THE EFFECT OF VITAMIN D_3 STATUS ON VITAMIN D_3 METABOLISM AND DISTRIBUTION OF METABOLITES.

BY

LESLIE RABINOWITZ

M.B.,Ch.B.(UCT),D.P.H.(UCT),

D.C.H.(R.C.P.& S.), F.R.C.P.(Edin.)

A dissertation submitted to the Faculty of Medicine,

University of the Witwatersrand, Johannesburg

for the Degree of Master of Science

August 1994

ABSTRACT

This dissertation discusses the results of a group of experimental studies performed on chickens to show the effects of vitamin D_3 status on vitamin D metabolism and the distribution of its metabolites in various organs. The chick was chosen as the experimental animal (Norman 1990a) as its vitamin D status can be easily manipulated and it appears that its vitamin D_3 metabolism is similar to that in man.

The oral requirements of vitamin D to produce a vitamin D replete state in vitamin D deficient chickens was assessed by dosing different groups of birds with varying doses of vitamin D_3 over a 15 day period. Plasma levels of 25-hydroxyvitamin D_3 [$25(OH)D_3$] were used to determine the vitamin D_3 status of the chickens. Vitamin D_3 at a dose of 1 ug every 3 days over the study period produced plasma levels of $25(OH)D_3$ of 20.6 ±5.6 ng/ml, which was considered to be within the physiological normal range.

The metabolism of vitamin D and its distribution of its various metabolites in different tissues were assessed in birds given normal, deficient or supraphysiological doses of vitamin D_3. At the end of the study, plasma , duodenal mucosa, kidney, liver, muscle and adipose tissue were extracted and the vitamin D_3 metabolites quantitated in each tissue, by assessing the conversion of radioactive vitamin to the various metabolites. Separation of the various metabolites was achieved by initial silicic acid chromatography followed by either isocratic or gradient high pressure liquid chromatography (HPLC)

The group of chicks receiving repeated doses of 4μ vitamin D_3 appeared to be the group receiving physiological amounts of the vitamin causing a vitamin replete state. In the plasma of these replete birds, $25(OH)D_3$ was the major metabolite present followed by vitamin D and the 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) which was

approximately 9 percent of the value of $25(OH)D_3$. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) was present at concentrations some 70 fold lower than $24,25(OH)_2D_3$. In the replete state, vitamin D_3 was present in all tissues. The largest amount was retained in adipose tissue. The amount of $25(OH)D_3$ present in adipose tissue was lower than that present in plasma.

In the deplete group of birds, all the plasma values of vitamin D_3 and its metabolites were either not detectable or present in very small amounts. The significant feature in the organs in this deficient state, was in the duodenal mucosa where the active metabolite $1,25(OH)_2D_3$ was the major metabolite.

In both the normal and deplete states, the plasma profile of the metabolites simulated those in humans.

The supraphysiological group recorded plasma levels of the vitamin and its metabolites greater than those present in the 4μ group, $25(OH)D_3$ again being the major metabolite. Vitamin D_3, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were also present, $24,25(OH)_2D_3$ being 9 percent of $25(OH)D_3$ and practically 80 fold greater than $1,25(OH)_2D_3$. Adipose tissue had a great affinity for vitamin D_3 and to a lesser extent $25(OH)D_3$. Liver and muscle also contained the vitamin, but not of the magnitude as that seen in adipose tissue.

The levels of putative vitamin D_3 esters in plasma and organs were highest in the supraphysiological group, but in both the replete and supraphysiological states the liver harboured much higher amounts than the other organs.

The possibility of adipose tissue being a storage organ for vitamin D in the replete state is very suggestive, judging from the quantity of vitamin located in this organ.

The plasma levels of calcium and phosphorus in chickens receiving physiological amounts of vitamin D_3, were similar to those reported in replete birds by other researchers. In the vitamin D deficient group, plasma calcium levels were low, while alkaline phosphatase values were elevated.

The studies confirm that the metabolism of vitamin D_3 and the distribution in tissues are influenced by the vitamin D_3 status of the chick. Further, plasma 25(OH)D values are a good indicator of the vitamin D_3 status of the animal.

The study also suggests that the adipose tissue in particular, and to a lesser extent muscle and liver may accumulate large amounts of vitamin D_3 when supraphysiological doses are given. High levels of putative vitamin D_3 esters were found in the liver of chickens on supraphysiological doses of vitamin D_3, however the role or function of this metabolite in these animals is unclear.

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in Faculty of Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in this or any other university.

.....3rd..... day of .....October..... 19.94.

.....

(LESLIE RABINOWITZ)

I certify that the studies contained in this dissertation have the approval of the Animal Ethics Committee of the University of the Witwatersrand (AEC Number 88/4/5).

..... Date: ...3/10/94

(signature of student)

TABLE OF CONTENTS.

CHAPTER 1 1

Vitamin D metabolism and mineral homeostasis.

1.1 Introduction. 2

1.2 Historical 3

1.3 The Vitamins D. 4

1.4 Metabolism of vitamin D. 14

1.5 Pharmacokinetics of vitamin D and its metabolites. 37

1.6 Vitamin D as a calcium regulating hormone. 42

1.7 Vitamin D as a phosphorus controlling hormone. 47

1.8 Serum levels of vitamin D and its metabolites. 50

1.9 Vitamin D metabolism in vitamin D deplete animals and man. 54

1.10 Vitamin D toxicity. 57

1.11 Discrimination of vitamin D by avian species and other animal species. 60

1.12 Distribution and storage of vitamin D and its metabolites in mammalian, avian and human tissues 63

1.13 Analysis of studies. 66

1.14 Vitamin D storage organs. 71

1.15 Summary of vitamin D_3 metabolism and its action on mineral homeostasis 73

1.16 Reason for undertaking of this study. 75

CHAPTER 2 76

Aims and methods.

2.1 Aims of study 77

2.2 Methods 78

2.2.1 Competitive protein-binding assay for measurement of plasma 25 hydroxyvitamin D_3 78

2.3 Validation of 25(OH)D_3 assay. 84

2.4 Extraction of specimens for determination of vitamin D_3 metabolites . 85

2.4.1 Plasma extraction prior to silicic acid chromatography. 85

2.4.2 Extraction of tissues by method of Bligh and Dyer. 86

2.5 Silicic acid chromatography for the separation of vitamin D_3 metabolites 88

2.5.1 Preparation of silicic acid for chromatography. 88

2.5.2 Silicic acid chromatography used to separate 25(OH)D_3 fraction from other metabolites 88

2.5.3 25-Hydroxyvitamin D_3 losses during extraction and chromatography. 91

2.5.4 Silicic acid column chromatography in the preliminary study 92

2.5.5 Silicic acid chromatography in the definitive study. 95

2.6 Isocratic high performance liquid chromatography of partially purified liquid extracts 96

2.6.1 Further purification of vitamin D_3, 25(OH)D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ 97

2.6.2 Quantitation of recovery of vitamin D_3 and metabolites during extraction and chromatography. 97

2.6.3 Concordance of unlabelled and radiolabelled metabolites. 101

2.7 High performance liquid chromatography used in definitive study . . 103

2.8 Quantitation of radioctive specimens 106

2.9 Quantitation of vitamin D_3 metabolites in plasma and tissues 106

2.10 Conversion of dpm to pmol/ml or pmol/g for plasma and tissue 108

CHAPTER 3 109

Vitamin D_3 requirements for physiological values of plasma 25-hydroxyvitamin D_3 in the chick.

3.1 Aims of study. 110

3.1.1 Rearing of chicks under rachitic conditions. 110

3.1.2 Vitamin D_3 preparation 110

3.1.3 Vitamin D_3 dosages for chickens. 112

3.1.4 Collection of samples. 114

3.2 Results of Study. 115

3.2.1 25-Hydroxyvitamin D_3 plasma values. 115

3.2.2 Effect of vitamin D_3 on plasma calcium, phosphorus and alkaline phosphatase concentrations 118

CHAPTER 4

121

Preliminary study: vitamin D_3 metabolism in the chicken given varying [^{3}H] vitamin D_3 concentrations.

4.1 Introduction. 122

4.1.1 Dosing of birds and preparation of tissues. 122

4.2 Results of the preliminary study. 123

4.2.1 Plasma results. 124

4.2.2 Target organs:-duodenal mucosa and kidney. 124

4.2.3 Organs:- liver, muscle and adipose tissue. 125

CHAPTER 5

137

Definitive study to determine vitamin D_3 and its metabolite distribution in chickens in different vitamin D_3 status.

5.1 Introduction 138

5.2 Investigation of chickens in definitive study. 138

5.2.1 Rearing of chicks and dosages of [^{3}H]vitamin$_3$. 139

5.2.2 Weights of chickens in definitive study 140

5.3 Results of definitive study 141

5.3.1 Plasma results. 141

5.3.2 Target organs: duodenal mucosa and kidney. 142

5.3.3 Liver. 143

5.3.4 Storage organs : Skeletal muscle and adipose tissue. 144

5.4 Vitamin D_3 Esters. 145

5.5 Unknown polar metabolites. 146

CHAPTER 6 162

Discussion and conclusions.

6.1 The chick as a suitable animal model to study vitamin D_3 metabolism 163

6.2 Vitamin D_3 deficient chicks 164

6.3 Weight of chickens in various groups 164

6.4 Is vitamin D a growth hormone? 165

6.5 Myopathy of vitamin D_3 deficient chicks 166

6.6 Effects of vitamin D_3 in mineral homeostasis 168

6.7 Vitamin D_3 and its metabolites in plasma and tissues in the physiological state 169

6.8 The effect of vitamin D_3 depletion on metabolites in plasma and organs. 172

6.9 Effects of supraphysiological doses of vitamin D_3 in plasma and tissues 174

6.10 The relationship of $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ in different vitamin D_3 status. 177

6.11 Vitamin D_3 storage organs 178

6.12 Vitamin D_3 esters 179

6.13 Polar metabolites of vitamin D_3 180

6.14 Comparison of the two vitamin D_3 deficient groups. 182

6.15 Vitamin D_3 requirements for chickens. 182

6.16 Conclusions from present study 183

LIST OF ABBREVIATIONS

$25(OH)D_3$	=	25 hydroxyvitamin D_3
$24,25(OH)_2D_3$	=	24,25 dihydroxyvitamin D_3
$1,25(OH)_2D_3$	=	1,25 dihydroxyvitamin D_3
DBP	=	vitamin D binding protein
HPLC	=	High pressure liquid chromotography
^3H	=	Tritium
cpm	=	counts per minute
dpm	=	disintergrations per minute
g	=	gram
mg	=	millegram
µg	=	microgram
ng	=	nanogram
pg	=	picogram
ml	=	millilitre
µl	=	microlitre
nm	=	nanometer
pmol	=	picomole
OD	=	optical density
CaBP	=	calcium binding proteins.

ACKNOWLEDGEMENTS

My sincerest thanks to Professor J.M. Pettifor, my supervisor, for his help, advice and time devoted to this project. Also his encouragement when the morale was at a low ebb.

I wish to thank Professor F.P. Ross for his expert advise given me with the various laboratory techniques.

Thanks also to Dr. Elvis D. Daniels for his assistance with the statistical evaluations.

I am also thankful to Mrs. Lynne Laishley for her typing and skill in compiling the charts.

Stan Ruch was a great help in finalising this dissertation.

I would especially like to thank my wife Esme for her support throughout this study.

TABLES

Table 1.

Serum Levels and Body Pools of vitamin D and its Metabolites in Adults (Marx 1989). 40

Table 2.

Serum 25(OH)D concentrations in various populations. 41

Table 3.

Serum $1,25(OH)_2D$ concentrations in normal persons. 41

Table 4.

Serum levels of vitamin D_2 and vitamin D_3 and their metabolites in five species of animals. 52

Table 5.

Percentages of vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ in various tissues of rachitic chicks after administration of radioactive Vitamin D_3. 69

Table 6.

The volume of solvents and water added per gram or ml of tissue during the Bligh and Dyer extraction 87

Table 7(i).

Recovery of [^{3}H]-25(OH)D_3 added to plasma prior to extraction and chromatography 92

Table 7(ii).

Recovery of [^{3}H]-25(OH)D_3 added to lipid extract prior to chromatography 92

Table 8.

Solvent system used in preliminary study 93

Table 9.

Solvent systems applied to silicic acid column in definitive study 96

Table 10.

Solvent systems used in HPLC in preliminary study 97

Table 11.

Masses of vitamin D_3 and metabolites added to samples and absorbance range utilized in HPLC definitive study 104

Table 12.

Rachitogenic diet 114

Table 13.

Plasma 25(OH)D obtained after dosing vitamin D deficient chickens with varying doses of vitamin D_3 116

Table 14.

Effect of vitamin D_3 status on plasma calcium, phosphorus and alkaline phosphatase values 119

Table 15.

Preliminary results: putative vitamin D_3 esters, vitamin D_3, $25(OH)D_3$, $24,25(OH)_2$, $1,25(OH)_2D_3$ and polar metabolites in plasma, duodenum, kidney, liver, skeletal muscle and fat in chickens which are a) vitamin D_3 deficient animals; b) animals receiving vitamin D_3 2μg doses, c) 4μg doses, d) 8μg doses and e) 16μg doses 127-129

Table 16.

Weights of chickens in definitive study 140

Table 17.

Definitive results: putative vitamin D_3, vitamin D_3, $25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$ and polar metabolites in plasma, duodenal mucosa, kidney, liver, skeletal muscle and adipose tissue.

a) vitamin D_3 deficient birds-single dose (SD);

b) vitamin D_3 deficient birds-multiple doses (MD);

c) birds receiving vitamin D_3 4μg doses;

d) birds receiving vitamin D_3 16μg doses

. 149-152

FIGURE LEGENDS

Figure 1.

Structure of a) vitamin D_2 (ergocalciferol), and

b) vitamin D_3 (cholecalciferol) compounds. 5

Figure 2.

Irradiation of ergosterol to form ergocalciferol. 7

Figure 3.

Photochemical reaction of provitamin D_3 to form previtamin D_3;

Thermal isomerization of previtamin D_3 to vitamin D_3. 9

Figure 4.

Photosynthesis of previtamin D_3 (Holick 1981). 11

Figure 5.

First hydroxylation of vitamin D_3 in liver by enzyme vitamin D 25-hydroxylase. 15

Figure 6.

Hydroxlation of 25(OH)D to form $1,25(OH)_2D$ and $24,25(OH)_2D$. 20

Figure 7.

C-24 oxidation pathway in catabolism of $1,25(OH)_2D_3$ in kidney. 33

Figure 8.

A typical standard curve for the measurement of $25(OH)D_3$ in chicken plasma samples. 82

Figure 9.

Silicic acid chromatography for elution of $25(OH)D_3$ (ether-hexane 95:5 v/v). . 90

Figure 10.

Preliminary study: Silicic acid chromatography for elution of vitamin D_3 and the main metabolites. 94

Figure 11.

Quantitation of authentic a) vitamin D_3 and b) $1,25(OH)_2D_3$. 99-100

Figure 12.

Concordance of unlabelled vitamin D_3 and [^{3}H]-vitamin D_3. 102

a) scan of vitamin D_3, b) dpm counts of [^{3}H]-vitamin D_3,

and dpm counts superimposed over scan of same sample.

Figure 13.

Definitive study: HPLC of i) vitamin D_3, ii) 25(OH)D_3, iii) 24,25$(OH)_2D_3$,

iv) 1,25$(OH)_2D_3$.

Solvent systems: isopropanol/hexane i) 1:99, ii) 4:96 iii) 5:95,

iv) 14:86 v/v.

Isocratic HPLC: vitamin D_3 and 25(OH)D_3.

Gradient HPLC: 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$. 105

Figure 14.

The quantitation of radioactive vitamin D_3 metabolites:

a) UV tracing of authentic vitamin D_3 metabolite,

b) dpm obtained in each fraction eluting off the column.

Sample 13 and 14 correspond to fractions 13 and 14 in figure (a). 107

Figure 15.

A typical UV scan of authentic vitamin D_3 using dual beam spectrophotometry

over the the wavelength range 300-220 nm. Maximum absorbance occurs at

264nm whilst minimum absorbance occurs at 228nm. 113

Figure 16.

Plasma 25(OH)D_3 values after dosing vitamin D_3 deficient chickens with varying amounts of vitamin D_3. 117

Figure 17.

Preliminary results: profiles of putative vitamin D_3 esters, vitamin D_3, 25(OH)D_3, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$ in i) plasma; ii) duodenum; iii) kidney; iv) liver; v) skeletal muscle; vi) fat in chickens, which are a) vitamin D_3 deficient, or; receiving b) 2ug; c)4ug; d) 8ug; e)16ug vitamin D_3. 130-136

Figure 18.

Silicic acid chromatography for elution of vitamin D_3. (ether : hexane 55 : 45 v/v). 147

Figure 19.

Definitive results: profiles of putative vitamin D_3 esters, vitamin D_3, 25(OH)D_3, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$ in i) plasma, ii) duodenum, iii) kidney, iv) liver, v) skeletal muscle, vi) fat in chickens which are a) vitamin D deficient, after one small dose of vitamin D_3, b) vitamin D deficient, after receiving multiple small doses of vitamin D_3, c) and d) repeated 4ug and 16ug amounts of vitamin D_3 respectively. 152-158

Figure 20.

Definitive study: pattern of a) vitamin D_3 esters, b) vitamin D_3, c) $25(OH)D_3$, d) $24,25(OH)_2D_3$, e) $1,25(OH)_2D_3$ in chicks in vitamin D_3 deficient state (0), and after administration of 4ug or 16ug of vitamin D_3 in plasma, fat, kidney, skeletal muscle and duodenum. Concentrations recorded in pmol/ml or pmol/g. 159-161

Chapter 1

Vitamin D metabolism and mineral homeostasis

1.1 Introduction.

Vitamin D is essential for life in higher animals. It is most important in the regulation of calcium metabolism in concert with two peptide hormones namely, parathyroid hormone and calcitonin. The vitamin also plays a role in phosphorus homeostasis.

Great progress has been made over the past four decades and it has now been established that the mode of action of vitamin D is similar to that of steroid hormones.

Presently the metabolism of vitamin D concerns the production of over 30 metabolites, while more than 20 tissues have been found to harbour the specific $1,25(OH)_2D$ receptor (Norman et al 1982), which is thought to be a prerequisite for the intracellular action of vitamin D.

The receptors are present in the expected target tissues, intestine, kidney and bone, but are also found in other organs not associated with mineral metabolism, giving the hormone a new perspective. (Editorial 1987; Dickson 1987). Disease states in man related to vitamin D, to quote some examples are, psoriasis (skin), sarcoidosis (lung), chronic renal disease (kidney), diabetes (pancreas), anticonvulsant therapy (bone) and leukaemia (bone morrow) (Norman 1990a).

It is certainly a far cry from the time when vitamin D was considered to be associated only with mineralization of bone and the development of rickets and osteomalacia, and when the author was confronted half a century ago in his final physiology examination with the question " Write notes of about one page on vitamin D".

1.2 Historical.

The scourge of vitamin D deficiency became apparent during the industrial revolution when people congregated into cities and the children played in sunless alleyways. Severe bone deficiencies became apparent due to the lack of sunlight. Young women were also affected with the development of deformities of the pelvis resulting in a high incidence of infant and maternal morbidity and mortality.

Palm in 1890, describing the geographic distribution of rickets, noted that children who lived in squalor in the cities of Japan, China and India rarely developed rickets whereas poor children who lived in industrialized cities of low-incident sunlight with air pollution were frequently affected. Countries with a high incidence of this disease included the British Isles, Europe and America. As a result Palm was able to surmise that lack of sunshine was the cause of this malady.

Sir Edward Mellanby (1919) produced rickets in dogs on an oatmeal diet, and cured the resulting condition by adding cod-liver oil to their diets.

It was initially thought that the antirachitic factor was vitamin A, but after destroying the vitamin A activity by heat and oxidation, cod liver oil still retained its antirachitic activity. The new fat soluble compound was named vitamin D (McCollum 1922)).

Huldshinsky (1919) reported that radiation from a mercury vapour arc lamp could cure rickets and in 1924 Steenbok and Black described the calcifying properties of food which had been exposed to ultraviolet light.

With the discovery of the antirachitic properties of vitamin D, the supplementation of food with the vitamin has been used to diminish the incidence of rickets in the developed world.

Advances in the understanding of the metabolism of vitamin D and the elucidation of its metabolites were made possible with the introduction of radio labelled vitamin D in the late 1960's with the detection of biologically active metabolites. Initially 25-hydroxyvitamin D [25(OH)D] was considered to be the biologically active form of vitamin D, but in 1968 Haussler et al reported that a more polar metabolite was present in the nuclear fraction of the intestine in the chick. This metabolite was later identified as 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) (Haussler 1972) , and was shown to be produced in the kidney (Fraser and Kodicek 1970). Norman (1968) was able to review that the biological action of vitamin D could be explained by a hormone-like action and $1,25(OH)_2D$ was the most potent form of vitamin D_3 (Norman and Wong 1972).

1.3 The Vitamins D.

Vitamin D compounds are defined as having anti-rachitic activity. Derived from the cyclopentanoperhydrophenanthrene ring system, they are related to other steroids. Several such compounds have been described but only two have nutritional significance, these being vitamin D_2 or ergocalciferol and vitamin D_3 or cholecalciferol. The latter compound is the naturally occurring form and generally the major form of the vitamin in the human.

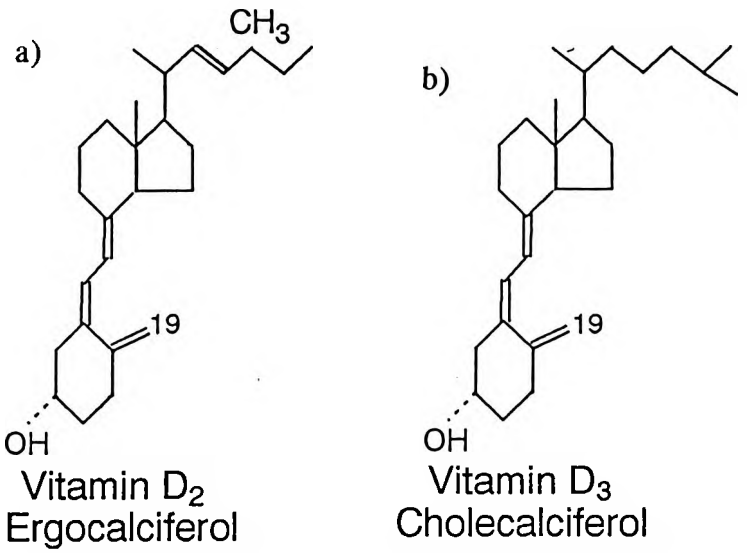


Figure 1.

Structure of (a) vitamin D_2 (ergocalciferol), and (b) vitamin D_3 (cholecalciferol) compounds.

1.3.1 Structure of vitamins D.

The structural difference between the two vitamin D compounds lies in their side chains, vitamin D_2 having a double bond between C-22 and C-23 and a methyl group attached to C-24 (figure 1a). Ergocalciferol is derived from the sterol ergosterol, a provitamin present in plants and lower organisms, as in fungi and yeasts (figure 2).

Cholecalciferol which is normally derived by exposure to sunlight (figure 1b), is formed from provitamin 7-dehydrocholesterol which is a by-product in the synthesis of cholesterol, and is present mainly in the Malpighian layer of the epidermis in skin.

1.3.2 Photochemical properties.

The prerequisite for the provitamin D is that it is a sterol with a ▲ 5-7 diene double bond system in ring B (Miller and Norman 1984). It is the conjugated double bond structure in this position that allows the absorption of light quanta at certain specific wavelengths in the ultra-violet (UV) range (between 290 and 315 nm UV-B radiation) with the maximum activity occurring at 305nm. With the absorption of UV radiation, the B ring of the molecule is broken due to fission of the C-9 C-10 bond by a non-enzymatic photochemical reaction forming a seco-steroid. Due to the "freeing" of the A ring, it becomes inverted from rings C and D, with rotation occurring around the bond between C-7 and C-8, thus the α and ß designations on the A ring must be drawn with reverse notations due to the inversion of this ring (Norman et al 1982). (figure 3).

As a result of the breakage of this bond, the A ring in solution is able to undergo interconversions several times per second between the two chair conformations

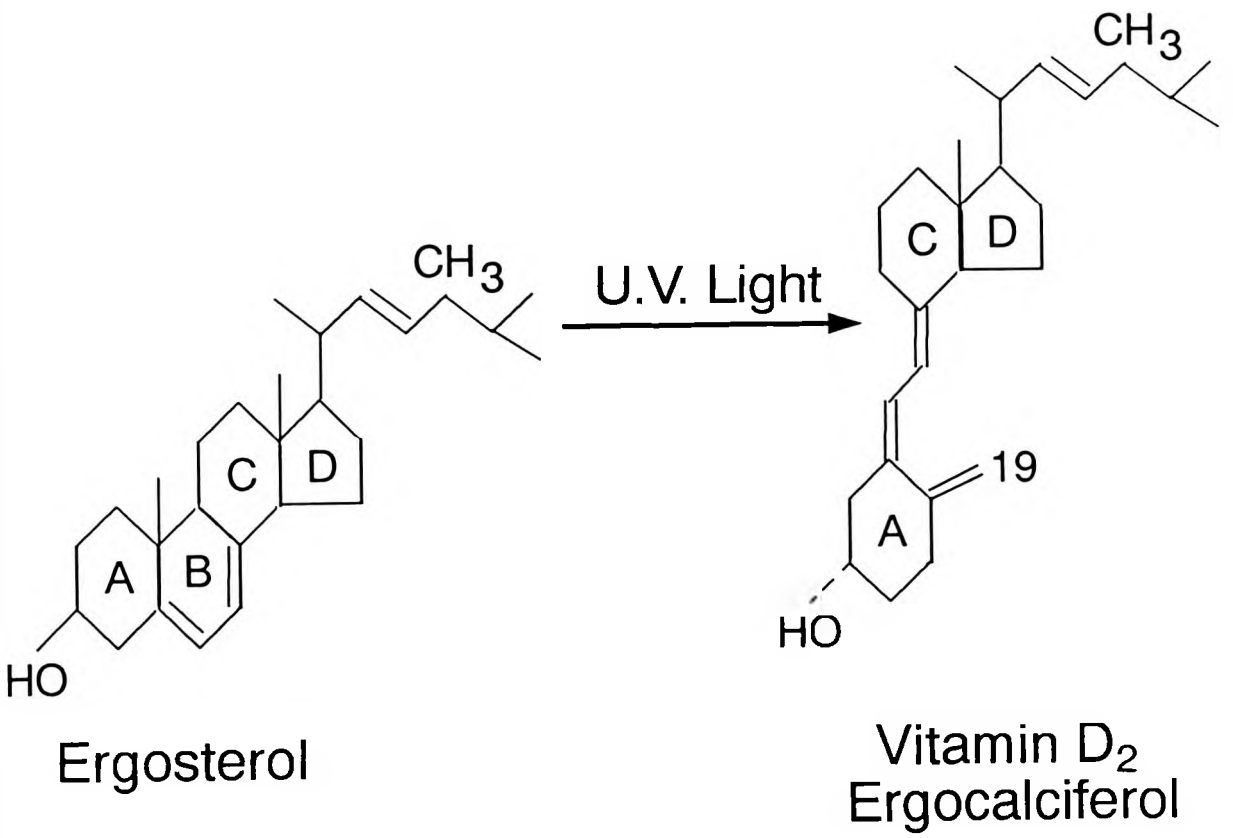


Figure 2.

Irradiation of ergosterol to form ergocalciferol.

alternating between the axial and equatorial positions (Wing et al 1974). As a result of this mobility, special problems of vitamin D receptors (VDR) are present that are not encountered by the receptors of other steroid hormones (Collins and Norman 1991). Provitamin D is thus converted to the seco-steroid previtamin D with an intramolecular hydrogen transfer from C-19 to C-9. (Miller and Norman 1984).

The photoproduction of previtamin D_3 in the skin is dependent on the concentration of 7-dehydrocholesterol in this tissue, and is decreased by the presence of chromaphores (melanin pigment) in the skin which compete with provitamin D for UV-B photons which have a spectral range of 290 nm to 320nm (Holick 1981). The amount of UV-B photons in the atmosphere which is able to penetrate the skin varies on the latitude, season of the year, time of day (Lu et al 1992), the degree of atmospheric pollution and the amount of melanin pigmentation in the skin (Holick 1981). Aging is an important factor influencing the production of vitamin D_3 in the skin. An inverse relationship exists between the amount of 7-dehydrocholesterol in the epidermis and age, thus the synthesis of vitamin D_3 is reduced in the elderly (MacLaughlin and Holick 1985; Holick 1986)). The topical application of a sunscreen with a sun protection factor of only eight, can completely block the photosynthesis of previtamin D_3 in human skin (Matsuoka 1989).

Previtamin D_3 in the skin slowly equilibrates to form vitamin D_3 (the process taking 36 to 48 hours) (Esvelt et al 1978) by means of a temperature-dependent thermal isomerization, thus permitting continuous synthesis of vitamin D_3 in the skin for up to four days after a single exposure to ultraviolet-B radiation (Holick 1984) (Figures 3; 4). Vitamin D is a seco-steroid in which the B ring is replaced by a 5,7-diene bridge. The 5,7-diene bridge together with the 10,19-double bond constitutes a cis-triene structure

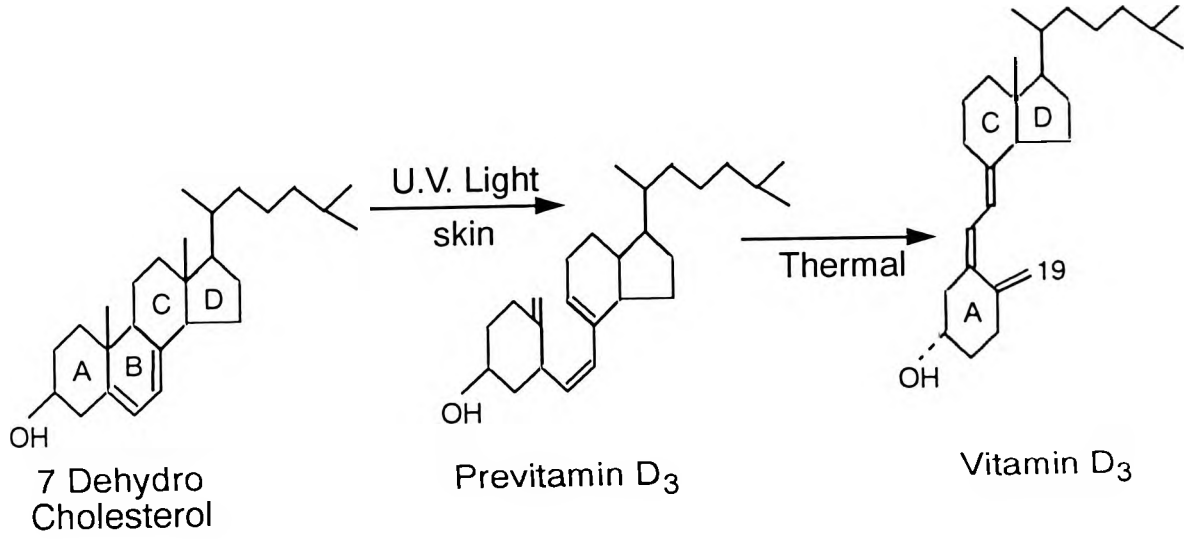


Figure 3.

Photochemical reaction of provitamin D_3 to form previtamin D_3.

Thermal isomerization of previtamin D_3 to vitamin D_3.

that provides the chromaphores for this molecule. It has a maximum UV absorption at 265nm with a minimum at 228nm.(refer 3.1.2). The molar extinction coefficient is 18,200 for all forms of vitamin D (DeLuca 1984). At 37^{0}C in equilibrium, the ratio of vitamin D_3 to previtamin D_3 is 89:11. Once formed in the skin, previtamin D_3 if exposed to further sunlight, absorbs excessive UV-B photons and is photoisomerized to biologically inert photoproducts, $lumisterol_3$ and $tachysterol_3$ (Webb et al 1989). This reaction provides protection against vitamin D_3 toxicity. Vitamin D_3 in the skin is also very sensitive to sunlight exposure and is rapidly degraded to 5,6 trans-vitamin D_3, suprasterol 1 and suprasterol 2, if it remains in the skin for too long a period of time.

1.3.3 Vitamin D_3

Vitamin D_3 which is formed in the skin is bound to vitamin D binding protein (DBP) and enters the dermal capillaries from where it is transported to the liver and storage sites (Holick 1981) (figure 4). This DBP has a higher affinity for vitamin D_3 than pro- or previtamin D_3.

It is apparent that as vitamin D_3 can be synthesized endogenously the term "vitamin" is a misnomer, the term applying to an essential nutrient that is included in the diet. Calciferol is a vitamin only when the individual or animal does not have access to sunlight or UV light (Norman 1982). Haussler et al (1988) are of the opinion that vitamin D is more appropriately classified as an exohormone.

Vitamin D_3 may also be available in a dietary form but usually is present in only small quantities in the diet, although liver and visceral oils in tuna and cod fish and the yolk of an egg may contain higher concentrations. Vitamin D_2 formed by the irradiation of ergosterol in its natural form, is taken in the diet or it may be added to fortify

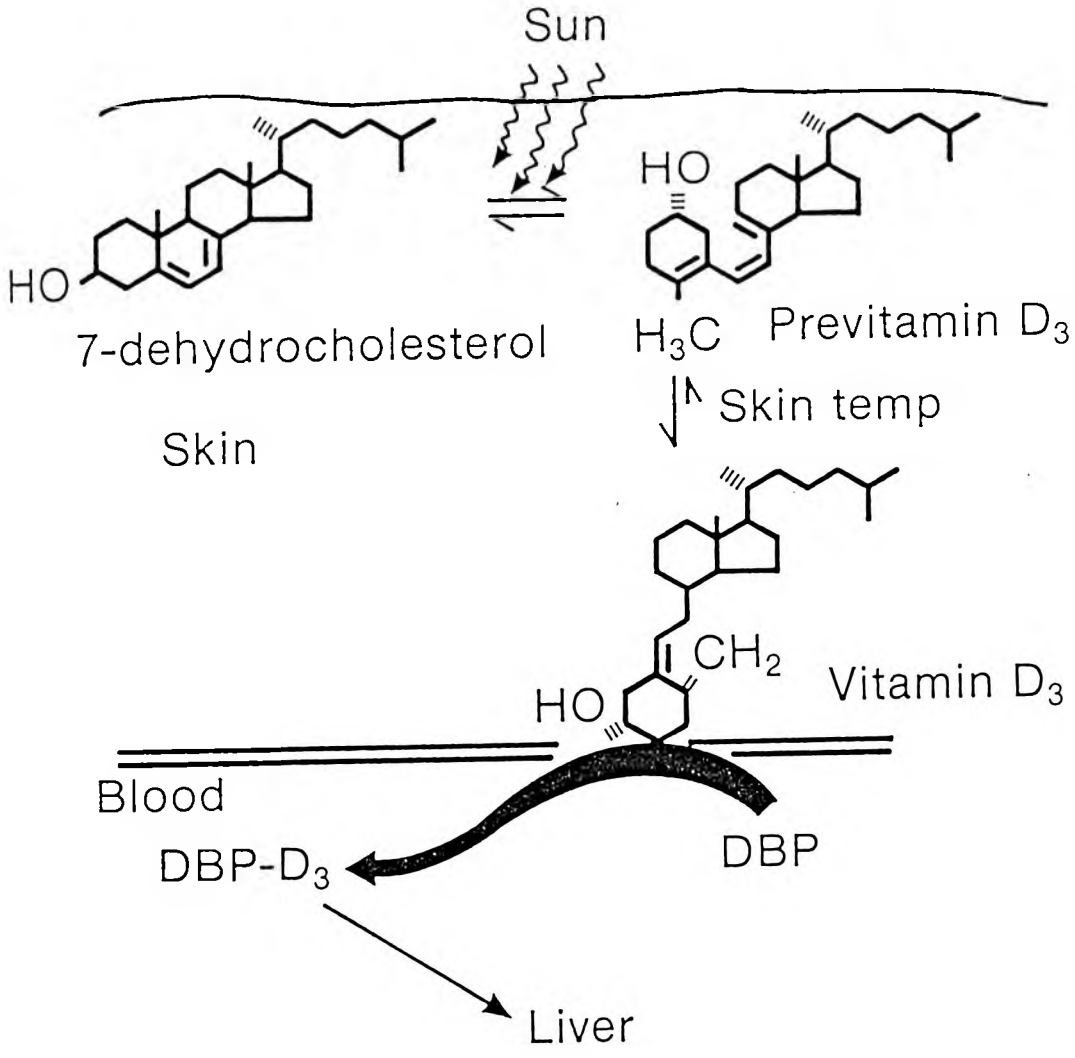


Figure 4.

Photosynthesis of previtamin D_3 (Holick 1981).

foodstuffs. Unsupplemented diets are generally a trivial source of vitamin D (Clements et al 1992).

1.3.4 Vitamin D absorption.

Gastro-intestinal absorption of vitamin D takes place in the small intestine, bile salts being essential for the process as vitamin D is fat-soluble. In birds most of absorption of cholecalciferol is completed in the upper jejunum (Bar et al 1980).

In humans it is absorbed into the lacteals of the lymphatic system of the intestine, 50 percent of the vitamin being present in the chylomicrons. (Dueland et al 1983) It is then transferred into the lipoprotein fraction of the plasma (Barragry et al 1979; Dueland et al 1982), and eventually in humans transferred to plasma α_2 globulins to reach the liver, where the first hydoxylation of the molecule occurs (figure 5).

1.3.5 Vitamin D binding proteins.

Like other steroids, vitamin D and its metabolites in plasma are bound noncovalently to a binding protein, in the case of vitamin D, to vitamin D binding protein (DBP), also termed transcalciferol. DBP was initially termed "group specific component" or "Gc protein," is synthesized in the liver and is present in most tissues. It is an abundant serum protein containing a single polypeptide chain, the molecular weight being 50,000 to 60,000 daltons. The sequence analysis of the cDNA shows homology with the α fetoprotein-albumin super family of proteins. All vitamin D metabolites bind DBP in a ratio of 1 mole of metabolite /mole of protein. The amino-

acid fragment representing the N-terminal of the protein contains the binding area for vitamin D and its metabolites. At normal concentrations of vitamin D and its metabolites, only 1 to 2 percent of the available vitamin D sterol binding sites on DBP are occupied by the vitamin at any given time (Ray et al 1991).

The binding protein is necessary to solubilize the vitamin D and its metabolites in plasma which is an aqueous environment, and to protect the steroid from oxidative inactivation (Chalk and Kodicek 1961). The uptake of the steroid by DBP is rapid and occurs with high affinity binding. Its highest affinity is towards 25-hydroxyvitamin D [25(OH)D], the major circulatory form of vitamin D. DBP also has a high affinity for 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] and 25,26-dihydroxyvitamin D [25,26$(OH)_2$D], which is greater than that for 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] or vitamin D which has the least affinity (Haddad and Cooke 1989).

The distance between the 3ß-hydroxy and another hydroxyl group on the molecule is of importance regarding the affinity of DBP for these metabolites (Kumar 1986). Both the natural and unnatural lactone derivatives of 25(OH)D and 1,25$(OH)_2$D exhibit 2 to 3 fold the ability to displace 25$(OH)D_3$ from DBP (Wilhelm et al 1984). The concentration of DBP influences the amount of free 1,25$(OH)_2$D in plasma, the latter being the functional fraction in vivo accessible to cells (Vieth 1990).

It was originally thought that only one type of protein was used for the transport of the vitamin and its metabolites in mammals. Hay and Watson (1976a) carried out an extensive phylogenetic study of 25(OH)D transport proteins. They showed that in 90 percent of mammals and in some birds studied, vitamin D metabolite binding was associated with an α globulin protein, the classical binding protein. In a few species of mammals, albumin is the transport protein for 25(OH)D, while in three species of Cebus

monkeys the protein has an albumin-like electrophoretic mobility (Bouillon et al 1976). The DBP in the rat is identical to that in humans namely, an α globulin while in most species of birds investigated, 25(OH)D is bound to α globulin but in a few species it is transported by albumin or α globulin. Chicken (Gallus gallus) serum contains two distinct types of binding protein, one of which (60000 daltons) binds predominantly cholecalciferol and the other (54000 daltons) 25(OH)D (Edelstein et al 1973). These binding proteins migrate with ß-globulin motility (Hay and Watson 1976b), and have a lower affinity for the metabolites of vitamin D_2 (Greenberg et al 1974). Fish with cartilaginous skeletons and amphibians use lipoproteins while bony fish and reptiles use α-globulins for 25(OH)D transport.

The human binding protein has similar affinities for both vitamins D_2 and D_3 (Miller and Norman 1984; Haddad and Cooke 1989).

1.4 Metabolism of vitamin D.

1.4.1 Vitamin D 25-hydroxylase.

The initial hydroxylation of vitamin D occurs mainly in the liver, but in the chick may also occur in the intestine and kidney (Mawer 1982). This hydroxylation is at carbon-25 and is catalyzed by the enzyme vitamin D 25-hydroxylase (25-hydroxylase) (figure 5) with the production of 25(OH)D.

In the liver this reaction takes place both in the hepatic microsomes (Bhattacharyya and DeLuca 1973; Madhok and DeLuca 1979) and hepatic mitochondria (Björkhem and Holmberg 1978) but the latter reaction is not identical to the mitochondrial 25-hydroxylase action on cholesterol.

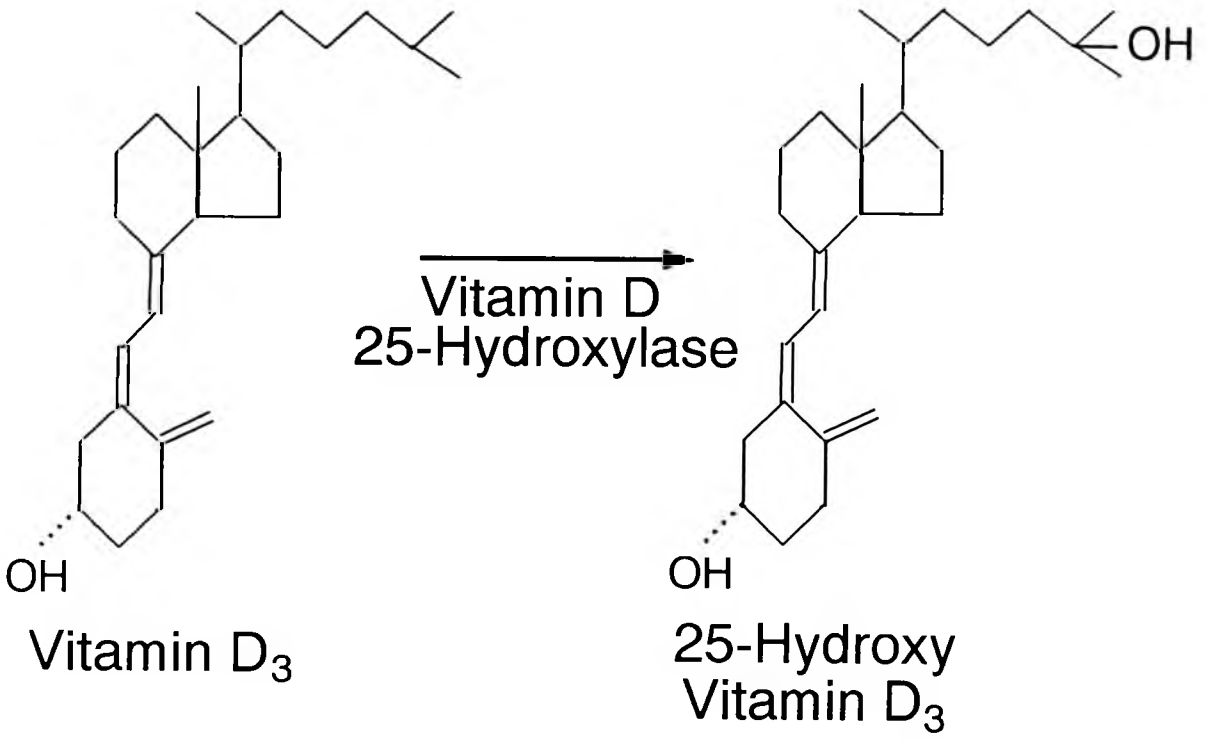


Figure 5.

First hydroxylation of vitamin D_3 in liver by enzyme vitamin D 25-hydroxylase

The microsomal 25-hydroxylase, is a mixed function oxidase enzyme consisting of a flavo-protein, a non-haeme iron sulphur protein and a NADPH-dependent cytochrome P-450 that utilizes molecular oxygen. The Michaelis constant (Km) of the microsomal enzyme is lower than that of the mitochondrial enzyme, so at low concentrations of vitamin D the microsomal enzyme is active. It is responsible for hydroxylation of physiological doses of calciferol and is easily saturated (Mawer 1982).

The mitochondrial enzyme with a higher Km, is active when large amounts of vitamin D are present in the circulation, and is poorly regulated resulting in increased amounts of plasma 25(OH)D when large amounts of vitamin D are administered. As the mitochondrial component of the 25 hydroxylase enzyme is active even with large amounts of the vitamin D substrate, the plasma 25(OH)D concentration is a good indicator of vitamin D status in an individual, as it bears a good reflection of the availability of the vitamin (Reichel et al 1989). It is the major circulating metabolite of vitamin D, and is biologically inert in physiological amounts.

1.4.2 Regulation of vitamin D 25-hydroxylase.

There is some controversy as to whether 25 hydroxylation is subject to feedback regulation or not. Bolt et al (1988) maintain that the concentration of vitamin D itself can modulate 25-hydroxylase activity, while Kumar (1991) states that the microsomal enzyme is better regulated than the mitochondrial enzyme and the activity of the former is negatively regulated by the quantity of 25(OH)D present in plasma.

Recent studies indicate that $1,25(OH)_2D$ may influence 25(OH)D catabolism and can contribute to vitamin D deficiency in some clinical disorders. Bell et al (1984)

observed that when large quantities of vitamin D were administered together with $1,25(OH)_2D$, the plasma levels of 25(OH)D were lower than when a similar amount of vitamin D was given alone. They postulated that $1,25(OH)_2D$ inhibited the production of 25(OH)D. However Kumar (1986) has shown that increasing the $1,25(OH)_2D$ plasma concentration does not suppress $25(OH)D_3$ levels.

Baran and Milne (1986) hypothesize that the possible mechanism by which elevated $1,25(OH)_2D$ levels decrease 25 hydroxylase enzyme is through the elevation of intracellular calcium (Baran et al 1989), as increased hepatocyte cytosolic calcium has been shown to inhibit 25(OH)D synthesis. Thus $1,25(OH)_2D$ through its action on intracellular calcium concentration may modify the production rate of 25(OH)D. However this view has not been supported by other workers.

Halloran et al (1986) maintain that $1,25(OH)_2D$ affects 25(OH)D concentration by increasing its metabolic clearance rate.(MCR). Halloran and Castro (1989) provide evidence that $1,25(OH)_2D$ given exogenously or increased endogenously through the induction of hypocalcaemia, affects 25(OH)D by increasing its MCR but not its production rate. $1,25(OH)_2D$ accelerates the degradation of 25(OH)D with increased biliary secretion and reduction of substrate available for further hydroxylation to $1,25(OH)_2D$ producing a relative vitamin D deficiency more rapidly (Clements et al 1987a; 1992). The site of the increased catabolism is unclear, although the catabolic products are excreted in the bile. By suppressing $1,25(OH)_2D$ levels with an increased calcium intake, Berlin and Björkhem (1987) were able to increase serum levels of 25(OH)D.

1.4.3 Conditions affecting vitamin D metabolism.

A decrease in 25(OH)D half-life has been observed in patients with malabsorption syndromes (Batchelor et al 1982) and on high fibre diets (Batchelor and Compston 1983). The mechanism by which it occurs is unclear but it is most probably through calcium malabsorption and the consequent increase in $1,25(OH)_2D$ levels. Arnaud et al (1975) suggested that the interruption of the enterohepatic circulation of the 25(OH)D might be responsible for the decreased levels of 25(OH)D in gut disorders, however this work has not been substantiated.

Clinically vitamin D deficiency in Asian individuals in Britain, who although exposed to UV light, might be produced through increased catabolism of 25(OH)D due to elevated levels of $1,25(OH)_2D$. These individuals consume a high fibre and high phytate diet which may interfere with calcium absorption causing a decreased concentration of plasma calcium. This results in hyperparathyroidism with increased plasma $1,25(OH)_2D$ resulting in low plasma 25(OH)D. Substituting white bread in place of the high chupatty diet in these patients with rickets or osteomalacia resulted in an improvement in their condition (Ford et al 1972). Asians may need larger exposures to sunlight than Caucasians for a similar vitamin D_3 response, but the capacity to produce vitamin D_3 is no different in Asian than Caucasian skin. (Lo et al 1986).

