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EFFECT OF VITAMIN D ON RIBONUCLEIC ACID SYNTHESIS IN BONE

by

SUE ANN SHELLEY

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

June

1971

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ABSTRACT

Abstract of the thesis entitled "EFFECT OF VITAMIN D ON RIBONUCLEIC ACID SYNTHESIS IN BONE" submitted by Sue Ann Shelley in partial fulfillment of the requirements for the degree of Master of Science, June, 1971.

The effect of vitamin D on the incorporation of ^3H -uridine into RNA of rat bone cells was studied using an in vitro system. Rachitic rats were treated with 5,000 I.U. of vitamin D_3 or with the vehicle only. In some experiments each rat received 100 μg of actinomycin D before treatment with vitamin D or vehicle. Five hours after the administration of the vitamin or vehicle the rats were sacrificed and the humeri, tibiae and femurs removed. Fragments of trabecular bone were dissected out, washed and incubated in medium containing 1 $\mu\text{Ci/ml}$ of ^3H -uridine for 4 hours. After incubation the bone fragments were washed repeatedly and the bone cells isolated.

In some experiments RNA was extracted from the bone cells with phenol-sodium dodecyl sulfate and the specific activity of the RNA determined. In other experiments the RNA was separated into two fractions by sequential treatment with phenol-SDS at 45°C and 65°C . The specific activities of both fractions of RNA were then determined. Comparison of the specific activities of the RNAs from vitamin D_3 treated and from control rats was used as the basis to determine the effect of vitamin D_3 , and vitamin D_3 following actinomycin D treatment, on the incorporation of ^3H -uridine into RNAs of rat bone cells.

Treatment with vitamin D₃ increased the incorporation of ³H-uridine into the cytoplasmic RNA of rachitic bone cells in this in vitro system. Prior treatment of the animals with actinomycin D largely inhibited this increased RNA synthesis, but the specific activity of the RNA from vitamin D treated animals was still greater than that of the controls.

Vitamin D treatment was found to stimulate the incorporation of ³H-uridine into both RNA which could be extracted at 45°C, which is believed to be a mixture of cytoplasmic and nucleolar RNAs, and RNA extracted at 65°C. The RNA extractable at 65°C is thought to be nucleoplasmic RNA with a base ratio similar to that of DNA, and is possibly a precursor to messenger RNA. In other tissues synthesis of the 45°C RNA has been shown to be inhibited by actinomycin D while the synthesis of the 65°C RNA is relatively insensitive to actinomycin D. However, it was found that actinomycin D did not inhibit the vitamin D stimulated increase in incorporation of ³H-uridine into either of these fractions of bone cell RNA.

Vitamin D appears to increase the synthesis of all types of bone cell RNA. The vitamin D stimulated synthesis of cytoplasmic RNA could be largely blocked by pretreatment with actinomycin D, but the vitamin D stimulated synthesis of nucleolar and nucleoplasmic RNA was unaffected by actinomycin D treatment. It appears from these experiments that one effect of vitamin D is the stimulation of RNA synthesis in bone cells, but the importance of this increased RNA synthesis in the mode of action of the vitamin is not yet clear.

LIFE

Sue Ann Eckhardt Shelley was born in Moweaqua, Illinois, on December 30, 1938.

She graduated from Moweaqua High School, Moweaqua, Illinois in June, 1956, and received a Bachelor of Arts degree from Millikin University, Decatur, Illinois, in June, 1959.

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In January, 1969, she began graduate study in the Department of Biochemistry and Biophysics, Loyola University Stritch School of Medicine.

On February 12, 1961, she married William E. Shelley.

ACKNOWLEDGEMENTS

The author is indebted to Dr. Maurice V. L'Heureux for his inspiring guidance throughout this research and in the preparation of this thesis, and to the other staff members and students of both the Department of Biochemistry and Biophysics and the Department of Pathology who have contributed to the author's work by the exchange of ideas.

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CHAPTER I

INTRODUCTION

Vitamin D plays an essential role in the absorption of calcium from the small intestine and in the mobilization of mineral from bone. By these actions it functions to elevate calcium and phosphate levels in the serum, which are necessary for normal bone mineralization. The primary end effect of vitamin D is, therefore, calcification of bone, although a direct effect of the vitamin on the calcification process has not yet been demonstrated. A deficiency of vitamin D then results in defective calcification of bone. In young animals, the deficiency disease is rickets, and in adults, this failure of calcification gives rise to osteoid seams which are characteristic of the condition known as osteomalacia.

An important characteristic of vitamin D action is the lag time after administration of vitamin D to a deficient animal before any physiological response is noted. The length of the lag time is from 4 to 24 hours depending on the dosage and the mode of administration. The events that occur during this time are currently under intensive investigation. Important discoveries in recent years that have partially answered the question of what happens during this time period are the isolation and characterization of biologically active metabolites of vitamin D.

Metabolites of vitamin D first isolated were soon identified as esters of vitamin D and long chain fatty acids (Fraser and Kodicek, 1965), but these now appear to be of minor functional importance (DeLuca, 1967).

Another metabolite, more polar than vitamin D₃ (cholecalciferol) proved to be of major interest. This metabolite, when given to rachitic animals, was more effective in the cure of rickets than the parent vitamin (Blunt et al., 1968b). It also stimulated intestinal calcium transport and bone mineral mobilization more rapidly than did vitamin D₃ (Morii et al., 1967). This active metabolite, isolated from plasma by silicic acid column chromatography, has been identified as 25-hydroxy-cholecalciferol (25-HCC) (Blunt et al., 1968a). Also, 25-HCC has been chemically synthesized and shown to be identical to the isolated material (Blunt and DeLuca, 1969). The corresponding metabolite from vitamin D₂ (ergocalciferol) has also been isolated and identified as 25-hydroxy-ergocalciferol (Suda et al., 1969). In addition, dihydrotachysterol₃, a compound structurally related to vitamin D₃ and with some biological activity like vitamin D, has been shown to be converted to 25-hydroxy-dihydrotachysterol₃ in vivo (Hallick and DeLuca, 1971).

Evidence that vitamin D must be hydroxylated at carbon 25 before it is effective was obtained in isolated end-organ experiments. Using an intestinal perfusion system in which calcium transport can be studied, Olson and DeLuca (1969) reported that direct infusion of as much as 250 µg of vitamin D₃ into the mesenteric artery of vitamin D deficient animals had little or no effect on calcium transport. On the other hand, when 0.25 µg of 25-HCC was infused, calcium transport rose to normal levels in about 1.5 hours. Other evidence that 25-hydroxylation is necessary for biological activity comes from studies of mineral mobilization in fetal bone cultures. Trummel et al., (1969) demonstrated

that as little as 0.9 International Units (I.U.) of 25-HCC per ml of culture media induced release of previously incorporated calcium-45 from fetal rat bone, while as much as 400 I.U. per ml of vitamin D₃ was without effect. These results provide strong evidence that 25-hydroxylation of the vitamin D molecule is essential for its activity.

It is also evident from the above data that both intestine and bone lack the ability to convert vitamin D₃ to 25-HCC. The liver has been indicated as the major, if not sole, site of the 25-hydroxylation of vitamin D₃ by experiments dealing with the disappearance of radioactivity from the plasma after ³H-vitamin D₃ injection and the appearance of 25-HCC in the plasma (Ponchon and DeLuca, 1969). Total hepatectomy resulted in the total loss of ability for the formation of 25-HCC (Ponchon et al., 1969). Both perfused liver and liver homogenates are able to form 25-HCC in vitro (Horsting and DeLuca, 1969).

That 25-HCC is the major form of the vitamin circulating in the blood has been shown for man as well as for experimental animals (Mawer et al., 1969). However, recently Cousins et al., (1970) have demonstrated that 25-HCC is metabolized very rapidly to metabolites more polar than 25-HCC. Previous work by Haussler et al., (1968) and Lawson et al., (1969b) had already indicated the presence of a metabolite more polar than 25-HCC in the nuclei of intestinal mucosal cells. This metabolite was also detected in liver, kidney, bone and blood (Lawson et al., 1969c). In the intestine, which contains the highest quantity of this metabolite, the concentration is very low, and it has been found never to be above 1 ng/g tissue. This metabolite, tentatively identified as 1,25-dihydroxy-

cholecalciferol, is the predominant form of the vitamin found in the intestine after a physiological dose of vitamin D₃ (Kodicek et al., 1970). The biological activity of this metabolite has been reported to be at least twice that of 25-HCC in stimulating intestinal calcium transport (Kodicek et al., 1970; Myrtle and Norman, 1971). Myrtle and Norman (1971) also report that this polar intestinal metabolite greatly shortens the lag time for maximum calcium transport as compared to 25-HCC. Omdahl et al., (1971) also report that this metabolite is very active in stimulating calcium absorption, demonstrating a shorter lag time than 25-HCC. However, they found that this metabolite, while demonstrating nearly equivalent bone mobilization activity, had less than one-half of the antirachitic activity of 25-HCC.

Fraser and Kodicek (1970) have identified the site of biosynthesis of 1,25-dihydroxycholecalciferol to be the kidney. They found that the intestine, liver, adrenals, parathyroid, thyroid, thymus, bone, erythrocytes and blood plasma all failed to convert 25-HCC to the "1-oxygenated 25-HCC". DeLuca (1971) has reported that his laboratory has confirmed the finding that this intestinally-active metabolite is generated exclusively in kidney tissue.

Another metabolite of vitamin D₃ has been isolated from plasma and identified as 21,25-dihydroxycholecalciferol. This metabolite is one-half as active as vitamin D₃ in rats in the cure of rickets and in intestinal calcium transport, but is more active in the mobilization of bone mineral (Suda et al., 1970). The site of synthesis of this metabolite which is preferentially active on bone has not yet been found.

At present, it is not possible to state conclusively what form of vitamin D is the form active at the target tissues. Hydroxylation at carbon 25 has been shown to be necessary for activity, but whether 25-HCC is active in the target tissues or whether it must be further metabolized is not yet clear. It is possible that both 25-HCC and more polar metabolites may be active at the target tissues.

The way in which vitamin D metabolites act at a cellular level to regulate calcium transport has been suggested to involve interaction with the genetic material of the cell stimulating RNA and subsequently protein synthesis. Several lines of evidence support this model of vitamin D action.

