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VITAMIN D METABOLISM IN THE FRUIT BAT (ROUSETTUS AEGYPTIACUS).

BY

MEROPI CAVALEROS

A dissertation submitted to the Faculty of Medicine,

University of the Witwatersrand, Johannesburg

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ABSTRACT

Rouesettus aegyptiacus, the fruit bat is a crepuscular frugivore with no obvious access to either exogenous or endogenous sources of vitamin D. Therefore this animal's vitamin D status and endocrine system was investigated.

Both captive and wild populations of fruit bats appear to be naturally in a vitamin D impoverished state. The serum concentration of the principle circulatory, metabolite [25(OH)D] is undetectable (< 4 ng/ml). Fruit bats possess the full compliment of enzymes associated with the vitamin D endocrine system. This was shown when labelled more polar metabolites were produced after the administration of ^{3}H vitamin D_3 and ^{3}H $25(OH)D_3$. Furthermore, a specific vitamin D binding protein (DBP) is present. After partial purification, it was revealed that this molecule is slightly larger in molecular mass than that of humans and baboons.

The intraperitoneal administration of $25(OH)D_3$ revealed enhanced 1α-hydroxylase activity such that 1.7 times more $1,25(OH)_2D_3$ was produced than $24,25(OH)_2D_3$. The ratio of these di-hydroxylated metabolites conform with the ratio of these 2 metabolites in states of vitamin D deficiency and thus confirm the impoverished vitamin D status. Undetectable serum concentrations of $25(OH)D_3$ might therefore be explained by a limited exogenous vitamin D substrate (rotting fruit peels and fungi). Given the elevated 1α hydroxylase activity, the small amounts of 25(OH)D produced would be rapidly converted to the active metabolite. The low concentration of active hormone appear adequate for the maintenance of mineral homeostasis as indicated by tightly controlled

serum calcium (2.26 ± 0.17 mmol/l), magnesium 01.16 ± 0.24 mmol/l) and inorganic phosphorus (2.93 ± 1.01 mmol/l).

Both vitamin D_2 and vitamin D_3 metaboiites were detected in bat serum albeit in very small amounts, suggesting that fruit bats exploit both exogenous plant sources (skins of fruit - vitamin D_2; fungi - vitamin D_3) and might indeed receive some U.V. light during their crepuscular forays to endogenously produce small amounts of vitamin D_3.

In conclusion, fruit bats appear to belong to a small group of animals that naturally have limited access to vitamin D, yet the vitamin D endocrine system in these animals is no different to that of other mammals. These animals have adapted their vitamin D endocrine system to function well at the low hormone concentrations and they exhibit no pathological problems associated with relative vitamin D depletion.

DECLARATION

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

.....fourteenthday of .....December.....19..92.

.....

(MEROPI CAVALEROS)

..... Date: ...14/12/92.....

(PROF. J.M. PETTIFOR)

(signature of supervisor)

I certify that the studies contained in this dissertation have the approval of the Animal Ethics Committee of the University of the Witwatersrand (AEC Number 88/189/4).

..... Date: ...14-12-92

(signature of student)

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LIST OF ABBREVIATIONS

25(OH)D	=	25 hydroxyvitamin D
$24,25(OH)_2D$	=	24,25 dihydroxyvitamin D
$1,25(OH)_2D$	=	1,25 dihydroxyvitamin D
DBP	=	vitamin D binding protein
HPLC	=	High pressure liquid chromotography
^3H	=	Tritium
cpm	=	counts per minute
dpm	=	disintergrations per minute
Pi	=	inorganic phosphorus
Ca	=	calcium
mg	=	magnesium
mg	=	microgram
ng	=	nanogram
pg	=	picogram
μl	=	microlitre
nm	=	nanometers
OD	=	optical density

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   American Society for Bone and Mineral Research.

   June 21-24 1986, Anaheim, CA.

   Meropi Cavaleros, F. Patrick Ross and John M. Pettifor.

   The fruit bat maintains 'normal' serum levels of 1,25 dihydroxyvitamin D in the face of very low levels of 25 hydroxyvitamin D.

2. 2nd Bone and Mineral Metabolic Congress.

   29 September-2 October 1986, Cape Town.

   F.P. Ross and M. Cavaleros.

   Partial characterization and purification of a putative bat serum vitamin D Binding Protein (oral communication presented by M. Cavaleros).

3. 7th Workshop on Vitamin D.

   24-27 April 1988, Rancho Mirage, Ca, USA.

   Cavaleros M., Pettifor J.M. and Ross F.P.

   Partial purification and properties of vitamin D binding protein in serum of the fruit bat.

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(b) Authentic $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$,

(c) human serum to which was added $1,25(OH)_2D_2$,

(d) human serum and

(e) bat serum.

The samples (b,c,d and e) were extracted and chromatographed by HPLC using the solvent system described in (a) and the eluted fractions (0.5ml) were measured in the calf thymus receptor assay for $1,25(OH)_2D$ activity. 100% binding of $^3[H]1,25(OH)_2D_3$ is equivalent to zero concentration of the $1,25(OH)_2D$ metabolite. 154

CHAPTER 1

The biology of Vitamin D and fruit bats: a literary perspective

1.1 Introduction

In all mammals studied to date, vitamin D appears essential for life, regulating many vital biological functions (Kumar, 1986). This fat soluble vitamin plays a major role in calcium and phosphorus homeostasis. Mineral balance is maintained by several processes; including the stimulation of active calcium transport in the intestine, the control of mineral excretion and the mobilization of minerals from bone. In this way plasma concentrations of minerals are tightly regulated. In addition vitamin D has several functions not directly related to mineral homeostasis, these include the activation of T lymphocytes (Tsoukas et al., 1984), the control of peptide hormone secretion e.g. parathyroid hormone (PTH) (Norman and Litwack, 1984), prolactin (Richard et al., 1991), and insulin (Kadowaki and Norman, 1984), and mediates the development and control of immunity (Haussler et al., 1988; Haddad, 1984; Manolagas et al., 1990).

The vitamin D system is unique amongst hormones in that it is dependent on environmental factors (e.g. sunlight) for the provision of the parent vitamin and on multi organ participation in both the activation and biological expression of the active metabolite (Stumpf, 1988; 1991). The principal circulating metabolite is 25-hydroxyvitamin D (25(OH)D). This metabolite is further hydroxylated to two more polar metabolites:- either the active hormone, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) or a second metabolite, 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) which is thought to be inactive. The kidney is the central endocrine gland that produces in regulated quantities the two key dihydroxyvitamin D metabolites (Norman, 1990).

1.2 Historical

The historical background to vitamin D and its function has been extensively reviewed (Norman et al., 1982; DeLuca, 1984; Kumar, 1986). Most of the early work on vitamin D examined the effects of vitamin D, or lack thereof, on bone diseases such as rickets and osteomalacia.

Vitamin D deficiency, as seen in rickets, was well documented from as early as the 17th century (Glisson and Whistler, 1929), however it was the nutritional studies of Sir Edward Mellanby in 1919 that demonstrated rickets to be a disease resulting from the deficiency of vitamin D. The work by McCollum in 1922 showed that there was more than one fat-soluble vitamin and he clearly differentiated between vitamin A and vitamin D deficiency. This early work thus established the nutritional importance of the fat-soluble vitamin D.

A most important discovery was that ultraviolet light could correct vitamin D deficiency (Huldschinsky, 1919; Hess and Gutman, 1922). Steenbock and Black in 1924 demonstrated that vitamin D, either synthesised in vivo in the skin of rats or available in rat food, was ultimately produced by irradiation with UV light. Shortly thereafter (in the 1930's) the chemistry of vitamin D was analysed by researchers such as Windaus et al., 1936 who identified the sterols, vitamin D_2 in 1932 and vitamin D_3 in 1936.

Since then the field of vitamin D research had advanced in leaps and bounds, both in respect of the function and the biochemical metabolism of vitamin D.

1.3 Sources of vitamin D

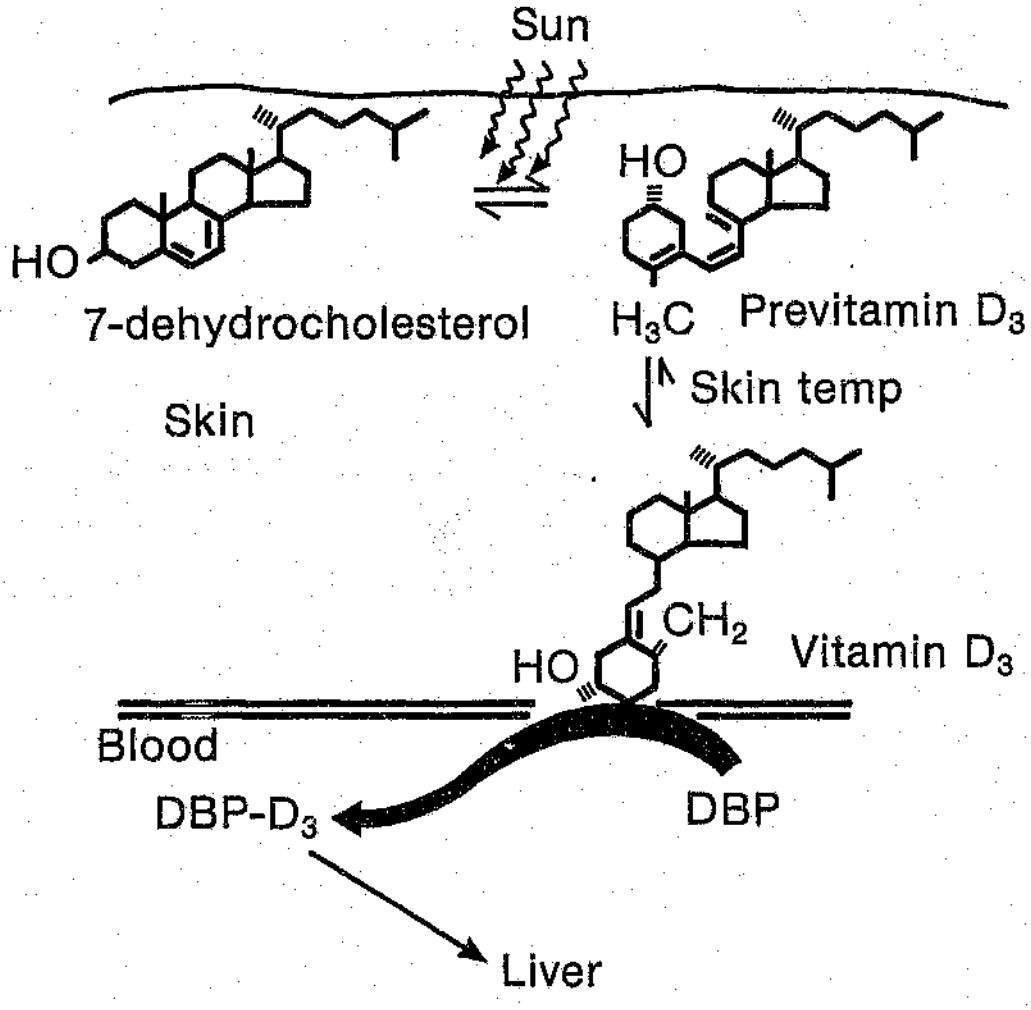
There are two major sources of vitamin D. In animals it may be endogenously produced in the skin in the presence of ultraviolet light or it may be acquired by dietary intake. Vitamin D_3 is the form naturally produced in the skin by the action of ultraviolet light. It may also be acquired from the ingestion of animal products. Vitamin D_2 is formed by the irradiation of the plant sterol (ergosterol) and this form may be ingested in the diet.

In man, both forms are usually present in the circulation though the concentration of $25(OH)D_3$ is generally substantially higher than $25(OH)D_2$. Higher serum concentrations of vitamin D_3 metabolites reflect the importance of skin synthesis in the maintenance of normal vitamin D status (Audran and Kumar, 1985; Horst et al., 1988; Reichel et al., 1990).

Vitamin D_3 is synthesised from 7-dehydrocholesterol, which is normally present in skin (Holick, 1984). When human skin is exposed to high energy ultraviolet photons, with energies between 290 and 315 nm, previtamin D_3 is formed from its precursor, provitamin D_3 (7-dehydrocholesterol). Previtamin D_3 is then thermally rearranged to form vitamin D_3 (Esvelt et al., 1978; Holick et al., 1979). This latter process takes place over a three day period (Figure 1).

Figure 1.

Photosynthesis of previtamin D_3 (Holick, 1981).


Sun
HO
HO
7-dehydrocholesterol
H_3C
Previtamin D_3
Skin temp
Skin
CH_2
HO
Vitamin D_3
Blood
DBP-D_3
DBP
Liver

The photolytic conversion of 7-dehydrocholesterol to vitamin D_3 occurs both in the epidermis and dermis in the ration of 80:20. With increasing age, vitamin D_3 production decreases in the epidermis with the resulting possible decrease in serum vitamin D_3 concentration. Other factors which influence the amount of vitamin D_3 produced include the duration and intensity of sun exposure, the wavelength and amount of ultraviolet light reaching the skin and the degree of skin pigmentation. Darker skin (increased melanin pigmentation) restricts vitamin D_3 production and a black person requires 20 times longer in the sun than a white person to achieve equivalent vitamin D_3 production (Holick et al., 1987).

Vitamin D_3 once synthesised is then transported away from the site of synthesis into the circulation bound to its specific carrier protein (Dueland et al., 1982). Previtamin D_3 has a low affinity for this protein and therefore remains behind in the skin.

Vitamin D_3 can be obtained by eating animal products (such as muscle and fat) however this source is usually inadequate to maintain a vitamin D replete state. Historically fish oils (e.g. cod, herring and sardines) have been acknowledged as an important source of vitamin D. Meat from all domestic mammals is also considered an importance source of vitamin D (Audran and Kuman, 1985).

In countries where adequate UV irradiation of the skin cannot be acquired, certain foods such as margarine or milk, have been fortified in an attempt to maintain an adequate intake of vitamin D.

In 1986 Ricardo Boland reported the presence of a vitamin D_3 like substance in plants in certain areas of Argentina and Brazil. It was initially found in the irradiated leaves only of species belonging to the family of Solanaceae, although recently it has been recorded in the leaves of a number of other plants including alfalfa (Boland, 1986). It appears that this form of vitamin D_3 can indeed be used by the animals, as animals ingesting the leaves develop hypercalcaemia.

Ergosterol, a plant sterol, can be converted to vitamin D_2 by exposure of UV light. It is not known whether D_2 is an important source of vitamin D in herbivores, but in man, vitamin D_2 generally appears to play a minor role in maintaining an adequate vitamin D status.

1.4 Transport of vitamin D and its metabolites in circulation

Dietary vitamin D is absorbed from the upper part of the gut in the same way as other fat soluble compounds (Dueland et al., 1982). It enters into the circulation through the thoracic duct in the chylomicron fraction of intestinal lymph (Dueland et al., 1982).

Like any other fat-soluble compound, only a small fraction of vitamin D and its metabolites circulate in the unbound state in plasma. Vitamin D and its metabolites are bound in the circulation to albumin or alternatively to a specific carrier protein (DBP), the vitamin D binding protein (Dueland et al., 1982; Smith and Goodman 1971; Haddad and Birge, 1975).

The specific DBP (also known as Gc-globulin) is a highly polymorphic serum glycoprotein synthesized by the liver. It is distributed in most tissues eg. liver, proximal gut, kidney, skeletal muscle and in lesser amounts in bone, brain and lung tissues (Harper et al., 1987). DBP is found in all animals with skeletons eg. great apes, new world monkeys, mouse, rat, horse, cow and dog (Hay and Watson, 1976 a and b) and is phylogenetically well conserved (Haddad, 1984; Bidmon and Stumpf, 1991).

D-binding protein has a strong homology with serum albumin and alpha-fetoprotein (Haddad, 1987), and has a higher molar concentration (5×10^{-6}M) in circulation than the major circulating metabolite, 25(OH)D (5×10^{-8}M). All the vitamin D metabolites are known to bind DBP in a ratio of one mole of metabolite/ mole of protein. The order of affinity of DBP for the sterols is thought to be the following:- 25(OH)D = $24,25(OH)_2D$ = $25,26(OH)_2D$ > $1,25(OH)_2D$ > vitamin D, with no distinction between D_2 or D_3 binding in man (Haddad and Cooke, 1989). In the physiological state, at any one time less than 5% of the binding sites of human DBP are occupied by vitamin D sterols. As 25(OH)D has the highest affinity and the greatest molar concentration of the vitamin D metabolites, it is the predominant form bound to DBP (Haddad, 1987).

Free metabolites, rather than those bound, are active in vivo (Bikle et al., 1985). The high affinity of DBP for the active metabolite is an important factor in determining the concentration of vitamin D metabolites available for use by the cells.

Human DBP has a molecular weight of 58 000 daltons while in the rat its molecular weight is slightly smaller at 52 000 daltons (Haddad, 1987). The DBP concentration in normal human serum samples is 422 ± 27 mg/L (Imawari and Goodman,1977; Bouillon et al., 1991). Human DBP has a half-life of 2.5 days which is significantly less than the 12 day half-life of 25(OH)D, and is produced at a rate of approximately 10 mg/kg/day. DBP is excreted in the urine after catabolism by proteases (Kumar, 1986).

Low serum levels of DBP occur in premature infants and in patients with liver disease, and nephrotic syndrome while an increase is seen in pregnant women and those receiving oestrogen therapy. The levels of DBP remain normal in a number of situations such as vitamin D deficiency, vitamin D intoxication, hyperparathyroidism, sarcoidosis, and hypophosphataemic rickets. Thus there appears to be no regulation of DBP by vitamin D or its sterols.

1.5 Vitamin D metabolism

Vitamin D is considered a prohormone in that it has no biological effect and exerts its actions only after undergoing further metabolism (DeLuca, 1984; Kumar, 1986). To date, more than 35 metabolites of vitamin D have been identified. The functional significance of most of these is unknown (Ikekawa, 1987; Henry and Norman, 1990).

The body is able to store vitamin D for relatively long period of time (Heymann, 1937; Rosenstreich et al., 1971). Body fat is the major storage site, although muscle, skin and

bone and liver are also important sites of storage (Mawer et al., 1972; Rosenstrich et al., 1971).

1.5.1 25-Hydroxylation of vitamin D in the liver

Activation of the prohormone vitamin D requires a series of hydroxylations. The first of these takes place primarily in the liver, although some hydroxylation may c cur in other tissues e.g. lung, kidney and intestine (Ichikawa et al., 1983). Vitamin D is hydroxylated at the C-25 position to 25-hydroxyvitamin D (25(OH)D) (Blunt et al., 1968) by the 25-hydroxylase enzyme. This enzyme is located in the microsomal and mitochondrial fractions of hepatocytes (Ponchon and DeLuca, 1969). The hepatic 25-hydroxylase enzyme, a cytochrome P-450, requires NADFH for its action. The reaction is inhibited by the absence of oxygen and the presence of carbon monoxide. The 25-hydroxyl oxygen atom is derived from molecular oxygen (Fraser, 1980).

25-hydroxyvitamin D is the major circulating vitamin D metabolite in blood. It has a relatively long half-life of about 5 days in humans. In physiological concentrations 25(OH)D is biologically inactive. Its metabolic pathways are unclear, but significant amounts are excreted in bile (Arnaud et al., 1984). Ponchon and DeLuca (1969) showed that in rats [^{3}H]-vitamin D_3 was taken up by the liver within 30 minutes of administration and that there was a dramatic increase in plasma [^{3}H]-25(OH)D_3 over the next 2 hours (Ponchon and DeLuca, 1969).

Although there is no evidence that the hydroxylation of vitamin D is controlled by serum levels of 25(OH)D, hydroxylation of vitamin D may be controlled by the serum concentration of $1,25(OH)_2D$ (Reichel et al., 1987). Bell et al. (1984), showed that the ingestion of large amounts of vitamin D_3 increased the concentration of $25(OH)D_3$ but when $1,25(OH)_2D_3$ and vitamin D_3 was administered together, there was no increase in $25(OH)D_3$ concentration. Serum calcium both directly and indirectly may also regulate various steps in vitamin D metabolism.

Clements et al. (1987) demonstrated a reduction in the half-life of 25(OH)D, in rats on a calcium deficient diet. Furthermore, addition of $1,25(OH)_2D$ to calcium replete rats produced a similar reduction in 25(OH)D half-life. These studies suggest that $1,25(OH)_2D$ leads to an increase in hepatic catabolism of 25(OH)D and a fall in circulating 25(OH)D.

1.5.2 Hydroxylation of $25(OH)D_3$ in the kidney

Further hydroxylation of $25(OH)D_3$ takes place primarily in the kidney. Two metabolizing enzymes, either 25(OH)D-1α-hydroxylase or 25(OH)D-24R-hydroxylase are involved (Kawashima and Kurokawa, 1983). The activities of both enzymes are very tightly controlled by ionic and hormonal balance. There is a reciprocal change in the activities of the two enzymes. During hypocalcaemia, hypophosphataemia and low vitamin D status, 25(OH)D-24-hydroxylase is completely absent (Fraser, 1980) and after $1,25(OH)_2D_3$ administration, the 24-hydroxylase activity increases (Kawashima and Kurokawa, 1983).

Both hydroxylating enzymes require the presence of oxygen for their activity. Oxygen is incorporated at either the C1 or C24 position on the sterosteroid molecule. Both enzymes are cytochrome P-450-dependent mono-oxygenases. These enzymes have similar biochemical properties to typical steroid hydroxylases, present in adrenal glands, testes and ovaries (Henry and Norman, 1984).

The activity of the 1α-hydroxylase is localized to the proximal convoluted and proximal straight tubules. Parathyroid hormone (PTH) stimulates adenylate cyclase and increases 1α-hydroxylase activity specifically in the proximal convoluted tubules, while calcitonin is responsible for its activation in the straight tubules (Zelikovic and Chesney, 1989). The mechanisms by which alterations in serum inorganic phosphorus concentration control the 1α-hydroxylase enzyme are unclear. Although hypocalcaemia stimulates 1α-hydroxylase activity through its stimulation of PTH secretion, there is evidence that alterations in serum calcium concentrations directly effect the activity of the 1α-hydroxylase enzyme.

The major metabolite formed from $25(OH)D_3$ while in a normo-to hypercalcaemic state is $24,25(OH)_2D_3$ (DeLuca, 1984). The functions of this metabolite are not known, although it is thought to be inactive in adult man (Boyle et al., 1973; Lam et al., 1973; Holick, 1984). Production of $24,25(OH)_2D_3$ appears to be a mechanism of regulating $1,25(OH)_2D_3$ and its production is stimulated by an increase in the serum concentration of $1,25(OH)_2D_3$. The kidney is the main site of $24,25(OH)_2D_3$ synthesis but it may also be produced in intestine, keratinocytes, cartilage and bone cell cultures (Knutson and DeLuca, 1974; Kumar et al., 1978).

1.6 Regulation of $1,25(OH)_2D_3$ production

The metabolite, 1,25-dihydroxyvitamin D, is the active form of vitamin D regulating many biological activities including gastro intestinal tract (G.I.T.) absorption of calcium, phosphate and magnesium, as well as the renal reabsorption of these minerals.

Serum levels of $1,25(OH)_2D_3$ are tightly regulated. Production of $1,25(OH)_2D_3$ is controlled primarily by the calcium requirements of the individual. The principal regulators of $1,25(OH)_2D_3$ production are PTH, $1,25(OH)_2D_3$ itself, and serum calcium and phosphorus concentrations (Fraser, 1980; Henry and Norman, 1984; Clements, 1987).

Parathyroid hormone (PTH) has both a direct and indirect effect on 1α-hydroxylase activity (Kronenberg, 1990). In its own right, PTH promotes tubular calcium reabsorption (Norman and Litwack, 1987) and the excretion of phosphate, which leads to changes in the serum concentrations of these minerals. Furthermore PTH promotes mineral mobilization from bone increasing serum calcium concentrations. Changes in serum mineral concentration in turn may lead to an alteration in $1,25(OH)_2D_3$ production. Besides stimulating 1α-hydroxylase activity, PTH inhibits 24-hydroxylase activity.

Serum calcium both directly and indirectly regulates 1α-hydroxylase activity. Indirect regulation occurs in response to hypocalcaemia stimulating PTH release which in turn promotes $1,25(OH)_2D_3$ synthesis (Carpenter, 1989). There is also evidence that

$1,25(OH)_2D$ synthesis might be directly stimulated by the effect of low serum calcium concentrates on the renal tubular cells.

Dietary phosphate intake modulates $1,25(OH)_2D_3$ renal production (Tanaka and DeLuca, 1973). Hypophosphataemia directly increases $25(OH)D_3$-1α-hydroxylase activity by PTH independent mechanisms. Low phosphorus concentrations increase the production of $1,25(OH)_2D_3$ and not the clearance of $1,25(OH)_2D_3$, thereby increasing the absolute amounts of the circulating $1,25(OH)_2D_3$ (Portale et al., 1984; Pettifor, 1990). Thus a decline in serum phosphate concentration produces the same effect as an increase in PTH, although the magnitude of the phosphate related effect is not as great (Norman and Litwack, 1987). Increases in serum phosphate oppose the actions of PTH. Phosphate increases 24-hydroxylase activity and decreases that of 1α-hydroxylase. Hyperphosphataemia, through its action on 1α-hydroxylase, decreases the amount of $1,25(OH)_2D_3$ being produced, and thus the absorption of intestinal phosphate resulting in a decrease in serum levels of phosphate.

$1,25(OH)_2D_3$ itself, is another modulator of vitamin D metabolism. It decreases 1α-hydroxylase activity and promotes 24-hydroxylase activity. These actions of $1,25(OH)_2D_3$ appear to result from changes in enzyme biosynthesis rather than allosteric end product inhibition (Norman and Litwack, 1987).

Other hormones e.g. oestrogen (Tanaka et al., 1976), prolactin (Zofkova et al., 1991), insulin (Kadowacki and Norman, 1984), growth hormone, and somatomedins (Dusso et al., 1988) stimulate 1α-hydroxylase, while heavy metals inhibit 1α-hydroxylase activity but it is unlikely that these are important regulators (Halloran et al., 1986).

1.7 Actions of $1,25(OH)_2D_3$

Over the last ten years, $1,25(OH)_2D_3$ has emerged as one of the most important regulators of cellular functions related to the maintenance of mineral homeostasis (Norman, 1990; Tsoukas et al., 1984), the differentiation of hematopoietic and immune cells (Manoglesdorf et al., 1984; Reichel et al., 1987) and the modulation of DNA replication and cell differentiation and proliferation (Haussler et al., 1988).

The steroid hormone, $1,25(OH)_2D_3$, is generally considered to exert its major effects by mechanisms that parallel classical sex steroid hormones. These mechanisms typically involve the binding of the hormone to specific intracellular receptors. The vitamin D receptor (a 48KD protein) has a ubiquitous distribution, found in all vertebrate phylla and in many invertebrates (Bidmon and Stumpf, 1991). All tissues studied to date in mammals possess this receptor (Stumpf et al., 1979; Bidmon and Stumpf, 1991; Minghetti and Norman, 1988). The consequent interaction of the hormone receptor complex with the nucleus thus promotes the expression by protein synthesis of specific target genes (Haussler et al., 1988; Minghetti and Norman, 1988).

Genomic actions are primarily involved in the classical functions of vitamin D and involve the formation of specific proteins facilitating the desired action (e.g. calcium binding proteins) (Nemere et al., 1986). In the nucleus, $1,25(OH)_2D_3$ increases chromatin template activity and DNA dependent RNA polymerase II activity which increases the synthesis of messenger RNA, for example, for calcium binding protein (CaBP).

Vitamin D may also exert its actions through non-genomic means (Nemere et al., 1986). Non-genomic actions are more rapid (as they do not involve protein synthesis) and are implicated in signal transduction, cell signalling and cell buffering (Reichel et al., 1987).

1.8 Vitamin D metabolism in animals

In all vertebrates studied to date, and also in some invertebrates, vitamin D and/or its metabolites are present in one form or another (Hay and Watson, 1976 a and b; Horst et al., 1988; Henry and Norman, 1984; Kobayashi et al., 1991).

In most mammals $25(OH)D_3$ is the principal circulating form and $1,25(OH)_2D_3$ the most active metabolite. The ratio of vitamin D_2/D_3 metabolites reflects the habitat and diet of animals as well as the rate of metabolism of the two vitamin D analogues.

The vitamin D status of a wide spectrum of animals has been examined and manipulated. In a comprehensive study undertaken by Horst et al. (1981) vitamin D and

its major metabolites were quantitatively measured in five species of domestic animals. These results are shown in Table 1. A number of differences were found in the vitamin D status of these five farm animals. All five species had detectable levels of vitamin D_3 and its more polar metabolites. Only sheep had detectable levels of vitamin D_2 although all other species had the more polar metabolite, $25(OH)D_2$, present albeit in much lower concentrations than in sheep. The higher levels of vitamin D_2 in sheep might reflect the higher intake of D_2 due to the ingestion of large amounts of UV-irradiated grass and the relative absence of vitamin D_3 formation in the skin due to the presence of a thick coating of wool.

Most animals (Horst et al., 1981; Hay and Watson, 1977) discriminate against vitamin D_2 in favour of vitamin D_3. The rat is however, the exception to the rule in that it preferentially uses vitamin D_2. It appears that this discrimination reflects differential metabolism of vitamin D. In both pigs and chicks the discrimination is quantitatively similar, however in the pig the discrimination is at the 25-hydroxylase level, whereas that of the chick occurs before 25(OH)D production (Horst et al., 1981).

