25(OH)_2D$ [Honma *et al.* 1983]. This phenomenon mainly results from the affinity constant of the vitamin D receptor for 1(OH)D, which is 100-1000 times less than that for $1,25(OH)_2D$ [Zerwekh *et al.* 1974]
A good alternative for administering $1,25(OH)_2D$ is the administration of DHT_2. Since the half-life of $1,25(OH)_2DHT_2$ is shorter than that of $1,25(OH)_2D$, the hypercalcemia in case of an overdose is of much shorter duration [Harrison *et al.* 1967]. This is obviously an advantage of DHT_2 administration over vitamin D administration. The metabolism of DHT_2 in vivo has only recently been elucidated. In the liver, 25-hydroxylation takes place [Hallick & DeLuca 1972], and both 1α- and 1β-hydroxylated metabolites have been found [Schroeder *et al.* 1994]. Although it is not yet clear where these latter hydroxylations take place, the probable site is the kidney. Because of the rotated A ring as compared to vitamin D, the 3β-hydroxyl can act as a pseudo 1α-hydroxyl, indicating that the 1-hydroxylation is not necessary for a vitamin D like action. This has been confirmed by an enhanced intestinal calcium absorption after 25(OH)DHT administration, which was unaffected by prior bilateral nephrectomy [Suda *et al.* 1970]. The most active metabolite of DHT_2 has not yet been established.
The assay most often used for the determination of $1,25(OH)_2D$ is a radioreceptorassay, using the calf thymus vitamin D receptor as binding protein [Reinhardt *et al.* 1984]. Because of the structural similarities between on the one hand 1(OH)D and DHT_2 and on the other hand $1,25(OH)_2D$ (figure 1.1), an interference of the binding during the assay may occur due to the former substances. Possible displacement of $1,25(OH)_2D$ from the receptor by 1(OH)D and DHT_2 was studied to find whether spurious results in the $1,25(OH)_2D$ assay during therapy are

possible due to this interference.

2A.2 MATERIALS AND METHODS

Dihydrotachysterol-2 was purchased from Solvay Duphar (Weesp, The Netherlands), 1(OH)D (alfacalcidol) was kindly donated by Solvay Duphar. Both substances were dissolved in ethanol. Solutions of $1,25(OH)_2D$ (kindly donated by Dr. U. Fisher; F. Hoffmann-La Roche & Co, Basel, Switzerland) were made in 25% ethanol in phosphate buffer (0.04 M Na_2HPO_4 , 0.01 M NaH_2PO_4 , 0.10 mM MTG)(pH = 7.4).

A dilution range was made of all three solutions ranging from 250 nmol/l to 60 pmol/l.

To 500 µl of calf thymus receptor solution, 50 µl of tritiated $1,25(OH)_2D$ (NEN Products, Dreieich, Germany) and 50 µl of vitamin D analog solution was added. After incubation for 3 hours at room temperature, 150 µl 1% charcoal suspension in 0.05 M phosphate buffer containing 0.1 M KCl and 0.1 % Dextran T70 was added to separate free from bound hormone. The tubes were centrifuged (10 min, 2800 g, room temperature) and radioactivity was determined in the supernatant.

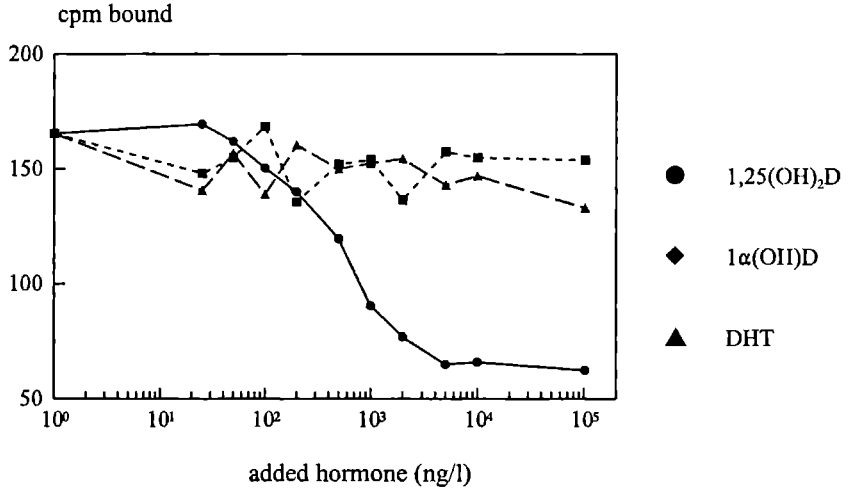

Figure 2a.1: Amount of ^{3}H-1,25(OH)$_2$D bound in the presence of increasing concentrations of different analogues.

2.A.3 RESULTS AND DISCUSSION

No displacement of ^{3}H-1,25(OH)$_2$D from the receptor by either 1(OH)D or DHT$_2$ was found, even at a concentration of 250 nmol/l. Since therapeutic concentrations of 1(OH)D and DHT$_2$ typically do not exceed 20 nmol/l, neither substance is expected to interfere in the 1,25(OH)$_2$D radioreceptorassay during therapy. Non-labelled 1,25(OH)$_2$D was capable of displacement of the ^{3}H-1,25(OH)$_2$D, with a 50% displacement at 1 pmol/l already (figure 2a.1).

Possible interference in the assay of 1,25(OH)$_2$D can still be expected of in vivo produced metabolites. Since 1(OH)D metabolizes to 1,25(OH)$_2$D, this can obviously not be seen as unwanted interference. It has been shown that DHT$_2$ is metabolized to 1,25(OH)$_2$DHT$_2$, although it isn't clear whether the active form is 1,25(OH)$_2$DHT$_2$ or 25(OH)DHT$_2$ [Schroeder *et al.* 1994]. These substances resemble 1,25(OH)$_2$D even more in their structure than DHT$_2$ and possible interference is therefore more likely. Unfortunately, it was impossible to obtain either one of these analogs for this study and it was therefore not possible to show tracer displacement. It has been shown that the calf thymus receptor does not seem to bind to any DHT$_2$ metabolite while the chicken receptor does, but no explanation for this phenomenon was found [Taylor & Norman 1987]. Bosch *et al.* found that 1,25(OH)$_2$DHT$_2$ comigrates with 1,25(OH)$_2$D during gas-chromatography, indicating that it is difficult to separate these two molecules by HPLC [Bosch *et al.* 1985]. Whether these metabolites can be separated by paper chromatography is unknown because of the lack of pure 1,25(OH)$_2$DHT$_2$. High 1,25(OH)$_2$D levels during DHT$_2$ therapy should therefore be looked at with great caution because of possible interference. On the other hand however, if 1,25(OH)$_2$DHT$_2$ does comigrate with 1,25(OH)$_2$D and is also measured in the radioreceptorassay, these results could be a better indication of biological vitamin D activity instead of only the 1,25(OH)$_2$D concentration. This may mean that it might be preferably to measure both metabolites.

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CHAPTER 3

Determination of non-protein bound plasma 1,25-dihydroxyvitamin D by symmetric (rate) dialysis

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3.1 ABSTRACT

Most of the total circulating 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) is bound to plasma proteins, mainly vitamin-D binding protein (DBP) and albumin. Only a small fraction in plasma exists in the free form. It is widely assumed that the non-protein bound, free hormone reflects the biologically active fraction. We describe a dialysis method for the determination of plasma free $1,25(OH)_2D$ which is relatively easy to perform. In this symmetric or 'rate' dialysis method, identical samples are placed at both sides of a membrane. At one side, tritiated $1,25(OH)_2D$ is added and the rate of transfer of this tritiated $1,25(OH)_2D$ through a dialysis membrane is directly related to the free fraction of plasma $1,25(OH)_2D$. This method is much less susceptible toward tracer impurities than indirect equilibrium dialysis and centrifugal ultrafiltration. Moreover, it requires much less tracer. The intraassay coefficient of variation for the determination of the free fraction is 1.0%; the interassay variation is 7.7%. Comparison of the free fraction of 23 samples assessed with both centrifugal ultrafiltration and symmetric dialysis showed much higher values using the former method. No significant correlation between the two methods was found. The free fraction of $1,25(OH)_2D$ in normal subjects as assessed with symmetric dialysis ranges from 0.049 to 0.103%.

3.2 Introduction

Vitamin D is mainly formed in the skin under the influence of light from 7-dehydrocholesterol. A small amount is absorbed directly from food. The first step in the activation of vitamin D, the 25-hydroxylation, takes place in the liver. In the kidney 25-hydroxyvitamin D is further hydroxylated to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the active form of the hormone. Both 25-hydroxyvitamin D and $1,25(OH)_2D$ can be deactivated via 24-hydroxylation in the kidney [Bouillon *et al.* 1995].

$1,25(OH)_2D$ plays an important role in calcium homeostasis and bone metabolism. It stimulates calcium uptake in the intestine and inhibits 1-hydroxylation of 25-hydroxyvitamin D in the kidney. Recently, evidence has been accumulated that $1,25(OH)_2D$ is also involved in cell differentiation, cell growth, insulin synthesis, and sex hormone synthesis [Frampton *et al.* 1983, Cade & Norman 1987, Meggouh *et al.* 1991, Pols *et al.* 1990].

Like steroid and thyroid hormones, $1,25(OH)_2D$ in plasma circulates mainly bound to a specific binding protein, i.e., vitamin D binding protein (DBP), and albumin [Bikle *et al.* 1985]. Furthermore, it has been suggested that a small fraction may also be bound to lipoproteins [Silver & Fainaru 1979]. $1,25(OH)_2D$ has a lower affinity for DBP than 25-hydroxyvitamin D [Bouillon & van Baelen 1981a]. The concentration of the latter is a thousand times higher than the

concentration of $1,25(OH)_2D$. However, since DBP has a very high binding capacity for both metabolites, the binding sites will not be saturated, so that most $1,25(OH)_2D$ is protein bound. Vieth [Vieth 1994] calculated that only 0.1% of the total $1,25(OH)_2D$ in plasma circulates in the free form. According to the so called "free hormone hypothesis" [Robbins & Rall 1957, Mendel 1989], the concentration of non-protein bound hormones in plasma reflects the biologically active hormone better than the concentration of total hormone in plasma [Ekins 1990].

Most authors use a free $1,25(OH)_2D$ index as an estimate of the free $1,25(OH)_2D$ concentration. This index is calculated as the molar ratio of $1,25(OH)_2D$ and DBP [Bouillon *et al.* 1981b] and is related to the free concentration. However, it neglects the concentrations of albumin and lipoproteins, which also bind $1,25(OH)_2D$. Although this probably is of minor importance in normal situations, it may lead to discrepancies between the actual free concentration and the free index in pathological situations.

The radioreceptorassay routinely used for the determination of total $1,25(OH)_2D$ in plasma is not sensitive enough to directly determine the very low concentration of free $1,25(OH)_2D$ found in dialysate or ultrafiltrate after separation of bound and free hormone [van Hoof *et al.*1993]. Therefore, to be able to determine the free $1,25(OH)_2D$ concentration, one must use an indirect approach, i.e. by measurement of the free fraction followed by multiplication of this fraction with the total concentration to yield the concentration of plasma free $1,25(OH)_2D$. Measurement of the free fraction using an indirect approach could be accomplished by adding a small amount of highly purified tracer to the plasma, followed by dialysis or ultrafiltration.

To our knowledge only one method for the determination of free $1,25(OH)_2D$ has been described so far [Bikle *et al.* 1984]. This method is based on the centrifugal ultrafiltration technique described by Hammond *et al.* [Hammond *et al.* 1980] and was adopted to measure free $1,25(OH)_2D$ by Bikle *et al.* [Bikle *et al.* 1984]. An attempt by the same authors to use equilibrium dialysis for this purpose failed due to adsorption of $1,25(OH)_2D$ to the dialysis cell walls and severe deterioration of the radioactive tracer. Using the ultrafiltration method, Koenig *et al.* [Koenig *et al.* 1992] showed that the free fraction of $1,25(OH)_2D$ in plasma was larger in patients with nephrotic syndrome, who lose DBP, than in healthy subjects. In patients with vitamin D intoxication and severe hypercalcemia, Pettifor *et al.* [Pettifor *et al.* 1995] found an elevated level of the free $1,25(OH)_2D$ together with a normal level of the total hormone. These reports confirm the free hormone hypothesis.

In this paper we describe an alternative dialysis method to assess free $1,25(OH)_2D$ in plasma which is reproducible and relatively easy to perform. In this symmetric or 'rate' dialysis method, the rate at which tritiated $1,25(OH)_2D$ migrates through a dialysis membrane from one compartment to the other containing the same plasma sample is a function of the magnitude of the free fraction of $1,25(OH)_2D$. With this method several disadvantages associated with

ultrafiltration and equilibrium dialysis are circumvented. Earlier, symmetric dialysis was successfully applied to assess free thyroid hormone [Ross 1978], free testosterone [Swinkels *et al.* 1987], and free progesterone and cortisol [Willcox *et al.* 1983]. symmetric dialysis requires calibration against (preferably) direct equilibrium dialysis using appropriate reference binding protein solutions.

3.3 MATERIALS AND METHODS

3.3.1 SUBJECTS

Plasma samples were obtained from healthy subjects [10 men, 10 non-pregnant women, 10 pregnant women (third trimester)]. None of the subjects were taking any medicine or oral contraceptives. The study was performed according to the standards of the ethical committee of the Academic Hospital Nijmegen, St. Radboud, The Netherlands. For assays comparison, 23 plasma samples were randomly obtained from healthy subjects.

3.3.2 ASSAY OF TOTAL 1,25(OH)$_2$D

The total concentration of 1,25(OH)$_2$D in plasma was assessed using a radioreceptorassay after extraction of the samples followed by paper chromatographic separation, as described previously [van Hoof *et al.* 1993]. The detection limit was 4 pmol/l, when 1 mL of plasma was assayed, recovery being taken into account. This value was obtained by subtracting 3 times the SD of the initial binding from the initial binding and reading the result from the standard curve. The intraassay coefficient of variation was 10.5% (at a level of 93.4 pmol/l (n=15)). The interassay coefficient of variation was assessed from 8 consecutive assays of one sample in duplicate and was 11.5% at 103.3 pmol/l. The concentration range of 1,25(OH)$_2$D in healthy, non-pregnant subjects was 80-200 pmol/l (n=60).

3.3.3 DIALYSIS APPARATUS

For the dialysis experiments, a Dianorm dialysis apparatus was used (Dianorm GmbH, München, Germany). With this apparatus 20 dialysis cells, each with a volume of either 2 x 1.0 mL or 2 x 200 µL, can be rotated at constant speed in a waterbath at 37°C. Diachema dialysis membranes (exclusion limit 10,000 Dalton) were used. The membranes were rinsed before use with 50 mmol/l phosphate buffer, 0.1 mol/l NaCl (pH 7.40) (PBS).

3.3.4 *EQUILIBRIUM DIALYSIS*

Symmetric dialysis was calibrated using indirect equilibrium dialysis of a human serum albumin (HSA) reference preparation. For this purpose 2.0 KBq 1α,25-[26,27]^{3}H-$(OH)_2D_3$, (NET 626, 6.3 TBq/mmol, NEN Dupont de Nemours, The Netherlands)(317 fmol) was dried under a stream of nitrogen and 1.0 mL HSA (2, 4, 10 and 20% w/v in PBS) (OHRA 20/21, Behringwerke AG, Marburg, Germany) was added. This solution was subsequently dialysed against 1.0 mL of PBS during 4 hours at 37°C, after which equilibrium had been reached (unpublished results). Radioactivity was determined in 400 µL aliquots of the dialysand and the dialysate during 10 minutes. During equilibrium dialysis, some adsorption of the radioactive $1,25(OH)_2D$ occurred. To assess the total amount of tracer present after dialysis, the amount of radioactivity was measured after dialysis and corrected for the volume shift that takes place due to osmosis. This shift was assessed beforehand by assaying the amount of protein in the dialysand before and after dialysis using an assay for total protein (BioRad, Richmond, CA). The effects of tracer contamination on the final result are minimized on the one hand by purification just before dialysis using high performance liquid chromatography (HPLC) on a LiChrosorb DIOL Column (Merck, Darmstadt, Germany). The $1,25(OH)_2D$ peak contained (98%) of the total radioactivity. On the other hand, analysis of the results obtained by indirect equilibrium dialysis of the serial dilutions of HSA in PBS permits assessment of, and appropriate correction for, non-protein-bound impurities that were still present or possibly were generated during dialysis. By virtue of the low affinity of HSA for $1,25(OH)_2D$, the ratio bound/free is directly proportional to the albumin concentration. Furthermore, the free fraction is independent of the $1,25(OH)_2D$ concentration. By extrapolation of the apparent free/bound ratio vs. dilution to zero dilution (infinite binding) an estimate of the percentage tracer that cannot bind to albumin is obtained.

