MECHANISMS OF CALCIUM TRANSPORT IN SMALL INTESTINE

Overall Review of the Contract

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ABSTRACT: The $1\alpha,25$ -dihydroxyvitamin D_3 was isolated and identified as the metabolically active form of vitamin D_3 in the intestine. It initiates intestinal calcium transport within one hour after its intravenous injection. This response is not blocked by actinomycin D but the conversion of 25-hydroxyvitamin D_3 to $1\alpha,25$ -dihydroxyvitamin D_3 is. The conversion takes place in the kidney and is strongly feed-back regulated indirectly by serum calcium concentration. This regulation is mediated by the parathyroid glands with the parathyroid hormone acting as a tropic hormone for $1\alpha,25$ -dihydroxyvitamin D_3 .

The adaptation of intestinal calcium absorption is mediated in part by regulation of $1\alpha,25$ -dihydroxyvitamin D_3 synthesis. Animals given $1\alpha,25$ -dihydroxyvitamin D_3 show high rates of intestinal calcium absorption independent of dietary calcium, suggesting the $1\alpha,25$ -dihydroxyvitamin D_3 as the terminal component of Nicolaysen's endogenous factor.

Strontium feeding and diphosphonates inhibit intestinal calcium absorption by inhibiting $1\alpha,25$ -dihydroxyvitamin D_3 synthesis. Administration of the $1\alpha,25$ -dihydroxyvitamin D_3 overcomes the inhibition of intestinal calcium absorption by these agents.

$1\alpha,25$ -Dihydroxyvitamin D_3 and its chemical analogs possessing a hydroxyl in the 1 position stimulate intestinal calcium absorption in nephrectomized rats while its precursor 25-hydroxyvitamin D_3 does not, establishing the $1\alpha,25$ -dihydroxyvitamin D_3 as the metabolically active form of vitamin D in intestine.

Possible precursor proteins for the intestinal calcium binding protein have been detected and isolated. A new high molecular weight protein in the brush borders of chick intestine after $1\alpha,25$ -dihydroxyvitamin D_3 administration has been detected. Its appearance correlates exactly with increased intestinal calcium absorption.

A vascularly perfused intestinal system has been developed which survives for 6-8 hours. With this system, a clear inhibition of calcium absorption by calcitonin administration, a stimulation by parathyroid hormone and a marked stimulation by $1\alpha,25$ -dihydroxyvitamin D_3 have been demonstrated.

1. Main Research Accomplishments:

The effort under this contract has been markedly hampered by the low level of funding and by markedly rising costs. We have been able to support one postdoctoral fellow equivalent on this contract and hence the research accomplishments must be viewed in this way. Furthermore, the research work carried out under this contract is interwoven with our research work on the metabolism and mechanism of action of vitamin D, which is supported by the National Institutes of Health. There will, therefore, be some repetition in the accomplishments achieved under this contract and some of the accomplishments listed with the National Institutes of Health.

Perhaps the greatest accomplishment of the joint efforts of personnel on this contract and the National Institutes of Health support is the isolation, identification and chemical synthesis of the $1\alpha,25$ -dihydroxyvitamin D_3 and the demonstration that it represents the metabolically active form of vitamin D which initiates intestinal calcium absorption. Work carried out under this contract established firmly the nature of its biological activity. Thus it can be shown to be much more rapidly acting than any form of vitamin D known and furthermore its activity disappears much more rapidly suggesting a rapid turnover of this metabolite. The $1\alpha,25$ -dihydroxyvitamin D_3 has also been demonstrated to be the metabolically active form of vitamin D in bringing about bone calcium mobilization. This, therefore, places in our hands the exact vitamin D tool which can be used to study intestinal calcium absorption.