The most frequently used experimental animal in vitamin D research is the chick. Its diet can be controlled as soon as the chick has hatched, thus making it easy to deplete this bird of its vitamin D reserves within a few weeks. Hughes et al. (1977) showed that the plasma concentrations of $25(OH)D_3$ in chicks decreased from 14.1 ng/ml at day 1 to undetectable levels within 3 weeks if the chicks were maintained on a vitamin D deficient diet. Their levels of $25(OH)D_3$ could be maintained at between 21-35 ng/ml and those of $1,25(OH)_2D_3$ between 51-75 pg/ml if a supplement of 1.4 IU/g of diet was

Table 1

Vitamin D_2 and D_3 and their metabolites in five species of animals

(from Horst et al., 1981)

	SPECIES				
	turkey	chicken	cow	sheep	pig
vit D_2* (ng/ml)	nd	nd	nd	0.52±0.35	nd
vit D_3 (ng/ml)	3.4±0.2	2.3±0.3	1.7±0.7	0.6±0.6	10.2±4.1
25(OH)D_2 (ng/ml)	0.8±0.7	1.6±1.5	5.8±1.7	13.9±3.7	0.8±0.3
25(OH)D_3 (mg/ml)	18.2±7.9	25.3±3.9	36.9±7.1	12.9±5.3	74.9±21.2
24,25$(OH)_2D_2$** (ng/ml)	nd	nd	0.7±0.2	6.4±1.0	nd
24,25$(OH)_2D_3$ (ng/ml)	1.2±0.1	2.3±0.2	2.6±1.3	4.4±0.7	20.2±10.3
25,26$(OH)_2D_2$** (ng/ml)	nd	nd	nd	0.5±0.2	nd
25,26$(OH)_2D_3$ (ng/ml)	0.6±0.4	1.1±0.2	2.5±1.0	1.0±0.3	4.1±0.3
25(OH)D_3-*** 26,23-lactone (ng/ml)	nd	1.5±0.5	nd	nd	2.0±1.2
1,25$(OH)_2D$ (pg/ml)	51.8±31.3	21.2±2.1	38.0±10.4	35.9±30.0	60.3±7.2

* nd (not detectable) = < 0.2 ng/ml.
** nd = < 0.2 ng/ml.
*** nd = < 0.1 ng/ml.

given. With a 50 fold increase in dietary supplementation, the levels of $25(OH)D_3$ increased to 87-100 ng/ml while the $1,25(OH)_2D_3$ remained at similar concentrations to that obtained with a 1.4 IU/g supplement. Similarly, in rats $25(OH)D_3$ concentration increased with supraphysiological vitamin D supplementation, but $1,25(OH)_2D_3$ levels fell significantly as vitamin D supplementation increased from 2 to 1000 IU/g diet. Similar results have been shown in mole rats (*Cryptomys damarensis*) (Buffenstein et al., 1991).

The hooded seal, a mammal which ingests large amounts of fish, showed no evidence of vitamin D toxicity despite the high vitamin D intake in its diet (Keiver et al., 1988). In studies using radio-isotopes, the uptake of [^{3}H]-vitamin D_3 resulted in an increase in [^{3}H]-$25(OH)D_3$ and [^{3}H]-$24,25(OH)_2D_3$ concentrations with no detectable increase in [^{3}H]-$1,25(OH)_2D_3$. The plasma level of the $1,25(OH)_2D_3$ in the seal was maintained at a similar level to those of other mammals and showed no increase even after vitamin D supplementation. The levels of $24,25(OH)_2D_3$ in both wild and supplemented seals were similar, but higher (4-33 ng/ml) than those reported in other mammals, and the metabolism of $25(OH)D_3$ to $24,25(OH)_2D_3$ was increased. The increase in vitamin D storage by seals in their large amounts of blubber seemed to protect these animals against vitamin D toxicity.

In most vertebrates studied to date, 25-OHD is the major circulating metabolite of vitamin D and is detectable using radio-receptor assays (Table 1) (Norman et al., 1982). However recent studies of fish, horses and mole rats have documented that this generalization does not always hold true (Takeuchi et al., 1991; Kobayashi et al. 1991;

Buffenstein et al., 1991). In these species, serum concentrations of 25(OH)D are undetectable yet $1,25(OH)_2D_3$ is present. Further studies using telecosts and cartilagenous fish reveal an absence of DBP and suggest that the active metabolite binds to other lipoproteins (Takeuchi et al., 1991; Kobayashi et al., 1991). The lower affinity binding to lipoproteins might maintain relatively high free metabolite concentrations.

Some invertebrates are also able to metabolise vitamin D_3. For instance the land snail is able to metabolise [^{3}H]-vitamin D_3 to more polar metabolites (Weiner et al., 1979). More recently [^{3}H]-$25(OH)D_3$ was shown to undergo metabolism to an even more polar metabolite in these animals; the functional importance of this metabolite is as yet unknown.

The mole-rat which lives in a vitamin D free environment on a herbivorous diet, appears to be naturally deficient in vitamin D, yet metabolises vitamin D_3 to $25(OH)D_3$ and the more polar metabolite, $1,25(OH)_2D_3$ (Buffenstein et al., 1991). This unusual mammal exhibits measurable levels of $1,25(OH)_2D$ (16.1 ± 1.98 pg/ml) in association with undetectable levels of $25(OH)D_3$ (< 4 ng/ml). Both mole rats and horses, have similar patterns of vitamin D metabolites (no 25(OH)D, yet detectable but low $1,25(OH)_2D_3$) and are thought to employ vitamin D independent modes of gastrointestinal mineral absorption.

Fruit bats (*Rousettus aegyptiacus*) are also thought to have no access to any obvious source of vitamin D for these animals are nocturnal, flying frugivores. Therefore this

fascinating mammal's vitamin D status and metabolism in both the absence of D_3 and with D_3 supplementation was investigated.

1.9 Fruit bat biology

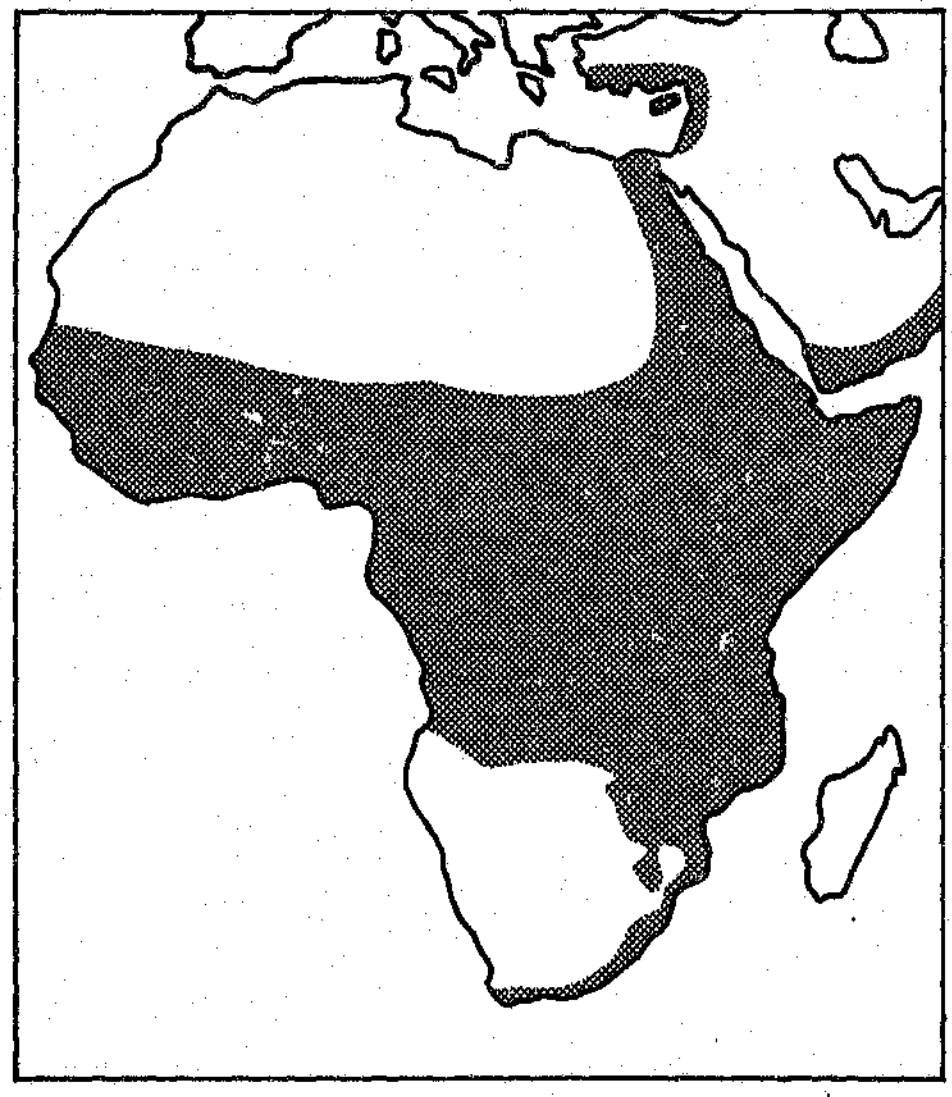
The Egyptian fruit bat (*Rousettus aegyptiacus*), (E. Geoffroy, 1810), derived its name after first being described from a specimen seen at the pyramids of Gaza in Egypt (Smithers, 1983). This bat is the second largest bat found in Africa and is gregarious, living in colonies of thousands in caves where it roosts during the day. At night it feeds on fruit.

These animals are widely distributed through Africa (Figure 2) from the southern Cape Province up through the Transvaal of South Africa, along the easter border up to Egypt and transversally across the central part of the continent (Smithers, 1983). The fruit bat is also found in parts of Pakistan and some Arab countries.

The adult animal has a mean weight of about 130 g, an approximate length of 15 cm and full flight wing span in the region of 60 cm. There is little sexual dimorphism, with both sexes having similar mass, size and colour. Skin colour can vary from dark brown to a light grey on their underbodies. Their eyes and ears are rather large for the body size and the face has an almost doglike appearance (Figure 3).

Figure 2

The distribution of the fruit bat (*R. aegyptiacus*)



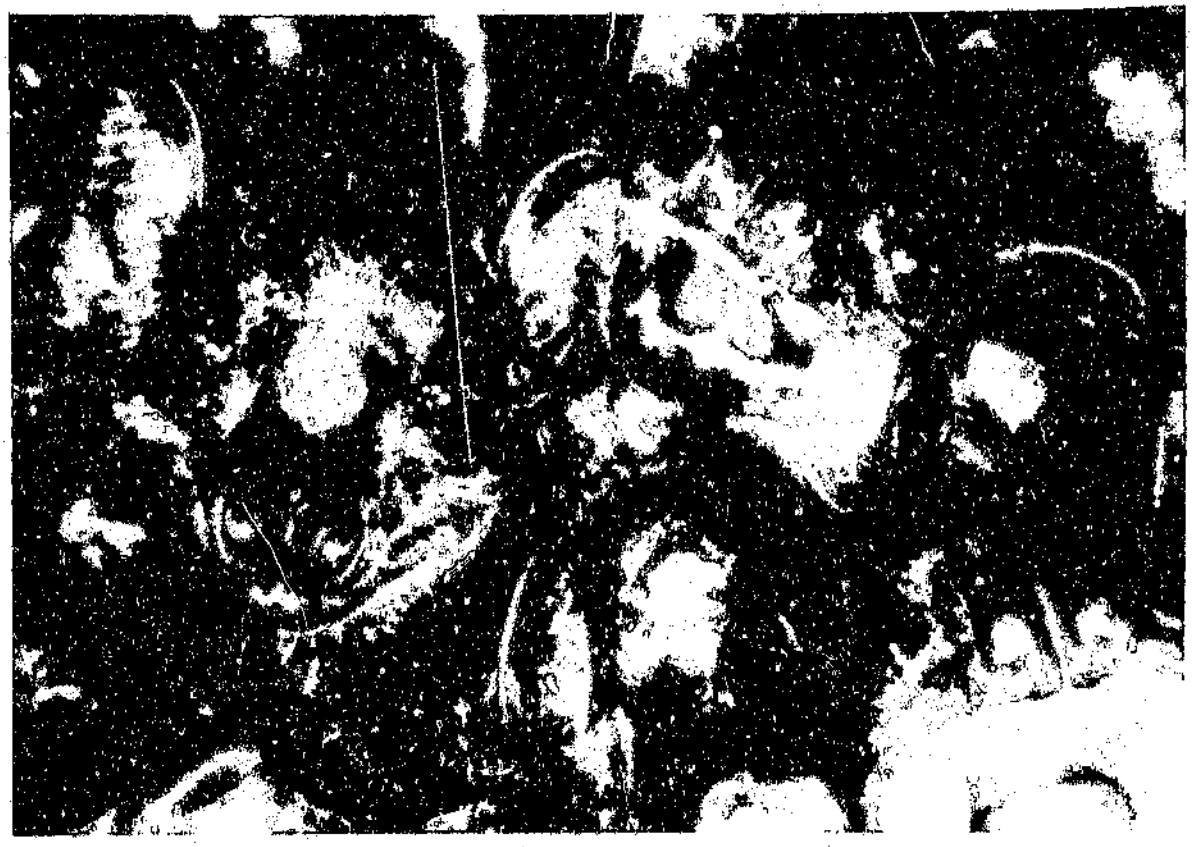
The female bat usually produces only one young after the mating season which occurs in early spring (June-September). During this time the males tend to group on their own and the female and young bats are separate, almost in a nursery fashion. The gestation period is about 106 days. At birth the young have closed eyes and flat ears, but within 10 days the eyes open and the ears lift up. The young are suckled for approximately six weeks during which time the mothers are often seen flying with the pup attached to her underbelly. The young bats will begin to fly at an age of between nine to ten weeks.

The fruit bat has a well established sense of smell, which is important in its nightly search for food. The large well developed eyes are more useful when looking for fruit at close range. This particular bat is the only one which has an echolocation system, thus allowing it free movement in total darkness. This system is totally different to that used by other bat species (Smithers, 1983). These animals are agile fast flyers and are able to negotiate narrow openings between the branches of trees with complete ease.

The fruit bat circles a suitable tree which has fruit on it from which it can feed and then settles on the branches. It will either use one of its feet to bring the fruit to its mouth, while hanging with the other or else will hang onto a branch with both its thumb claws and hold the fruit against the chest with both feet. Wild figs are by far the most appealing fruit to the bat. As the fruit begins to ripen, squabbles often occur as groups of bats feed in the same tree. The skins and seeds from the fruit are discarded and only the soft inner pulp is eaten. This pulp is not exposed to ultraviolet irradiation and is thus thought not to contain vitamin D_2 or vitamin D_3 in substantial quantities.

Figure 3.

The fruit bat (*Rousettus aegyptiacus*).



In Southern Africa figs (*Ficus capensis, Ficus petensii and Ficus sansibarica*) form the basis of their diet. These animals also eat the fruit off the Cape ash tree (*Kebergia capensis*), Saffronwood (*Cassine crocea*), Bushmans poison (*Acokanthera oppositifolia*) and Mistletoe (*Viscum obscarum*); soft garden and orchard fruits (e.g. pawpaw and bananas) are also eaten. Egyptian fruit bats have cheek pouches in which food can be temporarily stored and consumed later at their roost.

Grooming is done once feeding has been completed. The hair on the chest of the animals becomes matted with fruit juices and the bat uses the claws on its feet to comb out the hairs (Smithers, 1983), while the toes are cleaned thereafter in the mouth.

Being nocturnal and frugivorous, bats have no obvious access to vitamin D. Free living bats appear to suffer no ill effects attributed to vitamin D_3 deficiency, for they thrive in their chosen habitats.

A number of captive fruit bats used in vitamin B_{12} research, presented with signs of clinical vitamin D deficiency i.e. broken wing bones. We were therefore asked to investigate whether the animals had evidence of vitamin D deficiency. In initial blood samples which were analysed, 25(OH)D could not be detect .l (< 4 ng/ml). The subsequent measurement of $1,25(OH)_2D$ in bat serum samples indicated that this metabolite was present, and the whole question as to the vitamin D status, metabolism and transport in this fruit eating nocturnal mammal, *Rousettus aegyptiacus* was investigated further.

CHAPTER 2

The care and housing of fruit bats

2.1 Introduction

As the fruit bat, *Rousettus aegyptiacus*, has no apparent source of vitamin D, a number of experiments were designed to investigate the fruit bat's vitamin D status and to assess whether or not this animal has the ability to metabolise and transport vitamin D. In this regard, five major experiments were undertaken:

1. The measurement of circulating vitamin D metabolites.
2. An assessment of the in vivo conversion of vitamin D to 25(OH)D and following that the metabolism of 25(OH)D to the active hormone $1,25(OH)_2D$.
3. An in vitro study to determine the presence of the 25- hydroxyvitamin D 1α-hydroxylase enzyme in the kidney.
4. An investigation to determine whether or not the fruit bat has a specific vitamin D binding protein (DBP) for the transportation of vitamin D and its metabolites in blood.
5. An investigation into whether vitamin D_2 or D_3 is the major form of vitamin D in bats in the wild.

2.2 Methods

2.2.1 Housing and the care of experimental animals

A total of 81 bats were used for the various studies. The bats were divided into three groups:-

1. vitamin D supplemented animals (n=33),
2. animals fed on a vitamin D deficient diet (n=38)
3. wild animals studied immediately after capture (n=10)

Table 2: The number of bats used in the various experiments

Fruit bats 81	wild (n-10)	10	-	vitamin D metabolites
	vitamin D- (n=38)	22	-	vitamin D metabolites
		5	-	[^{3}H]-vitamin D_3 in vivo
		9	-	[^{3}H]-25(OH)D_3 in vivo
		2	-	kidneys
	vitamin D+ (n=33)	7	-	vitamin D metabolites
		26	-	purification of DBP

2.2.1.1 Captive animals

Captive fruit bats were cared for in the animal unit of the South African Institute for Medical Research (S.A.I.M.R.) Johannesburg, South Africa.

They were housed in a large room in which the windows had been completely blackened out. The sole source of light was a single incandescent light bulb which was switched on only while the animal unit staff entered the room for cleaning and feeding purposes.

The fruit bats' diet in captivity usually consisted of washed or peeled fruit, such as bananas, papaya (pawpaw) and apples. Occasionally unpeeled washed oranges were also fed to the animals. Each bat was allocated 150g of fruit per day and this was either increased or decreased according to their appetite. The food allocated per bat was more than sufficient to meet the animals nutritional requirements. A favourite fruit of these animals is wild figs which when available were included in their diet. The fruit was placed into the animals' enclosure daily in the early afternoon.

The water intake of the fruit bat is minimal, with the major source of water being from their intake of fruit. A shallow water trough was supplied at all times in the room even though it was not always used by the animals.

Bats maintained on both the vitamin D replete and deficient diets were injected with a vitamin B_{12} supplement (0.5 mg cyanocobalamin/100g body weight) every 14 days, to prevent vitamin B_{12} deficiency resulting from a peeled fruit diet (van der Westhuizen . al., 1982).

2.2.1.1.1 Vitamin D depleted bats

The bats required for the vitamin D deplete experiments were maintained in a dark room and fed on a vitamin D deficient diet described above for a minimum of one year prior to being used in the experiments.

The vitamin D deficient group were divided into four subgroups:

1. animals whose blood was collected to measure vitamin D metabolites (n=22);
2. bats used in the in vivo [^{3}H]-vitamin D_3 study (n=5);
3. bats used in the in vivo [^{3}H]-25(OH)D_3 study (n=9); and
4. bats whose kidneys were used in the in vitro 1-hydroxylase study (Table 2) (n=2).

2.2.1.1.2 Vitamin D supplemented bats

The bats required for the vitamin D replete studies were supplemented orally with a commercially available paediatric multivitamin syrup, [illegible] (Parke-Davis), which contained 100 IU vitamin D, 0.25 mg thiamin, 0.1 mg riboflavin, 0.12 mg vitamin B_6, 0.4 mg vitamin A, 0.12 mg niacin and 12.5 mg ascorbic acid per 150 $\mu\ell$.

The animals in the vitamin D supplemented group (n=33) were sub-divided into two further subgroups:

1. to measure circulating vitamin D metabolites (n=7);
2. to purify the vitamin D binding protein (n=26).

2.2.2 Wild caught bats

The wild bats used in the study were caught on the farm "Halfned Heights" at Matlapitsi in the Lebowa area, in the North Eastern region of the Transvaal. Nets were placed across the entrance of the roosting cave and the animals were caught on their return to the cave just before sunrise. Wild bats (n=10) were bled from the wing vein within 6 hours of their capture to determine vitamin D metabolite levels under natural environmental conditions.

The weights of vitamin D deficient and vitamin D deplete captive fruit bats used in this study were similar (Table 3, page 61) (vitamin D supplemented bats, 121.7g ± 14.1 (n=17); vitamin D deficient bats, 118.9g ± 16.9 (n=7)). Newly caught wild bats had a greater average weight, 135.6g ± 8.1 (n=10) (Table 3).

2.2.3 Collection of blood samples

Blood samples were collected from the wing vein of animals, by holding the bats down unanaesthetised and spreading the wing. A maximum of 1 ml of blood was collected into a heparinized syringe using a fine gauge needle (no. 25). Cardiac exsanguination was used to kill the animals, where necessary. As much blood as possible (± 6ml) was collected. The blood samples were kept on ice and spun as soon as possible at 3 000 rpm in a Beckman TJ6R for 15 minutes. Plasma was separated from the red blood cells and transferred into a microfuge storage tube. If the samples were not used immediately they were stored at -20°C until required.

2.2.4 Measurement of serum calcium, inorganic phosphorus, magnesium and alkaline phosphatase concentrations

Serum calcium, inorganic phosphorus, magnesium and alkaline phosphatase concentrations were measured by Mr G. Moodley in the MRC Mineral Metabolism Research Unit, Baragwanath Hospital, Johannesburg. The measurement of calcium and magnesium were performed by atomic absorption spectrophotometry on a Varian Techtron 1200, after diluting the samples in a lanthanum chloride solution (1% La). Inorganic phosphorus concentrations were assayed following the colorimetric methods described by Hurst (1967) and Kraml (1966) and commercially available from Technicon (Timepac Inorganic Phosphorus Reagent - Technicon No T40-0012). Plasma alkaline phosphatase was assayed colorimetrically following the method of Morgenstern, et al. (1965) and commercially available from Technicon (No T40-0009). A Technicon Auto Analyser was used for these measurements.

CHAPTER 3

Serum concentrations of vitamin D metabolites in the fruit bat

3.1 Methodology

3.1.1 The competitive protein binding assays to measure serum levels of 25(OH)D and 1,25$(OH)_2$D

As serum samples are not suitable for the direct assessment of the vitamin D metabolites due to the interference of various metabolites in serum, a reliable extraction and chromatographic procedure was necessary prior to their measurement by competitive protein binding assays. The methods used were modified from those of Haddad and Chyu (1971) and Reinhardt et al. (1982 and 1984).

The techniques described in section 3.1.1.1 to section 3.1.1.3 were used to purify and measure the circulating concentrations of 25(OH)D and 1,25$(OH)_2$D in serum samples collected from fruit bats.

3.1.1.1 Extraction and chromatography of serum samples

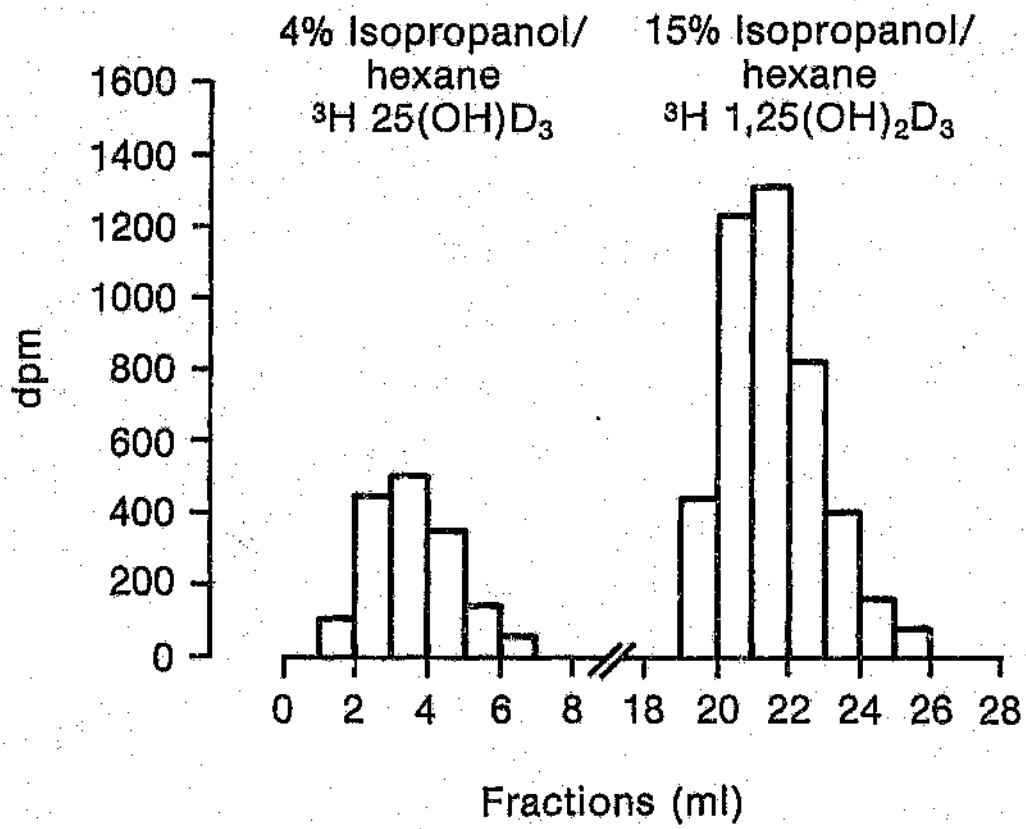
In order to assess recovery of vitamin D metabolites during the extraction procedure, 1000 cpm of [^{3}H]-25$(OH)D_3$ (3 pg, 160 Ci/mmol, Amersham) in 20 μl of ethanol and 1000 cpm of [^{3}H]-1,25$(OH)_2D_3$ (2.5 fg, 170 Ci/mmol) in 20 μl ethanol were added to a 500 μl aliquot of serum pipetted into a glass tube. The mixture was vortexed and allowed to stand at room temperature for 10 minutes.

The extraction and chromatography techniques used were those of Turnbull et al. (1982). All the solvents were either HPLC grade or redistilled prior to being used. Serum proteins were precipitated by the addition of acetonitrile (1 ml) (Riedel-de Haen, Holpro, South Africa), followed by vigorous vortexing of the tube. This was then centrifuged in a Beckman TJ6R benchtop centrifuge at 3000 rpm for 10 minutes to pellet the precipitated proteins. The supernatant which contained the extracted lipids plus the vitamin D metabolites was transferred to a tube containing 500 µl 0.4M K_2HPO_4 (pH 10.4) and vortexed. The mixture was loaded onto a C18 Sep-pak column (Waters, USA) which had been prewashed with 5 ml of each of the following solvents: hexane (Holpro), chloroform, methanol and finally distilled water. After loading the mixture onto the column, it was washed in the following sequence with 5 ml water, 3 ml methanol:water (7:3) and 3 ml acetonitrile, the latter fraction containing the eluted vitamin D metabolites was collected in a separate tube. The acetonitrile was evaporated off under a steady stream of nitrogen in a waterbath at 37°C, and the inside of the tube was washed down three times with approximately 0.25 ml hexane so as to concentrate all the vitamin D metabolites to the bottom of the tube, and the contents evaporated to dryness.

The vitamin D metabolites were redissolved in 0.25 ml 4% isopropanol in hexane and applied to a silica gel Sep-pak cartridge (Waters, USA), which had been prewashed sequentially with 5 ml of each of the following solvents: methanol, chloroform, hexane and 4% isopropanol in hexane. The sample tube was washed twice with 0.25 ml 4% isopropanol in hexane and each wash applied to the column. The 25(OH)D fraction, which eluted off the column with 10 ml 4% isopropanol in hexane (Figure 4) was

Figure 4.

The Silica Sep-pak cartridge was used to separate the vitamin D metabolites prior to their measurement in individual receptor binding assays. 25(OH)D eluted in fractions 2-7 and $1,25(OH)_2D$ eluted in fractions 20-26.


4% Isopropanol/
hexane
^{3}H 25(OH)D$_3$
15% Isopropanol/
hexane
^{3}H 1,25(OH)$_2$D$_3$
dpm
1600
1400
1200
1000
800
600
400
200
0
0 2 4 6 8 18 20 22 24 26 28
Fractions (ml)

evaporated to dryness and concentrated down under nitrogen in a waterbath. This 25(OH)D fraction was then stored in 0.5 ml hexane at -20°C until required for use in the binding assay.

The Sep-pak cartridge was then washed with 8 ml 6% isopropanol in hexane which eluted the $24,25(OH)_2D$ fraction which was discarded. Finally the cartridge was washed with 10 ml 15% isopropanol in hexane which eluted $1,25(OH)_2D$ (Figure 4). This fraction was placed in a waterbath at 37°C and the solvent evaporated off under nitrogen. Finally the metabolite was concentrated as described above and stored in 250 μl hexane at -20°C until required. The two fractions, 25(OH)D and $1,25(OH)_2D$ were now ready to be quantitatively assayed.