3.3.5 *SYMMETRIC DIALYSIS*

To assess the dialysis rate of samples in symmetric dialysis, identical plasma samples were placed on both sides of a dialysis membrane. The free fraction of $1,25(OH)_2D$ of an unknown sample (f_x) was assessed by comparing the dialysis rate of this sample (U_x) with the dialysis rate of a reference sample (U_r) with a known free fraction (f_r). The reference sample in symmetric dialysis was 1.0% *w/v* HSA in PBS, of which the free fraction had been determined by equilibrium dialysis as described above. In each experiment, the dialysis rate of this reference sample was assessed in quadruplicate. For this purpose, one compartment of the dialysis cell was filled with 1 mL HSA solution and the other compartment was filled with the same HSA solution to which 0.15 KBq ^{3}H-$1,25(OH)_2D_3$ (23.8 fmol) was added. Dialysis was performed in a waterbath at 37°C

for 16 h. After dialysis, radioactivity was determined in two 400 µL aliquots of the dialysate and of the dialysand for 10 minutes. Typical count rates in 400 µL of dialysate ranged from 200 to 300 cpm.

In the same way, the dialysis rates of unknown plasma samples were determined. Before dialysis, the plasma samples were diluted fifteen-fold in PBS. At this dilution the free fraction is such that about 20-30% of the tracer migrates through the membrane during dialysis for approximately 16 h. If the dialysis process is terminated when a smaller fraction has migrated, the effect of minor tracer impurities would be more pronounced. Conversely, a higher migrated fraction would result in lower precision of the measurement as the difference between the amount of radioactivity in the two compartments becomes too small. The amount of tracer added (23.8 fmol) corresponds to 347 pmol/l in undiluted plasma, which is in the order of magnitude of the endogenous total $1,25(OH)_2D$ concentration.

In symmetric dialysis, the rate at which the tracer migrates from one compartment to the other is related to the magnitude of the free fraction of hormone according to the relation:

$$2\frac{AD}{V}\times f = -\frac{1}{t}\ln\frac{(c_1-c_2)}{(c_1+c_2)} \qquad \text{Formula 3.1}$$

in which:

AD/V = cell permeability constant

A = membrane area

D = membrane diffusion constant

V = sample volume in the dialysis system

t = time of dialysis (hours)

c_1 = concentration of radioactivity in dialysis compartment 1

c_2 = concentration of radioactivity in dialysis compartment 2

f = free fraction.

The tracer distribution (I) in the system is indicated as: $I = (c_1 - c_2)/(c_1 + c_2)$;
the dialysis rate as: $U = (-1/t) \times ln(I)$, therefore $U = 2(AD/V) \times f$.
From this formula it follows that the log tracer distribution $ln(I)$ is a linear function in time (t). Moreover, the dialysis rate (U) is directly proportional to the free fraction.
The free fraction of $1,25(OH)_2D$ of the unknown sample can then be calculated using the equation: $f_x = (U_x/U_r) \times f_r$.
The free fraction of the original undiluted plasma was estimated according to the formula [Willcox *et al.* 1983]:

$$f'_0 = \frac{1}{(\frac{1}{f} - 1)\times\frac{V_t}{V_0} + 1}$$ Formula 3 2

in which

f'_0 = the estimated free fraction of the undiluted sample

f = the free fraction assessed with symmetric dialysis in diluted plasma

V_t = total volume of the dialysis system

V_0 = original volume of the plasma

This formula takes into account that, according to the law of mass action, it is actually the free/bound ratio that is proportional to dilution It is based on the assumption that a moderate degree of dilution does not change the saturation of the binding proteins

The free concentration was calculated by multiplying the free fraction (f'_0) by the total concentration of $1,25(OH)_2D$ in plasma

3 3 6 ASSESSMENT OF TRACER STABILITY

Tracer stability was investigated in two ways first, by HPLC of tracer before and after 16 h of symmetric dialysis, and second, by indirect equilibrium dialysis using 1 1 diluted pool plasma Chromatograms were run after diethylether extraction of tracer from plasma Subsequently, the relative amount of impurity was recorded

The conditions imposed on the tracer during symmetric dialysis of plasma samples were simulated by incubation of 2 x 180 µL of newly purified tracer in 1 15 diluted plasma in rotating dialysis cells at 37°C for 1, 2, 4, 8 and 16 h At the end of this period, 150 µL aliquots were taken and added to 150 µL of undiluted plasma Subsequently this mixture was dialysed against 180 µL of PBS for 2 h The true free $1,25(OH)_2$ D fraction of 1 1 diluted plasma is so low, that after equilibration, virtually all radioactivity present in the dialysate consists of unbound contaminants The effect of symmetric dialysis on the tracer purity during increasing time intervals could thus be monitored

3 3 7 CENTRIFUGAL ULTRAFILTRATION

Centrifugal ultrafiltration was performed as described by Bikle *et al* [Bikle *et al* 1984] on the same HSA solutions that had been measured by indirect equilibrium and symmetric dialysis and on 23 plasma samples at the same dilution as used for symmetric dialysis (1 15)

The actual ultrafiltration process took one hour

3.3.8 STATISTICS

The intraassay coefficient of variation of symmetric dialysis was estimated from the mean of variances of fourfold measurements on one sample performed in 20 consecutive runs. From the variance of the 20 quadruplicate means the interassay coefficient of variation was calculated, taking into account that this variance contains one-fourth of the intraassay variance.
Significances of differences between groups were calculated by ANOVA followed by t-testing with the Bonferroni correction for multiple comparisons (SAS statistical program on a VAX/VMS computer).

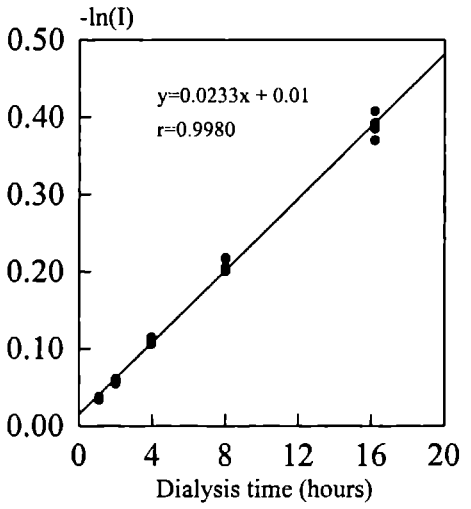

Figure 3.1: The tracerdistribution (-ln(I)) over both dialysis-compartments after symmetric dialysis of a 10% w/v HSA solution at 37° is proportional to the dialysis time.

3.4 RESULTS

Symmetric dialysis behaved according to theory (formula 3.1): a direct proportionality exists between ln(I) (log tracer distribution) and dialysis time (figure 3.1). The dialysis rate was directly proportional to the free $1,25(OH)_2D$ fraction ($r=0.995$, $p<0.0001$) (figure 3.2). The free $1,25(OH)_2D$ fractions of different concentrations (1, 2, 5 and 10%) of HSA in PBS were estimated by indirect equilibrium dialysis of 2, 4, 10 and 20% HSA against an equal volume of buffer. According to theory, a linear relation was obtained between the relative dilution factor (1% HSA: dilution factor =1) and the ratio free/bound $1,25(OH)_2D$ ($r=0.968$, $p<0.0001$). The

small but significant intercept observed at zero dilution, i e infinite binding protein concentration represents an estimate of an impurity present (0 14%) in the tracer that cannot be bound to albumin (figure 3 3)

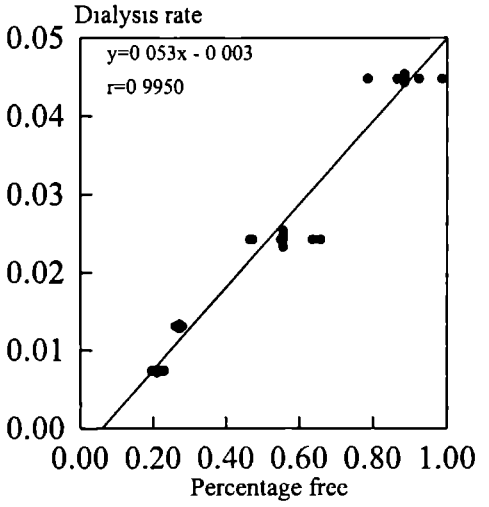

Figure 3 2 The dialysis rate (U) is proportional to the free fraction 1,25(OH)$_2$D after symmetric dialysis at 37° The free fractions of solutions of different concentrations of HSA in 0 1 M phosphate buffer, 0 1 M NaCl, pH 7 4 were assessed using indirect equilibrium dialysis and HPLC purified ^{3}H-1,25(OH)$_2$D$_3$

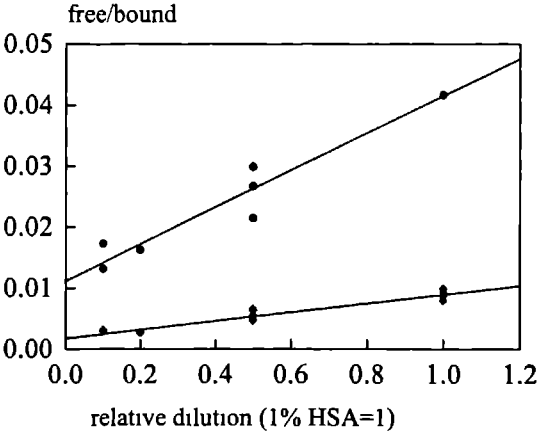

Figure 3 3 Free/bound ratio estimates in serial HSA dilutions by indirect equilibrium dialysis (◆ , y=0 007x+0 0014 , r=0 9672) and centrifugal ultrafiltration (● , y=0 030x+0 011 , r=0 9793) vs relative dilution (1% HSA=1)

Table 3.1

Mean, range, and standard deviation (s.d.) of the total concentration, free percentage, and free concentration of plasma 1,25(OH)$_2$D in men (n=10), non-pregnant women (n=10), and pregnant women (n=10).

		total conc. (pmol/l)	free %	free conc. (fmol/l)
men	mean	131	0.067	88
	s.d.	29.5	0.0092	19.6
	range	83-173	0.049-0.077	63-116
non-pregnant women	mean	128	0.083	107
	s.d.	18.4	0.0017	26.5
	range	95-158	0.063-0.103	67-139
pregnant women	mean	239	0.045	108
	s.d.	67.5	0.0039	27.9
	range	128-337	0.040-0.053	66-149

When tracer was dialysed for two hours in 1:1 diluted plasma against PBS after having been subjected to symmetric dialysis conditions in 1:15 plasma dilution for various time periods, a significant rise in dialysate radioactivity was observed. It ranged from undetectable without pretreatment to 4% of total activity after 8 h. A longer period of 16 h did not show any further effect. This is in accordance with other observations, such as the very slight contamination of less than 0.2% that was just detectable from the experiment with serial HSA dilutions as given above. Also the finding of 4% decrease in the main peak in the HPLC chromatogram after 16 h of symmetric dialysis is in line with this observation.

The condition for optimal precision of symmetric dialysis (when between 1/4 and 1/3 of tracer has passed through the membrane) is attained when dialysing 1 mL of 1:15 diluted plasma or 1% HSA for 16 h. Therefore the 1:15 dilution of plasma samples was chosen for routine measurements with 1% HSA as the calibrator. Dilutions of 1:10 up to 1:30 gave essentially the same results after correction for dilution.

From the quadruplicate measurements on a control plasma in 20 runs, a within-run CV of only 1% was obtained. The 20 quadruplicate means yielded a between-run CV of 7.7%, after correction for the within-assay component in the variance of these means.

With centrifugal ultrafiltration a linear relation between the reciprocal albumin concentration

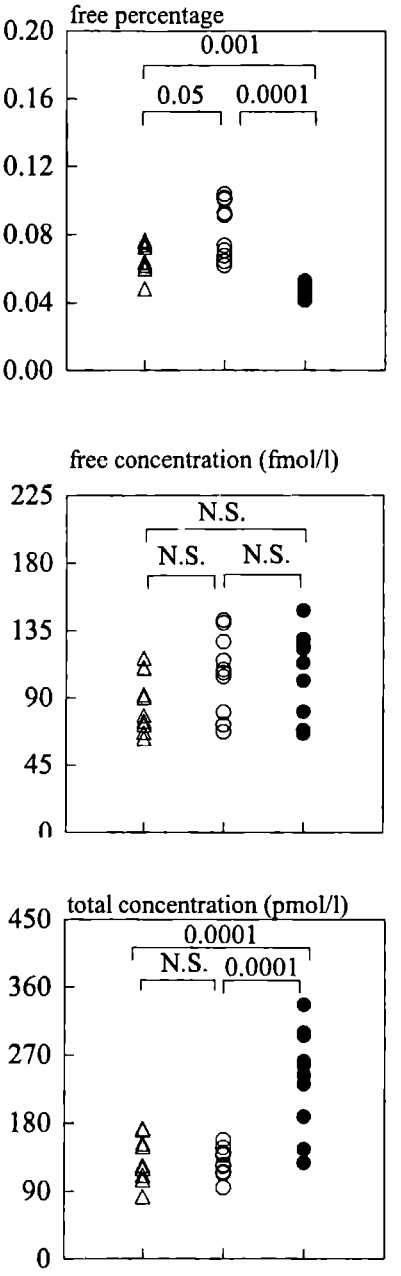

Figure 3.4: Free percentage, free concentration, and total concentration 1,25(OH)$_2$D in men (n=10), non-pregnant women (n=10), and pregnant women (n=10). △ *= men;* ○*=non-pregnant women;* ●*=pregnant women*

and the free $1,25(OH)_2D$ fraction estimate was also obtained ($r = 0.983$). However, both the slope and the intercept were much higher than for indirect equilibrium dialysis (figure 3.3). Likewise, the free $1,25(OH)_2D$ fraction estimates in 23 plasma samples were between 60 (at low values) and 10 (high values) times higher than for symmetric dialysis. No significant correlation between the two methods could be detected. In fact, the difference between the two methods became increasingly larger at lower free fractions as assessed by symmetric dialysis.

table 3.1 shows the results of measurements of the total concentration, the free fraction, and the free concentration of $1,25(OH)_2D$ in healthy men (n=10), non-pregnant women (n=10) and pregnant women (n=10).

ANOVA revealed a significant difference between the free fraction of $1,25(OH)_2D$ in pregnant women and the free fraction in both men and non-pregnant women, whereas the total $1,25(OH)_2D$ in pregnant women was significantly higher than that in men and non-pregnant women. There was no significant difference in the free concentration between any two of the three groups (figure 3.4).

3.5 DISCUSSION

Like the thyroid and steroid hormones, the three main metabolites of vitamin D (25-hydroxy-vitamin D, 24,25-dihydroxy-vitamin D, 1,25-dihydroxy-vitamin D) circulate in blood bound mainly to a specific binding protein, i.e. DBP. The affinity constant (K_a) for the binding of DBP to $1,25(OH)_2D$ has not yet been determined accurately. Reported values of the K_a for the binding of DBP range from 2.9×10^6 to 3.7×10^7 M^{-1}, the reported K_a of albumin is about 5.4×10^4 M^{-1} [Bikle *et al.* 1985, Brunvand & Haug 1994]. Therefore, the free fraction cannot be calculated accurately. Estimates of the fraction of $1,25(OH)_2D$ bound to DBP range from 85% to 95% and the estimates of the fraction bound to albumin range from about 15% to 5% [Bikle *et al.* 1985, Brunvand & Haug 1994]. Only a small fraction of $1,25(OH)_2D$ circulates in the free, non-protein bound form. According to the free hormone hypothesis [Robbins & Rall 1957, Mendel 1989] this free concentration governs the hormone's physiological effects. To assess the relevance of the free hormone hypothesis for $1,25(OH)_2D$, there is need for accurate assessment of the free concentration in plasma. Equilibrium dialysis of plasma followed by specific assessment of the free ligand in the dialysate (direct equilibrium dialysis) is considered as the gold standard method. In principle the same holds for ultrafiltration followed by free ligand assay in the filtrate.

However, the radioreceptorassay for $1,25(OH)_2D$ is relatively insensitive and is just barely suitable for measurement of total $1,25(OH)_2D$ in healthy persons [van Hoof *et al.* 1993]. Since the free concentration is less than 1% of the total concentration, this assay cannot be applied to directly assess the free concentration of $1,25(OH)_2D$ in dialysates or ultrafiltrates.