With this substance there is a lag of about 1 hour between its time of intravenous administration and the initiation of intestinal calcium transport at least in the rat. However, it can also be demonstrated that this action of $1\alpha,25$ -dihydroxyvitamin D_3 cannot be blocked by the prior administration of actinomycin D and probably cycloheximide. These results therefore strongly suggest that the intestinal calcium transport response to vitamin D does not involve protein synthesis and transcription of DNA once the exact metabolically active form of the vitamin is supplied. In other work carried out under the NIH grants it could be demonstrated that the previously detected actinomycin D sensitivity is at the level of conversion of 25-hydroxyvitamin D_3 to the $1\alpha,25$ -dihydroxyvitamin D_3 . These results therefore give us new insight into the nature of the mechanism initiated by vitamin D.

Another major accomplishment under the present AEC contract was the demonstration that the synthesis of $1\alpha,25$ -dihydroxyvitamin D_3 in the kidney from 25-hydroxyvitamin D_3 is under strong feed-back control by the serum calcium concentration. This has now been shown to be actually the result of the parathyroid hormone which is secreted by the parathyroid glands in response to hypocalcemia. The parathyroid hormone has now been demonstrated to serve as a tropic hormone for the generation of the $1\alpha,25$ -dihydroxyvitamin D_3 . It is therefore important to note that this demonstrates a new interrelationship between vitamin D and the parathyroid hormone, namely that the parathyroid hormone is essential for the conversion of 25-hydroxyvitamin D_3 to the terminal metabolically active form of vitamin D_3 , namely $1\alpha,25$ -dihydroxyvitamin D_3 ,

responsible for mobilization of calcium from both bone and intestine. This result also clearly suggests that the parathyroid hormone can affect intestinal calcium absorption probably through the medium of regulation of synthesis of the $1\alpha,25$ -dihydroxyvitamin D_3 . In thyroparathyroidectomized animals and man, it is likely that little $1\alpha,25$ -dihydroxyvitamin D_3 is synthesized and hence intestinal calcium absorption will as a result be low.

Another important accomplishment is the recognition that $1\alpha,25$ -dihydroxyvitamin D_3 and factors which stimulate its synthesis probably represent the long sought after Nicolaysen endogenous factor. When chicks are maintained on $1\alpha,25$ -dihydroxyvitamin D_3 as their source of the vitamin they fail to adapt to dietary calcium levels. On the other hand when they are maintained on 25-hydroxyvitamin D_3 as their source of vitamin D they adapt in the expected fashion showing a high rate of intestinal calcium absorption in the case of a low calcium diet and a low rate of intestinal calcium absorption in the case of a high calcium diet. The many factors which regulate the $1\alpha,25$ -dihydroxyvitamin D_3 synthesis are currently under study. In any case, it is apparent that many factors which regulate intestinal calcium absorption or which affect intestinal calcium absorption may well function through the regulation of production of the $1\alpha,25$ -dihydroxyvitamin D_3 , which in turn regulates intestinal calcium absorption.

Another major accomplishment is the demonstration that three factors previously believed to act directly on intestinal calcium absorption have now been shown to work through the medium of regulating the $1\alpha,25$ -dihydroxyvitamin D_3 synthesis. It has long been known that strontium feeding brings about a vitamin D resistant rachitic condition which has been shown by Wasserman and his colleagues to result in part from a depressed intestinal calcium absorption and depressed synthesis of calcium binding protein. We have now demonstrated that strontium feeding depresses very markedly the synthesis of $1\alpha,25$ -dihydroxyvitamin D_3 synthesis and that the $1\alpha,25$ -dihydroxyvitamin D_3 administered to strontium-fed animals will reverse completely the reduced intestinal calcium absorption restoring it to normal. A similar picture is anticipated with barium and other divalent cations which have been shown to produce a vitamin D-resistant rickets. The phosphonates have been demonstrated by Fleisch and his coworkers to inhibit intestinal calcium absorption. In collaboration with that group, this inhibition has now been demonstrated to result from a lack of synthesis of the $1\alpha,25$ -dihydroxyvitamin D_3 . When the EHDP (ethane-1-hydroxy-1,1-diphosphonate) is administered at a level which blocks calcification there is a transient hypercalcemia which undoubtedly shuts off secretion of the parathyroid hormone. In the absence of the parathyroid hormone the synthesis of $1\alpha,25$ -dihydroxyvitamin D_3 falls to very low levels. Intestinal calcium absorption falls which undoubtedly contributes to the rachitic state of the EHDP-treated animals. When $1\alpha,25$ -dihydroxyvitamin D_3 is administered to the EHDP-treated animals the intestinal calcium transport lesion is completely reversed restoring it to normal. Work is currently being carried out on the cortisone inhibition of intestinal calcium absorption, but thus far results are insufficient to draw conclusions.