The recovery of 25(OH)D and $1,25(OH)_2D$ during the extraction and chromatographic steps were assessed using authentic (cold) and radiolabelled vitamin D metabolites as markers. As described above, known amounts of [^{3}H]-$25(OH)D_3$ and [^{3}H]-$1,25(OH)_2D_3$ were added to normal serum samples, the sera were extracted as described above and an aliquot of the acetonitrile supernatant assessed for tritium. In separate experiments authentic $25(OH)D_3$ and $1,25(OH)_2D_3$ were added to serum samples in physiological concentrations, the recovery of added metabolites were measured by the appropriate receptor binding assay after extraction and chromatography.

3.1.1.2 The rat kidney receptor assay for the measurement of serum 25(OH)D concentrations

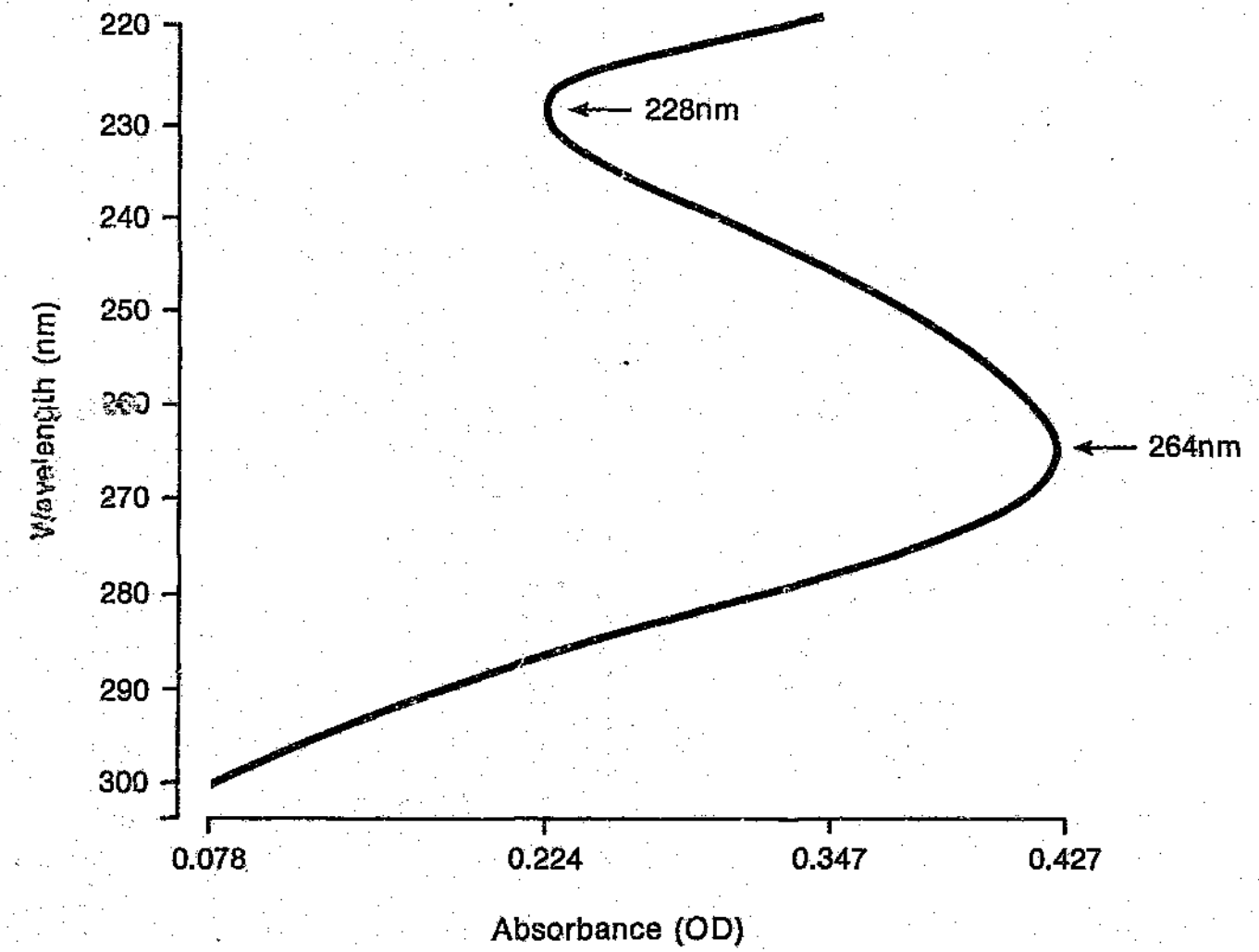
The 25(OH)D receptor was prepared and used as described by Haddad and Chyu (1971). Adult Sprague Dawley rats used for the kidney receptor preparation were sacrificed by cervical dislocation.

The kidneys were removed immediately, and were decapsulated and stored whole at -20°C prior to use. Kidneys (10-15g) were thawed, minced and homogenized in a Polytron in 40 ml ice cold 0.05M sodium phosphate buffer (pH 7.4), (Na_2HPO_4, 0.05M/L and NaH_2PO_4, 0.05M/L which were mixed in a ratio of 4 parts to 1 part) and centrifuged at 3 000 rpm for 10 minutes at 4°C. The supernatant was decanted and kept, while the pellet was re-suspended in another 40 ml sodium phosphate buffer and centrifuged as above. The two supernatants were combined and buffer added to a final weight (original kidney tissue): volume ratio of 1:10. This mixture was centrifuged at 4°C for 90 minutes at 18 000 rpm (HA-20.1 rotor; J2-21 Beckman ultracentrifuge). The supernatant was carefully poured through gauze to filter out any lipid and then lyophilized.

A stock solution of 25(OH)D_3 (Upjohn) at a concentration of approximately 1 mg/ml of ethanol which was stored at -20°C for up to 3 years was diluted 1/100 with ethanol to an approximate concentration of 10 μg/ml. The concentration and purity of this solution was assessed before use by scanning on a Pye Unicam 1800 dual beam spectrophotometer (Pye Unicam Limited, Cambridge, England), an example of such a

Figure 5.

A typical UV scan of authentic 25(OH)D_3 using dual beam spectrophotometry over the wavelength range 300-220nm. Maximum absorbance occurs at 264nm coupled to minimum absorbance at 228nm.


220
230
240
250
260
270
280
290
300
Wavelength (nm)
228nm
264nm
0.078
0.224
0.347
0.427
Absorbance (OD)

scan is shown in Figure 5. The purity of the 25(OH)D_3 was checked by determining the absorbance ratio at 265 nm/228 nm, which should be 1.8. The concentration was calculated using the known molar extinction coefficient of 18 500 (DeLuca, 1984). From this solution of known concentration two working solutions in ethanol were prepared, the first at a concentration of 10 ng/50 μl was serially diluted for use as standards and the second at 250 ng/50 μl was used as a non-specific binding standard. Ethanol was used as the blank which was included in the curve.

The receptor binding assay was performed in an ice bath at 4°C. By diluting the 10 ng/50 μl solution, nine 25(OH)D_3 standards in the concentration range 0-3 ng/50 μl were obtained. Two 50 μl aliquots of each standard were pipetted into polypropylene test tubes (12 x 75 mm, Elkay). In addition duplicate aliquots (50 μl) of the non-specific binding standard were included.

The 25(OH)D fraction (prepared as described in section 3.1.1.1) (obtained from 500 μl of serum) to be assayed was evaporated to dryness and redissolved in 500 μl ethanol. A 300 μl aliquot was pipetted directly into a counting vial to determine percentage recovery and 2 x 50 μl aliquots were pipetted into 12 x 75 mm polypropylene test tubes (assay tubes). To each assay and standard tube was added 6 000 cpm [^{3}H]-25(OH)D_3 in 50 μl ethanol and the mixture was vortexed. After a 30 minute pre-incubation, 1 ml of the reconstituted kidney cytosol receptor was added to all the tubes. The kidney cytosol receptor was reconstituted to a concentration of 0.2 mg receptor powder per ml in 0.05M sodium phosphate buffer. The tubes were incubated for a further two hours on ice.

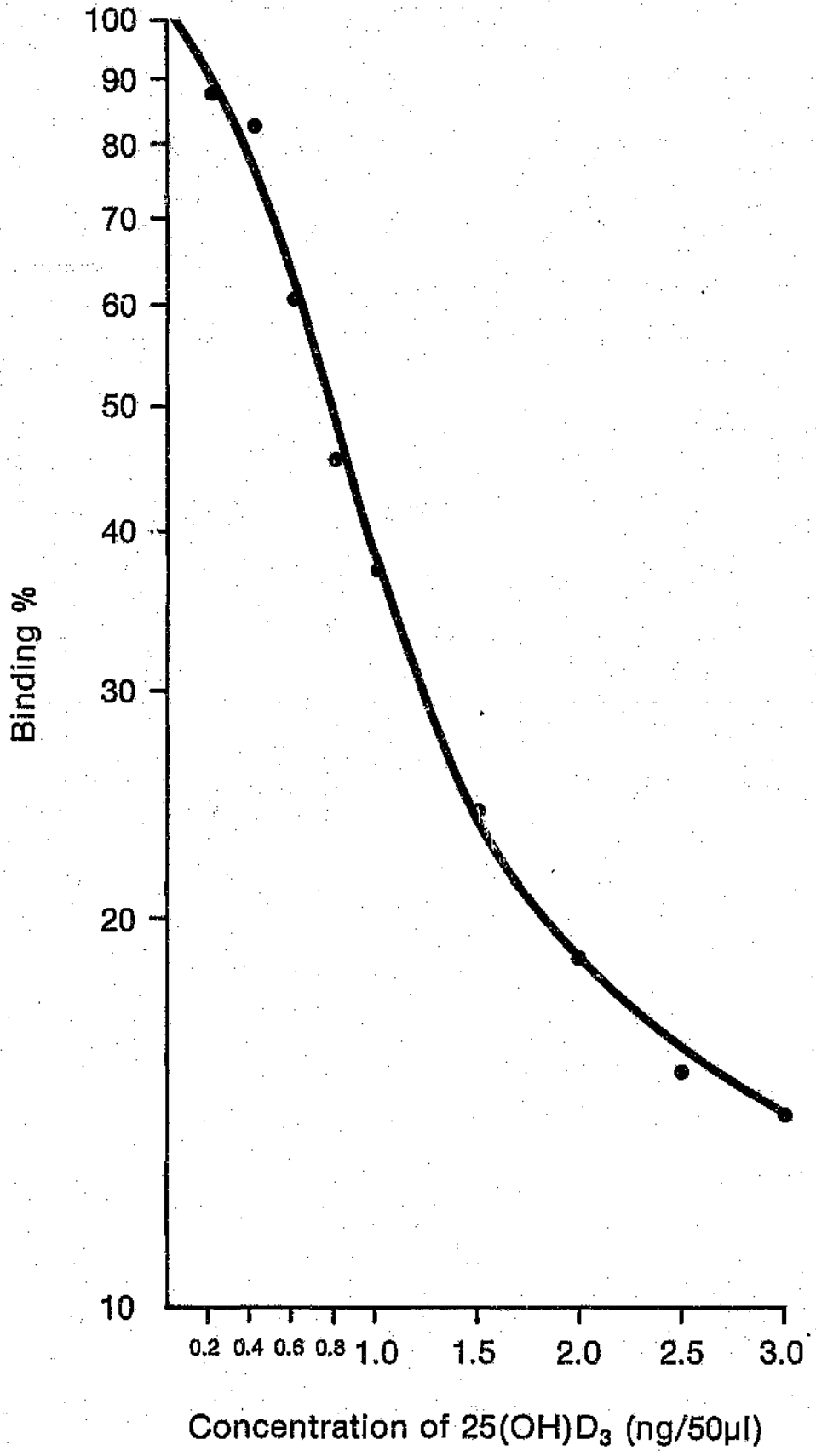
On completion of the two hour incubation 200 μl of a well mixed charcoal/dextran solution was added to each tube to separate the bound from free [^{3}H]-25(OH)D_3. This charcoal/dextran solution was prepared in the same phosphate buffer as had been used in the assay to produce a final concentration of 0.625% charcoal (Schwartsmann)/0.0625% T60 dextran (Sigma). The tubes were vortexed and allowed to stand for 18 minutes at 4°C, prior to centrifugation for 15 minutes at 3 000 rpm in a refrigerated benchtop centrifuge.

A 500 μl aliquot of each supernatant (containing the bound [^{3}H]-25(OH)D_3 was pipetted into a counting vial and 4.5 ml of scintillant (Aquagel: Cuemlab, South Africa) was added. The vials were mixed well by shaking and each vial was counted for 5 minutes in a Packard Tricarb 3255 beta liquid scintillation counter. Counting efficiency was determined using an external standard (AES ratio).

A standard curve was constructed by plotting the percentage binding of the standards against the concentration of the standards on semi log paper (Figure 6). The percentage binding of each of the standards was calculated as a percentage of the cpm obtained for the zero point. The 25(OH)D concentration in the unknown samples was calculated by reading off the percentage bound on the graph and correcting for the losses occurring during the extraction and chromatography which were calculated from the percentage recovery of the tracer added at the start of the extraction procedure. The percentage recovery was determined by dividing the cpm obtained in the aliquot at the end of the extraction procedure by the cpm added at the start of the extraction.

Figure 6.

A typical standard curve used in the measurement of 25(OH)D in bat serum samples.


100
90
80
70
60
50
40
30
20
10
Binding %
0.2
0.4
0.6
0.8
1.0
1.5
2.0
2.5
3.0
Concentration of 25(OH)D3 (ng/50µl)

CALCULATIONS:

a,b = cpm of duplicates of unknown or standard (500 μl)

c = cpm of blank

d = (cpm of zero point - c)

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

   [(a+b) ÷ 2) - c) ÷ d) x 100]

   = % binding of each sample.

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

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

   = % recovery

3. Final concentration of 25(OH)D (ng/ml)

$$\text{[ng of 25(OH)D off graph x } \frac{\text{dilution (vol of serum)}}{\text{1ml}} \text{ x 100] ÷ \% recovery}$$

   = ng 25(OH)D/ml serum.

In all the 25(OH)D assays performed, two control sera were included. One was a pooled serum sample from chicks fed on a laboratory prepared vitamin D deficient diet and kept in a light free environment and the other a serum pool from normal human subjects. The concentration of 25(OH)D was recorded as ng/ml of serum. The

validation of these control sera for use in the 25(OH)D assay is discussed in section 3.2.1.

3.1.1.3 The calf thymus receptor assay for the measurement of serum 1,25$(OH)_2$D levels

Thymus glands were removed from young calves between 5-20 weeks old. These were obtained from the Johannesburg Abattoir, South Africa. After removing the thymus gland, it was immediately rinsed in ice cold saline, sliced into small squares, snap frozen on dry ice and stored at -75°C until processed. An approximately 5g piece of each gland was thawed and individually processed to assess receptor activity. The tissue was minced through a stainless steel mincer and homogenized at 25% (w/v) with buffer, (0.05M Tris, 0.5M KCℓ, 0.0015M EDTA, 0.005M DDT, 0.01M Na_2MoO_4, pH 7.4) as described by Reinhardt et al. (1982). The homogenates were centrifuged at 45 000 rpm for 90 minutes using a 60Ti rotor in a Beckman L7-55 high speed centrifuge. The supernatants were removed, a note made of each volume and the proteins precipitated by slow addition of ground ammonium sulphate to 35% saturation (20.9 g/100 ml) with constant stirring. Stirring was continued for a further 30 minutes after the last of the ammonium sulphate has been added. Five ml aliquots were centrifuged for 20 minutes at 10 000 rpm. The supernantants were discarded and the wet pellets stored under nitrogen at -75°C. When used in the assay, the pellet was reconstituted to 5 ml with distilled water and diluted appropriately in the Tris buffer. This dilution varied from between 1:4 and 1:7 depending on the batch of thymus used. A large scale receptor preparation was performed on the remainder of the thymus gland

which showed the highest receptor binding of [^{3}H]-1,25$(OH)_2D_3$ at the zero point of the standard curve. The processed thymus was stable for up to 3 months when stored as a frozen pellet (Reinhardt et al., 1982 and 1984).

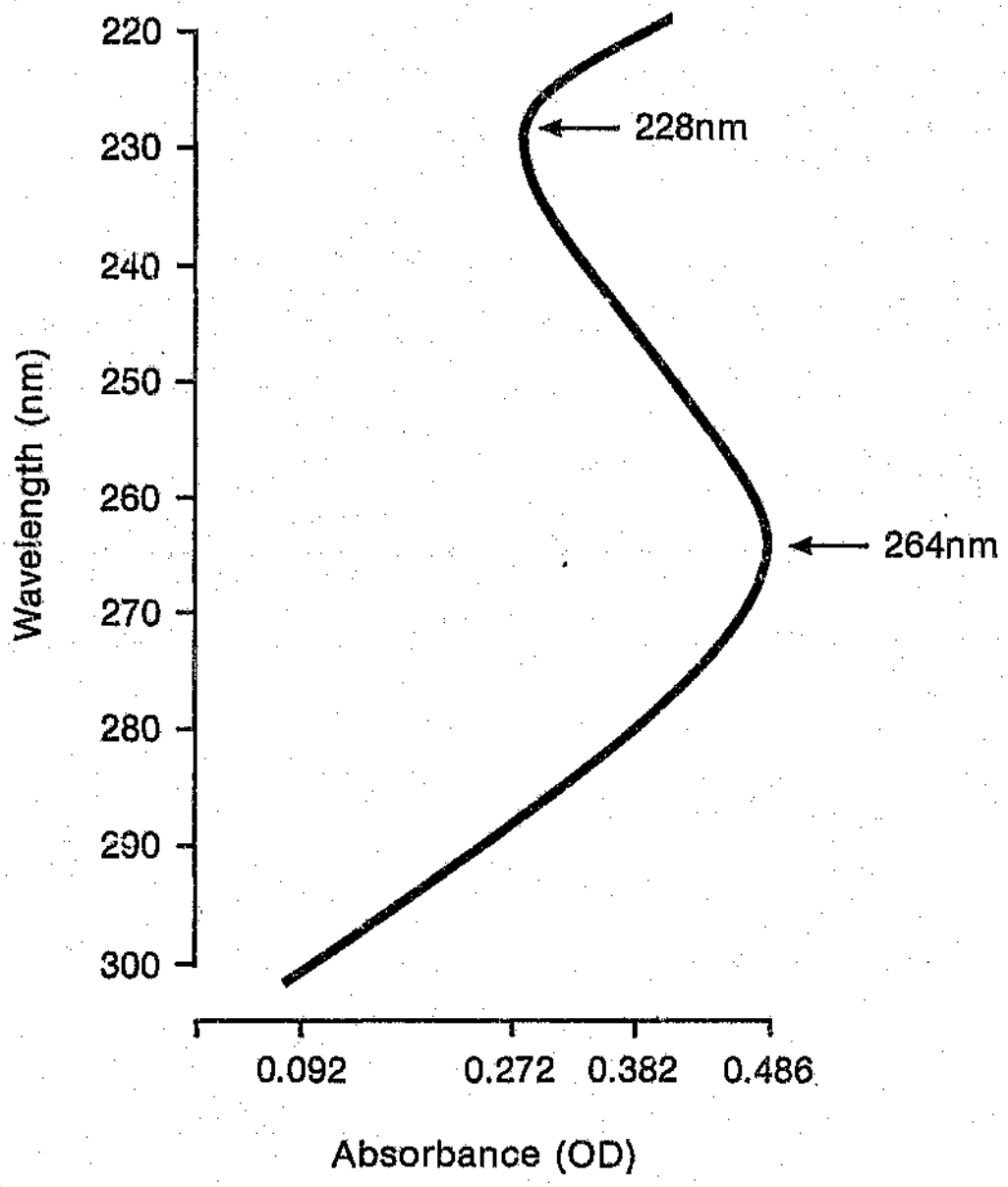
A stock solution of 1,25$(OH)_2D_3$ (Upjohn) in ethanol at an approximate concentration of 100 μg/ml, was diluted with ethanol to approximately 10 μg/ml. This was scanned on a UV spectrophotometer from 300 nm to 220 nm (DeLuca, 1984) and the purity and concentration were determined as described for the 25$(OH)D_3$ standards in section 3.1.1.2. A typical UV scan is shown in Figure 7.

This standard was diluted to a concentration of 2 000 ng/ml with ethanol and was stable for 6 months. When required, it was then diluted to a working standard (60 pg/30 μl) in 10 ml of ethanol. A non-specific binding standard in 1 ml of ethanol at concentration 900 pg/30 μl was also prepared. The 60 pg/30 μl standard was diluted to produce eight standards over the range 0-60 pg/30 μl with ethanol and the non-specific binding standard included at a concentration of 900 pg/30 μl to make up a standard curve. Each point in the curve was performed in duplicate. The standards and samples were kept in an ice bath at 4°C until the addition of the receptor protein.

The partially purified 1,25$(OH)_2D$ fractions obtained from the serum samples (500 μl), after Sep-pak chromatography as described in section 3.1.1.1 were evaporated to dryness and redissolved in 100 μl ethanol and vortexed. A 30 μl aliquot of each was pipetted into a counting vial to assess the percentage recovery during extraction and chromatography. Two 30 μl aliquots were pipetted into borosilicate glass test tubes

Figure 7.

A typical UV scan of authentic $1,25(OH)_2D_3$ using dual beam spectrophotometry over the wavelength range 300-220nm. Maximum absorbance occurs at 264nm coupled to minimum absorbance at 228nm.


220
230
240
250
260
270
280
290
300
Wavelength (nm)
228nm
264nm
0.092
0.272
0.382
0.486
Absorbance (OD)

(12 x 75 mm (Corning)). To each of the test tubes plus those containing the standards, 1 ml of the diluted receptor was added, and incubated for 45 minutes at 25°C in a shaking waterbath. After the 45 minute pre-incubation with the receptor, 30 μl of [^{3}H]-1,25(OH)$_2$D$_3$ (6 000 cpm/ 30 μl ethanol) was added to each of the tubes which were then incubated in a shaking waterbath for a further 15 minutes. 200 μl charcoal/dextran solution was prepared, as described in the 25(OH)D assay in section 3.1.1.2 and added to each tube to separate the bound from free 1,25(OH)$_2$D in this assay. The tubes were centrifuged at 3 000 rpm for 10 minutes and a 500 μl aliquot containing the bound [^{3}H]-1,25(OH)$_2$D$_3$ was taken for counting as described above.

A standard curve (Figure 8) was drawn on 3 cycle log paper, plotting percentage bound [^{3}H]-1,25(OH)$_2$D$_3$ against concentration of standards. The 30 μl aliquots pipetted into vials at the start of this assay were used to calculate the recoveries of [^{3}H]-1,25(OH)$_2$D$_3$ throughout the entire extraction procedure.

CALCULATIONS:

a,b = cpm of duplicates of unknown or standard (500 μl)

d = cpm of blank

c = cpm of zero point - c

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

   [(a + b) ÷ 2) - c) ÷ d) x 100]

   = % binding of each sample.

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

(cpm/30 μl aliquot X dilution of original serum vol X 100) ÷ total cpm added to serum sample

= % recovery

3. Final concentration of $1,25(OH)_2D$ (pg/ml.)

$$[\text{pg } 1,25(OH)_2D \text{ off graph x } \frac{\text{dilution (vol of serum) used}}{1 \text{ ml}} \text{ X } 100] \div \% \text{ recovery}$$

= pg $1,25(OH)_2D$ / ml of serum.

3.2 Results

3.2.1 Validation of the 25(OH)D and $1,25(OH)_2D$ assays

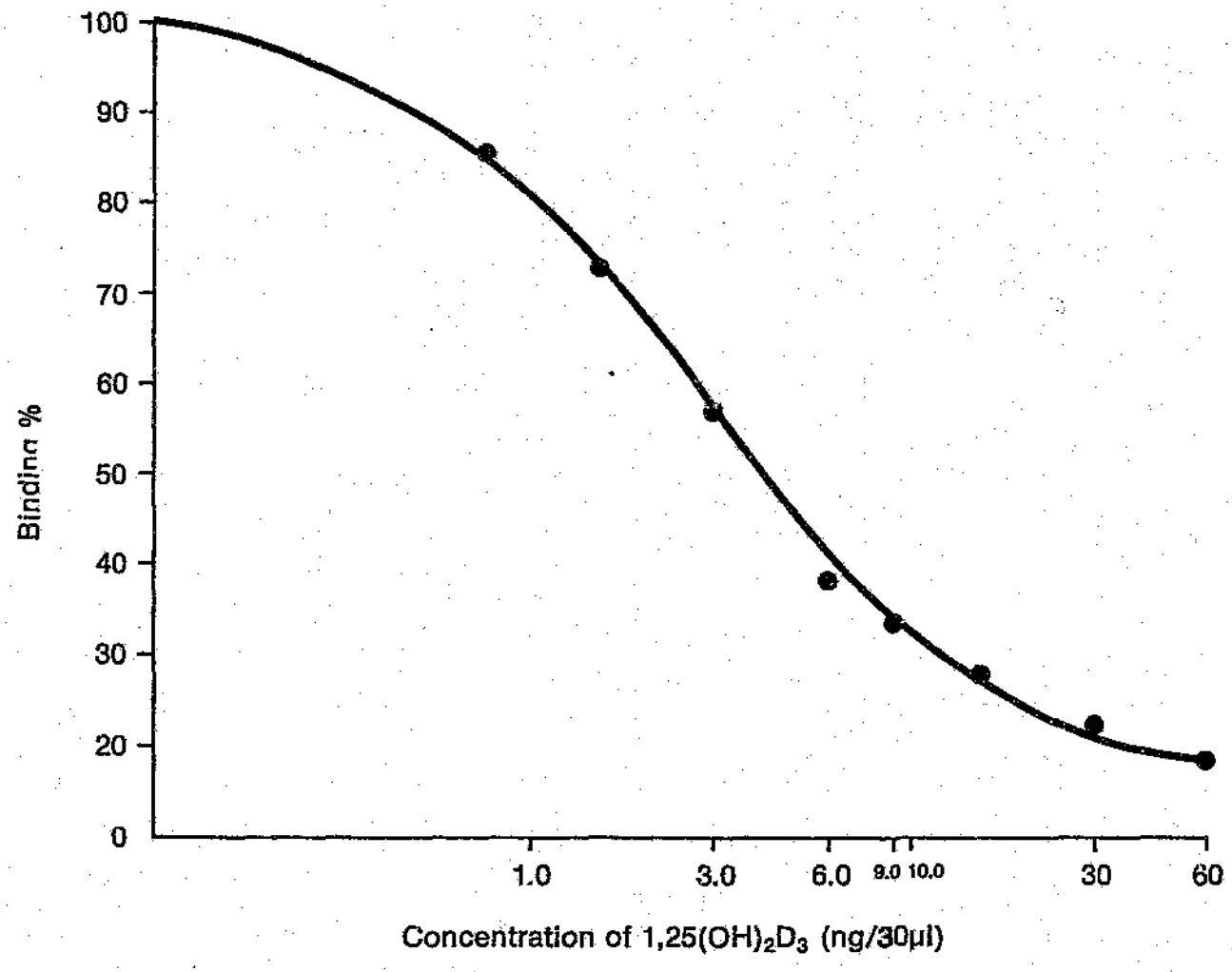
3.2.1.1 25(OH)D assay

A typical 25(OH)D binding curve is shown in Figure 6. With no added $25(OH)D_3$ (the zero point of the curve) approximately 20% of the total counts added was bound by the ligand.

The validation of the competitive protein binding assay was done by assaying the 25(OH)D concentration in 5 ml of (pooled) human serum by UV absorbance after HPLC chromatography. The 5 ml sample was extracted as described in section 4.2.2.1. The lipid extract was chromatographed on Sephadex LH-20 (section 4.2.3.2). The

Figure 8.

A typical standard curve used to determine the concentration of $1,25(OH)_2D$ in bat serum.


100
90
80
70
60
50
40
30
20
0
Binding %
1.0
3.0
6.0
9.0
10.0
30
60
Concentration of 1,25(OH)2D3 (ng/30µl)

pooled 25(OH)D region was evaporated to dryness, redissolved in 6% isopropanol/hexane, (solvent system (E) (section 4.2.4)) and chromatographed by HPLC. The absorbance was measured at 264 nm and the values of 25(OH)D calculated as ng/ml of serum.

The concentration of 25(OH)D in the pooled human serum when measured by HPLC was 19.4 ng/ml (n=1). This result was similar to the results obtained from the receptor binding assay (22.5 $\pm$ 3.5 ng/ml (n=9)). The accepted reference range for human sera is 10-40 ng/ml, as measured in this laboratory.

The vitamin D deficient chick serum sample consistently gave high percentage binding (95-105%) indicating, as expected, very low levels of 25(OH)D, < 4 ng/ml (n=5). These levels were indistinguishable from zero on the standard curve and was recorded as < 0.2 ng/tube or < 4 ng/ml of serum (when 0.5 ml of serum was extracted). The recovery of [^{3}H]-25(OH)D_3 which was added to each of the sera as a tracer before extraction, was 76.8 $\pm$ 7.9% (n=35).

3.2.1.2 1,25(OH)$_2$D assay

A typical 1,25(OH)$_2D_3$ standard curve is shown in Figure 8. Approximately 10% of the total counts was bound to the receptor at the top of the curve (0 pg/tube).

The mean 1,25(OH)$_2$D concentration of vitamin D deficient chick sera value was 13.0 $\pm$ 4.8 pg/ml (n=12). The values of the human control was 39.3 $\pm$ 12.9 pg/ml (n=7).

