THE UTILIZATION OF PHYTATE PHOSPHORUS BY CHICKS

by

ABDULLAH ABED AL-MONAM MOHAMMED

A Thesis Submitted to the

University of Southampton

for the Degree of

Doctor of Philosophy

Department of Nutrition
School of Biochemical and Physiological Sciences

July 1987

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE - DEPARTMENT OF NUTRITION

Doctor of Philosophy

THE UTILIZATION OF PHYTATE PHOSPHORUS BY CHICKS

by Abdullah Abed Al-Monam Mohammed

The effect of variations in dietary vitamin D_3, calcium and phosphorus on the utilization of phytate phosphorus for bone development and growth in chicks was studied. At normal intakes of calcium and vitamin D_3, reduced dietary levels of total phosphorus and increased percentage of dietary phosphorus as phytate phosphorus, led to a marked reduction in growth and bone mineralisation and induced a marked hypercalcaemia and hypophosphataemia. When a high level of vitamin D_3 was added to a low phosphorus diet, high levels of plasma $1,25(OH)_2D_3$ were associated with increased intestinal alkaline phosphatase, increased phytate digestibility, increased growth and increased bone mineralization. The digestibility of phytate was found to be influenced by a combination of intestinal phosphatases and dietary phytase activities and non-enzymatic gizzard hydrolysis. No role for gut microflora in phytate digestibility was found.

When the level of calcium was lowered in low phosphorus diets, and the level of vitamin D_3 was kept at the recommended level, phytate phosphorus digestibility was dramatically increased without any effect on circulating levels of $1,25(OH)_2D_3$. Thus low calcium diets may help to maintain plasma phosphorus at normal level by reducing the extent of formation of insoluble calcium-phytate complexes in the small intestine. The effect of calcium on bone mineralisation, was less than that achieved with vitamin D_3 while the improvement of the hypophosphataemia was better using a low calcium rather than high a vitamin D_3 diet. The most dramatic effects were seen when both low-calcium diets, and high-vitamin D_3 diets were fed. This led to maximal phytate phosphorus utilisation, correction of the hypophosphataemia and hypercalcaemia, and to maximal bone development and growth. Thus it is possible to remove all inorganic phosphorus supplements from the diet of chicks provided that the levels of calcium are reduced and the levels of vitamin D_3 are increased.

ACKNOWLEDGEMENTS

It is with great sincerity that I wish to express grateful appreciation to the former Head of the Department of Nutrition, the late Professor T.G. Taylor, for his encouragement and guidance throughout the period of this study. Professor T.G. Taylor guided the research program since the beginning and he was always a source of advice and encouragement even from his bed of illness.

I should like to express my condolences to his Wife and family for the great loss. He was a great scientific man, who devoted all his life to his work and will be sadly missed by his friends, and particularly his students, who will always remember him with great respect.

My deep gratitude is due to Dr.M.J. Gibney, who supervised the first year of this project, to whom I owe a considerable debt for the continuous encouragement, valuable ideas and help throughout the preparation of the manuscript.

My appreciation is expressed to my Supervisor Dr.R. F. Grimble, for his effort, valuable suggestions and guidance throughout my study. My thanks to Professor A. Jackson for the excellent research facilities and to Dr.D.A. York and all the staff of the Nutrition Department for their useful suggestions and couragement in my work.

I am deeply indebted to Dr. D. Hewitt and his colleagues (Food Research Institute, Shinfield, Reading) for providing the germ-free facilities and help throughout the experiment. My thanks also go to Mr. C. Bunce and Mrs. Ethel Mead for the supply and care of my chicks.

Thanks are also due to Dr. A. Hart, Dr. N. Saleh, Dr. P. Williams and Dr. C. Johner for advice and assistance on numerous occasions.

Special thanks to Mrs. Pam Dawson for her speedy and accurate typing of this thesis.

Many thanks are due to the University of Basrah for giving me the opportunity to do this degree study. The financial support from the ORS is highly acknowledged.

Finally, my immeasurable gratitude is due to my family in general, and my wife, Eman, in particular, for their tremendous patience and support.

TO THE MEMORY OF MY FATHER

LIST OF ABREVIATIONS

S.E.M.	Standard Error of the Mean
T.L.C.	Thin Layer Chromatography
H.P.L.C.	High Pressure Liquid Chromatography
h	hour
g	gram
l	liter
d	days
^{o}C	degrees centrigrade
$\pm$	mean
d.p.m.	disintegrations per minute
cAMP	cyclic adenosine -3'-5'-monophosphate
$^{3}[H]$	Tritium
NADP	Nicotinamide Adenine Dinucleotide Phosphate
i.u.	international units
Kg	Kilogram
m	milli
μ	micro
n	nano
p	pico
f	femto
M	molar
mol.	mole
V	volume
%	percentage
u.v.	ultra violet
PTH	parathyroid hormone
DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid
ATP	adenosine triphosphate
PG	Picogram

CONTENTS

PAGE NO

LIST OF TABLES

PAGE NO

LIST OF FIGURES

PAGE NO

PAGE NO

CHAPTER ONE

REVIEW OF RELEVANT LITERATURE

1.1 Phosphorus

Phosphorus is a non-metallic chemical element that plays a varied and important role in the chemistry of living organisms. It was first prepared in the free state in 1669 by Brandt, a German chemist, and first recognized as an essential constituent of bones by Gahn, a Swedish chemist, in 1769.

Although phosphorus was discovered in 1669, it was not until the mid 1800s that workers associated a dietary deficiency of phosphorus with certain symptoms of disease in animals. It was observed that animal diets could be deficient in mineral elements due to the inadequacies of the soil from which the feed was produced. Ewing, (1963) and Scott et al. (1982) reported that phosphorus is a mineral element which is required in large quantities by chicks, due to the need for phosphorus in bone formation and for the maintenance of a healthy skeleton.

In bone formation, phosphorus combines with calcium to form the mineral hydroxy apatite. When the apatite is deposited in adequate quantities, the bones possess the structural rigidity required for normal strength. Phosphorus is also involved in the storage, liberation and transfer of energy, in the maintenance of the acid-base balance of body fluids, and in the formation of phospho-protein. It is also an important part of the nucleic acids, DNA and RNA and is present in many coenzymes. Of the total phosphorus in the body, about 80% is present in the bones and about 20% in the soft tissues.

A deficiency of phosphorus in the diet of young growing chicks results in an abnormal skeletal development, known as rickets. One of the most noted symptoms of a phosphorus deficiency is retarded growth. This symptom has been reported by

many workers (Gardiner, 1969; Waldrop et al. 1974 and Scott et al. 1982). Most of these workers reported that a deficiency of phosphorus resulted in a reduction of the percentage of ash in the tibia, poorer feed efficiency, increasing mortality and decreased plasma phosphorus levels (Gonnerman et al. 1975; Ramp et al. 1974, Summers and Moran, 1972).

1.1.1 Phosphorus Requirement of Chicks

Most studies of the phosphorus requirement of chicks have analysed growth rate, percentage bone ash or the composition of the bone ash as the primary criteria for determining the requirement of phosphorus. The commonest form of expressing the phosphorus requirement has been as "inorganic phosphorus".

The National Research Council in the United States (1977) has suggested that the diet of the chick should contain 0.7% total phosphorus from 0-8 weeks of age. They have also suggested that at least 0.5% of the total phosphorus fed to the growing chick should be inorganic phosphorus. In proposing these figures, they have assumed that all of the phosphorus of non-plant feed ingredients may be considered to be inorganic, and that approximately 30% of the phosphorus of plant products is non-phytate phosphorus and may be considered part of the inorganic phosphorus requirement.

One of the most important single factors responsible for the variation in requirement of phosphorus is the metabolizable energy (ME) content of the diet.

The Agriculture Research Council of the United Kingdom (1975) recommended that the average level of inorganic phosphorus required, either for maximum growth or maximum percentage of bone ash should be 1.56 g inorganic phosphorus/M cal ME (0.37g/MJ). This corresponds to 4.7 g inorganic phosphorus/kg diet containing 3.0 Mcal ME/kg (12.6 MJ/kg).

The level of calcium in the diet can influence the phosphorus requirement.

Jensen and Edwards (1980) reported that a level of 0.8% total phosphorus and 0.7% calcium were required in the diet to produce maximum bone ash in broiler chicks, while 0.9% total phosphorus and 1.2% calcium were required to produce maximum bone ash in Leghorn chicks (layer). Paterson et al. (1978) showed that a level of 0.65% total phosphorus or 0.4% inorganic phosphorus for males and slightly lower levels for females, was adequate to support maximum growth and bone ash. Finally, Scott, et al. (1982) recommended that the diet of growing chicks contains only 0.45% inorganic phosphorus.

1.1.2 Phosphorus Sources for the Growing Chicks

In most commercial diets approximately one-third of the available phosphorus requirement is provided by feedstuff ingredients, the rest of it coming from supplementing the diet with mineral feed grade phosphates.

It has been recognised that inorganic phosphorus sources differ in their biological availability for the young chick. The chemical form of the phosphate molecule has a crucial influence on the availability. The phosphate must be in the ortho (PO_4^{-3}) form to be well absorbed and utilized by the chicken. The availability of phosphorus mineral has generally been estimated using biological methods. Standard and test diets are made-up by adding different phosphate materials to a phosphorus-deficient basal diet. In most cases, the body weight, the percentage ash in the fat-free dry tibia and the ash mineral contents, are determined.

In all these experiments the biological value of the standard diet was arbitrarily set at 100. Gillis et al. (1954) conducted an outstanding study on the availability for chicks of phosphorus

from various sources. The results of their study were used, together with reports of Damron and Harms (1970); McGillivray (1978) and Taylor (1979) for the evaluation of phosphorus presented in Table 1. These results show that phosphorus from orthophosphates, such as the sodium-, potassium-, ammonium- and monocalciumphosphates have the highest availabilities, giving biological values between 90 and 110.

The phosphorus from raw rock phosphates, soft phosphates, defluorinated rock phosphates and pyro-phosphates have lower biological values (between 30 and 90). Inorganic phosphorus, present in the rock phosphate, is relatively unavailable to chicks unless these rock phosphates are heat treated in such a way as to convert the native rock phosphates into available forms, such as mono- and tri-calcium phosphates. The dangers of using raw rock phosphates as a phosphorus supplement for chick feeds is that it could provide toxic levels of certain elements such as fluorine and vanadium.

The defluorination of rock phosphates has a positive effect and the biological value may increase to an average of about 85 with a large range, (Simons, 1979). Said, et al. (1979) used 0, 0.6, 1.2 and 1.8% raw rock phosphate diets; the fluorine levels added by the rock phosphate were 0, 216, 432 and 648 ppm for the four diets respectively. They found that the fluorine content of the diet had no significant effect on body weight, feed consumption, feed conversion and mortality of the chicks in early age. However, growth depression and reduced feed efficiency were observed amongst chicks on the highest dietary fluorine (648 ppm) at 20 weeks of age. Wozniak et al. (1977) found that the chicks fed the basal diet supplemented with dicalcium-phosphate produced significantly greater bone ash than when defluorinated rock phosphate was used. The biological availability of the phosphorus in these two feed phosphates was 93% of the dicalcium phosphate diet and 75% in the defluorinated rock phasphate diet. Soares et al. (1978) observed that the youngest age group of chicks (0 to 3

TABLE 1.1 : BIOLOGICAL VALUE OF INORGANIC PHOSPHORUS FOR THE CHICK

	biological value
ortho phosphates $(PO_4)^{-3}$	
Beta-tricalcium phosphate (anhydrous)	100
Dicalcium phosphate (hydrated)	110
Dicalcium phosphate (unhydrated)	90
Monocalcium phosphate (hydrated)	113
Dipotassium phosphate	108
Monosodium phosphate	106
Disodium phosphate	107
Monoammonium phosphate	95
Diammonium phosphate	117
Defluorinated rock phosphates	
Precipitated	90
Calcined	85
Fused	75
Raw rock phosphates	
Curacao Island phosphate	50-75
Tennessee brown rock	25
Low fluorine rock	45-60
Florida pebble	40
Soft rock	20-30
Polyphosphates	
Sodium tripolyphosphate	75-80
Ammonium polyphosphate	96
Pyrophosphate	
Sodium pyrophosphate	57
α, β and γ calcium pyrophosphates	0
Calcium acid pyrophosphate	60
Bone products	
Steamed bone meal	96
Bone ash	89
Metaphosphates $(PO_3)^{-3}$	
β and γ calcium metaphosphate	0
Sodium metaphosphate	2
Potassium metaphosphate	0
Vitreous calcium metaphosphate	45
Calcium phytate	0

weeks) appeared to completely utilize the phosphorus from defluorinated phosphate but the older chicks could utilize only 82-90% of this phosphorus.

It has been recognized for some time that biological availability of a given phosphate may be influenced by the composition of the diet in which it is fed. Scott, et al. (1962) reported that soybean meal would increase the bone ash of turkey poults fed certain diets containing inadequate levels of calcium phosphate. Anderson et al. (1971a) found that the inclusion of 3% alfalfa meal in the basal diet caused an increase in body weight and tibia ash. However, these findings are not explained by the phosphorus content alone. It was suggested that alfalfa meal might contain a factor that promotes phosphorus utilization from Curacao Island phosphate. Griffith, (1968) determined that the inclusion of ground soybean hulls in a purified diet improved the bone ash resulting from either calcium phosphate or Curacao Island phosphate.

1.2 Phytate

Since 1973, the price of mineral feed phosphorus has more than doubled. Consequently inorganic phosphorus is the most expensive mineral supplement in chick diets.

It is generally recognized that the commercial chick diet may have to increasingly rely on non-conventional sources for its phosphorus supply. It appears inevitable that a greater emphasis will be placed on using phosphorus from plant phytates. A minor part of the total phosphorus in plants is inorganic and thus available for the chicks. The major part, approximately 70%, consists of phytate phosphorus.

Much of the dietary phosphorus at present comes from supplements of inorganic phosphorus, but by increasing the proportion of phytate phosphorus hydrolysed cheaper cereal diets would be possible as less of the costly phosphorus supplement would have to be added to the diets.

1.2.1 Structure and Nomenclature of Phytate

Myo-inositol hexaphosphate or phytic acid has been the subject of several reviews (Nelson, 1967; Cheryan et al. 1980 and Maga, 1982).

In (1855-1856) Hartig isolated small particles or grains from the seeds of various plants. Pfeffer (1872) placed the grains described by Hartig into the following three categories:-

1 Crystals of calcium oxalate
2 Protein substance
3 Compound giving no reaction for protein, fat and inorganic salts, named globoides.

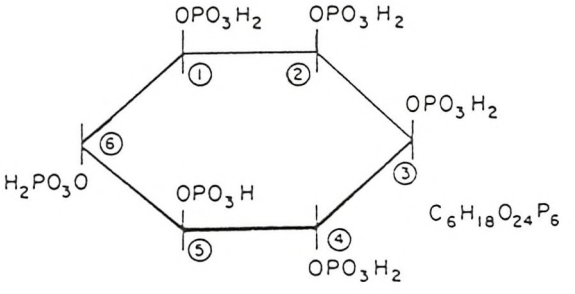
Palladin (1894), while studying the protein in the seeds of Indian mustard, obtained a non-nitrogenous substance, which was rich in phosphorus and also contained some calcium and magnesium. Schulze and Winterstin (1896) suggested that this substance was identical with Pfeffer's "globoid" particles. Winterstin (1897) proposed that the substance was inositol phosphoric acid since it yielded inositol and phosphoric acid on hydrolysis.

Posternak (1904, 1921) conducted extensive studies on the structure of this substance and proposed that it was an hydro-oxymethelene diphosphoric acid. He considered that the inositol arose from the hydrolysis of phytin. Strong support for the view that inositol was present in the original substance was obtained by Suzuki, et al. (1907).

The structure of phytic acid had been the subject of controversy. The controversy has centered around the structure proposed by Anderson (1914) (Figure 1.1A) and the structure suggested by Neubery (1908) (Figure 1.1B).

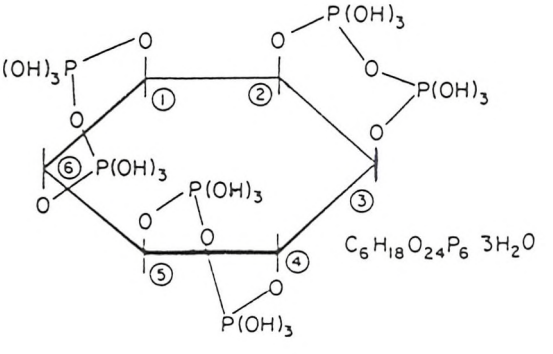
FIGURE 1.1 : COMPARISON OF ANDERSON (1914) AND THE NEUBERY (1908) PROPOSED STRUCTURES OF PHYTIC ACID

A



ANDERSON STRUCTURE

B



NEUBERG STRUCTURE

The controversial issue has been the isomeric conformation of the phosphate groups within the compound and whether three strongly bound water molecules were incorporated into the structure, which was distinguished mainly by having three P-O-P linkages between pairs of adjacent phosphates. Potentiometric titration of crystalline sodium phytate shows the presence of six strongly dissociated protons and six weakly dissociated protons (Hoff-Jorgenson et al. 1944). These results support the Anderson structure for phytic acid.

Brown et al. (1961) reported that six more hydrogens (to total 18) were titratable in aqueous and non-aqueous media. These last six hydrogens were too weakly acidic to be originally titratable in water. This work supports the Neubery structure as the correct one.

Although additional support for each structure has been published, most evidence points to the Anderson model (Cosgrove, 1966; Blank et al. 1971 and Costello et al. 1976) as the predominant form in plant materials. However, other additional evidence using nuclear magnetic resonance (Johnson and Tate, 1969) and x-ray crystal analysis (Blank et al. 1971), left little doubt that the structure proposed by Anderson was in fact the one found in plant seeds.

The term "phytin" was first employed by Posternak, (1903), to describe the major organic phosphate component of plant seeds and was determined to be inositol hexaphosphate.

The name "phytic acid" has been used interchangeably in the literature with the term "phytin" which more correctly refers to the mixed calcium and magnesium salt of the acid (Posternak, 1965). As noted by Oberleas (1973) and IUPAC-IUB (1968), the proper chemical designation for phytic acid is "myoinositol 1,2,3,4,5 and 6-hexa .(dihydrogen phosphate)", which is based on the cyclitol nomenclature and is presently utilized in some abstract and reference literature. This nomenclature could become

rather unwieldly in a discussion of this kind. Therefore, for simplicity, the term "phytate" is used as a general term.

1.2.2 Phytate Biological Function

Phytate occurs extensively in plant seeds, including cereals and oil seeds (Cosgrove, 1966). The physiological role of phytate has been described:

A) - Storage of phosphorus:

Phytates are considered the chief storage form of phosphate and inositol in almost all seeds (Hall et al. 1966 and Williams, 1970); the phytate molecule has six phosphate groups, and is so rich in phosphorus that 100g of the phytate contains over 28g of phosphorus. It has been generally assumed that phytate is utilized as a source of phosphorus at the germination (Asada et al. 1969).

B) - Storage of energy:

Biswas and Biswas (1965) confirmed the production of ^{32}P-guanosine triphosphate (^{32}P-GTP). After hydrolysis of germinating mung beans with 1 N HCl at 100°C for 7 minutes, more than 85% of the radioactivity is liberated as inorganic phosphate. The reaction may be written as follows:

$$\text{Inositol hexaphosphate} + \text{GDP} \xrightarrow[\text{-photransferase}]{\text{phosphoinositol phos-}} \text{Inositol pentaphosphate} + \text{GTP}$$

1.2.3 Distribution of Phytate Phosphorus in Feedstuffs

The distribution of phytate phosphorus in feedstuffs has been investigated by numerous research workers (McCance and Widdowson, 1935; Common, 1940; Temperton and Cassidy, 1964; Nott, 1967 and Nelson et al. 1968). Total phosphorus and phytate contents of cereals, legumes and other oil seeds are presented in Table 1.2.

TABLE 1.2: TOTAL PHOSPHORUS (T.P.) AND PHYTATE PHOSPHORUS CONTENT IN COMMON INGREDIENTS OF CHICKEN FEEDS

feed stuffs	Total phosphorus %	Phytate phosphorus %	Phytate P /T.P
Barley irrigated	0.42	0.25	60
Maize meal	0.3	0.2	67
Cotton seed meal dehulled	1.2	0.85	71
Oat meal	0.4	0.3	75
Rice polishings	1.4	1.24	88
Rice bran	1.5	1.29	86
Sesame meal	1.5	1.2	80
Soybean, unextracted	0.6	0.35	58
Sunflower seed meal	1.0	0.7	70
Wheat	0.4	0.27	68
Wheat bran	1.1	0.96	87
Wheat middlings	0.9	0.67	74

Cereal grains were shown to contain from 0.2 to 0.3 percent phytate phosphorus which constituted from 67% to 75% of the total phosphorus present. Cereal by-products such as wheat middlings, wheat bran and rice bran contained considerably higher levels from 0.67 to 1.29 percent phytate phosphorus which represented 74% to 87% of the total phosphorus. Oil seed by-products such as soybean oil meal and cotton seed meal were shown to contain from 0.35 to 1.2 percent phytate phosphorus which represented 58% to 80% of the total phosphorus.

In many cases, the phytate content is not considered to be absolute and may vary depending upon the variety and/or cultivar, climatic conditions, locations, irrigation conditions, type of soil and year during which the crop is grown. Miller et al. (1980) evaluated phytate levels in oats as influenced by variety, year and location of growth. Bassiri and Nahapetian (1977) observed that the wheat varieties grown under dry land conditions, had lower concentrations of phytate than the ones grown under irrigated conditions. Asada et al. (1969) demonstrated that the amount of phytate formed in rice grains is affected by the amount of phosphate available to the plant, and then postulated that excess phosphate could be stored in the form of phytate. Thus, excessive phosphate fertilization could result in high phytate levels.

Phytate concentration varies between different morphological components or parts of cereal grains, the endosperm of rice and wheat kernels is almost devoided of phytate, while the germ contains appreciable amounts of phytate. However, the major portion of phytate is in the bran. In rice, of the total phytate present, 84-88% was reported to be in bran (Resurreccion et al. 1979). O'Dell et al. (1972) found a level of 0.32% phytate in whole kernel wheat with approximately 87% of it being associated with the bran, 13% in the germ, 2% in the endosperm. Therefore, milling of wheats and subsequent separation of bran results in significant reduction of phytate in flours (Reddy, 1976).

Phytates in soybean appear to be unique in that although associated with protein bodies, they appear to have no specific site of localization (Tombs, 1967). Lolas et al. (1976) reported that the phytate content of 15 soybean varieties ranged from 1.00 to 1.47% dry weight which represented between 51.4 and 57.1% of the total phosphorus. But O'Dell et al. (1972) found that 90% of the phytate in corn was concentrated in the germ portion of the kernel, and corn endosperm had small amounts (3.2%) of phytate.

1.2.4 Effect of Phytate on Mineral Bioavailability

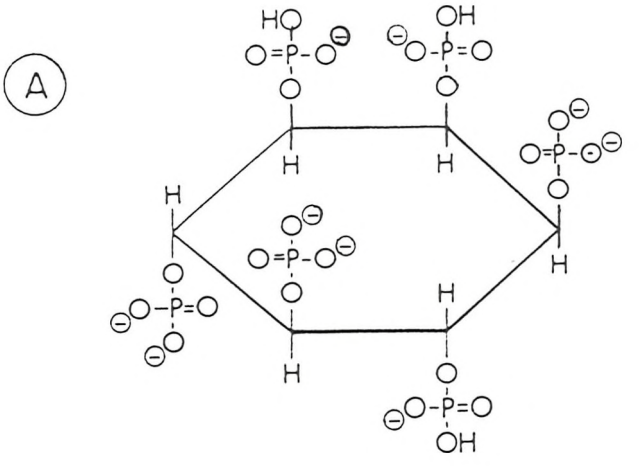
Numerous studies have led to the conclusion that phytate and its derivatives can bind essential dietary minerals, such as calcium, magnesium, zinc, copper, iron and others, thus making them unavailable or only partially available for absorption (Vohra et al. 1965). O'Dell, (1969) and Oberleas, (1975) suggested that the formation of inositol phytate metal complexes in the intestinal tract prevents the metals absorption.

An important factor in the precipitation of phytate salts is the synergistic effect of two or more cations, which, when present simultaneously, may act to increase the quantity of metallic phytate precipitated. This phenomenon has been demonstrated *in vitro* with zinc and calcium (Oberles and Prasad 1969).

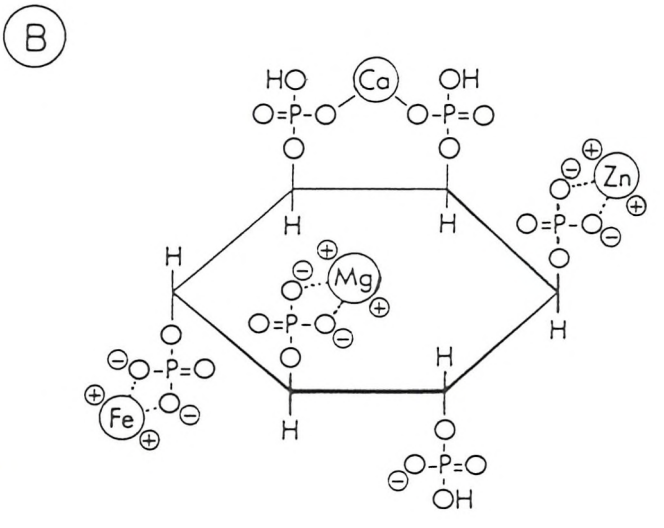
Taylor (1965) concluded that the solubility of such metal complexes of phytate are pH dependant-Weingartner and Erdman (1978) proposed that at neutral pH, the phosphate groups in phytate have either one or two negatively charged oxygen atoms, Figure 1.2A. It is apparent that various cations could strongly chelate between two phosphate groups or weakly within a phosphate group, Figure 1.2B.

Rackis and Anderson (1977) reported that reduced availability of essential minerals by phytate complexes in legumes depends on several factors.

FIGURE 1.2 : COMPARISON OF THE STRUCTURES OF PHYTIC ACID AND PHYTIC ACID CHELATE AT NEUTRAL pH. FROM WEINGARTNER AND ERDMAN (1978)



Structure of Phytic Acid



Structure of Phytic Acid Chelate at Neutral pH

1 - Ability of endogenous carriers in the intestinal mucosa to absorb essential minerals bound to phytate and other dietary substances.

2 - The concentration of phytate in food stuffs.

3 - The concentration of minerals in food stuffs. In the presence of high levels of calcium and magnesium, highly insoluble phytate sals are formed.

4 - The digestion or hydrolysis of phytate by phytase enzymes in the intestine. When phytate is digested, bound minerals are released.

5 - Method of processing.

Dietary factors other than phytate appear more responsible for reduced mineral availability in some situations. Dietary fibre accounts for much of the poor availability of minerals (Reinhold et al. 1975 and Ismail-beigi et al. 1977). Reinhold et al. (1976) attributed the depression of mineral absorption by humans fed wheat bread to the consumption of extra fibre.

The decreasing order of stability of the metal phytate complex has been reported to be $Zn^{++}>Cu^{++}>Co^{++}>Mn^{++}>Ca^{++}$ (Maddaiah et al. 1964). The higher stability of zinc was not surprising since similar findings have been reported by (Cheney et al. 1959). Vohra et al. (1965) reported that at pH 7.4, sodium phytate formed complexes with metals in the following decreasing order $Cu^{++}>Zn^{++}>Ni^{++}>Co^{++}>Mn^{++}>Fe^{++}>Ca^{++}$.

At this point it would be appropriate to discuss the nutritional implications of phytate interactions with specific minerals:-

A) - Calcium

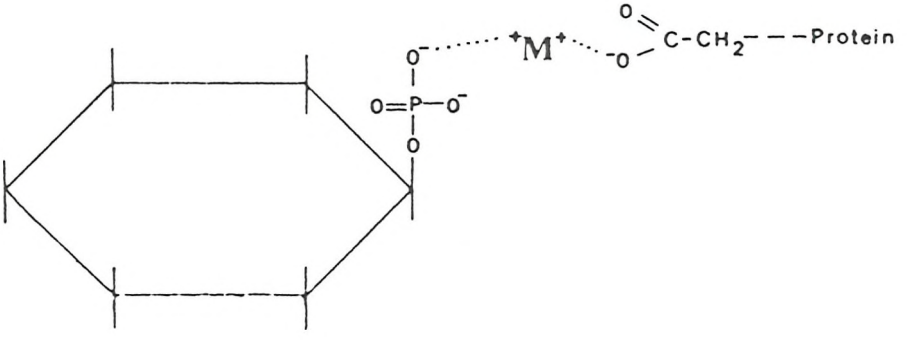
Calcium is the major divalent cation in most diets. Early reports indicated that cereals had anticalcifying and rachitogenic properties (Bruce and Callow, 1934; Harris and Bunker, 1935). They concluded that the compound responsible for such effects was phytate. Taylor (1965) suggested that the appearance of rachitic symptoms could be explained by the fact that metal ions, such as calcium or magnesium, present in the diet, are bound strongly by the soluble phytate, making them unavailable.

Calcium has been shown to have an adverse effect on the availability of phytate phosphorus. The phytate molecule has been observed to reduce the availability of calcium - six calcium ions can bind to each phytate molecule; the calcium phytate formed is insoluble at the near neutral pH found in the small intestine.

The formation of calcium phytate in the intestine could explain how dietary sodium phytate is able to reduce the absorption of calcium, resulting in a decreased plasma calcium concentration (Wills and Fairney, 1972). The level of calcium in the diet is probably the most underestimated factor contributing to the availability of phytate phoshorus. Published works indicate that high levels of calcium in the diets of rats (Taylor and Coleman, 1979) and in the diets of growing chicks and laying hens (Edwards, 1981; Ballam, 1985 and Nott et al. 1967), decrease the availability of phytate phosphorus.

Phytate is also able to bind to protein via divalent cation bridges at neutral pH (De Rham and Jost, 1979) (Figure 1.3). Nwokolo and Bragg (1977) demonstrated that the availability of calcium from protein supplements (soybean meal, cotton seed meal, rape-seed meal and palm kernel meal) was adversely affected by phytate in growing chicks.

FIGURE 1.3 : POSSIBLE STRUCTURE OF THE PHYTIC ACID-PROTEIN COMPLEX AT ALKALINE pH. M INDICATES MULTIVALENT ACTION. FROM DE-RHAM AND JOST (1979)



B - Zinc

Zinc has the greatest affinity for phytate and forms very stable complexes with it (Maddaiah et al. 1964; Vohra et al. 1965). This has been shown to be clinically important, since phytate-rich diets can complex with most of the zinc present in conventional diets, causing acute zinc deficiency which is characterized by depression of growth and skin lesions (Davies and Olpin, 1979). O'Dell and Savage, (1960) reported that phytate decreases the bioavailability of zinc in diets fed to animals. This observation has been confirmed in several animal species, including chicks, rats and pigs. Thus, the low bioavailability of zinc in cereal grains and legumes is explained at least in part by their relatively high content of phytate. Many investigators (Forbes, 1964; Likuski and Forbes, 1965 and Nwokolo and Bragg, 1977), have concluded that an inverse relationship exists between the level of phytate in the diet and zinc bioavailability.

Dietary calcium levels have long been associated with zinc bioavailability, but the detrimental effect of high calcium is dependent upon the presence of phytate in the diet (O'Dell, 1979). O'Dell et al. (1964) have shown that high levels of calcium in conjunction with phytate decreased zinc bioavailability in chicks indicating a calcium-zinc-phytate interaction which decreased biological availability. Adham and Song (1980) observed that high levels of dietary calcium inhibit zinc absorption. Forbes et al. (1983) showed that increasing calcium from 0.4 to 1.2 percent in the diet of rats, lowers zinc bioavailability in soy protein by approximately 50 percent, but has little or no effect when egg white supplies all of the protein. Presumably, the difference was due to the phytate in soy protein. However, increased dietary protein has been found to reduce the detrimental effect of dietary phytate on zinc absorption by humans (Sandstrom et al. 1980). O'Dell and Savage, (1960) showed that phytate decreased the availability of zinc in casein-based diets and produced a symptom similar to that observed amongst animals fed soybean protein containing a comparable level of phytate. Similar results were obtained by addition of phytate to diets based on free amino acids

(Likuski and Forbes, 1964). Forbes et al. (1979) suggested that the poor availability of zinc from soy concentrate was due to the presence of high phytate levels. Previous work by Forbes and Parker (1977) demonstrated that zinc added to rat diets in the form of whole fat soyflour was significantly less utilized than zinc added as zinc carbonate to an egg white diet.

Several studies have been reported about the possible role of fibre in zinc bioavailability in cereals and legumes (Reinhold et al. 1975, 1976). These investigators suggested that dietary fibre may also contribute, along with phytate to reduce minerals bioavailability from several cereals and legumes. Franz, (1979) studied the effects of processing on the zinc availability in white rice, corn and brown rice. He observed that zinc is more available from white rice than from corn or brown rice because the polishing process reduced the phytate content of white rice.

C) - Magnesium

Magnesium is an essential element for the chick. It is an activator of numerous enzyme systems that control many body functions and is a component of soft tissue and bone. Scott et al. (1982) reported that the chick requires 600 mg of dietary magnesium per kilogram diets for optimum growth and survival.

The bioavailability of magnesium in cereals depends on the presence or absence of certain nutritive and non-nutritive factors that either enhance or hinder magnesium absorption. Fibre and phytate are believed to adversely affect the bioavailability of magnesium (Nwokolo and Bragg, 1977 and Ismail-Beigi, et al. 1977). McWard (1969) observed that the addition of 4% phytate soy protein complex to the diet containing 75 p.p.m. of supplemental magnesium, depressed chick growth and increased mortality as a result of decreased bioavailability of magnesium.

He also demonstrated that in the absence of phytate, optimum growth was obtained at approximately 100 p.p.m. supplemental magnesium and he suggested the addition of phytate to the purified diet resulted in an increased requirements for magnesium by the chick.

The solubility of magnesium phytate complexes are pH dependant, Cheryan et al. (1983) observed that the magnesium phytate complexes were soluble below pH5 at molar ratios of between 0.5 and 12; above pH5, the solubility decreased rapidly. Roberts and Yudkin (1960) reported that soluble phytate can form insoluble mixed salts of calcium and magnesium. Signs of intoxication also shown by rats given diets rich in phytate, resemble those described in rats which were given diets deficient in magnesium.

The levels of calcium and phosphorus in the diet have a marked effect upon magnesium requirements. Nugara and Edwards (1963) reported that when either the calcium or phosphorus content of the chick diet was increased, the quantity of magnesium required in the diet for maximal weight gain increased. Likuski and Forbes (1965) showed that extra dietary calcium and phytate lowered magnesium absorption. Calcium and magnesium behave similarly, and a mixture of the two with phytate leads to the precipitation of a mixed phytate salt (Cheryan, 1980).

1.3 Phytase

In cereal grains, legumes and oil seeds, the major portion of the total phosphorus is present in the form of phytate. Since phytate phosphorus can only be absorbed from the small intestine as inorganic phosphorus after hydrolysis, the process of hydrolysis is of prime importance to the chick. Phytate can be hydrolysed in one of two ways.

1 - by a mineral acid 2 - enzymatically

Acid hydrolysis

Phytate can be dephosphorylated by heating in acid solution, with maximum velocity at pH 3.0 - 4.0 (Fleury, 1946). Dephosphorylation in neutral or alkaline solution is very slow. Desjobert (1954) prepared myoinositol 2-phosphate by heating of sodium phytate solution at pH 8.0. Mellanby (1950) showed that the hydrolysis of 280 mg sodium phytate was almost complete after boiling in 1% hydrochoric acid for 18 hours.

Enzymatic hydrolysis

The dephosphorylation of phytate by the action of the enzyme phytase (Bitar and Reinhold, 1972; Mandal et al. 1972 and Cosgrove, 1966) requires the soluble form of the substrate. Therefore phytate is poorly available from high-calcium diets due to formation of highly insoluble calcium phytate (Taylor, 1965).

The mode of action of phytase has remained controversial. Maiti, et al. (1974) reported that the degradation of phytate by phytase occurs in a stepwise manner, starting with dephosphorylation from position 6, which is followed by the removal of phosphate from position 5 and 4, 1 and 3 or 1 and 4, the phosphate at position 2 being stable.

myo-inositol hexaphosphate $\xrightarrow{\text{phytase}}$ myo-inositol pentaphosphate + H_3PO_4

followed by:

$$Ip_5 \xrightarrow{\text{phytase}} Ip_4 + P_i \text{ followed by}$$

$$Ip_4 \xrightarrow{\text{phytase}} Ip_3 + P_i \text{ followed by}$$

But this mode of action differs from that reported by Lim and Tate, (1973). They observed that the F-2 fraction from wheat bran phytase hydrolysed the phytate molecule at the 1, 4, and 2 or 5 positions of the myo-inositol ring. Maiti et al. (1974) postulated that differences in the mechanisms were probably due to the differences in the phytase origin.

After hydrolysis, the lower inositol phosphates are less able to precipitate with calcium at neutral pH *in vitro* (Kaufman and Kleinberg, 1971), so hydrolysis would lead to greater solubility of metals in the intestine.

Phytase (meso-inositol hexaphosphate phosphohydrolase, EC3.1.3.8) is widely distributed not only in various cereals and seeds (Maga, 1982); but also in the small-intestinal mucosae of many animals (Bitar and Reinhold, 1972); as well as in many species of fungi and bacteria (Cosgrove, 1966).

There are three sources of this enzyme of significance in nutrition.

1.3.1 - Plant phytase

Suzuki, et al. (1907) obtained inositol from phytate by the action of an enzyme in rice bran, which was capable of hydrolysing the substance into inositol and phosphoric acid, and named the enzyme "phytase".

Many investigators have isolated and characterized phytase from several different cereals and beans. It is widely distributed in cereals (Maga 1982); wheat (Peers, 1953; Lim and Tate, 1973); corn (Chang, 1967); barley (Lolas et al. 1976); rice (Asada and Kasai 1962) and beans (Mandal and Biswas, 1970; Mandal, et al. 1972; Lolas and Markakis, 1975). There are powerful phytases in wheat and rye. The enzyme in barley is considerably less active and oats and maize possess negligible enzyme activity (McCance and Widdowson, 1944). Mellanby (1920) observed that oat meal is more rachitogenic than wheat meal. However, it was not until 1934 that the rachitogenic factor in oats was identified by Bruce and Callow as a lack of phytase. McCance and Widdowson (1944) suggested that the lack of an active phytase in oats may explain why this cereal is more rachitogenic than wheat in low-calcium diets. Phytate is utilized as a source of inorganic phosphate during seed germination and the inorganic form becomes available for purposes of plant growth.

The liberation of phosphate from phytate occurs by enzyme hydrolysis.

Phytase activity usually increases on germination (Walker, 1974). This finding is confirmed by Chen and Pan 1977. They determined that the phytase apparently increased 227% in the soybean after five days of germination and the increase was inversely correlated with phytate content. Mollgaard (1946) reported that in two hours incubation, at pH 5 and 40°C the following degrees of hydrolysis took place; wheat, wheat bran and rye 100%; barley 69%; oats 8%; maize 2% and oil seeds 2-10%. But Hill and Tyler (1954) showed that at a given pH, the addition of calcium carbonate to wheat or wheat bran caused a reduction in phytate hydrolysis by the formation of calcium phytate and the inability of the phytase to hydrolyse this insoluble salt.

In general, the pH optimum for cereal phytase is around pH 5.[illegible]. However , Hill and Tyler (1954) showed some activity down to pH3, they also observed that the wheat phytase is completely destroyed at pH values of 2.5 and below.

The optimum temperature for phytase activity varies somewhat from source to source, but appears to be in the high temperature range 45-60°C. This high optimal temperature for the phytase activity may be important in food processing operations involving high temperatures. Ranhotra, (1972) showed that cereal phytase is active in dough for bread making but is soon destroyed in the baking process proper. The optimum pH and temperature for phytase from certain cereals and legumes are shown in the Table below.

phytase source	optimum pH	Temp.°C	Reference
Corn	5.6	50	Chang (1967)
Wheat flour	5.15	55	Peers (1953)
Wheat bran	5.0	50	Hill and Tyler (1954)
Beans	7.0	57	Mandal and Biswas (1970)

1.3.2 - Intestinal Phytase

That the intestine has the ability to hydrolyse phosphate from phytate was first shown by Patwarhan, (1937), who attributed this action to phytase enzyme (meso inositol hexaphosphate phosphohydrolase, EC 3.1.3.8). Patwarhan, believed this ability to be peculiar to the rat, but others have shown that phytate is hydrolysed by phytase in the intestines of the chick (Steenbock et al. 1953), rats; humans (Bitar and Reinhld, 1972) and hamster (Williams and Taylor, 1985).

The enzyme phytase is identical to one or more of the isoenzymes of alkaline phosphatase (EC 3.1.3.1), (Pileggi, 1959; Davies and Flett, 1978). Mixed-substrate studies were found, by Maddaiah et al. (1964), to indicate that chick intestinal phytase might be the same enzyme as alkaline phosphatase. This finding was confirmed by McCuaig et al. (1972), who observed that phytase activity was affected by the same factors as alkaline phosphatase.

Moog (1962) demonstrated that the small intestine is the site of the highest phytase activity in the body, and the enzyme is principally localized in the microvilli that cover the luminal surfaces of the epithelial cells. The enzyme was shown precisely in the microvillar membrane by (Clark, 1961) using the electron microscope. Roberts and Yudkin, (1961) reported that the maximal activity occurs in the proximal twelve inches of the rat intestine. Husbscher et al. (1965) showed that most intestinal alkaline phosphatase activity is present in the brush-border fraction. These findings are supported by (Davies and Flett, 1978), who found the specific activity of alkaline phosphatase and phytase in the brush-border fraction was approximately ten times greater than in whole homogenate.

Leblond and Stevens (1948) and Moog (1950) observed that alkaline phosphatase is not secreted from the duodenal wall, but seems to be released as the epithelial cells are sloughed off. Weiser, (1973), Padykula, (1962) and Fortin-Magana et al. (1969)

have shown that alkaline phosphatase is absent or at very low levels in the crypts with a gradual increase to a maximum at the villus tip. Chan and Atkins (1983) have found a similar pattern of distribution of alkaline phosphatase and phytase.

Intestinal alkaline phosphatase and phytase are influenced by diet particularly by dietary vitamin D_3. This was investigated by McCuaig et al. (1972) to determine the influence of dietary magnesium, calcium, phosphorus and thyroactive casein; high magnesium and calcium levels reduced the activity of both enzymes and high phosphate had no effect, whilst low dietary phosphorus greatly increased their activities. This latter finding agrees with earlier work from the same laboratory by Davies et al. (1970). Groth (1963) also observed that both calcium and phosphorus deficiencies caused increased alkaline phosphatase. McCuaig and Motzok (1974a) determined that a high-calcium diet decreased plasma inorganic phosphorus and mucosal alkaline phosphatase activity.

There was a positive correlation of alkaline phosphatase with plasma and mucosal inorganic phosphorus. McCuaig and Motzok (1974b) indicated that duodenal alkaline phosphatase might regulate calcium and zinc metabolism by the movement of inorganic phosphorus. Intestinal mucosal alkaline phosphatase activity is known to be zinc-dependent and in the rat, zinc deficiency brings about a marked reduction in enzyme activity (Williams, 1972). This finding is confirmed by Davies and Flett (1978), who reported that brush border alkaline phosphatase and phytase activities required both zinc and magnesium ions for maximal activity. But, when the diet contained calcium in excess of a 6:1 molar ratio with phytate, the mucosal activity of phytase was reduced (Bhandari, 1980).

It is interesting to note that intestinal phytase can be induced by vitamin D_3 (Holdsworth, 1970). It is possible to speculate that this response is of importance in enhancing phytate

hydrolysis in the intestine. In fact, plasma levels of 1-25 dihydroxy cholecalciferol are elevated in vitamin D replete rats given low phosphorus diets (Hughes et al. 1975) and this metabolite of vitamin D_3 is associated with enhanced intestinal phosphorus absorption and alkaline phosphatase levels (Peterlik and Wasserman, 1980). It has been suggested that the increase in intestinal phosphorus absorption during phosphorus deficiency may be related to enhanced synthesis of alkaline phosphatase in the intestinal mucosa (Kempson, et al. 1979; Birge and Avioli, 1981).

1.3.3 - Microbial phytase

The alimentary tract of the chick, like that of all animals in a normal environment, is densely populated with micro-organisms capable of intense metabolic activities. Consideration of the processes of digestion would be incomplete without taking account of possible interference by components of the gut microflora, the results of which might be of benefit, detriment, or no importance to the host. Microbial phytase, for example might facilitate digestion of dietary phytate phosphorus thereby contributing to the host's nutrition.

Ruminants such as sheep and cattle are able to utilize most of their dietary phytate (Nelson et al. 1976). These authors also observed that phytate hydrolysis occurred in the rumen and was completed before the feed reached other parts of the digestive system. This ability to utilise phytate phosphorus has been attributed to the ruminal microflora.

Microbial phytase has been the subject of controversy in different animals. Wise and Gilburt, (1982), demonstrated that germ-free rats were unable to hydrolyse phytate, but Yoshida et al. (1985) showed lower apparent digestibilities of calcium and phosphorus in the conventional rat groups than in the germ free groups. Similar results were obtained by Yosida et al. (1981), using mice. This observation supports the findings of Yoshida et al (1968), who observed that germ-free rabbits excreted a higher

percentage of ingested calcium and phosphorus in urine. However, in germ free young Japanese quails it was associated with an increase in the digestion of calcium, magnesium and phosphorus (Yoshida et al (1969).

The absence of a rat's viable intestinal microflora increased the digestion of calcium and magnesium and bone concentration of calcium, phosphorus and magnesium (Reddy, 1971) who also showed that germ free rats have altered activities of mucosal hydrolases enzymes. Similar results were obtained by Reddy and Wostmunn, (1966).

1.4 Phytate Phosphorus Availability in the Chick

The National Research Council (1977) stated that approximately 30% of the phosphorus in feedstuffs seeds could be utilized by non-ruminant animals. Two-thirds of the phosphorus of feedstuff of plant origin, particularly cereals, and by products of cereals and oil seed, is present in the form of phytic acid or its salts. As previously mentioned, it is of commercial interest to increase the availability of the phytate source of phosphorus in cereal diets. Most of the research has focussed on the availability of phosphorus from phytate phosphorus to the chick.

Several studies have been done in this area. Harms et al. (1962) and Wardrop et al. (1964) concluded that the phosphorus in phytate was highly available to the chick. Waldrop et al. (1967) and Singsen et al. (1969) reported that phytate phosphorus was found to be between 30 and 80% available to laying hens. Aston et al. (1960) fed ^{32}P labelled calcium phytate phosphorus and observed that four week-old chicks retained 20% and that six week-old chicks retained 36-49% of phytate phosphorus. They concluded that the chicks utilized one-fifth of phytate phosphorus.

Temperton and Cassidy (1964a) noted from balance studies that the chick could hydrolyse and absorb a large portion of ingested phytate phosphorus.

Phytate hydrolyses was enhanced when suboptimal amounts of non-phytate phosphorus were fed. Temperton and Cassidy (1964b) also demonstrated that 59-60% of the phytate phosphorus consumed by chicks was retained in the body. It was concluded that chicks could use phytate phosphorus for deposition in growing bones and that these bones appeared normal at 26 days of age when the diet carried neither animal protein nor phosphorus supplementation.

A wide disagreement has existed between investigators on the ability of chicks to utilize phytate. Some studies have found that phytate phosphorus was a poor source of phosphorus for chicks (Sunde and Bird, 1956). Nott (1967) fed chicks maize soya diets with 2g phytate/kg but a variable calcium and total phosphorus content. He showed that negligible (6%) phytate was digested. Nelson (1976a) used broiler chicks and laying hens and found that phytate phosphorus in corn diets was almost totally unavailable (0-8%).

The wide disagreement between investigators on the ability of chicks to utilize phytate is due to variations in their experimental methods and materials including species, age of the chicks, the source of phytate, the level of calcium, fibre and vitamin D_3 used in the experimental diets. Edwards (1981) using single comb white Leghorn (SCWL) chicks showed higher retention values for calcium, phosphorus and phytate phosphorus than the broiler chicks for all the diets tested. Ballam et al. (1984a) found that SCWL chicks hydrolysed 34 to 200% more phytate phosphorus than broiler chicks, depending upon the feed ingredients.

The level of calcium in the diet is probably the most important factor contributing to the availability of phytate phosphorus. Calcium reduces the availability of phytate phosphorus to chicks by binding to phytate, forming an insoluble calcium phytate complex (Taylor, 1965). Many investigators indicated that high levels of calcium in the diets of chicks decrease the availability of phytate phosphorus (Nott, 1967, Ballam et al. 1984a). Ballam et al. (1985) fed broiler chicks

cereal based diets with various calcium and phosphorus supplements. One of their findings was that, increasing the calcium content of the diet to 1.0% reduced ($p<0.05$) phytate hydrolysis regardless of phosphorus content.

Nelson et al. (1968) reported that phytate could be hydrolysed by Aspergillus ficuum and other moulds in soybean meal. Nelson et al. (1971) found that the addition of phytase to chick diets produced an increase in percent bone ash indicating hydrolysis of phytate by the added enzyme. Total hydrolysis of phytate occurred when 3g of phytase supplement were used per kilogram of diet.

Investigators have indicated that chicks can utilize plant phosphorus fed diets containing wheat (Temperton and Cassidy, 1964a,b), Temperton et al. 1965a,b, and Salman et al. 1969) which may be a potential source of phytase. In contrast, the phytate in chick diets without wheat is a poor source of phosphorus for young chicks (Nelson, 1976a). The situation is complicated because many feed ingredients high in fibre are also high in phytate and phytase.

Fibre sources have been shown to reduce the effective concentration of cations in the gastrointestinal tract and increase phytate hydrolysis (Ismail-Beigi et al. 1977). However, Heged et al. (1978) indicated that chicks given coarse wheat straw had longer villi and thicker muscular layers, that may also affect phytate hydrolysis. Ballam et al. (1984b) fed broiler chicks a corn-soybean meal diet or a corn-soybean diet containing either 15% rice bran, 15% wheat bran, 15% alfalfa meal, 10% cellulose or 10% cotton seed hulls. One of their findings was that the hydrolysis of phytate was influenced more by calcium than by fibre or by the level of phytate fed. Phytate hydrolysis is also closely associated with the vitamin D_3 content of the diet (see also Chapter 9).

1.5 Vitamin D

1.5.1 Introduction

Vitamin D is not a single compound but a small family of compounds which exhibit vitamin D like activity (DeLuca, 1979), the most important of which are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). Birds and mammals have the ability to produce vitamin D_3 from 7-dehydrocholesterol by ultra-violet (u.v.) light over the range of wave length 256-313 nm. In 1919 Sir Edward Mellanby demonstrated the vitamin-like activity of cholecalciferol when he showed that rickets can be produced by dietary manipulation and that this disease can be prevented by the administration of cod liver oil. Thus cholecalciferol can originate from either a dietary source, or from endogenous photochemical production in the skin.

Vitamin D_2 is formed in a similar fashion from ergosterol, which occurs only in plants. In most birds, vitamin D_2 is only about one tenth as active as vitamin D_3 (Chen and Bosman, 1964). In the following sections reference to vitamin D will be to cholecalciferol, not ergocalciferol, unless otherwise stated.

The importance of vitamin D in the regulation of calcium metabolism and phosphorus metabolism is emphasised by the effects of vitamin D deficiency. In young animals, vitamin D deficiency causes the disease rickets which is characterised by skeletal deformities, while in older animals it results in osteomalacia.

In recent years, it has become recognised that vitamin D plays a central role in the regulation of calcium and phosphorus metabolism with actions in several tissues including the intestine, bone and kidney. It has been established that vitamin D undergoes a two step activation prior to the production of the biologically active species 1,25 dihydroxycholecalciferol ($1,25(OH)_2D_3$). Active metabolite production is under a precisely regulated feedback control which is linked to plasma levels of calcium and phosphorus.

Thus for most practical purposes the term prohormone rather than vitamin is probably a more accurate description of vitamin D, whilst its active metabolite, $1,25(OH)_2D_3$, fulfils the necessary criteria for hormone identity. The metabolism of vitamin D, which has been subject of intensive study for some time, is discussed in the following sections (for reviews see Henry and Norman, 1984; Kumar, 1984, Taylor and Dacke, 1984).

