MECHANISMS OF CALCIUM TRANSPORT IN SMALL INTESTINE

Progress Report

for Period March 1, 1976--September 30, 1977

H. F. DeLuca

University of Wisconsin-Madison

Madison, Wisconsin 53706

NOTICE
This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Energy Research and Development Administration, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

Prepared for

THE U.S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

UNDER CONTRACT NO. EY-76-S-02-1668

MASTER

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED *eb*

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

ABSTRACT

Utilizing the previously reported chemically synthesized 25-OH-[23,24-³H]D₃ of 78 Ci/mmmole (last contract period), it was possible by means of enzymatic incubation to prepare 1,25-(OH)₂-[23,24-³H]D₃ having a specific activity of 78 Ci/mmmole. With this material it has now been possible to demonstrate unequivocally by autoradiography of frozen sections the specific localization of 1,25-(OH)₂D₃ in vivo in the nuclei of intestinal mucosal villus cells and crypt cells. It is not localized in submucosa, intestinal smooth muscle coat, lamina propria, liver, and skeletal muscle. It is, however, found specifically localized in the nuclei of osteoblasts, osteocytes and chondroblasts of bone. Some specific localization is found in some tubule preparations from kidney, but the exact anatomical location has not yet been determined. The localization of the ³H-1,25-(OH)₂D₃ in intestine is found at 2-1/2 to 6 hours post-injection, but is not found at 1 hour. Fifty percent of the villus cells and crypt epithelial cells become labeled with 1,25-(OH)₂D₃ giving a grain count of 4-5 per nucleus at 4 hours. These results strongly support the idea that 1,25-(OH)₂D₃ exerts its influence on intestinal calcium absorption by means of a nuclear mechanism.

Work with the high specific activity 1,25-(OH)₂-[23,24-³H]D₃ has permitted detailed investigation into the cytosolic receptor proteins for 1,25-(OH)₂D₃. In the chick the clear demonstration of a 3.7S cytosolic receptor can now be made following several important precautions. First, the intestinal cells must be thoroughly washed before homogenization to eliminate proteolytic degradation of the receptor. Secondly, the receptor is stabilized by .5 mM dithiothreitol. This receptor has been shown to be highly specific for 1,25-(OH)₂D₂ or 1,25-(OH)₂D₃. It shows much less affinity for 1,24,25-(OH)₃D₃ and still less activity for 1α-OH-D₃, 25-OH-D₃ and 24,25-(OH)₂D₃, both S and R isomers. 25-OH-D₃ is 1/5000 as active as 1,25-(OH)₂D₃ in competing for binding on the 3.7S chick cytosol receptor. By taking the above precautions and also by homogenizing intestinal mucosal cells in .3 M potassium chloride, the presence of a 3.2S cytosolic receptor protein for 1,25-(OH)₂D₃ can now be demonstrated in the intestine of rats, in calvaria of embryonic chicks and rats. This demonstration was only possible with the newly prepared high specific activity 1,25-(OH)₂D₃ under this contract.

From the studies on the specific cytosol receptor proteins found in intestinal mucosa, it has been possible to develop a specific competitive binding assay for 1,25-(OH)₂D₃ and a direct high-pressure liquid chromatographic method for determination of 25-OH-D₃ in human and animal plasma, permitting a number of experiments on factors which regulate the level of these two important hormonal substances in the plasma.

As chicks approach the egg laying cycle, they develop high rates of 25-OH-D₃-1-hydroxylation. These high rates are the result of stimulation by the sex hormones, estradiol, testosterone and progesterone. It is believed that the high levels of 1,25-(OH)₂D₃ stimulate intestinal calcium absorption, stimulate the mobilization of medullary bone and mediate the deposition of calcium as calcium carbonate in egg shell and calcium in egg albumin. The specific stimulation of the 25-OH-D₃-1-hydroxylase has been demonstrated in castrate male birds, in immature male and female birds, as

well as in castrate female birds. The greatest stimulation is obtained with a combination of estradiol, testosterone and progesterone, giving the highest rates of 25-OH-D₃-1-hydroxylation ever observed. Thus sex hormones exert a marked regulatory influence on vitamin D metabolism.

Calcitonin given in large doses to intact animals increases production of 1,25-(OH)₂D₃ and diminishes production of 24,25-(OH)₂D₃. However, this response is not obtained in thyroparathyroidectomized animals while parathyroid hormone clearly stimulates 1-hydroxylation in the thyroparathyroidectomized animals. Thus it appears that calcitonin does not regulate 25-OH-D₃-1-hydroxylation and that the previous report of its influence was the result of a secondary response of the parathyroid glands to hypocalcemia brought about by large doses of calcitonin.