These results were verified by an independent laboratory (R. Horst, Ames, Iowa, USA). The recovery of [^{3}H]-1,25(OH)$_2$D$_3$ added to the serum samples as a tracer was 61.0 ± 11.4% (n=26).

3.2.2 Results of the assays for vitamin D metabolites in fruit bats serum samples

3.2.2.1 Concentrations of 25(OH)D in bat serum

Serum samples from 39 fruit bats were assayed to determine their 25(OH)D values. In all cases, irrespective of time in captivity, the presence or absence of vitamin D$_3$ supplementation, or whether they were samples from wild animals, the bats had undetectable circulating levels (< 4 ng/ml).

To verify this, a known amount of unlabelled 25(OH)D$_3$ (200 ng) was added to a pooled bat serum sample (3 ml). When this sample was assayed, the concentration of 25(OH)D was 70 ng/ml, which showed that all the added material was recovered and very little other 25(OH)D was present in bat serum.

3.2.2.2 Concentrations of 1,25(OH)$_2$D in bat serum

Wild bats had a mean 1,25(OH)$_2$D concentration of 10 ± 4 pg/ml (n=10) (Table 3). This was significantly lower ($p<0.02$) than captive bats receiving a vitamin D supplement, which had 1,25(OH)$_2$D concentrations of 46 ± 37 pg/ml (n=7). The wild

bats' $1,25(OH)_2D$ levels were significantly higher than those of the bats kept on a vitamin D deficient diet; 5 ± 6 pg/ml (n=22) p = 0.000002 (Table 3).

3.2.3 Serum mineral and alkaline phosphatase concentrations

Mean plasma calcium levels (Table 3) were similar (p>0.05) in all three groups, although the unsupplemented captive bats (D-) tended to have a lower mean value (2.15 ± 0.34 mmol/L) (n=11) than the other two groups (2.26 ± 0.17 mmol/L, (n=10) (wild) and 2.30 ± 0.09 mmol/L, (n=7) (D+)) and their values had a greater variance than those of the wild and supplemented captive groups.

Plasma magnesium concentrations were also similar (p>0.05) in all these groups (Table 3).

Serum inorganic phosphorus (Pi) levels of wild bats were significantly higher than those of the vitamin D deficient ($p=1.47 \times 10^{-4}$), and vitamin D supplemented groups ($p=8.19 \times 10^{-5}$). Vitamin D supplementation had no effect (p=0.32) on Pi concentrations in the captive groups.

Alkaline phosphatase levels of the vitamin D+ group were higher than those of the other two groups. Wild animals had significantly lower alkaline phosphatase levels (p= 0.008) (576 ± 130 IU/L) compared to both captive populations.

Table 3: Vitamin D metabolite and other serum variable concentrations in the three groups of bats.

	wild		captive			
		(n)	vit D-	(n)	vit D+	(n)
weights (g.)	13[illegible].6±8.1		118.9±16.9		121.1±14.1	
$1,25(OH)_2D$ (pg/ml)	10.±4*	(10)	5±6*#	(22)	46±37#	(7)
Calcium (mmol/L)	2.26±0.17	(10)	2.15±0.34	(11)	2.30±0.09	(7)
Magnesium (mmol/L)	1.16±0.24	(10)	1.09±0.15	(11)	1.19±0.10	(7)
Inorganic Phosphorus (mmol/L)	2.93±1.01*	(10)	0.95±0.26	(7)	0.81±0.30*	(7)
Alkaline Phosphatase (IU/L)	576±130*	(10)	783±127	(6)	1032±216*	(7)

Values with similar superscripts are significantly different from each other.

* = significant ($p<0.05$)

(One way analysis of Variance)

\# = highly significant ($p<0.001$)

3.3 Discussion

3.3.1 Vitamin D status

Field observations suggest that fruit bats in the wild are not exposed to sunlight at any stage either on their departure from the roosting caves after dusk or on their return before dawn (Smithers, 1983). Freshly caught wild bats were used to the measure their

natural vitamin D status. They were bled within 6 hours of their capture thus giving an accurate assessment of vitamin D status in the wild (Table 3) for this mammal.

Like all vertebrates studied to date (Kobayashi et al., 1991; Horst et al., 1981; Takeuchi et al., 1991) the active metabolite of vitamin D was present in the serum of free ranging bats (Table 3). The concentrations of $1,25(OH)_2D$, although detectable are lower than the range (60-100 pg/ml) reported for most mammals (Audran and Kumar 1985; Horst et al., 1981; Halloran et al., 1986), and those for adult man (15-50 pg/ml). In fact, the levels found in the bat are consistent with those found in severe vitamin D deficiency in other mammals, although apparently normal free living mole rats have similar hormone concentrations to those found in the bat.

Despite low levels of $1,25(OH)_2D$, serum calcium concentrations are within the normal range for mammals (Clark et al., 1986). This might indicate that mineral metabolism in this mammal is independent of $1,25(OH)_2D$. Several mammals, most notably juvenile rats (Tanaka et al., 1972; Brommage and Baxter, 1988) and mole rats (Buffenstein and Yahav, 1991; Buffenstein et al., 1991; Skinner et al., 1991) show a positive mineral balance, despite low $1,25(OH)_2D$ levels. Furthermore studies of mole rats, suggests that mineral metabolism is independent of vitamin status (Buffenstein and Yahav, 1991; Yahav and Buffenstein, in press).

The role of vitamin D in mineral metabolism in the fruit bat has to date not been studied. However the tendency to lower serum calcium values in the vitamin D depleted captive bats suggest that small amounts of vitamin D might be necessary to maintain

normal mineral homeostasis. The small number of bats in which serum calcium was measured and the large scatter of the values might explain the lack of statistical significance in the serum calcium values between the vitamin D replete and vitamin D deplete animals.

In none of the 39 serum samples measured for vitamin D metabolites was 25(OH)D detected. That the levels of 25(OH)D were very low (< 4 ng/ml), was confirmed by the measurement of 25(OH)D after the addition of known amounts of unlabelled 25(OH)D_3 to bat serum (Section 3.2.2.1). The values obtained were not different from the amount of 25(OH)D added to the bat serum.

Serum concentrations of 25(OH)D were below the sensitivity of our assay (< 4 ng/ml). Undetectable concentrations of 25(OH)D usually reflect vitamin D deficiency (Audran and Kumar, 1985). This might indeed be the case, for bats have no obvious source of the precursor to 25(OH)D. Thus, one would expect to observe the pathological picture (Audran and Kumar,1985; DeLuca, 1984) of vitamin D deficiency in bats (such fractures, weakness and deformities), which was not noted in any of the bats (cap or wild) in the present study. As mentioned earlier, it is possible that the bat do require vitamin D for normal bone and mineral homeostasis.

Alternatively, undetectable plasma 25(OH)D concentrations might reflect a natural species specific phenomenon. Horses (Horst et al., 1988), mole rats (Buffenstein et al., 1988; Buffenstein et al., 1991) and sharks (Kobayashi, 1991) do not appear to employ 25(OH)D in a similar way to most mammals, as the principal circulating form of

vitamin D (Norman and Litwack, 1987). It is possible that bats, like these exceptions to the normal pattern, regulate vitamin D_3 metabolism at the level of the active metabolite ($1,25(OH)_2D_3$), using the inactive $24,25(OH)_2D_3$ as a hormone reservoir, which could then be further hydroxylated to $1,24,25(OH)_3D_3$ in situations of calcium lack (Kumar, 1984; Henry, 1992).

Another alternative is that vitamin D requirements of the fruit bat may have evolved into a finely tuned system whereby the metabolism of vitamin D occurs through the known biochemical pathways (Norman, 1990) to the final active hormone $1,25(OH)_2D$ but that substrate levels of 25(OH)D re[illegible]y the kidney to produce $1,25(OH)_2D$ are very much lower than in other mammals. All the 25(OH)D, available to the animals, could well be converted to $1,25(OH)_2D$. Thus all the vitamin D obtained by the bat, is utilized in the most efficient way possible. This would be highly adaptive as vitamin D is not obviously available to this nocturnal fruit eating mammal and perhaps only small quantities are indeed obtained.

A further possible explanation, could be that 25(OH)D is not transported bound to a vitamin D binding protein. Rather all 25(OH)D is found free, thus rendering it available in smaller amounts for the action of hydroxylating enzymes (Fraser, 1980).

3.3.1.1 Captive population of bats

The housing of the fruit bat in captivity simulated, as closely as possible, this mammal's environmental conditions in the wild. As the diet was considered to be vitamin D deficient, a group of animals were supplemented with vitamin D in captivity, to assess the effect of vitamin D supplementation on vitamin D metabolism and mineral homeostasis.

Animals not receiving a vitamin D supplement and maintained in captivity for long periods, showed similar serum mineral values to those of non-captive animals (Table 3), although $1,25(OH)_2D$ levels were lower, and there was a tendency (though not statistically significant) for serum calcium values to be lower.

Animals receiving a physiological supplement of vitamin D_3, has markedly raised (4 times) $1,25(OH)_2D$ levels compared to wild bats, although 25(OH)D levels still remained undetectable (Table 3). These data are at variance with vitamin D_3 supplementation studies of another mammal with no obvious vitamin D source (Buffenstein et al., 1991). Mole rats in the wild have undetectable levels of 25(OH)D, yet when given a vitamin D supplement, 25(OH)D concentrations are elevated to within the normal range for mammals (Buffenstein et al., 1991). Data obtained from the vitamin D_3 supplementation group in the present study, are thus surprising. It would be expected if the 25(OH)D substrate was naturally limiting, that when oral vitamin D_3 supplements were given, $25(OH)D_3$ would become detectable as was the case with the mole rats. A possible explanation is that the dose of vitamin D was sub-physiological,

thus preventing the accumulation of 25(OH)D to detectable levels in the animals. All available 25(OH)D would then be rapidly converted to more polar metabolites.

Bats in captivity receiving a vitamin D_3 supplement showed significantly higher serum concentration of $1,25(OH)_2D_3$ than those not supplemented. Vitamin D_3 supplementation brought the serum $1,25(OH)_2D_3$ concentrations into the same range as other mammals (Audran and Kumar, 1985; Horst et al., 1981). This implies that bats in the wild lack sufficient vitamin D_3 substrate and are compelled to maintain the active metabolite at below normal concentrations. They must therefore have either adjusted their mode of mineral homeostasis to function adequately at low $1,25(OH)_2D_3$ concentrations or alternatively maintain free $1,25(OH)_2D_3$ levels within the normal range but with a reduced total $1,25(OH)_2D_3$ concentration.

3.3.2 Mineral concentrations

Irrespective of vitamin D status, captive and wild bats tightly regulated serum calcium and magnesium levels (Table 3) within the normal range for mammals (Clark et al., 1986; Schaamfsma et al., 1988). This indicates the importance of these minerals in homeostatic functions and the need to tightly regulate them by whatever means. It is possible that serum concentrations of these minerals are not regulated by $1,25(OH)_2D_3$, as supplementation with vitamin D_3 did not alter their concentrations. However the tendency for lower calcium values in the vitamin D deficient captive animals suggests that the low concentrations of $1,25(OH)_2D$ are necessary for normal calcium homeostasis.

Inorganic phosphorus levels in captive animals were much lower than the free ranging animals. Vitamin D_3 supplementation had no influence on these concentrations. We therefore cannot attribute a decrease in Pi to vitamin D status, nor can Pi concentrations be the prime regulator of $1,25(OH)_2D$ levels. Experimental intervention must be the cause of the low values in captivity. Low Pi concentrations might be the main cause of the broken wing phenomena originally attributed to vitamin D deficiency. A normal phosphate concentration is essential for life in that it is intimately involved in ATP production and hence energy metabolism (Lehninger, 1975). The bone reservoir of this substance might therefore be drawn upon, to maintain serum Pi and thus result in a decrease in bone density. This in turn would increase the risk of bone fracture. Despite this, few bats in our study presented with these symptoms.

It is possible that the lower serum Pi values might reflect a dietary lack. Although the diet consisted of mainly peeled fruit, it was not possible to reproduce the wild bats' diet of mainly wild figs.

Alkaline phosphatase levels were lowest in wild caught animals and were markedly elevated in captive populations. It is possible that low serum Pi might result in the elevation of alkaline phosphatase levels through its effect on bone mineralization and turnover.

3.4 Conclusion

Not one of the serum samples from the 39 bats used for the measurement of 25(OH)D had detectable levels of 25(OH)D present. Despite this finding, detectable levels of the active hormone $1,25(OH)_2D$ were present in all the samples assayed. There was a clear distinction between the concentrations of the $1,25(OH)_2D$ levels in the three groups of bats assessed in these experiments. The levels in the vitamin D deficient group was significantly lower than those in the other two groups. The data indicate that despite unmeasurable levels of circulating 25(OH)D, the bat is able to maintain detectable levels of the $1,25(OH)_2D$ even in the wild state, suggesting that a small but significant amount of vitamin D is ingested by bats in their natural habitat.

Calcium and magnesium concentrations measured in the bats in the three groups showed no significant change with vitamin D status (Table 3), indicating that these minerals were tightly regulated in a manner possibly independent of vitamin D.

Inorganic phosphorus values did not follow the same pattern as those of calcium and magnesium (Table 3). Wild bats had significantly higher Pi concentrations than those held in captivity and this marked difference must be due to captive intervention treatments unrelated to vitamin D status.

Alkaline phosphatase concentrations in all three groups were high, possibly indicating a rapid rate of bone turnover. Values in wild bats were lower than in captive animals. Although alkaline phosphatase iso-enzymes were not measured it is possible that the

higher alkaline phosphatase values in the captive animals reflect the effect of hypophosphataemia on bone turnover.

Thus wild bats appear to have normal mineral homeostasis despite a lack of an obvious source of vitamin D. Furthermore the presence of serum $1,25(OH)_2D$ concentrations despite an absence of detectable 25(OH)D suggests that small amounts of vitamin D are obtained by these nor[illegible]al animals. It is possible that this is obtained from the skin of ingested fruits, whi[illegible]e been irradiated by sunlight.

Unlike many other mammals that have been studied, circulated 25(OH)D levels do not provide a good indicator of vitamin D status. The data from the present study suggest several possible mechanisms from this finding:-

1. 25(OH)D is not an intermediate metabolite in the metabolism of vitamin D to $1,25(OH)_2D$.

2. The fruit bat does not possess a vitamin D binding protein, thus levels of 25(OH)D are mainly in the free form and are too low to be detected in the receptor assay used in this study.

CHAPTER 4

The metabolism of tritium labelled vitamin D_3 and tritium labelled 25 hydroxyvitamin D_3 in the fruit bat

4.1 Introduction

Once it had been established that the fruit bat had measurable levels of circulating $1,25(OH)_2D$ but undetectable levels of 25(OH)D, more in depth studies were done to assess the bat's ability to metabolise vitamin D, and the metabolic pathways involved.

4.2 Methods

Two experiments were performed to determine vitamin D metabolism in fruit bats. The first experiment consisted of dosing a number of bats with ^3H vitamin D_3, while in the second experiment, different bats were dosed with unlabelled $25(OH)D_3$ and ^3H $25(OH)D_3$. This was done to assess the vitamin D metabolic pathway to the active hormone, $1,25(OH)_2D$, in the in vivo situation.

4.2.1 Tissue sample collection

Tissues necessary for the various experiments were dissected from the carcasses of freshly killed bats. Those which were not required immediately were stored in aluminium foil or plastic containers at -20°C. The kidneys used in the 25-hydroxyvitamin D 1α-hydroxylase experiments were processed as soon as they were removed from the animals. Blood samples were collected as described in section 2.2.3.

4.2.2 The extraction and chromatography of the lipid samples

The method of extraction of lipids from serum and bat tissue samples obtained for the different experiments was that developed by Bligh and Dyer (1959).

4.2.2.1 Extraction of serum

To serum (1ml) was added 5.75 ml methanol:chloroform:water (2.3:2.3:1.15) and the mixture well mixed. After centriguation for 15 minutes at 3000 rpm, the mixture separated into three phases. The top aqueous phase was transferred into a second tube, so that it would not interfere with access through the protein interface to the lower lipid containing chloroform phase. The chloroform lipid layer was transferred into a third test tube and evaporated to dryness under a stream of nitrogen at 37°C. The aqueous phase and the protein layer were re-extracted with chloroform (0.8 ml), and after centrifugation the two lipid extracts were combined and evaporated to dryness. The lipid extracts were then redissolved in 0.5 ml hexane and stored at -20°C until chromatographed.

4.2.2.2 Extraction of the bat kidney homogenate

Extraction of lipids from bat kidney followed the same procedure however in this case 1g of tissue was used with 3.8 ml of the ethanol:chloroform:water (2:2:1.8) mixture (Bligh and Dyer, 1959).

4.2.3 Chromatography of vitamin D metabolites

The extracted samples were either chromatographed on Bio-Sil Silicic Acid (Section 4.2.3.1) or LH-20 Sephadex (Section 4.2.3.2) as required. Bio-Sil Silicic Acid was used to chromatograph serum samples as they contained only small amounts of lipid. In the case of bat kidney tissue, the amount of lipid present was larger and therefore these samples required chromatography through a LH-20 Sephadex column.

4.2.3.1 Chromatography through Bio-Sil HA silicic acid

Silicic acid chromatography was used to separate a number of vitamin D metabolites, as described by Delvin et al. (1985). Prior to use, the Bio-Sil HA silicic acid powder (50g) (Bio-Rad, Richmond, CA) was washed extensively in re-distilled ether (BDH), the bulk of the ether was then aspirated off and the silicic acid allowed to air dry until no trace of ether was detected. The silicic acid was then activated at 100°C for 1 hour in a drying oven. Once the activation was completed, re-distilled hexane (Holpro, Johannesburg, South Africa) was added immediately to the powder and the slurry stored in this wet form until required for use.

4.2.3.1.1 Calibration of Bio-Sil HA silicic acid chromatography column

The column, a 0.5 x 8 cm pasteur pipette stoppered at the lower end with a cotton wool plug, was packed with a slurry of the silicic acid to a height of 4 cm. The column was calibrated using [^{3}H]-vitamin D_3, [^{3}H]-25(OH)D_3 and [^{3}H]-1,25$(OH)_2D_3$. Each

metabolite was initially chromatographed separately and finally in combination. Fifty microlitres of each metabolite containing approximately 1500 cpm was pipetted into a glass tube, the ethanol was evaporated off and 1 ml 20% ether/hexane added to the tube. The tube was well mixed by vortexing and the contents applied onto the column using a pasteur pipette. The tube was further washed with two consecutive 0.5 ml washes and each applied to the column as the previous application ran into the silicic acid. A third ml of the 20% ether/hexane was applied to the column. The column was then eluted successively with 5 ml of 55% ether/hexane, 5 ml of 95% ether/hexane and 5 ml 50% acetone/ether and each fraction collected separately as described by Delvin et al. (1985). Each of the eluted fractions was evaporated at 37°C under a steam of nitrogen. Thereafter the contents of each tube were transferred into scintillation counting vials with several washes of hexane. Scintillation fluid was added to each vial, which was counted for tritium in a beta liquid scintillation counter (Parkard Tricarb 3255).

Following chromatography, the metabolites eluted in the following fractions (Figure 9); vitamin D in the 55% ether/hexane (3-5 ml), 25(OH)D in the 95% ether/hexane region (7-9 ml), and the $1,25(OH)_2D$ in the 50% ether/acetone region (11 and 12 ml). The recovery of each of the three metabolites used, [^{3}H]-vitamin D_3, [^{3}H]-$25(OH)D_3$ and [^{3}H]$1,25(OH)_2D_3$ was 60%, 93% and 67% respectively. Other more polar metabolites would be eluted using 100% methanol (15-18 ml)

Figure 9.

The separation of vitamin D metabolites on Silicic Acid column chromatography after lipid extraction. The different metabolites were eluted with varying concentrations of ether/hexane; 20% (3ml), 55% (5ml) and 95% (5ml) followed by 5ml 50% acetone/ether.

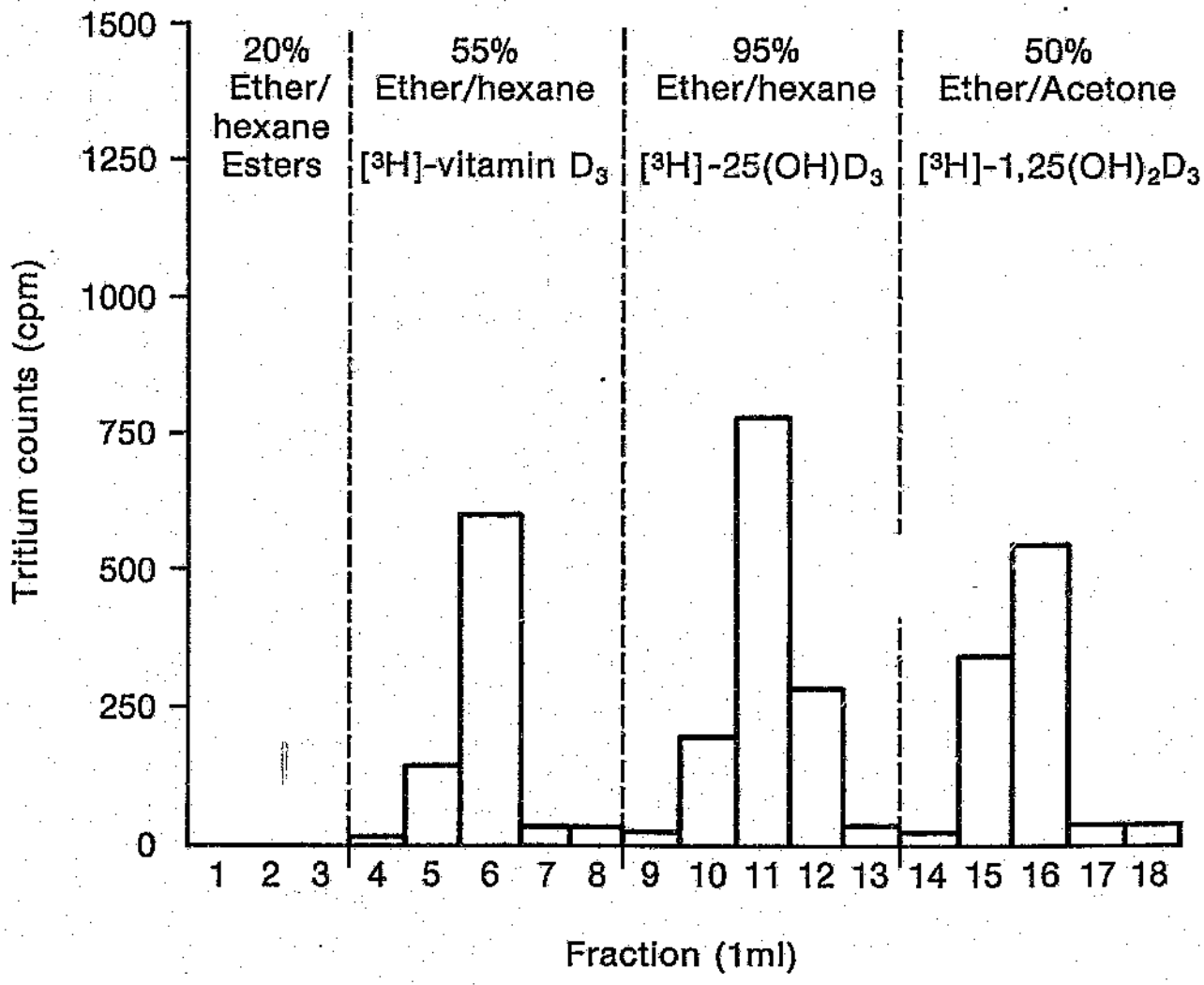

20% Ether/ hexane Esters
55% Ether/hexane
[3H]-vitamin D3
95% Ether/hexane
[3H]-25(OH)D3
50% Ether/Acetone
[3H]-1,25(OH)2D3
Tritium counts (cpm)
1500
1250
1000
750
500
250
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Fraction (1ml)

Figure 10.

LH 20 Sephadex chromatography separation of vitamin D metabolites (after lipid extraction) on a 2 x 20cm column eluted with 300 ml of hexane:chloroform:methanol (9:1:1). 5ml fractions were collected.

Vitamin D_3
$25(OH)D_3$
^3H $1,25(OH)_2D_3$
Absorbance at 264nm (OD)
5
4
3
2
0.6
0.4
0.2
0
300
250
200
150
100
50
0
Tritium concentration (cpm/µl)
0
10
20
30
40
50
60
Fractions (5ml)

4.2.3.2 LH-20 sephadex column chromatography

LH-20 Sephadex chromatography as described by Eisman et al. (1976), was used for the separation of vitamin D metabolites in samples containing larger amounts of lipid. A 2 x 20 cm colum was packed with a slurry of pre-swollen (hexane:chloroform:methanol) (9:1:1) LH-20 Sephadex (Bio-Rad, Richmond, CA). The column was calibrated using authentic vitamin D and $25(OH)D_3$ and 2 000 cpm of $[^3H]$-$1,25(OH)_2D_3$. The metabolites were premixed in a tube, the ethanol evaporated off and 1 ml of the eluting solvent added to the tube. The solution was well mixed and with two 0.5 ml washes of the same solvent, loaded onto the equilibrated column using a pasteur pipette. Sixty fractions (5 ml) were collected directly into counting vials. The solvent was evaporated off, scintillant added and each vial counted in a liquid scintillation counter for 5 minutes.

Authentic vitamin D_3 and $25(OH)D_3$ (assessed by absorbance peaks) eluted in fractions 4-10 (20-50 ml) and 11-19 (55-95 ml) respectively, and $[^3H]$-$1,25(OH)_2D_3$ used in the calibration of this column eluted between fractions 32-49 (160-245 ml) (Figure 10).

4.2.4 High pressure liquid chromatography systems

A number of different high pressure liquid chromatography (HPLC) solvent systems were used for the further purification and separation of vitamin D metabolites in the extracts from tissue and serum samples of the fruit bat.

Extensive HPLC purification was necessary to separate co-migrating vitamin D metabolites which might interfere with the determination of the amount of $1,25(OH)_2D$ produced (Chapter 5).

HPLC was performed either after preparative low pressure chromatography or directly after extraction if only very small amounts of lipid (< 0.5 mg) were present (Section 4.2.2).

All straight phase HPLC was done using a Spherisorb 10 μm, 250 mm silica column (Deeside Inst. Est. UK.). The pressure pump was a LDC Constametric (Milton Roy, Laboratory Data Control (LDC), Riviera Beach, Florida, USA), coupled to a LDC 1203 UV III monitor with a 245 nm filter and a Vitatron chart recorder.

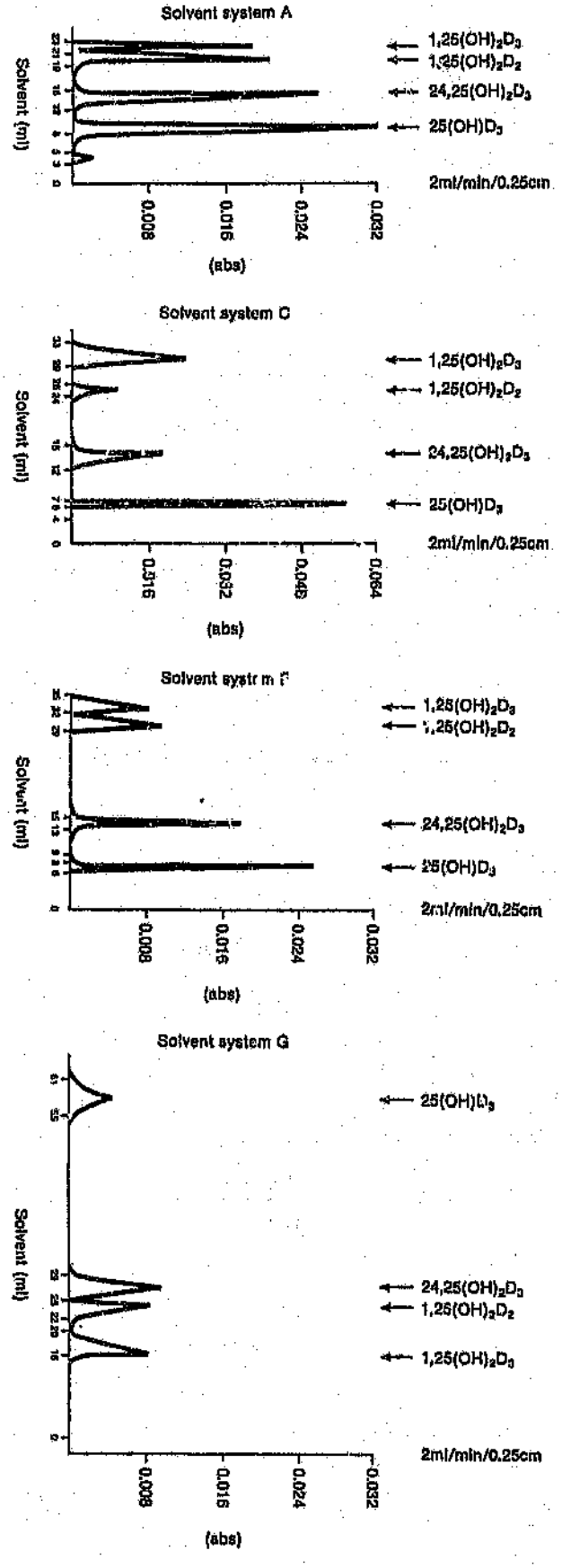
Reverse phase HPLC was performed using a Zorbax-ODS column attached to the same pressure pump. The flow rate for all the experiments was 2 ml/min and 1 ml fractions were collected. The absorbance sensitivity of the recorder was varied for each of the experiments.