Alternatives are the indirect equilibrium dialysis and indirect ultrafiltration. An advantage of these methods lies in the fact that they, like the direct methods, constitute *absolute* methods. Unfortunately these techniques are very susceptible to artefacts, especially those arising from tracer impurity and, in the case of ultrafiltration, the risk of protein leakage through the membrane [Dowsett *et al.* 1984]. Furthermore, adsorption of free (labelled) hormone is a potential risk.
To our knowledge, only one method for determination of free $1,25(OH)_2D$ has been described until now [Bikle *et al.* 1984]. This centrifugal ultrafiltration method is based on the previous described method of Hammond *et al.* for assessment of free steroid hormones [Hammond *et al.* 1980]. Apart from the disadvantages inherent to all indirect methods, only little ultrafiltrate is produced so that large amounts of -very expensive- tracer are necessary. The amount of tracer required exceeds the endogenous concentration ten times which is likely to lead to equilibrium displacement resulting in falsely elevated free $1,25(OH)_2D$ estimates. Furthermore, notwithstanding the large amounts of tracer used, the reported precision of this method is not high.
The problems encountered in these indirect methods can be eliminated or minimized in symmetric dialysis. This however, is a *relative* method: the dialysis rate of an unknown is compared to the dialysis rate of a reference solution with a known free fraction. Thus, a reference solution with a known free fraction must be used as a standard in symmetric dialysis. The only essential requirement for such a reference standard is that its free fraction can be accurately assessed by one of the afore-mentioned absolute methods. Ideally, direct equilibrium dialysis should be applied, as for this purpose the total concentration of $1,25(OH)_2D$ may be increased to attain free levels that can easily be assessed by the available radioreceptorassay method. Unfortunately, such concentrations cannot be attained due to the limited solubility of $1,25(OH)_2D$. Therefore one has to resort to one of the indirect methods, which implies that the afore mentioned artefacts have to be faced. At first sight, the advantages of symmetric dialysis would thereby disappear since the results ultimately will depend on the accuracy on an indirect method. This however ignores the fact that the accuracy that can be attained for a calibration solution -by optimal choice of the binding protein and design of the procedure- will by far exceed the possibilities with actual serum samples, primarily because there is almost no restriction to the amount of tracer added. Equilibrium displacement, if occuring, is not relevant, since all that matters is the free fraction in the presence of the same amount of tracer that is used in symmetric dialysis. Tracer contamination can be assessed and corrected for by extrapolating the free/bound ratio, as measured in serial dilutions of serum albumin to zero dilution (infinite binding protein concentration), where the free radioactivity only can represent non-vitamin D activity. This is possible because, due to the low affinity of albumin, the free/bound ratio is directly proportional to dilution. This, in addition to the fact that they can be very reproducibly prepared, makes serum

albumin solutions very suitable to serve as calibrators.

The effect of adsorption in the calibrator solution can be assessed and corrected for by measuring the total amount of unadsorbed tracer on both sides of the membrane. This also requires the assessment of total protein before and after dialysis to correct for osmotic shifts. This would not be feasible if using indirect equilibrium dialysis for straightforward assessment of the free fraction of $1,25(OH)_2D$ of unknown serum samples. The loss of tracer due to adsorption during equilibrium dialysis was very rapidly established and remained constant for 6 hours. The amount of tracer lost was about 12%, which is much lower than the 40% reported by Bikle *et al.* [Bikle *et al.* 1984]. The reason for this can presumably be found in the material of the cells used. Bikle *et al.* used plexiglass cells while we used teflon cells. This finding indicates that the material used when handling $1,25(OH)_2D$ may be of importance for the final results. In contrast, during symmetric dialysis there is no detectable adsorption to the cell walls as there is with equilibrium dialysis. This lack of adsorption to the cell walls during symmetric dialysis is probably caused by the presence of binding proteins on both sides of the membrane.

In symmetric dialysis, optimal precision is attained when dialysis is continued until between 1/4 and 1/3 of tracer has migrated to the other compartment. This distribution is reached with 1:15 diluted sera after about 16 hrs, which is considerably longer than for equilibrium dialysis or centrifugal ultrafiltration. In a special experiment addressing tracer stability during symmetric dialysis of 1:15 diluted plasma as well as seen on HPLC chromatograms, tracer deterioration after 16 h dialysis at 37°C was 4%. This indicates an overestimation of the free fraction of about 5.7%.

Next comes the question whether centrifugal ultrafiltration as described by Hammond could be an alternative to indirect equilibrium dialysis as the calibration method. Theoretically these methods would be equally well suited. In practice, however, the pressure exerted on the membrane in centrifugal ultrafiltration potentially may lead to protein leakage which, however small, results in spuriously elevated values [Dowsett *et al.* 1984]. When albumin solutions with tracer were subjected to both equilibrium dialysis and centrifugal ultrafiltration, either displayed linearity of the estimated free fraction with dilution. However, the intercept at zero dilution was 1.1% in the case of centrifugal ultrafiltration after one hour, such in contrast to the bias of 0.14% that is probably due to tracer impurities as mentioned above for indirect equilibrium dialysis. This is suggestive of the presence of protein leakage. One would expect, however, that such leakage of protein-bound tracer would be more or less independent of protein concentration so that the difference between dialysis and filtration would be the same for all albumin concentrations. Instead, with centrifugal ultrafiltration the slope was about 4 times as steep (figure 3.3). Therefore, one should also consider the possibility of preferential absorption of ^3H-$1,25(OH)_2D$ to ^{14}C-glucose by the filterpaper discs supporting the filtration membrane. In his original article, Hammond excluded this possibility for estradiol, testosterone and progesterone, but a similar

check for ^{3}H-1,25(OH)$_2$D cannot be found in Bikle's article. From the point of view of precision, in the case of indirect equilibrium dialysis more than 0.5 ml of dialysate is available for radioactivity counting, whereas only about 30 µl can be obtained from the centrifugal ultrafiltration. This can only partially be compensated for by increasing the amount of tracer added, the disadvantages of which have been discussed earlier. In an assessment of the free fraction of 1,25(OH)$_2$D in 23 plasma samples using both methods, no significant correlation between the two methods could be detected and the increasing difference at lower free fractions as assessed with symmetric dialysis again indicates a protein leak through the membrane during centrifugal ultrafiltration.

The t½ of the dissociation of 1,25(OH)$_2$D from DBP is 200 s [Levy *et al.* 1985]. This means that the fraction that dissociates per second from DBP is 3.47×10^{-3}. With a cell permeability constant of 5.3 h^{-1} (figure 3.2) the maximum fractional diffusion per second through the membrane is 1.47×10^{-5}, which is 2 orders of magnitude smaller than the dissociation from DBP. This means that during dialysis the dissociation from DBP will not be rate-limiting.

Rotating the cells during dialysis at different speeds did not result in a different dialysis rate (data not shown). Assuming that with faster rotation, the diffusion layer at the membrane/solution interface becomes thinner, this demonstrates that diffusion to the membrane surface also is not a rate limiting step in the dialysis process.

The intra- and interassay coefficients of variation of assessment of the free fraction using the symmetric dialysis method (1.0% and 7.7% respectively) are much lower than those using the centrifugal ultrafiltration (13% and 26% respectively)[Bikle *et al.* 1984]. This can obviously be seen as the consequence of the advantages of the method described in this paper.

The free fraction 1,25(OH)$_2$D found in healthy volunteers, determined using symmetric dialysis is somewhat below the values reported by Vieth [Vieth 1994], which values were calculated based on an experimentally found relation between the binding capacity of the DBP and the free fraction of the 1,25(OH)$_2$D. Our values are also about ten times lower than those reported by Bikle *et al.* [Bikle *et al.* 1984] and Koenig *et al.* [Koenig *et al.* 1992], which is in accordance with our own measurements using centrifugal ultrafiltration at small free fractions. This finding most likely results from the above mentioned limitations of the centrifugal ultrafiltration method.

The total 1,25(OH)$_2$D concentration in pregnant women was significantly larger than that in non-pregnant women. The free concentration however, showed no significant difference between the two groups (figure 3.4). This finding strongly supports the free hormone hypothesis, since during pregnancy there are no clinical signs of hypercalcemia.

The symmetric dialysis method offers the possibility to routinely and reproducibly assess plasma free 1,25(OH)$_2$D levels. The results of these measurements may shed more light on the biological role of 1,25(OH)$_2$D in plasma, as well as help to establish biologically active 1,25(OH)$_2$D levels

in situations where an abnormal DBP level can be expected like for example in pregnancy, liver cirrhosis, or renal failure.

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CHAPTER 4

Total and free 1,25-dihydroxyvitamin D in conditions of modified binding; especially in nephrotic syndrome

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4.1 ABSTRACT

It has been generally accepted that the non-protein bound fraction of a steroid hormone is a better representation of its biological activity than the total concentration. Using a novel, sensitive and accurate assay, the free 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) concentrations were studied in various populations known to differ in total $1,25(OH)_2D$ levels: 12 healthy women before and after 3 months of oral estrogen/progestagen treatment, 10 pregnant women, and 16 patients with a nephrotic syndrome and normal renal function.

In normal women, the concentrations of total $1,25(OH)_2D$, free $1,25(OH)_2D$ and vitamin D binding protein (DBP) were 132 ± 19 pmol/l, 92± 30 fmol/l, and 5.59 ± 0.43 µmol/l. After 3 months of estrogen/progestagen treatment, total $1,25(OH)_2D$ and DBP levels rose significantly to 175 ± 51 pmol/l and 8.32 ± 1.59 µmol/l ($p \leq 0.05$ and $p \leq 0.001$); the free $1,25(OH)_2D$ remained unchanged (105 ± 39 fmol/l). Compared to normal women, pregnant women had significantly higher levels of total $1,25(OH)_2D$ and DBP (239 ± 68 pmol/l and 11.32 ± 1.77 µmol/l; both $p \leq 0.001$); the free $1,25(OH)_2D$ level, however, was not different (104 ± 27 fmol/l; NS).

Unexpectedly, in patients with nephrotic syndrome no lowering of DBP levels was found (5.36 ± 0.84 µmol/l) as compared to those in healthy subjects. Despite this, levels of both total (69 ± 26 pmol/l, $p \leq 0.001$) and free $1,25(OH)_2D$ (53 ± 28 fmol/l; $p \leq 0.001$) were significantly lower than in healthy subjects. Serum albumin levels were lowered to about half the reference values. In patients with the nephrotic syndrome, positive correlations were found between serum albumin levels and both the total and free $1,25(OH)_2D$ concentrations ($r=0.54$, $p \leq 0.05$ and $r=0.52$, $p \leq 0.05$ respectively), and a negative correlation between urinary protein loss and serum 25-hydroxyvitamin D ($r=-0.55$; $p \leq 0.05$).

These results show that higher exogenous or endogenous premenopausal estrogen levels result in higher DBP levels with a parallel rise in total $1,25(OH)_2D$ levels, but do not change the biologically active moiety. The results in patients with nephrotic syndrome show that with increasing glomerular protein leakage, the free $1,25(OH)_2D$ concentration cannot be maintained, due to the loss of large amounts of both protein-bound 25(OH)D and $1,25(OH)_2D$.

4.2 INTRODUCTION

Vitamin D is an important hormone which is involved in many biological processes. Apart from vitamin D, three more major metabolites of vitamin D circulate in plasma, namely 25-hydroxyvitamin D (25(OH)D), 24-25 dihydroxyvitamin D ($24,25(OH)_2D$) and 1,25

dihydroxyvitamin D (1,25(OH)$_2$D). This latter hormone is considered to be the actual active metabolite [Norman 1971]. It stimulates calcium uptake through the intestine [Nicolaysen 1937] and is involved in the calcium homeostasis by regulating parathyroid hormone (PTH) [Chertow *et al.* 1975], calcitonin [Cote *et al.* 1987], osteocalcin [Zerwekh *et al.* 1985], and osteoclast formation [Reichel *et al.* 1989]. Besides this classical role, there have been many reports lately of involvement of vitamin D in cell differentiation [Stumpf *et al.* 1979], cell growth [Frampton *et al.* 1983] and apoptosis [Naveilhan *et al.* 1994].

All the vitamin D metabolites that circulate in blood are bound to carrier proteins. The active metabolite, 1,25(OH)$_2$D, has been shown to bind to a specific binding protein, vitamin D binding globulin (DBP) [Haddad 1979], to albumin [Bouillon *et al.* 1980], and perhaps also to lipoproteins [Silver & Fainaru 1979]. The DBP synthesis takes place in the liver and can be stimulated with oral estrogens [Haddad & Walgate 1976a], in the same fashion as other steroid binding proteins of hepatic origin, like steroid hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG). This increased DBP level is capable of binding more 1,25(OH)$_2$D. Whereas the binding of vitamin D to DBP occurs with an affinity similar to that for 1,25(OH)$_2$D [Imawari *et al.* 1976, Haddad & Walgate 1976a], the other hydroxylated metabolites bind to DBP with affinities that are about 100 times as high [Haddad & Walgate 1976b, Bouillon *et al.* 1976, Haddad *et al.* 1977]. The metabolites 25(OH)D and 24,25(OH)$_2$D circulate in blood in concentrations about 1000 times higher than 1,25(OH)$_2$D, but because of the remarkably high concentration of DBP in relation to all the vitamin D metabolites, many binding sites are still available for 1,25(OH)$_2$D and thus the percentage free hormone is very low (< 0.1%) [Vieth 1994]. However, it has been shown that in situations with extremely high 25(OH)D concentrations, the binding capacity for 1,25(OH)$_2$D can be reduced [Cooke & Haddad 1989]. Moreover, it has been shown that polyunsaturated fatty acids also bind to DBP and thus decrease the amount of vitamin D that can be bound [Calvo & Ena 1989, Bouillon *et al.* 1992]. For these reasons, the free 1,25(OH)$_2$D concentration cannot just be derived from the concentrations of total hormone and DBP alone, as is done by using the free index. This index is calculated as the molar ratio of 1,25(OH)$_2$D and DBP [Bouillon *et al.* 1981] and although it may often give an indication of the free concentration, it obviously is not an adequate substitute for the measurement of the free concentration.

According to the so called "free hormone hypothesis", it is the free moiety that determines both the biological effect and the turnover of the hormone [Robbins & Rall 1957, Mendel 1989, Ekins 1990]. This explains why modifications in transport by serum proteins may lead to modification of total hormone levels affecting neither the free concentration in serum nor the hormonal status. For example, rising levels of binding protein secondary to some independent condition or process, will lead to an initial drop of the free concentration. This

results in a reduction of the disposal rate and, with the production rate remaining constant, the total hormone level as well as the free hormone will start to rise until the free concentration reaches the level at which disposal again equals production. Simultaneously, but not necessarily, the decreased biological signal may trigger a feedback mechanism resulting in increased hormone production, which also contributes to restoration of the balance between production and disposal. In this new steady state the total hormone level and the circulating hormone pool are increased, whereas the free hormone concentration as well as the hormonal status are unaffected. So, measurement of the free hormone concentration may be advantageous since, in contrast to the total concentration, it is not dependent on processes or conditions that solely affect serum transport.

Since estrogens have no direct effect on the $1,25(OH)_2D$ status [Henry 1981], the free hormone hypothesis predicts that there should therefore be no effect of the estrogen induced elevated DBP levels on the free concentration. Indeed during premenopausal oral estrogen intake an increase in total $1,25(OH)_2D$ has been found [Bouillon *et al.* 1981, Aarskog *et al.* 1983, Buchanan *et al.* 1986], although others reported no increase [Baran *et al.* 1980, Zofkova & Kancheva 1996]. Only two studies investigated the free index and found it to remain constant [Bouillon *et al.* 1981, Aarskog *et al.* 1983]. To our knowledge, no study has been reported in which the true free concentration has been assessed during premenopausal estrogen intake.

During pregnancy there is a rise in both DBP and total $1,25(OH)_2D$ due to increased estrogen levels, while the free $1,25(OH)_2D$ remains constant [Bouillon *et al.* 1981, Vieth 1994, van Hoof *et al.* 1998], others however found increased free levels [Bikle *et al.* 1984, Markestad *et al.* 1986]. In nephrotic syndrome, where urine protein loss occurs, some authors have reported no change in free $1,25(OH)_2D$ concentrations [Koenig *et al.* 1992], whereas others found a decreased free index [Auwerx *et al.* 1986]. Reported osteomalacia and hyperparathyroid bone disease in patients with nephrotic syndrome [Malluche *et al.* 1979] suggest that the $1,25(OH)_2D$ status may be affected.

In the study presented here, novel assays are applied for free and total $1,25(OH)_2D$ that have been shown to be more accurate than previous ones [Bikle *et al.* 1984, Reinhardt *et al.* 1984]. These findings are combined with measurements of DBP and albumin in cases where total $1,25(OH)_2D$ is either elevated or low and modification of the $1,25(OH)_2D$ status is either improbable (oral contraceptive use and pregnancy) or uncertain (nephrotic syndrome).

4.3 MATERIALS AND METHODS

4.3.1 SUBJECTS

The control group consisted of samples from 12 healthy women, not taking any medications known to influence vitamin D or calcium metabolism. As shown before, there is no difference between healthy men and women in total $1,25(OH)_2D$ [van Hoof *et al.* 1993], free $1,25(OH)_2D$ [van Hoof *et al.* 1998], or DBP [Bouillon *et al.* 1977], indicating that the present control group can be used in all further statistical analyses.
From the afore mentioned 12 women, blood samples were also obtained after 3 months of oral contraceptive therapy, consisting of 20 µg ethinylestradiol with either 150 µg desogestrel (Mercilon®) or 75 µg gestodene (Harmonet®).
Further, blood samples were obtained from 10 pregnant women (third trimester) and 16 patients (14 male, 2 female) with a nephrotic syndrome and normal renal function. Again, none of these subjects were taking any medications known to influence vitamin D or calcium metabolism.
The study was performed according to the standards of the ethical committee of the Academic Hospital Nijmegen, St. Radboud, Nijmegen, The Netherlands.