The knowledge of the existence of the $1\alpha,25$ -dihydroxyvitamin D_3 has prompted our investigation into possible chemical synthesis which has just recently been achieved. This synthesis is however extremely expensive and will likely not yield a compound which will be useful in medicine. We have, therefore, synthesized a variety of analogs of the $1\alpha,25$ -dihydroxyvitamin D_3 and under this contract tested their biological activity. The 25-hydroxydihydrotachysterol which represents a close analog because of the fact that the ring A is rotated 180° placing the 3-hydroxyl of that compound in the position occupied by the 1-hydroxyl of $1\alpha,25$ -dihydroxyvitamin D_3 . This compound stimulates intestinal calcium absorption and bone calcium mobilization in animals which have been nephrectomized and hence have lost their ability to synthesize $1\alpha,25$ -dihydroxyvitamin D_3 . It is therefore evident that the dihydrotachysterols function following 25-hydroxylation without 1-hydroxylation and explain the observation that dihydrotachysterols are effective in patients suffering from chronic renal disease and in animals or man deprived of the parathyroid hormone and hence unable to synthesize significant quantities of the $1\alpha,25$ -dihydroxyvitamin D_3 .

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Other analogs have been synthesized/because of the rotation of ring A places the 3-hydroxyl in the 1-hydroxyl position of $1\alpha,25$ -dihydroxyvitamin D_3 . These compounds are 5,6-trans-vitamin D_3 and 5,6-trans-25-hydroxyvitamin D_3 . Both of these substances stimulate intestinal calcium absorption in nephrectomized animals illustrating that they do not have to be hydroxylated in the 1 position before they can function. They also indicate that these compounds may be of great interest for use in the treatment of chronic renal disease or hypoparathyroidism. In addition, the 5,6-trans-25-hydroxyvitamin D_3 fails to stimulate bone calcium mobilization whereas the 5,6-trans-vitamin D_3 does. It therefore appears possible to synthesize analogs of vitamin D which will specifically stimulate either bone or intestine without stimulating the other vitamin D responsive system. Furthermore, the 5,6-trans-25-hydroxyvitamin D_3 may be the compound of choice in the treatment of the bone disease associated with chronic renal disease.

A major advance was registered in our understanding of the intestinal calcium transport mechanism. During the tenure of the present contract we obtained evidence for the existence of a precursor protein for the calcium binding protein in the rat. Work currently in progress suggests that we now have similar evidence for the existence of such a protein in the chick. These proteins which are clearly changed in response to vitamin D are being isolated with the intent of studying them from a biochemical and physical chemical point of view. Furthermore, their possible conversion to the calcium binding protein is under investigation. Preliminary experiments suggest that we have in addition to the calcium binding protein and the calcium dependent ATPase which was described on the previous three-year AEC contract, a new large molecular weight protein which is found in the brush borders of intestine and which appears in response to vitamin D at exactly the time intestinal calcium absorption is stimulated. This protein is currently being isolated, has a molecular weight of about 200,000 and is very definitely a membrane component.