All the solvents used were HPLC grade and supplied by Waters, Millipore, SA. unless otherwise stated.

A number of different isocratic solvent systems were used and each system was calibrated using the major vitamin D metabolites:- Vitamin D_3, $25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ at a concentration of 100 μg/20 μl.

Figure 11.

The HPLC chromatography tracings using 4 solvent systems. A, hexane:isopropanol:methanol (90:6:4); C, 3% isopropanol/dichloromethane; F, 8% isopropanol/hexane; G, 80% methanol/H_2O. The flow rate for all the systems was set at 2 ml/minute.


Solvent system A
Solvent (ml)
1,25(OH)2D3
1,25(OH)2D2
24,25(OH)2D3
25(OH)D3
2ml/min/0.25cm
0.008
0.016
0.024
0.032
(abs)
Solvent system C
Solvent (ml)
1,25(OH)2D3
1,25(OH)2D2
24,25(OH)2D3
25(OH)D3
2ml/min/0.25cm
0.016
0.032
0.048
0.064
(abs)
Solvent system F
Solvent (ml)
1,25(OH)2D3
1,25(OH)2D2
24,25(OH)2D3
25(OH)D3
2ml/min/0.25cm
0.008
0.016
0.024
0.032
(abs)
Solvent system G
Solvent (ml)
25(OH)D3
24,25(OH)2D3
1,25(OH)2D2
1,25(OH)2D3
2ml/min/0.25cm
0.008
0.016
0.024
0.032
(abs)

The first system (A) was the ternary solvent system described by Jones (1980), using hexane:isopropanol:methanol in the ratio of 90:6:4(A). Three other systems were also used. These were binary solvent systems (B,C,D) described by Brown and DeLuca (1985) making use of varying concentrations of isopropanol/dichloromethane (DCM) (B:2%, C:3% and D:4% respectively). The calibration of each of these systems was performed using the same concentrations of vitamin D metabolites as described in (A). Thirty fractions (1 ml) were collected.

A further binary solvent system used was isopropanol/hexane at different concentrations of isopropanol i.e. 6%(E) and 8%(F). The calibration of these two systems was performed using the vitamin D metabolites described in (A). Forty fractions (1 ml) were collected.

A reverse phase system was also used, and was equilibrated with 80% methanol/water (G). The same vitamin D metabolites were used to calibrate the reverse phase column, and thirty fractions (1 ml) were collected.

The tracings of the four major solvent systems (A, C, F and G) used in the separation of the vitamin D metabolites are shown in Figure 11. The elution volumes of the authentic vitamin D metabolites were used to identify the putative metabolites in the various samples.

A number of reasons for the use of the different HPLC solvent system are mentioned in the following paragraphs. The purity of the putative $1,25(OH)_2D$ was increased by

the elimination of comigrating impurities by using consecutive HPLC solvent systems. With the elimination of these impurities (possibly other vitamin D metabolites) the possibility of the binding of other substances other than the putative $1,25(OH)_2D$, to the receptor in the receptor binding assay (Chapter 5) was avoided.

In the experiments in which the bats were injected with the radio-labelled vitamin D metabolites [^{3}H]-vitamin D_3 and [^{3}H]-$25(OH)D_3$, it was necessary to obtain good separation of the injected material from the more polar vitamin D metabolites produced in vivo by the bat. The number of counts present in the fractions collected off the HPLC systems was on occassion small and samples were therefore repurified to ensure that all the counts present were indeed those produced by the bat and not a contaminant.

In the experiments performed to try and determine the form of vitamin D utilized by the bat (i.e. vitamin D_2 or vitamin D_3) (Chapter 7), it was necessary to obtain baseline separation of authentic $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ so as to collect each metabolite separately off the HPLC system which was then included in the receptor binding assay.

With each solvent system used, interfering substances which showed up as absorbance peaks in the UV tracing were progressively eliminated.

4.2.5 Methods of the in vivo [^{3}H]-Vitamin D_3 metabolism study

Five fruit bats kept in captivity and fed on a vitamin D deficient diet of peeled or washed fruit for a minimum of one year, were injected intraperitoneally with 1.7 X 10^6 cpm/50 μl ethanol of [^{3}H]-vitamin D_3 (14 Ci/mmol, Amersham). Blood samples (all were less than 1 ml of blood) were collected from the wing vein of each of the animals 24 hours after the isotope was given. The five blood samples were centrifuged at 3 000 rpm for 15 minutes and the serum separated. The volume of each was recorded and a 5 μl fraction taken and counted for total tritium content. The sera were extracted as described in section 4.2.2, the weights of lipid were noted and the samples prepared for HPLC.

The first HPLC system used the ternary solvent system (A), described in section 4.2.4. Each of the five extracted samples was redissolved in 100 μl of this solvent, 90% was injected onto the column and the remaining 10% placed in a counting vial for calculation of the percentage recovery after lipid extraction. Of each fraction collected off the HPLC, 10% was pipetted into vial for calculating the recovery after chromatography. The fractions containing [^{3}H]-vitamin D_3 and [^{3}H]-25(OH)D_3 were separately pooled and concentrated down to dryness. The vitamin D_3 pool was redissolved in hexane and stored at -20°C. The 25(OH)D_3 pool was redissolved in 100 μl 2% isopropanol/dichloromethane for use on the first binary solvent system (B). Again 10% was taken for counting and the remaining 90% injected onto the column. Thirty fractions (1 ml) were collected and 10% of each counted, Fractions which eluted at the known elution volume for 25(OH)D_3 were concentrated dryness, redissolved in

100μl of solvent system (E), the final HPLC system used. As in the previous HPLC systems, 10% was taken for counting and the remaining 90% injected onto the column. Forty fractions (1 ml) were collected directly into vials for counting.

4.2.6 Methods used in vivo [^{3}H]-25(OH)D_3 study

Nine vitamin D deplete fruit bats were divided randomly into three groups of three bats each. Three solutions containing a fixed amount of [^{3}H]-25(OH)D_3 (5 X 10^{-5} cpm/50 μl ethanol, 1.5 ng, 160 Ci mmol Amersham) plus different amounts of authentic 25(OH)D_2 was prepared. The total amount of unlabelled 25(OH)D_3 received by each bat in the 3 groups was 2, 11 and 21 ng respectively. The 25(OH)D_3, dissolved in ethanol (50 μl), was injected intraperitoneally as a bolus. At 16 hours a blood sample was collected from the wing vein of each animal. The samples were centrifuged, the sera separated and a note made of each volume prior to storage at -20°C. The animals were sacrificed at 48 hours and as much blood as possible collected by cardiac puncture from each animal. The blood samples (utlizing 1.5 ml serum) were prepared for chromatography as described in section 4.2.2.1.

Silicic acid chromatography was performed on the serum samples, using a modification of the method as describe in section 4.2.3.1. The 95% ether/hexane fraction was eliminated, all the other fractions were assessed for radioactivity and only the 50% acetone/ether fraction which contained metabolites more polar than vitamin D (eluted in 55% either/hexane) was then collected for further processing. The column was stripped using 5 ml methanol which was collected, evaporated and the total fraction

counted for radioactivity. The latter fraction contained metabolites of vitamin D, more polar than $1,25(OH)_2D$.

The 50% acetone/ether fraction was evaporated down to dryness and redissolved in 100 μl of the ternary solvent (A), (section 4.2.4). A 10% aliquot was taken for counting and the remaining 90% was injected onto the HPLC column. Two peaks containing tritium were identified and each was pooled and evaporated down to dryness and stored in hexane at -20°C. The second peak (containing the putative $1,25(OH)_2D$) was redissolved in 100 μl of 4% isopropanol/dichloromethane (solvent (D)). A 10 μl aliquot was counted and the remaining 90 μl injected onto the column. Forty fractions (1 ml) were collected directly into vials for counting.

4.3 Results

4.3.1 Results of in vivo [^{3}H]-Vitamin D_3 metabolism study

The total tritium recovered prior to extraction and chromatography of the sera from the five bats which were dosed with [^{3}H]-vitamin D_3 is shown in Table 4.

Table 4

The recovery of tritium counts (cpm) in the sera of 5 bats 24 hours after they were injected with known amounts of [^{3}H]-vitamin D_3.

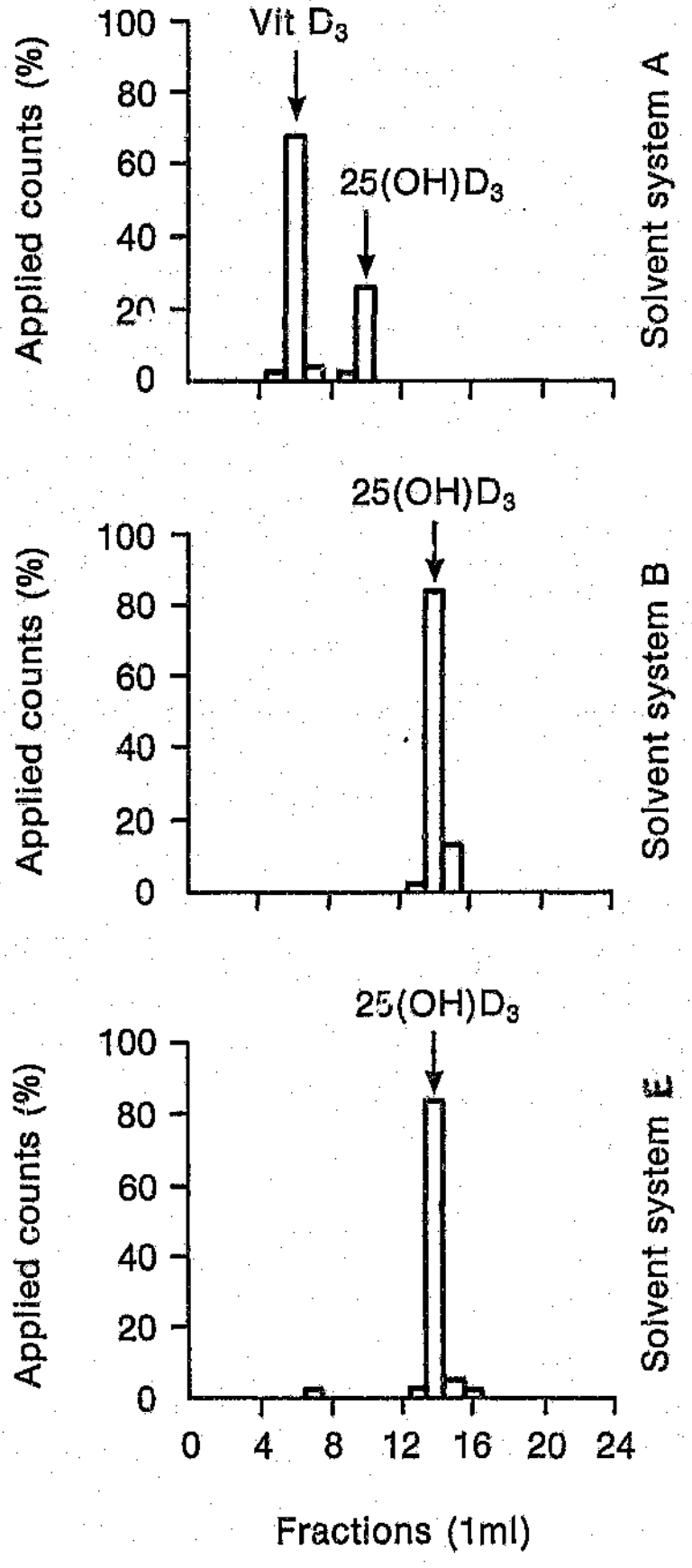
sample no.	vol. of serum	cpm/5μl serum	total cpm	cpm/ml	% of total dose admin/ml serum
1.	452μl	137.2	12412	27460	1.61
2.	335μl	78.0	5226	15600	0.92
3.	585μl	115.0	13455	23000	1.53
4.	352μl	121.3	8540	24251	1.42
5.	460μl	43.0	3956	8600	0.50

The results of the HPLC chromatography as described in section 4.2.5 are shown in Figure 12(a). The [^{3}H]-vitamin D_3 eluted in fractions 5-7, while [^{3}H]-25(OH)D_3 eluted in fractions 9 and 10. The more polar metabolites, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ would have eluted after fraction 16, but were not detected. In all five samples, the majority of the counts eluted in the same fractions as authentic vitamin D_3 with only a small number of counts eluting in the 25(OH)D_3 fraction. Further metabolism to the more polar metabolites was not detected in any of these serum samples.

The pooled 25(OH)D_3 region off HPLC system A, was injected onto the second HPLC system and eluted with solvent B described in section 4.2.4, and shown in Figure 12(b). The majority of the counts (60-90%) eluted in the same fractions as authentic 25(OH)D_3 (13-16). This fraction was analysed on a third solvent system E as described in section 4.2.4 and shown in figure 12(c) where only a small portion of the counts did not co-migrate with authenic material in fractions 13-15.

Figure 12.

The results of in vivo [^{3}H]-vitamin D_3 metabolism. Serum was extracted and metabolites separated using high pressure liquid chromatography with 3 different solvent systems. Fig. (a) shows the elution with solvent system A; [3H]-vitamin D_3 eluted in fractions 5-7, [^{3}H]-25(OH)D_3 eluted in fractions 9 & 10. Fig. (b) using solvent system B; [^{3}H]-25(OH)D_3 eluted in fractions 13-16. Fig. (c) using solvent system E; [^{3}H]-25(OH)D_3 eluted in fractions 13-15.


Vit D_3
25(OH)D_3
Applied counts (%)
Solvent system A
25(OH)D_3
Applied counts (%)
Solvent system B
25(OH)D_3
Applied counts (%)
Solvent system E
100
80
60
40
20
0
0
4
8
12
16
20
24
Fractions (1ml)

Thus it is clear that the bat is also able to metabolise vitamin D_3 to 25-hydroxyvitamin D_3, however because of the small amount of tritiated vitamin D_3 converted to $25(OH)D_3$ it was not possible to determine whether the bat was able to further metabolise $25(OH)D_3$ to the active metabolite $1,25(OH)_2D_3$.

4.3.2 In vivo [^{3}H]-$25(OH)D_3$ metabolism study

The acetone extracted serum had 10% or less counts present at both 16 and 48 hours. The total cpm of the lipid phase of each of the three groups of bats, at both times and at the different stages of the chromatography are shown in Table 5. Most of the extracted lipid eluted in 1-8 mls using Bio-Sil HA Silicic Acid, thus eliminating contamination of absorbance peaks on the HPLC sytems used.

The results from the silicic acid chromatography of the 16 hour samples, showed that only a small number of cpm were present in the 55% ether/hexane fraction (4-8 mls) containing the majority of lipid and unknown less polar metabolites than 25(OH)D, except for the groups of bats which received 11 ng of $25(OH)D_3$ per bat, which had higher counts (28.7%). The majority of the counts migrated in the 50% acetone/ether fraction.

The 16 hour serum samples had more mean total counts present than the 48 hour samples, although more counts were present in the 50% ether/acetone fraction at 48 hours than at 16 hours. The 100% methanol wash which was used to elute any more

polar metabolites than the active metabolite $1,25(OH)_2D$, from the columns, contained less than 9% of the counts.

Table 5

The tritiated metabolites (% of total cpm) eluted off the silicic acid column after the intraperitoneally injection of $[^3H]25(OH)D_3$

Time (hrs)	% of total counts in each fraction						
	Conc $25(OH)D_3$ admin ng/bat	Mean total cpm per ml of bat serum	Aqueous phase	Ether/ hexane 20% (1-3ml)	Ether/ hexane 55% (4-8ml)	Ether/ acetone 50% (9-13ml)	Methanol 100% (14-18ml)
16	2	13041	8.2	5.5	12.4	65.5	8.4
	11	15458	7.1	5.8	28.7	50.1	8.2
	21	14128	10.1	6.2	16.2	75.8	8.0
48	2	8869	3.9	2.2	5.7	84.7	3.4
	11	11669	3.5	1.5	4.0	86.9	4.1
	21	10234	3.3	1.9	7.9	84.5	2.5

The specific activity of each of the administered doses of 25(OH)D was 9.49×10^{-5} dpm/2 ng, 1.8×10^{-5} dpm/11 ng and 9.48×10^{-4} dpm/21ng. The results of HPLC chromatography of the 50% acetone/ether fractions (Table 6) show that the concentration of $25(OH)D_3$ measured in pg/ml, increased significantly from 16 to 48 hours and that this increase was related to the dose of authentic $25(OH)D_3$ with which each bat has been injected. The production of the more polar metabolites, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$, increased with administered dose of 25(OH)D. In all situations, the levels of $1,25(OH)_2D_3$ produced were greater than those of the $24,25(OH)_2D_3$.

Table 6

Metabolites formed after the administration of $25(OH)D_3$ to fruit bats.

Blood Sample Collection Time	Dose of $25(OH)D_3$ administered (ng/bat)	Metabolites (pg/ml)		
		25(OH)D	$24,25(OH)_2D$	$1,25(OH)_2D$
16 hrs	2	$27.3\pm13.2_{(3,4)}$*	1.9 ± 1.4	$4.3\pm3.1_{(2)}$
	11	$69.5\pm63.6_{(3,5)}$	9.2 ± 9.5	16.6 ± 12.8
	21	$530.9\pm190.3_{(4,5)}$	$22.5\pm9.4_{(1)}$	$38.6\pm11.3_{(2)}$
48 hrs	2	24.3 ± 20.2	1.1 ± 1.2	2.6 ± 2.7
	11	233.4 ± 105.3	5.6 ± 2.2	14.8 ± 9.5
	21	444.4 ± 306.0	13.0 ± 8.4	21.9 ± 22.7

* mean $\pm$ SD, n=3 in each group.

Values with the same superscript differ significantly from each other.

(1) $p = 0.05$, (2) $p = 0.02$, (3) $p = 0.01$, (4) $p = 0.00$, (5) $p = 0.00$

The peak regions of both $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ off solvent system A were re-chromatographed on a second HPLC solvent system D, but insufficient cpm were present to separate the peaks from baseline and thus the data could not be intepreted.

4.4 Discussion

Given the absence of 25(OH)D in serum of all 39 bats studied, we investigated whether vitamin D was indeed converted to its more polar metabolites, in a similar manner to that previously reported in other mammals (Henry, 1992).

The most efficient way of examining the metabolism of vitamin D to its more polar metabolites uses radioactive tracers (Mawer, 1972). Initially [^{3}H]-vitamin D_3 was used in vivo in five bats on a vitamin D deficient diet. We were able to confirm that the labelled vitamin D_3 was hydroxylated to 25(OH)D_3, for the counts co-migrated with authentic 25(OH)D_3 in all three solvent systems used (Figure 12 *a*,b,c) using solvent systems similar to those used by other researchers (Jones, 1980).

We were unable to document further hydroxylation or metabolism of 25(OH)D in the 24 hour samples as the counts recovered in the serum were too small to detect 1α- or 24-hydroxylation.

Thus, in order, to by-pass the 25 hydroxylation step, in the second experiment (section 4.2.6), [^{3}H]25(OH)D_3 was given intraperitoneally. In this study, we were able to confirm the further hydroxylation of 25(OH)D_3 to the active hormone, 1,25(OH)$_2D_3$ as well as to 24,25(OH)$_2D_3$ (Table 6). The amount of 1,25(OH)$_2D_3$ produced was directly proportional to the concentration of 25(OH)D_3 administered (Table 6) at both 16 and 48 hours. This implies that the amount of substrate available for conversion is the limiting step (Table 6).

Mean 25(OH)D, 24,25$(OH)_2$D and 1,25$(OH)_2$D concentrations rose progressively with the dose of 25(OH)D given, however considerable variation existed in these concentrations between animals given the same dose. These variations might reflect variable absorption from the intraperitoneal site, different sizes of the animals and therefore different volumes of distribution, and/or different rates of metabolism.

These data confirm that bats, like all other mammals (Norman et al., 1982; DeLuca, 1984; Henry and Norman, 1984) possess the complete complement of enzymes for metabolism of vitamin D to 25(OH)D and ultimately to the active hormone, 1,25$(OH)_2$D.

That the fruit bat does have the vitamin D metabolite, 1,25$(OH)_2$D, in circulation has been shown. It is also shown that this animal has the ability to convert vitamin D_3 to its more polar metabolites with the use of tritium labelled vitamin D. The 1α-hydroxylase enzyme must therefore be present and capable of converting exogenous 25$(OH)D_3$ to the active hormone, 1,25$(OH)_2D_3$, in the in vivo situation. Thus, in its natural habitat, the fruit bat must have small quantities of vitamin D in its circulation.

It is of interest to note that after 25(OH)D administration the fruit bat was able to produce serum concentrations of 1,25$(OH)_2$D, within the range detectable by our radio-immunoassay (> 10 pg/ml), and not dissimilar from those measured in recently caught wild bats. The mammal was able to do this at 25(OH)D concentrations well below the level of sensitivity of the radio-receptor assay (4 ng/ml). The peak levels of serum 25(OH)D obtained after 25(OH)D administration were approximately 0.5 ng/ml.

These results suggest that the fruit bat probably does have circulating 25(OH)D in its wild state, but that these levels are too low to be measured by the radio-receptor assay.

$24,25(OH)_2D$ concentrations rose progressively with the increase in administered of 25(OH)D. In man $24,25(OH)_2D$ levels are usually about 10% of the 25(OH)D concentration (Mawer et al., 1972). Thus values in the ng/ml range are typically found. The present study indicates that the fruit bat is able to produce $24,25(OH)_2D$, but the concentrations obtained are almost 10^2 times lower than those found in man. In vitamin D deficient states, 1α-hydroxylase activity is markedly greater than that of 24 hydroxylase (Henry, 1992). The proportionally lower concentrations of $24,25(OH)_2D$ (Table 6) after the administration of $25(OH)D_3$ therefore suggest that fruit bats naturally have an impoverished vitamin D status. In vitamin D replete animals more $24,25(OH)_2D$ should be produced than $1,25(OH)_2D$. Even at the highest administered dose of 25(OH)D (21 ng/bat) $1,25(OH)_2D$ concentration was 1.7 times that of $24,25(OH)_2D$ (Table 6), suggesting that 21 ng/bat is insufficient to alter the vitamin D status of these animals.

Elevated 1α-hydroxylated activity, as indicated by the ratio of $1,25(OH)_2D_3$ to $24,25(OH)_2D_3$ might lead to the rapid conversion of most of the available 25(OH)D to the active metabolite. This could explain the absence of measurable 25(OH)D yet the presence of $1,25(OH)_2D_3$ albeit in lower concentrations than most mammals (Chesney et al., 1981).

4.5 Conclusion

Bats do possess the full complement of enzymes necessary to metabolize vitamin D_3 to the more polar metabolites, including the active metabolite, $1,25(OH)_2D_3$.

CHAPTER 5

The production of 1,25 dihydroxyvitamin D by the bat kidney (in vitro)

5.1 Introduction

In mammals $1,25(OH)_2D$ production occurs in the mitochondria of the proximal tubular cells in the kidney. Once it had been established that the fruit bat was capable of metabolising [^{3}H]-$25(OH)D_3$ to more polar metabolites, it was appropriate to investigate the 1α-hydroxylase activity of the kidney in an in vitro experiment.

5.2 Methods

5.2.1 In vitro 1α-hydroxylase studies

Two bats were sacrificed, and the kidneys removed and weighed, following the method of Henry (1980). The conversion of 25(OH)D to $1,25(OH)_2D$ by a kidney homogenate was assessed by the detection of $1,25(OH)_2D$ on HPLC. Several different eluents were used to separate non-specific metabolites of 25(OH)D from $1,25(OH)_2D$.

The kidney tissue was minced and homogenized with a Potter Elvjehlm homogenizer in 50 ml 0.015M Tris buffer pH 7.4. The mixture was transferred to a 200 ml conical flask and preincubated in a shaking waterbath at 37°C for 10 minutes. The reaction began with the addition of 1.25 ml 1M sodium succinate, plus 2.5 mg $25(OH)D_3$ in 50 μl ethanol. The incubation was continued in the shaking waterbath for a further 30 minutes. The reaction was stopped with the addition of 100 ml of methanol:chloroform 2:1. As a marker during the purification of the unlabelled $1.25(OH)_2D$ produced during

Figure 13.

LH-20 Sephadex chromatography of the extract of the kidney homogenate after incubation with 25(OH)D_3. 1% aliquots of each fraction were counted for tritium. The [3H]-1,25$(OH)_2D_3$ eluted in fractions 34-50.

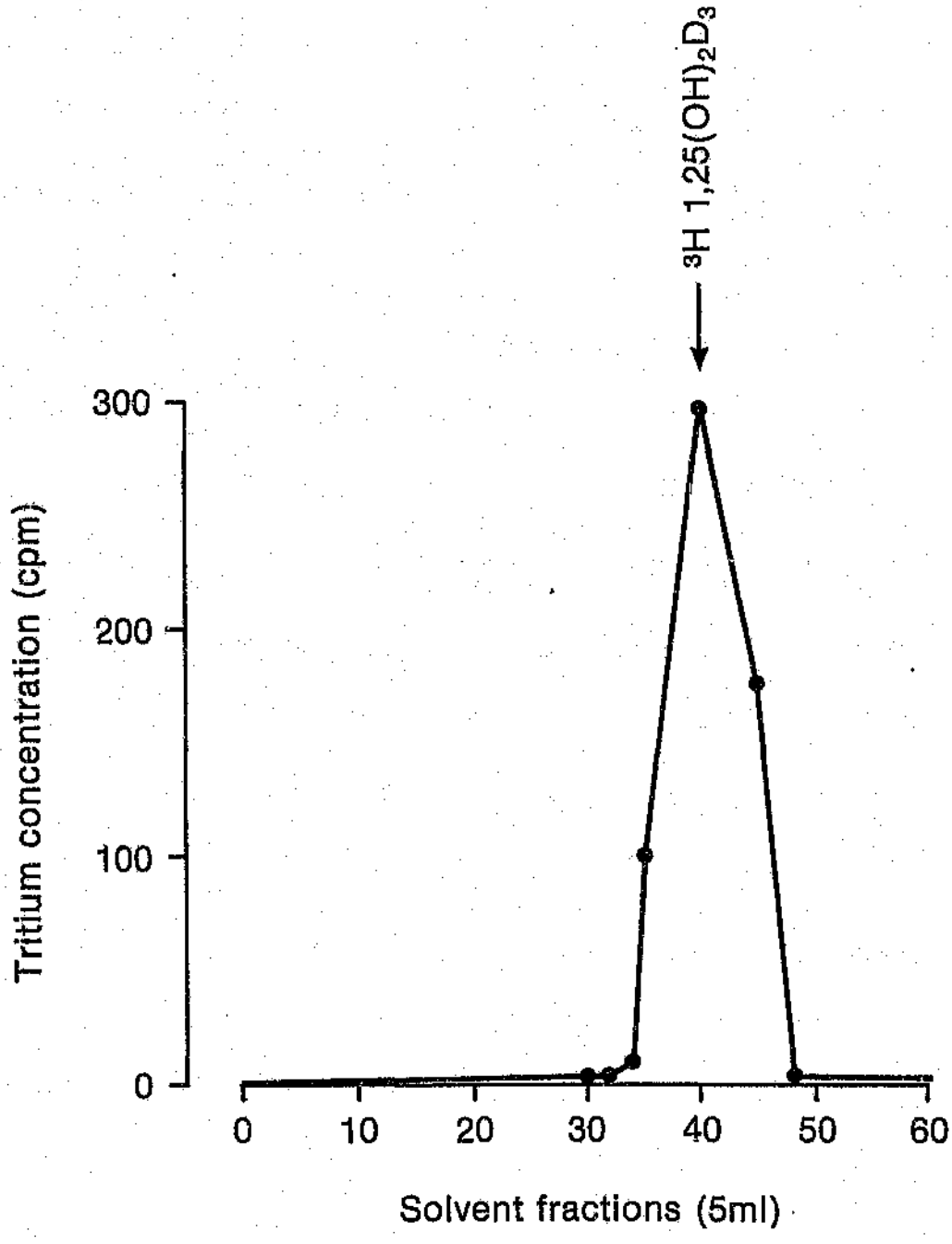

^{3}H 1,25(OH)$_2$D$_3$
Tritium concentration (cpm)
300
200
100
0
0
10
20
30
40
50
60
Solvent fractions (5ml)

Figure 14.

HPLC tracings of authentic metabolites as well as the tracings of the putatively purified $1,25(OH)_2D$ (as indicated on the graph by white arrows) using the 4 different solvent system A, C, F and G.

A



C



F



G



the reaction, 100 000 cpm (300 pg, 170 Ci/mmol) of [^{3}H]-1,25(OH)$_2$D$_3$ in 50 μl ethanol was added to the flask containing the total mixture once the reaction had been completed. The extraction was completed by the method of Bligh and Dyer (1959) as described in section 4.2.2.2.

The extract lipid was chromatographed on Sephadex LH-20 as described in section 4.2.3.2. Sixty fractions of 5 ml each were collected and a 1% aliquot of each fraction counted for tritium. The 1,25(OH)$_2$D$_3$ marker allowed for easy identification of the putative 1,25(OH)$_2$D region, namely fractions 34-50 (Figure 13). The two pools [fractions 1-32 (known 25(OH)D) and 34-50 (putative 1,25(OH)$_2$D)] were collected and evaporated down to dryness and the first pool stored in 10 ml hexane at -20°C until required.

