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VITAMIN D METABOLITES IN THE UTERUS
(SHELL GLAND) OF THE LAYING HEN

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VITAMIN D METABOLITES IN THE UTERUS (SHELL GLAND) OF THE LAYING HEN

By

Sandra I. Ambrus

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ABSTRACT

VITAMIN D METABOLITES IN THE UTERUS
(SHELL GLAND) OF THE LAYING HEN

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Single Comb White Leghorn hens, approximately one year of age were divided into two groups. One group received a control diet, the other received the same ration, except that the vitamin mix contained no vitamin D_3. All birds were fed their respective rations for three to ten weeks.

Egg production and shell quality measurements were taken. Parameters for shell quality included whole egg weight, shell weight, shell thickness, and egg length and breadth.

After hens had been on the deficient diet for at least three weeks, they were injected intravenously with two microcuries of ^3H-D_3. Liver, kidney and uterine tissues were collected 15 to 18 hours later and subjected to total lipid extraction. Lipid samples were chromatographed using Sephadex LH-20 column chromatography.

Egg production and shell quality were not markedly affected during the experimental period.

^3H-D_3 and 25-OH-D_3 were recovered from most of the lipid samples. 1,25-$(OH)_2$-D_3 was not recovered from any sample. Several liver, kidney and uterine samples had unidentified peaks appearing between 100 and 260 ml.

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INTRODUCTION

Thin-shelled eggs and egg breakage are of concern to the poultry industry. The incidence of poor quality egg shells tends to increase as the hen ages, with estimates up to 16%.

It is currently known that the liver and kidney are involved in vitamin D_3 metabolism, and ultimately calcium absorption from the birds' intestine. Research has also been done to determine what effects the addition of different calcium sources or vitamin D_3 metabolites to the hen's diet have on maintaining or improving egg shell quality. However, none of these studies has yet been able to elucidate the exact cause of declining shell quality in the aging hen.

This study will attempt to determine if the uterus of the laying hen contains metabolites of vitamin D_3 which vary in concentration during the egg-forming cycle and to determine if vitamin D_3 deficiency influences these metabolites.

OBJECTIVES

1. To feed hens a vitamin D_3 deficient diet until they begin laying eggs with poor quality shells.

2. To compare the chromatography patterns of vitamin D_3 metabolites in the liver, kidney and uterine tissue of vitamin D_3 deficient hens with hens that have been fed diets with adequate levels of vitamin D_3.

REVIEW OF LITERATURE

A hen's body store of calcium is approximately 20 grams. The major nutritional component involved in egg shell deposition is calcium, which comprises approximately 40% of the egg shell. If the hen lays an egg daily, which contains 2 grams of calcium, she will turn over approximately 10% of her body store of calcium each day (Hudson et al., 1971).

Egg breakage, shell-less egg, and thin-shell eggs are a persistent and continuing problem for the poultry industry. As the hen ages, her ability to maintain adequate shell quality decreases. In a study by Roland (1977), the percent of uncollectible eggs (shell-less and thin-shell) was found to increase as the hen aged. During the winter months, birds 8 months of age had 2.39% uncollectible eggs while birds 17 months of age had 14.74% uncollectible eggs. The percentages increased during the summer months with 2.86% for birds 8 months old and 16.11% for birds 17 months old. In addition, Roland determined that as the hen aged, the percent of eggs laid with cracks in the shell tended to increase. His study also indicated that out of every 100 eggs, 7.77 were uncollectible.

In addition to poor shell quality, egg handling practices can also result in fewer marketable eggs. Orr et al., (1977), reported that "the incidence of cracks which occurred during the laying, gathering and handling prior to shipment ranged from 1.1 to 1.7 percent of all eggs examined". There was also an increase in shell damage while the eggs were being graded, washed and packed. In addition, this study showed

that during laying, gathering and packing the highest percent of shell cracks were in the small end (27.1%), while during shipping, washing, grading and packing, most cracks occurred from the large end of the egg to the center (34.0%).

The metabolism of vitamin D to its active form, 1,25-dihydroxy-vitamin D_3, which promotes calcium absorption from the intestine and mobilization of calcium from bone, occurs in several sequential steps. This metabolic pathway is regulated by several factors, including diet, enzymes, and hormones.

Vitamin D_3 is produced in the epidermal layer of the skin. Pro-vitamin D_3, 7-dehydrocholesterol undergoes cleavage of the B-ring-carbon-carbon bond between C 9 and C 10 to form provitamin D_3. Ultraviolet light, at wavelengths of 280-305 nm, is required for the cleavage to occur (Editorial, Lancet, 1974). Provitamin D_3 is then slowly converted to vitamin D_3, without any further action by ultraviolet light. Vitamin D_3 is then carried to the liver by means of an alpha2-globulin. Vitamin D_3 may also be ingested in the diet. It is absorbed primarily from the duodenum and jejunum through the aid of bile salts and intraluminal lipids. Bile salts play an essential role in the intestinal absorption of vitamin D_3 and calcium. If a bile-salt deficiency occurs, the result could be a decreased absorption of dietary calcium. The absorbed vitamin D_3 is transported in lymph by means of chylomicrons (Avioli and Haddad, 1973; Holick and Clark, 1978).

At the liver, vitamin D_3 is first hydroxylated at the C-25 position on the side chain to produce 25-hydroxyvitamin D_3 [25-OH-D_3], which is the major circulating metabolite of vitamin D_3. Haussler and Rassmussen

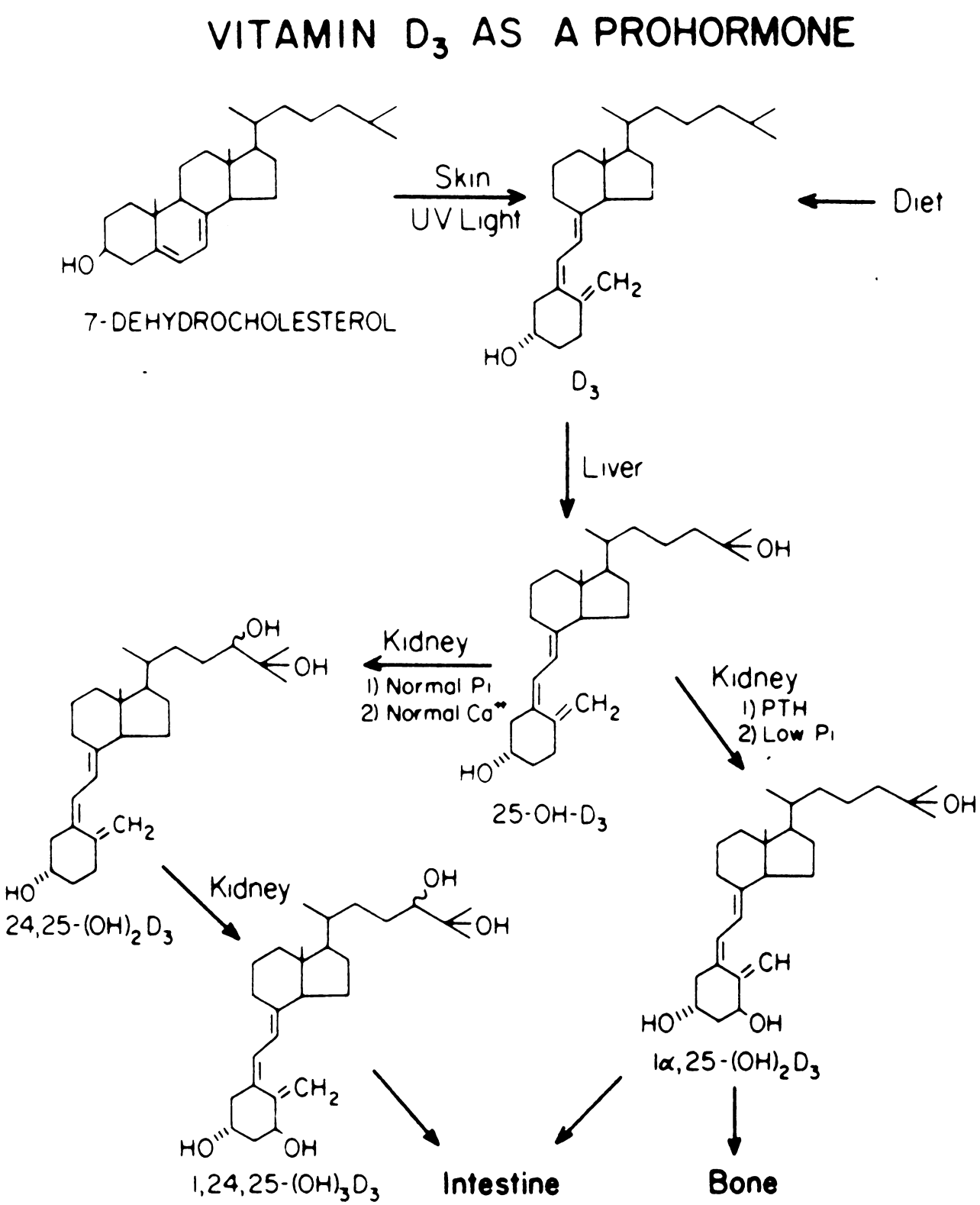
(1972) stated that the kidney is also capable of converting vitamin D_3 to 25-OH-D_3. 25-OH-D_3 is then transported to the mitochondria of the renal tubule cells, where it is hydroxylated at either the C-1 or C-24 position to form 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-D_3] (Figure 1) or 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2$-D_3] (Ghazarian and DeLuca, 1974; Henry and Norman, 1978; Swaminathan et al., 1977; Holick and Clark, 1978).

