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The Effect of Vitamin D₃ on the Incorporation of p3-H-orotic Acid Into Ribonucleic Acid of Rat Bone

Rodger Samuel Izzo
Loyola University Chicago

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THE EFFECT OF VITAMIN D₃ ON THE INCORPORATION OF
³H-OROTIC ACID INTO RIBONUCLEIC ACID OF RAT BONE

by

RODGER SAMUEL IZZO

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A Dissertation Submitted to the Faculty of the Graduate
School of Loyola University in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

February
1970
LIFE

Rodger Samuel Izzo was born in New Castle, Pennsylvania, on May 14, 1941.

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CHAPTER I
INTRODUCTION

Orr et al. (43) were among the first to suggest that a defective absorption of calcium and phosphate from the intestine, with a concomitant loss in the feces, was the result of vitamin D deficiency. Upon further investigation, Gyorgy (22) and Harris (24) concluded that this excessive loss of calcium in the fecal matter of vitamin D deficient animals was not due to faulty absorption, but rather to excessive excretion of calcium into the large intestine. Nicolaysen (36, 37) and Nicolaysen et al. (38) clarified this point by demonstrating conclusively that vitamin D increases the absorption of calcium and, secondarily, that of phosphorus in the small intestine, and that the effects of vitamin D could not be due to reduced excretion of calcium into the small intestine. Absorption of phosphate appeared to take place equally well in the absence and in the presence of vitamin D. Since 1953 various reviews on the intestinal absorption of calcium and concerned with many factors of influence including vitamin D have been published. This information is available in a book edited by Wasserman (60). There is little doubt that one of the basic physiological effects of vitamin D is to stimulate the absorption of calcium from the small intestine and, secondarily, that of phosphate.

The overwhelming evidence implicating vitamin D in the intestinal
absorption of calcium has promoted much research in an attempt to elucidate the mechanism of action of vitamin D on this absorption process. The everted gut sac technique of Schachter et al. (51) is the in vitro experimental procedure utilized in studying the role of vitamin D in calcium absorption. It involves taking a strip of intestine and turning it inside out so that the mucosal surface is now on the outside and the serosal surface is on the inside; then one or both ends of the intestinal strip are tied. This simulates the condition whereby the fluid bathing the mucosal surface would represent that existing in the lumen of the intestine. By adding equal concentrations of calcium to both sides of the sac, the rate of uptake of calcium by the sac can be established by determining the ratio of serosal calcium/mucosal calcium at various time intervals. The radioisotopes $^{45}$Ca and $^{47}$Ca are used to establish the rate of uptake of the calcium ion. Using this technique and the rat as the test animal, Schachter et al. (50) were able to demonstrate that calcium transport occurred against a concentration as well as an electrochemical gradient, and that this process was enhanced by prior administration of vitamin D$_2$; addition of the vitamin directly to the test medium had no effect. It might be added here that Wasserman et al. (63) also showed with in vivo studies that calcium absorption occurred against concentration and electrochemical gradients. Schachter
et al. (50) also found with in vitro studies that calcium transport decreased as the distance from the pylorus increased, and that metabolic inhibitors or a nitrogen atmosphere blocked the active transport of calcium by intestine. It was then suggested by Schachter et al. (49) that the uptake of calcium at the mucosal surface may be facilitated diffusion, and that its transport to the serosal side was dependent upon oxidative metabolism and the generation of phosphate bond energy. This two step process was confined primarily to the duodenum and was vitamin D dependent.

That metabolic inhibitors can block the active transport of calcium by intestine might be expected. However, the question arose whether the action of vitamin D itself was blocked. Schachter and co-workers (49, 50, 51) employed techniques which measured active transport but in no way affected the activity of vitamin D on the permeability of intestine to calcium. With in vitro techniques Harrison et al. (25) demonstrated that the transport of calcium across the intestine involves two processes: (a) penetration of the diffusion barrier of mucosal epithelium, and (b) the active transport of calcium across the mucosal cell; both of these processes were most active in the duodenal portion of the small intestine. Prior administration of vitamin D to animals a few hours before the intestinal preparations were made significantly influenced the
first step by reducing the diffusion barrier to calcium. They concluded that the primary action of the vitamin is to increase the permeability of intestine to calcium making it available to the active transport system. The in vivo experiments of Wasserman et al. (62, 64) showed that vitamin D increases the passage of calcium from intestinal lumen to blood and from blood to the lumen. From their work, they concluded that a specific effect of vitamin D on a unidirectionally oriented active transport is not entirely correct. The exact nature of the permeability change resulting from vitamin D action may be an alteration in membrane structure of mucosal cells and/or an increased synthesis of a "carrier" substance which enhances the movement of calcium in both directions.

There is also evidence that vitamin D alters the permeability of subcellular membranes to calcium (12). Calcium is actively taken up by mitochondria and vitamin D has no effect on this process. However, when the release of this calcium by the mitochondria is examined, vitamin D has a marked stimulatory effect whether it is given in vivo or in vitro. The stimulation of calcium release from mitochondria has been interpreted as an effect of vitamin D on membrane permeability to calcium. Microsomal preparations from chick intestinal mucosa bind calcium in vitro and the binding is depressed by addition of vitamin D.
The apparent depressed-binding could have been a consequence of increased release. All of these effects were found relatively specific for the vitamin D molecule and for calcium and appear to be in harmony with those of Harrison et al. (25) regarding intestinal permeability. More recently, Haskim et al. (27) demonstrated with the use of mucosal cell suspensions that vitamin D is involved in the release of calcium rather than in its uptake by mucosal cells. Although the exact site of accumulation of calcium within the epithelial cells was not determined, they suggested from their study that vitamin D could possibly prevent intracellular accumulation of calcium by increasing the membrane permeability of subcellular particles as well as the cellular membrane itself.

An interesting hypothesis proposed by Zull et al. (67) suggests a mechanism whereby vitamin D may alter calcium binding by membrane systems. It has been suggested frequently in the past that phospholipids bind calcium and other divalent cations at the surface of cell membranes. Using vitamin D and egg lecithin as a model system, these investigators studied the interaction of the vitamin and phospholipid and found that such an interaction would have the effect of displacing ions bound to the phospholipid. The net result in a cell membrane would be reduced binding of divalent cations such as calcium. In the presence of excess vitamin D then, such an effect would produce
a significant alteration in calcium binding by the cell membranes and possibly prevent the intracellular accumulation of the cation.

If vitamin D alters intestinal permeability to calcium, is it necessary to implicate vitamin D in some manner with the active transport mechanism? In other words, can the action of vitamin D be explained solely on the basis of an alteration of intestinal permeability? Evidence to the contrary arises because of a time lag between vitamin D administration and its observed action. For example, when small doses of \(^{3}\)H-vitamin D are given such as 10 I.U. (1 I.U. = 0.025 µg) to a rat radioactivity can be detected in the intestinal mucosa in 1 - 3 hours, but the apparent active transport of calcium does not take place until 10 - 12 hours later. If an active transport system were preformed, the response to vitamin D would appear much earlier than 10 hours. Also, actinomycin D blocks or prevents all physiological responses to vitamin D including active transport of calcium by intestine, whereas it does not block the vitamin's action on intestinal permeability to calcium (26, 66). Again, this would argue strongly against the idea that a transport system for calcium is totally preformed and all vitamin D does is make available the calcium to be transported.

Eisenstein and Passavoy (19) first noted that actinomycin D, an antibiotic which is known to block DNA transcription into RNA, blocks
the hypercalcemic response to large doses of vitamin D. Later it was demonstrated that administration of actinomycin D prior to vitamin D not only prevented a rise in serum calcium but also blocked the increased transport of calcium across everted gut sacs (39, 40, 65, 66).

This inhibition of vitamin D action was not due to a decrease in absorption of the vitamin; an injected dose of vitamin D was no more effective than an oral dose if the animal had been pretreated with actinomycin D. They also showed that the distribution of the vitamin was not affected by actinomycin D, since the antibiotic did not block the transport of vitamin D to small intestine, kidney, liver or bone in rats. Also, inhibition by the antibiotic could not be attributed to a general toxicity or cell necrosis, because photomicrographs of intestinal epithelial cells obtained from normal and actinomycin D-treated rats appeared to be identical in nature. Of interest here is the fact that the effect of actinomycin could be eliminated if vitamin D was administered to an animal 4 - 8 hours prior to the administration of the antibiotic. It would appear, therefore, that the antibiotic inhibits some early event in the chain of events necessary to bring about the physiological expression of vitamin D action.

More direct evidence that vitamin D acts on the chromatin material has been provided by Hallick et al. (23). Vitamin D was ad-
ministered to rats deficient in this vitamin and the intestinal mucosal chromatin was isolated. The isolated chromatin was then used as a template for DNA-directed RNA synthesis in an in vitro RNA-synthesizing system. Prior administration of vitamin D markedly increased the template activity for DNA-dependent RNA synthesis by the rat intestinal mucosal chromatin.

Pulse labeling experiments with $^3$H-orotic acid and $^3$H-uridine have produced additional evidence that vitamin D may act directly on the DNA - m-RNA complex. Stohs et al. (55) administered 2000 I.U. of vitamin D to rats; this was followed at various time intervals by a dose of $^3$H-orotic acid. One hour after the administration of the tracer the animals were sacrificed and various types of cellular RNA were isolated from intestinal mucosal cells. Vitamin D stimulated incorporation of $^3$H-orotic acid into nuclear RNA but not ribosomal or mitochondrial RNA within three hours; this effect was completely blocked by prior administration of actinomycin D. Norman (41) has obtained similar results upon showing that vitamin D stimulates the labeling of RNA in chicks using $^3$H-uridine. These data would imply that the expression of vitamin D action on intestinal epithelium in some manner involves DNA transcription into m-RNA and translation into functional protein(s).