During the tenure of the present AEC contract we developed an isolated vascularly perfused intestinal system for the possible use in the study of the calcium transport responses directly to various hormones. We have been able to demonstrate a direct inhibitory effect of calcitonin on intestinal calcium absorption, the direct stimulation of intestinal calcium absorption by vitamin D metabolites and a direct but somewhat delayed response of intestinal calcium absorption to the parathyroid hormone. Dr. E. B. Olson, who developed this vascularly perfused intestinal system has now left the laboratory and because of the low level of funding we have been unable to hire an additional person to carry out these studies. The system however is a very promising one for studying directly the role of hormones in the calcium transport process and must remain until adequate funding will permit the reinitiation of these experiments.

2. Plans for the Continuation of the Present Objectives and Possible New Objectives in Consideration of Past Results:

A number of new developments during the course of the last three years of my current AEC contract have opened many possibilities for the study of intestinal calcium transport. We would like very much to exploit further the fact that $1\alpha,25$ -dihydroxyvitamin D_3 is the metabolically active form of the vitamin biosynthesized in the kidney. We would like at this time to synthesize a variety of analogs of the $1\alpha,25$ -dihydroxyvitamin D_3 in an attempt to learn more about the relationship of its structure to function. Under the proposed AEC contract we would like to continue testing the effects of these vitamin D analogs and other metabolites of vitamin D which we are currently isolating in the intestinal calcium transport system.

The fact that the $1\alpha,25$ -dihydroxyvitamin D_3 is synthesized in the kidney and its production is strongly feed-back regulated by serum calcium through the parathyroid hormone opens up the possibility that many factors are involved in regulating intestinal calcium absorption primarily through regulation of the synthesis of $1\alpha,25$ -dihydroxyvitamin D_3 . During the course of the next AEC contract period we would like to investigate a number of known inhibitors of intestinal calcium absorption for possible effects on the kidney system which synthesizes $1\alpha,25$ -dihydroxyvitamin D_3 . We would like to determine, with the use of chemically synthesized $1\alpha,25$ -dihydroxyvitamin D_3 , whether the effects of any of these inhibitors can be overcome by merely supplying exogenous sources of the $1,25$ -dihydroxyvitamin D_3 . To accompany this investigation we would like to continue work on the vascularly perfused intestine in which we can study the direct effects of agents on intestinal calcium transport in a system which more closely approximates the in vivo situation. With the vascularly perfused intestine we would like to not only determine the effects of the vitamin D metabolites and analogs but would like to study possible effects of cortisone, parathyroid hormone, lactogenic hormone, strontium, barium and beryllium, directly on intestinal calcium absorption. By studying each agent in both directions one can obtain sound data in determining their site of action and possible reversal of its action by the supply of exogenous $1,25$ -dihydroxyvitamin D_3 .

We would like to continue our investigation on the molecular events which are followed in the transfer of calcium across intestinal mucosa. We would like to use the $1\alpha,25$ -dihydroxyvitamin D_3 to stimulate intestinal calcium absorption and to attempt to define those reactions which occur in its response. Possible precursor proteins to the calcium binding protein described by Wasserman and his colleagues will be isolated in pure form and studied biochemically and perhaps enzymatically to determine if, under the influence of $1\alpha,25$ -dihydroxyvitamin D_3 , they can be converted to the calcium binding protein.

A new protein which has been discovered in the brush borders of rats given $1\alpha,25$ -dihydroxyvitamin D_3 will be isolated in pure form and studied biochemically. This protein which appears to be of molecular weight of about 200,000 exactly correlates with the rise in intestinal calcium absorption in response to the $1\alpha,25$ -dihydroxyvitamin D_3 . We would like to study this substance as to its possible role in intestinal calcium absorption. Should this component or any of the other known components not correlate exactly with the increase in intestinal calcium absorption in response to $1\alpha,25$ -dihydroxyvitamin D_3 , other proteins or lipids which might be responsible for the vitamin D response will be looked for.

A more detailed description of our research plans appears in item 5 of this overall program review.

