

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF GHANA

APPLICATION OF COMPETITIVE BINDING ASSAY METHODS
TO SERUM VITAMIN D LEVELS IN
HEALTH AND DISEASE

THIS THESIS WAS PRESENTED TO THE
BIOCHEMISTRY DEPARTMENT,
UNIVERSITY OF GHANA
FOR THE MASTER OF
SCIENCE DEGREE
1979

BY
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DEDICATION

To

My Parents

Kwashie and Ashie

DECLARATION


The work presented here was undertaken by me at the Nuclear Medicine Unit, University of Ghana Medical School, Korle-Bu under the co-supervision of Dr. S. Dakubu and Dr. M.F. Chaplin.



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.....
(M.F. CHAPLIN)
.....

A C K N O W L E D G E M E N T

For introducing me to the realms of Competitive Binding Assay and related procedures and for guidance through the work, I wish to express my sincere gratitude to my supervisor, Dr. S. Dakubu, Nuclear Medicine Unit, University of Ghana Medical School, Korle-Bu. I wish to thank Dr. M.F. Chaplin, Biochemistry department, Legon, for his time to time useful hints and suggestions made available.

The work would not have taken off but for the help of Dr. J.C. Waterlow, Department of Human Nutrition, London School of Hygiene and Tropical Medicine who donated to us the rachitic rat serum, the standard 25-hydroxyvitamin D₃ and a protocol for the assay. His kindness is very much appreciated.

Through the help of Prof. A.K. Foli and Dr. D. Adu (Department of Medicine, Korle-Bu) serum samples were obtained from patients with cirrhosis of the liver and chronic renal failures.

Finally I wish to thank Mr. S.K. Caesar (Nuclear Medicine Unit), Mr. Defencer Anyetei Biochemistry department, Legon and all those through whose help

one way or another contributed to bring this
work to fruition.

I. S. AHENE

LEGON

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ABBREVIATIONS

B	- radioactivity bound at any concentration of the ligand.
B ₀	- radioactivity bound at zero ligand concentration.
bis MSB	- p _p bis (5-methylstyryl) benzene
BSA	- bovine serum albumin
CaBP	- calcium binding protein
CB	charcoal blank
Concn	- concentration
cpm	- counts per minute
CRF	- chronic renal failure
DBP	- vitamin binding protein
DCC	- dextran coated charcoal
E.Y	- extraction yield
HPLC	- high pressure liquid chromatography
IU	- international unit
IUPAC	- International Union of Pure and Applied Chemistry
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
NSB	- non specific binding
POPOP	- 1,4, - bis - 2 - (4-methyl - 5 - phenyl - oxazole)
PPO	- 2,5-diphenyloxazole
PTH	- parathormone

QC	- quality control
RNA	- ribonucleic acid
SD	- standard deviation
SN	- supernatant
STD	- standard
T	- Total
TA	- total activity
UV	- ultraviolet

APPLICATION OF COMPETITIVE BINDING ASSAY
METHODS TO SERUM VITAMIN D LEVELS
IN HEALTH AND DISEASE

ABSTRACT

Competitive Binding Assay methods have been used to determine the serum levels of 25-hydroxyvitamin D₃ in healthy Ghanaians and patients suffering from cirrhosis of the liver and chronic renal failure.

Although the assay followed fairly standard methods as used by several workers, an improvement in stability and sensitivity was obtained by the use of gelatin in the buffer; the final assay buffer being 0.02M phosphate buffer, pH 7.0 with 0.1% gelatin.

Though rachitic rat serum was used for the main part of the work, it was confirmed that normal rat serum contained the binding protein at a suitable titre for the assay as has been reported in the literature.

The normal circulating levels in 47 healthy Ghanaians was 129.0 ± 55.7 ng/ml serum, 46.0 ± 16.1 ng/ml ($\bar{x} \pm SD$) for 11 patients with cirrhosis of the liver and

83.5 \pm 82.7 ng/ml for 8 patients suffering from chronic renal failure. Both patients' levels were significantly different from the normal mean value ($p < 0.05$).

CHAPTER ONE

LITERATURE REVIEW

1.1. INTRODUCTION

It is well established that the deficiency of vitamin D, sometimes referred to as the 'sunshine vitamin' is the cause of rickets in infants and osteomalacia in adults. The basic functions ascribed to vitamin D are mineralisation of bone, absorption of calcium from the intestine, and renal reabsorption of calcium and phosphorus. Before these functions become operative, vitamin D undergoes at least two hydroxylation reactions, one in the liver followed by another in the kidney.

Calcium whose availability is controlled by vitamin D participates in a wide range of biochemical processes (nerve transmissions, blood clot formation, maintenance of membrane integrity, muscle contraction, egg shell formation and bone formation). The functions of phosphorus cannot be overemphasized. Its role in energy production, nucleotide formation, bone formation and presence in phospholipids are well known.

Obviously, diseased states may arise from the defects in the metabolic pathways of vitamin D and its control

mechanisms. The resultant effect will culminate in a change in one or more of vitamin D metabolites. In this chapter the chemistry, metabolism and methods of estimation of vitamin D will be considered. An attempt will be made to show the importance of vitamin D in some disease states. With this background a method for quantitation will be selected to establish the normal circulating levels in Ghanaians and a few disease states where vitamin D levels might be expected to change.

1.2. THE VITAMIN CONCEPT AND VITAMIN D

A vitamin may be defined as an organic compound of nutritional nature that is present in low concentrations as natural component of enzyme systems and therefore helps to catalyse reactions; it may be derived externally or by intrinsic biosynthesis in the tissues (1).

Vitamins have been arbitrarily referred to by the letters of the alphabet depending on the order in which they were discovered (2). Thus the letters A, B, C, D etc. were assigned to the vitamins awaiting the elucidation of their chemical nature. This has however appealed to many workers long after the chemical nature of the vitamins

has been determined.

Correlation between sunshine and the bone disease rickets was recognised as far back as 1882 (3). These observations led Mellanby (4) to conduct experiments to show that the lack of a dietary factor was involved in the production of rickets in puppies. Hess and Gutman (5) and McCollum and Davis (6) later identified the antirachitic factor as vitamin D. One can therefore refer to vitamin D as belonging to those 'food accessories that possess curative or prophylactic effect' - on rickets.

Foodstuffs irradiated with ultraviolet rays were also shown to possess antirachitic properties (7). At first cholesterol was regarded as the provitamin in the foodstuffs but in 1926 it was shown (8,9) that the biological activity depended on an 'impurity' in the cholesterol preparations.

The contaminant was found to be ergosterol. In 1930-31 potent crystalline preparations called vitamin D₁ (10) or calciferol were isolated from irradiated products of ergosterol. The pure vitamin was fully described in 1932

as vitamin D₂ (11, 12, 13) or ergocalciferol. The earlier preparation of vitamin D₁ was found to be a mixture of vitamin D₂ and lumisterol (14).

1.3. THE CHEMISTRY OF VITAMIN D

Vitamin D belongs to a group of chemical compounds that occur widely in nature, the steroids. The steroids possess a parent nucleus of perhydrocyclopentanophenanthrene which consists of three six membered rings A, B, C and a five membered ring D. The rings possessing a total of seventeen carbon atoms are joined as shown in fig. 1.1.

Apart from minor differences due to the presence of nuclear substituents and sometimes a degree of unsaturation, the diverse compounds classified as steroids arise mainly from variations in the side chains R₁, R₂ and R₃. R₁ and R₂ are generally methyl groups, although incompletely oxidised groups such as primary alcohol and aldehyde groups may be encountered. The side chain R₃ may be absent or may comprise two, four, five, eight, nine or ten carbon atoms. Many of these natural compounds have one or more alcoholic functional substituents. These secondary alcohols are known as sterols. The ring system may undergo fission to give an open structure. This open ring is denoted by seco in

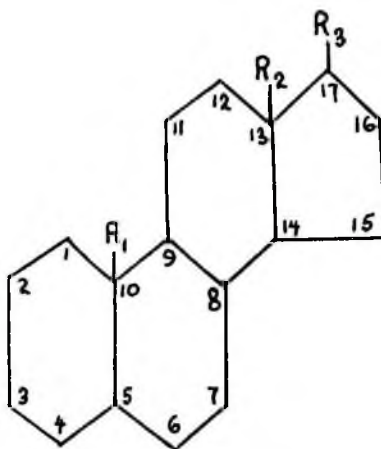


Fig. 1.1 : perhydrocyclopentanophenanthrene ring

R₁, R₂ and R₃ are substituents on

the parent ring

nomenclature for steroids. More detailed general chemistry of the steroids is described in the literature (15, 16, 17).

The compounds with vitamin D activity are seco-sterols. Their basic structure is shown in fig. 1.2. The differences among the group arise as a result of changes in the side chain R_3 , Figure 1.2b and 2c depict vitamin D_2 and D_3 respectively. Other substances that have demonstrated vitamin D activity are D_4 , D_5 , D_6 and D_7 . These are produced artificially by irradiating 22-dihydroergosterol, 7-dehydrositosterol, 7-dehydrostimaesterol and 7-dehydrocampesterol respectively. These have not yet been identified in animals (18).

Vitamin D_3 is the naturally occurring form found in animals. Vitamin D_2 is produced synthetically by ultraviolet irradiation of ergosterol, a plant sterol.

Henceforth, the term vitamin D refers to the group. Specific mention of various vitamins will be distinguished by the subscript, for example vitamin D_2 or D_3 .

<u>VITAMIN</u>	<u>FORMULA</u>	<u>STRUCTURE</u>
D		
D ₂	C ₂₈ H ₄₄ O	R =
D ₃	C ₂₇ H ₄₄ O	R =
D ₄	C ₂₈ H ₄₆ O	R =
D ₅	C ₂₉ H ₄₈ O	R =
D ₆	C ₂₉ H ₄₆ O	R =
D ₇	C ₂₈ H ₄₆ O	R =

Fig. 1.2: Members of vitamin D group. The secosterols which have been found to have vitamin D activity vary in the side chain at Carbon-17.

1.3.1. Nomenclature, units and some physical properties.

In addition to accepted generic names like ergocalciferol or calciferol for D_2 and cholecalciferol (D_3) the members of the group have been named following the system adopted by IUPAC (International Union of Pure and Applied Chemistry) (19). Under this scheme vitamin D_2 is 9, 10 - secoergosta - 5, 7, 10 (19) 22 tetraene - 3 β -ol, and vitamin D_3 as 9, 10 - secocholesta - 5, 7, 10 (19) - trien - 3 β -ol (20).

One International Unit (I.U.) of vitamin D is equivalent to 25ng of the crystalline vitamin.

Vitamin D_2 ($C_{28}H_{44}O$) has a molecular weight of 396.66, its melting point is 115-118°C. The wavelength of maximum absorption (λ_{max}) in hexane is 264nm Extinction coefficient $E_{1cm}^{1\%} = 459$

Vitamin D_3 ($C_{27}H_{44}O$) has a molecular weight of 384.65. It melts at 84-88°C. The λ_{max} is 264nm; in hexane; $E_{1cm}^{1\%} = 450$.

1.4. METABOLISM OF VITAMIN D₃

A vitamin has been defined in section 1.2. This takes into consideration the biosynthesis in the tissues. The simpler and earlier definition had considered a vitamin as a trace dietary component necessary for the normal functioning of physiological processes. Recent revelations of the metabolic pathways of some vitamins have necessitated the broadening of the primordial concept. A look at the metabolic pathway of vitamin D₃ identifies the skin as site of its synthesis. It is then converted to the most active form 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) in the kidney using 25-hydroxyvitamin D₃ (25-OH D₃) as a substrate. The 1,25 (OH)₂ D₃ has been regarded as a hormone (see later, section 1.4.6.).

1.4.1. Synthesis of 7-dehydrocholesterol.

Mammals including man are capable of synthesizing 7-dehydrocholesterol, the penultimate step in the biosynthesis of cholesterol. The details of the conversions are described in the literature (21,22). All tissues are capable of carrying out the cholesterol biosynthesis. Liver, skin, gastro-intestinal tract, adrenal glands and

the gonads are the most active with adipose tissues, muscle, aorta and adult brain of lower order of activity.

Acetate is the principal precursor of cholesterol. Of the 27 carbons of cholesterol 15 have been shown to arise from the methyl groups while 12 are derived from the carbonyl of the acetate. The synthesis involves the conversion of the acetate moieties to squalene. This is followed by the biosynthesis of lanosterol from squalene. The reaction requires molecular oxygen for the hydroxylation process at carbon-3. The enzyme which mediates the reaction needs NADPH as a co-factor. It converts squalene to 2, 3-oxidosqualene. A second enzyme, 2, 3-oxidosqualene sterol cyclase brings about the cyclisation of squalene. The enzymatic conversion of lanosterol to 7-dehydrocholesterol involves the loss of three methyl groups from carbons 4 and 14. This may be achieved by initial hydroxylation, followed by oxidation to carbonyl groups with subsequent decarboxylation, isomerisation of 8, 9-double bond to 7-8 position and the reduction of side chain double bond (fig. 1.3).