The nature of the interaction of vitamin D with the mucosal cell
has stimulated investigation on the subcellular localization of the vitamin. Vitamin D, when administered to animals at physiological levels, appears in high concentration as the vitamin or its metabolites associated with the nuclear chromatin material or nuclear membrane (28, 29, 34, 52). Upon isolation of the metabolites, in order to test for their biological significance, it was found that the metabolite of highest concentration appeared to be the biologically active form of vitamin D (5). This compound has been identified as 25-hydroxycholecalciferol (3, 4, 7). As compared to vitamin D₃, it is more active biologically in rats and chicks and acts more rapidly to induce bone mobilization and intestinal transport of calcium. It also induces bone mobilization in vitro, whereas vitamin D₃ is only minimally effective in culture (58). Whether the nuclear interaction represents a direct effect of the metabolite on the DNA - m-RNA complex or an indirect alteration of the permeability characteristics of the nuclear membrane is not known.

Wasserman and co-workers (31, 55, 64) have reported the presence of a vitamin D induced protein in intestine which will bind calcium. There is a close relation, in time of vitamin D administration, between the appearance of this calcium-binding protein and the enhanced absorption of calcium in vivo. Purification of the protein has revealed that its concentration in various soft tissues correlates well with their
respective ability to respond physiologically to vitamin D (55, 61). For example, the calcium absorptive capacity of the small intestine decreases in the following order, duodenum > jejunum > ileum; subsequent examination of the calcium-binding protein concentration in these intestinal segments showed a similar decrease. A preliminary analysis of the composition of the protein has been reported (61).

From these data, Wasserman and co-workers proposed the hypothesis that the vitamin D induced calcium-binding protein is in some manner intimately involved in the translocation of calcium across the intestinal epithelial cell. This would in part explain the lag period observed between the time when vitamin D is administered and the time when a physiological response can first be observed (48, 59).

Recently Martin et al. (35) have demonstrated that a calcium dependent adenosine triphosphatase located in the brush borders of the rat small intestine is markedly increased after the administration of vitamin D to vitamin D deficient rats. They propose that the increase in enzyme activity stimulated by the vitamin may in some way be an expression of vitamin D action on calcium transport resulting in the transfer of calcium across the brush border surface of the columnar epithelial cell.

At the present time it is evident that the calcium transport
process of the intestine, which is under the influence of vitamin D, cannot be attributed solely to active transport or to an increase in membrane permeability. The vitamin participates in some fashion in an active transport system, perhaps in the formation of it or some component thereof. It has, in addition, an effect on intestinal membrane permeability to calcium. Whether or not this is the primary event in the action of the vitamin remains to be determined, although it is clear that a mere increase in intestinal permeability to calcium cannot by itself account for the physiological effects of vitamin D. Observations to date appear to be consistent with the formation of a carrier protein system for calcium induced by vitamin D as suggested by Wasserman et al. (64).

In addition to the small intestine the physiological action of vitamin D is directed toward bone where it acts by promoting accretion. The gross and light microscopic changes that occur in the calcification of cartilage are well known. However, the exact biochemical processes underlying these changes are not fully understood.

The cartilage or epiphyseal disc of long bone consists of three zones, each characterized by both the arrangement and size of the cell (17). The immature or resting cell zone is a narrow band in close proximity to the epiphysis of the bone. The proliferating cell zone con-
sists of larger cells arranged in longitudinal columns separated from one another by cartilaginous matrix. The zone adjacent to the diaphysis (bone shaft) is the hypertrophic or mature cell zone and it is here that a line of calcification is observed in bone growth with subsequent disintegration of the cartilage cells.

In the course of endochondral calcification in the epiphyseal disc, cartilage cells from the basal proliferating layers transform in an orderly fashion into hypertrophic cells as the zone of calcification is reached. With growth and maturation of these hypertrophic cells, intracellular glycogen appears to increase \(20, 46\). In addition, phosphorylase is present in those cells which have hypertrophied most recently \(10\). Upon deposition of amorphous calcium phosphate in the ground substance about these cells in the zone of calcification, there appears to be glycogenolysis prior to or concomitant with disintegration of the hypertrophic cartilage cells \(57\).

In rickets produced in young rats by a low phosphate and vitamin D-deficient diet, the epiphyseal plate is markedly thickened because of widening and disorganization of the proliferating and hypertrophic cell zones. The cartilage cells fail to mature and disintegrate or be destroyed but instead continuously produce matrix material; this accounts for the resultant overgrowth of cartilage. Following administration of
vitamin D to a rachitic animal, a line of calcification is observed in the hypertrophic cell zone (18). In addition, a decrease in glycogen stainability was demonstrated in these cells (46), and this effect has been observed as early as 48 hours in rachitic rat cartilage following vitamin D administration (16). From these data it would appear that vitamin D has some effect on glycolysis, and that anaerobic metabolism and the calcification of cartilage are closely associated.

Changes in enzyme activity in the cartilage of the rachitic rat and dog following vitamin D administration have been reported (15, 30). Within 24 hours after vitamin D treatment citrate synthetase activity was significantly elevated; this was accompanied by an increase in the citrate content of the cartilage cells. It was not determined whether the vitamin acted directly upon the enzymatic reaction or indirectly by affecting the synthesis of the synthetase. In addition, the effect, if any, that a measurable increase in citrate content would have on the calcification process could not be discerned.

Cartilage cells synthesize mucopolysaccharides; the polysaccharide moiety of the molecule is composed of repeating disaccharide units of glucuronic acid and a hexosamine. These molecules are released by cartilage cells into the intercellular space and become an integral part of the ground substance necessary for cartilage calcification (6).
Following vitamin D administration, an elevation of hexosamine synthetase activity has been observed in rachitic cartilage (14). Since hexosamine is a component of the ground substance mucopolysaccharide, it is apparent that any factor affecting its level in cartilage cells may indirectly alter the calcification process.

Attempts to determine a characteristic pattern of enzymes in the various layers of the epiphyseal disc of rachitic cartilage, following vitamin D administration, have not been very rewarding. Epiphyseal cartilage is essentially avascular and the principal end product of glucose metabolism is lactic acid (33). Nevertheless, oxidative enzymes do exist in cartilage cells (1), since aerobic metabolism is necessary for the synthesis of matrix material. Upon vitamin D administration to D-deficient animals the activity of the oxidative enzyme isocitric dehydrogenase in the hypertrophic cell zone decreased (2). Activity of 6-phosphogluconic acid dehydrogenase was also found to decrease in both proliferative and hypertrophic zones following vitamin D treatment (17); however, the activity of lactic dehydrogenase was not significantly different in treated and untreated animals. The relevance of these findings to the calcification of cartilage matrix and degeneration of cells cannot be postulated at this time.

Recently, a study by Canas et al. (8) has demonstrated that one
of the early effects of vitamin D on rachitic chicks is to stimulate bone collagen synthesis by increasing the incorporation of $^3$H-proline. This increased incorporation was reported as early as 12 hours after vitamin D administration and before a rise in serum calcium was observed. The stimulatory action of the vitamin appeared to be specific for bone because collagen synthesis in the skin of these same animals was unaffected. Nevertheless, it could not be determined whether the action of the vitamin was on the DNA - m-RNA complex or on some enzyme(s) directly responsible for the synthesis of collagen. However, the results do suggest that stimulation of bone collagen synthesis by vitamin D would provide new matrix for the deposition of mineral made available by the renewed absorption of gut calcium.

In conclusion, it can be said that the role of vitamin D in the mineralization of bone matrix is still incompletely understood. It is known that vitamin D augments intestinal absorption of calcium and, to a lesser degree, that of phosphate; this raises the serum levels of calcium and phosphorus. The observed increase in serum calcium is largely the result of increased absorption. However, the increased level of serum phosphate following vitamin D administration probably represents the summation of increased absorption and retention of phosphate (mediated by high calcium levels with subsequent suppression
of parathyroid hormone activity). Beyond the systemic effects, vitamin D appears to have some local action for even when the supplies of calcium and phosphate are low, it is able, in the experimental animal, to increase bone crystal formation. At present, it can be stated only that in the presence of dietary vitamin D calcification of the matrix of bone occurs, while in vitamin D deficiency this does not take place.

STATEMENT OF THE PROBLEM

The physiological action of vitamin D is directed to bone and intestinal tissue. In bone the vitamin acts by promoting accretion and in the intestine its action is one of enhancing calcium absorption and secondarily, that of phosphate.

It is well established that rickets results from a deficiency of vitamin D with characteristic changes in the composition of bone such as increased water and decreased ash content. The development of these changes in bone has been attributed to a decrease in the intestinal absorption of calcium and secondarily, that of phosphate which leads to a fall in the blood \((Ca^{++})(HPO_4^2^-)\) product (12).

What remains unclear is the role of vitamin D, at the biochemical level, in the prevention and cure of rickets, particularly with respect to the action of vitamin D on bone.
Wasserman and Taylor (64) have shown that vitamin D induces the synthesis of a calcium-binding protein in the intestinal mucosa of the chick prior to an observable rise in serum calcium. This has also been reported in the rat (31) and dog (56).

Investigation into the nature of the interaction of vitamin D with the mucosal cell that leads to the formation of a calcium-binding protein suggests that vitamin D may be involved in some synthetic process. This concept is derived from studies by Corradino et al. (11), Zull et al. (66), and Norman (40), in which it was demonstrated that various inhibitors of protein synthesis partially or completely inhibit the action of vitamin D. Vitamin D, when administered to animals at physiological levels, appears in high concentration as the vitamin or its metabolites in the nuclei of intestinal mucosal cells, which may also reflect its molecular site of action (28, 29, 52). Whether this nuclear interaction represents a direct effect on the DNA - m-RNA complex or an indirect alteration of the permeability characteristics of the nuclear membrane is not known. Work by Stohs et al. (53) and Hallick et al. (23) has added support to the former hypothesis. Whatever is the mechanism, the product of the interaction has been proposed to be a translocase or a transport enzyme (65).

Such a proposal for the action of vitamin D in bone has not as yet
been suggested. The activities of various enzymes of the rachitic rat epiphyseal or cartilage disc, following vitamin D administration, have been investigated histochemically (2, 17). However, the data obtained do not appear to be consistent.

