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SOME ASPECTS OF CALCIUM METABOLISM IN BIRDS AND MAMMALS

by

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A thesis submitted to the
University of Southampton
for the degree of

Doctor of Philosophy

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School of Biochemical and Physiological Sciences

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ABSTRACT

FACULTY OF SCIENCE - DEPARTMENT OF NUTRITION

Doctor of Philosophy

SOME ASPECTS OF CALCIUM METABOLISM IN BIRDS AND MAMMALS

By Phillip John Williams

This thesis is divided into two distinct sections, both of which are concerned with aspects of calcium metabolism. In the first section, the role of various reproductive steroids on the metabolism of 25-hydroxycholecalciferol (25-HCC) in Japanese Quail was studied. Metabolism of 25-HCC was followed by incubating ^{3}[H]25-HCC with renal homogenates, and then separating the metabolites (consisting mainly of 1,25- and 24,25-dihydroxycholecalciferol) by a unique TLC system.

Quail raised on a short-day (SD) lighting regime of eight hours light, 16 hours dark (8L:16D) were extensively used in this work: these birds were somatically mature but sexually immature. Their reproductive steroid levels have been well monitored and are known to be minimal, and these birds proved to be an excellent model for studying the action of exogenous reproductive steroids on vitamin D metabolism. Both 17β-oestradiol and oestrone were shown to stimulate the renal 1-hydroxylase potently in the SD birds, while 17α-oestradiol and oestriol had weak stimulatory actions. This was the first time that oestrogens other than 17β-oestradiol had been implicated in regulating the 1-hydroxylase and their physiological importance is discussed. Plasma 17β-oestradiol and oestrone levels measured by RIA correlated well with **in vivo** renal hydroxylase activities. Oestrogens were shown to stimulate the 1-hydroxylase within 3 hours. Progesterone augmented and testosterone inhibited the oestrogenic stimulation of the 1-hydroxylase. Little is known of the mechanism of action of oestrogen on the 1-hydroxylase, but some evidence is presented suggesting that **de novo** protein synthesis is a requirement. Furthermore much circumstantial and some experimental evidence which does not necessarily contradict previous observations, suggests that prolactin may mediate the oestrogen induced stimulation of the 1-hydroxylase. In most experiments a reciprocal relationship was observed between the production of 1,25- and 24,25-dihydroxycholecalciferol, showing that hormonal treatments that stimulated the 1-hydroxylase inhibited the 24-hydroxylase and **vice versa**.

The second section studied the factors influencing phytate hydrolysis in the gastro-intestinal tract, and the great capacity for hydrolising phytate of the hamster compared to the rat. Phytate is a valuable but often wasted source of phosphorus, therefore this project has economic significance. Alkaline phosphatase and phytase activities were measured in the rat and hamster small intestines. The rat had higher alkaline phosphatase activity than the hamster, and considerable phytase activity, whereas the hamster had none. The disappearance of phytate was monitored during the passage of food through the rat and hamster gut, and it was shown that most of the phytate was hydrolysed in the stomach in both the rat and the hamster. As phytase activity could not be found in the stomach tissue of either species, the phytase had to be of either cereal or microbial origin. Cereal phytase activity was largely ruled out in the hamster by the use of a cereal diet, free of phytase activity, but this source of phytase appeared to be more important in the rat. The hamster is a 'pseudo-ruminant' with a well developed fore stomach and it was concluded that the hamster's superior ability to hydrolyse phytate was primarily due to extensive microbial hydrolysis of phytate in the fore stomach.

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LIST OF ABREVIATIONS

S.E.M.	Standard Error of the Mean
T.L.C.	Thin Layer Chromatography
H.P.L.C.	High Pressure Liquid Chromatography
r.f.	Relative Flow
h	hour
g	gram
l	liter
d	days
rpm	revolutions per minute
°C	degrees centrigrade
$t_{1/2}$	half life
%	percentage
±	mean
d.p.m.	disintergrations per minute
i.m.	intra-muscular
i.p	intra-peritoneal
cAMP	cyclic adenosine-3'-5'-monophosphate
B.W.	Bird Weight
^{3}[H]	Tritium
^{14}[C]	Carbon isotope 14
NADP	Nicotinamide adenine dinucleotide phosphate
i.u.	international units
prefix	-
k	kilo
m	milli
μ	micro
n	nano
p	pico
♀	female
♂	male
LH	Luteinizing Hormone
FSH	Follicle Stimulating Hormone
LH-RH	Luteinizing Hormone Releasing Hormone
Butyl PBD	2-4' tert butyl phenyl-5-4' biphenyl-1,3,4-oxadiazole
GF_{254}	Fluorescent Silica

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SECTION 1

The Role and Possible Mode of Action of Reproductive Steroids in the Regulation of the Renal 25-Hydroxycholecalciferol-1α-Hydroxylase in Japanese Quail

CHAPTER 1 - REVIEW OF RELEVANT LITERATURE

1.1 Calcium Homeostasis

1.1.1 Calcium an Overview

Calcium is one of the most important biological cations; it not only helps provide a rigid body structure in the form of bone, but it is also integral to the structure and functioning of all cells. Specific examples of its functions include the maintenance of nerve excitability, the release of acetylcholine at neuromuscular junctions and a role in muscle contraction and blood clotting. Intracellularly it seems likely that many of the functions of calcium are modulated by the protein calmodulin (Klee, Crouch and Richman, 1980). Calmodulin is a unique protein with no intrinsic enzyme activity; however it has four high affinity binding sites for calcium per molecule, and when these sites are occupied, calmodulin has the ability to regulate the activity of numerous enzymes, including adenylate cyclase, phosphorylase kinase, phosphodiesterases and calcium ATPase. These multiple functions necessitate a system capable not only of obtaining the element from the environment, but also of finely regulating the concentration in the body of the physiologically active calcium ion. The system that achieves these ends is the "Calcium Homeostatic System", and is composed of several components.

Calcium homeostasis in both avian and mammalian species, involves three main hormone systems and three major target tissues. Parathyroid hormone (PTH) and 1,25-dihydroxycholecalciferol (1,25-DHCC) are two of the hormones having hypercalcaemic actions, whereas the third hormone, calcitonin, has a hypocalcaemic one. The three major target tissues are bone, which contains 98-99% of the total body calcium, the small intestine and the kidney. In the following sections reference will be made primarily to avian and mammalian calcium homeostasis. It is important to realise that some differences exist in calcium metabolism between these two animal groups, some of which will be discussed in Chapter 1.1.5.

Avian species can be used to great advantage in research, as their huge requirement and rapid turnover of calcium can resolve various homeostatic controls too small to be easily detected in mammals.

1.1.2 The Role of PTH in Calcium Homeostasis

Bovine PTH (bPTH) has been extensively used and studied; its 84 amino acid sequence is the dominant hormonal form secreted (Habener, Segré, Powell, Murray and Potts, 1972), and is probably the active circulating form as well. bPTH has been found to be immunochemically heterogeneous (Berson and Yallow, 1968), mainly due to the renal and hepatic 'cleavage' enzymes. The precise relevance of the resultant fragments is at present a controversial one. Plasma PTH levels have been measured in mammals by various bioassay systems; however more sensitive radioimmunoassays have now been developed using bPTH. These assays give 'normal' plasma PTH levels between 40-120pg/ml (Papapoulos, Hendy, Manning, Lewin and O'Riordan, 1978). As yet there are no sensitive assays for avian PTH.

One of the major physiological actions of PTH is that of

overcoming hypocalcaemic states by increasing plasma calcium levels. PTH acts on the bone and the kidney. In the kidney it increases reabsorption of calcium from the distal renal tubules; PTH also inhibits phosphate reabsorption from the proximal and possibly distal renal tubules (Wideman and Braun, 1981). Increasing phosphate excretion helps to reduce deposition of calcium back into bone and helps, therefore, to maintain or elevate the plasma calcium level. PTH's action on bone is not fully understood. When large to moderate doses of PTH are given **in vivo** (Walker, 1971) and **in vitro** (Raisz, Lorenzo, Gworek, Kream and Rosenblatt, 1979; Howard, Bottemiller, Turner, Rader and Baylink, 1981) considerable bone resorption results. However when dogs were given continuous infusions of PTH, at a low dose, hypercalcaemia was observed, with considerable evidence suggesting that bone mass actually increased (Parsons and Reit, 1974). Howard et al (1981) in their **in vitro** studies also found that low doses of PTH caused bone accretion. It is quite possible therefore that PTH only causes increased bone resorption during extreme calcium stress as a result of hypersecretion of PTH.

The actual process of PTH induced bone resorption is thought to be mediated via increasing levels of cAMP (Chase and Aurbach, 1970; Rasmussen, Pechet and Fast, 1968). However, PTH stimulates cAMP production in both bone forming (osteoblastic) cells and bone resorbing (oesteoclastic) cells (Jilka and Cohn, 1981); presumably cAMP activates the osteoclasts and inhibits the osteoblasts. This view has been challenged by Martin and Partridge (1981), who believe that cAMP only increases in the oesteoblasts, and suggest that the small increase in cAMP production seen by other workers (Wong and Cohn, 1975; Jilka and Cohn, 1981) in the osteoclasts is due to osteoblastic contamination, a generally recognised problem. It is perhaps worth nothing that a 10% increase in the calcium concentration of the extracellular fluid could be accomplished by resorbing just 0.1% of the total bone mineral in man (Newman and Ramp, 1971). Therefore day to day regulation of

plasma calcium levels could be achieved easily by changing calcium fluxes through bone itself, renal calcium resorption or other mechanisms yet to be discussed, and without the real need to invoke the drastic bone resorption mechanism. Relatively recently a new function of PTH concerning calcium homeostasis was discovered. This was the direct stimulation of the renal 25-hydroxycholecalciferol-1α-hydroxylase, the enzyme that produces the hormonally active form of vitamin D. This specific action of PTH will be fully discussed in section 1.2.4a.

1.1.3 The Role of Vitamin D in Calcium Homeostasis

Rickets is a disease of children and young growing animals that has been recognised for centuries, and is characterised by skeletal deformities. It was shown unequivocally that rickets was caused by a deficiency of a trace compound in the diet (Mellanby, 1919 and 1921). It was demonstrated that this trace component was distinct from vitamin A, which had appeared to be the most likely factor and the new factor was called vitamin D (McCollum, Simmonds, Becker and Shipley, 1922). It is now known that vitamin D has a pivotal role in calcium metabolism, with actions on the bone, kidney and most importantly and uniquely on the intestine. After the initial discovery of vitamin D it took a further 50 years before it was discovered that vitamin D itself was physiologically inactive and had to be further metabolised to its active and now correctly considered hormonal form, namely 1,25-dihydroxycholecalciferol (1,25-DHCC). In recent years vitamin D metabolism has been intensively studied and will be discussed later in this chapter. However, this section focuses on the physiological actions of 1,25-DHCC and to a lesser extent other metabolites of vitamin D.

1.1.3a <u>Vitamin D and Intestinal Calcium Absorption</u>

Nicolaysen (1937a,b) was the first to report a direct relationship between vitamin D and calcium absorption from the gut. He noted that in vitamin D deficient rats the rate of calcium absorption was much lower than that of controls, and also that under conditions of dietary phosphorus deprivation vitamin D markedly stimulated intestinal calcium absorption. When radioactive calcium became available it was confirmed that vitamin D had a significant effect on intestinal calcium absorption in both the chick (Migicovsky and Emslie, 1949) - and the rat (Carlsson, 1951). It is now apparent that vitamin D possesses the unique ability to stimulate active intestinal calcium transport (Bikle, Morrissey, Zolock and Rasmussen, 1981). The problem still to be resolved is, mechanistically, how does 1,25-DHCC bring about this stimulation.

With this in mind it is worth considering the intestinal mucosa, as this is the barrier calcium has to cross to become absorbed. The first problem is entry into the intestinal mucosa cell through the brush border membrane (see Figure 1.1). Most evidence indicates that this is the rate-limiting step of calcium absorption and that 1,25-DHCC alters the calcium permeability of this membrane by a process as yet unknown but apparently not requiring <u>de novo</u> protein synthesis (Rasmussen, Fontaine, Max and Goodman, 1979). The ability of mucosa cells to rid themselves of excess calcium, induced by vitamin D, can be blocked by cycloheximide, thus suggesting a requirement for a protein to be made in response to vitamin D, that would increase the efficiency of the calcium 'pump' in the basolateral membrane (Bikle <u>et al</u>, 1981). This requirement fits in well with considerable data showing that 1,25-DHCC acts similarly to a classical steroid hormone (Norman, 1980). 1,25-DHCC is known to bind to specific high affinity cytosolic receptors that translocate the hormone to the nucleus, where specific RNA synthesis is stimulated. This ultimately leads to a new protein being made and the physiological response. The

Figure 1.1

A Simple Model Showing the Effects of 1,25-DHCC on a Gut Epithelial Cell

In Figure 1.1 the following abbreviations were made to simplify the diagram:

M	=	Mitochondria
DNA	=	Deoxyribonucleic Acid
N	=	Nucleus of cell
P	=	Calcium ATPase Pump
Ca^{++}	=	Ionic Calcium
Pi	=	Phosphate ion
CaBP	=	Calcium Binding Protein
R	=	1,25-DHCC Specific Receptor
C	=	Unknown Calcium Carrier
Ri	=	Ribosomal Translation
AC	=	Adenylate Cyclase
+	=	Stimulation

Brush Border Membrane

Basolateral Membrane

Tight Junction

Blood

Gut
Lumen

M

Ca Pi

M

N

DNA

Na^+

P

Ca^{++}

Ca

C

1,25DHCC+R

Ri

CaBP

c-AMP

ATP

AC

$[Ca^{++}]$
= 1-10mM

$[Ca^{++}]$ = 1μM

$[Ca^{++}]$ = 1.3mM

Figure 1.1

first protein to be identified as being produced <u>de novo</u> by the influence of vitamin D on the intestine was calcium binding protein (CaBP) (Wasserman and Taylor, 1966). The presence of this protein in the intestinal mucosa is totally dependant on the prior administration of vitamin D or one of its active metabolites, and it is absent from the intestine of vitamin D deficient chicks and rats (Bronner and Freund, 1975). Chick CaBP is relatively easily purified, has a high affinity for calcium ($Ka = 2x10^6 M^{-1}$), can bind four calcium atoms per molecule, and has a molecular weight of 28,000 daltons (Norman, 1979). Early work with CaBP disclosed a good correlation between levels of CaBP induced by vitamin D and the rate of calcium transport in the gut (Ebel, Taylor and Wasserman, 1969). They concluded that CaBP was the carrier protein responsible for calcium transport in the small intestine. More recent work by Spencer, Charman, Wilson and Lawson (1978), has clearly shown that 1,25-DHCC administration stimulates calcium transport at least 2 hours (h) before CaBP can be detected immunologically. It has been suggested, therefore, that CaBP protects cells from the potentially toxic surge of calcium that passes through epithelial cells when calcium transport is stimulated by 1,25-DHCC (Bikle <u>et al</u>, 1981). It is also likely that CaBP facilitates calcium exchange between certain subcellular organelles and the calcium pump at the basolateral membrane (Hamilton and Holdsworth, 1970). At the present time the precise role of CaBP in intestinal calcium transport is a contraversial one, but it does not appear to be directly responsible for increased calcium transport.

The problem of getting calcium out of the epithelial cells into the blood, which is against an electrochemical gradient, is thought to be carried out by a calcium ATPase 'pump'. This enzyme has been isolated in the basolateral membrane (Birge and Gilbert, 1974) and is most probably driven by the high concentrations of sodium in the extracellular fluid. Lane and Lawson (1978) observed a concommitant increase in calcium

absorption and calcium ATPase activity in vitamin D deficient chicks given 1,25-DHCC; they concluded that this enzyme has a direct role in the process of calcium absorption. They also suggest that 1,25-DHCC does not stimulate this enzyme directly (unpublished observations Lane and Lawson, 1978).

It should be pointed out that vitamin D appears to stimulate the activity of several other enzymes in the gut, for example alkaline phosphatase (Norman, Mircheff, Adams and Spielvogel, 1970), phytase (Steenbock, Krieger, Wiest and Pileggi, 1953), adenylate cyclase (Holdsworth, 1970) and ornithine decarboxylase (Shinki, Takahashi, Miyaura, Sumejima, Nishi and Suda, 1981). The most important of these enzymes is perhaps adenylate cyclase, as 1,25-DHCC administration causes increases in cAMP tissue levels after only 30-60 minutes. This stimulation of adenylate cyclase precedes most of the known actions of 1,25-DHCC on the gut, suggesting cAMP may possibly act as a secondary messenger for the action of vitamin D (Harrison and Harrison, 1970). An important action of 1,25-DHCC not fully explored as yet, is its effect on the brush border membrane lipid composition. Freeze fracture studies suggest that vitamin D and filipin (a calcium ionophore) increase calcium permeability at the brush border membrane by changes in membrane structure (Fontaine, Matsumota, Simoniescu, Goodman and Rasmussen, 1979).

Vitamin D has many effects on the intestinal epithelial cells, the relative importance of these effects and their inter-relationships are poorly understood, as is the process at the brush border membrane which presumably is the starting point for vitamin D induced calcium absoprtion. For a simplified model, showing the effect of 1,25-DHCC on a gut epithelial cell, see Figure 1.1.

1.1.3b The Action of Vitamin D on Bone

The primary defect leading to the discovery of vitamin D was the failure of bone mineralisation in vitamin D deficient animals. Surprisingly it has not yet been proved conclusively that vitamin D plays a direct role in bone mineralisation. This is because vitamin D can aid bone mineralisation indirectly by increasing serum calcium and phosphorus levels; hence direct effects on bone may be masked. Haavaldsen and Nicolaysen (1955) compared the effects of dietary vitamin D against both dietary and blood calcium and phosphorus levels. One of their conclusions was that vitamin D was required for normal bone accretion and possibly remodelling. **In vitro** studies using poorly mineralised metaphyseal slices from rachitic chicks, showed that there was significantly less mineralisation in these bones than from those of rachitic chicks fed calcium supplemented diets (Crenshaw, Ramp, Gonnerman and Toverud, 1974). They concluded that hypomineralisation of rachitic bone **in vivo** is due to reduced serum calcium levels. The same conclusion was reached by Yoshiki, Yanagisawa, Suda and Sasaki, (1974) studying the mineralisation of rachitic dentine.

While much early emphasis has been placed on looking at the role of vitamin D in bone mineralisation, it was also shown that vitamin D could have the opposite effect, that of bone resorption (Carlsson, 1952). It appears that vitamin D functions at the bone cell membranes, controlling the flux of calcium from the bone into the extracellular fluid (Talmage, 1969). Vitamin D and PTH both seem to be capable of stimulating bone resorption. The work of Garabedian, Tanaka, Holick and DeLuca (1974) even suggests that both hormones are required for bone resorption to occur. However, using bone organ cultures 1,25-DHCC can potently stimulate bone resorption alone. It was also shown that 25-hydroxycholecalciferol (25-HCC) could stimulate bone resorption but was 100 times less potent (Raisz, Trummel, Holick and DeLuca, 1972;

Reynolds, Holick and DeLuca, 1973). 1,25-DHCC induced resorption of bone can be inhibited by actinomycin D and other protein synthesis inhibitors (Tanaka and DeLuca, 1971), presumably because 1,25-DHCC normally directs **de novo** synthesis of protein or proteins responsible for the increased bone resorption. CaBP, a protein induced by 1,25-DHCC is found in bone tissue where 1,25-DHCC can cause a twenty fold stimulation of CaBP levels (Christakos, Friedlander, Frandsen and Norman, 1979). It may therefore have a role to play in 1,25-DHCC induced bone resorption.

1.1.3c The Actions of Vitamin D on the Kidney

The kidney plays an important part in calcium and phosphorus homeostasis, with both vitamin D and PTH playing key roles. The early work of Litvak, Moldawer, Forbes and Hennerman (1958) and Edwards and Hodgkinson (1965) suggested that vitamin D increased urinary excretion of calcium. They used pharmacological doses of vitamin D however, which would have increased serum calcium levels and hence increased the filtered load of calcium in the tubules, not surprisingly causing increased excretion of calcium (Steele, Engle, Tanaka, Lorenc, Dudgeon and DeLuca, 1975). Puschett, Beck, Jelonek and Fernadez (1974) found that 25-HCC, like PTH, caused increased tubular reabsorption of calcium in thyroparathyroidectomised dogs. Similar results were obtained from Costanzo, Sheehe and Weiner (1974) and Steele et al (1975), using thryoparathyroidectomised rats. It appears that vitamin D in low doses can stimulate tubular calcium reabsorption directly. The physiological importance of this is much less than that of vitamin D's actions on the gut and bone, as up to 99% of the calcium in the tubules is reasborbed even in the absence of PTH and 1,25-DHCC (Kleeman, Bernstein, Rockney, Dowling and Maxwell, 1961).

1.1.3d Role of Metabolites other than 1,25-DHCC in Calcium Homeostasis

The previous actions of vitamin D on the intestine, bone and kidney refer almost exclusively to the most active form of the vitamin, 1,25-DHCC. However, several other metabolites of vitamin D exist (see Figure 1.4). Of these 24,25-dihydroxy-cholecalciferol (24,25-DHCC) is produced in relatively large quantities with circulating plasma levels of around 2ng/ml. The precise biological function of 24,25-DHCC is uncertain, but as it is produced in greater quantities when calcium requirements are low it is considered by some to be an inactive metabolite. However, Canterbury, Lerman, Claflin, Henry, Norman and Reiss (1978) found that 24,25-DHCC caused a considerable decrease in PTH secretion in dogs, and it has also been shown to be necessary for correct bone mineralisation in rachitic chicks (Ornoy, Goodwin, Noff and Edelstein, 1978). However, convincing evidence has been provided that suggests 24,25-DHCC is not essential for any of vitamin D's major physiological actions (Halloran, DeLuca, Barthell, Yamada, Ohmori and Takayama, 1981). They gave weanling rats either 25-HCC, 24,24-difluoro-25-HCC or vehicle only as their only source of vitamin D for 16 days (d). 24,24-difluoro-25-HCC was made synthetically, the two fluorine atoms at the 24 position preventing the molecule from being hydroxylated at this site. Surprisingly, the fluorine atom is a similar size to the hydrogen atom and therefore steric effects are minimised; carbon-fluorine bonds are also very stable in bio-systems. This molecule therefore acts almost identically to 25-HCC except that it cannot undergo 24-hydroxylation. They found that 25-HCC and 24,24-difluoro-25-HCC were equally active in stimulating calcium transport, maintaining serum calcium and phosphate levels, and promoting bone growth and mineralisation, presumably through their 1-hydroxylated metabolites. Hens given only 1,25-DHCC as their source of vitamin D, while healthy themselves, often produced malformed chicks (Henry and Norman, 1978). Hatchability improved when

24,25-DHCC was given with 1,25-DHCC. The authors suggest this may be an important biological role for 24,25-DHCC. More recent work suggests that 25-HCC is an essential vitamin D metabolite with regards to egg hatchability, as this metabolite is much more easily transferred to the egg than either of the dihyroxy metabolites (Ameenuddin, Sunde, DeLuca, Ikekawa and Kobayashi, 1982).

Another metabolite of vitamin D of uncertain physiological importance is 1,24,25-trihydroxycholecalciferol (1,24,25-THCC). This metabolite does have very definite actions on the gut and on bone, and it has been suggested that any activity seen with 24,25-DHCC in **in vivo** experiments is due to its conversion to 1,24,25-THCC (Boyle, Omdahl, Gray and DeLuca, 1973). 1,24,25-THCC is an interesting metabolite as theoretically it could be produced by either the 1-hydroxylation of 24,25-DHCC or the 24-hydroxylation of 1,25-DHCC. Kumar, Schnoes and DeLuca (1978) believe that **in vivo** it is produced almost exclusively by the latter process, so implying that it is an inactivation product of the active 1,25-DHCC. It should be stressed however that 1,24,25-THCC is probably the second most potent natural metabolite of vitamin D, having half the activity of 1,25-DHCC in stimulating intestinal calcium transport and one tenth of its activity with respect to bone resorption.

Recently several other **in vivo** metabolites of vitamin D have been isolated and identified, for example 25,26-dihydroxycholecalciferol, 23,25,-dihydroxycholecalciferol, 24,oxo-25-hydroxycholecalciferol, and 25-hydroxycholecalcifcerol-26,23-lactone. In initial studies these metabolites have minimal physiological activity **in vivo** and what little they do have is probably due to their further metabolism to 1-hydroxylated metabolites (for example 25,26-DHCC to 1,25,26-THCC).

The results of this paper have recently been withdrawn.

1.1.4 Calcitonin and Calcium Homeostasis

The third hormone of calcium homeostasis is calcitonin. It has basically hypocalcaemic actions in contrast to the hypercalcaemic properties of PTH and 1,25-DHCC. Calcitonin is a 32 amino acid peptide that is secreted by the thyroid 'C' cells of mammals and by the ultimobranchial bodies of birds, reptiles and fish. It is interesting that a new peptide with calcitonin like actions has been discovered, which appears to be a 21 amino acid cleavage product of the calcitonin precursor peptide and is almost as potent as calcitonin itself (MacIntyre, Hillyard, Murphy, Reynolds, Gaines Das and Craig, 1982). Calcitonin's main target tissue is bone, where it can suppress bone resorption. (Friedman and Raisz, 1965; Milhaud and Moukhtar, 1966). **In vitro**, calcitonin stimulates bone adenylate cyclase and produces a measurable increase in bone cAMP (Heersche, Marcus and Aurbach, 1974). Calcitonin exerts its action by decreasing the number and activity of osteoclasts (Foster, Doyle, Bordier and Matrajt, 1966), and this is supported by the fact that calcitonin is most effective when the osteoclast population is high, as in young animals and adults with Paget's disease.

In egg laying birds calcitonin reduces ionised plasma calcium only when the bird does not have a calcifing egg in the oviduct (Luck and Scanes, 1982). This suggests that calcitonin is unable to overcome the enhanced bone resorption occurring during egg shell calcification which is presumably directed by PTH. This idea is supported by the work of Lloyd, Peterson and Collins (1970) and Sommerville (1978), who could only get a hypocalcaemic response with calcitonin in parathyroidectomised birds. **In vivo** calcium challenge can cause a seven fold increase in plasma calcitonin levels in young male Japanese quail (Boelkins and Kenny, 1973). In mammals calcitonin secretion responds not only to hypercalcaemia but also to gastro-intestinal hormones such as pancreozymin, cerulein and pentagastrin (Care, Bruce, Boelkins, Kenny, Conaway and

Anast, 1971). This is particularly relevant to postprandial hypercalcaemia, as calcitonin will suppress bone resorption so permitting maximum utilisation and deposition of dietary calcium into bone (Deftos, 1978). This mechanism does not appear to operate in birds (Luck, personal communication).

Olson, DeLuca and Potts (1972) reported that calcitonin inhibited vitamin D induced intestinal calcium absorption, but the work of Cramer (1973) did not support this finding. There is also some evidence to suggest that calcitonin acts directly on the kidney to increase the excretion of calcium and phosphate (Cochran, Peacock, Sachs and Nordin, 1970). However, this action is probably pharmacological rather than physiological. Calcitonin is the least well studied of the three main calcium homeostatic hormones, its main functions are probably to prevent dangerous hypercalcaemia and in times of calcium stress to quite literally stop animal's skeletons dissolving away.

1.1.5 The Avian Egg and Calcium Metabolism

There are three major components of an egg: the yolk, albumen and shell. Egg yolk is nearly 50% water and the dry matter consists almost entirely of proteins and lipoproteins. The liver is the site of synthesis of these proteins and their production is induced by oestrogens. Vitellogenin is the major precursor of the yolk proteins present in the plasma and it has been extensively studied. Vitellogenin is a large polypeptide with a molecular weight of 240,000 daltons: it is not only synthesised in the liver but also phosphorylated, glycosylated and associated with lipid there as well. It is carried in the blood as a dimer (molecular weight 500,000 daltons) to the ovary, where it is taken up by developing follicles. It is then cleaved specifically to form the egg yolk phosphoproteins, lipovitellin and phosvitins (Bergink and Wallace, 1974). The function of vitellogenin has never been

clearly elucidated, but it is assumed that it serves as a phosphate storage protein for the embryo and also as a metal ion transport protein. The protein is certainly heavily phosphorylated as each gram (approximately the amount in a chicken's egg) contains 150mg of high energy phosphate.

A considerable amount of calcium is bound to these egg yolk proteins, about 25mg per chicken egg. This is because the precursor protein vitellogenin has a very high calcium binding affinity; indeed this explains the increase in the bound plasma calcium fraction seen in laying birds.

Egg white or albumen, accounts for nearly two thirds of the weight of an egg. It is formed in the magnum of the oviduct and then secreted around the egg yolk. Albumen itself is nearly 90% water, the remainder being principally protein. There are many proteins in albumen, the most important being ovalbumin which accounts for about 60% of the total proteins; considerable amounts of ovotransferrin and ovomucoid are also present. Albumen also contains some calcium, albeit at only one tenth the concentration of that in the yolk.

The shell of an egg is often thought by many to have little importance, other than protecting the contents; however during incubation the shell is actively metabolised, as on hatching the chick contains five times more calcium than the egg contents, therefore 80% of its calcium has come from the egg shell (Simkiss, 1961). The shell of a chicken's egg is almost pure calcium carbonate; it weighs about 5g, 2g of which is calcium. Most of the calcium is laid down in just 16h in the shell gland, so that a deposition rate of approximately 125mg of calcium per hour is required. Hens only have about 25mg of calcium in their blood, so where does the calcium come from? The immediate source has to be the blood, but the ultimate source is the food. It has been shown that the intestine is usually unable to absorb the full requirement of calcium for egg shell production, so the deficit is made good

by the liberation of calcium from the skeleton (Taylor, 1970). The mobilisation of calcium from the skeleton increases as the dietary supply decreases and in extreme cases where a calcium free diet is fed, all the shell calcium comes from the bones. This cannot be repeated too often as each egg that is laid requires 10% of the bird's total body calcium.

This impressive ability to mobilise calcium from the skeleton is associated with a system of secondary bone in the marrow cavities of the ordinary bones, known as medullary bone. Medullary bone is produced under the combined influence of both oestrogen and androgen, and is only found in reproductively active female birds. Medullary bone is not naturally found in male birds but it can be induced by oestrogen administration. Much of the work on medullary bone metabolism has been performed on pigeons, and in these birds within a few hours of an egg entering the shell gland, changes in the medullary bone can be observed. Primarily there is a change in the bone cell population, from one dominated by osteoblasts, to one in which the bone-destroying osteoclasts predominate, resulting in resorption of medullary bone. This switch in bone cell types is probably mediated by PTH (Taylor, 1970). The breakdown of medullary bone persists for a few hours after the egg is laid, when a phase of bone formation begins in preparation for the next egg. It has been suggested that the actual process of medullary bone remineralisation is directly dependant on vitamin D (Takahashi, Shinki, Abe, Horiuchi, Yamaguchi, Yoshiki and Suda, 1983).

1.2 Vitamin D and its Further Metabolism

1.2.1 Vitamin D

Vitamin D is not a single compound but a small family of compounds exhibiting vitamin D like activity. Vitamins D_2, D_3, D_4, D_5 and D_6 have been identified (DeLuca, 1979), the most

important of these are Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_3 is produced in the skin of mammals non-enzymically by ultraviolet irradiation of 7-dehydrocholesterol (see Figure 1.2). Vitamin D_2 is formed in a similar fashion from ergosterol, which occurs only in plant tissues. Vitamin D_2 and D_3 are approximately equipotent in most mammals, with the exception of new-world monkey. In birds, vitamin D_2 is only one tenth as active as vitamin D_3 (Chen and Bosmann, 1964). In the following sections reference to vitamin D is to cholecalciferol, not ergocalciferol, unless otherwise stated.