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

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

- (1) Dr. Dennis Drescher (Ph.D. 1970)
- (2) Mr. Tai C. Chen (expects Ph.D. by December, 1972)
- (3) Miss Donna Brown (in third year of Ph.D. training)

Postdoctoral Students:

- (1) Dr. E. B. Olson, Jr. (January 1, 1970-July 1, 1971)
- (2) Dr. J. L. Omdahl (July 1, 1971-August 1, 1972)
- (3) Dr. Sachiko Moriuchi (July 1971-present)

4. Bibliography of Publications From the Present AEC Contract:

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- COO-1668-23 - DeLuca, H. F. The Function and Metabolism of Vitamin D in "Proceedings of the Eighth International Congress on Nutrition", Excerpta Medica International Congress Series No. 213, pp. 117-123 (1969).
- COO-1668-24 - Gray, R. W., and H. F. DeLuca. Metabolism of 25-Hydroxycholecalciferol and its Inhibition by Actinomycin D and Cycloheximide. *Arch. Biochem. Biophys.* 145, 276-282 (1971).
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- COO-1668-26 - Drescher, D., and H. F. DeLuca. Possible Precursor of Vitamin D Stimulated Calcium Binding Protein in Rats. *Biochemistry* 10, 2308-2312 (1971).
- COO-1668-27 - Melancon, M. J., Jr., H. Morii, and H. F. DeLuca. Physiologic Effects of Vitamin D, Parathyroid Hormone, and Calcitonin in "The Fat-Soluble Vitamins" (H. F. DeLuca and J. W. Suttie, eds.), The University of Wisconsin Press, Madison, pp. 111-123 (1970).
- COO-1668-28 - DeLuca, H. F. Vitamin D: Mode of Action in "Symposium Proceedings The Biochemistry, Metabolism and Chemical Analysis of Vitamin D and Related Compounds", Association of Vitamin Chemists, pp. 1-27 (1970).
- COO-1668-29 - DeLuca, H. F. The Role of Vitamin D and Its Relationship to Parathyroid Hormone and Calcitonin in "Recent Progress in Hormone Research", Vol. 27, Academic Press, New York, pp. 479-516 (1971).
- COO-1668-31 - DeLuca, H. F. Vitamin D: New Horizons. *Clin. Orthopaed. Related Res.* 78, 4-23 (1971).
- COO-1668-32 - Boyle, I. T., R. W. Gray, and H. F. DeLuca. Regulation by Calcium of in vivo Synthesis of 1,25-Dihydroxycholecalciferol and 21,25-Dihydroxycholecalciferol. *Proc. Nat. Acad. Sci. USA* 68, 2131-2134 (1971).

