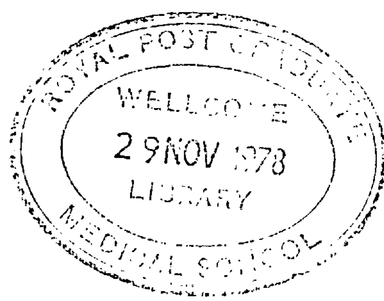


THE REGULATION OF VITAMIN D METABOLISM

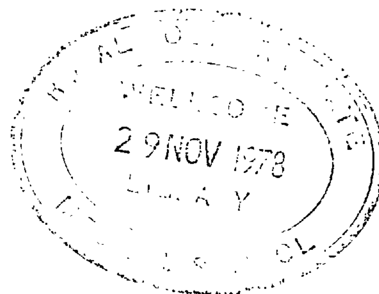
by

KAY WINIFRED COLSTON

Thesis submitted to the
UNIVERSITY OF LONDON
for the degree of
DOCTOR OF PHILOSOPHY

Endocrine Unit,
Royal Postgraduate Medical School, London.

1978.



ABSTRACT

The work presented in this thesis is divided into three sections.

Section I comprises Chapters One and Two. Chapter One is a literary review of vitamin D. This chapter recounts the major scientific developments from the initial observations of the vitamin's antirachitic properties to its recognition as a prohormone. Chapter Two describes the general methods used throughout the study.

Section II is made up of Chapters Three, Four and Five. Chapter Three describes the development of an *in vitro* assay for the renal 1-hydroxylase enzyme which converts 25 hydroxycholecalciferol (the major circulating form of vitamin D) to 1,25 dihydroxycholecalciferol, the most active metabolite. Chapters Four and Five describe how this assay has been utilized in *in vivo* studies to determine some of the factors which influence vitamin D metabolism in the whole animal.

Section III consists of Chapters Six, Seven and Eight. This section recounts *in vitro* experiments designed to investigate the intracellular mechanisms by which an acute control of vitamin D metabolism might be exerted. The studies described in Chapter Six were designed to investigate the *in vitro* properties of the renal hydroxylase enzymes. Chapter Seven describes studies with isolated mitochondria. Chapter Eight is a general discussion.

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S E C T I O N I

CHAPTER ONE

VITAMIN D - A REVIEW

Summary

Vitamin D is not a vitamin but a prohormone. Rickets was once considered to be a dietary deficiency disease, but sufficient vitamin D can be produced endogenously with a normal exposure to the ultraviolet light in the sun's rays. The anti-rachitic factor in cod-liver oil was finally identified in 1937, but it was not until the early sixties that the role of vitamin D as a prohormone began to be understood.

It is now recognised that vitamin D undergoes two metabolic conversions, in the liver and kidney, to a more active metabolite, 1,25 $(OH)_2D_3$. This steroid acts at its target organs (intestine, bone and kidney) by initiating nuclear changes which lead to new protein synthesis. The production of this hormone is regulated in an appropriate manner to maintain the required mineral content of the body. However, the exact mechanism of this regulation at the kidney level has been the source of some controversy over the past half decade.

1. Historical Review

Ths history of vitamin D begins with the history of rickets. Descriptions of infantile rickets were attributed to Homer in 900 B.C. and to Hippocrates. Soramus Ephesius (c. 130 A.D.) is credited with the classical description of rickets, referring to 'the back bone bending' and 'legs twisted at the thighs' in a disease noted to be commoner in smokey cities than in the country (Arneil, 1975). Later, in Leyden in 1645, Daniel Whistler drew attention to rickets in a dissertation "De morbo puerili Anglorum". A further description of the disease was published by Glisson in 1650. At this time, rickets became known as the English disease (Arneil, 1975).

The origin of the English word rickets is obscure: it may be derived from an Old English word 'wrick' meaning to twist, from 'wrygates' meaning crooked gate or from a Norman word 'riquets' meaning a hunchback. Alternatively, the word 'rachitic' probably is derived from 'rachis', which is the Greek word for the spine.

Rickets became prevalent in Britain with the advent of the industrial revolution, which led to an increasing concentration of poor people in the narrow, sunless alleys of factory towns and big city slums. The geographical relationship between rickets and industrial cities was noted in 1889 by the British Medical Association. In 1890, Palm published an essay on the distribution of rickets throughout the world and noted that rickets was common in large, crowded cities where people were deprived of sunlight. Leonard Findlay successfully induced rickets in experimental animals (Findlay, 1908), by confining puppies in cages. Control puppies which were not confined did not develop rickets. Findlay concluded that the development of rickets was not a dietary defect, but he wrongly attributed it to lack of exercise. It was Raczynski in 1912 who pointed to the lack of sunlight as the principal etiological factor in rickets (Loomis, 1970). In 1919, Huldschinsky showed that rickets could be treated by irradiation with ultraviolet (u.v.) light without alteration in the diet. He later extended this observation to show that, to be of value in healing rickets, u.v. light had to have a wavelength not greater than 302mμ. Huldschinsky further showed that an endocrine hormone must be involved. He irradiated one arm of a rachitic child with ultraviolet light and X-rayed both the child's arms. These X-rays

showed improved bone mineralization not only in the ultraviolet irradiated arm, but also in the other arm (Loomis, 1970).

Thus, by 1924, it had been shown that exposure to sunlight or ultraviolet irradiation of the body produced a hormonal factor which is capable of curing rickets. How was it then that this antirachitic factor became known as vitamin D? The concept of a dietary factor which was necessary only in small amounts to maintain health was propounded in 1912 by Casimir Funk at the Lister Institute in London, when he isolated an anti-beri beri substance. He proposed the name vitamine, an amine essential for life. The final 'e' is no longer used because many substances categorised as vitamins are not amines. There is evidence that by the 17th Century in Northern Europe and Scandinavia, cod-liver oil was used as a folk remedy for the prevention or cure of rickets. By 1807, cod-liver oil had been used for the treatment of osteomalacia and was the subject of a book by Bennet which was published in 1841. However, this author had confused scurvy with rickets (Arneil, 1975). It was not until 1917 that Hess showed unequivocally the effect of routine administration of cod-liver oil in a group of severely rachitic children (Hess and Unger, 1917).

However, this finding turned the attention of investigators away from the influence of sunlight and towards a dietary factor as the cause of rickets. In 1918, Mellanby produced rickets in dogs fed a diet of oatmeal, rice, salt and whole milk, and showed that cod-liver oil could cure the rickets so induced. The production of rickets in experimental animals by dietary means was in accord with

the new 'vitamine' theory of Funk, and was greeted with enthusiasm - even though Findley in Glasgow had produced experimental rickets in dogs 10 years earlier with no alteration in diet, but by simply confining the animals indoors. Mellanby further suggested that the efficacy of cod-liver oil was due to the newly discovered vitamin A. This theory was disproved by McCollum and co-workers in 1922 (McCollum, Simmonds, Becker and Shipley,1922), who treated cod-liver oil by bubbling oxygen through it. This destroyed the anti-xerophthalmic properties but left intact the anti-rachitic properties, indicating that the anti-rachitic factor was not identical with vitamin A. McCollum called this anti-rachitic factor 'vitamin D' and the name gained general acceptance. Rickets was thus considered as a vitamin-deficiency disease.

2. Characterization of the Anti-Rachitic Factor

The relationship between sunlight and vitamin D was not resolved until 1924 when Steenbock and Black discovered that a rachitogenic rat diet cured rickets when irradiated with ultraviolet light, and that rachitic rats themselves were cured when so irradiated (Steenbock and Black, 1924). Soon afterwards other workers (Hess and Weinstock, 1924) independently reported that foods containing no anti-rachitic activity (such as cottonseed oil, linseed oil, wheat or lettuce leaves) acquired anti-rachitic activity on irradiation with ultraviolet light. Further experiments (Hess, Weinstock and Helman, 1925; Steenbock and Black, 1925; Rosenheim and Webster, 1925) demonstrated that it is the sterols in foods that are activated,

particularly the cholesterol containing fraction, and that irradiation of apparently pure cholesterol confers upon it anti-rachitic activity. Extension of this work (Rosenheim and Webster, 1927; Hess and Windaus, 1927) showed that further purification of cholesterol led to the loss of ability to acquire the anti-rachitic activity on irradiation. Clearly, impure cholesterol contains a provitamin which is activated by ultraviolet light. It was at first postulated that this provitamin was similar in chemical nature to ergosterol, a sterol which had previously been found to be activated by ultraviolet irradiation (Rosenheim and Webster, 1926), or a highly unsaturated sterol of similar constitution.

Askew (Askew, Bourdillon, Bruce, Jenkins and Webster, 1930) and Windaus (Windaus, Linsert, Lüttringhaus and Weidlich, 1932) independently prepared the anti-rachitic sterol by irradiation of ergosterol. Askew called the product 'Calciferol'. Windaus called it 'Vitamin D_1'. However, it was discovered that the products from both laboratories were impure and that irradiation of ergosterol produced a succession of compounds, only one of which was anti-rachitic and some of which were toxic. Both laboratories succeeded in purifying the anti-rachitic compound; Askew retained the name 'calciferol' but Windaus called the purified product 'Vitamin D_2'. Thus there is now no such compound as Vitamin D_1.

At this time it was thought that ergosterol was the main provitamin D. However, Waddell (Waddell, 1934) showed that cod-liver oil and the irradiation product of non-purified cholesterol had a

greater anti-rachitic activity in chicks than did irradiated ergosterol. He concluded that the provitamin constituent of non-purified cholesterol was a substance differing from ergosterol. In 1936, Windaus succeeded in preparing the compound 7-dehydrocholesterol from cholesterol. Irradiation of this product produced another anti-rachitic substance which Windaus called 'Vitamin D_3' (Windaus, Lettré and Schenk, 1935). This work was extended by the isolation of 7-dehydrocholesterol from the skin (Windaus and Bock, 1937) and Schenk (Schenk, 1937) performed the final identification of Vitamin D_3.

3. The Biosynthesis of Vitamin D_3

Vitamin D_3 is normally produced in adequate amounts in man and animals with sufficient exposure to the sun's ultraviolet rays. It is only when exposure to sunlight is severely restricted that vitamin D becomes an essential dietary factor (Loomis, 1970). The study of the biosynthesis of vitamin D became concentrated on the synthesis and activation of its precursor, 7-dehydrocholesterol.

Cornforth, Popjak and co-workers (Cornforth, Hunter and Popjak, 1953) demonstrated the synthesis of cholesterol from slices of liver using C^{14}-labelled acetate and deduced that squalene was an intermediate in the cholesterol biosynthesis pathway. Later studies have shown cholesterogenesis in most mammalian tissues but the major sites are the liver and gastrointestinal tract. The basic routes are thought to be the same in all tissues.

The formation of 7-dehydrocholesterol (provitamin D_3) is the penultimate step in the conversion of lanosterol to cholesterol (Dempsey, Seaton, Schroepter and Trockman, 1964). However, some 7-dehydrocholesterol may arise in the intestine from dietary cholesterol (Glover, Glover and Norton, 1952). In 1954, Miller and Baumann identified the presence of 7-dehydrocholesterol in the skin of mammals (Miller and Baumann, 1954) and it was further shown that the main site of sterol biosynthesis in the skin is the epidermis (Nicolaides and Rothman, 1955). In 1964, Gaylor and Sault demonstrated that the 7-dehydrocholesterol can be synthesized locally in the skin. They showed that skin slices incubated in vitro incorporated ^{14}C-labelled acetate into 7-dehydrocholesterol. Thus the precursor to vitamin D_3 is found in the epidermis of the skin and can be synthesized there. It has further been demonstrated that any 7-dehydrocholesterol thus situated may be susceptible to ultraviolet irradiation. Loomis has postulated that negroes, who predominantly live in latitudes where there are relatively large amounts of ultraviolet light, are protected against forming excess vitamin D by a pigmented stratum corneum which absorbs some of the irradiation (Loomis, 1967).

4. The Chemistry of Vitamin D

According to the International Union of Pure and Applied Chemistry Commission on the nomenclature of Biological Chemistry, cholecalciferol (vitamin D_3) is defined as a steroid (IUPAC-IUB Commission, 1967). The parent saturated hydrocarbon from which cholecalciferol is derived is 5α-cholestane (Fig. 1a). This

tetracyclic structure is made up of 3 cyclohexane rings (A, B and C) and one cyclopentane ring (D). Attached to ring D at position 17 is an eight carbon side chain and there are two methyl groups at positions 10 and 13 which are of β configuration. The precursor of vitamin D_3, 7-dehydrocholesterol, differs from the parent molecule in containing a 3 β-OH group and two double bonds, one between carbon atoms 5 and 6 and another between carbon atoms 7 and 8 (Fig. 1b). When 7-dehydrocholesterol is irradiated with ultraviolet light, the bond between carbons 9 and 10 in ring B is broken and ring A rotates through 180^0 around the single bond between carbons 6 and 7. A double bond is then formed between carbons 10 and 19 (Fig. 1c). By convention, cholecalciferol is defined as a 9,10-seco-steroid, since it is formed by fission of the steroid ring B through the disruption of the bond between carbons 9 and 10. Thus, the chemical name of cholecalciferol is 9,10-seco-5,7,10(19)-cholestatrien-3β-ol.

The structure of ergocalciferol (vitamin D_2) and its precursor, ergosterol, is shown in Fig. 2. Ergocalciferol differs from cholecalciferol only in the nature of the side chain at carbon 17. Ergosterol is so named because it was discovered in 1879 by Tanet in ergot and Gerord noted its occurrence generally in the fungi. Ergosterol is the predominant provitamin D in plants. The biogenesis of this sterol in plants and fungi is established as occurring along similar pathways to those for cholesterol in mammalian tissue. Ultraviolet irradiation of ergosterol yields a number of products, namely lumisterol, tachysterol and calciferol. Further irradiation of calciferol yields three other identifiable products, toxisterol,

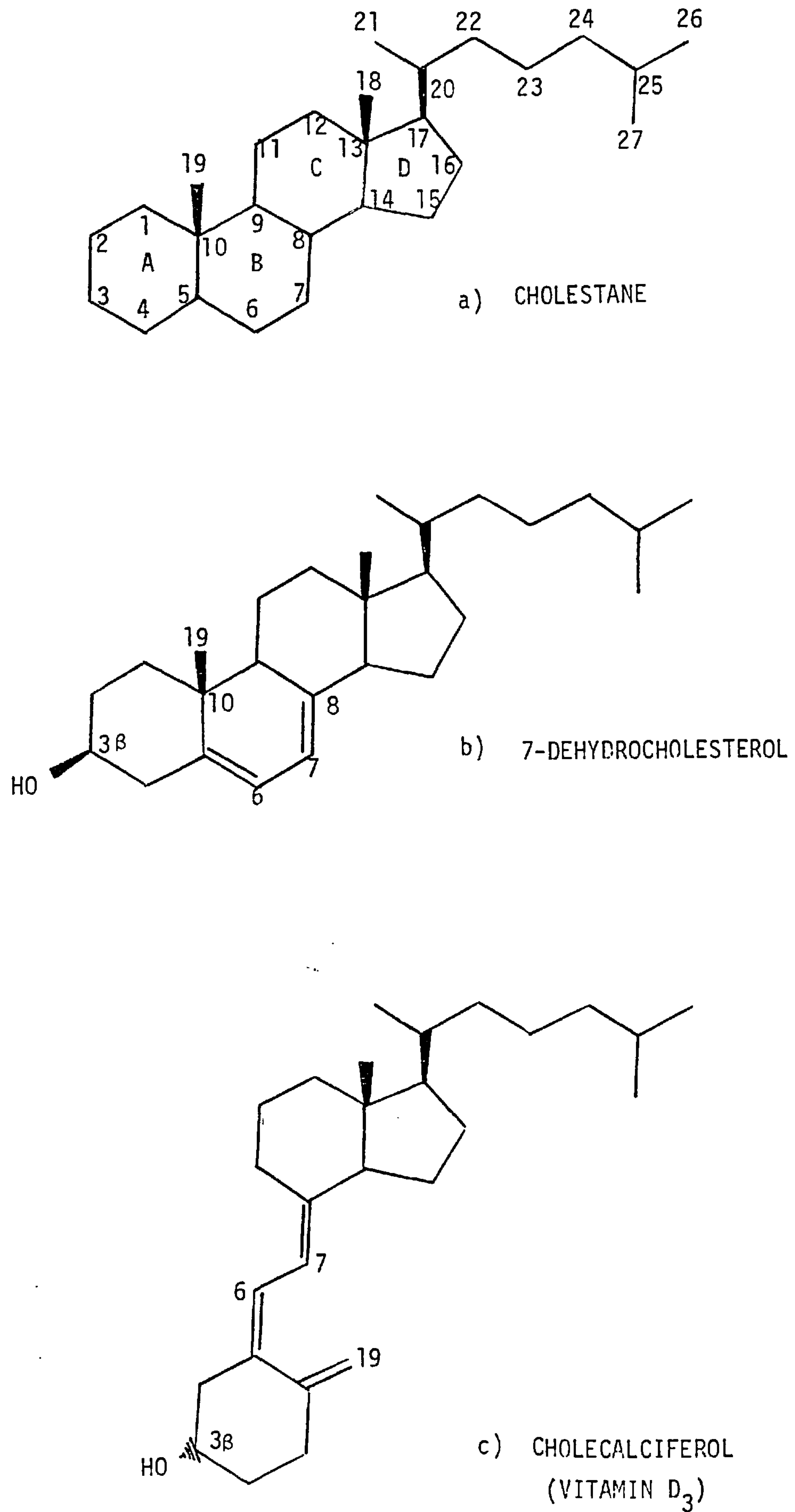


Fig. 1. The structure of cholestane, 7-dehydrocholesterol and cholecalciferol (vitamin D_3).

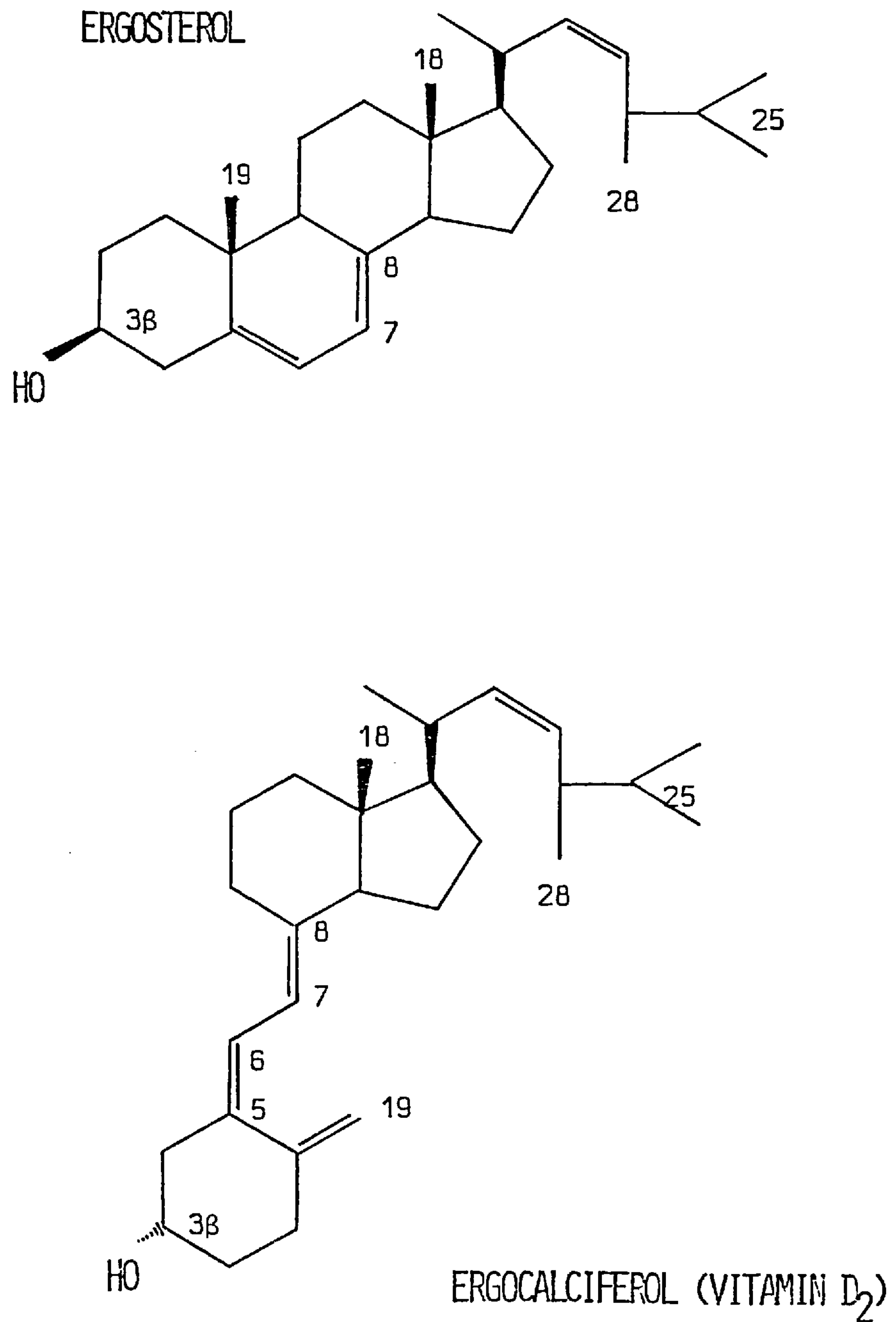


Fig. 2. The structure of ergocalciferol (vitamin D_2) and its precursor, ergosterol.

suprasterol I and suprasterol II. Of all these compounds, only calciferol has anti-rachitic activity. Tachysterol has among its properties the ability to raise the serum calcium concentration and lower phosphate concentration but it is toxic. A derivative, dihydrotachisterol is much less toxic and has been shown to increase intestinal absorption of calcium but has no action on bone (Harrison and Harrison, 1972).

5. Sources and Requirements of Vitamin D

One international unit of vitamin D is the amount that is equal in potency to 1 mg of the international standard of irradiated 7-dehydrocholesterol and is equivalent to 0.025 μg of pure crystalline calciferol. In man, the daily requirement has been estimated as 100 international units and for children, about 400 units (Fourman and Royer, 1968).

Very few foods provide adequate amounts of vitamin D. Although ergocalciferol is widely used in therapeutics, it occurs very rarely in nature. It is absent in almost all plant and animal tissue except for small amounts in certain fish oils. Ergosterol, from which it is derived by ultraviolet irradiation, occurs in only plants and fungi. The only rich sources of cholecalciferol, the natural form of the vitamin, are the liver oils of some fish. Egg yolks contain a small amount of vitamin D (0.1 - 1 unit per g) depending on the amount supplemented in the feed and the amount of sunlight to which the chickens have been exposed. A number of foods, including margarine, tinned milk and cereals are now supplemented with vitamin D. However,

many people get little or no vitamin D from diet and obtain their supply by synthesis from 7-dehydrocholesterol in the skin. Adequate supplies of vitamin D can be synthesized with sufficient exposure to sunlight. The value of sunlight depends on the latitude and the season. It had been estimated that the summer sunlight in Cape Town is roughly equivalent to 2,500 units of vitamin D daily in man (Irving and Schwartz, 1945). Knudson (1932) found that 20-30 minutes exposure to sunlight in June and July was sufficient to prevent rickets in rats but 270 minutes were needed in January and February. The amount needed to cure rickets in June and July was 90-120 minutes. There was not sufficient sunlight in the winter months to cure rickets.

The significance of such large amounts of vitamin D in the liver oils of some fish is not known. Cod-liver oil contains 60-300 units per gram, halibut-liver oil about 3,000 and the liver of the blue fin tuna 40,400 units per gram. It is known that fish can synthesize 7-dehydrocholesterol and can obtain sufficient dietary provitamin D from marine invertebrates. Bills (1927) working with cod and catfish estimated that neither irradiation of endogenous 7-dehydrocholesterol nor dietary intake can account for the high concentrations of vitamin D in these fish. However, attempts to investigate the possible synthesis of vitamin D by non-photochemical means by fish liver <u>in vitro</u> (Nes, Blondin, Scott, Hummer and Kulkarni, 1963; Blondin, Kulkarni and Nes, 1964 and 1967) were unsuccessful.

6. Vitamin D in the Body

6.1. Absorption

The absorption of vitamin D has been studied in the rat using ^{14}C-labelled vitamin D_3. It was found that maximum absorption occurred in the upper three-quarters of the small intestine (Bills, 1935; Taylor et al., 1935). Absorption takes place in two main steps. Firstly uptake by the mucosa and then transfer to the lymph. Bile salts are necessary for absorption and vitamin D malabsorption has been demonstrated in animals with bile fistulas (Schachter, Finkelstein and Kowarski, 1964).

6.2 Transport

Once vitamin D is absorbed, it becomes associated with a vitamin D binding protein. In man, rat and dog, this protein migrates with α-globulin on gel electrophoresis (Thomas, Morgan, Connor, Haddock, Bills and Howard, 1959; Chalk and Kodicek, 1961; Chen and Lane, 1965; Rikkers, Kletzein and DeLuca, 1969). Bouillon has recently isolated this protein from human serum and found its molecular weight to be 56,000 (Bouillon, van Baelen, Rombauts and De Moor, 1976). In chick serum, cholecalciferol is associated with a protein which migrates with β-globulin, while the vitamin D transporting protein in the New World monkey, Cebus albifrons, has albumin mobility (Edelstein, Lawson, and Kodicek, 1973). In the toad, Xenopus Laevis, vitamin D is transported by lipoproteins (Edelstein et al., 1973).

6.3 Storage

Heymann showed in 1937 (a) that 6 months after a single dose

of vitamin D, detectable amounts of the vitamin are still present in the livers of rats. It was later shown that vitamin D is stored not only in the liver, but also in the fat deposits throughout the body in rats (Rosenstreich, Rich and Volwiler, 1971).

6.4 Excretion

The primary excretory route for vitamin D is the bile (Heymann, 1937b). As much as 30% of a dose of vitamin D appears in the bile within 24-48 hours, whereas only 2% of the dose appears in the urine.

7. Studies on the Metabolism of Vitamin D

7.1 Early Studies

Following the identification of vitamin D as the anti-rachitic factor, Nicolaysen demonstrated that vitamin D acts to increase intestinal calcium absorption in rachitic rats (Nicolaysen, 1937). However, it was generally assumed that vitamin D must function in the body without alteration. In 1952, Carlsson noted a lag period between the time of vitamin D administration and the appearance of its physiological response. This fact was also noted by Kodicek and colleagues in Cambridge (Cruickshank and Kodicek, 1953; Kodicek and Ashby, 1954; Kodicek, 1955) who considered that this lag period could be explained by postulating that vitamin D is converted to more active metabolites or that vitamin D initiates some cellular mechanism

in the target tissue leading to the appearance of active macromolecules. Kodicek's group attempted to investigate the first possibility namely that vitamin D is altered to more active metabolites before exerting its physiological effect. Large doses of ergocalciferol were given to rachitic rats (Cruickshank and Kodicek, 1953; Kodicek and Ashby, 1954) but only 20% of the original dose could be accounted for when the various organs were bioassayed for anti-rachitic activity. It was assumed that the 70 to 80% of the administered dose of vitamin D had been converted to inactive metabolites.

7.2 The Identification of 25 hydroxycholecalciferol

In 1955, Kodicek reported the biosynthesis of ^{14}C-labelled vitamin D_2 but, because of the low specific activity of this material, he was unable to detect the presence of active metabolites (Kodicek, 1960). An important breakthrough came with the chemical synthesis of radiolabelled vitamin D_2 and D_3 with high specific activity (Norman and DeLuca, 1963a). Administration of physiological doses of tritiated cholecalciferol enabled DeLuca and colleagues to detect biologically active metabolites of vitamin D_3 in intestine and kidney (Norman, Lund and DeLuca, 1964) and in bone, liver and serum (Lund and DeLuca, 1966). At least 3 metabolites could be separated by silicic acid chromatography. One metabolite with anti-rachitic activity was more polar than cholecalciferol and was designated as peak IV. It was found that this material had a biological activity at least equal to the parent compound, but that it acted more rapidly than vitamin D_3

in stimulating intestinal calcium transport (Morii, Lund, Neville and DeLuca, 1967). This metabolite was subsequently isolated from hog plasma and identified as 25 hydroxycholecalciferol (25 OH D_3) (Blunt, DeLuca and Schnoes, 1968). The following year, 25 hydroxy-ergocalciferol (25 OH D_2) was isolated and identified (Suda, DeLuca, Schnoes and Blunt, 1969). 25 OH D_3 was found to be about 1.5 times more effective than an equal dose of vitamin D_3 in supporting calcification of rachitic bone in rats and chicks and its effects on intestinal calcium absorption and bone mobilization were more rapid than those of the parent compound (Blunt, Tanaka and DeLuca, 1968).

The chemical synthesis of 25 OH D_3 was achieved in 1969 (Blunt and DeLuca, 1969). The structure is shown in Fig. 3. The properties of the synthetic compound were shown to be identical to those of the isolated metabolite. In the same year, further studies of the metabolism of tritiated vitamin D_3 in rats provided evidence that 25 OH D_3 is formed primarily in the liver (Ponchon and DeLuca, 1969). Further evidence for the role of the liver in the formation of 25 OH D_3 was put forward by Horsting and DeLuca (Horsting and DeLuca, 1969) who reported the _in vitro_ synthesis of this metabolite by perfused liver and liver homogenates, showing that a hepatic enzyme is capable of performing the 25-hydroxylation. Furthermore, isolation of the liver from the circulation of rats almost completely eliminated their ability to convert tritiated D_3 to 25 OH D_3 (Ponchon, Kennan and DeLuca, 1969). However, recently a unique role for the liver in the 25-hydroxylation of vitamin D_3 has been challenged by the description of significant 25-hydroxylase enzyme activity in intestine

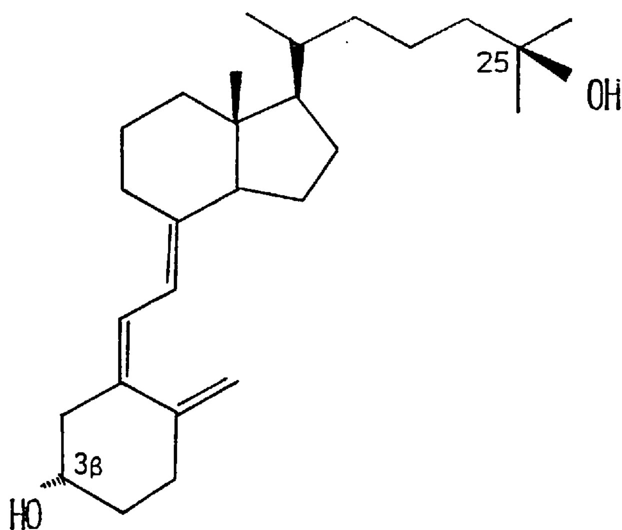


Fig. 3. The structure of 25-hydroxy-cholecalciferol

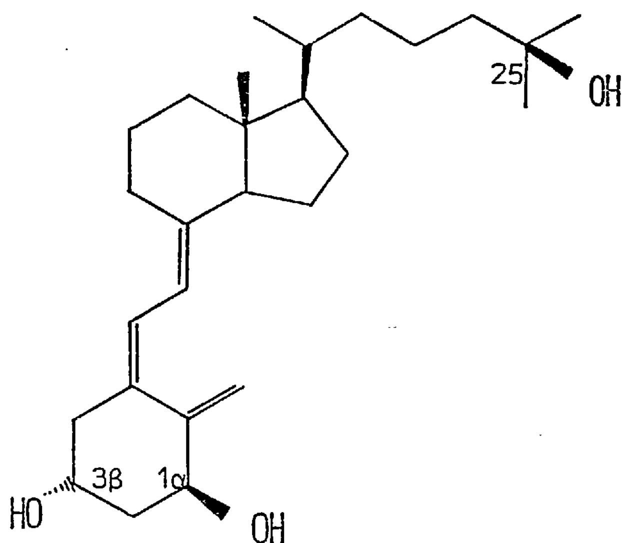


Fig. 4. The structure of 1α 25-dihydroxy-cholecalciferol

and kidney as well as liver in chicks (Tucker, Gagnon and Haussler, 1973). It is possible that species variation may account for these differences.

Further studies on the properties of 25 OH D_3 were reported to show a direct effect on calcium transport in perfused rat intestine (Olson and DeLuca, 1969) and this metabolite was also shown to stimulate bone resorption in tissue culture (Trummel, Raisz, Blunt and DeLuca, 1969). This calcium mobilising effect could not be reproduced by much larger concentrations of vitamin D_3. However, in both these two sets of experiments, supraphysiological doses of 25 OH D_3 were used.

7.3 The Discovery of 1,25 dihydroxycholecalciferol

Following the synthesis of 25 OH D_3, it was possible to prepare tritiated 25 OH D_3 of high specific activity. With this material it was demonstrated that 25 OH D_3 itself is metabolised both to less polar and to more polar compounds (Cousins, DeLuca and Gray, 1970). Furthermore, Haussler and colleagues could separate three metabolites of vitamin D_3 from intestinal nuclei by silicic acid and celite liquid-liquid chromatography. Only one of these metabolites, designated peak 4B, was associated with nuclear chromatin and was biologically active in stimulating intestinal calcium transport (Haussler, Myrtle and Norman, 1968). A major breakthrough in the identification of this new polar metabolite of vitamin D_3 came in 1969 from Kodicek's group in Cambridge. This group followed

the fate, *in vivo*, of doubly labelled vitamin D_3 with tritium in position C1 and carbon^{14} in position C4. A metabolite was isolated from the intestine which had a lowered tritium/carbon^{14} ratio, indicating a modification of the molecule involving the loss of hydrogen from the C1 position (Lawson, Wilson and Kodicek, 1969a). The polarity of this compound and loss of hydrogen from C1 position suggested the introduction of an oxygen function (Lawson, Wilson and Kodicek, 1969b). This polar metabolite was detected in high concentration in intestinal cell nuclei and also detected in bone and kidney cells.

A new method of gel liquid partition chromatography on Sephadex LH 20 was developed which could resolve the metabolites extracted from blood and tissues which were more polar than 25 OH D_3. This method permitted the isolation and identification of the polar metabolites of vitamin D in intestine (Holick and DeLuca, 1971). This new separation system enabled Holick and colleagues to isolate and identify the active polar metabolite from the intestines of 1500 chicks given tritiated vitamin D_3. 2 μg of pure metabolite were obtained and mass spectrometry and ultraviolet absorption spectra studies confirmed that this metabolite was 1α,25,dihydroxycholecalciferol (structure shown in Fig. 4).

The original suggestion of DeLuca's group was that this new active metabolite of vitamin D_3 was formed locally in the intestinal mucosa and bone cells (Cousins et al., 1970). However, this was disputed by the Cambridge group (Lawson, Pelc, Bell, Wilson and

Kodicek, 1971) who, from the time course of appearance in intestine and bone of rachitic rats and chicks, considered it more likely to originate outside the intestine. Furthermore, they considered that the presence of large amounts of this material in intestinal cytoplasm and its accumulation in the nuclei resembled the findings obtained on the intracellular distribution of the steroid hormones in their target tissues. In 1970, Fraser and Kodicek showed conclusively that 1,25 dihydroxycholecalciferol (1,25 $(OH)_2D_3$) was not formed in the intestine. Removal of intestine and successively other organs did not prevent the formation of the final metabolite of vitamin D_3. At the same time, homogenates of various tissues were studied for ability to synthesize 1,25 $(OH)_2D_3$ *in vitro*. It became clear both by *in vitro* studies and by nephrectomy that the kidney was the organ which finally introduced the hydroxyl group at position C1.

In 1972, the response of intestinal calcium transport to 25 OH D_3 and 1,25 $(OH)_2D_3$ was studied in nephrectomized rats (Boyle, Miravet, Gray, Holick and DeLuca, 1972). When the kidneys were removed from vitamin D deficient rats, physiological doses of 25 OH D_3 failed to stimulate intestinal calcium absorption, but the response to 1,25 $(OH)_2D_3$ was unimpaired. These experiments provided further evidence that 1,25 $(OH)_2D_3$ and not 25 OH D_3 is the final metabolically active form of vitamin D. Similar doses of vitamin D_3 and 25 OH D_3 were effective in stimulating intestinal calcium transport in ureamic rats, demonstrating that it was not uremia resulting from nephrectomy that prevented formation of the active

metabolite, but lack of kidney tissue (Gray, Boyle and DeLuca, 1971). Studies on isolated intestinal cells from chick also showed that 25 OH D_3 was not converted to 1,25 $(OH)_2D_3$ by this tissue *in vitro* and that 1,25 $(OH)_2D_3$ was not metabolised further, making it unlikely that a subsequent metabolite was responsible for the observed effects on calcium transport (Shain, 1972a).

The synthesis of 1,25 $(OH)_2D_3$ was later achieved by Semmler (Semmler, Holick, Schnoes and DeLuca, 1972) and also by Barton (Barton, Hesse, Pechet and Rizzardo, 1973).

7.4 The Biological Activity of 1,25 $(OH)_2D_3$

With the isolation, identification and synthesis of 1,25 $(OH)_2D_3$, studies were undertaken by many groups to document more fully its biological activity. It was generally agreed that 1,25 $(OH)_2D_3$ acts even more rapidly than 25 OH D_3 to stimulate intestinal calcium transport (Haussler, Boyce, Littledike and Rasmussen, 1971; Myrtle and Norman, 1971; Omdahl, Holick, Suda, Tanaka and DeLuca, 1971; Kodicek, Lawson and Wilson, 1970) but that its effect is short lived. 1,25 $(OH)_2D_3$ was found to mobilize bone calcium considerably faster than an equal dose of 25 OH D_3 or D_3 in the chick (Tanaka and DeLuca, 1971) and was found to be active in bone in anephric rats, while 25 OH D_3 and vitamin D_3 were ineffective (Wong, Myrtle, Tsai and Norman, 1972; Holick, Garabedian and DeLuca, 1972a). Studies of bone in tissue culture demonstrated that 1,25 $(OH)_2D_3$ was about 100 times more potent than 25 OH D_3 in releasing previously incorporated

^{45}Ca from foetal bone (Raisz, Trummel, Holick and DeLuca, 1972).

Long term studies on the biological activity of 1,25 $(OH)_2D_3$ indicated that, although this metabolite was more effective than 25 OH D_3 in acutely increasing intestinal calcium transport, it was less than half as effective as 25 OH D_3 when assayed for anti-rachitic activity in rats. Omdahl et al. (1971) suggested that the short half life of 1,25 $(OH)_2$ D_3 might be responsible for this finding. 1,25 $(OH)_2D_3$ was subsequently found to be effective in maintaining serum calcium levels in vitamin D deficient rats over longer periods if given parenterally (Tanaka, Frank and DeLuca, 1972) and that 1,25 $(OH)_2D_3$ given intraperitoneally was about 10 times as active as vitamin D_3 in the maintenance of serum calcium in rats.

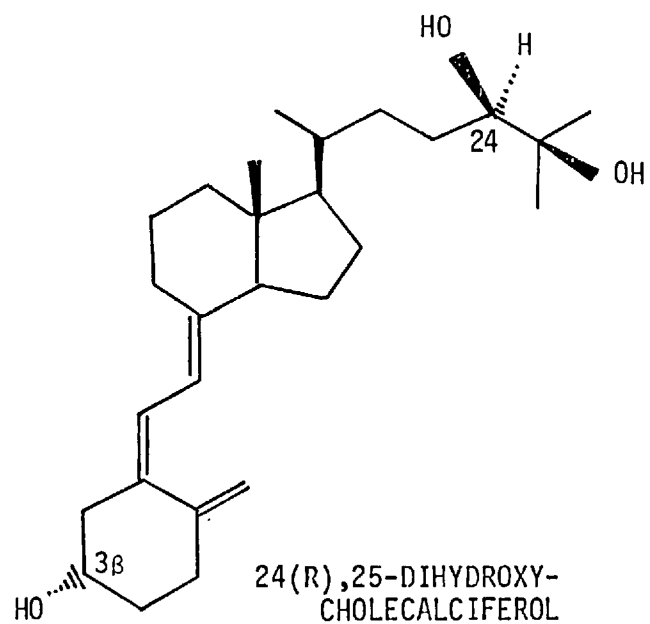
Brickman and colleagues compared the effect of 5 days treatment with 1,25 $(OH)_2D_3$ and 25 OH D_3 given intramuscularly in the rachitic dog, and found the dihydroxymetabolite to be 4 times more effective in raising serum calcium (Brickman, Reddy, Coburn, Passarro, Jowsey and Norman, 1973), while a comparison of the potency of oral 1,25 $(OH)_2D_3$ and vitamin D_3 in chronic experiments showed the renal metabolite to be 2 to 4 times as active as vitamin D_3 in promoting growth and 1 to 2 times as active in increasing calcium transport in rats and chicks. Recently, 1,25 $(OH)_2D_3$ has been reported to have a role in the maintenance of serum phosphorus (Tanaka and DeLuca, 1974b). Rats on a low phosphate, vitamin D deficient diet showed an elevation of serum phosphate which was maximal 10 hours after intravenous treatment with 1,25 $(OH)_2D_3$. The same dose of

25 OH D_3 produced a later response which was longer lived. In these acute experiments, bone calcification was noted after 25 OH D_3 treatment but not with 1,25 $(OH)_2D_3$. However, long term treatment with 1,25 $(OH)_2D_3$ led to a sustained increase in serum phosphate and produced bone calcification.

7.5 Other Renal Metabolites of Vitamin D_3

7.5.1 24,25 dihydroxycholecalciferol

In 1969, Lawson and colleagues noted that vitamin D supplemented chicks did not have the 'tritium deficient' vitamin D metabolite in their intestinal mucosa, but another metabolite which could be separated chromatographically from 25 OH D_3. This metabolite was isolated from the plasma of pigs given large doses of vitamin D_3 by the Wisconsin group (Suda, DeLuca, Schnoes, Ponchon, Tanaka and Holick, 1970). It was reported to be effective in mobilizing calcium from bone <u>in vivo</u>, but less than one third as active in the stimulation of intestinal calcium transport as vitamin D_3 and half as active as vitamin D in curing rickets. The Wisconsin group at first assigned this metabolite the structure of 21,25 dihydroxycholecalciferol. However, in 1972 it was reported that the correct structure was 24,25 dihydroxycholecalciferol (Holick, Schnoes, DeLuca, Gray, Boyle and Suda, 1972). It was further shown that this metabolite is synthesized by the kidney and represents the renal metabolite formed under circumstances where 1,25 $(OH)_2D_3$ is not formed (Omdahl, Gray, Boyle, Knutson and DeLuca, 1972). The structure of 24,25 $(OH)_2D_3$ is shown in Fig. 5. There are two possible conformational epimers of this molecule. The one formed



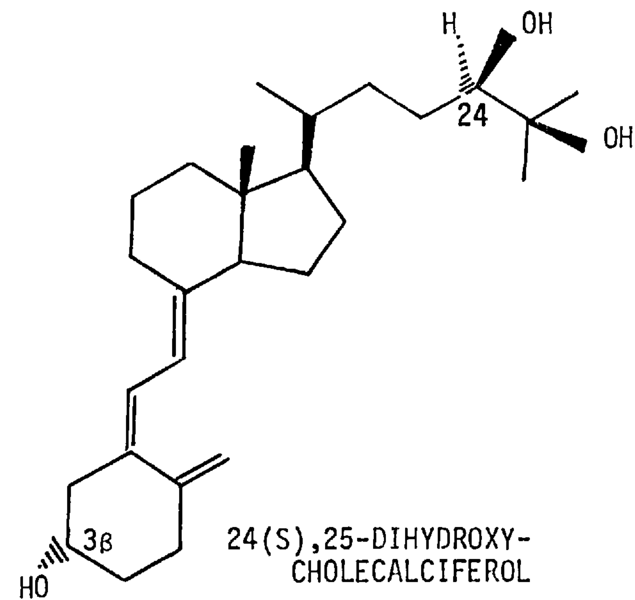


Fig. 5. The structures of the two possible epimers of 24,25-dihydroxycholecalciferol

in vivo is thought to be 24R,25 $(OH)_2D_3$ (DeLuca, 1976). Both epimers have been synthesized recently (Uskokovic, 1974; Seki, Koizumi, Morisaki and Ikekawa, 1975). Further studies of the biological activity of 24,25 $(OH)_2D_3$ on bone resorption in tissue culture have been reported by Atkins (Atkins, 1976). This worker found that neither 24R nor 24S $(OH)_2D_3$ had a marked effect on bone resorption *in vitro*. However, 24,25 $(OH)_2D_3$ enhanced the effect of a submaximal dose of 1,25 $(OH)_2D_3$.

7.5.2 1,24,25 trihydroxycholecalciferol

In 1973, Boyle and co-workers demonstrated that 24,25 $(OH)_2D_3$ can be metabolized further *in vivo* to 1,24,25 trihydroxy D_3 (Boyle, Omdahl, Gray and DeLuca, 1973). This trihydroxy derivative can also be formed *in vitro* from 24,25 $(OH)_2D_3$ in chick kidney homogenates. This compound has some biological activity but it is less active than vitamin D_3 in curing rickets and much less active than 1,25 $(OH)_2D_3$ in enhancing intestinal calcium absorption and bone calcium mobilization. Its physiological significance is not known (Holick, Kleiner-Bossaller, Schnoes, Kasten, Boyle and DeLuca, 1973).

7.6 Other naturally occurring metabolites of Vitamin D_3

7.6.1 25,26 dihydroxycholecalciferol

25,26 dehydroxycholecalciferol (25,26 $(OH)_2D_3$) was isolated from the plasma of pigs given high doses of vitamin D_3 (Suda, DeLuca, Schnoes, Tanaka and Holick, 1970). It can also be found in the serum of chick, rat and man (Mawer, 1973). Its site of origin is

not known, but it is not the kidney. This metabolite has modest activity in increasing intestinal calcium transport, but has little ability to cure rickets or mobilize calcium from bone. The physiological significance of this metabolite is not known.

7.6.2 Vitamin D esters

Following the administration of ^{3}H cholecalciferol to rats, Fraser and Kodicek (1965) noted the appearance of a peak less polar than vitamin D_3 present in liver and kidney. They subsequently showed that this peak represented fatty acid esters of vitamin D and that some esterification of the vitamin occurred during absorption from the alimentary tract (Fraser and Kodicek, 1966). The presence of vitamin D esters was also noted in the liver of rats 2 hours after vitamin D administration (Lund, DeLuca and Horsting, 1967). It is not known whether these esters are physiologically significant.