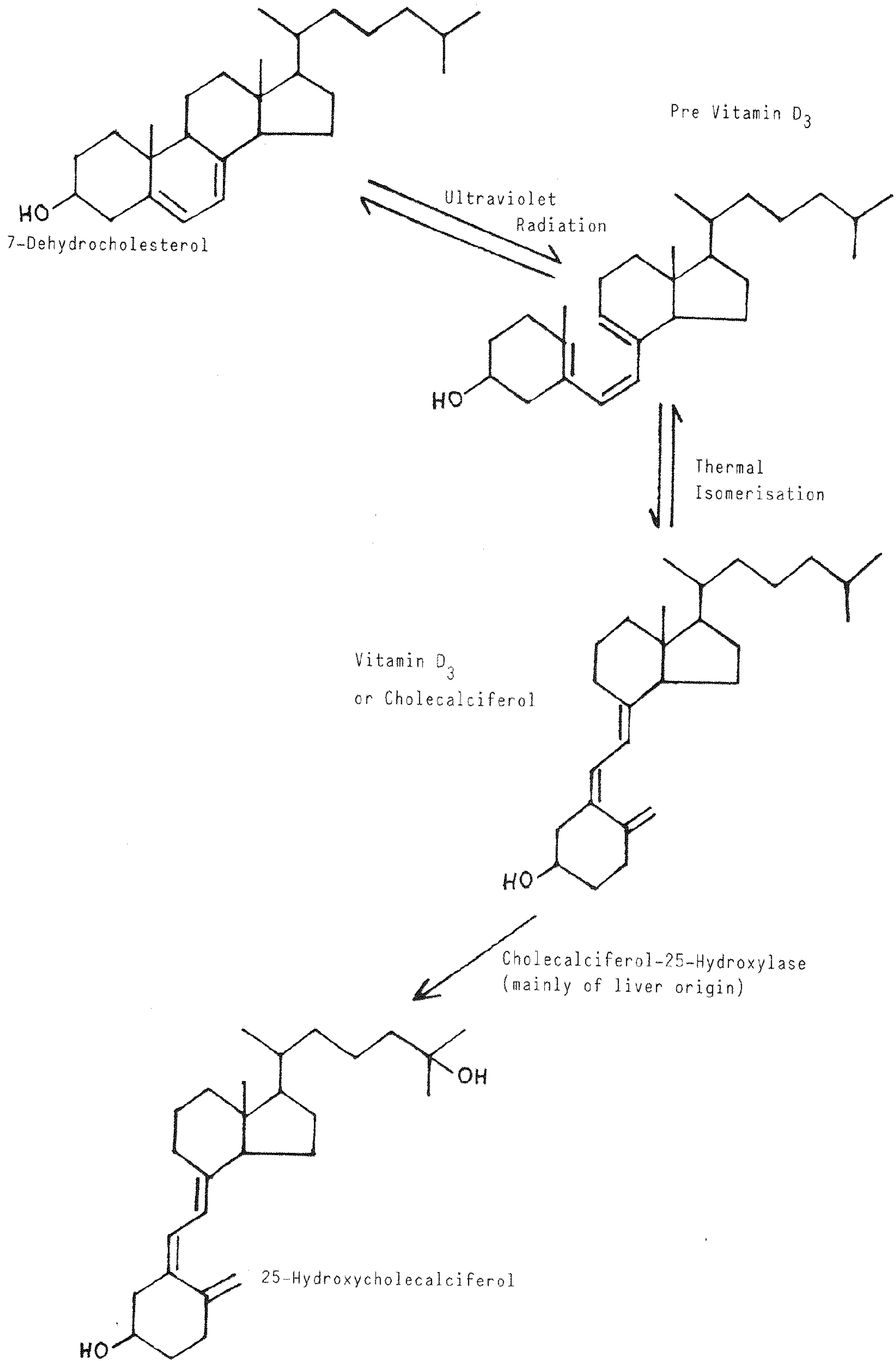
Between 1955-60 several laboratories became aware of the possibility that vitamin D might have to be further metabolised before it was physiologically active, in order to explain the considerable lapse of time between vitamin D administration and the observed response. This obviously led to the questions of what was the active metabolite of vitamin D and where was it synthesised. It was not until 1968 that a polar metabolite of vitamin D was isolated and characterised from porcine plasma (Blunt, DeLuca and Schnoes, 1968). The metabolite was 25-HCC and its structure is shown in Figure 1.2. Blunt et al (1968) also showed that this new metabolite was approximately twice as active physiologically as the parent vitamin.

1.2.2 25-Hydroxycholecalciferol and its Metabolism

25-HCC is synthesised in relatively large quantities by the liver enzyme cholecalciferol-25-hydroxylase (25-hydroxylase) (Ponchon, Keenan and DeLuca, 1969). This enzyme has also been detected in the small intestine and kidneys of avian species (Tucker, Gagnon and Haussler, 1973). The 25-hydroxylase requires NADPH and molecular oxygen for its activity and is therefore a mixed function monooxygenase (DeLuca, 1979). The spectral work of Cinti, Golub and Bronner (1976) strongly

Figure 1.2

Formation and Initial Metabolism of Vitamin D



suggests that a cytochrome P_{450} moiety is present in the 25-hydroxylase. The intracellular location of the 25-hydroxylase was in doubt for some time: Horsting (1970) had shown enzyme activity in the mitochondrial fraction while other workers showed that 25-hydroxylase activity was exclusively microsomal (Bhattacharyya and DeLuca, 1974). Recent work clarifies this anomaly as both microsomal and mitochondrical 25-hydroxylase activity has been detected (Bjorkhern and Holmberg, 1978).

The regulation of the 25-hydroxylase has been studied but not as fully as it perhaps deserves. Bhattacharyya and DeLuca (1973) observed an inverse relationship between the amount of vitamin D administered and 25-hydroxylase activity. This relationship was observed at physiological doses of vitamin D. When large pharmacological doses of vitamin D were tested, however, considerable increases in 25-HCC levels were observed (Haddad and Stamp, 1974). These previous observations can possibly be explained by the existence of both the mitochondrial and microsomal 25-hydroxylases. It has been suggested that the microsomal form may have a higher affinity for vitamin D and therefore will be most active at physiological doses, and that this form could be regulated by product inhibition. The other 25-hydroxylase being of lower affinity may have no such regulation, causing increased 25-HCC levels when large potentially toxic doses of vitamin D are given. It has been observed that oestradiol can stimulate the production of 25-HCC from vitamin D in Japanese quail (Nicholson, Akhtar, and Taylor, 1976) and it was suggested that when calcium demand was high, for example in the egg laying bird, that the 25-hydroxylase is 'switched on' directly by oestradiol.

25-HCC is the major circulating form of vitamin D, with plasma levels around 25-35ng/ml, however even at these concentrations 25-HCC has little if any physiological activity. Generally it is thought that the 25-hydroxylase is not stringently regulated, and that its main function is to

provide adequate quantities of 25-HCC which can then be further metabolised to more active metabolites.

25-HCC is the natural precursor from which several relatively new metabolites of vitamin D are formed. The most important of these metabolites is 1,25-DHCC produced by the 25-hydroxycholecalciferol-1α-hydroxylase (1-hydroxylase), as this metabolite is the major active hormonal form of the vitamin (see Chapter 1.1.3a, b and c). Another metabolite 24,25-DHCC is produced by the 25-hydroxycholecalciferol-24R-hydroxylase (24-hydroxylase) and the biological role of this metabolite has already been discussed (Chapter 1.1.3d). These two hydroxylases are at the heart of vitamin D metabolism, and therefore their properties, location and most importantly their regulation will be more fully discussed.

1.2.3 The 1-Hydroxylase Location and Properties

Fraser and Kodicek (1970) were the first to report the unique biosynthesis of "1α oxygenated-25-Hydroxycholecalciferol" by the kidney, and although they knew this metabolite to be biologically active (Kodicek, Lawson and Wilson, 1970), they were not certain of its structure. In very quick succession three separate groups confirmed that this new metabolite, produced exclusively by the kidney, and with biological activity in the intestine, had the structure of 1,25-dihydroxycholecalciferol (see Figure 1.4) (Lawson, Fraser, Kodicek, Morris and Williams, 1971; Holick, Schnoes and DeLuca, 1971; Norman, Myrtle, Midgett, Norwicki, Williams and Popják, 1971). Boyle, Miravet, Gray, Holick and DeLuca, (1972) demonstrated that no stimulation of intestinal calcium transport occurred when 25-HCC was administered to nephrectomised vitamin D deficient rats; however these animals did respond to 1,25-DHCC. Their work highlighted the importance of the kidney, and showed that 1,25-DHCC or a further metabolite of 1,25-DHCC is the active form of the vitamin in the gut.

For several years it was believed that the kidney was the only tissue to possess 1-hydroxylase activity, but this does not now appear to be the case. Two groups were able to show that nephrectomised pregnant rats could still produce 1,25-DHCC (Weisman, Vargus, Duckett, Reiter and Root, 1978; Gray and Lester, 1979). This extrarenal source of 1,25-DHCC was found to originate from the placental unit. Weisman et al (1978) also showed that the 1,25-DHCC produced by the placenta could be found in the maternal serum, and therefore this 1,25-DHCC produced by the placenta in their **in vitro** studies could be of considerable physiological significance in the pregnant mammal. Other workers have also found limited 1-hydroxylase activity in cultured bone cells of both avian and mammalian origin (Turner, Puzas, Forte, Lester, Gray, Howard and Baylink, 1980; Howard, Turner, Sherrard, and Baylink, 1981). Puzas, Turner, Forte, Kenny and Baylink (1980) have also detected considerable 1-hydroxylase activity in the chick chorioallantoic membrane, which in embryonic terms is analogous to the mammalian placenta.

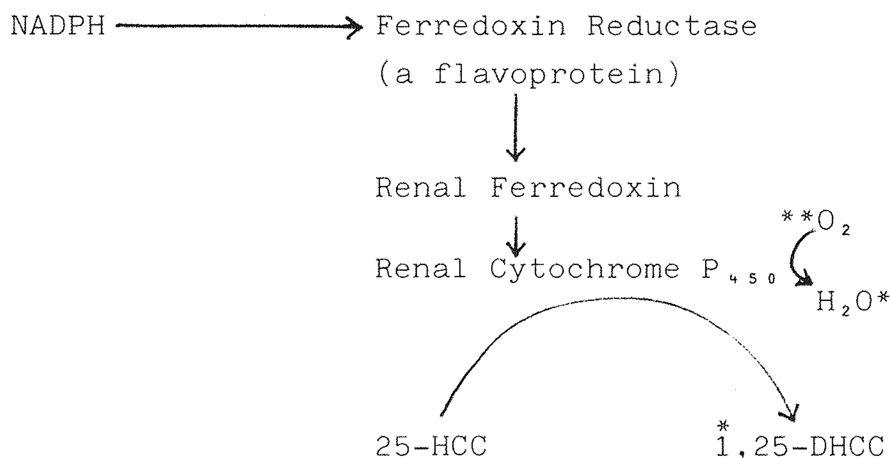
The importance of these extrarenal sites of 1-hydroxylase activity (placental 1-hydroxylase excepted) has been questioned. Reeve, Tanaka and DeLuca (1983) have shown convincingly that anephric vitamin D deficient male rats cannot produce detectable amounts of 1,25-DHCC, and therefore **in vivo** the kidney remains the major, if not the only site of 1,25-DHCC production in non pregnant animals. The location of the 1-hydroxylase in the kidney and also its intracellular location are of interest. The initial report of Fraser and Kodicek (1970) suggested that 1-hydroxylase activity may be associated with the mitochondrial fraction of the cell. Gray, Omdahl, Ghazarian and DeLuca (1972) have shown that the 1-hydroxylase is purely of mitochondrial origin. Midgett, Spielvogel, Coburn and Norman (1973) were able to show that in man, rat and dog, 1,25-DHCC was only produced by the renal cortex region. More recently Stumf, Sar, Narbaitz, Reid,

DeLuca and Tanaka (1980) have shown that the 1-hydroxylase is located primarily in the renal proximal tubule cells.

With regard to the properties of the 1-hydroxylase, Ghazarian, Schnoes and DeLuca (1973) were able to show that the oxygen inserted into the 1α position by the 1-hydroxylase was of molecular origin, so categorising the 1-hydroxylase as a mixed function oxidase. The 1-hydroxylase requires magnesium ions and NADPH in addition to molecular oxygen. In 1974 Henry and Norman were able to show that the 1-hydroxylase could be inhibited by carbon monoxide, and importantly they also showed that light of 450nm wavelength was capable of reversing the carbon monoxide inhibition. This provided strong evidence for the involvement of cytochrome P_{450} in the correct functioning of the 1-hydroxylase. Peterson, Ghazarian, Orme-Johnson and DeLuca (1976) revealed the presence of an iron-sulphur ferredoxin-like protein loosely associated with the cytochrome P_{450} unit purified from chick kidney mitochondria. It has since been shown that by combining this pure ferredoxin with a ferredoxin reductase and cytochrome P_{450} from rachitic chick kidney mitochondria, that 1,25-DHCC can be produced solely and very rapidly from 25-HCC (Yoon and DeLuca, 1980). A mechanism for the hydroxylation of 25-HCC to 1,25-DHCC has been proposed, which looks very similar to the steroidogenesis system of the adrenals and is shown in the following diagram.

Figure 1.3

Molecular Mechanism for the Hydroxylation of 25-HCC to 1,25-DHCC



1.2.4 Regulation of the Renal 1-Hydroxylase

The production of 1,25-DHCC is controlled by the strict regulation of the renal 1-hydroxylase. The regulation of the activity of this enzyme is poorly understood, and although several physiological factors have been implicated, few have been shown to have direct actions as determined by **in vitro** studies. These factors and their actions, where known, will be discussed in detail below:

a. Parathyroid Hormone

Parathyroid hormone (PTH) is possibly the most important regulator of the 1-hydroxylase. Garabedian, Holick, Boyle and DeLuca (1972) showed that parathyroidectomy decreased 1,25-DHCC production in the rat after 40h, and that this decrease could be prevented by administering PTH. Baksi and Kenny (1979), and Sedrani, Taylor and Ahktar (1981) have shown that **in vivo** administration of PTH stimulated **in vitro** production of 1,25-DHCC. As a polypeptide hormone, PTH would be expected to act by binding to membrane receptors of the

target tissue, thus stimulating the membrane bound enzyme, adenylate cyclase, which in turn would increase intracellular cAMP levels. cAMP, acting as a secondary messenger, activates specific protein kinases which may then phosphorylate enzymes resulting in altered enzyme activity. One major problem when applying this system to the 1-hydroxylase, is that this enzyme is located in the mitochondrial membrane and most enzymes regulated by dephosphorylation/phosphorylation are of cytoplasmic origin. However, recently just one case has been reported of a microsomal cytochrome P_{450} enzyme - the 7α-cholesterol hydroxylase - being regulated by such a mechanism (Goodwin, Cooper and Margolis, 1982). In the rat it has been shown that cAMP is involved in PTH stimulation of the 1-hydroxylase **in vivo** (Horiuchi, Suda, Takahashi, Shimazawa and Ogata, 1977); however there is some evidence that PTH may stimulate the 1-hydroxylase independantly of cAMP (Rost, Bikle and Kaplan, 1979).

Rasmussen, Wong, Bikle and Goodman (1972), were the first group to report that PTH stimulated the 1-hydroxylase when added to isolated chick renal tubules (an **in vitro** system). However, several workers, some even from the same laboratory were unable to repeat their experiment successfully (Shain, 1972a, b; Henry, 1979). In 1979 Trechsel, Bonjour and Fleisch were able to detect a small but significant increase in 1-hydroxylase activity after PTH administration to their culture system, but this only occurred when EGTA was added to the cultures to complex much of the calcium. Later still Henry (1981) obtained **in vitro** stimulation of the 1-hydroxylase with PTH but only if insulin was added to the cultures. Insulin alone had no effect. It has now been shown that PTH can stimulate the 1-hydroxylase directly, using **in vitro** chick kidney slices and a relatively short incubation time (Kaplan, Bikle and Rost, 1981). This group also found that theophylline (a phosphodiesterase inhibitor) potentiated the PTH stimulation adding weight to the involvement of cAMP.

In other systems where a hormone regulates another hormone's production, there is usually some form of feedback regulation. This has now been shown to be the case with PTH and 1,25-DHCC. Dietal, Dorn, Montz and Altenahr (1979) used parathyroid gland cultures and were able to show that 1,25-DHCC at low doses (30pg/ml) could cause a 40% inhibition in PTH secretion, suggesting a negative feedback role for 1,25-DHCC. It is interesting to note that, although PTH has a major tropic action on the production of 1,25-DHCC, its effect is not an absolute one, since even in the absence of any parathyroid tissue basal levels of 1,25-DHCC are still produced (Favus, Walling and Kimberg, 1974).

b. Calcium

1,25-DHCC can be considered as the calcium homeostatic steroid hormone. It is produced in relatively large quantities when calcium requirements are high and, conversely, when calcium needs are low 1,25-DHCC levels drop dramatically. It therefore does not seem unreasonable to expect that calcium should be strongly implicated in the regulation of the 1-hydroxylase. Using **in vitro** techniques such as renal cell cultures and isolated renal mitochrondria, most workers have found that calcium strongly inhibits the 1-hydroxylase even at concentrations as low as 50μM (Henry and Norman, 1974; Horiuchi, Suda, Sasaki, Ezawa, Sano and Ogata, 1974). However, lower concentrations still, appear to stimulate the 1-hydroxylase. (Horiuchi et al, 1974; Henry and Norman, 1976). Henry and Norman (1976) were able to show a partial reversal of the calcium inhibition of the 1-hydroxylase using the calcium ionophone A-23187, which effectively decreases mitochondrial calcium, implying that it is the intramitochondrial calcium level that regulates the 1-hydroxylase.

In considering the effects of calcium on the 1-hydroxylase **in vivo**, the actions of PTH must be taken into account, as PTH

secretion is very sensitive to varying calcium levels. Thus, hypocalcaemia will stimulate PTH release which will then stimulate the 1-hydroxylase and therefore calcium will be affecting the 1-hydroxylase indirectly. It has been shown, however, that thyroparathyroidectomised rats fed low calcium diets favoured the production of 1,25-DHCC from 25-HCC, suggesting that calcium has an effect on the 1-hydroxylase independant of PTH (Larkins, Colston, Galante, MacAuley, Evans and MacIntyre, 1973). Conflicting results though have been reported by Hughes, Brumbaugh, Haussler, Wergedal and Baylink (1975) who could find no evidence that low dietary calcium increases plasma 1,25-DHCC levels.

c. **Prolactin**

It has been shown that repeated injections of ovine prolactin for 7d into hens could produce a two-fold increase in the 1-hydroxylase activity when measured one hour after the last injection (Spanos, Colston, Evans, Galante, MacAuley and MacIntyre, 1976a): this finding was confirmed by Baksi, Kenny, Galli-Gallardo and Pang (1978). It was further shown that injections of ovine prolactin caused a two-fold increase in plasma 1,25-DHCC levels in hens (Spanos, Pike, Haussler, Colston, Evans, Goldner, McCain and MacIntyre, 1976). More recently Bikle, Spencer, Burke and Rost (1980) using vitamin D deficient chick renal tubules, or renal slices were able to show a two-fold stimulation of the 1-hydroxylase in these tissues, when incubated with turkey prolactin. Ovine prolactin was also tested but found to be inactive. Their results imply that prolactin stimulates the 1-hydroxylase directly as they had used **in vitro** techniques. This work has since been confirmed by similar **in vitro** experiments (Spanos, Brown, Stevenson and MacIntyre, 1981).

The physiological importance of the ability of prolactin to stimulate the 1-hydroxylase is not totally clear, however it may help to provide for the high calcium requirement of

lactation in mammals and egg production in birds. This idea is perhaps supported by the work of Goldsmith and Hall (1980) that showed an increase in pituitary prolactin content in maturing Japanese quail; they also found higher pituitary prolactin levels in females than males and in laying hens as opposed to non-layers. Further work implicating a relationship between vitamin D and prolactin comes from Murdock and Rosenfeld (1981) who found that 1,25-DHCC could feedback on pituitary GH_4 cell cultures to decrease basal prolactin production by 30%. In summary it appears that prolactin can cause a mild but direct stimulation of the 1-hydroxylase, which is of importance in the calcium stress associated with reproductive activity.

d. Oestrogens

There is considerable clinical evidence that sex hormones and in particular oestrogens play an important role in calcium metabolism (Rasmussen and Bordier, 1974), an important example of which is post menopausal osteoporosis, thought to be associated with decreasing oestrogen levels (Nordin, MacGregor and Smith, 1966). It has also been suggested that the change in vitamin D metabolism in maturing female Japanese quail is mediated by oestrogens (Turner, Rader, Eliel and Howard, 1979).

Nicholson, Akhtar and Taylor (1976) observed that exogenous oestradiol stimulated the production of 25-HCC from vitamin D in liver homogenates from immature Japanese quail of both sexes. It has also been shown that oestradiol, usually administered by injection of oestradiol benzoate, can stimulate **in vivo** 1-hydroxylase activity as determined by **in vitro** assay in both Japanese quail and chickens (Tanaka, Castillo and DeLuca, 1976; Baksi and Kenny, 1978a; Sedrani, Taylor and Akhtar, 1981) and in rats (Baksi and Kenny, 1978b). In addition oestradiol increases plasma 1,25-DHCC levels in the

bullfrog (Baksi and Kenny, 1978c).

The precise role of oestradiol in regulating the production of 1,25-DHCC has not yet been fully resolved. Tanaka et al (1976) found no stimulation of 1-hydroxylase activity in castrated male chickens injected with oestradiol benzoate alone, although a large stimulation was observed when testosterone was given together with the oestrogen. Tanaka, Castillo, Wineland and DeLuca (1978) later showed that progesterone and testosterone acted synergistically with oestradiol to stimulate the 1-hydroxylase, while neither progesterone nor testosterone alone or progesterone plus testosterone in the absence of oestrogen had any stimulatory effect. Baksi and Kenny (1978a) and Sedrani et al (1981) have failed to show a requirement for androgen in the oestrogenic stimulation of the 1-hydroxylase, in fact most of the latter authors' work showed that testosterone inhibited the oestrogenic stimulation. These contradictions are difficult to resolve but may hinge on the use of castrated male chickens and high testosterone doses (5-20mg/kg) by Tanaka et al, against immature Japanese quail and the lower dose (0.5-5.0mg/kg) of testosterone used by the other groups.

Baksi and Kenny (1977a) have shown that tamoxifen, an anti-oestrogen (competitive inhibitor), can inhibit the oestrogenic stimulation of the 1-hydroxylase in Japanese quail. They were surprised however when tamoxifen plus oestrogen caused an even greater inhibition of the 1-hydroxylase than tamoxifen alone. They suggested that this showed a latent inhibitory component of oestrogen with respect to the 1-hydroxylase that had been unmasked by the anti-oestrogen. In other fields tamoxifen has given unexpected results and it has been suggested that tamoxifen has its own specific receptor quite separate from the true oestrogen receptor (Sutherland, Murphy, Foo, Green, Whybourne and Krozowski, 1980) and that it has both oestrogenic and anti-oestrogenic properties.

With the use of renal cell cultures and other **in vitro** systems it is possible to determine whether or not any particular factor stimulates the 1-hydroxylase directly. To date no direct stimulation has been observed with oestrogen using these systems Spanos, Barrett, Chong and MacIntyre (1978) and Trechsel, Bonjour and Fleisch (1979), thus presenting strong but not conclusive evidence that oestrogens do not stimulate the 1-hydroxylase directly **in vivo**. Slight doubt remains since **in vitro** conditions are often extremely exacting in order to observe known **in vivo** actions. This point is well illustrated by the only recently observed **in vitro** stimulation of the 1-hydroxylase by parathyroid hormone: long known to stimulate the 1-hydroxylase **in vivo**. Of great interest also is the work of Murono, Kirdani and Sandberg (1979) showing the existence of high affinity low capacity 17β-oestradiol receptors in the rat kidney. In addition ^{3}[H] oestradiol binding has been observed in rat proximal tubule cells, where the 1-hydroxylase is located (Stumpf, Sar, Narbaitz, Reid, DeLuca and Tanaka, 1980). One very intriguing problem arises which is one of my research interests, and that is just how does oestrogen stimulate the 1-hydroxylase? More specifically if a direct stimulation occurs does it involve **de novo** synthesis of enzyme or some form of allosteric regulation? Alternatively if the oestrogenic stimulation is indirect through what factor or factors is this stimulation mediated?

e. **Phosphate**

The effect of phosphate on the 1-hydroxylase has been less thoroughly investigated than that of calcium. Tanaka and DeLuca (1973) using phosphate-depleted rats, were able to show higher plasma 1,25-DHCC levels in these animals than in normal rats. Most provocatively the serum 1,25-DHCC levels varied inversely with serum phosphate levels, whereas serum 24,25-DHCC levels varied directly. They suggested that low levels of phosphate in renal tubular cells stimulated the

1-hydroxylase and further showed that these actions occurred even in parathyroidectomised animals. Repeated attempts to show a stimulation **in vivo** or **in vitro** of the 1-hydroxylase by low phosphate levels have proved unsuccessful (Henry and Norman, 1974; Friedlander, Henry and Norman, 1977; Montecuccoli, Bar, Risenfeld and Hurwitz, 1977). Baxter and DeLuca (1976) showed that with long term phosphate deprivation it was possible to stimulate the 1-hydroxylase. However, this stimulation was only one tenth of the magnitude of the stimulation seen when calcium was restricted. An explanation for the high serum 1,25-DHCC levels observed by Tanaka and DeLuca (1973) have now apparently been given by Ribovich and DeLuca (1978). They found that phosphate deprivation resulted in a decreased turnover of 1,25-DHCC and an increased accumulation of 1,25-DHCC in both the plasma and intestines of these animals.

f. Vitamin D and its Metabolites

There is much evidence and considerable agreement among many laboratories that dietary vitamin D and 1,25-DHCC can decrease the specific activity of the 1-hydroxylase. Garabedian, Holick, DeLuca and Boyle (1972) were the first to note that dietary vitamin D could affect the production of 1,25-DHCC, although interpretation of their results was difficult as the rats had been thyroparathyroidectomised. The administration of 1,25-DHCC **in vivo** was shown to decrease the 1-hydroxylase activity in chickens (Horiuchi, Suda, Sasaki, Ezawa, Sano and Ogata, 1974; Tanaka, Lorenc and DeLuca, 1975); however it was not known if 1,25-DHCC acted directly on the kidney or through some other factor, possibly PTH. Horiuchi, Suda, Sasaki, Takahashi, Shimazawa and Ogata (1977) reported that a constant infusion of PTH into thyroparathyroidectomised rats overcame the suppressive effect of 1,25-DHCC on the 1-hydroxylase; and therefore concluded that the major effect of 1,25-DHCC **in vivo** on the 1-hydroxylase was mediated via PTH. In contrast, Omdahl (1977) reported that 1,25-DHCC decreased its own production and increased the production of

24,25-DHCC in thyroparathyroidectomised as well as intact rats, suggesting that PTH is not responsible for the action of 1,25-DHCC.

While these **in vivo** results were hard to resolve, Larkins, MacAuley and MacIntyre (1974) found a decrease in 1,25-DHCC production in renal tubule suspensions following preincubation with 1,25-DHCC itself. Henry (1977 and 1979) has shown, as have others, that 1,25-DHCC greatly decreases its own production in the **in vitro** chick renal cell culture system. All the **in vitro** data shows unequivocally that 1,25-DHCC does directly inhibit its own production in the renal cells. Colston, Evans, Spelsberg and MacIntyre (1977) have shown that actinomycin D, an inhibitor of mRNA transcription, and α-amanitin, a specific inhibitor of RNA polymerase II, can both prevent the induction of the 24-hydroxylase and the suppression of 1-hydroxylase activity associated with 1,25-DHCC administration. They therefore suggested that these effects of 1,25-DHCC are dependant on changes in gene transcription and protein synthesis. While it seems perfectly possible that 1,25-DHCC may stimulate **de novo** synthesis of the 24-hydroxylase, explaining how 1,25-DHCC through **de novo** protein synthesis decreases 1-hydroxylase specific activity is more difficult. Larkins, MacAuley and MacIntyre (1974) suggested that as in the intestine 1,25-DHCC increases the synthesis of proteins concerned with calcium transport in the kidney. These **de novo** synthesised proteins could then increase intracellular calcium thereby inhibiting the 1-hydroxylase. This hypothesis fits in well with 1,25-DHCC's known action in the kidney, to increase tubular reabsorption of calcium, and also the demonstration of a vitamin D dependant calcium binding protein in the kidney (Taylor and Wasserman, 1972). However further support for this hypothesis has not been forthcoming.

g. Calcitonin

Insufficient attention has been paid to the possible role

of calcitonin in regulating 25-HCC metabolism. Rasmussen, Wong, Bikle and Goodman (1972) reported that the addition of porcine calcitonin **in vitro** to isolated renal tubules from vitamin D deficient chicks, inhibited the conversion of 25-HCC to 1,25-DHCC. Galante, Colston, MacAuley and MacIntyre (1972) found that giving repeated large doses of salmon calcitonin into vitamin D deficient rats, while not affecting the plasma calcium levels, did cause an increase in the accumulation of 1,25-DHCC in the plasma and intestines. Lorenc, Tanaka, DeLuca and Jones (1977) in a more complete study found that while calcitonin increased 1,25-DHCC accumulation in plasma of vitamin D deficient intact rats, it had no effect in thyroparathyroidectomised vitamin D deficient rats. They concluded that calcitonin **in vivo** had no direct action on the 1-hydroxylase and that any response seen was probably secondary to the stimulation of the parathyroid glands.

Horiuchi, Takahashi, Matsumoto, Takahashi, Shimazawa, Suda and Ogata (1979) performed a complex balanced study that involved continuous infusions of calcium and calcitonin into thyroparathyroidectomised vitamin D deficient rats, and found a dose dependant increase in plasma 1,25-DHCC over time periods of 6-30h. Furthermore, they found that either PTH or cAMP infusions produced a similar stimulation, and that when PTH or cAMP was infused with calcitonin the two stimulations were additive. In addition concurrent infusions of calcitonin and theophylline did not potentiate the effect of calcitonin. These results strongly suggest that the rat kidney has a calcitonin sensitive 1-hydroxylase that is quite separate from the PTH/cAMP sensitive one. Kawashima, Torikai and Kurokawa (1981a) using defined nephron segments from vitamin D deficient rats found that the proximal convoluted tubule contained PTH sensitive 1-hydroxylase activity, and that 1-hydroxylase activity of a similar magnitude was found in the proximal straight tubules (PST), which could only be stimulated by calcitonin. They believe that calcitonin can recruit addition-

al tubule cells (those of the PST) to produce 1,25-DHCC when the calcium requirements are high, for example in the foetus where the calcitonin levels are known to be high (Garel, Martin-Rosset and Barlet, 1975)

h. Growth Hormone

Hanna, Harrison, MacIntyre and Fraser (1961) studying human patients observed an increase in both calcium and magnesium absorption from the gut and increased plasma calcium levels when the patients were treated with human growth hormone. It has since been shown that hypophysectomised rats have lower 1,25-DHCC tissue and plasma levels than controls, and that bovine growth hormone when administered at pharmacological doses can return 1,25-DHCC levels to those of the controls (Pavlovitch, Fontaine and Balsan, 1978; Yeh and Aloia, 1979).

The work of Lancer, Bowser, Hargis and Williams (1976) shows very clearly that when bovine growth hormone is administered to rats there is a two-fold increase in serum immunoreactive PTH. They suggest therefore that the hypercalcaemic action of growth hormone is mediated at least in part by the parathyroid glands via PTH. Spanos, Brown, Stevenson and MacIntyre (1981) however using **in vitro** chick renal cell cultures have shown that in just one hour large doses of chicken growth hormone can cause a small but significant stimulation of the 1-hydroxylase directly. In contrast, Bikle, Spencer, Burke and Rost (1980) using the **in vitro** renal tubule preparation from vitamin D deficient chicks were unable to get any stimulation of the 1-hydroxylase with turkey growth hormone.

In summary therefore, because of the large doses that have to be used often for several days or weeks, it seems unlikely that growth hormone can affect vitamin D metabolism except in extreme cases, for example in acromegaly.

i. <u>Insulin</u>

It has been reported that diabetes experimentally induced with alloxan and streptozotocin causes a decrease in calcium transport in the duodenum of rats (Schneider, Omdahl and Schedl, 1976). They also reported that this decreased calcium transport could be corrected by administration of 1,25-DHCC. It has also been shown that vitamin D deficient rats have decreased serum calcium and insulin levels, and that 1,25-DHCC injections can return these levels back to control (Clark, Stumf and Sar, 1981).

The requirement of calcium ions for adequate insulin secretion is well established (Curry, Bennett and Grodsky, 1968). It is possible therefore that the lower serum calcium levels in vitamin D deficient animals are directly responsible for the low serum insulin. However, an autoradiographic study by Clark, Stumpf, Sar, DeLuca and Tanaka (1980) has shown the presence of 1,25-DHCC in the nuclei of the insulin secreting β cells, suggesting that 1,25-DHCC has a direct genomic action on β cell functions including insulin secretion. It is intriguing also that diabetic rats have low serum 1,25-DHCC levels (Scheider, Schedl, McCain and Haussler, 1977) and that this has now been shown to be due to reduced 1-hydroxylase activity (Spencer, Khalil and Tobiassen, 1980).

