THE METABOLISM OF CHOLECALCIFEROL IN JAPANESE QUAIL

by

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To my dear Mother and Father

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

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THE METABOLISM OF CHOLECALCIFEROL IN THE JAPANESE QUAIL

The metabolism of 25 hydroxycholecalciferol (25 HCC) was studied in Japanese quail in relation to age and to reproductive activity and its regulation by sex hormones and parathyroid hormone alone and in combination was also investigated. This metabolism was studied using kidney homogenates incubated with ^{3}H-labelled 25 HCC and the labelled metabolites were separated by thin layer and column chromatography.

The results showed that the production of 1,25 dihydroxycholecalciferol (1,25 DHCC) increased and the biosynthesis of 24,25 dihydroxycholecalciferol (24,25 DHCC) decreased in female birds with the approach of sexual maturity. In male birds the production of 1,25 DHCC decreased with age and the activity of 25 HCC-1-hydroxylase was completely suppressed at sexual maturity.

Kidneys of birds that were laying regularly produced mainly 1,25 DHCC even during natural short pauses between clutches or during pauses artificially induced by feeding methallibure, an ovulation inhibitor, whereas in non-laying birds, ones having long natural or artificial pauses between laying sequences, adult males, and immature birds, 24,25 DHCC was the major metabolite. When laying birds were fed on a sulphanilamide-containing diet which inhibited egg shell calcification the activity of 25 HCC-1-hydroxylase in their kidneys was reduced significantly. Injection of parathyroid extract (PTE) into immature birds, adult male, and methallibure or sulphanilamide-fed laying females significantly increased the production of 1,25 DHCC and inhibited the biosynthesis of 24,25 DHCC. These results suggest that ovulation is unlikely to be involved in the stimulation of 1,25 DHCC production and indicate that PTH may be the key factor involved in controlling 1,25 DHCC production in laying birds.

Administration of oestrogen to immature birds stimulated the production of 1,25 DHCC and inhibited the biosynthesis of 24,25 DHCC, whereas testosterone inhibited the production of 1,25 DHCC whether given alone or in combination with oestrogen. PTE overcame the inhibition of 1,25 DHCC biosynthesis evoked by testosterone. It is suggested that the presence of PTH is required for oestrogen plus androgen to stimulate the production of 1,25 DHCC to the extent observed in normal laying birds.

The increased 25 HCC-1-hydroxylase activity occurring during the reproduction period of female birds may be of physiological significance in increasing the amounts of 1,25 DHCC needed to stimulate the absorption of calcium from the gut and in mobilizing calcium from the medullary bone under the influence of PTH for egg shell formation.

CHAPTER 1

CHAPTER 1. REVIEW OF RELEVANT LITERATURE

1.1 Introduction

Vitamin D is an antirachitic agent. The disease of rickets can be produced by maintaining animals on a fat-free diet, and can be prevented by cod liver oil supplements (Mellanby, 1919, a, b and c). In 1922 Zucker, Pappenheimer and Barnett found that the antirachitic substance responsible for the therapeutic action of cod liver oil was closely related chemically to cholesterol. Rickets can also be prevented by exposing patients (Huldschinsky, 1919) or animals (Hess and Gutman, 1922) to sunlight or more precisely to ultraviolet light of a particular wave length (256-313 mm). Vitamin D can be obtained therefore either from diet or from an endogenous photochemical reaction in the skin.

It has been firmly established that vitamin D_3 or cholecalciferol must be metabolized to active forms before exerting its physiological effects. The major pathways for vitamin D_3 metabolism involve hydroxylation in the liver (Ponchon, Kennan and De Luca, 1969) to give 25 hydroxycholecalciferol, followed by hydroxylation in the kidney (Fraser and Kodicek, 1970) to give 1,25 dihydroxcholecalciferol, the biologically active form of vitamin D_3, or 24,25 dihydroxycholecalciferol (Omdahl and De Luca, 1973).

The discovery of these pathways indicates that vitamin D_3 is a pro-hormone giving rise to at least one hormone which is produced in the kidney and which functions on the intestine and bone. The production of this hormone, 1,25 dihydroxycholecalciferol, is linked precisely to the calcium requirement of the animal. Results reported in this thesis indicate a role for sex hormones and parathyroid hormone and their interaction in regulating the production of 1,25 dihydroxycholecalciferol in the kidney of Japanese quail (Coturnix coturnix japonica).

1.2 Metabolism of Cholecalciferol in the Liver

The lag of 20 hours or more between the administration of vitamin D_3 and the expression of its physiological actions led some investigators to postulate that vitamin D_3 must be activated before becoming biologically effective.

The turning point in the concept of vitamin D_3 metabolism was made by Neville and De Luca (1966) who were able to synthesize tritiated vitamin D_3 with high specific activity (1,2 - ^3H D_3, S.A. 26,000 dpm/i.u.). Administration of this radioactive D_3 revealed that physiological amounts of D_3 are metabolized to biologically active metabolites (Lund and De Luca, 1966). 25 hydroxycholecalciferol (25 HCC) was the first metabolite of D_3 to be isolated from the plasma of pigs (Blunt, De Luca and Schnoes, 1968). It has been shown that the liver is the major site for the 25-hydroxylation, although evidence has been presented that some 25-hydroxylation occurs in the kidney and intestine of chicks (Tucker, Gagnon and Haussler, 1973).

The 25-hydroxylated derivative is the major circulating form of vitamin D_3 in normal subjects and its concentration in these subjects is about 12 ng/ml (range 4-33 ng/ml) (Preece, O'Riordan, Lawson and Kodicek, 1974).

1.3 The Metabolism of Cholecalciferol in the Kidney

The second and most important step of vitamin D_3 activation takes place in the kidney to convert 25 HCC to 1,25 DHCC.

For a few years, 25 HCC was considered to be the biologically active form of D_3. The studies of Lawson et al (Lawson, Wilson and Kodicek, 1969), of Haussler et al (Haussler, Myrtle and Norman, 1968) and of Blunt et al (Blunt, De Luca and Schnoes, 1968) revealed the existence of metabolites more polar than 25 HCC. The major metabolites extracted from intestinal homogenates was not 25 HCC, but a product of 25 HCC metabolism, and it acted more rapidly than 25 HCC. Lawson et al (1969) have reported that this metabolites possessed a modification on C-1, where $1\alpha^3H$ was lost from $1\alpha^3HD_3$ during the biosynthesis of this metabolite. A few months later, after the synthesis of 25 HCC, the structure of this metabolite was shown to be 1α,25 DHCC (Lawson, Fraser, Kodicek, Morris and Williams, 1971; Holick, Schnoes, De Luca, Suda and Cousins, 1971). The enzyme, 25 HCC-1-hydroxylase, responsible for the conversion of 25 HCC to 1,25 DHCC is localized exclusively in the mitochondrial fraction of kidney cortical cells (Midgett, Spielvogel, Coburn and Norman, 1973).

1,25 DHCC is considered to be the active form of vitamin D_3. Administration of this metabolite into nephrectomized animals initiates intestinal calcium transport (Boyle, Miravet, Gray, Holick and De Luca, 1972; Wong, Norman, Reddy

and Coburn, 1972), bone calcium mobilization and phosphate transport (Chen, Castillo, Korycka-Dahl and De Luca, 1974), whereas these animals do not respond well to 25 HCC when given in physiological amounts.

In addition to the 1-hydroxylation of 25 HCC, the kidney also catalyses a 24-hydroxylation (Holick, Schnoes, De Luca, Gray, Boyle and Suda, 1972). The kidney is not the only site of the 24-hydroxylation, since this hydroxylation occurs in nephrectomized rats (Garabedian, Pavlovitch, Fellot and Balsam, 1974a). Recent evidence for the existence of external 24-hydroxylase came from the studies of De Luca and associates (Kumar, Schnoes and De Luca, 1978) who found that the 24-hydroxylation of 25 HCC and of 1,25 DHCC occurs in vitro by rat intestinal homogenate.

Besides 1,25 DHCC, 24,25 DHCC and 25 HCC, there is a metabolite which has been isolated from the plasma of pigs and identified as 25,26 DHCC (Suda, De Luca, Schnoes, Tanaka and Holick, 1970). Tanaka et al (Tanaka, Shepard, De Luca and Schnoes, 1978b) have reported that kidney homogenate from chicks fed on vitamin D-supplemented diets produces 25,26 DHCC from 25 HCC, whereas kidney homogenates prepared from chicks fed on vitamin D-deficient diets do not produce this metabolite.

It has been reported that 24,25 DHCC and 1,25 DHCC can serve as substrates for the renal 25 HCC-1-hydroxylase and 25 HCC-24-hydroxylase respectively, yielding a more polar metabolite which, when isolated, was identified as 1,24,25-Trihydroxycholecalciferol (1,24,25 THCC) (Holick, Baxter, Schraufroget, Tavela and De Luca, 1976). The formation of this compound depends on the dietary conditions (Tanaka, Castillo and De Luca, 1977; Kleiner and De Luca, 1974; Friedlander and Norman, 1975) or on the physiological state of the animal (Baksi and Kenny, 1977).

Of some interest is the observation of Tanaka et al (Tanaka, Castillo and De Luca, 1977) that the renal 25 HCC hydroxylases is specific for a hydroxyl group on C-25 of the vitamin D_3 molecule before it can be 1- or 24-hydroxylated.

The approximate concentrations of the dihydroxylated derivatives of vitamin D_3 in the plasma of a normal individual subject have been reported to be:

60 pg/ml for 1,25 DHCC (Brumbaugh, Haussler, Bressler and Haussler, 1974).

0-4 ng/ml for 24,25 DHCC (Stanbury, 1975)
200-1000 pg/ml for 25,26 DHCC (Care, Bates, Richard, Peacock, Towlinson, O'Riordan, Mawer, Taylor, De Luca and Norman, 1975).

1.4 The Control of Cholecalciferol Metabolism

i. Regulation of 25-hydroxylation

The liver is a major site for 25-hydroxylation of D_3 to 25 HCC. The enzyme which converts the vitamin to its circulating form, 25 HCC, is localized in the endoplasmic reticulum of the liver, and requires molecular oxygen and NADPH for its full activity in cell free liver preparations (Horsting and De Luca, 1969; Tucker, Gagnon and Haussler, 1973).

The regulation of renal 25 HCC hydroxylases has been widely studied, but fewer data have been accumulated concerning the control of hepatic cholecalciferol-25-hydroxylase. Battacharry and De Luca (1973, 1974) using both *in vitro* and *in vivo* techniques in rat and chicken have shown an inverse relationship between the amount of D_3 administered to D-deficient animals and the activity of 25-hydroxylase. The relationship is observed only at physiological doses of D_3, while large doses can increase the level of circulating 25 HCC (Haddad and Stamp, 1974; Belsey, Clark, Bernat, Glowacki, Holick, De Luca and Potts, 1974; Clark and Potts, 1977). This change in 25-hydroxylase activity was not noted by pre-dosing with 25 HCC itself, 1,25 DHCC or 24,25 DHCC (Mawer and Reeve, 1977). These results suggest that the product inhibition is at the hepatic intracellular level and not by circulating level of 25 HCC (De Luca and Melacon, 1972).

Of great interest concerning the regulation of 25 HCC production are the experiments of Nicholson (1977) which show that oestrogens have a stimulatory effect on 25-hydroxylase activity in the kidney of Japanese quail. In addition, oestrogens play a role in protecting vitamin D_3 from the degradation which occurs in liver homogenates prepared from untreated male quail.

ii. Regulation of renal 25 HCC hydroxylases

The most important activation reaction of vitamin D_3 from a physiological point of view occurs exclusively in the kidney, and is catalyzed by 25-HCC-1-hydroxylase (Fraser and Kodicek, 1970; Gray, Boyle and De Luca, 1971). This enzyme

is found in a variety of animal species (Midgett et al, 1973).

The regulation of the production of 1,25 DHCC is extremely complex and controversial. Since 1971, great efforts have been exerted in many laboratories in an attempt to determine the factors which control the synthesis of 1,25 DHCC in the kidney.

The following factors have been implicated in the regulation of 25 HCC metabolism.

a. Dietary calcium
b. Dietary phosphorus
c. Parathyroid hormone
d. Calcitonin
e. Vitamin D_3 and its metabolites
f. Gonadal hormones
g. Prolactin
h. Growth hormone
i. Insulin
j. Glucocorticoids

a. Dietary calcium

Boyle et al (Boyle, Gray and De Luca, 1971) were the first to report a regulatory role for calcium in the production of 1,25 DHCC. The observations of these authors show that manipulation of dietary calcium (0.02-3% Ca) results in changes in the activity of 25 HCC-1-hydroxylase. In their experimental rats, low calcium diets markedly stimulated the activity of 1-hydroxylase, whereas high calcium diets suppressed the production of 1,25 DHCC. This suppression of 1,25 DHCC production by dietary calcium correlates with an increase in serum calcium concentration. They have also observed that these changes in the 1-hydroxylase activity occur only in animals given a source of vitamin D, whereas D-deficient animals show 1-hydroxylase activity independent of dietary calcium. In all cases, when synthesis of 1,25 DHCC is stimulated, the production of 24,25 DHCC is suppressed and vice versa.

In 1953 Nicolaysen, Eeg Larsen and Malm studied the adaptation processes to a low calcium diet, and came to a conclusion that an endogenous factor,

different from vitamin D and parathyroid hormone, regulates intestinal calcium transport when young rats are grown on a low calcium diet. They have shown, however, that this endogenous factor is a vitamin D-dependent one. In the light of Nicolaysen's observation, Boyle et al (1971) have suggested that 1,25 DHCC is the endogenous factor of Nicolaysen and that plasma calcium, or a factor which regulates serum calcium concentration, is the prime regulator of renal conversion of 25 HCC to 1,25 DHCC.

In two series of experiments, Fraser and Kodicek (1973) have reported that direct control of 1-hydroxylase by calcium ions is unlikely. In the first series, they have studied the activity of 1-hydroxylase at intervals up to 60h in 32 day-old, D-deficient chicks given 5 µg cholecalciferol. The results of this experiment indicated no correlation between the activity of 1-hydroxylase and serum calcium, since serum calcium concentration began to rise 14 h after the injection of cholecalciferol, but no decrease in the 1-hydroxylase occurred until about 60 h. In the second series of experiments, they measured the activity of 1-hydroxylase during the development of D-deficiency in chicks. The enzyme activity began to rise on day 6, and serum calcium concentration in these animals remained nearly normal. They also observed an inhibitory effect of calcium on 1-hydroxylase when added to the incubation medium. Following these results, Fraser and Kodicek (1973) have suggested that parathyroid hormone directly stimulates the renal production of 1,25 DHCC.

The observations of Boyle et al (1971) that dietary calcium can alter the activity of 1-hydroxylase, have been confirmed by others in vitamin D-replete (Swaminathan, Sommerville and Care, 1977; Friedlander, Henry and Norman, 1977; Sommerville, Swaminathan and Care, 1978), and vitamin D-deficient chicks (Omdahl and Evan, 1977).

On the other hand Swaminathan et al (1977) have reported a direct increase in the production of 24,25 DHCC, when the level of calcium was increased in the diet. This correlation between 24-hydroxylase and dietary calcium was not observed by other investigators (MacIntyre, Colston and Evans, 1975; Friedlander et al 1977; and Omdahl and Evan, 1977).

In an attempt to understand the mechanism by which calcium controls the 1-hydroxylase system, studies involving the addition of calcium to isolated mitochondria, isolated tubules, and other in vitro systems have been carried

out by a number of investigators (Bikle and Rasmussen, 1975, 1978; Bikle, Murphy and Rusmussen, 1975; Horiuchi, Suda, Sasaki, Ogata, Ezama, Sano and Shimazawa, 1975; Henry and Norman, 1976; Colston, Evans, Galante, MacIntyre and Moss, 1973; MacIntyre et al, 1975). The addition of calcium can either stimulate or suppress the 1-hydroxylase activity. Calcium stimulates the production of 1,25 DHCC, if the mitochondria are isolated from an EGTA media (Colston et al, 1973; Horiuchi et al, 1975), or inhibits the production when the mitochondria are isolated from normal media (Bikle and Rasmussen, 1975; Horiuchi et al 1975). When the mitochondria are washed several times with calcium-chelating agent, calcium has an inhibitory effect (Henry and Norman, 1976).

b. Dietary phosphorus

A role for phosphorus in the regulation of 1,25 DHCC production has been suggested by Tanaka and De Luca (1973) in rats, and by Baxter and De Luca, (1976), Friedlander et al (1977), and Sommerville et al (1978) in chicks.

Tanaka and De Luca (1973) observed an inverse relationship between serum inorganic phosphate and 1,25 DHCC production in thyroparathyroidectomized rats, but the stimulation by a low phosphorus diet was not as great as that observed with a low calcium diet. They have suggested that the intracellular phosphate concentration is the important regulator of the activity of 1-hydroxylase. Rasmussen and Bordier (1974) have explained these results by assuming that cellular phosphate depletion leads to an increase in the ionic calcium within the cell cytosol, and that ionic calcium is the regulator of 1-hydroxylase. These observations are in agreement with the idea that calcium absorption and accumulation of 1,25 DHCC in the intestine increases in animals receiving low phosphorus diets (Morrissey and Wasserman, 1971). On the other hand, Henry et al (Henry, Midgett and Morman, 1974) have reported that phosphate deprivation does not stimulate 1-hydroxylase activity. The diet of Henry et al (1974) has been critisized by Baxter and De Luca (1976) who claim that the diet was not low in phosphorus - the level of phosphorus should be no greater than 0.3% - nor was the length of time to which the animals were exposed to the low phosphorus diet sufficient to stimulate the 1-hydroxylase. In a recent report Norman's group reported an increase of 2.5 times in the activity of 1-hydroxylase, when serum phosphorus fell from 7 to 2 mg/100 ml (Friedlander et al, 1977). Sommerville et al (1978) have also shown a three-fold increase in the production of 1,25 DHCC, and the accumulation of this metabolite was doubled when young chickens

were fed a diet low in phosphorus. Booth et al (Booth, Tsai and Morris, 1977) have reported an inverse correlation between serum phosphorus and the activity of 1-hydroxylase in thyroparathyroidectomized, D-deficient chicks, not supplemented with vitamin D or its metabolite. These observations do not support the observations of De Luca and co-workers, that in a vitamin deficient state, modulation of 1-hydroxylase activity by the concentration of serum phosphate can occur only in the presence of 1,25 DHCC (De Luca, 1974).

In vitro studies on the effect of phosphorus on 1,25 DHCC production have revealed that addition of phosphate to the incubation media does alter the activity of 1-hydroxylase. Bikle and Rasmussen (1975), and Bikle et al (1975) observed that increasing the phosphate concentration in the incubation medium up to 6 mM stimulated the activity of 1-hydroxylase in the absence of calcium ions. At 1-2 mM Ca, phosphate had a biphasic effect on the production of 1,25 DHCC, increasing the concentration of Ca up to 4 mM resulted in blocking the stimulatory effect of phosphate. An inhibitory effect of 5×10^{-3} M phosphate has been reported by Henry and Norman (1976). However, a recent report (Bikle and Rasmussen, 1978) shows that the addition of phosphate stimulates the production of 1,25 DHCC in chick renal mitochondria when the ion concentration is raised above 10 mM.

Clearly, more work requires to be done to resolve the contradictions surrounding the role of calcium and phosphorus in the control of 1,25 DHCC production by the kidney.

c. Parathyroid hormone

Calcium deficiency or hypocalcemia leads to an increase in the secretion of PTH and in the production of 1,25 DHCC. It has been suggested by a number of investigators that PTH plays a role in the regulation of 1,25 DHCC production.

Parathyroidectomy of both rats (Garabedian, Holick, De Luca and Boyle, 1972; Horiuchi, Suda, Sasaki, Takahashi, Shimazawa and Ogata, 1976, 1977; Omdahl, Hynsaker and Aschenbrenner, 1977) and chicks (Fraser and Kodicek, 1973; Galante, Colston, Evans, Byfield, Mathews and MacIntyre, 1973; Booth et al, 1977) results in a decrease in the production of 1,25 DHCC by the kidney. In rats raised on a vitamin D-deficient, low-calcium diet supplemented with 1,25 DHCC the level of 1,25 DHCC in the blood was reduced 24 h after PTX (Garabedian et al,

1972). Administration of PTE 48 h after TPTX enhanced the production of 1,25 DHCC to the control level within 36 h after administration (Garabedian et al, 1972). These authors concluded that PTH serves as a tropic hormone for the production of 1,25 DHCC. Fraser and Kodicek (1973) using 28 d old, D-deficient chicks supplemented with cholecalciferol have reported a decrease in the 1-hydroxylase activity 14 h after parathyroidectomy. They came to the conclusion that PTH is a direct regulator of 1,25 DHCC production. Galante et al (1973) have studied the activity of renal 25 HCC hydroxylases in PTX, vitamin D-deficient chicks and concluded that PTH is not essential for the increase in the production of 1,25 DHCC, since the activity of 1-hydroxylase remained elevated and undiminished 20 h after PTX in chicks not supplemented with D_3. They argued that in the study of Fraser and Kodicek (1973) on the activity of 1-hydroxylase in the kidney of D-deficient chicks, the fall in the 1-hydroxylase activity after PTX is mainly due to the supplementation of vitamin D_3, since, they (Galante et al, 1973) have noted that removal of parathyroid and ultimobronchial glands has no significant effect on the activity of 1-hydroxylase in the absence of vitamin D_3. However, an inhibitory effect of D_3 and 1,25 DHCC on 1,25 DHCC production has been reported (Henry, Midgett and Norman, 1974; Tanaka and De Luca, 1974; MacIntyre, Colston, Evans, Lopez, MacAuley, Piegnoux-Deville, Spanos and Szelke, 1976; Spanos, Barrett, Chong and MacIntyre, 1978a). On the other hand, and even in the absence of vitamin D or its metabolites, the activity of 1-hydroxylase in vitamin D-deficient chicks decreased 24 h after PTX compared to that in sham-operated controls (Henry et al, 1974; Booth et al, 1977). These observations do not agree with those of De Luca's group (De Luca and Schnoes, 1976), that in vitamin D-deficient animals modulation of 1-hydroxylase by circulating PTH can occur only in the presence of 1,25 DHCC.

Horiuchi et al (1977) have reported that accumulation of 1,25 DHCC produced from 25 HCC in the plasma of vitamin D-deficient, TPTX rats was enhanced by infusion of cAMP (0.9 μmol/h) to a level similar to the maximum obtained by infusion of 2.5-7.5 UPTH/h. Accordingly, they have concluded that the stimulatory effect of PTH on 1-hydroxylase is mediated by cAMP, since PTH produces an increase in cAMP levels of renal cortical tissue of vitamin D-deficient animals (Forte, Nichols and Anast, 1976; KaKuta, Suda, Sasaki, Kimura and Nagata, 1975). Again, addition of cAMP to isolated renal tubules from D-deficient animals stimulates the conversion of 25 HCC to 1,25 DHCC (Rasmussen, Wong, Bikle and Goodman, 1972).

Administration of PTH into intact rats (Garabedian et al, 1972) or intact chicks (Fraser and Kodicek, 1973; Henry, 1977) stimulates the production of 1,25 DHCC. Henry has administered partially purified PTH intracardially at two hour intervals up to six hours into vitamin D-supplemented chicks and studied the renal 25 HCC hydroxylase activities one hour after the last injection. She observed that the low dose of PTH (7.5 U/dose) increased the activity of 1-hydroxylase only slightly, but large doses of PTH (15 U/dose) resulted in five-fold increase in its activity. These dose levels of PTH had no effect on 24-hydroxylase activity (Henry, 1977).

Attempts to study the direct effect of PTH on 1,25 DHCC production in vitro have been carried out by some investigators. Rasmussen et al (1972) have used renal tubules prepared from vitamin D-deficient chicks to study the effect of PTH. They have observed that PTH stimulates the production of 1,25 DHCC when added at small concentration, but higher doses lead to an inhibition of the conversion of 25 HCC to 1,25 DHCC. Henry (1977) has studied the effect of PTH on the biosynthesis of 1,25 DHCC by culture of chick kidney cells prepared from vitamin D-deficient chicks. She observed that PTH had no effect on the production of 1,25 DHCC during the 48 h of its presence in the cultures.

d. Calcitonin

Few data have been published on the role of calcitonin in the regulation of 25 HCC metabolism. Rasmussen et al (1972) reported that addition of porcine calcitonin in vitro to isolated renal tubules prepared from vitamin D-deficient chicks inhibited the conversion of 25 HCC to 1,25 DHCC. On the other hand, Shain (1972) using the same system has denied that calcitonin inhibits the production of 1,25 DHCC. Galante et al (Galante, Colston, MacAuley and MacIntyre, 1972) reported that injection of calcitonin into intact rats resulted in an increase in the accumulation of (^{3}H) 1,25 DHCC in the blood and intestine. The observations of Galante et al (1972) have been confirmed by Lorence, Tanaka, De Luca and Jones (1977), who have also observed that the effect of calcitonin on 1-hydroxylase is totally eliminated by TPTX under a variety of conditions. They have suggested that parathyroid glands mediate the effect of calcitonin in the intact animal, and at the same time they have denied any direct role of calcitonin on the production of 1,25 DHCC.

e. Vitamin D_3 and its metabolites

The idea that the regulation of renal 25 HCC hydroxylases does not occur in vitamin D-deficient rats (Boyle et al, 1971) and chickens (Galante et al, 1973) led some investigators to assume that a vitamin D derivative should be present for this regulation to occur. In vitamin D-deficient animals the activity of 1-hydroxylase predominates, whereas 24-hydroxylase is hardly detectable. Administration of D_3 or 1,25 DHCC into rats or chickens results in a decrease in 1-hydroxylase activity, and an increase in the activity of the alternative enzyme, 24-hydroxylase (Galante et al, 1973; Henry et al, 1974; Tanaka and De Luca, 1974; MacIntyre et al, 1976; Henry, Taylor and Norman, 1977; Horiuchi et al, 1976; Omdahl, 1977).

Attempts to study the direct effect of 1,25- and 24,25 DHCC on the metabolism of 25 HCC in vitro have been carried out by a number of investigators (Larkins, MacAuley and MacIntyre, 1974; Spanos et al 1978a; Henry, 1977). Larkins et al using isolated renal tubules have reported an inhibitory effect of large doses of 1,25 DHCC on its own production, but these doses had no effect on the activity of 24-hydroxylase. The suppression of 1-hydroxylase and the induction of 24-hydroxylase by the addition of 1,25 DHCC have been reported by Henry (1977) and Spanos et al (1978a) using the technique of chick kidney cells cultures. These results indicate that 1,25 DHCC acts directly on the kidney cells to regulate the metabolism of 25 HCC. On the other hand, studies on the effect of 1,25 DHCC on 25 HCC metabolism in the kidney of vitamin D-deficient TPTX rats, into which PTH was constantly infused revealed that infusion of PTH prevents the suppression of 1-hydroxylase by 1,25 DHCC (Horiuchi et al, 1976; Suda, Horiuchi, Fukushima, Nishii and Ogata, 1977). These results were interpreted that the effect of 1,25 DHCC on 1-hydroxylase is an indirect one, and 1,25 DHCC exerts its regulatory effect by suppressing PTH secretion. In contrast, Omdahl (1977) has reported that thyro/parathyroidectomy had no effect on the action of 1,25 DHCC to suppress the 1-hydroxylase activity, i.e. administration of 1,25 DHCC suppresses its own production and induces the production of 24,25 DHCC in the absence of parathyroid glands. Furthermore, he has also observed that PTE does not prevent the action of exogenous 1,25 DHCC on renal 25 HCC hydroxylases when given two days (10 U/8h) prior to 1,25 DHCC treatment. These results of Omdahl (1977), of Henry (1977), and of MacIntyre et al (1976) contradict the suggestion of Horiuchi et al (1976) and of Suda et al (1977).

Regarding the regulation of renal 25 HCC hydroxylases by 24,25 DHCC, Henry (1977) has reported that administration of this metabolite causes a decrease in the 1-hydroxylase activity 24 h after administration into vitamin D-deficient chicks fed a low calcium diet, but when animals were raised on a high calcium diet longer time is required for 24,25 DHCC to suppress the production of 1,25 DHCC. She did not observe any change in the activity of 24-hydroxylase after administration of 1,25 DHCC or 24,25 DHCC or a combination of these two steroids into her experimental animals on a low- and high-calcium intake.

f. Gonadal hormones

Kenny (1976) was the first to study the physiological changes in the metabolism of 25 HCC during the reproductive period of birds. He reported that the activity of the 1-hydroxylase in the kidney of female Japanese quail depends on the physiological state of the bird. He observed that tissue from birds with an egg in the oviduct produced mainly 1,25 DHCC, whereas kidneys of birds with an empty oviduct generated mainly 24,25 DHCC, when incubated in vitro with tritiated 25 HCC and he concluded that "ovulation in the Japanese quail leads to enhanced renal synthesis of 1,25 DHCC". These results led Kenny and others to suggest that sex hormones may be involved in the enhancement of 1,25 DHCC production.

De Luca and associates (Tanaka, Castillo and De Luca, 1976; Castillo, Tanaka, De Luca and Sunde, 1977) have reported that administration of pharmacological doses of oestrogen into male Japanese quail and chickens stimulates the production of 1,25 DHCC and inhibits 24,25 DHCC production, and claim that an androgen must be added for oestrogen to stimulate the production of 1,25 DHCC, since they have observed that oestrogen or androgen alone has little or no effect on the metabolism of 25 HCC. The permissive role of androgen in the stimulation of 1,25 DHCC production by oestrogen has been denied by Baksi and Kenny (1977a, b and c, 1978 a and b) and by MacIntyre (1977).

In a series of studies Kenny and Baksi have reported that oestrogen stimulates the production of 1,25 DHCC by the kidney of Japanese quail. They (1977a) have observed that the intramuscular injection of antioestrogen (tamoxifen citrate) at dose level of 30 mg/kg, 3 times weekly for four weeks into female Japanese quail results in an inhibition in the activity of

1-hydroxylase under normal or low calcium intake. A lower dose of tamoxifen citrate (10 mg/kg) inhibits 1-hydroxylase activity only under condition of low calcium feeding (Baksi and Kenny, 1977a). They have supported their previous observation by injecting oestradiol (3 mg/kg) or progesterone (5 mg/kg) intramuscularly for three days into immature Japanese quail of both sexes (Baksi and Kenny, 1977b). They found that administration of oestrogen or progesterone enhances the activity of 1-hydroxylase, but oestradiol is more effective than progesterone. Using physiological doses of oestradiol (0.01 mg/kg) Kenny and Baksi (1978) have reported a stimulatory effect at these doses on the production of 1,25 DHCC 12 and 24 h after injection, whereas higher doses (1 mg/kg) cause an increase in 1,25 DHCC production 4 h after injection. In order to extend their investigations, Baksi and Kenny (1978b) have studied the effect of ovariectomy and injection of pharmacological doses of sex hormones into ovariectomized female and intact male and female Japanese quail maintained on a low and normal calcium diet. Under normal calcium intake, they have observed that ovariectomy inhibits the production of 1,25 DHCC, when such birds were injected with oestradiol benzoate (1 mg/kg, 3 times weekly for four weeks), a significant increase in the production of 1,25 DHCC was observed compared to controls. Progesterone administration enhanced the production of 1,25 DHCC, but to a lesser degree than oestradiol. In intact males, the injection of oestradiol (3 mg/kg) led to an increase in the activity of 1-hydroxylase. When intact female birds were injected with testosterone (5 mg/kg) a decline in the production of 1,25 DHCC (from 14.4 $\pm$ 1.5 pmole/min/g kidney to 1.1 $\pm$ 1.0 pmole/min/g kidney) and an increase in the production of 24,25 DHCC has been observed by Baksi and Kenny (1978b). This regulatory effect of sex hormones on renal 25 HCC hydroxylases is eliminated when birds are raised on a low calcium diet.

MacIntyre (1977) using a synthetic oestrogen, diethyl stilboesterol, reported an increase in the 1-hydroxylase activity 24 h after administration into male chicks (weight 80-104 g). These results indicate that oestrogen alone is highly effective in stimulating the production of 1,25 DHCC in the kidney of immature quail and chick. On the other hand, Sommerville, Swaminathan and Scanes (1977) have observed that injection of 100 µg/oestrogen/d (a mixture of equal amounts of oestrone, oestradiol and oestriol) subcutaneously for 10 d into 10 day old, vitamin D-replete chicks results in an increase in serum calcium and calcium absorption, and has no effect on the in vitro production of 1,25 DHCC. However, in vivo the percentage of 1,25 DHCC accumulation in the

intestine was higher in oestrogen-treated chicks than in controls (Sommerville et al, 1977).

Attempts to study the effect of oestradiol on the metabolism of 25 HCC in vitro have been carried out (Spanos et al, 1978a). These investigators, who used chick kidney cells prepared from four-weeks old, vitamin D-deficient chicks, have reported that oestrogen had no effect on the production of 1,25 DHCC in vitro. Pre-treatment of these cells with 1,25 DHCC or the addition of this compound to the medium leads to an inhibition in the 1-hydroxylase activity. Spanos et al (1978a) found that under these conditions the addition of oestradiol to the incubation medium also had no effect. Thus, they have concluded that oestrogens have no direct effect on the kidney cells to regulate 25 HCC metabolism, but they stimulate the production of 1,25 DHCC by a mechanism in which the function of the liver is involved, since in chicks treated with ethylene dibromide, hepatotoxic agent, the stimulatory effect of oestrogens on 1,25 DHCC production was eliminated (Spanos et al 1978a).