A recent study by Canas et al. (8) has demonstrated that one of the early effects of vitamin D on rachitic chicks is to stimulate bone collagen synthesis; this would provide new matrix for the deposition of mineral made available by renewed absorption of gut calcium. In addition, the stimulatory action of the vitamin appeared to be specific for bone, because collagen synthesis in the skin of these same animals was unaffected.

The precise role of vitamin D in the mineralization of bone matrix is still incompletely understood. The biochemical events occurring after the administration of the vitamin and prior to accretion have not been investigated. In contrast to the action of vitamin D in the intestine, it is not known whether or not the action of the vitamin in skeletal tissue is directed upon the nuclear chromatin material or nuclear membrane. Since bone and the small intestine are target organs for the vitamin, a reasonable hypothesis would be that the vitamin may act in a fashion analogously to its action in the intestine and promote the synthesis of a protein(s) necessary for mineralization to occur.
It is the purpose of this dissertation to investigate this hypothesis by studying the effect of vitamin D on the synthesis of ribonucleic acid in rachitic bone cells. This information may possibly aid in establishing the effect of the vitamin prior to accretion and its possible role in curing or preventing rickets.
CHAPTER II
MATERIALS AND METHODS

EXPERIMENTAL DESIGN

There follows a brief description of the experimental approach employed in studying the effect of vitamin D on the rate of synthesis of ribonucleic acid in the bone cells of rachitic animals.

Male albino rats were maintained on a rachitogenic diet for four weeks and the rachitic state was evidenced by reduced growth and an increase in serum alkaline phosphatase activity. The animals were then divided into two groups one of which received vitamin D₃ whereas the control animals received the vehicle only. In those experiments employing actinomycin D to see whether or not the effect of the vitamin could be blocked, a dose of 100 µg of the antibiotic was administered intraperitoneally two hours prior to injection of either D₃ or the vehicle.

At various times after the administration of the vitamin or vehicle, 50 µg of ³H-orotic acid were injected intraperitoneally two hours prior to sacrifice. Upon sacrifice, the femurs, tibiae and humeri were excised from each animal and placed in 0.9% sodium chloride solution. The bones were then split longitudinally, scraped clean of bone marrow using a scalpel, frozen and then pulverized into a fine powder before homogenization. The homogenate was washed to remove acid-soluble
and lipid components and the ribonucleic acid (RNA) was extracted from the tissue residue with perchloric acid for 18 hours at 4°C.

In addition to RNA, the extracted fraction also contained protein. The majority of the protein was removed by using the sodium dodecyl sulfate-phenol method of Stohs et al. (53) except that the RNA was isolated in the last step by passing the aqueous layer through a Sephadex G-25 column, instead of precipitating the RNA from the aqueous layer with ethanol and sodium acetate. The RNA eluted from the column was detected with a Uvicord II Absorptiometer set at a wavelength of 254 mµ. The fraction eluted from the column containing RNA was flash evaporated to dryness and the residue dissolved in distilled water. Aliquots of this sample were used to determine the amount of RNA present and for liquid scintillation counting.

Absolute radioactivity determinations of ³H-RNA were accomplished with the use of a Beckman LS-250 liquid scintillation spectrometer. The specific activity of RNA was expressed as disintegrations per minute per milligram of RNA (DPM/mg RNA). A comparison of experimental and control specific activities showed whether or not vitamin D₃ enhanced the incorporation of ³H-orotic acid by increasing the rate of synthesis of RNA.

Positive identification of RNA was determined in the following
manner. The RNA remaining after flash evaporation, instead of being dissolved in water, was hydrolyzed in KOH at 95° C for one hour. The nucleotides were removed from the hydrolyzate with the anion-exchange resin Dowex 2X-8 and then eluted from the resin with HCl. Separation of the nucleotides was accomplished by placing them on a column containing the cation-exchange resin Dowex 50W-X4 and eluting first with HCl and then water. A Uvicord II Ultraviolet Absorptiometer was utilized for detection of the mononucleotides as they were eluted from the column. The fraction containing uridine monophosphate was flash evaporated and the residue dissolved in water.

Thin-layer chromatography and the Cary Model 15 recording spectrophotometer were employed for the positive identification of uridine monophosphate. Development of cellulose thin-layer sheets was carried out at room temperature by ascending chromatography. Along with a standard solution of uridine monophosphate, solutions of the monophosphates adenosine, guanosine and cytidine were also used for identification of components other than uridine monophosphate in the sample. The components were located on the thin-layer chromatogram with ultraviolet light. Uridine monophosphate was removed from the chromatogram and a spectral analysis was determined using the Cary Model 15 spectrophotometer.
A. Animal Maintenance Prior to Experiments

Three week old male albino rats, 50-60 grams in weight, (Holtzman Company, Madison, Wisconsin) were housed in suspended cages in an air-conditioned room from which sunlight was excluded. The animals were divided into two groups. One group was provided a normal diet (Rockland Laboratory Animal Diet) and tap water. The rats in the second group were placed on a rachitogenic diet (General Biochemicals, Chagrin Falls, Ohio) containing a high calcium to phosphorus ratio (Ca/P = 5.85) and no vitamin D; in addition, these animals were given only distilled water to drink. At the end of four weeks those animals on normal rat chow weighed approximately 200 g, whereas animals on the test diet exhibited a weight range of from 70 to 90 g.

B. Determination of Serum Alkaline Phosphatase Activity

In order to establish whether or not the animals were rachitic, two parameters were used. In addition to reduced growth, the level of serum alkaline phosphatase activity was determined; an increase in alkaline phosphatase activity would be indicative of the rachitic state.

p-Nitrophenylphosphate was used as the substrate for the determination of phosphatase activity. The substrate was dissolved in an alkaline buffer (pH - 9.4) and allowed to react with the serum phospha-
tase for 30 minutes at room temperature. The reaction was terminated
by the addition of NaOH. The p-nitrophenol liberated by the phosphatase
exhibits a yellow color in alkaline solution and the phosphatase activity
is directly proportional to the amount of p-nitrophenol liberated per unit
time.

Preparation of Solutions

1. p-Nitrophenol (5 x 10^{-3} M): Dissolve 696 mg of p-nitrophenol
   (Sigma) in 0.02 N NaOH and dilute to one liter with the base.
2. Working Standard (1.38 x 10^{-4} µm/µl): To 10 µl of 5 x 10^{-3}
   M p-nitrophenol, add 350 µl of 0.02 N NaOH.
3. Buffer Solution (pH - 9.4): Take 50 ml of 0.2 M Na₂CO₃ and
   to it add 200 ml of 0.2 M NaHCO₃.
4. Alkaline Buffer - Substrate Solution: Dissolve 20 mg of p-
   nitrophenylphosphosphate (Sigma) in 5.0 ml of doubly distilled water.
   To this add 175 ml of the alkaline buffer solution (pH - 9.4).
   Pipette 100 µl aliquots of this solution into micro test tubes
   (Beckman) and store at -10° C until ready for use.

The experimental approach used in determining serum alkaline
phosphatase began with the collection of four hundred microliters of
blood from rats which had been maintained for four weeks on a
particular dietary regimen. Three rats from each group were chosen for these experiments. Blood samples were obtained from the tail of the rat. The rat was placed in a restraining cage and the tail was severed approximately three-quarters of an inch from the tip. Bleeding was aided by "milking" the tail. Blood was collected in plastic microtubes and these were allowed to stand at room temperature for ten minutes. Centrifugation was performed in a Beckman/Spinco Microfuge for one minute. The serum was set aside until ready for use.

Micro test tubes containing 100 µl of the alkaline buffered substrate were removed from the freezer and left at room temperature for about one hour. To one of these tubes was added 5 µl of distilled water. Into a second tube was pipetted 5 µl of the working standard solution. Into a third tube was pipetted 5 µl of serum. The 5 µl additions were made at 30 second intervals. All samples were left at room temperature for 30 minutes and the reaction in each tube was stopped by the addition of 200 µl of 0.02 N NaOH. The Spectro-Colorimeter was set at zero absorbance against water and the optical density of the resulting solutions was determined at 410 mµ.

A unit of alkaline phosphatase activity was defined in these experiments as that amount of enzyme that will liberate 1.0 µm of p-nitrophenol in one hour at pH - 9.4 and 25° C. For comparison of sera
from normal and rachitic animals the activity was expressed as units of enzyme activity per ml of serum.

A standard curve for determining alkaline phosphatase activity was prepared by taking various aliquots of the working standard and measuring the optical density of the resulting solution at 410 mµ in the Beckman/Spinco 151 Spectro-Colorimeter. The data for this standard curve appear in Table I. A linear relationship between the optical density readings at 410 mµ and the concentration of p-nitrophenol is shown in Fig. 1.

C. Experiments with and without Actinomycin D

Preparation of Solutions

1. Vitamin D₃: Dissolve 5 mg of crystalline vitamin D₃ (Mann Laboratories, New York) in 0.5 ml of 95% ethanol and dilute to 10 ml with propylene glycol (1,2-propanediol). Five milligrams of vitamin D₃ are equivalent to 200,000 International Units (I. U.); therefore, one milliliter of this solution would contain 20,000 I. U.

2. Control Vehicle for Vitamin D₃: 0.5 ml of 95% ethanol was placed in a volumetric flask and diluted to 10 ml with propylene glycol.
TABLE I

Standard Curve Data for Determination of Alkaline Phosphatase Activity

<table>
<thead>
<tr>
<th>µm p-Nitrophenol x 10^{-3}</th>
<th>No. of Samples</th>
<th>O. D. 410 *</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.690</td>
<td>4</td>
<td>0.023 ± 0.005</td>
</tr>
<tr>
<td>1.38</td>
<td>4</td>
<td>0.044 ± 0.015</td>
</tr>
<tr>
<td>2.76</td>
<td>4</td>
<td>0.086 ± 0.017</td>
</tr>
<tr>
<td>5.52</td>
<td>4</td>
<td>0.175 ± 0.011</td>
</tr>
<tr>
<td>8.28</td>
<td>4</td>
<td>0.260 ± 0.022</td>
</tr>
<tr>
<td>11.04</td>
<td>4</td>
<td>0.341 ± 0.005</td>
</tr>
<tr>
<td>13.8</td>
<td>4</td>
<td>0.415 ± 0.028</td>
</tr>
</tbody>
</table>

* mean ± standard deviation
FIGURE 1
Standard Curve for Determination of Serum Alkaline Phosphatase Activity
3. Orotic Acid-5-T (\(^3\)H-orotic acid): \(^3\)H-orotic acid (specific activity = 14-16 Ci/mM) was obtained from Amersham/Searle, Chicago, and dissolved in physiological saline (0.9%) such that 50 µc were equivalent to 0.25 ml.