7.6.3 Uncharacterized metabolites

A variety of other metabolites have been reported using different chromatographic techniques in several laboratories, among them peak VI (Cousins, DeLuca and Gray, 1970) and peaks of radioactivity in material more polar than vitamin D_3 when separated by silicic acid chromatography (Mawer and Backhouse, 1969). Further characterization of these metabolites is necessary before their physiological significance, if any, can be determined.

7.7 Synthetic Analogues of Vitamin D

7.7.1 Dihydrotachysterol

Dihydrotachysterol is a reduction product of tachysterol. This vitamin D analogue has an altered configuration of the A ring of the molecule. The hydroxyl group in position 3 of dihydrotachysterol (DHT) occupies a position sterically similar to the hydroxyl group at carbon 1 in 1,25 $(OH)_2D_3$, which appears to confer biological activity. DHT has slight anti-rachitic activity and is capable of increasing calcium absorption in uremic man (Kaye, Chatterjee, Cohen and Saga, 1970) and nephrectomized rats (Harrison and Harrison, 1972). 25 OH dihydrotachysterol has been chemically synthesized and its biological activity assessed (Suda, Hallick, DeLuca and Schnoes, 1970). It was more effective than DHT in promoting mobilization of calcium from bone in organ culture (Trummel, Raisz, Hallick and DeLuca, 1971).

7.7.2 5,6, trans isomers of cholecalciferol and 25 OH D_3

Rotation of the A ring into the trans position in relation to the double bond between carbon 5 and 6 again brings the 3 hydroxyl group of cholecalciferol into the geometric position of the 1-hydroxyl group in the cis isomer of 1,25 $(OH)_2D_3$. 5,6, trans cholecalciferol and 5,6, trans 25 hydroxycholecalciferol have been synthesized. The pseudo 1α hydroxyl group confers 1,25 $(OH)_2D_3$ activity on the molecule and these two synthetic trans isomers have been shown to stimulate intestinal calcium transport in nephrectomized animals, whereas the corresponding 5,6, cis compounds are inactive in this situation (Holick, Garabedian and DeLuca, 1972b).

8. Physiological and Biochemical Basis of Vitamin D Action

8.1 Intestinal Calcium Absorption

In 1923, it was noted by Orr that there is a high fecal calcium loss in rachitic children. At this time Orr suggested that ultraviolet therapy cured rickets by increasing intestinal calcium absorption (Orr, Holt, Wilkins and Boone, 1923). Further, Nicolaysen showed that absorption of dietary calcium was impaired in vitamin D deficient rats (Nicolaysen, 1937) and demonstrated both by calcium balance studies and using isolated gut loops, that vitamin D treatment leads to increased intestinal calcium absorption in rachitic rats.

The action of vitamin D on the intestine is not a simple one. There is evidence for an effect on both active transport and passive diffusion. Wilson and Wiseman (1954) showed that the effect of vitamin D on the active transport of calcium in everted gut sacs was limited in capacity and dependent upon oxidative phosphorylation. Vitamin D deficiency impaired the capacity for active ^{45}Ca transport *in vitro*. Schachter and Rosen (1959) demonstrated that the intestinal transport system for calcium as studied in everted gut sacs was finite in capacity, required metabolic energy and moved calcium from the mucosal to the serosal surface against a concentration gradient.

An effect on passive diffusion also seems likely. Harrison and Harrison (1965) demonstrated that intestinal mucosa presents a diffusion barrier to calcium which is lessened by vitamin D treatment

A further series of experiments indicated the cellular mechanisms by which vitamin D might influence calcium transport. Zull and colleagues (Zull, Czarnowska-Misztal and DeLuca, 1965) demonstrated that the vitamin D dependent increase in calcium transport was blocked in rats by pretreatment with the transcriptional inhibitor, actinomycin D. This effect was later confirmed in the chick (Tsai, Midgett and Norman, 1973). Similarly, pretreatment with actinomycin D in organ cultured chick intestine blocked the effect of 1,25 $(OH)_2$ D_3, as well as its precursors, on calcium transport *in vitro*. These studies implied that the action of vitamin D and its metabolites on intestinal calcium transport requires a nuclear effect of the vitamin which involves new RNA synthesis. Other studies also demonstrated a nuclear mechanism of action of vitamin D on the intestine. Following a dose of tritiated vitamin D_3, the radioactivity in the intestinal mucosa is associated with the nuclear fraction (Haussler and Norman, 1967) and the site of nuclear binding has been localized to the chromatin fraction (Haussler, Myrtle and Norman, 1968). It has recently been demonstrated that 1,25 $(OH)_2D_3$ is initially bound to a cytoplasmic fraction (Tsai and Norman, 1973). Furthermore, an increase in RNA synthesis by intestinal cell nuclei in response to vitamin D has been demonstrated and this response is blocked by pretreatment with actinomycin D.

The next aspect of vitamin D action on the intestine to be investigated was the nature of the proteins which are synthesized as a result of the enhanced RNA synthesis in the intestinal mucosa. A

number of new proteins have been identified as being induced by vitamin D, but the most interesting are the vitamin D dependent calcium binding protein and a calcium stimulated ATPase-alkaline phosphatase enzyme complex.

8.1.1 Calcium binding protein

In 1966, Wasserman and Taylor discovered a vitamin D dependent intestinal calcium binding protein in the soluble fraction of chick mucosal cell homogenates. Further, these workers found a correlation between the time of appearance of the binding protein and the onset of increased calcium absorption following a dose of vitamin D_3. Under physiological conditions known to produce enhanced calcium absorption, such as a low calcium diet, there was an increase in the amount of calcium binding protein in the mucosa (Wasserman and Taylor, 1968). Calcium binding protein concentration was also greater in the intestines of rachitic chicks given a large dose of vitamin D_3 than in normally fed chicks, and was also elevated in laying hens. In 1968, Corradino and Wasserman showed that actinomycin D inhibited the appearance of calcium binding protein in response to a dose of vitamin D. Calcium binding protein has also been detected in the rat (Kallfelz, Taylor and Wasserman, 1972) and in primates (Wasserman and Taylor, 1971).

However, some studies have indicated that calcium binding protein may not be the only mechanism responsible for the increased calcium transport in response to vitamin D, since increased calcium transport has been shown to precede calcium binding protein synthesis (Harmeyer and DeLuca, 1969) and calcium binding protein continues to increase even after the rate of calcium absorption has plateaued.

Furthermore, cortisone-treated rats maintain high levels of calcium binding protein while calcium absorption is not increased (Kimberg, Baerg, Gershon and Graudicius, 1971).

There is also disagreement as to whether calcium binding protein is synthesized de novo in response to vitamin D or whether it is produced by transformation of a precursor product (Omdahl and DeLuca, 1973). However, Emtage, Lawson and Kodicek (1973) demonstrated the synthesis of calcium binding protein in incubates of isolated polysomes from mucosal cells prepared from chicks treated with vitamin D. Similar preparations from vitamin D deficient chicks were not capable of synthesizing calcium binding protein in vitro. The same group also showed that messenger RNA isolated from intestinal mucosa from vitamin D treated chicks could direct calcium binding protein synthesis in reticulocyte lysate preparations. These experiments demonstrate that calcium binding protein can be synthesized de novo in response to vitamin D by chick intestinal mucosa cells.

Studies with embryonic chick intestine in organ culture have shown that 1,25 $(OH)_2D_3$ is the most potent metabolite of vitamin D capable of inducing calcium binding protein in vitro, but vitamin D_3 and 25 OH D_3 also have an effect at higher concentrations (Corradino, 1973).

8.1.2 Calcium dependent adenosine triphosphatase

The presence of a vitamin D stimulated, calcium dependent

adenosine triphosphatase was first demonstrated in brush border preparations of rat small intestine (Martin, Melancon and DeLuca, 1969). Further, an increase in this ATPase activity followed the rise in calcium absorption after treatment with vitamin D (Melancon and DeLuca, 1970) which indicates a possible role for the ATPase in the intestinal absorption of calcium. Studies in chicks (Haussler, Nagode and Rasmussen, 1970) showed a corresponding induction of both intestinal alkaline phosphatase and calcium ATPase activity following treatment with vitamin D. Furthermore, both the induction of these two enzyme activities and the increase in calcium absorption were blocked by administration of actinomycin D and cycloheximide in vivo. Thus there appears to be induction of an ATPase-alkaline phosphatase complex associated with the increased calcium absorption in response to vitamin D. The part played by this enzyme complex in calcium transport is not known, but the fact that the complex is associated with the brush border region of the intestinal mucosa may indicate that the complex could function in the movement of calcium into the mucosal cell.

The manner in which the absorbed calcium is transported across the intestinal cell has not been fully elucidated. There is some evidence that mitochondria play a role in this process. Intestinal mitochondria from vitamin D treated rats were shown to contain numerous electron dense granules. These granules appear limited to the microvillus in rachitic animals (Omdahl and DeLuca, 1973). However, before this observed phenomenon can be assigned a physiological role,

several aspects must be considered. Among them, what is the mechanism by which the calcium is precipitated within the mitochondria then later solubilized for movement into the cytosol and across the basal membrane? Furthermore, can this method be fast enough to account for the time course of calcium absorption observed *in vivo*?

8.1.3 Vitamin D and Phosphate absorption

Nicolaysen (1937) demonstrated that phosphate absorption in rats was independent of vitamin D treatment, but was secondary to changes in calcium absorption. Later, *in vitro* work showed that calcium was required for the absorption of phosphate and it was suggested that an increased intracellular calcium activated the transport of phosphate (Harrison and Harrison, 1961). However, further evidence indicates that absorption of phosphate is sensitive to changes in dietary calcium (Taylor and Wasserman, 1972a). The exact role of vitamin D and its metabolites on phosphate transport remains uncertain.

8.2 The Action of Vitamin D on the Mineralization and Resorption of Bone

8.2.1 Mineralization of Bone

It has long been known that in rickets and in osteomalacia, a lesion occurs in the normal mineralization process of bone. This failure of bone to calcify results in the characteristic rachitic deformities of the skeleton, particularly in the long bones, ribs and skull. Vitamin D brings about normal calcification of bone,

while in states of vitamin D deficiency the calcification process fails to take place at a rate equal to the synthesis of new organic matrix of bone.

Many investigators have considered that vitamin D must function directly on the mineralization process but, as yet, experiments designed to test this theory have been open to other interpretations (Omdahl and DeLuca, 1973). However, it has been noted that in vitamin D deficiency the plasma calcium:phosphorus product is reduced (Neuman and Neuman, 1958). The suggestion has been put forward that blood is normally supersaturated with calcium and phosphate and that mineralization of bone can be viewed as a crystallization process. Thus in states of vitamin D deficiency, plasma calcium and/or phosphate concentrations are reduced and the low level of the calcium:phosphate product is responsible for the observed failure in bone calcification.

8.2.2 Bone calcium mobilization

In 1952, Carlsson conclusively demonstrated that one important action of vitamin D is the mobilization of calcium from previously formed bone. The administration of vitamin D to rats fed a low calcium diet led to a rise in serum calcium. Since increased calcium absorption from the gut had been excluded by the low calcium diet, it was reasoned that this increase in serum calcium was produced at the expense of bone (Omdahl and DeLuca, 1973). The details of the mechanism by which vitamin D mobilizes calcium from bone are not fully understood. There is evidence that 1,25 $(OH)_2D_3$ is the active form

in bone; after administration of tritiated vitamin D to rachitic chicks, the radioactivity in bone was found in the nuclei of the cells as 1,25 $(OH)_2D_3$ (Weber, Pons and Kodicek, 1971). This fact would indicate that some nuclear effect may be involved in the action of this vitamin D metabolite on bone. Further evidence for this hypothesis was put forward by Tanaka and DeLuca (1971) who found that the bone calcium mobilizing action of 1,25 $(OH)_2D_3$ was blocked by prior administration of actinomycin D.

As previously discussed, 1,25 $(OH)_2D_3$ mobilizes calcium from bone *in vitro* (Reynolds et al., 1973) and 24,25 $(OH)_2D_3$ has been shown to enhance the effect of 1,25 $(OH)_2D_3$ on bone calcium mobilization *in vitro* (Atkins, 1976). Parathyroid hormone also mobilizes calcium from bone. However, it has been demonstrated that parathyroid hormone cannot mobilize bone in vitamin D deficient animals (Rasmussen, DeLuca, Arnaud, Hawker and von Stedingk, 1963) and that small amounts of vitamin D act in a permissive way to allow the action of parathyroid hormone on bone. Au and Raisz (1967) have suggested that the failure of parathyroid hormone to initiate bone resorption in the absence of vitamin D is due to a lack of calcium, since responsiveness to parathyroid hormone could be restored by injecting calcium or by stimulating increased intestinal absorption with dietary lactose.

8.3 The Action of Vitamin D on the Kidney

Results obtained from early experiments to elucidate the action of vitamin D on the kidney were conflicting and open to other

interpretations (Omdahl and DeLuca, 1973). However, a direct action of vitamin D on the kidney was demonstrated in 1972 (Puschett, Fernandez, Boyle, Gray, Omdahl and DeLuca, 1972). Cholecalciferol, 25 OH D_3 and 1,25 $(OH)_2D_3$ significantly increased the reabsorption of phosphate, sodium and calcium when given to volume-expanded, thyroparathyroidectomized dogs. The mechanism of this effect of vitamin D and its metabolites remains to be elucidated. However, a vitamin D dependent calcium binding protein has been identified in kidney which may play a role in calcium reabsorption (Taylor and Wasserman, 1972b).

8.4 The Action of Vitamin D on Muscle

Although Vitamin D clearly has a marked effect on muscle, very little is known of its mechanism of action on this tissue. It has long been known that vitamin D deficiency is accompanied by a marked muscular myopathy (Smith and Stern, 1969) but the precise mechanism by which vitamin D affects muscular function is not known.

There is strong experimental evidence that muscle is a target organ of vitamin D. Kodicek demonstrated the presence of metabolites of vitamin D following administration of tritiated calciferol (Kodicek, 1963) and this was confirmed by DeLuca's group (Neville and DeLuca, 1966). Further, a binding protein for 25 OH D_3 has been identified in muscle tissue (Haddad and Birge, 1971). Recently Curry and colleagues have demonstrated an effect of vitamin D on

sarcoplasmic reticulum (Curry, Basten, Francis and Smith, 1974). In their experiments, the uptake of ^{45}Ca by preparations of sarcoplasmic reticulum isolated from vitamin D deficient rabbit muscle was less than with similar preparations from vitamin D replete animals.

8.5 Vitamin D and the Parathyroid Glands

Recently, evidence has been put forward that the parathyroid glands may be an additional target organ. 1,25 $(OH)_2D_3$ has been shown to bind to receptors in the parathyroid gland (Brumbaugh, Hughes and Haussler, 1975).

9. The Regulation of Vitamin D Metabolism

9.1 The control of 25-hydroxylation

The control of the cholecalciferol 25-hydroxylase enzyme is controversial. DeLuca's group have reported that the 25-hydroxylation is regulated by vitamin D status. Liver homogenates from vitamin D deficient rats showed higher rates of 25-hydroxylation than comparable homogenates from vitamin D replete rats *in vitro*. *In vivo*, a 100-fold increase in administered vitamin D_3 only led to a doubling of 25 OH D_3 concentration in plasma, indicating some feedback control mechanism (Omdahl and DeLuca, 1973).

However, other experiments failed to demonstrate a relationship between vitamin D status and 25-hydroxylation. No evidence was found for a feedback inhibition of enzyme activity in chick liver

homogenates (Tucker, et al., 1973). It is possible that a species difference could explain these conflicting results.

9.2. The regulation of 25 OH D_3 1-hydroxylation

Nicolaysen and colleagues established that the efficiency of the intestinal absorption of calcium is inversely related to dietary calcium and that this intestinal response is dependent upon the presence of vitamin D (Nicolaysen, Eeg-Larsen and Malm, 1953). With the discovery of 1,25 $(OH)_2D_3$ as the active metabolite of vitamin D_3, it was considered that this adaptation of calcium absorption to changes in dietary calcium levels was mediated via 1,25 $(OH)_2D_3$ production and that dietary calcium might in some way regulate the synthesis of 1,25 $(OH)_2D_3$ by the kidney.

In 1971, Boyle and colleagues demonstrated that the formation of this active metabolite was related to dietary and serum calcium levels (Boyle, Gray and DeLuca, 1971). Vitamin D deficient rats fed a low calcium diet were found to have 1,25 $(OH)_2D_3$ as the major renal metabolite in plasma while rats on a high calcium diet supplemented with vitamin D_3 produced 24,25 $(OH)_2D_3$. The production of 1,25 $(OH)_2D_3$ correlated with a low serum calcium, whereas in states of normo- or hypercalcaemia, 24,25 $(OH)_2D_3$ appeared in the serum (Boyle, Gray, Omdahl and DeLuca, 1972). Since a low serum calcium is associated with increased circulating levels of parathyroid hormone, it was suggested that parathyroid hormone might be a hormonal mediator of 1,25 $(OH)_2D_3$ production. To test this hypothesis, Galante and

colleagues administered parathyroid extract to rats which had been maintained previously on a low calcium diet and which were in the process of adapting to a high calcium diet. It was reasoned that if the observed decrease in 1,25 $(OH)_2D_3$ production in response to a high calcium diet is mediated by an inhibition of parathyroid secretion, then administration of exogenous parathyroid hormone in this situation would be expected to prevent this adaptation. However, rather than reversing the decrease in 1,25 $(OH)_2D_3$ production in response to the high calcium diet, parathyroid hormone was found to accelerate the adaptation process. There was a decrease in 1,25 $(OH)_2D_3$ production and an increase in 24,25 $(OH)_2D_3$ in the serum (Galante, MacAuley, Colston and MacIntyre, 1972a). These experiments showed that parathyroid hormone is not the sole regulator of 1,25 $(OH)_2D_3$ production, but did not exclude an important physiological role of parathyroid hormone, together with other controlling factors. Later in the same year, the Wisconsin group reported that parathyroidectomized rats on a low calcium diet lost the ability to synthesize 1,25 $(OH)_2D_3$, and that administration of parathyroid hormone to such rats restored the production of this active metabolite (Garabedian, Holick, DeLuca and Boyle, 1972).

Despite our experiments, this group concluded that 1,25 $(OH)_2D_3$ production by the kidney was controlled only by parathyroid hormone: thus DeLuca (1972) suggested that parathyroid hormone was essential for 1,25 $(OH)_2D_3$ production. It was against this background that the study described in this thesis was undertaken, with a view to elucidating more clearly the manner in which the renal metabolism of 25 OH D_3 is regulated and the cellular mechanisms involved in the regulation.

CHAPTER TWO

MATERIALS AND METHODS

Summary

This chapter is divided into three parts. The first part describes the methods employed for the extraction of tritiated vitamin D_3 metabolites from chick kidney homogenates, the separation of these metabolites by liquid-gel partition chromatography using Sephadex LH-20 and their estimation by liquid scintillation counting. Part 2 details other analytical procedures used in this study and Part 3 describes the routine maintenance of experimental animals and the operative techniques employed.

INTRODUCTION

After the discovery that vitamin D is converted to more active metabolites before exerting its physiological effects, a great deal of effort was directed towards elucidating the physiological regulation of this conversion. However, until very recently, such research has been hampered by the lack of a sensitive assay capable of measuring 1,25 $(OH)_2D_3$ levels in experimental animals. Changes in vitamin D metabolism have been determined in this situation by the injection of radiolabelled D_3 and the subsequent extraction and estimation of more active metabolites in tissue and serum. The limitations of this approach are discussed in Chapter Three.

In the study described in this thesis, a more direct method of determining changes in vitamin D metabolism has been adopted

which involves the *in vitro* measurement of the renal 1- and 24-hydroxylases in homogenates with tritiated 25 OH D_3 as substrate. The chick was found to be the experimental animal of choice since this animal has the highest levels of 1-hydroxylase activity of all vertebrates when assayed *in vitro* (Henry and Norman, 1975).

1. Extraction, Separation and Estimation of Vitamin D_3 Metabolites

The estimation of tritiated vitamin D_3 metabolites extracted from chick kidney homogenates involves a 3-stage procedure:

1. Extraction of metabolites with chloroform/methanol
2. Separation of metabolites by liquid-gel partition chromatography on Sephadex LH-20
3. Quantitation of tritiated metabolites by liquid scintillation counting.

1.1 Extraction of vitamin D_3 metabolites from chick kidney homogenates

The method of extraction of vitamin D metabolites from incubates of kidney tissue was a modification of that described by Bligh and Dyer (1959) for the extraction of unsaturated lipids from fish. Extraction was performed according to the following protocol:

To 3 ml of kidney homogenate were added 3 volumes of methanol: chloroform mixture (2:1 v/v, analytical grade reagents). The vessel was shaken briefly by hand and allowed to stand at 4°C overnight.

The monophasic homogenate was then separated into 2 phases by the addition of 1 volume of chloroform. The flask was shaken and allowed to stand at room temperature until 2 clear phases were formed. The lower, chloroform phase was removed with a pasteur pipette and 2 further additions of chloroform (0.5 volume) were made to the remaining aqueous-methanol phase to ensure total lipid extraction. All the chloroform extracts were pooled and the aqueous-methanol phase, containing tissue debris, was discarded. The chloroform extract containing the lipids was evaporated to dryness in a water bath at 40-45°C under a stream of nitrogen. In later experiments extracts were evaporated at room temperature in the forced draught of a fume cupboard. The extracts were then covered and stored at 4°C until the metabolites were separated by chromatography. Recovery of vitamin D metabolites from kidney homogenates was 85 - 95%.

1.2 Chromatography of the Vitamin D Metabolites

A number of chromatographical techniques have been described for the separation of vitamin D metabolites. Chromatography on silic acid (Norman and DeLuca, 1963b; Mawer and Backhouse, 1969) has been utilized, but the usefulness of this system is limited by failure to resolve a number of metabolites more polar than 25 OH D_3. Celite liquid-liquid chromatography has been used (Blunt and DeLuca, 1969) but the poor recovery (60 -80%) makes this system unsuitable for accurate quantitation of separated metabolites. Other methods include thin layer chromatography on silica gel (Norman and DeLuca, 1963b) and countercurrent distribution.

The standard chromatographical technique used in this study was an adaptation of the method of Holick and DeLuca (1971). These workers found that liquid gel partition chromatography on Sephadex LH-20 using a solvent of various percentages of chloroform in hexane allowed excellent resolution of vitamin D_3, 25 OH D_3 and their more polar metabolites.

Sephadex LH-20 was introduced in 1967 for gel filtration in organic solvents (Joustra, Söderqvist and Fischer, 1967) and was found to be particularly useful for separation of metabolites of similar size because, as well as separating substances according to molecular size, it could also be used in liquid-gel partition chromatography. Sephadex is a bead-formed dextran gel. The dextran chains are cross-linked to give a three-dimensional polysaccharide network. Sephadex LH-20 is prepared by hydroxypropylation of Sephadex G-25. The hydroxypropyl groups are attached by ether linkages to glucose units of the dextran chains. The introduction of hydroxypropyl groups does not alter the number of hydroxyl groups but increases the ratio of carbon to hydroxyl. The resultant gel therefore has both hydrophilic and lipophilic properties. In solvent mixtures containing both polar and non-polar organic solvents, the gel takes up predominantly the polar component, thereby creating a marked difference between stationary and mobile phases. In the application of Sephadex LH-20 to the separation of vitamin D metabolites using a mixture of chloroform and hexane as the solvent system, partition effects must be considered to play a major part

in the separation and gel filtration effects can usually be disregarded.

In this study, the metabolites of vitamin D were routinely separated according to the following protocol:

Sephadex LH-20 was weighed in 8.5 g aliquots and equilibrated in solvent (chloroform:hexane, 65:35 v/v). Analytical grade chloroform was used. Initially n-hexane (boiling point 67.5-68.5^{o}) was used, but later the petroleum distillation product with boiling range 60-68°C was used for reasons of economy. This solvent was equally satisfactory. The Sephadex was shaken to form a slurry and poured into glass columns (1.5 cm diameter, 20 cm length). The lower ends of the column were partially blocked by a small pad of glass wool. Solvent was pumped through the columns until they were well packed down and a layer of solvent was kept above the Sephadex to keep it moist. The columns were capped until used.

An automated pumping technique was devised so that the columns could be run with sufficient reproducibility to obviate the need for radioactive standards to be included in every run. The equipment is shown in Fig. 6. A displacement system was devised since the solvent system was found to corrode the tubing of the peristaltic pump. Water from a reservoir was pumped at 0.6 ml per minute through an 'AutoAnalyser' pump (Technicon) onto the top of a 2 litre reservoir which contained solvent. The reservoir was equipped with an outlet at the bottom through which the solvent was displaced. Solvent resistant nylon tubing was attached to the

reservoir outlet and the solvent was thus pumped onto the top of the columns at a rate of 0.6 ml per minute. Using this equipment it was possible to run 10 columns simultaneously.

The dried chloroform extracts of kidney homogenates were dissolved in 2 ml of the chloroform:hexane mixture and were shaken vigorously by hand. The solvent on top of the Sephadex column was removed and the redissolved homogenate extract was applied to the top of the column with a pasteur pipette. When the sample had run into the Sephadex, enough solvent was added to fill the space above the Sephadex to the top of the column. The top of the column was then attached to the solvent resistant tubing from the reservoir by means of a ground glass nozzle, and solvent was pumped through the column.

The eluate was collected directly into polythene scintillation vials (Intertechnique, Brighton, England) which were fitted into an LKB 340B circular fraction collector. Fractions were collected every 7½ or 15 minutes. The columns were run for 6½ hours which allowed all the radioactive metabolites to elute completely. These Sephadex columns could be reused satisfactorily about 10 times, but after this the background radioactivity became rather high and the sharpness of resolution declined. Thus columns were repacked with fresh Sephadex every 10 runs.

1.3 Preparation of the column eluate for scintillation counting

The scintillation vials were then evaporated to dryness in

preparation for liquid scintillation counting. A study was performed to determine the stability of the tritium associated with the eluted vitamin D metabolites with different methods of evaporation.

Tritiated 25 OH D_3 or 1,25 $(OH)_2D_3$ was aliquoted into methanol, chloroform or chloroform-hexane (65:35 v/v). The aliquots were then evaporated to dryness by a variety of methods and the relative recoveries of radioactivity compared. The results are summarized in Table 1. A significant loss of radioactivity was found to occur when samples were evaporated in the presence of light (e.g. on a windowsill in the sun) or in the presence of air at elevated temperatures (in an oven at 60^oC or on a hot plate at 55^oC). The loss of radioactivity was greater in chloroform or chloroform-hexane than in methanol. As slightly more loss occurred from 1,25 $(OH)_2D_3$ than from 25 OH D_3, the relative proportion of 1,25 $(OH)_2D_3$ would be underestimated in samples of column eluate dried this way. However, 100% recovery was found for both 25 OH D_3 and 1,25 $(OH)_2D_3$ in all three solvents when samples were dried in a forced draught. As a result of these experiments, the column eluates were routinely dried at room temperature in the forced draught of a fume cupboard. 5 ml of scintillation fluid was then added and the vials were vigorously shaken. The scintillation fluid used was either 6 g Butyl PBD (Butyl 2-phenyl 5-(4-buphenylyl)1,3,4-oxadiazole from Intertechnique, Brighton) or 4 g PPO (2,5 Diphenyloxazole, from Sigma, Norbiton, Surrey) in 1 litre toluene (analytical grade).

TABLE 1: The effect of the method of evaporation on the recovery of radioactivity associated with metabolites of vitamin D_3 in different solvents.

Method of evaporation	% Radioactivity remaining in vial after evaporation					
	Methanol		Chloroform		Chloroform/Hexane (65:35)	
	25 OH D_3	1,25 $(OH)_2D_3$	25 OH D_3	1,25 $(OH)_2D_3$	25 OH D_3	1,25 $(OH)_2D_3$
Fume cupboard (forced draught at room temp.)	100	100	100	100	100	100
Vacuum oven (55°C)	95	102	100	95	94	Not tested
Windowsill (room temp. in light)	93	95	79	67	76	Not tested
Oven in dark (60°C)	92	87	78	74	73	62
Hot plate in light (55°C)	69	57	50	42	56	49

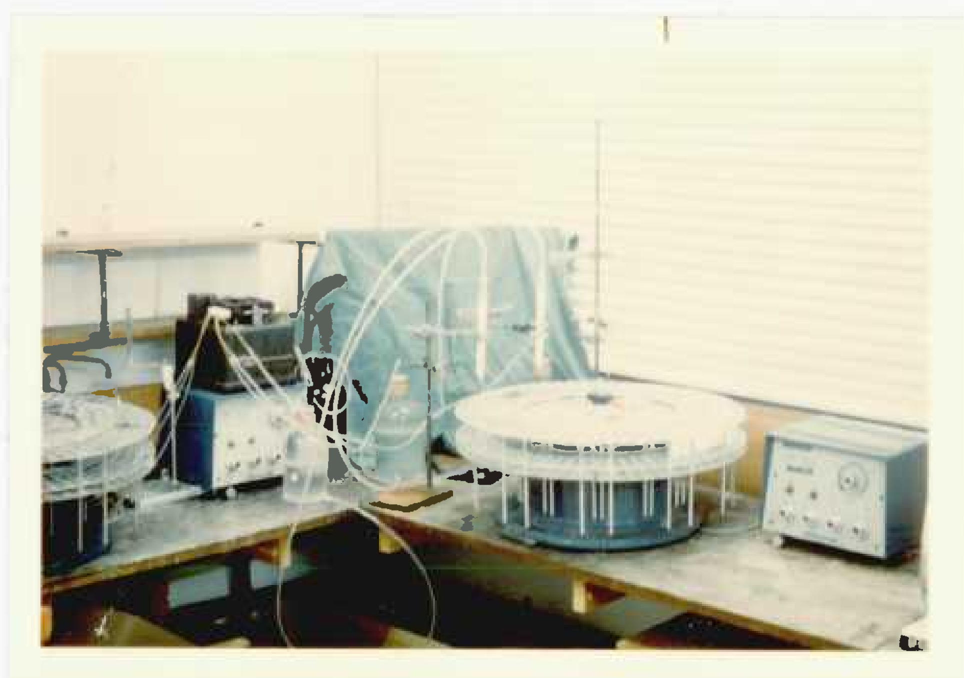


Fig. 6. Photograph of equipment used for the separation of vitamin D_3 metabolites using chromatography on Sephadex LH-20.

1.4 Scintillation Counting

The radioactivity was measured on an Intertechnique liquid scintillation counter (SL40 model) at $4^{o}C$. The counting efficiency was constant (55% for tritium) and quench was negligible since samples prepared as described were completely soluble in toluene.

Using the techniques described it was possible to resolve both 1,25 $(OH)_2D_3$ and 24,25 $(OH)_2D_3$ produced *in vitro* by incubations of chick kidney homogenates from the unconverted substrate, tritiated 25 OH D_3 (Fig. 7).

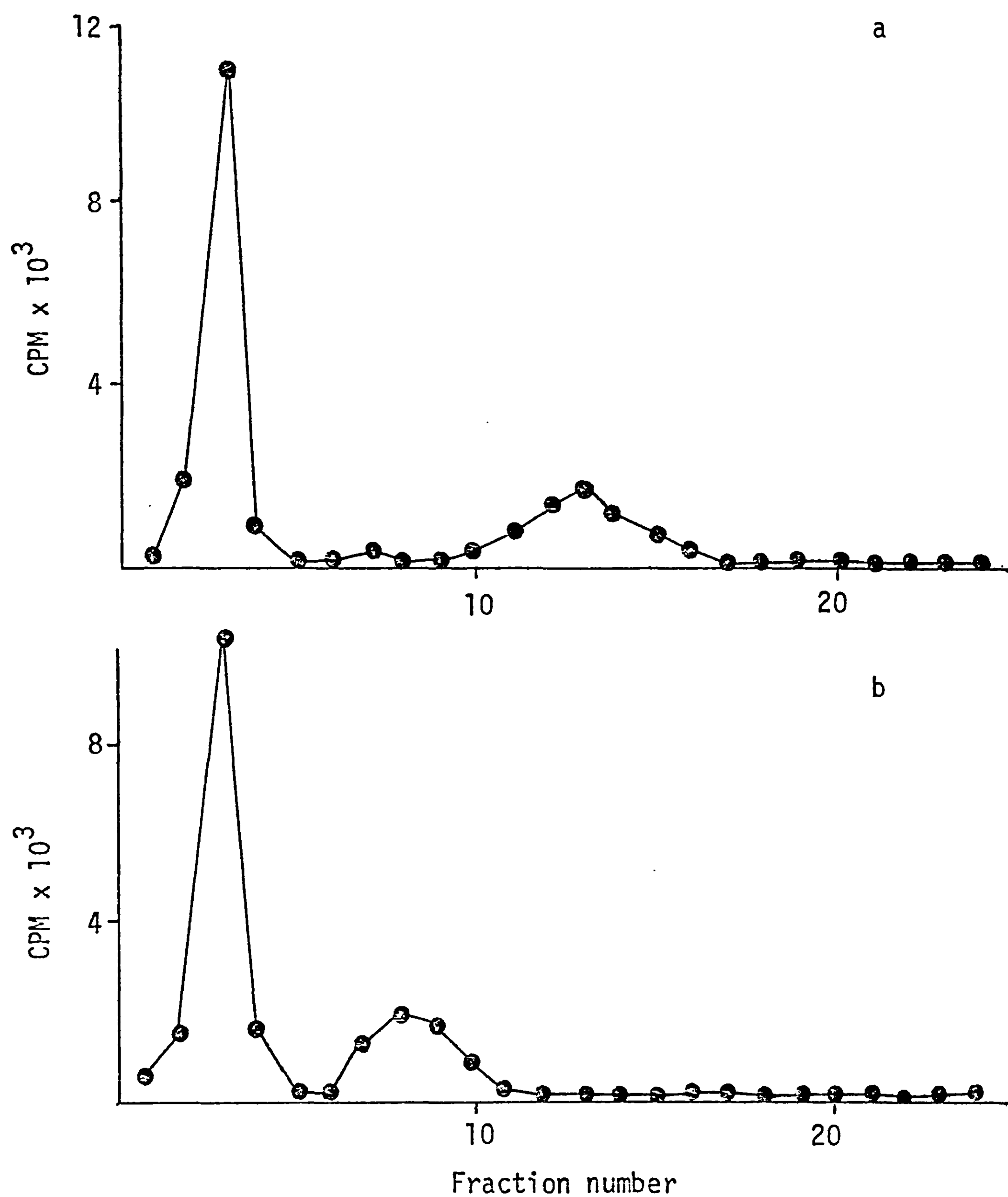


Fig. 7. Chromatography on Sephadex LH-20 of extracts of kidney homogenates incubated with ^3H 25 OH D_3.

a) from a vitamin D deficient chick. Fractions 1-5 contain 25 OH D_3, fractions 10-16 contain 1,25 $(OH)_2D_3$.

b) from a vitamin D deficient chick 38h after administration of 100 i.u. vitamin D_3. Fractions 1-5 contain 25 OH D_3, fractions 6-11 contain 24,25 $(OH)_2D_3$.

2. Analytical Methods

2.1 Measurement of Plasma Calcium

Blood was withdrawn from chicks by cardiac puncture, and was immediately transferred to plastic tubes containing EDTA as the anti-coagulant. The tubes were vigorously shaken and were then centrifuged at 2,000 r.p.m. for 10 min. The plasma was separated and stored at -20°C until analysed. Normally, 200 μl samples of plasma were diluted with 1.8 ml of plasma diluting fluid (which contained 55.5 ml of 60% perchloric acid and 10 ml 44.4 mM KH_2PO_4 in 1 litre of distilled water) in acid washed glass centrifuge tubes. The tubes were vigorously shaken and the precipitated plasma proteins were sedimented by centrifugation at room temperature at 2,000 r.p.m. for 10 min. Total plasma calcium was determined in the supernatant by emission flame photometry (MacIntyre, 1961) using a Zeiss PMQ II flame spectrophotometer with a flame attachment and a quartz double beam monochromater at a wavelength of 422.7 nm using a slit of 0.02 mm.

2.2 Measurement of Plasma Inorganic Phosphate

To avoid erroneous high values due to hydrolysis of phospholipids (Caraway, 1962) blood was centrifuged immediately after withdrawal and the plasma separated off. EDTA was used as anticoagulant. The plasma was stored at -20°C until analysed. Plasma inorganic phosphate was estimated by the method of Delsal and Manhouri (1958). The method is a variant of the phosphymolybdic

acid reaction. A substituted phenol is used as a reducing agent and the pH is controlled by an acetate buffer. Copper in the buffer hastens colour development which is complete after 5 minutes. The blue colour is stable for at least 30 min. Normally, 100 μl of plasma was diluted with 1.9 ml 5% trichloroacetic acid, mixed and centrifuged. To one ml of the supernatant was added 3 ml of acetate buffer, 0.5 ml 5% ammonium molybdate and 0.5 ml of metol mixing well after each addition. 1 ml of a standard phosphate solution (0.004 mg per ml) was treated in the same way and 1 ml of 5% T.C.A. served as a blank control. After allowing 7 min for the development of colour, readings were taken in a spectrophotometer at 880 mμ.

Calculation: Inorganic phosphate (mg/100 ml) =

$$\frac{\text{O.D. test}}{\text{O.D. Std.}} \times 0.004 \times \frac{100}{0.05}$$

$$= \frac{\text{O.D. test}}{\text{O.D. Std.}} \times 8$$

Reagents:

a) Trichloroacetic acid: 5 g per 100 ml in water

b) Phosphate standard: Analar KH_2PO_4 (0.004 mg per ml) in 5% T.C.A.

c) Acetate buffer pH 4.0: 2.5 g copper sulphate and 46 g of hydrated sodium acetate dissolved in 1 litre of 2N acetic acid and the pH adjusted to 4.0

d) Metol: 2 g of Metol (paramethyl aminophenol sulphate) in 80 ml of water with 10 g hydrated sodium sulphite added, the solution made up to 100 ml and filtered. The solution is then stored in a dark bottle at 4^oC.

e) Ammonium molybdate: 5 g per 100 ml in water.

2.3 Estimation of Protein Content of Homogenates

The method used in this study was a modification of the method of Lowry (Lowry, Rosebrough, Farr and Randall, 1951). Usually, a 1 ml sample of homogenate was solubilized with 0.1 ml normal sodium hydroxide. 9 ml of 0.15M sodium chloride was added and the solution well mixed. 0.1 ml of this was further diluted by the addition of 0.9 ml of 0.15M sodium chloride. 5 ml of reagent (C) was added to 1 ml of the diluted homogenate or to 1 ml of standard solution or to 1 ml of sodium chloride solution (blank) and mixed. After 10 min 0.5 ml of reagent (D) was added and mixed. The colour was left to develop for 1 hour and readings were taken in a spectrophotometer at 750 mμ. Since the colour thus obtained is not directly proportional to the concentration of protein, a series of protein standards (bovine albumin in 0.15M sodium chloride) were prepared with a range of 1.5 to 15 mg/100 ml. All these standards were included in every assay of homogenate protein content. The optical density corresponding to each of these standards was measured and the concentration of protein in the test solution was calculated from the standard curve thus obtained.

Reagents:

A) 2% sodium carbonate in 0.1N sodium hydroxide

B) 0.5% copper sulphate in 1% sodium citrate

C) 1 ml of reagent B mixed with 50 ml of reagent A

D) 5 ml Folin and Ciocalteu's reagent (BDH) diluted with 10 ml water.

3. Animals and Diets

3.1 Chicks

One day old chicks were obtained from Orchard Farm, Great Missenden, Bucks. Due to difficulty of supply, three breeds of chicks were used in this study. These were Light Sussex, Rhode Island Red/Light Sussex Crossbreed or White Leghorns. All three breeds proved to be equally satisfactory. The chicks were housed in wire cages with 15 to 30 chicks per cage, depending on their age. They were kept in the absence of ultraviolet light in a room illuminated by infra red lamps. All the chicks had free access to food and tap water. Table 2 shows the content of the vitamin D deficient diet. The calcium and phosphate content of the diet were normally 0.34% and 0.54% respectively, but in some experiments this was altered by the addition of calcium carbonate and sodium dihydrogen phosphate to give the desired calcium and phosphate content.

After three or four weeks, the chicks became severely rachitic and displayed profound muscle weakness. Fig. 8 shows a comparison between the appearance of four-week old chick maintained on this vitamin D deficient diet and a chick of the same age whose diet was supplemented with 0.5 μg vitamin D_3 thrice weekly. Fig. 9 shows an X-ray of a leg taken from a four-week old vitamin D deficient chick. There is marked decalcification of the bone. An X-ray of a leg from a vitamin D supplemented chick is included for comparison.

Fig. 8. Coloured photograph of 4-week old vitamin D deficient chick (right) with a vitamin D supplemented chick (left) as comparison.

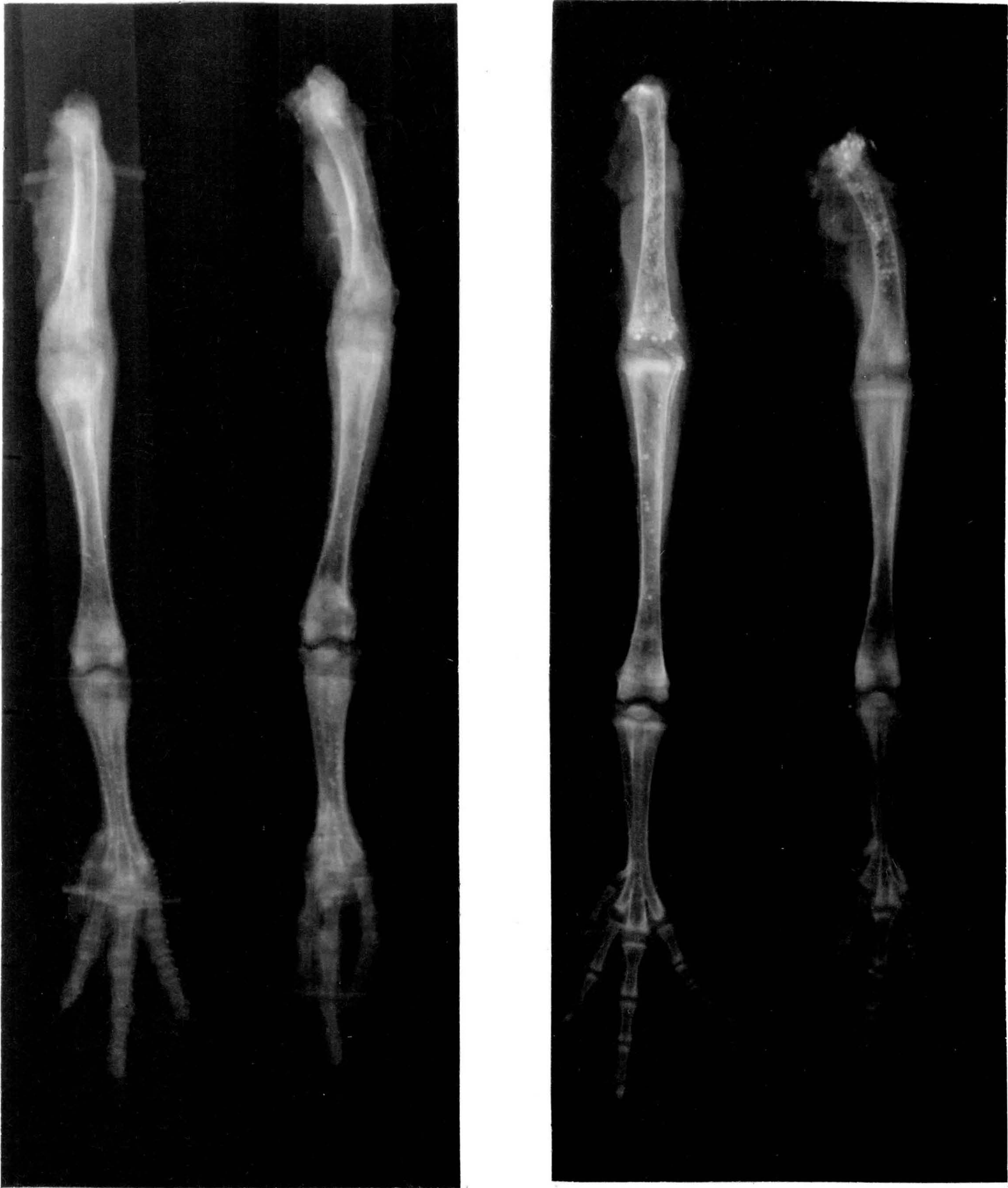


Fig. 9. X-ray of legs from 4-week old vitamin D deficient (left) and vitamin D supplemented (right) chicks.

TABLE 2: Composition of the Vitamin D deficient chick diet

	% by weight
Skimmed milk powder	21.0
Yeast	5.3
Maize	47.4
Wholemeal flour	17.5
Oats	8.8

3.2 Operative Techniques

In some experiments both the parathyroid and ultimobranchial glands were removed from vitamin D deficient chicks. Chicks of various ages and of varying states of vitamin D deficiency were lightly anaesthetized with ether and a longitudinal incision was made in the neck. Using blunt dissection, the crop was detached from the subcutaneous tissue and was deflected to one side in order to reveal the subclavicular air sacs. These were entered to reveal the thyroid gland and ultimobranchial body with the attached parathyroids. The ultimobranchial body is found on both sides posterior to the lower pole of the thyroid and above the brachio-cephalic artery where it branches into the carotid and brachial arteries. The glands are pink ovoid structures and generally lie on the jugular vein beneath the carotid artery. The ultimobranchial bodies with attached parathyroid glands were bilaterally excised using pointed forceps leaving both thyroid glands intact. The skin was then closed using fine surgical silk. The operative exposure is shown in Fig. 10.