Stimulation of 1,25 DHCC production by oestrogens occurs not only in birds, but also in amphibia (Kenny and Baksi, 1977) , and mammals (Baksi and Kenny, 1977c).

g. Prolactin

MacIntyre and associates (Spanos, Colston, Evans, Galante, MacAuley and MacIntyre, 1976a; Spanos, Pike, Haussler, Colston, Evans, Goldner, McCain and MacIntyre, 1976b; MacIntyre, 1977) were the first to report that prolactin may be involved in the regulation of 25 HCC metabolism. They have observed a marked increase in the production of 1,25 DHCC two hours after injection of 35 µg prolactin/12 h for seven days into vitamin D-deficient or D-supplemented chicks. A single dose of 70 µg prolactin caused a marked increase in the activity of 1-hydroxylase one hour after administration. This enhancement in 1-hydroxylase activity was absent three hours after injection. In another report, Spanos et al (1976b) have measured the circulating level of 1,25 DHCC in chicks, by radioreceptor assay, after subcutaneous injection of ovine prolactin (100 µg/d for 5 d). They found that this dose resulted in two-fold increase in the circulating level of 1,25 DHCC one hour after the last injection in both 3-week old, vitamin D-deficient or D-supplemented chicks, and caused an elevation in serum calcium of these birds. They suggested that the increase

in serum calcium by the administration of prolactin is madiated by the elevated level of 1,25 DHCC. Spencer and Tobiassen (1977) and Spanos et al (Spanos, Barrett, MacIntyre, Pike, Safilian and Haussler, 1978b) have reported that hypophysectomy decreases the amount of 1,25 DHCC in the intestinal mucosa and the blood of rats. Administration of prolactin into hypophysectomized rats has no effect on the metabolism of 25 HCC (Spanos et al, 1978b). On the other hand, Baksi and Kenny (1978b) failed to stimulate the 1-hydroxylase activity in the kidney of bullfrog by a hypercalcemic dose of prolactin.

The only well recognized physiological roles for prolactin in birds are in relation to the induction of broodiness (Nalbandov, 1945), and pigeons of both sexes to the development of the crop sac (Riddle, 1963) and neither of these conditions is associated with a high requirement for calcium. Furthermore, prolactin has a marked anti-gonadal action in hens (Nalbandov, 1945). These would not therefore appear, a priori, to be a strong case for the involvement of prolactin in the control of vitamin D metabolism in birds.

h. Growth hormone

As mentioned above, hypophysectomy reduces the production of 1,25 DHCC in rats (Spencer and Tobiassen, 1977; Spanos et al, 1978b). The first authors observed that removal of pituitaries resulted in a reduction in the amount of (^{3}H) 1,25 DHCC in rat intestinal mucosa and serum from 203 $\pm$ 55 fmoles/rat to 24 $\pm$ 3 fmoles/rat and from 40 $\pm$ fmoles/ml of serum to 25 $\pm$ 3 fmole/ml serum, respectively, and an increase in the production of 24,25 DHCC. Injection of rat growth hormone at dose level of 80 µg/d for six days to similar rats resulted in a significant increase in the accumulation of 1,25 DHCC in the intestine (Spencer and Tobiassen, 1977). Paradoxically, they have reported an inhibitory effect of growth hormone on 1,25 DHCC when given to intact animals. The observations of Spencer and Tobiassen have been confirmed by Spanos et al (1978b). They used purified human growth hormone and observed that administration of this hormone (100 µg/d for 7 days) into hypophysectomized rats elevated the circulating level of 1,25 DHCC to the normal level. Higher doses of growth hormone (500 µg/d for 6 days) resulted in an increase in the level of 1,25 DHCC, an increase in the levels of PTH, serum calcium, and phosphorus. These results correlate with the observation of Finkelstein and Schachter (1962) that hypophysectomy decreases intestinal

calcium absorption, and that the administration of growth hormone restores calcium transport.

i. Insulin

The pancreatic beta cells are considered to be the sole source of insulin production in mammals and administration of alloxan destroys the mammalian pancreatic beta cells within minutes. Birds are more resistant to alloxan than mammals. It has been reported that experimentally induced diabetes results in a decrease in calcium transport (Schneider and Schedl, 1972; Schneider, Omdahl and Schedl, 1976), and in calcium-binding protein (Schneider, Wilson and Schedl, 1973, 1974) in the duodenum of alloxan- and streptozotocin-treated rats. This depressed calcium transport can be corrected within 7 and 12 h by administration of 0.25 µg 1,25 DHCC or its synthetic analog $1\alpha(OH)D_3$ respectively (Schneider et al, 1976). Injection of D_3 or 25 HCC has no effect on calcium transport under these conditions, but all compounds, D_3, 25 HCC, 1,25 DHCC, and $1\alpha(OH)D_3$, stimulate duodenal calcium transport in control rats. In another report Schneider et al (Schneider, Schedl, McCain and Haussler, 1977b) have measured the circulating level of 25 HCC and 1,25 DHCC by radioactive ligand receptor assay, in control, streptozotocin diabetic rats and in streptozotocin diabetic rats injected with insulin. They observed that streptozotocin-induced diabetes resulted in a significant decrease in the circulating level of 1,25 DHCC. This decreased level of 1,25 DHCC in streptozotocin diabetic rats can be restored to a normal level by injecting 6 i.u. of porcine insulin (Schneider et al, 1977a).

j. Glucocorticoids

It is well established that corticosteroids have an inhibitory effect on intestinal calcium transport (Kimberg, Baerg, Gershon and Graudusius, 1971; Lukert, Stanbury and Mawer, 1973). Surprisingly, these steroid hormones have been reported to stimulate the production of the biologically active form of D_3, 1,25 DHCC, in rats (Lukert, et al 1973) and in chickens (Spanos and MacIntyre, 1977). In rats administration of 5 mg/d of 1-dehydrocortisol (prednisolone) for seven days resulted in a three-fold increase in the concentration of 1,25 DHCC in serum, but the concentration of this metabolite in the intestinal mucosa of control and glucocorticoid-treated rats was the same (Lukert et al,

1973). Spanos and MacIntyre (1977) working with chicks reported a disappearance of 24,25 DHCC and a 20-fold increase in the activity of 1-hydroxylase in D-supplemented chicks injected with a single dose of 2 mg cortisone. They have also reported a stimulatory effect of a single injection of 70 µg corticosterone within 5 h after injection. The concentration of the 1,25 DHCC-dependent CaBP in the intestinal mucosa is higher in glucocorticoid treated rats than in control animals (Kimberg et al, 1971). On the other hand, Carré et al (Carré, Ayigbedê, Miravet and Rasmussen, 1974) have reported that administration of 20 mg prednisolone/kg/d for five days into vitamin D-deficient rats has no effect on the rate of conversion of 25 HCC to 1,25 DHCC, but prednisolone treatment converts 1,25 DHCC to a more polar, inactive, and periodate-insensitive metabolite(s). Thus, they have suggested that the inhibition of intestinal calcium transport by large doses of adrenal corticoids is by converting 1,25 DHCC to inactive metabolite(s).

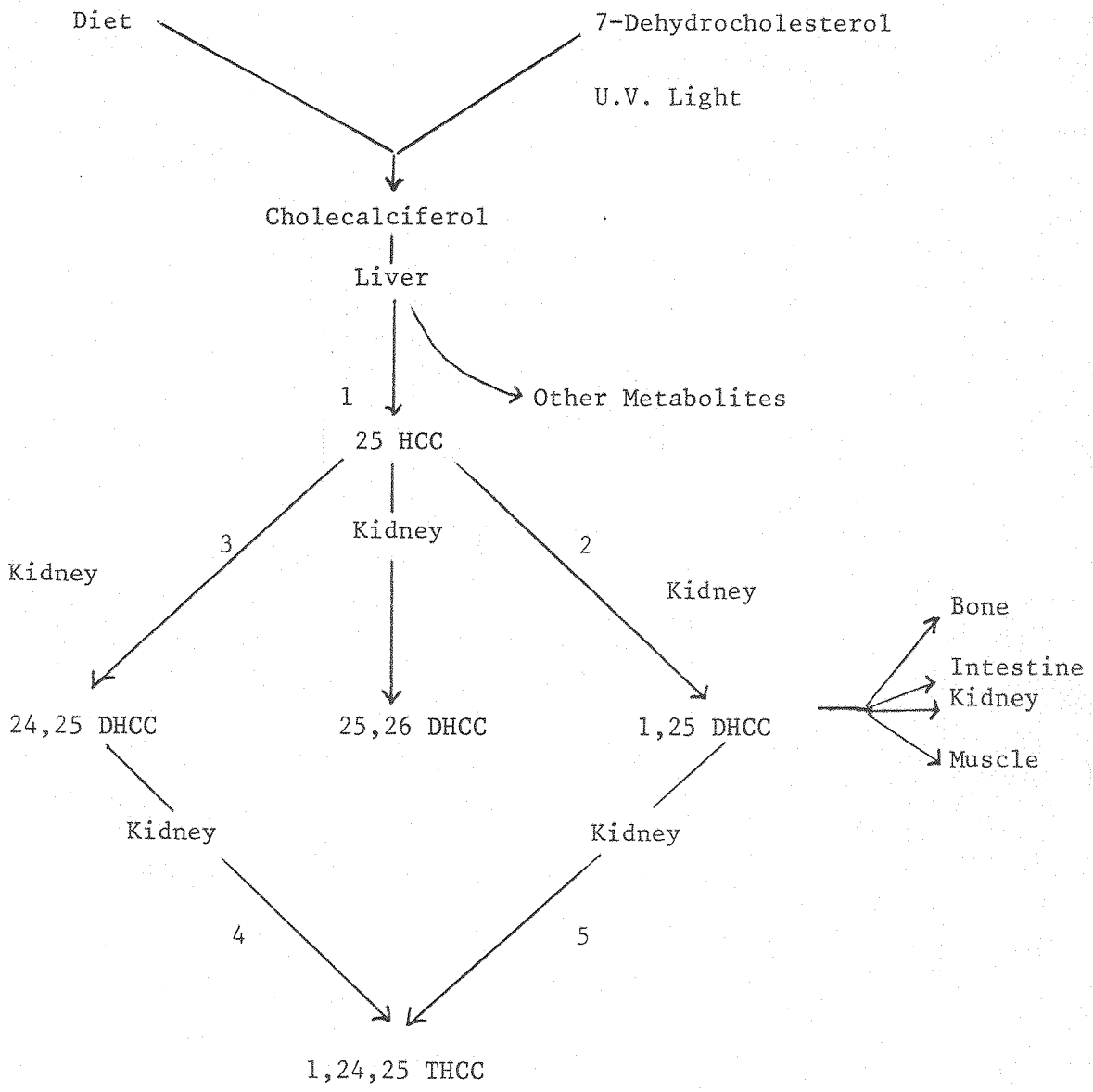
Other factors have been reported to influence vitamin D metabolism, such as, strontium and ethane-1-hydroxy-1, 1-diphosphonate (EHDP). Feeding strontium (2.35%) in the diet inhibits both 1,25 DHCC production and intestinal calcium transport (Omdahl and De Luca, 1971). Kidney mitochondria prepared from strontium fed chicks do not metabolize 25 HCC to 1,25 DHCC (Omdahl and De Luca, 1972). Treatment of animals with large doses of EHDP results in a reduction in the production of 1,25 DHCC (Hill, Lumb, Mawer and Stanbury, 1973; Baxter, De Luca, Bonjour and Fleisch, 1974).

At this time, it is not possible to clearly define the physiological relevance of all these observations and how prominently they feature in the control of 1,25 DHCC secretion by the kidney.

Figure 1 summarizes the pathways of vitamin D_3 metabolism, its regulation, and target organs of the active metabolite.

1.5 The Physiological and Biochemical Actions of Cholecalciferol

Intestinal mucosa and bone are considered to be primary target organs for vitamin D_3 action. It is not known for certain whether or not the kidneys are target organs for vitamin D_3, but recent evidence shows that vitamin D_3 metabolites may act on the parathyroid glands and muscle.



1 Oestrogens ↑

2 Low Ca, Low P ↑
PTH, Oestrogen
Prolactin?, Growth Hormone
Insulin

3 High Ca, High P ↑

4 As in Pathway No. 2 ↑

5 As in Pathway No. 3 ↑

Figure 1. The pathways of cholecalciferol metabolism, its regulation and the target organs of the active metabolite.

i. The action of cholecalciferol on the intestine

The role of cholecalciferol in calcium and phosphorus metabolism is exerted by its hormonal form, 1,25 DHCC, since this metabolite stimulates the movement of calcium and phosphorus from the intestinal mucosa into the blood stream (Myrtle and Norman, 1971; Omdahl, Holick, Suda, Tanaka and De Luca, 1971). Administration of 1,25 DHCC into animals or its addition to the incubation medium has been reported to cause the following changes:

a. Stimulation of the incorporation of ^{3}H uridine into intestinal RNA (Tsai and Norman, 1973; Corradino, 1977).

b. Stimulation of the nucleoplasmic DNA dependent RNA polymerase II activity (Zerwekh, Haussler and Lindell, 1974).

c. Modulation of the synthesis of a specific mRNA which codes for the de novo synthesis of a calcium-binding protein (CaBP) (Emtage, Lawson and Modicek, 1973; Charles, Martial, Zolock, Morrissey, Bikle and Baxter, 1977).

d. Administration of cholecalciferol (Cheesman, Copping and Prebble, 1964), or 1,25 DHCC in vivo (Bikle, Zolock, Morrissey and Herman, 1977), or in vitro (Moriuchi, Yoshizawa, Shimura, Oku and Hosoya, 1977) increases the activity of alkaline phosphatase.

e. Addition of 1,25 DHCC to organ-cultured chick embryo duodenum (Corradino, 1977), or its injection into vitamin D-deficient rats (Walling, Brasitus, and Kimberg, 1976) increases the production of cAMP.

f. Administration of cholecalciferol results in an increase in the activity of Ca ATPase (Martin, Melancon and De Luca, 1969).

Vitamin D-stimulated calcium transport is inhibited by pre-treatment with RNA and protein synthesis inhibitors (Tsai, Midgett and Norman, 1973; Lawson and Emtage, 1973). This inhibition of calcium transport suggests that the synthesis of new mRNA and protein(s) is required for 1,25 DHCC to stimulate the absorptive process of calcium. CaBP has been reported to play a role in calcium absorption (Wasserman and Corradino, 1973). However, recent reports have demonstrated that 1,25 DHCC stimulates calcium transport before the

appearance of CaBP (Spencer, Charman, Wilson and Lawson, 1976; Lawson, Spencer, Charman and Wilson, 1977, 1978; Lawson, 1978). Furthermore, 1,25 DHCC induces calcium transport even when CaBP synthesis is blocked by actinomycin D or cycloheximide (Tanaka, De Luca, Omdahl and Holick, 1971; Zolock, Morrissey and Bikle, 1977; Bikle et al, 1977).

The mechanism of action of 1,25 DHCC is not known, but recent reports confirm that this mechanism is nuclear in origin (De Luca and Schnoes, 1978) and involves specific alteration in the gene. In chick intestine, cytoplasmic and nuclear receptor proteins for 1,25 DHCC have been reported (Brumbaugh and Haussler, 1974 a and b, 1975; De Luca and Schnoes, 1978). The cytoplasmic receptor has been shown to transfer the 1,25 DHCC to the intestinal chromatin (Procsal, Okamura and Norman, 1975; Haussler, Hughes, McCain, Zerwekh, Brumbaugh, Jubiz and Wasserman, 1977), which then promotes the synthesis of functional proteins, such as, CaBP (Corradino 1973).

1,25 DHCC also stimulates the transfer of inorganic phosphate across the intestine of rats and chicks (Harrison and Harrison, 1961; Wasserman and Taylor, 1973; Walling and Kimberg, 1975; Walling, 1977; Bonjour, Preston, Rizzoli and Fleisch, 1978). At the present time no phosphate-binding protein has been detected.

ii. The role of cholecalciferol in bone metabolism

There is a general agreement that 1,25 DHCC is the functional form of vitamin D_3 with regard to its action on bone (Raisz, Trummel, Holick and De Luca, 1972; Reynold, Holick and De Luca, 1973). The major defect in the mineralization of bone in vitamin D deficiency appears to be a lack of supply of calcium and phosphorus to the mineralization centre (see review by Sebrell and Harris, 1971).

The specific role of vitamin D in bone metabolism was first demonstrated by Carlsson (1952, 1954) who showed that administration of vitamin D to D-deficient, hypocalcemic rats maintained on a low calcium diet resulted in an increase in both calcium and phosphorus in the plasma. The only source of this increase was the bone as confirmed by pre-labelling the bone with ^{45}Ca. Three years later Crawford, Gribetz, Diner, Hurst and Castleman (1957) have confirmed the observations of Carlsson, and have suggested that the effect of

vitamin D on bone required PTH, since administration of vitamin D to D-deficient, parathyroidectomized, hypocalcemic animals had no effect on blood chemistry. On the other hand, administration of PTH to D-deficient rats had no effect on plasma concentrations of either calcium or phosphorus (Harrison, Harrison and Park, 1958).

The stimulation of bone resorption by 1,25 DHCC (Tanaka and De Luca, 1971; Raisz et al, 1972; Peacock, Taylor and Norman, 1977) is inhibited by calcitonin (Reynold, 1974; Peacock et al, 1977) and actinomycin D (Tanaka and De Luca, 1971) suggesting that a transcriptional event is involved in this process. A recent report indicates that 1,25 DHCC is localized in the nuclei of osteoblasts and osteocytes (De Luca and Schnoes, 1978).

The action of 1,25 DHCC on bone in vivo requires the presence of PTH (Garabedian et al, 1974b), whereas in organ culture 1,25 DHCC functions without PTH. Recently, Reynolds, Pavloritch and Balsam (1976) have reported that 1,25 DHCC increases bone resorption independently of PTH.

iii. The role of cholecalciferol in the kidney

It is generally accepted that the kidney plays a very important role in the control of calcium and phosphorus homeostasis. The effect of cholecalciferol and its metabolites on the metabolism of calcium and phosphorus in the kidney is a somewhat contraversial subject. Earlier reports (Crawford, Gribetz and Talbot, 1955; Ney, Kelly and Bartter, 1968) indicate that cholecalciferol decreases renal tubular reabsorption of phosphate, but during the last six years a number of reports have shown that cholecalciferol and its metabolites increase the renal reabsorption of calcium and phosphorus (Puschett, Moranz and Kurnick, 1972a; Puschett, Fernandez, Boyle, Gray, Omdahl and De Luca, 1972b; Puschett, Beck, Jelonek and Fernandez, 1974; Popovtzer, Robinette, De Luca and Holick, 1974; Costanzo, Sheehe and Weiner, 1974; Steele, Engle, Tanaka, Lorence, Dodgeon, and De Luca, 1975; Sutton, Harris, Wong and Dirks, 1977). It is not known whether or not PTH is involved in the action of cholecalciferol on the kidney. Some investigators suggest that cholecalciferol or its metabolites act directly on the kidney to reduce the renal clearance of calcium and phosphorus (Puschett et al, 1972, a and b; Sutton et al, 1977), while others observed that this action requires the presence of PTH (Popovtzer et al, 1974). PTH has a phosphaturic effect on the kidney, induction of an

extensive phosphate diuresis, (Samiy, Hirsch and Ramsay, 1960, 1965), in a process which does not require cholecalciferol (Arnaud, Rasmussen and Anast, 1966).

A vitamin D-dependent CaBP has been identified in the kidney (Sands and Kessler, 1971; Taylor and Wasserman, 1972), which correlates with the action of 1,25 DHCC on this organ.

iv. Cholecalciferol and parathyroid glands

The stimulatory effect of PTH on the production of 1,25 DHCC by the kidney has been discussed above. This role of PTH in the pathways of vitamin D_3 metabolism led some investigators to speculate that vitamin D_3 and its metabolites may have effects on the parathyroid glands. Until 1974 these glands were not considered to be target organs for the vitamin and its derivatives, but the recent experiments of Norman and his associates suggest that these glands could be target tissue for at least one metabolite. This group has studied the interaction between parathyroid glands and cholecalciferol in 10-week old, D-deficient chicks in a series of experiments. In the first paper, Henry and Norman (1975) studied the accumulation of 1,25 DHCC in parathyroid glands 16 h after administration of 720 pmole of ^{3}H-25-HCC, and observed that these glands are capable of accumulating 1,25 DHCC to a level equivalent to that observed in the intestine, the primary target tissue for 1,25 DHCC, and four times that found in the plasma. The response of parathyroid glands of D-deficient chicks to cholecalciferol and its metabolites has been demonstrated by Henry et al (Henry, Taylor and Norman, 1977; Henry, Wecksler and Norman, 1978). Administration of cholecalciferol to such animals reduced the weight of the hyperplastic parathyroid gland by 50%, 1,25 DHCC at small dose (1.3 nmole/d) had no effect, but this dose in combination with small amounts of 24,25 DHCC reduced the parathyroid gland weight significantly. The subcellular localization of 1,25 DHCC in the parathyroid gland is in the chromatin, since the chromatin fraction demonstrated a selective and specific localization of 1,25 DHCC (Wecksler, Henry and Norman, 1977; Henry et al, 1978). Moreover, specific cytoplasmic and nuclear receptor for 1,25 DHCC have been identified in the parathyroid gland of chicks (Brumbaugh, Highes and Haussler, 1975; Henry et al, 1978), and human parathyroid adenoma (Haussler et al, 1977). Furthermore, administration of D_3 metabolites resulted in changes in the ultrastructure of parathyroid gland of D-deficient chicks, these changes

included regression in the weight and suppression of synthetic organelles (Capen, Henry and Norman, 1977; Capen, 1978).

There are conflicting results concerning the effect of cholecalciferol and its metabolites on the secretion of PTH. In dogs cholecalciferol had no effect on the secretion of PTH in vivo, 25 HCC and 24,25 DHCC suppressed, and 1,25 DHCC stimulated the secretion in both dogs (Canterbury, Larmen, Claflin, Henry, Norman and Reiss, 1978) and goats (Care et al, 1975). On the other hand, 1,25 DHCC suppressed the release of PTH from slices of bovine glands in vitro (Chertow, Baylink, Wergedal, Su and Norman, 1975), and in rats in vivo (Horiuchi, Suda, Sasaki, Takahashi, Shimazawa and Ogata, 1976). Hurst and Mayer (1977) have failed to demonstrate consistent results on the effect of 1,25 DHCC on the secretion of PTH in calves. Finally, Care et al (Care, Pickard, Papapoulos, O'Riordan and Redel, 1978) have reported an inhibitory effect of 25,26 DHCC on the secretion of PTH in goats.

1.6 Gonadal Hormones and Calcium Metabolism

It is well recognized that gonadal hormones are involved in calcium and phosphorus metabolism of normal avian reproductive activity. It has been reported that the absorption of calcium and phosphorus is greatly enhanced in birds shortly prior to the onset of lay and during the egg shell formation (Taylor and Moore, 1954; Taylor and Kirkly, 1967; Bar and Hurwitz, 1972). The absorbed minerals are stored in the marrow cavities of long bones as medullary bone (Stringer, 1962), which appears to act as a labile reservoir of calcium. These physiological changes, formation of the medullary bone, can be induced by the administration of oestrogen and androgen in combination but not by either hormone alone, since birds given oestrogen alone become hypercalcemic but they do not retain calcium (Common, Rutledge and Hale, 1948). The increase in serum calcium is restricted to the non-diffusible calcium. It has been reported that oestrogens have a poor hypercalcemic effect when administered into D-deficient cocks (Hertelendy and Taylor, 1965). Androgens alone (Testosterone) have no effect on serum calcium or phosphorus or the rate of retention of these minerals (Common et al, 1948; Jowsey, Oliver, Maw and Common, 1953). The changes in the blood chemistry is restricted to the effect of oestrogen, but not to that of androgen, since, when an androgen is given together with an oestrogen, the androgen does not affect the changes in serum calcium and phosphorus evoked by oestrogen alone, but it does increase the rate

of retention of these minerals (Common et al, 1948). Thus, according to these authors androgens play no role in the increase of blood calcium and phosphorus, but play a role in their retention. The enhancement of calcium absorption during reproduction in birds is accompanied by an increase in the duodenal calcium-binding protein (Bar and Hurwitz, 1972), which is 1,25 DHCC dependent.

Kenny (1976) has reported that 1-hydroxylase is the predominant enzyme in the kidney of birds having an egg in oviduct. Oestradiol has been reported to stimulate and testosterone to inhibit the production of 1,25 DHCC (Baksi and Kenny, 1978b). On the other hand, Tanaka et al (1976) and Castillo et al (1977) have reported that androgens play a permissive role in the stimulation of 1,25 DHCC production by oestrogens.

Recent evidence shows that oestrogens stimulate the conversion of cholecalciferol to 25 HCC in the liver of male and immature female Japanese quail (Nicholson, 1976).

One of the aims of this project was to study the effect of male and female sex hormones and their interaction on the biosynthesis of the biologically active form of D_3, 1,25 DHCC. It was hoped that these results would help in the understanding of the mechanism by which gonadal hormones regulate calcium metabolism in birds during the reproductive period.

CHAPTER 2

CHAPTER 2. MATERIALS AND METHODS

2.1 Animals

This research was performed using home-bred Japanese quail (Coturnix coturnix japonica). Fertilized quail eggs were incubated at 37°C and 60% humidity in a forced draught Westernette incubator (Messrs. Western Incubator Ltd., East Hanningfield, Essex, England). After hatching quail were housed for two weeks in wooden cages fitted with a wire-mesh roof and incorporating an infra-red lamp. They were raised on turkey starter diet containing (per kg) 10 g Ca, 8 g total phosphorus and 50 µg cholecalciferol (2000 i.u.).

2.2 Injection of Animals

(i) Sex hormones injection. Oestradiol dipropionate (β-oestradiol - 3,17, dipropionate) was dissolved in ethyl oleate at a concentration of 1 mg/ml and administered as a single intramuscular injection into the breast muscle at dose levels shown in Chapter 6. Testosterone propionate was obtained from CIBA (Messrs. Ciba Ltd., Horsham), and it was already dissolved in sterile oily solution to give 10 mg/ml. It was diluted by ethyl oleate to give 1 mg/ml. Testosterone propionate was also administered as a single intramuscular injection into the breast muscle, dose levels shown in Chapter 6. Care was taken to prevent leakage of the injected materials from the site of injection. The birds were killed 24 hours after injection of sex hormones except in the study of the time course effect of sex hormones on 25 HCC metabolism. The control birds received the ethyl oleate vehicle only.

(ii) Injection of parathyroid hormone. Beef parathyroid extract (Eli Lilly, Basingstoke) was injected as a single subcutaneous injection at a dose level of 10 usp unit/100 g body weight into immature Japanese quail. The birds were killed 0, 1, 2, 3, 5 and 8 hours after injection. Adult males were given 20 or 50 usp units/bird. The control birds received the carrier only, 0.2% phenol, 1.6% glycerol in isotonic solution. Pure MRC PTH Batch No. 72/286 (The ampoule contained approximately 200 µg of a preparation of purified bovine parathyroid hormone with 2.5 mg mannitol) was dissolved as recommended by the Medical Research Council (1st International reference preparation of parathyroid hormone, bovine, for bioassay, 1974) and administered as a single intravenous

injection through the wing vein into immature birds. The control birds received the carrier only, sodium acetate, 1.0 g; Crystalline bovine serum albumin, 0.1 g, dissolved in 100 ml distilled water.

2.3 Chemicals

The following chemicals were used in the study of 25 HCC metabolism during this research:

Butyl PBD	Koch-Light Laboratories, Bucks
Dimethyl POPOP	Koch-Light Laboratories, Bucks
2,2 Diphenyloxazol	Koch-Light Laboratories, Bucks
Dipotassium Hydrogen Ortho Phosphate	BDH Chemical Ltd., Poole, England
Ethyl Oleate	BDH Chemical Ltd., Poole, England
Glucose-6-Phosphate	Sigma Chemical Company, London
Glucose-6-Phosphate Dehydrogenase	Sigma Chemical Company, London
Magnesium Chloride	BDH Chemical Ltd., Poole, England
L-Malic Acid	Sigma Chemical Company, London
Methallibure ADM 49177/73	ICI Ltd., Macclesfield, Cheshire
Nicotinamide Adenine Dinucleotide Phosphate	Sigma Chemical Company, London
β-Oestradiol-3,7,Dipropionate	Sigma Chemical Company, London
Parathyroid Extract	Eli Lilly and Company, Basingstoke
Parathyroid Hormone	MRC, London
Sulphanilamide	BDH Chemical Ltd., Poole, England
Sephadex LH-20	Pharmacia Fine Chemicals, London
Silica Gel GF 254 Type 60	Merck
Sodium Periodate	BDH Chemical Ltd., Poole, England
Sucrose	BDH Chemical Ltd., Poole, England
Testosterone Propionate	CIBA Laboratories Ltd., Horsham, Sussex
Toluene	Koch-Light Laboratories, Bucks
Triton x-100	Koch-Light Laboratories, Bucks

All other common chemicals used in this study were obtained from either BDH Chemicals or Koch-Light Laboratories.

2.4 Radiochemicals

The tritiated 25 HCC (25-hydroxy {26(27) - methyl - ^{3}H} cholecalciferol) was obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England. The specific radioactivities of the different batches of 25 HCC used were as follows: 7.3, 7.7, 9, 9.4, 11.3 and 11.7 Ci/mmole. The chromatographic homogenity of the tritiated 25 HCC was assessed using t.l.c. before incubations were carried out. The radioactive 25 HCC was stored as recommended by The Radiochemical Centre.

2.5 Liquid Scintillation Fluids

The liquid scintillation fluids used throughout were a Toluene-Butyl PBD designed for lipid soluble samples and consisting of the following:

Toluene (Sulphur free)	2.5 l
Butyl PBD	20 g

and a triton x-100 scintillation fluid for aqueous samples and consisting of the following:

Toluene	1.0 l
Triton x-100	0.5 l
PPO	4.0 g
Dimethyl POPOP	0.5 g

2.6 Assay of Renal 25 HCC Hydroxylases Activity

Birds were killed by decapitation. Both kidneys were quickly removed, blotted, and placed in an ice-cold, pre-weighed glass homogenizer tube. A 10% (w/v) kidney homogenate was prepared by adding ice cold sucrose (300 mM). The tissue was homogenized by motor homogenizer at not more than 400 r.p.m. as described by Kenny (1976). 0.1 ml of this homogenate (10 mg kidney tissue) was added to an ice-cold 25 ml conical flask containing 1.9 ml phosphate buffer pH 7.4. The final 2 ml of the incubation mixture contained, KH_2PO_4 (0.2 M), Mg^{++} (4 mM), L-Malate acid (7.5 mM), glucose-6-phosphate (3.5 mM), NADP (0.3 mM), and glucose-6-phosphate dehydrogenase (1.2 unit). The reaction was initiated by

adding 13 pmole of 25-(26,27-^{3}H) HCC dissolved in 10 µl 95% ethanol. The flask was incubated in air in a metabolic shaking incubator at 37°C for 10 or 20 minutes with gentle agitation. The reaction was terminated by pouring the content of the flask into a 15 ml centrifuge tube containing 6 ml chloroform-methanol (1:2, v/v). The residue remaining in the flask was washed with 2 ml chloroform-methanol (1:2, v/v).

2.7 Extraction of Radioactive Sterols

The 15 ml centrifuge tube, and contents, was stoppered, shaken vigorously, and left to stand on ice for 30 minutes. The denaturated proteins and debris were removed by centrifugation at 2000 r.p.m. for 15 minutes. The supernatant was transferred into a 15 ml centrifuge tube and 2.5 ml chloroform and 1.25 ml distilled water were added. The tube was stoppered, shaken vigorously, and centrifuged at 2000 r.p.m. for 20 minutes as described by Kenny (1976). The upper aqueous phase was removed into a 25 ml separating funnel and re-extracted twice with 2.5 ml chloroform. Following phase separation, the lower chloroform layers were run off into a 100 ml round-bottomed flask. The three chloroform layers (extracts) were pooled and evaporated to dryness using a suction water pump. The lipid and the radioactive sterols were taken up in a small volume of chloroform for the chromatographic analysis. Care was taken during all extraction and chromatographic procedures to minimize exposure of 25 HCC and its metabolites to both natural and artificial lights.

2.8 Chromatographic Procedures

(i) Sephadex LH-20 Column. The dry extract was dissolved in 200 µl chloroform-petroleum ether, 40°-60° (65:35) in a 1.75 ml glass vial and applied to a 7 x 180 mm column of Sephadex LH-20 suspended in chloroform-petroleum ether 40°-60° (65:35) in a 7 x 200 mm glass column, the Sephadex LH-20 was treated with the solvent 24 hours before use. A further aliquot (100 µl) of the solvent was immediately added to the vial and applied to the column. The column was eluted with chloroform-petroleum ether 40°-60° (65:35) at a flow rate of not more than 40 drops per minute as described by Kenny (1976). A 200 ml separating funnel filled with the solvent was used as a reservoir and fifty 1 ml fractions were collected in test tubes. At the end of the chromatographic procedure the fractions were transferred into scintillation vials and

allowed to air-dry overnight. Figure 2 shows the behaviour of 25 HCC and its metabolites on Sephadex LH-20.

(ii) Thin Layer Chromatography. At the beginning of this research, column chromatography was used to separate 25 HCC and its metabolites. But for a number of reasons, thin layer chromatography was used to separate the radioactive sterols in most of this study. This technique was found to be completely satisfactory for samples containing small amounts of lipid. All thin layer plates used throughout were prepared by the following procedure:

A slurry of fluorescent silica gel (GF_{254} Type 60, Merck) was prepared in distilled water (50% w/v) and spread to 0.75 mm thickness on a 40 x 200 mm clean glass plate. The plate was allowed to stand for 1 hour at the laboratory temperature and then transferred to an oven at 100°C, where it was dried for 3 hours. It was then removed and allowed to cool before use. To some samples enough cold (non-radioactive) 25 HCC to be visible under u.v. light was added before applying the extract to the silica gel. The lipid extract was applied to the gel in a narrow band and the elution was carried out in the dark at 4°C. The major solvent system used in the study of 25 HCC metabolism in the kidney was ethyl acetate-n-heptane (1:1, v/v), although other solvent systems described later in this Theses were used to identify the metabolites produced in vitro from 25 HCC. At the end of the chromatographic procedure, the plate was removed from the tank and allowed to dry for 10 minutes in the dark at 4°C. Thereafter the plate was re-run again to the same distance. At the end of the chromatographic run, 5 mm bands were removed from the origin up to 10 mm above the 25 HCC region directly into scintillation vials (in samples where cold 25 HCC was not added, 5 mm bands were removed from the origin until the solvent front). The radioactivity in each fraction was determined and plotted against distance from the origin as a histogram. Figure 3 shows the profile obtained after chromatographing an extract of an incubation mixture of ^{3}H-25 HCC with a kidney homogenate.