4. Uridine-5-T (\(^3\)H-uridine): \(^3\)H-uridine (specific activity = 22.7 Ci/mM) was obtained from Amersham/Searle and dissolved in physiological saline (0.9%) such that 50 µc were equivalent to 0.25 ml.

5. Actinomycin D: Dissolve 1000 µg of actinomycin D (Calbiochem) in 5.0 ml of 0.9% saline:95% ethanol (9:1). The control vehicle for actinomycin D was the solution of saline:ethanol (9:1).

**Experiments without Actinomycin D:** Rachitic rats (70-90 g), maintained on the rachitogenic diet for four weeks, were fasted 13-15 hours before the start of each experiment. The fasting of these animals was arranged so that all the experiments began at 7:00 A.M. On the day of the experiment the animals were divided into two groups, each group comprising 4 to 5 animals. One group of animals received 5000 I.U. (0.25 ml) of vitamin D\(_3\) intraperitoneally whereas the control animals received only the vehicle. At various intervals (i.e., 1, 4, 7, 10 or 13
hours) after administration of the vitamin or vehicle, 50 µc of \(^3\)H-orotic acid were injected intraperitoneally; two hours after administration of the isotope, the animals were sacrificed. Both femurs, tibiae and humeri were excised from each animal, scraped clean of extraneous tissue and placed in ice-cold physiological saline. Each bone was then split longitudinally, scraped clean of bone marrow using a scalpel and then frozen.

Experiments with Actinomycin D: Actinomycin D was employed to see whether or not the enhanced incorporation of tritiated orotic acid into RNA by the vitamin was blocked. Animal preparation prior to the experiment was the same as described above. However, on the day of the experiment the animals were divided into four groups of four animals each, such that a dose of 100 µg of actinomycin D or its vehicle was injected intraperitoneally two hours prior to the administration of either vitamin \(\text{D}_3\) (5000 I.U.) or its vehicle. All experiments began at 5:00 A.M. Only one time interval (i.e., 9 hours) was used and either \(^3\)H-orotic acid (50 µc) or \(^3\)H-uridine (50 µc) was injected intraperitoneally two hours prior to sacrifice; this would mean that the isotope was administered on the seventh hour and the animals were sacrificed on the ninth hour. The removal and cleaning of the bone was the same as described above.
D. Preparation of Tissue Residue for Extraction of RNA (ribonucleic acid):

1. Pulverization of Bone: Before whole bones could be homogenized they had to be reduced to a fine powder. This was accomplished by using a stainless steel tissue pulverizer (Vollrath Company, Sheboygan, Wisconsin) packed with dry ice. Once the metal was cold whole bone samples were removed from the freezer and placed in the cylindrical portion of this apparatus. By repeated hammering a fine bone powder was obtained.

2. Homogenization and Preparation of Tissue Residue: The technique employed was essentially that of Park et al. (44). Powdered bone was homogenized with 20 ml of a mixture of ice cold 95% ethanol and 2% potassium acetate (2:1); the glass tissue homogenizer was placed in a beaker of ice and several passes were made before complete homogenization was accomplished. The homogenate was then centrifuged at 4000 r.p.m. in the Model PR-2 International Portable Refrigerated Centrifuge at 0°C for 15 minutes. After discarding the supernatant, the pellet was suspended in 15 ml of 0.5 N perchloric acid. After 30 minutes for decalcification at 0°C, the suspension was centrifuged at 4000 r.p.m. for 5 minutes at 0°C. Acid soluble and lipid components were extracted by twice washing the residue with 15 ml of cold 0.1 N perchloric acid in ethanol (95%), then with 15 ml of ethanol:ether (3:1)
first cold, then twice at 50° C. Finally, the residue was washed twice in 15 ml of cold 0.5 N perchloric acid to remove the remaining calcium matrix of bone.

E. Extraction of RNA

Extraction of RNA from the bone tissue residue was accomplished by using the combined methods of Ogur et al. (42) and Stohs et al. (53) with slight modifications.

Ogur et al. (42) Method: The RNA was extracted from the de-calcified bone tissue residue free from acid soluble and lipid components with 1.0 N perchloric acid for 18 hours at 4° C. The supernatant obtained after centrifugation was neutralized with 10 N KOH at approximately 4° C and then placed in the cold at 0° C for 30 minutes; the KClO₄ precipitate was then removed by centrifugation at 0° C.

Stohs et al. (53) Method: This method was employed to remove the protein that was extracted along with the RNA. After removal of KClO₄ in the above step, the supernatant was flash evaporated (Model PTFE-IGN Evaporator, Buchler Instruments) to dryness at 35° C. To the residue was added 5.0 ml of 0.01 M acetate buffer (pH - 5.0) followed by centrifugation at an International Clinical Centrifuge to re-
move insoluble material. Sodium dodecyl sulfate, 0.5 ml of a 10% solution, was added to the supernatant and the suspension was stoppered and placed in a shaker water bath (Elmac Engineering, Chicago, Illinois) at 65° C. In addition, a tube containing 5.0 ml of 88% phenol was simultaneously placed in the bath. After 5 minutes the hot phenol was added to the suspension and this mixture was shaken for another 5 minutes at 65° C. The emulsion was removed from the bath and rapidly cooled by placing it in a beaker of ice cold water. The emulsion was then broken by centrifugation (Servan SS-1) at 30,000 x g for 10 minutes. The aqueous layer was removed by pipetting and the phenol phase, pelleted material and interphase were discarded. The extraction procedure was repeated one more time with addition of sodium dodecyl sulfate and hot phenol. The aqueous phase remaining after the second extraction was flash evaporated to dryness at 35° C.

Removal of Phenol with Sephadex G-25 (21): To separate the RNA from any phenol contamination Sephadex G-25 was used. Fifty grams of Sephadex G-25 Coarse (Pharmacia Fine Chemicals) were placed in a beaker and washed several times with distilled water and the small particles were removed by decantation. After washing, the Sephadex beads were left at room temperature for one hour in distilled water. The swollen gel particles while being gently stirred were then poured
into a column (2.5 x 45 cm; Pharmacia). A sample applicator was placed on top of the column in direct contact with the Sephadex beads; the applicator is simply a cylindrical piece of plastic open at the top but whose bottom contains nets of nylon. A sample to be applied is first dissolved in a solvent (in this case 20% sucrose) whose density is greater than that of the eluent (distilled water) and then applied through a hole in the top of the column directly onto the nylon netting.

To the residue remaining after flash evaporation at 35°C, as described in the last step under the method of Stohs et al. (53), was added 4.0 ml of 20% sucrose; the insoluble material was removed by centrifugation (clinical centrifuge). The sucrose supernatant containing 3H-RNA was drawn up with a 5.0 ml syringe (an 18 gauge, 6 inch needle was attached to this syringe) and with the aid of a sample applicator was applied to the column. Distilled water was the eluent and the rate of flow was 3.0 ml/min. A Uvicord II Ultraviolet Absorptiometer set at a wavelength of 254 mµ was used for detection of RNA. The RNA was first detected with the Uvicord recorder after 75-80 ml of eluent had passed through the column; the next 30-40 ml of eluent were collected in a single flask. Collection of this single fraction was terminated when the recorder read essentially 100% transmission. This fraction containing RNA was then flash evaporated to dryness at 35°C.
The residue was dissolved in 2.0 ml of distilled water and aliquots of this sample were used to determine the amount of RNA present and for liquid scintillation counting. In those experiments designed to show that $^3$H-orotic acid was indeed being incorporated into RNA the residue remaining after flash evaporation was processed in a manner to be described later.

The amount of RNA was determined by using a standard yeast RNA (Sigma) sample and the Beckman DU spectrophotometer at a wavelength of 260 mµ. The Cary Model 15 spectrophotometer was also employed for a spectral analysis of each sample.

F. Liquid Scintillation Counting

Toluene Counting Solution: 5.0 g of PPO (2, 5-diphenyloxazole) were dissolved in one liter of toluene. To every 100 ml of this solution was added 20 ml of Beckman solubilizing reagent (Bio-Solv-BBS-3); this solution was the toluene cocktail used for counting purposes.

A 1.0 ml aliquot of the sample was placed in a liquid scintillation counting vial and to this was added 10 ml of the toluene counting solution. The samples were counted with the LS-250 Liquid Scintillation Spectrometer (Beckman Instruments). The specific activity of RNA was
expressed as disintegrations per minute per milligram of RNA (DPM/mg RNA). A comparison of experimental and control specific activities revealed whether or not vitamin D₃ enhanced the incorporation of ³H-uridine or ³H-orotic acid by increasing the rate of synthesis of RNA.

G. Hydrolysis of RNA

In order to show that ³H-orotic acid was indeed being incorporated into ³H-RNA, the final RNA residue remaining after flash evaporation was not dissolved in 2.0 ml of distilled water but instead was processed in a manner to be described in this and subsequent steps.

To the dry RNA sample was added 10 ml of 0.05 M KOH and the RNA was hydrolyzed to the mononucleotides by heating at 95°C in a water bath for 60 minutes. The hydrolyzate was cooled to 4°C and then neutralized with 5 N HClO₄. After precipitation at 0°C for 30 minutes the KClO₄ was removed by centrifugation. The supernatant was retained for purification of the ribonucleotides.