- COO-1668-33 -- Omdahl, J. L., and H. F. DeLuca. Strontium Induced Rickets: Metabolic Basis. Science 174, 949-951 (1971).
- COO-1668-34 -- DeLuca, H. F. Metabolism and Mechanism of Action of 25-Hydroxycholecalciferol in "Cellular Mechanisms for Calcium Transfer and Homeostasis", G. Nichols, Jr., and R. H. Wasserman (eds.), Academic Press, New York, pp. 421-440 (1971).
- COO-1668-35 -- DeLuca, H. F. 25-Hydroxy Vitamin D: A Hormone Derived from Vitamin D in "Hormonal Steroids", V. H. T. James, and L. Martini (eds.), Excerpta Medica, Amsterdam, pp. 346-356 (1971).
- COO-1668-36 -- DeLuca, H. F., and M. J. Melancon, Jr. 25-Hydroxycholecalciferol: A Hormonal Form of Vitamin D in "Biochemical Actions of Hormones" Vol. II, G. Litwack (ed.), Academic Press, New York, pp. 337-383 (1972).
- COO-1668-37 -- DeLuca, H. F. The Functional Metabolism of Vitamin D₃ in "Calcium, Parathyroid Hormone and the Calcitonins" Proceedings of the Fourth Parathyroid Conference, Chapel Hill, N.C. (March 15-19, 1971), R. V. Talmage, and P. L. Munson (eds.), Excerpta Medica, Amsterdam, pp. 221-235 (1972).
- COO-1668-38 -- Olson, E. B., Jr., H. F. DeLuca, and J. T. Potts, Jr. The Effect of Calcitonin and Parathyroid Hormone on Calcium Transport of Isolated Intestine in "Calcium, Parathyroid Hormone and the Calcitonins" Proceedings of the Fourth Parathyroid Conference, Chapel Hill, N.C. (March 15-19, 1971), R. V. Talmage, and P. L. Munson (eds.), Excerpta Medica, Amsterdam, pp. 240-246 (1972).
- COO-1668-39 -- Olson, E. B., Jr., H. F. DeLuca, and J. T. Potts, Jr. Calcitonin Inhibition of Vitamin D-Induced Intestinal Calcium Absorption. Endocrinology 90, 151-157 (1972).
- COO-1668-40 -- Boyle, I. T., L. Miravet, R. W. Gray, M. F. Holick, and H. F. DeLuca. The Response of Intestinal Calcium Transport to 25-Hydroxy and 1,25-Dihydroxy Vitamin D in Nephrectomized Rats. Endocrinology 90, 605-608 (1972).
- COO-1668-41 -- Cousins, R. J., and H. F. DeLuca. Vitamin D and Bone in "Biochemistry and Physiology of Bone", Vol. II, 2nd ed., Chapter 7, G. H. Bourne (ed.), Academic Press, New York, pp. 281-335 (1972).
- COO-1668-42 -- Boyle, I. T., R. W. Gray, J. L. Omdahl, and H. F. DeLuca. Calcium Control of the in vivo Biosynthesis of 1,25-Dihydroxyvitamin D₃: Nicolaysen's Endogenous Factor in "Endocrinology 1971" Proceedings of the Third International Symposium (London, July 19-22, 1971), S. Taylor (ed.), Wm. Heinemann Medical Books, Ltd., pp. 468-476 (1972).

- COO-1668-43 -- Olson, E. B., Jr., and H. F. DeLuca. Vitamin D, Metabolism and Mechanism of Action in "World Review of Nutrition and Dietetics" Vol. 17, G. H. Bourne (ed.), Karger, Basel, (1972) (in press).
- COO-1668-44 -- Omdahl, J. L., and H. F. DeLuca. Rachitogenic Activity of Dietary Strontium: I. Inhibition of Intestinal Calcium Absorption and 1,25-Dihydroxycholecalciferol Synthesis. J. Biol. Chem. (1972) (in press).
- COO-1668-45 -- Gray, R. W., J. L. Omdahl, J. G. Ghazarian, and H. F. DeLuca. 25-Hydroxycholecalciferol-1-Hydroxylase: Subcellular Location and Properties. J. Biol. Chem. (1972) (in press).

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

There is now little doubt that vitamin D must first be metabolized to its 25-hydroxy derivative by the liver and finally to its 1,25-dihydroxy derivative before it can carry out the well-known role in intestinal calcium absorption (1-3). Work in this laboratory (4, 5) and in the laboratories of others (6, 7) has demonstrated that the 1,25-dihydroxyvitamin D₃ will initiate intestinal calcium absorption much more rapidly than any other form of vitamin D. In any case, there is a lag of approximately 1 hour between its intravenous administration and the initiation of intestinal calcium transport. Actinomycin D and probably puromycin when given prior to the 1,25-dihydroxyvitamin D₃ do not prevent the initiation of intestinal calcium absorption, suggesting that transcription of DNA and protein synthesis are not involved in the initial response to the 1,25-dihydroxyvitamin D₃ (8, 9). The 1 hour lag would appear is due to some other mechanism. It will be one of the purposes of the present investigation to be covered by this proposal to investigate the reason for this one hour lag period in view of the actinomycin D insensitivity.