An isolated monkey kidney tissue culture system has been developed which will carry out 24-hydroxylation of 25-OH-D₃. This system is stimulated by 1,25-(OH)₂D₃ and high levels of ambient calcium concentration. The 24-hydroxylation is suppressed by either bovine or human parathyroid hormone. These cells do not carry out 25-OH-D₃-1-hydroxylation.

An examination of tissues taken from vitamin D-deficient rats given Solanum glaucophyllum has shown the presence of large amounts of 1,25-(OH)₂D₃ and virtually no 1,25-(OH)₂D₂. This substance has been very carefully demonstrated by co-chromatography in at least five different systems and by means of the specific competitive protein binding assay described above. Thus the active principle from Solanum glaucophyllum is undoubtedly a conjugate of either 1,25-(OH)₂D₃ or 1 α -OH-D₃ with a sugar component or some other conjugate making it water soluble.

Other work on analogs of 1,25-(OH)₂D₃ has revealed that 1,25-(OH)₂D₃ is rapidly converted to 1,24R,25-(OH)₃D₃. This hydroxylation occurs in nephrectomized animals, demonstrating the presence of a 24-hydroxylase in extra-renal tissue. 1,24,25-(OH)₃D₃ is a normal metabolite of vitamin D₃, but it is less active than the 1,25-(OH)₂D₃. Thus 24-hydroxylation may well be the initial event in the inactivation of the vitamin D hormones, 25-OH-D₃ and 1,25-(OH)₂D₃. Chemical synthesis of tritiated 24S and 24R-OH-D₃ has been completed and it has now been demonstrated that both compounds are rapidly converted to 24,25-(OH)₂D₃. However, the 1-hydroxylase discriminates against the 24S,25-(OH)₂D₃, yielding essentially no 1,24S,25-(OH)₃D₃, whereas the 24R,25-(OH)₂D₃ is rapidly 1-hydroxylated. Since 1,24S-³D₃ and 1,24R,25-(OH)₃D₃ are equally active, it appears that discrimination against the S configuration is at the level of 1-hydroxylation.

Two methods have been devised for the assessment of lead absorption from the small intestine. One is an in vitro everted sac technique and the other is an in vivo technique in which lead 210 absorption is followed after stomach tubing. Using these techniques it has been possible to show that the distal segments of small intestine (ileum) are the primary sites of lead absorption, ^{and} that it is markedly stimulated by vitamin D. The presence of calcium in the medium interferes with the lead absorption. On the other hand, calcium deprivation reduces lead absorption, whereas high calcium diets increase lead absorption.

Large doses of vitamin D do not markedly enhance lead absorption over animals receiving normal amounts of vitamin D. However, since nephrectomy prevents the response of intestinal lead absorption to vitamin D and 25-OH-D₃ but not to the 1,25-(OH)₂D₃, it seems that 1,25-(OH)₂D₃ is the active component in stimulating lead absorption.

I. Mechanism of Intestinal Calcium Transport

A. Subcellular location of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) in intestine. As described in the previous annual report, we were able to synthesize chemically and enzymatically 1,25-(OH)₂[23,24-³H]D₃ of high specific activity (78 Ci/mmol) to permit autoradiographic experiments with truly physiologic doses of this hormonal form of vitamin D. We then prepared rachitic chickens by methods described previously and they were then administered by intravenous injection into the wing vein 650 pmoles or 50 μCi of the 1,25-(OH)₂[23,24-³H]D₃ dissolved in .005 ml of absolute ethanol. At 1, 2-1/2, 4 and 6 hours post-injection, the chicks were killed and their duodena removed, cut into small segments and immediately frozen on Dry Ice blocks. The frozen sections of small intestine were then mounted on a freezing microtome and cut into 10 micron sections. The sections were then mounted on glass slides and dipped in a photographic emulsion. The glass slides were then packed in dark paper and maintained at -20° in the dark for 3-8 weeks. At various times the slides were removed, developed in Kodak developer and then the tissue sections were stained with hematoxylin and eosin. Good radioactive records were obtained at about 4-5 weeks of exposure. From the same animals samples of skeletal muscle, liver, kidney, smooth muscle from the intestine and tibia were taken. The soft tissues were processed as described for the small intestine while the bones were sent to Dr. J. L. Matthews at the Southwestern Medical School in Dallas for processing as undecalcified sections of bone. We have been able by this autoradiographic means to demonstrate that the intestinal epithelial crypt cells accumulate radioactive 1,25-(OH)₂D₃ in their nuclei. Similarly, we can show accumulation specifically of the tritium in the nuclei of the villus cells. No specific localization of the radioactivity could be found in the sub-mucosa, Lamina propria smooth muscle of intestine, skeletal muscle and liver. Some specific localization could be demonstrated in sections of renal tubules, but where these tubules originated was not determined. Other sections of the tubules showed no specific localization of tritium from the tritiated 1,25-(OH)₂D₃. Reports from Dr. Matthews' laboratory indicate localization of the tritium in osteoblasts osteocytes and chondroblasts, but no specific localization in osteoclasts.

