COO-1668-68

MECHANISMS OF CALCIUM TRANSPORT IN SMALL INTESTINE

Overall Review of the Contract

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ABSTRACT: During the past three years considerable advance has been registered in our understanding of the mechanism of intestinal calcium transport, which is activated in response to 1,25-$(OH)_2D_3$, the active form of the vitamin in the system. In brush borders isolated from vitamin D-deficient chicks, a 200,000 molecular weight protein has been found by disc gel electrophoresis which is not present in chicks given vitamin D. This protein does not bind calcium and does not possess calcium dependent adenosine triphosphatase activity. Following the administration of 1,25-$(OH)_2D_3$ to the deficient chicks this protein disappears from the disc gel profiles and a protein of molecular weight 220,000 appears in the gel profiles. This protein has been isolated and shown to possess calcium adenosine triphosphatase activity, alkaline phosphatase activity and it binds calcium. Work is progressing on the purification of these proteins with the ultimate aim of discerning what role they have in intestinal calcium transport.

An investigation of the subcellular localization of 1,25-$(OH)_2D_3$ has been partially completed using cellular fractionation techniques specifically adapted to the intestine. Eighty percent of the radioactivity found in intestinal mucosal homogenates of rats and chicks can be found in the crude nuclear debris fraction. However, when pure nuclei are isolated, only 20% of the total mucosal radioactivity can be accounted for in this fraction. Unfortunately, the yield of nuclei is only about 20%, which casts some doubt on this information. On the other hand, the isolation of pure chromatin using the procedures of Marushige and Bonner revealed that only 20-30% of the tissue radioactivity is found in this fraction. Thus some 50% of the radioactivity from 1,25-$(OH)_2D_3$ in the intestinal cells remains unaccounted for.

An investigation of possible receptor proteins for 1,25-$(OH)_2D_3$ in the intestine has been undertaken. In rat intestinal mucosal cytosol, a 6S macromolecule fraction has been found which will bind both 1,25-$(OH)_2D_3$ and 25-OHD_3. However, cytosol of a variety of non-target organs for vitamin D also show this 6S macromolecule. This protein prefers 25-OH-D_3 and *in vivo* saturation studies have shown that it does not bind 1,25-$(OH)_2D_3$ *in vivo*, but does bind 25-OH-D_3. This eliminates it as a possible receptor protein in the intestinal calcium transport system. So far in rat intestinal mucosal cytosol no evidence has been found for the existence of a small molecular weight macromolecule which would bind specifically 1,25-$(OH)_2D_3$ as has been reported in the chick. In the chick, evidence has been obtained that there is a 3.5S macromolecule in the cytosol which will bind rather specifically 1,25-$(OH)_2D_3$. Unfortunately the amount of this receptor varies widely from animal to animal which has hampered further investigation of it. Additionally, it is puzzling why the chick intestinal cytosol shows evidence of such a macromolecular binding agent for 1,25-$(OH)_2D_3$ and the rat does not.

Work has continued on the factors which regulate intestinal calcium transport by regulating the biogenesis of 1,25-$(OH)_2D_3$ in the kidney. It has now been shown by both *in vivo* measurements of 1,25-$(OH)_2D_3$ accumulation in blood and intestine and by means of *in vitro* measurements of the 25-OH-D_3-1α-hydroxylase in isolated chick kidney mitochondria that the following agents reduce intestinal calcium transport by reducing the biosynthesis of 1,25-$(OH)_2D_3$: high calcium diets, 1,25-$(OH)_2D_3$ itself, strontium diets, ethane-1-hydroxy-1,1-diphosphonate, high phosphate diets and parathyroidectomy. The administration of exogenous sources of 1,25-$(OH)_2D_3$ restores intestinal calcium transport of animals given high calcium diets, strontium

diets, treated with ethane-1-hydroxy-1,1-diphosphonate and animals whose parathyroids were removed by surgery, illustrating that these agents do function on intestinal calcium transport by depressing biogenesis of the 1,25-$(OH)_2D_3$.

Low phoshorus diets have also been shown to increase the accumulation of 1,25-$(OH)_2D_3$ in intestine and blood. Furthermore, chicks placed on a low phosphate diet show elevated levels of the 25-OH-D_3-1α-hydroxylase of isolated kidney mitochondria. However, the administration of exogenous sources of 1,25-$(OH)_2D_3$ to low phosphate rats and high phosphate rats fails to correct the difference in intestinal calcium transport which suggests that phosphate deprivation has some other effect on intestinal calcium transport other than stimulation of 1,25-$(OH)_2D_3$ biogenesis.

In conjunction with work carried out on my NIH grant dealing with the metabolism and function of vitamin D, a number of analogs of 1,25-$(OH)_2D_3$ and other metabolites of vitamin D_3 have been prepared. Of great importance is 1α-OH-D_3 which will approximate 1,25-$(OH)_2D_3$ in its biological activity in all systems known to be responsive to vitamin D. The synthesis of 6α-^{3}H-1α-OH-D_3 has been completed and this compound has been shown to be converted to 1,25-$(OH)_2D_3$ *in vivo*. However, it has not yet been determined whether 1α-OH-D_3 can stimulate intestinal calcium transport without 25-hydroxylation. On the other hand, 3-deoxy-1α-OH-D_3 has been synthesized and shown to be active in intestinal calcium transport which illustrates that the 3-hydroxyl group is not essential to intestinal calcium transport if the vitamin D molecule has a 1α-hydroxy group present. A number of side chain analogs have been synthesized which illustrate that shortening of the side chain by even one carbon on 1,25-$(OH)_2D_3$ markedly reduces its intestinal calcium transport activity and furthermore elimination of a large piece of the side chain will completely eliminate its activity in this system. Appreciably altering the side chain by removal of one or more carbons such as 26 and 27 also markedly reduces biopotency in the intestine. Other analogs which represent isomers of this cis-triene structure have revealed that alteration of this system also results in markedly reduced biological activity. So far it can be said that the intestinal calcium transport system requires an intact side chain, an intact cis-triene structure and the presence of a 1α-hydroxyl group. The 3-hydroxyl group is not required for this system if the 1α-hydroxyl group is present.

An extract of the plant *Solanum malacoxylon* has been studied for its effects on intestinal calcium absorption in nephrectomized rats. This extract will stimulate intestinal calcium transport in the vitamin D-deficient, nephrectomized rat very much like 1,25-$(OH)_2D_3$. However, unlike the metabolite of vitamin D, the extract will not stimulate bone calcium mobilization nor will it stimulate the elevation of serum inorganic phosphate and the cure of rickets. The active principle has been greatly purified and although not completely pure has a molecular weight of 2,000-2,500 as determined by Sephadex chromatography. The substance is stable to alkali and unstable to acid. Work is continuing on its purification for subsequent chemical identification.

The role of vitamin D in phosphate transport reactions in the intestine has been studied in some detail. Phosphate transport in the ileum and the jejenum in response to vitamin D takes place in the absence of calcium in the medium *in vitro*. Furthermore this transport system requires sodium and metabolic energy. Sodium is required for the uptake of phosphate from the brush border surface rather than for the extrusion of phosphate from the serosal or basement membrane. The ileal transport of phosphate is stimulated by 1,25-$(OH)_2D_3$ and 25-OH-D_3. Nephrectomy prevents the response to physiologic amounts of 25-OH-D_3, which illustrates that 1,25-$(OH)_2D_3$ is a phosphate transport hormone as well as a calcium transport hormone.

I. MAIN RESEARCH ACCOMPLISHMENTS

A. Regulation of Intestinal Calcium Transport

Perhaps the greatest accomplishment under the past three-year research support from the AEC has been the demonstration that a number of substances which have been previously thought to directly affect calcium transport have been shown to do so by regulating the synthesis of $1,25\text{-}(OH)_2D_3$, the active form of the vitamin which is synthesized exclusively in the kidney as described in the previous three-year overall review. The most significant of all of these is the well known regulation of intestinal calcium transport by dietary calcium levels. It had been demonstrated in 1937 by Nicolaysen and his collaborators in 1953 that animals or man on a low calcium diet develop high efficiencies of intestinal calcium absorption while those on a high calcium diet develop low efficiencies. Because the adaptation to low dietary calcium was found to continue until bones were completely calcified, Nicolaysen had postulated the existence of an endogenous factor or hormone which was secreted by the skeleton and which would instruct the intestine to absorb calcium. This endogenous factor could only be found in animals or man given a source of vitamin D. With the demonstration that $1,25\text{-}(OH)_2D_3$ is the metabolically active form of the vitamin in the intestine, research in this laboratory demonstrated clearly that low dietary calcium stimulates the biogenesis of $1,25\text{-}(OH)_2D_3$ whereas high calcium represses the synthesis of this hormone. When the synthesis of the $1,25\text{-}(OH)_2D_3$ was repressed another metabolite, $24,25\text{-}(OH)_2D_3$, was made instead. The function of the $24,25\text{-}(OH)_2D_3$ is not yet known. In any case, it appeared that $1,25\text{-}(OH)_2D_3$ might be the endogenous factor which controls intestinal calcium transport in response to the need for calcium. Conclusive demonstration has now been obtained in both chicks and rats that this is the case. Rats or chicks maintained on $1,25\text{-}(OH)_2D_3$ show high efficiencies of intestinal calcium transport quite independent of dietary calcium, whereas animals maintained on $25\text{-OH-}D_3$, the immediate precursor, as their source of vitamin D, show the expected adaptation to dietary calcium. It is, therefore, clear that the regulation of intestinal calcium transport by dietary calcium levels is mediated by regulation of biogenesis of $1,25\text{-}(OH)_2D_3$. In a similar fashion it has been demonstrated that strontium replacing calcium in the diet produces a vitamin D-resistant rickets and a markedly reduced intestinal calcium transport. This has been shown by work in this laboratory under the AEC contract to be due to a shut down in the biosynthesis of $1,25\text{-}(OH)_2D_3$. Administration of $1,25\text{-}(OH)_2D_3$ reverses the strontium-induced repression of intestinal calcium transport.

Ethane-1-hydroxy-1,1-diphosphonate, a pyrophosphate analog which is used in the treatment of Paget's disease and other bone diseases in man, causes rickets and repressed intestinal calcium absorption in both rats and chicks. This can be reversed by the administration of exogenous sources of $1,25\text{-}(OH)_2D_3$. Furthermore, it has been demonstrated that this diphosphonate given at 10-40 mg/kg body weight shuts down the biosynthesis of $1,25\text{-}(OH)_2D_3$. *In vitro* measurements of the $25\text{-OH-}D_3$-1α-hydroxylase of isolated mitochondria demonstrate that the enzyme has been shut down by the diphosphonate treatment. Furthermore, direct *in vitro* addition of the diphosphonate inhibits non-competitively the $25\text{-OH-}D_3$-1α-hydroxylase. In intact mitochondria this inhibition appears at about 0.5 mM diphosphonate. However, when the mitochondria are swollen to permit the use of NADPH for reducing equivalents,

the diphosphonate is inhibitory at much lower concentrations. In addition the inhibition of 1,25-$(OH)_2D_3$ synthesis by the diphosphonates in vivo cannot be reversed by parathyroid hormone administration and does occur even in phosphate depleted animals. Furthermore, the inhibition of the 25-OH-D_3-1α-hydroxylase by administration of the diphosphonate in both rats and chicks can be shown to take place in vitamin D-deficient animals. Therefore, it seems likely that the diphosphonate inhibits intestinal calcium transport by direct inhibition of the 25-OH-D_3-1α-hydroxylase.

It has long been known that phosphate depletion of animals brings about an elevated intestinal calcium transport. Work in this laboratory during the past contract period has shown that this is due to a stimulation of 1,25-$(OH)_2D_3$ synthesis even in parathyroidectomized animals. In fact the extent to which intestinal calcium transport is stimulated by low phosphate diets exactly correlates with the accumulation of 1,25-$(OH)_2D_3$ in the intestine. However, the exogenous administration of 1,25-$(OH)_2D_3$ is not effective in eliminating the difference between intestinal calcium transport of rats on a low phosphorus diet versus those on a normal phosphorus diet. It is, therefore, clear that phosphate depletion besides stimulating the biogenesis of 1,25-$(OH)_2D_3$ also has some other effect on intestinal calcium transport quite independent of its effect on the stimulation of 1,25-$(OH)_2D_3$.