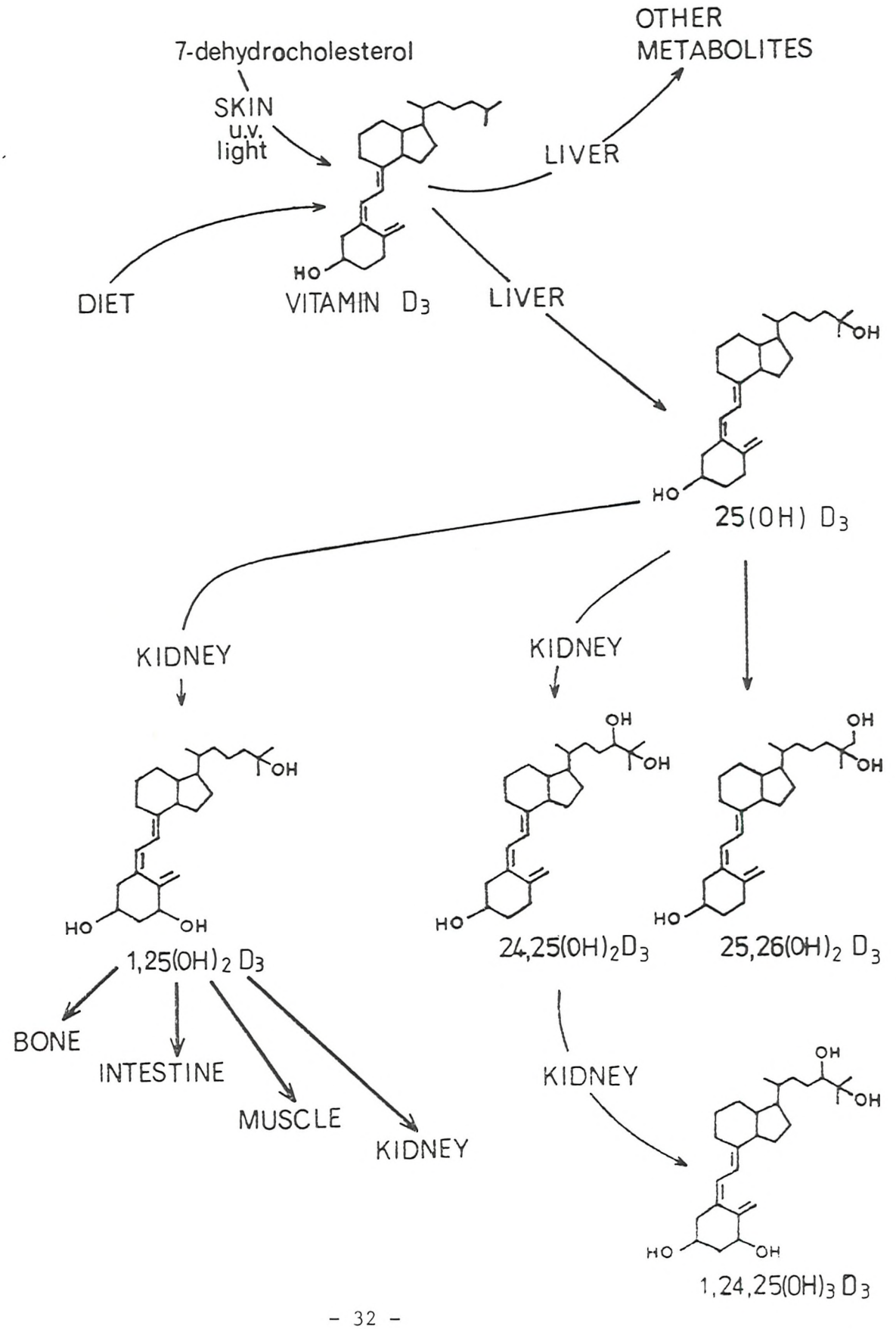
1.5.2 Vitamin D metabolism

Vitamin D may either be obtained from the diet or it can be synthesised from 7-dehydrocholesterol. It has been recognized since the early work of Mellanby (1919) that vitamin D is absorbed in the small intestine, with the most active rate of absorption occurring in the duodenum. However, because of the greater transit time of food in the ileum, the largest quantity of dietary vitamin D is absorbed through the distal portion of the small intestine (Schachter et al. 1964; Norman and DeLuca, 1963).

In mammals, cholecalciferol is produced in the malpighian layer of the skin by a non-enzymatic photolysis reaction (Holick, et al. 1979). Previtamin D_3 believed to be the intermediate metabolite. In birds, cholecalciferol may also be formed when 7-dehydrocholesterol present in the preen gland oil is spread over the feathers (Sebrell and Harris, 1954). Cholecalciferol obtained from the diet and ingested during preening, is absorbed with the fats in the small intestine and transported in chylomicra to the liver.

Vitamin D that reaches the blood compartment from all sources is rapidly cleared by the liver. In fact, Ponchon and DeLuca (1969) demonstrated that within 60 minutes the vitamin D in the plasma is cleared and accumulates in the liver. Once in the liver, cholecalciferol undergoes its first metabolic reaction, i.e. hydroxylation at the 25 position, to produce 25-hydroxycholecalciferol ($25(OH)D_3$) (see Figure 1.4). This reaction occurs primarily in the endoplasmic reticulum of liver cells by the liver

FIGURE 1.4 : THE PATHWAY FOR THE METABOLIC ACTIVATION OF VITAMIN D_3 AND TARGET ORGANS OF ACTIVE METABOLITES



enzyme cholecalciferol-25-hydroxylase (25-hydroxylase) (Ponchon et al. 1969). This enzyme has also been detected in the small intestine and kidneys of avian species (Tucker et al. 1973). The 25-hydroxylase requires NADPH and molecular oxygen for its activity and is therefore a mixed function mono-oxygenase (DeLuca, 1979). Following hydroxylation, $25(OH)D_3$ rapidly leaves the liver and is carried in the circulation bound to a plasma transport protein (Bouillon et al. 1980). The main site of metabolism of $25(OH)D_3$ is the kidney, where many different reactions are known to occur.

If there is a need for calcium, as signalled by high parathyroid hormone levels, or a need for phosphorus, as signalled by hypophosphatemia, or other factors that signal abnormal calcium requirements, $25(OH)D_3$ is hydroxylated on carbon 1 to yield 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$). The enzyme responsible for this conversion is localized exclusively in the mitochondria of cells of the kidney cortex (Midgett et al. 1973), probably in the proximal tubule cells (Stumpf et al. 1980).

The $1,25(OH)_2D_3$ formed in the kidney is then transported via a 52,000 molecular weight plasma protein to the target tissues of intestine, bone and elsewhere in the kidney where it functions on calcium and phosphorus transport reactions.

Following the identification of $1,25(OH)_2D_3$, Boyle et al. (1971) demonstrated that in animals given vitamin D and nutritionally adequate sufficient amounts of calcium, $1,25(OH)_2D_3$ was not the major product of $25(OH)D_3$ but instead another product somewhat less polar than $1,25(OH)_2D_3$ on Sephadex LH-20 columns was found (Boyle et al. 1971, 1972). This compound was isolated (Holick et al. 1972) and subsequently identified as 24,25 dihydroxy cholecalciferol ($24,25(OH)_2D_3$) (Boyle et al. 1973). There is a reciprocal relationship between the activities of the 25-OH-1-hydroxylase and the 25-OH-24-hydroxylase in the kidney.

Quantitatively, 24-hydroxylation of $25(OH)D_3$ is the second most important reaction which occurs in kidney and extensive 24-hydroyxlation has been shown to occur outside the kidney (Turner et al. 1984) in intestine, cartilage and bone (Kumar et al. 1978, Garabedian et al. 1978). The precise biological function of $24,25(OH)_2D_3$ is uncertain, but as it is produced in greater quantities when calcium requirements are low, it is considered by some to be an inactive metabolite. However, Canterbury et al. (1978) found that $24,25(OH)_2D_3$ caused a considerable decrease in pTH secretion, and it has also been shown to be necessary for correct bone mineralisation in rachitic chicks (Ornoy et al. 1978). These conclusions were based on the effects of administering combinations of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ to animals in vivo. Recently however, convincing evidence has been obtained which indicates that $24,25(OH)_2D_3$ is not required for any of the major actions of vitamin D. This became possible following the chemical synthesis of 24,24-difluoro-$25(OH)D_3$ (Kobayashi et al. 1979; Yamada et al. 1979), a compound very similar to $25(OH)D_3$ but which cannot be hydroxylated at the 24 position. Despite possessing this property, 24,24-difluor-$25(OH)D_3$ has been shown to have the same potency as $25(OH)D_3$ in several vitamin D responsive systems (Tanaka et al. 1979, Halloran et al. 1981 and Okamoto, 1981), and recently 24,24-difluoro-$25(OH)D_3$ has been shown to be capable of supporting normal growth and mineralization of bone and of maintaining plasma calcium and phosphorus levels in rats, which had been deprived of vitamin D for two generations (Brommage et al. 1983; Jarnagin et al. 1983).

Another metabolite of vitamin D of uncertain physiological importance is 1/24/25-trihydroxycholecalciferol ($1,24,25(OH)_3D_3$). This metabolite does have very definite actions on the intestine and on bone, and it has been suggested that any activity seen with $24,25(OH)_2D_3$ in in vivo experiments is due to its conversion to $1,24,25(OH)_3D_3$ (Boyle et al.1973). $1,24,25(OH)_3D_3$ can be produced either by the 1-hydroxylation of $24,25(OH)_2D_3$ (Holick et al. 1973) or by the 24-hydroxylation of $1,25(OH)_2D_3$ (Kleiner-Bossaler and DeLuca 1974). In view of the large amount of $1,25(OH)_2D_3$ taken

up by the intestine, it is interesting to note that intestine has the capacity to convert $1,25(OH)_2D_3$ to $1,24,25(OH)_3D_3$ (Ohnuma and Norman 1982). This observation is compatible with the suggestion of Kumar et al. (1978) that 24-hydroxylation may provide a mechanism for partially inactivating $1,25(OH)_2D_3$ in its target tissues and particularly in intestine. $1,24,25(OH)_3D_3$ is probably the second most active of the metabolites of vitamin D, having half the activity of $1,25(OH)_2D_3$ in stimulating intestinal calcium transport and one tenth of its activity with respect to bone resorption.

Recently many other metabolites of vitamin D have been isolated and identified (Figure 1.4). These metabolites are all devoid of biological activity and are undoubtedly formed as part of the process by which vitamin D is excreted via the bile (DeLuca and Schnoes, 1983). Of particular interest, however, is the 25,26-dihydroxycholecalciferol ($25,26(OH)_2D_3$). It was identified in 1970 by Suda and colleagues. This metabolite is produced in relatively large amounts when $25(OH)D_3$ is available in excess and it has, therefore, been suggested that 26-hydroxylation may be an important means of inactivating and removing $25(OH)D_3$ to prevent its accumulation to toxic levels. Horst and Littledike (1980) found the 25,26-hydroxylase was present not only in the kidney but also in other as yet undefined tissues.

1.5.3 Role of vitamin D in intestinal calcium and inorganic phosphate absorption

A - Calcium absorption

Nicolaysen (1937) was the first to establish that vitamin D improves intestinal calcium absorption. The most important site of action of vitamin D is the intestine, where it possesses the unique ability to stimulate calcium and phosphorus absorption (Bikel et al. 1981). While the requirement for vitamin D in intestinal calcium absorption is clear, the mechanisms involved are still poorly understood. Early studies of the mechanisms of

action of vitamin D showed that the stimulation of intestinal calcium absorption by vitamin D could be prevented by the addition of the protein synthesis inhibitor, actinomycin D (Norman, 1965).

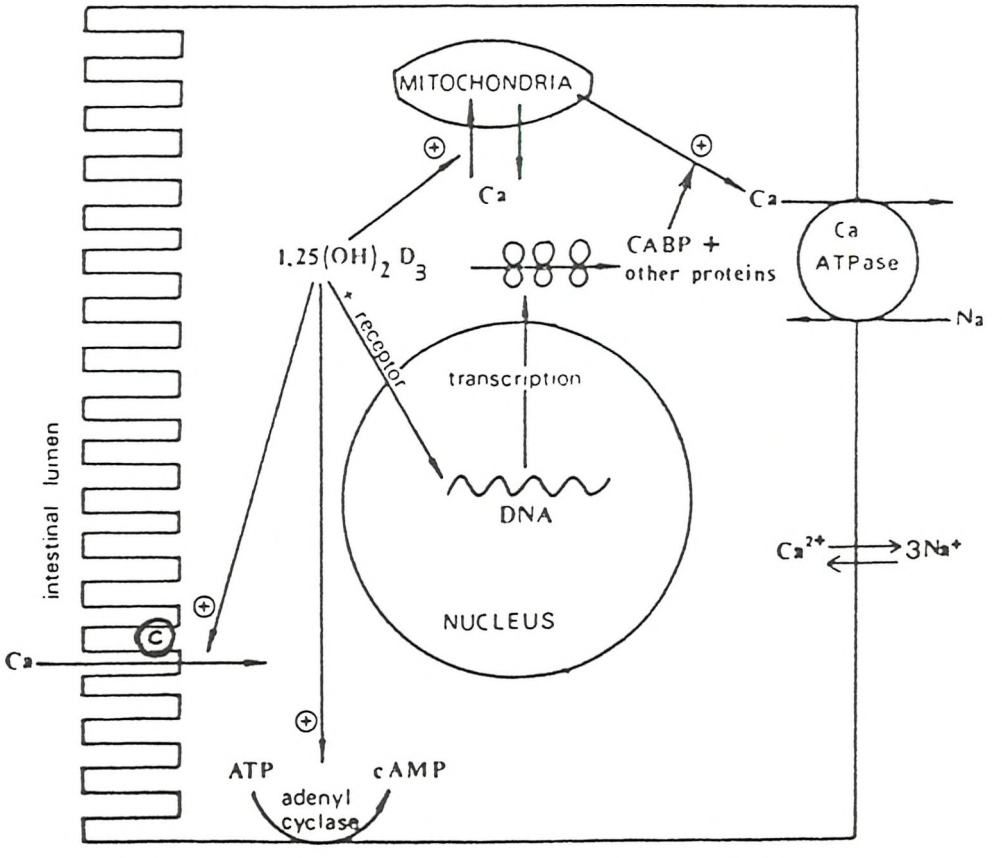
Following this observation, the first protein to be identified as being produced by the influence of vitamin D on the intestine, was calcium binding protein (CaBp) (Wasserman and Taylor 1966). The presence of this protein in the intestinal mucosa is totally dependent on prior administration of vitamin D or one of its active metabolites, and it is absent from the intestine of vitamin D deficient chicks and rats (Bronner and Freund, 1975).

In subsequent studies, however, it was found that the correlation between the rate of calcium transport and the concentration of CaBp in intestine was often poor and it has now been clearly demonstrated that vitamin D can stimulate calcium transport in the gut at least 2 hours before CaBp can be detected immunologically (Spencer et al. 1978). It would appear, therefore, that this protein is not directly responsible for the stimulation of calcium transport and it has been suggested that the CaBp may serve to buffer calcium entering the cell, to prevent the build-up of potentially toxic levels of free calcium that passes through epithelial cells when calcium transport is stimulated by $1,25(OH)_2D_3$ (Bikle, et al. 1981).

In passing from the intestinal lumen into the circulation, calcium ions must first pass through the brush border membrane into the mucosal cells before leaving through the baso-lateral membrane (Figure 1.5).

In view of recent studies, it would appear that one of the most important sites of action of vitamin D is at the brush border membrane. This has been demonstrated using isolated vesicles obtained from chicks (Rassmussen et al. 1979). Intestinal brush border membranes in which $1,25(OH)_2D_3$ has been shown to stimulate the transport of calcium. The mechanism involved is not known, but does not appear to require de novo protein synthesis (Rassmussen et al. 1979).

FIGURE 1.5 : A SIMPLE MODEL SHOWING THE EFFECTS OF $1,25(OH)_2D_3$ ON A GUT EPITHELIAL-CELL



A number of changes have been observed in the lipid composition of the brush border membrane, in response to $1,25(OH)_2D_3$, (Rassmussen et al. 1982), and as a result these authors have proposed that the increased calcium transport caused by $1,25(OH)_2D_3$, may be induced by changes in membrane fluidity. The way in which these changes in lipid composition could enhance calcium transport, however, is not at all clear and will undoubtedly be the subject of further research.

The problem of getting calcium out of the epithelial cells into the blood, against an electrochemical gradient, is thought to be carried out by a calcium ATPase "pump". This enzyme has been isolated in the basolateral membrane (Birge and Gilbert, 1974) and is most probably driven by the high concentrations of sodium in the extracellular fluid. In experiments using vitamin D deficient chicks, Lane and Lawson (1978) demonstrated a concomitant increase in calcium ATPase activity and calcium absorption following $1,25(OH)_2D_3$ administration.

B - Inorganic phosphate absorption

In addition to its effects on intestinal calcium transport, it is clear that vitamin D also stimulates the absorption of phosphate (Harrison and Harrison 1961). Kowarsky and Schacter (1969) reported that vitamin D given to intact rats was found to increase inorganic phosphate transport. They also reported that the effect of vitamin D on calcium absorption was maximal in the duodenum and independent of the presence of phosphate and, since phosphate absorption was found to be maximal in the jejunum and independent of the presence of calcium, they concluded that vitamin D separately influences these two intestinal transport mechanisms. Wasserman and Taylor (1973) and Taylor (1974) have also obtained additional evidence that phosphate absorption of intestine is independent of the calcium absorption system in the chick. Hurwitz and Bar (1972) also reported that vitamin D stimulates phosphate absorption independent of calcium absorption,

and that the vitamin D enhanced calcium absorption in the chick occurred mainly in the duodenum and that of phosphate mainly in the jejunum.

Chen et al. (1974) found that vitamin D and the metabolites $25(OH)D_3$ and $1,25(OH)_2D_3$, but not $24,25(OH)_2D_3$, stimulated phosphate absorption independently of calcium absorption in the rat. Furthermore, these workers concluded that, nephrectomy prevented the response to $25(OH)D_3$, but not to $1,25(OH)_2D_3$. Thus it appeared likely that $1,25(OH)_2D_3$ and not $25(OH)D_3$ was the metabolically active form in this system.

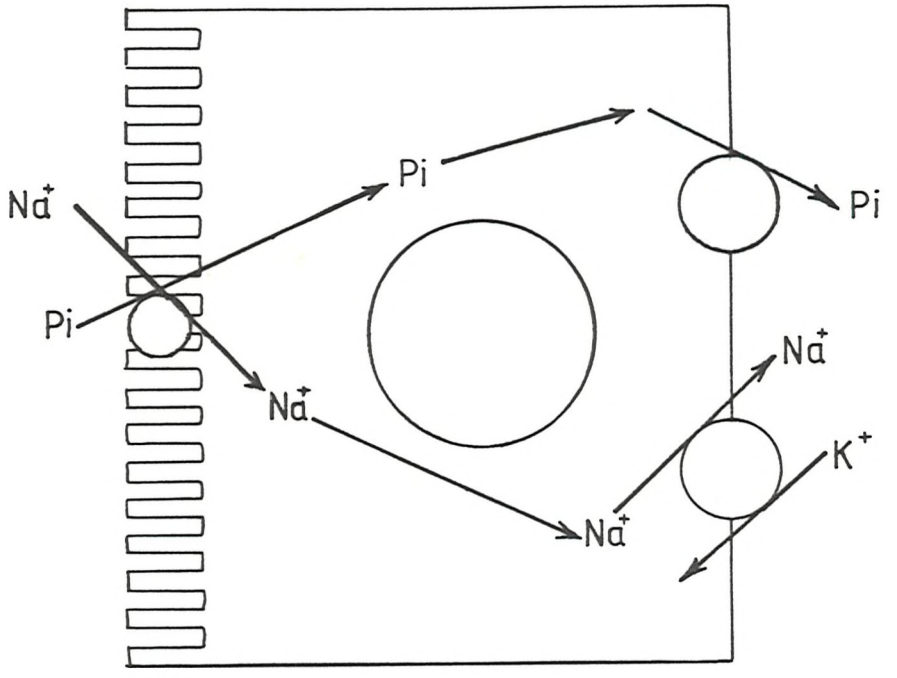
Walling (1977) observed that $1,25(OH)_2D_3$, increased the active absorption of both calcium and inorganic phosphate in all segments of the small intestine. Active calcium absorption was greatest in the duodenum > jejunum > ileum and active inorganic phosphate absorption was highest in the jejunum followed by the duodenum and then the ileum. Similar results were reported by Peterlik and Wasserman (1978), who observed that vitamin D increased the concentration ratio of inorganic phosphate across all intestinal segments, with greatest response occurring in the jejunum.

Berner et al. (1976), and Wasserman, (1981) reported that the phosphate absorption was sodium dependent. The essential features of the absorption of phosphate, in model form, are given in Figure 1.6 which shows the interaction of Na^+ and phosphate with a common component on the brush border of the intestine. The movement of Na^+ into the cytoplasm provides the energy for the absorption of phosphate in the same direction.

1.5.4 Role of vitamin D in bone

The primary defect leading to the discovery of vitamin D was the failure of bone mineralisation that results from vitamin D deficient animals. It is well known that an absence of vitamin D

FIGURE 1.6: MODEL OF PHOSPHATE TRANSPORT ON GUT EPITHELIAL CELL



A simple model showing phosphate (Pi) is transported across the brush border of the intestinal cell by a Na^+ dependent process. Phosphate moves through the cell without entering the cytoplasmic phosphate pool, and diffuses from cell to lamina propria, possibly by facilitative diffusion. The low intercellular Na^+ concentration is maintained by Na^+,k^+ extrusion pump on the basal lateral membrane. (Wasserman, 1981)

leads to rickets in the young and osteomalacia in the adult. Surprisingly it has not yet been proved conclusively that vitamin D plays a direct role in bone mineralisation. This is because vitamin D can aid bone mineralisation indirectly by increasing plasma calcium and phosphorus levels. However, Lamm and Neuman (1958) suggested that the role of vitamin D was to maintain plasma calcium and phosphorus levels by stimulating calcium and phosphorus absorption, reducing calcium and phosphorus excretion and increasing the mobilization of bone mineral. Once the concentration of these two ions reached appropriate levels, their deposition in bone matrix occurred spontaneously through a physiochemical process.

In vitro studies, using poorly mineralised metaphyseal slices from rachitic chicks, showed that there was significantly less mineral deposited than in slices from vitamin D-replete chicks or from those of rachitic chicks fed calcium supplemented diets (Crenshaw et al. 1974). They concluded that hypomineralisation of rachitic bone in vivo is due to reduce plasma calcium levels. A similar conclusion was reached by Yoshiki, et al. (1974) who studied the role of vitamin D in the mineralization of dentin in rats made rachitic.

Throughout the course of vitamin D investigation, emphasis has been placed on looking at the role of vitamin D in bone mineralisation. However, Carlsson (1952) produced convincing evidence that vitamin D plays a major role in bone resorption. He observed that a rachitic animal placed on a calcium free diet and given vitamin D, showed a rise in the plasma concentration of calcium and phosphorus. These ions entered the plasma from bone, thereby giving rise to the idea that bone was one of the target tissues of vitamin D for its homeostatic action.

It has been suggested that there is a functional bone cell membrane separating a bone fluid compartment from the extracellular fluid and that vitamin D acts upon the cells forming this membrane to alter the influx of calcium between these two compartments (Talmage, 1969).

Little is known about the process of bone mineral mobilization in which $1,25(OH)_2D_3$ has a direct role. It is clear that physiologic doses of $1,25(OH)_2D_3$ bring about the mobilization of mineral from bone (DeLuca and Schnoes, 1976). In vivo, this process required the presence of parathyroid hormone (PTH) (Garabedian et al. 1974), whereas in organ culture, $1,25(OH)_2D_3$ stimulates bone resorption in the absence of added PTH. It was also shown that $25(OH)D_3$ could stimulate bone resorption but was 100 times less potent than $1,25(OH)_2D_3$ whilst vitamin D itself was inactive (Raisz et al. 1972; Reynolds et al. 1973).

Very little is known of the actual mechanism of action of $1,25(OH)_2D_3$ in bone, but radiolabelled $1,25(OH)_2D_3$ has been shown to become concentrated in the nuclei of bone cells from rachitic chicks (Weber et al. 1971), and a specific receptor protein for $1,25(OH)_2D_3$ has been identified in chick calvaria cytosol (Kream et al. 1977). The stimulation of bone resorption by $1,25(OH)_2D_3$ has been shown to require de novo protein synthesis (Tanaka and DeLuca 1971; Norman and Henry, 1974).

CaBP, a protein induced by $1,25(OH)_2D_3$ is found in bone tissue where $1,25(OH)_2D_3$ can cause a twenty fold stimulation of CaBp levels, (Christakos et al. 1979). It may therefore have a role to play in $1,25(OH)_2D_3$ induced bone resorption.

1.5.5 Role of vitamin D in kidney

The role of vitamin D in the kidney is one of its most complex and poorly understood functions. The complexity arises because of three inter-related factors. First, and in contrast to the intestine, the most important action of vitamin D on the kidney is on phosphorus re-absorption. Although an effect on calcium excretion can be shown, it is much less significant since about 99% of calcium in the glomerular filtrate is normally re-absorbed (Chen and Newman 1955). Second, the 1-hydroxylase activity of the kidney is influenced to some extent by variations in intracellular calcium and phosphorus levels so that changes in

the concentration of these two ions are necessary for $1/25(OH)_2D_3$ synthesis (Horinchi et al. 1974; Baxter and DeLuca 1976). Third, PTH inhibits phosphorus re-absorption (Samiy et al. 1960 and Samiy et al. 1965) and, at the same time, seems to be necessary for the effect of $1,25(OH)_2D_3$ on this same process (Garabedian et al. 1972).

In experiments using thyroparathyroidectomised animals, Puschett et al. (1972) and Steele et al. (1975) have shown that relatively small doses of vitamin D and its metabolites can cause increased calcium re-absorption. However, even in the absence of $1,25(OH)_2D_3$ and PTH, calcium re-absorption from the glomerular filtrate is very efficient (Kleeman et al 1961) and the contribution of $1,25(OH)_2D_3$ stimulated calcium re-absorption in the kidney to calcium homeostasis is, therefore, likely to be small.

Attempts have been made to demonstrate that $1,25(OH)_2D_3$ is the most potent of the cholecalciferol metabolites in maintaining phosphorus homeostasis, but the effect of $1,25(OH)_2D_3$ on phosphorus re-absorption is controversial, with claims for a phosphaturic action of the hormone (Bonjour et al. 1977) and for no effect (Steele et al. 1975). Part of the explanation for the different response might be methodological: Bonjour and colleagues (1977) studied the chronic effect of small amounts of $1,25(OH)_2D_3$ in normal rats, whereas others assessed acute effects in vitamin D-deficient rats.

Plasma calcium concentration can affect the processing of phosphorus by the kidney. Lavender and Pullman (1963) showed that the short-term increase in plasma calcium into one renal artery was capable of inducing an increase in reabsorption of urinary phosphorus. Eisenberg (1965) found the opposite in the long-term increase in plasma calcium.

$1,25(OH)_2D_3$ induced CaBP has been identified in chick and rat kidney (Lawson and Wilson, 1974; Christakos and Norman, 1981 and Simpson et al. 1980) which supports the view that the kidney is a target tissue for this hormone.

1.5.6 Control of $1,25(OH)_2D_3$ synthesis

The production of $1,25(OH)_2D_3$ is catalysed in the kidney by the enzyme 1αoxygenated-25-hydroxycholecalciferol (1-hydroxylase). The activity of this enzyme is finely controlled by numerous hormones and ionic factors. Although several physiological factors have been implicated in the regulation of 1-hydroxylase activity *in vivo*, relatively few have been shown by *in vitro* studies to have direct effects on renal $1,25(OH)_2D_3$ synthesis. These factors and their actions, where known, will be discussed in detail below.

A. Calcium

$1,25(OH)_2D_3$ can be considered as the calcium homeostatic steroid hormone (Figure 1.7). It is produced in relatively large quantities when calcium requirements are high and, conversely, when calcium needs are low, $1,25(OH)_2D_3$ levels fall dramatically. Studies carried out *in vivo* showed that, at low plasma concentration of calcium, the 1-hydroxylase activity was high and that it declined as the calcium concentration increased. Concomitant with this declined in $1,25(OH)_2D_3$ formation, there was an increase in the amounts of $24,25(OH)_2D_3$ formation (Boyle et al. 1971). Attempts to reproduce these effects *in vitro* produced conflicting results, frequently being the reverse of those obtained *in vivo*. In some cases, the addition of calcium to kidney mitochondrial preparations at concentrations ranging from 10-100 μM inhibited the 1-hydroxylase by 30-50% (Fraser and Kodicek 1973; Henry and Norman, 1974). However, in other studies, calcium seemed to have a stimulatory effect on the 1-hydroxylase (Bickle et al. 1975; Horiuchi et al. 1975). A difference between these two sets of findings was the inclusion of EDTA in media used for the preparation of the mitochondria in the latter experiments.

In considering the effects of calcium on the 1-hydroxylase *in vivo*, the actions of PTH must be taken into account, as PTH secretion is very sensitive to varying calcium levels. Thus, hypocalcaemia will stimulate PTH release which will then stimulate

the 1-hydroxylase and therefore calcium will be affecting the 1-hydroxylase indirectly. It has been shown, however, that thyroparathyroidestomised rats fed low calcium diets favoured the production of $1,25(OH)_2D_3$ from $25OHD_3$ suggesting that calcium has an effect on the 1-hydroxylase independent of PTH (Larkins et al. 1973).

B. Phosphorus

The effect of phosphate on the renal 1-hydroxylase has been less thoroughly investigated than that of calcium. Low phosphorus diets and thus hypophosphataemia have been shown to stimulate the renal production of $1,25(OH)_2D_3$ *in vitro* (Trechsel et al. 1979; Baxter and DeLuca 1976), and to raise the circulating levels of $1,25(OH)_2D_3$ *in vivo* (Tanako and DeLuca 1973) (Figure 1.8).

Furthermore, thyroparathyroidectomised animals which were then made hypophosphatemic by dietary phosphorus deprivation had increased *in vivo* production of $1,25(OH)_2D_3$ even in the absence of parathyroid glands. This result has been confirmed by direct analysis of plasma levels in animals (Hughes et al. 1975; Haussler et al. 1977). An explanation for the high plasma $1,25(OH)_2D_3$ levels observed by Tanaka and DeLuca (1973) has now apparently been given by Ribovich and DeLuca (1978). They suggested that there is a decreased turnover of $1,25(OH)_2D_3$ and increased accumulation of both plasma and intestines of phosphate deprived animals.

The mechanism by which phosphate depletion stimulates the 1-hydroxylase is not known. However, it has been shown that the increase in plasma $1,25(OH)_2D_3$ in response to dietary phosphorus restriction can be completely blocked by hypophysectomy (Gray, 1981b). Gonadal hormones and PTH are apparently not required (Hughes et al. 1975; Gray 1981a), but recent studies (Gray and Garthwaite, 1985) indicated that the growth hormone is required for the stimulation by phosphate depletion of $1,25(OH)_2D_3$ synthesis. Although growth hormone is thought to be required, its

FIGURE 1.7 : A SIMPLE MODEL SHOWING THE CALCIUM HOMEOSTATIC MECHANISMS INVOLVING $1,25(OH)_2D_3$

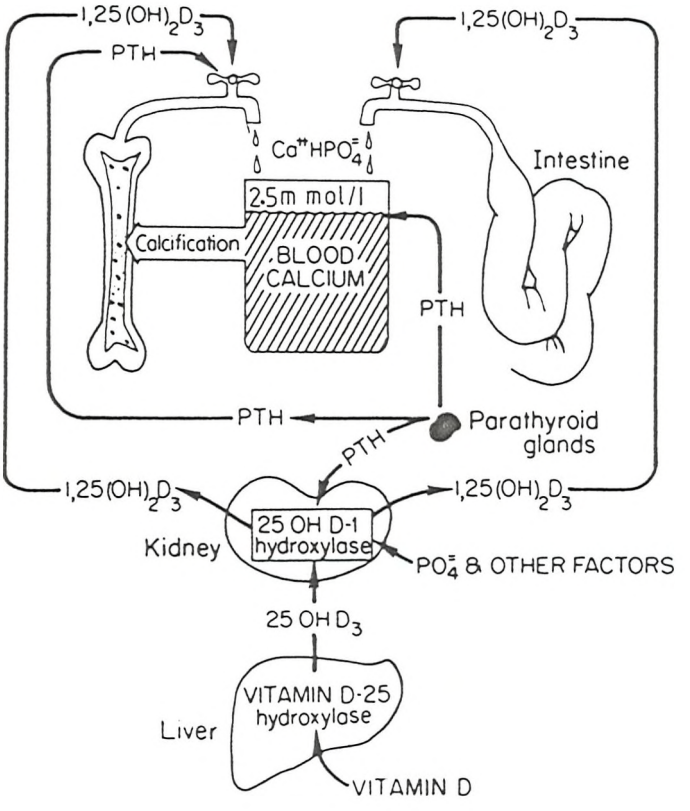
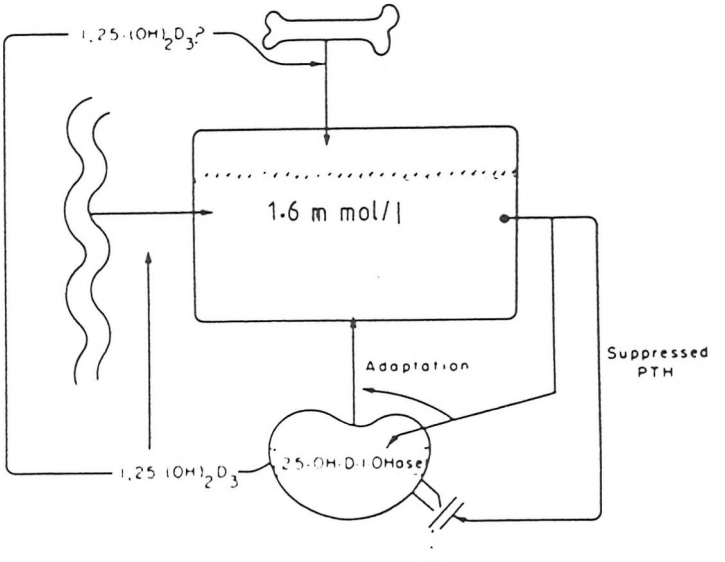


FIGURE 1.8 : A SIMPLE MODEL SHOWING THE PHOSPHORUS HOMEOSTATIC MECHANISMS INVOLVING $1,25(OH)_2D_3$



role may be purely permissive, since plasma levels of growth hormone are not elevated in phosphate deprived rats.

C. Parathyroid hormone

The single most important factor affecting the 1-hydroxylase activity is parathyroid hormone acting as a trophic hormone for $1,25(OH)_2D_3$. It seems, there is an endocrinological loop involving plasma calcium, PTH secretion and $1,25(OH)_2D_3$ formation. Any tendency for plasma calcium levels to fall results in an increased secretion of PTH (Garabedian et al. 1972; Rasmussen et al. 1972; Fraser and Kodicek 1973). This in turn stimulates the formation of $1,25(OH)_2D_3$ and there is a consequent rise in plasma calcium.

The evidence in favour of this loop is now quite extensive. Garabedian et al. (1972) showed that parathyroidectomy decreased $1,25(OH)_2D_3$ production in the rat after 40 hours, and that this decrease could be prevented by administering PTH. Baksi and Kenny (1979); and Sedrani et al. (1981) have shown that *in vivo* administration of PTH stimulated *in vitro* production of $1,25(OH)_2D_3$. Convincing evidence that PTH stimulates the 1-hydroxylase directly has also been obtained by *in vitro* studies using isolated renal tubules (Rassmussen et al. 1972), cultured kidney cells (Trechel et al. 1979) and kidney slices (Rost et al. 1981). In the latter study, evidence was obtained for the involvement of cyclic AMP in stimulating the 1-hydroxylases.

D. Vitamin D and its metabolites

The increased renal 1-hydroxylase observed in vitamin D-deficient animals can be inhibited by $1,25(OH)_2D_3$ and this has led to the hypothesis that $1,25(OH)_2D_3$ regulates its own synthesis (Larkins et al. 1975; DeLuca and Schnoes 1983). $1,25(OH)_2D_3$ when administered to chicks *in vivo*, inhibits 1-hydroxylase activity (Horiuchi et al. 1974; Tanaka et al. 1975). Co-incident with this

depression in 1-hydroxylase activity there was a rise in 24-hydroxylase (Tanaka et al. 1975; Colston et al. 1977, Trechsel et al. 1979). The mechanism of this production inhibition is poorly understood but the response to $1,25(OH)_2D_3$ are known to be blocked by RNA and protein synthesis inhibitors (Colston et al. 1977). The latter authors have suggested that $1,25(OH)_2D_3$ may act by inducing proteins concerned with calcium transport and thereby increasing intracellular calcium levels and inhibiting the 1-hydroxylase. This hypothesis, however, remains to be substantiated.

E. Sex steroids

There is a considerable clinical evidence that sex hormones and in particular oestrogens play an important role on calcium homeostasis by stimulating the renal production of $1,25(OH)_2D_3$ (Rasmussen and Bordiev, 1974). Oestradiol has been shown to act *in vivo* to stimulate 1-hydroxylase activity in both Japanese quail and chicks (Tanaka et al. 1976; Baski and Kenhy 1978; Sedrani et al. 1981). However, in experiments using castrated male chicks, Tanaka et al. (1976) could obtain no stimulation of the 1-hydroxylase in response to oestradiol benzoate alone, although a large stimulation was observed when testosterone was given together with the oestrogen. In subsequent experiments, Tanaka et al. (1978) showed that both progesterone and testosterone can act synergistically with oestradiol to stimulate the 1-hydroxylase.

While the importance of oestradiol as a major regulator of $1,25(OH)_2D_3$ synthesis is clear, the mechanism by which oestradiol stimulates the 1-hydroxylase is not known. Recent studies have resulted in the discovery of the high affinity, low capacity 17B-oestradiol receptors in rat kidney (Murono et al. 1979), which are probably located in the proximal tubule cells (Stumpf et al. 1980).

F. Prolactin and other hormones

Prolactin has been reported to stimulate the 1-hydroxylase (Baski and Kenny 1977) and to elevate plasma $1,25(OH)_2D_3$ levels in chicks (Spanos et al. 1976). The physiological importance of this effect is, however, only poorly understood.

Other factors which have been implicated in the regulation of the 1-hydroxylase include calcitonin, growth hormone/insulin and glucocorticoids. While these factors are being investigated, it seems unlikely that any of them will prove to be as important as PTH, calcium and phosphorus, and $1,25(OH)_2D_3$, which are at present considered to be the major regulators of $1,25(OH)_2D_3$ production.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Chicks

Day old male layer and broiler chicks were used in the following studies. Chicks were housed in an electrically heated battery unit. Artificial lighting was provided by tungsten filament bulbs throughout the entire series of experiments for 16 hours per day between 6.00 a.m. and 10.00 p.m. The chicks were raised until they were four weeks of age.

2.2 Diets

Various diets were used in the following studies. Calcium, total phosphorus and vitamin D_3 were the main variations in these diets. The composition of the basal diet that was fed to the chicks from 0 to 4 weeks is shown in Table 2.1. This basal diet contained no feedstuffs of animal origin and provided 12.6 Mj/kg metabolizable energy, 195 g/kg digestible crude protein, 1.5 g/kg calcium and 5 g/kg total phosphorus, of which 2.4 g/kg was phytate phosphorus. Mineral and vitamin mixtures provided vitamin A (2700 i.u/kg), ZnO (120 mg/kg) and $MnCO_3$ (120 mg/kg). Oystershell was used as a source of calcium and sodium dihydrogen phosphate ($NaH_2PO4^2H_2O$) was added as inorganic phosphorus to the control diets. Two grams of chromic oxide (Cr_2O_3)/kg was added and mixed well with all the diets. The chromic oxide was used as a marker to correct for bulk gut secretions and absorptions. The diets were mixed before the experiment was started, samples from the diets were assayed for calcium, total phosphorus, phytate phosphorus and chromium.

2.3 Sample collection

2.3.1 Blood sampling

Chicks were killed by decapitation and trunk blood was collected into heparinsed tubes. The tubes were centrifuged at

2000 g for 10 minutes and the plasma decanted from the blood cells and kept frozen at -20°C until required for assay. Where more than one blood sample was required from each chick, blood was collected from the wing vein using a 23 gauge needle and a 2 ml disposable plastic syringe primed with 50 iu of lithium heparin (see 2.5.1).

2.3.2 Sampling of droppings

The electrically heated battery unit has many cages, beneath each cage there is a tray. The droppings voided by the chicks of all groups were collected daily for three days. The trays were cleaned at 8.00 a.m. and the droppings were collected at 6.00 p.m. in polythene bags. Each days collection was stored at -20°C. Samples for three days collection from each groups were accumulated and oven dried (90°C for 24 hour) and ground in a Moulinex 531 grinder.

Droppings were analysed for calcium, total phosphorus, phytate phosphorus and chromium. In the determination of apparent digestibility of a nutrient by the indicator ratio technique, one needs to know only the concentrations of the indicators and nutrient in both the diet and the droppings. The digestibility of a nutrient is most accurately defined as that proportion which is not excreted in the droppings and which is, therefore, assumed to be absorbed by the chick. Apparent digestibility was calculated by using the formula of Mueller (1956). It is commonly expressed as a percentage.

$$\text{Digestibility} = \frac{\dfrac{\text{level of any substance in the diet}}{\text{level of chromium in the diet}} - \dfrac{\text{level of any substance in the dropping}}{\text{level of chromium in the dropping}}}{\dfrac{\text{level of any substance in the diet}}{\text{level of chromium in the diet}}} \times 100$$

2.4 Determination of total plasma calcium

Plasma total calcium measurements were made using a Perkin-Elmer 280 series atomic absorption flame spectrophotometer at 423 nm with a rich acetylene/air frame. 50 µl of plasma was added to 100 µl of 5% Lanthanum chloride and made up to 5 ml with de-ionized water and vigourously shaken. The samples were then read against calcium standards (0.5-4 µg/ml), prepared from analar calcium carbonate in a 0.1% Lanthanum chloride solution. The Lanthanum chloride is used to reduce interferences by the flame ionisations and phosphate contamination.

2.5 Measurement of plasma ionized calcium

Ionized calcium was measured in plasma samples by a method devised by Luck and Scanes (1979) which makes use of a calcium ion senstive electrode.

2.5.1 Collection of blood samples

Blood samples were collected using an anaerobic technique to draw 2 ml blood by plastic disposable syringe containing 50 i.u of lithium heparin in 100 µl of tris buffer (Table 2.2). Heart puncture was used to collect blood from layer chicks, because the veins were too small but in the broiler type of chick, wing vein puncture was used. Samples were collected by an anaerobic technique because ionized calcium measurements are sensitive to pH changes. After withdrawal, the syringes were capped, the samples well mixed, and the syringes centrifuged at 2000 g for 10 minutes with the cap towards the centre. The samples were then stored in the upright position either at room temperature and assayed within 2 hours or stored in the refrigerator for up to 10 hours and then brought the room temperature prior to ionized calcium measurement.

2.5.2 Assay procedure

Ionized calcium measurements were made using a Radiometer calcium selectrode (F2112) adapted for flow through operation which

TABLE 2.1: COMPOSITION OF EXPERIMENTAL DIET (g/kg)

maize	610
soybean meal	333
dried yeast	50
sodium chloride	5
methioine	1
mixed minerals and vitamins	1

TABLE 2.2: PLASMA IONIZED CALCIUM ASSAY BUFFER

Components	Weight g/I
Tris	13.31
Nacl	8.12
Kcl	0.36
$Mgcl_2$	0.26
Na/v_3	1

The mixture was equilibrated to pH 7.4 by Hcl and stored at room temperature

was placed in series with a reference calomel electrode. Both electrodes were connected to a digital pH meter set to read relative millivolts. The arrangement of the electrodes is shown in Figure 2.1. In between samples, tris buffer was flushed through the system by an LKB peristaltic pump at a rate of approximately 5 ml/min. This acted as a wash and as a zero reference. To make a sample reading, the pump was switched off and 0.25 ml of the sample was injected evenly over a period of 5 seconds, then the reading was taken after 5 seconds and the system flushed through once more. Samples were measured in duplicate. Calcium chloride at 0.5, 1.0, 1.5 and 2.0 mM in tris buffer was used as standards. Standards were injected in the same way as the samples at the beginning and at the end of each assay. Mean values were used to plot a standard curve of relative Mv against calcium ion concentration mM.

2.6 Measurement of total plasma phosphorus

Phosphorus in the plasma was measured by the method of Goldenberg and Fernandez (1966). This method employs two reagents:

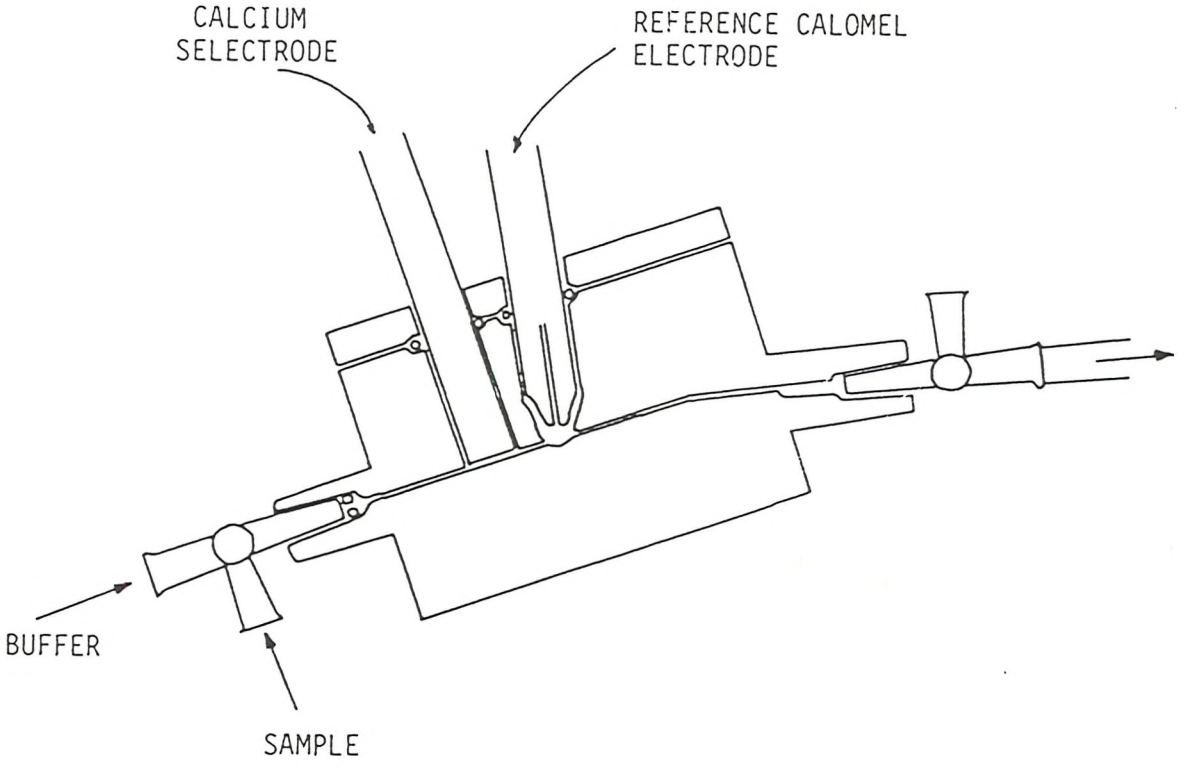
1 - Iron-TCA, reagent:
50 g of trichloroacctic acid was dissolved in 400 ml distilled water and 5 g thiourea was added followed by 15 g ferrous ammonium sulphate, the mixture was made up to 500 ml and stored in an amber bottle.

2 - Molybdate reagent:
45 ml concentrated sulphuric acid was added with cooling to 200 ml distilled water; 22 g ammonium molybdate was dissolved in 200 ml distilled water; the two solutions were mixed and made up to 500 ml. This reagent remains stable for several years.

Assay procedure

100-200 µl plasma was placed in the test tube and 5 ml of iron-TCA reagent was added to deproteinize the plasma. The mixture was shaken, then allowed to stand for 10 minutes and centrifuged. The

FIGURE 2.1 : THE ARRANGEMENT OF ELECTRODES USED FOR IONIZED CALCIUM MEASUREMENT



supernatant was decanted into a clean tube and 0.5 ml of molybdate reagent was added and mixed by inversion. The blue colour developed rapidly and was measured after 20 minutes against (2.5 - 10 µg/ml) of phosphorus standard, (0.2197 g $kH_2PO4/1$) plus 5 ml of iron-TCA and 0.5 ml of molybdate reagent. The SP8-400 spectrophometer was used to read the standards and the samples at 660 mu wavelength.

2.7 Determination of plasma zinc

Plasma was diluted with de-ionized water (1:4 v/v) and analysed for zinc by atomic absorption flame spectrophotometer (Perkin-Elmer 280 series) at 214 nm wavelength. Plasma concentrations of zinc were measured against zinc standard solutions ranging from 0.25 µg/ml to 1 µg/ml of Zn^{++} as zinc sulphate, $ZnSo_4.7H_20$ in de-ionized water.

2.8 Determination of plasma magnesium

Plasma magnesium measurements were made using a Perkin-Elmer 280 series atomic absorption flame spectrophotometer operating at 285 nm wavelength. 50 µl of plasma was added to 50 µl 5% Lanthanum chloride and made up to 1.5 ml with de-ionized water. Standards were prepared from Analar Magnesium Sulphase, ($MgSO_4.7H_20$), ranging from 0.25 µg/ml to 1 µg/ml of Mg^{++}.

2.9 Measurement of total phosphorus in diets and droppings

Total phosphorus was determinated by the method of Hanson (1950). The composition of Hanson reagent is:

1 - 140 ml concentrate nitric acid (HNO_3)
2 - 1.0 g ammonium vanadate ($NH4VO_3$)
3 - 20 g Analar ammonium molybdate ($(NH_4)_6MO_7O_{24}.4H_2O$)

The reagent was made up as follows:

The ammonium molybdate was dissolved in about 400 ml distilled water at about 50°C, and then the solution was cooled. The ammonium vanadate was dissolved in about 300 ml of boiling distilled water, cooled and the nitric acid added gradually with stirring. The ammonium molybdate solution was then added gradually to the other solution with stirring. The mixed solution was finally diluted to one litre with distilled water.

Assay procedure

It is possible to determine phosphorus, calcium and chromium, if necessary, in the same sample. A known weight of diet to gut digesta or droppings (100 - 1000 mg) was wet digested with (0.5 - 5 ml) of concentrated nitric acid in a 50 ml conical flask on a heating block. The sample was heated to near dryness and (0.4 - 4ml) 600 g/l of perchloric acid added. The sample was boiled gently for twenty minutes. This second digestion was necessary to oxidise the green insoluble chromic oxide to the soluble yellow/orange chromate and dichromate. The sample was cooled and approximately 20 ml distilled water was added. A few grains of acid washed sand was added to prevent bumping. The sample was gently reheated to hydrolyse any pyrophosphate that may have been formed. The sample was diluted (50 - 250 ml) with distilled water in a volumetric flask. To measure the phosphorus, an aliquot (0.2 - 0.5 ml) of the diluted sample was pipetted into small test tubes, made up to 2.5 ml with distilled water and 1.25 ml of Hanson (1950) reagent added. The mixture was allowed to stand for five minutes for development of the yellow colour and read in Sp-1800 Ultraviolet spectrophotometer at 400 mu. The spectrophotometer was zeroed on a distilled water blanch and a standard curve was prepared from 3.83 g/l Analar potassium dihydrogen phosphate (kH_2PO_4) solution. The range of the standard phosphorus solution was (5-25 µg/ml).

2.10 Determination of chromium in diets and droppings

0.5 ml from the same sample prepared to measure the total phosphorus was used to determine chromium by use of the atomic

absorpiton flame spectrophotometer (Perkin-Elmer 280) operating at 358 nm, using a rich air/acetylene flame. Samples were read against known standards in the range 0.5 - 4.0 µg/ml. The standards were prepared from Analar potassium chromate (3.735 g K_2CrO_4/l). All solutions contained 2% ammonium chloride to remove various ion flame interferences.

2.11 Measurement of calcium in diets and droppings

Diets or droppings were finely ground. Two grams of each sample was ashed in a furnace at 600°C overnight. Four ml of concentrated nitric acid was added to each sample. After considerable dilution with distilled water, samples were measured against standard calcium solutions (0.5 - 4.0 µg/ml) by atomic absorption flame spectrophotometer (Perkin-Elmer 280) at 423 nm in an air/acetylene flame. All solution to be finally made up in a 0.1% Lanthanum chloride to eliminate interference by phosphorus absorption.

2.12 Measurement of phytate phosphorus

Phytate-phosphorus was determined using a modified version of the micro-method developed by Oshima, et al. (1964). Phytate was extracted from ground samples of dried diet or dropping (0.5g) by shaking continuously with 10 ml of 0.5 M nitric acid for 3 hours at room temperature. The extract was centrifuged and 5 ml aliquots of the supernatant placed in test tubes. The tubes were heated for 15 minutes in a boiling water bath, then cooled to room temperature; one ml of ferric ammonium sulphate containing 40 µg of iron was added. The tubes were sealed and placed again in a boiling water bath for a further 60 minutes to speed up the formation of the ferric phytate complex which appeared as precipatate. The tubes were cooled and centrifuged for 10 minutes at 2000 g and the precipates were digested with a mixture of concentrate sulphuric acid and nitric acid (1ml of each). A few grains of acid washed sand were added to prevent bumping. The tubes were left to stand for one hour in a fume cupboard, then the

digestion was continued in a preheated drilled aluminium block at 275°C for a further 45 minutes, during which time all the nitric acid was volatilized. After the mixture had been cooled, water was added and the diluted digest, together with washings, were transferred to a 50 ml volumetric flask. The phosphorus was determined by the method of Hanson (1950), as described previously (Section 2.9).

2.13 Bone ash analysis

The chicks were sacrificed by decapitation. The right tibia bone was removed from the carcass and cleaned of all soft tissue. The tibia were dried in an oven and then were extracted for 24 hours with hot ethanol to remove the fat. The fat free tibia were dried at 80°C. The fat free tibias were weighed in preweight ashing crucibles and ashed at 600° for 8 hours. The ashed samples were then cooled in a dessicator and weighted. (A.O.A.C. Method, 1970).

A sample of the ash was then used for calcium, total phosphorus zinc and magnesium determination.

2.14 Measurement of intestinal phytase and alkaline phosphatase

2.14.1 Preparation of tissue

Phytase and alkaline phosphatase activities in the chicken small intestine were determined by the method of Davies and Flett (1978). Three sections were taken from the intestine (a) the first 10 cm of the duodenum (b) the first 10 cm of the jejunum (c) the first 10 cm of the ileum. These sections were carefully washed in assay buffer, turned inside out and washed. A mucosal scrape was taken using a glass microscope slide from the duodenum, jejunum and ileum. The mucosal samples were homogenised separately in five volumes of pH7.4 buffer (Table 2.3) in a Ten-Broeck glass homogeniser. For determination of alkaline phosphatase activity, a portion of these homogenates was diluted in a further 5 volume buffer (1:5). Small samples of both diluted

homogenate were taken and frozen at -20°C for later protein analysis.

2.14.2 Determination of phytase activity

The following incubation procedure was used; assay tubes contained 0.3 ml of 8.33 mM sodium phytate, 2.5 ml of pH7.4 50 mM Tris/succinate buffer, which also contained 0.5 mM $MgCl_2$ (table 2.3). The incubation was performed at 37°C in a water bath and was initiated by the addition of 0.2 ml of the concentrated homogenate in duplicate. The incubation lasted 30 minutes and was stopped by the addition of 1 ml of 200 g/l trichloroacetic acid (TcA) solution. After bench centrifugation, the supernatant was analysed for its phosphorus content by the same procedure described in section 2.9. Reagent blanks (no enzyme added) and enzyme blanks (no substrate added) were also performed and subtracted from the complete incubation. Protein in the concentrated homogenate was determined by Biuret method (see 2.14.4). Results were finally expressed as µmol of phosphorus liberated from phytate/mg protein/hour. The details of this assay procedure have been summarized diagrammatically in Figure 2.2.

