THE EFFECTS OF 24R, 25-DIHYDROXYVITAMIN D$_3$ AND 24S, 25-

DIHYDROXYVITAMIN D$_3$ ON PHOSPHATE TRANSPORT IN VIVO

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition

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UTAH STATE UNIVERSITY
Logan, Utah

2011

ABSTRACT

The Effects of 24R, 25-Dihydroxyvitamin D_3 and 24S, 25-Dihydroxyvitamin D_3 on

Phosphate Transport in Vivo

by

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Utah State University, 2011

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The steroid hormone 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] rapidly

stimulates the uptake of phosphate in isolated chick intestinal cells, while the steroid

24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] inhibits the rapid stimulation by

$1,25(OH)_2D_3$. Previous work in this laboratory has indicated that $24,25(OH)_2D_3$ inhibits

phosphate uptake in isolated intestinal cells and perfused duodenal loops. It is critical to

show this effect in the whole animal to determine the presence of any confounding

factors. Studies were therefore undertaken to determine if $24,25(OH)_2D_3$ had a similar

effect in vivo. $24,25(OH)_2D_3$ has two isomers which are 24R, 25dihydroxyvitamin D_3

[$24R,25(OH)_2D_3$] and 24S, 25dihydroxyvitamin D_3 [$24S,25(OH)_2D_3$]. We studied these

two isomers separately and tested them over a time course of 1, 5, 10, 15, and 18 h after

steroid using chicks on regular diet, but fasted, and chicks on a lower vitamin D diet. All

chicks were anesthetized prior to surgical exposure of the duodenal loop and injection of

a solution containing $H_3{}^{32}PO_4$ into the lumen. An initial time course study of phosphate

transport determined that 3 to 9 min of absorption in vivo was in a linear range, as judged by serum levels of radioactivity. Chicks were then injected with either 200 μg of 24R,25(OH)$_2$D$_3$, 20 μg of 24S,25(OH)$_2$D$_3$, or vehicle for control groups within the same time course studies. We found that the isomers had different effects on phosphate absorption. 24R,25(OH)$_2$D$_3$ had a hypophosphatemic effect in vivo. The serum levels of radionuclide revealed hypophosphatemic effects at 1, 5, 15, and 18 h time points with a decrease of 20%, 42%, 39%, and 43%, respectively, (P< 0.05) compared with controls; chicks raised on a low vitamin D diet also showed a decrease in phosphate absorption at 10 h time point by 33%. In contrast, 24S,25(OH)$_2$D$_3$ stimulated intestinal phosphate absorption at the 5-h time point by 64%, but had no other effects at the other time points tested. Because 24S,25(OH)$_2$D$_3$ was largely ineffective, dose-response studies were undertaken with only the 24R,25(OH)$_2$D$_3$ isomer. In comparing phosphate absorption in chicks fasted 18 h, and dosed with vehicle, 100 μg, 200 μg, or 300 μg of steroid 1 h prior to experimentation it was found that the lowest dose increased absorption to 99% of controls, while the 200 μg and 300 μg doses decreased phosphate absorption.

(48 pages)

PUBLIC ABSTRACT

The Effects of 24R, 25-Dihydroxyvitamin D_3 and 24S, 25-Dihydroxyvitamin D_3 on

Phosphate Transport in Vivo

This research studied the effects of the two isomers of $24,25(OH)_2D_3$ on phosphate absorption in vivo to increase our understanding of the effects of $24,25(OH)_2D_3$ on phosphate transport in vivo, and its two isomers. The current work demonstrates that $24R,25(OH)_2D_3$ is capable of decreasing phosphate absorption after a 1h injection in vivo and $24S,25(OH)_2D_3$ can increase phosphate absorption after a 5h injection, but this stimulation disappears at time points after 5h.

Since $24,25(OH)_2D_3$ is made in vitamin D-replete animals, the observation that $24R,25(OH)_2D_3$ decreases phosphate absorption suggests that over supplementation of feeds with vitamin D may be counterproductive. Whether or not the unexpected observation that $24S,25(OH)_2D_3$ briefly stimulates absorption off sets this inhibition is unknown. It is also likely that a lower level of supplementation with vitamin D would be adequate for bone growth. Thus, appropriate levels of vitamin D supplementation may result in more efficient utilization of phosphate in the diet, which would provide the benefits of reducing feed costs and reducing phosphorous excretion, thereby contributing to the sustainability of agriculture in the United States. Finally, these studies have implications for bone health in all animals, including humans to prevent rickets in children and osteoporosis in adults.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

Vitamin D is metabolized in the liver to 25-hydroxyvitamin D_3, and in the kidney to 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$]. $1,25(OH)_2D_3$ is well known for its hormonal activity in diverse cell types, and its ability to enhance intestinal calcium and phosphate absorption. By comparison $24,25(OH)_2D_3$ has not been as widely studied. However, a growing body of literature has indicated that $24,25(OH)_2D_3$ has important effects in development of bone mass. Somjen et al. [1983] reported that 24R, $25(OH)_2D_3$ but not 24S, $25(OH)_2D_3$ was active in bone while Ono et al. [1996] reported 24, $25(OH)_2D_3$ is found in fracture calluses in bone [Henry and Norman, 1978; Somjen et al., 1983; Ono et al., 1996].

One goal in poultry production is to increase the amount of meat on animals, which in turn requires sufficient support of bone structure. To date, strategies for improving bone strength have included increasing dietary calcium and phosphate, as well as vitamin D. Knowing the effects of $24,25(OH)_2D_3$ on phosphate and calcium absorption in vivo has great significance to approaching this goal. Previous work in our lab [Nemere, 1996a] indicated that $24R,25(OH)_2D_3$ inhibits the rapid stimulation of calcium and phosphate transport mediated by $1,25(OH)_2D_3$ in isolated chick intestinal cells and perfused duodenal loops. In addition Toffolon et al. [1975] found that $24,25(OH)_2D_3$ decreased calcium absorption in dogs and Maeda et al. [1987] reported that it caused hypocalcemia in rats. However the effects of $24,25(OH)_2D_3$ on phosphate transport in vivo remain unknown [Akiko et al., 1997].

The current study was undertaken to determine whether 24R, 25(OH)$_2$D$_3$ or 24S, 25(OH)$_2$D$_3$ alters phosphate transport in the whole animal and if there is any confounding factor present in vivo.

After establishing a time for linear transport in vivo, the experiments are designed in four parts.

#1 Chicks were raised on regular diet with variable times of fasting before experiments. They were then injected with 24R,25(OH)$_2$D$_3$ or 24S,25(OH)$_2$D$_3$ at different time points (1, 5, 10, 15, and 18 h) before experimentation and effects on phosphate transport analyzed.

#2 Chicks were raised on regular diet with a constant period of fasting before experimentation and they were then injected with 24R,25(OH)$_2$D$_3$ or 24S,25(OH)$_2$D$_3$ at different time points (1, 5, 10, 15, and 18 h) before experimentation and then the effects of the metabolites tested on phosphate transport.

#3 Chicks were raised on a low vitamin D diet without a constant 18 h of fasting before experiments. They were then injected with 24R, 25(OH)$_2$D$_3$ or 24S, 25(OH)$_2$D$_3$ at different time points (1, 5, 10, 15, and 18 h) before experimentation and then the effects of the metabolites tested on phosphate transport.

#4 After determining the optional time for observing steroid affects on phosphate transport, does-response studies were undertaken. Range finding were accomplished with does of 100, 200, and 300 μg 24R,25(OH)$_2$D$_3$.

CHAPTER II

LITERATURE REVIEW

Background

Vitamin D was discovered because of a disease named rickets in young animals; in adults this condition is known as osteomalacia. Professor Francis was the first to describe vitamin D deficiency as rickets in 1650. Rickets is now known as softening and weakening of the bone which results in leg bones bowing under the weight of children's upper torsos. This disease became the key to discovering vitamin D, which was considered a new nutritional factor [Hess, 1922; Mohr et al., 2008]. It was not initially realized that, unlike other vitamins, humans could synthesize vitamin D through exposure to UV light and did not require it in the diet, which meant vitamin D did not meet the definition of a vitamin. In addition, vitamin D metabolites can function through binding to specific protein receptors such as vitamin D receptor (VDR) or the $1,25D_3$-MARRS (membrane associated rapid response steroid binding) receptor to mediate calcium and phosphate transport. So $1,25(OH)_2D_3$ is more like a hormone rather than a vitamin.