The realization that 1,25-dihydroxyvitamin D₃ is the metabolically active form of the vitamin in the initiation of both intestinal calcium absorption (9-12) and bone calcium mobilization (12-14) opened many new possibilities regarding the investigation of intestinal calcium absorption. Besides supplying to us the form of vitamin D which acts directly on the intestine, it provided a new mechanism for regulating intestinal calcium absorption and another site whereby certain factors may interfere with intestinal calcium absorption, namely at the site of synthesis of the 1,25-dihydroxyvitamin D₃. Fraser and Kodicek (15) were the first to demonstrate that the kidney is the site of production of the 1,25-dihydroxyvitamin D₃. This was confirmed in our laboratory (16) and has been studied extensively. Of great importance is the realization that intestinal calcium absorption can be regulated by dietary calcium as first studied by Rottensten (17) and later firmly established by Nicolaysen and his coworkers (18, 19). Animals maintained on diets low in calcium show high rates of intestinal calcium absorption whereas those on high calcium diets show low rates of intestinal calcium absorption. In this laboratory it was shown that the rate of production of 1,25-dihydroxyvitamin D₃ in animals maintained on these diets correlates with intestinal calcium absorption (20, 21). That is, animals maintained on low calcium diets show rapid production of 1,25-dihydroxyvitamin D₃ whereas animals maintained on high calcium diets show a low rate of conversion of 25-hydroxyvitamin D₃ to the 1,25-dihydroxy derivative. Furthermore, it can now be shown that animals maintained on 1,25-dihydroxyvitamin D₃ as their source of the vitamin do not show the ability to adapt to dietary calcium, whereas animals maintained on 25-hydroxyvitamin D₃ still retain that capacity (J. L. Omdahl and H. F. DeLuca, unpublished results). It has also been shown that strontium feeding blocks the conversion of 25-hydroxyvitamin D₃ to the 1,25-dihydroxy derivative and results in low rates of intestinal calcium absorption which can be overcome by administration of exogenous 1,25-dihydroxyvitamin D₃ (22, 23). Similar results have been obtained with barium and very recently it has been demonstrated that the phosphonates which inhibit calcification (24) also bring about

a reduction in the production of 1,25-dihydroxyvitamin D₃ resulting in a low rate of intestinal calcium absorption. Again this can be corrected by an exogenous supply of 1,25-dihydroxyvitamin D₃ (L. Baxter, P. Bonjour, H. Fleisch, and H. F. DeLuca, unpublished results).

There are a number of other agents which are known to inhibit intestinal calcium absorption. For example, cortisone has been shown by a wide variety of investigators to inhibit intestinal calcium absorption (25, 26). Recently phenobarbital and dilantin likewise upon prolonged administration are known to inhibit intestinal calcium absorption (27, 28). Estrogens and other hormones also are known to influence this important process. During the course of the next three years we propose to investigate whether these agents regulate or affect intestinal calcium absorption by inhibiting or modifying both the 25-hydroxylation of vitamin D, which occurs in the liver, or the 1-hydroxylation, which occurs in the kidney. If evidence is found for this then the rate of intestinal calcium absorption will be studied in animals treated with the particular agent but given exogenous supplies of 1,25-dihydroxyvitamin D₃. In such a fashion the agents which have been suggested to work directly on intestine can be separated from those which work indirectly by affecting the metabolism of vitamin D to the terminal functionally active form of the vitamin or 1,25-dihydroxyvitamin D₃.