Therefore insulin appears to stimulate 1,25-DHCC production in the kidney, and 1,25-DHCC is probably capable of stimulating insulin secretion. The fact that these two endocrine systems are linked by positive feedback regulation is unusual, as negative feedback control is more useful in modulating hormone secretions. Little work has been performed on avian species, although Henry (1981) using chick renal cell cultures finds that insulin is required to observe PTH stimulation of the 1-hydroxylase. It is clear that more work is needed to determine the physiological role of insulin in

vitamin D metabolism.

j. Glucocorticoids

It has been known for some time that glucocorticoids can inhibit intestinal calcium absorption (Harrison and Harrison, 1960). Kimberg, Baerg, Gershon and Grandusius (1971) confirmed this observation and this group has since shown that glucocorticoids do not exert their effect by affecting vitamin D metabolism (Favus, Walling and Kimberg, 1973). Carré, Ayigbedé, Miravet and Rasmussen (1974) believe that glucocorticoids bring about the conversion of 1,25-DHCC to a slightly more polar but inactive metabolite, and that this results in the observed decrease in calcium absorption. In contrast 1-dehydrocortisol has been shown to increase serum 1,25-DHCC levels in the rat three-fold, even though, intestinal calcium transport is still inhibited (Lukert, Stanbury and Mawer, 1973). Spanos, Colston and MacIntyre (1977) found that three 70μg injections of corticosterone given to vitamin D replete chicks inhibited the 24-hydroxylase and stimulated the 1-hydroxylase in just five hours. They point out that their somewhat surprising result occurred rapidly and only required moderate doses of glucocorticoids, whereas the major inhibitory effects seen by other workers only develop after several days of treatment with pharmacological doses of the steroid.

k. Other factors

Several other factors have been shown to affect the renal 1-hydroxylase, some of these have only been poorly studied and are of limited physiological significance. Anticonvulsant drugs, for example phenobarbitone, appear to stimulate the 1-hydroxylase, long term treatment also results in decreased 25-HCC serum levels and the development of osteomalacia (Levison, Kent, Worth and Retallack, 1977). Human placental lactogen has been shown to cause a weak stimulation of the 1-hydroxylase in chick renal cell cultures (Spanos *et al*,

1981). Ethane-1-hydroxy-1,1-diphosphate which can be used in the treatment of Paget's disease (Smith, Russell and Bishop, 1971), interferes with vitamin D metabolism, probably by a direct inhibitory action on the 1-hydroxylase (Baxter, DeLuca, Bonjour and Fleisch, 1974) as well as inhibiting bone and cartilage mineralisation. Strontium disrupts calcium metabolism; high levels of strontium act similarly to high calcium levels and effectively halt calcium absorption from the gut resulting in a form of rickets by directly inhibiting the 1-hydroxylase (Omdahl and DeLuca, 1971). Both prostaglandin E_2 and $F_{2\alpha}$ give a mild stimulation of the 1-hydroxylase and inhibition of the 24-hydroxylase after six hours in chick renal cell cultures, at a concentration of 3-300nM (Trechsel, Taylor, Bonjour and Fleisch, 1980).

1.2.5 Localisation and Properties of the 24-Hydroxylase

The 25-hydroxycholecalciferol-24R-hydroxylase (24-hydroxylase) is responsible for the production of 24,25-DHCC from 25-HCC (see Figure 1.4). 24-hydroxylase activity can be found in the kidney (Knutson and DeLuca, 1974), the small intestine (Kumar, Schnoes and DeLuca, 1978) and certain bone cells (Turner et al, 1980). The localisation of the 24-hydroxylase in the rat nephron has been studied (Kawashima, Torikai and Kurokawa, 1981b). They found considerable 24-hydroxylase activity in the proximal convoluted tubule of normal rats, but when stimulated with 1,25-DHCC considerably more activity was detected in the proximal straight tubules.

Knutson and DeLuca (1974) reported that the 24-hydroxylase was localised exclusively in the mitochondria, and that the only cofactors required were NADPH and molecular oxygen, the NADPH being generated through the oxidation of succinate and malate as for the 1-hydroxylase. Magnesium ions were not required, and surprisingly they found that carbon monoxide did not inhibit the 24-hydroxylase, implying that cytochrome P_{450} was not involved. However, the more recent work of Kulkowski,

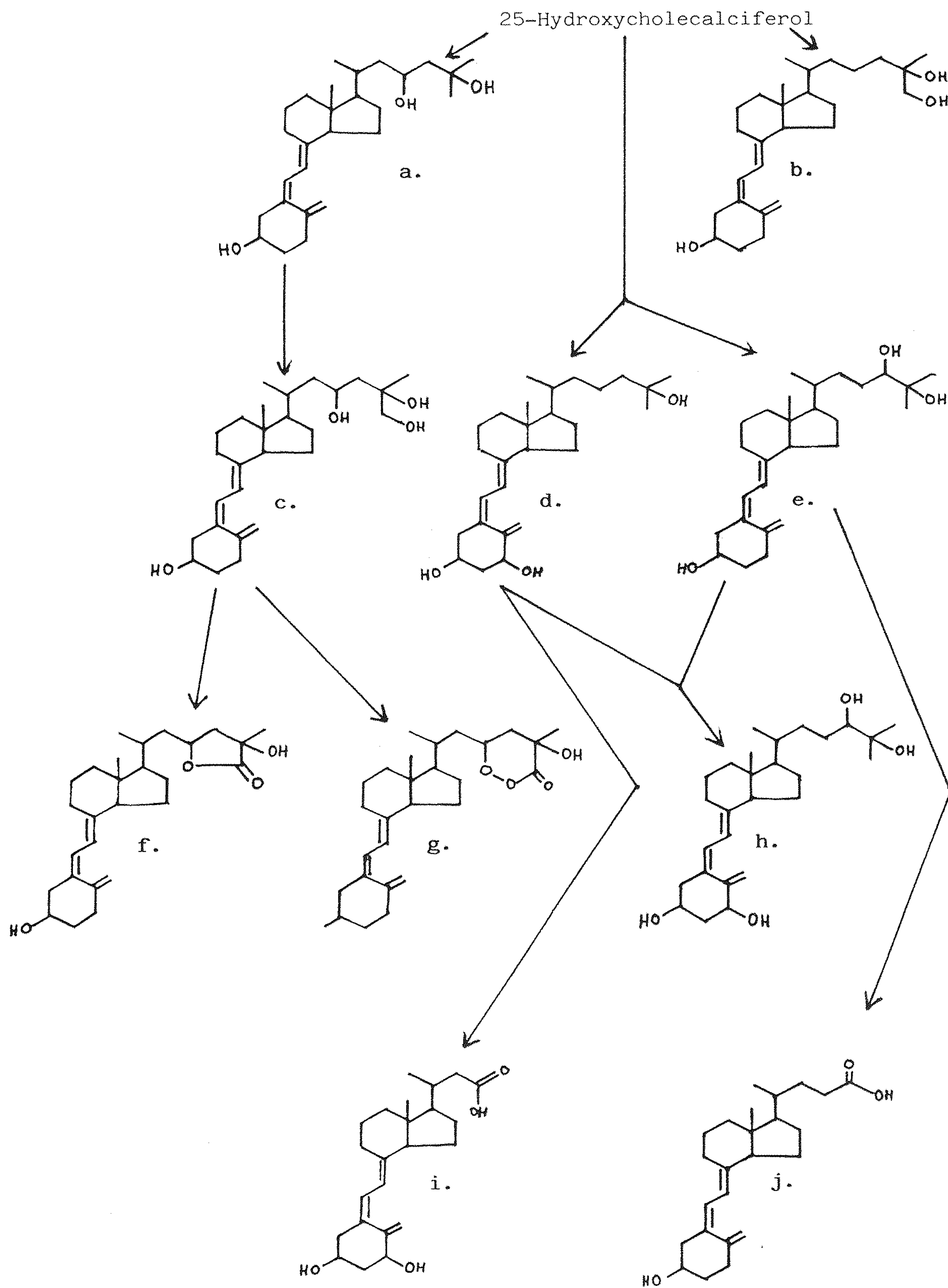
Figure 1.4

Important *in vivo* Metabolites of Vitamin D

In Figure 1.4 the vitamin D metabolites have been assigned the following subscripts:

a. 23,25-dihydroxycholecalciferol
b. 25,26-dihydroxycholecalciferol
c. 23,25,26-trihydroxycholecalciferol
d. 1,25-dihydroxycholecalciferol
e. 24,25-dihydroxycholecalciferol
f. 25-hydroxycholecalciferol-26,23-lactone
g. 25-hydroxycholecalciferol-26,23-peroxylactone
h. 1,24,25-trihydroxycholecalciferol
i. 1-hydroxy-24,25,26,27-tetranor-cholecalciferol-23-oic Acid
j. 25,26,27-trinor-cholecalciferol-24-oic Acid

Figure 1.4



Chan, Martinez and Ghazarian (1979) found that metyrapone and elipten, specific modifiers of cytochrome P_{450} and carbon monoxide all inhibited the 24-hydroxylase, so providing strong evidence that cytochrome P_{450} is an integral unit of the 24-hydroxylase. Madhok, Schnoes and DeLuca (1977) showed that the oxygen inserted into the '24' position was of molecular origin, and therefore like the 1-hydroxylase the 24-hydroxylase is a mixed function oxidase.

1.2.6 Regulation of the 24-Hydroxylase

Garabedian et al (1972) were the first group to propose a reciprocal relationship between the biological production of 1,25-DHCC and 24,25-DHCC; that is, factors that stimulate the production of 1,25-DHCC inhibit that of 24,25-DHCC and **vice versa.** This proposal has been proved correct in many cases; for example, PTH potently stimulates the 1-hydroxylase but inhibits the 24-hydroxylase (Howard, Turner, Bottemiller and Rader, 1979). Conversely 1,25-DHCC stimulates the 24-hydroxylase but inhibits the 1-hydroxylase both **in vivo** and **in vitro** (Henry, 1979). This stimulation of the 24-hydroxylase apparently requires **de novo** enzyme synthesis (Colston et al, 1977).

Kulkowski et al (1979) found that an injection into chickens of aminophylline, a cyclic nucleotide phosphodiesterase inhibitor, resulted in a ten-fold stimulation of 24-hydroxylase activity in just three hours, without any noticeable increase in kidney mitochondrial cytochrome P_{450} content. They suggest that the cytochrome P_{450} moiety is susceptible to phosphorylation/dephosphorylation which will result in altered enzyme activity. Obviously more effort has been made to understand how the 1-hydroxylase is regulated, as this enzyme produces the most active metabolite of vitamin D, even so the regulation of the 1-hydroxylase and 24-hydroxylase is still poorly understood. Hopefully though, even though the

1-hydroxylase and 24-hydroxylase are separate enzymes (Kawashima et al, 1981b) they are so closely related enzymically that when the regulation of one is fully understood it is likely that both will be.

1.2.7 Other Metabolites of Vitamin D

In addition to 25-HCC, 1,25-DHCC, 24,25-DHCC and 1,24,25-THCC already mentioned, several other metabolites of vitamin D are known. Most of these metabolites have only recently been isolated and identified, and hence their enzymic formation and physiological functions, if any, are as yet poorly studied, many as well have only been isolated in milligram, or even microgram, quantities.

The metabolite 25,26-dihydroxycholecalciferol (25,26-DHCC) was identified in 1970 by Suda, DeLuca, Schnoes, Tanaka and Holick, but only recently has this metabolite been further investigated. Horst and Littledike (1980) found the 25-HCC-26--hydroxylase was present not only in the kidney but also in other as yet undefined tissues. They also isolated a new metabolite 25,26-hydroxycholecalciferol-26,23-lactone (25-HCC-26,23-lactone) produced exclusively in the kidney which they believed was synthesised from 25,26-DHCC. However another new metabolite 23,25-dihydroxycholecalciferol (23,25-DHCC) is now thought to be the natural precursor for 25-HCC-26,23-lactone (Tanaka, Wichmann, Schnoes and DeLuca, 1981). Yet another metabolite 23,25,26-trihydroxycholecalciferol has been identified and is suggested to be an intermediate in the lactone formation (Ishizuka, Ishimoto and Norman, 1982a). The same group have also identified another metabolite 25-hydroxycholecalciferol-26,23-peroxylactone, which they now believe to be the naturally occurring form of the lactone, as when this metabolite is stored for short periods in the laboratory it decomposes to 25-HCC-26,23-lactone (Ishizuka, Ishimoto and Norman, 1982b).

The 'lactone' group of metabolites and 25,26-DHCC are only produced in detectable amounts in heavily vitamin D replete animals, and therefore the importance of these metabolites may be asociated with preventing vitamin D toxicity. Two further metabolites are 1-hydroxy-24,25,26,27-tetranor-cholecalciferol-23-oic acid and 25,26,27-trinor-cholecalciferol-24-oic acid. Both of these metabolites are made in the liver and appear predominantly in the bile and are most likely the polar excretory metabolites of 1,25-DHCC and 24,25-DHCC respectively (DeLuca and Schnoes, 1979). For full structures and pathways of formation of these metabolites see Figure 1.4.

In addition to these physiological vitamin D metabolites there exist several other metabolites that are produced when pharmacological doses of various vitamin D metabolites are given. These metabolites will be mentioned and their structures and tentative routes of formation are shown in Figure 1.5; however, their physiological relevance is considered to be minimal. For example large doses of 1,25-DHCC elicits the appearance of three new metabolites; 1,25,26-trihydroxycholecalciferol (Tanaka, Schnoes, Smith and DeLuca, 1981), 1,25-dihydroxy-23-oxocholecalciferol and 1,25,26-trihydroxy-23-oxocholecalciferol (Ohnuma, Kruse, Popjak and Norman, 1982). When 1.5×10^7 international units of vitamin D are given to chickens, in four days small quantities of the following metabolites can be detected in the plasma, 23,24,25-trihydroxycholecalciferol, 24,25,26-trihydroxycholecalciferol, 25-hydroxy-24-oxocholecalciferol, and 23-dehydro-25-hydroxycholecalciferol (Wichmann, Schnoes and DeLuca, 1981). Similar large dosing with vitamin D can produce the metabolite 25-hydroxy-23-oxocholecalciferol (Horst, Reinhardt and Napoli, 1982). A metabolite of possible clinical importance is 1,25-dihydroxycholecalciferol-26,23-lactone which is detected when a large dose of the clinically used 1α-hydroxycholecalciferol is given to rats (Ohnuma, Bannai, Yamaguchi, Hashimoto and Norman, 1980).

Figure 1.5

Metabolites of Vitamin D unlikely to be of any Physiological Significance

The names of the metabolites in this Figure have been given the following subscripts:

a. 1,25,26-trihydroxycholecalciferol

b. 1,25-dihydroxy-23-oxocholecalciferol

c. 1,25,26-trihydroxy-23-oxocholecalciferol

d. 23,24,25-trihydroxycholecalciferol

e. 24,25,26-trihydroxycholecalciferol

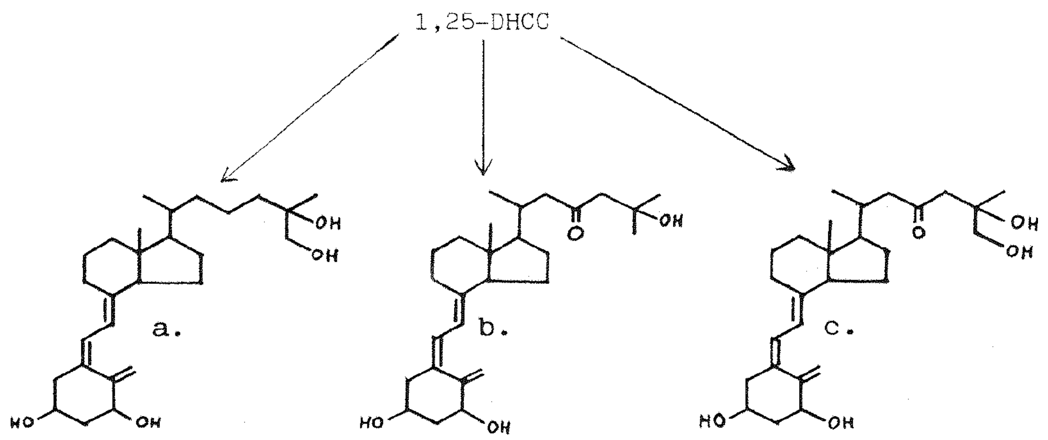
f. 25-hydroxy-24-oxocholecalciferol

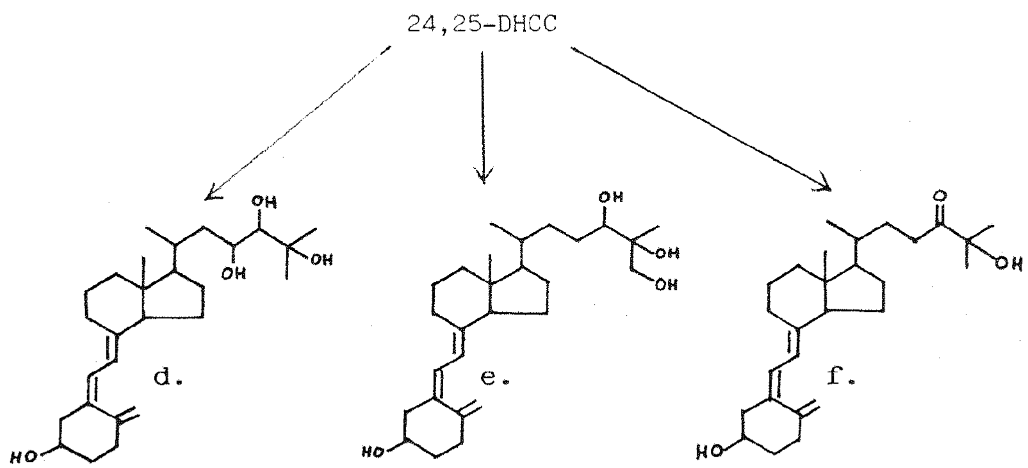
g. 23-dehydro-25-hydroxycholecalciferol

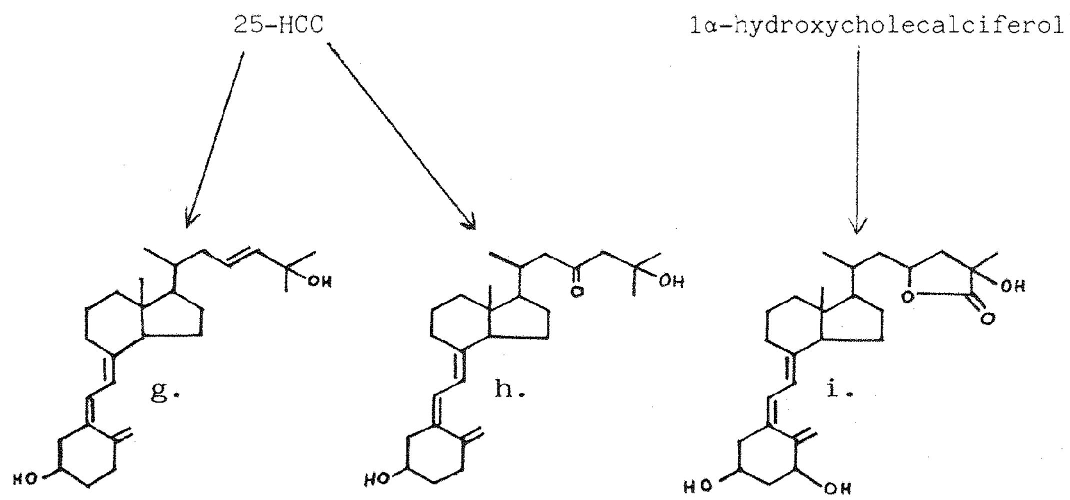
h. 25-hydroxy-23-oxocholecalciferol

i. 1,25-dihydroxycholecalciferol-26,23-lactone

Figure 1.5







CHAPTER 2 - MATERIALS AND METHODS

2.1 Animals

For all our studies on the metabolism of vitamin D Japanese Quail (Coturnix coturnix Japonica) were used. Fertile eggs were incubated at 37°C with 60% humidity in a forced draught Westernette incubator (Messrs Western Incubator Ltd., East Hanningfield, Essex, England), and they normally hatched after 17d. The chicks were housed under an infra-red lamp at approximately 25°C for the first fortnight, and from then on maintained at 20°C in standard stock cages (dimensions 100x50x50cm). They were raised on a commerical turkey starter diet which contained 10g calcium, 8g phosphorus and 50μg vitamin D per kilogram, and tap water ad libitum.

From hatching all quail were kept on one of three main lighting regimes:

1. Short Photoperiod (SD) (8L:16D): this system consisted of 8 hours of light followed by 16 hours of darkness: lights went on from 08.00-16.00h G.M.T. This regime resulted in production of somatically mature but sexually immature photosuppressed birds.

2. Long Photoperiod (LD) (16L:8D): this system was comprised of 16 hours of light followed by 8 hours of darkness; lights went on from 09.00-01.00h G.M.T. and sexually mature photostimulated birds resulted.

3. Transferred SD→LD (d70TLD): on this system birds were kept for 70d from hatching on the SD regime and then transferred to the LD regime to initiate sexual maturation.

For a review of the effects of photoperiods on the sexual development of Japanese Quail see Stein and Bacon (1976).

2.2 Blood Sampling

Birds were killed by decapitation and trunk blood was collected into heparinsed tubes. The blood was centrifuged at 2000rpm and the plasma decanted from the blood cells and kept frozen at -20°C until required for assay. Where more than one blood sample was required from each bird, samples were taken from the wing vein. To accomplish this the wing vein area of an unanaesthetised bird was plucked and the wing vein cut with a razor blade. Blood was then removed by Pasteur pipette into heparinised tubes until blood ceased to flow: 0.5-1.0ml of blood could be collected in this way. When anaerobic blood samples were required for ionised calcium measurements, blood was obtained by cardiac puncture.

2.3 Hormone Treatments

Unless otherwise specified 1.8mM solutions of reproductive steroids were injected intramuscularly (i.m.)into the birds at the equimolar dose of 0.18μmole/100g body weight (i.e. 0.1ml/100g body weight). Unless otherwise stated in the text the sex steroids were dissolved in ethyl oleate. The equimolar dose that we adopted approximates to 50μg steroid/100g body weight. Several other compounds were injected into the quail, full details of which will be found in the relevant chapters.

2.4 The Renal Hydroxylase Assay

The following assay system was used to measure the activity of the renal 25-hydroxycholecalciferol-1α and 24R-hydroxylases. It is based on the assay described by Kenny (1976) and is unsuitable for studying mammalian vitamin D hydroxylase systems.

2.4.i Incubation Procedure

The birds were decapitated, trunk blood collected, both

kidneys were removed to ice cold assay buffer (see Chapter 2.9). The kidneys were then lightly blotted and weighed wet and a 10% (weight/volume) homogenate was prepared, using five passes of a teflon pestle at 300rpm.

The incubation was started by the addition of 100µl of the homogenate to 1.9ml of assay buffer in a 25ml conical flask. The buffer contained an active NADPH generating system and Mg^{2+}, in addition to 6.5pmole of $^{3}[H]$25-HCC (the substrate). As direct sunlight will destroy 25-HCC the flasks were covered with tin foil, and then incubated at 37°C in a shaking water bath (80rpm) for 10 minutes.

2.4.ii Extraction of Metabolites

The incubation was stopped after 10 minutes by the addition of 8ml methanol:chloroform (2:1 v/v). The resultant mixture was shaken and then left in the dark at room temperature for 20 minutes. The mixture was then centrifuged at 2000rpm at room temperature to bring down tissue debris. The supernatant was decanted from the pellet and extracted four times with methylene chloride. The pooled extracts were evaporated under reduced pressure and transferred to a small glass vial. Total extraction of the $^{3}[H]$ compounds by this method was found to be 94.7±0.6% (n=4).

2.4.iii Separation of Vitamin D Metabolites

There are a variety of methods available to separate the tritiated metabolites of vitamin D. These methods include paper and thin layer chromotography, high pressure liquid chromatography and sephadex LH-20 columns. Initially the sephadex LH-20 system was used. The sephadex beads were allowed to swell for 24h at room temperature in the elution solvent, which was hexane:chloroform:methanol 9:1:1 (v/v/v). The 170x7mm column was packed wet and washed for 2h before use. The sample was applied to the column in 100µl of elution

solvent and eluted for 2-3h at room temperature in the dark. 0.8ml samples of eluate were collected, dried and the tritium counted using 5ml of toluene butyl PBD scintillant. For the vitamin D metabolite profile using LH-20 see Figure 2.1.

Subsequently thin layer chromatography (TLC) was used to separate the vitamin D metabolites. The plates are made with Kieselgel GF_{254} silica (type 60) which was made into a slurry (50g silica + 100ml distilled water) and then spread on 100x200mm plates to a thickness of 250μm. The plates were then activated by heating to 100°C for a minimum of 3h prior to use. The samples were applied to the plate in a fine line and eluted in the solvent system I developed of heptane:ethyl acetate:ethanol 12:12:1 (v/v/v) in the dark at 4°C. Non-radio-active standards of 25-HCC, 24,25-DHCC, 1,25-DHCC and 1,24,25--THCC were also applied to the plates and used to locate the ^{3}[H] metabolites under short wavelength ultraviolet. The respective r.f. values for the above metabolites are 0.60, 0.39, 0.25 and 0.14. Bands of silica 0.5cm wide were scraped from the plate into the scintillation vials and counted with 5ml toluene butyl PBD in a Philips 4800 series counter. For metabolite separation profile see Figure 2.2.

The dpm for each metabolite peak was then corrected for experimental loss, as only 40-60% of the total ^{3}[H] was normally be applied to the plate. Knowing the total dpm of a metabolite produced and its specific activity, which is the same as the substrates, it was possible to express the renal hydroxylase activities in the units of pmole metabolite produced/hour/gram wet weight kidney tissue. These units are used predominantly to express the renal 1α and 24R-hydroxylase activities.

2.5 Validation of the Renal Hydroxylase Assay

2.5.i Comparing the LH-20 and TLC Separating Systems

Initially the metabolites of vitamin D were separated by

Figure 2.1

Sephadex LH-20 Separation of Vitamin D Metabolites from a Long Day Female Quail

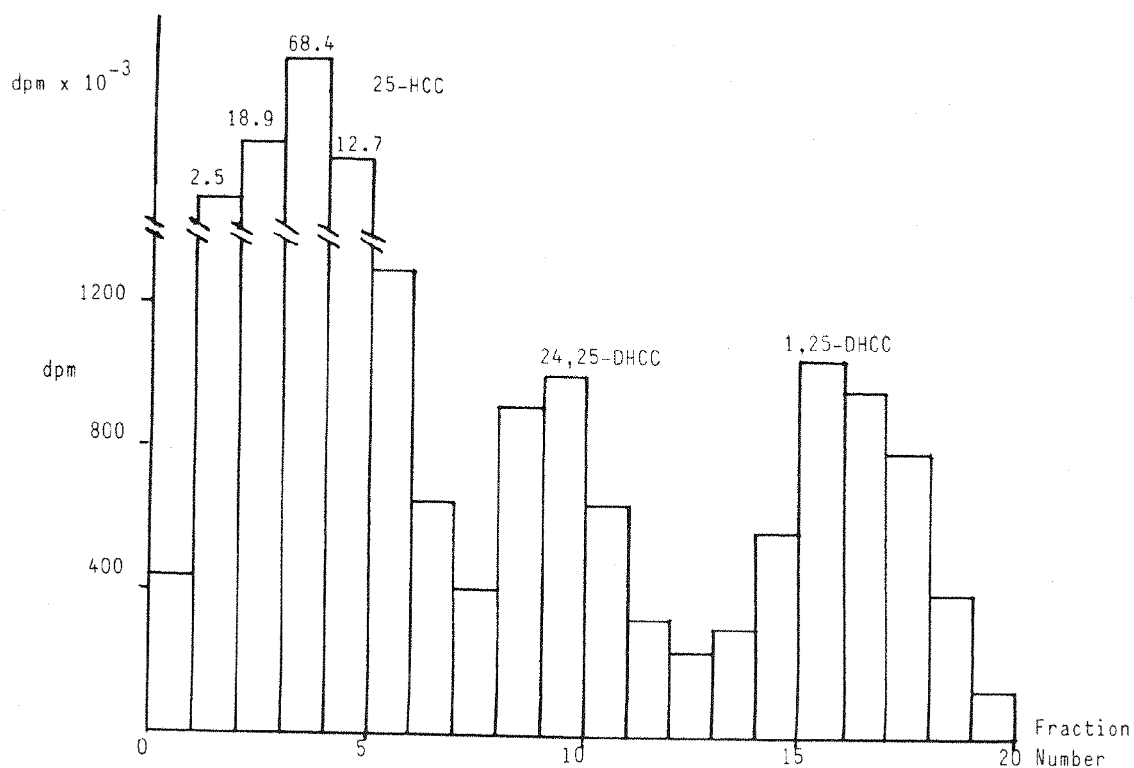
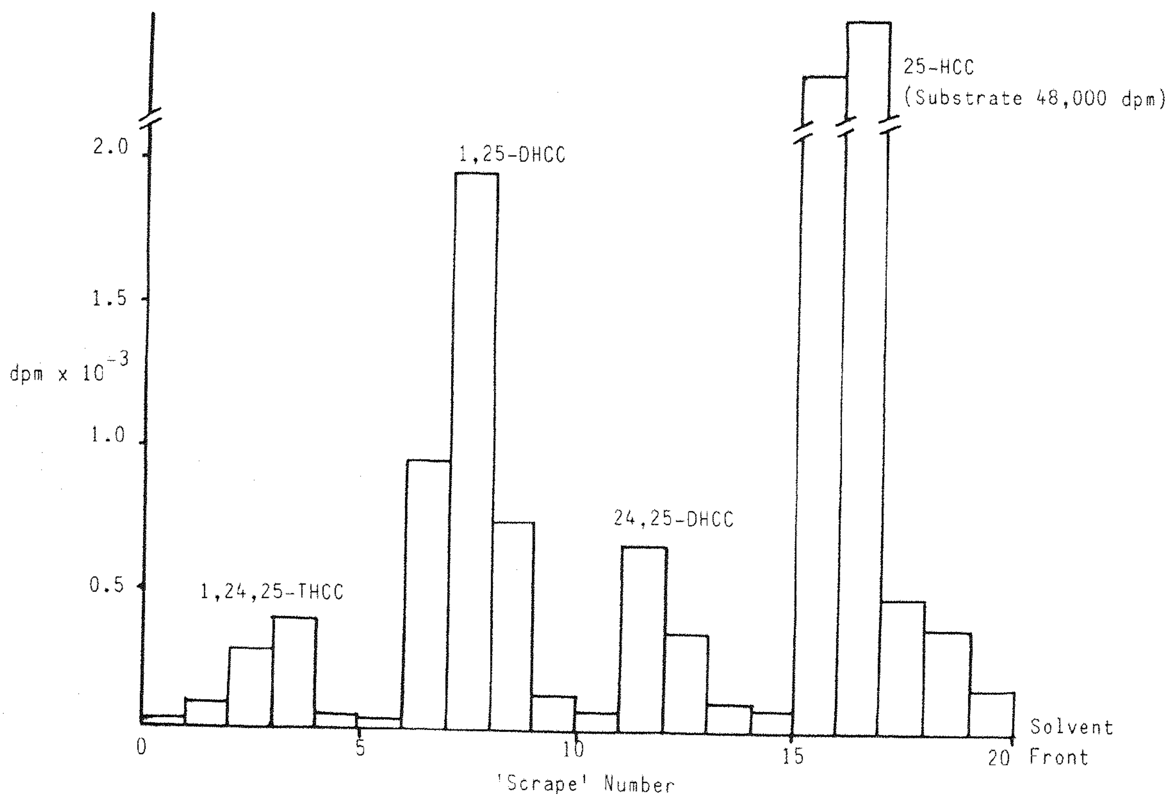


Figure 2.2

TLC Separation of Vitamin D Metabolites from an Oestrogen Treated Short Day Female Quail



both Sephadex LH-20 columns and by TLC plates. Having decided to adopt the TLC system it was necessary to show that the major vitamin D metabolites could be accurately resolved and identified by TLC. In an early experiment the TLC separation was compared with that of the LH-20 columns; the latter technique should have offered superior resolution, but with our relatively small columns this was not found to be the case. Incubation samples from 4 pairs of mature long day quail were prepared and the samples divided such that one half was analysed by TLC and the other by the LH-20 columns. Results are shown in Table 2.1.

Discussion

It was encouraging to see that all the females had greater 1-hydroxylase activities than the males reflecting the high calcium requirements of the laying birds; furthermore, most of the males had the greater 24-hydroxylase activity. There were differences between the two separatory techniques but these differences tended to be minor and entirely acceptable. The results from this experiment confirmed that the TLC system is of comparable accuracy to the LH-20 one, highlighting the fact that the rapid, cheap, non-labour intensive TLC system has no major faults.

2.5.ii Use of HPLC to Validate the TLC system

Recently we were able to further validate our TLC technique with HPLC. We used a single straight phase µ-porasil column with a solvent system of heptane 10:isopropanol 1 and a flow rate of 2ml/minute (800psi): this enabled excellent resolution of the vitamin D metabolite standards to be achieved (see Figure 2.3) especially 1,25-DHCC which eluted well clear of other metabolites. While we have not used HPLC extensively, two simple experiments have helped confirm the identity of our vitamin D metabolites as separated by TLC.