The sequence of the conversions is not yet clear but

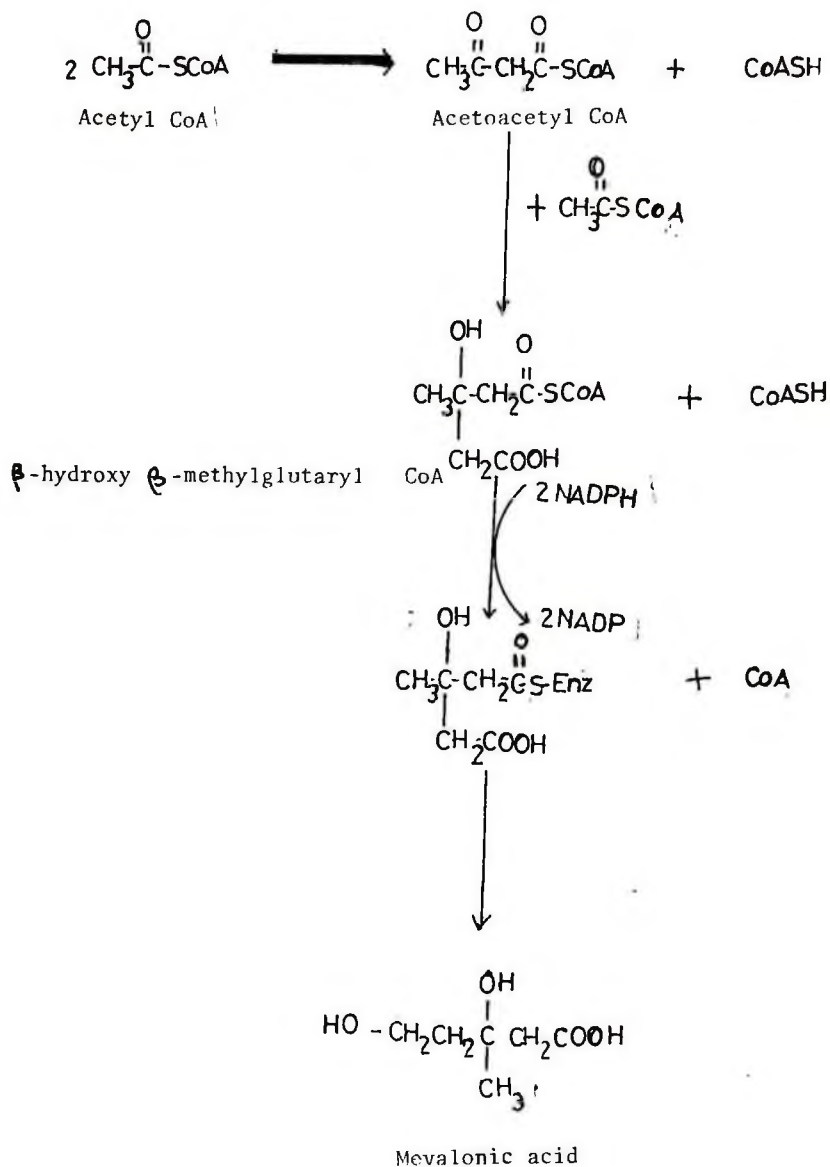


Fig. 1.3. Synthesis of vitamin D₃ from Acetyl CoA

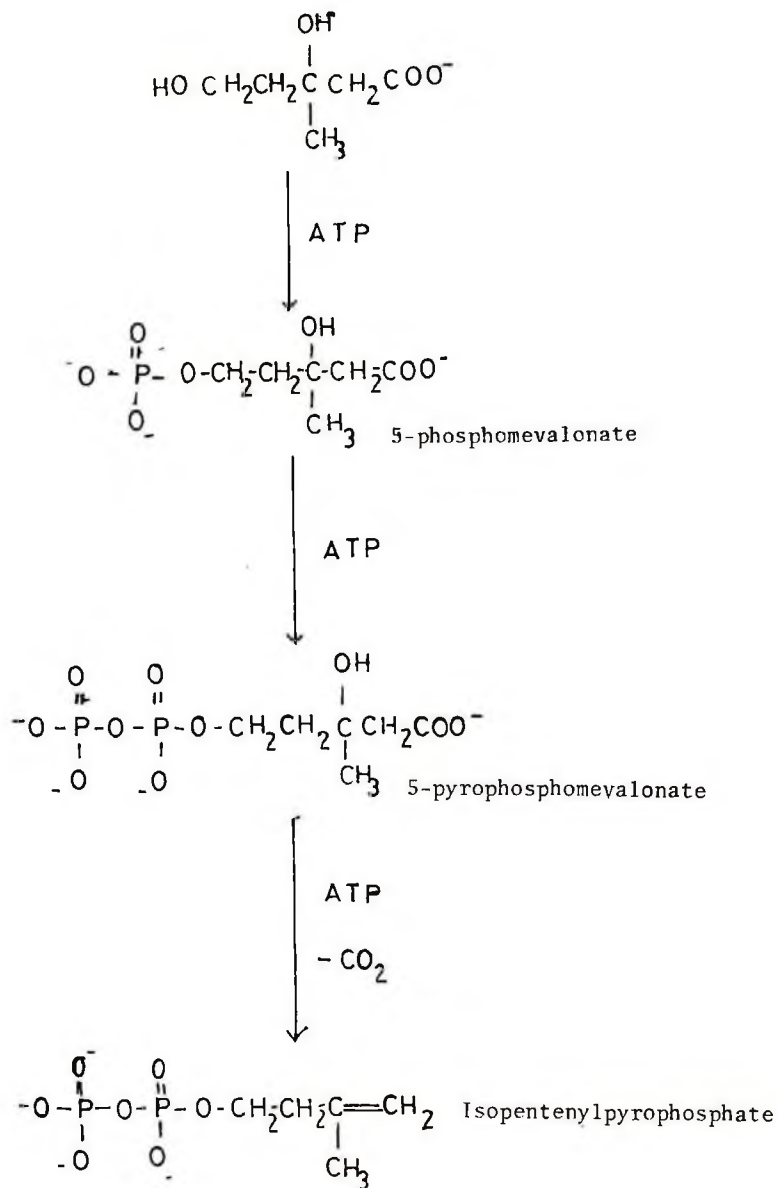


fig 1.3 continued

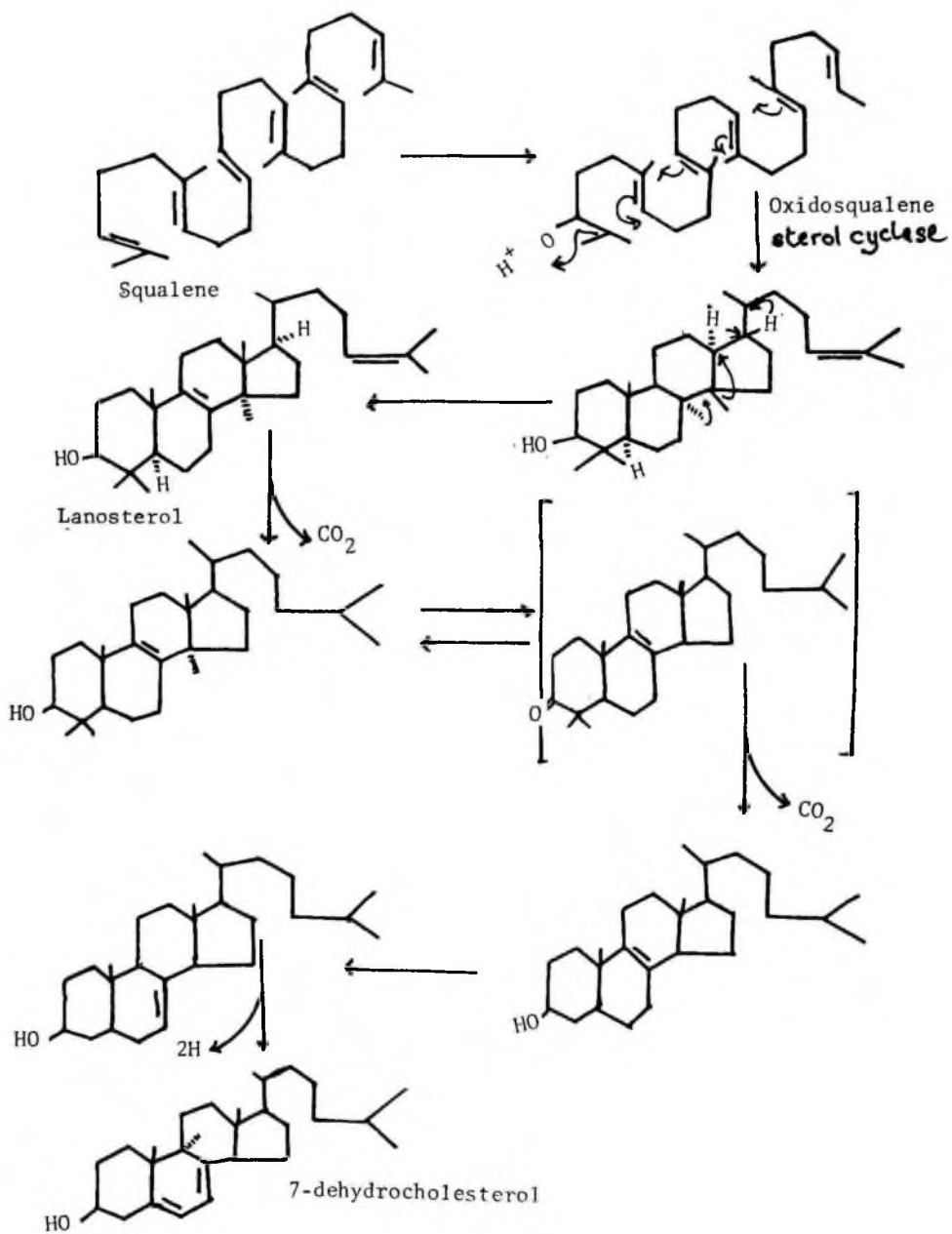


fig 1.3 continued

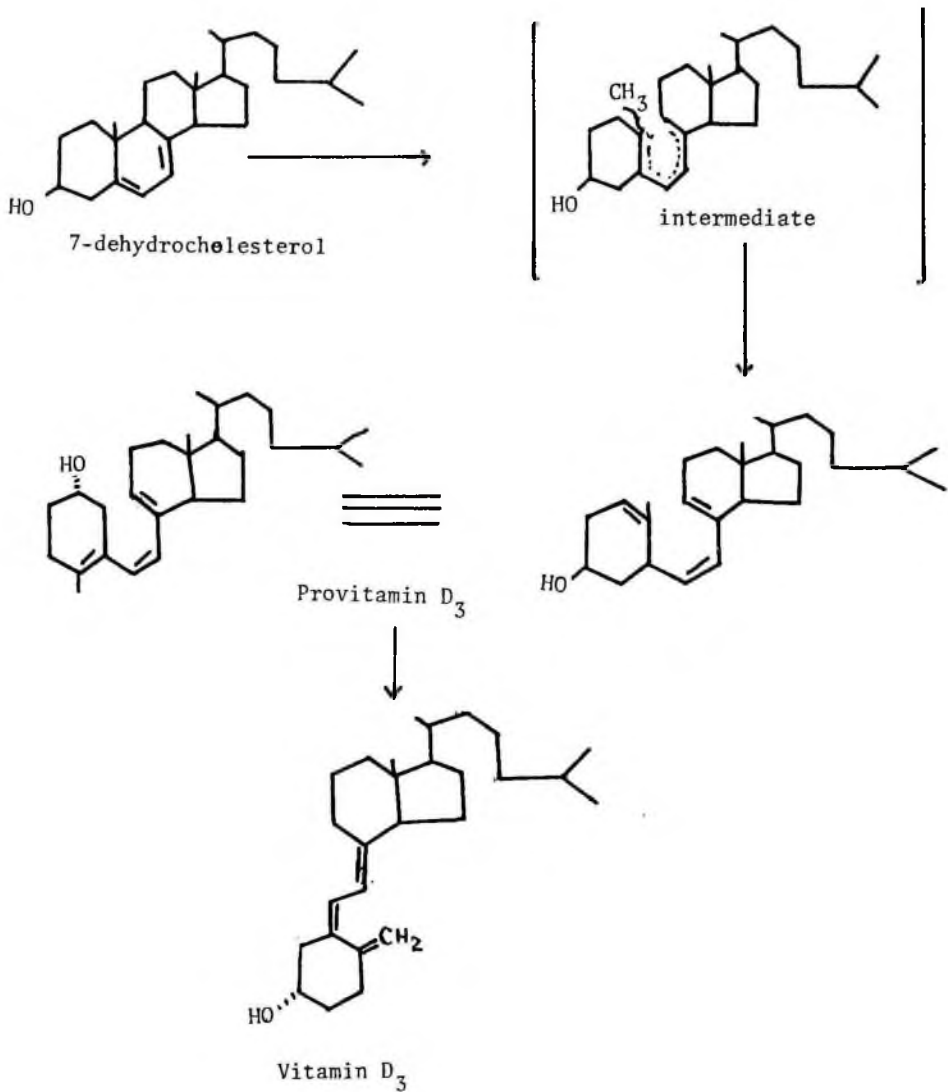


Fig 1.3 continued

the basic steps are the same in all tissues. The variations in levels of intermediates in various tissues may be attributed to the different rates. Thus the inability to isolate 7-dehydrocholesterol from the liver presumably points to a more active or greater concentration of the reductase. In contrast 7-dehydrocholesterol has been isolated as a normal component of skin sterols (23).

1.4.2. Production of vitamin D₃ in the skin

About 30% of the light in the 290-300nm wavelength range incident on the skin penetrates the stratum corneum in the epidermis and as much as 8% penetrates through the epidermis to the dermis. The estimates of course vary with skin pigmentation and keratinization of the underlying stratum corneum. Of the sterols found in the dermis, epidermis and surface lipids of the skin 7-dehydrocholesterol is the sole compound which is activable to vitamin D₃. There is a greater amount of sterols in the epidermis than the dermis and there is a more rapid appearance of ¹⁴C from ¹⁴C-labelled acetate in the sterol of the epidermis (24, 25). Thus, the site of biosynthesis of vitamin D₃ in the skin seems

to be the epidermis. The dermis does not appear to have the ability to synthesize sterols. The synthesis actually occurs in the sebaceous glands.

Though it was generally accepted that vitamin D₃ was made in the skin by the action of solar ultraviolet irradiation on 7-dehydrocholesterol the mechanism remained to be investigated until Holick et al. (26) presented evidence which suggested that the photoconversion of 7-dehydrocholesterol to vitamin D₃ in the skin occurred through an intermediate, previtamin D₃ which was further converted to vitamin D₃ by the thermal action of body heat.

1.4.3. 25-hydroxyvitamin D₃

It is now known that vitamin D₃ undergoes at least two steps of hydroxylations before it functions (27, 28, 29) with perhaps the sole exception of its action on the muscle (27). Vitamin D₃ taken in the diet is absorbed from the intestine probably through the lymphatic system. The vitamin D₃ is carried either by the specific binding protein or on β -lipoprotein but it is rapidly cleared from the plasma by the liver (30). In the liver vitamin D₃

is hydroxylated on carbon-25 (31) to give 25-hydroxyvitamin D₃ (25-OH D₃).

The subcellular location of the conversion is the endoplasmic reticulum (32). The hydroxylation system requires NADPH and molecular oxygen. Inhibitor studies show that the conversion is not catalyzed by cytochrome p-450. It is also not inhibited by lipid peroxidation inhibitors (33). There have been speculations on the existence of other sites of hydroxylation apart from the liver. Tucker and his colleagues (34) reported that homogenates of chick intestine and kidney carry out the 25-hydroxylation. Although this report has been confirmed (28) its physiological significance remains doubtful as similar conversions do not occur in rat tissues. In addition, hepatectomised rats fail to produce any measurable 25-hydroxyvitamin D₃ during the 4 hours of survival (27).

In conclusion, therefore, since the liver initially accumulates most of the vitamin D₃ and since the amount of hydroxylation in absence of the liver is negligible, it seems reasonable to assume that at least in mammalian species the liver is the major if not the sole site of 25-hydroxylations.

1.4.4. 1,25-dihydroxyvitamin D₃

For some time it was thought of the monohydroxylated vitamin D₃ as being the most active metabolite. However the amounts required for the mobilisation of calcium from bone in vitro (35) are too high to be of any physiological significance. Evidence adduced later indicated that 25-OH D₃ will not cause mobilisation of calcium from bone nor will it initiate intestinal calcium transport in nephrectomised animals (36, 37). Haussler et al. (38) recognised a vitamin D₃ polar metabolite in the intestine which was later identified as 1,25-dihydroxyvitamin D₃ (1,25 (OH)₂ D₃, 39, 40). 1,25-(OH)₂ D₃ was isolated from chick intestine (39, 40) and chick kidney homogenate (41). 1,25-(OH)₂ D₃ acts faster than ~~any~~ vitamin D₃ in the calcium mobilisation in bone and absorption from the intestine. It is also functional in nephrectomised animals whereas its immediate precursor 25-OH D₃ is not. It was therefore concluded that 1,25-(OH)₂ D₃ must be the metabolic^{ally} active form that brings about the functions that have been ascribed to vitamin D₃ (see 1.1.).

The kidney was found to be the site of the second hydroxylation of vitamin D₃ on the 1-position (42). The reaction takes place exclusively in the mitochondria of the kidney (42, 43). It appears the 1-hydroxylation needs cytochrome P-450 (44) and a requirement for a flavoprotein and another ferridoxin-like protein in order to incorporate molecular oxygen into the 1-position (45).

The 1-hydroxylase enzyme is a mixed function oxidase in which all the molecular oxygen incorporated into the 1-position on 25-OH D₃ arises from molecular oxygen. This eliminates any hydrogenase reaction followed by hydration as a mechanism (46).

1.4.5. Regulation of vitamin D₃ metabolism.

The action of 25-hydroxylase in the liver is regulated to some degree by the product of reaction 25-hydroxyvitamin D₃. When vitamin D₃ is administered to vitamin D deficient animals the hydroxylation is repressed (47). The degree and length of time of suppression is related to the size of the dose given and also the hepatic level of 25-hydroxyvitamin D₃.

Administration of vitamin D₃ to ^avitamin D deficient animal stimulates the enzymatic machinery towards the conversion of vitamin D₃ to 1,25(OH)₂ D₃ (48, 49). The appearance of 1,25(OH)₂ D₃ stimulates the synthesis of another hydroxylase, 25-OH D₃-24 hydroxylase also in kidney tissue (49). It converts 25-OH-D₃ to 24,25-dihydroxyvitamin D₃ ~~24,25-(OH)₂ D₃~~ which appears to be an inactivation product. 1,25 (OH)₂ D₃ will also be converted to 1,24,25-trihydroxyvitamin D₃ (1,24,25(OH)₃D₃ (50)). Like the 25-OH-D₃ 1-hydroxylase the 24-hydroxylase is found in renal mitochondria. Its appearance in response to 1,25 (OH)₂ D₃ brings about a feedback regulation in the renal hydroxylation systems. Under conditions of normal calcemia both 24,25(OH)₂ D₃ and 1,25(OH)₂ D₃ are produced but as the animal becomes hypocalcemic 1,25(OH)₂ D₃ is the major if not the sole dihydroxy metabolite appearing in the blood. In contrast 24,25(OH)₂ D₃ but not 1,25(OH)₂ D₃ is produced as the animal becomes hypercalcemic. Thus the need for calcium stimulates the production of calcium mobilising substance 1,25(OH)₂ D₃.

The hypocalcemic regulation of the 1-hydroxylase is

mediated by the parathyroid gland system. In this system the parathyroid glands monitor the serum calcium concentrations and in response to the hypocalcemic condition produce the parathyroid hormone (PTH). The parathyroid hormone proceeds to the kidney and the bone. In the kidney PTH causes phosphate diuresis, increases calcium reabsorption, stimulates the 1-hydroxylase and suppresses 24-hydroxylase. The result is an elevation of $1,25(\text{OH})_2\text{D}_3$ production. This form of vitamin D_3 then proceeds to the intestine where by itself ^{it} stimulates calcium absorption and together with PTH stimulates bone calcium mobilisation. There is the likelihood also that both $1,25(\text{OH})_2\text{D}_3$ and PTH increase renal reabsorption of calcium.

The resultant effect of these actions is the elevation of serum calcium concentration which represses the PTH secretion thus completing the feedback loop mechanism of the vitamin D endocrine system (27, see fig. 1.4.).

The level of serum calcium is not the only physiological regulator of the kidney hydroxylase system, phosphate also exerts some regulatory effect on the vitamin D

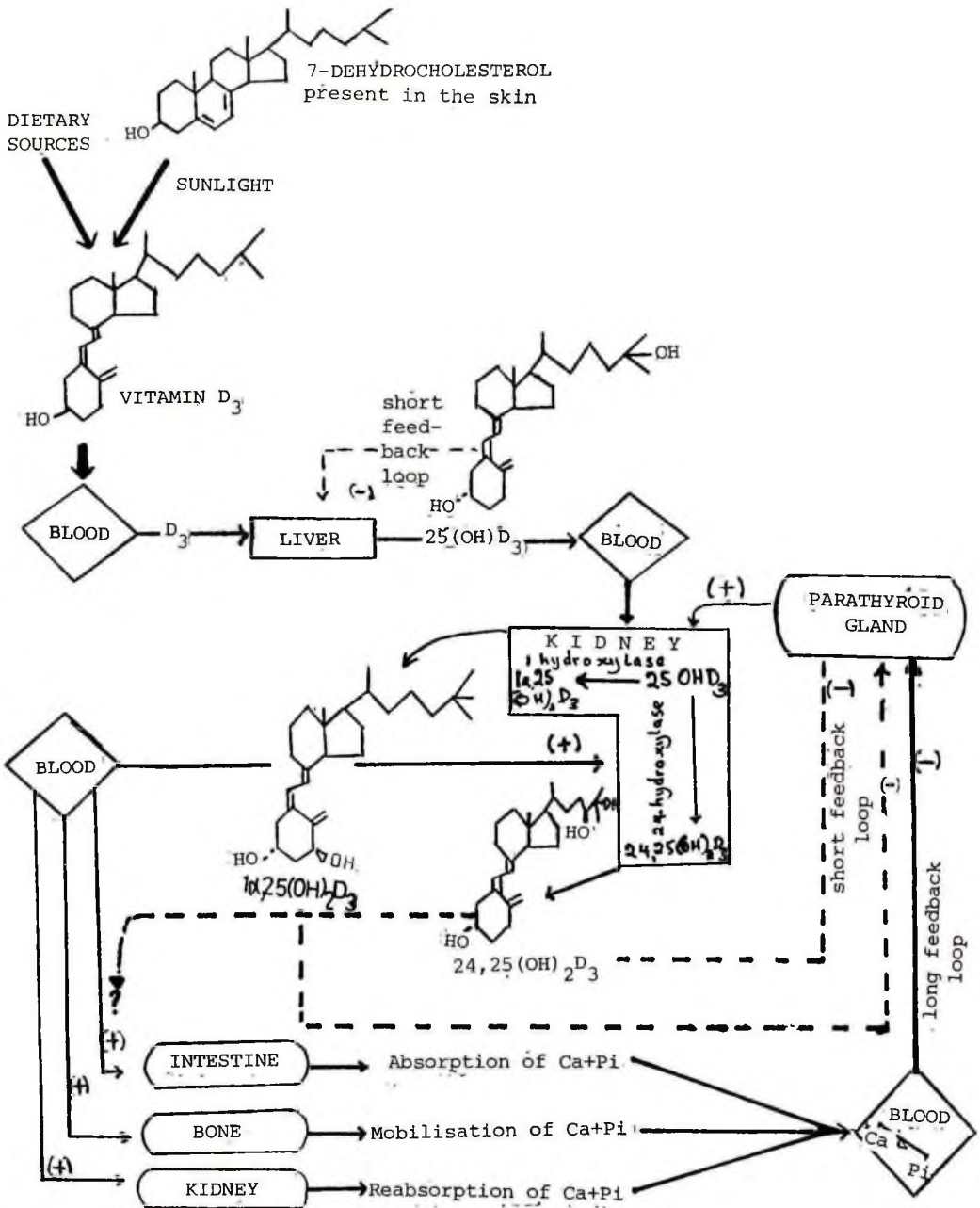


Fig. 1.4: Metabolism and control of vitamin D endocrine system. (Adapted from Norman and Henry(51));

hydroxylases. This can be demonstrated in rats which have been thyroparathyroidectomised. In which case the calcium regulatory system has been eliminated (52) and the animals produce mainly 24,25-dihydroxyvitamin D_3 and little 1,25-(OH) $_2$ D_3 . As the animals are made hypophosphatemic by dietary deprivation or glucose loading there is a turning on of the 1,25-(OH) $_2$ D_3 production and suppression of 24,25-(OH) $_2$ D_3 accumulation in the blood. Thus it is the combined effect of phosphate deprivation and presence of PTH that stimulate 1,25(OH) $_2$ D_3 production. It has however been suggested that the renal cell level of inorganic phosphorus is the determinant of whether the 1-hydroxylase or the 24-hydroxylase is active (52, 53).

The exact molecular mechanisms underlying these regulatory phenomena are not yet understood. However in vivo experiments on the regulatory effect may not be direct ionic activation or inhibition of the existing hydroxylases. The time course suggests rather a possible protein synthesis of a new enzyme and the degradation of an old one. There is also the possibility of the synthesis

of a regulatory substance or the degradation of one (27).

1.4.6. Vitamin D as a hormone.

The mode of action of vitamin D tends to render its classification as a vitamin a misnomer. Its metabolism delineates rather an endocrine system with the kidney as the sole organ (endocrine organ) which produces the metabolic active form $1,25\text{-(OH)}_2 \text{D}_3$ from its precursor 25OH D_3 . It is secreted and transported in the blood to the target organs (intestine, bone, muscle) where it acts at the level of gene transcription in a similar manner to other steroid hormones.

The action of $1,25\text{-(OH)}_2 \text{D}_3$ has been studied in more detail in intestinal cells than in any other target organ. The $1,25\text{-(OH)}_2 \text{D}_3$ initially binds to a cytosol receptor protein and this receptor complex migrates to the nucleus via a temperature dependent process, where it associates with the chromatin (54). Events that follow lead to the formation of messenger RNA which appears to be eventually attached to a polysomal array of 10-11 ribosomes for translation into calcium binding protein (CaBP).

Other hormonal attributes of the vitamin D endocrine

system is the concentrations of $1,25\text{-(OH)}_2\text{D}_3$ in target organs are in finite small quantities (270nM/nucleus in intestine, 140nM/nucleus in bone) and has an exacting control mechanism for its formation. Like other steroid hormones the 1-hydroxylase reaction in the kidney resembles that of 11β -hydroxylase for the corticosteroids and seems to be dependent on cytochrome P-450 (55).

1.5 THE VITAMIN D BINDING PROTEINS

It is of interest to know how vitamin D_3 and/or its metabolites are transported from one site to another in view of the isolated organs that process them (see section 1.4.). In 1954, Thomas and Co-workers found that serum proteins that were associated with antirachitic activity were α_1 and α_2 globulins in humans. Lesser amounts of activity was associated with the albumin fractions (56). These findings were confirmed by DeCrousaz *et al.* (57) and Chalk and Kodicek (58), Chen and Lane (59) observed that 80% of tritiated cholecalciferol (vitamin D_3) given to dogs sedimented when the serum was ultracentrifuged; 20% floated with the lipoprotein fraction. Rikkers and DeLuca (60) observed that vitamin D_3

associated rapidly with rat serum proteins in vivo. Four of them were shown to be lipoproteins while the fifth was an α -globulin.

The radioactivity first associated with the lipoproteins, the amount decrease with time and became attached to the α -globulin. It was therefore concluded that there is an α -globulin in the serum responsible for the transport of cholecalciferol.

1.5.1. The binding proteins, metabolite binders, species differences and target organ receptors.

It was established that there exists in human plasma a specific protein that is capable of binding both vitamin D₃ and 25-hydroxyvitamin D₃ (61, 62, 63). These findings did not answer the question whether different proteins (α -globulins) were responsible for each or not. It was through the work of Edelman *et al.* (64) that it was established that only one protein was responsible for vitamin D₃ and 25-OH-D₃ in the rat. Similar reports were made on the serum of man, pig and monkey (65). Thus in these animals and probably all mammals there is only a single binding protein for both vitamin D₃ and its 25-hydroxy derivative.