2.9 Determination of Radioactivity

The radioactive sterols either from Sephadex LH-20 column or from thin layer chromatographic fractions were dissolved in 10 ml toluene butyl PBD counting solution. The silica gel particles in the vials were allowed to settle before counting. The aqueous phase was extracted twice with chloroform as described in

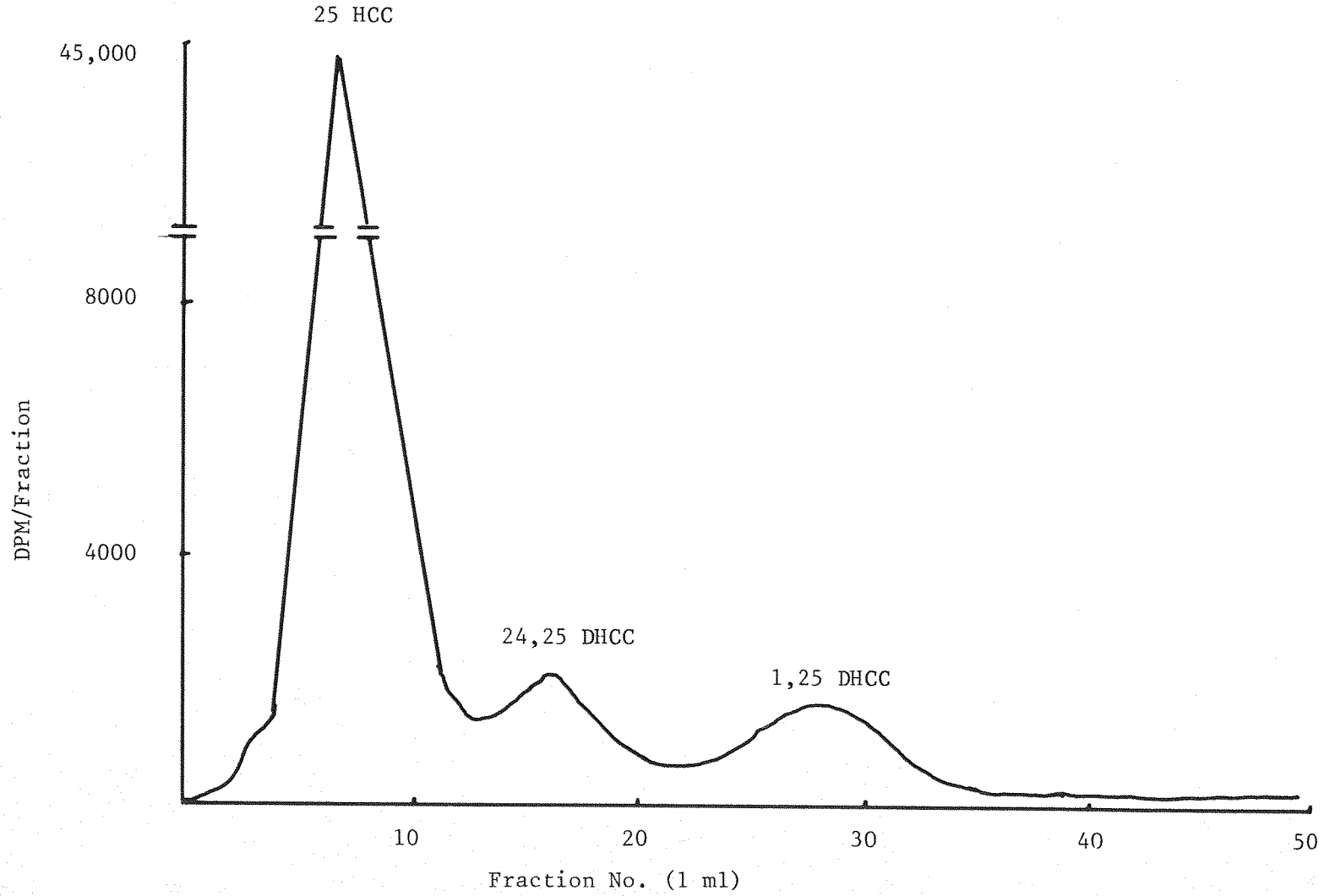


Figure 2. Chromatographic profile of extract prepared after incubating kidney homogenate from bird with an egg in the oviduct with ^{3}H-25 HCC. 7 x 200 mm Sephadex LH-20 column was used employing chloroform-petroleum ether (40-60^{o}) (65:35) as the eluting solvent.

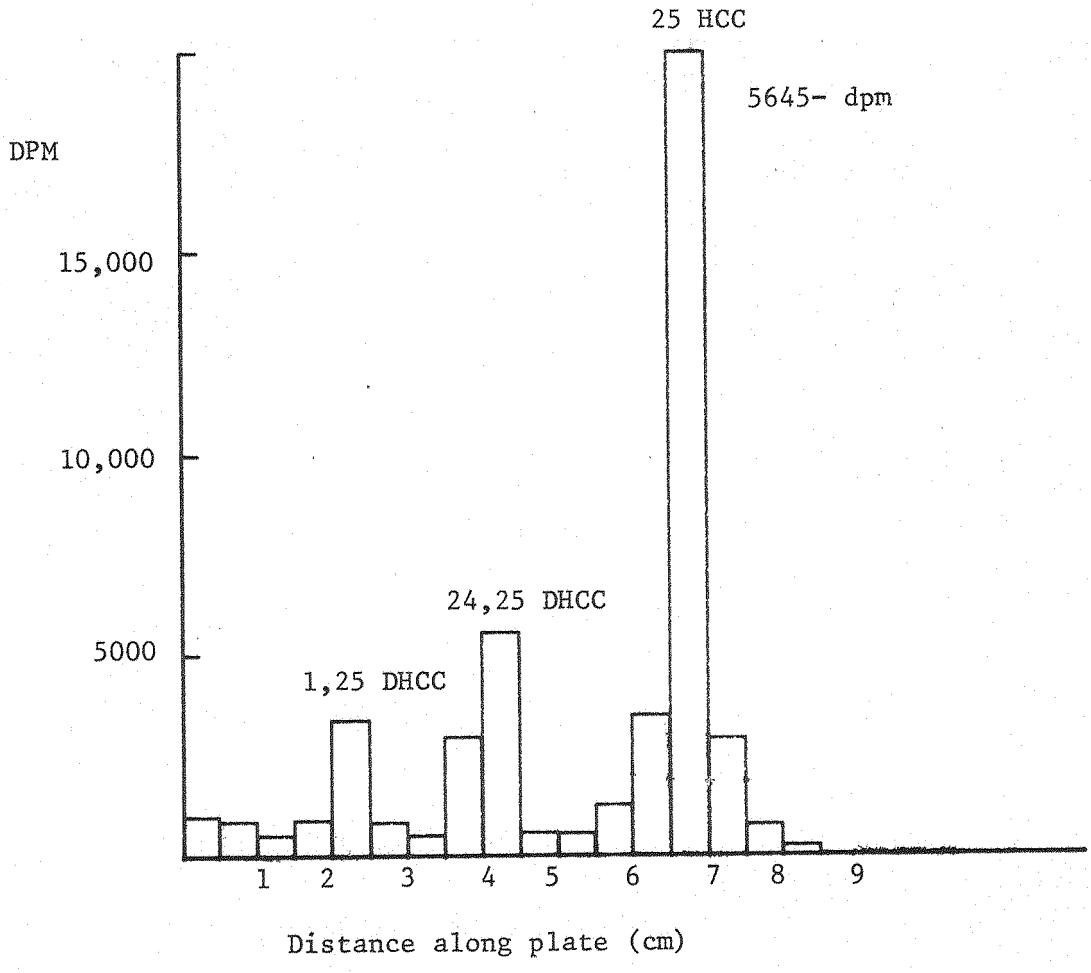


Figure 3. t.l.c. profile of extract prepared after incubating ^{3}H-25 HCC with a kidney homogenate of immature bird. Solvent system, ethyl acetate: n-heptane (1:1)

Section 2.7: 0.5 ml of this solution was transferred into a scintillation vial and 10 ml of a triton based scintillant was added. All samples were counted in a Philips Liquid Scintillation Analyser at 12°C with an automatic quench correction.

2.10 Determination of Plasma Calcium

Plasma calcium concentrations (total calcium) were determined using an Atomic Absorption Spectrophotometer (SP 90A Series 2, Atomic Absorption Spectrophotometer, Pye-Unicam Ltd., Cambridge, England) with specific calcium lamp. Samples of 0.1 ml were diluted with 50 volumes of a solution of lanthanum chloride containing 10 mmole $LaCl_3$ and 50 mmole HCl/l. Standards were prepared from Analar Calcium Carbonate and contained the same concentration of $LaCl_3$.

CHAPTER 3

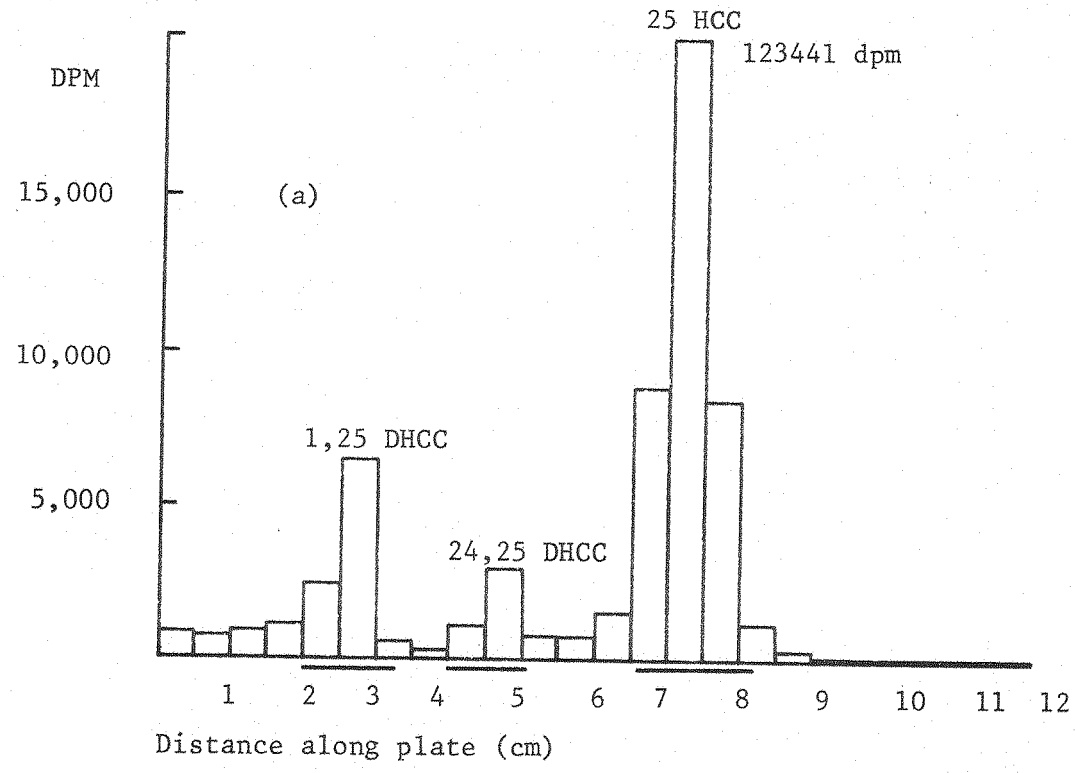
CHAPTER 3. THE IDENTIFICATION OF 1,25 AND 24,25 DHCC PRODUCED IN VITRO

The final extracts of the incubation mixtures of ^{3}H-25 HCC with kidney homogenates were chromatographed as described in section 2.8 (Figure 2 and 3). Cold synthetic 1,25 DHCC and 24,25 DHCC (obtained from Hoffmann-La Roche Inc, Nutley, by the courtesy of Dr. M.R. Uskokovic) were used to identify the biologically produced 1,25 DHCC and 24,25 DHCC. Enough non-radioactive 1,25 DHCC, 24, 25 DHCC and 25 HCC were added to the extract of the incubation mixture. The extract, including the cold compounds, were subjected to thin layer chromatography using fluorescent silica gel and two separate solvent systems, ethyl acetate: n-heptane (1:1) and chloroform; glacial acetic acid (95:5). The chromatographic profiles are displayed in Figure 4a and b. The chromatograms show that the radioactivity of the biologically produced 1,25 and 24,25 DHCC were recovered in the bands of the cold 1,25 and 24,25 DHCC respectively.

The suspected 1,25 DHCC and 24,25 DHCC fractions obtained from Sephadex LH-20 chromatography were pooled, combined and chromatographed separately with enough unlabelled 1,25 and 24,25 DHCC to be visible under u.v. light, using thin layer chromatography and the two different solvent systems, ethyl acetate: n-heptane (Figure 5a and b) (1:1) and chloroform; glacial acetic acid (95:5) (Figure 6a and b). The suspected ^{3}H-1,25 and 24,25 DHCC were shown to co-chromatograph with the non-radioactive 1,25 DHCC and 24,25 DHCC respectively in an indistinguishable mobility. The metabolites obtained from both the chromatographic techniques were further identified by treating them with sodium periodate. Periodate oxidizes any substance which has hydroxyl groups on adjacent carbon atoms with rupture of carbon-carbon bond. Figure 7 shows the periodate reaction with dihydroxylated derivatives of cholecalciferol.

The tritiated 1,25 DHCC and 24,25 DHCC produced in vitro from 25-hydroxy {26(27)-^{3}H} cholecalciferol and obtained either from Sephadex LH-20 chromatography or thin layer chromatography were dissolved in 2 ml methanol, and separately treated with 0.1 ml 5% aqueous $NaIO_4$ for seven hours. The radioactive compounds were extracted by adding 2 ml chloroform and 1 ml tap water. The aqueous phase was re-extracted with 1 ml chloroform. The radioactivity present in each phase was determined and the percentage loss of radioactivity from C-26 and C-27 was calculated (Table 1). The suspected ^{3}H-24,25 DHCC was

Figure 4. The comparison of the biologically produced 1,25 and 24,25 DHCC and cold synthetic 1,25 and 24,25 DHCC using t.l.c. Solvent system a (above) ethylacetate: n-heptane (1:1), b(below) chloroform, acetic acid glacial (95:5). The position of the synthetic hydroxylated compounds visualized under u.v. light are shown below the histogram.



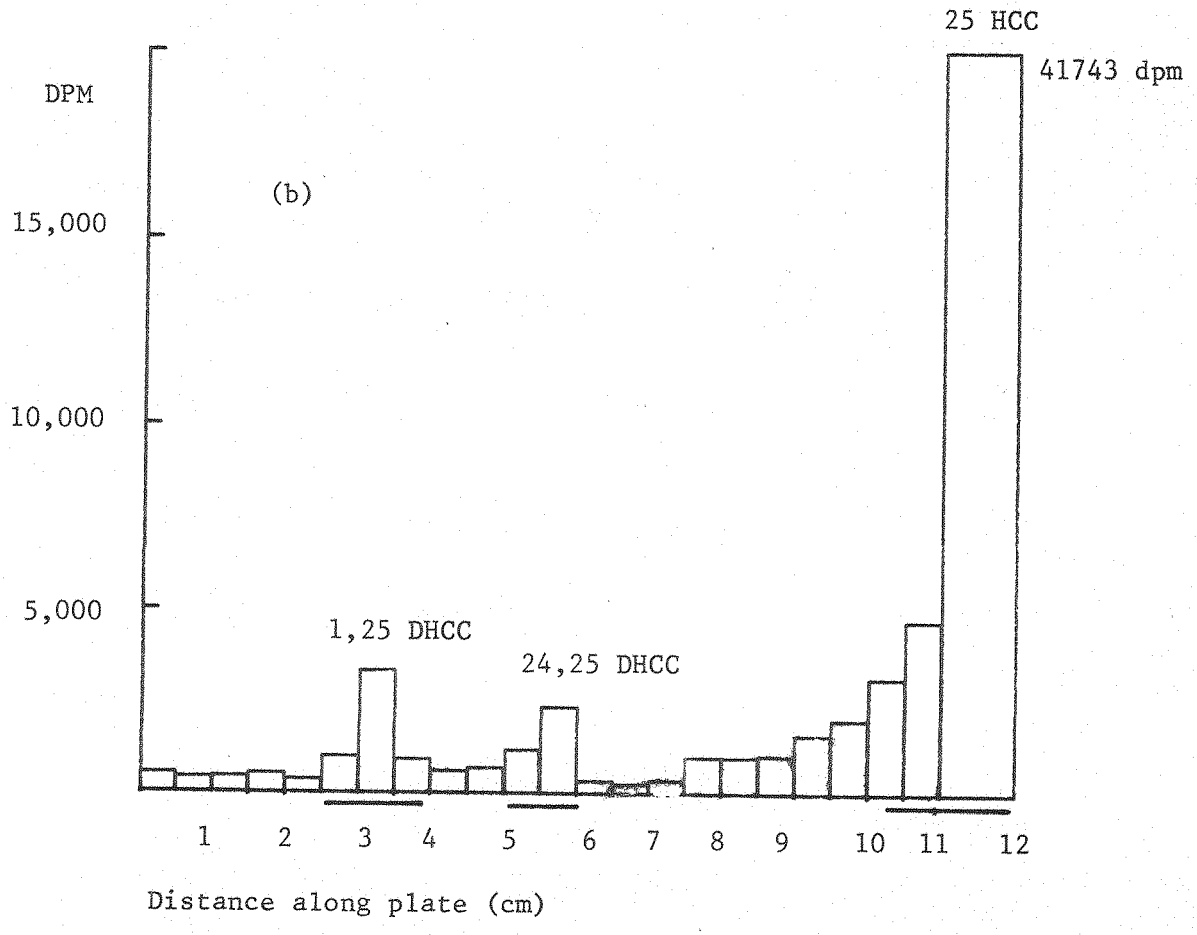
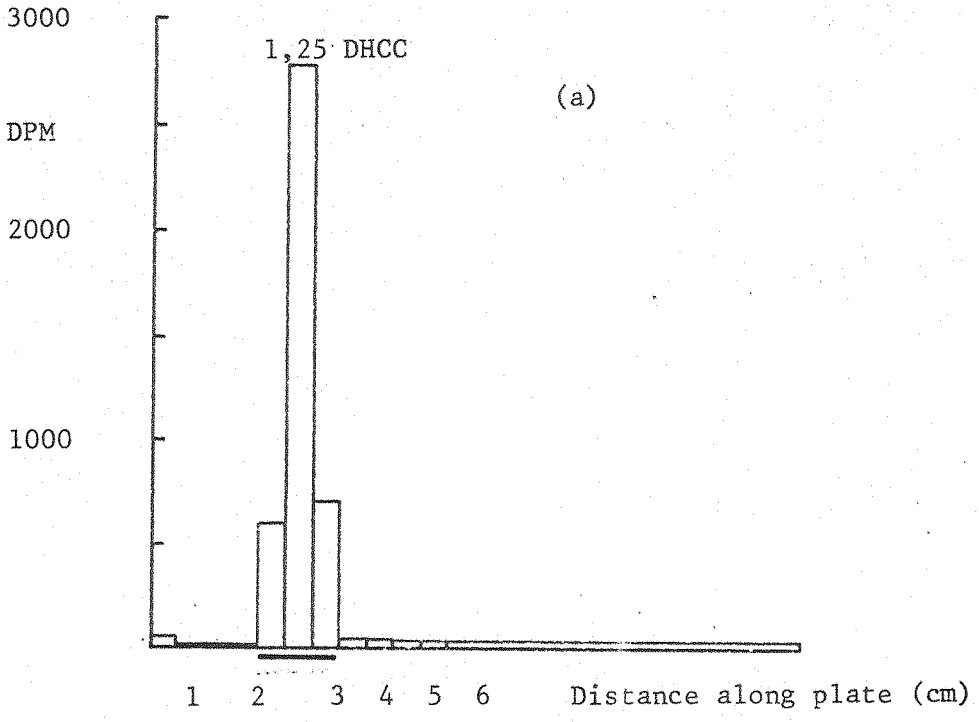


Figure 5. The t.l.c. of the biologically produced 1,25 and 24,25 DHCC pooled from their region of the Sephadex LH-20, and cold synthetic 1,25 and 24,25 DHCC. Solvent system, ethylacetate: n-heptane (1:1), a (above) 1,25 DHCC, b (below) 24,25 DHCC. The position of the synthetic dihydroxylated compounds visualized under u.v. light are shown below the histogram.



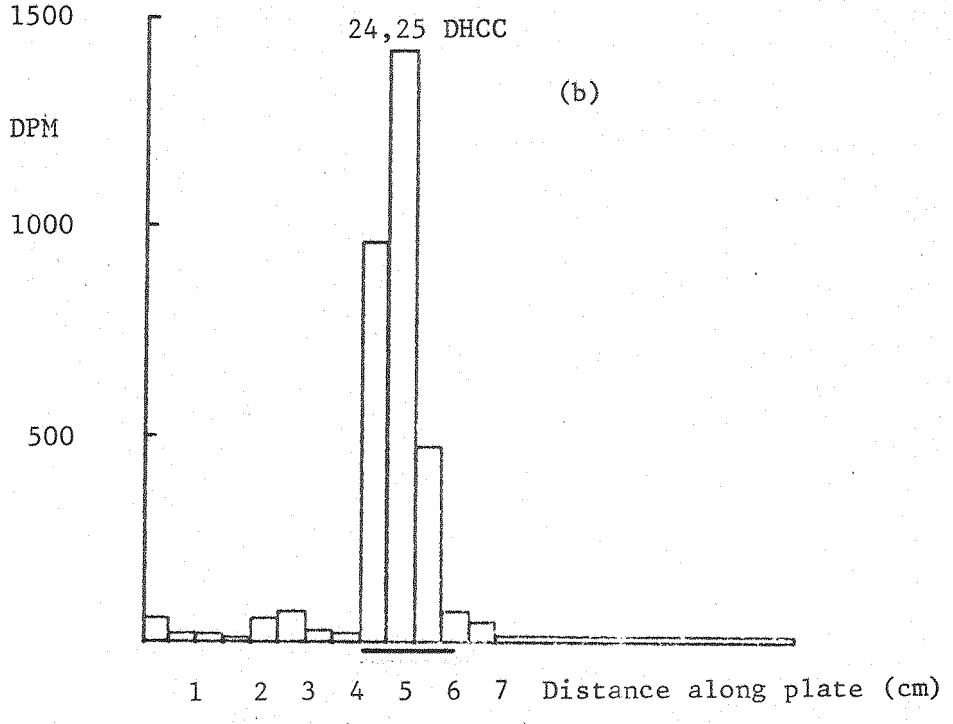
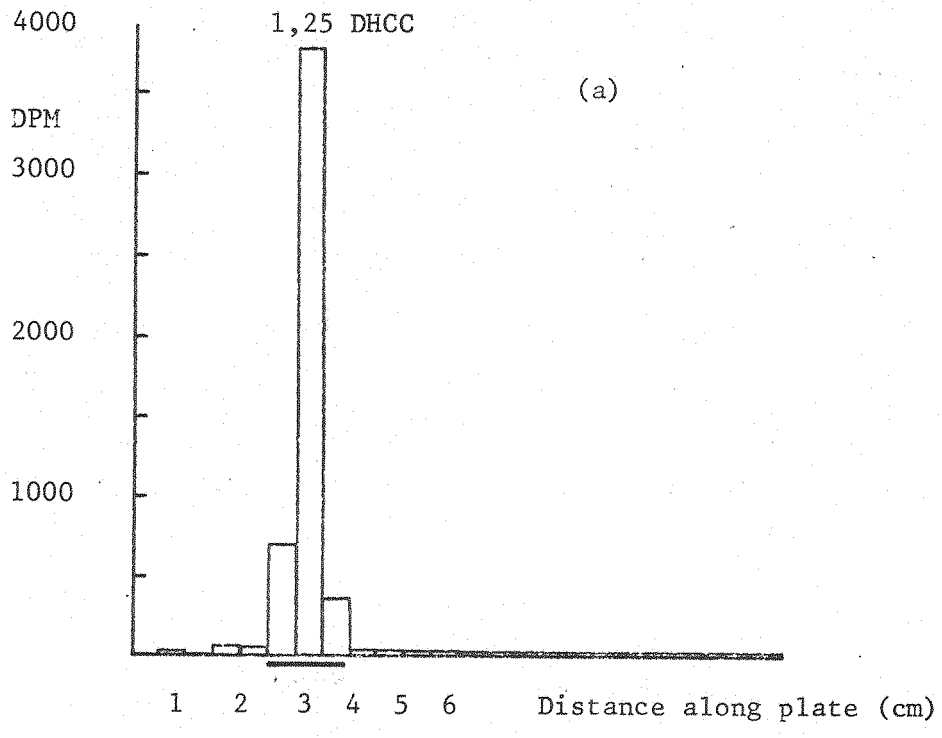


Figure 6. The t.l.c. of the biologically produced 1,25 and 24,25 DHCC pooled from their region of the Sepahdex LH-20, and cold synthetic 1,25 and 24,25 DHCC. Solvent system, chloroform: acetic acid glacial (95:5). a (above) 1,25 DHCC, b (below) 24,25 DHCC. The position of the synthetic dihyroxylated compounds visualized under u.v. light are shown below the histogram.



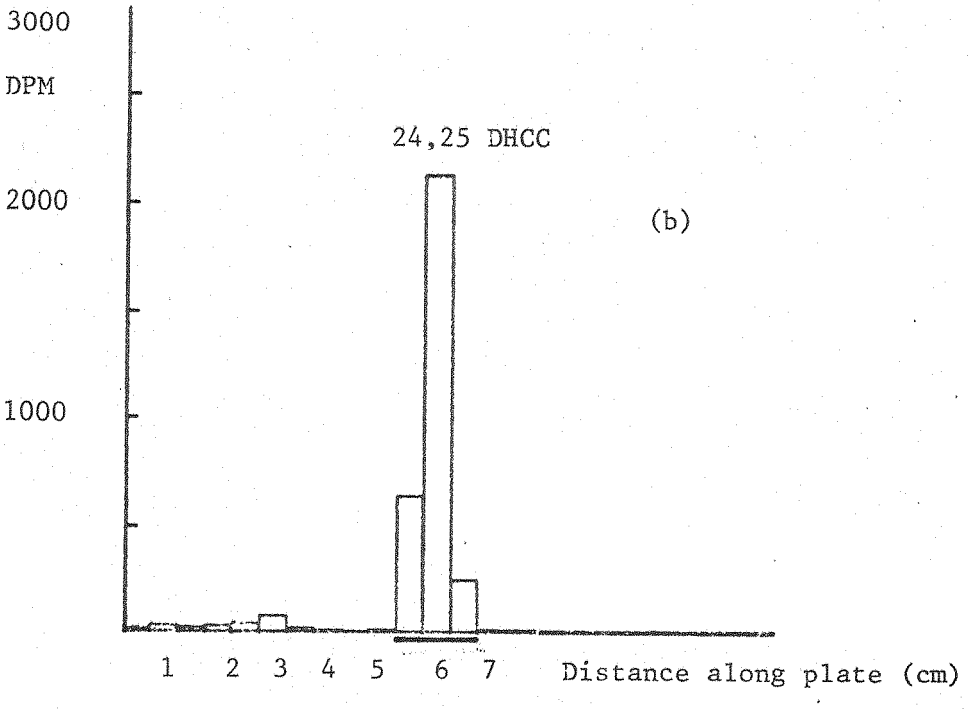
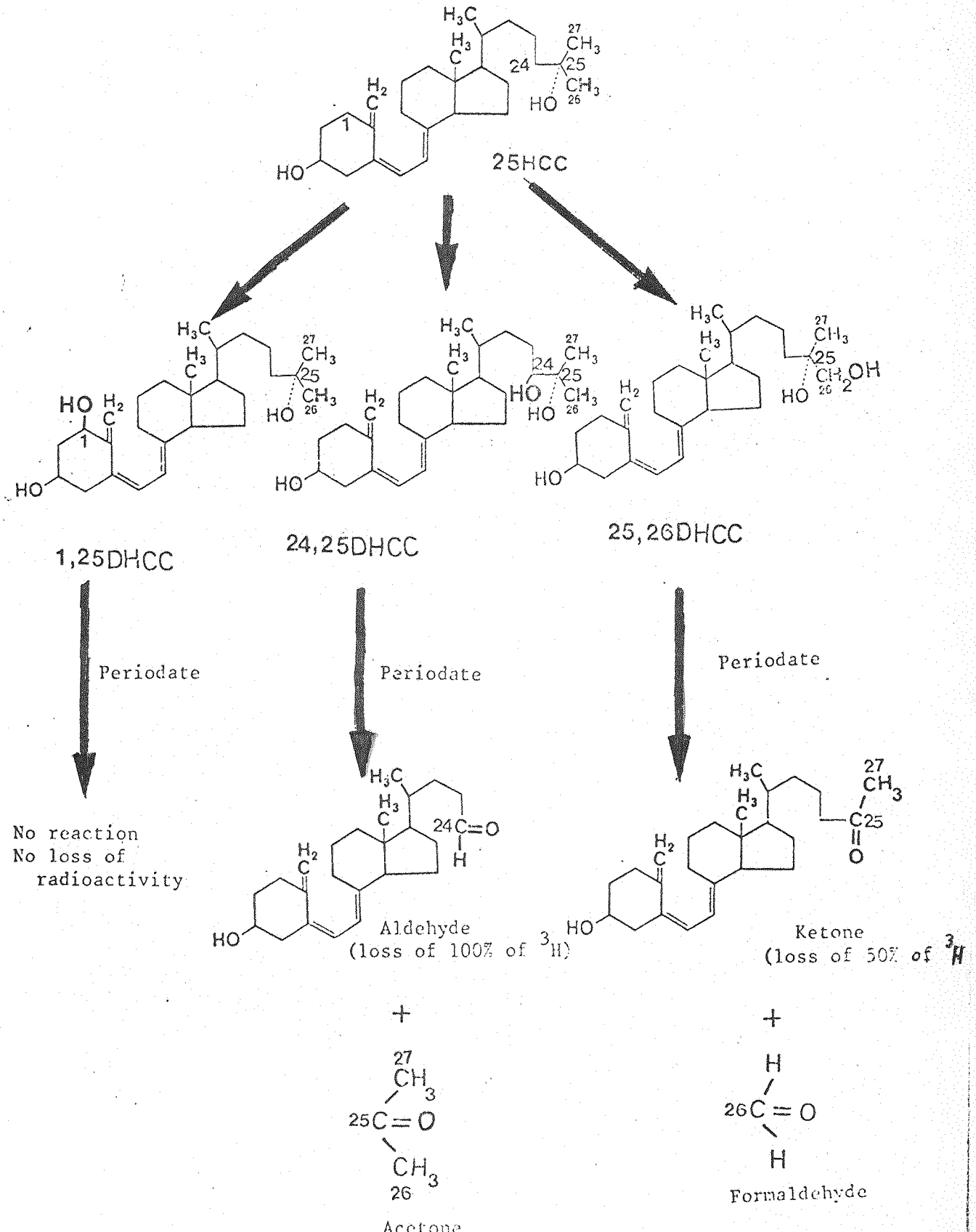


Figure 7. Periodate-sensitive and insensitive metabolites of cholecalciferol generated from 25 HCC. The tritium label on 25 HCC used as substrate for the renal hydroxylases is found on carbons 26 and 27.



about 70% sensitive to periodate cleavage, and about 30% remained as 24,25 DHCC. Incubation of 1,25 DHCC with $NaIO_4$ revealed that the major portion 89-96% remained as 1,25 DHCC. This treatment with periodate indicates that none of the major peaks produced in vitro after incubation of kidney homogenate with (26,27-^{3}H) 25 HCC is likely to be 25,26 DHCC, since this compound is sensitive to periodate oxidation with maximum 50% loss of radioactivity (Figure 7). Nevertheless, a slight possibility remains that peaks claimed to be 1,25 and 24,25 DHCC may be contaminated with cochromatographing metabolites. However, Tanaka et al (1976), Kenny (1976) and Castillo et al (1977) working with the same birds - Japanese quail - and using rigorous analytical techniques did not observe any unusual metabolites that chromatographed with 1,25 DHCC and 24,25 DHCC.

After using the above mentioned approaches, it was concluded that the main radioactive peak generated in vitro from 25 HCC by kidney homogenates prepared from laying females oestrogenized, and PTH-treated birds is 1,25 DHCC or very similar to it.

Table 1. The reaction with sodium periodate of tritiated 1,25 and 24,25 DHCC, produced in vitro by incubating tritiated 25 HCC with kidney homogenates of Japanese quail (numbers of samples in parentheses).

Metabolite	% of radioactivity recovered in $CHCl_3$ phase	% of radioactivity in aqueous phase
24,25 DHCC (column)	26.4 ± 5.9 (2)	73.6 ± 5.9 (2)
1,25 DHCC (plate)	89.3 ± 2.92 (3)	10.7 ± 2.92 (3)
1,25 DHCC (column)	96.17 ± 2.72 (3)	3.83 ± 2.72 (3)

CHAPTER 4

CHAPTER 4. THE METABOLISM OF 25 HCC BY THE KIDNEY OF JAPANESE QUAIL IN RELATION TO AGE

A group of 3 - 6 birds was killed weekly up to the fourth week after hatching, thereafter the remaining birds were weighed and housed in pairs (male and female) in wire cages. One group of females and another group of males were killed on the following days of age 30, 33, 35, 37, 39, 42 and 45. On day 45 the first egg appeared in the oviduct. The object of these experiments was to study the changes that occur in the metabolism of 25 HCC in the kidney of Japanese quail during development.

4.1 Female Birds

The results of this study are shown in Figure 8 and Table 2. The production of 1,25 DHCC was relatively high during the first two weeks after hatching. On day 21 it declined and remained fairly low and constant until day 33 of age, after which, the activity of 1-hydroxylase increased steadily reaching the highest value on day 45, when the first egg appeared in the oviduct.

From 7 - 39d of age, the 24-hydroxylase was more active than the 1-hydroxylase, but the activity of this enzyme was completely suppressed 3 days or so before the appearance of the first egg in the oviduct.

The production of 1,25 DHCC and 24,25 DHCC in relation to the ovarian development is shown in Figure 9 and Table 2. During the slow stage of the growth of the ovary the production of 1,25 DHCC was quite low and constant, but during the rapid growth of the ovary, there was an increase in the activity of 1-hydroxylase and a marginal decrease in the 24-hydroxylase activity. Figure 10 shows the individual values of conversion of 25 HCC to 1,25 DHCC plotted against the log weight of ovaries.