Vitamin D is also named cholecalciferol in animals and ergocalciferol in plants and yeast in its inactive state. To become active cholecalciferol, it must be converted into $25(OH)D_3$ in the liver where it is hydroxylated at carbon-25. Then in the kidney, $25(OH)D_3$ is either hydroxylated at carbon-1 or carbon-24 to form the hormonally active secosteroids $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, respectively, to function at target organs such as bone, intestine and kidney. Figure 1 shows the feedback inhibition by 24, $25(OH)_2D_3$.
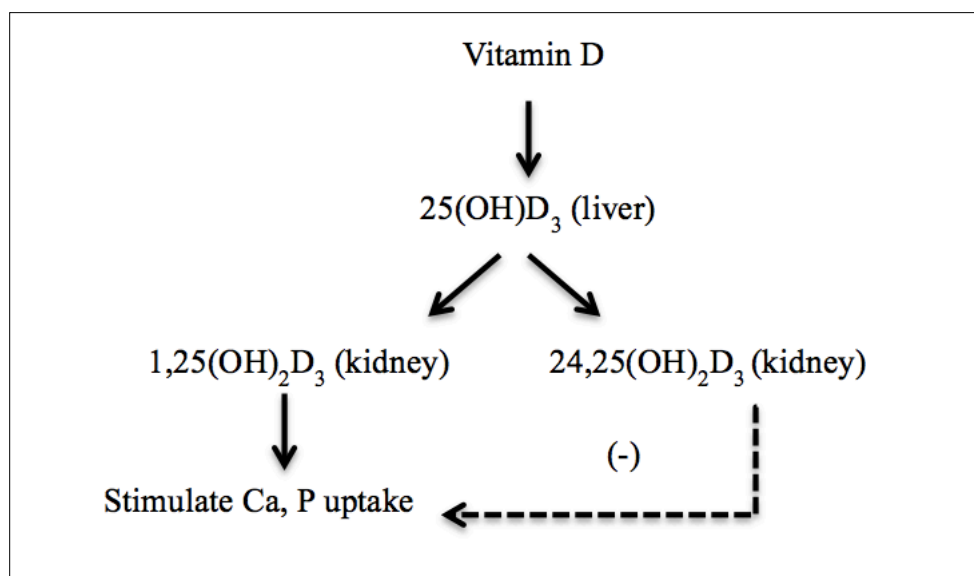
Fig. 1. 24, 25(OH)$_2$D$_3$ feedback loop.

Mechanisms of biological action of
vitamin D metabolites

The three active metabolites of vitamin D are 25(OH)D$_3$, 1,25(OH)$_2$D$_3$ and

24,25(OH)$_2$D$_3$ (see Table 1 below for summary of metabolites). They play an important

role in calcium and phosphate homeostasis in the body.

1,25(OH)$_2$D$_3$ is the most well studied metabolite of vitamin D. A major function

of 1,25(OH)$_2$D$_3$ is to enhance serum calcium and phosphate concentrations [Bachelet et

al., 1979]. This is accomplished by its action on three organ systems.

The first is intestine where it acts to enhance the dietary absorption of calcium and

phosphate [Corradino and Wasserman, 1968]. Second, in the kidney, it stimulates

reabsorption [Khanal and Nemere, 2007]. Third is in the bone to enhance the resorption

and storage of these two minerals. The production and degradation of 1,25(OH)$_2$D$_3$ are

processes regulated by feedback mechanisms resulting from ionic (Ca^{2+}, PO$_4^-$),

polypeptide hormones (PTH, calcitonin, FGF-23), and steroid factors [Bourdeau et al.,

1990; Rohe et al., 2007; Khanal and Nemere, 2008]. Nemere [1996b] has found that modulation of calcium and phosphate transport in the epithelia by $1,25(OH)_2D_3$ acts both through genomic and membrane-initiated actions. While the genomic regulation of phosphate homeostasis is mediated through the well-known vitamin D receptor (VDR) system; pre-genomic regulation involves the $1,25D_3$-MARRS receptor also identified as ERp57 and PDIA3 [Larsson and Nemere, 2003; Khanal et al., 2007; Tunsophon and Nemere, 2010]. In part, the nuclear action of $1,25(OH)_2D_3$ functions through the classical vitamin D receptor (VDR). VDR is a member of the nuclear receptor family of transcription factors.

Table 1. Vitamin D metabolite action summary

Metabolite	Site of activation	Proposed action	Reference
$25(OH)D_3$	Liver	Increased CA^{2+} uptake in rat and chick duodena	Olson and Deluca. 1969. 25-hydroxycholecalciferol; direct effect on calcium transport. Science. 165(891):405-7 Sterling and Nemere 2005 Seroids72:158-164
$1, 25(OH)D_3$	Kidney	Increased phosphate uptake and transport in intestine	Zhao and Nemere. 2002. $1,25(OH)D_3$-mediated phosphate uptake in isolated chick intestinal cells: effect of 24, $25(OH)D_3$, signal transduction activators, and age. J. Cell Biochem. 86(3): 497-508
$24,25(OH)D_3$	Kidney	Decreased phosphate uptake in presence of 1, $25(OH)D_3$ in intestine	Nemere. 1996a. apparent non-nuclear regulation of intestinal phosphate transport effects of 1,25-dihydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3, and 25-hydroxyvitamin D_3. Endocrinology. 137(6):2254-61

Upon activation by $1,25(OH)_2D_3$, the VDR forms a heterodimer with the retinoid-X receptor (RXR) and binds to vitamin D response elements (VDRE) on DNA resulting in expression or trans repression of specific gene products. These biological effects are achieved after a relatively long time.

Another receptor is the $1,25D_3$-MARRS protein which can mediate the rapid action of $1,25(OH)_2D_3$ [Nemere et al., 1994]. This separate membrane receptor was isolated from chick intestinal basal lateral membranes and was functionally linked to the rapid uptake of phosphate in intestinal cells by using ribozyme knockdown [Nemere et al., 2004.] A number of observations have contributed to the identification of a receptor different from the VDR. One of the observations is that vitamin D analogs that have limited ability to bind to the classical VDR can still stimulate the rapid actions analogous to those of $1,25(OH)_2D_3$ [Nemere and Norman, 1988]. Steroid hormone stimulated rapid uptake and transport of calcium and phosphate is mediated by the $1,25D_3$-MARRS protein. Receptor-ligand binding then activates the PKC pathway to stimulate phosphate uptake while the PKA pathway mediates calcium uptake [Nemere, 1996c; Rohe et al., 2005].

Functions attributed to $1,25(OH)_2D_3$ other than mineral ion homeostasis include regulation of gene transcription, promotion of cell proliferation, and stimulation of the immune system [Khanal and Nemere, 2009; Richard et al., 2010; Wu et al., 2010].

Olson and Deluca [1969] reported that $25(OH)D_3$ could increase calcium uptake in rat and chick duodena. Also Nemere [1996c] found that in perfused duodenal loops the mono-hydroxylated metabolite failed to stimulate phosphate transport at normal circulating levels and also abolished the stimulatory response to $1,25(OH)_2D_3$, while at 5-

fold lower than normal levels of $1,25(OH)_2D_3$ the appearance of ^{32}P in the venous effluent was stimulated. This may represent an adaption or protective mechanism for organisms experiencing vitamin D deficiency [Phadnis and Nemere, 2003].

The metabolite $24,25(OH)_2D_3$ is another active metabolite of vitamin D and is produced when an animal is calcium, phosphate, and vitamin D replete and represents an important feedback loop to provide a protective mechanism against excess $1,25(OH)_2D_3$ [Larsson et al., 1995]. Nemere [1996b] has found that $24, 25(OH)_2D_3$ suppresses a rapid action of $1,25(OH)_2D_3$ on phosphate and calcium transport in the chick intestine. At a cellular level, $24,25(OH)_2D_3$ has been found to inhibit the rapid membrane effects of $1,25(OH)_2D_3$ on opening of calcium channels in osteoblasts and osteosarcoma cells [Yukihiro et al., 1994; Khoury et al., 1995; Takeuchi and Guggino, 1996] and PKC activation in chick enterocytes [Nemere, 1999]. $24,25(OH)_2D_3$ has two isomers which are 24R, 25Dihydroxyvitamin D_3 and 24S,25-dihydroxyvitamin D_3. The physiological effect of the vitamin D metabolite 24R,25-dihydroxyvitamin D_3 on human osteoblastic cells was assessed. Physiological concentrations (10^{-9}-10^{-8} M) of 24R, $25(OH)_2D_3$ significantly increased the cyclic guanosine 5'-monophosphate (cGMP) content in the human osteoblastic cells by approximately 200% in 5 to 15 min. In contrast, 24S, 25-dihydroxyvitamin D_3 had only a weak effect on the cGMP content, and 1,25-dihydroxyvitamin D_3 did not affect the content [Yoshimoto and Norman, 1986]. These observations suggest that 24R, $25(OH)_2D_3$ has a unique activity of increasing cGMP content in osteoblastic cells, and that the increase in cGMP content may lead to the cooperative effect of 24R, $25(OH)_2D_3$ with 1, $25(OH)_2D_3$ on osteocalcin synthesis. These data support the hypothesis that 24R, $25(OH)_2D_3$ has a physiological role in human

bone and mineral metabolism.