4.3.2 Total $1,25(OH)_2D$ ASSAY

The assessment of $1,25(OH)_2D$ was performed as described before [van Hoof *et al.* 1993]. In short, 1 ml serum samples were extracted using 15 ml diethylether after which the extracts were subjected to paper chromatography, which separates the major vitamin D metabolites. A radioreceptorassay was then performed using calf thymus receptor, based on the assay as described by Reinhardt *et al.* [Reinhardt *et al.* 1984]. The sensitivity of the assay was 0.5 fmol/tube, corresponding to 4 pmol/l in serum, calculated as three times the standard deviation in the lowest standard point (0 pmol/l) and reading that from the standard curve. The intra- and interassay coefficient of variations were 10.5% (n=15 at 93.4 pmol/l) and 11.5% (n=8 at 103.3 pmol/l) respectively. The range of values measured in healthy controls was 80-200 pmol/l $1,25(OH)_2D$. There was no significant difference between men and women nor any correlation with age.

4.3.3 FREE 1,25(OH)$_2$D ASSAY

The free 1,25(OH)$_2$D concentration was assessed by measuring the free fraction and multiplying it with the total 1,25(OH)$_2$D concentration. The free fraction was measured using symmetric dialysis as described previously [van Hoof *et al.* 1998]. In short, before dialysis, the serum samples were diluted fifteen-fold in phosphate buffer (50 mmol/l phosphate, 0.1 mol/l NaCl; pH 7.40). At this dilution the free fraction is such that about 20-30% of the tracer migrates through the membrane during dialysis for approximately 16 h. To assess the free fraction, one compartment of a dialysis cell (Dianorm GmbH, München, Germany) was filled with 1 ml of this diluted serum and the other compartment was filled with the same diluted sample to which 0.15 KBq ^{3}H-1,25(OH)$_2$D$_3$ (23.8 fmol)(NET 626, 6.3 TBq/mmol, NEN Dupont de Nemours, The Netherlands) had been added. The amount of tracer added (23.8 fmol) corresponds to 347 pmol/l in undiluted plasma, which does not exceed the order of magnitude of the endogenous total 1,25(OH)$_2$D concentration. The rate at which the tracer migrates from one compartment to the other is directly proportional to the magnitude of the free fraction of hormone. The free fraction was determined by comparing the dialysis rate of the sample to that of the tracer in a reference preparation of which the free fraction was known. This reference preparation was a 1% human serum albumin (HSA)(OHRA 20/21, Behringwerke AG, Marburg, Germany) solution in phosphate buffer (50 mmol/l phosphate, 0.1 mol/l NaCl; pH 7.40), of which the free concentration had been assessed beforehand using indirect equilibrium dialysis. The range of values measured in healthy controls was 60-150 fmol/l.

4.3.4. OTHER ASSAYS

Serum calcium, albumin, creatinine, and total protein were kindly assessed in the Central Clinical Chemistry Laboratory of the Academic Hospital Nijmegen, using a Hitachi 747 automated analyzer. In nephrotic syndrome, calcium levels were corrected for the low albumin levels by the formula: $Ca_{corrected} = Ca_{assessed}$ - 0.025 albumin (µmol/l) + 1.00 mmol/l.
DBP levels were kindly measured in the Laboratory for Experimental Medicine of Prof. Dr. R. Bouillon at the Catholic University Leuven, Belgium, using single radial immunodiffusion [Bouillon *et al.* 1977].
25(OH)D was assessed using HPLC according to Dabek *et al.* [Dabek *et al.* 1981].
Intact PTH was measured using a commercial assay (Nichols, San Juan Capristano CA, USA).

4.3.5 STATISTICS

All values are given as means ± S.D.. Statistical analysis was performed using SPSS for Windows (version 6.0.1, SPSS Inc., USA). If Shapiro-Wilks testing revealed that data were not normally distributed, logarithmic transformation was performed. To compare groups, ANOVA and subsequent T-tests were performed. Correlation analysis was done by calculating the Pearson correlation coefficients.

4.4 RESULTS

Ranges and significant differences of total and free $1,25(OH)_2D$ as well as of DBP are shown in figure 4.1.

During the use of oral contraceptives, DBP levels rose from 5.59 ± 0.43 µmol/l to 8.32 ± 1.59 µmol/l ($p \leq 0.001$). The mean total $1,25(OH)_2D$ concentration rose significantly from baseline levels of 132.4 ± 19.1 pmol/l to 174.9 ± 50.8 pmol/l after 3 months of therapy ($p \leq 0.05$). The mean free $1,25(OH)_2D$ concentrations at both timepoints did not differ significantly and were

Table 4.1

Characteristics of patients with nephrotic syndrome (n=16; 14 male, 2 female).

		mean values ± S.D.	reference values
Age	(years)	44.5 ± 17.1	
serum DBP	(µmol/l)	5.35 ± 0.84	3.57-8.93
	(mg/l)	300 ± 47	200-500
serum creatinine	(µmol/l)	96.8 ± 25.5	50-110
serum albumin	(µmol/l)	300 ± 84	536-768
	(g/l)	20.7 ± 5.8	37-53
serum 25(OH)D*	(nmol/l)	29.8 ± 11.3	25-97.5
urine protein	(g/24h)	10.5 ± 3.2	<0.1
creatinine clearance	(ml/min)	119 ± 35	70-110
calcium	(mmol/l)	2.5 ± 0.1	2.2-2.6
PTH**	(pmol/l)	3.8 ± 1.7	<6.5

* n=14 ; ** n=7

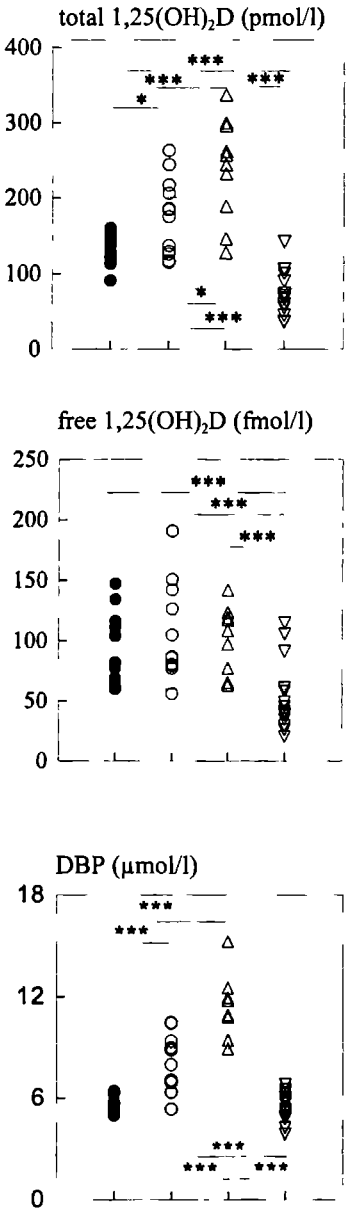

Figure 4.1: Total 1,25(OH)$_2$D, free 1,25(OH)$_2$D and DBP concentrations in healthy woen before (●), and after 3 months of estrogen/progestagen intake (○), in pregnant women (△), and in patients with nephrotic syndrome (▽) The dotted lines indicate reference ranges. (* $p \leq 0.05$ *** $p \leq 0.001$)

92.3 ± 29.9 fmol/l and 105.2 ± 39.5 fmol/l respectively. Albumin levels showed no significant change from 628 ± 38 µmol/l (43.3 ± 2.6 g/l) at baseline to 623 ± 43 µmol/l (43.0 ± 3.0 g/l) after 3 months. In pregnant women, a significant increase was found in DBP (11.32 ± 1.76 µmol/l, $p \leq 0.001$) and total $1,25(OH)_2D$ (239.1 ± 67.5 pmol/l, $p \leq 0.001$), but not in free $1,25(OH)_2D$ (103.8 ± 27.0 fmol/l).

Characteristics of the patients with nephrotic syndrome are summarized in table 4.1. In nephrotic syndrome, the mean DBP level was 5.35 ± 0.84 µmol/l, which was not significantly different from that in controls. The mean total $1,25(OH)_2D$ concentration was 68.7 ± 25.7 pmol/l and the mean free concentration was 53.3 ± 27.9 fmol/l. The total $1,25(OH)_2D$ concentration in these patients was significantly lower than the reference levels ($p \leq 0.001$, reference range: 80-200 pmol/l), as was the free concentration ($p \leq 0.001$, reference range: 60-150 fmol/l). Obviously, the albumin concentration was significantly lower than in controls (300 ± 84 µmol/l vs 628 ± 38 µmol/l; $p \leq 0.001$ *i.e.* 20.7 ± 5.8 g/l vs 43.3 ± 2.6 g/l). In patients with nephrotic syndrome, statistically significant positive correlations were found between the concentration of serum albumin and the levels of both total $1,25(OH)_2D$ ($r=0.54$; $p \leq 0.05$) and free $1,25(OH)_2D$ ($r=0.52$; $p \leq 0.05$), and a negative correlation was found with urine protein ($r=-0.50$; $p \leq 0.05$). DBP levels did not correlate with either total $1,25(OH)_2D$ or free $1,25(OH)_2D$, nor with albumin, but did correlate with total urine protein ($r=0.65$; $p \leq 0.01$). The levels of 25(OH)D were low (29.8 ± 11.3 nmol/l) and were correlated with DBP ($r=0.55$; $p \leq 0.05$) and urine protein ($r=-0.57$; $p \leq 0.05$), but not with total or free $1,25(OH)_2D$ nor with albumin levels.

4.5 DISCUSSION

To our knowledge this is the first report on the measurement of the free $1,25(OH)_2D$ concentration during premenopausal estrogen/progestagen intake. There have been previous studies investigating the effect of premenopausal estrogens/progestagen on the concentrations of total $1,25(OH)_2D$ and DBP, but these results were ambiguous. An increase in total $1,25(OH)_2D$ has been reported by some authors [Aarskog *et al.* 1983, Buchanan *et al.* 1986], whereas others found no such effect [Baran *et al.* 1980, Zofkova & Kancheva 1996]. Most studies report an increase in the concentration of DBP [Haddad & Walgate 1976a, Bouillon *et al.* 1977, Barragry *et al.* 1977, Aarskog *et al.* 1983], but not all [Buchanan *et al.* 1986]. Two studies report no change in the free $1,25(OH)_2D$ index during oral estrogen intake [Bouillon *et al.* 1981, Aarskog *et al.* 1983], but the actual free concentration was not assessed. The present study shows a significant increase in both total $1,25(OH)_2D$ and DBP levels, whereas the free $1,25(OH)_2D$ concentrations remain constant. Similar results were found in pregnancy, where

the increase in both total $1,25(OH)_2D$ and DBP levels is even higher than after 3 months of estrogen use, but the free concentration also remains unchanged. This increase in total $1,25(OH)_2D$ concentrations and DBP during pregnancy has been reported before [Haddad & Walgate 1976a, Barragry *et al.* 1977, Bouillon *et al.* 1981]. Reports on the free $1,25(OH)_2D$ are again equivocal. Some authors report an increase in the free $1,25(OH)_2D$ concentration [Bikle *et al.* 1984, Markestad *et al.* 1986], whereas others find no change in the free $1,25(OH)_2D$ index [Bouillon *et al.* 1981, Vieth 1994].

These results show that the free hormone hypothesis is valid for $1,25(OH)_2D$ as it is for the other steroid hormones and thyroid hormones [Willcox *et al.* 1983, Swinkels *et al.* 1987, Ross 1978] and therefore suggest that the free concentration of $1,25(OH)_2D$ is a more reliable marker for the biological availability of $1,25(OH)_2D$ than its total plasma concentration. It is also shown that supraphysiological estrogen levels in the premenopause do not result in increased levels of free $1,25(OH)_2D$. Any additional effects of the progestagenic compound in the oral contraceptives during the experiments are not known.

In patients with a nephrotic syndrome, many plasma proteins are lost in the urine. As a consequence it was expected to find lower levels of DBP in parallel with lower total $1,25(OH)_2D$ levels and, because of normocalcemic conditions, normal free $1,25(OH)_2D$ concentrations. However, the situation proved different. Although most patients had profound hypalbuminia, the DBP levels were normal and total $1,25(OH)_2D$ concentrations as well as free $1,25(OH)_2D$ levels were decreased. Literature data on the changes in DBP and total $1,25(OH)_2D$ in nephrotic syndrome are equivocal. Some authors report lower concentrations of DBP [Haddad & Walgate 1976a, Schmidt-Gayk *et al.* 1977, Grymonprez *et al.* 1995], and in some studies a correlation between serum albumin and DBP levels was found [Auwerx *et al.* 1986, Grymonprezv *et al.* 1995]. In contrast, other investigators report unchanged levels of DBP in patients with nephrotic syndrome [Chesney *et al.* 1984, Huang *et al.* 1992]. Likewise, total $1,25(OH)_2D$ levels have been found decreased [Goldstein *et al.* 1981, Lambert *et al.* 1982, Auwerx *et al.* 1986, Grymonprez *et al.* 1995], or unchanged [Korkor *et al.* 1983, Chesney *et al.* 1984, Huang *et al.* 1992] in patients with nephrotic syndrome.

A few investigators have measured free serum $1,25(OH)_2D$ levels in patients with nephrotic syndrome, using different methods. In one study, free levels of $1,25(OH)_2D$ as determined by assessing the free index were also found low [Auwerx *et al.* 1986]. However, using the same methodology, normal levels were reported by Chan *et al.* [Chan *et al.* 1983] and Grymonprez *et al.* [Grymonprez *et al.* 1995]. Koenig *et al.*, who used centrifugal ultrafiltration, also found normal levels of free $1,25(OH)_2D$ [Koenig *et al.* 1992]. It has been shown, however, that the centrifugal ultrafiltration method to assess the free $1,25(OH)_2D$ concentration is rather prone to overestimation of the free fraction [van Hoof *et al.* 1998]. The validity of the assessment of

the free index is questionable, since it especially neglects albumin variation [Bikle *et al.* 1984, van Hoof *et al.* 1998].

The lower levels of total $1,25(OH)_2D$ in nephrotic syndrome found in the present study can reflect a lower steady state, resulting from increased loss of $1,25(OH)_2D$ in the urine. This would also result in lower free $1,25(OH)_2D$ concentrations. However, one would expect increased synthesis of $1,25(OH)_2D$ after prolonged lower free $1,25(OH)_2D$ levels, because of lower intestinal calcium uptake and consequent changes in calcium metabolism. The fact that nonetheless lower free $1,25(OH)_2D$ levels were found in patients with nephrotic syndrome, indicates therefore that there is a problem with the $1,25(OH)_2D$ synthesis. This problem earlier has been attributed to lowered concentrations of 25-hydroxyvitamin D (25(OH)D) in patients with nephrotic syndrome, because of the loss in the urine of 25(OH)D bound to binding proteins [Auwerx *et al.* 1986, Mizokuchi *et al.* 1992]. Indeed total serum 25(OH)D levels were also decreased in the present study, and negatively correlated with urinary protein loss. Another strong argument for the hypothesis is the finding that 25(OH)D replacement therapy not only increases 25(OH)D levels, but also levels of total $1,25(OH)_2D$ [Haldimann & Trechsel 1983]. This would make a decrease of 25(OH)D levels a likely explanation for the decreased free $1,25(OH)_2D$ levels.

Another explanation for the decreased $1,25(OH)_2D$ levels in nephrotic syndrome could be impairment of 1-hydroxylase activity and blunting of the response to PTH because of defective proximal tubular function [Mizokuchi *et al.* 1992]. These possible mechanisms are the subject of further research.

Remarkably, a correlation of both total and free $1,25(OH)_2D$ with serum albumin and not with serum DBP was observed in this study, as well as in previous research [Lambert *et al.* 1982]. At first sight, this seems incomprehensible, because under normal circumstances at least 85% of all $1,25(OH)_2D$ is bound to DBP [Bikle *et al.* 1985]. However, this phenomenon can be explained by the continuous loss of albumin-bound $1,25(OH)_2D$ in the urine with both albumin and $1,25(OH)_2D$ synthesis lagging behind. Thus, both the albumin and the $1,25(OH)_2D$ remaining in the vascular compartment are inversely related to the severity of the disease, so that a correlation between those parameters in a group of patients with varying degrees of proteinuria should not be a complete surprise. The correlation between 25(OH)D, DBP and urine protein loss can be explained when assuming that DBP-bound 25(OH)D is lost in the urine. The DBP synthesis is just sufficient to maintain normal levels, whereas 25(OH)D synthesis is lagging behind, resulting in a lower total concentration. The whole picture is consistent with the assumption that $1,25(OH)_2D$ loss occurs primarily in albumin-bound form, whereas 25(OH)D leaks out mainly in DBP-bound form. Anyway, the conditions for the free hormone hypothesis do not apply with regard to turnover, since the disposal rate for the larger

part depends on the severity of proteinuria, rather than on the free hormone concentration. On the other hand, the reports of osteomalacia and hyperparathyroid bone disease in patients with nephrotic syndrome [Malluche *et al.* 1979] and increased bone resorption in rats with experimental nephrotic syndrome [Sierra *et al.* 1997] are in line with a reduced biological effect of $1,25(OH)_2D$ because of its lowered free concentration.