The enzymatic reaction involved in the hydroxylation of 25-OH-D_3 to 1,25-$(OH)_2$-D_3 has been studied extensively. 25-OH-D_3-1α-hydroxylase and 25-OH-D_3-24R hydroxylase are stereospecific mixed function oxidases that are located in the renal mitochondria. Reduced nucleotide (NADPH) and molecular oxygen are required for enzymatic activity. Cytochrome P_{450} is required by 25-OH-D_3-1α-hydroxylase for its activity. In vitamin D_3 metabolism, the rate limiting step is the renal 1α-hydroxylation of 25-OH-D_3 (Ghazarian and DeLuca, 1974; Holick and Clark, 1978; Avioli and Haddad, 1973; DeLuca, 1974).

The mode of action of 1,25-$(OH)_2$-D_3 is best summed up by Kumar (1982). He states that, "1,25-$(OH)_2$-D_3 probably enters the intestinal cell by diffusion across the cell membrane. In the cytosol it combines with a receptor.....that is specific for 1,25-$(OH)_2$-D_3. After it binds with the receptor, 1,25-$(OH)_2$-D_3 is translocated into the nucleus. Once in the nucleus, the 1,25-$(OH)_2$-D_3 receptor complex acts to increase the activity of the chromatin template and of a RNA II polymerase, an enzyme that makes messenger RNA that is specific for intestinal calcium binding protein (CaBP)".

1,25-$(OH)_2$-D_3 also has other effects on the intestinal cell. These

Figure 1. Metabolism of vitamin D_3 (DeLuca, 1974).



include an increase in the activity of alkaline phosphatase and calcium-dependent ATPase, and an increase in the uptake of calcium by the vesicles of the brush border membrane, the Golgi apparatus, and the basolateral membrane."

The CaBP formed in response to vitamin D metabolism which is responsible for the transfer of calcium across the intestinal wall, and into the bloodstream, is present in the intestinal mucosa of chicks. (Figures 2, 3) A similar CaBP is also present in the uterus and intestine of the laying hen. Bar and Hurwitz (1975) found that "calcium absorption and CaBP levels increase under conditions of long-term calcium depletion and at the onset of egg production. On the other hand, the increase in intestinal absorption and uterine secretion of calcium during periods of egg shell formation are not associated with any detectable changes of CaBP level in the two organs." In other words, the uterine and intestinal calcium-binding activity showed no significant change during the egg formation cycle. They also concluded that "the CaBP level of the different intestinal segments... also did not change significantly ($P>0.05$) between periods of egg shell calcification and periods of uterine inactivity." Finally, they determined that the reason for the increased absorption of calcium during shell formation was due primarily to an increase in permeability for calcium and not any change in CaBP.

Wasserman and Coombs (1978) studied calcium absorption in Japanese quail, and reported results similar to those by Hurwitz and Bar (1975). Wasserman and Coombs determined that there was no change in the efficiency of calcium absorption between the calcifying and non-calcifying stage in egg formation. They stated that "this finding is consistent with the observation that the kidney 25-hydroxycholecalciferol-1-hydroxylase activity does not differ during shell formation and in the noncalcifying

Figure 2. Mechanism of vitamin D_3 action (Kodicek, 1972).

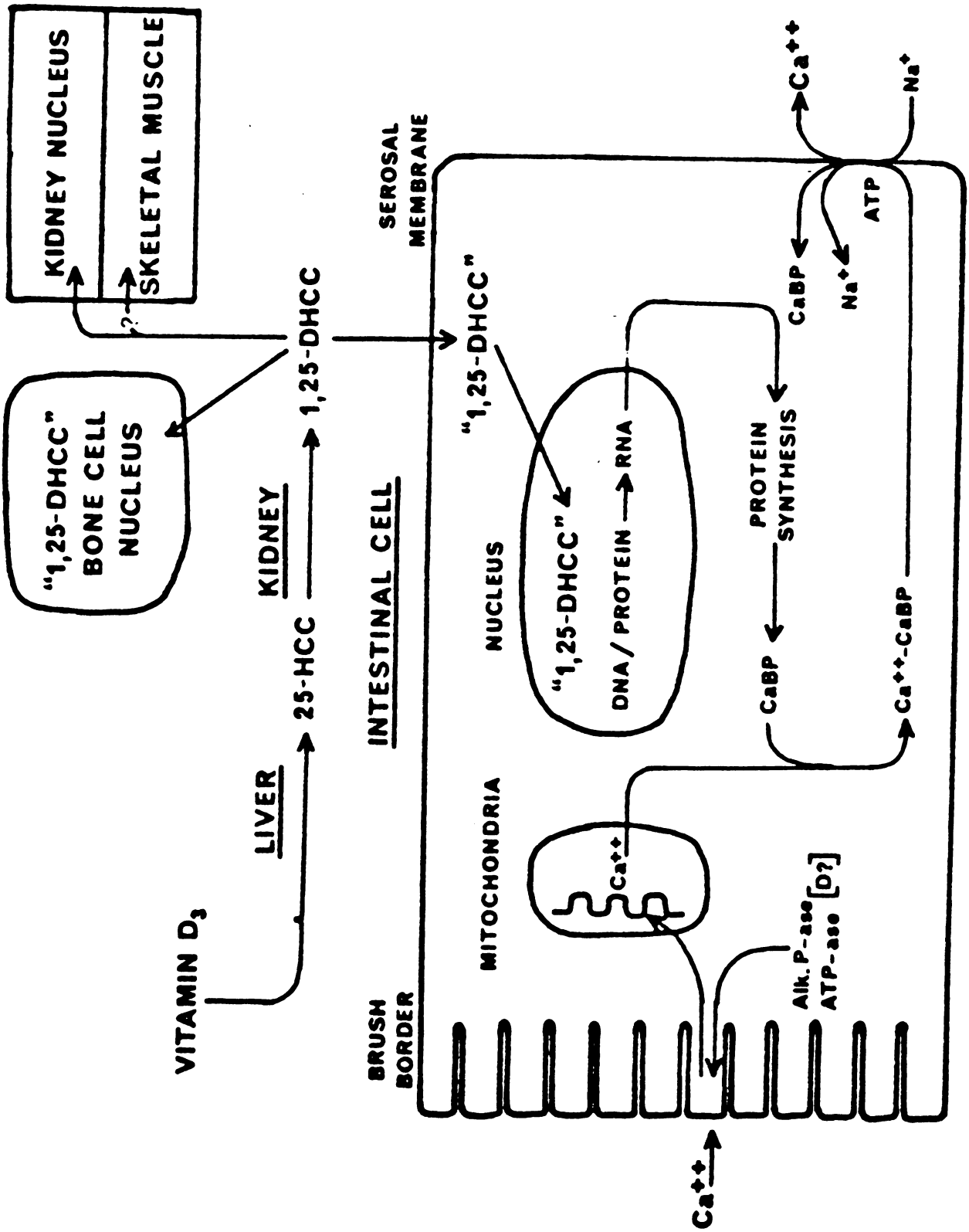
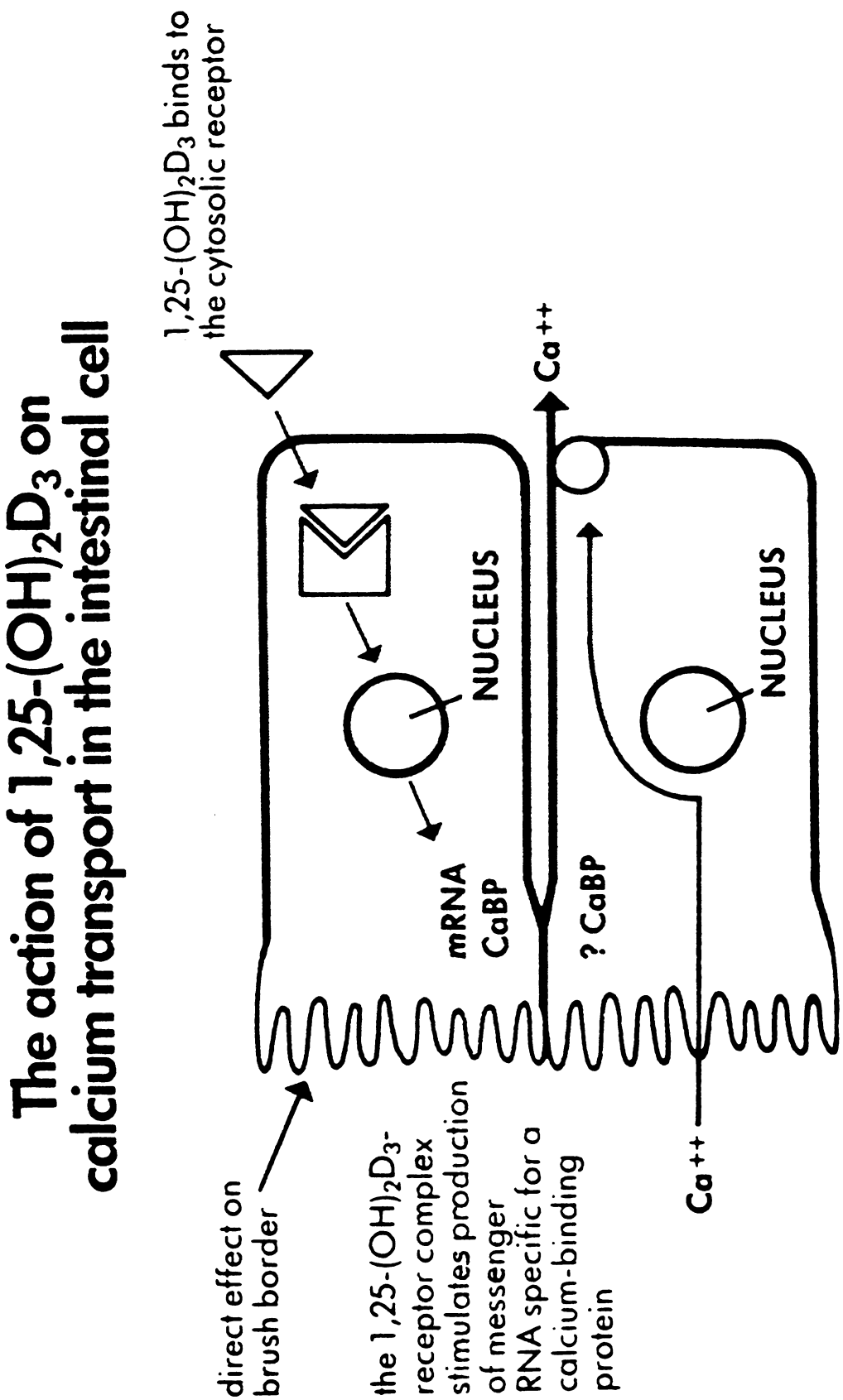