Stohs et al., (1967) have shown that administration of vitamin D₃ to rachitic rats stimulates two or threefold the incorporation of ³H-orotic acid into the RNA of the intestinal mucosa. This effect occurred within 3 hours after vitamin D₃ administration and was especially noted in the nuclear RNA. Izzo (1970) reported that vitamin D also increased the incorporation of ³H-orotic acid and ³H-uridine into bone RNA, with some increase after 3 hours and a maximal response 9 hours after administration of vitamin D₃. A careful study of the effect of vitamin D₃ on nuclear metabolism in chick intestinal nuclei by Lawson et al., (1969a) revealed that the proportions of RNA, DNA and protein of the isolated nuclei were unaffected by the vitamin D status of the animals. However, the incorporation of ³H-orotic acid into the intestinal nuclear RNA was increased in vitamin D deficient chicks only 10 minutes after a 125 µg dose of vitamin D₃. Also, they reported

that there was no stimulation of DNA-dependent RNA polymerase activity of the isolated nuclei from chicks treated with vitamin D₃. These results suggest that since vitamin D does not cause an increase in RNA polymerase activity, it must affect the other factor of the RNA polymerase system, namely the chromatin.

The polar metabolite, which now appears to be 1,25-dihydroxycholecalciferol, has been shown to be selectively localized within the nuclei of the intestinal mucosa cells (Haussler and Norman, 1967; Haussler et al., 1968). Lawson et al., (1969c) reported that isolated chromatin from intestinal mucosa cells contained 50% of the radioactivity 16 hours after injection of radioactively labeled vitamin D₃. It has been shown by Hallick and DeLuca (1969) more directly that vitamin D increases the template activity of chromatin from rat intestine. In these experiments chromatin, isolated from vitamin D deficient rat intestine and from intestine from rats given vitamin D at various times prior to killing, was incubated with highly purified *E. coli* RNA polymerase and nucleoside triphosphates containing ¹⁴C-ATP in an appropriate medium. Incorporation of ¹⁴C-ATP into the RNA during incubation was a measure of the template activity of the chromatin. In these experiments a very definite stimulation in template activity following vitamin D treatment was demonstrated. These results support the concept that vitamin D unmask specific genes which are then available for transcription by RNA polymerase. The messenger RNA thus produced could code for functional proteins involved in calcium metabolism.

Two such proteins have been identified in the intestine which may take part in the active transport of calcium. Wasserman and his colleagues have discovered, isolated and characterized a calcium-binding protein from chick intestinal mucosa which appears following vitamin D administration (Wasserman and Taylor, 1966; 1968; Wasserman et al., 1968). This protein has a high affinity for calcium and appears to be correlated with many aspects of intestinal calcium transport (Taylor and Wasserman, 1969). Another protein, a calcium-dependent adenosine triphosphatase has been demonstrated in the brush border isolated from the intestine of rats and chicks given vitamin D (Martin et al., 1969; Melancon and DeLuca, 1970). Enzymatic activity is minimal or absent in animals deficient in vitamin D, and there is a correlation between the time of appearance of the calcium-dependent ATPase and calcium transport. The interrelationship between these proteins is not known, but it is possible that both systems operate in calcium transport in the intestine or that the calcium-stimulated ATPase may be associated in some way with the action of the calcium-binding protein.

No such proteins have been found in bone where vitamin D also plays a role in calcium metabolism. In fact, Taylor and Wasserman (1969) have been unable to find a calcium-binding protein in bone. They report that if it is present it must be at a concentration of less than 10 $\mu\text{g/g}$ of bone cells on a dry weight basis, which is about 200 times less than that found in the intestinal mucosa of vitamin D treated rachitic chicks. Recently, Canas et al., (1969) have demonstrated that one of the early effects of vitamin D on bone in rachitic chicks is the

stimulation of bone collagen synthesis, which would provide new matrix for the deposition of mineral. This stimulatory action of the vitamin appeared to be somewhat specific for bone, because collagen synthesis in the skin of the same animal was unaffected.

The effect of actinomycin D, an inhibitor of DNA-directed RNA synthesis, on the actions of vitamin D is a subject of particular interest in attempting to understand the mode of action of vitamin D. Eisenstein and Passavoy (1964) first reported that the hypercalcemic response to large doses of vitamin D was blocked by actinomycin D. That actinomycin D blocks both the rise in serum calcium normally induced by vitamin D and the increased transport of calcium by the intestine was shown by Zull et al., (1965) and Norman (1965). Actinomycin D given after vitamin D administration did not block the effect of the vitamin, suggesting that it inhibits some very early event necessary for the expression of the physiological action of vitamin D. Stohs et al., (1967) found that actinomycin D completely blocked the vitamin D stimulated increase in RNA synthesis in intestinal mucosa. In the intestinal perfusion system described by Olson and DeLuca (1969), actinomycin D administered to the animal prior to 25-HCC blocked the rise in calcium transport but actinomycin D given after 25-HCC had no effect (DeLuca, 1969). Izzo, (1970), however, found that the stimulatory action of vitamin D on RNA synthesis in bone could not be completely blocked by pretreatment with actinomycin D.

Actinomycin D is known to block DNA-dependent RNA synthesis by binding to the double stranded DNA. Actinomycin D interacts with a

guanosine-cytosine nucleotide pair by intercalating its chromophore between two adjacent base pairs in the DNA (Muller and Crothers, 1968). However, little is known about the mechanism of binding of actinomycin D to the cell nucleus in vivo. Ringertz and Bolund (1969) found that preparations of deoxyribonucleoprotein bound only one molecule of actinomycin per 35-140 nucleotides whereas DNA preparations bound 1 actinomycin D per 14-20 nucleotides. The binding of actinomycin D to DNA in the chromatin of different eucaryotes showed properties similar to the binding to DNA, but this binding was restricted by the chromosomal proteins.

Low doses of actinomycin D have been shown to effectively suppress the formation of nucleolar and cytoplasmic RNAs, while leaving the synthesis of other nuclear RNAs relatively unaffected (Perry, 1963). Penman et al., (1968) have also studied this heterogeneous nucleoplasmic RNA, whose synthesis is relatively unaffected by doses of actinomycin D which completely inhibit synthesis of ribosomal RNA. This RNA has a unique base composition of 45 moles % guanine plus cytidine, resembling that of DNA, as compared with ribosomal RNA's 70 moles % guanine plus cytidine. This DNA-like RNA has been separated from other RNAs by successive phenol extractions at increasing temperatures (Georgiev et al., 1963). Treatment at 25°C yields cytoplasmic RNA which includes transfer RNA and ribosomal RNA. Treatment at 45°C yields nucleolar RNA which is believed to be the newly formed ribosomal RNA. Extraction at 65°C yields nucleoplasmic RNA, a heterogeneous DNA-like RNA, which may be a precursor to mRNA. This 65°C RNA fraction has been shown to have a

much greater template activity in a cell free system than the other RNA fractions (Mach and Vassalli, 1965), but whether a small portion or any of it is truly messenger RNA has yet to be shown.

An understanding of how actinomycin D blocks vitamin D action is important in determining the actions of vitamin D at the cellular level. The failure of actinomycin D to completely inhibit the vitamin D-stimulated increase in RNA synthesis in bone might reflect a relatively larger increase in the synthesis of this heterogeneous nucleoplasmic RNA in bone cells in response to vitamin D treatment than in the intestine.

STATEMENT OF THE PROBLEM

The end effect of vitamin D is the calcification of bone, but little is known about the direct action of the vitamin on bone. Most studies on vitamin D action have focused on its role in the intestinal absorption of dietary calcium. For many years it was widely accepted that this action was the sole basis for the antirachitic action of vitamin D. The stimulation of bone mineral mobilization by large doses of vitamin D was well known but was generally assumed to represent a toxic rather than a physiologic action of the vitamin. Carlsson (1952) provided indirect evidence that low doses of vitamin D can enhance the movement of calcium from skeletal tissue to blood by stimulating bone resorption. This has been confirmed by the findings of Blunt et al, (1968b) who showed that both vitamin D₃ and 25-HCC cause an increase in the serum calcium of vitamin D deficient rats on a calcium-free diet. There is now general agreement that vitamin D plays a role in the physiologic

regulation of bone resorption, but little is known about how the vitamin acts on bone cells to cause this response.

Parathyroid hormone (PTH) is also an important factor in stimulating bone resorption. Steinberg and Nichols (1968) found a very early stimulation by PTH of DNA-directed RNA synthesis in at least some bone cells. Owen and Bingham (1968) using autoradiography, found that PTH stimulated RNA synthesis in osteoclasts and inhibited RNA synthesis in osteoblasts. Raisz (1970) has reported that after a few hours of exposure to 25-HCC or PTH, either singly or together, bone cultures begin to show resorption and this process continues even when the agents are removed. Actinomycin D, when present during or immediately after the treatment with 25-HCC or PTH, abolishes the bone resorption response, but actinomycin D did not completely block the response when given two hours after 25-HCC or PTH. This indicates that an actinomycin D sensitive step, which is presumably RNA synthesis, is involved in the induction of the resorptive response.

Izzo (1970) using an in vivo system found that vitamin D₃ stimulated the incorporation of RNA precursors into bone RNA. Whether this increase in RNA synthesis is related to bone resorption or in some way to the calcification process is not known. However, he found that pretreatment with actinomycin D could not completely block the vitamin D stimulated increase in RNA synthesis. Since the synthesis of some nuclear RNAs are known to be insensitive to inhibition by actinomycin D, it appeared possible that vitamin D was selectively stimulating the incorporation of ³H-orotic acid and ³H-uridine into these nuclear RNAs, which are

postulated to be precursors of messenger RNA. This messenger RNA could conceivably code for some protein or proteins necessary for increased calcium mobilization, or possibly for some protein such as collagen, necessary for calcification of new bone.

The purpose of this research is to investigate the effect of vitamin D on the synthesis of various types of RNA in bone cells and to determine the effect of pretreatment with actinomycin D. An in vitro system will be used to permit isolation of RNA with a higher specific activity than that obtained by Izzo (1970), but will still allow sufficient time for vitamin D₃ to be metabolized to its active form in the intact animal.

CHAPTER II

MATERIALS AND METHODS

GENERAL EXPERIMENTAL DESIGN

Rachitic rats were obtained by maintaining 50-60 gram Holtzman rats on a vitamin D deficient diet for 4 to 5 weeks. Reduced growth and increased serum alkaline phosphatase levels were used as parameters to determine that the rats were rachitic.