Finally, it may be concluded that for the three conditions presented here free $1,25(OH)_2D$ adequately reflects its biological effect. Only under the pathological condition of the nephrotic syndrome, $1,25(OH)_2D$ turnover is not a direct function of the free concentration but depends on the severity of the protein loss.

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CHAPTER 5

Hormone replacement therapy increases serum 1,25-dihydroxyvitamin D; a 2 year prospective study

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5.1 ABSTRACT

Osteoporosis is a common disorder in postmenopausal women, which is probably due to decreased ovarian function. Currently, hormone replacement therapy (HRT), involving administration of estrogen and progestogen, is successfully applied to reduce bone resorption. In 23 postmenopausal women we studied the effect of HRT, consisting of a combination of 17β-estradiol and dydrogesterone, on the serum level of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) after 0, 6, 12, and 24 months. We have found mean serum concentrations (± S.D.) of $1,25(OH)_2D$ of 130.5 pmol/l (46.1), 152.7 pmol/l (45.1), 170.8 pmol/l (64.0), and 155.2 pmol/l (59.7), respectively. The baseline values in these women were found to be significantly lower than those during therapy ($p \leq 0.005$). No statistically significant differences were observed when comparing the estrogen-only phase with the combined estrogen-progestogen phase. It is concluded that HRT results in an increase in the serum $1,25(OH)_2D$ concentration which lasts for at least 2 years. This increase may explain in part the preventive effect of HRT on osteoporosis. Furthermore, these results suggest that dydrogesterone does not influence the estrogen-induced changes in serum $1,25(OH)_2D$ concentration.

5.2 INTRODUCTION

Osteoporosis is a common bone disorder, affecting millions of older people in the western world, especially women after menopause [Riggs & Melton III 1986]. With the menopause a significant decrease in bone density occurs [Albright *et al.* 1940], the exact causes of which are still unknown. Many therapies are used to try to halt or even to reverse this phenomenon, such as supplementation with fluoride [Riggs *et al.* 1990], as well as administration of calcitonin [Civitelli *et al.* 1988a], biphosphonates [Reginster *et al.* 1989], or 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) [Tilyard *et al.* 1992]. Probably the most effective therapy currently available is estrogen therapy [Lindsay *et al.* 1976]. The strong decline in estrogen concentrations after menopause suggests a direct relationship between estrogen concentration and bone density.
During estrogen therapy a rise in serum $1,25(OH)_2D$ has been observed [Prince *et al.* 1991, Lund 1982, Aloia 1991]. The latter hormone is capable of stimulating the intestinal absorption of calcium. However, literature on this issue is contradictory [Selby & Peacock 1986, Kalu *et al.* 1991], and most studies describe short treatment intervals of no more than six months.
Estrogen administration is often combined with progestogen to reduce the hyperplastic effects of estrogen on the endometrium. It has been postulated that progestogen is the cause of the enhanced bone mineralisation instead of estrogen [Lee 1991].
To gain a better insight in the effect of estrogen on serum $1,25(OH)_2D$ levels, we have studied

23 healthy postmenopausal women during a sequential 17β-estradiol - dydrogesterone treatment for up to 2 years. This treatment consisted of 24 cycles of 4 weeks, including a 2-week phase of 17β-estradiol - dydrogesterone administration and a 2-week phase of only 17β-estradiol administration. We assessed the serum 1,25(OH)$_2$ D concentration at several time intervals during the 17β-estradiol - dydrogesterone phase. Furthermore, to determine any additional effects of the progestogen on serum 1,25(OH)$_2$D concentration, we compared the estrogen-only with the combined estrogen-progestogen phase in the last cycle studied.

5.3 MATERIALS AND METHODS

5.3.1 SUBJECTS

In this study 23 healthy non-hysterectomized postmenopausal women, aged 49 to 59 years were included as described elsewhere [van der Mooren *et al.* 1993]. All women suffered climacteric symptoms and complaints and were amenorrhoeic for at least 6 months. They were screened to have follicle-stimulating hormone concentrations within the range characteristic for the postmenopausal phase (≥ 36 IU/l). Excluded were women using hormonal therapy for the previous two weeks. Characteristics of the study population are given in table 5.1.
All women were continuously treated with 2 mg daily of micronized 17β-estradiol (Zumenon^R), and with dydrogesterone (Duphaston^R), 10 mg daily for only the first half of each 28-days treatment cycle. Both analogs were administered orally (Solvay Duphar B.V., Weesp, The Netherlands).
Fasting venous blood sampling was performed before study entry and on day 12, 13, or 14 of the combined 17β-estradiol - dydrogesterone intake of cycles 6 (± 1), 12 (± 1), and 24 (± 1). Blood sampling took place after a 12-hour fast. To evaluate the possible differences in serum 1,25(OH)$_2$D between the combined estradiol-dydrogesterone phase and the estradiol-only phase blood was also sampled on cycle day 26, 27, or 28 of cycle 23 or 24.
Serum samples were stored at -80°C until measurement of 1,25(OH)$_2$D.

5.3.2 EXPERIMENTAL

Extraction and paper chromatography were performed as described elsewhere [van Hoof *et al.* 1993]. In short, to 1.0 ml of serum, 25 µl (10,000 dpm) ^{3}H-1,25(OH)$_2$D$_3$ (166.4 Ci/mmol, 26,27-^{3}H)(NEN Products, Dreieich, Germany) in ethanol was added. The samples were incubated and extracted with diethylether. The solvent was removed by drying under a stream of dry nitrogen,

and the samples were chromatographed in a descending paper-chromatography system [Bush 1961]. After locating the tracer, the appropriate paper area was cut out and the $1,25(OH)_2D$ was eluted with ethanol. The ethanol was then evaporated under a stream of dry nitrogen and the residue was dissolved in 200 µl of 25% ethanol in phosphate buffer in order to concentrate the sample.

The radioreceptorassay was performed as described by Hollis [Hollis 1986] with some modifications. A standard curve (0-1200 pmol/l) was set up in duplicate. To 50 µl of standard, control or unknown, 50 µl of ^3H-$1,25(OH)_2D_3$ (10,000 dpm) in 25% ethanol in phosphate buffer, and 500 µl receptor preparation from calf thymus in phosphate buffer were added. Following a 3 h incubation at room temperature, receptor-bound and unbound $1,25(OH)_2D$ were separated using 150 µl dextran-coated charcoal to adsorb the unbound fraction. After 3 min the samples were centrifuged at 2000 x g for 10 min at room temperature. The supernatants were decanted simultaneously into counting vials using the device described by Vecsei and Gless [Vecsei & Gless 1975]. After addition of 4 ml of scintillation liquid, the radioactivity was determined.

The intra- and interassay CV were 10% (n=15; at 93.4 pmol/l) and 11% (n=8; at 103.3 pmol/l) respectively.

The standard curve was fitted to a four-parameter model as described by Healey [Healey 1972], using a non-linear fit algorithm according to Marquardt [Marquardt 1963]. The concentration of hormone present in the sample eluate was read from the standard curve after correction of the total counts for the contribution of the radioactivity by the recovery tracer. The concentration was then corrected for mass-contribution of recovery tracer and reagent blank. Finally, a correction for procedural losses was performed.

Paired Student's t-tests were performed using the SAS program on a VAX/VMS computer.

Table 5.1

Characteristics of the study population[*].

Age	(years)	54.3 ± 3.5
Amenorrhoea	(months)	56 ± 40
FSH	(IU/l)	84 ± 26

[*] mean ± S.D. (n=23)

5.4 RESULTS

The basal mean serum concentration (± S.D.) of $1,25(OH)_2$ D was 130.5 pmol/l (± 46.1 pmol/l). At cycle 6, 12, and 24 the concentrations were 152.7 pmol/l (± 45.1 pmol/l), 170.8 pmol/l (± 64.0

pmol/l) and 155.2 pmol/l (± 59.7 pmol/l) respectively (figure 5.1). The concentrations during therapy were significantly elevated compared to the baseline level ($p \leq 0.005$). There was no significant difference between the serum concentrations during therapy. Mean serum $1,25(OH)_2D$ in the 17β-estradiol-only phase of cycle 23 or 24 was 154.2 (± 55.3) pmol/l. This was significantly higher than the baseline level ($p \leq 0.005$), but not significantly different from the concentration during the combined 17β-estradiol - dydrogesterone phase of the same cycle.

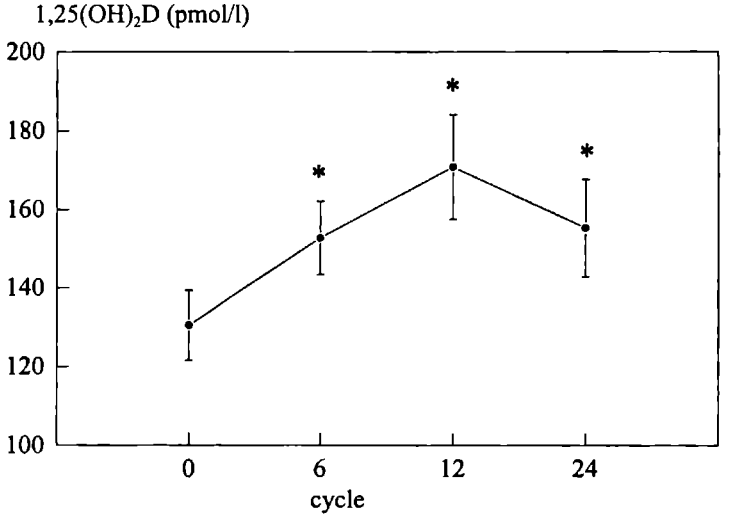

Figure 5.1: Mean concentration (± SEM) of $1,25(OH)_2D$ during hormone replacement therapy in postmenopausal women (n = 23) ($p \leq 0.005$ vs baseline).*

5.5 DISCUSSION

The present study discloses a significant rise in serum $1,25(OH)_2$ D during 2 years of hormone replacement therapy (HRT) in postmenopausal women. This is in accordance with other reports [Lund *et al.* 1982, Aloia *et al.* 1991], which however did not exceed study periods of more than 6 months. Civitelli *et al.* [Civitelli *et al.* 1988b] have reported elevated serum levels of $1,25(OH)_2D$ during estrogen therapy in postmenopausal osteoporosis after 1 year. Prince *et al.* [Prince *et al.* 1991] also studied the effect of HRT on the serum levels of $1,25(OH)_2D$ in postmenopausal women during 2 years (n=40). These authors, however, did not find a significant increase in serum $1,25(OH)_2D$ concentration after 2 years, although they reported changes in concentrations similar to those we have found. The underlying reason for the lack of any significance, may well be the result of their intra- and interassay CV of 21% and 24% respectively.

Literature data on the effects of HRT on the serum level of $1,25(OH)_2D$ are unequivocal though. When estrogen was applied transdermally, no change in serum $1,25(OH)_2$ D was observed [Selby & Peacock 1986], even though the serum estrogen concentration was significantly elevated. Further, a decline in the $1,25(OH)_2$ D serum concentration during estrogen therapy has been found in rats [Kalu *et al.* 1991].

These findings raise the question of whether estrogen exerts its effect on bone density only directly by inhibition of bone resorption or also via $1,25(OH)_2D$. It is generally believed that the estrogen induced elevation of the serum $1,25(OH)_2D$ is a secondary effect due to a stimulation of vitamin D binding protein (DBP) synthesis in the liver [Bikle *et al.* 1992, Hagenfeldt *et al.* 1991, Bouillon *et al.* 1981]. A higher concentration of DBP would result in an increase of the concentration of total serum $1,25(OH)_2D$ but would leave the free $1,25(OH)_2D$ index constant. This free index is derived by dividing the concentration of total serum $1,25(OH)_2D$ by the concentration of DBP, and is thought to reflect the biologically active moiety. However, reported results of measurements of total serum $1,25(OH)_2D$ and of the free $1,25(OH)_2D$ index during HRT have not been equivocal. In some studies a rise was found not only in DBP and total serum $1,25(OH)_2D$, but also in the free $1,25(OH)_2D$ index [Aloia *et al.* 1991, Cheema *et al.* 1989]. These results indicate that estrogen exerts its effect on bone density also via $1,25(OH)_2D$, and that the rise of serum $1,25(OH)_2D$ should not only be ascribed to an increase in DBP synthesis.

Progestogen is often combined with estrogen administration, to reduce the hyperplastic effects of estrogen on the endometrium. Lee [Lee 1991] suggests that progestogen and not estrogen is responsible for the increased bone density during HRT. This hypothesis cannot be dismissed on the basis of our results. However, we have found no difference between the serum $1,25(OH)_2D$ concentration during the combined estrogen-dydrogesterone phase and during the estrogen-only phase. Dydrogesterone is completely excreted in 72 hours [Kuhl 1990]. Any residual effects of dydrogesterone on the serum $1,25(OH)_2D$ after 2 weeks are therefore, although not completely impossible, highly unlikely. This indicates that dydrogesterone does not induce the change in serum $1,25(OH)_2D$ concentration and therefore also not a $1,25(OH)_2D$-induced change in bone density.

Trémollières *et al.* [Trésmollières *et al.* 1993] have reported estrogen to be a stronger inhibitor of postmenopausal bone loss than promegestone, a progestogen which also has no androgenic effects, similar to dydrogesterone. The results with medroxyprogesterone have been contradictionary [Bikle *et al.* 1992, Cheema *et al.* 1989]. Scheven *et al.* [Scheven *et al.* 1992] have reported that natural progestogen is a more potent stimulator of osteoblastic growth than estrogen, and Lee [Lee 1991] also used natural progesterone. It seems therefore likely that the effects of progesterone on bone density results from the androgenic characteristics of progesterone.

Estrogen has also been found to induce the synthesis of a cell/matrix surface-associated inhibitory factor of osteoclastic bone resorption from osteoblastic cells [Ishii *et al.* 1993].

The latter finding may indicate that the rise in serum $1,25(OH)_2D$ has two causes. First as a result of a higher concentration of DBP due to stimulation of its synthesis in the liver by estrogen, and secondly as a result of a lower level of blood Ca due to lower bone resorption. This may explain the contradictory results in assessing changes of serum $1,25(OH)_2D$ concentrations after estrogen therapy. The higher $1,25(OH)_2D$ level will generate a higher level of blood Ca through higher Ca uptake in the intestine, which subsequently can suppress the $1,25(OH)_2D$ synthesis via a feed-back mechanism.

In conclusion, HRT induces a rise in serum $1,25(OH)_2D$ concentration, which lasts at least for 2 years. This rise appears to be a result of the estrogen alone. Literature data indicate that progestogen seems able to improve bone density only as a result of its androgenic characteristics whereas analogs with no androgenic effects such as dydrogesterone apparently have no effects on the serum $1,25(OH)_2D$ concentration and consequently on the bone density.

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CHAPTER 6

Hormone Replacement Therapy increases Serum Free 1,25-Dihydroxyvitamin D; a 1 year prospective study

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6.1 ABSTRACT

Osteoporosis is a common disorder in postmenopausal women, which probably is partly due to decreased ovarian function. Currently, hormone replacement therapy (HRT), involving administration of estrogen and progestagen, is successfully applied to reduce bone resorption. In 36 postmenopausal women we studied the effect of HRT, consisting of a combination of 17β-estradiol and norethisteroneacetate (NETA) on the serum level of total and free 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) after 0, 3, 6, and 12 cycles. We have found mean serum total $1,25(OH)_2D$ concentrations (± S.D.) of 106.4 pmol/l (27.5), 155.0 pmol/l (49.5), 176.7 pmol/l (70.0), and 161.1 pmol/l (55.3), respectively. Serum free $1,25(OH)_2D$ concentrations were 68 fmol/l (± 22), 107 fmol/l (± 35), 120 fmol/l (± 43), and 108 fmol/l (± 37) respectively. Baseline values of both total and free $1,25(OH)_2D$ were found to be significantly lower than those during therapy ($p \leq 0.001$). It is concluded that both the serum total $1,25(OH)_2D$ and the free $1,25(OH)_2D$ concentrations are increased during HRT for a year. Assuming that the free concentration $1,25(OH)_2D$ reflects the biologically active fraction, this rise may in part explain the preventive effect of HRT on osteoporosis.