Another major accomplishment during the past contract period is the demonstration that the need for calcium, which stimulates the synthesis of 1,25-$(OH)_2D_3$, has been shown to be mediated by the parathyroid glands. Work carried out in our laboratory has shown that parathyroidectomy of rats maintained on a low calcium diet is followed by a shut-down in 1,25-$(OH)_2D_3$ synthesis, which could be restored by the administration of exogenous parathyroid hormone. It is, therefore, clear that parathyroid glands sense slight hypocalcemia which in response secretes parathyroid hormone. The parathyroid hormone progresses to the kidney where it stimulates 1,25-$(OH)_2D_3$ synthesis. The 1,25-$(OH)_2D_3$ then journeys to the intestine and bone where it mobilizes calcium, restoring serum calcium to normal and shutting off parathyroid hormone secretion.

Because of the finding that the parathyroid glands regulate 1,25-$(OH)_2D_3$ synthesis in response to a need for calcium, work was carried out to test whether the well established effects of parathyroid hormone on intestinal calcium absorption and bone calcium mobilization might be due solely to its stimulation of 1,25-$(OH)_2D_3$ synthesis which must be regarded as a calcium mobilizing hormone. It was shown in a series of experiments that the intestinal calcium transport response to 1,25-$(OH)_2D_3$ does not require the presence of parathyroid hormone and furthermore this effect is not enhanced by the presence of the peptide hormone. On the other hand the mobilization of calcium from bone requires the parathyroid hormone as well as 1,25-$(OH)_2D_3$. It is, therefore, clear that the stimulation of intestinal calcium transport by parathyroid hormone is mediated entirely by the stimulation of 1,25-$(OH)_2D_3$ synthesis.

In all of these regulation studies the accumulation of 1,25-$(OH)_2D_3$ in the tissues in vivo from radioactive 25-OH-D_3 have been confirmed by direct measurements in vitro of the 25-OH-D_3-1α-hydroxylase in isolated mitochondria from chicks. Thus we have shown that high calcium diets, diphosphonate, high strontium diets, parathyroidectomy and high phosphate diets repress the level of 25-OH-D_3-1α-hydroxylase in the kidney on the other hand, parathyroid hormone, the lack of calcium and the lack of phosphate results in enhanced levels of the 25-OH-D_3-1α-hydroxylase in isolated chick kidney mitochondria.

Finally, it should be noted that regulation of the 25-OH-D_3-1α-hydroxylase is greatly minimized in vitamin D deficiency. Thus vitamin D-deficient animals regardless of their dietary calcium and phosphorus levels have high levels of 25-OH-D_3-1α-hydroxylase. In animals fed either a high calcium or a high phosphate diet, the administration of vitamin D brings about a shut-down in the 25-OH-D_3-1α-hydroxylase and an increase in the 25-OH-D_3-24-hydroxylase. This shut-down has been shown to be due to the 1,25-$(OH)_2D_3$ in some way playing a direct role in the regulation of its own biogenesis. The exact mechanism of this regulation is unknown but since the shut-down of the 1α-hydroxylase requires hours, it does not appear to be due to 1,25-$(OH)_2D_3$ stimulating or working through ionic inhibition or activation. Thus it is clear that physiologically, the calcium and phosphorus levels of the blood, the parathyroid hormone and 1,25-$(OH)_2D_3$ itself all interact to regulate the 25-OH-D_3-1α-hydroxylase in the kidney by some complex and unknown mechanism.

B. Mechanism of Calcium Transport

The main thrust of this investigation has been an examination of the mechanism whereby 1,25-$(OH)_2D_3$ will stimulate the intestine to begin transporting calcium. Thus examination of the subcellular location of tritium labeled 1,25-$(OH)_2D_3$ was undertaken. Within one hour following the administration intravenously of radioactive 1,25-$(OH)_2D_3$, 80% of the intestinal mucosa ^3H is found in the nuclear fraction. The remainder is found primarily in the cytosol with small amounts appearing in the mitochondria and microsomes. A further examination of the nuclear fraction revealed that the intestine does not fractionate very easily by standard methods. Repeated attempts to develop methods which would allow for quantitative isolation of pure nuclei has met with failure. However, a procedure for isolating 20% of the mucosal DNA in the form of pure nuclei has been devised. With this method it is possible to show that only 20-30% of the nuclear fraction radioactive 1,25-$(OH)_2D_3$ can be accounted for in pure nuclei. This agrees with our finding that only 20-30% of the cellular tritium labeled 1,25-$(OH)_2D_3$ is found in the chromatin fraction when it is isolated in pure form by the method of Marushige and Bonner. Thus approximately 50% of the cellular radioactivity from tritiated 1,25-$(OH)_2D_3$ in intestinal mucosa is unaccounted for.

Because of a report from another laboratory that 1,25-$(OH)_2D_3$ functions in the intestine in the manner of a steroid hormone, attempts have been made to locate a cytosolic receptor protein in intestinal mucosa which would be specific for 1,25-$(OH)_2D_3$. In the intestinal mucosal cytosol obtained from vitamin D-deficient rats, a 6S macromolecular component could be located by sucrose density centrifugation which binds both tritium 25-OH-D_3 and tritium labeled 1,25-$(OH)_2D_3$. No other protein could be located which would bind specifically 1,25-$(OH)_2D_3$ as has been reported in the chick. The 6S macromolecular component, however, shows binding ability only for tritium labeled 25-OH-D_3 and not labeled 1,25-$(OH)_2D_3$ when administered *in vivo*. Furthermore, saturation studies have shown that the 6S macromolecular component has a higher affinity for 25-OH-D_3 than for 1,25-$(OH)_2D_3$. By administering to nephrectomized animals non-radioactive 25-OHD_3 or non-radioactive 1,25-$(OH)_2D_3$ in saturating amounts, it was possible to test whether *in vivo* loaded 6S macromolecular component could bind 1,25-$(OH)_2D_3$ *in vitro*. Density gradient sucrose analysis revealed clearly that *in vivo* saturation with 25-OH-D_3 prevents *in vitro* binding of either tritium labeled 1,25-$(OH)_2D_3$ or tritium labeled 25-OH-D_3 to the 6S macromolecular component. On the other hand, the *in vivo* administration of 1,25-$(OH)_2D_3$ in saturating

amounts does not prevent the binding in vitro of either of the radioactive metabolites of vitamin D. These results clearly show that the 6S macromolecular component in the intestinal cytosol is not a receptor for 1,25-$(OH)_2D_3$ in the stimulation of intestinal calcium transport.

More recent work has centered on a 3.5S cytosolic protein in chick intestinal mucosa which binds 1,25-$(OH)_2D_3$. This cytosolic component does not bind 25-OH-D_3 and binds 1,25-$(OH)_2D_3$ rather specifically. It is strange, however, that this protein does not make its appearance in rat intestinal mucosa and furthermore all chick tissues including non-target organs contain this component. It has not been established as yet, but it appears unlikely that this component represents a "receptor".

Despite the fact that some 20% of the tritium labeled 1,25-$(OH)_2D_3$ of intestinal mucosa is found in the nuclear fraction, it has not been possible to block the intestinal calcium transport response to 1,25-$(OH)_2D_3$ with actinomycin D, despite the fact that this identical treatment will block 1,25-$(OH)_2D_3$-induced bone calcium mobilization. Thus it is not at all convincing that 1,25-$(OH)_2D_3$ stimulates intestinal calcium transport by the unmasking of genetic information which brings about the synthesis of specific proteins which function in intestinal calcium transport. Much additional work is necessary before this hypothesis can be either excluded or be supported by experimental fact.

In a continuing effort to elucidate the mechanism whereby 1,25-$(OH)_2D_3$ will stimulate intestinal calcium transport, the brush borders have been isolated from chick intestinal mucosa, solubilized by butanol extraction and then separated by disc gel electrophoresis utilizing sodium dodecyl sulfate gels. Using this technique it has been possible to demonstrate that vitamin D-deficient brush borders contain a protein with a molecular weight of 200,000, which disappears following treatment with 1,25-$(OH)_2D_3$ and instead there appears a protein of molecular weight 220,000. This change occurs at the same time intestinal calcium transport is initiated. Work has continued on the isolation of these two proteins. The 200,000 molecular weight protein has some alkaline phosphatase activity, but no calcium dependent adenosine triphosphatase activity and no ability to bind calcium. The 220,000 molecular weight protein binds calcium and has calcium dependent adenosine triphosphatase activity. The time course of appearance of this substance suggests that it may well play an important role in intestinal calcium transport in response to 1,25-$(OH)_2D_3$.

C. Analogs and Metabolites of 1,25-$(OH)_2D_3$

Following the demonstration that 1,25-$(OH)_2D_3$ is the metabolically active form of vitamin D_3 in the intestine, work has begun to prepare analogs of 1,25-$(OH)_2D_3$ to learn about the relationship between the structure of the 1,25-$(OH)_2D_3$ molecule and its role in intestinal calcium transport. Much of the earlier work carried out on analogs of vitamin D must be disregarded inasmuch as discrimination against an analog may have been at the level of 25-hydroxylation or 1-hydroxylation. In any case the following synthetic analogs have been prepared and the testing has been carried out under the present contract. One of the most important analogs of 1,25-$(OH)_2D_3$ is 1α-hydroxyvitamin D_3 (1α-OH-D_3). This substance, which lacks the 25-hydroxyl, is almost as active as 1,25-$(OH)_2D_3$ in stimulating intestinal calcium

transport in the chick and about one-half as active in the rat. The possibility, however, exists that this compound can be hydroxylated on carbon 25 in vivo before it functions. To learn more about this, tritiated 1α-OHD$_3$ has just been prepared and although it is clearly evident that the 1α-OH-D$_3$ is converted to 1,25-(OH)$_2$D$_3$, it has not yet been decided whether 1α-OH-D$_3$ initiates intestinal calcium transport without conversion to the 1,25-(OH)$_2$D$_3$. This work will undoubtedly continue and be elucidated under the continuation of the contract. More recently, however, the 3-deoxy-1α-OH-D$_3$ has also been synthesized and is found to be active on intestinal calcium transport. This result demonstrates that once the 1α-hydroxyl group is in position, the 3-hydroxyl is no longer necessary to support intestinal calcium transport. However, the critical compound, 3-deoxy-1α,25-(OH)$_2$D$_3$, has not yet been produced and is being synthesized at the present time.

The synthesis of side chain analogs of 1,25-(OH)$_2$D$_3$ have been made. They include 5,6-trans-vitamin D$_3$, 5,6-trans-25-OH-D$_3$, 26-nor-25-OH-D$_3$, 5,6-trans-26-nor-25-OH D$_3$, 26,27-bisnor-25-OH-D$_3$, 5,6-trans-26-27-bisnor-25-OH-D$_3$, 24-nor-5,6-trans-25-OH-D$_3$ and 24-nor-1,25-(OH)$_2$D$_3$. In addition, the 22-27-pentanor-1,25-(OH)$_2$D$_3$ has also been synthesized. A detailed study of their intestinal calcium transport activity has led to the conclusion that an intact and extended side chain is ncessary for optimal intestinal calcium transport activity. Even the omission of a single carbon markedly reduces intestinal calcium transport activity. The 24-nor-1,25-(OH)$_2$D$_3$ has about 1/1000 the activity of 1,25-(OH)$_2$D$_3$. On the other hand the 22-27-pentanor-1,25-(OH)$_2$D$_3$ is totally devoid of intestinal calcium transport activity. It is, therefore, clear at this stage that a 1-hydroxyl and a full side chain is ncessary for intestinal calcium transport activity, whereas the 3-hydroxyl is not necessary once the 1-hydroxyl is in position. Even slight modification of the side chain brings about drastic reduction in intestinal calcium transport activity of 1,25-(OH)$_2$D$_3$.

Natural metabolites of vitamin D$_3$, namely 24,25-(OH)$_2$D$_3$, 25,26-(OH)$_2$D$_3$ and 1,24,25-(OH)$_3$D$_3$ have been examined for their intestinal calcium transport activity. All these compounds have intestinal calcium transport activity, although 1,24,25-(OH)$_3$D$_3$ is the only one with activity approaching 1,25-(OH)$_2$D$_3$. This compound appears to be approximately 60% as active as 1,25-(OH)$_2$D$_3$. The 24,25-(OH)$_2$D$_3$ is less than 1/5 as active and the 25,26-(OH)$_2$D$_3$ is approximately 1/10 as active as the 1,25-(OH)$_2$D$_3$ compound.