4.2 Male Birds

The production of 1,25 and 24,25 DHCC by the kidney of male bird is shown in Figure 11 and Table 3. During the sexual development of these birds, there was a steady decrease in the activity of 1-hydroxylase reaching zero value on

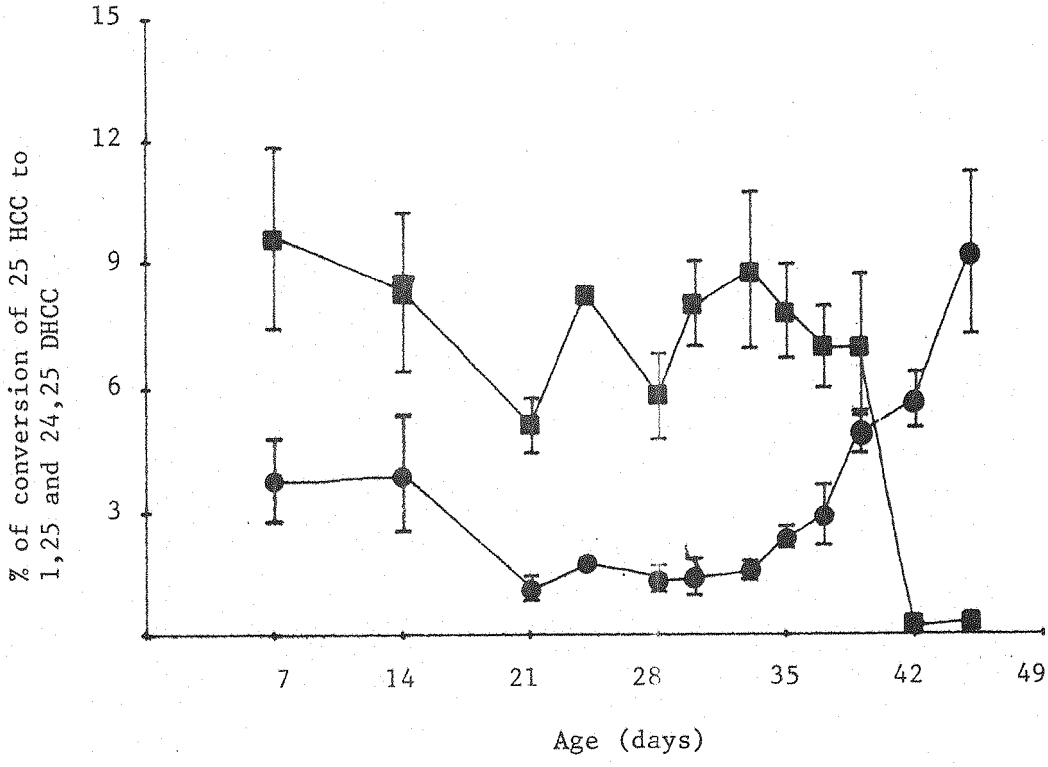


Figure 8. Changes with age in the mean percentage (± SEM) of 25 HCC converted to 1,25 (●), and 24,25 DHCC (■) in vitro by kidney homogenates of FEMALE birds.

Age (d)	No. of Birds	1,25 DHCC	24,25 DHCC	Mean weight of ovaries (mg)	$\log_{10}$ weight of ovaries	Plasma Ca (mg/100 ml)
*7	6	3.8 ± 1.02	9.60 ± 2.19	-	-	10.8 ± 0.10
*14	3	3.96 ± 1.42	8.30 ± 1.93	-	-	10.7 ± 0.10
21	3	1.13 ± 0.32	5.10 ± 0.68	44	1.643	10.4 ± 0.17
24	2	1.70 ± 0.10	8.20 ± 0.20	49	1.690	10.0 ± 0.29
28	2	1.35 ± 0.35	5.80 ± 1.05	60	1.778	10.4 ± 0.23
30	5	1.40 ± 0.47	8.00 ± 1.04	83	1.919	14.7 ± 0.26
33	5	1.57 ± 0.26	8.80 ± 1.90	111	2.045	16.1 ± 0.59
35	4	2.40 ± 0.30	7.80 ± 1.13	119	2.076	18.3 ± 0.52
37	3	2.93 ± 0.76	6.96 ± 1.00	144	2.158	22.7 ± 0.47
39	5	4.93 ± 0.53	7.00 ± 1.70	913	2.960	24.3 ± 0.35
42	3	5.7 ± 0.68	0.20 ± 0.05	3118	3.494	34.0 ± 1.00
†45	4	9.2 ± 1.96	0.35 ± 0.20	4958	3.695	22.7 ± 0.52

* mixed sexes
† first egg appeared in the oviduct

Table 2. Changes with age in the mean percentage (± SEM) of 25 HCC converted into 1,25 and 24,25 DHCC in vitro by kidney homogenates of <u>female</u> Japanese quail and serum calcium values.

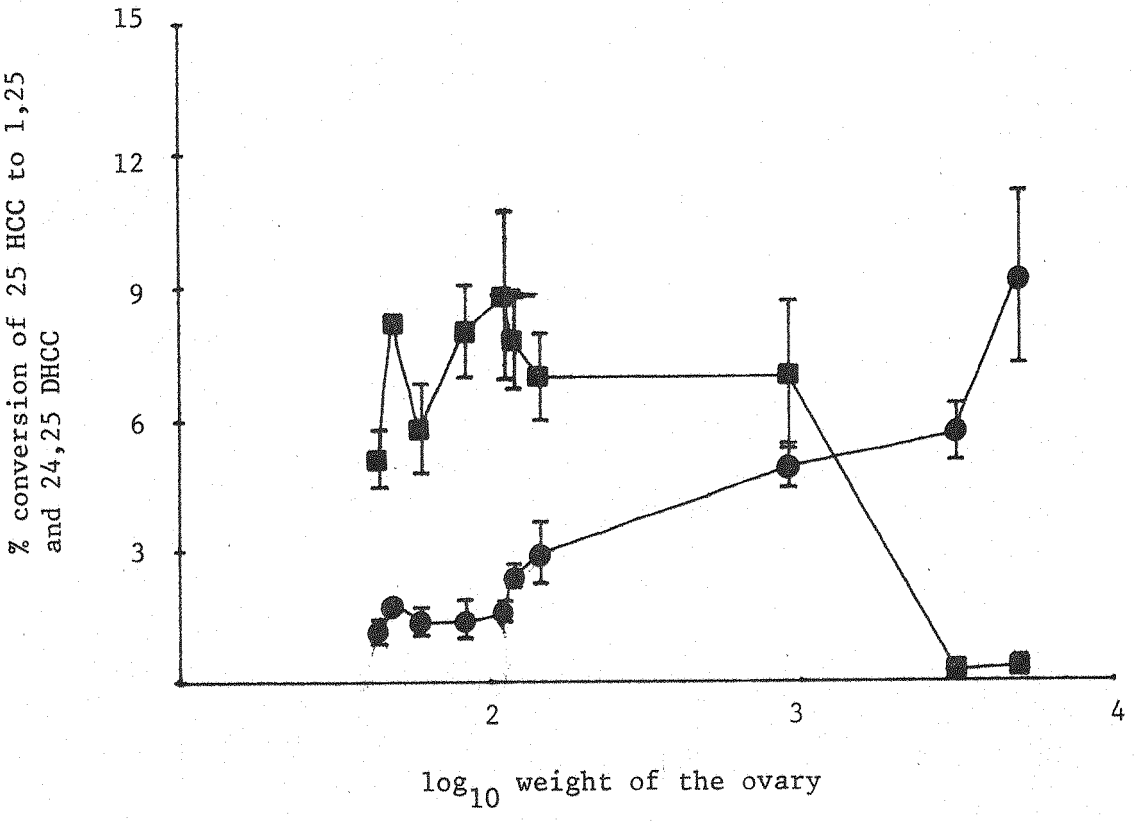


Figure 9. Production of 1,25 DHCC (●), and 24,25 DHCC (■), in relation to ovarian development

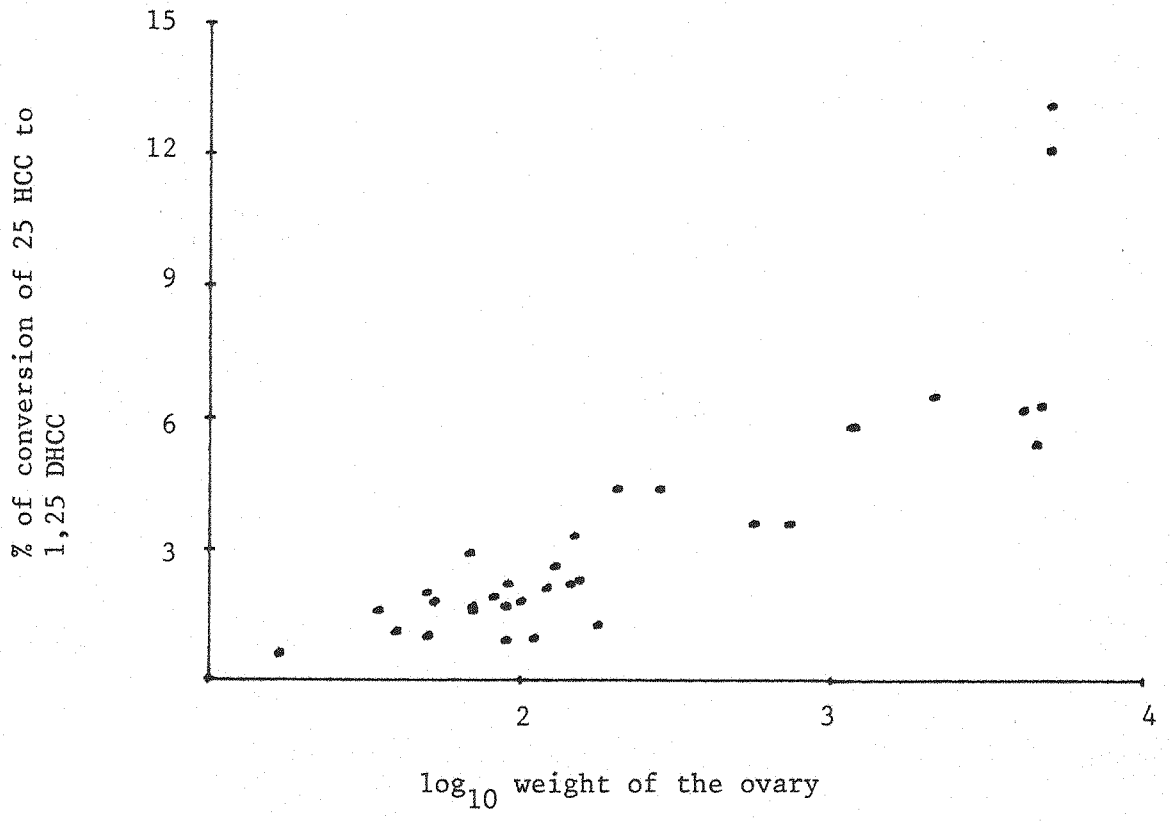


Figure 10. The individual values of percentage of conversion of 25 HCC to 1,25 DHCC in relation to the ovarian development.

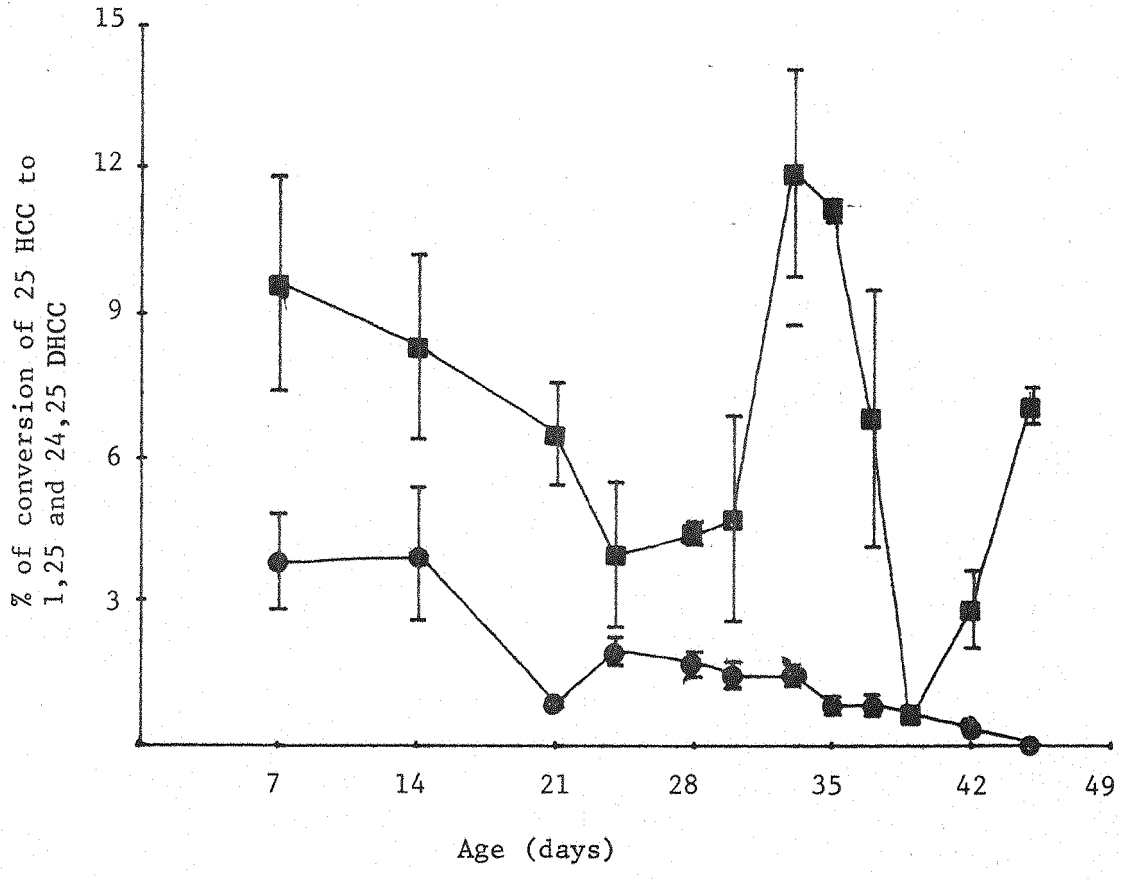


Figure 11. Changes with age in the mean percentage (± SEM) of 25 HCC converted to 1,25 DHCC (●), and 24,25 DHCC (■) in vitro by kidney homogenates of MALE birds

Age (d)	No. of Birds	1,25 DHCC	24,25 DHCC	Mean weight of testes (mg)	$\log_{10}$ weight of testes	Plasma Ca (mg/100 ml)
7	6	3.8 ± 1.02	9.60 ± 2.19	-	-	10.80 ± 0.10
14	3	3.96 ± 1.42	8.30 ± 1.93	-	-	10.70 ± 0.10
21	3	0.83 ± 0.08	6.50 ± 1.09	70	1.845	10.50 ± 0.12
24	2	1.9 ± 0.30	3.95 ± 1.55	78	1.892	9.80 ± 0.25
28	3	1.63 ± 0.28	4.40 ± 0.25	90	1.956	10.20 ± 0.06
30	3	1.4 ± 0.28	4.70 ± 2.20	136	2.132	9.80 ± 0.25
33	3	1.4 ± 0.23	11.90 ± 2.15	245	2.389	10.10 ± 0.05
35	3	0.80 ± 0.2	11.10 ± 0.23	395	2.597	10.40 ± 0.15
37	3	0.80 ± 0.23	6.80 ± 2.70	784	2.894	10.20 ± 0.08
39	3	0.60 ± 0.15	0.50 ± 0.08	1693	3.229	10.00 ± 0.15
42	3	0.3 ± 0.08	2.80 ± 0.84	1936	3.287	10.03 ± 0.03
45	3	0.0 ± 0.0	7.10 ± 0.40	2198	3.342	10.03 ± 0.03

Table 3. Changes with age in the mean percentage ($\pm$ SEM) of 25 HCC converted into 1,25 and 24,25 DHCC in vitro by kidney homogenates of male Japanese quail and serum calcium values.

day 45 of age, the time in which the first egg appeared in the oviduct of female birds from the same batch. The production of 24,25 DHCC fluctuated reaching a very low level on day 39, then increasing again on days 42 and 45. The activity of 1-hydroxylase in relation to the weight of testes is shown in Figure 12 and Table 3. There was an inverse relationship between the development of the testes and the production of 1,25 DHCC.

4.3 Changes in Plasma Calcium and Weight of Gonads of Japanese Quail in Relation to Their Age

The plasma calcium level of females began to rise on day 30 of age (Fig. 13a and Table 2) and continued to increase reaching a peak value on day 42, three of more days before the calcification of the first egg took place. On day 45 and during the existence of a calcified egg in the oviduct the level of plasma calcium was reduced.

The rapid growth of follicles in the ovary started on day 39 (Fig. 13b and Table 2), five or more days before the first ovulation. In contrast to the female birds, no changes in the plasma calcium occurred in relation to age or to weight of testes of male birds (Fig. 14a, b and Table 3).

4.4 Kinetic Studies on the Production of 1,25 DHCC

In order to determine the most suitable incubation conditions under which the production of 1,25 DHCC is near maximum, it was essential to carry out a simple kinetic study using a fixed substrate concentration of 13 pmole, incubation time of up to 20 minutes, and homogenate volume of up to 0.2 ml.

The conversion of 25 HCC to 1,25 DHCC was linear with respect to the incubation time up to 5 minutes (Fig. 15a), after which it slightly departed from linearity. With respect to the homogenate volume, the production of 1,25 DHCC was approximately linear up to 0.1 ml (Fig. 15b) after which, it marginally departed from linearity reaching a plateau at 0.2 ml homogenate volume. The results show that using 0.1 ml kidney homogenate and an incubation of 10 minutes duration to be suitable for the study of 25 HCC metabolism. Increasing the homogenate volume over 0.1 ml or using an incubation time of longer than 10 minutes resulted in a very slight increase in the percentage of conversion of 13 pmole of 25 HCC to 1,25 DHCC (Fig. 15a, b). Thus it was decided to assay the

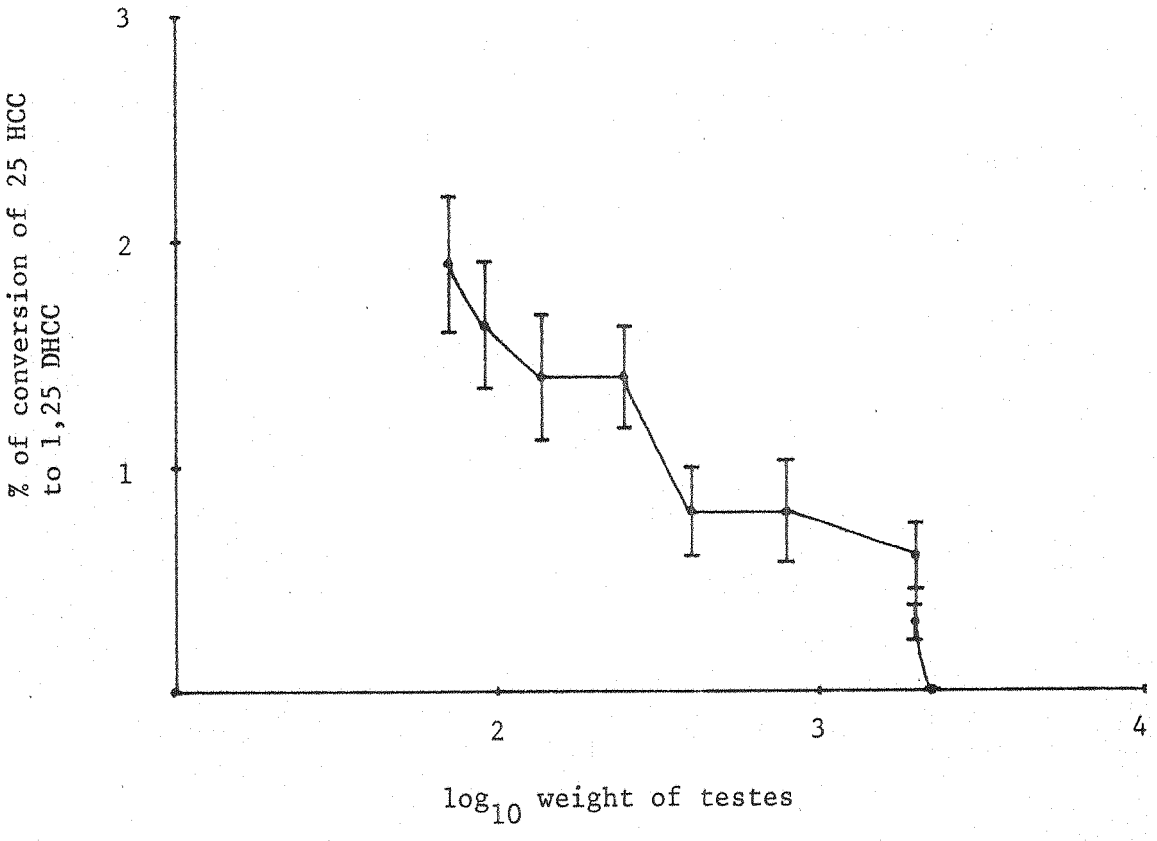


Figure 12. In vitro production of 1,25 DHCC by the kidney of male birds in relation to the development of gonads.

Figure 13. Changes with age in, a (above) plasma calcium, and b (below) weight of gonads of female Japanese quail.

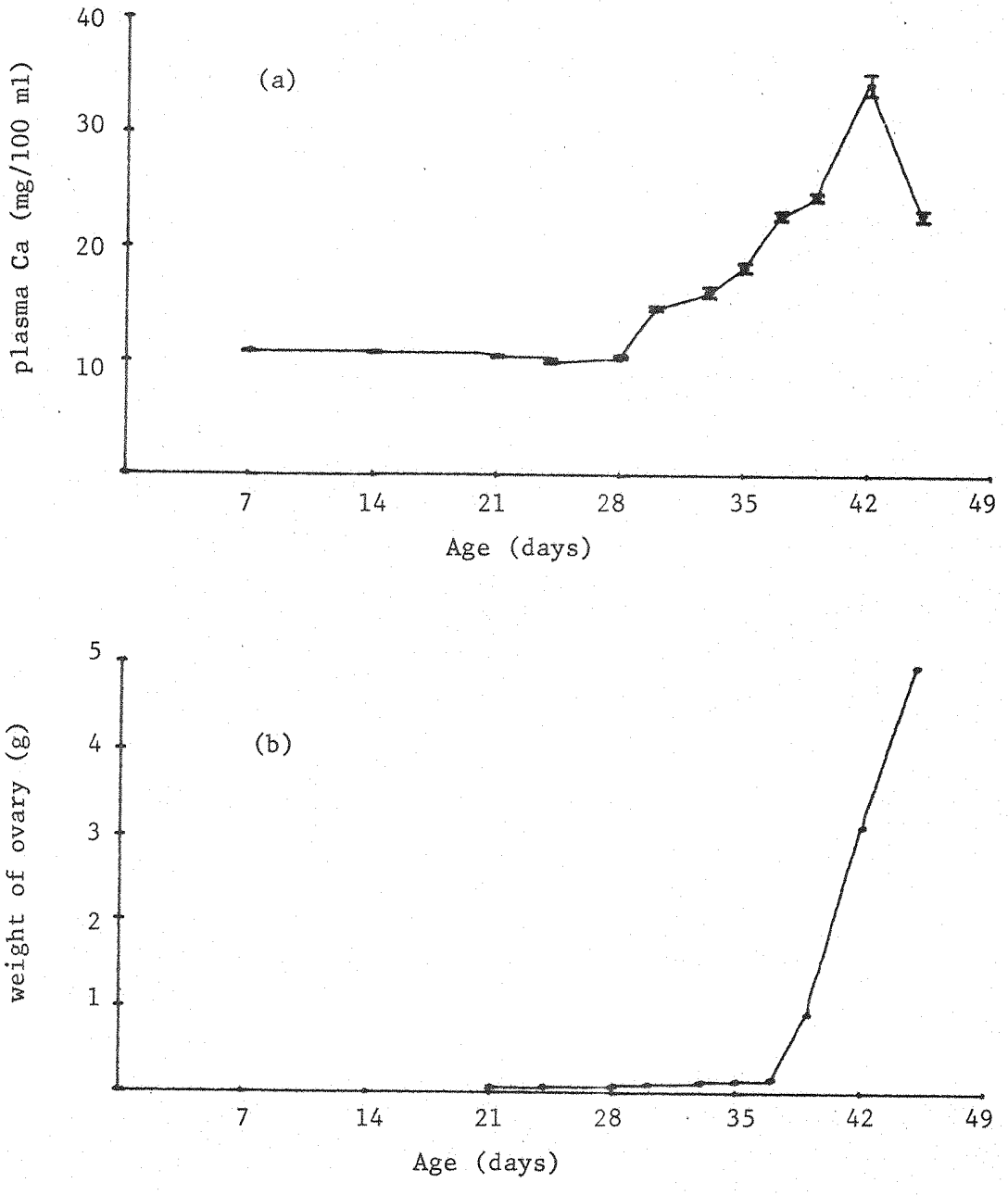


Figure 14. a (above) plasma calcium and b (below) weight of gonads of male Japanese quail in relation to age.

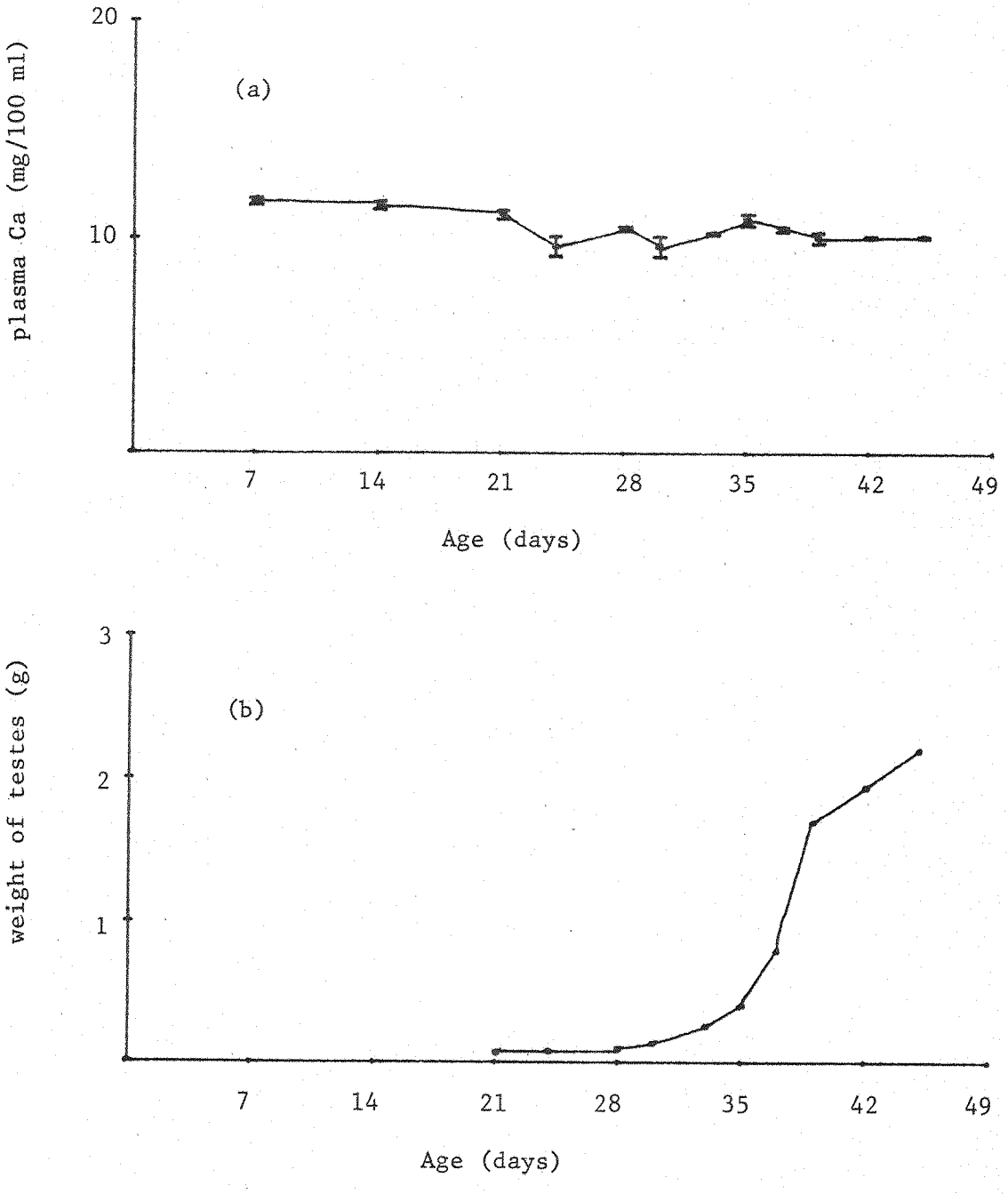
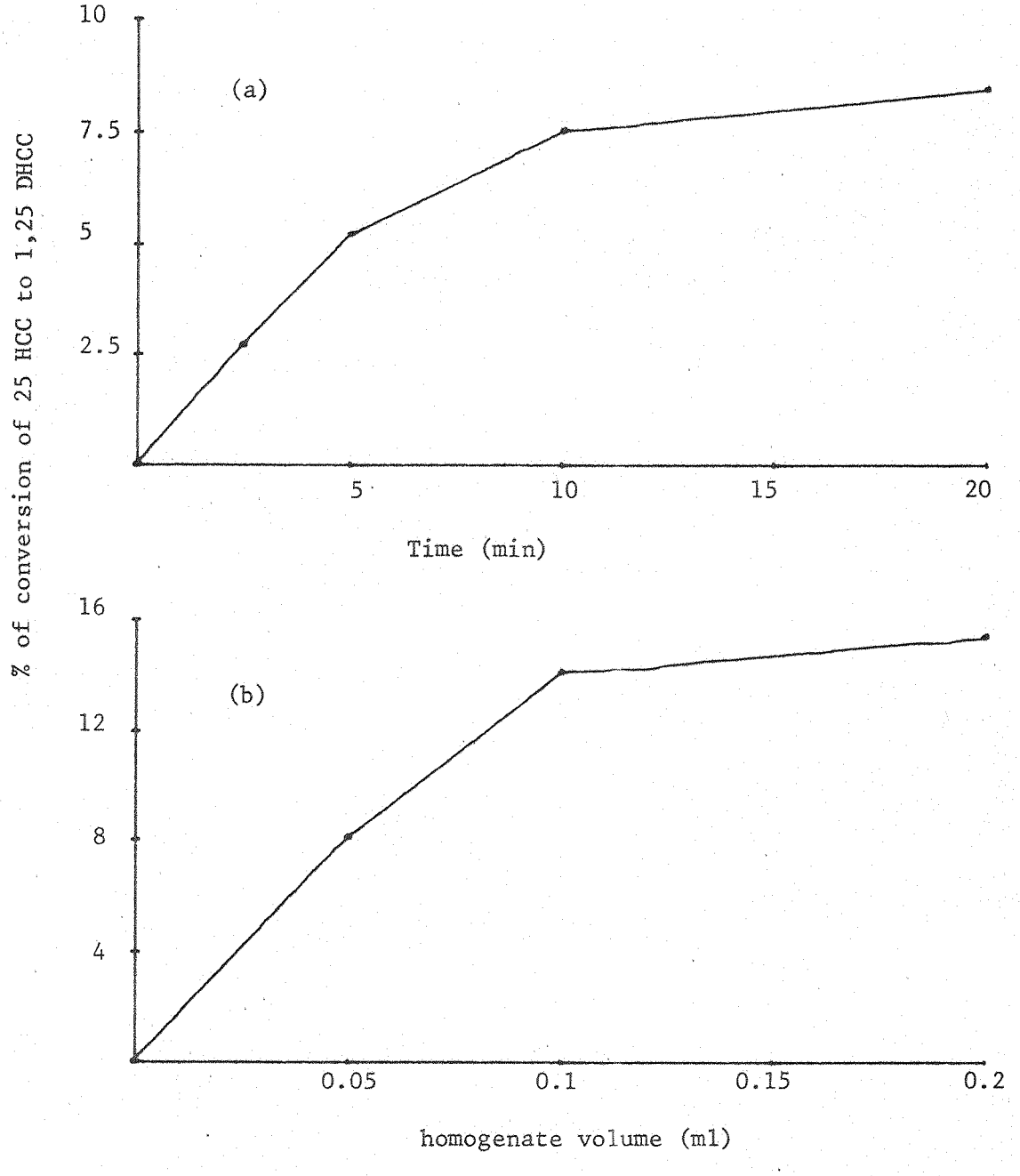


Figure 15. a (above) A time course study on the percentage of conversion of 25 HCC to 1,25 DHCC, using 0.1 ml kidney homogenate, b (below) The effect of varying homogenate volume on the percentage of conversion of 25 HCC to 1,25 DHCC, using an incubation period of 10 minutes.

The homogenates were prepared from birds with an egg in the oviduct, and a substrate concentration of 13 pmole was used



renal 25 HCC hydroxylases activity by incubating 13 ρmole of ^{3}H-25 HCC with 0.1 ml of 10% kidney homogenate for 10 minutes. In some experiments, however, (Chapter 5: 5.1, 5.2, 5.3 and a few experiments reported in Chapter 6: 6.1, and Chapter 7: 7.2) a 20 minutes incubation period was used.

CHAPTER 5

CHAPTER 5. THE METABOLISM OF 25 HCC IN RELATION TO REPRODUCTION IN THE FEMALE BIRDS

Kenny (1976) reported that the activity of the 1-hydroxylase in the kidney of female Japanese quail depends on the physiological state of the bird. He observed that tissue from birds with an egg in the oviduct produced mainly 1,25 DHCC, whereas kidneys of birds with an empty oviduct generated mainly 24,25 DHCC when incubated in vitro with ^{3}H-25 HCC and he concluded that "ovulation in the Japanese quail leads to enhanced renal synthesis of 1,25 DHCC". However he observed a wide range of values for 1,25 DHCC production (from 0 - 100%) in one series of birds without an egg in the oviduct. He did not comment on this variability but it seemed probable that the birds in this series represented a mixed population: some of the birds may have been out of lay altogether, while others may have been having long or short pauses in lay before beginning the next sequence of ovulation.