The rats were divided into two groups; in one group each rat received an injection of 5,000 I.U. of vitamin D₃, and each rat in the other group received an injection of the vehicle only. In some experiments, each rat received an injection of 100 µg of actinomycin D two hours prior to treatment with vitamin D or the vehicle. Five hours after the administration of the vitamin or vehicle the rats were sacrificed and the humeri, tibiae and femurs removed. Fragments of trabecular bone were dissected out, washed and incubated in Krebs-Ringer bicarbonate buffer containing 1 µCi/ml of ³H-uridine at 37°C in an atmosphere of 5% CO₂ and 95% O₂ for 4 hours. After incubation the bone fragments were washed repeatedly and the bone cells isolated.

In some experiments RNA was extracted from the bone cells and the specific activity of the RNA was determined to see if vitamin D stimulated the incorporation of ³H-uridine into RNA in this in vitro system. The RNA was extracted from the bone cell pellets with phenol-sodium dodecyl sulfate (SDS). The RNA was precipitated from solution with sodium chloride and ethanol at -20°C for 16 hours and collected by centrifugation at

12,000 g for 20 minutes. After treatment with DNase, residual protein was removed by extraction with phenol-SDS, and precipitating and centrifuging as above. A Sephadex G-25 column was used to free the RNA from the phenol and DNA nucleotides. The RNA was then precipitated and centrifuged and the final precipitate dissolved in a small volume of sodium acetate buffer for spectrophotometric measurement and for scintillation counting. A comparison of the specific activities of the treated and control samples was made to determine whether or not vitamin D enhanced the incorporation of ^3H -uridine into bone cell RNA.

In other experiments, the RNA was separated into two fractions by sequential treatment with phenol and SDS at 45°C and 65°C . Each fraction of RNA was purified and concentrated as above. The specific activities of the RNA fractions from treated and control animals were compared to determine the effect of vitamin D and actinomycin D on the synthesis of the various types of RNA.

ANIMAL MAINTENANCE

Male albino rats (Holtzman Company, Madison, Wisconsin) weighing 50 to 60 grams were maintained in a room without sunlight on a vitamin D deficient diet with a calcium/phosphorus ratio of 5.85 (Rachitogenic Test Diet, U.S.P., General Biochemicals, Chagrin Falls, Ohio). Animals fed a normal diet (Wayne Lab Blox, Allied Mills, Chicago, Illinois) served as controls. A total of twenty control rats, used at various times over a period of about one year, ranged in weight from 230 to 270 grams after maintenance for four weeks on the normal diet. One hundred thirty-four

rats fed the vitamin D deficient diet, used as various times over the same period of one year, ranged in weight from 70 to 90 grams.

DETERMINATION OF SERUM ALKALINE PHOSPHATASE

In addition to reduced growth, serum alkaline phosphatase levels were measured to confirm that the rats were rachitic. Increased serum alkaline phosphatase is one of the early signs of a rachitic state.

The method used for determination of serum alkaline phosphatase is that of Bessey et al., (1946). Alkaline phosphatase catalyzes the hydrolysis of the substrate, p-nitrophenylphosphate into p-nitrophenol and phosphate. p-Nitrophenylphosphate absorbs in the ultraviolet region but not in the visible range, whereas p-nitrophenol in alkaline medium is yellow and has an absorption maximum at 400 nm. The phosphatase activity is directly proportional to the amount of p-nitrophenol liberated per unit time.

Preparation of Solutions

1. Alkaline buffer solution - 0.2 M Na_2CO_3 - NaHCO_3 buffer, pH 9.4

1.06 grams of Na_2CO_3 and 3.36 grams of NaHCO_3 are dissolved in distilled water and the solution diluted to 250 ml.

2. Substrate - p-nitrophenylphosphate

20 mg of p-nitrophenylphosphate (Sigma Chemical Company, St. Louis, Missouri) is dissolved in 5 ml of distilled water and to this solution is added 175 ml of the alkaline buffer solution, pH 9.4.

3. Standard - p-nitrophenol, 5 mM

69.6 mg of p-nitrophenol (Sigma Chemical Company) is dissolved

in 0.02 N NaOH and diluted to 100.0 ml with 0.02 N NaOH.

Five microliters of various dilutions of the standard solution of p-nitrophenol were added to 100 μ l of substrate and 200 μ l of 0.02 N NaOH. After mixing, the optical density was measured at 410 nm in the Beckman/Spinco 151 Spectro-Colorimeter. This data which was used in the preparation of a standard curve is shown in Table I. The linear relationship between the optical density at 410 nm and the concentration of p-nitrophenol is shown in Figure 1.

Serum alkaline phosphatase measurements were carried out using serum obtained from blood samples collected from the tail vein of the rats. Five microliters of serum were added to 100 μ l of substrate and incubated at 25°C for 30 minutes. A blank was prepared by using 5 μ l of distilled water and a standard was prepared with each determination using 5 μ l of 1.667 mM p-nitrophenol.

At the end of the incubation period, the reaction was terminated by adding 200 μ l of 0.02 N NaOH to each tube. The optical density was measured at 410 nm in the Beckman/Spinco 151 Spectro-Colorimeter. A correction for the yellowish color of serum was made by adding 5 μ l of serum to 300 μ l of 0.02 N NaOH and subtracting the optical density at 410 nm from that of the sample.

A unit of alkaline phosphatase activity is defined as the amount of enzyme that will liberate 1 μ m of p-nitrophenol in 1 hour at pH 9.4 and 25°C. For comparison of normal and vitamin D deficient values, the alkaline phosphatase activity is expressed as units per milliliter of serum.

TABLE I

STANDARD CURVE DATA FOR DETERMINATION OF SERUM ALKALINE PHOSPHATASE

$\mu\text{m p-Nitrophenol}$ $\times 10^{-3}/\text{ml}$	Number of Samples	O.D. 410 nm*
2.2	4	0.020 ± 0.005
4.5	4	0.043 ± 0.005
9.1	4	0.084 ± 0.001
18.2	4	0.161 ± 0.009
27.3	4	0.238 ± 0.016
36.4	4	0.314 ± 0.002
45.4	4	0.398 ± 0.016

* Mean \pm Standard Deviation

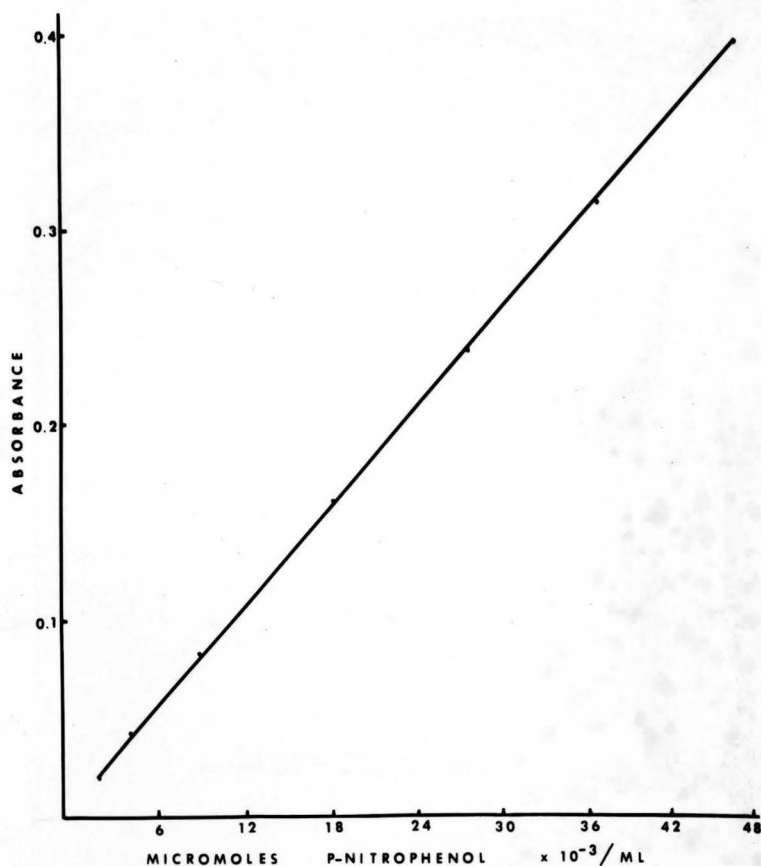


FIGURE 1. Standard Curve for Determination of Serum Alkaline Phosphatase

TREATMENT WITH VITAMIN D AND ACTINOMYCIN D

Five milligrams of crystalline vitamin D₃ (Mann Laboratories, New York, New York) were dissolved in 0.5 ml of 95% ethanol and then diluted to 10 ml with 1,2-propanediol. Five milligrams of vitamin D₃ are equivalent to 200,000 International Units; therefore, one milliliter of this solution contained 20,000 I. U. of vitamin D₃. Each rat to be treated with vitamin D received an intraperitoneal injection of 0.25 ml of this solution containing 5,000 I. U. of vitamin D₃.

The control animals were injected with 0.25 ml of the vehicle. This solution was prepared by mixing 0.5 ml of 95% ethanol and 9.5 ml of 1,2-propanediol.

A solution of actinomycin D was prepared by dissolving actinomycin D (Calbiochem, Los Angeles, California) to a concentration of 200 µg/ml in 0.9% saline. Each rat to be treated with actinomycin D received an intraperitoneal injection of 100 µg (0.5 ml) of this solution.

In each experiment, equal numbers of rachitic rats were injected with vitamin D and with the vehicle. The rats were injected at 20 minute intervals so that each animal could be sacrificed and the bone samples prepared for incubation exactly 5 hours after injection. In some experiments actinomycin D was injected 2 hours prior to the treatment with vitamin D or vehicle.

INCUBATION OF BONE FRAGMENTS WITH ³H-URIDINE

Five hours after treatment with vitamin D₃ or vehicle the animals were sacrificed by decapitation. The humeri, tibias and femurs were

quickly removed and placed in cold Krebs-Ringer bicarbonate buffer solution. After all the bones from one rat were collected, each bone was freed from extraneous tissue and split longitudinally. The bone marrow was washed out with pressure by using a syringe containing Krebs-Ringer bicarbonate buffer solution and a 25 gauge needle. Small pieces of trabecular bone were dissected out and washed again. The cleaned bone fragments had a slight creamy color, but no visible evidence of bone marrow contamination.