The chick, unlike the rat has two binding proteins circulating in the serum. These were named 25-hydroxycholecalciferol binding protein and cholecalciferol binding protein (64). Evidence adduced strongly suggests that 25-(OH) D₃ synthesized in the liver is released into the bloodstream bound to its specific binding protein.

Toads, lower in the rank of the evolutionary ladder do require vitamin D (66). A binding protein as found in man, rats, monkeys, and chicks is not present in the serum. The transportation is done on β -lipoprotein (64). Attempts to identify a specific binding protein for 1,25-dihydroxycholecalciferol (1,25-(OH)₂ D₃) circulating in the serum of rats have not proved successful. The reason may stem from the very minute circulating level if any. Up to date the question as to whether there is a specific binding protein for 1,25-(OH)₂ D₃ is still open (67).

Target organs for vitamin D₃ metabolites have been shown to possess specific receptor binding proteins. Haddad and Birge (68) found a receptor protein for 25-OH D₃ in kidney and muscle homogenates. Edelstein (69)

made similar findings in rachitic rat kidney, muscle, skin and bone. With the exception of the kidney the significance of tissue receptors for 25-OH D₃ in other tissues is not yet clear.

Receptors for the hormonally active 1,25(OH)₂ D₃, (Cytoplasmic receptor protein) have been found on intestinal mucosa (70, 71, 72). The binding of 1,25(OH)₂ D₃ to this protein is an obligatory step for the subsequent localisation of the hormone in the chromatin fraction (see 1.4.6.).

1.6. QUANTITATION OF VITAMIN D₃

Some of the functions of vitamin D were considered in section 1.1. These are two-fold-curative and prophylactic. The minimum daily requirement of vitamin D₃ for man is 400 I.U. (51). Curative doses are higher depending on the vitamin D status of the patient. Prolonged and excessive daily use of the vitamin however have been shown to cause undesirable side effects like headache, thirst, lassitude, urinary frequency, anorexia, nausea, vomiting, diarrhoea, abdominal discomfort, localised osteoporosis, anaemia, convulsions, renal impairment and

death (73). In cognisance of these side effects, before vitamin D therapy is administered the endogenous circulating level must be ascertained.

Individuals exposed to sunlight have high circulatory levels of vitamin D₃ (74). Thus it is expected that higher circulating levels of the vitamin D₃ will be encountered in the tropics in general and Ghana in particular.

The dietary contribution to vitamin D₃ level is small. The foodstuffs in the Ghanaian context that are likely to provide the vitamin are egg yolk, and fortified food like milk ('Ideal milk', 1.0 I.U./g), 'Milo' (2.4 I.U./g) and 'Blue Band' margarine and fan milk (concentration unquoted). These foodstuffs which may contain some vitamin D₃ are only taken habitually by a small fraction of the Ghanaian population. Therefore in Ghana the major source of vitamin D₃ is that from the biosynthesis in the skin (section 1.4.2.).

The vitamin D₃ from the skin is hydroxylated at the 25 position in the liver (section 1.4.3.). The liver also

stores vitamin D_3 (34). One therefore expects the malfunction of the liver to alter the level of $25\text{-OH-}D_3$ hence the importance of its measurement in liver diseased patients. The levels of $25\text{-OH-}D_3$ in cirrhosis have been determined by various workers in regions of relatively subdued sunlight (75). It is therefore of interest to establish here the level in liver disease patients (cirrhotics).

The hepatic product is the substrate for 1-hydroxylase of the kidney (section 1.4.4.). Thus if the kidney is functioning well $25\text{-OH-}D_3$ will be removed during the formation of $1,25(\text{OH})_2 D_3$. However with a defective kidney the level of $25\text{-OH-}D_3$ is expected to be high if 1-hydroxylase activity is lowered and low if it is increased. Thus the measurement of the level of $25\text{-OH-}D_3$ in renal diseases gives information concerning the clinical state of the kidney provided the liver is normal. $25\text{-OH-}D_3$ levels determined in chronic renal failure (CRF) patients indicated a rising or falling pattern (52). Thus the picture in these patients is not clear. Chronic renal failure (CRF) patients are therefore included for study in this work.

In spite of the fact that $1,25(\text{OH})_2 \text{D}_3$ is the most active form of vitamin D, 25-OH-D_3 measurements are still used to assess vitamin D_3 status because of the relative simplicity of the assay as compared to determination of $1,25(\text{OH})_2 \text{D}_3$ (86).

1.6.1. Non-Competitive binding assay methods

Methods that have been used to determine pharmaceutical preparations of vitamin D include biological and physico-chemical methods. The bioassays that have been widely employed until more recently are the chick and the rat methods (77). These were based on the quantification of the healing effect of vitamin D after it has been supplied to the rachitic rat or chick. Mention has also been made to microbiological methods capable of measuring vitamin D (77).

Earlier physico-chemical methods used for determinations in pharmaceutical preparations were mainly spectrophotometric and colorimetric. In the spectrophotometric methods interfering substances, notably vitamin A, have to be removed by chromatography (78). The colorimetric methods follow the general colour reactions of sterols, hence it

is unspecific. The colorimetric reagents that have been used, as cited in the 'Extra Pharmacopoeia' (77), are those employing:-

- (i) antimony chloride;
- (ii) glycerol dichlorohydrin and acetylchloride;
- (iii) production of carbonium salts by treatment with perchloric acid and subsequent reaction with an aldehyde e.g. vanillin;
- (iv) iodine trichloride and carbon tetrachloride.

These physico-chemical methods just outlined suffer from either non-specificity or insensitivity to the levels of vitamin D that circulate in the serum.

Earlier laboratory methods used to diagnose vitamin D deficiencies in humans therefore did not measure the amount of the vitamin D directly. The inadequacy of the vitamin is reflected in the blood levels of calcium, inorganic phosphorus and alkaline phosphatase, each of which could be determined adequately. Calcium is precipitated as the oxalate and then titrated against permanganate (79).

The inorganic phosphate is determined in protein-free filtrate of blood or serum by the method of Fiske and Subarow (80) or the modification by Gomori (81) may be used. The alkaline phosphatase activity is determined either by the methods of Kay (82) or King and Armstrong (83).

In human rickets there is nearly always a decreased level of inorganic phosphorus and an increase in the level of alkaline phosphatase. The change in the level of serum calcium is less constant but usually it is below normal. There are other conditions which will produce these changes, consequently they should be looked upon as non-specific but may be useful. This is especially true when calcium and phosphorus levels are considered together.

More powerful physico-chemical methods have been developed recently. These are capable of measuring serum levels of vitamin D and its metabolites directly. The methods have been reviewed by Sheppard et al. (94, 85). Of these the most significant is the use of high pressure liquid chromatography (HPLC) to measure the various vitamin D₃ metabolites in serum by Eisman et al. (86) and Lambert et al. (87). In a novel procedure Bjorkhem and Holmberg (88) applied mass fragmentography to measure 25-OH D₃.

1.6.2. Competitive Binding Assay Methods for 25 OH D₃

With the availability of more specific labelled radioactive 25-OH D₃ and the discovery by Thomas et al. (see section 1.5.) that there exists in mammalian plasma a protein capable of binding specifically circulating vitamin D₃ and 25-OH D₃ the way was paved for Belsey et al. (89) to develop the Competitive Binding Assay for Vitamin D₃ and 25-OH D₃. This method uses the delicate sensitivity of radioactive measurement and the high specificity of a binding reagent to measure biologically active substances (vitamins, hormones, drugs, etc.) which occur in nanogram down to the picogram range. The general principles and other considerations pertaining to the method have been described extensively in the literature (90, 91, 92, 93, 94).

The original method for measuring vitamin D₃ as developed by Belsey and others (89) involved extraction of the serum sample with a chloroform-methanol mixture followed by separation of vitamin D₃ from 25-OH D₃ by chromatography on silicic acid columns. This is because both vitamin D₃ and 25-OH D₃ are bound by the binding protein. The two fractions were then incubated independently with weanling rachitic rat serum. The assay buffer was

barbital-acetate, pH 8.6. Human β -lipoprotein was added as a solubilizer for the vitamins because of their limited solubility in aqueous media. An improvement upon the procedure for measuring 25-OH D₃ was the elimination of the preparative chromatographic step (95). From kinetic studies it was established that the binding of tritiated 25 OH D₃ ($[^3\text{H}]$ - 25-OH D₃) to the binding protein occurred quite rapidly. The binding half-time was a matter of minutes. In contrast, the binding half-time for $[^3\text{H}]$ - vitamin D₃ was measured in days. It was further shown that large amounts of $[^3\text{H}]$ - D₃ would not interfere with the early association of $[^3\text{H}]$ - 25-OH D₃ with the binding protein. Thus 120 minutes was chosen as the suitable incubation time that allowed 25-OH D₃ in the medium to come to complete equilibrium with the binding protein without significant contribution from vitamin D₃.

Percent cross-reactivity (ie $x/y \times 100$), where x is the weight of 25-OH D₃ and y the weight of the interfering substance required to produce 50% inhibition of binding of $[^3\text{H}]$ - 25-OH D₃ as measured by Okano et al. (53) in a similar assay was 2.18% for vitamin D₃, 0.70% for 1,25-(OH)₂ D₃, less than 0.28% for 1 - OH D₃ and less than

0.06% for dihydrotachysterol, cholesterol and cortisol.

The initial extraction step prior to incubation with the binding protein therefore was to deproteinise the serum from proteins known to bind vitamin D and its analogues (see section 1.5.). These might interfere with the assay system.

Several variants of the method for measuring 25-OH D₃ based on Competitive Binding assay have been published since 1971. They differ mainly in the source of the binding protein, the extraction procedure, the presence or the absence of a solubilizer and the inclusion or omission of a chromatographic step. A survey of the methods has been given by De Nayer et al. (75).

1.7. CONCLUSION ON LITERATURE REVIEW

The review of the literature indicates that vitamin D deficiency is commonly a problem in a region of subdued sunlight unless the requirement is augmented by supplies from the diet. In the presence of adequate sunlight, as obtains in Ghana, incidence of ultraviolet (U.V.) rays on the skin maximises vitamin D₃ contribution from the skin. Maladjustment in the level of the vitamin may arise from

disease states of the liver and kidney despite abundant sunlight.

Older methods for assessing vitamin D₃ status either failed to measure vitamin D₃ directly or were too insensitive to the levels that circulate in man. In recent times the introduction of radioimmunoassay and related procedures make it possible to measure the low levels of biologically active compounds. These new procedures are exploited in the determination of 25-hydroxyvitamin D₃ levels in sera from healthy Ghanaians (in order to establish the local normal value) as well as selected diseased states of the liver and the kidney, namely cirrhosis and chronic renal failure (CRF) respectively.

CHAPTER TWO

2. DEVELOPMENT OF THE ASSAY SYSTEM

The assay of 25-hydroxyvitamin D₃ or 25-hydroxycholecalciferol described here is based on the materials and method supplied through the good offices of Professor J.C. Waterlow of the Department of Human Nutrition, London School of Hygiene and Tropical Medicine. The method is a modification of that of Belsey et al. (95). The materials supplied along with the procedure to be followed were rachitic rat serum as a source of the vitamin D₃ binding protein (DBP) and standard 25-hydroxyvitamin D₃. The method is described here.

2.1. MEASUREMENT OF 25-HYDROXYCHOLECALCIFEROL IN PLASMA

A. Reagents

1. The standard 25-hydroxyvitamin D₃ supplied along with the method was obtained from Philips-Duphar, Amsterdam.

5.00 mg was weighed out and was either dissolved in ethanol and various dilutions made down to a 'master' dilution of 10ng/ml, or it was dissolved in 50ml analar

benzene and distributed in 50 μ l aliquots (50 μ g) into stoppered vials which were kept frozen for future use. When needed for use one 50 μ g aliquot was blown dry with nitrogen, redissolved in 1 ml ethanol and this solution was used for further dilutions. All the standards should be kept in the deep freezer (The standard received was 100 μ g/ml in benzene. This was distributed into 250 μ l aliquots and kept frozen).

1. The Binding Protein.

The serum from rats fed for two generations on a vitamin D-free diet was used as a source of the vitamin D₃ binding protein. Waterlow and coworkers found a final dilution of 1:20,000 suitable for the assay. If higher concentrations of 25-hydroxyvitamin D₃ in plasma were expected it was better to use a stronger solution. This had to be established. The buffer solution diluted in 0.02M phosphate buffer, pH 7.6 must be kept in a refrigerator and made up fresh about once a week.

B. Procedure

1. Extraction

2 ml Analar ethanol was added to 500 μ l plasma

(or serum) in a plastic centrifuge tube. The tube was stoppered and the contents mixed for 10 seconds on a shaker. The tube was then allowed to stand for 30 minutes in an ice-bath. The mixture was then shaken briefly again and centrifuged at 0-4°C for 20 minutes at 2500rpm (1,540 g).

2. Competitive protein binding assay

(a) Standards

A standard curve had to be done with each assay. 100 μ l aliquots of each standard (ranging between 0.00ng/ml and 10ng/ml) were pipetted in duplicate into 3x $\frac{1}{2}$ in. glass (or plastic) test-tubes. 100 μ l of ethanol was also pipetted in duplicate for the 'charcoal blank'.

(b) Samples

100 μ l aliquots of ethanolic extracts from (1) were pipetted in triplicate into 3x $\frac{1}{2}$ in. test-tubes for assay and a further 100 μ l for the 'sample blank'.

The racks of standards were kept in an ice-bath.

(c) (i) 500 μ l of 0.02M phosphate buffer pH 7.6 containing 0.1% albumin was added to the 'sample blank' and 'charcoal blank' tubes.

(ii) 500 μ l of the phosphate buffer (c (i) above)

containing the binding protein (1:10,000 dilution) was added to the remaining tubes of standards (including zero standards) and samples.

- (iii) 500 μ l buffer containing labelled 25-hydroxyvitamin D₃ (see section 2.2.a) was added to all tubes.
- (iv) The contents of the tubes were mixed briefly on the shaker and the tubes were allowed to stand for 2 hours in an ice-bath.
- (v) After 2 hours, 100 μ l charcoal suspension (see section 2.2.b) which was kept stirring while addition was made was added to each tube. The tubes were shaken again and set aside in the ice-bath for further 30 minutes. They were then centrifuged for 20 minutes at 0^o-4^oC at 2500rpm (1540 g).
- (vi) 500 μ l aliquots were then taken from the supernatant in each tube into 4ml scintillation fluid in polyethylene minitubes for counting. The samples were counted for 4,000 counts or 10 minutes in order to keep to schedule. Longer counting times were probably advisable.

Notes

The following comments were given at the end of the method given by J.C. Waterlow and coworkers.

1. The reproducibility of the counts (especially those for the standards) was much improved by having 0.1% bovine serum albumin (BSA) present in the buffer (except that used for making up the charcoal suspension). This does not keep indefinitely, and it was added each time a new dilution of the binding protein was made. However not all kinds of albumin work. Sigma Fraction V was used but even then not all batches worked. The reasons for this was unknown but it was suggested that possibly binding was inhibited.

It was suggested that a whole procedure without albumin must be tried. If it works it could then be checked to see if reproducibility can be improved by addition of 0.1% albumin. This amount was chosen because it seemed to have the desired effect without increasing the blank too much.

2. In any assay of different samples, one blank from each sample was taken and the average found. That

represented the non-specific binding. The charcoal blank under those conditions amounts to 50cpm and the sample blank about 60cpm.

2.2. MATERIALS

In addition to the standard 25-hydroxyvitamin D₃ in benzene (100µg/ml) and the rachitic rat serum supplied by Prof. Waterlow, the following materials were also used.

a. 25-hydroxy-26(27)-methyl-³H-cholecalciferol

The tritiated 25-hydroxycholecalciferol (³H-25 OH D₃) was purchased from the Radiochemical Centre, Amersham, England, their product TRK 396, batch No. 24. The specific activity on receipt was 11.3 Curies/mol or 28mCi/mg.

The ampoule containing 0.5ml benzene ethanol (9:1) solution of [³H]-25-OH D₃ was made up to 10ml with benzene : ethanol (9:1) mixture and distributed in 250µl portions and kept at -20°C. For use the benzene : ethanol solution was thawed and then blown dry in a stream of nitrogen. The residue was then dissolved in 7-10ml of 95% ethanol. The aim was to accumulate 4000cpm in 100µl of the ethanolic solution.

Before each assay the appropriate volume of the ethanolic [^3H]-25-OH D_3 was diluted 10 times with the assay buffer.

b. Dextran coated charcoal.

0.25g activated charcoal ('Norit A' from Sigma, 'Norit GSX' or 'Norit OL' from Hopkin-Williams) was stirred with 0.025g dextran C (molecular weight range was 60,000-90,000; obtained from Sigma and Hopkin-Williams) in 10ml phosphate buffer (no albumin) for 16-24 hours in the cold unless otherwise stated. This suspension was kept in the refrigerator.

Commercial dextran coated charcoal was obtained from CIS, France.

c. Buffers and solubilizers

(1) 0.02M phosphate buffer, pH 7.6.

A. Na_2HPO_4 : 7.10g dissolved in 250ml deionised distilled water.

B. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$: 2.76g was dissolved in 100ml of deionised distilled water.

C. 174ml A was mixed with 26ml B and made up to 2 litres with deionised distilled water.

- (ii) 0.02M phosphate buffer, pH 7.6 with 0.1% albumin or gelatin as solubilizer

0.1g bovine serum albumin (BSA) or gelatin was weighed into buffer C (see above) and stirred gently to dissolve.

It was necessary to warm gently in order to dissolve the gelatin.

- (iii) 0.02M phosphate buffer, pH 7.6 with saline

Where sodium chloride was used 0.88g was dissolved in 100ml of buffer C.

- (iv) 0.02M phosphate buffer, pH 7.4

Slight adjustment in pH was achieved by addition of a few drops of Na_2HPO_4 or NaH_2PO_4 solutions.

- (v) 0.05M carbonate-bicarbonate buffer, pH 9.6

1.59g Na_2CO_3 and 2.93g NaHCO_3 were dissolved in 1 litre deionised distilled water.

d. Tubes

- (i) Mini polypropylene test-tubes were obtained from CEA, France (CIS catalogue Code No. REAC 30).
- (ii) Pyrex glass tubes (10mm x 75mm).
- (iii) Vacutainer glass tubes.

e. Counting vials

Low potassium counting vials were used.

f. Scintillation fluids

The scintillation cocktails used contained

- (i) 4g PPO, 200mg POPOP and 100g naphthalene
in ^qlitre 1,4-dioxan or
- (ii) 5g PPO, 120g naphthalene and 0.5g bis MSB
benzene made up to 1 litre with 1,4-dioxan.

g. Equipment

- (i) Packard Liquid Scintillation Spectrometer
model 3320
- (ii) MSE Mistral 4L refrigerator centrifuge.

h. Rats

Hertman albino rats strain 116 were obtained from the animal house at the Korle-Bu Teaching Hospital, Accra and the Biochemistry department, University of Ghana, Legon.

2.3. PRELIMINARY EXPERIMENTS.

The initial effort was to set up a standard curve using the materials and procedure as given in section 2.1.

These early attempts were not successful. Further experiments were subsequently conducted to check the suitability of the dextran coated charcoal (DCC), the constitution of the buffer and the titre of the rachitic rat serum used in these experiments.

2.3.1. Trial standard curve for 25-hydroxyvitamin D₃

A. Reagents

a. Standards: The 10ng/ml stock solution of the standard 25-hydroxyvitamin D₃ was prepared by blowing dry one tube of the stored standard in benzene in a stream of nitrogen. (It contains 25µg. 25 OH D₃). The residue was dissolved in 1ml 95% ethanol. 10ml of the ethanolic solution was made up to 25ml to give a stock solution of 10ng/ml.

The 10ng/ml ethanolic solution was double diluted with 95% ethanol to give standards of 5, 2.5, 1.25, 0.625 and 0.315ng/ml.

b. Tritiated 25-hydroxyvitamin D₃ (³H-25-OH D₃)

The tritiated 25-hydroxyvitamin D₃ solution used was prepared according to the procedure given in section 2.2.1.

c. Buffer

0.02M phosphate buffer, pH 7.6 was used. The buffer contained 0.1% bovine serum albumin (BSA) as solubilizer.

d. Binding protein (DBP)

The rachitic rat serum was diluted 1 : 20,000 with the phosphate buffer (see c. above). This served as a source of the vitamin D binding protein.

e. Dextran coated charcoal

Norit A charcoal was used in the preparation of the dextran coated charcoal. The procedure followed is given in section 2.2.6.

B. Assay

(1) Table 2.1 shows the tubes disposition for the trial standard curve. 100 μ l each of the ethanolic standards were pipetted in triplicate into the plastic tubes.

Total activity tubes and zero standards received triplicate aliquots of 95% ethanol. 500 μ l aliquots of buffer only were pipetted into total activity (T.A.) and charcoal blank tubes.