Since respiration is impaired when the subclavicular air sacs are ruptured, it was necessary to perform the whole operation under positive pressure ventilation. The chicks were intubated by means of a narrow plastic tube which was passed into the trachea of the chicks. The tube was equipped with a small hole so that oxygen pressure could be regulated by opening or closing the hole with a finger. The birds were intubated with 100% oxygen which passed from a cylinder through a simple barometer filled with water and so to the

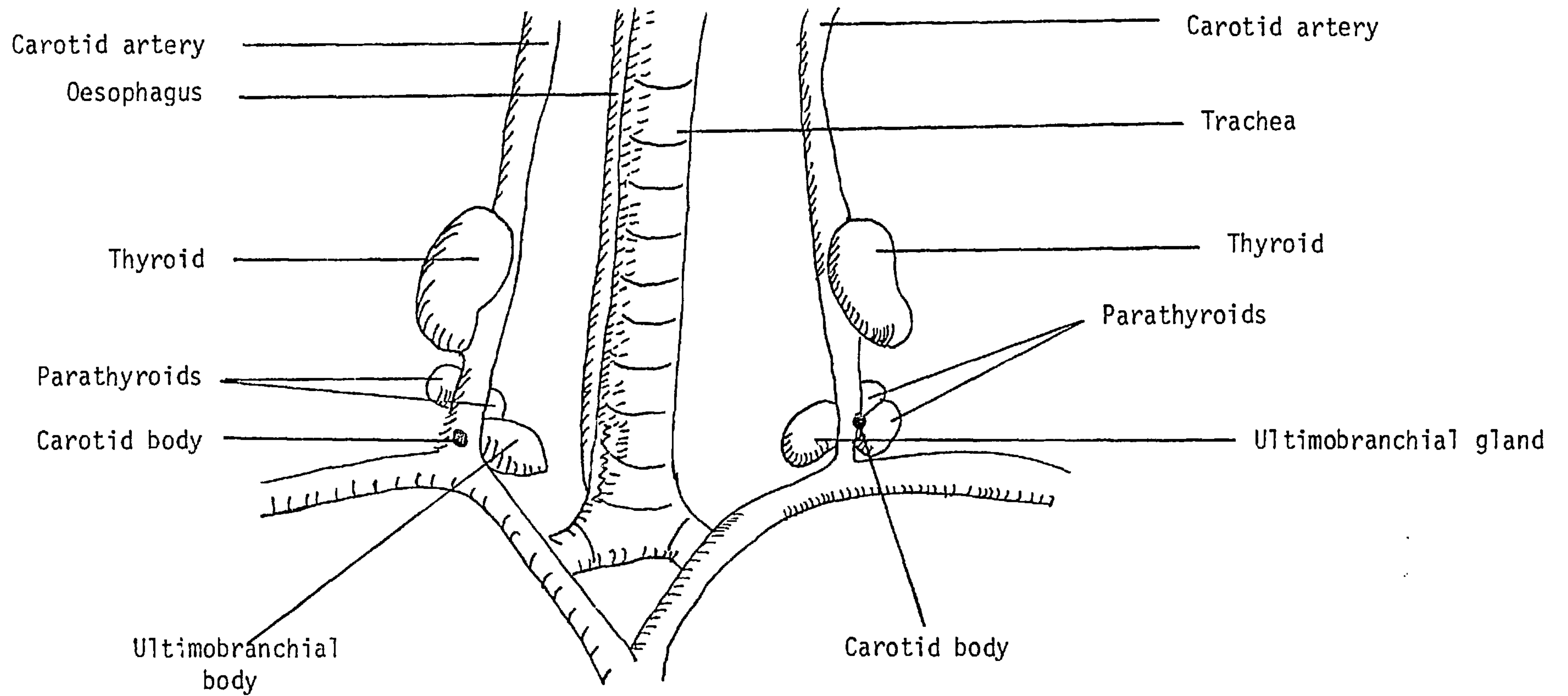


Fig. 10. Operative exposure

intubation tube. The barometer served the dual purpose of humidifying the oxygen and as a measure that the pressure of oxygen remained the same. An illustration of the intubation equipment is shown in Fig. 11. Sham operations were performed in an identical fashion, except that the ultimobranchial bodies and parathyroid glands were left _in situ_.

The chicks were kept in plastic cages with free access to water and were warmed by means of an infra red lamp. Post-operative survival was found to be no more than 50%. The high mortality rate was due to either massive haemorraghing from accidental damage to thyroid blood vessels or from a precipitous fall in serum calcium. Various methods were instituted to counteract this fall in serum calcium. In some experiments the birds were treated with calcium supplements and in others chicks were treated pre-operatively with vitamin D_3. The significance of these measures will be discussed later in this thesis.

Fig. 11. Photograph of equipment used for intubating chicks during parathyro-ultimobranchialectomy.

SECTION II

CHAPTER THREE

THE ASSAY OF 25 HYDROXYCHOLECALCIFEROL-1-HYDROXYLASE

Summary

This chapter describes experiments designed to standardize conditions for the assay of 25 hydroxycholecalciferol-1-hydroxylase activity in chick kidney homogenates. Other experiments were designed to demonstrate that the peak from an extract of homogenate which elutes as 1,25 $(OH)_2D_3$ from Sephadex LH-20 is homogeneous.

INTRODUCTION

The presence in vertebrate kidney of an enzyme system which converts 25 OH D_3 to its most active metabolite, 1,25 $(OH)_2D_3$, was reported by Fraser and Kodicek (1970). They located an hydroxylating system capable of converting 25 OH D_3 to 1,25 $(OH)_2D_3$ _in vitro_ in 'large' mitochondria which sedimented with the nuclear fraction of 700 g. Gray and colleagues concluded, on the basis of a somewhat different centrifugation procedure, that the 25 OH D_3-1-hydroxylase enzyme is present in 'heavy' mitochondria (Gray, Omdahl, Ghazarian and DeLuca, 1972).

The renal 1-hydroxylase enzyme (25-hydroxycholecalciferol 1-monooxygenase; 25-hydroxycholecalciferol, NADPH oxygen oxido reductase (1-hydroxylating), EC 1.14.13.13) is classed as a monooxygenase (or mixed function oxidase). This class of enzyme catalyses the incorporation of one atom of the oxygen into a substrate. The other

atom is reduced to water. The reaction requires an additional electron donor or co-substrate, e.g.,

$$R - H + ZH_2 + O_2 \longrightarrow R - OH + Z + H_2O$$

Many of the enzymes involved in steroid synthesis by the adrenal gland are mixed function oxidases, utilizing NADPH as a co-substrate. Ghazarian and DeLuca (1974) have confirmed that NADPH is a necessary cofactor for the in vitro activity of the 1-hydroxylase enzyme.

The 1-hydroxylase enzyme shows certain properties associated with cytochrome P450-dependent hydroxylases. For example, microsomal hydroxylase enzymes of the liver, which are concerned with drug detoxification, are inhibited by carbon monoxide. The activity of the renal 1-hydroxylase enzyme has also been shown to be inhibited by carbon monoxide (Henry and Norman, 1974), an observation which implicates the involvement of this hemeprotein as a component of the chick kidney 1-hydroxylase. It has also been demonstrated that all the oxygen inserted by this mitochondrial enzyme into the 1α position of 25 OH D_3 in vitro is derived from molecular oxygen (Ghazarian, Schnoes and DeLuca, 1973). 1-hydroxylase activity has been detected in kidney mitochondria isolated from 25 vertebrate species. However, kidney mitochondria from rachitic chicks were found to have the highest levels of enzyme activity of all the species tested (Norman and Henry, 1974). Fraser and Kodicek (1970) also demonstrated that whole homogenates of chick kidney could perform this hydroxylation in vitro.

In the study described in this thesis, the capacity of chick kidney homogenates to convert 25 OH D_3 to 1,25 $(OH)_2D_3$ (or to 24,25 $(OH)_2D_3$) has been utilized to provide an *in vitro* assay for the assessment of the renal metabolism of 25 OH D_3 in a variety of *in vivo* situations. In this way it was possible to elucidate the role played by various factors in the regulation of vitamin D metabolism by investigating their effects on the activity of the renal 1-hydroxylase enzyme assayed *in vitro*. This *in vivo/in vitro* approach has a number of advantages over the more usual method of estimating changes in vitamin D metabolism. This involves the injection of radio-labelled vitamin D_3 or 25 OH D_3 and subsequent extraction of serum or other tissues in order to determine the percentage conversion of tracer to active metabolites.

One disadvantage of this method is that conversion to more active metabolites takes time (from 8 to 12 hours) making it unsuitable for short term investigations. Secondly, when serum is being analysed, it is impossible to separate 1,25 $(OH)_2D_3$ from other metabolites, such as 25,26 $(OH)_2D_3$, by conventional chromatographic techniques. Pool size is also a problem, since the animals used must be almost completely depleted of vitamin D in order to detect the low levels of conversion of the injected radioactive tracer.

The kidney homogenate system largely overcomes these disadvantages. Since the conversion of 25 OH D_3 to dihydroxy metabolite is performed *in vitro*, it is possible to determine changes in enzyme activity at any time after administration of a test substance to the

whole animals. Restriction of pool size is also less important since it is the capacity of the renal enzyme to convert substrate which is being estimated rather than the absolute production of 1,25 $(OH)_2D_3$.

The experiments described in this section were designed to standardize conditions for the assay of 25 OH-1-hydroxylase activity in chick kidney homogenates and to demonstrate that the peak from an extract of homogenate which elutes as 1,25 $(OH)_2D_3$ on Sephadex LH-20, is homogeneous.

1. Methods

1.1. Preparation of kidney homogenates

One-day old chicks were fed the vitamin D deficient diet for three to four weeks. The chicks were killed by decapitation and the kidney tissue was removed and placed on ice. Homogenates (5% w/v) were prepared in a Potter-Elvehjem homogenizer in ice-cold Tris-acetate buffer (15 mM, pH 7.4), supplemented with magnesium (1.9 mM), sodium succinate (5 mM) and sucrose (200 mM).

1.2 Assay of 25 hydroxycholecalciferol-1-hydroxylase activity

Aliquots (3 ml) of homogenate were preincubated at 37°C for 5 or 10 min. The substrate, tritiated 25 OH D_3 (25-hydroxy 26(27)-Me-^{3}H -cholecalciferol, specific activity 6.3 to 19.7 Ci/mmol; Radiochemical Centre, Amersham, Bucks.) dissolved in absolute ethanol, was added to each incubation in a volume of 10 to 20 µl. The final concentration of added substrate varied from 15.2 to 75

p moles per 3 ml incubate, depending on the specific activity. The reaction mixtures were flushed with 100% oxygen for 30 to 60 sec and incubated for 10 min at $37^{o}C$ with gentle shaking. The reaction was stopped by the addition of 9 ml methanol/chloroform (2:1 v/v) and then 25 OH D_3 and its metabolites were extracted and chromatographed as described in 'Materials and Methods'. In early experiments, results were expressed in terms of the percentage radioactivity eluting as 1,25 $(OH)_2D_3$. Later, 1-hydroxylase activity was expressed as p mol 1,25 $(OH)_2D_3$ produced per mg of homogenate protein per minute of incubation.

2. Preliminary Studies

2.1 Time course of 1,25 $(OH)_2D_3$ production

Under these assay conditions, the production of 1,25 $(OH)_2D_3$ was linear with time up to 20 min and fell off rapidly beyond this time. At 40 min no further increase in 1,25 $(OH)_2D_3$ production was seen (Fig. 12). This fall off in production was not due to substrate utilization since at this time, less than 30% of the substrate was converted to product. On the basis of these results, an incubation time of 10 min was adopted for routine assays during experiments.

2.2 pH optimum

The activity of the 25 OH D_3-1-hydroxylase enzyme was markedly

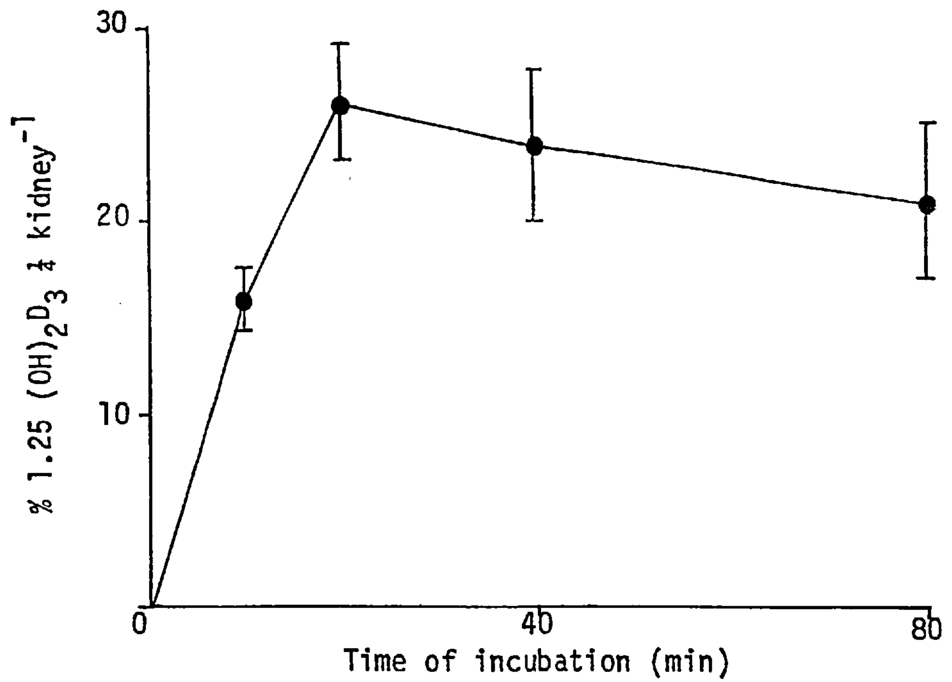


Fig. 12. Production of 1,25 $(OH)_2D_3$ by chick kidney homogenates with time of incubation. Each point represents the mean of 3 estimations ± S.E.M.

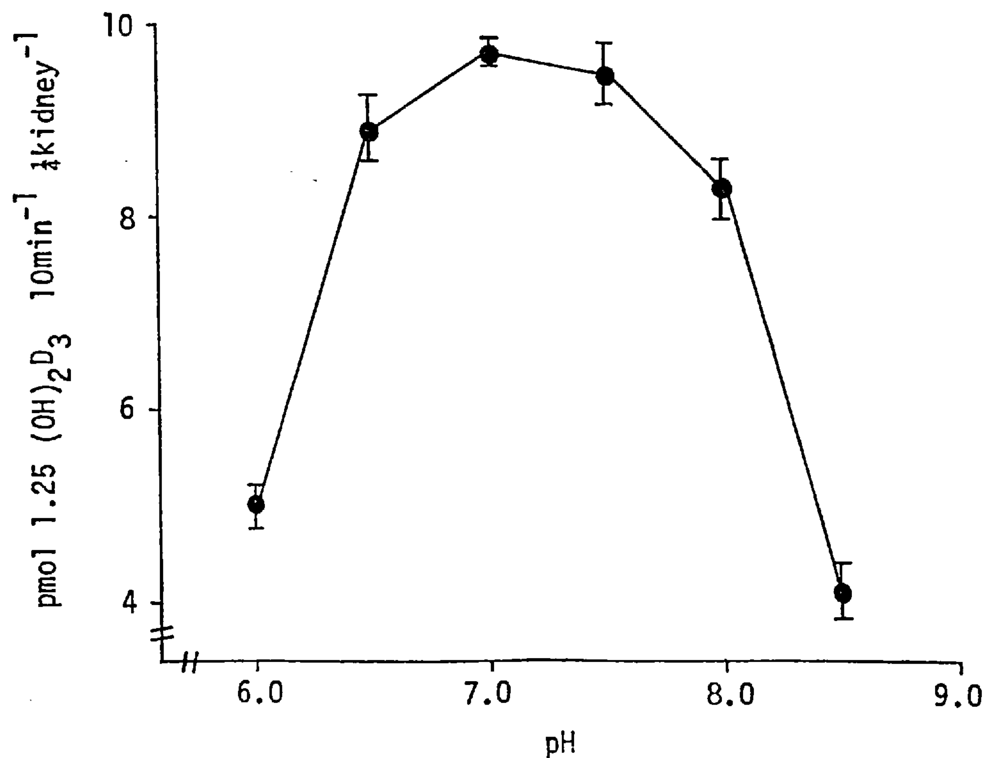


Fig. 13. Effect of pH on 25 OH-1-hydroxylase activity. Kidneys from 3-week old vitamin D deficient chicks were pooled, roughly chopped and placed on ice. The tissue was divided into 6 equal portions by weight and each portion was homogenized in Tris-acetate buffers of varying pH. Each homogenate was equivalent to 1 kidney in 12 ml buffer.

influenced by the hydrogen ion concentration of the incubation medium. This is shown in Fig. 13. Formation of the dihydroxy metabolite was found to be maximal between pH 7.0 and pH 7.5, and consequently an incubation medium with a pH of 7.4 was used in later assays.

2.3 Relationship between 1-hydroxylase activity and protein concentration

There was found to be a linear relationship between the production of 1,25 $(OH)_2D_3$ and homogenate protein concentration in the range 2.7 to 20.7 mg protein per 3 ml incubate. However, a doubling of protein concentration only resulted in a 50% increase in enzyme activity. Little further increase in enzyme activity was seen at higher concentrations, while no conversion of 25 OH D_3 to 1,25 $(OH)_2D_3$ could be detected with protein concentrations of 1.2 mg or less (Table 3). On the basis of these results, a protein concentration of approximately 15 mg protein per 3 ml was chosen in later assays. This protein concentration corresponds to approximately one quarter of a kidney from a 3 week old chick.

TABLE 3

Effect of increasing protein concentration on 25 OH D_3-1-hydroxylase activity.

mg protein per 3 ml incubate	1-hydroxylase activity
45.6	2.70 ± 0.11
20.7	2.41 ± 0.02
10.8	1.96 ± 0.05
2.73	1.31 ± 0.19
1.20	No detectable activity

A 20% w/v homogenate was made from the kidneys of 3-week old vitamin D deficient chicks. Various dilutions of this homogenate were made in Tris-acetate buffer, pH 7.4 and 3 x 3ml incubates of each dilution were assayed for enzyme activity. In addition, 3 ml of each dilution was used for estimation of protein content. Enzyme activity is expressed as mean (± S.E.M.) pmoles 1,25 $(OH)_2D_3$ produced per min. The substrate concentration was 75 pmol 25 OH D_3 per 3 ml incubate.

3. Separation of 1,25 $(OH)_2D_3$ from other dihydroxy-metabolites by high pressure liquid chromatography

As previously mentioned, it is not possible to separate 1,25 $(OH)_2D_3$ from 25,26 $(OH)_2D_3$ by conventional chromatographic techniques. Since the site of 25,26 $(OH)_2D_3$ production has not been identified, it was necessary to confirm that 1,25 $(OH)_2D_3$ is not contaminated by this other dihydroxy-metabolite following extraction from chick kidney homogenates and separation on Sephadex LH-20.

A new chromatographic system was devised for the separation of the hydroxylated derivatives of vitamin D_3, using high pressure liquid chromatography.

3.1 High pressure liquid chromatography

The separation system employed the use of a Du Pont 830 liquid chromatograph fitted with a gradient elution accessory and fitted with a steel tube (500 mm x 2.1 mm) backed with octadecyl-silane permanently bonded to glass beads (ODS-Permaphase, Du Pont De Nemours, Wilmington, Delaware, USA). The apparatus was maintained at $40^{o}C$ and samples (in 1 to 10 μl methanol) were injected into the separation column and eluted by a linear gradient from water to methanol in which the methanol concentration increased by 5% v/v per min. The flow rate was 1 ml per min with a pressure drop of 700 pounds per square inch. The absorption of the effluent was recorded continuously at 254 nm and fractions were collected for determination of radioactivity.

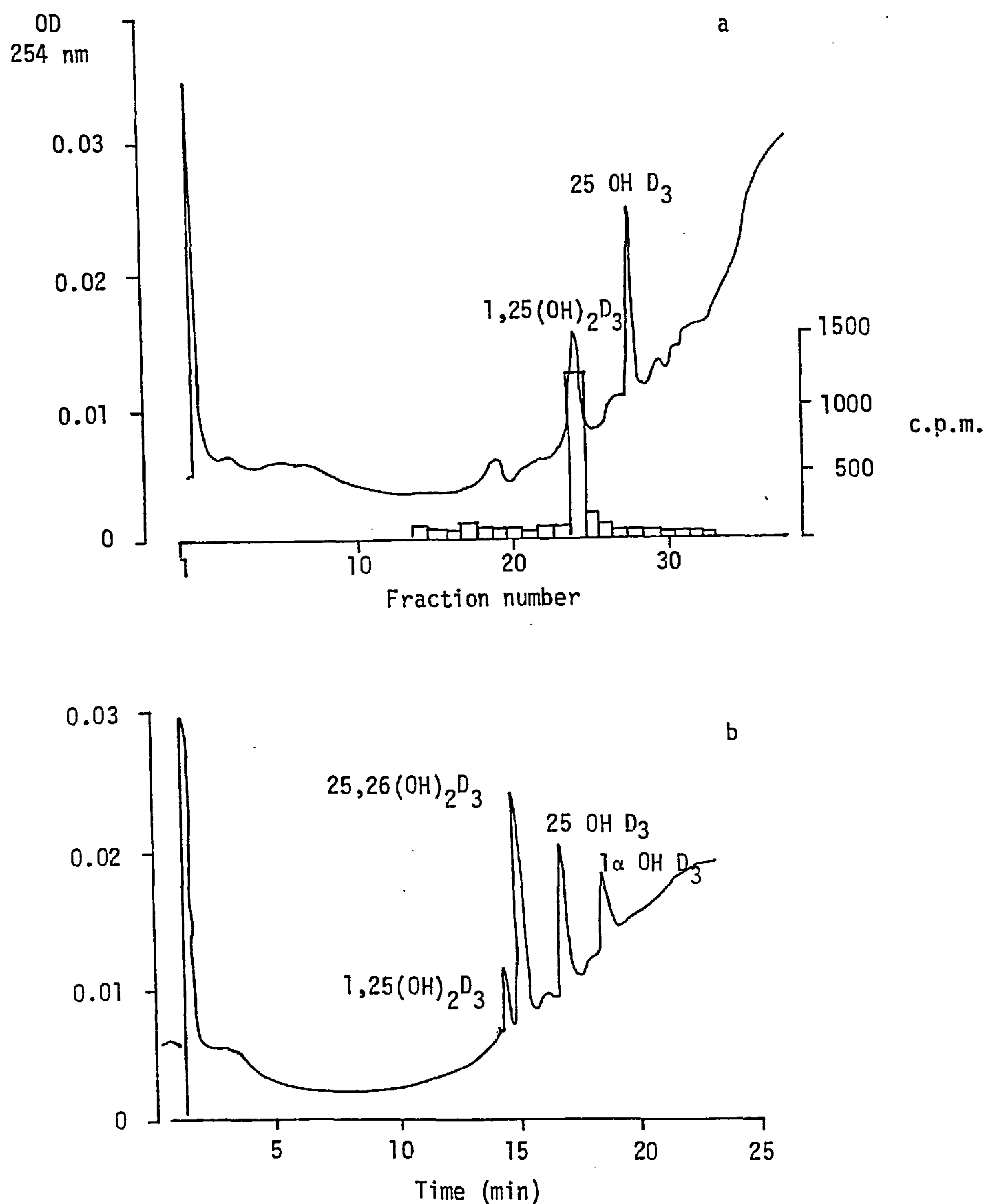


Fig. 14. a) the identification as 1,25 $(OH)_2D_3$ of a radioactivity peak from Sephadex LH-20 chromatography of an extract of chick kidney homogenate.

b) high pressure liquid chromatography of a mixture of 1,25 $(OH)_2D_3$, 25,26 $(OH)_2D_3$, 25 OH D_3 and 1α OH D_3.

3.2 Co-chromatography of synthetic 1,25 $(OH)_2D_3$ with a radioactivity peak eluting from Sephadex LH-20.

The peak of radioactivity eluting as 1,25 $(OH)_2D_3$ from Sephadex LH-20 was evaporated to dryness under a stream of air and redissolved in methanol. 75 ng synthetic 1,25 $(OH)_2D_3$ and 100 ng 25 OH D_3 were added and the mixture was then applied to the liquid chromatograph. Fig. 14a shows that the radioactivity coincides exactly with the peak of added synthetic 1,25 $(OH)_2D_3$ and is homogeneous on this system. Fig. 14b demonstrates that this system is capable of separating synthetic 1,25 $(OH)_2D_3$ from synthetic 25,26 $(OH)_2D_3$.

4. Discussion

These experiments demonstrate that chick kidney homogenates are capable of converting 25 OH D_3 to 1,25 $(OH)_2D_3$. The production of the active metabolite was linear with time up to 20 minutes but fell off rapidly beyond this time.

In subsequent experiments, an incubation time of 10 minutes was chosen to allow a sensitive estimation of the initial rate of reaction while still maintaining a high enough percentage conversion of substrate to product to be accurately detectable when separated by LH-20 chromatography.

The pH optimum for the enzyme was found to be 7.4 and in all subsequent experiments, Tris-acetate buffer of this hydrogen ion concentration was used as the incubation medium.

The K_m of the 1-hydroxylase enzyme has been reported as 1×10^{-6} (Gray et al., 1972) or 0.8×10^{-7} (Chapter Six of this thesis). When assaying any enzyme activity it is generally desirable to employ a substrate concentration of approximately 4-5 times the K_m value so that the enzyme is working at maximum velocity. However, in the case of the 1-hydroxylase enzyme, the product of the enzymic reaction cannot be detected directly. As the ratio of substrate to product increases, so the accuracy of detection of the product decreases. At high concentrations of substrate, the percentage conversion of substrate to product will be too low to detect accurately. For this reason it was not possible to assay 1-hydroxylase activity using a saturating concentration of substrate.

CHAPTER FOUR

PARATHYROID HORMONE AND VITAMIN D METABOLISM

Summary

A study was carried out to determine whether parathyroid hormone is essential for the continued activity of the 25 OH D_3-1-hydroxylase enzyme.

The results show that, in the absence of vitamin D, removal of the parathyroid glands has no effect on renal-1-hydroxylase activity. However, administration of vitamin D_3 caused a significant decrease in the 1-hydroxylase activity and this effect was potentiated by the removal of the parathyroid gland.

It is concluded that parathyroid hormone is not essential for the continued activity of the 1-hydroxylase enzyme, but may play a role in physiological situations.

INTRODUCTION

In 1971, Boyle and his co-workers found that rats maintained on a low calcium diet had 1,25 $(OH)_2D_3$ as the major circulating renal metabolite of vitamin D_3, while rats on a high calcium diet produced 24,25 $(OH)_2D_3$. It was thought at first that this effect of dietary calcium was mediated via parathyroid hormone. However, Galante and co-workers (1972b) gave exogenous parathyroid extract to intact rats and found that parathyroid extract suppressed the conversion of 25 OH D_3 to 1,25 $(OH)_2D_3$ and enhanced the conversion to 24,25 $(OH)_2D_3$. Further conflict arose when, later the same year, DeLuca's group in Wisconsin reported that thyroparathyroidectomized vitamin D deficient rats showed a decrease in 1,25 $(OH)_2D_3$ production compared with intact

controls (Garabedian et al., 1972) and observed that administration of parathyroid hormone reversed this effect. However, administration of glucose also reversed the inhibitory effect of thyroparathyroidectomy on 1,25 $(OH)_2D_3$ production (Tanaka and DeLuca, 1973).

To further investigate the role of parathyroid hormone on vitamin D metabolism, a series of experiments was undertaken to elucidate the effect of acute parathyroidectomy and ultimobranchialectomy upon the activity of the 1-hydroxylase enzyme in chick kidney.

1. <u>Preliminary Experiments to Investigate the Effect of Removal of the Parathyroültimobranchial Complex on 1-hydroxylase Activity</u>

Experimental protocol:

3-week old vitamin D deficient chicks were parathyroidectomized and ultimobranchialectomized as described in 'Materials and Methods'. The ultimobranchial glands were removed since they contain accessory parathyroid tissue.

Results:

It was found that the removal of the parathyroid glands superimposed upon the hypocalcaemia of vitamin D deficiency caused rapid death. In the first series of experiments, 24 3-week old chicks were parathyro-ultimobranchialectomized. All but 2 died in tetany within 6hr of this operative procedure. In an attempt to lessen the rapid fall in plasma calcium following parathyroidectomy, fat free milk powder was allowed <u>ad libit um</u> after the operation. This

post-operative procedure markedly improved the survival rate, and was henceforth adopted in another series of experiments designed to investigate possible changes in 1-hydroxylase activity with time after operation.

2. Effect of Parathyroidectomy and Ultimobranchialectomy on 1-hydroxylase Activity with Time After Operation

Experimental protocol:

Parathyroid and ultimobranchial glands were removed from 30 chicks which had been fed the standard vitamin D deficient diet for 3 weeks before the operation. The chicks were allowed fat free milk powder post-operatively. 14 of the original 30 birds survived the operation, while all 14 sham operated birds survived. At various times after operation, the chicks were killed by decapitation and blood was removed by cardiac puncture for the estimation of plasma calcium concentration. Kidneys were then removed and the level of 1-hydroxylase activity was assayed as described previously.

Results:

The results of this experiment are summarized in Table 4. There was no significant decrease in the activity of the renal 1-hydroxylase enzyme up to 20h after parathyroidectomy and ultimobranchialectomy.

Following the completion of this preliminary series of experiments, apparently conflicting results on the effect of parathyroidectomy on 1-hydroxylase activity were reported by Kodicek's group in Cambridge (Fraser and Kodicek, 1973). This group showed

TABLE 4

Effect of parathyroidectomy and ultimobranchialectomy on 25 OH D_3-1-hydroxylase activity.

Time after operation (h)	No. of birds	1-hydroxylase activity	Plasma Ca (mmol l^{-1})
0 (Sham)	14	49 ± 0.1	2.5 ± 0.045
1	1	46	1.6
3	3	38 ± 4.9	1.5 ± 0.06
6	2	23 ± 0.5	1.85 ± 0.33
9	2	42 ± 6.5	1.4 ± 0.1
20	6	42 ± 7.1	1.3 ± 0.09

Parathyroid and ultimobranchial glands were removed under ether anaesthesia. Kidneys were removed and homogenized in Tris-acetate buffer at $4^{o}C$. The assay was completed by the addition of substrate (^{3}H 25 OH D_3, 75 pmol per 3 ml incubate). The incubation time was 10 min. Enzyme activity is expressed as mean (± S.E.M.) percentage ^{3}H as 1,25 $(OH)_2D_3$ in extracts of homogenates from one-quarter kidney. Four replicate estimations were made from each bird. The plasma Ca is the mean ±S.E.M. of samples taken immediately before the birds were killed.

that removal of the parathyroid and ultimobranchial glands almost completely abolished the activity of the renal 1-hydroxylase enzyme. The experimental procedure adopted by the Cambridge group differed in a number of ways, and a series of experiments was designed to investigate the possibility that procedural differences could explain these apparently conflicting results.

The main difference between the two sets of experiments was the procedure adopted to ensure post-operative survival. The Cambridge group elected to administer vitamin D_3 24h pre-operatively rather than giving oral calcium supplements as previously described. Other differences were the protein, calcium and phosphate content of the vitamin D deficient chick diet and the use of an assay for 1-hydroxylase activity which did not permit the concomitant estimation of 24,25 $(OH)_2D_3$ production.

3. The Influence of Experimental Procedures on the Effect of Parathyroidectomy and Ultimobranchialectomy on 1-hydroxylase Activity

3.1 The influence of diet

Experimental protocol:

Chicks were maintained for 3 weeks on a vitamin D deficient diet. In some experiments the calcium and phosphate content of the diet was altered as shown in Table 5a. Chicks were parathyroidectomized and ultimobranchialectomized or sham operated. Blood was withdrawn by cardiac puncture for estimation of plasma calcium and phosphate.

TABLE 5a

Effect of parathyroidectomy and ultimobranchialectomy (PTUX) on 25 OH D_3-1-hydroxylase activity in chick kidney homogenates.

Operation	No. of birds surviving	Treatment	Pre-operative treatment	1-hydroxylase activity	Plasma Ca (mmol l^{-1})	Plasma PO_4 (mmol l^{-1})
Sham	3/3	$-D_3$ + Ca	Diet 1	15.97 ± 2.78	2.47 ± 0.25	1.80 ± 0.15
PTUX	4/6	$-D_3$ + Ca	Diet 1	19.02 ± 2.18	1.43 ± 0.27	2.38 ± 0.25
Sham	4/4	$-D_3$	Diet 3	21.02 ± 3.79	2.46 ± 0.1	2.58 ± 0.13
PTUX	2/4	$-D_3$	Diet 3	21.21 ± 6.16	1.6 ± 0.3	3.19 ±±0.16
Sham	3/3	$-D_3$	Diet 2	32.59 ± 9.92	2.53 ± 0.20	1.93 ± 0.1
PTUX	2/6	$-D_3$	Diet 2	9.06 ± 2.23	1.05 ± 0.17	3.1 ± 0.65
Sham	2/2	$-D_3$ + Ca	Diet 1	54.51 ± 5.23	2.75 ± 0.23	2.42 ± 0.25
PTUX	5/5	$-D_3$ + Ca	Diet 1	33.08 ± 8.65	1.58 ± 0.29	2.49 ± 0.11

Enzyme activities are expressed as percentage ^3H (±S.E.M.) as 1,25 $(OH)_2D_3$ in extracts of homogenates of one-quarter kidney. Incubation time for the assay was 10 min and substrate concentration was 75 pmol 25 OH D_3 per 3 ml incubate.

TABLE 5b

The effect of parathyroidectomy and ultimobranchialectomy (PTUX) and of vitamin D_3 on 25 OH D_3-1-hydroxylase activity in chick kidney homogenates.

Operation	No. of birds surviving	Treatment	Pre-operative treatment	1-hydroxylase activity	Plasma Ca (mmol l^{-1})	Plasma PO_4 (mmol l^{-1})
Sham	3/3	$+D_3$	Diet 4	16.61 ± 4.08	2.95 ± 0.28	2.15 ± 0.28
PTUX	3/4	$+D_3$	Diet 4	2.36 ± 0.92	1.92 ± 0.42	2.49 ± 0.19
Sham	3/3	$+D_3$	Diet 3	24.16 ± 1.37	2.8 ± 0.2	3.14 ± 0.11
PTUX	2/5	$+D_3$	Diet 3	10.81 ± 0.96	1.98 ± 0.09	2.96 ± 0.30
Sham	3/3	$+D_3$	Diet 2	21.97 ± 1.82	2.93 ± 0.02	1.73 ± 0.06
PTUX	4/6	$+D_3$	Diet 2	4.51 ± 1.46	2.15 ± 0.13	1.93 ± 0.17
Sham	3/3	$+D_3$	Diet 4	25.56 ± 1.04	2.51 ± 0.02	1.76 ± 0.17
PTUX	3/4	$+D_3$	Diet 4	8.36 ± 3.19	1.56 ± 0.21	2.35 ± 0.32

6.25 nmol vitamin D_3 was given in 0.1 ml ethanol to all birds 24h before PTUX or sham operation. Enzyme activities were assayed 14h after operation. Other experimental details were as described under Table 5a.

TABLE 6 - Details of chick diets

DIET 1 Basic diet
(% by weight)

whole wheat flour	17.5
maize	47.5
oats	8.8
milk powder	21.0
yeast	5.2
Ca content	0.34
PO_4 content	0.54

DIET 2 Basic diet with added $CaCO_3$ (2%) and $Al(OH)_3$ (3.2g/Kg)

Ca content	2.34
PO_4 content	? low

DIET 3

maize	54.0
whole wheat flour	20.0
oats	10.0
$CaCO_3$	0.5
NaH_2PO_4	0.5
iodised salt	1.0
$MnSO_4.4H_2O$	0.02
yeast	2.48
low vitamin casein	11.5
Ca content	0.68
PO_4 content	0.95

DIET 4 Basic diet with added $CaCO_3$ and NaH_2PO_4

Ca content	1.0
PO_4 content	0.8

Two or three replicate kidney homogenates were prepared for enzyme assay from each bird. Details of the various diets are shown in Table 6.

In this series of experiments, no vitamin D_3 was given pre-operatively, but in some experiments, calcium supplements were given by mouth post-operatively (+Ca).

3.2 The effect of pre-operative vitamin D_3 treatment.

Experimental protocol:

Chicks were maintained for 3 weeks on vitamin D deficient diets. In some experiments, the calcium and phosphate content of the diet was altered as shown in Table 5b: 100 i.u. (6.25 nmol) vitamin D_3 in 0.1 ml ethanol was given subcutaneously to all birds 24h before parathyro-ultimoboranchialectomy or sham operation. Enzyme activities were assayed 14h after operation and blood was withdrawn by cardiac puncture at this time for estimation of plasma calcium and phosphate concentrations.

Results:

As shown in Table 5, alteration in the calcium and phosphate content of the diet had little or no influence on the effect of parathyro-ultimobranchialectomy on 1-hydroxylase activity. In 3 of the 4 experiments, no decrease in enzyme activity was seen 14h after the operation. In one experiment a slight decrease in enzyme activity was seen. However, when the birds were pretreated with 100 i.u.

TABLE 7

Effect of vitamin D_3 and of parathyroidectomy and ultimobranchial ectomy (PTUX) on 25 OH D_3-1-hydroxylase and 25 OH D_3-24-hydroxylase activities in chick kidney homogenates

Operation	No. of birds surviving	Treatment	1-hydroxylase activity	24-hydroxylase activity	Plasma Ca (mmol l^{-1})	Plasma PO_4 (mmol l^{-1})
Sham	4/4	-Ca $-D_3$	140.6 ± 7.33	3.6 ± 0.87	2.3 ± 0.09	1.55 ± 0.04
	4/4	-Ca $+D_3$	57.8 ±12.60	75.7 ±12.0	2.45± 0.09	2.32 ± 0.19
PTUX	4/4	-Ca $+D_3$	5.0 ± 1.71	120.0 ± 7.64	1.55± 0.21	4.1 ± 0.84
	2/4	-Ca $-D_3$	111.0 ±16.73	3.35± 0.84	1.75± 0.22	2.23 ± 0.03
Sham	3/4	+Ca $-D_3$	86.6 ±11.16	2.25± 0.92	2.6 ± 0.18	1.52 ± 0.06
	4/4	+Ca $+D_3$	11.15± 3.02	94.7 ±10.21	2.65± 0.05	2.48 ± 0.27
PTUX	4/4	+Ca $+D_3$	4.15± 1.32	101.45± 7.69	1.9 ± 0.21	2.0 ± 0.06
	4/4	+Ca $-D_3$	62.85±12.28	3.3 ± 1.3	2.0 ± 0.05	1.8 ± 0.12

Enzyme activities are expressed as fmol dihydroxy metabolite produced (±S.E.M.) min^{-1} mg $protein^{-1}$. Vitamin D_3 has a significant effect ($P < 0.01$) in decreasing 1-hydroxylase and enhancing 24-hydroxylase activity. PTUX has no effect ($P > 0.05$) in the $-D_3$ group, but has a marked effect ($P < 0.01$) in the $+D_3$ group.

vitamin D_3 24h before operation, a marked decrease in 1-hydroxylase activity was seen in the operated birds compared to intact controls.

The results are also depicted graphically in Fig. 15. Clearly, pre-operative treatment with vitamin D_3 has a significant effect on 1-hydroxylase activity following parathyro-ultimobranchial-ectomy. Only birds given vitamin D_3 showed an effect of operation.

Since a variety of treatment regimens was used in these experiments, all the groups are not strictly comparable and a confirmatory experiment was necessary.

3.3 The effect of vitamin D_3 and parathyro-ultimobranchialectomy on 1-hydroxylase activity

Experimental protocol:

In this series of experiments, less severely vitamin D deficient chicks were used. 32 chicks were maintained for 10 days on the standard vitamin D deficient diet. They were then divided into two equal groups: one group received 6.25 nmol (2.5 μg) cholecalciferol in 0.05 ml ethanol subcutaneously while the second group received ethanol alone. After 24h the groups were further subdivided into sham or operated (PTUX). Each of these 4 subgroups contained 8 birds. After operation, four of each subgroup were given 10 mg of calcium in 15% glucose solution orally every hour and the remaining 4 birds received water. 14h after operation, blood was withdrawn by cardiac puncture for estimation of plasma calcium and phosphate and the birds were then killed by decapitation. Three replicate kidney homogenates were prepared for enzyme assay. The results are shown in Table 7 and Fig. 16.

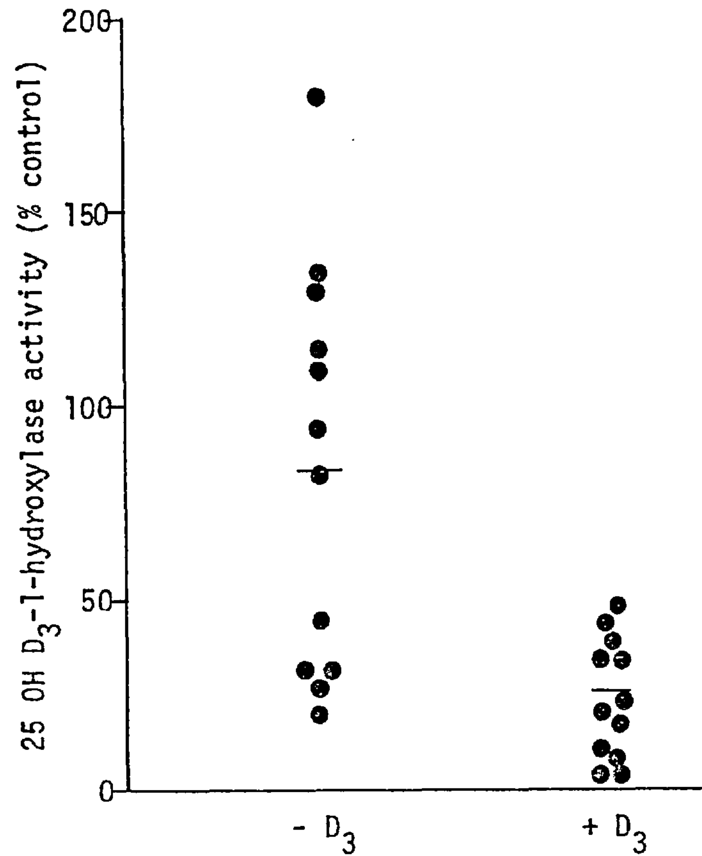


Fig. 15. 25 OH D_3-1-hydroxylase activity after parathyroidectomy and ultimobranchialectomy (PTUX).

Each point is the mean of two to four replicate estimations from 1 bird as a percentage of sham operated controls. One group ($+D_3$) were given 6.25 nmol cholecalciferol subcutaneously 24h before operation. Mean plasma Ca and PO_4 as a percentage of sham operated controls were: D_3 + PTUX, Ca 69.64% ± 0.26, PO_4 106.11% ± 0.46

PTUX only, Ca 57.97% ± 0.29, PO_4 120.12% ± 0.45

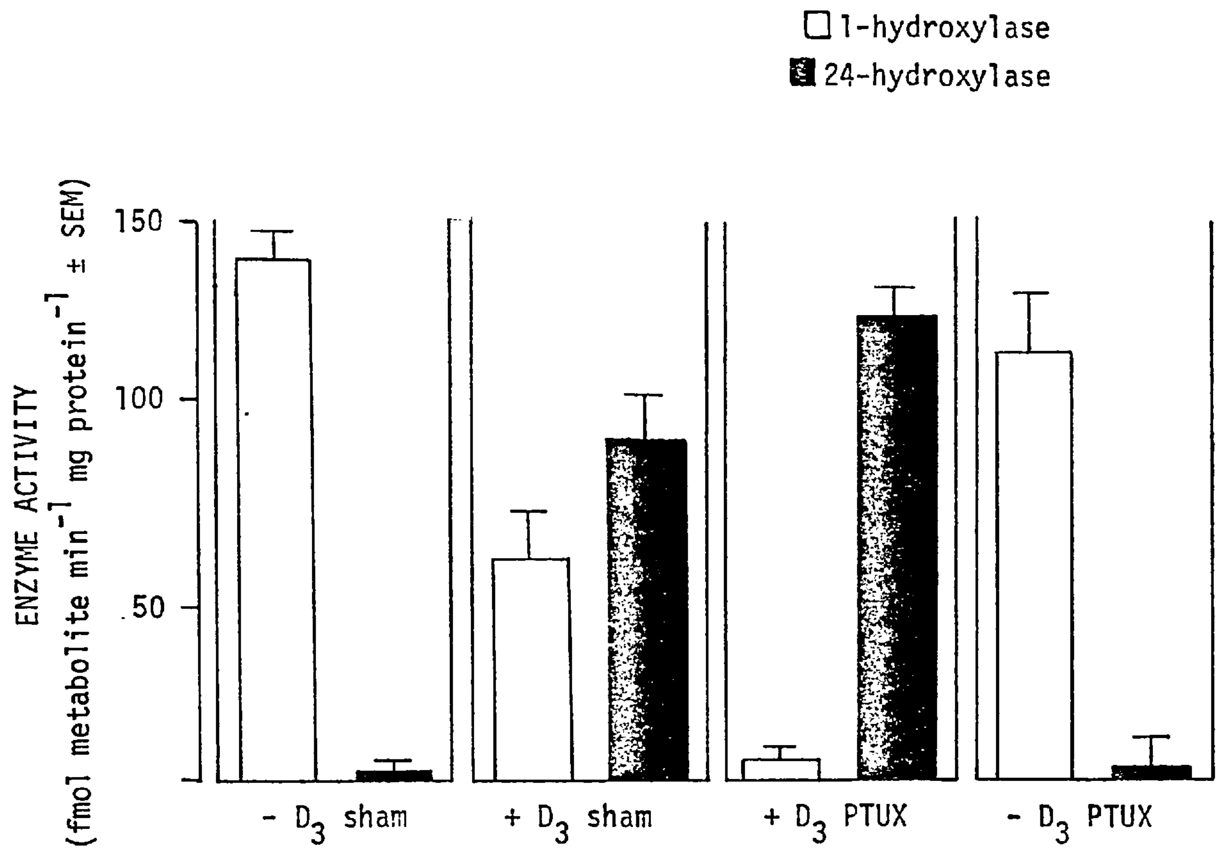


Fig. 16. Effect of cholecalciferol on renal 1- (□) and 24-(■)-hydroxylase activities.

Parathyroidectomy and ultimobranchialectomy (PTUX) or sham operation was carried out 24h after subcutaneous injection of 6.25 nmol cholecalciferol in ethanol (+ D_3) or ethanol alone (- D_3). Renal enzyme activities were assayed 14h after operation. Vitamin D_3 has a significant effect ($P < 0.01$) in increasing 24-hydroxylase activity and in decreasing 1-hydroxylase activity in both sham and PTUX groups. PTUX decreased 1-hydroxylase activity only in the + D_3 group ($P < 0.01$).

Results:

Vitamin D_3 treatment alone caused a striking inhibition of 1-hydroxylase activity and an enhancement of 24-hydroxylase activity. Removal of the parathyroid and ultimobranchial glands from chicks given vitamin D_3 pre-operatively produced a further depression of 1-hydroxylase activity. However, the operation had no significant effect in the absence of vitamin D_3.