Biological effect and mechanism of
24,25(OH)$_2$D$_3$ action

Evidence has shown that 24,25(OH)$_2$D$_3$ has distinctly different biological effects

and mechanisms from 1,25(OH)$_2$D$_3$. 24,25(OH)$_2$D$_3$ inhibits the rapid stimulation by

1,25(OH)$_2$D$_3$. Earlier work in the Nemere lab has indicated that a cellular binding protein

for 24,25(OH)$_2$D$_3$ is the enzyme the catalase [Larsson et al., 2006]. A functional

consequence of 24,25(OH)$_2$D$_3$ binding to catalase was found to be a decrease in enzyme

activity, with concomitant increase in H$_2$O$_2$ production [Nemere et al., 2006]. One

mechanism of inhibition was then found to be oxidation of the 1,25D$_3$-MARRS receptor

and inhibition of 1,25(OH)$_2$D$_3$ binding [Nemere et al., 2006]. Antioxidant diets nearly

doubled phosphate absorption in vivo [Nemere et al., 2006]. Peery and Nemere [2007]

then proved the hypothesis that anti-oxidant conditions, such as an anti-catalase antibody

or excess exogenous catalase protein, could block the inhibitory action of 24,25(OH)$_2$D$_3$,

whereas pro-oxidant conditions, such as a triazole inhibitor of catalase activity, could

mimic the inhibitory action of the secosteroid. It was further recognized that H$_2$O$_2$ could

also influence the signal transduction pathway beyond ligand binding to the 1,

25(OH)$_2$D$_3$-MARRS receptor [Peery and Nemere, 2007].

The model for the interaction between the 1,25D$_3$-MARRS receptor and catalase

is described in Fig. 2. Upon binding 1,25(OH)$_2$D$_3$, the 1,25D$_3$-MARRS receptor

dimerizes and increases signal transduction through PKA for enhanced calcium uptake

and PKC for enhanced phosphate uptake. Feedback inhibition of this stimulatory pathway

is initiated when 24,25$(OH)_2D_3$ binds to catalase, thereby decreasing enzymatic activity. The increased H_2O_2 levels are known to inhibit stimulated ion uptake and in this way the rapid stimulation by 1, 25$(OH)_2D_3$ is inhibited by 24, 25$(OH)_2D_3$.

Phosphate absorption and bone health

One of the most important roles of vitamin D is to maintain skeletal phosphate balance by promoting phosphate absorption in the intestines, promoting bone resorption by increasing osteoclast number, maintaining calcium and phosphate levels for bone formation, and allowing proper functioning of parathyroid hormone to maintain serum calcium and phosphate levels [Tatsuo, 2004; Adams and Hewison, 2010].
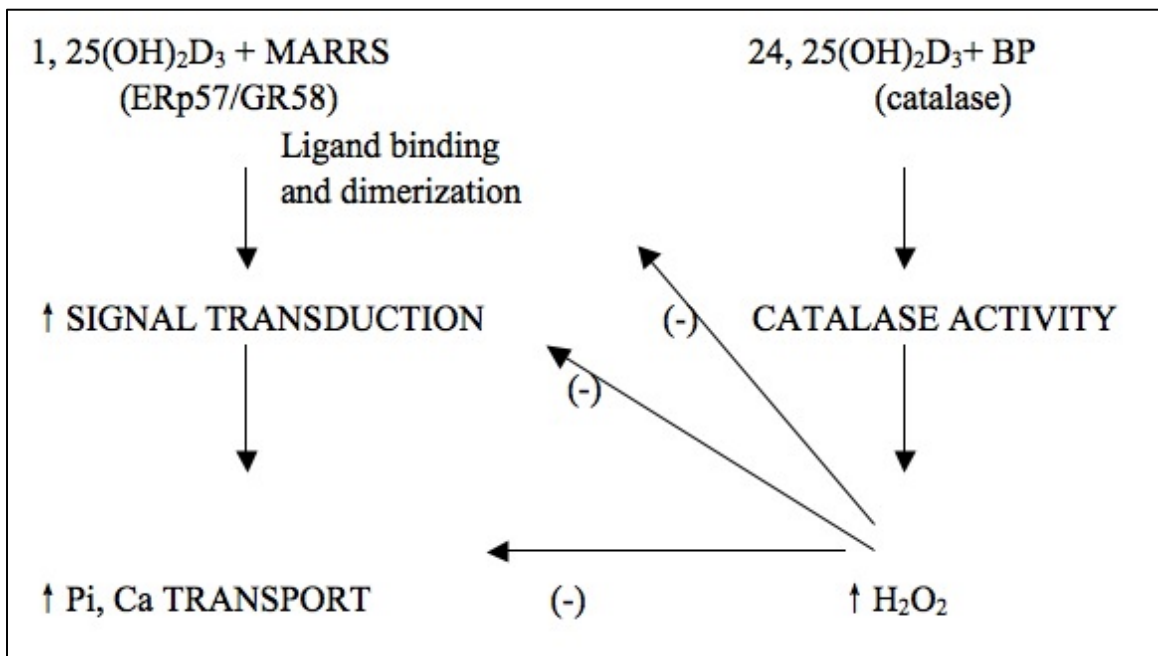
Fig. 2. Model of endocrine feedback of 24, 25$(OH)_2D_3$ on the rapid action of 1,25$(OH)_2D_3$.

The reason why vitamin D deficiency can result in lower bone mineral density and an increased risk of bone loss (osteoporosis) or bone fracture is because a lack of vitamin D alters mineral metabolism in the body [Lips, 2010]. This suggests that phosphate plays a very important role in bone health either in bone formation and bone mineral density.

Phosphates are normally absorbed from food and are important chemicals in the body. They are involved in cell structure, energy transport and storage, vitamin function, and numerous other processes essential to health [Holick, 2005]. White et al. [2011] tested the effect of oral phosphate and alendronate on bone mineral density when given as adjunctive therapy to growth hormone replacement (GHR) in adult growth hormone deficiency [Marry et al., 1976]. Phosphate and alendronate therapy given in combination with GHR confer an advantage in terms of bone mineral density (BMD) increase. Phosphate appears to exert its effect by increasing PTH target-organ action, whereas alendronate acts primarily through reduction in bone resorption [Nemere, 2007]. It was also recently reported that phosphorus has a unique role in bone formation and osteocyte maturation recently. In addition phosphate levels in blood are also very important to heart and kidney disease. And $24,25(OH)_2D_3$ as a hormone that mediates phosphate absorption is also important in bone health.

Hypothesis

The hypothesis to be tested is that $24,25(OH)_2D_3$ decreases phosphate transport in vivo. The experiments conducted to test the hypothesis:

1. Determining a period of linear phosphate absorption.

2. Determining phosphate absorption with variable times of fasting in chicks on a normal vitamin D diet.

3. Determining phosphate absorption after constant 18 h fasting in chicks on a normal vitamin D diet.

4. Determining phosphate absorption with chicks on a low vitamin D diet, no fasting.

5. Dose-response studies of $24R,25(OH)_2D_3$ on phosphate absorption with chicks on a regular diet but fasted.

Thesis Proposal

With sufficient study results suggesting that there are pregenomic actions of $24,25(OH)_2D_3$ in vitro, I tested the hypothesis that $24, 25(OH)_2D_3$ can also alter phosphate transport in the whole animal and if its two isomers $24R,25(OH)_2D_3$ and $24S,25(OH)_2D_3$ have the same effects on phosphate transport in vivo. The specific aims were as follows:

1. To determine the effect of $24R,25(OH)_2D_3$ on phosphate transport in chicks raised on regular diet with variable or constant times of fasting before experiments.

2. To determine the dose-response of $24R,25(OH)_2D_3$ on phosphate transport in chicks raised on regular diet with a constant interval of fasting before experiments

3. To determine the effect of $24R,25(OH)_2D_3$ on phosphate transport in chicks raised on low vitamin D diet with variable times of fasting before experiments.

4. To determine the effect of $24S,25(OH)_2D_3$ on phosphate transport in chicks raised on regular diet with a variable or a constant interval of fasting before experiments.

5. To determine the effect of $24S,25(OH)_2D_3$ on phosphate transport in chicks raised on low vitamin D diet with variable times of fasting before experiments.

CHAPTER III

MATERIALS AND METHODS

Animals and surgical procedures

Utah State University Institutional Animal Use and Care Committee approved all

procedures. White leghorn cockerels were obtained on the day of hatch (Privett Hatchery,

Portales, NM) and raised on a regular diet (800 IU vitamin D /kg) or low vitamin D diet

(200 IU vitamin D/kg) for 3 weeks prior to experimentation. On the day of use, chicks

were anesthetized with 0.3 ml Chloropent /100g of body weight (Fort Dodge

Laboratories, Fort Dodge, IA). The abdominal cavity was surgically opened and the

duodenal lumen injected with 1 ml Grey's Balanced Salt Solution (GBSS, 119 mM

NaCL, 4.96 mM KCl, 0.22 mM KH_2PO_4, 0.89 mM Na_2HPO_4, 1.03 mM $MgCl_26H_2O$,

0.28 mM $MgSO_4$ $7H_2O_2$, 0.9 mM $CaCl_2$ $2H_2O$ buffered to PH 7.4) mixed with 1µCi

$H_3{}^{32}PO_4$ (GBSS; $H_3{}^{32}PO_4$ 1000:1). An initial time course of phosphate transport

determined that 5 min transport period was within a linear range. After 5 min of

transport, blood was collected by decapitation before preparation of serum, and

radioactivity determined by liquid scintillation spectrophotometry.