TUBE NO.	CODE OR STD	VOL. ETHANOLIC STD IN μ l.	VOL. BUFFER IN μ l.	VOL. D B P IN μ l	[3 H]-25-OH D ₃ IN μ l.
1 2 3	T.A 0.00	100	500	-	500
4 5 6	CB OR NSB	100	500	-	500
7 8 9	0.00 ng/ml	100	-	500	500
10 11 12	0.312 ng/ml	100	-	500	500
13 14 15	0.625 ng/ml	100	-	500	500
16 17 18	1.25 ng/ml	100	-	500	500
19 20 21	2.50 ng/ml	100	-	500	500
22 23 24	5.00 ng/ml	100	-	500	500
25 26 27	10.0 ng/ml	100	-	500	500

TABLE 2.1 : Tubes disposition for establishment of standard curve for 25-OH D₃ / STD = standard; T.A = Total activity; CB = Charcoal blank; NSB - non specific binding; DBP = vitamin D binding protein.

500 μ l aliquots of buffer containing the diluted binding protein (1 : 20,000) were put into the remaining tubes.

500 μ l of buffer containing [^3H]-25-OH D_3 (about 2000 cpm) was drawn into each tube.

(ii) The contents of the tubes were shaken and the tubes were incubated in an ice-bath at 0-4 $^{\circ}$ C in the dark for 2 hours.

(iii) At the end of the incubation period 0.1ml dextran coated charcoal suspension which had been stirring all day was added to all tubes except for total activity.

The tubes were shaken on an automatic shaker and set aside in an ice-bath for a further 30 minutes. They were then centrifuged at 0-4 $^{\circ}$ C for 20 minutes at 2500 rpm.

500 μ l aliquots of each tube was drawn into 10ml scintillant for counting.

C. Results

Table 2.2. shows the results obtained following the method given in section 2.1. The counts for the non-specific binding (NSB) or the charcoal blank (CB)

STANDARDS IN ng/ml OR CODE	A RESIDUAL RADIO- ACTIVITY IN 500 μ l OF S.N. /cpm	B RESIDUAL RADIO- ACTIVITY IN 500 μ l OF S.N. /cpm
T.A	252	632
CB OR NSB	I5I	324
0.00	I36	2I6
0.3I2	I52	335
0.625	I3I	304
I.25	I26	43I
2.50	I29	304
5.00	I35	378
IO.0	II5	258

TABLE 2.2 : A and B are two separate experiments conducted in an attempt to obtain a standard curve for 25-OH D₃. Higher total activity was used in B. The charcoal blanks (CB) counts were too high. The samples were counted for only I minute in order to see the trend. Longer counting times are of no advantage.

were too high. The DCC was not removing the ^3H -25-OH D_3 . The CB counts were of the same order as the counts for the standards. Thus, it was not possible to subtract the non-specific binding counts from the counts for the standards to get any meaningful values. In short, no standard curve was obtained.

The absorption properties of the dextran coated charcoal used were therefore suspected. The buffer composition was also another factor that had to be checked.

2.3.2. Charcoal experiments

In this section a series of investigations were carried out to find dextran coated charcoal with suitable absorption properties.

a. The absorption properties of Norit 'A', Norit 'OL' and Norit 'GSX' charcoals.

0.25g charcoal was stirred in the cold in 2.5ml phosphate buffer, pH 7.6 containing 0.025g dextran C (Sigma). This gave 10% charcoal in 1% dextran solution. The capacity of each type of charcoal to absorb $[\text{}^{-3}\text{H}]-25\text{-OH } \text{D}_3$ was conducted following the procedure given in section 2.3.B(iii). The charcoal (DCC) dilutions used were

10%, 5%, 2.5%, 1.25% and 0.625%. (These dilutions were obtained by double diluting the 10% charcoal preparation above).

The pattern of residual radioactivity with increasing dextran coated charcoal concentration as depicted in fig. 2.1 suggests that the different types of charcoals tested (in Norit 'A', Norit 'OL' and Norit 'GSX') had similar absorption properties for $[^{-3}\text{H}]-25\text{ OH D}_3$.

b. Suitabilities of dextran from different sources

Dextran C (from Sigma and Hopkin-Williams) were used to coat (2.3.2a) Norit 'OL' charcoal. Commercial dextran coated charcoal and uncoated charcoal were also used for comparison. The capacities of these preparations in removing $[^{-3}\text{H}] - 25\text{-OH D}_3$ were tested.

Fig. 2.2. shows plots of residual radioactivity versus the charcoal concentration. There is no difference basically between the absorption capacities of the charcoals. Strikingly, the uncoated charcoal had about the same properties as the coated ones. This tends to suggest poor coating onto charcoal surface. However, with the newly acquired commercial dextran coated charcoal

exhibiting the same pattern of absorption it was doubtful whether the results obtained were due to poor coating. It was nevertheless decided to reactivate the charcoals before coating.

c. Re-activated charcoals

The charcoal to be reactivated was spread thinly on a clean metal tray and heated in a thermostated oven. Norit GSX was reactivated at 105°C and 200°C for 24 hours. Norit A charcoal was also activated at 200°C for 24 hours.

The capacities of these charcoals to adsorb $[^{-3}\text{H}_7 - 25\text{-OH D}_3]$ were examined. For comparison commercial dextran coated charcoal purchased from CIS was also included.

The adsorption patterns are shown in figure 2.3. The residual radioactivity in the supernatant was still high. This pattern was even shown by the commercial dextran coated charcoal. Thus activation of the charcoal did not improve the adsorption properties. It was also evident that different types ^{of} charcoal tested had about the same capacity for the adsorption of $[^{-3}\text{H}_7 - 25\text{OH D}_3]$

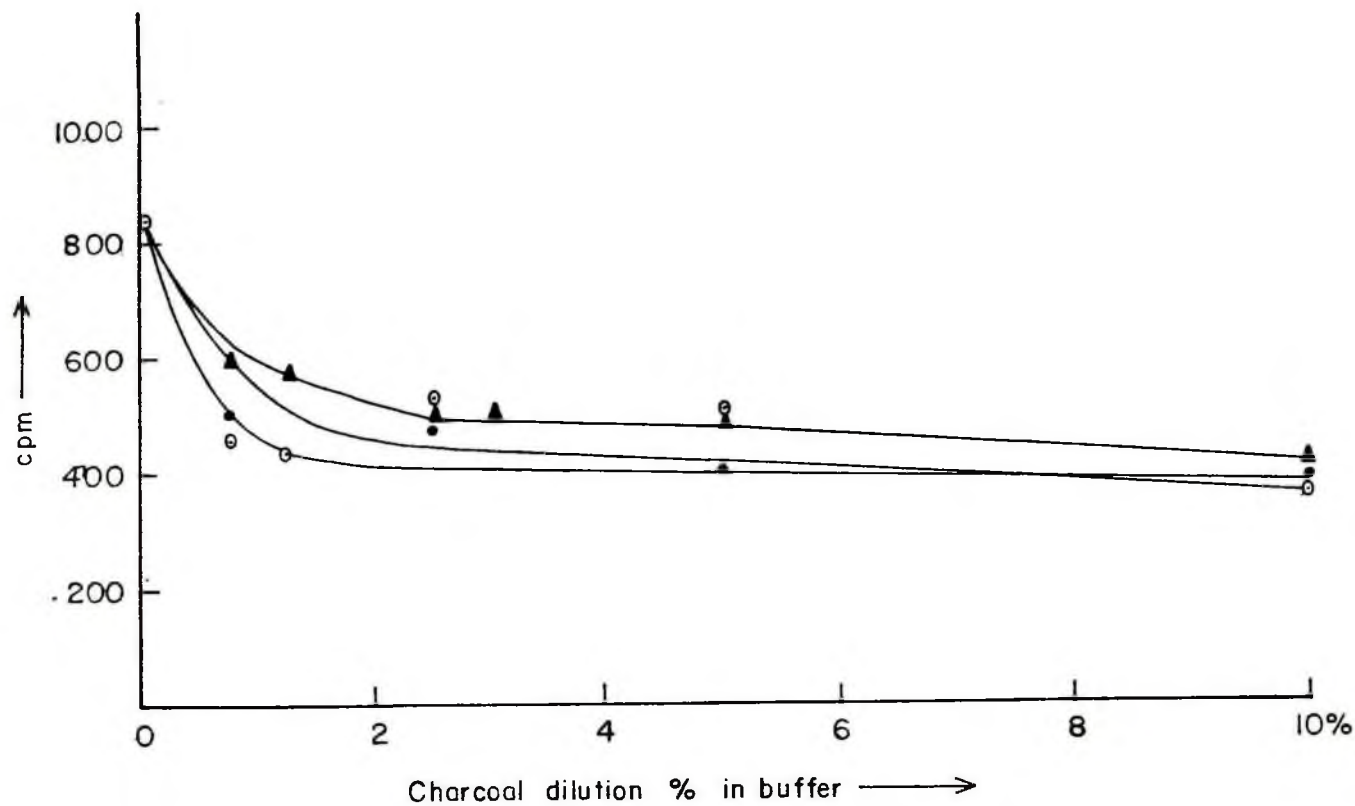


Fig. 2. 1. Adsorption of ^3H -25-OH D_3 by dextran coated Norit 'A' (\blacktriangle - \blacktriangle - \blacktriangle), Norit 'OL' (\circ - \circ - \circ) and Norit GSX (\bullet - \bullet - \bullet) charcoals .

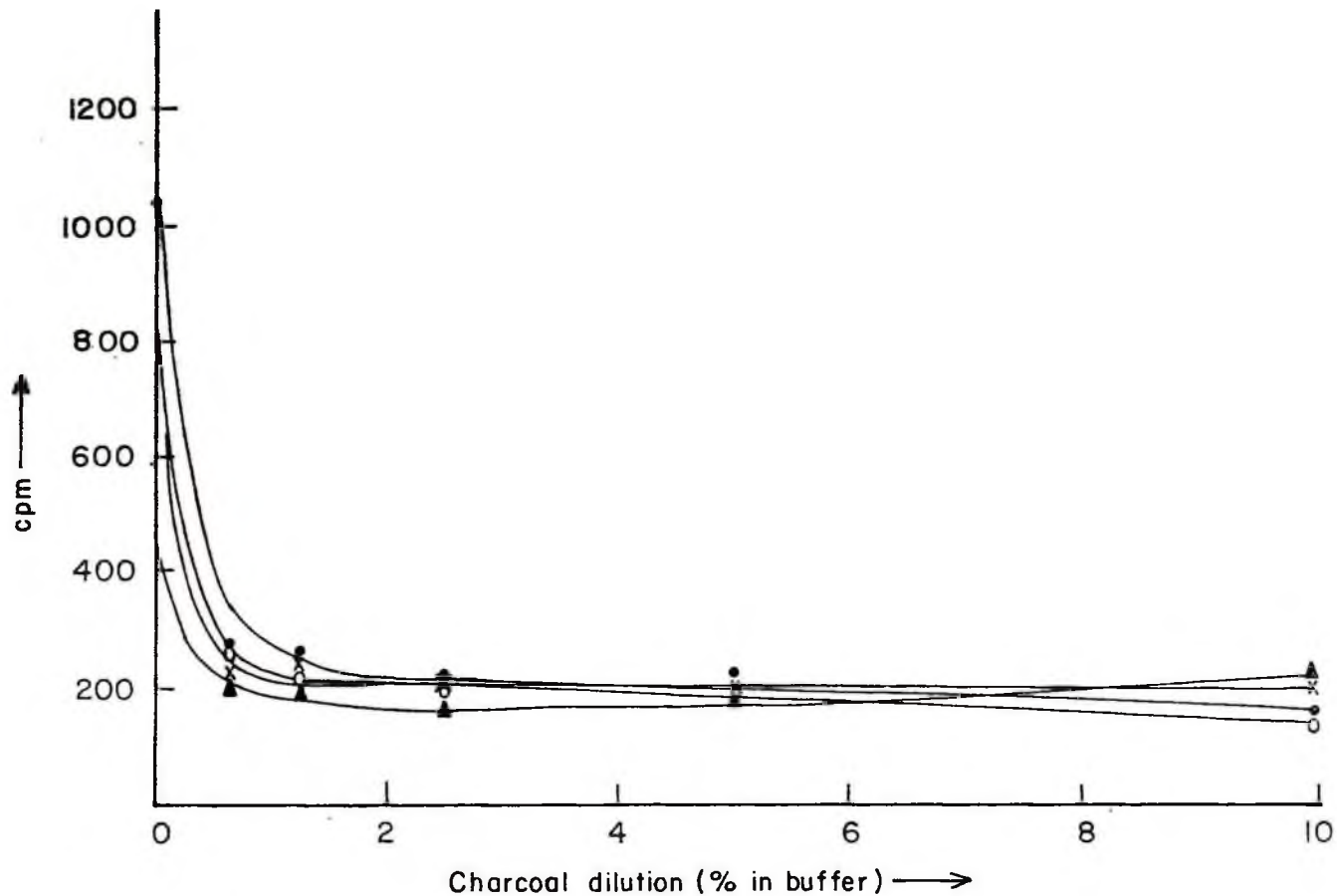


Fig. 2-2 Adsorption of $^3\text{H} - 25\text{OHD}_3$ uncoated Norit 'OL' charcoal (o-o-o), Norit 'OL' coated with dextran from Hopkin Williams (x-x-x), Norit 'OL' coated with dextran from sigma (•-•-•), and commercially dextran coated charcoal from CIS (Δ-Δ-Δ)

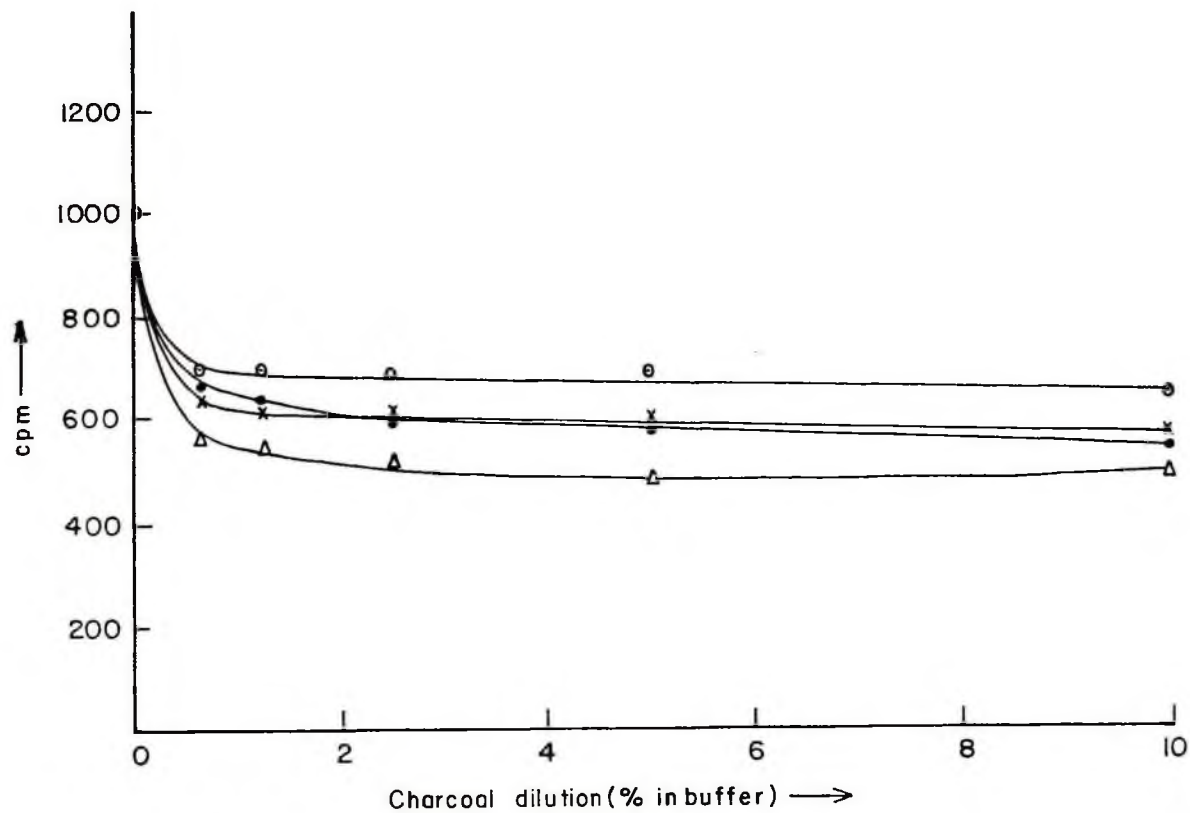


Fig. 2.3 Adsorption of ^3H -25 OH D_3 by

- a) Norit GSX reactivated at 150°C for 24hrs (o-o-o)
- b) Norit GSX reactivated at 200°C for 24hrs (•-•-•)
- c) Norit A reactivated at 200°C for 24hrs (Δ - Δ - Δ)
- d) Commercial dextran coated charcoal purchased from CIS*

under the conditions of assay. These experiments tend to suggest that the charcoal preparations were suitable. The fault with the assay lay with some other factor. The next likely parameter to consider was the constitution of the assay buffer.

2.3.3. Constitution of the buffer

It was noted in the methods given in section 2.1 that only few batches of albumin gave successful assays. An assay was therefore run without bovine serum albumin as a solubilizer.

a. Assay without BSA

The format of the assay procedure is as given in section 2.3.1. The buffer used is 0.02M phosphate, pH 7.6. No albumin was incorporated.

Figure 2.4. shows an assay curve obtained using phosphate buffer without BSA. The counts for the various points are given in table 2.3. The charcoal blank (non-specific binding) counts were lower than what had hitherto been obtained. The B/Bo (B = counts for a particular point; Bo = counts at the point where $[25\text{-OH } D_3] = 0$) for some points were above 100%. The

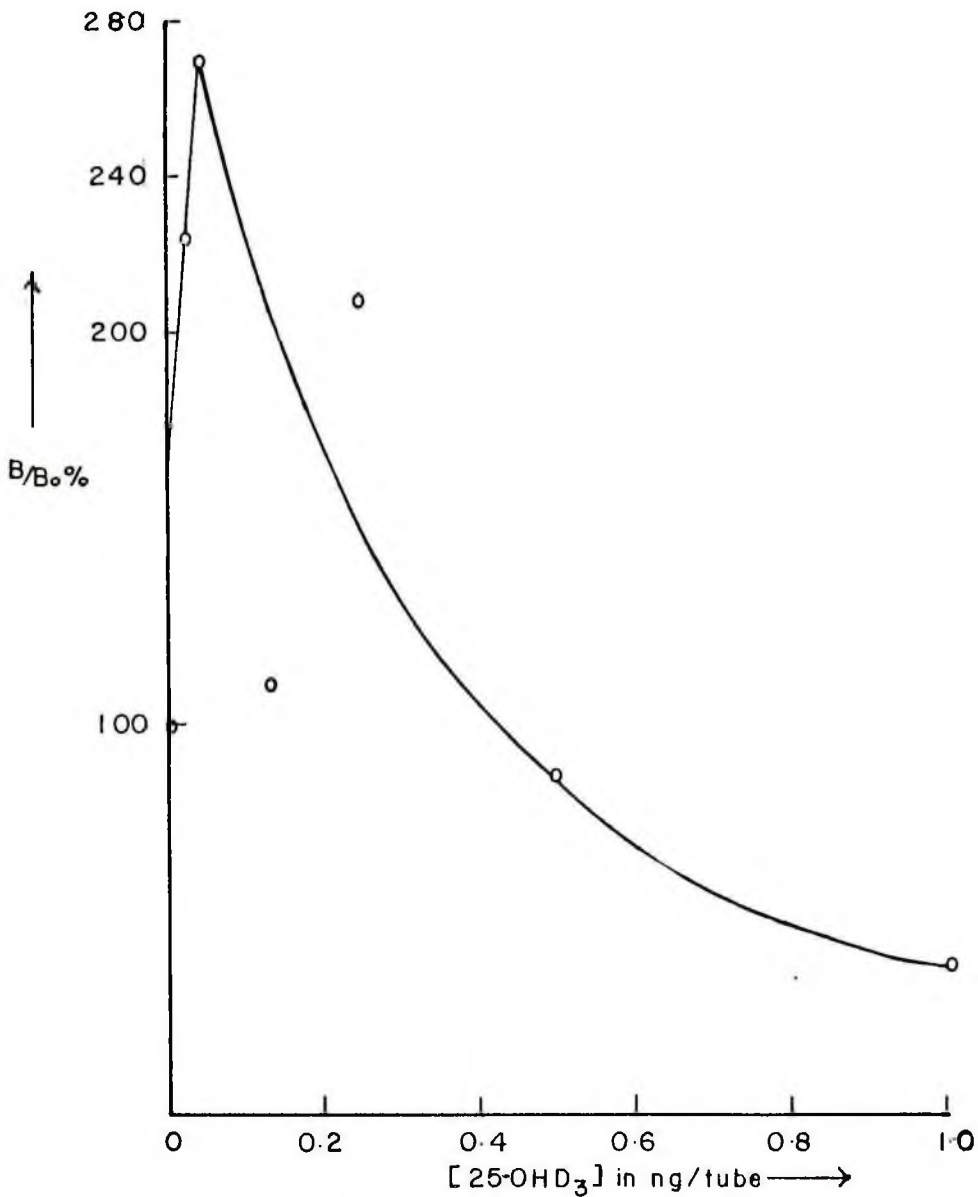


Fig. 2.4 Assay curve of ^3H -25-OH D_3 . The assay was conducted in 0.02M phosphate buffer pH 7.6 with no BSA solubilizer.