4. Discussion

The experiments reported in this chapter show that removal of the ultimobranchial and parathyroid glands does not inhibit renal 1-hydroxylase activity in the vitamin D deficient chick. This observation indicates that parathyroid hormone is not the sole regulator of 1,25 $(OH)_2D_3$ production and therefore cannot be considered as the 'trophic hormone' for the production of this active vitamin D metabolite as has been suggested by DeLuca (DeLuca, 1972). However, the nature of these experiments does not allow any conclusions to be drawn as to the role of parathyroid hormone in the regulation of vitamin D metabolism under normal physiological conditions. These studies were not designed to test the hypothesis that a modulation of parathyroid hormone secretion may, along with other factors, influence 1,25 $(OH)_2D_3$ production in normal physiological situations. However, the results do indicate that parathyroid hormone is not an absolute requirement for the activity of the 1-hydroxylase enzyme.

Other experimental evidence indicates that the parathyroid glands are not essential for the increased production of 1,25 $(OH)_2D_3$ in response to a low calcium diet. Larkins and colleagues thyroparathyroidectomized vitamin D deficient rats and maintained them on

a high calcium diet for 4 days after the operation, After this time, a low calcium diet was substituted and the ability of the thyroparathyroidectomized rats to initiate 1,25 $(OH)_2D_3$ production was tested. In these experiments, removal of the parathyroid glands did not impair 1,25 $(OH)_2D_3$ production in response to this dietary challenge (Larkins, MacAuley, Colston, Evans, Galante and MacIntyre, 1973).

Other workers have attempted to elucidate the role of parathyroid hormone in the regulation of both calcium absorption and vitamin D metabolism. Earlier experiments using everted gut sacs demonstrated that vitamin D was necessary for the adaptation of calcium absorption to a low calcium diet, but that parathyroid hormone was not (Kimberg, Schachter and Schenker, 1961). If it is assumed that this increase in calcium absorption was mediated via the action of the active metabolite, then these experiments also indicate that increased production of 1,25 $(OH)_2D_3$ can be initiated in the absence of parathyroid hormone.

In apparent conflict with this view are the results of Garabedian et al. (1972) who demonstrated that thyroparathyroidectomy acutely decreased 1,25 $(OH)_2D_3$ production in rats, while administration of parathyroid extract restored the ability to produce the active metabolite. Tanaka and DeLuca have suggested that renal phosphate concentration may influence 1,25 $(OH)_2D_3$ production. They prevented hyperphosphataemia following thyroparathyroidectomy either by giving a low phosphate diet or by giving glucose and milk powder, and in these circumstances the inhibition of 1,25 $(OH)_2D_3$ did not occur, (Tanaka and DeLuca, 1973).

Several groups have attempted to show an effect of parathyroid hormone on 25 OH D_3 metabolism <u>in vitro</u>. Shain (1972b) could demonstrate no effect of parathyroid hormone on 1,25 $(OH)_2D_3$ production in isolated renal tubules, and similar results have been reported by Larkins (Larkins, MacAuley, Rappoport, Martin, Tulloch, Byfield, Matthews and MacIntyre, 1974) and Spanos (Spanos, 1976). However, Rasmussen has reported that parathyroid hormone causes a stimulation of 1,25 $(OH)_2D_3$ production by isolated renal tubules (Rasmussen, Wong, Bikle and Goodman, 1972).

In the experiments reported in this chapter, and in all those in which the thyroid or ultimobranchial glands as well as the parathyroids are removed, the possible influence of removal of calcitonin must be considered. Early experiments in rats showed that administration of calcitonin to vitamin D deficient rats enhanced the conversion of 25 OH D_3 to 1,25 $(OH)_2D_3$ <u>in vivo</u> (Galante, Colston, MacAuley and MacIntyre, 1972b; Hill and Mawer, 1973). Recent experiments from DeLuca's group have fully confirmed these findings. However, thyroparathyroidectomy prevented the effect of calcitonin on 1,25 $(OH)_2D_3$ production (Lorenc, Tanaka, DeLuca and Jones, 1977). These later findings indicate that this effect of calcitonin is not a direct one, but is dependent upon the presence of the parathyroid glands and may be mediated via changes in parathyroid hormone secretion. Thus, it remains to be established whether calcitonin influences vitamin D metabolism under normal physiological conditions.

An unexpected finding from the studies described in this chapter is

that vitamin D_3 itself causes a significant decrease in 1-hydroxylase activity and this effect is potentiated by the removal of the parathyroid and ultimobranchial glands. Vitamin D_3 treatment also causes a striking increase in 24-hydroxylase activity. These observations are in contrast to those reported by Fraser and Kodicek (1973) since this group found no effect of vitamin D_3 treatment on the renal enzymes. However, this apparent conflict may be explained by the fact that these workers used only a small number of birds in each of their treatment groups and also because they did not estimate 24,25 $(OH)_2D_3$ production.

The mechanism by which vitamin D_3 produces these marked changes in renal enzyme activities is not immediately apparent, nor is the manner in which parathyroidectomy and ultimobranchialectomy potentiates this effect at all clear. Vitamin D is reported to increase intracellular calcium (Matthews, Martin, Arneuis, Eisenstein and Kuettner) and calcium ions have a powerful inhibitory effect on 1-hydroxylase activity *in vitro*, as will be discussed later in this thesis. Increased plasma phosphate levels by vitamin D treatment may also, in part, explain this decrease in 1-hydroxylase activity since hyperphosphataemia has been reported to suppress 1,25 $(OH)_2D_3$ production (Tanaka and DeLuca, 1973). Furthermore, parathyroidectomy would be expected to potentiate the effect of vitamin D on plasma phosphate and the absence of calcitonin may have enhanced any increase in intracellular calcium induced by vitamin D. However, the effect of vitamin D in inhibiting 1-hydroxylase activity and causing an induction of 24-hydroxylase activity is so striking that it is likely that some as yet unsuspected

action of the vitamin on the kidney is the explanation. In the following experimental section of this thesis, the effect of vitamin D and some of its metabolites on the renal metabolism of 25 OH D_3 is reported in more detail.

CHAPTER FIVE

FEEDBACK REGULATION OF VITAMIN D METABOLISM

Summary

This chapter describes experiments designed to study the feedback regulation of vitamin D metabolism.

Vitamin D deficiency increased the activity of the renal 1-hydroxylase enzyme. Conversely, treatment with 100 i.u. cholecalciferol led to a disappearance of 1-hydroxylase activity and an appearance of 24-hydroxylase activity.

The synthetic analogue, 1α OH D_3 had similar effects but acted more rapidly. Even more striking changes were seen with the natural metabolite, 1,25 $(OH)_2D_3$.

The effect of 1,25 $(OH)_2D_3$ on the renal enzymes was abolished by pre-treatment with the transcriptional inhibitors actinomycin D and α-amanitin.

Treatment with 1,25 $(OH)_2D_3$ also had a rapid effect on RNA polymerase I and II activities in isolated kidney nuclei.

INTRODUCTION

The experiments discussed in the previous chapter of this thesis indicate that neither parathyroid hormone nor calcitonin *per se* is essential for the activity of the 1-hydroxylase enzyme in chick kidney. However, it is apparent that treatment with vitamin D_3 itself has a profound effect on the renal metabolism of 25 OH D_3 and that removal of the parathyroid and ultimobranchial glands exacerbates this effect. Vitamin D_3 causes a marked inhibition of 1-hydroxylase

activity and an appearance of 24-hydroxylase activity. This observation indicates that feedback regulation may be an important factor in the control of 1,25 $(OH)_3D_3$ in vivo.

In this chapter, experiments will be described which were designed to further characterize this major effect of vitamin D_3 and its more active metabolites on renal 1- and 24-hydroxylase activities and to investigate the mechanisms involved in this feedback control system.

1. The Effect of Vitamin D Deficiency on 1-hydroxylase Activity

Experimental protocol:

30 chicks were maintained on the vitamin D deficient diet from the day of hatching (day 1). At four day intervals, blood was withdrawn from 3 chicks by cardiac puncture for the estimation of plasma calcium and phosphate. The chicks were killed by decapitation and kidney tissue was removed immediately and placed on ice. Homogenates (2% w/v) were prepared as previously described and assayed for 1- and 24-hydroxylase activity.

Result:

Vitamin D deficiency caused a marked increase in the activity of the renal 1-hydroxylase enzyme (Fig. 17). 1-hydroxylase levels were increased 4-fold as early as 4 days after restriction of vitamin D intake with a concomitant decrease in 24-hydroxylase activity. At this time there was no significant change in plasma calcium or phosphate levels (Table 8).

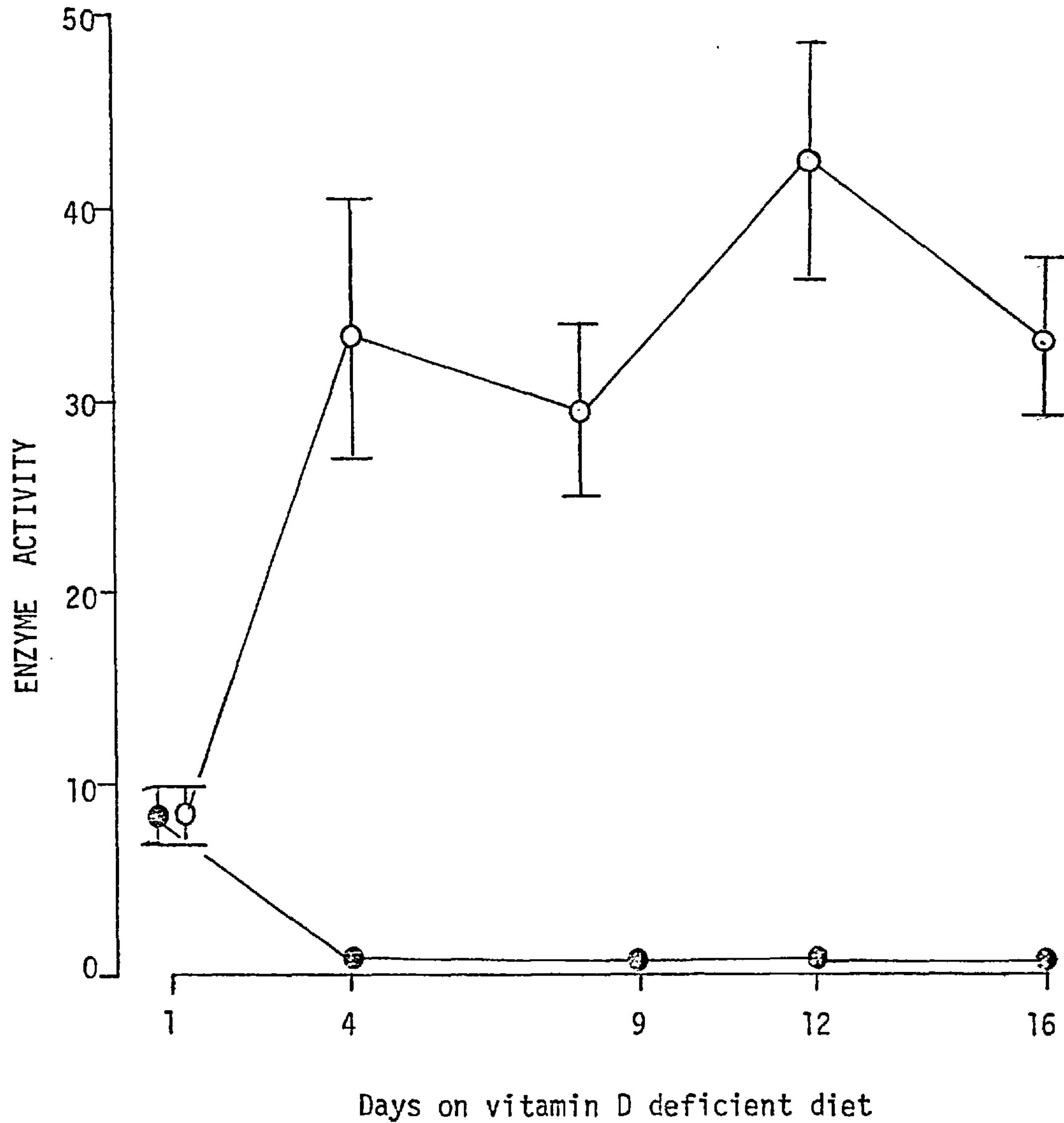


Fig. 17. The effect of dietary vitamin D restriction on the activities of renal 1- (○) and 24- (●) hydroxylase enzymes.
Enzyme activity is expressed as fmol dihydroxy metabolite produced min^{-1} mg $protein^{-1}$ ± S.E.M. with 3 birds per time and 2 duplicate estimations of enzyme activity per bird.
The substrate concentration was 75 pmol per 3 ml incubate.

TABLE 8

Plasma calcium and phosphate concentrations of vitamin D deficient chicks.

Days on vitamin D deficient diet	No. of birds	Ca (mmol l^{-1}) ± s.e.m.	PO_4 (mmol l^{-1}) ± s.e.m.
1	3	2.14 ± 0.05	2.14 ± 0.03
4	3	1.81 ± 0.10	2.39 ± 0.14
9	3	2.17 ± 0.10	2.43 ± 0.13
12	3	1.68 ± 0.09	2.62 ± 0.40
16	2	2.58 ± 0.08	2.63 ± 0.13
23	2	1.94 ± 0.34	2.81 ± 0.61
26	2	1.39 ± 0.28	2.60 ± 0.01
32	2	1.86 ± 0.49	2.15 ± 0.13

The observation that 1-hydroxylase activity is stimulated before there is a marked decrease in plasma calcium or phosphate levels would indicate that this effect of vitamin D deficiency on renal 1-hydroxylase activity is not mediated via changes in these plasma electrolytes. Increased parathyroid hormone secretion, which is known to accompany vitamin D deficiency, is unlikely to be responsible for this stimulation of enzyme activity. Neither parathyroid hormone nor calcitonin are essential for the maintenance of these high levels of 1-hydroxylase activity seen in states of vitamin D deficiency, since removal of the parathyroid and ultimobranchial glands from vitamin D deficient chicks has no effect on 1-hydroxylase activity as demonstrated by the experiments described in the previous chapter.

Hence some other mechanism must be responsible for this enhanced activity of the 1-hydroxylase enzyme in states of vitamin D deficiency, perhaps lack of the vitamin per se causes this increase in enzyme activity. Conversely, as already discussed, administration of vitamin D_3 to vitamin D deficient chicks causes a decrease in 1-hydroxylase levels and stimulates 24-hydroxylase activity.

2. The Effect of Vitamin D on 1-hydroxylase Activity

2.1 Preliminary study.

Experimental protocol:

Chicks were maintained on the vitamin D deficient diet from the day of hatching. Vitamin D_3 (cholecalciferol, Sigma, London) was

administered subcutaneously in 0.1 ml ethanol to 10-day old chicks at a dose of 6.25 nmol (2.5 μg, 100 i.u.) per bird with 3 birds per treatment group. Control birds received ethanol alone. 38h after treatment, the birds were killed by decapitation and renal 1- and 24-hydroxylase activities were assayed as previously described.

Results:

Administration of this dose of vitamin D_3 to vitamin D deficient chicks induced an appearance of 24-hydroxylase activity and a significant decrease in 1-hydroxylase activity at 38h (Fig. 18).

Since vitamin D_3 exerts a profound effect on the renal metabolism of 25 OH D_3, a series of experiments was undertaken to elucidate the effect of the active metabolite of vitamin D_3 on renal 1- and 24-hydroxylase activities.

2.2 A comparison of the effect of equal doses of vitamin D_3 and 1α OH D_3 on renal 1- and 24-hydroxylase activities

At the start of this investigation, the natural active metabolite, 1,25 $(OH)_2D_3$, was not easily available and hence it was necessary to use the synthetic analogue 1α OH D_3 for these studies. 1α OH D_3 has been shown to have similar actions to the natural kidney metabolite in man (Chalmers, Davie , Hunter, Szaz, Pelc and Kodicek, 1973; Peacock, Gallagher and Nordin, 1974) and has been used to study the mechanism of action of 1,25 $(OH)_2D_3$ in experimental animals (Haussler, Zerwekh, Hesse , Rizzardo and Pechet, 1973).

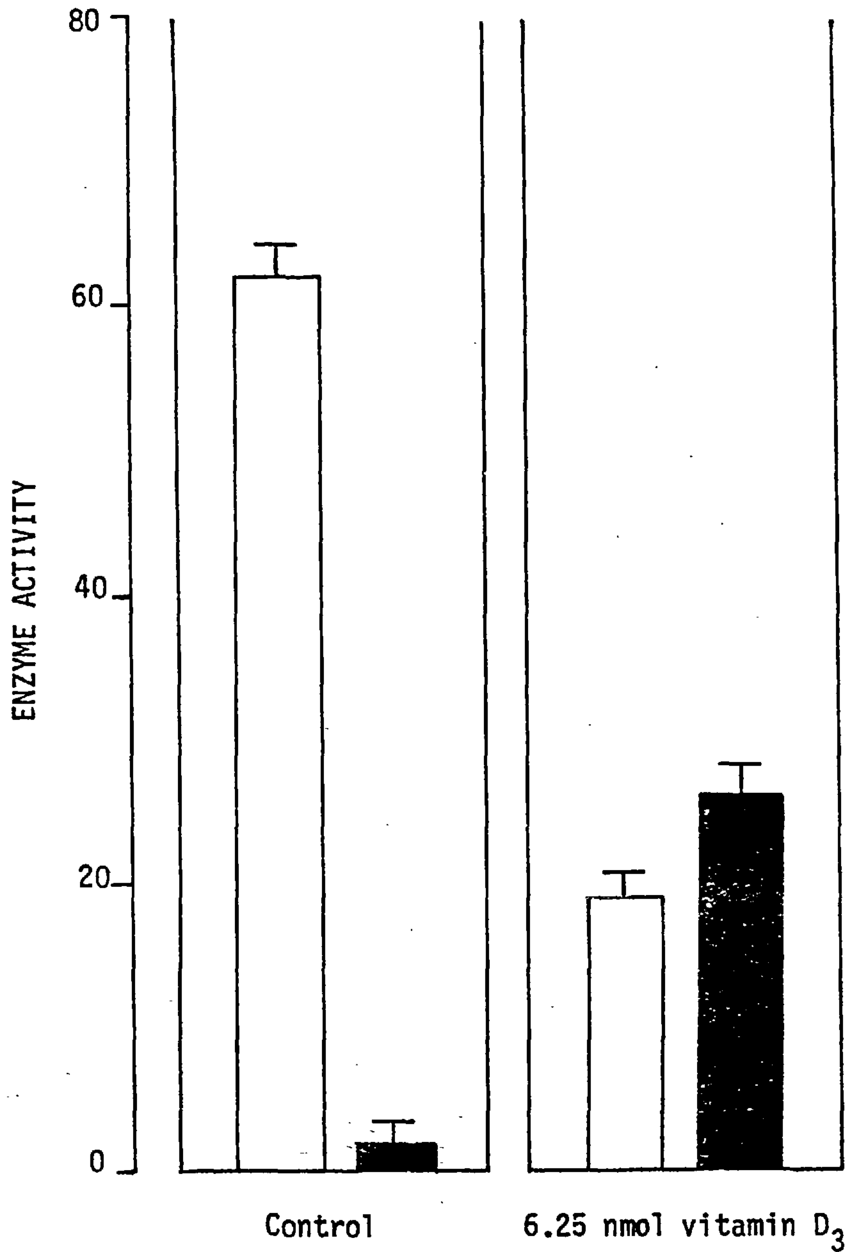


Fig. 18. The effect of 6.25 nmol vitamin D_3 on renal 1-(□) and 24-(■) hydroxylase activities at 38h.

Enzyme activity is expressed as fmol dihydroxy metabolite produced min^{-1} mg $protein^{-1}$. Each bar represents the mean (± S.E.M.) of 3 replicate estimations of enzyme activity from 1 bird with 3 birds per treatment group. The substrate concentration was 25 pmol per 3ml incubate of kidney homogenate. Vitamin D_3 treatment caused a significant decrease in 1-hydroxylase activity and a significant increase in 24-hydroxylase activity ($P < 0.01$).

Experimental protocol:

Vitamin D_3 (cholecalciferol) was administered subcutaneously in 0.1 ml of ethanol to 8 day old vitamin D deficient chicks at a dose of 6.25 nmol (2.5 μg) per bird. Synthetic 1α OH D_3 (a gift from Professor B. Lythgoe, University of Leeds) was administered subcutaneously in 0.1 ml ethanol in the same dose. Control birds received ethanol alone. The chicks were killed by decapitation at various times after treatment and kidney homogenates were assayed for 1- and 24-hydroxylase activity as previously described.

Results:

As shown in Table 9, 6.25 nmol 1α OH D_3 has a profound inhibitory effect on 1-hydroxylase activity at 24h. Vitamin D_3 was less effective. Control birds had no detectable renal 24-hydroxylase activity while 1α OH D_3, and to a lesser degree vitamin D_3, induced an appearance of this enzyme.

2.3 Dose related response of 1- and 24-hydroxylase activities to 1α OH D_3 treatment

Experimental protocol:

Chicks were maintained on the vitamin D deficient diet for 8 days from the day of hatching. Synthetic 1α OH D_3 was administered subcutaneously in 0.1 ml ethanol in a dose of either 625 nmol or 62.5 nmol. Control chicks received ethanol alone. The chicks were then killed by decapitation at various times after treatment as indicated in Table 10, and homogenates prepared for estimation of

TABLE 9

A comparison of the effect of 6.25 nmol vitamin D_3 and 1α OH D_3 on the activities of the renal 1- and 24-hydroxylases in chick kidney homogenates

	Control		6.25 nmol vitamin D_3		6.25 nmol 1α OH D_3	
Time (h)	1-hydroxylase	24-hydroxylase	1-hydroxylase	24-hydroxylase	1-hydroxylase	24-hydroxylase
6	20.58 (13.0 -28.5)	Nil	25.47 (16.05-39.87)	Nil	22.4 (18.8 -27.34)	Nil
12	21.37 (13.69-28.79)	Nil	17.94 (14.43-22.14)	Nil	25.01 (31.74-17.22)	2.60 (0-5.62)
24	25.49 (20.99-31.67)	Nil	14.63 (12.2 -16.7)	2.5 (1.0 -5.0)	4.57 (6.4 - 2.28)	3.92 (0-9.85)
48	24.78 (20.1 133.45)	Nil	17.52 (12.63-22.41)	0.82 (1.63-0.1)	6.43 (4.6 -10.03)	5.94 (3.14-4.68)

Enzyme activities are expressed as mean % ^3H (with range) in an extract of an homogenate of one-third kidney as the dihydroxy metabolite, with 3 birds per treatment group and 2 estimations of renal enzyme activity per bird.

TABLE 10

Effect of 2 doses (62.5 nmol and 625 nmol) 1α OH D_3 with time on 25 OH D_3-1-hydroxylase and 25 OH D_3-24-hydroxylase activities in chick kidney homogenates

	Control		62.5 nmol 1α OH D_3		625 nmol 1α OH D_3	
Time (h)	1-hydroxylase	24-hydroxylase	1-hydroxylase	24-hydroxylase	1-hydroxylase	24-hydroxylase
3	46.98 (39.1-56.22)	Nil	12.85 (3.07-22.84)	Nil	31.12 (19.81-49.9)	4.35 (0 -11.16)
6	53.0 (45.7-64.21)	Nil	54.35 (36.1-71.54)	Nil	21.2 (6.2 -49.0)	25.05 (1.0 -52.64)
9	64.86 (55.9-71.4)	Nil	Nil	40.97 (23.72-51.94)	0.8 (0 -4.6)	37.6 (37.6-45.84)
12	50.0 (28.08-64.26)	Nil	3.5 (0 -13.0)	27.80 (18.0 -39.26)	Nil	39.05 (28.67-50.4)

Enzyme activities are expressed as mean % ^3H (with range) as the dihydroxymetabolite in an extract of an homogenate of one-third kidney, with 3 birds per treatment group and duplicate estimations of renal enzyme activities per bird. Incubation time for the *in vitro* assay was 10 min and the substrate concentration was 75 pmol per 3 ml incubate.

renal enzyme activities as previously described.

Results:

The larger dose of 1α OH D_3 (625 nmol) induced the appearance of 24-hydroxylase activity at 6h and the smaller dose (62.5 nmol) at 9h. Control birds had no 24-hydroxylase activity. In all cases there was a concomitant decrease in 1-hydroxylase activity.

2.4 Discussion

Both vitamin D_3 and 1α OH D_3 have a profound inhibitory effect on 25 OH D_3-1-hydroxylase activity. Control birds have no detectable renal 24-hydroxylase activity, while both vitamin D_3 and 1ᾱ OH D_3 induce the activity of this enzyme. Treatment with 6.25 nmol cholecalciferol produced an appearance of 24-hydroxylase activity at 24h. An equal weight of 1α OH D_3 also induced 24-hydroxylase activity but with a more marked depression of 1-hydroxylase activity. Higher doses of 1α OH D_3 produced an even more striking effect much more quickly; 625 nmol of the synthetic analogue induced a dramatic appearance of 24-hydroxylase activity as early as 6h after treatment. In all cases the appearance of 24-hydroxylase activity was accompanied by a marked decrease in 1-hydroxylase activity (Fig. 19).

These results suggest that vitamin D_3 or its metabolites may play a role in the regulation of the renal metabolism of 25 OH D_3. Although the most rapid and dramatic effects were seen with very large doses of the synthetic analogue 1α OH D_3, cholecalciferol itself was also active and physiological doses of the vitamin markedly influenced the activities of the renal 25 OH D_3 hydroxylases.

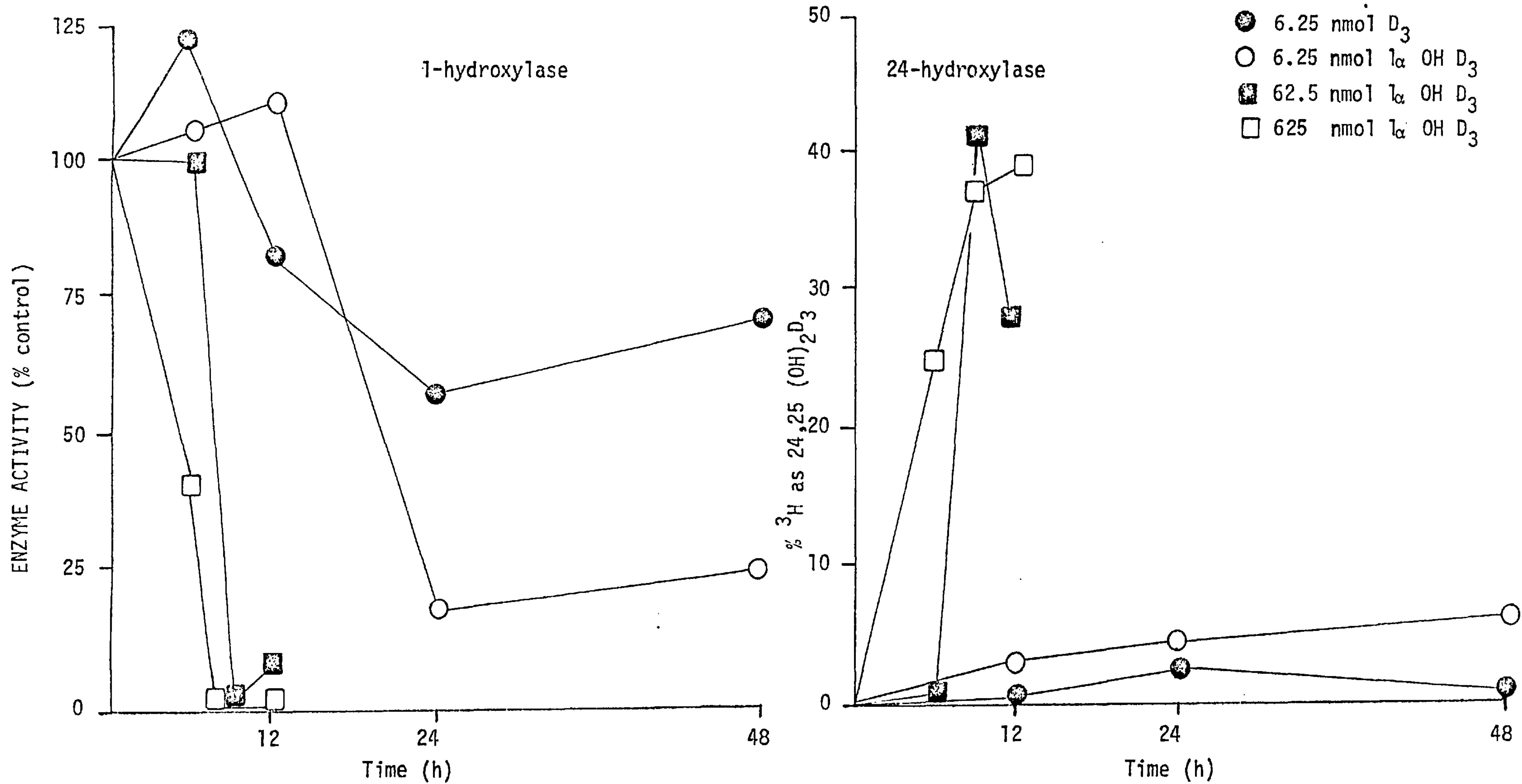


Fig. 19. Effect of vitamin D_3 and 1α OH D_3 on 1- and 24-hydroxylase activity in chick kidney homogenates. Each point represents the average of duplicate estimations from each of 3 birds.

TABLE 11

Effect of actinomycin D (40 nmol) on the induction of 24-hydroxylase activity by 1α OH D_3 (62.5 nmol)

No. of birds surviving	-6hr	0hr	+6hr	+12hr	1-hydroxylase activity	24-hydroxylase activity
2/3	-	1αOH D_3	-	-	0.4 (0 - 2.6)	29.6 (27.6-31.5)
2/3	-	1αOH D_3 + actinomycin D	-	-	Nil	46.5 (54.3-35.7)
3/3	-	actinomycin D	-	-	32.6 (19.4-22.8)	0.47 (0 - 1.4)
3/3	-	0	-	-	59.2 (42.4-69.7)	0.35 (0 -1.05)
3/3	-	1α OH D_3	actinomycin D	-	Nil	45.8 (43.5-48.2)
2/3	-	0	actinomycin D	-	23.7 (12.3-35.0)	0.4 (0 -1.17)
3/3	-	1α OH D_3	-	actinomycin D	0.36 (0 -1.09)	35.3 (31.4-39.9)
3/3	-	0	-	actinomycin D	18.1 (20.5-14.3)	0.4 (0 -1.23)
3/3	actinomycin D	1α OH D_3	-	-	Nil	42.95 (33.2-49.3)
2/3	actinomycin D	0	-	-	45.0 (34.91-55.1)	0.45 (0 - 0.9)
3/3	-	0	-	-	58.7 (53.00-64.4)	1.2 (1.0 1 1.5)

1α OH D_3 (or ethanol) was given at time 0hr (24h before enzyme assay) in all cases. Enzyme activity is expressed as % ^3H (with range) in extract of kidney homogenate as the dihydroxymetabolite with 2 estimations of enzyme activity per bird and 2 to 3 birds per treatment group.

3. Effect of Actinomycin D on the Induction of 25 OH D_3-24-hydroxylase Activity by 1α OH D_3

Vitamin D_3 and the synthetic analogue, 1α OH D_3, have a profound effect on the renal metabolism of 25 OH D_3. Both steroids inhibit 25 OH D_3-1-hydroxylase activity and induce the appearance of 24-hydroxylase activity. Very large doses of 1α OH D_3 produce an effect at 6h, but this relatively slow change over from 1- to 24-hydroxylase indicates that the effect is not simply one of product inhibition but may reflect a change in amount of enzyme or a change in the activity of existing enzyme molecules dependent on new protein synthesis. To test this hypothesis, the effect of the transcriptional inhibitor actinomycin D was studied on the induction of 24-hydroxylase by 1α OH D_3.

3.1 Effect of a single dose of actinomycin D on renal 1- and 24-hydroxylase in the presence and absence of 1αOH D_3.

Experimental protocol:

33 chicks were maintained on the vitamin D deficient diet for 8 days from the day of hatching, when they were divided into two groups, one of 15 and another of 18 chicks. All of the 15 chicks in the smaller group received 62.5 nmol 1α OH D_3 in 0.1 ml ethanol subcutaneously. The other group received ethanol alone at the same time. Actinomycin D was given in a single dose at 30, 24, 18 or 12h before death. 1α OH D_3 (or ethanol) was given 24h before death in all cases. Actinomycin D (Dactinomycin, Merck, Sharp and Dohme International) was given in 0.1 normal saline at a dose of 40 nmol (50 μg) per bird intravenously into a wing vein. The protocol for the experiment is

given in Table 11. 24h after administration of 1α OH D_3 the birds were killed by decapitation and renal enzyme activities were assayed as previously described.

Results:

As shown in Table 11, a single intravenous dose of 40 nmol actinomycin D did not prevent the induction of 24-hydroxylase activity by 62.5 nmol 1α OH D_3. The time of administration of actinomycin D had no effect on the induction of 24-hydroxylase activity since the transcriptional inhibitor was given 6h prior to, at the same time and 6 and 12h following 1α OH D_3 treatment. Actinomycin D had no effect *per se* on 1-hydroxylase activity at any time of administration. However, Tsai and co-workers found that to effectively block 1,25 $(OH)_2D_3$ stimulated intestinal calcium transport, it was necessary to give actinomycin D in repeated doses at 2 hourly intervals (Tsai, Midgett and Norman, 1973). For this reason a further series of experiments was designed to test the effect of repeated doses of actinomycin D on the induction of renal 25 OH D_3-24-hydroxylase activity.

3.2 The effect of repeated doses of actinomycin D

Experimental protocol:

36 8-day old vitamin D deficient chicks were divided into 2 equal groups. One group received 62.5 nmol 1α OH D_3 subcutaneously in 0.1 ml ethanol and the other group received ethanol alone. Actinomycin D was given every 2h for a total of 3 or 9h beginning 30 min before 1α OH D_3 (or ethanol) treatment. Actinomycin D, in a total

dose of either 40 nmol (50 μg) or 400 nmol (500 μg) per bird, was given intravenously in normal saline. Control birds received saline alone. The experimental protocol is given in detail in Table 12, and the results are shown in Tables 13 and 14.

Results:

62.5 nmol 1α OH D_3 induced the appearance of 24-hydroxylase activity at 9h but not at 3h. At 3h neither dose of actinomycin D had a significant effect on 1-hydroxylase activity. However, at 9h the larger dose of actinomycin D (total dose 400 nmol given in 5 equal doses of 80 nmol at 2 hourly intervals) prevented the induction of 24-hydroxylase activity by this dose of 1α OH D_3. The smaller dose of actinomycin D (total dose 40 nmol) did not prevent the appearance of 24-hydroxylase activity in response to 62.5 nmol 1α OH D_3. In the absence of 1α OH D_3, both doses of actinomycin D caused a slight decrease in 1-hydroxylase activity.

A further experiment was designed to confirm these results, using the regimen of actinomycin D administration found to prevent 1α OH D_3 induced 24-hydroxylase activity in this first experiment.

3.3 Experiment 2

Experimental protocol:

Two groups of birds (3 per group) received 1α OH D_3 in a dose of 125 nmol (50 μg) per bird and one group in addition received a total dose of 400 nmol (500 μg) per bird actinomycin D. The actinomycin D was given intravenously in 0.1 ml normal saline in five

TABLE 12

Effect of repeated doses of actinomycin D on 1α OH D_3 induced 24-hydroxylase activity; experimental protocol.

No. of birds	Dose of 1α OH D_3	Dose of actinomycin D	Time (after 1α OH D_3) of assay
3	62.5 nmol	2 x 20 nmol total 40 nmol	3h
3	62.5 nmol	2 x 200 nmol total 400 nmol	3h
3	62.5 nmol	Nil	3h
3	Nil	2 x 20 nmol total 40 nmol	3h
3	Nil	2 x 200 nmol total 400 nmol	3h
3	Nil	Nil	3h
3	62.5 nmol	5 x 8 nmol total 40 nmol	9h
3	62.5 nmol	5 x 80 nmol total 400 nmol	9h
3	62.5 nmol	Nil	9h
3	Nil	5 x 8 nmol total 40 nmol	9h
3	Nil	5 x 80 nmol total 400 nmol	9h
3	Nil	Nil	9h

TABLE 13

Effect of repeated doses of actinomycin D on the induction of 24-hydroxylase activity by 1α OH D_3 at 3h.

1α OH D_3	Total dose of actinomycin D	1-hydroxylase activity	24-hydroxylase activity
62.5 nmol	40 nmol	24.27 (19.6-30.6)	Nil
62.5 nmol	400 nmol	29.94 (18.5-39.0)	Nil
62.5 nmol	Nil	27.88 (19.9-35.4)	Nil
Nil	40 nmol	37.77 (23.5-35.4)	Nil
Nil	400 nmol	18.02 (13.76-23.1)	Nil
Nil	Nil	46.17 (20.9-58.3)	Nil

Enzyme activities are expressed as mean % ^3H (with range) as the dihydroxy metabolite in an extract of homogenate of one-third kidney with 2 estimations of enzyme activity per bird and 3 birds per treatment group. The substrate concentration was 75 pmol and the incubation time 10 min.

TABLE 14

Effect of repeated doses of actinomycin D on the induction of 24-hydroxylase activity by 1α OH D_3 at 9h.

1α OH D_3	Total dose of actinomycin D	1-hydroxylase activity	24-hydroxylase activity
62.5 nmol	40 nmol	14.75 (11.78-28.19)	8.22 (5.9-12.9)
62.5 nmol	400 nmol	24.89 (9.6 -49.0)	Nil
62.5 nmol	Nil	26.94 (13.9 - 39.6)	9.7 (5.6-14.4)
Nil	40 nmol	34.74 (24.6 - 46.8)	0.8 (0.2- 2.5)
Nil	400 nmol	34.94 (24.9 -49.84)	Nil
Nil	Nil	55.10 (49.2-60.3)	1.0 (0 - 4.0)

Enzyme activities are expressed as mean % ^3H (with range) as the dihydroxy metabolite in an extract of homogenate of one-third kidney with 2 estimations of enzyme activity per bird and 3 birds per treatment group. The substrate concentration was 75 pmol and the incubation time 10 min.

equal doses of 80 nmol (100 μg) per bird at 2 hourly intervals beginning 30 min before administration of 1α OH D_3. A third group received actinomycin D alone and a fourth group served as controls. Renal enzyme activities were assayed 9h after administration of 1α OH D_3.

Results:

Fig. 20 shows that the effect of 1α OH D_3 on both enzymes was completely abolished by pre-treatment with actinomycin D. This effect indicates that the action of 1α OH D_3 on both enzymes is dependent upon synthesis of new messenger RNA. It can be postulated that these results are due to an effect of 1α OH D_3 on the renal nuclei which results in transcription and new protein synthesis, leading to the appearance of 24-hydroxylase activity and the disappearance of 1-hydroxylase activity. The observation that the transcriptional inhibitor actinomycin D prevents the action of 1α OH D_3 on both enzymes is consistent with this interpretation. However, although actinomycin D is a highly effective inhibitor of RNA synthesis (Reich and Goldberg, 1964; Waring, 1968; Beard, Armentrout and Weisberger, 1969), it is not specific. Since large doses of the antibiotic were necessary to demonstrate the changes in hydroxylase activities, it is possible that an unsuspected effect of actinomycin D was the explanation. Furthermore, there is some evidence to suggest that 1α OH D_3 requires 25-hydroxylation to 1,25 $(OH)_2D_3$ for activity (DeLuca, Holick and Holick, 1976). If this hydroxylation is indeed necessary for full recognition by the renal receptor, inhibition of the activity of this hepatic enzyme by actinomycin D

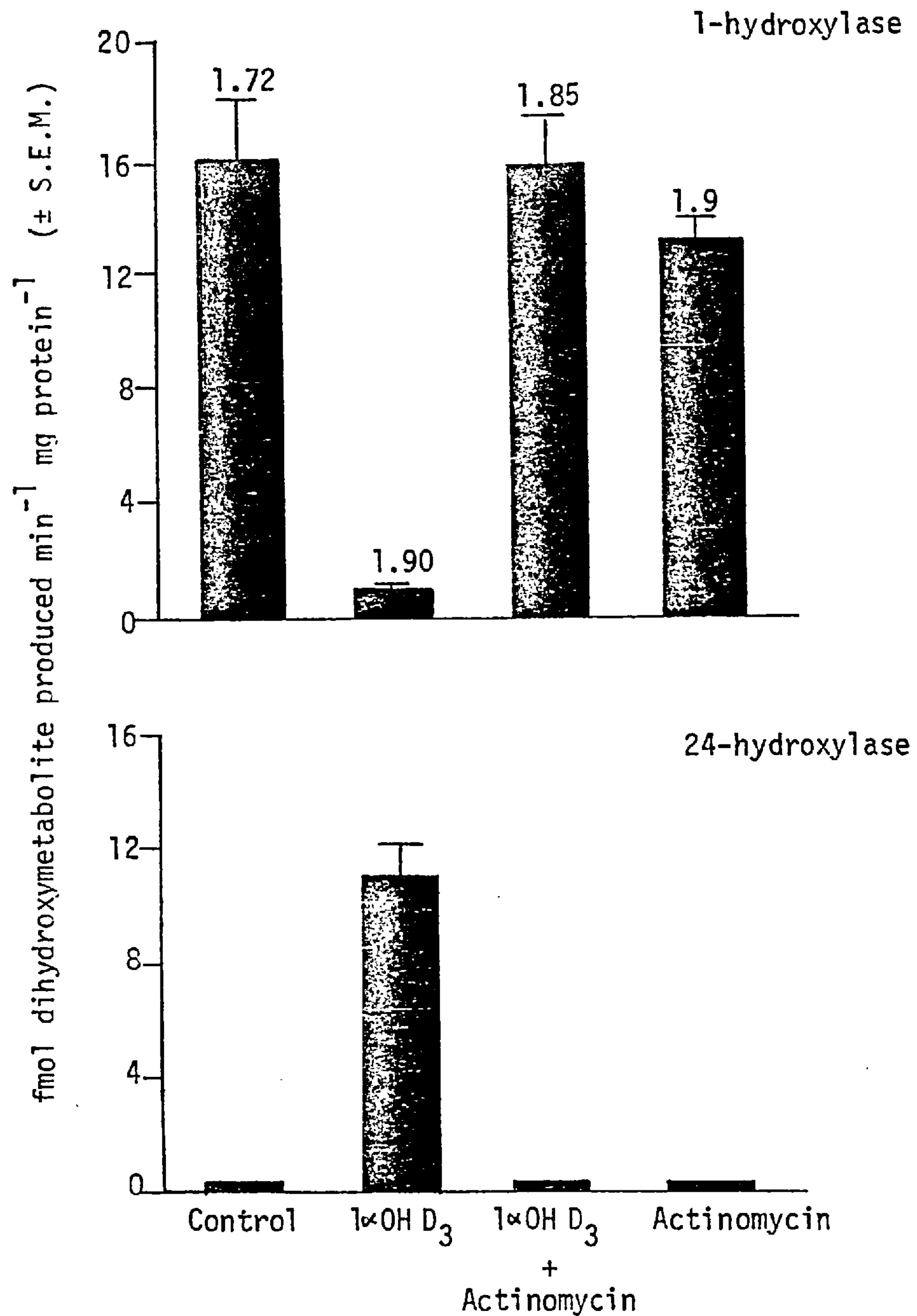


Fig. 20. Effect of actinomycin D (400 nmol per bird) on the induction of 24-hydroxylase activity by 1α OH D_3 (125 nmol per bird) at 9h. Mean plasma calcium levels in each group (m mol l^{-1}) are given above the histograms.

may have been responsible for these results (Haussler et al., 1973).

4. Feedback Regulation by 1,25 $(OH)_2D_3$

4.1 Introduction

As previously discussed, both vitamin D_3 and the synthetic analogue, 1α OH D_3, depress renal 25 OH D_3-1-hydroxylase activity and enhance 24-hydroxylase activity. This effect of vitamin D_3 has subsequently been confirmed by Horiuchi and colleagues (Horiuchi, Suda, Sasaki, Ezawa, Sano and Ogata, 1974) and substantiated by Tanaka and DeLuca (1974b) using biosynthetic 1,25 $(OH)_2D_3$. However, neither of these reports included any experimental evidence indicating the mechanism by which vitamin D_3 or its most active metabolite might regulate its own metabolism.

The observation that the transcriptional inhibitor, actinomycin D prevents the effect of 1α OH D_3 on renal hydroxylase activities indicates the involvement of a nuclear action in this process. However, since a synthetic analogue rather than the natural kidney hormone was used in this study, a series of confirmatory experiments was performed when supplies of synthetic 1,25 $(OH)_2D_3$ became more readily available.

4.2 Effect of 1,25 $(OH)_2D_3$ on renal hydroxylase activities: dose related response.

Experimental protocol:

Chicks were maintained on the vitamin D deficient diet for 11 to 17 days from the day of hatching. Synthetic 1,25 $(OH)_2D_3$ (a gift

TABLE 15

Effect of increasing doses of 1,25 $(OH)_2D_3$ on renal 1- and 24-hydroxylase activities

Time	1,25 $(OH)_2D_3$ dose	No. of birds	1-hydroxylase	24-hydroxylase
3h	2.5 nmol	3	35.36 ± 6.06	4.38 ± 2.58
3h	6.25 nmol	4	75.32 ± 3.74	9.00 ± 2.10
3h	control	7	51.65 ± 7.24	5.00 ± 2.72
9h	2.5 nmol	3	29.10 ± 2.16	17.96 ± 2.08
9h	6.25 nmol	4	35.38 ± 5.82	15.08 ± 2.46
9h	62.5 nmol	4	17.00 ± 5.20	36.20 ± 3.64
9h	control	11	58.36 ± 6.48	6.84 ± 2.14

Enzyme activities are expressed as fmol dihydroxy metabolite produced min^{-1} mg $protein^{-1}$ with 3 estimations per bird (± S.E.M.)

from Dr. Uskokovik, Hoffman La Roche, Nutley, New Jersey) was administered subcutaneously in 0.1 ml ethanol in a dose of either 2.5, 6.25 or 62.5 nmol. Control birds received ethanol alone. The chicks were killed by decapitation at either 3 or 9h after treatment and homogenates prepared for estimation of renal enzyme activities as previously described.