Primary hyperparathyroidism which is characterized by increased serum $1,25(OH)_2D$ is also associated with an increased MCR of 25(OH)D with an increased excretion of the metabolite in the faeces (Clements et al 1987b: 1992).

The anticonvulsants phenobarbital and primidone induce hepatic microsomal enzymes by increasing activity of cytochrome P450 like enzymes, which enhance

metabolism of sterols. (Audran and Kumar 1985). This leads to an increased rate of hydroxylation of vitamin D in the synthesis of 25(OH)D and enhanced degradation and excretion of metabolites in bile. These metabolites are mainly water soluble conjugates and more polar than 25(OH)D, including glucuronides (Silver et al 1974). Rats given prolonged phenobarbital treatment, deplete their tissues of vitamin D.

Liver disease may interfere in the synthesis of 25(OH)D. Mawer et al (1985b) reported the impairment of 25-hydroxylation in patients with alcoholic liver disease resulting from hepatocellular dysfunction.

1.4.4 Hydroxylation of 25(OH)D in the kidney. 25-Hydroxyvitamin D 1α-hydroxylase.

Once 25(OH)D is synthesized, it rapidly leaves the liver bound to DBP and is transferred to the kidney where the second hydroxylation occurs at C-1 or C-24 positions with the production of the two daughter metabolites $1,25(OH)_2D$ or 24,25 dihydroxyvitamin D [$24,25(OH)_2D$] by the enzyme systems 25-hydroxyvitamin D-1α-hydroxylase (1α hydroxylase) or 25-hydroxyvitamin D-24R-hydroxylase (24 hydroxylase) respectively (Kawashima and Kurokawa 1983) (figure 6).

The kidney is recognised as the major site in the synthesis of the hormone $1,25(OH)_2D$, which is the predominant mediator for the biological responses of vitamin D. The enzyme 1α hydroxylase is situated in the mitochondria of the proximal convoluted and straight tubules in the renal cortex. It is a mixed function oxidase, resembling 11ß hydroxylase of the adrenal cortex. It requires internally generated NADPH, magnesium ions and molecular oxygen and is composed of three components

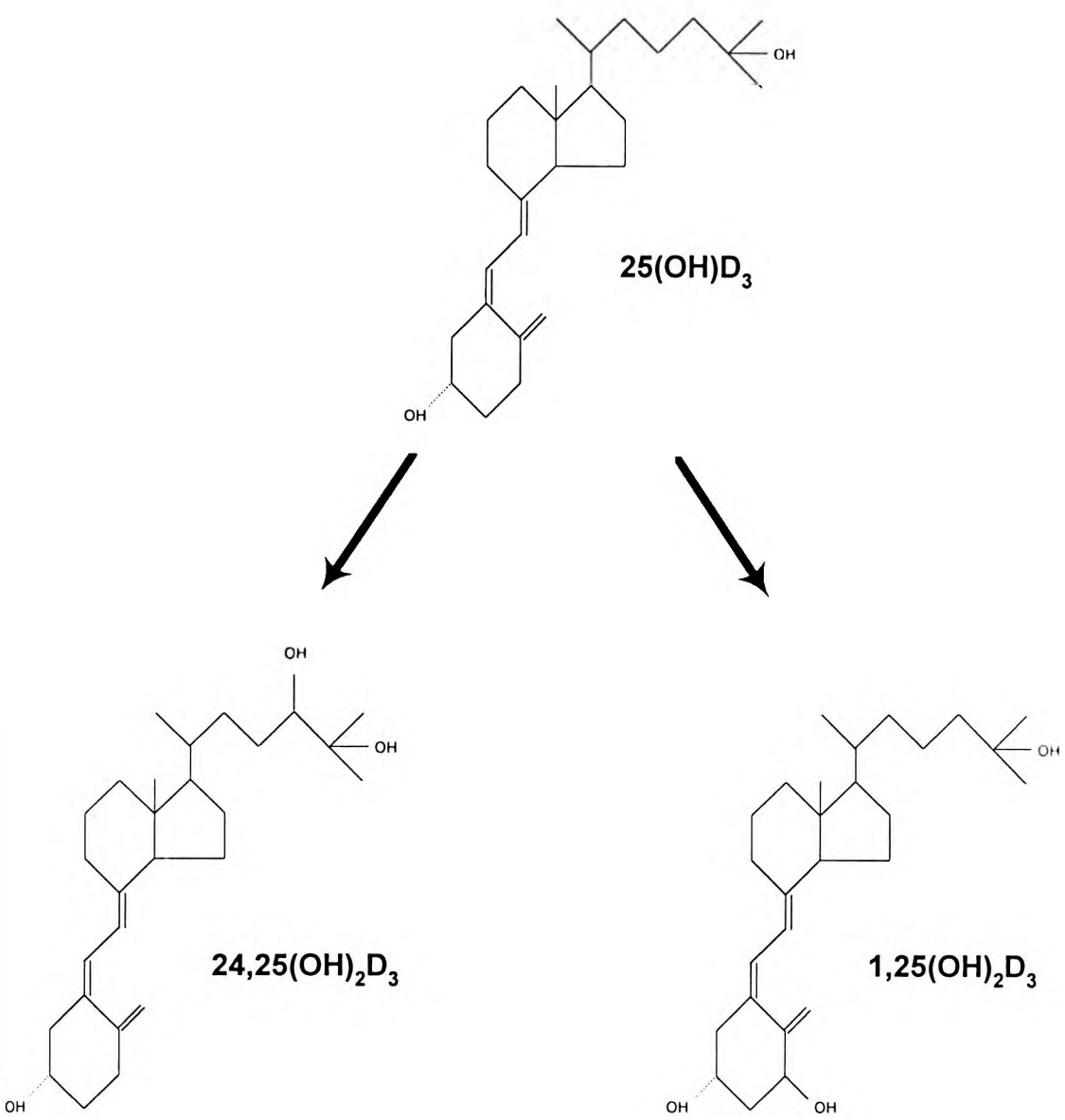


Figure 6. Hydroxylation of $25(OH)D_3$ to form $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$

which are internal compounds of the inner mitochondrial membrane, two of which are a flavoprotein - renal ferredoxin reductase and a non-haeme iron sulphur protein, ferredoxin. NADPH serves as an integral part of the inner membrane, ferredoxin reductase is loosely bound to this membrane while ferredoxin is a matrix protein. These latter two proteins serve as an intermediate in transporting electrons from NADPH to the terminal component P-450, which provides the substrate with a combining site and the catalytic site where the oxygen atom is introduced at the C-1 position for the production of $1,25(OH)_2D$.

Thus one molecule of oxygen is utilized per molecule of 25(OH)D and one hydroxyl molecule is incorporated into the steroid at a specific site and the remaining atom of oxygen is reduced to water by the hydrogen donor-NADPH.

1.4.5 Extra renal sites of $1,25(OH)_2D$ production.

Sites other than the kidney producing $1,25(OH)_2D$ include placenta and decidual cells, which contribute to the high levels of $1,25(OH)_2D$ during pregnancy (Delvin et al 1985). Activated macrophages, as occurs in the granulomata of sarcoidosis and tuberculosis (Bikle 1992), are able to synthesize $1,25(OH)_2D$ in sufficient quantities to produce hypercalcaemia (Papapoulos et al 1979). Hypercalcaemia with increased production of $1,25(OH)_2$ has also been described in lymphomas, disseminated candidiasis and silicon-induced granulomas. (Bell 1985).

It is still undecided whether anephric but otherwise normal patients are able to synthesize $1,25(OH)_2D$ at sites other than the kidney in quantities sufficient to produce measurable levels in plasma. It has been reported that $1,25(OH)_2D$ levels are undetected

in anephric patients (Shepard et al 1979), but Lambert et al (1982) recorded serum $1,25(OH)_2D$ levels of 14.1 ± 0.6 pg/ml after the daily administration of 50,000 or 100,000 IU vitamin D_2, the level prior to vitamin D administration being 5.8 ± 1.9 pg/ml. There was a positive correlation between serum 25(OH)D and $1,25(OH)_2D$ in these treated patients. In a similar study by Jongen et al (1984), levels of serum $1,25(OH)_2D$ in anephric patients rose from < 5 pmol/l (<2.08 pg/ml) to 14-36 pmol/l (5.8 - 14.9 pg/ml) after the administration of 15,000 or 30,000 IU units daily of vitamins D_2 or D_3

1.4.6 Actions of $1,25(OH)_2D$.

The biological actions of vitamin D are executed by the active hormone $1,25(OH)_2D$, which is the most highly potent form of vitamin D_3 (Norman and Wong 1972). Its main mineral function is assisting in the control of serum calcium concentrations through the absorption of calcium from the proximal small intestine and indirectly the mineralization of bone by maintaining an adequate plasma calcium concentration. $1,25(OH)_2D$ is also involved in the absorption of phosphorus.

With the isolation of receptors for this metabolite in over 20 different tissues, it has became apparent that the functions of $1,25(OH)_2D$ are more complex than initially documented (Norman 1990a; Bell 1985)).

Controversy exists regarding the effect of vitamin D on calcium absorption in the kidney. This is due to a variety of factors, notably serum calcium concentration, nutritional vitamin D status and PTH activity which are not independently controlled.

When these factors are controlled vitamin D deficiency reduces calcium reabsorption (Yamamoto et al 1984).

Vitamin D deficiency results in decreased insulin secretion in response to glucose, calcium being necessary for insulin secretion (Bikle 1992). Receptors for $1,25(OH)_2D$ are present in the pancreas (Christakos and Norman 1981) as are calcium-binding proteins (Morrissey et al 1975). A combination of $1,25(OH)_2D$ and calcium is necessary for the optimal insulin secretion. Bikle (1992) is of the opinion that $1,25(OH)_2D$ has a direct role in promoting insulin secretion but nutrition and serum minerals also play an indirect role.

Although not directly involved in this dissertation, mention must be made of the role played by $1,25(OH)_2D$ not pertaining to calcium metabolism but associated with cell growth and differentiation and also immunoregulation.

The literature contains many references regarding these topics, DeLuca (1988) noting that 60% or more of all cancer cell lines having large amounts of vitamin D receptors. Walters (1992) in a review of this subject quotes $1,25(OH)_2D$ induction of cell growth in the duodenum and the correlation between receptor levels and the rate of growth in cultured osteoblasts. However the majority of the effects of $1,25(OH)_2D$ on growth and differentiation have been reported from in vitro systems.

The effects of the active hormone of vitamin D on the immune system has been shown to be inhibitory at times, however in vitamin D deficiency there is an increased risk of infection (Bikle 1992). Thus Bar-Shavit et al (1981) noted reduced chemotaxis and phagocytosis of peritoneal macrophages from vitamin D deficient mice which could be corrected by vitamin D treatment. The inhibitory action in which immunoglobulin production is depressed appears to be on the helper function of the T cells rather than

a direct action on B cells. This latter effect may prove useful in controlling autoimmune states.

1.4.7 Control of 1α-hydroxylase.

The stringent control of the activity of the enzyme 1α-hydroxylase regulates the vitamin D endocrine system, the synthesis of $1,25(OH)_2D$ being modulated to the calcium needs of the individual. PTH is the most important endocrine hormone in the control of $1,25(OH)_2D$ production. Low calcium levels activate PTH production which in turn stimulates activity of 1α-hydroxylase and causes a reduction of 24R-hydroxylase activity. Serum phosphorus concentration also plays a role in the regulation of $1,25(OH)_2D_3$ production, but not via PTH stimulation.

The presence of a short feedback loop allows $1,25(OH)_2D$ to directly modulate or reduce secretion of PTH (Norman et al 1982), the parathyroid gland which contains intracellular receptors for $1,25(OH)_2D$ (Brumbaug et al 1975), may be affected directly or indirectly by $1,25(OH)_2D$ (Norman et al 1982). The direct action of $1,25(OH)_2D$ has a direct depressing effect on preproparathyroid mRNA by inhibiting PTH gene transcription (Silver et al 1985). $1,25(OH)_2D$ also acts indirectly on the parathyroid gland by increasing serum calcium concentrations through its effect on calcium absorption from the small intestine and mobilization of the ion from bone (Norman 1982).

The serum concentration of phosphorus has a direct action on 1α hydroxylase, low levels of the ion increasing the activity of the enzyme with increased production of the hormone. (Pettifor 1990). $1,25(OH)_2D$ itself is able to depress the activity of 1α hydroxylase and activate 24 hydroxylase with increased production of $24,25(OH)_2D$ (Henry et al 1992).

In the chick 1α hydroxylase activity exhibits diurnal activity, the maximum activity is observed at noon and the minimum at night (Miller and Norman 1979).

1.4.8 Hormonal control of $1,25(OH)_2D$.

Various endocrine glands play a role in the control of the hormone, as already mentioned the most important being the parathyroid gland. Oestrogen, growth hormone, prolactin, insulin and calcitonin modulate the production of $1,25(OH)_2D$.

Oestrogens are reported to enhance 1α hydroxylase activity (Pike et al 1978) with increased concentration of serum $1,25(OH)_2D$ and suppressed production of $24,25(OH)_2D$. It is also believed that it may be an indirect effect via increased formation of DBP by the liver thus decreasing the level of free $1,25(OH)_2D$ with resultant stimulation of 1α hydroxylase production. It is also reported that oestradiol increases the number of PTH receptors in the kidney, which could increase 1α hydroxylase activity, with normal circulating PTH concentrations (Forte et al 1983).

Prolactin is also considered to have an effect on $1,25(OH)_2D$ synthesis as administration to chicks of ovine prolactin elevated the activity of 1α hydroxylase with increased $1,25(OH)_2D$ production (Spanos et al 1976 as reported by Norman 1979). Rats subjected to ovine prolactin therapy developed elevated plasma calcium concentration and intestinal calcium absorption, suggesting an increase in $1,25(OH)_2D$ production. However contrary to this hypothesis, Fraser (1980) showed that prolactin increased serum calcium in vitamin D deficient chicks indicating a process not involving vitamin D.

Calcitonin has hypocalcaemic actions and is stimulated in hypercalcaemic conditions. The interrelationship between vitamin D and calcitonin is not well defined (Norman 1982). The hormone inhibits osteoclast activity and the efflux of calcium from bone and increases excretion of calcium from the kidney. It appears that calcitonin plays a major role in preventing post-prandial hypercalcaemia (Haussler et al 1988) by inhibiting efflux of calcium from bone. Rasmussen and Tenenhouse (1989) feel that calcitonin has an action on the proximal straight renal tubules.

The relationship of $1,25(OH)_2D$ and insulin secretion has been discussed (refer section 1.4.6). It appears that in experimental animals rendered diabetic the serum half - life of $1,25(OH)_2D$ is reduced, suggesting that there is a defect in production of this active hormone.

1.4.9 25-Hydroxyvitamin D 24(R)-hydroxylase.

The 24 hydroxylation of the substrate 25(OH)D by the enzyme 24-hydroxylase producing $24(R),25(OH)_2D$, is catalysed by a cytochrome P-450-dependent mixed

function oxidase requiring NADPH. The kidney is the major site of production of this metabolite (Holick et al 1972) which is produced in the mitochondria of the proximal tubules but may also be synthesized in cartilage, bone cells, intestine and keratinocytes (Knutson and DeLuca 1974).

1.4.10 Functions of $24,25(OH)_2D$

Controversy exists regarding the biological importance of $24,25(OH)_2D$ as the metabolite is considered by some to be the initial event in the inactivation of 25(OH)D.

Receptors for this metabolite have been isolated in the chondrocytes of bone (Sömjen et al 1982) and parathyroid gland (Merke and Norman 1981).

$24,25(OH)_2D$ is reputed to have a number of functions in concert with $1,25(OH)_2D$, including a reduction of parathyroid size following hypocalcaemia in vitamin D depletion (Henry et al 1977), stimulation of skeletal mineralization in man and chick (Dickson et al 1984), production of normal hatchability of eggs in the chick (Henry and Norman 1978) and quail (Norman et al 1983).

Jarnagin et al (1985) dispute these results, as by using a $1,25(OH)_2D$ analogue which was unable to be hydroxylated at C-24, they were able to produce normal growth, reproduction and skeletal mineralization in vitamin D deficient rats. Brommage and DeLuca (1985) believe that $1,25(OH)_2D$ is the sole active vitamin D_3 metabolite.

1.4.11 Activity of 24,25(OH)$_2$D.

The bioactivity of 24,25(OH)$_2$D may possibly be due to the formation of 1,24,25(OH)$_3$D which is two to five fold less active than 1,25(OH)$_2$D in stimulating intestinal calcium transport, bone resorption and mobilization. Its action is also much shorter than 1,25(OH)$_2$D (Holick et al 1976). This product appears to be the initial product of 25(OH)D or 24,25(OH)$_2$D degradation.

In normo- and hypercalcaemic states and normo- and hyper- phosphataemic states, 25(OH)D is converted mainly to 24,25(OH)$_2$D, but in hypocalcaemic and hypophosphataemic situations, 24,25(OH)$_2$D is undetectable or present in low concentrations in plasma.

1.4.12 Control of dihydroxyvitamin D hydroxylases.

Explaining the variable reaction rates of the 1α and 24 hydroxylases, Fraser (1980) suggested there were two points of control, the one point being within the mitochondria which controls the entry of the substrate 25(OH)D, while the second point controls the activity of 1α hydroxylase. With no particular demand for 1,25(OH)$_2$D, both points of control are unstimulated with only small amounts of 1,25(OH)$_2$D synthesized and the bulk of the substrate available for 24 hydroxylation. When plasma calcium levels decline, both points of the system are activated which allows more 25(OH)D to enter the hydroxylase compartment stimulating mainly 1α hydroxylase with small amounts available for 24 hydroxylation.

Henry et al (1992) noted that vitamin D deficient animals have elevated 1α hydroxylase activity which decreased after the administration of $1,25(OH)_2D$, and concomitantly the activity of 24-hydroxylase increased while 1α hydroxylase declined, causing the metabolite $24,25(OH)_2D$ to become the dominant dihydroxyvitamin metabolite. The effects were reversed if $1,25(OH)_2D$ was withdrawn.

As it takes several hours for these changes to become apparent, it suggested that it occurs through a genomic effect, producing a protein which stimulates 24 hydroxylase activity whilst depressing the enzyme 1α hydroxylase. The genes involved included those which code for the heat stable inhibitor protein (PKI) and the proteins necessary for 1α and 24-hydroxylase activity. Cyclic AMP dependant protein kinase A (PKA) is decreased (Rudack-Garcia and Henry 1981) and the inhibitor protein PKI elevated in vitamin D deficient chicks (Al-Abdaly and Henry 1989). $1,25(OH)_2D$ administration abolishes PKI activity within 48 hours, allowing PKA and protein kinase C (PKC) to react with the newly synthesized proteins inducing phosphorylation compounds which are able to activate or depress the two hydroxylases for the desired effects (Henry 1992).

1.4.13 $1,25(OH)_2D$ receptors.

Steroid hormones, including $1,25(OH)_2D$, produce their biological responses in selected target organs and tissues due to their stereoselective interaction with a macromolecule effector, the steroid receptor (Haussler and Norman, 1969). Target tissues possess these specific high affinity binding receptors. The molecular mass of avian $1,25(OH)_2D$ receptor (VDR) is 60000 daltons. This receptor is a member of the supergene nuclear transacting receptor family, others being the oestradiol, progesterone, cortisol, aldosterone, thyroxine (T_3) and retinoic acid receptors.

The control of the activity of a steroid hormone involves the regulated production by the endocrine gland of the hormone followed by its systemic delivery to the target tissues where it moves into the cell and binds avidly with its specific receptor. All receptor forms contain a hormone ligand binding site and a DNA binding domain. Once the ligand has interacted with the receptor, some type of conformational change occurs, so that the steroid-receptor complex has a high affinity for the selected domain of the nuclear DNA.

As mentioned (refer 1.1) more than 20 tissues have been found to harbour the specific $1,25(OH)_2D$ receptor. (Norman et al 1990a; Dickson 1987).

1.4.14 Other actions of $1,25(OH)_2D$.

One of the actions of $1,25(OH)_2D$ is to stimulate the vitamin D dependent calcium binding proteins in cells, mainly calbindin D_{28K}. Some other vitamin D dependant proteins are calbindin D_{9K}, alkaline phosphatase, calcium-stimulated ATPase, bone Gla-containing proteins (osteocalcin) in osteoblasts and 24 hydroxylase catabolizing enzyme in target tissues (Lohnes and Jones 1987).

Vitamin D has many different actions including cell proliferation and differentiation through its action on the vitamin D responsive genes (Haussler et al 1988). Perhaps the best studied is its action on vitamin D calcium binding proteins.

1.4.15 Transcaltachia.

1,25 Dihydroxyvitamin D action may also be of relatively short duration which may not involve its nuclear receptor. Nemere et al (1984) describe a rapid unidirectional calcium transport response within minutes from the intestinal lumen in vitamin D replete animals after the administration of $1,25(OH)_2D$. Norman (1990b) has termed this process transcaltachia which involves rapid transfer of calcium across cell membranes. This is a nongenomic response involving the interaction of $1,25(OH)_2D$ linking up with a membrane-active antibiotic filipin, which reacts with cholesterol components of membranes, and the time of biological response after $1,25(OH)_2D$ administration may be 2 to 5 minutes.

Nemere and Norman (1991) also postulated that the seco-steroid reacts with a membrane associated receptor stimulating protein kinase (PK) activity and phosphorylation, resulting in activation of voltage operated calcium channels situated on basal-lateral membranes. Influx of calcium stimulates exocytosis of vesicles bearing calcium ions and also initiates transport across the enterocyte.

1.4.16 Catabolism and excretion of vitamin D metabolites.

Over 30 vitamin D metabolites representing the degradation products of the main metabolites have been described besides vitamin D and its main metabolites 25(OH)D, $1,25(OH)_2D$ and $24,25(OH)_2D$.

In the replete state the concentration of plasma $1,25(OH)_2D$ remains fairly constant due to the stringent control of the enzyme 1α-hydroxylase. However the rate

of catabolism is also important in maintaining this stability and acts as a possible defence mechanism guarding against excessive levels of the active metabolite which has great biological potency. Both $1,25(OH)_2D$ and $24,25(OH)_2D$ appear to have the same catabolic pathway (Henry and Norman 1984). Makin et al (1989) were able to demonstrate that kidney and bone cells are able to inactivate $1,25(OH)_2D$ as they possess 24-hydroxylase necessary for the first stage of the C-24 oxidation pathway. After 24-hydroxylation the next intermediate metabolite formed is C-24-oxo followed by C-23 hydroxylation and side chain cleavage. Target cells also contain the full complement of enzymes enabling $1,25(OH)_2D$ to be degraded to calcitroic acid which is the major side chain cleaved water soluble metabolite of $1,25(OH)_2D$ (Esvelt et al 1979; Reddy and Tserng 1989) (figure 7).

Radioactive $1,25(OH)_2D$ is catabolised by a variety of metabolic processes and rapidly removed from the organism. In the rat it appears in the bile within 30 minutes after administration, a quarter of the total activity being excreted in the faeces within 24 hours.

Glucuronides and glucosides of vitamin D_3 may become biologically active in the intestine following hydrolysis to the free sterol (Kumar 1986). They are biologically inactive in systems that lack glucuronidase activity. $25(OH)D_2$ glucuronides have been isolated as a major metabolite of $25(OH)D_2$ in birds, which may be due to a rapid conversion of vitamin D_2, as birds show discrimination against the compound (DeLuca and Schnoes 1983). Products of lower polarity than those secreted in bile appear in faeces, due to deconjugation by bacterial glucuronidase activity and other enzymatic processes in the large bowel (Clements et al 1984).

Approximately one third of the degradation metabolites of $1,25(OH)_2D$ is accounted for by the process of oxidation involving either the loss of four carbon atoms in the side chain or formation of a lactone compound on the side chain, both pathways inactivating $1,25(OH)_2D$.

1.4.17 Calcitroic acid.

Hamden (1976) examining radioactive CO_2 in expired air of rats within four hours of administration of [26,27-^{14}C] vitamin D_3, demonstrated oxidation of the 26-27-carbon atoms of $1,25(OH)_2D$. Three years later, Esvelt et al (1979) isolated a side chain cleaved metabolite of 1,25(OH)D from the liver of rats identified as calcitroic acid (1-OH-23-COOH-24,25,26,27-tetranor-vitamin D) (figure 7). It has little activity in healing rickets or causing bone mineral mobilization and has no effect on intestinal calcium transport (Esvelt and DeLuca 1981). The metabolite could be detected in kidney and blood, while the highest concentrations were in the liver and intestinal mucosa, reaching higher levels than $1,25(OH_2D$. In each tissue examined, calcitroic acid increased in amount four to twelve hours after labelled $1,25(OH)_2D$ administration, accounting for 20 percent of the administered tritium compared with 30 percent as $1,25(OH)_2D$. 25(OH)D appears to follow the same metabolic pathways as does $1,25(OH)_2D$, the final metabolite of 25(OH)D in this catabolic pathway is 25,26,27-trimor-24-COOH-vitamin D (Norman et al 1982).

Calcitroic acid circulates in the blood, and is more polar than the active metabolite. It is concentrated in the liver and excreted in the faeces via the bile in the form of glucuronides and sulphates (Kumar 1984). Hepatic excretion of 25(OH)D

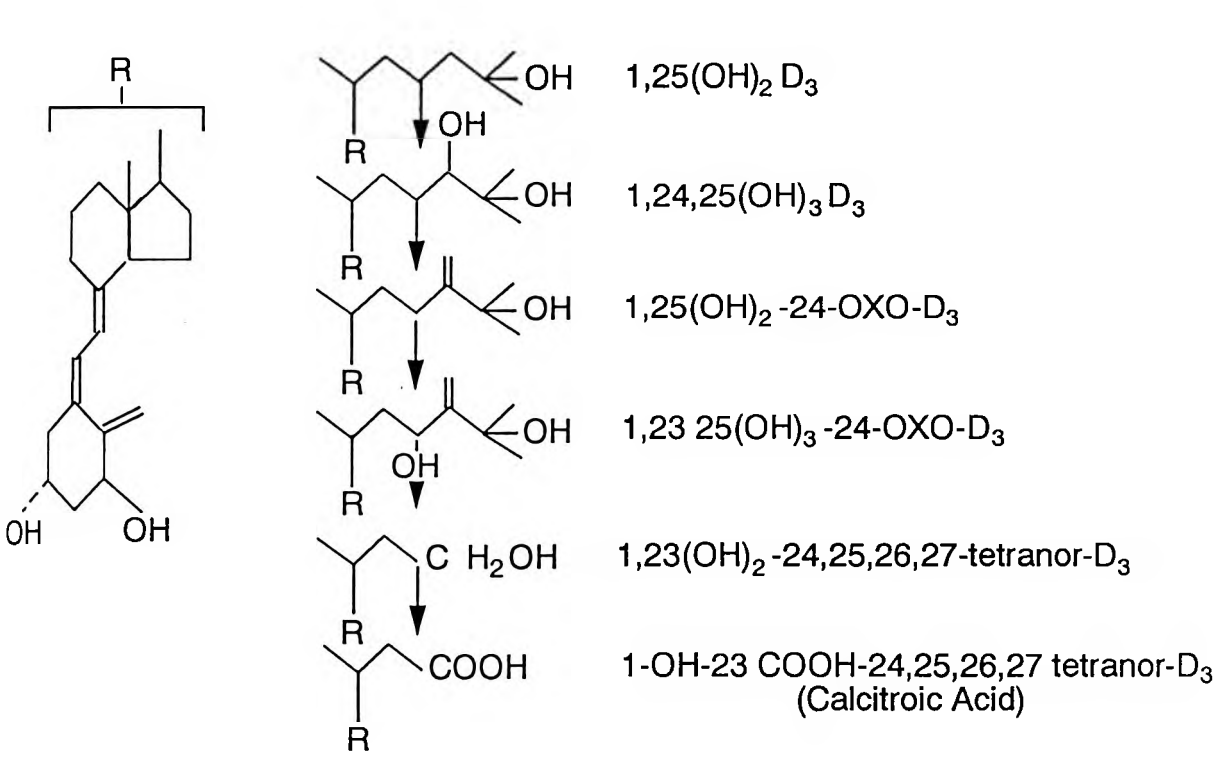


Figure 7.

C-24 oxidation pathway in catabolism

of $1,25(OH)_2D_3$ in kidney.

metabolites is much slower than $1,25(OH)_2D$ (2 to 3 percent versus 25 to 30 percent) (Clements et al 1984). The hepatic process is virtually non-saturable and even with the addition of large amounts of $1,25(OH)_2D$, the same percentage is excreted (Kumar 1991; Gray et al 1978).

1.4.18 Other vitamin D metabolites.

Other metabolites with lower concentrations than calcitroic acid have been detected. The catabolic metabolite $1,24,25(OH)_3D$ is generated from 25(OH)D and also from $24,25(OH)_2D$ by chick kidney homogenates. This polar metabolite is 60 percent as active as vitamin D in curing rickets and is two to five fold less active than $1,25(OH)_2D$ in stimulating and sustaining intestinal calcium transport, bone resorption and mobilization. Its duration of action is also much shorter than $1,25(OH)_2D$ (Holick et al 1976).

Another metabolite of both $1,25(OH)_2D$ and 25(OH)D, is the lactone $1,25(OH)_2$ 26,23-lactone D and 25(OH)D 26,23-lactone (Horst 1979), oxidation at C-23 being the initial step preceding C-26 hydroxylation. This reaction occurs in the kidney and possibly in the intestine as well. Under physiological conditions this metabolite is a minor product of $1,25(OH)_2D$ degradation, but chicks, rats and pigs receiving pharmacological doses of $1,25(OH)_2D$ for several days produce large amounts of this compound in plasma (Wilhelm et al 1984). The natural form of this compound contains the C-23 in the 23S position unlike the C-25 which is always in the R position. It is five times more potent than $25(OH)D_3$ in binding with rat plasma vitamin D binding protein. The 25-hydroxy-lactone has some biological activity in the chick as it can increase

intestinal calcium absorption, while 1,25$(OH)_2$-lactone can mediate intestinal calcium absorption and bone calcium mobilization responses, probably through acting with 1,25$(OH)_2$D specific receptors in these target tissues (Wilhelm et al 1984).

Hydroxylation of 25(OH)D at C-26 forming 25,26$(OH)_2$D produces a metabolite less active than the substrate (Suda et al 1970) with some activity on intestinal calcium transport (Lam et al 1974). It is inactive in curing rickets and mobilization of bone minerals. A strong positive correlation exists between the concentration of serum 25(OH)D and that of 25,26$(OH)_2$D. In man the production of this dihydroxylated metabolite depends on the concentration of its precursor 25(OH)D (Fraser et al 1980). Patients with osteomalacia due to dietary vitamin D deficiency have low or undetectable concentration of 25,26$(OH)_2$D. Sites of synthesis in man are not yet known, but the chick kidney can carry out 26-hydroxylation from the substrate 25(OH)D. This metabolite was also isolated by Thierry-Palmer et al (1983) when examining kidney microsomes in rats fed vitamin D.

The vitamin D metabolite, 25(OH)5,6-E vitamin D_3, (5-6-trans D_3), has the A-ring rotated 180° around the 5,6 double bond converting the 3-hydroxyl group to a pseudo 1-hydroxyl compound (Kumar 1991). If pharmacological doses of 25(OH)D are given to animals, large amounts of 5,6-trans-25(OH)D are present in the plasma. As this compound now acts as a pseudo 1-hydroxyl metabolite, its affinity for the 1,25$(OH)_2$ D receptor could activate hormonal responses. This metabolite may play an active role in vitamin D toxicity.

A lipid-soluble metabolite of 24,25$(OH)_2D_3$, 24-oxo-25$(OH)D_3$ has been identified, its biological activity being similar to 24,25$(OH)_2D_3$. 24-Keto-1,23,25-trihydroxyvitamin D is a major metabolite synthesized in intestine mucosal cells of rats,

dosed chronically with $1,25(OH)_2D$ (Napoli and Horst 1983), the pathway of production is via $1,24,25(OH)_3D_3$ and 24-keto-$1,25(OH)_2D_3$ which are physiological metabolites of $1,25(OH)_2D_3$. The ketone is as active as the parent compound $24,25(OH)_2D_3$ in the stimulation of intestinal calcium transport in the rat (Takasaki et al 1981) and has the same potency as $1,25(OH)_2D_3$ in binding to its cytosolic receptor (Napoli and Horst 1983).

Another plasma metabolite isolated when chicks are given large doses of vitamin D_3 is 24(R)-hydroxyvitamin D_3 (Wichman et al 1981), but is not detected when physiological doses of the vitamin are administered. Also detected in plasma of rats fed physiological amounts of vitamin D is $24,26(OH)_2D$. It is the major pathway for further metabolism of 24(OH)D and being a deactivation product possess very little biological activity (Kosjewski 1988).

Vitamin D_3-sulphate has been identified as the 3ß sulphate ester of vitamin D_3 (Reeve et al 1981). Tests for biological activity shows that it has less than five percent of the activity of vitamin D_3 mobilizing calcium from bones, and approximately one percent of the activity in stimulating calcium transport.

1.4.19 Catabolism of vitamin D_3 metabolites.

Due to the the availability of radioactive vitamin D_3 metabolites, the majority of studies have concentrated on the metabolism of vitamin D_3. metabolites. The structural differences of the side chains of vitamins D_2 and D_3 cause the catabolic pathways of their metabolites to be dissimilar (figure 1), the difference occurring after the C-24 hydroxylation step. Hydroxylation at C-23, C24-ketonization and C23:26 lactonization

reactions do not occur with vitamin D_2 metabolites. Reddy and Tserng (1986; 1990) have isolated and identified tri- and tetrahydroxy metabolites of 25(OH)D_2 and 1,25$(OH)_2D_2$ respectively in the degradation pathways of these metabolites.

1.4.20 Vitamin D esters.

Fraser and Kodicek (1965), isolated and identified esters of vitamin D in the liver and kidney of rats. They are less polar metabolites than vitamin D, the palmitate being most abundant. Lund et al (1967) described the rapid formation of esters in the liver in vivo after [^{3}H]vitamin D_3 administration. This was also described by Haussler and Rasmussen (1972) after chicks were given vitamin D.

Ratzkowski et al (1982) examining intestine, liver, kidney and bone showed the presence of these compounds in these tissues when chicks were given physiological and toxic doses of the vitamin. The levels of esters were greater with the larger doses of vitamin D_3 and in both categories the liver accumulated the greatest amount of the metabolite. Lund et al (1967), regard vitamin D esters as a "medical curiosity to which little or no functional importance can be ascribed".

1.5 Pharmacokinetics of vitamin D and its metabolites.

When discussing the pharmacokinetics of vitamin D and its metabolites, one has to consider the vitamin D status of the animal or individual at that time.

The metabolic disposal of the parent vitamin entering the body thus depends on the nutritional state of that vitamin rather than the primary disease of the patient (Mawer

et al 1971). Most of vitamin D storage in the body is in adipose tissue with smaller amounts of 25(OH)D and $1,25(OH)_2D$. Skeletal muscle also acts as a storage depot for vitamin D and 25(OH)D.

More than 99 percent of the metabolite is bound to DBP in the serum. According to Marx (1989) the normal daily turnover of vitamin D is approximately 30 μg/day. There is a discrepancy in this statement as this amount does not tally with the recommended daily allowances of 400 IU (10 μg) which can maintain an adequate vitamin D status without any additional UV light. Most of this is degraded through catabolic pathways with only 1 μg/day utilized in the daily turnover of $1,25(OH)_2D$. However in vitamin D deficient states during repletion with vitamin D, the percentage conversion to 1,25(OH) D is higher than in normal replete subjects, due to a smaller serum pool of the vitamin turning over more rapidly than in individuals who are vitamin replete.

Mawer et al (1971) noted that the serum decay curve of vitamin D consisted of two components; the half-life of the first or fast phase was much shorter in vitamin D deficiency (0.41 days) than in replete individuals (0.71 days). There was no statistically significant difference in half-life in the second or slow phase of the two groups (6 to 40 days).

25(OH)D is the major circulating form of vitamin D. The range of serum concentrations in man depends on the season of the year and latitude of the region in which he resides. The level of this metabolite lowest in late winter and early spring (Lu et al 1992), is due to the amount of UV irradiation to which an individual is exposed.

After vitamin D administration, the serum concentration of 25(OH)D reaches its maximum more rapidly in deplete compared to replete individuals with a higher

percentage of conversion of vitamin D to 25(OH)D (Mawer et al 1971). The half-life of serum 25(OH)D is shorter in deplete subjects (15.9 ± 2.7 days) than in replete subjects (30.7 ± 3.4 days). The plasma half-life of [^{3}H] 25(OH)D in normal individuals varies between 19 and 34 days, the average being 27.5 ± 2.1 days (mean ± SEM) (Batchelor and Compston 1983). The large variation is likely to be at least partly due to the differences in vitamin D status of the subjects studied (Mawer et al 1971) and in calcium and phosphorus homeostasis.

The plasma clearance of the dihydroxyvitamin metabolites 1,25 and 24,25$(OH)_2$D follows a two compartment model (Jarnagin et al 1985). When administered intravenously 1,25$(OH)_2$D is rapidly cleared from the circulation of man, and within five minutes less than fifty percent of the initial dose is present in the plasma. This is followed by a more gradual disappearance of the hormone. The half-life of this metabolite is approximately 5 hours (Marx 1989). However Kumar (1986) is of the opinion that the half-life of this metabolite is approximately 10 hours.

The half-life of the first phase of 24,25$(OH)_2$D decay is 0.55 hours compared with 0.5 hours for this phase in 1,25$(OH)_2$D. These similar rates of degradation may indicate a common rate limiting step for immediate clearance of 1,25$(OH)_2$D and 24,25$(OH)_2$D. However in the second slow phase, the half-life for 24,25$(OH)_2$D is 73.8 hours versus 6.4 hours for 1,25$(OH)_2$D.

The higher production rate of 24,25$(OH)_2$D (26 ± 7ug/day) (Kumar et al 1982) compared with 0.3-1.0ug/day for 1,25$(OH)_2$D (Gray et al 1978) may explain the higher levels of plasma 24,25$(OH)_2$D. Stanbury (1977) quotes the half-life of 24,25$(OH)_2$D as being 40 days.

The cumulative excretion of $1,25(OH)_2D$ in faeces and urine is 54 ± 6 percent (within 6 days) and 7 ± 2 percent (within 2 days) in the latter respectively, while the excretion of $24,25(OH)_2D$ over similar periods is 49 ± 11 and 16 ± 3 percent (Kumar 1986).

Maierhofer et al (1981) maintain that low or normal serum $1,25(OH)_2D$ concentrations are principally determined by renal synthesis, but with increased levels, the metabolic clearance of the hormone is accelerated. When endogenous production of $1,25(OH)_2D$ increases, the serum concentration is elevated to a lesser extent due to the accelerated clearance of the hormone, and the level eventually plateaus suggesting enhanced metabolic clearance of hormone at high production rates.

The serum levels and body pools of vitamin D and its metabolites in adults as quoted by Marx (1989) are presented in table 1. However the half-life of serum 25(OH)D given as 15 days is lower than that found in the study quoted by Bachelor and Compston (1983).

Table 1. Serum Levels and Body Pools of vitamin D and its Metabolites in Adults (Marx 1989).

Metabolite	Serum Conc. ng/ml	Half-time in serum (days)	Pool size in body (μg)	Turnover in body (μg/day)
Vitamin D	10	30	1000	30
25(OH)D*	25	15	500	15
$1,25(OH)_2D$	0.03	0.2	0.5	1.0
$24,25(OH)_2D$	1.0	2.0	10	10

* Typical summer mean in temperate climate.

Table 2. **Serum 25(OH)D concentrations in various populations.**

		25(OH)D ng/ml (mean ± SD)	Reference.	
S.Africa	Children	30.7 ± 9.9	Pettifor et al	1976.
U.S.A.	Adults	27.3 ± 11.8	Haddad and Chyu	1977.
	Adults	13.0 ± 1.7	Preece et al	1975.
Children:	winter	23.5	Arnaud et al	1977.
	summer	41.6.		
	Children	27.0 ± 10.0	Chesney et al	1980b
U.K.	Adults	15.2 ± 5.6	Edelstein et al	1974
France	Adults	15.0 ± 4.2	Bayard et al	1972

Table 3. **Serum 1,25(OH)$_2$D concentrations in normal persons.**

	Serum 1,25(OH)$_2$D (pg/ml)	Reference.	
Normal adults	33.9 ± 1.8 (M ± SD)	Eisman et al	1977
Adults > 18 years	34 ± 8 (M ± SD)	Haussler et al	1977
Adolescents 12-16 yrs	55 ± 2 (M ± SEM)	Chesney et al	1980a
Children	37 ± 11.9 (M ± SEM)	Scriver et al	1978
Children <12 years	38 ± 3.0 (M ± SEM)	Chesney et al	1980a
Children 5-10 years	64 ± 9 (M ± SD)	Haussler et al	1977
Neonates	21 ± 2 (M ± SD)	Streichen et al	1978

1.6 Vitamin D as a calcium regulating hormone.

Calcium is an essential element for survival of life in higher organisms. Together with phosphorus it is responsible for the formation of calcium hydroxyapatite, essential for the normal mineralization of the skeleton which contains 99 percent of the calcium content in the body. It is also important in neuro- muscular activity and necessary for blood coagulation and intracellular functions.

Vitamin D, through its active metabolite $1,25(OH)_2D$, is intimately involved in maintaining calcium homeostasis and the maintenance of normal serum ionised calcium concentrations. The latter is achieved through increased absorption of intestinal calcium and mobilization of bone calcium. PTH also helps in this ionic control through mobilization of bone and increased calcium absorption in the kidney and through its stimulation of $1,25(OH)_2D$ production.

Calcium within the extracellular pool, equilibrates with the intracellular and bone pool, maintaining the plasma ionised calcium within narrow limits, as deviation from these limits, results in life threatening consequences. The vast amount of filtered calcium in the glomerulus is mainly reabsorbed in the renal tubules and the amount excreted in the urine reflects the net absorption from the intestine in the adult in the steady state.

Dairy products are responsible for the major portion of our dietary calcium. Only a minor portion of the mineral taken in the diet is retained in the body, the remainder being excreted in the faeces. In the human this retention is increased during growth, pregnancy and lactation. This adaptation is controlled by the vitamin D endocrine system (Norman 1990b). After the third to the fourth decade the retention decreases and calcium balance may become negative at times.

1.6.1 Effect of 1,25(OH)$_2$D on calcium transport in the small intestine.

1,25(OH)$_2$D administration is associated with a rapid increase in calcium entry into the enterocyte and a rise in concentration of intracellular vitamin D dependant CaBP and a number of enzymes.

The specific mechanism whereby 1,25(OH)$_2$D increases calcium transport is unknown, but it appears that both genomic and nongenomic processes play a role. The transfer of calcium from the intestinal lumen into the extracellular space entails both active and passive processes. The movement of calcium out of the intestinal lumen into the enterocyte is a passive process down an electrical and concentration gradient (Kumar 1991), but the extrusion of the ion across the basolateral membrane is against an electro-chemical concentration requiring energy. 1,25(OH)$_2$D increases the activity of calcium dependent ATPase pump present in the basolateral membrane (Ghijsen and VanOs 1982).

Calcium movement across the cytosol of the intestinal cell is enhanced by a genomic process synthesizing vitamin D induced-calcium binding protein (CaBP). Sodium is also necessary for calcium transport across the cell and in the absence of this ion, transport is depressed (Martin and DeLuca 1969). 1,25(OH)$_2$D has a role in increasing the rate of extrusion of calcium from the cell into the extra cellular fluid space (Kumar 1991).

With the administration of 1,25(OH)$_2$D, calcium transport in the brush border of the intestine shows a biphasic response reaching a maximum in 6 hours then declining and reaching another maximum 12 to 24 hours later. This is probably due to the two types of cells present in the mucosal layer of the small intestine, one being at the tip of

the villus and the other in the crypts, the latter migrating rapidly towards the tip of the villus and replacing the depleted cells. Brush border membrane vesicle uptake of calcium is temperature dependent and does not involve changes in membrane fluidity (Bikle et al 1984).

The rapid action of $1,25(OH)_2D$ enhancing intestinal absorption of calcium termed transcaltachia, is considered a nongenomic response (Norman 1990b). This has recently been linked to protein kinase C activity (Walters 1992). However it has also been demonstrated that calcium entry into the cell invokes modulation of dihydropyridine-sensitive L-type calcium channels and activation of the Na/Ca exchange pathway, a reaction that is genomic and requires protein synthesis. Walters (1992) is of the opinion that the action of $1,25(OH)_2D$ on phospholipid transduction is suggestive of a multiplicity of pathways and mechanisms and include both genomic and nongenomic actions of $1,25(OH)_2D$.

Ionised calcium concentration in extra cellular fluid directly influences PTH secretion. Thus the stimulation of intestinal calcium absorption in situations of low dietary calcium intake is probably an indirect response to a fall in ionized calcium and stimulation of 1α hydroxylase activity in the kidney through the action of PTH. (Pettifor et al 1981).

In hypocalcaemic states, PTH is liberated in increased amounts. This stimulates cAMP production which is presumed to act on modulators activating 1α hydroxylase enzyme (Henry et al 1992), resulting in increased $1,25(OH)_2D$ synthesis. The hormonal effects on the target organs result in increased transport in the intestine, increased mobilization of calcium from bone due to PTH acting in concert with $1,25(OH)_2D$ and

diminished excretion of calcium from the kidney. These three reactions attempt to revert the plasma calcium back to normal.

The response of the vitamin D system to hypercalcaemia is the reverse to that which has just been described. Parathyroid hormone secretion is diminished causing a decrease in plasma $1,25(OH)_2D$. Calcium absorption is decreased, and its mobilization from bone is diminished while loss of calcium from the kidney is increased. Calcitonin release also occurs in this state, diminishing the activity of the osteoclasts (refer 1.4.8).

There is evidence that $1,25(OH)_2D$ also affects intracellular calcium content within other cells beside the gut, an example being in cartilage cells where it has been shown that $1,25(OH)_2D$ reduces intracellular concentration of calcium and increases activation of Ca-ATPase (Lidor and Edelstein (1987). It appears that calcitriol regulates intracellular calcium by modulating activity of Ca-ATPase.

1.6.2 Effect of $1,25(OH)_2D$ on calcium and phosphorus in bone.

Constant remodeling occurs in bone tissue and an equilibrium exists in which osteoclast mediated resorption occurs alongside osteoblast mediated formation of new bone. The hormone $1,25(OH)_2D$ is essential in this process for mineralization of new bone and bone resorption.

The process of mineralization of bone is not a direct action of $1,25(OH)_2D$ but rather an indirect one as the hormone provides calcium and phosphorus through intestinal absorption for addition to bone matrix. In vitamin D deficiency, osteoid produced by osteoblasts remains unmineralized resulting in the bone becoming soft and pliable (DeLuca 1988) causing rickets or osteomalacia.

Osteoblasts are the primary target cells for $1,25(OH)_2D$ and amongst the effects $1,25(OH)_2D$ has on these cells, are an increase in alkaline phosphatase activity in cultured osteoblasts, increased synthesis of bone carboxyglutamic acid protein (osteocalcin) and a depressed production of type 1 collagen.

$1,25(OH)_2D$ increases bone resorption by increasing the number and activity of osteoclasts, but there is evidence that the hormone has no direct effect on osteoclasts which originate from a haemopoietic cell of early macrophage lineage. It is feasible that the hormone has a maturation effect on myeloid haematopoietic precursor cells to differentiate into osteoclasts, or it may induce the release of osteoblast-derived resorption factors which stimulate osteoclast activity.

Several hours after administration, $1,25(OH)_2D$ may release calcium from bone, but the maximal calcium release requires the combined presence of PTH and $1,25(OH)_2D$ acting in concert.

1.6.3 Effect of $1,25(OH)_2D$ on kidney.

$1,25(OH)_2D$ receptors have been isolated in the distal renal tubular cells while the hormone has been shown to be present in the nucleus of these cells. Studies on vitamin D and its metabolites have been recorded as increasing, decreasing or causing no change in renal calcium absorption. This may be due to factors such as serum calcium concentration, vitamin D nutrition state and PTH activity not being independently controlled. Yamamto et al (1984) have shown that both PTH and vitamin D are necessary for optimal renal tubular calcium absorption.

The suppression of 1α hydroxylase activity by $1,25(OH)_2D$ itself, may occur in the proximal convoluted cells accompanied by induction of 24,25 hydroxylation. In vitamin D and calcium deplete states, 1α-hydroxylase activity is activated whilst 24R-hydroxylase is suppressed.

