THE EFFECTS OF NUTRIENT MODIFICATION ON TWO BONE DISORDERS OF POULTRY

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Declaration

Acknowledgements

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DECLARATION

This thesis is my own composition, and the work presented in it entirely my own. All references and assistance from other individuals have been acknowledged.

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ABSTRACT

The aims of the project described in this thesis were to investigate the effects of nutrient modification on the development of osteoporosis in the laying hen and tibial dyschondroplasia in broiler chickens.

For the osteoporosis study, a large scale experiment was carried out to determine the effects of nutrition, bird strain and lighting pattern during rearing on egg production, plasma calcium, inorganic phosphate and alkaline phosphatase activity during the laying period, and degree of osteoporosis at the end of the laying period when the birds were 68 weeks old. The nutritional treatments were dietary oystershell (to contribute 50% of dietary calcium), low crude protein & high vitamin K (150 g/kg + 20 mg/kg), low phosphorus (4.5 g/kg), and supplements of fluoride (200 mg/kg), ascorbic acid (300 mg/kg), $1,25(OH)_2D_3$ (5 μg/kg). There was also a control treatment.

The improved strain's (Hi-sex) egg production was unaffected by nutrition or lighting pattern. The unimproved strain (J-line) laid fewer eggs than birds from the improved (Hi-sex) strain.

Some of the treatments affected the plasma measurements, and dietary fluoride and oystershell increased plasma alkaline phosphatase activity, indicating increased bone synthesis.

Analysis of bone sections from sites in the axial and appendicular skeleton (free thoracic vertebra and proximal tarsometatarsus respectively) revealed that strain influenced the degree of osteoporosis more than nutrition. The J-line birds' bones contained more trabecular (load bearing) bone than the bones of the Hi-sex birds. Amongst the Hi-sex birds, dietary fluoride and oystershell increased the amount of medullary (labile calcium source) bone in the proximal tarsometatarsus.

There was no effect of treatment on trabecular bone content, and this was thought to be related to reports of lack of osteoblastic activity in trabecular bone in sexually mature hens.

Some treatments were associated with a decrease in wing bone fracture. Fluoride decreased the incidence of fracture in the humerus and ulna. Oystershell, ascorbic acid, low crude protein & high vitamin K and low phosphorus reduced the incidence of fracture in the humerus.

Young, fast growing broiler chickens develop tibial dyschondroplasia (TD). The TD lesion is found in the growth plate of the long bones, and leads to bowing of the bones and lameness. TD is caused by a failure of chondrocyte hypertrophy.

A series of experiments using broiler chicks reared until three weeks old showed that feeding a imbalanced calcium/phosphorus (7.5 g/kg Ca, 7.6 g/kg P)) diet could induce a high incidence of TD. Supplementing an imbalanced or balanced (12 g/kg Ca, 6 g/kg P) calcium diet with $1,25(OH)_2D_3$ (2.5-10 μg/kg) reduced the incidence of TD. The mechanism was thought to involve dietary $1,25(OH)_2D_3$ increasing chondrocyte differentiation *in vivo*. Dietary ascorbic acid (250 or 500 mg/kg) was effective only when added to the normal calcium diet, and did not increase renal 1-hydroxylase activity. The beneficial effect of ascorbic acid when added to the normal calcium diet was thought to be related to its role in collagen, and hence extracellular matrix, synthesis

The effects on TD of two experimentally developed $1,25(OH)_2D_3$ analogues with increased cell-differentiating properties *in vitro* were tested *in vivo* by orally dosing chicks, and comparing results to control and $1,25(OH)_2D_3$-dosed chicks. Neither analogue reduced TD incidence appreciably, and a further experiment showed that this was due to greatly reduced binding of both analogues to plasma vitamin D binding protein and chondrocyte $1,25(OH)_2D_3$ receptors.

A final experiment showed that dietary $1,25(OH)_2D_3$ interacted with dietary calcium, resulting in kidney tubule deposits of calcium phosphate when low levels of $1,25(OH)_2D_3$ were fed. When high levels of $1,25(OH)_2D_3$ were fed, there were heavy deposits of urates and calcium phosphate in the kidney tubules, hypercalcaemia and blood changes indicative of kidney damage.

Abbreviations

ADP	adenosine diphosphate
ANOVA	analysis of variance
AP	alkaline phosphatase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BHT	butylated hydroxytoluene
B_{max}	maximum amount of ligand which can bind specifically to the receptors in a cell preparation
BSA	bovine serum albumin
BTM	bromotetramisole
cAMP	cyclic adenosine monophosphate
CaBP	calcium-binding protein
cDNA	complementary deoxyribosenucleic acid
CK	creatine kinase
CP	crude protein
D_2	ergocalciferol
D_3	cholecalciferol
DBP	vitamin D-binding protein
df	degrees of freedom
DNA	deoxyribosenucleic acid
$1\alpha(OH)D_3$	1α-hydroxycholecalciferol
$25(OH)D_3$	25-hydroxycholecalciferol
$1,25(OH)_2D_3$	1,25-dihydroxycholecalciferol
$24,25(OH)_2D_3$	24,25-dihydroxycholecalciferol
EBDA	equilibrium binding data analysis
EDTA	ethylenediamine tetraacetic acid
EM	electron microscopy
FTV	free thoracic vertebra
glucose-6-P	glucose-6-phosphate
GDH	glutamate dehydrogenase
h^2	heritability estimate

hOST	human osteocalcin
HPLC	high performance liquid chromatography
HRE	hormone response element
IU/l	international units per litre
K	vitamin K
K_a	affinity constant
K_d	dissociation constant
kDa	kilodalton
LDH	lactate dehydrogenase
MB	medullary bone
MJ	megajoules
mOST	mouse osteocalcin
mRNA	messenger ribosenucleic acid
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidised and reduced)
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate (oxidised and reduced)
NAF	nuclear accessory factor
p	probability
P	dietary phosphorus (inorganic and organic)
PBS	phosphate-buffered saline
Pi	inorganic phosphate
PTH	parathyroid hormone
PTM	proximal tarsometatarsus
PTT	proximal tibiotarsus
r^2	correlation coefficient
RAR	retinoic acid receptor
RARE	retinoic acid response element
REML	residual maximum likelihood
RIA	radioimmunoassay
RO23-6474	1,25,28-trihydroxy-ergocalciferol
RO23-7553	1,25-dihydroxy-16-ene-23-yne-cholecalciferol
rOST	rat osteocalcin
RXR	retinoid-X-receptor
sed	standard error of the difference

sem	standard error of the mean
TB	trabecular bone
TD	tibial dyschondroplasia
T_3R	thyroid hormone receptor
TRE	thyroid hormone response element

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Modern Conventional Egg and Broiler Production

Broiler and egg laying chickens are now derived from different strains of bird. Up until about 40 years ago, both meat and eggs were produced from dual-purpose strains of bird, with males being reared for meat and females for eggs. But the desire for increased productivity and efficiency lead to the development of different strains by breeding companies.

Current practice is as follows. Fertilised eggs from both strains are artificially incubated for 21 days under conditions of controlled temperature and humidity. After hatching, laying strain chicks are sexed by differences in feather development or colour of down and the unwanted males disposed of. Female chicks are brooded for about 3 weeks in artificially heated pens. They are then moved to rearing cages until they are approaching lay at about 16 weeks of age. They are then moved to laying accommodation, usually in the form of battery cages. These cages provide a hygienic, disease-free environment for the hens, as the wire floors of the cages allow the droppings to pass through. Newly laid eggs roll away to a collecting trough at the front of the cage. Feed is contained in a feed trough also at the front of the cage and water is piped in through nipple drinkers. Temperature and lighting are controlled. Lighting patterns and intensity are important as chickens are photo-periodic and their reproductive physiology is affected by day length. Generally speaking, reproductive performance is enhanced by long day length and adversely affected by short day length. The most common procedure is for pullets to receive 10 hours light / day until 16 weeks old and then for day length to be increased by 30 minutes per week

up to 18 hours / day. This ensures that birds come into lay uniformly and produce good quality, well-sized eggs from the start. Hens remain in their cages for about 12 months by which time their rate of egg production has started to decline. Most commonly, they are then removed from their cages by teams of workers and transported to factories for slaughter and processing into products such as chicken soup, pate etc.

Broiler production is more straightforward. Chicks are hatched as described above and both male and female chicks are reared. Birds are grown up in large litter floored houses with brooder lamps to provide supplemental heat until enough feathers have developed to provide insulation. Food and water are provided *ad libitum*, and lights are on 23 hours / day to encourage maximum food intake. Slaughter weight (about 2.5 kg) is achieved by 6 weeks of age.

1.2 Production Disease in Farm Animals

Production disease in farm animals can be defined as a breakdown in the various metabolic systems of the animal's body under the combined strain of high production and modern intensive husbandry systems. It is therefore a condition of fast growing or high yielding animals. Production disease is largely a man-made problem, often occurring at a very early stage in agricultural improvement. For example, parturient hypocalcaemia or milk fever of dairy cows was first observed in 1793 when selection for high milk yields first began, but it was nearly 150 years before the cause was found to be a temporary but severe calcium deficiency due to a failure of the cow to mobilise calcium reserves quickly enough to meet the demands of lactation (Payne, 1972).

Many of the poultry diseases of 'unknown or uncertain aetiology' (Randall, 1991) can be classified under the heading of production diseases. Massive selection

pressure on broiler chickens has resulted in a bird that will grow to 2.34 kg in 6 weeks, with a food conversion ratio of 1.88, and a breast meat yield of 15.7% by weight (Euribrid Hybro). This very rapid growth has lead to increased physiological demands particularly on the cardiovascular and skeletal systems. A problem arising with the former is ascites, a condition of broilers, the main symptom of which is the presence of serous fluid in the abdomen. A small liver, dilation of the right ventricle of the heart and passive venous congestion of the lungs and viscera are also seen. Chronic hypoxia is the cause of the broiler ascites seen at both high and low altitudes (Maxwell *et al.*, 1993).

The skeleton, particularly the bones of the leg, is the site of much of the production-related disease of modern, fast-growing poultry. The aetiology of leg disorders is complex, but these problems are important from both an economic and welfare point of view. Valgus leg deformity ('twisted leg') is seen fairly commonly in growing broilers. Males are affected more often than females. The disorder is caused by a lateral tilting of the distal tibiotarsal condyles. Similarly, the two skeletal disorders described in the main body of this thesis could also be categorised as production diseases, namely tibial dyschondroplasia in broilers and osteoporosis in laying hens. The aetiology and pathology of each will be described fully in the appropriate sections. Briefly, both have been observed only following selection for increased growth rate and egg production respectively and are therefore due to high output under intensive husbandry conditions.

1.3 Endochondral Ossification

Endochondral ossification is the term used to describe the processes by which long bones, such as the bones of the leg, increase in length. It occurs in the growth plate or physis, and is a co-ordinated sequence of cellular processes i.e. the

multiplication, growth, maturation and degeneration of chondrocytes in the growth plate, the vascularisation of the hypertrophic cartilage, and the replacement and subsequent remodelling of cartilage by bone (Sissons, 1971).

The avian growth plate contains layers of chondrocytes of different developmental stages, proximally arranged into ordered columns, but with the arrangement becoming more randomly orientated further down the growth plate. At the top are columns of small disc-shaped proliferating chondrocytes, although this columnar arrangement becomes disordered as the cells enlarge and become separated by larger amounts of extra-cellular matrix. Further down, the cells develop into pre-hypertrophic chondrocytes which are more spherical in shape and more voluminous. They have prominent, sometimes branching cytoplasmic processes which extend further into the matrix than those of the proliferating chondrocytes. Finally, the chondrocytes differentiate fully and hypertrophy to form large (up to 50 μm diameter) cells occupying prominent lacunae. The growth plate is approximately 200 cells deep in the 4-7 week old chicken, compared with about 25 cells deep in the growing rat (Howlett, 1979).

Extracellular matrix is secreted by and surrounds chondrocytes all through the growth plate. It is composed of types II, VI, IX, X and XI collagen and proteoglycans, macromolecules consisting of a protein core onto which large side chains of chondroitin sulphate, keratin sulphate or dermatan sulphate are attached. The composition of the extracellular matrix varies according to the developmental stage of the chondrocytes secreting it. That secreted by the proliferating chondrocytes is rich in type II collagen and proteoglycans, whereas the matrix secreted by hypertrophic chondrocytes contains type X collagen (Kwan *et al.*, 1989) with much less type II collagen.

The energy status of chondrocytes changes according to their state of differentiation (Haynes, 1990). Cells in the proliferating zone generate energy through glycolysis, and cells in the hypertrophic zone show a decreased reliance on oxidative phosphorylation. Changes in enzyme activity are associated with these changes in energy status, with maximal lactate dehydrogenase (EC 1.1.1.27) activity occurring in the hypertrophic zone (Shapiro *et al.*, 1991).

Mineralisation in the growth plate is thought to be mediated by matrix vesicles. These are 100-200 nm membrane-bound particles which arise from the processes of the chondrocyte plasma membrane (Marks and Popoff, 1991). Matrix vesicles are not normally evident in the upper proliferating zone of the growth plate where cells are rapidly undergoing mitotic division, but in the lower proliferating zone, empty matrix vesicles can be seen. In the transitional zone vesicles are seen to contain needle shaped crystals of mineral, thought to be generated from calcium from sequestration by phospholipids combined with inorganic phosphate released by alkaline phosphatase activity, with ATP providing energy for the processes. As crystals grow within the vesicles their membranes are disrupted, the crystals protrude and grow further by accretion eventually forming quite large crystals of hydroxyapatite (Ali, 1992). However, some studies have shown deposition of mineral in areas poor in, or devoid of matrix vesicles (Landis and Glimcher, 1982). Proteoglycan monomers have been implicated as initiators of matrix calcification and have been found using imaging techniques to be associated with areas with high calcium concentrations (Pines and Hurwitz, 1991). Therefore both could be necessary for complete mineralisation with matrix vesicles supplying high phosphate concentrations to areas rich in proteoglycan-associated calcium. In the next stage of calcification, the crystal clusters and mineral nodules get larger and coalesce to calcify the longitudinal septum. Crystals are seen to be associated with fibrils of

collagen, and finally, as the matrix becomes saturated with mineral, the crystals appear to align themselves in between the long collagen fibrils in the direction of the long axis of the fibres.

1.4 Avian bone

The skeleton of the bird is unusual amongst vertebrates in that the bones have developed to become particularly light and strong due to the evolution of the power of flight. Some of the bones (e.g. the humerus, and most of the cervical and thoracic vertebrae) are pneumatised, the air sacs connecting with the relatively small, compact lungs. In common with other vertebrates, birds possess cortical bone and trabecular bone which confer mechanical strength. Trabecular bone consists of interconnected plates and can be seen on X-rays of pnematised bones such as the humerus. Adult female birds also possess a third type of bone called medullary bone, a form of woven bone which is formed under the influence of oestrogens and androgens at sexual maturity about 10-14 days before the first egg is laid (Taylor *et al.*, 1971). Male birds will lay down medullary bone if injected with oestrogen. Medullary bone is found in quantity within the long bones particularly the femur and tarsometatarsus. It appears to be formed from the re-compartmentalisation of the endosteal surface of existing cortical bone. Medullary bone can be differentiated from cortical and trabecular bone histologically. Silver stains such as Von Kossa which stain for calcium (or more correctly the phosphate associated with calcium) do not differentiate between the two types of bone (Lynch and Maxwell, 1991), but other stains such as Alcian blue, hematoxylin and acid Giemsa, and periodic acid-Schiff can differentiate between the two types of bone, due to differences in chemical composition of the organic parts (Taylor *et al.*, 1971). Collagen is found predominantly in trabecular bone, but the proportions of hexosamine and uronic acid

components of chondroitin sulphate are higher in medullary bone. The inorganic part of all three types of bone consists of hydroxyapatite crystals embedded in organic matrix.

In common with other animals, chickens possess small basophilic osteoblasts which deposit new bone, and large multi-nucleated osteoclasts which resorb bone. The ultra-structure and organelles of active osteoblasts reflect their role in the synthesis of bone matrix. There are numerous cytoplasmic processes on the osteoblast surface next to the developing bone which may extend deep into the adjacent collagen fibrils of the osteoid. The cell cytoplasm contains a large amount of rough endoplasmic reticulum, with both free and bound ribosomes. Mitochondria are numerous and randomly scattered. The Golgi apparatus is also prominent, consisting of flattened sacs arranged in stacks (Hodges, 1974). Osteoblasts form bone by laying down organic matrix called osteoid, which is composed of Type I collagen, osteocalcin, osteonectin, bone sialoprotein and other proteins. The synthesis of bone consists of two stages. Firstly, osteoid is synthesised and laid down, and secondly, mineral salts are deposited and calcification occurs. The processes are similar to those described in the section on endochondral ossification with matrix vesicles and the enzyme alkaline phosphatase again playing an important role. Medullary bone osteoblasts possess receptors for oestrogen, and oestradiol stimulates the proliferation and collagen synthesis of osteoblasts *in vitro* (Ernst *et al.*, 1988) reflecting the role of this hormone in the induction of medullary bone (Ohashi *et al.*, 1990, 1991).

Osteoclasts are multi-nucleated cells of variable size. An active osteoclast is found in a pit or groove of the underlying bone, and the area next to the bone possesses a ruffled border which interdigitates with the bone. The osteoclast contains many mitochondria and lysosymes in the region of the ruffled border, and the

cytoplasm contains vacuoles which give it a foamy appearance. Osteoclasts have high levels of acid phosphatase, carbonic anhydrase (Mueller *et al.*, 1973), n-acetyl glucose aminidase, glucuronidase and succinate dehydrogenase activity. Acid phosphatase from other body tissues is inhibited by tartrate, whereas that from osteoclasts generally is not. H^+ ions are generated by carbonic anhydrase and pumped out of the cell by a H^+-ATPase pump. The resorbing area under the ruffled border therefore becomes acidic, which enables dissolution of bone mineral (Norman and Hurwitz, 1993). Osteoclasts also posses oestrogen receptors, but there is little evidence to suggest that osteoclasts are direct target cells of this hormone (Ohashi *et al.*, 1990, 1991)

1.5 Medullary bone and the egg laying cycle

The role of medullary bone has been shown to be that of a labile calcium source for the formation of eggshell (Taylor *et al.*, 1971). Each eggshell contains about 2.3g calcium (Etches, 1987) in the form of calcite ($CaCO_3$) crystals. Early workers disagreed about the proportion of eggshell calcium derived from dietary sources, but a good estimate is 50% with the remainder being provided by the dynamic turnover of primarily medullary bone (Rowland and Foutz, 1990). The exact proportions probably depend on a number of factors including dietary calcium intake, and may vary between species which lay eggs in different-sized clutches and at different times of the day (Miller, 1992)

The egg laying cycle of the hen is about 24 hours long. The cycle starts with the release of the ovum from the follicular hierarchy into the oviduct. The ovum travels down through the magnum and isthmus where it has albumen secreted around it. After about 5 hours it reaches the shell gland. Eggshell formation takes place between 7 and 20 hours post-ovulation, and finally the egg is laid. The processes

within the egg-laying cycle of active shell formation and no shell formation can be differentiated metabolically (Miller, 1977). During the active period, the percentage of active osteoclasts in medullary bone (identified histologically as multi-nucleated giant cells with a clear zone and ruffled border facing the bone) is increased. During the inactive period (no eggshell formation) the active osteoblast surface (cuboidally-shaped osteoblasts with the Golgi apparatus often clearly visible) is increased (Hodges, 1974, Van de Velde *et al.*, 1984).

The total quantity of medullary bone remains constant throughout the daily egg-laying cycle, and it is thought that matrix formation and mineralisation are separately regulated but closely controlled (Van de Velde *et al.*, 1985).

The amount of shell enclosing an egg is not directly correlated to medullary bone metabolism. An investigation was conducted using lines of hens bred to lay eggs with either thick or thin eggshells containing on average 2.3 or 1.7 g calcium (Stout and Buss, 1980). There was no difference in femoral calcium content between the two lines, but the thick eggshell line had a greater ability to absorb calcium from the small intestine, higher serum calcium and higher serum oestrogen-induced calcium binding protein (vitellogenin) than the thin eggshell line (Buss and Guyer, 1984).

1. 6 Calcium, Phosphate and Vitamin D metabolism in chickens.

Calcium and phosphate homeostasis in chickens, as with other birds and animals, is closely related to bone metabolism and is a tightly regulated process involving interactions between three hormones, namely parathyroid hormone, calcitonin, and 1,25$(OH)_2$cholecalciferol. This section describes the salient points. The subjects of calcium and phosphate homeostasis, and vitamin D metabolism are reviewed by other authors e.g. Anderson and Consuegra, 1970, Wideman, 1990,

Norman and Hurwitz, 1993, and Henry and Norman, 1992. See also *Figure 1a* for a summary.

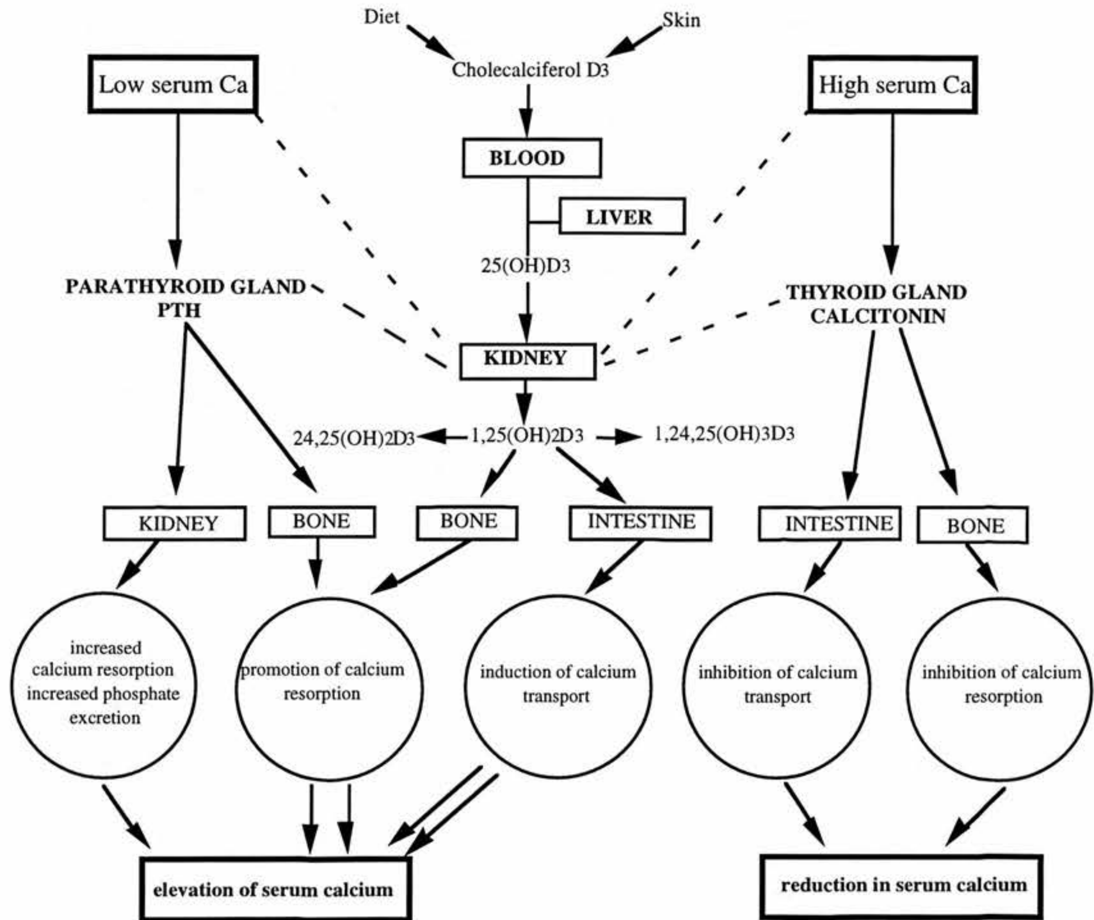

Figure 1a Calcium Homeostasis (from Norman, 1979).

1.6.1 Parathyroid Hormone

Parathyroid hormone (PTH) is an 84 amino acid linear polypeptide synthesised in cells of the parathyroid glands. In the chicken it is initially synthesised in a precursor form called pro-PTH (MacGregor *et al.*, 1973). In mammalian species

an even larger form pre-pro-PTH is known to be synthesised. The hydrophobic 23 amino acid 'pre' sequence and 6 amino acid 'pro' sequence help transport the molecule from the endoplasmic reticulum where it is synthesised to the Golgi apparatus where it is packaged into secretory granules before being released by the cell. Appropriate stimuli for the release of PTH are a fall in blood calcium or magnesium levels, although magnesium is physiologically much less important. In mammals, PTH circulates in the blood in more than one form, namely the entire peptide and a variety of fragments derived mostly from the carboxy terminal region, although also from the amino terminal region of the molecule. There is debate as to the biological activity of these forms and as to their diagnostic significance in serum (Marx, 1989a).

The major effect of PTH on bone is that of osteoclast-mediated resorption. However, the effect is probably indirect as several studies have failed to demonstrate receptors for PTH on osteoclasts. When osteoclasts are cultured, addition of PTH to the medium has no effect on cell mobility or bone-resorbing ability. These findings suggest that the effect of PTH on bone is mediated through another cell type, probably osteoblasts, which do possess receptors for PTH. Suggested mechanisms include either a paracrine interaction between the two cell types with the production of an osteoclast resorption stimulating activity factor (Chambers, 1988) or a possible requirement for physical contact between osteoblasts and osteoclasts (Marks and Popoff, 1991).

The complete amino acid sequence for chicken PTH is unknown, although it has been elucidated to position 20 at the amino terminal end (MacGregor *et al.*, 1973). It has been extracted and purified from the hyperplastic parathyroid glands of chickens made vitamin D_3 deficient, and was found not to cross react with antisera to bovine PTH (Pines *et al.*, 1984). Bioassays to measure avian PTH activity based

on production of cAMP by renal tubular cells have been developed, but no radioimmunoassay (the preferred technique) has been reported as yet.

The parathyroid glands become enlarged with the onset of sexual maturity in the egg-laying bird, but also if birds are fed diets deficient in calcium or vitamin D_3. PTH acts to increase blood calcium levels. It increases calcium absorption from the small intestine by stimulating 1-hydroxylase-catalysed production of 1,25-dihydroxycholecalciferol in the kidney, which in turn stimulates the synthesis of calbindin-28K. In the proximal tubule of the kidney it enhances calcium reabsorption and phosphate excretion. It also stimulates osteoclastic resorption of bone.

The response of laying hens to injection of mammalian PTH, measured as a rise in plasma calcium, is very rapid, and is larger than in cockerels treated identically. This suggests the presence of cell receptors in medullary bone, or that PTH prevents the shell gland from depositing calcium onto the eggshell. It seems that the target organ receptors are more sensitive to this hormone than those of mammals (Dacke, 1979). There is indirect evidence for cyclical fluctuation in PTH levels throughout the ovulatory cycle of the hen, based on the differing morphologies of the osteoclasts and osteoblasts in medullary bone at different stages in the cycle (see earlier section), and in the variation seen in both blood calcium and $1,25(OH)_2D_3$ levels.

1.6.2 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$)

The importance of $1,25(OH)_2D_3$ in calcium homeostasis in the chicken is the subject of a review by Soares (1984). Cholecalciferol (vitamin D_3) is obtained either from the diet or synthesised in the skin from the U.V. irradiation of the precursor 7-dehydrocholesterol. In the case of the chicken, their confinement indoors means that essentially all D_3 used by the bird is supplied in the diet. Cholecalciferol is first

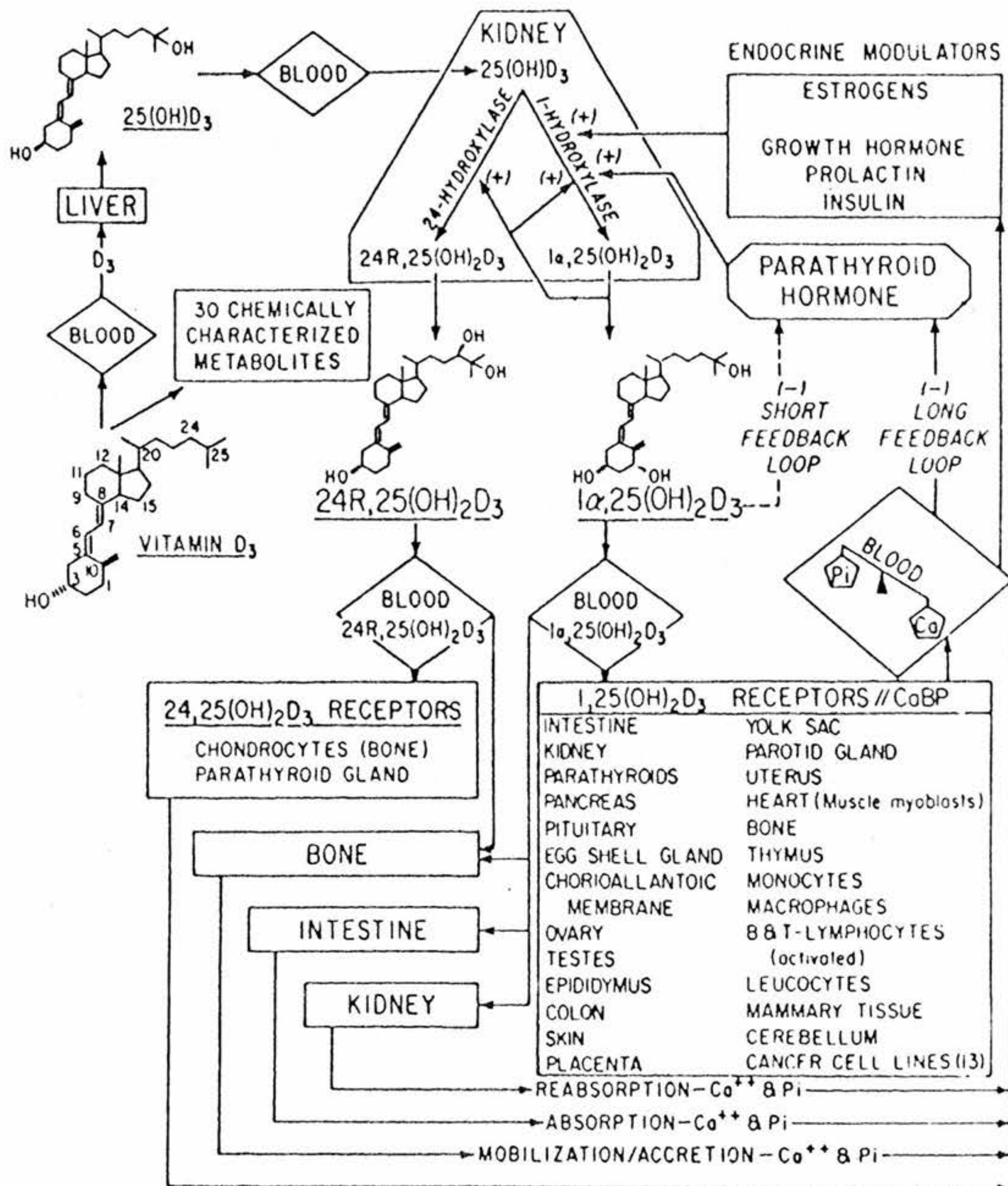

Figure 1b. Vitamin D metabolism (from Henry and Norman, 1992)

hydroxylated in the liver by the enzyme-complex 25-hydroxylase to form 25-hydroxycholecalciferol ($25(OH)D_3$), which is quantitatively the major circulating metabolite (ng/ml) within the blood. A second hydroxylation takes place within the kidney. A mitochondrial 1-hydroxylase system catalyses the synthesis of $1,25(OH)_2D_3$, generally accepted to be the metabolically active, truly hormonal form of vitamin D. Circulating levels are typically pg/ml. $25(OH)D_3$ can also be converted to $24,25(OH)_2D_3$ by a second kidney enzyme system, 24-hydroxylase. 1-hydroxylase and 24-hydroxylase are reciprocally regulated (See *Figure 1b*). Whether $24,25(OH)_2D_3$ has a specific biological role is still uncertain (Norman *et al.*, 1982). Henry and Norman (1978) demonstrated that although fertile eggs were laid by hens administered only $1,25(OH)_2D_3$, they failed to hatch. However, when $24,25(OH)_2D_3$ was also provided, hatchability returned to normal. Others have argued for a role of $24,25(OH)_2D_3$ in chondrocyte development (Corvol *et al.,* 1978, Somjen *et al.*, 1983), but there is little evidence that it has a role in bone mineralisation (Dickson *et al.*, 1984).

1.6.2.1 Vitamin D receptor

$1,25(OH)_2D_3$ acts classically as a steroid hormone in that it binds to a specific receptor (vitamin D receptor; VDR) in the cell nucleus. The hormone-receptor complex is then transported specific regions of DNA and regulates genes controlling protein synthesis. $1,25(OH)_2D_3$-dependent genes include those coding for the calbindins D-9K and D-28K (which are thought to be involved in the transport of calcium across the small intestine against an electrochemical gradient), bone proteins such as osteocalcin and osteopontin, PTH, alkaline phosphatase and 25(OH)-24-hydroxylase (Pike, 1992). A fuller list is given by Darwish and DeLuca (1993) and is partly reproduced in the table below.

Table I.i $1,25(OH)_2D_3$-regulated genes

Gene name	Type of Regulation
Calbindin D-9k	positive
Calbindin D-28k	positive
Rat & human osteocalcin	positive
Mouse osteopontin	positive
C-myc	negative
Parathyroid hormone	negative
Myeloblastin	negative
Collagen type I	negative
Protein kinase C	positive
Metallothionein	positive
Alkaline phosphatase	positive
Mitochondrial ATP synthetase	positive
Cytochrome oxidase subunits I and III	positive
Rat & human $25(OH)D_3$-24-hydroxylase	positive
Vitamin D receptor	positive

The molecular mechanisms describing the regulation of some of these genes are described below. In addition to the genomically-regulated effects, $1,25(OH)_2D_3$ induces other effects e.g. the rapid, non-genomic activation of Ca^{2+} channels linked to protein kinase C activity (transcaltachia), resulting in a very fast movement of calcium across intestinal cells. (Walters, 1992).

The vitamin D receptor (VDR) is a member of a receptor super-family, which includes the thyroid hormone, steroid hormone and retinoid receptors, as well as

orphan receptors.[1] Nucleotide sequencing of human, rat and partial chicken VDR clones has confirmed structural homology between species (Darwish and DeLuca, 1993). The VDR is a protein of about 50 kDa molecular mass in the human (Norman *et al.*, 1992), and 55 kDa in the rat, mouse and pig. In the chicken, there appear to be two forms, a predominant species of about 60 kDa and a less abundant form of 58 kDa. The 58 kDa form does not appear to result from proteolysis of the larger form. VDR is extremely labile, in part due to its extreme sensitivity to elevated temperature, and also its sensitivity to endogenous and exogenous proteolysis (Pike, 1992). VDR possesses several functional domains, namely -

a). a DNA binding domain at the amino-terminal end of the protein, a highly basic region which contains a cysteine rich cluster with two zinc-finger structures (Nishikawa *et al.*, 1993), which are thought to form α-helices. The double Zn-finger motif is characteristic of the steroid receptor family. The first Zn-finger directs the specificity of the sequence, and the second, through a symmetrical interface of amino acids which is aligned by DNA binding, modulates co-operativity (Cheskis and Freedman, 1994)

b). a steroid binding domain at the carboxyl-terminal end of the protein. Mutations introduced at this region stop the receptor binding ligand, not only in the case of VDR, but for other steroid receptors too. The steroid binding region may also possess other functional domains. Other steroid hormone receptors possess short peptide regions that are directly involved in gene promoter transactivation, and peptide surfaces that influence protein-protein interactions, either with the receptor, or with currently uncharacterised proteins.

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[1] Orphan receptors are those members of the steroid-receptor gene family identified by cDNA probes for other previously identified receptors, for which known hormonal ligands have not been characterised. It is as yet unknown whether active ligands exist for orphan receptors.

c). a hinge region connecting the two binding domains. The sequences of the DNA binding domains of the different hormone receptors are highly conserved, and those of the steroid binding domains are moderately conserved (Nishikawa *et al.,* 1992).

The VDR binds to a region of DNA adjacent to the target gene called the vitamin D response element (VDRE). Other members of the receptor super-family are also associated with response elements, collectively known as hormone response elements (HREs). The VDRE is a small sequence of DNA that mediates the target gene promoter responsiveness to $1,25(OH)_2D_3$ through the VDR, resulting in either activation or repression of mRNA transcription. VDREs are made up of two functional halves consisting of nucleotide hexamers, separated by a small, non-conserved sequence. An example of a VDRE involved in activation of transcription are situated in the promoter regions of the human and rat osteocalcin genes. The VDRE is situated 400-500 nucleotides upstream of the site of transcriptional initiation. An example of a VDRE involved in the suppression of transcription is situated in the promoter region of the human PTH gene. This VDRE mediates the suppression of this gene by $1,25(OH)_2D_3$, and is located much closer to the site of transcriptional initiation - 125 nucleotides upstream.

The various HREs differ in the number of nucleotides that separate the two functional halves. Thyroid response elements have four nucleotides between the two halves, and retinoic response elements have five. VDREs have three nucleotides separating the two halves as shown by the sequences of the VDREs for calbindin D-9k and human osteocalcin below.

Calbindin D-9k	GGGTGG CGG AAGCCC
Osteocalcin	GGGTGA AGG AGGTCA

Figure 1c Structure/function of the Vitamin D receptor (VDR). From Darwish and DeLuca, 1993.

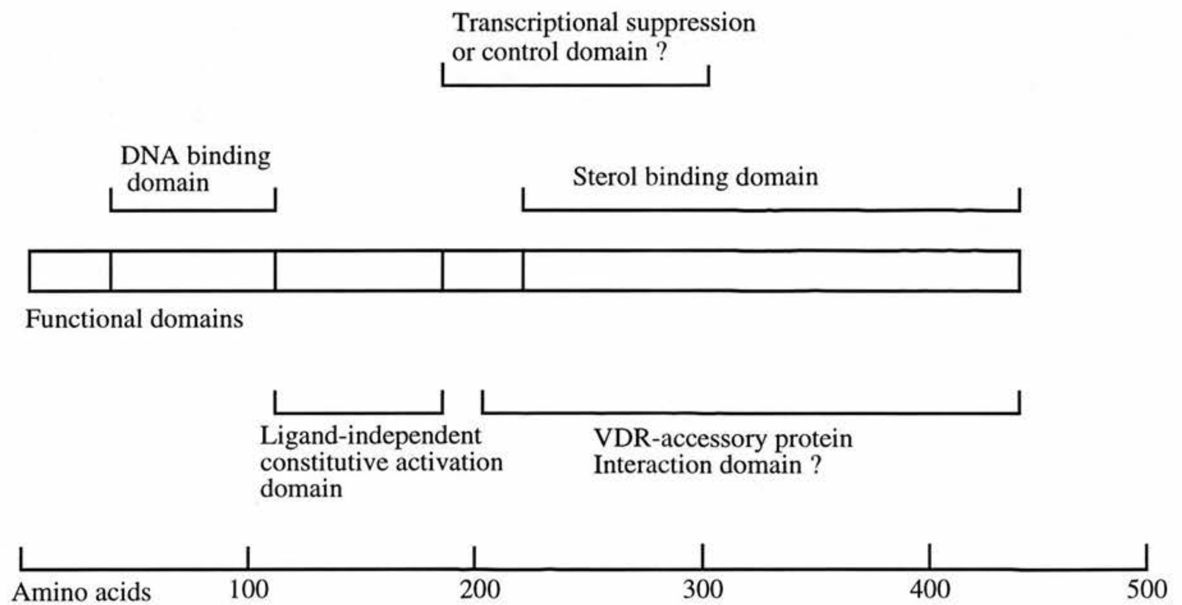
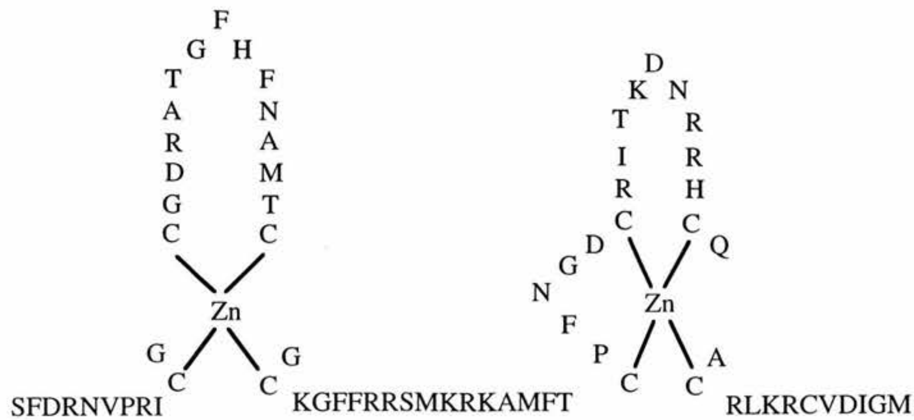


Figure 1d Amino acid sequence of the human VDR DNA binding domain, showing the two Zn-finger regions amid cysteine-rich clusters (From Freedman *et al.*, 1994).



The *in vitro* interaction between human VDR and the human osteocalcin gene VDRE has been reported to be dependant on the presence of 1,25$(OH)_2D_3$. However, pig and rat VDRs bind rat osteocalcin VDRE in the absence of 1,25$(OH)_2D_3$, although 1,25$(OH)_2D_3$ may increase the affinity of the receptor to DNA.

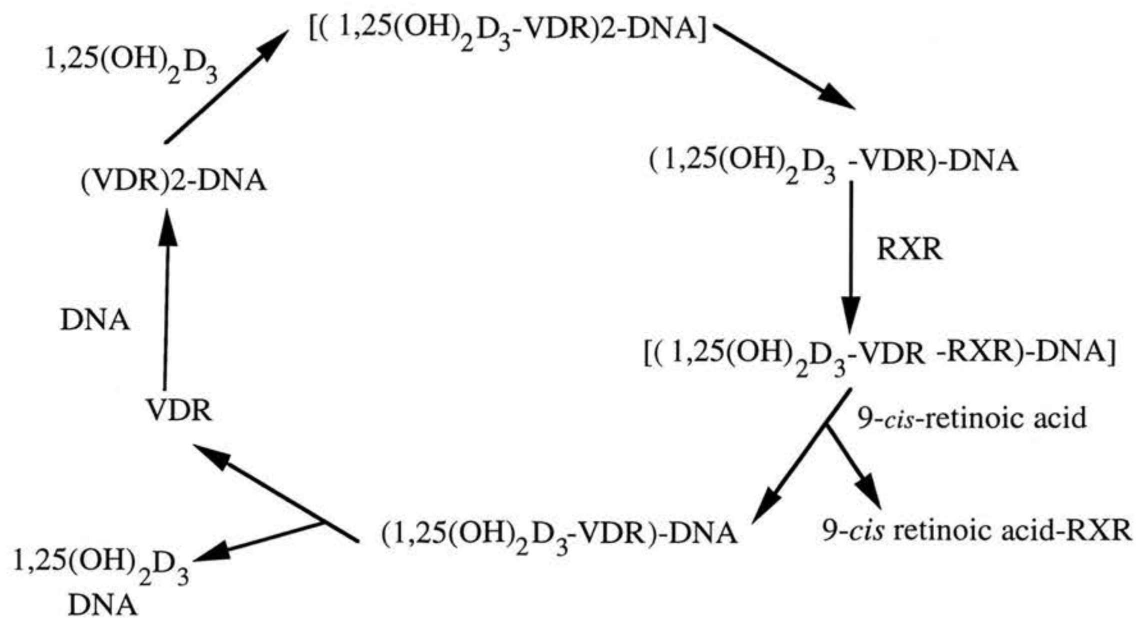
It is now realised that the concept that each steroid hormone binds to a specific receptor heterodimer is an oversimplification and, in a similar fashion to T_3Rs and RARs, other factors are involved in the interaction of VDR with DNA. A nuclear accessory factor, or factors (NAF) are required to generate the receptor-DNA complex. An NAF with a molecular mass of 55 kDa extracted from monkey kidney cells was shown to increase the strength of interaction of VDR and VDRE *in vitro* (Sone *et al.*, 1991). This requirement for an NAF for optimal DNA binding has also been reported for the retinoic acid and thyroid hormone receptors. Sone *et al.* (1991) speculated that the NAF required for optimal binding of VDR to VDRE was identical to those required for RARs and T_3Rs, and discussed the similarity in size of the VDR NAF to that associated with the RAR of cultured HeLa cells, compared with the NAF for T_3R, which has a molecular mass of 63 kDa.

A number of naturally occurring metabolites of retinol including all-*trans*-retinoic acid, 9-*cis*-retinoic acid and 3,4-didehydroretinoic acid are believed to function as signalling molecules that regulate cell behaviour during embryonic development and adult life. Receptors have a modular structure of six domains, designated A to F. Binding of retinoic acid to the E domain converts the receptor into an active transcription factor that can regulate gene transcription *via* promoters containing a retinoic acid response element (RARE). Dimerised receptors bind to RAREs via their C domains. Two classes of nuclear retinoid receptors have been identified in vertebrates, firstly, the retinoic acid receptors (RARs) and secondly, the

retinoid-X receptors (RXRs). There are three RAR and 3 RXR genes - RAR/RXR-α, -β, and -γ. The DNA-C binding domain is highly conserved between the RAR and RXR receptors, but there is no significant homology between their other domains. As a consequence of the differences in the ligand-binding domains between the RAR and RXR receptors, they have different retinoid-binding specificities. RARs bind and respond to all-*trans*-retinoic acid and 9-*cis*-retinoic acid, but RXRs bind and respond only to 9-*cis*-retinoic acid. RXRs form heterodimers with thyroid hormone receptor, VDR and peroxisome proliferator activated factor (Seleiro *et al.*, 1994). It is believed that the presence of RXR not only allows VDR to bind to VDREs which have an imperfect direct repeat of nucleotides such as rat osteocalcin VDRE (in the absence of RXR, K_d too low to measure, in the presence of RXR, K_d= 0.88 nM), but also increase the binding affinity of the VDR for those VDREs which contain direct nucleotide repeats such as mouse osteopontin VDRE (in the absence of RXR, K_d= 1.7 nM, in the presence of RXR, K_d= 0.68 nM; Nishikawa *et al.*, 1994). The chicken RXR-γ gene gives rise to two mRNA species - RXR-γ1 and RXR-γ2 - that differ at their 5' ends, and have different embryonic distributions. The predicted protein products of the two genes differ in amino acid composition at their amino-terminal regions, thought to be important in the transcription transactivation modulation by the receptor. Schrader *et al.* (1994) have shown that VDR will form heterodimers not only with RXRs, but also RARs and thyroid receptors (T_3Rs). All three heterodimers increased the *in vitro* binding affinity of VDR to VDRE, compared with VDR homodimers, but the binding affinities were altered to varying degrees depending on the class of VDRE. mOST VDRE was dominated by the VDR.RXR heterodimer pathway, and that the formation of this heterodimer induced a 3-fold higher binding affinity for the VDRE, and that stimulation with both $1,25(OH)_2D_3$ and 9-*cis*-retinoic acid also provided a 3-fold higher induction of gene activity. In contrast, for

the hOST VDRE, the VDR.VDR homodimer, VDR.T_3R and VDR.RAR heterodimers varied in both their DNA binding affinities and their induction of gene activity less than 2-fold. Schrader *et al.* (1994) suggest that the expression levels of VDRs, RXRs, RARs and T_3Rs and their specific ligands may interact to regulate the transcription of 1,25$(OH)_2D_3$-responsive genes, and that the interactions between these nuclear receptors are extremely complex.