2.14.3 Determination of alkaline phosphatase activity

A very similar incubation procedure was used as for the phytase assay. 0.3 ml of 50 mM B-glycerophosphate was used as substrate instead of the phytate, and 0.2 ml of the dilute homogenate was used. The incubation time was only 15 minutes, but in all other respects the assays were identical. The units of alkaline phosphotase activity were expressed as µmol of phosphorus liberated from B-glycerophosphate/mg protein/ hour.

TABLE 2.3: PHYTASE AND ALKALINE PHOSPHATASE ASSAY BUFFER

50 mM Tris/succinate buffer pH 7.4

Compounds	Weight g/l
Tris	6.06
Nacl	24.81
Kcl	1.28
Succinic acid	5.90
$Mgcl_2$	0.10

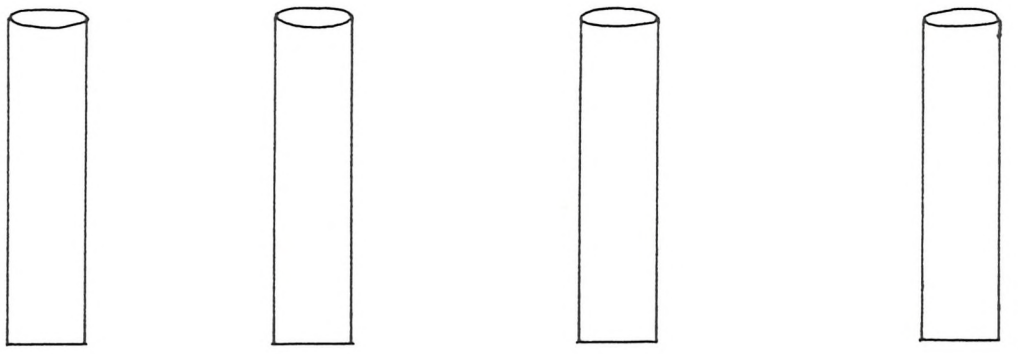
2.14.4 Determination of protein

Protein concentration in the intestinal mucosal homogenates was measured by the Biuret method. 1 ml of normal or diluted homogenate containing 1-10 mg protein was added to 4 ml of Biuret reagent, mixed using a Rotamix and then to stand at room temperature for 30 minutes. A standard was constructed using the same procedure but using 1-10 mg of BSA as the standard protein instead of homogenate. All tubes were read in a spectrophotometer at 540 nm, and from the optical densities, protein concentrations in the homogenates were determined. The Biuret reagent was made up of 1.5 g hydrated cupric sulphate ($CUSO_4$-$5H_2O$) plus 6 g sodium/potassium tartrate and made up to 500 ml with distilled water and then mixed with 500 ml of 60 g/l sodium hydroxide. This reagent was then stored at 4^oC.

2.15 Statistical analysis

All the results were analysed by one-way and two-way analysis of variance (ANOVA). The treatment means were tested for significant differences by Duncan's new multiple range test at 1% and 5% level of probability (Duncan, 1955). Kramer, (1956) method's were used to compare the effects of treatments which have been replicated unequally.

FIGURE 2.2: SCHEME OF PROCEDURE FOR THE STUDY OF THE INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE ACTIVITIES



Tubes for estimation		Reagent blank-tube (no enzyme added)	Enzyme blank tube (no substrate added)
2.5 ml buffer	2.5 ml buffer	2.5 ml buffer	2.5 ml buffer
+0.3 ml phytate	0.3 ml phytate	0.3 ml phytate	0.3 ml H_2O
+0.2 ml homogenat	0.2 ml homogenat	0.2 ml H_2O	0.2 ml homogenate

CHAPTER THREE

PRELIMINARY INVESTIGATION OF THE INFLUENCE OF VARYING CONCENTRATION OF CALCIUM AND VITAMIN D_3 ON THE UTILIZATION OF PHYTATE PHOSPHORUS BY LAYER CHICKS

3.1 Introduction

Phosphorus is derived by the hydrolysis of a number of organic compounds present in cereals and seeds of which phytate is the predominant form. This process is one of the least understood and most debated subject in the field of mineral nutrition. However, phytate phosphorus is poorly available to chicks, thus making it necessary for costly inorganic forms of phosphorus to be added to the diets. If the organic phytate phosphorus source could be utilized to a greater extent, less supplementary phosphorus would be needed resulting in considerably cheaper cereal based diets. It is generally accepted that chicks use only one-third of the phosphorus in cereals and seeds. This is based on the report by NRC (1977), that 30% of the phosphorus in plant materials is non-phytate and considered to be well utilized by chicks. Many reports have been made concerning the extent of the hydrolysis of phytate phosphorus in chicks. However, these reports present conflicting results on the extent of the utilization of phytate in chicks. This study, therefore, was undertaken to investigate the effect of varying the concentrations of dietary calcium and vitamin D_3 on the utilization of phytate phosphorus in growing chicks fed the (NRC) recommended level of total phosphorus.

3.2 Chicks and Treatments

One hundred and sixty day-old male layer strain chicks (Ross Brown) were used in this experiment. The chicks were completely randomized into four treatments. Each treatment was replicated four times, ten chicks were used per group. Treatments and the composition of each treatment are shown in Table 3.1. On arrival,

TABLE 3.1 : THE CALCIUM, PHOSPHORUS AND VITAMIN D_3 CONTENT OF THE DIETS

Treatments	Normal Calcium		Low Calcium	
	normal vit.D_3	high vit.D_3	normal vit.D_3	high vit.D_3
Calcium (g/kg)	10	10	5	5
Total phosphorus (g/kg)	6.9	6.9	6.9	6.9
Phytate phosphorus (g/kg)	2.4	2.4	2.4	2.4
Vitamin D_3 (1.U/kg)	500	50,000	500	50,000
Oystershell (g/kg)	21.21	21.21	8.71	8.71

the chicks were randomised, wing-banded and placed in an electrically heated battery; their initial weights were recorded. Diet and water were given ad-libitum for twenty eight days (see Chapter 2.2) for the basal diet used). This basal diet was supplied with ground oystershell as a source of calcium and sodium dihydrogen phosphate ($NaH_2Po_4.2H_2O$) as a source of inorganic phosphorus. Dropping collections were made daily (16.00 hours) and frozen at -20°C. At intervals of seven days, the accumulated droppings from each group (10 chicks) were dried and ground together.

Feed and dropping samples were analysed for calcium and chromium (Perkin-Elmer 280 series atomic absorption flame spectophotometer), total phosphorus (see Hanson, 1950) and phytate phosphorus by the method described by Oshimia et al. (1964).

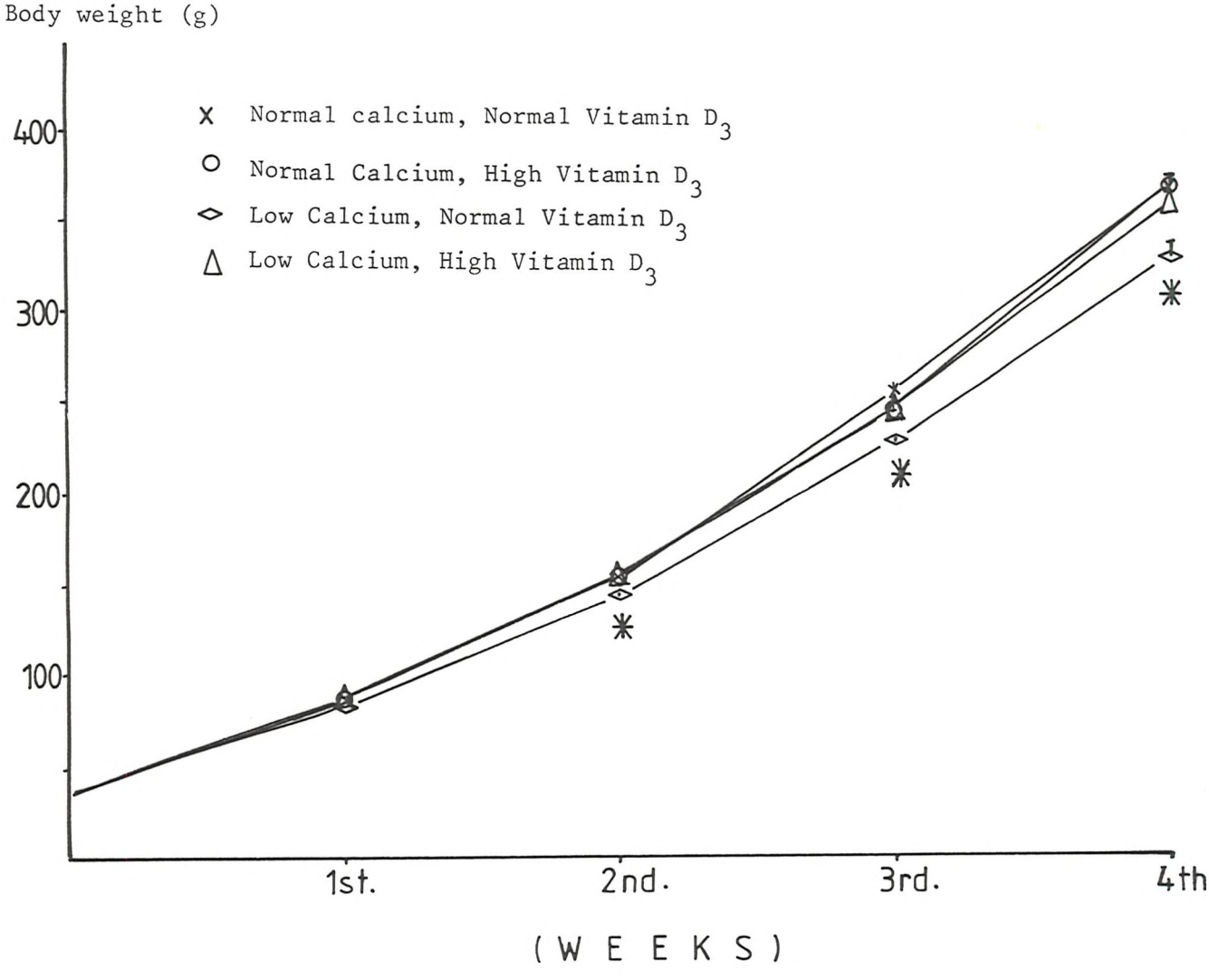
The nutrient digestibilities were determined using chromic oxide as an inert digestibility indicator (see Chapter 2.3.2). After twenty eight days, chicks were weighed, killed by decapitation and the right tibia from each chick was removed, cleaned of tissue and defatted. The tibias were dried to a constant weight and ashed. The ash percentages were calculated, and calcium phosphorus, magnesium and zinc contents were determined. These methods are described in detail in Chapter 2. All the results were subjected to statistical analysis by analysis of variance, (randomized complete block design). The results were then tested for significant differences by Duncan's new multiple range test at 1% and 5% levels of probability, (Duncan, 1955) and Kramer, (1956).

3.3 Results

3.3.1 Growth

The chicks were individually weighed weekly and the data is presented (see Figure 3.1). No significant differences were found at Week 1. However, results obtained in the 2nd, 3rd and 4th week show that only one treatment (low calcium-normal vitamin D_3)

FIGURE 3.1 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS ON THE GROWTH OF LAYER CHICKS UP TO 4 WEEKS



✳ All values for normal calcium, normal vitamin D_3 group are significantly lower than values for all other dietary treatment ($P<0.05$)

(mean ± S.E.M.)

produced a significant reduction ($P<0.01$) in weight compared with the control (normal calcium-normal vitamin D_3). In the other treatment with low calcium-high vitamin D_3, the difference in body weight was non-significant.

3.3.2 Bone growth and calcification

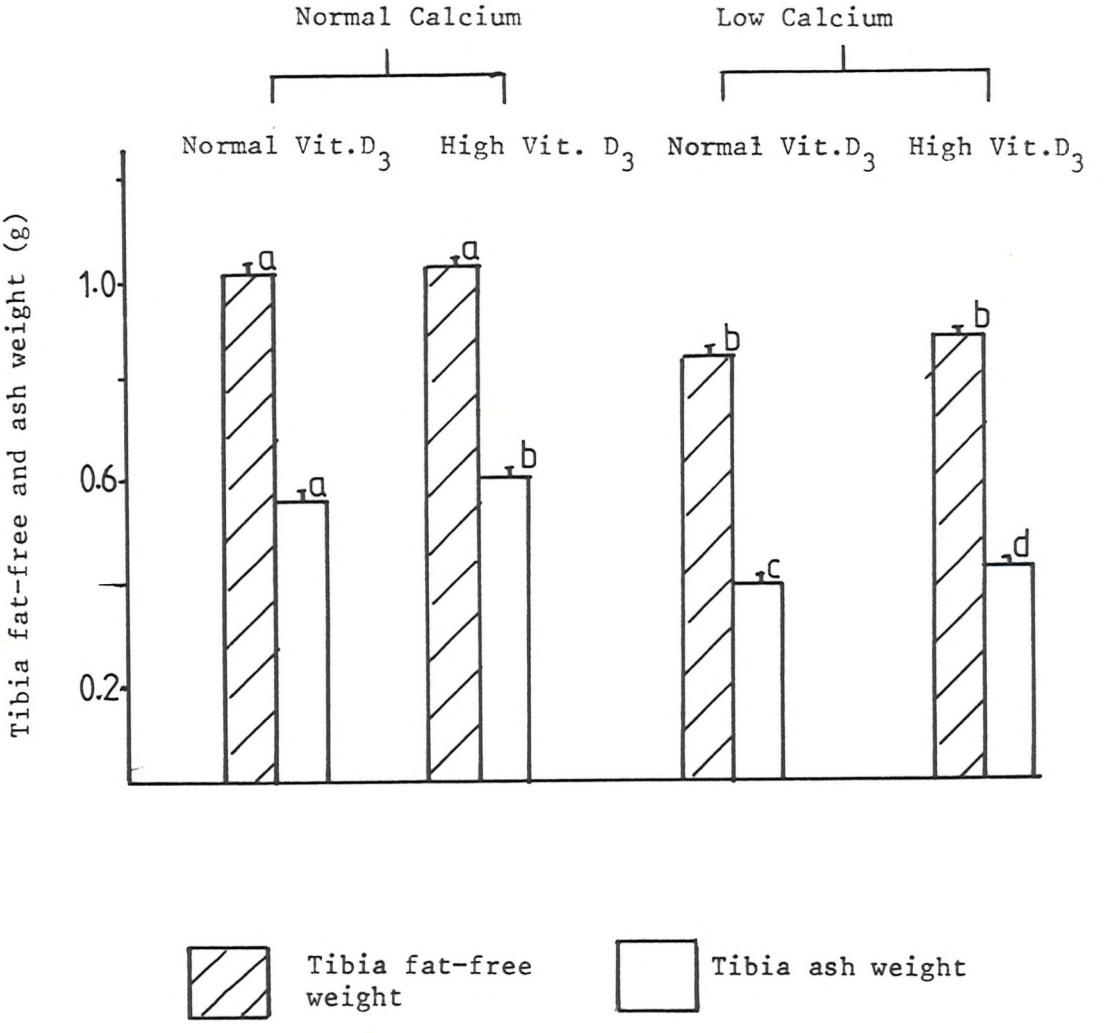
A - Tibia fat-free weight, tibia ash weight and ash percentage.

The major effects observed were associated with the level of dietary calcium. Thus, the bones from the low-calcium chicks were significantly smaller and they contained less ash than the bones from the chicks fed the diets normal with respect to calcium (Figure 3.2). Furthermore, the percentage of ash in the tibias of the low-calcium chicks was significantly less than in the normal calcium birds (Figure 3.3). Increasing the level of vitamin D had no significant effect on bone weight at either calcium level but tibia ash weight was significantly increased in both the normal- and the low-calcium chicks. Another effect of additional vitamin D was to increase the tibia ash percentage in the low-calcium chicks but not in the chicks fed on the normal-calcium diets.

B - Mineral composition of the tibia of layer chicks at 28 days of age

Varying the concentrations of calcium and vitamin D_3 in the diet of chicks did not significantly influence the levels of total phosphorus, magnesium and zinc in tibia (Table 3.2). However, chicks fed a low calcium and normal vitamin D_3 diet produced a significant ($P<0.05$) reduction in calcium levels in tibia, compared with all other treatments values.

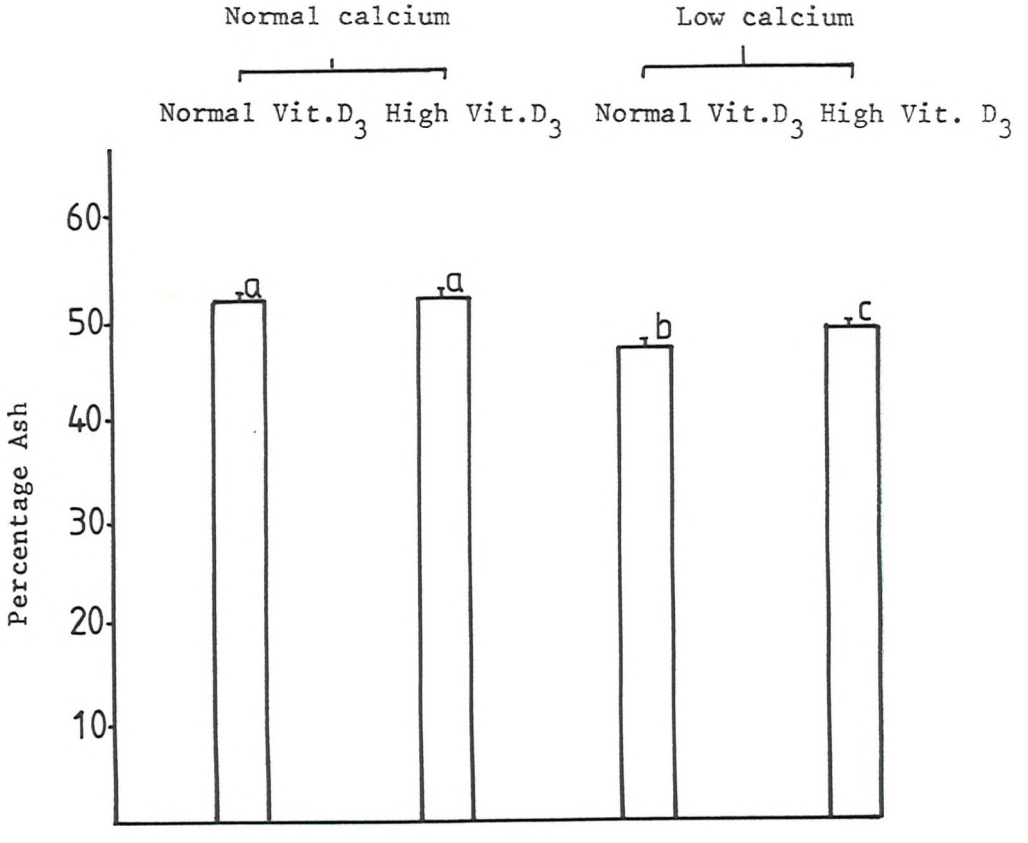
FIGURE 3.2 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS ON A TIBIA FAT-FREE AND ASH WEIGHT OF LAYER CHICKS AT 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.05$)

(Mean ± S.E.M.)

FIGURE 3.3 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS ON A TIBIA ASH PERCENTAGE OF LAYER CHICKS AT 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.05$)

(Mean ± S.E.M.)

TABLE 3.2 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS ON TIBIA MINERAL COMPOSITION OF LAYER CHICKS AT 28 DAYS OF AGE

Variables	Normal Calcium		Low Calcium	
	Normal Vit.D_3	High Vit.D_3	Normal Vit.D_3	High Vit.D_3
Calcium (mg/g dry fat-free tibia)	184.5 ± 3.74^{a}	186 ± 2.76^{a}	174.6 ± 3.06^{b}	180.7 ± 2.95^{ab}
Phosphorus (mg/g dry fat-free tibia)	87 ± 2	89 ± 2	84 ± 2	87 ± 2
Magnesium (µg/g dry fat-free tibia)	4980 ± 86	4801 ± 39	4995 ± 84	4997 ± 109
Zinc (µg/g dry fat-free tibia)	223 ± 5	224 ± 4	211 ± 5	214 ± 4

Groups without common subscripts are significantly different from each other ($P<0.05$)

Values are expressed as the mean ± S.E.M.

3.3.3 Percentage digestibility of phytate phosphorus and percentage retention, total phosphorus and calcum by analysis of droppings.

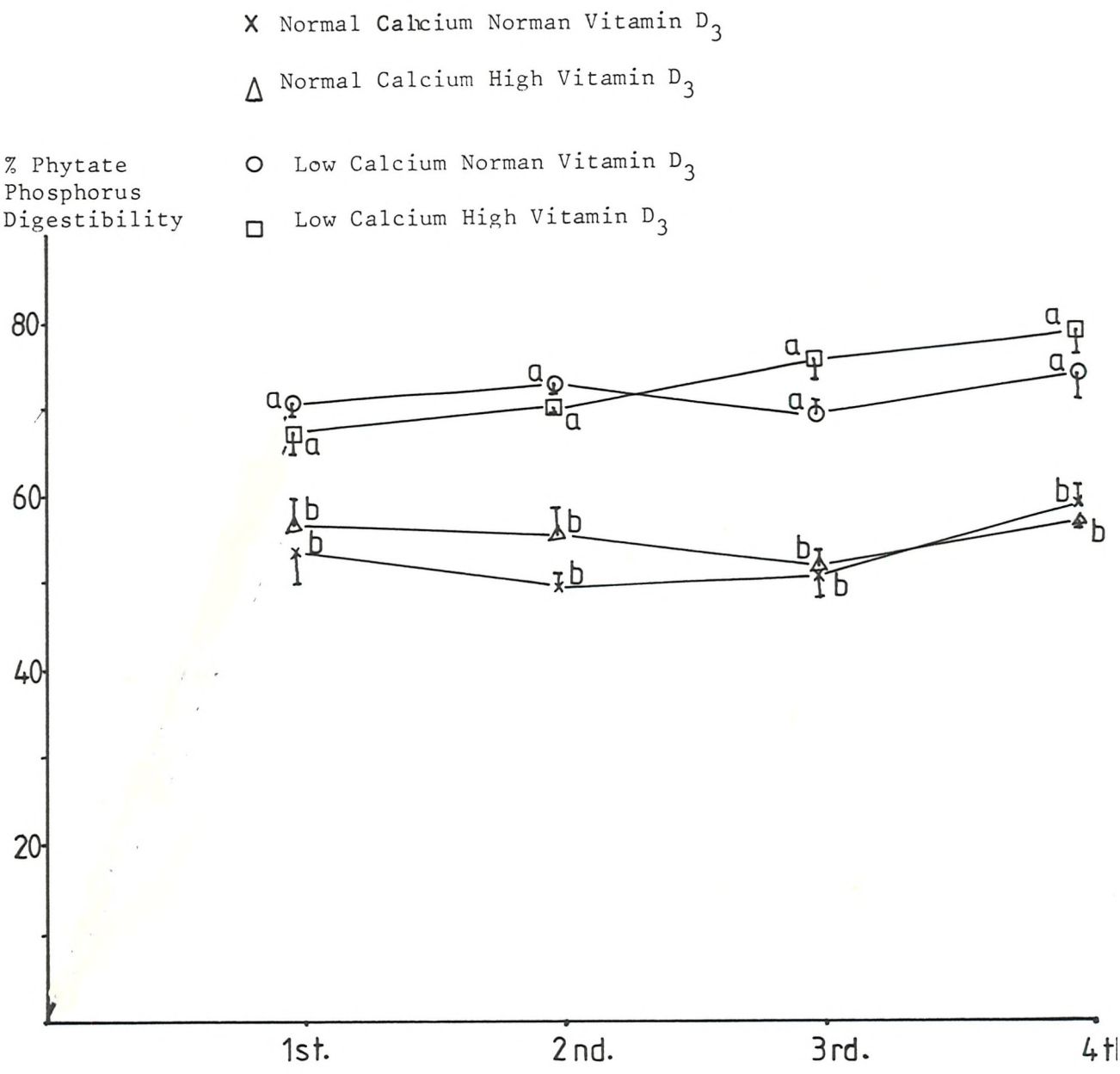
A - Phytate digestibility and total phosphorus retention

The digestibility of phytate phosphorus increased by decreasing the concentration of calcium in diet (see Figure 3.4). This effect of varying calcium concentration is observed in the 1st, 2nd, 3rd and 4th weeks of testing. The increase in phytate phosphorus digestibility with low calcium (5g/kg) treatments is significantly different ($P<0.01$) from values obtained from treatments with normal calcium (10g/kg) throughout the four weeks of experiments. Varying the concentration of vitamin D_3 (500-50000 i.u/kg) did not influence the digestibility of phytate phosphorus (Figure 3.4). However, the percent retention of total phosphorus in chicks was not significantly influenced by varying the concentration of calcium and vitamin D_3 (Figure 3.5).

B - Calcium retention

Reducing the concentration of calcium, from the (NRC) recommended 10g/kg to 5g/kg diet significantly ($P<0.01$) enhanced the percent retention of calcium in the chicks throughout the four weeks of experiment (see Figure 3.6). Increasing the concentration of vitamin D_3 in the diet only influenced the digestibility of calcium during the 4th week. The percent retention of calcium was significantly ($P<0.01$) enhanced with low levels of dietary calcium.

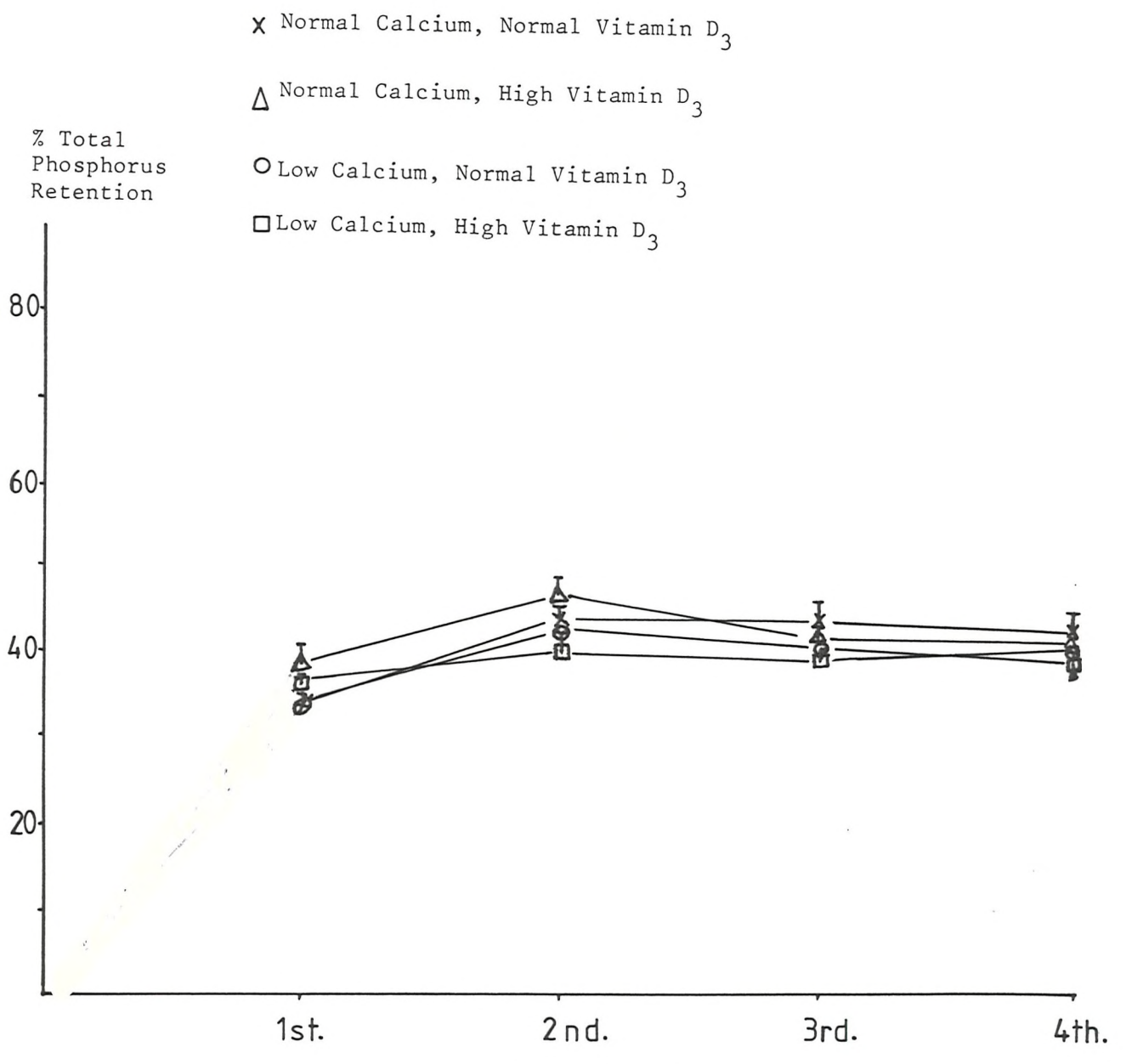
FIGURE 3.4 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS ON THE PHYTATE DIGESTIBILITY (%) OF LAYER CHICKS



Groups without common subscripts are significantly different from each other ($P<0.01$)

(Mean ± S.E.M.)

FIGURE 3.5 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS ON TOTAL PHOSPHORUS RETENTION (%) OF LAYER CHICKS



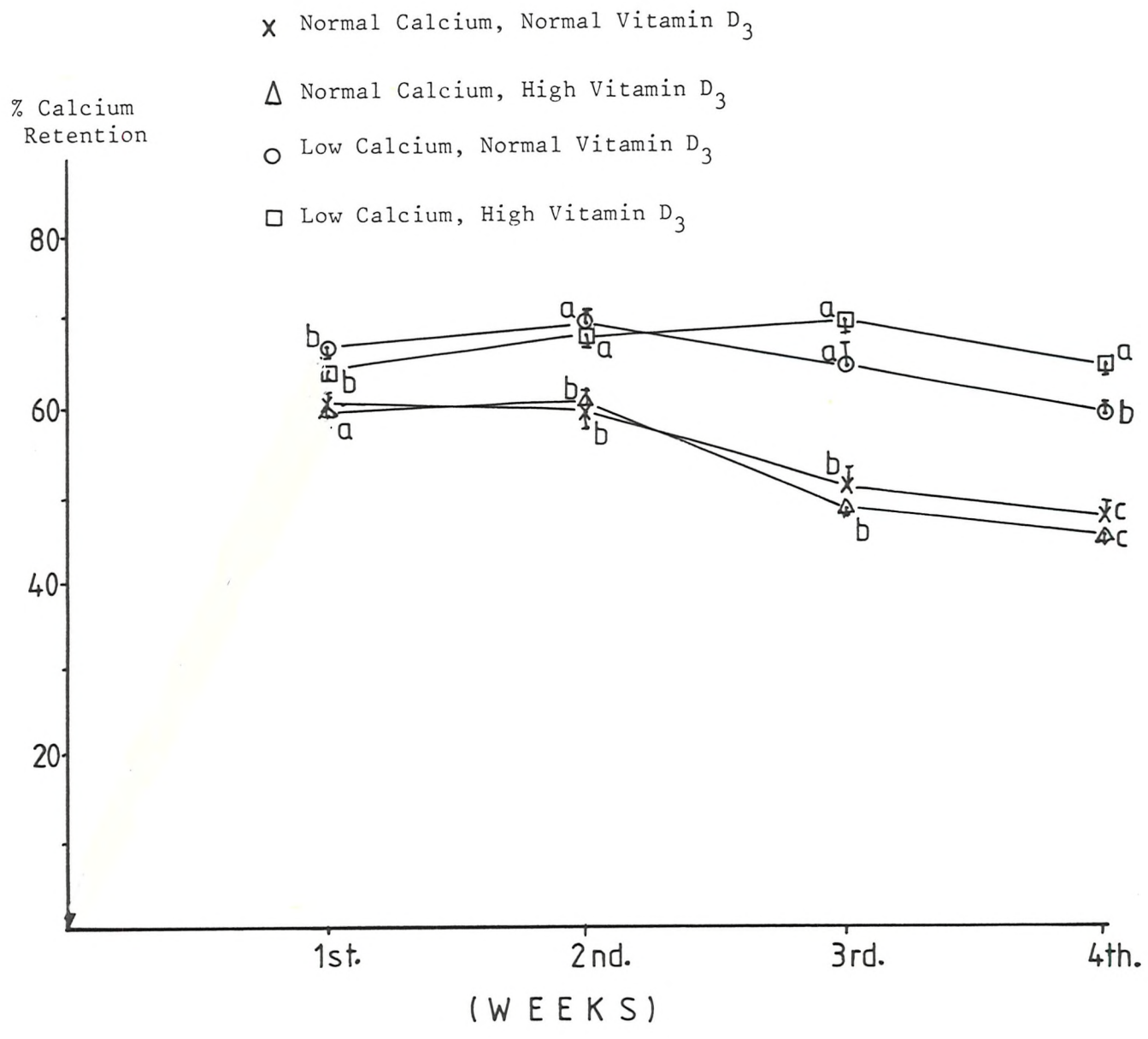
No significant difference between the treatments

(Mean ± S.E.M.)

3.4 DISCUSSION

The results of the present study show that high dietary levels (50,000 1.u/kg) of vitamin D_3 did not significantly influence the growth of layer chicks fed diets containing the recommended levels of phosphorus and calcium (NRC 1977). Waldroup et al. (1965) have also reported that high levels (7920 1.u/kg) of vitamin D_3 in an otherwise normal diet, did not influence the growth of chicks up to 8 weeks of age. The growth suppressing effect of low calcium diets containing recommended levels of vitamin D_3 did not become apparent until the second week of study, presumably due to the high concentration of vitamin D_3 in egg yolk i.e. ten times hens plasma vitamin D_3 levels (Fraser and Emtage 1976). The delay in the appearance of this growth reducing effect of low-calcium, normal-vitamin D_3 diet has also been observed by Scott et al. (1982). The reduction in growth of chicks fed diets containing low levels of calcium and normal levels of vitamin D_3, is in agreement with the data of Waldroup et al. (1963) and Nott (1967), as is the observation that high levels of vitamin D_3 will reverse this growth-reducing effect of low calcium diets. These high levels of dietary vitamin D_3 are considerably below the level of dietary vitamin D_3 (50,000 1.u/kg) previous shown to retard the growth of chicks who appear to be very resistant to vitamin D_3 toxicity (Taylor et al. 1968). Whilst high intakes of vitamin D_3 reversed the growth suppression effect of low calcium diets, they only partially improved tibia ash percent. This effect has also been observed by Waldroup et al. (1963, 1965) and Nott (1967). These authors did not examine the effects of these dietary changes on the composition of tibia ash. However, the results of the present study show that high intakes of vitamin D_3 restore the level of calcium in tibia ash in chicks fed low-calcium diets to that observed for chicks given normal-calcium diets, normal-vitamin D_3 diets, even though the tibia ash content is not fully restored. Thus, conservation of tibia ash calcium by high intakes of vitamin D_3 on low calcium diets is due to the increased absorption of calcium by chicks fed

FIGURE 3.6 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS ON THE CALCIUM RETENTION (%) OF LAYER CHICKS



Groups without common subscripts are significantly different from each other ($P<0.01$)

(Mean $\pm$ S.E.M.)

these diets. This effect is extensively reported in the literature which is reviewed by Taylor and Clarke (1984). Whilst variations in the dietary levels of calcium and vitamin D_3 did not influence total phosphorus availability, phytate phosphorus digestibility was significantly increased by low calcium diets, independent of vitamin D_3 levels. These results have also been reported by Vandepopuler et al. (1961); Harms et al. (1962) and Ballam et al. (1984a) for the chick and by Taylor and Coleman (1979) for rats.

Thus the results of this experiment show that phytate phosphorus is available to the chick and that this availability is considerably influenced by the level of dietary calcium. The potentially undesirable effects of the low calcium intakes required to maximise phytate phosphorus availability, are largely overcome by increasing vitamin D_3 intakes. These increased vitamin D_3 intakes overcome the growth suppressing effects and the lowered tibia ash calcium levels of low calcium diets. They do not, however, quite fully compensate for the reduction in tibia ash which also occurs with low calcium intakes.

CHAPTER FOUR

AN INVESTIGATION INTO THE INFLUENCE OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON THE UTILIZATION OF PHYTATE PHOSPHORUS BY YOUNG LAYER CHICKS

4.1 Introduction

The ability of chickens to utilize phytate phosphorus depends on phytate being hydrolyzed (digested) to inositol and ortho phosphoric acid or its salts. Many investigators have studied the biological availability of phytate phosphorus to the chicken and results of the efficiency of utilization have been quite variable. It was shown in the previous experiment 1 that when the level of calcium in the diet was increased towards the requirement (10g/kg) the average digestion of phytate phosphorus was 50.90 and 50.76% in the high and normal vitamin D_3 diets respectively. Phytate digestibility was greater with the lower level of calcium (5g/kg), 71.85 and 70.72% respectively with either high or normal vitamin D_3 diets. Furthermore, the chickens were fed the recommended level of inorganic phosphorus. The results of this experiment, indicated that chickens during the first 28 days are able to use phytate phosphorus derived from feedstuffs of plant origin for growth and bone formation. This raises the question as to the possibility of further increasing phytate phosphorus digestibility in chickens by feeding diets low in calcium (5g/kg) and containing low total phosphorus with a high concentration of vitamin D_3. The combined effect of these dietary modifications should be to increase the activity of intestinal phytase and to lower the concentration of calcium in the digestive tract without decreasing the amount of calcium absorbed. This possibility was investigated in the present experiment.

4.2 Chicks and treatments

One hundred and sixty day-old male layer strain chicks (Ross

Brown) were used in this experiment. Chicks were completely randomized into four treatments. Each treatment was replicated four times, ten chicks were used per group. On arrival, the chicks were randomised, wing-banded and placed in an electrically heated battery; their initial weights were recorded. Diet and water were given ad-libitum for twenty eight days (see Chapter 2.2 for basal diet used). This basal diet contained neither animal protein nor phosphorus supplements, and it was supplied with ground oystershell as a source of calcium. All these treatments contained low level of total phosphorus (5g/kg), 2.4 g/kg of which was phytate phosphorus. The remainder, 2.6 g/kg, was present as inorganic phosphorus, (Table 4.1). Plasma was collected into heparinised syringe from the heart on day 26 to measure the ionized calcium (see Chapter 2.5). Droppings were collected on days 25, 26 and 27 at 16.00 hrs and frozen at -20°C. The accumulated droppings from each group (10 chicks) were dried and ground together. Feed and dropping samples were analyzed to calculate the retention of calcium and total phosphorus and the digestibility of phytate phosphorus (see Chapter 2.3.2). After twenty eight days, chicks were weighed, killed by decapitation and plasma and right tibia was collected. The tibias were cleaned of tissue, defatted and dried to constant weight and ashed. The ash percentages were calculated. Plasma and tibia ash calcium, total phosphorus, magnesium and zinc contents were determined. These methods are described in detail in Chapter 2.

4.3 Results

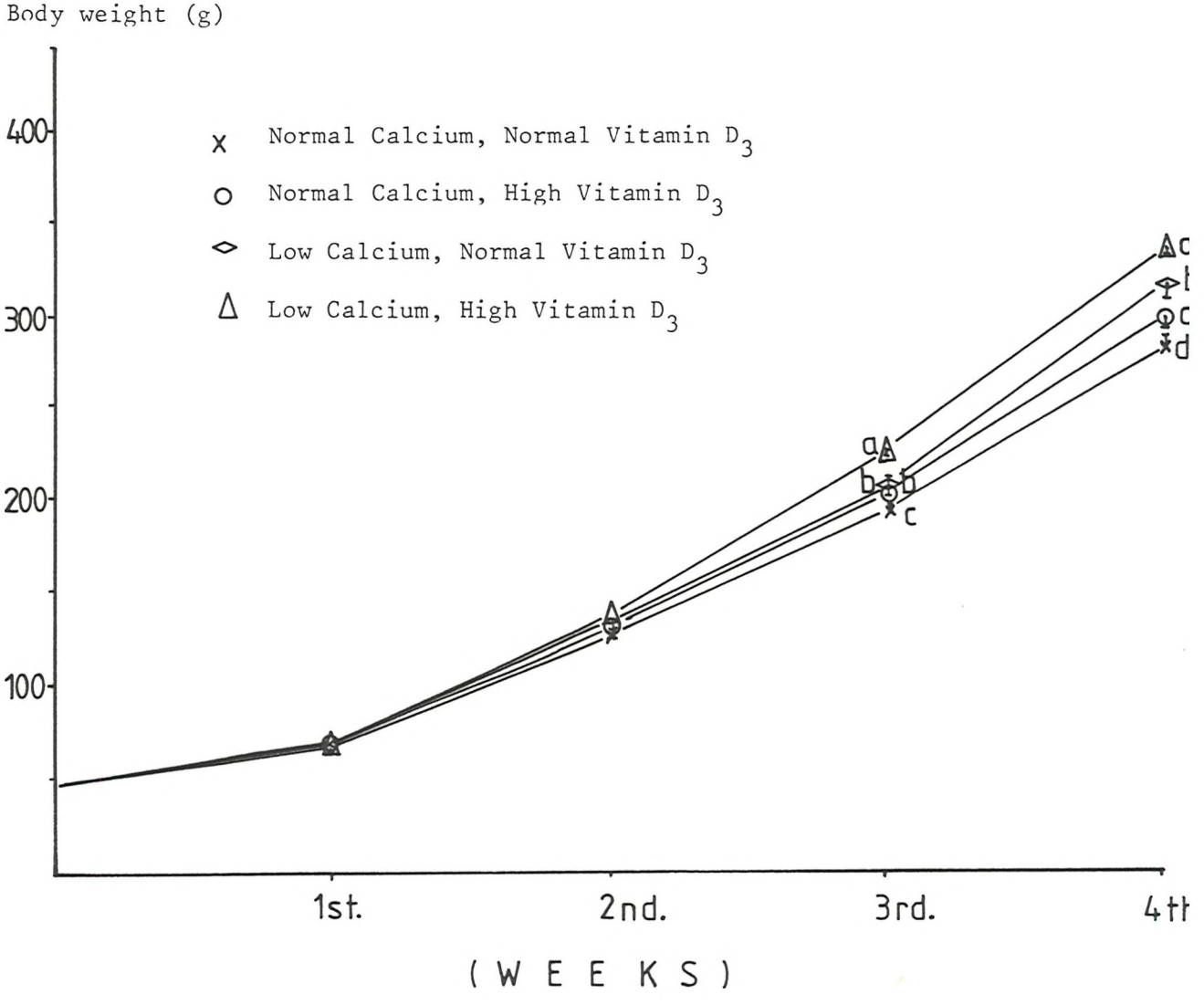
4.3.1 Growth (Figure 4.1)

There was no significant differences between treatments in body weight at the first week. Results obtained in the second week show that only the normal calcium diet with either high or normal vitamin D_3 produced a significant reduction in body weight compared with the low calcium high vitamin D_3 diet ($P<0.01$). However, results from in the third and fourth weeks show that both high vitamin D_3 treatments, led to significantly greater weight

TABLE 4.1 : THE CALCIUM, PHOSPHORUS AND VITAMIN D_3 CONTENT OF THE DIETS

Treatments	Normal Calcium		Low Calcium	
	normal vit.D_3	high vit.D_3	normal vit.D_3	high vit.D_3
Calcium (g/kg)	10	10	5	5
Inorganic phosphorus (g/kg)	2.6	2.6	2.6	2.6
Phytate phosphorus (g/kg)	2.4	2.4	2.4	2.4
Total phosphorus (g/kg)	5	5	5	5
Vitamin D_3 (I.U/kg)	500	50,000	500	50,000
Oystershell (g/kg)	21.21	21.21	8.71	8.71

FIGURE 4.1 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON BODY WEIGHT OF LAYER CHICKS UP TO 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.01$)

(Mean ± S.E.M.)

gains (P<0.01) than observed with both normal vitamin D_3 treatments. Furthermore, the increase in body weight observed with the low calcium - high vitamin D_3 was significantly higher (P<0.01) than all the other treatments.

4.3.2 Bone calcification

A - Tibia fat-free weight, tibia ash weight, ash percentage and tibia length

The data obtained for tibia fat-free weight, tibia ash weight, ash percentages and tibia length, is given in Table 4.2. Chicks fed normal calcium - normal vitamin D_3 diets, showed significantly (P<0.01) decreased tibia fat free weights, tibia ash weights, ash percentages and tibia lengths compared with other treatments. The low calcium-high vitamin D_3 treatment significantly (P<0.01) increased the tibia fat free weight, tibia ash weight and tibia length compared with other treatments.

B - Mineral composition of the tibia at 28 days of age

The differences in the mineral composition of the tibias was found to vary with vitamin D_3 level on both low or normal calcium (Table 4.3). The normal calcium - normal vitamin D_3 diet produced significant reductions P<0.01) in tibia calcium, phosphorus and magnesium compared with others. Rachitic symptoms were apparent in chicks fed the normal calcium - normal vitamin D_3 diet reflecting the lower mineral content of tibia ash (see Figure 4.2). Increasing the level of vitamin D significantly increased the calcium, phosphorus and magnesium contents of the bones at both dietary levels of calcium. The tibia phosphorus and magnesium of the normal calcium - high D_3 chicks were significantly lower than in the low calcium - high D_3 birds but no leg weakness appeared. There were no marked differences between treatments in the tibia content of zinc.

TABLE 4.2 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON TIBIA CALCIFICATION IN LAYER CHICKS AT 28 DAYS OF AGE

	Normal Calcium		Low Calcium	
	Normal Vit.D_3	High Vit.D_3	Normal Vit.D_3	High Vit.D_3
Tibia fat free weight (mg)	658 ± 13^{a}	723 ± 15^{b}	744 ± 22^{b}	810 ± 22^{c}
Tibia ash weight (g)	288 ± 8^{a}	357 ± 9^{b}	355 ± 15^{b}	411 ± 11^{c}
Tibia ash percentage	43.43 ± 0.84^{a}	48.37 ± 0.57^{bc}	47.33 ± 1.02^{b}	50.9 ± 0.55^{c}
Tibia length (cm)	5.58 ± 0.05^{a}	5.73 ± 0.05^{b}	5.79 ± 0.06^{b}	5.97 ± 0.04^{c}

Groups without common subscripts are significantly different from each other ($P<0.01$)

Values are expressed as the mean ± S.E.M.

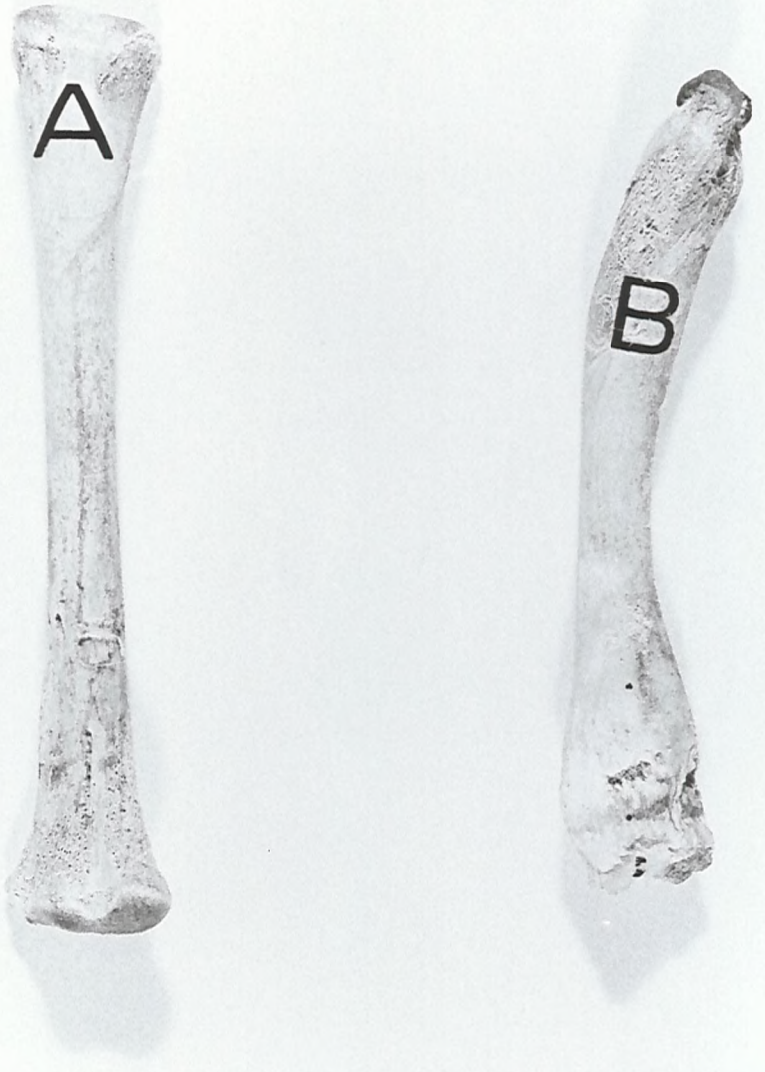
TABLE 4.3 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON TIBIA MINERAL COMPOSITION IN LAYER CHICKS AT 28 DAYS OF AGE

	Normal Calcium		Low Calcium	
Variables	Normal Vit.D_3	High Vit.D_3	Normal Vit.D_3	High Vit.D_3
Calcium (mg/g dry fat-free tibia)	170 ± 4^{a}	186 ± 3^{bc}	179 ± 4^{ab}	196 ± 4^{c}
Total phosphorus (mg/g dry fat-free tibia)	79 ± 2^{a}	89 ± 2^{b}	89 ± 2^{b}	97 ± 0.93^{c}
Magnesium (µg/g dry fat-free tibia)	3586 ± 145^{a}	4633 ± 144^{b}	5089 ± 191^{c}	5587 ± 135^{d}
Zinc (µg/g dry fat-free tibia)	219 ± 5	215 ± 5	207 ± 5	224 ± 4

Groups without common subscripts are significantly different from each other ($P<0.01$)

Values are expressed as the mean ± S.E.M.

FIGURE 4.2 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON TIBIA STRUCTURE OF LAYER CHICKS AT 28 DAYS OF AGE



A — Normal tibia (Low Calcium, Low Phosphorus and high Vitamin D_3 diet)

B — Rachitic tibia (Normal Calcium, Low Phosphorus and normal Vitamin D_3 diet)

4.3.4 Mineral Composition of the Plasma

Total calcium and ionized calcium in the plasma showed a high dependence upon the dietary concentration of calcium in the diet (Table 4.4). In normal calcium diets with either normal or high lervels of vitamin D_3, there was a highly significant ($P<0.01$) increase in both plasma total and ionized calcium levels compared to the corresponding low calcium diets. Thus the level of vitamin D_3 in the diet did not influence the effect of dietary calcium on these parameters. Plasma total phosphorus levels were significantly increased ($P<0.01$) in chicks given the low calcium diets. Equally, diets high in vitamin D_3 led to consistently higher levels ($P<0.01$) of total phosphorus at both levels of dietary calcium. Thus the effects of low calcium diets and of high vitamin D_3 diets in elevating plasma phosphorus were independent. Plasma magnesium and zinc were not significantly effected by the diets.

4.3.4 The Digestibility of Phytate Phosphorus and the Retention Total Phosphorus and Calcium by Analysis of Droppings

The level of calcium and vitamin D_3 in the diet had a remarkable effect on the digestibility of phytate phosphorus and on the retention of calcium and total phosphorus (see Figure 4.3). Chicks fed the diet containing low calcium and high vitamin D_3 showed a significant ($P<0.05$) increase in the digestibility of phytate phosphorus and on the retention of total phosphorus and calcium than chicks fed on the other three treatments.

At the high vitamin D_3 level (50,000 i.u/kg) there was a significant ($P<0.05$) increase in the digestibility of phytate and on the retention of the calcium and total phosphorus at both levels of dietary calcium.

4.4 Discussion (See Chapter 5.4)

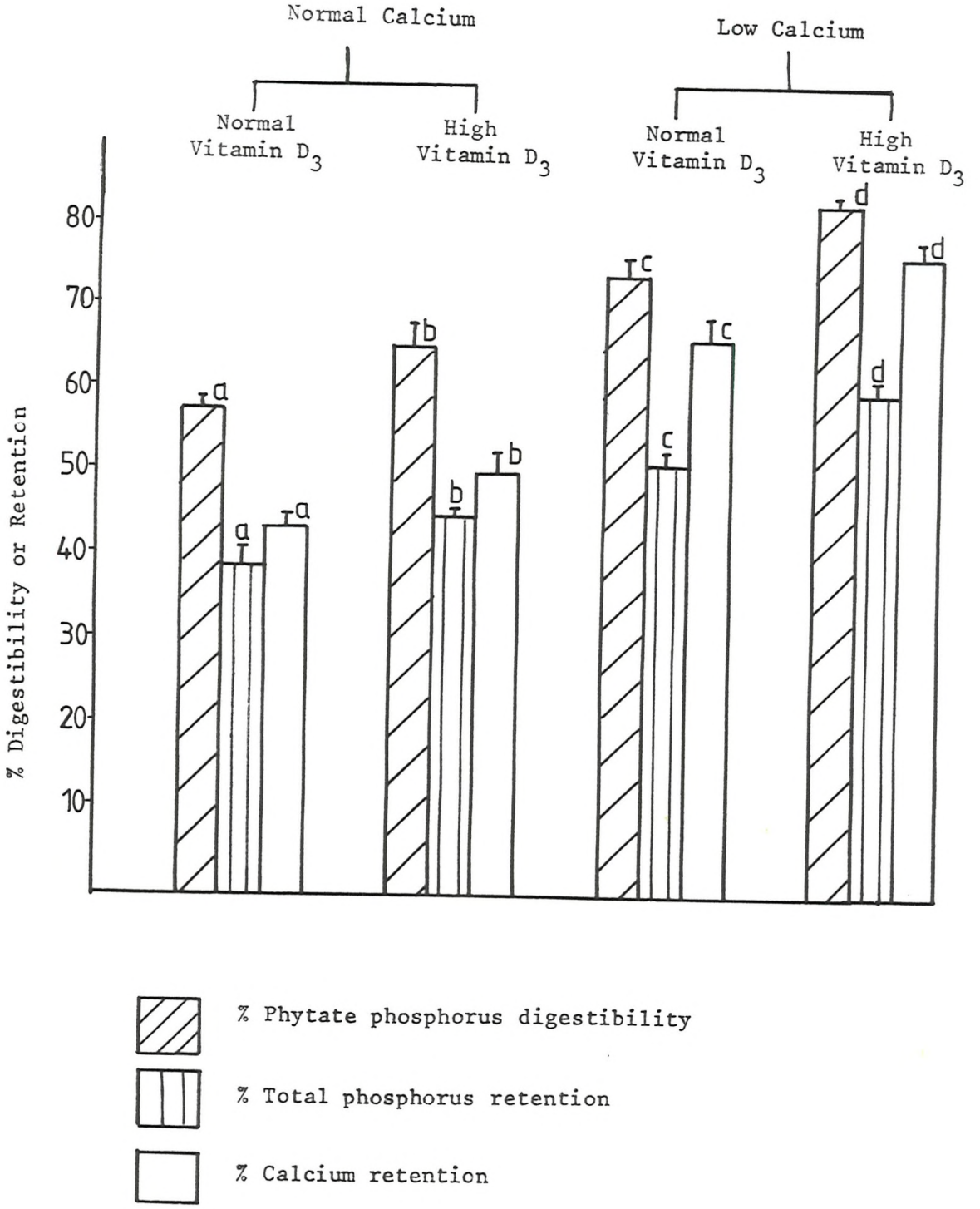
TABLE 4.4 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON PLASMA MINERAL COMPOSITION IN LAYING CHICKS AT 28 DAYS OF AGE

Values are expressed as the mean ± S.E.M.