1.7 Vitamin D as a phosphorus controlling hormone.

Phosphates in association with calcium are necessary for mineralisation of the skeleton. Phosphates are also essential for certain cell functions some of which consist of ATP synthesis, introduction of phosphates into organic compounds DNA and RNA, incorporation into membrane phospholipids, phosphorylation of proteins for cellular function and regulation of cellular calcium metabolism.

Phosphates are plentiful in natural foods and 80 to 90 percent of ingested phosphate is absorbed in the small intestine, but in vitamin D deficiency the percentage absorption decreases to between 50 and 60 percent due to the reduced absorption of calcium which lowers the free phosphate concentration in the bowel lumen. Unlike calcium absorption from the intestine which has a stringent hormonal control, hormonal regulation plays a minor role in phosphate absorption. Phosphate homeostasis and plasma concentrations depend on dietary phosphate intake, intestinal absorption and its reabsorption by renal tubules. Extra-cellular fluid phosphate represents less than one percent of total body content, so excess tissue destruction or bone mobilization will temporarily increase the plasma content.

Cellular mechanisms of transport across intestinal cells and renal tubular cells are similar but hormonal control is quite different.

1.7.1 Effect of 1,25$(OH)_2$D on phosphorus absorption in intestine.

Vitamin D increases intestinal absorption of phosphorus from the bowel lumen, the active hormone 1,25$(OH)_2$D acting on the brush border. It has been shown that brush border membrane vesicles (BBMV) pretreated with 1,25$(OH)_2$D have an increased uptake of phosphate and sodium salts. It is also suggestive that this hormone may stimulate Na-dependent phosphate transport (Kurnik et al 1987) by modification of the lipid cell membrane, the sodium dependent phosphate flux being responsible for maintaining an increased transepithelial phosphate transport. Karsenty et al suggests that 1,25$(OH)_2$D has an early effect on phosphate fluxes in the enterocyte, the action being non genomic with a possible modification of lipid structure of the plasma membrane.

Fox and Care (1979) have shown that 1,25$(OH)_2$D increases absorption of calcium and phosphorus from the intestine, the calcium ion being absorbed more rapidly. Investigating the 1,25$(OH)_2$D response on 20 day old cultured embryonic chick intestine, Cross and Peterlik (1988) noted that adding triiodo-thyronine increased the response on calcium and phosphorus transport, the latter ion being more responsive.

No intracellular phosphate binder is present in the mucosal cell analogous to CaBP (Wasserman and Taylor 1973) and the efflux of phosphate from the mucosal cell into the extracellular space via the basolateral membrane is a diffusional process. Vitamin D dependent alkaline phosphatase has been shown not to be required for phosphate transport. The physiological responses to plasma phosphorus changes are dissimilar to that which has been described in the response to hypo- and hypercalcaemic states. Hypo- and hyperphosphataemic states have effects on 1,25$(OH)_2$D formation and catabolism, but not directly on PTH secretion (Haussler and McCain 1977).

Marx et al (1983) noted three differences in the transport of calcium and phosphate ions. [1] Although $1,25(OH)_2D$ stimulated the uptake of calcium and phosphate into explants of chick intestine, the uptake of calcium occurred after 20 minutes while phosphate uptake occurred after 60 minutes (Birge and Miller 1977). [2] The active flux from lumen to serosa in duodenal mucosa was greater for calcium than phosphate, $1,25(OH)_2D$ stimulating the process. In the jejunum the opposite applied, again with $1,25(OH)_2D$ stimulation. [3] In vivo, $1,25(OH)_2D$ stimulates the maximal uptake of calcium and phosphate in the chick brush border membrane vesicle, the saturable uptake of phosphate being sodium dependent, but not so in the case of calcium (Matsumoto 1980).

In hypophosphataemia there is a reciprocal increase in plasma calcium due to direct stimulation of 1α hydroxylase in the kidney. Increased $1,25(OH)_2D$ levels result in an increased absorption in phosphorus from the intestine and increased mobilization of bone and soft tissue, thus liberating phosphate into the plasma. The secondary elevation of plasma calcium level depresses PTH excretion resulting in diminished phosphate loss in the urine. These responses allow the plasma phosphate level to increase (Kumar 1991).

In hyperphosphataemic situations, which may occur in renal failure, 1α hydroxylase activity is suppressed diminishing $1,25(OH)_2D$ synthesis, and lowering serum $1,25(OH)_2D$ concentrations. There is a tendency to hypocalcaemia due to the diminished calcium transfer from the intestine into the enterocyte and diminished mobilization of calcium from bone due to the low serum $1,25(OH)_2$ levels. The decreased plasma steroid hormone affects the parathyroid gland by increasing the preproPTH mRNA (Silver et al 1985), diminishes the number of $1,25(OH)_2D$ receptors,

and alters the calcium set point, resulting in higher plasma levels than normal being necessary to reduce PTH values (Pettifor 1990). These effects cause an increase in PTH secretion, the net result in the kidney is a lowering of the tubular maximum phosphorus, resulting in hyperphosphaturia and hypophosphataemia, through the action of increased PTH concentrations.

1.7.2 Effect of $1,25(OH)_2D$ on phosphate in bone.

The activity of $1,25(OH)_2D$ on phosphate in bone is similar to that as already described in calcium control.(refer 1.6.2). Resorption of bone by $1,25(OH)_2D$ aided by PTH, results in both calcium and phosphorus being transported into plasma with an increase in their plasma concentrations.

1.7.3 Effect of $1,25(OH)_2D$ on phosphate handling by the kidney.

It is unclear whether or not $1,25(OH)_2D$ has a direct action on the handling of phosphorus excretion in the kidney. If it does, its effects are minor compared with other hormones such as PTH.

1.8 Serum levels of vitamin D and its metabolites.

The profile of vitamin D and its metabolites in plasma vary in the replete, deplete and toxic state.

1.8.1 Serum levels of vitamin D and its metabolites in the vitamin D replete state in various species.

It has been accepted that plasma 25(OH)D, the major circulating metabolite, is a good indicator of the vitamin D status in humans and animals. However problems arise when dealing with certain animals such as the fruit bat and the mole rat. Cavaleros (1992) and Buffenstein et al (1988) investigating the fruit bat and mole rat respectively, found undetectable levels of 25(OH)D in wild animals. Whether this reflects either a natural impoverished vitamin D status or an altered vitamin D metabolism in these animals is unclear. Walters (1992) states that there are complex differences among different states of vitamin D deficiency and that physiological levels of $1,25(OH)_2D$ can exist in animals/humans that are deficient in vitamin D and 25(OH)D.

The normal plasma values of 25(OH)D vary in different species. Horst et al (1981) quantitated plasma vitamin D and its metabolites in five species of animals namely chicken, turkey, cow, sheep and pig. All the animals received supplementation of calcium, phosphorus and vitamin D (as vitamin D_3) to meet with the normal requirements. All the species were of adult age. The turkeys and chickens were housed inside with no exposure to direct sunlight. The sheep, cattle and pigs were housed outside on concrete or in a drylot. The results are summarized in table 4.

Serum vitamin D_2 is detectable only in the sheep, its concentration practically equal to vitamin D_3. As expected the 25(OH)D is the major circulating metabolite in these animals. Again the vitamin D_2 metabolite, $25(OH)D_2$ is present in significant amounts in the sheep, constituting 52 percent of the total metabolites. This was quite unexpected, as the sheep were kept away from the fields, were continuously exposed to

the sunlight and also given vitamin D_3 supplementation. Vitamin D_2 present in natural foodstuffs, apparently supplies a major portion of vitamin D to sheep. The heavy covering of wool and lanolin probably makes the sheep inefficient producers of vitamin D_3. The cow also exposed to the sunrays and receiving supplemental vitamin D_3 obviously imbibed vitamin D_2 from the cow's foodstuffs as evidenced by the level

Table 4. Serum levels of vitamin D_2 and vitamin D_3 and their metabolites in five species of animals. (Horst et al 1981).

	Species				
Sterol	Turkey	Chicken	Cow	Sheep	Pig
Vit D_2 (ng/ml)	ND	ND	ND	0.5 ±0.3	ND
Vit D_3 (ng/ml)	3.4 ±0.2	2.3 ±0.3	1.7 ±0.7	0.6 ±0.5	10.2 ±4.1
$25(OH)D_2$ ng/ml	0.8 ±0.7	1.6 ±1.5	5.8 ±1.7	13.9 ±3.7	0.8 ±0.3
$25(OH)D_3$ ng/ml	18.2 ±7.9	25.3 ±3.9	36.9 ±7.1	12.9 ±5.3	74.9 ±21.2
24,25** $(OH)_2D_2$	ND	ND	0.7 ±0.2	6.4 ±1.0	ND
24,25** $(OH)_2D_3$	1.2 ±0.1	2.3 ±0.2	2.6 ±1.3	4.4 ±0.7	20.2 ±10.3
1,25 $(OH)_2D$ pg/ml	51.8 ±31.3	21.2 ±2.1	38.0 ±10.4	35.9 ±30.0	60.3 ±7.2

** ng/ml ND (Not Detectable) < 0.2ng/ml.

of plasma $25(OH)D_2$, which was approximately 16 percent of the total 25(OH)D. In the chick and pig, $25(OH)D_2$ was only 6 and 1 percent respectively of the total 25(OH)D.

The conversion of $25(OH)D_3$ to $24,25(OH)_2D_3$ was more efficient in some farm animals than others. In the cow, turkey and chick the conversion ranged from 7 percent to 9 percent, whereas higher conversions of 27 and 34 percent were observed in the pig and sheep respectively. $24,25(OH)_2D_2$ was not detectable in the turkey, chick or pig, but was very evident in the sheep, the conversion being 46 percent of $25(OH)D_2$. There was a large variation in concentrations of the active metabolite $1,25(OH)_2D$ between the five animals. The chick recorded the lowest level, while the concentrations in the turkey and pig were two and a half and three fold greater respectively than the chick.

Hughes et al (1977) noted the influence of dietary vitamin D_3 on the circulating concentration of its active metabolites in the chick and rat. In one day old chicks, the plasma $25(OH)D_3$ and $1,25(OH_2D_3$ were 14.1 ng/ml and 8.9ng/100ml (89pg/ml). Chicks receiving optimal supplements of vitamin D_3 recorded plasma levels of 21 to 35 ng/ml and 5.1-7.5ng/100ml (51-75 pg/ml) respectively after 3 to 4 weeks.

Changes in serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ with age and vitamin D status in chicks, were studied by Sedrani (1984) in chicks between 3 and 7 weeks of age, fed a normal diet. The serum levels of $25(OH)D_3$ was approximately 25.5nmol/l (10.2 ng/ml), and $1,25(OH)_2D_3$ 160pmol/l (66.6 pg/ml).

Bony fish which store vitamin D (cod, halibut) receive very little vitamin D in their diet (Copping 1934 as quoted by Oizumi and Monder 1972), and ultraviolet radiation does not lead to an increase in vitamin D in fish (Bills 1927 as quoted by Oizumi and Mondre 1972). Oizumi and Monder (1972) observed that vitamin D administered to the goldfish accumulated mainly in intestine and liver but insignificant amounts of 25(OH)D or related metabolites were present in blood. They concluded that goldfish can accumulate vitamin D, but cannot metabolize it.

Henry and Norman (1975) were able to detect 1α hydroxylase activity in both fresh and salt water fish and also in 25 of 28 vertebrate species studied which included reptiles and amphibians, thus demonstrating further metabolism of vitamin D in this vertebrate class. It is possible that the synthesis and metabolism in fish differ from that in mammals.

The plasma concentrations of vitamin D and its metabolites in human adults, have already been documented when discussing pharmacokinetics of vitamin D and its metabolites (refer tables 1, 2 and 3). Of the five animals reviewed, the chick has plasma metabolite concentrations which are fairly similar quantitatively to the plasma metabolites of the human adult (Hughes et al 1977).

1.9 Vitamin D metabolism in vitamin D deplete animals and man.

Vitamin D deficiency, through a lack of UV light exposure with an inadequate dietary intake leads to the clinical syndrome of rickets in the child or osteomalacia in the adult.

In the vitamin D deplete state, serum levels of 25(OH)D are characteristically low. In the rachitic chicken, $25(OH)D_3$ values are less than 4ng/ml, and in man the values are also generally less than 4 ng/ml.

Low values of $1,25(OH)_2D$ are described in experimentally induced vitamin D deficiency (Hughes et al 1977). However Sedrani (1984), has reported elevated levels in vitamin depleted chicks. In humans, $1,25(OH)_2D$ levels have been reported to be low (Drezner and Feinglos 1977; Marx et al 1983), normal or even elevated (Markestad et al 1984).

Describing vitamin D concentrations in vitamin D deficiency in three children, Chesney et al (1981) found zero serum levels in two and a low value in the third. The values of 25(OH)D and $24,25(OH)_2D$ were within the range of the control subject. Markestad et al (1984) investigating 17 children with vitamin D deficiency rickets, also found the serum $1,25(OH)_2D$ levels to vary within the reference range, but increased to well above the upper limit of normal within one week of treatment with vitamin D and reverted back to normal levels after 10 weeks of therapy.

Hughes (1977) noting the presence of $25(OH)D_3$ and $1,25(OH)_2D_3$ in one day old chicks, found undetectable amounts of these metabolites when the chicks were raised under rachitic conditions for three weeks.

The concentration of the parent compound vitamin D, has not been reported in the situation of vitamin D deficiency in chickens, but would be expected to be undetectable.

The finding of variable $1,25(OH)_2D$ levels in vitamin D deficiency is difficult to explain as it would be expected that values would fall if the availability of the substrate 25(OH)D was diminished. Furthermore if $1,25(OH)_2D$ is the only active metabolite in physiological conditions, it is difficult to reconcile the finding of hypocalcaemia in a situation of elevated $1,25(OH)_2D$ levels unless calcium availability in the gastrointestinal tract was impaired.

Several explanations have been put forward to explain the elevated $1,25(OH)_2D$ levels; firstly the elevated levels represent the result of a small amount of vitamin D being obtained and rapidly converted to $1,25(OH)_2D$, and secondly, that the free levels of $1,25(OH)_2D$ might actually be reduced due to increased binding of $1,25(OH)_2D$ to DBP as a result of reduced amounts of 25(OH)D and other metabolites.

The vitamin D deficient chick activates only 1α hydroxylase enzyme in the kidney (Henry et al 1992), serum 24,25(OH)$_2$D not being detected. Treatment with vitamin D causes activation of the 24-hydroxylase enzyme and suppression of 1α hydroxylase, resulting in higher values of serum 24,25(OH)$_2$D than 1,25(OH)$_2$D$_3$.

1α hydroxylase activity is at its maximum when vitamin D deficiency is most marked (Mawer 1982). This was also noted by Henry and Norman (1975) that the highest enzyme activity occurred in birds severely rachitic. As already mentioned Sedrani (1984) feeding chicks on a low but not a fully deficient vitamin D diet, noted a low serum 25(OH)D$_3$ level and a seven fold higher level of 1,25(OH)$_2$D$_3$ compared with normal chicks.

The associated lowered concentration of plasma calcium has a direct effect on PTH causing secondary hyperparathyroidism. The increased PTH stimulates the activity of the enzyme 1α hydroxylase in the mitochondria of the kidney, but due to the absence or paucity of the substrate 25(OH)D, the synthesis of the active hormone is typically decreased. Although PTH increases mobilization of calcium from bone, the diminished transfer of calcium from the bowel results in hypocalcaemia which leads to a failure of mineralization, causing rickets in childhood and osteomalacia in adults. The deformities seen are due to the inability of the bones to withstand the stresses exerted upon them.

Increased secretion of PTH also affects the renal reabsorption of phosphorus as the tubular maximum for phosphorus is decreased, resulting in hypophosphataemia and hyperphosphaturia. The product of calcium and phosphorus expressed in mg/100ml is decreased, and is associated with diminished mineralization of bone.

The profile of plasma vitamin D metabolites in chicks is very similar to that seen in humans in the vitamin D deplete state (Collins and Norman 1991). The plasma

mineral profile is also similar, plasma calcium and phosphate levels being decreased and the plasma enzyme alkaline phosphatase raised.

1.10 Vitamin D toxicity.

Vitamin D toxicity is a hypercalcaemic and hypercalciuric state suggestive of excess $1,25(OH)_2D$ activity. The usual cause of toxicity is iatrogenic due to ingestion of excessive amounts of vitamin D usually in the form of vitamin D_2 (Mawer et al 1985a). Other predisposing causes in situations of normal or only moderate vitamin D intakes include sarcoidosis (Papapoulas et al 1979) and other vitamin D hypersensitivity syndromes, a high calcium diet with excessive vitamin D intake and tertiary hyperparathyroidism as occurs in renal insufficiency. Vitamin D toxicity has also been recorded in farm animals especially cows, when large doses of the vitamin were administered (Vitamin Tolerance of Animals 1987).

Excessive exposure to sunlight is not a cause of vitamin D toxicity. Control over the production of endogenously produced cholecalciferol (20.3 ± 1.4 ng/ml Hollis et al 1981) and only modest elevation of plasma $25(OH)D_3$ (up to about 80ng/ml) is evident from prolonged solar exposure (Mawer 1982). Also during prolonged exposure to the sun, the accumulation of previtamin D_3 is limited to about 10 to 15% of the original 7-dehydrocholesterol content because the previtamin photoisomerizes to the inert photoproducts $lumisterol_3$ and $tachysterol_3$ (Holick 1981). It was originally thought that the pigmentation of individuals exposed to excessive sunlight protected them against toxicity, but it has been shown that skin pigment is not an essential regulator of vitamin D production (Holick 1981).

Controversy exists as to the actual mechanism by which an excess of vitamin D causes the metabolic disturbances in vitamin D toxicity. In this state $1,25(OH)_2D$ concentrations are either normal, only mildly increased (Kumar 1991) or even decreased (Shepard and DeLuca 1980) as the hormone 1α hydroxylase is depressed due to the negative feedback mechanism caused by high levels of plasma calcium (Kumar 1991).

Kumar (1991) considers two possibilities for the enigma of hypercalcaemia and relatively normal plasma $1,25(OH)_2D$ concentrations. Firstly, although 25(OH)D has a lower affinity than $1,25(OH)_2D$ for the vitamin D receptor, 25(OH)D concentrations in the toxic state may reach plasma levels of 400ng/ml or higher due to the 25-hydroxylase enzyme not being stringently controlled. It is possible that at these high levels, enough 25(OH)D binds to the receptor to stimulate calcium absorption from the intestine producing hypercalcaemia. The second possibility is the increased synthesis of 5,6-trans 25(OH)D observed when excessive amount of 25(OH)D are administered to animals. Due to cis-trans isomerization of vitamin D, the A-ring undergoes 180° rotation placing the 3-hydroxyl group in the position of 1 hydroxyl. In this position the metabolite is actually a pseudo-1-hydroxyl analogue of $1,25(OH)_2D$ which may activate the $1,25(OH)_2D$ receptor with increased absorption of calcium from the intestine.

Mawer et al (1985a) has suggested another possible mechanism by which excessive vitamin D ingestion might cause hypercalcaemia. In their study, they found $1,25(OH)_2D$ concentrations to be elevated by some four to seven fold, and thus believe that the synthesis of $1,25(OH)_2D$ might be increased through the mass action effect on renal 1α hydroxylase of very high concentrations of 25(OH)D.

Veith (1990) disputes the above theories as he believes it is not clear whether 25(OH)D or any other agonist besides $1,25(OH)_2D$ acts on the vitamin D receptor in

vivo. Rather he presents the hypothesis that free or unbound $1,25(OH)_2D$ is the metabolite responsible for causing toxicity. Vitamin D binding protein has a greater affinity for 25(OH)D and $24,25(OH)_2D$ than $1,25(OH)_2D$, and any increase in these former two metabolites will result in a greater amount of free or unbound $1,25(OH)_2D$.

The increased free $1,25(OH)_2D$ that is accessible to cells and thus functional in vivo (Bikle et al 1985), would be available to combine with the vitamin D receptor resulting in increase bone mobilization and intestinal absorption. Support for this hypothesis, is provided by Shepard and DeLuca (1980) who showed that the calculated amount of plasma free $1,25(OH)_2D$ in intoxicated rats was greatly increased.

Wichman et al (1981) isolated 24(R)-hydroxyvitamin D_3 from the plasma of chicks given large doses of vitamin D_3. The metabolite was not detected when the chicks received physiological doses of the vitamin. They feel that the presence of compounds such as 24(R)-hydroxyvitamin D_3 or other unidentified metabolites may be responsible for the toxic effects of the vitamin.

Shepard and DeLuca (1980) are of the opinion that the hypercalcaemia is the result of mobilization of calcium from bone rather than through an increase of calcium absorption from the intestine. Lindquist (1952) showed that mobilization of calcium from bone increases with massive amounts of vitamin D, whereas intestinal response is saturated at very low doses of vitamin D.

The metabolic profile in vitamin D toxicity reveals that plasma 25(OHD), $24,25(OH)_2D$ (Mawer et al 1985a) and 25(OH)D-26,23-lactone are present at greatly elevated levels (Wichman et al 1981). Plasma 25(OH)D-26,23-lactone increases from low to abundant levels as the dose of vitamin D increases, while $25,26(OH)_2D$ is present in small amounts. It was considered that these last two metabolites serve as a route of

inactivation and excretion. Kosjewski (1988) investigating the metabolites in hypervitaminosis D in rats found significant amounts of plasma 24(OH)D and 25(OH)D, the former being catabolized further through conversion to $24,26(OH)_2D$.

Young rats are more susceptible to vitamin D intoxication than older rats, the possible cause being due to a diminished rate of degradation of $1,25(OH)_2D$ (Dostal and Toverud 1983). Stern et al (1981) are of the opinion that this susceptibility may be due to $1,25(OH)_2D$ production being only loosely regulated in children, thus the plasma levels of $1,25(OH)_2D$ are proportional to those of 25(OH)D.

Discrimination against vitamin D_3 or vitamin D_2 may play a role in the severity of vitamin D toxicity. Rats appear to be less toxic to 1α hydroxyvitamin D_2 compared to 1α hydroxy-vitamin D_3 (Sjoden et al 1985). This is rather unexpected as Horst and Napoli (1982) have shown that the rat exhibits discrimination against vitamin D_3. Similarly Rhesus monkeys appear to be less affected by hypervitaminosis D caused by vitamin D_2 compared with that caused by vitamin D_3 (Hunt et al 1972).

1.11 Discrimination of vitamin D by avian species and other animal species.

Vitamins D_2 and D_3 have equal bioactivity in most mammals but vitamin D_2 is approximately 1/100 as potent as D_3 in preventing or curing rickets in young chicks (Belsey et al 1974; Rambeck et al 1984).

Horst et al (1982) gave vitamins D_2 and D_3 to chickens and were able to demonstrate that the major point of discrimination between vitamins D_2 and D_3 is before their hydroxylation to 25(OH)D. This may be the result of less efficient absorption of vitamin D_2 or alternatively plasma vitamin D_2 may be more efficiently removed from the

blood. Jones et al (1976) were of the opinion that the point of discrimination in the chick against ergocalciferol was at the 25(OH)D level. Another possibility was that vitamin D_2 was presented to the liver more rapidly, catabolized and cleared from the circulation.

Hay and Watson (1976b) investigating vitamin D binding proteins in various species of animals, noted that avian sera contain two distinct transport proteins which have a lower affinity for the metabolites of vitamin D_2 than vitamin D_3. Belsey et al (1974) also showed reduced binding of vitamin D_2 and 25(OH)D_2 by blood proteins in chicks, thus reducing delivery of substrate to liver. Old world monkey tissue receptor protein exhibited a higher affinity towards 25(OH)D_3 than the corresponding protein in New world monkeys. (Hay and Watson 1977).

The human vitamin D binding protein has identical affinities for both vitamins.(Haddad and Cooke 1989). In vitro studies show vitamin D_2 hydroxylation reactions in the chick to be identical to that occurring with vitamin D_3 and it appears that this species possess the required enzymatic machinery to synthesize 1,25$(OH)_2D_2$ (Jones et al 1976; Chen and Bossman 1964). It has also been revealed that the chick duodenal receptor binds 1,25$(OH)_2D_2$ with the same capacity as 1,25$(OH)_2D_3$ (Rambeck et al 1984).

A species of mammals which discriminate against vitamin D_2 is the New World primate (Cebidae). Hunt (1967) showed that in the cebus monkey, vitamin D_2 had no effect in the transfer of calcium from the intestinal lumen into the blood. In contrast to the chick, they have a single binding protein which is either albumin or α globulin fraction, while Hay and Watson (1975) have shown that their binding properties for 25(OH)D_2 and 25(OH)D_3 are similar.

Horst and Napoli (1982) were also able to show that the pig discriminated against vitamin D_2 at the vitamin D 25-hydroxylase level and the rat showed discrimination against vitamin D_3. This may be due to rats evolving as nocturnal animals, consume grain which contain mainly ergosterol. It has also been observed that 1α hydroxyvitamin D_2 is less toxic to the rat than 1α hydroxy-vitamin D_3 (Sjöden et al 1985). As mentioned earlier, this is unexpected as showing discrimination to vitamin D_3, it would be expected that vitamin D_3 would be better tolerated than vitamin D_2.

Tjellesen et al (1985) have described different effects in patients on anticonvulsive therapy treated with vitamin D_2 or vitamin D_3. They found vitamin D_2 to be more efficacious as it produced greater bone mass.

The structural differences between the vitamins D_2 and D_3 molecules (refer figure 1) may be a factor in the causation of vitamin D discrimination. It is well known that $1,25(OH)_2D_3$ is converted into various metabolites in the kidney which do not occur with $1,25(OH)_2D_2$. Reddy and Tserng (1986;1990) have isolated new metabolites of $1,25(OH)_2D_2$ which they feel require further studies to help explain the differences between vitamins D_2 and D_3, regarding their bioactivity and toxicity both in avian and mammalian species.

Therapeutically it is very fortunate that the human does not appear to show any discrimination against ergocalciferol, as this antirachitic compound has proved to be an invaluable agent medicinally and in the fortification of food.

1.12 Distribution and storage of vitamin D and its metabolites in mammalian, avian and human tissues.

Over the past 40 years great advances have been made in studying the tissue distribution of vitamin D and its metabolites.

Initially large unphysiological amounts of vitamin D_2 were administered to the animals studied, and biological assays, particularly the rat line test were utilized to assess activity. Paper chromatographic techniques, which were less effective than more modern techniques such as HPLC, in separating the various metabolites were also used. The rat line test measured the bioactivity of vitamin D and its metabolites present but not the concentration of nonactive metabolites present. No separation of the metabolites was carried out. Thus Cruikshank et al (1953;1954) utilized 1 mg (40000 IU) oral ergocalciferol in rachitic rats, and Kodicek (1954) administered graded doses of vitamin D_2 (0.25 - 8.0 mg) also to rachitic rats.

However with the synthesis of radioactive vitamin D, progress was made on the tissue distribution and subcellular localization of the metabolites. Initially the specific activities of these preparations were too low for detection of the vitamin or its metabolites using physiological doses, thus high doses of the vitamin were used. Total concentrations of radioactivity were measured in these studies as separation of the metabolites was not possible. The disadvantage of this technique was that it mainly demonstrated the stored or non functional vitamin D (Neville and DeLuca 1966). Using this technique Kodicek (1955) administered 1 mg (40,000 IU) ^{14}C-ergocalciferol to rachitic rats which were killed 24 hours later.

With the advent of techniques in which radioactive vitamin D of high specific activity was synthesized, it was now possible to study the distribution of the vitamin and its metabolites in the various tissues and their subcellular localization more accurately without disturbing the vitamin D status of the animal or subject. These were now more meaningful experiments.

An extensive study was performed by Neville and DeLuca (1966), investigating the distribution of radioactivity in target tissues, liver, blood, muscle, the contents of small and large intestine and faeces in vitamin D deplete rats after receiving [^{3}H] vitamin D (10.7 IU) injected into the intrajugular vessels. The tissues were examined at intervals from 15 minutes to 48 hours. They calculated the percentage of radioactivity in each organ compared with the administered dose and also the relative incorporation into each tissue expressed as dpm/100mg dry weight. Imrie et al (1967) examined the amount of radioactivity in blood, skeletal muscle, liver and bone over 24 hours in rachitic chicks. Haussler and Norman (1969) did a similar study after an intracardiac dose of [^{3}H] vitamin D 50 IU (3.25 nmol), the tissues investigated which included liver, skeleton, kidneys, intestinal mucosa and serum, were examined at intervals between 1 and 48 hours.

In more recent studies actual metabolic concentrations have been assessed using chromatographic methods to separate out the various metabolites.

The studies by Haussler and Norman (1967) and Haussler and Rasmussen (1972) are fairly similar, as combined use was made of physiological doses of labelled vitamin D_3 with high specific activity and of chromatographic studies to examine metabolism of vitamin D_3 in rachitic chicks. They were now able to ascertain not only the radioactivity

in each organ, but also the distribution of the parent vitamin, $25(OH)D_3$ and $1,25(OH)_2D_3$ metabolites in these tissues. $24,25(OH)_2D_3$ was not measured.

Haussler and Rasmussen (1972) utilized 20 IU [^{14}C] vitamin D^3 given orally or intracardially in rachitic chicks whose intestinal tissues were examined after 16 hours. In the Haussler and Norman (1967) study, 0.65 nmol (10 IU) [^{14}C] vitamin D_3 was injected intracardially into rachitic chicks which were killed after 18 hours. The tissues studied were plasma, kidney, bone, small intestine and liver. Ratzkowski et al (1982) administered rachitic chicks a total of 4ug [^{3}H] vitamin D_3 in 4 equal divided doses, an interval of 3 days separating each dose. The chickens were killed 2 days after receiving the last dose. The tissues examined were similar to those in the Haussler and Rasmussen (1972) study, but also included valuation of $24,25(OH)_2D_3$ and vitamin D_3 esters along with the other metabolites.

Mawer et al (1972) carried out a study by combining the use of intravenous radioactive cholecalciferol (either [^{14}C] or [^{3}H]) and biological assays (by line test) in human tissue. These tissue extracts which were subjected to silicic acid chromatography revealed the distribution and storage of vitamin D and metabolites between 4 and 90 days after vitamin D administration. The patients investigated were divided into two groups, those who were treated with vitamin D before study (treated) and the untreated group which received no treatment beforehand.

It is of interest to note that initially only rats were utilized when large doses of vitamin D_2 were administered. This may have been due to the knowledge that chickens discriminated against vitamin D_2 or else the choice was fortuitous. Later chickens were studied and were given physiological doses of vitamin D_3.

Initially even with the use of large unphysiological amounts of vitamin D_2, either unlabelled or later labelled, and the use of crude assays compared to the subsequent advanced techniques, the pattern in these studies was basically similar compared with the later studies. Cognisance of this fact is noted by Neville and DeLuca (1966) when they compared their study in bone using 10.7 IU [^{3}H] vitamin D_3 the results being practically identical to those observed by Norman and DeLuca (1963) when an amount of 500 IU vitamin D_2 was administered.

1.13 Analysis of studies.

Analyzing the various studies discussed above, certain conclusions were evident following the administration of unlabelled or labelled vitamins D_2 or D_3 :-

i) The rapidity of clearance of the vitamin from the blood and the rapid distribution of the steroid in all tissues examined.

In the study of Neville and DeLuca (1966), following intravenous injection of physiological doses of the vitamin with high specific activity, radioactivity was present in liver, bone, kidney and small intestine 15 minutes after administration. One hour after oral or intraperitoneal administration of [^{3}H] vitamins D_2 or D_3, Haussler and Norman (1969) demonstrated rapid distribution of radioactivity in all tissues examined. This showed a rapid and efficient mechanism for systematically transporting vitamin D in the body (Norman 1979). At this stage, the serum contained approximately 10 percent of the injected dose, 90 percent of this being unmetabolised.

Mawer et al (1972) noted a consistent pattern of distribution of the parent vitamin and its metabolites despite different diseases in the patients.

Mawer et al (1969) observed that vitamin D_3 was eliminated more rapidly from plasma, and the percentage of [^{3}H] 25(OH)D_3 derived from [^{3}H] vitamin D_3 was higher when the patients were vitamin D deficient compared with the individuals who were vitamin replete. Patients with osteomalacia had a higher mean ratio of serum 25(OH)D_3 to vitamin D_3 compared with normal people four days after vitamin administration.

ii) Early accumulation of vitamin D in liver and its rapid elimination as 25(OH)D.

In all the studies mentioned, there was rapid accumulation of vitamin D in the liver after administration of vitamin D. This was the first clue of the major involvement of this organ in vitamin D metabolism. Neville and DeLuca (1966) noted that the vitamin accumulated in the liver within 15 minutes after administration, while 24 hours later only 15 per cent of the original amount was present. Haussler and Norman (1969) after detecting the presence of the vitamin in the liver within one hour, noted 86 percent decrease of the steroid 23 hours later in this organ.

iii) 25(OH)D is the major circulating metabolite in vitamin D metabolism.

The studies of Haussler and Norman (1967) and Haussler and Rasmussen (1972) confirm that 89 per cent and 74 per cent of the metabolites in blood estimated after 18 and 16 hours respectively, were 25(OH)D (table 5). Similar findings were described by Ratzkowski et al (1982).

iv) 1,25(OH)$_2$D is the major metabolite in the intestine in vitamin D deplete state.

In the vitamin D deplete state, 76 per cent of the metabolites present in the intestine, were identified as 1,25(OH)$_2$D (Haussler and Norman 1967), compared with 66 per cent in a similar study by Haussler and Rasmussen (1972).

In these studies, 1,25(OH)$_2$D levels may not be reflecting the true concentration of this metabolite, as the methods of separation were relatively crude and did not separate out all the metabolites from each other. Therefore the measurements particularly of the more polar metabolites may have been complicated by the inclusion of other metabolites. In these studies the active metabolite may have been contaminated by 25,26(OH)$_2$D and 1,24,25(OH)$_3$D metabolites.

Ratzkowski et al (1982) found 25(OH)D$_3$ to be the major metabolite in the intestine when the chickens were given physiological amounts of vitamin D$_3$.

A summary of the distribution of vitamin D, 25(OH)D, and 1,25(OH)$_2$D$_3$ in various organs from the studies of Haussler and Rasmussen (1972) and Haussler and Norman (1967) is presented in table 5. The results of these two studies are similar qualitatively and fairly similar quantitatively.

Table 5. Percentages of vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ in various tissues of rachitic chicks after administration of radioactive Vitamin D_3

		Vitamin D_3	$25(OH)D_3$	$1,25(OH)_2D_3$
(1) H/N	Intestine	4.4	19.6	76
(2) H/R		16	18	66
H/N	Blood	2	89	9
H/R		12	74	14
H/N	Bone	15	59	26
H/R		18	62	20
H/N	Kidney	11	52	37
H/R		25	42	33
H/N	Liver	41	41	18

(1) H/N Haussler and Norman (1967) after 18 hours.

(2) H/R Haussler and Rasmussen (1972) after 16 hours.

v) High content of radioactivity in intestine and bone.

Bone and intestinal mucosa accumulate radioactivity early after administration of labelled vitamin D, but if a correction is made for the noncellular material in bone then skeletal tissue is most radioactive. Similarly if the presence of smooth muscle is not

included in the radioactive counts in small intestine, then the mucosal contents also contain a high relative incorporation of radioactivity (dpm/100gm dry weight) (Neville and DeLuca 1966).

Haussler and Norman (1969) also showed high incorporation of radioactivity in the skeleton of the chicken, the amount of radioactivity increased over 24 hours. This was also borne out in the studies of Haussler and Rasmussen (1972).

vi) Early appearance of radioactivity in the bowel.

The presence of a small quantity of radioactivity in the small intestine 15 minutes after vitamin D was injected, could not be accounted for by Neville and DeLuca (1966). It appears that this early appearance could be due to excretion of products from the liver via bile into the intestine (Kumar 1991).

vii) High levels of ergocalciferol or radioactivity in the large bowel and faeces.

In studies of rats receiving ergocalciferol (1 mg or 40,000 IU) orally (Cruikshank et al 1953), faeces contained the highest percentage of the vitamin at 24 hours. This amount diminished rapidly within 48 hours after the given dosage. Similar results were obtained in Kodicek's study (1954) and also by Neville and DeLuca (1966).

With the knowledge that the bulk of degradation products are excreted into the small intestine from the liver via the bile, it appears that this process is responsible for these results. However considering the massive doses of vitamin D_2 which were administered orally in the studies of Cruikshank et al (1953) and Kodicek (1956), it is

feasible that unabsorbed vitamin D_2 plays a part in the amount of ergocalciferol excreted in the faeces.

viii) Other Metabolites.

Ratzkowski et al (1982) in their study included values of $24,25(OH)_2D_3$ in plasma and tissues and vitamin D_3 esters in the tissues. In the replete state the metabolite $24,25(OH)_2D_3$ was 23 per cent the value of plasma $25(OH)D_3$ and exceeded the values of $1,25(OH)_2D$ in the tissues, except in the intestine where it was less in amount. Vitamin D_3 esters were present in all the tissues examined, the largest quantity being in the liver with smaller amounts in bone, kidney and intestine.

Rosenstreich et al (1971), showed that esters of vitamin D_3 accumulated in adipose tissue. There was a progressive increase in the total quantity of this metabolite in fat after cessation of supplementation, and 6 weeks later had doubled its content to 20 percent of all radioactivity in that organ. As this represented a large amount compared to esters in other sites, the authors suggested that this metabolite was formed from vitamin D in fat.

1.14 Vitamin D storage organs.

In studies conducted in human adult patients over a period of 90 days after administration of labelled vitamin D_3, Mawer et al (1969), were able to show that adipose tissue and voluntary muscle together contained a greater fraction of the administered dose than was recovered totally from all the other tissues. They suggested

that these tissues acted as storage organs due to the relatively long half-life of vitamin D_3, and its prolonged retention in adipose tissue and muscle and subsequent slow release into the circulation. During times of vitamin D deprivation the retained vitamin was used biologically.

Rosenstreich et al (1971), after supplementing rachitic rats for two weeks with vitamin D_3, and following the accumulation of the vitamin and its metabolites in adipose tissue over the following 6 weeks, noted that half of the radioactivity in adipose tissue was unaltered vitamin D_3. However over the next five week period the amount of radioactivity fell at a slow rate, the half-life of the vitamin being 81 days. Throughout the entire study, vitamin D_3 remained the most abundant compound in adipose tissue.

From this description it appeared that adipose tissue acted as a storage organ for vitamin D_3. $25(OH)D_3$ which made up the next largest fraction of the total radioactivity was also stored in this organ. The rates of decrease of these two metabolites were similar.

The rate of decline of biological activity in human plasma (half-life 3 to 4 months) after a pharmacological dose of vitamin D was similar to that found in rats (half-life 80 days).

1.15 Summary of vitamin D metabolism and its action on mineral homeostasis.

Two vitamin D compounds that are important from the nutritional point of view are vitamins D_2 and D_3. The structural difference between these compounds lies in their side chains, which may be partly responsible for the discrimination against vitamin D_2 by chicks and New World monkeys.

Shortly after administration of labelled vitamin D to animals, radioactivity is present in all tissues examined, the major portion located in the liver. Over the next few hours, radioactivity decreases fairly rapidly in this organ.

The liver 25-hydroxylase enzyme appears not to be rigidly controlled, although some workers have suggested that 25(OH)D or $1,25(OH)_2D$ might influence its activity. Intracellular calcium has also been incriminated.

In contrast, $1,25(OH)_2D$ synthesis is stringently controlled. The agents responsible for this control are PTH, serum calcium, serum phosphorus and $1,25(OH)_2D$ itself.

The actions of $1,25(OH)_2D$ can be divided into two distinct groups, namely the well recognised effect on mineral metabolism, and the effects on cell growth and differentiation.

Receptors for $1,25(OH)_2D$ have been located in over 20 different tissues, and it is apparent that the functions of $1,25(OH)_2D$ are more complex than initially documented.

The half-life of $1,25(OH)_2D$ is short and it induces its own catabolism in the target tissues by increasing enzymes such as 24-hydroxylase and 23-hydroxylase.

Controversy still exists regarding the role of $24,25(OH)_2D$. Some authors feel that it plays a part in bone mineralization, while others maintain that it is a breakdown product in vitamin D metabolism.

The profile of vitamin D and its metabolites in deplete and replete states, indicate that the level of serum 25(OH)D provides good evidence of the vitamin D status in humans and animals, while the serum concentration of the active metabolite $1,25(OH)_2D$ in physiological conditions indicates the state of calcium homeostasis.

Adipose tissue and muscle both possess the ability of acting as vitamin D storage organs, adipose tissue being more aggressive in this function. Storage is not only necessary for the provision of vitamin D during times of deprivation, but may also help to prevent vitamin D toxicity and to avoid great variations in the plasma vitamin levels.

$1,25(OH)_2D$ functions as a hypercalcaemic agent in concert with PTH and calcitonin to regulate bone mineral metabolism. In prolonged states of high calcium requirements such as growth, pregnancy and lactation, $1,25(OH)_2D$ functions with PTH to enhance renal calcium conservation and bone resorption, but is also able to increase intestinal calcium absorption, thus sparing the mineral content of the skeleton. $1,25(OH)_2D$ also plays a minor part in homeostasis of phosphorus but other factors, such as the amount of this mineral taken in the diet and its renal control are of much greater significance.

1.16 Reason for undertaking of this study.

Although considerable advances have been made in our understanding of vitamin D metabolism and actions over the past thirty years, there is still little information on the changes in vitamin D metabolite profiles in tissues and blood in animals maintained on diets deficient or replete in vitamin D.

The studies reported in this dissertation address this question and provide some information on the changes that occur in the chicken with changing vitamin D_3 intakes.

Chapter 2

Aims and methods.

2.1 Aims of study.

The study was designed to establish 1) the physiological daily vitamin D_3 requirements by comparing plasma 25(OH)D_3 levels to normal reference concentrations and 2) the profile of vitamin D_3 and its main metabolites and their distribution in various tissues in both vitamin D deficient and replete chickens.

In order to fulfil these aims it would be necessary to rear chicks under rachitic conditions and to administer varying vitamin D_3 dosages, ranging from small to large, to groups of chicks in order to determine the plasma 25(OH)D_3 levels in each group. These levels would then be compared to plasma 25(OH)D_3 concentrations obtained from chicks reared normally and exposed to sunlight and receiving normal feeds. From these comparisons it would be able to determine which group received a dose of vitamin D_3 with plasma 25(OH)D_3 levels equivalent to that of normal chicks and so deduce the daily amount of vitamin D_3 required for normal vitamin D_3 metabolism.

Having established the daily requirements of vitamin D_3 in the chick to prevent a deficiency state, it would be possible to study the distribution of vitamin D_3 and its metabolites in plasma and selected organs. These profiles would be studied after the administration of physiological, deficient and supraphysiological doses of the vitamin.

2.2 Methods.

2.2.1 Competitive protein-binding assay for measurement of plasma 25-hydroxyvitamin D_3.

Plasma 25(OH)D_3 concentrations were measured by a competitive protein-binding technique using the method described by Haddad and Chyu (1971). The source of the specific binding protein was the cytosolic fraction of rat kidney.

2.2.1.1 Materials.

25-(26,27 ^{3}H) hydroxycholecalciferol (157Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. and stored in ethanol at 4°C.

Unlabelled 25-hydroxycholecalciferol (gift from Upjohn, Kalamazoo) was stored as a stock solution at -20°C for up to 3 years.

Sodium phosphate buffer was made by adding $Na_2HPO.2H_2O$ (Saarchem) (0.05M/l) to $NaH_2PO.H_2O$(Saarchem) (0.05M/l) in a ratio of 4 parts to 1 part, to achieve pH 7.4. The buffer was stored in a brown bottle in the refrigerator.

Rat kidney cytosol which was to be used as the 25(OH)D ligand, was prepared by the method described by Haddad and Chyu (1971). The kidneys were obtained from adult Sprague Dawley vitamin D replete rats. After excision, the kidney capsule was removed and 10-15g of the organ minced and homogenized in a Polytron in 40ml ice cold sodium phosphate buffer (pH 7.4), using short bursts at 1500 rpm for 2 minutes. The sample was centrifuged at 3000 rpm for 10 minutes at 4°C, in a Beckman bench top

TJ 6R centrifuge. The supernatant was decanted and retained, while the pellet was resuspended with a second amount of 40 ml sodium phosphate buffer, homogenised and centrifuged as before. The two supernatants were combined, buffer being added to produce a final weight of the original kidney tissue : volume ratio of 1:10. This mixture was centrifuged at 18000rpm for 90 minutes at 4°C in a Beckman ultracentrifuge (HA-20.1 rotor; J2-21). The final supernatant was carefully filtered through gauze to filter out any lipid and later lyophylised. The ligand preparation was performed on ice. The kidney cytosol was reconstituted by adding 1 ml sodium phosphate buffer to 0.4 mg lyophylised receptor when the assay was performed.

Dextran-coated charcoal was prepared under constant magnetic stirring. Phosphate buffer (100ml)(refer 2.2.1.1) was added to produce a final concentration of 0.625% charcoal (radio-immunoassay grade, Schwartzmann)/ 0.0625% T60 dextran (Sigma).

2.2.1.2 Methods.

The extracted samples to be assayed and which had been stored at -20°C, were thawed and after removing the hexane by evaporation, were concentrated down by washing the sides of the tube with 0.5ml ether and evaporating to dryness on three occasions after which 500 ul ethanol was added to each tube. The mixture was well vortexed and two aliquots of 50 µl each were pipetted into 12x75 mm tubes (Elkay) for the assay. 300 µl of the mixture, acting as a tracer, was removed to monitor recovery during the extraction procedure. It was placed in a miniature scintillation vial and after drying, 5ml toluene based scintillant (Aquagel from Chemlab, Johannesburg, South Africa) was added and the [^{3}H] 25(OH)D_3 counted on a Packard Tri-carb 3255 beta

liquid scintillation system fitted with an Automatic external standardization system for checking counting efficiency.

A stock solution of 25(OH)D_3 at a concentration of approximately 1mg/ml of ethanol was prepared as described in section 3.1.2 and accurately quantitated by scanning on a spectrophotometer over the wavelength range 220 to 300nm. From this stock solution of known concentration, a working solution of 10ng/50µl ethanol was prepared and diluted further to give a series of standards containing 0, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 7.0 ng 25(OH)D_3/50µl ethanol. To determine the non-specific binding in the assay, a separate solution containing 250 ng 25(OH)D_3/50µl ethanol was also constituted. Both the working and non-specific binding solutions were stored in the freezer at -20°C and remade every 4 weeks. During the assay all samples were stored on ice.

Aliquots of 50µl from the standards, non-specific binding solutions and extracted samples in duplicate were pipetted into 5ml tapered polypropylene tubes (Elkay Scientific Cat. No.0002080/001). Duplicate tubes for the "blank" determination received 50µl ethanol. Approximately 6000 cpm of [^{3}H] 25(OH)D_3 in 50µl ethanol was added into each tube, and directly into 3 scintillation vials to monitor the total counts added. The tubes were vortexed and allowed to stand for 30 minutes. One millilitre reconstituted kidney cytosol (0.4mg/ml phosphate buffer refer 2.2.1.1) was pipetted into each of the tubes except the "blank" tubes to which 1ml cold buffer was added. All the preparations were gently vortexed and kept on ice at 4°C for 2 hours. The charcoal/dextran suspension which had been mixing for at least 30 minutes in a the refrigerator beforehand, was added (150 µl) to each polypropylene tube, to separate the bound from the free [^{3}H]-25(OH)D_3. The tube was then vigorously vortexed and kept on ice for a further 18 minutes. The tubes were centrifuged in a Beckman TJ-6 at 3000 rpm

for 15 minutes at 2°C. An aliquot (500 µl) of supernatant fluid was pipetted off, and placed in a miniature counting vial. After the addition of 5ml scintillant (Aquagel; Chemlab, South Africa) to each vial, which was mixed by shaking, counting was carried out for 5 minutes in a Packard Tricarb 3255 beta liquid scintillation system.

A standard curve was constructed on semi-logarithmic paper by plotting the percentage binding of the total [^{3}H] 25(OH)D_3 added to each tube (refer section 2.2.1.3) versus the 25(OH)D_3 standards in nanograms/50µl ethanol (figure 8).

The concentration of 25(OH)D_3 in the unknown samples was read off the standard curve having determined the percentage binding of that specimen. The final concentration of 25(OH)D_3 was calculated, allowing for the percentage recovery of each specimen and the dilution factor (refer 2.2.1.3).