Figure 1e Model of VDR heterodimer formation, taken from Cheskis and Freedman (1994)



Cheskis and Freedman (1994) propose an interesting model, which shows how RAR and RXR may modulate the dimerisation state of VDR in a cyclical manner (*see Figure 1e*). 1,25$(OH)_2D_3$ binds to VDR homodimer, forming a transient, unstable quaternary complex that gives way to a DNA- and 1,25$(OH)_2D_3$-bound VDR monomer. In the presence of RXR, this monomer preferentially forms a

VDR-RXR heterodimer. 9-*cis*-retinoic acid in turn destabilises the heterodimeric complex, giving free VDR, which is available to repeat the cycle.

During the course of its action, serine residues of VDR are phosphorylated by a kinase. This phosphorylation is thought to be an important step in the signal transduction of $1,25(OH)_2D_3$-dependent gene regulation. The particular kinase involved has not been definitely identified, although candidates are protein kinase A and C, and casein kinase-II. Protein kinase C has been shown to phosphorylate serine residues 51, 119 and 125. Mutation of serine 51, which lies between the two zinc fingers of the DNA binding domain (see *Figure 1d*) inhibits transcriptional activation by $1,25(OH)_2D_3$ (Hsieh *et al.*, 1991). Casein kinase II phosphorylates serine residues at the amino-terminal border of the steroid binding domain (Jones *et al.,* 1991). It has been suggested that VDR is subject to phosphorylation at multiple sites, and that perhaps each phosphorylation modulates different functions of VDR such as hormone and DNA binding, transactivation and desensitisation (Hsieh *et al.*, 1991). Phosphorylation possibly also improves binding of VDR to DNA, interaction with other transcription factors (as described above), or even VDR turnover and degradation (Pike, 1992).

The effects of $1,25(OH)_2D_3$ on cell growth and differentiation have been shown to be associated with altered proto-oncogene expression. In rat osteosarcoma cell lines exhibiting high or low levels of VDR, there is a good correlation between expression of receptor and *c-myc* mRNA (Walters, 1992). Treatment of HL-60 leukaemia cells with $1,25(OH)_2D_3$ results in a decrease in *c-myc* mRNA levels (Simpson *et al.*, 1987). Molecular studies have shown that the promoter regions of the rat osteoblast osteocalcin and alkaline phosphatase genes contain an activator protein AP-1 sequence. This sequence provides a site that is capable of binding the protein products encoded by the *c-fos* and *c-jun* proto-oncogenes. The importance

of the AP-1 sequence is that binding of the *c-fos* and *c-jun* proteins causes the osteocalcin and alkaline phosphatase genes to be negatively regulated during cell proliferation. These genes are finally expressed after the development and maturation of extra-cellular matrix and completion of cell differentiation (Owen *et al.*, 1990).

1.6.2.2 Hormonal and cellular factors influenced by 1,25-dihydroxycholecalciferol

There are profound changes in vitamin D metabolism in female chickens approaching sexual maturity and the onset of egg laying, coinciding with the appearance of medullary bone. The primary event is the increase in circulating levels of 17-β oestradiol following follicle maturation. 17-β oestradiol and circulating androgens stimulate the formation of medullary bone (Castillo et *al.*, 1979). The changes in calcium requirement due to the formation of medullary bone secondarily induce a physiological calcium deficiency. This stimulates 1-hydroxylase either directly, or indirectly due to rising PTH concentration. The result is increased synthesis of $1,25(OH)_2D_3$ which in turn induces the synthesis of calbindin-28 K resulting in increased absorption of dietary calcium (Bar and Hurwitz, 1979). Circulating levels of $1,25(OH)_2D_3$ increase 10-fold and plasma total calcium rises from 2.5 to 5-6 mM. This large amount of calcium is able to be transported in the blood due to the oestrogen-stimulated hepatic production of vitellogenin at sexual maturity (Wideman, 1990). Oestrogen also up-regulates target tissue parathyroid hormone receptors.

$1,25(OH)_2D_3$ stimulates osteoclastic bone resorption *in vivo* and *in vitro* (Merke *et al.*, 1986). The administration of the hormone to rabbits or mice causes osteoclast numbers to rise, an effect which is independent of PTH. Specific cytosolic

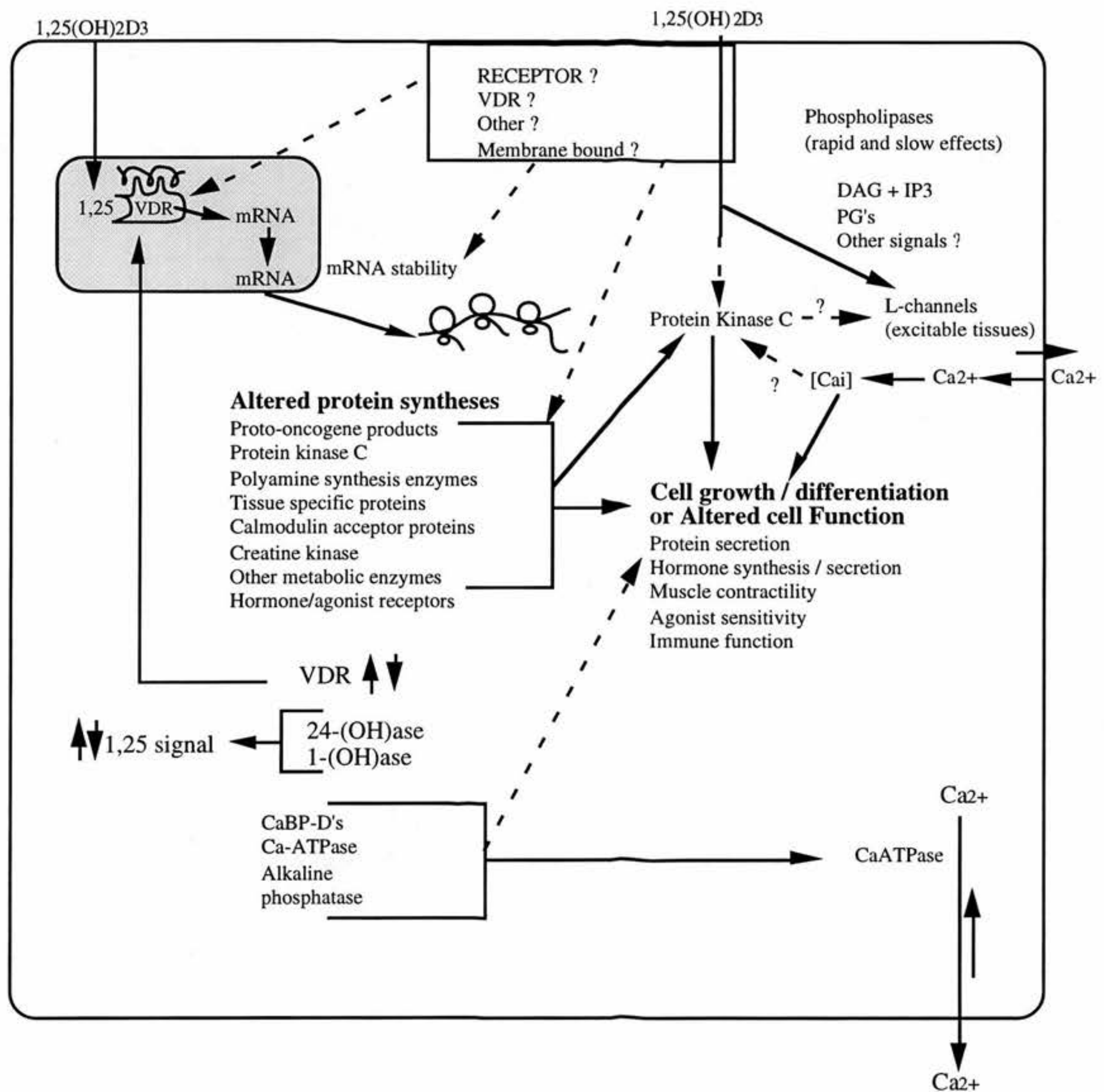


Figure 1f Recently discovered actions of 1,25$(OH)_2D_3$

(from Walters, 1992)

receptors for 1,25$(OH)_2D_3$ have been demonstrated only in osteoblasts and pre-osteoblasts, and not on osteoclasts, although monocytes, the presumed osteoclast precursors, do possess receptors (Merke *et al.*, 1986). The resorptive effects of 1,25$(OH)_2D_3$ probably involve a local regulatory mechanism within the bone microenvironment, similar to PTH (Marks and Popoff, 1991). Osteoblasts also possess receptors for 1,25$(OH)_2D_3$ (Braidman, 1990).

In addition to its role in calcium and phosphate homeostasis, 1,25$(OH)_2D_3$ has recently been found to stimulate a variety of cellular processes through genomic and non-genomic mechanisms. These are summarised in *Figure 1f.*

1.6.3 Calcitonin

Calcitonin is a 32 amino acid polypeptide containing a 1-7 disulphide bridge. It is synthesised and secreted by cells of the ultimobranchial gland in the chicken. In mammals it is rapidly released into the circulation in response to small increases in plasma ionic calcium. It can therefore be considered to be a counter-regulator to PTH. There are receptors for calcitonin on kidney cell membranes. Calcitonin also inhibits bone osteoclasts. *In vivo* administration of calcitonin in mammals causes a decrease in osteoclast numbers, and rapid ultrastructural changes are induced in organ culture following addition of the hormone to the medium. These include flattening of the ruffled border, loss of the cytoplasmic coating of the ruffled border, and a physiological separation from the underlying bone surface (Marks and Popoff, 1991).

The role of calcitonin in the egg-laying bird, as in mammals, is not very well understood. In the mature female quail, there are modest fluctuations in plasma calcitonin inversely correlated with plasma total calcium throughout the ovulatory cycle with a peak occurring about 7 hours after ovulation and a trough during

eggshell calcification (Dacke *et al.*, 1972). Chronic intra-muscular injection of calcitonin in egg-laying hens receiving a high calcium diet were associated with a decrease in the total amount of medullary bone, and an increase in the density of cortical bone in which were found several large resorption cavities indicating increased remodelling (Belanger and Copp, 1972). There appears to be no obvious effects of calcitonin on eggshell deposition - injections of the hormone at the onset of calcification had no effect on eggshell thickness (Dacke, 1979). Dacke considers the only possible role of calcitonin in the adult female to be to prevent hypercalcaemic overshoot by the fast - acting parathyroid system at the end of eggshell calcification. Alternatively, calcitonin may have a more important endocrine role in the embryo, where a large amount of calcium is mobilised from the eggshell in a short space of time. Evidence for this hypothesis is based on the observations that circulating levels of the hormone rise in the embryo from day 14 to day 19, and then drop. No calcitonin could be detected in plasma taken from chicks the day after hatching (Simkiss and Dacke, 1971).

1.7 Vitamin D, bone formation and chondrocyte differentiation

Cholecalciferol is generally regarded as playing a major role in the processes of endochondral ossification. In the absence of cholecalciferol, calcification is severely reduced, and rickets results. However, the degree of involvement is uncertain. For example, Underwood and DeLuca (1984) found that cholecalciferol-deficient, young growing rats continually infused with calcium and phosphate had blood calcium and phosphate levels identical to those of cholecalciferol-sufficient non-infused rats, and that food consumption and growth also increased to normal rates. Infused animals also had significantly higher femur ash than the

cholecalciferol-supplemented rats, and no signs of rickets (osteoid seam and epiphyseal growth plate widths were normal).

The hydroxylated metabolites of cholecalciferol are the active forms. Receptors for $25(OH)D_3$, $1,25(OH)_2D_3$ (Suda *et al.*, 1985), and $24,25(OH)_2D_3$ (Somjen *et al.*, 1982) have all been found in growth plate chondrocytes, the cells are ultimately responsible for endochondral ossification. However, the roles of the individual metabolites are still not clear. Many studies have been carried out using chondrocytes in different culture systems and at different stages of development, investigating the addition of cholecalciferol metabolites. Results have been conflicting, possibly reflecting differences in culture conditions and time, tissues from which the cells were derived, and the concentration of metabolites used (Takigawa *et al.*, 1988). Briefly, for example, in the chicken, Gerstenfeld *et al.* (1990) found that 10^{-10} to 10^{-8} M $1,25(OH)_2D_3$ caused chondrocytes derived from embryonic chick caudal sterna to dose-dependently acquire a more mature morphology, and to increase their synthesis of $\alpha 1(X)$ collagen, found *in vivo* only in hypertrophic chondrocytes. Proteoglycan synthesis was also increased, but not as dramatically. The altered cell morphology seemed to be the primary event, with biochemical changes in protein synthesis secondary to this. However, Inao and Conrad (1992) speculated that $1,25(OH)_2D_3$ was in fact capable of instigating de-differentiation. They found that in primary cultures of hypertrophic chondrocytes from embryonic chick tibial growth plate, 10^{-10} to 10^{-7} M $1,25(OH)_2D_3$ caused time and dose dependent inhibitory effects on alkaline phosphatase activity and collagen synthesis, with types X and XI being affected more than types II and IX.

1.8 Loss of bone (osteoporosis) in the laying hen

A syndrome named cage layer fatigue was described by Couch (1955), characterised by a combination of leg weakness and acute death. Bell and Siller (1962) described the pathology of the acute syndrome more fully. *Post mortem* radiographs showed virtual disappearance of rib shadows and thinning of the shafts of long bones. The ribs and shafts of the long bones were atrophic and reduced in diameter. No vertebral deformities were observed, and *ante mortem* fractures were seen in only two cases. In contrast to the findings of Couch (1955), cortical bone was not brittle. Histologically, the cortical bone was very reduced in thickness with moderately extensive resorption cavities. Trabeculae had all but disappeared. Osteoclasts were numerous and morphologically appeared to have increased activity. The number of osteoblasts also appeared to be increased and they had a cuboidal appearance again suggesting increased activity. A very small amount of uncalcified osteoid was occasionally seen on residual trabeculae. Plasma alkaline and acid phosphatase activity was increased compared with normal pullets. Urist and Deutsch (1960) described osteoporosis in White Leghorn hens during the first year of egg production following sexual maturity at about 20 weeks old. In the tibias of immature pullets there was solid compact cortical bone with no secondary Haversian systems or medullary bone. The cortical bone was 0.7-1.0 mm thick. Hens in peak lay had cortical bone that was 0.2-1.0 mm thick with enlarged vascular channels and medullary bone lining the marrow cavity and the enlarged Haversian canals. Total calcium levels varied between 2.4-3.0 mM and 3.2-8.9 mM in immature pullets and laying hens respectively. Ultra-filterable calcium levels (an approximation of ionised calcium concentration) varied between 1.3-1.8 mM and 1.6-2.2 mM in the immature pullets and laying hens respectively. By the time the birds went into a natural moult following a laying period of 1 year, cortical width was reduced to 0.2-0.5 mm. At

necroscopy, tibias were very light and brittle, shattering when cut with a knife, but bone tissue was fully calcified.

In common with humans, the consequence of osteoporosis is the greatly increase likelihood of fracture of thinning, fragile bones. Estimates of the incidence of fracture in hens are as high as 30% (Wokac, 1989). Wokac found that 81% of caged hens showed bony changes indicative of osteoporosis when killed at 17 months of age. Gregory and Wilkins (1989) showed that 29% of hens had broken bones prior to processing, with the keel, ischium and pubis showing highest incidences of fracture. By the end of processing, this figure rose to 98%. Fractures detected during this study were new and caused by removal of the hens from cages and hanging from the shackling line. Fracture rates in end of lay caged hens seem to be influenced by rearing systems. Gregory *et al.* (1991) found that 6.4% of hens reared to 18 weeks in cages had breaks compared with 16.3% of hens reared on the floor. 5% of end of lay caged hens were found to have old breaks. However, alternative production systems also had a part to play in fracture incidence. 25% of hens kept in a perchery system had fractures, and this was attributed this to flight and landing accidents.

The high rate of fractures, both old and new, in end of lay hens is important not only from the welfare aspect in the case of the former, but also because the latter can cause contamination of processed meat products by bone splinters (Gregory and Wilkins, 1992).

1.8.1 Management factors and osteoporosis

Osteoporosis in the laying hen is considered to be due to interactions between egg production, nutrition, and management systems including housing.

Forty years of selection for increased production and reduced nutrient input means that the modern hybrid lays an average of 283 eggs/year consuming 112g/day feed, compared with 207 eggs/year with a feed intake of 145 g/day. Average egg weight has also increased (Beckett, 1992). The fact that only about 50% of calcium required for egg shell synthesis is derived directly from the feed means that medullary bone turnover is ever more important in the desire for maximum egg output. By doing some simple calculations, the contributions of feed and skeletal calcium can be put into context. Assuming the feed contains about 35 g/kg calcium, this means that the average daily intake of calcium is about 3.9g. However, only about 50% of this calcium is used to form eggshell, i.e. about 1.9g, meaning that the rest must be supplied by bone, i.e. about 0.4g. Over a year's production, this contribution of skeletal reserves totals about 113g calcium. The average skeleton contains about 20g calcium (Soares, 1984).

Much work has been done on the nutrition of birds from rearing through to production, moulting and the final weeks pre-slaughter. Roland and Rao (1992) described a typical system of limiting calcium intake of hens just entering lay by continuing the pre-lay, low calcium diet until 5% of the flock commenced lay, to make sure that later-maturing birds did not consume excess calcium, putting them at risk from kidney stones and blocked ureters. The authors clearly showed that this was detrimental to bone quality in terms of reducing bone weight and bone-breaking strength and concluded that because a hen is basically in peak production as soon as lay starts, the lack of dietary calcium will place an undue emphasis on bone resorption which may predispose to skeletal problems later on. Hens in lay are fed a diet containing about 30-35 g/kg calcium usually in the form of ground limestone ($CaCO_3$). Adding extra calcium to the diet is not necessarily of benefit as absorption of dietary calcium from the small intestine is largely an active process

under the direct control of $1{,}25(OH)_2D_3$. However, Moore *et al.* (1977) found that increasing dietary calcium from 32.2 to 37.8 g/kg, and dietary phosphorus from 6.5 to 10 g/kg significantly increased radius breaking strength in caged hens, but only after 12 months. There were no significant differences after 4 months. In contrast Wilson (1991) found that increasing dietary calcium from 36 to 42 g/kg and dietary phosphorus from 5.2 to 6.9 g/kg during the last 8 weeks of production had no significant effect on tibial breaking strength and force. The only group which showed significant improvement were birds which had ceased to lay for three or more weeks before testing. The explanation put forward for this finding was the formation of 'stable' medullary bone within the tibias of these non-producing hens, thus implying a biomechanical role for medullary bone. There is evidence to suggest that feeding forms of particulate calcium may be beneficial in that dissolution in the crop and gizzard may be slowed down. The result would be that calcium was still being absorbed from the gut in the dark period during shell formation when the hen is not feeding. Similarly, Farmer *et al.* (1986) showed that tube-feeding birds 2.5 g calcium during the last 14 hours of shell formation reduced the eggshell calcium derived from the skeleton to 15%.

Phosphorus nutrition has also been implicated in laying hen osteoporosis. Phosphorus is present in diets in inorganic and organic forms. Inorganic supplements, usually dicalcium phosphate, are added to boost the phosphorus content of diets, and are generally assumed to have an availability of 100%. Phosphorus in materials derived from plants is mainly in an organic form, phytate, and is generally assumed to have an availability of 30%. Adding dicalcium phosphate to the diet increases the cost, so there has been a trend to cut the total phosphorus content of poultry diets. However, there is evidence to suggest that the availability of inorganic sources of phosphorus may be less than 100%, which in the

case of hens, means that their lower phosphorus intake might be marginal. A low intake of dietary phosphorus stimulates 1-hydroxylase, and $1,25(OH)_2D_3$ enhances calcium and phosphate absorption from the intestine and bone resorption. Raised plasma and urine calcium are seen (Roland and Rao, 1992). Hence a diet marginal in phosphorus could accelerate bone loss in hens.

Laying hen rearing and housing systems have been shown to influence bone characteristics. The great majority of laying hens in Great Britain are housed in battery cages, but the success of the animal welfare lobby has meant that the public is now willing to pay a premium for eggs produced in alternative systems such as barns containing terraces or percheries, with or without access to outdoor runs, which are perceived to provide the birds with higher welfare standards. Rowland *et al.* (1968) compared tibial breaking strength and ash at 5 and 10 months of age and found that both were significantly higher in hens kept in floor pens compared with hens kept in battery cages, at two levels of dietary phosphorus. An interesting result from this experiment was that caged cockerels also had reduced tibial breaking strengths compared with cockerels kept in floor pens, evidence that restriction of movement plays a part in the development of bone fragility in birds in common with humans and other animals. Similar results were obtained in a later experiment (Rowland and Harms, 1970b). Norgaard-Nielson (1990) found that humerus breaking strength was influenced by the frequency of vigorous wing flapping movements which in turn was related to the housing system used. The strongest bones and most wing flapping movements were found in hens kept on deep litter, followed by those kept in a system using wire floors, and lastly those from battery cages. However, it is of note that the deep litter and perch hens laid fewer eggs. Wilson *et al.* (1993) found a small benefit, in terms of tarsometatarsal trabecular bone volume, in hens kept in cages fitted with perches, compared with hens kept in

cages without perches, although all birds were judged to be osteoporotic, and the mechanism by which the rate of loss of bone was reduced was unclear.

1.8.2 Measuring osteoporosis

The accepted definition of osteoporosis is a reduction in the amount of bone present, where the chemical and biochemical composition of the remaining bone is normal and unaltered in any way. Many techniques have been used over the years in human and animal studies to directly or indirectly quantify the degree of osteoporosis or to measure the rate of loss (or indeed gain, following therapeutic treatment) of bone. Simple examination of radiographs from human osteoporotics reveals sequential loss of trabeculae within weight-bearing bones such as the femoral head (Jowsey and Gordan, 1971). In the human spine, the lumbar vertebrae show loss of spongiosa following almost complete mobilisation of the trabeculae, resulting in a hollow box-like appearance of the vertebrae. The vertebrae become deformed in a biconcave manner, and partial (wedge) or complete (crush) fractures may result. Indirect measurements of bone mineral content can be made using radiographs incorporating a standard such as an aluminium step-wedge, or vials of increasing concentrations of calcium phosphate solution. More sophisticated techniques have evolved in recent years such as single photon absorptiometry (Wahner *et al.*, 1983, Safadi *et al.*, 1988), dual energy X-ray absorptiometry and quantitated computed tomography (Murby and Fogelman, 1987, Pacifici *et al.*, 1990, Genant *et al.*, 1982). The advantage of all these techniques in both human and animal studies lies in their non-invasiveness. Direct and therefore invasive techniques for the quantitative measurement of bone are based on histomorphometric analysis of bone samples. Histomorphometry is defined as the quantitative measurement of tissue components using geometrical probabilities (Arnala, 1991). Thus, three-dimensional structures

can be assessed on the basis of two-dimensional images. Samples of bone (obtained by biopsy from humans) may be decalcified or preferably left undecalcified before being cut and stained. Decalcified bone is easier to section, but the morphological details of osteoblasts, osteoclasts and osteocytes are lost. Various parameters can be calculated using histomorphometry including cancellous bone volume, osteoid volume, osteoid surface and resorption surface. Tetracycline double labelling gives dynamic information such as rates of bone formation and resorption.

The comparatively sophisticated techniques described above have been used in the study of human idiopathic and iatrogenic osteoporosis, as well as in animal studies modelling the human condition. Unfortunately, they have not been used in the published work on avian osteoporosis to any great extent. This is largely due to the presence of medullary bone within many of the long bones of the avian skeleton which, using stains such as Von Kossa, is indistinguishable from the trabecular bone with which it is intimately associated. It is also impossible to differentiate bone types radiologically. Studies on the bone quality of laying hens have made use of comparatively unsophisticated techniques such as the measurement of ash content of bone and bone breaking strength. These techniques have clear disadvantages. Measurement of ash is essentially a measure of bone mineral content, but gives no information as to whether the bone is normally or abnormally mineralised (as in osteomalacia), or the compartmentalisation of the bone between cortical, trabecular or medullary components. Measurement of bone breaking strength by techniques such as tension, compression, shear and torsion tests are considered to evaluate mechanical properties of the bone and to be superior to the more commonly used three point loading test (Wilson, 1991). Bone breaking strength measurements have been made in experiments investigating the effects of housing in laying hens and cockerels (Rowland *et al.* 1968, Rowland and Harms, 1970b), oestrogen treatment of

caged layers and roosters (Rowland and Harms, 1970a) etc. These studies are described in a later section. Bone breaking strength measurement in hens does not take into account the contribution of medullary bone, as yet unquantified, although researchers consider it to confer no mechanical strength, due to its diffuse nature and rapid turnover. It is also important to note that in human studies, it is well known that fracture risk is not completely predictable from measurement of bone mineral content. Material and geometric properties of the bone are also important. Material properties include the brittleness and indeed breaking strength of the bone. Also of importance are the accumulation of cement lines over time, maturation of the mineral crystal and changes in collagen cross-linking. Changes in trabecular microstructure are also relevant. Loss of trabeculae leads to deficient trabecular connections resulting in increased porosity and weakening of the structure (Marcus, 1991).

1.8.3 How quickly do birds become osteoporotic ?

The development of bone fragility in hens has been the subject of little study. Cox and Balloun (1971) found that depletion of femoral bone (measured as ash) commenced with the start of egg production, and that it increased linearly over the first 25-30 eggs produced by the hen. However, Rowland and Harms (1972) found that tibial breaking strength in hens caged at 4 weeks of age gradually declined over a 20 week period, with a greater rate of fall after 16 weeks. In this study, birds kept in floor pens showed a progressive rise in tibial breaking strength until 12 weeks of age followed by a fall starting around 16 weeks of age. At all ages tested, caged birds had a lower tibial breaking strength than birds kept in floor pens. The authors do not give information as to whether any of the hens in this study reached sexual maturity and began to lay eggs. They do suggest that placing pullets in cages

prevents their skeletal system from developing sufficient strength to allow a gradual decrease in bone mass during the laying period. In other words, in a concept familiar in the study of human osteoporosis, peak bone mass is not being attained.

1.8.4 The aetiology of osteoporosis

In summary, osteoporosis in the laying hen is due to interactions between egg production, nutrition and management systems. The modern bird lays nearly 40% more eggs per year and consumes about 25% less feed than the old-fashioned type. Selection for increased egg production has resulted in birds which do not lay eggs in clutches with pauses between clutches, but rather lay nearly an egg a day. The development of battery houses with controlled environmental conditions means that birds are exposed to long day-length all year round, which stimulates a daily ovulation. Under natural conditions, the shorter day-lengths as winter approaches cause birds to stop laying.

As discussed earlier, nutrition of birds, particularly calcium and phosphorus nutrition, is thought to be involved in osteoporosis in hens. As absorption of dietary calcium is under strict hormonal control, increasing the amount of calcium in the feed does not decrease the reliance on medullary bone turnover for the synthesis of eggshell.

Lighting and housing are likely to be important in other ways. The practice of lighting up birds at ever decreasing ages is likely to cause repartioning of a more significant proportion of structural bone, if the immature pullets have not reached 'peak bone mass'. The stocking densities in commercial systems are so high as to encourage inactivity, which may lead to decreased bone loading. Birds kept four to a cage at a currently legal stocking density can barely move, let alone flap their wings or engage in any kind of locomotory activity.

1.9 Tibial dyschondroplasia in broilers

Tibial dyschondroplasia (TD) is a disorder primarily of rapidly growing broiler chickens, although it has also been described in turkeys and meat strains of duck. It was first described by Leach and Nesheim (1965). TD affects the long bones, in particular the proximal tibiotarsus and is characterised by the presence there of an avascular mass of cartilage. The lesion varies in size and shape from a small localised plug of cartilage located towards one side of the growth plate to a large symmetrical mass extending into the metaphysis. Often, the only gross visible sign of TD is a thickening of the growth plate. Histologically, the lesion consists of pre-hypertrophied chondrocytes. In TD the process of chondrocyte differentiation is arrested at the pre-hypertrophic stage. Lowther *et al.* (1974) analysed cartilage matrix components from chickens with and without TD and found that there were no significant changes in the cartilage content of total collagen and proteoglycan in affected birds compared with unaffected controls. Likewise, the proteoglycan composition was virtually identical between the two groups of birds. However, they did find that the rate of synthesis of proteoglycans, as measured by incorporation of ^{3}H-acetate into the sulphated polysaccharide fraction of cartilage, was greatly reduced. They suggested that inhibition of proteoglycan destruction may result in a blockage to blood vessel penetration of cartilage. Gay *et al.* (1985) studied histochemically the activities of alkaline phosphatase and carbonic anhydrase within the growth plate and lesion of birds affected with TD. Alkaline phosphatase (a vitally important enzyme in calcification of matrix) activity appeared as chondrocytes began to hypertrophy as in normal growth plates. The chondrocytes within the main part of the lesion showed hardly any alkaline phosphatase activity, but this was felt to be due to necrotic changes secondary to cell death and proteolysis of cell contents. Lilburn and Leach (1980) found biochemical changes within the

TD lesion suggestive of reduced oxidative capacity, and Gay and Leach (1985) found that the development of TD was not due to a fault of chondrocyte proliferation.

In normal broiler flocks, up to 10% of birds may be clinically affected and many more may be sub-clinically affected. Affected birds may show bowing or even fracture of the tarsometatarsus, and become so lame that they sit on their hocks and have difficulty in reaching food and water. They must then be culled. The development of TD has been followed radiologically (Lynch *et al.,* 1992) and areas of radiolucency corresponding to the presence of lesions can first be seen at two weeks of age.

1.10 Experimental Induction of Tibial Dyschondroplasia

See also the reviews of Leach and Lilburn (1992) and Cook *et al.* (1994).

1.10.1 Genetic Selection

Genetic selection of broilers has been used to produce lines of broilers with high and low incidences of TD. Selection of lines can be done rapidly over 3 or 4 generations using progeny testing (Sheridan *et al.*, 1978), or more satisfactorily, non-invasive testing of parent stock using imaging techniques (Bartels *et al.*, 1989, Wong-Valle *et al.*, 1993) or X-rays (Riddell, 1976). Wong-Valle *et al.* (1993) estimated the heritabilty of TD of 7 week old birds in their study at 0.74. Phenotypic characteristics which have a heritability score of 0.5 or over are highly heritable. Sheridan *et al.* (1978) claimed from their experimental results that a major sex-linked recessive gene was involved in the production of TD. Breeding companies are currently selecting against TD with some success (Dr. A. Tinch, Ross Breeders, personal communication).

1.10.2 Manipulation of Dietary Cations and Anions

A high incidence of TD can be induced in a variety of ways. Many studies have been conducted over the years investigating the effects of feeding diets containing altered ratios of cations to anions since Leach and Nesheim (1972) reported that dietary manipulation appeared to influence the incidence of TD. Manipulation of dietary cation/anion ratio can be achieved fairly easily. Concentrating on the common ions Na^+, K^+ and Cl^-, a high Na^+/K^+ intake relative to Cl- can be achieved by feeding metabolisable salts of the cations e.g. sodium citrate or sodium lactate. Similarly, a high intake of Cl^- relative to Na^+/K^+ can be achieved by feeding either $CaCl_2$ or NH_4Cl. In the case of $CaCl_2$, some of the dietary calcium is now provided in this form rather than $CaCO_3$. In the case of NH_4Cl, the NH^{4+} ion is excreted as uric acid, leaving Cl^-. A high intake of Na^+/K^+ relative to Cl^- results in a metabolic alkalosis, and a high intake of Cl^- relative to Na^+/K^+ results in a metabolic acidosis (Ruiz-Lopez and Austic, 1993). This ability of dietary cations and anions to influence acid-base balance is due to homeostatic mechanisms within the distal tubule of the kidney involving active absorption of Na^+, passive absorption of Cl^- and H^+, and the enzymatic synthesis of HCO_3^- (Bowman and Rand, 1980). Sauveur and Mongin (1978) found that metabolic acidosis induced by excessive intake of Cl^- or NH_4Cl, increased the incidence of TD, whilst raising the intake of Na or K reduced it. Other similar results have been reported (Ruiz-Lopez *et al.*, 1993, Simons *et al.*, 1987). It was speculated by Mongin and Sauveur (1977) that a direct relationship existed between acid-base balance (measured as plasma bicarbonate) and incidence of TD, but others have found no such relationship (Ruiz-Lopez and Austic, 1993). Mongin (1981, 1989) developed the theory that animals try to regulate the blood base excess equivalent to zero to maintain homeostasis and that deviation in dietary cations and anions which affect homeostasis may also affect

growth or production by affecting metabolic pathways. It was speculated that NH_4Cl-induced metabolic acidosis may affect the renal synthesis of $1,25(OH)_2D_3$ (Mongin and Sauveur, 1977). Many of the cation/anion studies have focused on the dietary content of mEq (Na + K) - Cl, as these are the most important in maintenance of acid/base balance in the kidney. But Nelson *et al.* (1981) found that other cations and anions influence chick performance. Halley *et al.* (1987) extended the range of cations and anions under investigation to look at the cations Ca^{2+} and $Mg^{2+}$and the anions PO_4^{2-}, Cl^- and SO_4^{2-}. In a series of experiments using 3 week old broiler chicks and measuring blood pH, pCO_2 and base excess they found that narrowing the cation/anion ratio (essentially by lowering dietary calcium but keeping dietary phosphorus constant) from 1.31:1 to 0.9:1 increased the incidence of TD. Raising total dietary levels of calcium and phosphorus, but keeping cation/anion ratios similar still resulted in some birds with TD although the percentage of affected birds was reduced. In another experiment, reducing blood base excess (i.e. inducing metabolic acidosis) by feeding chloride or sulphate increased the incidence of TD. Blood pH was reduced, although not significantly. In a third experiment, increasing the cation/anion ratio to 2.2:1 with Ca^{2+} or K^+ reduced the high incidence of TD seen when it was lowered to 1.6:1 by addition of PO_4^{2-}. The anions Mg^{2+} and Na^+ had no beneficial effect.

1.10.3 Calcium and Phosphorus

Edwards and Veltmann (1983) turned their attentions to calcium and phosphorus in the experimental induction of TD. They used a practical ration based on maize and soya meal containing normal amounts of Na, Cl, and K (1.8, 2.5 and 9 g/kg) and supplemented it with calcium carbonate and dicalcium phosphate to give a range of diets containing 6.3 and 16.7 g/kg calcium and 5.3 to 10.9 g/kg phosphorus.

Broiler chicks were examined at 2 weeks of age for TD (gross examination of sagittal sections of growth plate only) and tibias were ashed. pH, pCO_2, pO_2, base excess, bicarbonate and total CO_2 were measured in blood samples, but there were no differences between treatments from the normal literature values. Optimum growth occurred when the chicks were fed the 14.9 g/kg calcium, 10.1 g/kg phosphorus diet. Bone ash was depressed on the low phosphorus diets. 21.5 g/kg calcium and 11.2 g/kg phosphorus produced the highest bone ash. The highest incidences of TD were seen in chicks receiving the low calcium / high phosphorus diets. It was concluded that the ratio of calcium to phosphorus in the diet is very important in the induction of TD, and that high dietary phosphorus may act similarly to high dietary Cl (although without inducing an acidotic state).

Riddell and Pass (1987) confirmed this study in an experiment using diets containing 8 to 14 g/kg calcium and 5.5 to 7.5 g/kg available phosphorus (equivalent to 7.2 to 9.8 g/kg total phosphorus). Birds fed 8 g/kg calcium and 7.5 g/kg phosphorus had the highest incidence of TD at both 4 and 7 weeks of age. However, they speculated that the thickened growth plates seen in the 2 week old chickens fed low calcium / high phosphorus diets were caused by hypocalcaemic rickets, and quoted Long *et al.* (1984) as saying that calcium deficiency rickets can proceed to tibial dyschondroplasia, possibly as an attempt to adapt to the calcium deficiency by increased hypertrophic growth of chondrocytes.

1.10.4 Administration of Mycotoxin

Walser *et al.* (1982) first reported that young broiler chickens consuming the fungus *Fusarium equiseti* (sometimes referred to in the earlier literature as *F. roseum* 'Graminearum') developed TD. Chu *et al.* (1988) tested the effects of a variety of fungi on the development of TD and found that only F. equiseti # 15 was able

significantly and repeatedly to induce TD (60-70% when fed 20 g/kg air-dried fungus in the diet). The water soluble active principle of the fungus was isolated and named fusarochromanone (Lee *et al.*, 1985). Wu *et al.* (1993) found that 100% of broiler chicks fed 75 mg/kg fusarochromanone developed TD. 200 mg/kg added Zn or Cu slightly reduced the TD-inducing effects of fusarochromanone. The authors speculated that fusarochromanone-induced TD may be the result of deranged copper metabolism, on the basis that dietary copper deficiency has been associated with skeletal lesions grossly similar to TD (Carlton and Henderson, 1964) and depressed superoxide dismutase activity (Bettger *et al.*, 1979). However, in their experiment, they found that activities of the copper-containing enzymes superoxide dismutase and caeruloplasmin were unchanged. Walser *et al.* (1988) speculated that selenium might interact with fusarochromanone to reduce the incidence of TD in dosed chicks, but found this was not the case. Krogh *et al.* (1989) describe the isolation of fusarochromanone from pelleted feed samples from two broiler farms with a high incidence (up to 56%) of TD. But, Wu *et al.* (1993) conclude that fusarochromanone intoxication is unlikely to be a major cause of field cases of TD, as analysis of a number of compound feeds revealed only tiny concentrations of naturally-occurring fusarochromanone.

Lawler *et al.* (1987) confirmed that fusarochromanone-treated chickens developed TD, and also demonstrated histochemically that defective chondroclasis is not the cause of accumulation of cartilage.

1.10.5 Thiuram and Disulfiram

Vargas *et al.* (1983) demonstrated that addition of 30 mg/kg diet of thiuram (bis[dimethylthiocarbamyl] disulphide, a fungicide used for treatment of seeds and fungal infestations in plants) could induce severe TD in broiler chickens. Similar

results were reported by Veltmann *et al.*, (1985). Edwards (1985) confirmed these studies, finding that 30 mg/kg significantly raised the incidence of TD from 42% to 75% and reduced plasma total calcium from 2.1 mM to 1.9 mM in 3 week old chicks fed a TD inducing diet low in calcium and high in phosphorus. Interestingly, thiuram was found to be much less effective in inducing TD when the basal diet contained adequate levels of calcium. 16% of chicks fed the calcium-adequate diet developed TD compared with 42% of chicks fed this diet with added thiuram. In a further study, Edwards (1987) tested the effects of the closely related compound disulfiram (bis[diethylthiocarbamyl] disulphide; Antabuse) on TD compared with thiuram, again feeding the imbalanced calcium/phosphorus diet, to chicks for 3 weeks supplemented with 30 mg/kg of either compound. In the first of two experiments 43% of chickens receiving the basal diet developed TD, compared with 75% and 90% of chickens supplemented with thiuram and disulfiram respectively. In the second identical experiment, the figures were 50%, 64% and 92%. There was no significant improvement to be gained from supplementation of any of the diets with a trace element mixture containing all the trace elements shown to have a role in animal nutrition - B, Ni, Al, Sr, Br, V, Si, Sn, Cr, F, Mo, Li, Mn, Fe, Zn, Cu and I. However, both compounds were found to reduce the absorption of orally-administered ^{47}Ca, and Edwards concluded that disulfiram, a known inhibitor of microsomal enzymes of the cytochrome P450 electron transport chain, may decrease calcium absorption from the duodenum by inhibiting 25-hydroxylation and 1-hydroxylation of cholecalciferol, and thus reducing synthesis of calbindin 28-K Presumably thiuram, a molecule with a similar structure, is capable of acting similarly.

1.10.6 Cysteine and Homocysteine

Orth *et al.* (1992) found that addition of sulphur-containing amino acids to a normal diet adequate in calcium and phosphorus significantly influenced the incidence of TD in broiler chickens. Levels of greater than 7.5 g/kg cysteine, 5 g/kg l-homocysteine and 4.5 g/kg dl-homocystine were required to produce the effect, far greater than would be seen in standard diets. The TD-inducing effects of these amino acids was shown not to be due to their sulphur content. 250 mg/kg supplemental Cu to 4 weeks of age was shown to have some beneficial effects in reducing TD when added to the diet containing dl-homocystine. Cysteine and homocysteine are known to alter the composition of extracellular matrix by affecting collagen cross-linking. Homocysteine can inhibit lysyl hydroxylase and is thought to induce changes in the structure of the collagen fibril network. The net result of supplements of these amino acids may be the production of chemically and structurally abnormal matrix (Orth *et al.*, 1991). The authors hypothesised that the beneficial effects of Cu seen in this experiment may be due to the chelation of homocysteine by Cu, thus knocking out its TD-inducing properties by reducing absorption and uptake or increasing excretion. Assuming an intake of 100 g/day of feed, the daily intake of Cu would be 25 mg or 4 mmol. 7.5 g/kg cysteine would result in a daily intake of 0.75 g cysteine, or 6 mmol. A mole of cysteine will chelate at least one mole of Cu, so this explanation is feasible.

1.10.7 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$)

Edwards (1989a) studied $1,25(OH)_2D_3$ in relation to TD. He tested the effects of adding D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ to a basal diet containing 7.5 g/kg calcium, 7.6 g/kg phosphorus, 3.2 g/kg Cl, either with or without disulfiram. The basal diet contained no cholecalciferol (D_3). It was found that addition of 10 μg/kg

$1,25(OH)_2D_3$ significantly reduced the incidence of TD in the broiler chicks, both with or without addition of disulfiram, although there was not such a marked effect in the birds receiving disulfiram. There was no effect of supplemental D_3 on either performance or TD, which is rather surprising as Edwards based his diagnosis of TD on naked eye examination of growth plates, and in this experiment it is very likely that some of the chicks thought to have TD had in fact hypocalcaemic rickets. The minimum requirement for D_3 of broiler chickens is 5 µg/kg. In an earlier paper (Edwards, 1984), Edwards had reported no such beneficial effect of $1,25(OH)_2D_3$ on TD, but in this work chicks were dosed orally with 10 ng/day in propylene glycol, compared with an estimated intake of over 100 ng/day when fed a diet containing 10 µg/kg. Edwards speculated (1989a) that the effect of supplemental $1,25(OH)_2D_3$ was due to either innate inadequate synthesis of the hormone in young broilers, or to high dietary D_3 supplements in the female breeders suppressing 1-hydroxylase activity in the young bird. Stevens *et al.* (1984) showed that maternal dietary D_3 could influence turkey poult 1-hydroxylase activity. High D_3 resulted in peaking of 1-hydroxylase at 10-20 days post-hatch, whereas low D_3 resulted in high 1-hydroxylase activity from 1-10 days. Innate inadequate 1-hydroxylase activity is unlikely, as enzyme activity is switched on very early on in the chick embryo, with maximal activity seen in the 18-day old embryo prior to hatch at 21 days (Moriuchi and DeLuca, 1974, Bishop and Norman, 1975). It is likely that 1-hydroxylase would rise in tandem with increased bone growth and hence increased calcium requirement, maximum allometric bone growth occurring at three weeks (Thorp, 1988). Therefore the low levels of 1-hydroxylase seen in the very young chick or turkey poult may be physiologically normal.

Sanders and Edwards (1990) reported an experiment investigating the effects of $1,25(OH)_2D_3$ on TD in growing turkeys. Using diets similar to those in his broiler

experiments i.e. containing marginal calcium, they found that a very high proportion of the turkeys developed rickets (as assessed by gross changes visible in the growth plate) and that 1,25$(OH)_2D_3$ did little to alleviate this situation. The incidence of TD at 14 weeks was low, but this was undoubtedly influenced by the low growth rate and final weight of these birds. When diets were used containing more normal (NRC recommended) levels of calcium and either 22.5 (low) or 67.5 (high) µg/kg D_3, the incidence of rickets was lowered. Turkeys fed these diets initially had a low incidence of TD - 0-3% at 3 weeks, but at 14 weeks this rose to 65-83 %, and several of the lesions showed signs of necrosis. In birds fed diets containing NRC recommended levels of calcium and 67.5 µg/kg D_3, 71% of 14 week old turkeys had TD, compared with 54% receiving an identical diet supplemented with 10 µg/kg 1,25$(OH)_2D_3$. It is possible, given the high growth rate of the Large White turkey, and the longer rearing time of these birds compared with broiler chickens, that a higher dose of 1,25$(OH)_2D_3$ may have given improved results.

Thorp *et al.* (1991) confirmed that diets imbalanced in calcium and phosphorus could induce a high incidence of TD in broilers, and showed for the first time the importance of histological examination of growth plate sections for the accurate diagnosis of TD within the context of nutritional experiments. A significant proportion of birds raised on this diet had growth plates that showed signs of hypocalcaemic rickets - an increase in the width of the proliferating zone containing disorganised, morphologically-abnormal chondrocytes - in the absence of other signs such as lowered plasma calcium or raised plasma alkaline phosphatase.