Table 2.1

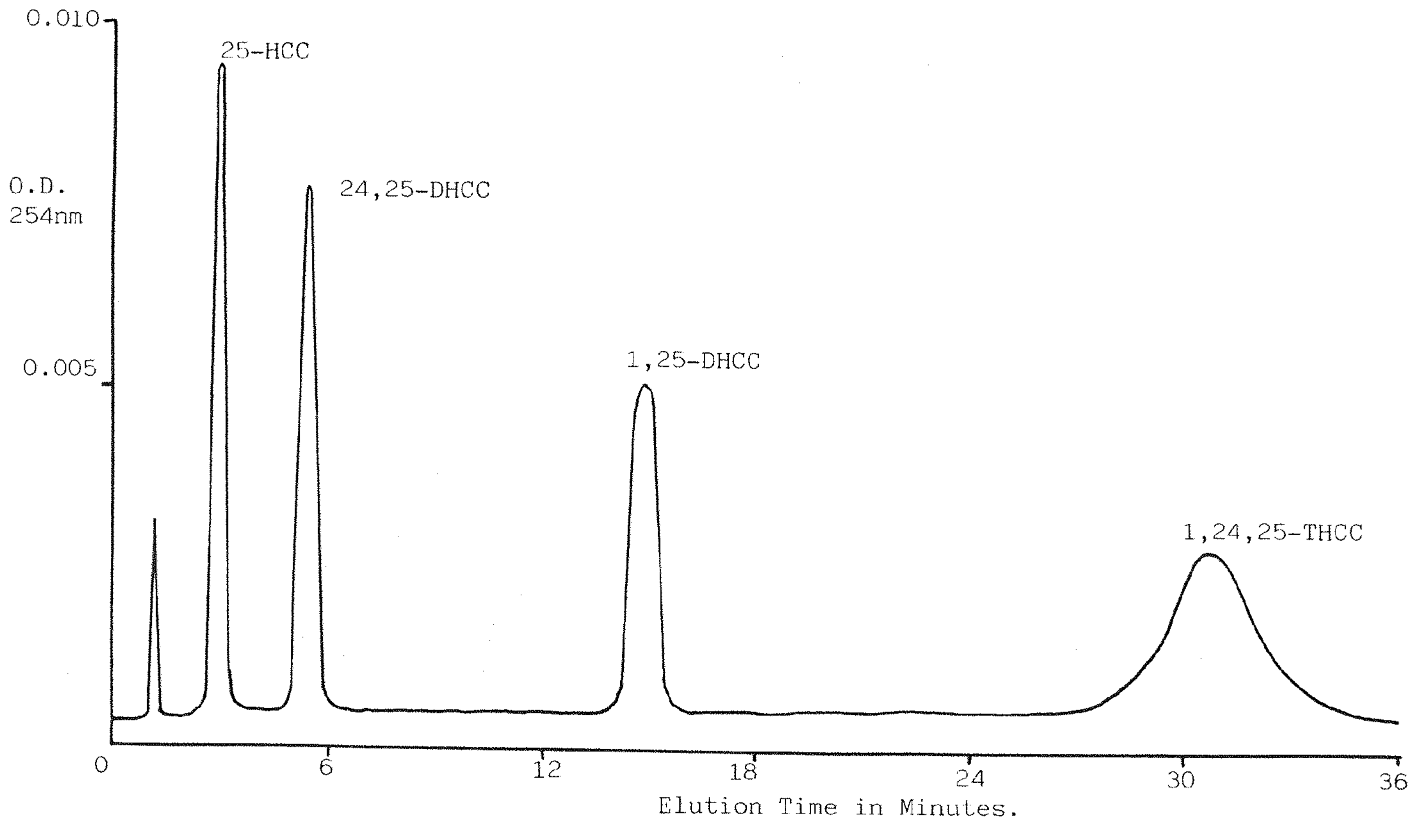
Comparison of TLC and LH-20 Separatory Techniques Using Four Pairs of Mature Quail

Pair Number			1-Hydroxylase Activity $\frac{\text{dpm metabolite}}{\text{Total dpm}} \times 100$	24-Hydroxylase Activity $\frac{\text{dpm metabolite}}{\text{Total dpm}} \times 100$
1	TLC	♀	2.86	ND
	LH-20		2.80	ND
	TLC	♂	1.76	6.00
	LH-20		1.60	6.18
2	TLC	♀	2.36	3.13
	LH-20		3.11	3.13
	TLC	♂	0.48	5.36
	LH-20		0.98	5.27
3	TLC	♀	1.73	4.09
	LH-20		1.85	4.67
	TLC	♂	0.40	2.27
	LH-20		0.69	2.67
4	TLC	♀	5.97	2.30
	LH-20		4.36	2.10
	TLC	♂	ND	3.03
	LH-20		ND	2.05

ND = No Detectable Activity

Figure 2.3

HPLC Separation of Vitamin D Metabolites



a. In the first experiment two separate incubations were performed, one from an oestrogen injected female which we expected to have a large 1-hydroxylase activity and the other from a control male which had high 24-hydroxylase activity. The metabolites were then separated by TLC and the bands of silica corresponding to the cold metabolites (as visualised under u.v. light) scraped into small elution funnels, the silica should therefore have contained specifically TLC purified 1,25-DHCC or 24,25-DHCC. The silica was eluted with five 1ml aliquots of diethyl ether through a glass wool filter in the funnels. The eluate was evaporated to dryness and the metabolites taken up in the HPLC solvent system. These samples were run individually on the HPLC with the addition of 5nmole cold 1,25-DHCC or 24,25-DHCC. The eluate from the HPLC was collected in 30 second samples and a note taken of the vials in which the u.v.$_{254nm}$ absorption peak of cold metabolite appears. Toluene butyl PBD was added to the vials and the vials counted for tritium. The tritium appearing in the specific metabolite peak (as determined by u.v. absorbance) was then expressed as a percentage of the total tritium eluted on the run.

Results

Metabolite Studied	Purity $\frac{\text{dpm } ^{3}[\text{H}]\text{metabolite} \times 100}{\text{dpm } ^{3}[\text{H}]\text{total}}$
24,25-DHCC	97.7%
1,25-DHCC	91.4%

Purity values are means of duplicate determinations.

b. In the second experiment attempts were made to separate all the vitamin D metabolites from an extended incubation and to compare the TLC and HPLC separation profiles. A single incubation was performed that contained both 1- and

24-hydroxylase activity. The sample was roughly divided into two and both halves separated by TLC; one half was carried through the normal assay procedure and the metabolites expressed as % total tritium recovered from the plate. The silica scraped from the origin to just above the 25-HCC band on the other plate was handled in exactly the same way as in 2.5.ii.a and then run on HPLC. The initial TLC run acted as a purification step as there was much neutral lipid above the 25-HCC band. Previously, the incubation extract was applied directly onto the HPLC column, but the neutral lipids overloaded the column and took two days to wash out completely.

Results

Separation System Used	**Metabolities as % of total ^{3}[H]**		
	24,25-DHCC	1,24,25-THCC	1,25-DHCC
TLC	2.2	3.6	11.2
HPLC	1.6	3.9	12.9

Discussion

The first experiment showed that the tritiated metabolite peaks separated on the TLC plates and believed to be 1,25-DHCC and 24,25-DHCC appeared to be 91.4% and 97.7% pure, respectively, when extracted and separated by HPLC. It appears, therefore, that neither peak was greatly contaminated with another vitamin D metabolite, of which 25,26-DHCC would perhaps have been the most likely. The second experiment showed that there was very little difference in the elution patterns of the three main metabolites of 25-HCC separated by TLC and by HPLC. 1,24,25-THCC did not appear that frequently in these incubations; this is partly due to its relatively poor separation on TLC, running close to the origin. In addition, the substrate concentration (1,25-DHCC and 24,25-DHCC) for its formation is very low, only 5-10% that of

25-HCC. If 1,24,25-THCC was normally produced in significant quantities this would introduce some error into the assay, as 1,25-DHCC and/or 24,25-DHCC metabolite levels would be decreased slightly in the incubation mixture, so reducing the apparent hydroxylase activities. The HPLC elution profile also failed to show up any unknown metabolite peaks, so indicating that in our birds the 1α and 24R-hydroxylases are the major active vitamin D renal hydroxylases.

2.5.iii Effect of Substrate Concentration on the Assay

Usually 6.5pmole of ^{3}[H] 25-HCC was used in the 2ml incubation volume (i.e. 3.25nM). In a simple experiment the renal homogenate from a single mature female quail, was incubated at four different substrate concentrations and the hydroxylase activities measured. Results are shown in Figure 2.4.

Discussion

The results show that by doubling the substrate concentration it was possible to increase the apparent renal hydroxylase activities by a factor slightly less than two. This indicates that the hydroxylases are not saturated and that it may be possible to increase the assay sensitivity by increasing the substrate concentration; however, this would have to be weighed against increased background counts and cost. It would be possible to add cold 25-HCC to the label, thus increasing the substrate concentration and reducing its specific activity, but errors may be introduced when different batches of label are manipulated. With this in mind it was decided to continue using our incubation concentration of 3.25nM ^{3}[H] 25-HCC.

Figure 2.4

Effect of Substrate Concentration on the Activity of Renal Hydroxylases

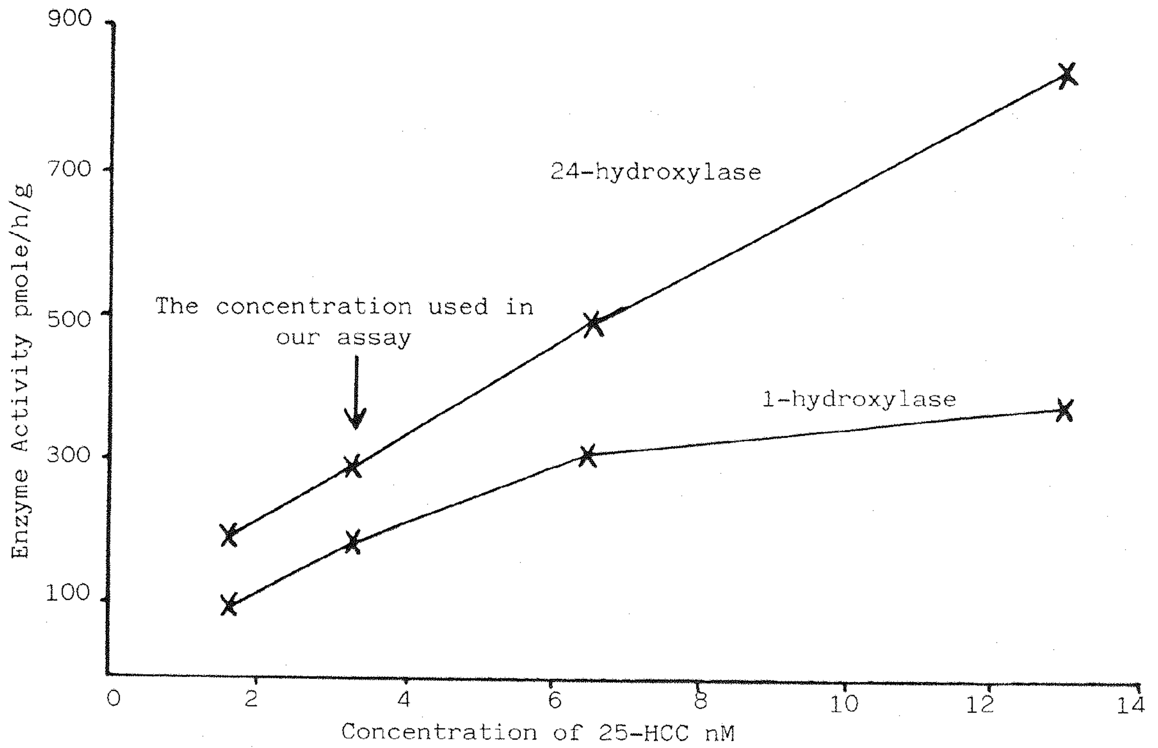
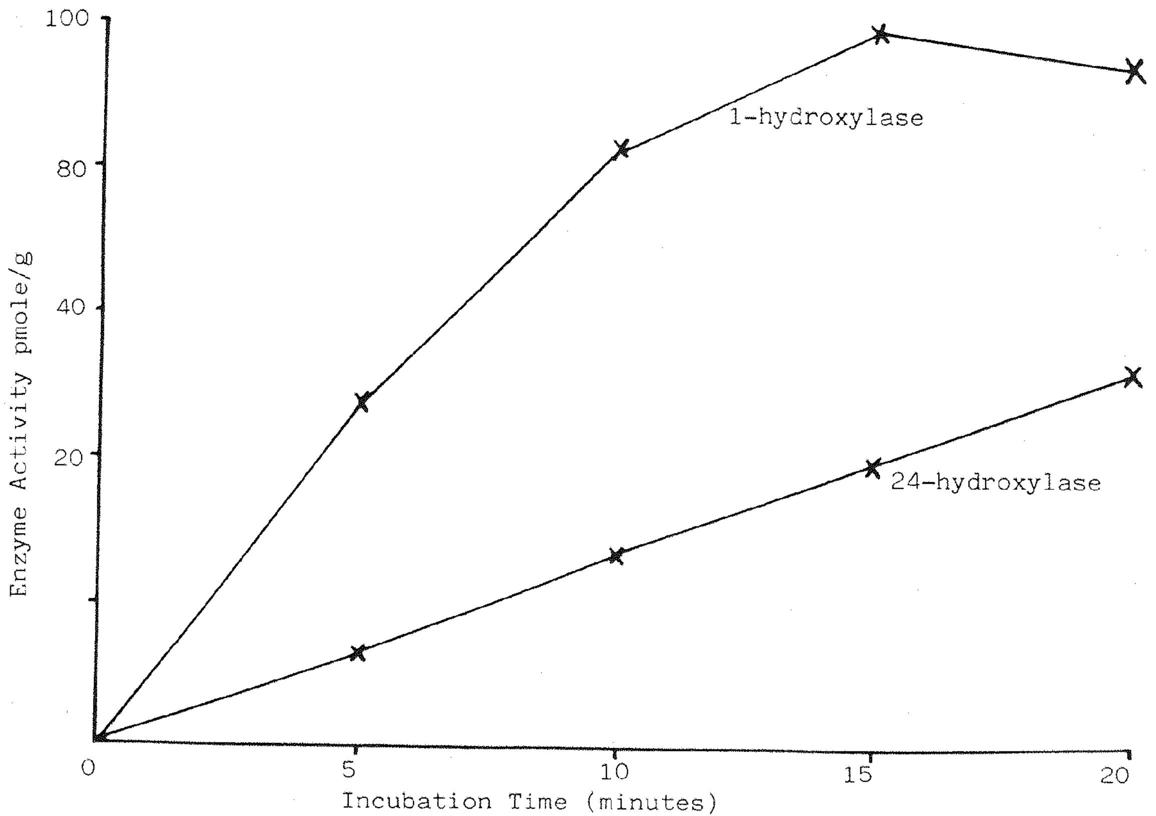


Figure 2.5

Time Course Study of Renal Hydroxylase Activities Using 3.25 nM Substrate



2.5.iv Incubation Time

This is an important variable that must be considered, for if extended incubation times are used it may be possible to get product inhibition of enzyme activity and even a significant decrease in substrate concentration which will decrease the apparent enzyme activities. Ideally, the incubation time should be such that the rate of metabolite production is linear with time throughout the incubation, but it must also be lengthy enough to ensure the production of adequate quantities of metabolite to permit their later quantification. Therefore in this experiment 1- and 24-hydroxylase activities were measured with incubation time being the only variable. Two birds were used: the first, a female, with large 1-hydroxylase but no detectable 24-hydroxylase activity; the second, a male, with an active 24-hydroxylase.

Results

See Figure 2.5.

From the graph it can be seen that 24,25-DHCC production and hence 24-hydroxylase activity was linear throughout the time course of the experiment, while 1,25-DHCC production was only linear up to 10-15 minute incubation times. The 1-hydroxylase activity in this specific case was extremely high and therefore represents a most stringent test; it was therefore decided to adopt the 10 minute incubation time.

2.5.v Assay Reproducibility

Throughout this thesis renal hydroxylase activities are determined from a single incubation only. To test the reproducibility of the assay five identical incubations were performed. The results are shown below:

Table 2.2 **Results from Five Separate Incubations Using the same Renal Homogenate**

	Enzyme Activity pmole/h/g	
	1-Hydroxylase	24-Hydroxylase
1	290.8	38.7
2	306.0	49.9
3	284.9	54.7
4	289.9	53.5
5	287.7	52.5
Mean ± S.E.M	291.9 ± 3.7	49.9 ± 2.9

The results show that the assay is very reproducible especially when elevated hydroxylase activities are being measured. Overall it must be stated that some of the assay errors could have been reduced if the incubations had been performed in duplicate; however, in view of these results and the huge 10-20 fold changes in hydroxylase activities observed in many of the studies, duplicating incubations did not seem to be justified.

2.5.vi Effect of Oestrogen Dose on Renal Hydroxylase Activites

In the following experimental chapters reproductive steroids were injected at the equimolar dose of 1.8μmole/kg BW, this approximates to 50μg of oestrogen per quail. This particular dose was chosen arbitarily although we wanted a low dose, as unlike most workers, we always injected the free steroids. Since much of the work in this thesis involves stimulating the 1-hydroxylase with oestrogen, it was necessary to show that our adopted dose was neither too extreme, nor for the ease of measuring responses, too weak.

SD female quail were injected i.m. with varying amounts of 17β-oestradiol in ethyl oleate. Birds were killed 6h post

injection and renal hydroxylase activities determined. The results are expressed as a dose response curve. (Figure 2.6)

It can be seen that our adopted dose (490μg/kg BW) produced a submaximal stimulation of the 1-hydroxylase; however a four-fold increase in oestrogen dose only produced a slightly bigger response. The lowest dose (31μg/kg BW) produced no response while the 122μg/kg BW dose produced small but significant responses. Our dose of 1.8μmole/kg BW is therefore quite adequate with respect to the stimulation of the 1-hydroxylase by oestrogen.

2.6 Radioimmunoassay of Reproductive Steroids

In some experiments plasma levels of $17\beta\text{-}E_2$ and E_1 were measured. Specific radioimmunoassays were used to determine these steroid levels, the actual assays were performed by Dr's Brain or Peddie, for experimental details, see Brain (1982).

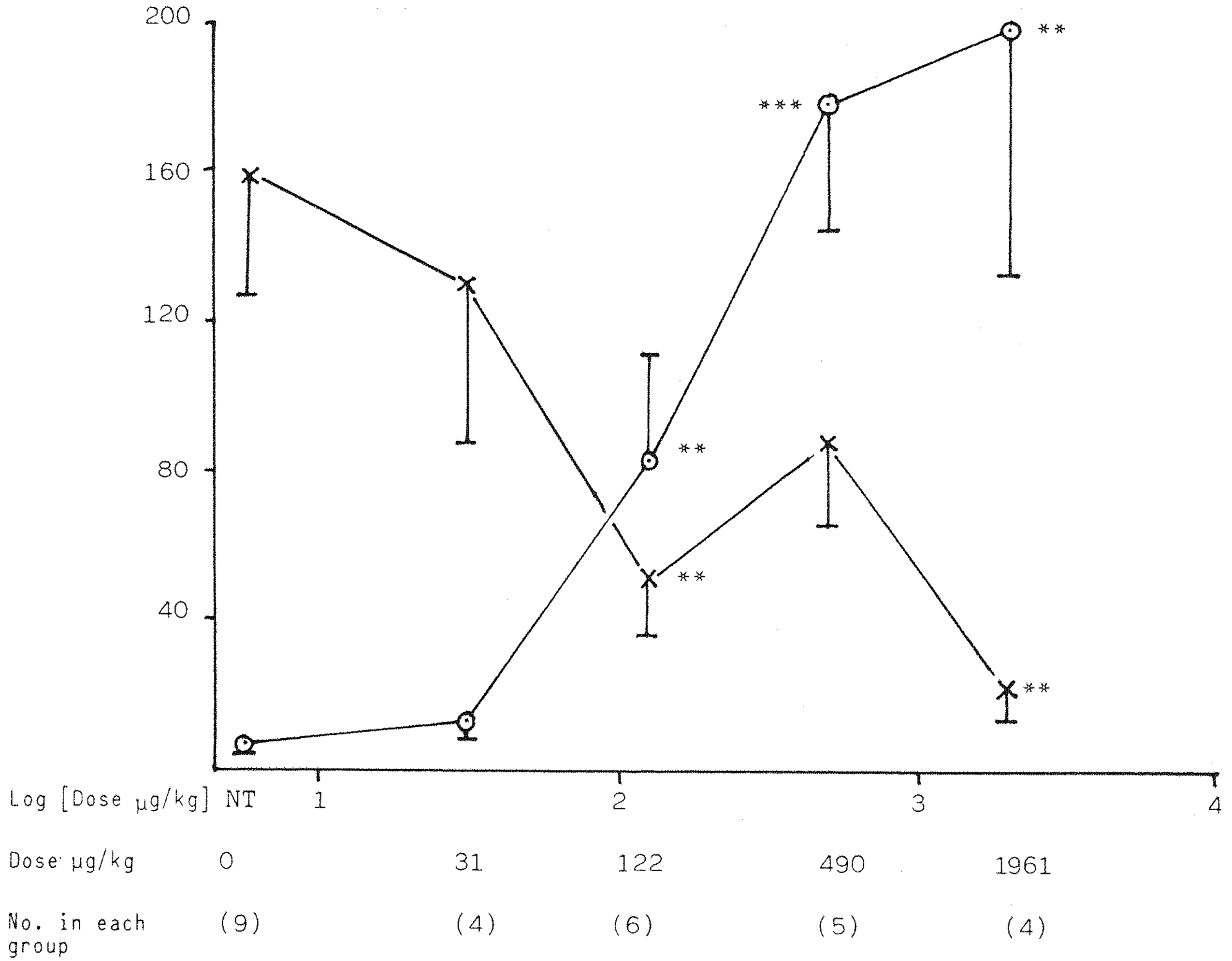
2.7 Measurement of Total and Ionised Plasma Calcium

To measure total plasma calcium 50μl of plasma was added to 100μl of 5% Lanthanum Chloride and 4.85ml of distilled water and vigourously shaken. The samples were then read against calcium standards (0-4μg/ml calcium), prepared from Analar calcium carbonate in a 0.1% Lanthanum Chloride solution. The measurements were made on a Perkin-Elmer 280 series atomic absorption flame spectrophotometer at 423nm with a rich acetylene air flame. The 0.1% Lanthanum is used to reduce interferences by the flame ionisations and phosphate contamination.

To measure ionised calcium levels a calcium specific glass electrode is required. The sample must be kept anaerobic as

Figure 2.6

Dose Response Curve for the Renal Hydroxylase Activities Following Oestrogen Treatment



NT = Group that received no oestrogen treatment.

All points are mean values with error bars.

Significant differences are from the control (NT) group only at the following levels:

** $p<0.01$

*** $p<0.001$

⊙ - 1-hydroxylase

X - 24-hydroxylase

measurements are very sensitive to pH changes; another problem is that 2ml of blood are required for each determination which for quail can present problems. Ionised calcium measurements were performed by Dr. Luck: for experimental details, see Luck and Scanes (1979).

2.8 Measurement of Acid Labile Plasma Phosphate

To 450 µl distilled water and 500 µl of 200g/l trichloroacetic acid (TCA) was added 50 µl of plasma. The mixture was shaken and left for 30 minutes, followed by a quick bench spin. Two 400 µl aliquots of supernatant were assayed; 350µl of TCA (100g/l) was added to the aliquots followed by 750 µl of molybdate solution. These samples were then read against phosphate standards at 700nm in a spectrophotometer exactly 10 minutes after the addition of the molybdate solution. The molybdate solution was made fresh each day and contained 30ml of ammonium molybdate (6g/l) in Perchloric acid (50g/l) plus 10ml ascorbic acid (2g/l).

2.9 Chemicals, Scintillants and Assay Buffer

The chemicals used in this research and their commercial sources are listed below.

Chemical	Source
K_2HPO_4	BDH
KH_2PO_4	BDH
$MgCl_2.6H_2O$	BDH
D-Glucose-6-Phosphate	Sigma Chemical Co
NADP	Sigma Chemical Co
L-Malic Acid	Sigma Chemical Co
Analar Calcium Carbonate	BDH
Analar Lanthanium Chloride	BDH
Sodium Sulphate	BDH
Analar Ammonium Molybdate	BDH
L-Ascorbic Acid	BDH
Kieselgel 60 GF_{254}	BDH

Steroids

$17\alpha-E_2$	Steraloids Inc
$17\beta-E_2$	Sigma Chemical Co
E_3	Sigma Chemical Co
E_1	Sigma Chemical Co
Testosterone	Sigma Chemical Co
Progesterone	Sigma Chemical Co
$^3[H]$25-HCC	Amersham Radio Chemicals
25-HCC	(Gifts from Uskokovic
1,25-DHCC	(Hoffman-La Roche
24,25-DHCC	(
1,24,25-THCC	(
$^{14}[C]\ E_1\ \ ^{14}[C]E_2$	Amersham Radio Chemicals
$^3[H]17\beta E_2\ ^3[H]E_1$	Amersham Radio Chemicals

Solvents

Ethanol	Koch-Light Labs
Heptane (I.P. grade)	BDH
Hexane (60°-80°)	BDH
Isopropanol	Koch-Light Labs
Ethyl Acetate	Koch-Light Labs
Chloroform	Koch-Light Labs
Methanol	Koch-Light Labs
Methylene Chloride	Koch-Light Labs
Diethyl Ether	Koch-Light Labs
Benzene	Koch-Light Labs

Scintillants

Toluene-Butly PBD was used to count non-aqueous samples: this scintillant was superceded later by Beckman's Ready-Solv^{TM}NA.

The composition of Tolune-Butyl PBD was as follows:

Toluene (Sulphur free) 2.5l	Koch-Light Labs
Butyl PBD (2-4' tert butyl phenyl-5-4' biphenyl)-1,3,4-oxadiazole 20g	C & G Chemicals

Assay Buffer Composition

KH_2PO_4	0.2 M	
$MgCl_2.6H_2O$	4 mM	
L-Malic Acid	7.5 mM	
D-Glucose-6-P	3.5 mM	
NADP	0.3 mM)	added at time of assay
G-6-PΔH	600u/l)	

2.10 Statistical Methods

The Wilcoxon rank sum test was used extensively to compare two groups of independant samples. This is a non-parametric test and is probably a more valid test than the 't' test as there was no evidence that our data was normally distributed; this is especially true for the renal hydroxylase data ('probit' test performed).

The following two tail significance levels were adopted:

* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

Some sex steroid data is compared by the 't' test, two tail significance levels quoted are as below:

† = $p < 0.05$
†† = $p < 0.01$
††† = $p < 0.001$

Some group data was suitable for treatment by analysis of variance; this was performed and significance assessed following Duncan's Multiple range test.

Where applicable all data is expressed as mean ± Standard Error of the Mean (S.E.M.).

CHAPTER 3 - EFFECT OF *in vivo* ADMINISTRATION OF FOUR DIFFERENT OESTROGENS ON RENAL HYDROXYLASES AND OTHER VARIABLES

3.1 Introduction

As mentioned in Chapter 1, single injections of oestradiol esters **in vivo** can result in increased 1-hydroxylase activity as measured **in vitro** (Sedrani, 1979).

It has also been shown that renal 1-hydroxylase activity increases prior to egg lay (Sedrani, 1979; Castilo et al, 1979). Since oestrogen levels also increase considerably at this time (Brain, 1982), several groups have suggested that oestrogen causes the observed stimulation of the 1-hydroxylase activity seen in egg laying birds (Castillo, Tanaka, DeLuca and Sunde, 1977; Bar and Hurwitz, 1979; Baksi and Kenny, 1979). However, the situation is probably more complicated than this, as it has been shown that medullary bone formation, which requires increased circulating levels of both androgen and oestrogen (Stringer, 1962), occurs 2-4 weeks prior to any increase in 1-hydroxylase activity in maturing female chickens (Castillo et al, 1979). Brain (1982) had also observed that the main increase in plasma 17β-oestradiol levels measured by RIA in maturing female Japanese quail did not coincide with the increase in 1-hydroxylase activity observed by Sedrani (1979) in similar birds.

One possible explanation for these anomalies may be the existence and specific actions of other oestrogens, since in addition to 17β-oestradiol, oestrone has been detected in hen and quail plasma (Peterson and Common, 1972; Doi, Takai, Nakamura and Tanabe, 1980). 17α-oestradiol and oestriol have also been isolated and identified from hen plasma, but in relatively smaller amounts (O'Grady and Heald, 1965; Chan and Common, 1974).

Therefore, changes in renal hydroxylase activity in sexually maturing Japanese Quail could possibly be influenced by changes in the plasma levels of these other oestrogens. Although oestrogens, other than 17β-oestradiol, have poorly defined physiological actions they have been shown to build up in the plasma several weeks before the onset of lay in chickens, considerably earlier than the main rise in 17β-oestradiol levels just prior to lay (Peterson and Webster, 1974).

The comparative effects of these four oestrogens on the renal hydroxylases was therefore studied. In addition, ovary, oviduct and body weights were recorded and plasma total calcium, acid labile phosphate, 17β-oestradiol and oestrone levels were determined.

This experiment was performed jointly with Miss P. C. Brain and the results have been published in her thesis (Brain, 1982).

3.2 Methods

Female Japanese quail that were 50-70 days old and raised on the photosuppressive short photoperiod (8L:16D) were used. These birds were somatically mature, but sexually immature and hence ideal to use in studies involving sex steroids. Birds were divided into five groups and body weights recorded. Quail were injected intramuscularly (i.m.) once daily in their 2nd or 3rd hour of light with either oestrone, 17β-oestradiol, 17α-oestradiol or oestriol, using ethyl oleate as vehicle, or with vehicle only for 3 days. All sex steroids were administered at the equimolar dose of 1.8 μ mole/kg body weight. Blood was collected into heparinised tubes from the wing vein on days 1, 2 and 3 of the experiment prior to injection. All quail were killed on the fourth day by decapitation 24h after the last injection and trunk blood was collected. Ovaries and oviduct were removed and their weight recorded, kidney tissue

was also removed for the estimation of 1 and 24-hydroxylase activities. Plasma calcium, phosphate, 17β-oestradiol and oestrone levels were measured in the plasma samples at a later date.

3.3. Results

Ovary weights remained unchanged following oestrogen treatment; however all four oestrogens caused an increase in oviduct weight (Table 3.1). The largest response was seen in quail injected with 17β-oestradiol or oestrone, in which oviduct weights were significantly greater than controls ($p<0.01$ and $p<0.001$ respectively). 17α-oestradiol and oestriol both caused smaller increases than the other two oestrogens ($p<0.01$ and $p<0.05$ respectively).

Total plasma calcium levels 24h after a single oestrogen injection were significantly elevated from the control value only by 17β-oestradiol ($p<0.01$). Oestrone caused a small non-significant rise (see Table 3.2). Plasma calcium continued to rise following the two subsequent injections in birds treated with 17β-oestradiol and oestrone, reaching 10.0±1.9mmole/l($p<0.01$) and 6.7±0.9mmole/l($p<0.01$) respectively compared to control values of 2.8±0.1mmole/l. Plasma calcium levels in quail treated with 17α-oestradiol and oestriol, remained low. Showing small non-significant increases compared to the control value to, 3.2±0.2 and 3.1±0.1 mmole/l respectively on day 4. Ionised (free) calcium levels had not been measured in these plasma samples as an anaerobic plasma sample of at least 1ml is required; however, a brief supplementary study was performed checking the effect of oestrogen on ionised calcium levels (see Chapter 3.5).

Acid labile plasma inorganic phosphorus levels were measured on the day 4 samples only (Table 3.2). The largest stimulation was seen with 17β-oestradiol injections

Table 3.1

Effect of Intramuscular Injections (1.8μmole/kg Body Weight) of Four Different Oestrogens, 17α-Oestradiol (17α-E_2), 17β-Oestradiol (17β-E_2), Oestrone (E_1) and Oestriol (E_3), on Ovary and Oviducts Weights

Group	Body wt. (g)	Ovary wt. (mg)	Oviduct wt. (mg)
1. Control	100.7 ± 2.4	35.3 ±3.4	17.3 ± 1.2
2. 17α-E_2	100.3 ± 2.9	35.8 ±3.0	64.9 ±10.9**
3. 17β-E_2	94.7 ± 3.0	35.5 ±2.5	178.6 ±40.2**
4. E_1	97.4 ± 1.8	39.7 ±2.7	135.8 ±10.3***
5. E_3	99.7 ± 3.2	37.0 ±3.2	42.5 ± 9.2*

Results are expressed as Mean ± S.E.M
6 or 7 birds/treatment.

Significant stimulations from control values are shown

*$p<0.05$ **$p<0.01$, *** $p<0.001$

Table 3.2

Effect of Four Different Oestrogens on Total Plasma Calcium and Phosphorus Levels and on the Activities of the Renal 1α and 24R-Hydroxylases

Oestrogens were injected daily for 3 days, blood was collected from the wing vein prior to injections on Days 1, 2 and 3. Trunk blood was collected on Day 4 and Kidneys removed for assay of hydroxylase activities. Results are expressed as Mean ± S.E.M. with n = 6 in each group unless otherwise stated (x). Significant changes from control values are shown *$p<0.05$ and **$p<0.01$.