6.2 INTRODUCTION

Osteoporosis is a common bone disorder, affecting millions of elderly in the western world, especially women after menopause [Riggs & Melton III 1986]. With the menopause a significant decrease in bone density occurs [Albright *et al.* 1940]. Simultaneously, a significant drop in estrogen levels occurs, which suggests a relation between these two phenomena. Hormone replacement therapy (HRT) is used as a therapy to halt or even reverse the rapid bone loss after menopause [Lindsay *et al.* 1976]. Despite the fact that this therapy is now widely used, the exact mechanism with which estrogen exerts its effect has not yet been fully elucidated. Estrogen receptors have been found in the kidney [Hagenfeldt & Eriksson 1988], the intestine [Arjmandi *et al.* 1993], osteoblasts [Eriksen *et al.* 1988] and recently in osteoclasts [Mano *et al.* 1996]. This may indicate that estrogen has direct effects on calcium retention, calcium uptake or bone remodelling. Apart from these effects, several researchers have reported an increase in 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) during HRT [Prince *et al.* 1991a, van Hoof *et al.* 1994]. The increased level of this hormone would increase the calcium uptake through the intestine and thus exert an effect on bone.

Vitamin D binding protein (DBP) production in the liver is stimulated by estrogens [Haddad & Walgate 1976]. The higher concentration of binding protein in turn leads to a higher concentration of $1,25(OH)_2D$ in blood. Controversy exists whether the increase in $1,25(OH)_2D$

during HRT actually represents an increase in its biological activity. The free hormone hypothesis states that the non-bound fraction of a hormone best reflects the biologically active moiety [Ekins 1990]. This has already been shown for several steroid and thyroid hormones [Baumann *et al.* 1975, Kelstrup 1973]. During pregnancy, total $1,25(OH)_2D$ is increased but the free concentration is normal. Since there is no hypercalcemia during pregnancy, this clearly indicates that the free hormone hypothesis also is valid for $1,25(OH)_2D$ [van Hoof *et al.* 1998]. The measurement of the free concentration of $1,25(OH)_2D$ is rather difficult due to its low values of typically less than 0.1%. Therefore, many researchers use the free hormone index as an indicator of the free fraction [Bouillon *et al.* 1981]. This index is the molar ratio of the total concentration of $1,25(OH)_2D$ and the concentration of DBP, and obviously not a very precise assessment. To our knowledge, measurement of the free concentration of $1,25(OH)_2D$ during HRT has only been published three times. Dick *et al.* [Dick *et al.* 1995] did not find a change in the free concentration during HRT. Cheema *et al.* [Cheema *et al.* 1989] and Bikle *et al.* [Bikle *et al.* 1992] reported a rise in the free concentration during HRT, but they studied the effects for only one and three months respectively. All authors used an ultrafiltration method to assess the free fraction of $1,25(OH)_2D$ [Bikle *et al.* 1984]. This method is known to be rather prone to disturbances. When very low free concentrations are concerned, like with $1,25(OH)_2D$, there is a real risk of protein leakage resulting in overestimation of the free fraction. Recently we described a new method for determining the free fraction of $1,25(OH)_2D$ using a symmetric dialysis method [van Hoof *et al.* 1998]. In this paper we use this method to assess the free fraction of $1,25(OH)_2D$ during one year of HRT to investigate whether the therapy has a long lasting effect on the free concentration of $1,25(OH)_2D$.

6.3 MATERIALS AND METHODS

6.3.1 SUBJECTS

Selected were 40 healthy non-hysterectomised women (mean age ± SD: 52.1 ± 4.0 years) who were amenorrhoic for at least six months and who had a serum FSH concentration of 36 IU/l or more and a serum estradiol concentration of 150 pmol/l or less. Women with any contraindication for HRT were excluded.

After screening women were treated with Trisequens® (28-day cycle consisting of 12 days of 2 mg 17β-estradiol, 10 days of 2 mg 17β-estradiol combined with 1 mg norethisteroneacetate (NETA), and 6 days of 1 mg 17β-estradiol; Novo Nordisk Farma BV, Alphen a.d. Rijn, The Netherlands) for 1 year. The assays were perfomed in the samples of 36 women, who completed the whole 12 cycles.

Blood samples were obtained after an overnight fast before treatment and after three, six, and twelve 28-day cycles of treatment during day 8-10 of the combined estradiol-NETA administration.
Serum samples were frozen at -20°C within one hour after sampling.
This study was approved by the Institutional Review Board of the University Hospital Nijmegen and the participating women gave their written informed consent before enrollment in the study.

6.3.2 TOTAL 1,25(OH)$_2$D ASSAY

The total concentration of 1,25(OH)$_2$D in serum was assessed using a radioreceptorassay after extraction of the samples followed by paper chromatography, as described previously [van Hoof *et al.* 1993]. The detection limit was 4 pmol/l, when 1 ml of serum was assayed, recovery being taken into account. This value was obtained by subtracting 3 times the SD of the initial binding from the initial binding and reading the result from the standard curve. The intraassay coefficient of variation was 10.5% (at a level of 93.4 pmol/l (n=15)). The interassay coefficient of variation was assessed from 8 consecutive assays of one sample and was 11.5% at 103.3 pmol/l. The concentration range of 1,25(OH)$_2$D in healthy subjects was 80-200 pmol/l (n=60).

6.3.3 FREE 1,25(OH)$_2$D ASSAY

The free fraction of 1,25(OH)$_2$D was measured using symmetric dialysis. Serum samples were diluted 15 times before assaying [van Hoof *et al.* 1998]. Intra- and interassay CVs of this assay were assessed by measuring one sample 4 times during each of 20 consecutive assays. The values were 1.0% and 7.7% respectively. The free concentration of 1,25(OH)$_2$ D was calculated by multiplying the total concentration of 1,25(OH)$_2$ D with the free percentage, correcting for dilution.

6.3.4 STATISTICS

Significances were determined with ANOVA, followed by subsequent paired Student's *t*-tests in case significances were indicated.
Statistical calculations were performed with the SPSS statistical program.

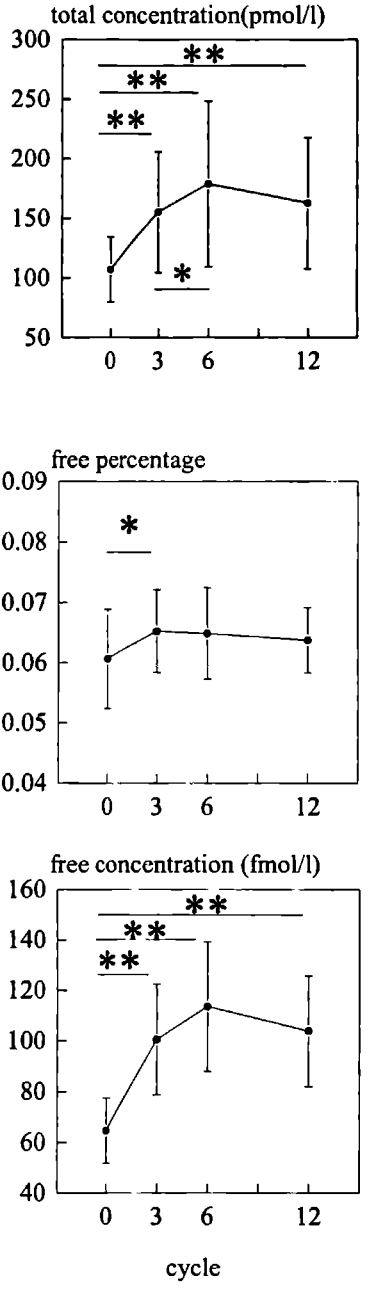

*Figure 6.1: Mean concentrations (± S.D.) of total concentration, free percentage, and free concentration of 1,25(OH)$_2$D during hormone replacement therapy in postmenopausal women. (n=36) (*p ≤0.05 **p ≤0.001)*

6.4 RESULTS

The basal mean serum concentration (± S.D.) of total $1,25(OH)_2$ D was 106.4 pmol/l (± 27.5 pmol/l). At cycle 3, 6, and 12 the concentrations were 155.0 pmol/l (± 49.5 pmol/l), 176.7 pmol/l (± 70.0 pmol/l) and 161.1 pmol/l (± 55.3 pmol/l) respectively (figure 6.1). The total concentrations during therapy were significantly elevated compared to the baseline level ($p \leq 0.001$).There was also a small significant difference between the serum concentrations at cycle 3 and cycle 6 ($p \leq 0.05$). The basal mean serum concentration of free $1,25(OH)_2$ D was 68 fmol/l (± 22 fmol/l), which was significantly lower than the free $1,25(OH)_2$D concentrations during therapy ($p \leq 0.001$). At cycle 3, 6, and 12 the free concentrations were 107 fmol/l (± 35 fmol/l), 120 fmol/l (± 43 fmol/l), and 108 fmol/l (± 37 fmol/l) respectively. There were no significant differences between the free concentrations during therapy. The free percentage also showed no significant differences during therapy, save from a small significant difference between the free percentage at baseline and at cycle 3 ($p \leq 0.05$).

6.5 DISCUSSION

To study whether HRT has a lasting effect on the free $1,25(OH)_2$D concentration, symmetric dialysis was used to assess the free fraction of $1,25(OH)_2$D during 12 cycles of HRT. A significant rise in both the total and free $1,25(OH)_2$D concentrations was found during HRT. Other methods to determine free $1,25(OH)_2$D during HRT have been described previously. The centrifugal ultrafiltration method of Bikle *et al.* [Bikle *et al.* 1984] is the commonest. Using this method, Bikle *et al.* also reported an elevated level of free $1,25(OH)_2$D during HRT lasting three months [Bikle *et al.* 1992]. Dick *et al.* [Dick *et al.* 1995] however did not find a significant rise in free $1,25(OH)_2$D employing centrifugal ultrafiltration. The reported intra- and interassay variation coefficients for their assay of the free $1,25(OH)_2$D fraction were 19 and 26% respectively. Since our intra- and interassay coefficients of variation are only 1 and 7.7% respectively, it is highly likely that using this more precise assay instead of centrifugal ultrafiltration, a significant rise would have been found in their study. Moreover, the symmetric dialysis method has several advantages over the centrifugal ultrafiltration method [van Hoof *et al.* 1998]. Since 20-30% of the added radioactivity is measured during symmetric dialysis, small impurities will have only a negligible effect on the size of the assessed free fraction, whereas they can lead to major increases of the free fraction measured by ultrafiltration. The amount of radioactive hormone added in symmetric dialysis is of the same order of magnitude as the endogenous $1,25(OH)_2$D concentration, while in centrifugal ultrafiltration ten times as much has to be added. This latter amount is much more likely to disturb the equilibrium of $1,25(OH)_2$D

distribution and increase the free fraction, leading to spuriously high results.

Another way to assess the free concentration of $1,25(OH)_2D$ is to calculate it from the concentration of total $1,25(OH)_2D$, the concentration of DBP and the affinity constant of DBP for $1,25(OH)_2D$. Using this method, Selby *et al.* reported no rise in the free $1,25(OH)_2D$ concentration during HRT while the total concentration did show a significant rise [Selby *et al.* 1985]. While this method in theory could yield the correct results, it has several limitations. First, $1,25(OH)_2D$ is also bound to albumin and, perhaps, plasma lipoproteins [Silver & Fainaru 1979], which means that taking only DBP into account as binding protein will never lead to the correct value of the free concentration. Second, the affinity constant of DBP for $1,25(OH)_2D$ is not yet very exactly established, varying by a factor 10 in different reports. Therefore, this calculation cannot be very accurate. Moreover, Bouillon *et al.* showed that unsaturated free fatty acids can decrease the binding capacity of DBP for $1,25(OH)_2D$ [Bouillon *et al.* 1992], while Cooke and Haddad [Cooke & Haddad 1990] showed that in situations with an excessive amount of 25(OH)D present, the availability of unoccupied binding sites on DBP for $1,25(OH)_2D$ may be reduced. These findings suggest that it will be necessary to assess more parameters to be able to correctly calculate the true free fraction of $1,25(OH)_2D$.

The same problems will also arise when as an indication of the free fraction the free $1,25(OH)_2D$ index is calculated. This index is the molar ratio of the total $1,25(OH)_2D$ concentration and the DBP concentration [Bouillon *et al.* 1981]. This may explain the unequivocal results obtained while assessing the free $1,25(OH)_2D$ index during estrogen therapy. Cheema *et al.* reported a rise in the free $1,25(OH)_2D$ index after one month of estrogen therapy [Cheema *et al.* 1989]. Hagenfeldt *et al.* [Hagenfeldt *et al.* 1991] and Hartwell *et al.* [Hartwell *et al.* 1990] however did not find any change in the free $1,25(OH)_2D$ index after respectively 3 and 12 months.

Bikle *et al.* [Bikle *et al.* 1992] observed a return to baseline free and total $1,25(OH)_2D$ concentrations when the estrogen was opposed by medroxyprogesterone during 13 days of a 25/30 days cycle, after a rise in both total and free $1,25(OH)_2D$ during unopposed estrogen. This finding indicates that changes in the level of total and free $1,25(OH)_2D$ may also be influenced by the progestagenic component of HRT. This effect can be the result of either the duration of the progestagen period or of the character of the progestagen used. Hartwell *et al.* [Hartwell *et al.* 1990] used continuous NETA as progestagen and did not observe a change in the free $1,25(OH)_2D$ index. Using NETA for 10 days of a 28 days cycle, the study here presented did show a rise in the free $1,25(OH)_2D$ concentration. However, as explained before, the free index assessment has several drawbacks. The different effects on the $1,25(OH)_2D$ seem therefore more likely to be related to the type of the progestagen. The differences in the length of the progestagen period between the study here presented and that of Bikle *et al.* [Bikle *et al.* 1984] are rather small. Therefore, these results seem to indicate a difference between the effect of NETA and of

medroxyprogesterone on the concentration of $1,25(OH)_2D$. As observed earlier [van Hoof *et al.* 1994], there may be different effects on the DBP concentration depending on the androgenic characteristics of the progestagen, thus leading to differences in the free and total $1,25(OH)_2D$ concentrations. This would explain the absence of a rise in total $1,25(OH)_2D$ as found by Bikle *et al.* during HRT [Bikle *et al.* 1992]. This phenomenon will need more research.

From the results presented in this study and the data from the literature, we conclude that during oral HRT with sequential progestagen both the total and free concentration of $1,25(OH)_2D$ are elevated. A direct stimulation by estrogen of the $1,25(OH)_2D$ production seems unlikely, since no effect of estradiol on the 1α-hydroxylase in the kidney has been found [Henry 1981]. Moreover, a higher $1,25(OH)_2D$ would lead to lower PTH levels, which have not been reported. During HRT a raised PTH has been found by McKane *et al.* [McKane *et al.* 1995] and Prince *et al.* [Prince *et al.* 1991a], even though it is still unclear whether there are estrogen receptors in the parathyroid [Saxe *et al.* 1992, Prince *et al.* 1991b]. If this rise in PTH is not due to a direct stimulation of the PTH production by estrogen, it could occur as a result of inhibited bone resorption and following lower plasma calcium levels. Recently, estrogen receptors have been found in osteoclasts [Mano *et al.* 1996] which may indicate that estrogen directly inhibits bone resorption, resulting in a lower plasma calcium concentration. This decreased plasma calcium concentration will be directly counteracted by a rise in $1,25(OH)_2D$ [Suda *et al.* 1973] as well as by a rise in PTH [Sherwood *et al.* 1968]. These higher PTH levels will also result in higher $1,25(OH)_2D$ levels [Garabedian *et al.* 1972].

In conclusion this means that oral hormone replacement therapy leads to a rise in the amount of calcium absorbed through the intestine, by a probably indirect raising of the serum free $1,25(OH)_2D$ concentration. As shown in this study, the elevation of the free $1,25(OH)_2D$ concentration during HRT persists for at least one year. The possible effects of progestagens on the DBP concentration and thus the free $1,25(OH)_2D$ concentration need further study.

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CHAPTER 7

General discussion

7.1 General discussion

Vitamin D plays a crucial role in maintaining calcium homeostasis as well as in the regulation of cell growth and differentiation [Bouillon *et al.* 1995]. Much about this hormone is still unknown, partly because of the lack of a reliable assay. The assessment of total $1,25(OH)_2D$ is complicated because of the very low concentration and the occurrence of several vitamin D metabolites. For the latter reason, it is necessary to separate vitamin D from its main metabolites prior to most assays to eliminate interference. Obviously, a reliable assay for $1,25(OH)_2D$ must have both a high sensitivity and specificity. Most assays available at the moment either lack one of these properties or are too expensive or too cumbersome to be of much use in daily practice.

The paper chromatographic method introduced in the present study to separate the main vitamin D metabolites is in many respects an improvement over existing purification techniques, like HPLC [Watson *et al.* 1991, Eisman *et al.* 1976] and solid phase extraction [Reinhardt *et al.* 1984, Hollis 1986]. Although analytically satisfying, they do have several drawbacks, the most important being the rather long time needed per sample and the higher costs, both financial and environmental, resulting from the use of large amounts of organic solvents. When expressed per sample, paper chromatography is over ten times as fast as HPLC and uses ten times smaller volumes of organic solvents than HPLC or solid phase extraction. Furthermore, with paper chromatography it is possible to separate all three major vitamin D metabolites, whereas solid phase extraction using C18 columns only separates 25(OH)D from $24,25(OH)_2D$ and $1,25(OH)_2D$. The $24,25(OH)_2D$ remaining in this case in the $1,25(OH)_2D$ fraction will not interfere in the radioreceptorassay because the affinity of the receptor for $24,25(OH)_2D$ is much lower, but it may well be a disturbing factor in radioimmunoassays [Clemens *et al.* 1979].