1.11 The aetiology of tibial dyschondroplasia

Researchers have speculated over the years that field cases of tibial dyschondroplasia are due to a variety of causes e.g. copper deficiency, mycotoxin

contamination of feed, etc. Others have speculated that the decreased vascular penetration of the growth plate is due to synthesis of abnormal cartilage, or that the relatively deep growth plate of the broiler chicken compared to that of the Leghorn strain predisposes to hypoxia. Similar lesions have been produced experimentally by surgical interference (Riddell, 1975c). But, the variety of disparate ways in which a high rate of TD can be induced experimentally in broiler chickens leads to the suggestion that TD is in fact the end result of failure of one or more biochemical processes. It has been suggested that an analogy can be drawn to cirrhosis of the liver (Dr. B. Thorp, personal communication), where several factors such as alcohol, infection with viruses or bacteria result in degeneration of hepatic tissue and resultant fibrosis.

The very fast growth of broilers is without doubt a major factor in the aetiology of TD, as a reduction in the rate of growth by feed restriction or daily periods of feed withdrawal is known to reduce the incidence of TD (Poulos *et al.*, 1978; Huff, 1980; Edwards and Sorensen, 1987). Interestingly, in view of evidence of a genetic involvement in TD, an obvious difference in a line of birds bred for high and low susceptibility to TD is a difference in body weight, with the low susceptibility line having a reduced body weight (Leach and Nesheim, 1972).

CHAPTER 2

NUTRITION AND BONE QUALITY

Nutritional manipulation is an attractive concept in the desire to improve bone quality in poultry. It is comparatively easy to effect, unlike modification of production systems which requires high capital input and may not be cost effective to today's producers working on increasingly tight profit margins. The effects of nutrition of both broiler chickens and laying hens on bone metabolism are wide-ranging. Both macro- and micro-nutrients are known to be important for optimum bone quality, and will be discussed within the context of both the nutrition of broiler chickens and laying hens. Also discussed will be the potential role of certain chemically synthesised vitamin D derivatives.

2.1 Protein

Dietary protein provides amino acids and nitrogen for the synthesis of body protein and nitrogen containing compounds. In the chicken, 10 amino acids are essential (arginine, lysine, histidine, leucine, isoleucine, valine, methionine, threonine, tryptophan and phenylalanine, Scott *et al.*, 1982) and must be supplied by the diet, the rest can be synthesised by the animal. Bone consists of 70% mineral and 5-8% water, with the balance being made up of matrix proteins (Einhorn, 1990). The matrix proteins consist largely of collagen, with lesser amounts of proteoglycans, glycoproteins, phosphoproteins and lipoproteins. Dietary protein is therefore important to the structural integrity of bone as it provides the amino acids necessary to synthesise the matrix on which bone mineral is laid down. Both a deficiency and excess of dietary protein can have deleterious effect on bone. Severe protein

deficiency in monkeys produced histological osteoporosis, decreased endochondral bone formation and decreased appositional bone growth. El-Maraghi *et al.* (1965) found in protein deficient rats radiographic signs of osteoporosis caused by a decrease in matrix production. It has been suggested that the decrease in matrix production seen in protein deficiency is due to deficiency of specific amino acids such as lysine, critical in the formation of collagen cross-links. Several unusual manipulations of dietary protein or amino acids will precipitate the development of bone abnormalities in poultry. If purified diets insufficient in l-glutamate are fed, birds will develop a high incidence of leg abnormalities (de Moraes *et al.*, 1984). Chicks fed a valine-deficient diet develop a rickets-like condition (Farran and Thomas, 1988).

Experimental feeding of acidogenic diets has been shown to induce hypercalciuria (Barzel, 1969). The hypercalciuria is reduced by increasing dietary calcium. The hypercalciuric effects of high protein intake may be caused by reduced fractional renal tubular reabsorption of calcium, increased glomerular filtration of calcium, or by the use of skeletal calcium as a physiological buffer of the acid products of methionine and cysteine oxidation. In human nutrition, it has been suggested that the excessive consumption of protein by the majority of Westerners leads to calcium loss sufficient to induce osteoporosis, although it is likely that other factors are also involved. A high protein diet fed to turkeys causes an increase in the incidence of leg disorders, particularly twisted and weak legs (Stevens and Salmon, 1988, Ferket and Sell, 1989). A possible explanation for this is that the induced hypercalciuria leads to weakening of the bones, and that feeding a low protein diet corrects the hypercalciuria and causes a reduced growth rate during the critical period of bone formation. Excess levels of homocysteine or cysteine have been implicated in tibial dyschondroplasia (Bai *et al.*, 1989).

2.2 Fluoride

Excessive intake of fluoride has long been known to result in abnormal bone growth. Fluoride intoxication may result from consumption of water containing high levels of fluoride or in animals from grazing land contaminated by industrial emissions or following volcanic activity with release of fluoride-rich phosphate ash (Araya *et al.*, 1990). Fluoritic bone is dense and chalky in appearance, and exostoses are frequently seen (Prince and Navia, 1983). Histological changes are seen in bone chronically exposed to fluoride. Trabecular bone is coarsened and condensed, fibrous bone is formed sub-periosteally, cortices become thickened and matrix formation is irregular (Edwards, 1992). Affected animals are lame and sometimes kyphotic (Araya *et al.*, 1990). However, more moderate intake has been shown epidemiologically to result in a lower incidence of fracture and osteoporosis in humans consuming water that has been fluoridated (to protect against dental caries) or where drinking water naturally contains moderate (>1 mg/ml) fluoride.

Fluoride causes new bone growth by stimulating osteoblastic synthesis of matrix (Hodsman and Drost, 1989). It also appears to have molecular effects in that it increases adenyl cyclase activity and inhibits magnesium-calcium ATP-ase activity in osteoblast-like cells in culture, leading to speculation that fluoride enhances cytosolic calcium concentrations (Pak, 1989). Fluoride ions are also incorporated into bone mineral with the formation of fluoroapatite from the substitution of hydroxyl ions by fluoride ions. Fluoroapatite is more crystalline and less soluble in acid than hydroxyapatite.

The positive effects of fluoride on bone synthesis measured as an increase in bone mineral density have led to its long-term use in the treatment of human osteoporosis (Lundy *et al.*, 1989, Dure-Smith *et al.*, 1991, Hodsman and Drost, 1989). Slow release forms of sodium fluoride have been developed in an attempt to

overcome side effects such as gastro-intestinal disturbance, nausea and bone pain, and it is sometimes given with supplemental calcium and cholecalciferol as there is evidence that in the absence of optimum levels of these nutrients, the newly-synthesised bone is inadequately mineralised leading to the development of osteomalacia (Kragstrup *et al.*, 1989).

Several studies have been published regarding the effects of fluoride on bone in poultry. Chickens are relatively resistant to fluoride compared with other species. Van Toledo and Combs (1983) found that feed consumption was reduced in 52 week old hens fed graded doses of sodium fluoride for 8 weeks followed by a basal diet for 11 weeks, but only in birds receiving the maximum dose of 1200 ppm fluoride (equivalent to 2655 mg/kg sodium fluoride). Egg shell breaking strength and shell weight were also reduced. Femur weight, volume, specific gravity and breaking strength were all unaffected. Merkely (1981b) found that hens given 100 ppm fluoride (equivalent to 221 mg/l sodium fluoride) in drinking water from 0-20 weeks (rearing period) and 0-45 weeks (rearing and laying period) had significantly increased bone ash and tibia and humerus breaking strength. Interestingly, fluoride provided only during the laying period (20-45 weeks) had no effect on bone breaking strength.

2.3 Calcium

Laying hens must be fed a calcium-rich diet to ensure production of adequately shelled eggs (Narbaitz *et al.*, 1987). However, considerable variation exists in the nutritional management of hens and current feeding practices may not be best in terms of optimising bone quality. Immature pullets are fed a rearing diet containing moderate levels of calcium. When they reach sexual maturity (contemporaneous with the laying down of medullary bone) they are transferred to a

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high calcium diet (35-40 g/kg calcium). However, for reasons of economy, some producers feed a pre-lay diet at around the time of sexual maturity containing an intermediate level of calcium until 5% of pullets have come into lay. (Roland and Rao, 1992). Hybrid egg-laying strains reach maximum egg production very quickly, and therefore early maturing birds would potentially not be being supplied with adequate dietary calcium under this system, placing high demand on bone calcium.

Calcium in hen feed is generally supplied in the form of ground limestone, although crushed oyster shell, sea shell or eggshell are sometimes used. All consist chiefly of calcium carbonate. Keshavarz (1991) investigated the use of calcium sulphate (gypsum) which is cheaply available in parts of the USA, and found that 2/3 of the calcium content of the ration of laying hens could be provided in this form with no reduction in eggshell quality, provided that the remainder was in the form of crushed oyster shell.

The total intake, timing and form of calcium can influence eggshell quality and the proportion of eggshell calcium derived from bone. Farmer *et al.* (1986) found that 96 % of eggshell calcium was derived from bone when the diet contained 0.8 g/kg calcium, dropping to 30 % when the diet contained 37.5 g/kg. Intubation of birds during the last 14 hours of eggshell calcification with 2.5 g extra calcium in the form of feed supplemented with finely ground limestone reduced this further to 15%. Similarly, Etches (1987) found that hens offered free choice oyster shell particles accrued 1.8g calcium, compared with 0.3g accrued when the calcium was homogeneously incorporated into the feed. Farmer and Roland (1986) found that eggshell quality in hens intubated with ground limestone was only improved in birds that already had feed in their crops, and speculated that the presence of other nutrients or dry matter may be needed to facilitate the passage of calcium from the gizzard.

2.4 1,25-dihydroxycholecalciferol

As described in an earlier section, several workers have found beneficial effects on TD of adding the metabolically active form of D_3, $1,25(OH)_2D_3$, to the diet of broiler chickens. Studies have also been carried out investigating the effects of dietary $1,25(OH)_2D_3$ on bone parameters and eggshell quality in laying hens. Egg size increases and shell quality declines in older hens (Joyner *et al.*, 1987). It has been postulated that the ability of the kidney to hydroxylate $25(OH)D_3$ declines with age (Abe *et al.*, 1982, Joyner *et al.*, 1987). A reduction in renal synthesis of $1,25(OH)_2D_3$ is known to occur in elderly humans and is thought to be a factor in the development of Type II osteoporosis (Riggs and Melton, 1986, 1990). Feeding a low phosphorus diet to older hens is known to result in a transient improvement in shell quality, thought to be due to increased synthesis of $1,25(OH)_2D_3$ in response to hypophosphataemia. Frost *et al.* (1990) did not find any benefit in feeding up to 4.5 μg/kg $1,25(OH)_2D_3$ in addition to 55 μg of D_3 for 12 weeks on egg production, shell quality or tibial strength in 53 week old laying hens. In a second experiment however, they found a positive effect on these parameters of feeding smaller amounts of $1,25(OH)_2D_3$, 0.5 or 1.0 μg/kg, to 65 week old hens for 10 weeks, but only at levels of dietary D_3 of 0 or 12.5 μg/kg. They concluded that the commercial laying hen synthesises enough $1,25(OH)_2D_3$ from dietary D_3 to maintain shell quality but not tibia strength. In contrast, Tsang *et al.* (1990) looked at the effects of adding 5 μg/kg $1,25(OH)_2D_3$ to a diet containing 27.5 μg/kg D_3 in 32 week old hens for a period of 9 weeks. They found that eggs laid by this group had a higher specific gravity and percentage shell compared with those from the group receiving D_3 alone, and that egg production was maintained rather than gradually declining. Plasma calcium was also elevated. Similar results for egg production and shell quality were obtained by Rambeck *et al.* (1991).

2.5 1,25-dihydroxycholecalciferol analogues

The classical actions of $1,25(OH)_2D_3$ in calcium and phosphate homeostasis have already been described. Yet it now appears that this hormone has wide ranging effects on a whole variety of cells, tissues and systems. Receptors for $1,25(OH)_2D_3$ have been found in tissues as diverse as heart, mammary gland, testis, spinal cord, lung, liver and skin, using sensitive biochemical techniques such as Northern analysis and autoradiography (Walters, 1992, Cancella *et al.*, 1988).

It has been suggested that, given the wide ranging distribution of $1,25(OH)_2D_3$ receptors, that the hormone/receptor complex regulates intra-cellular calcium homeostasis by affecting the cell membrane, calcium sequestration/release in cell organelles, calcium binding proteins, or in other unknown ways (Walters, 1992).

However, $1,25(OH)_2D_3$ has also been implicated in the processes controlling the proliferation and differentiation of cells. Attention was focused firstly on the effects *in vitro* of $1,25(OH)_2D_3$ on the differentiation of vitamin D receptor-containing human myeloid leukaemia cells (Suda *et al.*, 1985), but soon differentiating effects were being studied in non-cancerous cells such as keratinocytes (MacLaughlin *et al.*, 1985). This led to clinical application such as the experimental use of $1,25(OH)_2D_3$-containing ointment for topical application to affected areas in patients suffering from psoriasis, a skin disorder characterised by hyperproliferation of keratinocytes (Holick, 1989).

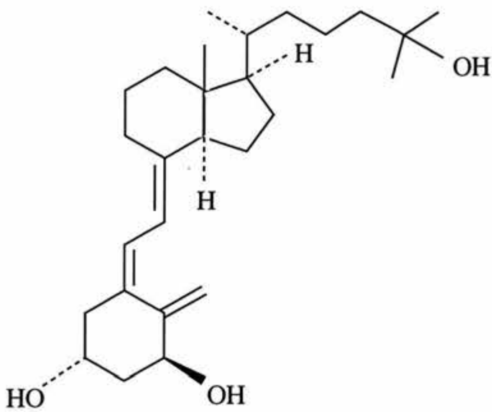
A possible side effect of treatment with $1,25(OH)_2D_3$ was its potential effect on calcium metabolism. Hypercalcaemia was found in some studies using the hormone in the treatment of human osteoporosis (Jones and Calverley, 1993). The rationale for using $1,25(OH)_2D_3$ was that it would promote calcium absorption and bone deposition. Attention turned to the development of chemical analogues of

$1,25(OH)_2D_3$, for clinical applications such as treatment of osteoporosis, osteopetrosis (a syndrome where there is failure to resorb bone), psoriasis and treatment of cancer. The rational behind using analogues for osteoporosis would be to increase bone mineral absorption from the gut, but also to try to enhance osteoblast differentiation and function. Similarly for osteopetrosis, the aim would be to enhance osteoclast differentiation, and in the treatment of cancer, to decrease cell proliferation, but increase cell differentiation (Bikle, 1992).

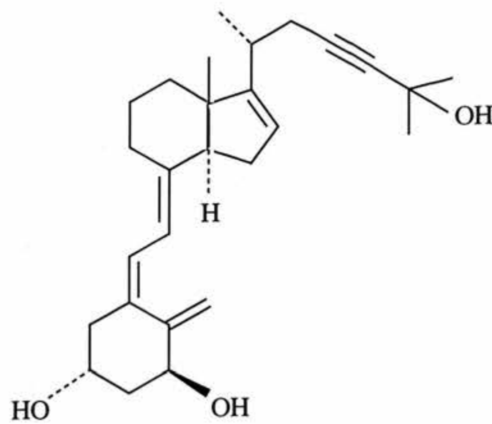
The synthesis of a large number of analogues with different properties has been achieved by complex chemical synthesis largely based on modifications of the side chain of the molecule. Replacement of H atoms with F produces compounds with more pronounced effects on calcium metabolism (more calcaemic) than $1,25(OH)_2D_3$. Modifications aiming towards the production of non-calcaemic analogues have concentrated on carbon atoms 22, 23 and 24, because changes here are not thought to affect binding to the receptor. The analogues are thought to work similarly to $1,25(OH)_2D_3$ i.e. by binding to specific receptors and gene activation. An example of the specific properties of one analogue are those of 1,25-dihydroxy-16-ene-23-yne cholecalciferol (RO23-7553). This analogue has, *in vitro*, 70% affinity for rat duodenal receptor, and less than 0.1% affinity for plasma vitamin D-binding protein compared with $1,25(OH)_2D_3$. *In vivo* (3 week old rachitic chick model), it has less than 0.1% effect on the synthesis of calbindin 28 K and serum osteocalcin, compared with $1,25(OH)_2D_3$. (Bouillon *et al.*, 1991a). It prolongs the survival time of leukaemic mice by inhibiting proliferation and increasing differentiation of myeloid leukaemia cells injected into BALB/c mice. $1,25(OH)_2D_3$ increased differentiation but caused hypercalcaemia, and did not increase survival time (Zhou *et al.*, 1990).

The chemical modifications that define analogues of $1,25(OH)_2D_3$ are potentially important in the study of tibial dyschondroplasia in broilers, where the defect lies in the processes controlling chondrocyte hypertrophy in the growth plate.

Figure 2a. Structures of parent molecule, 1,25-dihydroxycholecalciferol, and of two analogues, RO23-7553 and RO23-6474.

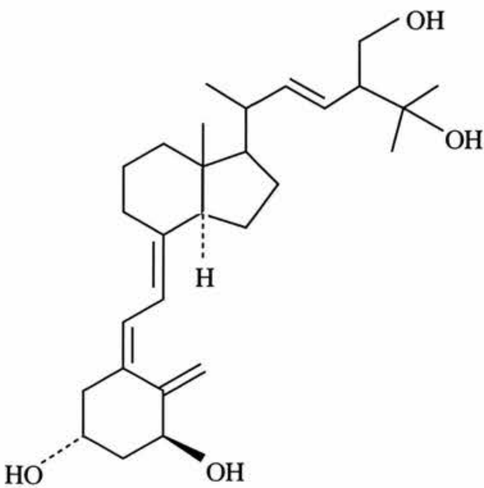


$1,25(OH)_2D_3$



1,25-16-ene-23-yne-$(OH)_2D_3$ (RO23-7553)

(Norman *et al.*, 1992)



1,25,28$(OH)_3$-ergocalciferol (RO23-6474; Henry, 1992)

2.6 Ascorbic Acid

Ascorbic acid or vitamin C is an essential nutrient for only a few vertebrates, including man, monkeys, and guinea-pigs. Nearly all other animals, including poultry, can synthesise ascorbic acid by the glucuronic acid pathway. The enzyme l-gulonolactone oxidase catalyses the synthesis of 2-oxo-l-gulonate from l-gulonolactone, which then undergoes spontaneous tautomerisation to form l-ascorbic acid. The basic functional property of ascorbic acid is its redox potential of + 0.166 V (Jaffe, 1984). Ascorbate can reduce Cu^{2+} and Fe^{3+} and oxygen to H_2O. It is therefore a vital cofactor for many metallo-enzymes such as the cytochromes and lysyl and prolyl hydroxylase. Classic symptoms of deficiency in animals unable to synthesise ascorbic acid are structural degeneration of connective tissue in different organs and glands, and within muscle, skin and the vascular system, due to reduced lysyl and prolyl hydroxylase activity and probably also impaired glycosylation and sulphation reactions. The end result is reduced collagen synthesis and bone formation, with loss of basement membrane integrity (Chattergee, 1990).

Although it was stated above that all classes of poultry are able to synthesise their own ascorbic acid, it has been widely demonstrated that supplementing poultry diets with ascorbic acid can be beneficial to both broilers and laying hens under conditions such as heat stress (Njoku *et al.*, 1990, Pardue *et al.*, 1985). Other studies have demonstrated improvements in bone and egg shell quality in hens fed ascorbic acid under normal environmental conditions, but results have often been ambiguous. For example, in an early study, Rowland *et al.* (1973) reported that spent hens given 15-1000 mg/kg ascorbic acid in feed for 1, 2 or 4 weeks showed no improvement in bone or egg shell quality. However, Orban *et al.* (1993) ran four experiments in total. The first two used broilers up to 7 weeks of age, and the other two laying hens of 76 and 96 weeks of age. In the two hen experiments, the bone mineral content and bone

density of the older hens increased as the ascorbic acid content of the diet increased, although conversely to the broilers, bone breaking strength was unaltered. In the younger hens, egg weight increased, possibly due to increased egg shell thickness, but there were no changes in bone characteristics. Only one of the broiler experiments showed any improvement in bone quality, as measured by a 16% increase in femoral breaking strength, although surprisingly in view of this result, there was no effect on either bone mineral content or bone density in the tibia, femur, or metatarsus.

Ascorbic acid has also been tested for efficacy in tibial dyschondroplasia in broiler chickens, although the experiments reported in the literature have generally reported negative results (Edwards, 1989b).

2.7 Vitamin K

Vitamin K or phylloquinone is a necessary co-factor for the post-translational modification of the important bone protein osteocalcin. A specific enzyme catalyses the carboxylation of glutamyl residues to form γ-carboxyglutamyl residues. Chicken osteocalcin has a molecular weight of 6000-12000 daltons and contains 4 γ-carboxyglutamyl residues per 57 amino acids (Suttie, 1981). It is synthesised by osteoblasts largely under the control of 1,25-dihydroxycholecalciferol. Osteocalcin binds ionised calcium and hydroxyapatite, but has a weak affinity for amorphous calcium phosphate. Its precise role in mineralisation is not clear. It is not involved in initial mineral formation but may be more connected with the maturation of the mineral phase in bone (Ali, 1992). Plasma concentrations of osteocalcin reflect metabolic activity and are raised in disorders such as rickets, Paget's disease and hyperparathyroidism. In vitamin K deficiency an abnormal form of osteocalcin is synthesised lacking γ-carboxyglutamyl residues which is unable to bind

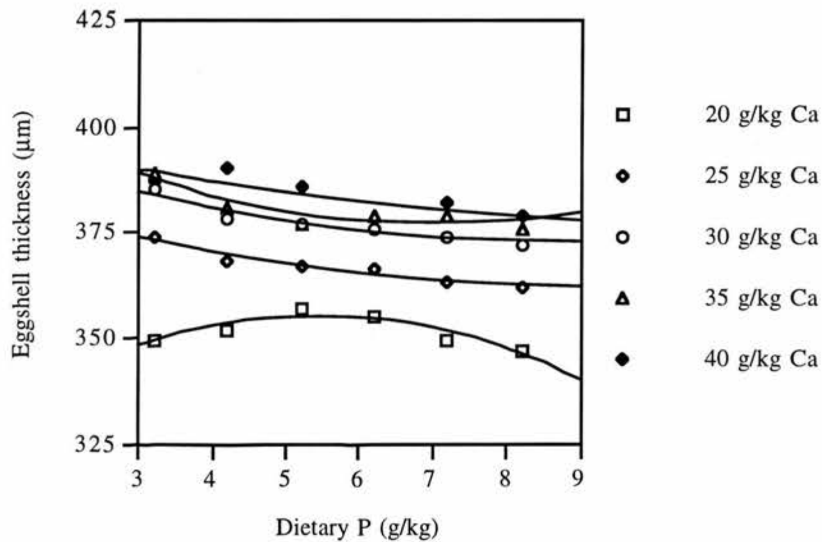
hydroxyapatite. However, in animals made experimentally deficient, no abnormalities of bone histology or mineralisation are seen (Root, 1990). Newly hatched chicks from hens fed a vitamin K-deficient diet had increased prothrombin times and reduced metaphyseal tibiotarsal osteocalcin, but no obvious disorders of skeletal development (Lavelle *et al.*, 1994). Chickens have a minimum vitamin K requirement of about 530 μg/kg (Suttie, 1981).

2.8 Phosphorus

A large amount of research has been carried out on phosphorus in the nutrition of the egg-laying hen. Particular attention has focused on the fact that a reduction in dietary phosphorus is sometimes associated with an improvement in shell quality. This is important in older birds where an increase in egg size results in a drop in shell quality, with a higher proportion of rejects due to cracks and breakages. The hen is believed to be in negative calcium balance during the night, the period when shell is being laid down, and so draws upon skeletal reserves. Calcium is withdrawn from the skeleton together with phosphate, resulting in an increase in plasma phosphate, which inhibits further bone resorption limiting further calcium release. The rational behind feeding a low phosphorus diet is that it leads to a drop in plasma phosphate and allows maximum use of skeletal calcium. However, as Roland and Farmer (1986) discuss, these results have not been constantly replicated in experiments, and both low and high phosphorus diets are known to stimulate bone resorption. A diurnal rise in plasma phosphate at night, such as has been measured in hens due to lay the following morning, has also been observed to occur in rats. In an experiment investigating the relationship between dietary calcium and phosphorus and egg production, they found that plasma phosphate was only reduced when the diet contained marginal calcium (27.5 g/kg), and egg production

was unaffected by treatment. They suggest that the improvement in eggshell quality obtained by reducing dietary phosphorus may be influenced by the source of dietary calcium. In another similar experiment Roush *et al.* (1986) found that although egg production and egg weight were unaffected by the range of experimental treatments (25, 37.5 and 50 g/kg calcium and 3.5, 5.0 and 6.5 g/kg available phosphorus corresponding to 4.5, 6.5 and 8.4 g/kg total phosphorus), percentage eggshell was affected. Reducing dietary phosphorus only increased percentage eggshell when the diet contained the very high level of 50 g/kg calcium.

Figure 2b Interaction between dietary calcium, phosphorus and eggshell thickness (redrawn from the data of Hartel, 1989).



Hartel (1989) carried out an experiment using a wider range of treatments to investigate further the potential interaction between dietary calcium and phosphorus and egg shell quality. This work (see *Figure 2b*) showed benefits to eggshell

thickness, amongst other parameters, to be gained from reducing dietary phosphorus when the diet contained > 20 g/kg calcium, but the magnitude of response was similar over 20-40 g/kg calcium.

2.9 Basis for project to determine nutrient effects on bone disorders.

Nutrition plays an important role in bone growth, development and quality. The general aims of this project were to investigate whether nutritional modification could influence the two skeletal disorders seen most commonly in modern poultry, osteoporosis and tibial dyschondroplasia.

For the experiment involving laying hens, nutritional treatments were chosen to be fed during the laying period that were expected to positively and negatively influence bone composition and quality, based on studies in the literature of human osteoporosis, animal models, and avian nutrition as related to bone. It was decided to confine the nutritional treatments to one strain of bird typical of the hybrid hens used for egg production in Great Britain. As genetic strain has been shown to influence bone breaking strength (Rowland *et al.*, 1972; Knowles *et al.*, 1993), it was thought that a strain effect might influence the development of osteoporosis. It was hypothesised that a bird not selected for a high level of production might have a lower incidence of osteoporosis, due to a smaller demand being made on bone turnover. Therefore it was decided to introduce a strain comparison into the general aims of the project. It was also decided to investigate the potential effects of earlier attainment of sexual maturity, and therefore laying down of medullary bone, on osteoporosis, as it was hypothesised that any factor preventing the hen reaching the bird equivalent of 'peak bone mass' might be deleterious.

For the broiler investigations, the general aims were to investigate more closely the effects of only a few nutritional modifications on the development of TD.

Despite the variety of experimental methods for the induction of a high incidence of TD, it was decided to use only one in the experiments described below, namely modification of dietary calcium and phosphorus. This was partly for convenience, as diets based on conventional ingredients could easily be formulated and mixed to reproducible standards, without the need for purified or uncommon ingredients, and partly because it was felt that the TD induced under this dietary regime was comparable to that seen in the field. Reports in the literature suggested that $1,25(OH)_2D_3$ was a potentially very positive and important factor in TD, and that addition of the purified hormone to broiler diets could be of benefit. It was decided to investigate these claims more fully, and to use analogues of $1,25(OH)_2D_3$ to deduce the mechanism of its effect. As ascorbic acid was known to be essential for collagen synthesis and had also been suggested to be involved in renal hydroxylation of $25(OH)D_3$, it was decided to investigate also the effects of addition of ascorbic acid to broiler diets on TD.

CHAPTER 3

3.1 LAYING HEN EXPERIMENT

EXPERIMENTAL AIMS

The aims of this experiment were:

1. To investigate and quantify osteoporosis in hens at the end of lay.
2. To carry out studies into the effect of nutrition, strain and level of production on the development of osteoporosis.

3.2 MATERIALS AND METHODS

3.2.1 Birds

The birds for this experiment were of 2 strains. The first was the Hi-sex strain, a modern laying strain with a high rate of egg production. The second was the J-line, an unimproved Leghorn-type strain with a low rate of egg production, maintained at the Poultry Research Centre, Roslin since the 1950's.

3.2.2 Reagents and Equipment

Ascorbic acid and sodium fluoride were purchased from BDH, Thornliebank, Glasgow. Feed grade $1,25(OH)_2D_3$ was provided by Dr. Weiser, Hoffmann-La Roche, Basle. Blood tubes were purchased from Sarstedt Ltd., Beaumont Leys, Leicester. Euthatal was purchased from Veterinary Drug Company, Falkirk. Wako clinical chemistry kits were purchased from Alpha Laboratories, Eastleigh, Hampshire. Kodak X-OMAT S film was purchased from H. A. West, Edinburgh. $1,25(OH)_2D_3$ assay kits were purchased from Incstar Ltd., Wokingham, Berkshire.

Plate 1 J-line (left) and Hi-sex (right) hens, as used in the laying hen osteoporosis experiment.

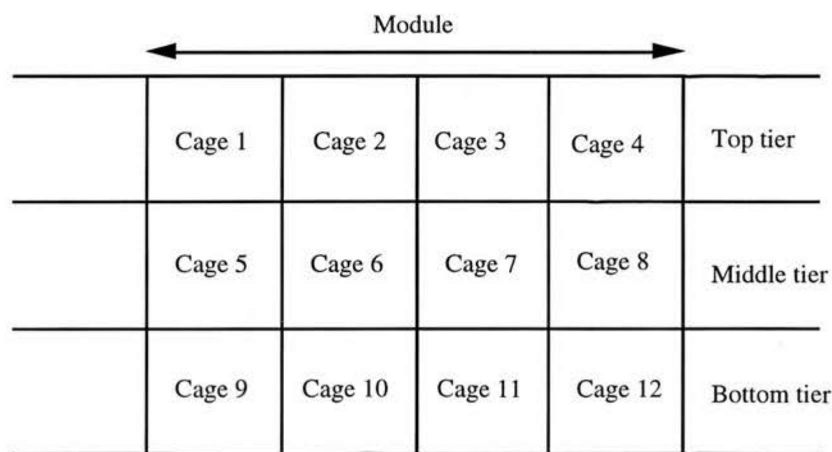
HPLC grade solvents were purchased from Rathburn Chemicals, Walkerburn, Peeblesshire. All other chemicals were purchased from Sigma Chemical Company, Poole, Dorset.

3.2.3 Rearing and Housing

Chicks from both strains were reared following standard rearing procedures in floor pens from day old until 16 weeks old. They were all fed a standard starter and rearer diets (see Appendix 1) *ad libitum* and had free access to water. The lighting pattern was 8L:16D. At 16 weeks old, the birds were transferred and randomly allocated to individual cages of a battery. The lighting pattern was stepped up by 0.5 hours light per week to induce lay at 20 weeks.

3.2.4 Experimental Design and Treatments during the Laying Period

Figure 3a Arrangement of cages in a laying hen battery unit



Birds were housed in single cages (48cm x 30cm x 46cm) of 3 tier battery units, subdivided into modules consisting of 4 cages in each tier. The experimental design consisted of 8 modules x 12 birds over 3 tiers, giving a total of 96 hens per

treatment for the control treatment, and 8 modules x 8 birds over 2 tiers for the other treatments, giving a total of 64 birds per treatment (See *Figure 3a*). However, an outbreak of Marek's disease amongst the J-lines during rearing meant that their numbers were severely reduced by the time they were allocated to cages.

There were 9 experimental groups within this experiment. There were 7 diets consisting of a basal diet and this basal diet either modified or supplemented with nutritional additives (See Appendix 1). The basal diet was formulated using FORMULATE program for the Prime computer and was low in fat, and based on wheat, soya bean meal and fishmeal, with the calcium content provided by limestone flour and feed-grade dicalcium phosphate It contained 170 g/kg crude protein, 11.4 MJ/kg metabolisable energy, 35 g/kg calcium and 6 g/kg phosphorus. This basal diet was fed to the control, early-lighting and the J-line birds. For the nutritional comparisons, the basal diet was supplemented with ascorbic acid (300 mg/kg), sodium fluoride (200 mg/kg), and 1,25-dihydroxycholecalciferol (1,25$(OH)_2D_3$, 5 μg/kg diet, in a stabilised powder containing 200 mg 1,25$(OH)_2D_3$ per kg powder).

There were 3 modified diets. The first was a low phosphorus (4.5 g/kg) diet. The second modified diet contained 150 g/kg crude protein (CP) and 20 mg/kg sodium menadione bisulphite (vitamin K) supplement. This treatment is subsequently referred to as the low CP, high vitamin K treatment. These first two modified diets were also formulated using FORMULATE program. The third diet was such that half of the total calcium content of 35 g/kg was derived from crushed limestone and dicalcium phosphate, the balance being made up by the addition of crushed oyster shell with a known calcium content and a mean particle size of approximately 6 mm.

The final experimental treatment used a group of Hi-sex birds that had been brought into lay two weeks before all the other Hi-sex birds by moving them out of

the rearing accommodation into the laying accommodation and stepping up number of hours of light in the 24 hour cycle a fortnight before the other birds. In the chapters that follow, this treatment is described as the early-lighting group.

Proximate analysis was routinely carried out on newly-mixed batches of feed to ensure that the nutrient content did not deviate from calculated values.

Diets were fed *ad libitum* to all birds in each of the experimental groups with the exception of the diet containing $1,25(OH)_2D_3$, which was only fed to the birds on the middle tier due to financial constraints.

3.2.5 Egg Records

Egg records were updated daily onto cards and transferred to the Vax computer. Whole eggs and soft shelled eggs were recorded using different codes. Whole eggs and cracked eggs were not differentiated.

3.2.6 Tissue sampling

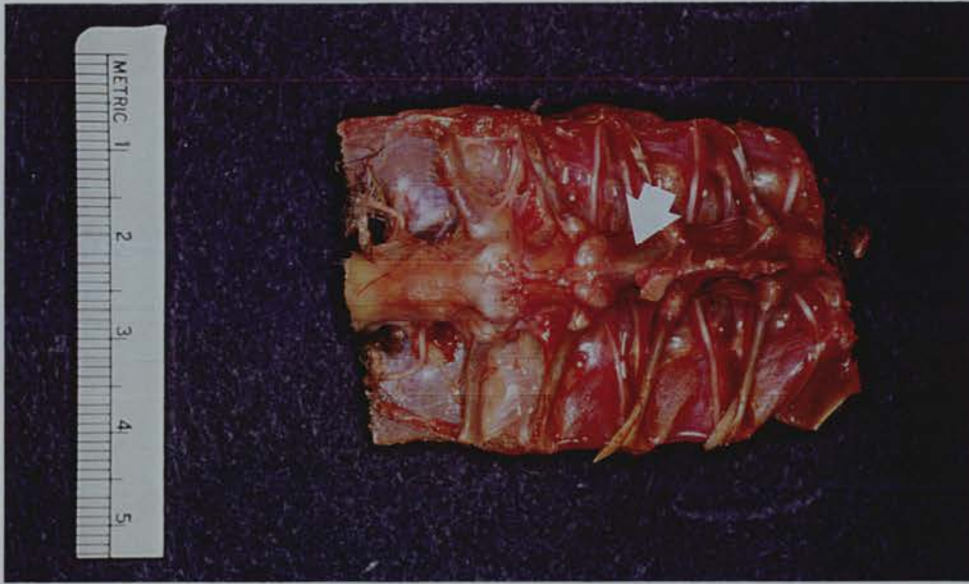
Blood samples were taken from all birds on the control treatment and also from other treatment groups birds from the middle tiers of the battery. Four blood samples were taken altogether, at 12-weekly intervals. All hens were sampled between 0830 and 1130. Hens were carefully removed from cages with both hands and gently restrained. 5 ml blood was collected from the wing vein of individuals using a 23 gauge needle into polystyrene tubes containing 50 units sodium heparin per ml blood. Samples were kept on ice prior to centrifugation at 4 C and 750 g in a Damon centrifuge with swing-out buckets. Plasma was drawn off and pooled from adjoining pairs of birds and subsampled into different polythene tubes for each set of assays to be carried out, to overcome the problem of repeated freeze-thawing of samples. All samples were stored at -20 C pending analysis.

3.2.7 Bone Collection and Processing

Hens were kept until 68 weeks of age. Only Hi-sex birds from the middle tier still in lay and all J-lines were selected for further study. The birds were carefully removed from their cages and immediately killed by barbiturate (Euthatal) overdose. The reproductive state of each hen was checked by examining the ovary. Bones were only selected from birds with an active ovary. The free thoracic vertebra (FTV) and proximal tarsometatarsus (PTM) were dissected free and fixed in neutral buffered formalin (NBF). Wings only were removed for X-ray study of fracture incidence due to the impracticality of storing large numbers of frozen carcasses. The fixed bones were embedded undecalcified in methylmethacrylate resin. Sections were cut using a microtome, mounted on glass microscope slides and stained with toludine blue. Three sections were prepared from each bone.

3.2.8 Image Analysis

The amounts of trabecular and medullary bone in individual FTVs were measured using a Macintosh Quadra running the public domain software package NIH Image 1.47 connected to a Panasonic WVBL600 monochrome video camera. The slides were placed on a light box and illuminated. The video camera produced a magnified image of the section on the computer screen which was then captured. Toludine blue stains medullary bone purple and trabecular bone a paler blue, which translated to dark grey and a much paler grey on the black and white image. The region of interest, essentially the body of the FTV minus the cortical and subchondral bone was selected, and the density thresholds of the 2 types of bone set. The software then calculated, in terms of pixels, the 2-dimensional area occupied by each type of bone, and used these data to express the result as a percentage area.



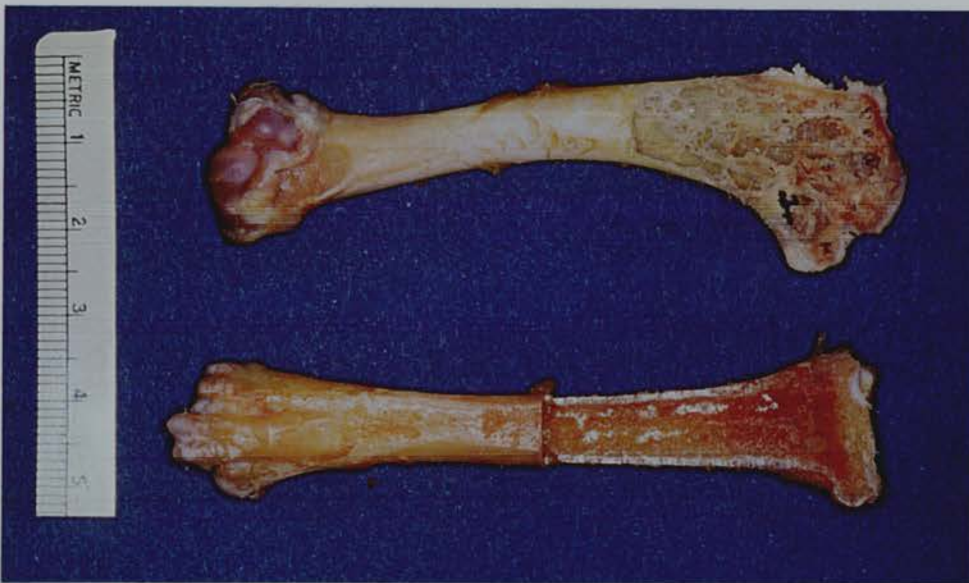
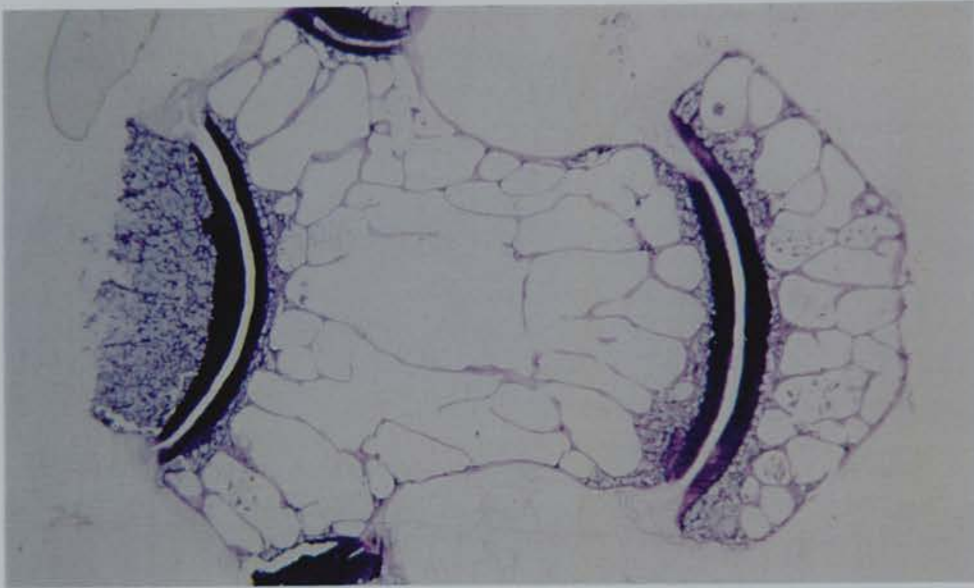


Plate 2a Part of the spinal column of a hen, showing the free thoracic vertebra (arrow) *in situ.*

2b Humerus (top) and tarsometatarsus (bottom) from a hen. The pneumatised humerus contrains trabecular bone, but the tarsometatarsus contains both trabecular and medullary bone.



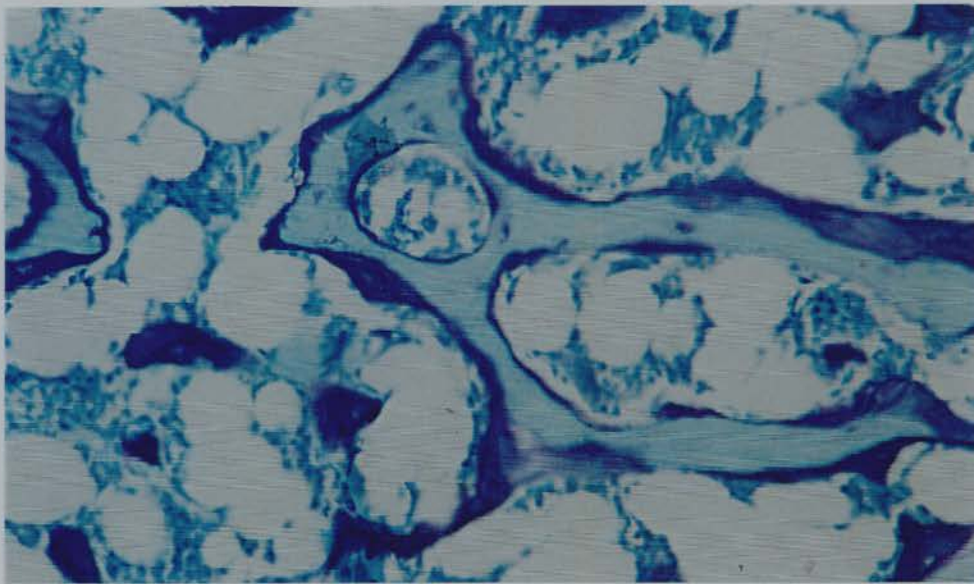


Plate 3a Free thoracic vertebra section from a 68 week old Hi-sex hen, stained with toluidine blue. Note the sparse, thin trabeculae. Magnification x7.

3b Photomicrograph of a section of proximal tarsometatarsus from a 68 week old Hi-sex hen, stained with toluidine blue. Trabecular bone stains turquoise, and medullary bone stains purple.

3.2.9 Point Counting

The amounts of trabecular and medullary bone in PTMs were quantified using point counting as described by Kimmel and Jee, (1983).

3.2.10 Radiographic studies

Dissected wings were placed extended on a cassette containing X-OMAT S (Kodak) film and placed in a Faxitron closed source X-ray machine. Following a 2 second exposure at 50 kV, plates were developed using a fully automated system. Developed X-rays were then placed on a light-box and studied for fractures, which were classified as old or new (i.e. by the amount of callus formation) and whether they were spiral, simple or compound.

3.2.11 Biochemical assays.

Total calcium, inorganic phosphate and alkaline phosphatase were measured using Wako kits adapted for use with a plate-reader (Titertek Twin-Reader Plus, ICN).

3.2.11.1 Total Calcium

Total calcium was measured in plasma that had been diluted two-fold with Millipore extra-pure water. Diluted plasma (4 μl), was mixed with buffer (200 μl), and colour reagent (20 μl). A standard curve was prepared over the range 0-5 mM by dilution with water of standards provided with the kit. Samples were read at 560 nm after 20 minutes.[2] An estimate of assay variation was calculated by running an

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[2] Calcium and cresolphthalein complexone in ethanolamine-borate buffer form a purple calcium-cresolphthalein complexone complex. 8-hydroxyquinoline in the reaction mixture makes the test specific for calcium.

aliquot of pooled plasma on every plate. Within assay variation was 2.7%, and between assay variation 6.5%.

3.2.11.2 Inorganic Phosphate

Inorganic phosphate was also measured in plasma again diluted two-fold with water. Diluted plasma (5 µl)was mixed with colour reagent (125 µl). A standard curve was again constructed by dilution of standards provided with the kit over the range 0-1.05 mM, and samples read after 20 minutes at 690 nm.[3] Within assay variation was 3.1% and between assay variation 13.0%.

3.2.11.3 Total Alkaline Phosphatase

Plasma alkaline phosphatase (AP, EC 3.1.3.1) is a measure of osteoblastic activity in the chicken as in other species (Bell, 1971). There is a relationship between plasma alkaline phosphatase activity and dietary calcium in the hen (Hurwitz and Griminger, 1961) and radius breaking strength at 38 weeks (Reichmann and Connor, 1977) due to low dietary calcium increasing bone turnover and loss.

Total alkaline phosphatase activity was measured in plasma diluted ten-fold with extra-pure water. Samples were kept on ice until they could be assayed. A standard curve was prepared by diluting 5 mM p-nitrophenol in assay buffer to give a range of standards from 0-62.5 µM. Enzyme activity was measured by adding diluted plasma (10 µl) to p-nitrophenyl phosphate substrate (250 µl), and measuring absorbance at 405 nm for 10 minutes. The mean change in absorbance per minute

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[3] phosphate + ammonium molybdate → ammonium phosphomolybdate
ammonium phosphomolybdate + aminonaphtholsulphonic acid → heteropolymolybdenum (blue colour)

was calculated and related to p-nitrophenol concentration. Alkaline phosphatase activity was calculated after correction for dilution, and expressed as international units/l (IU/l). One IU of enzyme activity is defined as the amount of enzyme that will catalyse the transformation of one µmole of substrate per minute under standard conditions.