Variables	Normal Calcium		Low Calcium	
Treatments	Normal Vit.D_3	High Vit.D_3	Normal Vit.D_3	High Vit.D_3
Total calcium (mmol/l)	2.93 ± 0.11^a	2.94 ± 0.09^a	2.14 ± 0.03^b	2.24 ± 0.05^b
Ionized calcium (mmol/l)	1.72 ± 0.04^a	1.65 ± 0.04^a	1.22 ± 0.03^b	1.20 ± 0.01^b
Total phosphorus (mmol/l)	1.1 ± 0.07^a	1.47 ± 0.14^b	2.11 ± 0.11^c	2.66 ± 0.04^d
Magnesium (mmol/l)	0.93 ± 0.05	0.79 ± 0.03	0.88 ± 0.01	0.89 ± 0.07
Zinc (µmol/l)	30.27 ± 1.65	30.97 ± 1.59	27.96 ± 1.09	32.46 ± 1.20

Groups without common subscripts are significantly different from each other ($P<0.01$)

FIGURE 4.3 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE DIGESTIBILITY OF PHYTATE PHOSPHORUS AND ON THE RETENTION OF TOTAL PHOSPHORUS AND CALCIUM OF LAYER CHICKS AT THE FINAL THREE DAYS OF THE 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.05$)
(Mean $\pm$ S.E.M.)

CHAPTER FIVE

AN INVESTIGATION INTO THE INFLUENCE OF VARYING THE CONCENTRATION OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON THE DIGESTIBILITY OF PHYTATE PHOSPHORUS BY BROILER CHICKS

5.1 Introduction

Unlike in the laying chick, the potential of broiler chicks for growth rate and maximum weight has undergone considerable changes in the last few decades. Several studies have demonstrated that various breeds and/or strains of chicks responded differently to the levels and the source of dietary phosphorus. Using four weeks old layer (single comb white leghorn, SCWL) and broiler-type chicks, Gardiner (1969) reported that broilers and SCWL responded differently to suboptimal levels of dietary phosphorus indicating a differential phosphorus response in body weight, feed consumption, percent bone ash, plasma inorganic phosphorus and percent livability for the two breeds of chicks. Andrews, et al. (1971b) observed that the broiler-type chick was able to differentiate between defluorinated phosphate and soft phosphate for growth and mineralization, whereas SCWL chicks were not able to differentiate between these phosphate sources. Further Edwards (1981) reported that, the SCWL chick showed higher retention values for calcium, total phosphorus and phytate phosphorus than the broiler chick. Thus, layer and broiler breeds of chick may differ in their response to varying the level and source of phosphorus. Therefore, the present study was undertaken to investigate more comprehensively the influence of varying the concentration of dietary calcium, vitamin D_3 and total phosphorus on the digestibility of phytate phosphorus by broiler chicks.

5.2 Experimental Procedure-

Sixty day-old male broiler strain chicks (Ross one) were used in this experiment. Chicks were completely randomized into five

treatments. Each treatment was replicated three times; four chicks were used per group. On arrival, the chicks were randomised, wing-banded and placed in an electrically heated battery; their initial weights were recorded. Diet and water were given ad-libitum for forty-two days (see Chapter 2.2 for the basal diet used). This basal diet was supplied with oystershell as a source of calcium. No animal protein or inorganic phosphorus was provided except for the control diet which was supplied with 10 g sodium dihydrogen phosphate/kg diet as a source of inorganic phosphorus. The control treatment contained the NRC (1977) recommended amount of calcium, inorganic phosphorus and vitamin D_3, (Table 5.1). Plasma was collected into heparinised syringes from the wing vein on day 26 to measure the ionized calcium (see Chapter 2). Droppings were collected on days 25, 26 and 27 at 16.00 hrs and frozen at -20°C. The accumulated droppings from each group (4 chicks) were dried and ground. Feed and droppings samples were analysed to measure the retention of calcium and total phosphorus and phytate phosphorus hydrolyses, (see Chapter 2). Seven chicks were killed by decapitation from each treatment on day 28 and five on day 42 and samples of plasma taken together with the right tibia from each chick. Tibias were cleaned of tissue, defatted, dried to constant weight and ashed. The ash percentages were calculated. Plasma and tibia ash calcium, total phosphorus, magnesium and zinc contents were determined. These methods are described in detail in Chapter 2.

5.3 Results

5.3.1 Growth

The growth of chicks given all of the four diets containing less than the NRC recommended level of dietary phosphorus, was less than that of control chicks where the diet contained the recommended level of phosphorus. However, this differential in growth due to dietary phosphorus levels, was considerably influenced by dietary calcium levels and to some extent by vitamin

TABLE 5.1 : THE CALCIUM, PHOSPHORUS AND VITAMIN D_3 CONTENT OF THE DIETS

	Control	Normal Calcium		Low Calcium	
Treatments	Recommended diet	Normal Vit.D_3	High vit.D_3	Normal Vit.D_3	High Vit.D_3
Calcium (g/kg)	10	10	10	5	5
Inorganic phosphorus (g/kg)	4.5	2.6	2.6	2.6	2.6
Phytate phosphorus (g/kg)	2.4	2.4	2.4	2.4	2.4
Total phosphorus (g/kg)	6.9	5.0	5.0	5.0	5.0
Vitamin D_3 (1u/kg)	500	500	50,000	500	50,000
Oystershell (g/kg)	21.21	21.21	21.21	8.71	8.71

D_3 levels but only on the lower calcium diets. Thus when the low phosphorus diets were also low in calcium, the body weights of chicks were not significantly different throughout the 6 weeks of study from the body weight of control chicks. In contrast when the diets low in inorganic phosphorus contained normal levels of calcium, the body weights of chicks were consistently lower than those achieved by chicks fed the control diet ($P<0.01$). These results are illustrated in Figure 5.1.

Growth rates (increase of body weight, grams per week) during weeks 4, 5, and 6 for each of the treatments is shown in Figure 5.2. When the diets contained low phosphorus levels and normal levels of calcium, the 4th and 5th weeks growth rates of the chicks were significantly less than those achieved with the control diet ($P<0.05$). However, when the diets containing low levels of phosphorus were also low in calcium with high vitamin D_3, this depression in growth rate compared with the control was not seen at 4th and 5th weeks. Interestingly, the chicks given diets which were low in both phosphorus and calcium but which were high in vitamin D_3, had growth rates higher but not significant than control chicks at the end of the 6th week.

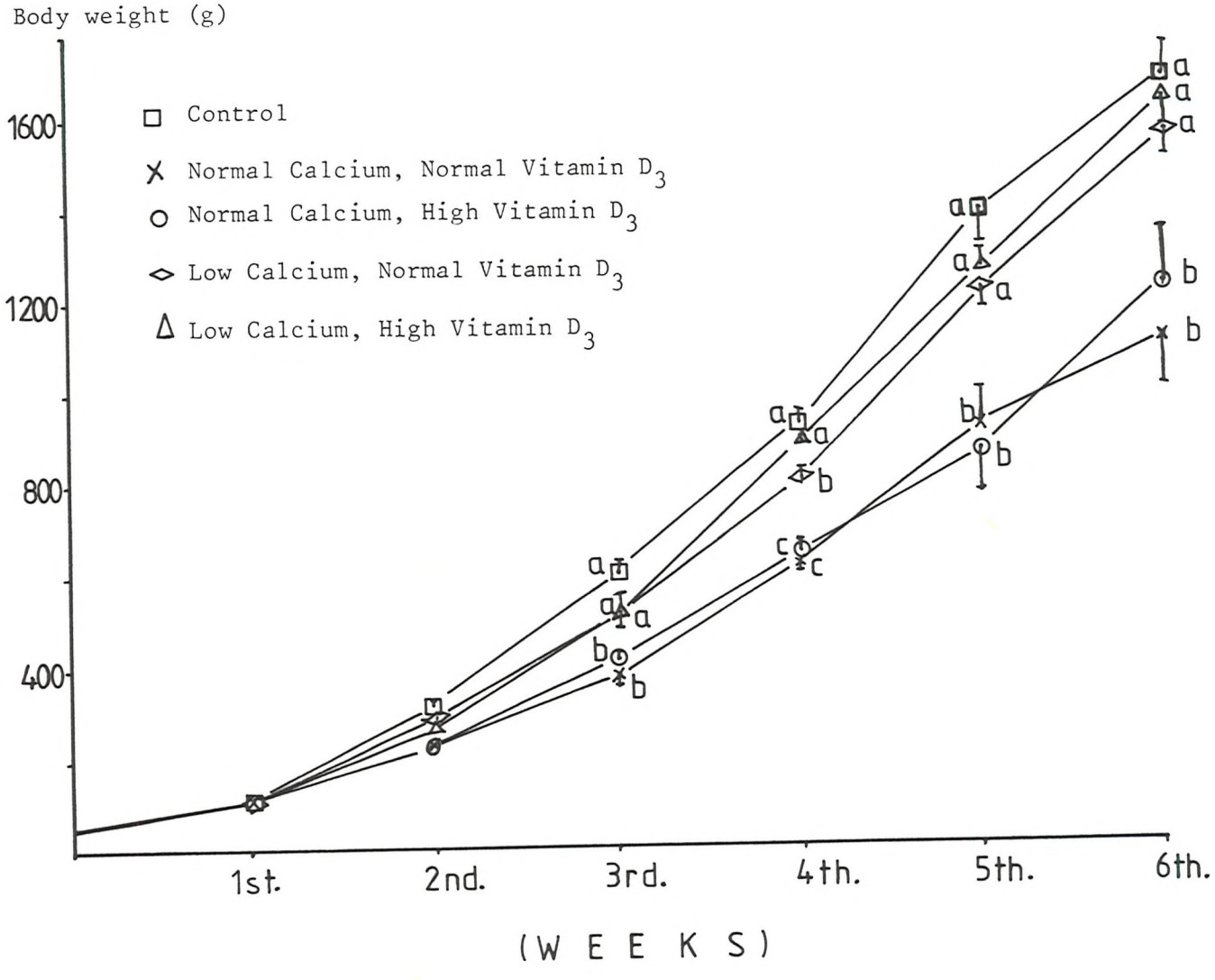
5.3.2 Tibia Calcification

5.3.2.1 Tibia calcification at 28 days of age

A - Tibia fat free weights, tibia ash weights and percentage ash in tibia.

Each of these parameters was significantly reduced ($P<0.01$) with all of the low-phosphorus diets, compared to controls (Table 5.2). In general, these reductions were least with low calcium, high vitamin D_3 diets and greatest with diets containing normal levels of both calcium and vitamin D_3.

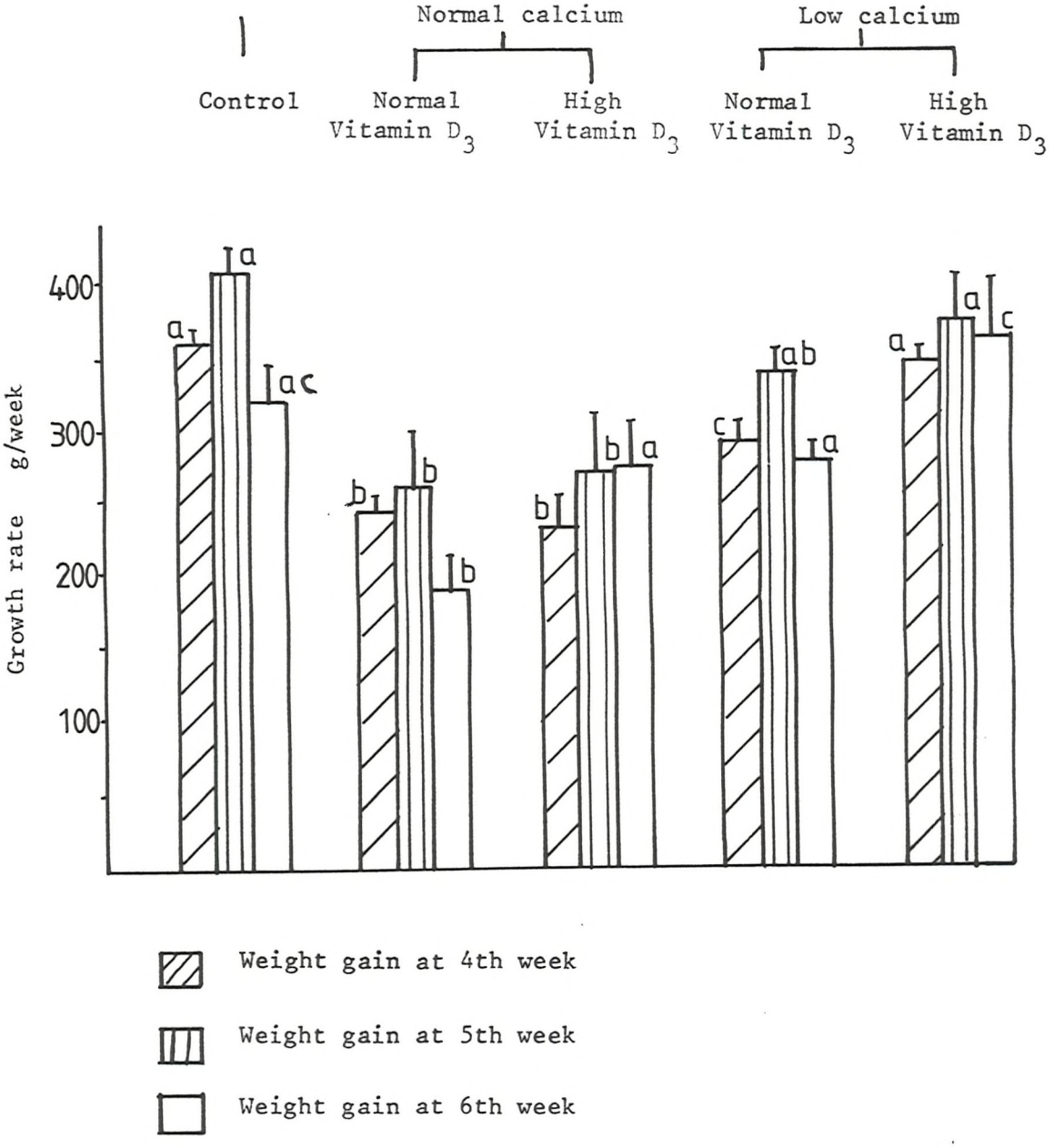
FIGURE 5.1 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE BODY WEIGHT IN BROILER CHICKS UP TO 6 WEEKS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.01$)

(Mean $\pm$ S.E.M.)

FIGURE 5.2 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON GROWTH RATES OF BROILER CHICKS AT 4TH, 5TH AND 6TH WEEKS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.05$)

(Mean ± S.E.M.)

TABLE 5.2 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON TIBIA CALCIFICATION IN BROILER CHICKS AT 28 DAYS OF AGE

Variables	Control	Normal Calcium		Low Calcium	
	Recommended diet	Normal Vit.D_3	High vit.D_3	Normal Vit.D_3	High Vit.D_3
Tibia fat free (g)	2.68 ± 0.15^{a}	1.45 ± 0.04^{b}	1.60 ± 0.06^{bc}	1.83 ± 0.1^{c}	1.91 ± 0.11^{c}
Tibia ash weight (g)	1.41 ± 0.09^{a}	0.60 ± 0.02^{b}	0.69 ± 0.02^{bc}	0.80 ± 0.4^{cd}	0.92 ± 0.06^{d}
Tibia ash percentage	52.44 ± 0.41^{a}	41.28 ± 0.08^{b}	43.37 ± 0.09^{b}	43.94 ± 1.85^{b}	48.17 ± 0.9^{c}

Groups without common subscripts are significantly different from each other ($P<0.01$)

Values are expressed as the mean ± S.E.M.

B - Mineral composition of the tibia at 28 days of age

Again, the levels of calcium, phosphorus and magnesium in tibia, on a fat-free basis, were significantly lowered ($P<0.01$) with diets containing low levels of phosphorus with the exception of the chicks fed diets which were also low in calcium and which were high in vitamin D_3 (Table 5.3). The level of tibia zinc did not vary between dietary treatments. Diets which were low in phosphorus but which had normal levels of both calcium and vitamin D_3 led to a typically rachitic tibia structure (Figure 5.3).

5.3.2.2 Tibia calcification at 42 days of age

A - Tibia fat free weights, tibia ash weight and percentage ash in tibia and tibia length

There were no significant differences in any of these parameters between chicks given the control diet and those given diets low in both phosphorus and calcium and high in vitamin D_3. However, all other low phosphorus diets resulted in a signficant reduction compared to control diets ($P<0.01$) in all of these parameters except tibia length. For the latter parameter, the adverse effects of low phosphorus intakes were ameliorated by low calcium diets, irrespective of vitamin D_3 levels (Table 5.4).

B - Mineral composition of the tibia at 42 days of age

Chicks fed the low phosphorus diets showed significant reductions ($P<0.01$) in the calcium, phosphorus and magnesium levels in tibia ash compared with the chicks fed control diets with the exception of the chicks on the low calcium high vitamin D_3 diet (Table 5.5). The reductions in tibia ash levels of these minerals with low phosphorus diets was greatest in chicks fed diets which were normal in both vitamin D_3 and calcium levels and this was again reflected in rachitic tibia in these chicks (Figure 5.4).

TABLE 5.3 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON TIBIA MINERAL COMPOSITION IN BROILER CHICKS AT 28 DAYS OF AGE

	Control	Normal Calcium		Low Calcium	
Variables	Recommended diet	Normal Vit.D_3	High vit.D_3	Normal Vit.D_3	High Vit.D_3
Calcium (mg/g dry tibia fat free)	191 ± 3^a	156 ± 3^b	155 ± 6^b	168 ± 6^b	186 ± 8^a
Total phosphorus (mg/g dry tibia fat free)	91 ± 0.7^a	65 ± 1.3^b	67 ± 2^b	76 ± 2^c	86 ± 1^a
Magnesium (µg/g dry tibia fat free)	4993 ± 89^a	3031 ± 120^b	3788 ± 70^c	4187 ± 238^c	4919 ± 113^a
Zinc (µg/g dry tibia fat free)	258 ± 16	258 ± 12	236 ± 14	262 ± 7	244 ± 8

Groups without common subscripts are significantly different from each other ($P<0.01$)

Values are expressed as the mean ± S.E.M.

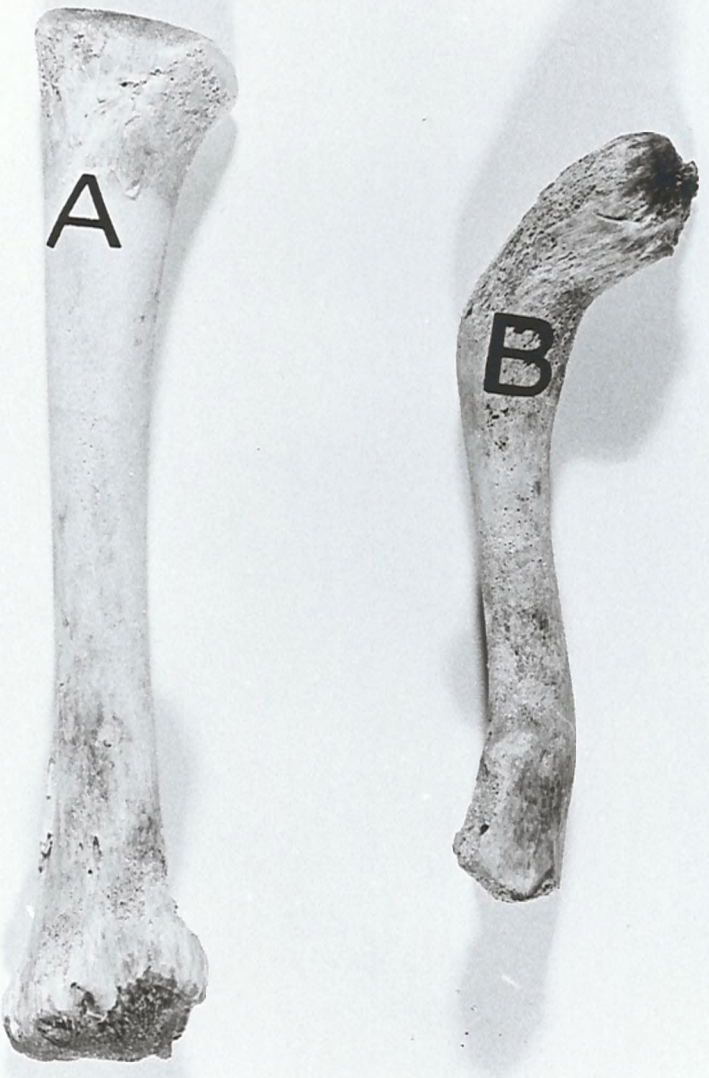
TABLE 5.4 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON TIBIA CALCIFICATION IN BROILER CHICKS AT 42 DAYS OF AGE

Variables	Control	Normal Calcium		Low Calcium	
	Recommended diet	Normal Vit.D_3	High vit.D_3	Normal Vit.D_3	High Vit.D_3
Tibia fat free (g)	4.45 ± 0.3^{a}	3.05 ± 0.2^{b}	3.06 ± 0.3^{b}	3.39 ± 0.2^{bc}	3.88 ± 0.2^{ac}
Tibia ash weight (g)	2.26 ± 0.15^{a}	1.30 ± 0.11^{b}	1.41 ± 0.14^{b}	1.52 ± 0.07^{b}	1.91 ± 0.13^{a}
Tibia ash percentage	50.8 ± 0.58^{a}	42.3 ± 1.02^{b}	46.0 ± 0.85^{c}	45.07 ± 1.37^{cb}	49.12 ± 0.43^{a}
Tibia length (cm)	9.1 ± 0.1^{a}	7.7 ± 1.2^{b}	7.9 ± 0.3^{b}	8.6 ± 0.1^{a}	8.9 ± 0.1^{a}

Groups without common subscripts are significantly different from each other ($P<0.05$)

Values are expressed as the mean ± S.E.M.

FIGURE 5.3 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON TIBIA STRUCTURE OF BROILER CHICKS AT 28 DAYS OF AGE



A — Normal tibia (Low Calcium, Low Phosphorus and high Vitamin D_3 diet)

B — Rachitic tibia (Normal Calcium, Low Phosphorus and normal Vitamin D_3 diet)

5.3.3 Mineral Composition of Plasma

A - At 28 days of age

Diets which were low in phosphorus and which contained normal levels of calcium led to significant ($P<0.01$) elevations in both plasma total and ionized calcium compared to all other treatments (Figure 5.5). The levels of dietary vitamin D_3 did not influence plasma calcium levels, total or ionized. Plasma phosphorus levels were significantly reduced ($P<0.01$) in chicks given diets containing low levels of phosphorus with the exception of those given diets which were also low in calcium but high in vitamin D_3. In general, the hypophosphataemia induced by low phosphorus diets was significantly greater ($P<0.05$) with normal vitamin D_3 diets, irrespective of calcium levels.

B - At 42 days of age

A significant ($P<0.01$) elevation in plasma calcium was observed with diets containing low levels of phosphorus and normal levels of calcium, irrespective of vitamin D_3 levels (Table 5.6). This group also showed a significant reduction in plasma total phosphorus ($P<0.01$) compared with controls as did the group on comparable intakes of phosphorus but with low intakes of calcium and normal intakes of vitamin D_3. No significant effect of diet was observed for either the magnesium or zinc plasma levels.

5.3.4 Retention of Calcium and Total Phosphorus and Digestibility of Phytate

Marked variation in phytate digestibility betwen the five dietary treatments was found (Figure 5.6). Reducing the level of phosphorus in the diet increased phytate phosphorus digestibility compared to the control in all treatments ($P<0.01$) except with the

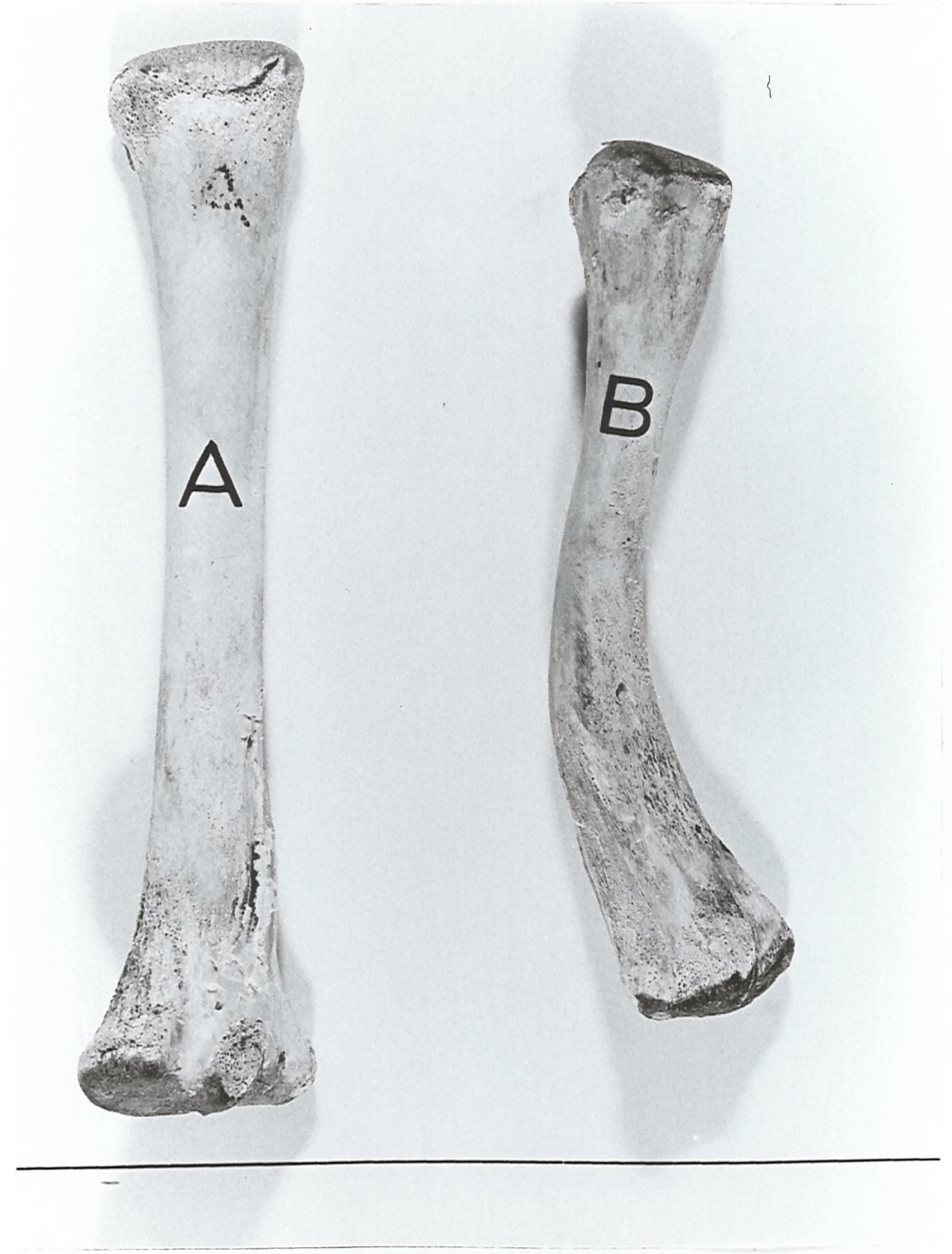
TABLE 5.5 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON TIBIA MINERAL COMPOSITION IN BROILER CHICKS AT 42 DAYS OF AGE

	Control	Normal Calcium		Low Calcium	
	Recommended diet	Normal Vit.D_3	High vit.D_3	Normal Vit.D_3	High Vit.D_3
Calcium (mg/g dry tibia fat free)	196 ± 2.5^{a}	155 ± 6^{b}	173 ± 1.25^{c}	169 ± 6^{bc}	184 ± 4^{ac}
Total phosphorus (mg/g dry tibia fat free)	88 ± 0.96^{a}	63 ± 2^{b}	72 ± 3^{c}	72 ± 3^{c}	86 ± 2^{a}
Magnesium (µg/g dry tibia fat free)	4837 ± 109^{a}	3124 ± 184^{b}	4002 ± 47^{c}	3404 ± 241^{b}	4535 ± 75^{a}
Zinc (µg/g dry tibia fat free)	245 ± 11	241 ± 17	235 ± 6	262 ± 10	242 ± 9

Groups without common subscripts are significantly different from each other ($P<0.01$)

Values are expressed as the mean ± S.E.M.

FIGURE 5.4 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON TIBIA STRUCTURE OF BROILER CHICKS AT 42 DAYS OF AGE



A _ Normal tibia (Low Calcium, Low Phosphorus and high Vitamin D_3 diet)

B _ Rachitic tibia (Normal Calcium, Low Phosphorus and normal Vitamin D_3 diet)

group fed on normal levels of vitamin D_3 and calcium. Increasing the vitamin D_3 level of the diet increased significantly ($P<0.01$) phytate phosphorus digestibility, irrespective of the calcium content. In turn, low calcium diets significantly increased phytate digestibility ($P<0.01$) irrespective of the level of dietary vitamin D_3.

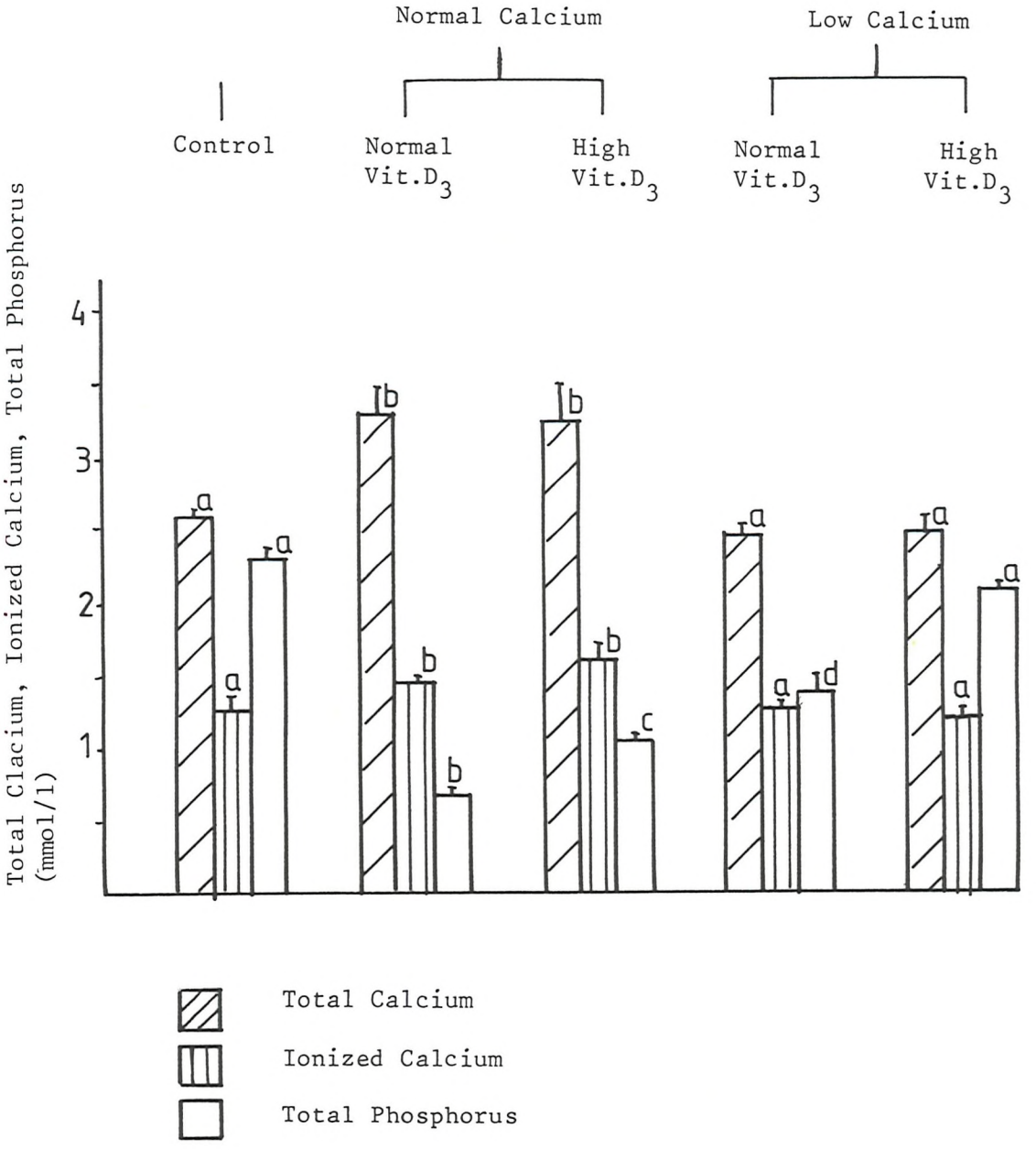
Chicks given the low phosphorus low calcium diets showed significantly higher ($P<0.01$) total phosphorus retention compared to chicks given the low phosphorus diet with normal level of calcium, or to control chicks (Figure 5.6). High level of dietary vitamin D_3 led to significantly greater ($P<0.01$) total phosphorus retention at both levels of dietary calcium.

Percentage retention of calcium by chicks given the low calcium, low phosphorus high vitamin D_3 diet was significantly greater ($P<0.05$) than for the other treatments (Figure 5.6). At both levels of calcium treatments there was a significant ($P<0.05$) increase in calcium retention when the vitamin D_3 level was increased from 500 to 50,000 i.u/kg.

5.4 DISCUSSION

In the previous experiment, the effects of calcium and vitamin D_3 on growth of chicks was studied using diets in which all the dietary requirement for phosphorus was provided by inorganic phosphorus. In contrast, the diets used in the present experiments were low in total phosphorus (5g/kg) and all of this phosphorus was provided by phosphorus present in the feed. A deficiency of dietary phosphorus is characterised by poor growth, abnormal skeletal development and by a combined hypophosphataemia and hypercalcaemia (Scott et al. 1982). Hypophosphataemia is associated with a reduction in circulating growth hormone (Carew et al. 1985) and Sommerville et al. (1985) concluded that the first adaptive response of the chick to a low dietary phosphorus is a marked fall in plasma level of growth hormone.

FIGURE 5.5 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE PLASMA TOTAL CALCIUM, IONIZED CALCIUM AND TOTAL PHOSPHORUS OF BROILER CHICKS AT 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.05$)

(Mean ± S.E.M.)

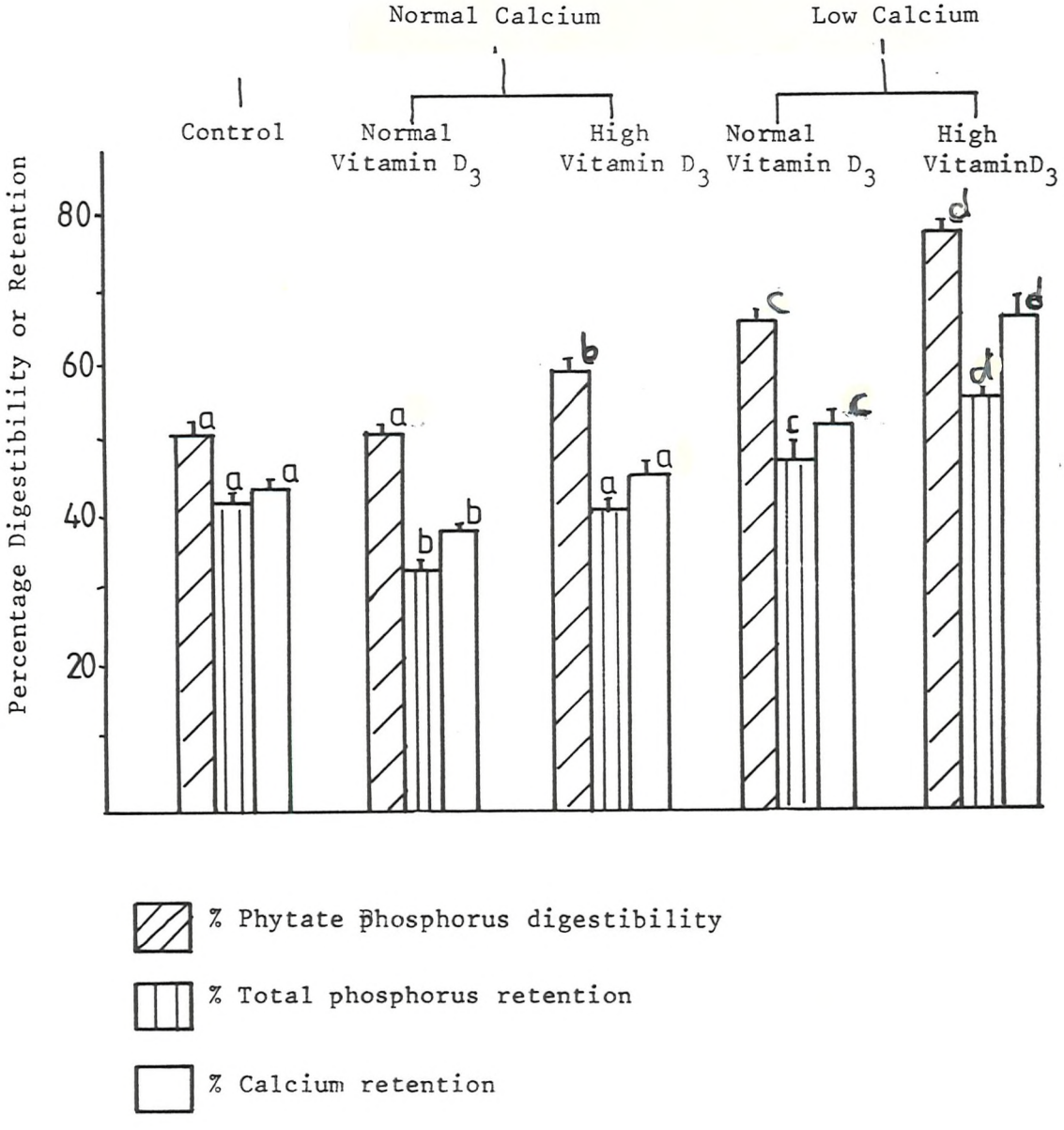
TABLE 5.6 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON PLASMA MINERAL COMPOSITION IN BROILER CHICKS AT 42 DAYS OF AGE

	Control	Normal Calcium		Low Calcium	
	Recommended diet	Normal Vit.D_3	High vit.D_3	Normal Vit.D_3	High Vit.D_3
Calcium in the plasma (mmol/l)	2.65 ± 0.06^{a}	3.33 ± 0.17^{b}	3.53 ± 0.06^{b}	2.58 ± 0.04^{a}	2.55 ± 0.06^{a}
Total phosphorus in the plasma (mmol/l)	2.26 ± 0.19^{a}	0.65 ± 0.06^{b}	$0.90 \pm 0.1b^{c}$	1.26 ± 0.26^{c}	1.94 ± 0.19^{a}
Magnesium in the plasma (mmol/l)	0.79 ± 0.03	0.90 ± 0.03	0.92 ± 0.07	0.74 ± 0.08	0.76 ± 0.04
Zinc in the plasma (µmol/l)	34.72 ± 0.077	28.87 ± 2.78	30.21 ± 2.39	31.74 ± 1.41	33.27 ± 1.69

Groups without common subscripts are significantly different from each other ($P<0.01$)

Values are expressed as the mean ± S.E.M.

FIGURE 5.6 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE DIGESTIBILITY OF PHYTATE PHOSPHORUS AND ON THE RETENTION OF TOTAL PHOSPHORUS AND CALCIUM OF BROILER CHICKS BETWEEN 24 AND 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.05$)

(Mean ± S.E.M.)

In the present studies, low intakes of dietary phosphorus reduced growth only in those chicks given diets containing normal levels of calcium. Whilst the level of dietary vitamin D_3 did not influence this response in broilers, the higher intake of vitamin D_3 did improve growth in layers. This different response of breeds to variation in dietary levels of phosphorus, calcium and vitamin D_3 have also been reported by Gardiner (1969), Fritz et al. 1969 and Christmas and Harms (1978).

In contrast, low intakes of phosphorus did not impede the growth of chicks given low calcium diets, and in the case of broilers the combination of low-calcium, low-phosphorus diets produced body weights comparable to those observed in chicks fed diets containing all nutrient at the recommended level. The 4 week growth of chicks given low-calcium, low-phosphorus diets was enhanced by the higher intakes of vitamin D_3. This is in agreement with the data of Edward Jr.(1976) who concluded that it is difficult to rationalise the continued use of high-calcium, low vitamin D_3 diet to meet calcium requirements when low calcium, high vitamin D_3 diets are both nutritionally adequate and economically favourable.

Hyperculcaemia was found in both layer and broiler chicks which were fed on the low-phosphorus, normal-calcium diets, irrespective of vitamin D_3 intakes. A marked increase in the efficiency of absorption of dietary calcium with low phosphorus diets has been reported for pigs (Fox et al. 1978, Engstrom et al. 1982), for chicks (Friedlander et al. 1977, Sommerville et al. 1978, 1985) and rats (Ribovich and DeLuca, 1975). Low phosphorus diets led to enhanced production of $1,25(OH)_2$ vitamin D_3 by 1-hydroxylase in chicks (Baxter and DeLuca, 1976) which as discussed in Chapter (1.5.3B) would greatly increase calcium absorption.

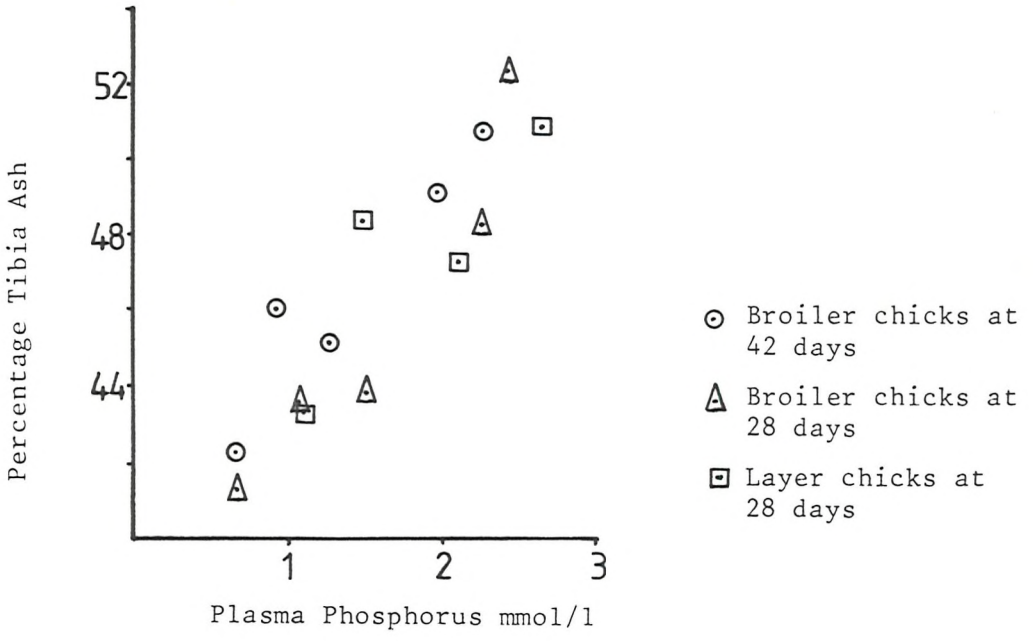
Hypophosphataemia was found in both layer and broiler chicks which were fed on the low phosphorus, normal-calcium diets, the effect being greatest with recommended intakes of vitamin D_3. Thus plasma phosphorus levels are a good indicator of phosphate

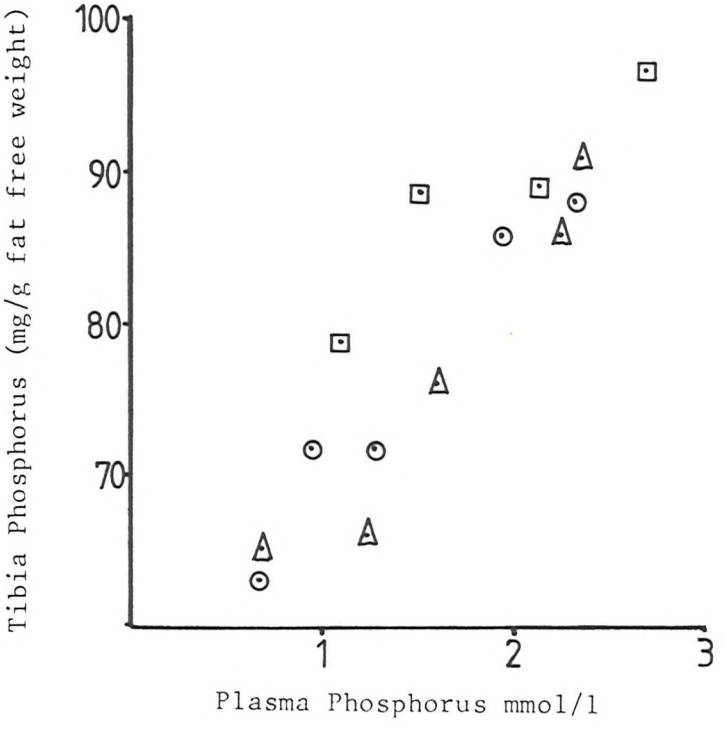
status in growing chicks and as has been proposed by Gardner (1962), plasma phosphorous measurements should replace tibia ash phosphorus in studies of phosphorus nutrition of the chick.

The results of the present studies show that tibia mineralization in chicks given a low-phosphorus diet is maximised by reducing the intake of calcium and increasing the intake of vitamin D_3. Tibia mineralization in such chicks is minimised by feeding normal levels of both calcium and vitamin D_3. While this effect of dietary calcium is evident for both breeds at all ages, the effect of vitamin D_3 supplementation is not evident with younger broilers. In this group of chicks, at this age, the growth of soft tissue is maximum and hence creates an additional demand on plasma phosphorus which does not occur with the slower growing layer or with the older broiler. In addition the layer chicks tend to make greater utilization of phytate phosphorus than broiler (Edwards Jr., 1981). However, in the present study, the effect of breed on phytate phosphorus availability was much less than the various effects of dietary phosphorus, calcium and vitamin D_3.

The marked hypercalcaemia contrast with the low level of tibia calcium while the hypophosphataemia is parallelled by low levels of tibia phosphorus. As the hypophosphataemia improves, tibia phosphorus levels increase and tibia ash % increases. This is seen in Figure 5.7. Whilst plasma magnesium and zinc were not influenced by diet, the tibia content of magnesium, but not zinc, was influenced by diet. There is a close relationship between calcium and magnesium absorption as they share a common intestinal transport mechanism (Clarkson et al. 1967, Care and Van's-Klooster, 1965). However, no relationship was observed in the present study between plasma magnesium and plasma total or ionized calcium. Tibia magnesium tended to parallel tibia phosphorus and tibia calcium. The lowest levels of tibia magnesium were observed at the higher levels of dietary calcium which agrees with data of Chicco et al. (1967). Other workers have also shown the plasma magnesium is not influenced by high-calcium, low-phosphorus

FIGURE 5.7: THE RELATIONSHIP BETWEEN PLASMA PHOSPHORUS AND TIBIA ASH (%) AND TIBIA PHOSPHORUS IN LAYER AND BROILER CHICKS





diets (Scott et al. 1982). There was no effect of diet on either plasma zinc levels or tibia zinc levels. Bafunto et al. (1984) observed that high zinc intakes are required to effect the plasma zinc lowering effects of excessive intakes of either calcium or phytate.

In both breeds, phytate digestibility, and the retention of both calcium and total phosphorus were reduced by increasing dietary calcium to the recommended level. These results agree with previous work by Ballam et al. (1984a,b), Edwards, Jr., (1982), Nelson and Kirby (1979) and Oberlears (1973). These illustrate the classical interactions between calcium and phytate phosphorus. The traditional explanation for these interactions is that six calcium ions bind to each phytate phosphorus molecule forming an insoluble phytate-calcium complex, thus reducing the available substrate for intestinal phytase and alkaline phosphatase (Taylor, 1965).

High levels of vitamin D_3 markedly increased phytate digestibility as well as the retention of both calcium and phosphorus, being more pronounced at the low calcium intakes. These results are consistent with the early work of Krieger and Steenbock (1940) and Mellanby (1950) who reported that dietary vitamin D_3 increased the digestibility of phytate phosphorus and reduced the rachitogenicity of phytate when low calcium diets were given. This is achieved by either an increase in the solubility of phytate or by an increase in intestinal phytase (Pileggi et al. 1955). In addition, high intakes of vitamin D_3 will lead to increased calcium absorption, as previously discussed (Chapter 3).

Thus the results of the present study show that normal growth and normal tibia mineralisation can be achieved on low phosphorus diets, provided that the calcium level is also lowered and the vitamin D_3 intake is elevated. The reduction in the level of dietary calcium directly increases phytate phosphorus availability, and the increase in vitamin D_3, increases both phytate

digestibility and calcium and phosphorus retention. In the absence of reduced dietary calcium level, a marked hypercalcaemia and hypophosphataemia results. Increasing the dietary intake of vitamin D_3 has no effect on this hypercalcaemia but partially improves the hypophosphataemia, presumably through its effect on improving phytate digestibility and through the effect of $1,25(OH)_2D_3$ on venal phosphate reabsorption.

CHAPTER SIX

THE EFFECT OF DIETARY PHYTATE PHOSPHORUS, PHOSPHORUS, CALCIUM AND VITAMIN D_3 LEVELS ON THE DIGESTIBILITY OF PHYTATE PHOSPHORUS, TOTAL PHOSPHORUS AND CALCIUM BY BROILER CHICKS

6.1 Introduction

The utilization of phytate phosphorus by the broiler chick requires that phytate be digested to inositol and phosphate. The ability of chicks to hydrolyse phytate is influenced by a number of factors. Some feed ingredients such as wheat bran have phytase activity while other such as corn contain little or none (McCance and Widdowson, 1944; Mollgaard, 1946; Abernethy et al. 1973). The phytase in wheat bran may improve the digestion of phytate phosphorus in diets when fed to chicks (Nelson, 1967). Several investigators have reported that the chick and rat can digest plant phosphorus when fed diets based on wheat (Temperton and Cassidy, 1964; Salman et al. 1969; Nelson and Kirby, 1979). The level of calcium in the diet is, however, the main factor influencing the digestibility of phytate phosphorus. Published data indicates that high levels of calcium in the diets of the rat and hamster (Taylor and Coleman, 1979); and in the diet of chicks (Nott 1967) reported a decrease in the digestibility of phytate phosphorus, due to the formation of an unabsorbable complex of calcium with phytate (Taylor, 1965).

Many feed ingredients high in fiber are also high in phytate, which have been shown to reduce cation availability to chicks (Nelson et al. 1968). Reinhold et al. (1975) suggested that fiber rather than phytate largely determines the availability of cations to the chick. The cation exchange properties of added fiber may reduce the effective concentration of cations in the gastro-intestinal tract and increase phytate digestibility. The following experiment was conducted to investigate the effect on digestibility of phytate phosphorus, and the retention of phosphorus and calcium by broiler chicks of different dietary levels of phytate phosphorus achieved by adding extra wheat bran

to two of the diets. The concentration of calcium was increased to 7.5 g/kg in three diets (Table 6.2).

6.2 Experimental Procedure

The experiment design and organisation were similar to that described for experiment 3 Chapter 5. The formulations and compositions of the diets are given in Tables 6.1, 6.2, respectively.

Ionized calcium was measured in plasma on day 26. Droppings were collected on days 25, 26 and 27 at 16.00 hrs and frozen at -20°C. The chicks were weighed and killed on day 28 by decapitation. Plasma and the right tibia were collected from each chick. These samples were assayed as a previous experiment. The methods are described in Chapter 2.

6.3 Results

6.3.1 Growth

The chicks were weighed individually each week (see Figure 6.1). The results show that only one treatment reduced body weight significantly compared with control, namely the medium-calcium, normal phytate diet.