The object of this study was to extend the work of Kenny (1976) by investigating birds in a number of non-laying states.

Quails lay one egg a day consequtively for 4 or 5 or more days before starting another sequence or clutch of eggs after a pause of one or more days.

Laying quail were housed in plastic cages fitted with a wire-mesh roof. Each cage contained one male and one female. Egg production was recorded daily and six birds with a pause of one day between laying sequences and four birds with a pause of three days were identified. (None of the birds examined had a break of 2 days). Other birds without an egg in the oviduct were selected from the laying flock by external palpation and those with a quiescent ovary were taken for the study. Birds with an egg in the oviduct and adult male were also investigated.

5.1 Laying Female with an Egg in the Oviduct

Kidneys from birds with an egg in the oviduct, when incubated with ^{3}H-25 HCC, produced a high conversion of 25 HCC to 1,25 DHCC (85%, as % of total 1,25 + 24,25 DHCC) as shown in Figure 16 and Table 4.

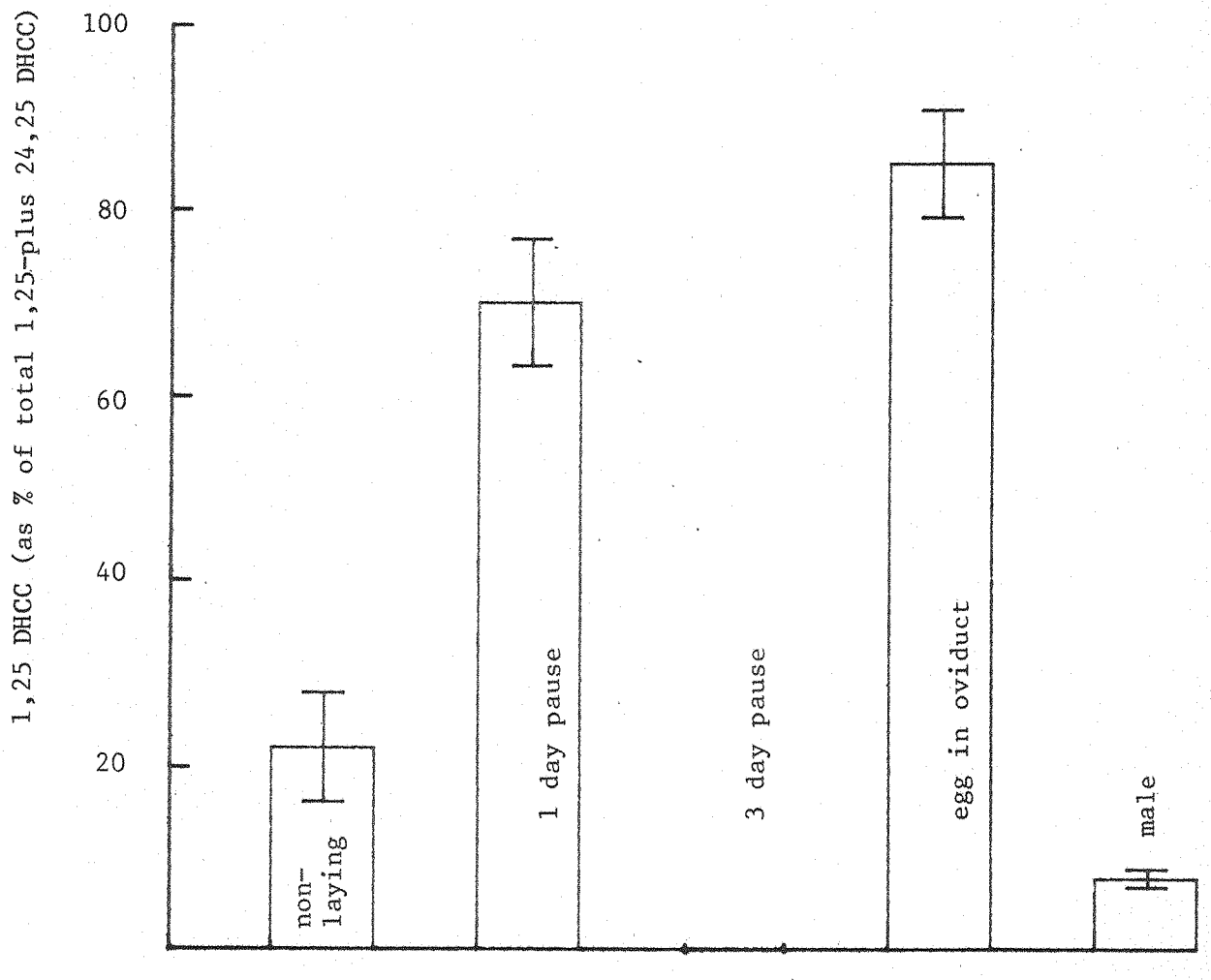


Figure 16. Production of 1,25 DHCC (as % of total 1,25-plus 24,25 DHCC) by kidney homogenates of female Japanses quail in a variety of physiological states, and in adult male.

Physiological state	No. of Birds	Mean weight of ovary (g)	1,25 DHCC Production
No egg in Oviduct			
Non-laying	7	0.76 ± 0.34	22.0 ± 5.8
1d pause	6	6.45 ± 0.94	70.0 ± 6.8 N.S.
3d pause	4	6.08 ± 0.82	0
Egg in Oviduct	7	4.97 ± 0.56	85.0 ± 5.8
Male	4	-	7.9 ± 1.02

N.S. Not significantly different from birds with an egg in the oviduct.

Table 4. In vitro production of 1,25 DHCC (as % of total 1,25-plus 24,25 DHCC) by the kidney homogenates of female Japanese quail in a variety of physiological states and of adult male (values are means ± SEM)

5.2 Laying Female Having One Day Pause

Kidneys of birds that were having a laying pause of one day still produced a high proportion of 1,25 DHCC (70%) (Fig. 16 and Table 4). This percentage of 1,25 DHCC production is not significantly different from the percentage of 1,25 DHCC (85%) produced by the kidney of birds with an egg in the oviduct.

5.3 Laying Female Having Three Days Pause

As shown in Figure 16 and Table 4, birds with an empty oviduct showed wide differences in the proportion of 1,25 DHCC produced (from 0 - 70%), the percentage declining with the length of time since the last oviposition. In birds that were having a long pause (3d) between clutches, in non-laying ones, and in adult males 24,25 DHCC was the major product (Fig. 16, Table 4).

These results show that the kidneys of birds that are laying regularly convert 25 HCC mainly to 1,25 DHCC, even during pauses between egg sequences of one day, whereas in non-laying females, in ones having a long pause (3d) between clutches and in adult male 24,25 DHCC is the major metabolite.

The weight of ovaries of birds examined is shown in Table 4.

5.4 The Effect of Methallibure on 25 HCC Metabolism in the Kidney of Laying Female

The object of this study was to investigate the role that ovulation plays in the metabolism of 25 HCC in the kidney of laying birds, and what is the fate of 25 HCC during the induction of artificial pauses of varying length.

Regular laying female quail were fed turkey starter diet containing 50 mg methallibure/Kg. Higher doses caused ovarian atresia. Methallibure (ADM 49177/73 Messrs. ICI Ltd.), an ovulation inhibitor, was added to the diet as a suspension in 75% ethanol and mixed thoroughly by an automatic mixer. After two days on the diet laying ceased. The third, fourth and fifth day on the methallibure diet were considered to correspond to one day, two days, and three days artificial pauses respectively. Three to five days after removing the methallibure from the diet some birds began to lay.

The results of this study are in agreement with those reported above (i.e. production of 1,25 DHCC in relation to natural pauses). Kidneys of birds that were having a one day artificial pause converted 25 HCC to an extent (79%) similar to that (85%) produced by the kidney of birds with a calcified egg in the oviduct (Fig. 17 and Table 5). The percentage declined significantly with the length of the artificial pause. Birds having a two day pause produced 55% 1,25 DHCC, and this percentage declined to only 22% in birds having a 3 day pause. Administration of 10 usp unit PTE, into birds having a 3 day pause, 3 hours before sacrifice restored the activity of the 1-hydroxylase and completely inhibited the 24-hydroxylase (Fig. 17 and Table 5). As shown in Table 5, all birds under investigation had well-developed ovaries.

These results suggest that the decline in the production of 1,25 DHCC with the length of the break is probably due to a decline in the level of PTH, since administration of PTE into birds having a 3 day pause, whose kidneys produce a low proportion of 1,25 DHCC, enhances the activity of the 1-hydroxylase and suppresses the 24-hydroxylase activity.

5.5 The Effect of Sulphanilamide on 25 HCC Metabolism in the Kidney of Laying Female

Sulphanilamide is an egg-shell calcification inhibitor. The drug inhibits the action of carbonic anhydrase, the enzyme responsible for breaking down carbonic acid and working in reverse, for the hydration of carbon dioxide and the production of carbonate ions. Thus the effect of this drug on egg-shell calcification is brought about by a reduction in the production of these ions (for review see Taylor and Stringer, 1965).

The object of these experiments is to study the effect of partial inhibition of egg-shell calcification in laying female quail on the metabolism of 25 HCC.

Regular laying quail were raised on diet containing three different concentrations of sulphanilamide (0.3, 1.0 and 3 g/Kg) for two weeks and a series of laid eggs were collected. Only the diet containing 3 g sulphanilamide/Kg caused a significant reduction in the weight of shells. Quail are more resistant to the drug than laying hens, since in hens 0.3 g sulphanilamide/Kg diet causes a 28 - 32% reduction in the total thickness of shells (Cooke, 1978).

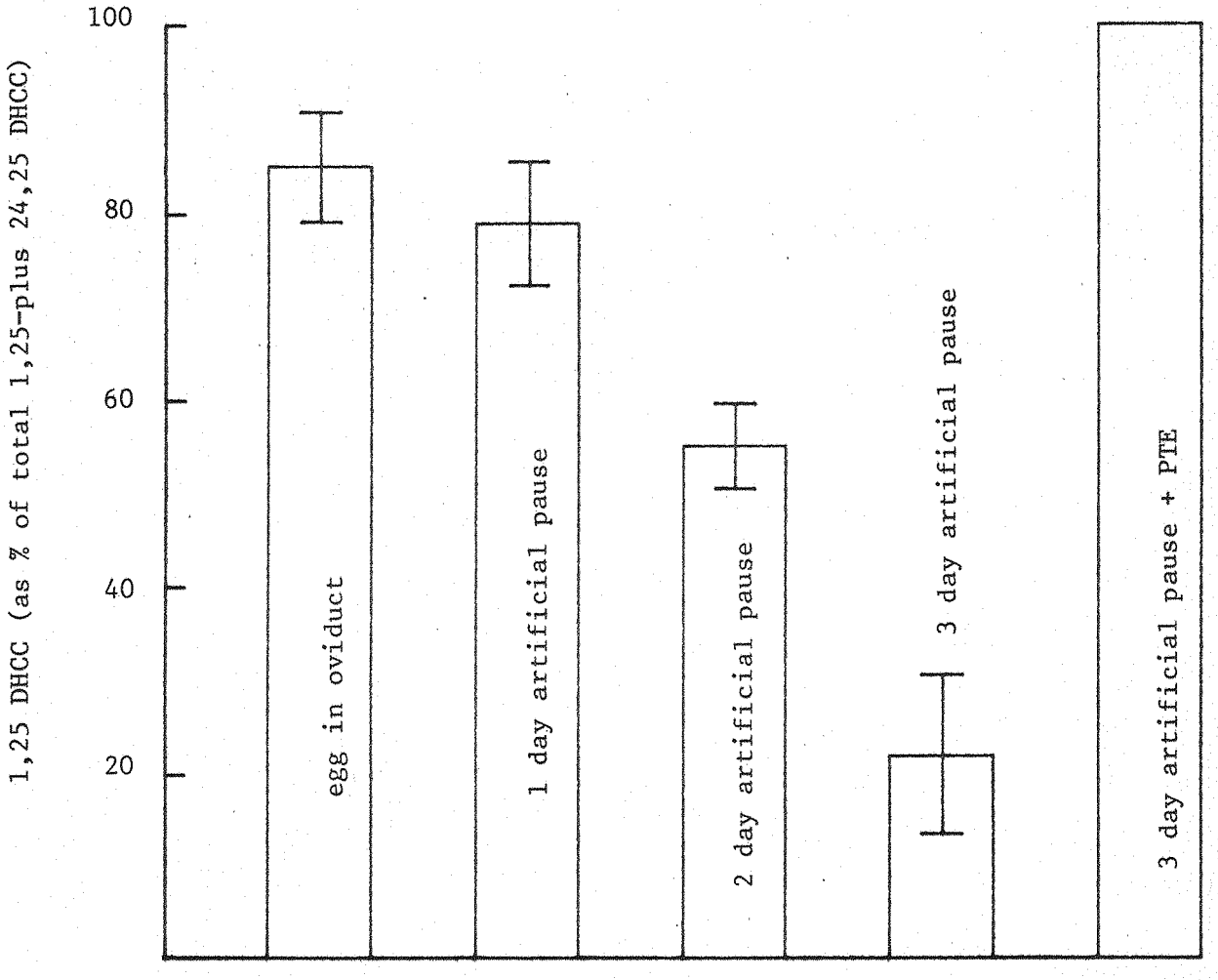


Figure 17. The effect of artificial pauses induced by feeding methallibure (0.005%) an ovulation inhibitor, on the production of 1,25 DHCC (as % of total 1,25-plus 24, 25 DHCC) in the kidney of laying female, and the influence of the administration of PTE into birds which have not ovulated for 3d.

Length of the Artificial Pause	No. of Birds	Mean weight of ovaries	1,25 DHCC Production
Egg in Oviduct[a]	7	4.97 ± 0.56	85.0 ± 5.8
1d pause[b]	3	4.59 ± 0.28	79.0 ± 6.56 N.S.
2d pause[b]	3	5.09 ± 0.20	55.1 ± 4.57^{x}
3d pause[b]	3	7.26 ± 0.19	22.1 ± 8.5^{xxx}
3d pause + PTE[c]	6	5.73 ± 0.65	100.0 ± 0.00

[a] birds were fed normal turkey starter diet.

[b] birds were laying regularly three weeks before feeding the methallibure diet.

[c] 10 usp units PTE 3 hours before sacrifice.

N.S. Not significantly different from birds with an egg in the oviduct.

[x] Significantly different from bird with an egg in the oviduct ($p < 0.02$)

[xxx] Significantly different from bird with an egg in the oviduct ($p < 0.001$)

Table 5. The effect of feeding of Methallibure (50 mg/Kg diet) an ovulation inhibitor, on the production of 1,25 DHCC (as % of total 1,25-plus 24,25 DHCC) in the kidney of laying female, and the influence of parathyroid extract (PTE) administration (values are means ± SEM).

The effect of sulphanilamide (3 g/Kg) on egg-shell weight is shown in Table 6. Administration of this drug to regular laying female for two weeks resulted in a significant reduction in the weight of shell, the percentage varying from 20 to 31.5%. This reduction in the weight of shell caused a significant decline in the activity of 1-hydroxylase (64%) compared to that (90%) in birds raised on normal diet (Fig. 18 and Table 7). Administration of PTE (15 usp unit/bird) into these birds partially restored the activity of the 1-hydroxylase (Fig. 18 and Table 7) and the increase was statistically significant.

Bird No.	Mean weight of shell (g)		% of inhibition
	Normal diet[a]	sulphanilamide diet[b]	
1	0.689 ± 0.038 (4)	0.551 ± 0.02^{x} (4)	20
2	0.734 ± 0.027 (5)	0.526 ± 0.02^{xxx} (4)	28.4
3	0.724 ± 0.006 (5)	0.563 ± 0.012^{xxx} (4)	22.6
4	0.758 ± 0.019 (5)	0.585 ± 0.003^{xxx} (4)	22.8
5	0.741 ± 0.019 (5)	0.525 ± 0.02^{xxx} (4)	29.1
6	0.708 ± 0.040 (5)	0.485 ± 0.058† (2)	31.5
7	0.827 ± 0.038 (3)	0.600 ± 0.017^{xx} (3)	27.4

[a] Turkey starter diet

[b] Sulphanilamide was added to the diet at concentration level of 3 g/Kg. The birds were maintained on the diet for two weeks

† Significantly different from normal diet ($p < 0.05$)

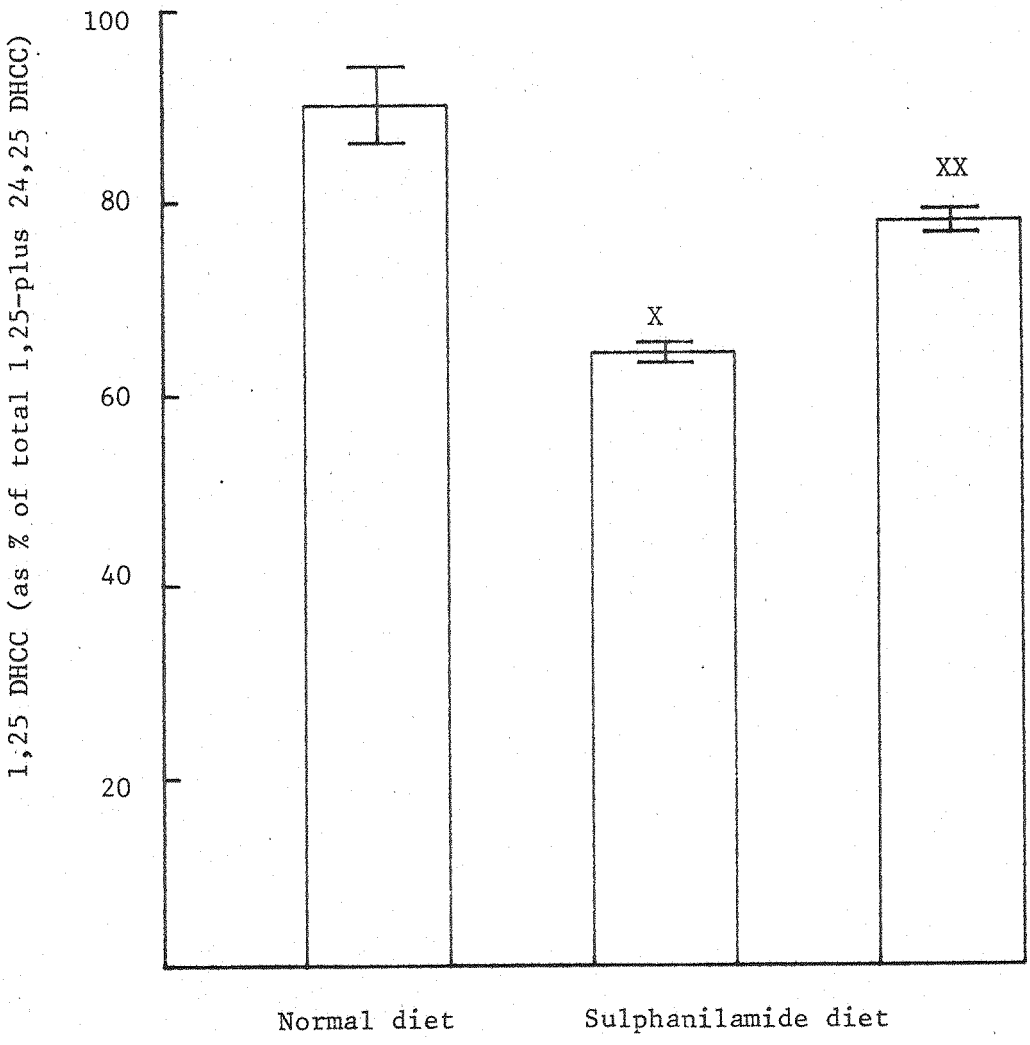
x Significantly different from normal diet ($p < 0.02$)

xx Significantly different from normal diet ($p < 0.01$)

xxx Significantly different from normal diet ($p < 0.001$)

Table 6. Effect on egg-shell weight of including sulphanilamide in the diet of Japanese quail values are mean ± SEM. (Number of shells in parentheses).

Figure 18. The effect of feeding of sulphanilamide (3 g/Kg) an egg-shell calcification inhibitor, on the production of 1,25 DHCC (as % of total 1,25-plus 24,25 DHCC) by the kidney of laying Japanese quail, and the influence of the administration of PTE (15 usp unit) into bird fed sulphanilamide diet for 2 weeks on that production. All birds had a calcified egg in the oviduct at the time of sacrifice.



X Fed sulphanilamide diet for two weeks
XX Fed sulphanilamide diet for two weeks and then given 15 usp unit PTE 3 h before sacrifice

Condition	No. of Birds	1,25 DHCC Production
Control diet [a]	3	90.1 ± 3.98
Sulphanilamide diet [b]	3	64.3 ± 1.00^{xx}
Sulphanilamide diet + PTE [c]	3	77.9 ± 1.15^{xxx}

[a] Turkey starter diet

[b] Sulphanilamide was added to the diet at concentration level of 3 g/Kg. The birds were maintained on that diet for two weeks.

[c] Birds were killed 3h after the subcutaneous injection.

[xx] Significantly different from control diet ($p < 0.01$)

[xxx] Significantly different from sulphanilamide diet ($p < 0.001$)

Table 7. The effect of sulphanilamide (3 g/Kg) on the production of 1,25 DHCC (as % of total 1,25-plus 24,25 DHCC) by the kidney of laying Japanese quail, and the influence of PTE (15 usp unit/bird) administration.

CHAPTER 6

CHAPTER 6. THE EFFECT OF SEX HORMONES ON THE METABOLISM OF 25 HCC

The aim of these experiments was to study the effect of sex hormones on 25 HCC metabolism, because of the important role of these hormones in influencing calcium metabolism in laying birds. During the period 10 - 14 days before a female bird comes into lay, the absorption of calcium and phosphorus from the gut is greatly increased (Taylor and Moore, 1954; Taylor and Kirkly, 1967) and the absorbed minerals are stored in a system of secondary bone, known as medullary bone (Stringer 1962), that develops in the marrow cavities of many bones of the skeleton. These physiological changes can be induced by the administration of oestrogen and androgen in combination but not by either alone, and I wished to study the possibility that the increased absorption of calcium and phosphorus is brought about by stimulating 1,25 DHCC production by the kidney.

6.1 Immature Birds

Immature birds (2 - 4 weeks old) of both sexes were given a single intramuscular injection of three dose levels of oestradiol dipropionate (0, 50 and 100 µg/100 g body weight) and testosterone propionate (0, 25 and 50 µg/100 g body weight) in a 3 x 3 factorial experiment. The doses of hormones used were considered to be physiological, unlike the dose levels used by some other workers (Castillo et al, 1977, Tanaka et al, 1976, 1978b and Baksi and Kenny, 1977b). The activity of renal 25 HCC hydroxylases were assayed 24 h after the injection, and the results are shown in Figure 19 and Table 8.

Administration of oestradiol as little as 50 µg/100 g body weight markedly stimulated the production of 1,25 DHCC. Increasing the dose of oestradiol up to 100 µg resulted in a further increase in the biosynthesis of 1,25 DHCC. On the other hand, testosterone alone at dose level of 25 µg/100 g body weight had no significant effect on 1,25 DHCC production, but at higher doses (50 µg) testosterone suppressed this production significantly. The effect of the interaction between oestradiol and testosterone on the 1-hydroxylase activity is also shown in Figure 19 and Table 8. Testosterone inhibited the stimulation of 1,25 DHCC production evoked by oestrogen alone. This reduction in 1-hydroxylase

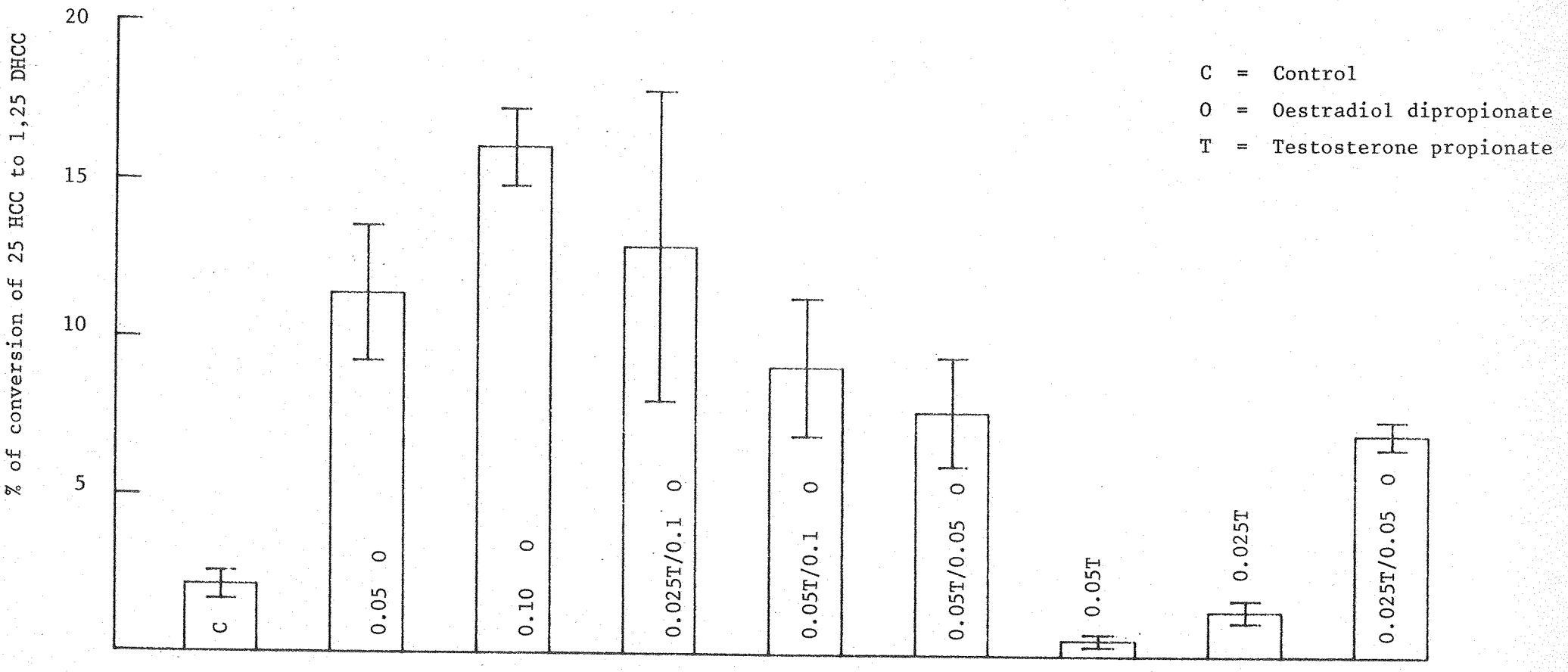


Figure 19. Changes in the mean percentage (± SEM) of 25 HCC converted to 1,25 DHCC in vitro by kidney homogenates of sex hormones-treated immature birds.

Oestradiol dipropionate (µg/100 g BW)

Testosterone propionate (µg/100 g BW)	0	50	100
0	2.10 ± 0.44 (12) Control	11.40 ± 2.15^{xxx} (3)	16.00 ± 1.2^{xxx} (8)
25	1.42 ± 0.36 (6)	7.04 ± 0.94^{xxx} (6)	12.90 ± 4.88^{xxx} (3)
50	0.50 ± 0.20 ^{xx} (7)	7.70 ± 1.74^{xx} (7)	9.1 ± 2.18^{xx}† (8)

^{xx} Significantly different from control ($p < 0.01$)

^{xxx} Significantly different from control ($p < 0.001$)

† Significantly different from group given 100 µg O alone ($p < 0.02$)

Table 8. Changes in the mean percentage (± SEM) of 25 HCC converted into 1,25 DHCC in vitro by kidney homogenates of sex hormones-treated immature Japanese quail of both sexes (Number of birds in parentheses).

activity was significant when 50 µg testosterone was injected together with 100 µg oestrogen.

The effect of gonadal hormones on 24,25 DHCC production was also studied (Fig. 20 and Table 9). Oestradiol administration inhibited the activity of 24-hydroxylase, whereas testosterone had no effect on 24,25 DHCC production.

6.2 Adult Male

Adult male quail were given a single intramuscular injection of oestradiol dipropionate at dose levels of 0.25, 0.5, 1.0 and 5 mg/birds. The activity of the 1-hydroxylase was assayed 24 h after injection.

The results of these experiments are shown in Figure 21a and Table 10. Administration of oestradiol into adults significantly stimulated the production of 1,25 DHCC. This production was further enhanced by increasing the dose of oestradiol from 0.25 up to 5 mg/bird. To shift the activity of 1-hydroxylase in the adult male to that of laying female (10% conversion of substrate to product) a high and unphysiological dose of oestradiol was required. This dose was 100 times more than the dose which was required for oestradiol to stimulate the activity of 1-hydroxylase in immature birds (Table 8). These differences in response to oestradiol between immature bird and adult male may be due to the endogenous testosterone present in the adult male.

6.3 The Hypercalcemic Effect of Oestradiol on Japanese Quail

When 0.25 mg oestradiol was administered into adult male a marked increase in the plasma calcium was induced (Fig. 21b and Table 10). Increasing the dose of oestradiol up to 5 mg/bird caused a further increase in the plasma levels of total calcium.

6.4 A Time Course Study on the Effect of Oestradiol Dipropionate Alone and Oestradiol Dipropionate Plus Testosterone Propionate on 25 HCC Metabolism

Immature birds (3 - 4 weeks old) of both sexes were given a single intramuscular injection of oestradiol dipropionate (100 µg/100 g body weight) alone, or together with testosterone propionate (50 µg/100 g body weight). The renal

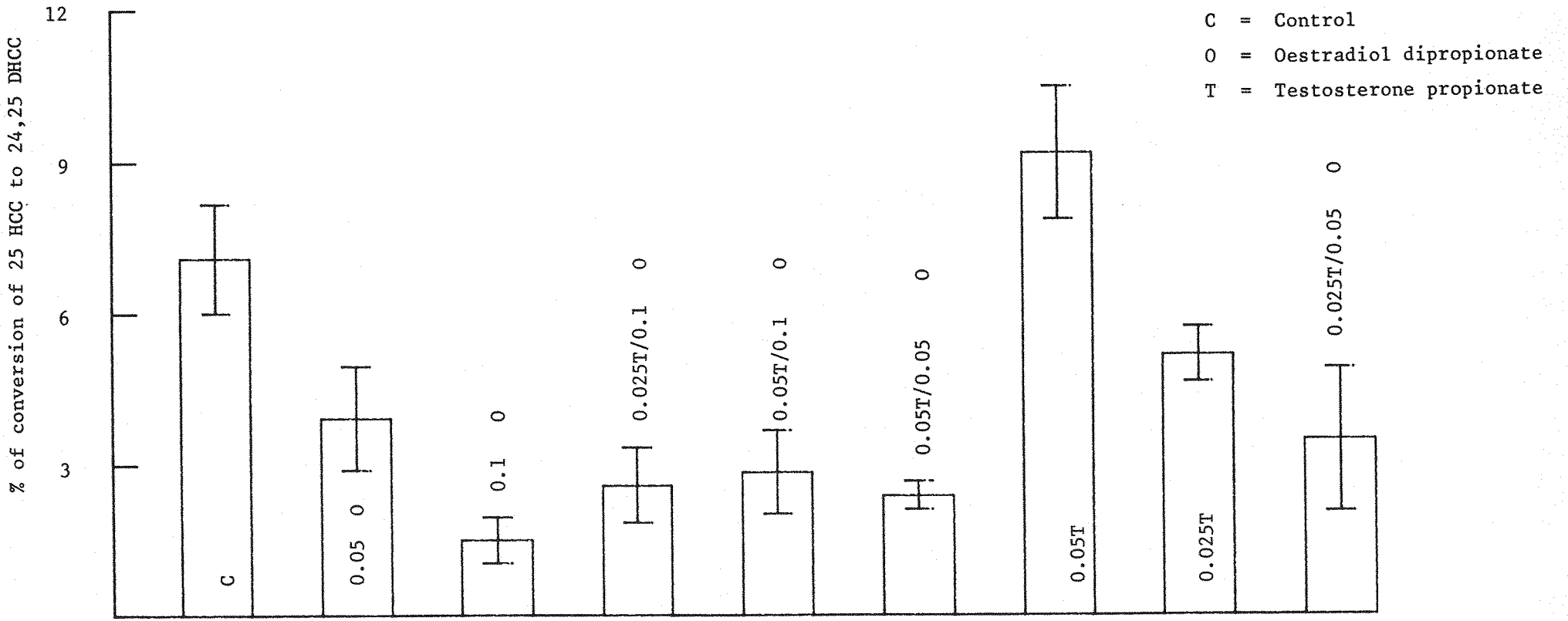


Figure 20. Changes in the mean percentage (± SEM) of 25 HCC converted to 24,25 DHCC in vitro by kidney homogenates of sex hormones-treated immature birds.