3.2.11.4 Alkaline phosphatase iso-enzymes

Iso-enzymes of alkaline phosphatase are present in plasma derived from bone, small intestine and liver. They can be separated and visualised by electrophoresis. Wheat germ *(Triticum vulgaris)* lectin will precipitate bone alkaline phosphatase from human plasma, and can therefore be used to quantify both the bone iso-enzyme and those from other tissues (Rosalki and Foo, 1984). Similarly, l-p-bromotetramisole (BTM) will inhibit liver and bone enzymes, and so can be used to measure alkaline phosphatase of intestinal origin (Kuwana and Rosalki, 1991).

Pooled broiler and laying hen plasma was prepared from two birds from each category by taking blood samples into heparinised tubes, centrifuging at 750 g for 15 minutes, and drawing off and mixing the plasma. Lectin precipitation of AP was carried out essentially by the method of Rosalki and Foo (1984). Wheat germ lectin (5mg), was dissolved in de-ionised water (1ml). Plasma was diluted also with de-ionised water. Hen plasma was diluted 1:5 and the broiler plasma was diluted either 1:25 or 1:40. Lectin solution (50µl) was mixed with diluted plasma (50 µl) in an Eppendorf tube and incubated for 30 minutes in a water bath at 37 C. The tubes were then centrifuged at 5000g for 10 minutes. A precipitate was clearly visible. The supernatant was drawn off and placed in a clean tube. The precipitate was re-suspended in 9 g/l NaCl (100µl). The AP activity of the two fractions, and also of control plasma diluted 1:10 (hen), 1:50 or 1:80 (broiler) was measured in the usual

way. BTM inhibition of AP was measured by the method of Kuwana and Rosalki (1991), by adding the inhibitor, as the oxalate salt, to the substrate solution so that the final concentration of the inhibitor was 0.1mM. The AP activity of diluted plasma was then measured both with and without inhibitor added to the substrate.

3.2.12 Plasma $1,25(OH)_2D_3$

$1,25(OH)_2D_3$ was measured in plasma using an Incstar kit based on calf thymus cell receptor. This assay is based on that developed by Reinhardt *et al.*. (1984) and is the preferred assay system for $1,25(OH)_2D_3$, as the receptor does not bind to other hydroxylated metabolites of cholecalciferol. This assay has been validated for use in poultry (Ruschkowski *et al.*, 1993).

All solvents used were HPLC grade and Millipore water was used in buffers. Recovery tracer (50 μl, containing a small amount of ^3H-$1,25(OH)_2D_3$) was added to plasma (1 ml) which was then extracted by vortexing with acetonitrile (1 ml). The tubes were then centrifuged at 750 g for 15 minutes and the supernatant was drawn off and mixed with 0.4 M potassium phosphate buffer, pH 7.5, prepared with Millipore water (1 ml). $C_{18}OH$ columns were conditioned by washing through sequentially with iso-propanol (5 ml), methanol (5 ml), and water (5 ml). The mixture was applied to the columns and washed through with water (2 ml). The sample was cleaned up by washing through the column with methanol/water 70:30 (5 ml), hexane/dichloromethane 90:10 (5 ml), and hexane/iso-propanol 99:1 (3 ml). $1,25(OH)_2D_3$ was eluted by washing through with hexane/iso-propanol 96.5:3.5 (6 ml). This solvent mixture was evaporated from the sample by drying down under vacuum at 37 C. The samples were reconstituted in assay buffer (200 μl), and an aliquot (50 μl) was removed and mixed 3 ml Optiphase Hi-safe. The ^3H content was measured using liquid scintillation counting.

A protein binding assay utilising calf thymus cell receptor was then performed on the samples. Briefly, sample or standard (50 μl) were pipetted into borosilicate glass tubes. Receptor preparation (400 μl) was added to tubes, which were then mixed, and incubated at room temperature for one hour. Assay tracer (50 μl, containing a larger amount of ^3H-1,25$(OH)_2D_3$) was added to the tubes. Tubes were mixed again, and incubated for a further hour at room temperature. Tubes were then placed on ice for 10 minutes, and bound and free ^3H separated by the addition of ice-cold charcoal suspension. Tubes were kept at 4 C for a further 15 minutes, and centrifuged for 20 minutes at 1000 g. The supernatant was pipetted into scintillation vials and Optiphase Hisafe (3 ml) added to the vials. The ^3H content of the vials was measured in a liquid scintillation counter. Data were processed using Assayzap computer program supported by a Macintosh IIsi, and corrected for recovery. A quality control sample was provided with each kit, the 1,25$(OH)_2D_3$ content of which varied from batch to batch. The assayed content of the quality control sample was always within the limits quoted by the manufacturers.

3.3 BROILER EXPERIMENTS

EXPERIMENTAL AIMS

The aims of the experiments described below were :

1. To confirm that use of a diet containing imbalanced amounts of calcium and phosphorus could induce a high incidence of TD in small scale experiments using broiler chickens kept until 3 weeks of age, and was a suitable model to use in further experiments.

2. To investigate the effect on TD and calcium metabolism of incorporating the active cholecalciferol metabolite $1,25(OH)_2D_3$ into both the imbalanced Ca/P and standard Ca/P diets.

3. To investigate the effect of ascorbic acid either with or without $1,25(OH)_2D_3$ on TD and calcium metabolism.

4. To investigate the effects of chemical analogues of $1,25(OH)_2D_3$ on TD and calcium metabolism.

5. To investigate the possible interaction between dietary calcium and dietary $1,25(OH)_2D_3$ in terms of potential toxicity.

3.3.1 Birds and Husbandry

A series of broiler experiments was carried out using day-old, male Ross I or Cobb strain broiler chicks. They were housed in electrically-heated wire-floored compartments of a brooder unit until 3 weeks old. Blood samples were taken from the wing-vein into heparinised tubes (sodium heparin, 50 IU/ml), and chicks were killed either by barbiturate injection or cervical dislocation.

3.3..2 Diets

Diets of varying calcium and phosphorus concentrations (see Appendix 2) were formulated using Acufeed formulation program (copyright University of Minnesota, courtesy of Professor Paul Waibel). The experiments investigating the effects of ascorbic acid and $1,25(OH)_2D_3$ on TD were carried out feeding birds diets containing either 12 g/kg Ca, 6 g/kg P or 7.5 g/kg Ca, 7.6 g/kg P. Other experiments used birds fed the standard Institute broiler starter diet which contained 10 g/kg Ca, 6 g/kg P.

3.3.3 Reagents

Feed grade $1,25(OH)_2D_3$ was provided by Dr. Weiser, Hoffmann-La Roche, Basle. Ascorbic acid was purchased from BDH, Thornliebank, Glasgow. Blood tubes were purchased from Sarstedt Ltd., Beaumont Leys, Leicester. Euthatal was purchased from Veterinary Drug Company, Falkirk. Wako clinical chemistry kits were purchased from Alpha Laboratories, Eastleigh, Hampshire. $1,25(OH)_2D_3$ assay kits were purchased from Incstar Ltd., Wokingham, Berkshire. HPLC grade solvents were purchased from Rathburn Chemicals, Walkerburn, Peeblesshire. All other chemicals were purchased from Sigma Chemical Company, Poole, Dorset. Ionised calcium was measured in blood using a 634 Ca^{2+}/pH analyser, purchased from Ciba-Corning Diagnostics Ltd., Halstead, Essex. 25-[26,27-^{3}H]hydroxycholecalciferol in toluene:ethanol solution (specific activity 165 Ci/mmol) was supplied by Du Pont-NEN. 1α,25-dihydroxy[26,27-methyl-^{3}H] hydroxycholecalciferol in toluene:ethanol solution (specific activity 163 Ci/mmol) was supplied by Amersham PLC.

3.3.4 Plasma Assays

Plasma assays were carried out as previously described, except that plasma was assayed without dilution.

3.3.4.1 Ionised calcium

In later broiler experiments, the concentration of ionised calcium in whole blood was measured using a Ciba-Corning 634 Ca^{2+}/pH analyser. Blood samples (1ml) were collected into tubes containing 50 units Li-heparin. Li-heparin was used as Na-heparin will bind calcium. Li-heparin will also bind calcium, but at a much higher concentration than 50 units/ml (D. Utomo and Dr. Malcolm Mitchell,

unpublished data).The tubes were capped, mixed and kept on ice to reduce respiration by erythrocytes (nucleated and with mitochondria in avians, Harris, 1983). Measurements were taken within 1 hour of collection.

3.3.4.2 Validation of techniques for measurement of ionised and total calcium.

Calcium exists in blood in two different forms. The first is ionised or 'free' calcium and the second is 'bound' calcium. Ionised calcium is regulated to remain within a tightly defined range since it affects a wide range of biological processes including nerve conduction, muscle contraction etc. The bound form is attached to plasma proteins, mainly albumin, and citrate, bicarbonate and phosphate. In mammals about 50% of the total blood calcium is ionised. In birds, the picture is slightly more complex. Egg-laying females need to transport large amounts of calcium to the shell gland for synthesis of egg shell. A special protein called vitellogenin is synthesised in the liver largely under the influence of oestrogen at sexual maturity. It is transported to the ovary where it is split to form two yolk proteins, phosvitin and lipovitellin. Vitellogenin is found in the serum where it binds calcium and allows it to be transported in the blood (Guyer *et al.*, 1980). Therefore in birds, the plasma level of total calcium increases with the attainment of sexual maturity. The concentration of ionised calcium also varies cyclically in the reproductively active female only. Ionised calcium is highest when the shell gland is empty, and then declines following the entry of the egg into the shell gland, and reaches a minimum level 16 hours before the next oviposition (Parsons and Combs, 1981).

As total calcium (see Section 3.2.11.1) and ionised calcium (see Section 3.3.4.1) were being measured in samples using widely differing methodologies, it was important to validate these two techniques. A stock 5 mM calcium chloride

standard was made up in 0.05 M Tris-HCl buffer, pH 7.4, and diluted with Tris-HCl to make a range of standards from 0.5-5 mM. A further set of standards was made up over the same range that contained 3% (w/v) bovine serum albumin. As calcium chloride is ionised in solution, the results from the two sets of measurements using calcium chloride in buffer solution alone were directly comparable. The aim of using buffer solution containing albumin was to investigate a) whether a physiological protein concentration would affect measurements of total calcium, and b) to check that the 634 analyser would correctly measure reduced ionised calcium levels as the albumin bound up the calcium. The two sets of standards were then measured using both techniques as described above. Duplicate measurements were made of each standard.

3.3.4.3 Plasma enzymes and metabolites

Enzymes present in the plasma are due to leakage from blood or somatic cells. Measurement of plasma enzyme activity can be used diagnostically, as damage to an organ or tissue, or altered metabolic activity is reflected in increased levels of marker plasma enzymes. Increased plasma aspartate aminotransferase (AST, EC 2.6.1.1) is often due to skeletal or cardiac muscle damage, whereas increased creatine kinase (CK, EC 2.7.3.2) reflects skeletal muscle damage. Increased lactate dehydrogenase (LDH, EC 1.1.1.28) reflects liver and muscle damage. LDH, AST, CK, and uric acid were measured in a broiler study using Wako kits adapted for use with the plate reader. [4]

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[4] lactate dehydrogenase assay is based on the following reaction. pyruvate + NADH → lactate + $NAD^+ + H^+$

aspartate aminotransferase assay is based on the following reactions. a. aspartate + 2-oxoglutarate → oxaloacetate + l-glutamate b. oxaloacetate + NADH → l-malate + $NAD^+ + H^+$

3.3.4.4 Plasma osmolality

Plasma osmolality was measured in one experiment using a Fison's Osmometer Model 3DII. This method uses the relationship between freezing point and osmolality, and plasma osmolality was expressed as mOsm/kg (osmolality refers to mOSm/unit weight and osmolarity to mOsm/unit volume). Osmolality was measured following the observation that blood samples were harder to draw from chicks that had been given $1,25(OH)_2D_3$ in the diet.

3.3.5 Bone ash

Toe ash was measured as an estimate of bone calcification (Fritz and Roberts, 1968). The middle toe of the left foot was removed and frozen until the analysis could be done. Once thawed, the middle phalange of the toe was dissected free and adhering tissue removed. The individual bones were solvent-extracted in petroleum ether, dried at 100 C, and ashed at 550 C overnight. Ash was expressed on a percentage wet weight basis.

3.3.6 Preparation of tissues for light microscopy and electron microscopy

Samples of growth plate, liver, kidney, heart, spleen, proximal duodenum, lung and skeletal muscle were fixed in neutral buffered formalin for light microscopy. Sections of all tissues were cut and stained with hematoxylin and eosin. Sections of kidney were also cut and stained with Von Kossa with Neutral Red counterstain. Samples of kidney for electron microscopy (EM) were fixed in osmium tetroxide.

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creatine kinase assay is based on the following reactions. a. ADP + creatine phosphate $\rightarrow$ creatine + ATP b. ATP + glucose $\rightarrow$ glucose -6-phosphate + ADP c. glucose-6-phosphate + $NADP^+$ + H^+ $\rightarrow$ 6-phosphogluconate + NADPH

3.3.7 Confirmation of concentration of pure vitamin D metabolites and analogues

The concentration of ethanol solutions of vitamin D metabolites and analogues was routinely checked by measuring UV absorption at 265 nm in silica cuvettes after zeroing with ethanol. The molar absorbance coefficient for a solution of D_3 and 25-OHD_3 in ethanol at this wavelength is 18 300 cm^{-1}. Hence a solution containing 2.1 μg/ml will have an absorption of 0.1 units. 1,25$(OH)_2D_3$ and vitamin D analogues were assumed to have identical molar extinction coefficients.

3.3.8 Renal 25$(OH)D_3$ 1- and 24-hydroxylase activity

Renal hydroxylase activity was measured by the following method (Dr. M. Hayes, personal communication). Birds were killed at 3 weeks old by intravenous injection of Euthatal. Kidneys were rapidly excised, rinsed in NaCl (9g/l), and frozen in liquid N_2. Samples were stored at -70 C until enzyme activity was assayed. Homogenates were prepared by homogenising kidney with 5 volumes of ice-cold 0.25 M sucrose, 0.05 M Hepes, 2 mM $MgCl_2$ buffer, pH 7.4, using a motor-driven Potter homogeniser. An aliquot was kept for protein assay. Homogenate (1.5 ml) was pipetted into a 20 ml stoppered glass tube, and gassed with 100 % O_2 for 30 seconds. The oxygenated homogenate was warmed in a 37 C bath for 5 minutes, and the reaction initiated by the addition of ^3H-25-OHD_3 in ethanol (5 μl). The reaction was terminated after 1 hour by the addition of chloroform/methanol 3:2 (4 ml) and the metabolites extracted into the solvent mixture by shaking. The phases were separated by centrifuging at 4 C for 20 minutes at 1000 g. The lower chloroform layer containing the metabolites was pipetted off and transferred to a clean tube. The solvent was removed using a vacuum oven at 37 C, and the residue re-dissolved in ethanol (300 μl) and frozen at -20 C pending chromatographic analysis. Prior to

assay, ethanol was removed using the vacuum oven and the metabolites redissolved in mobile phase (300 μl). The metabolites were separated using normal-phase HPLC. A mobile phase consisting of hexane/iso-propanol 96.5:3.5 was pumped through a Waters Nova-Pak Silica column at a rate of 1 ml/minute. Aliquots (100 μl) were injected into a sample loop, and loaded onto the column using a rheodyne valve. Fractions (0.5 ml) were collected using a fraction collector. Optiphase Hi-safe (3 ml) was added to samples and ^3H was counted using an LKB liquid scintillation counter. ^3H-1,25$(OH)_2D_3$ or ^3H-24,25$(OH)_2D_3$ peaks were identified by injecting authentic ^3H-1,25$(OH)_2D_3$ onto the column. Hydroxylase activity was expressed as pmol ^3H-1,25$(OH)_2D_3$ or ^3H-24,25$(OH)_2D_3$ formed per minute by 1 mg protein.

3.3.8.1 Protein assay

Samples of homogenate were diluted with deionised water. Protein standards were made over the range 0-90 μg/ml by diluting a stock standard of bovine γ-globulin (2 mg/ml) with deionised water. Sample or standard (160 μl) was pipetted into microplate wells. Reagent (Bio-rad protein assay reagent; 40 μl) was added to the wells, and the mixture thoroughly mixed. After 10 minutes, the absorbance of the wells was measured at 595 nm, using a plate reader.

3.3.9 Administration of 1,25$(OH)_2D_3$ analogues to chicks.

Analogues (see *Figure 2a* ;1 mg) had been supplied by Hoffmann-La Roche in amber vials. Absolute ethanol (1 ml) was added to each vial to give a concentration of 1 mg/ml. A few crystals of butylated hydroxytoluene (BHT) were added to 100 ml freshly opened maize oil to ensure there was no fatty acid oxidation. For week 1, stock alcohol solution of each analogue or 1,25$(OH)_2D_3$ (6 μl) was mixed into maize oil (5 ml) to give an oil solution containing 1.2 μg/ml. Each chick

was dosed on alternate days with 250 µl oil solution, equivalent to a daily intake of 300 ng of analogue or $1,25(OH)_2D_3$. For week 2, stock solution (8 µl) was dissolved in oil (5 ml), giving an equivalent daily dose of 400 ng. For week 3, stock solution (10 µl) was added to oil (5 ml), giving an equivalent daily intake of 500 ng. Each oil solution was made freshly as required, and the stock solutions of analogues and $1,25(OH)_2D_3$ were gassed with N_2 before being replaced in the freezer at -20 C. Chicks were dosed by transferring 250 µl aliquots of oil solution of analogue or $1,25(OH)_2D_3$ to fine tipped plastic disposable pipettes (Alpha) and letting the oil solution dribble down their throats, with care being taken to avoid it going down the trachea. The control group received the BHT-containing maize oil only. The diet fed to the chicks during the experiment was a standard broiler starter ration (see Appendix 2).

3.3.10 Binding of $1,25(OH)_2D_3$ and analogues to growth plate chondrocytes

3.3.10.1 Preparation of chondrocytes

3-4 week old chicks raised on a standard broiler starter ration (see Appendix 2) were killed by cervical dislocation. Whole, normal proximal tibiotarsi from 4 birds were dissected out under sterile conditions and thin sections of growth plate were shaved off using a scalpel. Articular and calcified cartilage were removed, and the remaining tissue, containing proliferating through to hypertrophic chondrocytes was transferred to a Petri dish containing a small amount of Minimal Essential Medium (MEM). Collagenase (1 mg/ml in MEM + 10% foetal calf serum) was used to digest extracellular matrix and free the chondrocytes by overnight incubation at 37 C in a shaking water bath. Any fragments of bone were removed by filtering through a stainless steel mesh filter and cells were washed with MEM and left for a further

24 hours at 18 C in order to re-synthesise cell-surface proteins stripped off by the collagenase treatment. A 50 μl aliquot of well-mixed cell suspension was mixed with 50 μl 0.2% (w/v) Trypan Blue and left for 10 minutes. Cells were counted using a haematocytometer. Viability was greater than 90% (dead cells take up Trypan Blue). The remainder of the cell suspension was spun down at 500 g for 5 minutes at room temperature. Chondrocytes were re-suspended in 2 ml assay buffer, containing 50 mM Tris, 500 mM KCl, 10 mM Na_2MoO_4), 1.5 mM EDTA, 5 mM dithiothreitol, 0.1 % (w/v) gelatin, pH 7.5, and kept on ice. The suspension was sonicated using 3x10 second bursts with 20 seconds on ice between bursts. Enough assay buffer was added to give an equivalent concentration of 2×10^6 cells per ml or 1×10^6 cells per assay tube. The preparation was left on ice till required.

3.3.10.2 Preparation of dextran-coated charcoal for separation of bound and free ^3H-1,25$(OH)_2D_3$

Dextran coated charcoal was prepared by mixing 0.3 g activated charcoal and 30 mg dextran with 25 ml 50 mM Tris, 500 mM KCl, 10 mM Na_2MoO_4, 1.5 mM EDTA, 5 mM dithiothreitol, pH 7.5 and stirring for 2 hours at 4 C. The mixture was centrifuged for 5 minutes at room temperature and the supernatant drawn off. Fresh buffer (25 ml) was added with constant stirring until use.

3.3.10.3 Assay

The assay was set up in duplicate in 12x75 mm borosilicate glass tubes. Blanks contained 500 μl Tris/KCl/gelatin buffer and 20 μl absolute ethanol. Zeros contained 500 μl cell preparation and 20 μl ethanol. Standards contained 500 μl cell preparation and 20 μl 1,25$(OH)_2D_3$ solution in ethanol (2.5-8000 pg/tube) or analogue solution in ethanol (2.5-10 000 pg/tube). 1,25$(OH)_2$[26,27-methyl-^3H]D_3

(10 μl, containing 6000 dpm, prepared by dilution with ethanol of stock solution, specific activity 163 Ci/mmol, Amersham PLC) was added to all tubes, which were mixed and incubated for one hour at room temperature in the dark. Dextran-coated charcoal (100 μl) was added to all tubes to separate bound from free ^3H-$1,25(OH)_2D_3$. The tubes were mixed and left to stand for 15 minutes at 4 C then centrifuged at 1000 g for 15 minutes at 4 C. Supernatant (500 μl) was drawn off from each tube and added to Optiphase Hi-Safe (3 ml), and ^3H counted using a liquid scintillation counter.

Data from the study of binding of ^3H-$1,25(OH)_2D_3$ to chondrocyte receptors were analysed using EBDA (Equilibrium Binding Data Analysis; MacPherson, 1983) program supported by a Macintosh IIsi. The Scatchard and Hill plots gave estimates of K_d and B_{max}. K_d (dissociation constant) of a ligand is the concentration of the ligand such that 50% of the receptors are occupied. Ka (affinity constant; sometimes seen in the literature) is the reciprocal of K_d. B_{max} is the maximum amount of ligand which can bind specifically to the receptors in a cell preparation. From B_{max} can be calculated the average number of receptors per cell.

3.3.11 Affinity of $25(OH)D_3$, $1,25(OH)_2D_3$ and analogues for plasma vitamin D binding protein (plasma DBP).

3.3.11.1 Production of vitamin D-deficient plasma.

Broiler chicks were raised from day old until 3 weeks of age on a vitamin D deficient diet formulated using Accufeed program (see Appendix 2). The D_3 content of the diet was essentially zero. Vitamin D_2, synthesised by plants, has only a fraction of the activity of D_3 in chickens, as in other birds. Therefore, under this dietary regime, binding sites on plasma DBP should not have been occupied by

endogenously synthesised vitamin D metabolites. Blood samples were taken from the chicks at 3 weeks old into heparinised tubes. The blood samples were centrifuged and plasma was drawn off and stored frozen at -20 C until use. The total calcium content of plasma samples from individual chicks was measured to confirm that the chicks were vitamin D-deficient and hence hypocalcaemic. Plasma samples were then pooled for use in the assay.

3.3.11.2 Assay

Pooled plasma was diluted two-fold with phosphate-buffered saline, pH 7.4 (PBS) to a final dilution of 1:32 768. Diluted plasma (0.5 ml) was pipetted into borosilicate glass tubes and incubated with ^3H-25(OH)D_3 in ethanol (10 μl; 6000 dpm, prepared by dilution of a stock solution with a specific activity of 165 Ci/mmol) for 1 hour at room temperature. Tubes were then placed on ice for 15 minutes. Bound and free ^3H-25(OH)D_3 were separated by the addition of ice-cold dextran-coated charcoal in PBS (0.1 ml). Tubes were thoroughly mixed and left on ice for 20 minutes. The tubes were centrifuged at 1000 g for 10 minutes. The supernatant was drawn off and mixed with 4 ml Optiphase Hi-safe. ^3H was counted using an LKB liquid scintillation counter. The dilution of plasma which bound 40% of added ^3H-25(OH)D_3 was determined to be 1:128 (2^7) and was used for the affinity study. The affinity of ^3H-25$(OH)_2D_3$ for plasma DBP was calculated using EBDA program supported by a Macintosh IIsi. A similar exercise was carried out using ^3H-1,25$(OH)_2D_3$. Aliquots (10 μl, 6000 dpm, prepared by dilution of a stock solution of specific activity 163 Ci/mmol) were added to diluted plasma as described above.

For the affinity study, pooled plasma was diluted 1:128 with PBS. Aliquots (0.5 ml) of plasma were pipetted into borosilicate glass tubes. Stock solutions of

$25(OH)D_3$, $1,25(OH)_2D_3$, $1,25(OH)_2$-16-ene-23-yne-cholecalciferol (RO23-7553) and 1,25,28-$(OH)_3$-ergocalciferol (RO23-6474) were diluted with ethanol to give a range of concentrations. 10 μl of each concentration of each metabolite or analogue was added in duplicate to the tubes of diluted plasma. ^3H-$25(OH)D_3$ (10 μl, 6000 dpm) was added to each tube. Zero tubes contained ethanol instead of metabolite or analogue, total tubes contained only ^3H-$25(OH)D_3$ and non-specific binding tubes contained PBS instead of diluted plasma. Tubes were incubated for 1 hour at room temperature, then placed on ice for 15 minutes. Ice-cold dextran-coated charcoal (0.1 ml) was added to all tubes, which were thoroughly mixed. The tubes were left on ice for 20 minutes and centrifuged at 1000 g for 10 minutes. The supernatant was drawn off, and mixed with 4 ml Optiphase Hi-safe. ^3H was counted using an LKB liquid scintillation counter. B/B_0 was calculated, and the amount of added metabolite or analogue that was sufficient to displace 50% of ^3H-$25(OH)D_3$ from plasma DBP was estimated from a graph of the results.

3.4 Histological analysis of growth plates

Birds were killed by intravenous injection of Euthatal at 3 weeks of age. Samples of proximal tibiotarsus were collected into buffered neutral formalin. They were decalcified, processed and cut. Sections were stained with hematoxylin and eosin and examined microscopically for changes characteristic of tibial dyschondroplasia or hypocalcaemic rickets. Growth plates of birds with TD show an increase in the width of the pre-hypertrophic zone, with or without necrotic changes secondary to oxygen and nutrient starvation due to lack of vascularisation. The cells themselves are smaller, stain more strongly and have shrunken pyknotic nuclei. The staining properties of the persistent cartilage are altered (Riddell, 1975b). In the normal bird, the pre-hypertrophic zone is only 4-5 cells thick (Howlett, 1979). In

calcium-deficiency rickets, the growth plate is thickened but vascularised. The thickness of the proliferating zone is thickened, and the chondrocytes show a characteristic disordered, crescent-shaped morphology (Itakura *et al.*, 1978).

3.5 Data analysis

Data from the large scale hen experiment such as percentages of trabecular and medullary bone, plasma minerals, enzymes and metabolites was analysed using REML (Residual Maximum Likelihood; Patterson and Thompson, 1971), supported by a Vax computer. REML was used to overcome the problems of missing data. The assumption has been made that treatment does not affect variance around the mean for experimental results. Therefore, apart from egg production data, experimental data are given in the form of mean values for each variable in its treatment group, with a standard error of the difference (sed) for each variable. This is preferable to giving standard errors of the mean or standard deviations for each set of data. Possible linear trends with time for plasma variables were assessed by calculating the deviance change for the data, and comparing this value with the appropriate chi-squared (χ^2) statistic.

Data from the smaller scale broiler such as bird weights, concentrations of plasma minerals, metabolites or enzyme activity were analysed using analysis of variance (ANOVA) by Genstat (Genstat 5 Committee, 1993) supported by a Vax computer. Data, apart from the activities of the kidney hydroxylase enzymes, are presented as a mean for each treatment group with a standard error of the difference for each variable.

CHAPTER 4

RESULTS

4.1 Validation of analytical techniques

The purpose of the first set of experiments was to check the accuracy of the measurements of total calcium, and to cross calibrate the measurements of total and ionised calcium, using the colorimetric and ion electrode methods described in Section 3.3.4.2. Measurements were made in duplicate, and averages of the two measurements are given in *Figures 4a, 4b* and *4c* below.

4.1.1 Measurement of total and ionised calcium in blood and plasma

Figure 4a. The measurement of total calcium in buffered solutions of $CaCl_2$, with or without 3% (w/v) added BSA.

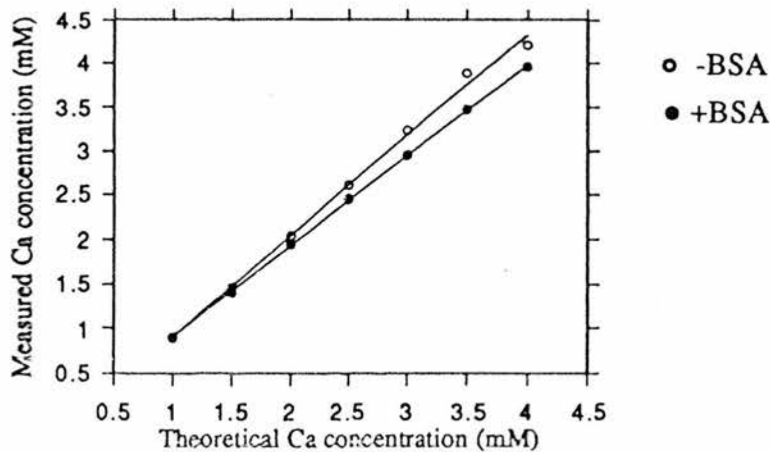
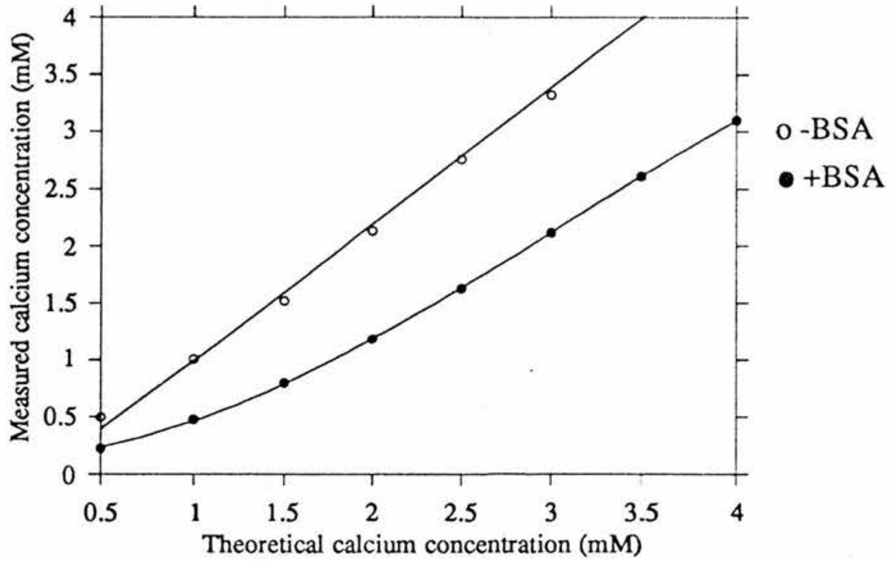
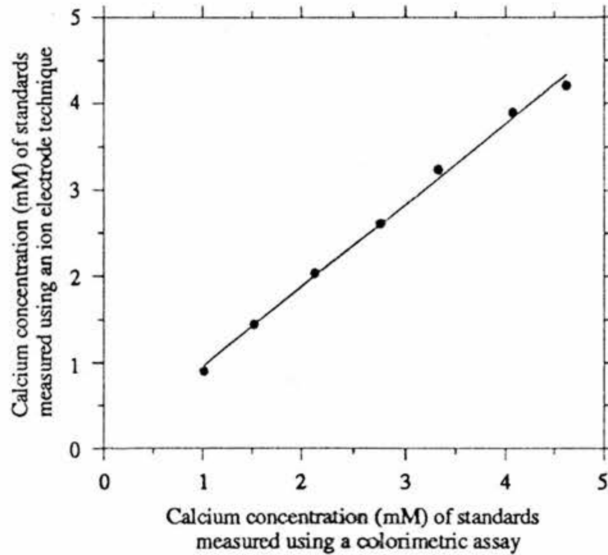


Figure 4b. The measurement of ionised calcium in buffered solutions of $CaCl_2$, with or without 3% (w/v) added BSA.



As expected, BSA caused a large (about 50%) reduction in ionised calcium content of samples. The percentage ionised calcium dropped as the total calcium fell, from 67% at 4 mM to 46% at 0.5 mM.

Figure 4c. Correlation over a range of concentrations between measurements of calcium obtained using a colorimetric method or a calcium-specific ion electrode.



The overall correlation between the two methods over the range 1.0-4.5 mM was satisfactory . r^2 was 0.99 assuming a linear response, and the slope of the regression line was 0.93.

Bovine serum albumin (BSA) had no detrimental effect on the measurement of total calcium using the Wako kit on the plate reader (see *Figure 4a)*. Addition of BSA improved the accuracy of the results, as the method tended to overestimate calcium chloride in solution alone.

The ionised calcium meter was very accurate at estimating over the physiologically important range i.e. 0.5-2 mM, but then overestimated at higher concentrations (see *Figure 4b*). This was not surprising as only two standards (1.25

and 2.5 mM), supplied with the 634 Ca^{2+}/pH analyser, are used to calculate concentrations of unknowns.

In conclusion, it was demonstrated that the Wako method was completely satisfactory for measuring total calcium in plasma over a wide range (see *Figure 4a*), and the ionised calcium meter was also completely satisfactory for measuring ionised calcium in blood over a much narrower range (see *Figure 4b*).

The purpose of the second set of experiments was to investigate whether the bone alkaline phosphatase iso-enzyme could be specifically measured by measuring the reduction in enzyme activity following wheat germ lectin precipitation of the bone iso-enzyme, and to partition the bone, small intestine and liver enzymes in plasma by a combination of BTM and wheat germ lectin treatments using the colorimetric method described in Section 3.3.4.2. All measurements were made in duplicate, and results given in *Tables IV.i and IV.ii.*

4.1.2 Plasma alkaline phosphatase (AP) activity

Table IV.i Alkaline phosphatase (AP) activity in hen and broiler plasma, with or without wheat germ lectin treatment of plasma.

Sample	AP (IU/l)	*% activity of total*
broiler	1149	
broiler	905	
hen	912	
broiler lectin supernatant	0	0
broiler lectin supernatant	0	0
hen lectin supernatant	0	0
broiler lectin precipitate	816	71
broiler lectin precipitate	772	85
hen lectin precipitate	513	56

There was no alkaline phosphatase activity in the supernatant of either lectin-treated hen or broiler plasma. The alkaline phosphatase activity of the re-suspended pellet varied between 56 and 85% of the activity of the untreated plasma. These results imply that virtually all the plasma alkaline phosphatase activity is bone-derived in both broilers and hens, as wheat germ lectin will precipitate the bone iso-enzyme.

Table IV.ii Alkaline phosphatase (AP) activity in hen and broiler plasma, with or without l-p-bromotetramisole (BTM) treatment of plasma.

sample	*AP (IU/l)*	*% activity of total*
broiler	1442	
broiler	1364	
hen	860	
broiler+BTM	432	30
broiler+BTM	287	21
hen+BTM	137	16

The alkaline phosphatase activity of the plasma was between 16 and 30% of total when BTM was added to the reaction mixture. This implies that there is a substantial amount of alkaline phosphatase in plasma derived from small intestine, because BTM inhibits the liver and bone iso-enzymes (see *Table IV.ii*). The results from this assay are therefore in conflict with the results described above using lectin (see *Table IV.i*).

Therefore, the only conclusion that can be firmly drawn is that chicken alkaline phosphatase iso-enzymes do not respond to inhibitors in the same way as human iso-enzymes. However, experiments done with rapidly growing broilers with high rates of bone growth and turnover have consistently shown much higher levels of plasma total alkaline phosphatase compared with adult birds (Matzuzawa, 1981). In the absence of data to suggest otherwise, for the purposes of this project, it was assumed that plasma total alkaline phosphatase activity was largely derived from osteoblastic activity and was therefore a marker of bone turnover.

4.2. Laying Hen Experiment

4.2.1 Egg Production

Table IV.iii Production of whole and soft shelled eggs[5] by hens on different treatments over a 48 week laying period.

Treatment	*n*	*whole eggs / 48 week laying period*	*soft shelled eggs / 48 week laying period*
Control	96	276 (5.7)	2.1 (0.343)
Oystershell	64	294 (3.5)	1.8 (0.331)
Fluoride	64	297 (2.4)	2.3 (0.410)
Ascorbic acid	64	278 (5.9)	3.1 (0.968)
$1,25(OH)_2D_3$	32	286 (10.2)	2.0 (0.560)
Low CP & high vitamin K	64	283 (4.4)	2.5 (0.939)
Low Phosphorus	64	291 (3.6)	1.8 (0.348)
Early Lighting	64	280 (6.4)	1.7 (0.542)
J-line	31	142 (13.9)***	0.1 (0.054)

Data given as mean (standard error of the mean). *** $p<0.001$

Data were analysed using one-way analysis of variance. All Hi-sex birds laid at a good rate during the course of the experiment with an average daily production of between 0.8-0.9 eggs per hen/day. There were no significant differences ($p>0.05$) in egg production between treatments. As expected, the J-line birds laid significantly fewer ($p<0.001$) eggs with an average of 0.42 eggs per hen/day.

Birds on all treatments laid a small proportion of soft shelled eggs. Amongst the Hi-sex birds, this ranged from 0.004-0.01 soft shelled eggs per hen/day, with no significant differences between treatments ($p>0.05$). The J-line birds laid an insignificant proportion of soft shelled eggs.

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[5] Soft shelled eggs are eggs with an imperfectly formed shell . The shell can either be very thin, or non existant.

4.2.2 Blood Samples

REML was used to analyse data to take into account missing values where blood samples were, for example, clotted or haemolysed or where a pair of birds was missing. Statistical analysis of results from assays of blood samples for total calcium, inorganic phosphate and alkaline phosphatase, measured using the colorimetric methods described in Sections 3.2.11.1, 3.2.11.2 and 3.2.11.3, gave the following results.

4.2.2.1 Control Treatment - Tier Effects

Results from each sampling period were used to analyse whether there was any linear component of trend amongst the variables measured from control birds from each tier.

Table IV.iv a,b,c Plasma total calcium, inorganic phosphate and total alkaline phosphatase activity in control hens from different tiers at four sampling times.

Table IV.iv .a

Total Calcium (mM) Sampling Time

Tier	*1*	*2*	*3*	*4*
top	6.80	6.11	6.60	6.12
middle	7.96	6.35	6.70	6.12
bottom	7.16	6.80	6.99	6.25
sed	0.542	0.419	0.308	0.438
deviance change	3.61	2.520	1.639	0.105
χ^2 (2 df)	5.991			

n=168/tier

Table IV.iv .b

Inorganic Phosphate (mM)	Sampling Time			
Tier	*1*	*2*	*3*	*4*
top	0.774	0.825	0.803	0.968
middle	0.794	0.892	0.830	0.823
bottom	0.807	1.034	0.884	1.000
sed	0.060	0.066	0.036	0.071
deviance change	0.339	7.92	4.409	5.65
χ^2 (2 df)	5.991			

n=168/tier

Table IV.iv .c

Alkaline Phosphatase (IU/l)	Sampling Time			
Tier	*1*	*2*	*3*	*4*
top	442	897	1247	473
middle	459	619	1079	462
bottom	551	738	1347	541
sed	101.3	147.3	143.1	110.8
deviance change	1.38	3.168	3.122	0.692
χ^2 (2 df)	5.991			

n=168/tier

These data were used to estimate the linear component of trend for each of the variables. There was no evidence of any linear relationship with time for any of the variables. The deviance changes for calcium, inorganic phosphate, and alkaline phosphatase were always less than 5.991 (where $p>0.05$), apart from inorganic phosphate measurements in plasma samples from the second sampling time .

Table IV.v Grand means of plasma total calcium (total Ca), inorganic phosphate (Pi) and total alkaline phosphatase activity (AP) in control hens from different tiers.

Variable	*Total Ca (mM)*	*P i (mM)*	*AP (IU/l)*
Tier			
top	6.35	0.821	664
middle	6.74	0.829	626
bottom	6.85	0.916**	792
sed (average)	0.409	0.028	112.7
p	>0.05	<0.02	>0.05

n=168/tier **p<0.02

There was little evidence of a 'tier effect' in birds on the control diet. Birds from the bottom tier had values for plasma inorganic phosphate that were significantly (p<0.02) higher than those from either the top or middle tier.

The overall observed lack of effect of tier on the concentrations of plasma total calcium and inorganic phosphate and the activity of alkaline phosphatase suggests that bone turnover and quality in birds from different tiers in the battery unit would not be influenced by tier.

4.2.2.2 Effects of treatment or strain on plasma variables in laying hens

Blood samples were taken from birds from the middle tier only, and the results from assays for plasma total calcium, inorganic phosphate and alkaline phosphatase compared with those obtained from birds from the middle tier of the control treatment only. Again, data from individual sampling periods were analysed

to see whether there was any linear component of trend. Results from each sampling period were also averaged to determine whether there were any significant differences between treatments.

Table IV.vi Plasma total calcium (mM) from laying hens on different treatments sampled at regular intervals.[5]

	Sampling time			
Treatment	*1*	2	*3*	*4*
Control	7.96	6.35	6.70	6.12
Oystershell	8.10	6.61	7.51	6.38
Fluoride	8.29	7.13	7.73	6.96
Ascorbic Acid	7.86	6.66	7.82	6.97
$1,25(OH)_2D_3$	-	6.82	8.20	6.43
Low CP & high vitamin K	8.00	6.52	7.67	6.61
Low Phosphorus	8.02	6.70	7.75	6.05
Early Lighting	7.96	6.76	7.54	6.65
J-line	7.60	7.24	7.64	6.41
df (full model)	78			
sed (average)	0.016			
deviance change	9.594			
χ^2 6 df	12.592			

For control and nutritional treatments, n=16. J-line, n=8.

Table IV.vii Plasma inorganic phosphate (mM) from laying hens on different treatments sampled at regular intervals.[5]

Sampling Time

Treatment	*1*	*2*	*3*	*4*
Control	0.793	0.892	0.830	0.823
Oystershell	0.932	0.998	0.752	0.881
Fluoride	0.901	1.092	0.728	0.992
Ascorbic Acid	0.833	0.932	0.717	1.043
$1,25(OH)_2D_3$	-	0.966	0.776	1.068
Low CP & high vitamin K	0.957	0.946	0.698	1.073
Low Phosphorus	0.800	0.934	0.642	0.819
Early Lighting	0.887	0.854	0.690	0.798
J-line	1.063	1.218	0.845	0.781
df (full model)	78			
sed (average)	0.0027			
deviance change	9.594			
χ^2 6 df	12.592			

For control and nutritional treatments, n=16. J-line, n=8.

Table IV.viii Plasma total alkaline phosphatase activity (IU/l) from laying hens on different treatments sampled at regular intervals.[6]

Sampling Time

Treatment	*1*	*2*	*3*	*4*
Control	442	619	1079	462
Oystershell	694	1003	1306	761
Fluoride	1022	1252	1595	1178
Ascorbic Acid	725	896	896	670
$1,25(OH)_2D_3$	-	830	1111	617
Low CP & high vitamin K	806	829	953	642
Low Phosphorus	787	825	1188	1097
Early Lighting	778	1116	1368	575
J-line	335	242	452	457
df (full model)	78			
sed (average)	4.611			
deviance change	10.39			
χ^2 6 df	12.592			

For control and nutritional treatments, n=16. J-line, n=8.

There was no indication of any linear component of trend. The deviance change for calcium, inorganic phosphate and alkaline phosphatase were 6.962, 9.594, and 10.390 respectively. To reach significance ($p<0.05$), this value would have to be greater than 12.592.

[6] Feeding of the 1,25-dihydroxycholecalciferol-supplemented diet was started only after the first blood sample was taken

Table IV.ix Mean plasma concentrations of total calcium (Ca), inorganic phosphate (Pi) and total alkaline phosphatase activity (AP), in hens from different experimental treatments over the whole experiment.

Treatment	*Ca (mM)*	*P i(mM)*	*Total AP (IU/l)*
Control	6.81	0.819	613
Oystershell	7.13	0.877	975*
Fluoride	7.62*	0.928*	1269***
Ascorbic acid	7.19	0.887	806
$1,25(OH)_2D_3$[a]	7.02	0.927*	845
Low CP & high vitamin K	7.20	0.922*	808
Low Phosphorus	7.04	0.840	970*
Early Lighting	7.24	0.823	930*
J-line	7.19	0.995***	313*
sed (average)	0.298	0.0403	137.2
df 78			

[a] means calculated from three sampling times

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

There were significant differences between treatments for the grand mean. Replacement of a proportion of the ground limestone in the diet with oystershell significantly ($p<0.05$) raised plasma alkaline phosphatase activity. Supplementation with sodium fluoride significantly raised plasma total calcium($p<0.05$), inorganic phosphate ($p<0.05$) and alkaline phosphatase ($p<0.001$) activity. Feeding a low crude protein, high vitamin K diet significantly raised plasma inorganic phosphate ($p<0.05$). Feeding a diet containing $1,25(OH)_2D_3$ significantly increased plasma

inorganic phosphate ($p<0.05$). Feeding a low phosphorus diet significantly increased plasma alkaline phosphatase activity ($p<0.05$). Birds brought into lay early by stepping up the lighting significantly raised plasma alkaline phosphatase activity ($p<0.05$). The J-line birds had increased plasma inorganic phosphate activity ($p<0.001$) and reduced plasma alkaline phosphatase activity ($p<0.05$). There was no effect on any of the variables measured of supplementation of the diet with ascorbic acid.

Table IV.x Plasma $1,25(OH)_2D_3$ in hens from selected treatments sampled at regular intervals.

	plasma $1,25(OH)_2D_3$ (pg/ml)		
Sampling time	*2*	*3*	*4*
Treatment			
Control	173	232**	163
Ascorbic Acid	180	213**	158
$1,25(OH)_2D_3$	160	209**	148
sed	26.0		
p diet	0.474		
sampling time	<0.001		
diet*sampling time	0.968		

n=16 per treatment ** $p<0.01$

Plasma $1,25(OH)_2D_3$ levels were measured using the protein binding assay described in Section 3.2.12.

Plasma $1,25(OH)_2D_3$ increased significantly between sampling periods 2 and 3 over all three treatments ($p<0.01$), and then declined. At separate sampling periods, there were no significant differences ($p=0.474$) between treatments.

However, the birds given $1,25(OH)_2D_3$ in the diet had consistently lowered plasma circulating $1,25(OH)_2D_3$ compared with birds from the other two treatments.