6.3.2 Tibia calcification

A - Tibia fat free weight, tibia ash weight, percentage of tibia ash and tibia length

There was a significant reduction in tibia fat free weight, tibia ash weight and tibia ash percentage in all treatments compared with controls ($P<0.01$). Tibia length was significantly reduced compared to control ($P<0.05$) with moderate levels of calcium (7.5 g/kg) but not with the low calcium diet (Table 6.3). In general, the reductions in each

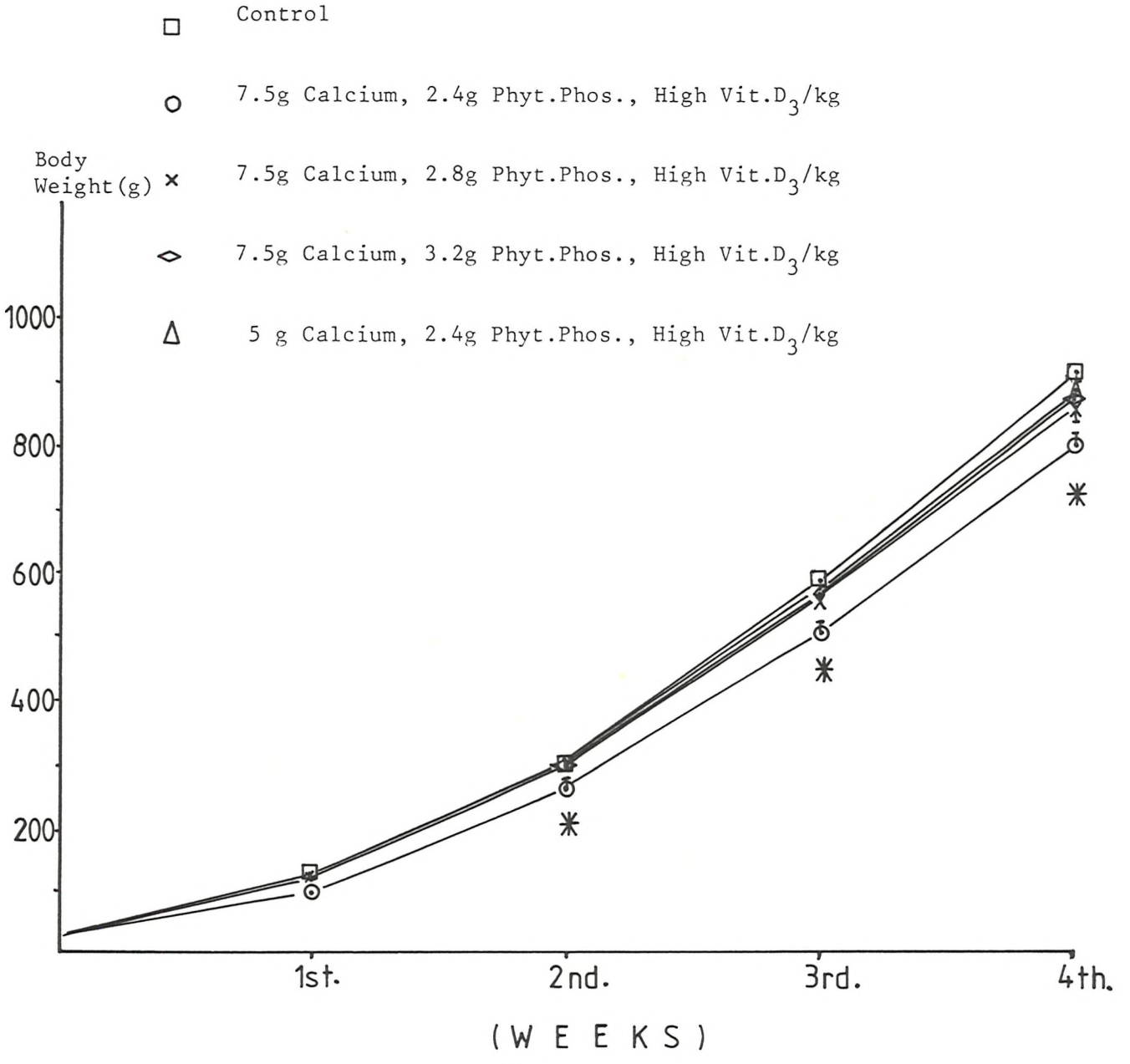
TABLE 6.1 : THE FORMULATIONS OF THE TREATMENTS

Substance	Control	Moderate calcium in the diet (7.5 g/kg)			Low calcium diet
	Recommended diet				
Maize meal	600	610	560	510	610
Soybean meal	333	333	333	333	333
Dried yeast	50	50	50	50	50
Wheat bran	0	0	50	100	0
$NaH_2 PO_4 2 H_2O$	10	0	0	0	0
Sodium chloride	5	5	5	5	5
Methionine	1	1	1	1	1
Mixed mineral and vitamin	1	1	1	1	1

TABLE 6.2: THE CALCIUM, TOTAL PHOSPHORUS AND VITAMIN D_3 CONTENT OF THE DIET

	Control				
	Recommended diet	Moderate calcium in the diets			Low calcium
Calcium (g/kg)	10	7.5	7.5	7.5	5.0
Inorganic phosphorus (g/kg)	4.5	2.6	2.7	2.8	2.6
Phytate phosphorus (g/kg)	2.4	2.4	2.8	3.2	2.4
Total phosphorus (g/kg)	6.9	5.0	5.5	6.0	5.0
Vitamin D_3 (1.u/kg)	500	50,000	50,000	50,000	50,000
Oystershell (g/kg)	21.21	14.95	14.95	14.95	8.71

FIGURE 6.1 : THE EFFECTS OF DIETARY PHYTATE PHOSPHORUS, PHOSPHORUS AND CALCIUM LEVELS IN HIGH VITAMIN D_3 DIETS ON BODY WEIGHT IN BROILER CHICKS UP TO 4 WEEKS OF AGE



* All values for 7.5g calcium/kg, 2.4g Phytate Phosphorus/kg group are significantly lower than values for all other dietary treatments ($P<0.05$)

(Mean ± S.E.M.)

of these parameters on moderate calcium diets was greatest with the lowest levels of phytate phosphorus.

B - Mineral composition of the tibia

Phytate phosphorus (2.4g/kg) and high vitamin D_3 diet produced a significant ($P<0.05$) decrease in calcium, total phosphorus and magnesium contents in tibia ash compared with other treatment values (Table 6.4). Varying the concentration of calcium, phytate phosphorus and vitamin D_3 in the diet of chicks, did not significantly affect the concentration of zinc in tibia ash.

6.3.3 Mineral composition in the plasma

There were not significant effects of diet on plasma levels of magnesium and zinc (Table 6.5). Equally, there were no significant differences between chicks fed the control diet and those receiving the low calcium, high vitamin D_3 in the levels of any of the plasma minerals which were studied. However, significant differences ($P<0.05$) were observed in plasma total and ionized calcium and plasma total phosphorus between control chicks and those given the moderate calcium-high vitamin D_3 with the two lowest levels of phytate phosphorus.

6.3.4 Phytate phosphorus, total phosphorus and calcium digestibility

Chicks fed the control diet showed a highly significant ($P<0.01$) decrease in phytate phosphorus digestibility compared with all other treatments (Figure 6.2). Chicks fed low phytate diets with low levels of calcium showed of total phosphorus retention significantly higher ($P<0.01$) than for the other diets. Chicks given the low calcium - low phytate phosphorus diet showed significantly higher ($P<0.01$) calcium retentions compared to all other treatments (see Figure 6.2).

TABLE 6.3 : THE EFFECT OF DIETARY CALCIUM, PHOSPHORUS AND VITAMIN D_3 IN VARIOUS LEVELS OF PHYTATE PHOSPHORUS ON TIBIA CALCIFICATION IN BROILER CHICKS AT 28 DAYS OF AGE

	Control	Moderate calcium in the diet (7.5g/kg)			low calcium
Level of phytate phosphorus in diet g/kg	2.4	2.4	2.8	3.2	2.4
Tibia fat free weight(g)	2.60 ± 0.13^a	1.97 ± 0.08^b	2.16 ± 0.9^b	2.01 ± 0.4^b	2.16 ± 0.07^b
Tibia ash weight (g)	1.35 ± 0.07^a	0.94 ± 0.04^b	1.08 ± 0.06^b	1.01 ± 0.2^b	1.05 ± 0.04^b
Tibia ash percentage	51.86 ± 0.19^a	47.56 ± 0.47^b	49.76 ± 0.64^c	50.27 ± 0.49^c	49.19 ± 0.26^c
Tibia length (cm)	7.33 ± 0.6^a	6.76 ± 0.09^b	7.03 ± 0.09^c	6.97 ± 0.06^c	7.27 ± 0.6^a

Groups without common subscripts are significantly different from each other ($P<0.05$)

Values are expressed as the mean ± S.E.M.

TABLE 6.4 : THE EFFECT OF DIETARY CALCIUM, TOTAL PHOSPHORUS AND VITAMIN D_3 IN VARIOUS LEVELS OF PHYTATE PHOSPHORUS ON TIBIA MINERAL COMPOSITION IN BROILER CHICKS AT 28 DAYS OF AGE

	Control	Moderate calcium in the diet (7.5g/kg)			low calcium
Level of phytate phosphorus in diet (g/kg)	2.4	2.4	2.8	3.2	2.4
Calcium (mg/g dry fat-free tibia)	198 ± 5[a]	171 ± 3[b]	186 ± 4[a]	189 ± 5[a]	186 ± 6[a]
Total phosphorus (mg/g dry fat-free tibia)	96 ± 2[a]	87 ± 2[b]	93 ± 2[a]	93 ± 3[a]	94 ± 2[a]
Magnesium (µg/g dry fat-free tibia)	4765 ± 54[a]	4244 ± 77[b]	4519 ± 56[a]	4516 ± 122[a]	4699 ± 102[a]
Zinc (µg/g dry fat-free tibia)	211 ± 6	199 ± 6	199 ± 5	208 ± 7	204 ± 6

Groups without common subscripts are significantly different from each other ($P<0.05$)

Values are expressed as the mean ± S.E.M.

TABLE 6.5 : THE EFFECT OF DIETARY CALCIUM, TOTAL PHOSPHORUS AND VITAMIN D_3 IN VARIOUS LEVELS OF PHYTATE PHOSPHORUS ON PLASMA MINERAL COMPOSITION IN BROILER CHICKS AT 28 DAYS OF AGE

	Control	Moderate calcium in the diet (7.5g/kg)			low calcium
Level of phytate phosphorus (g/kg	2.4	2.4	2.8	3.2	2.4
Total calcium (mmol/l)	2.57 ± 0.06^{a}	3.09 ± 0.09^{b}	2.98 ± 0.14^{b}	2.83 ± 0.15^{bc}	2.74 ± 0.05^{c}
Ionized calcium (mmol/l)	1.22 ± 0.04^{a}	1.41 ± 0.07^{bc}	1.44 ± 0.06^{b}	1.26 ± 0.03^{ca}	1.23 ± 0.05^{a}
Total phosphorus (mmol/l)	2.42 ± 0.06^{a}	1.76 ± 0.09^{b}	1.9 ± 0.08^{b}	2.24 ± 0.08^{a}	2.24 ± 0.05^{a}
Magnesium (mmol/l)	0.66 ± 0.03	0.76 ± 0.04	0.75 ± 0.03	0.65 ± 0.01	0.68 ± 0.02
Zinc (µmol/l)	34.87 ± 2.12	33.65 ± 1.28	32.83 ± 2.14	34.21 ± 2.15	32.97 ± 1.32

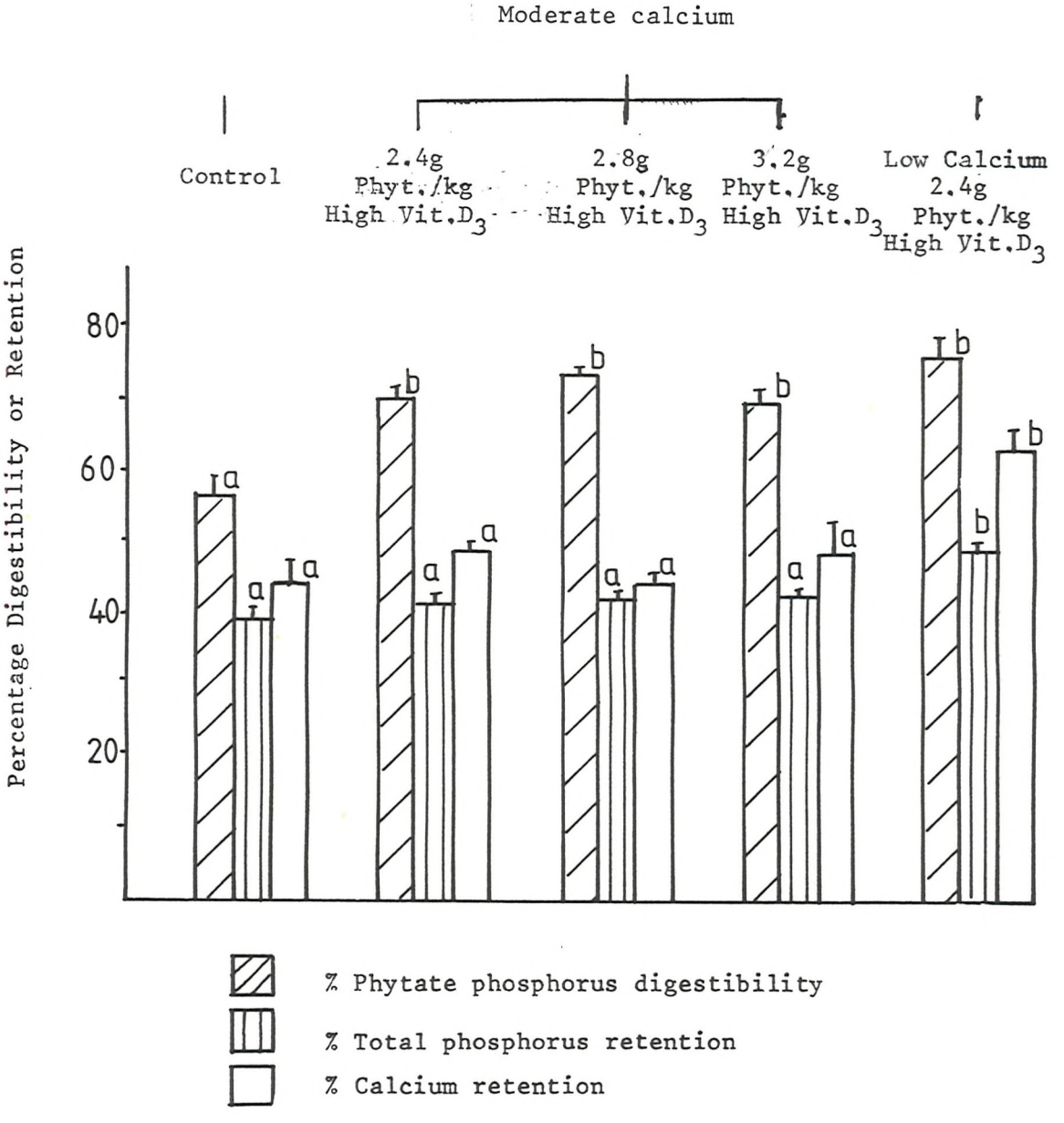
Groups without common subscripts are significantly different from each other ($P<0.01$)

Values are expressed as the mean ± S.E.M.

6.4 DISCUSSION

The results of the present study confirm the findings of the previous experiment with broilers, that is, that on low-calcium, high vitamin D_3 diets, the growth of chicks is greatest when calcium intake is least. At a given level of dietary calcium (7.5g/kg) increasing the dietary concentration of wheat bran, increased growth, tibia mineralisation and improved the hypophosphataemia and hypercalcaemia observed at the lowest level of phytate supplementation. Hedge et al. (1978) also observed this growth promoting effect on wheat bran in chicks and the data of Thompson and Weber (1981) agrees with our observation that the growth of chicks given wheat bran supplements, did not differ from that of control chicks for whom the phosphorus requirement was met with inorganic phosphorus. It can also be seen from the present study that when the level of dietary calcium is increased from 5.0 to 7.5 g/kg, the level of dietary phytate also has to be increased to maintain adequate growth rates. All of these results are consistant with the observation of previous experiments. By lowering calcium intake, the availability of phytate phosphorus to intestinal phytase improves (mcCuaig and Motzok, 1974a,b) allowing a reduction in the level of dietary inorganic phosphorus. These changes are only possible when high levels of vitamin D_3 are included in the diet to stimulate intestinal phytase (Holdsworth, 1970; Kempson et al. 1979) enhance calcium and phosphorus absorption (Lane and Lawson, 1978; Moog and Glazier, 1972) and reduce phosphorus excretion (DeLuca, 1979). The digestibility of phytate phosphorus was approximately 70% in diets containing 5.0 or 7.5 g/kg at dietary calcium. This agrees with findings of the previous study for comparable diets. Although the digestibility of phytate phosphorus did not increase with increasing intakes of phytate phosphorus, the concentration of available phytate did increase. This is also true of total dietary phosphorus. The increased concentration of available dietary phosphorus is reflected in the increase in plasma total phosphorus. This in turn is reflected in the response of tibia mineralisation to increasing availability of plasma phosphorus.

FIGURE 6.2 : THE EFFECTS OF DIETARY PHYTATE PHOSPHORUS, PHOSPHORUS AND CALCIUM LEVELS IN HIGH VITAMIN D_3 DIETS ON THE DIGESTIBILITY OF PHYTATE PHOSPHORUS AND ON THE RENTENTION OF TOTAL PHOSPHORUS AND CALCIUM OF BROILER CHICKS BETWEEN 24 AND 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.05$)

(Mean $\pm$ S.E.M.)

Thus the results of the present study show that in the use of low phosphorus (5-6 g/kg), moderate calcium (7.5 g/kg), high vitamin D_3 (50,000 1.u/kg) diets, increasing intakes of phosphorus can be achieved with sources of phytate phosphorus. However, these moderate intakes of calcium, even with higher intakes of phytate phosphorus, do not represent any improvement over the best diet observed in this and in the previous study i.e. low calcium (5 g/kg), low phosphorus (5 g/kg), high vitamin D_3 (50,000 1.u/kg). These diets yielded values which were very similar to those obtained by the control diet.

CHAPTER SEVEN

THE INFLUENCE OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON THE SMALL INTESTINAL ACTIVITIES OF PHYTASE AND ALKALINE PHOSPHATASE AND ON INTESTINAL PHYTATE HYDROLYSIS

7.1 Introduction

Intestinal phytase and alkaline phosphatase are considered to be important factors in the utilization of dietary phosphorus by the chick. The mechanism by which phytate digestibility is increased in chicks given low phosphorus diets, is unknown but may be related to the activity of phytase (EC3.1.3.8) and alkaline phosphatase (Ec.3.1.3.1) present in the intestine (Pileggi, 1959; Davies and Flett, 1978). Although it has been stated (Cheryan, 1980) that single stomached animals do not possess these intestinal enzymes, the data of Cooper and Gowing (1983); Reinhold (1972) and McCuaig et al. (1972) show that intestinal phytase and alkaline phosphatase do occur in such animals although the enzyme activity varies between different species.

As has been shown in previous chapters, the chick can digest and utilize phytate phosphorus as a source of dietary phosphorus. The aim of the following work was to understand just how the chick was so efficient in this respect. The first obvious approach was to see if phytates digested in the chick could be explained by greater intestinal phytase or alkaline phosphatase activities. The second approach was to measure the phytate digestibility and the disappearance of total phosphorus and calcium in the intestinal segments.

7.2 Materials and Methods

Twenty four day old broiler type chicks (Ross one) were used for this experiment. These chicks were randomized into three treatments. Each treatment was replicated twice with four chicks per replicate. The basal diet was similar to that used in the previous experiments and contained no feedstuffs of animal origin.

The mineral composition of the three diets is shown in Table 7.1. One major problem with such a study is to overcome the confounding effects of gut secretions and absorptions. Therefore chromic oxide, a non-absorbable gut marker that is associated with the solid phase, was added to the diets ($2g/Cr_2O_3/kg$). This marker made it possible to correct for the various gut absorptions and secretions. The chickens were maintained on this diet for 4 weeks and were then killed. The small intestines were removed and whole digesta were taken from the gizzard and the intestine. These samples were then dried in an oven at 90°C on tin foil sheets. The dried samples were then divided into two weighted aliquots. One aliquot was then extracted in diluted nitric acid for the determination of phytate by the method of Oshima et al. (1964). (See materials and methods Chapter 2). The other aliquot was wet digested in boiling concentrated nitric acid followed by boiling perchloric acid for total phosphorus determination by the method of Hanson (1950) and the total calcium and chromium by atomic absorption flame spectrophotometry. All these methods are described in detail in Chapter 2.

The small intestines were removed and the first 10 cm of the duodenum, jejunum and ileum were taken. These sections were washed in 50 mM Tris succinate (pH 7.4) buffer and mucosal scrapings, taken from each section. Alkaline phosphatase and phytase activities were then determined on these tissue samples by the method of Davies and Flett (1978), (see materials and methods Chapter 2.14). Right tibia bones were collected to determine the mineral contents. Three day's droppings were collected for analysis of phytate, total phosphorus and calcium (see materials and methods Chapter 2).

7.3 Results

7.3.1 Growth

The low phosphorus diet with a normal level of calcium led to a significant reduction ($P<0.01$) in body weight compared to the

TABLE 7.1: THE CALCIUM, PHOSPHORUS AND VITAMIN D_3 CONTENT OF THE DIET

	Recommended diet (control)	Norman calcium high vitamin D_3	Low calcium High vitamin D_3
Calcium (g/kg)	10	10	5
Inorganic phosphorus (g/kg)	4.5	2.6	2.6
Phytate phosphorus (g/kg)	2.4	2.4	2.4
Total phosphorus (g/kg)	6.9	5	5
Vitamin D_3 (1.u/kg)	500	50,000	50,000
Oystershell (g/kg)	21.21	21.21	8.71

control (see Figure 7.1). However, the body weight of the chicks fed on the low phosphorus low calcium diet with high vitamin D_3, were not significantly different from the chicks on the control diet.

7.3.2 Tibia calcification

A - Tibia fat free weights, tibia ash weight and percentage of ash in tibia

Tibia fat-free weight, tibia ash weights and percentages were significantly lower ($P<0.01$) with the two experimental diets compared to control (Table 7.2). However, the extent of these effects was significantly greater with the normal level of calcium.

B - Tibia mineral composition

There were no signficant differences in the tibia ash content of calcium, phosphorus, magnesium or zinc between control diet and those low in calcium, but high in vitamin D_3. However, these parameters were significantly reduced ($P<0.01$) compared to other treatment in chicks fed the high vitamin D_3 - normal calcium diets (Table 7.3).

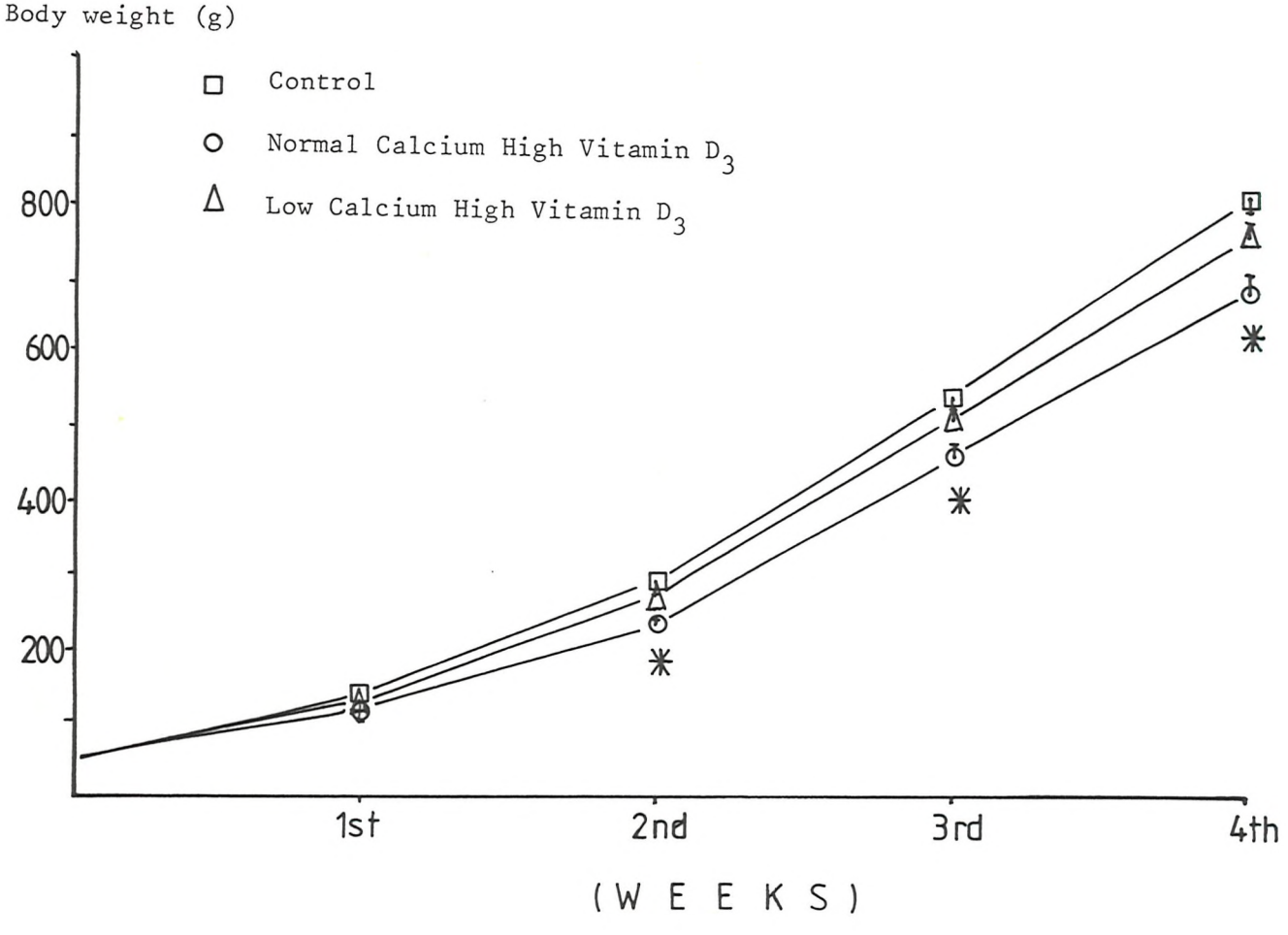
7.3.3 Intestinal phytase activity

There were no significant treatment effects on intestinal phytase activity which was found to decrease in moving from the proximal to distal small intestine (Figure 7.2).

7.3.4 Intestinal alkaline phosphatase activity

Alkaline phosphatase was also found to decrease in moving from the proximal to distal regions of the small intestine. In the ileum, there were no significant treatment effects on intestinal alkaline phosphatase activity. However, in both the

FIGURE 7.1 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON BODY WEIGHT OF BROILER CHICKS UP TO 28 DAYS OF AGE



* All values for normal calcium, normal vitamin D_3 with low phosphorus group are significantly lower than values for all other dietary treatment ($P<0.05$)

(Mean $\pm$ S.E.M.)

TABLE 7.2: THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON TIBIA CALCIFICATION IN BROILER CHICKS AT 28 DAYS OF AGE

	Recommended diet (control)	Normal calcium High vitamin D_3	Low calcium High vitamin D_3
Tibia fat free weight (g/kg)	2.22 ± 0.11^a	1.50 ± 0.07^b	1.73 ± 0.14^b
Tibia ash weight (g/kg)	1.15 ± 0.06^a	0.68 ± 0.03^b	0.85 ± 0.07^c
Tibia ash (percentage)	51.94 ± 0.47^a	45.36 ± 0.62^b	49.28 ± 0.38^c

Groups without common subscripts are significiantly different from each other ($P<0.01$).

Values are expressed as the mean $\pm$ S.E.M.

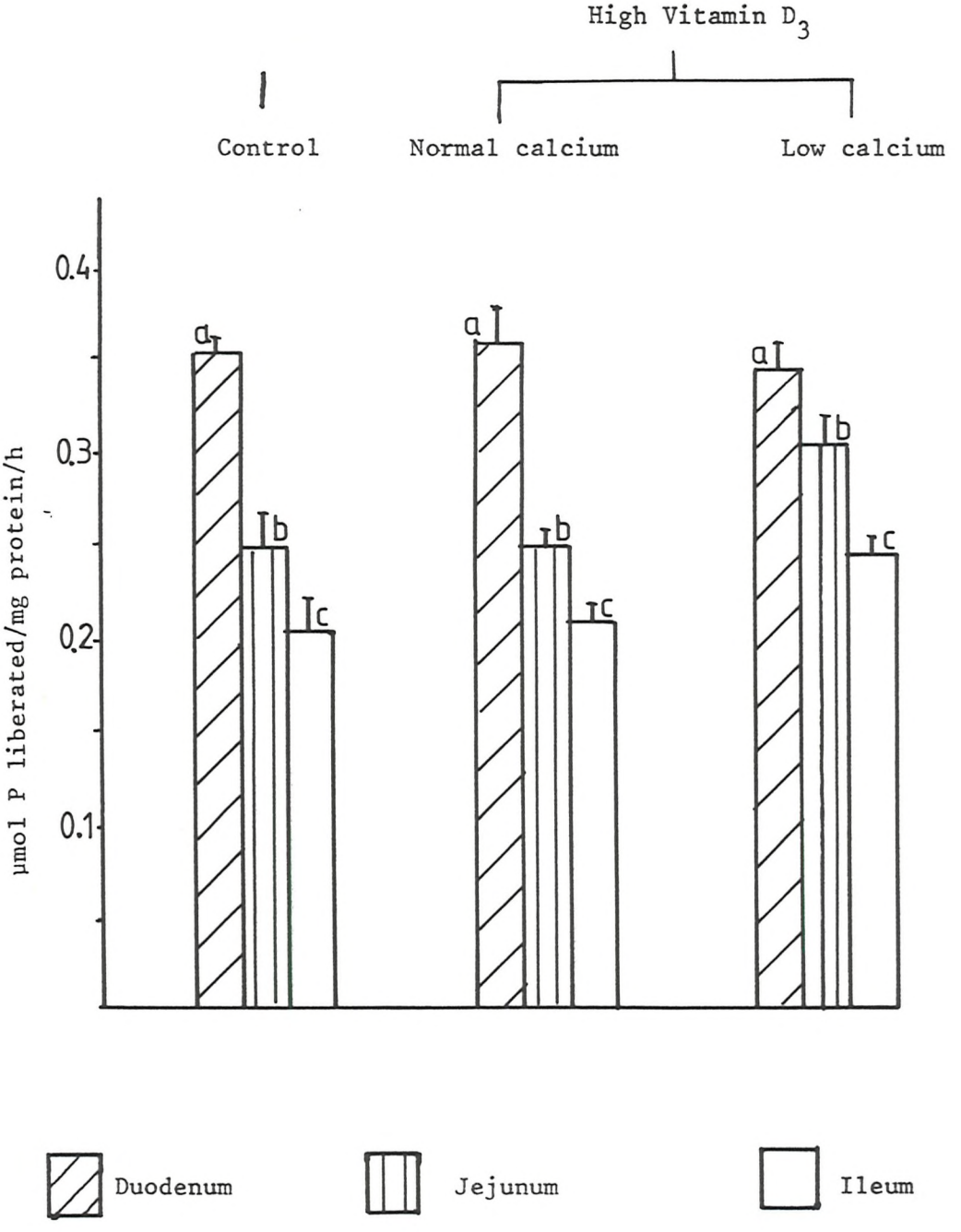
TABLE 7.3: THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON TIBIA MINERAL COMPOSITION IN BROILER CHICKS AT 28 DAYS OF AGE

	Recommended diet (control)	Normal calcium High vitamin D_3	Low calcium High vitamin D_3
Calcium (mg/g dry fat free tibia)	188 ± 2^a	166 ± 3^b	182 ± 3^a
Total phosphorus (mg/g dry fat free tibia)	94 ± 1^a	77 ± 3^b	91 ± 2^a
Zinc (µg/g dry fat free tibia)	256 ± 8	243 ± 11	262 ± 13
Magnesium (µg/g dry fat free tibia)	5147 ± 84^a	3807 ± 104^b	4929 ± 115^a

Groups without common subscripts are significiantly different from each other ($P<0.01$).

Values are expressed as the mean $\pm$ S.E.M.

FIGURE 7.2 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE ACTIVITY OF INTESTINAL PHYTASE PHOSPHORUS OF BROILER CHICKS AT 28 DAYS OF AGE IN VARIOUS SECTIONS OF THE SMALL INTESTINAL MUCOSA



Non-significant differences in various sections of the small intestinal mucosa

(Mean ± S.E.M.)

duodenum and jejunum, the activity of alkaline phosphatase was significantly higher ($P<0.05$) in chicks fed both high vitamin D_3 diets compared with those fed the control diet (see Figure 7.3). In the duodenum, the activity of this enzyme was significantly higher ($P<0.05$) in chicks fed low calcium - high vitamin D_3 diet compared to the other treatments. The total intestinal alkaline phosphatase activity was also significantly higher ($P<0.01$) in chicks fed the high vitamin D_3 diets compared with control (Figure 7.4).

7.3.5 Digestibility of phytate phosphorus and total phosphorus and calcium in the gizzard and small intestine

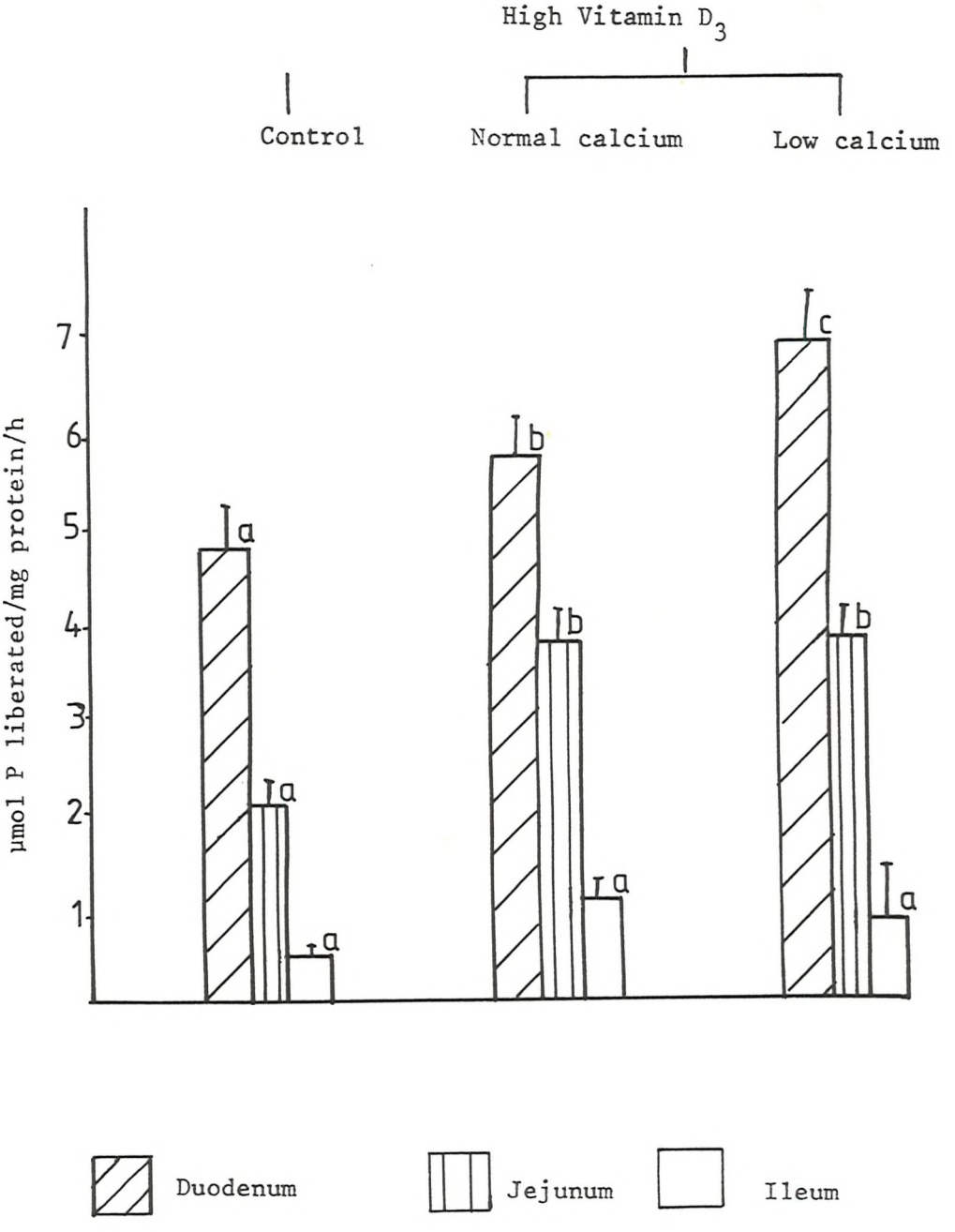
A - Phytate digestibility

The data in (Figure 7.5) show cogently that much of the dietary phytate is digested before it leaves the gizzard. There were, however, no significant differences in gizzard phytate phosphorus digestibility between the three dietary treatments. The high vitamin D_3 diets increased intestinal phytate digestibility compared to control. This effect was more pronounced in chicks fed low calcium diet ($P<0.01$).

B - Disappearance of total phosphorus

Chicks fed the control diet showed a significantly greater ($P<0.05$) disappearance of total phosphorus in the gizzard (28.9%) compared with chicks fed the high vitamin D_3 diets, irrespective of calcium levels (Figure 7.6). In the small intestine the disappearance of total phosphorus in the normal calcium high vitamin D chicks was less than in the control diets. In the case of the low calcium high vitamin D_3 diet this value was significantly higher ($P<0.05$) than in the control chicks.

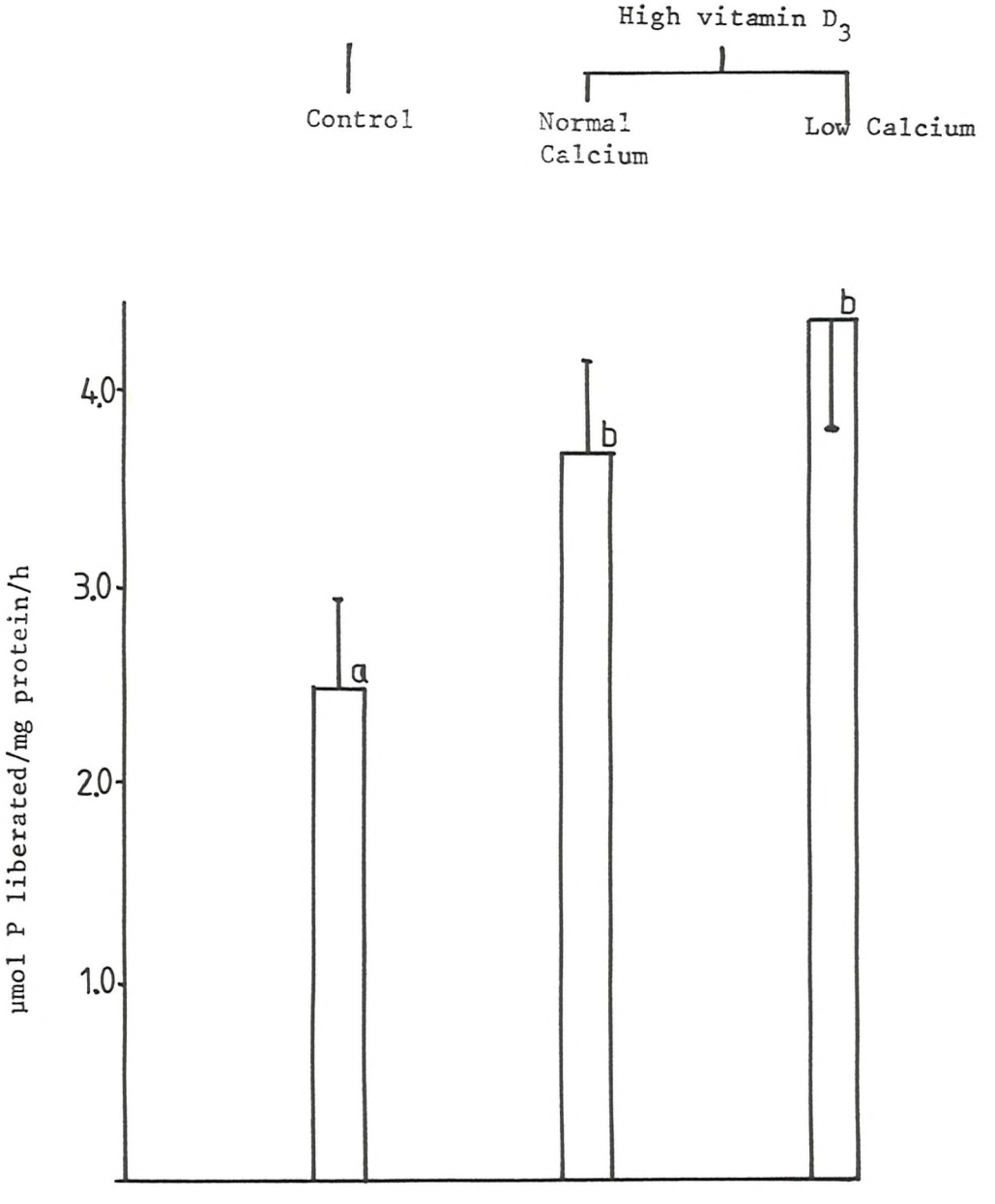
FIGURE 7.3 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE ACTIVITY OF INTESTINAL ALKALINE PHOSPHATASE OF BROILER CHICKS AT 28 DAYS OF AGE IN VARIOUS SECTIONS OF THE SMALL INTESTINAL MUCOSA



Intestinal segments without common subscripts are significantly different from each other ($P<0.05$)

(Mean $\pm$ S.E.M.)

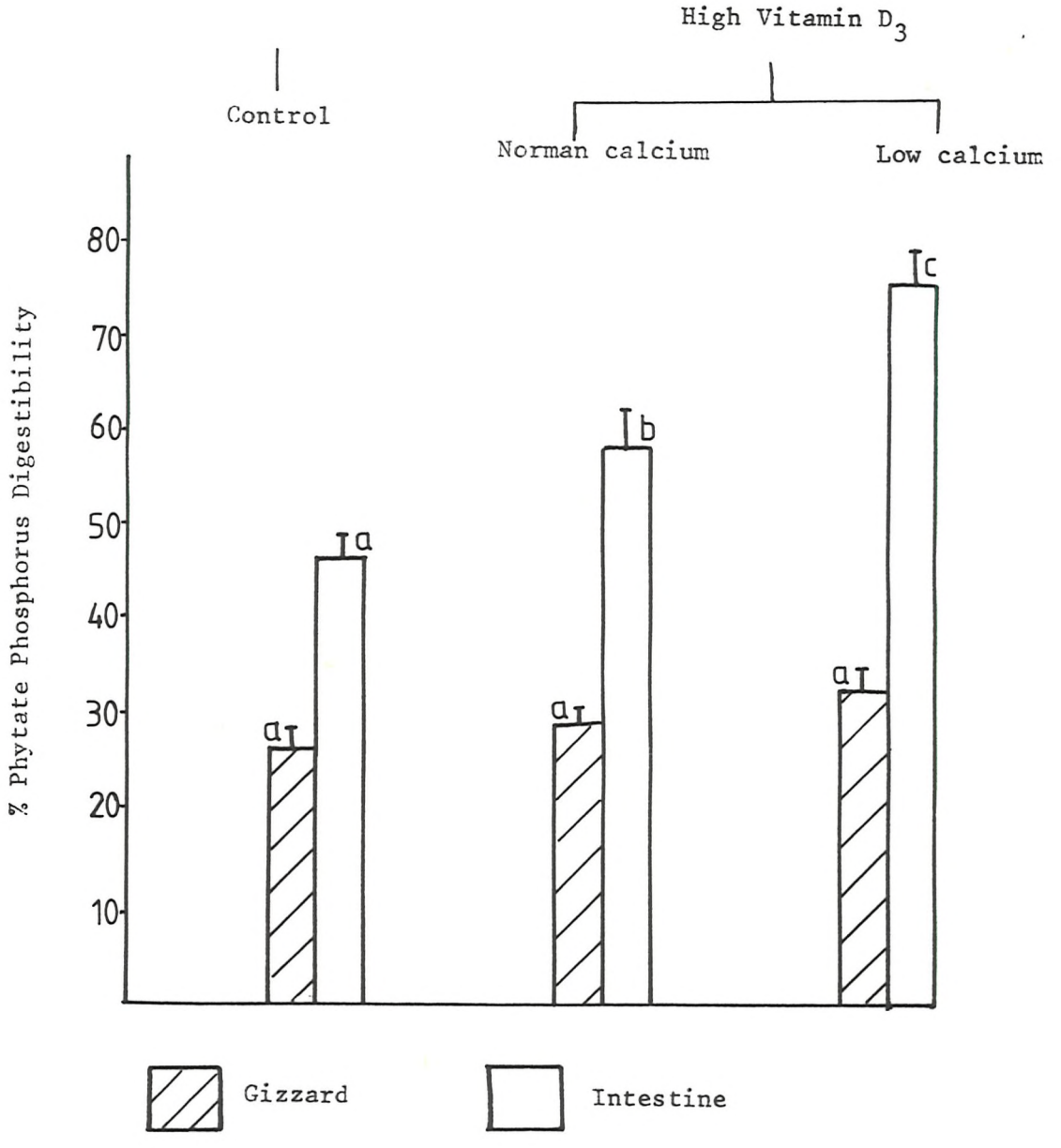
FIGURE 7.4 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE ACTIVITY OF TOTAL INTESTINAL ALKALINE PHOSPHATASE OF BROILER CHICKS AT 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.01$)

(Mean $\pm$ S.E.M.)

FIGURE 7.5 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE GIZZARD AND SMALL INTESTINAL PHYTATE DIGESTIBILITY OF BROILER CHICKS AT 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.05$)

(Mean $\pm$ S.E.M.)

C - Disappearance of Calcium

Approximately 30% of the calcium disappeared in the gizzard in all treatments with no significant differences between them (Figure 7.7). Intestinal calcium disappearance was significantly higher ($P<0.01$) compared with the gizzard values for all the treatments. High vitamin D_3 low phosphorus diets led to significantly greater ($P<0.01$) calcium disappearance compared to control diets.

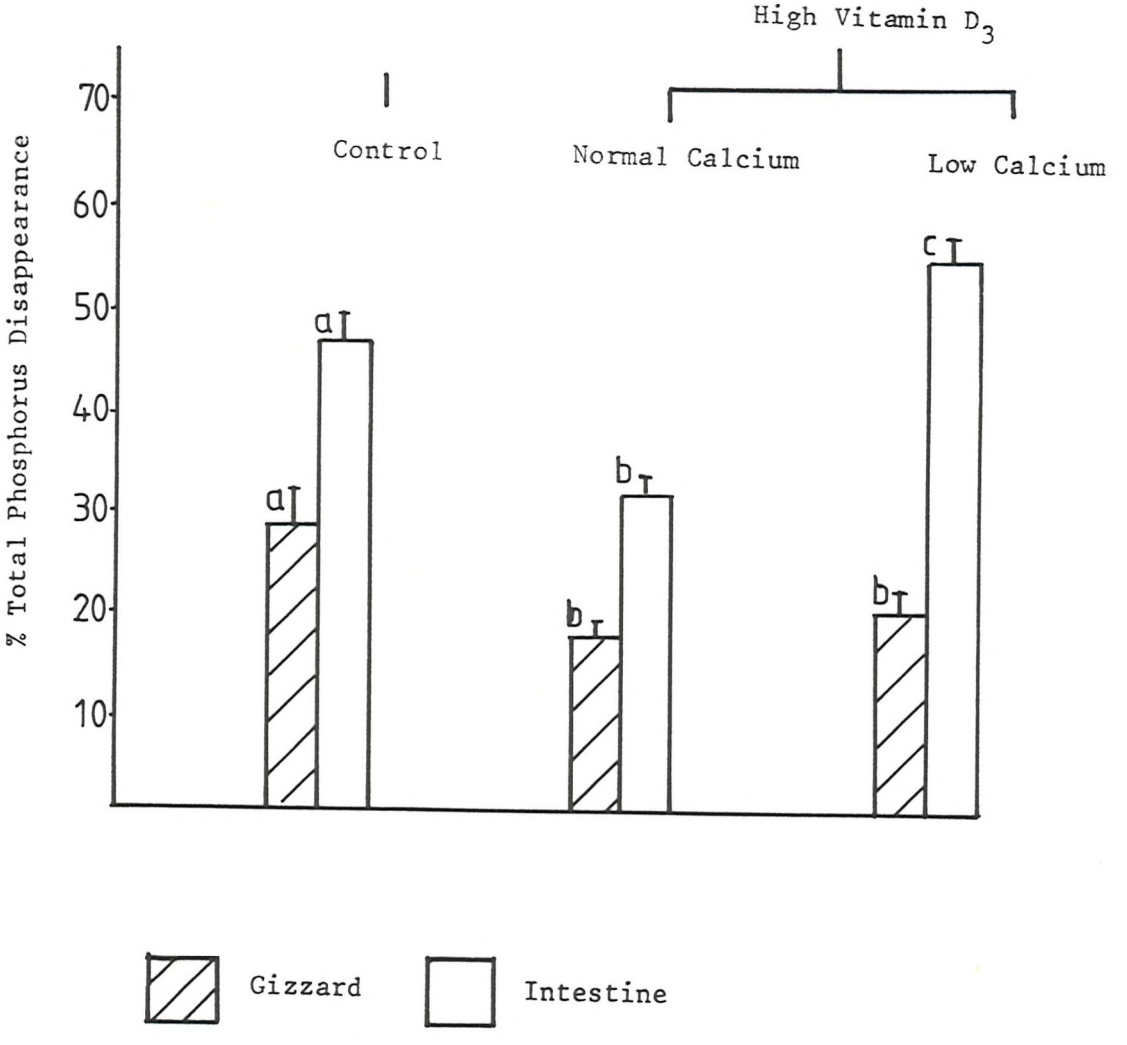
7.3.6 Total digestibility of phytate and retention of total phosphorus and calcium from analysis of droppings

These data are given in (Figure 7.8) and are in agreement with comparable data in previous chapters (see Figures 5.6, 6.2). However, in this experiment, because only two groups of chicks were used for each treatment, the data were not subject to statistical analysis.

7.4 DISCUSSION

The results of the present study for body weight, tibia mineralization and the availability of calcium and phosphorus are comparable to those found in the previous experiments. The decline in intestinal phytase in the distal small intestine observed in the present study has also been reported for rats (Williams 1984) but was not observed with pigs (Poinfillared et al. 1984). The lack of an effect of low-phosphorus diets on intestinal phytase has also been observed by Moore and Veum (1983 in rats and Moser et al. (1982) in pigs. In contrast, Davies et al. (1972), working with very low phosphorus diets (1.6 g/kg) did find elevated duodenal activity of phytase in the chick. The activity of intestinal phytase is however much lower than that of intestinal alkaline phosphatase and this has been previously reported for the rat and hamster (Williams and Taylor 1985) and pig (Poinfillared et al. 1985). The data of Williams et al. (1984) confirms

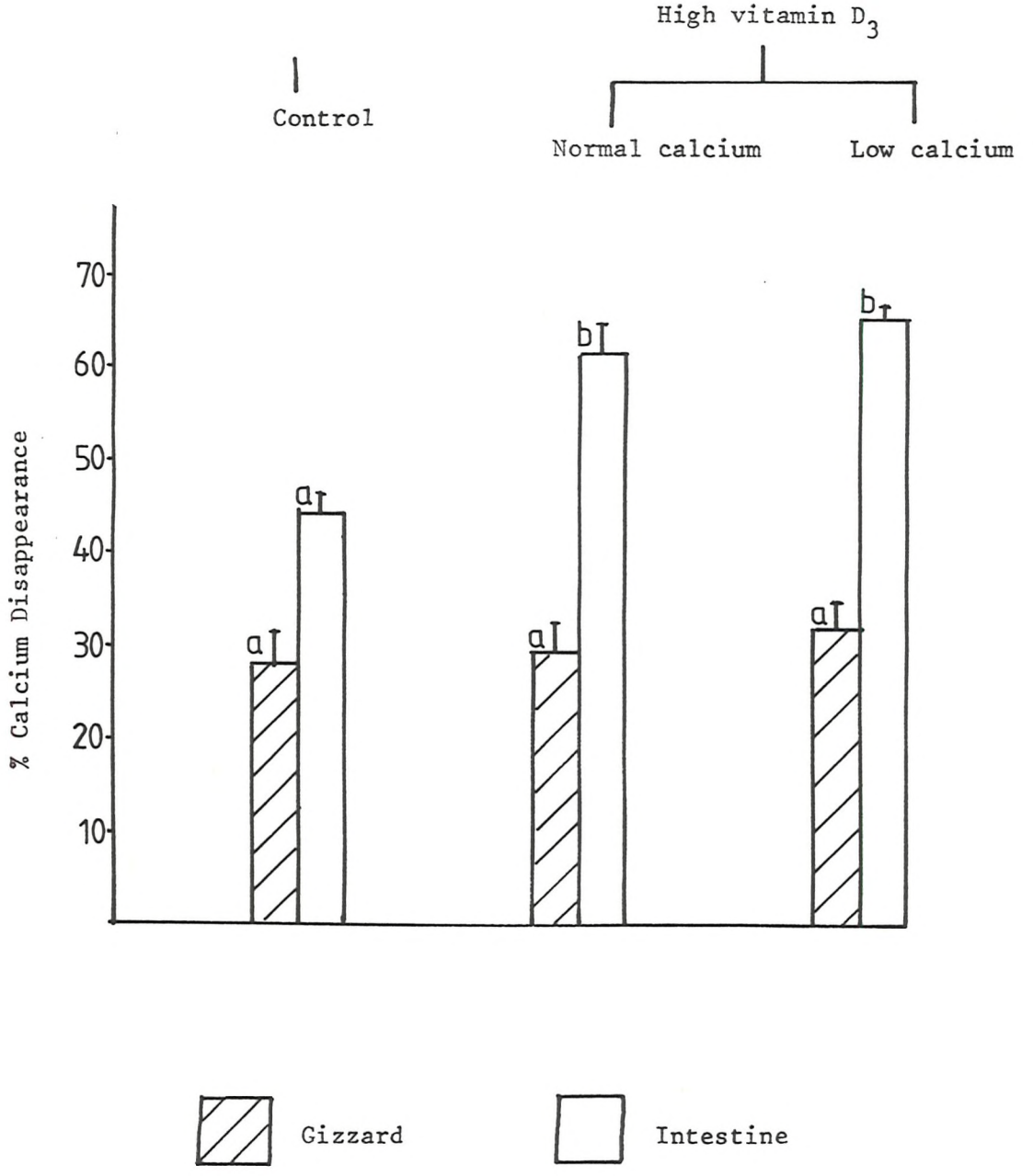
FIGURE 7.6 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE DISAPPEARANCE OF TOTAL PHOSPHORUS FROM THE GIZZARD AND SMALL INTESTINE OF BROILER CHICKS AT 28 DAYS OF AGE



Groups without common subscripts are significantly different from each other ($P<0.05$)

(Mean ± S.E.M.)