In one set of experiments chicks were anesthetized with ether. Although no

significant differences were eventually found between results with ether or chloropent

anesthesia, ether was not used because of potential oxidant formation.

Time courses studies

The chicks were divided into three groups in each time course study. They were

injected with vehicle (1:1 ethanol: propanediol), 200 µg 24R,25(OH)$_2$D$_3$ [Maeda et al.,

1987] in vehicle, or 20 µg 24S,25(OH)$_2$D$_3$ [Ishizuka et al. 1984] in vehicle several hours

before experimentation. The extended time course points were 1, 5, 10, 15, and 18 h.

During these time points the chicks were fed water only (the chicks which were on

regular diet had an 18 h fast before experiments).

Effect of 24R,25(OH)$_2$D$_3$ or 24S,25(OH)$_2$D$_3$ on ^{32}P
uptake by intestine

After waiting for 5 min for phosphate transport, the blood was collected after

decapitation. The blood was collected in tubes, labeled, sealed and stored at 4°C

overnight for clot separation from serum. An aliquot of serum (200µl) was pipetted into

vials and scintillation fluid added. ^{32}P was then determined by using liquid scintillation

spectrophotometry.

Dose-response studies

Chicks were divided into four groups with a constant fasting period of 18 h. They

were injected with vehicle (1:1 ethanol: propanediol), 100 µg 24R,25(OH)$_2$D$_3$ in vehicle,

200 µg 24R,25(OH)$_2$D$_3$ in vehicle or 300 µg 24R,25(OH)$_2$D$_3$ in vehicle 1 hour before

experimentation.

Statistical analyses

Statistical comparisons between treatment groups were made using ANOVA.

Statistical significance was set at $P<0.05$. Results are expressed as Mean $\pm$ SEM for each

set of independent experiments. See Appendix B for a detailed summary of statistical

measures.

CHAPTER IV

RESULTS

Linear transport range (3-12 min)

Figure 3 shows the results of experiments designed to determine a suitable time

point for ^{32}P transport in intestine. Time course studies on ^{32}P uptake in chick intestine

were undertaken with a range of time points (3, 6, 9 and 12 min). The results show a

linear increase of ^{32}P in serum (R^2 =0.9757) from 3 to 9 min, reflecting absorption, as

judged by serum levels of radioactivity. At 12 min there was a decrease in ^{32}P level in

serum, most likely due to a decrease in available phosphate. Since phosphate uptake is

stably increasing from 3 to 9 min, 5 min was chosen as the phosphate transport time in

the subsequent experiments.

Phosphate absorption with variable times of
fasting in chicks on a normal vitamin D diet

Figure 4 illustrates the results of experiments designed to test whether

24R,25(OH)$_2$D$_3$ has a hypophosphatemic effect in vivo. In this series of experiments,

chicks were raised on regular diet for three weeks. Prior to the day of use, chicks (n=12)

in the control groups (absence of hormone) were injected with vehicle (1:1 ethanol:

propanediol) at selected hours before experimentation, and at the same time the chicks

(n=12) of the treated groups (with hormone) were injected with 24R,25(OH)$_2$D$_3$ in

vehicle. Before experimentation, chicks were only fed water. After the selected hours, all

chicks were anesthetized prior to surgical exposure of the duodenal loop and injection of

a solution containing H$_3$^{32}PO$_4$ into the lumen. According to the initial time course study,
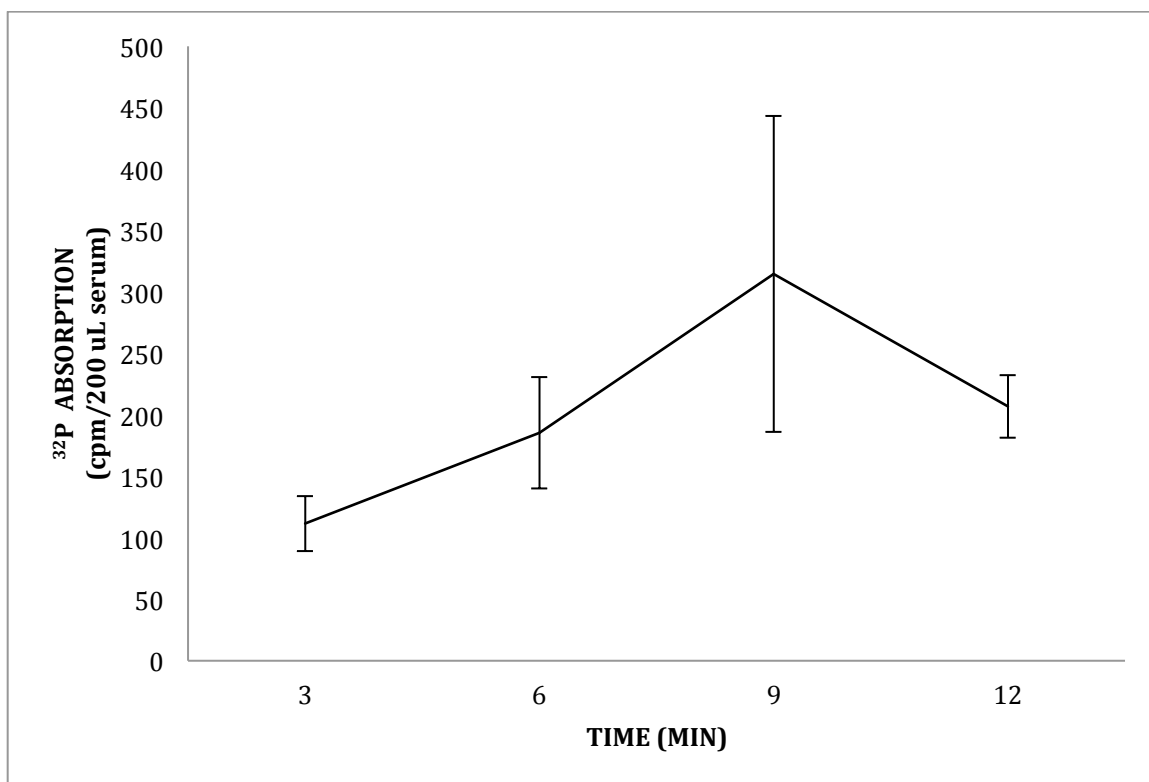
Fig. 3. Time course study of ^{32}P transport in chick intestine. 18 chicks were divided into four groups. They were anesthetized with 0.3 ml chloropent /100g of body weight. The abdominal cavity was surgically opened and the duodenal loop injected with 1 ml GBSS containing 1μCi of ^{32}P. After waiting for (1) T=3 min (n=6); (2) T=6 min (n=6); (3) T=9 min (n=6); (4) T=12 min (n=3), blood was collected by decapitation. Serum from the samples was obtained after storage at 4 C for several hours to allow clot formation and radioactivity determined by liquid scintillation spectrophotometry. Data are presented as mean ± SEM.

after 5min of transport, serum levels of radioactivity were used to determined phosphate

absorption.The results show 1 h and 5 h after injection of 24R,25(OH)$_2$D$_3$ there was no

difference in phosphate absorption between the control and treated groups. A slight

inhibition was found at the10-hour time point; and a dramatic response was obtained at

15 and 18 h (Fig. 4) after steroid. Statistical comparison by ANOVA confirmed that

phosphate absorption in chick intestine was not different between control and treated

groups at 1, 5, and 10 time points, but at the 15- and 18-hour time points phosphate

transport was significantly decreased by $24,25(OH)_2D_3$ (P<0.05) by 33% and 25%, respectively. Figure 4 further reveals that variable fasting times had an effect on phosphate absorption, since the control group of 15 h was 2990.8 ± 431.6 cpm/200μl serum while the 1 h was only 1184.7 ± 121.9 cpm/200μl serum, suggesting that food consumption in general maybe a confounding factor for phosphate transport in vivo.