Group	Total Plasma Calcium mmole/l				Day 4		
	DAY 1	DAY 2	DAY 3	DAY 4	Plasma Phosphorus mmole/l	1-hydroxylase pmole/h/g	24-hydroxylase pmole/h/g
1. Control	2.85 ± 0.05 (5)	2.90 ± 0.08	2.83 ± 0.13	2.80 ± 0.13	0.58 ± 0.03 (5)	12.3 ± 2.2	145.1 ± 33.0
2. 17α-E_2	2.75 ± 0.20	2.98 ± 0.15	3.23 ± 0.10	3.23 ± 0.15	0.77 ± 0.05	23.5 ± 10.3	93.6 ± 15.3
3. 17β-E_2	2.90 ± 0.15 (5)	3.78 ± 0.18	6.35 ± 1.05	10.08 ± 1.93	1.58 ± 0.22	36.9 ± 7.7*	246.2 ± 90.3
4. E_1	2.90 ± 0.18	3.35 ± 0.25	4.35 ± 0.45 (7)	6.73 ± 0.90 (7)	1.13 ± 0.17	25.7 ± 4.4* (7)	162.0 ± 24.5 (7)
5. E_3	2.75 ± 0.08 (5)	3.00 ± 0.05	3.18 ± 0.25	3.10 ± 0.10	0.63 ± 0.09	24.9 ± 8.4	144.1 ± 29.2

Table 3.2

1.6±0.2mmole/l($p<0.01$) against the control groups 0.6±0.03mmole/l. A significant increase was also seen in the oestrone and 17α-oestradiol groups with levels of 1.1±0.2 ($p<0.05$) and 0.8±0.1mmole/l($p<0.05$) respectively. Oestriol had no effect on plasma phosphorus levels.

Significant increases in the activity of the renal 1-hydroxylase were seen after injection of 17β-oestradiol and oestrone, from 12.3±2.2pmole/h/g in the controls to 36.9±7.7pmole/h/g ($p<0.02$) and 25.7±4.4pmole/h/g ($p<0.05$) respectively (Table 3.2). Injections of 17α-oestradiol and oestriol caused non-significant increases in 1-hydroxylase activity. None of the four oestrogens had any significant effect on the 24-hydroxylase.

Plasma 17β-oestradiol levels as measured by specific RIA, were found to be elevated significantly only in the birds injected with 17β-oestradiol on the last day, $10.5\pm1.4\times10^{-13}$mole/ml ($p<0.05$) against a control value of $6.0\pm0.5\times10^{-13}$mole/ml (Table 3.3). The only oestrone level to significantly change was in the 17α-oestradiol group on the last day 1.5±0.2pmole/ml ($p<0.05$) against a control value of 3.3±0.7pmole/ml.

3.4 Discussion

All four oestrogens showed the ability to induce an increase in oviduct weights, when injected into photosuppressed quail. This particular specific oestrogenic response has been demonstrated in birds since 1940 (Witschi and Fugo). In this study 17β-oestradiol and oestrone were the most effective oestrogens at increasing oviduct weight and in fact in all the other major variables studied.

The most exciting results from this experiment show that an oestrogen other than 17β-oestradiol, namely oestrone, can

Table 3.3

Plasma 17β-Oestradiol and Oestrone Levels in Sexually Immature Female Quail Before (Day 1) and After (Day 2-4) i.m. Injections of Four Different Oestrogens

Sex steroid levels were measured by specific RIAs, results are expressed as Mean ± S.E.M., n = 5 in each group unless otherwise stated (x). Significant changes from control values are shown † $p<0.05$.

Group	17β-Oestradiol x 10^{-13}mole/ml Plasma				Oestrone x 10^{-12}mole/ml Plasma			
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 1	DAY 2	DAY 3	DAY 4
1. Control	7.7 ± 0.8 (3)	5.4 ± 0.4 (4)	5.8 ± 0.7 (3)	6.0 ± 0.5 (6)	3.3 ± 0.5 (4)	2.9 ± 0.5	3.7 ± 0.3 (3)	3.3 ± 0.7 (6)
2. 17α-E_2	6.4 ± 2.6 (4)	5.3 ± 0.9	4.7 ± 0.7	5.6 ± 1.9 (6)	4.3 ± 1.6 (4)	3.5 ± 0.6 (6)	4.6 ± 1.1	1.5 ± 0.2†
3. 17β-E_2	5.2 ± 0.5 (4)	7.9 ± 1.0	9.2 ± 2.1	10.5 ± 1.4† (6)	2.9 ± 0.8	2.7 ± 1.0	4.5 ± 0.9 (4)	3.6 ± 0.3
4. E_1	7.8 ± 1.1 (4)	7.5 ± 1.2 (4)	4.9 ± 1.3 (4)	5.4 ± 0.7 (7)	4.9 ± 1.8 (4)	2.6 ± 0.8 (4)	3.8 ± 1.0	2.4 ± 0.7 (7)
5. E_3	4.3 (2)	6.0 ± 1.3	8.0 (2)	5.3 ± 0.9 (6)	5.5 (2)	2.7 ± 0.2	3.5 ± 0.8 (3)	2.5 ± 0.5 (6)

Table 3.3

effect a significant stimulation of the 1-hydroxylase. In addition 17α-oestradiol and oestriol caused a doubling of the 1-hydroxylase activity; however this was not statistically significant. Throughout this experiment it is worth noting that 17β-oestradiol consistently produced a larger response than oestrone. One major problem that is encountered when considering the possibility that oestrone may have a new action, such as that observed on the 1-hydroxylase, is the enzyme 17β-hydroxysteroid-dehydrogenase (17β-OHSDHase). This enzyme is abundant, being found in many tissues and it is capable of converting oestrone to 17β-oestradiol and **vice versa**. There are therefore three basic possibilities regarding the oestrogenic stimulation of the 1-hydroxylase:

a). only 17β-oestradiol is active and oestrone has to be converted to 17β-oestradiol before it has any effect,

b). only oestrone is active and that conversely 17β-oestradiol has to be converted to oestrone, and

c). both 17β-oestradiol and oestrone are physiologically active.

There is some support for the later possibility as Ruh, Katzenellenbogen, Katzenellenbogen and Gorski (1973) have shown that oestrone in its own right is a biologically active oestrogen in the rat, although it is somewhat less potent than 17β-oestradiol.

Plasma levels of 17β-oestradiol and oestrone were measured to see if the relatively large injections of oestrogen caused a physiological increase in the plasma levels of these hormones. It is surprising therefore, that no large increases in plasma oestrogen levels were detected. Most workers use oestrogen esters, that are slowly hydrolysed to yield the free steroid. In the present study the free steroids were used, since only these are detected by the RIA. However, the plasma oestrogen results suggest that the free steroids used were

either rapidly excreted and/or further metabolised, for example to their respective conjugates, which are not detected in the assay.

17β-oestradiol and oestrone both caused an elevation in total plasma calcium and acid labile phosphate levels and the 17α-oestradiol group also had significantly elevated phosphate levels. It has been known for some time that both endogenous and exogenous oestrogen can stimulate the avian liver to produce egg yolk phospholipoproteins (Griminger, 1976). These proteins bind calcium and phosphate with high affinity, so increasing total plasma calcium and phosphate levels. This oestrogenic action is quite separate from any action of oestrogen on the 1-hydroxylase, which may, through elevated 1,25-DHCC levels, stimulate calcium transport and possibly ionised plasma calcium levels. It is interesting that 17α-oestradiol apparently stimulates phosphate levels but not plasma calcium levels; this could be explained if these two parameters were independent, and may therefore be individually stimulated.

Finally these results support the work of Baksi and Kenny (1977) and Sedrani (1979) who both found that oestradiol benzoate given in vivo caused a stimulation of the 1-hydroxylase as measured in vitro, without any requirement for testosterone, which had been suggested by Tanaka et al (1976) to be necessary. This was also the first time that photo-suppressed Japanese quail had been used in a study of vitamin D metabolism, their barely detectable endogenous sex steroid levels making them ideal for the such studies.

3.5 Ionised Calcium Levels during Oestrogen induced Hypercalcaemia

In the last experiment the oestrogenic hypercalcaemia induced by 17β-oestradiol and oestrone was thought to be due to an increase in the protein bound fraction of calcium only, and not in the diffusible or ionic calcium (Urist et al, 1958). To see if this was the case in our birds, ionised calcium levels were measured using the relatively new and very sensitive calcium specific electrodes.

Short day males 50-70 days old were injected with 1.8μmole/kg B.W. of 17β-oestradiol in ethyl oleate or with carrier only once daily for 3 days, and just as in the previous experiment birds were killed on the fourth day. However, blood was collected by cardiac puncture, since anaerobic plasma is required for ionised calcium measurements. The blood was centrifuged in the capped syringes and ionised calcium determined by the method of Luck and Scanes (1979). These measurements were made by Dr M. Luck and Mr C. Joyner and total calcium was measured on the residual plasma as described in Materials and Methods (Chapter 2.7).

Results

	Plasma Calcium (mmole/l)		
	Ionised	Total	n
Control	1.02 ± 0.03	2.28 ± 0.05	6
17β-E_2	1.15 ± 0.06	4.48 ± 0.31***	7

All Mean ± S.E.M
*** $p < 0.001$ from Control

These results show that 17β-oestradiol caused a non-significant 12.7% increase in plasma ionised calcium levels and that total plasma calcium increased significantly by nearly 100%. The large increase in total plasma calcium was expected but the small rise in ionised calcium was not, and it may have been due, to the elevated bound calcium fraction being in close equilibrium with the ionised one, however the poor correlation (r=0.45) between individual total and ionised calcium levels does not substantiate this. Alternatively and more probably it may be due to an independent effect, through the stimulation of the 1-hydroxylase by 17β-oestradiol, leading to increased calcium absorption from the gut.

CHAPTER 4 TIME COURSE STUDY OF THE RESPONSE OF THE RENAL HYDROXYLASES TO A SINGLE INJECTION OF OESTRONE OR 17β-OESTRADIOL

4.1 Introduction

Following on from the previous experiment, it appeared likely that a short time course study of renal hydroxylase activity in oestrone and 17β-oestradiol treated birds might resolve some uncertainties. Firstly, the scale of the oestrogenic stimulation of the 1-hydroxylase seen in Chapter 3 was considerably less than that seen by other workers. Our use of 'free' steroids may have meant that we had missed the main response, as we measured hydroxylase activities 24h after the last injection. More importantly such a study might be expected to show a difference in response between oestrone and 17 -oestradiol with respect to the 1-hydroxylase; a delayed response, for example, might indicate that one steroid had to be further metabolised before it became active. It might also be possible to measure more dramatic changes in plasma sex steroid levels in a time course study.

In the following study only oestrone and 17β-oestradiol were tested as they appeared to be the two most active oestrogens with respect to the 1-hydroxylase.

4.2 Methods

Short day female Japanese quail that were 50-70 days old were injected i.m. with 1.8μmole/kg body weight of oestrone or 17β-oestradiol in ethyl oleate, in the second hour of their light period. Birds were killed by decapitation 3, 6, 12 or 24h after the injection; trunk blood was collected into heparinised tubes and the kidneys removed into ice cold assay buffer for the determination of renal hydroxylase activity. Blood was spun down and plasma collected and stored at -20°C

until assayed for 17β-oestradiol and oestrone levels.

4.3 Results

The activity of the renal 1-hydroxylase increased significantly in both oestrone and 17β-oestradiol injected groups 3h post injection, and continued to rise to a maximum at 6h post injection. The activity of the 1-hydroxylase had almost returned to control levels by 24h post injection.

In contrast, 24-hydroxylase activity decreased significantly in the 17β-oestradiol and oestrone groups by 3 and 6h respectively. Maximum inhibition of the 24-hydroxylase was seen at 12h post injection in both groups, but by 24h the activity had increased in both groups above the control values, but not significantly. All hydroxylase data are presented in Tables 4.1 and 4.2 and Figures 4.1 and 4.2.

Plasma oestrone and 17β-oestradiol levels also changed dramatically in this experiment (see Tables 4.3 and 4.4, and Figures 4.3 and 4.4.). In the oestrone injected birds, oestrone levels 3h post injection were significantly higher than control values, returning to pre-injection levels after 12h. In this group 17β-oestradiol levels also increased significantly at 3 and 6h post injection. In the 17β-oestradiol injected birds, 17β-oestradiol levels were significantly raised at 3, 6, and 12h with a maximum at 3h; by 24h control levels had been re-established. Plasma oestrone levels in these birds showed a significant peak at 3-6h post injection and had returned to control levels by 12h.

4.4 Discussion

These results go some way to explain some of the problems encountered in the last experiment. Firstly, much more potent and significant stimulations of the 1-hydroxylase occurred,

Results of a Time Course Study of Renal Hydroxylase Activities Following a Single Injection of Oestrogen

Table 4.1

Injecting 17β-Oestradiol

Time after Injection h	Renal Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxylase	
0	6.3 ± 1.9	159.4 ± 32.7	9
3	31.7 ± 7.5**	69.5 ± 9.4**	5
6	178.0 ± 34.3***	87.3 ± 24.8	5
12	129.6 ± 24.3***	35.1 ± 5.9***	5
24	45.9 ± 7.8**	246.3 ± 89.7	4

All values are Mean ± S.E.M

Significances from 0h Group * $p<0.05$
** $p<0.01$
*** $p<0.001$

Table 4.2

Injecting Oestrone

Time after Injection h	Renal Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxylase	
0	6.3 ± 1.9	159.4 ± 32.7	9
3	100.9 ± 21.0***	138.1 ± 66.1	5
6	172.9 ± 36.9***	53.8 ± 16.8*	5
12	62.8 ± 10.8**	28.5 ± 4.6**	4
24	80.9 ± 23.4**	214.8 ± 61.3	4

Figure 4.1

Renal Hydroxylase Activities After a Single Injection of 17β-Oestradiol

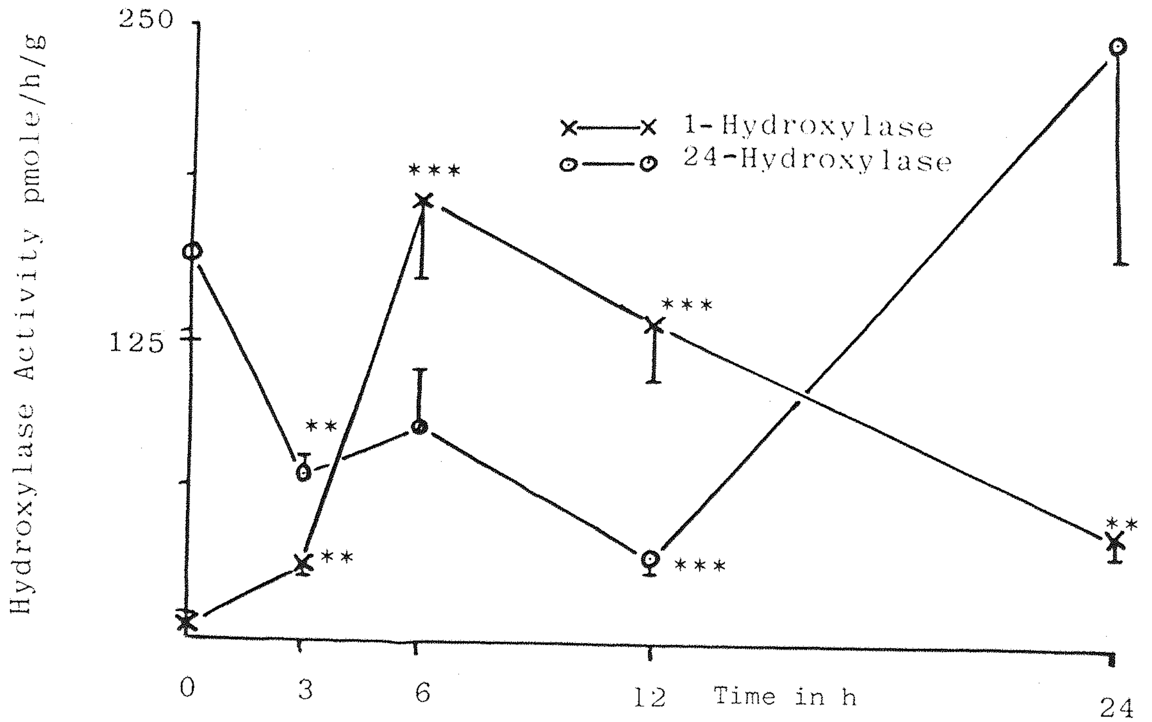


Figure 4.2

Renal Hydroxylase Activities After a Single Injection of Oestrone

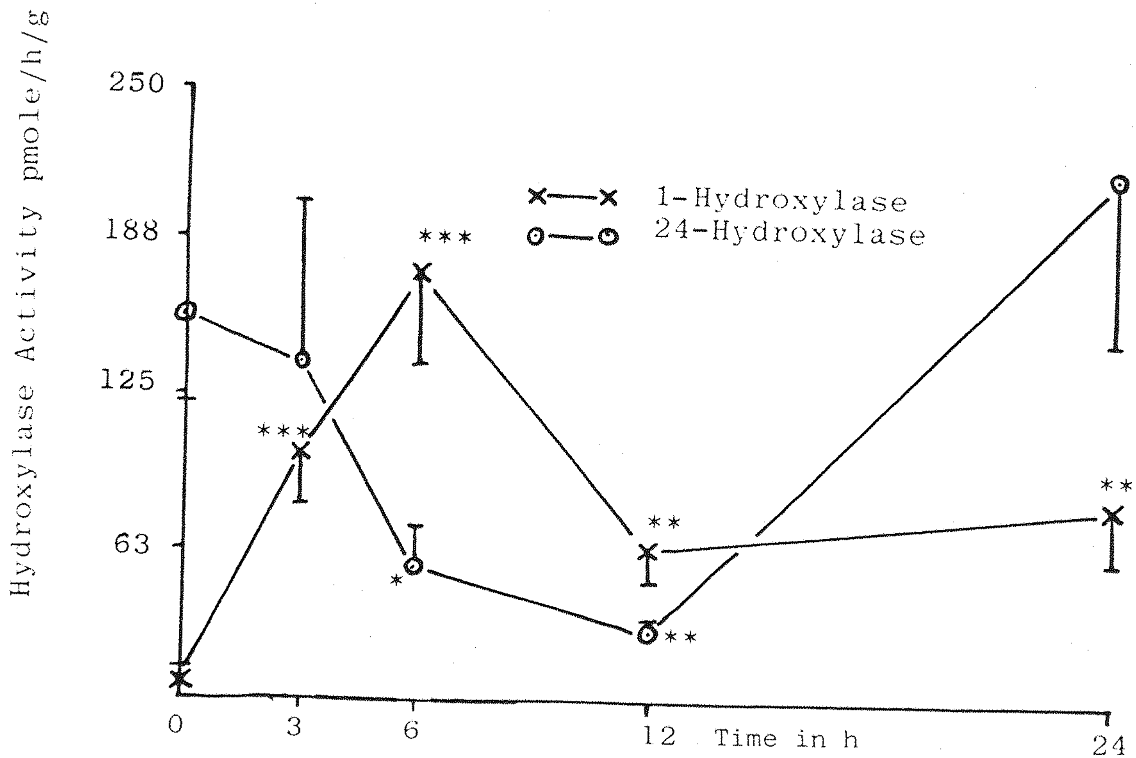


Table 4.3

Plasma 17β-Oestradiol (17β-E_2) and Oestrone (E_1) Levels Following a Single Injection of 17β-Oestradiol

Time after Injection h	17β-E_2 pmole/ml	E_1 pmole/ml	n
0	1.54 ± 0.29	3.98 ± 0.58	5
3	12.45 ± 1.86†††	12.54 ± 0.86†††	5
6	7.99 ± 2.44†	12.48 ± 2.70†	5
12	3.67 ± 0.26†††	3.34 ± 0.54	5
24	1.94 ± 0.29	3.01 ± 0.49	4

All values are Mean ± S.E.M

† $p < 0.05$
†† $p < 0.01$
††† $p < 0.001$

Table 4.4

Plasma 17β-Oestradiol (17β-E_2) and Oestrone (E_1) Levels Following a Single Injection of Oestrone

Time after Injection h	17β-E_2 pmole/ml	E_1 pmole/ml	n
0	1.54 ± 0.29	3.98 ± 0.58	5
3	4.06 ± 0.73†	19.86 ± 3.08††	4
6	3.54 ± 0.43††	16.13 ± 1.63††	5
12	1.30 ± 0.46	4.75 ± 1.87	4
24	1.79 ± 0.23	4.14 ± 0.45	4

Figure 4.3

Plasma 17β-Oestradiol and Oestrone Levels After a Single Injection of 17β-Oestradiol

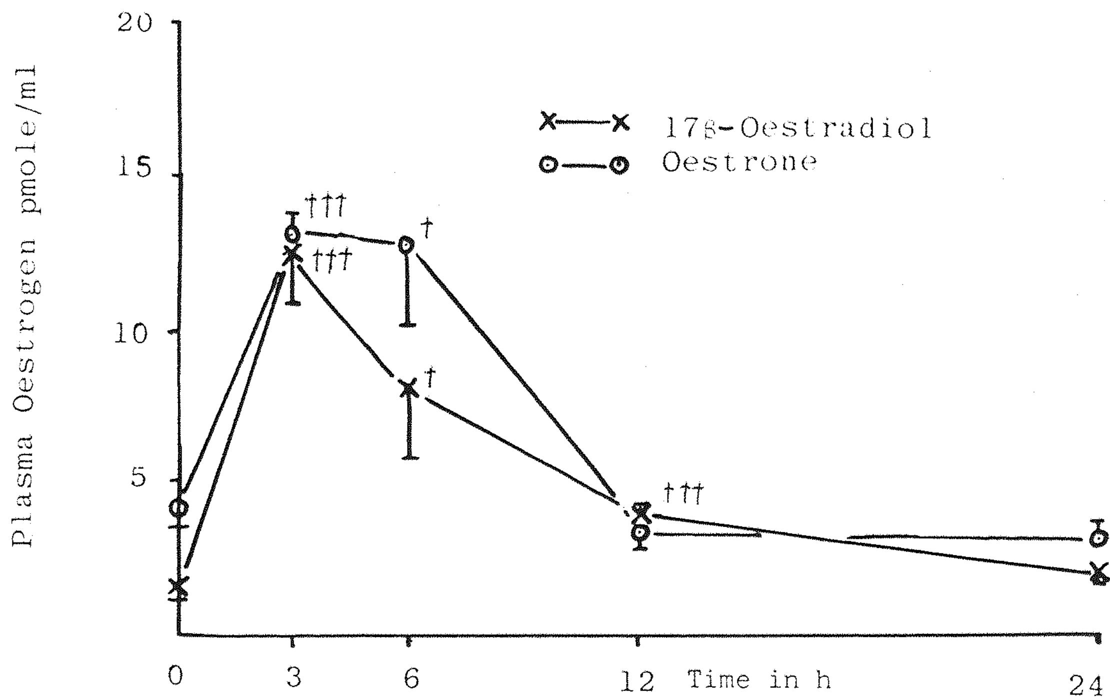
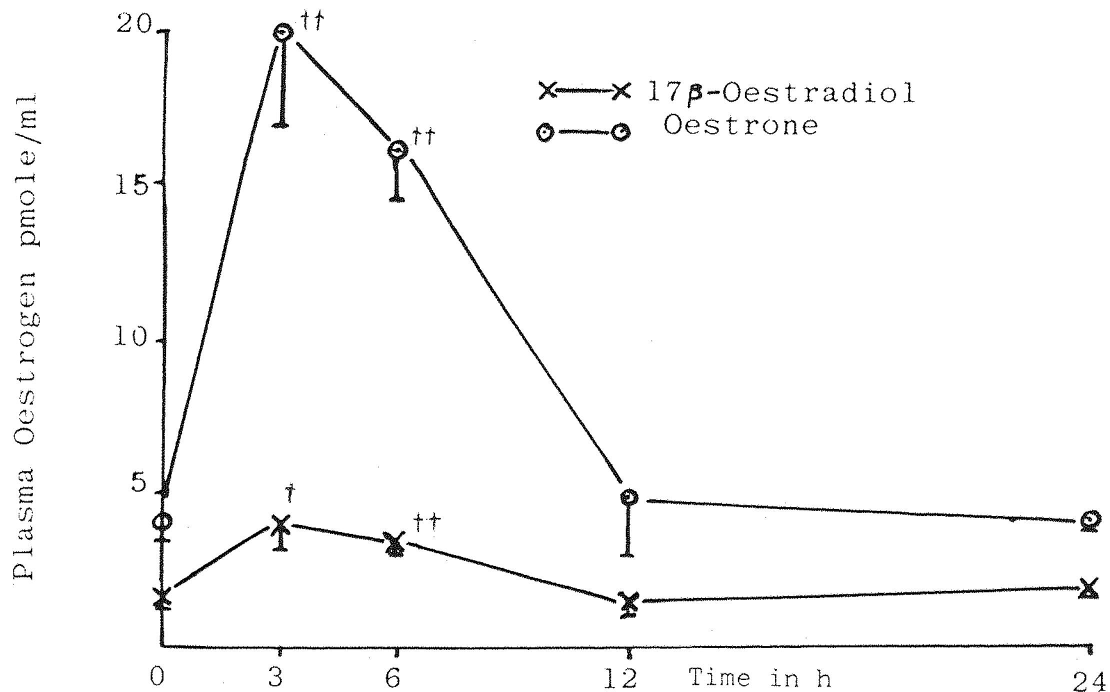


Figure 4.4

Plasma 17β-Oestradiol and Oestrone Levels After a Single Injection of Oestrone



which were comparable to the responses observed by other workers, thus confirming our fears that in the last experiment we had missed the main oestrogenic stimulation of the 1-hydroxylase by waiting 24h after the last oestrogen injection.

The plasma concentrations of 17β-oestradiol and oestrone showed that our injections had succeeded in raising plasma oestrogen levels, and that there was considerable interconversion between the two steroids studied. It is also apparent that 12h after the injections oestrone and 17β-oestradiol levels had almost always returned to pre-injection control levels, suggesting that the free oestrogens are rapidly metabolised (probably to glucuronides or sulphates) and/or excreted. Unfortunately our data did not show any real significant 'lag' in response between 17β-oestradiol and oestrone and their stimulation of the 1-hydroxylase, and so the question posed at the end of the previous experiment, as to which oestrogen was active remains unanswered (it is still quite possible that both are active).

However, some clue is probably given by the plasma oestrone and 17β-oestradiol levels, because the antisera used in the RIA to measure oestrone and 17β-oestradiol cross react with compounds structurally similar to those they are meant to measure. The cross reactivity at the 50% displacement level of the 17β-oestradiol antisera to oestrone is 25% and that of the oestrone antisera to 17β-oestradiol is 22% (Brain, 1982). This means that if a sample containing 17β-oestradiol only was measured for oestrone, the assay would detect oestrone at about one quarter the level of that of 17β-oestradiol even though no oestrone were present. It can now be seen that the small but significant increase in 17β-oestradiol levels in the oestrone injected birds at 3 and 6h could be due to the cross reaction of oestrone with the 17β-oestradiol antisera. However, the increase in oestrone levels in the 17β-oestradiol

injected birds was large enough to suggest that the cross reactivity of 17β-oestradiol with the oestrone antisera could not produce the total response observed. Therefore, in summary, the evidence suggests that there is little or no <u>in vivo</u> conversion of oestrone to 17β-oestradiol but considerable <u>in vivo</u> conversion of 17β-oestradiol to oestrone. If this were extrapolated further - which is dangerous with the limited evidence available - it would imply that oestrone itself is capable of stimulating the 1-hydroxylase. The possibility also exists that 17β-oestradiol has to be converted to oestrone before it can actively stimulate the 1-hydroxylase.

One possible weakness in the design of this experiment was that the large stimulation of the 1-hydroxylase could possibly have been due to the use of an incorrect control group. For example there may be some diurnal variation in 1-hydroxylase activity centred around the onset of darkness in our 'short day' birds (i.e. 6h post injection), or alternatively the ethyl oleate vehicle may affect the 1-hydroxylase. The control group in this experiment were killed in their 2nd hour of light and received no carrier injection; therefore another control group was added that received the normal dose of carrier and were killed 6h post injection to check on diurnal variation (see Table 4.5). The results show conclusively that neither diurnal variation or ethyl oleate account for any of the oestrogenic stimulation of the 1-hydroxylase.

As well as the large stimulation of the 1-hydroxylase the 24-hydroxylase was inhibited strongly in both groups, with the maximum inhibition following the maximum stimulation of the 1-hydroxylase. Presumably while oestrogen is stimulating the 1-hydroxylase a similar mechanism must be inhibiting the 24-hydroxylase: the lag in this response is probably due to the 24-hydroxylase enzyme and its mRNA already present in the tissue. A crude approximation of the enzyme half life and the half-life of its mRNA can be obtained from Figures 4.1 and 4.2 and is approximately 4½h. Tanaka <u>et al</u> (1975) followed the suppress-

Table 4.5

Additonal Control Groups to See if Either Ethyl Oleate or Diurnal Variation Can Effect the 1-Hydroxylase

Group	Renal Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxylase	
Previous Control	6.3 ± 1.9	159.4 ±32.7	9
Killed at 3pm (No injection)	9.5 ± 6.4	210.7 ±47.6	3
Killed at 3pm (6h post Ethyle Oleate)	3.1 ± 1.7	233.8 ±83.9	3

All values Mean ± S.E.M.

Table 4.6

Renal Hydroxylase Activites 2h After an Injection of 17β-Oestradiol into 50 day-old SD Female Quail

Group	Renal Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxylase	
Injected with Ethyl Oleate	6.6 ± 4.0	149.2 ±26.0	4
1.8μmole/kg 17β-E_2 in Ethyl Oleate	10.0 ± 14.1	114.6 ±45.8	4

ion of the 24-hydroxylase activity after parathyroid extract had been given to chicks and they found that the $t_{1/2}$ for the enzyme was about 1h and that of the mRNA to be about 5h. Therefore our crude $t_{1/2}$ of 4½h may reflect that the mRNA for the 24-hydroxylase in our birds was still being translated and it would therefore follow that oestrogen inhibits the 24-hydroxylase at the level of mRNA transcription. The later stimulation of the 24-hydroxylase 12-24h post injection, was almost certainly due to the elevated levels of 1,25-DHCC following the stimulation of the 1-hydroxylase (1,25-DHCC is one of the most potent stimulators of 24-hydroxylase both in vivo and in vitro). This may be an important control point for regulating 1,25-DHCC levels in vivo, as the 24-hydroxylase will convert 1,25-DHCC to the less active 1,24,25-THCC so modulating the activity of any remaining 1,25-DHCC. The higher levels of 1,25-DHCC that probably exist in the kidney will have a strong inhibitory effect on the 1-hydroxylase. This may account for most of the decrease in 1-hydroxylase activity after 6h, although falling plasma oestrogen levels must also have played a role.

In this experiment the rapidity of the oestrogenic stimulation of the 1-hydroxylase was exceptional; the transient nature of the stimulation also highlights just how responsive this enzyme is in vivo. Much previous data showed that oestrogen esters caused long term, large but 'sluggish' stimulations of the 1-hydroxylase (Baksi and Kenny, 1978; Tanaka et al, 1978). This appears to be an artefact of their use of esters: our use of free steroids, while possibly giving unphysiologically high levels initially, exposes the sensitivity of this enzyme. The rapid and short lived stimulation of the 1-hydroxylase by oestrogen in this experiment is unlikely to have had much effect on calcium metabolism; however it might account for the small rise in ionised calcium levels seen in Chapter 3.5.

The stimulation of the 1-hydroxylase after only 3h is the

earliest that such a response has been observed: in a later experiment birds were killed just 2h after an oestrogen injection and no stimulation of the 1-hydroxylase was seen (see Table 4.6). Therefore the actual oestrogenic stimulation of the 1-hydroxylase requires somewhere between 2-3h. This prompts the intriguing question of just how does oestrogen stimulate the 1-hydroxylase, as such a rapid response makes de novo synthesis of the 1-hydroxylase unlikely, particularly as the cytochrome P_{450} unit, which contains the active site of the 1-hydroxylase is a large protein that even when synthesised has to be cleaved before it is inserted into the renal mitochondria (Dubois, Simpson, Tuckey, Lambeth and Watermann, 1981). In addition, it has been shown that the de novo synthesis of the 24-hydroxylase, an enzyme very similar to the 1-hydroxylase, takes 4-6h before the slightest increase in activity is observed. Two major possibilities remain, if de novo synthesis does not occur:

a. that oestrogen acts on the kidney either directly or through some cellular messenger to activate enzyme already present in the kidney cells, or

b. that oestrogen stimulates the production and/or release of another hormonal factor such as prolactin or parathyroid hormone, both polypeptide hormones that have a known rapid direct stimulatory action on the 1-hydroxylase.

The latter suggestion will be further explored and discussed in Chapter 8.

CHAPTER 5 FURTHER STUDIES WITH REPRODUCTIVE STEROIDS

5.1 Effect of Endogenous Oestrogen on the Renal Hydroxylases in transferred Male Japanese Quail

5.1.1 Introduction & Procedure

When male Japanese quail are transferred from the short non-photostimulatory photoperiod (8L:16D) to the long photo-stimulatory photoperiod (16L:8D) a small but significant increase in their plasma 17β-oestradiol levels can be detected (Brain, Peddie and Taylor, 1980). It was considered that such birds would make an ideal model for investigating whether or not this elevated endogenous, and therefore physiological oestrogen level, stimulates the 1-hydroxylase in the same way as the rather large exogenous doses normally administered.