FIGURE 7.7 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE DISAPPEARANCE OF CALCIUM FROM THE GIZZARD AND SMALL INTESTINE OF BROILER CHICKS AT 28 DAYS OF AGE



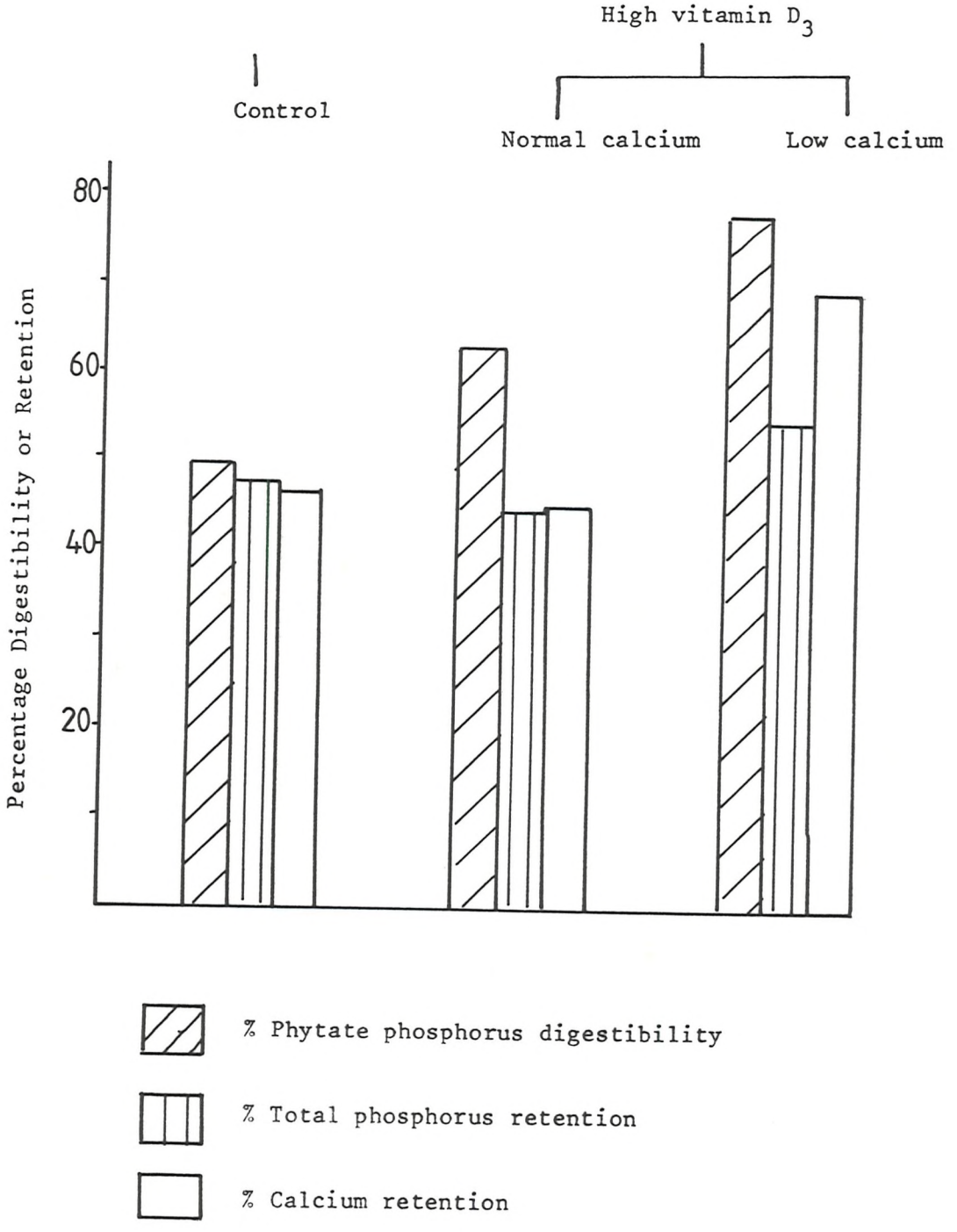
Groups without common subscripts are significantly different from each other ($P<0.01$)

(Mean $\pm$ S.E.M.)

the finding of the present study that alkaline phosphatase activity declines towards the distal regions of the small intestine. The present study shows that unlike intestinal phytase, intestinal alkaline phosphosphatase is influenced by dietary levels of calcium, phosphorus and vitamin D_3. McCuaig and Moztok (1972) and Kempson et al. (1979) indicated that the functional adaptation of low phosphorus diets is accompanied by an increase in the activity of alkaline phosphatase, localised in the intestinal bruch border membrane. This remarkable adaptive change in a single enzyme leads to the suggestion that alkaline phosphatase plays an important role in the enhanced transport of inorganic phosphorus in the intestine in chicks deprived of dietary phosphorus. Calcium deficiency also increases alkaline phosphatase activity (McCuaig and Moztok, 1974) but excess calcium or magnesium was found to reduce the activity of alkaline phosphatase (McCuaig et al. 1972; McCuaig and Motzok 1973). Zinc deficiency also leads to a marked fall in rat intestinal alkaline phosphatase which is a zinc dependent enzyme (Williams, 1972; Davies and Flett, 1978). High intakes of vitamin D_3 were found in the present study to increase duodenal and jejunal alkaline phosphatase activity. Other workers have observed this effect of vitamin D_3 (Holdsworth 1970 and Haussler *et al.* 1970). It should be noted that the effects of both calcium and vitamin D_3 on alkaline phosphatase activity observed in the present study, were anatomically variable. For example, no effect of diet was observed in ileum alkaline phosphatase. Jejunal alkaline phosphatase was elevated by the low-phosphorus, high vitamin D_3, normal-calcium diet and when the calcium content of this diet was lowered, this effect then became apparent in duodenal alkaline phosphatase activity. When the overall intestinal alkaline phosphatase activity was considered, the low-phosphorus, high-vitamin D_3 effect was still apparent but there was no direct effect due to calcium reduction.

In the present experiment, a new approach was adapted to measure the phytate content in the gizzard and the small intestine which meant that instead of directly measuring the enzymes phytase

FIGURE 7.8 : THE EFFECTS OF DIETARY CALCIUM AND VITAMIN D_3 LEVELS IN LOW PHOSPHORUS DIETS ON THE OVERALL DIGESTIBILITY OF PHYTATE PHOSPHORUS AND ON THE RETENTION OF TOTAL PHOSHORUS AND CALCIUM OF BROILER CHICKS BETWEEN 24 AND 28 DAYS OF AGE



and alkaline phosphatase, an indirect measurement of the disappearance of the substrate, phytate was made. The most interesting result in this experiment was that about 30% of the phytate appeared to be hydrolysed in the gizzard of chicks, irrespective of different treatment. Overall, about a half to two thirds of the total phytate was hydrolysed by the chicks in the small intestine. This result is in agreement with the report of Simons (1979). Tyler (1946) has shown that pH plays a critical role in phytase hydrolysis and in the disappearance of both calcium and total phosphorus in the gizzard. According to Hill and Tyler (1954), the activity of cereal phytase is destroyed at a pH of 2.5 or lower. This level of pH does not exist in any part of the chick gastrointestinal tract. A pH between 3.5 - 7.5 is found in the crop (Bell and Freeman, 1971) and 3.6 - 4.96 in the gizzard (Tyler, 1946).

Apparently, the gizzard retains larger feed particles longer than smaller ones (Hurwitz and Bar, 1965). There is, however, evidence that the release of food from the full crop only occurs when the gizzard has discharged its contents into the duodenum (Pastea et al. 1968). As long as food remains in the gizzard, onward movement of ingesta does not occur. The inference from these observations is that the gizzard is the major regulator of the movement of ingesta through the digestive tract. It is clear from the present study that some phytate is released in the gizzard probably by the hydrolytic action of hydrocholoric acid (HCl) in the gastric juice, with some of the phytate being leached out to the small intestine. Tyler (1946) showed evidence of regurgitation of acid into the crop with a consequent leaching of soluble calcium and phosphorus from feeds.

Leaching of soluble calcium and phosphorus is also very pronounced in the gizzard. Thus there will be a more rapid flow of soluble calcium and phosphorus from the gizzard leaving behind ingredients lower in calcium and phosphorus. This concept is supported by the fact that the chicks receiving inorganic phosphorus in their diet (control), showed more total phosphorus disappearance from the gizzard than other treatments which did not

receive inorganic phosphorus supplementation. There is a close relationship between intestinal phytate digestibility and the intestinal disappearance of calcium and phosphorus. Intestinal calcium disappearance was increased in low-phosphorus diets. This is in accordance with the findings of Morrisy and Wasserman (1971; Fox et al. (1978) and Sommerville et al. (1985)). These workers reported that the low phosphorus or low calcium diets led to an increased intestinal calcium absorption and may be related to both the synthesis of calcium binding protein (Peterlik and Wasserman, 1980), and an increase in the $1,25(OH)_2D_3$ production (Henery et al. 1974; Baxter and DeLuca, 1976; Swaminathan et al. 1978). $1,25(OH)_2D_3$ acts on the small intestine to stimulate intestinal calcium transport. Most evidence indicates that $1,25(OH)_2D_3$ alters the calcium permeability of brush border membrane by a process as yet unknown, but apparently not requiring protein synthesis (Rasmussen et al. 1979). CaBP seems to protect the cell against the surge of calcium passing through the intestinal epithelium when calcium transport is stimulated by $1,25(OH)_2D_3$ (Bikle et al. 1981).

Subsequent to the high calcium disappearance on low-phosphorus, high vitamin D_3 diets, the concentration of calcium in the small intestine is reduced. Therefore, the formation of calcium phytate may also be reduced in the intestinal digesta, thus increasing the availability of phytate to the elevated levels of alkaline phosphatase observed on these diets.

The disappearance of calcium on low-phosphorus, high vitamin D_3 diets was not influenced by the levels of dietary calcium. Both levels of dietary calcium (5 - 10 g/kg) showed disappearance rates of about 60%. From this it can be calculated that the unavailable calcium concentrations in the high and low calcium diets were 3.9 and 1.8 g respectively. This is reflected in the digestibility of phytate phosphorus.

Subsequent to the lower phosphorus absorption, growth and tibia calcification and tibia mineral composition were reduced. The control diet where all of the dietary requirement was met by sodium dihydrogen phosphate, produced a high intestinal phosphorus

disappearance compared with the chicks fed a normal-calcium, low-phosphorus, high-vitamin D_3 diet. This finding is related to the high biological values of sodium dihydrogen phosphate. Friz et al. (1969) reported that the biological value of sodium dihydrogen phosphate is 100%. In the present study, total phosphorus retention by the chicks fed the normal-calcium, low phosphorus, high-vitamin D_3 diet was higher than the intestinal phosphorus disappearance. However, calcium retention showed the opposite effect. Low levels of phosphorus in the diet resulted in hypophosphataemia, hypercalcaemia as well as hypercalciuria and hypophosphaturia. Thus urinary excretion of calcium would be high and that of phosphorus would be low. Therefore, calcium recovery in the droppings (faeces and urine) was high, while phosphorus recovery was low. This finding agrees with the results of Moore et al. (1984) who found that rats given a diet containing 1.5 g phosphorus/kg showed hypercalciuria and hypophosphaturia compared with rats receiving 4.5 g phosphorus/kg diet.

The results of the present study show that intestinal alkaline phosphatase is a quantitatively more important enzyme than intestinal phytase and that the activity of both enzymes declines towards the distal small intestine. Alkaline phosphatase, but not pytase, is influenced by variation in the levels of dietary phosphorus, calcium and vitamin D_3. The results also show that about 30% of the intestinal disappearance of calcium, total phosphorus and phytate phosphorus occur in the gizzard and the remainder in the small intestine. These disappearances from the intestine are variably influenced by dietary calcium, phosphorus and vitamin D_3.

CHAPTER EIGHT

A STUDY OF THE POSSIBLE ROLE OF THE INTESTINAL MICROFLORA IN PHYTATE HYDROLYSIS IN CHICKS

8.1 Introduction

Chicks can digest considerable amounts of phytate in their gizzard (approximately 30%) prior to food reaching the known intestinal phytase in the duodenum. A further increase in phytate digestibility occurs in the intestine. The origin of their gizzard phytase is thus uncertain but the only real possibilities are for cereals and microbes to provide the phytase activity or for this digestion of phytate to occur by non-enzymatic cleavage (Hegsted et al. (1954).

Several reports have suggested that phytase, which releases inorganic phosphorus from phytate, is active in the guts of rats (Pileggi, 1959; Ramarkrishnan and Bhandari, 1979) and other animals (Steenback, et al. 1953; Welch et al. 1974).

Most attention has, however, been given to the endogenous enzyme. The highest hydrolytic activity for sodium phytate has been found in the brush border of the duodenal mucosa (Davies and Flett, 1978) of rats. The relative contributions of endogenous, plant and bacterial phytase on phytate digestibility are of nutritional significance, because any decrease in phytate concentration in the small intestine would reduce its influence over the availability of metals absorbed there., Oral administration of antibiotics are known to increase tibial deposition of Ca (Migicovsky, et al. 1951) and to improve the utilization of calcium and phosphorus (Lindblad et al. 1952).

Work with germ free rats (Yoshida et al. 1985), mice (Yoshida et al. 1982) and quails (Yoshida et al. 1969) have shown that the apparent digestibility of calcium, total phosphorus and magnesium were greater than that seen in their conventional counterparts. Therefore, it is possible that phytate hydrolysis is altered by gut microflora.

To date, the comparative effects of the three possible sources of phytase (dietary, intestinal and microbial) on the hydrolysis of phytate phosphorus by chicks, has not been studied.

The aim of this experiment was to determine the relative contribution of these three sources of the phytase to phytate digestion. This was attempted by feeding phytase containing and phytase-free diets to conventional and germ-free chicks.

8.2 Experimental Procedure

8.2.1 Isolators

Stainless-steel isolators designed by Gustafsson (1959) were used (Figure 8.1). The isolator consisted essentially of a stainless steel tank, 34 inches long, 18 inches wide and 20 inches deep, covered by a plate-glass window. Into one of the sides a pair of heavy rubber sleeves was sealed, to which could be fitted rubber gloves. A third sleeve was provided on the opposite side. At one corner of the tank was a stainless steel U-trap to be filled with germicide, through which objects could be passed into or out of the tank. The air supply was sterilized by passing it through a steel cylinder heated electrically to 350°; it was cooled by passing through a tube along the side of the tank. The exhaust air was similarly treated. Heat was supplied by three 250 watt lamps clamped about 12 inches below the floor of each tank. Light was provided by a 20 watt fluorescent tube fixed along one of the long sides of the plate-glass window. It was controlled by a time-switch to give 12 hr illumination a day.

8.2.2 Chicks

Germ-free chicks were produced and reared from the National Institute for Research in Dairying's flock of Rhode Island RedX Light Sussex Chicks. Eggs were incubated for 18 days in a commercial incubator and then the outsides of the eggs were disinfected with peracetic acid (Harrison 1969). Those destined

FIGURE 8.1 : GUSTAFSSON'S ISOLATOR, USED IN THE REARING OF GERM-FREE CHICKS

Two views of the Gustafsson aparatus for germ-free chicks

A : Rebber sleeves with gloves for manipulations

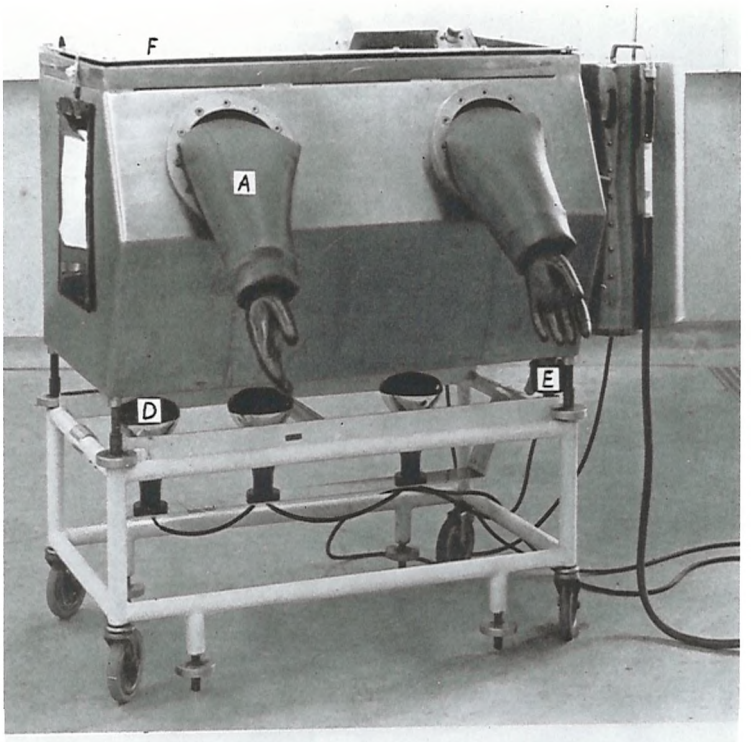
B : Trap for germicide

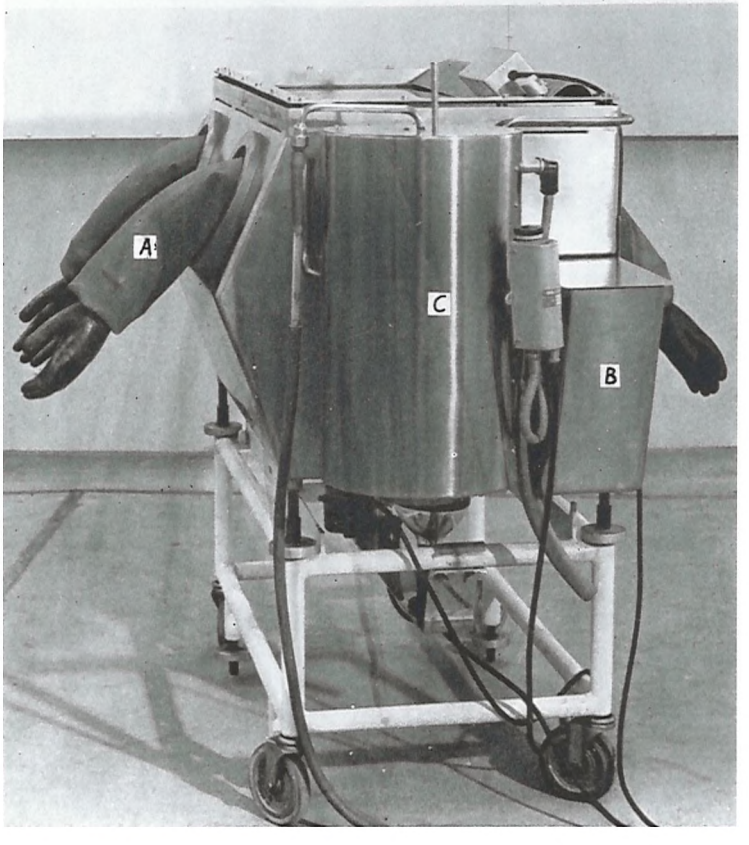
C : Air sterilizer

D : Heating lamps

E : Control for thermostat

F : Fluorescent lamp holder


F
A
D
E


A
C
B

for the production of germ-free chicks were transferred to Gustafson stainless-steel isolators to hatch. After hatching, eight pairs germ free chicks were housed in wire cages with wire mesh floors. The cages rested about 2 inches above a solid droppings-tray, which allowed sufficient space for the droppings to accumulate throughout the whole 4-week test period. Each isolator housed two pairs and to ensure identical environmental conditions, one pair was fed diet containing plant phytase, while the other received the diet without plant phytase. During the experiment, swabs were taken from chicks in the germ-free isolators to check for contamination by micro-organisms, (Fuller, 1968). A further four pairs were studied in a clean but non-sterile isolator to provide conventional controls.

8.2.3 Food and water

Sufficient sterilized drinking water, packed in pint bottles, were placed in the tank at the beginning of each experiment. The reservoirs were filled when necessary and the empty bottles taken out through the germicidal trap.

Two diets of similar composition were used in this study (Table 8.1). One diet contained a source of plant phytase activity, which came from the wheat bran. This enzyme is widely distributed in the bran (Lim and Tale, 1973) and may improve the utilization of phytate phosphorus in diets when fed to chicks (Nelson, 1967). The other diet contained the same amount of wheat bran, which had been wet-autoclaved to destroy phytase. The diets provided 12.6 Mj/kg metabolizable energy, 195g/kg crude protein, 6g/kg total phosphorus of which 3.2g/kg was phytate phosphorus, 5g/kg calcium and 50,000 i.u/kg vitamin D_3. Oyster-shell was added as a source of calcium.

To counteract possible losses of vitamins during sterilization by irradiation, the customary supplement was quadrupled. The diet was treated by Y-irradiation. It was packed

TABLE 8.1: COMPOSITION OF EXPERIMENTAL DIET (g/kg)

Ingredients	
Maize meal	510
Soybean meal	333
Dried yeast	50
Wheat bran	100
Sodium chloride	5
Methionine	1
Mixed minerals and vitamins	1

in polythene bags each containing 500 g diet, which were evacuated, sealed and then placed in a second bag, which was also sealed. Finally, the sealed packets were put into large black polythene bags and loaded into boxes in which they were sterilized by gamma-radiation at 5 Mrad. After treatment the packets were left inside the boxes in a cool room until required.

8.2.4 Introduction of materials into the tanks

During the experiment, it was necessary to pass several objects, such as packets of diet or bottles of water, aseptically into the tanks by way of the germicidal trap. The risk of introducing micro-organisms during this procedure was high. Stainless steel lids covered the traps to minimize the amount of dust falling into the germicide. To reduce the risk of surface contamination, all objects to be passed into the tanks were doubly wrapped and then sterilized. The outer one was removed at the trap, allowing the container with the inner cover still in place, to sink into the germicide. The second cover was then removed inside the tank. The boxes of irradiated diet were similarly brought to the tanks before the lid was opened, a packet of diet was then removed, the outer bag was slit open, and the inner packet of diet was slipped into the germicide. Throughout these procedures, materials introduced into the tank were handled only by operators wearing sterile rubber gloves.

8.2.5 Experimental procedure

The experiment lasted for 4 weeks. All chicks were weighed at the end of the experiment immediately after the tanks had been opened. The chicks were killed by decapitation. Plasma from each chick was collected to determine plasma levels of calcium, total phosphorus, magnesium and zinc. The accumulated droppings in each cage were mixed. Samples were taken and stored at -20°C until analysed to determine the phytate and total phosphorus and calcium (see Chapter 2 for methods used).

8.2.6 Preparation of Negligible Phytase Wheat Bran

Wheat bran was placed in a tray and the tray was autoclaved for 10 minutes at 121°C to inactivate the endogenous phytase. The autoclaved wheat bran was mixed with diet ingredients to produce a dietary low phytase activity. To determine the dietary phytase activity in the diets, the method of Hill and Tyler (1954) was used. 500 mg of dry diet which contained a plant phytase as wheat bran was added to 9 ml of distilled water and the pH adjusted to 5.0 with dilute hydrochloric acid. This mixture was then shaken and incubated at 37°C. The incubation was then stopped by the addition of 1 ml of 5 M nitric acid at 30, 60 and 90 minutes after commencing incubation, and was then shaken for 3 hours to extract the remaining phytate. This phytate was measured by the method of Oshima et al. (1964) (see Chapter 2.12). 21.79% of the dietary phytate was hydrolysed in the 90 minutes incubation, by the phytase containing diet. The phytase free diet had negligible dietary phytase activity, when measured under similar conditions. Approximately 0.3% of the dietary phytate was hydrolysed in 90 minutes by the low dietary phytase. These results are illustrated in Figure 8.2.

8.3 Results

8.3.1 Growth

For the four week experimental period, there was no significant differences in the mean final body weights of chicks in any of the four treatments (see Figure 8.3).

8.3.2 Plasma mineral composition

Calcium, total phosphorus, zinc and magnesium concentrations in the plasma were similar in germ-free and conventional chicks and were not influenced by the presence or absence of dietary phytase (Table 8.2).

FIGURE 8.2 : THE EFFECT OF INCUBATING THE EXPERIMENTAL DIET WITH NORMAL OR AUTOCLAVED WHEAT BRAN ON THE LEVEL OF PHYTATE IN THE DIET (Mean ± S.E.M.)

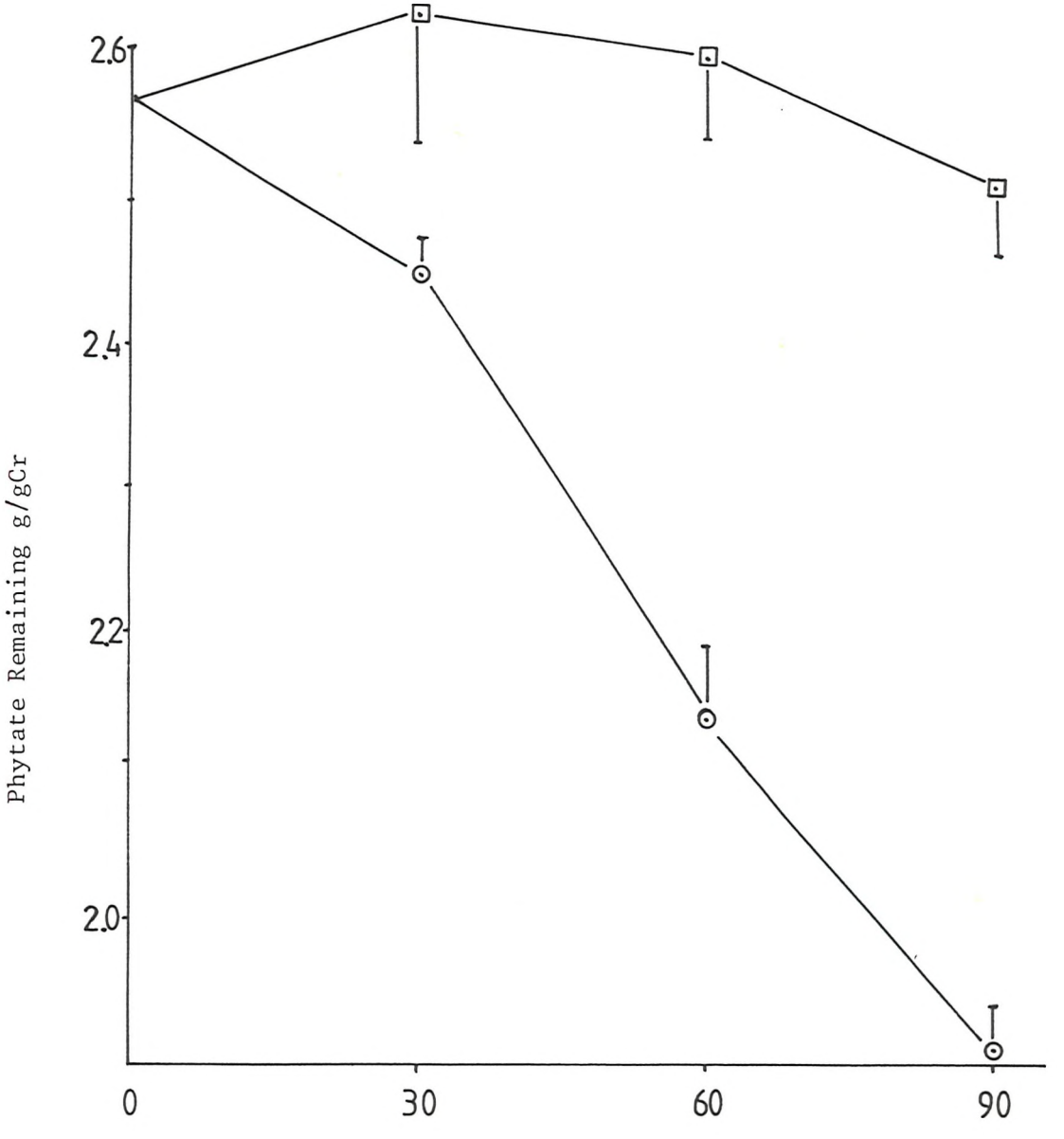
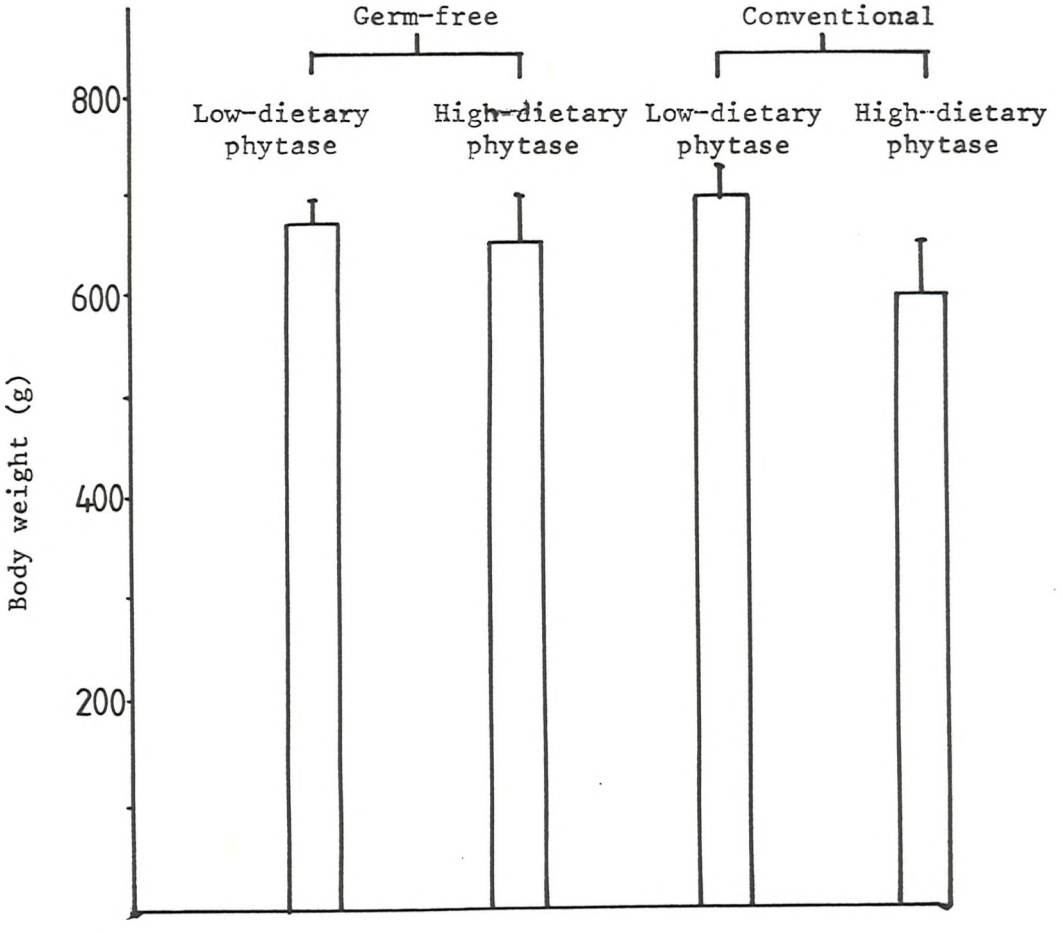


FIGURE 8.3 : THE GROWTH OF GERM-FREE AND CONVENTIONAL CHICKS FED DIETS WITH HIGH OR LOW LEVELS OF DIETARY PHYTASE ACTIVITY



Non-significant difference between the treatments

(Mean $\pm$ S.E.M.)

8.3.3 Digestibility of phytate phosphorus and retention of total phosphorus and calcium

The data for the digestibility of phytate phosphorus and for retention of total phosphorus and calcium are given in Figure 8.4. Microbial phytase activity did not significantly alter phytate digestibility while dietary phytase increased phytate digestibility ($P<0.001$) in both conventional and germ-free chicks. The relative importance of plant and microbial phytase is shown in Table 8.3. The net change in digestibility due to plant phytase was +12.45% while the net change in digestibility due to microbial phytase was -1.55%.

There were no significant treatments effects on the retention of either total phosphorus or calcium (see Figure 8.4).

8.4 DISCUSSION

The purpose of the present study was to compare the relative roles of dietary phytase, microbial phytase and intestinal phosphatases in the digestion of dietary phytate. When both plant and microbial phytases are excluded from exerting an effect on phytate digestibility, the dominant effect of intestinal phytase can be seen, accounting for approximately 80% of phytate digestibility. When chicks were given diets containing wheat bran with active phytase, the level of phytate digestibility increased significantly to 92.94%. This confirms the observation of Mollgaard (1946) that wheat bran contains a powerful phytase. This phytase will exert its effect mainly in the crop and gizzard and because of the large particle size of wheat bran, its relatively longer retention in these organs will also maximal effect of wheat bran phytase to be expressed. The 12 to 14% increase in digestibility of phytate seen with phytase-containing, as opposed to phytase-low diets, is due solely to the effect of dietary phytase. Any contribution made by mechanical grading or HCl leaching, as discussed in Chapter Seven, will appear in the figure for the phytase-low diet.

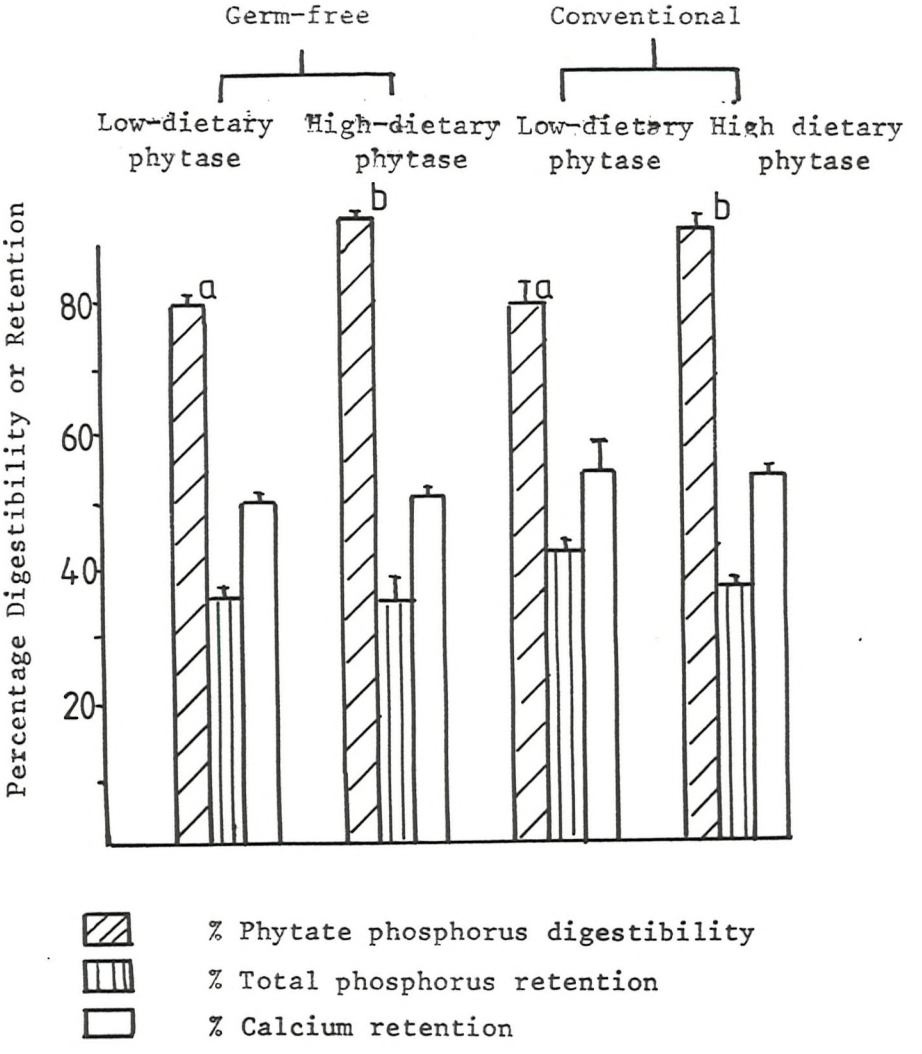
TABLE 8.2: THE EFFECT OF DIETARY PHYTASE ACTIVITY ON PLASMA MINERAL COMPOSITION OF GERM-FREE AND CONVENTIONAL BROILER CHICKS AT 28 DAYS OF AGE

Chicks status		germ-free		conventional	
Dietary phytase		low	high	low	high
Calcium	(mmol/l)	2.39 ± 0.05	2.33 ± 0.13	2.46 ± 0.06	2.31 ± 0.1
Total phosphorus	(mmol/l)	1.92 ± 0.04	1.78 ± 0.07	1.90 ± 0.04	2.00 ± 0.05
Magnesium	(mmol/l)	0.93 ± 0.04	0.95 ± 0.04	0.88 ± 0.07	0.91 ± 0.07
Zinc	(µmol/l)	32.89 ± 1.54	28.81 ± 2.84	30.40 ± 3.98	28.49 ± 2.03

No significant treatment effects

Values are expressed as the mean ± S.E.M.

FIGURE 8.4 : THE EFFECTS OF HIGH OR LOW LEVELS OF DIETARY PHYTASE ACTIVITY ON DIGESTIBILITY OF PHYTATE PHOSPHORUS AND RETENTION OF TOTAL PHOSPHORUS AND CALCIUM OF GERM-FREE AND CONVENTIONAL BROILER CHICKS



Groups without common subscripts are significantly different from each other ($P<0.001$).

(Mean $\pm$ S.E.M.)

There was no evidence forthcoming from the present study to support the hypothesis that microbial phytases contribute to phytate digestibility in the chick. This contracts with work on mice and rats where major differences in mineral absorption was recorded between germ-free and conventional animals. Yoshida et al. (1982) working with mice observed that the absence of an intestinal micro-flora, significantly increased the absorption of both calcium and total phosphorus. This increased absorption was paralleled by increased rentions of both calcium and phosphorus. Similar results were obtained with germ-free and conventional rats by Yoshida et al. (1985) and Reddy (1971). All of these studies, however, while demonstrating an effect of intestinal micro-flora on mineral absorption, confirm to an extent the findings in the present study : the presence of an intestinal flora does not contribute to phytate phosphorus absorption, the highest levels of absorption having been found in the absence of a gut micro-flora. It should, however, be stated that none of these authors attempted to differentiate between the availability of inorganic phosphorus and the digestibility of phytate phosphorus. Wise and Gilburt (1982) examined the digest-ibility of phytate in germ-free and conventional rats and observed negligible digestibility in germ-free rats and phytate digestibilities of 22 and 56% respectively for high- and low-calcium diets. Thus, these authors concluded that any effect of intestinal phytase was negligible. This contrasts directly with studies which have shown that both alkaline phosphatase and phytase are active in the small intestine of rats (Pileggs 1959; Ramakrishman and Bhandari 1979). Such enzymes have also been observed in the present series of experiments (Chapter Seven) for the chick, confirming the data of McCuaig and Motzok (1974). Indeed, Williams (1984) has also shown these enzymes to be active in the hamster.

The findings of Wise and Gilburt (1982) are therefore difficult to explain. However, the experimental method adapted by these authors differs from that normally used for comparing germ-free and conventional animals. The normal approach is to take a group of conventional animals from which germ-free animals are reared and directly compare with a group of germ-free animals. Wise and Gilburt (1982) used germ-free animals for the first part of their

TABLE 8.3: THE RELATIVE EFFECTS OF DIETARY, MICROBIAL AND INTESTINAL PHYTASES ON THE DIGESTIBILITY OF PHYTATE PHOSPHORUS BY GERM-FREE OR CONVENTIONAL CHICKS FED DIETS WITH HIGH OR LOW DIETARY PHYTASE ACTIVITY

	Dietary Phytase			
	low	high	mean	difference
Conventional	91.1 ± 1.86	79.6 ± 2.61	85.35	1.55
Germ-free	93.6 ± 0.09	80.20 ± 0.9	86.9	
Mean	92.35	79.9		
Difference	12.45*			

Values are pressed as the mean ± S.E.M.

* Significantly different ($P<0.001$)

study and then "conventionalised" them for a period of 7 days. Whether the sudden introduction of an intestinal micro-flora, with all its associated intestinal immunological changes, fairly represents a conventional animal is open to question.

The increase in calcium and phosphorus absorption which has been observed by Yoshida et al. (1982) Yoshida et al. (1985) and Reddy (1971) in germ-free rats and mice may be explained on several grounds. The intestines of germ-free animals are thinner than those of conventional animals (Abrams et al. 1963) so that calcium and phosphorus absorption may be more rapid across the thinner gut. Furthermore, in germ-free animals the activities of many digestive and absorptive enzymes are raised e.g. Ca^{++} - ATPase, alkaline phosphatase, calcium-binding protein (Reddy (1971). Finally the lower mitotic activity of intestinal cells in germ-free animals may result in a higher proportion of more mature intestinal cells (Palmer and Rolls 1983).

In conclusion, the results of the present study show that the main contributory factor to phytate digestibility in the chick are the intestinal enzymes with some contribution by the non-enzymatic effects of the crop and gizzard. Dietary phytase can make a substantial contribution to phytase digestibility while the intestinal micro-flora makes no contribution.

CHAPTER NINE

DETERMINATION OF $1,25(OH)_2D_3$ IN PLASMA FROM 28 DAYS BROILER CHICKS

9.1 Introduction

The $1,25(OH)_2D_3$ metabolite is considered to be the most active form of the vitamin D_3 derivatives in stimulating calcium and phosphorus absorption (Boy et al. 1972), in stimulating calcium and phosphorus mobilization from the bone (Raisz et al. 1972), in inducing the synthesis of a specific CaBP (Spencer et al. 1978) and in enhancing the activity of several enzymes located in the brush border membrane of intestinal cells, such as calcium-ATPase and alkaline phosphatase (Lane and Lawson 1978). It is also reported that vitamin D status affects the level of intestinal phytase (Steenbock and Herting, 1955; Davies et al. 1970), although the significance of this enzyme is unclear. It has been proposed that phytase is an iso-enzyme of alkaline phosphatase (Davies and Flett, 1978) and that it functions by hydrolysing phosphate residues on the non-absorbable phytate in the diet, thus reducing the chelation of divalent metals (Ca^{++}, Zn^{++}, Ca^{++}, Mn^{++}) and making these metals available for absorption. The circulating concentration of $1,25(OH)_2D_3$ is significantly higher in laying hens than in immature or in non-laying adult hens. The concentration in immature pullets is 30-50 pg/ml and in laying birds is 120-300 pg/ml (Spanos et al. 1976, Abe et al. 1979). However, Abe et al. (1979) have reported that the plasma concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$ in laying hens with an egg in the shell gland are 14.0 ± 2 ng/ml and 0.63 ± 0.18 ng/ml respectively.

The objectives of the present study were to compare the effects of dietary calcium, total phosphorus and vitamin D_3 levels on the plasma level of $1,25(OH)_2D_3$ in broiler chicks at 28 days of age.

9.2 Normal rabbit intestinal cytosol as a source of binding protein for the $1,25(OH)_2D_3$ assay

A competitive binding assay to measure $1,25(OH)_2D_3$ in human and rat plasma using rachitic chick intestinal cytosol has been described by Brumbaugh et al. (1974), Eisman et al. (1976) and Mallon et al. (1980). These chicks are usually raised for 4 to 12 weeks on a rachitogenic diet prior to preparation of the binding protein. Rachitogenic chicks are expensive to maintain, and in some experiments may die before the vitamin D deficient intestinal mucosa can be obtained. Duncan et al. (1983) examined intestinal cytosol from non vitamin D deficient rabbits as a source of binding protein for the competitive binding assay for $1,25(OH)_2D_3$. It appears that the $1,25(OH)_2D_3$ binding protein from rabbit intestinal cytosol is very similar to that described by Eisman et al. (1976) and Mallon et al. (1980), and is suitable for use in a sensitive assay for $1,25(OH)_2D_3$.

Cytosol binding protein preparation

Two New Zealand white rabbits raised on a standard rabbit diet from the animal house were killed by an intravenous injection of sodium pentabarbital (50 mg/kg). The small intestine was immediately removed to ice cold phosphate buffered saline pH 7.4 (Table 9.1a), divided into 5 segments and flushed with cold buffer. All subsequent steps were performed at 5°C. The mucosa was scraped from the intestinal wall and washed twice with cold buffer. The washed mucosa was homogenized with a motor-driven Teflon pestle at 300 rpm in two volumes of Tris buffer pH 7.4 (Table 9.1b). The homogenate was centrifuged at 25,000 g for 10 minutes and the crude supernatant was recentrifuged at 100,000 g for 60 minutes. The clear cytosol supernatant, under the thin lipid upper layer, was collected with a Pasteur pipette. An aliquot of the pooled supernatants was used to determine the protein concentration using the method of Lowry et al. (1951). Aliquots (1ml) of the cytosol were frozen in small tubes under argon at -70°C.

TABLE 9.1 : CYTOSOL BINDING PROTEIN PREPARATION BUFFERS

A - phosphate buffer

Compounds	Weight g/l
NaH_2PO_4	1.2
Nacl	8.76

The mixture was equilibrated to pH 7.4 by NaOH.

B - Tris buffer

Compounds	Weight g/l
Tris	3.14g
Dithiothreitol	0.771
EDTA	0.336
Kcl	22.368
Sodium molybdate	2.059

The mixture was equilibrated to pH 7.4 by Hcl.

9.3 Steroids

Crystalline vitamin D_3 $25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$ and $25,26(OH)_2D_3$ were gifts from Hoffman-La Roche, Nutley, NJ, U.S.A. Tritiated ^{3}[H]25-OH and ^{3}[H] $1,25(OH)_2D_3$ were purchased from Amersham Radio Chemicals. ^{3}[H] $25,26(OH)_2D_3$ was generously provided by Professor A.D. Care from Department of Animal Physiology and Nutrition, University of Leeds.

9.4 ^{3}[H] $24,25(OH)_2D_3$ production

The following method, which is based on the assay described by Kenny (1976), was used to produce ^{3}[H] $24/25(OH)_2D_3$ from ^{3}[H] $25(OH)D_3$ in a sample of a quail kidney.

Assay procedure

Three male quails were decapitated and samples of kidney removed into ice cold assay buffer (see Table 9.2). The kidney was weighed and a 10% (weight/volume) homogenate prepared. 200 µl of this homogenate was added, with 1.8 ml of incubation buffer, to a 10 ml foil-covered conical flask containing 65 pmole of ^{3}[H] $25(OH)D_3$ and incubated at 37°C in a shaking water bath for 20 minutes. The incubation was stopped after 20 minutes by the addition of 8 ml of methanol:chloroform (2:1 V/V). The mixture was shaken and left in the dark at room temperature for 20 minutes and then centrifuged at 2000 g for 10 minutes to bring down the tissue debris. The supernatant was transferred to a 50 ml separating funnel and extracted with methylene chloride (4x4ml).

The volume of the extract was then reduced by rotary evaporation to about 30 µl. The extract was spotted onto an activated thin layer chromatography (TLC) plate to separate the ^{3}[H]$24,25(OH)_2D_3$ from the other metabolites. The plates were made

TABLE 9.2 : ^{3}[H] 24,25(OH)$_2$D$_3$ PRODUCTION ASSAY BUFFER

Compounds	Weight g/l	
KH_2PO_4	27.218	
$Mgcl_2.6H_{20}$	0.813	
L-Malic Acid	1.005	
D-Glucose-6-p	0.987	
NADp	0.270	added at time of assay
G-6-p H	600 U/l	

The mixture was equilibrated to pH. 7.4 by KOH.

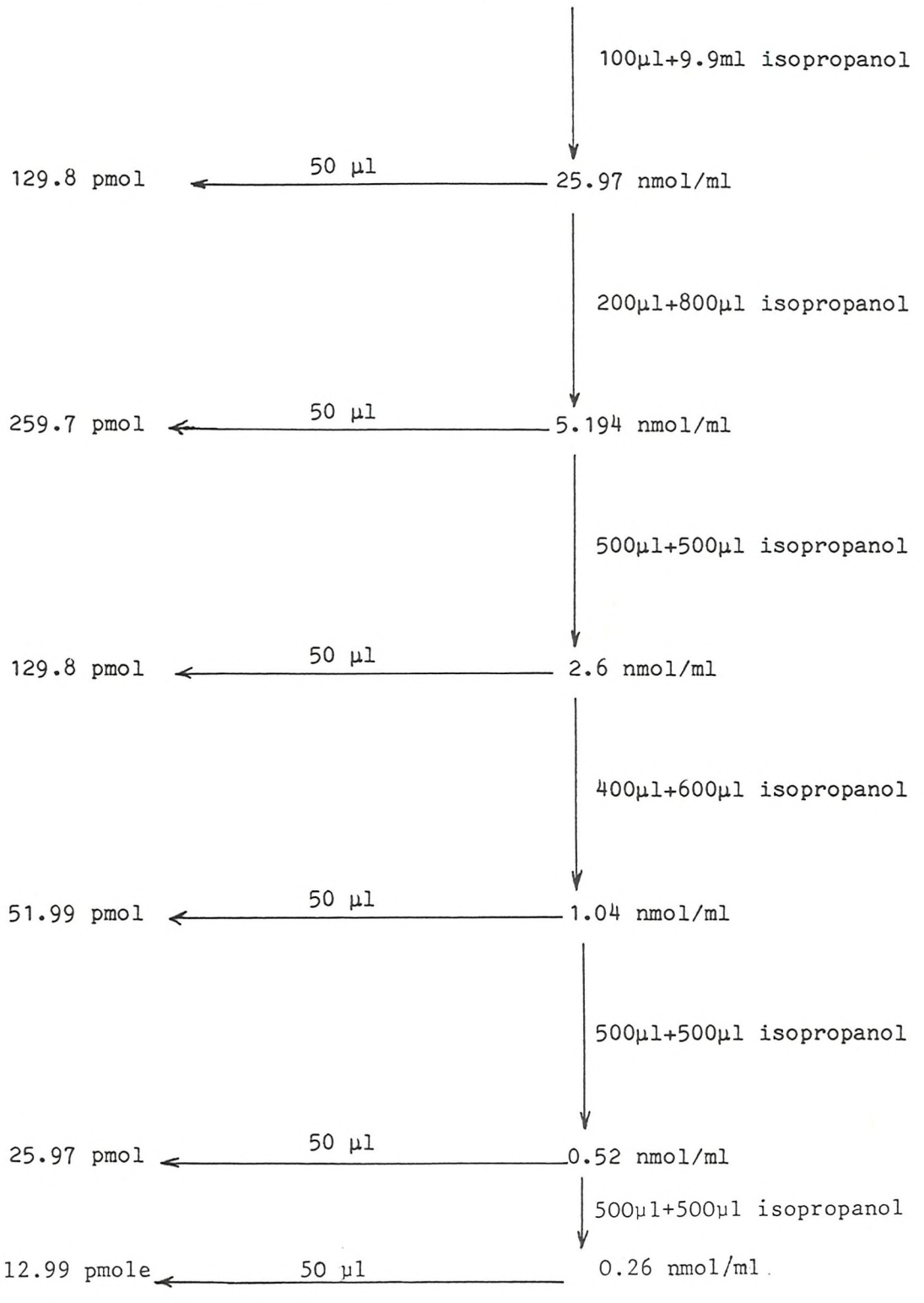
with Kineselgel GF254 silica (type 60) which was made into a slurry (50 g silica + 100 ml distilled water) and then spread on the plates to a thickness of 250 μm. TLC plate was activated by heating to 100°C for at least 3 hours before use. The sample was applied to the plate in a fine line and the plate was placed in a tank containing ethyl acetate (60 ml), n-heptane (60 ml) and ethanol (5 ml). The metabolites were chromatographically separated in the dark at 4°C. Non radioactive standard of $24,25(OH)_2D_3$ was also applied to the plate. This could be visualised under short wavelength U.V. light and was used to identify the position of the corresponding ^{3}[H]$24,25(OH)_2D_3$, which was then scraped the plate into a clean vial. 3 ml of ethanol was added to the vial to extract the ^{3}[H]$24,25(OH)_2D_3$ from the silica. The sample was then centrifuged at 2000 g for 5 minutes and the supernatant was decanted from the silica. The volume of the supernatant was then reduced to about 300μl under a stream of nitrogen and kept in a small tube at -20°C until needed.

9.5 Competitive protein binding assays

9.5.1 Competitive displacement of ^{3}[H[$1,25(OH)_2D_3$ from rabbit intestinal cytosol binding protein by unlabelled vitamin D_3

Incubations were carried out in microcentrifuge tubes. To each assay tube was added 50 μl of isopropanol containing various concentration of standard unlabelled vitamin D_3 (Figure 9.1), and 50 μl of isopropanol containing approximately 12000 - 14000 Dpm ^{3}[H] $1,25(OH)_2D_3$. The incubation was initiated by the addition of 1 ml of cold buffer containing 0.2 mg of cytosol binding protein. Prior to the assay the 1 ml/frozen protein was diluted with 14 ml cold buffer to produce 0.2 mg/ml cytosol binding protein. The tubes were vortexed for several seconds and then incubated at 4°C for 3 hours. The free and bound steroids were separated by adding 0.25 ml of a cold dextran-coated charcoal suspension (1.25 mg charcoal and 0.125 mg of Dextran T_{70} in 100 ml buffer) to the cytosol sterol solution. After incubation for 10 minutes at 4°C and centrifugation at 1000 g for 10 minutes at 4°C, 1 ml of the supernatant was transferred to a scintillation vial and counted

FIGURE 9.1 : SCHEME FOR THE DILUTION OF UNLABELLED VITAMIN D_3 FOR USE IN COMPETITIVE BINDING STUDIES WITH THE $1,25(OH)_2D_3$ BINDING PROTEIN FROM RABBIT MUCOSAL CYTOSOL



with 7 ml toluene butyl PBD in a Philips PW4700 series scintillation counter. Figure 9.4A shows the competitive displacement of ^{3}[H]1,25(OH)$_2$D$_3$ from rabbit intestinal cytosol binding protein by unlabelled vitamin D$_3$.

9.5.2 Competitive displacement of ^{3}[H]1,25(OH)$_2$D$_3$ from rabbit cytosol binding protein by unlabelled 25(OH)D$_3$

The assay procedure was similar to that described above, although the concentration of unlabelled 25(OH)D$_3$ standard was different (Figure 9.2). The results of the competitive displacement is shown in Figure 9.4B.

9.5.3 Competitive displacement of ^{3}[H]1,25(OH)$_2$D$_3$ from rabbit intestinal cytosol binding protein by unlabelled 24,25(OH)$_2$D$_3$ and 25,26(OH)$_2$D$_3$

The various concentrations of the standard of the unlabelled 24,25(OH)$_2$D$_3$ and 25,26(OH)$_2$D$_3$, diluted from 1.06 mg/ml (2550 nmol /ml) and 199.68 µg/ml (480 nmol /ml) stock respectively, was similar to those given in Figure 9.2. The competitive displacement is shown in Figure 9.4C for 24,25(OH)$_2$D$_3$ and Figure 9.4D for 25,26(OH)$_2$D$_3$.