Figure 3. The action of 1,25-$(OH)_2$-D_3 on calcium transport in the intestinal cell (Kumar, 1982).



period, i.e., up to 4 hours after ovulation."

Bar et al. (1978) stated that "intestinal CaBP synthesis is controlled by the concentration of 1,25-dihydroxycholecalciferol in the cells of this organ." They found that uterine and intestinal CaBP increased at the onset of egg production in birds, but that uterine CaBP concentration did not change, while intestinal CaBP concentration increased when laying hens were subjected to calcium restriction. Also, kidney-1-hydroxylase activity, as well as, intestinal CaBP levels were higher in the calcium restricted hens, while no change in uterine or renal CaBP was noted. However, the increased production of 1,25-$(OH)_2$-D_3 did not result in a corresponding increase in calcium deposition into shells. They therefore concluded that, "calcium deposition into egg shell is not dependent upon cholecalciferol metabolism."

In addition to calcium ingested from the diet, Hurwitz and Bar (1966) found that the ends of the femurs of the hen were the principal site of storage of available calcium that could be utilized during early egg production, even when the hen's diet contained a sufficient level of calcium. When the hens were fed diets that were depleted in calcium (1.7%), a progressive decrease in blood and egg shell calcium resulted. Both ends and medullary segments of the femur showed a marked decrease in calcium content during the period of calcium restriction, however, the calcium level in the cortical segment of the bone was not affected to a great extent. When the birds were placed on a repletion diet that contained 3.7% calcium, the researchers found that the egg shell calcium returned to normal levels after 6-8 days on the repletion-level diet. They also found that the bones returned to normal in 3 weeks on the repletion diet, despite the rapid increase in egg shell calcium levels.

The recovery of the bones led them to suggest that the hen's ability to retain calcium following a period of calcium depletion was greater than normal. This assumption was based on a trial in which hens were fed either a high-calcium (3.7%) or low-calcium (1.7%) diet for one week. Then, all birds were fed calcium "free" diets for two days, and then returned to their initial diets for one week. The excreta were collected and analyzed for calcium content. In both the low-calcium and high-calcium groups, the calcium excretion was markedly reduced while the repletion diet was being fed, resulting in a higher calcium retention. Hurwitz and Bar (1966a) further found that, "a greater absorption of calcium seems to be responsible for the increased retention, since the magnitude of this increase exceeded the total amount of calcium of 140-200 mg per day normally found in the urine of laying hens".

In a previous study, Hurwitz and Bar (1965) determined that the majority of calcium and phosphorus absorption occurred in the anterior portion of the intestinal tract, and that the absorption and phosphurus appeared to be related to, as well as influenced by, the absorption of calcium. They also reported that, "the absence of a calcifying shell was associated with a reduced rate of calcium absorption". More recently, Hurwitz et al. (1973) reported that a duirnal fluctuation in calcium absorption occurred, which is generally associated with the laying cycle; also that the absorption of calcium tended to increase during shell calcification in all intestinal segments, except the lower ileum.

Older hens tend to have a greater percent of cracked or broken eggs. This problem is more apparent in warm weather. McLaughlin and Soares (1976) showed that "hen-sized calcium carbonate, regardless of the source had a definite influence on improving shell quality during warm weather". These

researchers also experimented with feeding different calcium sources (limestone or oyster shell) at 3.5 or 4.0% of diet, in combination with either 600 or 3000 I.U. of vitamin D_3 or 25-OH-D_3 per kg. of diet. This study showed that "...600 I.U. of 25-OH-D_3 per kg. when fed in combination with either limestone or oyster shell as the calcium source to laying hens in an advanced state of production results in an improvement ($P<.05$) in shell quality as measured by specific gravity and shell thickness." In addition, they determined that 25-OH-D_3 was more effective than vitamin D_3 in promoting the mobilization of calcium in older hens and also "in the formation of CaBP in the uterus during egg shell formation." Their study led them to postulate that the reason for the more efficient effect of 25-OH-D_3 on the mobilization of calcium and subsequent egg shell calcification might be due to a decreased ability of the older hen to hydroxylate vitamin D_3 to 25-OH-D_3.

In another study involving the effect of calcium source on shell quality, Scott et al. (1971) demonstrated that when hen-sized oyster shell was substituted for a portion of pulverized limestone in the diet of laying hens, an improvement in shell strength resulted. The improvement appeared to be due to the fact that the oyster shell allowed for a constant metering of calcium from the gizzard, therefore allowing the hen to absorb calcium during the entire day. This was compared to the fact that hens with the pulverized limestone in their diet must absorb all their calcium during the 14-16 hours of light.

The metering of calcium from the crop and gizzard was studied by Roland et al. (1972a, 1972b). Most of the calcium source was metered from the gizzard during the early morning hours, with little at night. In addition, it was observed that when granular limestone was the calcium source,

it was metered fairly uniformly, but at a decreasing rate during the night. They concluded that "less total calcium is metered into the lower digestive system from the crop and gizzard during the night when oyster shell is fed than when fine granular limestone is fed".

Many factors are responsible for the regulation of calcium absorption and vitamin D_3 metabolism. Hurwitz et al. (1973) believe that there exist two types of regulation for intestinal calcium absorption. These differ in stimulus, site and time of response. The first type is based on the increase of calcium absorption during egg shell formation. The time of response to this stimulus is very short, about 2 hours or less. This first type of regulation is "characterized by a general increase in calcium absorption at all levels of the intestine, except for the ileum. Within the small intestine, the response is greatest in the upper jejunum. This mechanism seems to be available during the first few days of egg production, and is found at similar intensity in both young and old hens".

The second type of regulation described occurs only in the duodenum and is stimulated by the onset of egg production from a nonproducing state, and also by the amount of body calcium stores. The response time for duodenal regulation is 1.5 days. Also, the duodenum had a higher level of calcium absorption in the older lyaing hen than in the younger laying hen (Hurwitz et al., 1973).

Polin and Ringer (1977) conducted a study to determine the effects of 25-OH-D_3, vitamin D_3 and varying levels of phosphorus on egg shell quality. While D_3 is involved in the transport of phosphorus across the intestinal wall, the absorption of phosphorus is not dependent upon calcium absorption. This study found that when no phosphorus was added, or when the level of added available phosphorus was 0.28%, adding 25-OH-D_3 to the diet appeared

to yield better quality shells than when vitamin D_3 was added. The D_3 forms resulted in an increased availability of phosphorus from diets that consisted primarily of plant-type ingredients. Both D_3 forms resulted in comparable quality egg shells when targeted levels of 0.42 or 0.56% phosphorus was included in the diet. In addition, "25-OH-D_3 did not protect shell quality any better than D_3 during hot weather." Both D_3 forms were unable to prevent the decrease in shell quality, brought on by aging and hot weather, that occurred when the hens were 32 to 52 weeks of age. And, when both D_3 forms were added at 25ug/kg of diet, 25-OH-D_3 tended to result in higher egg production and better shell quality at submarginal levels of phosphorus.