Figure 5 depicts the effects of $24S,25(OH)_2D_3$ on phosphate transport in vivo with variable fasting times. Conditions were the same as Fig. 4, but instead of injecting $24R,25(OH)_2D_3$, $24S,25(OH)_2D_3$ was injected prior to experimentation. No differences
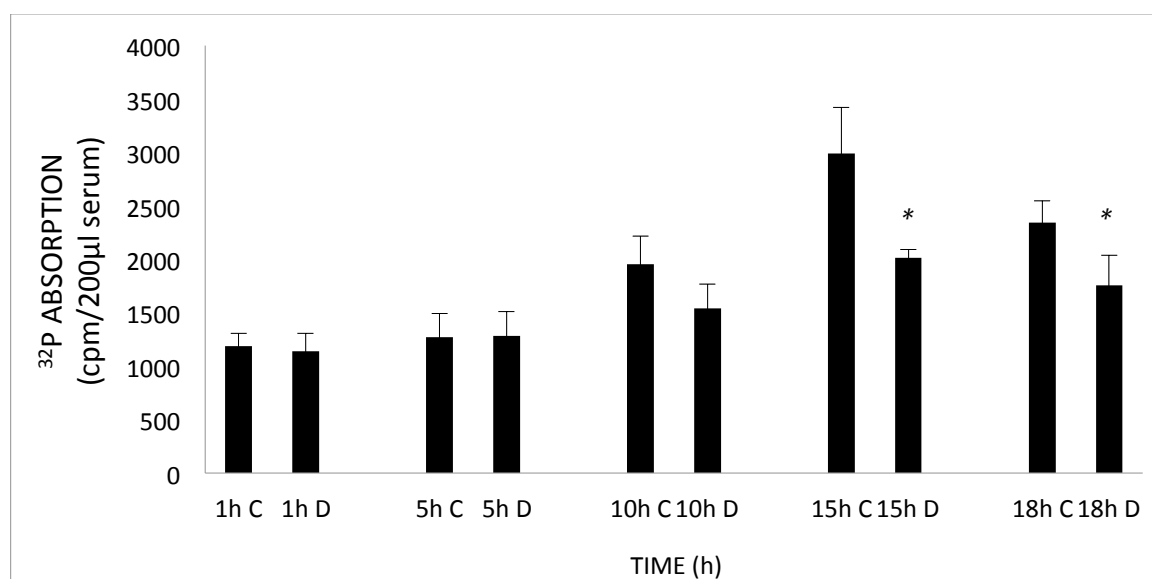
Fig. 4. Time course of $24R,25(OH)_2D_3$ effect on ^{32}P transport in chick intestines with variable times of fasting. Chicks were divided into five groups, 24 in each group and each group further divided into two groups, control (C, n=12) and treated (D, n=12). They were raised on a regular diet (800 IU vitamin D /kg) generally for 3 weeks prior to experimentation. On the day of use, chicks in control groups were injected with vehicle at the indicated hours before experimentation and at the same time the chicks of the treated groups injected with $24R,25(OH)_2D_3$ in vehicle. After selected hours (1, 5, 10, 15, and 18h) of fasting, they were anesthetized with a 0.3 ml chloropent /100g of body weight. The abdominal cavity was surgically opened and the duodenal loop injected with1 ml GBSS containing 1μCi of ^{32}P. After 5min, blood was collected by decapitation. Serum from the samples was obtained after storage at 4 C for several hours to allow clot formation and radioactivity determined by liquid scintillation spectrophotometry. Data are presented as mean $\pm$ SEM. C, vehicle control; D, 200μg $24R,25(OH)_2D_3$.

were observed between control and treated groups at any of the time points (P>0.2). Also,

in this figure, it is evident that there was a large range in phosphate absorption from 1 h

to 15 h, which further indicates that diet is one of the confounding factors in phosphate

transport. General nutrient uptake may affect gastrointestinal hormones (GI hormone),

which may increase the phosphate uptake. The increase in phosphate absorption in

controls at the later time points may be due to an increase in transporters at the brush

border surface.

Fig. 5. Time course of 24S,25(OH)$_2$D$_3$ effect on ^{32}P transport in chick intestines with variable times of fasting. Chicks were divided into two groups, control and treated, 60 in each group and each group further divided into four time groups, 1h (n=3), 5 (n=3), 10 (n=30), 15 (n=30). They were raised on a regular diet (800 IU vitamin D /kg) generally for 3 weeks prior to experimentation. On the day of use, chicks in control groups were injected with vehicle at the indicated times before experimentation, and at the same times the chicks of the treated groups were injected with 24S,25(OH)$_2$D$_3$ in vehicle. After selected hours (1, 5, 10, and 15 h) of fasting, they were anesthetized with 0.3 ml chloropent /100g of body weight. The abdominal cavity was surgically opened and the duodenal loop injected with1 ml GBSS containing 1μCi ^{32}P. After 5 min, blood was collected by decapitation. Serum from the samples was obtained after storage at 4 C for several hours to allow clot formation and radioactivity determined by liquid scintillation spectrophotometry. Data are presented as mean $\pm$ SEM. C, vehicle control; D, 20μg 24S,25(OH)$_2$D$_3$.

Phosphate absorption after constant 18 h fasting
in chicks on a normal vitamin D diet

Clearly the variable times of fasting had an impact on phosphate absorption (Figs.

4, 5). To exclude this confounding factor shown in Fig. 4 and Fig. 5, chicks in the next

series of experiments were raised on regular diet for three weeks but prior to use, they

were all fasted for 18 h before experimentation. In these studies, chicks were injected

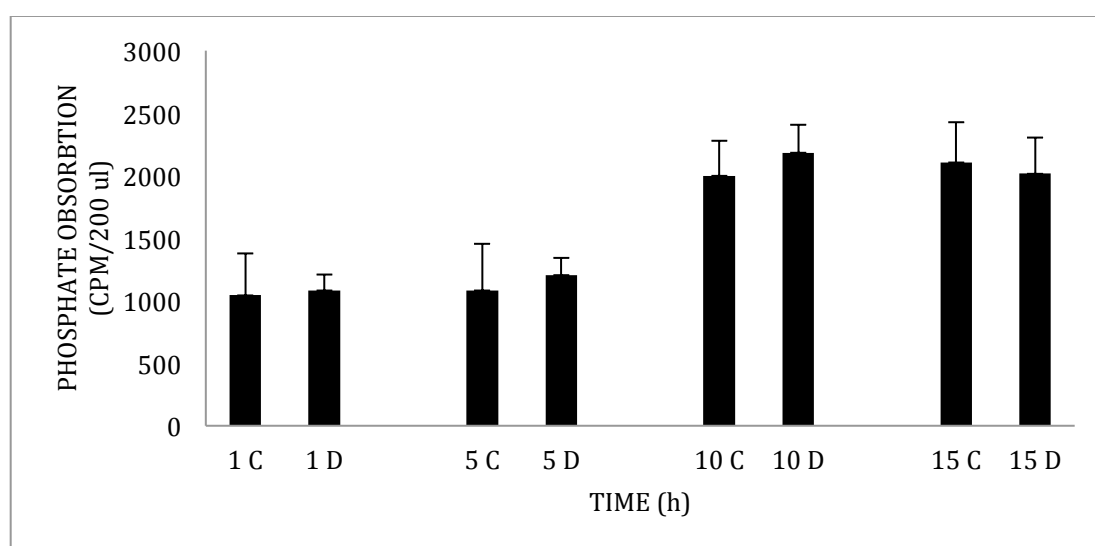with 24R,25(OH)$_2$D$_3$ or vehicle.

Fig. 6. Time course of 24R,25(OH)$_2$D$_3$ effect on ^{32}P transport in chick intestines with a constant 18 hours of fasting. Chicks were divided into six groups, control (n=17), 1h (n=7), 5 h (n=10), 10h (n=9), 15h (n=13) and 18h (n=15). They were raised on a regular diet (800 IU vitamin D /kg) for 3 weeks prior to experimentation. On the day of use, chicks in control groups were injected with vehicle 18 hours before experimentation at the same time the chicks of the treated groups began fasting and were served only water. At the indicated hours before experimentations chicks of the treated groups were injected with 24R,25(OH)$_2$D$_3$ in vehicle. After selected hours (1, 5, 10, 15 and 18 h) they were anesthetized with 0.3 ml /100g of body weight chloropent. The abdominal cavity was surgically opened and injected with1 ml GBSS containing 1μCi ^{32}P in the duodenal loop. After 5 min of absorption, blood was collected by decapitation. Serum from the samples was obtained after storage at 4 C for several hours and radioactivity determined by liquid scintillation spectrophotometry. Data are presented as mean $\pm$ SEM. *P<0.05, relative to vehicle controls.