H. Ribonucleotide Purification with Dowex-2

Preparation of Anion-Exchange Resin: One and a half grams of Dowex-2-X8 (200-400 mesh; Bio-Rad Laboratories) were alternately treated with 30 ml of 1 N NaOH and 30 ml of 1 N HCl. The resin was
then washed five times with 1 N HCl (30 ml) to remove the very fine particles. Finally, the washing procedure was performed twice with doubly distilled water and the resin was kept wet until needed.

Adsorption and Elution of the 2', 3'-Ribonucleotides: To the neutralized solution of mononucleotides (i.e., supernatant remaining after KClO₄ precipitation as described above) was now added an equal volume of 0.05 M Tris-HCl buffer (tris hydroxymethyl aminomethane hydrochloride) pH - 7.8. To this solution was added the washed Dowex-2-X8 and the mixture was left at room temperature with occasional stirring for 30 minutes. After centrifugation (clinical centrifuge) the supernatant was discarded and the resin was transferred to an ultra-fine sintered glass filter (50 ml) with 0.025 M Tris-HCl buffer, pH-7.8. The buffer was removed by suction and discarded and to the resin was added 1.0 ml of 1.0 N HCl. After 30 minutes, and with occasional stirring, the mixture was filtered by suction. The filtrate contained the ribonucleotides in 1.0 N HCl.

I. Separation of 2', 3'-Ribonucleotides (32)

The separation of the 2', 3'-ribonucleotides on a cation exchange resin by elution first with HCl and then water takes advantage of the fact that below pH - 2.0 the guanosine, adenosine and cytidine mono-
phosphates (GMP, AMP and CMP) have charged amino groups and are exchanged on the column whereas uridine monophosphate (UMP) passes through. Since the pK of the amino group of GMP of 3.2 is approximately 1.3 pH units below CMP and AMP, it is eluted immediately after water is passed through the column. The CMP and AMP with similar pK's were eluted together.

**Preparation of Cation-Exchanger Dowex 50W-X4**: Fifty grams of Dowex 50W-X4 (200-400 mesh; Bio-Rad Laboratories) were washed five times with doubly distilled water and then twice with 0.05 N HCl. The resin was then mixed with 0.05 N HCl and with gentle stirring was poured into a column (0.9 x 10 cm). The flow rate was 0.76 ml/min and detection of the nucleotides as they were eluted from the column was again performed with the Uvicord II Ultraviolet Absorptiometer at a wavelength of 254 mµ.

The sample (nucleotides in 1.0 ml of 1.0 N HCl) was applied to the column and allowed to drain to the top of the resin. The sides of the column were then washed down with 1.0 ml of 0.05 N HCl, which was allowed to drain to the top of the resin. Three milliliters of 0.05 N HCl were then added to the top of the resin and the column was attached to a reservoir containing 0.05 N HCl. UMP was detected after 4.0 ml of HCl effluent were collected; the UMP was then
collected as one fraction (approximately 3.0 ml). The HCl was now allowed to drain to the top of the resin and 3.0 ml of water were added. The column was attached to a reservoir containing distilled water and GMP was detected after 5.0 ml of water effluent were collected; GMP was collected over the next 3 - 4 ml. The next 25 - 30 ml of water passed through the column eluted CMP and AMP together. The various fractions (i.e., UMP, GMP or AMP-CMP) were then flash evaporated to dryness at 32°C.

J. Thin-Layer Chromatography

To identify the UMP fraction thin-layer chromatography was employed in conjunction with the Cary Model 15 recording spectrophotometer.

The solvent system used for thin-layer chromatography (9) was isopropyl alcohol-concentrated HCl-water (65:16.7:18.3). Development of the cellulose thin-layer sheet containing fluorescent indicator was carried out at room temperature by ascending chromatography with chromatographic tanks purchased from the Kensington Scientific Corporation, Oakland, California. The various mononucleotide fractions were dissolved in 0.3 ml of 1.0 N HCl and 20 µl aliquots were applied to the cellulose sheets. Standard solutions of the 2' and 3' mono-
phosphate (mixed isomers; Schwarz Bioresearch, Inc., New York) of GMP, AMP, CMP and UMP were employed for identification of the components in the $^3$H-RNA hydrolysate. The concentration of the standard solutions was approximately 2.5 $\mu$g/$\mu$l and 10 $\mu$l aliquots were applied to the cellulose sheets.

Development of the chromatogram took one hour; the sheet was then air dried and the components were identified by ultraviolet light (UVS-11 Mineralight; Ultraviolet Products, San Gabriel, California). The standard UMP spot was cut out and the cellulose scraped from the sheet into a test tube. Two milliliters of 0.1 N HCl were then added and the tube was mixed and left at room temperature for 30 minutes. After centrifugation the supernatant containing UMP was retained for spectral analysis. The UMP from the RNA hydrolysate was cut out and processed in the same manner as just described. A blank was prepared by cutting out a spot on the cellulose sheet of approximately equal area and with the same $R_f$ value as the UMP spots; it was treated in the same manner as the UMP samples.
CHAPTER III
EXPERIMENTAL RESULTS

'Experiment 1: Determination of Serum Alkaline Phosphatase Activity in Normal and Rachitic Rats.

As described in the section on Materials and Methods, the level of serum alkaline phosphatase was used as a parameter to determine whether or not rats were rachitic. Prior to all experiments, three rats were chosen at random for these determinations from a population of 30 animals maintained on the rachitogenic diet for approximately four weeks. Serum samples for phosphatase assay were also obtained from control animals fed normal rat chow during the same four week period.

The values in Table II represent the total number of determinations that were made. In all cases the serum of rachitic rats exhibited a higher level of phosphatase activity and this difference is significant at \( P < 0.01 \).

Experiment 2: Effect of Vitamin D\(_3\) on the Incorporation of \(^3\)H-Orotic Acid into RNA of Rachitic Rat Bone.

Rachitic rats were fasted 15 hours prior to the start of the experiment which began at 7:00 A.M. On the day of the experiment five animals received 5000 I.U. of vitamin D\(_3\) intraperitoneally and five
TABLE II

SERUM ALKALINE PHOSPHATASE ACTIVITY
OF NORMAL AND RACHITIC RATS
(units/ml serum)

<table>
<thead>
<tr>
<th>Normal</th>
<th>Rachitic</th>
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<tbody>
<tr>
<td>1.94</td>
<td>3.92</td>
</tr>
<tr>
<td>2.80</td>
<td>3.56</td>
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<tr>
<td>2.20</td>
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<tr>
<td>1.68</td>
<td>3.86</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation. The difference between means is significant at $P < 0.01$. 
received the vehicle only. Two hours prior to sacrifice at 3, 6, 9 or 12 hours all animals received 50 µc of 3H-orotic acid. Upon sacrifice, the femurs, tibiae and humeri were removed from each animal, placed in 0.9% saline and then split open and cleaned as described under Materials and Methods. The bones of five animals within a group were pooled. The bones were then pulverized and homogenized and the RNA was extracted. The extracted RNA was separated from phenol on a Sephadex G-25 column. The results of this separation are illustrated in Figure 2. The RNA fraction was eluted from the column, flash-evaporated to dryness and then dissolved in distilled water. A spectrum of the RNA so isolated is shown in Figure 3. Aliquots of RNA dissolved in distilled water were used to determine the concentration and the absolute radioactivity of the 3H-RNA. The concentration of RNA was determined by using a standard yeast RNA (Sigma Chemical Company) sample and the Beckman DU spectrophotometer set at a wavelength of 260 mµ. The total amount of RNA isolated in this and subsequent experiments exhibited a concentration range of from 200 µg to 250 µg, and the specific activity is expressed as disintegrations per minute per milligram of RNA (DPM/mg RNA). From the data given in Table III, it is seen that the vitamin enhances the incorporation of 3H-orotic acid into RNA, when administered 1, 4 and 7 hours before
Separation of RNA from Phenol on Sephadex G-25 Column. (I) RNA Obtained from Bone of Rachitic Rats, (II) Unidentified Fraction, (III) Phenol.
FIGURE 3

(I) Spectrum of RNA Isolated from Bone of Rachitic Rats,
(II) Spectrum of Yeast RNA (Sigma Chemical Company).
### TABLE III

**INFLUENCE OF 5000 I.U. OF VITAMIN D₃ ON THE INCORPORATION OF ³H-OROTIC ACID INTO RNA OF RACHITIC RAT BONE***

Specific Activity of ³H-RNA  
(dpm/mg RNA)**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control</th>
<th>Vitamin D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1660 ± 50</td>
<td>1840 ± 55</td>
</tr>
<tr>
<td>6</td>
<td>2480 ± 74</td>
<td>3010 ± 90</td>
</tr>
<tr>
<td>9</td>
<td>2920 ± 88</td>
<td>3960 ± 119</td>
</tr>
<tr>
<td>12</td>
<td>4100 ± 123</td>
<td>3790 ± 114</td>
</tr>
</tbody>
</table>

*Vitamin D₃ deficient rats maintained on a diet with a high Ca:P ratio were sacrificed 3, 6, 9 and 12 hours after having received 5000 I.U. of vitamin D₃ or vehicle intraperitoneally. All rats received 50 µc of ³H-orotic acid 2 hours before sacrifice. The specific activity of the labeled RNA at each time interval was determined on pooled samples of bone obtained from 4 or 5 animals.

**Specific activity ± 3% counting error.
the tracer. The specific activity of the labeled RNA increases as the exposure time of the animals to the vitamin is increased. However, it appears that the incorporation of $^3$H-orotic acid into RNA is not influenced by vitamin D$_3$ when administered ten hours before the tracer.

Experiment 3: Use of the Ion-Exchange Resins Dowex 2 and Dowex 50 to Isolate and Separate the 2', 3'-Ribonucleotides of RNA Obtained from the Bone of Rachitic Rats.

This experiment was performed to demonstrate that $^3$H-orotic acid was being incorporated into RNA.

To a dry sample of extracted RNA, free from any contamination with phenol, was added 0.05 M KOH. The RNA was hydrolyzed to mononucleotides by heating it at 95°C for 60 minutes. The pH of the resultant solution was adjusted to 7.8 and the 2', 3'-ribonucleotides were adsorbed on Dowex 2 anion-exchange resin and then eluted from this resin with 1.0 N HCl.