D. An Active Calcium Transport Substance from the Plant Solanum malacoxylon

A plant in South America which produces menastatic calcification in grazing cattle has been studied in regard to intestinal calcium transport. An extract of these dried plants has been made and in agreement with work carried out in the field, has been shown to contain marked intestinal calcium transport activity. Solanum malacoxylon water extract stimulates intestinal calcium absorption in vitamin D-deficient rats and furthermore this stimulation occurs in nephrectomized animals which suggests that it can substitute for 1,25-(OH)$_2$D$_3$. However, unlike 1,25-(OH)$_2$D$_3$ the plant extract cannot stimulate the mobilization of calcium from bone nor can it stimulate the elevation of serum inorganic phosphate and the cure of rickets in rats. This compound nevertheless represents a substance of marked interest in view of its effect in stimulating intestinal calcium transport. The extract has been subjected to silicic acid column chromatography, Sephadex

chromatography and thin-layer chromatography. The active principle has been greatly purified and has a molecular weight in the neighborhood of 2,000-2,500. It is acid labile and alkaline stable. It can be acetylated and made lipid soluble for purification. Work is now in progress on continued purification with the ultimate aim of isolation and identification.

E. Intestinal Phosphate Transport

As a corollary to intestinal calcium transport, intestinal phosphate transport has been studied in response to vitamin D_3. In work carried out in other laboratories it has been shown that vitamin D activates an intestinal phosphate transport mechanism in the jejenum and the ileum. This transport mechanism requires metabolic energy and is supported by glucose and sodium ions. Sodium ions are required for the uptake of the phosphate rather than the extrusion into the serosal fluid as has been shown to be the case for intestinal calcium transport. The intestinal phosphate transport mechanism can be stimulated by 25-OH-D_3 and by 1,25-$(OH)_2D_3$. Nephrectomy prevents the response to 25-OH-D_3 but not to 1,25-$(OH)_2D_3$. Since phosphate deprivation and hypophosphatemia stimulates 1,25-$(OH)_2D_3$ biosynthesis and this compound also stimulates intestinal phosphate transport, this substance must also be regarded as a hormone involved in phosphate mobilization as well as calcium mobilization.

II. PLANS FOR CONTINUATION OF THE PRESENT OBJECTIVES AND POSSIBLE NEW OBJECTIVES IN CONSIDERATION OF PAST RESULTS

In general past results and new developments which have occurred in the field will not alter the general aim of the contract, namely to elucidate the molecular mechanism of intestinal calcium transport. Many new developments in the area of vitamin D metabolism and function have made the prospects for realization of the aims much greater than before. The realization that 1,25-$(OH)_2D_3$ works directly on the intestine has greatly simplified the task of studying the mechanism of intestinal calcium transport. In addition much new chemistry has become available in the synthesis of active analogs of vitamin D which have provided an important new tool for the synthesis of highly radioactive 25-OH-D_3 and hence highly radioactive 1,25-$(OH)_2D_3$ which will permit for the first time a clear investigation into the subcellular location of the active form of vitamin D in the intestine and its possible combination with receptor proteins in cytosol and the nucleus. In addition, the development of high pressure liquid chromatography system has provided the most powerful resolution of all the metabolites of vitamin D_3 which allows assignment of unambiguous structures to radioactive peaks. Finally, the discovery of proteins in the brush border which are altered in response to 1,25-$(OH)_2D_3$ has opened an entire new approach to understanding the mechanism whereby calcium is transported across the small intestine. It is primarily using these tools that the mechanism of calcium transport will be investigated. In keeping with the general aims of the agency, we will also examine the possibility that lead, strontium, barium and cadmium may be absorbed in a manner similar to calcium or perhaps are absorbed by identical carriers which transport calcium. An understanding of how these compounds are absorbed would aid greatly in prevention of toxicity or would help in suggesting methods of coping with patients once toxicity has been determined. Furthermore, the synthesis of an anti-1,25-$(OH)_2D_3$ may provide a tool for counteracting anti-lead poisoning.

III. Graduate Students Trained, Degrees Granted and Postdoctoral Tenures:

The following students have been trained under the present contract, at least in part:

Graduate Students

Chen, Tai C. (7/1/72-12/31/72)

Frolik, Charles A. (1/1/73-5/31/73)

Knutson, Joyce C. (9/1/72-12/31/72)

Postdoctoral Tenures:

Knutson, Joyce C. (4/1/73-6/30/73)

Kream, Barbara (currently)

Moriuchi, Sachiko (9/1/73-12/31/73)

Reynolds, Robert D. (11/1/73-4/30/74)

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V. Status of Knowledge of Intestinal Calcium Absorption, the Role of Vitamin D, Parathyroid Hormone, and General Aims of the Proposal

The major thrust of this contract is to determine the molecular mechanism of intestinal calcium transport in the intestine; to determine what factors control the transport and does this transport reaction accommodate any of the environmental pollutants to which man is now exposed. There are many factors which are known to markedly influence intestinal calcium absorption. The most notable, of course, is the vitamin D status. It has long been known that vitamin D plays a central role in intestinal calcium absorption. This was defined primarily through the work of Nicolaysen and his collaborators during the 1930-1950 era (1). Additional work by Migicovsky and Nielson (2) and Harrison and Harrison (3) helped place this phenomenon on a firmer base at the physiologic level. It was Schachter and his coworkers, however, that provided the first important breakthrough in our understanding of the mechanism whereby calcium is transported across the small intestine (4). Utilizing the everted intestinal sac method originally devised for glucose transport by Wilson and Wiseman (5), Schachter and Rosen first showed that calcium is transported actively against a concentration gradient and that this system was in some way activated by vitamin D. Schachter and collaborators extended their observations to show that this system could be used as a method of assay for vitamin D biological activity (6). They furthermore provided evidence that vitamin D activates not only the uptake of calcium from the brush border surface, but also the extrusion of calcium into the serosal fluid (7). Harrison and Harrison also studied the intestinal calcium transport mechanism by the everted sac technique and in addition to the observations of Schachter and colleagues provided evidence that vitamin D changes the permeability of the intestinal mucosal membrane and suggested that the active transport portion of the process was not vitamin D dependent (8, 9). This question has not been totally resolved and probably will not be resolved until the mechanism whereby intestinal calcium transport is delineated at the molecular level. Wasserman and his colleagues (10) and Schachter (11) were the first to show that calcium is transported against an electrical gradient as well as a chemical gradient. Furthermore, using *in vivo* techniques, Wasserman and Kallfelz (12) were able to show that vitamin D stimulates the transfer of calcium not only from mucosal to serosal but also from serosal to mucosal surfaces. However, the rate constant for the mucosal to serosal surface is very much larger than the reverse direction. Wasserman and Taylor (13) reported that sodium is not required for intestinal calcium transport. This, however, was disproved by the work of Martin and DeLuca (14) and by Harrison and Harrison (15) in which a clear requirement for sodium in the process could be demonstrated. The sodium ions, however, in contrast to amino acid and glucose transport are required for the extrusion of calcium across the basal membrane surface (16). The question of whether vitamin D plays a role at the brush border surface as well as the basal surface has come under scrutiny and is not settled. Schachter and his colleagues have provided evidence based on 15 minute incubation periods that both processes are affected by vitamin D (7). On the other hand, Martin and DeLuca (16) demonstrated that the uptake of calcium from the brush border surface is affected by vitamin D and probably not the basal membrane handling of calcium. The fact that the brush border surface is affected has been supported by the work of Walling and Rothman in which very short time periods of incubation illustrate that the initial uptake of calcium by the brush borders is influenced by vitamin D (17).

One of the most important breakthroughs in our understanding of intestinal calcium transport has been the discovery of a calcium binding protein in the intestine in response to vitamin D treatment (18). A great deal of work has been carried out on this important substance in the laboratory of Wasserman and his colleagues defining the characteristics of the protein and the kinetics of its appearance (19-21). In the chick this protein has a molecular weight of approximately 24,000 and it binds four calcium ions per mole of protein. In the rat and other mammals, the protein that binds calcium and which appears in response to vitamin D has a molecular weight of between 8,000-12,000 (21, 22). Exactly how the calcium binding protein participates in intestinal calcium transport has not been settled. It is also possible that the calcium binding protein does not participate in the transport process but may represent an ancillary substance responsible for concentrating the calcium at the brush border surface. Taylor and Wasserman (23) have shown that the primary source of calcium binding protein in the intestine appears to be the goblet cell with localizations observed along the brush border surface of the villus cell. In general calcium transport correlates quite well with the level of calcium binding protein under a variety of circumstances (19, 24-27). However, there have been disturbing points where there is no quantitative relationship between the level of calcium binding protein and intestinal calcium transport level. For example, there is no real quantitative relationship between the appearance of calcium binding protein and the level of calcium transport in response to a single dose of vitamin D (25, 28). In the case of 1,25-$(OH)_2D_3$ chicks respond by showing the calcium binding protein and increased intestinal calcium absorption. However, when the intestinal calcium transport is allowed to decay to predosage levels, the calcium binding protein remains high (21). In addition there have been reports whereby cortisone depresses intestinal calcium transport but increases the calcium binding protein level (29, 30). Therefore, intestinal calcium transport and calcium binding protein levels in the small intestine cannot be equated until transport is more fully delineated.

During the past several years it has been clearly established that vitamin D_3 must be metabolically altered in the liver to 25-OH-D_3 and subsequently in the kidney to 1,25-$(OH)_2D_3$ before it can function in the small intestine (31, 32). Evidence has been presented which convincingly demonstrates that 1,25-$(OH)_2D_3$ is the metabolically active form in this system (33-36). It is clear that for future investigation it is the 1,25-$(OH)_2D_3$ which must be used in order to understand the molecular mechanisms which are responsible for responses to vitamin D in intestinal calcium transport. It, therefore, becomes important to determine what is the nature of the responses to 1,25-$(OH)_2D_3$ in the small intestine. There is disagreement as to whether the 1,25-$(OH)_2D_3$ stimulation of intestinal calcium transport is prevented by the prior treatment with actinomycin D. Although actinomycin D will block in rats the response of the intestine to vitamin D_3, the same dose of actinomycin D given one or two hours prior to 1,25-$(OH)_2D_3$ will not block this response (37, 38). On the other hand, Norman and his colleagues have reported that dosage of actinomycin D every two hours prior to and during treatment with 1,25-$(OH)_2D_3$ to chicks will prevent their intestinal calcium transport response (39). In our hands this treatment results in chicks which are near death and it is not surprising that their intestines do not respond under this circumstance. It is, therefore, not entirely clear whether 1,25-$(OH)_2D_3$ functions in the young growing animal by derepression or by the induction

of a calcium transport substance. Corradino has developed an intestinal organ culture system in which metabolites of vitamin D can be added and the stimulation of calcium binding protein appearance can be observed (40, 41). Under these circumstances 1,25-$(OH)_2D_3$ is the most active substance known, although some activity is observed with 25-OH-D_3, 1α-OH-D_3 and vitamin D_3 itself (42). These responses are all blocked by the addition of α-aminitin and actinomycin D which suggests in the embryonic intestine that an induction process is required for the appearance of the calcium binding protein. The relationship, however, of these studies to intestinal calcium transport responses in a vitamin D-deficient animal is not entirely clear.

Haussler and his colleagues have championed the idea that 1,25-$(OH)_2D_3$ functions by interacting with a 3.5S macromolecule or receptor in intestinal cytosol which undergoes change as it enters the nucleus to become a larger protein and that this protein functions by binding to chromatin and unmasking genes which code for calcium transport protein (43-45). Haussler and his colleagues have shown that incubation of radioactive 1,25-$(OH)_2D_3$ with cytosol will bring about the transfer of the radioactivity into isolated chromatin. This has been used as a method of assay for 1,25-$(OH)_2D_3$ but the success of this method has not yet been established (46, 47). Lawson and colleagues in Great Britain have isolated polysomes from the intestines of chicks either given vitamin D or not given vitamin D and studied their ability to synthesize calcium binding protein *in vitro* from radioactive amino acids and using calcium binding protein antibody to isolate the synthesized protein (48, 49). They have shown that polysomes from vitamin D-treated animals synthesize the calcium binding protein while those from vitamin D-deficient animals do not. Although this represents an interesting experiment it does not constitute proof that 1,25-$(OH)_2D_3$ functions in intestinal calcium transport by unmasking genes which code for calcium binding protein. Experiments in the rat have not supported either the receptor hypothesis or the calcium binding protein synthesis experiments of Lawson. For example, Drescher and DeLuca (50) attempted to show *de novo* synthesis of calcium binding protein by the injection of radioactive amino acids at the time that the intestine was responding to various forms of vitamin D. However, they detected no incorporation of radioactive amino acids into the calcium binding protein. Similar work has been reported by Bruns and Avioli (51) again casting doubt on the idea that the active form of vitamin D brings about the rise in intestinal calcium transport by stimulating calcium binding protein biosynthesis *de novo*. On the other hand, MacGregor and his colleagues have been able to show incorporation of amino acids into the chick calcium binding protein in response to vitamin D (52). However, they found it necessary to maintain chicks on a level of vitamin D in order to observe such incorporation. It is very possible that their experiments merely show that calcium binding protein turnover is greater in animals given vitamin D than in those that are maintained on marginal amounts of vitamin D_3. The question then of the molecular mechanism whereby 1,25-$(OH)_2D_3$ stimulates intestinal calcium transport remains open. The approach being used in this laboratory is to examine the characteristics of the brush border membrane to determine what changes are taking place at the time intestinal calcium transport is initiated. Initially we are examining the proteins which can be separated by disc gel electrophoresis. So far we have discovered two proteins or macromolecular components which are altered following 1,25-$(OH)_2D_3$ administration. We intend to continue this approach and to isolate and study the proteins which appear altered or

proteins which appear in response to the 1,25-$(OH)_2D_3$ inasmuch as it seems that there is universal agreement that at least one site of vitamin D alteration in the mucosal cells is at the brush border surface. Furthermore, a considerable amount of work will be expended in studying the subcellular location of 1,25-$(OH)_2D_3$ and the way in which it progresses from the plasma through the cytosol or endoplasmic reticulum to its primary subcellular location. In general a great deal of work is planned in the area of the receptor proteins for 1,25-$(OH)_2D_3$, if there are any, and whether they function in the manner suggested for other steroid hormones.