4.2.3 *Post Mortem* wing radiographic study

X-rays of wing bones were made as described in Section 3.2.10.

A significant proportion of Hi-sex birds had broken wing bones. Fractures were found in all the wing bones - humerus, radius and ulna. Some bones displayed more than one fracture. Most fractures were of a spiral type, although in some cases the bones were completely broken and the broken ends were quite badly displaced. None of the fractures had occurred after death as all showed evidence of healing by a greater of lesser degree of callus formation. In contrast to the Hi-sex birds, none of the J-line birds had any fractured wing bones.

4.2.3.1 Effect of treatment on percentage of broken bones in hen wing bones

Individual wing bones were classified as either broken or unbroken. Fracture data were analysed using Genstat. A binomial model was fitted to the percentage data which were logit transformed (Snedcor and Cochran, 1967)[7] Significant differences were established by calculating differences in incidences of fracture by treatment.

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[7] logit n = loge [(n/100-n)]

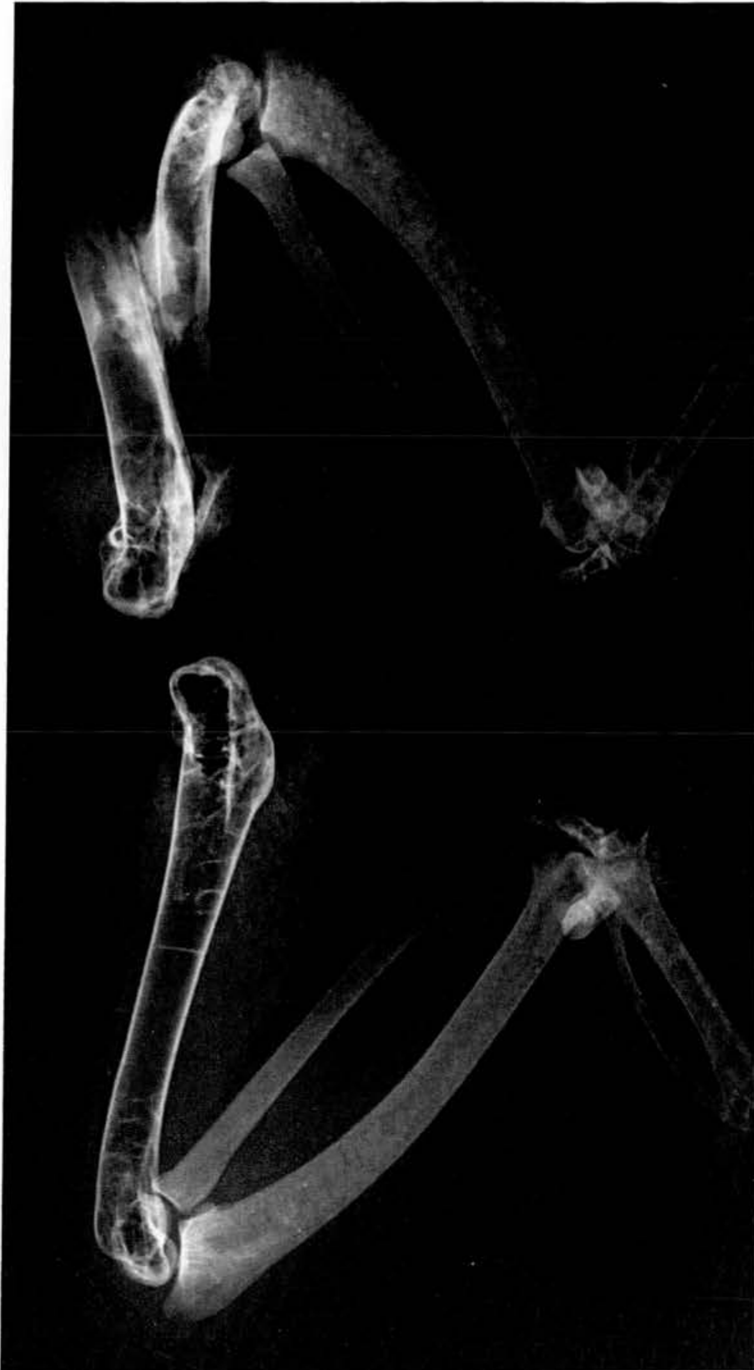


Plate 4 Photograph of a radiograph of the wings of a 68 week old Hi-sex hen. The upper humerus is badly fractured. Note the thinness of the cortices and the sparse trabeculae in the pneumatised humerus. The radius and ulna appear opaque due to the presence of medullary bone.

Table IV.xi Effect of treatment on the incidence of wing bone fracture.

	%age of birds with fractured wing bones			
Treatment	*humerus*	*radius*	*ulna*	*(n)*
Control	43	0	43	14
Oyster shell	16*	21	26	19
Fluoride	6**	6	12*	17
Ascorbic acid	7*	26	26	15
1,25$(OH)_2D_3$	23	18	23	17
Low CP & high vitamin K	13*	7	33	15
Low Phosphorus	7*	13	13	15
Early lighting	28	7	14	14
J-line	0	0	0	10

*p<0.05, ** p<0.02

The results of the analysis showed that in the control group, the humerus had a high rate of fracture, and that the oystershell, ascorbic acid, low CP & high vitamin K treatments (all $p<0.05$), and fluoride and low dietary phosphorus treatments (both $p<0.02$), resulted in significantly lowered rates of fracture of this bone. There was no effect of early lighting on the rate of fracture of the humerus.

In contrast, no radii were fractured in the control group, but all the treatment groups of Hi-sex birds had a few individuals with broken radii. No treatment produced a statistically significantly different (i.e. worse) rate of fracture.

There was a moderate rate of fracture of the ulna on the control group. The only treatment which resulted in a reduced rate of fracture was the group of Hi-sex birds fed the fluoride-supplemented diet ($p<0.05$).

None of the J-line birds had any fractured bones. However, there was a smaller number of birds in this group to incorporate into the statistical model, which meant that this result was not deemed to be significant.

4.2.4 Effect of treatment on the percentages of trabecular bone and medullary bone in the free thoracic vertebra (FTV) in 68 week old hens.

Histological sections of bone were prepared and analysed for trabecular and medullary bone content as described in Sections 3.2.8 and 3.2.9.

REML was used for statistical analysis of data. Data were log transformed (natural logs) as they were not normally distributed. Individual t-tests were performed by comparing treatment means and standard errors of difference from test groups with those from birds on the control treatment.

Table IV.xii Treatment and percentage of trabecular bone (TB) and medullary bone (MB) in the FTV in 68 week old hens.

Treatment	*FTV*			
	TB %	log fractional TB	MB (%)	log fractional MB
Control	12.15	-2.032	0.49	-5.638
Oystershell	11.40	-2.101	0.70*	-5.075
Fluoride	11.92	-2.029	0.54	-5.494
Ascorbic acid	12.01	-1.936	0.40	-5.280
$1,25(OH)_2D_3$	11.14	-2.117	0.44	-5.278
Low CP & high vitamin K	13.19	-1.944	0.84	-5.227
Low Phosphorus	10.00	-2.129	0.63	-5.208
Early lighting	11.94	-2.055	2.43*	-4.988
J-line	16.43	-1.829	1.13	-4.795
df (full model)		96		96
sed (average)+		0.128		0.362

+ calculated from log transformed data, * p<.0.05

Table IV.xiii Treatment and percentage of trabecular bone (TB) and medullary bone (MB) in the PTM of 68 week old hens.

Treatment	*PTM*			
	TB %	log fractional TB	MB (%)	log fractional MB
Control	12.98	-1.934	11.85	-2.238
Oystershell	12.38	-1.991	19.85**	-1.469
Fluoride	12.87	-1.957	17.53*	-1.617
Ascorbic acid	12.05	-2.023	13.39	-2.060
1,25$(OH)_2D_3$	13.47	-1.936	15.12	-1.845
Low CP & high vitamin K	12.58	-1.992	12.45	-2.066
Low Phosphorus	12.60	-1.943	13.54	-1.966
Early lighting	12.24	-2.018	14.89	-1.854
J-line	18.14 *	-1.537	2.38 ***	-4.232
df (full model)	117		117	
sed (average)+	0.138		0.362	

+ calculated from log transformed data

* p<.0.05, ** p<0.01, *** p<0.001

All Hi-sex birds were judged to be osteoporotic by current criteria (Whitehead and Wilson, 1992) i.e. all the PTM's and FTV's measured contained less than 16% trabecular bone (*Tables IV.xii and IV.xiii*). Using these criteria, J-line birds were not judged to be osteoporotic. There were significant differences between treatments. The J-line birds had a larger percentage of trabecular bone within both the FTV and PTM than any of the Hi-sex birds, but this difference was only

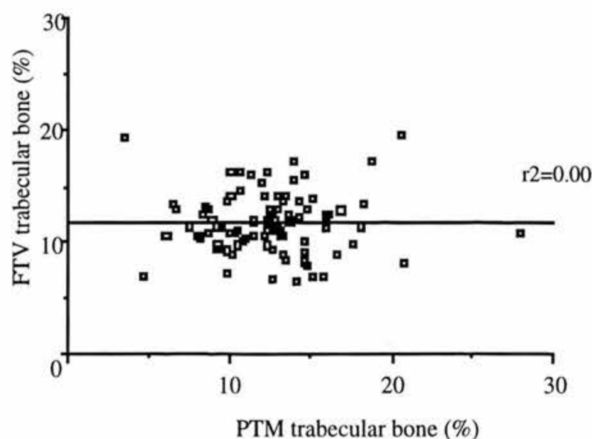
significant for the PTM ($p<0.05$). The J-line birds had significantly lower amount of medullary bone within the PTM ($p<0.001$) compared with the control Hi-sex birds.

Within the Hi-sex treatment groups, there were no significant differences within the trabecular bone contents of the FTV's or PTM's. However, the early lighting and oystershell had a significantly higher amount of medullary bone within the FTV ($p<0.05$), and the oystershell and fluoride groups had a significantly higher amount of medullary bone within the PTM ($p<0.01$ and $p<0.05$ respectively).

4.2.5 Relationships between trabecular and medullary bone, and egg output.

As there was no positive evidence of nutritional treatment on quantities of trabecular bone within the Hi-sex birds, graphs were drawn of PTM trabecular bone against FTV trabecular bone to determine whether there was any relationship between the quantities of structural bone in the two bones.

Figure 4d. Relationship between PTM trabecular bone and FTV trabecular bone in all 68 week old Hi-sex hens.



As can be seen from the graph (*Figure 4d*), there appears to be no relationship between the amounts of structural bone in the FTV compared with the PTM (r^2=0.00).

Graphs were then drawn of trabecular against medullary bone for the PTM and FTV to see whether there were any stronger relationships between the two bone types indicating possible links between resorption of trabecular bone and formation of medullary bone.

Figure 4e. Relationship between PTM trabecular and medullary bone in all 68 week old Hi-sex hens.

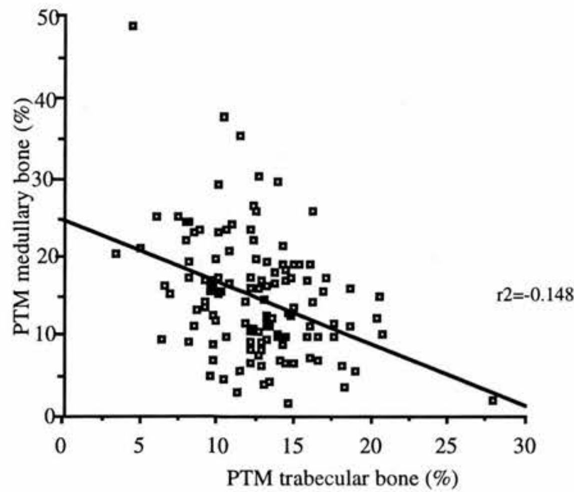
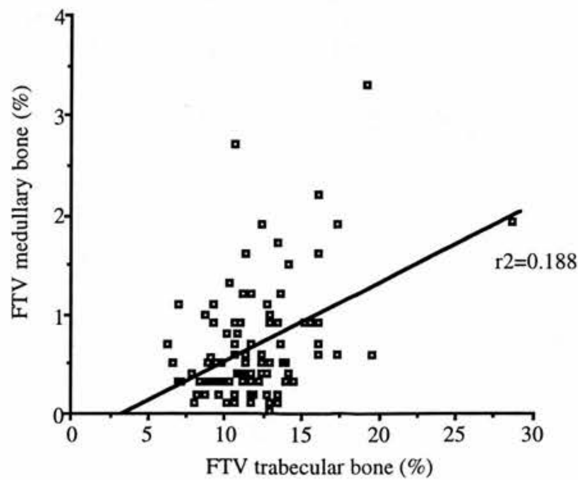


Figure 4f. Relationship between FTV trabecular and medullary bone in all 68 week old Hi-sex hens.



There was a weak negative relationship between the amounts of trabecular and medullary bone in the PTM (*Figure 4e*, r^2=-0.148), and a weak positive correlation between trabecular and medullary bone in the FTV (*Figure 4f,* r^2=0.188).

Similar plots were drawn to establish whether there was any obvious relationship between egg output and the amounts of trabecular bone in the FTV and PTM.

Figure 4g. Lack of relationship between PTM trabecular bone and egg output in all Hi-sex birds.

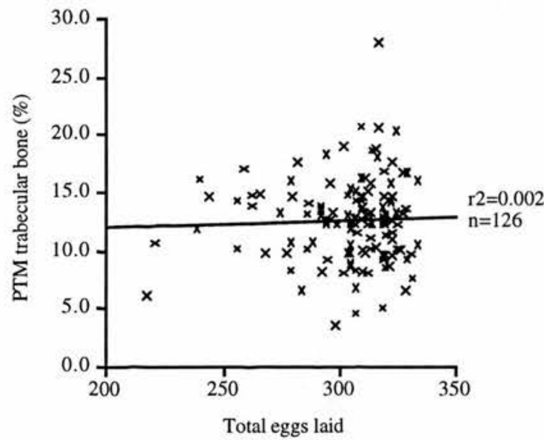
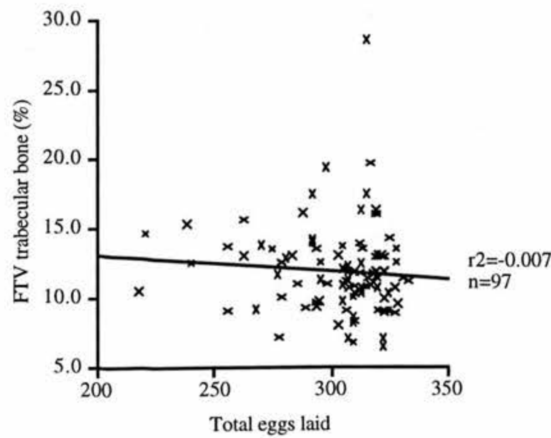


Figure 4h. Lack of relationship between FTV trabecular bone and egg output in all Hi-sex birds.



There was no evidence of a relationship between total number of eggs laid and PTM trabecular bone (*Figure 4g*, r^2=0.002), or between total number of eggs laid and FTV trabecular bone (*Figure 4h,* r^2=-0.007).

4.3. Broiler Experiments

In Experiments 1-5, measurements of plasma total calcium, inorganic phosphate and alkaline phosphatase activity were made using the colorimetric assays described in Sections 3.2.11.1, 3.2.11.2 and 3.2.11.3. Plasma $1,25(OH)_2D_3$ levels were measured using the protein binding assay described in Section 3.2.12. Bone ash was measured as described in Section 3.3.5. Histological sections of growth plate were assessed for tibial dyschondroplasia or rickets lesions as described in Section 3.3.6.

4.3.1 Experimental induction of TD using nutritional modification

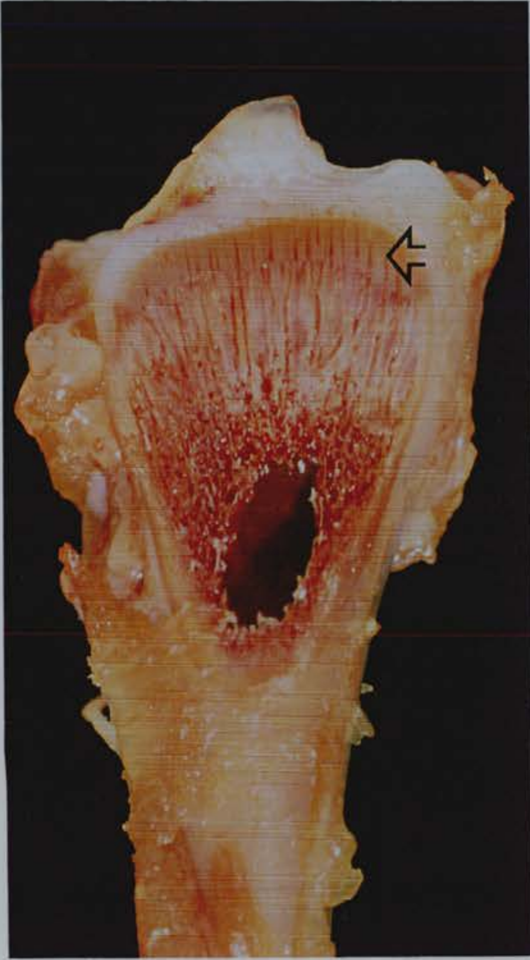
Table IV.xiv Experiment 1. Effect of imbalanced Ca/P diet on bird weight, the experimental incidence of TD, rickets, plasma chemistry (total calcium [Ca], inorganic phosphate [Pi] and $1,25(OH)_2D_3$), compared with a standard Ca/P diet in 3 week old chicks.[8]

Diet					*plasma*		
Ca, P (g/kg)	*D_3 (µg/kg)*	*weight (g)*	*TD (%)*	*rickets (%)*	*Ca (mM)*	*P i (mM)*	*$1,25(OH)_2D_3$ (pg/ml)*
12, 6	25	476	14	0	2.59	0.760	37
7.5, 7.6	25	535	21	14	2.99	0.999	42
7.5, 7.6	75	533	46	0	2.94	0.881	42
sed		16.4			0.232	0.0809	13.3
p		<0.001			0.191	0.019	0.690

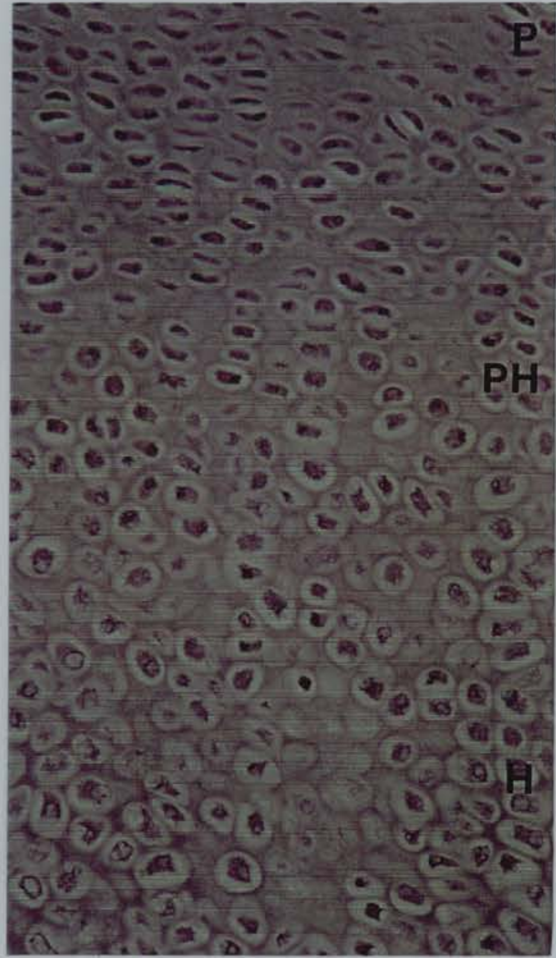
n=30 per group (bird weight, percentage incidence of TD and rickets), n=15 per group (plasma Ca and Pi), n=10 per group (plasma $1,25(OH)_2D_3$)

[8] As there was no beneficial effect of the higher level of 75 µg/kg cholecalciferol on TD, all subsequent broiler experiments using either balanced or imbalanced Ca/P diets contained a more conventional level of 25 µg/kg cholecalciferol.

a



b



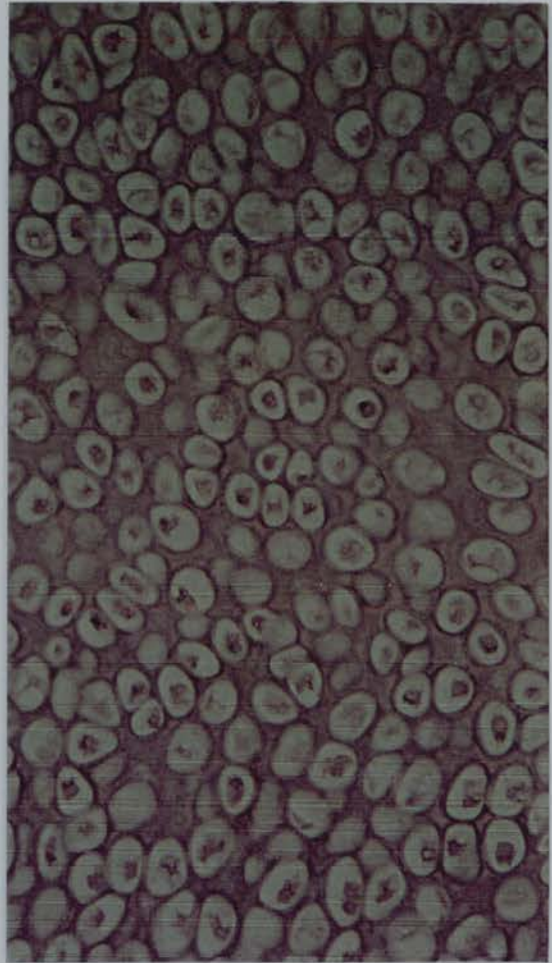
150 μm

Plate 5a A sagittal section of the tibiotarsus from a normal 3 week old broiler chicken, showing the growth plate (arrow). Magnification x4

5b Photomicrograph of the proximal tibiotarsus growth plate from a normal 3 week old broiler chicken, stained with hematoxylin and eosin. Note the ordered sequence of proliferating (P), pre-hypertrophic (PH) and hypertrophic (H) chondrocytes.

a

b



150 μm

Plate 6a A sagittal section of the tibiotarsus from a 3 week old broiler chicken affected by tibial dyschondroplasia, showing the accumulation of avascular cartilage in the growth plate (arrow). Magnification x4

6b Photomicrograph of the proximal tibiotarsus growth plate from a 3 week old broiler chicken affected by dyschondroplasia, stained with hematoxylin and eosin. Note the mass of pre-hypertrophic chondrocytes

The results from the first experiment (*Table IV.xiv*) confirmed that feeding diets imbalanced in calcium and phosphorus to birds can induce a high incidence of TD. The control group had a 14% incidence of TD, as assessed histologically, compared to a 21% incidence of TD and a 14% incidence of rickets in the group fed the imbalanced Ca/P diet containing 25 μg/kg D_3, and a 46% incidence of TD and 0% incidence of rickets in the group fed the imbalanced Ca/P diet containing 75 μg/kg D_3. Bird weight was significantly lower ($p<0.001$) on the standard Ca/P diet, and plasma inorganic phosphate was significantly higher ($p<0.05$) in birds on the imbalanced Ca/P diet. There were no differences in plasma calcium or $1,25(OH)_2D_3$ concentrations between treatments. Alkaline phosphatase measurements were unsuccessful in this experiment as the plasma samples were insufficiently diluted.

4.3.2 Addition of $1,25(OH)_2D_3$ to the experimental diet.

Table IV.xv Experiment 2. Effect of adding 10 µg/kg $1,25(OH)_2D_3$ to the imbalanced Ca/P diet on bird weight, the incidence of TD, rickets, and plasma chemistry (total calcium [Ca], inorganic phosphate [Pi] and alkaline phosphatase activity [AP]) in 3 week old chicks, compared with the standard Ca/P diet.

Diet						*plasma*		
Ca , P (g/kg)	*D_3 (µg/kg)*	*$1,25(OH)_2D_3$ (µg/kg)*	*weight (g)*	*TD (%)*	*rickets (%)*	*Ca (mM)*	*P i (mM)*	*AP (IU/l)*
12, 6	25	0	647	29	0	1.55	0.606	17329
7.5,7.6	25	0	650	29	0	1.58	0.564	15080
7.5,7.6	25	10	552	0	0	1.62	0.638	13136
sed			24.4			0.093	0.0344	3804.8
p			<0.001			0.614	0.126	0.496

n=20 per group (bird weight, percentage incidence of TD and rickets), n=10 per group (plasma Ca and Pi).

The second experiment (*Table IV.xv*) showed that addition of $1,25(OH)_2D_3$ to a diet imbalanced in calcium and phosphorus can reduce the incidence of TD. The control group had a 29% incidence of TD, as did the group fed the imbalanced diet. The incidence dropped to 0 in the group fed the imbalanced diet supplemented with 10 µg/kg $1,25(OH)_2D_3$. There was a significant difference in body weights between treatments, with the group receiving $1,25(OH)_2D_3$ having a significantly lower mean body weight than birds on the other two treatments. There were no significant differences in plasma calcium, inorganic phosphate or alkaline phosphatase between treatments.

Table IV.xvi Experiment 3. Effect of adding graded doses of $1,25(OH)_2D_3$ (0, 2.5, 5 and 10 μg/kg) to the imbalanced Ca/P diet on bird weight, the incidence of TD and rickets, plasma chemistry (total calcium [Ca], inorganic phosphate [Pi], and $1,25(OH)_2D_3$, and plasma alkaline phosphatase activity [AP]) in 3 week old chicks.

Diet				*plasma*			
Ca, P (g/kg)	*$1,25(OH)_2D_3$ (μg/kg)*	*weight (g)*	*TD and rickets (%)*	*$1,25(OH)_2D_3$ (pg/ml)*	*Ca (mM)*	*P i (mM)*	*AP (IU/l)*
12, 6	0	546	0, 11	40	2.18	0.601*	17111
7.5, 7.6	0	532	36, 0	65*	1.90	0.700	17736
7.5, 7.6	2.5	549	19, 0	49	2.07	0.632	18578
7.5, 7.6	5	516	0, 0	53	2.19	0.695	17687
7.5, 7.6	10	522	0, 0	67	2.32	0.708	13047
7.5, 7.6	10+	544	24, 0	52	1.91	0.756	17390
sed		23.9		8.7	0.186	0.0394	3369.8
p		0.678		0.026	0.177	0.004	0.834

+ for days 1-7 only   * $p<0.05$

n=20 per group (bird weight , TD and rickets), n=10 per group (plasma Ca, Pi, AP and $1,25(OH)_2D_3$).

The third experiment (*Table IV.xvi*) showed that the response to dietary $1,25(OH)_2D_3$ is influenced by dose, in that the group receiving no $1,25(OH)_2D_3$ had a 36% incidence of TD, and the groups receiving 2.5, 5, or 10 μg/kg had 19, 0 , and 0% incidences respectively. The group receiving 10μg/kg $1,25(OH)_2D_3$ for week 1 had a 24% incidence of TD. In this experiment there were no significant differences in body weights between treatments, although there was a trend towards lower body

weight at the higher levels of supplementation of 5 and 10 μg/kg of $1,25(OH)_2D_3$. There were no significant differences in plasma calcium or alkaline phosphatase between treatments, but plasma inorganic phosphate was significantly lower ($p<0.05$) in chicks fed the standard Ca/P diet. There was a trend towards hypercalcaemia with increasing dietary $1,25(OH)_2D_3$. Feeding imbalanced Ca/P diet significantly increased ($p<0.05$) mean circulating $1,25(OH)_2D_3$ levels in this experiment from 40 to 65 pg/ml. Concentrations increased from a mean of 49 pg/ml in birds fed 2.5 μg/kg $1,25(OH)_2D_3$, to a mean of 67 pg/ml in birds fed 10 μg/kg, although these changes were not statistically significant.

4.3.3 Addition of ascorbic acid and 1,25$(OH)_2D_3$ to the experimental diets.

Table IV.xvii Experiment 4. Effect of addition of ascorbic acid or 1,25$(OH)_2D_3$ to a diet either imbalanced or standard in Ca/P on the incidence of TD, percentage bone ash, and plasma chemistry (total calcium [Ca], inorganic phosphate [Pi], and alkaline phosphatase activity [AP]) in 3 week old chicks.

Diet			*plasma*		
Ca, P (g/kg) and supplement	*TD (%)*	*Bone Ash (%)*	*Ca (mM)*	*P i (mM)*	*AP (IU/l)*
7.5, 7.6	42	41.9	1.19*	0.586	15927
+200mg/kg ascorbic acid	53	41.3	1.70*	0.686*	15682
+5µg/kg 1,25$(OH)_2D_3$	0	41.0	1.43*	0.663*	10333*
+ both	0	42.0	1.42	0.592	15341
12, 6	41	41.9	1.53	0.576	14468
+200mg/kg ascorbic acid	11	41.7	1.49	0.661*	14954
+5µg/kg 1,25$(OH)_2D_3$	0	40.7	1.88*	0.482*	16178
+ both	0	42.5	1.97	0.470	11959
sed		1.006	0.099	0.0381	2490.3
p basal		0.641	<0.001	0.003	0.960
ascorbic acid		0.798	0.057	0.372	0.891
1,25$(OH)_2D_3$		0.724	0.007	0.006	0.305
basal*ascorbic acid		0.364	0.112	0.712	0.229
basal*1,25$(OH)_2D_3$		0.182	0.003	0.015	0.517
1,25$(OH)_2D_3$*ascorbic acid		0.193	0.199	0.017	0.930

n=20 per group *p<0.05

The results of the fourth experiment (*Table IV.xvii*)showed that feeding either the imbalanced Ca/P (7.5 g/kg Ca, 7.6 g/kg P) or the standard Ca/P diet (12 g/kg Ca, 6 g/kg P) diet induced a high incidence of TD (42 and 41% respectively).

Supplementation of the imbalanced Ca/P diet with 200 mg/kg ascorbic acid had no effect on the incidence of TD, but supplementation with 5 μg/kg $1,25(OH)_2D_3$ reduced the incidence to 0%. Supplementation with both ascorbic acid and $1,25(OH)_2D_3$ again resulted in a 0% incidence of TD. However, supplementation of the standard Ca/P diet with 200 mg/kg ascorbic acid resulted in a drop in the incidence of TD to 11%. Supplementation with 5 μg/kg $1,25(OH)_2D_3$ either alone or in conjunction with 200 mg/kg ascorbic acid again resulted in a 0% incidence of TD.

In this experiment, there were significant differences in blood chemistry results. Birds fed the imbalanced Ca/P diet had significantly lower ($p<0.05$) plasma calcium than birds fed the standard Ca/P diet, but plasma inorganic phosphate and alkaline phosphatase were unchanged. Adding 200 mg/kg ascorbic acid to the imbalanced Ca/P diet resulted in a significant rise ($p<0.05$) in plasma calcium and inorganic phosphate, but no change in alkaline phosphatase. Addition of 5 μg $1,25(OH)_2D_3$ to the imbalanced Ca/P diet resulted in a significant rise in plasma calcium and inorganic phosphate ($p<0.05$), but a significant fall ($p<0.05$) in plasma alkaline phosphatase.

Addition of 200 mg/kg ascorbic acid to the standard Ca/P diet resulted in no change in plasma calcium or alkaline phosphatase, but a significant rise ($p<0.05$) in plasma inorganic phosphate. Addition of 5 μg/kg $1,25(OH)_2D_3$ to the standard Ca/P diet resulted in a significant rise ($p<0.05$) in plasma calcium a significant fall ($p<0.05$) in plasma inorganic phosphate, but no change in plasma alkaline phosphatase. There was no interaction between ascorbic acid and $1,25(OH)_2D_3$ on plasma calcium, inorganic phosphate or alkaline phosphatase ($p>0.05$) although there was a trend to even higher values of plasma calcium in chicks given the standard Ca/P diet with both supplements.

There were no significant differences in toe ash between treatments.

The results of Experiment 4 were so positive with regard to the effect of ascorbic acid on the incidence of TD, it was repeated in order that the results could be replicated, using a larger dose of ascorbic acid. As there seemed to be no benefit of adding ascorbic acid to a diet containing 5 μg/kg $1,25(OH)_2D_3$, this treatment was not repeated.

Table IV.xviii Experiment 5. Effect of adding ascorbic acid or $1,25(OH)_2D_3$ to a diet either imbalanced or standard in Ca and P on bird weight, incidence of TD, and plasma chemistry (total calcium [Ca], inorganic phosphate [Pi], and alkaline phosphatase activity [AP]) in 3 week old chicks.

				plasma	
Ca, P (g/kg) and Supplement	*Weight (g)*	*TD (%)*	*Ca (mM)*	*P i (mM)*	*AP (IU/l)*
7.5, 7.6	576	47	1.59	0.761	17120*
+ 500 mg/kg ascorbic acid	623	72	1.68	0.782	13589
+ 5 μg/kg $1,25(OH)_2D_3$	641	5	1.62	0.773	7510*
12, 6	588	25	1.66	0.796	12253
+ 500 mg/kg ascorbic acid	626	5	1.80	0.743	9300
+ 5 μg/kg $1,25(OH)_2D_3$	521	0	2.21*	0.604*	11664
sed	32.6		0.084	0.0402	1762.5
p basal	0.044		<0.001	0.060	0.343
supplement	0.167		0.005	0.045	0.036
basal*supplement	0.002		0.004	0.069	0.031

n=20 per group

The results of the fifth experiment (*Table IV.xviii*) showed again that supplementation of the imbalanced Ca/P diet with 500 mg/kg ascorbic acid had no

effect on the incidence of TD, whereas supplementation of the standard Ca/P diet reduced the incidence from 25 to 5%. Supplementation of either diet with 5 μg/kg $1,25(OH)_2D_3$ reduced the incidence of TD virtually to zero.

In this experiment there were significant effects of treatment on blood chemistry. There was no significant effect of basal diet (imbalanced Ca/P or standard Ca/P) on plasma calcium or inorganic phosphate ($p>0.05$), but chicks given the imbalanced Ca/P diet had a significantly higher ($p<0.05$) plasma alkaline phosphatase. When either of the basal diets were supplemented with ascorbic acid, there were no significant differences in plasma calcium, inorganic phosphate or alkaline phosphatase ($p>0.05$). When the imbalanced Ca/P diet was supplemented with $1,25(OH)_2D_3$ there were no significant effects on plasma calcium or inorganic phosphate ($p>0.05$), but plasma alkaline phosphatase was significantly ($p<0.05$) reduced. When the standard Ca/P diet was supplemented with $1,25(OH)_2D_3$, there was a significant ($p<0.05$) rise in plasma calcium, a significant fall ($p<0.05$) in plasma inorganic phosphate, but no change in plasma alkaline phosphatase. There seemed to be a potential interaction between small doses of $1,25(OH)_2D_3$ and ascorbic acid.

4.3.4 Dietary supplements and renal hydroxylase activity

Vitamin D metabolites and ascorbic acid were added to the diet of broiler chickens in order to determine whether these supplements altered renal hydroxylase activity.

Renal hydroxylase activity was measured in kidney homogenates using ^{3}H-$25(OH)D_3$ as substrate and HPLC of chloroform-methanol extracts as described in Section 3.3.8.

Figure 4i High performance liquid chromatography of authentic standards of ^{3}H-$25(OH)D_3$ and ^{3}H-$1,25(OH)_2D_3$.

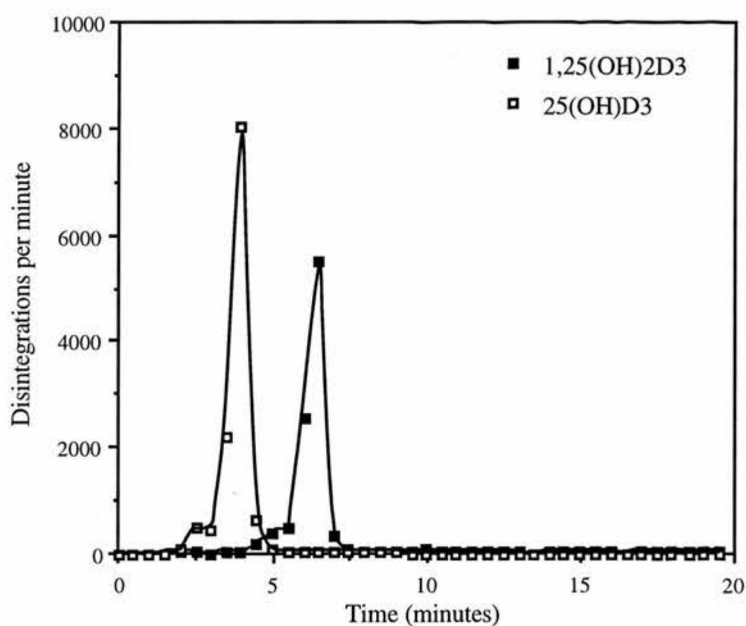


Figure 4j. High performance liquid chromatography of extract of kidney homogenate incubated with $^{3}H\text{-}25(OH)D_3$

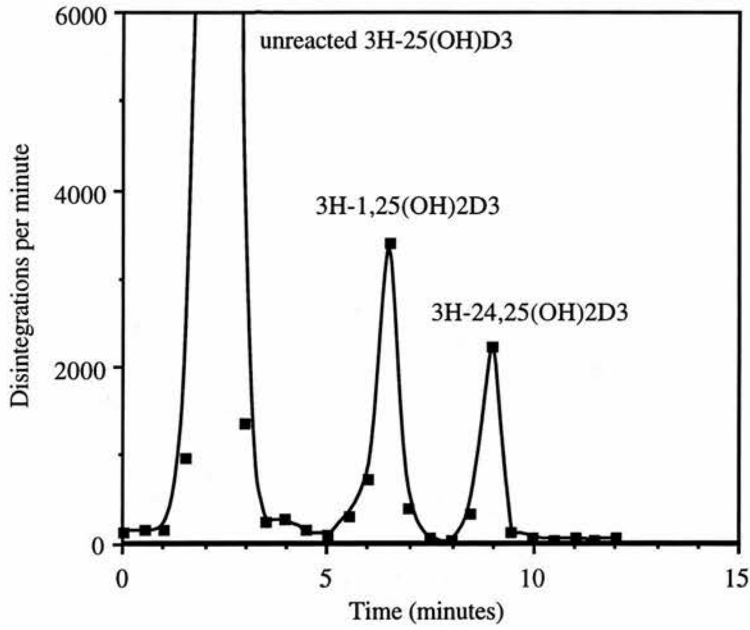


Table IV xix The effect of adding vitamin D metabolites or ascorbic acid to a diet imbalanced in Ca/P on the activities of renal 1-hydroxylase and 24-hydroxylase in 3 week old chicks.

		pmol synthesised/min/mg protein	
Ca, P (g/kg)	*Supplement*	*1,25(OH)$_2$D$_3$*	*24,25(OH)$_2$D$_3$*
7.5, 7.6	none	0.98 (0.125)	0.76 (0.078)
7.5, 7.6	2 μg/kg 1,25(OH)$_2$D$_3$	1.11(0.248)	1.24 (0.251)
7.5, 7.6	5 μg/kg 24,25(OH)$_2$D$_3$	1.12 (0.069)	1.01(0.093)
7.5, 7.6	5 μg/kg 1,24,25(OH)$_3$D$_3$	1.21 (0.185)	1.25 (0.171)
7.5, 7.6	250 mg/kg ascorbic acid	1.30 (0.095)	1.95* (0.129)
7.5, 7.6	1000 mg/kg ascorbic acid	0.70 (0.110)	1.05 (0.135)

Data given as mean (sem), n=4 samples per treatment, pooled from different birds. *$p<0.05$

Sample sizes in this experiment were small and therefore analysis of data showed little significant effect of treatment on enzyme activities. However, there were definite trends. Feeding the imbalanced Ca/P diet resulted in a higher rate of 1-hydroxylase activity compared with 24-hydroxylase activity. Supplementing this diet with 2 μg/kg 1,25(OH)$_2$D$_3$ did not affect 1-hydroxylase activity but caused 24-hydroxylase activity to increase.

A supplement of 5 μg/kg 24,25(OH)$_2$D$_3$ did not alter 1-hydroxylase activity, but again increased 24-hydroxylase activity. 5 μg/kg 1,24,25(OH)$_3$D$_3$ increased both 1-hydroxylase and 24-hydroxylase activities.

The effects of ascorbic acid supplementation were not clear. Either 250 or 1000 mg/kg seemed not to have much effect on 1-hydroxylase activity, whereas 250 mg/kg only stimulated 24-hydroxylase ($p<0.05$).

4.3.5 Analogues of $1,25(OH)_2D_3$

The purpose of this experiment was to investigate the effects of two analogues of $1,25(OH)_2D_3$ with enhanced cell differentiating properties *in vitro*, on chondrocyte differentiation *in vivo*, by dosing broiler chicks with equal quantities of either $1,25(OH)_2D$, either analogue, or vehicle, every 2 days, for 3 weeks, as described in Section 3.3.9.

Plasma total calcium, inorganic phosphate and alkaline phosphatase measurements were made using the colorimetric assays described in Sections 3.2.11.1, 3.2.11.2 and 3.2.11.3. Ionised calcium levels were measured in whole blood using an ion electrode technique as described in Section 3.3.4.1.

Table IV.xx. Effect of dosing at two-day intervals with 300-500 ng/chick $1,25(OH)_2D_3$ and analogues of $1,25(OH)_2D_3$ on bird weight, incidence of TD, plasma chemistry (total calcium [total Ca], inorganic phosphate [Pi], and alkaline phosphatase [AP]), and blood ionised calcium [Ca^{2+}] and pH in 3 week old chicks.[9]

				blood		*plasma*		
Treatment	*n*	*weight (g)*	*TD (%)*	*Ca^{2+} (mM)*	*pH*	*Ca (mM)*	*P i (mM)*	*AP (IU/l)*
$1,25(OH)_2D_3$	20	557	10	1.29	7.47*	1.66	0.601*	15283
RO 23-6474	20	522	35	1.29	7.47*	1.80	0.674	19622
RO 23-7553	20	564	50	1.32	7.42	1.88	0.755	17629
Control	20	524	55	1.32	7.43	1.83	0.762	17808
sed		32.1		0.051	0.019	0.128	0.0668	2148.7
p		0.435		0.927	<0.01	0.400	0.07	0.267

[9] All birds were fed the Institute starter diet, which contained 10 g/kg Ca, 6 g/kg P and 15 μg/kg cholecalciferol.

Administration of either of the analogues had limited effects on the histological incidence of TD. Incidences were 55, 10, 50 and 35% for control, $1,25(OH)_2D_3$, RO23-7553 and RO23-6474 groups respectively (see*Table IV.xx*).

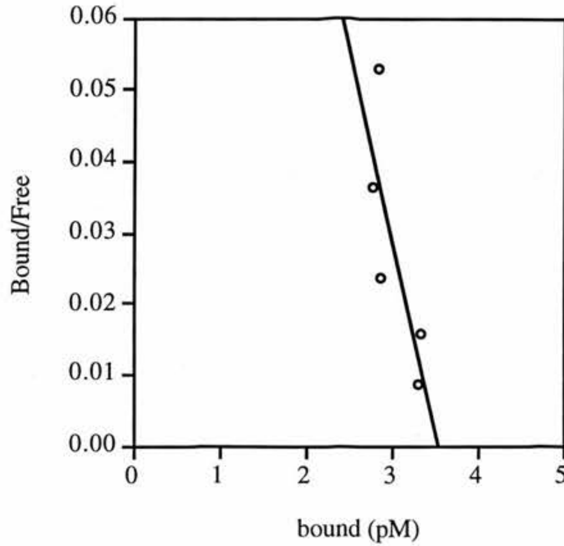
There were no significant effects of analogues on growth, blood ionised calcium, plasma total calcium or alkaline phosphatase activity, but inorganic phosphate was significantly reduced ($p<0.05$) in the group administered $1,25(OH)_2D_3$, and blood pH was significantly raised ($p<0.05$) in the birds administered $1,25(OH)_2D_3$ and RO23-6474 (see*Table IV.xx*).

4.3.5.1 Binding of $1,25(OH)_2D_3$ and analogues to growth plate chondrocyte receptors

The purpose of this experiment was to measure the relative binding affinities of $1,25(OH)_2D_3$ and two analogues of $1,25(OH)_2D_3$ to growth plate chondrocyte receptors from 3 week old broiler chicks fed a standard diet containing 12 g/kg Ca and 6 g/kg P, to clarify the reasons for the lack of effect of $1,25(OH)_2D_3$ analogues on TD *in vivo*.

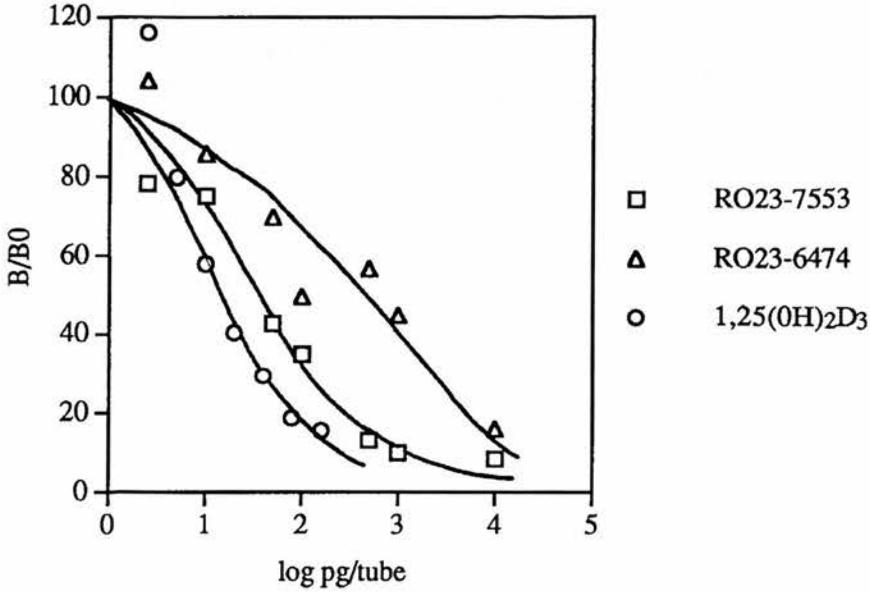
Chondrocytes were isolated from growth plates using collagenase digestion as described in Section 3.3.10.1. The competitive binding assay was carried out as described in Section 10.3, using dextran coated charcoal, prepared as in Section 10.2, to separate free from bound ^3H-$1,25(OH)_2D_3$.