STANDARD OR CODE	cpm/0.5ml SUPERNATANT	MEAN cpm/0.5ml SUPERNATANT
T.A. *	663 656 653	657
CB †	90 103 51	81
0.00ng/ml	238 192 137	189
0.312 "	428 275 262	322
0.625 "	414 240 472	375
1.25 "	265 256 93	201
2.50 "	143 288 469	300
5.00 "	269 123 127	173
10.0 "	127 84 151	121

TABLE: 2.3 The counts for the standard curve of 25-OH D₃ using 0.02M phosphate buffer, pH 7.6 containing no BSA as solubilizer. The charcoal blank (CB*) counts were low but the counts for the standards were erratic. [T.A* = total activity]

counts were also erratic.

Though the precision was poor in this experiment -it provided a clue that an assay could be set up with the materials at hand if a suitable buffer system could be found.

b. Gelatin and sodium chloride in assay buffer

Gelatin has been incorporated with great success into buffers for steroid hormone assays (WHO Programme for the provision of matched assay reagents for radioimmunoassay of hormones in Reproductive physiology). By virtue of structural similarities between 25 OH D₃ and the steroid hormones gelatin was adopted in the assay.

Two 0.02M phosphate buffers pH 7.4 and 7.6 each containing 0.1% gelatin and 0.38% sodium chloride were prepared. The dilution for the standard 25-OH D₃ is as given in Table 2.4. The assay followed the procedure given in section 2.3.1B.

Using the phosphate buffer, pH 7.4, the assay curve in fig. 2.5. was obtained. The points determined

STANDARD	CONCN. in ng/ml	Volume of 10ng/ml stock used (ml)	Vol. 95% ethanol (ml)
A	0.00	0.00	2.00
B	0.50	0.10	1.90
C	1.00	0.20	1.80
D	1.50	0.3 of I	1.20
E	2.00	0.40	1.60
F	3.00	0.60	1.40
G	4.00	0.80	1.20
H	5.00	1.00	1.00
I	7.50	1.50	0.50
J	10.0	2.00	0.00

TABLE 2.4 : Preparations of standard 25-hydroxyvitamin D₃ solutions from stock of 10ng/ml.

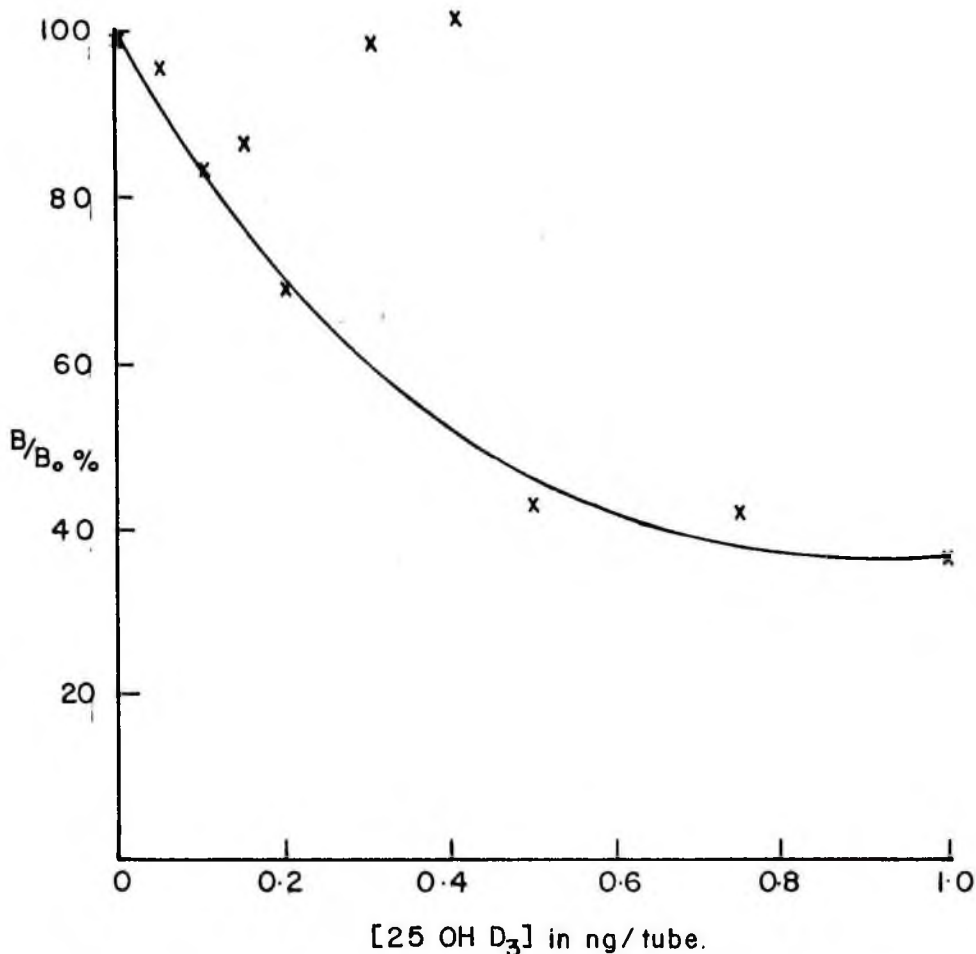


Fig. 2. 5. Assay curve for 25-OH D₃. The constituents of the buffer were 0.02M phosphate buffer pH 7.4 containing 0.88% NaCl and 0.1% gelatin .
(NSB= 55 cpm)

between 0-0.5 ng/tube were imprecise. It appeared the curve had a shoulder between these points. The charcoal blank (or non-specific binding) counts of 55cpm was quite low. The effect of pH change from 7.4 to 7.6 is shown in figure 2.6. The shoulder seen in fig. 2.5. has been reduced. The charcoal blank count was 50cpm.

Thus, inclusion of 0.1% gelatin and 0.88% sodium chloride into 0.02M phosphate buffer improved the assay.

c. An assay buffer containing only gelatin

The assay buffer given in section 2.1. does not incorporate sodium chloride. It was also observed from 2.3.3b that the inclusion of 0.88% sodium chloride gave rise to cloudiness in the scintillant. This brought about higher quenching of the counts accumulated.

An assay buffer containing gelatin was therefore compared with one containing both gelatin and 0.88% sodium chloride.

Figures 2.7. and 2.8. show the assay curves obtained

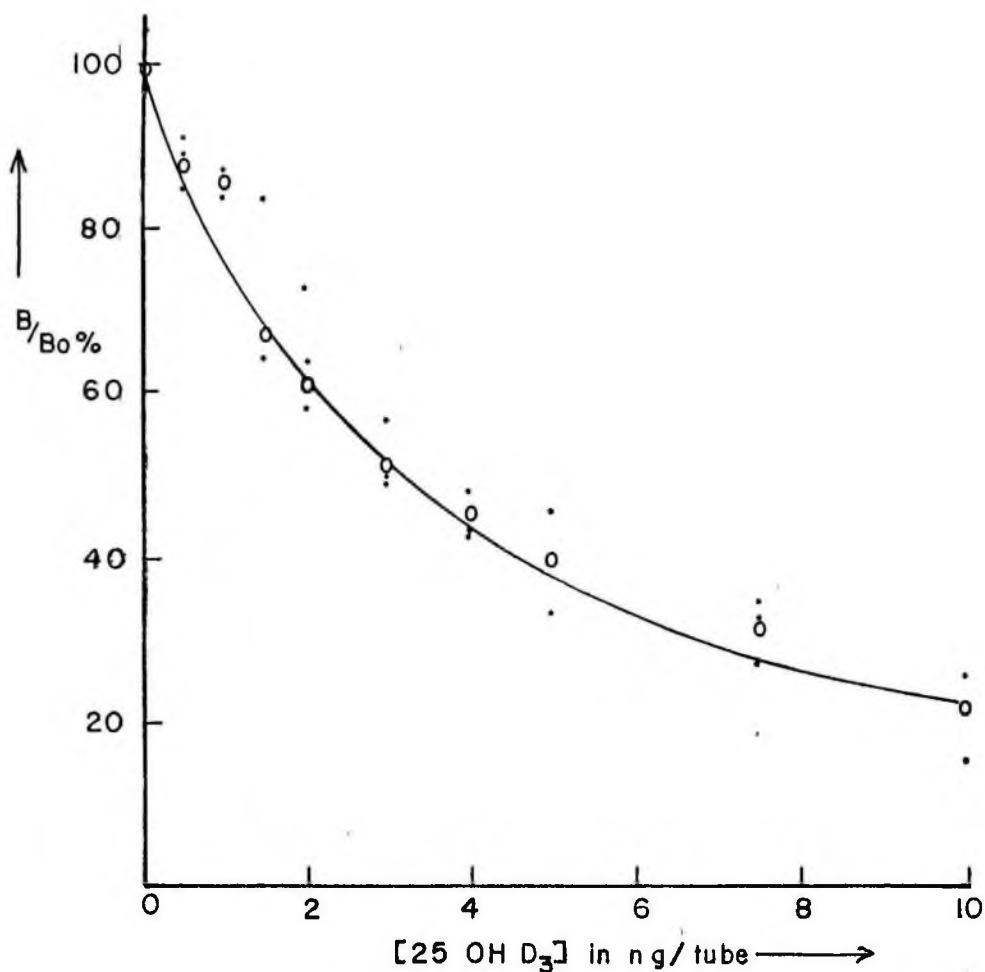


Fig.2. 6 . Standard curve for the assay of 25-OH D₃.The pH of the phosphate buffer was adjusted from 7.4 to 7.6.The buffer contained 0.1% gelatin and 0.88% NaCl .

($B/T = 38.9$; NSB = 50 cpm :for individual points,
O. for the mean points)

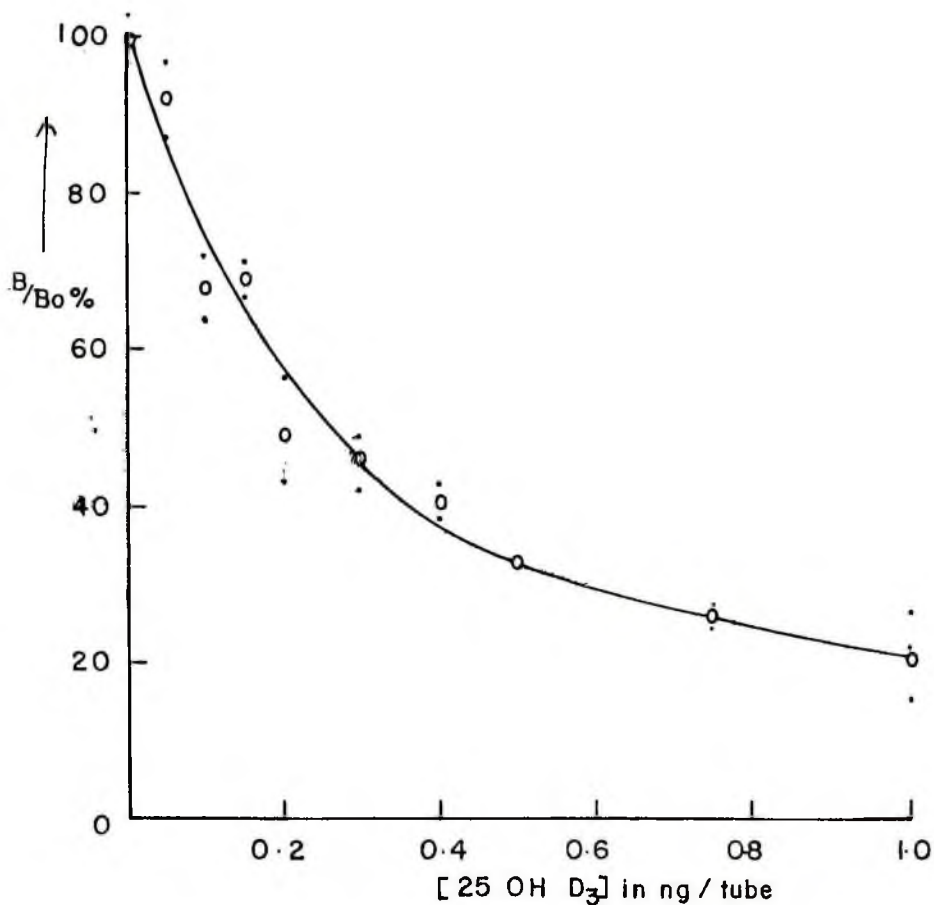


Fig. 2. 7. Standard curve for 25-OH-D₃ assay using 0.02M phosphate buffer pH 7.6, containing only 0.1% gelatin (B₀/T=37.0%, NSB=62.8 cpm)

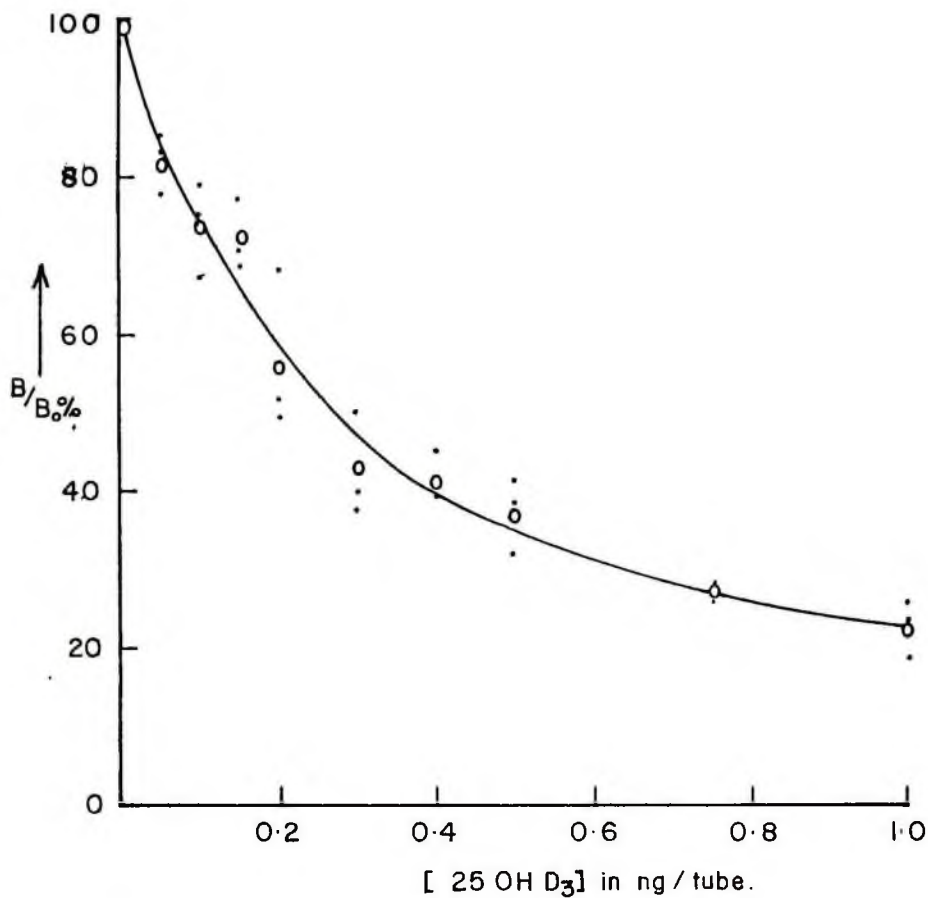


Fig. 2. 8. Standard curve for 25-OH D₃ assay using 0.02 M phosphate buffer pH 7.6, containing 0.1% gelatin and 0.88% NaCl (B₀/T=49.5%; NSB = 49 cpm).

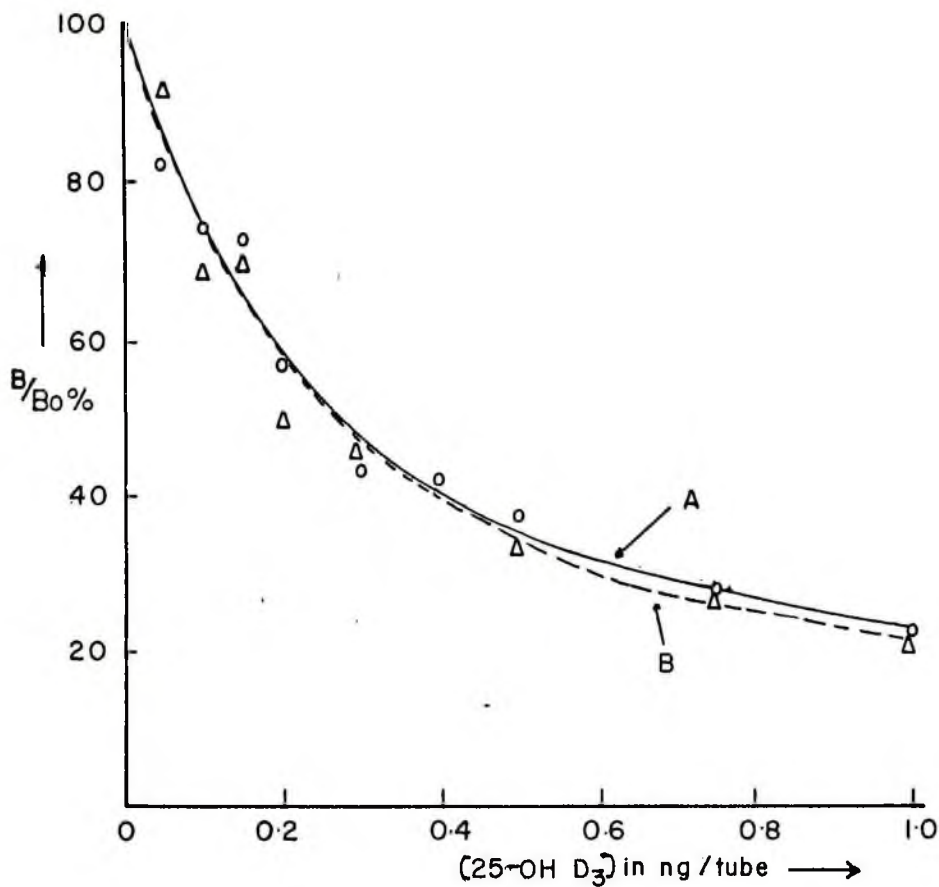


Fig.2 9. Two standard curves superimposed on each other. Curve A: Assay was performed on 0.02M phosphate buffer pH 7.6 containing 0.1 gelatin and 0.88% NaCl. Curve B: The assay was conducted in 0.02M phosphate buffer pH 7.6 containing only 0.1% gelatin.

respectively. Figure 2.9. shows the two curves superimposed on each other.

Basically there was no difference between the assay standard curves. To cut down the degree of quenching 0.02M phosphate buffer, pH 7.6 containing only gelatin as solubilizer was used in subsequent assays.

2.3.4. Suitable dilution of rachitic rat serum

It was suggested in section 2.1. that a rachitic ^{Serum} rat dilution of 1 : 20,000 or 1 : 10,000 could be suitable. The actual titre to be used however had to be determined.

5 μ l of thawed rachitic rat serum was taken into 5ml 0.02M phosphate buffer, pH 7.6 containing 0.1% gelatin. This gave a dilution of 1 : 1000 which was further diluted to 1 : 5000, 1 : 10,000, 1 : 20,000 and 1 : 40,000. Each dilution was incubated with [³H]- 25-OH D₃ and the capacity to bind the radioactive analogue of 25-OH D₃ was determined. The details of the methods of incubation and separation of free radioactivity from the bound radioactivity was given in section 2.1.B (as for zero standards).