The second fraction containing the putative 1,25(OH)$_2$D was prepared for HPLC by re-dissolving in 100 μl of the ternary solvent (A) described in section 4.2.4. Forty fractions of 1 ml were collected, of which 1% was counted, while a simultaneous UV scan was done at full scale absorbance of 0.128 (Figure 14). The fractions containing [H^3]-1,25(OH)$_2$D$_3$ were pooled and concentrated down to dryness, re-dissolved in 100 μl 3% isopropanol/dichloromethane (C), and re-injected onto the HPLC column, using solvent system C.

Thirty fractions (1 ml) were collected with the UV absorbance scale set to 0.064, and a 1% aliquot of each fraction was counted. The [H^3]-1,25(OH)$_2$D$_3$ fractions were pooled and concentrated down to dryness and re-dissolved in 100 μl 8% isopropanol/hexane

(F) and re-injected onto the HPLC column, using solvent system F. Forty fractions (1 ml) were collected and 1% of each fraction counted. Full scale absorbance of 0.032 was set for the UV monitoring. The [H^3]-1,25$(OH)_2D_3$ fractions were pooled and concentrated.

Prior to the reverse phase HPLC (solvent system G), the pooled putative 1,25$(OH)_2D_3$ fractions were concentrated down to dryness, re-dissolved in 0.5 ml ethanol and scanned over the range 300 nm to 220 nm in a Pye-Unican spectrophotometer (DeLuca, 1984). Three authentic 1,25$(OH)_2D_3$ standards were prepared at concentrations 0.5 μg/ml, 1.0 μg/ml and 5.0 μg/ml in ethanol, and scanned at the same time as the putative 1,25$(OH)_2D$ fraction. The concentration of 1,25$(OH)_2D$ was calculated from the absorbance. The ethanol was evaporated off the scanned material, re-dissolved in solvent system (G), and the remaining HPLC was performed. The putative 1,25$(OH)_2D_3$ fractions off the final HPLC solvent system G were pooled concentrated to dryness and re-dissolved in 0.5 ml ethanol.

The putative 1,25$(OH)_2D_3$ was diluted to a concentration of approximately 20 ng/ml ethanol. An aliquot was set aside for receptor binding studies. This was further diluted to two concentrations of approximately 60 pg/30 μl and the second to 900 pg/30 μl with ethanol. The 60 pg/30 μl solution was diluted to the same concentrations as those of the standards used to generate the standard curve in the receptor binding assay for the measurement of 1,25$(OH)_2D_3$ in serum samples. The 900 pg/30 μl standard was used undiluted in the curve as a non-specific binding standard. An authentic standard curve was generated and run in parallel to the putative 1,25$(OH)_2D_3$ curve as described in

section 3.1.1.3. Comparative graphs were drawn of the two receptor binding assays as described by Reichel et al. (1987).

5.3 Results

5.3.1 Kidney 1-hydroxylase In vitro Studies

The cpm in each of the resultant fractions off the LH-20 Sephadex column after chromatography of the extract from the incubated kidney homogenate are show in Figure 13. The radioactive peak which eluted in fractions 35-47, represents 0.1% of the added [^{3}H]-1,25(OH)$_2$D$_3$ counts.

Fractions 35-47 were pooled, concentrated down and injected onto the four consecutive HPLC solvent systems as shown in Figure 14.

Using solvent system A, a number of absorbance peaks were noted, of which two eluted in fractions 19-22 and 23-24 and migrated in the region of radiolabelled 1,25(OH)$_2$D$_3$ marker. Unlabelled authentic metabolites 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$ eluted in fractions 19-21 and 22-23 respectively when the system was being calibrated.

These fractions containing the [H^3]-1,25(OH)$_2$D$_3$ and putation 1,25(OH)$_2$D$_3$ were pooled, concentrated and injected onto the next solvent system C, which showed fewer absorbance peaks. Two absorbance peaks (fractions 24-30 and 31-33) eluted and

Figure 15.

(a) The UV tracing of authentic $1,25(OH)_2D_3$.

(b) The putative $1,25(OH)_2D$ scanned before reverse phase high pressure liquid chromatography. Note the similarity of the putative $1,25(OH)_2D$ tracing to that of authentic $1,25(OH)_2D_3$.

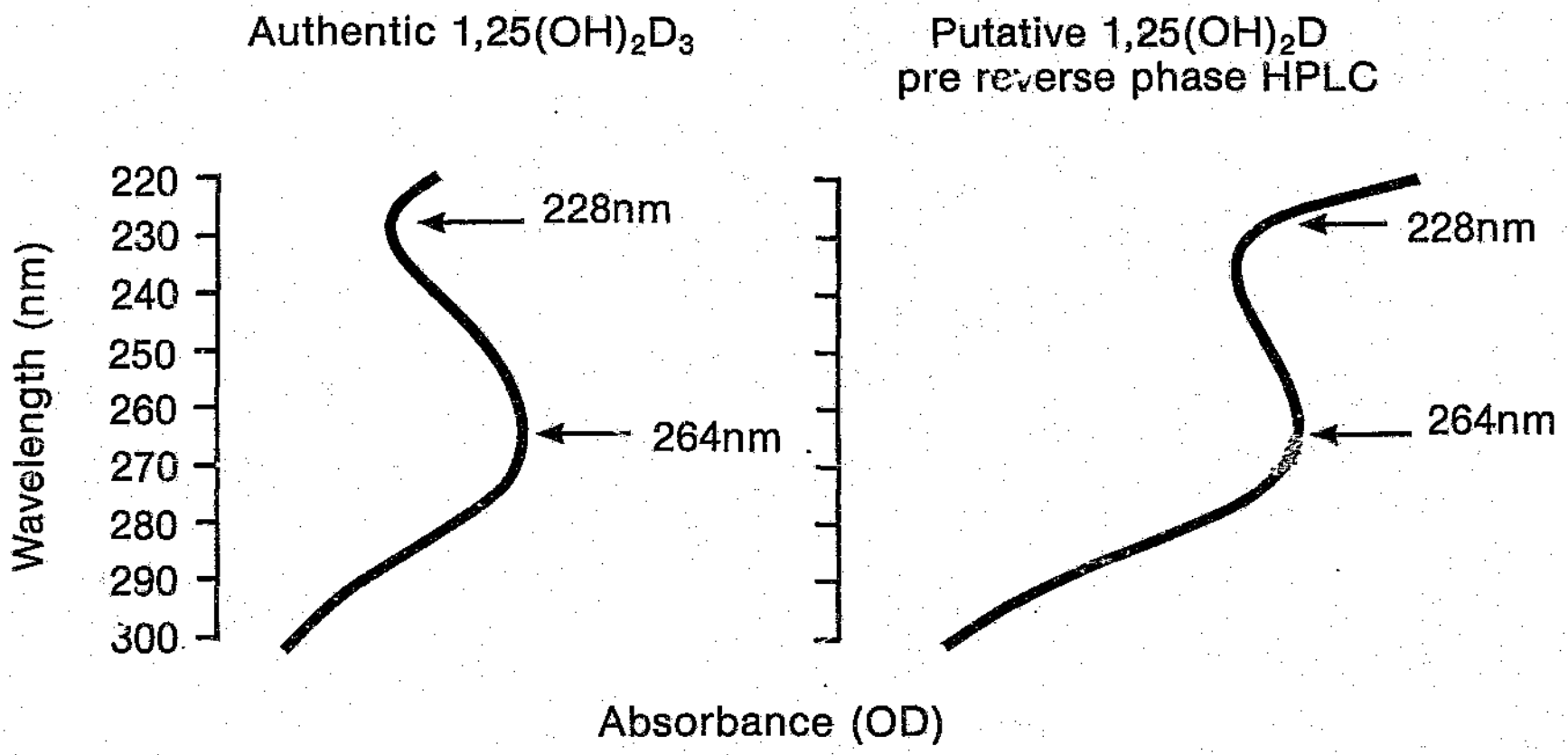
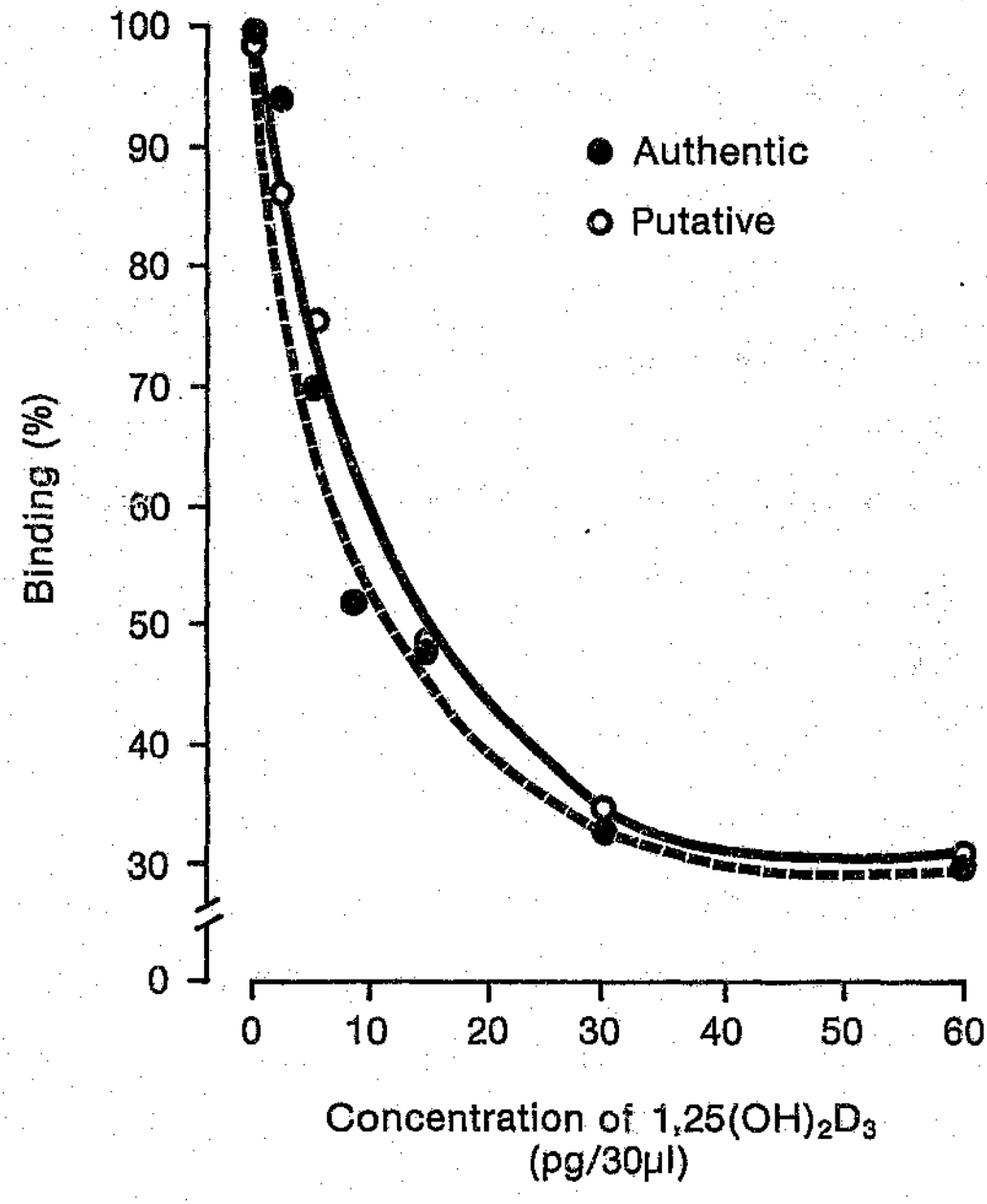

Authentic 1,25(OH)2D3
Putative 1,25(OH)2D
pre reverse phase HPLC
Wavelength (nm)
220
230
240
250
260
270
280
290
300
228nm
264nm
228nm
264nm
Absorbance (OD)

Figure 16.

The comparison of authentic $1,25(OH)_2D_3$ (●) and the putative $1,25(OH)_2D$ (o) obtained from the kidney homogenate incubation in a calf thymus receptor binding assay.


100
90
80
70
60
50
40
30
0
Binding (%)
Authentic
Putative
0
10
20
30
40
50
60
Concentration of 1,25(OH)2D3
(pg/30µl)

migrated in the same region as the [H^3]-1,25$(OH)_2D_3$. During calibration of the system, unlabelled 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ eluted in fractions 24-26 and 29-33 respectively.

Fractions 24-33 off system C were pooled and injected on to solvent system (F). Two major UV peaks eluted in fractions 2-5 and fractions 28-32. The latter peak co-migrated with [H^3]-1,25$(OH)_2D$. On calibration of system F, authentic 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ eluted in fractions 29-32 and 33-35 respectively. Fractions 28-32 containing the [H^3]-1,25$(OH)_2D_3$ from the above system were pooled and chromatographed on reverse phase HPLC using solvent system (G).

Only a single peak eluted in fractions 18-25. The [H^3]-1,25$(OH)_2D_3$ marker also eluted in this region. Unlabelled 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ eluted in fractions 16-20 and 21-24 respectively.

The scan of the putative 1,25$(OH)_2D_3$ obtained off the HPLC after the third solvent system, prior to the reverse phase HPLC was similar to authentic 1,25$(OH)_2D_3$ (Figure 15 a and b). In addition the putative 1,25$(OH)_2D$ showed similar binding characteristics to the authentic metabolite in the calf thymus receptor binding assay (Figure 16).

5.4 Discussion

Given the absence of detectable 25(OH)D in the serum of fruit bats but the presence of circulating $1,25(OH)_2D$ (Chapter 3) and the fact that bats can convert tritiated vitamin D_3 to the more polar metabolites (Chapter 4), we speculated that the bat has the renal 1α-hydroxylase enzyme necessary to convert 25(OH)D to the active metabolite, $1,25(OH)_2D$.

When homogenised renal tissue was given an excess of $25(OH)D_3$, as described by Henry (1980), the substrate was converted to putative $1,25(OH)_2D$, which by several methods appeared to be $1,25(OH)_2D$. The substance produced was purified using the techniques of Jones (1980). Furthermore the material was found to have a number of similarities to that of authentic $1,25(OH)_2D_3$ (DeLuca, 1984) when scanned over the UV range 300-220 nm (Figure 15).

We further confirmed that the substance was $1,25(OH)_2D_3$ following the methods of Reichel et al. (1987). They showed that the receptor binding assay used for measuring $1,25(OH)_2D$ metabolites in serum, was a reliable technique to compare the substance produced to authentic $1,25(OH)_2D_3$ (Table 6).

These findings therefore confirm that the 1α-hydroxylase enzyme was present and active in the kidney and was able to convert $25(OH)D_3$ to $1,25(OH)_2D_3$.

The 1α-hydroxylase enzyme has been found in the kidney of frogs (Kobayashi et al., 1991), reptiles (Takeuchi et al., 1991; Kobayashi et al., 1991), mammals and birds (Tanaka et al., 1976; Norman et al., 1984). Indeed all animals studied to date possess this enzyme. Although always present in the kidney, this enzyme and thus $1,25(OH)_2D_3$ production may occur extrarenally (Dusso et al., 1988). The enzyme has been found to be present in the liver, lung, macrophage cells, placenta, bone cells, embryonic intestine, and aortic endothelial cells (Dusso et al., 1988; Henry, 1992; Manoglesdorf et al., 1984). The control of the extrarenal 1α-hydroxylase enzyme is not PTH sensitive (Henry, 1992). It thus appears that the 1α-hydroxylase has a ubiquitous distribution and it is thus not surprising that it is present in bat renal tissue.

5.5 Conclusion

The experiments provided data which indicate that fruit bats possess an active 1α-hydroxylase enzyme in kidney tissue, and are able to convert 25(OH)D to $1,25(OH)_2D$.

CHAPTER 6

The purification of the vitamin D binding protein from the fruit bat

6.1 Introduction

In section 3.2.2, the bat was shown to have measurable levels of serum $1,25(OH)_2D$, but undetectable levels of 25(OH)D. Possible explanations for this phenomenon could include:

1) that the bat does not metabolise vitamin D in the usual way, possibly by-passing the formation of 25(OH)D,
2) that the bat does not possess a vitamin D binding protein, thus markedly reducing the circulating levels of 25(OH)D or
3) that the binding properties of the vitamin D binding protein (DBP) are different from those of DBP in other mammals.

The first explanation appears unlikely as in section 4.3 it was shown that the bat is able to metabolize [^{3}H]-vitamin D_3 to [^{3}H]-25(OH)D_3 and is able to convert [^{3}H]-25(OH)D_3 to [^{3}H]-$1,25(OH)_2D_3$. Furthermore the kidney possesses the 1α-hydroxylase enzyme, whose substrate is 25-OHD.

Thus attention was directed to determining whether or not the bat had a specific vitamin D binding protein, and if so, to characterise the binding protein.

Pilot experiments to assess the most appropriate techniques to purify DBP from serum were performed using baboon serum which was readily available in our laboratory. Later, experiments were conducted on pooled bat serum samples. Serial column

chromatographic techniques were employed in conjunction with a number of DBP identification techniques. The column chromatography techniques used were Affigel Blue chromatography followed by DEAE Sephacel chromatography (Gianazza et al., 1982; Taylor, 1986). The DBP identification techniques used were immunodiffusion, [^{3}H]-25(OH)D_3 binding, and finally SDS gel electrophoresis combined with western blot analysis.

6.2 Methods used in the Immunodiffusion Studies

Immunodiffusion studies were performed on human, baboon, chick, rabbit and bat serum samples. Furthermore, during the purification steps of the baboon and bat DBP fractions off the columns were assessed using this technique for the presence of DBP.

6.2.1 Ouchterlony Immunodiffusion

Ouchterlony immunodiffusion (Garvey et al., 1977) was used to test the cross reactivity of the DBP from different species with an anti-human DBP antibody.

A 2% agarose solution was prepared by dissolving two grams of agarose (Litex, Denmark) in 100 ml of a 0.05M sodium barbital buffer pH 8.6. Ouchterlony immunodiffusion plates were prepared in 6 cm disposable Petri dishes, by pouring a 1% agarose gel which was prepared by mixing 3.3 ml of 2% agarose and an equal volume of the sodium barbital buffer. The agar was allowed to set overnight at 4°C in a hydrated container. A circle of six wells, each with a 5 μl capacity was punched, using

a cutter and template kit (Miles Laboratories, England). A seventh well was punched in the centre of this circle, and into this well the antibody (5 μl), anti-human Gc-globulin (60 μl/ml) (Dako Laboratories, Denmark), was pipetted. The fractions (5 μl) to be assayed for DBP activity were pipetted into the outer wells.

6.2.2 Radial Immunodiffusion (RID)

To quantitatively measure DBP, a 1% radial immunodiffusion (RID) plate (Garvey et al., 1977), was poured as described in section 6.2.1 except that anti-human Gc-globulin was added to the agarose, (3.3 ml of the 2% agarose, 3.2 ml 0.05M sodium barbital buffer pH 8.6 and 100 μl of the anti-DBP antibody). The agar was allowed to set before 32 wells (5 μl) were punched. A human Gc-globulin standard (Sigma, St Louis) was diluted with distilled water to produce a range of standards from 5-60 μg/ml. The fractions to be assayed for DBP activity were applied neat and diluted 1/10, 1/100 and 1/500 into different wells so as to ensure the detection of a possible ring.

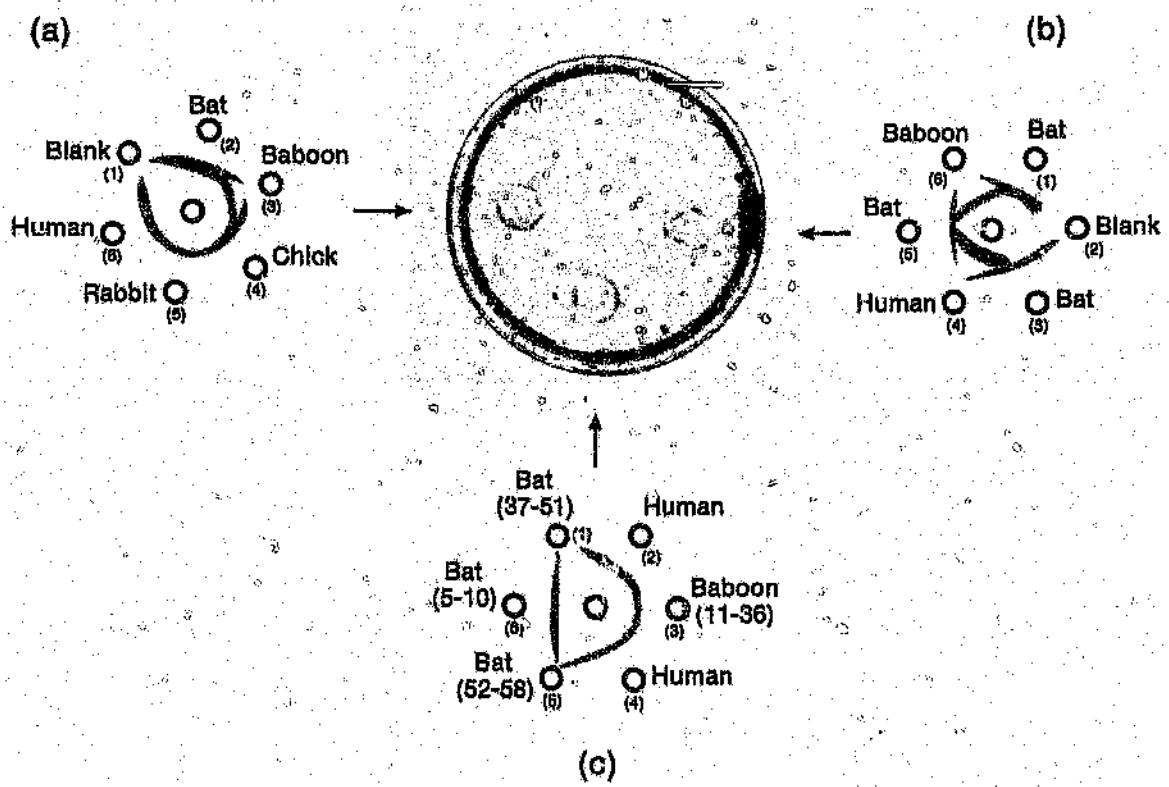
For both the Ouchterlony and RID plates, maximum diffusion of protein into the agar was completed after a 48 hour incubation at 4°C in the hydrated container. The wells were then filled with molten agarose and the plates washed in 5L 0.3M NaCl for 12 hours. The solution was changed to 5L 0.15M NaCl for a further 12 hours and the plates were then extensively washed in distilled water for two hours, prior to staining with 0.1% Coomassie blue in 45% absolute alcohol, 10% acetic acid and 45% distilled water for 15 minutes. The plates were de-stained in 10% acetic acid, 25% ethanol, 65% H_2O until all background staining has been removed.

Figure 17.

(a) The cross reactivity of bat, baboon, chick, rabbit and human sera to antihuman DBP antibody using the ouchterlony immunodiffusion technique. The bat and chick sera (wells 2 and 4 respectively) show a spur effect.

(b) Purified bat DBP fractions in wells 1 (37-51), 5 (52-58) and 6 (5-10). Human and baboon DBP are in wells 2, 3 and 4 respectively. Purified bat DBP (fraction 5-10) in wells 1, 3 and 5. A blank, human DBP and baboon DBP are in wells 2, 4 and 6 respectively, and spur effects between bat and baboon as well as bat and human are evident.

(c) Wells 2 and 4 contain human serum, well 3 contains the purified baboon DBP. The 3 bat pools off the Affigel Blue column are in wells 5,6 and 1 (52-59, 5-10 and 37-51), cross reactivity with human DBP antibody, occurred in bat pool present in well 6 (fraction 5-10).


(a)
Bat
(2)
Blank
(1)
Baboon
(3)
Human
(6)
Chick
(4)
Rabbit
(5)
(b)
Baboon
(6)
Bat
(1)
Bat
(5)
Blank
(2)
Human
(4)
Bat
(3)
Bat
(37-51)
(1)
Human
(2)
Bat
(5-10)
(6)
Baboon
(11-36)
(3)
Bat
(52-58)
(5)
Human
(4)
(c)

A precipitation line on the Ouchterlony plates became visible between the antibody and antigen in the agar (Figure 17). The rings around the wells in the RID plates were measured on a TG Calibrating Viewer (Transidyne General Corporation, Ann Arbor, Michigan) and the square of each of the diameters calculated. A graph was drawn of the concentration of human DBP standards against the square of the ring size of each of the standards. The concentration of DBP in the serum samples was read off the graph, having calculated the square of the diameters for each of the samples.

6.2.3 Identification techniques to determine DBP activity

6.2.3.1 [^{3}H]-25(OH)D_3 binding studies

The fractions collected off the Affigel Blue chromatography column (see Section 6.2.4 and 6.3.3.1) were assessed for [^{3}H]-25(OH)D_3 binding activity (Imawari and Goodman, 1977).

Aliquots (400 μl) from appropriate fractions were pipetted into test tubes and incubated with 10 000 cpm [^{3}H]-25(OH)D_3 (30 pg (160 Ci/mmol Amersham) in 50 μl ethanol). Included in this assay was a blank containing only 400 μl 0.03M sodium phosphate buffer pH 7.0 plus [^{3}H]-25(OH)D_3. The tubes were incubated at 4°C for 2 hours, after which 200 μl charcoal/dextran solution (Section 3.1.1.2) was added to each of the tubes except for a second blank pair to which was pipetted 200 μl of buffer. The tubes were centrifuged at 3 000 rpm for 10 minutes. Aliquots (500 μl) from each were transferred into counting vials, scintillant was added and each vial counted for 5 minutes.

6.2.3.2 Albumin determination

Albumin was measured by the method described by Doumas et al. (1971). A 0.06M solution of bromacresol green (BCG) pH 4.2 and 0.1M succinate buffer pH 8.4 were prepared. One part BCG was diluted with three parts succinate buffer to produce a working dye solution. A 25 μl sample of each fraction to be measured was diluted with 5 ml working dye solution. Included in the experiment was a known albumin standard (34 g/L) and a reagent blank. The tubes were vortexed and incubated for 5 minutes at room temperature before absorbances were measured at 628 nm in a dual beam spectrophotometer. The albumin concentration of the fractions was calculated and reported as g/L.

6.2.3.3 Total protein estimation

The total protein was measured using the Lowry Folin method of Lowry et al. (1951), which was modified to measure microgram concentrations of protein. A bovine serum albumin standard (Sigma, St Louis, 0.3 mg/ml) was diluted over the range 0-0.3 mg/ml. The fractions to be measured for total protein were diluted 1/50 with H_2O. To 100 μl of each of the standards and fractions in duplicate, 500 μl of a mixture of 2% disodium hydrogen carbonate ($Na_2(HCO_3)_2$, 1% copper sulphate and 2% sodium potassium tartrate in the ratio of 50:0.5:0.5 was added.

Exactly 15 minutes later 50 μl of Folin's reagent diluted 1:1 with H_2O was added. The tubes were vortexed and incubated in a dark cupboard for 30 minutes. Absorbances

were read at 750 nm and a graph drawn. The total protein was calculated taking into account the dilution factor of each of the fractions and their concentrations were reported in μg/ml.

An estimation of protein concentrations was also determined by measuring the absorbances of fractions in a dual beam spectrophotometer at 280 nm.

6.2.4 Purification of DBP from serum using affigel blue chromatography

To purify DBP a 5 ml pool of either baboon (Ross et al., 1982) or bat serum was dialysed against 4 litres of a 0.03M sodium phosphate buffer pH 7.0, over 24 hours. The serum was then centrifuged at 3 000 rpm for 10 minutes at 4°C, to remove any precipitated material.

A 2.6 x 9 cm column was packed with an Affigel Blue slurry (50-100 mesh, Bio-Rad) and equilibrated with 0.03M sodium phosphate buffer pH 7.0 (Gianazza et al., 1982). The dialysed serum sample was loaded onto the column at a flow rate of 1 ml/minute and 4 ml fractions collected. The column was eluted firstly with 0.03M sodium phosphate (120 ml). Thereafter a linear gradient prepared with 20 ml 1M NaCl in 0.03M sodium phosphate buffer and 20 ml phosphate buffer, was used to elute further proteins. This was followed by 20 ml of 1M NaCl. The final elution was performed with 100 ml of 0.5M sodium thiocyanate in 0.03M phosphate buffer pH 7.0.

To determine total protein content of each fraction, its absorbance was measured on a dual beam UV Spectrophotometer Pye Unicam at 280 nm (Fruton et al., 1958). DBP was measured using RID (Section 6.2.2), [^{3}H]-25(OH)D_3 binding was assessed as described in Section 6.2.3.1 and albumin measured as described in Section 6.2.3.2.

The fractions which contained the DBP were pooled and lyophilized and stored at -20°C until required for further purification.

6.2.5 Ion exchange chromatography

A 1 x 30 cm Pharmacia column was packed with DEAE-Sephacel (Pharmacia) using 0.5M Tris pH 7.5 as the equilibrating buffer at a flow rate of 0.3 ml/minute, as described by Taylor (1986). The lyophilysed fraction from the Affigel Blue chromatography containing DBP was dissolved in starting buffer (2 ml) and loaded onto the column. Ten fractions (10 ml) were collected and this was followed by a 0-0.4M NaCl gradient in the Tris buffer, 20 fractions (10 ml) were collected and the protein determined in all the fractions by absorbances at 280 nm.

The fractions were assayed for DBP activity using RID (Section 6.2.2) and by Western immunoblotting (Section 6.2.7).