Separation of ribonucleotides was accomplished with the use of a column containing the cation-exchange resin Dowex 50. UMP was eluted with 0.05 N HCl; GMP, AMP together with CMP were eluted with distilled water. Figure 4 shows the pattern of elution of the nucleotides as they were detected with the Uvicord II Absorptiometer set at a wavelength of 254 m.$\mu$. The spectra of the UMP and GMP separated from
Separation of 2', 3'-Ribonucleotides from the Alkaline Hydrolysis of RNA Obtained from Bone of Rachitic Rats. (I) Uridine Monophosphate, (II) Guanosine Monophosphate, (III) and (IV) are Mixed Isomers of the Monophosphates of Cytidine and Adenosine. (I) Was Eluted with 0.05 N HCl, (II), (III) and (IV) Were Eluted with Doubly Distilled Water. Separation of Nucleotides was performed on a Dowex 50W-X4 Column.
the mononucleotides appear in Figures 5 and 6. They were determined on aliquots taken from the fractions represented as Peak I and Peak II in Figure 4.

Experiment 4: Identification by Thin-Layer Chromatography of 2',3'-Ribonucleotides Isolated from the Alkaline Hydrolysis of RNA Obtained from Bone of Rachitic Rats.

The experiment was undertaken to obtain evidence, in addition to that already obtained, that \(^3\)H-orotic acid was indeed being incorporated into RNA.

The various mononucleotide fractions of (I) UMP, (II) GMP and (III - IV) AMP together with CMP were separated from the alkaline hydrolysate of RNA obtained from bone as described in Experiment 3 and illustrated in Figure 4. After elution from a column of Dowex 50, these fractions were flash-evaporated to dryness at 32°C and the dry material in each case was dissolved in 0.3 ml of 1.0 N HCl. Aliquots of the different nucleotides were applied to a thin-layer cellulose sheet with fluorescent indicator and development of the chromatogram was accomplished in one hour with a solvent system composed of isopropyl alcohol-concentrated HCl - water (65:16.7:18.3). Following ascending chromatography, the nucleotides were identified by ultraviolet light.
FIGURE 5

(I) Spectrum of UMP Separated from Mononucleotides Obtained from the Alkaline Hydrolysis of RNA Isolated from Bone of Rachitic Rats, (II) Spectrum of Standard UMP (Schwarz BioResearch)
FIGURE 6

(I) Spectrum of Standard GMP (Schwarz BioResearch)

(II) Spectrum of GMP Separated from the Alkaline Hydrolysis of RNA Isolated from Bone of Rachitic Rats.
The chromatogram illustrated in Figure 7 shows that the spots represented by V and VI are UMP and GMP respectively. Additional evidence for the isolation of UMP was obtained by cutting out the spots represented by I (standard UMP) and V (UMP obtained from RNA of bone) in Figure 7 and eluting them from the cellulose sheet with 0.1 N HCl. The spectra of the nucleotides are illustrated in Figure 8.

Although it is not described in this experiment, resolution of spot VII in Figure 7 into AMP and CMP was accomplished by using an additional solvent system containing saturated ammonium sulfate - 0.1 M sodium phosphate buffer (pH 6.0) - isopropyl alcohol (79:19:2).

Experiments 5 and 6: Effect of Vitamin D<sub>3</sub> on the Incorporation of <sup>3</sup>H-Orotic Acid into RNA of Rachitic Rat Bone.

The following two experiments were performed to delineate further the time interval at which a maximum response to vitamin D<sub>3</sub> could be observed. The experiments were conducted in the same fashion as in Experiment 2 except that the rats were sacrificed at 6, 9, 12 and 15 hours after having received vitamin D<sub>3</sub> or the vehicle. Table IV shows that any increase in the rate of incorporation of <sup>3</sup>H-orotic acid into RNA produced by vitamin D<sub>3</sub> is questionable. However, the results in Table V appear to indicate that vitamin D<sub>3</sub> enhanced the incorporation
FIGURE 7: Identification by Thin-Layer Chromatography of 2', 3'-Ribonucleotides Isolated from an Alkaline Hydrolysate of $^3$H-RNA Obtained from Bone of Rachitic Rats. Standards of UMP (I), CMP (II), AMP (III) and GMP (IV) were obtained from Schwarz BioResearch. The Nucleotides (V) UMP, (VI) GMP, and (VII) AMP-CMP were initially separated from the alkaline hydrolysate using a Dowex 50 column as illustrated in Figure 4. Development of the chromatogram takes one hour using an ascending solvent system composed of isopropyl alcohol-concentrated HCl-water (65:16.7:18.3).
FIGURE 8: (I) Spectrum of Standard UMP (Schwarz BioResearch), (II) Spectrum of UMP Isolated from an Alkaline Hydrolysate of $^3$H-RNA Obtained from Bone of Rachitic Rats. (I) and (II) were chromatographed on a cellulose thin-layer sheet using an ascending solvent system of isopropyl alcohol-concentrated HCl-water (65:16.7:18.3) and then eluted from the sheet with 0.1 N HCl.
TABLE IV

INFLUENCE OF 5000 I. U. OF VITAMIN D₃ ON THE INCORPORATION OF $^{3}H$-OROTIC ACID INTO RNA OF RACHITIC RAT BONE*

Specific Activity of $^{3}H$-RNA (dpm/mg RNA)**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control</th>
<th>Vitamin D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2020 ± 61</td>
<td>2370 ± 71</td>
</tr>
<tr>
<td>9</td>
<td>2640 ± 79</td>
<td>2790 ± 84</td>
</tr>
<tr>
<td>12</td>
<td>2330 ± 70</td>
<td>2500 ± 75</td>
</tr>
<tr>
<td>15</td>
<td>2270 ± 68</td>
<td>2300 ± 69</td>
</tr>
</tbody>
</table>

*Vitamin D₃ deficient rats maintained on a diet with a high Ca:P ratio were sacrificed 6, 9, 12 and 15 hours after having received 5000 I. U. of vitamin D₃ or vehicle intraperitoneally. All rats received 50 µc of $^{3}H$-orotic acid 2 hours before sacrifice. The specific activity of the labeled RNA at each time interval was determined on pooled samples of bone obtained from 4 or 5 animals.

**Specific activity ± 3% counting error.
TABLE V

INFLUENCE OF 5000 I. U. OF VITAMIN D₃ ON THE INCORPORATION OF $^3$H-OROTIC ACID INTO RNA OF RACHITIC RAT BONE*

Specific Activity of $^3$H-RNA

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control</th>
<th>Vitamin D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1340 ± 40</td>
<td>1910 ± 57</td>
</tr>
<tr>
<td>9</td>
<td>1600 ± 48</td>
<td>2370 ± 71</td>
</tr>
<tr>
<td>12</td>
<td>1760 ± 53</td>
<td>2280 ± 68</td>
</tr>
<tr>
<td>15</td>
<td>1700 ± 51</td>
<td>2940 ± 88</td>
</tr>
</tbody>
</table>

*Vitamin D₃ deficient rats maintained on a diet with a high Ca:P ratio were sacrificed 6, 9, 12 and 15 hours after having received 5000 I. U. of vitamin D₃ or vehicle intraperitoneally. All rats received 50 µc of $^3$H-orotic acid 2 hours before sacrifice. The specific activity of the labeled RNA at each time interval was determined on pooled samples of bone obtained from 4 or 5 animals.

**Specific activity ± 3% counting error.
of $^3$H-orotic acid and that the specific activity increased with time.

The results of Experiments 2, 5 and 6 are summarized in Table VI and Figure 9. These data indicate that the highest specific activity is observed nine hours after the administration of vitamin $D_3$ with a decrease being observed by the 12th hour.

Experiments 7 and 8: Effect of Vitamin $D_3$ on the Incorporation of $^3$H-Orotic Acid into RNA of Rachitic Rat Bone Nine Hours after the Administration of the Vitamin.

In order to discern whether or not the response produced by vitamin $D_3$ observed in Figure 9 was indeed significant at 9 hours, two additional experiments were performed at this time interval only.

On the day of the experiment the rachitic animals were divided into two groups of five animals each. Five animals received 5000 I.U. of vitamin $D_3$ and five received the vehicle only. Seven hours later all animals received 50 µc of $^3$H-orotic acid and two hours later they were sacrificed. Upon sacrifice, the femurs, tibiae and humeri were removed from each animal, placed in ice-cold 0.9% saline and then split open and cleaned. The bones were then pulverized and homogenized and the RNA was extracted.

The results of Experiments 7 and 8 and those of Nos. 2, 5 and
TABLE VI

INFLUENCE OF 5000 I. U. OF VITAMIN D₃ ON THE INCORPORATION OF ³H-OROTIC ACID INTO RNA OF RACHITIC RAT BONE*

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>No. of Experiments</th>
<th>Specific Activity of ³H-RNA (dpm/mg RNA) **</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>1660 ± 49 ( \pm ) 3%</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>1947 ± 53 ( \pm ) 3%</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>2387 ± 72 ( \pm ) 3%</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>2730 ± 82 ( \pm ) 3%</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>1985 ± 60 ( \pm ) 3%</td>
</tr>
</tbody>
</table>

*Vitamin D₃ deficient rats maintained on a diet with a high Ca:P ratio were sacrificed 3, 6, 9, 12 and 15 hours after having received 5000 I. U. of vitamin D₃ or vehicle intraperitoneally. All rats received 50 µc of ³H-orotic acid 2 hours before sacrifice. For each experiment at each time interval the specific activity of labeled RNA was determined on pooled samples of bone obtained from 4 or 5 animals.

**The values represent the mean \( \pm \) 3% counting error.
Vitamin D$_3$ Stimulated Increase in Rate of RNA Synthesis in Bone of Rachitic Rats. 5000 I. U. of Vitamin D$_3$ or Vehicle were administered intraperitoneally followed by 50 µc of $^3$H-orotic acid two hours prior to sacrifice at 3, 6, 9, 12 or 15 hours. (•—•—•) Vitamin D$_3$-treated, (○—○—○) Controls.
6 are summarized in Table VII. In all cases the data show an increase in the rate of RNA synthesis, as evidenced by a higher specific activity, 9 hours after the administration of vitamin D₃. The difference between control and D₃-treated animals is significant at P = 0.05.