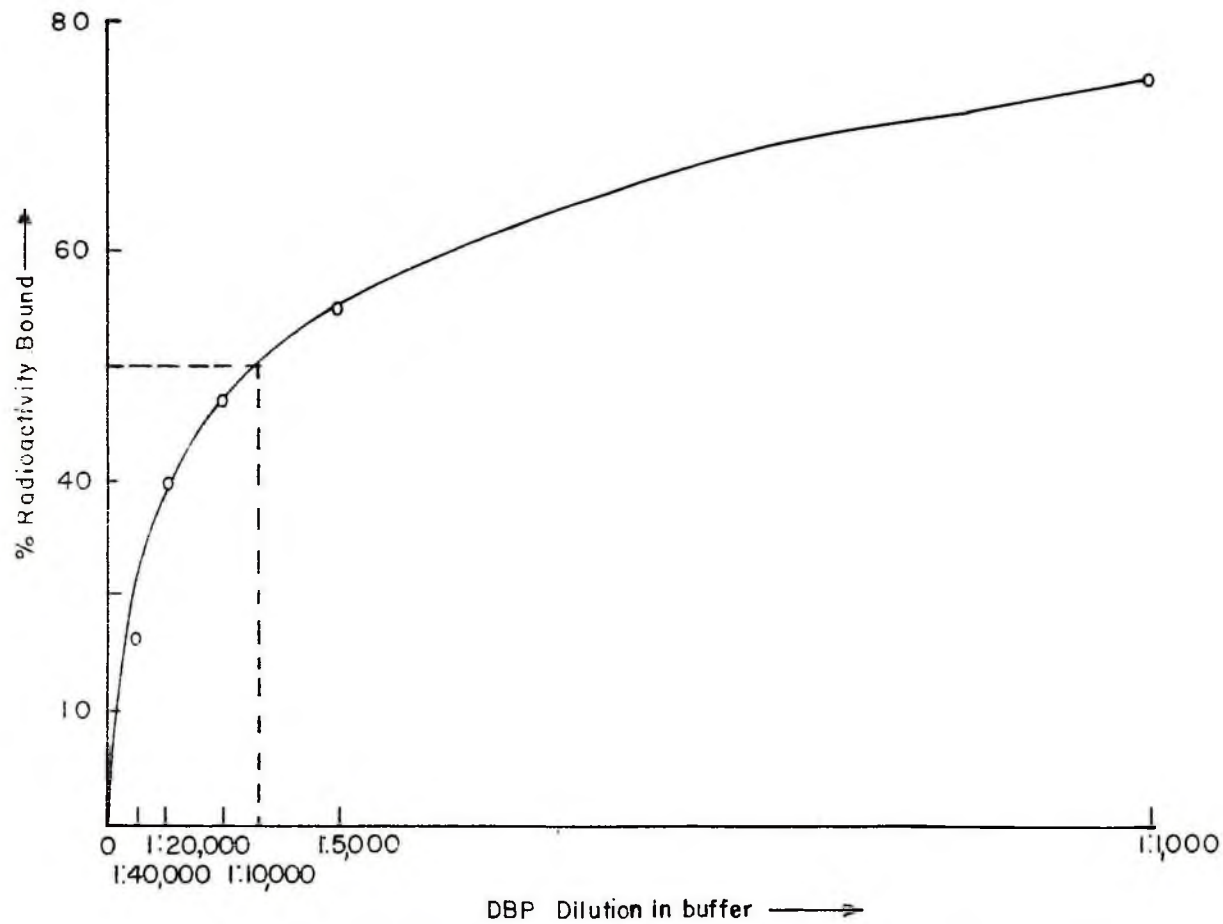


Fig. 2.10. Titre of rachitic rat serum curve showing % radioactivity bound versus DBP dilution.

Fifty percent binding of [^3H]- 25-OH D_3 was observed at 1 : 7,600 dilution (Fig. 2.10.). Thus, 1 : 10,000 dilution of the rachitic rat serum was chosen as a convenient titre for the assay.

2.4. THE FINAL FORM OF THE ASSAY

It was established from the preliminary investigations

- that :
1. dextran coated charcoal preparation of 0.25g charcoal stirred in a solution of 0.025g dextran in 10ml phosphate buffer was adequate for the assay;
 2. a suitable assay buffer was 0.02M phosphate buffer, pH 7.6 containing 0.1% gelatin as a solubilizer;
 3. the workable titre of rachitic rat serum was 1 : 10,000.

These are the main points to be noted in the final form of the assay. Some minor points to note are the following:

The volume of the scintillant used for counting was 8ml. This was chosen because it was observed that there was no difference in the count rate when the

scintillant volume was between 7-10ml.

The assay reagents were prepared fresh for use each day. That is the benzene-ethanol mixture containing [^3H]-25-OH D_3 and the solution of benzene containing the standard 25-OH D_3 were blown dry with nitrogen and reconstituted with ethanol on the day of assay. The 0.1% gelatin phosphate buffer was also prepared fresh for use. The plain phosphate buffer could be kept for 2 - 3 weeks.

The 25-OH D_3 standards for the standard curve were prepared according to Table 2.4. With these points in view the performance of the final form of the assay is as given in section 2.3.1.B.

2.4.1. The Standard Curve

There are a variety of ways to process the counts obtained in order to draw a standard curve from which unknown values could be interpolated manually or automatically by use of computer programmes (96). Only two of such methods will be considered here. The properties of the standard curve are also described.

a. Data Processing

The charcoal blank counts are averaged and subtracted from all counts. The radioactivity bound at each concentration of the ligand is expressed as a percentage of the radioactivity bound at zero 25 OH D₃ concentration. This is written as $B/B_0 \times 100$ (where B is the counts at any ligand concentration and B₀ the counts at zero ligand concentration). A plot of $B/B_0 \times 100$ versus ligand concentration is then drawn. Figure 2.11. is a standard curve for the assay for 25 OH D₃. B/B₀% was plotted against 25 OH D₃ concentration in ng per tube.

For convenient dose interpolation attempts were made to linearize the standard curve. It was found that a straight-forward logit-log transformation (97) was adequate for this purpose in the range of interest.

The logit function is defined as

$$\text{logit } (y) = \ln \frac{y}{(1-y)}$$

In this instance $y = B/B_0$. When the logit (y) is plotted against the dose x, a linear dose response curve is obtained. This transformation finds use in

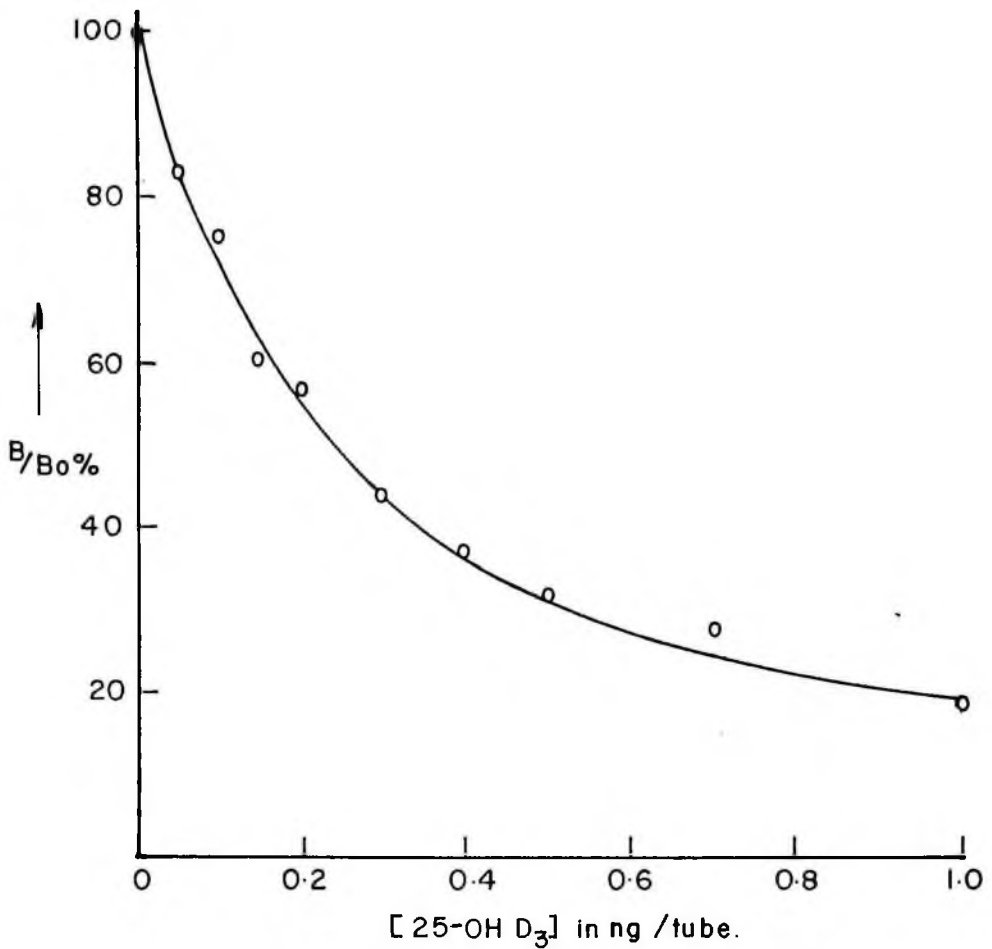


Fig. 2.11 Established standard assay curve for 25-OH D₃ (NSB = 48 cpm, B₀/T=64.6%)

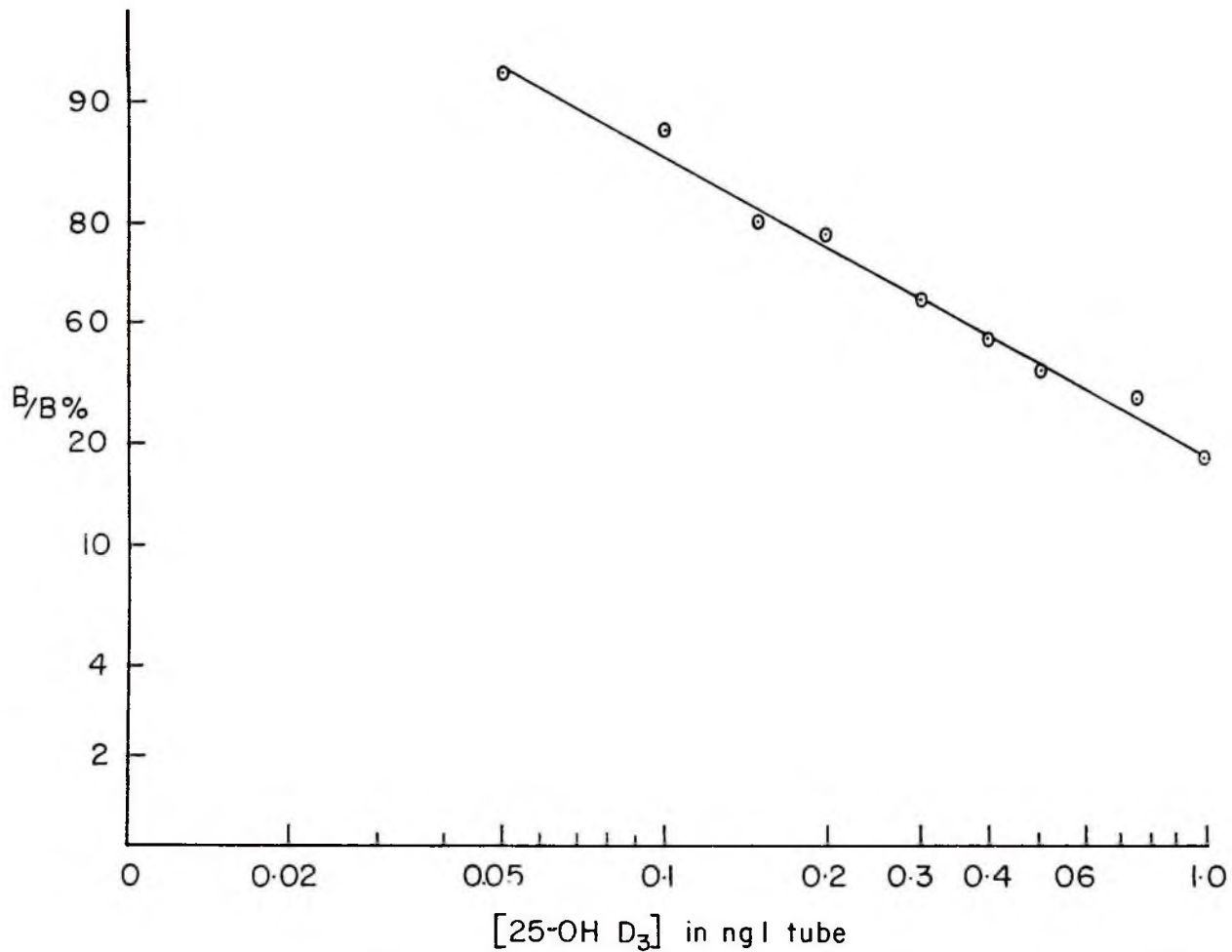


Fig. 2.12. Linearization of assay standard curve (the graph was drawn on logit-log paper obtainable from CIS).

computer-curve fitting programmes. Manual interpolation of assay results is facilitated by its use. To this end logit-log graph paper is available commercially to cut down the the calculations involved.

Logit-log transformation of figure 2.11. is given in fig. 2.12. The graph was drawn on commercial logit-log paper obtainable from CIS (CEA-IRE-SOBIF logit-log paper for linearization of the radioimmunoassay standard curve). The graph is linearised between 0.05 - 1.0ng/tube.

b. The sensitivity, precision and the range of the Assay standard curve.

In order to determine the precision of the standard curve replicate determinations were carried out at 25 OH D₃ standard concentration of 0.00, 0.05, 0.15, 0.30, 0.50 and 1.00ng/tube; triplicate determinations were included at 0.10, 0.20, 0.40 and 0.75ng/tube in order to obtain a smoother curve.

The plot of the points obtained is shown in figure 2.13. The 'charcoal blank' (CB) counts was 48 cpm/0.5ml

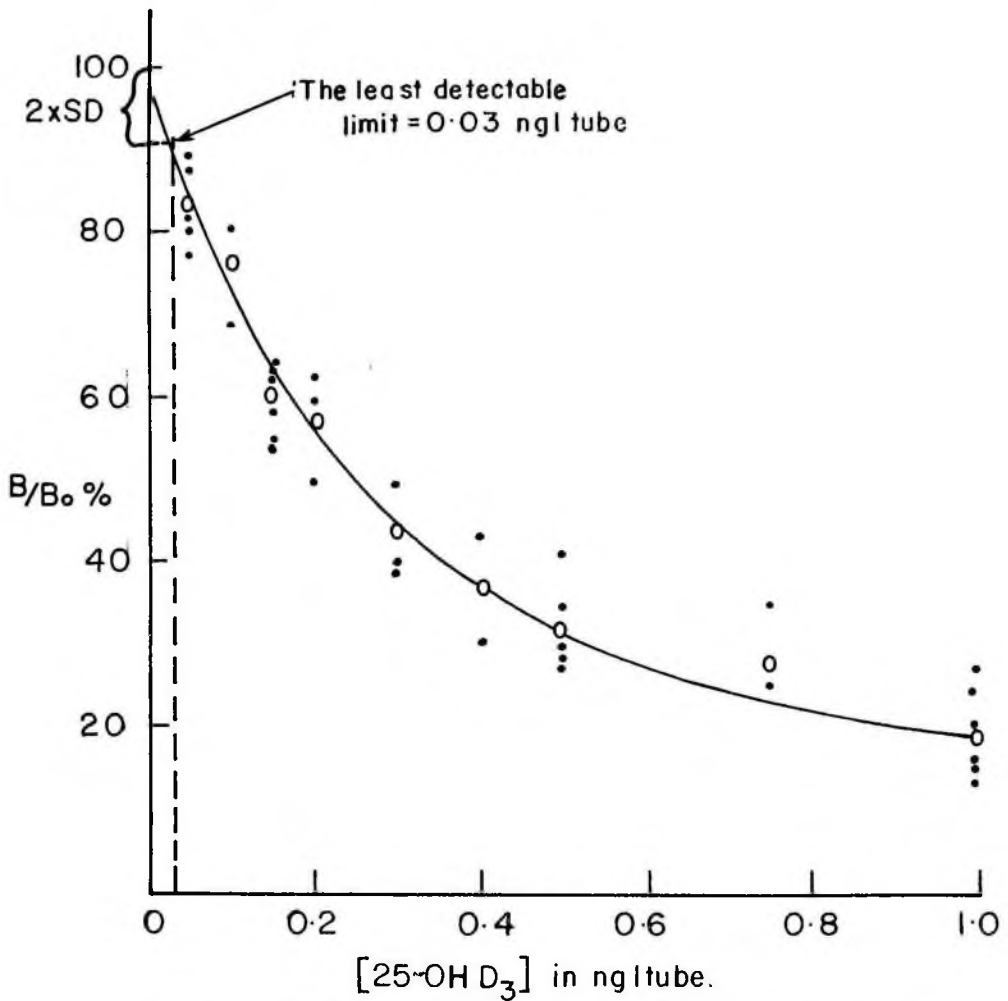


Fig. 2.13. Standard curve showing the distribution of points about each concentration of 25-OH D₃ [Individual points; O mean points]

CONCN. IN ng/tube	n	MEAN B/Bo %	STANDARD DEVIATION	2 x STANDARD DEVIATION
0.00	10	100	4.92	9.84
0.05	9	83.6	4.01	8.02
0.15	9	60.5	3.80	7.60
0.30	8	43.2	3.12	6.24
0.50	9	31.5	4.04	8.08
1.00	10	18.3	4.35	8.70

TABLE 2.5 : The means, standards deviations (SD) and 2 x SD of B/Bo x 100% of the points 0.00, 0.05, 0.15, 0.30, 0.50 and 1.00 ng/tube:

of supernatant counted. The ratio of counts at zero ligand concentration to the total activity (B./T) expressed as a percentage was 64.6%. The $B/B_0 \times 100$ for the various points are given in Table 2.5. The standard deviations about the means are also given. The standard deviation at zero point is ± 4.92 . Using twice the standard deviation to determine the sensitivity defined as the least detectable limit the sensitivity may be placed at 0.03ng/tube (see fig. 2.13). By similar considerations the precisions of other points on the assay standard curve may be found. The detectable range of the assay therefore was between 0.03 - 1.00ng/tube. Figure 2.14. is logit-log transformation of fig. 2.13. The curve was linearized between 0.05 - 1.00ng/tube, which lies within the working range of the assay curve. Thus the assay standard curve obtained was satisfactory in terms of sensitivity, precision and range.

2.5. ALTERNATIVES TO FINAL FORM OF ASSAY

One of the major difficulties encountered in setting up this assay was the lack of some of the assay

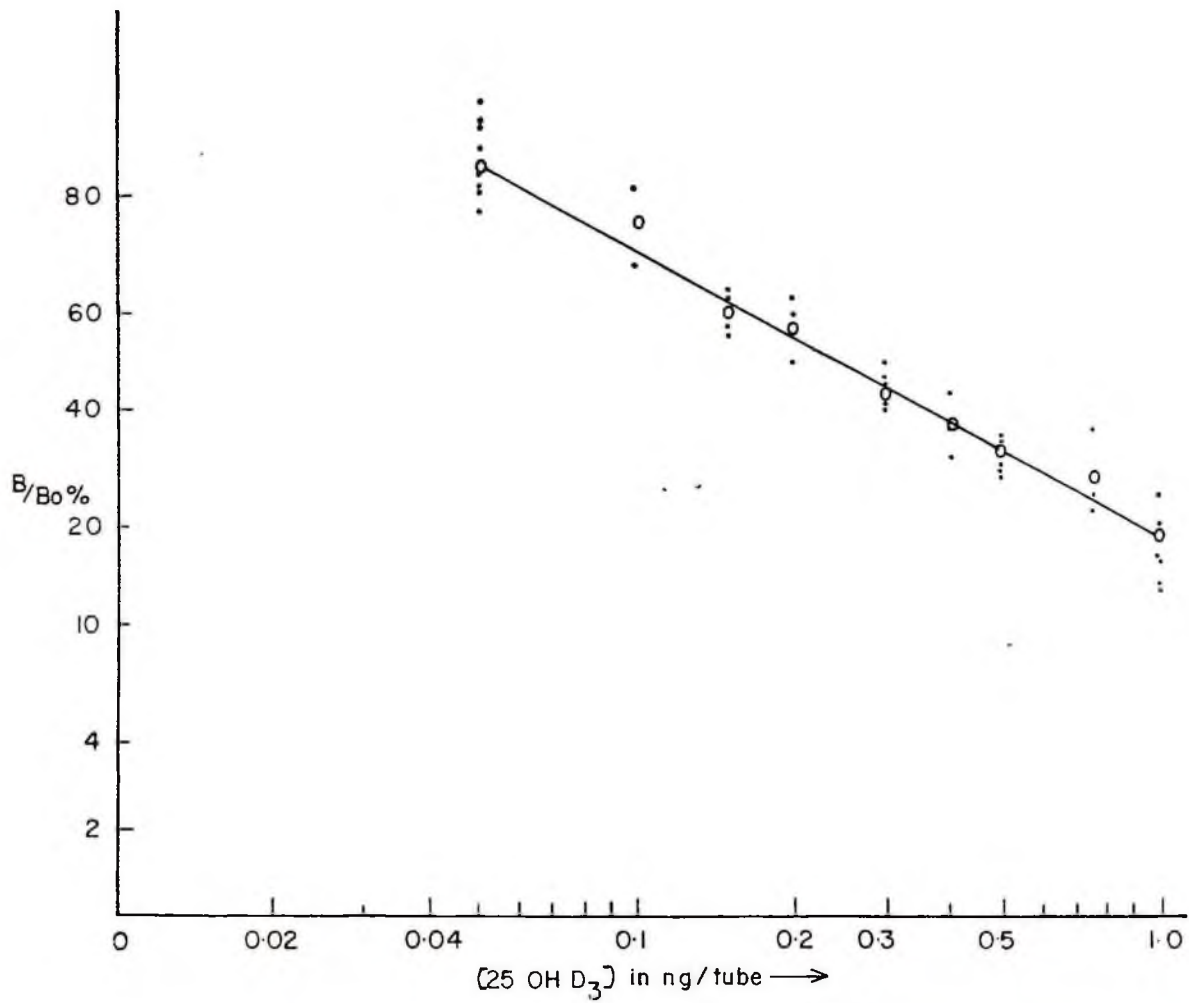


Fig. 2.14. Linearization of standard assay curve (• are individual points
○ are the mean values)

materials locally and the ease with which they could be processed before use. Also an assay with fewer and easily operated steps is less prone to errors. These factors were looked at as alternatives to the final form of assay.