In the intestine the localization of the tritium was not found at 1 hour but appeared at 2-1/2 hours, was maximal at 4 hours and was diminishing at 6 hours. It has been demonstrated in this laboratory and elsewhere that maximal intestinal calcium absorption occurs 9 hours post-injection of 1,25-(OH)₂D₃ to rachitic chicks.

A count of the grains over the nuclei indicate that approximately 50% of the nuclei are labeled with the tritium from ³H-1,25-(OH)₂D₃ and each nuclei has about 4.5 grains per nucleus at maximum labeling. Thus one of our major aims has been accomplished, namely the unequivocal in vivo demonstration of nuclear location of ³H-1,25-(OH)₂D₃ prior to the initiation of the target organ response of the intestine. It, therefore, appears that

1,25-(OH)₂D₃ must function at least in part by stimulating nuclear transcription of DNA to form messenger RNA. This *in vivo* experiment provides the necessary information to determine clearly the subcellular location of the 1,25-(OH)₂D₃ and, therefore, obviates the need for studying biochemical subcellular location of ³H-1,25-(OH)₂D₃ in isolated crypt and villus cells.

B. Study of the receptor proteins in intestine for 1,25-(OH)₂D₃.
In the previous report with the 9 Ci/mole 1,25-(OH)₂-[26,27-³H]D₃ it was shown that chick intestine contains a protein which binds specifically 1,25-(OH)₂D₃ with high affinity and low capacity. We have continued studying this particular protein, which is considered a receptor for 1,25-(OH)₂D₃, by comparing the ability of unlabeled 1,25-(OH)₂D₃ to compete with the labeled 78 Ci/mole 1,25-(OH)₂-[23,24-³H]D₃ for the 3.7S binding site. We have carried out these studies by means of polyethylene glycol precipitation of the receptor-1,25-(OH)₂D₃ complex and counting the precipitated pellet or we have carried out sucrose density gradient sedimentation studies to determine the displacement from the 3.7S cytosol receptor. Intestinal cytosol from rachitic chicks was prepared by means of high speed centrifugation and then incubated with .25 nM 1,25-(OH)₂-[23,24-³H]D₃ in the presence and absence of unlabeled 1,25-(OH)₂D₃ or one of its analogs. The amount of unlabeled analog required to bring about 50% displacement was computed. Using this as a basis it was possible to demonstrate that 1,25-(OH)₂D₂ was equal to 1,25-(OH)₂D₃ in competing for the receptor protein. However, no other analog approached the activity of the native hormone. The closest competitor was 1,24R,25-trihydroxyvitamin D₃ (1,24R,25-(OH)₃D₃) and 1,24S,25-trihydroxyvitamin D₃ (1,24S,25-(OH)₃D₃). The 1,24,25-(OH)₃D₃'s were about 100 times less competent than the native hormone, 1 α -hydroxyvitamin D₃ (1 α -OH-D₃) was about 1,000 times less effective and 25-hydroxyvitamin D₃ (25-OH-D₃) about 5,000 times less effective. Other less competent metabolites include the 24R,25-dihydroxyvitamin D₃ (24R,25-(OH)₂D₃), 24R-hydroxyvitamin D₃ (24R-OH-D₃), whereas the least competent were the 24S-hydroxyvitamin D₃ (24S-OH-D₃) and 24S,25-dihydroxyvitamin D₃ (24S,25-(OH)₂D₃). Vitamin D₃ itself did not compete even at 10⁻⁶M. Thus the 3.7S receptor protein is obviously highly specific for 1,25-(OH)₂D₃ or 1,25-(OH)₂D₂, discriminating against all of the other known metabolites of vitamin D. With this protein it has been possible to develop a competitive protein binding assay for the 1,25-(OH)₂D₃ provided the extracts of tissue or serum are first prepurified by chromatography on Sephadex LH-20 followed by high-pressure liquid chromatography. By using a small amount of radioactive 1,25-(OH)₂D₃ of high specific activity, one can monitor recovery of the 1,25-(OH)₂D₃ during extraction and chromatography prior to competitive binding assay examination. Using this recovery figure and the competency in the competitive binding assay, it is possible to estimate the level of 1,25-(OH)₂D₃ in the plasma of experimental animals and of human subjects. As an off-shoot of this continued investigation we have been able to develop a method of directly assessing 25-OH-D₃ in human plasma by means of high-pressure liquid chromatography and optical density at 254 nm. In this procedure tracer 25-OH-D₃ of high specific activity is added to a plasma samples and then subjected to extraction. The extracted sample