The bone fragments from each rat were placed in a small vial containing 2.5 ml of the incubation medium and loosely covered with aluminum foil. The samples were incubated in a shaking water bath at 37°C in an atmosphere of 5% CO₂ and 95% O₂ for four hours. At the end of the incubation period the samples were chilled in an ice bath and the medium immediately removed. The bone fragments were then washed 4 times with cold Krebs-Ringer bicarbonate buffer and 4 times with cold phosphate-buffered saline (PBS) and kept in cold PBS until all samples in an experimental group were collected.

Preparation of Solutions

1. Krebs-Ringer bicarbonate buffer with 11 mM glucose, pH 7.4

1000 ml of 0.90% NaCl

40 ml of 1.15% KCl

30 ml of 1.22% CaCl₂

10 ml of 2.11% KH₂PO₄

10 ml of 3.82% MgSO₄ · 7H₂O

The above solutions are mixed and to this mixture is added 210 ml of 1.30% NaHCO_3 and 2.58 grams of glucose. Five percent CO_2 is bubbled through the solution for 10 minutes to obtain a pH of 7.4. The buffer solution is stored in glass stoppered bottles in the cold.

2. Incubation medium

^3H -uridine (Uridine-5-T) with a specific activity of 24.9 Ci/mM (Amersham/Searle, DesPlaines, Illinois) was obtained at a concentration of 1 mCi/ml. A stock solution of 100 $\mu\text{Ci/ml}$ was prepared by diluting 1.0 ml of the ^3H -uridine to 10.0 ml with distilled water. Incubation media was made by diluting 1.0 ml of the stock solution of ^3H -uridine to 100.0 ml of Krebs-Ringer bicarbonate buffer, giving a concentration of 1 $\mu\text{Ci/ml}$ of ^3H -uridine.

3. Phosphate buffered saline (PBS)

A solution of 2.90 g/l of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in 0.15 M NaCl and a solution of 0.768 g/l of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ in 0.15 M NaCl were mixed in proportions to give a pH of 7.4.

ISOLATION OF BONE CELLS

The method employed for the isolation of bone cells is based on the procedure described by Nichols et al., (1965). The bone fragments from 6 to 12 animals on a like treatment were pooled and transferred to a cold mortar. The bone fragments were ground by hand for 3 minutes with about 10 ml of a solution of PBS containing 0.0673 grams of calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) per 100 ml of PBS. The liquid was then transferred to conical

centrifuge tubes. The grinding of the residue was repeated 2 more times, adding the liquid to the previously collected solution. This solution was then allowed to stand in the conical tubes in the cold for 20 minutes. The calcified matrix material settles to the bottom of the tube and the cells remain in solution. The supernatant was carefully removed, placed in other tubes, and bone cells were collected in a pellet by centrifugation at 750 g for 10 minutes (Sorvall, Refrigerated Model RCB-2, with SS-1 Rotor). The bone cell pellets were kept frozen at -20°C until extraction of RNA.

To confirm that we were isolating bone cells and not bone marrow cells, both were examined by light microscopy. Bone marrow was collected from bones which had been split longitudinally by scooping out the bone marrow and collecting it in a tube containing cold PBS. The cells were collected by centrifugation at 750 g for 10 minutes. Both bone cell and bone marrow cell pellets were fixed in osmic acid and embedded in Epon. One micron sections were stained with toluidine blue and examined by light microscopy.

EXTRACTION OF RNA

The method used for extraction of RNA was the widely used phenol extraction method described by Kirby (1956) which depends on the partition of RNA between aqueous buffer and 88% phenol. Steinberg and Nichols (1967) used this method to isolate RNA from normal bone cells. The method outlined below is the same as that used by Steinberg and Nichols except for the use of a Sephadex G-25 column to separate RNA from phenol.

The pellet of bone cells was homogenized in 9 ml of a sucrose medium composed of 0.25 M sucrose and 1 mM MgCl_2 in 10 mM Tris-HCl buffer, pH 7.4, using a ground glass homogenizer. The homogenate was made 0.5% with respect to SDS by adding 1 ml of a 5% solution of SDS. After stirring for 10 minutes at room temperature, an equal volume (10 ml) of phenol was added and the sample stirred at 4°C for 30 minutes. Centrifugation at 12,000 g for 10 minutes was used to break the emulsion. The upper aqueous phase and the material at the interface were removed and reextracted with 5 ml of phenol for 10 minutes at 4°C and centrifuged. The phenol layer obtained in the first centrifugation was reextracted with 10 ml of the sucrose medium for 10 minutes at 4°C and centrifuged. The aqueous phases from each of these two latter centrifugations were carefully removed and combined.

The RNA was precipitated from solution by adding 0.1 volume of 2 M NaCl and 2 volumes of 95% ethanol and storing overnight at -20°C in a freezer. The RNA was collected by centrifugation at 15,000 g for 20 minutes. The precipitate was resuspended in 8 ml of 0.02 M MgSO_4 in 0.02 M Tris-HCl buffer, pH 7.4. This solution of RNA was treated with deoxyribonuclease (Worthington Biochemical Corp., Freehold, New Jersey), at a concentration of 10 $\mu\text{g/ml}$ for 20 minutes at room temperature with stirring. The residual protein was removed by extracting again with 0.5% SDS and 0.5 volume of phenol for 15 minutes at 4°C. After centrifugation to break the emulsion the aqueous phase was removed and the RNA was precipitated from solution with sodium chloride and ethanol as before. After 90 minutes or longer, the precipitate was collected by centrifugation.

This precipitate was dissolved in 1 to 2 ml of distilled water and was passed through a Sephadex G-25 column to remove the DNA nucleotides and phenol. The eluent from the column was monitored in a Beckman DB spectrophotometer, using a 5 mm flow cell, by observing the increase in optical density at 258 nm. The RNA collected from the column was again precipitated with sodium chloride and ethanol and the final precipitate dissolved in a small volume of 0.25 M sodium acetate buffer, pH 5.3.

The amount of RNA present was determined by using a standard yeast RNA sample (Sigma Chemical Company) and the Beckman DB spectrophotometer at a wavelength of 258 nm.

PHENOL FRACTIONATION OF RNA

RNA was extracted in the same way as described before, except that after stirring the bone cell homogenate-phenol-SDS mixture for 30 minutes at 4°C, this mixture was heated and stirred for 15 minutes at 45°C. After centrifugation to break the emulsion the upper phase was removed without disturbing the interface and the lower phase and interface were reextracted with sucrose media and SDS for 15 minutes at room temperature. After centrifugation the upper phase was carefully removed and combined with the previous aqueous phase. The combined aqueous phases were reextracted with 5 ml of phenol for 15 minutes at room temperature. After centrifugation, the upper phase was removed and the RNA precipitated from solution as described in the previous section.

Sixty-five degree RNA was extracted by adding fresh phenol to the interface material and homogenizing with SDS and sucrose media. After stirring for 10 minutes at room temperature, the sample was heated and stirred at 65°C for 15 minutes. It was then centrifuged, reextracted and precipitated in the same manner as the 45°C RNA.

Each of these samples, 45°C RNA and 65°C RNA, were then treated with DNase, extracted again with phenol, separated on a Sephadex G-25 column and the amount of RNA present determined as described in the previous section.

LIQUID SCINTILLATION COUNTING

A 0.2 or 0.5 ml aliquot of the sample of RNA was placed in a liquid scintillation counting vial and to this was added 15 ml of the toluene counting solution. The toluene counting solution was prepared by dissolving 5 grams of PPO (2,5-diphenyloxazole) in one liter of toluene. To every 100 ml of this solution was added 20 ml of Beckman solubilizing reagent Bio-Solv BBS-3. The samples were counted in the LS-250 Liquid Scintillation Spectrometer using the automatic quench correction. A quench curve was prepared by adding various amounts of RNA isolated from bone cells in 0.25 M sodium acetate buffer, pH 5.3 to 15 ml of the toluene counting solution containing 0.1 μ Ci of ^3H -uridine. These samples were counted with the LS-250 Liquid Scintillation Spectrometer (Beckman Instruments) using the automatic quench correction. The per cent efficiency was plotted against the external standard ratio (Figure 2).

The specific activity of the RNA was expressed as disintegrations per minute per milligram of RNA (dpm/mg RNA).

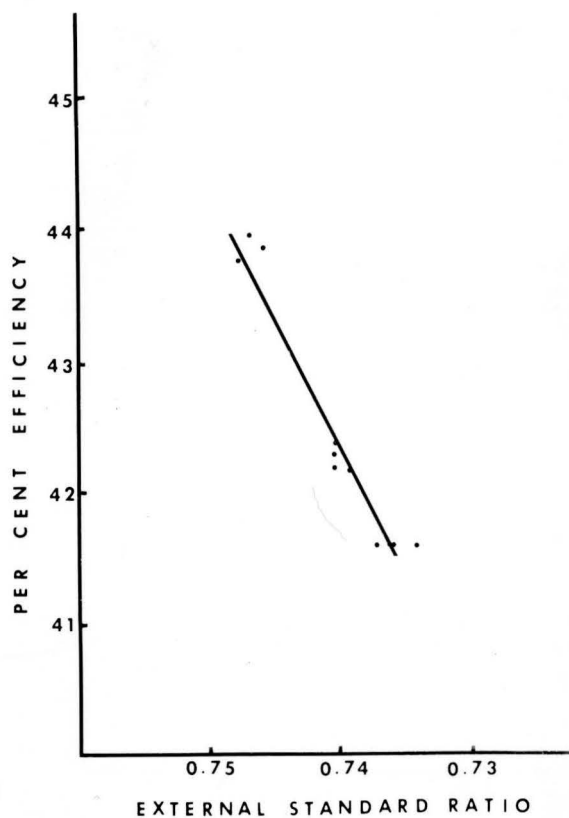


FIGURE 2. ^3H -Uridine Quench Curve

CHAPTER III

EXPERIMENTAL RESULTS

SERUM ALKALINE PHOSPHATASE ACTIVITY IN NORMAL AND RACHITIC RATS

Serum alkaline phosphatase measurements were made on two rats fed a normal diet and two or three rats fed a vitamin D deficient for 4 weeks selected at random from each group of animals to be used for a given experiment. As shown in Table II, the serum alkaline phosphatase levels of the vitamin D deficient rats were significantly higher than those of rats fed a normal diet. These results, in accord with the much lower body weight, show that the rats fed a vitamin D deficient diet are indeed rachitic. Additionally, this conclusion was confirmed by the appearance of the bones when the rats were sacrificed.