Experiments 9 and 10: Effect of Actinomycin D on Vitamin D₃ Stimulated Incorporation of ³H-Orotic Acid into RNA of Rachitic Rat Bone.

Once it was established that the stimulatory action of the vitamin was significant at 9 hours, experiments with actinomycin D were designed to see whether or not the effect produced by the vitamin within this time interval could be blocked by the antibiotic. This information would reveal whether or not the action of the vitamin was directed upon the nuclear chromatin material of the rachitic bone cells.

At 5:00 A.M. on the day of the experiment, the animals were divided into four groups of four animals each, such that a dose of 100 µg of actinomycin D or its vehicle was injected intraperitoneally two hours prior to the administration of either vitamin D₃ (5000 I.U.) or its vehicle. Seven hours after the administration of the vitamin or vehicle all animals received 50 µc of ³H-orotic acid and two hours later they were sacrificed.

The data in Table VIII and Table IX again indicate that there was
TABLE VII

INFLUENCE OF 5000 I. U. OF VITAMIN D₃ ON THE INCORPORATION
OF ³H-OROTIC ACID INTO RNA OF RACHITIC RAT BONE*

Specific Activity of ³H-RNA
(dpm/mg RNA)

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Control</th>
<th>Vitamin D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2920 ± 88</td>
<td>3960 ± 119</td>
</tr>
<tr>
<td>5</td>
<td>2640 ± 79</td>
<td>2790 ± 84</td>
</tr>
<tr>
<td>6</td>
<td>1600 ± 48</td>
<td>2370 ± 71</td>
</tr>
<tr>
<td>7</td>
<td>1920 ± 58</td>
<td>3340 ± 100</td>
</tr>
<tr>
<td>8</td>
<td>2250 ± 68</td>
<td>3200 ± 96</td>
</tr>
<tr>
<td></td>
<td>2266 ± 68**</td>
<td>3132 ± 94**</td>
</tr>
</tbody>
</table>

*Vitamin D₃ deficient rats maintained on a diet with a high Ca:P
ratio were sacrificed 9 hours after having received 5000 I. U. of
vitamin D₃ or vehicle intraperitoneally. All rats received 50 µc of
³H-orotic acid 2 hours before sacrifice. The specific activity of
labeled RNA was determined for each experiment on pooled samples
of bone obtained from 4 or 5 animals.

**Mean ± 3% counting error. The difference between means is signifi-
cant at P = 0.05.
TABLE VIII

EFFECT OF ACTINOMYCIN D ON VITAMIN D₃ STIMULATED INCORPORATION OF ³H-OROTIC ACID INTO RNA OF RACHITIC RAT BONE*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Actinomycin D</th>
<th>Vitamin D₃</th>
<th>Specific Activity of ³H-RNA (dpm/mg RNA)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>1540 ± 46</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>2020 ± 60</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>300 ± 9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>460 ± 13</td>
</tr>
</tbody>
</table>

*Vitamin D₃ deficient rats maintained on a diet with a high Ca:P ratio were given 100 µg of actinomycin D or a control solution. Two hours later they were given 5000 I. U. of vitamin D₃ or vehicle intraperitoneally. Each animal received 50 µc of ³H-orotic acid seven hours after vitamin D₃ or vehicle and all were sacrificed two hours later. The specific activity of the labeled RNA was determined on pooled samples of bone obtained from 4 animals given the treatment specified.

**Specific activity ± 3% counting error.
**TABLE IX**

EFFECT OF ACTINOMYCIN D ON VITAMIN D₃

STIMULATED INCORPORATION OF ³H-OROTIC ACID INTO RNA OF RACHITIC RAT BONE*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific Activity of ³H-RNA (dpm/mg RNA)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D</td>
<td>Vitamin D₃</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Vitamin D₃ deficient rats maintained on a diet with a high Ca:P ratio were given 100 µg of actinomycin D or a control vehicle. Two hours later they were given 5000 I.U. of vitamin D₃ or vehicle intraperitoneally. Each animal received 50 µc of ³H-orotic acid seven hours after vitamin D₃ or vehicle and all were sacrificed two hours later. The specific activity of the labeled RNA was determined on pooled samples of bone obtained from 4 animals given the treatment specified.

**Specific activity ± 3% counting error.
a stimulated incorporation of $^3$H-orotic acid into RNA nine hours after the administration of vitamin D$_3$ in those animals that did not receive any actinomycin D. That actinomycin D was effective in decreasing the incorporation of $^3$H-orotic acid is evidenced by the decrease in specific activity of $^3$H-RNA upon prior administration of the antibiotic. However, the results also show that the stimulatory effect of the vitamin could not be blocked even upon prior treatment of animals with actinomycin D. The specific activities of the tritiated RNA from the vitamin-treated animals, instead of being the same, were still higher than those from the control animals. This would imply that the action of the vitamin is probably not on the nuclear chromatin material.

Experiment 11: Effect of Vitamin D$_3$ on the Incorporation of $^3$H-Uridine into RNA of Rachitic Rat Bone.

This experiment was performed to see whether or not vitamin D$_3$ induced the stimulated incorporation of $^3$H-uridine, in addition to that of $^3$H-orotic acid, into RNA. Also, actinomycin D was employed to determine whether or not the stimulatory action of the vitamin could be blocked, in the event that such an effect was produced.

Rachitic rats were fasted 15 hours before the experiment which began at 5:00 A.M. The animals were divided into four groups of
four animals each and received 100 µg of actinomycin D or vehicle 2 hours before the administration of 5000 I. U. of D₃ or vehicle. Seven hours after the injection of vitamin D₃ or vehicle, all animals received 50 µc of ³H-uridine and 2 hours later the animals were sacrificed. The bones were removed and ³H-RNA extracted as previously described.

The results in Table X indicate that vitamin D₃ does stimulate the incorporation of ³H-uridine into RNA of rachitic rat bone. However, the data observed with actinomycin D support the results obtained previously that the antibiotic did not block the enhanced incorporation of ³H-orotic acid into RNA. Of interest, in addition, is the fact that the specific activities reported in Table X for control and D₃-treated animals without prior administration of actinomycin D are smaller than those reported in Tables VIII and IX when the isotope ³H-orotic acid was employed instead of ³H-uridine.
TABLE X

EFFECT OF ACTINOMYCIN D ON VITAMIN D₃ STIMULATED INCORPORATION OF ³H-URIDINE INTO RNA OF RACHITIC RAT BONE*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Actinomycin D</th>
<th>Vitamin D₃</th>
<th>Specific Activity of ³H-RNA (dpm/mg RNA)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>639 ± 19</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>896 ± 26</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>219 ± 6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>338 ± 10</td>
</tr>
</tbody>
</table>

*Vitamin D₃ deficient rats maintained on a diet with a high Ca:P ratio were given 100 µg of actinomycin D or a control solution. Two hours later they were given 5000 I.U. of vitamin D₃ or vehicle intraperitoneally. Each animal received 50 µc of ³H-uridine seven hours after vitamin D₃ or vehicle and all were sacrificed two hours later. The specific activity of the labeled RNA was determined on pooled samples of bone obtained from 4 animals given the treatment specified.

**Specific activity ± 3% counting error.
CHAPTER IV
DISCUSSION AND CONCLUSIONS

The physiological expression of vitamin D action in skeletal tissue results in accretion. The biochemical events occurring after the administration of the vitamin and prior to accretion have not been delineated. In contrast to its action in intestine, it has not been demonstrated whether or not the vitamin can induce protein synthesis in bone, although recent work by Canas et al. (8) suggests that this may be the case.

Administration of 2000 I.U. of vitamin D$_3$ intraperitoneally to rats stimulates the incorporation of $^3$H-orotic acid into RNA obtained from the mucosal cells of intestinal tissue (53). This effect is blocked by prior administration of actinomycin D. The metabolic inhibitor is believed to exert its effect by combining with the guanine moieties of DNA. The accepted concept for its mode of action is that it blocks DNA-directed synthesis of m-RNA. Therefore, the results reported by Stohs et al. (53) strongly suggest that the action of the vitamin may be on the DNA - m-RNA complex of intestinal mucosal cells.

Such a proposal for the action of vitamin D on bone has not been suggested. However, since bone and intestine are target organs for the vitamin, a reasonable hypothesis would be that the vitamin may
act in a manner similar to its action in the intestine and stimulate the rapid labeling of skeletal tissue RNA by a precursor such as $^3$H-orotic acid. This has now been demonstrated.

Care was taken in these experiments in the isolation of the RNA before it was counted for tritium content. The RNA was extracted initially from skeletal tissue with perchloric acid followed by extraction with phenol. The RNA was then freed from any contamination with phenol by passing it through a column of Sephadex G-25 (Figure 2). That RNA was really isolated is shown first by the ultraviolet spectra, routinely checked, (Figure 3) and by hydrolysis of the RNA with subsequent isolation of the pyrimidine mononucleotide UMP (Figures 7 and 8).

From Table VI and Figure 9 it can be seen that an early event in the action of vitamin $D_3$ is an increased synthesis of RNA in the skeletal tissue of rachitic rats. A maximal response is obtained 9 hours after the intraperitoneal administration of 5000 I.U. of the vitamin and this response is significant at $P = 0.05$ (Table VII). Various interpretations may be advanced to account for the decrease in specific activity observed twelve hours after the administration of vitamin $D_3$ and illustrated in Figure 9. It has been reported that upon the intravenous injection of 10 I.U. of 1,2-$^3$H-vitamin $D_3$ (10 I.U., or
0.25 µg, is considered to be a physiological dose for the rat) 24 per cent of the original dose still remained in the plasma of rats after 6 hours and this amount was essentially the same even after 12 hours (13). Also, 10 per cent of the original dose could be found in the skeleton 4 hours after the administration of the vitamin; the time study did not exceed 4 hours. In the experiments reported here no attempt was made to determine the effective concentration of vitamin D$_3$ in bone cells. However, although the route of injection was different (intraperitoneal versus intravenous), the administration of 5000 I.U. of vitamin D$_3$ makes it seem unlikely that the concentration of the vitamin present in skeletal tissue is not sufficient to produce an effect even after 12 hours.