Compared with Figs. 4 and 5, the range of ^{32}P absorption is considerably less than that depicted in Fig. 6. In addition, the 1 h and 5 h steroid treated groups also showed a significant decrease (P<0.02) in phosphate transport relative to controls, instead of only at 15 h and 18 h. At 10 h 24R,25(OH)$_2$D$_3$ again failed to inhibit phosphate transport even after 18 h of constant fasting before experimentation. As seen previously (Fig. 4), however, 24R,25(OH)$_2$D$_3$ inhibited phosphate absorption at the 15- and 18-h time points (Fig. 6) .
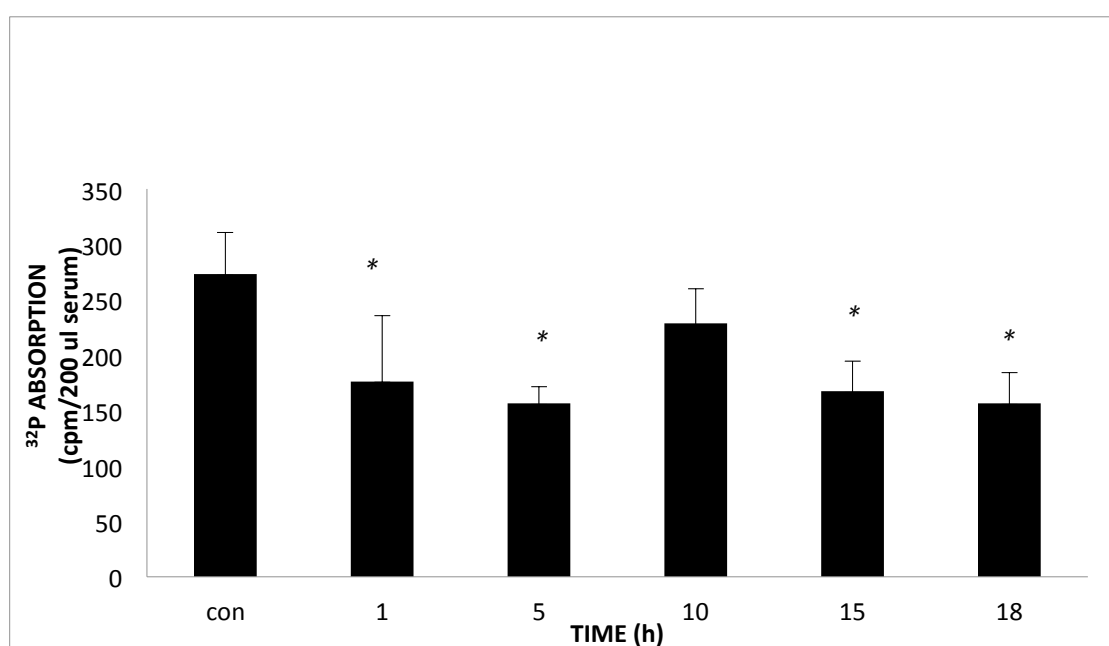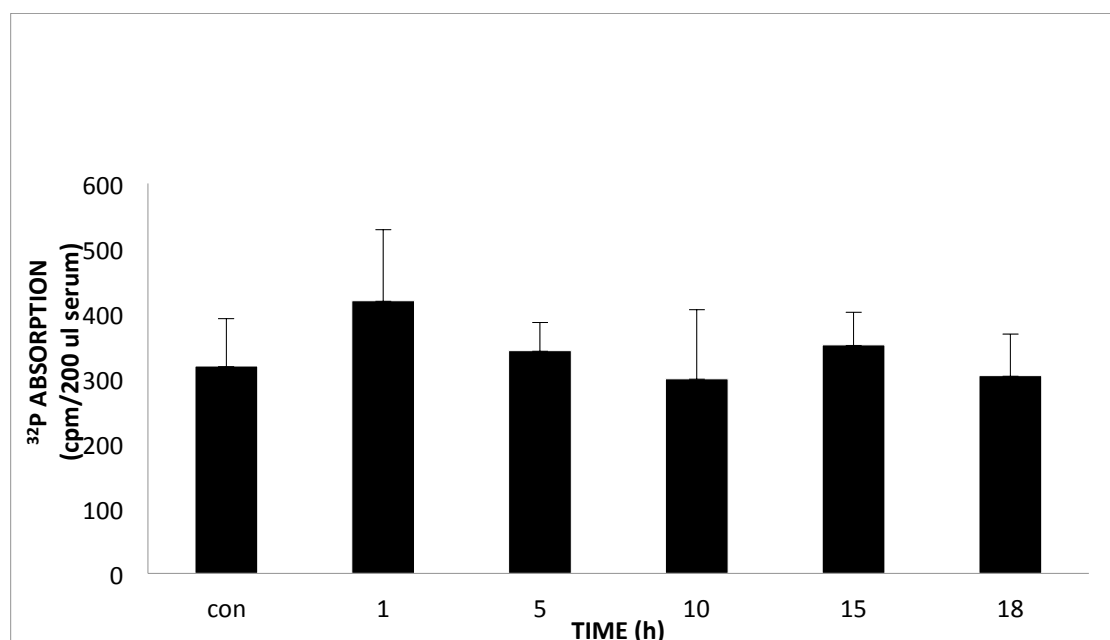

Fig. 7. Time course of 24S,25(OH)$_2$D$_3$ effect on ^{32}P transport in chick intestines with constant 18 hours of fasting. Chicks were divided into six groups, control (n=12), 1 h (n=9), 5 h (n=10), 10 h (n=8), 15 h (n=8) and 18 h (n=8). They were raised on a regular diet (800 IU vitamin D /kg) for 3 weeks prior to experimentation. On the day of use, chicks in control groups were injected with vehicle 18 h before experimentation and at the same time the chicks of the treated groups began fasting and were only fed on water. At selected hours before experimentation chicks of the treated groups were injected with 24S,25(OH)$_2$D$_3$ in vehicle. After selected hours (1, 5, 10, 15, and 18 h) they were anesthetized with 0.3 ml chloropent /100g of body weight. The abdominal cavity was surgically opened and the duodenal lumen injected 1 ml GBSS containing 1μCi ^{32}P. After 5min of absorption, blood was collected by decapitation. Serum from the samples was obtained after storage at 4 C for several hours and radioactivity determined by liquid scintillation spectrophotometry. Data are presented as mean $\pm$ SEM.

In Fig. 7, a slight stimulation by $24S,25(OH)_2D_3$ was found in the 1 h and 15 h groups but this was still not significant when compared by ANOVA (P>0.05). After 18 h constant fasting in chicks, $24S,25(OH)_2D_3$ also failed to alter in phosphate transport in vivo.

Dose-response studies of $24R,25(OH)_2D_3$

After 18 h constant fasting in chicks, $24S,25(OH)_2D_3$ failed to alter phosphate transport in vivo (Fig. 7) whereas $24R,25(OH)_2D_3$ decreased phosphate transport 1, 5, 15, and 18 h after injection (Fig. 6). After determining the optimal time for observing steroid affects on phosphate transport, dose-response studies of $24R,25(OH)_2D_3$ were undertaken. Chicks were injected with vehicle (1:1 ethanol: propanediol), 100 μg $24R,25(OH)_2D_3$ in vehicle, 200 μg $24R,25(OH)_2D_3$ in vehicle or 300 μg $24R,25(OH)_2D_3$ in vehicle respectively 1 h before experimentation, and phosphate absorption was determined from serum by liquid scintillation spectrophotometry. Results are shown in Fig. 8. ANOVA confirmed that the 100 μg group exhibited an increased phosphate absorption compared with control group (P<0.05). In the 100 μg group, phosphate absorption increased by 99% compared with the chicks in control group. The stimulation may be because lower level of $24R,25(OH)_2D_3$ could affect Parathyroid hormone (PTH) secretion [Nemere, 1996b] which further stimulates phosphate absorption.
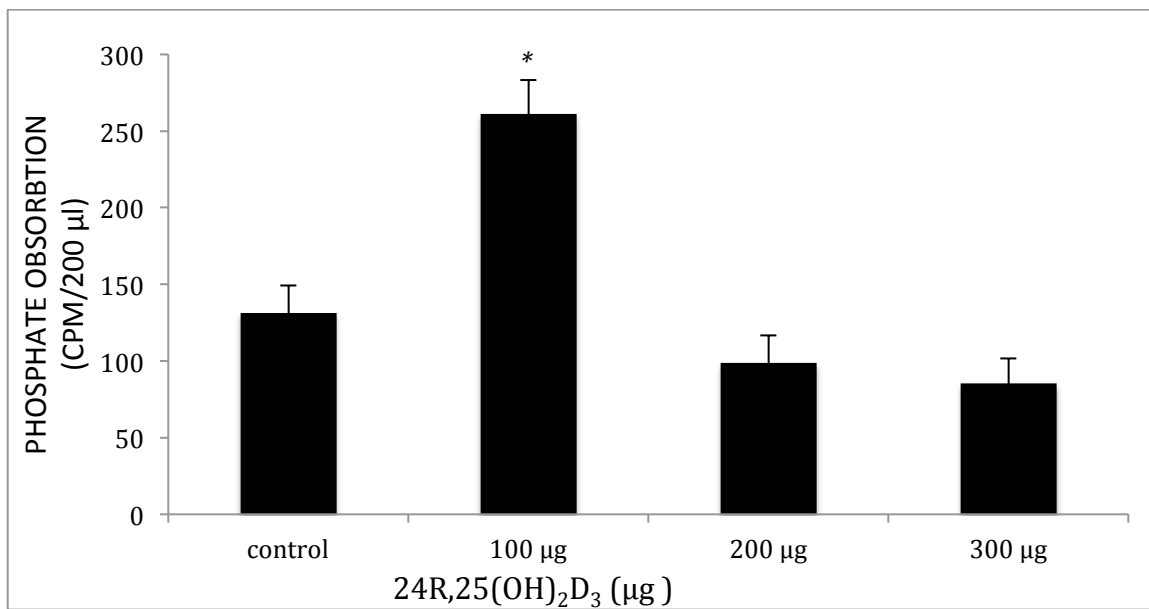
Fig. 8. Dose-response study of 24R,25(OH)$_2$D$_3$ effect on ^{32}P transport in chick intestines with a constant 18 hours of fasting. Chicks were raised on a regular diet (800 IU vitamin D /kg) for 3 weeks prior to experimentation. Chicks were divided into four groups, control (n=5), 100 µg (n=3), 200 µg (n=3) and 300 µg (n=). They were injected with vehicle (1:1 ethanol: propanediol), 100 µg 24R,25(OH)$_2$D$_3$ in vehicle, 200 µg 24R,25(OH)$_2$D$_3$ in vehicle or 300 µg 24R,25(OH)$_2$D$_3$ in vehicle, respectively, 1 h before experimentation. On the experimentation they were anesthetized with 0.3 ml chloropent /100g of body weight. The abdominal cavity was surgically opened and injected with1 ml GBSS containing 1µCi ^{32}P in the duodenal loop. After 5min of absorption, blood was collected by decapitation. Serum from the samples was obtained after storage at 4 C for several hours and radioactivity determined by liquid scintillation spectrophotometry. Data are presented as mean $\pm$ SEM. *P<0.05, relative to vehicle controls.