Figure 4k. Scatchard plot of $1,25(OH)_2D_3$ binding to chondrocyte receptors.



Estimates of K_d and B_{max} were 18.9 pM and 3.54 pM, respectively (see *Figure 4k*). The average number of receptors per cell was 1129. The slope of the Hill plot was 0.782 (data not shown). Slopes of less than 1 may indicate heterogeneity of binding sites or negative cooperativity (i.e. binding of ligand by receptor reduces binding of further ligand by further receptors). $1,25(OH)_2D_3$ is thought to interact cooperatively with its receptor (Minghetti and Norman, 1988). The modulation of the cooperativity may regulate the affinity of the ligand for the receptor and, in turn, the affinity of the receptor-ligand complex for the DNA binding region.

Figure 4l. Binding affinity of $1,25(OH)_2D_3$ analogues for chondrocyte receptors.



From *Figure 4l*, neither analogue had as great an affinity for the chondrocyte receptor as $1,25(OH)_2D_3$. However, RO23-7553 bound more strongly than RO23-6474, with an affinity of the same order of magnitude as $1,25(OH)_2D_3$. RO23-7553 is based on a modification of cholecalciferol (D_3). RO23-6474 bound much more weakly with an affinity at least one order of magnitude lower. The side chain of RO23-6474 is based on a modification of ergocalciferol (D_2), but this type of modification is not thought to affect binding to the cell receptor.

4.3.5.2 Binding of $25(OH)D_3$, $1,25(OH)_2D_3$ and analogues to plasma vitamin D binding protein (DBP).

The purpose of this experiment was to determine the relative binding affinities of $1,25(OH)_2D_3$ and two analogues of $1,25(OH)_2D_3$ for plasma DBP, given the lack of effect of either analogue *in vivo*, and their lowered affinity for the chondrocyte VDR.

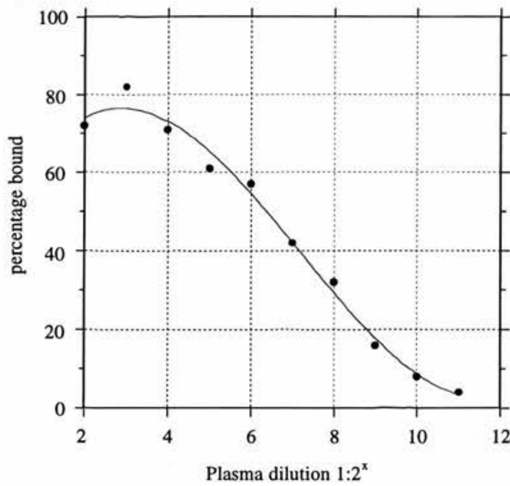
DBP was prepared from the plasma of vitamin D-deficient chicks as described in Section 3.3.11.1. The binding assay was carried out as described in Section 3.3.11.2.

4.3.5.2.1 Production of vitamin D-deficient plasma.

Plasma samples from four very lame, rachitic 3 week old chicks raised on the vitamin D deficient diet (see Appendix 2) were analysed for total calcium. The results were 2.18, 1.56, 1.60, and 1.29 mM for individual chicks. Plasma from the three chicks with the lowest plasma calcium was pooled and used in the assay.

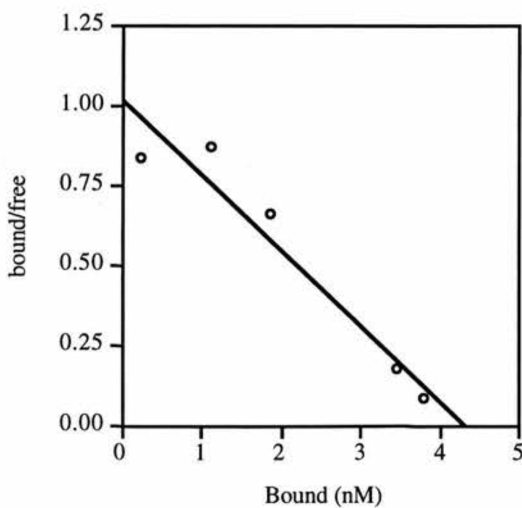
4.3.5.2.2 Dilution Assay

Figure 4m. Binding curve of ^{3}H-25(OH)D_3 to plasma vitamin D binding protein.



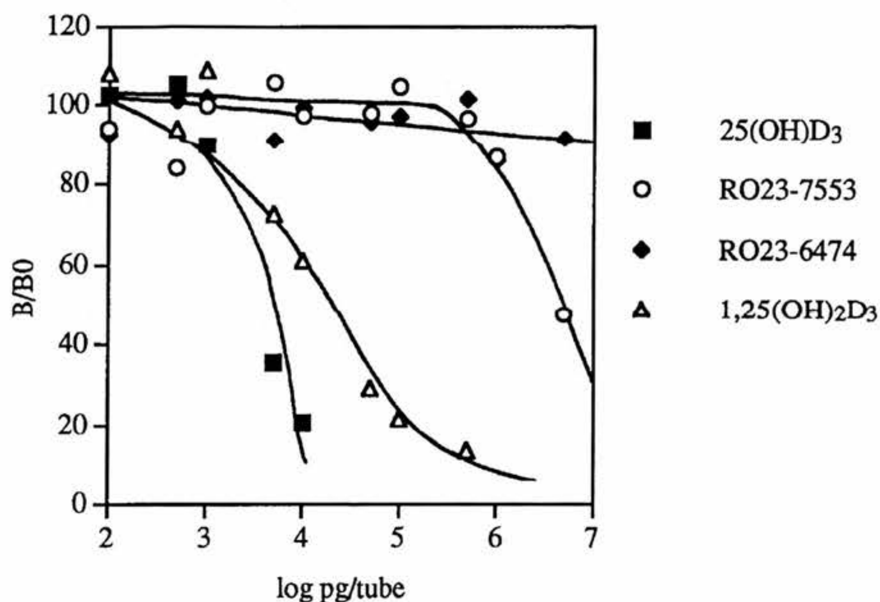
A dilution of 1:128 (i.e. 2^7) was used in the assay.

Figure 4n. Scatchard plot of 25(OH)D_3 binding to plasma vitamin D binding protein.



An estimate of K_d for $25(OH)D_3$ binding to plasma DBP was obtained of 4.27 nM. An estimate for B_{max} was obtained of 4.31 nM (see *Figure 4n*). The slope of the Hill plot was 0.94 (data not shown).

Figure 4o. Comparative binding affinity of $25(OH)D_3$, $1,25(OH)_2D_3$, and the analogues RO23-7553 and RO23-6474 to plasma vitamin D binding protein.



From *Figure 4o*, it was clear that the different vitamin D metabolites and analogs had differing affinities for chick plasma vitamin D binding protein. $25(OH)D_3$ bound most strongly, with approximately 5000 pg added before displacement of 50% of ^3H-$25(OH)D_3$ occurred. $1,25(OH)_2D_3$ bound next most strongly, with approximately 20 000 pg added before displacement of 50% of ^3H-$25(OH)D_3$ occurred. RO23-7553 was the only analogue which displayed any

binding over the ranges tested, with approximately 5 000 000 pg added before displacement of 50% of ^3H-25(OH)D_3 occurred.

4.3.6 The toxicology of 1,25$(OH)_2D_3$.

The aim of this experiment was to define the nature of the toxicity of 1,25$(OH)_2D_3$ when the metabolite was added to the calcium-adequate diets of young chicks.

Plasma total calcium, inorganic phosphate and alkaline phosphatase activity were measured using the colorimetric assays described in Sections 3.2.11.1, 3.2.11.2 and 3.2.11.3. Blood ionised calcium levels were measured using an ion electrode technique as described in Section 3.3.4.1. LDH, CK and AST activities were measured using the kinetic assays described in Section 3.3.4.3, involving the oxidation of NADH (LDH and AST), and reduction of $NADP^+$ (CK). Plasma osmolality was measured using a freezing point technique as described in Section 3.3.4.4.

Histological sections of soft tissues were prepared and examined using light and electron microscopy for signs of calcification or other pathological changes, as described in Section 3.3.6.

Table IV.xxi a,b Toxicological effects of 20 μg/kg dietary $1,25(OH)_2D_3$ on bird weight, and blood (ionised Ca and pH), and plasma chemistry (total calcium [Ca], inorganic phosphate [Pi], protein, uric acid, alkaline phosphatase [AP], lactate dehydrogenase [LDH], creatine kinase [CK], and aspartate aminotransferase [AST]) in 3 week old chicks.[10]

Table IV.xxi a

$1,25(OH)_2D_3$ (μg/kg)	*weight (g)*	*blood: ionised Ca (mM)*	*blood: pH*	*plasma: osmolality (mOsm/kg)*	*plasma: Ca (mM)*	*plasma: P i (mM)*	*plasma: protein (mg/ml)*
0	535	1.46	7.52	301	2.91	0.700	42.0
20	290	2.01	7.59	297	2.89	0.411	48.3
sed	41.2	0.095	0.026	2.60	0.176	0.0510	1.38
p	<0.001	<0.001	0.021	0.123	0.929	<0.001	<0.001

Table IV.xxi b

$1,25(OH)_2D_3$ (μg/kg)	*plasma: uric acid (mM)*	*plasma: AP (IU/l)*	*plasma: LDH (IU/l)*	*plasma: CK (IU/l)*	*plasma: AST (IU/l)*
0	18.49	4850	271	178	71.5
20	46.03	3156	253	107	63.2
sed	5.70	826.6	27.6	82.2	3.94
p	<0.001	0.056	0.512	0.400	0.052

n=10 for all variables.

[10] All birds were fed the Institute starter diet, which contained 10 g/kg Ca, 6 g/kg P and 15 μg/kg cholecalciferol.

Compared with the control group fed no $1,25(OH)_2D_3$, the weight of the chicks fed 20 µg/kg $1,25(OH)_2D_3$, was significantly ($p<0.001$) reduced, blood ionised calcium and pH were significantly increased ($p<0.001$ and $p=0.021$), plasma protein and uric acid significantly increased ($p<0.001$ for both variables) and plasma inorganic phosphate significantly reduced ($p<0.001$). Plasma osmolality, total calcium, AP, LDH and CK were unaffected by treatment ($p>0.05$). Plasma AST was reduced, but the difference just failed to reach significance ($p=0.052$)

There were no significant abnormalities in the sections of growth plate, proximal duodenum, liver, heart, skeletal muscle or spleen when examined by light microscopy. Lung tissue contained some cartilage nodules, as described by Maxwell *et al.*, (1993), but such nodules are often seen in the lungs of birds kept in conditions of reduced pO_2 (Maxwell *et al.*, 1993).

When the sections of kidney stained with hematoxylin and eosin were examined the proximal and distal tubules contained amorphous deposits (see Plate 7). These samples were recut and stained with Von Kossa stain with neutral red counterstain, with or without a saturated lithium carbonate wash, as saturated lithium carbonate will dissolve urates but not calcium phosphate (Drury and Wallington, 1967).

The sections still stained strongly, and so the deposits were tentatively identified as calcium phosphate, based on the histological evidence and the hypercalcaemia observed in the chicks' plasma.

EM revealed the structure and location of the deposits in greater detail. Deposits were both intra- and extra-cellular, and were generally quite electron-dense. Some of the deposits had the characteristic 'bulls-eye' appearance of urates (see Plate

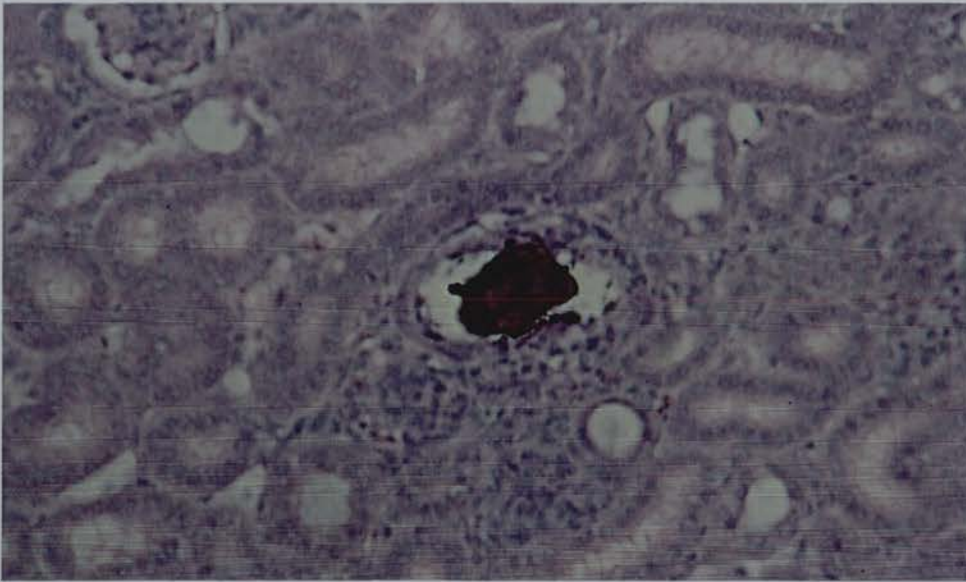


Plate 7 A photomicrograph of a section of kidney containing tubular deposits from a 3 week old broiler chicken fed a diet containing 10 g/kg calcium and 20 μg/kg 1,25-dihydroxycholecalciferol.

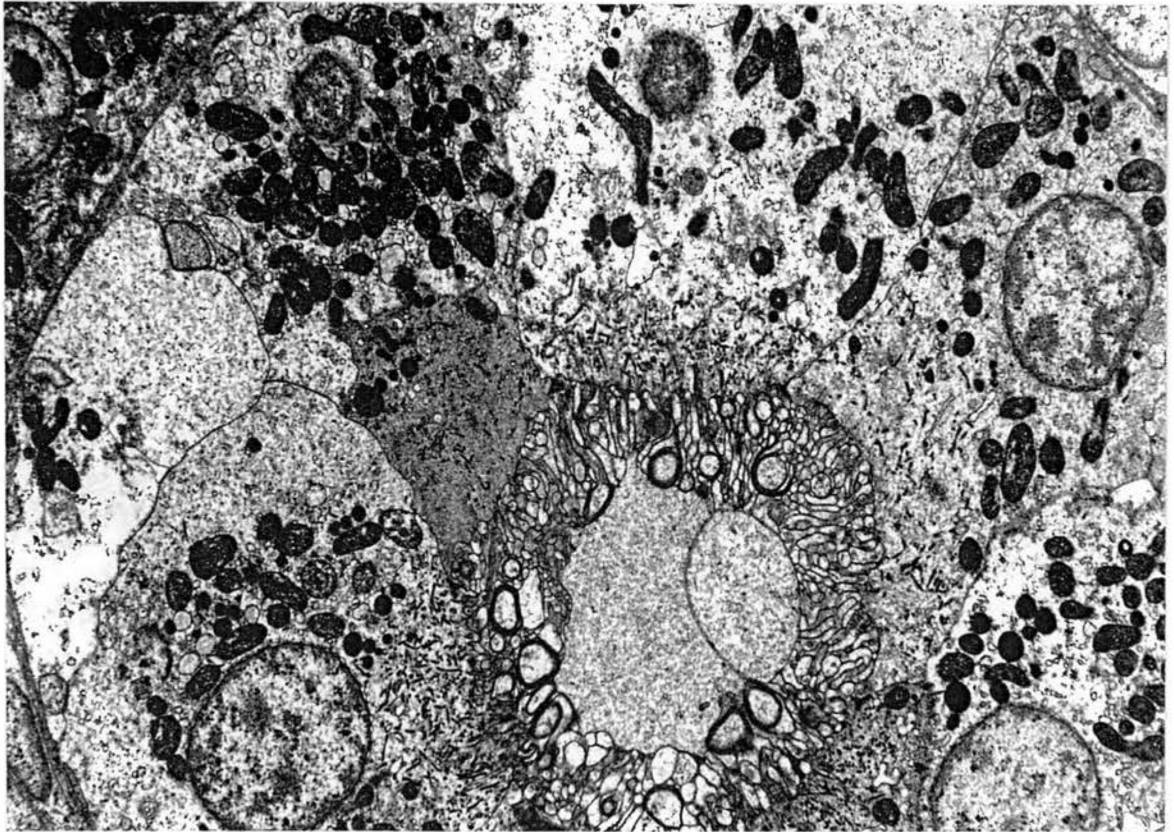


Plate 8 Electron micrograph of normal chick kidney, showing proximal convoluted tubule cells and lumen.
Magnification x 6000

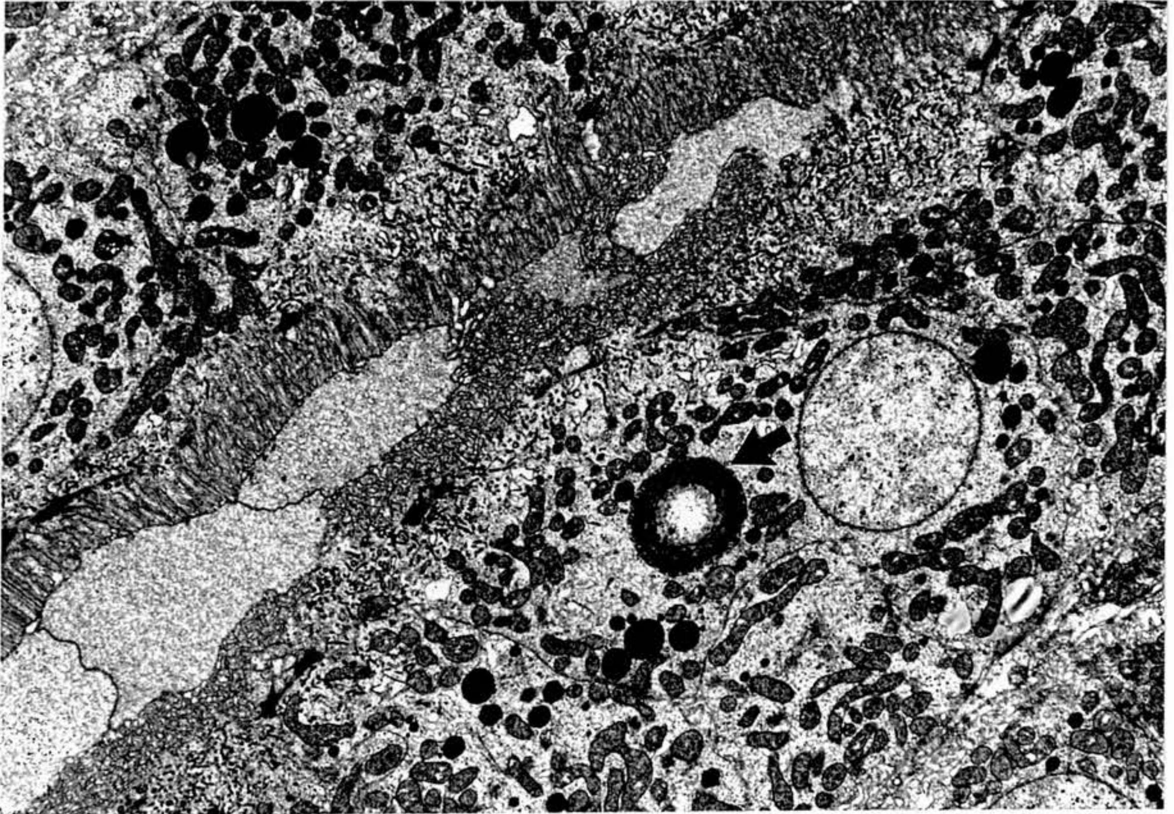


Plate 9 Electron micrograph of kidney from a chick fed 10 g/kg calcium and 5 μg/kg 1,25-dihydroxycholecalciferol. The proximal convuluted tubule cell contains a deposit of calcium phosphate (arrow).

Magnification x 6000

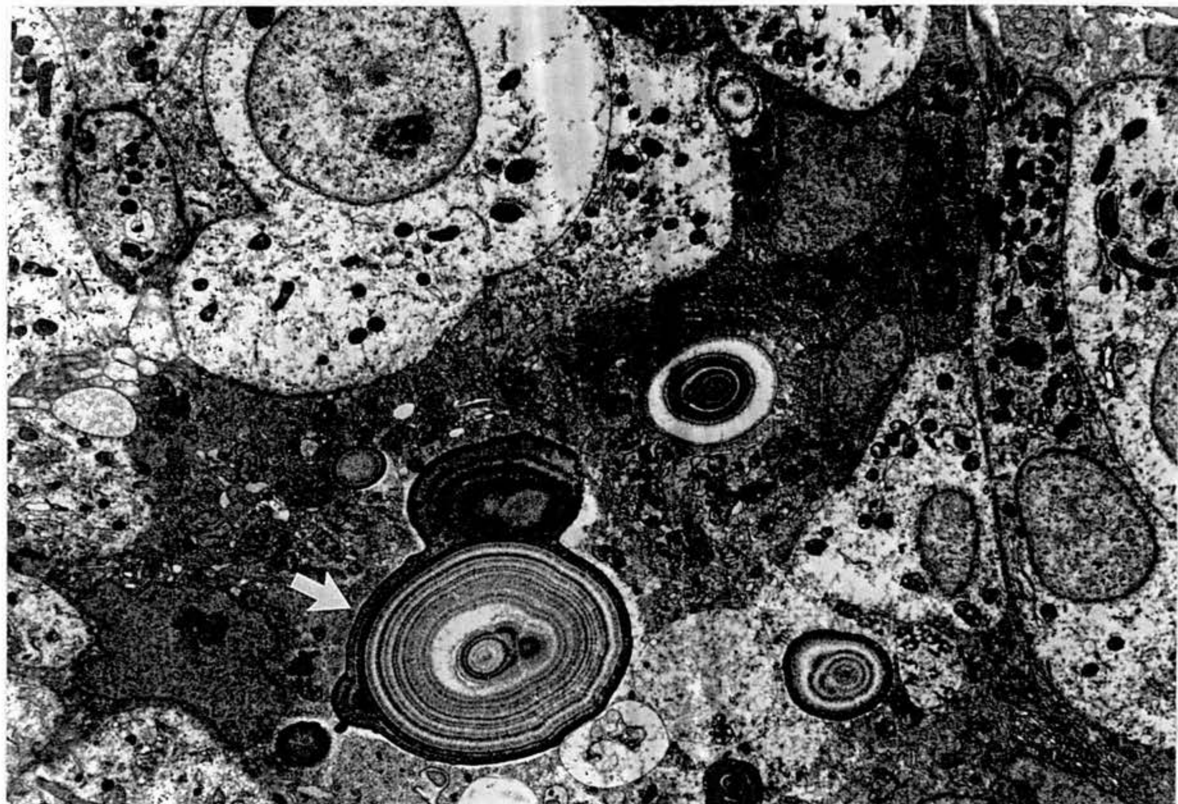


Plate 10 Electron micrograph of kidney from a chick fed 10 g/kg calcium and 20 μg/kg 1,25-dihydroxycholecalciferol. The proximal convuluted tubule cells and lumen contain deposits of urate (arrow).

Magnification x 6000

10) which can also be formed both intra- and extra-cellularly (Dr. M. Maxwell, personal communication), but others did not.

Kidney samples were then taken from 3 week broiler chickens from another experiment fed a diet containing 10 g/kg calcium and 6 g/kg phosphate supplemented with only 5 μg/kg $1,25(OH)_2D_3$. There was no evidence of growth depression or hypercalcaemia in these birds. The samples were fixed and processed as before for EM, and revealed a few deposits, which did not have the structure of urates (see Plate 9). These were identified as calcium phosphate.

CHAPTER 5

DISCUSSION

5.1 Laying Hen Experiment

The results obtained from this experiment are of two types. The measurements of proportions of trabecular and medullary bone in the PTM and FTV at the end of the experimental period give information of a 'static' kind i.e., they only tell us what amounts of bone are there at one point in time, and do not give any information as to the dynamics and rates of bone turnover over the course of the experiment.

However, the measurement at discrete time intervals of plasma total calcium, inorganic phosphate, and in particular alkaline phosphatase provide more 'dynamic' information. Alkaline phosphatase as secreted by bone-forming osteoblasts gives an indication of the amount of bone formation and therefore bone turnover can be implied. In this experiment, it was disappointing that the scale of the experiment made impossible measurements of serum acid phosphatase, a marker of osteoclastic activity, as these measurements would have added to the information available. Assay of this enzyme requires the preparation of serum rather than plasma, which is problematical and unreliable, due to the presence of differently-behaving clotting factors in avian blood compared with mammalian blood (Archer, 1971, my own experience, and Dr. M. Maxwell, personal communication). Serum must be stabilised within one hour of collection with an acid reagent for measurement of tartrate-resistant acid phosphatase activity, which is the serum iso-enzyme thought to be derived from osteoclasts, otherwise activity is greatly reduced. However, avian

blood must be incubated undisturbed for 24 hours at 4 C before a stable clot is formed and serum can be drawn off. If the process is rushed, 'serum' will be produced, but will often gel at room temperature or during storage in a refrigerator or freezer.

Finally, egg production information can also be considered to be of a dynamic nature, in that as continued egg production in highly-selected birds is physiologically 'normal', then any treatments interrupting or causing egg production to cease could also be disturbing calcium homeostasis and bone turnover.

The effects of nutrition on egg production, plasma assays and quantities of trabecular and medullary bone within the FTV and PTM will be discussed in this section.

5.1.1 Egg production

The overall high rate of lay and the lack of significant differences in egg production amongst the Hi-sex birds on the different experimental treatments showed that none of the treatments limited egg production. If egg production had been depressed, it might have been expected to affect bone turnover and incorporation of medullary bone calcium into eggshell calcium. As expected, the J-line hens laid on average significantly fewer eggs than the Hi-sex birds, although there was far greater variation in the rate of lay amongst this group (see *Table IV.iii*). Some individuals had a rate of lay approaching that of the Hi-sex birds. There was no obvious effect of tier on egg production.

5.1.2 Potential tier effects in hens maintained on the control treatment

Tier had no major effect on plasma calcium, inorganic phosphate or alkaline phosphatase, both in mean values over the course of the experiment (see *Table IV.v*) and in values taken at the discrete time intervals (see *Tables IV.iv.a, b and c*). The results of this experiment were important as any alterations in circulating levels might have had implications for bone turnover. For example, it is well known that hypocalcaemia is the main stimulus for PTH secretion, which results in osteoclastic bone resorption, as well as stimulating increased absorption of dietary calcium via PTH-mediated synthesis of $1,25(OH)_2D_3$. Although light levels within the house were not measured, it was obvious to the observer that light levels were higher in the top tier of the battery, whereas cages in the bottom tier of the battery were comparatively dimly lit. Ultra-violet light is necessary for the conversion of 7-dehydrocholesterol to cholecalciferol in the skin, but the importance of this source of vitamin D in hens with good feather cover, and fed diets more than adequate in cholecalciferol, is not likely to be high. Increased light levels more importantly might also be expected to result in increased activity of hens, which could result in increased patterns of loading and alterations in bone turnover. That loading is important in turnover of bone in avians was demonstrated by Rubin *et al.* (1992) using the elegant preparation of functionally isolated ulnas from young (1 year old) male turkeys. They found that loading to a physiological level resulted in increased bone formation, which was not found in old birds (3 years old) subjected to the same treatment.

5.2 Treatments in Hi-sex birds

Plasma variables and bone measurements were affected by only a few of the experimental treatments (see *Tables IV.ix, IX.x1, IV.x11 and IV.xiii).*

5.2.1 Sodium fluoride

Administration of sodium fluoride increases bone mass in both humans and other animals in a dose-dependent manner. In some human studies, beneficial results have been confined to the axial skeleton, with effects on the appendicular skeleton not always so predictable (Kleerkoper and Mendlovic, 1993). In this study, 200 mg/kg sodium fluoride significantly increased plasma calcium, inorganic phosphate and alkaline phosphatase (see *Table IV.ix).* Fluoride is known to stimulate bone formation in animals, and evidence suggests that the mechanism involves increased recruitment of active, normally functioning osteoblasts. Normal bone remodelling processes appear to be interrupted in that osteoblastic activity is not necessarily preceded by osteoclastic activity (Kleerkoper and Mendlovic, 1993). Fluoride (1-100 μM) stimulates osteoblast-like cells in culture, increasing cellular proliferation, alkaline phosphatase activity and collagen synthesis and deposition (Farley *et al.*, 1983). In the treatment of human osteoporosis, high doses of fluoride may result in a rise in serum alkaline phosphatase activity (Farley *et al.*, 1987). Hence the rise in alkaline phosphatase activity can be accounted for by increased osteoblastic activity, and is in agreement with results obtained in growing chickens given drinking water containing 176 or 706 mg/l sodium fluoride (Lundy *et al.*, 1992), or turkeys, although in this latter experiment, increased enzyme activity was only seen when the dietary content was 885 mg/kg sodium fluoride (Nahorniak *et al.*, 1983). The reason for the rise in plasma calcium and phosphate are not so clear. In human studies, administration of fluoride caused no changes in intestinal absorption of calcium, or alterations in serum calcium or phosphate (Pak, 1989). It has been suggested that foods rich in calcium (Pak, 1989) or protein (Mariano-Menez *et al.*, 1990) tend to bind fluoride causing less absorption from the gastro-intestinal tract.

In view of the biochemical evidence of fluoride stimulation of osteoblastic activity, it was expected that increased bone formation would be seen from image analysis of bone samples taken from treated birds. It was therefore rather unexpected to find that sodium fluoride only appeared to increase the amount of medullary bone within the PTM, and had no effect on the amount of trabecular bone within the PTM (see *Table IV.xiii*), or the amounts of trabecular or medullary bone within the FTV (see *Table IV.xii*). It is unlikely that this was due to either the fact that 200 mg/kg was an ineffective dose or was bound by the high dietary content of calcium as there was a measurable and significant increase in plasma alkaline phosphatase activity. However, other studies with poultry have used much higher dose rates as the chicken is supposedly resistant to fluoride toxicity, and adult birds can tolerate 400 ppm fluoride (equivalent to 850 mg/kg sodium fluoride) before food intake and production drop (Guenter, 1979). Van Toledo and Combs (1984) used up to 2560 mg/kg sodium fluoride in an experiment investigating tolerance, although some birds receiving this dose showed signs of toxicity such as feather loss and diarrhoea.

A possible explanation is that the bones of the sexually mature female chicken are resistant to the anabolic actions of fluoride. There is evidence of a lack of osteoid (Wilson *et al.*, 1992) and functional osteoblasts on the trabeculae of mature egg-laying hens although medullary bone possesses both osteoclasts and osteoblasts (Taylor *et al.*, 1971, Wilson *et al.*, 1993). The experiments of Hudson *et al.* (1993) used histomorphometry to measure the amount of trabecular bone in the proximal femur and found it was reduced in 39.5 week old White Leghorn hens compared with 20 week old hens. Osteoblasts were present in the older birds, but in fewer numbers. When fluorochrome was administered to the older birds, label was deposited diffusely into medullary bone of the proximal femur, indicating active mineralisation, but none was deposited into the cortex, indicating cessation of

modelling activity. Hence, fluoride administration following sexual maturity would target only medullary bone osteoblasts, accounting both for the rise in plasma alkaline phosphatase activity and the increased amounts of medullary bone within the PTM. The FTV normally contains negligible medullary bone, so any increase stimulated by fluoride would be unmeasureable. This conclusion is supported by the work of Merkely (1981b) in which pullets were reared either with or without access to fluoridated water (100 ppm fluoride, equivalent to 221 μg/l sodium fluoride w/v). Following sexual maturity treatments were either maintained or reversed. Birds given fluoride before, or before and after sexual maturity had significantly increased tibial bone ash (raised by about 10%), and significantly increased humeral and tibial breaking strengths (both were doubled) at 45 weeks. But birds given fluoride only after they reached sexual maturity (at 20 weeks) had values no different from control birds given untreated water all through the experiment.

The results of the radiographic study suggested that birds given fluoride had a lower rate of fracture in the bones of the wing compared with other groups (see *Table IV.xi*). This implies either a structural role for medullary bone in the radius and ulna, as the humerus is generally pneumatised and therefore does not contain medullary bone, or an effect, not necessarily involving osteoblasts on the cortical and trabecular bone. Medullary bone is widely considered to have no structural role as it consists of tiny spicules and is not formed into any kind of structural framework (Taylor *et al.*, 1971). Medullary bone is also turned over, with remodelling occurring at a very high rate (Etches, 1987). It has been suggested that fluoride decreases resorption of existing bone as well as increasing osteoblastic formation of new bone, resulting in a dually-mediated increase in bone mass (Lundy *et al.*, 1992). However the results from image analysis and point counting show no net gain of bone. Assuming the rate of loss of bone from the bones of the wing would be

similar to that of the PTM and FTV, then the wing bones are likely to be osteoporotic, and this was borne out by their radiological appearance. However, from human studies it is known that factors other than global bone mineral density influence the rate of fracture of bones, including both material and geometric properties of bone (Heaney, 1993). Material properties include both the brittleness and breaking strength of the bone. Material properties of bony matter are influenced by factors such as the accumulation of cement lines, maturation of the mineral crystal, and the degree of collagen cross-linking (Marcus, 1991). Geometric properties include the degree of trabecular connectivity. Perhaps fluoride is incorporated into remaining bone by some means not necessarily involving osteoblasts, resulting in a more stable crystal lattice and an improvement in its bio-mechanical properties (Lundy *et al.*, 1992). Another possibility is that it chemically increases collagen cross linking, improving its 3-dimensional stability (Professor A. Bailey, personal communication).

5.2.2 Oystershell

Over the whole experiment, plasma alkaline phosphatase was significantly raised by the oystershell treatment, in which 50% of the dietary calcium was in the form of oystershell particles. There were no effects of this treatment on plasma calcium and inorganic phosphate (see *Table IV.ix).* It had been hypothesised that the particles of oystershell would remain in the crop and gizzard and be slowly broken down and released in the dark period as the egg shell was being laid down (Wideman, 1990). A diurnal variation in blood calcium is known to exist in egg-laying hens (Luck and Scanes, 1979; Singh *et al.*, 1986), with lowest levels of ionised calcium being found during the period of eggshell calcification. A concurrent rise in circulating levels of blood calcium might therefore be expected following

administration of this 'slow-release' form of calcium, as indeed was found by Nys and Sauveur (1983) in blood samples measured 0-5 hours post oviposition. The effect of oystershell in increasing plasma alkaline phosphatase activity (see *Table IV.ix*) would imply that bone formation is increased by this treatment as this enzyme is a marker of osteoblastic activity. This is interesting as it implies that the form and/or timing of calcium administration can influence its fate. The implication is that supplying calcium in this form results in increased bone formation, although of course what type of bone is not known. Guinotte and Nys (1991) found that particulate calcium increased both eggshell breaking strength and tibial breaking strength. Farmer *et al.*, (1983) found the timing of calcium administration determined its fate. They administered a bolus of ^{45}Ca to hens in either the evening or morning and found that the greater proportion of the evening bolus seemed to bypass bone and instead be deposited on the next morning's eggshell, but the greater proportion of the morning bolus was incorporated into the skeleton.

The oystershell diet had no effect on the amounts of trabecular and medullary bone within the FTV (see *Table IV.xii*), or trabecular bone within the PTM, but was associated with an increased amount of medullary bone within the PTM (see *Table IV.xiii*). This is consistent with the increased plasma alkaline phosphatase activity measured in this group, and leads to the suggestion that the 'slow release' calcium in oystershell is incorporated into medullary bone rather than going straight from the gut to eggshell. However, plasma alkaline phosphatase activity was measured from blood samples drawn in the morning, after the day's egg had been laid, and when medullary bone was presumably being regenerated. It would have been of interest to investigate whether a variation existed in the activity of this enzyme in control birds and birds given the oystershell ration, and particularly whether the activities were different at night-time when there was release of oystershell calcium from the

gizzard at the same time as medullary bone would be turned over to provide calcium for the eggshell.

As the X-ray data suggest there is also no benefit to the birds in terms of rate of fracture by giving them oystershell (see *Table IV.xi*), there seems to be no effects of this form of dietary calcium on bone quality. However, numerous other studies have demonstrated an improvement in shell quality in birds given oystershell or other forms of particulate calcium, for example Watkins *et al.*, (1977), McLoughlin and Soares (1976) who found a small increase in shell thickness in 62 week old hens, and Scott *et al.*, (1971), who found that substitution of 2/3 dietary calcium by oystershell particles improved eggshell breaking strength in birds of around 60 weeks of age. This improvement in shell quality has always been assumed to be due to the reduced rate of dissolution of particulate calcium allowing a steady flow of protein-bound blood calcium to the shell gland where it is laid down on eggshell.

5.2.3 Low dietary phosphorus

Giving the hens a low phosphorus diet increased plasma alkaline phosphatase activity, but had no effect on plasma calcium or inorganic phosphate (see *Table IV.ix*). This is interesting as a low phosphorus/normal calcium diet is associated with lowered plasma inorganic phosphate with unchanged plasma calcium in laying hens (Rao and Roland, 1990). Feeding a low phosphorus diet to older hens results in an apparent improvement in shell quality, thought to be mediated through a $1,25(OH)_2D_3$ stimulated increase in absorption of both minerals from the small intestine (Frost *et al.* 1991) or increased $1,25(OH)_2D_3$-stimulated osteoclastic bone resorption providing more mineral at night when the eggshell is being laid down. This effect seems to be independent of PTH, as studies with phosphate-deprived parathyroidectomised animals have shown increased renal production of

$1,25(OH)_2D_3$ (Hughes *et al.*, 1975).Whilst egg shell quality was not measured during this experiment, the results from the assay of plasma alkaline phosphatase activity imply altered bone turnover in birds fed this diet, and suggest that bone is the more significant source of eggshell mineral rather than any increase in absorption in dietary mineral.

It has been suggested that although a low phosphorus diet may be beneficial in terms of a potential improvement in egg quality, this improvement might be at the expense of bone (Roland and Rao, 1992). However, results from this experiment showed no difference in the amounts of trabecular or medullary bone compared with the control treatment which contained a more conventional level of phosphorus (see *Tables IV.xii and IV.xiii*). Remaining bone was histologically normal with no evidence of thickened osteoid seams, indicating that the level of dietary phosphorus was not low enough to result in osteomalacia.

5.2.4 Low crude protein, high vitamin K

Protein (and therefore amino acid) nutrition of the laying hen is important as it directly influences egg output. There are many factors which affect the protein requirements of hens e.g. dietary energy, age and state of lay of the bird, genetic strain and management (e.g. cage *versus* floor, lighting etc.). The protein requirement of the hen can be related to the energy content of the diet, with diets lower in protein also lower in energy content. Commonly, laying birds are fed a diet containing about 170 g/kg crude protein and about 12.7 MJ/kg metabolisable energy throughout lay. However, during the early stages of egg production, hens are gaining weight and depositing protein as well as laying eggs. Further on in lay, it has been argued that the protein requirement may be reduced, as, although egg output is maintained and egg size is increased, the bird's requirement for protein is reduced to

that required for maintenance (Austic and Nesheim, 1990). In other species, a high protein diet induces hypercalciuria, thought to be due to the buffering of acidic products of methionine and cysteine oxidation by skeletal calcium reserves. Feeding hens a diet low in crude protein (150 g/kg, compared with 170 g/kg in the control group), and high in vitamin K resulted in a significant rise in plasma inorganic phosphate (see *Table IV.ix*). The reasons for this are not clear. However, there was no effect of diet on the bone variables measured (see *Tables IV.xii and IV.xiii*). Neither was there any effect on egg production (see *Table IV.iii*). Although egg production was not changed by reducing dietary crude protein, other variables such as egg weight and egg quality were not measured. Effects may well have been seen if the scale of the experiment had allowed such measurements. Pesti (1991), investigating protein and energy requirements of laying birds, found that when dietary energy was maintained at 12.55 MJ/kg, a reduction in dietary crude protein from 180 to 160 g/kg significantly reduced egg production, but not egg weight, from 20-44 weeks of age. Summers (1993) found that reducing dietary protein from 170 to 130 g/kg and keeping metabolisable energy at 11.72 MJ/kg had no effect on egg production, but decreased egg weight. Keshavarz (1984) found a combination of both reduced egg production and egg weight from 20-48 weeks of age, but neither from 48-72 weeks, when dietary crude protein was reduced from 165-145 g/kg and the diet contained 12.27 MJ/kg metabolisable energy. Although there appeared to be no beneficial effect on bone in feeding a low crude protein diet, it would have been interesting to measure urinary calcium in birds from this and the control treatment, to determine the effects of lowered dietary protein on bone, but in birds this is technically complicated.

This diet also contained a high level of vitamin K. Vitamin K is a co-factor in the post-translational modification of osteocalcin, and therefore might be expected to

influence bone. However, as the main stimulus for osteocalcin synthesis is $1,25(OH)_2D_3$ (Zerweckh *et al.*, 1985, Pike, 1992) any effect of vitamin K would be secondary. Also, vitamin K deficiency does not seem to affect skeletal development in newly hatched chicks (Lavelle *et al.*, 1994). One would expect a developing embryo to be more sensitive to such a deficiency than an adult bird which is turning over bone rather than synthesising bone *de novo*.

5.2.5 1,25-dihydroxycholecalciferol

Giving birds 5 μg/kg $1,25(OH)_2D_3$ had no effect on plasma total calcium or alkaline phosphatase, but significantly increased plasma inorganic phosphate (see *Table IV.ix*). If any effects on mineral homeostasis had been anticipated, these would have been that $1,25(OH)_2D_3$ would have induced hypercalcaemia and hypophosphataemia, as in some of the broiler studies described in a further section (see *Tables IV.xvii and IV.xviii*). The reasons for the elevation in plasma inorganic phosphate measured in this experiment are not clear. Perhaps if the feed had contained a larger amount of $1,25(OH)_2D_3$, more easily-explained results would have been seen. $1,25(OH)_2D_3$ has been used in the treatment of human osteoporosis, and there is evidence that its main effect is an increase in intestinal absorption of calcium (Lambarg-Allardt, 1991). The presence of vitellogenin in blood plasma means that hens theoretically would be able to make use of any increase in the absorption of dietary calcium that $1,25(OH)_2D_3$ may induce. However, it has been demonstrated using computer simulation that birds absorbing extra calcium dietary $1,25(OH)_2D_3$ treatment, also increase their excretion of calcium (Hurwitz *et al.*, 1984). It was interesting in this study that despite the fact that the feed contained $1,25(OH)_2D_3$, there was no increase in circulating concentrations of the hormone

(see *Table IV.x*). Similar results were also obtained in the broiler studies, and further experiments with broiler plasma gave a possible explanation for this.

In this experiment, there were no significant effects of $1,25(OH)_2D_3$ on quantities of trabecular and medullary bone within the PTM and FTV(see *Tables IV.xii and IV.xiii)*. Nor were there any significant effects on fracture incidence in the wing bones (see *Table IV.xi*).

It has been suggested that although plasma levels of $1,25(OH)_2D_3$ and the concentration of calbindin-28K do not appear to alter with age (Bar and Hurwitz, 1987), that the aged laying hen (over 1 year old) loses its ability to modulate $1,25(OH)_2D_3$ production i.e. it cannot boost 1-hydroxylase activity in response to low dietary calcium. There is also evidence that the binding capacity of the $1,25(OH)_2D_3$ receptor protein in the small intestine is reduced with age, and that $1,25(OH)_2D_3$ may be more rapidly cleared from the circulation and degraded (Abe *et al.*, 1982). In hens, therefore the fine tuning of the homeostatic processes controlling calcium homeostasis begins to suffer. Thought to be associated with this is the drop in eggshell quality seen in older birds. Several workers have found an improvement in eggshell quality in old birds given either $1,25(OH)_2D_3$ in the diet (Bar *et al.*, 1988), or $1\alpha(OH)D_3$, which is hydroxylated in the kidney to form $1,25(OH)_2D_3$ (Soares *et al.*, 1988). McLoughlin and Soares (1976) found that 15 µg/kg $25(OH)D_3$ significantly increased shell thickness in eggs from 62 week old hens. Soares *et al.* (1988) also found that $1\alpha(OH)D_3$ increased tibial breaking strength in 40 week old hens. Similarly, Frost and Roland (1991b) found that bone density was significantly correlated to breaking strength, and that supplementing the diet of 75 week old hens with 0.5 or 1 µg/kg $1,25(OH)_2D_3$ significantly and linearly increased tibial bone density (measured using a Norland densitometer) and breaking strength. As bone density is related to bone content, the findings of Frost and Roland in particular

imply that $1,25(OH)_2D_3$ increases tibial bone content in older laying hens. The fact that breaking strength is increased also implies that the effect is not confined to an increase in medullary bone. Newbrey *et al* (1992) found that 1.0 μg/kg $1,25(OH)_2D_3$ increased mineralised medullary bone area in 65-75 week old hens, measured by histomorphometry, but do not mention trabecular or cortical bone.

5.2.6 Ascorbic Acid

Ascorbic acid is not added routinely to poultry diets in this country, although there is ample evidence to suggest that it may be useful in conditions of heat stress. There is also evidence to suggest that it may improve eggshell quality. As ascorbic acid has been suggested to boost renal production of $1,25(OH)_2D_3$ (Weiser *et al.*, 1988), supplementation may be of particular benefit in older birds where the endocrine mechanisms controlling production of the hormone are not so effective. However, in this experiment, there was no evidence of any alteration in circulating levels of $1,25(OH)_2D_3$ in hens supplemented with ascorbic acid at discrete time intervals, although levels did fluctuate (see *Table IV.x*).

Ascorbic acid is also a necessary co-factor for the synthesis of the collagenous proteins of bone matrix (osteoid). In animals with a dietary requirement for the vitamin, such as man and guinea-pigs, deficiency leads to synthesis of structurally abnormal collagen lacking in cross-links. A strain of rat unable to synthesise ascorbic acid develops osteopenia, with a striking reduction in trabecular bone volume. The defects are due to a decreased matrix formation but with no mineralisation defect (Tsunenari *et al.,* 1991). Ascorbic acid supplementation might therefore be beneficial for matrix synthesis, and in turn for the laying down of new bone. However, the results of this experiment indicated no benefit to the birds of ascorbic acid supplementation in terms of quantities of bone quantity or quality (see

Tables IV.xii, IV.iii, and IV.xi), or egg production (see *Table IV.iii*), although eggshell quality was not measured.