As previously mentioned, vitamin D$_3$ has been shown to stimulate the incorporation of $^3$H-orotic acid into RNA of intestinal mucosal cells. In that study Stohs et al. (53) demonstrated that the stimulatory effect produced by the vitamin was abolished after 8 hours. They proposed that the decrease in the response may have resulted from an increase in the level of intracellular calcium with the cation subsequently acting as a feedback inhibitor, blocking the synthesis of more RNA following the initial burst. Since bone and intestine are target tissues for the vitamin, the idea that some regulatory mechanism may also exit in
skeletal tissue that controls the action of vitamin D₃ cannot be dismissed.

Finally, depletion of the RNA precursor ³H-orotic acid following the initial increase in RNA synthesis may be an additional explanation for the decrease in specific activity observed 12 hours after the administration of vitamin D₃.

Figure 9 illustrates that there is an increase in the rate of RNA synthesis at 3, 6 and 9 hours following the administration of 5000 I. U. of vitamin D₃ intraperitoneally. However, this increase in specific activity of ³H-RNA with time is paralleled by a similar increase, although of smaller magnitude, in the control animals. A possible explanation for this may arise upon closer examination of the vehicle utilized for these experiments. The solution containing vitamin D₃ was prepared by first dissolving 5 milligrams of the vitamin in 0.5 ml of 95% ethanol and then diluting this solution to 10 ml with propylene glycol such that 0.25 ml of this solution was equivalent to 5000 I. U. Oxidation of one of the components in the vehicle namely, propylene glycol, results in the production of lactic and pyruvic acids. The rachitic rats used in these experiments were fasted approximately 15 hours before the start of each experiment. Therefore, the possibility exists that propylene glycol, and more specifically its metabolites, may
have served as a source of food for these animals. The incorporation of these metabolites into the general metabolism of bone cells may have favored an increase in the rate of RNA synthesis. Consequently, an effect, in addition to that of vitamin D₃, may have been produced because of the vehicle employed.

In addition to propylene glycol, the use of ethanol in the vehicle merits consideration. Recently, Ponchow et al. (45) have studied the fate of vitamin D₃ in vitamin D-deficient rats following the intravenous injection of 10 I.U. of 1,2-³H-vitamin D₃ dissolved either in 0.05 ml of absolute ethanol or 0.05 ml of blood plasma. Their study revealed that ethanol accelerates the disappearance of vitamin D₃ from the plasma and that this disappearance may have been reflected in the observed increased fecal excretion of tritium in the ethanol-treated rats. Also, they proposed that ethanol may interfere directly with the intracellular metabolism of the vitamin in the liver by decreasing the rate of conversion of vitamin D₃ to its active metabolite 25-hydroxycholecalciferol. In the current experiments the route of administration and the dose of vitamin D₃ were different from those utilized by Ponchow et al. (45). Also, approximately 0.01 ml of ethanol (as compared to 0.05 ml reported above) was contained in 0.25 ml of the injection medium. Therefore, although it is possible, it seems unlikely that this amount
of ethanol administered intraperitoneally along with 5000 I.U. of vitamin D₃ would have any noticeable effect on the metabolism of the vitamin in these experiments.

From Tables VIII and IX it appears that the stimulated incorporation of ³H-orotic acid into RNA nine hours after the administration of vitamin D₃ is not abolished by prior administration of actinomycin D. This suggests that the vitamin may act in some fashion other than directly upon the nuclear chromatin material. There is evidence to suggest that vitamin D₃ acts on cellular membranes of intestinal mucosal cells (25, 27, 67) as well as on the subcellular membranes of the nucleus (52) and mitochondria (12). This is in contrast to the apparent action of the vitamin on the nuclear chromatin material (23, 28, 29). Therefore, the vitamin may well exhibit multiple sites of action. If this is the case, then one may postulate that vitamin D may act analogously to its action in the intestine and alter the membrane permeability of skeletal tissue cells. This could ultimately result in an increase in the availability of the RNA precursor ³H-orotic acid, and this increase may be reflected in a greater incorporation of the isotope into RNA.

Upon histochemical examination of skeletal tissue obtained from normal and rachitic rats, it has been observed that the activity of
certain enzymes was altered following the administration of vitamin D 
(2, 14, 15, 17). However, these studies did not reveal whether or not 
the vitamin acted directly upon a particular enzymatic reaction or 
indirectly by affecting the synthesis of the enzyme. If the vitamin can 
affect enzyme reactions of skeletal tissue directly, then it is possible 
to assume that the vitamin may increase the synthesis of RNA in bone 
indirectly by stimulating the activity of an enzyme such as DNA-
dependent RNA polymerase. Such a stimulation has actually been 
demonstrated (54) in the case of several hormones (e.g., thyroid 
hormone, growth hormone, testosterone). However, the mechanism of 
the stimulatory action of these hormones on the RNA polymerase is 
not known.

The dose of 5000 I.U. of vitamin D₃ used in the above experi-
ments is several orders of magnitude larger than that considered to be 
a physiological dose (10 I.U.) for the rat. However, this is not to say 
that the response obtained (i.e., an increase in the rate of RNA 
synthesis) is an apparent dissociation from the physiological action of 
the vitamin. It is probable that such a dose of the vitamin may have 
partially overcome the inhibition by actinomycin D. That this may be 
the case is suggested by studies of certain hormones, in which it was 
reported that an increase in the dose of a hormone partially overcame
prior inhibition by actinomycin D (54).

Table X shows that vitamin D₃ stimulated the incorporation of ³H-uridine into RNA in those animals that had not been previously treated with actinomycin D. In addition, the stimulatory action of the vitamin was still observed upon treatment of the animals with actinomycin D prior to the administration of vitamin D₃. Therefore, it appears that the action of the vitamin is not on the nuclear chromatin material. Several explanations are possible for the action of vitamin D₃ and these are the same as those mentioned above relative to the experiments in which ³H-orotic acid was used instead of ³H-uridine. Of interest, also, is the fact that the specific activities reported in Table X for control and D₃-treated animals without prior administration of actinomycin D, are smaller than those reported in Tables VIII and IX when ³H-orotic acid was employed instead of ³H-uridine. Examination of possible pathways for the enzymatic synthesis of pyrimidines not involving orotic acid, has revealed that isotopic uridine serves as a poor substrate for the synthesis of UMP in mammals as evidenced by a decrease in the utilization of the isotope for polynucleotide synthesis (47). In the above experiments the ³H-orotic acid or ³H-uridine was administered two hours prior to sacrifice. Since uridine is not the substrate of choice in the synthesis of UMP, a possible explanation for
the lower specific activities reported in Table X would be that the conversion of $^3$H-uridine to $^3$H-UMP requires a pulse labeling period longer than two hours to produce an amount of $^3$H-UMP equivalent to that formed when $^3$H-orotic acid is used as a precursor instead.

In this study no attempt was made to distinguish between the various types of RNA isolated from the bone of rachitic animals. However, the evidence suggests that vitamin D$_3$ does not affect the synthesis of mRNA directly even though the overall rate of RNA synthesis was increased. Nevertheless, it is still possible that the vitamin may have acted at the nuclear level indirectly by stimulating the activity of an enzyme(s) essential for the synthesis of mRNA or by altering nuclear membrane permeability such that the precursors for RNA synthesis are more readily available. At present it is difficult to discern the full importance of this stimulation in the sequence of events which occurs following the administration of vitamin D$_3$. It does seem, however, that an early event in the expression of vitamin D$_3$ action in rachitic bone involves the increase in the rate of RNA synthesis.
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ABSTRACT


The effect of vitamin D₃ on the incorporation of ³H-orotic acid into the RNA of bone obtained from rachitic rats was determined. Rickets was produced in young animals by maintaining them for four weeks on a diet containing a high Ca:P ratio and deficient in vitamin D₃. Reduced growth and an elevation in the level of serum alkaline phosphatase were used as parameters to establish that the rats were rachitic.

The administration of 5000 I.U. of vitamin D₃ intraperitoneally to rachitic animals, previously fasted for 15 hours, was followed by sacrifice at 3, 6, 9, 12 and 15 hours. Two hours prior to sacrifice all animals received 50 µc of ³H-orotic acid. The femurs, tibiae and humeri were removed from each animal and the RNA was extracted.

Care was taken in these experiments in the isolation of the RNA before it was counted for tritium content. The RNA was extracted initially from skeletal tissue with perchloric acid followed by extraction with phenol. The RNA was then freed from any contamination with phenol by passing it through a column of Sephadex G-25. That RNA
was really isolated was shown first by the ultraviolet spectra, routinely checked, and by the hydrolysis of the RNA with subsequent isolation of the pyrimidine mononucleotide UMP.

At all time intervals studied, treatment with vitamin D₃ increased the incorporation of ³H-orotic acid into the ribonucleic acid of rachitic bone with a maximal response being observed by the ninth hour. Prior treatment of the animals with an antibiotic, actinomycin D, did not abolish the stimulatory action of vitamin D₃. In a similar study utilizing ³H-uridine instead of ³H-orotic acid, it was found that vitamin D₃ stimulated the incorporation of the isotope into the RNA of bone. Again, as was reported above, prior administration of actinomycin D did not abolish the stimulatory effect of the vitamin. This would suggest that the action of the vitamin is not on the nuclear chromatin material.

At present it is difficult to discern the full importance of this stimulation in the sequence of events which occurs following the administration of vitamin D₃. It does seem, however, that an early event in the expression of vitamin D₃ action in the bone of rachitic animals involves the increase in the rate of RNA synthesis.
APPROVAL SHEET

The dissertation submitted by Rodger Samuel Izzo 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 dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

January 19, 1970

Date

Signature of Advisor: [Signature]