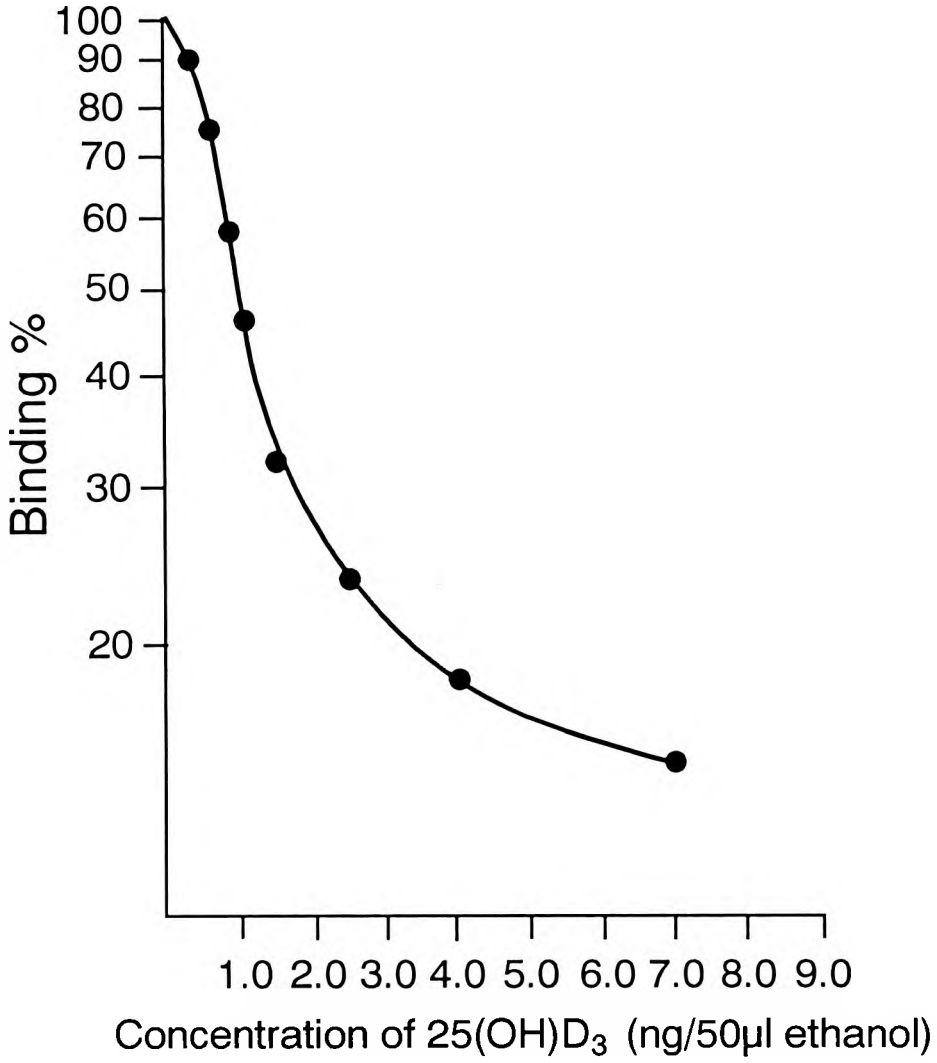


Figure 8.

A typical standard curve for the measurement of $25(OH)D_3$ in chicken plasma samples.

2.2.1.3 Calculations involved in determining results of $25(OH)D_3$ assay.

1. Percentage binding of each standard point or unknown sample:

([{(A + B) ÷ 2} - C] ÷ D) x 100

= % binding of each sample.

2. Recovery of added counts at the start of the extraction:

$$\frac{\text{cpm/300µl aliquot x dilution of original serum vol x 100}}{\text{total cpm added to serum sample}}$$

= % recovery.

3. Concentration of $25(OH)D_3$ in ng/ml of serum

$$[\text{ng of } 25(OH)D_3 \text{ off graph}^{\bullet} \times \frac{\text{dilution (vol of serum)}}{1 \text{ ml}} \times 100] \div \% \text{ recovery}$$

= ng $25(OH)D_3$/ml serum.

•Determined from percentage binding on standard curve.

Notes:

A,B = cpm of duplicates of unknown or standard (500µl).
C = cpm of "*blank*"
D = cpm of "*zero*" point - C

"Blank" Average counts in tubes containing 1ml buffer.

"*Zero*" Average counts in tubes containing only 1ml buffer + 1ml rat kidney cytosol (i.e. maximal binding)

In all the $25(OH)D_3$ assays performed, two control sera were included. One was the pooled sera from chicks reared under rachitogenic conditions, and the other was pooled sera from normal staff members. The concentration of $25(OH)D_3$ was recorded as ng/ml of plasma.

2.3 Validation of $25(OH)D_3$ assay.

A typical $25(OH)D_3$ binding curve is shown in figure 7. The zero point of the curve representing the cpm absorbed without the addition of $25(OH)D_3$ was 21.5 ± 0.4 (n= 5) percent of the total counts added, indicating the amount bound by the ligand.

In our laboratory the validation of the competitive protein binding assay was done by assaying the 25(OH)D concentration in 5 ml of pooled human serum by UV absorbance after HPLC chromatography. The 5ml sample was extracted as described in the method of Bligh and Dyer (refer section 2.4.2). The lipid extract was chromatographed on Sephadex LH-20, the eluate was evaporated to dryness then redissolved in 6 percent isopropanol/hexane and chromatographed by HPLC. The absorbance was measured at 264nm and the values of $25(OH)D_3$ calculated as ng/ml of serum.

The concentration of 25(OH)D in the pooled human serum when measured by HPLC was 19.4 ng/ml (n=1). This result compares favourably with the results obtained with the method of competitive protein-binding assay (22.5 ± 3.5 ng/ml n= 9).

The percentage binding observed in the vitamin D_3 deplete chicks varied between 94 and 107 percent, indicating very low levels of $25(OH)D_3$, < 4 ng/ml. As these levels could not be differentiated from zero on the standard curve, they are recorded as < 0.2

ng/tube or < 4ng/ml of plasma when 0.5ml of plasma was extracted.

Extraction efficiency, evaluated by determining the percent recovery of[^{3}H]-25(OH)D, added prior to the extraction procedure was 73.4 ± 2.2% (n= 5).

The intra-assay CV for 25(OH)D is ± 10%.

2.4 Extraction of specimens for determination of vitamin D_3 metabolites.

2.4.1 Plasma extraction prior to silicic acid chromatography.

This method was used in the study to determine plasma 25(OH)D_3 levels in the chick.

A 2ml plasma aliquot was added to a pyrex tube (16x125mm) fitted with screw cap. The volume of plasma was adjusted to 2ml by adding normal saline where necessary. A tracer consisting of 1000cpm [^{3}H]-25-(26,27) hydroxycholecalciferol (160Ci/mmol) was added to each sample in order to measure recovery after extraction. The sample was vortexed, allowed to stand for 20 minutes, after which 400µl heparin (5000 IU/ml) (Glaxo-Allenbury) was added and the mixture vortexed. Manganese chloride (250 µl) at a concentration of 1.0 M was added to the sample and vortexed and then allowed to stand for a further 15 minutes. Heparin and manganese chloride were added to the sample to separate the bound vitamin D_3 from the unbound vitamin D_3 by precipitation of the lipoprotein fraction (Norman, 1979). Freshly redistilled ether (9 ml) was added to the tube and mixed by rotation on an orbital mixer for 20 minutes. The tubes were then centrifuged at 3000 rpm in a Beckman TJ-6 for 15 minutes at 2°C. The supernatants from each, were removed with a pasteur pipette into a clean tube. Re-

extraction of the pellet with ether was repeated twice more, the supernatents were combined and evaporated to dryness under a stream of nitrogen in a waterbath at a temperature of 35°C. The combined lipid extracts of each sample were concentrated by washing the sides of the tubes three times, each with 0.5ml ether after the latter had been evaporated to dryness on each occasion. Finally the lipid extract was redissolved in 1 ml hexane and stored at -20°C until analysed later.

2.4.2 Extraction of tissues by method of Bligh and Dyer.

The extraction of plasma and tissues obtained from both the preliminary and final studies when the chicks received [^{3}H] vitamin D_3, was by the method of Bligh and Dyer (1959) using 50ml polypropylene tubes fitted with a screw top. The water content of each tissue sample was assumed to be as follows: for mucosa, muscle, kidney and liver 80 percent, plasma 92 percent and fat, water free.

To approximately 1g of wet tissue, methanol, chloroform and water (with fat) were added to produce a ratio of 2:1:0.8. The tissue was then homogenised using a Ika-Werk Ultra Turrex homogeniser fitted with a 18N shaft resulting in a single phase mixture.

After homogenation additional chloroform and water were added to produce a final ratio of methanol: chloroform: water of 2:2:1.8. Further homogenization (3 bursts of 15 seconds each) resulted in a phase separation into an upper methanolic layer containing the water soluble compounds and lower chloroform zone containing the hydrophobic lipids (table 6). Separation of the phases was enhanced by centrifugation of the mixture for 20 minutes at 3000rpm at 2°C in a Beckman TJ-6.

Table 6. The volume of solvents and water added per gram or ml of tissue during the Bligh and Dyer extraction.

	INITIAL(ml)			SUBSEQUENT(ml)	
TISSUE	Methanol	Chloroform	Water	Chloroform	Water
Liver, kidney, duodenal mucosa, muscle.	2.0	1.0	0.0	1.0	1.0
Plasma	2.3	1.15	0.0	1.15	1.15
Fat	2.0	1.0	0.8	1.0	1.0

Initial Ratio : Methanol : Chloroform : Water 2 : 1 : 0.8; Final ratio : 2 : 2 : 1.8

The upper methanol-water layer was aspirated and retained while the chloroform layer under the interface was also removed by aspiration and stored separately. The aspirated upper phase was returned to its original tube, and rehomogenized with additional chloroform, the amount being equal to the volume previously removed. After centrifugation the chloroform layer was removed and combined with the original lipid phase and then dried in a waterbath under nitrogen. On complete evaporation, 1ml hexane was added to each sample and the sample stored at -20°C.

The methanolic layer was discarded, as in two separate experiments using liver and kidney no radioactive vitamin D metabolites were detected in this fraction.

2.5 Silicic acid chromatography for the separation of vitamin D_3 metabolites.

2.5.1 Preparation of silicic acid for chromatography.

All solvents (supplied by Halpro SA) were redistilled in glass prior to being utilised.

Batches of 100g silicic acid (BIO-RAD HA minus 325 mesh) were washed with redistilled ether. The bulk of the material was allowed to settle and the supernatant was removed using an extractor pump. Following five such washings, the silicic acid was allowed to dry in room air for two hours, then dried overnight with gentle heating above the laboratory oven set at 60°C. The following day the powder was activated in an oven at 105°C for 1 hour, after which it was cooled and stored in a dry brown bottle with a quick fit glass top until required. At this stage redistilled hexane was added to form a slurry which was allowed to stand for at least 24 hours before use. The suspension was stored in this form at room temperature for up to two weeks. With each fresh batch, the elution of the various vitamin D metabolites was checked using known amounts of authentic radioactive vitamin D metabolites.

2.5.2 Silicic acid chromatography used to separate 25(OH)D_3 fraction from other metabolites.

Pasteur pipettes (14.5 x 0.4cm) were gently plugged with a square (0.5cm) of folded surgical gauze and a few threads of cotton wool. The columns were packed by adding

a slurry of silicic acid and ether-hexane mixture (20:80 v/v) until a column of silicic acid 4.0 x 0.4 cm was established. The columns were washed with 5ml ether-hexane (20:80 v/v) prior to the addition of the sample. The extracted lipids from the various tissues (refer section 2.4.2) which were stored in hexane, were allowed to reach room temperature and blown down to dryness under a stream of nitrogen. The extracts of the samples were concentrated down as described in section 2.4.1. Finally 1 ml of ether-hexane (20:80 v/v) was added to each sample, vortexed and pipetted onto the column. The sample tube was rinsed with 0.5ml ether-hexane mixture (20:80 v/v) and finally with 1ml ether-hexane of a similar mixture, each being added to the column. Elution was continued by sequentially washing the columns with 5ml ether-hexane (55:45 v/v) and 5ml ether-hexane (95:5 v/v)(figure 9). This last eluate containing the 25(OH)D fraction, was evaporated to dryness under nitrogen, concentrated by three washings of the tube each with 0.5ml ether and dried under nitrogen. The final elution of the column was the addition of 5ml ether-acetone (50:50 v/v) (figure 9). If the $25(OH)D_3$ assay was performed immediately, 500ul absolute ethanol was added to the dried eluate but if the assay was performed the following day, the extracted $25(OH)D_3$ was solubilized in 1 ml of hexane and stored at -20°C.

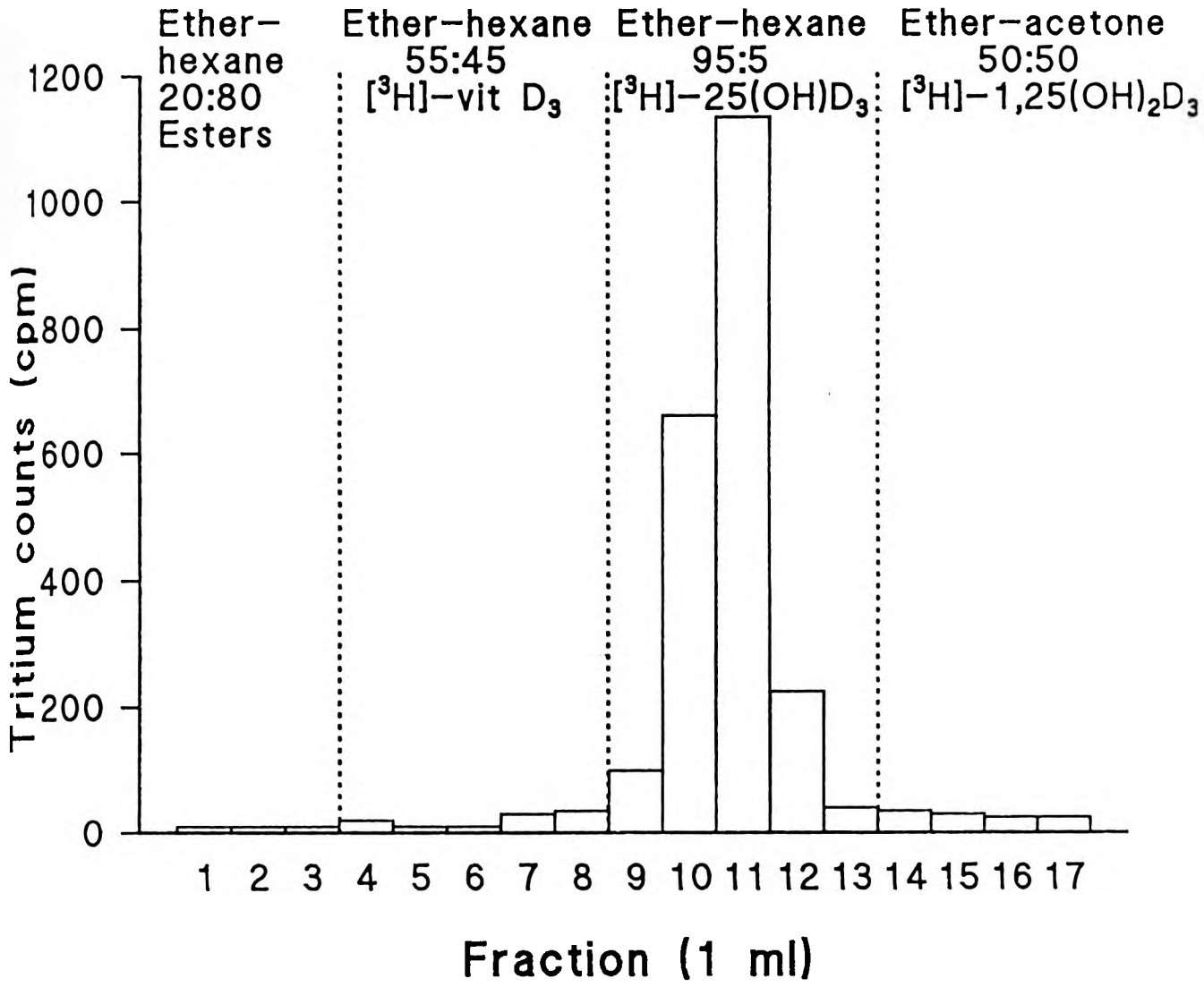


Figure 9.

Silicic acid chromatography for elution of 25(OH)D_3 (ether-hexane 95:5 v/v).

2.5.3 25-Hydroxyvitamin D_3 losses during extraction and chromatography.

Assessment of $25(OH)D_3$ losses during extraction and chromatography was evaluated by determining the recovery of a known quantity of added [^{3}H] $25(OH)D_3$ either to the plasma sample before extraction, or directly to the liquid extract immediately prior to loading onto the silicic acid column as already described in section 2.5.2. The fraction containing $25(OH)D_3$ which eluted off the column with 5ml ether-hexane (95:5 v/v) was collected in its entirety directly into a macro-polyethylene scintillation counting vial. The contents were evaporated to dryness under nitrogen, 10ml Aquagel was added to the vial and the radio-activity assessed. Recoveries were expressed as a percentage of the counts recorded in the eluates compared to the initial counts added to the plasma.

Three serum samples were spiked with approximately 11000 cpm [^{3}H] $25(OH)D_3$ prior to extraction and chromatography. After extraction and chromatography the ether-hexane (95:5 v/v) fraction contained 91.2 ± 4.1 percent of the added cpm. Less than three percent of the added [^{3}H] $25(OH)D_3$ was recorded in ether-acetone fraction (50:50 v/v) [table 7(i)]. Losses during chromatography were small, with 97.7 ± 0.9 percent [^{3}H]-$25(OH)D_3$ added prior to chromatography being recovered in the $25(OH)D_3$ fraction [table 7(ii)].

Table 7(i). Recovery of [^{3}H]-25(OH)D_3 added to plasma prior to extraction and chromatography.

[^{3}H]-25(OH)D_3 ADDED BEFORE EXTRACTION					
SAMPLE (cpm)	E-H* 20:80 (cpm)	E-H* 55:45 (cpm)	E-H 95:5* (cpm)	E-Ac 50:50 (cpm)	Recovery from E-H 95:5 %
A (9474)	0	0	8695.8	174	91.7
B (11991)	0	21	10239	303	85.9
C (13426)	0	8.0	13005	295	96.0
				Recovery (Mean ± SD)	91.2 ±4.1

Table 7(ii). Recovery of [^{3}H]-25(OH)D_3 added to lipid extract prior to chromatography.

[^{3}H]-25(OH)D_3 ADDED BEFORE CHROMATOGRAPHY					
SAMPLE (cpm)	E-H* 20:80 (cpm)	E-H* 55:45 (cpm)	E-H* 95:5 (cpm)	E-Ac# 50:50 (cpm)	Recovery from E-H 95:5 %
A (9474)	0	6	9437	14	99.0
B (11991)	5	1	11647	70	97.0
C (13426)	0	9	13047	44	97.0
				Recovery (Mean ± SD).	97.7 ±0.9

E-H* Ether-hexane. E-Ac# Ether-acetone.

2.5.4 Silicic acid column chromatography in the preliminary study.

To obtain a more complete profile of the vitamin D metabolites formed after the administration of vitamin D_3 to chicks in the preliminary study, silicic acid chromatography was carried out using a system similar to that described in section 2.5.2,

except that the solvent mixtures used were altered to separate the vitamin D_3 metabolites more completely. The solvent mixtures used are shown in table 8, and were added consecutively onto the columns after loading and packing with silicic acid. Consecutive fractions were collected separately into scintillation vials, blown down to dryness under a stream of nitrogen and counted after the addition of 5 ml Aquagel. Figure 10 shows the separation of the vitamin D metabolites after being added to plasma. Clear separation of the added metabolites was obtained.

Table 8. Solvent system used in preliminary study.

Volume ml	Solvents	Eluate
3	Ether-hexane(20:80v/v)	$PtVitD_3$ Esters
8	Ether-hexane(55:45v/v)	Vitamin D_3
8	Ether- hexane(70:30v/v)	$25(OH)D_3$
12	Ether- hexane(95:5 v/v)	$24,25(OH)_2D_3$
6	Ether-acetone(50:50v/v)	$1,25(OH)_2D_3$
3	Methanol	Polar metabolites

$PtVitD_3$ Esters Putative vitamin D_3 esters

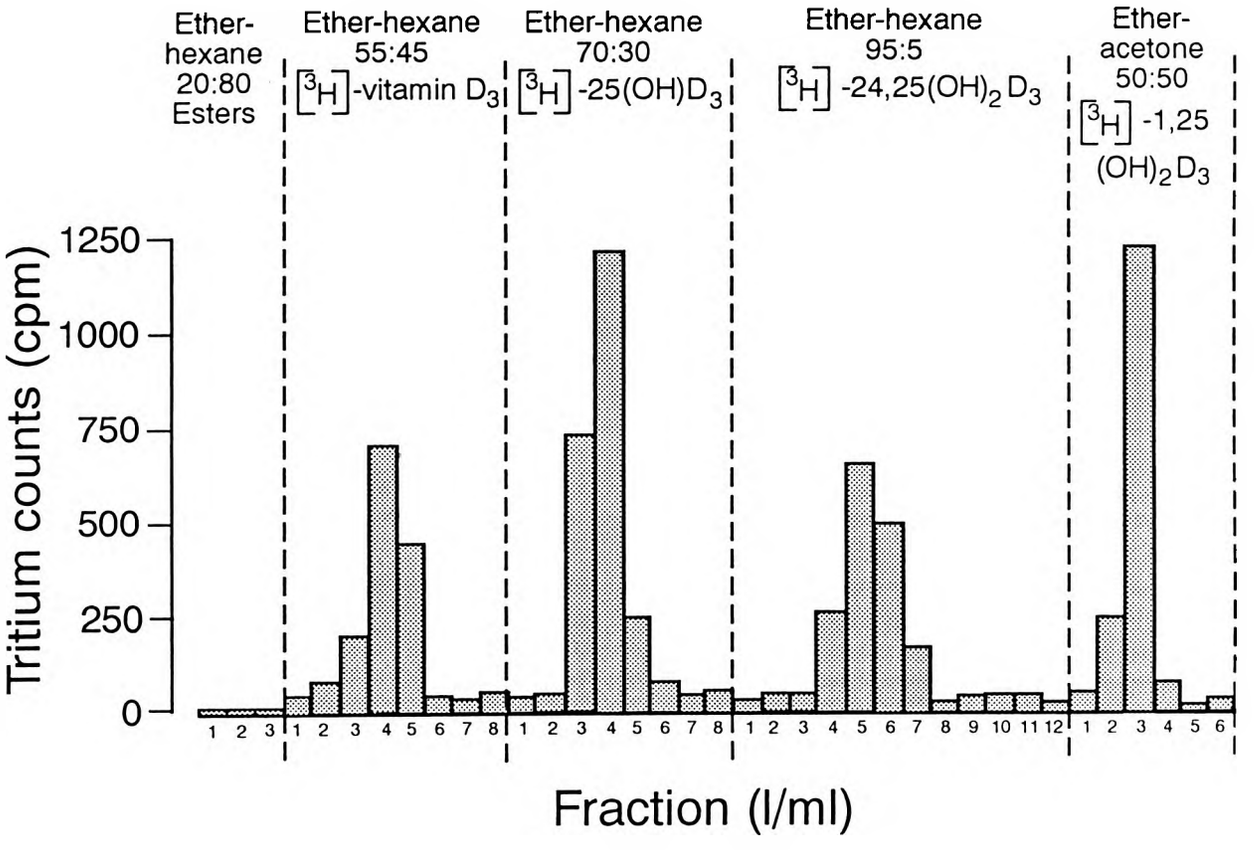


Figure 10.

Preliminary study. Silicic acid chromatography for elution of vitamin D_3 and the main metabolites.

2.5.5 Silicic acid chromatography in the definitive study.

Certain modifications in silicic acid chromatography procedure were made for separation of metabolites in the definitive study, as silicic acid chromatography was used only as a preliminary procedure to remove excessive lipid. Vitamin D_3 and its metabolites eluted, were later separated by high performance liquid chromatography (HPLC).

As obstruction to the flow of solvents through the columns prepared from pipette tubes was encountered in the preliminary study, especially when loaded with the lipid extracts from tissues containing a high content of lipid, a new column was constructed 140 mm in length with the diameter increased to 8 mm (compared to 4mm of the pasteur pipette). A sintered glass disc was inserted to prevent the escape of silicic acid from the column.

As described previously (refer section 2.5.2) during the packing of the column with silicic acid (40 x 8 mm), a mixture of ether-hexane (20:80 v/v) was perfused until the required height was obtained. For the elution of putative vitamin D_3 esters, vitamin D_3 and 25(OH)D_3 the solvents used are stated in table 9. The dihydroxyvitamin D_3 metabolites were eluted with a mixture of ether-acetone (50:50 v/v), and the column was stripped using methanol (table 9).

As described in section 2.5.2 all the eluates were collected and evaporated to dryness under a stream of nitrogen. If stored at -20°C, 1ml of hexane was added to each sample.

Table 9. Solvent systems applied to silicic acid column in definitive study.

Volume ml	Solvents.	Eluate.
3	Ether-hexane(20:80v/v)	$PtVitD_3$ Esters
8	Ether-hexane(55:45v/v)	Vitamin D_3
8	Ether- hexane(75:25v/v)	$25(OH)D_3$
12	Ether-acetone(50:50v/v)	$24,25(OH)_2D_3$ $1,25(OH)_2D_3$
3	Methanol	Polar metabolites

$PtVitD_3$ Esters Putative vitamin D_3 esters

2.6 Isocratic high performance liquid chromatography of partially purified liquid extracts.

High performance liquid chromatography (HPLC) (Koenigberger) was carried out using a LDC/Milton Roy Constamatic II metering pump coupled to a Universal Liquid Chromatograph injector Model 6 K fitted with a 2ml injector loop (Waters Associates). The injector was connected to a Spherisorb Phase Sep HPLC column S5W 250x4.6mm packed with a Spherical Silica 5µ particle size, fitted with a Brownlee Guard Cartridge 30x4.6mm packed with 5µ spherical silica held in a Brownlee Cartridge holder. Mass detection of metabolites flowing from the column was performed using a LKB 2158 Uvicord detector fitted with a 8 µl x 1 cm path flow cell and a UV filter at 254nm. The detector was coupled to a LKB 2210 recorder, set at a full sensitivity deflection of 100 mV with the chart speed set at 5mm per minute and the flow rate regulated at 2ml per minute. All solvents utilized were of HPLC grade (Rathburn Chemicals).

2.6.1 Further purification of vitamin D_3, $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$.

All HPLC investigations performed in the preliminary study were carried out using an isocratic solvent system. Before submitting the samples obtained from plasma and the various tissues for HPLC, extraction was performed by the method of Bligh and Dyer (1959)(refer section 2.4.2) and then submitted for silicic acid chromatography to eradicate extraneous metabolites. Thus the samples were purified as much as possible before being subjected to HPLC (2.6).

The concentrations of the solvents, isopropanol and hexane, used for the isolation of the metabolites (HPLC) in the preliminary study, are as shown in table 10.

Table 10. Solvent systems used in HPLC in preliminary study.

Metabolite.	Isopropanol-hexane ratios.
Vitamin D_3	1:99v/v
25-OHD_3	4:99v/v
$24,25(OH)_2D_3$	6:94v/v
$1,25(OH)_2D_3$	8:92v/v

2.6.2 Quantitation of recovery of vitamin D_3 and metabolites during extraction and chromatography.

Recovery of vitamin D_3 and its metabolites during extraction and chromatography was assessed by adding known amounts of authentic unlabelled vitamin D_3, $25(OH)D_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$ to the plasma or tissues prior to extraction.

After extraction and chromatography (both silicic acid and HPLC), the amount

of the specific vitamin D_3 and its metabolites recovered, was determined by calculating the area under the peak of the UV tracing by the method of triangulation, and comparing it to a standard curve constructed by adding known amounts of the specific metabolite directly to the HPLC system (figures 11a and 11b). The calculated mass of the different radioactive metabolites obtained after extraction and chromatography of plasma and the various tissues, was corrected for losses which occurred during the procedure, as assessed by the recoveries obtained from the method described above. Figures 11a) and 11b) illustrate quantitations for authentic vitamin D_3 and $1,25(OH)_2D_3$. Similar results were obtained when quantitations for $25(OH)D_3$ and $24,25(OH)_2D_3$ were performed.

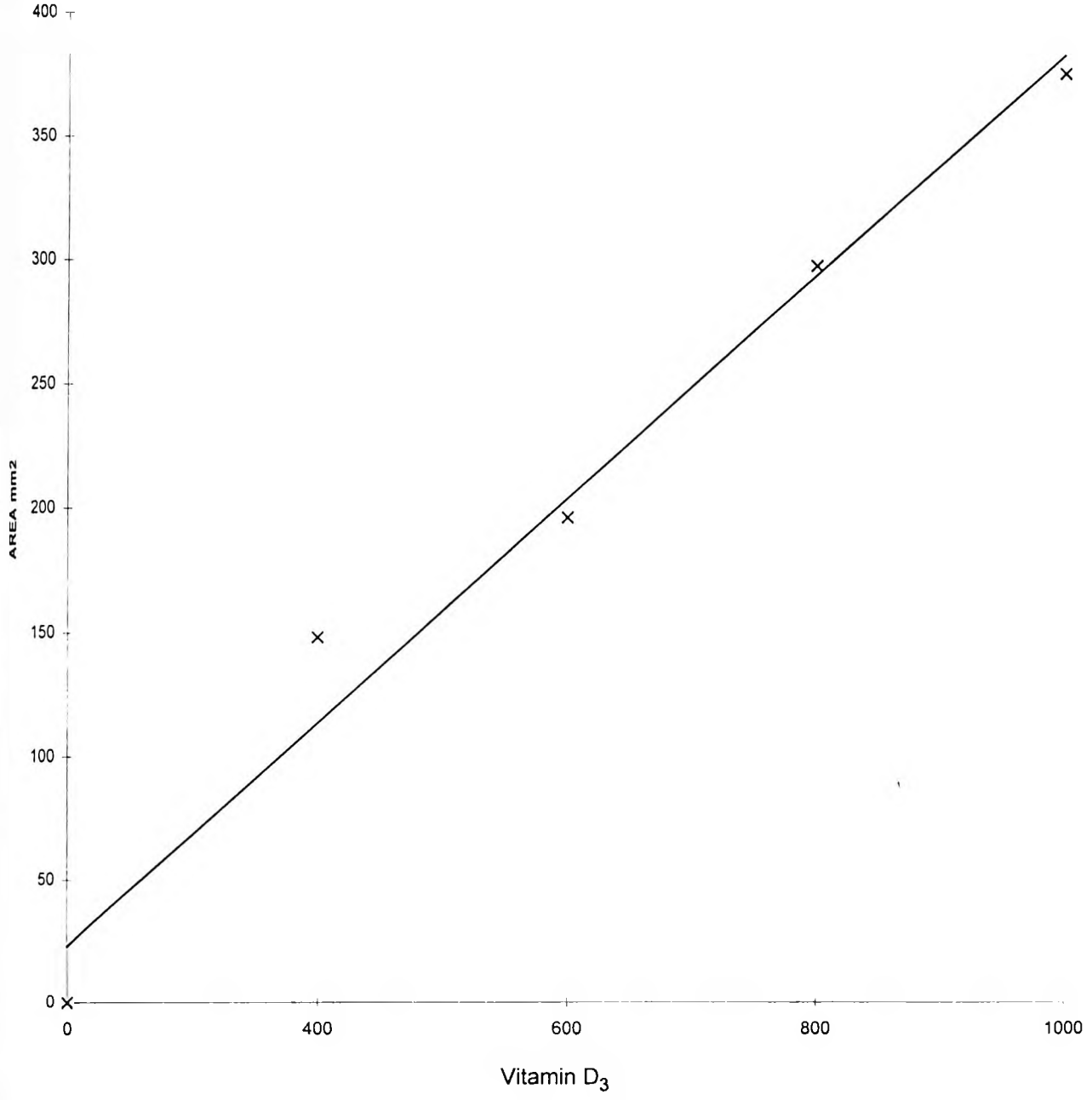


Figure 11a).
Quantitation of authentic vitamin D3.

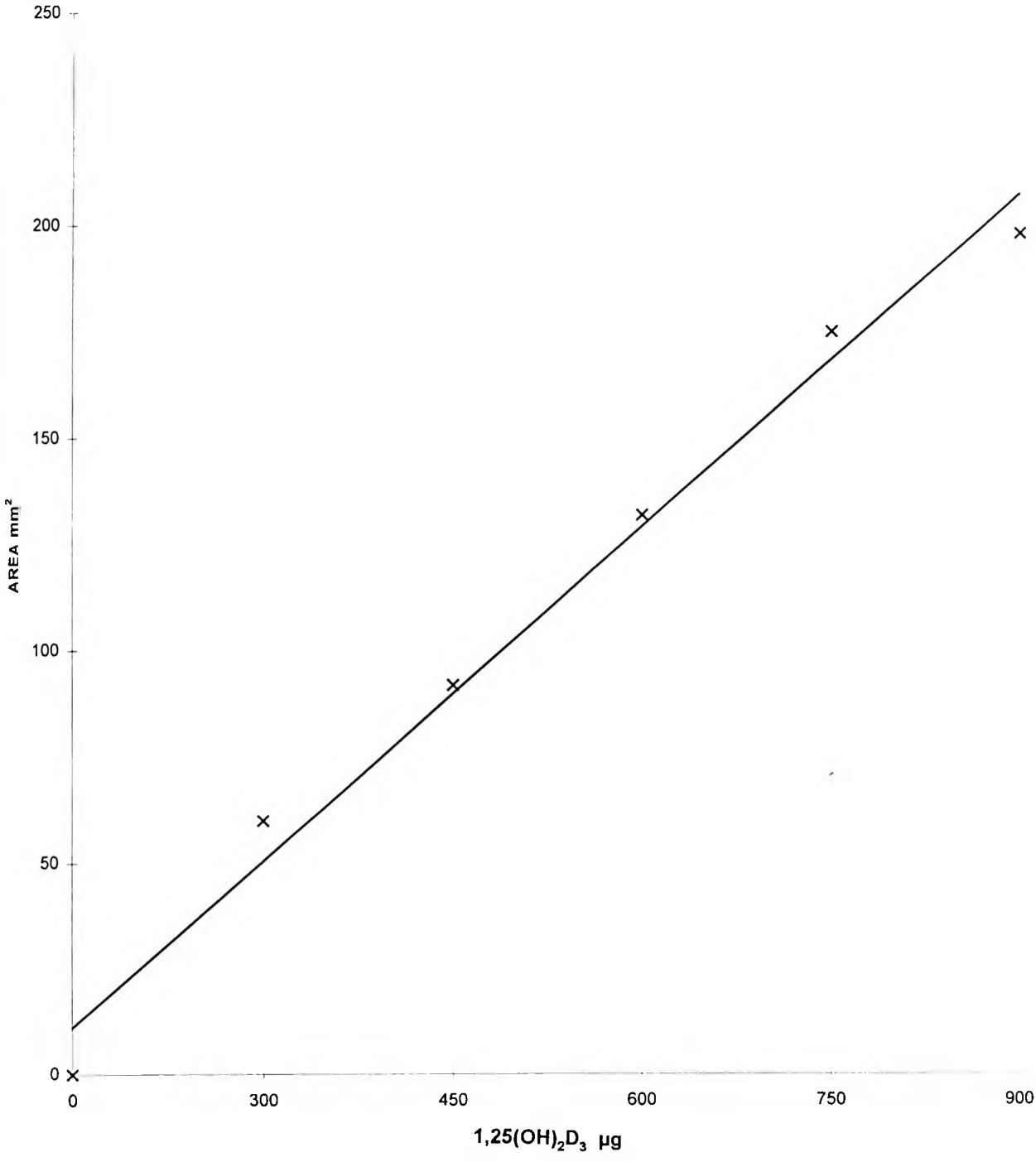


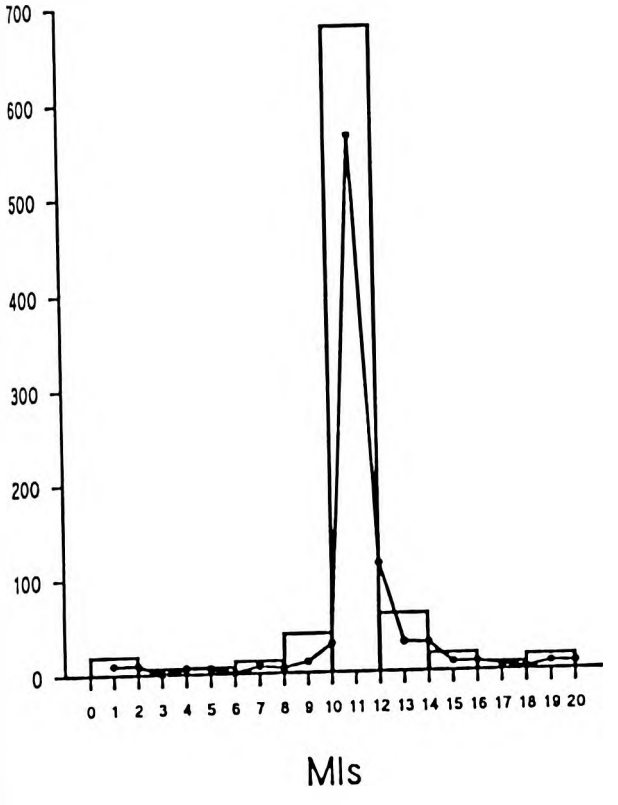
Figure 11b).
Quantitation of authentic $1,25(OH)_2D_3$.

2.6.3 Concordance of unlabelled and radio labelled metabolites

The concordance of the unlabelled and radio labelled metabolites was verified by simultaneously injecting a mixture of unlabelled and radio-labelled metabolites onto the HPLC column. The unlabelled metabolites were detected by UV absorption at 254nm while the [^{3}H]-metabolites were quantitated by liquid scintillation counting. The consecutive 1 ml fractions off the column in the region where the metabolites were expected to elute, were collected and dried.

Figures 12(a) and (b) depict the elution of unlabelled 25(OH)D_3 and [^{3}H]-25(OH)D_3 on the HPLC system used. The unlabelled metabolite was detected by UV absorbance as shown in figure 12 (a). The UV absorbance peak appeared at the end of the 5th minute after the injection of the sample onto the column, which was synchronous with the end of the 10th ml of eluate. The corresponding radioactive metabolite elution is shown in figure 12 (b) which indicates that the peak of radio-activity appeared between the end of the 5th and 6th minute during elution of the 11th and 12th ml collected in the 11th and 12th vials.

(a)



Numbers (0–20) equals Eluate in mls.
Each bar represents 1 minute

(b)

Vial number	Time (minutes)	Eluate (mls)	dpm
1		1st	9.36
2	1	2nd	9.39
3		3rd	0.34
4	2	4th	6.21
5		5th	5.91
6	3	6th	1.05
7		7th	7.34
8	4	8th	5.61
9		9th	11.12
10	5	10th	29.30
11		11th	564.18
12	6	12th	113.51
13		13th	30.52
14	7	14th	29.60
15		15th	9.04
16	8	16th	9.22
17		17th	5.53
18	9	18th	2.66
19		19th	8.08
20	10	20th	7.66

Pump: Rate of flow 2 mls/min
Speed of chart: 5 mm/min

Figure 12.
Concordance of unlabelled vitamin D_3 and [^{3}H]-vitamin D_3.
(a) scan of vitamin D_3 (b) dpm counts of [^{3}H]-vitamin D_3.
dpm counts superimposed over scan of same sample.

2.7 High performance liquid chromatography used in the definitive study.

Certain modifications to the above procedure were instituted during the definitive study. Because of interference from unidentified UV peaks during the separation of the various metabolites in the preliminary study, it was difficult to determine the exact peak pertaining to known metabolites. Also the base lines were not always linear making it difficult to determine the area of the peak. It was apparent that some of the quantitative results especially for the dihydroxyvitamin D_3 metabolites recorded high values, perhaps because of inadequate separation of various metabolites.

To improve the detection of vitamin D_3 and its metabolites, increased amounts of unlabelled vitamin D_3 and metabolites were added to the specimens prior to extraction, while the sensitivity of UV detection was also decreased (refer table 11). As mentioned in section 2.5.5 and table 9, both $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were collected in a single eluate off the silicic acid chromatography column, which was subjected to gradient HPLC to separate the two metabolites. Isocratic HPLC was still used for the final determination of vitamin D_3 and $25(OH)D_3$ concentrations, the isopropanol-hexane combinations being 1:99 v/v and 4:96 v/v respectively (refer section 2.6.1; table 10). These last two procedures were performed individually, the samples having been collected separately off the silicic acid column (figure 13).

Table 11. Masses of vitamin D_3 and metabolites added to samples and absorbance range utilized in HPLC in definitive study.

	Unlabelled Mass Added	Absorbance Range
Vitamin D_3	50μg	1.0
25-(OH)D_3	5μg	0.1
24,25$(OH)_2D_3$	4μg	0.05
1,25$(OH)_2D_3$	3μg	0.05

For gradient HPLC a Milton Roy Gradient Master 1601 was utilized, the pumps being Constametric I, IIG and II. One pump was connected to resevoir (A) containing hexane, while the other was connected to reservoir (B), containing isopropanol-hexane(25:75 v/v). A gradient range was obtained from [(A):(B)] 80:20 v/v at the commencement of solvent flow, reaching to [(A):(B)] 45:55 v/v after 10 minutes, the latter concentration being maintained for another 15 minutes. This was equivalent to commencing with a mixture of isopropanol/hexane 5:95 v/v and reaching a concentration of isopropanol/hexane 14:86 v/v. The recorder was set at a full sensitivity deflection of 100mV, and the flow rate maintained at 2ml/min. All other aspects were similar to that described in section 2.6.

A number of 1ml aliquots were collected consecutively in miniature scintillation vials on a Fraction Collector ISCO Model 328 (Instrumentation Specialties Co). Twenty such aliquots were collected during HPLC for vitamin D_3 and also for 25(OH)D_3. For the dihydroxylated metabolites 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$, 50 1ml aliquots were collected. Aquagel (5ml) was added to each vial and radioactivity was assessed on a Packard tri-carb spectrometer (figure 13).

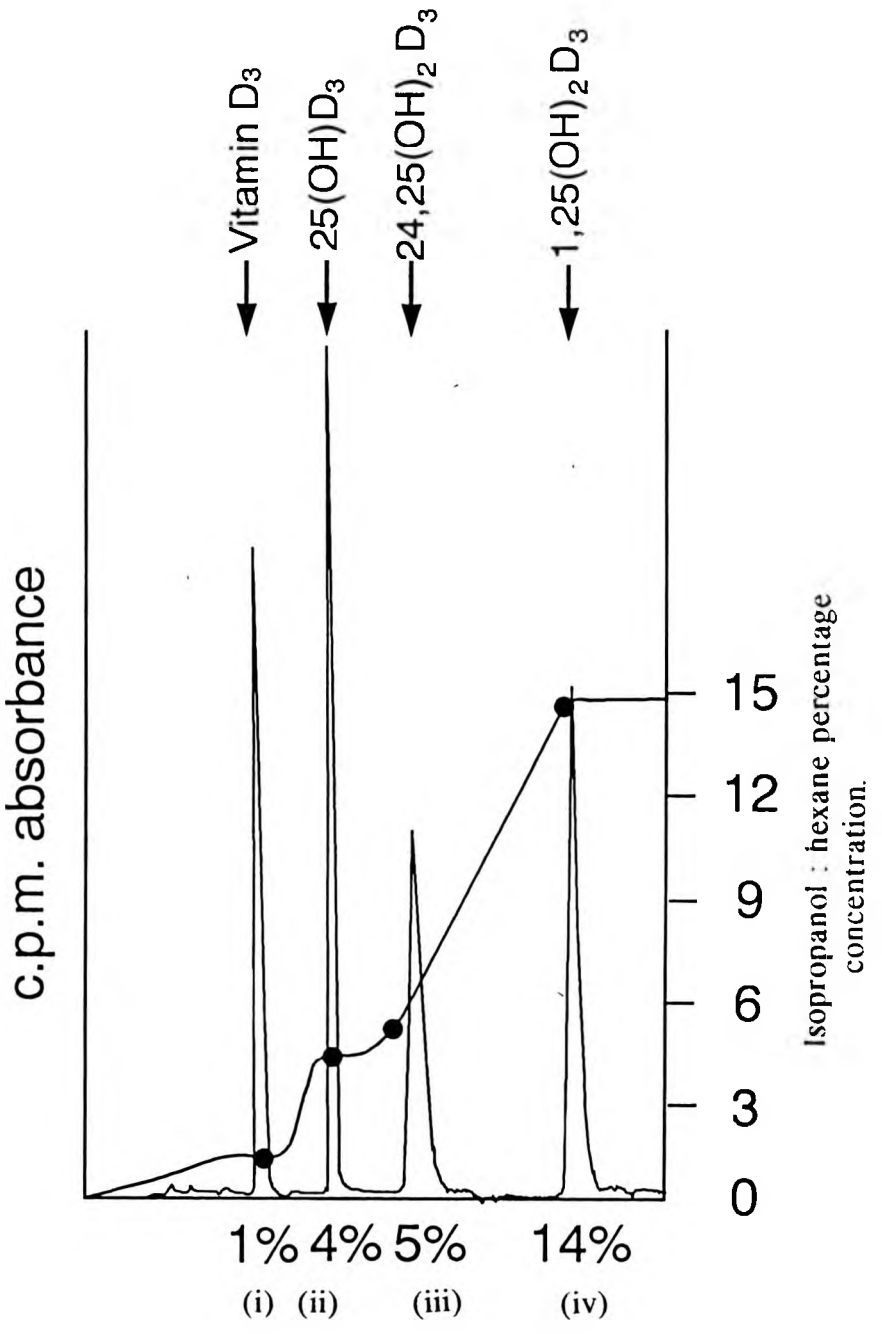


Figure 13.

Definitive study: HPLC of i) vitamin D_3, ii) 25(OH)D_3, iii) 24,25(OH)$_2$$D_3$, iv) 1,25(OH)$_2$$D_3$.

Solvent systems: isopropanol/hexane i) 1:99, ii) 4:96 iii) 5:95, iv) 14:86 v/v.

Isocratic HPLC: vitamin D_3 and 25(OH)D_3.

Gradient HPLC: 24,25(OH)$_2$$D_3$ and 1,25(OH)$_2$$D_3$.

2.8 Quantitation of radioactive specimens.

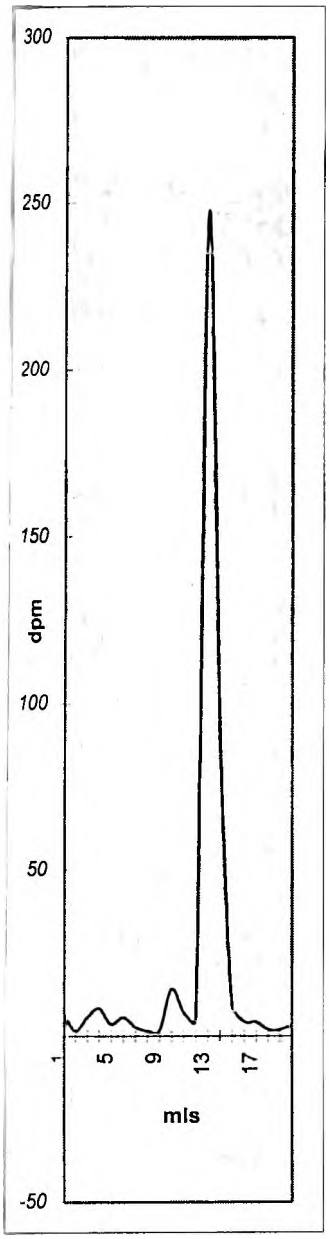
Radioactivity eluting off the column in the elution volume of a known vitamin D metabolite was considered to be significant if the cpm (or dpm) increased more than 15 percent above the background cpm (or dpm), which was determined by averaging the cpm (dpm) in the two fractions immediately preceding and following the elution volume of the specific metabolite (figure 14). The total cpm (dpm) recovered in a particular peak was calculated by subtracting the average background cpm (dpm) from the cpm (dpm) in the relevant fractions and adding the resultant counts together. Counts per minute for cpm were converted to dpm by utilizing a quench correction curve which was calculated monthly.

$$\frac{\text{cpm} \times 100}{\% \text{ efficiency}} = \text{dpm}$$

2.9 Quantitation of vitamin D_3 metabolites in plasma and tissues.

The masses of vitamin D_3 and its metabolites, contained in plasma and tissues, which were extracted and processed, were determined by measuring the radioactivity obtained off the HPLC column at the elution volumes of authentic vitamin D metabolites. The radioactivity contained in plasma or tissue (refer 2.8 and 2.6.2), was corrected for the amount of tissue extracted and the losses incurred during the extraction and chromatography procedures. As the specific activity of the orally administered vitamin D_3 was known, it was possible to convert the cpm in the preliminary study and dpm in the definitive study obtained in a particular fraction to actual mass, which was then coverted to pmol/ml or pmol/g.