Results:

The larger dose of synthetic 1,25 $(OH)_2D_3$ (62.5 nmol) caused a significant enhancement of 24-hydroxylase activity over control values at 9h which coincided with a virtual abolition of 1-hydroxylase activity. The lower doses of the steroid (2.5 and 6.25 nmol) also caused changes in the activity of the renal enzyme at 9h, but their effects were less marked. No effect was seen with the lower doses at 3h (Table 15 and Fig. 21).

4.3 A comparison between the effect of equal doses of 1,25 $(OH)_2D_3$ and 25 OH D_3 on renal hydroxylase activities

In order to investigate the comparative activities of 1,25 $(OH)_2D_3$ and its precursor, 25 OH D_3 on the renal enzymes, the smallest dose of the natural kidney hormone which had been previously shown to be effective (2.5 nmol) was compared with an equal dose of 25 OH D_3. Fig. 22 shows that at 9h, 2.5 nmol 1,25 $(OH)_2D_3$ caused a depression of 1-hydroxylase activity and an increase in 24-hydroxylase activity, while the same dose of 25 OH D_3 had no effect.

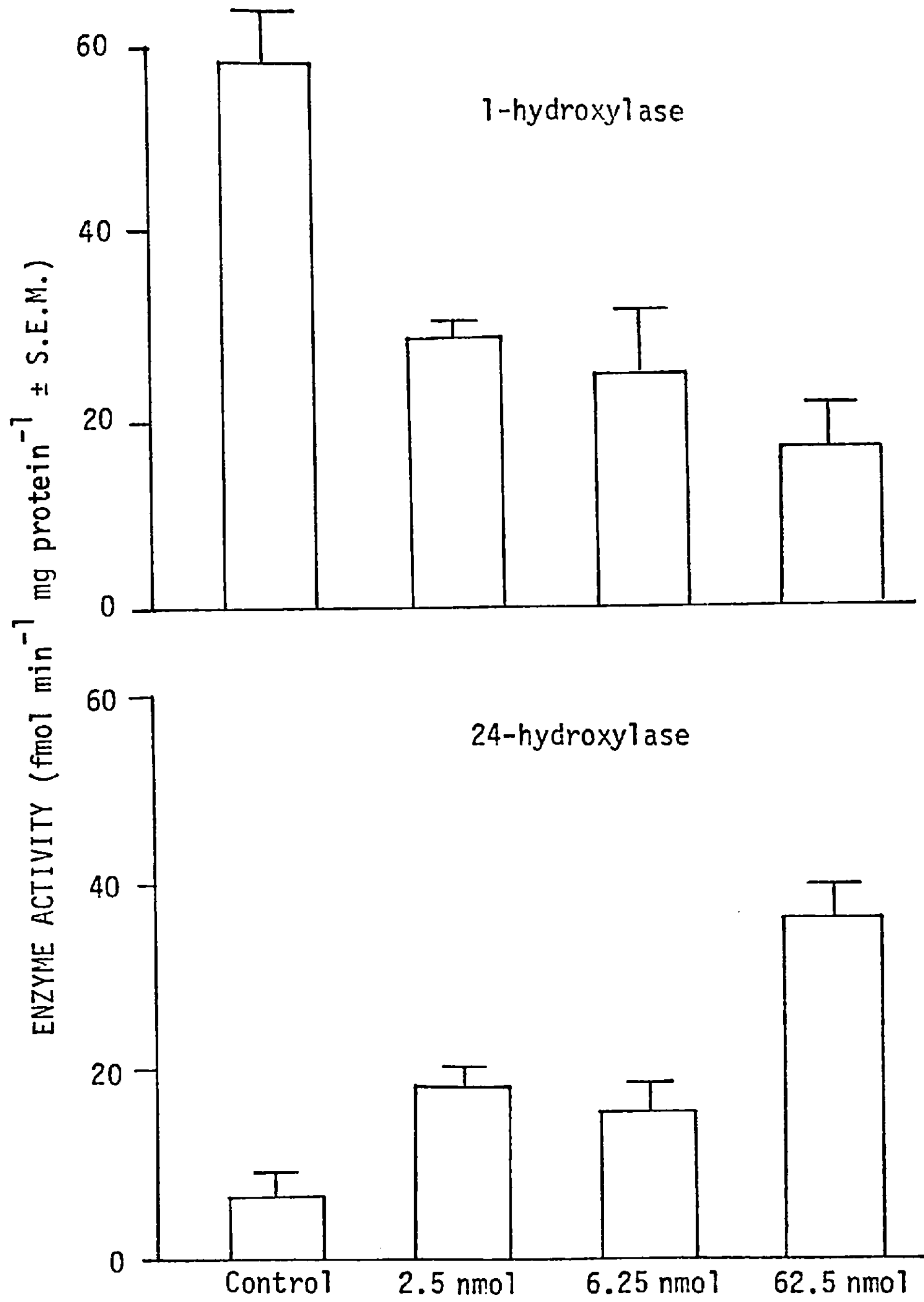


Fig. 21. Effect of increasing doses of 1,25 $(OH)_2D_3$ on renal 1- and 24-hydroxylase activities at 9h.

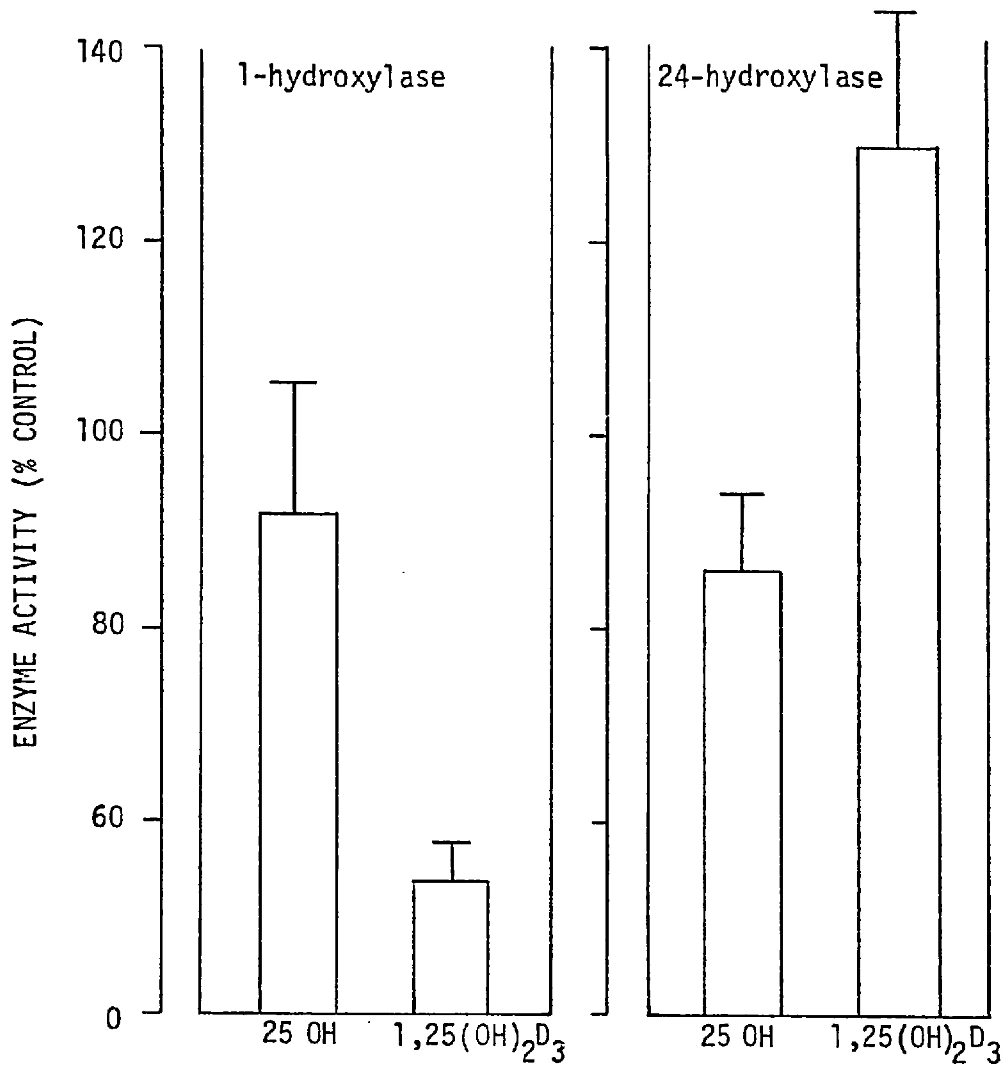


Fig. 22. Effect of equal doses (2.5 nmol) of 1,25 $(OH)_2D_3$ and 25 OH D_3 on renal 1- and 24-hydroxylase activities at 9h. Enzyme activities are expressed as a percentage (± S.E.M.) of corresponding enzyme activities in untreated control birds.

4.4 Discussion

As might be expected, administration of 1,25 $(OH)_2D_3$, the natural kidney metabolite, produced the same effects on the renal hydroxylases as those seen with the parent vitamin and the synthetic analogue, 1α OH D_3, but smaller doses were needed to observe these effects. Thus, 2.5 nmol 1,25 $(OH)_2D_3$ produced an increase in 24-hydroxylase and caused a depression in 1-hydroxylase activity. However, the most striking effects on renal enzyme activities were seen with larger doses of the steroid. An effect of 62.5 nmol was seen on both enzymes: this dose of 1,25 $(OH)_2D_3$ induced a dramatic appearance of 24-hydroxylase activity and a virtual disappearance of 1-hydroxylase activity at 9h. In order to allow analysis of the changes in the renal enzyme activities by means of transcriptional inhibitors, it was necessary to give large doses of the steroid to ensure that gross changes in enzyme activities were seen within hours of administration of 1,25 $(OH)_2D_3$. Large doses of these antibiotics would prove fatal to the experimental animals over longer periods of time.

5. <u>The Effect of Transcriptional Inhibitors on the Induction of 24-hydroxylase Activity by 1,25 $(OH)_2D_3$</u>

5.1 The effect of actinomycin D

Experimental protocol:

16 chicks were maintained on the vitamin D deficient diet for 8 days from the day of hatching. Two groups of birds (4 per group) received 125 nmol synthetic 1,25 $(OH)_2D_3$ and one group in addition

received actinomycin D to a total dose of 400 nmol (500 μg) per bird given intravenously in 0.1 ml saline in five equal doses of 80 nmol (100 μg) at 2 hourly intervals. The first injection of actinomycin D was given 30 min before administration of 1,25 $(OH)_2D_3$. A third group received actinomycin alone and a fourth group served as controls. Renal enzyme activities were assayed 9h after administration of 1,25 $(OH)_2D_3$.

Results:

The results are shown in Fig. 23. Actinomycin D abolished both the increase in 24-hydroxylase and the inhibition of 1-hydroxylase activity in response to 1,25 $(OH)_2D_3$; moreover, actinomycin D had little effect on either enzyme activity per se. These results indicate that renal enzyme activities seen in response to 1,25 $(OH)_2D_3$ are dependent upon transcription and therefore new protein synthesis, since treatment with actinomycin D, which inhibits transcription by binding to the DNA template, abolished the effect of this steroid.

A more specific inhibitor of messenger RNA synthesis is α-amanitin, the toxic octapeptide of the poisonous mushroom, Amanita phalloides. α-Amanitin exerts its effect on transcription by specifically inhibiting the activity of RNA polymerase II which catalyses the formation of messenger RNA (Tata, Hamilton and Shields, 1972).

5.2 The effect of α-amanitin

The effect of α-amanitin on the induction of 24-hydroxylase

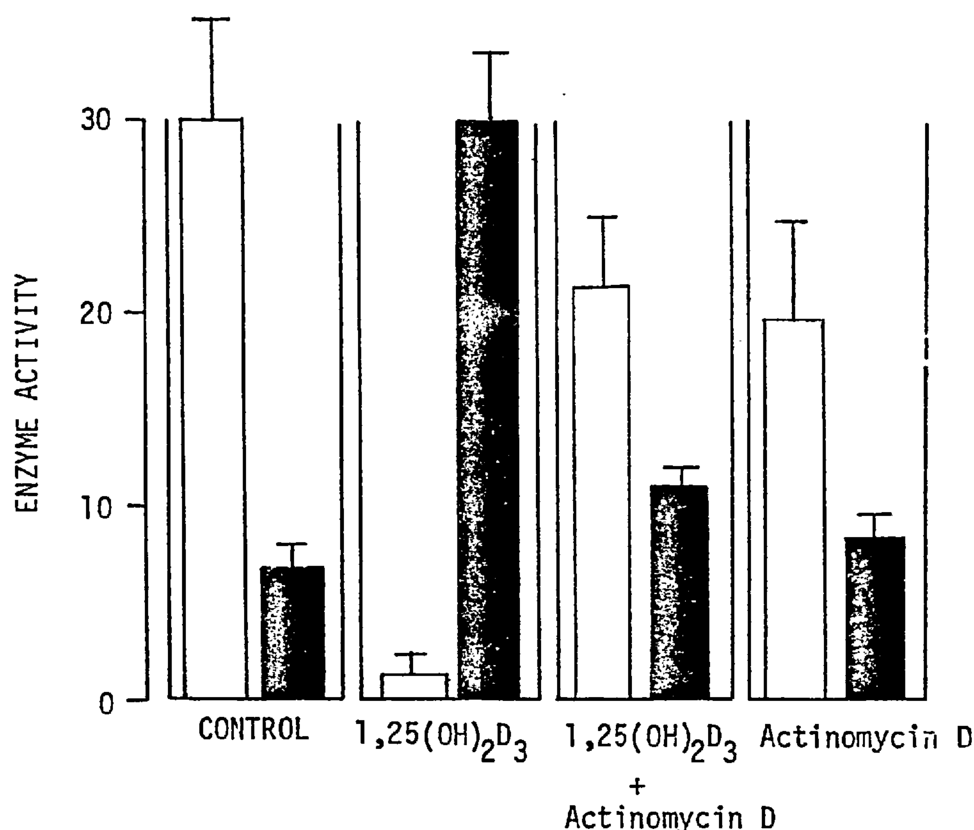


Fig. 23. Effect of Actinomycin D (400 nmol total per bird) on the induction of 24-hydroxylase activity by 1,25 $(OH)_2D_3$ at 9 hr.

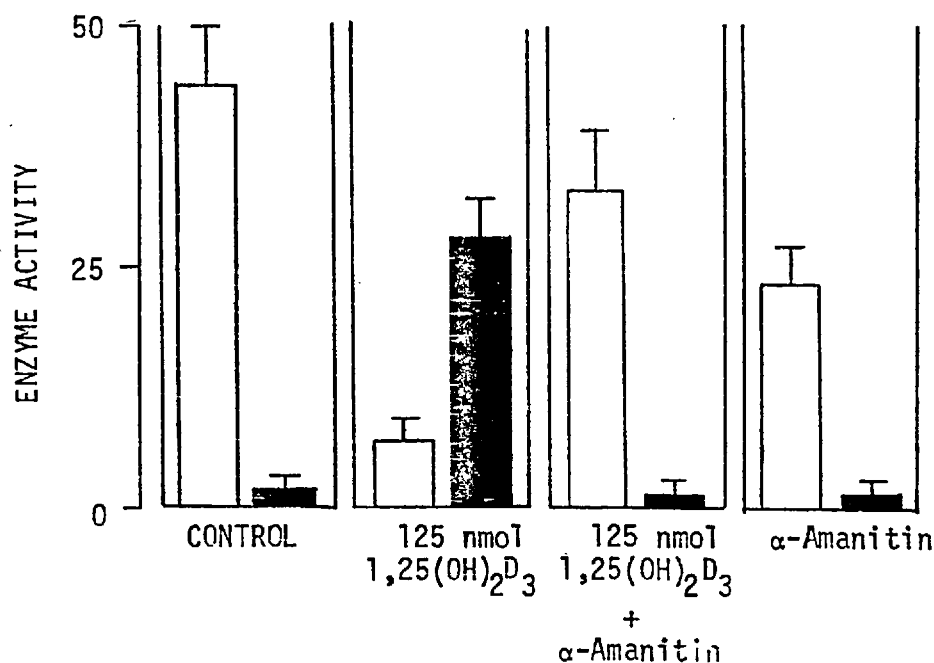


Fig. 24. Effect of α-amanitin (100 nmol per bird) on the induction of 24-hydroxylase activity by 1,25 $(OH)_2D_3$ (125 nmol per bird) at 9 hr. Enzyme activities are expressed as fmol dihydroxymetabolite produced min^{-1} mg $protein^{-1}$ (± S.E.M.) with 3 replicate estimations from each bird and 3 birds per treatment group. Open bars represent 1-hydroxylase and solid bars 24-hydroxylase activity. The concentration of substrate (^3H 25 OH D_3) was 25 pmol per 3 ml incubate.

activity by 1,25 $(OH)_2D_3$ was assessed in a similar experiment to that described using actinomycin D. In addition, the same dose of α-amanitin used in the experiment was assessed for its effect on RNA polymerase II activity.

Experimental protocol:

Sixteen chicks were maintained on the vitamin D deficient diet for 17 days from the day of hatching. Two groups of birds (3 per group) received 125 nmol synthetic 1,25 $(OH)_2D_3$ per bird and one of these groups in addition received a single dose of 100 nmol α-amanitin (100 μg) per bird. This dose of α-amanitin was chosen as being equivalent to that used in rats by Tata et al. (1972). α-Amanitin was given intravenously in 0.1 ml normal saline 30 min before treatment. A third group of birds (5 per group) received α-amanitin alone and a fourth group (5 birds) served as controls. 9h after 1,25 $(OH)_2D_3$ treatment, the chicks were killed by decapitation and one kidney from each bird was removed for the estimation of renal 1- and 24-hydroxylase activities. The second kidney was snap frozen in liquid nitrogen and stored at $-20^{\circ}C$ until assayed for RNA polymerase activity. In addition, two birds receiving α-amanitin alone and two control birds were killed 30 min after α-amanitin treatment and kidneys were removed for estimation of RNA polymerase II activity.

Assay of RNA polymerases:

These assays were kindly performed by Dr. T.C. Spelsberg and his staff at the Department of Molecular Medicine, Mayo Clinic, Rochester, Minnesota, U.S.A. The isolation of nuclei and assay for endogenous RNA polymerase I (nucleolar) and II (nucleoplasmic)

activities were performed as previously described (Glasser, Chytil and Spelsberg, 1972; Spelsberg, Knowler and Moses, 1974; Spelsberg and Cox, 1976). Freezing the whole tissue has previously been shown to have only mild effects on subsequent RNA polymerase activities compared with unfrozen tissue in the isolated nuclei of chick oviducts and liver (Knowler, Moses and Spelsberg, 1973).

Results:

Pretreatment with α-amanitin greatly diminished the effect of 1,25 $(OH)_2D_3$ on the renal hydroxylase. Although α-amanitin alone had a slightly inhibitory effect on 1-hydroxylase activity, the effect of 1,25 $(OH)_2D_3$ was much greater and was largely blocked by the combined α-amanitin/1,25 $(OH)_2D_3$ treatment (Fig. 24). Moreover, the dose of α-amanitin used in the experiment caused a dramatic decrease in RNA polymerase II activity as early as 30 min after treatment. The activity of this nuclear enzyme remained depressed at 9h. Thus, 100 nmol α-amanitin produced over 90% inhibition of total RNA polymerase II activity during the period of the experiment (Table 16). 1,25 $(OH)_2D_3$ had no significant effect on RNA polymerase II as assayed in isolated kidney nuclei 9h after 1,25 $(OH)_2D_3$ treatment (Table 16).

5.3 Discussion

The observation that two transcriptional inhibitors abolish the changes in renal hydroxylase activities seen in response to 1,25 $(OH)_2D_3$ would seem to indicate that this effect of the steroid involves gene transcription and protein synthesis. However, in preliminary studies

TABLE 16

Effect of α-amanitin and 1,25 $(OH)_2D_3$ on the activities of RNA polymerases I and II in chick kidney nuclei.

Treatment	Polymerase I	Polymerase II
a) At 9h		
125 nmol 1,25 $(OH)_2D_3$	116.0	95.0
125 nmol 1,25 $(OH)_2D_3$ + 100 nmol α-amanitin	59.0	4.4
100 nmol α-amanitin	70.6	4.3
control	100.0	100.0
b) At 30 min		
100 nmol α-amanitin	89.6	2.9
control	100.0	100.0

One kidney from each of 3 chicks per treatment group were pooled. Results are a single estimation of RNA polymerase activities per treatment group, and are expressed as % control levels.

1,25 $(OH)_2D_3$ treatment had no effect at 9h on RNA polymerase II activity, the enzyme involved in the production of messenger RNA. This observation is not surprising since at this time after treatment, effects on renal enzyme activities are already manifest. It might be expected that any change in nuclear polymerase activities in response to this steroid would be seen at a much earlier time. Thus, it was of interest to study in more detail the effect of 1,25 $(OH)_2D_3$ on the activities of the polymerase enzymes involved in RNA synthesis.

6. The Effect of 1,25 $(OH)_2D_3$ on RNA polymerases

A series of experiments was performed to assess the actions of 1,25 $(OH)_2D_3$ on the activities of RNA polymerases I and II which are involved in the synthesis of ribosomal RNA and the synthesis of messenger RNA respectively. The effect of 2 doses of this steroid on the activities of both enzymes was assessed at various times after treatment.

Experimental protocol:

10-day old vitamin D deficient chicks each received either 12.5 or 125 nmol 1,25 $(OH)_2D_3$ subcutaneously in 0.1 ml ethanol at time zero. A further group of birds (controls) received ethanol alone. 1,25 $(OH)_2D_3$ treated birds were killed at 30 min, 1h, 2h and 4h and control birds were killed at 4h only. Both kidneys were snap frozen in liquid nitrogen and stored at -20^oC until assayed for RNA polymerase activities. A further 3 birds from each dose group were killed 9h after injection and kidney homogenates were prepared for the assay of renal 1- and 24-hydroxylase activities.

TABLE 17

Effect of 12.5 nmol and 125 nmol 1,25 $(OH)_2D_3$ on renal 1- and 24-hydroxylase activites

Dose of 1,25 $(OH)_2D_3$	Time (h)	1-hydroxylase	24-hydroxylase
0	9	81.93 ± 5.01	0
12.5 nmol	9	0	55.05 ± 2.24
125.0 nmol	9	0	62.54 ± 9.49

Renal 1- and 24-hydroxylase activities are expressed as fmol dihydroxy metabolite produced min^{-1} mg $protein^{-1}$ and are a mean of 2 replicate estimations from each of 3 birds per treatment group (± s.e.m.)

Results:

Fig. 25 compares the effects of 12.5 nmol and 125 nmol 1,25 $(OH)_2D_3$ over a 4h period after injection. RNA polymerase I activity was inhibited up to 2h after treatment with both doses of the steroid. However, by 4h an enhancement of this enzyme activity was seen.

Both doses of 1,25 $(OH)_2D_3$ also inhibited RNA polymerase II activity up to 2h after injection. This inhibition of enzyme activity persisted up to 4h with the higher dose, but with the lower dose, enzyme activity had returned to control levels by 4h. Fig. 26 shows the effect of the higher dose (125 nmol) on RNA polymerase I and II activities over a 9h period. A decrease in the activity of both polymerases was seen 30 min after injection and by 4h RNA polymerase I activity was markedly elevated, while RNA polymerase II activity was still depressed at this time. At 9h the activities of both enzymes approached control levels. Table 17 shows the corresponding renal 1- and 24-hydroxylase activities assayed 9h after injection of 12.5 and 125 nmol 1,25 $(OH)_2D_3$. The higher dose abolished 1-hydroxylase and induced the appearance of high levels of 24-hydroxylase activity at this time. The smaller dose (12.5 nmol) had a less marked effect.

7. Discussion

The experimental data described in the chapter strongly suggests that 1,25 $(OH)_2D_3$, the most active metabolite of vitamin D_3, plays an important role in regulating the metabolism of 25 OH D_3 by

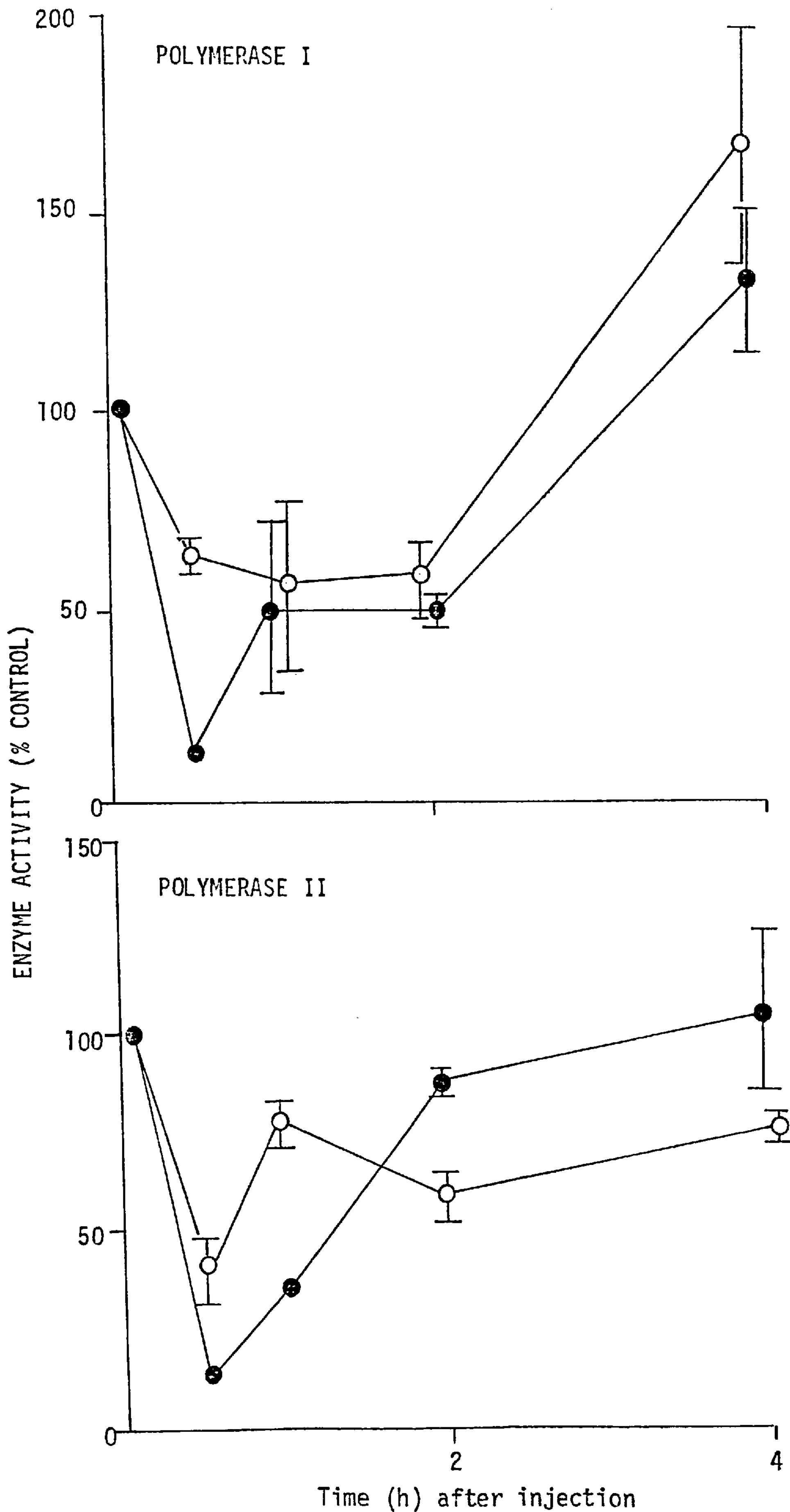


Fig. 25. Effect of 12.5 (●) and 125 nmol (○) 1,25 $(OH)_2D_3$ on the activities of RNA polymerases I and II over a 4h period after injection. Enzyme activities are expressed as a percentage of corresponding enzyme activities in untreated control birds.

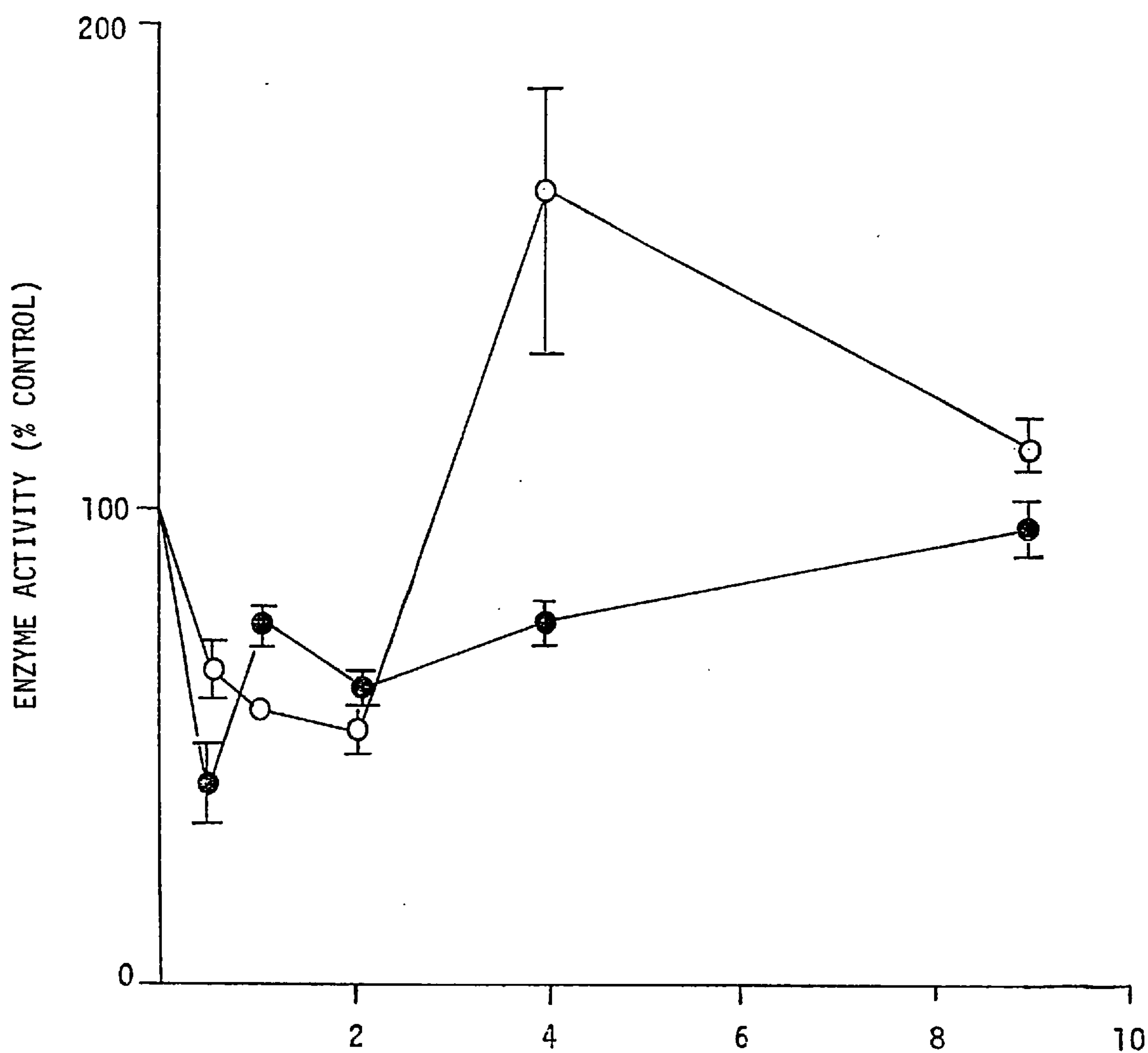


Fig. 26. Effect of 125 nmol 1,25 $(OH)_2D_3$ on the activities of RNA polymerases I (○) and II (●) over a 9h period after injection. Both enzyme activities are expressed as a percentage of corresponding enzyme activities in control (untreated) birds.

kidney. In states of vitamin D deficiency, renal 1-hydroxylase activity is markedly increased whereas administration of a physiological dose of vitamin D_3 depresses 1-hydroxylase activity and induces the appearance of 24-hydroxylase activity at 28h. The same dose of 1,25 $(OH)_2D_3$ has the same effect on the activities of the renal enzymes, but at an earlier time. Large doses of 1,25 $(OH)_2D_3$ act even more rapidly, such that at 9h 1-hydroxylase activity is abolished and there are high levels of 24-hydroxylase activity.

One possible explanation of this effect is an alteration of 1-hydroxylase activity consequent upon an increase in serum calcium concentration. There is evidence that calcium may have an influence on 1,25 $(OH)_2D_3$ production (Boyle et al., 1971). However, an effect via serum calcium is unlikely to be an essential part of the observed action of 1,25 $(OH)_2D_3$ on the kidney enzyme activities. Serum calcium levels were not routinely measured in the experiments using 1,25 $(OH)_2D_3$, but the synthetic analogue, 1α OH D_3 produced the same effects at 9h without changes in serum calcium. Thus, 9h after administration of 125 nmols 1α OH D_3, no change was observed in plasma calcium levels (Fig. 20).

Although an inverse relationship between serum calcium and 1,25 $(OH)_2D_3$ production has been reported (Boyle et al., 1971), this is not invariably the case. Some factors can alter the activity of the renal enzyme by means other than a change in extracellular calcium concentration. Thus, Horiuchi and co-workers demonstrated a modulation in 1-hydroxylase activity in the face of a constant serum calcium level (Horiuchi et al., 1974). These workers found that 1-hydroxylase

activity varied inversely with the amount of vitamin D in the diet. Combined oestrogen/testosterone treatment has also been reported to influence 1-hydroxylase activity. The mechanism of this stimulatory effect is not known, but it cannot be due to a fall in extracellular calcium since plasma calcium in fact is increased in response to this treatment (Tanaka, Castillo, and DeLuca, 1976). Prolactin also stimulates 1-hydroxylase activity while producing hypercalcaemia (Spanos, Pike, Haussler, Colston, Evans, Goldner, McCain and MacIntyre, 1976b).

However, there is evidence that the mechanism by which the activities of the renal enzymes are modulated in response to 1,25 $(OH)_2D_3$ is via some nuclear effect. Both the disappearance of 1-hydroxylase activity and the appearance of 24-hydroxylase activity seem to be dependent upon transcription and therefore new protein synthesis. Treatment with actinomycin D which inhibits transcription by binding to the DNA template, abolishes the effect of 1,25 $(OH)_2D_3$ on these renal enzymes. Similarly, α-amanitin, which inhibits transcription by binding to RNA polymerase II, also prevents the changes in renal enzyme activities in response to 1,25 $(OH)_2D_3$.

The studies on the activities of RNA polymerases I and II would seem to confirm that 1,25 $(OH)_2D_3$ exerts an immediate effect on transcription. Even 12.5 nmol causes a marked decrease in the polymerase activities within 30 min. The rapid inhibition of RNA polymerase II activity by 1,25 $(OH)_2D_3$ is similar to the effects seen with other steroid hormones, notably the action of progesterone on the chick oviduct (Spelsberg and Cox, 1976). The overall reduction

in RNA polymerase II activity in response to 1,25 $(OH)_2D_3$ does not necessarily imply that new messenger RNAs and thus new proteins are not being synthesised, but merely indicates that total gene transcription in the cell is being reduced by this vitamin D metabolite. The transcription of new genes could be masked by the massive decrease in the transcription of genes which were being transcribed before 1,25 $(OH)_2D_3$ administration. This data provides evidence that one important control of vitamin D metabolism is feedback regulation at the kidney level by 1,25 $(OH)_2D_3$, and that this active vitamin D metabolite regulates its own production via an effect on the renal cell nuclei. This nuclear effect involves changes in gene transcription and new protein synthesis with the appearance of 24-hydroxylase and disappearance of 1-hydroxylase activity.

Without further data it is only possible to speculate upon the nature of the new proteins which are the product of this observed change in gene transcription, but one such protein may be the 24-hydroxylase enzyme molecule itself or some protein which may modulate its activity. The observation that treatment with the transcriptional inhibitors actinomycin D and α-amanitin alone has little or no effect on 1-hydroxylase activity, indicates that the observed decrease in 1-hydroxylase activity induced by 1,25 $(OH)_2D_3$ cannot be explained by an inhibition of transcription of the messenger RNA for the 1-hydroxylase molecule. Were this the case, both actinomycin D and α-amanitin would be expected to mimic the effects of the steroid and cause a depression of this enzyme activity at 9h. It may be that one further protein synthesised in response to 1,25 $(OH)_2D_3$ inhibits 1-hydroxylase activity by enhancing the breakdown of the molecule or by preventing its translation.

This experimental data is consistent with the view that vitamin D regulates its own metabolism in a feedback manner. The observed effect of 1,25 $(OH)_2D_3$ is probably an acceleration of a slower physiological regulation of major importance. The most likely mechanism of this effect is a direct nuclear action on the kidney cell of a similar nature to that established for other steroid hormones. Thus the active form of the vitamin exerts an effect on the target organ (the kidney) by action on the cell nucleus involving changes in total gene transcription. Such a nuclear feedback control would explain how the kidney responds to the prevailing vitamin D status of the animal. Thus variation in the amount and type of renal enzyme with intake of vitamin D may prove to be an important factor in the physiological regulation of vitamin D metabolism.

S E C T I O N III

CHAPTER SIX

SOME IN VITRO PROPERTIES OF THE 25 HYDROXY-CHOLECALCIFEROL 1- AND 24-HYDROXYLASES

Summary

A comparison of some *in vitro* properties of the renal 1- and 24-hydroxylase enzymes was undertaken. Conditions for optimal activity of the two enzymes were found to be similar. The response of these two renal enzymes to calcium and to other divalent cations was also studied.

INTRODUCTION

The metabolism of 25 hydroxycholecalciferol was first demonstrated to be influenced by dietary factors in 1971 by Boyle (Boyle et al., 1971). Young rats fed a vitamin D restricted, low calcium diet and receiving 1 i.u. vitamin D_3 daily were found to have 1,25 $(OH)_2D_3$ as the predominant renal dihydroxy metabolite in their blood following administration of ^3H 25 OH D_3 tracer. However, rats fed a high calcium diet were found to have a less polar metabolite in their serum following administration of the radioactive tracer. This less polar metabolite, eventually identified as 24,25 $(OH)_2D_3$, was later shown to be the major metabolite of 25 OH D_3 in the vitamin D supplemented chick (Knutson and DeLuca, 1974) and man (Gray, Weber and Lemann, 1973).

The kidney has been shown to be the major site of production of 24,25 $(OH)_2D_3$ (Omdahl, Gray, Boyle, Knutson and DeLuca, 1972) as well as the sole site of 1,25 $(OH)_2D_3$ production (Fraser and

Kodicek, 1970). It was of interest to compare some of the *in vitro* properties of these two renal enzymes.

1. In Vitro Properties of the 1- and 24-hydroxylase Enzymes in Whole Kidney Homogenates: Preliminary Studies

Experimental:

One-day old chicks were fed the standard vitamin D deficient diet for two to three weeks. For the induction of 24-hydroxylase activity, 2- to 3-week old vitamin D deficient chicks were treated with 100 i.u. cholecalciferol given subcutaneously in 0.1 ml ethanol daily for 3 days.

The preparation of homogenates, incubation conditions, extraction procedure and chromatography were performed as described previously.

Results:

As previously described, the production of 1,25 $(OH)_2D_3$ by chick kidney homogenates was linear with time up to 20 min and with protein concentration over the range 2.7 to 20.7 mg protein per 3 ml incubate of kidney homogenate. Formation of 1,25 $(OH)_2D_3$ was maximal between pH 7.0 and 7.5. Similar incubation conditions were found to be suitable for optimal production of 24,25 $(OH)_2D_3$ by kidney homogenates prepared from vitamin D deficient chicks treated with cholecalciferol (Fig. 27).

An apparent k_m of 0.87 x 10^{-7}M was calculated from the linear regression of the reciprocal of the rate of 1-hydroxylation on the

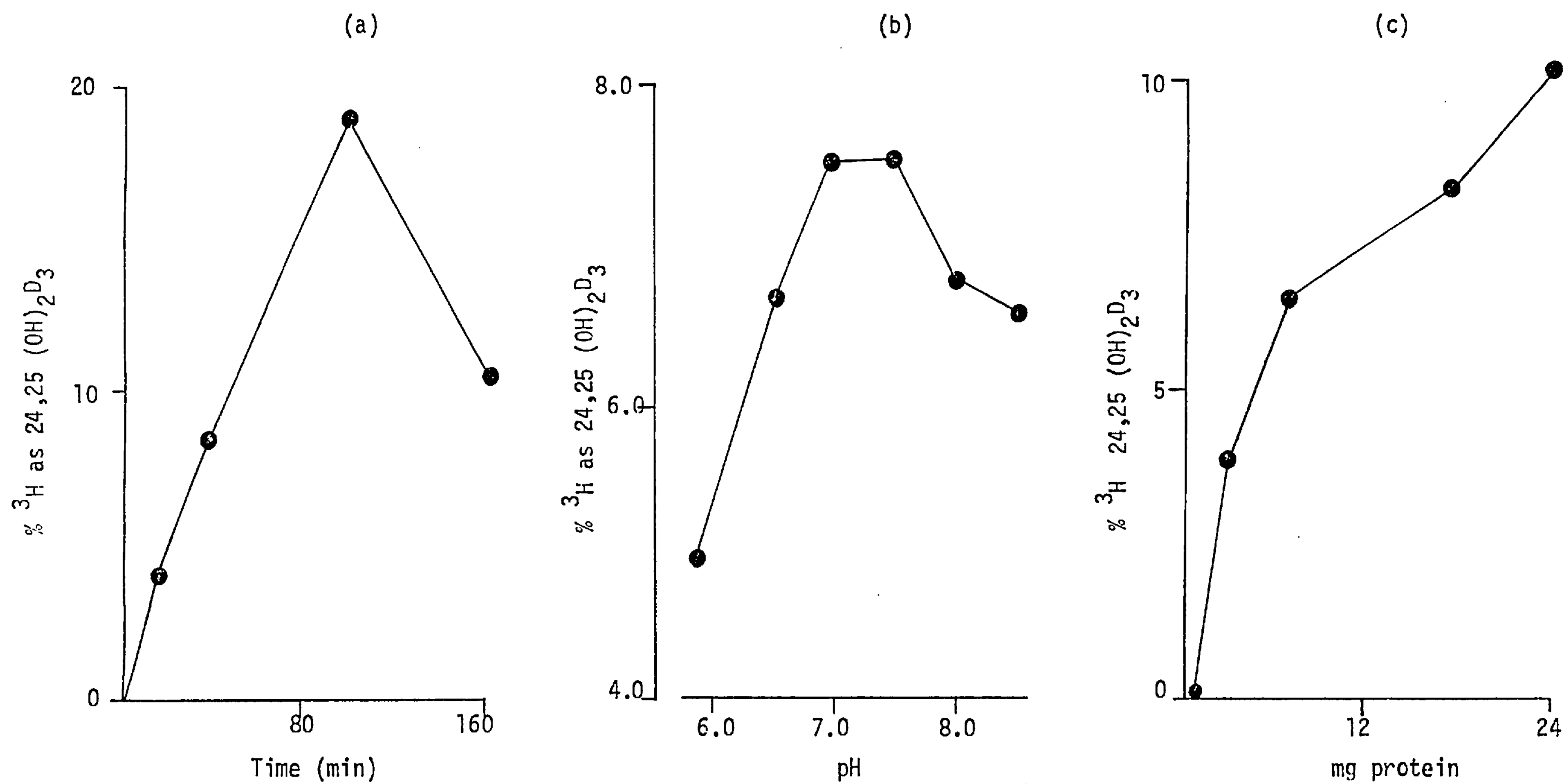


Fig. 27. The production of 24,25 $(OH)_2D_3$ by chick kidney homogenates as a function of (a) time, (b) hydrogen ion concentration and (c) protein concentration

reciprocal of the concentration of added 25 OH D_3, over a substrate concentration range of 3 - 202 nM (Table 18 and Fig. 28). A similar plot of the dependence of 24-hydroxylation on the concentration of substrate revealed an apparent k_m of 5×10^{-7}M of this reaction (Table 18 and Fig. 29). V_{max} values for the 1- and 24-hydroxylase reactions were 0.477 and 2.897 pmol min^{-1} mg $protein^{-1}$, respectively.

2. A Comparison of Succinate and Malate as Supporters of 1- and 24-hydroxylase Activities

Succinate and malate supported 1-hydroxylase activity to a similar extent. Malate supported 24-hydroxylase activity was higher than that supported by succinate (Table 19). The reason for this observation is not immediately apparent.

3. The Effect of Ions on Renal 1- and 24-hydroxylase Activities

The effect of several cations and anions were tested to determine whether changes in their concentrations might affect in some regulatory fashion the activities of these two renal enzymes. In view of the role played by the metabolites of vitamin D in calcium homeostasis, it was of particular interest to investigate the interrelationship of Ca^{++} and PO_4^{3-} ions on 1- and 24-hydroxylase activities.

3.1 Effect of Ca^{++} and PO_4^{3-} on 1- and 24-hydroxylase activities

Experimental:

1- and 24-hydroxylase activities were assessed as previously

TABLE 18

Effect of increasing substrate concentrations on 1- and 24-hydroxylase activities.

1-HYDROXYLASE

Substrate (nM)	$\frac{10^{-7}}{S}$	v (pmol min^{-1} mg protein^{-1})	$\frac{1}{v}$
3.17	31.55	0.016	59.52
6.32	15.82	0.030	33.33
12.64	7.91	0.068	14.81
25.28	3.96	0.109	9.17
50.56	1.98	0.135	7.41
101.00	0.99	0.292	3.42
202.00	0.49	0.412	2.43

24-HYDROXYLASE

Substrate (nM)	$\frac{10^{-7}}{S}$	v (pmol min^{-1} mg protein^{-1})	$\frac{1}{v}$
3.17	31.55	0.018	54.95
6.32	15.82	0.037	27.40
12.64	7.91	0.069	14.49
25.28	3.96	0.133	7.55
50.56	1.98	0.286	3.49
101.00	0.99	0.475	2.10
202.00	0.49	1.09	0.92

The value of v is a mean of 3 replicate estimations of enzyme activity.