BONE CELL PREPARATIONS

Since the spaces in trabecular bone are filled with bone marrow cells, it was necessary to determine that the cells released by grinding trabecular bone fragments were bone cells and not bone marrow cells. Bone cell pellets were a creamy white color. The absence of a grossly red color as one sees with bone marrow cells was one of the criteria used to determine that bone cells and not bone marrow cells were being isolated. Histological examination of bone cell and bone marrow cell preparations showed that although the bone cell pellets contained a heterogenous mixture of cells (Figure 3), there were very few cells typical of bone marrow such as red blood cells, megakaryocytes and myeloid cells (Figure 4).

SERUM ALKALINE PHOSPHATASE ACTIVITY OF NORMAL AND RACHITIC RATS

(units/ml serum)

<u>Normal</u>	<u>Rachitic</u>
3.09	6.44
1.75	5.17
1.96	4.87
2.49	5.78
2.74	5.83
2.65	4.30
2.44	4.66
2.46	4.16
2.73	5.19
2.49	5.53
2.84	5.17
2.26	4.54
2.48	5.42
2.56	4.64
2.83	3.95
<u>3.05</u>	6.24
* 2.56 ± 0.32	4.75
	7.30
	<u>4.93</u>
	* 5.20 ± 0.84

* Mean \pm Standard Deviation.

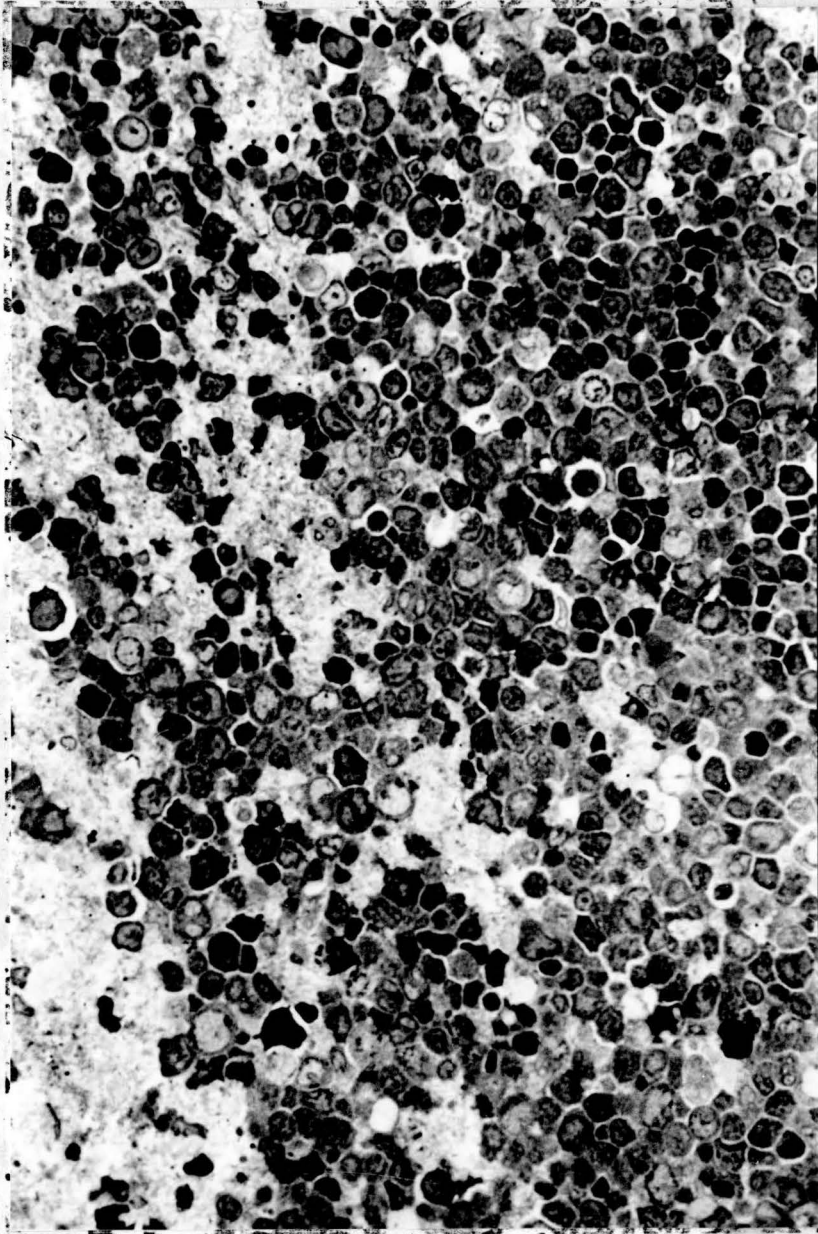


FIGURE 3. Isolated Bone Cells (x 600)

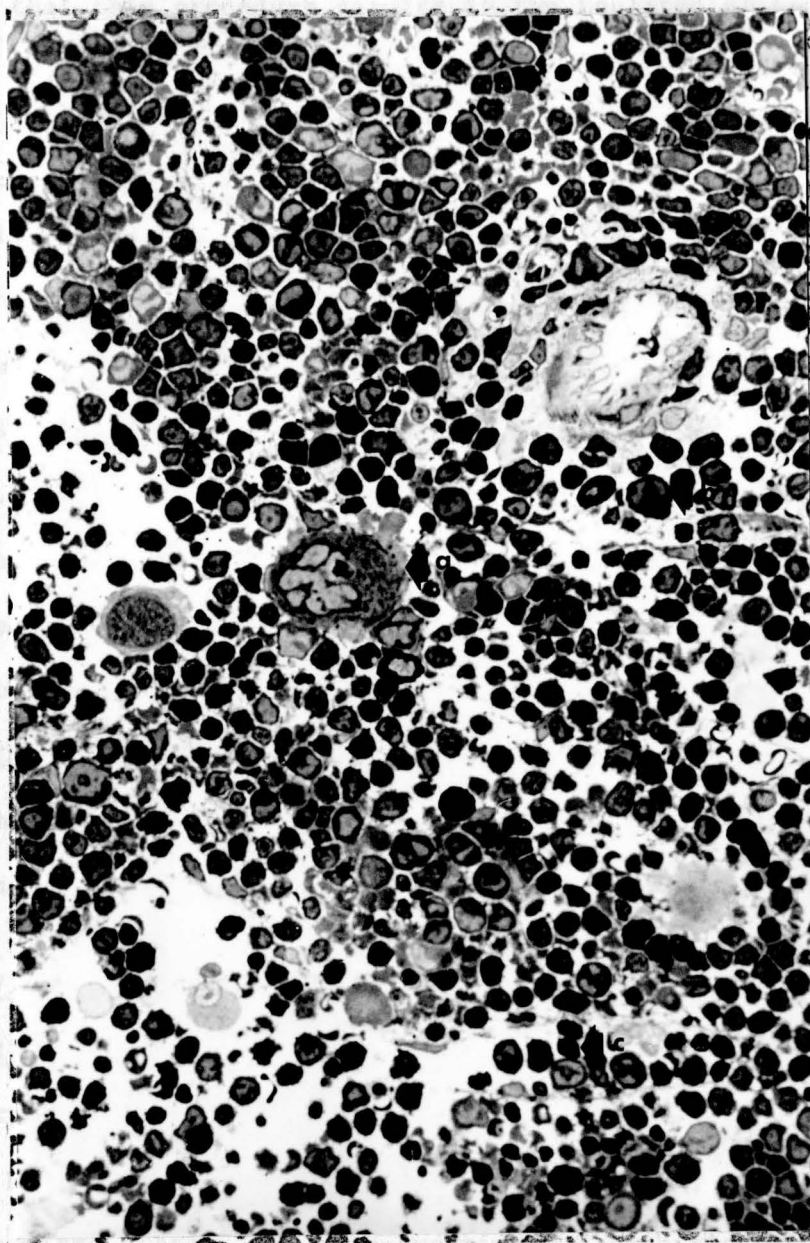


FIGURE 4. Bone Marrow Cells (x 600). (a) megakaryocyte, (b) myeloid cell, (c) red blood cell.

It appears that this procedure results in the isolated of bone cells relatively uncontaminated with bone marrow cells.

RNA FROM BONE CELLS

A typical separation of RNA from phenol on a Sephadex G-25 column is shown in Figure 5. To establish that any DNA digested by DNase treatment would be separated on this column, an excess of DNA (Worthington Biochemical Corp.) was digested with DNase and passed through the column (Figure 6). No such peaks were observed between RNA and phenol in any of the experiments, indicating that little or no DNA was extracted.

The spectrum of RNA isolated from bone cells of eleven rachitic rats is shown in Figure 7 and for comparison a spectrum of purified yeast RNA (Sigma Chemical Company) is also shown. The amounts of RNA in each sample in the various experiments ranged from 5 to 25 μ g. The optical density at 258 nm was used to calculate the amount of RNA present.