In clinical practice, other interferences in the $1,25(OH)_2D$ assay may occur due to the presence of pharmaceuticals in samples. The first artificial vitamin D analogue that was widely used was calcipotriol (figure 1.1). This analogue is very effective in the treatment of psoriasis [Kragballe *et al.* 1988]. The affinity of DBP for this analogue is many times lower than the affinity for $1,25(OH)_2D$, which results in a rapid transformation of the analogue into inactive metabolites [Kissmeyer & Binderup 1991]. The affinity of the vitamin D receptor for calcipotriol however, is comparable to that for $1,25(OH)_2D$. This means that when the analogue occurs in blood, it will be a potentially interfering factor in the radioreceptorassay. In general practice, this is not a great problem, since the analogue is mostly used in topical applications and no detectable transdermal transport into the bloodstream occurs [Mortensen *et al.* 1993]. Another widely used analogue that has a structure similar to $1,25(OH)_2D$ is

dihydrotachysterol (DHT_2) (figure 1.1). Together with 1α(OH)D, these two substances are currently often used in low $1,25(OH)_2D$ conditions. In the body, DHT_2 is hydroxylated to $1,25(OH)_2DHT_2$ [Schroeder *et al.* 1992], which has a shorter half life than $1,25(OH)_2D$. This ensures that the hypercalcemia in case of an overdose is of much shorter duration than it would be with $1,25(OH)_2D$ treatment [Harrison *et al.* 1967], which makes DHT_2 treatment safer. It has been shown in this study that DHT_2 per se does not interfere in the radioreceptorassay, although unfortunately, interference in the assay by $1,25(OH)_2DHT_2$ could not be ruled out due to unavailability for testing of the appropriate substance. Abnormal $1,25(OH)_2D$ levels during DHT_2 therapy should therefore be looked upon with caution because of this possible interference.

The radioreceptorassay after paper chromatography as described in this study has intra- and interassay coefficients of variation of 10.5% and 11.5% respectively, which are similar to those reported by Hollis [Hollis 1986]. The sensitivity of the assay is 0.5 fmol/tube, calculated as three times the standard deviation of the counts of the zero standard. This is favourable if compared to the assay of Reinhardt *et al.* who, using two times the standard deviation of the zero sample, reported a sensitivity of 3.6 fmol/tube [Reinhardt *et al.* 1984], and compared to the assay of Hollis, who reported a sensitivity of 1.7 fmol/tube, also using two times the standard deviation [Hollis 1986].

Reference values of total $1,25(OH)_2D$ in healthy controls, obtained with the assay described here, range from 80-200 pmol/l which are comparable to those found in the literature [Reinhardt *et al.* 1984, Bouillon *et al.* 1987]. Some authors report much lower reference values for healthy controls [Tsai *et al.* 1984, Hollis 1986], which may be related to the use of a different reference preparation. With the present method, no difference was found in total $1,25(OH)_2D$ levels between men and women, which is in agreement with literature data [Reinhardt *et al.* 1984, Bouillon *et al.* 1987].

In accordance with Sherman *et al.* [Sherman *et al.* 1990] and Orwoll and Meier [Orwol & Meier 1986], we found no relation between age and the total concentration of $1,25(OH)_2D$ in plasma. This is in contrast with the findings of some authors, who did report decreasing $1,25(OH)_2D$ levels with age [Bouillon *et al.* 1987, Tsai *et al.* 1984]. However, the latter two studies were not confined to healthy volunteers, but also comprised diseased subjects. This could very well be the reason for the dissimilarity of the results. Indeed, the production of $1,25(OH)_2D$ is an enzymatic process with no known age-related decrease [Cantatore & Carrozzo 1990]. Furthermore, when only healthy subjects are used for the assessment of the reference values, the substrate for the 25(OH)D 1-hydroxylase should be available in sufficient amounts. In this case, no relation between the total concentration of $1,25(OH)_2D$ and age is to be expected.

Possible differences in reference values at different ages of $1,25(OH)_2D$ levels might also be ascribed to differences in DBP levels, since higher protein levels are capable of binding more $1,25(OH)_2D$. When there is no change in hormone synthesis and degradation, these higher protein concentrations would thus lead to higher hormone concentrations, as would lower DBP levels lead to decreased total $1,25(OH)_2D$ concentrations. However, no age or illness related difference in DBP has been found that could account for the reported differences in total $1,25(OH)_2D$ levels [Toss & Sorbo 1986].

With the $1,25(OH)_2D$ assay as described in this study, high levels of total $1,25(OH)_2D$ were found during use of oral contraceptives and during pregnancy. These results are in accordance with previous studies during premenopausal oral estrogen administration [Bouillon *et al.* 1981, Aarskog *et al.* 1983, Buchanan *et al.* 1986] and during pregnancy [Bouillon *et al.* 1981, Bikle *et al.* 1984, Wilson *et al.* 1990]. Since in the premenopause, estrogens increase hepatic DBP synthesis [Haddad 1979], and have no direct effect on $1,25(OH)_2D$ synthesis and turnover, the increased $1,25(OH)_2D$ levels can exclusively be ascribed to the increased DBP levels. According to the free hormone hypothesis, the free $1,25(OH)_2D$ level should remain normal under these conditions and therefore is a better indicator of the biological activity of this hormone than the total concentration is [Robbins & Rall 1957, Mendel 1989, Ekins 1990]. The hypothesis stems from the fact that only the non-protein bound hormone can directly enter the cells. Of course, the protein-bound fraction will dissociate when free hormone is taken up by the cells lining the bloodvessels, so that the fractional uptake during the passage through an organ can be (much) larger than the free fraction. This latter phenomenon has led some authors to suggest other mechanisms. Pardridge has proposed that the interaction of the albumin-hormone complex with capillary endothelia results in an increased dissociation constant as compared to the one found *in vitro* [Pardridge 1987]. Several authors however, have shown that the difference between the free fraction and the fractional uptake can readily be explained in terms of the free hormone hypothesis, and that there is therefore no need for the postulation of a completely new kind of mechanism [Mendel *et al.* 1988, Ekins 1990]. Others state that at least in some cases, the protein-hormone complex is actively transported into the cells [Keller *et al.* 1969, Siiteri *et al.* 1982], but although this may be the mechanism of uptake of some hormones, no receptors for the complete DBP-$1,25(OH)_2D$ complex have been found and this therefore seems not a likely alternative.

It has been found that $1,25(OH)_2D$ in serum is bound to several binding proteins and calculations show that the high concentration of DBP (6-10 μM) results in less than 0.1% non-protein bound $1,25(OH)_2D$. This generally corresponds to a free $1,25(OH)_2D$ concentration of less than 0.1 pmol/l [Vieth 1994].

Until now, only a few methods to study the free $1,25(OH)_2D$ concentration have been

described. Due to the very low concentrations involved, direct assessment of the free $1,25(OH)_2D$ concentration in an ultrafiltrate or an equilibrium-dialysate is actually not feasible with the methods presently available. Because of this problem, many authors rely on the use of the free index as an indicator of the free concentration. This index is calculated as the molar ratio of $1,25(OH)_2D$ and DBP [Bouillon *et al.* 1981] but, as explained before in Chapter 1, is very imperfect as an estimation of the free concentration.

This means that indirect approaches are the only alternative to assess the free $1,25(OH)_2D$ concentration. Measuring the free $1,25(OH)_2D$ fraction and multiplication of this fraction with the total concentration will yield the free concentration. The only such method published thus far is a centrifugal ultrafiltration technique [Bikle *et al.* 1984]. The main problems with this technique are the susceptibility to tracer impurities and protein leakage through the membrane. Because the free fraction of $1,25(OH)_2D$ is extremely low, namely below 0.1%, an impurity of only 0.1% might already result in a 100% overestimation of the free fraction. Moreover, the pressure exerted on the membrane in centrifugal ultrafiltration potentially may lead to protein leakage which, however small, will likewise result in spuriously elevated values [Dowsett *et al.* 1984]. Consequently, reported results obtained with this method must be considered with caution.

In the present study a new approach is described, using a symmetric dialysis technique to determine the free $1,25(OH)_2D$ fraction. This procedure uses the rate of tracer migration through a membrane as an measure of the free fraction, and has already succesfully been applied to assess the free fraction of other hormones [Ross 1978]. An advantage of this method over the centrifugal ultrafiltration technique consists of the measurement of about a third of the total amount of tracer added compared to less than 1%, which substantially decreases the susceptibility of the method to tracer impurities. Further, since there is neither centrifugal nor osmotic pressure exerted on the membrane the occurrence of leakage of protein-bound tracer is much less likely.

The intra- and interassay coefficients of variation of the assessment of the free $1,25(OH)_2D$ fraction using the symmetric dialysis method were 1.0% and 7.7% respectively. These values are much lower than those reported for the assessment of the free fraction using centrifugal ultrafiltration, namely 13% and 26% respectively [Bikle *et al.* 1984].

From the data obtained in the present study, it is possible to calculate the dissociation constants of albumin and DBP for $1,25(OH)_2D$. Using the concentrations obtained from the experiments with 1% serum albumin and substituting them in formula 1.2, results in a K_d of albumin for $1,25(OH)_2D$ of 1.3×10^{-6} M. The value reported in the literature is about 1.9×10^{-5} M [Bikle *et al.* 1986]. When using this calculated K_d of albumin together with experimental data in formula 1.4, a K_d of DBP for $1,25(OH)_2D$ of about 5×10^{-9} M is found. Again this

figure is about ten times lower than the results published in literature (6.7×10^{-8})[Bouillon *et al.* 1980]. This latter figure however, was established using Scatchard analysis, where the bound and free hormone were separated using dextran-coated charcoal. It is known that this method may lead to overestimated free levels, since charcoal easily disturbs the equilibrium by stripping the hormone from the binding proteins. Since in the present study the free fraction has been assessed without disturbing the equilibrium and with a superior assay, it can be concluded that the K_ds here calculated probably are closer to the true K_ds than those previously reported.

The values of the free fractions reported here are also about ten times lower than those reported before using centrifugal ultrafiltration [Bikle *et al.* 1984, Koenig *et al.* 1992], which is in accordance with our own experiments using centrifugal ultrafiltration at small free fractions. It is most likely that this discrepancy results from the above mentioned limitations of the centrifugal ultrafiltration method. Since it is therefore clear that symmetric dialysis is both theoretically and practically superiour to centrifugal ultrafiltration, it must be concluded that the symmetric dialysis method should be the method of choice for the determination of the free $1,25(OH)_2D$ fraction.

In pregnant women as well as in women using oral contraceptives, the increased estrogen concentration induces higher DBP levels. Assuming this is the only effect of increased estrogen levels, the free hormone hypothesis predicts on the one hand elevated total $1,25(OH)_2D$ and on the other unchanged free $1,25(OH)_2D$ levels. This is exactly what has been found and indeed, no signs of altered calcium or bone metabolism are observed under these conditions. Nevertheless, previous studies reported an increase in the free $1,25(OH)_2D$ concentration during pregnancy [Bikle *et al.* 1984, Wilson *et al.* 1990], but in these studies the ultrafiltration method was used, which - as shown before - may lead to spuriously elevated results. This is especially true in cases where DBP levels are elevated. Since the free fraction will be lower, the relative error induced by impurities or protein-bound tracer leakage will increase and the free fraction will be overestimated even more.

Decreased DBP levels have been reported in nephrotic syndrome, with an unchanged free $1,25(OH)_2D$ index [Chan *et al.* 1983, Grymonprez *et al.* 1995]. In the study presented here however, surprisingly no decrease in DBP levels was found in nephrotic syndrome but instead a decrease in the free $1,25(OH)_2D$ concentration. These results are reminiscent of those of Auwerx *et al.*, who also reported a lack of change in DBP levels and a lower free index [Auwerx *et al.* 1986]. Lower free $1,25(OH)_2D$ levels must be explained by either diminished production and/or increased disposal. Increased $1,25(OH)_2D$ disposal is a likely explanation, because albumin carries about 15% of the circulating hormone. The albumin loss into the urine therefore forms an additional route for $1,25(OH)_2D$ disposal, far exceeding the normal

pathway that depends only on the very small free fraction. The $1,25(OH)_2D$ loss is parallel to the albumin loss, which also explains the correlation between serum albumin and free $1,25(OH)_2D$, as both reflect the severity of proteinuria in nephrotic syndrome. An alternative or additional explanation could be forwarded, namely that the hyperlipidaemia which is encountered in nephrotic syndrome would offer an additional pathway for disposal by virtue of the excellent solubility of $1,25(OH)_2D$ in formed micelles that are readily excreted.

Arguments for decreased synthesis can also be found. The 25(OH)D concentration is also decreased and its level correlates with DBP and proteinuria. This suggests that 25(OH)D is lost along with DBP, although the loss of DBP is not so huge compared with the synthesizing capability, that it leads to lower serum levels, as is the case with albumin. Lower levels of 25(OH)D mean less substrate availability for the 1-hydroxylation, so that this also may be a possible cause for the decreased $1,25(OH)_2D$ concentration. Indeed, the decreased $1,25(OH)_2D$ levels in patients with nephrotic syndrome can at least be partially reversed by administration of 25(OH)D [Haldimann & Trechsel 1983].

The lowered free $1,25(OH)_2D$ may very well explain the reported osteomalacia and hyperparathyroid bone disease in nephrotic patients [Malluche *et al.* 1979] as well as increased bone resorption reported in nephrotic rats [Sierra *et al.* 1997]. Thus, the free $1,25(OH)_2D$ in serum may still reflect its biological effect, although it is no longer a direct measure of its removal. This is because in nephrotic syndrome, a significant proportion of hormone disposal is accounted for by the protein-bound fraction. Under this pathological condition, the free hormone hypothesis appears to be still valid with regard to the biological effect.

The aforementioned observations clearly indicate that the measurement of the free $1,25(OH)_2D$ concentration rather than of the total $1,25(OH)_2D$ concentration reflects the true $1,25(OH)_2D$ status and therefore should preferably be used. The free index should preferably not be employed in these circumstances, as it ignores the effect of the low albumin levels. The lower albumin concentrations should lead to a higher percentage of free $1,25(OH)_2D$, which is adequately reflected in the fact that the average percent free $1,25(OH)_2D$ in patients with nephrotic syndrome just exceeds the upper limit for healthy males as reported in Chapter 3.

After it had been shown that the symmetric dialysis assay can successfully be used to assess the free concentration of $1,25(OH)_2D$ in challenging situations, it was utilized to measure free $1,25(OH)_2D$ during HRT. During the menopause a significant accelerated loss of bone density occurs [Albright *et al.* 1940], the exact causes of which are still unknown. Many therapies are used to try to halt or even to reverse this phenomenon, but still the most effective therapy currently available is estrogen replacement therapy [Lindsay *et al.* 1976]. During oral estrogen therapy a rise in total serum $1,25(OH)_2D$ has been observed [Prince *et al.* 1991, Lund *et al.*

1982, Aloia *et al.* 1991]. However, literature on this issue is contradictory [Selby & Peacock 1986, Kalu *et al.* 1991], and most studies describe short treatment intervals of no more than six months. The increased estrogen levels will lead to increased DBP production [Dick *et al.* 1995] and, according to the free hormone hypothesis, this rise would lead to increased total $1,25(OH)_2D$ levels, but will only result in a change in free $1,25(OH)_2D$ when there is also an effect on $1,25(OH)_2D$ synthesis or degradation. Previous results on this subject are contradictory, probably because of problems with the assays used [Cheema *et al.* 1989, Bikle *et al.* 1992, Dick *et al.* 1995].

In the present study, the total $1,25(OH)_2D$ concentration in subjects receiving HRT was significantly elevated as compared to baseline during the whole two years of the study. A significant rise in both the total and free $1,25(OH)_2D$ concentrations was found during one year of therapy. These findings indisputably show that HRT increases the biologically active $1,25(OH)_2D$ and it seems plausible that this rise in free $1,25(OH)_2D$ forms at least part of the mechanism by which HRT exerts its effect on bone metabolism. In contrast, premenopausal estrogen administration has no effect on bone formation, nor on plasma PTH [Zofkova & Kancheva 1996]. The apparent contradictory effects of estrogens before and after the menopause may be explained by assuming that stimulation by estrogens of $1,25(OH)_2D$ synthesis is at its maximum at normal premenopausal estrogen levels, so that further stimulation has no effect. In the postmenopausal state, however, estrogens are low and restoration to premenopausal values leads to an increase of free $1,25(OH)_2D$ to premenopausal levels. However, this is only plausible when there is a decrease in 1-hydroxylase activity and thus a decrease in the free $1,25(OH)_2D$ concentrations after menopause, when estrogen levels drop dramatically. Although this has not been reported until now, comparing the postmenopausal free $1,25(OH)_2D$ values before the start of treatment with the premenopausal levels presented in chapters 3 and 4 (68 fmol/l vs 98 fmol/l; $p \leq 0.0001$), it can only be concluded that there indeed is a significant decrease of $1,25(OH)_2D$ synthesis around the climacterium. This decrease might even play a role in the genesis of postmenopausal osteoporosis, in concert with other factors known to be of importance in the cause of the disease [Wark 1996].