Polin and Sturkie (1957) studied the effect of the parathyroid glands on blood calcium levels and shell deposition. They found that a decrease in the level of diffusible calcium occurred at the time of shell deposition, but that total calcium level showed no change. When the parathyroid glands were removed from laying hens, a marked decrease in plasma calcium was noted, and subsequently, eggs that were present in the uterus were expelled prematurely and showed little or no evidence of shell calcification. They further found that "shell deposition is dependent upon the maintenance of the diffusible calcium level in the blood and that the parathyroid glands are involved in the maintenance of this level." When the rate of calcium mobilization was less than the rate of shell deposition, the lowered concentration of diffusible calcium acts as a stimulus for an increase in calcium mobilization.

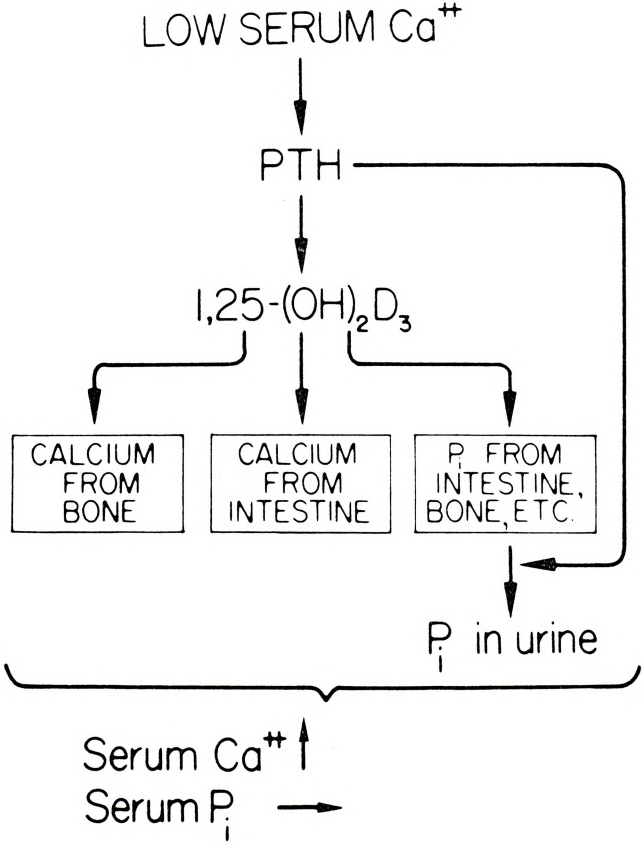
Another study by Polin and Sturkie (1958) showed that a rise in the non-diffusible calcium level in blood occurred in response to estrogen-like substances. The level of non-diffusible calcium was found to be

higher in laying birds than in non-laying birds. Parathyroid hormone was involved primarily with the regulation of the level of diffusible calcium. When the parathyroids were removed, the diffusible level decreased, with or without the administration of estrogen. Their study concluded that "parathyroid hormone and estrogen are indirectly related in their action on blood calcium. Parathyroid hormone maintains diffusible calcium levels so that estrogens can increase non-diffusible calcium levels."

Many physiological mechanisms are involved in the control of vitamin D_3 metabolism. Colston et al. (1973) proposed two possible mechanisms. First, that hydroxylase activity is regulated by the concentration of intracellular calcium. The hydroxylase activity responds rapidly and inversely to changes in calcium concentration. The intracellular calcium concentration is influenced by parathyroid hormone and calcitonin. The second regulatory mechanism is based on the concentration of 25-hydroxycholecalciferol-binding protein in the cytoplasm. This leads to the accumulation of 25-OH-D_3, thereby raising its concentration in the region of the enzyme, 25-hydroxycholecalciferol-1-hydroxylase, resulting in an increased rate of formation of 1,25-$(OH)_2$-D_3.

The plasma calcium levels control the parathyroid activity. When the bird is in a state of hypocalcemia, the production and release of parathyroid hormone is stimulated (Figure 4). When parathyroid hormone is secreted, the increased amount stimulates the activity of 25-hydroxycholecalciferol-1-hydroxylase, thereby resulting in an increased production of 1,25-$(OH)_2$-D_3 by the kidney. It has been shown that 1,25-$(OH)_2$-D_3 is 13-15 times as effective as vitamin D_3 in stimulating intestinal absorption of calcium, and 5.5 times as effective as vitamin D_3 in stimulating the elevation of serum calcium. When the calcium level returns to normal, the para-

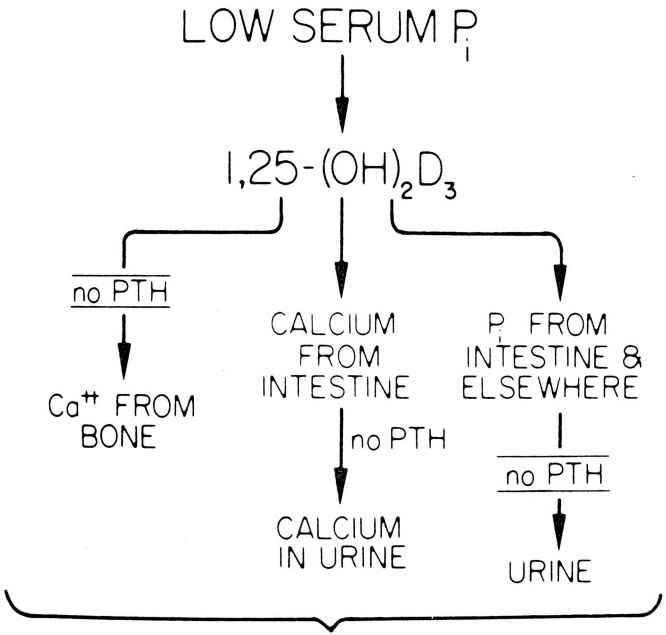
Figure 4. Effects of low serum calcium (DeLuca, 1974).



thyroid hormone secretion is suppressed, the activity of 25-hydroxycholecalciferol-1-hydroxylase decreases while the activity of the enzyme 25-hydroxycholecalciferol-24-hydroxylase increases. Once formed, the 24-hydroxylated metabolites are rapidly metabolized and excreted (Swaminathan et al., 1977, Holick and Clark, 1978, Suda et al., 1973, Henry and Norman, 1978, Norman and Henry, 1974). Holick and Clark (1978) also demonstrated that in rats constantly infused with calcitonin, no direct role of calcitonin in vitamin D metabolism could be elucidated.

The role of phosphate depletion in vitamin D_3 metabolism was studied by Haussler et al. (1977) (Figure 5). They observed that 1,25-$(OH)_2$-D_3 is able to mobilize phosphate in gut, bone, and possibly kidney, but whether phosphate could control the formation of 1,25-$(OH)_2$-D_3 was undetermined. Also, several controls on the stimulation activity of 25-hydroxycholecalciferol-1-hydroxylase were noted, including, low dietary phosphate, vitamin D_3 deficient diet, and hypophosphatemia and hypocalcemia in relation to improper bone calcification. On the other hand, birds with a normal level of vitamin D_3 and a low phosphorus level in the diet, had low enzyme activity. They also found that a high level of enzyme activity was present in birds on a vitamin D_3 deficient diet, regardless of the calcium level in the diet (0.2, 0.7, or 3.0%).

Figure 5. Effects of low serum phosphorus (DeLuca, 1974).



PROCEDURES

This experiment was conducted in two trials. Both studies were to determine which vitamin D_3 metabolites, if any, were present in the uterus of the laying hen. The first trial was conducted primarily to determine how the experimental diet affected shell quality and the uterine metabolites of vitamin D_3. The second trial utilized the information gathered in the first and, additionally, investigated the fate of vitamin D_3 in the liver, kidney, as well as in the uterus of the laying hen.

Single Comb White Leghorn mature laying hens, approximately one year of age, were subjected to one of two dietary regimes. The rations were either a control diet, which contained an adequate level of vitamin D_3 (1500 ICU/kg of diet) or a diet that was nutritionally inadequate in vitamin D_3 (no vitamin D_3 added to diet). Feed and water were supplied *ad libitum*.

The diets were formulated to equal or exceed the nutritional requirements of laying hens (Tables 1 and 2) according to values given in Nutrition of the Chicken (Scott *et al.*, 1976). The values used to formulate the vitamin mixture were based on recommendations from the Department of of Animal Science, Michigan State University. In the deficient diet, a separate vitamin premix was used, which had no vitamin D_3 added. The hens were fed diets with or without adequate D_3 for a minimum of three weeks to a maximum of ten weeks.