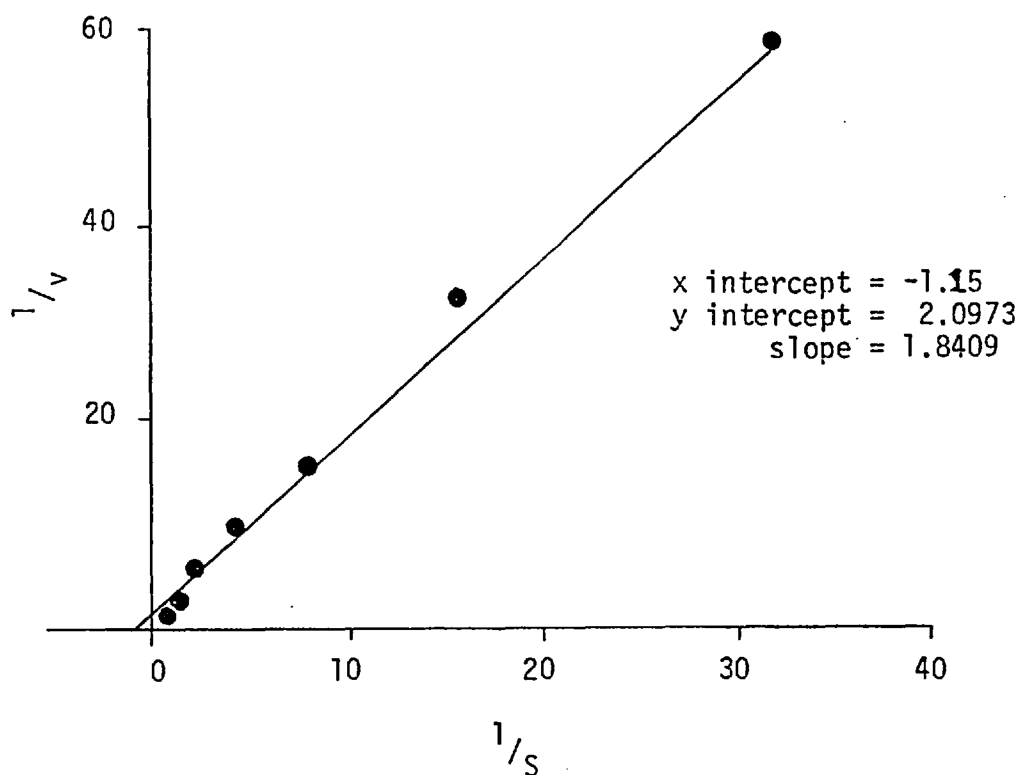


Fig. 28. Plot of $^1/_v$ by $^1/_S$ for 1-hydroxylase enzyme.

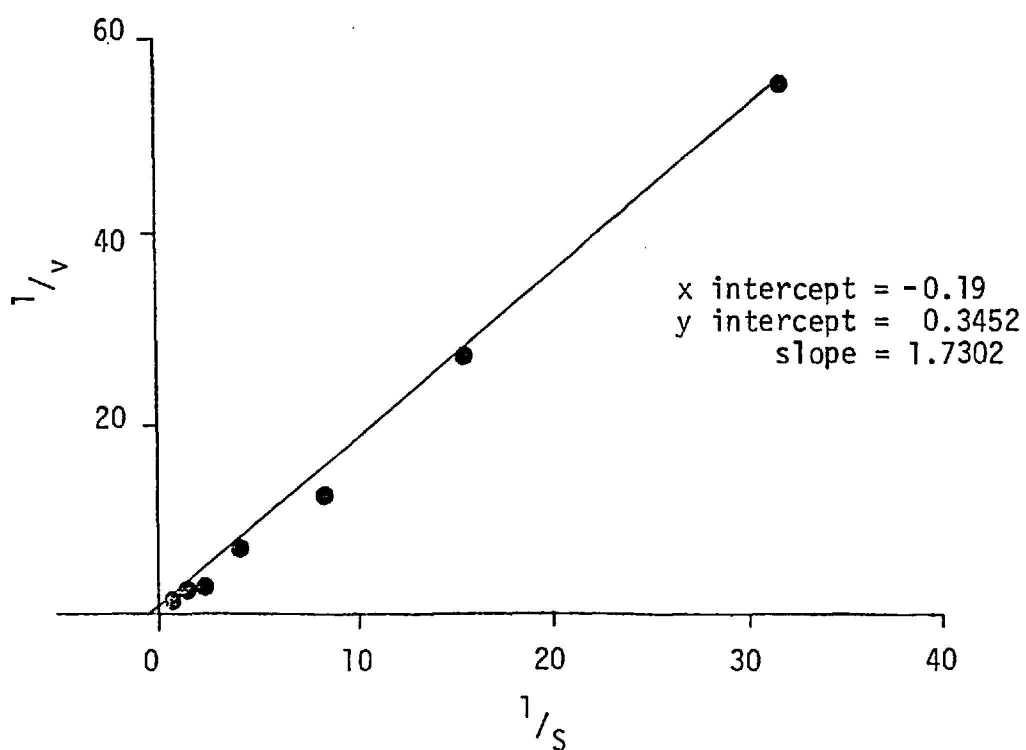


Fig. 29. Plot of $^1/_v$ by $^1/_S$ for 24-hydroxylase enzyme.

TABLE 19

A comparison of succinate and malate as supporters of 1- and 24-hydroxylase activities in chick kidney homogenates.

	Source of Reducing Equivalents	Enzyme Activity
1-hydroxylase	succinate	42.89 ± 2.2
	malate	39.85 ± 6.0
24-hydroxylase	succinate	99.41 ± 4.1
	malate	166.5 ± 22.1

The final concentration of sodium succinate or malate was 5mM. Enzyme activity is expressed as fmol dihydroxy metabolite (± s.e.m.) produced min^{-1} mg $protein^{-1}$. The substrate concentration was 75 pmol per 3 ml incubate.

TABLE 20

Effect of ions on 1-hydroxylase activity.

	Concentration	% Control Activity
Calcium	2.5×10^{-4}	45.0
	5×10^{-4}	14.8
Strontium	5×10^{-4}	25.6
	5×10^{-3}	29.2
Magnesium	1×10^{-3}	134.2
	8×10^{-3}	131.2
EGTA	2×10^{-4}	134.5
Phosphate	1×10^{-3}	88.6
	1×10^{-2}	87.3

Control incubations contained 1.9mM $MgCl_2$, 5mM sodium succinate, 15mM Tris acetate and all incubations were at pH 7.4. The substrate concentration was 75 pmol ^3H 25 OH D_3, and cation or anions were added at the indicated concentration. The chloride salt of cations (except strontium, which was added as strontium gluconate) and the sodium salt of anions were used.

TABLE 21

Effect of ions on 24-hydroxylase activity.

	Concentration (M)	% Control Activity
Barium	5×10^{-5}	89.35
	5×10^{-4}	94.81
	5×10^{-3}	102.87
Calcium	5×10^{-4}	92.3
	1×10^{-3}	91.0
EGTA	2×10^{-4}	192.8
Manganese	5×10^{-5}	115.13
	5×10^{-4}	69.99
	5×10^{-3}	42.04
Strontium	5×10^{-5}	55.39
	5×10^{-4}	54.73
	5×10^{-3}	46.62
Magnesium	1×10^{-3}	136.3
	8×10^{-3}	100.8

Control incubations contained 1.9mM $MgCl_2$, 5mM sodium succinate, 15 mM Tris acetate and all incubations were at pH 7.4. The substrate concentrations were 75 pmol ^3H 25 OH D_3. Cations were added as the chloride salt except strontium, which was added as strontium gluconate.

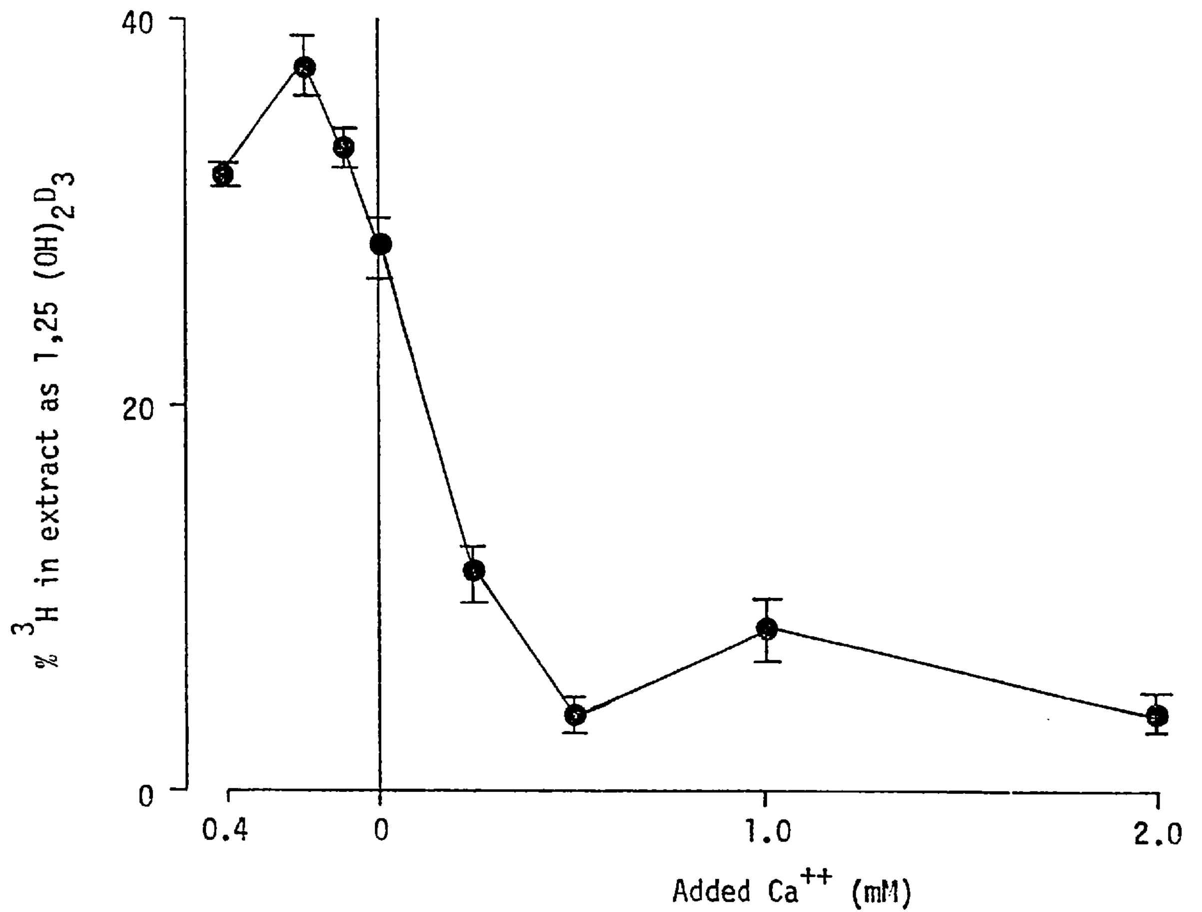


Fig. 30. Inhibition of 1-hydroxylase activity in chick kidney homogenates by Ca^{++}. Points to the left of the zero index on the abscissa were obtained in the presence of added EGTA up to 0.4 mM. Points represent the mean of 4 determinations of enzyme activity ± S.E.M.

described. Additions of Ca^{++} (as $CaCl_2$), PO_4 (as 1:4 w/w mixture of NaH_2PO_4 and Na_2HPO_4) and EGTA (ethanedioxybis(ethylamine)-NNN'N'-tetra-acetate) to the incubation mixtures were made to the final concentrations indicated.

Results:

Increasing the concentration of Ca^{++} in the assay medium produced a pronounced inhibition of 1-hydroxylation. Addition of the chelating agent EGTA enhanced the formation of 1,25 $(OH)_2D_3$ so that the maximum rate of production was obtained at a final concentration of EGTA of 0. 2mM. A decline in the rate of 1-hydroxylation was observed at EGTA concentrations greater than 0.2mM (Fig. 30).

In the absence of added Ca^{++}, addition of increasing amounts of PO_4 up to a final concentration of 20mM had no effect on the rate of 1-hydroxylation. However, when Ca^{++} was present in an inhibitory concentration (0.2mM), PO_4 concentrations above 8mM reversed this effect (Fig. 31).

In contrast, no such inhibition of enzyme activity with Ca^{++} was seen with the 24-hydroxylase system (Fig. 32). However, addition of EGTA to the incubation medium stimulated 24-hydroxylate activity. Fig. 33 compares the effect of increasing Ca^{++} concentrations up to 4mM added Ca^{++} on the activities of both enzymes.

3.2 Effects of other divalent cations

The effects of other divalent cations on renal 1- and 24-hydroxylase activities were tested to determine whether changes in

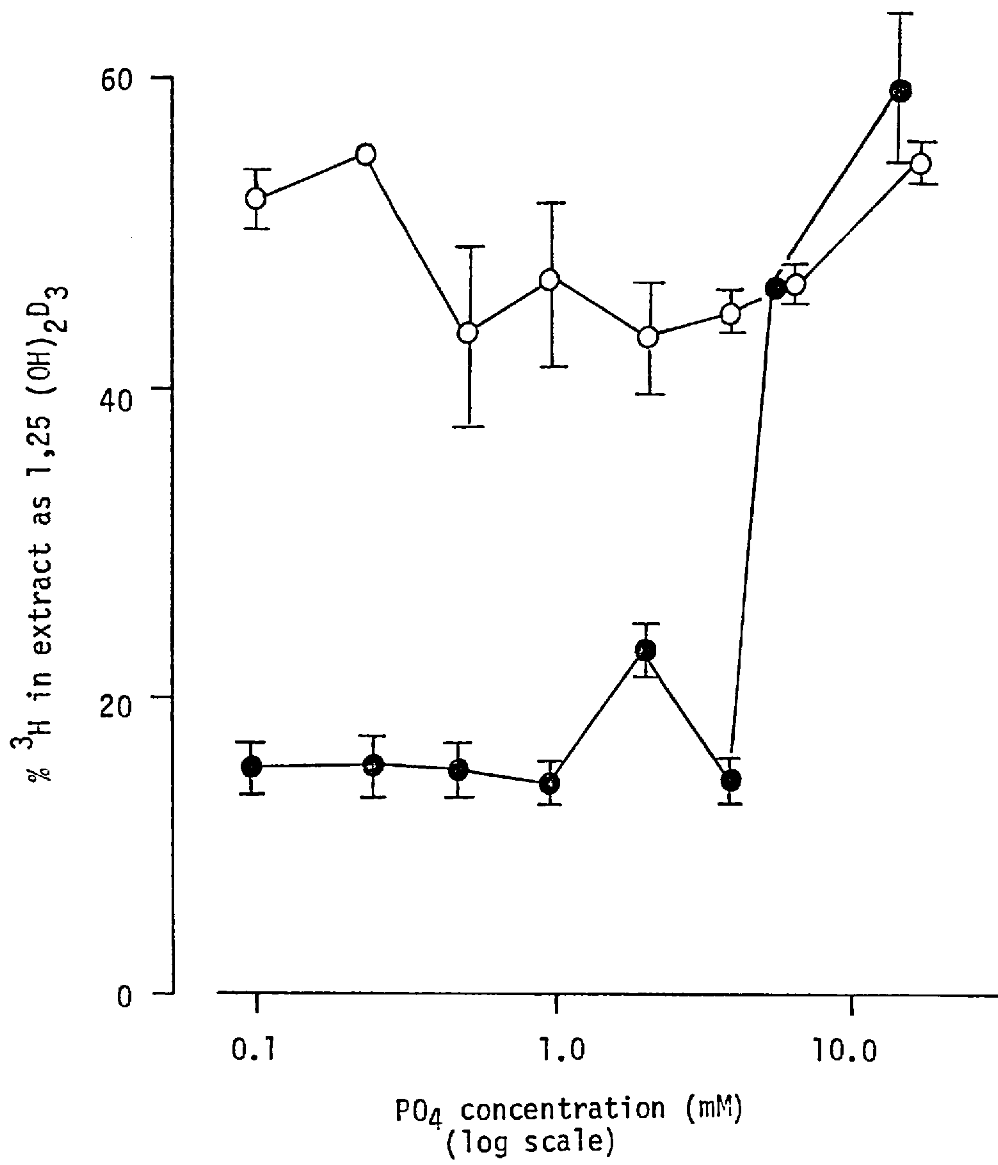


Fig. 31. Effect of PO_4 on the inhibition of 1-hydroxylase activity by Ca^{++}.

○ 0.2 mM EGTA

● 0.2 mM Ca^{++}

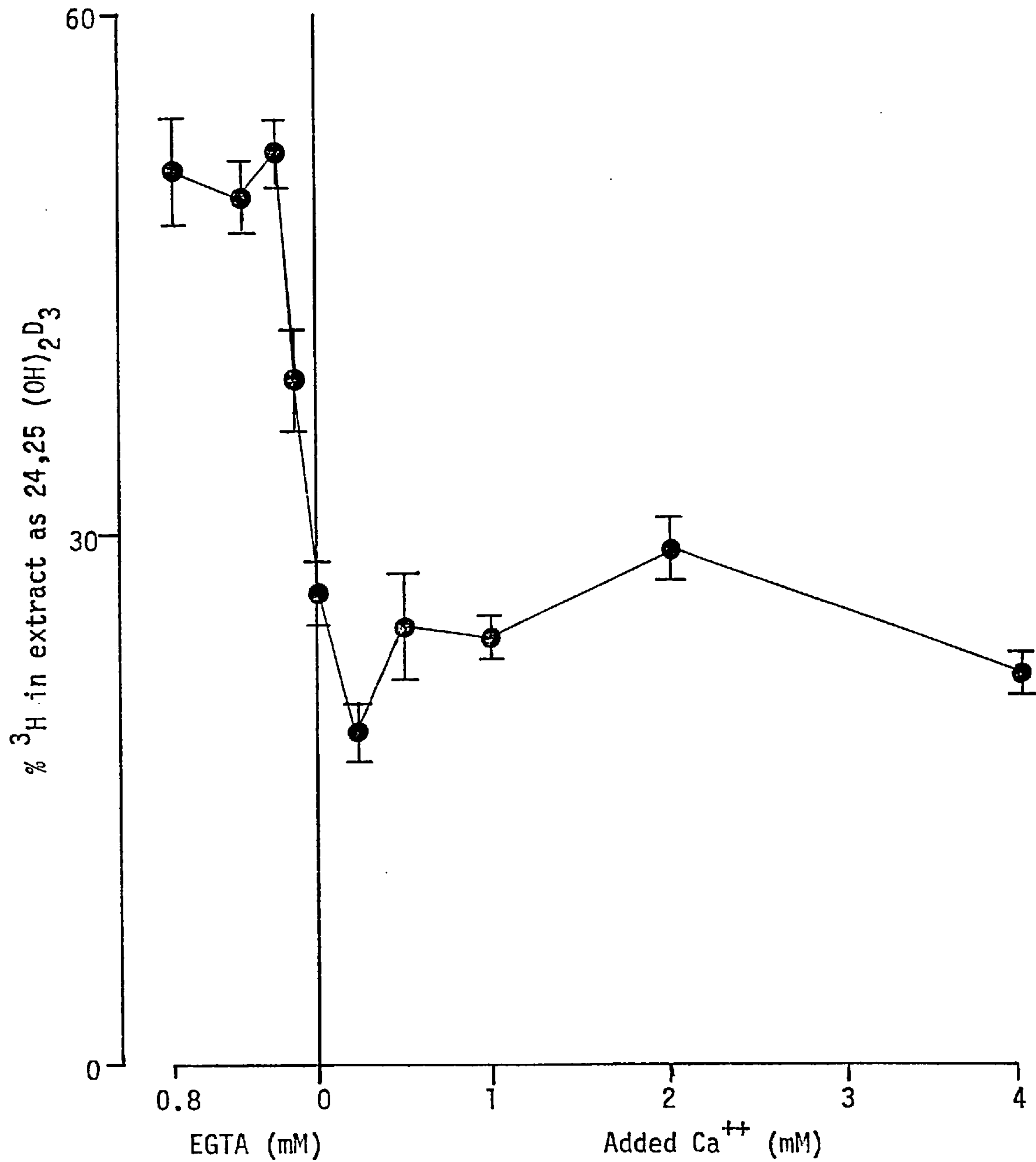


Fig. 32. Effect of Ca^{++} on 24-hydroxylase activity in chick kidney homogenates. Points to the left of the zero index on the abscissa were obtained in the presence of added EGTA up to 0.8 mM. Points represent the mean of 3 determinations of enzyme activity ± S.E.M.

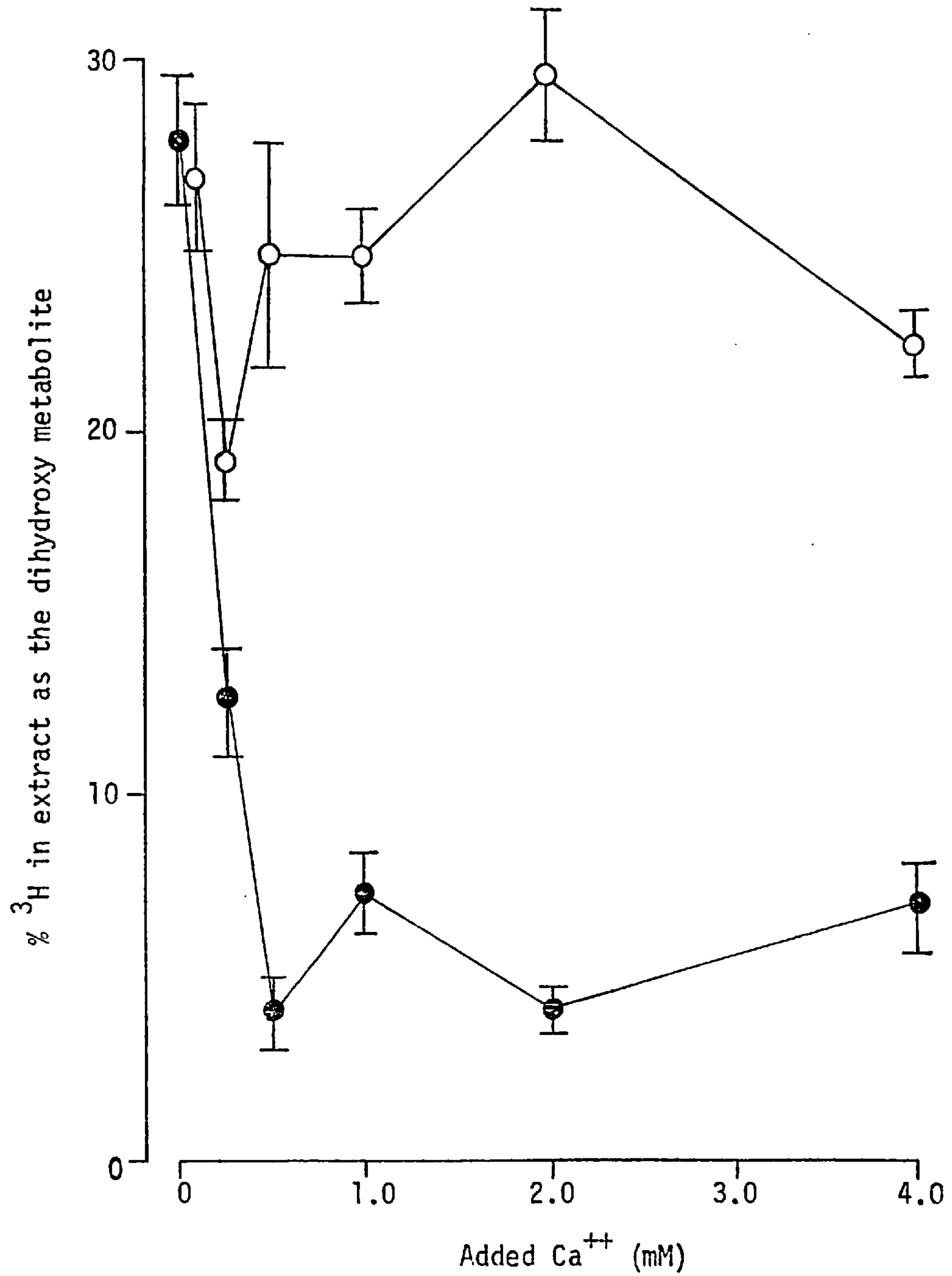


Fig. 33. Effect of Ca^{++} on 1- and 24-hydroxylase activity in chick kidney homogenates.

● 1-hydroxylase

○ 24-hydroxylase

their concentrations might also have differing effects on the two enzymes. The results of these studies are shown in Tables 20 and 21. Strontium (5×10^{-4}M) caused an inhibition of both enzymes. Increasing concentrations of magnesium had no marked effect on either enzyme activity. Manganese (5mM) inhibited 24-hydroxylase activity whereas this concentration of barium has no effect on this enzyme.

4. Discussion

Table 22 summarizes the in vitro properties of the two renal hydroxylase enzymes. The apparent K_m calculated for the 1-hydroxylase enzyme is in agreement with that reported by Henry and Norman (1974) for isolated mitochondria, but differs from that of Gray (Gray et al., 1972). A K_m of 10^{-6} has been reported for the 24-hydroxylase enzyme in isolated mitochondria (Knutson and DeLuca, 1974) which is somewhat higher than the value of 5×10^{-7}M calculated from the experimental data described in this section. It is possible that the discrepancies in apparent K_m calculated for both enzymes are due to differing experimental conditions. The results reported in this thesis were obtained with whole homogenates, whereas other workers (Gray et al., 1972; Knutson and DeLuca, 1974; Henry and Norman, 1974) have used isolated mitochondria.

The comparative studies of the effects of cations and anions on the 1- and 24-hydroxylase enzymes in homogenates are of interest. Ca^{++} inhibits 1-hydroxylase activity in this system and removal of calcium ions already present in the homogenate by addition of the chelating agent EGTA stimulated the activity of this enzyme.

Inhibition of 1-hydroxylase activity by Ca^{++} has also been reported by other workers (Henry and Norman, 1974). In contrast, 24-hydroxylase activity appears to be unaffected by increasing Ca^{++} concentrations. This observation is in conflict with that reported by Horiuchi (Horiuchi et al., 1974). Interestingly, two other divalent cations, Mn^{++} and Str^{++} inhibit 24-hydroxylase activity. However, the effects of Ca^{++} on these mitochondrial enzymes should be interpreted with caution. Mitochondria actively take up Ca^{++} and loss of mitochondrial integrity through swelling and changes in mitochondrial pH can occur with high concentrations of calcium. In order to elucidate the possible physiological role of Ca^{++} on the renal hydroxylase system, a detailed study of the effects of this ion on isolated mitochondria was undertaken.

TABLE 22

Summary of the properties of the 1- and 24-hydroxylases.

	1-hydroxylase	24-hydroxylase
K_m	0.87×10^{-7}M	5×10^{-7}M
pH optimum	7.0 - 7.4	7.0 - 7.4
2.5×10^{-4}M Ca^{++}	55% inhibition	No inhibition
2×10^{-4} EGTA	33% stimulation	92% stimulation
5×10^{-4} Str	75% inhibition	45% inhibition

CHAPTER SEVEN

STUDIES WITH ISOLATED KIDNEY MITOCHONDRIA

Summary

This chapter describes experiments with isolated chick kidney mitochondria. These studies were designed to investigate the effects of Ca^{++} and PO_4^{3-} ions on 1-hydroxylase activity.

The response of the 1-hydroxylase enzyme to Ca^{++} is biphasic. Concentrations of added Ca^{++} up to 0.1 mM caused an inhibition of enzyme activity while at higher levels of Ca^{++} a reversal of inhibition was seen.

Inhibitors of mitochondrial Ca^{++} uptake prevented the inhibition of 1-hydroxylase activity induced by Ca^{++}.

PO_4^{3-} ions modified the inhibition of enzyme activity induced by low concentrations of Ca^{++}.

It is concluded that changes in intracellular levels of Ca^{++} and PO_4^{3-} ions and their uptake by renal mitochondria may play a role in the short-term regulation of vitamin D_3 metabolism.

INTRODUCTION

As previously discussed, many factors have been suggested as regulators of the renal metabolism of 25 OH D_3, among them serum calcium concentration (Boyle et al., 1971), renal phosphate concentration (Tanaka and DeLuca, 1973) and parathyroid hormone as well as feed-back regulation by 1,25 $(OH)_2D_3$. However, the precise mechanisms by which many of these factors exert their effects at a subcellular level remain to be clarified. All the above mentioned effects can

be considered as 'long-term' regulators since their effects take hours or days to become apparent. It is likely that one further regulatory mechanism is the modulation of existing enzyme molecules through a direct interaction with calcium and perhaps phosphate ions. Such a system would offer an immediate means of control. The location of the renal 25 OH-1- and 24-hydroxylases in mitochondria, organelles that are known for their ability to accumulate calcium and thereby control cytosolic calcium concentrations, would ensure that the enzymes sense fluctuations in the concentrations of calcium ions within the cytosol.

There have been several attempts to study the direct effect of Ca^{++} and PO_4^{3-} ions on the activity of the 25 OH D_3-1-hydroxylase enzyme in isolated chick kidney mitochondria, but the reports emerging from these studies are conflicting in that some report a stimulation of enzyme activity (Horiuchi, Suda, Sasaki, Ogata, Ezawa, Sano and Shimazaw, 1975; Bikle, Murphy and Rasmussen, 1975) and some a decrease (Henry and Norman, 1974). This disparity between the findings of these various groups can probably be accounted for, in part, by the different experimental conditions prevailing since isolated mitochondrial experiments need to be carefully designed so that any observed changes in enzyme activity are not the results of a change in mitochondrial integrity through swelling or alterations in intramitochondrial pH.

1. Kinetic Aspects of Calcium Uptake by Mitochondria

The uptake of calcium is a property common to mitochondria

of most eucaryotic cells. The uptake is an energy-linked process, which can be supported by either the oxidation of respiratory substrates, or by the hydrolysis of added ATP. In both cases the transport is abolished by uncouplers of oxidative phosphorylation (Carafoli, Malstrom, Capano, Sigel and Crompton, 1975). Rottenberg and Scarpa (1974) have postulated that ATP and the activity of the respiratory chain energize the uptake of calcium by generating a membrane potential across the inner membrane which is the immediate pulling force for the uptake of calcium. The uptake of Ca^{++} does not require the simultaneous uptake of permanent anions, although the uptake of phosphate increases the total level of Ca^{++} uptake, (Carafoli et al., 1975). One unanswered question is whether Ca^{++} accumulated in the absence of anions is stored in the matrix as a soluble species or whether it remains bound to fixed anionic sites on the inner side of the inner membrane. Also, it is not known whether the endogenous Ca^{++} pool is in the form of an insoluble precipitate.

When Ca^{++} alone is present in the medium, a maximum of 100 to 150 nmoles Ca^{++} is taken up per mg of mitochondrial protein, but when PO_4 is also present a reported maximum of 2-3 μmoles Ca^{++} is taken up per mg mitochondrial protein. Since high levels of PO_4 are present in the cytosol, Lehninger has suggested that PO_4 could play an essential role in the uptake of Ca^{++} (Carafoli et al., 1975).

Mitochondria have a high affinity for Ca^{++}. The K_m for Ca^{++} uptake is in the order of 1-50 μM (Scarpa, 1975). It is also of

interest that there are specific inhibitors of mitochondrial calcium uptake, Lanthanum (Mela, 1969) and ruthenium red (Vasington, Gazotti, Tiozzo and Carafoli, 1972). These inhibitors act at very low concentrations and do not impair the ability of mitochondria to phosphorylate ADP. Ruthenium red is a histochemical stain with some specificity for carbohydrates, and this may indicate that carbohydrates, possibly glycoproteins, may be components of the calcium transporting system.

Calcium is known to influence many cellular reactions, and the regulation of intracellular calcium is of great importance. Mitochondria create a concentration gradient of calcium with respect to the soluble phase of the cell. If these calcium fluxes between cytosol and mitochondria are reversible, mitochondria may regulate cytosolic Ca^{++} concentrations and thus also regulate Ca^{++} dependent cellular processes. It has long been known that Ca^{++} accumulated by mitochondria can be promptly discharged upon interruption of the energy flow with respiratory inhibitors or uncouplers, or by adding to the medium specific Ca^{++} ionophoric antibiotics. The release of Ca^{++} accumulated in the absence of permanent anions is very rapid but that taken up with PO_4 is presumably stored as an insoluble precipitate and is released slowly. Of great interest is the fact that the endogenous mitochondrial pool of Ca^{++} can be rapidly mobilized and discharged with the calcium ionophore A23187 (Carafoli, Malstrom, Sigel and Crompton, 1976). This would suggest that most of the endogenous Ca^{++} is not stored in mitochondria as an insoluble salt and so mitochondria may be very dynamic reservoirs for cell Ca^{++}.

Thus it is clear that mitochondrial Ca^{++}, both endogenous and accumulated, can be rapidly discharged by artificial means, but it is still not known how Ca^{++} is 'naturally' released. Recently it has been reported that Ca^{++} release from mitochondria can be induced by prostaglandins and also by sodium ions (Carafoli et al., 1975).

2. Methods

2.1 Preparation of mitochondria

One-day old chicks were maintained on the vitamin D deficient diet for 2 to 3 weeks. Chicks were then killed by decapitation and the kidneys immediately removed and placed in ice-cold medium (200 mM sucrose, 15 mM Tris-acetate buffer, pH 7.4 supplemented with 2 mM EGTA, latterly supplemented with 0.2% bovine serum albumin) that had been depleted of oxygen by gassing with nitrogen for 2 hours. The tissue (approximately 25 g) was homogenized in 100 ml of the buffer and the homogenate was centrifuged for 10 min at 500 g at $4^{o}C$. The precipitate was discarded and the supernatant was transferred to clean centrifuge tubes and recentrifuged at 9000 g for 10 min.

The second supernatant was discarded and the precipitated mitochondria were resuspended by homogenization (a few strokes by hand) in an equal volume of Tris-acetate buffer supplemented with 10 mM EGTA. The mitochondria were resedimented by centrifugation at 10,000 g for 10 min and were washed once more with the Tris-acetate containing 10 mM EGTA and finally with the Tris buffer alone. The isolated mitochondria were then suspended in approximately 5 ml of the

Tris-acetate buffer containing 1 mg/ml bovine serum albumin to give a mitochondrial protein concentration of 15-30 mg per ml. The anaerobic conditions and the presence of EGTA were designed to minimize the uptake of calcium by the mitochondria during isolation.

2.2 Assay of 1-hydroxylase activity in isolated mitochondria

Aliquots (0.1 ml) of the mitochondrial suspension were preincubated for 10 min at 37°C in a total volume of 1.5 ml buffer (15 mM Tris-acetate pH 7.4, 1.9 mM $MgCl_2$, 200 mM sucrose, 5 mM sodium succinate). In some experiments, $CaCl_2$ or Na_2HPO_4 was added to the required concentration. The substrate, ^3H 25 OH D_3 (6.25 pmol to 15 pmol), was added to each incubate in 5 µl ethanol. After a further 10 min incubation, the reaction was stopped by the addition of 4.5 ml methanol/chloroform (2:1 v/v). Samples were then extracted, chromatographed and counted as previously described.

2.3 Estimation of mitochondrial respiration

The oxygen consumption was measured polarographically at room temperature using an oxygen electrode (Chance and William, 1955). The oxygen electrode has a platinum cathode and silver anode in saturated KCl. Oxygen diffuses to the surface of the platinum cathode which is depolarized, allowing the flow of a current that is proportional to the amount of O_2 present.

The electrode is immersed in the medium which is agitated with a stirrer. It is assumed that air saturated buffer at 26°C contains

240 nmoles dissolved oxygen per ml. For calibration purposes zero oxygen is obtained by adding sodium dithionite. The rate of oxygen consumption in nmoles/min can be calculated from the slope of the recorder trace.

2.4 Estimation of mitochondrial calcium content

0.2 ml of the concentrated mitochondrial suspension was diluted with 2 ml plasma diluting fluid, supplemented with 2.5 mM NaCl. Precipitated proteins were removed by centrifugation at 2000 g for 10 min and the calcium content of the supernatant was measured by flame photometry using the method of MacIntyre (1962).

3. Preliminary Studies with Isolated Kidney Mitochondria

3.1 Measurement of P/O ratios

Kidney mitochondria prepared in this way were found to be coupled and had a P/O ratio of approximately 1.8 (Fig. 34) for succinate supported respiration.

3.2 1-hydroxylase activity

Such mitochondria convert 25 OH D_3 to 1,25 $(OH)_2D_3$ *in vitro*. The production of 1,25 $(OH)_2D_3$ with mitochondrial protein content was linear over a range 1 to 4 mg per 1.5 ml (Fig.35).

3.3 Effect of freezing the mitochondria

1-hydroxylase activity was almost completely abolished when

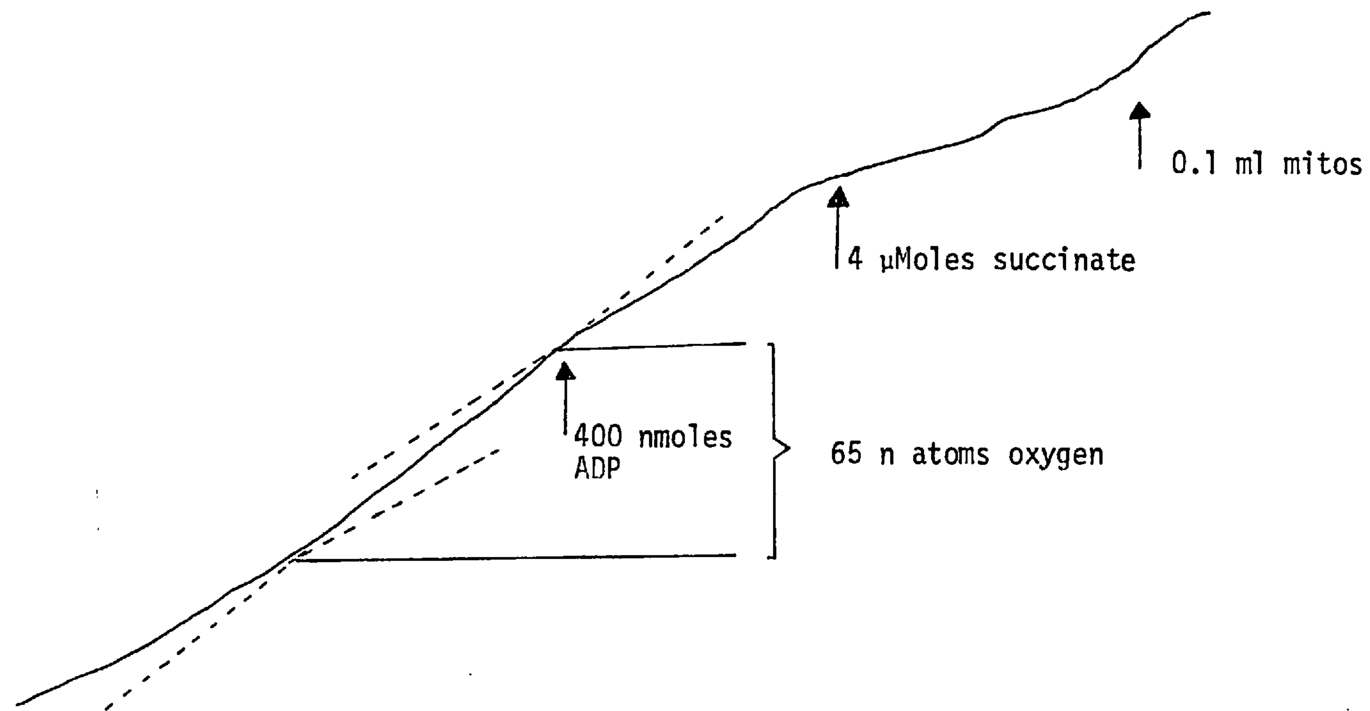


Fig. 34. Calculation of P/O ratio for chick kidney mitochondria. The incubation medium contained 200 mM sucrose, 15 mM Tris-acetate pH 7.4 and 3 mg mitochondrial protein in a total volume of 3.5 ml.

$$\text{P/O ratio} = \frac{400}{65} \times 3.5 = 1.76$$

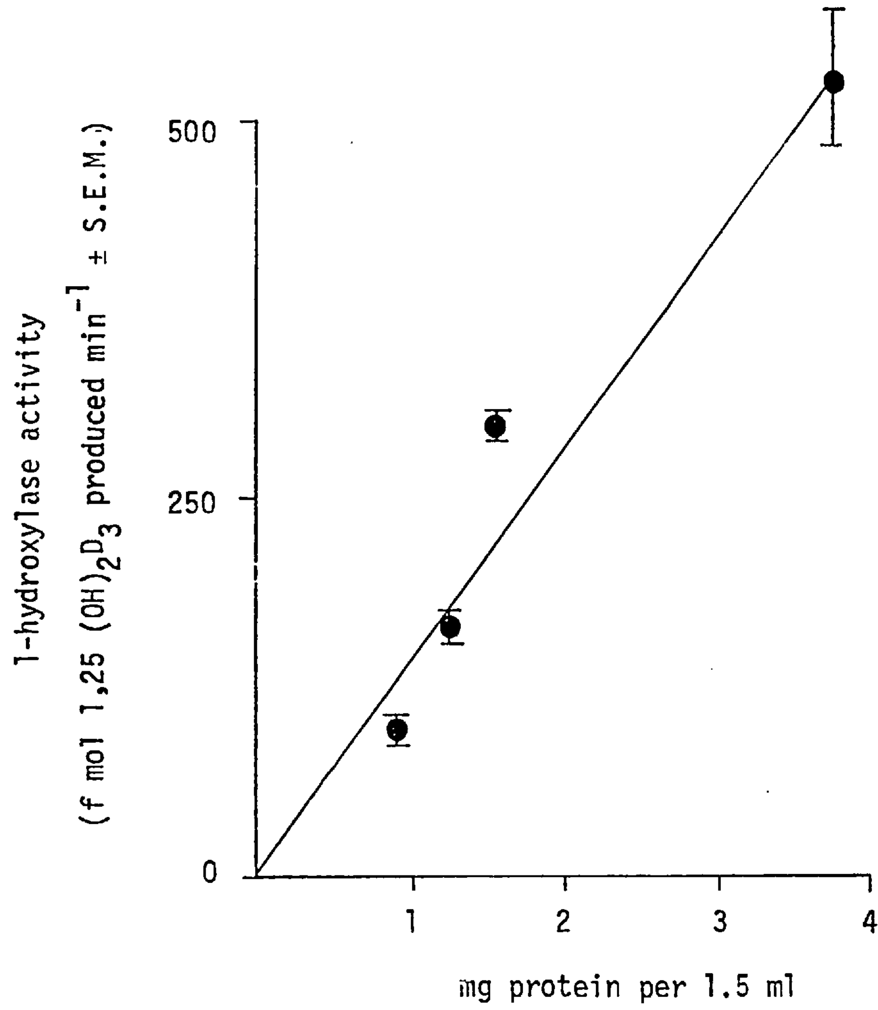


Fig. 35. Effect of increasing protein concentration on 1-hydroxylase activity in isolated mitochondria. The substrate concentration was 15 pmol per 1.5 ml incubate.

TABLE 23

Effect of freezing on 1-hydroxylase activity in isolated chick kidney mitochondria

		1-hydroxylase activity
Freshly isolated mitochondria	1.	111.7
		93.3
		103.3
	2.	183.3
		133.3
		150.0
Mitochondria stored at -70°C		33.3
		0
		43.3

1-hydroxylase activity is expressed as fmol 1,25 $(OH)_2D_3$ produced min^{-1} mg $protein^{-1}$. The substrate concentration was 75 pmol per 1.5 ml incubate.

mitochondrial pellets were frozen at -70°C and subsequently thawed before assay (Table 23). Respiration studies showed that such treatment uncoupled the mitochondria.

4. Effect of Calcium Ions on Kidney Mitochondria

4.1 The effect of Ca^{++} on 1-hydroxylase activity in intact mitochondria

Figure 36 shows the effect of $CaCl_2$ on 1-hydroxylase activity in intact mitochondria. The response to calcium was biphasic. Additions of increasing concentration of Ca^{++} up to 0.117 mM caused a marked decrease in enzyme activity; more than 50% inhibition was seen with 0.058 mM added $CaCl_2$. At higher concentrations of Ca^{++} a reversal of this inhibition was seen. Furthermore, this reversal of inhibition by higher concentrations of Ca^{++} did not seem to be dependent upon an exogenous supply of NADPH (Table 24). Similar effects of calcium ions were seen both when calcium was present during the time of preincubation and when calcium was added with the substrate after the 10 min preincubation period.

4.2 Effect of Ca^{++} on mitochondrial respiration

The inhibition of 1-hydroxylase activity with 0.058 mM Ca^{++} was not due to a change in mitochondrial integrity since respiration was not inhibited following uptake of Ca^{++} (Fig.37a). In contrast, respiration was stimulated transiently by the pulse of Ca^{++} as expected (Carafoli et al., 1975; Severson, Denton, Pask and Randle, 1974). However, with the higher levels of Ca^{++} associated

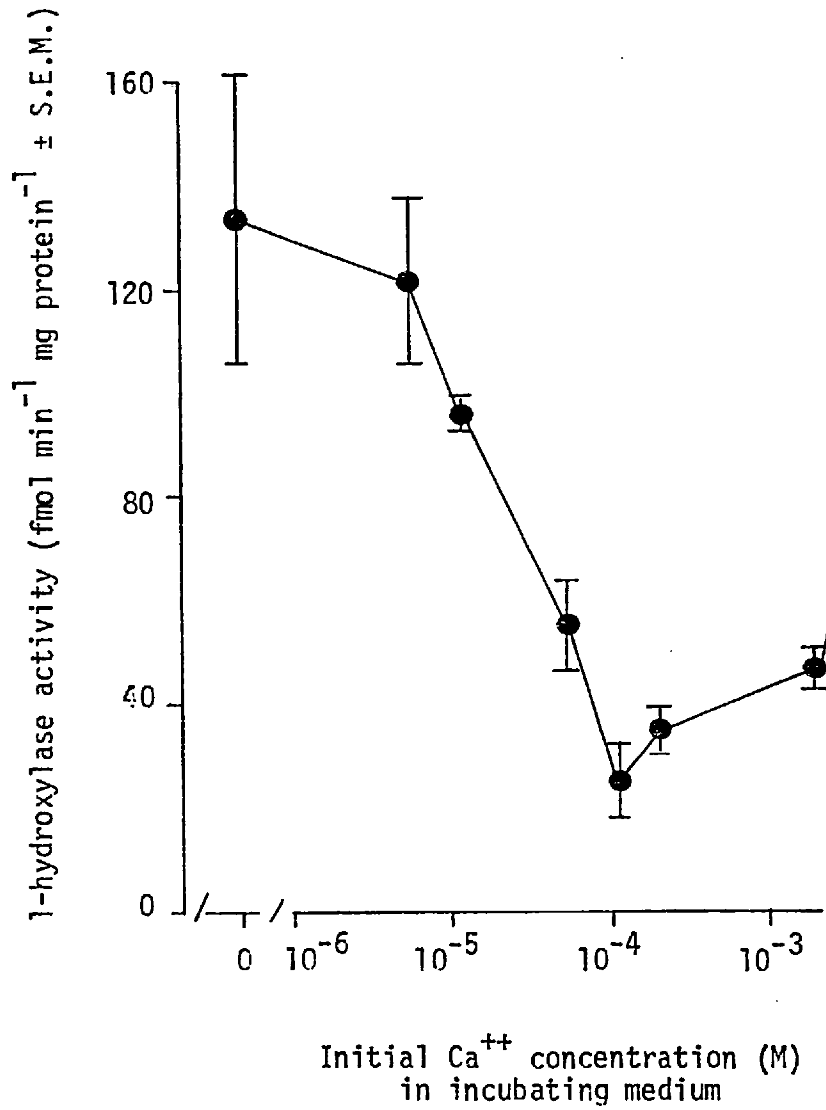


Fig. 36. Effect of increasing Ca^{++} concentration on 1-hydroxylase activity.