which is usually extracted with methanol-chloroform is placed on a Sephadex LH-20 column followed by a short reverse-phase lipidex column. The 25-OH-D₃ region is then injected into the high-pressure liquid system and the optical density at 254 nm or peak height is determined. Peak height is related linearly to the 25-OH-D₃ level in the sample and thus can be directly taken from a standard curve. The 25-OH-D₃ fraction from high-pressure liquid chromatography is then counted and the value obtained from the peak height standard curve is corrected for losses during extraction and chromatography using the recovery figure obtained by counting the radioactivity. This highly precise method will certainly provide a new and advanced technique for measuring this metabolite in plasma.

Work has continued on mammalian intestinal cytosol receptors since previously both in this laboratory and elsewhere was not possible to demonstrate clearly the existence of a cytosol receptor to 1,25-(OH)₂D₃. By scraping the mucosa from vitamin D-deficient rats and washing the mucosa with buffered sucrose, it was possible to eliminate a good share of the proteolytic enzymes which destroy the receptor. If the mucosal cells are then homogenized in high salt, Tris buffer containing .5 mM dithiothreitol, the presence of a 3.2S cytosol receptor type protein can be easily demonstrated using sucrose density gradient analysis. When the cytosol is incubated with .25 nM 1,25-(OH)₂-[23,24-³H]D₃, binding can be observed by a 3.2S component and a 6S component. Addition of non-radioactive 25-OH-D₃ displaces the radioactivity from the 6S component onto the 3.2S component. Furthermore, addition of non-radioactive 1,25-(OH)₂D₃ will displace the radioactivity from the 3.2S protein to the 6S protein. This mammalian receptor has been found only in intestine and bone, but cannot be demonstrated in liver, kidney, testes, spleen and other non-target tissues.

We have looked very carefully for the presence of a receptor for 1,25-(OH)₂D₃ in bone. Twenty-day old embryonic chicks or rats were used. Calvaria were removed from the embryos, homogenized in .3% potassium chloride, 0.001 M Tris-chloride, pH 7.4, and .5 mM dithiothreitol. Sedimentation at high speeds yielded a cytosol which when incubated with the tritiated 1,25-(OH)₂D₃ clearly demonstrated the presence of a 3.2S binding protein as well as a 6S binding protein. The 3.2S binding could be eliminated by addition of excess non-radioactive 1,25-(OH)₂D₃ whereas the 6S binding was eliminated by addition of non-radioactive 25-OH-D₃. Thus the characteristics of the 3.2S binder from embryonic bone is similar to that of the rat intestinal cytosol binding protein for 1,25-(OH)₂D₃.

Concern about the 6S binding protein led us to examine its sensitivity to an antibody which had been directed toward the plasma transport protein for vitamin D and its metabolites. The 6S 25-OH-D₃ binding protein reacted with the antibody directed to the 4.1S transport protein of the plasma. However, it did not cross react with the 3.2S rat or the 3.7S chick intestinal cytosol binders for 1,25-(OH)₂D₃. Addition of serum to cytosol brought about a conversion of 4S serum binding to the 6S binding peak. Heating of the 6S binding peak to 60° brought about a regeneration of the 4.1S component. According to reports from Belgium

as well, it seems that the 6S protein is an artifact of contaminating serum transport protein combining with an undetermined cytosol protein to form a 6S binder. This 6S binder is found in every tissue and is directly related to the vascularization of that tissue. Tissue culture cells grown in the absence of serum or plasma do not show the 6S binding protein. Thus the 6S protein appears not to be a receptor but is an artifact of preparation.