9.5.4 Competitive displacement of ^{3}[H]1,25(OH)$_2$D$_3$ from rabbit intestinal cytosol binding protein by unlabelled 1,25(OH)D$_3$

The assay is based on competition between unlabelled 1,25(OH)$_2$D$_3$ and tritiated ^{3}[H]1,25(OH)$_2$D$_3$ for binding to a rabbit intestinal cytosol protein which has a low specificity for unlabelled vitamin D$_3$, 25(OH)$_2$D$_3$, 24,25(OH)$_2$D$_3$ and 25,26(OH)$_2$D$_3$ (see Figures 9.4,A,B,C and D).

The various concentrations of standard 1,25(OH)$_2$D$_3$ were produced from (58 µg/ml (139-423 pmol /ml)) stock dilutions are illustrated in Figure 9.3. The results of the competitive displacement is shown in Figure 9.4E. Figure 9.5 summarises the

FIGURE 9.2 : SCHEME FOR THE DILUTION OF UNLABELLED $25(OH)D_3$ FOR USE IN COMPETITIVE BINDING STUDIES WITH THE $1,25(OH)_2D_3$ BINDING PROTEIN FROM RABBIT MUCOSAL CYTOSOL

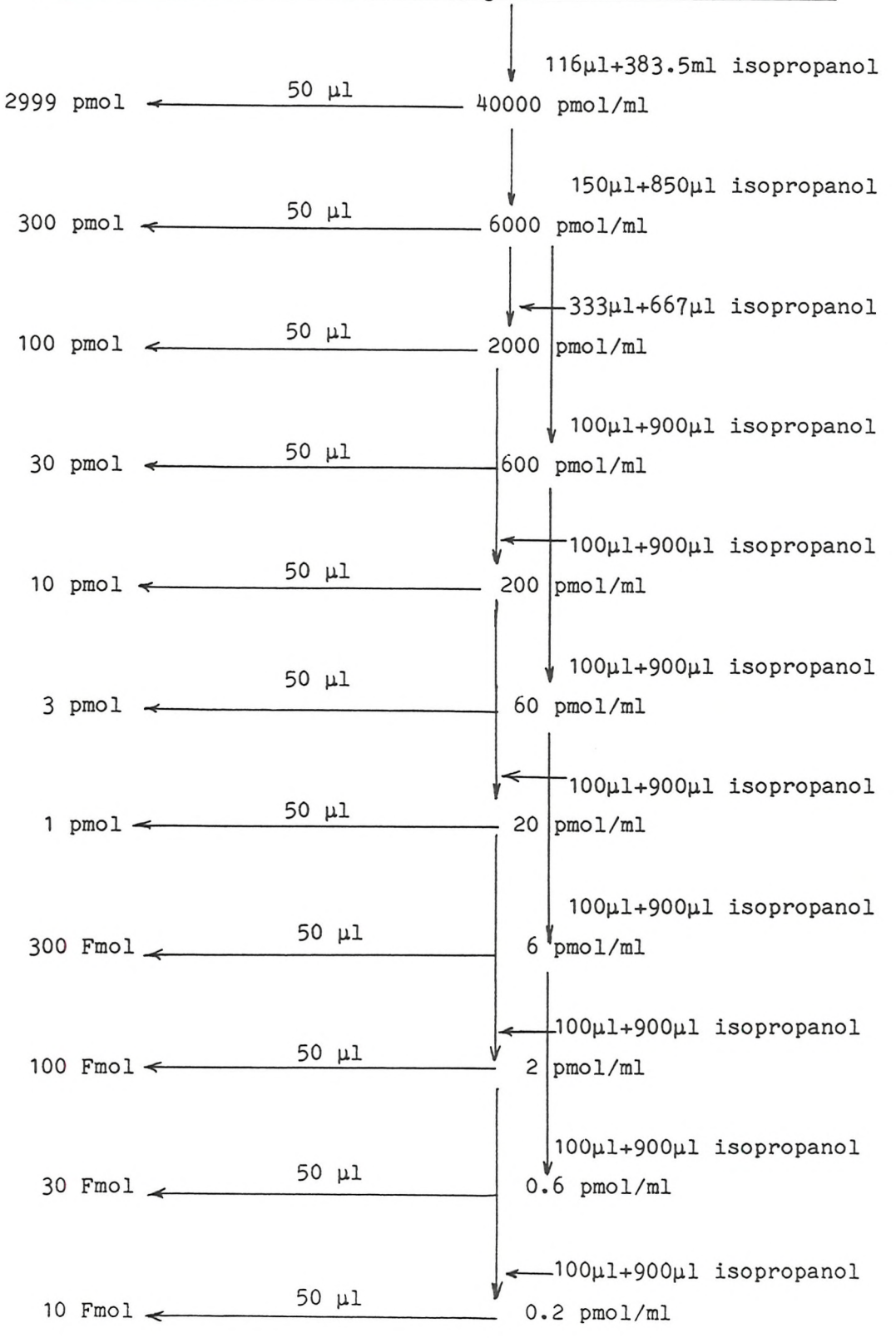


FIGURE 9.3 : SCHEME FOR THE DILUTION OF UNLABELLED $1,25(OH)_2D_3$ FOR USE IN COMPETITIVE BINDING STUDIES WITH THE $1,25(OH)_2D_3$ BINDING PROTEIN FROM RABBIT MUCOSAL CYTOSOL

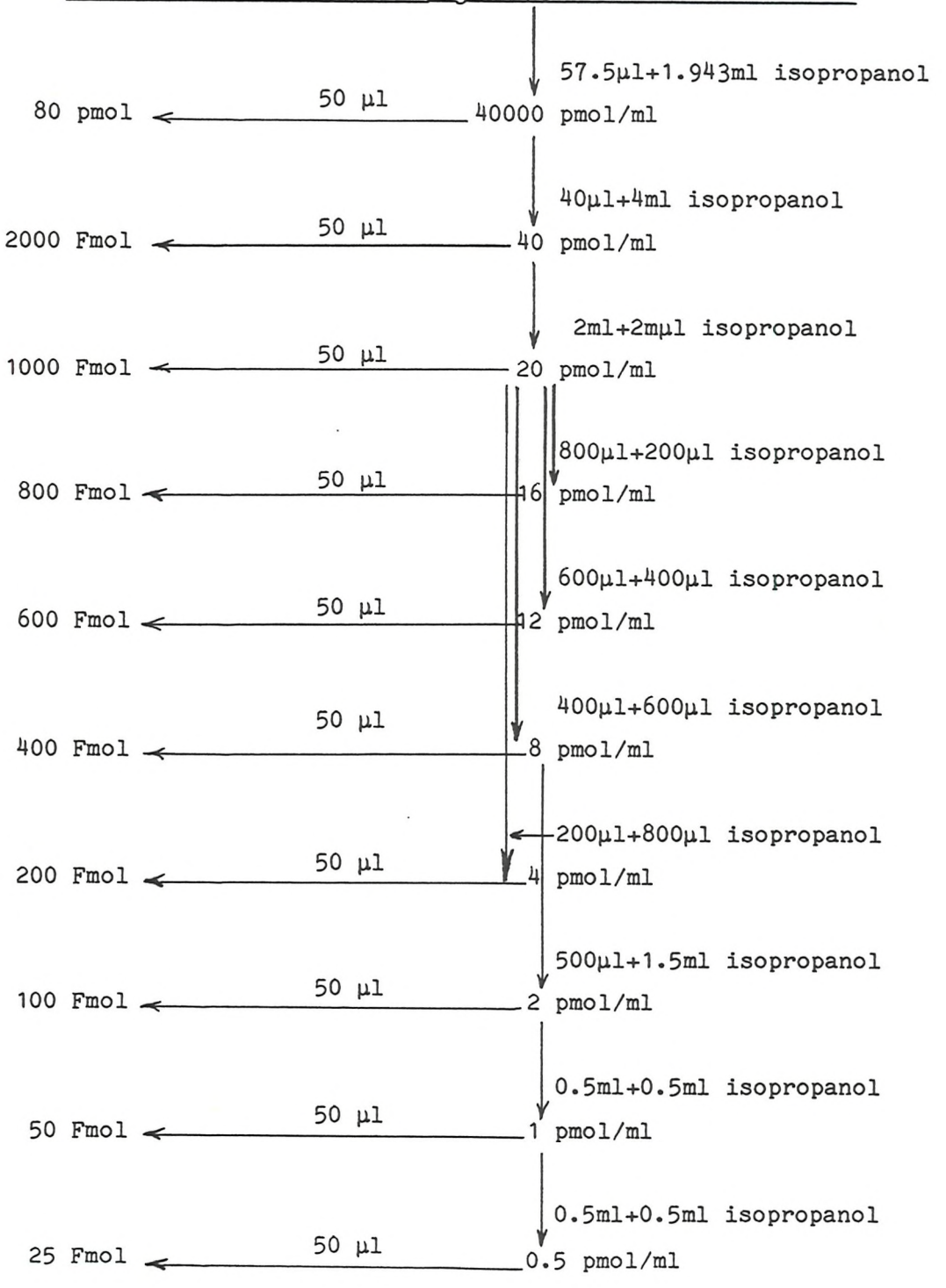


FIGURE 9.4 : COMPETITION BY VITAMIN D_3 AND ITS METABOLITES WITH ^{3}[H]-1,25(OH)$_2D_3$ FOR THE 1,25(OH)$_2D_3$ BINDING PROTEIN OF RABBIT INTESTINAL CYTOSOL

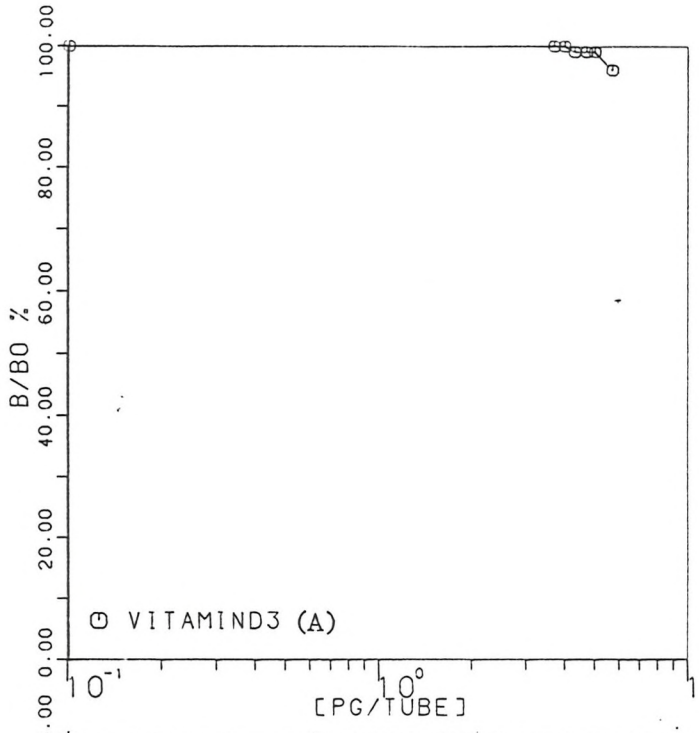
A - Vitamin D_3 (Cholecalciferol)

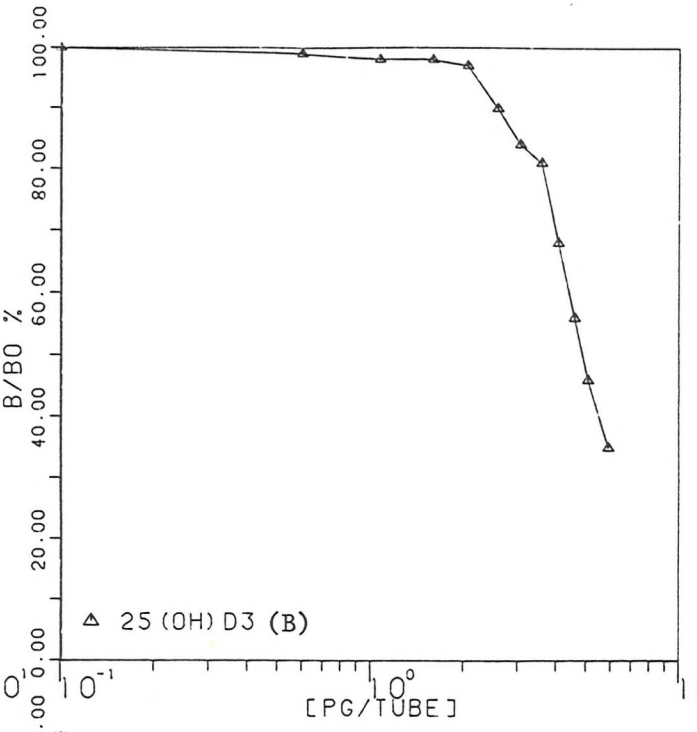
B - 25(OH)D_3 (25-Hydroxycholecalciferol)

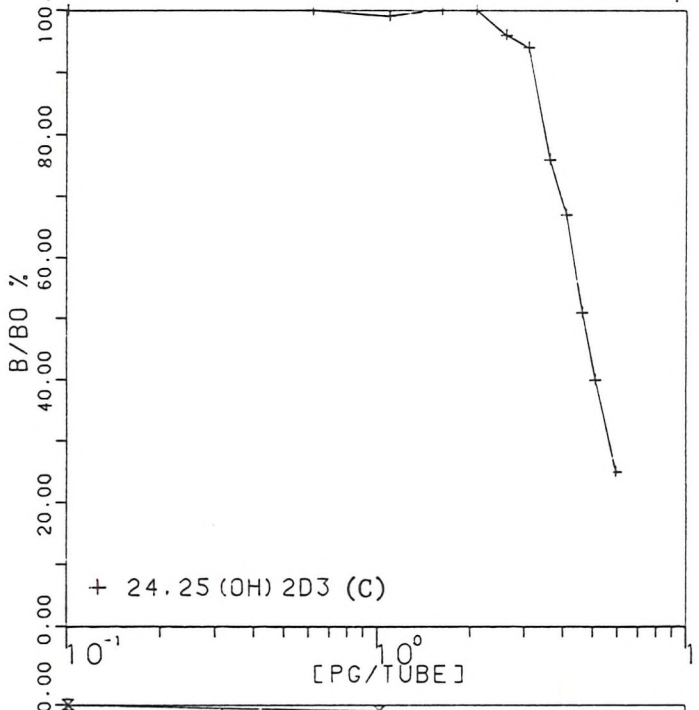
C - 24,25(OH)$_2D_3$ (24,25-Dihydroxycholecalciferol)

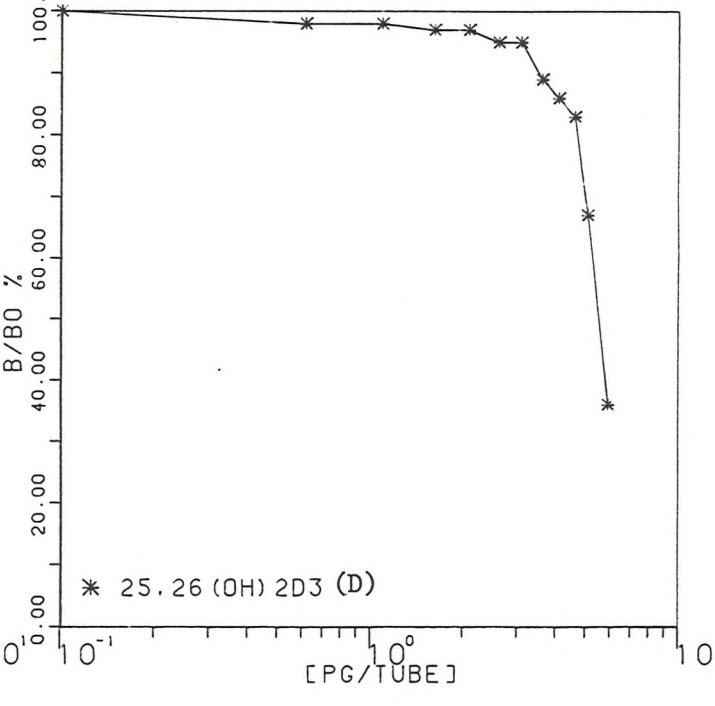
D - 25,26(OH)$_2D_3$ (25,26-Dihydroxycholecalciferol)

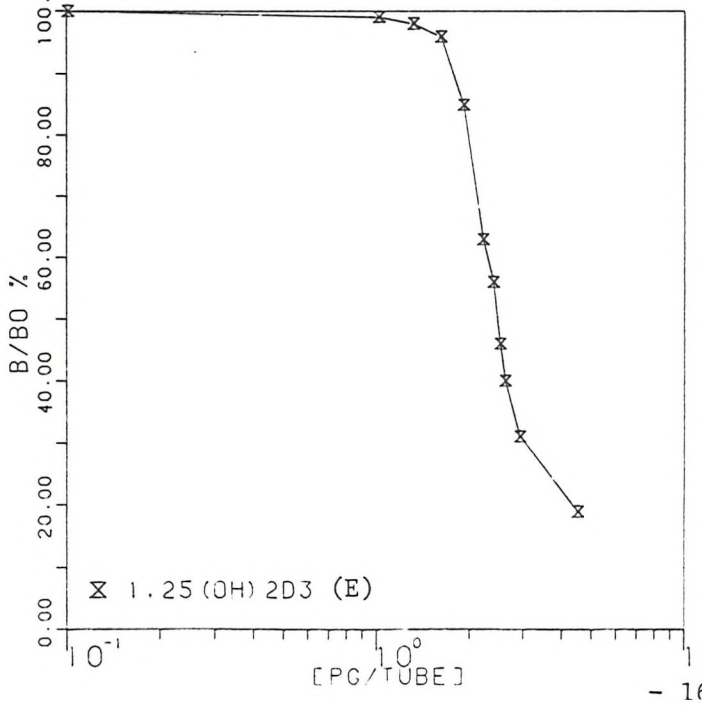
E - 1,25(OH)$_2D_3$ (1,25-Dihydroxycholecalciferol)


B/BO %
100.00
80.00
60.00
40.00
20.00
0.00
VITAMIND3 (A)
10^{-1}
10^{0}
[PG/TUBE]


B/BO %
100.00
80.00
60.00
40.00
20.00
0.00
25(OH)D3 (B)
10^{-1}
10^{0}
[PG/TUBE]


B/BO %
100.00
80.00
60.00
40.00
20.00
0.00
24,25(OH)2D3 (C)
10^{-1}
10^{0}
[PG/TUBE]


B/BO %
100.00
80.00
60.00
40.00
20.00
0.00
25,26(OH)2D3 (D)
10^{-1}
10^{0}
[PG/TUBE]


B/BO %
100.00
80.00
60.00
40.00
20.00
0.00
1,25(OH)2D3 (E)
10^{-1}
10^{0}
[PG/TUBE]

competitive binding studies with all the metabolites of vitamin D_3. The specificity of this binding protein for $1,25(OH)D_3$ is evident from Figure 9.5.

The specificity of the intestinal cytosolic protein for $1,25(OH)_2D_3$ was further examined in the presence of its closest chromatographic metabolites $25,26(OH)_2D_3$ and $24,25(OH)_2D_3$. The incubations were carried out in microcentrifuge tubes. To each assay tube was added 50 µl of isopropanol containing different concentrations of either unlabelled $25,26(OH)_2D_3$ or unlabelled $24,25(OH)_2D_3$ (0, 500, 1000 and 5000 Fmol), and 50 µl of isopropanol containing 500 Fmol of unlabelled $1,25(OH)_2D_3$ was added to each assay tube. 50 µl of isopropanol containing 12000 - 14000 DpM $^3[H]1,25(OH)_2D_3$ was used in each assay tube. The incubations were initiated by addition of 1 ml cold buffer containing 0.2 mg cytosol binding protein. The results are shown in Table 9.3 for $25,26(OH)_2D_3$ and Table 9.4 for $24,25(OH)_2D_3$. There was no significant decline in the binding of $1,25(OH)_2D_3$ due to either of these metabolites.

9.6 Extraction of vitamin D metabolites from plasma

2 ml of saline (0.9% w/v) was added to 2 ml plasma to which had been added 50 µl of isopropanol containing $^3[H]1,25(OH)_2D_3$ (approximately 12000 dpm) or its relevant metabolite. 8 ml of isopropanol-toluene (2 ml isopropanol + 6 ml toluene) were added to the mixture. Samples were extracted by shaking for one minute, followed by centrifugation at 1000 g for 15 minutes. The two solvent layers were separated by freezing the aqueous layer for 45 seconds in liquid nitrogen. The supernatants were decanted into scintillation vials, evacuated under steam of nitrogen and the radioactivity counted in a Philips liquid scintillation counter, using toleuene butyl pBD as the scintillant. The recovery of $^3[H]OHD_3$, $^3[H]24,25(OH)_2D_3$, $^3[H]25,26(OH)_2D_3$ and $^3[H]1,25(OH)_2D_3$ from plasma were respectively, 89.03%, 84.05%, 75.98% and 94.8% ± 1.21%. Each value represents the mean of duplicates except for $^3[H]1,25(OH)_2D_3$, the mean of six replicates (n=6).

FIGURE 9.5: COMPETITION BY VITAMIN D_3 AND ITS METABOLITES WITH ^{3}[H]-1,25(OH)$_2D_3$ FOR THE 1,25(OH)$_2D_3$ BINDING PROTEIN OF RABBIT INTESTINAL CYTOSOL

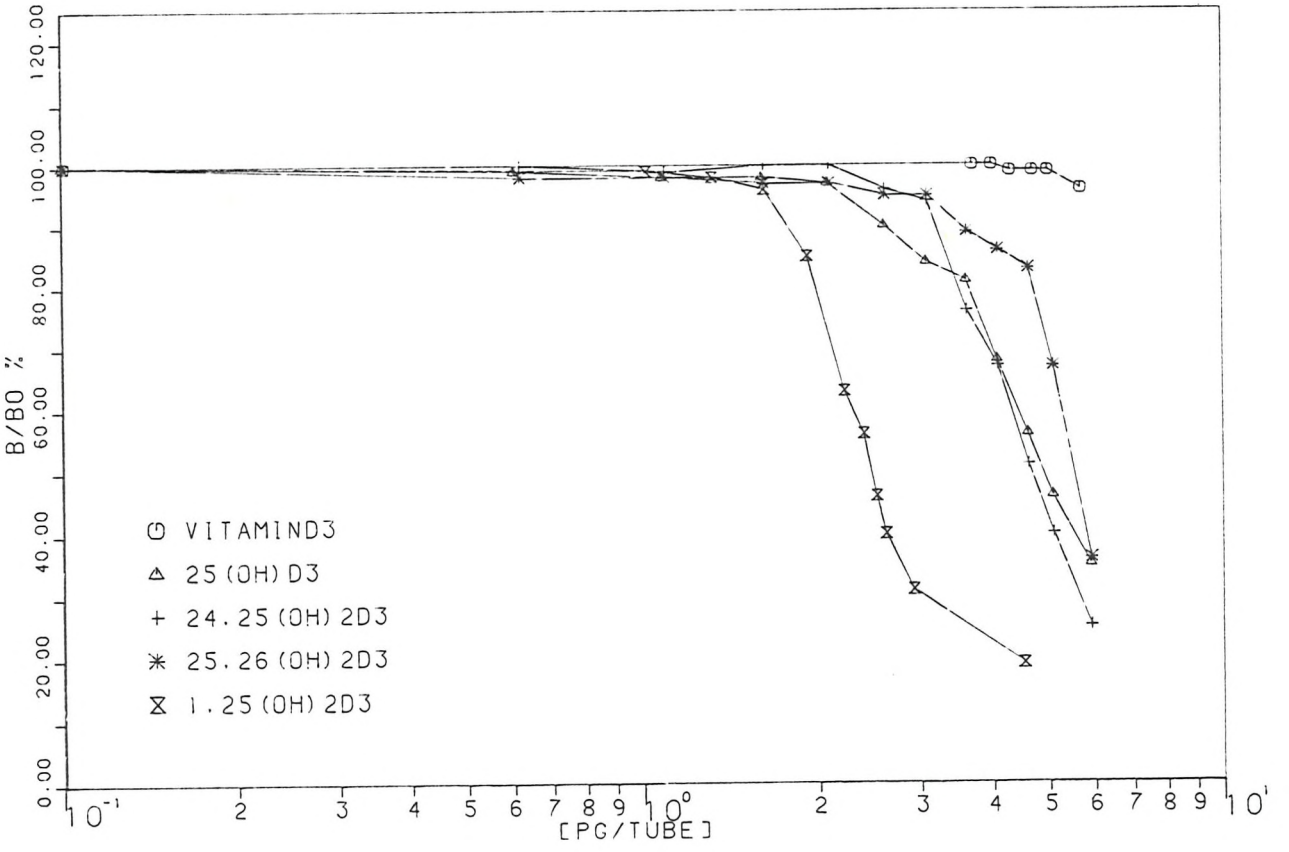


TABLE 9.3 : THE EFFECT OF INCREASING CONCENTRATIONS OF $25,26(OH)_2D_3$ ON THE COMPETITIVE BINDING OF LABELLED AND UNLABELLED $1,25(OH)_2D_3$ WITH RABBIT CYTOSOL BINDING PROTEIN

Concentration	500 F1,25-$(OH)_2D_3$	500 F1,25-$(OH)_2D_3$ + 500F25,26$(OH)_2D_3$	500 F1,25-$(OH)_2D_3$ + 1000F25,26$(OH)_2D_3$	500 F 1,25-$(OH)_2D_3$ + 5000F25,26$(OH)_2D_3$
dpm	1445 ± 12	1438 ± 6	1448 ± 33	1288 ± 19

Values are Expressed as the mean ± S.E.M.

TABLE 9.4 : THE EFFECT OF INCREASING CONCENTRATIONS OF 24,25$(OH)_2D_3$ ON THE COMPETITIVE BINDING OF LABELLED AND UNLABELLED 1,25$(OH)_2D_3$ WITH RABBIT CYTOSOL BINDING PROTEIN

Concentration	500 F1,25-$(OH)_2D_3$	500 F1,25-$(OH)_2D_3$ + 500F24,25$(OH)_2D_3$	500 F1,25-$(OH)_2D_3$ + 1000F24,25$(OH)_2D_3$	500 F1,25-$(OH)_2D_3$ + 5000F24,25$(OH)_2D_3$
dpm	1445 ± 12	1407 ± 44	1431 ± 39	1277 ± 134

Values are expressed as the mean ± S.E.M.

9.7 Chromatography

There are a variety of methods available to separate the metabolites of vitamin D. These methods including thin layer chromography (TLC), high pressure liquid chromatography (HPLC), and sephadex LH20. Most current assays require a preliminary extraction, followed by a two step chromatographic separation on sephadex LH20 and high pressure liquid chromatography, (Eisman et al. 1976; Cleman et al. 1979 and Lage et al. 1980).

Adams et al. (1981), and Redhwi et al. (1982) have developed a rapid chromatographic technique using disposable silica cartridges to achieve separation of vitamin D_3, $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ from extract plasma. The major advantages of silica sep-pak cartridges are, in the efficiency of sample recovery, and in the time saved in column packing and standardization.

Assays for measuring $1,25(OH)_2D_3$ still require its extensive purification before assay. However, the assay used in the present study does not require such extensive prelininary purification because the rabbit binding protein is highly specific for $1,25(OH)_2D_3$.

The high specificity of the binding protein means that complete removal of other vitamin D metabolites such as $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ is not essential. Removal of vitamin D_3 and $25(OH)D_3$ are still required because, they circulate at concentrations much higher than $1,25(OH)_2D_3$ itself, and these can be easily carried out by using sep-pak silica cartridge or the Sephadex LH20 column.

Procedure

The method used for separating the metabolites of vitamin D_3 was based on that of Redhwi et al. (1982) with slight modifications. The method has been developed using Sep-Pak disposable silica cartridges to achieve separation of vitamin D_3, $25(OH)D_3$, $24,25(OH)_2D_3$, $25,26(OH)_2D_3$ and $1,25(OH)_2D_3$.

The Sep-Pak cartridges were equilibrated after 2 h with 5 ml of hexane prior to their use. A suitable mobile phase was established for each vitamin D_3 metabolite by using solvent mixtures of increasing polarity. These were:

1 - 10 ml isopropanol 0.1% v/v in hexane, to remove lipids
2 - 20 ml isopropanol 1% v/v in hexane, to remove vitamin D_3
3 - 20 ml isopropanol 3% v/v in hexane, to remove $25(OH)D_3$
4 - 5 ml Ethanol 2.5% v/v in dichloromethane, to remove $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$
5 - 10 ml Ethanol 50% v/v in toluene followed by 5 ml Ethanol, to remove $1,25(OH)_2D_3$

The recovery in each of the above fractions was then checked with extracts of plasma containing known amounts of labelled standards. Appoximately 12000 dpm of $^3[H]1,25(OH)_2D_3$ and different amounts of $^3[H]^{25}OHD_3$, $^3[H]24,25(OH)_2D_3$ and $^3[H]25,26(OH)_2D_3$ were added separately into 2ml aliquots of pooled chick plasma which were then extracted as previously described. Each extract was dried under a steam of nitogen, resolved in 0.1% isopropanol in hexane (2 ml) and applied to the top of a Sep-Pak cartridge, which were held in a purpose built rack. Solvents were dispensed into the barrel of a glass syringe attached to each Sep-Pak. The percent recovery of each metabolite in fractions 1 to 5 is shown in Table 9.5. It can be seen that the recovery of $1,25(OH)_2D_3$ in fraction 5 was 79.72±1.24%. This fraction was contaminated by 27.45% of $25,26(OH)_2D_3$. However, given the very negligible quantities of this metabolite in plasma and given its very low affinity for the binding protein, it is clear that the specifity of the assay for $1,25(OH)_2D_3$ is not impaired by $25,26(OH)_2D_3$. The same applies to the other minor contaminants.

9.8 Determination of $1,25(OH)_2D_3$ in plasma from 28 days broiler chicks

Results

Plasma was collected from 28 day chicks fed diets containing various levels of calcium, total phosphorus and vitamin D_3 (see

TABLE 9.5 THE RECOVERY (%) OF LABELLED METABOLITES OF VITAMIN D_3 IN EACH OF 5 FRACTIONS ELUTED FROM SEP-PAK COLUMNS

fraction	25,OH)D_3	24,25(OH)$_2D_3$	25,26(OH)$_2D_3$	1,25(OH)$_2D_3$
1	trace	trace	trace	trace
2	24.75	trace	trace	trace
3	58.69	36.71	10.37	8.32 ± 0.1
4	10.63	52.25	61.45	8.48 ± 0.62
5	5.01	10.95	27.45	79.72 ± 1.24

n = 6 for 1,25(OH)$_2D_3$ and n = 2 for all other metabolites

TABLE 9.6 : THE EFFECT OF DIETARY CALCIUM AND VITAMIN D_3 IN LOW PHOSPHORUS DIETS ON THE CONCENTRATIONS OF $1,25(OH)_2D_3$ IN BROILER CHICKS PLASMA AT 28 DAYS OF AGE (PG/ML)

	Control (recommended diet)	Normal Calcium		Low Calcium	
		Normal Vit. D_3	High Vit. D_3	Normal Vit. D_3	High Vit. D_3
Concen.of $1,25(OH)_2D_3$ pg/ml	11.07 ± 2.66^a	58.99 ± 8.24^b	90.04 ± 17.07^c	48.20 ± 4.5^b	87.0 ± 9.15^c

Groups without common subscripts are significantly different from each other ($P<0.05$)

Values are expressed as the mean ± S.E.M.

Chapter Five for details to the treatments, number of chicks per treatment and diet composition). $1,25(OH)_2D_3$ levels were measured as described above. 2 ml of plasma was mixed with 2 ml of saline (0.9%), extracted with 8 ml isopropanol : toluene (2:6 v/v) and chromatographed on sep-pak cartridges. The $1,25(OH)_2D_3$ fraction was collected and measured by the competitive protein binding assay using the rabbit intestinal cytosol as the source of the specific binding protein. The concentrations of the standard curve ranged from 25 to 2000 Fmol/tube. The results are shown in Table 9.6. Plasma levels of $1,25(OH)_2D_3$ were elevated significantly ($P<0.05$) in chicks fed a diet containing low levels of phosphorus compared with chicks given the control diet irrespective of the levels of calcium or vitamin D_3. When the level of vitamin D_3 in the diets was increased 100 times, there was a significant increase ($P<0.05$) in plasma $1,25(OH)_2D_3$ with both normal and low levels of calcium.

9.9 DISCUSSION

The use of normal rabbit intestinal cytosol as a source of $1,25(OH)_2D_3$ binding protein has been described by Duncan et al. (1983). In evaluating the specificity of this protein for $1,25(OH)_2D_3$, these authors only used vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$. In the present study the evaluation of the specificity of this protein for vitamin D_3 metabolites has been extended to include $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$. The result shows that the affinity of these metabolites for the $1,25(OH)_2D_3$ binding protein is equal to or less than that of $25(OH)D_3$. These di-hydroxy metabolites, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ are difficult to separate completely from $1,25(OH)_2D_3$ by any method. However, the data in tables 9.1 and 9.2 clearly show that $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ even at molar concentrations twice that of $1,25(OH)_2D_3$, do not inhibit the binding of $1,25(OH)_2D_3$ to the rabbit binding protein. In plasma, $25,26(OH)_2D_3$ does not exist in anything but trace quantities, while $24,25(OH)_2D_3$ may exist at 3 to 4 times the concentration of $1,25(OH)_2D_3$ in the chick. However, following the sep-pak separation used in the present study, the ratio of $24,25(OH)_2D_3$ to $1,25(OH)_2D_3$ in an extract of plasma will be only 0.14:1.

Two modifications were made to the method described by Redhwi et al. (1982) for purifying $1,25(OH)_2D_3$. In the method described by these authors the extraction of vitamin D_3 metabolites from plasma uses dry-ice. In the method employed in the present study, liquid nitrogen was used in preference to dry-ice. This is a more convenient method of separating the solvent and aqueous phases and gave extraction rates comparable to that of Redhwi et al. (1982) i.e. 94.8 ± 1.21% v. 94 ± 2.7%. The chromatographic separation of vitamin D_3 metabolites, described by Redhwi et al. (1982) was also modified. In the present study, the first three extractions from the sep-pak columns were the same as used in the original method. The fourth extraction was modified to decrease the concentration of ethanol in dichloromethane from 3% v/v to 2.5% v/v. This modification allowed the majority of $25,26(OH)_2D_3$ to be co-eluted with $24,25(OH)_2D_3$ and to reduce the contamination of the subsequent $1,25(OH)_2D_3$ rich extraction with $25,26(OH)_2D_3$. The final extraction was also modified by increasing the volume of the ethanol/toluene applied to the column from 6 to 10 mls, and was further modified by subsequently applying 5 ml ethanol to the column. This increased the recovery of $1,25(OH)_2D_3$ from the sep-pak column. The data of Redhwi et al. (1982) do not permit calculation of the recovery of $1,25(OH)_2D_3$ from plasma using sep-pak columns. In the present study this was found to be 79.72 ± 1.2% which compares very favourably with other methods of purifying $1,25(OH)_2D_3$ e.g. Sephadex LH-20 plus HPLC 68 ± 1.2% Eismann et al. (1976), 69 ± 17% Caldas et al. (1978) and 75 ± 5% Sedrani (1984). Thus sep-pak separation of vitamin D_3 metabolites offers a major advantage over other methods. It is quick, convenient, reproducible, and relatively cheap.

The results of the present study show that low phosphorus diets lead to a dramatic increase in the circulating levels of $1,25(OH)_2D_3$ compared with diets containing recommended levels of phosphorus. Several studies have also observed this effect with low phosphorus diets and have also shown increased renal 1-hydroxylase, increased intestinal calcium binding protein and increased calcium absorption (Bar and Wasserman, 1973; Baxter and DeLuca 1976; Sommerville et al. 1978 and Sommerville et al. 1985). Low

calcium diets also lead to increased levels of circulating $1,25(OH)_2D_3$ and to increases in both calcium binding protein and calcium absorption (Taylor and Dacke (1983); Ribovich and DeLuca 1978). However, the effects of low phosphorus diets on these parameters are much greater than those seen with low calcium diets. This difference in the $1,25(OH)_2D_3$ response to calcium and phosphoprus levels in the diet was also widened in the present study. At the recommended intake of vitamin D_3, the low phosphorus diets led to a five fold increase in circulating $1,25(OH)_2D_3$ while there was no difference between low and normal levels of dietary calcium.

Increasing the dietary level of vitamin D_3 from 500 to 50,000 iv/kg led to a doubling of the circulating levels of $1,25(OH)_2D_3$. The combined effect of a low-phosphorus, high vitamin D_3 diet led to a 9 fold increase in $1,25(OH)_2D_3$ in plasma compared with control diets with recommended levels of phosphorus and vitamin D_3. This dramatic increase in plasma $1,25(OH)_2D_3$ on low-phosphorus, high vitamin D_3 diets is most likely responsible for the increased phytate phosphorus digestibility, calcium and phosphorus retention and intestinal alkaline phosphatase activity observed on these diets in preceding experiments. It will also directly contribute to increased bone mineralisation.

CHAPTER TEN

GENERAL DISCUSSION

The purpose of the experiments described in this thesis was to examine the dietary factors leading to an increased utilisation of dietary phytate by the chick. It is clear from the results of these experiments, that a considerable improvement in phytate utilisation by the chick can be achieved without influencing growth. The results of the first experiment (Chapter 3) show that phytate digestibility is considerably increased (55% to 70%) on low calcium diets containing adequate levels of total phosphorus. In this first experiment phytate provided only 35% of the total phosphorus (6.9 g/kg). In subsequent studies, phytate phosphorus comprised 50% of a total dietary phosphorus level of 5.0 g/kg. These reductions in the level of phosphorus and the proportion of that phosphorus as phytate, considerably altered the response of tibia growth to dietary clacium and vitamin D_3. These changes in dietary phosphorus also led to the first adaptation by chicks to dietary factors, namely a five-fold increase in $1,25(OH)_2D_3$. In the first experiment (Chapter 3), at adequate phosphorus intakes, there was a considerable improvement in bone growth as the dietary calcium was increased from 5 to 10 g/kg. In this first experiment, therefrore, calcium was the limiting factor to bone growth. Subsequently, as the level of both total phosphorus and % of total phosphorus as phytate was lowered, phosphorus became the element to limit bone growth and this limitation was made worse at the higher (normal) levels of calcium. These changes in dietary phosphorus also influenced the effects of dietary vitamin D_3. When phosphorus intakes were adequate , high vitamin D_3 levels in the diet exerted only modest effects on bone development and then, only on low calcium diets.

The use of low-phosphorus diets provided the greatest challenge to the chick in phytate phosphorus utilisation.

Two factors accentuated this challenge; a normal level of dietary calcium and a normal level of vitamin D_3. By increasing vitamin D_3 to 50,000 1.u/kg,- circulating levels of $1,25(OH)_2D_3$ increased by 90%. As has been pointed out in the introduction, this increase in circulating $1,25(OH)_2D_3$ will exert a number of effects. In both broiler and layer chicks, this increased intake of vitamin D_3 led to increased phytate digestibility. The data in Chapter 7 shows that this increased intake of vitamin D_3 led to an increase in the activity of intestinal alkaline phosphatase but did not influence intestinal phytase levels. The former is, however, quantitatively the more active enzyme of two phosphatase.

The data in Chapter 8 shows that intestinal digestibility of phytate is achieved only through intestinal phosphatase and that microbial phytases are not responsible. However, the data also shows that dietary phytases do make a small but significant contribution to phytate digestibility and the data in Chapter 7 suggests that some contribution is also made in the gizzard through the action of gastric hydrochloric acid. Nonetheless, the main hydrolytic activity resides in alkaline phosphatase, which responds to high circulating of $1,25(OH)_2D_3$. The same chapter also shows that high intakes of dietary vitamin D_3 also improve calcium absorption, presumably by its effect on mucosal calcium binding protein. These effects of vitamin D_3 on phytate digestibility and calcium absorption must be independent. Although high levels of dietary vitamin D_3 on low-phosphorus diets containing normal levels of calcium, increased phosphorus absorption and improved the hypophosphataemia, the extent of this improvement in plasma phosphorus was insufficient to achieve normal tibia growth. Further improvement in tibia growth could only be achieved when the second dietary modification was introduced, i.e. a reduction in dietary calcium from 10 to 5 g/kg.

This introduction of low calcium diets, further increased phytate utilisation. Total phosphorus absorption increased by 100% with the introduction of the low-calcium diet. In contrast to vitamin D_3 levels, this improvement of phytate utilisation on

low-calcium diets was not mediated by changes in either intestinal phosphatase activity or by circulating levels of $1,25(OH)_2D_3$. Clearly, the beneficial effects of lowering dietary calcium lies in the fact that at these lower levels, less insoluble phytate complexes are formed thus increasing the available substitute for the intestinal phosphatases. Thus low calcium diets exerted a marked improvement in the hypophosphataemia of low-phosphorus diets. However, the increase in plasma phosphorus achieved by lowering dietary calcium (at normal intakes of vitamin D_3) is much greater than the increase in plasma phosphorus achieved by raising dietary vitamin D_3 (at normal calcium intakes) i.e. 0.65 to 1.26 v 0.65 to 0.90, mmol/l. This differential in the improvement in plasma phosphorus, was not, however, reflected in an equal differential in tibia mineralisation i.e. 42.3 to 45.1 v 42.3 to 46.0 (% ash). Clearly the increased absorption of phosphorus due to higher dietary levels of vitamin D_3, is reflected in increased bone formation due to the higher levels of circulating $1,25(OH)_2D_3$. When this increased absorption is achieved by lowering dietary calcium, no real improvement in bone mineralisation occurs because lowering dietary calcium does not alter circulating levels of $1,25(OH)_2D_3$.

The most dramatic improvement in all parameters is achieved when these two dietary manipulations occur simultaneously. Thus the highest plasma levels of phosphorus are achieved with low-calcium, high-vitamin D_3 diets. Because the levels of $1,25(OH)_2D_3$ are also high on these diets, bone mineralisation is maximised. Indeed, the mineral absorption, growth and bone development on low-calcium, high-vitamin D_3 diets equal that achieved when chicks are given all nutrients at recommended intakes. Thus the marked hypophosphataemia that arises when only phosphorus is lowered is fully corrected by modifying, vitamin D_3 and calcium. Utilization of plant phytate phosphorus is maximised.

REFERENCES

Abe, E., Tanabe, R. and Yoshiki, S. (1979): Circadian Rhythm of 1,25-dihydroxy vitamin D_3 production in egg-laying hens. Biochem. Biophys. Res. Commun., 88: 500-507.

Abernathy, R.H., Paulsen, O.M. and Ellis, R. Jr. (1973): Relationship among phytic acid, phosphorus, and zinc during maturation of winter wheat. J. Agric. Food Chem. 21: 282-284.

Abrams, G.D., Bauer, H. and Sprinz, H. (1963): Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. Lab. Invest., 12: 355-364.

Adams, J.S., Clemen, T.L. and Holick, M.F. (1981): Silica sep-pak preparative chromatography for vitamin D and its metabolites. J. Chromatography, 226: 198-201.

Adham, N.F. and Song, M.K. (1980): Effect of calcium and copper on zinc absorption in the rat. Nutr. Metab. 24: 281-290.

Anderson, R.J. (1914): A contribution to the chemistry of phytin. J. Biol. Chem., 17: 171-190.

Andrews, T.L., Damron, B.L. and Harms, R.H. (1971a): The influence of dietary alfalfa meal on the availability of phosphorus from Curacao Island phosphate to the chick. Poult. Sci. 50: 104-108.

Andrews, T.L., Damron, B.L. and Harms, R.H. (1971b): Single comb white leghorn cockerels versus broiler chicks for use in phosphorus assays. Poult. Sci. 50: 1485-1488.

A.O.A.C. (1970): Official methods of analysis (11th Ed.). Association of Official Agricultural Chemists, Washington, D.C.

ARC, (1975): Nutrient requirements of farm livestock. No.1. Poultry Agricultural Research Council, London.

Asada, K. and Kasai, Z. (1962): Formation of myo-inositol and phytin in ripening rice grains. Plant Cell Physiol. 3: 397-402.

Asada, K., Tanaka, K. and Kasai, Z. (1969): Formation of phytic acid in cereal grains. Ann. N.Y. Acad. Sci. 165: 801-808.

Aston, W.M., Evans, C. and Williams, P.C. (1960): Phosphorus compounds of oats. 11. The utilization of phytate phosphorus by growing chicks. J.Sci. Food Agric. 11: 722-727.

Bafundo, K.W., Baker, D.H. and Fitzgerald, P.R. (1984): Zinc utilization in the chick as influenced by dietary concentrations of calcium and phytate and by Eimeria acervulina infection. Poult. Sci. 63: 2430-2437.

Ballam, G.C., Engster, H.M. and Snetzinger, D.C. (1984a): Effect of calcium level on the ability of broiler and single comb white leghorns to hydrolyze phytate phosphorus. Poult. Sci. 63: 61 (Abstr.).

Ballam, G.C., Nelson, T.S. and Kirby, L.K. (1984b): Effect of fiber and phytate source and of calcium and phosphorus level on phytate hydrolysis in the chick. Poult. Sci. 63: 333-338.

Ballam, G.C., Nelson, T.S. and Kirby, L.K. (1985): Effect of different dietary levels of calcium and phosphorus on phytate hydrolysis by chicks. Nutr. Report Interna. 32: 909-913.

Baksi, S.N. and Kenny, A.D. (1978): Acute effect of estradiol on the renel vitamin D hydroxylase in Japanese quail. Biochem. Pharmacol. 27: 2765-2768.

Baksi, S.N. and Kenny, A.D. (1979): Parathyroid hormone stimulation of 1,25-dihydroxy vitamin D_3 production in antiestrogen-treated Japanese quail. Mol. Pharmacol. 16: 932-940.

Bar, A. and Wasserman, R.H. (1973): Control of calcium absorption and intestinal calcium binding protein synthesis. Biochim. Biophys. Res. Commun. 54: 191-195.

Bassiri, A. and Nahapetian, A. (1977): Difference in concentrations and inter-relationships of phytate, phosphorus, magnesium, calcium, zinc and iron wheat varieties grown under dryland and irrigated condition. J. Agri. Food Chem. 25: 1118-1122.

Baxter, L.A. and DeLuca, H.F. (1976): Stimulation of 25-hydroxy Vitamin D_3 - 1 -hydroxylase by phosphate depletion. J. Biol. Chem. 251: 3158-3161.

Bell, D.J. and Freeman, B.M. (1971): In Physiology and Biochemistry of the domestic fowl, pp.25.

Berner, W., Kinne, R. and Murer, H. (1976): Phosphate transport into brush-border membrane vesicles isolated from rat small intestine. Bicohem. S., 160: 467-474.

Bhandari, S.D. (1980): Effect of phytate feeding with and without protein and vitamin D deficiencies on intestinal phytase activity in rat. Indian J. Biochem. Biophys. 17: 309-312.

Bikle, D.D., Murphy, E.W. and Rasmussen, H. (1975): The ionic control of 1,25-dihydroxy vitamin D_3 synthesis in isolated chick renal mitochondria, the role of calcium as influenced by inorganic phosphate and hydrogeniun. J. Chin. Invest., 55: 299-304.

Bikle, D., Morrissey, R., Zolock, D. and Rasmussen, H. (1981): The intestinal response to vitamin D. Rev. Physiol. Biochem. Pharmacol. 89: 63-119.

Birge, S. and Gilbert, H. (1974): Identification of an intestinal sodium and calcium release from intestinal mucosal cells. J. Clin. Invest. 54: 710-717.

Birge, S.J. and Avioli, R.C. (1981): Intestinal phosphate transport and alkaline phosphatase activity in the chick. Am. J. Physiol. 240: E384-E390.

Biswas, S. and Biswas, B.B. (1965): Enzymatic synthesis of guanosine triphosphate. Biochem. Biophy. Acta, 108: 710-713.

Bitar, K. and Reinhold, J.G. (1972): Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf and man. Biochim. Biophys. Acta 268: 442-452.

Blank, G.E., Pletcher, J. and Sax, M. (1971): The structure of myo-inositol hexaphosphate dodecasodium salt octama-contahydrate. A single crystal x-ray analysis. Biochem. Biophys. Res. Comm. 44: 319-325.

Bonjour, J.P., Preston, C. and Fleisch, H. (1977): Role of $1,25(OH)_2D_3$ in renal handling of inorganic phosphate in rats. Experientia 33: 777. (Abstr.).

Bouillon, R., Van Baelen, H. and Demoor, P. (1980): Comparative study of the affinity of the serum vitamin D-binding protein. J. Steroid Biochem. 13: 1029-1034.

Boyle, I.T., Gray, R.W. and DeLuca, H.F. (1971): Regulation by calcium of *in vivo* synthesis of 1,25-dihydroxycholecalciferol and 21,25-dihydroycholecalciferol. Proc. Natl. Acad. Sci. 68: 2131-2134.

Boyle, I.T., Miravet, L., Gray, R.W., Holick, M.F. and DeLuca, H.F. (1972): The response of intestinal calcium transport to 25-hydroxy and 1,25-dihydroxy vitamin D in nephrectomized rats. Endocrin. 90: 605-608.

Boyle, I.T., Omdahl, J.L., Gray, R.W. and DeLuca, H.F. (1973): The biological activity and metabolism of 24,25-dihydroxy vitam D_3. J. Biol. Chem. 248: 4174-4180.

Bronner, F. and Freund, T. (1975): Intestinal CaBP: a new quantitive index of vitamin D deficiency in the rat. Am. J. Physiol. 229: 689-694.

Brommage, R., Jarnagin, K., DeLuca, H.F., Yamada, S. and Takayama, H. (1983): 1-But not 24-hydroxylation of vitamin D is required for skeletal mineralization in rats. Am.J. Physiol. 244: E298-304.

Bruce, H.M. and Callow, H.K. (1934): Cereals and rickets - The role of inositol hexaphosphoric acid. Biochem. J. 28: 517-528.

Brumbaugh, P., Haussler, D., Bursac, K. and Haussler, M. (1974): Filter assay for 1,25-dihydroxy vitamin D_3. Utilization of the hormone's target tissue chromatin receptor. Biochemistry, 13: 4091-4097.

Brown, E.C., Heit, M.L. and Ryan, D.E. (1961): Phytic acid : An analytical investigation. Can. J. Chem. 39: 1290-1294.

Caldas, A.E., Gray, R.W. and Lemann, J. Jr. (1978): The simultaneous measurement of vitamin D metabolites in plasma: Studies in healthy adults and in patients with calcium nephrolithiasis. J. Lab. Clin. Med. 91: 840-849.

Canterbury, J., Lerman, S., Claflin, A., Henry, H., Norman, A. and Reiss, E. (1978): Inhibition of parathyroid hormone secretion by 25-hydroxydolecalciferol and 24,25-dihydroxycholecalciferol in the dog. J. Clin. Invest. 61: 1375-1383.

Care, A.,D. and Van't Klooster, A.T. (1965): *In vivo* transport of magnesium and other cations across the wall of the gastro-intestinal tract of sheep. J. Physiol. 177: 174-191.

Carew, L.B. Jr., Gestone, T.A. and Alster, F.A. (1985): Effect of a phosphorus deficiency on thyroid function and growth hormone in the white leghorn male. Poult. Sci. 64: 2010-2012.

Carlsson, A. (1952): Tracer experiments on the effect of vitamin D on the skeletal metabolism of calcium and phosphorus. Acta Physiol. 26: 212-220.

Chan, S.D.H. and Atkins, D. (1983): Distribution and properties of Ca^{++} ATpase, phytase and alkaline phosphatase in isolated enterocytes from normal and vitamin D-deficient rats. Gut, 24: 886-892.

Chang, C.W. (1967): Study of phytase and fluoride effects in germinating corn seeds. Cereal. Chem. 44: 129 -

Chen, P.S. and Neuman, W.F. (1955): Renal excretion of calcium by the dog. Amer. J. Physiol. 180: 623-632.

Chen, P.S. Jr. and Bosmann, H.B. (1964): Effect of vitamin D_2 and D_3 in serum calcium and phosphorus in rachitic chicks. J. Nutr. 83: 133-139.

Chen, T.C., Castillo, L., Korycka-Dahl, M. and DeLuca, H.F. (1974): Role of vitamin D metabolites in phosphate transport of rat intestine. J. Nutr. 104: 1056-1060.

Chen, L.H. and Pan, S.H. (1977): Decrease of phytates during germination of pea seeds. Nutr. Rep. Int. 16: 125-131.

Cheney, E.G., Fernando, Q. and Freiser, H. (1959): Metal chelates of mercaptosuccineic acid. J. Phys. Chem. 63: 2055-2057.

Cheryan, M. (1980): Phytic acid interactions in food systems. Rev. Food Sci. Nutr. 13: 397-335.

Cheryan, M., Anderson, F.W. and Orynspan, F. (1983): Magnesium-phytate complexes: Effect of pH and molar ratio on solubility characteristics. Cereal Chem. 60: 235-237.

Chicco, C.F., Ammerman, C.B., Van Walleghem, P.A., Waldroup, P.W. and Harms, R.H. (1967): Effects of varying dietary ratios of magnesium, calcium and phosphorus in growing chicks. Poult. Sci. 46: 368-373.

Christmas, R.B. and Harms, R.H. (1978): Relative phosphorus requirements of white leghorn cockerels. Poult. Sci. 57: 489-491.

Christakos, S., Friedlander, E., Frandsen, B. and Norman, A.W. (1979): Studies on the mode of action of calciferol. XIII. Development of a radioimmunoassay for vitamin D-dependent chick intestinal calcium-binding protein and tissue distribution. Endocrinology, 104: 1495-1503.

Christakos, S. and Norman, A.W. (1981): Studies on the mode of action of calciferol. Biochemical characterization of 1,25-Dihydroxy vitamin D_3 receptors in chick pancreas and kidney cytosol. Endocrinology, 108: 140-149.

Clark, S.L. (1961): Alkaline phosphatase of the small intestine studied with electron microscopects in suckling and adult mice. Anat. Record, 139: 216 (Abstr.).

Clarkson, E.M., Warren, R.L., McDonald, S.J. and De Wardener, H.E. (1967): The effect of a high intake of calcium on magnesium metabolism in normal subjects and patients with chronic renal failure. Clin. Sci. 32: 11-18.

Clemens, T.L., Hendy, O.N., Papapoules, S.E., Fraher, L.J., Care, A.D. and O'Riodan, J.L.H. (1979): Measurement of 1,25-dihyroxy-cholecalciferol in man by radioimmunoassay. Clin. Endocrinol. 11: 225-234.