Male Japanese quail were kept for 70d after hatching on the short photoperiods and killed at varying times after transferring to the long photoperiod. The activity of the renal hydroxylases and the plasma 17β-oestradiol levels were measured. In addition groups of 70d old short day males were injected with 17β-oestradiol (1.8μmole/kg B.W.) using the same experimental design as in Chapter 4. This separate experiment was to see if the renal hydroxylases of these birds were capable of responding to oestrogen, as this was the first time that short day male quail had been used in a vitamin D related study.

5.1.2 Results

Plasma 17β-oestradiol levels showed a significant but transient increase 2d after photostimulation. Renal 1-hydroxylase activity increased almost two-fold after 2d, but was only significantly stimulated after 4d of photostimulation.

There were no significant changes in 24-hydroxylase activity, although levels certainly fluctuated (Table 5.1)

The birds injected with 17β-oestradiol responded in a manner very similar to the females in the previous experiment. A very large stimulation of the 1-hydroxylase was seen 6h post injection which was still just significant after 12h and control values were restored after 24h. No significant change in 24-hydroxylase activity was seen, when compared to the control value, although at 6h and 12h the activity was considerably less than control (Table 5.2).

5.1.3 Discussion

These results show that the 1-hydroxylase of male Japanese quail does indeed undergo a small but significant increase in activity, following their transfer from a non-photostimulatory to a photostimulatory lighting regime. The major increase does however occur after the main rise in 17β-oestradiol levels and hence it cannot be conclusively said that endogenous 17β -oestradiol is responsible for the increase in 1-hydroxylase activity observed at day 4. The time course study does, however, show that male quail do have a renal 1-hydroxylase responsive to exogenous 17β-oestradiol; the possibility therefore remains that endogenous 17β-oestradiol is causing the observed stimulation of the 1-hydroxylase.

Alternatively some other factor or factors that are fluctuating during photostimulation may affect the 1-hydroxylase. Urbanskie and Follett (1982) have shown that both LH and FSH increase 10-20 fold in castrated male Japanese quail that undergo the same photoperiod transfer. It remains to be seen if these gonadotrophins have either direct or indirect actions on the avian 1-hydroxylase. It has been shown that 1,25-DHCC can inhibit LHRH-stimulated LH release from an **in vitro** pituitary cell incubation system (Luck and Scanes, 1980), so

Table 5.1

Renal Hydroxylase Activities and Plasma 17β-Oestradiol Levels in Transferred Male Quail

Days of Photostimulation	Hydroxylase Activity pmole/h/g		Plasma 17β-Oestradiol pmole/ml	n
	1-hydroxylase	24-hydroxylase		
0	12.9 ± 6.0	69.2 ±33.9	1.2 ± 0.3	5
2	21.1 ± 4.6	200.9 ±61.0	4.7 ± 1.1 ††	5
4	67.7 ± 6.2**	109.4 ±32.1	1.2 ± 0.3	6
9	27.7 ±10.5	50.5 ±18.5	2.7 ± 0.8	5
15	Samples Lost		1.1 ± 0.6	6
22	5.8 ± 1.7	38.5 ± 7.7	0.6 ± 0.1	6

** †† = p<0.01
* = p<0.05

All values Mean ± S.E.M

Table 5.2

Responsiveness of the Renal Hydroxylases of Short Day Male Quail Following an Injection of 17β-Oestradiol

Time after Injection h	Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxylase	
0	13.1 ± 6.2	137.2 ±38.4	5
3	70.5 ±33.5	119.9 ±36.7	5
6	412.7 ±63.6**	56.5 ±16.3	5
12	63.9 ± 3.9*	48.8 ± 9.8	4
24	32.7 ± 5.8	172.0 ±25.5	6

that the possibility that some metabolic control exists between gonadotrophins and 1,25-DHCC **in vivo** is a real one.

5.2 Action of a Single Dose of 17α-oestradiol, Oestriol, Progesterone or Testosterone on the Renal Hydroxylases.

5.2.1 Introduction and Procedure

In Chapter 3 it was shown that only 17β-oestradiol and oestrone cause a significant stimulation of the 1-hydroxylase, while oestriol and 17α-oestradiol cause a two-fold stimulation of 1-hydroxylase activity. In view of the later findings, that the main oestrogenic stimulation of the 1-hydroxylase occurs 3-12h post injection (Chapter 4), it was decided to see if 17α -oestradiol or oestriol could significantly stimulate the 1-hydroxylase 6h post injection. Two other reproductive steroids, testosterone and progesterone were also studied.

All hormones were injected as the free steroids, and for ease only they were dissolved in ethanol, and injected i.m. into 50-70d old short day female quail at our standard equimolar dose of 1.8μmole/kg B.W.

5.2.2 Results

The results are shown in Table 5.3, no significant changes were seen in 1-hydroxylase activity, primarily due to the high degree of variation within the groups and small numbers in each group. All four groups had significantly lower 24-hydroxylase activity than the control.

<u>Table 5.3</u>

Renal Hydroxylase Activities from S.D Female Quail 6h after a Single Hormone Injection

Treatment	Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxlyase	
Control (Ethanol only)	16.1 ± 5.9	327.4 ±17.6	4
17α-Oestradiol	84.2 ±54.3	82.3 ± 7.7*	4
Oestriol	46.4 ±17.6	164.1 ±68.4*	4
Progesterone	3.6 ± 2.6	79.2 ±23.8*	4
Testosterone	10.6 ± 6.8	165.4 ±27.6*	5

All values Mean ± S.E.M

* = $p<0.05$

5.2.3 Discussion

17α-oestradiol caused a large stimulation of the 1-hydroxylase and although this stimulation was not statistically significant it could quite possibly have been due to the conversion of 17α-oestradiol to either 17β-oestradiol or oestrone; alternatively it may have acted directly. Oestriol on the other hand cannot be converted to 17β-oestradiol or oestrone and its slight stimulation of the 1-hydroxylase may therefore have been a direct one. This experiment makes it seem quite likely that the whole group of structurally related oestrogens are capable of stimulating the renal 1-hydroxylase to varying degrees.

Testosterone and progesterone had little effect on the 1-hydroxylase in these birds. Other workers have also found progesterone either to have no effect, or a very slight stimulatory action on the 1-hydroxylase (Baksi and Kenny, 1977b; Pike, Spanos, Colston, MacIntyre and Haussler, 1978), while testosterone has been shown to be primarily inhibitory (Sedrani, 1979). It is quite possible that testosterone and even progesterone inhibit the 1-hydroxylase in our short day birds; however these birds already have minimal 1-hydroxylase levels and therefore such an inhibition could not be clearly demonstrated. Short day birds are an ideal model to use when looking for a stimulation of the 1-hydroxylase, but of little use when studying its possible inhibition.

The 24-hydroxylase activity decreased significantly in all groups, possibly due to the exceptionally high activity in the control group, which was more than double that of previous control values, so that little weight can be attached to these decreases.

5.3 Studies on the *in vivo* interconversion of Oestrone and 17β-oestradiol in Short Day Female Quail

5.3.1 Introduction

In Chapter 4 it was apparent from the plasma oestrogen levels that there was some conversion of the 17β-oestradiol to oestrone, but only poor evidence in support of the converse. It is of relevance to these investigations to study the interconversions of oestrogens taking place in short day sexually immature quail, as it may influence our understanding of the oestrogenic stimulation of the 1-hydroxylase in these birds. We therefore injected tritiated oestrone and 17β-oestradiol into quail and followed the metabolism of these steroids in a time dependent manner. Unfortunately, initial experiments showed that the labelled oestrogens (injected together with cold oestrogen) were very rapidly metabolised, resulting in insufficient counts to be detected in the plasma even 1h post injection. Hawkins and Taylor (1967) showed that the initial half life of tritiated oestradiol in the plasma of laying hens was only one minute, so that for this study to succeed large quantities of radioactivity and a short *in vivo* incubation time would have to be used.

5.3.2 Methods

50-70d old short day female quail were injected i.m. with a known amount of either ^{3}[H]oestrone or ^{3}[H]-oestradiol; approximately 10μCi in ethanol. The ethanol also contained cold oestrogen at a concentration that gave 1.8μmole/kg B.W. The injected ^{3}[H]-oestrogen was purified by TLC immediately before use and the purity was always found to be greater than 96%.

Birds were killed 30 minutes post injection and the trunk blood was immediately centrifuged and the plasma collected.

Known volumes of plasma were then denatured with 3 volumes of ethanol and ^{14}[C] tracer oestrogen was added; (approximately 5,000dpm per sample) in order to monitor work-up losses. The denatured plasma proteins were then spun down and the supernatant extracted twice with re-distilled diethyl ether, using distilled water to effect phase separation. The upper ether layers were pooled, evaporated under reduced pressure, dried with anhydrous sodium sulphate and taken up again in ether prior to application to TLC plates. The samples were co-eluted with cold oestrone and 17β-oestradiol on 400μm thick GF_{254} TLC plates, using benzene: ethyl acetate (3:1v/v) as the solvent system. Good separations of oestrone and 17β-oestradiol were obtained with respective r.f. values of 0.65 and 0.40; for a separation profile see Figure 5.1. Bands of silica 7.5mm wide were scraped into vials and counted with PBD scintillant using a dual label program. 70μl of glacial acetic acid were added to the vials to stop chemiluminescence.

5.3.3 Results

The results are shown in Table 5.4 and are expressed, in the oestradiol injected birds as the amount of oestrone produced, as a percentage of the sum of the oestrone and 17β-oestradiol peaks. Similarly in the oestrone injected birds, results are expressed as the amount of 17β-oestradiol produced as a percentage of the sum of the oestrone and 17β-oestradiol peaks. The results show that in just 30 minutes there was considerable conversion not only of 17β-oestradiol to oestrone but also of oestrone to 17β-oestradiol. There was no significant difference in the rate of oestrogen metabolism between the two groups. Considerable radioactivity was regularly seen near the origin of the plates, indicating that more polar oestrogen metabolites had also been produced. The mean half life of the two injected oestrogens in the plasma is also given in Table 5.4.

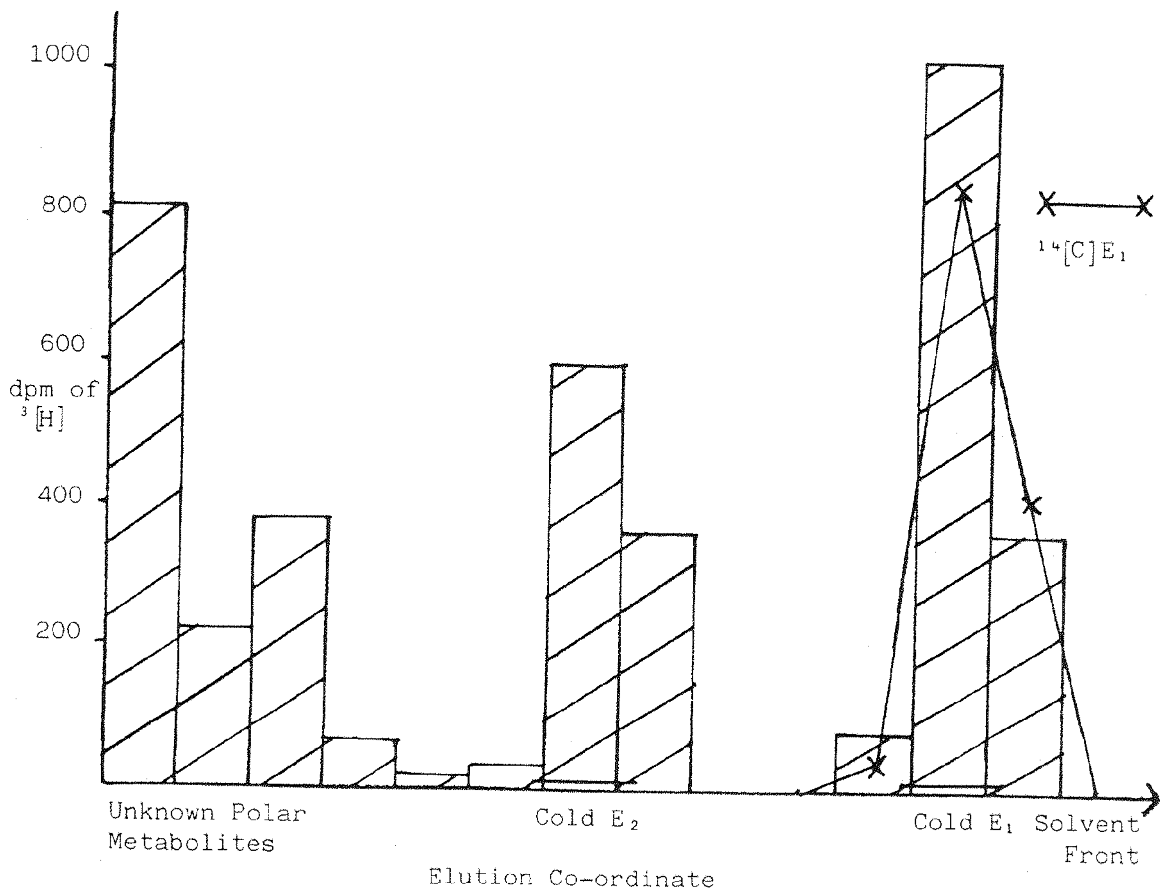
Table 5.4

Results of *In Vivo* Oestrogen Interconversion Studies Using ^{3}[H]-17β E_2 and ^{3}[H] E_1

	Oestrogen Injected		
	17β-Oestradiol	Oestrone	
	$\frac{\%\ E_1\ dpm}{E_1 + E_2\ dpm}$	$\frac{\%\ E_2\ dpm}{E_1 + E_2\ dpm}$	
	19.5	26.7	
	20.2	28.0	
	23.7	38.8	
	21.0	35.8	
	34.8	23.4	
Mean ± S.E.M	23.8% ± 2.8%	30.5% ± 2.9%	n = 5
$t_{1/2}$ for Oestrogen injected	3min 25sec	2min 55sec	n = 3

Figure 5.1

Elution Profile for a Short Day Female Quail Injected with ^{3}[H]-E_1



5.3.4 Discussion

From Figure 5.1 it can be seen that when $^{3}[H]$-oestrone was injected only $^{14}[C]$-oestrone tracer was added. Both $^{14}[C]$-oestrone and $^{14}[C]$-oestradiol gave recoveries of greater than 95% from ether, and therefore to prevent cross-over of counts from the $^{14}[C]$ to $^{3}[H]$ channel producing a 'ghost metabolite' peak, only $^{14}[C]$ tracer of the same oestrogen as the $^{3}[H]$ oestrogen under test was used. It was possible to calculate approximate half lives for the injected oestrone and 17β-oestradiol in the plasma, by using the $^{14}[C]$dpm to correct for losses by assuming that each 100g of bird weight contained 4.3ml of plasma (Sturkie, 1976), and by knowing how much tritium was initially injected. It should be noted that these half life values are calculated over a 30 minute time period, and metabolism and tissue uptake of the injected oestrogens may not have been linear over this time. The half lives of 17β-oestradiol and oestrone are short and quite similar, thus indicating that both steroids are rapidly removed from the plasma through tissue uptake, excretion and/or further metabolism. Polar metabolites such as steroid glucuronides and sulphates were not measured in this experiment.

It had been hoped that this experiment might show that the interconversion between oestrone and 17β-oestradiol was unidirectional, and so shed some light on the problem of whether for example oestrone has to be converted to 17β-oestradiol to become active with respect to the 1-hydroxylase. These data suggest that the equilibrium of the enzyme 17β-hydroxysteroid dehydrogenase is not vastly shifted to favour the production of either oestrone or 17β-oestradiol, as considerable interconversion is seen. The only clear way to elucidate whether oestrone, 17β-oestradiol or both hormones can stimulate the 1-hydroxylase may be through the use of a specific 17β-hydroxysteroid dehydrogenase inhibitor. Such inhibitors are being developed (Tobias, Covey and Strickler, 1982), but as yet they have only limited success in vitro and have not yet been tested in vivo.

CHAPTER 6 DOES THE STIMULATION OF THE 1-HYDROXYLASE BY 17β-OESTRADIOL REQUIRE *de novo* PROTEIN SYNTHESIS?

6.1 Introduction

Since the stimulation of the 1-hydroxylase by oestrogens occurs within 3h, a relatively rapid stimulation, it must be questioned whether *de novo* protein synthesis is involved in this response. One way to approach this problem is by using protein synthesis inhibitors, such as cycloheximide. Cycloheximide disrupts polypeptide elongation at the ribosomes and hence effectively inhibits protein synthesis; however this type of inhibition is slowly reversible.

6.2 Methods

50-70d old female Japanese quail that were raised on the short photoperiod were used. They were given cycloheximide intra-peritoneally (i.p.) at a dose of 250μg/100g B.W. in 0.1ml of 0.9% saline. Thirty minutes after this injection 17β-oestradiol was given i.m. 1.8μmole/kg B.W. in ethyl oleate: 4½h after the oestrogen injection the birds were killed and the renal hydroxylase activities measured. Another group received saline plus 17β-oestradiol while the control group received cycloheximide and ethyl oleate. It was also necessary to see if our dose of cycloheximide was actually inhibiting protein synthesis. This was done by measuring ^{3}[H]-leucine uptake by the kidney of short day birds 1h after an injection of cycloheximide at the above dosage, using the method of Bolton, Munday and Parsons (1977).

6.3 Results

The results are shown in Table 6.1, and clearly show that cycloheximide, a protein synthesis inhibitor can prevent the oestrogenic stimulation of the 1-hydroxylase. In addition the uptake of the ^{3}[H]-leucine in the two birds studied, shows that protein synthesis had only been inhibited by about 40%. Therefore although some inhibition of protein synthesis had occurred, it was far from complete. The dose of cycloheximide used would normally give a greater than 90% inhibition of protein synthesis in the rat (Bolton *et al*, 1977).

6.4 Discussion

The results imply that oestrogens are responsible for either the rapid *de novo* synthesis of the 1-hydroxylase, or of a protein ultimately involved in the functioning of the 1-hydroxylase, e.g. a mitochondrial membrane transport protein, or possibly a protein that can directly stimulate the 1-hydroxylase such as PTH or prolactin. Our understanding of the enzymic regulation of the 1-hydroxylase is very poor while that of cytochrome P_{450} enzymes in general is not much better. Wiseman, Lim and Woods (1978) studied the regulation of cytochrome P_{450} production by yeast protoplasts. They were primarily concerned with the regulation of cytochrome P_{450} by cyclic nucleotides, but they also showed that *de novo* synthesis of their specific cytochrome P_{450} enzyme occurred 2-4h after the initial stimulation, with a peak response around 6h. Their data therefore shows that the stimulation of our renal cytochrome P_{450} 1-hydroxylase by oestrogens, could indeed be due to *de novo* synthesis of enzyme when considering the constraint of time. The regulation of cytochrome P_{450} enzymes is obviously complex: very recently Goodwin, Cooper and Margolis (1982) have shown conclusively that the activity of the cytochrome P_{450} enzyme cholesterol-7α-hydroxylase is

Table 6.1

Effect of Cycloheximide on the Oestrogenic Stimulation of the 1-Hydroxylase

Treatment	Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxylase	
Cycloheximide and Ethyl Oleate (Control)	14.4 ± 8.5	118.7 ± 23.4	4
Cycloheximide and 17β-E_2	11.2 ± 5.4	153.8 ± 26.8	4
Saline and 17β-E_2	58.2±13.8*	40.7 ±12.4*	4

All values Mean ± S.E.M.

* = $p<0.05$ from Control

^{3}[H]-Leucine uptake into 2 birds given cycloheximide was found to be inhibited by 29 and 51% of that of control birds. Hence cycloheximide did not totally abolish protein synthesis.

modulated by its phosphorylation state. This enzyme is of microsomal origin and it remains to be seen if a mitochondrial cytochrome P_{450} enzyme such as the 1-hydroxylase can be similarly regulated. The intriguing problem still remains - do oestrogens stimulate **de novo** synthesis of the 1-hydroxylase directly, and if not through what factor or factors do they act?

In this experiment cycloheximide also prevented the oestrogenic decrease in 24-hydroxylase activity, as well as inhibiting the stimulation of the 1-hydroxylase. Presumably the usual decrease in 24-hydroxylase activity following oestrogen administration is due to oestrogen or a protein whose activity is directed by oestrogen, preventing **de novo** 24-hydroxylase synthesis. More specifically, this is probably achieved by inhibiting transcription of new mRNA rather than inhibiting mRNA translation, as the half life of the mRNA (5h) as opposed to that of the 24-hydroxylase (1h) follows the observed decay of the 24-hydroxylase activity more closely (see Chapter 4).

One anomaly is that the 24-hydroxylase levels are still elevated in the control and the cycloheximide plus 17β-oestradiol groups, as cycloheximide would be expected to inhibit **de novo** protein synthesis across the board. As there is considerable 24-hydroxylase activity in these groups (remembering the enzyme has a half life of only 1h), one can only assume that the 24-hydroxylase is one of the enzymes still produced, as a result of the incomplete inhibition of protein synthesis by cycloheximide in these birds.

CHAPTER 7 - STUDIES ON VITAMIN D METABOLISM FOLLOWING INJECTIONS OF MORE THAN ONE REPRODUCTIVE STEROID.

7.1 Introduction

Maturing female quail, in addition to showing a rise in 17β-oestradiol concentrations in their plasma prior to lay, exhibit an even earlier elevation in progesterone levels (Brain, 1982). For this reason it was decided to examine the possible synergistic action of injections of both oestrogens (17β-oestradiol and oestrone) and progesterone on renal hydroxylase activities. Furthermore, since testosterone can also be detected in the plasma of maturing female quail, although levels fluctuate very little, testosterone was included in the group of reproductive steroids to be tested. In addition, as mentioned in the introduction (Chapter 1.2.4d), some workers have found that testosterone has a positive synergistic effect with oestrogen on the 1-hydroxylase (Tanaka et al, 1978) while others have found the reverse to be true (Sedrani et al, 1981; Baksi and Kenny, 1978a). It was hoped that this study would help resolve this controversy. Therefore all possible combinations of the following reproductive steroids were tested; progesterone, testosterone, oestrone and 17β-oestradiol, which, it was hoped, would mimic in vivo conditions more precisely.

7.2 Methods

50-70d old SD female Japanese quail were injected with combinations of the above steroids, which were injected i.m. into breast muscle at a dose of 1.8μmole/kg B.W. in ethyl oleate carrier. All birds were killed 6h post injection and renal tissue was removed for determination of hydroxylase activities. As several batches of quail were needed for this

experiment, birds within a test group were from more than one batch so as to minimise any possible effect of the different batches.

7.3 Results

The results are shown in Table 7.1 and are arranged in order of increasing 1-hydroxylase activity. Statistical analysis was performed by analysis of variance and significances ascribed between groups following Duncan's Multiple Range Test. In an attempt to simplify the statistical data Table 7.2 was constructed, from which it is possible to compare any statistical differences existing between groups. The significant stimulation of the 1-hydroxylase by various combinations of reproductive steroids can be clearly seen in Table 7.2 and are too numerous to be individually mentioned.

The 24-hydroxylase activity in all groups was significantly lower than control. In addition all groups except the control had significantly lower 24-hydroxylase activity than the group injected with testosterone plus oestrone.

7.4 Discussion

The largest stimulation of the 1-hydroxylase was seen in the oestrone plus progesterone group. This stimulation was 70% greater than with oestrogens alone (E_2+E_1 group $p < 0.05$), thus implying that progesterone had a positive synergistic effect. (It is important to note that previous results in Chapter 5.2 have shown that progesterone had no stimulatory action of its own on the 1-hydroxylase after 6h). Similarly the second largest stimulation of the 1-hydroxylase was seen in the oestrone + 17β-oestradiol + progesterone group, which was greater (but not significantly) than that of the oestrone + 17β-oestradiol group. This also supports the possibility that

Table 7.1

Renal Hydroxylase Activities Following Various Sex Steroid Treatments

Treatment	Hydroxylase Activity pmole/h/g	
	1-Hydroxylase	24-Hydroxylase
Control	7.8 ± 3.8	202.0 ± 50.4
T+P	15.2 ± 9.9	69.5 ± 13.9
$P+T+E_1+E_2$	48.2 ± 14.0	66.1 ± 9.0
$T+E_1+P$	87.0 ± 23.4	42.7 ± 9.6
E_1+T	96.1 ± 23.2	132.2 ± 25.5
E_2+T	102.8 ± 37.0	64.7 ± 14.3
$T+E_2+P$	141.1 ± 62.7	43.0 ± 11.2
E_2+P	162.8 ± 36.7	28.2 ± 3.0
E_2+E_1	168.6 ± 47.0	40.2 ± 6.4
$T+E_1+E_2$	186.3 ± 36.3	48.8 ± 15.5
E_1+E_2+P	268.5 ± 34.6	22.4 ± 9.3
E_1+P	285.4 ± 45.4	34.8 ± 20.2

T = Testosterone

P = Progesterone

E_1 = Oestrone

E_2 = 17β-Oestradiol

All values are Mean ± S.E.M (n=5 in each group)

Table 7.2

A Summary of the Statistical Differences in Hydroxylase Activities Between all Groups Studied

The top right hand section of Table 7.2 deals with significances between 1-hydroxylase activities between the various groups. The lower left section similarly deals with significant differences in 24-hydroxylase activities.

Groups are arranged in the top row in order of ascending 1-hydroxylase activity.

To use the table, for example, to see if the 1-hydroxylase activity of the E_1E_2 group is greater than the PTE_1E_2 group, locate E_1E_2 on the top row and look down the column to the PTE_1E_2 row, which shows * significant difference between these two groups.

	C	PT	PTE_1E_2	TE_1P	TE_1	TE_2	TE_2P	E_2P	E_1E_2	TE_1E_2	E_1E_2P	E_1P
C	–						*	**	**	**	**	**
P	**	–					*	**	**	**	**	**
PTE_1E_2	**		–					*	*	*	**	**
TE_1P	**			–							**	**
TE_1	*	*	*	**	–						**	**
TE_2	**				*	–					**	**
TE_2P	**				**		–				**	**
E_2P	**				**			–			*	*
E_1E_2	**				**				–			*
TE_1E_2	**				*					–		*
E_1E_2P	**				**						–	
E_1P	**				**							–

← 24-Hydroxylase Significances → (lower left of diagonal)

← 1-Hydroxylase Significances → (upper right of diagonal)

* $p<0.05$
** $p<0.01$
All empty boxes = No Significant Difference

Table 7.2

progesterone acts synergistically with oestrogens, or perhaps more accurately as the evidence suggests, with oestrone, with respect to the stimulation of the 1-hydroxylase. 17β-oestradiol + progesterone only produced an oestrogenic stimulation of the 1-hydroxylase of average magnitude.

Testosterone seemed to play a role in these combination treatments. With the exception of the oestrone + 17β-oestradiol + testosterone group, testosterone caused significant reductions in the stimulation of the 1-hydroxylase by oestrogens and oestrogens + progesterone. The most impressive inhibitory effect is seen when comparing the oestrone + progesterone group (285pmole/h/g) with the oestrone + progesterone + testosterone group (87pmole/h/g). It therefore appears that the predominant actions of testosterone on the oestrogenic stimulation of the 1-hydroxylase are inhibitory. Testosterone plus progesterone had no stimulatory effect on the 1-hydroxylase, but an inhibitory effect cannot be ruled out in these short day birds, as they already have minimal 1-hydroxylase levels.

All 24-hydroxylase activities were significantly lower in the test groups when compared to the control and this is to be expected whenever the 1-hydroxylase is stimulated by oestrogen. It was therefore surprising to see a reduction of the 24-hydroxylase activity in the progesterone + testosterone group which experienced no real stimulation in 1-hydroxylase activity. It is possible that progesterone and/or testosterone have specific inhibitory actions on the 24-hydroxylase, quite independent of the oestrogenic mechanism of inhibition that is linked to the stimulation of the 1-hydroxylase.

The respective positive and negative synergistic properties of progesterone and testosterone on the stimulation of the 1-hydroxylase by oestrogen, may help modulate renal hydroxylase activity <u>in vivo</u>; certainly these reproductive steroids are found in measurable quantities in both male and

female Japanese quail (Brain, 1982). Therefore, the controversy regarding the testosterone requirement for the stimulation of the 1-hydroxylase by oestrogen, could possibly be accounted for by the levels of other reproductive steroids in the different birds used by the various workers.

CHAPTER 8 - IS THE STIMULATION OF 1-HYDROXYLASE ACTIVITY BY OESTROGENS MEDIATED BY PROLACTIN?

8.1 Introduction

It has been mentioned in previous chapters that the mechanism by which oestrogen stimulates renal 1-hydroxylase activity is not known. Renal tissue culture studies and other *in vitro* techniques suggest that the oestrogen stimulation is indirect and we must, therefore, address ourselves to the problem of through what factor or factors does oestrogen work. Two of the most likely factors are prolactin and PTH, as both of these polypeptide hormones are known to act rapidly and directly in avian species to stimulate the 1-hydroxylase.

It is not known whether oestrogen causes an increase in plasma PTH levels in avian species, due to the extreme difficulty of measuring this hormone in birds. However, it has been shown that an injection of 1mg oestradiol into non-laying turkey hens produces a three-fold increase in plasma prolactin levels 30minutes-2h post injection (McNeilly, Etches and Friesen, 1978). Laying turkey hens showed no such elevation in plasma prolactin levels following the same treatment, but their plasma prolactin levels were already ten-fold greater than in the non-layers. It is also well documented in mammalian species, that oestrogen can stimulate both prolactin synthesis and release *in vivo*, (Lu, Koch and Meites, 1971) and *in vitro* (Nicoll and Meites, 1962).

In mammals prolactin is secreted by the anterior pituitary and its release is controlled by an inhibitory factor thought to be dopamine, derived from the hypothalamus (Labrie, Ferland, Deniseau and Beulieu, 1980). In avian species, control of prolactin secretion is not so clear. It appears to be mainly under stimulatory control by an unknown factor from the hypothalamus; however, inhibitory dopamine control in

addition has not been ruled out (Harvey, Chadwick, Border, Scanes and Phillips, 1982).

In this experiment bromo-ergocryptine, a potent long lasting dopamine agonist, was given prior to oestrogen treatment, to see if it could interfere with the usual stimulation of the 1-hydroxylase by oestrogen.

8.2 Methods

50-70d old SD female and male Japanese quail were used (4 of each sex per group). They were injected i.p. with 500μg of 2-bromo-α-ergocryptine methane sulfonate/100g B.W., in 100μl of dimethylformimide:water (1:9v/v) as carrier, or with carrier only. Thirty minutes later 1.8μmole/kg B.W. of 17β-oestradiol was injected i.m. in ethyl oleate into these birds, while others received ethyl oleate only. 5½h after the oestrogen injection the birds were killed and renal tissue removed for renal hydroxylase activity determination.

8.3 Results

The results are shown in Table 8.1. Statistical analysis was performed by analysis of variance, and significances between groups measured by Duncan's Multiple Range test. The only group with a significantly elevated 1-hydroxylase activity was the group that received oestrogen only ($p<0.01$ from all other groups). Thus, bromo-ergocryptine has prevented the oestrogenic stimulation of the 1-hydroxylase, while having no significant effects when injected alone. 24-hydroxylase activity was significantly reduced in both the bromo-ergocryptine plus 17β-oestradiol group and, to a greater extent, in the 17β-oestradiol only group.

Table 8.1

Renal Hydroxylase Activities Following Bromo-Ergocryptine and/or 17β-Oestradiol Treatments

Group	Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxylase	
Carrier + Carrier	8.4 ± 3.5^{a}	260.4 ± 32.4^{a}	8
Ergot + Carrier	11.8 ± 3.4^{a}	294.3 ± 57.6^{a}	8
Ergot + 17β-E_2	53.5 ± 10.6^{a}	175.1 ± 53.2^{b}	8
Carrier + 17β-E_2	210.5 ± 46.8^{c}	51.7 ± 11.5^{c}	8

All values are Mean ± S.E.M

Significant Differences exist between the following subscripts:

a → b, b → c $p<0.05$

a → c $p<0.01$

Ergot = Bromo-Ergocryptine

8.4 Discussion

A major criticism of this experiment is that prolactin secretion in avian species appears to be under the control of the hypothalamic stimulatory releasing factor (SRF), so that our use of an inhibitory dopamine agonist must be justified.

The identity of the avian SRF is unknown, but it seems likely that several factors such as thyroid stimulating hormone, serotonin, histamine and some unidentified polypeptides are responsible for SRF activity (Harvey et al, 1982); this uncertainty makes any study involving prolactin release extremely difficult. There is also considerable evidence that a prolactin inhibitory factor may co-exist with SRF (Tixier-Vidal and Gourdji, 1972); indeed Harvey et al (1982), have shown that dopamine and its precursor L-Dopa have strong inhibitory actions on prolactin release in chickens. Therefore, although it is thought that avian prolactin release is predominantly under stimulatory control, a possible inhibitory control involving dopamine has not been excluded.