II. Control of Intestinal Calcium Transport

Work has continued in this laboratory on factors which control intestinal calcium transport by regulating the biogenesis of the principle active hormone derived from vitamin D_3 , namely $1,25-(OH)_2D_3$. A most important breakthrough has been registered in our examination of regulation of the $25-OH-D_3$ -1-hydroxylase in kidneys as related to the reproductive cycle in birds. It has been demonstrated in our laboratory that female birds approaching the egg laying phase of their lives show a marked enhancement of the $25-OH-D_3$ -1-hydroxylase. This enhancement appears at the time that medullary bone is mobilized for deposit in egg albumin and in the egg shells. The stimulation of the 1-hydroxylase occurs at the time progesterone, estradiol and testosterone levels are high in the egg laying birds. Injection of estradiol to mature male birds which have low levels of 1-hydroxylase show a marked enhancement of the 1-hydroxylase within 6-24 hours post-injection of the estradiol. Injection of the estradiol to immature males brings about little or no response, but injection of both estradiol and testosterone to immature males brings about a marked stimulation. Similarly, progesterone can replace testosterone or all three hormones together produce a marked stimulation of the 1-hydroxylase. Castrate adult male birds also show very marked enhancement of their 1-hydroxylase in response to a combination of estradiol and testosterone or estradiol and progesterone or estradiol, progesterone and testosterone. Thus the sex hormones markedly stimulate the $25-OH-D_3$ -1-hydroxylase in the egg laying cycle of birds and accounts for marked enhancement of intestinal calcium absorption, the mobilization of medullary bone and the deposition of calcium carbonate in egg shell.

We have examined the question of whether calcitonin might play a regulatory role on the $25-OH-D_3$ -1-hydroxylase, thereby modifying intestinal calcium transport. We were able to confirm the experiments of Galante *et al.*, which demonstrated that injections of large amounts of calcitonin to intact rats causes an enhancement of $1,25-(OH)_2D_3$ production from $25-OH-D_3$ and a suppression of 24-hydroxylation of $25-OH-D_3$. However, when the same experiment was done in thyroparathyroidectomized animals, no response was observed. Parathyroid hormone on the other hand caused a marked stimulation of the 1-hydroxylase in the thyroparathyroidectomized rats. From these results it was concluded that calcitonin stimulated production of $1,25-(OH)_2D_3$ by causing a hypocalcemia which in turn stimulated secretion of the parathyroid hormone. This in turn stimulated the $25-OH-D_3$ -1-hydroxylase. Our experience, therefore, is that calcitonin does not regulate $25-OH-D_3$ -1-hydroxylation.

We have attempted to develop a tissue culture method which would be able to carry out 1-hydroxylation of $25-OH-D_3$ and show sensitivity to the factors which are known to stimulate $1,25-(OH)_2D_3$ production and hence intestinal calcium absorption. Although we have not succeeded in

obtaining a culture which produces $1,25-(\text{OH})_2\text{D}_3$, we have been able to obtain a monkey kidney cell culture which produces $24,25-(\text{OH})_2\text{D}_3$ from 25-OH-D_3 . This system is sensitive to parathyroid hormone, ambient calcium concentration and $1,25-(\text{OH})_2\text{D}_3$. $1,25-(\text{OH})_2\text{D}_3$ stimulates production of $24,25-(\text{OH})_2\text{D}_3$, high calcium stimulates production of this compound whereas parathyroid hormone suppresses 24-hydroxylation.

III. Analog of $1,25-(\text{OH})_2\text{D}_3$

We have continued our work on the isolation and identification of the active principle of Solanum glaucophyllum, the South American plant which causes metastatic calcification and death to grazing animals. It had been shown previously from a number of laboratories that this material has $1,25-(\text{OH})_2\text{D}_3$ -like activity despite the fact it is water soluble. Work by Wasserman and his colleagues has been able to show that nephrectomy does not prevent response of the animal to Solanum glaucophyllum nor does the feeding of high strontium diets which blocks 1-hydroxylation. Preliminary evidence has been offered by that laboratory that the compound which is active is a water soluble complex of $1,25-(\text{OH})_2\text{D}_3$. We have been able to feed Solanum glaucophyllum to completely vitamin D-deficient rats and by means of our competitive protein binding assay and sophisticated chromatography demonstrate that the tissues of the affected animals accumulate large amounts of $1,25-(\text{OH})_2\text{D}_3$ in intestine and blood despite the fact that they have no other source of vitamin D_3 . Thus it is very likely indeed that the active material of the plant Solanum glaucophyllum is a water soluble or glycosidic form of $1,25-(\text{OH})_2\text{D}_3$ or its analog $1\alpha\text{-OH-D}_3$ which is known to be rapidly converted to the $1,25-(\text{OH})_2\text{D}_3$.