Another important area of investigation is the regulation of intestinal calcium transport by agents other than vitamin D. Agents such as cortisone, high dietary calcium, high dietary strontium, dietary barium, low dietary phosphate and dietary lead are known to influence intestinal calcium transport. It has long been known that animals fed a low calcium diet show marked elevations in efficiency of intestinal calcium absorption whereas those on a high calcium diet show low efficiencies (53). Furthermore, there is a relationship between the need for calcium in the skeleton and the efficiency of intestinal calcium transport which lead to the suggestion by Nicolaysen of the existence of an endogenous factor which would inform the intestine of the skeletal needs for calcium. With the realization that 1,25-$(OH)_2D_3$ is the active form of the vitamin in the intestine came the hypothesis that this substance might represent the endogenous factor (54, 55). Boyle *et al.* demonstrated that animals on a low calcium diet have marked ability to synthesize 1,25-$(OH)_2D_3$ *in vivo* whereas those on a high calcium diet have a markedly reduced ability to make this metabolite. In vitamin D-deficient animals, 1,25-$(OH)_2D_3$ is synthesized regardless of the level of calcium in the diet. Omdahl and DeLuca have demonstrated that chicks maintained on 1,25-$(OH)_2D_3$ from exogenous sources show markedly efficient intestinal calcium absorption independent of dietary calcium whereas the same animals maintained on 25-OH-D_3 as their source of vitamin D show the expected adaptation to dietary calcium (38). In extensive work carried out under the present contract Ribovich and DeLuca (unpublished results) have demonstrated that 1,25-$(OH)_2D_3$ eliminates the ability of the rat to adapt to dietary calcium levels. All rats regardless of dietary levels of calcium show markedly efficient intestinal calcium absorption when given 1,25-$(OH)_2D_3$. In contrast, when given 25-OH-D_3 or vitamin D_3, the intestinal adaptation to dietary calcium is observed. It seems likely that 1,25-$(OH)_2D_3$ represents the endogenous factor at least as far as the intestine is concerned.

Strontium in the diet is known to inhibit intestinal calcium absorption and produce a vitamin D-resistant rickets as shown by Wasserman and his colleagues (56, 57). Omdahl and DeLuca were able to show that this was due to an inhibition of 1,25-$(OH)_2D_3$ production as measured by both *in vivo* and *in vitro* techniques (58, 59). Furthermore, the inhibition of intestinal calcium absorption by strontium feeding could be eliminated by the administration of 1,25-$(OH)_2D_3$ from exogenous sources. Similar experiments have been carried out with ethane-1-hydroxy-1,1-diphosphonate in which reduced intestinal calcium transport is brought about by the administration of this diphosphonate at a level of 10 mg/kg to 40 mg/kg (60, 61). Administration of exogenous 1,25-$(OH)_2D_3$ in this case will overcome the reduced intestinal calcium absorption brought about by the diphosphonate. *In vitro* experiments with chicks given the diphosphonate have shown that in fact the 25-OH-D_3-1α-hydroxylase is essentially shut-off by the administration of the diphosphonate (61-63). The

exact nature of this inhibition is not entirely settled although preliminary results suggest that it may operate by direct inhibition of the hydroxylase. Work by Hill et al. (63) has shown that rachitic rats given diphosphonate will respond quite well to a single dose of vitamin D_3 but following the initial dose a second dose of vitamin D_3 produces no response in the diphosphonate-treated animals. This suggests that vitamin D_3 is necessary to condition the animal to a response to the diphosphonate. Unfortunately work in our laboratory has failed to confirm these findings and in fact one can demonstrate that diphosphonate given to vitamin D-deficient rats and chicks will shut off the 25-OH-D_3-1α-hydroxylase (61, Baxter and DeLuca, unpublished results). The reason for the discrepancy between results from the two groups is unknown at the present time.

Because the need for calcium stimulates synthesis of 1,25-$(OH)_2D_3$, an examination of the relationship between serum calcium concentration and synthesis of 1,25-$(OH)_2D_3$ in rats and in chicks has been carried out in this laboratory (54, 55, 64). Hypocalcemia brings about an increased synthesis of 1,25-$(OH)_2D_3$ whereas normal to hypercalcemia will shut off the synthesis of this metabolite. This led to the concept that parathyroid gland might be monitoring the serum calcium concentration and secreting parathyroid hormone in response to hypocalcemia. This in turn would stimulate 1,25-$(OH)_2D_3$ production. Experiments in the rat have shown that in fact parathyroidectomy eliminates the ability of animals on a low calcium diet to make 1,25-$(OH)_2D_3$ (65). This ability is restored by the administration of exogenous sources of parathyroid hormone. Additionally, mitochondria isolated from chicks given injections of parathyroid hormone show a marked elevation in the 25-OH-D_3-1α-hydroxylase as measured in vitro (66). It therefore seems clear that an important role of the parathyroid hormone is the stimulation of 1,25-$(OH)_2D_3$ synthesis. It seems possible, therefore, that the role of parathyroid hormone in in intestinal calcium transport is entirely mediated by the stimulation of 1,25-$(OH)_2D_3$. Experiments carried out by Garabedian et al. (67) show that the intestinal calcium transport response to 1,25-$(OH)_2D_3$ does not require the presence of parathyroid hormone whereas its effect on bone calcium mobilization does. Therefore, it is clear that reduced intestinal calcium transport in the hypoparathyroid state is due to a lack of 1,25-$(OH)_2D_3$ synthesis. The administration of exogenous 1,25-$(OH)_2D_3$ restores intestinal calcium transport levels to normal in parathyroidectomized rats (67) and in hypoparathyroid patients (Fraser and DeLuca, and Neer and DeLuca, unpublished results).

Carlsson (68) first discovered that phosphate deprivation markedly stimulates intestinal calcium absorption. This work was confirmed by the work of Morrissey and Wasserman (27) and later by the work of Tanaka et al. (69). Tanaka and DeLuca, however demonstrated that the increased intestinal calcium transport brought about by phosphate deprivation correlates very well with the accumulation of 1,25-$(OH)_2D_3$ in the intestine. Furthermore, Tanaka and DeLuca (70) have been able to show that extreme hypophosphatemia will stimulate 1,25-$(OH)_2D_3$ production even without the presence of the parathyroid hormone. However, Ribovich and DeLuca (unpublished results) have found that rats maintained on 1,25-$(OH)_2D_3$ from exogenous sources will nevertheless show a marked elevation in intestinal calcium transport under conditions of phosphate deprivation suggesting that some other factor is involved in the hypophosphatemic stimulation of intestinal calcium absorption. A similar conclusion was reached by Wasserman and colleagues using an analog of 1,25-$(OH)_2D_3$, namely dihydrotachysterol$_3$ (71). Work will continue on the mechanism whereby hypophosphatemia stimulates intestinal calcium absorption in 1,25-$(OH)_2D_3$-treated animals. Attention will be

focused on the metabolism of 1,25-$(OH)_2D_3$ of hypophosphatemic versus normal phosphatemic animals and in addition a possible phosphate shut down of intestinal transport *in vitro* will be studied as a possible mechanism.

There has been very little attention paid to the idea that lead is transported by a mechanism similar to those used for calcium. The incidence of lead poisoning remains as a major problem, especially among children in old and substandard housing. Information which might be helpful in excluding lead from being absorbed is needed. Although this author is not familiar with the massive literature relating to lead toxicity and lead metabolism, we hope to examine the mechanism of lead absorption as to whether it is related to the calcium transport system and whether it is activated by vitamin D. Thus the methodology used in studying intestinal calcium transport by everted sacs, *in situ* loops and in different segments of the small intestine will be used. Hopefully some insight into the conditions under which lead absorption is favored or conditions under which it is not, will be useful in prevention and treatment of chronic lead toxicity.

With the realization that 1,25-$(OH)_2D_3$ is the metabolically active form in the intestine has come renewed interest in the structural requirements for the vitamin D molecule in the initiation of intestinal calcium transport. The first analog of major importance to be synthesized is the 1α-OH-D_3 (72), which was prepared in the synthetic exercise prior to the synthesis of 1,25-$(OH)_2D_3$. This compound was found to approach 1,25-$(OH)_2D_3$ in its activity in the stimulation of intestinal calcium transport (72, 73), but it is unknown at the present time whether this substance acts directly without 25-hydroxylation or whether it undergoes 25-hydroxylation prior to function (72, 74). With the synthesis of [6α-^{3}H]-1α-OH-D_3 this question is currently under intensive investigation under this contract. Although it can be shown that 1α-OH-D_3 is converted to 1,25-$(OH)_2D_3$ *in vivo*, it is not certain whether this hydroxylation occurs rapidly enough to account for the initial response of the intestine to 1α-OH-D_3. This is currently under examination.

The synthesis of 3-deoxy-1α-OH-D_3 has been completed and it has been found to be active on intestinal calcium transport (75, 76). Thus the 3-hydroxyl is no longer necessary in vitamin D compounds possessing the 1α-hydroxyl group. Kaneko (personal communication) have prepared 2α-$(OH)_2D_3$ and 1α,2β-$(OH)_2D_3$ and have shown them to be inactive in the stimulation of intestinal calcium transport. At the present time, therefore, evidence is clear that the 1-hydroxyl of ring A is required whereas the 3-hydroxyl is not required and further modification of the molecule by hydroxylation on carbon 2 results in inactivation of the molecule. A determination of the effect of side chain modification has also been carried out and briefly one can state that appreciable shortening of the side chain or elimination of many of the carbons of the side chain results in complete elimination of intestinal calcium transport activity. It can, therefore, at this stage be said that an intact side chain, a 1-hydroxyl, a 5,6-cistriene system are all required for intestinal calcium transport whereas a 3-hydroxyl is not required once the vitamin D molecule has a 1α-hydroxyl group. However, much remains to be learned regarding the structural requirements for stimulation of intestinal calcium transport and furthermore these compounds will be of great use in elucidating the correct receptor molecules which mediate the intestinal calcium transport function. Under separate support we are planning to synthesize the 3-deoxy-1,25-$(OH)_2D_3$, the 25Aza-1α-OH-D_3, the 25-fluoro-1α-OH-D_3 and 25-methoxy-1α-OH-D_3 and the 1-methoxy and 1-fluoro-25-OH-D_3.

Under this contract these compounds will be tested for their biological activity not only in stimulating intestinal calcium transport, but as a possible anti-$1,25\text{-}(OH)_2D_3$. Note that the 25Aza, 25-fluoro and 25-methoxy compounds will reveal whether a 25-hydroxyl group is essential for calcium transport since they cannot be hydroxylated in this position.

In South America a disease among grazing cattle has been noted in certain seasons in which the animals consume the plant Solanum malacoxylon (77). Scientists in Argentina and Brasil and elsewhere have shown that this substance increases intestinal calcium absorption, causes hypercalcemia and soft tissue mineralization (77). In this laboratory and in the laboratory of Wasserman, it has been shown that the Solanum malacoxylon extracts stimulate intestinal calcium absorption in vitamin D-deficient rats and chicks and furthermore nephrectomy does not prevent the response to the Solanum malacoxylon (77-79). Wasserman and his colleagues have shown that this substance stimulates the appearance of calcium binding protein, both in vivo and in vitro in the organ culture systems and they have suggested that this substance represents a plant source of $1,25\text{-}(OH)_2D_3$ (78, 80). In this laboratory it has been shown that although the Solanum malacoxylon stimulates intestinal calcium absorption, it does not stimulate the mobilization of calcium from bone in vitamin D-deficient animals suggesting that it is not $1,25\text{-}(OH)_2D_3$ (79). Work has already been started on the isolation of this substance and its chemical identification. It is proposed that work will continue on the isolation and identification of the active principle in the Solanum malacoxylon extract. In addition, further work will be carried out on the possible relationship of this compound or substance to the well known functions of vitamin D in phosphate metabolism and in mineralization of bone.