Table 1. Layer Ration for Vitamin D_3 Experiment

Ingredients	Parts per 1000
Corn #2 yellow, grnd.	573.5
Soybean meal, 40%	240.0
Alfalfa	42.0
Corn oil, stabl.[a]	42.0
Limestone	74.0
Defluorinated phosphate	18.0
Salt, iodized	3.0
Methionine hydroxy analogue	1.0
Choline chloride, 50%	1.5
Vitamin mix[b]	3.0
Mineral mix[c]	0.5
Selenium mix[d]	0.5

a. Ethoxyquin added to supply 125 mg/kg diet.

b. Supplies per kg diet: vitamin A-12,000 I.U.: vitamin D_3-1500 I.C.U.: vitamin E-15 I.U.: Menadione sodium bisulfite complex-1.5 mg: Thiamine-2.4 mg: Riboflavin-6.6 mg: Pantothenic acid 6.6 mg: Niacin 30 mg: Pyridoxine-9.0 mg: Biotin-0.3 mg: Folic acid-1.25 mg: B_{12}-.009 mg: carrier of corn gluten meal with 4% corn oil to 3 grams. Vitamin D_3 omitted from vitamin premix in ration fed to hens on D_3 deficient diet.

c. Trace mineral premix obtained from Calcium Carbonate Company (CCC), Quincy, Illinois, 62301.

d. From CCC.

Table 2. Calculated Analysis of Layer Ration for Vitamin D_3 Experiment

Nutrient	Value
Metabolizable energy - Kcal/g	2.91
Protein - %	16.40
Calcium - %	3.55
Phosphorus, available - %	0.46
Fat - %	6.78
Fiber - %	3.59
Lysine - %	0.84
Methionine - %	0.36
Methionine - % of protein	2.19
Methionine plus cystine - % of protein	3.89

Feed intake was monitored weekly for each group of birds during the experimental period. Feed intake (grams/bird/day) was calculated from the weekly consumption measurements.

During the summer months, the hens were housed in individual cages in house number 5 at the Michigan State University Poultry Research and Teaching Center. When the weather turned cooler along with significant day-night temperature fluctuations, the birds were moved into cages in the cage room at Anthony Hall and housed one per cage. There, the temperature was maintained at $22 \pm 2^{o}C$. In both housing situations, the hens were subjected to a photoperiod of 15 hours light, 9 hours dark.

Eggs were collected at day 0 and once weekly during the experimental period. These eggs were then subjected to shell quality measurements. Production records were kept for each hen according to dietary regime. Shell quality was measured based on the following parameters: whole egg weight (grams), shell weight (grams), and egg length and breadth (cm). These values were used in a formula (Carter, 1975) to calculate mg of calcium per square millimeter of shell surface area. To determine shell weight, eggs were broken at the equator, rinsed and allowed to air dry. Shell membranes were left intact, and the entire shell was weighed. Shell thickness was also determined, and in the following manner. The shell, with the membranes attached, was measured using a micrometer (Federal, Providence, R.I.) at two places along the equator. The average of these two measurements was used as the value for the thickness of the shell. Egg length and breadth, in cm, were measured using vernier calipers. The length was based on the distance between the large and small end of the egg, and the breadth was taken at the widest point along the egg's equator.

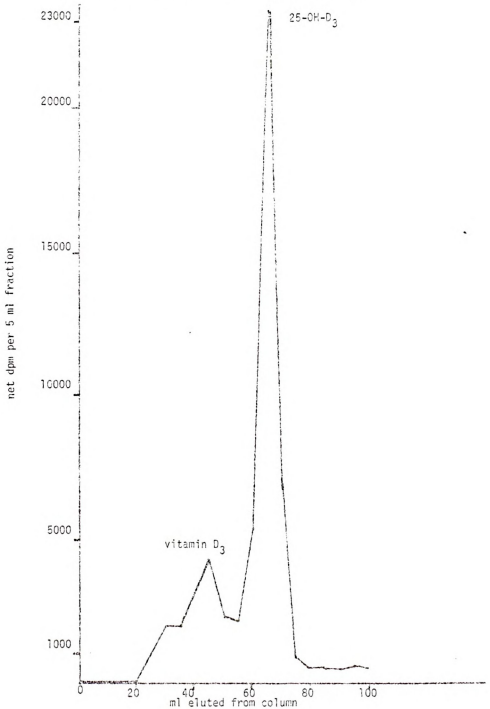
Column Preparation and Standardization

Chromatography columns were prepared according to the procedure of Holick and DeLuca (1971). Twenty grams of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was slurried in 70 mls of chloroform-hexane, 65:35 (v/v). The slurry was allowed to equilibrate for 24 hours. It was then poured into a glass column measuring 60 x 1.5 cm, that contained 20 ml of the solvent. The Sephadex was allowed to settle by gravity, with solvent flowing until it was completely settled. Each column was washed with 50-100 ml of solvent prior to the application of any sample.

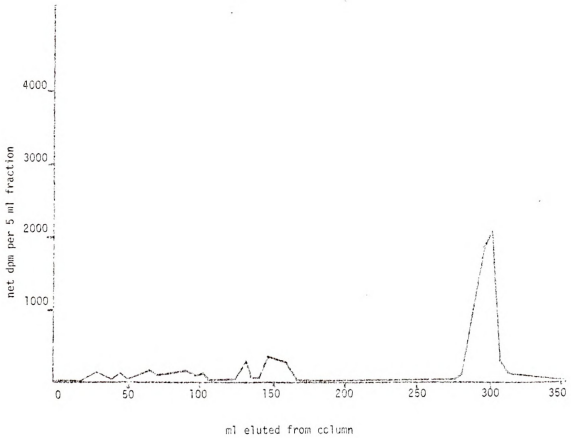
To determine the elution pattern of vitamin D_3 (D_3) and its metabolites, 25-OH-D_3 and 1,25-$(OH)_2$-D_3, known radioactive, tritiated standards (Amersham, Arlington Heights, Illinois.) were applied to the chromatography columns and eluted with $CHCl_3$-hexane. Up to 75 elution fractions, 5 ml each, were collected after the standard(s) were applied to the column. The fractions were then transferred to scintillation vials, and the solvent was allowed to evaporate by air drying in a hood. Ten mls of Scinti-verse® Liquid scintillation counting solution (Fisher Scientific Company, Pittsburgh, PA) was added to each vial. Radioactivity of each sample was determined by liquid scintillation counting in a Searle Isocap 300 counter, for one minute per vial.

Using radioactive standards, vitamin D_3 was found to elute off the column at 40-45 ml, 25-OH-D_3 at 65-70 ml, and 1,25-$(OH)_2$-D_3 at 295-300 ml of solvent flowing through the chromatography column (Graphs 1, 2, and 3). An additional peak was present at 140-150 ml. It was initially apparent in the chromatography pattern of the 1,25-$(OH)_2$-D_3 standard, and appeared again when D_3 and both metabolites were concurrently chromatographed. The identity and significance of this peak is unknown at this time.

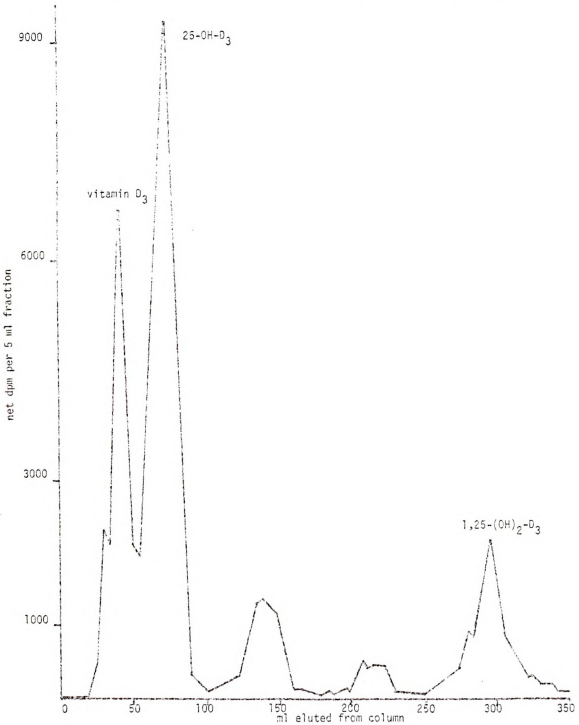
Graph 1. Column standardization-vitamin D_3 and 25-OH-D_3



Graph 2. Column standardization-1,25-$(OH)_2$-D_3



Graph 3. Column standardization-vitamin D_3. 25-OH-D_3, 1,25-$(OH)_2$-D_3



Injection of Hens

Preliminary feeding trials using D_3-deficient diets showed that hens which had been fed the deficient diet for a minimum of three weeks had lowered egg production as compared with the control birds (Table 4). A similar trend was not seen for egg production and shell quality in the second trial (Tables 5, 6, 7, and 8). When hens being fed the D_3-deficient diet during the second trial period began showing lowered egg production and an apparent decrease in shell quality, 6 control and 6 deficient hens were injected intravenously (brachial vein) with two microcuries of ^3H-D_3. A non-injected hen fed a diet adequate in D_3 was sacrificed for use as a control.