EFFECT OF VITAMIN D ON THE INCORPORATION OF ³H-URIDINE INTO BONE CELL RNA IN VITRO

Two experiments were carried out in order to determine the effect of vitamin D₃ on the incorporation of ³H-uridine into bone cell RNA using an in vitro system. In each experiment 8 rachitic rats were injected intraperitoneally with 5,000 I.U. of vitamin D₃ and 8 rachitic rats were injected with the vehicle only. Rats were injected at 20 minute intervals so that each animal could be sacrificed and the tibias, femurs and humeri removed and cleaned exactly 5 hours after treatment. Bone

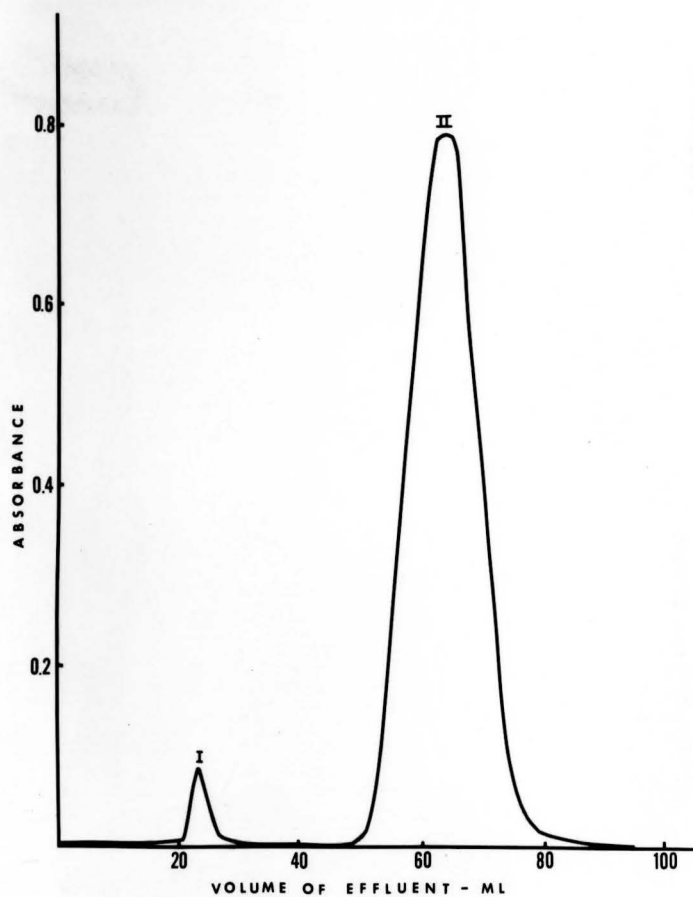


FIGURE 5. Separation of RNA from Phenol on a Sephadex G-25 Column. (I) RNA, (II) Phenol

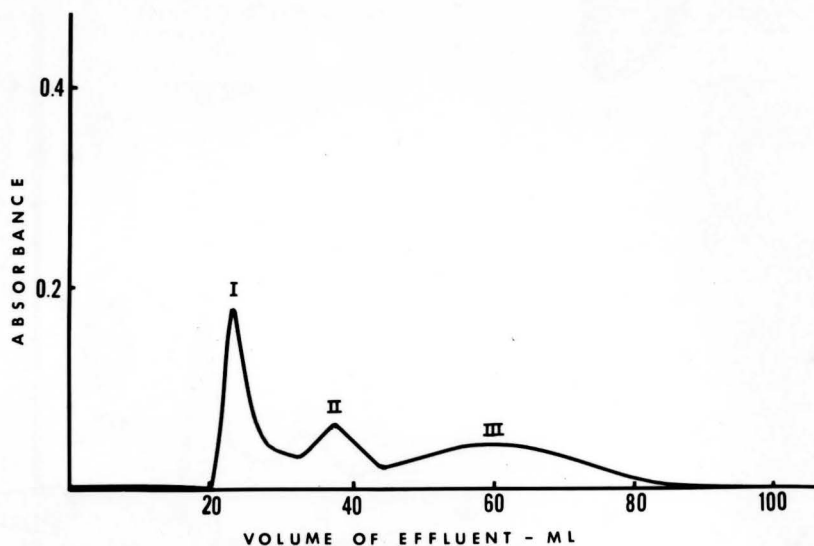


FIGURE 6. Separation of DNA and Nucleotides after DNase Digestion, on a Sephadex G-25 Column. (I) DNA, (II) and (III) Fragments of DNA recovered after DNase treatment.

FIGURE 7. Spectra of Bone Cell RNA and Purified Yeast RNA. (I) Bone Cell RNA; (II) Yeast RNA.

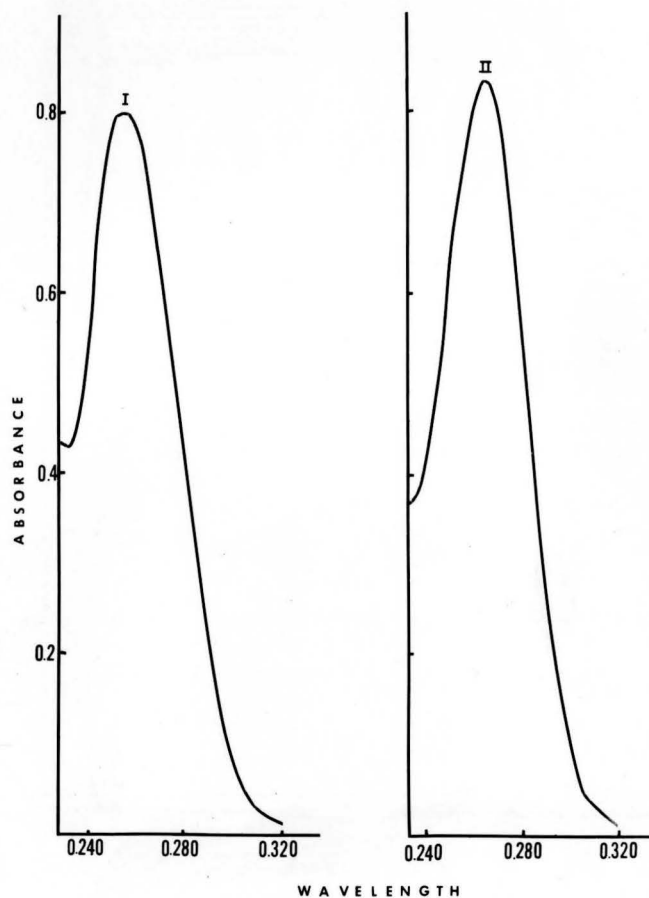


FIGURE 7. Spectra of Bone Cell RNA and Purified Yeast RNA.
(I) Bone Cell RNA, (II) Yeast RNA

fragments were incubated for 4 hours with ^3H -uridine and then washed and chilled in an ice-bath to stop the incorporation of the isotope. A total of 9 hours from the time of treatment with vitamin D or vehicle to the end of the exposure to ^3H -uridine was chosen because Izzo (1970) found that 9 hours was the time of maximal incorporation of precursors into RNA in bone using an in vivo system.

The bone fragments from the 8 rats in each experimental group were pooled and bone cells isolated. RNA was extracted and measured as described in Chapter II. Aliquots were then used for scintillation counting and the specific activity expressed as disintegrations per minute per milligram of RNA.

In both experiments, the specific activity of the RNA from the vitamin D treated animals was greater than that of the controls (Table III). The ratios of the specific activity of the RNA from vitamin D treated rats and the specific activity of the RNA from rats treated with the vehicle only (T/C ratio) were comparable to the results reported by Izzo (1970) which were 1.36, 1.06, 1.48, 1.74 and 1.42 giving an average of 1.4. The specific activity of the RNA in these experiments was about forty times higher than those of the in vivo experiments by Izzo (1970).

It can be concluded from these results that, in agreement with the experiments of Izzo (1970), vitamin D does enhance the incorporation of ^3H -uridine into RNA into bone in this in vitro system.

TABLE III

EFFECT OF VITAMIN D ON THE INCORPORATION OF ^3H -URIDINE INTO BONE CELL
RNA IN VITRO^a

Experiment		Specific Activity of RNA (dpm/mg RNA)	<u>Treated</u> ^b <u>Control</u>
1	Control	71,708 \pm 1076 ^c	1.86
	Vitamin D	133,248 \pm 1999	
2	Control	88,507 \pm 1328	1.33
	Vitamin D	117,962 \pm 1769	

^a Rachitic rats were sacrificed 5 hours after injection with 5,000 I.U. of vitamin D₃ or vehicle. Bone fragments were incubated for 4 hours with ^3H -uridine. RNA was extracted from bone cells isolated from these bone fragments.

^b Ratio of the specific activity of RNA from vitamin D treated rats and the specific activity of RNA from rats treated with the vehicle only.

^c Specific activity \pm 1.5% counting error.

EFFECT OF ACTINOMYCIN D ON THE VITAMIN D STIMULATED INCORPORATION OF
³H-URIDINE INTO BONE CELL RNA IN VITRO

The next experiment was designed to determine the effect of actinomycin D on the increased incorporation of ³H-uridine into bone cell RNA following vitamin D treatment in this in vitro system. The procedure was the same as outlined in the previous experiment. Each rat received two intraperitoneal injections of 50 µg each of actinomycin D, one 6 hours before and one 2 hours before treatment with vitamin D or vehicle. Each of the two experimental groups consisted of eight animals.

The results shown in Table IV indicate that actinomycin D was effective in decreasing the incorporation of ³H-uridine into RNA in both the vitamin D treated and control animals as compared with those animals not treated with actinomycin D (Table III). However, the specific activity of the bone cell RNA from vitamin D treated animals was still greater than that of the controls.

EFFECT OF VITAMIN D ON THE INCORPORATION OF ³H-URIDINE INTO BONE CELL
RNAS EXTRACTED AT 45°C AND 65°C

The synthesis of some nuclear RNAs, which are thought to be precursors to messenger RNA, have been shown to be unaffected by actinomycin D pretreatment. These RNAs can be isolated by sequential fractionation with phenol-SDS. Treatment at 45°C should yield cytoplasmic and nucleolar RNA. Following treatment at 45°C these nuclear RNAs, insensitive to actinomycin D, can be obtained by extraction at 65°C.

The next two experiments were designed to determine the effect of vitamin D on the incorporation of ³H-uridine into these two fractions of

TABLE IV

EFFECT OF ACTINOMYCIN D ON THE VITAMIN D STIMULATED INCORPORATION OF
³H-URIDINE INTO BONE CELL RNA IN VITRO^a

	Specific Activity of RNA (dpm/mg RNA)	<u>Treated</u> ^b <u>Control</u>
Control	13,557 ± 407 ^c	1.68
Vitamin D	22,816 ± 684	

^aRachitic rats were given two intraperitoneal injections of 50 µg of actinomycin D 6 hours and 2 hours before an injection of 5,000 I.U. of vitamin D₃ or vehicle. Bone fragments were incubated for 4 hours with ³H-uridine. RNA was extracted from bone cells isolated from these bone fragments.

^bRatio of the specific activity of RNA from vitamin D treated rats and the specific activity of RNA from rats treated with the vehicle only.

^cSpecific activity ± 3.0% counting error.

RNA. Two experimental groups, each consisting of eight animals, were used. The experiments were carried out as previously described, but the RNA was separated into two fractions as described in the chapter on Materials and Methods.

The results presented in Table V show that vitamin D stimulated the incorporation of ^3H -uridine into both 45°C and 65°C RNA. In both experiments, the treated/control ratio of specific activities was greater for the 65°C than for the 45°C RNA.