Although attention has focused almost exclusively on the role of vitamin D in calcium metabolism, more recently attention has become focused also on the role of vitamin D in phosphate metabolism (81). Harrison and Harrison (82) first demonstrated that vitamin D stimulates the transport of phosphate in the distal small intestine. Although they could not exclude the possibility that it was related to vitamin D activation of calcium transport, more recent work by Kowarski and Schachter (83) and by Wasserman and Taylor (84) has shown in fact that the phosphate transport system is unrelated to the calcium transport system responsive to vitamin D. This has been confirmed by work in our laboratory which has shown that the phosphate transport system in the ileum and jejenum is not stimulated by the addition of calcium (85). Taylor (86) has shown quite clearly that the sodium ions function in the initial uptake of phosphate rather than its extrusion across the basal membrane. In our laboratory we have demonstrated that $1,25\text{-}(OH)_2D_3$ and $25\text{-}OH\text{-}D_3$ stimulate intestinal phosphate transport and that nephrectomy eliminates the response to the $25\text{-}OH\text{-}D_3$ illustrating that the $1,25\text{-}(OH)_2D_3$ compound is probably the active form (85). During the proposed tenure of the contract we expect to do considerable work on the phosphate transport mechanism to delineate as far as possible the nature of the transport process and to demonstrate conclusively that it is independent or unlike calcium transport system responsive to vitamin D. We would further like to determine more fully the nature of the process, the requirements and inhibitors,

Methods of Procedure

The proposed projects which will be worked on during the next contract period are listed according to their priority.

1. Synthesis of High Specific Activity ^{3}H-1,25-$(OH)_2D_3$ and Its Subcellular Location in Intestine

The top priority investigation will center upon the subcellular location of tritium labeled 1,25-$(OH)_2D_3$ and the mechanisms whereby the tritium labeled 1,25-$(OH)_2D_3$ are transported to these subcellular sites. In short, an investigation of the possibility that 1,25-$(OH)_2D_3$ functions like the steroid hormones through a cytosolic receptor and nuclear receptor mechanism to activate transcription of DNA which codes for calcium transport proteins will be pursued further. Initially it will be necessary to synthesize radioactive 1,25-$(OH)_2D_3$ of a specific radioactivity of the order of 100 Curies/mM. The most radioactive vitamin D compound currently available is 25-OH-D_3 of about 10 Curies/mM radioactivity. The ^{3}H-25-OH-D_3 can be converted *in vitro* to ^{3}H-1,25-$(OH)_2D_3$ with kidney mitochondria isolated from rachitic chicks followed by purification on Sephadex LH-20 columns. Unfortunately, it is not possible to make a more radioactive 25-OH-D_3 using the procedures employed in making the 10 Curies/mM substance. However, we have succeeded in synthesizing large amounts of an intermediate in 25-OH-D_3 synthesis which is 3-acetoxy-Δ^{22}-homocholenic methylester whose structure is shown in Figure 1.

We propose to react this compound by means of methyl magnesium iodide to form the $\Delta^{22,23}$-25-hydroxycholesterol. Following acetylation to protect the 3 and the 25-hydroxyl we plan to allylically brominate the 7 position and dehydrohalogenate with trimethylphosphite to yield the Δ22,23-7-dehydro-25-hydroxycholesteryl diacetate. We will then react this substance with a dieneophile to protect the 5-7 double bond system. This will be followed by a catalytic reduction with tritium gas to yield at least 100 Curies/mM material. The triazoline can be removed by lithium aluminum hydride, regenerating the highly radioactive 25-hydroxy-7-dehydrocholesterol which can be converted by ultraviolet irradiation to ^{3}H-25-OH-D_3. This can then be converted by the chick kidney mitochondrial system to the 1,25-$(OH)_2D_3$ which can be purified by Sephadex LH-20 chromatography followed by liquid-liquid partition chromatography. This compound will permit higher resolution microautoradiography in collaboration with Dr. Les Matthews at Dallas and ordinary autoradiography in our own laboratory using frozen sections. We will be able for the first time to give injections of physiologic amounts of the 1,25-$(OH)_2D_3$ and obtain sufficient amount of localized radioactivity to permit detection by this technique.

With this substance we can also begin a much more intensive search of the subcellular location of the tritiated metabolite. Before we can progress with the subcellular location, it will be necessary to devise methods whereby pure nuclei can be obtained. The major problem in the standard subcellular fractionation techniques is the presence of excessive amounts of mucous. However, there are procedures in which crypt cells can be isolated as well as can the villus cells (87-89). Furthermore, Dr. Patrick O'Doherty, an expert in this technique, is a postdoctoral fellow in our laboratory and will be with us for at least two additional

years. During this procedure the goblet cells are lost and the villus and crypt cells can be independently isolated and washed. These washed cells will then be used for subcellular fractionation. It is anticipated that the mucous problem will be much less if not nonexistent in these preparations. These could then be subjected to standard subcellular fractionation techniques (90, 91) which should yield us information as to where the tritiated 1,25-$(OH)_2D_3$ concentrates in the villus cells or in the crypt cells. We will then be able to isolate pure fractions from these cells to determine how much is truly associated with a particular fraction, especially the nuclear-debris fraction. Techniques for isolation of pure intestinal nuclei could then be applied using the methods described by Lawson et al. (92) and by Blobel and Potter (93) and others. Certainly one of these techniques will allow us to determine the exact amount of tritiated 1,25-$(OH)_2D_3$ found in the nuclei. We would then proceed with sub-fractionation of the nuclei if sufficient amounts of radioactive 1,25-$(OH)_2D_3$ are found in this subcellular fraction.

Chromatin will be isolated by the exact procedure of Marushige and Bonner (94) rather than by modifications of this method which gives contaminated preparations (95, 96, 104). Procedures are also available for isolation of histones, acidic proteins and etc.

Brush borders will be isolated by the procedure of Forstner et al. (97), which has been found successful in our hands. Attempts will be made to isolate other cell fractions as well. If a cell fraction shows remarkable accumulation of the ^3H, it will be fully characterized by marker enzymes and EM, the latter being available to us in our department. Throughout these studies marker enzymes will be used whenever possible. Also the ^3H will be extracted and chromatographed on Sephadex LH-20 and high pressure liquid chromatography to establish whether the ^3H remains as 1,25-$(OH)_2D_3$ or has been changed to another compound.

2. Possible Receptors for 1,25-$(OH)_2D_3$ in Intestinal Mucosa

Independently of the above set of investigations, the tritiated 1,25-$(OH)_2D_3$ will be utilized in the study of the cytosolic and nuclear receptors, if they are present. Rat and chick intestinal cytosol will be prepared and studied for their ability to bind 1,25-$(OH)_2D_3$. This will be done by incubation at 0° of the cytosol with tritiated 1,25-$(OH)_2D_3$ for a period of between 1-7 hours. The cytosolic mixture will then be subjected to density gradient centifugation on sucrose gradients as described by Jensen and his coworkers (98). The gradients will be varied acccording to where the peaks of radioactivity sediment. Additionally, the proteins which bind the 1,25-$(OH)_2D_3$ will be analyzed with Sephadex G-200 column chromatography. So far we have found two macromolecular components of interest in the intestine. A 6S macromolecular component has been found in the rat preparations which will bind 1,25-$(OH)_2D_3$ (99, Knutson, Reynolds and DeLuca, unpublished results). We have been able to exclude this as a receptor in the calcium transport system and since 1,25-$(OH)_2D_3$ is the active form, we will have to look further in this species for a way in which the 1,25-$(OH)_2D_3$ is transported to its subcellular site of action. In the chick we have found a 3.5S protein in agreement with Haussler's work (43, 46) which binds 1,25-$(OH)_2D_3$. In disagreement with Haussler's work we have found that this protein or macromolecule when present binds 1,25-$(OH)_2D_3$ exclusively and does not bind 25-OHD_3. Unfortunately, the variability of its

appearance in the mucosa from rachitic chicks has hampered our work with it. We have learned recently, however, that if great care is taken in washing the intestine initially, reproducible amounts of this protein can be obtained. We propose to isolate this protein by Sephadex chromatography and by ion exchange chromatography hopefully to homogeniety. We intend then to incubate this protein component with purified intestinal nuclei (as described above) to determine if we can obtain a transfer into the nuclear fraction. If so we will try to extract from these nuclei, protein within them that binds $1,25\text{-}(OH)_2D_3$ to determine if it is identical or changed from the receptor protein of the cytosol. We will, of course, carry out experiments to determine whether the cytosolic protein or macromolecule is necessary for the transfer of the $1,25\text{-}(OH)_2D_3$ into the nucleus. However, if we learn by the subcellular fractionation method described above that the nucleus is not the major site of deposition of $1,25\text{-}(OH)_2D_3$, we will attempt to learn whether a cytosolic component plays a role in the transfer of $1,25\text{-}(OH)_2D_3$ to whatever fraction accumulates the $1,25\text{-}(OH)_2D_3$.

Ultimately we hope to isolate each of the $1,25\text{-}(OH)_2D_3$ binding proteins in pure form and by means of physical measurements determine their binding characteristics, their molecular weight and how they interact in the transfer of the $1,25\text{-}(OH)_2D_3$ in the intestinal cell. We will also try from time to time to determine by in vivo saturation experiments whether these proteins play a role in vivo in the transfer of the 1,25- compound. These saturation experiments are carried out in nephrectomized vitamin D-deficient rats as follows: inject into them non-radioactive and saturating amounts of either $1,25\text{-}(OH)_2D_3$ or $25\text{-}OH\text{-}D_3$. The subcellular component which binds the $1,25\text{-}(OH)_2D_3$ will be isolated and incubated with the radioactive $1,25\text{-}(OH)_2D_3$ to determine if the sites on that protein are saturated. This type of approach has already been used to exclude the 6S protein as playing a role in the binding and transfer of $1,25\text{-}(OH)_2D_3$.

It is possible that the cytosol plays no role in the transfer of $1,25\text{-}(OH)_2D_3$ to its ultimate sites. If we succeed in isolating the villus cells, we will attempt to incubate those cells with radioactive $1,25\text{-}(OH)_2D_3$ to determine a time course of what happens to the $1,25\text{-}(OH)_2D_3$ as it progresses through the cell by both autoradiography of frozen sections and subcellular fraction techniques. It is anticipated that work on subcellular fractionation and the receptor proteins for the $1,25\text{-}(OH)_2D_3$ will consume much of our effort in the next three-year contract period.

3. Identification and Isolation of Calcium Transport Components

A third and important area of investigation will be a study of the components of the brush border of the small intestine as they are changed in response to $1,25\text{-}(OH)_2D_3$. The approach taken here will be to isolate the brush borders from the intestinal villi cells of rats and chicks at various times following a single intravenous injection of $1,25\text{-}(OH)_2D_3$. The isolated brush borders will be solubilized by a variety of techniques and subjected to disc gel electrophoresis to determine what are the separable components that can be observed. The separable components will be observed as a time course following $1,25\text{-}(OH)_2D_3$ administration and in relation to intestinal calcium transport. The qualitative changes in the profile will be taken as a possible change in the membrane which may play a role

in calcium transport. The components which are found changed in response to $1,25\text{-}(OH)_2D_3$ will be isolated and studied for its activities such as calcium dependent ATPase, alkaline phosphatase, calcium binding activity and whether they contain $1,25\text{-}(OH)_2D_3$. Furthermore, these components may be incubated with the fraction which finally contains the $1,25\text{-}(OH)_2D_3$ as determined in the previous section.

Finally it will be determined whether actinomycin D prevents the changes in the protein profiles and again this will be correlated with the intestinal calcium transport response under identical conditions. We will continue our work on the isolation of the 200,000 molecular weight protein from the brush borders which is found in vitamin D-deficient chicks. We will also isolate the 220,000 molecular weight protein which appears within 6 hours following the administration of $1,25\text{-}(OH)_2D_3$ to rachitic chicks. Ultimately it is hoped that these proteins will be useful in our understanding of the intestinal calcium transport mechanism.