6.2.6 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis as described by Laemmli (1970), was performed using a Mini PROTEAN II dual slab cell system (Bio-Rad, Richmond, CA). Two 15% separating gels, 75 mm thick were poured using the following reagents: 5.0 ml acrylamide, N,N-methylene-bis-acrylamide (bis:acrylamide, 0.8:30 g/100 ml) (Bio-Rad), 3.75 ml 1M Tris pH 8.8, 0.65 ml H_2O, 0.1 ml 10% sodium dodecyl sulphate (SDS) (Serva) and 0.5 ml 1.5% ammonium per- sulphate (APS) (Bio-Rad). To polymerize the gel 7.5 μl tetramethylethylenediamine (TEMED) was added. The solution was syringed as quickly as possible to a height of 120 mm between two glass plates, which were clamped together in the Bio-Rad gel pouring system. To allow for even polymerization a small volume of distilled water was carefully layered over the top of the gel. Once the gel had set this was removed.

A 4% stacking gel solution was prepared using the following reagents: 1 ml acrylamide (bis:acrylamide, 0.8:30 g/100 ml), 0.95 ml 1M Tris pH 6.8, 4.65 ml H_2O, 0.5 ml 80% glycerol (BDH), 0.075 ml 10% SDS and 0.35 ml 1.5% APS. To polymerize the gel 20 μl TEMED was added. A ten space comb was placed between the glass plates 2 mm above the separating gel. The stacking solution was syringed in between the glass plates and around the spacer which formed the wells (20 μl) into which the samples were loaded.

The samples for electrophoresis were diluted to an approximate concentration of 10-25 μg protein per well. An equal volume of sample buffer containing 0.125M Tris, 10%

b-mecaptoethanol, 4.6% SDS, 40% sucrose pH 6.8 (BDH) and 0.01% bromphenol blue as a tracking dye was added to each sample and the proteins denatured by heating in a boiling water bath for 5 minutes. The samples were then cooled and loaded into the wells with a micro syringe. The running buffer (0.025M Tris, 0.192M glycine and 0.1% SDS) was placed into the lower and upper buffer chambers and the gel was run under constant voltage with a Bio-Rad power pack model 3000/300, at 200 volts until the dye front was just visible at the bottom of the gel. The gel was removed from the system and stained for 10 minutes in a Coomassie blue stain (1.25 g Coomassie blue, 250 ml methanol, 50 ml acetic acid and 200 ml H_2O) prior to de-staining in a 10% acetic acid, 10% methanol and 80% H_2O solution until all background colour had been removed and only the protein bands were visible.

6.2.7 Immunoblotting; Western Blot Analysis

Fractions to be analyzed were diluted to a final protein concentration of 1 mg/ml. Two 15% SDS acrylamide gels were poured and parallel sets of the fractions loaded into corresponding wells in each of the gels. The proteins were electrophoresed and one of the gels was stained with Coomassie blue. The other was electrophretically transferred to a 0.45 μm nitrocellulose sheet using the standard Bio-Rad immunoblotting system (Tsang, 1983). The nitrocellulose sheet was presoaked in the transfer buffer (0.024M Tris, 0.192M glycine, 20% methanol), lowered on the gel which in turn had been placed on filter paper on a fibre pad. A second filter paper and fibre pad were overlaid and the cassette closed. The gel holder was placed into the tank together with an ice

pack and filled with transfer buffer. The proteins were transferred at 80 mA for 90 minutes.

The nitrocellulose sheet was removed and washed using 0.14M phosphate buffered saline (PBS), 10% Carnation Instant Non-Fat Dry Milk and 0.3% Tween*20 while gently shaking at room temperature for 1 hour. The strip was incubated for 1 hr in 40 μl DBP antibody, 20 ml PBS, 10% dry milk and 540 μl Tween*20 with gentle shaking and then washed three times with 25 ml PBS, 0.3% Tween*20. The final incubation solution, containing 200 μl peroxidase-conjugated swine anti-rabbit immunoglobulin complex (Dako) diluted with 20 ml PBS, 10% dry milk and 540 μl Tween*20, was added to the strip and was left shaking for 1 hour at room temperature. Following three 5 minute washes with 25 ml PBS, the colour was developed with 3 mg diaminobenzidine tetrahydrochloride (DAB) (Merck) and 8 μl H_2O_2 per 15 ml PBS. The colour development was stopped with extensive distilled water washes.

6.3 Results

6.3.1 Ouchterlony immunodiffusion of five different serum samples

The serum samples(bat, baboon, chick, rabbit and human) which were assessed for DBP activity against the human antibody (Gc-globulin, Dako) showed that all five sera cross reacted to this antibody. This result is shown in Figure 17(a). The bat and chick serum samples in wells number 2 and 4 respectively showed a spur effect to the human

antibody. The other samples (baboon, rabbit and purified human DBP) in wells 3, 5 and 6 showed cross reactivity to the antibody without the spur effect visible.

Thus bat serum contained a protein which cross reacted immunologically with the human DBP antibody. This result suggested that the bat has a vitamin D binding protein, which has immunological similarity to the human protein.

6.3.2 Results of the purification of baboon DBP

The fractionation of baboon serum on Affigel column chromatography gave four protein peaks which eluted in fractions 5-10, 37-42, 43-51 and 52-59, as shown in Figure 18.

Baboon DBP identified by RID (Section 6.2.2) was present in fractions 10-45. The maximum DBP activity was measured in fractions 18 and 42 with concentrations of 60 mg/L and 57 mg/L respectively. Using the binding of [^{3}H]-25(OH)D$_3$ (Section 6.3.1) as another indicator of DBP activity, there were two distinct binding peaks (Figu
The first occurred between fractions 8 and 36, and the second between fractions

The SDS PAGE electrophoresis results of the five pooled fractions 5-10, 11-36, 37-42, 43-51 and 52-59 are shown in Figure 19. A protein of similar molecular weight to pure baboon DBP was present in the pooled fractions 11-36 and 37-42. The last two absorbance peaks (43-51, 52-59) eluted off the column were predominantly albumin (Figure 18). Using the RID technique and human DBP as a standard (60 mg/L), most

Figure 18.

Affigel Blue column chromatography of baboon serum. A 2.6 x 9cm column was packed with Affigel Blue and 70 fractions (4ml) were collected. The fractions were each assessed for total protein at 280nm (—), albumin (- -) and [3H]-25(OH)D_3 binding (---). 5 peaks; fractions 5-10, 11-36, 37-42, 43-51 and 52-59 were noted.

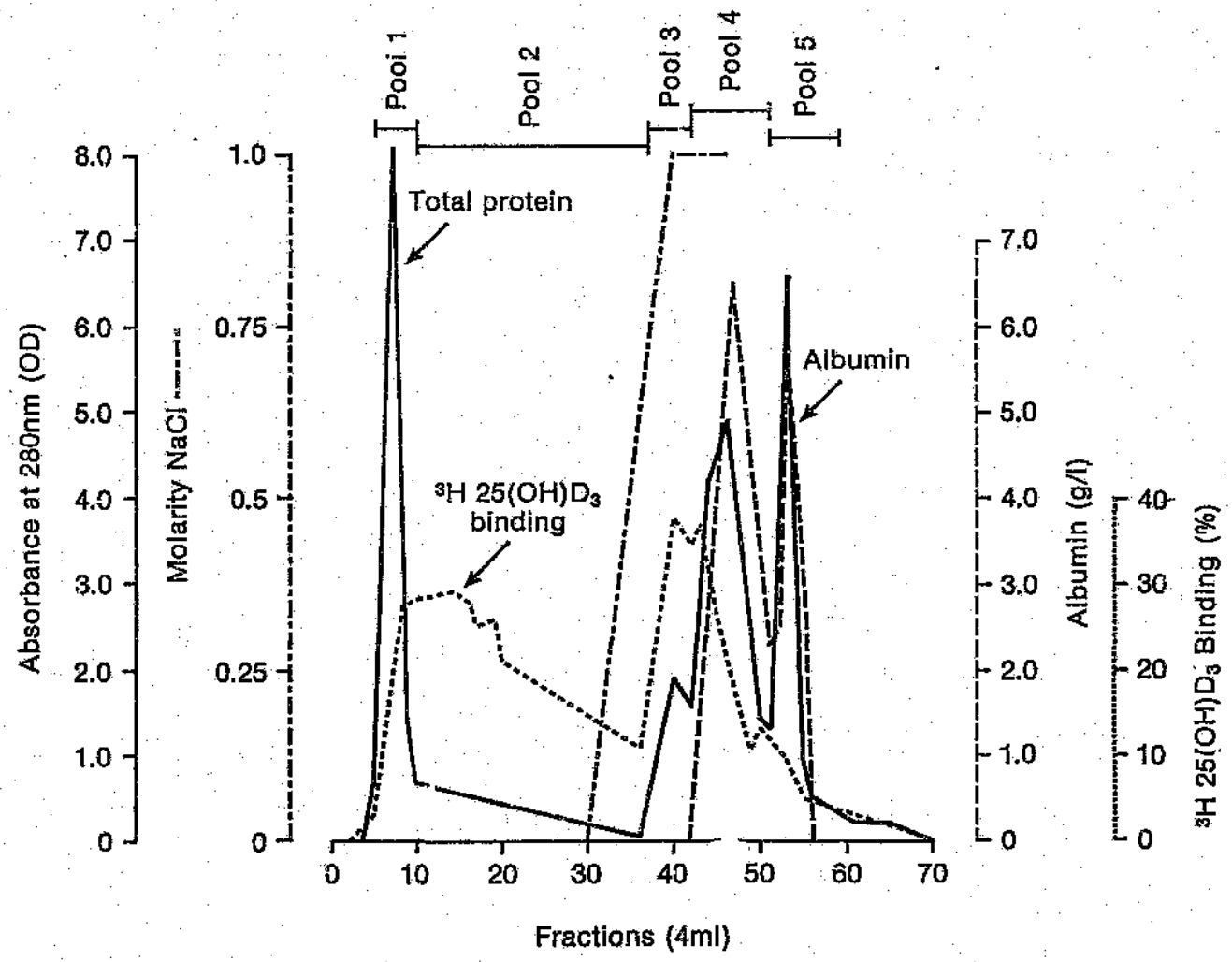
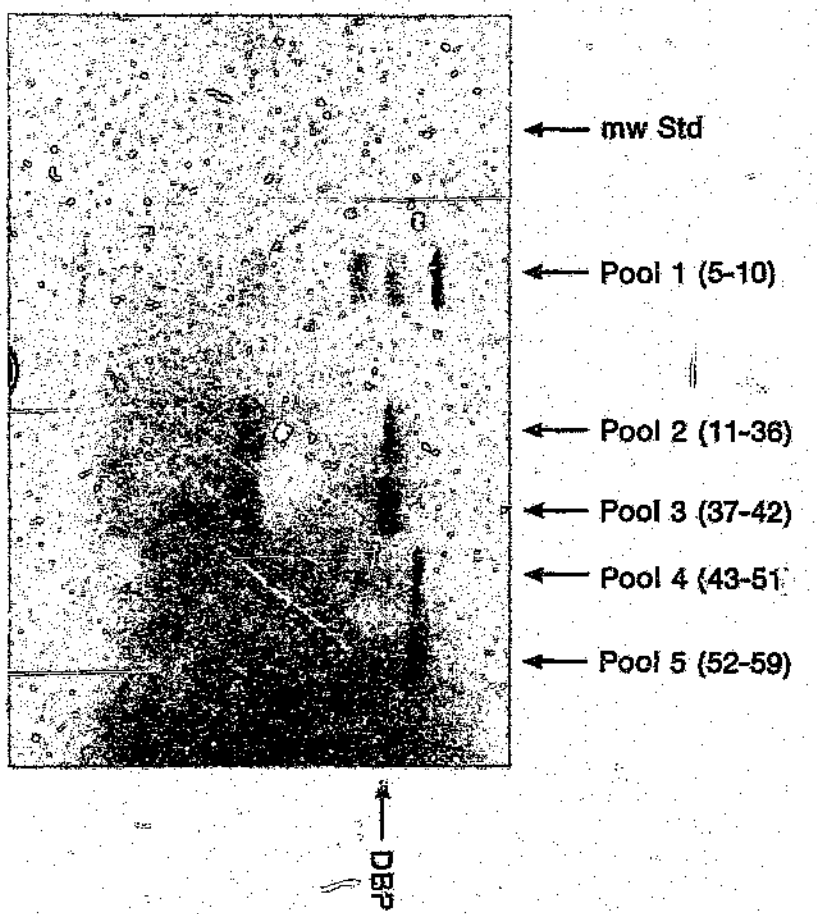

Pool 1
Pool 2
Pool 3
Pool 4
Pool 5
Total protein
Albumin
^{3}H 25(OH)D$_3$ binding
Absorbance at 280nm (OD)
Molarity NaCl
Albumin (g/l)
^{3}H 25(OH)D$_3$ Binding (%)
Fractions (4ml)
0
10
20
30
40
50
60
70
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1.0
0.75
0.5
0.25
0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
40
30
20
10
0

Figure 19.

15% SDS-PAGE electrophoresis of the pooled fractions obtained after Affigel Blue chromatography of baboon serum. Pool 2 (11-36) contained a single band which was identified as DBP by RID.


mw Std
Pool 1 (5-10)
Pool 2 (11-36)
Pool 3 (37-42)
Pool 4 (43-51)
Pool 5 (52-59)
DBP

of the DBP eluted in fractions 11-36. The apparent concentration of baboon DBP in this pooled fraction was 47.5 mg/L. The cross reactivity of the baboon DBP to the human antibody is clearly shown in Figure 17 (b and c) of a Ouchterlony immunodiffusion plate.

The total protein content of the pooled fraction 2 (11-36), measured by the Lowry method (Section 6.2.3.3) was 36.3 g/L.

6.3.3 Purification of fruit bat DBP

6.3.3.1 Affigel chromatography of bat serum

The analogous Affigel column separation of bat serum yielded three protein absorbance peaks, fractions 5-10, 37-51 and 52-58 (Figure 20). The second and third peaks were again identified mainly as albumin (Figure 20). Binding studies using [^{3}H]-25(OH)D_3 (Section 6.2.3.1) showed binding widely distributed in fractions 4-55, with poorly defined peaks present indicative of poor binding to [^{3}H]-25(OH)D_3. Using RID as a tool for identification of DBP activity, immuno reactivity was not detected in any of the individual fractions.

However when the three pools (pool 1 (5-10), pool 2 (11-36) and pool 3 (37-58)) were concentrated and assayed separately for DBP activity using Ouchterlony immunodiffusion, cross reactivity to the human DBP antibody was shown to be present in pool 1, fractions 5-10 (Figure 17 (b)).

Figure 20.

Affigel Blue column chromatography of bat serum. 70 fractions (4ml) were collected and each assessed for total protein absorbance at 280 nm (—), ablumnin (--) and [3H]-25(OH)D_3 activity (·-·). The 3 fractions pooled were 5-10, 11-36 and 37-58.

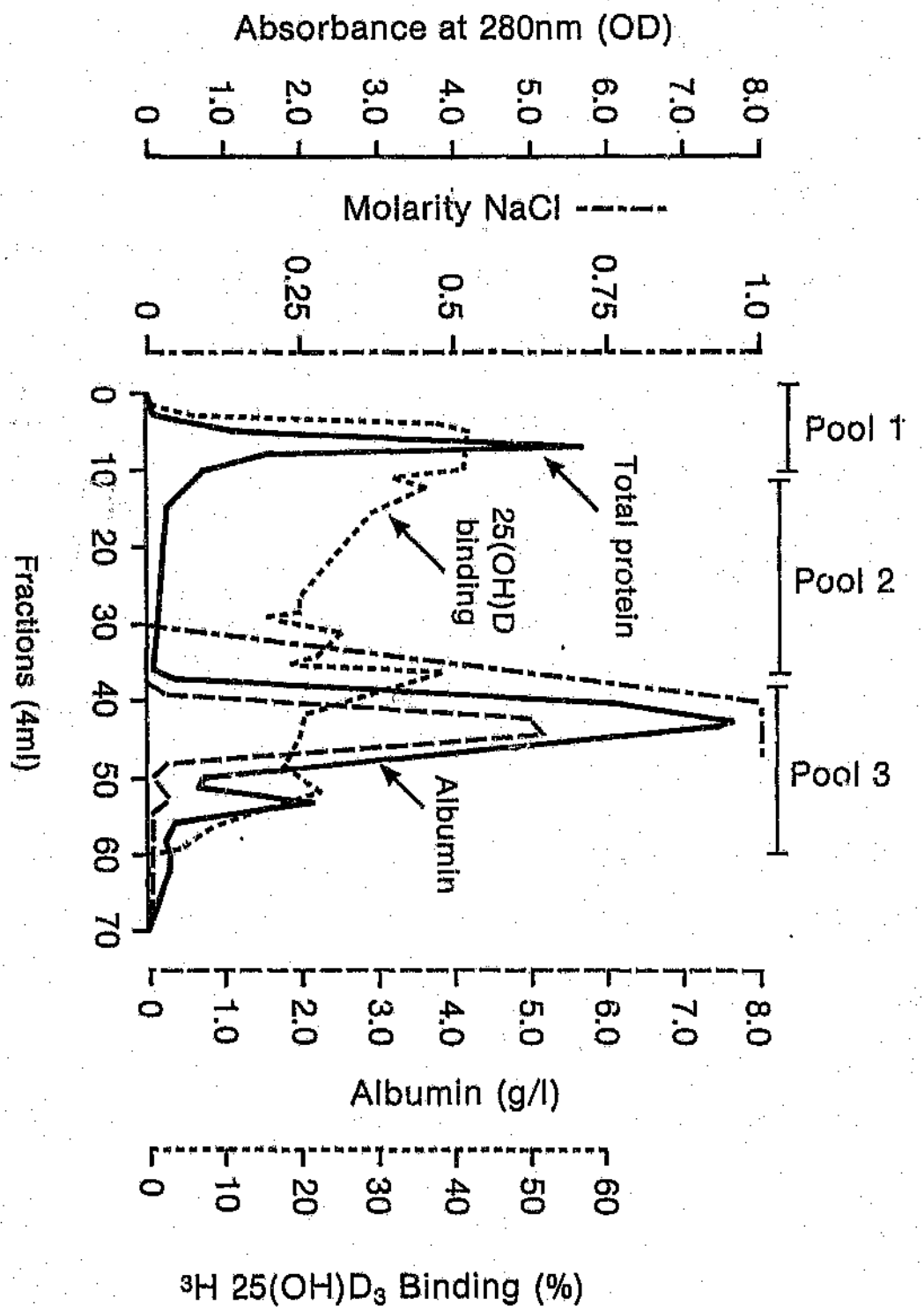

Absorbance at 280nm (OD)
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Molarity NaCl
0
0.25
0.5
0.75
1.0
Fractions (4ml)
0
10
20
30
40
50
60
70
Total protein
25(OH)D binding
Albumin
Pool 1
Pool 2
Pool 3
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Albumin (g/l)
0
10
20
30
40
50
60
^{3}H 25(OH)D$_3$ Binding (%)

Figure 21.

15% SDS-PAGE electrophoresis of the pooled fractions obtained after Affigel Blue chromatography. The bat DBP eluted in pool 1 (5-10).

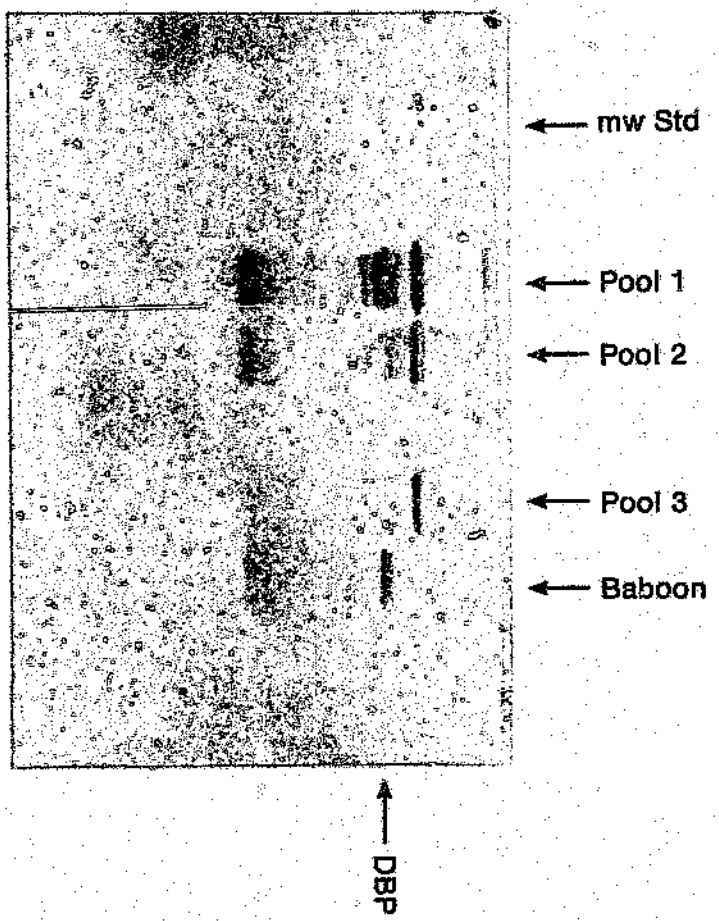

mw Std
Pool 1
Pool 2
Pool 3
Baboon
DBP

Figure 22.

15% SDS-PAGE electrophoresis of human DBP (lane 5) and bat DBP (lane 9), run against molecular weight standards (lane 2).

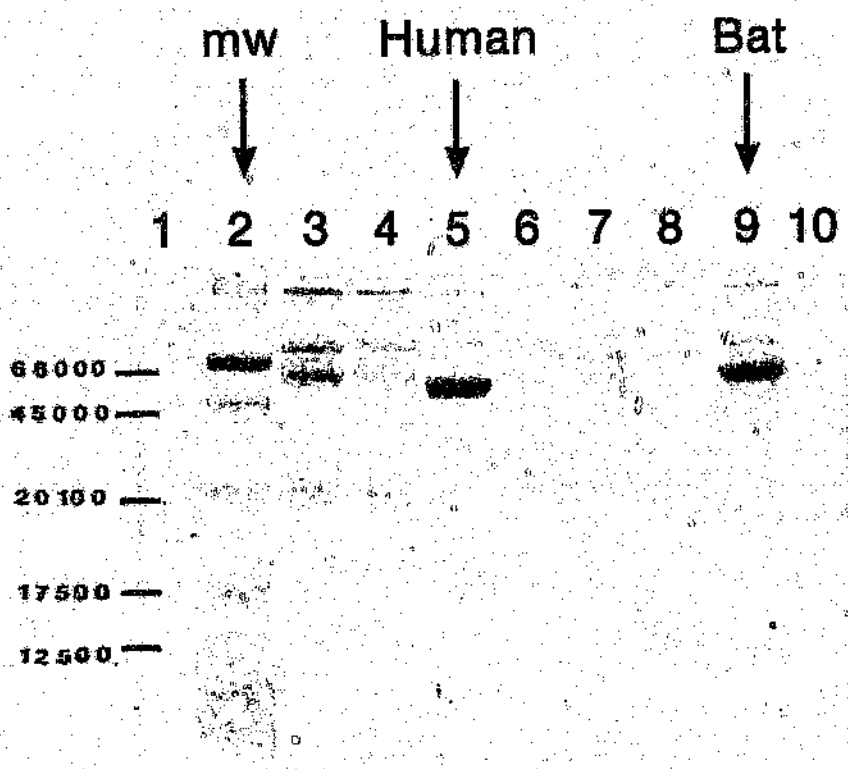
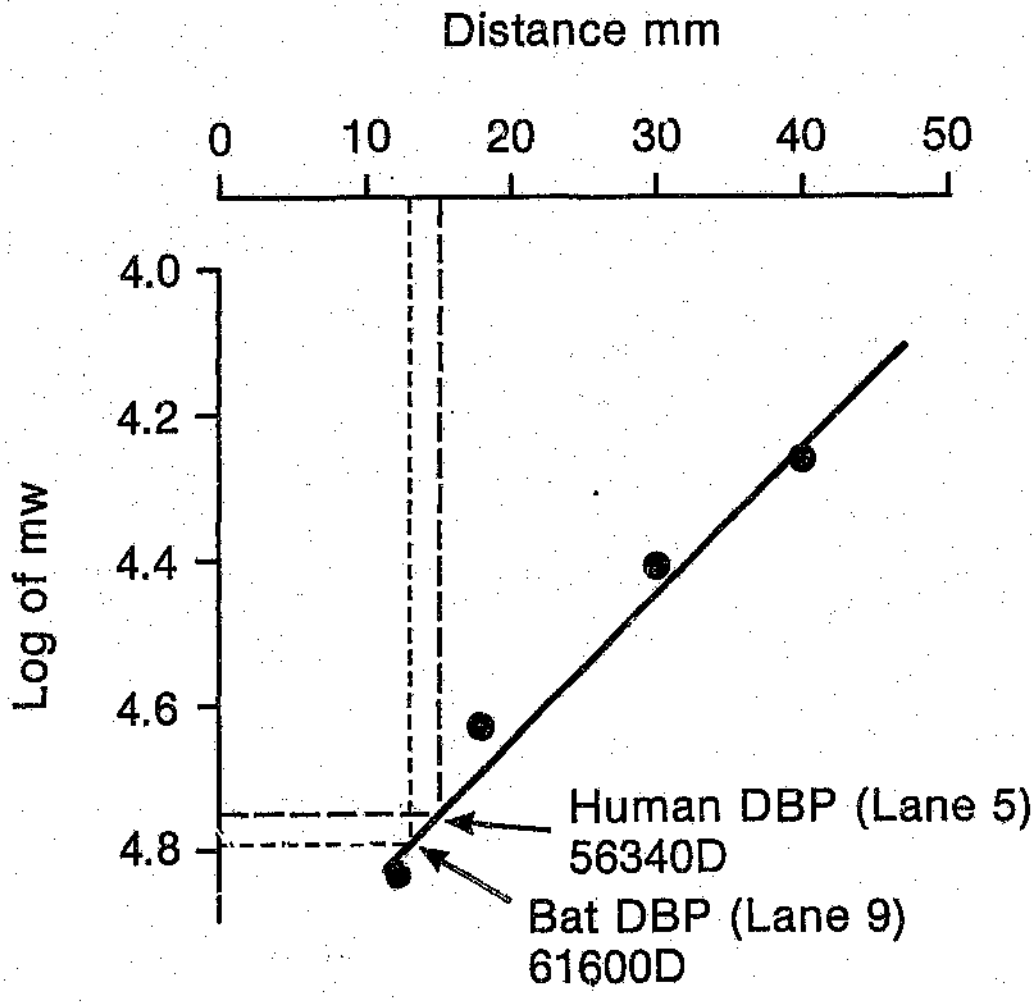

mw
Human
Bat
1 2 3 4 5 6 7 8 9 10
68000
45000
20100
17500
12500

Figure 23.

The determination of the relative molecular weights of the bat and human DBP determinated by SDS-PAGE electrophoresis. Human DBP had a mw of . .J and bat DBP had a mw of 61 600, using information extrapolated from figure 22).


Distance mm
0
10
20
30
40
50
4.0
4.2
4.4
4.6
4.8
Log of mw
Human DBP (Lane 5)
56340D
Bat DBP (Lane 9)
61600D

Electrophoresis was performed using known molecular weight standards, commercial human DBP and the partially purified baboon DBP, plus the three fractions off the bat Affigel column chromatography (Figure 21 and 22). There appeared to be a major protein band present in pool 1 (fractions 5-10) which migrated differently to the human DBP (Figure 22). This pool showed cross reactivity to the human DBP antibody as shown in Figure 17(b).

The molecular weight of this major protein was determined by the known MW marker proteins to be 61 600 Daltons as shown in Figure 23. This protein was considered to be fruit bat DBP by its immuno cross reactivity to the human DBP antibody (Figure 17). The size of the human DBP as determined in this same experiment was 56 340 Daltons (Figure 23).

6.3.3.2 Identification of the bat DBP after DEAE sephacel chromatography using immunodiffusion

In a further purification step of the pooled fraction 5-10 off the affigel column, the bat DBP eluted off the DEAE Sephacel column in fractions 18-21 as shown in Figure 24. These fractions showed poor immuno-reactivity to the antibody by RID.

Figure 24.

DEAE-Sephacel chromatography of the partially purified bat DBP. 30 fractions (10ml) were collected and absorbance of each measured at 280nm. Fractions were assessed for DBP activity by RID. Immuno-cross-reactivity was shown to be present in fractions 18-21.