EFFECT OF ACTINOMYCIN D ON THE VITAMIN D STIMULATED INCORPORATION OF ^3H -URIDINE INTO BONE CELL RNAS EXTRACTED AT 45°C AND 65°C

The next experiment tested the effect of actinomycin D on the vitamin D stimulated incorporation of ^3H -uridine into the two RNA fractions. The experiment was done as previously described except that 12 rats were used in each experimental groups and each rat received an intraperitoneal injection of $100\text{ }\mu\text{g}$ of actinomycin D two hours before treatment with vitamin D or vehicle.

The results given in Table VI show that actinomycin D did not inhibit the vitamin D stimulated incorporation of ^3H -uridine into either 45°C or 65°C RNA. In fact, the treated/control ratio of specific activities of the 45°C RNA appears to be increased by actinomycin D pretreatment.

Vitamin D increased the incorporation of ^3H -uridine into both fractions of bone cell RNA and this response was not inhibited by actinomycin D.

EFFECT OF VITAMIN D ON THE INCORPORATION OF ^3H -URIDINE INTO BONE CELL
RNAs EXTRACTED AT 45°C AND 65°C ^a

Experiment	Temperature of Extraction of RNA		Specific Activity of RNA (dpm/mg RNA)	<u>Treated</u> ^b Control
1	45°C	Control	$24,078 \pm 482$ ^c	1.22
		Vitamin D	$29,334 \pm 587$	
	65°C	Control	$34,473 \pm 689$	1.45
		Vitamin D	$49,902 \pm 998$	
2	45°C	Control	$56,954 \pm 1139$	1.35
		Vitamin D	$76,722 \pm 1534$	
	65°C	Control	$17,447 \pm 349$	1.62
		Vitamin D	$28,276 \pm 566$	

^a Rachitic rats were sacrificed 5 hours after injection with 5,000 I.U. of vitamin D₃ or vehicle. Bone fragments were incubated for 4 hours with ^3H -uridine. After isolation of bone cells, RNA was fractionated into RNAs extractable at 45°C and 65°C .

^b Ratio of the specific activity of RNA from vitamin D treated rats and the specific activity of RNA from rats treated with the vehicle only.

^c Specific activity $\pm 2.0\%$ counting error.

EFFECT OF ACTINOMYCIN D ON THE VITAMIN D STIMULATED INCORPORATION OF
³H-URIDINE INTO BONE CELL RNAs EXTRACTED AT 45°C AND 65°C^a

Temperature of Extraction of RNA		Specific Activity of RNA (dpm/mg RNA)	<u>Treated</u> ^b <u>Control</u>
45°C	Control	20,379 ± 408 ^c	1.85
	Vitamin D	37,663 ± 753	
65°C	Control	33,333 ± 667	1.49
	Vitamin D	49,583 ± 992	

^aRachitic rats were given 100 µg of actinomycin D intraperitoneally 2 hours before 5,000 I.U. of vitamin D₃ or vehicle. Bone fragments were incubated for 4 hours with ³H-uridine. After isolation of bone cells, RNA was fractionated into RNAs extractable at 45°C and 65°C.

^bRatio of the specific activity of RNA from vitamin D treated rats and the specific activity of RNA from rats treated with the vehicle only.

^cSpecific activity ± 2.0 % counting error.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Vitamin D has been reported to cause many metabolic changes including alterations in bone citrate content (Carlsson and Hollunger, 1954), phospholipid metabolism (Thompson and DeLuca, 1964; Neville and Holdsworth, 1968), Ca^{2+} release by mitochondria (Engstrom and DeLuca, 1964) and adenyl cyclase activity (Neville and Holdsworth, 1969), but the relation of these changes to the physiologic actions of vitamin D are not known. The question that arises here is whether the increased incorporation of nucleic acid precursors into RNA observed in bone under the stimulus of vitamin D is directly involved in the mode of action of the vitamin.

The effect of vitamin D on RNA synthesis in other tissues is of particular interest in respect to this work. In the intestine, Stohs et al., (1967), found that after the administration to rats of a 2,000 I.U. dose of vitamin D_3 , maximal incorporation of ^3H -orotic acid into nuclear RNA occurred at 3 hours with a T/C ratio of 2.81; however, this increase could be completely blocked by actinomycin D given 2 hours before treatment with vitamin D. Lawson et al., (1969a) found in chicks that 125 μg of vitamin D_3 increased the incorporation of ^3H -orotic acid into nuclear RNA; in three experiments they found T/C ratios of 1.83, 1.13 and 5.02, one hour after intracardial injection.

Stohs et al., (1967) also studied the effect of vitamin D on ^3H -orotic acid incorporation into nuclear RNA of rat liver and kidney,

tissues that are not known to be affected by vitamin D. They found T/C ratios of 1.30 for liver and 1.53 for kidney. These values are in the same range as those found for bone.

A possible explanation for the increased RNA synthesis in kidney can be found on the basis of a recent report by Tanaka and DeLuca (1971). They found that actinomycin D administered prior to radioactive 25-HCC blocked the metabolism of 25-HCC to the polar metabolite(s) that accumulate in the intestinal tissue. The site of biosynthesis for at least one of these metabolites is the kidney (Fraser and Kodicek, 1970). Actinomycin D, however, did not prevent the 25-hydroxylation of vitamin D₃ in the liver.

There is considerable evidence that vitamin D or its metabolites can interact with DNA to stimulate RNA synthesis in the intestine, and possibly in the kidney. There is also some evidence from organ cultures that this interaction precedes bone resorption. The results of our experiments indicate that vitamin D₃ does increase the rate of synthesis of both ribosomal and nucleoplasmic RNA in bone cells. The increased synthesis of RNA found in our system may be related to resorption, but since vitamin D is needed for normal calcification in addition to the resorption process even in the presence of an adequate dietary supply of calcium and phosphorus (DeLuca, 1967), the increase could be related to the calcification process.

The synthesis of the RNA extracted at 4°C in these experiments, which is most likely cytoplasmic RNA, was found to be largely inhibited by actinomycin D; however, the specific activity of the RNA from vitamin D treated animals was still larger than that of the controls. The two fractions

of RNA (45°C and 65°C) obtained in these experiments showed increased incorporation of ^3H -uridine into RNA, and this increased incorporation was not blocked by actinomycin D pretreatment. This indicates that vitamin D stimulated the synthesis of all nuclear RNAs in bone cells and not specifically the DNA-like nucleoplasmic RNA resistant to actinomycin D inhibition. In fact, the synthesis of RNA which can be extracted at 45°C, which others have found to be completely blocked by actinomycin D (Georgiev, 1967), appears to be increased in animals treated with actinomycin D and vitamin D (T/C ratio = 1.85) over those animals not treated with actinomycin D (T/C ratio = 1.22 and 1.35). Whether these numbers are significant is not known, but it is of interest to note that Izzo (1970) found that in each of three experiments where RNA was extracted at 65°C only, the T/C ratio was higher in the animals treated with actinomycin D than those treated with only the vitamin (T/C ratio 1.53 as compared to 1.31; 1.54 to 1.40; and 1.49 to 1.14).

Why actinomycin D does not inhibit the vitamin D stimulated increase in 45° RNA synthesis is puzzling. One possibility is that the dose of vitamin D₃ of 5,000 I.U. which is 500 times that considered physiological (10 I.U.) may be overcoming the effect of actinomycin D. Corradino and Wasserman (1968) reported that while actinomycin D inhibits the formation of vitamin D induced calcium-binding protein in the intestinal mucosa, this inhibition can be overcome by large doses of vitamin D. Complete inhibition of formation of calcium-binding protein was achieved when the vitamin D₃ dose was 100 I.U., but with 250 and 500 I.U. of vitamin D₃ progressively less inhibition was seen. Stohs et al., (1967), however,

used doses of 2,000 I.U. of vitamin D₃ and found that the increase in RNA synthesis caused by vitamin D₃ in the intestine could be completely inhibited by actinomycin D.

In summary, it was found that vitamin D increased the incorporation of ³H-uridine into all types of bone cell RNA. The vitamin D stimulated synthesis of cytoplasmic RNA could be largely blocked by pretreatment with actinomycin D, but vitamin D stimulated synthesis of nucleolar and nucleoplasmic RNA remained unaffected following actinomycin D treatment. It appears from these experiments that one effect of vitamin D is the stimulation of RNA synthesis in bone cells, but the importance of this increased RNA synthesis in the mode of action of the vitamin is not yet clear.

BIBLIOGRAPHY

- Bessey, O.A., O.H. Lowry and M.J. Brock. 1946. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J. Biol. Chem.* 164: 321-329.
- Blunt, J.W. and H.F. DeLuca. 1969. The synthesis of 25-hydroxycholecalciferol. A biologically active metabolite of vitamin D₃. *Biochemistry* 8: 671-675.
- Blunt, J.W., H.F. DeLuca and H.K. Schnoes. 1968a. 25-Hydroxycholecalciferol. A biologically active metabolite of vitamin D₃. *Biochemistry* 7: 3317-3322.
- Blunt, J.W., Y. Tanaka and H.F. DeLuca. 1968b. The biological activity of 25-hydroxycholecalciferol, a metabolite of vitamin D₃. *Proc. Nat. Acad. Sci. U.S.A.* 61: 1503-1506.
- Canas, F., J.S. Brand, W.F. Neuman and A.R. Terepka. 1969. Some effects of vitamin D₃ on collagen synthesis in rachitic chick cortical bone. *Amer. J. Physiol.* 216: 1092-1096.
- Carlsson, A. 1952. Tracer experiments on the effect of vitamin D on the skeletal metabolism of calcium and phosphorus. *Acta Physiol. Scand.* 26: 212-220.
- Carlsson, A. and G. Hollunger. 1954. The effect of vitamin D in the citric acid metabolism. *Acta Physiol Scand.* 31: 317-333.
- Corradino, R.A. and R.H. Wasserman. 1968. Actinomycin D inhibition of vitamin D₃-induced calcium-binding protein (CaBP) formation in chick duodenal mucosa. *Arch. Biochem. Biophys.* 126: 957-960.
- Cousins, R.J., H.F. DeLuca, T. Suda, T. Chen and Y. Tanaka. 1970. Metabolism and subcellular location of 25-hydroxycholecalciferol in intestinal mucosa. *Biochemistry* 9: 1453-1459.
- DeLuca, H.F. 1967. Mechanism of action and metabolic fate of vitamin D. *Vitam. Horm.* 25: 315-367.
- DeLuca, H.F. 1969. Recent advances in the metabolism and function of vitamin D. *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 28: 1678-1689.
- DeLuca, H.F. 1971. Role of kidney tissue in metabolism of vitamin D. *N. Engl. J. Med.* 284: 554-555.