In this regard normal rat serum was used as the source of the binding protein. A coated tube method was also attempted. This is one of the solid phase methods used to ease the separation step in assays. In the coated tube assay, the binder (here DBP) is bound to plastic tube surfaces and the assay is carried out in the tube. The method, originated by Catt and Tregear (98) appears to have additional advantages in terms of cost, speed and potential for automation. Its disadvantages include variation in the quality of plastic characteristics, maximal amount of tracer bound is less than what obtains in liquid phase methods; Dakubá et al. (99) have indicated that it might even be necessary to search for new assay conditions quite different from what was used in the

liquid phase methods.

2.5.1. Normal rat serum in assay

Indications that normal rat serum could provide a suitable binding protein has been given by Ph. DeNayer et al. (75).

Serum from albino rats (obtained from the animal house at Korle-Bu Teaching Hospital, Accra) fed on normal diet was used as a source of the vitamin D binding protein (DBP). The procedure for the determination of the titre is as given in section 2.3.4. The serum from rachitic rat was also run concurrently for comparison.

The titre of normal rat serum that bound 50% of the [^3H]-25-OH D_3 was 1 : 10,000 dilution (see fig. 2.15). The 50% titre for rachitic rat serum was 1 : 12,000. Approximately, 1 : 10,000 was the 50% titre for both types of sera. The slightly higher binding capacity for the rachitic rat serum under the same conditions ^{was} to be expected as the binding sites were relatively less occupied naturally by the endogenous 25-OH D_3 .

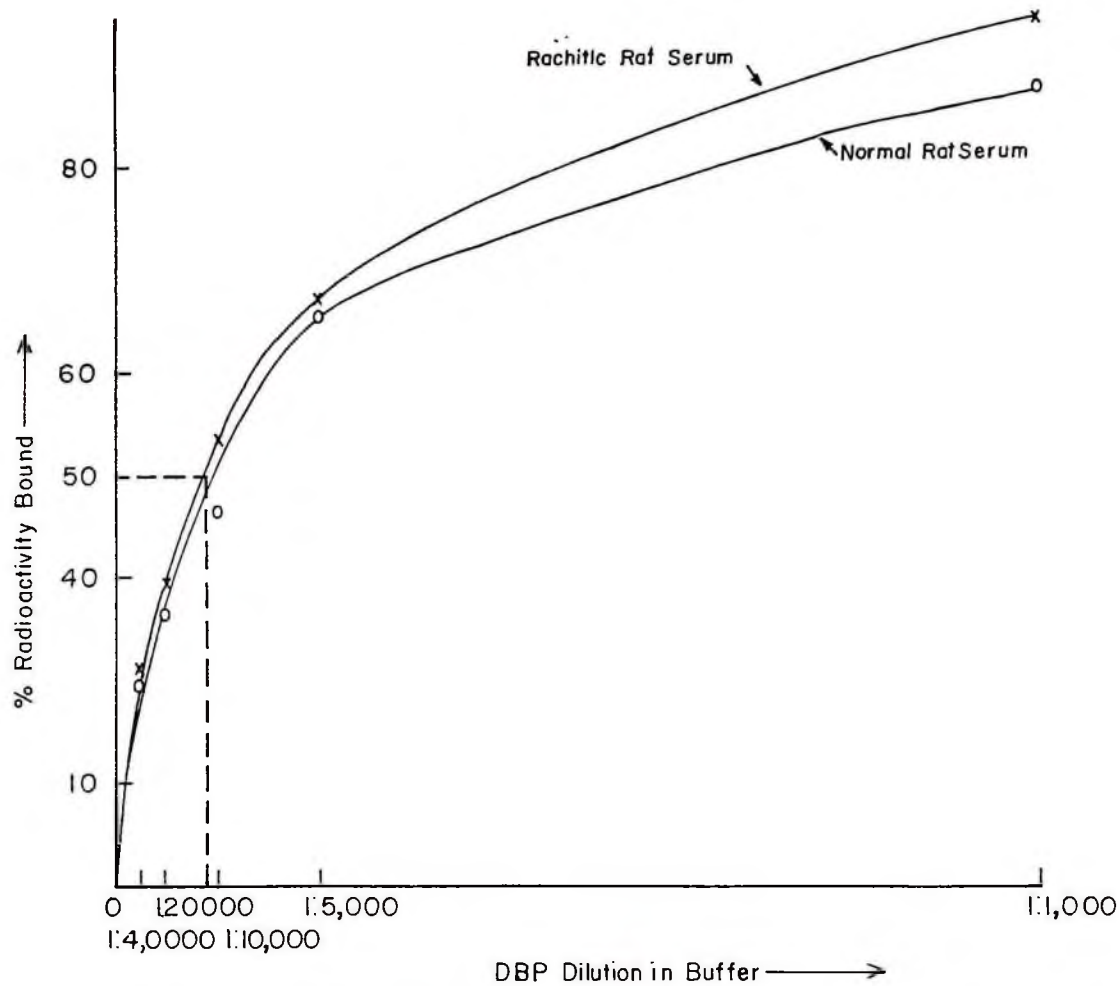


Fig. 2-15 Curves showing the suitability of normal rat serum in assay of 25 OH D_3
 50 % titre of rachitic rat serum = 1:12,000 % titre of normal rat serum
 = 1:10,000

It has therefore been confirmed that normal rat serum was suitable for the assay of 25-OH D₃. It was not necessary to put the rats on a rachitogenic diet before the serum could be used in assays. In this work, however, the rachitic rat serum was used for further assays for consistency.

2.5.2. Solid Phase Method : The Coated Tube Assay

The merits and demerits of the coated tube assay have been discussed (2.5.). An attempt was made to simplify the final form of the assay to a solid phase method. The procedure for coating adopted is as given by Catt et al. (100).

a. Coating

The serum from rachitic rat was diluted 1 : 1000 with 0.05M carbonate-bicarbonate buffer, pH 9.6. 500µl portions were carefully dropped at the bottom of polypropylene tubes. The tubes were divided into three batches and each group incubated at a different temperature. The incubation temperatures were 4°C,

25°C and 37°C. The tubes were incubated for 24 hours.

At the end of the incubation the tubes were washed once with 1% BSA and then twice with physiological saline (0.9% NaCl). In a second attempt ~~at~~ coating the tubes were washed three times with normal saline only (see later for reasons). The tubes were inverted and dried in an oven at 37°C.

b. Binding of [^3H]-25-OH D₃

After drying the tubes received 500 μ l of the working solution of [^3H]-25-OH D₃. The tubes were incubated at 0-4°C in the dark for 2 hours.

c. Counting

At the end of the incubation period the contents of the tubes were tipped into 8ml of scintillant and counted.

d. Results and discussion

The counts in the supernatant are shown in Table 2.6. These represent the unbound radioactivity. The counts in 2.6A represents tubes which were washed once with 1% BSA and then twice with physiological saline. Those in

TUBE NO.	DESCRIPTION	A cpm in SN ⁺	B cpm in SN
1	TOTAL ACTIVITY	1572	1664
2		1691	1630
3		1583	1590
4	UNCOATED TUBES	665	-
5		780	-
6		792	-
7	UNCOATED TUBES WASHED WITH BSA OR SALINE	984	1019
8		1074	980
9		1042	1028
10	TUBES COATED AT 4°C	1102	1129
11		1113	1132
12		971	1078
13		1039	1075
14		988	1168
15	TUBES COATED AT 22°C	1037	1090
16		923	1041
17		1079	1091
18		997	1037
19		1118	1133
20	TUBES COATED AT 37°C	1069	1075
21		1032	1074
22		994	1087
23		1055	1091
24		1062	1124

TABLE 2.6 : Coated tube assay : Binding of ^3H -25-OH D_3 to coated and uncoated tubes surfaces. A - the tubes were washed once with 1% BSA and then twice with 0.9% NaCl solution; B - tubes were washed 3 times with physiological saline (0.9% NaCl) SN⁺ = supernatant

2.6B were washed with saline (0.9%) only. The results show a decrease in counts for all tubes. Uncoated tubes which were not washed with albumin recorded the lowest counts. Uncoated tubes which were the same order as the coated tubes. The counts accumulated for the coated tubes were indistinguishable from the counts given by uncoated tubes washed with albumin. Incubation at different temperatures did not show any significant difference.

These observations tend to suggest that plain plastic tubes adsorb onto their surfaces higher quantities of $[^3\text{H}]-25\text{-OH D}_3$ (resulting in lower counts in the supernatant). When the tubes were washed with albumin the $[^3\text{H}]-25\text{-OH D}_3$ that binds to the plastic surface is decreased because the coated surface had lower affinity for $[^3\text{H}]-25\text{-OH D}_3$. The similarity between counts in supernatant of coated tubes and that of uncoated tubes washed with albumin points to the fact that counts of the coated tubes only reflect non-specific binding due to albumin.

In short, therefore it was not possible to conduct a coated tube assay under the conditions employed.

2.6. SUMMARY

1. The main factor that was hampering the initial progress of the assay conducted according to the protocol described in section 2.1 was the incorporation of albumin as a solubilizer. Its replacement by gelatin greatly improved the assay.

2. A suitable dilution of rachitic rat serum to be used for assays was 1 : 10,000. Normal rat serum was also found to be suitable for use in the assays.

3. It was not possible to convert the assay to a coated tube method.

CHAPTER THREE

3. ASSAY OF SERUM 25 HYDROXYVITAMIN D₃-

With the establishment of the standard curve as described in the preceding chapter, the normal serum levels of 25-OH D₃ in Ghanaians could be determined. The departure from the normal of 25-OH D₃ levels in patients with cirrhosis of the liver and chronic renal failures could also be found.

3.1. THE ASSAY

The assay of serum 25-OH D₃ was carried out according to the format for the final form of the assay given in section 2.4. A flow-chart of the procedures involved is given in fig. 3.1.

a. Collection and storage of serum samples.

The normal human venous blood samples were collected randomly from healthy looking Ghanaians on regular diet between July and October 1977. During the same period, whole blood samples from patients with liver cirrhosis and chronic renal failures were obtained from the Third Floor of the Medical Block, Korle-Bu Teaching Hospital, Accra,

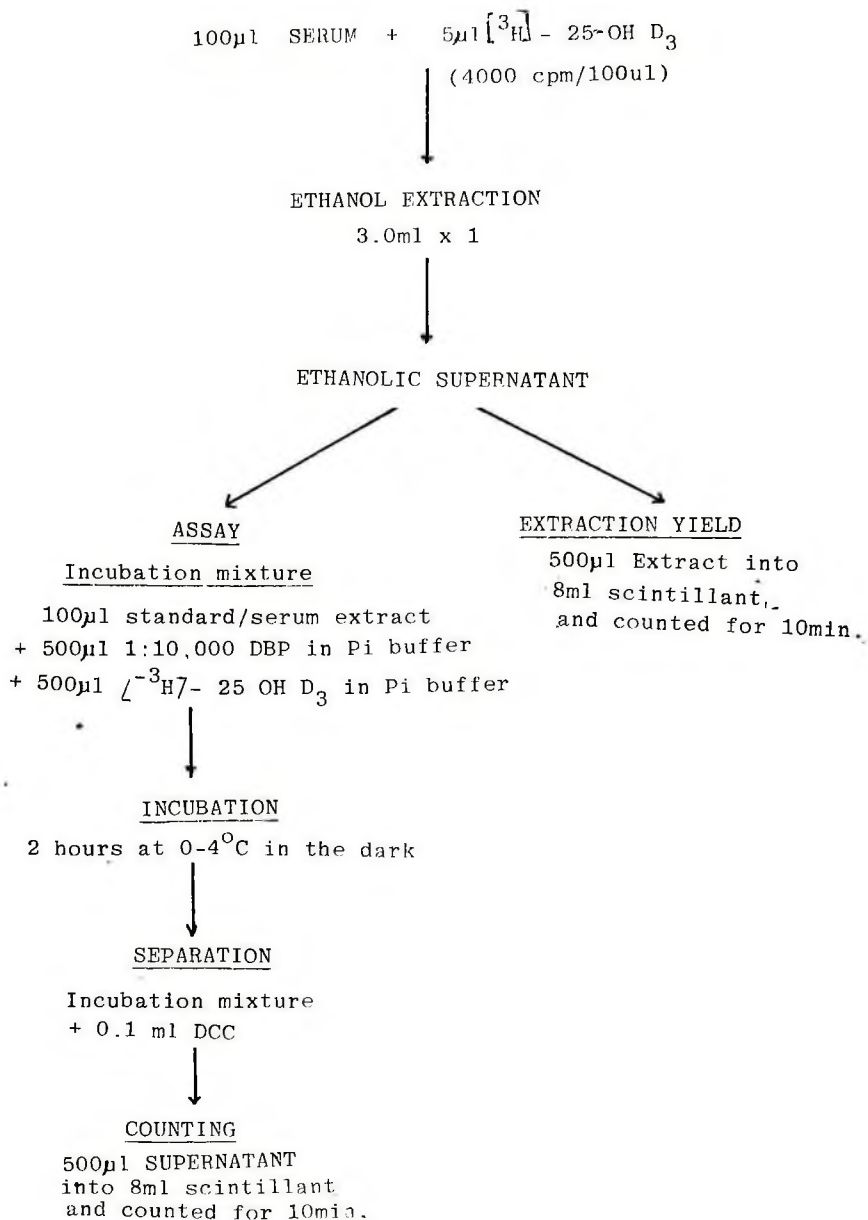


Fig. 3.1 FLOW-CHART OF THE ASSAY

through the indispensable help of Prof. A.K. Foli and Dr. D. Adu (both physicians at Korle-Bu Hospital).

5-10ml whole blood samples were collected into plain vacutainer tubes. The clotted blood samples were spun at 2,500rpm for 5 minutes and the sera aliquoted into plastic tubes and stored away at -20°C until required for use.

b. Extraction of 25 OH D_3

100 μl portions of sera to be extracted were drawn into plastic tubes or glass tubes. 5 μl aliquots of ethanolic [^{-3}H]-25-OH D_3 (2.2.a) was put into all tubes and incubated at 37°C for 30 minutes.

3.0ml portions of absolute ethanol were drawn into the tubes. The tubes were stoppered and shaken for 10 seconds. The tubes were allowed to stand in ^{an}ice-bath ($0-4^{\circ}\text{C}$) for 30 minutes. They were then shaken and centrifuged at 2500rpm for 20 minutes at $0-4^{\circ}\text{C}$.

For extraction yield 5 μl of the ethanolic [^{-3}H]-25-OH D_3 was put directly into scintillant for counting.

500 μ l ethanol was added to the same scintillant fluid.
500 μ l of the alcoholic extract was pipetted into another vial of scintillant.

It should be noted that it was found necessary to use 3.0ml of ethanol instead of 2.0ml as suggested in 2.1. for the extraction of the serum samples. Table 3.1. shows result of an experiment conducted to find the suitable volume of ethanol to be used.

c. Tubes disposition

For the standard curve, the reagents were dispensed as given in section 2.4. The serum samples were prepared as follows for the assay.

100 μ l of ethanolic extract was pipetted in triplicate into plastic tubes.

500 μ l aliquots of the binding protein in phosphate buffer was pipetted into all tubes.

500 μ l aliquots of [3 H]- 25-OH D₃ were also put into all tubes.

The tubes disposition for the standards and the samples are given in Table 3.2

CONCN. OF 25 OH D ₃	VOL. OF SERUM/VOL. OF ETHANOL (in ml)		
	0.5 / 2.0 c/10 min.	0.1 / 2.0 c/10 min.	0.1 / 3.0 c/10 min.
0.00 ng/tube	2064 2188 1838	2027 1999 2127	2553 2529 2429
1.00 ng/tube	950 1073 847	925 849 920	747 940 948
UNKNOWN SERUM 25-OH D ₃	645 760 630	726 848 797	1453 1558 1532

TABLE 3.1 : The suitable volume of ethanol used to extract serum 25-OH D₃. The range of the assay curve was 0.00 - 1.00 ng/tube. The figures represent counts / 10min. When 0.1 ml of serum was extracted with 3.0ml ethanol the counts for the unknown serum 25 OH D₃ was within range.

TUBE No.	CODE OR NAME	Vol STD OR SAMPLE IN ul	Vol PHOSPHATE BUFFER IN ul	Vol. DBP in Pi BUFFER IN ul	Vol 25-OH D ₃ IN ul
I - 3	T.A.	↑ 100 Et OH	↑ 500	-	↑
4 - 6	C.B.	↓	↓	-	↓
7 - 9	A	↑	↑	↑	↑
10 - 12	B	↑	↑	↑	↑
13 - 15	C	↑	↑	↑	↑
16 - 18	D	↑	↑	↑	↑
19 - 21	E	↑	↑	↑	↑
22 - 24	F	100	↑	↑	↑
25 - 27	G	↓	↑	500	500
28 - 30	H	↓	↑	↓	↓
31 - 33	I	↓	NIL	↓	↓
34 - 36	J	↓	↓	↓	↓
37	E.Y. ₁ *	-	↓	-	-
38 - 40	SAMPLE ₁	100	↓	500	500
41	E.Y. ₂	-	↓	-	-
42 - 44	SAMPLE ₂	100	↓	500	500
etc.			↓	↓	↓

TABLE 3.2 : Disposition of tubes showing the distribution of reagents into assay tubes

[* E.Y. += extraction yield]

d. Sample calculations

(i) Extraction Yield

Data: counts in 0.5ml extract = 203 c/10 min.

Activity added to serum before extraction = 1493 c/10min.

Vol. of extract = 3.1ml.

Calculation of % Extraction Yield

$$\begin{aligned} \text{Total counts in 3.1 ml extract} &= \frac{3.1}{0.5} \times 203 \\ &= 1258 \end{aligned}$$

$$\begin{aligned} \% \text{ Extraction yield} &= \frac{1258}{1493} \times 100 \\ &= 84.7\% \end{aligned}$$

(iii) Concentration of 25-OH D₃ in serum sample

Data: vol. of extract used in assay = 0.1ml.

vol. of serum extracted = 0.1ml.

interpolated 25-OH D₃ value from graph 0.2 ng/tube.

Calculation

Thus 0.1ml extract contains 0.2 ng 25-OH D₃

$$\text{Amount in 3.1ml extract} = 0.2 \times \frac{3.1}{0.1} \text{ ng}$$

$$\text{Amount in 0.1ml serum} = 0.2 \times \frac{3.1}{0.1}$$

$$\text{Hence 1 ml serum contains } 0.2 \times \frac{3.1}{0.1} \times \frac{1.0}{0.1} \text{ ng}$$

But the Extraction was 84.7% efficient.

$$\begin{aligned}
 &\text{Actual concentration of 25-OH D}_3 \text{ in serum} \\
 &= 0.2 \times \frac{3.1}{0.1} \times \frac{1.0}{0.1} \times \frac{100}{84.7} \\
 &= 73.4 \text{ ng/ml serum.}
 \end{aligned}$$

3.2. RELIABILITY CRITERIA

The reliability of an assay depends on the stability of the standard curve obtained from assay to assay, the similarities in the manner of binding of serum 25-OH D₃ to the DBP and the percentage radioactivity bound when 25-OH D₃ concentration is zero. These factors were controlled and monitored in the three assays in which the levels of 25-OH D₃ in the human samples were estimated.

a. Quality Control sample

To check the variability in the levels of 25-OH D₃ within assays and between assays a particular serum sample was determined each time an assay was carried out. This was the quality control sample (QC). After extraction of the QC sample (see 3.1.b) the ethanolic solution was double diluted^{to} one-eighths (1/8)^{of} its full strength. The estimated values are shown in Table 3.3.