Collection of Tissue Samples

Hens were sacrificed by cervical dislocation 15 to 18 hours after injection with ^3H-D_3. This was to allow time for egg shell calcification to be occurring in each hen. The following data were collected for each hen at the time of sacrificing - presence of an egg in the uterus, and weight of liver, kidney and uterine tissues (Table 9). After weighing, the tissues were washed in physiological saline and then frozen individually until time of analysis.

Sample Processing and Lipid Extraction

The frozen tissue samples were processed as follows. The entire tissue sample was placed in a Waring Blendor® that contained 200 ml of methanol and 100 ml of chloroform. The tissue was homogenized for two to three minutes. The homogenate was transferred to a beaker, and 100 ml of distilled water and 50 ml of chloroform were added to the homogenate 2

hours later. This procedure allowed for the separation of the lipid phase from the water soluble phase. The beaker contents were transferred to a separatory funnel and the lipid (chloroform) phase was collected in an Erlenmyer flask. An equal volume of water was added to the flask, and it was stored overnight at 4°C. The next day, the flask contents were transferred to a separatory funnel, the chloroform phase was collected and allowed to air-dry under a hood at room temperature.

Sample Analysis by Column Chromatography

Each dried lipid sample was redissolved in 0.3 to 0.5 ml of $CHCl_3$-hexane solvent. The entire sample was then applied to a Sephadex LH-20 chromatography column. Four hundred ml of $CHCl_3$-hexane was run through the column to elute D_3 and/or its metabolites. The 400 ml was collected in 5 ml fractions. The elution fractions were handled in the manner previously stated.

Table 3. Feed Intake (grams per bird per day) Control Diet vs. D_3 Deficient Diet

Week of Trial	Intake-g/b/d Control	Deficient	Deficient as % of Control
1	99	103	104
2	127	155	122
3	117	118	101
4	108	95	88
5	107	84	79
6	111	85	76
7	111	94	85
8	116	86	74
9	130	85	65
10	123	97	79

Table 4. Total Weekly Egg Production of Hens in Trial 1.

Treatment	Hen No.	Week 1	Week 2	Week 3	Week 4	Week 5
$+D_3$	61	4	5	4	3	5
	62	5	5	4	5	5
	63	5	5	5	5	6
	64	6	4	3	5	4
	65	5	5	3	5	5
	66	5	6	2	5	6
	67	5	4	4	5	5
	68	5	6	4	6	6
	69	5	5	5	5	5
	70	6	5	4	6	6
	71	4	5	4	3	5
	72	5	4	4	4	6
	Mean ± S.D.	5 ± 2	5 ± 1	4 ± 1	5 ± 1	5 ± 1
$-D_3$	1	6	4	1	0	3
	2	5	3	1	3	0
	3	5	5	1	0	0
	4	4	4	4	3	4
	5	6	5	5	4	4
	6	5	5	2	1	0
	7	5	5	4	3	3
	8	7	5	5	2	5
	9	6	1	0	1	1
	10	0	4	5	5	4
	11	6	3	1	1	1
	12	5	1	0	0	0
	Mean ± S.D.	5 ± 2	4 ± 1	2 ± 2	2 ± 2	2 ± 2

Table 5. Relationship of Diet to Eggs Collected on a Once-per-Week Basis in Table 2.

Hen No.	Diet	Day 0	Day 7	Day 14	Day 21
57	Control	+[a]	+	+	+
58	Control	+	+	+	-
59	Control	+	-	-	+
60	Control	-	+	-	-
93	Control	+	+	+	+
94	Control	+	+	+	+
95	Control	+	+	+	-
96	Control	+	+	+	+
	Total #	7	7	6	5
% of Production		87.5	87.5	75.0	62.5
65	Deficient	+	+	+	-
66	Deficient	-	+	+	-
67	Deficient	+	+	+	+
68	Deficient	+	+	-	-
69	Deficient	+	+	-	-
70	Deficient	-	+	+	+
71	Deficient	-	+	-	-
72	Deficient	+	+	+	+
89	Deficient	+	+	+	+
90	Deficient	+	-	-	+
91	Deficient	+	+	+	+
92	Deficient	+	-	-	-
	Total #	9	10	7	6
% of Production		75.0	83.3	58.3	50

+[a] = laid egg; - = no egg laid on day of collection.

Table 6. Calcium (mg) per Shell Surface Area (mm^2) Calculated from Egg Quality Measurements[a] (Trial 2).

Treatment	Hen No.	Calcium (mg)/S (mm^2) Day 0	Day 7	Day 14	Day 21
$+D_3$	57	.30	.29	.32	.32
	58	.32	.32	.34	no egg
	59	.32	no egg	no egg	.33
	60	no egg	.30	no egg	no egg
	93	.28	.27	.31	.32
	94	.32	.30	.32	.30
	95	.32	.34	.38	no egg
	96	.34	.33	.34	.37
	Mean ± S.D.	.31 ± .02	.31 ± 0.3	.34 ± .02	.33 ± .02
	% Production	87.5	87.5	75.0	62.5
$-D_3$	65	.30	.29	.32	no egg
	66	no egg	.30	.34	no egg
	67	.32	.30	.32	.30
	68	.27	.26	no egg	no egg
	69	.34	.37	no egg	no egg
	70	no egg	.30	.34	.34
	71	no egg	.31	no egg	no egg
	72	.30	.30	.34	.31
	89	.30	.30	.34	.31
	90	.31	no egg	no egg	.34
	91	.32	.29	.34	.34
	92	.32	no egg	no egg	no egg
	Mean ± S.D.	.31 ± .02	.30 ± .03	.33 ± .01	.32 ± .02
	% Production	76.0	83.3	58.3	50.0

[a] = Carter, British Poultry Science, 1975.

Table 7. Shell Quality Change as Related to Egg Shell Thickness (Trial 2).

		Egg Shell Thickness (mm)				
Treatment	Hen No.	Day 0	Day 7	Day 14	Day 21	Day 21-Day 0
$+D_3$	57	37	36	38	36	-1
	58	39	38	42	--	*[b]
	59	40	--	--	39	-1
	60	-[a]	36	--	--	*
	93	36	33	36	36	0
	94	39	36	36	36	-3
	95	42	44	47	--	*
	96	43	40	39	40	-3
	Mean	39.4	37.6	39.7	37.2	-2.0
$-D_3$	65	48	37	38	--	*
	66	--	38	39	37	*
	67	40	36	36	37	-3
	68	34	32	--	--	*
	69	45	45	--	--	*
	70	--	38	42	39	*
	71	--	35	--	--	*
	72	39	38	36	38	-1
	89	40	39	40	35	-5
	90	40	37	40	37	-3
	91	40	37	40	37	-3
	92	42	--	--	--	*
	Mean	41	37.5	38.8	37.0	-3.0

[a] = no egg laid or collected on that day.

[b] = no data on day 21 to allow calculation.

Table 8. Changes in Percent Shell Weight as Compared with Whole Egg Weight Based on Dietary Regime.

(Shell Weight/Whole Egg Weight) x 100

Treatment	Hen No.	Day 0	Day 7	Day 14	Day 21	Day 21-Day 0
$+D_3$	57	10.2	9.7	11.8	11.9	1.7
	58	10.4	10.3	12.1	--	*[b]
	59	11.1	--	--	12.2	1.1
	60	-[a]	10.1	--	--	*
	93	9.7	9.2	11.8	12.1	2.4
	94	11.4	10.2	12.1	11.1	-0.3
	95	10.8	11.3	13.7	--	*
	96	11.3	10.9	12.9	13.9	2.6
	Mean	10.7	10.2	12.4	12.2	+1.5
$-D_3$	65	9.9	9.6	11.8	--	*
	66	--	9.4	12.0	12.4	*
	67	10.8	10.2	12.0	10.6	-0.2
	68	8.9	8.5	--	--	*
	69	11.2	11.9	--	--	*
	70	--	10.3	12.3	13.2	*
	71	--	10.0	--	--	*
	72	10.1	9.8	12.2	11.5	1.4
	89	9.7	9.5	12.6	10.9	1.2
	90	10.6	--	--	12.6	2.0
	91	10.1	9.3	12.7	12.3	2.2
	92	10.2	--	--	--	*
	Mean	10.2	9.8	12.2	11.9	+1.3

[a] = no egg laid or collected on that day.

[b] = no data on day 21 to allow calculation.

RESULTS AND DISCUSSION

Feed Consumption

Table 3 shows that from the fourth week of the trial, through the termination, hens being fed a diet that contained no vitamin D_3 ate less feed than did the control birds. The deficient birds ate 65% to 88% of the quantity consumed by the control group. The decrease in feed consumption was brought about by the vitamin D_3 deficiency which led to a decreased calcium absorption. The decrease in calcium absorption, in turn, resulted in less feed consumption.

Egg Production

In the preliminary feeding trial (Table 4) the data show that after hens had been fed a vitamin D_3 deficient diet for 3 weeks, their weekly egg production was less than that of hens fed the diet which had an adequate level of vitamin D_3. In the second trial, in which eggs were collected once weekly, (Table 5) the vitamin D_3 deficient hens again showed a lower percent production on the days that eggs were collected.