About 80 vitamin D-deficient rats were each given, by stomach tube, an extract from 100 mg Solanum glaucophyllum leaves. Eighty vitamin D-deficient control rats were treated in the same way except they received a stomach tube of the vehicle without the leaf material. Both groups of animals were killed at 48 hours, some were used to determine intestinal calcium transport and serum calcium concentration showing clearly the response to Solanum glaucophyllum. Intestinal tissue and blood were removed from both groups and each was spiked with a small amount of high specific activity [^3H]- $1,25-(\text{OH})_2\text{D}_3$ to follow purification, and extracted with methanol-chloroform. The extracts were then partitioned between hexane and 90% methanol/10% water which eliminated much of the neutral lipids in the hexane fraction. The 90% methanol-water fraction was subjected to Sephadex LH-20 chromatography using a solvent system of 65% chloroform, 35% Skellysolve B. The $1,25-(\text{OH})_2\text{D}_3$ fraction which would also contain $1,25-(\text{OH})\text{-D}_2$ was subjected to further chromatography on Sephadex LH-20 developed with a solvent system of 90% hexane, 10% chloroform, and 10% methanol. Following chromatography on a reversed phase hydroxyalkoxy propyl Sephadex column, the extracts were then subjected to high pressure liquid chromatography on fine grain silica using 10% isopropynol in hexane and assay by competitive protein binding utilizing the 3.7 S chick intestinal cytosol receptor. Intestinal tissue from animals treated with Solanum glaucophyllum had large amounts of $1,25-(\text{OH})_2\text{D}_3$ present of the order of 230 pg/g of tissue. Vitamin D deficient tissue had no detectable $1,25-(\text{OH})_2\text{D}_3$. Similarly, blood values of $1,25-(\text{OH})_2\text{D}_3$ were in the neighborhood of 300 pg/ml in the Solanum glaucophyllum-treated

animals and nondetectable in the controls. In addition, the activity was entirely as $1,25(\text{OH})_2\text{D}_3$ with no detectable $1,25(\text{OH})\text{-D}_2$. These experiments demonstrate that Solanum glaucophyllum extracts are a source of $1,25(\text{OH})_2\text{D}_3$ and the most plausible explanation is that it exists as a conjugated form with some glycosidic residue in the plants. Wasserman and his colleagues have recently hydrolyzed Solanum glaucophyllum extracts with a glucuronidase and succeeded in demonstrating a mass spectrum of confirming the existence of $1,25(\text{OH})_2\text{D}_3$ in the Solanum material.

Other analog work has been a study of the biological activity and competitive protein binding of analogs versus $1,25(\text{OH})_2\text{D}_3$ using the chick intestinal cytosol receptor. By adding essentially saturating amounts of [^3H]- $1,25(\text{OH})_2\text{D}_3$ to a constant amount of chick intestinal cytosol receptor it was possible to add increasing amounts of a variety of analogs. The amount was increased until 50% of the tritiated $1,25(\text{OH})_2\text{D}_3$ could be displaced from the 3.7 S cytosol receptor. This was detected both by sucrose density gradient analysis and by precipitation with polyethylene glycol. Using these two techniques it was possible to show that $1,25(\text{OH})\text{-D}_2$ and $1,25(\text{OH})_2\text{D}_3$ were equal in their activity in displacing radioactive $1,25(\text{OH})_2\text{D}_3$. About 300 fold less competent was $1,24\text{R-}25(\text{OH})_3\text{D}_3$ and somewhat less competent was $1,24\text{S-}25(\text{OH})_3\text{D}_3$. About two to three thousand times less competent was $1\alpha\text{-OH-D}_3$ and 25-OH-D_3 was approximately 5000 times less competent. $24,25\text{R-}(\text{OH})_2\text{D}_3$ was approximately equal to 25-OH-D_3 in its ability to compete for the cytosol receptor. The least competent analogs were the 24 S and the $24\text{S,}25(\text{OH})_2\text{D}_3$. Vitamin D_3 itself did not show activity even when added at 10^{-6} molar. This demonstrates the great selectivity of the intestinal cytosol receptor for the $1,25(\text{OH})_2\text{D}_3$. It also demonstrates that if the concentration of 25-OH-D_3 can be made sufficiently high, it will interact with the receptor. This may account for vitamin D toxicity at very high dosages because of the generation of high circulating levels of 25-OH-D_3 acting as an analog of $1,25(\text{OH})_2\text{D}_3$.