4. The Chemical Synthesis and Testing of Analogs of $1,25\text{-}(OH)_2D_3$

This project will be carried out in conjunction with my National Institutes of Health program-project grant on the fat-soluble vitamins. Under the NIH program-project grant the compounds will be synthesized, chemically characterized and prepared for biological activity testing. Under this contract the compounds will be tested for their biological activity. There are two types of compounds which are currently considered of extreme importance to our elucidation of the structure-function relationship between $1,25\text{-}(OH)_2D_3$ and intestinal calcium transport. One of the most important questions to be answered is whether $1\alpha\text{-OH-}D_3$ must be 25-hydroxylated before it can function. The synthesis of tritiated $1\alpha\text{-OH-}D_3$ has been completed but results with this compound have not been able to resolve this important question. The reason is that small amounts of $1,25\text{-}(OH)_2D_3$ appear quite rapidly following an injection of tritiated $1\alpha\text{-OH-}D_3$. Whether this is a sufficient amount of $1,25\text{-}(OH)_2D_3$ to stimulate intestinal calcium transport is unknown. We, therefore, propose to synthesize analogs of $1,25\text{-}(OH)_2D_3$ which are minus the 25-hydroxyl group and which cannot be hydroxylated on the 25 position because of structural constraints. Thus we plan to synthesize the following compounds for this project: 25-az-1α-OH-D_3, 25-methoxy-1α-OH-D_3 and 25-fluoro-1α-OH-D_3. These compounds cannot be hydroxylated on carbon 25 and hence one can test whether they can stimulate intestinal calcium transport. If they can stimulate intestinal calcium transport, one can safely conclude that 25-hydroxylation is not essential to stimulation of intestinal calcium transport once the 1-hydroxyl group is in position. The structures of the compounds which are proposed for synthesis are shown in Figure 2. The chemical synthesis will be carried out by well-known feasible reactions shown in Figure 3. Following their synthesis, the response of intestinal calcium transport in the rat measured by the everted sac technique *in vitro* commonly used in our laboratory, will be studied. In addition, the isolated loop method of measuring intestinal calcium absorption in the vitamin D-deficient chick will also be used (100).

Two different types of bioassays will be run. One will be a time course of response of intestine to a single dose of an anlog as compared to a similar dose of either 1,25-$(OH)_2D_3$ or 1α-OHD_3. If there is a response then another type of bioassay will be carried out in which daily injections of the compound will be given for a period of one week to vitamin D-deficient rats. At the end of the week the level of intestinal calcium transport will be determined. A plot of the log daily dose of analog versus the response will be constructed. We will, therefore, try to arrive at the daily dose of analog that will maintain the same intestinal calcium transport level as a known daily dose of 1α-OH-D_3, 1,25-$(OH)_2D_3$ or vitamin D_3 (101). Besides testing of intestinal calcium transport activity another measurement will be made such as serum calcium elevation in rats maintained on a low calcium diet which is considered an in vivo measurement of bone calcium mobilization (102). In addition the antirachitic calcification line test for cure of rickets and the elevation of serum inorganic phosphorus level of rats on a low phosphorus diet will be measured in response to the analog (101, 103). These latter tests will be incidental to the calcium transport measurement, however. In addition to these tests the compounds will be supplied to Dr. John J. Reynolds of the Strangeways Research Laboratory in Cambridge, England, who will test them for activity in the embryonic intestinal culture system by measurement of immunoreactive calcium binding protein produced and by measurement of intestinal calcium uptake. The test involves culturing embryonic intestine at 19 days of embryonic life, the addition of small amounts of metabolites or analogs and the measurement quantitatively of calcium binding protein produced 24 or 48 hours later. These measurements will give another assessment of the biological activity of these compounds in the intestinal calcium absorption system. With these assessments it may be possible then to decide on the necessity or lack of necessity for the 25-hydroxylation to carry out intestinal calcium absorption.

Besides these compounds, another series of compounds will be made which hopefully will be anti-metabolites of vitamin D. We consider that blockage of either the 25-hydroxyl group or the blockage of the 1-hydroxyl position should result in a form of vitamin D which will serve as an anti-1,25-$(OH)_2D_3$ compound in the intestine. Thus we propose to synthesize 1-methoxy or 1-fluoro-25-OH-D_3 and additionally 25-fluoro or 25-methoxy-1α-OH-D_3. We will test for anti-intestinal calcium transport activity by the administration of increasing levels of the compounds followed by a single injection of 65 pmoles of 1,25-$(OH)_2D_3$ and the measurement of the extent of intestinal calcium transport by one of the methods described above. Should one of these compounds block the response to 1,25-$(OH)_2D_3$ it would suggest that it may well serve as an anti-1,25-$(OH)_2D_3$ in the intestine. Similarly the compounds will be tested for anti-1,25$(OH)_2D_3$ activity in bone calcium mobilization and in the cure of rickets in rats. An anti-1,25-$(OH)_2D_3$ may be very useful in medicine and of importance to the agency. It may be useful in inhibiting absorption of Pb, Sr and other environmental pollutants.

5. The Isolation of the Active Principle from the Plant Solanum malacoxylon and Its Chemical Identification

A large amount of the dried plant Solanum malacoxylon has been obtained from South American colleagues. These leaves will be extracted with water and the water extract treated with alcohol as already described (79). The alcoholic extract will then be subjected to a series of purification procedures. The first

and most obvious are the gel filtration methods which involve not only the Sephadex series but also the polyacrylamide molecular sieves, which are readily available on the market. In addition, the molecular sieve Bio-Beads SX-8 will be used. Following this, the active principle will be acetylated with acetic anhydride, catalyzed by a small amount of pyridine and the acetylated project which now becomes lipid soluble can then be subjected to silicic acid column chromatography, Sephadex LH-20 chromatography, and high pressure liquid chromatography. Throughout all of these separations samples of the acetylated products will be deacetylated by alkaline hydrolysis which we know does not interfere with or destroy the biological activity of the Solanum malacoxylon and then subjected to testing in the vitamin D-deficient rat by measurement of intestinal calcium transport by the everted sac following an oral dose. If the active principle is obtained in homogeneous form, as determined by mass spectrometry, high pressure liquid chromatography and gas liquid chromatography of the acetylated and trimethylsilyl derivatives, they will be subjected to mass spectrometry. Since it has a molecular weight of over 2,000, it seems likely that it could be a conjugate of some material with a glucuronide or some other sugar molecule. Should this prove to be the case which should be clearly evident on mass spectrometry, attempts will be made to hydrolyze the compound to yield the sugar and the other component. The non-sugar component will then be subjected to isolation and identification. If on the other hand it does not show evidence of conjugation, such a large molecule will be subjected to degradative cleavage by periodate or oxidative breakdown with chromic acid or other oxidation agents. Which procedures will be used can only be determined once preliminary results are obtained in our structural analysis. Ultimately it is hoped that the structure will be determined and its relationship to the vitamin D series determined on a chemical basis.

6. Study of Phosphate Transport in the Small Intestine

If time permits work will be carried out on the phosphate transport system in vitro primarily characterizing the requirements for transport of phosphate across intestinal membrane and a study of the inhibitors which will block the transport (methods as described in Chen et al. (85)). This will follow a standard everted gut sac technique in which inhibitors will be added in vitro or in some cases protein synthesis inhibitors will be given prior to the administration of $1,25\text{-}(OH)_2D_3$ to the animal. Such inhibitors as actinomycin D or cycloheximide could be used for this study in which case control experiments will have to be done in which the actinomycin D or cycloheximide is given after the $1,25\text{-}(OH)_2D_3$ to eliminate the possibility that they are inhibiting by a mechanism not involving their expected modes of action. Beyond this no further experiments are planned under the present contract.

7. Study of Pb Absorption

As we progress in our work on the intestinal calcium transport mechanism we will apply our findings to a study of the transport of Pb^{210}. We have already carried out preliminary experiments to reveal that lead 210 is transported actively throughout the small intestine. We hope to begin looking into the possibility that lead 210 is transported across the intestinal membrane by the same carrier that transfers calcium. To test this we will test lead 210 transport in the presence of excesses of non-radioactive calcium and vice versa, although undoubtedly lead will serve as a metabolic poison under that circumstance. We will further determine whether the lead transport is responsive to vitamin D in the vitamin D-

deficient animals and if so we will proceed with determination of whether it is responsive to the active metabolites of vitamin D. We will also obtain some chick calcium binding protein to determine what the binding affinity is for lead 210. We will also use the techniques described by Martin and DeLuca (14) to determine whether vitamin D changes the permeability of the brush border membrane to lead and hence determine in another circumstance the possibility that lead is being transferred by a mechanism related to the one activated by vitamin D for calcium.

It is clear that there is more work described in the contract than could be logically carried out during this entire grant period. However, we will concentrate very heavily on the intestinal calcium transport mechanism and will concentrate on the mechanism whereby $1,25\text{-}(OH)_2D_3$ carries out its well known function in the intestine.

Summary

Major emphasis during the proposed three-year continuation of this contract will be placed on the mechanism whereby $1,25\text{-}(OH)_2D_3$ stimulates intestinal calcium transport. The chemical synthesis of $^3H\text{-}1,25\text{-}(OH)_2D_3$ of 100 Curies/mmole is planned. Autoradiography will be feasible with this preparation and in addition, a fresh approach is planned for subcellular location studies using isolated villus cells and isolated crypt cells and cell fractionation techniques. Special emphasis will be placed on the nuclear and intranuclear location of $1,25\text{-}(OH)_2D_3$.

A study of "receptor" proteins in the cytosol and nucleus of intestine from rats and chicks using sucrose density gradient analysis and Sephadex chromatography is planned. Proteins which might be considered receptors will be isolated and studied in detail.

Alterations in the brush border components of intestinal mucosa of rats and chicks separable by SDS disc gel electrophoresis in response to $1,25\text{-}(OH)_2D_3$ will be looked for. A 200,000 molecular weight protein present in brush border from rachitic chicks but not in vitamin D-treated chicks will be isolated as will a 200,000 molecular weight protein which appears in response to $1,25\text{-}(OH)_2D_3$. The physical properties, calcium activated by ATPase, alkaline phosphatase and calcium binding properties will be determined.

Chemical analogs of $1,25\text{-}(OH)_2D_3$ will be synthesized under other support and will be tested for biological activity under the proposed contract to allow a delineation of the structural requirements for intestinal calcium transport activity. Attempts to synthesize an anti-$1,25\text{-}(OH)_2D_3$ in intestine will be made.

The active substance from the plant Solanum malacoxylon will be isolated and its structure identified to determine what relation it has to the $1,25\text{-}(OH)_2D_3$ induced intestinal calcium transport.

Work will be initiated to determine if Pb and Cd are absorbed by the same or an analogous system which transports calcium. Possible use of an anti-$1,25\text{-}(OH)_2D_3$ to block absorption of these cations will be examined.

The ultimate aim of the project is to define the molecular mechanisms whereby calcium and other divalent cations are absorbed. With this information control of absorption of unwanted atmospheric pollutants will become more feasible.

Significance

This project should add a great deal of information to our knowledge of (1) function of vitamin D, (2) mechanism of intestinal calcium absorption and (3) regulation of calcium homeostasis. It has practical significance in helping devise methods of minimizing absorption of environmental pollutants such as Pb, Cd, plutonium and radio-strontium. It also will provide basic information and tools such as analogs of $1,25\text{-}(OH)_2D_3$ which will help in preventing and treatment of disorders of bone and calcium metabolism.