(a)



(b)

Sample No.	dpm					
1	6.61					
2	1.70					
3	5.77					
4	8.50					
5	3.75					
6	5.77					
7	2.70					
8	1.70					
9	1.71					
10	14.33					
11	7.13	Average				
12	4.44					
13	246.81	-	5.79	=	241.025	
					+	= 325.6
14	91.76	-	7.18	=	84.585	
15	9.92					
16	4.43	Average				
17	4.40					
18	2.04					
19	2.03					
20	3.41					

Figure 14.

The quantitation of radioactive vitamin D_3 metabolites:

a) UV tracing of authentic vitamin D_3 metabolite,

b) dpm obtained in each fraction eluting off the column.

Sample 13 and 14 correspond to fractions 13 and 14 in figure (a).

2.10 Conversion of dpm to pmol/ml or pmol/g for plasma and tissues.

$$\text{dpm} \times \frac{100}{\%\ \text{recovery}} \times \frac{1}{\text{mass}^{*}} = \text{dpm/ml or g}$$

$$\frac{\text{dpm}}{\text{Sp. Ac.}} = \mu\text{g/ml or g}$$

$$\frac{\mu\text{g}}{\text{Mol wt of metabolite}} = \mu\text{mol/ml or g}$$

$$\mu\text{mol} \times 10^{6} = \text{pmol/ml or g}$$

Chapter 3

Vitamin D_3 requirements for physiological values of plasma 25-hydroxyvitamin D_3 in the chick.

3.1 Aims of study.

Having established the methodology necessary for determining the vitamin D_3 dosage that would produce physiological concentrations of plasma 25 hydroxyvitamin D_3 [25(OH)D_3], it was decided to administer varying doses of vitamin D_3 to vitamin D deficient chicks. The average daily dose of vitamin D_3 required to produce levels of plasma 25(OH)D_3 equivalent to that of normal chicks, would be accepted as being the physiological dose necessary for normal development.

Having established the amount of oral vitamin D necessary to produce physiological concentrations of plasma 25(OH)D, it will be possible to deduce the dosages of the vitamin that would create a deplete or supraphysiological state in the chick. Thus from the available data it will be possible to determine the effect of vitamin D status on the metabolism and distribution of its metabolites in these various states.

3.1.1 Rearing of chicks under rachitic conditions.

Fifty, one day old chicks obtained from Bergvliet, Transvaal were housed in wire cages in the Central Animal Unit of the Faculty of Medicine in an electrically heated brooder at 35°C in a room lit by incandescent light operating on a 12 hour on and 12 hour off cycle. The chicks were fed a semi-synthetic vitamin D_3 free diet (table 12) containing 0.6% calcium and 0.4% phosphorus. The feed and water were available ad libitum.

3.1.2 Vitamin D_3 preparation.

The concentration and purity of vitamin D_3 utilized for dosing the various groups of chicks was prepared as follows:-

A stock solution of vitamin D_3 in absolute alcohol was prepared by adding approximately 1.0 mg cholecalciferol (obtained from Phillips du Pont) to 10ml absolute ethanol, which was then stored at -20°C until examined. The concentration and purity of this solution was assessed before use.

An aliquot from this stock solution (20µl) was diluted 1/100 with ethanol and scanned in the wavelength range from 300 to 200nm, using a Pye Unican 1800 double beam spectrophotometer (Pye Unicam Limited, Cambridge, England), with ethanol as a blank. The maximum absorbance was at 264nm, whilst the nadir was at 228nm. An example of such a scan is shown in figure 15.

The purity of the material was assessed by determining the ratio of absorbance at 264 and 225 nm (λ max: λ min) which should be in the range of approximately 1.8 (Horst 1979).

The absolute concentration of the sample was calculated knowing, 1) the maximum absorbance measured at 264nm, 2) the molar extinction coefficient of vitamin D_3 (18,200) (DeLuca 1984) and 3) molecular weight of vitamin D_3 (384). Using the above data, the concentration of the stock solution was determined as follows:-

maximum absorption at 264nm (max)	0.886
minimum absorption at 228nm (min)	0.466
λ max./λ min.	1.90

Vitamin D_3 concentration = (max.absorption x mol.wt.) ÷ ε^*

= (0.886 x 384) ÷ 18200

= 0.01859g/l

= 18.6 ug/ml (Sample diluted 100 fold).

Concentration of Vitamin D_3 = 1.86mg/ml.

*(molar extinction coefficient=18200)

Dilutions of the stock solution were made by the addition of a mixture consisting of ethanol and propylene glycol (1:2v/v) to produce five different concentrations of vitamin D_3 (ranging from 0.05 to 100µg/250µl) (refer section 3.1.3).

3.1.3 Vitamin D_3 dosages for chickens.

After 17 days on a rachitogenic diet (table 12) the chicks were divided into 6 groups of 5 birds each. The birds in 5 of the groups received varying doses of unlabelled vitamin D_3 on days 17, 20, 23, 26 and 29 whilst the birds in the 6th group only received vehicle (ethanol/propylene 1:2 v/v).

The dosage of vitamin D_3 administered orally by pipette was 0.05µg, 0.1µg, 0.5µg, 1.0µg or 100µg, each dose was dissolved in 250µl ethanol/propylene glycol(1:2 v/v). All the birds were killed by decapitation on day 31.

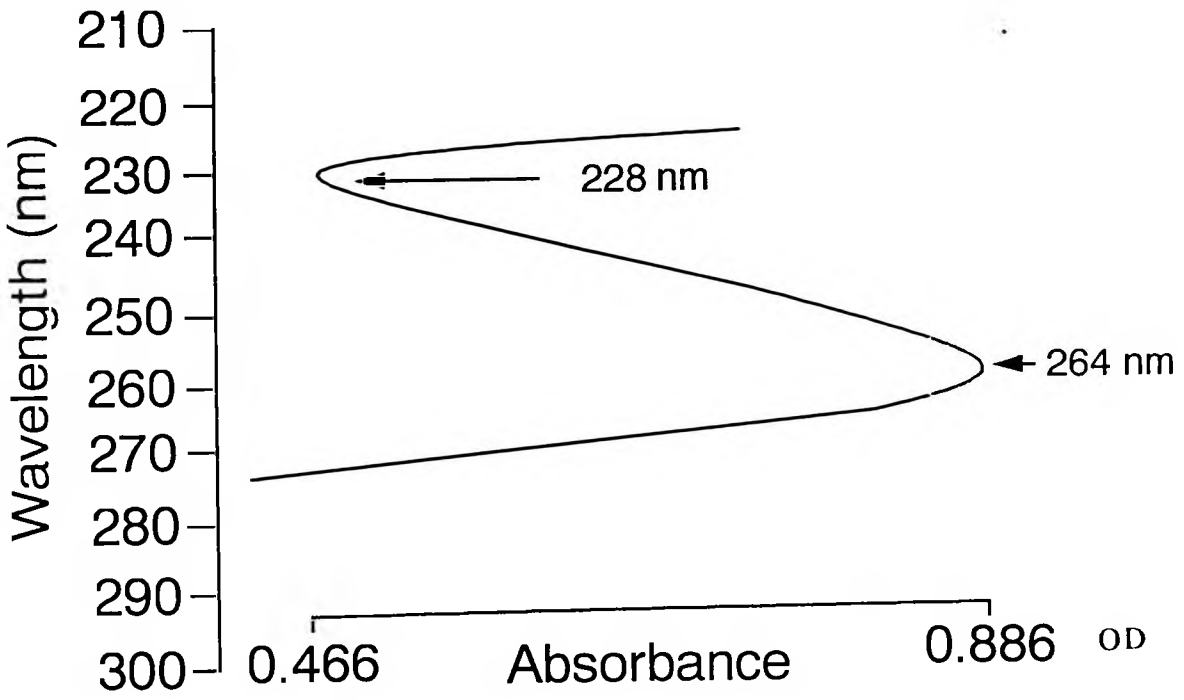


Figure 15.

A typical UV scan of authentic vitamin D_3 using dual beam spectrophotometry over the the wavelength range 300-220 nm. Maximum absorbance occurs at 264nm whilst minimum absorbance occurs at 228nm.

Table 12. Rachitogenic diet.

Maize meal	10.00 Kg
Soya bean	3.336 Kg
$CaCO_3$	0.320 Kg
Na_2HPO_4	0.294 Kg
Alfafa	0.200 Kg
Sodium Chloride	66.40 g
$MnSO_4.4H_2O$	29.60 g
Choline chloride	12.00 g
Vitamin A palmitate	200.00 µl
Na or Ca pentothenate	60.00 mg
Nicotinamide	30.00 mg
Riboflavin	24.00 mg
Vitamin B_{12}	120.00 µg
Vitamin K_2 (methyl 4naphthoquione)	25.00 mg
Sunflower oil	750.0 ml

3.1.4 Collection of samples.

On decapitation, blood was collected in heparinised tubes, allowed to stand for 30 minutes and then centrifuged at 3000rpm for 15 minutes at 4°C. The plasma was then pipetted off and stored at -20°C until required for the various assays. The method used for the measurement of plasma 25(OH)D_3 content, was a competitive-binding technique

described by Haddad and Chyu (1971) (refer section 2.2.1).

Plasma calcium, phosphorus and alkaline phosphatase concentrations were measured by Mr. G.P. Moodley in the Mineral Metabolism Research Unit, Baragwanath Hospital, Johannesburg. The measurement of plasma calcium was performed on a Varian Techtron 1200 by atomic absorption spectrophotometry, after diluting the samples in a lanthanum chloride solution (1% La). Inorganic plasma phosphorus and alkaline phosphatase values were determined by colorimetric methods using a Technicon Auto Analyser II, the former by the method described by Hurst (1967) and Kraml (1966) and the latter value by the modified method of Morgenstern et al (1965).

3.2 Results of study.

3.2.1 25-Hydroxyvitamin D_3 plasma levels.

The results of this study when the different groups of chicks were given repeated doses of vitamin D_3 ranging from 100 to 0.05 μg are shown in table 13. Group 6 in the table received no vitamin D_3.

Samples of plasma $25(OH)D_3$ from chicks of the same age, and on a normal vitamin D containing diet were examined, the level of this metabolite being 23.2 ± 2.7 ng/ml (mean ± SD) (table 13). This result which served as a reference value, was compared with the different groups of chicks in this study.

With repeated vitamin D_3 dosages less than 0.5μg, plasma 25-hydroxyvitamin D_3 concentrations were not detected (<4ng/ml). Chicks receiving repeated doses of 1.0 μg vitamin D_3, had plasma $25(OH)D_3$ levels similar to the reference value ($p= 0.85$) and

were considered to be receiving physiological doses of vitamin D_3. The group receiving repeated 100μg doses had significantly higher levels than the group receiving 1.0μg and the levels were above those in the reference range. The only other group (0.5μg) with detectable plasma 25(OH)D_3 levels, had levels which were considered to be in the vitamin D_3 deficiency range (table 13).

Table 13. **Plasma 25(OH)D_3** obtained after dosing vitamin D deficient chickens with varying doses of vitamin D_3.

Group	Dose Vit. D_3 (μg) per dose	Total Vit. D_3 (μg)	Average Vitamin D_3/day (μg)	IU	Plasma 25(OH)D_3 ng/ml
1	100	500	33.3	1333	111 ±29.0*(1)
2	1.0	5.0	0.33	13	20.6 ±5.6*(2)
3	0.5	2.5	0.17	6.7	7.0 ±2.7*
4	0.1	0.5	0.03	1.3	N.D.
5	0.05	0.25	0.02	0.7	N.D
6	0.0	0.0	0.0	0.0	N.D.
Reference value					23.7 ±2.7

N.D. Not demonstrable. * mean ± S.D (5)
(1) significantly greater than reference value ($p = 0.0001$), and groups 2 and 3.
(2) similar to reference value ($p = 0.85$).

Figure 16 depicts the values of plasma 25(OH)D_3 in chicks, corresponding to the dosages of vitamin D_3 administered.

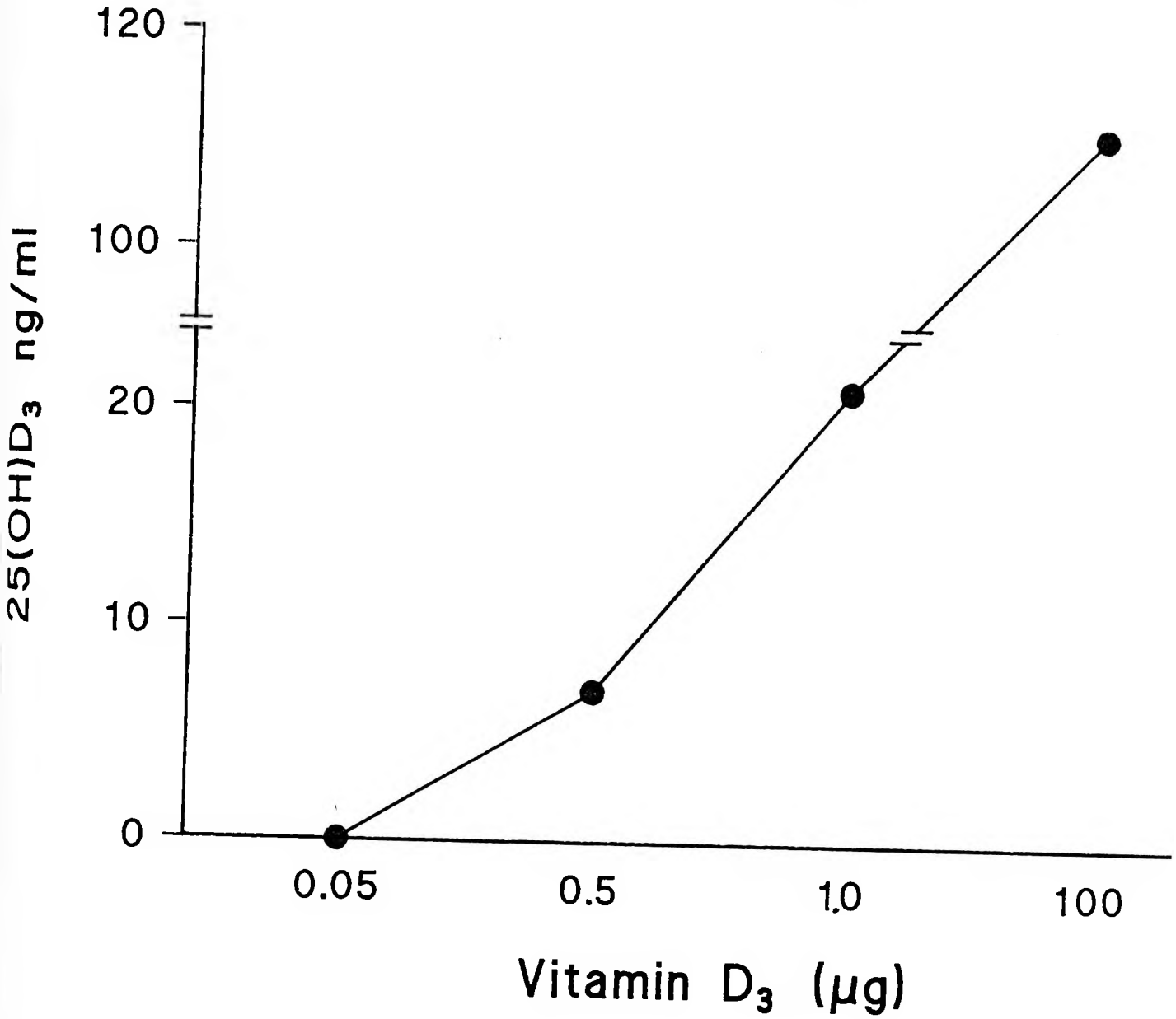


Figure 16.

Plasma 25(OH)D_3 values after dosing vitamin D

deficient chickens with varying amounts of vitamin D_3.

3.2.2 Effect of vitamin D_3 on plasma calcium, phosphorus and alkaline phosphatase concentrations.

Plasma calcium results obtained in this study, were compared with the concentrations recorded in normal chicks of a similar age (Egleton 1991), and plasma results of phosphorus were compared with those reported in the study by Sedrani (1984) in female chicks at 8 weeks of age. These results served as reference values for these two minerals in this study (table 14).

A plasma calcium result equivalent to the reference values was obtained when the chicks were given repeated 1μg vitamin D_3 doses ($p = 0.83$). With 100μg doses the plasma calcium levels were abnormally raised ($p = 0.007$) and were associated with high plasma $25(OH)D_3$ values. All the groups receiving 0.5μg or less vitamin D_3 had significantly lowered plasma calcium levels ($p = 0.0001$). The plasma calcium levels were not corrected for plasma albumin content.

The plasma phosphorus level of 1.78 ± 0.16 mmol/l in the group receiving repeated 1μg vitamin D_3 doses at 5 weeks of age, was slightly higher than the value 1.65 ± 0.1 mmol/l recorded by Sedrani (1984) in chicks at 8 weeks of age. However Sedrani (1984) noted a fall in serum phosphorus from 1 week after hatching to approximately one-half the original value at 15 weeks, thus it is possible that the value obtained in this study would decline over the next 3 weeks to approximate the value given at 8 weeks of age. Statistically the chicks in the groups receiving repeated 100ug and 1ug of vitamin D_3 were not significally different from the plasma reference value ($p > 0.50$ (100μg) and $p > 0.10$ (1.0μg) respectively. The plasma phosphorus values in the chicks receiving repeated 0.5μg or 0.05μg of the vitamin were less than the reference group ($p < 0.05$), but due to the wide scatter of the standard deviation in the birds administered 0.1μg doses, the difference was not significant ($p > 0.20$).

Table 14. Effect of vitamin D_3 status on plasma calcium, phosphorus and alkaline phosphatase values.

Group	Vitamin D3 Dosage (μg)	Calcium (mmol/l)	Calcium (mg/dl)	Phosphorus (mmol/l)	Phosphorus (mg/dl)	Alk. Phos. (I.U.)
1	100	3.10 ±0.1[a]	12.4 ±0.2	1.61 ±0.19[d]	4.6 ±0.9	1915 ±191
2	1.0	2.74 ±0 3[b]	10.9 ±1.0	1.78 ±0.16[d]	5.4 ±0.5	2060 ±128
3	0.5	1.83 ±0.6[c]	7.3 ±1.8	1.45 ±0.13[e]	4.5 ±0.4	2240 ±149
4	0.1	1.91 ±0.2[c]	7.6 ±0.8	1.48 ±0.28	4.6 ±0.9	2250 ±132
5	0.05	1.88 ±0.3[c]	7.5 ±1.3	1.33 ±0.23	4.1 ±0.7	3533 ±1378
6	0.0	1.48 ±0 2[c]	6.0 ±1.0	1.43 ±0.25	4.4 ±0.8	3206 ±1310
	Ref. values	2.72 ±0.2[1]	10.9 ±1.1	1.65 ±0.1[2]	5.1 ±0.3	

All values represent the mean and standard deviation (n = 5).

[a] significant difference from reference value (p = 0.007)(calcium).

[b] no difference from reference value (p = 0.83)(calcium).

[c] significant difference from reference value (p < 0.0001).

[d] not significantly different from reference value (p > 0.05 (group 1) and > 0.10 (group 2)). (phosphorus).

[e] less than reference value (p < 0.05) (phosphorus).

[1] from Egleston (1993)

[2] from Sedrani (1984)

The alkaline phosphatase values had a broad scatter, the lowest values were obtained with the 100μg and 1μg dosages while the groups receiving lower levels than 1μg of the vitamin had higher amounts of the enzyme.

It was therefore concluded from the data involving the plasma values of $25(OH)D_3$, and plasma calcium, phosphorus and alkaline phosphatase data, that the dose of vitamin D_3 capable of producing physiological concentrations of plasma $25(OH)D_3$ was evident in the group which received repeated doses of 1μg of vitamin D_3. This group was able to maintain normal mineral homeostasis and so prevent a vitamin D deficiency state in these chickens.

Chapter 4

Preliminary Study.

Vitamin D_3 metabolism in the chick given varying [^{3}H]vitamim D_3 concentrations.

4.1 Introduction.

Analysing the results of vitamin D_3 study (refer table 13), it is evident that for the prevention of vitamin D deficiency, a repeated dose between 1μg and 100μg vitamin D_3 was required. On this basis it was decided to administer the birds in the experimental groups repeated doses of either 2, 4, 8, or 16μg [^{3}H]-vitamin D_3.

4.1.1 Dosing of birds and preparation of tissues.

Thirty five, one day old chicks were raised on a vitamin D_3 deficient diet for 17 days (refer section 3.1.1; table 12). On day 17 the chicks were divided into 5 groups. Four of the groups received [^{3}H]vitamin D_3 (Specific activity 4.24×10^5 dpm/μg) in 2, 4, 8, or 16μg doses on days 17, 21, 24 and 28. The fifth group received vehicle only for the first 3 doses and then on day 28, received a single dose of 3.17×10^6 dpm [^{3}H]vitamin D_3 (Specific activity 1.06×10^8 dpm/μg), each chick receiving 0.03μg (1.2 IU) [^{3}H]vitamin D_3. Two days later all the chicks were killed by decapitation. Plasma, liver, skeletal muscle, kidney, scrapings of duodenal mucosa and adipose tissue were obtained and individually frozen in plastic vials and stored at -20°C for further analysis.

Plasma was collected as outlined in section 3.1.4 while duodenal mucosa was prepared as follows: the duodenal loop was removed, the pancreas excised, the loop sliced open and rinsed with ice-cold saline. The mucosa was scraped free from the serosa with a microscope slide and transferred to a plastic vial and stored. Abdominal fat could not be investigated in the vitamin deficient birds, as it was not detected after a careful examination of the abdominal cavity.

Before analysis, 1 to 2ml of plasma or 1 to 2 grams of tissue were thawed, homogenised and the metabolites extracted as outlined in section 2.4.2. (Bligh and Dyer 1959)). The samples were then submitted to silicic acid chromatography (refer section 2.5.4). The fractions containing vitamin D_3 (E-H 55:45), $25(OH)D_3$ (E-H 70:30) $24,25(OH)_2D_3$ (E-H 95:5) and $1,25(OH)_2D_3$ (E-Ac 50:50) elutions were collected separately off the silicic acid column (figure 10) and then subjected individually to isocratic HPLC (refer section 2.6). The various radio-active metabolites collected off HPLC, were authenticated by their elution volumes compared to those of authentic vitamin D metabolites applied to the column.

The elutions of putative vitamin D_3 esters (E-H 20:80) and metabolites more polar than $1,25(OH)_2D_3$ (methanol), were collected off the silicic acid column and counted on a Packard Tri-carb liquid scintillation system.

4.2 Results of the preliminary study.

The pattern of distribution of the parent vitamin D_3, its main metabolites, putative vitamin D_3 esters and polar metabolites in the chick in its deplete state, compared to those groups after receiving four repeated doses of 2, 4, 8, or 16μg [^{3}H]vitamin D_3 over 12 days is presented in table 15 and figure 17.

Studying the results and histogram patterns it is possible to divide the 6 organs into three categories. The first category is plasma on its own, whilst the target organs, duodenal mucosa and kidney form the second, with the organs liver, skeletal muscle and adipose tissue forming the third.

4.2.1 Plasma results.

The results and histogram profiles of plasma, reveal that $25(OH)D_3$ (E-H 70:30) was the dominant circulating metabolite with added vitamin D_3, the levels increasing with the higher vitamin dosage.

The plasma level of the parent vitamin (E-H 55:45) was not detected in the deplete state, but increased with the addition of the vitamin, and was 60 percent of $25(OH)D_3$ concentration in the group receiving 16μg doses.

In the deficient state, putative $1,25(OH)_2D_3$ (E-Ac 50:50) was present in small amounts. With the addition of oral vitamin D_3 its values increased, but putative $24,25(OH)_2D_3$ (E-H 95:5) not detected in the deficiency group, now exceeded $1,25(OH)_2D_3$ in all the replete groups. The values of $24,25(OH)_2D_3$ varied between 29 and 35 percent compared with $25(OH)D_3$ in the four replete groups.

Putative vitamin D_3 esters were not detected in the deficient chickens, but increased in value with the larger doses of vitamin D_3.

4.2.2 Target Organs:-duodenal mucosa and kidney.

The profile of the target organs duodenum and kidney in the deficiency state showed that $1,25(OH)_2D_3$ was the most dominant metabolite in its particular organ. This was more evident in the duodenal mucosa.

In the kidney, $25(OH)D_3$ was 66 percent the value of the putative $1,25(OH)_2D_3$ in the deplete state, but with the addition of vitamin D_3, $25(OH)D_3$ exceeded the value of $1,25(OH)_2D_3$ in both the kidney and duodenal mucosa.

Vitamin D_3 was the dominant steroid in the 8 and 16μg groups in both these organs. Vitamin D_3 esters were not detected in the deplete state but with the administration of the vitamin, increased in value with the two highest dosages.

4.2.3 Organs:- liver, muscle and adipose tissue.

The presence of the parent vitamin D_3 and vitamin D_3 esters in these organs was very evident in the replete groups, most noticeably in the 8 and 16μg groups.

Most of the metabolites examined in liver, muscle and adipose tissue increased in value with vitamin D_3 administration, the highest amounts were present in the group receiving 16μg doses of the vitamin. Adipose tissue dominated the increase in these metabolites.

Vitamin D_3 was greater than the other metabolites in the 8 and 16μg groups, the increase in vitamin D_3 in adipose tissue in the 16μg group being practically 30 fold greater compared to that in the 2μg group in the same tissue. Adipose tissue also harboured the highest amounts of $25(OH)D_3$ in each replete group of chicks. The increase in the dihydroxyvitamins D_3 was apparent in all tissues with the addition of vitamin D_3, but the values of these metabolites were highest in adipose tissue in all groups examined.

In the deficient state, vitamin D_3, $24,25(OH)_2D_3$ and vitamin D_3 esters were not detected in any of the organs, and only small quantities of $25(OH)D_3$ and $1,25(OH)_2D_3$ were present except muscle in which these metabolites were not detected.

In all groups, the values of vitamin D_3 esters were higher in liver than in the other organs, and was the only organ in the 2μg group in which this compound was

demonstrated.

The kidney and intestinal mucosa contained the highest percentage of polar metabolites of all tissues in each group examined, adipose tissue being the lowest in the 4μg and 16μg groups. The percentage content of these metabolites in all groups of plasma was fairly equal.

During the course of the study it became apparent that difficulties were encountered. The flow of the solvents through the narrow pipette tubes used for the silicic acid chromatography was impeded due to the lipid content of the specimens.

It is possible that due to this intermittent' flow with the various solvents, radioactive metabolites of vitamin D_3 were not effectively separated and thus gave spurious low readings of the parent vitamin D_3 and $25(OH)D_3$, while the dihydroxyvitamin metabolites recorded high values. The latter spurious results were possibly due to inadequate separation of various unknown metabolites from the major metabolites being quantitated.

This study was therefore considered to be of a preliminary nature, to be followed by the definitive study, after the problems encountered in this study had been resolved.

Table 15.

Preliminary results: putative vitamin D_3 esters, vitamin D_3, $25(OH)D_3$, $24,25(OH)_2$, $1,25(OH)_2D_3$ and polar metabolites in plasma, duodenum, kidney, liver, skeletal muscle and fat in chickens which are a) vitamin D_3 deficient animals; b) animals receiving vitamin D_3 2μg doses, c) 4μg doses, d) 8μg doses and e) 16μg doses

PRELIMINARY RESULTS

Table 15 (a). **VITAMIN D_3 AND METABOLITES**

Vitamin D_3 deficient. (pmol/g or pmol/ml).						
	E-H 20:80	E-H 55:45	E-H 70:30	E-H 95:5	E-Ac 50:50	Polar**
P*	ND	ND	0.10 ±0.003	ND	0.02 ±0.005	5.7 ±1.7
I	ND	ND	0.01 ±0.001	ND	0.06 ±0.02	8.3 ±1.9
K	ND	ND	0.02 ±0.005	ND	0.03 ±0.01	20.7 ±7.7
L	ND	ND	0.015 ±0.006	ND	0.01 ±0.004	7.8 ±3.8
M	ND	ND	ND	ND	ND	5.4 ±3.0
F	-	-	-	-	-	-

Table 15 (b).

Vitamin D_3 2µg doses. (pmol/g or pmol/ml).						
	E-H 20:80	E-H 55:45	E-H 70:30	E-H 95:5	E-Ac 50:50	Polar**
P*	ND	0.11 ±0.08	6.93 ±1.44	2.03 ±0.64	1.58 ±0.22	8.2 ±0.9
I	ND	0.15 ±0.06	0.54 ±0.23	0.26 ±0.08	0.29 ±0.06	15.7 ±9.3
K	ND	0.31 ±0.29	1.63 ±0.26	0.68 ±0.22	1.04 ±0.22	34.2 ±21.2
L	0.53 ±.12	0.75 ±0.20	0.92 ±0.19	0.34 ±0.14	0.34 ±0.16	14.8 ±8.5
M	ND	0.3 ±0.07	0.55 ±0.15	0.22 ±0.10	0.21 ±0.08	6.5 ±4.1
F	ND	2.33 ±0.34	1.39 ±0.33	0.46 ±0.09	1.29 ±0.30	-

Table 15 (c).

Vitamin D_3 4µg doses (pmol/g or pmol/ml)						
	E-H 20:80	E-H 55:45	E-H 70:30	E-H 95:5	E-Ac 50:50	%Polar**
P*	0.03 ±0.02	0.34 ±0.05	8.6 ±0.67	2.96 ±0.23	1.67 ±0.33	8.4 ±1.7
I	ND	0.64 ±0.12	1.16 ±0.13	0.55 ±0.29	0.50 ±0.22	17.6 ±10.0
K	0.11 ±0.05	0.73 ±0.16	3.09 ±0.48	0.88 ±0.16	1.42 ±0.30	18.5 ±5.0
L	1.56 ±0.37	1.79 ±0.51	1.82 ±0.39	0.68 ±0.26	0.67 ±0.16	8.8 ±4.3
M	0.23 ±0.13	1.44 ±0.58	1.34 ±0.26	0.33 ±0.13	0.62 ±0.38	5.7 ±3.8
F	0.03 ±0.02	5.87 ±1.97	3.22 ±0.61	2.02 ±0.57	3.34 ±0.41	2.7 ±1.28

Table 15 (d).

Vitamin D_3 8µg doses. (pmol/g or pmol/ml)						
	E-H 20:80	E-H 55:45	E-H 70:30	E-H 95:5	E-Ac 50:50	Polar**
P*	0.08 ±0.02	1.76 ±1.27	11.2 ±2.6	3.32 ± 1.43	2 .43 ±0.55	7.0 ±.7
I	0.12 ±0.07	2.94 ±1.21	1.26 ±.23	0.62 ±0.39	1.0 ±0.23	15.0 ±1.9
K	0.30 ±0.16	3.66 ±1.54	2.77 ±.77	1.12 ±0.32	1.73 ±0.87	16.0 ±8.2
L	4.0 ±1.0	5.03 ±1.36	2.11 ±.58	0.89 ±0.31	1.1 ±0.71	4.0 ±1.3
M	0.96 ±0.48	6.12 ±1.57	1.53 ±.42	0.40 ±0.07	0.8 ±0.5	3.0 ±1.9
F	1.54 ±0.73	24.2 ±6.07	8.69 ±2.87	5.01 ±2.05	5.21 ±1.46	-

Table 15 (e).

Vitamin D_3 16μg doses. (pmol/g or pmol/ml)						
	E-H 20:80	E-H 55:45	E-H 70:30	E-H 95:5	E-Ac 50:50	Polar**
P*	0.29 ±0.08	9.1 ±2.3	15.1 ±3.0	5.4 ±0.7	2.8 ±0.7	7.0 ±.8
I	0.28 ±0.15	7.0 ±0.9	1.7 ±0.2	1.0 ±0.3	1.1 ±0.2	12.0 ±3.5
K	0.44 ±0.15	13.1 ±2.9	6.6 ±0.8	3.2 ±0.6	6.3 ±2.6	11.0 ±6.2
L	8.73 ±3.22	14.5 ±3.0	3.8 ±0.4	1.7 ±0.2	2.1 ±0.4	4.0 ±3.0
M	2.2 ±0.9	17.2 ±4.1	2.0 ±1.0	1.2 ±0.6	0.9 ±0.3	2.0 ±.8
F	2.4 ±0.7	58.5 ±21.2	21.9 ±5.3	18.0 ±2.0	16.4 ±2.8	1.1 ±0.5

*P Plasma; I Duodenal mucosa; K Kidney; L Liver; M Muscle;
F Adipose Tissue; E Ether; H Hexane; Ac Acetone.
** Polar: Metabolites eluted with Methanol (100%) and expressed as a percentage of the total counts encountered in that tissue.
E-H 20:80 = putative vitamin D_3 esters; E-H 55:45= vitamin D_3;
E-H 70:30 = $25(OH)D_3$; E-H 95:5= $24,25(OH)_2D_3$;
E-Ac 50:50 = $1,25(OH)_2D_3$. ND Not detected.

Figure 17.

Preliminary results:

profiles of putative vitamin D_3 esters, vitamin D_3,

$25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$

in

i) plasma, ii) duodenum, iii) kidney, iv) liver,

v) skeletal muscle, vi) fat in chickens

which are

a) vitamin D deficient or receiving b) 2μg,

c) 4μg, d) 8μg, e) 16μg vitamin D_3.

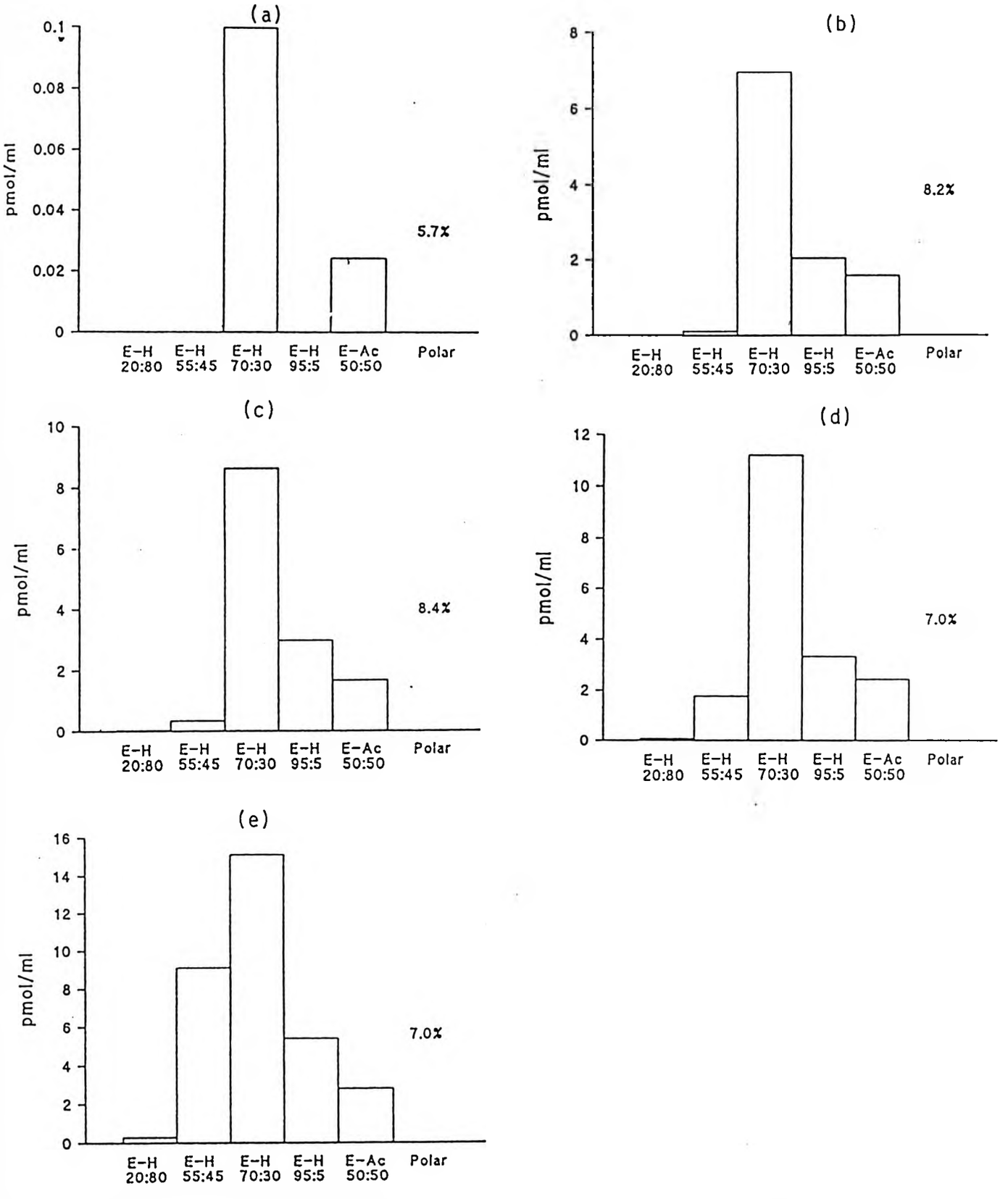


Figure 17 i). **Vitamin D metabolite concentrations in plasma in the various groups of chickens.**

a) vitamin D_3 (deficient)
b) vitamin D_3 (2μg)
c) vitamin D_3 (4μg)
d) vitamin D_3 (8μg)
e) vitamin D_3 (16μg)

E:H 20:80 (putative vitamin D_3 esters)
E:H 55:45 (vitamin D_3)
E:H 70:30 (25-(OH)D_3)
E:H 95:5 (24,25-$(OH)_2D_3$)
E:Ac 50:50 (1,25-$(OH)_2D_3$)

E = Ether; H = Hexane; Ac = Acetone

Y axis enlarged 100 times in vitamin D deficient chicks (a).

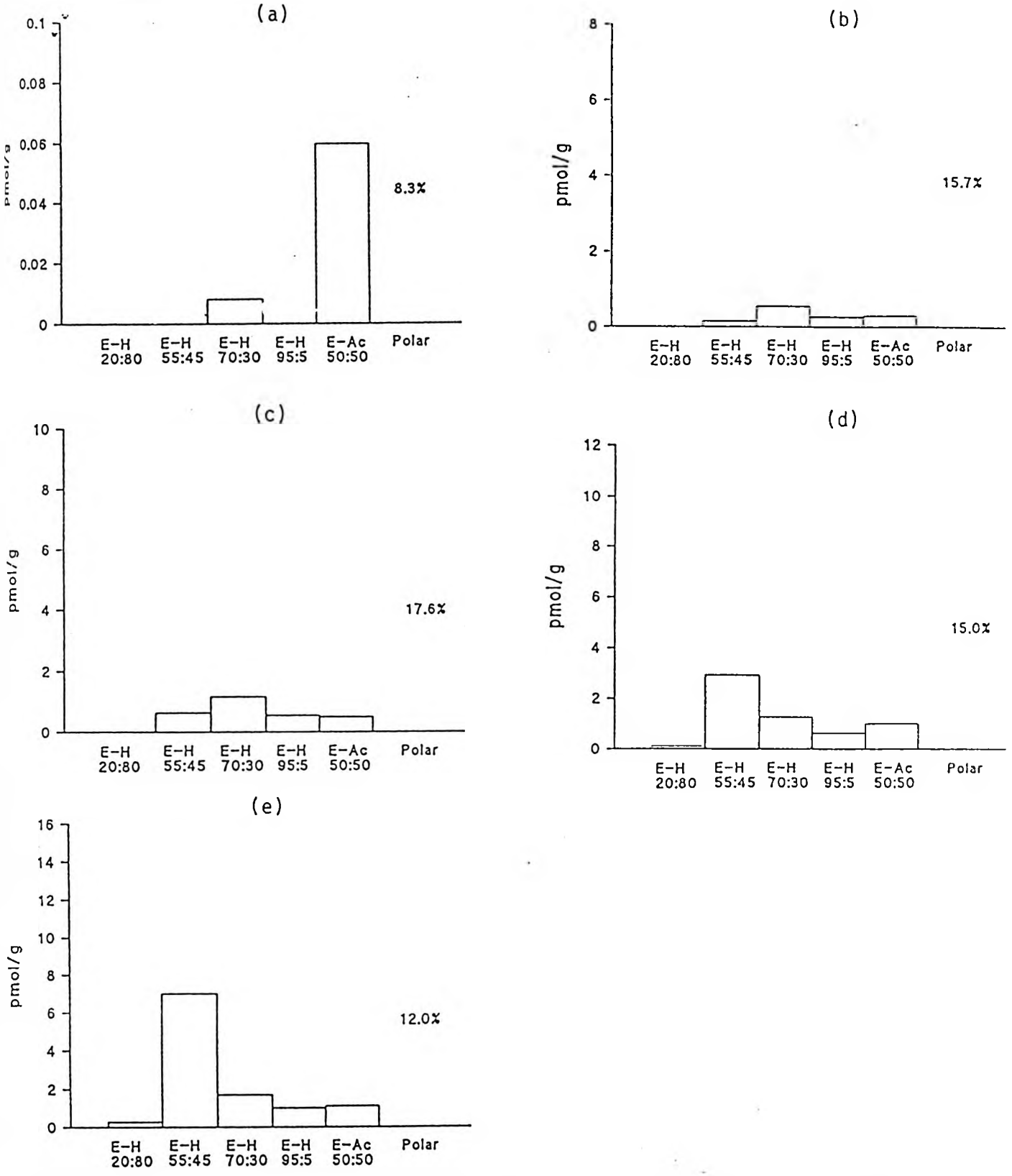


Figure 17 ii). **Vitamin D metabolite concentrations in duodenum in the various groups of chickens.**

a)	vitamin D_3 (deficient)	E:H	20:80 (putative vitamin D_3 esters)
b)	vitamin D_3 (2μg)	E:H	55:45 (vitamin D_3)
c)	vitamin D_3 (4μg)	E:H	70:30 (25-(OH)D_3)
d)	vitamin D_3 (8μg)	E:H	95:5 (24,25-$(OH)_2D_3$)
e)	vitamin D_3 (16μg)	E:Ac	50:50 (1,25-$(OH)_2D_3$)

E = Ether; H = Hexane; Ac = Acetone

Y axis enlarged 100 times in vitamin D deficient chicks (a).

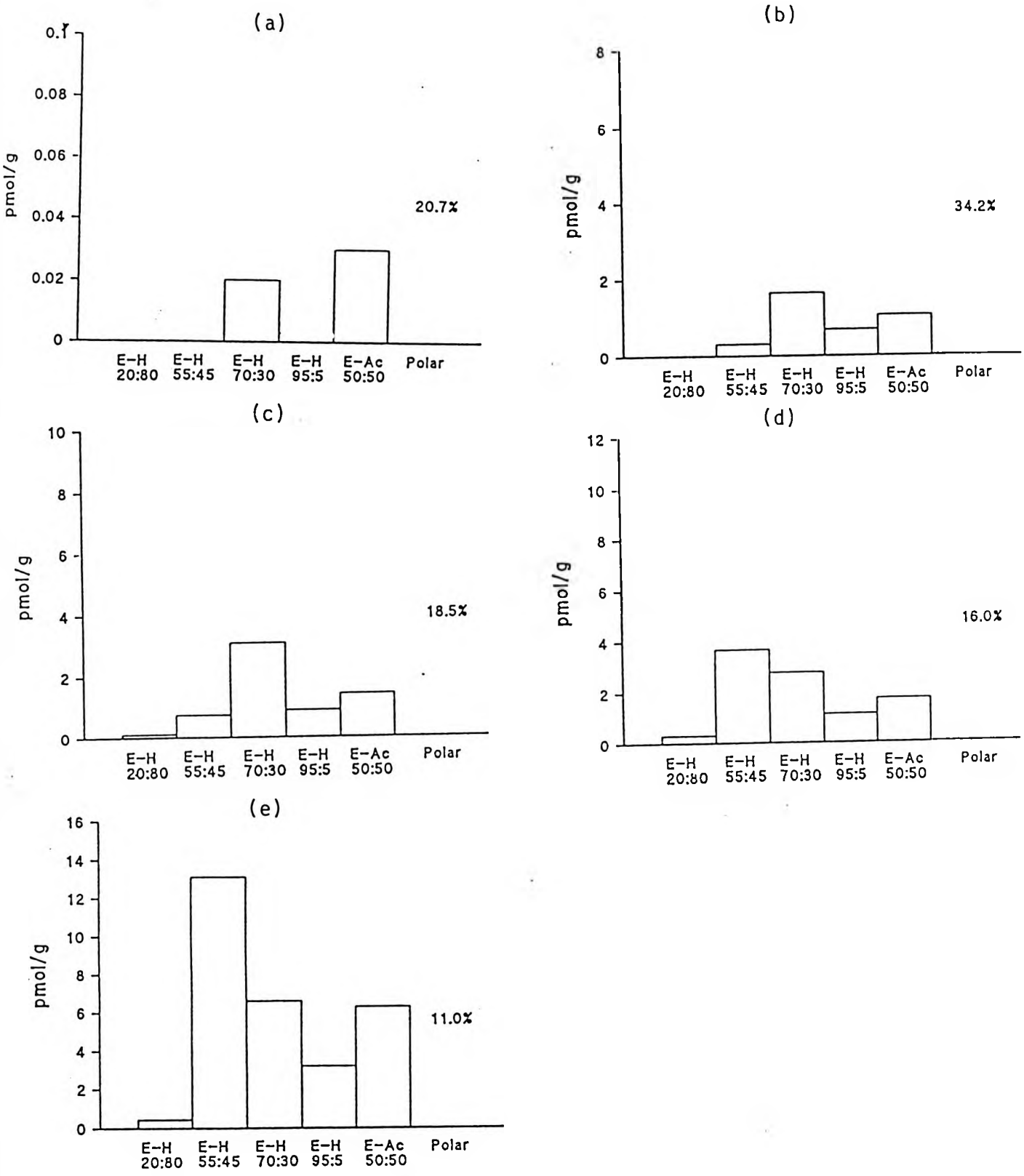


Figure 17 iii). **Vitamin D metabolite concentrations in kidney in the various groups of chickens.**

a)	vitamin D_3 (deficient)	E:H	20:80 (putative vitamin D_3 esters)
b)	vitamin D_3 (2μg)	E:H	55:45 (vitamin D_3)
c)	vitamin D_3 (4μg)	E:H	70:30 (25-(OH)D_3)
d)	vitamin D_3 (8μg)	E:H	95:5 (24,25-$(OH)_2D_3$)
e)	vitamin D_3 (16μg)	E:Ac	50:50 (1,25-$(OH)_2D_3$)

E = Ether; H = Hexane; Ac = Acetone

Y axis enlarged 100 times in vitamin D deficient chicks (a).