Another factor of interest is formed by the free fatty acids [Jensen *et al.* 1994]. Fatty acids have been shown to affect the calcium uptake through the intestine [Kreutter *et al.* 1983, Coetzer *et al.* 1994], to decrease the calcium binding of albumin [Ladenson & Shyong 1977, Aguanno & Ladenson 1982], and to bind calcium themselves [Stevens & Olson 1984, Watras *et al.* 1984]. Through these actions, they thus may indirectly alter $1,25(OH)_2D$ synthesis. Moreover, it has been reported that free fatty acids decrease the binding affinity of albumin for several steroid hormones [Watanabe *et al.* 1990], and that polyunsaturated free fatty acids

decrease the binding affinity of DBP for $1,25(OH)_2D$ [Calvo & Ena 1989, Bouillon *et al.* 1992]. Oral contraceptive use increases the proportion of polyunsaturated free fatty acids [Hagenfeldt *et al.* 1977] and continuous estrogen/progestogen treatment in postmenopausal women results in an increase of the polyunsaturated arachidonic and linoic acids in serum lecithin ester [Mattson *et al.* 1986]. Thus lowering of binding affinity may be expected to occur during HRT. The binding affinity for $1,25(OH)_2D$ was not assessed in the study here presented. However, according to the law of mass action, in this case of an insaturable amount of binding sites, the free fraction should increase when the affinity of the binding proteins decreases. The facts that the DBP concentration during HRT is either normal or elevated and the free $1,25(OH)_2D$ fraction increases during HRT indeed point towards a decrease of the affinity of DBP for $1,25(OH)_2D$ by the free fatty acids.

Furthermore, there have been reports of effects of $1,25(OH)_2D$ *per se* on polyunsaturated fatty acid metabolism [Adamek *et al.* 1987, Bellido *et al.* 1987, Drittanti *et al.* 1988], which suggest an even more direct link between both $1,25(OH)_2D$ and the fatty acids.

Interestingly, although hepatic DBP production can be stimulated by estrogens [Haddad 1979], no decrease has been found in DBP levels during menopause as a result of the decreasing estrogen levels [Falch *et al.* 1987, Hartwell *et al.* 1990a]. The same effects can be found with corticosteroid-binding globulin, another steroid binding protein in plasma [Moore *et al.* 1978]. In contrast, the levels of a third hepatic, estrogen stimulated, hormone binding protein, namely sex hormone binding globulin, do fall after menopause. Apparently, the effects that occur during withdrawal of estrogens are not always opposite to the effects that occur during the addition of estrogens. Again, effects of estrogen may be related to its starting level.

At the moment, it may be concluded that changes of estrogen levels from the normal premenopausal to the postmenopausal state do affect $1,25(OH)_2D$, but probably not DBP synthesis. However, differences between normal premenopausal and oral contraceptive or pregnancy induced elevated levels do affect DBP but not $1,25(OH)_2D$ activity. The net result of changes in $1,25(OH)_2D$ binding conditions is conveniently reflected by the fraction or percent $1,25(OH)_2D$ that is in the free form.

It has been suggested by Lee that progestogen and not estrogen *per se* is responsible for the increased bone density during HRT [Lee 1991]. To reduce the hyperplastic effects of estrogen on the endometrium, estrogen administration is often combined with progestogen. In literature there have been many contradicting reports concerning the effects of progestagens. Trémollières *et al.* reported estrogen to be a stronger inhibitor of postmenopausal bone loss than promegestone [Trésmollières *et al.* 1993]. Bikle *et al.* observed a return to baseline of increased free and total $1,25(OH)_2D$ concentrations when the estrogen was opposed by

medroxyprogesterone during 13 days of a 25/30 days cycle [Bikle *et al.* 1992]. Hartwell *et al.* used continuous NETA as progestagen and did not observe a change in the free $1,25(OH)_2D$ index at all [Hartwell *et al.* 1990b]. Scheven *et al.* have reported that natural progestogen is a more potent stimulator of osteoblastic growth than estrogen [Scheven *et al.* 1992]. In the present study, no effect of dydrogesterone on the total $1,25(OH)_2D$ concentration was found, while NETA did not prevent a rise in the free $1,25(OH)_2D$ concentration. There are several reasons why it is very difficult to compare all reported data on this subject. The progesterone analogues used are not the same, the lengths of the studies differ, and the length of the progestagenic period in each study is different. Nevertheless, it seems that the hypothesis of Lee (see above) is rather doubtful. A more likely hypothesis would be that the effects of progesterone on the calcium metabolism and thus bone density depend on the androgenic characteristics of the progesterone used. This view has been supported by the recent finding of Roux *et al.* that dydrogesterone, a progestagen without androgenic effects, does not prevent bone loss due to ovariectomy in rats [Roux *et al.* 1996]. Nevertheless, a thorough study of the possible effects of progesterone on bone metabolism should be performed to fully elucidate this subject.

In the present study, reliable assays for the assessment of both total and free $1,25(OH)_2D$ concentrations in plasma are described, that are superior to existing methods. It has been shown that the assessment of the free $1,25(OH)_2D$ concentration is preferable to the assessment of the total $1,25(OH)_2D$ concentration, as anticipated by the free hormone hypothesis. This is because by assessment of only total $1,25(OH)_2D$, the true status may be partially or wholly obscured by effects on protein binding, such as occur during oral contraceptive use, pregnancy or nephrotic syndrome. The rise in total $1,25(OH)_2D$ during HRT and the beneficial effect of this therapy on bone metabolism in postmenopausal women has been interpreted as purely coincidentally due to side effects on hormone binding [Dick *et al.* 1995]. However, the rise in free $1,25(OH)_2D$ as we have found, shows that the effect of HRT on bone formation could at least partially have been mediated by the biological effect of $1,25(OH)_2D$.

7.2 References

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CHAPTER 8

Summary

8.1 SUMMARY

This thesis ultimately aims at gaining more insight into the possible role of $1,25(OH)_2D$ in the genesis and prevention of postmenopausal osteoporosis by estrogens.

In the introduction (Chapter 1) an extensive overview is given of the ontogenesis, biochemistry, epidemiology and clinical relevance of the hormone.

In approaching the desired insight, a new specific and sensitive method for serum total $1,25(OH)_2D$ has been developed, which is based on radioreceptorassay preceded by extraction and paper chromatographic prepurification (Chapter 2). This method has superiour characteristics over previously published methods and also significantly reduces both the financial and environmental costs per sample. In addition, symmetric dialysis was adopted for the measurement of the free $1,25(OH)_2D$ fraction in serum. This resulted in an assay which is superior in accuracy and precision to the previously described ultrafiltration technique. This new assay was applied to assess for the first time the free $1,25(OH)_2D$ in control and pregnancy serum samples. The results demonstrate that the free moiety is unaltered during pregnancy in spite of the elevated total $1,25(OH)_2D$ levels, induced by the rise of the vitamin D binding protein (DBP). Thus, the validity of the free hormone hypothesis in the case of $1,25(OH)_2D$ is demonstrated (Chapter 3). This is further explored by measurements performed in samples from subjects using oral contraceptives and from patients with nephrotic syndrome. During use of oral contraceptives, the findings were comparable to those obtained during pregnancy, and also support the free hormone hypothesis (Chapter 4). In nephrotic syndrome, both total and free $1,25(OH)_2D$ were decreased in spite of unaltered DBP. This could be explained by massive loss of the hormone into the urine in albumin-bound form. According to the free hormone hypothesis, these low free levels of $1,25(OH)_2D$ mean low biological $1,25(OH)_2D$ activity , which is in accordance with reports suggesting bone malformation with long-standing proteinuria (Chapter 4). Chapters 5 and 6 deal with the effects of postmenopausal hormone replacement therapy (HRT) on total and free $1,25(OH)_2D$. Initially, low levels significantly rise to slightly elevated values concomitant with the beneficial effect on bone metabolism.

The general discussion (Chapter 7) compares the effects of estrogen treatment before and after the menopause. Without estrogen treatment, postmenopausal free $1,25(OH)_2D$ is reduced to about 70% of the premenopausal level, which partially might explain the onset of osteoporosis. Starting at the normal premenopausal estrogen levels, further elevation of these levels has no effect on free $1,25(OH)_2D$. In contrast, estrogen suppletion has a considerable effect on the subnormal free $1,25(OH)_2D$ level in the estrogen deficient postmenopausal state, leading to free $1,25(OH)_2D$ values even higher than normal. This not only suggests that the

subnormal postmenopausal free $1,25(OH)_2D$ levels may play a role in the genesis of osteoporosis, it also suggests a significant role for vitamin D as a mediator of the effect of HRT on bone formation, notwithstanding the fact that other effects of HRT have been described that affect calcium metabolism and thereby PTH synthesis. HRT is expected to affect $1,25(OH)_2D$ binding to DBP and/or albumin, thus potentially obscuring the true $1,25(OH)_2D$ status when assessed through measurements of total hormone only. Assessment of the free moiety now has proven to be a useful aid in exploring the involvement of $1,25(OH)_2D$ in the genesis and prevention of postmenopausal osteoporosis.

CHAPTER 9

Samenvatting

9.1 SAMENVATTING

Dit proefschrift heeft tot doel om meer inzicht te verkrijgen in de mogelijke rol van $1,25(OH)_2D$ bij het ontstaan en de preventie van postmenopausale osteoporose.
In de Inleiding (Hoofdstuk 1) wordt een uitgebreid overzicht gegeven van de ontogenese, biochemie, epidemiologie en klinische relevantie van dit hormoon.
Voor het bereiken van het gewenste doel werd een nieuwe specifieke en gevoelige methode voor het bepalen van totaal $1,25(OH)_2D$ in serum ontwikkeld, gebaseerd op een radioreceptorassay welke wordt vooraf gegaan door extractie en papier-chromatografische voorzuivering (Hoofdstuk 2). Deze methode is niet alleen gevoeliger dan de bestaande methoden, maar brengt ook lagere kosten met zich mee per monster, zowel financieel als vanuit milieu-oogpunt. Verder werd symmetrische dialyse toegepast voor de meting van de fractie vrij $1,25(OH)_2D$ in serum. Dit resulteerde in een bepalingsmethode die in juistheid en precisie de eerder in de literatuur beschreven ultrafiltratietechniek verre overtreft. De symmetrische dialyse werd toegepast om voor het eerst het vrij $1,25(OH)_2D$ in serum te meten van gezonde zwangere en niet-zwangere proefpersonen. Hierbij werd aangetoond dat de concentratie van de vrije vorm gelijk blijft tijdens de zwangerschap, ondanks de verhoogde totale $1,25(OH)_2D$ spiegel. Deze verhoging wordt veroorzaakt door de stijging van het vitamine D bindend eiwit (DBP), dat op zijn beurt geïnduceerd wordt door de verhoogde oestrogenenspiegel tijdens de zwangerschap. De geldigheid van de vrije-hormoon-hypothese wordt hierdoor ondersteund (Hoofdstuk 3). Dit werd verder onderzocht met behulp van metingen uitgevoerd in serum van gezonde vrouwen die orale anticonceptiva gebruiken en van patiënten met het nefrotisch syndroom. De resultaten bij vrouwen die orale anticonceptiva gebruiken waren vergelijkbaar met die verkregen bij zwangeren en ondersteunen ook de vrije hormoon hypothese (hoofdstuk 4). Daarentegen waren bij patiënten met het nefrotisch syndroom zowel de totale als de vrije $1,25(OH)_2D$ spiegels verlaagd, ondanks gelijk blijvende DBP spiegels. Dit kan worden verklaard door een sterk verlies van het hormoon in albumine-gebonden vorm naar de urine aan te nemen. De vrije hormoon hypothese stelt dat de lage vrije $1,25(OH)_2D$ spiegel een lage biologische $1,25(OH)_2D$ activiteit betekent, wat past in het beeld van een mogelijk verminderde botaanmaak bij patiënten met nefrotisch syndroom (Hoofdstuk 4). De hoofdstukken 5 en 6 behandelen de effecten van postmenopausale hormoon substitutie (HRT) op de totale en vrije $1,25(OH)_2D$ spiegels in serum. Aanvankelijk lage vrije hormoonspiegels stijgen tot licht verhoogde waarden hetgeen gepaard gaat met gunstige effecten op de botstofwisseling. In de algemene discussie (Hoofdstuk 7) wordt een vergelijking gemaakt tussen het effect van oestrogenenbehandeling voor en na de menopauze. Zonder oestrogenenbehandeling ligt het postmenopausale vrij $1,25(OH)_2D$ op ongeveer 70%

van het premenopausale niveau, welke verlaging ten dele een verklaring zou kunnen zijn voor het ontstaan van de osteoporose. Verdere verhoging van reeds normale premenopausale oestrogenenspiegels heeft geen effect op de vrije $1,25(OH)_2D$ concentratie. Daarentegen heeft, zoals eerder vermeld, oestrogeensuppletie in de postmenopausale oestrogenendeficiente periode een aanzienlijk effect op het verlaagde vrije $1,25(OH)_2D$ niveau, dat hierdoor zelfs tot boven premenopausale waarden kan stijgen. Dit suggereert een belangrijke rol van het $1,25(OH)_2D$ als intermediair bij het effect van HRT op de botvorming, naast andere effecten van HRT welke de calciumstofwisseling en daarmee de PTH synthese beïnvloeden. HRT beïnvloedt waarschijnlijk de binding van $1,25(OH)_2D$ aan DBP en albumine, en belemmert daardoor het zicht op de werkelijke $1,25(OH)_2D$ status, wanneer deze alleen aan de hand van de meting van totaal hormoon zou plaatsvinden. Meting van de vrije vorm is aldus een nuttig hulpmiddel bij de bestudering van de betrokkenheid van $1,25(OH)_2D$ bij het ontstaan en het voorkomen van postmenopausale osteoporose.

LIST OF ABBREVIATIONS

1(OH)D	1α-hydroxyvitamin D
$1,25(OH)_2D$	1α,25-dihydroxyvitamin D
$24,25(OH)_2D$	24,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
CBG	corticosteroid binding globulin
cpm	counts per minute
DBP	vitamin D binding protein
DHT_2	dihydrotachysterol
HPLC	high-performance liquid chromatography
HRT	hormone replacement therapy
HSA	human serum albumin
MC901	calcipotriol
MS	mass spectrography
NETA	norethisteroneacetate
NS	not significant
p	probability
PBS	phosphate buffer
PTH	parathyroid hormone
r	correlation coefficient
SD	standard deviation
SEM	standard error of the mean
SHBG	steroid hormone binding globulin
TBG	thyroid hormone binding globulin
VDR	vitamin D receptor

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 18 juni 1966 te Waalwijk. In 1984 behaalde hij het VWO diploma aan het Dr. Mollercollege te Waalwijk en begon daarna aan de studie Scheikunde aan de Katholieke Universiteit Nijmegen. Zijn hoofdvakstage heeft hij gedaan bij de vakgroep Biochemie onder leiding van Dr. W. de Jong en behelste 'Onderzoek aan posttranslationele modificaties van α-crystalline'. Zijn bijvakstage Klinische Chemie werd uitgevoerd op het Laboratorium Endocrinologie en Voortplanting in het St. Radboud ziekenhuis onder leiding van Prof. Dr. Th. Benraad en betrof het onderzoek naar 'Biologisch beschikbaar testosteron'. Het doctoraal examen werd behaald in 1990. Meteen hierna werd hij aangesteld op het Laboratorium Endocrinologie en Voortplanting als wetenschappelijk medewerker. Zijn onderzoek was eerst een voortzetting van dat van de bijvakstage. Daarna heeft hij geholpen bij het implementeren van een nieuwe bepaling voor catecholamines en later werd het $1,25(OH)_2$ vitamine D onderzoek gestart, wat beschreven is in dit proefschrift.
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Frontpage illustration:
Amulet found in the tomb of Tutankhamen, depicting the Eye of Horus.
Horus was an ancient Egyptian sun god. He was the son of Osiris, the god of the air and the Earth and the mother goddess Isis. Osiris was killed by his brother Seth who wanted to seize his throne. When Horus grew up, he challenged Seth, and in the following fight, Horus lost one eye, but won the war. Thoth, the God of Wisdom, managed to heal the eye, after which Horus offered his recovered eye to Osiris and by doing so resurrected him.
To the ancient Egyptians, the eyes of Horus were depicting the sun (right) and the moon (left). Because of the myth, it was a powerful amulet believed to ward of disease and ensure safety.

A symbol of the sun, thought to protect health almost makes one imagine the ancient Egyptians already knew all about 1,25-dihydroxyvitamin D.