It is apparent in this experiment that bromo-ergocryptine has significantly reduced the oestrogen response on both renal hydroxylases, even though it has not completely abolished it. The results therefore imply quite strongly that in our SD birds the stimulation of the 1-hydroxylase by oestrogen is closely linked to a dopamine sensitive process. In mammals it has been repeatedly shown that bromo-ergocryptine can block oestrogen induced prolactin release (D'Agata and Scapagnini, 1979). Bromo-ergocryptine is a D_2 dopamine agonist (Kebabian and Calne, 1979) that may possibly have effects systemically. However its best known action is in inhibiting prolactin release and it is even used clinically to this end.

Evidence supporting the idea that oestrogen acts through prolactin to stimulate the 1-hydroxylase includes the observation of elevated prolactin levels in avian species following

oestrogen treatment. Other work shows that prolactin can directly stimulate the avian renal 1-hydroxylase, and this present study implicates a dopamine sensitive mechanism for the oestrogenic stimulation. It has also been shown that testosterone can directly inhibit prolactin release from quail pituitary incubations (Tixier-Vidal and Gourdji, 1972), a fact that might explain our observed inhibition of the oestrogen induced stimulation of the 1-hydroxylase when testosterone is also given (see Chapter 7).

There is one major problem when considering the previous evidence namely, the fact that elevated prolactin levels in the chicken and probably other avian species initiate and maintain broodiness (Bedrak, Harvey and Chadwick, 1981). Turkeys are apparently an exception, as no elevation in prolactin levels are seen in broody turkey hens (Harvey, Bedrak and Chadwick, 1981). Since no eggs are laid by broody hens, their calcium requirements must be greatly reduced, but, paradoxically, with elevated prolactin levels one would expect the 1-hydroxylase to be stimulated.

It is possible that the 1-hydroxylase is somehow desensitised to prolactin during broodiness; alternatively, other hormonal effects such as the decreased plasma 17β-oestradiol, progesterone and LH levels associated with broodiness may be involved (Bedrak et al, 1981). A more attractive and simple possibility is that elevated prolactin levels initially stimulate the renal 1-hydroxylase which then results in elevated ionised calcium levels. If the calcium level continues to increase (since no calcium is laid down in medullary bone or on the egg shell) it will directly inhibit the 1-hydroxylase in a way that prolactin cannot overcome. Therefore the stimulation of the 1-hydroxylase by prolactin is likely to be of physiological importance only during calcium stress, i.e. when low ionised calcium levels, associated with shell formation or with the calcification of medullary bone, occur.

Another possibility is that, since the food intake of broody hens decreases dramatically (Savory, 1979), it may be beneficial to them to maintain elevated 1-hydroxylase activity, to ensure maximum utilisation of dietary calcium. Renal hydroxylase activities do not appear to have been measured in broody birds, which would help to clarify this point.

8.5 A further Experiment Examining the Role of Dopamine in Avian Prolactin Release

8.5.1 Introduction and Procedure

If dopamine plays any **in vivo** inhibitory role in avian prolactin secretion, it should be possible to remove this inhibition by using a specific dopamine antagonist. In an attempt to investigate this possibility renal hydroxylase activities were measured after the administration of a dopamine antagonist. An elevation in 1-hydroxylase activity would then imply that dopamine had some inhibitory control over prolactin secretion **in vivo**.

50-70d old SD male and female quail were injected i.p. with 500μg/100g B.W. of sulpiride ($\pm$enantiomers) - a specific dopamine antagonist - dissolved in a dilute NaH_2PO_4 solution. 6h after the injection birds were killed and renal tissue removed for hydroxylase activity determination. In a similar experiment 60d old LD female quail, all with an egg in the oviduct, were used. These birds received the identical dose of sulpiride and experimental work up as the SD birds.

8.5.2 Results

The results are shown in Tables 8.2 and 8.3. The SD males and females showed no significant change in 1- or 24-hydroxy-

Table 8.2

Renal Hydroxylase Activities in SD Quail Following a Single Injection of the Dopamine Antagonist Sulpiride

Group	Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxylase	
Control Females only	6.6 ± 4.0	149.2 ± 26.0	4
Females + Sulpiride	14.1 ± 3.7	85.3 ± 14.2	4
Males + Sulpiride	27.7 ± 11.1	130.9 ± 54.0	4

All values are Mean ± S.E.M

No Significant Differences from Control

Table 8.3

Renal Hydroxylase Activities in LD Laying Female Quail Following a Single Injection of the Dopamine Antagonist Sulpiride

Group	Hydroxylase Activity pmole/h/g		n
	1-Hydroxylase	24-Hydroxylase	
Control Na H_2PO_4 only	270.5 ± 48.1	87.2 ± 11.5	4
Sulpiride injected	206.9 ± 28.8	70.2 ± 32.1	4

lase activity although slight increases in 1-hydroxylase activity were seen. The mature LD females similarly showed no significant change in hydroxylase activities from controls, although a slight reduction in 1-hydroxylase activity was apparent.

8.5.3 Discussion

In the experiment using SD quail it was thought that the low 1-hydroxylase activity may be stimulated if the possible inhibitory dopamine control on prolactin secretion was removed. No such stimulation was seen and hence the low plasma prolactin levels in immature and non-laying birds (McNeilly et al, 1978) can be assumed to be due to a lack of stimulatory control rather than being under inhibitory dopamine control.

The LD group, in contrast to the SD birds, should have considerably elevated plasma prolactin levels (McNeilly et al, 1978) and their regulation of prolactin release may balance on stimulatory-inhibitory control. It was hoped that the removal of the inhibitory control with sulpiride may cause an increase in prolactin secretion, which in turn could be detected by increased 1-hydroxylase activity. From the results it can be seen that no such stimulation was observed; in fact a slight inhibition was detected. In laying birds, therefore, prolactin secretion does not appear to be under any inhibitory dopaminergic control.

These combined SD and LD quail results do not necessarily conflict with those of Table 8.1, which simply suggest that the possible stimulation of prolactin release by oestrogen is dopamine sensitive, and says nothing of more normal **in vivo** control.

CHAPTER 9 - SUMMARY AND CONCLUSIONS

This thesis has been primarily concerned with the **in vivo** regulation of the renal hydroxylases by reproductive steroids, particularly oestrogen. Results presented in this thesis have increased our knowledge in this field and have also suggested possible productive areas for future research. Each experimental chapter in this thesis contains its own discussion and hence this chapter will only summarise some of the most salient points.

For the first time it has been shown that oestrone is as potent as 17β-oestradiol in stimulating plasma calcium and acid labile phosphate levels, in activating the renal 1-hydroxylase and in inhibiting the renal 24-hydroxylase. This may be of physiological importance as oestrone is the major circulating oestrogen in immature female quail prior to the onset of lay. A major problem in being 100% certain that oestrone is active with respect to the 1-hydroxylase, is the enzyme 17β-hydroxysteroid dehydrogenase, which catalyses the inter-conversion of oestrone and 17β-oestradiol. As stated earlier this makes it possible that oestrone or 17β-oestradiol is the only oestrogen capable of stimulating the 1-hydroxylase, although quite possibly all oestrogens have this ability. The latter possibility is supported in some measure by the weak stimulation of the 1-hydroxylase by 17α-oestradiol and oestriol, particularly as oestriol cannot be metabolised to either oestrone or 17β-oestradiol. It seems most likely then, that this group of structurally similar compounds (the oestrogens) can stimulate the 1-hydroxylase to varying degrees, with oestrone and 17β-oestradiol being the most potent. The matter may be resolved in the future with the use of 17β-hydroxysteroid dehydrogenase inhibitors, which at the present time are being developed. It is also possible that when we know more about the precise mechanism of the stimulation of the 1-hydroxylase by oestrogen, some direct

oestrogen specificity, if any, will be easier to observe.

The time course studies involving single oestrogen injections, showed good correlations between elevated plasma oestrogen levels and elevated 1-hydroxylase activity and decreased 24-hydroxylase activity. This is the first time that **in vivo** plasma oestrogen levels have been monitored in such a vitamin D metabolism study. These experiments also showed that the 1-hydroxylase responds very rapidly to the oestrogen stimulus. Much previous work using oestrogen esters assumed that a 24h lag period was necessary in order to detect any increase in 1-hydroxylase activity. Our data show that the initial stimulation of the 1-hydroxylase by oestrogen can occur just 2-3h post injection (this is the fastest time interval over which such stimulation has ever been observed), and that by 12h the response is almost over. Since the decrease in 24-hydroxylase activity was rather slower to develop than the increase in 1-hydroxylase activity, it seems likely that oestrogen acts by inhibiting mRNA synthesis for the 24-hydroxylase, rather than by inhibiting mRNA translation or by directly inhibiting the 24-hydroxylase. The impressive overshoot in 24-hydroxylase activity (presumably **de novo** synthesis) by 24h, was probably due both to the removal of the oestrogenic stimulation and to stimulation by the elevated levels of 1,25-DHCC in the kidney and plasma.

Possible doubts of the relevance of what are quite large **in vivo** (but still less than most workers have used) oestrogen doses, were partly dispelled when the levels of endogenous oestrogen secreted in 'transferred' male quail were shown to increase the renal 1-hydroxylase activity, implying that even small physiological increases in plasma oestrogen have some effect on the 1-hydroxylase.

The synergism studies using all combinations of testosterone, progesterone, oestrone and 17β-oestradiol, showed quite clearly that oestrogen and more specifically oestrone plus

progesterone produced the greatest stimulation of the 1-hydroxylase. As progesterone alone had no stimulatory action on the 1-hydroxylase, progesterone can be said to have a true synergistic action with oestrogen; just how this is achieved is unknown but it may be as simple as progesterone 'protecting' oestrone from further metabolism, thus increasing its relative potency. Testosterone generally had an inhibitory action when given together with oestrogen, but again we do not know the mechanism of this inhibition: perhaps it is by increasing oestrogen metabolism and excretion. Another possible mechanism for the action of testosterone and progesterone will be mentioned later. The testosterone plus oestrogen results also support the work of Baksi and Kenny, and Sedrani, as opposed to those of DeLuca's group, that suggested a testosterone requirement for the oestrogenic stimulation of the 1-hydroxylase.

The synergism study was also an attempt to imitate the **in vivo** reproductive steroid environment in females prior to egg lay, where hormones other than oestrogens will also be fluctuating. We know that plasma levels of progesterone and 17β-oestradiol increase several days before the onset of lay, whereas oestrone levels remain relatively constant but at a higher level than the initial 17β-oestradiol levels. Our experimental data suggest that oestrogen and progesterone may be important in increasing the 1-hydroxylase's activity prior to egg lay. It must also be recognised that before the onset of lay medullary bone is laid down, with a consequent increase in calcium demand, so that PTH levels are also likely to be elevated. One can imagine that the increase in 1-hydroxylase activity prior to lay is controlled, not by just one factor but by several interacting factors.

A major problem still to be answered is the mechanism whereby oestrogens stimulate the 1-hydroxylase. Some evidence is given in this thesis to suggest that **de novo** protein synthesis is required, but as pointed out earlier this does

not necessarily mean de novo synthesis of the 1-hydroxylase. It could be that a protein such as a mitochondrial membrane substrate transport protein has to be made, or a peptide hormone or some other unknown protein. The best way to prove whether de novo synthesis of the 1-hydroxylase is taking place, is to purify the enzyme, raise antibodies to it, and then to accurately measure the actual amount of 1-hydroxylase present under various physiological and experimental conditions by immunoprecipitation. This procedure has not yet been successfully applied to the 1-hydroxylase.

The idea that oestrogen could be acting indirectly through a peptide hormone is quite attractive. It has not been shown that oestrogen has a direct stimulatory action on the renal 1 -hydroxylase as studied in in vitro systems; therefore it seems quite likely that some other factor is stimulated by oestrogen to cause the response. A peptide hormone fits in well with the available evidence; its production could be inhibited by preventing de novo protein synthesis, peptide hormones can stimulate the 1-hydroxylase rapidly, and indeed two peptide hormones are already known that can stimulate the 1-hydroxylase directly (PTH and prolactin). PTH is something of a problem in avian species as it has yet to be directly measured. In addition it is impossible to inhibit PTH secretion except by often fatal surgical removal of the parathyroid glands. It is not known if oestrogen injections can cause in vivo release and de novo synthesis of PTH. Prolactin, however, can be measured by radioimmunoassay, although this assay has not yet been set up in our laboratory. It is possible though to inhibit prolactin secretion quite specifically by the use of an ergot alkaloid such as 2-bromo-ergocryptine (a dopamine agonist). In this thesis it has been shown that 2-bromo-ergocryptine can significantly reduce the oestrogenic stimulation of the 1-hydroxylase, thus providing strong evidence that oestrogen acts through a dopamine sensitive process, such as prolactin secretion. Unfortunately this result is not definitive, since prolactin

secretion in birds, unlike mammals, is not thought to be primarily under inhibitory dopaminergic control, a fact that was supported by our experiments with sulpiride.

Further studies are therefore necessary. These could include measuring prolactin levels in quail after a single oestrogen injection; in addition, surgical removal of the pituitary followed by oestrogen administration could indicate whether or not a pituitary hormone was involved in the stimulation of the 1-hydroxylase by oestrogen. Finally, studies with isolated anterior pituitaries could be used to investigate the possible release of prolactin by individual oestrogens. The observed actions of testosterone and progesterone could also be explored with this technique, in particular, to see if these hormones can modulate prolactin release. The question of how does oestrogen stimulate the 1-hydroxylase may thus be answered.

An equally exciting research area is the mechanism of regulation of the 1-hydroxylase in the renal cells. Little is known of the regulation of mitochondrial P_{450} enzymes; possible mechanisms include allosteric regulation, secondary messenger regulation through cAMP or phosphatidyl-inositol hydrolysis or de novo enzyme synthesis. Indeed the vast array of factors known to stimulate the 1-hydroxylase indicate that more than one type of regulation may well exist.

Hopefully some of these problems will be answered, when it is possible to isolate and maintain a responsive multicomponent membrane enzyme such as the 1-hydroxylase.

9.1 Conclusions

1. Oestrone and 17β-oestradiol both cause large increases in renal 1-hydroxylase activity and in total plasma calcium levels in Japanese quail. Oestriol and 17α-oestradiol produce a weak stimulation of the 1-hydroxylase and have no effect on total plasma calcium levels.

2. The renal 1-hydroxylase is a very responsive enzyme and it is stimulated by oestrogen just 2-3h post injection. Its activity also correlates well with **in vivo** plasma oestrogen levels.

3. The respective synergistic and inhibitory effects of progesterone and testosterone on the oestrogenic stimulation of the 1-hydroxylase may be of prime importance in modulating 1-hydroxylase activity **in vivo**.

4. The stimulation of 1-hydroxylase activity by oestrogen involves **de novo** protein synthesis, though this does not necessarily mean **de novo** synthesis of the 1-hydroxylase itself.

5. Much circumstantial and some experimental evidence suggests that prolactin mediates the oestrogenic stimulation of the 1-hydroxylase.

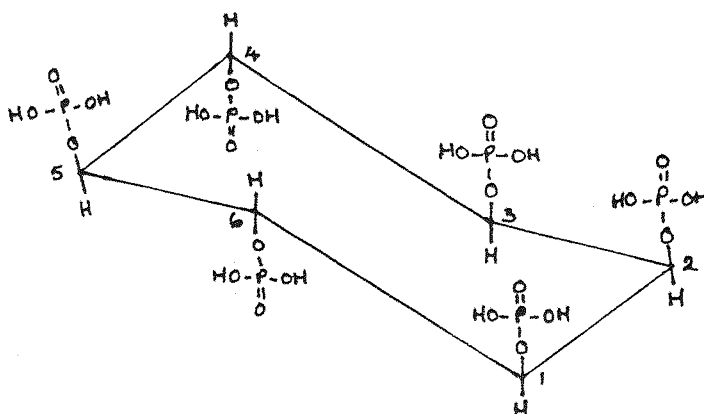
SECTION 2

An Investigation of the Phytate Hydrolysing Capacity of the Gastro-Intestinal Tract of the Rat and Hamster

CHAPTER 1 - REVIEW OF RELEVANT LITERATURE

1.1 Phytate

Phytate is more correctly called myo-inositol 1,2,3,4,5,6 -hexakis (dihydrogen phosphate), and has the following structure.



The six phosphate groups can lose hydrogen ions so that they can become negatively charged (maximum of minus 2); this process is pH dependent. The most striking aspect of this molecule is its number of phosphate groups; it is so rich in phosphorus that 100g of the molecule above contains over 28g of phosphorus.

Phytate is the highest member of a series of inositol phosphates that are widespread in nature. Phytate occurs extensively in plant seeds and grains, where indeed it is regarded as the primary storage form of both phosphate and inositol (Cosgrove, 1966); it is also found in roots and

tubers (McChance and Widdowson, 1935). Myo-inositol 1,3,4,5,6 -pentakis (dihydrogen phosphate) is found in the nucleated erythrocytes of birds and some turtles (Johnson and Tate, 1969). Still lower inositol phosphates, have been isolated from human and animal tissue (Burns and Conney, 1960). Immature plant seeds also contain lower inositol phosphate esters, but mature seeds contain almost exclusively phytate (Boland, Garner and O'Dell, 1975).

1.2 Effects of Phytate on Mineral Bioavailability

Although phytate has been known to occur in seed and grains since the early 1900s, more recent nutritional observations have renewed an interest in this molecule. The negatively charged phosphates in phytate can complex very strongly with divalent metal cations in particular. Therefore animal feeds rich in phytate may have considerable amounts of metal ions complexed to phytate, thus making these minerals extremely hard to absorb and essentially unavailable. If, however, the phytate in this complex is hydrolysed the valuable minerals will be released. Zinc has the greatest affinity for phytate and forms very stable complexes with it. This has been shown to be clinically important, as high phytate diets can complex with most of the trace levels of dietary zinc so causing acute zinc deficiency which is characterised by depression of growth and skin lesions (Davies and Olpin, 1979). Although calcium has a considerably lower affinity for phytate than zinc it is the major divalent cation in most diets, so considerable amounts of calcium phytate are often formed. Magnesium also complexes with phytate and often does so in conjunction with calcium, to form the mixed ion complex of calcium magnesium phytate, known as phytin. Phytate can also form complexes with copper, nickel, cobalt, manganese, iron and lead, and has been shown to be beneficial in protecting against acute lead toxicity (Wise, 1981).

Phytate is closely associated with calcium, phosphorus and vitamin D in nutrition. It is linked to calcium quite literally as up to six calcium ions can bind to each phytate molecule. This calcium phytate is quite insoluble at the near neutral pH found in the small intestine, but it exhibits a high degree of solubility in acid solution, and can therefore probably dissolve in the stomach (Wise, 1983). Mellanby (1925) was the first to note that cereal diets could be rachitogenic, although he was unable to identify this rachitic factor. McChance and Widdowson (1942) reported, that in humans, calcium absorption could be increased if phytate was removed from the diet. It seems most likely that phytate is rachitogenic by its action of strongly binding to calcium, so making it poorly available for absorption (Taylor, 1965), although other possibilities have been investigated.

Vitamin D is linked to phytate indirectly, as it enhances calcium and phosphate absorption from the gut and in so doing may effect phytate phosphorus availability and gut solubility, but more directly vitamin D stimulates the activity of the intestinal mucosal enzyme phytase which is capable of hydrolysing phytate (Steenbock, Krieger, Wiest and Pileggi 1953).

High dietary phytate can therefore cause a problem of mineral availability, but in livestock cereal diets this problem can be easily overcome by adding cheap calcium, and vitamin D supplements (and zinc if needed). The problem in commercial diets today centres more around how to increase the availability of the phosphorus in phytate. While phytate is very rich in phosphorus it is usually a very poor dietary source of this element. Phytate is not absorbed directly through the gut and has to be hydrolysed to inositol-2-phosphate and inorganic phosphates before any absorption can occur. If phytate could be hydrolysed to a greater extent, cheaper cereal diets would be possible as less of the costly phosphorus supplement would have to be added to the diets.

1.3 Phytases.

In vivo hydrolysis of phytate occurs through the action of the enzyme phytase. Phytase dephosphorylates only free inositol phosphates. Phytase has been isolated from several different sources; it is widespread in cereals and beans such as wheat, barley, rice and mung beans. It is also found in the small intestine mucosa of rats, chickens, calves and humans and finally in fungi, bacteria and even plant leaves (Reddy, Sathe and Salunkhe, 1982).

The importance of the animal phytase in the small intestine is uncertain. Certainly *in vitro* preparations of this enzyme can hydrolyse phytate. However, it has not conclusively been shown to be active *in vivo* as it remains in the mucosal cells and is not secreted into the intestinal lumen. It has even been suggested that phytase plays an important role in phosphoinositide metabolism in the mucosal cells themselves (Ramakrishan and Bhandari, 1977). It is interesting to note that the intestinal phytase can be induced by vitamin D in both rats and chickens (Steenbock, Krieger, Wiest and Pileggi, 1953). It is possible to speculate that this response is of importance in enhancing phytate hydrolysis in the intestine, although this has not clearly been shown to be the case. In fact vitamin D is known to stimulate *de novo* synthesis of various mucosal brush border membrane proteins, loosely defined as alkaline phosphatases (of which phytase is said to be an isoenzyme) and it appears that these may be directly responsible for active intestinal calcium transport. The optimum pH for intestinal phytase activity is pH7.4-8.0.

It is not surprising that as plant seeds are often so rich in phytate that phytase activity is also found in the seeds. The pH optimum for cereal phytase is more acidic, around pH5.0. It is also extremely heat insensitive when dry, but in moist germinating seeds where maximum activity is found, heat

in excess of 70°C can destroy its activity. It has been shown that cereal phytase is active in dough for bread making but is soon destroyed in the baking process proper (Ranhotra, 1972). Cereal phytase still has some activity at pH3 but is destroyed below pH2.5. It is therefore thought that some hydrolysis of phytase by cereal phytase does occur in cereal diets during the time from food ingestion to complete acidification of the food by the stomach (Hill and Tyler, 1954). A consideration worth noting is that high calcium diets reduce phytate phosphorus availability by complexing with it so preventing enzymic digestion. This is certainly true for the neutral intestines but in the acid stomach phytate will be much more soluble. The converse is also true: low calcium diets leave phytate more open to enzymic digestion.

The final important source of phytase is of microbial origin. Ruminants such as sheep and cattle are able to utilise most of their dietary phytate (Nelson, Daniels, Hall and Shields, 1976). They also showed that phytate hydrolysis occurred in the rumen and was complete before the feed reached other parts of the digestive system. This ability to utilise phytate phosphorus has been attributed to the ruminal microflora. The microflora found in rat and human faeces have also been shown to be capable of hydrolysing phytate. It certainly appears that the gut flora of the rat are important when considering phytate hydrolysis; as it has been shown that germ-free rats are unable to hydrolyse phytate appreciably whereas the conventional rats are (Wise and Gilbert, 1982). These findings are supported by those of Yoshida, Shinoda, Matsumoto and Watarai (1982), who showed that germ-free mice excreted more faecal phosphorus than conventional mice. Raum, Cheng and Burroughs (1956) demonstrated that ruminal bacteria could hydrolyse particulate calcium phytate, but that the proportion hydrolysed was inversely related to substrate concentration. It has been assumed that microbial hydrolysis of phytate in non-ruminants is negligible in the stomach and small intestines but that it occurs predominantly in the

caecum and colon, so that animals practising coprophagy probably derive benefit from the hydrolysed phytate. In the chicken matters are complicated, in that their gut microflora have been shown to synthesise phytate (Jenkins, 1965).

1.4 Phytate Phosphorus Availability

As previously mentioned it is of commercial interest to increase the availability of the phytate source of phosphorus in cereal diets. Phytate phosphorus availability has been studied in various forms of livestock and experimental animals under widely variable dietary conditions.

Moore and Tyler (1955) fed pigs cereal based diets with various calcium and phosphorus supplements. One of their findings was that phytate content in the pigs' stomachs decreased by about 50% 2h after a meal. As their diet contained considerable phytase activity, they concluded that it was cereal phytase that accounted for the observed hydrolysis of phytate. Pierce, Doige, Bell and Owen (1977) found that only limited phytate hydrolysis (less than 20%) occurred in pigs fed either wheat or maize based diets. This is surprising as wheat diets, as opposed to maize, should have high cereal phytase activities; their data thus suggests that cereal phytase has little effect on in vivo phytate hydrolysis.

Phytate phosphorus availability in the chicken has been extensively studied; early work showed that phytate was a poor source of phosphorus for poultry (Sunde and Bird, 1956). Nott (1967) fed hens maize-soya diets with 2g phytate/kg but variable calcium and total phosphorus contents. He showed that negligible (6%) hydrolysis of phytate occurred when dietary calcium was 35g/kg - a level that assures maximum egg shell thickness. When calcium was reduced to 15g/kg 40% of the phytate was hydrolysed. In another experiment Nott saw no

apparent phytate hydrolysis when broiler chicks were fed a diet with only 8.7g/kg of calcium, in strong contrast to the 40% observed in the layers on the 15g/kg calcium diet. It is likely that the layers being on a relatively low calcium diet absorbed all or most of their dietary calcium, so leaving phytate as the free acid and more open to hydrolysis. In addition layers quite possibly have greater intestinal phytase activity than the broiler chicks. Nott's work clearly shows, however, that when layers are given adequate calcium in their diet, their utilization of phytate as a phosphorus source is negligible.

Talmage (1976) also used broiler chicks and laying hens and found that phytate in corn diets was almost totally unavailable (0-8%), and that wheat diets rich in cereal phytase were only slightly more advantageous with 8-13% of the phytate being hydrolysed. This supports the idea that cereal phytases have minimal effects in vivo.

Pileggi, DeLuca and Steenbock (1955) showed that rats fed a diet very low in calcium could hydrolyse about 90% of their ingested phytate. However, when the calcium was increased to 10g/kg phytate hydrolysis decreased to about 50%, and a 30g calcium/kg diet caused a further reduction to just 30% of the phytate hydrolysed. They assumed that intestinal phytase was responsible for all phytate hydrolysis, although no consistent correlation between phytate hydrolysis and gut phytase activity could be seen.

In a more recent study Taylor and Coleman (1979) measured phytate availability and calcium absorption in rats and hamsters, with two dietary levels of calcium 5.2 and 10.8g/kg and two levels of phytate phosphorus 1.6 and 3.4g/kg. They found that hamsters were far more efficient at absorbing calcium and hydrolysing phytate than the rat, and that phytate hydrolysis was greater in both the rat and hamster when the low calcium diets were fed. It is tempting to assume that the

hamsters greater ability to hydrolyse phytate is due to their greater ability to absorb calcium, so making phytate more vulnerable to enzymic attack. Rowland, Mallet and Wise (in press) believe that it may be due to the hamster's larger caeca in relation to body weight, and hence great microbial metabolism.

Overall it can be seen that dietary calcium levels have an important control over phytate phosphorus availability, and that the various discrepancies between different groups can often be accounted for by differing dietary calcium levels.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Animals

The only animals used in the following studies were mature Wistar rats and Golden hamsters. Rat and hamster weights varied between 200-300g and 90-140g respectively. All dietary studies were carried out in the animal house. Animals were killed in the laboratory, rats by a blow to their head followed by dislocation of their neck, while hamsters were subdued in a chloroform vapour tank before having their necks broken.

2.2 Diets

Various diets were used in the following studies. The standard 'PRD' diet, was the most commonly used base. This diet contained 6.5-8.0g Calcium/kg, 1g of free phosphorus/kg, total phosphorus varied between 5-7g/kg and 100iu of vitamin D/kg. This diet could be milled and chromic oxide (Cr_2O_3) added if a non-absorbable marker were needed, and then thoroughly mixed repelleted and dried. Other diet variations will be dealt with in the relevant chapters. Chromic oxide was used as a marker to correct for bulk gut secretions and absorptions.

2.3 Measurement of Intestinal Phytase and Alkaline Phosphatase Activities

Phytase and Alkaline phosphatase activities in the rat and hamster small intestine were measured by the method of Davies and Flett (1978). Lengths of small intestine were removed from the dead animals, slit longitudinally, carefully washed in assay buffer and then blotted dry. A mucosal scrape was then

taken using a glass microscope slide, and the samples homogenised moderately in approximately five volumes of 0.25M sucrose. The homogenate was then divided and one half further diluted (about five times) with the sucrose solution. Small samples of both diluted and normal homogenate were taken and frozen at -20°C for later protein analysis.

i. Determination of Phytase Activity:

The following incubation procedure was used; assay tubes contained 0.3ml of 8.33mM sodium phytate, 2.5ml of pH7.4 50mM Tris/Succinate buffer, which also contained 0.5mM MgCl . The incubation was performed at 37°C in a water bath and was initiated by the addition of 0.2ml of the concentrated homogenate. The incubation lasted 30 minutes and was stopped by the addition of 1ml of 200g/l trichloroacetic acid (TCA) solution. After bench centrifugation the supernatant was analysed for its phosphate content by the same procedure described in Chapter 2.6. Reagent blanks (no enzyme added) and enzyme blanks (no substrate added) were also performed and subtracted from the complete incubations. Protein in the homogenate was determined by the Biuret Method (Chapter 2.4). Results were finally expressed as μmole of phosphorus liberated from phytate/mg total protein/h.

ii. Determination of Alkaline Phosphatase Activity:

A very similar incubation procedure was used as for the phytase assay. 0.3ml of 50mM β-glycerophosphate was used as substrate instead of the phytate, and 0.2ml of the dilute homogenate was used, not the more concentrated one. The incubation time was only 15 minutes but in all other respects the assays were identical. Once again the units of alkaline phosphatase activity were expressed as μmole of phosphorous liberated from β-glycerophosphate/mg total protein/h.

2.4 Determination of Protein

Protein concentration in the gut mucosal homogenates was measured by the Biuret method. 1ml of normal or diluted homogenate containing 1-10mg protein was added to 4ml of Biuret reagent, mixed and then left for 30 minutes. A standard curve was constructed using the same procedure but using 1-10mg of BSA as the standard protein instead of homogenate. All tubes were read in a spectrophotometer at 540nm, and from the optical densities, protein concentration in the homogenates was determined. The Biuret reagent was made up of 1.5g hydrated cupric sulphate plus 6g sodium/potassium tartrate made up to 500ml and then 500ml of 60g/l sodium hydroxide added. This reagent was then stored at 4°C.

2.5 Measurement of Dietary Calcium

Calcium was only measured in the diets for quantification; no experimental studies on calcium absorption were attempted. However a separate project by Mr. J. King ran concurrently to compare calcium and phosphate absorption in the rat and hamster using more refined techniques.

Test diets were finely ground and samples wet digested in concentrated nitric acid. After considerable dilution with distilled water, samples were measured against standard calcium solutions by atomic absorption flame spectroscopy at 423nm in a lean air/acetylene flame. All solutions were finally made up in 0.1% Lanthanum Chloride solution to eliminate interference by phosphorus absorption.

2.6 Measurements of Total Dietary Phosphorus and Chromium

It is possible to determine phosphorus, chromium and calcium, if necessary, in the same sample. A known weight of

food or gut digesta (100-200mg) was wet digested with 2ml of concentrated nitric acid in a conical flask on a heating block. The sample was heated to near dryness and then 1.5ml of 600g/l perchloric acid was added and the sample boiled gently for 20 minutes. This second digestion was necessary to oxidise the green insoluble chromic oxide to the soluble yellow/orange chromate and dichromate; care was taken not to boil to dryness when explosive chlorates would be produced. The sample was then cooled and diluted with distilled water and then gently reheated to hydrolyse any pyrophosphate that may have been formed. The sample was now diluted 50-500 times with distilled water so that the chromium concentration was between 1-4μg/ml and that of the phosphorus was 0.05-0.5μmole/ml.

To measure the phosphorus an aliquot (100-750μl) of the diluted sample was taken in a disposable LP3 tube and if necessary made up to 750μl with distilled water. 750μl of a mixture of 3 parts 6g/l ammonium molybdate in 50ml/l perchloric acid and 1 part 2g/l ascorbic acid was added. This solution was made fresh daily from 2 stock solutions. 15 minutes after the addition of the mixture the optical density of the blue colouration was measured at 700nm and samples read against a standard (0.05-0.5μmole K_2HPO_4) phosphorus solution.

Chromium was determined by use of the atomic absorption flame spectrophotometer at 357nm using a rich air/acetylene flame. Samples were read against known chromium standards in the range 0.5-4.0μg/ml, all solutions contained 20g/l ammonium chloride to remove various ion and flame interferences.