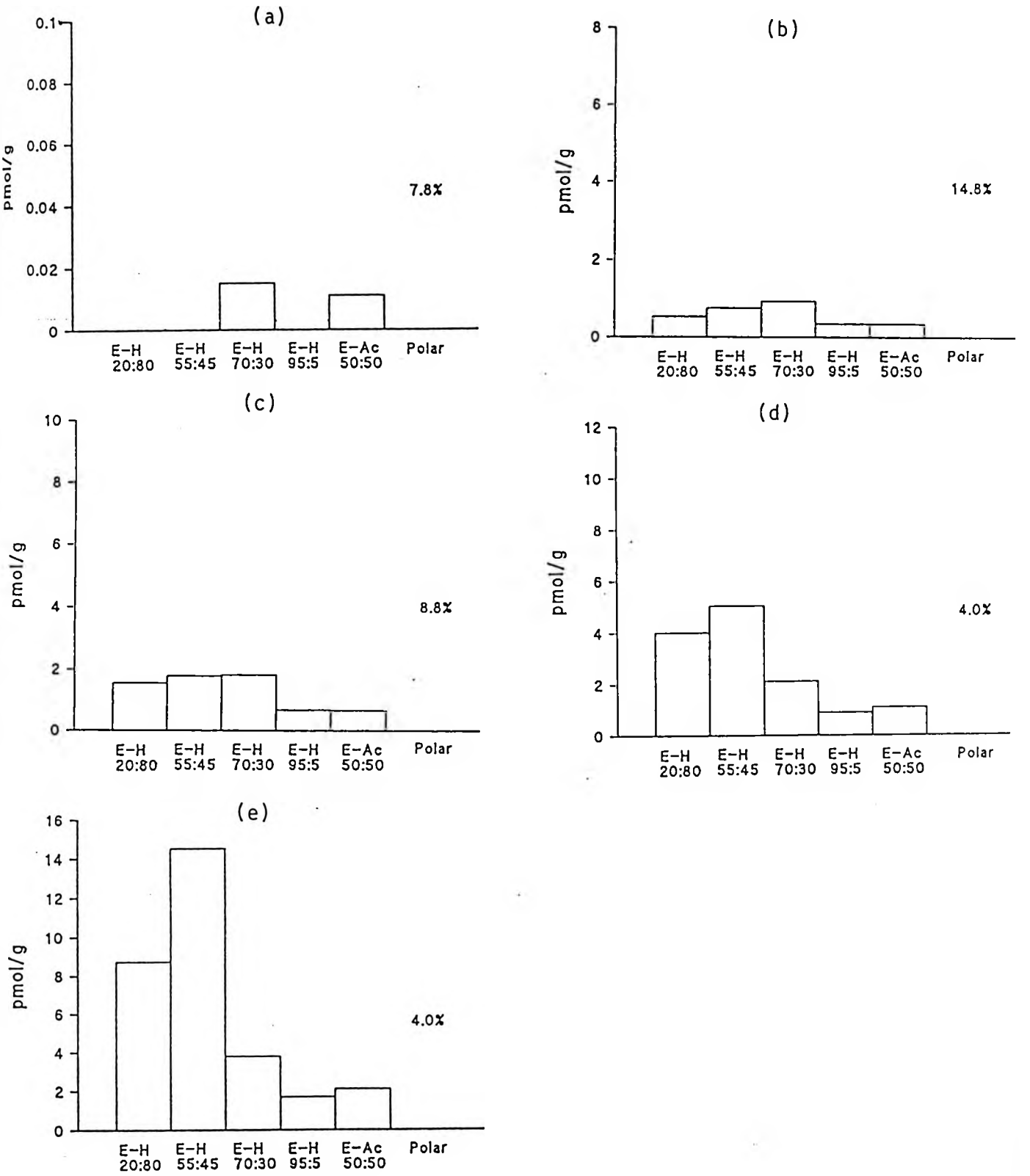


Figure 17 iv). **Vitamin D metabolite concentrations in liver in the various groups of chickens.**

a) vitamin D_3 (deficient)
b) vitamin D_3 (2μg)
c) vitamin D_3 (4μg)
d) vitamin D_3 (8μg)
e) vitamin D_3 (16μg)

E:H 20:80 (putative vitamin D_3 esters)
E:H 55:45 (vitamin D_3)
E:H 70:30 (25-(OH)D_3)
E:H 95:5 (24,25-$(OH)_2D_3$)
E:Ac 50:50 (1,25-$(OH)_2D_3$)

E = Ether; H = Hexane; Ac = Acetone

Y axis enlarged 100 times in vitamin D deficient chicks (a).

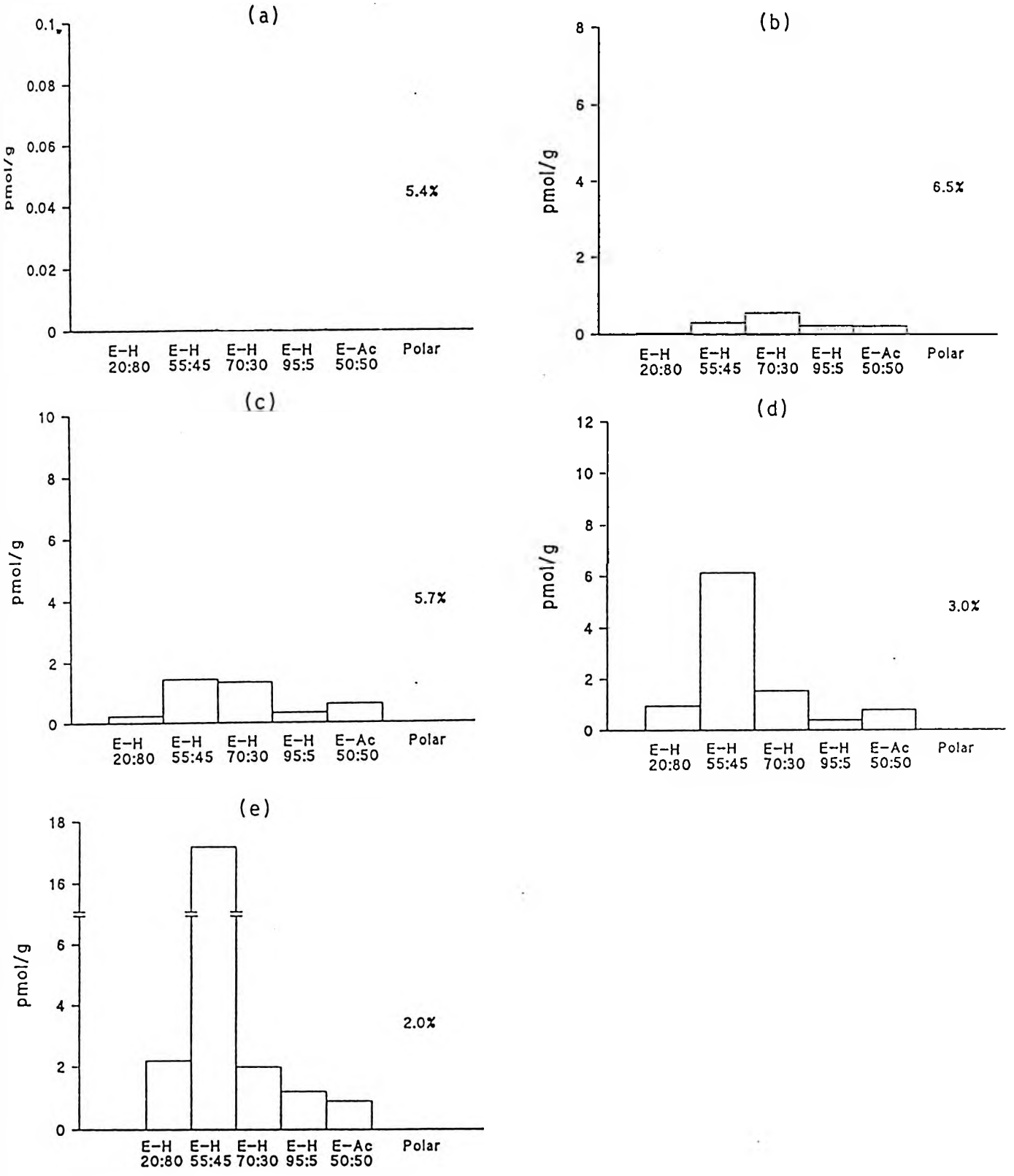


Figure 17 v). **Vitamin D metabolite concentrations in muscle in the various groups of chickens.**

a)	vitamin D_3 (deficient)	E:H	20:80 (putative vitamin D_3 esters)
b)	vitamin D_3 (2μg)	E:H	55:45 (vitamin D_3)
c)	vitamin D_3 (4μg)	E:H	70:30 (25-(OH)D_3)
d)	vitamin D_3 (8μg)	E:H	95:5 (24,25-$(OH)_2D_3$)
e)	vitamin D_3 (16μg)	E:Ac	50:50 (1,25-$(OH)_2D_3$)

E = Ether; H = Hexane; Ac - Acetone

Y axis enlarged 100 times in vitamin D deficient chicks (a).

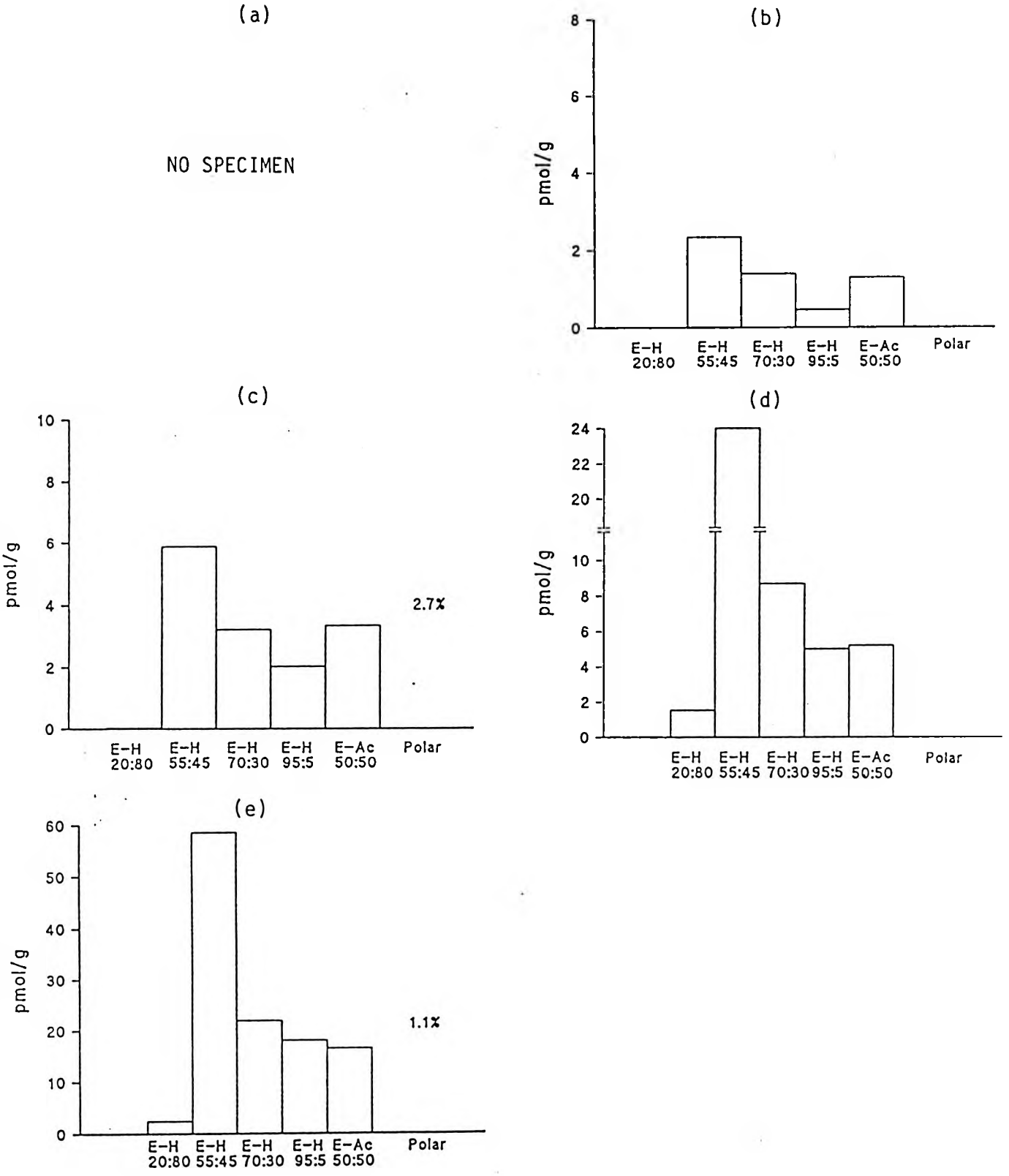


Figure 17 vi). **Vitamin D metabolite concentrations in fat in the various groups of -chickens.**

a)	vitamin D_3 (deficient)	E:H	20:80 (putative vitamin D_3 esters)
b)	vitamin D_3 (2μg)	E:H	55:45 (Vitamin D_3)
c)	vitamin D_3 (4μg)	E:H	70:30 (25-(OH)D_3)
d)	vitamin D_3 (8μg)	E:H	95:5 (24,25-$(OH)_2D_3$)
e)	vitamin D_3 (16μg)	E:Ac	50:50 (1,25-$(OH)_2D_3$)

E = Ether; H = Hexane; Ac = Acetone

Y axis enlarged 100 times in vitamin D deficient chicks (a).

Chapter 5

Definitive study.

To determine vitamin D_3 and its metabolite distribution in chickens in different vitamin D_3 status.

5.1 Introduction

It is evident from the preliminary study that qualitatively the results of the group receiving repeated 2 μg doses of vitamin D_3, show a similarity to the group receiving repeated 4 μg dosages. There also existed a qualitative similarity between the groups receiving 8μg and 16μg doses of vitamin D_3. It was decided to eliminate one of the dosage regimens in each of the two groups and to concentrate on the groups receiving repeated 4μg and 16μg vitamin D_3 dosages, these being representative of a vitamin D replete group receiving physiological amounts of vitamin D_3 and a group receiving excessive vitamin D_3 respectively.

5.2 Investigations of chickens in definitive study.

It was also decided to investigate the vitamin D_3 deficient chicks in two different ways. The one group would receive a single small dose of [^{3}H]-vitamin D_3 (Group SD) 48 hours before decapitation, while the other group would receive multiple small tracer amounts of [^{3}H]-vitamin D_3 (Group MD) on the days when the other two replete groups received their vitamin D_3 dosages. The group receiving a single bolus would supply information regarding the metabolism and distribution of a single minute dose of vitamin D_3 given acutely, while the group receiving repetitive small doses, although not influencing the vitamin D status of the animal significantly, would allow assessement of more chronic metabolism of vitamin D_3 in the deficiency state.

5.2.1 Rearing of chicks and dosages of [^{3}H]vitamin D_3.

Twenty four chicks were reared on a rachitogenic diet (refer table 12) administered for 17 days under the same conditions as described in section 3.1.1. They were then divided into 4 groups, each group consisting of 6 birds. The chicks received the alloted treatment on days 17, 21, 25, 29 and 33 and were decapitated on day 35. All treatments were given orally with the aid of a pipette.

The birds in group SD were administered 100µg vehicle (ethanol-propylene glycol 1:2v/v) for the first 4 doses and finally $3.1x10^6$ dpm [^{3}H]-vitamin D_3 (Specific Activity $4.51x10^7$ dpm/µg) which contained 0.07µg (2.8 IU) vitamin D_3 on day 33. The chicks in group MD each received a similar dose of $3.1x10^6$ dpm [^{3}H]-vitamin D_3 containing 0.07µg vitamin D_3 in vehicle on each of the five days alloted for treatment. The birds in the other two groups received either 4µg or 16µg [^{3}H]-vitamin D_3 (specific activity $7x10^5$ dpm/µg) in 100µl vehicle per dose respectively. The collection and storage of the various organs were similar to that already described (refer section 4.1.1). Extraction of the tissues for the metabolites was by the method of Bligh and Dyer (1959)(refer section 2.4.2), and the preliminary chromatography on silicic acid columns as discussed in section 2.5.5. Isocratic HPLC for the identification of vitamin D_3 and 25(OH)D_3 is described in sections 2.6.1; 2.7, while gradient HPLC was performed for the identification of 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ metabolites (refer section 2.7).

5.2.2 Weights of chickens in definitive study.

The weights of the two vitamin D_3 deficient groups (SD and MD) were not statistically significantly different (p= 0.8), but the group which received multiple small vitamin D_3 doses (group MD) weighed approximately 20 percent more than the other deficient group(SD). The birds, which received 4μg and 16μg vitamin D_3 doses, were of similar weights, but were significantly heavier than the D deficient groups (p = 0.0001). (table 16).

Table 16. Weights of chickens in definitive study.

Group:	**1**	**2**	**3**	**4**
Dosage:	Vitamin D^- (SD)	Vitamin D^- (MD)	4μg	16μg
Weight: (gm)	214[1] ± 37	255[1] ± 43	308 ± 32	313 ± 42

[1] Weight significantly different from Groups 3 and 4 (p=0.0001).

Results of Definitive Study

5.3 Distribution of vitamin D_3 and metabolites in plasma and tissues.

The distribution of vitamin D_3 and its main metabolites, $25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$ and putative vitamin D_3 esters in plasma and the tissues examined, are documented in table 17 and figures 19 and 20. Metabolites more polar than $1,25(OH)_2D_3$, were eluted from the silicic acid chromatography column with methanol and the counts expressed as dpm/ml or dpm/gm of plasma or tissue respectively, and also as a percentage of the total counts of radioactivity in that particular specimen.

5.3.1 Plasma results.

All the plasma values in both the SD and MD vitamin D_3 deplete groups were either very low or not detectable. The concentrations of vitamin D_3 and the metabolites in the two groups were fairly similar and there appeared to be no difference between these two groups.

The parent vitamin was not demonstrated in either the SD or MD groups, however with 4μg vitamin D_3 dose, the level rose to 3.9 ± 0.9pmol/ml (1.5 ± 0.03 ng/ml) and progressed further (33.4 ± 2.7pmol/ml ; 12.8 ± 1.0 ng/ml) to practically half the level of $25(OH)D_3$ in the group receiving 16μg doses.

$25(OH)D_3$ was the major circulating metabolite in plasma. Concentrations were low in vitamin D_3 deplete groups: 0.33 ± 0.08 and 0.28 ± 0.02 pmol/ml(0.13 ± 0.03 and

0.11 ± 0.01 ng/ml). With an increase in vitamin D_3 dosage, circulating $25(OH)D_3$ attained values of 48.4 ± 7.1 pmol/ml (19.36 ± 2.8 ng/ml) with 4μg doses and 75.5 ± 8.9 pmol/ml (30.2 ± 3.5ng/ml) with 16μg doses.

In the deficient state, $1,25(OH)_2D_3$ concentration was 0.014 ± 0.006 and 0.016 ± 0.001 pmol/ml(6.0 ± 0.41 and 6.4 ± 0.4pg/ml) in the SD and MD groups respectively. The metabolite concentration rose with the higher dose of vitamin D_3 reaching 0.06 ± 0.01 pmol/ml(26.6 ± 5.4pg/ml) and 0.09 ± 0.03pmol/ml (39.9 ± 12.5pg/ml) with 4 μg and 16μg doses. $24,25(OH)_2D_3$, not detected in either of the deficient groups, increased with addition of the vitamin and was 9.1 percent of the value of $25(OH)D_3$ in both the 4μg and 16μg groups, recording 4.4 ± 1.1 and 6.9 ± 1.3 pmol/ml(1.8 ± 0.45 and 2.86 ± 0.54ng/ml) respectively, the metabolite being greater in amount than $25(OH)_2D_3$.

Putative vitamin D_3 esters were present in small amounts in the replete groups.

5.3.2 Target organs: duodenal mucosa and kidney.

$1,25(OH)_2D_3$ was the major metabolite in both deficient groups in duodenal mucosa (0.033 ± 0.007 and 0.055 ± 0.01pmol/g) while all other metabolites were less than half that amount. With the addition of vitamin D_3 to the chickens, $1,25(OH)_2D_3$ increased in concentration, but the pattern altered, $25(OH)D_3$ and the parent vitamin became the major metabolites, the latter steroid being most dominant in the 16μg group, recording 8.6 ± 1.3 pmol/g.(3.3 ± 0.5ng/g).

The dihydroxyvitamin metabolites $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were practically equal in the 4μg group, but with the highest vitamin dosage $24,25(OH)_2D_3$ (0.42 ± 0.12 pmol/g) was approximately four times that of $1,25(OH)_2D_3$.

In the kidney, the metabolite $1,25(OH)_2D_3$ was present in both deficiency groups. The levels were not as high as that recorded in the duodenal mucosa, being 0.02 ± 0.004 and 0.014 ± 0.006 pmol/g respectively, while $25(OH)D_3$ was the major metabolite in this organ in the deficient state. In both replete groups, $1,25(OH)_2D_3$ was not detected, while $24,25(OH)_2D_3$ increased with the administration of the vitamin.

With the addition of 4μg vitamin D_3, the concentration of the parent vitamin was 68 percent that of $25(OH)D_3$ and was four fold greater than $25(OH)D_3$ (48.6 ± 3.0pmol/g) in the 16μg group. In the replete states the content of $25(OH)D_3$ was greater than the amount encountered in liver but less in value than adipose tissue.

Vitamin D_3 esters were present in both replete groups, the values being higher in the group receiving 16μg dosages.

5.3.3 Liver.

The profile of metabolites in the liver in all groups showed vitamin D_3 esters to be prominent in the replete state with small quantities present in the deplete groups. Liver contained higher levels of vitamin D_3 esters than the other organs in each group examined.

Vitamin D_3 presented as the chief form of the steroid in both replete groups, the concentration being 6.8 ± 2.5pmol/g in the 4μg group and ultimately 61.3 ± 13.9pmol/g with the larger dosage. $25(OH)D_3$ also increased in the replete phase with larger doses of the vitamin, its highest level being 7.86 ± 1.08 pmol/g (3.1 ± 0.4ng/g) S m a l l amounts of vitamin D_3 esters observed in the deficient chicks, were more than twice that of vitamin D_3 and four fold greater than $25(OH)D_3$ in the 4μg group. With the highest

vitamin D_3 dose administered, the value of this metabolite was almost 70 percent of vitamin D_3.

The dihydroxyvitamin metabolites were present in the replete tissues, $24,25(OH)_2D_3$, although not detected in the deplete groups, was greater than $1,25(OH)_2D_3$ when the chicks received added vitamin D_3.

5.3.4 Storage organs:

skeletal muscle and adipose tissue.

The parent vitamin was the major steroid in both the 4 and 16μg groups in skeletal muscle, reaching 41.2 ± 6.8pmol/g (16.5 ± 2.7ng/g) with the maximum dosage. The amounts of $25(OH)D_3$ were low in the deplete groups but increased with the administration of vitamin D_3, the levels not being as high as those recorded in kidney, liver and adipose tissue with 16μg vitamin doses.

The dihydroxylated metabolite $24,25(OH)_2D_3$ not detected in the deplete state, recorded 0.52 ± 0.11pmol/g with 4μg and 0.78 ± 0.27pmol/g with 16μg doses, while $1,25(OH)_2D_3$ was not detected in both replete states.

In the deficient state, vitamin D_3 esters were not detectable in muscle but increased to 1.65 ± 1.14 and 6.65 ± 0.99pmol/g in the 4μg and 16μg groups respectively, but not reaching the high levels seen in the liver.

Adipose tissue proved to have a strong affinity for vitamin D_3 and $25(OH)D_3$ metabolites. No fat was visible in the vitamin D deficient chick (group SD) when a single dose of vitamin D_3 was administered 48 hours before death, however in the other deplete group (MD) where adipose tissue was obtainable, $25(OH)D_3$ was present in the

vitamin D_3 deficient state at a low level, the other metabolites being present in small quantities except $24,25(OH)_2D_3$ which was not detected in this stage.

With the administration of vitamin D_3, the parent vitamin was most prominent in both replete groups recording four and three fold the quantity stored in liver and muscle respectively, with physiological doses of vitamin D_3. With the 16μg dose, the concentration of the vitamin was two and three times greater than in those organs.

In the replete state the dihydroxyvitamin metabolite, $24,25(OH)_2D_3$ was present but $1,25(OH)_2D_3$ was not detected in either of these groups. Vitamin D_3 esters were observed in the groups examined, being slightly higher than that in muscle but not reaching the high values found in liver.

Each section in figure 20, illustrates the amount of a metabolite contained in organs for that particular metabolite. Plasma contains the highest values of $25(OH)D_3$ and $24,25(OH)_2D_3$, adipose tissue acting as a storage organ for the parent vitamin, with liver recording the largest amount of putative vitamin D_3 esters. The high level of $1,25(OH)_2D_3$ in the liver is difficult to explain.

5.4 Vitamin D_3 Esters.

Unfortunately it was not possible to verify the presence of vitamin D_3 esters due to the lack of any authentic compounds. However it is very suggestive that these compounds being less polar than vitamin D_3, which eluted from the silicic acid chromatography column with the addition of ether: hexane (20:80 v/v), were vitamin D_3 esters.

The study of Mawer and Backhouse (1965) appears to confirm this suggestion,

as the separation of metabolites of isotopically labelled vitamin D_3 on silicic acid columns, revealed three peaks. The third peak represented cholecalciferol, the second pre-cholecalciferol and peak 1 was the presence of vitamin D_3 esters.

When checking the purity of the [^{3}H]vitamin D_3 in silicic acid chromatography, no radioactivity was present in the eluate when the column was perfused with a solvent mixture of ether : hexane (20:80 v/v) (refer figure 18). Thus the radioactivity present in the eluate from samples after perfusion of the column with ether: hexane 20:80 v/v, was very suggestive of vitamin D_3 esters.

The amount of putative vitamin D_3 esters in the liver was very insignificant in both groups of vitamin D_3 deficient chicks. In the replete groups, the liver harboured the greatest amount of these metabolites, adipose tissue containing the next largest. In the 16μg group, the amount in skeletal muscle was practically equal to that present in adipose tissue.

5.5 Unknown polar metabolites.

As already mentioned, the results of these polar metabolites are recorded as total dpm per ml/gram or as a percentage of the total counts per ml/gram of tissue.

In the majority of tissues, these metabolites represent less than 10% of the radioactivity found in the tissues.

The quantity of metabolites present were similar in the two plasma deplete groups, but doubled in amount with the addition of 4μg vitamin D_3 doses. With 16μg doses, the plasma concentration was practically treble that present in the physiological group. The percentage of these metabolites present in plasma was almost equal in all four groups.

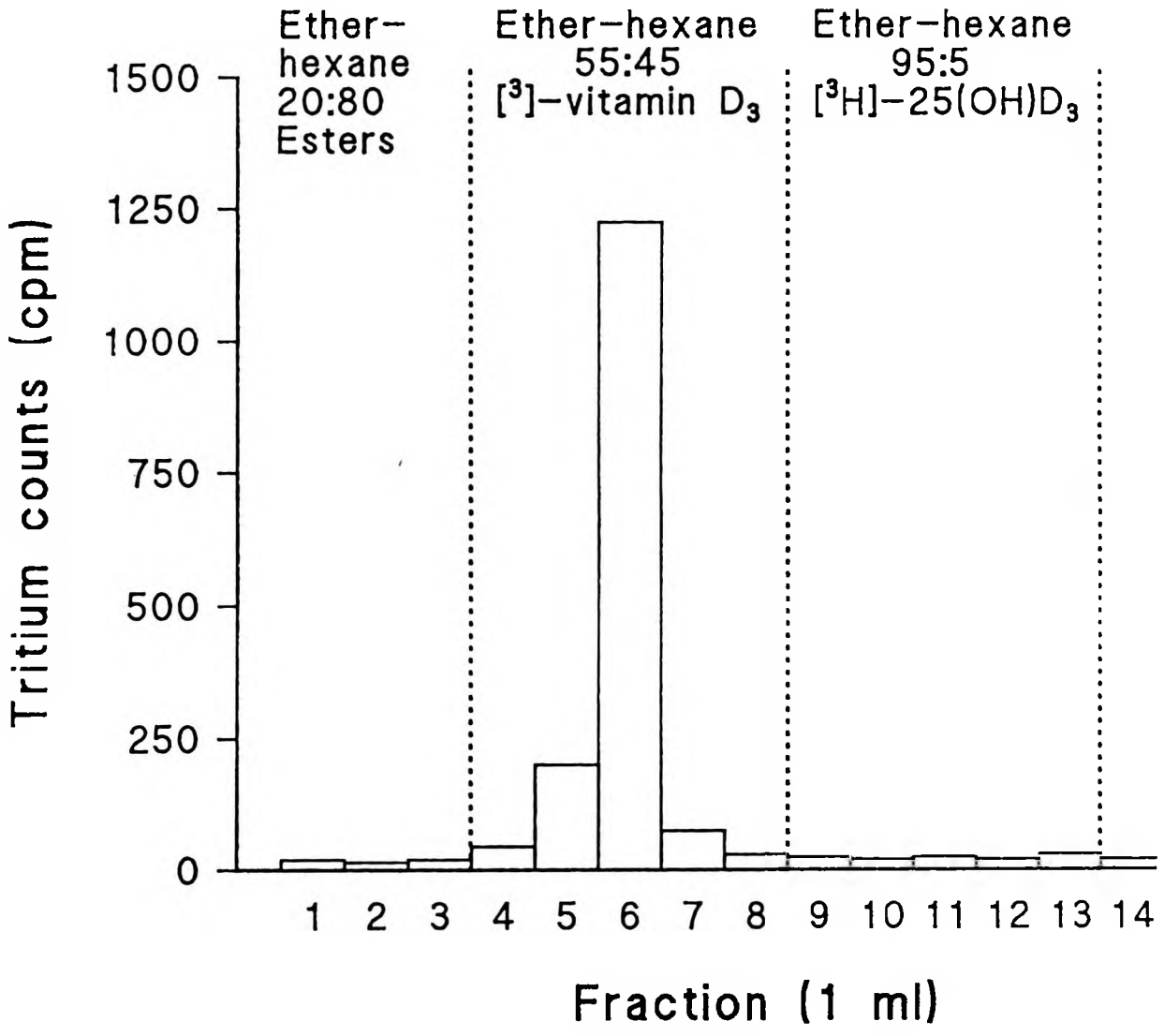


Figure 18.

Silicic acid chromatography for elution of vitamin D_3. (Ether:hexane 55:45 v/v).

The kidney recorded the highest counts of all tissues in both the 4μg and 16μg groups, while the target organs, kidney and duodenal mucosa, had the highest percentage counts per gram of tissue in both the above groups.

Adipose tissue had the lowest percentage of these metabolites per gram of tissue in three groups, skeletal muscle being less in the 4μg group.

Table 17.

Definitive results: putative vitamin D_3, vitamin D_3, $25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$ and polar metabolites in plasma, duodenal mucosa, kidney, liver, skeletal muscle and adipose tissue.
a) vitamin D_3 deficient birds-single dose (SD);
b) vitamin D_3 deficient birds-multiple doses (MD);
c) birds receiving vitamin D_3 4μg doses;
d) birds receiving vitamin D_3 16μg doses

Table 17 a)

Vitamin D_3 Deficient (Single Dose). (pmol/g or pmol/ml)							
	Pt VitD_3 Esters	VitD_3	25 $(OH)D_3$	24,25 $(OH)_2D_3$	1,25 $(OH)_2D_3$	#Polar Metab	##Polar Metab %
P*	ND	ND	0.33 ±0.08	ND	0.014 ±0.006	301 ±92	5.5 ±2.1
I	ND	0.01 ±0.003	0.01 ±0.003	ND	0.033 ±0.007	210 ±41	15.4 ±2.9
K	ND	0.02 ±0.008	0.08 ±0.011	ND	0.02 ±0.004	394 ±63	18.5 ±4.1
L	0.04 ±0.012	0.03 ±0.01	0.07 ±0.017	ND	0.01 ±0.005	164 ±88	3.6 ±0.4
M	ND	0.01 ±0.004	0.05 ±0.01	ND	ND	46 ±11	3.4 ±0.5
F	NO SPECIMEN						

Table 17(b).

Vitamin D_3 Deficient (Multiple Doses). (pmol/g or pmol/ml)							
	Pt$VitD_3$ Esters	$VitD_3$	25 $(OH)D_3$	24,25 $(OH)_2D_3$	1,25 $(OH)_2D_3$	#Polar Metab	##Polar Metab %
P*	ND	ND	0.28 ±0.02	ND	0.016 ±0.001	253 ±67	4.9 ±1.5
I	ND	ND	0.015 ±0.005	ND	0.055 ±0.01	84 ±33	5.2 ±1.8
K	ND	ND	0.06 ±0.01	ND	0.014 ±0.006	176 ±114	6.5 ±1.2
L	0.04 ±0.01	ND	0.06 ±0.01	ND	0.01 ±0.002	59 ±9	4.0 ±0.98
M	ND	ND	0.045 ±0.01	ND	ND	192 ±10	16.6 ±1.4
F	0.02 ±0.003	0.06 ±0.005	0.14 ±0.02	ND	0.01 ±0.005	136 ±56	2.7 ±0.88

Table 17(c).

Vitamin D_3 4μg doses (pmol/g or pmol/ml)							
	PtVitD_3 Esters	VitD_3	25 (OH)D_3	24,25 $(OH)_2D_3$	1,25 $(OH)_2D_3$	#Polar Metab	##Polar Metab %
P*	0.16 ±0.03	3.9 ±0.9	48.4 ±7.1	4.4 ±1.1	0.06 ±0.01	621 ±138	5.5 ±2.4
I	0.35 ±0.14	0.95 ±0.3	1.18 ±0.22	0.19 ±0.03	0.16 ±0.03	65.0 ±10.2	13.8 ±2.1
K	0.83 ±0.38	4.3 ±0.73	5.83 ±1.15	0.67 ±0.21	ND	807 ±196	17.2 ±8.2
L	15.28 ±4.97	6.8 ±2.5	3.95 ±0.93	1.13 ±0.19	0.11 ±0.03	146 ±48	3.8 ±0.3
M	1.65 ±1.14	9.5 ±1.9	3.8 ±0.58	0.52 ±0.11	ND	66 ±19	2.0 ±0.8
F	3.13 ±0.82	29.22 ±3.43	7.79 ±0.88	0.97 ±0.23	ND	219 ±37	2.4 ±0.7

Table 17(d).

	Vitamin D_3 16µg doses (pmol/g or pmol/ml)						
	$PtVitD_3$ Esters	$VitD_3$	$25(OH)D_3$	$24,25(OH)_2D_3$	$1,25(OH)_2D_3$	#Polar Metab	##Polar Metab %
P*	0.6 ±0.2	33.4 ±2.7	75.5 ±8.9	6.9 ±1.3	0.09 ±0.03	1770 ±311	5.6 ±1.3
I	0.72 ±0.08	8.6 ±1.3	1.64 ±0.43	0.42 ±0.12	0.12 ±0.03	279 ±44	10.9 ±1.7
K	2.76 ±0.48	48.6 ±3.0	12.44 ±3.37	2.7 ±0.2	ND	869 ±120	6.4 ±2.0
L	36.26 ±11.84	61.3 ±13.9	7.86 ±1.08	1.2 ±0.17	0.9 ±0.3	547 ±122	2.5 ±0.3
M	6.65 ±0.99	41.2 ±6.8	5.16 ±1.01	0.78 ±0.27	ND	196 ±116	1.8 ±1.4
F	7.77 ±0.68	127 ±11	20.6 ±2.4	0.9 ±0.2	ND	401 ±96	1.2 ±0.2

$PtVitD_3$ Esters Putative vitamin D_3 esters; * P Plasma; I Duodenal mucosa; K Kidney; L Liver; M Muscle; F Adipose Tissue; #Polar metabolites eluted with Methanol(100%), expressed as dpm/ml or dpm/g; ## Polar metabolites-percentage of counts per ml of plasma or per gram of tissue. ND Not detected.

Figure 19.

Definitive results:

profiles of putative vitamin D_3 esters, vitamin D_3,

$25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$

in

i) plasma, ii) duodenal mucosa, iii) kidney, iv) liver,

v) skeletal muscle, vi) fat

which are

a) and b) vitamin D_3 deficient

receiving c) 4μg d) 16 μg vitamin D_3

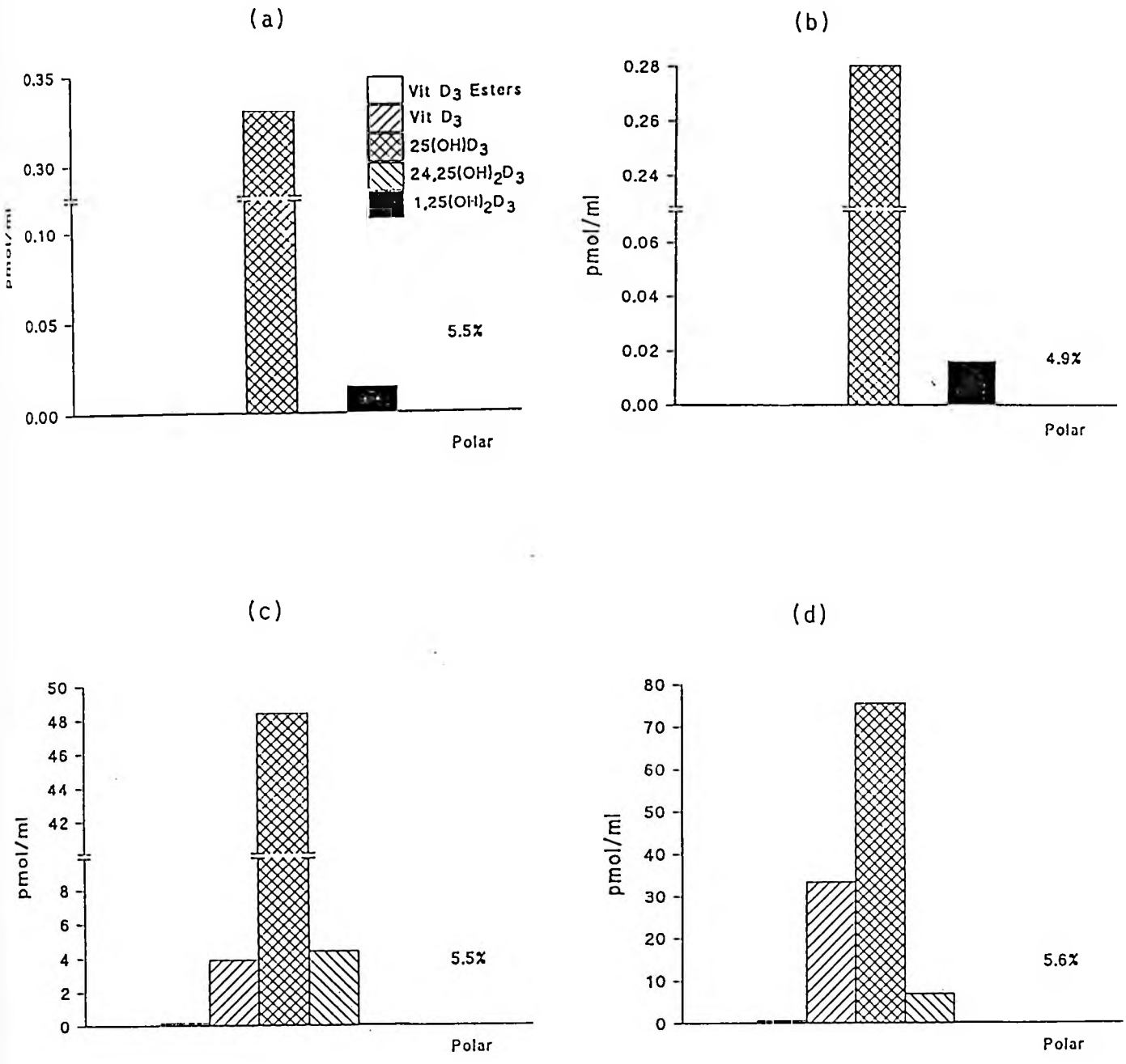


Figure 19 i). **Vitamin D metabolite concentrations in plasma in the various groups of chickens.**

a) **vitamin D_3 deficient (single dose)**
b) **vitamin D_3 deficient (multiple dose)**
c) **vitamin D_3 (4μg dose)**
d) **vitamin D_3 (16μg dose)**

Y axis enlarged 100 times in vitamin D deficient chicks (a and b).

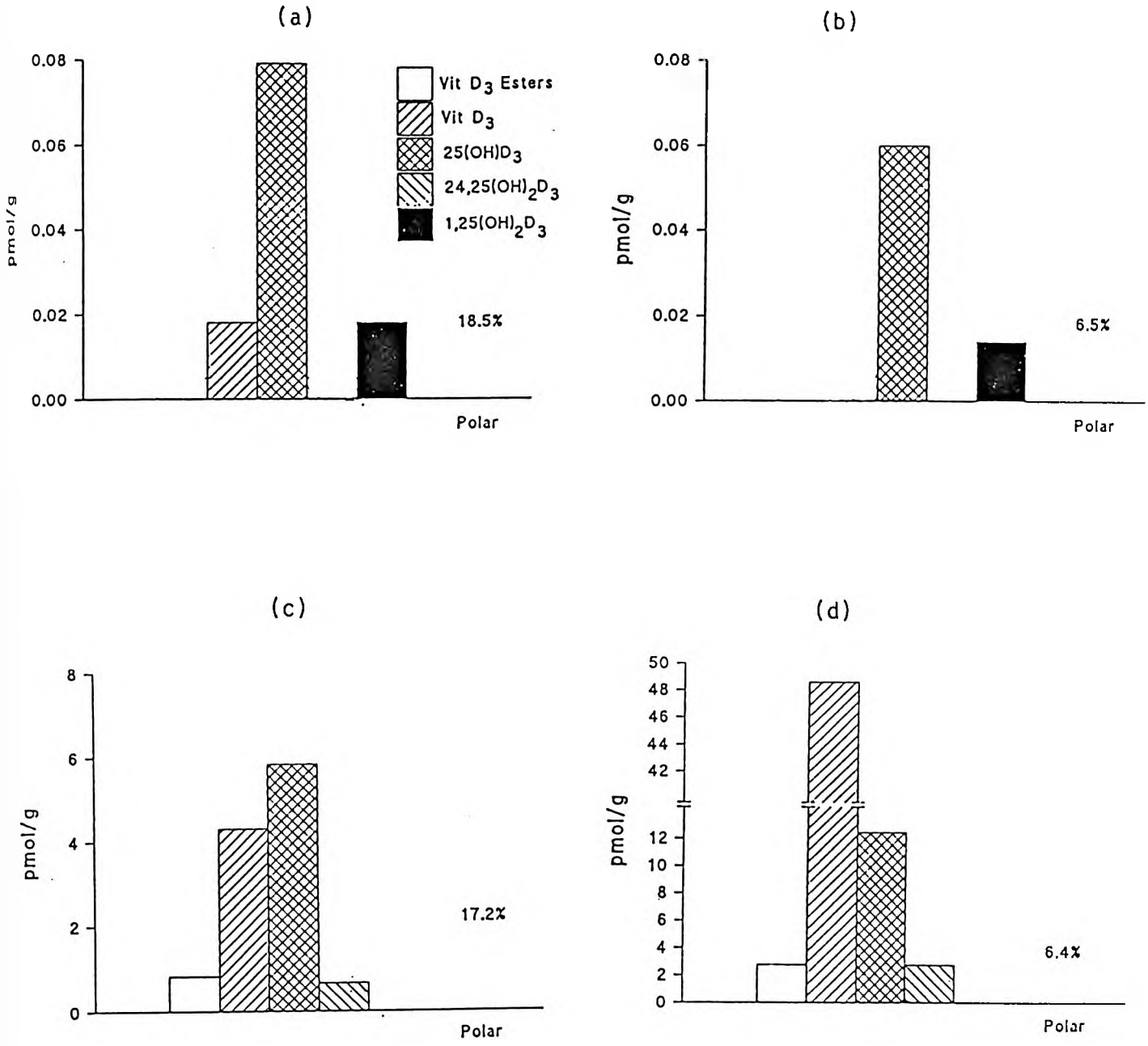


Figure 19 iii). **Vitamin D metabolite concentrations in kidney in the various groups of chickens.**

a) vitamin D_3 deficient (single dose)
b) vitamin D_3 deficient (multiple dose)
c) Vitamin D_3 (4μg dose)
d) Vitamin D_3 (16μg dose)

Y axis enlarged 100 times in vitamin D deficient chicks (a and b).

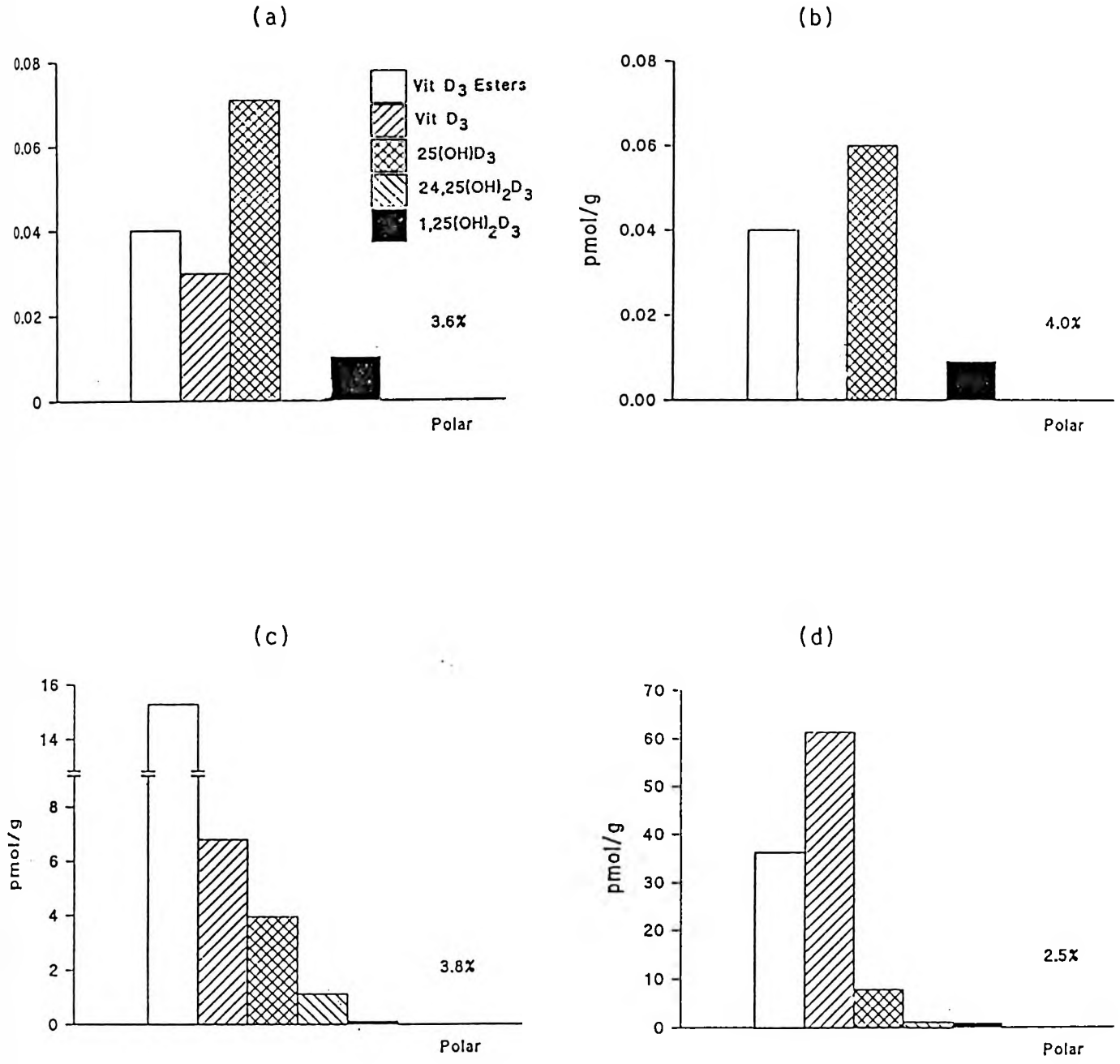


Figure 19 iv). **Vitamin D metabolite concentrations in liver in the various groups of chickens.**

a) **vitamin D_3 deficient (single dose)**
b) **vitamin D_3 deficient (multiple dose)**
c) **Vitamin D_3 (4μg dose)**
d) **Vitamin D_3 (16μg dose)**

Y axis enlarged 100 times in vitamin D deficient chicks (a and b).