Phosphate absorption with chicks on a low
vitamin D diet, no fasting

To further exclude the diet's effect on phosphate absorption, chicks in this series

of experiments were raised on a low vitamin D diet for 3 weeks before the experiments

with no fasting before experimentation. Previous experiments showed 24R,25(OH)$_2$D$_3$

has a hypophosphatemic effect in vivo after 1, 5, 15, and 18 h following injection with 18

h constant fasting before experimentation. Figure 9 shows the results of absorption in

chicks raised on a low vitamin D diet, and ANOVA confirmed that the 1-h, 5-h, 10-h, and

15-h groups all exhibited a decreased phosphate absorption compared with control groups (P<0.02). In the 1-h group, phosphate absorption decreased by 50% compared with the chicks that were raised on regular diet in which phosphate transport decreased by 30%. However the latter decrease was not significantly different than control, which indicates that the vitamin D level in the diet is one of the confounding factors that may affect phosphate transport in vivo.

Fig. 9. Time course of 24R,25(OH)$_2$D$_3$ effect on ^{32}P transport in chick intestines in chicks raised on a low vitamin D diet but not fasted. Chicks were divided into six groups, control (n=12), 1 h (n=6), 5 h (n=9), 10 h (n=9), 15 h (n=10) and 18 h (n=8). They were raised on a low vitamin D diet (200 IU vitamin D /kg) for 3 weeks prior to experimentation. On the day of use, chicks of control groups were injected with vehicle 18 h before experimentation. At selected hours (1, 5, 10, 15, and 18 h) before experimentation chicks of the treated groups were injected with 24R,25(OH)$_2$D$_3$ in vehicle. On the day of experimentation they were anesthetized with 0.3 ml chloropent /100g of body weight. The abdominal cavity was surgically opened and injected with 1 ml GBSS containing 1μCi ^{32}P into the lumen of duodenal loop. After 5min, blood was collected by decapitation. Serum from the samples was obtained after storage at 4 C for several hours and radioactivity determined by liquid scintillation spectrophotometry. Data are presented as mean ± SEM. * P<0.05, relative to vehicle controls.

Figure 10 illustrates the results of comparable experiments with chicks fed a low vitamin D diet, and injected with 24S,25(OH)$_2$D$_3$ or vehicle. 24S,25(OH)$_2$D$_3$ stimulated the phosphate transport at the 5-h time point after injection (P<0.05), but no significant differences were observed at the other time points.
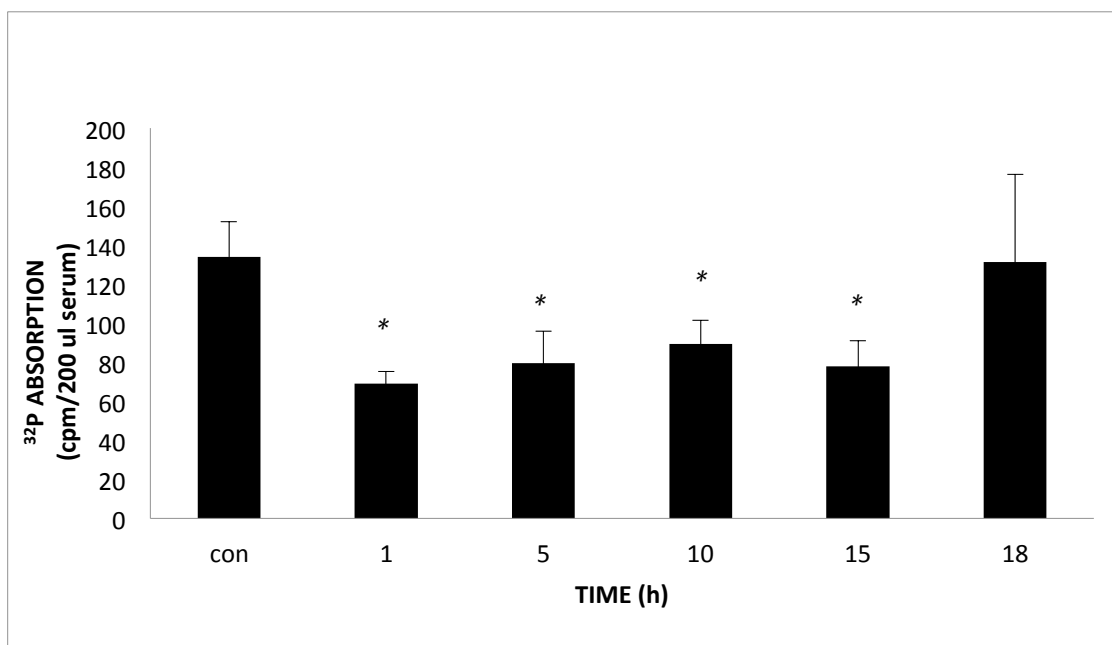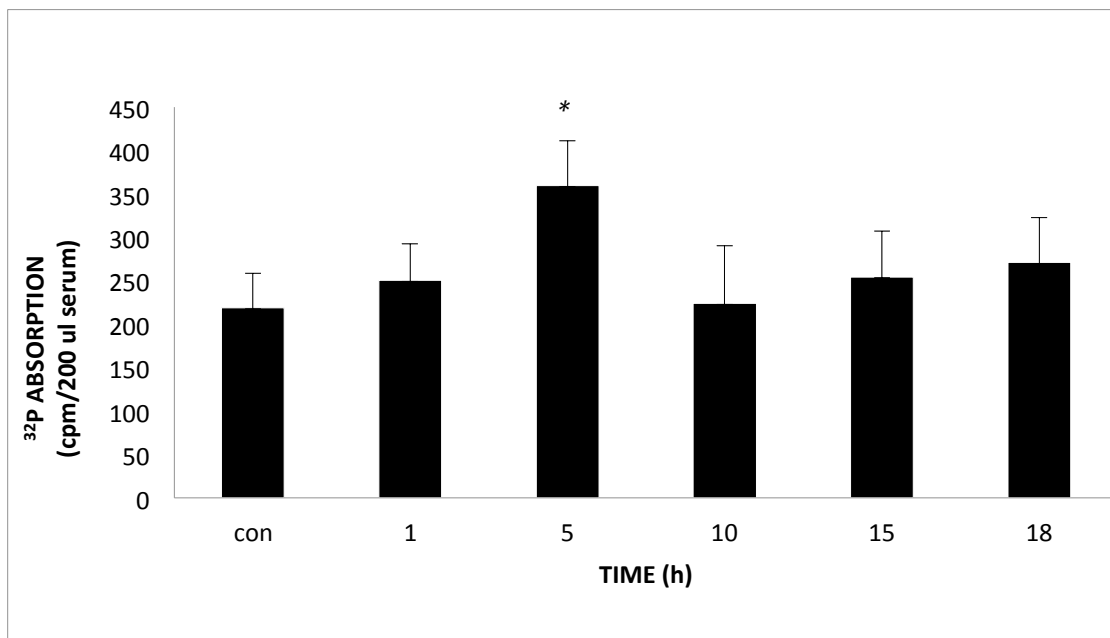

Fig. 10. Time course of 24S,25(OH)$_2$D$_3$ effect on ^{32}P transport in chicks raised on a low vitamin D diet but not fasted. Chicks were divided into six groups, control (n=9), 1 h (n=9), 5 h (n=9), 10 h (n=8), 15 h (n=9) and 18 h (n=10). They were raised on a low vitamin D diet (200 IU vitamin D /kg) generally for 3 weeks prior to experimentation. On the day of use, chicks of control groups were injected with vehicle 18 h before experimentation. And the treated groups were injected with 24S,25(OH)$_2$D$_3$ in vehicle1, 5, 10, 15, and 18 h before experimentation. After selected hours (1, 5, 10, 15, and 18 h), they were anesthetized with a 0.3 ml chloropent /100g of body weight. The abdominal cavity was surgically opened and injected with 1 ml GBSS containing 1µCi of ^{32}P into the lumen of duodenal loop. After 5 min, blood was collected by decapitation. Serum from the samples was obtained after storage at 4 C for several hours and radioactivity determined by liquid scintillation spectrophotometry. Data are presented as mean ± SEM. * P<0.05, relative to vehicle controls.

CHAPTER V

DISSCUSSION

The current work demonstrates that $24R,25(OH)_2D_3$ is capable of decreasing phosphate absorption after a 1-h injection in vivo and $24S,25(OH)_2D_3$ can increase phosphate absorption after a 5-h injection, but this stimulation disappears at the time points tested after 5 h.