Celston, K.W., Evans, I., Spelsberg, T.C. and Macintyre, I. (1977): Feedback regulation of vitamin D metabolism by 1,25-dihydroxycholecalciferol. Biochem. J. 164: 83-89.

Common, R.H. (1940): The phytic acid content of some poultry feeding stuffs. The Analyst, 65: 79-83.

Cooper, J.R. and Gowing, H. (1983): Mammalian small intestinal phytase (EC3.1.3.8). Bri. J. Nutri. 50: 673-678.

Cosgrove, D.J. (1966): The chemistry and biochemistry of inosital poly phosphates. Rev. Pure Apple. Chem. 16: 209-234.

Costello, A.J.R., Glonek, T. and Myers, T.C. (1976): Phosphorus-31-nuclear magnetic resonance-pH titrations of myoinositol hexaphosphate. Carbohydr. Res. 46: 159-171.

Crenshaw, M., Ramp, W., Gonnerman, W. and Toverud, S. (1974): Effects of dietary vitamin D levels on the *in vitro* mineralization of chick metaphyses. Proc. Soc. Expt. Biol. Med. 146: 488-493.

Damron, B.L. and Harms, R.H. (1970): A comparison of phosphorus assay techniques with chicks: comparison of the relative performance of eight phosphate sources. Poult. Sci. 49: 1541-1545.

Davies, M.I., Ritcey, G.M. and Motzok, I. (1970): Intestinal phytase and alkaline phosphatase of chicks: influence of dietary calcium, inorganic and phytate phosphorus and vitamin D_3. Poult. Sci. 49: 1280-1286.

Davies, N.T. and Flett, A.A. (1978): The similarity between alkaline phosphatase (EC3.1.3.1) and phytase (EC3.1.3.8) activities in rat intestine and their importance in phytate-induced zinc deficiency. Br. J. Nutr. 39: 307-316.

Davies, N.T. and Olpin, S.E. (1979): Studies on the phytate: Zinc molar contents in diets as a determinant of zinc availability to young rats. Br. J. Nutr. 41: 591-603.

DeLuca, H.F. and Schnoes, H.K. (1976): Metabolism and mechanism of action of vitamin D. Ann. Rev. Biochem. 45: 631-666.

DeLuca, H.F. (1979): In "vitamin D metabolism and functions", Springer-Verlag, p.8.

DeLuca, H.F. and Schnoes, H.K. (1983): Vitamin D : Recent advances. Ann. Rev. Biochem. 52: 411-439.

De-Rham, O. and Jost, T. (1979): Phytate protein interactions in soybean extracts and low-phytate soyprotein products. J. Food Sci. 44: 596-600.

Desjobert, A. and Fleurent, P. (1954): Influence delareaction dumileen sur l hydrolyse chimique de linositol - hexaphosphate. Considerations sur la constitution dece derive. Bull. Soc. Chim. Biol. 36: 475-482.

Duncan, D.B. (1955): Multiple range and multiple F test. Biometerics, 11: 1-42.

Duncan, W.E., Aw, T.C., Walsh, P.O. and Haddan, J.G. (1983): Normal rabbit intestinal cytosol as a source of binding protein for the 1,25-dihydroxy vitamin D_3 assay. Analy. Biochem. 132: 209-214.

Eisenberg, E. (1965): Effects of serum calcium level and parathyroid extracts on phosphate and calcium excretion in hypoparathyroid patients. J. Clin. Invest. 44: 942-946.

Eisman, J.A., Hamstra, A.½J., Kream, B.E. and DeLuca, H. (1976): A senstive, precise, and convenient method for determination of 1,25-dihydroxy vitamin D in human plasma. Arch. Biochem. Biophys. 176: 235-243.

Engstrom, O.W., Horst, R.L., Reinhardt, T.A. and Littledike, E.T. (1982): In: vitamin D, chemical, biochemical and clinical endocrinology of calcium metabolism.

Ewing, E.R. (1963): Poultry nutrition (5th ed.). The Ray Ewing Co., Pasadena, CA.

Edwards, H.M. Jr. (1976): Are-Examination of the nutritional role of vitamin D. Proc. Georgia Nutr. Conf. 77-83.

Edwards, H.M. Jr. (1981): Phosphorus requirements of growing chickens. Proc. Georgia Nutr. Conf. 73-77.

Edwards, H.M. Jr. (1982): Phosphorus and calcium for broilers. Proc. Georgia Nutri. Conf. 161-167.

Edwards, H.M. Jr. (1982): Effect of bred and strain on utilization of suboptimal levels of phosphorus in the ration. Poult. Sci. 62: 77-84.

Fleury, P. (1946): Study of chemical hydrolysis of phytic acid. Bull. Soc. Chim. Biol. 28: 282-286.

Forbes, R.M. (1964): Mineral utilization in the rat. III. Effects of calcium, phosphorus, lactose and source of protein in zinc-deficient and in zinc-adequate diets. J. Ntr. 83: 225-233.

Forbes, R.M. and Parker, H.M. (1977): Biological availability of zinc in and as influenced by whole fat soy flour in rat diets. Nutr. Rep. Int. 15: 681-688.

Forbes, R.M., Weingartner, K.E., Parker, H.M., Bell, R.R. and Erdman, J.W. Jr. (1979): Bioavailability to rats of zinc, magmesium and calcium in casein-, egg-, and soy protein-containing diets. J. Nutr. 109: 1652-1660.

Forbes, R.M., Endman, J.W., Parker, H.M., Kondo, H. and Ketelsen, S.M. (1983): Bioavailability of zinc in coagulated soyprotein to rats and effect of dietary calcium at a constant phytate:zinc ratio. J. Nutr. 113: 205-210.

Fox, J., Pickard, D.W., Care, A.D. and Murray, T.M. (1978): Effect of low phosphorus diets on intestinal calcium absorption and the concentration of calcium binding protein in intact and parathyroidectomised pigs. J. Endocr. 78: 379-387.

Franz, K.B. (1979): Bioavailability of zinc from selected cereals and legumes. Diss. Abstr. B. 39: 3766-3775.

Fraser, D.R. and Kodicek, E. (1973): Regulation of 25(OH)-1-hydroxylase activity in kidney by parathyroid hormone. Nature New Biol. 241: 163-166.

Frazer, D.R. and Emtage, J.S. (1976): Vitamin D in the avian egg, its molecular identity and mechanism of incorporation into yolk. Biochem. J. 160: 671-682.

Friedlander, E.J., Henry, H.L. and Norman, A.W. (1977): Studies on the mode of action of cholecalciferol. J. Biol. Chem. 252: 8677-8683.

Fritz, J.S., Roberts, T., Boehne, J.W. and Hore, E.L. (1969): Factors affecting the chicks requirements for phosphorus. Poult. Sci. 48: 307-320.

Garabedian, M., Holick, M.F., DeLuca, H.F., Boyle, I.T. (1972): Control of 25-hydroxycholecalciferol metabolism by the parathyroid glands. Proc. Natl. Acad. Sci. 69: 1673-1676.

Garabedian, M., Tanaka, Y., Holick, M.F., and DeLuca, H.F. (1974): Response of intestinal calcium transport and bone calcium mobilization to 1,25-dihydroxy vitamin D_3 in thyroparathyroid-ectomized rats. Endocrinolgy 94: 1022-1027.

Gardiner, E.E. (1962): The relationship between dietary phosphorus level and the level of plasma inorganic phosphorus of chicks. Poult. Sci. 41: 1156-1163.

Gardiner, E.E. (1969): Response of two breeds of chickens to graded levels of dietary phosphorus. Poult. Sci. 48: 986-993.

Gillis, M.B., Norris, L.C. and Heuser, G.F. (1954): Studies on the biological value of inorganic phosphates. J. Nutr. 52: 115-125.

Goldenberg, H. and Fernandez, A. (1966): Simplified method for the estimation of inorganic phosphorus in body fluids. Clinical Chemis. 12: 871-882.

Gonnerman, W.A., Ramp, W.K. and Toverud, S.U. (1975): Vitamin D, dietary calcium and parathyroid hormone interactions in chicks. Endocrinology 96: 275-281.

Gray, R.W. (1981a): Effect of age and sex on the regulation of plasma $1,25(OH)_2D_3$ by phosphorus in the rat. Calc. Tiss. Int. 33: 477-484.

Gray, R.W. (1981b): Control of plasma $1,25-(OH)_2-$ vitamin D concentrations by calcium and phosphorus in the rat: Effect of hypophysectomy. Calc. Tiss. Int. 33: 485-488.

Gray, R.W. and Garthwaite, T.L. (1985): Activation of renal 1,35-dihydroxy-vitamin D_3 synthesis by phosphate deprivation: evidence for a role for growth hormone. Endocrinology 116: 189-193.

Griffith, M. (1968): Influence of soybean products on the bone ash of chicks fed certain phosphorus deficient purified diets. Poult. Sci. 47: 765-771.

Groth, W. (1963): The osteopathies of calcium and phosphorus deficiency and their relation to parathyroid function and to themineral content of the blood in growing chicks. Nutr. Abst. and Rev. 33: 1206.

Hall, J.R. and Hodges, T.K. (1966): Phosphorus metabolism of germinating oat seeds. Plant. Physiol. 41: 1459-1464.

Halloran, B.P., DeLuca, H.F., Barthell, E., Yamada, S., Ohmori, M. and Takayama, H. (1981): An examination of the importance of 24-hydroxylation to the function of vitamin D during early development. Endocrinology, 108: 2067-2071.

Hanson, W.C. (1950): The photometric determination of phosphorus in fertilizers using the phosphovandaomolybdate complex. J. Sci. Food Agr. 1: 172-173.

Harms, R.H., Waldroup, P.W., Shirley, R.L. and Ammerman, C.B. (1962): Availability of phytic acid phosphorus for chicks. Poult. Sci. 41: 1189-1191.

Harris, R.S. and Bunker, T.W.M. (1935): The phytin phosphorus of the corn component of a rachitogenic diet. J. Nutr. 9: 301-306.

Harrison, H.E. and Harrison, H.C. (1961): Intestinal transport of phosphate: action of vitamin D, calcium, and potassium. Am. J. Physiol. 201: 1007-1012.

Harrison, G.F. (1969): Production of germ-free chicks: A comparison of the hatchability of eggs sterilized externally by different methods. Lab. Anim. 3: 51-59.

Hartig, T. (1855): Uber das klebermehl. Bot. Ztg. 13,881. (Cited from Reddy 1982).

Hartig, T. (1856): Weitere mittheilungen uber klebermehl. Bot. Ztg. 14,257. (cited from Reddy 1980).

Haussler, M.R., Nagode, L.A. and Rasmussen, H. (1970): Induction of intestinal brush border alkaline phosphatase by vitamin D_3 and identity with Ca-ATpase. Nature 228: 1199-1201.

Haussler, M.R., Hughes, M., Baylink, D., Littledike, E.T., Cork, D. and Pitt, M. (1977): Influence of phosphate depletion on the biosynthesis and circulating level of 1,25-dihydroxy vitamin D, In "phosphate metabolism", pp.233-250.

Henry, H.L. and Norman, A.W. (1974): Studies on calciferol metabolism, renal 25(OH)D_3-1-hydroxylase. Involvement of cytochrome P-450 and other properties. J. Biol. Chem. 249: 7529-7535.

Henry, H.L., Midget, R.J. and Norman, A.W. (1974): Regulation of 25-hydroxy vitamin D_3-1-hydroxylase in vivo. J. Biol. Chem. 249: 7584-7592.

Henry, H.L., and Norman, A.W. (1984): Vitamin D, metabolism and biological actions. Ann. Rev. Nutr. 4: 493-520.

Hedge, S.N., Rolls, B.A., Turvey, A. and Coates,M.E. (1978): The effects on chicks of dietary fibre from different sources as growth factor in wheat bran. Brit. J. Nutr. 40: 63-69.

Hegested, D.M., Trulson, M.F. and Stare, F.J. (1954): Role of wheat and wheat products in numan nutrition. Physiol. Rev. 34: 221-258.

Hill, R. and Tyler, C. (1954): The influence of time, temperature, pH and calcium carbonate on the activity of the phytase of certain cereals. J. Agr. Sci. 44: 306-310.

Hoff-Jorgensen, E. (1944): Kgl. Danske videnskab., selskab mat. nat. medd. 21:7 (Cited from Hoff-Jorgensen, 1946).

Holdsworth, E.S. (1970): The effect of vitamin D on enzyme activities in the mucosal cells of the chick small intestine. J. Member. Biol. 3: 43-53.

Holick, M.F., Kleiner-Bossaller, A., Schnoes, H.K., Kasten, P.M., Boyle, I.T. and DeLuca, H.F. (1973): 1,24,25-trihydroxy vitamin D_3, a metabolite of vitamin D_3 effective on intestine. J. Biol. Chem. 248: 6691-6696.

Holick, M.F., Richtand, N.M., McNeill, S.C., Holick, S.A., Fromer, J.E., Henley, J.W. and Potts, J.T. Jr. (1979): Isolation and identification or pre-vitamin D_3 from the skin of rats exposed to ultraviolet irradiation. Biochemistry, 18: 1003-1008.

Horiuchi, N., Suda, T., Sasaki, S., Ezawa, I., Sano, Y. and Ogata, E. (1974): Direct involvement of vitamin D in the regulation of 25-hydroxycholecalciferol metabolism. Febs. lett. 43: 353-356.

Horiuchi, N., Suda, T., Sasaki, S., Ogata, E., Ezawa, I., Sanyo, Y. and Shimazawa, E. (1975): The regulatory role of calcium in 25-hydroxycholecalciferol metabolism in chick kidney in vitro. Arch. Biochem. Biophys. 171: 540-548.

Horst, R. and Littledike, T. (1980): 25-OHD_3-26-23 lactone: Demonstration of kidney-dependent synthesis in the pig and rat. B.B. Res. Comm. 93: 149-154.

Hughes, M.R., Brumbaugh, P.F. and Haussler, M.R. (1975): Regulation of serum 1,25 di-hydroxy vitamin D_3 by calcium and phosphorus in the rat. Science, 190: 578-580.

Hughes, M.R., Haussler, M.R., Wergedal, J. and Baylink, P.J. (1975): Regulation of serum 1,25-dihydroxy vitamin D_3 by calcium and phosphate in the rat. Science 190: 578-580.

Hurwitz, S. and Bar, A. (1965): Absorption of calcium and phosphorus along the gastrointestinal tract of the laying fowl as influenced by dietary calcium and eggshell formation. J. Nutrition 85: 433-438.

Hurwitz, S. and Bar, A. (1972): Site of vitamin D action in chick intestine. Am. J. Physiol. 222: 761-767.

Husbscher, G., West, G.R. and Brindley, D.N. (1965): Studies on the fractionation of mucosal homogenates from the small intetine. Biochem. J. 97: 629-642.

Ismail-Beigi, F., Faradji, B. and Reinhold, J.G. (1977): Binding of zinc and iron to wheat bread, wheat bran and their components. Amer. J. Chin. Nutr. 30: 1721-1725.

IUPAC-IUB, (1968): The nomenclature of cyclitols. Eur. J. Biochem. 5: 1-12.

Jarnagin, K., Brommage, R., DeLuca, H.F., Yamada, S. and Takayama, H. (1983): I - but not 24-hydroxylation of vitamin D is required for growth and reproduction in rats. Am. J. Physiol, 244: E290-E297.

Jensen, L.S. and Edwards, H.M. Jr. (1980): Availability of phosphorus from ammonium polyphosphate for growing chickens. Poult. Sci. 59: 1280-1283.

Johnson, L.F. and Tate, M.E. (1969): Structure of phytic acids. Can. J. Chem. 47: 63-73.

Kaufman, H.W. and Kleinberg, I. (1971): Effect of pH on calcium binding by phytic acid and its inositol phosphoric acid derivatives and on the solubility of their calcium salts. Arch. Oral Biol. 16: 445-460.

Kempson, S., Kim, J., Northrup, T., Know, F. and Dousa, T. (1979): Alkaline phosphatase in adaptation to low dietary phosphate intake., Am. J. Physiol. 237: E465-E473.

Kenny, A.D. (1976): Vitamin D metabolism: physiological regulation in egg-laying Japanese quail. Am. J. Physiol. 230: 1609-1615.

Kleeman, C., Bernstein, D., Rockeney, R., Dowling, J. and Maxwell, M. (1961): in "The parathyroids", Springfield, p.353-387.

Kleiner-Bossaller, A. and DeLuca, H.F. (1974): Formation of 1,24,25-trihydroxy vitamin D_3 from $1,25(OH)_2D_3$. Biochim. Biophys. Acta 338: 489-495.

Kobayashi, Y., Taguchi, T., Terada, T., Oshida, J., Morisaki, M. and Ikekawa, N. (1979): Synthesis of 24,25-difluoro and 24-fluoro-25-hydroxy vitamin D_3. Tetrahendron Lett. 22: 2023-2026.

Kowarski, S. and Schachter, D. (1969): Effects of vitamin D on phosphate transport and incorporation into mucosal constituents of rat intestinal mucosa. J. Biol. Chem. 244: 211-217.

Kramer, C.Y. (1956): Extension of multiple range tests to group means with unequal numbers of replications. Biometrics 12 : 307-310.

Kream, B.E., Jose, M., Yamada, S. and DeLuca, H.F. (1977): A specific high-affinity binding macromolecule for 1,25-dihydroxy vitamin D_3 in fetal bone. Science, 197: 1086-1088.

Krieger, C.H. and Steenbock, H. (1940): The effect of calcium and vitamin D_3 on the availability of phosphorus. J. Nutr. 20: 7-14.

Kumar, R., Schnoes, H.K. and DeLuca, H.F. (1978): Rat intestinal 25-hydroxy vitamin D_3 - and 1,25-dihydroxy vitamin D_3 -24-hydroxylase. J. Biol. Chem. 253: 3804-3809.

Kumar, R. (1984): Metabolism of 1,25-dihydroxy vitamin D_3. Physiol. Rev. 64: 478-504.

Lage, A. (1980): Quantitation of the main metabolites of vitamin D in a single serum sample - 1, Extraction, separation and purification of metabolites. Clin. Chim. Acta, 104: 133-146.

Lamm, M. and Neuman, W.F. (1958): On the role of vitamin D in calcification. Arch. Pathol. 66: 204-209.

Lane, S. and Lawson, D. (1978): Differentiation of the changes in alkaline phosphatase from calcium ion-activated adenoisine triphosphatase activities associated with increased calcium absorption in chick intestine. Biochem. J. 174: 1067-1070.

Larkins, R.G., Colston, K., Galante, L., Macauley, S.G., Evans, I. and Macintyre, I. (1973): Regulation of vitamin D-metabolism without parathyroid hormone. Lancet 2: 289-291.

Larkins, R.G., Macauley, S.G. and Macintyre, I. (1975): Inhibitors of protein and RNA synthesis and 1,25-hihydroxycholecalciferol formation in vitro. Mol. Cell Endocrinol. 2: 193-202.

Lawson, D.E.M. and Wilson, P.W. (1974): Intranuclear localization and receptor proteins for $1,25(OH)_2D_3$ in chick intestine. Biochem. J. 144: 573-583.

Lavender, A.R. and Pullman, T.N. (1963): Changes in inorganic phosphate excretion induced by renal arterial infusion of calcium. Am. J. Physiol. 205: 1025-1032.

Leblond, C.P. and Stevens, C.E. (1948): The constant renewal of the intestinal epithelium in the albino rat. Anat. Rec. 100: 357-377.

Likuski, H.J.A. and Forbes, R.M. (1964): Effect of phytic acid on the availability of zinc in amino acid and casein diets fed to chicks. J. Nutr. 84: 145-148.

Likuski, H.J.A. and Forbes, R.M. (1965): Mineral utilization in the rat. IV. Effects of calcium and phytic acid on the utilization of dietary zinc. J. Nutr. 85: 230-234.

Lim, P.E. and Tate, M.E. (1973): The phytase. II. properties of phytase fractions F1 and F2 from wheat bran and myoinositol phosphates produced by fraction F2. Biochim. Biophys. Acta 302: 316-328.

Lindblad, G.S., Slinger, S.J., Anderson, G.W. and Motzok, I. (1952): Effect of aureomycin on the calcium and phosphorus requirements of chicks. Poult. Sci. 31: 923-924.

Lolas, G.M. and Markakis, P. (1975): Phytic acid and other phosphorus compounds of beans. J. Agric. Food. Chem. 23: 13-15.

Lolas, G.M., Palamidis, N. and Markakis, P. (1976): The phytic acid-total phosphorus relationship in barley, oats, soybeans and wheat. Ceral Chem. 53: 867-871.

Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Luck, M.R. and Scanes, C.G. (1979): Plasma levels of ionized calcium in the laying hen. Comp. Bioch. Physio. 63: 177-181.

Maddaiah, V.T., Kurnick, A.A. and Reid, C.L. (1964): Phytic acid studies. Proc. Soc. Exp. Biol. Med. 115: 391-393.

Maga, J.A. (1982): Phytate - its chemistry, occurence, food interaction, nutritional significance and methods of analysis. J. of Agr. and Food Chem. 30: 1-9.

Maiti, I.B., Majumdar, A.L. and Biswas, B.B. (1974): Purification and mode of action of phytase from phaseolus vulgaris. Phytochemistry 13: 1047-1051.

Maddaiah, V.T., Kurnick, A.A., Hulett, B.J. and Reid, C.L. (1964) Nature of Intestinal Phytase Activity. Proc. Soc. Exp. Biol. Med. 115: 1054-1057.

Mallon, J.P., Hamilton, J.G., Nauss-Karol, C., Karol, R.J., Ashley, C.J., Matuszewski, D.S., Tratnyek, C.A., Bryce, G.F. and Miller, O.N. (1980): An improved competitive protein binding assay for $1,25(OH)_2D_3$. Arch. Biochem. Biophys. 201: 277-285.

Mandal, N.C. and Biswas, B.B. (1974): Metabolism of inositol phosphates. 1. phytase synthesis during germination of cotyledons of mung beans. Plant Physiol. 45: 4-7.

Mandal, N.C., Burman, S. and Biswas, B.B. (1972): Isolation, purification and characterization of phytase from germinating mung bean. Phytochemistry 11: 495-502.

Mellanby, E. (1919): An experimental investigation on rickets. Lancet 1: 407-412.

Mellanby, E. (1920): Vitamins and infant feeding. Lancet 1: 856-862.

Mellanby, E. (1950): "A story of nutrition research" p.263. Williams and Wilkins, Baltimore, Maryland.

Midgett, R.J., Speilrogel, A.M., Coburn, J.W. and Norman, A.W. (1973): Studies on calciferol metabolism. VII. The renal production of the biologically cholecalciferol. J. Clin. Endocr. Metab. 36: 1153-1161.

Migicovsky, B.B., Neilson, A.M., Gluck, M. and Burgess, R. (1951): Penicillin and calcium absorption. Arch. Biochim. Biophys. 34: 479-480.

Miller, G.A., Youngs, V.L. and Oplinger, E.S. (1980): Environmental and cultivar effects on oat phytic acid concentration. Cereal Chem. 57: 189-191.

Mollgaard, H. (1946): On phytic acid, its importance in metabolism and its enzymic cleavage in bread supplemented with calcium. Biochem. J. 40: 589-603.

Moog, F. (1950): The functional differentiation of the small intestine. 1 - The accumulation of alkaline phosphatase in the duodenum of the chick. J. Exptl.2001. 115: 109-129.

Moog, F. (1962): Developmental adaptations of alkaline phosphatase in the small intestine. Federation Proc. 21: 51-56.

Moog, F. and Glazier, H.S. (1972): Phosphate asborption and alkaline phosphatase activity in the small intestine of the adult mouse and of the chick embryo and hatched chick. Comp. Biochem. Physiol. 42A: 321-326.

Moore, R.J. and Veum, T.L. (1983): Adaptive increase in phytate digestibility by phosphorus-deprived rats and the relationship of intestinal phytase (EC3.1.3.8) and alkaline phosphatase (EC3.1.3.1) to phytate utilization. Bri. J. Nutr. 49: 145-152.

Moore, R.J., Philip, G.R. and Veum, T.L. (1984): Influence of dietary phosphorus and sulphaguanidine levels on p utilization in rats. Bri. J. Nutr. 51: 453-465.

Morrissey, R.L. and Wasserman, R.H. (1971): Calcium absorption and calcium-binding protein in chicks on differing calcium and phosphrous intakes. Am. J. Physio. 220: 1509-1515.

Moser, R.L., Peo, E.R.Jr., Moser, B.D. and Lewis, A.J. (1982): Effect of grain source and dietary level of oat hulls on phosphorus and calcium utilization in the growing pigs. J. Anim. Sci., 54: 800-805.

Mueller, W.J. (1956): Feasibility of the chromic oxide and the lignin indicator method for metabolism experiments with chickens. J. Nutr. 58: 29-36.

Murono, E., Kirdani, R. and Sandberg, A. (1979): Specific estradiol-17B binding component in adult rat kidney. J. Ster. Biochem. 11: 1347-1351.

McCance R.A. and Widdowson, E.M. (1935): Phytin in human nutrition. Biochem. J. 29B: 2694-2699.

McCance, R.A. and Widdowson, E.I. (1944): Activity of phytase in different cereals and its resistance to dry heat. Nature, 153: 650.

McGillivary, J.J. (1978): Biological availability of phosphorus sources. First Annal. Inter-miner. Conf. pp.73-85.

McCuaig, L.W., Davies, M.I. and Motzok, I. (1972): Intestinal alkaline phosphatase and phytase of chicks: Effect of dietary magnesium, calcium phosphorus and thyroactive casein. Poult. Sci. 51: 526-530.

McCuaig, L.W. and Motzok, I. (1973): Interactions of Ca, P, Zn and alkaline phosphatase in the chick. Poultry Science 52: 1896-1902.

McCuaig, L.W. and Motzok, I. (1974a): Interactions of calcium, phosphorus, zinc and alkaline phosphatase in the chick. II. Effect of dietary calcium level. Can. J. Physiol. Pharmaco 1. 52: 92-95.

McCuaig, L.W. and Motzok, I. (1974b): Interactions of calcium, phosphorus, zinc and alkaline phosphatase in the chick. III. Effects of dietary phosphase, sodium chloride and theophylline. Comp. Biochem. Physio. 48A: 663-674.

McWard, G.W. (1969): Effects of phytic acid and ethylenediamine-tetraacetic acid (EDTA) on the chick's requirement for magnesium. Poult. Sci. 48: 791-794.

Nelson, T.S. (1967): The utilization of phytate phosphorus by poultry - A review. Poult. Sci. 46: 862-871.

Nelson, T.S. and Ferrara, L.W. (1968): Phytate phosphorus content of feed ingredients derived from plants. Poult. Sci. 47: 1372-1374.

Nelson, T.S., Shieh, T.R., Wodzinski, R.J. and Ware, J.H. (1971): Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. J. Nutr. 101: 1289-1293.

Nelson, T.S. (1976a): The hydrolysis of phytate phosphorus by chicks and laying hens. Poult. Sci. 55: 2262-2264.

Nelson, T.S., Daniels, L.B., Hall, J.R. and Shields, L.G. (1976b): Hydrolysis of natural phytase phosphorus in the digestive tract of calves. J. Animal Sci. 42: 1509-1512.

Nelson, T.S. and Kirby, L.K. (1979): Effect of age and diet composition on the hydrolysis of phytate phosphorus by rats. Nutr. Rep. Int. 20: 729-734.

Neuberg, C. (1908): Biochem. Z.2, 557 (Cited from Johnson and Tate, 1969).

Nicolaysen, R. (1937): Studies upon the mode of action of vitamin D. III. The influence of vitamin D on the absorption of calcium and phosphorus in the rat. Biochem. J. 31: 122-129.

Norman, A.W. and DeLuca, H.F. (1963): The preparation of H^3-vitamin D_2 and D_3 and their localization in the rat. Biochemistry 2: 1160-1168.

Norman, A.W. (1965): Actinomycin D and the response to vitamin D. Science 149: 184-186.

Norman, A.W. and Henry, H.L. (1974): The role of the kidney and vitamin D metabolism in health and disease. Clin. Orthop. Res. 98: 258-286.

Nott, H. (1967): Ph.D. Thesis, Reading University.

NRC. (1977): Nutrient requirement of poultry. National Academy of Science - National Research Council, Washington, D.C.

Nugara, D. and Edwards, H.M. Jr. (1963): Influence of dietary calcium and phosphorus levels on the magnesium requirement of chick. J. Nutr. 80: 181-184.

Nwokolo, E.N. and Bragg, D.B. (1977): Influence of phytic acid and crude fibre on the availability of minerals from four protein supplements in growing chicks. Can. J. Anim. Sci. 57: 475-477.

Oberleas, D. and Prasad, A.S. (1969): Growth as affected by zinc and protein nutrition. Am. J. Clin. Nutr. 22: 1304-1314.

Oberleas, D. (1973): Phytates. In "Toxicants occurring naturally in foods", 2nd ed., p.363, Natl. Acad. Sci. Washington, D.C.

Oberleas, D. (1975): Factors influencing availability of minerals. Proc. West. Hemisphere Nutr. Congr., 4th 1974 p.156.

O'Dell, B.L. and Savage, J.E. (1960): Effect of phytic acid on zinc availability. Proc. Soc. Exp. Biol. Med. 103: 304-306.

O'Dell, B.L., Yohe, J.M. and Savage, J.E. (1964): Zinc availability in the chick as affected by phytate, calcium, and ethylenediamine-tetra-acelate. Poult. Sci. 43: 415-419.

O'Dell, B.L. (1969): Effect of dietary components upon zinc availability: A review with original data. Am. J. Clin. Nutr.22: 1315-1322.

O'Dell, B.L., de Boland, A. and Koirtyohann, R. (1972): Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. J. Agric. Food Chem. 20: 718-721.

O'Dell, B.L. (1979): Effect of soy protein on trace mineral bioavailability. In 'soy protein and human nutrition". Academic Press, New York.

Ohnuman, N. and Norman, A.W. (1982): Isolation and chemical characterization of two new vitamin D metabolites produced by the intestine. J. Biol. Chem. 257: 5097-5102.

Okamoto, S., Tanaka, Y., DeLuca, H.F., Yamada, S. and Takayama, H. (1981): 24,24-difluoro-25-hydroxy vitamin D_3- enhanced bone mineralization in rats comparison with 25-hydroxy vitamin D_3 and vitamin D_3. Arch. Biochem. Biophys. 206: 8-14.

Ornoy, A., Goodwin, D., Noff, D. and Edelstein, S. (1978): 24,25-dihydroxy vitamin D is a metabolite of vitamin D essential for bone formation. Nature, 276: 517-519.

Oshima, M., Taylor, T.G. and Williams, A. (1964): Variations in the concentration of phytic acid in the blood of the domestic fowl. Bioch. J. 92: 42-46.

Padykula, H.A. (1962): Recent functional interpretation of intestinal morphology. Fed. Proc. 21: 873-879.

Palladin, W.Z. (1894): Fur biologite 31, 191 (cited from Cosgrove 1966).

Palmer, M.F. and Rolls, B.A. (1983): The activities of some metabolic enzymes in the intestines of germ-free and conventional chicks.

Pastea, E., Nicolau, A., Popa, V. and Rosca, I. (1968): Dynamics of the digestive tract in hens and ducks. Acta Physiol. Hung. 33: 305-310.

Paterson, J.A., Waller, J.C. and Sullivan, T.W. (1978): Influence of strain and sex on the dietary phosphorus requirement of broilers. Poult. Sci. 57: 1179 (Abstract).

Partwardham, V.N. (1937): The occurrence of phytine splitting enzyme in the intestines of albino rats. Bioch. J. 31: 560-563.

Peers, G.F. (1953): The phytase of wheat. Biochem. J. 53: 102-110.

Peterlik, M. and Wasserman, R.H. (1978): Effect of vitamin D on transepithelial transport in chick intestine. Am. J. Physiol. 234: E379-E388.

Peterlik, M. and Wasserman, R.H. (1981): Regulation by vitamin D_3 of intestinal phosphase absorption. Horm. Metab. Res. 12: 216-219.

Pfeffer, E. and Pringsheims, J.W. (1872): Bot., 8, (429-475) (cited from Cosgrove, 1966).

Pileggi, V.J., DeLuca, H.F. and Steenbock, H. (1955): The role of vitamin D and intestinal phytase in the prevention of rickets in rats on cereal diets. Archs. Biochem. Biophys.58: 194-204.

Pileggi, V.J. (1959): Distribution of phytase in the rat. Arch. Biochem. Biophys. 80: 1-8.

Pointillarf, A., Fontaine, N. and Thomasset, M. (1984): Phytate phoshorus utilization and intestinal phosphorus in pigs fed low phosphorus wheat or corn diets. Nutrition Reports International 29: 473-483.

Pointillart, A., Fontaine, N., Thomasset, M. and Jay, M.E. (1985): Phosphorus utilization, intestinal phosphatase and hormone control of calcium metabolism in pigs fed phytic phosphorus, soybean or rapeseed diets. Nutrition Reports International 32: 155-167.

Ponchon, G., Kennan, A.L. and DeLuca, H.F. (1969): "Activation" of vitamin D by the liver. J. Clin. Invest. 48: 2032-2037.

Ponchon, G. and DeLuca, H.F. (1969): Metabolites of vitamin D_3 and their biologic acitivity. J. Nutr. 99: 157-167.

Posternak, S. (1903): Compt. Rend. Soc. Biol. 55: (Cited from Johnson and Tate 1969).

Posternak, S. (1904): Bull. Soc. Chim. 33: 116 (Cited from Rose 1912).

Posternak, S. (1921): The synthesis of inosito-hexaphosphoric acid. Chem. Abstr. 15, 1891.

Posternak, T. (1965): The cyclitols, p.227, Holder Day, San Francisco, California.

Puschett, J.B., Fernandez, P.C., Boyle, I.T., Gray, R.W., Omdahl, J.L. and DeLuca, H.F. (1972): The acute renal tubular effects of 1,25-dihydroxycholecalciferol. Proc. Soc. Exp. Biol. Med. 141: 379-384.

Rackis, J.J. and Anderson, R.L. (1977): Mineral availability in soy protein products. Food Prod. Dev. 11: 38-44.

Raisz, L.G., Trummel, C.L., Holick, M.F. and DeLuca, H.F. (1972): 1,25-dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. Science 175: 768-769.

Ramakrishnan, C.V. and Bhandari, S.D. (1979): Diffential developmental pattern of acid and alkaline phosphatase activities in rat intestine. Experientia 35: 994-995.

Ramp, W.K., Toverud, S.U. and Gonnerman, W.A. (1974): Effects of cholecalciferol on bone formation and serum calcium, phosphate and magnesium in chicks. J. Nutri. 104: 803-809.

Ranhotra, G.S. (1972): Hydrolyusis during breadmaking of phytic acid in wheat protein concentrate. J. Food Sci. 37: 12 -

Rasmussen, H. and Bordier, R. (1974): In "physiological and cellular basis of metabolic bone disease". Williams and Wilkins, Baltimore, Maryland.

Rassmussen, H., Fontaine, O., Max, E. and Goodman, D.B.P. (1979): The effect of 1, -hydroxy vitamin D_3 administration on calcium transport in chick intestinal brush border membrane vesicles. J. Biol. Chem. 254: 2993-2999.

Rassmussen, H., Matsumoto, T., Fontaine, O. and Goodman, D.B.P. (1982): Role of changes in membrane lipid structure in the action of 1,25-dihydroxy vitamin D_3. Fed. Proc. 41: 72-77.

Reddy, B.S. and Wostmann, B.S. (1966): Intestinal disaccharidase activities in the growing germ-free and conventional rats. Arch. Biochem. Biophys. 113: 609-616.

Reddy, B.S. (1971): Calcium and magnesium absorption: Role of intestinal microflora. Fed. Proc. 30: 1815-1821.

Reddy, N.R. (1976): Milling and biochemical characteristics of triticale. J. Food Sci. 45: 1708-1713.

Reddy, N.R., Sathe, S.K. and Salunke, D.K. (1982): Phytate in legumes and cereals. Advances in Food Research, 28: 1-11.

Redhwi, A.A., Anderson, D.C. and Smith, G.N. (1982): A simple method for the isolation of vitamin D_3 metabolites from plasma extracts. Steriods, 39: 149-154.

Reinhold, J.G., Ismail-Beigi, F. and Faradji, B. (1975): Fibre vs phytate as determinant of the availability of calcium, zinc and iron of bread stuffs. Nutr. Rep. Int. 12: 75-83.

Reinhold, J.G., Faradji, B., Abadi, P. and Ismail-Beigi, F. (1976): Decreased absorption of calcium, magnesium, zinc, and phosphorus by humans due to increased fiber, and phosphorus consumption as wheat bread. J. Nutr. 106: 493-503.

Resurreccion, A.P., Juliano, B.O. and Tanaka, Y. (1979): Nutrient content and distribution in milling fractions of rice grain. J. Sci. Food Agric. 30: 475-481.

Reynolds, J.J., Holick, M.F. and DeLuca, H.F. (1973): The role of vitamin D metabolites in bone resorption. Calcif. Tissue Res. 12: 295-301.

Ribovich, M. and DeLuca, H.F. (1978): 1,25-dihydroxy vitamin D_3 metabolism. The effect of dietary calcium and phosphorus. Arch. Biochem. Biophys. 188: 164-171.

Roberts, A.H. and Yudkin, J. (1960): Dietary phytate as a possible cause of magnesium deficiency. Nature 185: 823-825.

Roberts, A.H. and Yudkin, J. (1961): Effect of phytate and other dietary factors on intestinal phytase and bone calcification in the rat. Br. J. Nutr. 15: 457-471.

Rose, A.R. (1912): A resume of the literature on inosite phosphoric acid, with special reference to the relation of the substance to plants. Biochem. Bull. 2: 21-32.

Rost, C.R., Bickle, D.D. and Kaplan, R.A. (1981): In vitro stimulation of 25-hydroxycholecalciferol 1 -hydroxylation by parathyroid hormone in chick kidney slices: Evidence for a role for adenosine 3,5-monophosphate. Endocrinology 108: 1002-1006.

Said, N.W., Sunde, M.L., Bird, H.R. and Stuttie, J.W. (1979): Raw phosphate as a phosphorus supplement for growing pullets and layers. Poult. Sci. 58: 1557-1563.

Salman, A.J., Ali, M.S. and McGinnis, J. (1969): Effect of level and sources of phosphorus and different levels on productivity and phosphorus utilization by laying hens. Poult. Sci. 48: 1004-1009.

Sandstrom, B., Arvidsson, B., Cederblad, A. and Bjorn-Rasmussen, E. (1980): Zinc absorption from composite meals. The significance of wheat extraction rate, zinc, calcium, and protein content in meals based on bread. Am. J. Clin. Nutr. 33: 739-745.

Samiy, A.H., Hirsch, P.F., Ramsay, A.G., Giordana, C. and Merrill, J.P. (1960: Localization of the renal tubular action of parathyroid hormone. Endocrinology 67: 266-269.

Samiy, A.H., Hirsch, P.F. and Ramsay, A.G. (1965): Localization of phosphaturic of parathyroid hormone in nephron of the dog. Amer. J. Physiol. 208: 73-77.

Schachter, D., Finkelstein, J.D. and Kowarski, S. (1964): Metabolism of vitam D. 1. preparation of radioactive vitamin D and its intestinal absorption in the rat. J. Clin. Invest. 43: 787-796.

Schulze, E. and Winterstein, E. (1896): Hoppe-Seyler's Z. Physiol. Chem. 40: 120 (Cited from Rose, 1912).

Scott, M.L., Butters, H.E. and Ranit, G.O. (1962): Studies on the requirements of young poults for available phosphorus. J. Nutr. 78: 223-230.

Scott, M.L., Nesheim, M.C. and Younge, R.J. (1982): In "nutrition of the chicken".

Sebrell, W.H. and Harris, R.S. (1954): In "The vitamins", Academic Press, 131-266.

Sedrani, S.H., Taylor, T.G. and Ahktar, M. (1981): The regulation of 25-hydroxycholecalciferol metabolism in the kidney of Japanese quail by sex hormones and by parathyroid extract. Gen. Comp. Endo. 44: 514-523.

Sedrani, S.H. (1984): Changes in serum levels of 1,35-dihydroxy vitamin D_3, calcium and phosphorus with age and vitamin D status in chickens. Brit. j. Nutr. 52: 329-334.

Simons, P.C.M. (1979): Calcium and phosphorus in poultry nutrition. 2nd Europen Symposium on Poultry Nutrition pay 51-59.

Simpson, R.H., Franceschi, R.T. and DeLuca, H.F. (1980): Characterization of a specific, high affinity binding macromolecule for 1,25-dihydroxy vitamin D_3 in cultured chick kidney cells. J. Biol. Chem. 255: 10160-10166.

Singsen, E.P., Matherson, L.D., Tlustohowicz, J.J. and Pudelkiewicz, W.J. (1969): Phosphorus in the nutrition of the adult hen. II. The relative availability of phosphorus from several sources for caged layers. Poult. Sci. 48: 387-393.

Soares, J.H. Jr., Swerdel, M.R. and Bossard, E.H. (1978): Phosphorus availability I. The effect of chick age and vitamin D_3 metabolites on the availability of phosphorus in defluorinated phosphate. Poult. Sci. 57: 1305-1312.

Sommerville, B.A., Fox, J. and Cave, A.D. (1978): The *in vitro* metabolism of 25-hydroxycholecalciferol by pig kidney_ effect of low dietary levels of calcium and phosphorus. J. Nutr. 40: 159-162.

Sommerville, B.A., Blahos, J., Harvey, S., Chadwick, A. and Spencer, G.S.G. (1985): The time sequence of adaptive changes to dietary phosphorus deficiency in the chick. Horm. Metabol. Res. 17: 247-250.

Sommerville, B.A., Maunder, E., Ross, R., Care, A.D. and Brown, R.C. (1985): Effect of dietary calcium and phosphorus depletion on vitamin D metabolism and calcium binding protein in the growing pig. Horm. Metabol. Res. 17: 78-81.

Spanos, E., Pike, J., Haussler, M., Colston, K., Evans, I., Goldner, M., McCain, T. and Macintyre, I. (1976): Circulating 1 ,25-dihydroxy vitamin D in the chicken: Enhancement by injection of prolactin and during egg laying. Life Sci. 19: 1751-1756.

Spencer, R., Charman, M., Wilson, P. and Lawson, E. (1978): The relationship between vitamin D-stimulated calcium transport and intestinal calcium-binding protein in the chick. Biochem. J. 170: 93-101.

Steele, T.H., Engle, J.E., Tankak, Y., Lorens, R.S., Dudgeon, D.L. and DeLuca, H.F. (1975): Phosphatemic action of $1,35(OH)_2D_3$. Am. J. Physiol. 229: 489-495.

Steenbock, H., Krieger, C.H., Wiest, W.G. and Pileggi, W.J. (1953): Vitamin D and intestinal phytase. J. Biol. Chem. 205: 993-999.

Steenbock, H. and Herting, D.C. (1955): Vitamin D and growth. J. Nutr. 57: 449-468.

Stumpf, W., Sar, M., Narbaitz, R., Reid, F., DeLuca, H.F. and Tanaka, Y. (1980): Cellular and subcellular localization of $1,25\text{-}(OH)_2$ - vitamin D_3 in rat kidney: Comparison with localization of parathyroid hormone and estradiol. Proc. Nat. Acad. Sci. 77: 1149-1153.

Suda, T., Hallick, R.B., DeLuca H.F. and Schnoes, H.K. (1970): 25-hydroxydihydrotachysterol-synthesis and biological activity. Biochemistry, 9: 1651-1657.

Sunde, M.L. and Bird, H.R. (1956): A critical need of phosphorus for the young pheasant. Poult. Sci. 35: 424-430.

Summers, J.D. and Moran, E.T. Jr. (1972): Interaction of dietary vanadium, calcium and phosphorus for the growing chicken. Poult.Sci. 51: 1760-1961.

Suzuki, V., Yoshimura, K. and Takaishi, M. Bull. Coll. Agric. Tokyo Imp. Univ. (1907), 7: 503. (Cited from Maga, J.A. 1982).

Swaminathan, R., Sommerville, B.A. and Care, A.D. (1978): Metabolism in vitro of 25-hydroxycholecalciferol in chicks fed on phosphorus deficient diets. Clin. Sci. Mol. Med.54: 197-200.

Talmage, R.V. (1969): Calcium homeostasis - calcium transort parathyroid action. The effects of parathyroid hormone on the movement of calcium between bone and fluid. Clin-Orthop. 67: 210-224.

Tanaka, Y. and DeLuca, H.F. (1971): Bone mineral mobilization activity of 1,25-dihydroxycholecalciferol. A metabolite of vitamin D. Arch. Biochem. Biophys. 146: 574-578.

Tanaka, Y. and DeLuca, H.F. (1973): The control of 25(OH)D_3 metabolism by inorganic phosphorus. Arch. Biochem. Biophys. 154: 566-574.

Tanaka, Y., Lorens, R. and DeLuca, H.F. (1975): The role of 1,25-dihydroxy vitamin D_3 and parathyroid hormone in the regulation of chick renal 25-hydroxy vitamin D_3 - 24-hydroxylase. Arch. Biochem. Biophys. 171: 521-526.

Tanaka, Y., Castillo, L. and DeLuca, H.F. (1976): Control of renal vitamin D hydroxylases in birds by sex hormones. Proc. Natl. Acad. Sci. 73: 2701-2705.

Tanaka, Y., Castillo, L., Wineland, M.J. and DeLuca, H.F. (1978): Synergistic effect of progesterone, testosterone, and estradiol in the stimulation of chick renal 25-hydroxy vitamin D_3 -1 - hydroxylase. Endocrinology 103: 2035-2039.

Tanaka, Y., DeLuca, H.F., Kobayashi, Y., Taguchi, T., Ikekawa, N. and Morisaki, M. (1979): Biological activity of 24,24-difluoro-25-hydroxy vitamin D_3. J. Biol. Chem. 254: 7163-7167.

Taylor, A.N. (1974): In vitro phosphate transport in chick ileum: Effect of cholecalciferol, calcium, sodium and metabolic inhibitors. J. Nutr. 104: 489-494.

Taylor, T.G. (1965): The availability of the calcium and phosphorus of plant materials for animals. Proc. Nutr. Soc. 24: 105-112.

Taylor, T.G. (1979): Availability of phosphorus in animal feeds: In recent advances in animal nutrition - pp.23135 (W. Haresign and D. Lewis, editors). London: Butterworths.

Taylor, T.G. and Coleman, J.W. (1979): A comparative study of the absorption of calcium and the availability of phytate-phosphorus in the golden hamster and the laboratory rat. Br. J. Nutr. 42: 113-119.

Taylor, T.G. and Dacke, C.G. (1983): Calcium metabolism and its regulation. Physio. Biochem. Domestic Fowl Vol. 5: 126-169.

Temperton, H.F. and Cassidy, J. (1964a): Phosphorus requirements of poultry. I. The utilization of phytin phosphorus by the chick as indicated by balance experiments. Br. Poult. Sci. 5: 75-80.

Temperton, H.F. and Cassidy, j. (1964b): Phosphorus requirements of poultry. II. The utilization of phytin phosphorus by the chick for growth and bone formation. Br. Poult. Sci. 5: 81-86.

Temperton, H.F., Dudley, J. and Pickering, G.J. (1965a): Phosphorus requirements of poultry. IV. The effects on growing pullets of feeding diets containing no animal protein or supplementary phosphorus. Br. Poult. Sci. 6: 125-133.

Temperton, H.F., Dudley, J. and Pickering, G.J. (1965b): Phosphorus requirements of poultry. V. The effects during the subsequent laying year of feeding growing diets containing no animal protein or supplementary phosphorus. Br. Poult. Sci. 6: 135-141.

Thompson, S.A. and Weber, C.W. (1981): Effect of dietary fiber on tissue mineral levels in chicks. Poult. Sci. 60: 840-845.

Tombs, M.P. (1967): Protein bodies of the soybean. Plant Physiol. 42: 797-813.

Trechsel, V., Bonjour, J.P. and Fleisch, H.J. (1979): Regulation of the metabolism of 25-hydroxy vitamin D_3 in primary cultures of chick kidney cells. J. Clin. Invest. 64: 206-217.

Tucker, G., Gagnon, R.E. and Haussler, M.R. (1973): vitamin D_3-25-hydroxylase tissue occurrence and apparent lack of regulation. Arch. Biochem. Biophys. 155: 47-57.

Turner, R.T., Avioli, R.C. and Bell, N.H. (1984): Extrarenal metabolism of 25-hydroxycholecalciferol in the rat: Regulation by 1,25-dihydroxycholecalciferol. Calc. Tiss. Int. 36: 274-278.

Tyler, C. (1946): Studies in the absorption and excretion of certain minerals by poultry. II. Changes in the composition of food material as it moves along the alimentary tract of the hen. J. Agric. Sci. 36: 275-282.

Vandepopuliere, J.M., Ammerman, C.B. and Harms, R.H. (1961): The relationship of calcium-phosphorus ratios to the utilization of plant and inorganic phosphorus by the chick. Poult. Sci. 40: 951-957.

Vohra, P., Gray, G.A. and Kratzer, F.H. (1965): Phytic acid-metal complexes. Proc. Soc. Exp. Biol. Med. 120: 447-449.

Waldroup, P.W., Ammerman, C.B. and Harms, R.H. (1963): The relationship of phosphorus, calcium and vitamin D in the diet of broiler-type chicks. Poult. Sci. 42: 982-989.

Waldroup, P.W., Ammerman, C.B. and Harms, R.H. (1964): The availability of phytic acid phosphorus for chicks. II. Comparison of phytic phosphorus sources. Poult. Sci. 43: 426-432.

Waldroup, P.W., Stearns, J.E., Ammerman, C.B. and Harms, R.H. (1965): Studies on the vitamin D_3 requirement of the broiler chick. Poult. Sci. 44: 543-547.

Waldroup, P.W., Simpson, C.F., Damron, B.L. and Harms, R.H. (1967): The effectiveness of plant and inorganic phosphorus in supporting egg production in hens and hatchability and bone development in chick embryos. Poult. Sci. 46: 659-664.

Waldroup, P.W., Mitchell, R.J. and Hazen, K.R. (1974): The phosphorus needs of finishing broilers in relationship to dietary nutrient density levels. Poult. Sci. 53: 1655-1663.

Walker, K.A. (1974): Changes in phytic acid and phytase during early development of phaseolus vulgaris. Planta 116: 91-98.

Walling, M.W. (1977): 1,25-dihydroxy vitamin D_3 and intestinal phosphate absorption. In: Vitamin D: Biochemical, Chemical and Clinical Aspects related to Calcium Metabolism. pp.321-330.

Wasserman, R.H. and Taylor, A.N. (1966): Vitamin D_3 induced calcium-binding protein in chick intestinal mucosa. Science 152: 791-793.

Wasserman, R.H. and Taylor, A.N. (1973): Intestinal absorption of phosphate in the chick: Effect of vitamin D_3 and other parameters. J. Nutr. 103: 586-599.

Wasserman, R.H. (1981): Intestinal absorption of calcium and phosphorus. Federation Proc. 40: 68-72.

Weber, J.C., Pons, V. and Kodicek, E. (1971): The localization of 1,25-dihydroxycholecalciferol in bone cell muclei of rachitic chicks. Biochem. J. 125: 147-153.

Weiser, M.M. (1973): Intestinal epithetial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation. J. Biol. Chem. 248: 2536-2541.

Weingartner, K.E. and Erdman, J.W. Jr. (1978): Bioavailability of minerals in human soybean foods. Illinois Research, 20: 4-10.

Welch, R.M., House, W.A. and Allaway, W.H. (1974): Availability of zinc from pea seeds to rats. J. Nutr. 104: 733-740.

Williams, R.B. (1972): Intestinal alkaline phosphatase and inorganic pyrophosphatase activities in the zinc-deficient rat. Br. J. Nutrition, 27: 121-130.

Williams, S.G. (1970): The role of phytic acid in the wheat grain. Plant Physiol. 45: 376-381.

Williams, P.J. (1984): Ph.D. Thesis, University of Southampton.

Williams, P.J. and Taylor, T.G. (1985): A comparative study of phytate hydrolysis in the gastro-intestinal tract of the golden hamster and laboratory rat. Bri. J. Nutr. 54: 429-435.

Wills, M.R. and Fairney, A. (1972): Effect of increased dietary phytic acid on cholecalciferol requirements in rats. Lancet, 2: 406-408.

Wise, A. and Gilburt, D.J. (1982): Phytate hydrolysis by germ-free and conventional rats. Applied and Envir. Microb. 43: 753-756.

Winterstein, E. (1897): Ber. Dtsch. Chem. Ges.30, 2299 (Cited from Cosgrove 1966).

Wozniak, L.A., Pensack, J.M., Stryeski, V. and Wilbur, R.D. (1977): Biological availability of feed grade phosphates using a corn-soybean meal basal diet. Poult. Sci. 56: 366-369.

Yamada, S., Ohmori, M. and Takayama, H. (1979): Synthesis of 24,24-difluoro-25-hydroxy-vitamin D_3. Tetrahedr on Lett. 21: 1859-1862.

Yoshida, T., Pleasants, J.R., Reddy, B.S. and Wostmann, B.S. (1968): Efficiency of digestion in germ-free and conventional rabbits. Br. J. Nutr. 22: 723-737.

Yoshida, T., Hirano, K., Kamizono, M. and Kuramasu, S. (1969): Nutrition of germ-free Japanese quail, on the ratio of retention of nitrogen, calcium, magnesium and phosphorus during the growth. Japan. J. Food Nutr. 22: 213-218.

Yoshida, T., Shinoda, S., Urano, T. and Maejima, K. (1981): Role of gastrointestinal microflora in nitrogen and mineral balances in young mice fed on autoclaved and irradiated diets. J. Nutr. Sci. Vitaminol, 27: 341-352.

Yoshida, T., Shinoda, S., Matsumoto, T., and Watarai, S. (1982): Comparison of mineral balances in germ-free and conventional mice when sodium phytate is added to purified diet. Agric. Biol. Chem. 46: 3093-3095.

Yoshida, T., Shinoda, S., Kawaai, V., Iwabuchi, A. and Mutai, M. (1985). a. The effect of gut flora on the utilization of Ca, P and Zn in rats fed a diet containing phytate. Agric. Biol. Chem. 49: 2199-2202.

Yoshiki, S., Yanagisawa, T., Suda, T. and Sasaki, S. (1974): The role of vitamin D in the mineralization of dentin in rats made rachitic by a diet low in calcium and deficient in vitamin D_3. Cal. Tiss. Res. 15: 295-302.