2.7 Phytate Analysis

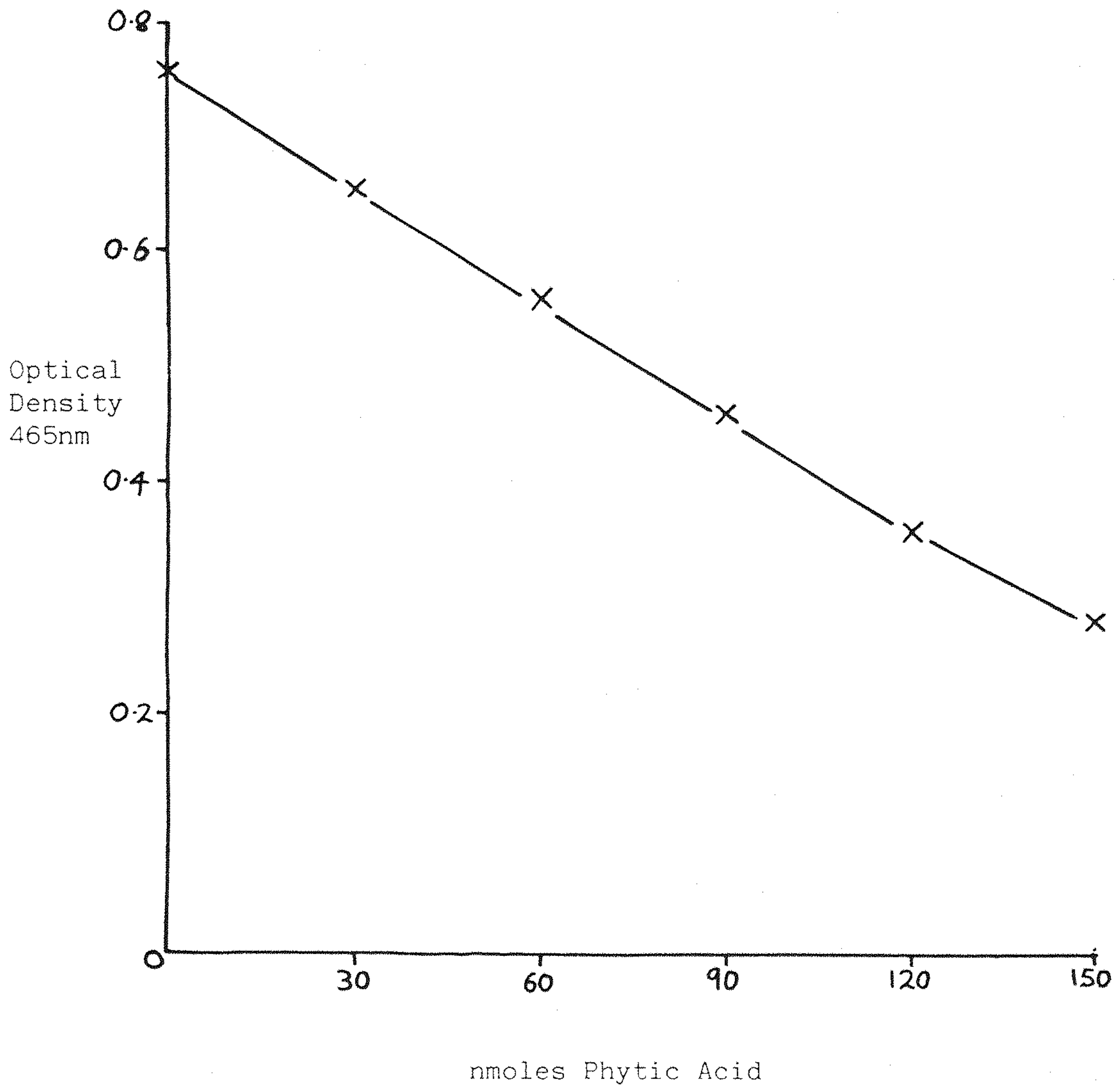
Phytate content of food, faeces and gut digesta was determined by the modified "Holt (1955)" method described by Davies and Reid (1979). Samples were dried to constant weight and 50-200mg of dry material was accurately weighed, and

extracted with 5ml of 0.5M nitric acid for 3-4h with continuous shaking at room temperature. The extract was centrifuged and an aliquot of the supernatant examined for its phytate content as follows.

100µl-1000µl of supernatant was made up to 1ml with distilled water and 0.4ml of 0.5M nitric acid added. 1ml of a solution of ferric ammonium sulphate containing 40µg of iron was added. Test tubes were then sealed and placed in a boiling water bath for 20 minutes, thus speeding up the formation of the ferric phytate complex. The tubes were then cooled and 7ml of amyl alcohol was added to each tube, to extract the red colour, which was formed when 0.1ml of a 100g/l solution of ammonium thiocyanate was added. The tubes were mixed by several gentle inversions, and the intensity of the red colouration measured at 465nm in a spectrophotometer (upper amyl alcohol layer measured only). The spectrophotometer was zeroed on an amyl alcohol blank and a standard curve was formed using 20-200nmoles of phytate per tube from a standard solution. This phytate assay works on the principal that the ferric ion complexed with phytate at pH2 cannot react with the thiocyanate ion to give the characteristic red-coloured ferric thiocyanate. Therefore an inverse linear relationship is found between phytate and optical density see Figure 2.1. Samples and standards were always measured in triplicate, as being an indirect assay some errors were experienced.

Figure 2.1

Inverse Standard Curve Used for Phytate Determination



2.8 Buffer and Chemicals

i. Phytase and Alkaline Phosphatase Assay Buffer

50 mM Tris/Succinate Buffer pH7.4

Components	Weight g/l
Tris	6.06
NaCl	24.81
KCl	1.28
Succinic Acid	5.90
$MgCl_2$	0.10

ii Chemicals Used

Chemicals Used	Supplier
Trichloroacetic Acid	Sigma Chemical Co
60% Perchloric Acid	BDH
Amyl Alcohol	BDH
Chloroform	Koch-Light Labs
Chromic Oxide	BDH
Analar 'Tris'	BDH
Potassium Chloride	BDH
Succinic Acid	BDH
Sucrose	BDH
Cupric Sulphate	BDH
Sodium Potassium Tartrate	BDH
Sodium Phytate	Sigma Chemical Co
β-Glycero-Phosphate	BDH
Calcium Carbonate	BDH
Lanthanum Chloride	BDH
Ammonium Molybdate 'Analar'	BDH
Ascorbic Acid	BDH
Ferric Ammonium Sulphate	BDH
Ammonium Thiocyanate	BDH
Dipotassium Hydrogen Phosphate	BDH
Concentrated Nitric Acid	BDH

CHAPTER 3 - MEASUREMENT OF PHYTASE AND ALKALINE PHOSPHATASE ACTIVITY IN THE RAT AND HAMSTER SMALL INTESTINE

3.1 Introduction

In the first chapter it was mentioned that the hamster could hydrolyse and utilise phytate as a source of dietary phosphorus to a much greater extent than the rat. One of the main aims of the following work was to understand just how the hamster was so efficient in this respect. It was hoped that what we might learn could be applied to improving dietary phytate phosphorus availability for important commercial animals such as poultry and pigs. Financial savings through a reduction in dietary phosphate supplements could be considerable.

The first obvious approach was to see if the difference in hamster and rat phytate hydrolysis could simply be accounted for by greater intestinal phytase activity in the hamster. While rat intestinal phytase has been studied previously, the hamster's has not. Alkaline phosphatase activity was also measured as phytase is believed to be an isoenzyme of alkaline phosphatase.

3.2 Methods

Male Wistar rats (250-300g) and male Golden hamsters (100-120g) were used for this experiment. Both rats and hamsters were fed the standard pelleted chow which contained 8.0g/kg calcium, 5.0g/kg phosphorus and 100iu of vitamin D/kg. The small intestines were removed and divided into three sections of equal length, section 1 represented the intestine distal of the pylorus, section 2 was in the middle and section 3 was attached to the caecum. Mucosal gut scrapes were taken from the various sections of the gut after they had been

washed with buffer. Alkaline phosphatase and phytase activities were then determined on these tissue samples by the method of Davies and Flett (1978), (see Materials and Methods 2.3).

An additional experiment was performed on one rat only. 10cm lengths of its small intestine were examined for alkaline phosphatase and phytase activities; in this way ten sections of gut were obtained. This format would have been too time consuming to perform on a larger scale, but it does produce a higher resolution profile of enzyme activities through the rat small intestine.

3.3 Results

The rats showed considerable alkaline phosphatase activity in the first section of the small intestine, which decreased rapidly distally; the hamster, however, had relatively constant alkaline phosphatase activities throughout the small intestine (Table 3.1). Phytase activity was found mainly in section 1 of the rat small intestine but no activity was observed in the hamster. The experiment with the single rat, shows more precisely that rat intestinal phytase is found almost exclusively in the first 20cm of small intestine (i.e. in the duodenum), whereas alkaline phosphatase activity, while maximal in the this small area, is found throughout the length of the rat's small intestine (see Figures 3.1 and 3.2).

3.4 Discussion

The activities of alkaline phosphatase and phytase in the rat are in close agreement to those found by Davies and Flett (1978). There was a considerable decrease in both enzyme activities towards the caecum. However, phytase activity decreased earlier than alkaline phosphatase, thus indicating

Table 3.1

Phytase and Alkaline Phosphatase Activities in Various Sections of Rat and Hamster Small Intestine

		Rat (n=5)	Hamster (=4)
Alkaline	1	5.16 ± 0.50	1.78 ± 0.42
Phosphatase	2	1.48 ± 0.34	1.88 ± 0.35
Activity	3	0.28 ± 0.03	1.42 ± 0.30
Phytase	1	1.05 ± 0.10	ND
Activity	2	0.06 ± 0.03	ND
	3	0.02 ± 0.02	ND

Both Enzyme Activities are expressed as μmoles phosphorus liberated/mg protein/h.

1, 2 and 3 refer to the 3 sections of gut studied.

All values are Mean ± S.E.M.

ND = No Enzyme Activity detectable.

Figure 3.1

Alkaline Phosphatase Activity in Rat Small Intestine

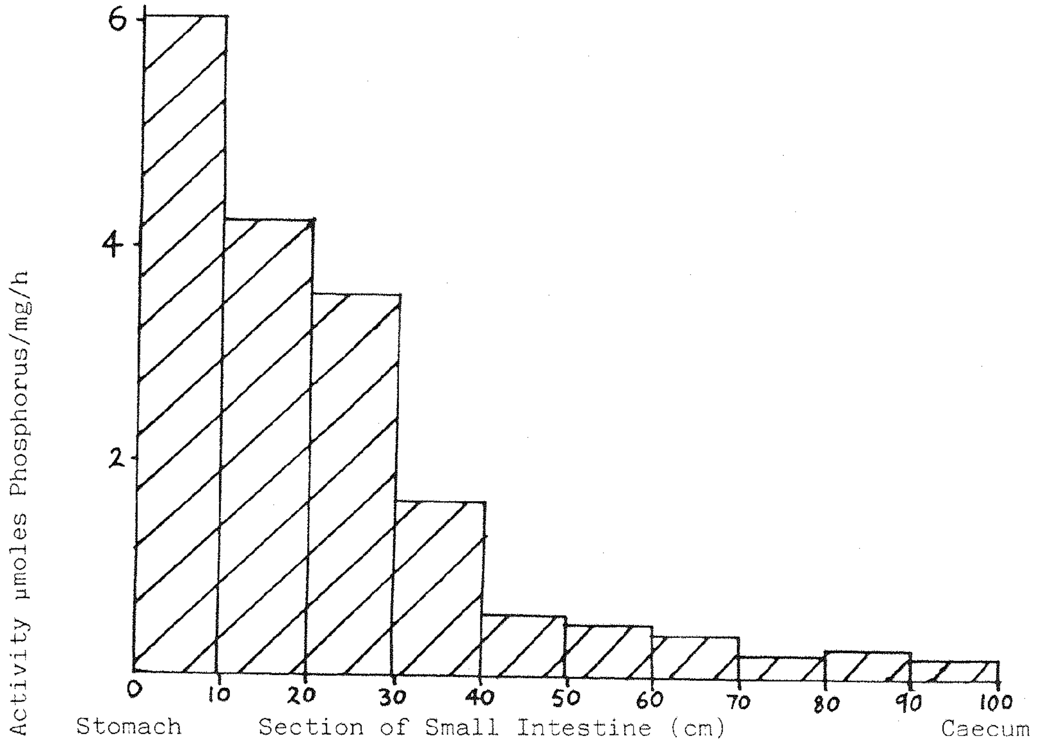
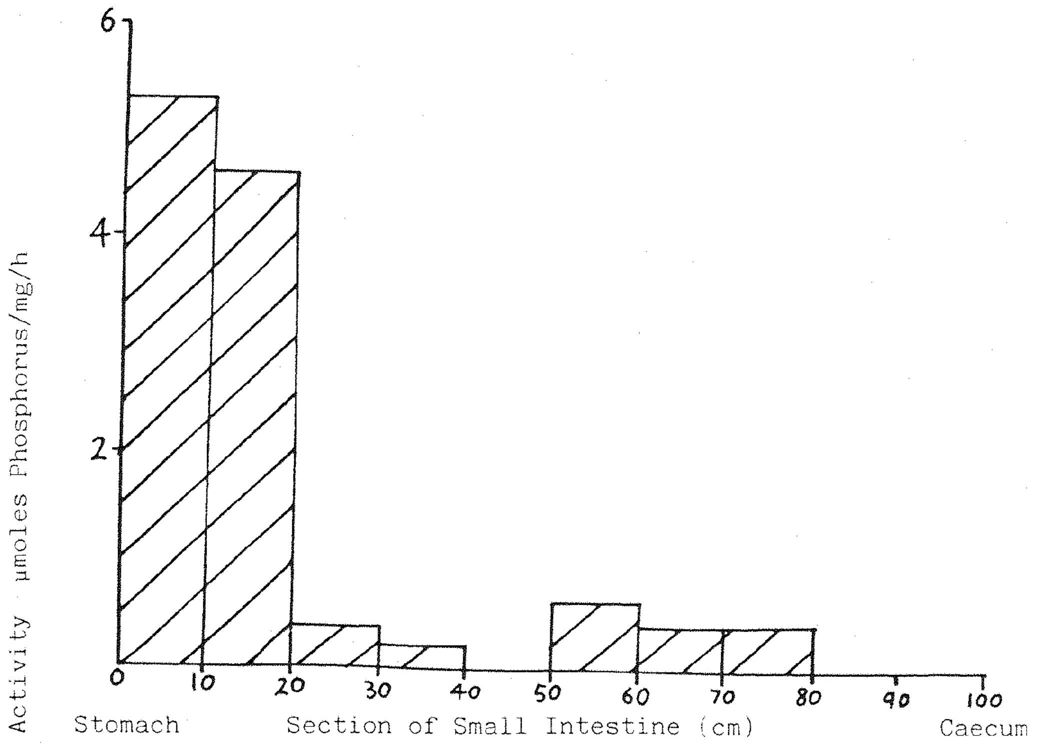


Figure 3.2

Phytase Activity in Rat Small Intestine



that these two enzymes, although closely related in terms of their substrates, are quite separate enzymes as determined by their distribution. Alkaline phosphatase activity in the hamster showed no such gradation in activity, with maximum activity being approximately one third of the maximum alkaline phosphatase activity in the rat; the significance of this is unknown.

Most surprisingly phytase activity was entirely absent from the hamster small intestine, as it was expected that considerable activity would be found there. This finding therefore made it even more interesting to discover just how the hamsters could be so efficient at hydrolysing phytate. In later small scale experiments, tissue was taken from hamster cheek pouches, stomach, caecum and various sections of large intestine and incubated with phytate but no phytase activity was found in any of these tissues either. Clearly a new approach to the problem was needed.

CHAPTER 4 - A STUDY OF PHYTATE HYDROLYSIS THROUGH THE RAT AND HAMSTER GUT.

4.1 Introduction

The new approach we adopted was to measure the phytate content in various sections of the gut. This meant that instead of looking directly for the enzyme phytase, we would do so indirectly by observing the disappearance of the substrate, phytate. Samples of food/gut digesta were to be taken from the fore stomach, true stomach, caecum and lower large intestine. One major problem with such a study is to overcome the effects of gut secretions and more importantly absorptions. Therefore chromic oxide a non-absorbable gut marker that is associated with the solid phase was added to the standard stock diets (2gCr_2O_3/kg). This marker made it possible to correct for the various gut absorptions and secretions.

4.2 Methods

Adult male hamsters and rats were fed the standard 'PRD' diet, which had been milled and had 2g/kg of chromic oxide added to it, and then repelleted. They were maintained on this diet for three whole days and were killed on the fourth day. The animals' guts were removed and samples of digesta removed from various sections of the gut. These samples were then dried to constant weight in an oven at 90°C on tin foil sheets. Insufficient dry matter could be found in either rat or hamster small intestines, but adequate samples were obtained from the true and fore stomachs, caecum and lower large intestine. The dried samples were then divided into two and accurately weighed. One sample was then extracted in dilute nitric acid for the determination of phytate and the other wet digested in boiling concentrated nitric acid

followed by boiling perchloric acid for chromium and total phosphorus determinations. All these methods are described in greater detail in Chapter 2.

The dry weight of sample needed to make the above determinations was 200-300mg, and as this was usually about the total dry weight of sample taken duplicates could not be performed. In a few cases where excess sample remained, free phosphorus levels were determined and usually found to be very close to the difference between total and phytate phosphorus.

4.3 Results

The chromium content increased from 1.5g/kg in the food to approximately 5g/kg in the soft faeces, showing that approximately 70% or more of the ingested food was absorbed. The results are given in Tables 4.1 and 4.2, and show most forcefully that much of the dietary phytate is hydrolysed before it leaves the stomach of both rat and hamster (46% and 70% hydrolysed respectively). In the hamster a further small increase in phytate hydrolysis after the stomach was seen. This response was mirrored in the rat but to a slightly greater degree. Overall phytate phosphorus availability (phytate hydrolysis) was 82% in the hamster and 61% in the rat. Total phosphorus levels were quite similar in both the rat and hamster, with the rats apparently absorbing more of their dietary phosphorus than the hamsters.

4.4 Discussion

The most interesting result in this experiment was that phytate appears to be hydrolysed predominantly in the stomach in both the rat and the hamster. This finding helps explain how the hamster, although devoid of intestinal phytase activity, can hydrolyse phytate. The phytase activity in the

Table 4.1

Total Phosphorus and Phytate Phosphorus Content of Gut Digesta from Different Regions of the Hamster Gut

Sample	Mg Phosphorus/mg Chromium	
	Total	Phytate
Food	3.4 ± 0.1	2.6 ± 0.1
Fore Stomach	3.4 ± 0.2	1.6 ± 0.2
True Stomach	1.8 ± 0.1	0.8 ± 0.2
Caecum	3.3 ± 0.2	0.7 ± 0.1
Soft Faeces	2.4 ± 0.1	0.5 ± 0.1

All values Mean ± S.E.M (n=4)

Table 4.2

Total Phosphorus and Phytate Phosphorus Content of Gut Digesta from Different Regions of the Rat Gut

Sample	Mg Phosphorus/mg Chromium	
	Total	Phytate
Food	3.4 ± 0.1	2.6 ± 0.1
Fore Stomach	2.9 ± 0.3	1.6 ± 0.2
True Stomach	1.8 ± 0.2	1.4 ± 0.3
Caecum	2.4 ± 0.2	1.3 ± 0.1
Soft Faeces	1.9 ± 0.1	1.0 ± 0.1

All values Mean ± S.E.M (n=4)

stomach can presumably be of cereal or microbial origin. This experiment was not designed to distinguish between these possibilities. However, evidence was found from other workers to suggest that the active phytase is most likely to be microbial. Wise and Gilbert (1982) showed that germ-free rats could not hydrolyse phytate to any great extent and hence cereal and intestinal phytases were essentially inactive **in vivo**. They thought that microbes in the caecum were responsible for normal phytate hydrolysis, but our data for the rat implies that the likely major site is the stomach. This does not necessarily exclude the caecum. The hamster seemed to have an even greater potential for hydrolysing phytate in its stomach than the rat.

Hoover, Mannings and Sheerin (1969) showed that the hamster with its stomach clearly divided into two distinct sections could be classified as a 'pseudo' ruminant. The fore stomach or gastric pouch was found to have a pH of 5.1 as opposed to pH2.0 for the true stomach, and as such was quite capable of supporting micro-organisms. They also found considerable production of volatile fatty acids, indicative of microbial fermentation in the fore stomach. Thus it seems quite plausible to suggest that the hamsters great ability to hydrolyse phytate comes from a microbial culture in their fore stomachs, in a fashion similar to that of the true ruminants. It must also be recognised that some phytate is hydrolysed in the hamsters true stomach prior to the digested matter being fully acidified. It is also quite likely that the hamsters caecum contains microbes capable of hydrolysing phytate, although most of the phytate will have already been hydrolysed by the time the food reaches this region of the gut.

CHAPTER 5 - A TIME COURSE STUDY OF PHYTATE HYDROLYSIS BY THE HAMSTER.

5.1 Introduction

Phytate hydrolysis was measured in a time dependant manner primarily in the hamster. In this experiment it was possible to check that no coprophagy occurred at least in the ½h group, thus eliminating a potentially confusing variable. It was hoped that this experiment would confirm previous findings showing that the primary site of phytate hydrolysis in both the rat and hamster was the stomach.

5.2 Methods

Adult female hamsters and male rats were fed the 'PRD' diet containing 2gCr_2O_3/kg for 4 days. They were then starved overnight for 16h and then given continuous access to their diet once again. They fed voraciously and the hamsters were then killed either ½h, 1h or 2h after commencing feeding, while rats were killed after 2h only. Samples of food were taken from the hamster fore and true stomachs, while one sample only was taken from the less clearly defined stomachs of the rats. These samples were dried and assayed as before for their chromium, total phosphorus and phytate phosphorus content.

5.3 Results

The results are shown in Tables 5.1 and 5.2.

Total phosphorus increases in the fore stomach of the hamsters ½h after the start of their feed, presumably due to secretions of phosphate in the saliva or gastric juice. Total

Table 5.1

Total Phosphorus Content of Rat and Hamster Stomach Contents

Group	Total Phosphorus (mg/mgCr)			
	½h	1h	2h	n
Diet	5.3	5.3	5.3	8
Hamster Fore Stomach	5.7 ± 0.5	4.6 ± 0.2	5.0 ± 0.1	4
Hamster True Stomach	2.6 ± 0.6	0.8 ± 0.2	1.5 ± 0.2	4
Rat Stomach	-	-	4.2 ± 0.3	4

Table 5.2

Phytate Phosphorus Content of Rat and Hamster Stomach Contents

Group	Phytate phosphorus (mg/mgCr)			
	½h	1h	2h	n
Diet	3.9	3.9	3.9	8
Hamster Fore Stomach	1.6 ± 0.2	1.4 ± 0.2	1.5 ± 0.1	4
Hamster True Stomach	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	4
Rat Stomach	-	-	2.0 ± 0.1	4

phosphorus values for the fore stomach were lower in the 1 and 2h groups. In the true stomachs of the hamsters, total phosphorus had decreased considerably, as if phosphate had been absorbed in the stomach. The use of chromic oxide as a solid phase marker was probably responsible for this result; phosphorus in the true stomach will be almost exclusively in the form of free soluble phosphate, and as such will be associated with the more mobile aqueous phase and hence will be leached out into the small intestine, rather than being absorbed in the stomach. In the hamster over 50% of the phytate was hydrolysed in all the fore stomach samples with no significant differences with time. The food in the true stomachs contained less than 20% of the original dietary phytate and again there was no difference between phytate content of the true stomach at the various times after feeding. The results for the rats are similar, except that only 50% of the dietary phytate had been hydrolysed after 2h in the stomach.

5.4 Discussion

None of the total phosphorus results are unusual; the high level in the ½h group is presumably due to saliva and gastric secretions in the fore stomach, and the rapid decrease in true stomach phosphorus can be accounted for by leaching of phosphate into the duodenum. The phytate levels are, however, surprising, as just ½h after the beginning of the hamsters feed, 59% of the phytate in their diet had been hydrolysed. Further hydrolysis then occurred until, in the true stomach only, 13-18% of the original phytate remained. These results confirm that hamsters have a very efficient phytate-hydrolysing system. Coprophagy was ruled out for the ½h group and in addition, chromium levels in the fore stomachs of the 1 and 2h groups were only slightly greater than levels in the food, strongly supporting the fact that no chromium rich faeces had been consumed. The fore stomachs of nearly all the hamsters in

the 2h group were almost empty, and as the hamster only fed for 45 minutes-1h it seems unlikely that food remains in the fore stomach much longer than 1h. This experiment demonstrates that 1h is more than long enough to ensure considerable phytate hydrolysis. The single 2h rat-group also confirm that considerable (although less than in the hamster) hydrolysis of dietary phytate occurs in the rat stomach, even though rats do not possess a gastric pouch with its associated microflora. A possible error in experimental technique was recognised after this experiment had been completed. Since the digesta samples from the stomach were dried in an oven and took over 1h to dry fully, it seemed possible that cereal or microbial phytase could still be active for some time after the actual sampling time of ½, 1 and 2h. To see if this were the case the following experiment was performed.

5.5 Additional Experimentation

In this short experiment, samples of food from the fore and true stomachs were taken from hamsters that had been allowed to feed for either ½ or 1h after being starved overnight. The experimental details were identical to those in Chapter 5.2 except that to determine the phytate content of the samples, digesta were taken straight from the stomachs into a preweighed test tube containing 5ml of 0.5M nitric acid. This meant that the phytate was extracted straight away and at this pH of 0-1 any phytase activity would be destroyed very rapidly. To determine the dry weight of sample taken, the preweighed test tubes were gently heated to dryness.

Only two hamsters were studied in each group and therefore, S.E.M could not be calculated and means only are given (Table 5.3).

The results show clearly that phytate had still been rapidly hydrolysed and to a very similar extent as before: therefore the previous results (Table 5.2) must be considered to be genuine.

Table 5.3

Total Phosphorus and Phytate Levels in Hamster Stomach Contents Measured by an Improved Procedure

Group	Total Phosphorus (mg/mgCr) Time after start of meal		n
	½h	1h	
Diet	5.3	5.3	8
Fore Stomach	3.8	4.5	2
True Stomach	1.4	1.2	2

Group	Phytate Phosphorus (mg/mgCr) Time after start of meal		n
	½h	1h	
Diet	3.9	3.9	8
Fore Stomach	1.3	1.4	2
True Stomach	1.2	1.1	2

Values given are Means only

CHAPTER 6 - INVESTIGATING THE ORIGIN OF PHYTASE ACTIVITY IN THE RAT AND HAMSTER STOMACHS.

6.1 Introduction

Although it seemed most likely that the phytase activity in the hamster fore stomach was of microbial origin, this had not been conclusively proven. In addition the rats have shown their ability to hydrolyse considerable amounts of phytate in their stomach, prior to the food reaching the known intestinal phytase in the duodenum. The origin of their stomach phytase is thus uncertain but the only real possibilities are for cereals or for microbes to provide the phytase activity. While microbial phytase could be difficult to study and indeed it rarely has been, cereal phytase is somewhat easier. Firstly the standard 'PRD' diet was assayed for intrinsic phytase activity, and secondly a cereal phytase free diet was prepared and phytate hydrolysis measured in both rat and hamster stomachs with this new diet.

6.2 Methods

To determine the cereal phytase activity in our standard 'PRD' diet the method of Hill and Tyler (1954) was used. 500mg of dry crushed diet was added to 9ml of distilled water and adjusted to pH5.0 with dilute hydrochloric acid, this mixture was shaken and incubated at 37°C for 1h. The incubation was stopped by the addition of 1ml of 5M nitric acid, and then shaken for 3h to extract the remaining phytate which was measured as in the previous experiment.

In the second experiment hamsters and rats were fed a diet devoid of cereal phytase activity (no activity apparent after a 1h incubation as above). This diet was maize-based, as maize has negligible phytase activity except when germinating (Hill

and Tyler, 1954; Mollgaard, 1946). The diet was for short term use only and contained the following ingredients:

Ingredient	Weight g/kg
Maize	500
Toasted Soya	383
Sucrose	100
CaCO	10
NaCl	5
Cr_2O_3	2

This diet contained only 2.31g phytate phosphorus/g chromium, as opposed to the 'PRD' diets 3.89g/gCr.

Female hamsters and male rats were fed the stock 'PRD' diet for 4 days and then starved overnight (16h), they were then fed the cereal phytase free diet and the hamsters killed after 1h and the rats 1 and 2h post feeding. Phytate phosphorus, total phosphorus and chromium levels were determined on all the stomach contents.

6.3 Results

The 'PRD' diet had some cereal phytase activity, approximately 10% of the dietary phytate was hydrolysed in 1h (see Table 6.1). It was interesting that when activity was also looked for after just 30 minutes, none was found, implying that an activation period for the 'dry' phytase was required.

When fed on the phytase-free diet, hamsters still retained their ability to hydrolyse substantial amounts (90%) of phytate in just 1h. The rats however could only hydrolyse about 10% of their dietary phytate in 1 or 2h (see Table 6.2).

Table 6.1

Cereal Phytase Activity of PRD Diet

	Diet	After 1h Incubation
Phytate Content mg/mgCr	3.89 ± 0.24	3.50 ± 0.18

Therefore 9.0% of the dietary phytate was hydrolysed presumably

by cereal phytase in 1h

Table 6.2

Total Phosphorus and Phytate Phosphorus Content of Rat and Hamster Stomachs Fed a Cereal Phytase Free Diet

Group	Phytate Pmg/mgCr	Total Pmg/mgCr	n
Diet	2.31 ± 0.14	2.88 ± 0.03	4
Rat 1h	2.19 ± 0.04	2.84 ± 0.12	4
Rat 2h	2.18 ± 0.07	2.89 ± 0.08	4
Hamster 1h Fore Stomach	0.51 ± 0.07	2.13 ± 0.13	4
Hamster 1h True Stomach	0.22 ± 0.05	0.96 ± 0.07	4

All values are Mean ± S.E.M

6.4 Discussion

The 'PRD' diet appeared to possess limited intrinsic phytase activity, which is almost certainly cereal phytase. The relatively low activity cannot, however, account for the considerable hydrolysis of phytate seen in the hamster stomach, especially when it is remembered that the food remains for only about 1h in the fore stomach. This is also confirmed by the second experiment using a maize-soya phytase-free diet, as the hamster could hydrolyse nearly all of its dietary phytate in the stomach. This experimental data coupled with that of Hoover _et al_ (1969), showing that the hamster behaved as a 'pseudo' ruminant and the more detailed studies of Kunstyr (1974) showing specific microbial species and their abundance in the hamster fore stomach suggests, that the hamster's great ability to hydrolyse phytate is due to the action of a microbial phytase in their fore stomach.

The situation in the rat, however, appears to be slightly different, as only about 10% of the dietary phytate was hydrolysed when they were fed the phytase-free diet. Previously using the 'PRD' diet about 50% of the dietary phytate was hydrolysed in 2h. It therefore appears that cereal phytase activity may play an important role in phytate hydrolysis in the rat stomach.

CHAPTER 7 - DISCUSSION AND CONCLUSIONS

The main aim of this project was to discover how the hamster hydrolyses phytate so much more efficiently than the rat. It was hoped that by understanding this exceptional ability, we may be able to apply this knowledge to improve phytate phosphorus availability in other animals, particularly farm livestock such as poultry and pigs.

Initial experiments looked at the most probable explanation, that of intestinal phytase. Phytase activity is found in the intestinal mucosa of various animals including rats and it seemed quite possible that the hamster possessed a very active intestinal phytase. However, while we could detect intestinal phytase activity in the rat none could be found in the small intestine of the hamster. Intestinal alkaline phosphatase activity was detectable in both rat and hamster, although the greater activity was found in the rat. It is thought by some workers that phytase activity, is, in effect, non-specific alkaline phosphatase activity, while others believe that phytase is a specific enzyme in its own right. The fact that we found alkaline phosphatase activity but no phytase activity in the hamster, supports the latter possibility.

In the following experiment dietary phytate hydrolysis was monitored through the rat and hamster gut using chromic oxide as a non-absorbable marker. This experiment provided the most surprising result, as it was found that the majority of the dietary phytate was hydrolysed in the stomach of both the rat and the hamster. Phytase activity was unsuccessfuly looked for in the hamster mouth pouches and stomach linings. It appeared, therefore, that an external source of phytase such as cereal or microbial was responsible.

When the hydrolysis of phytate in the hamster stomach was

followed in a time dependant manner, it was found that in just ½h after commencing to feed most of the available phytate had been hydrolysed.

Finally to distinguish between cereal and microbial phytase, a cereal phytase-free diet was prepared and fed to rats and hamsters. This diet did not affect the hamster's ability to hydrolyse phytate, whereas that of the rats was severely restricted. The stock diet used in all previous studies being wheat based, was shown to have some cereal phytase activity. It therefore seems that phytate is hydrolysed primarily in the rat stomach by the action of cereal phytase when present, and that the hamsters exceptional ability to hydrolyse phytate is due to their stomach microflora. Furthermore the microflora exist primarily in the hamsters fore stomach, thus making them effectively 'pseudo' ruminants; and as such it will be impossible to exploit the hamster's phytate hydrolysing ability because of their unique stomach compartmentation.

7.1 Conclusions

1. The hamster has no intestinal phytase.

2. Dietary phytate is hydrolysed primarily but not exclusively in the stomach of both the rat and the hamster.

3. The importance of the rat intestinal phytase with respect to dietary phytate hydrolysis must be questioned.

4. The active phytase in the rat stomach is probably of cereal origin.

5. The active phytase in the hamster stomach is almost certainly of microbial origin.

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