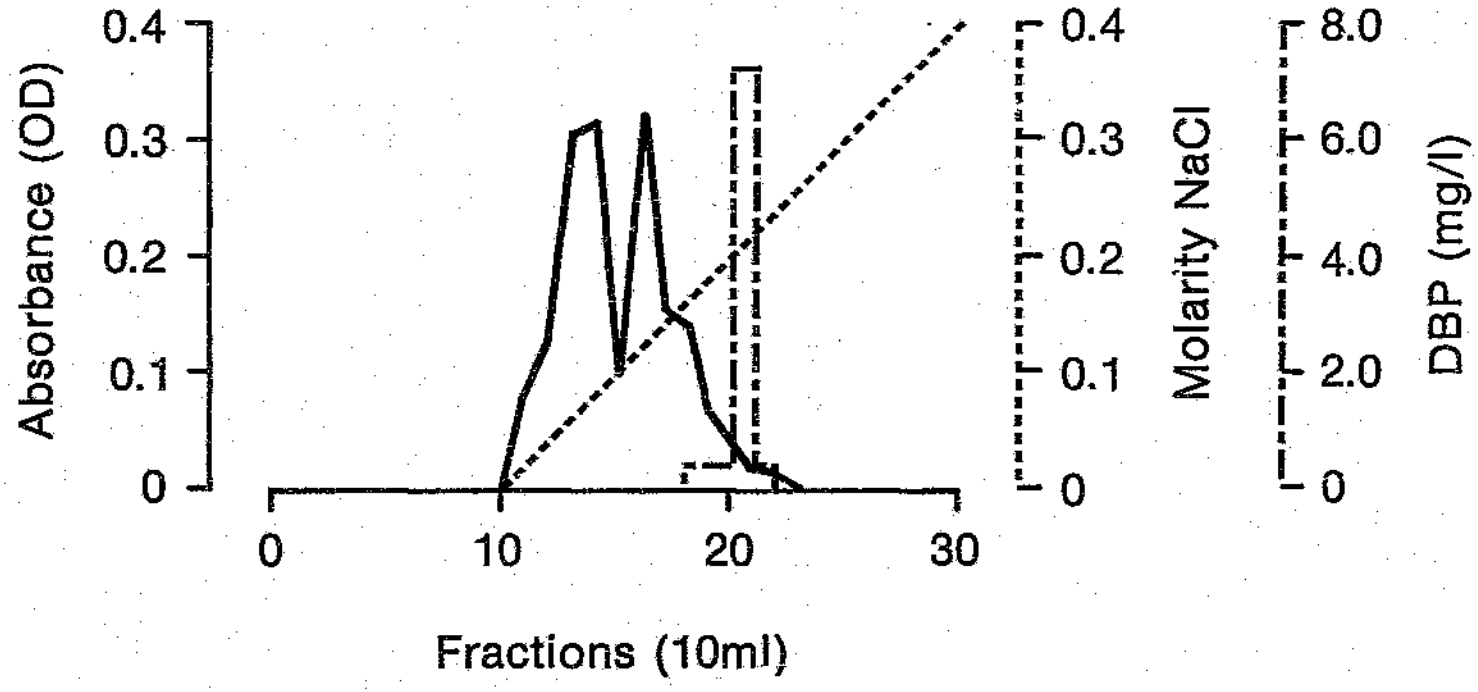
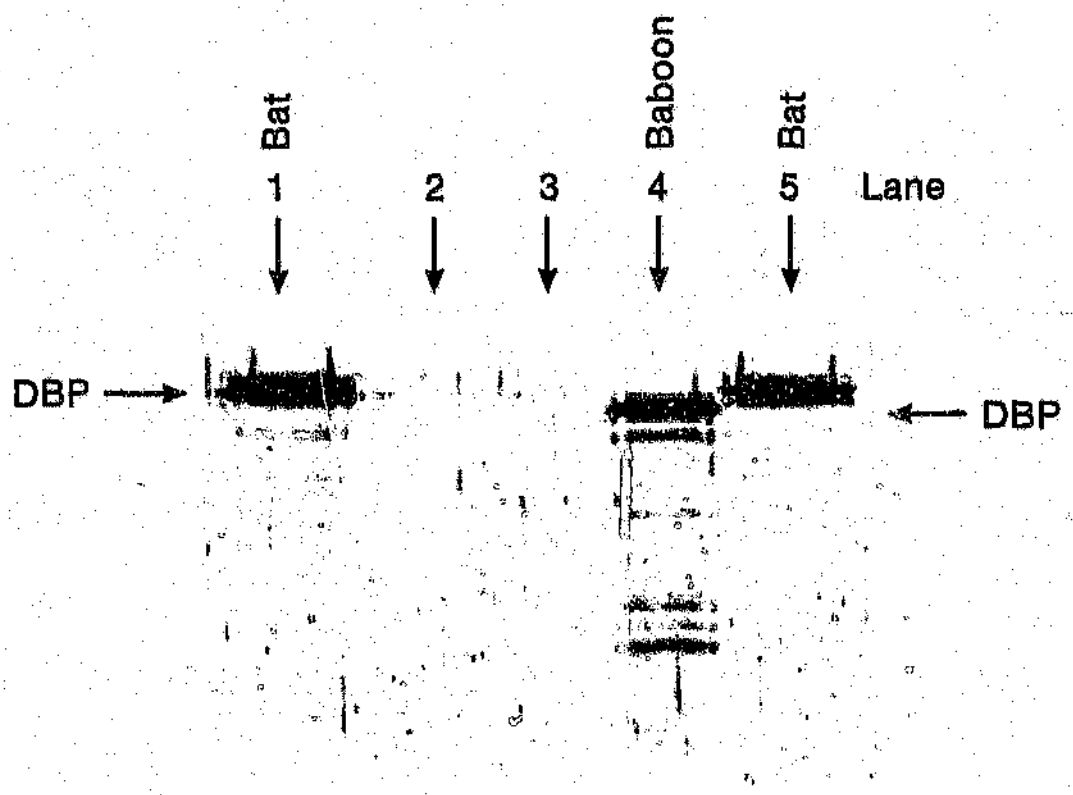

Absorbance (OD)
0.4
0.3
0.2
0.1
0
0
10
20
30
Fractions (10ml)
0.4
0.3
0.2
0.1
0
Molarity NaCl
8.0
6.0
4.0
2.0
0
DBP (mg/l)

Figure 25.

Western Immunoblot of the partially purified bat and baboon DBP.


Bat
Baboon
Bat
1
2
3
4
5
Lane
DBP
DBP

6.3.3.3 Bat DBP identification by Western Blot

Fractions 18-21 off the DEAE Sephacel column were electrophoresed and western immunoblotted onto nitrocellulose. Fractions 18 and 19 showed strong immuno cross reactivity to the human DBP antibody (Figure 25).

6.4 Discussion

Vitamin D and its metabolites in the serum are bound to a specific carrier protein. On investigation, the bat was found to possess a vitamin D binding protein. Fruit bat vitamin D binding protein appears to be different from those of other mammals, in that it eluted differently to baboon DBP on Affigel chromatography, on SDS electrophoresis, and on Western immunoblotting.

Immunodiffusion and immunoblotting confirm the presence of a substance that cross reacts with human DBP antibody and thus it is assumed to be DBP. Bats therefore like all other animals with skeletons have a circulating DBP that is capable of sterol binding with the various vitamin D metabolites (Hay and Watson, 1976 a and b; Haddad, 1984).

Although DBPs are phytogenetically conserved (Hay and Watson, 1976 a and b; Haddad, 1984), small differences in DBP structure and their binding properties may occur (Hay and Watson, 1976 a and b; Haddad, 1984). Fruit bat partially purified DBP

(61 600D) has a higher molecular weight than that reported for humans which was assessed at 58 000D by Haddad (1987) or at 56 340D in our laboratory.

Furthermore, cross reactivity of fruit bat DBP with the human antibody was poor, showing a spur effect on immunodiffusion which again confirms the fruit bat DBP is not identical to that of primates.

The fruit bat like many other animals such as fish, reptiles, birels and monotremes appears to have a DBP present (Hay and Watson, 1976 a and b). Fruit bat DBP does show poor binding to 25(OH)D (Figure 20), thus it is possible that 25-OHD in the bat is circulating largely in its free form, which could account for the undetectable serum concentration.

Low affinity of DBP for vitamin D metabolites could lead to an increase metabolic clearance of 25(OH)D (Bouillon, 1991) primarily through catabolism in the liver.

No comment can be made about the affinity of the bat DBP for any of the vitamin D metabolites as studies in this direction were not undertaken.

6.5 Conclusion

The fruit bat does have a detectable vitamin D binding protein. This binding protein has a molecular weight of 61600, which is higher than that of the human (56 340D). It does however have some similarity to human DBP. This fact is demonstrated by its cross reactivity (although poor) to the human DBP antibody by immunodiffusion analysis.

CHAPTER 7

The Utilisation of Vitamin D_2 or Vitamin D_3 by the Fruit Bat

7.1 Introduction

Having demonstrated that the bat has measurable levels of serum $1,25(OH)_2D$ and that the animal is able to convert vitamin D to 25(OH)D and $1,25(OH)_2D$, the source of vitamin D to nocturnal free-living frugivores was still unclear.

Vitamin D_3 is exclusively formed in the skin of animals under the influence of UV light, while the vitamin D_2 is dietarily obtained by its formation from the UV irradiation of ergosterol, a plant and fungal sterol. If the latter was shown to be the major circulating form of the vitamin, it would suggest that the source of vitamin D in the fruit bat is from plant material.

The question as to the source of vitamin D for the fruit bat remains unanswered.

It is possible that the low ultraviolet light intensity during crepuscular foraging is sufficient to facilitate vitamin D production. Evidence for this is provided by an in vitro study of vitamin D synthesis from 7-dehydrocholesterol. In Johannesburg although maximum vitamin D synthesis occurs around midday, significant amounts of previtamin D were formed between 08h00-09h00 and 16h00-17h00. It is thus possible that during the summer months, sufficient UV light might be available to produce vitamin D in the skin of bats in the early morning and evening (Pettifor and Holick - unpublished data pers comm). Alternatively, the bat might use a dietary source of this vitamin to maintain circulatory levels of $1,25(OH)_2D$. Possibly dietary sources are the fruit, that

these animals eat which might contain ergosterol. Fungi are known to produce vitamin D_3 (Gardner, 1988) and these fruit symbionts might be the vitamin D_3 source.

In order to determine whether endogenous vitamin D and metabolites in the serum of the fruit bat was derived from a vitamin D_2 or vitamin D_3 source, serum $1,25(OH)_2D$ was separated into either D_2 or D_3 metabolites by HPLC.

7.2 Methods

7.2.1 Preparation of the serum samples

The experiment was done on three serum samples each prepared differently. These were:- a pool sample of wild bat (1 ml) serum, a human (1 ml) sample and a human (1 ml) serum sample to which was added 200 pg $1,25(OH)_2D_2$/50 μl ethanol. A standard of 200 pg $1,25(OH)_2D_2$ plus 200 pg $1,25(OH)_2D_3$ diluted in 50 μl ethanol in 1 ml saline was used as a control.

The samples and standard were extracted, chromotographed as described in Section 3.1.1.1, and then subjected to HPLC (straight phase silica gel) using the solvent system (hexane:methanol: dichloromethane (85:5:7.5)) described by Jones (1980) which is known to separate $1,25(OH)_2D_2$ from $1,25(OH)_2D_3$. Forty fractions (0.5 ml) were collected, and evaporated to dryness.

Figure 26.

(a) The separation of authentic $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ eluted off HPLC using the solvent system hexane:methanol:dichloromethane (85:5:7,5) as measured by absorbance at 264nm.

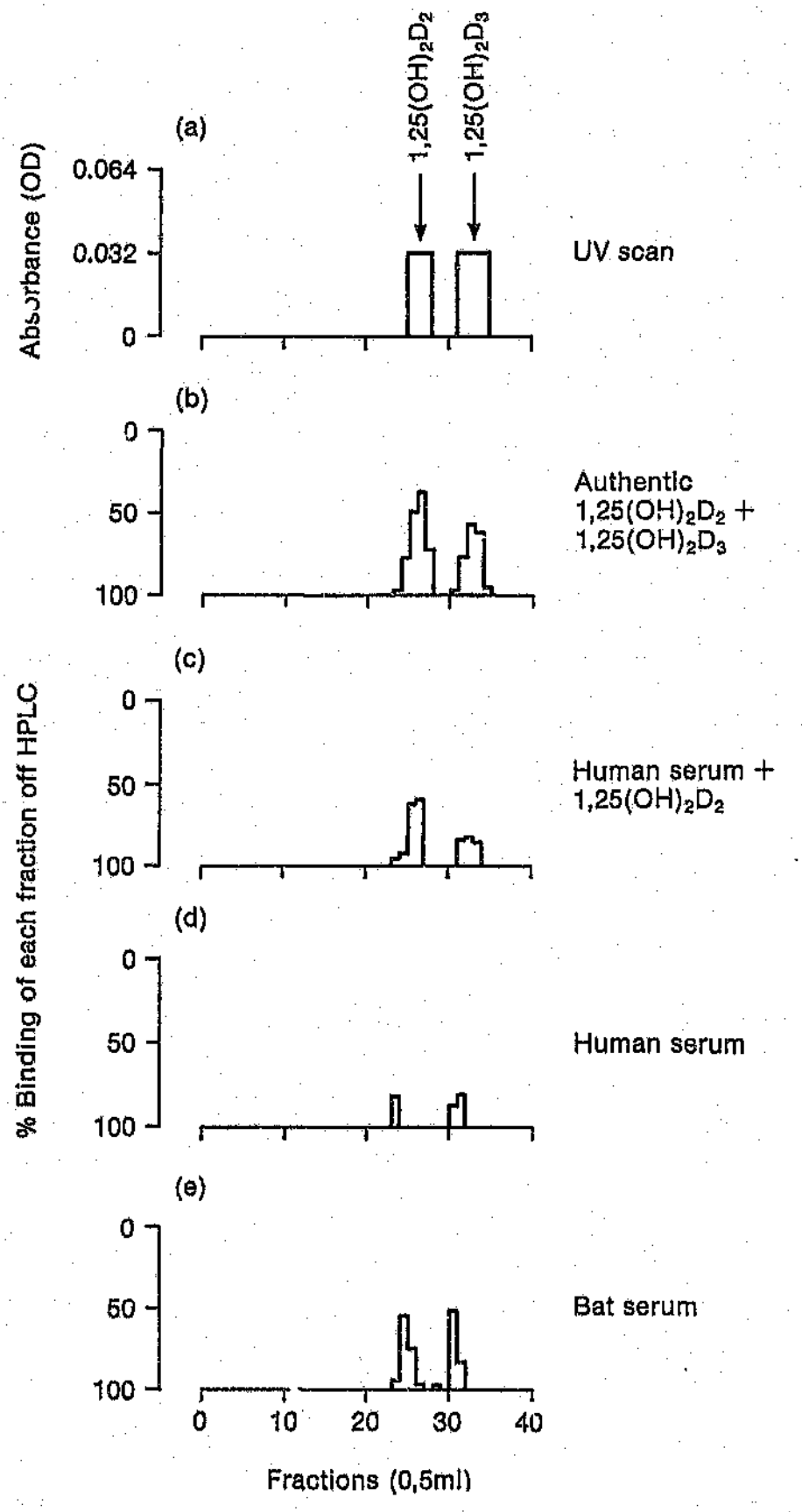
(b) Authentic $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$,

(c) human serum to which was added $1,25(OH)_2D_2$,

(d) human serum and

(e) bat serum.

The samples (b,c,d and e) were extracted and chromatographed by HPLC using the solvent system described in (a) and the eluted fractions (0.5ml) were measur[illegible] in the calf thymus receptor assay for $1,25(OH)_2D$ activity. 100% binding of ^{3}[H]$1,25(OH)_2D_3$ is equivalent to zero concentration of the $1,25(OH)_2D$ metabolite.


(a)
1,25(OH)2D2
1,25(OH)2D3
Absorbance (OD)
0.064
0.032
0
UV scan
(b)
0
50
100
Authentic
1,25(OH)2D2 +
1,25(OH)2D3
(c)
0
50
100
Human serum +
1,25(OH)2D2
(d)
0
50
100
Human serum
(e)
0
50
100
Bat serum
% Binding of each fraction off HPLC
0
10
20
30
40
Fractions (0,5ml)

As no radiolabelled $1,25(OH)_2D_2$ was available, the presence of $1,25(OH)_2D_2$ was detected by the use of the receptor binding assay. Each fraction was redissolved in 100 μl ethanol. Two 30 μl aliquots of each fraction were assayed for $1,25(OH)_2D$ activity using the calf thymus receptor binding assay as described in Section 3.1.1.3.

7.3 Results

7.3.1 Utilisation of vitamin D_2 or D_3 by the fruit bat

Figure 26(a) depicts the UV tracing of the fractions eluting off the HPLC column, after having been loaded with authentic $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$. $1,25(OH)_2D_2$ eluted in fractions 25-28 while $1,25(OH)_2D_3$ eluted in fractions 31-35. A clear separation of the two metabolites was obtained.

The radio-receptor binding assay, utilized to detect $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ yielded similar results (Figure 26 (e)). Once again, clear separation of the two metabolites was achieved.

Extraction and separation of the $1,25(OH)_2D$ metabolites in normal human serum yielded two distinct peaks, the first smaller peak in fraction 23 and the larger peak in fractions 30-31 (Figure 26(d)). Addition of authentic $1,25(OH)_2D_2$ to the serum prior to extraction, resulted in a marked increase in the size of the early peak, indicating that this peak was $1,25(OH)_2D_2$ (Figure 26(c)).

The extraction and chromotography of bat serum yielded two almost equally size peaks at the elution position of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ (Figure 26(e)).

7.4 Discussion

Although it has been established in Chapters 3, 4, 5 and 6 that fruit bats possess the enzymes for D_3 metabolism and naturally have measurable $1,25(OH)_2D$, the source of vitamin D is still not known. In the preceeding experiments an attempt was made to establish possible sources of vitamin D.

Vitamin D_3 is obtained from the consumption of animal products namely muscle and fat (Mawer, 1972) or endogenously synthesized after UV irradiation of the skin (Holick, 1984), whereas vitamin D_2 is obtained from the consumption of irradiated plant tissue (Boland, 1986). Recently bacteria have been shown to produce metabolites (oxy- and glycosylated) of vitamin D_3 (Gardner et al., 1988) and these metabolites are thought to be a source of vitamin D_3 in herbivorous mammals (Gardner et al., 1988).

Man, being an omnivore (consuming both plant and animal products) naturally has serum concentrations of both vitamin D_2 and D_3 metabolites (Mawer, 1972). Vitamin D_3 levels are usually substantially higher than the vitamin D_2 as man maintains his vitamin D status primarily by obtaining the required vitamin D from the conversion of 7-dehydrocholesterol to vitamin D under the influence of UV light. The diet generally contains little vitamin D unless the food is fortified (see Section 1.3). Human serum in

this study, showed measurable amounts of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ (Figure 26(d)); with $1,25(OH)_2D_3$ levels being approximately twice that of $1,25(OH)_2D_2$.

Some mammals discriminate against vitamin D_2 metabolites; the turkey (Horst et al., 1981) and pig (Horst et al., 1982) have no detectable $25(OH)D_2$, but measurable $25(OH)D_3$ levels (17.9 and 44.4 ng/ml respectively). The cow (Sommerfeldt et al., 1980) and chick (Horst et al., 1981) do have some $25(OH)D_2$, but appear to favour the $25(OH)D_3$ form of vitamin D. The horse has both $25(OH)D_2$ and $25(OH)D_3$, both of which being < 4 ng/ml (Horst et al., 1981), almost at non-detectable concentrations when measured using acceptable assay methodology. Sheep on the other hand appear to utilize both forms of vitamin D (Horst et al., 1981), although $25(OH)D_2$ appears to be favoured.

When authentic $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were eluted off the HPLC (Figure 26(a)) and assayed (Figure 26(b)) the peak $1,25(OH)_2D_2$ occurred at fractions 25-28 and the $1,25(OH)_2D_3$ at fractions 31-34. Eat serum shows similar peaks (Figure 26(e)) in fractions 24-27 and 31-32. These data clearly suggest the presence of both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ in bat serum.

Thus, bats like many other animals (Horst et al., 1981) probably acquire vitamin D_2 through the diet, but also produce some vitamin D_3 endogenously. The skin of fruit, a possible source of vitamin D_2, is generally discarded by these animals. However it is possible that during and after a meal while grooming (Chapter 1, Section 1.9),

vitamin D_2 is swallowed and although not directly chosen for the meal, the fruit skin could be the source of this vital substance.

Sources of vitamin D_3 may be more varied, gut flora (e.g. bacteria) might serve as a source of vitamin D_3, as they do in other herbivorous mammals (Gardner et al., 1988). Fungal growth on rotting fruit might also produce this vitamin which could then be dietarily acquired (Gardener et al., 1988). Another possibility is that bats might be unique amongst mammals and produce vitamin D_3 by endogenous means in processes that do not require the presence of ultraviolet light. This has yet to be tested.

7.5 Conclusion

The results of the preceeding study indicate that the serum of wild bats contain both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$. It is unclear how the bat obtains these two forms. It is possible that vitamin D_2 is obtained by dietary means from the skin of fruits. How the bat obtains vitamin D_3 is unclear. It is possible that fungi are a dietary source of D_3. Alternately fruit bats might receive some U.V. light during their dawn and dusk forrays to endogenously produce this substance. Further work in this area needs to be performed.

CHAPTER 8

Vitamin D metabolism in fruit bats: a synopsis

All animals [invertebrates (Kiever et al., 1988) and vertebrates (Norman, 1990)] require and utilize vitamin D. The fruit bat is no exception to this generalization in that it has the full complement of enzymes needed to use this endocrine system. However in the wild, the fruit bat has no obvious source of this vital hormone and appears to be naturally vitamin D deficient, without any pathological evidence that this presents problems to the animal.

8.1 Vitamin D Status

The principal circulating form [25(OH)D; chapter 3] was undetectable. Serum levels of 25(OH)D are routinely used as clinical indicators of vitamin D status (Audran and Kumar, 1985; DeLuca, 1984) and the lack of detectable 25(OH)D serum concentrations suggest an impoverished vitamin D status. Further evidence for this is the relative proportions (1:1.7) of the more polar metabolites ($24,25(OH)_2D$ and $1,25(OH)_2D$) produced after labelled $25(OH)D_3$ supplementation (Table 6). The greater degree of 1α-hydroxylase activity than 24 hydroxylase activity is indicative of a vitamin D deficient state. Elevated 1α-hydroxylase activity could lead to the rapid conversion of available $25(OH)D_3$ and explain undetectable levels of this, the principal circulating metabolite.

Recently, however, undetectable levels (<4 ng/ml) of 25(OH)D have been reported in several free ranging mammals [e.g. horses (Horst et al., 1981) and bathyergid mole rats (Buffenstein et al., 1991)] with no obvious pathological effects. Other vertebrate phyla most notably fish [cyclostomes (Lampreys), cartilaginous fish (sharks), teleosts (carp)] and amphibia [bull frogs] also have members with undetectable 25(OH)D concentrations

(Kobayashi et al., 1991; Takeuchi et al., 1991). These data suggest that 25(OH)D may not be as accurate an indicator of vitamin D status (Walters, 1991), but rather, the active hormone, $1,25(OH)_2D$ should be used as an indicator of vitamin D status. In fruit bats, $1,25(OH)_2D$ was present in both captive and wild bats (Table 3). These serum concentrations, however, (Table 3) are at the lower end of the normal range for mammals (Horst et al.,1981; Audran and Kumar, 1985). This could reflect a natural vitamin D deficient state, to which bats have adapted and which does not present pathological problems. The low levels of $1,25(OH)_2D$, naturally present, are therefore adequate to regulate physiological systems dependent on vitamin D and might indeed reflect the animal's evolutionary status and long term adaptation to their habitat.

8.2 Vitamin D endocrine system

The vitamin D endocrine system appears to be phylogenetically conserved throughout the evolutionary time frame, in that even invertebrates (Weiner et al., 1979) and primitive vertebrates appear to employ the full complement of enzymes (Kobayashi, 1991; Takeuchi et al., 1991). It is not surprising therefore that bats are similar to all other mammals studied to date (DeLuca, 1984; Haddad, 1987; Norman, 1982; Skinner et al., 1991). They possess the full complement of enzymes, as shown by the conversion of [^{3}H] vitamin D_3 to [^{3}H]-$25(OH)D_3$. This inturn was further metabolized to the active hormone, [^{3}H]-$1,25(OH)_2D_3$ (Chapters 4 and 5). Furthermore the binding protein (DBP), (Chapter 6) necessary for a functional vitamin D endocrine system, was present, and found to have a molecular weight of 61 600D (Chapter 6). This binding protein is slightly larger than that known for man (Haddad, 1987) and for baboon (Ross

et al., 1982). Size no doubt reflects species differences, as most species studied to date possess DBPs with unique molecular weights. The differences in size are, however, not thought to influence the functional properties of DBP (Haddad, 1987).

Opperman et al. (1990) have shown that fruit bats possess vitamin D dependant calbindins in the classic target organs. These findings confirm that the vitamin D endocrine system in fruit bats is not vestigial but rather is active, albeit at lower levels of active metabolite than normal vitamin D replete mammals. These concentrations may be sufficient to regulate mineral homeostasis.

8.3 Vitamin D metabolism

Vitamin D metabolism in fruit bats might be different to other mammals, in that 25(OH)D is undetectable in serum. These undetectable serum concentrations might be explained by: 1) a limited vitamin D substrate and 2) stimulated 1α-hydroxylase activity. As the animals are not exposed to sunlight and only forage before dawn and after dusk, they do not appear to have an endogenous source of vitamin D. The only dietary source of vitamin D could be that obtained from skins of fruit and fungal activity in rotting fruit. As there is only an intermittent and scarce supply of vitamin D, the vitamin D substrate could thus be limiting and the small amounts of 25(OH)D produced from this substrate would be rapidly converted to the more polar metabolites. Evidence that this is indeed the case, is that there is a graded increase in the production of both the renal dihydroxylated metabolites with increased 25(OH)D substrate (Table 6, Chapter 4).

It is well documented that the 1α-hydroxylase activity is elevated and the 24-(R)-hydroxylase suppressed when the vitamin D supply is limited (Henry, 1992). Relative ratios of the two renal vitamin D metabolites produced confirmed this to be true in the fruit bats, for twice as much $1,25(OH)_2D$ is produced in comparison to $24,25(OH)_2D$ (Table 6, Chapter 4). Elevated 1α-hydroxylase activity would use up the available 25(OH)L substrate and may explain the undetectable concentrations of 25(OH)D.

The regulation of vitamin D_3 homeostasis in the fruit bat appears similar to that reported for other mammals (Fraser, 1980) including; subterranean mole rats, (Buffenstein et al., 1991). In mole rats, which naturally have an impoverished vitamin D status the 1-hydroxylase activity was significantly increased compared to that of the 24-hydroxylase activity (Buffenstein et al., in press). The production of both $24,25(OH)_2D$ and $1,25(OH)_2D$ increased with increasing doses of 25(OH)D administered when vitamin D depleted animals received $25(OH)D_3$ (Chapter 4). The levels at the lower dose of $25(OH)D_3$ given (2.1 ng/bat) were below the detectable levels of $1,25(OH)_2D_3$ when measured in our receptor binding assay (section 3.1.1.3). The concentrations of the active hormone $1,25(OH)_2D_3$ produced during that experiment (Table 6) was similar to those found in freshly caught wild bats (Chapter 3). This finding suggests that wild populations of bats receive some vitamin D for animals given the highest $25(OH)D_3$ supplement (21 ng/bat) had higher $1,25(OH)_2D$ concentrations than freshly caught bats.

In captivity the diet of the bats was strictly controlled in that most of the fruit was peeled and/or was washed. Fruit bats, kept on this diet without vitamin D_3 exhibited serum $1,25(OH)_2D$ concentrations, which were 4 x less than that of the wild caught animals; suggesting that the fungal contamination of the unwashed fruit skin is a source of vitamin D. In the serum of wild bats both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were detected (Chapter 7), thus showing that there are two sources of vitamin D for the fruit bat. It is well documented that vitamin D_2 is present in plants tissue, Boland (1986), also found vitamin D_3-like substances present in plants which might explain the presence of both vitamin D_2 and D_3 in bat serum samples (Chapter 7). Alternately fungi, present in rotting fruit skins might be the source of vitamin D_3 (Gardner et al., 1988).

One can conclude that in the wild, vitamin D substrate is limited. Bats most likely do not endogenously synthesize vitamin D but rather rely on dietary sources for this vital compound.

8.4 Mineral homeostasis

Serum concentrations of calcium and magnesium in both freshly caught and long term captive colonies (irrespective of vitamin D_3 supplementation), were tightly regulated (Table 3). These data confirm that even in the absence of vitamin D_3 supplementation and with the naturally undetectable 25(OH)D serum concentrations, the endocrine system controlling mineral homeostasis functions adequately. This could imply that calcium homeostasis in the fruit bat is independent of vitamin D status, however the

tendency for serum calcium levels to be lower and to have greater variability in those animals kept in captivity on a vitamin D deplete diet suggests that vitamin D might be necessary, although the concentrations required appear to be lower than found in other mammals.

8.5 Conclusion

Fruit bats appear to belong to a small group of animals that naturally have limited access to vitamin D and have to be parsimonious in their vitamin D metabolism. All these animals are faced with no obvious source of vitamin D and/or inadequate exposure of the dermal layer of skin to the ultraviolet light. The primary source of vitamin D in fruit bats is therefore thought to be dietary, rather than endogenous (Chapter 7). This source is however limiting, with the result that the available substrate is converted to the active metabolite and little, if any, vitamin D is present in circulation as 25(OH)D (Chapter 3). The quantity of $1,25(OH)_2D$ produced, although comparatively lower than in most mammals, is sufficient to ensure adequate function despite the impoverished vitamin D status. The vitamin D endocrine system in fruit bats is thus finely regulated and no different in (a) its complement of enzymes (Chapter 4 and 5), (b) its mode of serum transport (DBPs, Chapter 6), and (c) action [CaBP (Opperman et al., 1990) serum mineral homeostasis, (Chapter 3)] to that in other mammals.

The fruit bat is thus well adapted to face the ecophysiological problems encountered in its milieu, especially with respect to an absence of an obvious source of vitamin D.

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