We have continued to examine the question of whether 24-hydroxylation of the vitamin D compounds has functional physiologic significance. We have completed our study of the biological activity of the 24-hydroxylated vitamins on intestinal calcium absorption. In the rat, the 24-hydroxylated vitamins are almost as active as their non-24-hydroxylated counterparts. On the other hand, in the chicken, the 24-hydroxylated compounds are much less active. These results taken in total argue that the 24-hydroxylation reaction may be an initial event in the inactivation of the potent vitamin D molecules. We were concerned with the observation that $24\text{R,}25(\text{OH})_2\text{D}_3$ and 24R-OH-D_3 were considerably more active than the $24\text{S,}25(\text{OH})_2\text{D}_3$ and the 24S-OH-D_3 . We prepared radioactive 24S and 24R- OH-D_3 's and upon their administration learned that they are rapidly converted to corresponding 25-hydroxylated compound but that at the renal 1-hydroxylation site the S configuration is not recognized. Thus there is a discrimination against the 24 S-hydroxyl configuration by the 25-OH-D_3 -1-hydroxylase in the kidney. This accounts for the lack of activity of the $24\text{S,}25(\text{OH})_2\text{D}_3$ and the 24S-OH-D_3 .

Of considerable interest is that nephrectomy did not prevent 24-hydroxylation in vivo of $1,25(\text{OH})_2\text{D}_3$. Thus it appears there is an extra renal site of 24-hydroxylation.

IV. Intestinal absorption of lead.

During the past year we have made considerable progress in studying intestinal lead absorption. We have developed two techniques for assessment of lead transport in vitro in small intestine and lead absorption in vivo. In the in vitro technique, intestines are removed from the animals and everted in 5.5 cm segments. The segments are then filled with a physiological solution buffered with Tris chloride at pH 7.4. The segments are tied into sacs and incubated under 100% oxygen in a medium containing the buffered physiological salt solution with the addition of .01 mM lead acetate labeled with lead 210. The time course of lead appearing into the serosal sac shows a linear progression for up to 45 min. Using this technique, it has been possible to demonstrate that the distal ileum shows the greatest ability to transport lead. Furthermore, vitamin D deficiency results in a diminished capacity of all segments to transport lead but especially the ileal segment. Calcium in the incubation medium diminishes lead appearing in the serosal fluid, but of considerable interest, animals fed a low calcium diet show low rates of lead absorption whereas those on a high calcium diet show high rates of lead transport. The latter observation is in contrast to what has been observed in terms of calcium transport; that is, low calcium diet animals show high rates of calcium transport, whereas high calcium rats show low rates of calcium transport. These results would argue against the idea that lead is transported by a mechanism similar to calcium.

Because of the problem of lead uptake by intestinal smooth muscle in in vitro measurements, we developed an in vivo technique for the assessment of lead absorption. This technique involves stomach tubing .45 ml of lead acetate solution (.01 mM) containing lead 210. The animals are then allowed to absorb lead over a period of 18 to 24 hr. The entire G.I. tract plus fecal excretions are counted to determine the amount of lead 210 remaining. Knowing the amount of lead 210 present initially, the percent lead absorption can be calculated. Using this in vivo technique, one can readily demonstrate that the distal ileum is the site of major lead absorption and that it is heavily vitamin D-dependent. Vitamin D deficiency results in a marked decrease in lead absorption. Furthermore, calcium present in intubation solution diminishes lead absorption. On the other hand lead absorption is highest in animals on a high calcium diet and lowest in animals fed a low-calcium diet, again supporting the in vitro experiments and suggesting that lead and calcium are not transported by the same mechanism.

The vitamin D dependency of lead absorption has been investigated more completely. Although physiologic amounts of vitamin D markedly enhance lead absorption, much higher levels of vitamin D do not appreciably increase further the rate of lead absorption. Nephrectomy prevents the response of the intestine to vitamin D₃ in terms of stimulation of lead

absorption whereas it does not prevent the response to $1,25-(OH)_2D_3$. These results suggest that $1,25-(OH)_2D_3$ is the active component in stimulating lead absorption and therefore opens the possibility that factors which suppress $1,25-(OH)_2D_3$ production might well suppress lead absorption.