Egg Quality

The data presented in Tables 6, 7, and 8 show that only slight changes in shell quality occurred during the course of the second trial. The quantity of calcium per mm of shell surface area was .01 mg less in the vitamin D_3 deficient group in days 7, 14, and 21, than for the control group. Egg shell thickness decreased for both the control and vitamin D_3 deficient hens. The thickness decreased by an average of 2.0 mm for the control group; for the deficient hens an average decrease of 3.0 mm resulted.

The data on the change in % shell of total weight (Table 8), again show the minimal changes that took place during the second experimental period. The average total change from day 0 to day 21 for the control group was an increase in shell weight of 1.5%; the change for the deficient birds was an increase of 1.3%. These slight differences suggest that the hens were not subjected to the vitamin D_3 deficient diet for an adequate length of time to bring about the classical changes of vitamin D_3 deficiency: thin-shelled and soft-shelled eggs and a decrease in egg production. These symptoms have been reported to occur about 1-2 months after the birds are on a deficient diet (Scott et al., 1976).

The results of the second trial would be improved had egg quality data been collected for a longer period of time, i.e. 28 or 35 days.

Another reason for the minimal changes might be that although the hens were being fed a vitamin D_3 deficient diet, they attempted to maintain shell quality during the experimental period by producing fewer eggs, that were smaller in size, and whole egg weight. In this manner, they were able to conserve available calcium for shell deposition onto the eggs that were produced.

Recovery of Radioactivity

The data in Table 10 show that, although all birds were injected intravenously with equal amounts (2 microcuries) of ^{3}H-vitamin D_3, the recovery of radioactive metabolites was low and varied with each hen. One control hen and one deficient hen, both sacrificed with an egg present in the uterus, had the best recovery of ^{3}H-vitamin D_3, with 3.02% and 3.87%, respectively. The data from the remaining birds followed no predictable patter, in that

Table 9. Tissue Weights and Presence of Egg in Uterus, Collected at Time of Sacrificing

Hen No.	Diet	Egg in Uterus	Liver	Kidney	Uterus
57	Control	+	29.0	11.0	12.5
58	Control	+	21.8	10.9	13.8
59	Control	-	40.8	12.9	11.3
*60	Control	-	19.8	11.2	12.0
93	Control	-	27.0	11.2	10.5
94	Control	-	20.0	11.2	13.0
Mean ± S.D.			26.4 ± 8.0	11.4 ± 0.7	12.2 ± 1.2
66	Deficient	-	24.0	10.5	15.0
*67	Deficient	-	24.8	9.3	10.0
68	Deficient	+	24.5	11.5	17.5
69	Deficient	+	22.8	9.5	10.0
70	Deficient	+	25.5	9.1	12.0
91	Deficient	+	34.5	14.5	15.5
Mean ± S.D.			26.0 ± 4.2	10.7 ± 2.0	13.3 ± 3.1
Non-injected hen -----		+	44.0	14.5	18.0

*Hens 60 and 67 laid eggs 5-10 minutes prior to sacrificing.

Table 10. Recovery of Radioactivity in Tissues Collected From Injected Hens

Hen No.	Diet	Egg In Uterus	Tissue	Tissue Weight g.	Total dpm Recovered	dpm per g. tissue	$\left(\frac{\text{Total dpm recovered}}{\text{Total dpm injected}}\right) \times 100$
57	Control	+	Liver	29.0	9152	316	
			Kidney	11.0	2696	245	3.02
			Uterus	12.5	2831	226	
58	Control	+	Liver	21.8	74	3	
			Kidney	10.9	300	28	.12
			Uterus	13.8	192	14	
59	Control	-	Liver	40.8	166	4	
			Kidney	12.9	176	14	.09
			Uterus	11.3	80	7	
60	Control	-	Liver	19.8	0	0	
			Kidney	11.2	142	13	.05
			Uterus	12.0	86	7	
93	Control	-	Liver	27.0	2543	94	
			Kidney	11.2	4524	404	2.57
			Uterus	10.5	5359	510	
94	Control	-	Liver	20.0	1268	63	
			Kidney	11.2	742	66	.54
			Uterus	13.0	604	46	
66	Deficient	-	Liver	24.0	5848	244	
			Kidney	10.5	2665	254	2.33
			Uterus	15.0	2790	186	
67	Deficient	-	Liver	24.8	3340	135	
			Kidney	9.3	2496	268	2.23
			Uterus	10.0	4938	494	

Table 10. (continued)

Hen No.	Diet	Egg In Uterus	Tissue	Tissue Weight g.	Total dpm Recovered	dpm per g. tissue	$\left(\frac{\text{Total dpm recovered}}{\text{Total dpm injected}}\right) \times 100$
68	Deficient	+	Liver	24.5	0	0	
			Kidney	11.5	40	3	.01
			Uterus	17.5	0	0	
69	Deficient	+	Liver	22.8	0	0	
			Kidney	9.5	64	7	.01
			Uterus	10.0	0	0	
70	Deficient	+	Liver	25.5	11056	434	
			Kidney	9.1	3464	381	3.87
			Uterus	12.0	4191	349	
91	Deficient	+	Liver	34.5	3199	93	
			Kidney	14.5	2956	204	1.51
			Uterus	15.5	1123	72	

percent recovery appeared to be independent of diet and presence of egg in the uterus at time of sacrificing. These results may be due to individual bird variation, inability of these hens to metabolize the ^{3}H-vitamin D_3 or improper length of time between injection of ^{3}H-vitamin D_3 and sacrificing of birds to allow for accumulation radioactive vitamin D_3 or its metabolites in the liver, kidney, or uterus.

The data presented in Table 11a indicates that a greater percent recovery of radioactivity occurred in uterine tissue from the hens on the control diet than from the uterine tissue of deficient hens. The data in Table 11b indicate that a greater percent recovery of radioactivity occurred in hens sacrificed without an egg than those birds which had an egg. This table also reveals that 0% recovery of radioactivity was associated with 2 deficient hens sacrificed when an egg was present in the uterus. This result was not expected as it seems more probable that hens with calcifying shells present at time of sacrificing would also have ^{3}H-D_3 or its metabolites present, as opposed to those birds sacrificed without an egg.

Chromatography of Tissues

The results of the column chromatography of the lipid extract from the liver, kidney and uterine tissue collected from the hens are depicted in Graphs 4a, 4b, 5 and 6. Except for the liver of one bird, all other samples showed recovery of some of the injected ^{3}H-vitamin D_3. This was usually the prominent peak in the chromatographic pattern.

The liver patterns all had evidence of the 25-OH-D_3 metabolite. These peaks were usually less than 500 dpm and were eluted between 60 and 80 ml. The two control hens showed 25-OH-D_3 peaks of 1000 dpm and 2500 dpm, respectively. One of these hens also had a peak of 700 dpm appear at 100

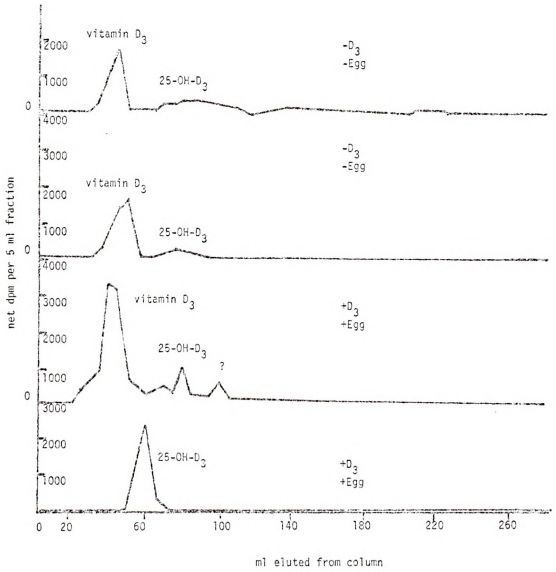
Table 11a. Uterine dpm as Percent of Total dpm Recovered, as Related to Dietary Treatment

Treatment	Hen No.	Egg In Uterus	% Uterine dpm
$+D_3$	57	+	19
	58	+	34
	59	-	19
	60	-	38
	93	-	43
	94	-	23
	Mean ± S.D.		29 ± 10
$-D_3$	66	-	25
	67	-	46
	68	+	0
	69	+	0
	70	+	22
	91	+	15
	Mean ± S.D.		18 ± 17

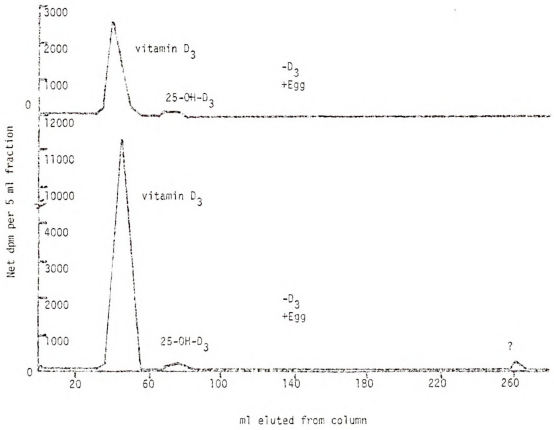
Table 11b. Uterine dpm as Percent of Total dpm Recovered as Related to Presence of Egg in Uterus