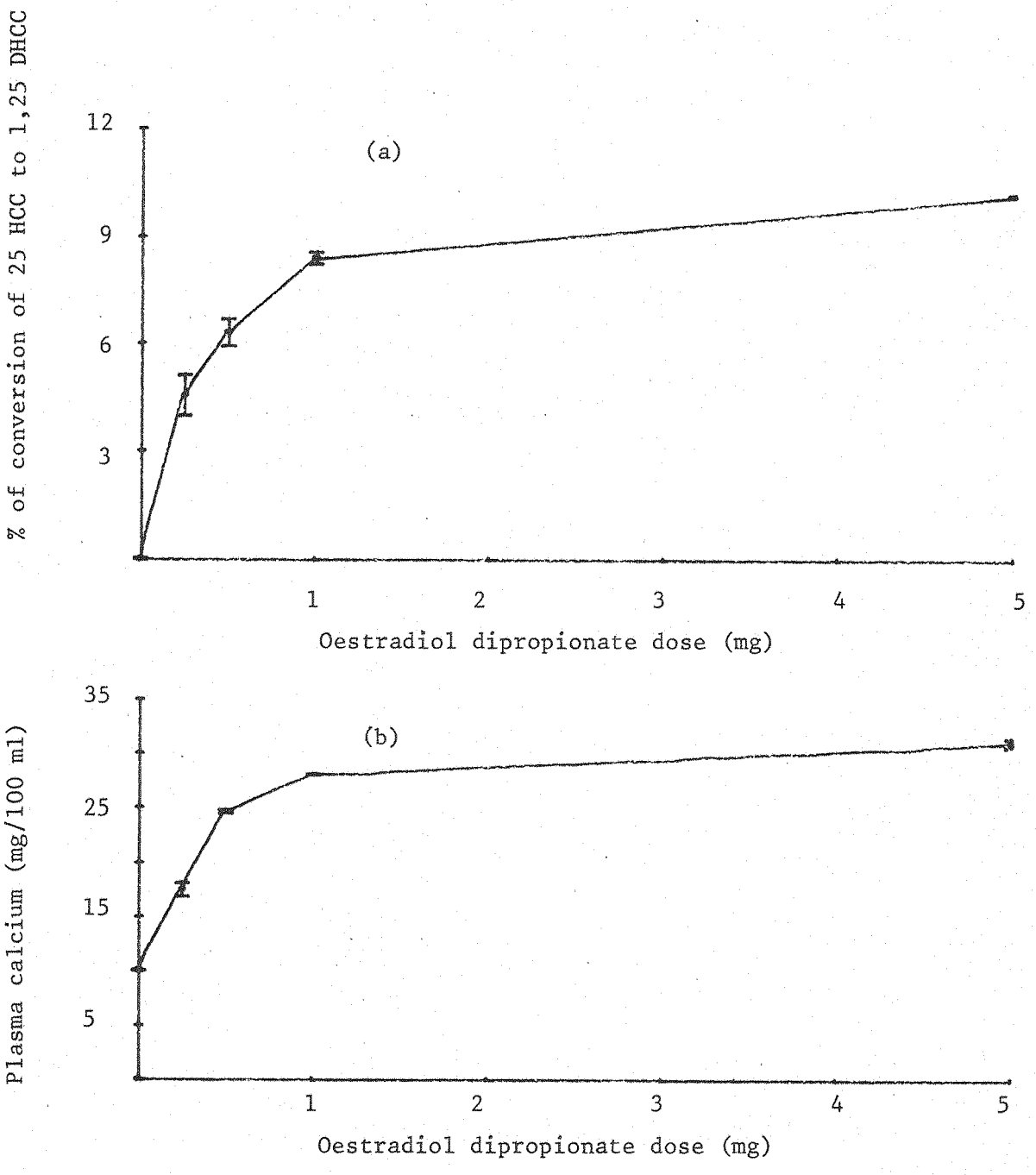
Oestradiol dipropionate (µg/100 g BW)

Testosterone propionate (µg/100 g BW)	0	50	100
0	7.10 ± 1.08 (12) Control	3.93 ± 1.03 (3)	1.5 ± 0.46 (8)
25	5.2 ± 0.55 (6)	3.5 ± 1.43 (6)	2.6 ± 0.75 (3)
50	9.2 ± 1.3 (7)	2.4 ± 0.28 (7)	2.86 ± 0.83 (8)

All oestrogen treatment are significantly lower than the control ($p < 0.05$ or greater).
Responses to testosterone are all non-significant.

Table 9. Changes in the mean percentage (± SEM) of 25 HCC converted into 24,25 DHCC in vitro by kidney homogenates of sex hormones-treated immature birds of both sexes (Number of birds in parentheses).

Figure 21, a, changes in the mean percentage (± SEM) of 25 HCC converted to 1,25 DHCC in vitro by kidney homogenates of oestrogen-treated adult male, and
b, in plasma calcium in relation to the dose of hormone.



Oestrogen dose (mg)	1,25 DHCC	plasma Ca (mg/100 ml)
0.00	0 (3)	10.1 ± 0.06
0.25	4.53 ± 0.58 (3)	17.5 ± 0.66
0.50	6.30 ± 0.40 (3)	24.6 ± 0.18
1.00	8.40 ± 0.20 (3)	28.0 ± 0.06
5.00	10.20 ± 0.05 (3)	31.0 ± 0.46

Table 10. Changes in the mean percentage (± SEM) of 25 HCC converted to 1,25 DHCC by kidney homogenates of oestrogen-treated adult male quail, and plasma calcium values (Number of birds in parentheses).

1- and 24-hydroxylases were assayed at intervals up to 7 days following the injection.

As shown in Figure 22 and Table 11, a, b, the peak 1-hydroxylase activity was detected as early as one day after the treatment. The peak value was higher when oestradiol was given alone than when it was administered together with testosterone. On day 2 the activity of 1-hydroxylase declined and by day 4 all birds under investigation showed a typical control response.

The production of 24,25 DHCC (Fig. 23, Table 11 a, b) was greatly decreased one day after injection of sex hormones, after which it started to increase and by day 7 all birds given oestradiol plus testosterone showed typical control values.

6.5 The NADPH - Dependence of Oestrogen-Stimulated 25 HCC-1-Hydroxylase

The inclusion of an NADPH-generating system in the incubation medium of ^{3}H-25 HCC with kidney homogenates prepared from oestrogen-treated immature bird caused a marked stimulation in the production of both 1,25 and 24,25 DHCC (Table 12); a 15 and 4 fold increase in the production of 1,25 DHCC and 24,25 DHCC, respectively, was noted. On heating the homogenates for 10 minutes at 100^{o}C, the production of these metabolites was abolished and all the radioactivity was recovered as unchanged substrate (Table 12).

6.6 A Comparison Between the Metabolism of 25 HCC by Kidney Homogenates Prepared from Laying, Oestrogen-Treated, Immature and Adult Male Birds

The metabolism of 25 HCC in the kidney of such birds has been reported in the previous chapters. The aim of these experiments was to compare the fate of ^{3}H-25 HCC, in homogenates prepared from these birds, in the following fractions 1,25 DHCC, 24,25 DHCC, unchanged 25 HCC substrate, and the radioactivity remaining in the aqueous phase of the incubation mixture extract. The results in Table 13 show that the percentage of the radioactivity remaining in the aqueous phase was very low in all cases. In birds having an egg in the oviduct and in oestrogen-treated immature birds, the 25 HCC was mainly metabolised to 1,25 DHCC in a high percentage and only 78% remained unchanged in the extract of oestrogenised

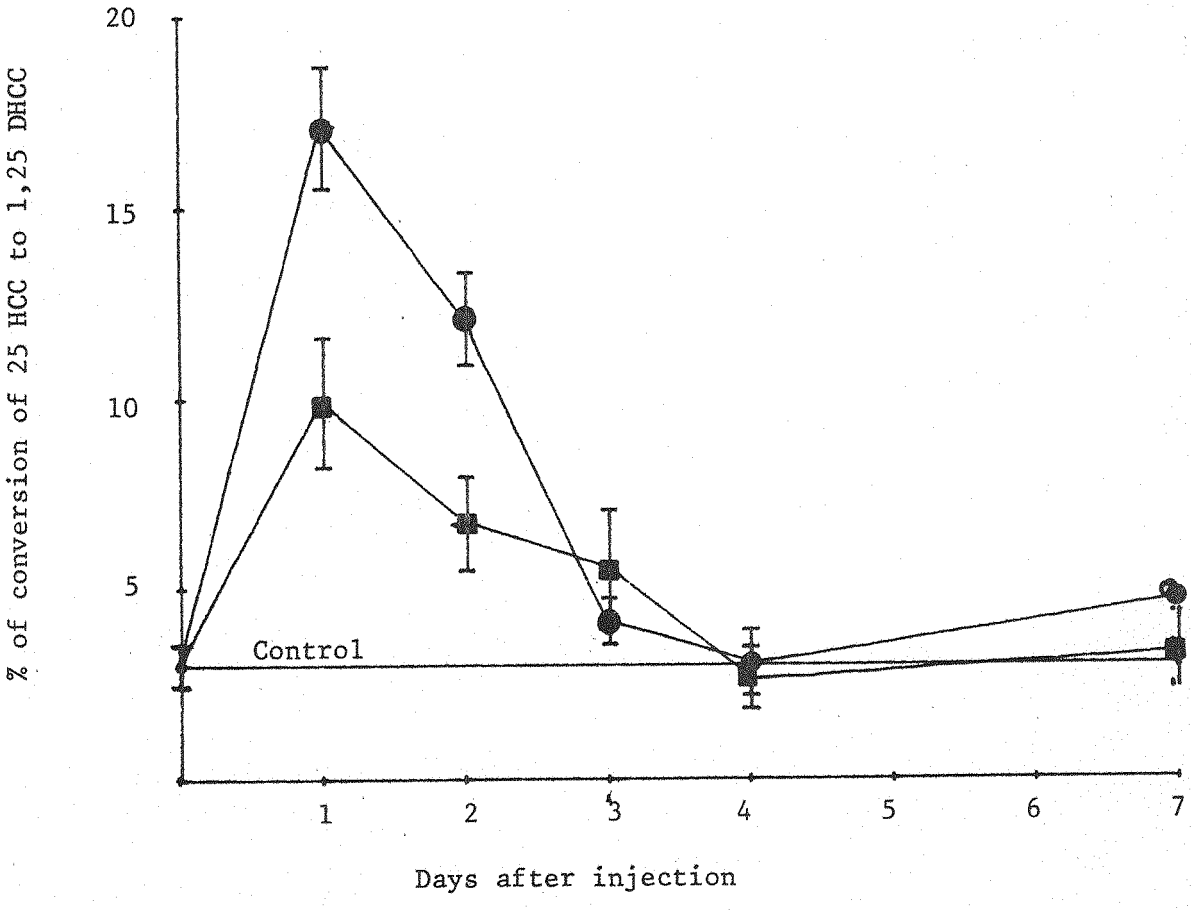


Figure 22. A time course study on the effect of single injections of oestradiol dipropionate (●), and oestradiol dipropionate plus testosterone propionate (■) on 1,25 DHCC production by kidney homogenates of immature Japanese quail.

(a) Oestrogen alone (100 µg/100 g BW)

Days after Injection	No. of Birds	1,25 DHCC	24,25 DHCC
0	7	2.94 ± 0.55	9.6 ± 1.3
1	8	17.1 ± 1.58	0.62 ± 0.24
2	3	12.1 ± 1.23	2.8 ± 0.36
3	4	4.1 ± 0.62	4.0 ± 0.84
4	3	3.0 ± 0.87	6.2 ± 1.33
7	2	4.7 ± 00	6.5 ± 0.4

(b) Oestrogen (100 µg/100 g BW) + Androgen (50 µg/100 g BW)

0	7	2.94 ± 0.55	9.6 ± 1.3
1	7	9.9 ± 1.7	1.9 ± 0.65
2	4	6.7 ± 1.25	3.9 ± 1.11
3	4	5.5 ± 1.56	4.4 ± 0.97
4	3	2.6 ± 0.83	6.6 ± 0.74
7	2	3.3 ± 1.00	9.7 ± 1.35

Table 11. A time course study on the effect of oestradiol dipropionate alone (a, above), and oestradiol dipropionate plus testosterone propionate (b, below) on the mean percentage (± SEM) of 25 HCC converted into 1,25- and 24,25 DHCC (Age of birds, 20 - 26d)

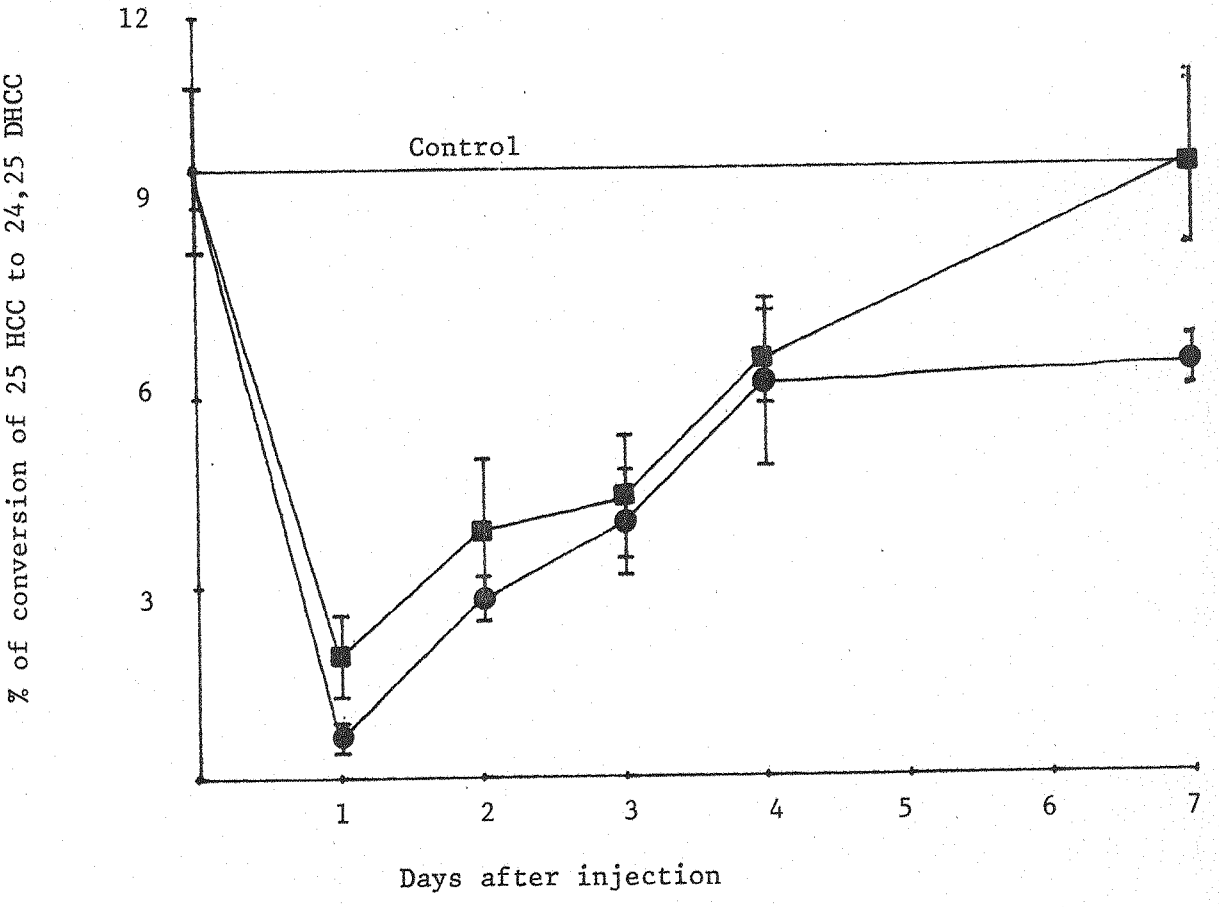


Figure 23. A time course study on the effect of single injections of oestradiol dipropionate (●), and oestradiol dipropionate plus testosterone propionate (■) on 24,25 DHCC production by kidney homogenates of immature Japanese quail.

Radioactivity as	with NADPH (n = 2)	without NADPH (n = 2)	Boiled (n = 2)
1,25 DHCC	17.8 ± 1.1	1.2 ± 0.4	0.2 ± 0.2
24,25 DHCC	2.1 ± 0.3	0.5 ± 0	0.0 ± 0.0
Unchanged 25 HCC	78.8 ± 1.3	96.9 ± 0.1	99.15 ± 1.06
Radioactivity in Aqueous phase	1.3 ± 0.1	1.4 ± 0.3	0.65 ± 0.05

Kidney homogenates were prepared from 3-week old immature birds injected with 100 µg oestradiol dipropionate/100 g body weight 24 hour before sacrifice.

Table 12. The NADPH-dependent oestrogen-stimulated 25 HCC-1-hydroxylase (values as % of total counts recovered ± SEM).

Fraction	% radioactivity in aqueous phase	% 25 HCC → 1,25 DHCC	% 25 HCC → 24,25 DHCC	% of unchanged 25 HCC
Condition				
Egg in oviduct	0.9	9	0	90.1
Oestrogenised[a]	1.3 ± 0.1	17.8 ± 1.1	2.1 ± 0.3	78.8 ± 1.3
Immature	0.9 ± 0.1	1.55 ± 0.45	3.8 ± 0.5	93.75 ± 0.85
Adult Male	0.85 ± 0.05	0.5 ± 0.3	5.5 ± 0.7	93.15 ± 1.05
Boiled[b]	0.8	0.4	0	98.8

[a] Immature birds injected with 100 µg oestradiol/100 g BW.

[b] Boiled homogenate prepared from bird with an egg in oviduct

Table 13. A comparison between the metabolism of 25 HCC by kidney homogenates prepared from laying, oestrogen-treated immature and adult male birds (values as % of total counts recovered ± SEM)

birds, whereas in immature (control) and adult male 24,25 DHCC was the major product, but a high percentage of 25 HCC remained unchanged (93%). The percentage of the radioactivity in the aqueous phase was unaffected by boiling these homogenates for 10 minutes at 100°C, whereas the conversion of 25 HCC to 1,25- and 24,25 DHCC was completely inhibited and about 99% of the radioactivity remained as unchanged substrate.

CHAPTER 7

CHAPTER 7. THE EFFECT OF PARATHYROID EXTRACT (PTE, LILLY) ON 1,25- AND 24,25 DHCC PRODUCTION BY THE KIDNEY OF IMMATURE AND ADULT MALE BIRDS

7.1 Immature Birds

Immature birds (3 - 4 weeks old) were given a single subcutaneous injection of 10 usp units PTE/100 g body weight and the activities of 1- and 24-hydroxylase were assayed at intervals up to 8 hours.

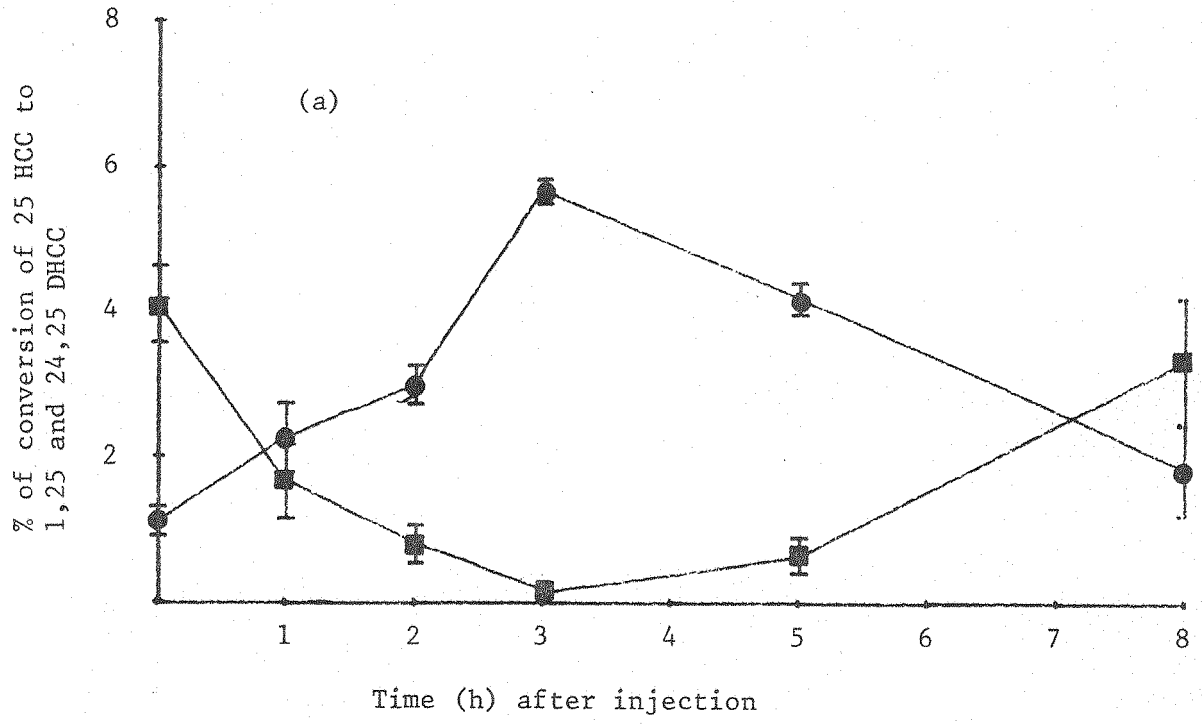
The results of this study are shown in Figure 24a and Table 14a. Administration of PTE resulted in an increase in the activity of 1-hydroxylase and a decrease in the activity of 24-hydroxylase as early as one hour after injection. The peak value of 1,25 DHCC production was detected 3 hours after administration, and at the same time the activity of the 24-hydroxylase was suppressed almost completely. After 8 hours the activities of both enzymes were near control values. The hypercalcemic effect of PTE was detected one hour after its administration (Fig. 24b and Table 14a) and it persisted up to 8 hours.

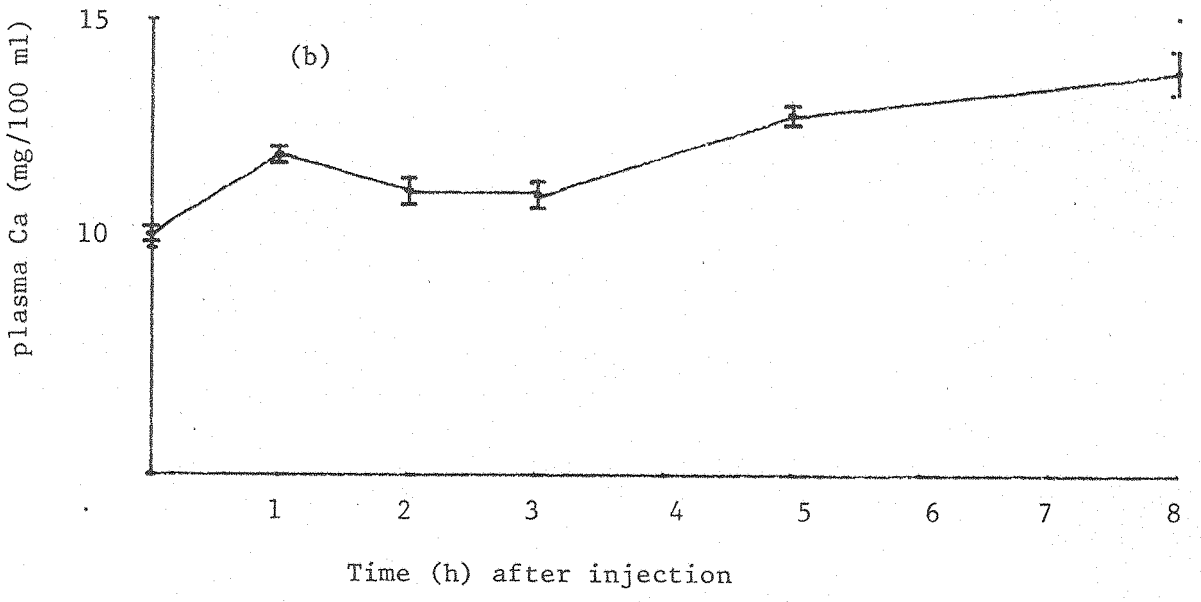
The effect of pure MRC parathyroid hormone was also investigated. The results in Table 14b show that purified PTH had no effect on the metabolism of 25 HCC either 3 h after its administration subcutaneously or 20 minutes after intravenous injection, nor did it influence plasma calcium values (Table 14c) when given intravenously at a dose level of 20 MRC unit/bird. Plasma calcium was determined 10, 20 or 60 minutes after intravenous injection and 3 h after subcutaneous injection.

7.2 Adult Male

As shown in Figure 25 a, b and Table 15, 20 usp unit PTE enhanced the production of 1,25 DHCC from 0.5 to 1.7% within 3 h. After 5 h the activity of 1-hydroxylase was still significantly greater than the control value. Increasing the dose of PTE up to 50 usp unit resulted in a further increase in the percentage of 25 HCC converted to 1,25 DHCC at both 3 and 5 hours after injection.

Figure 24, a, changes in the mean percentage of 25 HCC converted to 1,25 DHCC (●) and 24,25 DHCC (■), b, changes in plasma calcium after administration of 10 usp units PTE/100 g BW to immature birds.





(a)

Time (h) after Injection	No. of Birds	1,25 DHCC	24,25 DHCC	Plasma Ca mg/100 ml
0	4	1.1 ± 0.21	4.08 ± 0.51	10.31 ± 0.19
1	4	2.25 ± 0.47^{NS}	1.65 ± 0.51^{x}	12.05 ± 0.20^{xxx}
2	4	2.96 ± 0.27^{xx}	0.8 ± 0.27^{xx}	11.25 ± 0.32^{x}
3	4	5.65 ± 0.18^{xxx}	0.15 ± 0.15^{xxx}	11.16 ± 0.31^{NS}
5	4	4.15 ± 0.23^{xxx}	0.65 ± 0.25^{xxx}	12.92 ± 0.23^{xxx}
8	3	1.8 ± 0.61^{NS}	3.3 ± 0.88^{NS}	13.85 ± 0.49^{xxx}

^{x}Significantly different from control ($p < 0.05$ or greater), ^{xx} $p < 0.01$, ^{xx} $p < 0.001$, ^{NS} Not significant.

(b)

Condition	No. of Birds	1,25 DHCC	24,25 DHCC
Control^{x}	3	2.7 ± 0.87	4.6 ± 0.44
PTH-treated^{x}	4	2.9 ± 0.64^{NS}	4.4 ± 0.70^{NS}
Control$^{\Psi}$	5	1.98 ± 0.38^{NS}	4.38 ± 0.93^{NS}
PTH-treated$^{\Psi}$	5	2.0 ± 0.33^{NS}	4.52 ± 0.94^{NS}

^{x}Birds killed 20 minutes after intravenous injection of pure PTH (20 MRC unit) or carrier.

$^{\Psi}$Birds killed 3 h after subcutaneous injection of pure PTH (20 MRC unit) or carrier

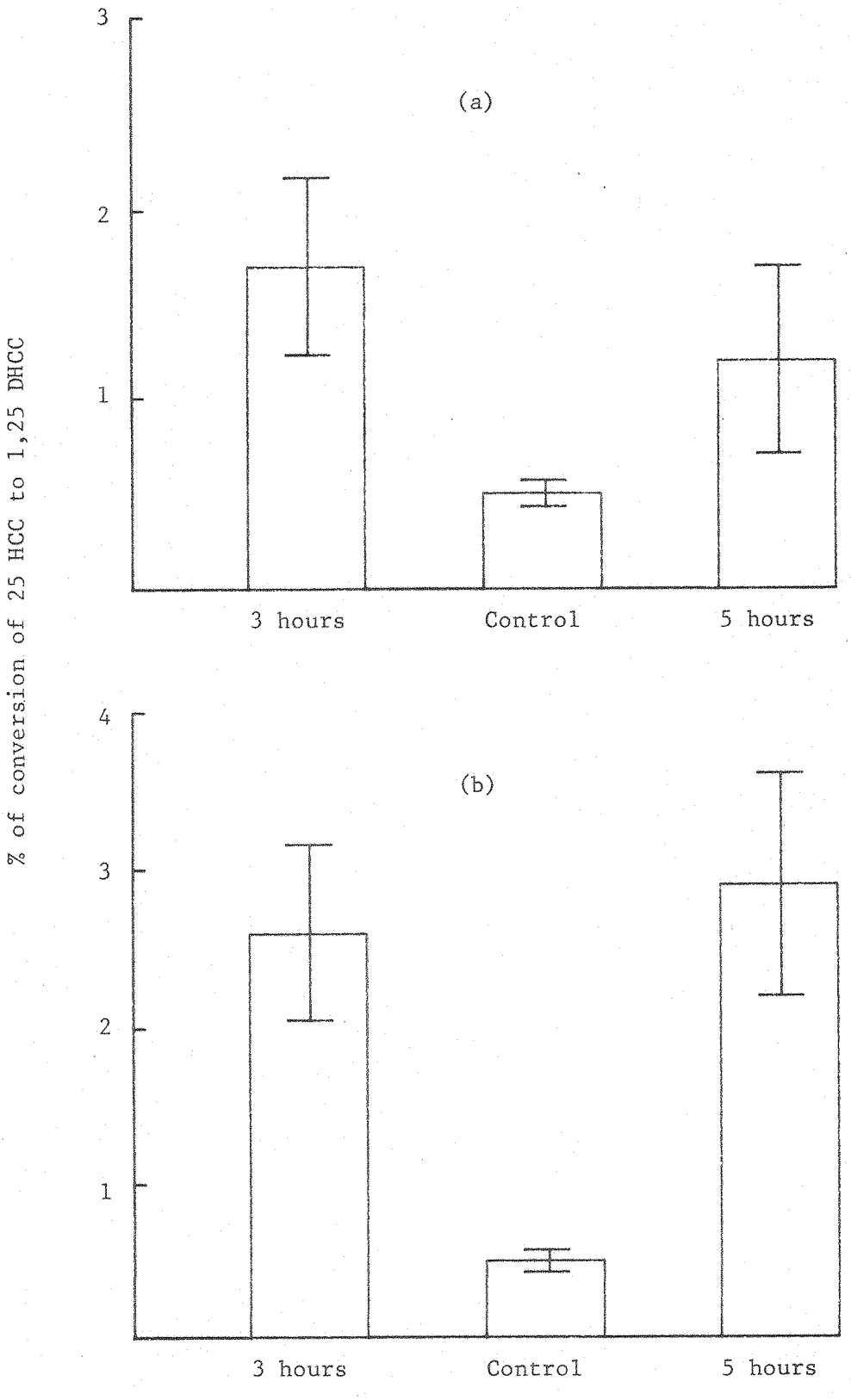
(c)

Time (min) after injection	No. of Birds	Plasma Calcium (mg/100 ml)
0	11	10.81 ± 0.23
10	3^{x}	10.3 ± 0.15^{NS}
20	4^{x}	10.4 ± 0.16^{NS}
60	3^{x}	10.43 ± 0.43^{NS}
180	5$^{\Psi}$	10.7 ± 0.23^{NS}

^{x}PTH administered intravenously $^{\Psi}$PTH administered subcutaneously

Table 14. The effect of parathyroid extract (PTE) and pure parathyroid hormone on the mean percentage (± SEM) of 25 HCC converted into 1,25- and 24,25 DHCC by the kidney of immature birds given
(a) 10 usp units PTE/100 g BW, subcutaneously,
(b) 20 MRC unit pure PTH/bird intravenously and
(c) the effect of pure parathyroid hormone on plasma calcium concentrations at various intervals

Figure 25. Stimulation of 1,25 DHCC production by parathyroid extract in adult male Japanese quail, a (above) 20 usp unit, b (below) 50 usp unit/bird.



Dose of PTE usp unit/bird	Time (h) after Injection	No. of Birds	1,25 DHCC	Plasma Ca mg/100 ml
0	3	4	0.5 ± 0.07	9.9 ± 0.19
20	3	3	1.7 ± 0.47$^{\Psi}$	12.6 ± 0.58^{xx}
20	5	3	1.2 ± 0.30$^{\Psi}$	12.7 ± 0.35^{xxx}
50	3	4	2.6 ± 0.55^{xx}	11.6 ± 0.59$^{\Psi}$
50	5	3	2.9 ± 0.70^{xx}	12.3 ± 0.78^{x}

$^{\Psi}$ Significantly different from control ($p < 0.05$)

^{x} Significantly different from control ($p < 0.02$)

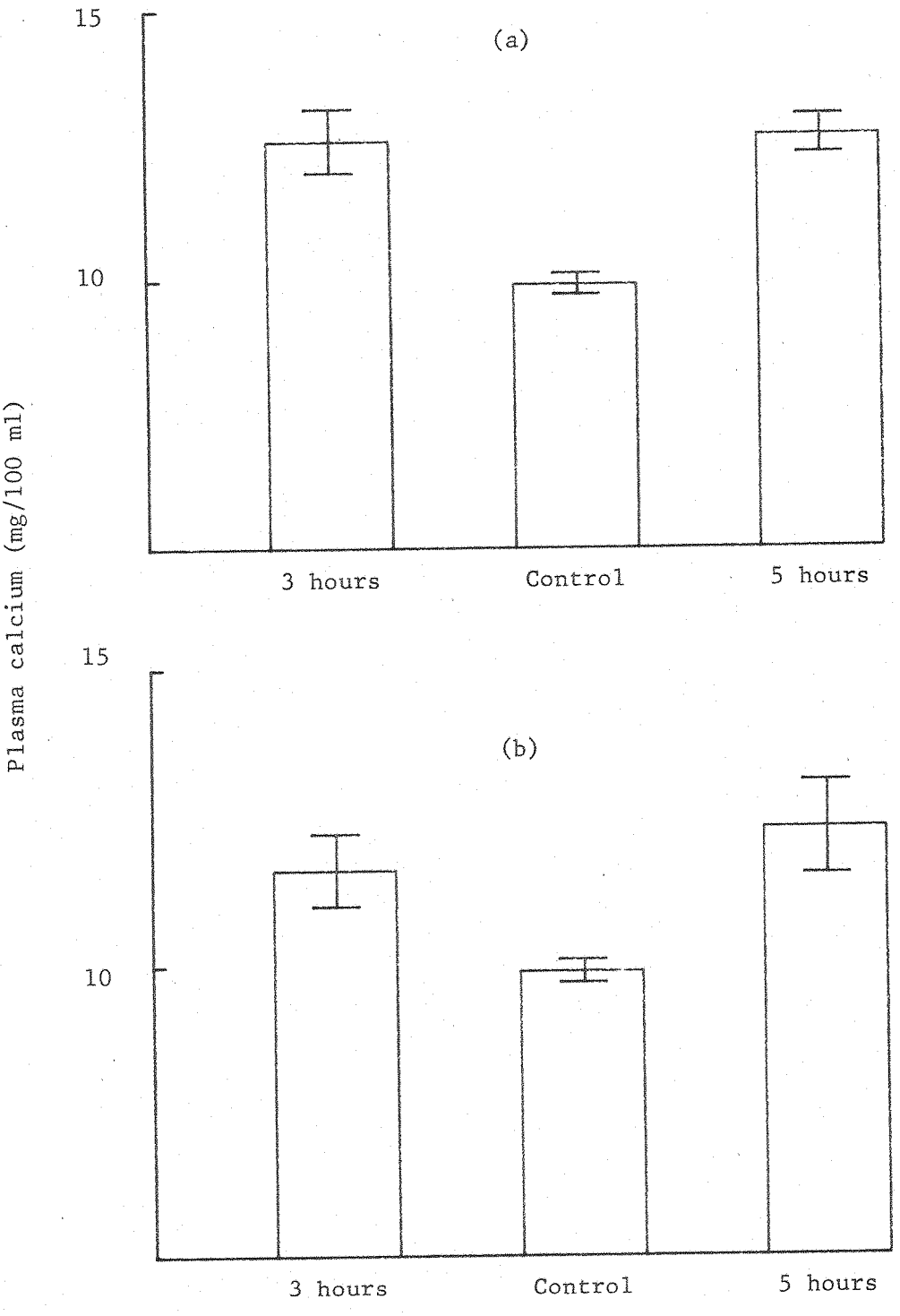
^{xx} Significantly different from control ($p < 0.01$)

^{xxx} Significantly different from control ($p < 0.001$)

Table 15. The effect of parathyroid extract on the mean percentage (± SEM) of 25 HCC converted into 1,25 DHCC by the kidney of adult male.