DILUTION OF QUALITY CONTROL SAMPLE	EXPERIMENT I CONCN. OF 25 OH D ₃ in ng/ml	EXPERIMENT II CONCN. OF 25 OH D ₃ in ng/ml	EXPERIMENT III CONCN. OF 25 OH D ₃ in ng/ml	MEAN + S.D. ng / ml.
100%	82.1, 88.8, 96.4	73.9, 74.2, 96.1	72.3, 78.9, 80.3	82.5 ± 9.5
50%	21.4 *	33.3, 44.3, 59.1	39.7, 43.0, 46.9	41.1 ± 11.7
25%	21.4, 25.4, 27.8	18.5, 25.8, 29.7	21.7, 25.1, 25.3	24.5 ± 3.4
12.5%	10.7, 12.3, 13.6	7.4, 11.1, 19.8	10.7, 10.8, 14.4	11.7 ± 2.5

TABLE 3.3 : The Quality control samples determined once a week for three weeks (24 days). The values were obtained from the three assays in which the serum samples were determined. (* Only one point was available, the other tubes were spilled)

Generally, there was a two fold decrease in the values with double dilution of the QC sample. The samples of the same strength consistently gave the same range of values in the assays.

The three standard assay curves used to determine the values of 25-OH D₃ in human samples are shown in fig. 3.2.

b. Parallelism between samples and standards

Fig. 3.3. shows a logit-log transformation of the standard curve and the curve for the quality control samples. Within the range of the quality control samples the two curves are parallel. This is an indication that the assay is satisfactory within the range and also the serum extracts behaved just like the standards.

c. Total activity, Bo/T, and charcoal blank counts

To ensure that values determined from the series of assays were comparable a strict routine was observed as to the values of the total activity counts (T). This and the assay conditions reflect on the percentage radioactivity bound at zero 25-OH D₃ concentration (Bo/T) and the counts for the charcoal blank (CB).

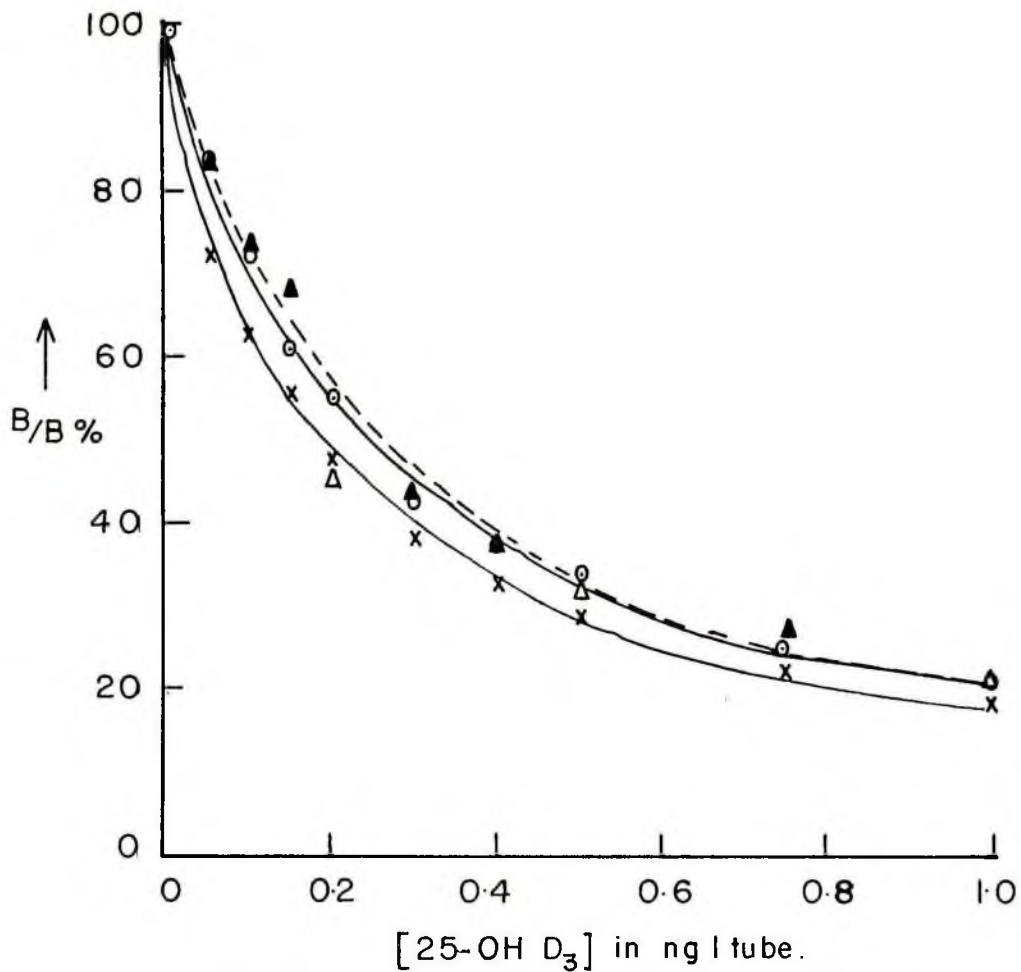


Fig. 3.2: The three standard curves which were used to estimate serum 25-OH D₃ levels.

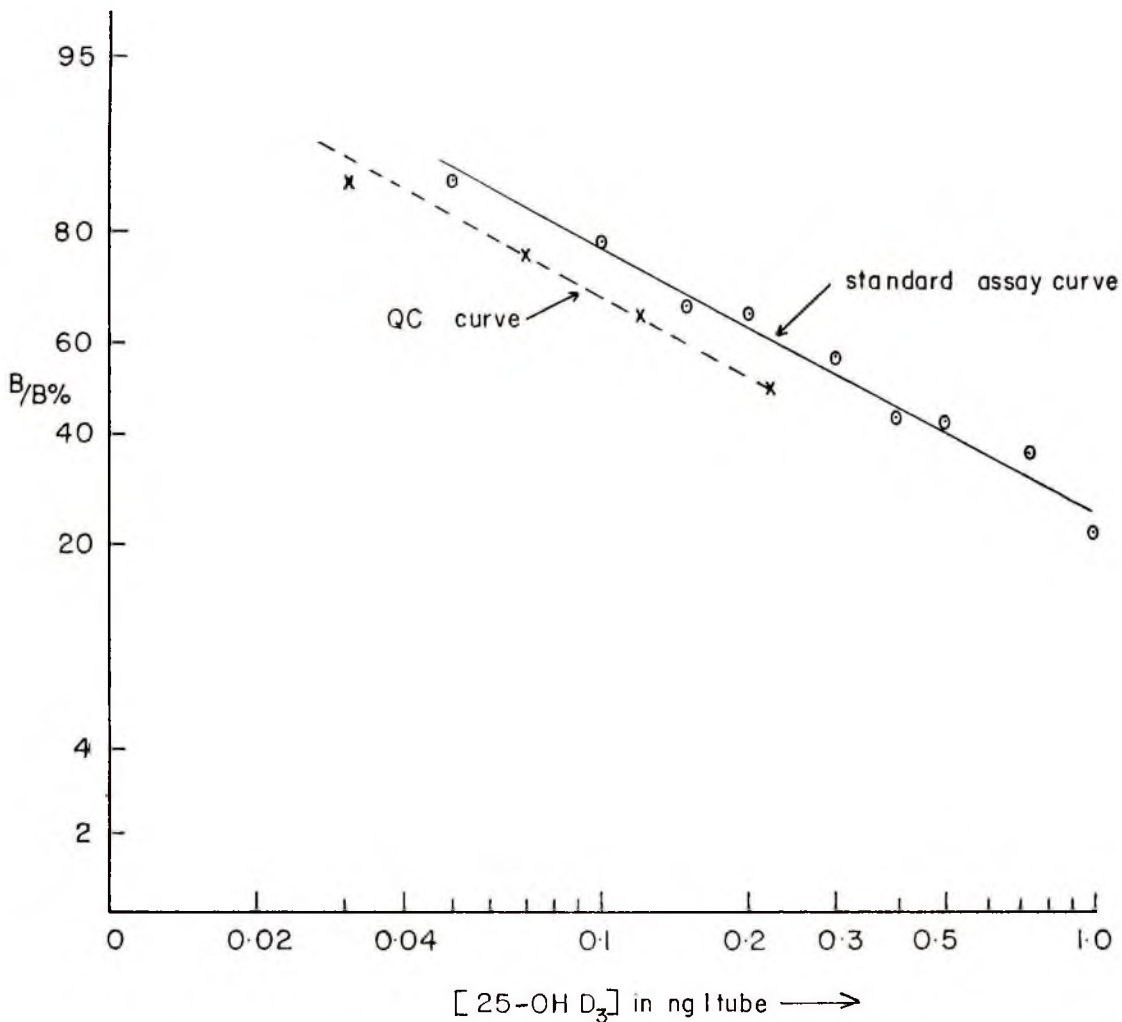


Fig. 3.3: Parallelism between standard assay curve and quality control curve (QC), (the points represent mean values, the graphs were drawn on CIS Logit-log paper for linearization of radioimmunoassay standard curve)

Table 3.4. shows the values obtained for T, Bo/T and CB in the assays which were used to determine the serum levels of 25-OH D₃.

As the [³H]- 25-OH D₃ solution was prepared fresh for use (2.4.) each time an assay was carried out the fluctuations in T, Bo/T and CB was to be expected under the circumstances.

3.3. SERUM 25-OH D₃ LEVELS

The levels of serum 25-OH D₃ in 47 healthy Ghanaians aged between 11 and 50 years are shown in Table 3.5. The levels encountered fell within a wide range of 33.2 - 284.7 ng/ml serum. The normal mean value was 129.0 ± 55.7 ng/ml (mean ± SD).

The patient levels of 25-OH D₃ in serum are shown in Table 3.6. The mean value for 11 patients with cirrhosis of the liver was 45.0 ± 16.1 ng/ml serum; while that for 8 patients with chronic renal failures was 83.5 ± 82.7 ng/ml serum.

The statistical significance of difference of the grouped means were determined by student's t test. The serum 25-OH D₃ levels in patients with cirrhosis of

ASSAY	TA c/min/0.5ml/SN	Bo/T %	CB c/min/0.5ml/SN
I	415.8	47	68
II	300.2	48	56
III	397.5	59	48

TABLE 3.4 : The values of total activities (TA), percentage radioactivity bound at zero 25-OH D₃ Concentration (Bo/T %) and the charcoal blank (CB) in the three assay which were used to estimate 25-OH D₃ levels in serum samples.

the liver was significantly decreased ($p < 0.05$) as compared to normal values. Chronic renal failure patients showed a higher mean value of 25-OH D₃ with a wider spread (83.5 ± 82.7 ng/ml). The level was significantly different from the normal at 0.05 level of significance.

3.3.1. Comments on the 25-OH D₃ levels

The normal levels of 25-OH D₃ determined here were higher than any value that had been noted in the literature. There was an initial concern as to whether these levels represented the actual levels circulating in the serum of Ghanaians. A look at the serum levels determined by others elsewhere may probably lend support to the levels found. The range of mean values of serum 25-OH D₃ determined in countries like U.S.A., France, Belgium, U.K., Germany, Japan and South Africa was 11.9 - 39.5 ng/ml (75). Indication was not given of levels in individuals residing in purely tropical regions. However, circulating levels in negroes have been determined in the U.S.A. The values obtained did not vary much from caucasian levels though they were slightly higher (26 ng/ml for negroes and 25 ng/ml for

caucasians). It must be noted that the negroes in this study were living under the same climatic conditions as the caucasians (101, 102). Individuals particularly exposed to large amounts of U.V. light have higher levels of 25-OH D₃ circulating in their blood (103). In this regard Hollard et al. (104) found the levels in volunteers in winter to be 31.5 \pm 15.8 ng/ml. In summer the level rose to 62.7 \pm 36.5 ng/ml. It is gratifying to note that a subset of the volunteers who were well exposed to sunlight had levels as high as 84.8 \pm 30.4 ng/ml. These levels indicate the capability of the skin to contribute to 25-OH D₃ levels.

Serum 25-OH D₃ also correlates with vitamin D intake in the diet (103). The dietary intake then augments the major contribution by the skin. In cases of vitamin D intoxication, levels as high as 500 ng/ml have been encountered. When the vitamin D ingestion was stopped in the case in point 25-OH D₃ level fell to 160 ng/ml after 8 months.

The level of 25-OH D₃ found in 47 healthy Ghanaians on a regular diet was 129.0 \pm 55.7 ng/ml. This is justifiable in the face of abundant exposure to intense sun's irradiation all the year over, a condition which

is tenable in Ghana. If volunteers exposed to the sun for relatively a brief period during summer could have circulating levels as high as 84.8 ± 30.4 ng/ml (104) then the Ghanaian level of 129.0 ± 55.7 ng/ml should be acceptable. The level is also physiologically acceptable since up to 160 ng/ml 25-OH D_3 have been found in individuals recuperating after vitamin D_3 intoxication (103).

These high levels of 25-OH D_3 found in Ghanaians are contributed mainly by the skin upon the sun's irradiation. Vitamin D intake in popular Ghanaian diet appears to be little. What there is may be found in egg yolk, margarine, milk and 'Milo' (1.6.). The fraction of the population who take these frequently is rather small.

Very high levels of vitamin D lead to various diseased states (1.6.). The high level of incidence of sun's radiation on the skin maximizes the biosynthesis of vitamin D_3 . However, the melanized African skin serves as a protective device against vitamin D intoxication by cutting down the amount of light

(in 300-400nm wavelength range) that reaches the cutaneous site of vitamin D biosynthesis. Only 18% as compared to 64% in European skin is transmitted (105). Thus the dark skin of Ghanaians limits the amount of 25-OH D₃ which could otherwise be formed.

The 25-OH D₃ levels in patients with cirrhosis of liver were lower than normal levels (Tables 3.5 and 3.6). These levels were of the same order as normal values determined by others (75). Hollard et al. (104) and Okano et al. (53) reported a similar decrease in cirrhotic patients.

The level of 25-OH D₃ in chronic renal failure patients was more difficult to interpret. The range was 7.4 - 232.5 ng/ml with a mean value of 83.5 ± 82.7 ng/ml. There was significant difference between the normal values of CRF values at 0.05 significant level. Similar findings were reported by Shen et al. (106) and Okano et al. (53) in renal patients. Bayard et al. (107) found a decrease in 25-OH D₃ level. However, Hollard et al. (104) and Ph. DeNayer et al. (75) were cautious in interpreting the levels observed in renal patients.

The means of the diseased levels of 25-OH D3 though higher than the normal levels measured in other areas were lower than the Ghanaian normal level. This reflects the peculiar effect of sunlight in Ghana and presumably other tropical regions in general. It is still not clear in the case of CRF patients whether the levels were high or low judging from the wide dispersion about the mean (i.e. 83.5 ± 82.7 ng/ml). Thus the problem has to be looked into further.

SAMPLE NO.	25 OH D ₃ ng / ml Serum	SAMPLE NO.	25 OH D ₃ ng / ml Serum
1	152.9	25	102.9
2	245.4	26	149.1
3	106.7	27	99.1
4	180.6	28	77.1
5	72.9	29	195.6
6	147.0	30	79.8
7	91.3	31	78.2
8	128.0	32	131.6
9	193.2	33	225.1
10	64.0	34	173.5
11	78.2	35	155.0
12	75.9	36	166.0
13	184.8	37	88.6
14	109.2	38	210.4
15	126.8	39	284.7
16	97.2	40	88.6
17	256.1	41	132.9
18	80.6	42	81.2
19	100.8	43	33.2
20	109.2	44	177.1
21	115.0	45	121.8
22	79.8	46	140.2
23	100.8	47	99.6
24	73.5		
n = 47			
MEAN 129.0			
STANDARD DEVIATION (SD) = 55.7			

TABLE 3.5 : The normal levels of 25-OH D₃ in 47 healthy Ghanaians on regular diet. The mean level found circulating in the serum was 129.0 ± 55.7 ng/ml.

PATIENTS WITH CIRRHOSIS OF LIVER		PATIENTS WITH CHRONIC RENAL FAILURE	
SAMPLE NO.	25 OH D ₃ ng / ml SERUM	SAMPLE NO.	25-OH D ₃ ng / ml SERUM
I	64.6	I	232.5
2	25.1	2	103.3
3	53.8	3	177.4
4	50.2	4	7.4*
5	71.7	5	11.1
6	46.6	6	62.7
7	39.5	7	55.4
8	21.5	8	18.5
9	57.4		
10	46.6		
11	28.7		
n = 11		n = 8	
MEAN = 46.0		MEAN = 83.5	
STANDARD DEVIATION = 16.1		STANDARD DEVIATION = 82.7	

TABLE 3.6 : The levels of 25-OH D₃ in serum of patients with cirrhosis of the liver and Chronic renal failure (CRF). The levels found were 46.0 ± 16.1 ng/ml and 83.5 ± 82.7 ng/ml.

* This sample value fell just outside the range but it was estimated.

3.4 GENERAL DISCUSSION

The method (2.1.) on which this work was based used albumin as a solubilizer in the phosphate buffer. A buffer with BSA as a component was found to fluctuate considerably in suitability for the assay of 25-OH D₃. In this assay gelatin was incorporated into the assay buffer with great success. Thus the earlier suspicion that dextran coated charcoal did not have suitable adsorption properties was unfounded. Other buffer systems have been used for the assay. In the original method by Belsey et al (95), barbital-acetate buffer, pH 8.6 with β -lipoprotein solubilizer was used. Triton X-405 (108) solubilizer has been employed by others. From these findings it is advisable to check the buffer constitution if the initial assay is not giving favourable results.

The least detectable limit (sensitivity) of the assay may be placed at 0.03 ng/tube or 0.3 ng/ml. In terms of sensitivity the assay developed here was comparable or even better than similar assays reported. The sensitivity quoted by Belsey et al. was 1 - 2 ng/ml in a latex assay (95). 4 ng/ml by Hollard et al. (104) and 0.22 ng/tube by Okano et al. (53).

The Ghanaian serum levels of 25-OH D₃ though higher than levels determined under different climatic conditions were quite reasonable judging from the point of view of constant incidence of sunlight on the skin of individuals living in Ghana. These high levels may be augmented by dietary sources in a few individuals who take eggs, milk and 'Milo' (a beverage). The quantitative contribution however has to be assessed. The high level 25-OH D₃ observed in the test samples therefore reflect significant delivery of vitamin D from the skin to the liver where the 25-hydroxylation takes place.

The levels of 25-OH D₃ in disease could be used to assess ^{some} the vitamin D status of a patient hence throw [^]light on the concomitant deficiency symptoms

associated. The data must however be used with caution especially the levels found circulating in CRF patients. These levels fluctuate through a wide range, some individual values being as high as the upper limit obtainable in normal Ghanaians. As has been stated in section 3.3.1., the level of 25-OH D₃ in the two diseased conditions considered were as high as normal levels determined elsewhere. Thus, before 25-OH D₃ values could be used to any advantage in a different geographical area the local normal level should be established.

3.5. CONCLUSIONS

The assay of 25 hydroxyvitamin D₃ was improved when 0.02M phosphate buffer, pH 7.6 containing 0.1% gelatin solubilizer was used.

The sensitivity of the assay was 0.03 ng/tube or 0.3 ng/ml serum.

It was also confirmed that non-rachitic rat serum could be used as a source of the vitamin D₃ binding protein in these assays.

The mean normal level of 25-OH D₃ in Ghanaians

was 129.0 ± 55.7 ng/ml serum. The patients with cirrhosis of the liver had an average level of 45.0 ± 16.1 ng/ml which was significantly lower than normal value ($p < 0.05$). Chronic renal failure patients showed circulating level of 83.5 ± 32.7 ng/ml which was also significant at the five percent level of significance ($p < 0.05$).

3.6. FUTURE WORK

The strikingly high levels of 25-OH D₃ obtained in these assays necessitates that a wider sample size be considered for confirmation of the results. The level of vitamin D in average Ghanaian diet should be assessed as its contribution to the high level is only on speculative basis. When these studies are carried out one may end up confirming further the major role played by the skin in the production of the vitamin or a significant contribution from the diet. Further survey involving the levels in the diseased states studied here may throw more light on the actual levels in CRF patients which are at present not clear cut.

The volume of 100 μ l serum used in the assay makes the procedure amenable to pediatric studies. Moreover rickets are found in children hence with a tool capable of ^{measuring} 25-OH D₃ in minute volumes (0.1 ml) the studies could be extended to children.

In future assays one can dispose of the rachitic rat serum as normal, non rachitic rat serum has been shown to contain binding protein at a suitable titre. It may even be worthy to try normal human serum in place of rat serum.

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