5.2.7 J-line birds (strain comparison)

It had been anticipated that the J-lines, an unimproved line with a low rate of egg production, would have better bones than the Hi-sex birds. Indeed, this was the case. J-line birds had more trabecular bone in both the FTV and PTM(see *Tables IV.xii and IV.xiii*) although the differences in the FTV were not significant, due to the smaller number of samples from these birds compared with the other groups for reasons already explained. The J-lines also had less medullary bone in the PTM compared with the Hi-sex birds. A feature of the lower rate of lay in these birds is that they lay eggs in definite groups or clutches, which may reduce the intense demand for calcium and subsequent very rapid bone turnover. The fact that bone turnover is reduced in these birds is shown by the very low plasma alkaline phosphatase activities in these birds (see *Table IV.ix*). Of course, blood samples may have been taken on mornings when an egg was not laid, and so medullary bone renewal was not occurring, which might account for the lowered values, as plasma alkaline phosphatase activity varies with the different stages of the egg laying cycle (Solomon, 1970). It would be therefore have been of interest to compare the activities of this enzyme both when an egg had been laid and when an egg had not been laid, to establish whether these birds truly have a lower baseline rate of bone turnover.

5.3 Relationships between trabecular and medullary bone in the proximal tarsometatarsus (PTM) and free thoracic vertebrae (FTV).

When all Hi-sex dietary treatments were grouped together, the results of image analysis of relative quantities of trabecular bone in the two sites suggested no general relationship between the trabecular bone content of the PTM and FTV, at the time point chosen when the hens were 68 weeks old (see *Figure 4d*). This suggests that the rate of bone loss in the two bones is not the same. Measurement of bone volume to assess osteoporosis in human and animal subjects is often done at one site, such as the spine (axial skeleton) or distal radius, iliac crest or calcaneum (appendicular skeleton). In human studies especially, the sites most commonly measured are those with the highest rates of fracture (hip and vertebrae). The implication is that if one area shows evidence of bone loss, then osteoporosis is likely to be generalised, and from this, that rate of bone loss is similar at different skeletal sites. However, some human studies have shown that axial bone loss starts earlier, and that rate of loss at different sites may be altered by physiological state e.g. post-menopause. The fact that the hen bones sampled for this experiment contained different proportions of trabecular to medullary bone may be the cause of the lack of relationship between the amounts of trabecular bone at the end of lay. The PTM contains a relatively large amount of medullary bone, whereas the FTV contains negligible medullary bone. Medullary bone is known to be turned over very quickly, to provide calcium for the eggshell, and trabecular bone may be resorbed to provide the necessary mineral requirements for medullary bone formation (Wilson *et al.*, 1992). This was borne out by *Figure 4e* which showed a weak negative relationship between the amount of trabecular and medullary bone within the PTM.

The reason for the weak positive relationship between the amount of trabecular and medullary bone within the FTV can only be the subject of speculation.

Although the mean results for percentage trabecular bone in both the PTM and FTV for all nutritional treatments were below 16% in the Hi-sex birds, taken to indicate osteoporosis, there was comparatively wide variation in the amount of trabecular bone amongst individuals birds. Even ignoring those birds which had a low proportion of trabecular bone in the PTM, and a high proportion in the FTV, or *vice versa*, there were individuals with a relatively high proportion of trabecular bone at both sites. Simple plots of trabecular bone percentages against total egg output suggested that the amount of bone remaining after 48 weeks in lay was not directly related to egg output (see *Figures 4g* and *4h*). Therefore, there may be genetic factors controlling loss of bone and development of osteoporosis. Information as to the heritability of bone characteristics on poultry is scarce. However, of interest to the present study into osteopenia in laying hens is the work of Mandour *et al.* (1989a,b). In the first paper (Mandour *et al.*, 1989a), it was shown that cage reared broilers could be selected for increased humerus strength. Male broilers kept in cages suffer more broken wing bones, presumably due to disuse osteoporosis. After three generations of selection, humerus elastic force and ulna elastic energy were significantly increased. In the second paper (Mandour *et al.*, 1989b), heritability estimates (h^2) were calculated based on half-sib analysis for the various strength characteristics. h^2 for humerus elastic force was 0.67, elastic stress 0.80, and length 0.51, but h^2 estimates for radius and ulna characteristics were lower. As discussed in an earlier section, bone strength (i.e. its material properties) makes an important contribution to overall bone quality and fracture risk. But how these heritability estimates relate to measurements of bone quality/osteoporosis in laying

hens can only be the subject of speculation. However, the results show that in principle, birds can be bred for improved bone quality.

In human studies, there also appear to be genetic factors which influence the incidence of osteoporosis in women, and although multiple factors determine peak bone mass, inheritance appears to account for about 80% of the variance in bone mass in healthy young women (Kelly and Eisman, 1993).

5.4 Cellular factors in laying hen osteoporosis

The type of osteoporosis seen in the adult laying hen selected to produce a large number of eggs has some factors in common with human osteoporosis. Inactivity leading to a reduction in skeletal loading, dietary calcium (Marcus, 1987; Arnaud and Sanchez, 1990) and phosphorus (Arnaud and Sanchez, 1990) have all been shown to be important in the development of human osteoporosis, and are likely to be important in the severity of osteoporosis in the laying hen.

However, in two key areas, which may be linked, the factors influencing the development of osteoporosis are very different. In the human condition, osteoporosis results from an imbalance or uncoupling in the synthesis/resorption of bone by bone remodelling units (Parfitt, 1977). Over a period of time, there is a net loss of bone. Osteoporosis is most widespread in post-menopausal women, and it is well known that oestrogen-replacement therapy can reverse the effects of the menopause. The mechanism for the protective effects of oestrogen on human bone is not clear, but oestrogen deficiency is known to result in increased bone remodelling and a net loss of bone (Johnston, 1989). Oestrogen receptors have been identified on osteoblast-like cells in culture, and oestrogen may actually influence the differentiation of new osteoblasts (Heersche and Aubin, 1990), or increase cellular activity including collagen synthesis (Ernst *et al.,* 1988). Alternatively, some studies have shown that

oestrogen reduces bone resorption (Selby and Peacock, 1986), perhaps acting indirectly to alter production of one or more local regulators of bone metabolism.

In the high-producing laying hen, the effects of oestrogen on structural bone appear to be negative. This is the first difference in factors involved in the development of human and avian osteoporosis. The increase in circulating oestrogen concentrations around the time of sexual maturity from a mean of less than 100 pg/ml to about 350 pg/ml (Senior, 1974) stimulates the formation of medullary bone. Medullary bone is thought to be formed from existing cortical bone, and is actively turned over during the egg-laying cycle. Both osteoblasts and osteoclasts are involved in the turnover of medullary bone. Osteoid is seen on, and injected fluorochrome is deposited in, medullary bone. In contrast, trabecular bone in the egg-laying hen appears not to have any osteoid associated with it, and no fluorochrome is deposited in it. This implies a lack of osteoblastic activity. Interestingly, osteoid seams on trabeculae are seen in birds which have gone out of lay in which the ovary has regressed (Whitehead and Wilson, 1992). As trabecular bone is lost throughout lay, there must be resorption. In bones which contain a high proportion of medullary bone such as the femur or PTM, this implies perhaps that osteoclasts are indiscriminate in the bone that they resorb. In bones such as the FTV, which contain very small, localised amounts of medullary bone, the reason for the loss of bone with time is not clear. It is generally accepted that there are very close paracrine links between osteoblasts and osteoclasts, and that cells of an osteoblastic lineage are required to initiate osteoclastic bone resorption (Chambers, 1988). PTH and $1,25(OH)_2D_3$ stimulate bone resorption via osteoblasts, and osteoclasts have no receptors for these hormones. It has been suggested that osteocytes, the bone cells derived from osteoblasts which have become embedded in calcified tissue, have a role in the loss of trabecular bone seen throughout lay (Wilson *et al.*, 1992).

Belanger (1969) first suggested that osteocytes possess osteocytic activity, but this theory is not widely accepted at the present time. Osteocytes interconnect and communicate with surface osteoblasts by means of cell processes which extend through numerous canaliculae in the bone matrix. Current theories as to their role are either that they 'report' on the internal state of the bone or that they are involved in calcium regulation in bone extra-cellular fluid (Byers, 1994). However, Marks and Popoff (1991) hold a differing view, and state that osteocytes can possess either osteoclastic or osteoblastic activity, as they consider them to be derived from both types of cell, and that changes in metabolic activity of osteocytes can be detected in the area of the lacuna.

The cellular effects of oestrogen on osteoblasts in the egg laying hen need to be fully investigated. Circulating levels of the hormone increase as the pullet becomes sexually mature, coinciding with the proliferation and differentiation of cells that line the endosteal bone surface to form osteoblasts (Miller and Bowman, 1981, Turner *et al.*, 1993), and subsequent medullary bone formation (Ohashi *et al.*, 1991). Oestrogen is known to have direct effects on osteoblastic-like cultured cells, and certain cell lines have specific oestrogen receptors (Heersche and Aubin, 1990). It is possible that this rise in hormone levels at sexual maturity result in a shift in osteoblastic activity with increased osteoblast differentiation and activity in medullary bone, and a cessation in osteoblast activity in trabecular bone. If trabecular bone osteoblasts were inactivated by oestrogen, this would account for the lack of effect of fluoride on trabecular but not medullary bone formation, and the reappearance of osteoid in reproductively inactive females (Whitehead and Wilson, 1992). Does this mean that trabecular and medullary bone osteoblasts are derived from different osteoprogenitor cells ? Oestrogen may also be the stimulus for the altered quality of matrix/osteoid produced by the medullary bone osteoblast. The

matrix has different staining properties from that of trabecular or cortical bone, due to its altered organic content. It is richer particularly in hexosamine and uronic acid (Taylor *et al.*, 1971). These alterations in organic content may reflect its rapid turnover, as there is *in vitro* evidence to suggest that osteoclast precursors may be chemotactically attracted to hexosamine-rich matrix. However, in work on human osteoporosis, the implication is that oestrogen decreases the hexosamine/collagen ratio in matrix, and that the matrix found in oestrogen-deficient post-menopausal women is in fact richer in hexoses, sialic and uronic acids (Simmonds and Grynpas, 1991).

Oestrogen may be responsible also for the differences in bone structure, seen particularly clearly in sections under a polarising microscope. Medullary bone is a type of woven bone containing a loose arrangement of collagen fibrils. The altered 3-dimensional structure of woven bone is thought to be due to a rapid, un-coordinated and non-polarised synthesis of matrix by randomly arranged osteoblasts. In human bone biology, five operationally distinct types of osteoblast activity have been described, reflecting differences between the immature and mature state, and the synthesis of lamellar and woven bone (Parfitt, 1976). Whether these activities are mediated by different cell types, or by the same cell type responding to different stimuli and different micro-environments is not known (Parfitt, 1990). Hence, there exists the possibility of an analogous situation in the chicken. Either there exist two different populations of osteoblasts in trabecular and medullary bone, or the population is identical, but some respond positively, and some negatively to oestrogen. Alternatively, the oestrogen-stimulated increase in synthesis of $1,25(OH)_2D_3$ at the onset of egg laying may be responsible for the shift in osteoblastic activity. $1,25(OH)_2D_3$ receptors are present in osteoblasts and some of their progenitor cells and $1,25(OH)_2D_3$ is considered to increase osteoblast

differentiation thereby promoting mineralisation and bone formation (Braidman, 1990). Note that this property of $1,25(OH)_2D_3$ results in the opposite effect to that seen on addition of $1,25(OH)_2D_3$ to bone organ culture.

CHAPTER 6

DISCUSSION

6.1 Broiler Experiments

6.1.1 Model for experimental induction of tibial dyschondroplasia

Experiment 1 (see *Table IV.xiv)* confirmed that the use of a diet imbalanced in calcium and phosphorus (imbalanced Ca/P) and containing 25 μg/kg D_3 could give a higher incidence of TD than a control diet containing normal amounts of calcium (standard Ca/P). That TD is not caused by a dietary deficiency of D_3 *per se* was demonstrated by the fact that supplementation of the imbalanced Ca/P diet to give a total D_3 content of 75 μg/kg did not reduce the incidence of TD although it did prevent rickets. The experiment also confirmed the importance of examining growth plates histologically in order to confirm accurately the presence or absence of TD. The growth plates of some chicks appeared to the naked eye to be thickened, but upon histological examination there was an accumulation of pre-hypertrophic chondrocytes but none of the pathological changes associated with TD (Thorp *et al.*, 1993). Some of the thickened growth plates were assumed to be due to TD but in fact showed signs of hypocalcaemic rickets i.e. an increase in the thickness of the proliferating zone with disorganised and misshapen chondrocytes. There was no obvious relationship in these few chicks between the histological appearance of the growth plate and blood chemistry i.e. there was no hypocalcaemia or raised plasma alkaline phosphatase activity.

6.1.2 Effect of supplementation with $1,25(OH)_2D_3$ on tibial dyschondroplasia

Experiment 2 (see *Table IV.xv)* showed that a supplement of 10 μg/kg of $1,25(OH)_2D_3$ could prevent the TD induced in this model. However, there was a significant depression of body weight with this diet, and a lower rate of growth is known to be associated with a decreased incidence of TD (Huff, 1980). In broiler chicks fed *ad libitum*, growth accelerates rapidly from two weeks of age, with maximum allometric growth in the proximal tibiotarsus occurring between two and three weeks of age (Thorp, 1988). Therefore, during this crucial period, any factor reducing growth rate will also reduce chondrocyte differentiation and therefore the likelihood of TD. The possible toxic effects of $1,25(OH)_2D_3$ were considered as a cause of the lowered body weight in this group.

6.1.3 Dose-response to $1,25(OH)_2D_3$

Experiment 3 (see *Table IV.xvi)* showed that a lower dose was also effective in reducing the incidence and severity of TD, and that dietary contents of 5 and 10 μg/kg were effective in preventing TD. There was little advantage to chicks in receiving 10 μg/kg for one week after hatching only. In this experiment, there was no significant growth depression at the higher supplemental levels. Plasma inorganic phosphate and alkaline phosphatase were unaffected by supplementation whereas plasma total calcium tended to be elevated, although the changes were not statistically significant. Circulating concentrations of $1,25(OH)_2D_3$ were elevated among birds given the TD-inducing diet compared with the control diet, but did not show any obvious response to dietary supplementation of the TD-inducing diet with $1,25(OH)_2D_3$. Similar results were obtained by El Deeb and Soares (1987). It is well known that calcium deficiency increases the renal synthesis of $1,25(OH)_2D_3$ (Cancella *et al.,* 1988) and in a recent study (Goff *et al.,* 1990) circulating

concentrations achieved after dietary restriction of calcium (392 pg/ml) were comparable to those seen after injection of $1,25(OH)_2D_3$ (261 pg/ml), both being several times greater than plasma $1,25(OH)_2D_3$ concentrations in control rats (53 pg/ml). In the present study, $1,25(OH)_2D_3$ was administered orally. Its efficiency of absorption is not known, but absorption must have occurred in view of the profound effect on the incidence of TD. However, plasma concentrations of $1,25(OH)_2D_3$ are apparently not related to the development of TD.

It was also of interest in the third experiment that supplementation with 10 μg/kg of $1,25(OH)_2D_3$ for the first week after hatching only did not prevent TD. This implies that supplementation is needed over the whole period, when rapid bone growth is likely to make the growth plate more susceptible to TD. It also suggests that the problem does not originate at the breeder level, with inadequate carryover of cholecalciferol and its metabolites to the hatching chick.

The results of these experiments imply that the supplement of $1,25(OH)_2D_3$ added to the imbalanced Ca/P diet promotes or increases the differentiation of growth plate chondrocytes. This hypothesis is plausible given the findings from work carried out *in vitro*. Firstly, chondrocytes have been shown to possess receptors for $1,25(OH)_2D_3$ and secondly, addition of $1,25(OH)_2D_3$ to cultures of chondrocytes has biological effects such as enhanced $^{35}SO_4$ incorporation into proteoglycans (Corvol *et al.,* 1978) and in most studies decreases proliferation and increases differentiation as in a variety of other cell types. The work of Farquharson *et al* (1993) supports the putative role of $1,25(OH)_2D_3$ in the induction of chondrocyte differentiation *in vivo*. In this study, chicks were fed diets supplemented with various concentrations of $1,25(OH)_2D_3$. They were injected with bromodeoxyuridine (BrdU) which is incorporated into the nuclei of proliferating chondrocytes and can be detected immunofluorimetrically. After 21 hours, chicks were killed and normal

growth plates only sectioned for study. The proportion of BrdU-positive cells was counted and alkaline phosphatase activity was measured cytochemically in different zones of the growth plates as a marker of chondrocyte differentiation. $1,25(OH)_2D_3$ was observed to have no effect on chondrocyte proliferation, but speeded up chondrocyte differentiation in a dose dependent manner as assessed by the appearance of alkaline phosphatase activity nearer the top of the growth plate in supplemented chicks. There was also a significant increase in alkaline phosphatase activity in the pre-hypertrophic chondrocytes of supplemented chicks compared with unsupplemented chicks.

The experimental results described in this thesis may imply some failure of the processes involving the interaction of $1,25(OH)_2D_3$ with growth plate chondrocytes *in vivo* in chickens affected with tibial dyschondroplasia. Circulating levels of the hormone are within normal ranges, implying no failure of hormone synthesis by the mitochondrial enzymes of the kidney. A later experiment measured the activities of 1-hydroxylase and 24-hydroxylase in pooled samples of kidney taken from birds with or without TD, and supplemented with vitamin D metabolites including $1,25(OH)_2D_3$ (see *Table IV.xix)*. There were no obvious differences in enzyme activity in birds with or without TD, so data were pooled to measure any treatment effect. Activities of both enzymes were within normal ranges for young chicks (Swaminathan *et al.*, 1977, Somerville *et al.*, 1978). The activities of the two hydroxylases are reciprocally regulated, and $1,25(OH)_2D_3$ feeds back to inhibit its own synthesis. Dietary $1,25(OH)_2D_3$ did not appear to have any effect on 1-hydroxylase but increased 24-hydroxylase activity. Dietary $1,25(OH)_2D_3$ stimulated production of $24,25(OH)_2D_3$ in vitamin D deficient rats (Tanaka and DeLuca, 1974). Dietary $24,25(OH)_2D_3$ did not appear to have any effect on the activities of 1-hydroxylase, but 24-hydroxylase was again higher. Tanaka and DeLuca (1974)

found that dietary 24,25$(OH)_2D_3$ had no effect on the activities of either hydroxylase in rats. Dietary 1,24,25$(OH)_3D_3$ also appeared to have little effect on 1-hydroxylase activity, whilst being associated with raised 24-hydroxylase activity. 1,24,25$(OH)_3D_3$ is synthesised *in vitro* from 1,25$(OH)_2D_3$ by rat kidney homogenates, but its biological significance is not clear. The fact that in the one experiment reported in this thesis, dietary 1,24,25$(OH)_3D_3$ appeared to reduce the incidence of TD indicates a potential, non-physiological effect, possibly mediated through the hydroxyl group on C1.

Defects of human cholecalciferol metabolism are known to occur at every step from defective synthesis of 1,25$(OH)_2D_3$ due to either 25-hydroxylase or 1-hydroxylase deficiency, deficient 1,25$(OH)_2D_3$ receptor function or abnormal binding of the hormone-receptor complex to nuclear DNA (Marx, 1989b). Clinical signs are widespread, severe and the result of generalised vitamin D deficiency - hypocalcaemia, rickets and secondary hyperparathyroidism. Interestingly, there is evidence again from human studies that some tissues or cells can display a selective resistance to 1,25$(OH)_2D_3$ (Marx and Barsony, 1988). MacLaughlin *et al* (1985) examined the responsiveness to 1,25$(OH)_2D_3$ of cultured dermal fibroblasts from affected or unaffected skin taken from patients suffering from psoriasis, a skin disorder characterised by hyper-proliferation of skin cells, compared with fibroblasts from control patients. They found that although K_d and DNA-cellulose binding of cytosol extracts from psoriatic patients were normal, the concentrations of 1,25$(OH)_2D_3$ required to inhibit proliferation were higher than in controls. They suggested that a biochemical defect existed in the dermal fibroblasts of psoriatic patients, the rest of whose vitamin D metabolism was presumably completely normal.

K_d for $1,25(OH)_2D_3$ was found to be within normal ranges in receptors from growth plate chondrocytes taken from TD-affected chicks (Soares *et al.*, 1990, J. Berry, unpublished data). The K_d for chondrocyte receptors is similar to that of other tissues possessing $1,25(OH)_2D_3$ receptors - 10^{-10} - 10^{-11} M (Minghetti and Norman, 1988) . Although Soares *et al.* (1990) found a reduction in the mean number of receptors per cell in TD chicks compared with controls, this is likely to be due to the fact that the lesion contains areas of necrosis and cell death.

6.2 Effect of ascorbic acid without added $1,25(OH)_2D_3$ on tibial dyschondroplasia

Feeding the imbalanced Ca/P diet again induced a high incidence of TD, but supplementation with 200 mg/kg ascorbic acid had no effect on the incidence of TD (see *Table IV.xvii).* This is in accordance with the results of Edwards (1989b) who found essentially no effect of 250, 500 or 1000 mg/kg ascorbic acid on the incidence of TD in broilers fed diets low in calcium, but containing normal amounts of phosphorus (6.5 g/kg and 7.1 g/kg respectively). However, supplementation of the standard Ca/P diet with 200 mg/kg ascorbic acid strikingly reduced the incidence of TD without having any effect on blood chemistry. This result, which was replicated (see *Table IV.xviii),* is particularly interesting, as it is in contrast to those found by other workers. For example Leach and Burdette (1985) found no effect of either 100 or 250 mg/kg ascorbic acid on TD when added to 2 diets, each containing 11 g/kg calcium and 5 g/kg available phosphorus (equivalent to 6.5 g/kg total phosphorus), but differing amounts of K and Cl. However, assessment of the incidence of TD was once again by naked eye examination of sagittal sections of the proximal tibiotarsus. Also, indexed scores for each experimental group were calculated by dividing the total of the individual lesion scores by the number of chicks in the group, rather than

simply giving the number of birds in each group affected by TD. The size of the lesion, visible grossly, is not necessarily related to its severity, as assessed histologically. However, it was of interest that in this experiment, birds fed the high Cl, low K diet had consistently lower plasma ascorbic acid concentrations and a higher apparent rate of TD than birds fed the low Cl, high K diet.

The results of the experiments described in this thesis indicating possible benefits of ascorbic acid on TD are important in that another factor in the aetiology of TD may be uncovered (*see Tables IV.xvii and IV.xviii)* . As was stated in the introduction to this work, TD is likely to be the end result of a multiple set of factors. That ascorbic acid supplementation of the imbalanced Ca/P diet failed to reduce TD in contrast to supplementation of the standard Ca/P diet suggests an effect on collagen or another component of the extracellular matrix surrounding the developing chondrocytes. Ascorbic acid is a cofactor for prolyl and lysyl hydroxylase. It also has a major influence on, for example, the composition of the extracellular matrices formed by cultured smooth muscle cells and fibroblasts. *In vitro*, a deficiency of ascorbic acid promotes elastin formation and inhibits collagen formation. When it is supplied, the amount of elastin synthesised is reduced and collagen synthesis is increased. However, the effect of ascorbic acid on elastin and collagen metabolism varies a great deal, depending on the source and species of cells used in culture experiments (Chattergee, 1990).

It is well known that addition of ascorbic acid (50-100 μg/ml) to chondrocyte culture medium is necessary for the development of the hypertrophic phenotype as measured biochemically by maximal alkaline phosphatase activity, production of type X collagen and histologically by the appearance of areas of mineralisation (Gerstenfeld and Landis, 1991; Wu *et al*, 1989). Chondrocytes cultured in the absence of ascorbic acid synthesise under-hydroxylated, non-helical procollagen

molecules that are transported very slowly and accumulate in the rough endoplasmic reticulum of the cell (Pacifici, 1990). Ascorbic acid is of course a co-factor for prolyl hydroxylase, situated in the rough endoplasmic reticulum and the enzyme which catalyses the modification of the pro-collagen chains. The mechanism by which ascorbic acid promotes chondrocyte differentiation *in vitro* is not clear. Gerstenfeld and Landis (1991) suggest that its effects on chondrocyte phenotype are secondary to its actions on the synthesis and deposition of a normal extra-cellular matrix, and that the extra-cellular matrix in turn a) helps to maintain a normal, rounded chondrocyte morphology and b) helps to establish normal cell-extracellular matrix interactions. This idea is backed up by studies in which chondrocyte differentiation towards a more hypertrophic phenotype is seen when cells are cultured in a 3-dimensional system utilising collagen or agarose gels.

Interestingly, abnormalities of matrix composition have been found in birds affected by TD. Bashey *et al.* (1989) found that the TD lesion contained much less type X collagen than normal hypertrophic cartilage. Kwan (1994) found that in chick growth plate hypertrophic chondrocytes immunostaining of normal cartilage revealed strong pericellular and matrix staining for Type X collagen. However, in growth plates from TD-affected chicks, Type X collagen was located intra-cellularly with only weak pericellular staining. But Type X collagen mRNA was actually elevated in TD-affected chicks, suggesting that a defect in secretion or incorporation of the collagen into extracellular matrix existed, rather than reduced synthesis. So, given the importance of ascorbate in chondrocyte differentiation in culture, it could be postulated that the TD seen in chicks fed diets containing normal amounts of calcium and phosphorus, and which responds to supplementation with ascorbic acid could be due to some degree of deficiency in ascorbic acid affecting the composition of the chondrocyte extracellular matrix which in turn affects the cell morphology.

Chickens do synthesise ascorbic acid, but there is evidence to suggest that the rate of synthesis is not maximised until about 2 weeks post-hatch. Hornig and Frigg (1979) found that the biosynthetic capability of the broiler chick to synthesise ascorbic acid varied with age, and reached a maximum at when the chicks were about 30 days old. This was reflected in plasma and liver ascorbate concentrations. The authors considered that the amount of ascorbic acid available to the chick was particularly low for the first 15 days of life, when demand might be high due to various stresses. Long bone growth is most rapid when the broiler chick is 2-3 weeks of age (Thorp, 1988), and therefore the cells contributing to this process are possibly more sensitive to marginal circulating ascorbate concentrations.

Other workers have described a gallinaceous bird that appears to have a dietary requirement for ascorbic acid (Hannsen *et al.*, 1979). The kidney of the willow ptarmigan (*Lagopus lagopus lagopus)* is capable of synthesising ascorbic acid from the precursor, but unless the diet of the growing chick contains plant material rich in the vitamin, deficiency symptoms such as reduced body weight, leg and muscle weakness, spontaneous fracture of the leg and wing bones are seen. Histologically, the metaphysis of the proximal tibia was abnormally vascularised, the blood vessels being dilated and irregular. There were areas of necrosis in the hypertrophic zone of the growth plate. Other authors (Edwards, 1989b) have likened the pathological changes seen in this ascorbic acid-deficient state to those seen in broilers affected with tibial dyschondroplasia, but in fact there are quite clear differences, in terms of the structure and degree of penetration of the metaphyseal vessels into the growth plate, and the structures of cortical and trabecular bone in the metaphysis. However, this work is interesting in that it appears that the growing chicks of this species have a requirement for dietary source of ascorbic acid to

supplement that synthesised endogenously, whereas the amount of ascorbic acid synthesised endogenously by adult birds is satisfactory.

Another possibility to be considered returns to the hypothesis that $1,25(OH)_2D_3$ is important in the processes of chondrocyte differentiation. It has been suggested that production of $1,25(OH)_2D_3$ is inadequate in young growing broiler chicks, and that endogenous production may be stimulated by administering ascorbic acid. In man, acute injections of ascorbic acid raised serum $1,25(OH)_2D_3$ after 30 minutes, and the rise was maintained for at least 1 hour. Other blood parameters (ionised Ca, P, $25(OH)D_3$ and PTH) were unchanged (Cantatore *et al*, 1991). Weiser and Schlacter (1987) found that addition of 100 mg/kg ascorbic acid to feed containing 4, 16 or 80 µg/kg cholecalciferol was associated with significantly higher circulating $1,25(OH)_2D_3$ and enhanced calcium binding capacity of gut CaBP, and suggested that this was due to increased 1-hydroxylation. The 1-hydroxylase enzyme complex is located in the mitochondria of the kidney tubule. It is a typical mixed function oxidase with NADPH, ferridoxin, ferridoxin reductase and cytochrome P450 acting together to form a mini electron transport chain. The oxygen atom added to $25(OH)D_3$ in the reaction is supplied by molecular O_2, the surplus atom of which is reduced to form H_2O (Henry, 1992). Barnes and Kodicek (1972) suggested that the source of reducing power in the reaction was ascorbic acid. The results of the experiment described earlier in this thesis (see *Table IV.xix)*, showed no clear effect of ascorbic acid supplementation on the synthesis of $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$. However, other studies have shown that ascorbic acid deficiency in guinea pigs is associated with reduced 1-hydroxylase activity and reduced binding of $1,25(OH)_2D_3$ to target tissues (Sergeev *et al.*, 1990).

Ascorbic acid has also been shown to be associated with the energy status of chondrocytes *in vitro* as well as changes in alkaline phosphatase activity (Shapiro *et*

al., 1991). Addition of 40 μg/ml ascorbic acid to cultured cells was associated with a reduction in lactate formation and maintenance of iso-citrate dehydrogenase activity, suggesting that ascorbate promotes oxidative metabolism by inhibiting reduction of pyruvate to lactate. It has been suggested that a factor in the development of TD is reduced availability to chondrocytes of O_2 leading to anaerobically-induced changes in cell metabolism. However, Farquharson *et al.* (1992) showed no differences in lactate dehydrogenase activity in the tibial growth plate pre-hypertrophic zones of birds with or without TD. Another interpretation of the results of Shapiro *et al.* (1991) is that ascorbic acid promotes cell differentiation, and the changes in energy metabolism are secondary to this.

6.3 Effects of $1,25(OH)_2D_3$ analogues on TD

Initially, the results of the $1,25(OH)_2D_3$ analogues RO23-7553 and RO23-6474 *in vivo* were unexpected. The former analogue had essentially no effect on the incidence of TD, and the latter only slightly reduced the incidence of TD (See *Table IV.xx).* These two analogues have been chemically synthesised for clinical application, their structures based on that of $1,25(OH)_2D_3$. They have enhanced cell differentiating and reduced cell proliferating effects *in vitro* compared with $1,25(OH)_2D_3$. The analogues also have much reduced calcaemic properties. It was hypothesised that the pre-hypertrophic chondrocytes that fail to differentiate and accumulate to form the TD lesion might respond *in vivo* to the analogues as they appeared to do with $1,25(OH)_2D_3$. However, histological examination of sections of PTT growth plate showed that this was not the case. Many of the thickened growth plates from chicks which had been administered the analogues had clear pathological changes indicating TD. There was no effect of either analogue on either blood ionised calcium or plasma total calcium (see *Table IV.xx*), unlike $1,25(OH)_2D_3$

which in previous experiments had been associated with hypercalcaemia. Blood pH was significantly raised in chicks administered RO23-6474 and $1,25(OH)_2D_3$ (see *Table IV.xx*) but the reason for this is unknown.

The dosing experiment had been carried out administering equal quantities of $1,25(OH)_2D_3$ or either analogue to the chicks. It was assumed that all would be equally well absorbed from the gut, and would in turn be equally well transported to the target tissues, in this case the cells of the PTT growth plate. However, analogues are thought to have differing affinities for plasma vitamin D binding protein and tissue receptors compared with $1,25(OH)_2D_3$. These differing affinities are thought possess to influence biological activity (Bouillon *et al.*, 1991a).Therefore, the lack of effect of the analogues on TD compared with $1,25(OH)_2D_3$ could be due to reduced affinity for either the plasma vitamin D binding protein or chondrocyte receptor.

The *in vitro* studies of Farquharson and Whitehead (unpublished data) showed that when PTT growth plate chondrocytes were cultured in suspension, $1,25(OH)_2D_3$ and both analogues increased differentiation (measured as increased alkaline phosphatase activity) and inhibited proliferation (measured as reduced ^{3}H-thymidine uptake). Both analogues seemed to have potencies similar to that of $1,25(OH)_2D_3$.

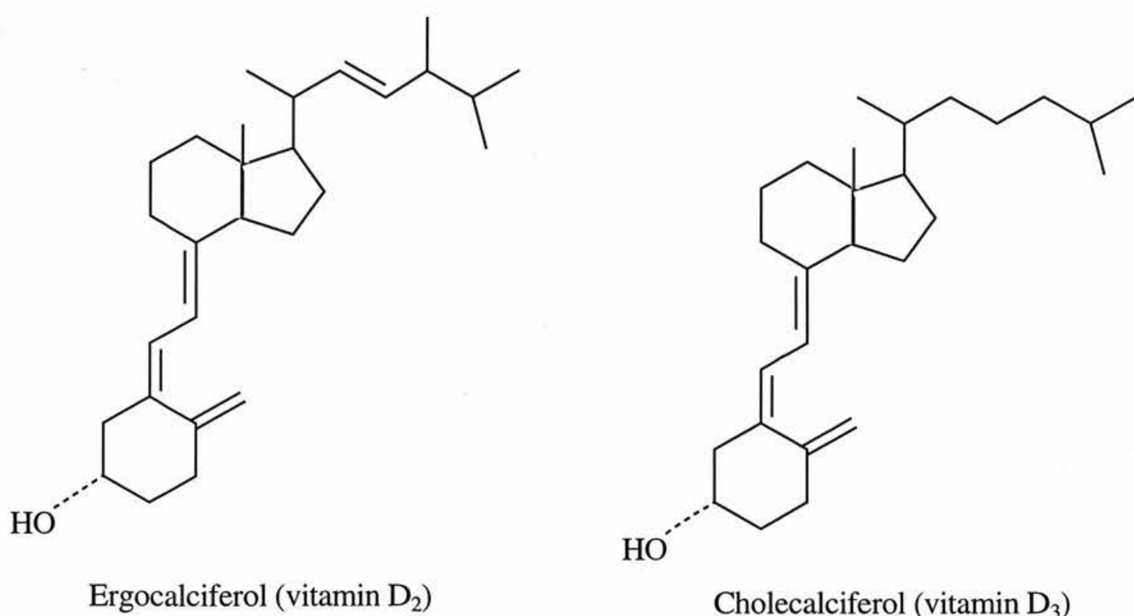
The results of the plasma vitamin D binding protein affinity study and the *in vitro* chondrocyte $1,25(OH)_2D_3$ binding study gave some insight into the results obtained *in vivo*.

6.4 Plasma DBP affinity study

Chick serum contains two vitamin D binding proteins, a β-globulin which binds primarily vitamin D and another protein which binds primarily $25(OH)D_3$

(Edelstein *et al.*, 1972, 1973) with a dissociation constant which varies slightly according to different reports (e.g. Kd of 2.3 nM; Lawson, 1978, Bouillon *et al.*, 1980; K_d of 3 nM; Norman, 1979). Diluted chick serum will also bind $1,25(OH)_2D_3$. The inability of chickens to utilise ergocalciferol (vitamin D_2) to any great extent (estimates of potency of D_2 vary from one-tenth to one-fortieth of that of D_3; Webb and Taylor, 1976) can be explained by the fact that chicken plasma vitamin D binding protein does not bind D_2 and metabolites as strongly as D_3 and metabolites (Haddad, 1992). This is probably due to the differing chemical structures of the side chain.

Figure 5a. Diagram to show the structures of ergocalciferol (D_2) and cholecalciferol (D_3)



The affinity of either analogue for plasma vitamin D binding protein was greatly reduced compared with either $1,25(OH)_2D_3$ or $25(OH)D_3$. Of the two

vitamin D metabolites, $25(OH)D_3$ had the greater affinity for the plasma binding protein. K_d was estimated at 4.27 nM (see *Figure 4m*). These results are of the same magnitude as those of other authors (Norman, 1979, Lawson, 1978, Bouillon *et al.*, 1980). Dusso *et al.* (1991) showed that $1,25(OH)_2D_3$ bound about one-tenth as strongly as $25(OH)D_3$ to the binding protein in rat serum (measured as the concentration required to displace 50% of ^3H-$25(OH)D_3$). The present study for the binding protein in chick plasma gave an estimate of about one-sixth (see *Figure 4n*). Over the range of concentrations tested, a relative binding estimate could only be obtained for RO23-7553. This analogue was found to bind about 1/1260 times as strongly as $25(OH)D_3$ (see *Figure 4n*). This compares with a figure of about 1/10 000 times for normal rat serum (Dusso *et al.*, 1991). Species differences for relative binding affinities of analogues to plasma vitamin D binding protein may occur (Dusso *et al.*, 1991). No estimate of relative binding affinity could be obtained for RO23-6474, the structure of which is derived from $1,25(OH)_2D_2$.

6.5 Chondrocyte receptor binding study

The chondrocytes receptors bound $1,25(OH)_2D_3$ with high affinity as shown by the Scatchard plot, with a K_d of 18.9 pM, and B_{max} of 3.54 pM (see *Figure 4j*). Soares *et al.*, (1990) obtained estimates of 70 - 700 pM for binding of $1,25(OH)_2D_3$ to growth plate chondrocytes. Neither analogue bound as well to the receptor, with RO23-7553 binding better than RO23-6474 (see *Figure 4k*).

The results of both these studies imply that uptake of analogue into chondrocytes *in vivo* would be reduced compared with $1,25(OH)_2D_3$, and may be insufficient to induce differentiation. Structure-function studies of vitamin D analogues suggest that the affinity of an analogue for the vitamin D receptor in cells

does not necessarily parallel its affinity for plasma vitamin D binding protein (Bikle, 1992). It is generally considered that the reduced affinity of analogues with side-chain modifications such as the two used in the present study means that the circulating concentration of 'free' molecule is raised, and therefore rapid degradation takes place (Bouillon *et al.*, 1991b). However, tissue uptake may also be increased. The rapid clearance of vitamin D analogues *in vivo* may account in part for their selective effects in different organs and tissues. For example, raising plasma calcium (an effect which analogues with modifications of the side chain do not generally possess) via increased absorption of dietary calcium and bone resorption are processes which require *de novo* protein synthesis, and therefore might require a more prolonged circulating concentration of vitamin D metabolite or analogue.

Many of the growth plates from chicks administered the analogues appeared visually to be thickened due to TD. However, when they were histologically examined, although accumulations of cells were seen, there was terminal differentiation of chondrocytes, and an absence of the pathological changes seen in TD. These findings are consistent with a reduction in cell differentiation, but not severe enough to result in TD.

6.6 Toxicological study

This study showed that 20 μg/kg $1,25(OH)_2D_3$ added to chick diets containing normal levels of calcium and phosphorus was toxic, as evidenced by the severely reduced body weight, hypercalcaemia and hypophosphataemia, raised plasma protein and uric acid (see *Table IV.xxi a* and *b*). These findings are similar to the effects of vitamin D intoxication. Few studies have been carried out on the possible toxicity of $1,25(OH)_2D_3$ in animals although there are reports in the literature of hypercalcaemia in human patients treated with $1,25(OH)_2D_3$ for skin

disorders and osteoporosis (Jones and Calverly, 1993). Mullen *et al.* (1979) studied the toxicology of $1\alpha(OH)D_3$ in calves and found increased serum GOT (glutamate oxaloacetate transferase; now known as aspartate aminotransferase) and GDH (glutamate dehydrogenase), indicative of liver damage, and increased serum calcium and phosphate concentrations. Visual and histological examination of the heart, pulmonary artery and kidney revealed areas of calcification.

A possible explanation for the presence of deposits of both urate and calcium phosphate (see *Plates 9* and *10*) is that supplementation with 5 μg/kg $1,25(OH)_2D_3$ was sufficient to cause the precipitation and deposition of small amounts of calcium phosphate in the kidney. However 20 μg/kg $1,25(OH)_2D_3$ caused hypercalcaemia and larger and more numerous calcium phosphate deposits. This possibly compromised normal kidney function, leading to increased protein and uric acid in the plasma. The uric acid, being practically insoluble in aqueous solution, was also eventually precipitated in the kidney instead of being filtered and excreted.

The results of this experiment demonstrated practically the potency of $1,25(OH)_2D_3$ in influencing calcium metabolism, and demonstrated the potential for toxic interaction with dietary calcium. The experiment also showed that $1,25(OH)_2D_3$ could act in the kidney at a sub-microscopic level, in the absence of any other pathological changes capable of influencing production. Conventional broiler chicken diets would contain about 8-10 g/kg calcium (NRC, 1984). Under normal physiological conditions, cholecalciferol is enzymically converted into $1,25(OH)_2D_3$ in two stages in the liver and the kidney, largely under the control of parathyroid hormone (PTH). PTH secretion is stimulated by low serum calcium. $1,25(OH)_2D_3$ directly influences the absorption of calcium from the small intestine by in turn stimulating the synthesis of calcium binding protein (CaBP). Serum

calcium rises, and negatively feeds back on PTH secretion. $1,25(OH)_2D_3$ also increases bone mobilisation which boosts serum calcium (Norman, 1979). Therefore, intestinal calcium absorption is adaptively regulated by the animal so that there is increased efficiency of calcium absorption under conditions where demand is high, such as low dietary calcium. Friedlander *et al.* (1977 a,b) showed in the chick that intestinal CaBP levels change when dietary calcium and phosphorus alter, when cholecalciferol (1.6 nmol/day) was administered from 14-28 days of age. CaBP was highest when the diet contained 0.5 g/kg calcium and 7 g/kg phosphorus, and lowest when the diet contained 12 g/kg calcium and 11 g/kg phosphorus. However, this mechanism was overridden when 1 nmol/day $1,25(OH)_2D_3$ was administered, and levels were similar over the ranges of calcium and phosphorus tested. This infers that supplementing chicks with $1,25(OH)_2D_3$ disrupts the normal mechanisms of calcium homeostasis resulting in hypercalcaemia due to excess absorption from the gut. Hypercalcaemia due to excess cholecalciferol intake is known to produce certain physiological effects including increased blood pressure, decreased tubular reabsorption of Na and Mg, reduced ability to concentrate urine due to nephrocalcinosis rather than reduced glomerular filtration rate (Norman, 1979). Morissey *et al.* (1977) saw clear pathological changes in chicks given 10 mg/kg D_3 or 100 µg/kg $25(OH)D_3$ indicative of calcification of kidney distal convoluted tubules in the absence of any changes in blood calcium, and suggested that histopathological examination of kidney was a more sensitive indicator of toxicity of vitamin D and its metabolites than measurement of blood parameters. The results of the present study would seem to confirm this.

CHAPTER 7

CONCLUSIONS AND AREAS FOR FURTHER STUDY

The work presented in this thesis demonstrates that manipulation of nutrition can influence bone characteristics of poultry. The results could be of practical benefit in terms of welfare and production in the case of tibial dyschondroplasia, although the practical benefits for osteoporotic birds are not quite so apparent.

7.1 Studies on osteoporosis in hens

The results of the laying hen experiments showed that structural bone quantity (i.e. the amount of trabecular bone remaining at the end of lay) is not influenced in terms of increase by nutrition, but that non-structural, labile bone quantity (i.e. the amount of medullary bone) can be. The lack of effect of nutrition appears to be due to the fact that in the sexually-mature hen, there is a lack of osteoblastic activity in trabecular bone. However, the experiment did not attempt to measure the detrimental effects that very inadequate nutrition is known to have on bone.

However, there was evidence that nutrition might influence the material properties of bone in that there was a reduced rate of wing-bone fracture in birds from certain dietary treatments. Scope exists for further investigations into the effects of nutrition on the biochemical factors influencing the material properties of bone, such as collagen crosslinking and crystal stability, and to assess the contribution of medullary bone to overall bone strength and likelihood of fracture.

An obvious difference in quantity of trabecular bone at the end of lay existed between the two strains of birds used in the experiment. This difference was

thought to be related to level of egg production, and therefore it would be important to see whether any differences existed in the amount and quality of bone within individuals of a high producing strain.

The timescale of this project did not allow a full examination of biochemical markers of bone turnover, which could have given some additional information e.g. any correlations between bone markers, quantity of bone and egg output. Assay of the peptide calcium-regulating hormones would have been interesting, but avian peptide hormones have different amino-acid compositions from mammalian versions, and cross react poorly with antibodies raised against mammalian hormones. A bioassay for PTH based on the release of cAMP from renal tubule cells has been used to measure PTH responses to calcium stress during eggshell calcification (Van de Velde *et al.*, 1984), but again the scale of the experiment made such an assay impractical. During the course of the project, a radioimmunoassay (RIA) was developed for avian calcitonin, similar to that published by McMurtry and Steele (1989) based on a rabbit polyclonal antibody to salmon calcitonin. Salmon calcitonin has a structure similar to that of the avian peptide, and is very immunogenic. The RIA gave good standard curves, and good estimates of calcitonin in pooled plasma stored at -70 C from broilers which had been fed a diet high in calcium (2.2 ng/ml; 60 g/kg calcium), or low in calcium (1.0 ng/ml; 10 g/kg calcium). However, plasma samples from experimental hens had been stored at -20 C prior to assay, and this was suspected not to be a sufficiently low temperature to prevent decomposition of calcitonin, as the results were generally too low to detect.

It would also have potentially been of interest to measure osteocalcin, as this peptide is a good marker of bone turnover. A double antibody RIA is commercially available (e.g. Incstar), but would fail on avian samples for the same

reasons as for the peptide hormones. Another marker of bone turnover is the measurement of collagen crosslinks, using either high performance liquid chromatography, or enzyme-linked immunosorbent assay. But, in the bird, urine and faeces are voided together, making such measurements impractical.

Had the potential existed to measure markers of bone turnover, the results might not have given any clear indication of what was happening to trabecular bone, given the presence of rapidly-turning over medullary bone. However, there is evidence that the extracted collagen of mature bone contains different crosslinks to that of immature, recently laid down bone (Bailey *et al.*, 1992), but such differences may not be subsequently apparent in products of crosslink metabolism excreted in urine.

From a practical view, possibilities exist for maximising bone mass in immature birds by selective breeding either by using radiographic techniques to directly measure bone, or using image analysis on samples of bone from sibs. Another possibility concerns recent reports which have shown a relationship between vitamin D receptor alleles and the genetics of human osteoporosis (Morrison *et al.*, 1992, 1994). Could the same also be true of pullets ?