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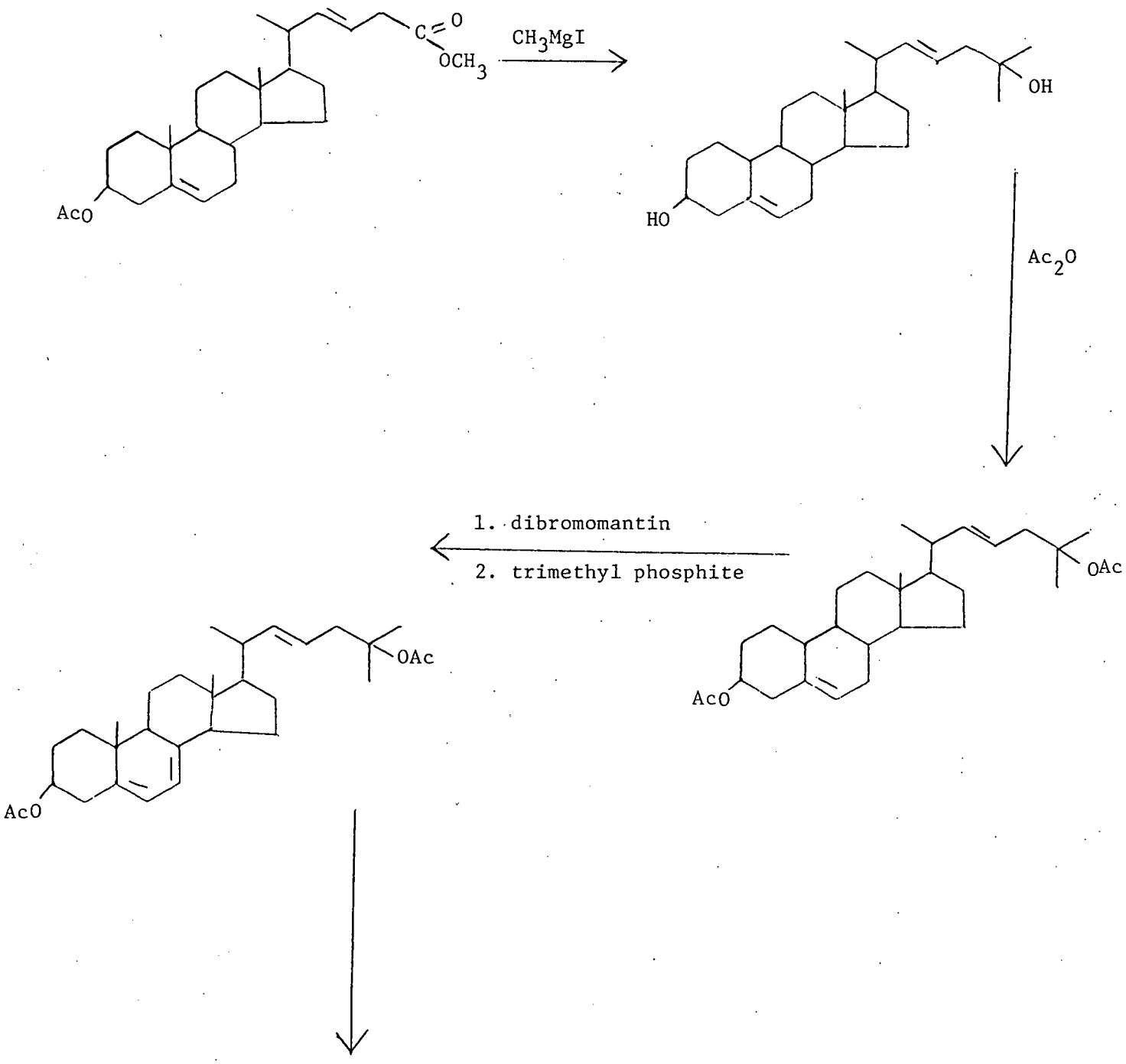
FIGURE 1



to next page (Figure 1 continued)

FIGURE 1 CONTINUED

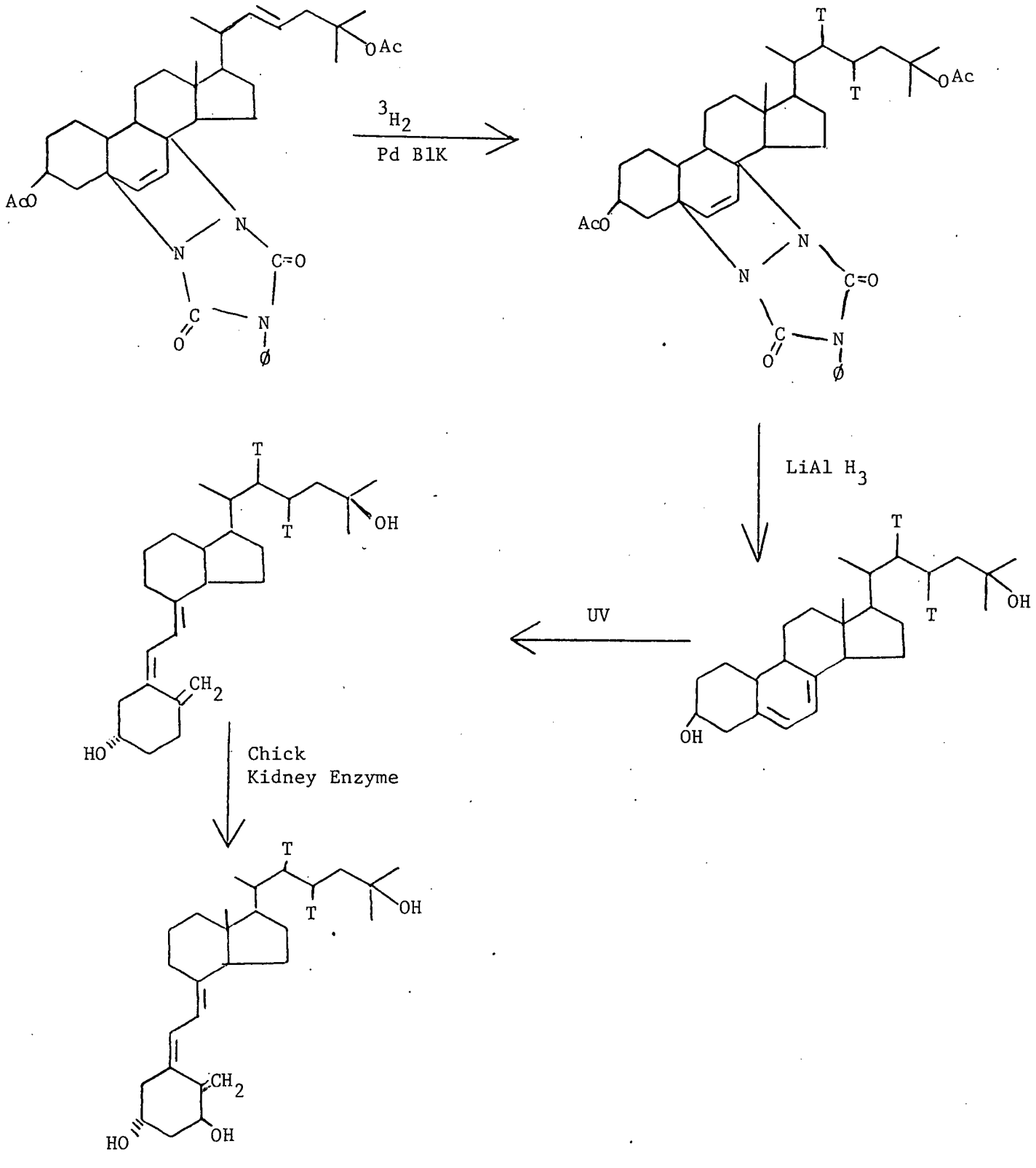
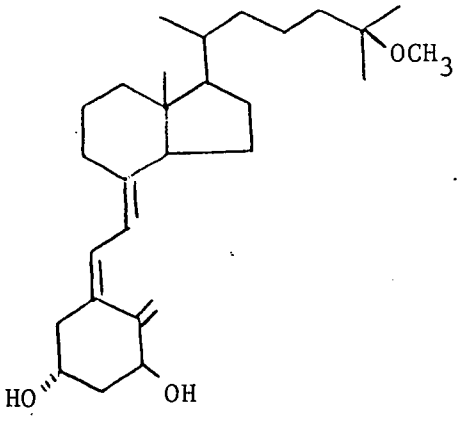
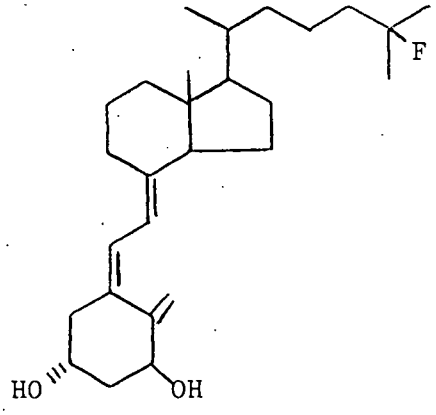


FIGURE 2

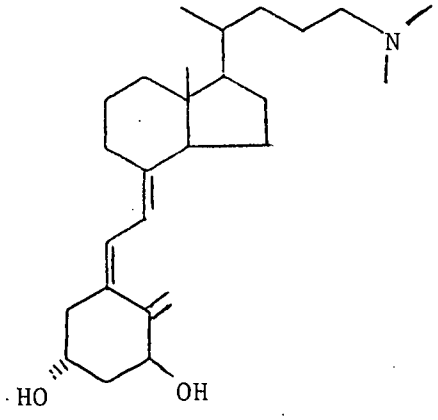
Structural Analogs of 1α,25-Dihydroxyvitamin D_3 Modified at C-25



1: 1α-Hydroxy-25-methoxy-vitamin D_3



2: 1α-Hydroxy-25-fluoro-vitamin D_3



3: 1α-Hydroxy-25-aza-vitamin D_3

FIGURE 3

Synthesis

Scheme 1: Possible synthesis of 1α-hydroxy-25-methoxy-vitamin D_3 (1).

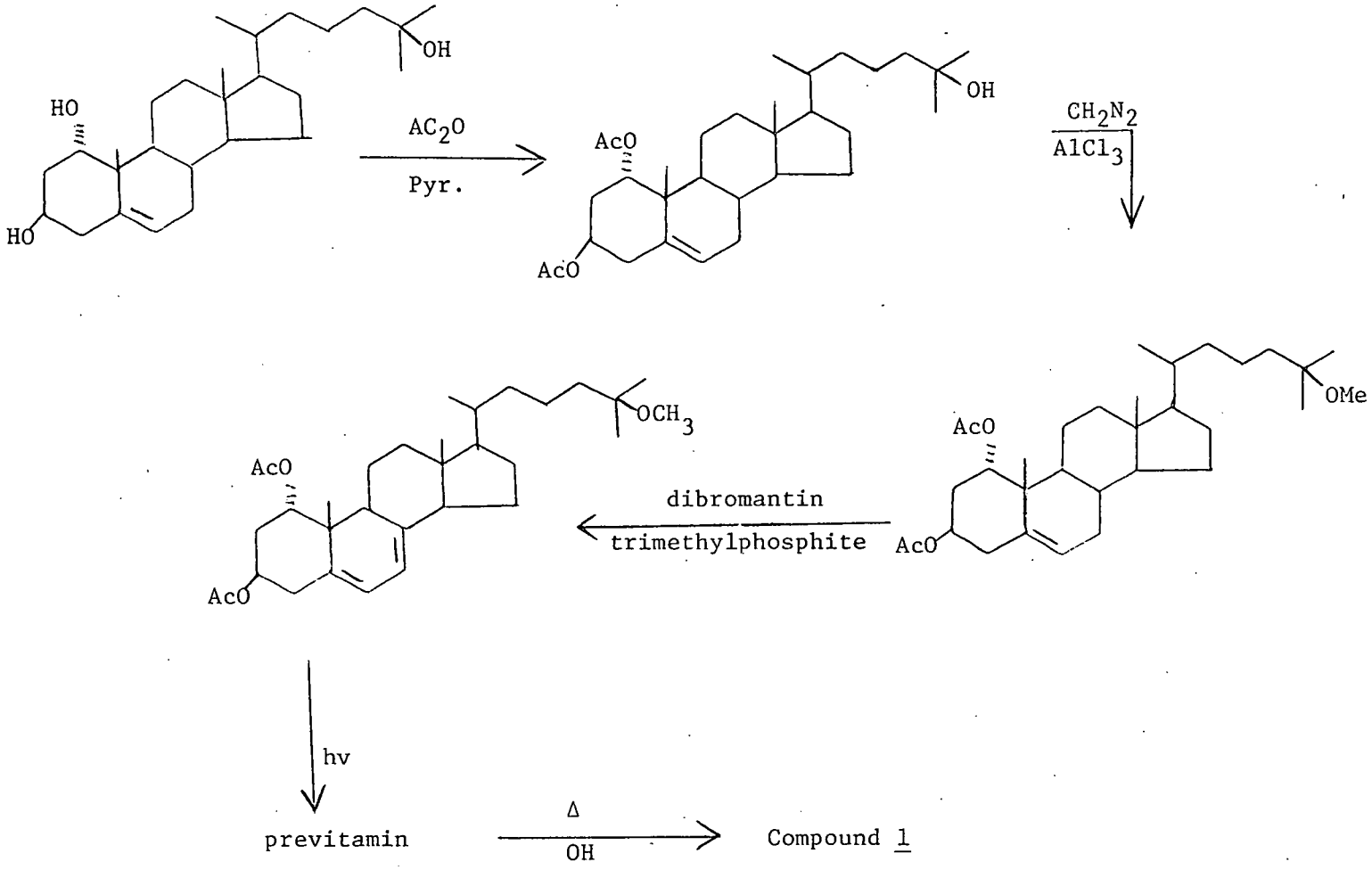
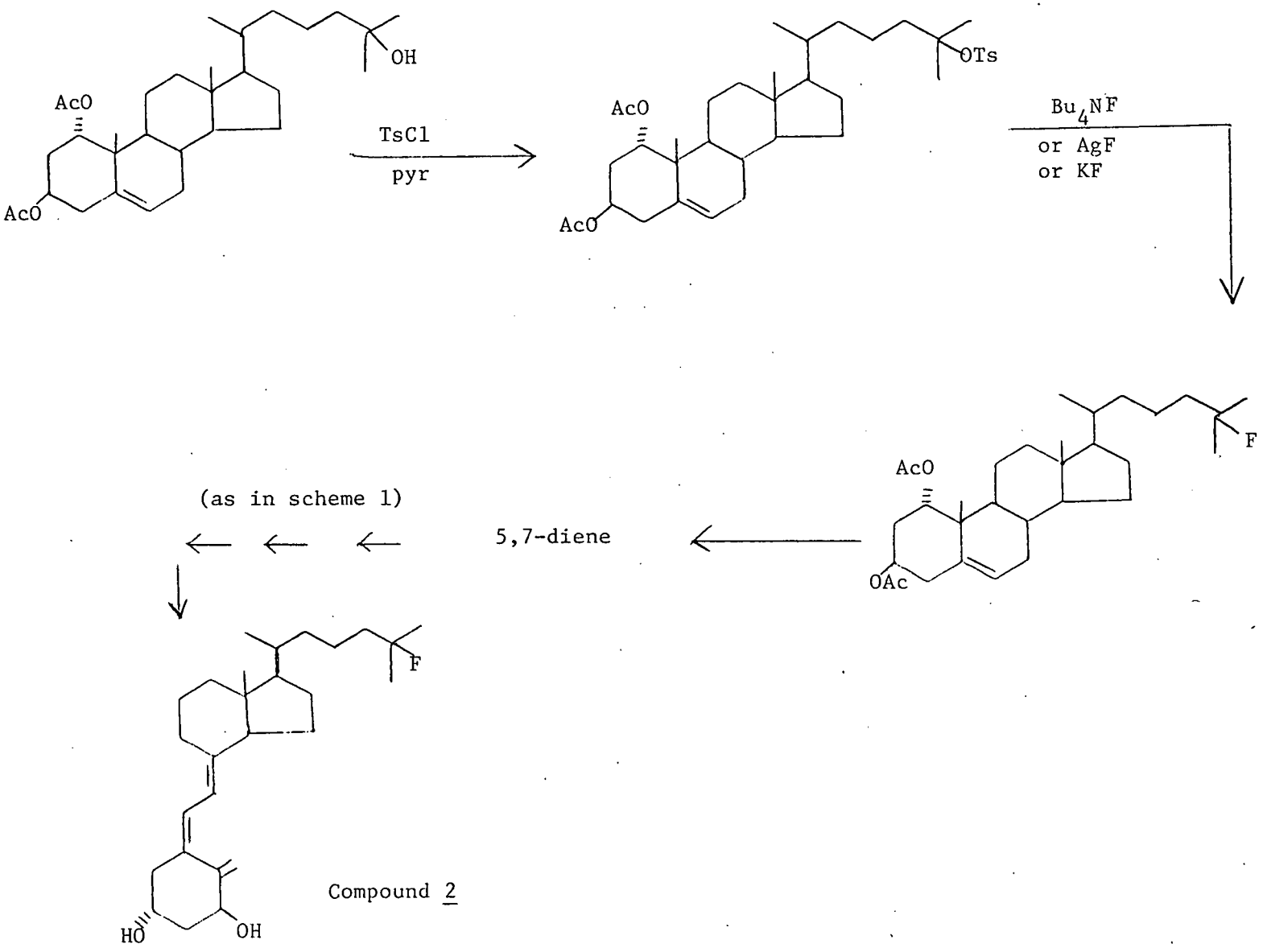