In previous reports, the enzyme catalase was proved to be a cellular binding protein for $24,25(OH)_2D_3$ [Larsson et al., 2006]. Catalase activity could be inhibited by $24,25(OH)_2D_3$ and production of H_2O_2 increased in intestinal cells [Nemere et al., 2006]. Hydrogen peroxide, in turn, oxidizes thiols within the $1,25D_3$-MARRS receptor to decrease binding of $1,25(OH)_2D_3$, and also inhibit the rapid stimulation of the protein kinase C signaling pathway [Peery and Nemere, 2007].

In the present study, it was found that the two isomers of $24,25(OH)_2D_3$ had different effects on phosphate transport in vivo suggesting that the inhibitory effect might be mainly performed by $24R,25(OH)_2D_3$ while $24S,25(OH)_2D_3$ might have another receptor or in some manner stimulate other pathways to mediate phosphate absorption in vivo. Somjen et al. [1983] reported that $24R,25(OH)_2D_3$ but not $24S,25(OH)_2D_3$ was active in bone. However, in this work, $24S,25(OH)_2D_3$ was found to be active in chick intestine in vivo and increased phosphate transport at 5h but this effect disappeared at 10 h and later time points.

Another observation is $24R,25(OH)_2D_3$ decreased phosphate absorption after 1 h indicating that the phosphate inhibition might not occur because of $24R,25(OH)_2D_3$ binding to catalase or blocking the $1,25D_3$-MARRS receptor. Previous work in the

perfused duodenal loop system proved that the antagonism of the stimulatory effect of $1,25(OH)_2D_3$ by $24,25(OH)_2D_3$ was seen 10 min after the onset of perfusion [Nemere, 1996b]. There might be a separate nuclear receptor for $24,25(OH)_2D_3$ which could induce genomic change in cells resulting in a decreased absorption of phosphate after 1 h. Another mechanism maybe catalase binding to STAT3 [Ndubuisi et al., 1999]. In addition, this effect disappeared after 18 h. Alternatively, $24R,25(OH)_2D_3$ might mediate the production of separate hormones that regulate phosphate absorption, such as fibroblast growth factor-23 (FGF 23) which is a recently identified member of the FGF family involved in phosphate homeostasis [Razzaque et al., 2005].

Early work with isolated cells or perfused duodenal loops could not completely apply in vivo because of potential confounding factors that have yet to be determined. In the current work using individual chicks and studies of the effects of $24,25(OH)_2D_3$ in vivo, we found that the duration of fasting and vitamin D level in the diet were two factors that had a major influence on phosphate absorption. The chicks fed on a low vitamin D diet showed more significant decreases in phosphate transport than chicks fed on regular diet. While chicks on the regular diet but with different fasting times had a large range of serum phosphate values between each time point. The greater phosphate absorption could be due to a generalized increase in nutrient absorption, in which phosphate is bound to other components. Another possibility could be due to the release and action of GI hormones. Also, the dose response experiment suggested low level of $24R,25(OH)_2D_3$ could stimulate phosphate absorption while a higher level could not increase phosphate absorption but decreased it instead. The stimulation could due to a low level of $24R,25(OH)_2D_3$ that may increase PTH secretion which led to increasing

phosphate absorption in vivo [Nemere and Szego, 1981a; Nemere and Szego, 1981b]. As noted earlier, the higher cpms at longer fasting times might be due to increased phosphate transporters appearing at the brush border membrane.

This research increases our understanding of the effects of $24,25(OH)_2D_3$ on phosphate transport in vivo, and its two isomers. By understanding the action of these two isomers, progress will be made in enhancing phosphate absorption in production animals to supply the minerals for adequate bone growth. Moreover, more efficient utilization of phosphate in the diet will provide the benefits of reducing feed costs and reducing phosphorous excretion, thereby contributing to the sustainability of agriculture in the United States. Also these studies have implications for bone health in all animals, including humans to prevent rickets in children and osteoporosis in adults.

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APPENDICES

Appendix A. Abbreviations

μCi……………………..Microcurie, a term used to quantify radioactivity

μg………………………Micrograms

μl……………………….Microliter

$1,25(OH)_2D_3$…..………..1, 25-dihydroxyvitamin D_3

$1,25D_3$-MARRS………..1,25-dihydroxyvitamin D_3 membrane-associated, rapid-response
receptor   steroid-binding protein

$24R,25(OH)_2D_3$…..…….24R, 25-dihydroxyvitamin D_3

$24S,25(OH)_2D_3$…..……..24S, 25-dihydroxyvitamin D_3

$25(OH)_2D_3$…..………….25-hydroxyvitamin D_3

^{32}P……………………….Radioisotope of phosphorous from abbreviated notation of
$H_3^{32}PO_4$

ANOVA…………………Analysis of Variants

Ca^{2+}………………….....Calcium

cpm/ μg…………………Counts per minute over micrograms of protein

GBSS……………………Gey's Balanced Salt Solution

H_2O_2……………………..Hydrogen peroxide

mM………………………Millimolar.

P………………………….P-value statistical measure

PKA………………………Protein Kinase A

PKC………………………Protein Kinase C

SEM………………………Standard Error of Mean

T…………………………..Abbreviation of Time

VDR………………………Vitamin D Nuclear Receptor

Appendix B. Statistical Analyses

Table A. ANOVA for figure 4. Time course of $24R,25(OH)_2D_3$ effect on ^{32}P transport in chick intestines with variable times of fasting.

Time	1h C	1h D	5h C	5h D	10hC	10hD	15hC	15hD	18hC	18hD
Average	1184	1137	1272	1281	1954	1538	2990	2016	2341	1755
SE	121.8	171.5	222.1	232.5	265.2	230.3	431.6	73.7	207.3	284.9
DF	12	12	12	12	12	12	12	12	12	12
T test		0.22		0.028		1.19		2.23		1.67
P value		P>0.1		P>0.1		P>0.1		P<.05		P<.05

Table B. ANOVA for figure 5. Time course of $24S,25(OH)_2D_3$ effect on ^{32}P transport in chick intestines with variable times of fasting.

Time	1h C	1h D	5h C	5h D	10h C	10hD	15h C	15h D
Average	1043.2	1081.6	1081.6	1203.2	1999.4	2184	2105.8	2015.74
S.E.	332.8	128	371.2	140.8	280.96	220.96	322.56	289.68
DF	3	3	3	3	30	30	30	30
T test		0.11		0.31		0.52		0.21
P value		P>0.05		P>.05		P>.05		P>0.05

Table C. ANOVA for figure 6. Time course of $24S,25(OH)_2D_3$ effect on ^{32}P transport in chick intestines with a constant 18 hours of fasting.

Time	Control	1 h	5h	10h	15h	18h
Average	318.64	419.67	342.78	299.80	351.40	303.98
Standard Error	72.87	109.46	43.15	105.57	50.85	64.10
DF	12	9	10	8	8	8
T test		0.77	0.28	0.14	0.37	0.15
P value		P>0.2	P>0.2	P>0.2	P>0.2	P>0.2

Table D. ANOVA for figure 7. Time course of $24S,25(OH)_2D_3$ effect on ^{32}P transport in chick intestines with a constant 18 hours of fasting.

Time	Control	1 h	5h	10h	15h	18h
Average	318.64	419.67	342.78	299.80	351.40	303.98
Standard Error	72.87	109.46	43.15	105.57	50.85	64.10
DF	12	9	10	8	8	8
T test		0.77	0.28	0.14	0.37	0.15
P value		P>0.2	P>0.2	P>0.2	P>0.2	P>0.2

Table E. ANOVA for figure 8. Dose-response of $24R,25(OH)_2D_3$ effect on ^{32}P transport in chick intestines at 1h.

Time	Control	100 μg	200 μg	300 μg
Average	131.12	261.20	98.90	85.33

Standard Error	18.09	22.19	17.7	16.37
DF	5	3	3	3
T test		3.32	1.15	1.86
P value		P<0.05	P>0.05	P>0.05

Table F. ANOVA for figure 9. Time course of 24R,25(OH)$_2$D$_3$ effect on ^{32}P transport in chick intestines without fasting on a low vitamin D diet.

Time	Control	1 h	5h	10h	15h	18h
Average	134.03	68.93	79.43	89.47	77.90	131.35
Standard Error	17.94	6.28	16.38	12.17	13.28	45.04
DF	12	6	9	9	10	8
T test		3.66	2.35	2.16	2.8	0.06
P value		P>0.2	P<0.05	P<0.05	P<0.05	P>0.2

Table G. ANOVA for figure 10. Time course of 24S,25(OH)$_2$D$_3$ effect on ^{32}P transport in chick intestines without fasting on a low vitamin D diet.

Time	Control	1 h	5h	10h	15h	18h
Average	218.64	250.22	359.5	223.75	254.44	270.8
Standard Error	40.00	42.63	52.03	66.85	53.05	52.08
DF	9	9	9	8	9	10
T test		1.15	2.15	1.5	1.45	0.99
P value		P>0.2	P<0.05	P>0.2	P>0.2	P>0.2