Treatment	Hen No.	Egg In Uterus	% Uterine dpm
$+D_3$	59	-	19
$+D_3$	60	-	38
$+D_3$	93	-	43
$+D_3$	94	-	23
$-D_3$	66	-	25
$-D_3$	67	-	46
	Mean $\pm$ S.D.		32 $\pm$ 11
$+D_3$	57	+	19
$+D_3$	58	+	34
$-D_3$	68	+	0
$-D_3$	69	+	0
$-D_3$	70	+	22
$-D_3$	91	+	15
	Mean $\pm$ S.D.		15 $\pm$ 13

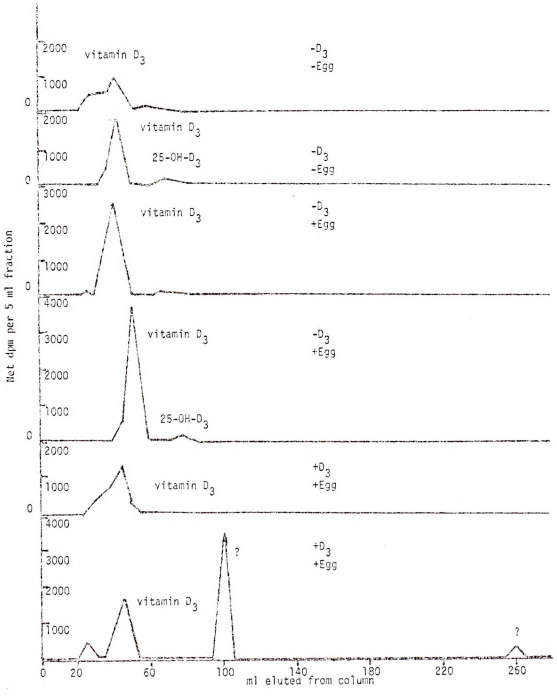
Graph 4a. Chromatography pattern-liver



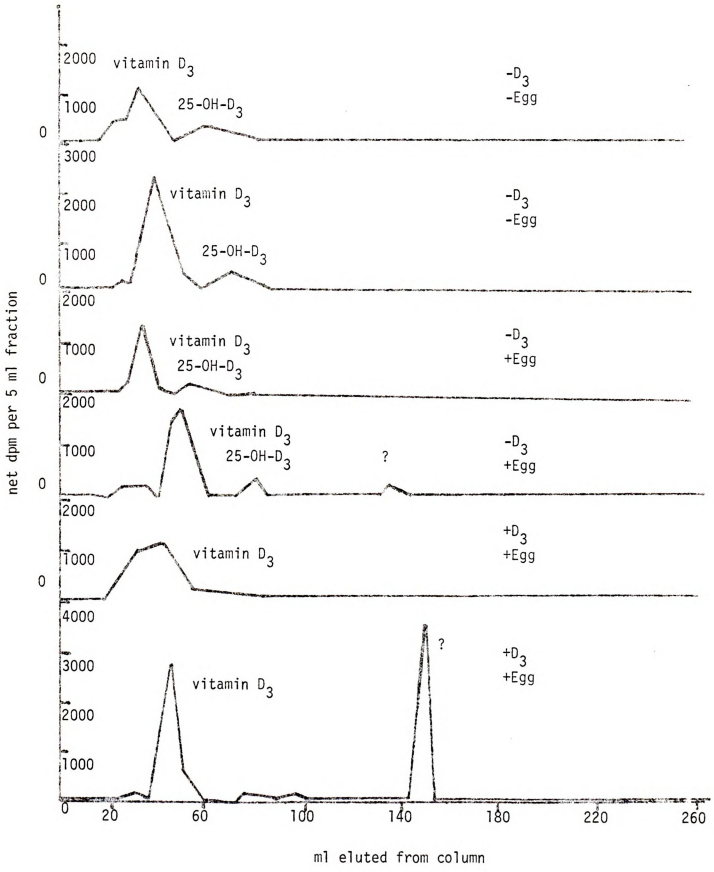
Graph 4b. Chromatography pattern-liver



Graph 5. Chromatography pattern-kidney



Graph 6. Chromatography pattern-uterus



ml. One deficient bird had a small peak of 300 dpm at 260 ml. The identity of this peak and the peak at 100 ml are unknown at this time. These peaks may be significant due to their relative prominence in the elution patterns of 2 hens, both sacrificed with an egg present in the uterus.

The elution pattern for the kidneys shows 25-OH-D_3 peaks in only two of the birds. As in the liver there peaks are less than 500 dpm. The pattern from one control bird has two additional peaks, one of 3400 dpm at 100 ml and one of 350 dpm at 260 ml.

The uterine chromatography pattern appears to follow the trend of the previous two tissues, in that ^{3}H-vitamin D_3 was recovered from all samples and that 4 of 6 showed small 25-OH-D_3 peaks (Graph 6). One control bird had a prominent peak (3500 dpm) at 150 ml. This was the same bird which had a significant peak at 100 ml in the kidney.

These results show that the injected vitamin D_3 was transported to all tissues examined. In addition, some metabolism occurred, which resulted in the production of the 25-OH-D_3 metabolite. However, because of the relative small recovery of dpm in the 25-OH-D_3 peaks, the time interval allowed between injection and sacrificing may have been incorrect. The hens were either sacrificed too soon to allow for complete metabolism of the injected sample, or too late to allow for metabolite recovery. This is probably also the reason why none of the 1,25-$(OH)_2$-D_3 metabolite was recovered. A study by Holick et al. (1976) did show that after a single intravenous injection of 0.125 mg of either 25-OH-26, 27-^{3}H-D_3, or 24,25-$(OH)_2$[26,27-^{3}H]D_3, the tissue concentrations of 25-OH-D_3 and 1,25-$(OH)_2$-D_3 were greater at 24 hours than at 48 hours. The liver concentration of

25-OH-D_3 was 77 pg-gram of tissue at 24 hours, and 28 pg/g at 48 hours. Similar results were obtained for the 1,25-$(OH)_2$-D_3 metabolite in the liver and intestine.

The peaks at 100 ml, 150 ml, and 260 ml were observed in hens that were sacrificed with an egg in the uterus. It is possible that these peaks represent the 24-hydroxy metabolites which are produced when no additional 1,25-$(OH)_2$-D_3 is required for calcium absorption and subsequent egg shell deposition. Work done by Holick et al. (1976) shows that 24,25-$(OH)_2$-D_3 is eluted from a Sephadex LH-20 column at 72 ml, and the 1,24,25-$(OH)_2$-D_3 metabolite is eluted between 180 and 250 ml, with the peak occurring at 216 ml.

Many factors influence calcium absorption, transport, and deposition. The parathyroid glands are involved in the maintenance of the diffusible calcium level in the blood. They therefore have an indirect influence on egg shell deposition (Polin and Sturkie, 1957).

The metabolism of vitamin D_3 results in the production of a calcium-binding protein, which mediates the active transport of calcium in the intestine. A similar calcium-binding protein is also present in the uterus of hens (Corradino et al., 1968). The activity of both intestinal and uterine calcium-binding protein was found not to change during the egg formation cycle or during periods of uterine inactivity (Bar and Hurwitz, 1975).

This study shows that the uterus of the laying hen is able to take up vitamin D_3. The presence of 25-OH-D_3 in the uterine lipid extract indicates that the uterus is either able to take up the metabolite or metabolize vitamin D_3.

The presence of a peak at 150 ml in the chromatography pattern of the uterine tissue of one hen suggests that the uterus may be involved in the metabolism of vitamin D_3. The identity of this peak is undetermined at this time, however, it is possible that it is the 1,24,25-$(OH)_2$-D_3 metabolite, since it occurred in a position on the elution pattern similar to that described by Holick et al., (1976). In addition, the peak occurred in a hen that was sacrificed with an egg in her uterus.

This study therefore suggests that the uterus of the hen may be able to regulate the production of its own calcium-binding protein. Further, the uterus would then be able to regulate the transport of calcium it requires for egg shell calcification.

SUMMARY

The purpose of this experiment was to determine if the uterus of the laying hen is able to metabolize the vitamin D_3 to its active form, 1,25-$(OH)_2$-D_3, and therefore regulate its own calcium transport. The effects of vitamin D_3 deficiency on egg production and shell quality were also noted.

Production records showed that vitamin D_3 deficiency will cause a decreased egg production. Shell quality analysis was inconclusive, but eggs from hens on deficient diets tended to have thinner shells, with less calcium per mm of surface area, and the shell weight comprised less of the whole egg weight.

It was observed on column chromatography of lipid extracts from liver, kidney and uterus, that these tissues were all able to take up vitamin D_3. Most of these organs were also either able to participate in the metabolism of vitamin D_3 of take up the metabolites from the blood as evidenced by the presence of 25-OH-D_3 and other undetermined metabolites in their chromatographic patterns. The extent to which the uterus of the laying hen is able to regulate its calcium transport is unknown at this time, but is an area that merits further investigation.

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