Papers Published Under This Contract Period

1. DeLuca, H. F., and H. K. Schnoes. Metabolism and Mechanism of Action of Vitamin D. *Ann. Rev. Biochem.* 45, 631-666 (1976).
2. DeLuca, H. F. Vitamin D Endocrinology. *Ann. Internal Med.* 85, 367-377 (1976).
3. Tanaka, Y., L. Castillo, and H. F. DeLuca. Control of Renal Vitamin D Hydroxylases in Birds by Sex Hormones. *Proc. Natl. Acad. Sci. USA* 73, 2701-2705 (1976).
4. Kream, B. E., R. D. Reynolds, J. C. Knutson, J. A. Eisman, and H. F. DeLuca. Intestinal Cytosol Binders of 1,25-Dihydroxyvitamin D₃ and 25-Hydroxyvitamin D₃. *Arch. Biochem. Biophys.* 176, 779-787 (1976).
5. Bonjour, J.-P., U. Trechsel, U. Trohler, H. Fleisch, L. A. Baxter, and H. F. DeLuca. Influence of a Diphosphonate and 1,25-Dihydroxycholecalciferol on Calcium Metabolism, in "Calcium Metabolism, Bone and Metabolic Bone Diseases" Proceedings of the X European Symposium on Calcified Tissues. F. Kuhlencordt and H.-P. Kruse (eds.), Springer-Verlag, Berlin, pp. 251-255 (1975).
6. Tanaka, Y., H. F. DeLuca, A. Akaiwa, M. Morisaki, and N. Ikekawa. Synthesis of 24S and 24R-Hydroxy-[24-³H]vitamin D₃ and Their Metabolism in Rachitic Rats. *Arch. Biochem. Biophys.* 177, 615-621 (1976).
7. DeLuca, H. F., Y. Tanaka, and L. A. Baxter. The Interrelationship Between Vitamin D Metabolites and Phosphate Metabolism, in "Phosphate Metabolism Kidney and Bone" Proceedings of the International Workshop on Phosphate. L. Avioli, Ph. Bordier, H. Fleisch, S. Massry, and E. Slatopolsky (eds.), Nouvelle Imprimerie Fournie, Toulouse, France, pp. 223-237 (1976).
8. Lorenc, R., Y. Tanaka, H. F. DeLuca, and G. Jones. Lack of Effect of Calcitonin on the Regulation of Vitamin D Metabolism in the Rat. *Endocrinology* 100, 468-472 (1977).
9. Tanaka, Y., L. Castillo, H. F. DeLuca, and N. Ikekawa. The 24-Hydroxylation of 1,25-Dihydroxyvitamin D₃. *J. Biol. Chem.* 252, 1421-1424 (1977).
10. Castillo, L., Y. Tanaka, H. F. DeLuca, and M. L. Sunde. The Stimulation of 25-Hydroxyvitamin D₃-1 α -Hydroxylase by Estrogen. *Arch. Biochem. Biophys.* 179, 211-217 (1977).
11. Kream, B. E., M. J. L. Jose, and H. F. DeLuca. The Chick Intestinal Cytosol Binding Protein for 1,25-Dihydroxyvitamin D₃: A Study of Analog Binding. *Arch. Biochem. Biophys.* 179, 462-468 (1977).
12. Napoli, J. L., L. E. Reeve, J. A. Eisman, H. K. Schnoes, and H. F. DeLuca. *Solanum Glaucophyllum* as a Source of 1,25-Dihydroxyvitamin D₃. *J. Biol. Chem.* 252, 2580-2583 (1977).

13. Juan, D., and H. F. DeLuca. The Regulation of 24,25-Dihydroxyvitamin D₃ Production in Cultures of Monkey Kidney Cells. *Endocrinology*, in press (1977).
14. Eisman, J. A., R. M. Shepard, and H. F. DeLuca. Determination of 25-Hydroxyvitamin D₂ and 25-Hydroxyvitamin D₃ in Human Plasma Using High-Pressure Liquid Chromatography. *Anal. Biochem.*, in press (1977).
15. Kream, B. E., S. Yamada, H. K. Schnoes, and H. F. DeLuca. A Specific Cytosol Binding Protein for 1,25-Dihydroxyvitamin D₃ in Rat Intestine. *J. Biol. Chem.*, in press (1977).
16. Kream, B. E., M. Jose, S. Yamada, and H. F. DeLuca. A Specific High Affinity Binding Macromolecule for 1,25-Dihydroxyvitamin D₃ in Fetal Bone. *Science*, in press (1977).
17. Kream, B. E., and H. F. DeLuca. A Specific Binding Protein for 1,25-Dihydroxyvitamin D₃ in Rat Intestinal Cytosol. *Biochem. Biophys. Res. Commun.*, in press (1977).
18. DeLuca, H. F. Vitamin D Metabolism. *Clin. Endocrinol.*, in press (1977).

Reprints + Preprints removed