- Eisenstein, R. and M. Passavoy. 1964. Actinomycin D inhibits parathyroid hormone and vitamin D activity. *Proc. Soc. Exp. Biol. Med.* 117: 77-79.
- Engstrom, G.W. and H.F. DeLuca. 1964. Vitamin D-stimulated release of calcium from mitochondria. *Biochemistry* 3: 203-209.
- Fraser, D.R. and E. Kodicek. 1965. Vitamin D esters: their isolation and identification in rat tissues. *Biochem. J.* 96: 59P-60P.
- Fraser, D.R. and E. Kodicek. 1970. Unique biosynthesis by kidney of a biologically active vitamin D metabolite. *Nature (London)* 228: 764-766.
- Georgiev, G.P. 1967. The nature and biosynthesis of nuclear ribonucleic acids. *Progr. Nucl. Acid Res. Mol. Biol.* 6: 259-351.
- Georgiev, G.P., O.P. Samarina, M.I. Lerman, M.N. Smirnov and A.N. Severtzov. 1963. Biosynthesis of messenger and ribosomal ribonucleic acids in the nucleolochromosomal apparatus of animal cells. *Nature (London)* 200: 1291-1294.
- Hallick, R.B. and H.F. DeLuca. 1969. Vitamin D₃-stimulated template activity of chromatin from rat intestine. *Proc. Nat. Acad. Sci. U.S.A.* 63: 528-531.
- Hallick, R.B. and H.F. DeLuca. 1971. 25-Hydroxydihydrotachysterol₃: in vivo 25-hydroxylation of dihydrotachysterol₃. *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 30: 407Abs. No. 1201.
- Haussler, M.R. and A.W. Norman. 1967. The subcellular distribution of physiological doses of vitamin D₃. *Arch. Biochem. Biophys.* 118: 145-153.
- Haussler, M.R., J.F. Myrtle and A.W. Norman. 1968. The association of a metabolite of vitamin D₃ with intestinal mucosa chromatin in vivo. *J. Biol. Chem.* 243: 4055-4064.
- Horsting, M. and H.F. DeLuca. 1969. Enzymatic conversion of cholecalciferol to 25-hydroxycholecalciferol. *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 28: 351. Abstract No. 522.
- Izzo, R.S. 1970. The effect of vitamin D₃ on the incorporation of ³H-orotic acid into ribonucleic acid of rat bone. Doctoral Dissertation, Loyola University of Chicago.
- Kirby, K.S. 1956. A new method for the isolation of ribonucleic acids from mammalian tissues. *Biochem. J.* 64: 405-408.

- Kodicek, E., D.E.M. Lawson and P.W. Wilson. 1970. Biological activity of a polar metabolite of vitamin D₃. *Nature (London)* 228: 763-764.
- Lawson, D.E.M., P.W. Wilson, D.C. Barker and E. Kodicek. 1969a. Isolation of chick intestinal nuclei. Effect of vitamin D₃ on nuclear metabolism. *Biochem. J.* 115: 263-268.
- Lawson, D.E.M., P.W. Wilson and E. Kodicek. 1969b. New vitamin D metabolite localized in intestinal cell nuclei. *Nature (London)* 222: 171-172.
- Lawson, D.E.M., P.W. Wilson and E. Kodicek. 1969c. Metabolism of vitamin D. A new cholecalciferol metabolite, involving loss of hydrogen at C-1, in chick intestinal mucosa. *Biochem. J.* 115: 269-277.
- Mach, B. and P. Vassalli. 1965. Template activity of RNA from antibody-producing tissues. *Science* 150: 622-626.
- Martin, D., M.J. Melancon, and H.F. DeLuca. 1969. Vitamin D stimulated, calcium-dependent adenosine triphosphatase from brush borders of rat small intestine. *Biochem. Biophys. Res. Commun.* 35: 819-823.
- Mawer, E.B., G.A. Lumb and S.W. Stanbury. 1969. Long biological half-life of vitamin D₃ and its polar metabolites in human serum. *Nature (London)* 222: 482-483.
- Melancon, M.J. and H.F. DeLuca. 1970. Vitamin D stimulation of calcium-dependent adenosine triphosphatase in chick intestinal brush borders. *Biochemistry* 9: 1658-1664.
- Morii, H., J. Lund, P.F. Neville and H.F. DeLuca. 1967. Biological activity of a vitamin D metabolite. *Arch. Biochem. Biophys.* 120: 508-512.
- Muller, W. and D.M. Crothers. 1968. Studies of the binding of actinomycin and related compounds to DNA. *J. Mol. Biol.* 35: 251-290.
- Myrtle, J.F. and A.W. Norman. 1971. Vitamin D: a cholecalciferol metabolite highly active in promoting intestinal calcium transport. *Science* 171: 79-82.
- Neville, E. and E.S. Holdsworth. 1968. Phosphorus metabolism during transport of calcium. *Biochim. Biophys. Acta* 163: 362-373.
- Neville, E. and E.S. Holdsworth. 1969. A "second messenger" for vitamin D. *FEBS Letters* 2: 313-316.

- Nichols, G., B. Flanagan and J.F. Woods. 1965. Parathyroid influences on bone biosynthetic mechanisms. In: The Parathyroid Glands. Ultrastructure, Secretion and Function. Editors: Gaillard, P.J., R.V. Talmage and A.M. Budy. Chicago: University of Chicago Press. pp. 243-260.
- Norman, A.W. 1965. Actinomycin D and the response to vitamin D. Science 149: 184-186.
- Olson, E.B. and H.F. DeLuca. 1969. 25-Hydroxycholecalciferol: direct effect on calcium transport. Science 165: 405-407.
- Omdahl, J.L., Y. Tanaka and H.F. DeLuca. 1971. Mode of action of vitamin D₃. Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30: 407 Abs. No. 1196.
- Owen, M. and P.J. Bingham. 1968. The effect of parathyroid extract on RNA synthesis in osteogenic cells in vivo. In: Parathyroid Hormone and Thyrocalcitonin. Editors: Talmage, R.V. and L.F. Belanger. New York: Excerpta Medica Foundation. pp. 216-225.
- Penman, S., C. Vesco and M. Penman. 1968. Localization and kinetics of formation of nuclear heterodisperse RNA, cytoplasmic heterodisperse RNA and polyribosome-associated messenger RNA in HeLa cells. J. Mol. Biol. 34: 49-69.
- Perry, R.P. 1963. Selective effects of actinomycin D on the intracellular distribution of RNA synthesis in tissue culture cells. Exp. Cell Res. 29: 400-406.
- Ponchon, G. and H.F. DeLuca. 1969. Role of the liver in the metabolism of vitamin D. J. Clin. Invest. 48: 1273-1279.
- Ponchon, G., A.L. Kennan and H.F. DeLuca. 1969. Activation of vitamin D by the liver. J. Clin. Invest. 48: 2032-2037.
- Raisz, L.G. 1970. Physiologic and pharmacologic regulation of bone resorption. N. Engl. J. Med. 282: 909-916.
- Ringertz, N.R. and L. Bolund. 1969. Actinomycin binding capacity of deoxyribonucleoprotein. Biochim. Biophys Acta 174: 147-154.
- Steinberg, J. and G. Nichols. 1967. Synthesis of ribonucleic acid in normal bone in vitro. Biochem. J. 105: 843-856.

- Steinberg, J. and G. Nichols. 1968. Stimulation of bone RNA synthesis by parathyroid hormone. In: Parathyroid Hormone and Thyrocalcitonin. Editors: Talmage, R.V. and L.F. Belanger. New York: Excerpta Medica Foundation. pp. 226-241.
- Stohs, S.J., J.E. Zull and H.F. DeLuca. 1967. Vitamin D stimulation of ³H-orotic acid incorporation into ribonucleic acid of rat intestinal mucosa. *Biochemistry* 6: 1304-1310.
- Suda, T., H.F. DeLuca, H.K. Schnoes and J.W. Blunt. 1969. 25-Hydroxy-ergocalciferol: a biologically active metabolite of vitamin D₂. *Biochem. Biophys. Res. Commun.* 35: 182-185.
- Suda, T., H.F. DeLuca, H.K. Schnoes, G. Ponchon, Y. Tanaka and M.F. Holick. 1970. 21,25-Dihydroxycholecalciferol. A metabolite of vitamin D₃ preferentially active on bone. *Biochemistry* 9: 2917-2922.
- Tanaka, Y. and H.F. DeLuca. 1971. Inhibition of the metabolism of 25-hydroxycholecalciferol by actinomycin D and cycloheximide. *Proc. Nat. Acad. Sci. U.S.A.* 68: 605-608.
- Taylor, A.N. and R.H. Wasserman. 1969. Correlations between the vitamin D-induced calcium-binding protein and intestinal absorption of calcium. *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 28: 1834-1838.
- Thompson, V.W. and H.F. DeLuca. 1964. Vitamin D and phospholipid metabolism. *J. Biol. Chem.* 239: 984-989.
- Trummel, C.L., L.G. Raisz, J.W. Blunt and H.F. DeLuca. 1969. 25-Hydroxycholecalciferol: stimulation of bone resorption in tissue culture. *Science* 163: 1450-1451.
- Wasserman, R.H. and A.N. Taylor. 1966. Vitamin D₃-induced calcium-binding protein in chick intestinal mucosa. *Science* 152: 791-793.
- Wasserman, R.H. and A.N. Taylor. 1968. Vitamin D-dependent calcium-binding protein. Response to some physiological and nutritional variables. *J. Biol. Chem.* 243: 3987-3993.
- Wasserman, R.H., R.A. Corradino, and A.N. Taylor. 1968. Vitamin D-dependent calcium-binding protein. Purification and some properties. *J. Biol. Chem.* 243: 3978-3986.
- Zull, J.E., E. Czarnowska-Misztal and H.F. DeLuca. 1965. Actinomycin D inhibition of vitamin D action. *Science* 149: 182-184.

APPROVAL SHEET

The thesis submitted by Sue Ann Shelley has been read and approved by a committee from the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 21, 1971

Date

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Signature of Advisor