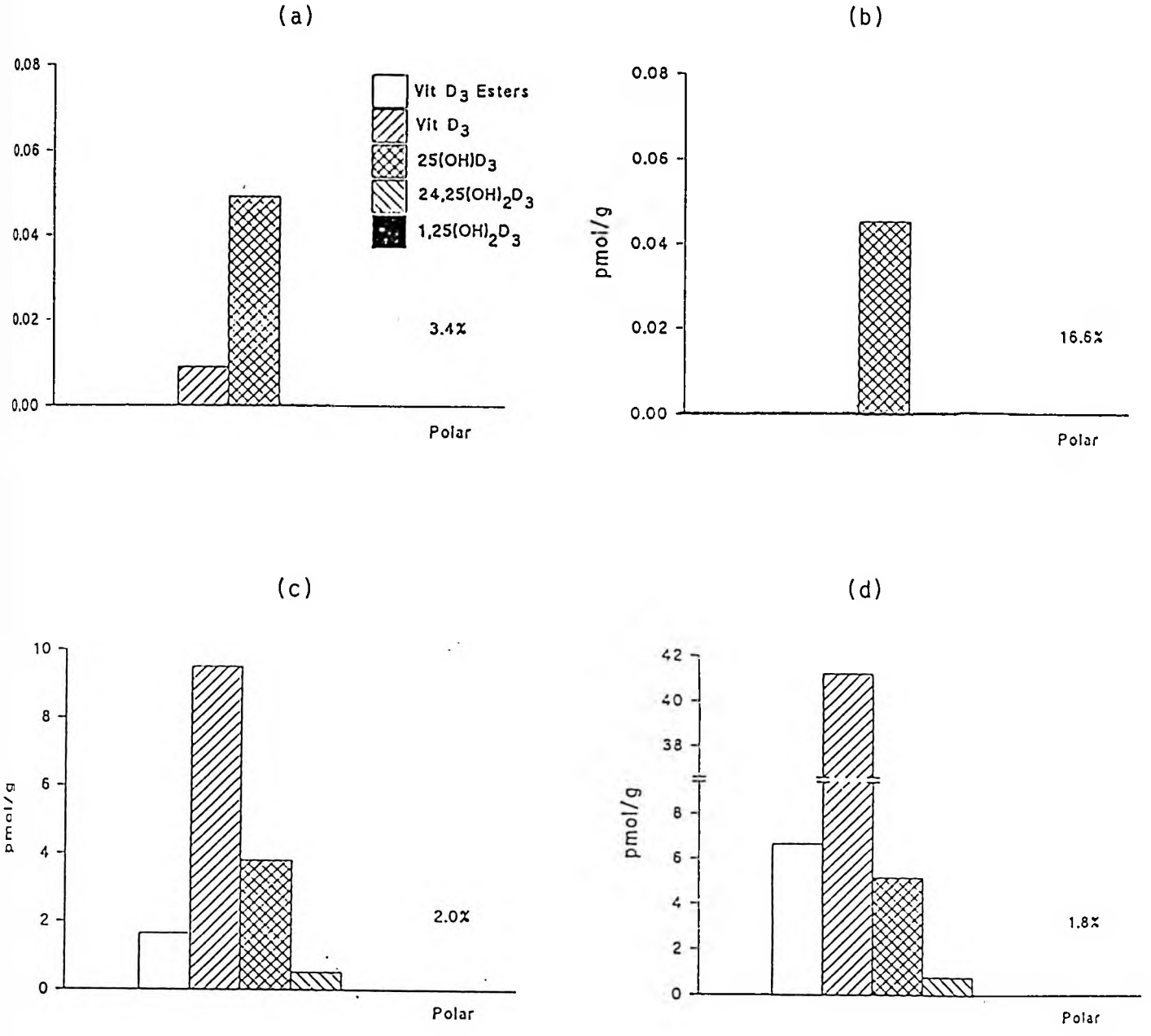


Figure 19 v). **Vitamin D metabolite concentrations in muscle in the various groups of chickens.**

a) **vitamin D_3 deficient (single dose)**
b) **vitamin D_3 deficient (multiple dose)**
c) **Vitamin D_3 (4μg dose)**
d) **Vitamin D_3 (16μg dose)**

Y axis enlarged 100 times in vitamin D deficient chicks (a and b).

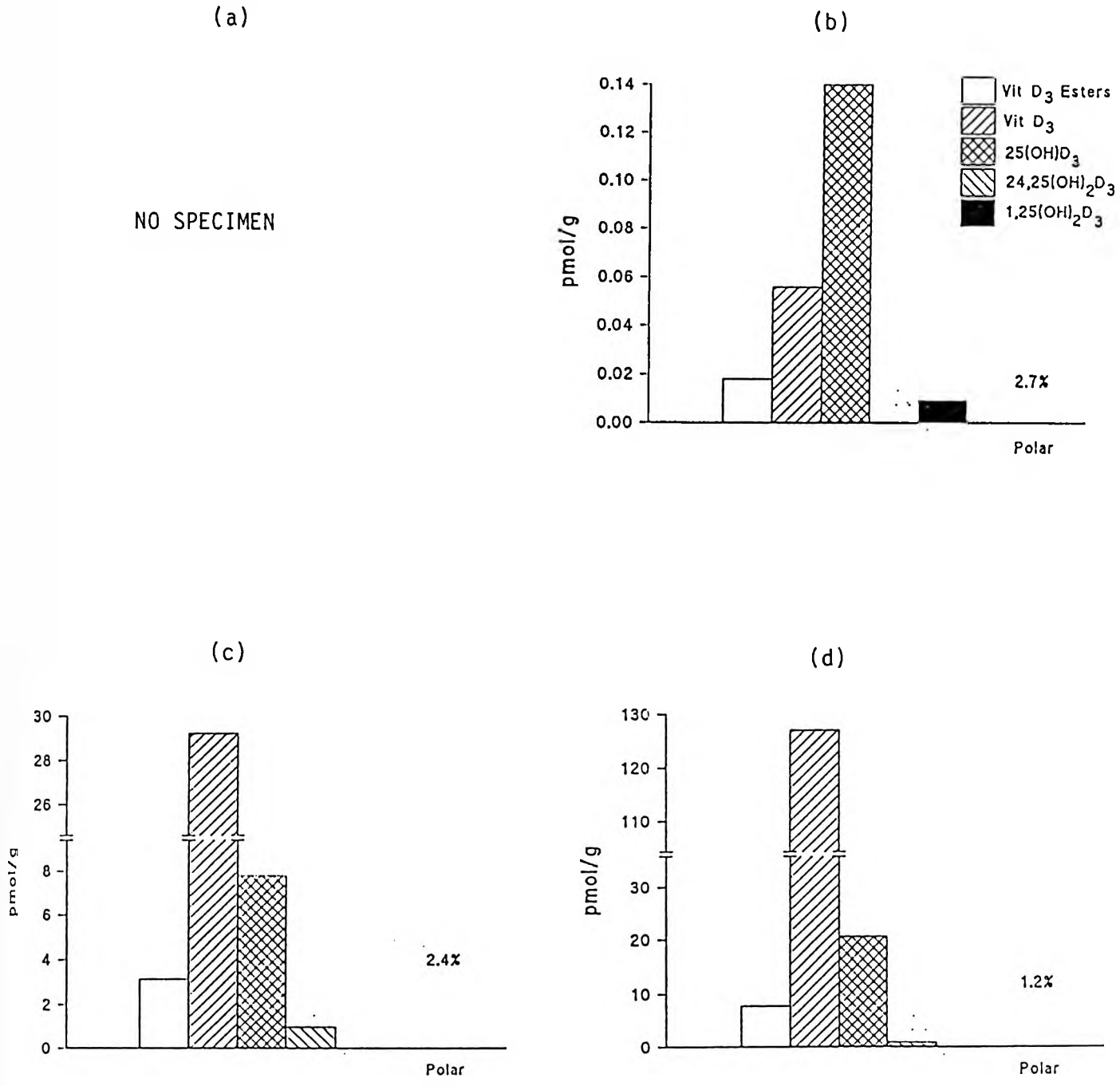


Figure 19 vi). **Vitamin D metabolite concentrations in fat in the various groups of chickens.**

a) **vitamin D_3 deficient (single dose)**
b) **vitamin D_3 deficient (multiple dose)**
c) **Vitamin D_3 (4μg dose)**
d) **Vitamin D_3 (16μg dose)**

Y axis enlarged 100 times in vitamin D deficient chicks (a and b).

Figure 20.

Definitive results:

pattern of

(a) putative vitamin D_3 esters, (b) vitamin D_3, (c) $25(OH)D_3$

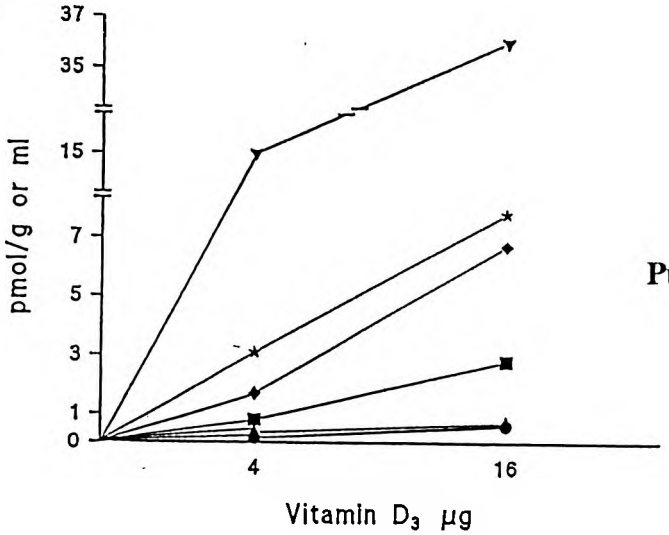
(d) $24,25(OH)_2D_3$ (e) $1,25(OH)_2D_3$

in chicks in a vitamin D_3 deficient state (0)

and after administration of 4 μg or 16 μg of vitamin D_3

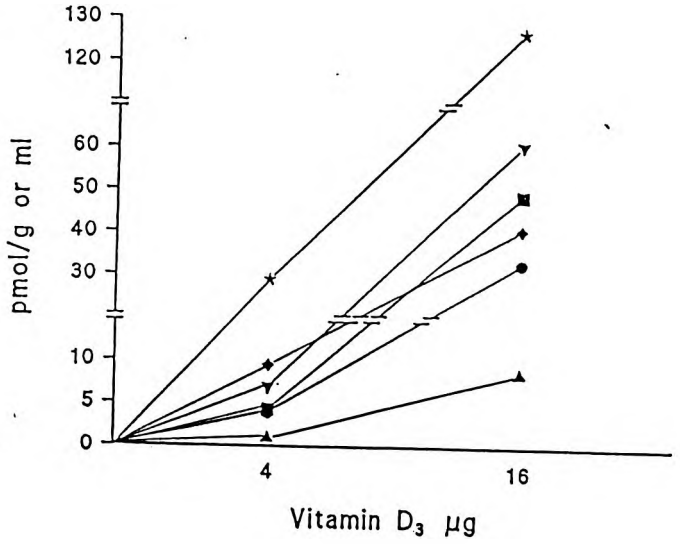
in plasma, duodenum, kidney, liver, muscle and fat.

(a)



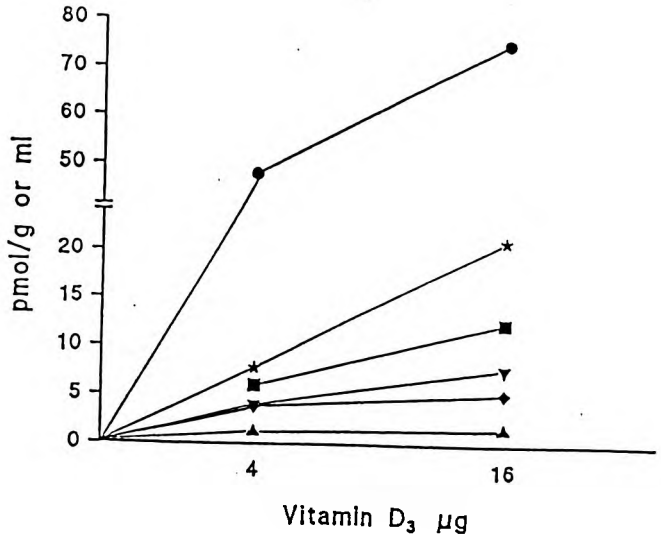
Putative vitamin D_3 esters

(b)



Vitamin D_3

(c)



25-(OH)D_3

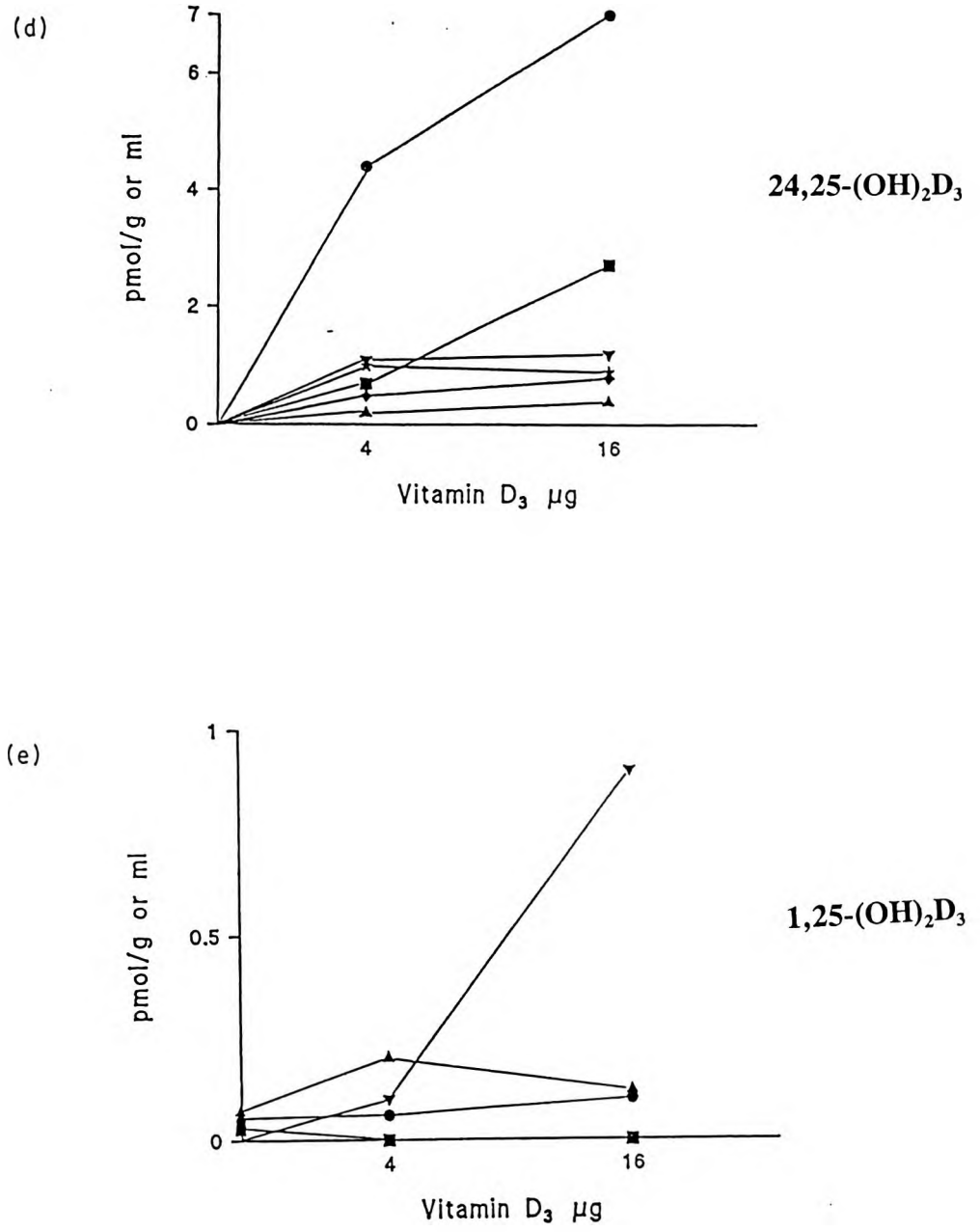


Figure 20. a) Vitamin D_3 esters; b) Vitamin D_3; c) $25(OH)D_3$; d) $24,25(OH)_2D_3$ and e) $1,25(OH)_2D_3$ at 4µg and 16µg.

● = Plasma
* = Fat
■ = Kidney
▼ = Liver
◆ = Muscle
▲ = Duodenum

Chapter 6

Discussion and conclusions.

6.1 The chick as a suitable animal model to study vitamin D_3 metabolism.

The suitability of the chick as an animal model for the study of the vitamin D_3 endocrine system appears to be most satisfactory. Of more than 30 metabolites isolated in the metabolic pathway of the vitamin, at least half has been isolated from an avian system. Specific receptors for $1,25(OH)_2D_3$ have been found in no less than 23 tissues and at least 10 were identified in avian studies. CaBP has also been demonstrated in some chick tissues (Norman 1990a).

An advantage of the chick as an animal model is that it can easily be rendered vitamin D deficient. In approximately 14 days after hatching, the chick if not exposed to light containing UV-B rays and a diet containing vitamin D_3, develops rachitic symptoms. (Norman 1966). On hatching, the plasma concentration of 25(OH)D is approximately 14 ng/ml, and if confined to rachitic conditions, within three weeks the plasma level of this metabolite becomes undetectable (Hughes et al 1977). At this stage the plasma $24,25(OH)_2D_3$ is absent or present in minute quantities and $1,25(OH)_2D_3$ is also present in subnormal amounts.

Discrimination against vitamin D_2 by the chick could be a disadvantage to its use as an animal model, but most experimental work performed has been with vitamin D_3, and generally in the human, the main form of vitamin D is the naturally occurring vitamin D_3. Thus this disadvantage is not significant. Furthermore, the present study only used vitamin D_3 and its metabolites.

The minimum daily requirements of chicks is between 5 and 10 IU (0.125 and 0.25μg) vitamin D_3 (Haussler and Rasmussen 1972). In the definitive study the amounts of

vitamin D_3 administered were as follows: Group SD received 0.07μg (2.8 IU) 48 hours before being sacrificed, Group MD averaged 0.018μg (0.72 IU) per day, while the 4μg and 16μg groups each averaged 1.05μg (42 IU) and 4.2μg (168 IU) daily respectively for a period of 16 days. Thus the first two groups were on a vitamin D_3 deficient regimen, the third group received a physiological amount and the last group was on a supraphysiological programme.

6.2 Vitamin D_3 deficient chickens.

During the studies it was apparent to the author when inspecting the chickens in their cages, which birds were vitamin D_3 deficient. The chicks receiving sufficient vitamin D_3 were very mobile, eating well and making active attempts to avoid being captured. This was in contrast to the deficient groups, where the chicks were smaller in size, immobile and sitting on their haunches (de Boland 1983). Patches of skin were visible due to the absence of feathers. Further, several deaths occurred in the D deficient groups of birds in the early experiments. The amount of blood collected from deficient birds was less than that recovered from vitamin D_3 replete birds, while the former's organs were also smaller in size.

6.3 Weights of chicks in various groups.

The mean weights of the two vitamin D_3 deficient groups were statistically similar, as were the mean weights of the two groups receiving adequate doses of the vitamin. However the difference in mass between the deplete and replete groups was very significant

($p= 0.0001$)(refer table 16).

It was noticed that growth in chicks raised under rachitic conditions plateaus by the fourth week, and weight does not increase. (Norman 1966). The administration of vitamin D_3 to chicks causes an enhanced whole body growth. This raises the question of whether or not the vitamin D system acts as a growth hormone. Another possible explanation is that the deficient birds suffered from muscle weakness and anorexia which reduced the food intake and thus the growth of the birds.

6.4 Is vitamin D a growth hormone?

The mechanisms by which $1,25(OH)_2D_3$ affects growth are difficult to determine due to its effects not only on other hormones, on calcium and phosphorus metabolism but also on cell proliferation and differentiation. The association between vitamin D and insulin secretion may be a factor in the growth effects that have been reported (Bikle 1992). Vitamin D depletion diminishes the secretion of insulin and the addition of $1,25(OH)_2D$ results in rapid normalization of insulin release in vitamin D deficient rats. Steenbock and Herting (1955) noted that the marked growth promoting effects of vitamin D were not confined only to skeletal tissue but also involved soft tissue metabolism. The indirect role of $1,25(OH)_2D$ in skeletal growth is well accepted, as is its role in many other organs (Walters 1992).

Clark et al (1986) evaluated the role of somatomedin-C/insulin-like growth factor 1 (Sm-C/IGF-1) in young rats comparing growth rates when receiving different amounts of food and under hypocalcaemic and vitamin D deficient states. They concluded that in vitamin D deficient states, alterations in Sm-C/IGF-1 concentrations in growing rats were

not related to lack of vitamin D but rather to the poor nutrition resulting from deficient intake of food. In hypopcalcaemic states even with serum $1,25(OH)_2D$ levels raised three to four fold higher than normal, the serum levels of Sm-C/IGF-1 levels were depressed as was weight gain which appeared to be related to the anorexia.

In the present study, the groups which received only one dose of vitamin D_3, had no visible adipose tissue despite a careful examination. This was in contrast to the chicks which developed fat after receiving repeated subnormal doses of the vitamin (refer 5.3.4). The mean weight of the chicks in the latter group was nearly twenty percent higher than that of the deficient group which received a single dose of vitamin D_3 but the difference was not statistically significant (refer 5.2.2). One must consider whether the administration of repeated minute doses caused these chicks to be less anorexic and possibly ate more than the birds given a single dose of vitamin D_3 shortly before death.

6.5 Myopathy of vitamin D_3 deficient chickens.

Muscular weakness is a prominent feature in vitamin D deficient rickets and osteomalacia.(Dent and Smith 1969; Marsden 1973).

The immobility of the deficient chicks is reminiscent of the features of nutritional rickets in babies kept indoors and not exposed to sunlight. These children when hospitalized lay in one position in a pseudoparetic state and the nurses were instructed to change their position at regular intervals.

The association of vitamin D or its active biological metabolite $1,25(OH)_2D_3$ on muscle metabolism is well documented. The types of muscle involved include smooth (Wakasugi et al 1991), cardiac (O'Connell et al 1994) and skeletal muscle.

The effects of $1,25(OH)_2D$ on embryonic myoblasts include the stimulation of protein synthesis (Drittanti et al 1993), phosphate accumulation (Bellido and Boland 1991), and the non-genomic action with generation of inositol triphosphate and diacylglycerol (Morelli et al 1993). Mature skeletal muscle fibres in the rat, in which no $1,25(OH)_2D$ receptors have been detected, demonstrated $1,25(OH)_2D_3$ dependent Ca^{2+} uptake (Massheimer and deBoland 1992; deBoland et al 1991) and the presence of vitamin D dependent CaBP involved in muscle fibre function (Hietanen-Peltola et al 1992).

Birge and Haddad (1975) and Pointon et al (1979) postulated that the possible effect of vitamin D on muscle is mediated through metabolites other than $1,25(OH)_2D$ such as 25(OH)D, as analysis of muscle cytosol revealed a protein fraction which specifically bound 25(OH)D and whose affinity for $1,25(OH)_2D$ was less. Birge (1978) noted that phosphate depletion was associated with myopathy of osteomalacia and considered that 25(OH)D at physiological concentrations might exert important biological effects. It has been suggested that 25(OH)D may have a direct influence on intracellular accumulation of phosphate in muscle and is important in maintenance of muscle metabolism and function.

de Boland et al (1983) examining muscle in vitamin D deficient conditions, found altered protein contents of mitochondria, and modified distribution pattern of components of actinomycin contractile complex.

The vitamin D deficient myopathy, which responds rapidly to vitamin D therapy, is considered by Kumar (1991) not to be related to changes in concentration of the divalent ions.

It is difficult to come to a definite conclusion as to the cause of this myopathy associated with vitamin D rickets and osteomalacia. However the lack of the active hormone $1,25(OH)_2D$, appears to be the main factor in the causation of this condition,

possibly due to involvement of both the mineral and other changes that occur in the organelles of the cell and its fibres.

6.6 Effects of vitamin D_3 in mineral homeostasis.

The results showing the effects of vitamin D_3 on calcium metabolism (refer table 14) indicate that the plasma calcium levels are very dependent on vitamin D status.

The chicks which received a physiological dose of vitamin D_3 (1μg) had a plasma calcium value equivalent to the reference value ($p=0.83$) while the chicks administered supraphysiological amounts of vitamin D_3 (100μg) had abnormally raised plasma calcium concentrations ($p=0.007$). All the groups which received repeated doses of 0.5μg or less of vitamin D_3, had plasma values of calcium significantly lower than the concentration of the reference value for chicks of the same age ($p<0.0001$) (refer table 14).

Plasma phosphorus values (refer table 14) in the replete groups were not significantly different from those in the reference value ($p > 0.05$ in the 100μg group, and $p > 0.10$ in the 1.0μg group), but the values in the chicks receiving no vitamin D_3 or very small amounts were lower than those recorded in the reference range.

A failure of bone mineralization due to mineral disturbance as a result of vitamin D_3 deficiency, was borne out in this study; the long bones in the deficient chicks were pliable and difficult to fracture. These weakened bones were unable to support the weight of the body, resulting in deformities.

Higher concentrations of plasma alkaline phosphatase were evident with the lower administered dosages of vitamin D (refer table 14). All groups receiving repeated doses of 0.5μg or less of vitamin D_3 had higher levels of the enzyme than the groups receiving

physiological and supraphysiological amounts of the vitamin.

The actual role of plasma alkaline phosphatase in bone mineralization has not been finalised. The activity of this enzyme appears to correlate with calcium and phosphate metabolism, osteoblast activity and bone turnover.

6.7 Vitamin D_3 and its metabolites in plasma and tissues in the physiological state.

The concentrations of metabolites in the plasma, reflect the net effect of synthesis and catabolism of that particular metabolite. The various plasma values measured at one moment in time do not reflect the dynamics or flux of the biological system, thus one is not aware whether the production or catabolism of a particular compound is increased or repressed. However a great deal of information can be obtained from measuring plasma 25(OH)D and $1,25(OH)_2D$ levels in humans and animals. Plasma 25(OH)D levels indicate the vitamin D status of the subject, while $1,25(OH)_2D$ probably reflects calcium homeostasis. These metabolites are helpful in defining the pathogenesis of some calcium related syndromes.

In the replete state (4μg group), vitamin D_3 and the three major metabolites were all present in plasma. The most abundant metabolite in the circulation in the physiological state was $25(OH)D_3$ which as already mentioned, serves as a good indicator of vitamin D status in the body and its reserves. In the physiological replete state, plasma $25(OH)D_3$ was 48.4 ± 7.1 pmol/ml (19.4 ± 2.9ng/ml).

The level of the active metabolite $1,25(OH)_2D_3$ was 0.06 ± 0.01 pmol/ml (24.9 ± 5.9pg/ml), while the other dihydroxylated metabolite $24,25(OH)_2D_3$ present in vitamin D

replete animals, was 9 percent of $25(OH)D_3$, the concentration being 4.4 ± 1.1 pmol/ml (1.8 ± 0.47ng/ml). The parent vitamin D_3 was also detected in the physiological state at a level of 3.9 ± 0.9 pmol/ml (1.51 ± 0.33ng/ml) compared with 2.3 ± 1.1 ng/ml in normal humans (Hollis et al 1981) .

Comparing the results in the present study with those obtained by Horst et al (1981) who analysed the plasma concentrations of vitamin D and its metabolites in five species of animals including chickens aged 10 to 12 months, it was noted that the normal values vary in different species, the concentration of serum $25(OH)D_3$ in the chick being 25.3 ±3.9ng/ml. In Sedrani's series (1984), the value of this metabolite in chicks (3 to 7 weeks of age) on a normal vitamin D_3 diet, was 12.4 ± 2.1 ng/ml. Hughes et al (1977) noted that one day old chicks had serum $25(OH)D_3$ levels of approximately 14 ng/ml and when maintained on a vitamin D_3 replete diet recorded 21 to 35 ng/ml between 3 to 4 weeks of age. These different values in chickens probably reflect diet variations in the actual amount of vitamin D_3 the chicks received, and the calcium and phosphorus content of the diets and possibly their age.

The serum levels of the active metabolite $1,25(OH)_2D$ also show great variations in different species of animals (Horst et al 1981). Levels of $1,25(OH)_2D$ are dependent not only on the vitamin D status, but also on the calcium and phosphorus content of the diet, and the calcium requirements of the animal.

In the present study the plasma $1,25(OH)_2D_3$ concentration was 0.06 ± 0.01 pmol/ml (24.9 ± 5.9pg/ml) compared with 21.2 ± 2.1pg/ml in the study of Horst et al (1981) and 66.6pg/ml in Sedrani's series (1984). In the study by Hughes et al (1977), the plasma levels of this metabolite in chicks varied between 51 and 75 pg/ml at 3 to 4 weeks of age, the level being 89 pg/ml in the first day after hatching.

The levels obtained for circulating $24,25(OH)_2D_3$ (1.8 ± 0.45ng/ml) correlate well with that reported by Horst et al (1981) (2.3 ± 0.2ng/ml), the values being approximately 10 percent of the serum $25(OH)D_3$ levels. A similar relationship has been reported in man (Mawer et al 1971).

The serum vitamin D_3 levels in the replete state in this study concur with those reported by Horst et al (1981), where the serum vitamin D_3 concentration in chicks was 2.3 ± 0.3ng/ml. Ratzkowski et al (1982) recorded a plasma vitamin D_3 concentration of approximately 0.2ng/ml after the chicks averaged 14.5 IU vitamin D_3 daily over 11 days.

The author was unable to find reports of vitamin D esters being measured in the plasma of animals and thus comparisons could not be made with other studies.

In this study, examination of the organs in the physiological group, revealed that $25(OH)D_3$ was the major metabolite in the duodenal mucosa and kidney. However in liver, muscle and adipose tissue, it was exceeded by vitamin D_3, adipose tissue containing three and four times more vitamin D_3 than muscle and liver respectively. It has already been noted that liver accumulates a large amount of vitamin D after administration for the conversion to 25(OH)D, adipose tissue and muscle acting as depots for the storage of the vitamin. The content of $25(OH)D_3$ for conversion to dihydroxyvitamins D_3 in the kidney, was higher than liver, and was next in value to adipose tissue.

Small amounts of the dihydroxyvitamin D_3 metabolites were present in tissues, $24,25(OH)_2D$ being larger in amount than $1,25(OH)_2D$, the latter metabolite being undetected in kidney, muscle or adipose tissue in the replete state.

The profile of vitamin D_3 and its metabolites in tissues in chicks receiving a daily average of 14.5 IU vitamin D_3 (Ratzkowski et al 1982) was fairly similar to the results obtained in this study. The tissues examined were intestine, kidney, liver and bone.

$25(OH)D_3$ was the major metabolite in the organs they examined, whereas vitamin D_3 was the chief form of the steroid in liver in the present study. Smaller quantities of cholecalciferol were present in the kidney and liver than in the present study, the same applying to $25(OH)D_3$ and $24,25(OH)_2D_3$. The profiles of $1,25(OH)_2D_3$ in kidney and liver in the two studies were fairly similar.

The findings in the present study reveal that the plasma profile of vitamin D_3 and its metabolites in the replete state in the chick, is similar to that found in humans and thus concur with the findings of Hughes et al (1977). Also the presence of vitamin D_3 and its metabolites in the various organs, is very suggestive of the findings of Mawer et al (1972) that adipose tissue and muscle serve as storage organs for vitamin D and 25(OH)D.

6.8 The effect of vitamin D_3 depletion on metabolites in plasma and organs.

A deficient supply of vitamin D due to lack of exposure to UV-B photons present in the solar system and a lack of dietary intake of vitamin D results in a specific plasma profile in the metabolites. As both groups SD and MD did not receive the minimum amount of daily vitamin D_3 required for the chick (between 5 and 10 IU Haussler and Rasmussen 1972), it must be assumed that both groups SD and MD received a vitamin D_3 deficient diet.

Jones and DeLuca (1988) have defined low plasma concentrations of vitamin D less than 1ng/ml, 25(OH)D less than 4ng/ml and $1,25(OH)_2D$ less than 15pg/ml in humans. Pettifor (1991) states that plasma levels of 25(OH)D less than 10ng/ml in humans generally indicate vitamin D deficiency. These results correlate well with those found in vitamin D_3

deficient chicks.

The profile of plasma metabolites in the deficient groups SD and MD show that all the results were below the levels just described. The concentrations of plasma $25(OH)D_3$ in both deficient groups were 0.33 ± 0.08 and 0.28 ± 0.02 pmol/ml (0.13 and 0.11 ng/ml) respectively. The active hormone $1,25(OH)_2D_3$ was present in minute amounts (0.014 ± 0.006 and 0.016 ± 0.001 pmol/ml (5.8 ± 0.4 and 6.7 ± 0.3 pg/ml) respectively, while $24,25(OH)_2D_3$ was not detected in either group of deficient chicks.

The results in this study correlate well with those described by Hughes et al (1977) who recorded non detectable serum levels of 25(OH)D and $1,25(OH)_2D_3$ when chicks were on a vitamin D free diet for 3 weeks after hatching. Norman (1966) noted that chicks bred under vitamin D deficient conditions, developed rachitic symptoms after 14 days. Sedrani (1984) reared chicks on a low but not a free vitamin D diet, (5.5 μg vitamin D_3/kg food) and between 2 and 7 weeks the serum $25(OH)D_3$ concentration was low (10.6 nmol/l or 4.2ng/ml). However serum $1,25(OH)_2D_3$ in adult male chicks, was seven times (604 pmol/l or 251pg/ml) greater than chicks on a normal vitamin D_3 diet, indicating the high activity of the enzyme 1α hydroxylase associated with vitamin D deficiency.

The serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ in Sedrani's study (1984), are higher than the values reported in this study. This may be explained by the diet administered in his study. In this present study the chickens were subjected to conditions and a diet which were fully vitamin D deficient. In Sedrani's study (1984), as already noted, the chicks were reared on a low but not completely deficient vitamin D_3 diet, which would explain the serum $25(OH)D_3$ level substantially higher than the result in this study. The high serum $1,25(OH)_2D_3$ value was possibly due to the high activity of the 1α-hydroxylase enzyme reacting to a diet which is vitamin D deficient. This may have been

compounded by the chicks receiving a diet which was calcium deficient, but this can be excluded by the normal serum calcium values reported in these birds.

In both vitamin D_3 deficient groups, $1,25(OH)_2D_3$ was the major metabolite present in duodenal mucosa demonstrating the role of the active hormone in effecting the transfer of calcium and phosphorus from the lumen of the small intestine into the organism. This is essential to maintain mineral homeostasis and mineralization of the skeleton. The concentration of $25(OH)D_3$ in the other deplete tissues was very low but higher than the other metabolites in each tissue.

The profile of vitamin D deficiency is characterized by the low levels of plasma $25(OH)D_3$ and $1,25(OH)_2D_3$. This is in association with diminished values of plasma calcium and phosphorus and increased plasma alkaline phosphatase. In this condition the product of calcium and phosphorus expressed in mg/100ml of plasma, is lower than that necessary for mineralization of bone to occur.

The results in this study are consistent with the diagnosis of vitamin D_3 deficiency in chicks in groups SD and MD, as evidenced by the low plasma levels of $25(OH)D_3$ and $1,25(OH)_2D_3$. This is also consistent with the low plasma calcium, phosphorus and high plasma alkaline phosphatase findings in chickens receiving subnormal amounts of vitamin D_3 (table 14), thus confirming that the minimal daily requirements of vitamin D_3, were not obtained for normal vitamin D_3 metabolism.

6.9 Effects of supraphysiological doses of vitamin D_3 in plasma and tissues.

With repeated doses of 16μg vitamin D_3, the metabolic profile of vitamin D_3 and its

metabolites in plasma and the organs increased in magnitude compared with the administration of physiological doses of the compound. Thus the values in plasma and organs in chicks receiving the larger amounts of vitamin D_3 were higher than those found in chicks receiving repeated 4μg vitamin D_3 dosages.

High values of vitamin D_3 were recorded in plasma and tissues. The amount in plasma (33.4 ± 2.7pmol/ml or 12.8 ± 1.0ng/ml) was higher than the normal upper limit of 10ng/ml described by Jones and DeLuca (1988) in humans. The parent vitamin was greater than all metabolites in each tissue examined, its highest being in adipose tissue (127 ± 11pmol/g; 48.7 ± 4.2 ng/g).

The level of plasma 25(OH)D_3 (75.5 ± 8.9 pmol/ml or 30.2 ± 3.6 ng/ml) was higher than in chicks receiving physiological doses of the vitamin. However it was not in the range associated with vitamin D toxicity when individuals may have a 15 fold increase in plasma 25(OH)D as compared with normal subjects. The concentration of this metabolite is still within the physiological range, less than 50ng/ml, as described by Jones and DeLuca (1988).

The administration of 50 fold excess of vitamin D_3 to chicks (Hughes et al 1977) produced a high concentration of serum 25(OH)D_3 namely 87 to 130ng/ml, and in the study of Ratzkowski et al (1982), a total dose of 1200 μg vitamin D_3, administered in four doses each of 300μg over nine days, produced a plasma 25(OH)D_3 level of approximately 350pmol/ml (145ng/ml). The highest value of 25(OH)D_3 in tissues in this study was also in adipose tissue, with a value of 20.6 ± 2.4pmol/g (8.2 ± 0.9ng/g) which was four fold greater than that in skeletal muscle. As also noted in the physiological group, the content of 25(OH)D_3 was greater in the kidney than liver, but did not attain the high values of adipose tissue. Mawer et al (1972) dealing with human tissues concluded that muscle

serves as the main storage site for 25(OH)D (Norman 1979). This may also be the case in the chick as the mass of muscle is so much greater than adipose tissue.

The concentration of the active metabolite $1,25(OH)_2D_3$ in plasma was greater in the 16μg group (0.09 + 0.03pmol/ml; 37.4 ± 12.5pg/ml) than in the 4μg chickens. The metabolite was not detected in kidney, muscle and adipose tissue. This plasma concentration was not considered to be high, as the mean (± SD) of this metabolite in chicks (Sedrani 1984) was 65.5± 11.4 pg/ml, whereas in humans the level is 36.9 ± 12.7pg/ml with a range of 17 to 59pg/ml (Jones as quoted by Jones and DeLuca (1988). This result in the study was not unexpected, as it has been documented that in hypervitaminosis D, even though serum calcium and the metabolites may reach very high levels, $1,25(OH)_2D_3$ is not substantially altered (Hughes et al 1977).

This paradox of a high plasma calcium (table 14) in the face of a normal plasma $1,25(OH)_2D_3$ in vitamin D toxicity, has been the subject of a number of speculations. It is unclear whether $1,25(OH)_2$ or another metabolite is responsible for the hypercalcaemia in vitamin D toxicity. (Vieth 1990).

The other dihydroxyvitamin D_3 metabolite, $24,25(OH)_2D_3$ was within the normal plasma range in the physiological group of chicks. It was 11 percent of the value of plasma $25(OH)D_3$ in both the 4 and 16μg groups and consequently increased in amount with higher $25(OH)D_3$ values. In vitamin D toxicity the level of plasma $24,25(OH)_2D$ is raised and is next in value after 25(OH)D and vitamin D. In this study it was present in all tissues in the replete groups. Of the organs examined, kidney tissue contained the highest amount of $24,25(OH)_2D_3$ in the 16ug group, while $1,25(OH)_2D_3$ was not detectable. This suggests that of the two hydroxylases, 24-hydroxylase was the major enzyme system functional in the kidney in vitamin D repletion.

In the supraphysiological group, esters of vitamin D_3 were present in small amounts in plasma, the organs containing larger quantities. Concentrations in both plasma and tissues were greater in the 16μg group than 4μg group. In both groups the liver contained the largest concentration of vitamin D_3 esters which was approximately five fold greater than the concentration in fat, which contained the second highest amount of vitamin D_3 esters.

The results in this study confirm that the chickens administered repeated doses of 16μg vitamin D_3, did not receive toxic amounts of the vitamin, but rather supraphysiological quantities as evidenced by the plasma levels of $25(OH)D_3$ which did not attain toxic levels.

6.10 The relationship of $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ in different vitamin D_3 status

The relationship between the two dihydroxyvitamins in plasma and the kidney is interesting. In both groups of vitamin D deficiency, plasma levels of $1,25(OH)_2D_3$ were present in small amounts (5.8 and 6.6 pg/ml), but $24,25(OH)_2D_3$ was not detected. With the administration of vitamin D, the relationship was altered in both groups in the replete state, $24,25(OH)_2D_3$ now being 70 fold greater than $1,25(OH)_2D_3$. A similar profile between these two metabolites occurred in the kidney in the deplete and replete phases.

The relationship of these two metabolites appears to follow the metabolic pathway of vitamin D, when synthesis of $24,25(OH)_2D_3$ is depressed in the deplete state, but is activated by $1,25(OH)_2D_3$ in the replete phase with suppression of 1α hydroxylase activity.

The presence of the high level of $25(OH)D_3$ in the kidney, may be an indication of metabolism of the substrate in the synthesis of the dihydroxyvitamin D_3 metabolites. In the

replete phase conversion to $24,25(OH)_2D_3$ is greater than $1,25(OH)_2D_3$. This was prevalent in both the physiological and supraphysiologal groups.

6.11 Vitamin D_3 storage organs.

In replete animals, high concentrations of vitamin D were found in liver, muscle and fat. The presence of vitamin D in muscle and fat which are not actively involved in vitamin D metabolism, may reflect the storage facility of these organs. This is substantiated in the studies performed by Mawer et al (1972) where they observed that adipose tissue and skeletal muscle acted as storage organs.

However the results in this study, revealing that the parent vitamin had a higher value than $25(OH)D_3$ in muscle in the physiological state, do not fully concur with the findings of Mawer et al (1972) who described $25(OH)D_3$ as being the chief steroid in this tissue (Norman 1979). However due to the large mass of muscle in the chick, it may also act as a storage site for $25(OH)D_3$

Rosenstreich et al (1971) noted in the rat that adipose tissue served as a storage depot for vitamin D which has a long biological half-life (Mawer et al 1969). Thus it is in keeping with the present study and the presence of high amounts of the parent vitamin in these tissues after receiving physiological amounts of the compound, indicates that fat acts as a storage organs for vitamin D.

The presence of high levels of vitamin D_3 in the liver is not surprising. As already mentioned, the parent vitamin is rapidly taken up into the liver after administration (Norman and DeLuca 1963; also refer 1.11 section ii). Originally it was thought that as retinol, also a fat soluble vitamin, was stored in the liver, the same applied to vitamin D. The amount

of 6.8 ± 2.5 pmol/g (2.6 ± 0.9ng/g) vitamin D_3 in the liver (in the 4μg group) could be the accumulation of the seco-steroid in the organ awaiting the first hydroxylation to occur at C-25 position of the compound. From the profile of the rapid accumulation of vitamin D in the liver soon after administration, and the continued loss of this compound from that organ in a relatively short space of time, is suggestive that the liver does not act as a storage organ for the parent vitamin, which is present as the precursor for 25(OH)D.

6.12 Vitamin D_3 esters.

In the physiological group, the amount of vitamin D esters in the liver is five and nine fold greater than that present in adipose tissue and muscle respectively. Small amounts of this compound were present in the liver in both deplete groups.

The dominance of this compound in the liver may be due to the many metabolic processes that occur in this organ, as the liver is well known to esterify other lipids such as cholesterol. It is generally felt that vitamin D esters do not play an active role in vitamin D metabolism, but little work has been done in assessing the role of vitamin D esters in the liver. It is unknown if this reflects a catabolic process.

Levels of vitamin D esters in intestine, kidney and liver were studied by Ratzkowski et al (1982). Their values in the organs studied were 0.4 pmol/g in the intestine, 0.3 pmol/g in the kidney and 0.7 pmol/g in the liver. The amounts in this study were 0.35, 0.8 and 15.2 pmol/g in the above tissues respectively. Both studies found that the liver harboured the largest amount of this compound, compared with the other organs. The average daily amount of vitamin D_3 received by the chicks was similar in both studies.

Rosenstreich et al (1971) demonstrated that vitamin D_3 esters were present in adipose tissue in rats shortly after vitamin D_3 administration. Six weeks after administration of the vitamin, 20 percent of all radioactivity in this tissue was due to vitamin D esters. Following supplementation with vitamin D_3 there was a progressive increase in the total quantity of vitamin D_3 esters in fat, while the concentration of all other metabolites of vitamin D_3 was decreasing. The amount of esters present in adipose tissue after 60 days was very large compared with the quantity found outside this tissue, leading the authors to suggest that these compounds may be synthesized from vitamin D in adipose tissues.

6.13 Polar metabolites of vitamin D_3

It is important that $1,25(OH)_2D_3$ is not allowed to accumulate in the various tissues where it is active, due to the deleterious effects it may have on the cell. The plasma half-life of this metabolite is approximately 10 hours (Collins and Norman 1991) however some workers estimate that it is less (Marx 1989). In the present study the metabolites more polar than $1,25(OH)_2$ were not identified, and only measured as the amount of radioactivity eluting with methanol (100 percent) off the silicic acid chromatography column.

The actual concentration of these metabolites could not be determined as the molecular weights were not known, but dpm/ml or dpm/g tissue does reflect the concentration although not accurately. The percentage polar metabolites reflect the percentage of these metabolites compared to the sum of vitamin D_3 and its metabolites within a given organ.

The polar metabolites (dpm/ml) in plasma in both deficient groups were lower than in the other two groups receiving larger amounts of vitamin D_3. This is to be expected as

the polar metabolites are either the catabolic or oxidative products of $1,25(OH)_2D_3$ or $25(OH)D_3$ and calcitroic acid, the major excretory product of $1,25(OH)_2D_3$ circulates in the blood (Kumar 1984).

As the concentrations of the main metabolites were lower in the vitamin D deficient groups, the concentration of the polar metabolites would also be expected to be lower. The plasma dpm/ml of polar metabolites in the deplete groups were two fold lower than in the group receiving physiological doses and seven times lower than in the group receiving the supraphysiological dose. The fraction of polar metabolites (%) making up the total vitamin D metabolites was similar in plasma in all four groups.

In all the organs, the kidney contained the largest amount of polar metabolites in the replete groups of animals. It is possible that as the main synthesis of the active hormone occurs in the kidneys, the metabolic pathways following on from $1,25(OH)_2D$ are very active in this organ causing the large quantity of polar metabolites to be synthesized. The major portion of these excretory products are eliminated in the gastrointestinal tract via their excretion in bile (Kumar 1986), but the kidney also plays a role (Kumar 1986). It is thus feasible that some of the polar metabolites present in kidney tissue, represent excretory products formed in other tissues.

It is of interest that in both replete groups, the kidney and duodenal mucosa, contained a high percentage of vitamin D metabolites in the form of polar metabolites compared to other organs. This might reflect the active conversion of the active metabolite of vitamin D to non-active polar metabolites in the classical target organs of vitamin D.

The skeletal muscle and adipose tissues have a low percentage polar metabolite value in both replete groups, possibly reflecting that it may be due to the slow turnover rate of vitamin D_3 in the vitamin D depot organs.

6.14 Comparison of the two vitamin D_3 deficient groups.

The results of the study to determine whether vitamin D metabolism differed when deplete chicks were given an acute small bolus of the vitamin compared with repeated minimal doses, appeared to show no obvious difference.

The plasma levels of $25(OH)D_3$ in both groups were less than 4ng/ml, while vitamin D_3 and $24,25(OH)_2D_3$ were not detectable. The values of the active hormone $1,25(OH)_2D_3$ were very low, 0.014 and 0.016pmol/ml (5.8 and 6.6pg/ml) showing no difference between the two groups.

The low plasma levels of $1,25(OH)_2D_3$ in the deplete state in both groups SD and MD, do not correspond with the very high values of this metabolite recorded by Sedrani (1984; refer section 6.8), due to the higher activation of the enzyme 1α hydroxylase in the vitamin D deficient state. It is apparent that the chickens in Sedrani's study (1984) received larger amounts of vitamin D_3 as revealed by the plasma $25(OH)D_3$ level (4.2ng /ml), which was 30 fold greater than in the present study. Thus the small amounts of vitamin D_3, even when given as a single bolus or in repeated small doses in the study, were too low to activate the enzyme 1α hydroxylase with the resultant low values of plasma $1,25(OH)_2D_3$.

6.15 Vitamin D_3 requirements for chickens.

The results in this study do not fully support the findings of Haussler and Rasmussen (1972) the average daily minimum requirement in chickens for normal vitamin D_3 metabolism is 5 to 10 IU (0.125-0.25μg).

With the average daily administration of 6.7 IU (0.17μg) vitamin D_3, (refer group

3 in table 13) the plasma 25(OH)D_3 value was 7.0 ± 2.7 ng/ml which was considered to be in the vitamin D_3 deficiency range. The plasma calcium level (7.3 mg/dl) and phosphorus (4.5 mg/dl) in this group, were also both less than the reference values (refer 3.2.2 and table 14).

It appears that a minimum daily amount of 10 IU (0.25μg) vitamin D_3 for chickens would be more appropriate.

6.16 Conclusions from present study.

The studies reported in this dissertation demonstrate that the profile of vitamin D_3 and its metabolites in plasma and tissues changes with the vitamin D_3 status of the chick.

In the vitamin D deficient state, vitamin D_3 was not detected in plasma, and was present in only very small quantities in the tissues examined in Group SD and only in adipose tissue in group MD.

With improvement of the vitamin D status, the concentration of vitamin D_3 increased markedly in plasma and in a number of tissues, but particularly so in adipose tissue, which appears to act specifically as a storage site for vitamin D.

25-Hydroxyvitamin D_3 is present mainly in plasma, and serum levels provide a good indicator of the vitamin D status of the animal. Vitamin D deficient birds had very low circulating levels (<4ng/ml), while those on a supraphysiological vitamin D_3 intake had concentrations some 200 times greater. 25(OH)D_3 was also taken up by fat when the vitamin D intake was adequate, although concentrations in that tissue did not reach levels achieved by vitamin D_3.

Due to its large mass compared to other organs in the chick, muscle may act as a

storage organ for vitamin D_3 and $25(OH)D_3$.