Another approach could be to use bone anabolic agents such as fluoride or bisphosphonates (Thorp *et al.*, 1993) during rearing. During rearing, birds are only laying down trabecular bone, on the osteoblasts and osteoclasts of which fluoride and bisphosphonates would be able to act respectively (Sietsma *et al.*, 1989, Cariano *et al.*, 1990).

7.2 Studies on tibial dyschondroplasia in broilers

It is now clear that feeding a diet moderately low in calcium and high in phosphorus can induce a high incidence of dyschondroplasia in young growing

broiler chickens. However, it is still not clear how such a nutritional modification can interact with the bird's genotype to result in the development of the lesion, but the paper of Goff *et al.* (1990) is of interest within the context of this thesis. They describe the effects of a diet very low in calcium in rats (0.2 g/kg) on plasma $1,25(OH)_2D_3$ and vitamin D receptor, and concluded that a feeding a low calcium diet resulted in down regulation of kidney vitamin D receptor.

The finding that supplementation of the chicken's low-calcium diet with $1,25(OH)_2D_3$ reproducibly lowers the incidence of dyschondroplasia, together with the work of Farquharson *et al.* (1993) showing that the rate of chondrocyte differentiation is speeded up in supplemented birds, point to a failure in the processes controlling chondrocyte differentiation in this model of TD. (Modifications of other nutrients, or administration of drugs which result in dyschondroplasia, as described in Chapter 1, may be related to a primary failure of chondrocyte differentiation or matrix abnormalities which subsequently influence cell differentiation. Cook *et al.* (1994) suggest that these manipulations result in decreased growth plate cartilage degradation and hence reduced vascular penetration). Recent studies (Farquharson *et al.*, 1993, and Loveridge *et al.*, 1993) are of interest as they demonstrate reduced levels of growth factors in the growth plate of birds with TD. Growth factors such as insulin-like growth factor-1, basic fibroblast growth factor and transforming growth factor-β have been shown to be important autocrine and paracrine signallers in the avian growth plate (Leach and Twal, 1994). The production of some of these factors may be influenced by $1,25(OH)_2D_3$ (Pfeilschifter and Mundy, 1987).

Circulating levels of endogenously synthesised $1,25(OH)_2D_3$ and renal 1-hydroxylase were normal in TD affected birds, indicating no lack of ability to synthesise the hormone. Interestingly, dietary supplementation with $1,25(OH)_2D_3$

did not result in any rise in circulating plasma concentration. Preliminary investigations (Rennie, unpublished data) suggest that only a small fraction of intubated ^3H-1,25$(OH)_2D_3$ is absorbed, but rapidly, from the gut. It is then cleared quickly from the circulation, with little evidence of storage in body tissues.

Receptors in growth plate chondrocytes were found to have normal affinity for 1,25$(OH)_2D_3$, although in order to fully rule out the possibility of a failure of the classical steroid hormone mode of action, the stability of the hormone/receptor complex and its subsequent binding to nuclear DNA merit investigation. There are some interesting studies in the human field demonstrating both global defects of 1,25$(OH)_2D_3$ interactions with cells, leading to a generalised, functional vitamin D deficiency (Marx, 1989b, Liberman *et al.*, 1983, 1986, Hirst *et al.*, 1985), but also evidence of limited defective 1,25$(OH)_2D_3$ interactions in certain cell types such as in keratinocytes in patients with psoriasis (McLaughlin *et al.*, 1985).

Analogues of 1,25$(OH)_2D_3$ with reduced calcaemic properties and enhanced differentiating properties *in vitro* were administered to growing chicks in an attempt to determine whether the effects of 1,25$(OH)_2D_3$ on TD were mediated genomically or non-genomically. The mechanism of action of the analogue 1,25-16-ene-23-yne-dihydroxycholecalciferol is thought to be mainly genomic (however, other analogues with different structures may possess other properties such as stimulating trans-membrane calcium flux, Farach-Carson *et al.*, 1991). There was little effect of either analogue *in vivo*, and subsequent studies revealed the reason for this - very poor binding to plasma DBP and chondrocyte receptors. However, the work of Farquharson *et al.* (unpublished data) does confirm that the analogues' ability to induce differentiation in chondrocytes in physiological concentrations *in vitro* was comparable to that of 1,25$(OH)_2D_3$, showing that this property appears to be constant in different cell types, and that the effects of 1,25$(OH)_2D_3$ *in vivo* are at

least partly due to genomic effects. These analogues are developed using such cell types as osteoblast-like cells from human osteosarcoma or leukaemia cell lines. It is possible that the administration of larger doses of the analogues, RO23-7553 in particular, would have elicited a biological response, but the small quantities available of analogues meant that this was not possible.

The possibility still remains that some of the effects of $1,25(OH)_2D_3$ on growth plate chondrocytes are mediated through pathways not involving gene transcription. Boyan *et al.* have demonstrated non-genomic effects of $1,25(OH)_2D_3$ on cultured chondrocytes including alterations in membrane fluidity (Boyan *et al.* 1991) and stimulation of growth zone chondrocyte protein kinase C activity (Boyan *et al.*, 1994). $1,25(OH)_2D_3$ also appears to induce changes in calcium flux and prostaglandin production in isolated matrix vesicles, which have no DNA (Boyan *et al.*, 1994). Further *in vivo* and *in vitro* experiments are necessary to elucidate the mechanism of action in alleviating TD, which could in turn highlight possible biochemical / genetic defects in broiler chickens. *In vivo* experiments could extend the preliminary work mentioned earlier, and determine the pharmacokinetics of administered $1,25(OH)_2D_3$. These results would give information as to how much dietary $1,25(OH)_2D_3$ is actually absorbed, how quickly it is cleared from the blood stream, how it is excreted, and whether any at all is stored in the body. The results of the plasma vitamin D binding protein study suggest that in both hens and broilers, supplemental $1,25(OH)_2D_3$, following absorption from the gut, would not be transported very efficiently to target tissues, as binding sites would be preferentially occupied by endogenous $25(OH)D_3$. Autoradiography would determine which cells in the growth plate take up $1,25(OH)_2D_3$, and whether the process is interrupted in TD. Wezeman (1976) reported that in growing rats administered ^3H-$25(OH)D_3$, autoradiographs of the growth plate showed incorporation of $25(OH)D_3$ into the

hypertrophic chondrocytes of the growth plate, with the cells of the proliferating zone showing negligible uptake. However, as Garabedian *et al.* (1978) comment in their paper showing that chondrocytes metabolise $25(OH)D_3$ to more polar derivatives, it is not clear from Wezeman's study whether the radioactive material is $25(OH)D_3$ or a further metabolite. From our work on chondrocyte receptors and the role of $1,25(OH)_2D_3$ in chondrocyte differentiation, it is possible that the autoradiographs do in fact show uptake of $1,25(OH)_2D_3$ into hypertrophic chondrocytes. However, Klaus *et al.* (1991) demonstrated immunohistochemically the presence of receptors for $1,25(OH)_2D_3$ in rabbit tibia growth plate, localised mainly in the proliferating zone, with far fewer in the hypertrophic zone .

In vitro experiments could focus on likely candidates for non-genomic mechanisms of $1,25(OH)_2D_3$ such as any effects on intracellular calcium. Also of interest would be the effects of calcium channel blockers (e.g. verapamil or nifepidine) which have been shown to block the differentiating effects of $1,25(OH)_2D_3$ in keratinocytes (McLane *et al.*, 1994)

The results of such experiments could give valuable information to breeding companies, who have had success in selecting against the TD-phenotype in grandparent and parent stock as part of general programs to improve leg health and bird welfare.

END

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APPENDIX 1

Composition of experimental layer diets (g/kg)

Ingredient	*Layer starter (0-4w)*	*Layer rearer (4-16w)*	*Basal diet ** (16w-end)*
Barley	100	300	
Wheat	245	280	755
Maize	300	200	
Soya meal	220	100	50
Fish meal	50	35	80
Meat & bone meal			23
Grass meal	50	50	
Limestone	5.3	10	70
Dicalcium phosphate	21.7	17.5	15
Salt	2.5	2.5	2
Vitamin & mineral supplement	5*	5*	5+
Calculated analyses (g/kg)			
Metabolisable energy	11.7 MJ/kg	11.1 MJ/kg	11.4 MJ/kg
Crude protein	200	155	170
Ca	12	11	35
P (total)	7	6	6

Ingredient	*Oystershell diet*	*Low CP, high vitamin K diet*	*Low P diet*
Wheat	755	767	758
Wheatfeed		23	330
Soya meal	50	40	50
Fish meal	80	54	80
Meat & bone meal	23	20	23
Limestone	35	70	76
Crushed oystershell	35		
Dicalcium phosphate	15	19	6
Salt	2	2	2
Vitamin and mineral supplement	5+	5++	5+
Calculated analyses (g/kg)			
Metabolisable energy	11.4 MJ/kg	11.4 MJ/kg	11.4 MJ/kg
Crude protein	170	150	170
Ca	35	35	35
P (total)	6	6	4.5

**Supplemented with ascorbic acid (300 mg/kg), sodium fluoride (200 mg/kg) or 1,25-dihydroxycholecalciferol (5 μg/kg).

* Provides (per kg diet) retinol, 1376μg, cholecalciferol, 25μg, α-tocopherol, 10mg, menadione, 1.3 mg, thiamine, 1.3mg, riboflavin, 3.3mg, pyridoxine, 1.7mg, cyanocobalamin, 0.6μg, folic acid, 0.7mg, biotin, 267μg, pantothenic acid, 5.3mg, nicotinic acid, 23.3mg, zinc, 40mg, copper, 2.9mg, iodine, 320μg, iron, 64mg, manganese, 80mg, selenium, 120μg.

+ Provides (per kg diet) retinol, 1720 μg, cholecalciferol, 20μg, α-tocopherol, 25 mg, menadione, 1.3 mg, riboflavin, 4mg, nicotinic acid, 28mg, pantothenic acid, 10mg, and minerals as above.

++ Provides (per kg diet) retinol, 1720 μg, cholecalciferol, 20μg, α-tocopherol, 25 mg, menadione, 21.3 mg, riboflavin, 4mg, nicotinic acid, 28mg, pantothenic acid, 10mg, and minerals as above.

APPENDIX 2

Composition of experimental broiler diets (g/kg)

Ingredient	*Control diet*	*Imbalanced diet*	*Standard starter*	*Vitamin D-deficient diet*
Wheat	559	563	250	622
Maize			330	
Soya meal	350	350	298	303
Vegetable oil	45	50	28	36
Fish meal			21	
Meat & bone meal			46	
Limestone	13	-	20	15
Dicalcium phosphate	21	25		15
Salt	4	4	2	3
Methionine	2	2		2.5
Lysine	2	2		0.5
Vitamin and mineral supplement	4[a]	4[a]	5[b]	5*
Calculated analyses (g/kg)				
Metabolisable energy	12.2 MJ/kg	12.2 MJ/kg	12.4 MJ/kg	12.2 MJ/kg
Crude protein	220	220	220	220
Ca	12	7.5	10	10
P (total)	6	7.6	6	7

[a] Provided (per kg diet) retinol, 1376μg, cholecalciferol, 25μg, α-tocopherol, 10mg, menadione, 1.3 mg, thiamine, 1.3mg, riboflavin, 3.3mg, pyridoxine, 1.7mg, cyanocobalamin, 0.6μg, folic acid, 0.7mg, biotin, 267μg, pantothenic acid, 5.3mg, nicotinic acid, 23.3mg, zinc, 40mg, copper, 2.9mg, iodide, 320μg, iron, 64mg, manganese, 80mg, selenium, 120μg.

[b] Provided (per kg diet) retinol, 688 μg, cholecalciferol, 15 μg, α-tocopherol, 25 mg, menadione, 1.3 mg, riboflavin, 4 mg, nicotinic acid, 28 mg, pantothenic acid, 10 mg, biotin, 50 μg, zinc, 50 mg, copper, 3.6 mg, iodide, 400 μg, iron, 80 mg, manganese, 100 mg, selenium, 150 μg.

* As in (b) above, but without cholecalciferol.

APPENDIX 3

Publications arising from this thesis

Rennie, J.S., Whitehead, C.C. and Thorp, B.H. (1993). The effect of dietary 1,25-dihydroxycholecalciferol in preventing tibial dyschondroplasia in broilers fed on diets imbalanced in calcium and phosphorus. British Journal of Nutrition 69, 809-816.

Rennie, J.S., Thorp, B.H. and Whitehead, C.C. (1992). The effect of 1,25-dihydroxycholecalciferol on tibial dyschondroplasia in broiler chickens. Proceedings of the 14th Annual Meeting of the American Society for Bone and Mineral Research, Minneapolis, September 30-October 4. Journal of Bone and Mineral Research 7 (supplement 1) S169.

Rennie, J.S., Thorp, B.H. and Whitehead, C.C. (1992). Responses of tibial dyschondroplasia to dietary supplementation with $1,25(OH)_2D_3$ as assessed by histopathology and gross examination. Bone Biology and Skeletal Disorders in Poultry. Proceedings of the 23rd Poultry Science Symposium, Edinburgh, September 18-20, p. 355.

The effect of dietary 1,25-dihydroxycholecalciferol in preventing tibial dyschondroplasia in broilers fed on diets imbalanced in calcium and phosphorus

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Three experiments were carried out to investigate the effects of supplemental dietary 1,25-dihydroxycholecalciferol (1,25(OH)$_2$cholecalciferol) and a low dietary Ca:P ratio on the occurrence of tibial dyschondroplasia (TD) in 3-week-old broilers. Histopathology was used to diagnose TD. In the first experiment, feeding a diet containing 7·5 g Ca and 7·6 g P/kg gave a higher incidence of TD than a control diet containing normal amounts of Ca and P (12 and 6 g/kg respectively). Increasing the dietary supplement of cholecalciferol in the imbalanced diet prevented rickets but did not decrease the incidence of TD. In the second experiment, supplementing the imbalanced diet with 10 μg 1,25(OH)$_2$cholecalciferol/kg prevented TD completely but also gave a slight growth depression. In the third experiment the imbalanced diet was supplemented with 0, 2·5, 5 or 10 μg 1,25(OH)$_2$ cholecalciferol/kg. The supplement of 2·5 μg/kg depressed and the higher supplements prevented the occurrence of TD, this time without a growth depression. Feeding the 10 μg/kg supplement for the first week only did not prevent TD. Plasma total Ca, inorganic P and alkaline phosphatase (*EC* 3.1.3.1) were unaffected by diet but 1,25(OH)$_2$cholecalciferol was higher on the imbalanced than on the control diet. Supplementation of the imbalanced diet with 1,25(OH)$_2$cholecalciferol did not increase plasma levels. It is concluded that 1,25(OH)$_2$cholecalciferol is exerting a powerful biological effect in this model of TD, but the mechanism is unclear.

Tibial dyschondroplasia: Cholecalciferol: 1,25(OH)$_2$cholecalciferol: Calcium–phosphorus balance: Broilers

Tibial dyschondroplasia (TD) is a well-recognized disorder of rapidly growing broilers, first described by Leach & Nesheim (1965). TD affects the long bones, particularly the proximal tibiotarsus (PTT), and is characterized by the presence there of an avascular plug of cartilage. If the lesion is large, affected birds may become lame due to weakening, bowing or fracture of the PTT (Lynch *et al.* 1991). Less severely affected birds show thickening of the growth plate. Estimates of clinically affected birds are as high as 10% and many more birds may be sub-clinically affected.

Histologically the lesion consists of a mass of pre-hypertrophied chondrocytes, due to a failure, for as yet unknown reasons, of the normal sequence of chondrocyte differentiation through proliferating, hypertrophying and calcifying stages (Farquarson *et al.* 1991).

A high incidence of TD can be induced experimentally in a number of ways: for example, feeding diets with an altered cation:anion ratio or acid–base balance (Leach & Nesheim, 1972; Halley *et al.* 1987), fusarochromanone administration, or by feeding certain organic sulphur compounds (Vargas *et al.* 1983; Veltmann *et al.* 1985; Edwards, 1987). Edwards & Veltmann (1983) reported that feeding diets imbalanced in Ca–P could induce a high

incidence of TD as assessed by sectioning of bone extremities and gross examination of the growth plate. However, Riddell & Pass (1987), using histological techniques, found that chickens fed on diets containing high concentrations of P developed thickened growth plates identical to those seen in Ca-deficiency rickets before the development of typical TD.

An involvement of cholecalciferol metabolites in TD has been reported (Edwards, 1989, 1990). Using basal diets low in Ca (7·5 g/kg) and high in P (7·6 g/kg), the addition of 1,25-dihydroxycholecalciferol (1,25(OH)$_2$cholecalciferol) to a diet containing supplemental cholecalciferol in the presence and absence of disulfiram was found to result in a considerable increase in bone ash and a decreased incidence and severity of TD. However, in none of these experiments was this or any other metabolite of cholecalciferol found to prevent TD.

In these studies (Edwards, 1989, 1990) the occurrence of TD was assessed by visual examination of slices of PTT and identification of thickening of the growth plate. However, naked eye identification of TD is not so reliable as histopathological methods. This is because thickening of the growth plate can occur in the absence of cellular changes characteristic of TD (Thorp *et al.* 1991). Visual identification of TD lesions would be complicated further if rickets were also induced by the feeding of Ca–P imbalanced diets, as rickets will also cause thickening of the growth plate.

The aim of the present study was to carry out a detailed histological investigation of the effects on feeding 1,25(OH)$_2$cholecalciferol on the occurrence of TD and rickets in young broilers fed on diets containing an imbalance of Ca and P. Diagnosis of growth plate lesions was based on histopathology and biochemical characteristics of birds were also assessed.

MATERIALS AND METHODS

Birds and husbandry

Three experiments were conducted using groups of 1-d-old broiler chicks (Cobb strain; H. Morrison, Duns, Berwickshire) reared to 3 weeks of age in wire-floored electrically-heated brooder units. Food and water were available *ad lib.* and diets, based on wheat and soya-bean meal, were either a control diet calculated to contain (g/kg) Ca 12, P 6, or a TD-inducing diet calculated to contain (g/kg) Ca 7·5 and P 7·6 (Table 1). The TD-inducing diet was supplemented with various amounts of 1,25(OH)$_2$cholecalciferol, 0·2 g/kg in a stable matrix, kindly donated by Dr H. Weiser, Hoffmann–La Roche, Basle.

The aim of Expt 1 was to confirm, using histology, that diets imbalanced in Ca–P could induce a high incidence of TD, and to test the effects of giving birds extra cholecalciferol. Three groups of birds were used, the first receiving the control diet and the second and third groups receiving the TD-inducing diet containing either 25 or 75 μg cholecalciferol/kg.

Expt 2 assessed the effects of dietary supplementation of the TD-inducing diet with 1,25(OH)$_2$cholecalciferol. Three groups were again used, the first receiving the control diet, the second the TD-inducing diet, and the third the TD-inducing diet containing 10 μg added 1,25(OH)$_2$cholecalciferol/kg.

Expt 3 assessed the effects of supplementing the TD-inducing diet with various amounts of 1,25(OH)$_2$cholecalciferol, and consisted of a control group and four experimental groups receiving the TD-inducing diet supplemented with either 0, 2·5, 5, or 10 μg 1,25(OH)$_2$ cholecalciferol/kg. A further group was fed the last diet up to 7 d of age before being transferred to the unsupplemented TD-inducing diet.

At 3 weeks of age birds were weighed and blood samples were taken from the wing vein of ten to fifteen birds per treatment, using heparin as anticoagulant. Plasma was separated and stored at −20° until it could be analysed. After blood sampling, all birds were killed by cervical dislocation and the PTT was examined and scored visually for growth plate

Table 1. *Composition of experimental diets (g/kg)*

Ingredient	Control diet	Imbalanced diet
Wheat	559	563
Soya-bean meal	350	350
Vegetable oil	45	50
Limestone	13	—
Dicalcium phosphate	21	25
Salt	4	4
Methionine	2	2
Lysine	2	2
Vitamin and mineral supplement*	4	4
Calculated analyses (g/kg)		
Ca	12	7·5
P	6	7·6

* Provided (mg/kg diet): retinol 1376 μg, cholecalciferol 25 μg, α-tocopherol 10, menadione 1·3, thiamine 1·3, riboflavin 3·3, pyridoxine 1·7, cyanocobalamin 0·6 μg, folic acid 0·7, biotin 267 μg, pantothenic acid 5·3, nicotinic acid 23·3, zinc 40, copper 2·9, iodine 320 μg, iron 64, manganese 80, selenium 120 μg.

thickening. Sections of PTT were taken for histological examination to confirm TD and to characterize growth plate pathology.

Laboratory procedures

Total Ca, inorganic phosphate and alkaline phosphatase (*EC* 3.1.3.1) were measured using Wako kits (Alpha Laboratories, Eastleigh, Hants.) adapted for use with a plate-reader (Titertek Twin-Reader Plus; ICN, High Wycombe, Bucks.). Total Ca was measured in undiluted plasma, using 4 μl plasma, 200 μl buffer and 20 μl colour reagent. A standard curve was prepared over the range 0–5 mM by dilution with extra-pure water (Millipore, Watford, Herts.) of standards provided with the kit. Samples were read after 20 min at 560 nm. Inorganic phosphate was also measured in undiluted plasma, using 5 μl plasma and 125 μl mixed colour reagent. A standard curve was again constructed by dilution of standards provided with the kit over the range 0–100 mg/l, and samples read after 20 min at 690 nm. Alkaline phosphatase was measured in plasma diluted eighty times with extra-pure water. Samples were kept on ice until they could be assayed. A standard curve was prepared by diluting 5 mM-*p*-nitrophenol (Sigma, Poole, Dorset) in assay buffer to give a range of standards from 0 to 62·5 μM. Enzyme activity was measured by adding 10 μl diluted plasma to 250 μl substrate, and measuring absorbance at 405 nm for 10 min. The mean change in absorbance per min was calculated and related to *p*-nitrophenol concentration. Alkaline phosphatase activity was calculated after correction for dilution, and expressed as units (U). 1,25(OH)$_2$cholecalciferol was measured in plasma using an Incstar kit (Incstar Ltd, Wokingham, Berks.).

Samples of PTT were fixed in buffered neutral formalin before decalcification in formic acid–formalin. Paraffin embedded sections were then prepared and stained with haematoxylin and eosin and examined. TD was diagnosed when characteristic changes to the chondrocytes of the proliferating, pre-hypertrophied and hypertrophied zones were seen, including accumulation of avascular matrix and degenerative changes in cytoplasm and nuclei. Rickets was diagnosed where changes in the growth plate included a widening of the proliferating zone and disturbances in vascularity within the growth plate (Lacey & Huffer, 1982).

Statistical analyses

Data were analysed by analysis of variance using Genstat statistical program.

RESULTS

The results from the first experiment confirm that feeding diets imbalanced in Ca and P to birds can induce a high incidence of TD (Table 2). The control group had a 14% incidence of TD, as assessed histologically, compared with a 21% incidence of TD and a 14% incidence of rickets in the group fed on the imbalanced diet containing 25 μg cholecalciferol/kg, and a 46% incidence of TD and 0% incidence of rickets in the group fed on the imbalanced diet containing 75 μg cholecalciferol/kg. There were no significant differences between treatments in bird weights, or between plasma Ca, P or 1,25(OH)$_2$ cholecalciferol concentrations. Alkaline phosphatase measurements were unsuccessful in the first experiment as the samples were not sufficiently diluted.

The second experiment showed that addition of 1,25(OH)$_2$cholecalciferol to a diet imbalanced in Ca and P can reduce the incidence of TD. The control group had a 29% incidence of TD compared with 19% in the group fed on the imbalanced diet. The incidence dropped to zero in the group fed on the imbalanced diet supplemented with 10 μg 1,25(OH)$_2$cholecalciferol/kg. There was a significant difference in body weights between treatments, with the group receiving 1,25(OH)$_2$cholecalciferol having a significantly lower mean body weight than birds on the other two treatments. Again, there were no significant differences in plasma Ca, P or alkaline phosphatase between treatments.

The third experiment showed that the response to dietary 1,25(OH)$_2$cholecalciferol is influenced by dose, in that the group receiving no 1,25(OH)$_2$cholecalciferol had a 36% incidence of TD, and the groups receiving 2·5, 5, or 10 μg/kg had 19, 0, and 0% incidences respectively. In the present experiment there were no significant differences in body weights between treatments, although there was a trend towards lower body weight at the higher levels of supplementation of 5 and 10 μg 1,25(OH)$_2$cholecalciferol/kg. There were no significant differences in plasma Ca, P or alkaline phosphatase between treatments, although there was a trend towards hypercalcaemia with increasing dietary 1,25(OH)$_2$cholecalciferol. Feeding a diet imbalanced in Ca and P significantly increased mean circulating 1,25(OH)$_2$cholecalciferol levels in Expt 3 (although not in Expt 1) from 40 to 65 pg/ml. Concentrations increased from a mean of 49 pg/ml in birds receiving 2·5 μg 1,25(OH)$_2$ cholecalciferol/kg, to a mean of 67 pg/ml in birds receiving 10 μg 1,25(OH)$_2$cholecalciferol/kg.

DISCUSSION

These experimental results demonstrate that dietary supplementation with 1,25(OH)$_2$ cholecalciferol can prevent the occurrence of TD in broilers fed on a diet imbalanced in Ca and P. The present study extends the observations of Edwards (1989, 1990) who demonstrated in several experiments substantial decreases in the incidence of TD after dietary supplementation with this metabolite. The observations of complete prevention of TD at the higher supplementation rates in the present study are perhaps the result of the use of histology to make a specific diagnosis of TD in the presence of other growth plate abnormalities, including rickets, which were also found to occur with the experimental diets used.

The first experiment confirmed that the use of a diet imbalanced in Ca and P and containing 25 μg cholecalciferol/kg could give a higher incidence of TD than a control diet containing normal amounts of Ca, although this was not always the case in later experiments. That TD is not caused by a dietary deficiency of cholecalciferol *per se* was

Table 2. *Effects of different dietary calcium, phosphorus, cholecalciferol* (D_3) *and 1,25-dihydroxycholecalciferol* (1,25(OH)$_2$D$_3$) *concentrations on the growth, incidences of tibial dyschondroplasia and rickets, and plasma Ca and P in broilers*

(Mean values with their standard errors)

Expt no.	Ca (g/kg)	P (g/kg)	D$_3$ (μg/kg)	1,25(OH)$_2$D$_3$ (μg/kg)	Wt (g) Mean	Wt (g) SE	Wt (g) n	Percentage TD	Percentage rickets	Plasma Ca (mM) Mean	Ca SE	Ca n	Plasma P (mg/l) Mean	P SE	P n	Plasma AP (U) Mean	AP SE	AP n	Plasma 1,25(OH)$_2$D$_3$ (pg/ml) Mean	SE	n
1	12	6	75	0	476[a]	9·8	30	14	0	2·59[a]	0·19	15	72·2[a]	4·6	15				37[a]	3·4	8
	7·5	7·6	25	0	535[b]	13·2	30	21	14	2·99[a]	0·13	15	94·9[a]	6·0	15				42[a]	5·4	6
	7·5	7·6	75	0	533[b]	11·8	29	46	0	2·94[a]	0·16	15	83·7[a]	5·6	15				42[a]	4·9	10
2	12	6	25	0	647[a]	13·9	28	29	0	1·55[a]	0·06	25	57·6[a]	2·0	25	17329[a]	3093	25			
	7·5	7·6	25	0	650[a]	14·7	29	29	0	1·58[a]	0·06	17	53·6[a]	2·4	16	15080[a]	2274	17			
	7·5	7·6	25	10	552[b]	22·6	29	0	0	1·62[a]	0·06	26	60·6[a]	2·2	25	13136[a]	1877	26			
3	12	6	25	0	546[a]	15·5	17	0	11	2·18[a]	0·11	10	57·1[a]	2·1	10	17111[a]	1760	10	40[a]	4·0	10
	7·5	7·6	25	0	532[a]	19·1	17	36	0	1·90[a]	0·16	9	66·5[a]	2·9	9	17736[a]	2174	9	65[b]	7·8	9
	7·5	7·6	25	2·5	549[a]	9·7	16	19	0	2·07[a]	0·13	10	60·1[a]	2·5	10	18578[a]	2613	10	49[a]	5·6	9
	7·5	7·6	25	5	516[a]	17·9	19	0	0	2·19[a]	0·11	10	66·0[a]	3·0	10	17687[a]	3038	10	53[a]	5·6	9
	7·5	7·6	25	10	522[a]	23·8	16	0	0	2·37[a]	0·17	9	67·3[a]	2·9	9	13047[a]	2837	9	67[b]	7·4	9

AP, alkaline phosphatase (*EC* 3.1.3.1).

[a,b] Within a column, values having different superscript letters were significantly different ($P < 0·05$).

demonstrated by the fact that supplementation of the imbalanced diet to give a total cholecalciferol content of 75 μg/kg did not reduce the incidence of TD, although it did prevent rickets.

The second experiment showed that a supplement of 10 μg 1,25(OH)$_2$cholecalciferol/kg could prevent the TD induced in this model. However, there was a significant depression of body weight with this diet, and a lower rate of growth is known to be associated with a decreased incidence of TD (Huff, 1980). In broiler chicks fed *ad lib.*, growth accelerates rapidly from 2 weeks of age, with maximum allometric growth in the PTT occurring between 2 and 3 weeks of age (Thorp, 1988). Therefore, during this crucial period, any factor reducing growth rate will also reduce chondrocyte differentiation and, therefore, the likelihood of TD. The toxic effects of cholecalciferol metabolites were considered as a cause of the lowered body weight in this group, but as this result was not duplicated in the third experiment it was felt to be due instead to a factor outwith the experiment. The standard error of the mean for body weight was much higher in this group compared with other treatments.

The third experiment showed that a lower dose was also effective in reducing the incidence and severity of TD, and that dietary contents of 5 and 10 μg/kg were effective in preventing TD. In this experiment there was no significant growth depression at the higher supplemental levels. Plasma P and alkaline phosphatase were unaffected by supplementation whereas plasma Ca tended to be elevated, although the changes were not statistically significant. Circulating concentrations of 1,25(OH)$_2$cholecalciferol were elevated among birds given the TD-inducing diet compared with the control diet, but did not show any obvious response to dietary supplementation of the TD-inducing diet with 1,25(OH)$_2$ cholecalciferol. It is well known that Ca deficiency increases the renal synthesis of 1,25(OH)$_2$cholecalciferol (Cancela *et al.* 1988) and in a recent study (Goff *et al.* 1990) circulating concentrations achieved after dietary restriction were comparable with those seen after injection of 1,25(OH)$_2$cholecalciferol, both being approximately eight times normal. In the present study 1,25(OH)$_2$cholecalciferol was administered orally. Its efficiency of absorption is not known, but absorption must have occurred in view of the profound effect on the incidence of TD. However, plasma concentrations of 1,25(OH)$_2$ cholecalciferol are apparently not related to the development of TD.

It was also of interest in the third experiment that supplementation with 10 μg 1,25(OH)$_2$cholecalciferol/kg for the first week after hatching only did not prevent TD. This implies that supplementation is needed over the whole period when rapid bone growth is likely to make the growth plate more susceptible to TD. It also suggests that the problem does not originate at the breeder level, with inadequate carryover of cholecalciferol and its metabolites to the hatching chick.

It is clear from the experimental results that 1,25(OH)$_2$cholecalciferol is exerting a powerful biological effect in this TD model. *In vitro* studies have demonstrated receptors for 1,25(OH)$_2$cholecalciferol on chondrocytes (Suda *et al.* 1985) and the metabolite has been shown to have a powerful inhibitory effect on cell proliferation and a stimulatory effect on cell differentiation in chondrocytes derived from embryonic chicken caudal sterna (Gerstenfeld *et al.* 1990). A basis for the involvement of 1,25(OH)$_2$cholecalciferol, therefore, exists in this model of TD, either related to the supply of the metabolite to the chondrocyte and/or its effects on cellular activity. Soares *et al.* (1990) found a trend towards reduced numbers of 1,25(OH)$_2$cholecalciferol receptors in growth plate and intestine of TD-affected birds, but normal affinity for the hormone, as measured by Scatchard analysis. However, it is known from human studies (Marx, 1989) that defects related to cholecalciferol metabolism are complex, and may occur at every stage of the hormone acting on its target cell, from locating its receptor to binding to the nuclear DNA.

It is also possible that the observed response to 1,25(OH)$_2$cholecalciferol is mediated through a further metabolite of the hormone.

The role of 1,25(OH)$_2$cholecalciferol in preventing TD in this model is as yet uncertain, and its significance in field cases can only be the subject of speculation. Further *in vitro* and *in vivo* studies are necessary to elucidate the mechanism by which broilers develop TD, and to clarify the role of cholecalciferol metabolism in the occurrence of this important condition.

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RENAL 25-HYDROXYVITAMIN D-1α-HYDROXYLASE ACTIVITY FOLLOWING UNINEPHRECTOMY IS REDUCED IN AGED C57BL6J MICE. C.W. Bales, K.L. Currie*, P. Lin*, and M.K. Drezner, Duke University Medical Center, Durham, NC 27710.

Evidence of age-related changes in vitamin D metabolism, including reductions in calcium absorption and serum $1,25(OH)_2D_3$ (calcitriol) levels, have raised concerns regarding the regulation of calcium homeostasis in elderly individuals who donate or receive a kidney transplant. Thus, we examined the capacity of the aged kidney to undergo metabolic/endocrine adaptation. In these studies we compared calcium homeostasis in young (aged 2 mos) and old (aged 18 mos) C57BL6J mice at various times following uninephrectomy. We measured renal 25(OH)D-1α-hydroxylase activity (in the remaining kidney) and serum calcium and $1,25(OH)_2D_3$ levels according to previously described techniques. Our data indicate that in contrast to young mice, which show approximately a 60% increase ($P<0.01$) in 25(OH)D-1α-hydroxylase activity at 1 day following surgery (9.86 ± 1.43 versus 6.21 ± 0.56 fmoles/mg/min in sham-operated controls), enzyme levels in aged mice are unchanged at the same timepoint (5.53 ± 0.70 fmol/mg/min in treated mice versus 6.14 ± 0.58 in shams). Moreover, while serum calcium levels do not change in either animal model (8.95 ± 0.12 vs 9.08 ± 0.16 mg/dl in young shams versus treated; 9.21 ± 0.15 vs 9.19 ± 0.41 mg/dl in aged shams versus treated), serum calcitriol levels are lower ($P<0.05$) in aged than in young animals at 8 days post-uninephrectomy (13.4 ± 1.8 vs 21.2 ± 1.3 pg/ml; $p<0.01$), which is the half-life of serum $1,25(OH)_2D_3$. We conclude that the ability of the aged kidney to restore normal vitamin D metabolism following uninephrectomy is impaired. These results may have important implications for the pathophysiology and treatment of elderly kidney donors and isograft recipients.

AFFINITY LABELLING OF 1,25-DIHYDROXYVITAMIN D_3 RECEPTOR. R. Ray*, S. Ray*, P.N. MacDonald, M.R. Haussler and M.F. Holick, Vitamin D Laboratory, Boston University School of Medicine, Boston, MA 02118, and Department of Biochemistry, The University of Arizona Health Sciences Center, Tucson, AZ 85724.

The structure of 1,25-dihydroxyvitamin D_3 receptor (VDR), particularly in relation with its function, has been of great interest for many years. Recently the cDNA-derived amino acid structures of human and avian VDRs have been determined, and it has been shown that VDR molecule contains separate cysteine-rich hormone and DNA-binding domains. However, these molecular domains are quite large, and little information is currently available to obtain a more focussed view of these segments of the VDR molecule.

In the past efforts to covalently label VDR by photo-affinity labelling have met very limited success and future applicability due to low efficiency of labelling. Recently we have synthesized an affinity analog of 1,25-dihydroxyvitamin D_3 ($1,25\text{-}D_3\text{-}X$) containing a reactive group capable of forming covalent bond with VDR. This analog (a) competed with $1,25(OH)_2D_3$ for the binding of the latter to chick intestinal VDR in a dose-dependent manner, and (b) blocked, upon incubation with chick intestinal cytosol, the binding of $^3H\text{-}1,25(OH)_2D_3$ to VDR. When an extract from Sf-9 cells, infected with VDR recombinant baculovirus, was incubated (0°C/3 hr) with $^3H\text{-}1,25\text{-}D_3\text{-}X$, the radiolabelled version of $1,25\text{-}D_3\text{-}X$, a single protein band (52 KDa), which cross-reacted with monoclonal antibody against human VDR, was labelled. No labelling was observed in a control experiment with cells infected with wild-type baculovirus. These results confirm covalent labelling of VDR molecule with $^3H\text{-}1,25\text{-}D_3\text{-}X$. Hence, we describe here, for the first time, development and successful application of an affinity analog of $1,25(OH)_2D_3$.

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THE EFFECTS OF LATITUDE, SEASON, AND TIME OF DAY ON THE SYNTHESIS OF PREVITAMIN D_3. T.C. Chen*, Z. Lu*, L. Kline*, T. Markestad*, M Ladizesky*, C. Mautalen*, and M.F. Holick, Boston University, Boston, MA, USA, University of Alberta, Edmonton, Alberta, Canada, Haukeland Hospital, Bergen, Norway, University of Witwatersrand, Bertsham, South Africa, and Hospital De Clinicas, Buenos Aires, Argentina.

The amount of solar UVB reaching the earth depends on the solar zenith angle and, therefore, is influenced by latitude, season, and time of day. To investigate their effects on the cutaneous production of previtamin D_3 ($preD_3$), we employed a model with 7-dehydrocholesterol (7-DHC) in ethanol sealed in ampules under argon to determine the sunlight conversion of 7-DHC to $preD_3$ on an hourly basis once a month in Boston (42°N) and once a month in six other locations at different latitudes in both hemispheres. Our results indicate that in the midsummer, more than 1% of $preD_3$ was produced before 8:30 am and after 3:30 pm. In the northern hemisphere, the maximal conversion was found at noon time in June and July. Very little or no conversion was found in the months of December and January in Boston. This period is extended from October through March in Bergen (61°N). In the southern hemisphere, no conversion of 7-DHC to $preD_3$ occurred in the months of April through July in Ushuaia (55°S). In summary, the results demonstrate that (1) the dramatic influence of changes in solar UVB radiation on the cutaneous vitamin D_3 synthesis, (2) exposure to natural sunlight for a short period of time in the morning or mid afternoon in the summer months is sufficient for adequate cutaneous synthesis of preD and (3) the latitudinal increase in the length of the "vitamin D winter" during which dietary supplementation of the vitamin may be advisable.

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THE EFFECT OF 1,25 DIHYDROXYCHOLECALCIFEROL ON TIBIAL DYSCHONDROPLASIA IN BROILER CHICKENS. JS Rennie, B Thorp and CC Whitehead (Intr. by SJ Robins). AFRC Institute of Animal Physiology and Genetics Research, Roslin, Midlothian, EH25 9PS, Scotland.

Four experiments were carried out to study the effect of supplementing diets with various amounts of 1,25 dihydroxycholecalciferol ($1,25(OH)_2D_3$) on the incidence of tibial dyschondroplasia (TD) in 3 week old broiler chicks. Chicks were fed diets from hatching to 3 weeks of age, blood sampled and killed. All proximal tibiotarsi were examined histologically. Plasma total Ca, inorganic P, and alkaline phosphatase were measured colorimetrically, and $1,25(OH)_2D_3$ using IncStar kits.

The first experiment showed that feeding a diet imbalanced in Ca and P (7.5 g/kg Ca and 7.6 g/kg P) resulted in a higher incidence of TD than a diet containing normal amounts of Ca and P (12 g/kg Ca and 6 g/kg P) (21% vs. 14%). In the second experiment, chicks fed the imbalanced diet supplemented with 10 μg/kg $1,25(OH)_2D_3$ did not develop TD unlike chicks fed the unsupplemented imbalanced diet (0% vs. 29%). In the third experiment 2.5μg/kg lowered the incidence (19%) and 5 and 10 μg/kg prevented TD (both 0%) compared to chicks fed the unsupplemented imbalanced diet (36%). In the final experiment, the effect of supplementing a normal diet with $1,25(OH)_2D_3$ was investigated. 5μg/kg completely prevented TD in chicks fed the normal diet (0% vs. 41%) as well as the imbalanced diet (0% vs. 42%).

In all the experiments plasma total calcium, inorganic phosphate and alkaline phosphatase were not significantly affected by treatment. In the third experiment dietary supplementation with $1,25(OH)_2D_3$ did not affect plasma levels of the metabolite. Feeding the diet imbalanced in Ca and P tended to raise plasma $1,25(OH)_2D_3$ as shown in the first (42 vs. 37 pg/ml) and third (65 vs. 40 pg/ml) experiments.

It is concluded that supplementing diets with $1,25(OH)_2D_3$ has a potent effect on the incidence of TD. In vitro studies have shown that chondrocytes possess receptors for $1,25(OH)_2D_3$ and also that the metabolite appears to have a powerful inhibitory effect on cell proliferation and stimulatory effect on differentiation. It may be that TD is a result of failure of chondrocyte hypertrophy due to lack of endogenously synthesised metabolite or failure of the metabolite to act at cellular or even receptor level.

dyschondroplastic and non-dyschondroplasic tibias in males of a breeding flock (ANOVA, $P = 0.03$, correlation $r = 0.23$, $n = 289$, $P = 0.01$).

Rotation was constant during 10 to 22 weeks in breeding males. In week 16, it was 37.8° ± 11.9°, $n = 102$. Rotation was not associated with dyschondroplastic lesions (ANOVA). In females, axial rotation was the same as in male and did not vary with age (43.3° ± 10.9°, $n = 100$, in week 16).

Effects of dietary element level on tibial dyschondroplasia in chicks fed Chinese practical diets

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Two experiments were conducted to assess the effects of varying dietary levels of magnesium, chloride and non-phytate phosphorus on the performance, occurrence of tibial dyschondroplasia (TD) and tissue element contents of broilers fed Chinese practical maize-soyabean meal diets. Two levels of dietary chloride (1.5 and 3.5 g/kg) and three of magnesium (2, 4 and 6 g/kg) were fed in a factorial design in Expt. 1. In Expt. 2, there two levels each of chloride (1.5 and 3.5 g/kg), magnesium (3 and 6 g/kg) and non-phytate phosphorus (4.5 and 9 g/kg) in a 2 × 2 × 2 factorial. Both experiments lasted for 21 days.

The results suggested that the maize and soyabean meals produced in northeastern China may predispose broilers to TD. High dietary chloride and/or non-phyate phosphorus levels induced a high incidence of TD ($P < 0.05$) which could be lowered by dietary magnesium supplementation, but at the cost of decreased growth rate. Dietary magnesium plays an anti-rachitic role by improving the bioavailability of copper and zinc and improving the deposition of these elements in bone. The optimum combination of 6 g magnesium, 3.5 g chloride and 4.5 g non-phytate phosphorus/kg diet is recommended for broiler diets in northeast China.

Responses of tibial dyschondroplasia to dietary supplementation with $1{,}25(OH)_2$ vitamin D as assessed by histopathology and gross examination

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In a series of experiments, Edwards (1989, 1990) has shown that adding $1{,}25(OH)_2$ vitamin D_3 to diets containing an imbalance of Ca/P can decrease the incidence and severity of tibial dyschondroplasia (TD). As has been common in many studies, assessment of TD was based on examination of the proximal tibiotarsus with the naked eye. However TD can be difficult to distinguish visually from rickets and other growth plate defects.

In the present study, 3 experiments were carried out to study the effects of dietary Ca/P imbalance and supplementation with $1{,}25(OH)_2D_3$ on the occurrence of TD as assessed both visually and histologically. The control diet contained 12 g Ca and 6 g P/kg, the imbalance diet had 7.5 g Ca and 7.5 g P/kg and both diets contained 25 µg D_3/kg. Broilers fed these diets up to 3 weeks of age showed incidences

(30–75%) of accumulations of growth plate cartilage that grossly resembled TD. However histological examination revealed that several different lesions were involved that could be classified as TD, hypocalcaemic rickets or accumulations of hypertrophied chondrocytes not characteristic of TD (Thorp *et al.*, 1991).

Supplementing the Ca/P imbalanced diet with graded levels of $1,25(OH)_2D_3$ resulted in a progressive decline in growth plate abnormalities. Histology revealed that supplements of 5 and 10 µg $1,25(OH)_2D_3$ had completely prevented TD and rickets but not the third abnormality. Supplementing with vitamin D_3 itself prevented only rickets. These studies show that histology is needed for an accurate diagnosis of TD and that when this technique is used, supplemental dietary $1,25(OH)_2D_3$ can be found to have prevented completely the TD induced by diets with imbalanced Ca/P.

It is thus apparent that $1,25(OH)_2D_3$ is playing a powerful rôle in preventing the failure of chondrocyte hypertrophy that is the cause of TD. The problem is not caused by a lack of vitamin D itself, since supplementation with this vitamin did not affect TD. It was curious that plasma levels were not elevated in response to dietary supplementation with $1,25(OH)_2$ vitamin D. The rôle of this metabolite in preventing TD is thus uncertain, but may be related to turnover, cell reception or conversion of other metabolites.

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Studies on the effect of strain, calcium, and fasting on growth, tibial dyschondroplasia, and plasma $1,25(OH)_2D_3$ and $25(OH)D_3$ in 16-day old male chickens

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Four experiments were conducted on the effects of strain, calcium, and 8-hour fasts on weight gain (WG), food efficiency (FE), tibia bone ash (BA), tibial dyschondroplasia (TD), and plasma $1,25(OH)_2D_3$ and $25(OH)D_3$ (Expts. 1 & 3). Five strains (Athens-Canadian Randombred (ACR) Single-Comb White Leghorn (SCWL) Peterson × Hubbard (PH), Ross × Arbor Acre (RAA), and Peterson × Arbor Acre (PAA) were used. Experiment 1 used all five and Expts. 2–4 used the three broiler strains. The strains in Expts. 1, 2, and 4 were fed TD-inducing basal diets. In Expt. 3 the birds were fed 6.0 or 9.5 g calcium/kg and in Expt. 4 the birds were fed the basal diet and were either fasted or not fasted. In Expt. 1 the ACR and SCWL chicks had poorer WG and FE that the three broiler strains and a lower incidence and severity of TD and higher $25(OH)D_3$. The PH chicks had a lower WG and a higher severity of TD than the other two broiler strains. There was no strain effect on $1,25(OH)_2D_3$. In Expt. 2 the PH birds had a lower WG and a higher incidence and severity of TD. The RAA birds had a higher BA than the other two strains. In Expt. 3 WG, FE, and $25(OH)D_3$ were not