The hypercalcium effect of PTE, in all birds examined, is shown in Figure 26 a, b and Table 15.

Figure 26. Changes in plasma calcium after administration of PTE into adult male birds, a (above) 20 usp unit, b (below) 50 usp unit/bird.



CHAPTER 8

CHAPTER 8. THE EFFECT OF THE INTERACTION OF SEX HORMONES AND PARATHYROID EXTRACT ON 25 HCC METABOLISM BY THE KIDNEY OF IMMATURE BIRDS

The effect of gonadal hormones and parathyroid extract on 25 HCC metabolism was studied in the experiments reported in Chapters 6 and 7. The object of these experiments was to study the effect of the interaction of these three hormones on the production of 1,25- and 24,25 DHCC by the kidney of immature birds.

Immature birds (2 - 4 weeks old) were given a single injection of oestradiol dipropionate (100 µg/100 g body weight), testosterone propionate (50 µg/100 g body weight), parathyroid extract (10 usp unit/100 g body weight), or the three hormones together. The activity of renal 25 HCC hydroxylases was assayed 24 h after administration of sex hormones and 3 h after parathyroid extract.

As shown in Figure 27 and Table 16, administration of oestradiol or parathyroid extract enhanced the activity of 1-hydroxylase significantly, whereas testosterone inhibited its activity in confirmation of previous observations. In comparison with birds given oestradiol alone, parathyroid extract together with oestradiol did not significantly affect the 1-hydroxylase, whereas parathyroid extract restored the inhibition of 1-hydroxylase evoked by testosterone. There was a dramatic increase in the activity of 1-hydroxylase when the three hormones, parathyroid extract, oestradiol, and testosterone, were given together (Fig. 27, Table 16).

The production of 24,25 DHCC in response to the hormonal treatments is shown in Figure 28 and Table 16. The activity of 24-hydroxylase was suppressed in birds given parathyroid extract, oestradiol, parathyroid extract plus oestradiol and in birds given these two hormones together with testosterone, whereas its activity was not affected by testosterone treatment.

The changes in plasma calcium after administration of these hormones are shown in Figure 29 and Table 16. Parathyroid extract and oestradiol each caused

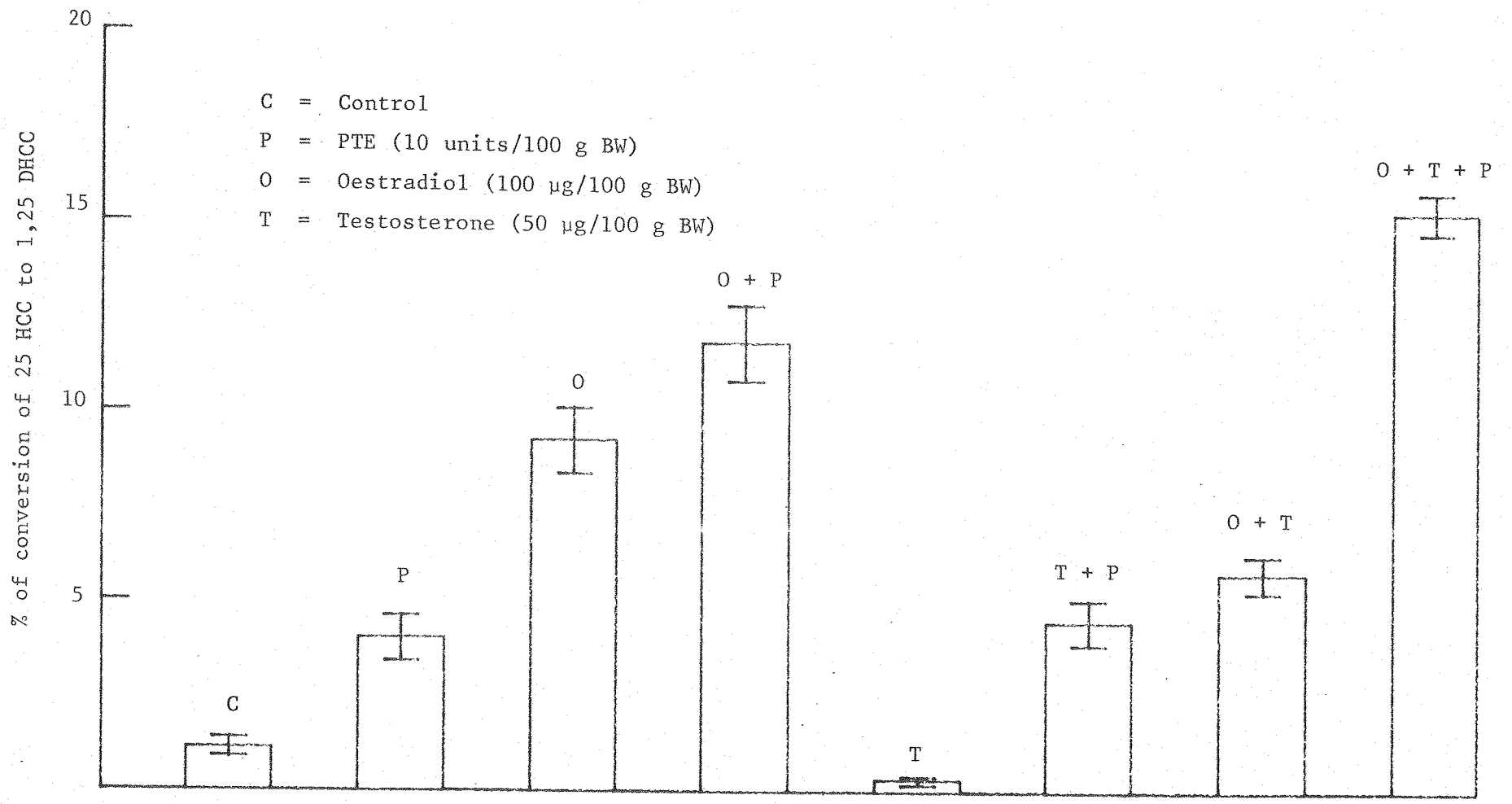


Figure 27. The effect of oestradiol dipropionate, testosterone propionate, and parathyroid extract, and their interaction on the production of 1,25 DHCC by the kidney of immature birds.

Condition	No of Birds	1,25 DHCC	24,25 DHCC	Plasma Ca mg/100 ml
C	9	1.11 ± 0.25	3.9 ± 1.02	9.96 ± 0.55
P	6	4.02 ± 0.6	0.4 ± 0.25	13.27 ± 0.29
O	6	9.23 ± 0.86	0.93 ± 0.49	22.8 ± 4.19
O + P	6	11.8 ± 0.99	0.77 ± 0.36	24.61 ± 3.15
T	6	0.3 ± 0.1	5.02 ± 0.98	10.1 ± 0.21
T + P	5	4.46 ± 0.59	0.46 ± 0.25	10.93 ± 0.41
O + T	5	5.72 ± 0.48^{xx}	2.42 ± 0.62	21.67 ± 1.76
O + T + P	6	15.23 ± 0.53^{xxx}	0.28 ± 0.17	26.9 ± 5.3

C = Control
P = Parathyroid extract (10 units/100 g body weight)
O = Oestradiol dipropionate (0.1 mg/100 g body weight)
T = Testosterone propionate (0.05 mg/100 g body weight)

All sex hormones and PTE treatments are significantly different from control ($p < 0.001$ except in group given testosterone $p < 0.05$).

xx Significantly different from group given oestradiol alone ($p < 0.01$)

xxx Significantly different from group given oestradiol plus testosterone ($p < 0.001$)

Table 16. The effect of oestradiol, testosterone, parathyroid extract, and their interaction on the mean percentage (± SEM) of 25 HCC converted into 1,25- and 24,25 DHCC by the kidney of immature birds. (Birds were killed 24 and 3 h after administration of sex hormones and PTE respectively).

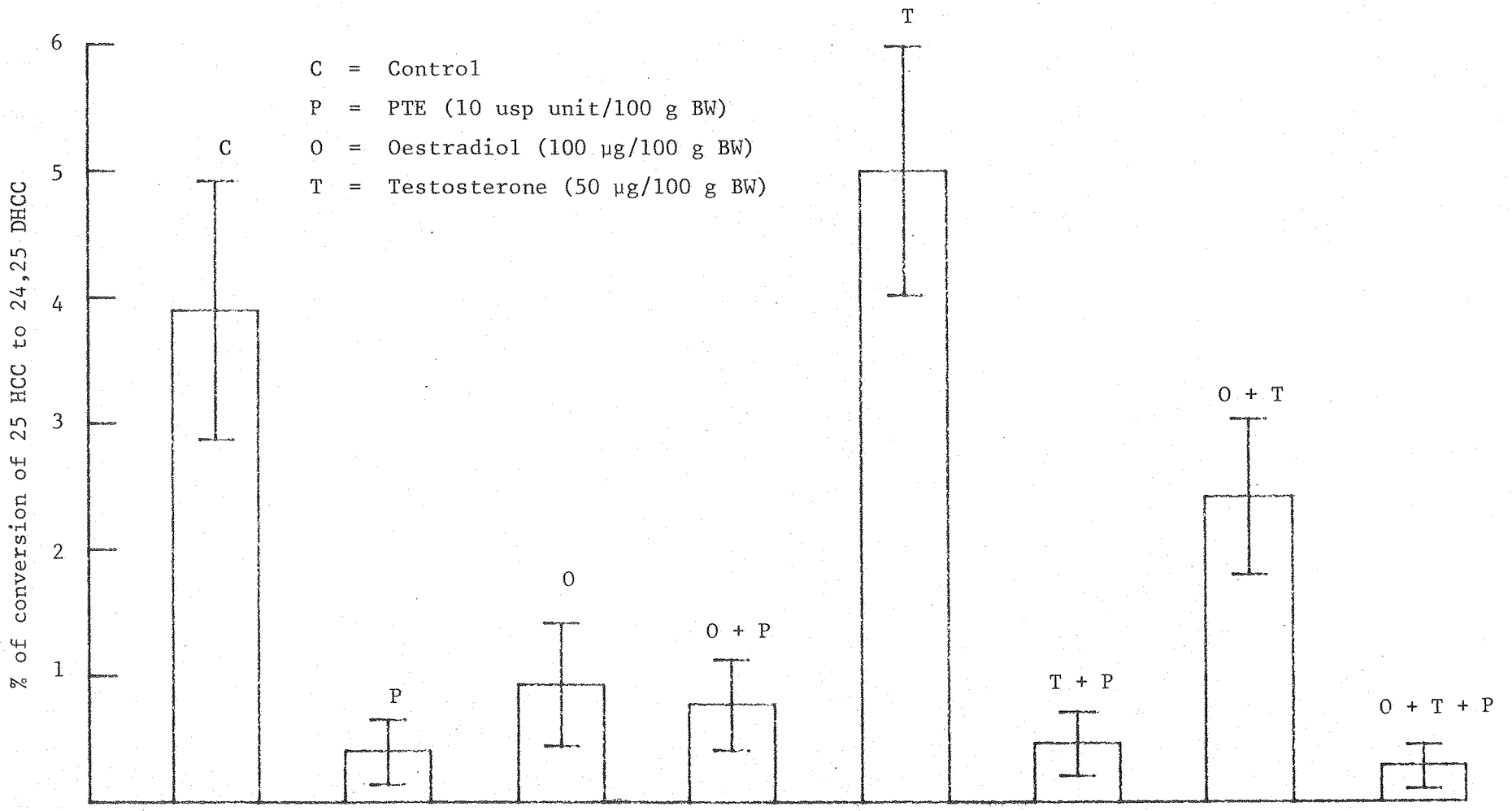


Figure 28. The effect of oestradiol dipropionate, testosterone propionate and parathyroid extract, and their interaction on the production of 24,25 DHCC by the kidney of immature birds.

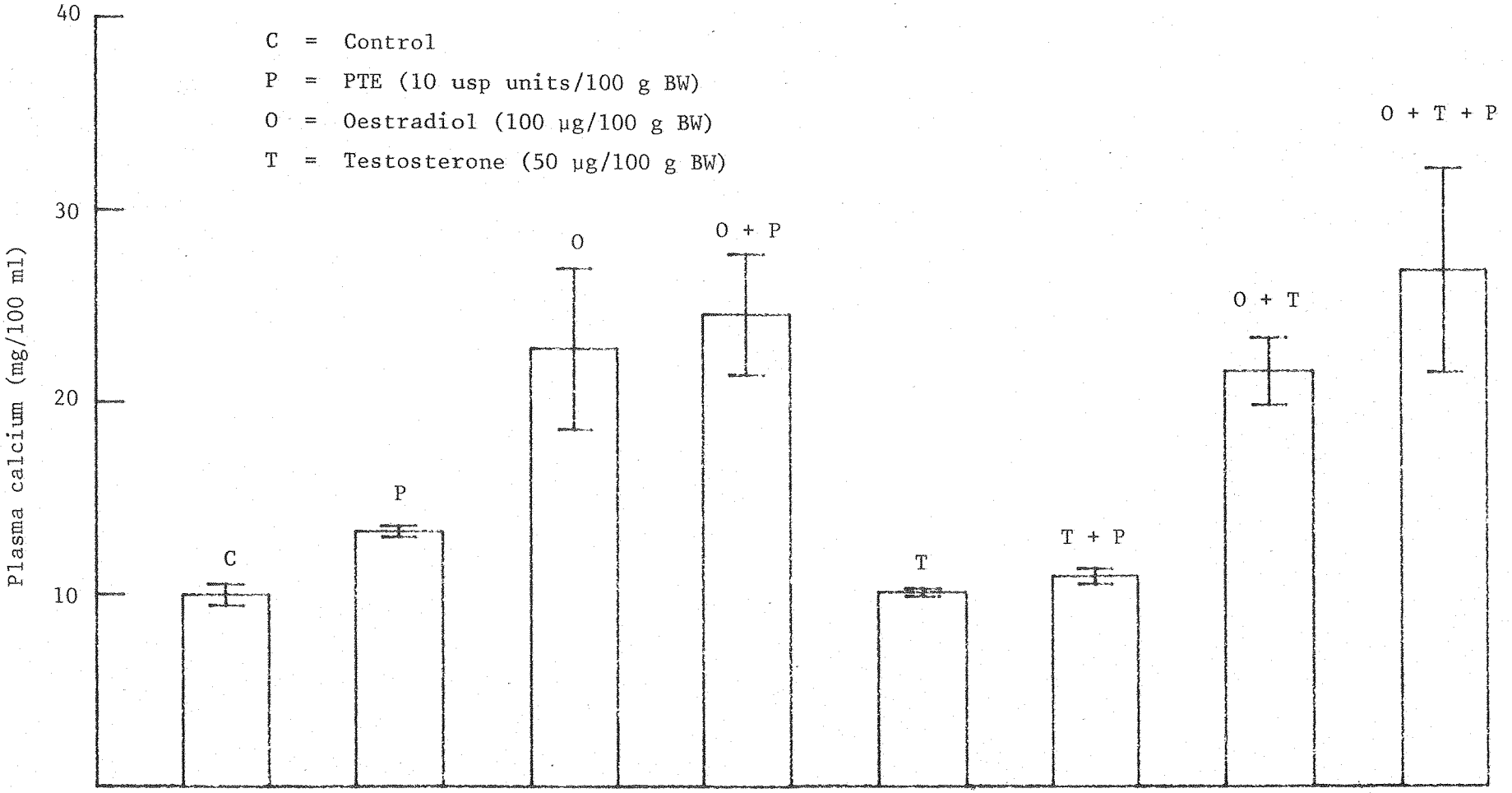


Figure 29. The effect of oestradiol dipropionate, testosterone propionate and parathyroid extract, and their interaction on plasma calcium concentration of immature birds.

an elevation in total plasma calcium and there was no significant augmentation of the effect when both hormones were given together. Testosterone had no effect on the plasma calcium either alone or when given together with parathyroid extract and/or oestradiol.

CHAPTER 9

CHAPTER 9. DISCUSSION AND CONCLUSIONS

The results reported in this thesis relate to the metabolism of 25 hydroxycholecalciferol (25 HCC) in the Japanese quail from hatching until the first egg appeared in the oviduct and thereafter in relation to reproduction and its regulation by sex hormones and parathyroid hormone.

The results of this study clearly show that both oestradiol and parathyroid hormone have the ability to stimulate the production of 1,25 dihydroxycholecalciferol (1,25 DHCC) and to inhibit the production of 24,25 dihydrixycholecalciferol (24,25 DHCC), whereas testosterone has an inhibitory effect on the biosynthesis of 1,25 DHCC.

The embryonic chick liver and kidney contain the enzymes which convert cholecalciferol to its hormonally active form, 1,25 DHCC, essential for calcium homeostasis. Moriuchi and De Luca (1974) have indicated that the conversion of cholecalciferol to 25 HCC, 24,25 DHCC, and 1,25 DHCC can be demonstrated by day 18. Bishop and Norman (1975) have confirmed these results and reported that the enzyme 1-hydroxylase is present as early as day 9 of incubation. The chick intestinal calcium-binding protein (CaBP) is not detectable until the day of hatch (Corradino, Taylor and Wasserman, 1969; Moriuchi and De Luca, 1974), in spite of the presence of cholecalciferol hydroxylases in the liver and kidneys of embryos and of the fact that egg yolk contains cholecalciferol (Gilbert, 1971; Fraser and Kodicek, 1976; Nicholson, 1977) and 25 HCC (Nicholson, 1977), and that the embryonic intestinal cytosolic receptor can be detected by day 17 of incubation (Wasserman, Fuller and Taylor, 1978). The reason for the inability of embryonic intestine to produce detectable amount of CaBP is not known, but it was suggested that insufficient amounts of 1,25 DHCC are present in the intestine to induce its synthesis at this site (Wasserman et al., 1978), since the injection of a large dose of the hormone into 18 d old embryos or its addition to an embryonic organ culture can induce the synthesis of duodenal CaBP (Moriuchi and De Luca, 1974; Wasserman et al, 1978).

The production of 1,25 DHCC in the kidney of Japanese quail of both sexes was relatively high during the first two weeks after hatching, when the birds were actively growing and probably had a high requirement for calcium. By the third week, when the demand for calcium was presumably less, there was a fall in the activity of 1-hydroxylase in the female birds that continued until they were 33 d old. The decline in the 1-hydroxylase activity in these immature

birds was probably due to the availability of calcium and cholecalciferol in their diet in excess of their demand, since they were raised on a diet adequate for egg laying birds. It has been reported that diets high in calcium (Boyle et al., 1971) and in cholecalciferol (Galante et al., 1973; Henry et al., 1974) reduce the activity of 1-hydroxylase. Surprisingly the livers of immature quail do not produce significant amounts of 25 HCC from cholecalciferol in vitro (Nicholson, 1977). Tucker et al., (1973) have discovered that the kidney and intestine of birds are able to produce 25 HCC and as suggested by Nicholson (1977) these organs may play a more important role in these birds than the liver in the production of small amounts of 25 HCC, to be converted to 1,25 DHCC in the kidney, and this small amount of 1,25 DHCC is probably enough for these birds in order to regulate their calcium balance.

When female birds approached their sexual maturity the production of 1,25 DHCC increased significantly reaching a peak value at the onset of lay (Fig. 8); at this time the activity of the 24-hydroxylase was almost completely suppressed. These changes in the renal 25 HCC hydroxylases coincide with the rapid growth of the ovarian follicles (Fig. 9) and the increased secretion of oestrogen by the developing ovary as judged by the increase in the concentration of plasma calcium (Fig. 13a) and the increase in the weight of the oviduct. This correlation between 1,25 DHCC production and the weight of ovaries is maintainted until the appearance of the first egg in the oviduct; thereafter no such correlation can be observed.

In the male birds the activity of 1-hydroxylase declined gradually with age and it was completely suppressed when the birds were sexually mature (Fig. 11) The gradual decrease in the 1-hydroxylase activity may reflect a gradual increase in the level of testosterone secreted by the developing testes, since testosterone has an inhibitory effect on this enzyme (Table 8).

As mentioned earlier Kenny (1976) studied the metabolism of 25 HCC in the Japanese quail at different physiological states and he observed that birds with an egg in the oviduct produced mainly 1,25 DHCC (64% as % of total 1,25 - plus 24,25 DHCC) and he concluded that ovulation is the factor triggering the enhancement of 1,25 DHCC production since, if oviposition was not followed by a further ovulation the activity of 1-hydroxylase fell rapidly (20%) within six hours. Our experiments clearly show that the kidneys of birds with an egg in the oviduct convert 25 HCC

mainly to 1,25 DHCC as reported by Kenny (1976), but birds with an empty oviduct showed wide differences in the proportion of 1,25 DHCC produced, the percentage declining with the length of time since the last oviposition. The birds that were having a laying pause of 1 d still produced a high proportion of 1,25 DHCC (70%) compared with 20% observed by Kenny (1976) for birds that had not ovulated in the past 24 hours. These differences may be related to the fact that his birds were given a diet containing 20 g Ca/Kg, whereas our diet provided only 10 g Ca/Kg. However, it is unlikely that ovulation is the trigger stimulating the synthesis of 1,25 DHCC, since in birds having a pause of 1 d, 1,25 DHCC was still the major product. It is more probable the egg-shell calcification is the stimulus to 1,25 DHCC production and that parathyroid hormone is the key factor involved (Fraser and Kodicek, 1973). Of great interest is the findings of MaCowan (1931) that the parathyroid glands undergo structural changes related to the size of the egg in the oviduct and to changes in plasma calcium.

These suggestions are supported by the results of the experiments in which artificial pauses were induced by feeding methallibure, an ovulation inhibitor, and in which sulphanilamide, an inhibitor of egg-shell calcification was given. These two drugs have different modes of action. The ovulation inhibitor inhibits LH release (Paget, Walpole and Richardson, 1961; Sykes, 1963) and it would be expected therefore to reduce the plasma levels of both oestrogen and parathyroid hormone and possibly testosterone. Sykes (1963) used the changes in blood calcium levels of laying hens after feeding the ovulation inhibitor as an indication of ovarian oestrogen production. He observed a fall in blood calcium level from 20 mg/100 ml to 15 mg/100 ml the day after a single dose of 10 mg/kg and to 10 mg/100 ml after further doses. Over a period of 10 days after removing the inhibitor from the diet the blood calcium levels rose from non-laying levels (10 mg/100 ml) and egg production commenced.

Sulphanilamide does not interfere with the ovulation or with the production of ovarian steroids, but it is effective in inhibiting egg-shell calcification by reducing the rate of shell deposition (Cooke, 1978). It acts to inhibit the action of carbonic anhydrase on the hydration of the metabolic carbon dioxide, thus reducing the production of carbonate ions for the formation of the calcite crystals of which the shell mineral is formed (see Taylor and Stringer, 1965, for review), so oestrogens, testosterone, and progesterone levels in birds fed sulphanilamide should be unchanged, but parathyroid

hormone level would be expected to fall. These results may emphasize that PTH is the key factor involved in the stimulation of 1,25 DHCC production, since administration of parathyroid extract (PTE) into birds raised on methallibure or sulphanilamide diets restores the inhibitory effect of these drugs on 1,25 DHCC biosynthesis. These results may also indicate that the maximum stimulation of 1,25 DHCC production by oestrogen plus androgen requires the presence of PTH. The changes in the activity of renal 1-hydroxylase in relation to egg-shell formation may thus provide a particular example of the mechanism by which calcium absorption from the gut adapts to calcium requirements in normal animals.

As far as quail are concerned, unfortunately, nothing is known about the levels of sex hormones in relation to age or even to the ovulatory cycle, but research on this subject is in progress in this department. Senior (1974) has studied the changes in the level of oestradiol in the domestic hen at weekly intervals from 7 weeks of age until the time of sexual maturity; he reported a mean value 94 pg/ml plasma 7 weeks before the first egg was laid, after which the concentration began to rise sharply reaching a mean peak value of 355 pg/ml 2-3 weeks before laying began. This level declined to 138 pg/ml when egg laying was established. The peak value of oestradiol coincides with the enhancement of calcium absorption from the gut (Taylor and Moore, 1954) and the increase in the level of duodenal CaBP (Bar and Hurwitz, 1972) which is 1,25 DHCC dependent.

Williams and Sharp (1977) determined the concentration of progesterone in the plasma of hens at different stages before and during sexual maturity. They found that the level is low from eight weeks of age to just before the onset of lay, but a significant increase in the concentration occurred when the first egg appeared in the oviduct and remained within this level while the birds were in lay (4 weeks after the first egg was laid).

In relation to ovulatory cycle it has been reported (Peterson and Common, 1971; Kappauf and Tienhoven, 1972; Furr, Bonney, England and Cunningham, 1973) that plasma progesterone level reaches a peak 2-7 hours before ovulation. Similar results have been reported for oestradiol, oestrone (Peterson and Common, 1972) and androgen (Peterson, Henneberry and Common, 1973).

The calcium requirements of laying birds for egg shell formation are extremely high and the skeleton has an important role to play in providing calcium for egg shell formation when the absorption of calcium from the gut is inadequate to support this function (see Taylor and Stringer, 1965, for review).

The increase in the activity of the kidney 25 HCC-1-hydroxylase during the reproductive period in female birds is most likely to be of physiological significance in providing the increased amounts of 1,25 DHCC needed for the absorption of calcium from the intestine and for the mobilization of bone calcium under the influence of parathyroid hormone when dietary supplies are inadequate for egg shell calcification.

The control of skeletal metabolism in relation to egg shell formation involves a number of hormones in particular, oestrogen, androgen, and parathyroid hormone and all these hormones are concerned in the metabolism of cholecalciferol (Chapter 6 and 7). Oestrogens are also involved in the synthesis of calcium-rich yolk protein by the liver and ten days or so before a female bird lays her first egg the concentration of plasma calcium rises as the secretion by the liver of phospholipoprotein for the developing follicle is initiated. The absorbed calcium is stored in the medullary bone, a secondary system of trabecular bone that occupies the marrow cavity of many bones of the skeleton of laying birds before and during reproductive period. Androgen acts synergistically with oestrogen in the induction of the medullary bone.

It is clear that gonadal hormones have the ability to influence the renal production of 1,25 DHCC and 24,25 DHCC. Oestradiol stimulates the production of 1,25 DHCC and inhibits the synthesis of 24,25 DHCC, whereas testosterone has an inhibitory effect on the biosynthesis of 1,25 DHCC whether given alone or in combination with oestradiol. These results do not support the hypothesis that oestrogen plus androgen stimulate calcium absorption from the gut during the pre-laying period by increasing the production of 1,25 DHCC unless it is postulated that PTH secretion is also increased during this period. However, the primary role of androgen in the pre-laying and laying birds may be in relation to the formation of the organic matrix of medullary bone rather than to 1,25 DHCC production. Some of our results are supported by the observation of Baksi and Kenny (1978b). De Luca and his associates (Tanaka

et al., 1976, 1978a; Castillo et al., 1977) have reported that the stimulation of 1-hydroxylase does not occur unless testosterone or progesterone is also given. They claim a permissive role for androgen in the response to oestrogen and that progesterone acts synergistically with androgen and oestrogen to greatly enhance the 1-hydroxylase. In the first report (Tanaka et al., 1976) their study was conducted using chicken and Japanese quail. The latter birds were injected with very high doses of oestrogen (5 mg/bird) and androgen (10 mg/bird) and the possibility of unphysiological changes cannot be excluded. Nevertheless, it is unlikely that the suggestion of Tanaka et al (1976) is applicable in the case of Japanese quail given physiological doses of sex hormones since, as reported in this thesis (Chapter 6 and 8) and in the reports of other workers (MacIntyre, 1977; Baksi and Kenny, 1978b) oestrogen alone can stimulate the synthesis of 1,25 DHCC. Furthermore, the work of Nicholson (1977) on the metabolism of cholecalciferal in the liver of Japanese quail revealed that the injection of oestrogen alone into immature birds enhances the production of 25 HCC and induces the formation of CaBP in the intestine. This increase in the amount of 25 HCC is needed to match the large increase in the 1-hydroxylase activity in the kidney after oestrogen treatment.

Our results clearly show a stimulatory role of PTH on the production of 1,25 DHCC and an inhibitory effect on 24,25 DHCC synthesis. These results are in concert with others observations (Fraser and Kodicek, 1973; Henry, 1977). Parathyroid hormone also has the ability to overcome the inhibition of 1,25 DHCC production evoked by testosterone and when given together with this hormone and oestradiol a further increase in the synthesis of 1,25 DHCC is observed.

The effects of PTH and oestrogen on blood calcium in birds are quite different. Parathyroid hormone affects only the ultrafilterable calcium, the ionic calcium (Urist, Deutsch, Pomerantz and McLean, 1960) whereas the increase in blood calcium after oestrogen treatment is restricted to the nonultrafilterable fraction (Urist et al., 1960), ionic calcium being largely unaffected. The dramatic increase in the blood calcium level of oestrogenized birds is associated with the appearance of a phospholipoprotein with a high capacity for the binding of calcium (Urist, Schjeide, and McLean, 1958). This phospholipoprotein is synthesized in the liver, under the effect of oestrogen, secreted into the blood and transported to the developing follicles. Its

synthesis does not normally occur in immature birds or in adult males.

The mechanism of action of oestrogen on the renal 25 HCC hydroxylases in birds is not known. Whether the enhancement of 1,25 DHCC production by oestrogen results from stimulation of de novo synthesis of 1-hydroxylase, activation of pre-existing enzyme, or a secondary phenomenon mediated by other factors is open to conjecture. Certainly Spanos, et al., (1978a) have been unable to show an effect of oestrogen on 1-hydroxylase in chick cells grown in culture.

It is known that oestrogens stimulate protein synthesis in different classes of vertebrates. In amphibia oestrogen causes hypercalcemia, hyperproteinemia and lipemia. The hyperproteinemia results from the synthesis by the liver of lipoprotein (Urist, and Schjeide, 1961). Administration of oestradiol into Xenopus laevis (South African toad) leads to the appearance in the plasma of lipophosphoprotein (Ansari, 1970) known as xenoprotein. The liver is the site of its synthesis and was characterized as calcium-binding lipophosphoprotein and its calcium and phosphorus contents were 0.84 g and 1.66 g/100 g xenoprotein respectively.

Oestrogens also increase the totel protein in the serum of freshwater turtles (Clark, 1967) and in the lizard Uta stansburiana (Hahn, 1976). The liver of birds also responds to oestrogen administration by synthesising two proteins designated as x_1 (a phosphoprotein) and x_2 (a phospholipid lipoprotein). These proteins are liberated into the blood and transported to the ovarian follicles (Urist et al, 1958; Urist and Schjeide, 1961).

In mammals, oestradiol accelerates the uterine protein synthesis both in vivo and in vitro and causes a rapid incorporation of labelled amino acid, glycine-2-C^{14} into protein (Mueller, 1953). The activity of the uterine amino acid-activating enzymes increased dramatically within a few hours in response to a single injection of oestradiol into ovarectomized rats (McCorquodale and Meuller, 1958) and a striking acceleration occurred in the incorporation of in vivo of tritiated uridine into RNA of the rat uterus (Ui and Mueller, 1963). These authors concluded that the early response to oestrogen depends on the synthesis of new RNA and that the primary action of oestrogen is to facilitate the utilization of this RNA by the protein synthetic

mechanisms. Hamilton (1968) also observed that the incorporation of tritiated uridine into RNA was increased by more than 500% within minutes after oestrogen treatment. Furthermore the effect of oestrogen on two DNA-dependent RNA polymerases has been studied by Widnell and Tata (1966). Nevertheless, it is more probable that the action of oestradiol on renal 25 HCC hydroxylases is mediated by parathyroid glands. This possibility is supported by the following facts.

a) Parathyroidectomy reduces the production of 1,25 DHCC and injection of parathyroid hormone restores this production (Garabedian et al., 1972; Horiuchi et al., 1976, 1977; Omdahl et al., 1977; Fraser and Kodicek, 1973; Galante et al., 1973; Booth et al., 1977).

b) Birds having a long pause and very well developed ovaries did not produce significant amounts of 1,25 DHCC. The levels of sex hormones in these birds would be expected to be equivalent to those of laying female with an egg in the oviduct, but the level of parathyroid hormone would almost certainly have been reduced in these birds. Unfortunately no assay for avian PTH is presently available.

c) Sulphanilamide, an egg-shell calcification inhibitor, which reduces the level of parathyroid hormone without affecting the levels of sex hormones, resulted in a significant reduction in the production of 1,25 DHCC. Administration of parathyroid extracts, into birds raised on a sulphanilamide containing diet, enhanced the activity of 1-hydroxylase significantly (Chapter 5).

d) Injection of PTE overcomes the inhibitory effect of testosterone on 1,25 DHCC production (Chapter 8).

e) When testosterone was given together with oestrogen, it inhibited the stimulatory effect evoked by oestrogen alone but administration of PTE into such birds caused a 3-fold increase in the activity of 1-hydroxylase (compared to control birds given oestrogen plus testosterone) (Chapter 8).

Clearly the best experimental proof for this possibility requires the direct measurement of circulating oestrogen and PTH at different ages and at different stages of the ovulatory cycle and the measurement of PTH levels at time intervals after administration of oestrogen to immature or ovarectomized birds, but this experiment has not yet been carried out.