Like $25(OH)D_3$, $24,25(OH)_2D_3$ is present mainly in plasma. Concentrations were not detected in deficient birds, but in the replete groups, the levels were approximately 10 percent of $25(OH)D_3$. The lack of any apparent control of $24,25(OH)_2D_3$ concentrations, supports the generally held view that it is not primarily a physiological active form of vitamin D_3.

The active metabolite of vitamin D_3, $1,25(OH)_2D_3$ is present in plasma and tissues at very much lower concentrations than any of the other measured metabolites in the replete state. Unlike the other metabolites, $1,25(OH)_2D_3$ concentrations were highest in the intestine in the groups receiving vitamin D_3 deficient diets. These findings support the important role that the metabolite plays in intestinal calcium absorption. It is unclear why the liver should have the highest levels of all the tissues in the group receiving supraphysiological doses of the vitamin.

The study also provides interesting information on a metabolite (vitamin D_3 esters) that has received little attention in the past. The high concentrations found in the liver in the two vitamin D_3 replete groups, suggest that the liver may either store the vitamin or be involved in the esterification of vitamin D_3. The role of vitamin D_3 esters in the physiology of vitamin D_3 is unclear.

No definite conclusions can be made concerning the metabolites more polar than $1,25(OH)_2D_3$, as no attempt was made to separate or quantitate these. In all the studies, they only formed a small proportion of the total radioactive metabolites.

The results in this study indicate that the minimum daily vitamin D_3 requirements in the chick is at least 10 IU (0.25μg).

Finally it is apparent that the chick is a good animal model in the study of vitamin

D_3 metabolism, as shown by the different metabolite profiles assumed dependent on varying vitamin D_3 status, and also the similarity to that occurring in humans in these varying states.

REFERENCES

Al-Abdaly, L.Y. and Henry, H.L. (1989). Hormonal regulation of chick kidney inhibitor of cAMP dependent protein kinase. *Endocrinology* **124:** 2901-2906.

Arnaud, S.B., Goldsmith R. S., Lambert, P.W., Go,V.L.W.(1975). 25- hydroxyvitamin D_3 : Evidence of an enterohepatic circulation in man. *Proc. Soc. Exp. Biol. Med.* **149:** 570-572.

Audran, M. and Kumar, R. (1985). The physiology and pathophysiology of vitamin D. *Mayo Clin. Proc.* **60:** 851-866.

Bar, A., Sharvit, D., Noff, D., Edelstein, S., Hurwitz, S. (1980). Absorption and excretion of cholecalciferol and 25 hydroxy- cholecalciferol and metabolites in birds. *J. Nutr.* **110:** 1930-1934.

Bar-Shavit, Z., Noff, D., Edelstein, S., Meyer, M., Shibolet, S., Goldman, R. (1981) 1,25 Dihydroxyvitamin D_3 and the regulation of macrophage function. *Calcif. Tissue. Int.* **33:** 673-676.

Baran, D.T. and Milne M.L. (1986). 1,25-dihydroxyvitamin D increases hepatocyte cytosolic calcium level-a potential regulator of vitamin D-25-hydroxylase. *J. Clin. Investig.* **77:** 1622-1626.

Baran, D.T., Sorense, A.M., Honeyman, T.W., Ray, R. Holick, M. F. (1989). Rapid action of 1 25-dihydroxyvitamin D on Ca^{2+} and phospholipids in isolated rat liver nuclei. *FEBS Lett.* **259:** 205-208.

Barragry, J.M., France, M.W., Boucher, B.J. and Cohen, R.D. (1979). Metabolism of intravenously administered cholecalciferol in man. *Clin. Endocrinol.* **11:** 491-495.

Batchelor, A.J., Watson, G.,and Compston J.E. (1982). Changes in plasma half-life and

clearance of ^{3}H-25-hydroxyvitamin D_3 in patients with intestinal malabsorption. *Gut* **23:** 1068-1071.

Batchelor, A.J. and Compston, J.E. (1983). Reduced plasma half-life of radio-labelled 25-hydroxyvitamin D in subjects receiving a high fibre diet. *Br. J. Nutr.* **49:** 213-216.

Bell, N.H., Shaw, S., and Turner, R.T. (1984). Evidence that 1,25-dihydroxyvitamin D_3 inhibits the hepatic production of 25-hydroxyvitamin in man. *J. Clin. Invest.* **74:** 1540-1544.

Bell, N.H. (1985) Vitamin D-endocrine system. *J.Clin.Invest.* **76:** 1-6.

Bellido,T. and Boland,R. (1991). Effects of 1,25-dihydroxy-vitamin D_3 on phosphate accumulation by myoblasts. *Horm Metab Res* **23:** 113-116.

Belsey,T., DeLuca, H.F. and Potts, J.T. (1974). Selective binding properties of the vitamin D transport protein in chick plasma in vitro. *Nature*. **247:** 208-209.

Berlin, T. and Björkhem. (1987). On the regulatory importance of 1,25-dihydroxyvitamin D and dietary calcium on serum levels of 25-hydroxyvitamin D in rats. *Biochem. Biophys. Res. Commun.* **144:** 1055-1058.

Bhattacharyya, M. H. and DeLuca, H.F. (1973). Comparative studies on the 25-hydroxylation of vitamin D and dihydrotachysterol. *J. Biol. Chem.* **248:** 2960-2973.

Bikle, D.D. (1992). Clinical counterpoint: Vitamin D. New actions, new analogues, new therapeutic potential. *Endocr. Rev.* **13:** 765-784.

Bikle,D.D., Whitney, J,. and Munson,S. (1984). The relationship of membrane fluidity to calcium flux in chick intestinal brush border membrane. *Endocrinology* **114:** 260-267.

Bikle, D.D., Siiteri, P.K., Ryzen, E.,and Haddad, J.G., with the technical assistance of

Gee,E. (1985). Serum protein binding of 1,25-dihydroxyvitamin D; A re-evaluation by direct measurement of free metabolite levels. *J. Clin. Endocrinol. Met.* **61:** 969-975.

Birge, S.J. and Haddad, J.G. (1975). 25-hydroxycholecalciferol stimulation of muscle metabolism. *J. Clin. Invest.* **56:** 1100-1107.

Birge, S.J., and Miller, R. (1977). The role of phosphate in the action of vitamin D on the intestine. *J. Clin. Invest.* **60:** 980-988.

Birge, S.J. (1978). Vitamin D, muscle and phosphate homeostasis. *Miner. Electrolyte Metab.* **1:** 57-64.

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

Bligh, E. G. and Dyer W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.* **37:** 911-917.

Bolt, M.J.G., Meredith, M.C. and Rosenberg, I.H. (1988). Suppression of rat hepatic vitamin D-25-hydroxylase by cholecalciferol, but not by 25-hydroxy- or 1,25-dihydroxymetabolites. *Calcif. Tissue. Int.* **42:** 273-278.

Bouillon, R., Van Baelen, H., and DeMoor, P. (1976). The transport of 25-hydroxyvitamin D in cebus monkey. *Biochem. J.* **159:** 463-466

Brommage, R. and DeLuca, H.F. (1985). Evidence that 1,25 dihydroxyvitamin D_3 is the physiologically active metabolite of vitamin D_3. *Endocr. Rev.* **6:** 491-511

Brumbaugh, P.F., Hugues, M.R.,and Haussler, M.R. (1975). Cytoplasmic and nuclear binding components for 1,25-dihydroxyvitamin D in chick parathyroid glands. *Proc. Natl. Acad. Sci. USA.* **72:** 4871-4875.

Buffenstein, R., Jarvis, J.U.M., Opperman, L.A., Cavaleros,M., Hough, S., and Ross, F.P. (1988). Vitamin D metaboloism and expression in chthonic naked mole rats (abstract). *J. Bone Miner. Res.* **3:** S119.

Cavaleros, M. (1992). Vitamin D in the fruit bat (Rousettus Aegyptiacus). Thesis. Univ. Witwatersrand.

Chalk, K.J.I. and Kodicek,E. (1961). The association of ^{14}C-labelled vitamin D with rat serum proteins. *Biochem. J.* **79:** 1-7.

Chen, P.S. Jr. and Bosmann, H.B. (1964). Effects on vitamin D_2 and D_3 on serum calcium and phosphorus in rachitic chicks. *J. Nutr.* **83:** 133-139.

Chesney, R.W., Mazess, R.B., Rose, P., Hamstra, A.J.and DeLuca, H.F. (1980a). Supranormal 25-hydroxyvitamin D and subnormal 1,25-dihydroxy-vitamin D. *Am. J. Dis. Child.* **134:** 140-143.

Chesney, R.W., Rosen, J,F., Hamstra, A.J., and DeLuca, H.F.(1980b). Serum 1,25(OH)-dihydroxyvitamin D levels in normal children and in vitamin D disorders. *Am. J. Dis. Child.* **34:** 135-139.

Chesney, R.W., Zimmerman, J., Hamstra, A., DeLuca, H.F., and Mazess, R.B. (1981). Vitamin D metabolite concentrations in vitamin D deficiency. *Am. J. Dis. Child.* **135:** 1025-1028.

Christakos, S. and Norman A.W. (1981). Studies on the mode of action of calciferol. XXIX. Biochemical characterization of 1,25-dihydroxyvitamin D receptors in chick pancreas and kidney cytosol. *Endocrinology* **108:** 140-149.

Clark,S.A., D'Ercole, A.J. and Toverud, S.U. (1986). Somatomedin-C/insulin like growth factor I and vitamin D-induced growth. *Endocrinology* **119:** 1660-1665.

Clements, M.R., Chalmers ,T.M. and Fraser, D.R. (1984). Enterohepatic circulation of vitamin D: a reappraisal of the hypothesis. *Lancet* **1:** 1376-1379.

Clements, M.R., Johnson, L.and Fraser, D.R. (1987a). A new mechanism for induced vitamin D deficiency in calcium deprivation. *Nature* **325:** 62-65.

Clements, M.R., Fraser, D. R., Lumb, A., Mawer, E.B.,and Adams, P.H. (1987b). Metabolic inactivation of vitamin D is enhanced in primary hyperparathyroidism. *Clin. Sci.* **73:** 659-664.

Clements, M R., Davies, M., Hayes, M. E., Jickey, C. D., Lumb,G.A. Mawer, E.B., and Adams, P.H.(1992). The role of 1,25-dihydroxyvitamin D in the mechanism of acquired vitamin D deficiency. *Clin. Endocrinol.* **37:** 17-27.

Collins, E.D. and Norman, A.W. (1991). *Handbook of vitamins* p 60-93. Edited by L.J. Machlin. Published Marcel Dekker Inc. New York.

Cross, H.S. and Peterlik, M. (1988). Calcium and inorganic phosphate transport in embryonic chick intestine: triiodothyronine enhances the genomic action of 1,25-dihydroxycholecalciferol. *J. Nutr.* **118:** 1529-1534.

Cruikshank, E.M., Kodicek,E. and Armitage, P. (1953;1954). Bone structure and metabolism p161-174. Ciba Foundation Symposium.(1956) (Eds. Wolstenholme G.E.W. and O'Connor C.M.).

de Boland A.R., Albornos, L. E. and Boland R. (1983). The effect of calciferol in vivo on proteins and lipids of skeletal muscle from rachitic chicks. *Calcif Tissue Int* **35:** 798-805

deBoland,A.R., Flawia, M., Coso,O. and Boland,R. (1991). A guanine nucleotide-binding protein mediates 1,25-dihydroxyvitamin D_3 dependent rapid stimulation of Ca^{2+} uptake in skeletal muscle. *Biochim Biophys Acta* **1094:** 238-242.

DeLuca, H.F. (1984). The metabolism, physiology and function of vitamin D. In: *Vitamin D: Basic and Clinical Aspects.* (Ed. Kumar, R.), Martinus Nijhoff Publishing, Boston.

DeLuca, H.F. (1988). The vitamin D story: a collaborative effort of basic science and clinical medicine. *FASEB J.* **2:** 224-236.

DeLuca, H.F. and Schnoes, H.K. (1983). Vitamin D: Recent advances. *Ann. Rev. Biochem.* **52:** 411-439.

Delvin, E.E., Avabian, A., Glorieux,F H. and Mamer, O.A. (1985). In vitro metabolism of 25-hydroxycholecalciferol by isolated cells from human decidua. *J Clin. Endocrinol. Metab.* **60:** 880-885.

Dent, C.E. and Smith, R. (1969). Nutritional osteomalacia. *Q.J.Med.* **38:** 195

Dickson, I. (1987). New approaches to vitamin D. *Nature* **325:** 18.

Dickson, I.R., Hall, A.K. and Jande, S.S. (1984). The influence of dihydroxylated vitamin D metabolites on bone formation in the chick. *Calcif. Tissue Int.* **36:** 114-122.

Dostal, L.A. and Toverud, S.U. (1983). Enhanced sensitivity of young suckling rats to the toxic effects of 1,25-dihydroxyvitamin D_3. *Calcif. Tissue Int.* **35:** 432-437.

Drezner, M.K. and Feinglos, M.N. (1977). Osteomalacia due to 1,25 dihydroxycholesterol deficiency. *J. Clin. Invest.* **60:** 1046-1053.

Drittanti, L., deBoland, A.R. and Boland, R. (1993). Involvement of the 3',5'-cyclic AMP pathway in the induction of calmodulin synthesis in myoblasts by 1,25$(OH)_2$-vitamin D_3. *Biochem Biophys Res Comm* **192:** 886-892.

Dueland, S. (1983). Absorption and distribution of vitamin D and 25-hydroxyvitamin

D in rat. *Am. J. Physiol.* **245:** E463-467.

Dueland, S., Peterson, J.L. and Helgerud, P. (1982). Transport of vitamin D_3 from rat intestine : Evidence for transfer of vitamin D_3 from chylomicrons to α-globulins. *J. Biol. Chem.* **257:** 146-150.

Edelstein, S., Lawson, D.E.M.,and Kodicek, E.(1973). The transporting proteins of cholecalciferol and 25-hydroxycholecalciferol in serum of chicks and other species. *Biochem. J.* **135:** 417-426.

Edelstein, S., Charman, M., Lawson, D.E.M. and Kodicek, E. (1974). Competitive protein-binding assay for 25-hydroxycholecalciferol. *Clin.Sci.Mol.Med.* **46:** 231-240.

Editorial. (1987). Vitamin D: New perspective. *Lancet* **1:** 1122-1123.

Egleton. (1991). Personal Communication (Rainbow Chickens, Natal).

Eisman, J.A., Shepard, R.M. and DeLuca, H.F. (1977).
Anal. Biochem. **89:** 298.

Esvelt, R.P., Schnoes, H.K. and DeLuca, H.F. (1978). Vitamin D_3 from rat skins irradiated in vitro with ultravioletlight. *Arch. Biochem. Biophys.* **188:** 282-286.

Esvelt, R.P., Schnoes, H.K. and DeLuca, H.F. (1979). Isolation and characterization of 1 Hydroxy-23 carboxytetranorvitamin D_3; A major metabolite of 1,25 dihydroxyvitamin D_3. *Biochemistry* **18:** 3977-3983.

Esvelt, R.P and DeLuca H.F. (1981). Calcitroic acid: biological activity and tissue distribution studies. *Arch. Biochem. Biophys.* **206:** 403-413.

Ford, J.A., Colhoun, E. M., McIntosh, W.B.,and Dunnigan, . (1972). Biochemical response of late rickets and osteomalacia to a chupatty-free diet. *Br. Med. J.* **3:** 446-447.

Forte, L.R., Langeluttig, S.G., Biellier, H.V., Poelling, R.E. (1983). Up-regulation of kidney adenylate cyclase in egg-laying hen. Role of estrogens. *Am. J. Physiol.* **245:** E273-E280.

Fox, J. and Care, A.D.(1979). Effects of hydroxlated derivatives of vitamin D_3 and of aquous extracts of Solonus malacoxylon on the absorption on calcium, phosphate, sodium, potassium and water from the jejunum of pigs. *J. Endocrinol.* **82:** 417-424.

Fraser, D.R. (1980). Regulation of the metabolism of vitamin D. *Physiol. Rev.* **60:** 551-602.

Fraser, D.R. and Kodicek, E. (1965). Vitamin D esters: Their isolation and identification in rat tissues. *Proc. Biochem. Soc.* 59P-60P.

Fraser,D.R and Kodicek, E. (1970). Unique biosynthesis of kidney of a biological active vitamin D metabolite. *Nature* **228:** 764-766.

Ghijsen, W.E.J.M. and VanOs O.H. (1982). 1,25-dihydroxyvitamin D regulates ATP-dependent calcium transport in basolateral plasma membrane of rat enterocyte. *Biochim. Biophys. Acta.* **689:** 170-172.

Gray,R.W., Caldas,A.E., Wilz, D.R., Lemann, J.Jr., Smith, G.A. and DeLuca, J.F. (1978). Metabolism and excretion of ^{3}H-1,25-$(OH)_2$-vitamin D_3 in healthy adults. *J. Clin. Endocrinol. Metab.* **46:** 756-765.

Greenberg P.B., Hillyard, C.J., Galante, L.S., Colston, K.W., Evans, I.M.A. and MacIntyre,I. (1974). Affinity of calciferol analogues for 25 Hydroxyvitamin D receptors. *Clin. Sci. Mol. Med.* **46:** 143-147.

Haddad, J.G. and Chyu, K.G. (1971). Competitive protein binding radioassay for 25-

hydroxycholecalciferol. *J. Clin. Endocrinol.* **33:** 992-995.

Haddad, J.G. and Cooke N.E. (1989). Vitamin D binding protein (Gc-globulin). *Endocr. Rev.* **10:** 294-307.

Halloran, B.P., Bikle, D.D., Levens, L.J., Castro, M.E., Globus, R.J.,and Holton, E. (1986). Chronic 1,25-dihydroxyvitamin D administration in the rat reduces the serum concentration of 25-hydroxyvitamin D by increasing metabolic clearance rate. *J. Clin. Invest.* **78:** 622-628.

Halloran, B.P. and Castro M.E.(1989). Vitamin D kinetics in vivo: effect of 1,25-dihydroxyvitamin D administration. *Am. J. Physiol.* **256:** E686-E691.

Hamden, (1976). Side chain metabolism of 25-hydroxy-[26,27- C] vitamin D_3 and 1,25-dihydroxy-[26,27-^{14}C] vitamin D_3 in vivo. *Science* **193:** 493-494.

Haussler, M.R., (1972) Characterization of the metabolites in the chick. *Steroids* **20:** 639-650.

Haussler, M.R. and Norman, A.W. (1967). Subcellular distribution of physiological doses of vitamin D_3. *Arch. Biochem. Biophys.* **118:** 145-153.

Haussler, M.R., Myrtle, J.F. and Norman, A.W. (1968). The association of a metabolite of vitamin D_3 with intestinal mucosa chromatin in vivo. *J. Biol. Chem.* **243:** 4055-4064.

Haussler, M.R. and Norman, A.W. (1969). Chromosomal receptor for a vitamin D metabolite. *Proc. Natl. Acad. Sci. USA.* **26:** 155-162.

Haussler, M.R. and Rasmussen, H. (1972). The metabolism of vitamin D_3 in the chick. *J. Biol. Chem.* **247:** 2328-2335.

Haussler, M.R. and McCain, T.A. (1977). Basic and clinical concepts related to vitamin

D metabolism and action. *N. Engl. J. Med.* **297:** 1041-1050.

Haussler,M.R., Mangelsdorf,D.J., Komm,B.S., Terpening,C.M., Yamaoka, K., Allegretto,E.A., Bakwe, A.R., Shine, J., McDonnel, D.P., Hughes, M., Weigel, G.L., O'Malley,B.W. and Pike, J.W.(1988). Molecular biology of the vitamin D hormone. *Recent Prog. Horm. Res.* **44:** 263-305.

Hay, A.W.M. and Watson, G. (1975). Binding of 25-hydroxyvitamin D_2 to plasma proteins in New World monkeys. *Nature* **256:** 150.

Hay, A.W.M. and Watson, G. (1976a). The plasma transport proteins of 25-hydroxycholecalciferol in mammals. *Comp. Biochem. Physiol.* **53B:** 163-166.

Hay, A.W.M. and Watson,G. (1976b). The plasma transport proteins of 25-hydroxycholecalciferol in fish, amphibians, reptiles and birds. *Comp. Biochem. Physiol.* **53B:** 167-172.

Hay, A.W.M. and Watson, G. (1977). The binding of 25-hydroxy- cholecalciferol and ergocalciferol to receptor proteins in a New World and an Old World Primate. *Comp. Biochem. Physiol.* **56B:** 131-134.

Henry, H.L. (1992). Vitamin D hydroxylases. *J. Cell. Biochem.* **49:** 4-9.

Henry, H.L. and Norman A.W. (1975). Presence of renal 25-hydroxyvitamin D-1 hydroxylase in species of all vertebrate classes detected in 25 of 28 vertebrate species studied. *Comp. Biochem. Physiol.* **50B:** 431-434.

Henry, H.L., Taylor, A.N., and Norman. A.W. (1977). Response of chick parathyroid glands to the vitamin D metabolite, 1,25 dihydroxycholecalciferol, and 24,25 dihydroxycholecalciferol. *J. Nutr.* **107:** 1918-1926.

Henry, H.L. and Norman, A.W. (1978).Vitamin D: Two hydroxylated metabolites are

required for normal chicken egg hatchability. *Science* **201:** 835-837.

Henry, H.L. and Norman A.W. (1984). Vitamin D : Metabolism and biological actions. *Ann. Rev. Nutr.* **4:** 493-520.

Henry, H.L., Dutta, C., Cunningham, N., Blanchard, R., Penny, R., Tang, C., Marchetto, G. and Chai,S-Y. (1992). The cellular and molecular regulation of $1,25(OH)_2D$ production. *J. Steroid Biochem. Molec. Biol.* **44:** 401-407.

Hietanen-Peltola,M., Pelto-Huikko, M., Rechardt, L.,Emson, P and Hokfelt, T. (1992). Calbindin D_{28k}-immunoreactivity in rat muscle spindle. *Brain Research* **479:** 327-332.

Holick, M.F. (1981). The cutaneous photosynthesis of pre-vitamin D_3: a unique photoendocrine system. *J. Invest. Dermatol.* **76:** 51-58.

Holick, M.F. (1984). The photobiology of vitamin D_3 in man. In: *Vitamin D: Basic and Clinical Aspects.* (Ed. Kumar,R.), Martinus Nijhoff Publishing, Boston pp 197-216.

Holick, M.F. (1986). Vitamin D requirements for the elderly.
Clin. Nutr. **5:** 121-129

Holick, M.F., Schnoes, H.K., DeLuca, H.F. Gray, R.W., Boyle, I.I. and Suda, T. (1972). Isolation and identification of 24,25-Dihydroxyvitamin D_3, a metabolite of vitamin D_3 made in the kidney. *Biochemistry* **11:** 4251-4255.

Holick, M.F., Baxyter, L.A., Schraufrogel, P.K., Tavela, T.E., and DeLuca, H.F. (1976). Metabolism and biological activity of 24,25 dihydroxyvitamin D in the chick. *J. Biol. Chem.* **251:** 397-402.

Holick, M.F., Mac Laughlin, J.A. and Doppett, S.H. (1981). Regulation of cutaneous previtamin D in man: skin pigment is not an essential regulator. *Science* **211:** 590-593.

Hollis, B.W., Roos, B.A. and Lambert, P.W. (1981). Vitamin D in plasma: quantitation by a nonequilibrium ligand binding assay. *Steroids* **37:** 609-619.

Horst, R.L. (1979). 25-OHD_3 -26,23-lactone. *Biochem. Biophys. Res. Comm.* **89:** 286-293.

Horst, R.L., Littledike, E.T., Riley, J.L. and Napoli, J.L. (1981). Quantitation of vitamin D and its metabolites and their plasma concentration in five species of animals. *Anal. Biochem.* **116:** 189-203.

Horst, R.L., Napoli, J.L.,and Littledike, E.T. (1982). Discrimination in the metabolism of orally dosed ergocalciferol and cholecalciferol by the pig, rat, and chick. *Biochem. J.* **204:** 185-189.

Hughes, M.R., Baylink, D.J., Gonnerman, W.A.,Toverud, S,U., Ramp,W.K.,and Haussler, M. R. (1977). Influence of dietary D on the circulating concentration of its active metobolites in the chick and rat. *Endocrinology* **100:** 799-806.

Huldschinsky, K. (1919). Heilung von Rachitis durch kuenstliche Hohensonne. *Dtsch. Med. Wochenschr.* **45:** 712.

Hunt, R.D., Garcia, F,G., Hegsted,D.M., and Kaplinsky, N. (1967). Vitamins D_2 and D_3 in New World Primates: Influence on calcium absorption. *Science* **157:** 943-945.

Hunt, R.D., Garcia, F.G., and Walsh, R.J. (1972). A comparison of the toxicity of ergocalciferol and cholecalciferol in rheusus monkeys (macaca mulatta). *J. Nutr.* **102:** 975-986.

Hurst, R.O.(1967). A simplified approach to the use of determinants and calculations of complex enzymes. *Can. J. Biochem.* **45:** 2015-2016.

Imrie, M.H., Neville, P,F., Snellgrove, A. W.,and DeLuca, H.F. (1967). Metabolism of

D_2 and D_3 in the rachitic chick. *Arch. Biochem. Biophys.* **120:** 525-532.

Jarnagin, K., Zeng, S-Y., Phelps, M.,and De Luca, H.F.(1985). Metabolism and pharmacokinetics of 24,25$(OH)_2$D in vitamin D replete rats. *J. Biol. Chem.* **260:** 13625-13630.

Jones, G., Schnoes, H.K. and DeLuca. (1976). An in vitro study of vitamin D_2 hydroxylases in the chick. *J. Biol. Chem.* **251:** 24-28.

Jones, G. and DeLuca, H.F. (1988). High-performance liquid chromatography of vitamin D and its application to endocrinology. *Monogr. Endocrinol.* 95-139.

Jongen, M.J.M., van der Vijgh, W.J.F., Lips, P. and Netelenbos,J.C. (1984). Measurement of vitamin D metabolites in anephric subjects. *Nephron.* **36:** 230-234.

Karsenty, G. Lacour, B. Ulmann, A., Pierandrei, E. and Drueke, T. (1985). Early effects of vitamin D metabolites on phosphate fluxes in isolated rat enterocytes. *Am. J. Physiol.* **248:** G40-G45.

Kawashima, H. and Kurokawa K. (1983). Unique hormonal regulation of vitamin D metabolism in the mammalian kidney. *Min. Elect. Metab.* **9:** 227-235.

Knutson, J.C. and DeLuca, H.F. (1974). 25-Hydroxyvitamin D-24-hydroxylase; subcellular location and properties. *Biochemistry* **13:** 1543-1548.

Kodicek, E. (1954;1955). Bone structure and metabolism. *Ciba Foundation Symposium.(1956).* (Eds. Wolstenholme G.E.W. and O'Connor C.M.) p161-174.

Koenigberger, R. High pressure liquid chromatography. *Scientific Foundations of Clinical Biochemistry* 165-185.

Kosjewski, N.J., Reinhardt, T. A., Nopoli, J.L., Beitz, D.C., Horst, R.L. (1988). 24,26-

dihydroxyvitamin D_2: A unique physiological metaboloite of vitamin D_2. *Biochemistry* **27:** 5785-5790.

Kraml, M. (1966). A semiautomated determinations of phospholipids. *Clin. Chim. Acta.* **13:** 442-444.

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

Kumar, R. (1986). The metabolism and mechanism of action of 1,25-dihydroxyvitamin D_3. *Kidney Int.* **30:** 793-803.

Kumar, R. (1991). Vitamin D and calcium transport. *Kidney Int.* **40:** 1177-1189.

Kumar, R., Wiesner, R., Scott, M., and Go,V.L.W. (1982). Physiology of 24,25-dihydroxyvitamin D_3 in normal human subjects. *Am. J. Physiol.* E370-E374.

Kurnik, B.R.C., Huskey, M., and Hruska, K.A. (1987). 1,25 dihydroxycholecalciferol stimulates renal phosphate transport by directly altering membrane phosphocholine composition. *Biochim. Biophys. Acta.* **917:** 81-85.

Lam, H.Y. Schnoes, H.K. and DeLuca H.F. (1974). Synthesis and biological action of 25,26-dihydroxycholecalciferol. *Steroids* 247-256.

Lambert, P.W., Stern, P.H., Avioli, R.C., Brackett, N.C., Turner R.T., Greene, A. and Fu I.Y. (1982). Evidence for extrarenal production of 1,25-dihydroxyvitamin D in Man. *J. Clin. Invest.* **69:** 722-725.

Lidor, C. and Edelstein, S. (1978). Calcitriol increases Ca-ATPase activity. *Biochem. Biophys. Res. Commun.* **144:** 713-717.

Lindquist, B. (1952). Effect of vitamin D on metabolism of radiocalcium in rachitic rats. *Acta. Paediatr.* **41:** 599-603.

Lo, C.W., Paris, P.W. and Holick, M.F. (1986). Indian and Pakistani immigrants have the same capacity as Caucasians to produce vitamin D in response to ultraviolet irradiation. *Am. J. Clin. Nutr.* **44:** 683-685

Lohnes,D., and Jones, G. (1987). Side chain metabolism of vitamin D_3 in osteosarcoma cell line UMR-106. *J. Biol. Chem.* **262:** 14394-14401.

Lu, Z., Chen, T.C., Markestad T., Pettifor, J.F., Ladizesky, M., Mautalen, C. and Holick, M.F. (1992). Photosynthesis of previtamin D in cities around the world; Influence of season and time of day on the synthesis of vitamin D. Biologic effects of light. Ed. Holick, M.F. Kligman A.M. Walter de Gruyter, Berlin. p 27-52; p 53-56.

Lund, J., DeLuca H.F. and Horsting, M. (1967). Formation of vitamin D esters in vivo. (1967). *Arch. Biochem. and Biophys.* **120:** 513-517.

MacLaughlin, J. and Hollick M.F. (1985) Ageing decreases the capacity of human skin to produce vitamin D. *J. Clin. Invest.* **76:** 1536-1538.

Madhok, T.C. and DeLuca H.F. (1979). Characteristics of the rat liver microsomal enzyme system converting calciferol into 25- hydroxycholecalciferol: Evidence for the participation of cytochrome P-450. *Biochem. J.* **184:** 491-499.

Maierhofer,W.J., Gray, R.W., Adams,N.D., Smith,G.A. and Lemann, Jr, J.(1981). Synthesis and metabolic clearance of 1,25-dihydroxy- vitamin D as deteminants of serum concentrations: a comparison of two methods. *J. Clin. Endocrinol. Metab.* **53:** 472-475.

Makin, G., Lohnes, D., Byford, V., Ray, R and Jones, G. (1989). Target cell metabolism of 1-25-dihydroxyvitamin D to calcitroic acid. Evidence for a pathway in kidney and bone involving 24-oxidation. *Biochem. J.* **262:** 173-180.

Markestad, T., Halvorsen, S., Halvorsen, K.S., Aksnes, L. and Aarskog, D. D. (1984). Plasma concentrations of vitamin D metabolites before and during treatment of vitamin

D deficiency rickets in chidren. *Acta. Paediatr. Scand.* **73:** 225-231.

Marsden, C.D., Reynolds, E. H., Parsons, V., Harris, R and Duchen, L. (1973). Myopathy associated with anticonvulsant osteomalacia. *Br. Med. J.* **4:** 526-527.

Martin, D.L. and DeLuca H.F. (1969). Influence of sodium on calcium transport by the rat small intestine. *Am. J. Physiol.* **216:** 1351-1359.

Marx, S.J, Liberman, U.A., and Eil, C. (1983). Calciferols: actions and deficiencies in action. *Vitam. horm.* **40:** 235-308.

Marx,S.J., The Metabolic Basis of Inherited Diseases. Eds. Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. McGraw-Hill Information Services. 6th Edition (1989) Book II, 2029-2045.

Massheimer,V., Fernandez, L.M., Boland, R and de Boland, A.R. (1992). Regulation of Ca^{2+}, uptake in skeletal muscle by 1,25-dihydroxyvitamin D_3. *Molecular and Cellular Endocrinology* **84:** 15-22.

Matsumoto, T., Fontaine, O.,and Rasmussen, H. (1980). Effect of 1,25-DHVD on phosphate uptake in chick intestinal brush border membrane vesicle. *Biochim. Biophys. Acta.* **599:** 13-23.

Matsuoka, L.Y., Wortsman, J., Haddad, J.G.and Hollis,B.W. (1989). In vivo threshold for cutaneous synthesis of vitamin D. *J. Lab. Clin. Med.* **114:** 301-305.

Mawer, E.B. (1982). Functional control over the metabolic activation of calciferol. Endocrinology of calcium metabolism. Edited by John A. Parsons. Raven Press, New York.

Mawer, E.B. and Backhouse, J. (1965). An improved system for the separation of

metabolites of isotopically labelled vitamin D_3 on silicic acid columns. *Biochem. J.* **112:** 255-256.

Mawer, E.B., Lumb, G.A. and Stanbury, S.W. (1969). Long biological half-life of vitamin D and polar metabolites in serum. *Nature.* **222:** 482-483.

Mawer, E.B., Lumb, G.A., Schaefer, K. and Stanbury, S.W. (1971). The metabolism of isotopically labelled vitamin D_3in man: The influence of the state of vitamin D nutrition. *Clin. Sci.* **40:** 39-53.

Mawer, E.B., Backhouse, J., Holman, C.A., Lumb, G.A.and Stanbury,S.W. (1972). The distribution and storage of vitamin D and its metabolites in human tissues. *Clin. Sci.* **43:** 413-431.

Mawer, E.B., Hann, J.T., Berry, J.L. and Davies, M. (1985a). Vitamin D metabolism in patients intoxicated with ergocalciferol. *Clin. Sci.* **68:** 135-141.

Mawer, E.B., Klass, H.J., Warnes, T.W., and Berry, J.L. (1985b). Metabolism of vitamin D in patients with primary biliary cirrhosis and alcoholic liver disease. *Clin. Sci.* **69:** 561-570.

Mellanby, E. (1919) A further demostration of the part played by accessory food factors in the aetiology of rickets. *Proc. Physiol. Soc. London* p.liii

Merke, J. and Norman, A.W. (1981). Studies of the mode of action of calciferol XXX11. Evidence for a 24(R),25(OH)D_3-vitamin D receptor in the parathyroid gland of the rachitic chick. *Biochem. Biophys. Res. Commun.* **100:** 551-558.

Miller, B E. and Norman, A.W. (1984). The Handbook of Vitamins, Nutritional, Biochemical and Clinical Aspects. Ed. L.J. Machlin p 45-97.

Miller, B.E. and Norman, A.W. (1979). Studies on the metabolism of calciferol. XIV:

Evidence of a circadian rhythm in the activity of the 25-hydroxyvitamin D_3-1-hydroxylase. *Biochem. Biophysics. Res. Commun.* **88:** 730-734.

Morgenstein, S.G., Kessler, J., Auerback, R.V., Flow and Klein, B. (1965). An automated p-nitrophenylphosphate serum alkaline phosphatase procedure for the autoanalyser. *Clin. Chem.* **11:** 876-878.

Morelli, S., de Boland A.R. and Boland, R.L. (1993). Generation of inositol phosphates, diacylglycerol and calcium fluxes in myoblasts treated with 1,25-dihydroxyvitamin D_3. *Biochem J.* **289:** 675-679.

Morrissey, R.L., Bucci, T.J., Empson, R.N.and, Lufkin,E.G. (1975). Calcium binding protein: its cellular localization in jejunum, kidney and pancreas. *Proc. Soc. Exp. Biol. Med.* **149:** 56-60.

Napoli, J.L. and Horst, R.L. (1983). C(24)- and C(23)- oxidation, converging pathways of intestinal 1,25-dihydroxyvitamin D_3 metabolism : Identification of 24-keto-1,23,25-trihydroxy-vitamin D_3. *Biochemistry* **22:** 5853-5858.

Nemere, I., Yoshimoto, Y., and Norman. A.W. (1984). Calcium transport in perfused duodena from normal chicks: Enhancement within fourteen minutes of exposure to 1,25-dihydroxyvitamin D. *Endocrinology* **115:** 1476-1483.

Nemere, I. and Norman, A.W. (1991). Steroid hormone actions at the plasma membrane: induced calcium uptake and exocytic events. *Mol. Cell. Endocrinol.* **80:** C165-C169.

Neville, P.F. and DeLuca H.F. (1966). The synthesis of [1,2-^{3}H] vitamin D and tissue localization of a 0.25ug (10 IU) dose per rat. *Biochemistry* **5:** 2201-2207.

Norman, A.W. (1966). Actinomycin D effect on lag in vitamin D-mediated calcium absorption in the chick. *Am. J. Physiol.* **211:** 829-834.

Norman, A.W. (1968). The mode of action of vitamin D. *Biol. Rev. Cambridge Philos. Soc.* **43:** 97-137

Norman, A.W. (1979). Vitamin D. The calcium homeostatic steroid hormone. Academic press. New York.

Norman, A.W. (1982). Fundamental and Clinical Bone Physiology. Ed. Urist, M.R. Published J.B.Lippincot Company, Philadelphia.

Norman, A.W. (1990a). The avian as as an animal model for the study of the vitamin D system. *J. Expt. Zool. Supplement.* **4:** 37-45.

Norman, A.W. (1990b). Intestinal calcium absorption: a vitamin D-hormone-mediated adaptive response. *Am. J. Clin. Nutr.* **51:** 290-300.

Norman, A.W. and DeLuca H.F.(1963). The preparation of ^{3}H- vitamins D_2 and D_3 and their localization in the rat. Biochemistry 2: 1160-1163.

Norman, A.W. and Wong, R.W. (1972). Biological activity of the vitamin D metabolite 1,25 dihydroxycholecalciferol in chickens and rats. *J. Nutr.* **102:** 1709-1718.

Norman, A.W., Roth, J. and Orci, L (1982) The vitamin D endocrine system : Steroid metabolism, Hormone receptors, and biological response (calcium binding proteins). *Endocrine Review* **3:4:** 331-366.

Norman, A.W. Leathers, V.L. and Bishop, J.E. (1983). Normal egg hatchability requires the simulataneous administrattion to the hen of 1,25 dihydroxyvitamin D and 24R,25-dihydroxyvitamin D. *J. Nutr.* **113:** 2505-2515.

O'Connell, T.D., Weishaar, R.E. and Simpson, R U.(1994). Regulation of myosin isozyme expression by vitamin D_3 deficiency and 1,25-dihydroxyvitamin D_3 in the rat heart. *Endocrinology* **134:** 899-905.

Oizumi, K. and Monder, C. (1972). Localization and metabolism of 1,2-^{3}H vitamin D_3 and 26,27-^{3}H-25-hydroxycholecalciferol in goldfish (Carassius auratus L.). *Comp. Biochem. Physiol.* **42B:** 523-532.

Palm, T.A. (1890) The geographical distribution and etiology of rickets. *Practitioner* **45:** 270.

Pan, L.C., and Price, P.A. 1986. *J. Bone Miner. Res.* 1(Suppl 1) Abstr. 20

Papapoulas, S.E., Clemens, T.L., Fraher, L.J., Lewin,I.G., Sandler, L.M.and O'Riordan. J.L.H. (1979). 1,25 dihydroxycholecalciferol in the pathogenesis of the hypercalcaemia of sarcoidosis. *Lancet* **(i):** 627-630.

Pettifor, J.M., Ross, F.P., Travers, R., Glorieux, F.H.and DeLuca H.F. (1981). Dietary calcium deficiency: A syndrome associated with bone deformitiesand elevated serum 1,25 dihydroxyvitamin D concentration. *Metab. Bone Disease Relat. Res.* **2:** 301-305.

Pettifor,J.M. (1990). Recent advances in paediatric metabolic bone disease: the consequences of altered phosphate homeostasis in renal insufficiency and hypophosphataemic vitamin D-resistant rickets. *Bone Miner.* **9:** 199-214.

Pike, J. W., Spanos, E.,Colston, K.W., MacIntyre, I. and Haussler, M.R. (1978). Influence of oestrogens on renal vitamin D hydroxylases and serum 1 25-$(OH)_2D_3$ in chicks. *Am. J. Physiol.* **235:** E338-E343.

Pointon, J.J., Francis, M.J.O. and Smith, R. (1979). Effect of vitamin D deficiency on sarcoplasmic reticulum function and troponin C concentration of rabbit skeletal muscle. *Clin. Sc.* **57:** 257-263.

Rambeck,W.A., Weiser,H. and Zucker, H. (1984). Biological activity of 1 alpha, 25-dihydroxyergocalciferol in rachitic chicks and in rats. *Int. J. Vitam. Nutr. Res.* **54:** 135-139.

Rasmussen, H. and Tenenhouse, H.S. (1989). Metabolic Basis of Inherited Disease II. (6th Edition). Ed.Scriver, C.R. Beaudet, A.L. Sly, W.S. WcGraw-Hill Inc.

Ratzkowski, C., Fine, N.,and Edelstein, S. (1982). Metabolism of cholecalciferol in vitamin D intoxicated chicks. *Isr. J. Med. Sci.* **18:** 695-700.

Ray, R., Bouillon, R., Van Baelen, H. and Holick, M.F. (1991). Photoaffinity labeling of human serum vitamin D binding protein and chemical cleavages of the labeled protein : Identification of an 11.5 kDa peptide containing the putative 25-hydroxyvitamin D binding site. *Biochemistry* **30:** 7638-7642.

Reddy, G.S and Tserng, K-Y. (1986). Isolation and Identification of 1,24,25-Trihydroxyvitamin D 1,24,25,28-Tetrahydroxyvitamin D and 1,24,25,26-Tetrahydroxyvitamin D: New Metabolites of 1,25- dihydroxyvitamin D produced in Rat Kidney. *Biochemistry* **25:** 5328-5336.

Reddy, G.S. and Tserng, K-Y. (1989). Calcitroic acid, end product of renal metabolism of $1,25(OH)_2D_3$ through C-24 oxidation pathway. *Biochemistry* **28:** 1763-1769.

Reddy, G. S. and Tserng, K-Y. (1990). 24,25,28-trihydroxyvitamin D_2 and 24,25,26-trihydroxyvitamin D_2 : Novel metabolites of vitamin D_2. *Biochemistry* **29:** 943-949.

Reeve, L.E., DeLuca, H. F. and Schnoes, H. K. (1981). Synthesis and biological activity of vitamin D_3-sulfate. *J. Biol. Chem.* **256:** 823-826

Reichel, H., Koeffler, H.P.,and Norman, A.W. (1989). The role of the vitamin D system in health and diseaes. *N. Engl. J. Med.* **320:** 980-991.

Rosenstreich, S J., Rich, C. and Vilwiler, W. (1971). Deposition in and release of vitamin D_3 from body fat: Evidence for a storage site in the rat. *J. Clin. Invest.* **50:** 679-687.

Rudack-Garcia, D. and Henry, H.L. (1981). Effect of vitamin D status on cyclic AMP dependent protein kinase activity and its heat stable inhibitor in the chick kidney. *J. Biol. Chem.* **256:** 10781-10785.

Scriver, C.R., Reade,T.M., DeLuca, H.F. and Hamstra, A.J. (1978). Serum 1,25-dihydroxyvitamin D levels in normal subjects and in patients with hereditary rickets or bone disease. *N. Engl. J. Med.* **299:** 976-979.

Sedrani,S.H. (1984). Changes in serum levels of 1,25-DHVD, calcium and phosphorus with age and vitamin status in chickens. *Br. J. Nutr.* **52:** 329-334.

Shepard, R. M., Horst, R.L., Hamstra,A.J. and DeLuca, H.F. (1979). Determination of vitamin D and its metabolites in plasma from normal and anephric man. *Biochem. J.* **182:** 55-69.

Shepard,R.M. and DeLuca H. (1980). Plasma concentration of vitamin D and its metabolites in the rat as influenced by vitamin D or 25 hydroxyvitamin D intakes. *Arch. Biochem. Biophys.* **202:** 43-53.

Silver, J., Neale, G. and Thomson, G.R. (1974). Effect of phenobarbitone treatment on vitamin D metabolism in mammals. *Clin. Sci. Mol. Med.* **46:** 433-448.

Silver, J., Russel, J., and Sherwood, L.M. (1985). Regulation by vitamin D metabolites of messenger ribonucleic acid for preproparathyroid hormone in isolated bovine parathyroid cells. *Proc. Natl. Acad. Sci. USA.* **82:** 4270-4273.

Sjoden, G., Smith, C., Lindgren, U., and DeLuca, H.F. (1985). 1 Hydroxyvitamin D_2 is less toxic than 1 hydroxyvitamin D_3 in the rat. *Proc. Soc. Exp. Biol. Med.* **178:** 432-436.

Somjen, D., Binderman, I., Harell. and Weisman, Y. (1982). Specific receptors for 24R $25(OH)_2D$ in the epiphyses of long bones in vitamin-depleted rats (p 185-192). Current

advances in skeletogenesis: Development, Biomimeralization, Mediators and Metabolic Bone Disease (Eds. Silberman,M. and Slavkin, H.C.) Elsevier, New York).

Stanbury, S.W. (1977). The role in vitamin D in renal bone disease. *Clin. Endocrinol. Supp.* **7:** 255-305.

Steenbock, H. and Black, A. (1925). The induction of growth promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. *J. Biol. Chem.* **64:** 263-298.

Steenbock, H. and Herting, D.C. (1955). Vitamin D and growth. *J. Nutr.* **57:** 449-4468.

Stern, P.H., Taylor, A.B., Bill, N.H. and Epstein, S.(1981). Renal production of $1,25(OH)_2D$ in normal children loosely regulated. *J. Clinic. Invest.* **68:** 1374-1377.

Suda, T., DeLuca, H.F., Schnoes, H.K., Tanaka, Y.,and Holick, H.F. (1970). 25,26-dihydroxycholecalciferol, a metabolite of vitamin D_3 with intestinal calcium transport activity. *Biochemistry* **9:** 4776-4780.

Takasaki. Y., Suda, T., Yamada, S., Takayama, H.,and Nashii, Y. (1981). Isolation, identification and biological activity of 25-hydroxy-24-oxovitamin D_3: a new metabolite of vitamin D_3 generated by in vitro incubation with kidney homogenates. *Biochemistry* **20:** 1681-1686.

Thierry-Palmer, M. (1983). Rat kidney microsomes convert 25-hydroxyvitamin D to an unidentified metabolite. *Biochem. Biophys. Res. Commun.* **110:** 766-771.

Tjellesen, L., Gotfredsen, A. and Christiansen, C. (1985). Different actions of vitamin D_2 and D_3 in bone metabolism treated with phenobarbitone/phenytoin. *Calcif. Tissue Int.* **37:** 218-222.

Vieth, R. (1990).The mechanism of vitamin D toxicity. *Bone Miner.* **11:** 267-272.

Vitamin Tolerance of Animals, (1987). Committee on animal nutrition National Research Council. National Academy Press, Washington, D.C.

Wakasugi,M., Noguchi,T., Inoue,M., Kazama,Y., Tawta,M.,Kanemaru,Y. and Onaya,T. (1991) Vitamin D_3 stimulates the production of prostacyclin by vascular smooth muscle cells. *Prostaglandins* **42:** 127-136.

Walters, M.R. (1992). Newly identified actions of the vitamin D endocrine system. *Endocr. Rev.* **13:** 719-763.

Wasserman, R.H. and Taylor A.N. (1973). Intestinal absorption of phosphate in the chick: Effect of vitamin D and other parameters. *J. Nutr.* **103:** 586-599.

Webb, A.R., DeCosta B.R.,and Holick M.F. (1989). Sunlight regulates the cutaneous production of vitamin D by causing its photo- degradation. *J. Clin. Endocrinol. Metab.* **68:** 943-949.

Wichman,J., Schnoes, H.K. and DeLuca, H.F. (1981). Isolation and identification of 24(R)-hydroxyvitamin D_3 from chicks given large doses of vitamin D_3. *Biochemistry* **20:** 2350-2353.

Wilhelm, F., Mayer, E., Norman, A.W. (1984). Biological activity assessment of the 26,23-lactones of 1,25-dihydroxyvitamin D and 25-hydroxyvitamin D and their binding properties to chick intestinal receptor and plasma vitamin D binding protein. *Arch. Biochem. Biophys.* **233:** 322-329.

Wing, R.M., Okamura, W.H., Pirio M R., Sine, S.M.and Norman, A.W. (1974). Vitamin D in solution: Conformations of vitamin D 1,25 dihydroxy-vitamin D and dihydrotachysterol. *Science* **186:** 939-941.

Yamamoto, M., Kawanobe, Y., Takahashi, H., Kimura, S. and Ogata, E. (1984). Vitamin D deficiency and renal calcium transport in the rat. *J. Clin. Invest.* **74:** 507-513.