FIGURE 3

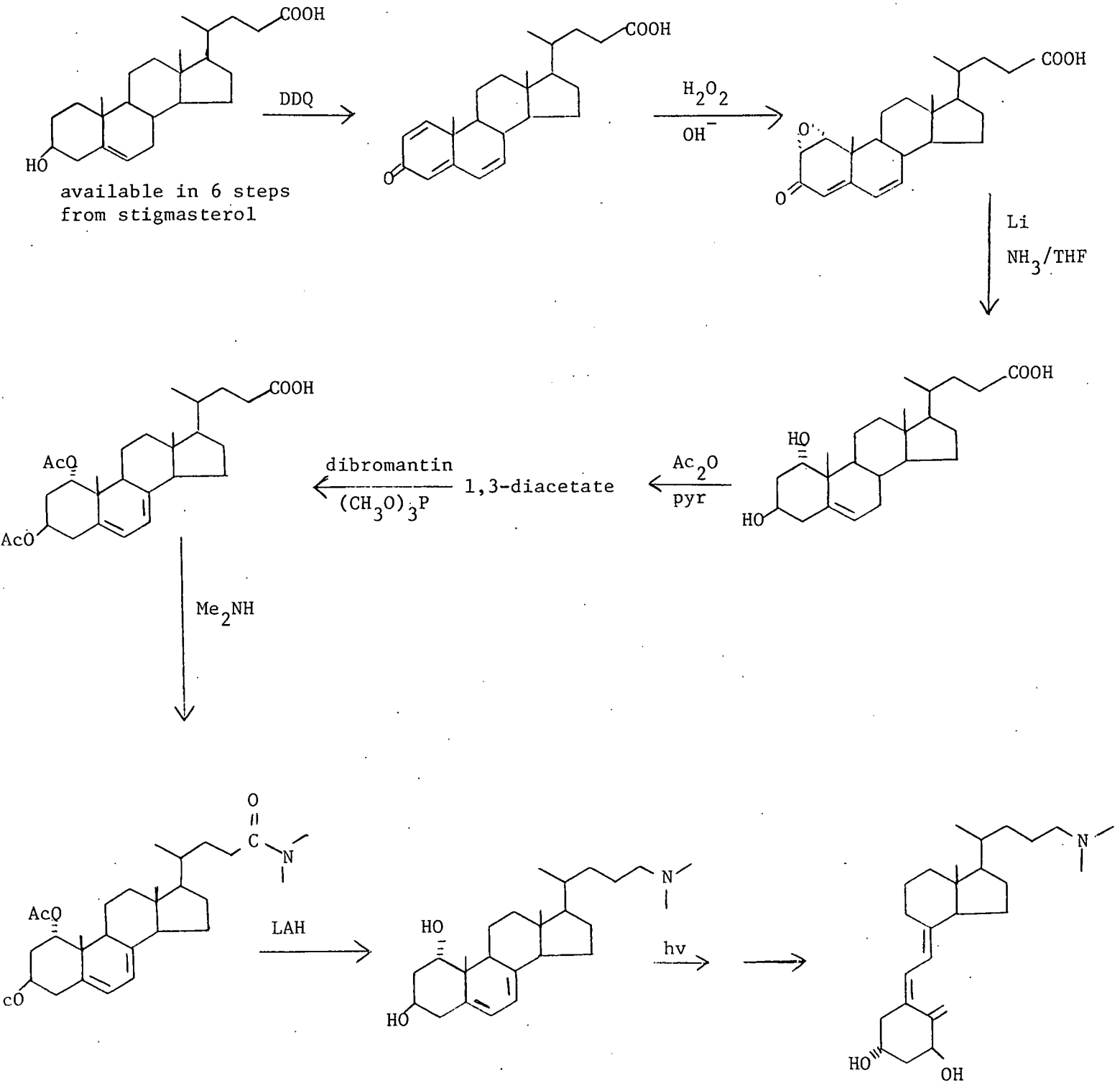
Scheme 2: Possible synthesis of 1α-hydroxy-25-fluoro-vitamin D_3



Compound 2

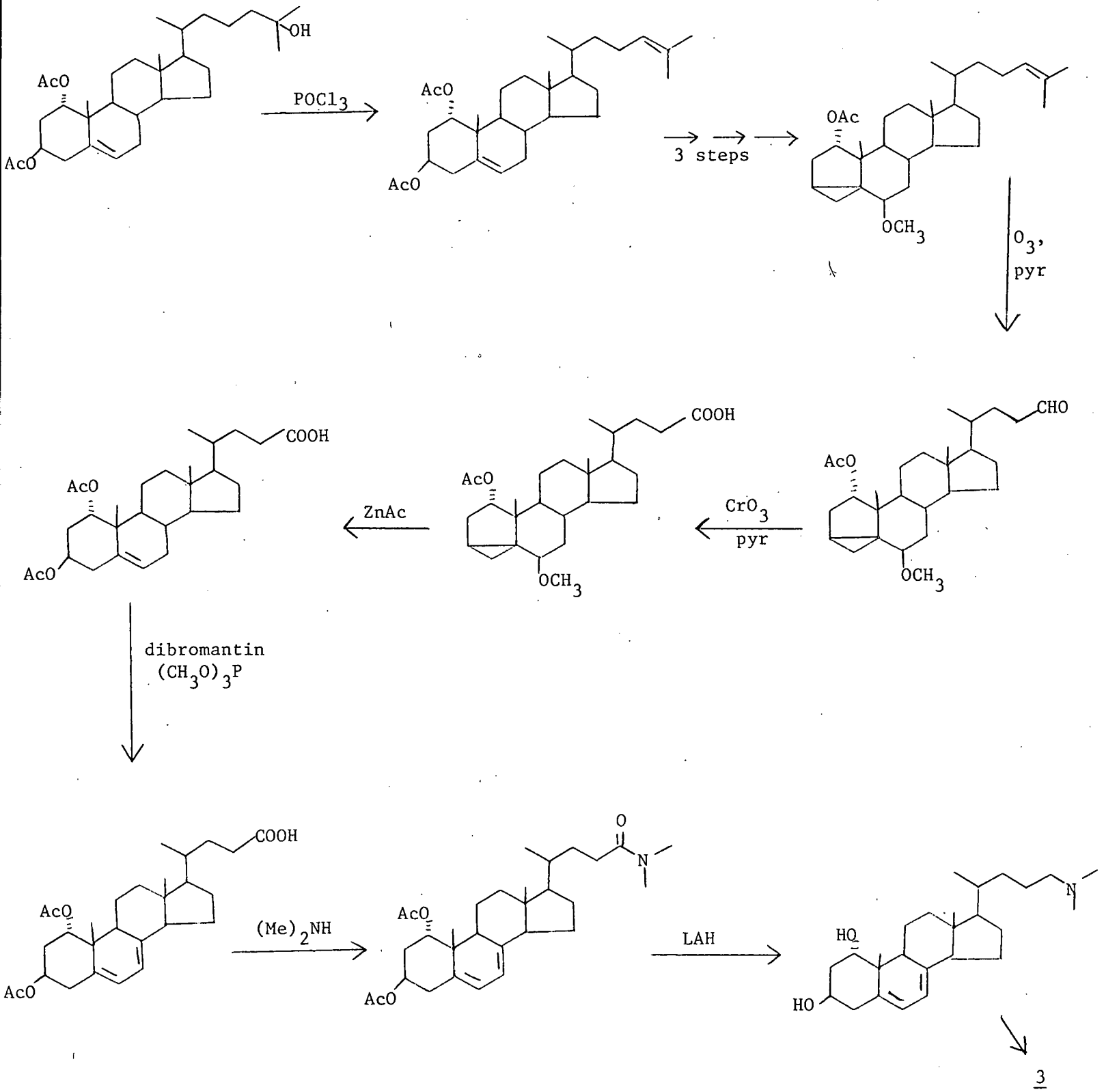
FIGURE 3

Scheme 3: Possible syntheses of 1α-hydroxy-25-azavitamin D_3
(a) from stigmasterol



Compound 3

Scheme 3: (b) alternate route



VI. FEDERAL SUPPORT FOR OVERALL RESEARCH PROGRAM

National Institutes of Health (AM-14881): "Metabolism and Function of the Fat-Soluble Vitamins" (Component of program-project grant on metabolism of vitamins A and D) H. F. DeLuca's portion $90,000

National Institutes of Health (Renewal of AM-15512 applied for, pending as yet) "Metabolic Consequences of Uremia" (Program-Project Grant)

Food & Drug Administration, Contract to study "The Mechanisms of Absorption of Lead and Cadmium from the Gastrointestinal Tract" (Joint between H. F. DeLuca and W. G. Hoekstra) (one year only) $28,808

National Institutes of Health (NO1 AM-2-2226): "Preparation of Vitamin D Metabolites and Analogs for Clinical Evaluation" (contract will end 9/30/75 and will not be renewed, this is jointly between H. F. DeLuca and H. K. Schnoes) H. F. DeLuca's portion $9,000

NASA Ames Research Center (one year contract): "Development of Methods of Determination of 1,25-$(OH)_2D_3$ in Blood" $16,000 (includes overhead)