It is well known that postmenopausal women often develop osteoporosis apparently due to oestrogen deficiency. In patients with postmenopausal

osteoporosis the impaired calcium absorption is probably due to decreased production of 1,25 DHCC. Recent evidence shows that the circulating levels of this hormone are lower in osteoporotic postmenopausal when compared with non-osteoporotic postmenopausal woman (Riggs and Gallagher, 1978). They also observed that postmenopausal osteoporotic patients respond to 1,25 DHCC. This suggests a possible defect in the conversion of cholecalciferol to its hormonally active form, 1,25 DHCC. It is of interest that the treatment of patients with postmenopausal osteoporosis with oestrogen leads to an increase in serum immunoreactive parathyroid hormone (Riggs, Howsey, Goldsmith, Kelly, Hoffman and Arnaud, 1972 and 1973). On the other hand, in pregnant women the circulating levels of both oestrogens (Roy and MacKay, 1962) and parathyroid hormone (Cushard, Creditor, Cantebury and Riess, 1977) increase during pregnancy and lactation. This is associated with an increase in the circulating level of 1,25 DHCC (Kumar, Cohen, Silva and Epstein, 1979). However, increased secretion of prolactin in these physiological conditions has also been implicated in this increased 1,25 DHCC (Spanos et al., 1976a).

Conclusions

1. Production of 1,25 DHCC in female birds increases with the approach of sexual maturity and production of 24,25 DHCC decreases.

2. In male birds biosynthesis of 1,25 DHCC decreases with age and the activity of the 1-hydroxylase is completely suppressed at sexual maturity.

3. Kidneys of birds that are laying regularly convert 25 HCC mainly to 1,25 DHCC even during short pauses between clutches.

4. Ovulation, therefore, is unlikely to trigger the 1-hydroxylase.

5. Non-laying birds, ones having a long pause between laying sequences, adult male, and immature birds produce mainly 24,25 DHCC.

6. The production of 1,25 DHCC increases with the increase in the weight of ovary until the first egg appears in the oviduct.

7. Oestrogen stimulates the production of 1,25 DHCC and inhibits the synthesis of 24,25 DHCC.

8. Androgen inhibits the 1-hydroxylase without affecting the 24-hydroxylase.

9. Oestrogen plus androgen do not stimulate the absorption of calcium from the gut by increasing the production of 1,25 DHCC.

10. The primary role of androgen in the pre-laying and laying birds may be in relation to the formation of organic matrix of medullary bone.

11. Parathyroid hormone stimulates 1,25 DHCC production and overcomes its inhibition by testosterone.

12. The stimulation of 1,25 DHCC by oestrogen plus androgen requires the presence of parathyroid hormone.

13. The stimulation of 1,25 DHCC production and the inhibition of 24,25 DHCC synthesis by oestrogen is probably mediated by parathyroid hormone.

These experiments are consistent with the view that 24,25 DHCC is an inactive metabolite of 25 HCC produced when the demands of the body for calcium are low.

CHAPTER 10

CHAPTER 10. REFERENCES

ANSARI, A.Q., (1970) Ph.D. Thesis, Southampton University.

ARNAUDE, C.D., RASMUSSEN, H. and ANAST, C. (1966). J. Clin. Invest. 45, 1955-1964

BAKSI, S. and KENNY, A.D., (1977). J. Reprod. Fert. 50, 175-177.

BAKSI, S.N. and KENNY, A.D., (1977a). Biochem. Pharmacol. 26, 2439-2443.

BAKSI, S.N. and KENNY, A.D., (1977b). Endocrinol. 101, 1216-1220 and In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V., eds.) pp 89-91.

BAKSI, S.N. and KENNY, A.D., (1977c). Pharmacologist (Abstract) 19, 186.

BAKSI, S.N. and KENNY, A.D., (1978a). In: Endocrinology of Calcium Metabolism (Copp, D.H. and Talmage, R.V., eds.) p 370.

BAKSI, S.N. and KENNY, A.D., (1978b). Am. J. Physiol. 234, E622-E628.

BAR, A. and HURWITZ, S., (1972). Comp. Biochem. Physiol. 41, 735-744.

BATTACHARRYA, M. and DE LUCA, H.F., (1973). J. Biol. Chem. 248, 2969-2973.

BATTACHARRYA, M. and DE LUCA, H.F., (1974). Biochem. Biophys. Res. Commun. 59, 734-741.

BAXTER, L.A. and DE LUCA, H.F. (1976). J. Biol. Chem. 251, 3158-3161.

BAXTER, L.A., DE LUCA, H.F., BANJOUR, J.-P. and FLEISCH, H. (1974). Arch. Biochem. Biophys. 164, 655-662.

BELSEY, R., CLARK, M.B., BERNAT, M., GLOWACKI, J., HOLICK, M.F., DE LUCA, H.F. and POTTS, J.T. (1974). Am. J. Med. 57, 50-56.

BIKLE, D.D., MURPHY, E.W. and RASMUSSEN, H. (1975). J. Clin. Invest. 55, 299-304.

BIKLE, D.D. and RASMUSSEN, H. (1975). J. Clin. Invest. 55, 292-298.

BIKLE, D.D. and RASMUSSEN, H. (1978). Biochem. Biophys. Acta, 538, 127-138.

BIKLE, D.D., ZOLOCK, D.T., MORRISSEY, R.L. and HERMAN, R.H. (1977). In: Vitamin D, Biochemical, Chemical, and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V., eds.) pp 223-225.

BISHOP, J.E. and NORMAN, A.W. (1975). Arch. Biochem. Biophysics. 167, 769-773.

BLUNT, J.W., DE LUCA, H.F. and SCHNOES, H.K. (1968). Biochemistry 7, 3317-3322.

BONJOUR, J.-P., PRESTON, C., RIZZOLI, R. and FLEISH, H. (1978). In: Endocrinology of Calcium Metabolism (Copp, D.H. and Talmage, R.V. eds.) pp 172-177.

BOOTH, B.E., TSAI, H.C. and MORRIS, Jr. R.C. (1977). J. Clin. Invest. 60, 1314-1320.

BOYLE, I.T., GRAY, R.W. and DE LUCA, H.F. (1971). Proc. Natl. Acad. Sci. USA, 68, 2131-2134.

BOYLE, I.T., MIRAVET, L., GRAY, R.W., HOLICK, M.F. and DE LUCA, H.F. (1972). Endocrinol. 90, 605-608.

BRUMBAUGH, P.F. and HAUSSLER, M.R. (1974a). J. Biol. Chem. 249, 1251-1257.

BRUMBAUGH, P.F. and HAUSSLER, M.R. (1974b). J. Biol. Chem. 249, 1258-1262.

BRUMBAUGH, P.F. and HAUSSLER, M.R. (1975). J. Biol. Chem. 250, 1588-1594.

BRUMBAUGH, P.F., HAUSSLER, D.H., BRESSLER, R.M. and HAUSSLER, M.R. (1974). Science, 183, 1089-1091.

BRUMBAUGH, P.F., HUGHES, M.R. and HAUSSLER, M.R. (1975). Proc. Natl. Acad. Sci. USA. 72, 4871-4875.

CANTERBURY, S.M., LARMEN, S., CLAFLIN, A.J., HENRY, H., NORMAN, A.W. and REISS, E. (1978). J. Clin. Invest. April, 1375-1383.

CAPEN, C.C. (1978). In: Endocrinology of Calcium Metabolism (Copp, D.H. and Talmage, R.V. eds.) p. 367.

CAPEN, C.C., HENRY, H.L. and NORMAN, A.W. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 101-104.

CARE, A.D., BATES, R.F.L., RICHARD, D.W., PEACOCK, M., TOWLINSON, S., O'RIORDAN, J.L.H., MAWER, E.B., TAYLOR, C.M., DE LUCA, H.F. and NORMAN, A.W. (1975). Proceedings of the XIth European Symposium on Calcified Tissue (Pors Nielsen, S. and Hjørting-Hansen, E. eds.) pp 142-146.

CARE, A.D., PICKARD, D.W., PAPAPOULOS, S.E., O'RIORDAN, J.L.H. and REDEL, J. (1978). J. Endocrinol. 78, 303-304.

CARLSSON, A. (1952). Acta. Physiol. Scand. 26, 212-220.

CARLSSON, A. (1954). Acta. Physiol. Scand. 31, 308-311.

CARRÊ, M., AYIGBEDÊ, O., MIRAVET, L. and RASMUSSEN, H. (1974). Proc. Natl. Acad. Sci. USA. 71, 2996-3000.

CASTILLO, L., TANAKA, Y., DE LUCA, H.F. and SUNDE, M.L. (1977). Arch. Biochem. Biophys. 179, 211-217.

CHARLES, A., MARTIAL, J., ZOLOCK, D., MORRISSEY, R., BIKLE, D. and BAXTER, J. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Harrath, D.V. eds.) pp 227-229.

CHEESMAN, E.M., COPPING, A.M. and PREBBLE, P.M. (1964). Br. J. Nutr. 18, 147-152.

CHEN, T.C., CASTILLO, L., KORYCKA, D.M. and DE LUCA, H.F. (1974). J. Nutr. 104, 1056-1060.

CHERTOW, B.S., BAYLINK, D.J., WERGEDALE, J.E., SU, M.H. and NORMAN, A.W. (1975) J. Clin. Invest. 56, 668-678.

CLARK, M.B. and POTTS, J.T. (1977). Calci. Tiss. Res. 22, (Suppl.) pp 29-34.

CLARK, N.B. (1967). Comp. Biochem. Physiol. 20, 823-834.

COLSTON, K.W., EVANS, I.M.A., GALANTE, L., MACINTYRE, I. and MOSS, D.W. (1973). Biochem. J. 134, 817-820.

COMMON, R.H., RUTLEDGE, W.A. and HALE, R.W. (1948). J. Agric. Sci. 38, 64-80.

COOKE, A.S. (1978). Br. Poult. Sci. 19, 267-272.

CORRADINO, R.A. (1973). Science, 179, 402-405.

CORRADINO, R.A. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 231-240.

CORRADINO, R.A., TAYLOR, A.N. and WASSERMAN, R.H. (1969). Fed. Proc. 28, (Abstract) 760.

COSTANZO, L.S., SHEEHE, P.R. and WEINER, I.M. (1974). Am. J. Physiol. 226, 1490-1495.

CRAWFORD, J.D., GRIBETZ, D., DINER, W.C., HURST, P. and CASTLEMAN, P. (1957). Endocrinol. 61, 59-71.

CRAWFORD, J.D., GRIBETZ, D. and TALBOT, N.B. (1955). Am. J. Physiol. 180, 156-162.

CUSHARD, W.G., CREDITOR, M.A., CANTERBURY, J.M. and RIESS, E. (1972). J. Clin. Endocrin. Metab. 34, 767-771.

DE LUCA, H.F. (1974). Fed. Proc. 33, 2211-2219.

DE LUCA, H.F. and MELACON, M.J. (1972). In: Biochemical Action of Hormones (Litwack, G. ed.), 2, 337-383, Academic Press.

DE LUCA, H.F. and SCHNOES, H.K. (1976). Ann. Rev. Biochem. 45, 631-666.

DE LUCA, H.F. and SCHNOES, H.K. (1978). In: Endocrinology of Calcium Metabolism (Copp, D.H. and Talmage, R.V. eds.) pp 178-185.

EMTAGE, J.S. LAWSON, D.E.M. and KODICEK, E. (1973). Nature. 246, 100-101.

FINKELSTEIN, J.D. and SCHACHTER, D. (1962). Am. J. Physiol. 203, 873-880.

FORTE, L.R. NICHOLS, G.A. and ANAST, C.S. (1976). J. Clin. Invest. 57, 559-568.

FRASER, D.R. and EMTAGE, J.S. (1976). Biochem. J. 160, 671-682.

FRASER, D.R. and KODICEK, E. (1970). Nature, 228, 764-766.

FRASER, D.R. and KODICEK, E. (1973). Nature, N.B. 241, 163-166.

FRIEDLANDER, E.S., HENRY, H.L. and NORMAN, A.W. (1977). J. Biol. Chem. 252, 8677-8683.

FRIEDLANDER, E.J. and NORMAN, A.W. (1975). Arch. Biochem. Biophys. 170, 731-738.

FURR, B.J.A., BONNEY, R.C., ENGLAND, R.J. and CONNINGHAM, F.J. (1973). J. Endocrinol. 57, 159-169.

GALANTE, L., COLSTON, K.W., EVANS, I.M.A., BYFIELD, P.G.H., MATHEWS, E.W. and MACINTYRE, I. (1973). Nature, 244, 438-440.

GALANTE, L., COLSTON, K.W., MACAULEY, S.G. and MACINTYRE, I. (1972). Nature, 238, 271-272.

GARABEDIAN, M., HOLICK, M.F., DE LUCA, H.F. and BOYLE, I.T. (1972). Proc. Natl. Acad. Sci. USA, 69, 1673-1676.

GARABEDIAN, M., PAVLOVITCH, H., FELLOT, C. and BALSAM, S. (1974a). Proc. Natl. Acad. Sci. USA, 71, 554-557.

GARABEDIAN, M., TANAKA, Y., HOLICK, M.F. and DE LUCA, H.F. (1974b). Endocrinol. 94, 1022-1027.

GILBERT, A.B. (1971). In: Physiology and Biochemistry of the Domestic Fowl (Bell, D.J. and Freeman, M.D. eds.) 3, 1389, Academic Press.

GRAY, R., BOYLE, I. and DE LUCA, H.F. (1971). Science, 172, 1232-1234.

HADDAD, J.G. and STAMP, T.C. (1974). Am. J. Med. 57, 57-62.

HAHN, W.E. (1967). Comp. Biochem. Physiol. 23, 83-93.

HAMILTON, T.H. (1968). Science, 161, 649-661.

HARRISON, H.E. and HARRISON, H.C. (1961). Am. J. Physiol. 201, 1007-1012.

HARRISON, H.C., HARRISON, H.E. and PARK, E.A. (1958). Am. J. Physiol. 192, 432-436.

HAUSSLER, M.R., HUGHES, M.R., MCCAIN, T.A., ZERWEKH, J.E., BRUMBAUGH, P.F., JUBIZ, W. and WASSERMAN, R.H. (1977). Calci. Tiss. Res. 22, (Supp.) pp 1-18.

HAUSSLER, M.R., MYRTLE, J.F. and NORMAN, A.W. (1968). J. Biol. Chem. 243, 4055-4064.

HENRY, H.L. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 125-133.

HENRY, H.L., MIDGETT, R.J. and NORMAN, A.W. (1974). J. Biol. Chem. 249, 7584-7592.

HENRY, H.L. and NORMAN, A.W. (1975). Biochem. Biophys. Res. Commun. 62, 781-788.

HENRY, H.L. and NORMAN, A.W. (1976). Arch. Biochem. Biophys. 172, 582-589.

HENRY, H.L., TAYLOR, A.N. and NORMAN, A.W. (1977). J. Nutr. 107, 1918-1926.

HENRY, H.L., WECKSLER, W.R. and NORMAN, A.W. (1978). In: Endocrinology of Calcium Metabolism (Copp, D.H. and Talmage, R.V. eds.) pp 197-203.

HERTELENDY, F. and TAYLOR, T.G. (1965). Br. Poult. Sci. 6, 339-343.

HESS, A.F. and GUTMAN, M.B. (1922). J. Am. Med. Assoc. 78, 29-31.

HILL, L.F., LUMB, G.A., MAWER, E.B. and STANBURY, S.W. (1973). Clin. Sci. 44, 335-347.

HOLICK, M.F., BAXTER, L.A., SCHRAUFROGEL, P.K., TAVELA, T.E. and DE LUCA, H.F. (1976). J. Biol. Chem. 251, 397-402.

HOLICK, M.F., SCHNOES, H.K., DE LUCA, H.F., GRAY, W.R., BOYLE, I.T. and SUDA, T. (1972). Biochemistry, 11, 4251-4255.

HOLICK, M.F., SCHNOES, H.K., DE LUCA, H.F., SUDA, T. and COUSINS, R.J. (1971). Biochemistry, 10, 2799-2804.

HORIUCHI, N., SUDA, T., SASAKI, S., OGATA, E., EZAMA, I., SANO, Y. and SHIMAZAWA, E. (1975). Arch. Biochem. Biophys. 171, 540-548.

HORIUCHI, N., SUDA, T., SASAKI, S., TAKAHASHI, S., SHIMAZAWA, E. and OGATA, E. (1976). Biochem. Biophys. Res. Commun. 73, 869-875.

HORIUCHI, N., SUDA, T., TAKAHASHI, H., SHIMAZAWA, E. and OGATA, E. (1977). Endocrinol. 101, 969-974.

HORSTING, M. and DE LUCA, H.F. (1969). Biochem. Biophys. Res. Commun. 36, 251-256.

HULDSCHINSKY, K. (1919). Deut. Med. Wochschr. 45, 712-713.

HURST, J.G. and MAYER, G.P. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 139-141.

JOWSEY, J.R., OLIVER, W.F., MAW, W.A. and COMMON, R.H. (1953). Can. J. Agric. Sci. 33, 216-224.

KAKUTA, S., SUDA, T., SASAKI, S., KIMURA, N. and NAGATA, N. (1975). Endocrinol. 97, 1288-1293.

KAPPAUF, B. and VAN TIENHOVEN, A. (1972). Endocrinol. 90, 1350-1355.

KENNY, A.D. (1976). Am. J. Physiol. 230, 1609-1615.

KENNY, A.D. and BAKSI, S.N. (1977), (Abstract). Fed. Proc. 36, 1097.

KENNY, A.D. and BAKSI, S.N. (1978), (Abstract). Fed. Proc. 37, 926.

KIMBERG, D.V., BAERG, R.D., GERSHON, E. and GRANDUSIUS, R.T. (1971). J. Clin. Invest. 50, 1309-1321.

KLEINER, A. and DE LUCA, H.F. (1974). Biochem. Biophys. Acta. 338, 489-495.

KUMAR, R., COHEN, W.R., SILVA, P. and EPSTEIN, F.H. (1979). J. Clin. Invest. 63, 342-344.

KUMAR, R., SCHNOES, H.K. and DE LUCA, H.F. (1978). J. Biol. Chem. 253, 3804-3809.

LARKINS, R.G., MACAULEY, S.J. and MACINTYRE, I. (1974). Nature, 252, 412-414.

LAWSON, D.E.M. (1978). In: Vitamin D, (Lawson, D.E.M. ed.) pp 167-200, Academic Press (London).

LAWSON, D.E.M., CHARMAN, M., WILSON, P.W. and SPENCER, R. (1978). In: Endocrinology of Calcium Metabolism (Copp, D.H. and Talmage, R.V. eds.) pp 191-196.

LAWSON, D.E.M. and EMTAGE, J.S. (1973). In: Metabolism and Function of Vitamin D, Biochem. Soc. Spec. Publ. No. 3.

LAWSON, D.E.M., FRASER, D.R., KODICEK, E., MORRIS, H.R. and WILLIAMS, D.H. (1971). Nature, 230, 228-230.

LAWSON, D.E.M., SPENCER, R., CHARMAN, M. and WILSON, P. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 265-275.

LAWSON, D.E.M., WILSON, P.W. and KODICEK, E. (1969). Biochem. J. 115, 269-277.

LORENCE, R., TANAKA, Y., DE LUCA, H.F. and JONES, G. (1977). Endocrinol. 100, 468-472.

LUKERT, B.P., STANBURY, S.W. and MAWER, E.B. (1973). Endocrinol. 93, 718-722.

LUND, J. and DE LUCA, H.F. (1966). J. Lipid. Res. 7, 739-744.

MACINTYRE, I. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 155-164.

MACINTYRE, I., COLSTON, K.W. and EVANS, I.M.A. (1975). In: Proceedings of the XIth European Symposium on Calcified Tissues (Neilsen, S.P. and Hjørting-Hensen, E. eds.) pp 136-141.

MACINTYRE, I., COLSTON, K.W., EVANS, I.M.A., LOPEZ, E., MACAULEY, S.J., PIEGNOUX-DEVILLE, J., SPANOS, E. and SZELKE, M. (1976). Clin. Endocrinol. Suppl. 5, 85S-95S.

MACOWAN, M.M. (1931). Quart. J. Exp. Physiol. 21, 383-391.

MARTIN, D.L., MELANCON, M.J. and DE LUCA, H.F. (1969). Biochem. Biophys. Res. Commun. 35, 819-823.

MAWER, E.B. and REEVE, A. (1977). Calci. Tiss. Res. 22, (Suppl.) pp 24-28.

MCCORQUODALE, D.J. and MUELLER, G.C. (1958). J. Biol. Chem. 232, 31-42.

MELLANBY, E. (1919a). J. Physiol. 52, xi-xii.

MELLANBY, E. (1919b). J. Physiol. 52, liii-liv.

MELLANBY, E. (1919c). The Lancet 1, 407-412.

MIDGETT, R.J., SPEILROGEL, A.M., COBURN, J.W. and NORMAN, A.W. (1973). J. Clin. Endocrinol. Metab. 36, 1153-1161.

MORINCHI, S. and DE LUCA, H.F. (1974). Arch. Biochem. Biophys. 164, 165-171.

MORIUCHI, S., YOSHZAWA, S., SHIMURA, F., OKU, T. and HOSOYA. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 285-287.

MORRISEY, R.L. and WASSERMAN, R.H. (1971). Am. J. Physiol. 22, 1509-1515.

MUELLER, G.C. (1953). J. Biol. Chem. 204, 77-90.

MYRTLE, J.F. and NORMAN, A.W. (1971). Science, 171, 79-82.

NALBANDOV, A.V. (1945). Endocrinol. 36, 251-258.

NEVILLE, P.F. and DE LUCA, H.F. (1966). Biochemistry, 5, 2201-2207.

NEY, R.L., KELLY, G. and BARTTER, F.D. (1968). Endocrinol. 82, 760-766.

NICHOLSON, R.A. (1977). Ph.D. Thesis, Southampton University.

NICOLAYSEN, R., EEG-LARSEN, N. and MALM, O.J. (1953). Physiol. Rev. 33, 424-444.

OMDAHL, J.L. (1977). In; Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 183-185.

OMDAHL, J.L. and DE LUCA, H.F. (1971). Science, 174, 949-951.

OMDAHL, J.L. and DE LUCA, H.F. (1972). J. Biol. Chem. 247, 5520-5526.

OMDAHL, J.L. and DE LUCA, H.F. (1973). Physiol. Rev. 53, 327-372.

OMDAHL, J.L. and EVAN, A.P. (1977). Arch. Biochem. Biophys. 184, 179-188.

OMDAHL, J.L., HOLICK, M.F., SUDA, T., TANAKA, Y. and DE LUCA, H.F. (1971). Biochemistry 10, 2935-2940.

OMDAHL, J.L., HUNSAKER, L.A. and ASCHENBRENNER, V.A. (1977). Arch. Biochem. Biophys. 148, 172-178.

PAGET, G.E., WALPOLE, A.L. and RICHARDSON, D.W. (1961). Nature, 192, 1191.

PEACOCK, M., TAYLOR, G.A. and NORMAN, A.W. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 411-413.

PETERSON, A.J. and COMMON, R.H. (1971). Can. J. Zool. 49, 599-604.

PETERSON, A.J. and COMMON, R.H. (1972). Can. J. Zool. 50, 395-404.

PETERSON, A.J., HENNEBERRY, G.O. and COMMON, R.H. (1973). Can. J. Zool. 51, 753-758.

PIKE, J.W., TOVERUD, S., BOASS, A., MCCAIN, T. and HAUSSLER, M.R. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 187-189.

PONCHON, G., KENNAN, A.L. and DE LUCA, H.F. (1969). J. Clin. Invest. 48, 2032-2037.

POPOVTZER, M.M., ROBINETTE, J.B., DE LUCA, H.F. and HOLICK, M.F. (1974). J. Clin. Invest. 53, 913-921.

PREECE, M.A., O'RIORDAN, J.L.H., LAWSON, D.E.M. and KODICEK, E. (1974). Clin. Chem. Acta. 54, 235-242.

PROCSAL, D.A., OKAMURA, W.H. and NORMAN, A.W. (1975). J. Biol. Chem. 250, 8382-8388.

PUSCHETT, J.B., BECK, W.S., JELONEK, A. and FERNANDEZ, P.C. (1974). J. Clin. Invest. 53, 756-767.

PUSCHETT, J.B., FERNANDEZ, P.C., BOYLE, I.T., GRAY, R.W., OMDAHL, J.L. and DE LUCA, H.F. (1972b). Proc. Soc. Exp. Biol. Med. 141, 379-384.

PUSCHETT, J.B., MORANZ, T. and KURNICK, W.S. (1972a). J. Clin. Invest. 51, 373-385.

RAISZ, L.G., TRUMMEL, C.L., HOLICK, M.F. and DE LUCA, H.F. (1972). Science, 175, 768-769.

RASMUSSEN, H. and BORDIER, P. (1974). In: The Physiological and Cellular Basis of Metabolic bone disease, pp 207-249 (Williams and Wilkins Co., Baltimore).

RASMUSSEN, H., WONG, M., BIKLE, D. and GOODMAN, D.B.P. (1972). J. Clin. Invest. 51, 2502-2504.

REYNOLDS, J.J. (1974). Biochemical Society Special Publication No. 3, p 91 (Fraser, D.R. ed.).

REYNOLDS, J.J., HOLICK, M.F. and DE LUCA, H.F. (1973). Calci. Tiss. Res. 12, 295-301.

REYNOLDS, J.J., PAVORITCH, H. and BALSAM, S. (1976). Calci. Tiss. Res. 21, 207-212.

RIDDLE, P. (1963). Animal Behaviour, 11, 419-432.

RIGGS, B.L., ARNAUD, C.D., JOWSEY, J., GOLDSMITH, R.S. and KELLY, P.J. (1973). J. Clin. Invest. 52, 181-184.

RIGGS, B.L. and GALLAGHER, J.C. (1978). In: Endocrinology of Calcium Metabolism (Copp, D.H. and Talmage, R.V. eds.) pp 43-47.

RIGGS, B.L., JOWSEY, J., GOLDSMITH, R.S., KELLY, P.J., HOFFMAN, D.L. and ARNAUD, C.D. (1972). J. Clin. Invest. 51, 1659-1663.

*ROY, E.J. and MACKAY, R. (1962). J. Obstet. Gynaecol. Br. Commonw. 69, 13-17.

SAMIY, A.H., HIRSCH, P.F. and RAMSAY, A.G. (1960). Endocrinol. 67, 266-270.

SAMIY, A.H., HIRSCH, P.F. and RAMSAY, A.G. (1965). Am. J. Physiol. 208, 73-77.

SANDS, H. and KESSLER, R.H. (1971). Proc. Soc. Exp. Biol. Med. 137, 1267-1273.

SCHNEIDER, L.E., NOWOSIELSKI, L. and SCHEDL, H.P. (1977a). Endocrinol. 100, 67-73.

SCHNEIDER, L.E., OMDAHL, J. and SCHEDL, H.P. (1976). Endocrinol. 99, 793-799.

SCHNEIDER, L.E. and SCHEDL, H.P. (1972). Am. J. Physiol. 223, 1319-1323.

SCHNEIDER, L.E., SCHEDL, H.P., MCCAIN, T. and HAUSSLER, M.R. (1977b). Science, 196, 1452-1454.

SCHNEIDER, L.E., WILSON, H.D. and SCHEDL, H.P. (1973). Nature, 245, 327-328.

SCHNEIDER, L.E., WILSON, H.D. and SCHEDL, H.P. (1974). Am. J. Physiol. 227, 832-838.

SEBRELL, W.H. and HARRIS, R.S. (1971). In: The vitamins, New York, Academic Press, Chapter 7, pp 155-301.

SENIOR, B.E. (1974). J. Reprod. Fert. 41, 107-112.

SHAIN, S.A. (1972). J. Biol. Chem. 247, 4404-4413.

SOMMERVILLE, B.A., SWAMINATHAN, R. and CARE, A.D. (1978). Br. J. Nutr. 39, 411-414.

SOMMERVILLE, B.A., SWAMINATHAN, R. and SCANES, C.G. (1977). Calci. Tiss. Res. 22, (Suppl.) 51-54.

SPANOS, E., BARRETT, D.I., CHONG, K.T. and MACINTYRE, I. (1978a). Biochem. J. 174, 231-236.

SPANOS, E., BARRETT, D., MACINTYRE, I., PIKE, J.W., SAFILIAN, E.F. and HAUSSLER, M.R. (1978b). Nature, 273, 246-247.

SPANOS, E., COLSTON, K.W., EVANS, I.M.A., GALANTE, L.S., MACAULEY, S.J. and MACINTYRE, I. (1976a). Molec. Cell. Endocrin. 5, 163-167.

SPANOS. E. and MACINTYRE, I. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 191-195.

SPANOS, E., PIKE, J.W., HAUSSLER, M.R., COLSTON, K.W., EVANS, I.M.A., GOLDNER, A.M., MCCAIN, T.A. and MACINTYRE, I. (1976b). Life Science, 19, 1751-1756.

SPENCER, R., CHARMAN, M., WILSON, P. and LAWSON, E. (1976). Nature, 263, 161-163.

SPENCER, E.M. and TOBIASSEN, O. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D. Grigoleit, H.G. and Herrath, D.V. eds.) pp 197-199.

STANBURG, W.A. (1975). In: Calcium Regulating Hormones (Talmage, R.V. ed.) Excerpta Medica, Amsterdam, pp 431-438.

STEELE, T.H., ENGLE, J.E., TANAKA, Y., LORENCE, R.S., DODGEON, K.L. and DE LUCA, H.F. (1975). Am. J. Physiol. 229, 489-495.

STRINGER, D.A. (1962). Ph.D. Thesis, University of Reading.

SUDA, T., DE LUCA, H.F., SCHNOES, H.K., TANAKA, Y. and HOLICK, M.F. (1970). Biochemistry, 9, ~~2701-2705.~~ 4776 – 4780

SUDA, T., HORIUCHI, N., FUKUSHIMA, M., NISHII, Y. and OGATA, E. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 201-210.

SUTTON, R.A.L., HARRIS, C.A., WONG, N.L.M. and DIRKS, J.H. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 451-453

SWAMINATHAN, R., SOMMERVILLE, B.A. and CARE, A.D. (1977). Br. J. Nutr. 38, 47-54.

SYKES, A.H. (1963). J. Rep. Fert. 6, 319.

TANAKA, T., CASTILLO, L. and DE LUCA, H.F. (1976). Proc. Natl. Acad. Sci. USA. 73, 2701-2705.

TANAKA, T., CASTILLO, L. and DE LUCA, H.F. (1977). J. Biol. Chem. 252, 1421-1424.

TANAKA, Y., CASTILLO, L., WINELAND, M.J. and DE LUCA, H.F. (1978a). Endocrinol. 103, 2035-2039.

TANAKA, Y. and DE LUCA, H.F. (1971). Arch. Biochem. Biophys. 146, 574-578.

TANAKA, Y. and DE LUCA, H.F. (1973). Arch. Biochem. Biophys. 154, 566-574.

TANAKA, Y. and DE LUCA, H.F. (1974). Science, 183, 1198-1200.

TANAKA, Y., DE LUCA, H.F., OMDAHL, J. and HOLICK, M.F. (1971). Proc. Natl. Acad. Sci. USA. 68, 1286-1288.

TANAKA, Y., SHEPARD, R.A., DE LUCA, H.F. and SCHNOES, H.K. (1978b). Biochem. Biophys. Res. Commun. 83, 7-13.

TAYLOR, T.G. and KIRKLEY, J. (1967). Br. Poult. Sci. 8, 289-295.

TAYLOR, T.G. and MOORE, J.H. (1954). Br. J. Nutr. 8, 112-124.

TAYLOR, T.G. and STRINGER, D.A. (1965). In: "Avian Physiology", Second edition, by Sturkie, P.D., Comstock Publishing Associates, Cornell University Press, Ithaca, N.Y., pp 485-501.

TAYLOR, A.N. and WASSERMAN, R.H. (1972). Am. J. Physiol. 223, 110-114.

TSAI, H.C., MIDGETT, R.J. and NORMAN, A.W. (1973). Arch. Biochem. Biophys. 157, 339-347.

TSAI, H.C. and NORMAN, A.W. (1973). Biochem. Biophys. Res. Commun. 54, 622-627.

TUCKER, G., GAGNON, R.E. and HAUSSLER, M.R. (1973). Arch. Biochem. Biophys. 155, 47-57.

UI, H. and MUELLER, G.C. (1963). Fed. Proc. 22, 409 (Abstract).

URIST, M.R., DEUTSCH, N.M., POMERANTZ, G. and MCLEAN, F.C. (1960). Am. J. Physiol. 199, 851-855.

URIST, M.R., SCHJEIDE, A.O. and MCLEAN, F.C. (1958). Endocrinol. 63, 570-585.

WALLING, M.W. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 321-330.

WALLING, M.W., BRASITUS, T.A. and KIMBERG, D.V. (1976). Endocrine. Res. Comm. 3, 83-91.

WALLING, M.W. and KIMBERG, D.V. (1975). Endocrinol. 97, 1567-1576.

WASSERMAN, R.H. and CORRADINO, R.A. (1973). Vitamins and Hormones, 31, 43-103.

WASSERMAN, R.H., FULLER, C.S. and TAYLOR, A.N. (1978). In: Vitamin D (Lawson, D.E.M. ed.) pp 144, Academic Press, London.

WASSERMAN, R.H. and TAYLOR, A.N. (1973). J. Nutr. 103, 586-599.

WECKSLER, W.R., HENRY, H.L. and NORMAN A.W. (1977). Arch. Biochem. Biophys. 183, 168-175.

WIDNELL, C.C. and TATA, J.R. (1966). Biochem. Biophys. Acta. 123, 478-492.

WILLIAMS, J.B. and SHARP, P.J. (1977). J. Endocrinol. 75, 447-448.

WONG, R.G., NORMAN, A.W., REDDY, C.R. and COBURN, J.W. (1972). J. Clin. Invest. 51, 1287-1291.

ZERWEKH, J.E., HAUSSLER, M.R. and LINDELL, T.J. (1974). Proc. Natl. Acad. Sci. USA. 71, 2337-2341.

ZOLOCK, D.T., MORRISSEY, R.L. and BIKLE, D.D. (1977). In: Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., De Luca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V. eds.) pp 345-347.

ZUCKER, T.F., PAPPENHEIMER, A.M. and BARNETT, M. (1922). Proc. Soc. Exp. Biol. Med. 19, 167-169.

* The original reference was not consulted.