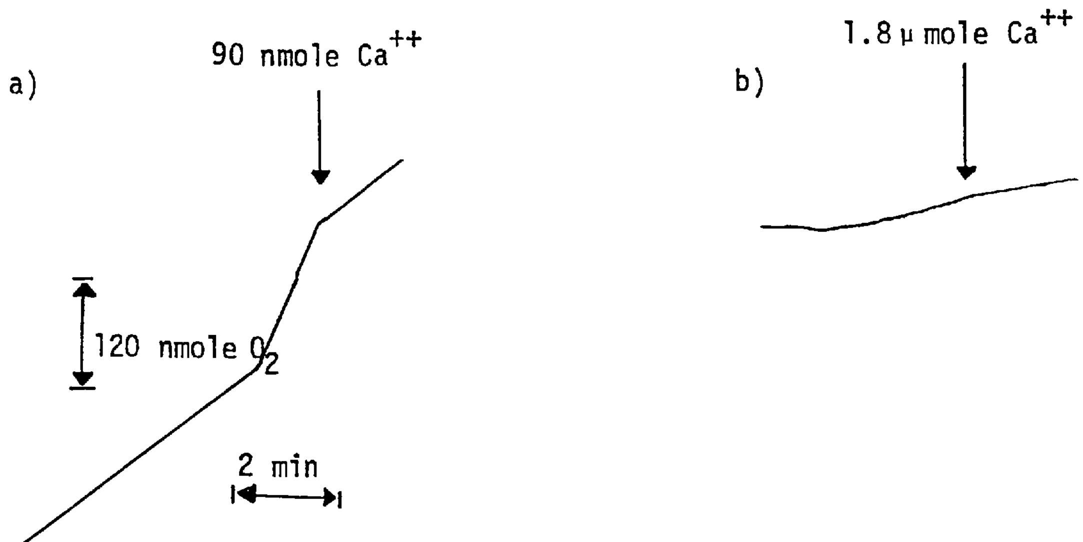


Fig. 37 a & b. Effect of Ca^{++} on the respiration of chick kidney mitochondria. The incubation medium consisted of 200 mM sucrose, 15 mM Tris acetate buffer, pH 7.4, 4 mM Na succinate and 3 mg of mitochondrial protein in a total volume of 2.5 ml. Ca^{++} was added in a pulse at the point shown. The oxygen consumption was measured polarographically.

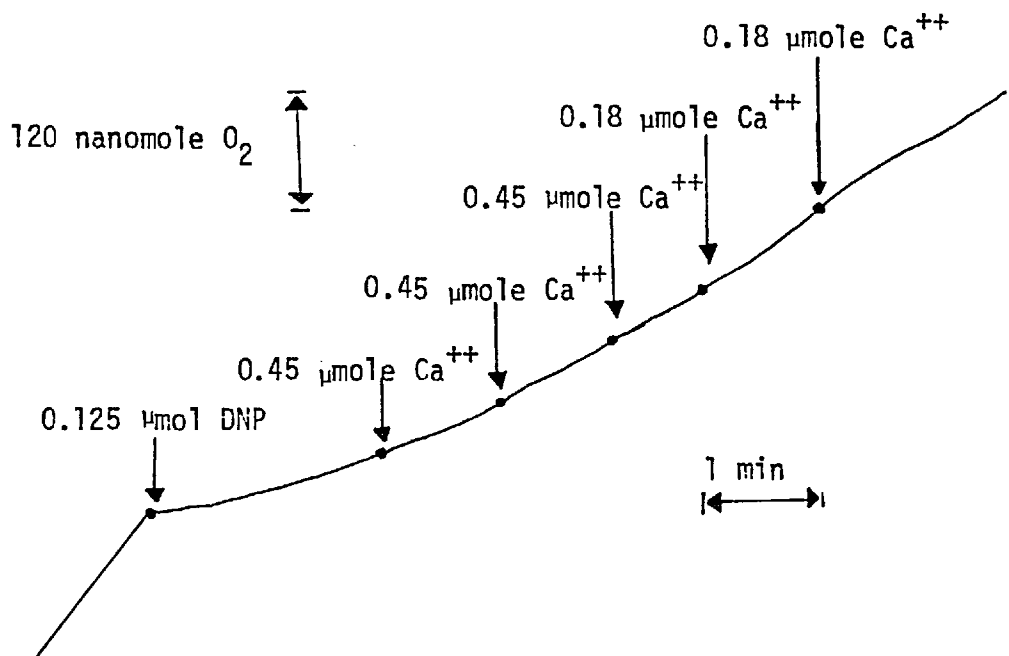


Fig. 37c. Effect of higher levels of Ca^{++} on the respiration of chick kidney mitochondria. The conditions were similar to those in Fig. 37a except that the total volume of the incubation medium was 4.4 ml containing 6.2 mg of mitochondrial protein.

TABLE 24

Effect of an exogenous supply of NADPH on 1-hydroxylase activity in the presence of high concentrations of added Ca^{++}

	Added Ca^{++} (mM)	1-hydroxylase activity (% control)
1) NADPH generating system added	* 0	100
	* 0.117	25.55
	* 0.58	10.41
	* 1.75	14.22
	* 3.5	29.11
2) No NADPH generating system	0	103.9
	1.75	36.38
	3.50	32.10

* Incubation medium supplemented with NADPH generating system (glucose-6-phosphate; 5 mM, $NADP^{+}$:0.33 mM and glucose-6-phosphate dehydrogenase: 600 units l^{-1}).

with reversal of enzyme inhibition, mitochondria showed a disturbance in respiration following Ca^{++} uptake (Fig.37b). This inhibition of respiration was relieved by the addition of the uncoupler DNP (Fig.37c).

4.3 The effect of modulators of mitochondrial calcium uptake

4.3.1 Ruthenium red and lanthanum

Further evidence for the involvement of mitochondrial levels of Ca^{++} on 1-hydroxylase activity was obtained with experiments using inhibitors of calcium uptake. Mitochondria were preincubated for 10 min in the presence of various concentrations of Ca^{++} and the inhibitors before addition of substrate. Both $LaCl_3$ and ruthenium red prevented the inhibition of 1-hydroxylase activity induced by low levels of Ca^{++} (Fig.38). As expected, the addition of ruthenium red after Ca^{++} had already been accumulated by the mitochondria did not alleviate Ca^{++} inhibition of enzyme activity (Table 25a). In this experiment, mitochondria were preincubated in the presence of 60 µM Ca^{++} for 10 min before the addition of ruthenium red and substrate. Furthermore, ruthenium red prevented the stimulation of oxygen uptake of kidney mitochondria by Ca^{++} (Fig. 39). However, attempts to show a reversal of Ca^{++} induced inhibition of hydroxylase activity by treatment with the ionophore A23187 (1.25 µM) were unsuccessful because the antibiotic was itself found to be inhibitory. Respiration studies showed that the ionophore acted as an uncoupler under these experimental conditions.

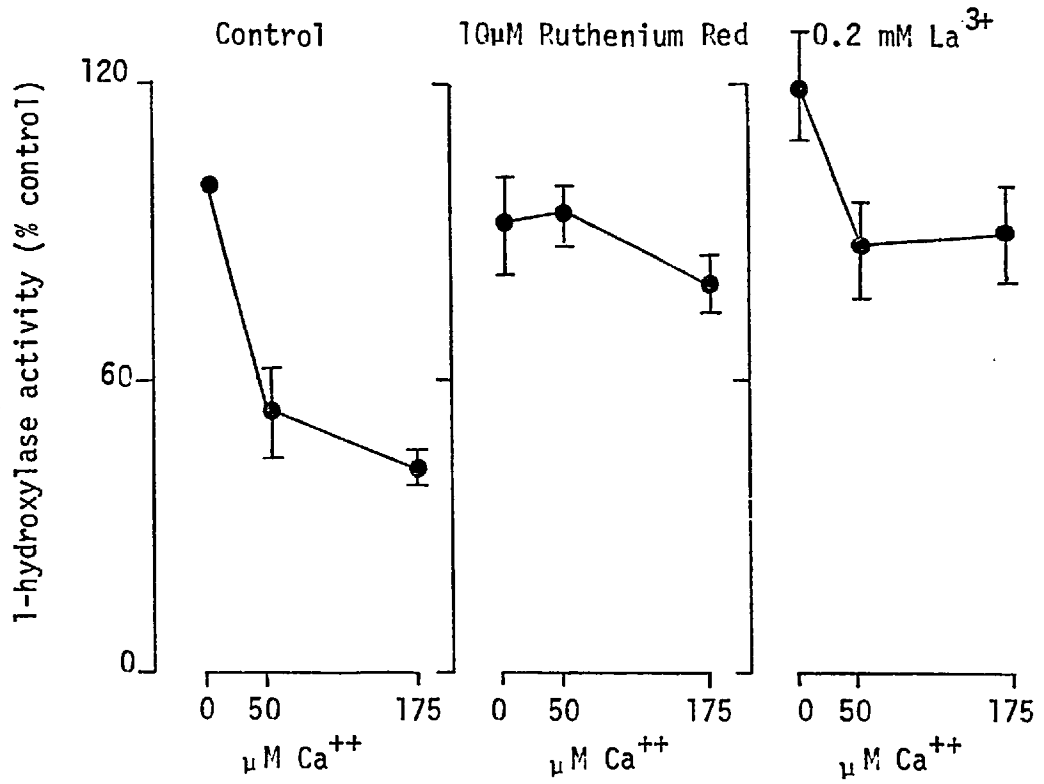


Fig. 38. Effect of Ruthenium Red and La^{3+} on inhibition of 1-hydroxylase activity by Ca^{++}. Mitochondria were preincubated for 10 min in the presence of Ca^{++} and the inhibitors before addition of substrate.

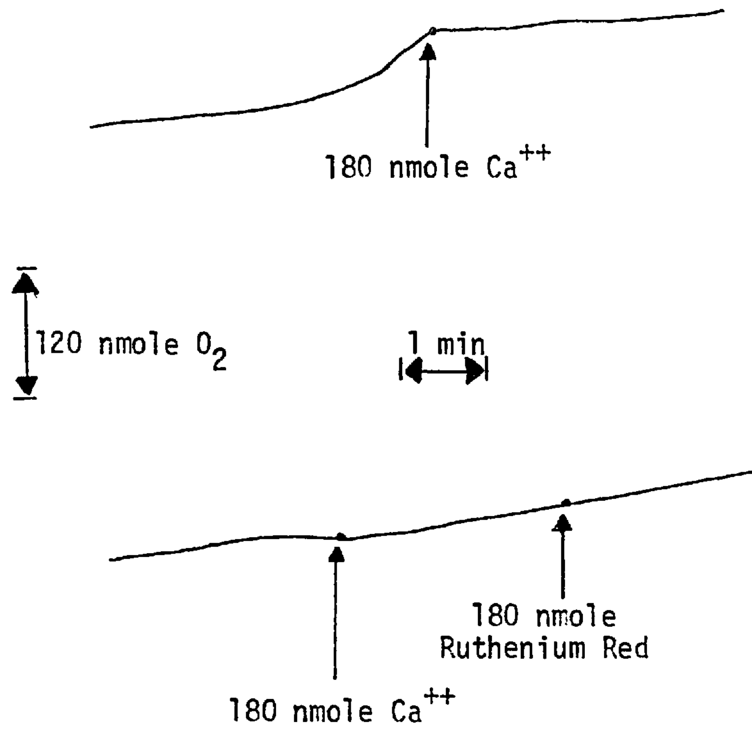


Fig. 39. Effect of Ruthenium Red on the stimulation of oxygen uptake of kidney mitochondria by Ca^{++}.

TABLE 25

Effect of modulators of mitochondrial Ca^{++} uptake on 1-hydroxylase activity

a) Ruthenium Red

Additions	Total Ca^{++} (μM)	1-hydroxylase activity (% control ± S.E.M.)
Nil	60	56.2 ± 2.9
Ru. Red (60 μM)	60	93.7 ± 9.3
Ru. Red (60 μM) added after Ca^{++}	60	61.0 ± 9.8
A23187 (1.25 μM) + *Ru. Red (60 μM)	30	2.7 ± 0.85
A23187 (1.25 μM) + *Ru. Red (60 μM)	0	8.6 ± 2.4

* Ruthenium Red was included in the incubation media to prevent reaccumulation of Ca^{++}.

TABLE 25b - Mg^{++} and K^+

Experiment No.	1-HYDROXYLASE ACTIVITY (% CONTROL)				
	Ca^{++}	K^+	Ca^{++} + Mg^{++}	Ca^{++} + Mg^{++} + K^+	K^+ + Ca^{++}
1	36.92	95.04	-	-	39.56
2	56.24	93.71	66.74	41.26	-
3	51.94	103.89	90.64	106.4	-
4	34.83	85.29	60.52	95.04	-
5	25.10	-	70.70	97.18	102.2
6	66.68	119.04	91.02	126.71	115.79

Results are a mean of 5-6 separate estimations of enzyme activity. The incubation medium contained Tris-acetate (15 mM, pH 7.4), Na succinate (5 mM), $MgCl_2$ (1.9 mM), sucrose (100 mM) supplemented, as indicated above, with $CaCl_2$ (60 μM), KCl (80 mM) or $MgCl_2$ (5 mM final concentration).

TABLE 25c - Prostaglandins and Na^+

	Additions	Total Ca^{++} (μM)	1-hydroxylase activity (% control)
Experiment I			
	-	60	51.94
	*$PGF_{2}\alpha$ (10μM) + *Ru Red (60μM)	60	53.92
	*PGE_2 (10μM) + *Ru Red (60μM)	60	71.18
Experiment II			
	-	60	25.10
	*Ru Red (60μM)	60	25.43
	+NaCl (54mM) + *Ru Red (60μM)	60	26.66

* Added after 10 min pre-incubation with $CaCl_2$

4.3.2 Magnesium and potassium ions

There have been reports that calcium uptake by mitochondria is inhibited by Mg^{++}. Carafoli (Carafoli et al., 1975) found that 5 mM $MgCl_2$ inhibited calcium uptake by heart, but not by liver, mitochondria. However, other workers have recently reported that Mg^{++} inhibited the stimulation of mitochondrial respiration induced by calcium using liver mitochondria, and that K^+ overcame this inhibition (Hutson, Pfeiffer and Lardy, 1976).

Bikle and co-workers have found a stimulatory effect of calcium on 1-hydroxylase activity and that increasing K^+ concentrations in the incubating medium enhanced this effect (Bikle, Murphy and Rasmussen, 1976).

A study was carried out to investigate the influence of changes in extra-mitochondrial $Mg^{++}:K^+$ ratios on the response of the 1-hydroxylase enzyme to added Ca^{++}. Increasing $MgCl_2$ in the incubation medium from 1.9 mM to 5 mM partially reversed the inhibition produced by 60 μM Ca^{++}in all experiments. The results with K^+ were more variable. In some experiments, the presence of 80 mM KCl in the incubation medium negated the effect of Ca^{++} on 1-hydroxylase activity (Table 25b). 80 mM KCl in the absence of Ca^{++} had no effect on enzyme activity.

4.3.3. Prostaglandins and Na^+

Calcium accumulated within mitochondria can be released upon the addition of an uncoupler, such as DNP.

TABLE 26

Composition of Ca/EGTA buffers

$MgCl_2$ (mM)	$CaCl_2$ (mM)	EGTA (mM)	Ca^{++}free (μM) at pH 7.4
1.9	-	5.0	-
1.9	3.5	5.0	0.08
1.9	4.25	5.0	2.63
1.9	4.5	5.0	3.16
1.9	5.0	5.0	12.7
1.9	5.5	5.0	50.0

Free Ca^{++} concentrations were calculated by the method of Portzehl, Caldwell and Ruegg (1964).

Other agents can induce rapid loss of Ca^{++} from mitochondria, such as the specific calcium ionophore A23187. More recently two more agents of a more physiological nature have been reported to induce release of accumulated calcium. These agents are the prostaglandins PGB_2 and PGE_1 and sodium ions.

Carafoli et al. (1975) have demonstrated release of mitochondrial calcium by 50 mM NaCl using heart mitochondria, but report that this effect is far less evident with mitochondria isolated from liver. The same group have shown that PGB_2 can accelerate the loss of Ca^{++} from liver mitochondria. Kirtland and Baum (1972) had previously shown a similar effect of PGE_1 on heart mitochondria. A preliminary study was performed to investigate whether these agents could reverse the inhibition of 1-hydroxylase activity by calcium ions.

The results were variable, although in one experiment there was some indication of a reversal of calcium induced inhibition of 1-hydroxylase activity with 10 μM prostaglandin PGE_2 but not with $PGF_{2\alpha}$. NaCl (54 mM) did not reverse the inhibition of enzyme activity (Table 25c).

4.4 The effect of Ca^{++}/EGTA buffers

Bikle and colleagues (Bikle, Murphy and Rasmussen, 1975) have argued that in determining the effects of calcium ions on 1-hydroxylase activity in isolated mitochondria, attempts should be made to regulate free calcium concentration using calcium/EGTA buffers. Using such buffers, this group have shown an increase in 1-hydroxylase activity with increasing calcium concentration at pH 6.8, but no effect of calcium at pH 7.4. They measured the pH of the medium at $37^{o}C$.

In an attempt to repeat the findings of this group, calcium/EGTA buffers were prepared (Table 26) over a range of free calcium concentrations from 0.08 μM to 50 μM. Zero calcium concentration corresponded to a final concentration of 5 mM EGTA. An experiment was designed to compare the effects of calcium/EGTA buffers with buffer containing free Ca^{++} over the same range of free calcium concentrations. As shown in Fig.40 , 5 mM EGTA caused an approximately 50% decrease in 1-hydroxylase activity, while calcium in the presence of EGTA had a profound inhibitory effect. The most likely explanation for this inhibition by calcium even in the presence of EGTA is that the mitochondria actively take up the free calcium ions, leading to a dissociation of calcium from the calcium/EGTA complex which, in turn, is taken up by the mitochondria. Eventually this process would lead to the accumulation of large amounts of calcium within the mitochondria, leading to the loss of mitochondrial integrity. Hence, although the calculated free concentration is very low, the level of calcium available for accumulation within the mitochondria is in the region of 5 mM.

In view of these results, it is difficult to reconcile the stimulation of 1-hydroxylase activity by calcium reported by Bikle and colleagues with the findings reported here of an inhibition of enzyme activity by low levels of calcium. It may be that differences in experimental techniques may explain these opposing findings. Bikle did not report any attempt to ascertain the effects of calcium upon mitochondrial integrity.

In the experiments reported in this chapter, the inhibition of 1-hydroxylase activity occurs at initial concentrations of calcium in

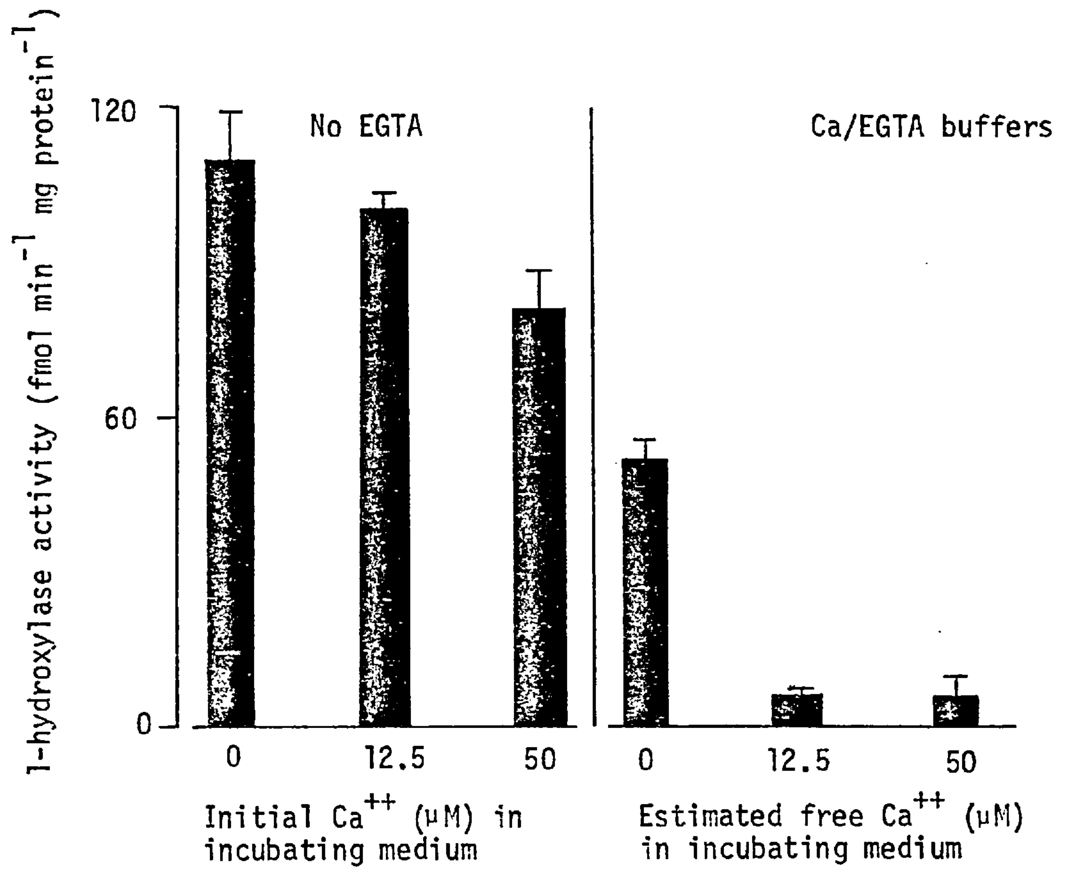


Fig. 40. Effect of Ca/EGTA buffers.

the incubation medium which are very low (0.058 mM) and which may by physiologically significant. Furthermore, uptake of these amounts of Ca^{++} (29 nmol Ca^{++} per mg mitochondrial protein, Fig.41) would lead to a total mitochondrial calcium load of approximately 65 nmoles calcium per mg mitochondrial protein, since the intra-mitochondrial calcium levels were found to be about 35 nmoles Ca^{++} per mg protein (mean of a determination of intra-mitochondrial calcium levels from 7 separate experiments). The results of estimations of mitochondrial calcium content vary, but a concentration of less than 100 nmoles calcium per mg protein is generally accepted. Carafoli et al. (1975) have reported a value of 15 nmoles Ca^{++} per mg protein for heart mitochondria and Van Rossum and colleagues (Van Rossum, Smith and Beeton, 1976) found a value of 47 nmoles Ca^{++} per mg protein for rat liver mitochondria.

4.5 The effect of DNP treatment

Horiuchi et al. (1975) have also reported a calcium stimulated increase in 1-hydroxylase activity. The experimental design employed by these workers differed in that they isolated mitochondria in the presence of the uncoupler DNP in order to grossly calcium-deplete the mitochondria.

These calcium depleted mitochondria had very low levels of 1-hydroxylase activity when incubated in medium containing 0.5 mM EGTA. However, 1-hydroxylase activity was maximally stimulated at an added Ca^{++} concentration of 0.2 mM. Increasing Ca^{++} beyond this concentration led to an inhibition of enzyme activity.

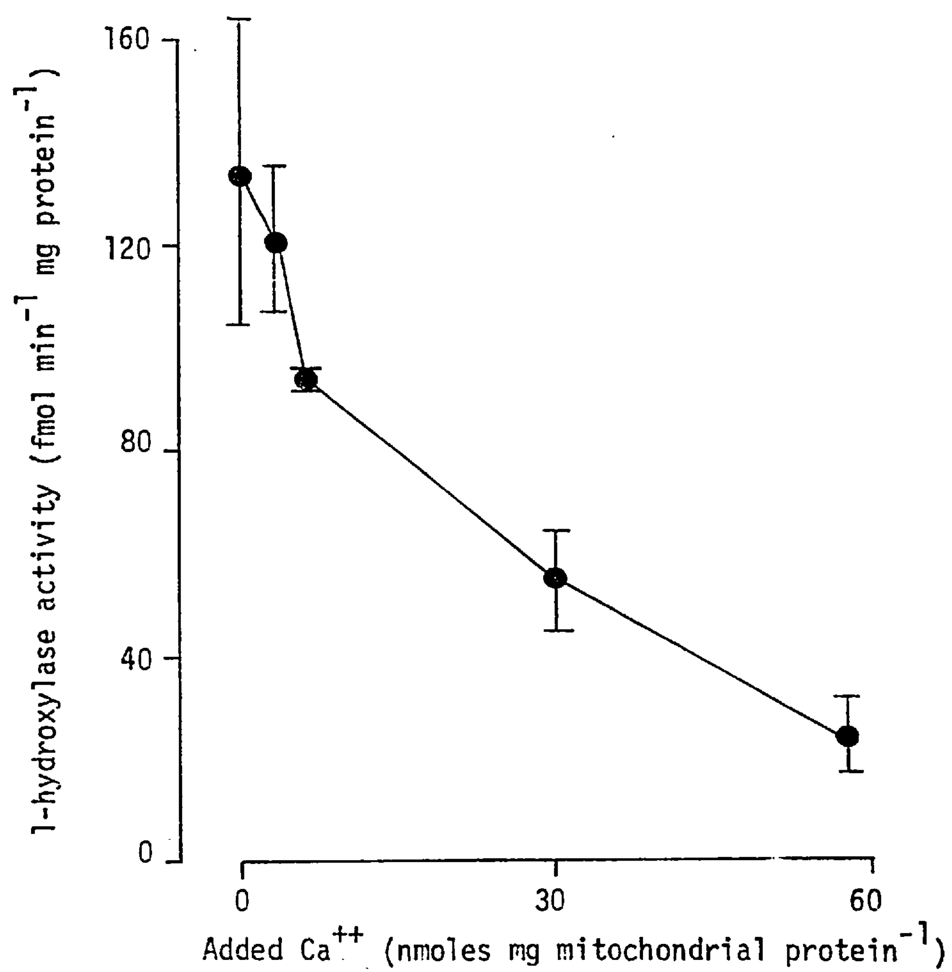


Fig. 41. Effect of Ca^{++} on 1-hydroxylase activity.

The experiment described by Horiuchi was repeated to test the hypothesis that a very low concentration of intra-mitochondrial calcium may be required for 1-hydroxylase activity.

Experimental protocol:

Kidney tissue was removed from 20 2-week old vitamin D deficient chicks. The tissue was homogenized in 100 ml of the Tris-acetate buffer containing 0.2% bovine serum albumin (B.S.A.) and 2 mM EGTA. The homogenate was centrifuged at 500 g at $4^{o}C$ for 10 min. The supernatant was then centrifuged at 9000 g for 10 min. The mitochondria were resuspended in Tris-acetate buffer supplemented with 10 mM EGTA and divided into two equal parts. The mitochondria were resedimented by centrifugation at 10,000 g for 10 min. Half of the mitochondria ('DNP treated') were incubated for 5 min at room temperature in 50 ml of the Tris-acetate buffer supplemented with 10 mM KCl, 1 mM $MgCl_2$ 0.9 mM AMP, 3.2 mM EGTA, 0.1 mM Dinitrophenol and 0.1% B.S.A. The other half ('Control') were resuspended in the Tris-acetate buffer supplemented with 10 mM EGTA and 0.2% B.S.A. Both mitochondrial suspensions were resedimented by centrifugation at 10,000 g for 10 min. Finally, both mitochondrial sediments were resuspended in Tris-acetate buffer alone and resedimented. Each pellet was then suspended in 5 ml of Tris-acetate buffer containing 1 mg/ml B.S.A.

The incubation conditions were as previously described.

DNP treated mitochondria were incubated at $37^{o}C$ in the incubation medium supplemented with either 2 mM EGTA, 30 μM $CaCl_2$,

60 μM $CaCl_2$ or with no additions. Control mitochondria were incubated in the presence or absence of 60 μM $CaCl_2$. 1-hydroxylase activity was assayed as previously described.

DNP treatment alone markedly inhibited 1-hydroxylation (Table 27) and DNP treated mitochondria incubated in the presence of 30 μM or 60 μM had even lower levels of enzyme activity. Thus Ca^{++} did not stimulate 1-hydroxylase activity in DNP treated mitochondria.

Respiration studies showed that oxygen uptake by DNP treated mitochondria was markedly depressed compared with that of control mitochondria.

Thus it is likely that DNP treatment leads to some loss of mitochondrial integrity. Horiuchi and co-workers do not comment in any detail as to the effect of this drastic treatment on the integrity of their mitochondria. This group also did not attempt to determine the effect of added calcium on the integrity of these mitochondria, but it is of interest that their mitochondria are capable of accumulating a maximum of approximately 18 nmoles of calcium per mg of mitochondrial protein. Carafoli (1975) has reported that heart mitochondria incubated in the absence of phosphate ions are normally capable of accumulating up to 150 nmoles calcium per mg protein and in the presence of phosphate ions up to 2-3 μmoles. As shown in Fig. 37c, kidney mitochondria isolated by the procedure described in the methods section of this chapter and incubated in the absence of phosphate ions, are capable of accumulating up to 200 nmoles calcium per mg protein before a disturbance of respiration is seen. Addition

TABLE 27

Effect of DNP treatment on 1-hydroxylase activity

a) DNP treated mitochondria

Additions	1-hydroxylase activity (% control)
--	51.38
2 mM EGTA	28.11
30 μM $CaCl_2$	7.21
60 μM $CaCl_2$	1.97

b) Control mitochondria

Additions	
--	100
60 μM $CaCl_2$	6.91

DNP treated mitochondria were prepared as described in section 4.5 of the text. The incubation medium contained 15 mM Tris-acetate pH 7.4, 1.9 mM $MgCl_2$ and 5 mM Na succinate with the various additions listed above.

of uncoupler restores respiration, presumably by causing the release of accumulated calcium.

In vivo, it is improbable that Ca^{++} can be accumulated by mitochondria without a simultaneous uptake of phosphate ions (Carafoli et al., 1975). Thus a study of the inhibition of the hydroxylase by Ca^{++} ions in the presence of PO_4 may be a realistic guide to the nature of any direct regulation by calcium.

4.6 The effect of Ca^{++} and PO_4 on 1-hydroxylase activity

The inhibition of enzyme activity with low concentrations of Ca^{++} was found to be modified, but not completely reversed, when PO_4 was present in the incubation medium (Fig.42). Higher levels of PO_4 alone had an inhibitory effect on the enzyme (Fig.43).

5. Discussion

The results described in this chapter confirm the sensitivity to calcium of the renal 1-hydroxylase enzyme in isolated renal mitochondria. Low concentrations of calcium in the incubation medium cause a marked decrease in enzyme activity. That this calcium inhibition is not due merely to loss of mitochondrial integrity is shown by the studies of mitochondrial respiration in the presence of calcium. Furthermore, the studies with ruthenium red and La^{3+} suggest that it is an increase in intra-mitochondrial calcium levels, or some consequence of mitochondrial calcium uptake, which is responsible for the inhibition. These inhibitors of mitochondrial calcium uptake reverse the inhibition of enzyme activity by calcium, and have been shown to prevent the uptake of calcium by these mitochondria as assessed by

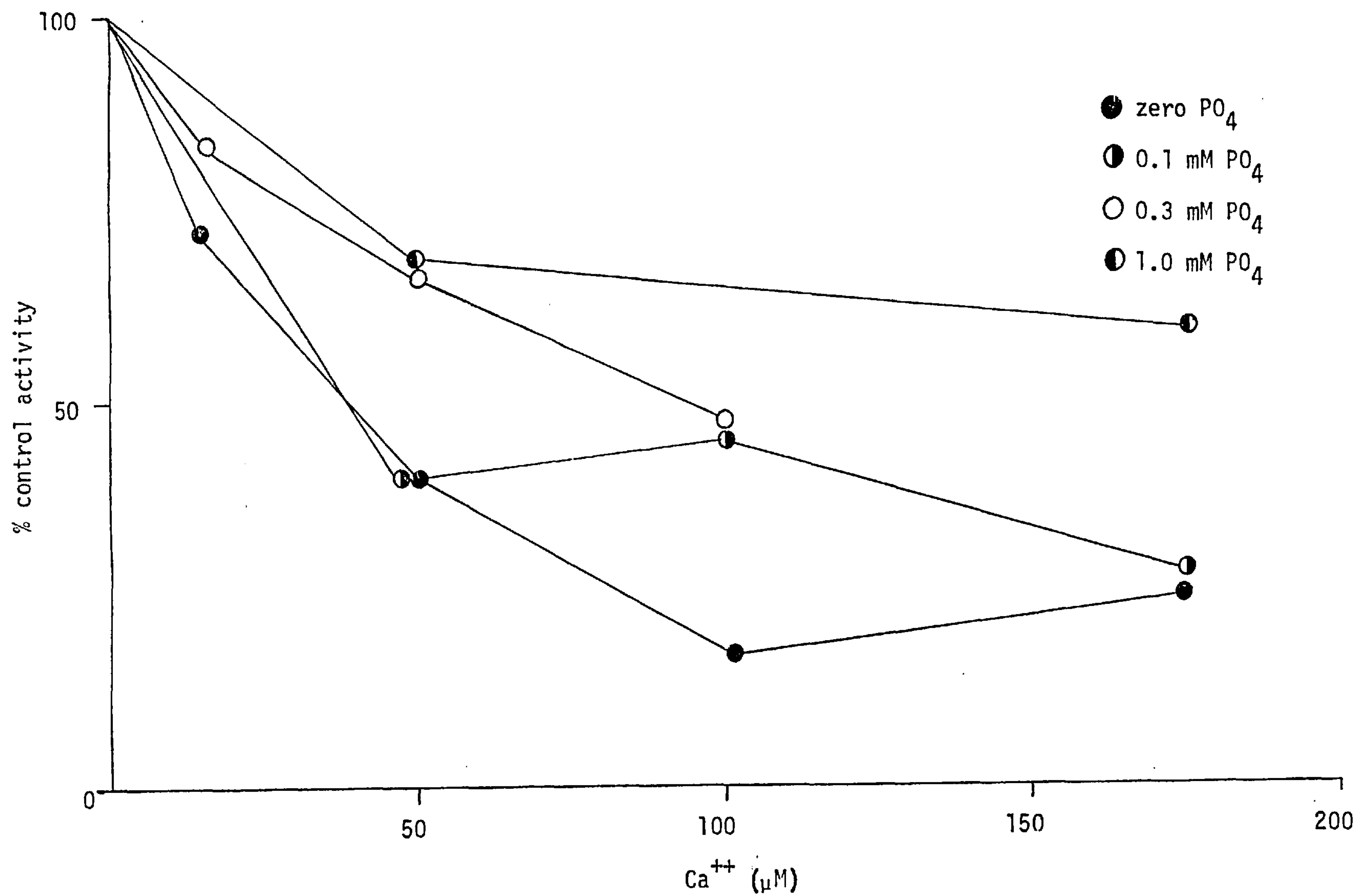


Fig. 42. Effect of Ca^{++} and PO_4 on 1-hydroxylase activity. Enzyme activities are expressed as a percentage of control. Zero Ca^{++} for each PO_4 concentration equals 100. Each point is the mean of results from two separate experiments.

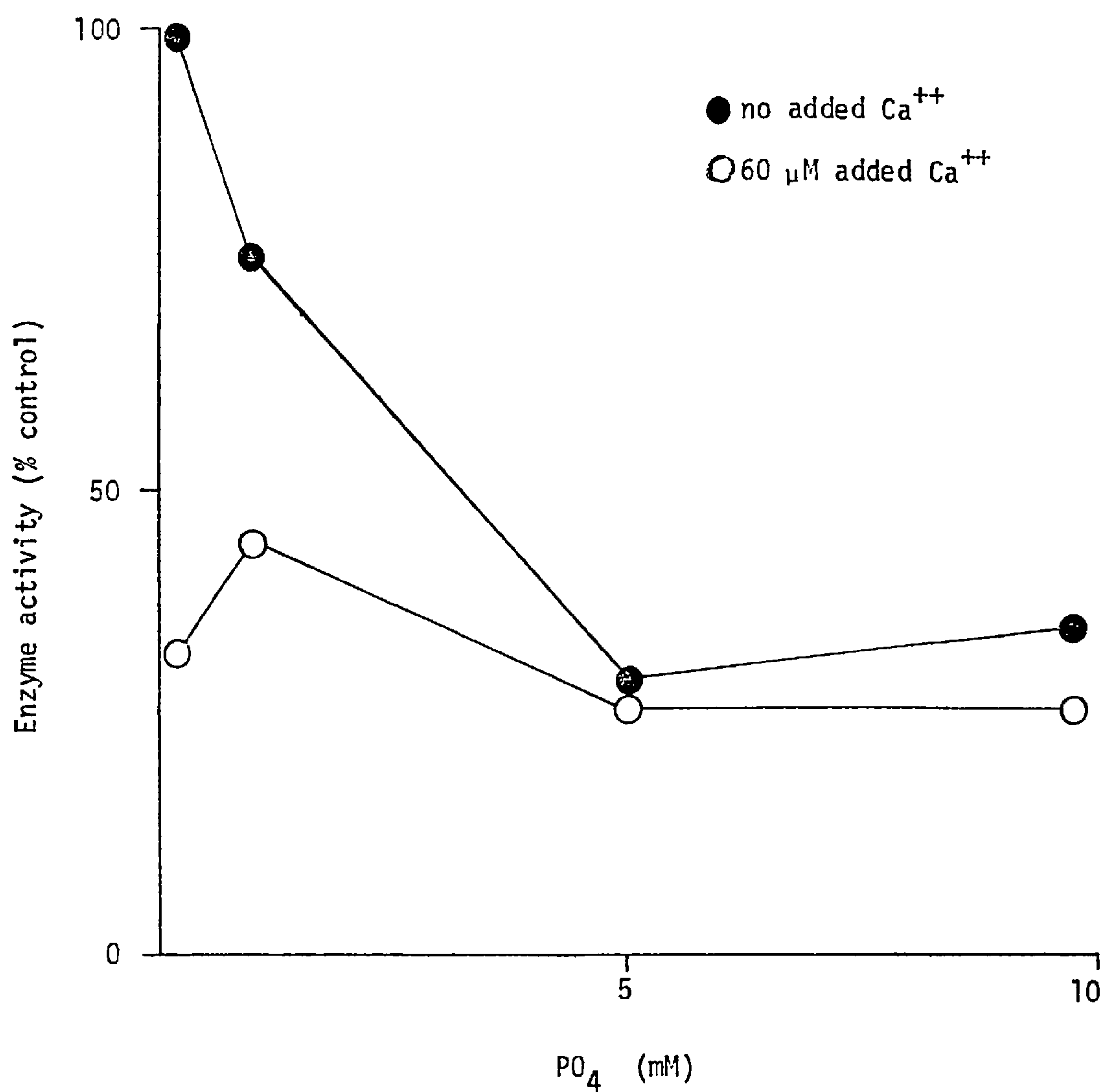


Fig. 43. Effect of PO_4 on 1-hydroxylase activity in the presence and absence of Ca^{++}. Activities are expressed as percentage of control (zero Ca^{++}, zero PO_4) levels.

respiration studies. Moreover, the total calcium load of these mitochondria following uptake of these low concentrations of calcium is likely to be in the accepted physiological range, and this might suggest that 1-hydroxylase activity is regulated by changes in intracellular or intra-mitochondrial levels of this ion.

However, in vivo it is improbable that calcium can be accumulated by mitochondria without a simultaneous uptake of phosphate. Thus the results of the studies of the inhibition of the hydroxylase enzyme by calcium ions in the presence of phosphate may be a more realistic guide to the nature of any direct regulation by calcium ions. Simultaneous uptake of Ca^{++} and PO_4^{3-} may result in storage of some of the Ca^{++} in an insoluble form that is unable to interact with the enzyme and so the inhibition is relieved. Another explanation could be some interaction between PO_4^{3-} ions and the 1-hydroxylase enzyme that lessens the sensitivity to calcium. Whatever the mechanism, 1-hydroxylase activity seems to be buffered against wide fluctuations in calcium ions by the presence of phosphate. Since the activity of this enzyme is inhibited by PO_4^{3-} in the absence of Ca^{++}, it is likely that this ion can interact with the enzyme molecule directly. As phosphate ions can be accumulated by mitochondria in the absence of Ca^{++}, it may be that this anion can have a regulatory role in addition to that of buffering Ca^{++} inhibition.

In view of the reports of the varied effects of Mg^{++} and K^{+} on calcium transport by mitochondria isolated from different tissues, the studies with these ions are more difficult to interpret. There have been few studies on the kinetics of calcium transport in renal mitochondria, and until the effects of Mg^{++} and K^{+} on calcium uptake by these mitochondria have been established, few conclusions can be

drawn as to the possible physiological regulation of 1-hydroxylase activity by changes in cytosolic concentrations of these ions.

If intra-mitochondrial Ca^{++} concentration is to be an efficient short term regulator of 1-hydroxylase activity *in vivo*, accumulated Ca^{++} must be stored in a form which can be rapidly mobilized. Carafoli and colleagues (1976) have demonstrated that endogenous Ca^{++} can be rapidly released from heart mitochondria *in vitro* on addition of the uncoupler, DNP. Other recent reports have suggested that prostaglandins and sodium ions can induce Ca^{++} release from mitochondria but results vary according to the tissue source of the organelles. Whether or not this effect can occur *in vivo* remains to be established, but these observations suggest that most of the endogenous Ca^{++} is not stored within mitochondria as an insoluble salt and hence can be rapidly mobilized, and that 'physiological' agents can induce the release of Ca^{++} from mitochondria. Whether such agents can release accumulated Ca^{++} from renal mitochondria and, further, whether release of accumulated Ca^{++} can reverse the inhibition of 1-hydroxylase activity remains to be established.

In summary, the experiments described in this chapter represent a preliminary investigation of the possible role of intra-mitochondrial calcium levels in the acute regulation of 1-hydroxylase activity. However, before any such regulatory role can be established, more rigorous studies are needed, particularly with regard to the kinetics of calcium transport in renal mitochondria, the influence of other ions, especially Mg^{++} and K^{+}, on this transport system and the reversibility of this process by 'physiological' agents.

CHAPTER EIGHT

GENERAL DISCUSSION

The rapid advances made in the knowledge of vitamin D have meant that cholecalciferol must now be regarded as a prohormone, the inactive precursor of a potent steroid hormone, 1,25-dihydroxycholecalciferol, which is secreted by the kidney. The overall action of this hormonal form of vitamin D is the maintenance of calcium and phosphate concentrations in plasma and extra-cellular fluids and the promotion of normal calcification of bone. Thus, this active steroid can be considered, along with parathyroid hormone and calcitonin, as one of the three main hormone systems involved in the control of calcium homeostasis. Intracellular calcium concentration plays a crucial role in a number of vital cellular mechanisms such as muscle contraction, the modulation of enzyme activity, the propagation of nervous impulses and the exocytosis of hormones and neural transmitters. The control of cytosolic levels of this ion relies in part upon the maintenance of a constant extracellular concentration of calcium. This is achieved by a complex interaction between parathyroid hormone, calcitonin and 1,25 $(OH)_2D_3$.

In the space of a decade, 1,25 $(OH)_2D_3$ has achieved the status of a potent calcium regulating hormone and the discovery that the kidney is the sole site of production of this steroid has revealed a further endocrine function of this organ. The kidney also produces a less active metabolite, 24,25-dihydroxycholecalciferol, and alters its production of

active and inactive metabolite in an appropriate manner to maintain the required mineral content of the body.

The regulatory mechanism has not been fully elucidated, but it is likely that the kidney responds to two main types of regulatory factors, short term or acute regulators which act within minutes, and long term regulators which take hours or days to produce their effects. The studies with isolated kidney mitochondria described in this study strongly suggest that the 25 OH D_3-1-hydroxylase enzyme is sensitive to the level of calcium within the mitochondria. Thus, 1-hydroxylase activity is inhibited over a range of intra-mitochondrial calcium concentrations which are likely to be within the physiological range *in situ*. Mitochondria actively accumulate calcium and by this mechanism the 1-hydroxylase enzyme might sense fluctuations in cytosolic calcium concentrations within the renal tubule cell, thus providing a short term means of control.

It seems likely that feedback regulation is an important factor in the long term control of vitamin D metabolism. Thus, 1-hydroxylase activity is stimulated within days of dietary vitamin D restriction. It is unlikely that this enhancement of enzyme activity is secondary to increased parathyroid hormone secretion since, over this short period of time, plasma calcium concentration is unchanged. Further, removal of the parathyroid and ultimobranchial glands does not inhibit the high levels of 1-hydroxylase activity seen in vitamin D deficiency providing steps are taken to prevent gross changes in plasma calcium and phosphate levels.

A physiological dose of vitamin D_3 given to vitamin D deficient chicks produces a change in the pattern of renal enzyme activities. 1-Hydroxylase activity is inhibited and there is a concomitant appearance of 24-hydroxylase activity. More rapid effects are seen with large doses of the hormonally active form, 1,25 $(OH)_2D_3$. This action is not simply one of product inhibition, rather it depends on a nuclear action of the steroid involving changes in total gene transcription. Pretreatment with transcriptional inhibitors prevents the changes in renal hydroxylase activities in response to 1,25 $(OH)_2D_3$. Furthermore, treatment with 1,25 $(OH)_2D_3$ has a rapid effect on renal nuclear RNA polymerase II activity, the enzyme involved in the synthesis of messenger RNA. Although, of necessity, large doses of the steroid were used to ensure a rapid effect on renal hydroxylase activities when using these potent transcriptional inhibitors, it would seem reasonable to postulate that this feedback mechanism may be important physiologically. Such a regulatory process would allow the kidney to maintain a constant production of 1,25 $(OH)_2D_3$ in the face of large variations in circulating 25 OH D_3 levels such as would be expected to occur with dietary vitamin D restriction or with long exposure to sunlight.

It is interesting to reflect that, within the relatively short time since the discovery of 1,25 $(OH)_2D_3$ as the active metabolite of vitamin D, a number of once widely held views as to the regulation of the production of this potent calcium regulating hormone are now no longer tenable. Parathyroid hormone was once described as the 'essential trophic hormone for 1,25 $(OH)_2D_3$ production' (DeLuca, 1972). It is now clear that, although parathyroid hormone does influence vitamin D

metabolism in some or even most physiological settings, its role is not likely to be that of sole or single regulator.

Another once widely held view was that 1,25 $(OH)_2D_3$ production is inversely related to serum calcium concentration. This is clearly not the case in all physiological settings since 1-hydroxylase activity is enhanced under conditions where serum calcium is either unchanged (Fraser and Kodicek, 1973) or is elevated (Spanos et al., 1976b; Tanaka et al., 1976). The regulation of the renal metabolism of 25 OH D_3 is extremely complex and the view that 1,25 $(OH)_2D_3$ production is regulated solely by changes in parathyroid hormone secretion secondary to changes in serum calcium is clearly an over-simplification.

Although the effect of a low dietary calcium intake on 1,25 $(OH)_2D_3$ production has been well documented (Boyle et al., 1971; Haussler, Baylink, Hughes, Brumbaugh, Wergedal, Shen, Nielsen, Counts, Bursac and McCain, 1976), until very recently little attention had been paid to other physiological states of calcium stress, such as pregnancy, lactation and growth, in which changes in vitamin D metabolism might play an important role. Recently, it has been reported that in each of these situations, circulating 1,25 $(OH)_2D_3$ levels are elevated (Pike, Toverud, Boass, McCain and Haussler, 1977) indicating that it may be this steroid hormone which is the physiological agent responsible for the observed changes in intestinal absorption of calcium and phosphorus.

Experimental evidence has indicated that both prolactin and growth hormone influence the metabolism of vitamin D_3 and that by this mechanism, these hormones may modulate calcium metabolism under physiological states of calcium stress. Prolactin enhances the production of

1,25 $(OH)_2D_3$ in the chick (Spanos et al., 1976a and 1976b), and there is also evidence that prolactin is of importance in mammals: lactating rats have a fourfold higher level of circulating 1,25 $(OH)_2D$ compared to their non-lactating counterparts (Boass, Toverud, McCain, Pike and Haussler, 1977). Other experiments have shown that when prolactin secretion in lactating rats is inhibited by administration of bromocryptine, a twofold decrease in circulating 1,25 $(OH)_2D$ levels is seen. Furthermore, administration of ovine prolactin (MacIntyre, Colston, Robinson and Spanos, 1977) reverses this effect of bromocryptine. These results implicate prolactin as a regulator of 1,25 $(OH)_2D$ production but do not allow any conclusions to be drawn as to whether this effect of prolactin is a direct one or whether some intermediary factor is involved.

The similarities in sequence between prolactin and growth hormone suggest that these effects of prolactin might also be seen with growth hormone. Plasma 1,25 $(OH)_2D$ levels are increased in the young hen compared to its adult non-laying counterpart. Further, hypophysectomized rats have circulating 1,25 $(OH)_2D$ levels which are twofold lower than those of sham operated controls, and administration of human growth hormone can restore plasma 1,25 $(OH)_2D$ to control levels (MacIntyre et al., 1977). Such an action of growth hormone on 1,25 $(OH)_2D$ production would account for the enhanced intestinal absorption of calcium during growth. It is possible that this effect is also the basis of the disturbances in calcium metabolism seen in acromegaly (Hanna, MacIntyre, Harrison, and Fraser, 1961) and the observation that growth hormone stimulates intestinal calcium absorption in the growth hormone deficient dwarf (Beck, McGarry, Dyrenfurth and Venning, 1957).

There is now some experimental evidence to implicate sex hormones as modulators of vitamin D metabolism. Oestrogens are reported to increase calcium absorption and serum calcium in birds (Simkiss, 1961) and Kenney has shown that 1-hydroxylase activity is increased during the reproductive period in the Japanese quail (Kenney, 1976). Thus, the increased levels of oestrogen which are seen prior to egg laying may be responsible for the observed increase in circulating 1,25 $(OH)_2D$ levels in the laying hen (Spanos et al., 1976b). There has been one report of an enhancement of 1-hydroxylase activity with combined oestrogen/testosterone treatment in birds (Tanaka et al., 1976). However, the doses of steroids given were very large (5 mg diethyl-stilbesterol plus 5 mg testosterone) and no corresponding measurements of circulating 1,25 $(OH)_2D$ levels were reported. Thus, these results should be treated with caution. It may be that oestrogen influences vitamin D metabolism in birds, but this effect may not be a direct one: no effect of oestrogen on 1-hydroxylase activity could be demonstrated in vitro (Spanos, Barrett and MacIntyre, 1977). Further, other steroids exert a similar effect to those of oestrogen in vivo: cortisol and corticosterone markedly stimulate 1-hydroxylase activity in chicks (Spanos, Colston and MacIntyre, 1977).

Future considerations

Research into the physiological regulation of vitamin D metabolism has, until recently, been hampered by the lack of a suitably sensitive assay for 1,25 $(OH)_2D$. The difficulty in measuring circulating levels of the active metabolite of vitamin D has meant that experiments designed to investigate the regulation of vitamin D metabolism have been performed in

vitamin D deficient animals in order to detect the low levels of conversion of radioactive tracer to more active metabolites.

In 1974, Brumbaugh and colleagues described a competitive protein binding assay for 1,25 $(OH)_2D$ levels in plasma, utilizing a chick intestinal receptor system (Brumbaugh, Haussler, Bressler and Haussler, 1974; Brumbaugh, Haussler, Bursac and Haussler, 1974). Using this assay this group have reported a normal range of 3-5 ng/dl in adults. Hypoparathyroid patients had 1,25 $(OH)_2D$ levels which were slightly lower than normals, whereas hyperparathyroid subjects tended to have higher levels of the active metabolite (Haussler et al., 1976). However, this assay has a number of important disadvantages. Firstly, relatively large volumes (up to 20 ml) of plasma are required to allow for the 50-70% losses incurred during the chloroform extraction procedure and the three chromatographic steps which are necessary to separate 1,25 $(OH)_2D$ from 25 OH D as the cytosol-chromatin receptor has some affinity for 25 OH D. Secondly, the cytosol-chromatin receptor is unstable and must be freshly prepared immediately prior to use. Lastly, the necessity for three chromatographic steps for each plasma sample means that assays are laborious to perform, the length of time for analysis is long and the number of samples which can be processed at one time is limited.

A somewhat simplified assay procedure has been reported (Eisman, Hamstra, Kream and DeLuca, 1976). By the use of high pressure liquid chromatography, only one chromatographic step is required to separate 1,25 $(OH)_2D$ from 25 OH D. A modified method is also used for the isolation of the intestinal receptor, with the added advantage that this receptor system can be stored for several weeks. However, relatively large volumes of plasma are required for this assay also, which makes its application to studies using small experimental animals unsuitable.

The chief disadvantage of both these competitive protein binding assays for 1,25 $(OH)_2D$ is the necessity for one or more chromatographic step to separate the active metabolite from 25 OH D. This chromatographic step is essential since the intestinal receptor system exhibits appreciable binding to 25 OH D.

Although the interaction of 1,25 $(OH)_2D$ with the cytosol protein is greater than that of 25 OH D, since circulating 25 OH D levels are more than 100 times that of 1,25 $(OH)_2D$, erroneously high results would be obtained if unseparated plasma extracts were assayed. A greatly simplified assay could be achieved if a specific antibody to 1,25 $(OH)_2D$ were available which exhibited negligible binding to 25 OH D. However, as yet attempts to raise such an antibody have met without success. In the next decade, the application of a specific assay for circulating 1,25 $(OH)_2D$ may prove to be the most important approach to the advancement in our knowledge of vitamin D metabolism and its regulation under physiological situations such as pregnancy, lactation and growth and in pathological states.

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ACKNOWLEDGEMENTS

I wish to thank Professor Iain MacIntyre for his advice, guidance and encouragement throughout this study.

I am grateful to Drs. Leonora Galante and Imogen Evans for their collaboration during the early part of this study.

I should also like to thank Professor D.W. Moss for his helpful suggestions and Dr. Peter Butterworth of the Department of Biochemistry, Chelsea College for his help and advice on the preparation of kidney mitochondria.

I am very grateful to Mr. Eric Matthews who devised the separation system for vitamin D metabolites on high pressure liquid chromatography.

I wish to thank the Medical Research Council and the Wellcome Trust who provided financial support for this work.

I am very grateful to Mrs. Brenda Salvage for her help in finalising and typing the manuscript.

Finally, I wish to thank my husband and all friends at the Royal Postgraduate Medical School for their help and encouragement throughout this study.

Molecular and Cellular Endocrinology 5 (1976) 163–167
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EFFECT OF PROLACTIN ON VITAMIN D METABOLISM

E. SPANOS, Kay W. COLSTON, Imogen M.S. EVANS, Leonora S. GALANTE, S.J. MACAULEY and I. MACINTYRE

Endocrine Unit, Royal Postgraduate Medical School, Ducane Road, London W12 OHS, U.K.

Received 3 March 1976

The effect of ovine prolactin on the renal 25-hydroxycholecalciferol-1-hydroxylase was studied in the chick. Prolactin was found to increase the activity of this enzyme in both long-term and short-term experiments. In the long term, 7 days treatment with prolactin caused a marked stimulation of the 1-hydroxylase activity, however this effect was only seen when the enzyme was assayed 2–3 hours after the final injection of prolactin. A single subcutaneous injection of prolactin was also effective in increasing the 1-hydroxylase activity, this effect was maximal at one hour and had largely disappeared 3 hours after prolactin administration.

Keywords: calcium absorption; 1,25-dihydroxycholecalciferol; 25-hydroxycholecalciferol-1-hydroxylase.

Vitamin D_3 (cholecalciferol) is hydroxylated in the liver and other tissues to the major circulating form, 25-hydroxycholecalciferol (25-OH-D_3) (Blunt et al., 1968). A further hydroxylation takes place in the kidney to produce the active form of the vitamin, 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$ D_3) (Fraser and Kodicek, 1970). 1,25-$(OH)_2$ D_3 is now regarded as a steroid hormone which controls the absorption of calcium and possibly phosphorus from the diet (Kodicek, 1972). Vitamin D_3 itself (Galante, et al., 1973; Evens et al., 1975), parathyroid hormone (Garabedian et al., 1972), and the calcium and phosphorus content of the diet (Boyle et al., 1971) are each thought to be important in the control of 1,25-$(OH)_2$ D_3 production by the kidney. However, the mechanism by which these factors influence the activity of the renal 25-hydroxycholecalciferol 1-hydroxylase enzyme, which converts 25-OH D_3 to 1,25-$(OH)_2$ D_3 has not been fully elucidated. In addition, none of the regulatory factors mentioned account for the increased intestinal calcium absorption seen in certain physiological settings. Thus, the mechanism for the marked increase in calcium and phosphate absorption during pregnancy and lactation (Horrobin, 1974) remains unexplained and it is also unclear why vitamin D requirements fall in pregnant hypoparathyroid patients (Evans, unpublished observations). It would seem reasonable that hormonal changes during pregnancy might af-

fect the metabolism of vitamin D and we now wish to present experimental evidence showing that prolactin markedly enhances the production of 1,25-$(OH)_2$ D_3 by the kidney.

MATERIALS AND METHODS

Animals

One-day-old chicks, Light Sussex–Rhode Island Red cross breed were maintained on a vitamin D-deficient diet (0.34% Ca, 0.54% P).

Materials

Ovine prolactin (25–35 IU/mg) was obtained from Sigma Chemicals, St. Louis, MO, USA. 25-Hydroxycholecalciferol (25-OH D_3), [26-(27)-*Me*-^{3}H]25-hydroxycholecalciferol 9 Ci/mmol was obtained from the Radiochemical Centre, Amersham, England.

Methods

Prolactin was given subcutaneously in 0.1 ml 0.9% sodium chloride adjusted to pH 8.6 with sodium hydroxide either every 12 hours throughout the duration of the experiment, or as a single dose. Blood was withdrawn by cardiac puncture after prolactin administration and the chicks were killed by decapitation. Control animals received the injection vehicle only. In some experiments chicks received a sub-optimal maintenance dose of vitamin D_3 (5 IU/day) administered into the crop in 0.1 ml arachis oil.

Preparation of kidney homogenates, assay of the 25-OH D_3-1-hydroxylase enzyme, and measurement of homogenate protein content were as previously described (Lowry et al., 1951; Colston et al., 1973; Galante et al., 1973).

RESULTS

Both long-term and short-term (acute) experiments were performed. In the first series of long-term experiments the chicks were divided into two main groups, both groups were maintained on a vitamin D-deficient diet but in addition one group received a sub-optimal maintenance dose of vitamin D_3 (5 IU D_3/day). Each of these groups was subdivided into two further groups; control and prolactin-treated. The prolactin-treated groups received 35 μg prolactin every 12 hours for seven days while the control birds received the injection vehicle only. The chicks were killed 2–3 hours after the final injection of prolactin. Prolactin administration markedly increased the renal 25-OH D_3-1-hydroxylase activity in both the vitamin D-deficient and in the vitamin D-supplemented birds and this increase is highly significant ($P < 0.01$) (table 1).

Table 1
Effect of prolactin on 25-OH D_3-1-hydroxylase activity in chick kidney homogenates. Enzyme activity was assayed following 7 days of prolactin treatment (35 μg every 12 hours). All the chicks were kept on a vitamin D-deficient diet for 7 days and, in addition, the vitamin D-supplemented group received a submaintenance dose of 5 IU D_3 daily.

Group	Treatment	No. of birds	25-OH D_3-1-hydroxylase activity (fmol/min/mg protein ± S.E.M.)	
Vitamin D-deficient	Control	7	99.39 ± 13.84	$P < 0.001$
	Treated *	4	188.20 ± 6.19	
Vitamin D-supplemented	Control	8	37.77 ± 4.41	$P < 0.001$
	Treated *	8	69.44 ± 3.53	

* 35 μg prolactin every 12 h.

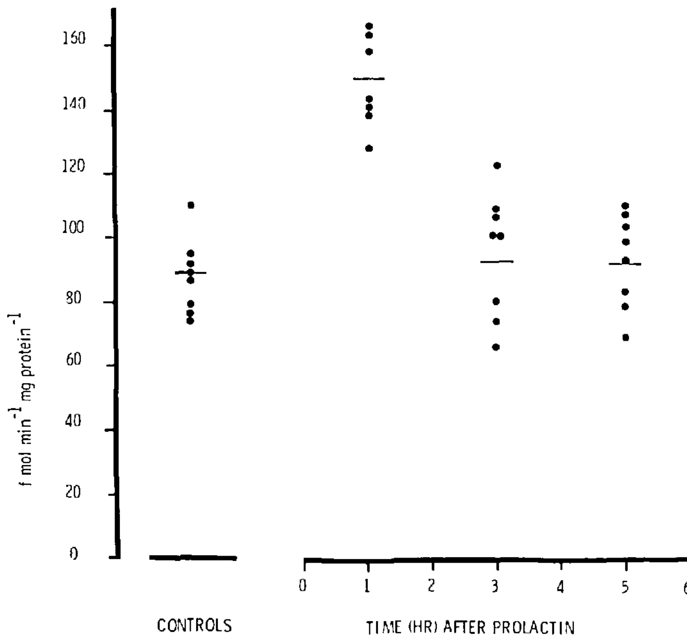

Fig. 1. Shows the levels of 25-OH D_3-1-hydroxylase enzyme in chick kidney homogenates following a single subcutaneous injection of 70 μg prolactin. The chicks were killed at 1, 3 and 5 hours after administration of the hormone. The control levels represent the enzyme levels obtained for birds receiving the injection vehicle only but killed at the same times as the chicks in each separate experimental group. Each point represents a single homogenate and the solid bar the mean for each group.

Table 2
Effect of different doses of prolactin on 25-OH D_3-1-hydroxylase in chick kidney homogenates. Experiment 1. All birds were maintained on a vitamin D-deficient diet for 7 days and received 5 IU D_3 daily. The 25-OH D_3-1-hydroxylase activity was measured in the homogenate 1 hour after the administration of prolactin.

Treatment (μg protactin/bird)	No. of birds	25-OH D_3-1-hydroxylase activity (fmol/min/mg protein ± S.E.M.)
0	5	54.01 ± 7.36
10	5	82.45 ± 15.52
50	5	119.40 ± 7.29
250	5	104.30 ± 7.28

In the second series of long-term experiments chicks maintained on a vitamin D-deficient diet were treated with a large dose of prolactin (150 μg every 12 hours for seven days). These birds were killed 18 hours after the last injection of prolactin. In this situation there was no marked stimulation of 25-OH D_3-1-hydroxylase activity following prolactin administration.

In the first of the acute experiments a single dose of 70 μg prolactin was given subcutaneously and the chicks were killed after 1, 3 and 5 hours. This single dose also markedly increased 25-OH D_3-1-hydroxylase activity (fig. 1). This increase was maximal at 1 hour but had disappeared 3 hours after prolactin administration. Bearing this in mind, a dose–response experiment was performed at the 1-hour time. In this experiment all the birds were maintained on a vitamin D-deficient diet for seven days and received 5 IU vitamin D_3 daily. Three doses of prolactin were used: 10, 50 and 250 μg per bird, given as a single injection. As shown in table 2, 10 μg of prolactin caused a marked increase in 25-OH D_3-1-hydroxylase activity after 1 hour, and this response appeared to be maximal at 50 μg.

DISCUSSION

These experiments show that prolactin causes a marked stimulation in the activity of the 25-OH D_3-1-hydroxylase enzyme in chick kidney. This response was seen both in the acute situation and with longer term prolactin treatment. It was also noted that more prolonged administration of the hormone produced an effect on the activity of the renal enzyme which persisted for longer than that produced by a single injection.

Care must be taken in the interpretation of these results, since an action of ovine prolactin in chicks may not necessarily reflect an action of the hormone in mammals. Prolactin is known to have a wide range of action in various species which may not be represented in others (Nicoll, 1974). These include teleost sodium-retaining activity, the EFT water-drive-stimulating activity, pigeon-crop-stimulating

activity, luteotropic activity and mammary-secretion-stimulating activity.

However the chick has proved to be a good model for the study of the major factors controlling vitamin D metabolism. Using chicks vitamin D and its active metabolites (Galante et al., 1973; Evans et al., 1975) have been shown to have a marked effect on the renal metabolism of 25-OH D_3. It has also been demonstrated in the chick that parathyroultimobranchialectomy per se does not influence renal 25-OH D_3-1-hydroxylase activity and this lack of effect has been confirmed in mammals. Thus, the thyroparathyroidectomised rat is able to increase its production of 1,25-$(OH)_2$ D_3 in response to a low dietary calcim (Larkins et al., 1973) and similarly the parathyroidectomixed pig can increase intestinal calcium absorption when challenged with a hypocalcaemic stimulus (Swaminathan et al., 1974).

Therefore, extrapolation to mammals of this action of prolactin on vitamin D metabolism seems reasonable and may well explain the marked increase in calcium and phosphorus absorption seen in late pregnancy and lactation, when plasma prolactin levels are high. This hypothesis cannot be finally confirmed or refuted until a plasma assay for 1,25-$(OH)_2$ D_3 in man becomes generally available, but our results, whatever their precise interpretation, document an important addition to the already formidable list of the actions of prolactin.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Wellcome Trust and Medical Research Council. IMAE is in receipt of a Medical Research Council Clinical Research Fellowship.

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Life Sciences Vol. 19, pp. 1751-1756, 1976. Pergamon Press
Printed in the U.S.A.

CIRCULATING 1α,25-DIHYDROXYVITAMIN D IN THE CHICKEN: ENHANCEMENT BY INJECTION OF PROLACTIN AND DURING EGG LAYING

E. Spanos, J. W. Pike*, M. R. Haussler*, K. W. Colston,
I.M.A. Evans, A. M. Goldner*, T. A. McCain*, and I. MacIntyre

Endocrine Unit
Royal Postgraduate Medical School
Ducane Road, London W12 OHS

and

*Departments of Biochemistry and Physiology
University of Arizona College of Medicine
Tucson, Arizona 85724

(Received in final form October 22, 1976)

Summary

In order to investigate possible modulation of vitamin D metabolism by prolactin, circulating 1α,25-dihydroxyvitamin D (1α,25-$(OH)_2$D) was measured by radioreceptor assay in chicks given injections of prolactin for five days. At a dose of 100 μg/day, the lactogenic hormone elicited a two-fold increase in plasma 1α,25-$(OH)_2$D. This effect may explain the known action of prolactin in producing hypercalcemia and could be physiologically important in birds. The laying hen represents a physiologic state in which calcium absorption is known to be stimulated and prolactin has been reported to be elevated. Assay of serum 1α,25-$(OH)_2$D in the laying hen demonstrates a nine-fold enhancement over non-laying controls. Since this marked increase during egg laying is at least partially mimicked by injecting prolactin, a possible causative relationship between elevated prolactin and 1α,25-$(OH)_2$D is suggested.

Prolactin is known to exert a significant effect on calcium metabolism (1,2). The hormone produces hypercalcemia in rats (3) and chicks (4). No satisfactory hypothesis to explain this has been proposed until recently when Spanos et al. (5) found that prolactin stimulates renal 25-hydroxyvitamin D-1α-hydroxylase. Since this enzyme produces 1α,25-dihydroxyvitamin D (1α,25-$(OH)_2$D)[1], the active calcemic sterol, it was suggested that this effect of prolactin on vitamin D metabolism could be responsible for the influence of the lactogenic hormone on calcium homeostasis (5). To fully document this action of prolactin, the amplification of the renal enzyme must be shown to produce an increase in the total circulating concentration of 1α,25-$(OH)_2$D. We here report studies in the chicken using a specific assay for 1α,25-$(OH)_2$D that prolactin elicits a pronounced increase in the plasma level of this sterol hormone. Moreover there is a possible correlation between this observation and a known

[1] Abbreviation used: 1α,25-$(OH)_2$D, 1α,25-dihydroxyvitamin D.

physiologic state of calcium modulation in birds, namely, egg laying. Estrogens, known to be increased prior to and during reproduction (6,7), stimulate the release of prolactin in mammals (8,9). Since both prolactin and serum calcium levels are reported to be elevated in the laying hen, it is reasonable to suppose that the 25-hydroxyvitamin D-1α-hydroxylase activity might be enhanced, and the circulating level of 1α,25-$(OH)_2D$ may be elevated. Kenny has recently shown that 25-hydroxyvitamin D-1α-hydroxylase activity is increased during the reproductive period in Japanese quail (10) and he and his associates (11) have reported a similar phenomenon in the egg laying hen. We therefore measured total circulating 1α,25-$(OH)_2D$ in laying hens and report that the level is strikingly enhanced compared to non-laying birds. It appears likely therefore that prolactin may mediate at least part of the alteration in vitamin D metabolism seen during egg laying.

Materials and Methods

Prolactin Injection Experiment. One day old male Light Sussex-Rhode Island Red chicks (120 animals) were kept two weeks on a vitamin D deficient diet (0.34% calcium, 0.54% phosphorus). During this time 50 IU of vitamin D_3 daily was injected into the crop. The chicks were then divided into three groups with the control group receiving vehicle and the treatment groups receiving injections of 10 μg or 50 μg of ovine prolactin (25 IU/mg, Sigma Chemicals, St. Louis, MO). The birds were injected subcutaneously twice daily for five days. Prolactin was prepared fresh before each injection. Three mg of hormone was dissolved with 300 μl of 2 mM NaOH and then diluted to 6 ml with physiologic saline. Blood was obtained by intracardial puncture 1 hr after the last injection. Plasma was prepared and pooled from 10 chickens and stored at -20° C until assay for 1α,25-$(OH)_2D$.

Laying Hen Experiment. Blood was drawn via brachial vein from the following three groups of normal White Leghorn chickens: Laying hens, their non-laying counterparts, and immature (approximately two months of age) hens. Laying hens contained an egg in the shell gland and records showed that the non-layers had not produced eggs for five weeks. Blood was collected and pooled from two to six animals per group. After clotting, serum was prepared and frozen until assayed for 1α,25-$(OH)_2D$.

Radioreceptor Assay of 1α,25-$(OH)_2D$. Ten to 20 ml of plasma or serum were processed for triplicate assay of 1α,25-$(OH)_2D$. After adding 1000 cpm of 1α,25-$(OH)_2[^3H]D_3$ (6 - 8 Ci/mmole) to quantitate hormone yields after purification, 1α,25-$(OH)_2D$ is extracted and isolated via a three step chromatography procedure (Sephadex LH-20, silicic acid, and microCelite) as detailed by Hughes et al. (12). Final yields range from 50 - 70% and 1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_2$ are not routinely resolved by these chromatographic procedures. Assays are carried out by competitive binding utilizing the chick intestinal receptor system as described by Brumbaugh et al. (13,14). This receptor has an equal affinity for binding 1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_2$ and therefore the assay measures total 1α,25-$(OH)_2D$ (12). The minimum sensitivity of the assay is 17 pg (nonradioactive 1α,25-$(OH)_2D_3$ standard was obtained from Dr. M. Uskokovic of Hoffmann-La Roche) and triplicate assays result in a 10 - 15% interassay variation. Preparation of the intestinal receptor system and the binding assay has been modified since the original reports by Brumbaugh et al. (13,14) as follows: Intestinal mucosa (2 g) from one rachitic chick is homogenized in 25 ml of 0.25 M sucrose in 0.05 M Tris-HCl (pH 7.4), 0.025 M KCl, 0.005 M $MgCl_2$. Cytosol fraction is obtained by centrifugation at 100,000 xg for 1 hr. Chromatin is prepared from crude nuclei (isolated from original homogenate by centrifugation at 1000 xg for 10 min) by homogenizing successively in one 25 ml portion of 0.8 mM EDTA, 25 mM NaCl, pH 8; one 25 ml portion of 1% Triton X-100, 0.01 M Tris-HCl, pH 7.5; and one 25 ml portion of 0.01 M Tris-HCl, pH 7.5. The chromatin is harvested by sedimentation

at 30,000 xg for 10 min after each wash. The entire chromatin pellet from 2 g of mucosa is reconstituted with half the cytosol fraction by homogenization to create a cytosol-chromatin receptor system for the competitive binding assay. The reconstituted homogenate is then forced through a 22 G needle. All operations are performed at 0 - 4° C and receptor system is prepared immediately prior to use. To each assay tube containing 1α,25-$(OH)_2[^3H]D_3$ and unlabeled sterol (dried together with a stream of nitrogen) is added 10 µl of distilled ethanol and 100 µl of reconstituted cytosol-chromatin system (containing about 100 µg DNA). The final concentration of 1α,25-$(OH)_2[^3H]D_3$ is 4.3 nM. After incubation for 30 min at 25° with vigorous shaking in a water bath, the quantity of labeled sterol bound to chromatin is determined by filtration. To each assay tube, 1 ml of cold 1% Triton X-100 in 0.01 M Tris, pH 7.5, is added and the entire mixture applied to a Gelman Type A/E glass fiber filter at very low vacuum. After 2 - 4 min the vacuum is increased to achieve uniform flow rates of <u>ca.</u> 1 ml/min and each of the filters is washed with 2 ml of 1% Triton X-100, 0.01 M Tris, pH 7.5. Following filtration, the filters are placed in liquid scintillation vials with 5 ml of methanol-chloroform (2:1, v/v). After 20 min the methanol-chloroform is evaporated and the sterols are solubilized in a standard toluene based cocktail and counted via liquid scintillation procedures.

Results and Discussion

The influence of prolactin injection on circulating 1α,25-$(OH)_2D$ is summarized in Table 1. These data demonstrate that prolactin, administered in a physiologic dose of 100 µg/day (J. Meites, personal commun.) is capable of doubling the circulating level of 1α,25-$(OH)_2D$, the hormonally active vitamin D metabolite. The 20 µg/day injection was apparently without effect. The present finding parallels our earlier experiments on 25-hydroxyvitamin D-1α-hydroxylase activity under similar treatment. In both vitamin D-deficient and vitamin D-supplemented birds 100 µg of prolactin for seven days was capable of eliciting a two-fold increase in the enzyme activity (5).

TABLE 1

Circulating 1α,25-$(OH)_2D$ in Prolactin Treated Chicks

Group	No. of Sets	No. of Birds Per Set	Plasma 1α,25-$(OH)_2D$ (ng/dl ± SD)
Control	4	10	15.9 ± 2.1
20 µg Prolactin/day	4	10	14.8 ± 2.5
100 µg Prolactin/day	4	10	28.6 ± 4.5*

*Significantly different from control, $P < 0.005$.

These findings may be important relative to calcium metabolism. The hypercalcemic effect of prolactin appears to be mediated through the action of 1α,25-$(OH)_2D$. As circulating levels of 1α,25-$(OH)_2D$ are elevated by prolactin or a consequence thereof, the transport of calcium into the extracellular fluid is enhanced. This is the result of the action of the sterol hormone on its receptors in intestine and possibly kidney. Measurement of serum calcium levels verified this notion since 100 µg of prolactin per day for seven days in chicks elevated plasma calcium from 8.0 to 9.1 mg% in D-withdrawn and 8.9 to 9.4 mg% in D-replete chicks.

To further investigate the possible activity of prolactin in birds we measured the circulating level of $1\alpha,25-(OH)_2D$ in immature and mature hens. The laying hen represents a biologic situation in which a calcium stress (i.e., eggshell formation) is imposed upon the animal. Therefore one might expect an increase in circulating $1\alpha,25-(OH)_2D$ as a result of altered demand for calcium. The results seen in Table 2 indicate a nine-fold enhancement of active vitamin D metabolite in laying birds over non-laying controls. It is probable that the augmented calcium absorption found in the laying hen is elicited by the action of this elevated $1\alpha,25-(OH)_2D$, but the results must be interpreted with caution since total $1\alpha,25-(OH)_2D$ was measured and estrogen is known to stimulate the synthesis of a number of plasma binding proteins. In addition, serum $1\alpha,25-(OH)_2D$ is also significantly increased in the young hen as compared to its older non-laying counterpart (Table 2), intimating that $1\alpha,25-(OH)_2D$ is also enhanced during the added calcium requirements of the active growing period. It should be noted that the circulating $1\alpha,25-(OH)_2D$ level in the three week old male chicks (Control - Table 1) was markedly higher than that observed in eight week old hens (Young hen - Table 2). This discrepancy could be accounted for by age, sex, or strain differences, but could also be explained by the environmental and dietary variations in the two experiments. The young male chicks were raised on a diet containing 0.34% calcium and received 50 IU of vitamin D_3 daily while the females were routinely obtained from a poultry farm and were maintained on a high calcium (1 - 3%) diet. Since a low calcium diet in rats has been shown to increase circulating levels of $1\alpha,25-(OH)_2D$ (15), the higher levels of sterol in the male birds could be accounted for by this phenomenon.

TABLE 2

Enhancement of Circulating $1\alpha,25-(OH)_2D$ in the Laying Hen

Group	No. of Sets	No. of Birds Per Set	Serum $1\alpha,25-(OH)_2D$ (ng/dl ± SD)
Non-laying Hen	4	3	2.0 ± 0.8
Laying Hen	5	2	17.4 ± 3.5*
Young Hen	4	6	4.2 ± 0.4*

*Significantly different from non-laying adult hens, $P < 0.005$.

Estrogens, which increase dramatically several days prior to laying, have long been known to increase the intestinal absorption of calcium and to elevate total serum calcium from ~ 10 mg% to 20 - 30 mg% (6,7). Recently, it has been demonstrated that injections of estrogen and testosterone act synergistically to increase the 25-hydroxyvitamin D-1α-hydroxylase activity in birds (16,17). In addition, ovariectomy in Japanese quail on normal calcium diets results in a loss in hydroxylating activity, though low calcium diets tend to stimulate the enzyme (18). These data suggest that estrogen may influence or modulate the conversion of 25-(OH)-D to $1\alpha,25-(OH)_2D$, though other factors may be involved. Since estrogens are known to stimulate the release of prolactin in mammals, they may influence circulating prolactin in birds, and explain both the increased 25-hydroxyvitamin D-1α-hydroxylase activity and the hypercalcemic effect of estrogen. Bolton _et al_. (19) have indicated that prolactin levels are high in laying hens and March and McKeown (20) have shown elevated prolactin levels in wild pigeons prior to ovulation. The present experiments extend previous findings in chickens of increased renal 25-hydroxyvitamin D-1α-hydroxylase

activity after prolactin administration (5) and during egg laying (11) to include a measurable enhancement of circulating $1\alpha,25$-$(OH)_2D$ hormone. The results, along with data from other studies suggest, but do not prove, that prolactin is an important modulator of the biosynthesis of $1\alpha,25$-$(OH)_2D$. Estrogen may accomplish its effects on calcium metabolism via prolactin influence on $1\alpha,25$-$(OH)_2D$, although a direct action of estrogenic hormones on the kidney 25-hydroxyvitamin D-1α-hydroxylase is also possible. Conversely, since prolactin has been shown to alter vitamin D metabolism only in intact animals, its operation could be mediated by some other hormonal factor. Regardless, it is likely that prolactin should be added to the list of known physiologic regulators of vitamin D metabolism which includes $1\alpha,25$-$(OH)_2D$, parathyroid hormone, phosphate, and calcium (15,21).

A similar hypercalcemic action of prolactin has been observed in mammals (1,2,22). Thus it is tempting to speculate on the relevance of our results to calcium metabolism in mammals. The enhanced prolactin levels during the latter part of pregnancy and in lactation (23) might well contribute to augmented calcium absorption observed in these states. Indeed it may well be that elevated prolactin levels might be characteristic of any normal calcium stress, be it in birds or mammals. Although direct evidence is necessary before concluding that our observation of prolactin-mediated stimulation of $1\alpha,25$-$(OH)_2D$ is general, it is clear that this effect of prolactin is an important addition to the known actions of the hormone. It is likely to be physiologically important in birds, and gives a hint of an unsuspected role for prolactin in mammals.

Acknowledgments

This research was supported in part by grants from the Wellcome Trust and Medical Research Council and NIH Grant AM 15781. We thank Joseph Zerwekh and Adrienne Sainten for help in processing the plasma for $1\alpha,25$-$(OH)_2D$ assay, and Jean Hruska of the University of Arizona Department of Animal Sciences for assistance in obtaining blood samples from hens. Please direct all correspondence to Professor Iain MacIntyre at the Royal Postgraduate Medical School.

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EFFECT OF GLUCOCORTICOIDS ON VITAMIN D METABOLISM

E. SPANOS, K. W. COLSTON and I. MACINTYRE

Endocrine Unit, Royal Postgraduate Medical School, Ducane Road, London W12 OHS, England

Received 5 January 1977

1. Introduction

Vitamin D_3 is hydroxylated in the liver and other tissues to 25-hydroxycholecalciferol ((25-OH)D_3) [1] and further converted by the kidney to 1,25-dihydroxycholecalciferol ((1,25-OH)$_2$$D_3$) the hormonal form of vitamin D_3 [2].

Evidence has been presented to show that the calcium and phosphorus content of the diet [3,4] 1,25(OH)$_2$$D_3$ itself [5], parathyroid hormone [6] and prolactin [7,8] regulate the production of (1,25-OH)$_2$$D_3$ by the kidney. Recently Kenny [9] suggested that steroid hormones stimulate the 25-hydroxycholecalciferol-1α-hydroxylase, the enzyme responsible for the production of (1,25-OH)$_2$$D_3$ and Tanaka et al. [10] reported that large doses of oestrogens in the presence of androgens have a profound stimulatory effect on the activity of this enzyme. Our results are slightly different since we found that the presence of androgens is not essential for oestrogens to exert their effect. Thus oestrogens alone, or when combined with androgens or even with progesterone, markedly stimulate 1α-hydroxylase activity [11].

The purpose of this report is to present evidence that glucocorticoids also have a profound stimulatory effect on the activity of 1α-hydroxylase.

2. Materials and methods

One-day-old cockerels, Light Sussex–Rhode Island cross-breed were raised on a vitamin D-deficient diet containing 0.34% calcium and 0.54% phosphate. Vitamin D_3 supplements (5 or 20 IU daily injected into the crop in 0.1 ml arachis oil) were given to some chicks beginning at one-day after hatching.

Cortisol was dissolved in propylene glycol and corticosterone in ethanol to the required concentration. The steroids were injected subcutaneously either every 12 h throughout the duration of the experiment or as a single dose. The chicks were killed by decapitation at varying times after the last injection and enzyme activity was assayed in kidney homogenates.

Renal tubules were prepared by a slight modification of the method of Burg and Orloff [12]. Protein estimation was performed by the Folin-Lowry method [13]. Preparation of kidney homogenates and assay of the 25-hydroxycholecalciferol-1α-hydroxylase were as previously described [14,15].

3. Results

The effect of chronic administration of cortisol on the 1α-hydroxylase activity is shown in fig.1. Similarly isolated renal tubules, prepared from vitamin D-deficient chicks, responded to physiological cortisol concentration in the medium by increasing the rate of (1,25-OH)$_2$$D_3$ production (fig.2). Pentagastrin served as a negative control while the lack of an effect of oestrogens under these conditions was probably due to a very short preincubation time.

When vitamin D-supplemented chicks were used, a single injection of cortisol resulted in the disappearance of 24-hydroxylase activity and in a 20-fold stimulation of the 1α-hydroxylase activity (fig.3).

An early response to steroid hormone treatment was seen using a moderate dose of corticosterone, the chief glucocorticoid in chicks [16] (fig.4).

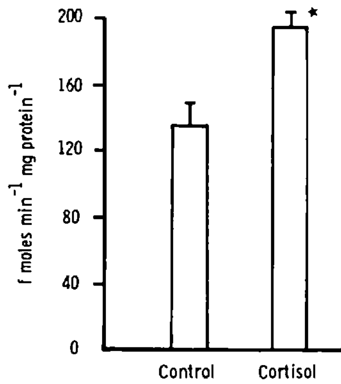

Fig.1. Effect of cortisol on 1α-hydroxylase activity from vitamin D-deficient chicks. 8-Day-old chicks, on a vitamin D-deficient diet, were treated with 500 μg of cortisol every 12 h for 7 days. Kidney homogenates were prepared 18 h after the last injection. Incubation for 10 min at 37°C was carried out in the presence of 12 ng of tritiated $(25\text{-OH})D_3$.

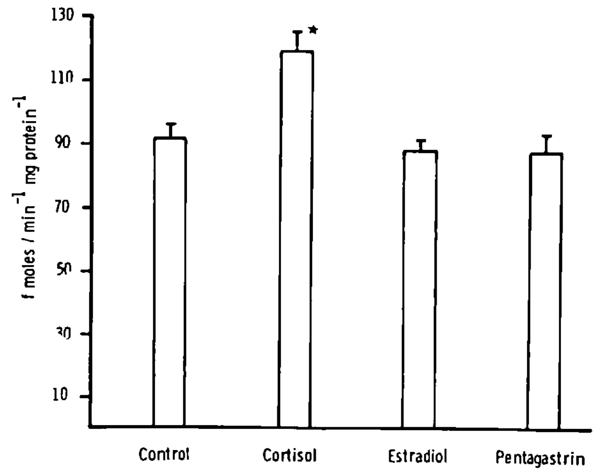

Fig.2. Effect of cortisol on 1α-hydroxylase activity in vitro. Renal tubules were prepared from 24-day-old D-deficient chicks. The renal cells were preincubated for 4 h in the presence of hormones. This was followed by a 10 min incubation in the presence of 12 ng of tritiated $(25\text{-OH})D_3$. Hormone concentrations were as follows: cortisol 50 ng/ml, β-oestradiol 60 ng/ml and pentagastrin 40 ng/ml.

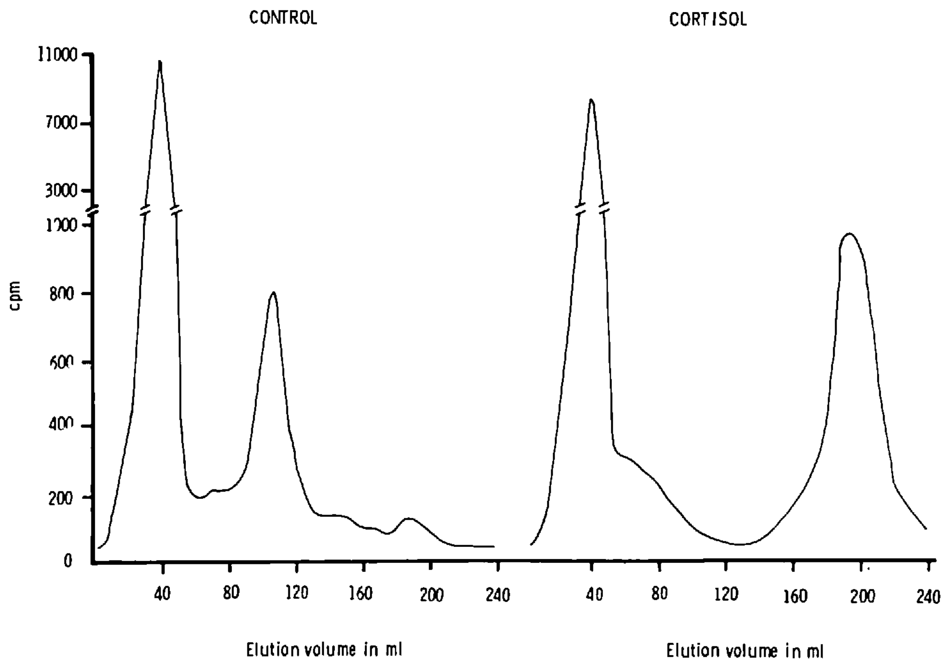

Fig.3. Stimulation of renal 1α-hydroxylase and suppression of 24-hydroxylase by cortisol. A single injection of 2 mg of cortisol was given to 15-day-old chicks, supplemented with 20 IU vitamin D daily. The chicks were killed 16 h later. Kidney homogenates were prepared and incubated for 12 min in the presence of 125 ng tritiated $(25\text{-OH})D_3$. The lipid extracts were chromatographed on a 12 g Sephadex LH-20 column. Typical elution profiles are shown in fig.3a for controls and fig.3b for cortisol treated animals.

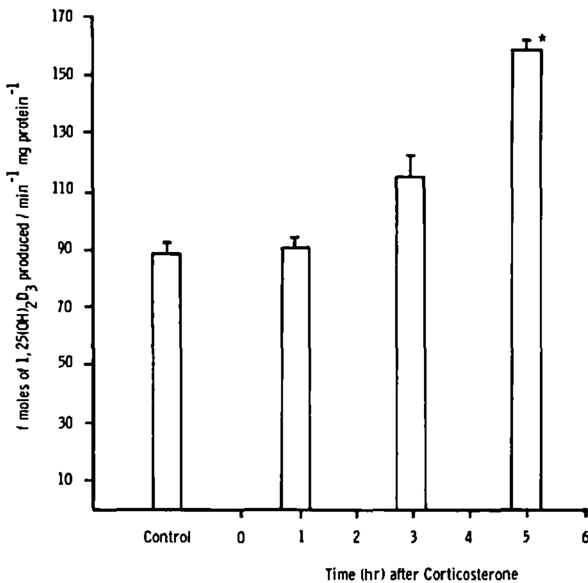

Fig.4. Effect of corticosterone on 1α-hydroxylase activity. 8-Day-old chicks, supplemented with 5 IU vitamin D daily, were given a single injection of 70 μg of corticosterone at 45 min, 3 h and 5 h before sacrifice. Incubation for 10 min was carried out in the presence of 12 ng of tritiated $(25\text{-}OH)D_3$.

4. Discussion

The present results clearly show that glucocorticoids stimulate the 1α-hydroxylase activity both in vitamin D-deficient and vitamin D-supplemented chicks either in vivo or in vitro. The differing degree of stimulation observed between these two groups of birds is explained by the fact that in the vitamin D-deficient chicks this enzyme is already stimulated by the lack of vitamin D in the diet [17].

This evidence seems to be in conflict with the well documented inhibitory effect of glucocorticoids on intestinal calcium absorption [18,19]. However, this inhibitory effect is only seen with pharmacological doses of steroids and takes days to develop [20], while the stimulation of the 1α-hydroxylase activity is demonstrated with moderate doses of glucocorticoids within a matter of hours.

Since the effect of glucocorticoids on intestinal calcium absorption is opposite to that of vitamin D, it has been suggested that glucocorticoids may act by antagonizing the action of vitamin D [18,12], or by interfering with the metabolism of vitamin D [21,22]. Avioli and his colleagues [21] reported in 1968 that prednisone administration in man resulted in diminished production of 'active metabolites' (peak IV) and increased production of 'inactive metabolites' (peak V). Since the presumed inactive metabolite in peak V was subsequently identified as $(1,25\text{-}OH)_2D_3$ these results seem to be in excellent agreement with our observations.

Kimberg et al. and Favus et al. found that glucocorticoids do not interfere with the metabolism of vitamin D or with the cellular and subcellular localization of $(1,25\text{-}OH)_2D_3$ in the intestinal target tissue [20,23,24]. The inability of these investigators to show a stimulatory effect of glucocorticoids on the conversion rate of $(25\text{-}OH)D_3$ to $(1,25\text{-}OH)_2D_3$ is possibly due to severely D-deficient animals used in their studies. This explanation is supported by our findings as well as by their own observations in vitamin D-supplemented animals, in which the levels of CaBP and bioassayable vitamin D activity in intestinal mucosa from cortisone-treated animals were higher than control animals [20]. A number of studies in vitamin D-supplemented or moderately vitamin D-deficient animals indicates that glucocorticoids may cause an enhanced production of $(1,25\text{-}OH)_2D_3$ despite the observed inhibition of intestinal calcium absorption [25–28]. These observations are entirely consistent with, and are explained by, our own findings.

This report presents evidence which rules out the suggestion that the inhibition of intestinal calcium transport by glucocorticoids is due to interference with vitamin D metabolism. Futhermore, these results clearly demonstrate that glucocorticoids stimulate 1α-hydroxylase activity before any inhibition of intestinal calcium transport is observed. The physiological significance of enhanced production of $(1,25\text{-}OH)_2D_3$ before and possibly during inhibition of intestinal calcium transport by glucocorticoids remains to be established.

Acknowledgements

This work was supported in part by the Wellcome Trust.

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