The mechanism of calcium transport by the intestine received its great initial push at the hands of Schachter and his coworkers (29-31), who first used the everted gut sac technique to study this transport process. Schachter and his coworkers, Harrison and Harrison (25, 32-35), and Wasserman and his colleagues (36-39) were responsible for the demonstration that calcium is transported actively against a concentration gradient and that metabolic energy is required. Other groups, including our own, have provided additional evidence that the transport process is cation oriented and occurs against an electrochemical potential gradient with phosphate as the normal accompanying anion (40-43). Schachter and his colleagues (31) have provided evidence that vitamin D affects both the entry of calcium across the brush border surface to the intestinal cell and the exit of calcium from the absorption cell across the lateral-basal membrane. Work in this laboratory has suggested that the primary site of vitamin D action is the initial uptake of calcium from the brush border surface (41). Both Harrison and Harrison (33) and our own laboratory (40) have established that sodium is required for calcium to be extruded from the absorption cell into the serosal fluid. Adams and Norman (42) have studied very thoroughly the intestinal calcium absorption mechanism using the short-circuit method originally described for sodium transport by Ussing and have provided additional strong evidence that calcium is transported against an electrical as well as a concentration gradient. They have also used the polyene antibiotic filipin to show that vitamin D-deficient intestine will transport calcium at rates equal to that of their vitamin D sufficient counterparts by the addition of the polyene antibiotic which apparently rearranges the lipoprotein brush border membrane permitting calcium to enter (43). This suggests that the primary function of vitamin D is at the brush border surface as well. Thus it is evident that there is uniform agreement that vitamin D somehow affects the initial entry of calcium into the mucosal cell and that sodium is required for the exit of

calcium at the serosal surface. The exact mechanism whereby calcium is taken up by the mucosal cells, carried across the cell and extruded at the basal/lateral membrane remains relatively unknown.

Wasserman and his colleagues carried out pioneering investigations resulting in the demonstration of the existence of a calcium binding protein in the mucosa of animals given vitamin D (44-47). Wasserman and his colleagues have further provided evidence that the rate of calcium absorption correlates under a variety of circumstances with the level of calcium binding protein in the intestinal mucosa (48-52). They have isolated the protein from chicks in pure form, prepared fluorescent antibodies to it and demonstrated that the probable site of its production is the goblet cell (53). Its primary location in the absorption cells appears to be on the surface of the brush borders (53). The mere fact that calcium binding protein is present following vitamin D and that vitamin D stimulates intestinal calcium absorption and that this protein has a high affinity for calcium makes it almost certain to be a participant in the calcium transport process. Exactly how it participates, however, remains to be determined.

In this laboratory following the work of Martin under a previous portion of this AEC contract, evidence was provided that vitamin D must stimulate the rate of transfer of calcium across the brush border surface by an energy dependent process (40). We, therefore, looked for a calcium dependent adenosine triphosphatase in the brush border which would appear in response to vitamin D. This proved to be successful and a calcium dependent ATPase appeared in the mucosa or brush borders of both rats and chicks following vitamin D (54, 55). The correlation of its appearance appeared to be approximately as good as that of the calcium binding protein. Work in Rasmussen's laboratory has suggested that this protein system is identical with or at least related to the well-known alkaline phosphatase of the brush border which has long been known to be stimulated by vitamin D (56).

With the $1\alpha,25$ -dihydroxyvitamin D_3 in this laboratory the following appears to be true. There is a poor correlation between both the calcium dependent ATPase and the calcium binding protein appearance in the mucosa and the initiation of intestinal calcium absorption. Furthermore, calcium absorption drops rather rapidly after peaking in response to the $1,25$ -dihydroxyvitamin D_3 , whereas the calcium binding protein level remains rather high. It therefore appears that some other factor must play a role in the initial events resulting in intestinal calcium absorption following the $1,25$ -dihydroxyvitamin D administration. We have recently found a new protein in the brush borders of intestine which appears immediately following the $1,25$ -dihydroxyvitamin D. Its appearance correlates exactly with the increased calcium transport reaction. Its molecular weight is of the order of 200,000 and is currently being isolated in pure form for study of its physical characteristics, as well as its potential catalytic effects. It may well represent a complex between the calcium adenosine triphosphatase and the calcium binding protein. Only continued investigation will be able to determine this. This protein as well as the calcium binding protein appears to be formed from preexisting proteins.

Evidence for a precursor protein for the calcium binding protein in rats was first obtained by Dr. D. Drescher of this laboratory under the current AEC contract (57, 58). Evidence is now being obtained for the existence of a similar precursor protein for the chick calcium binding protein. In addition, there is a precursor protein for the large molecular weight protein of brush border which appears following vitamin D₃. If these represent true precursor proteins this could well explain the observation that actinomycin D does not block the responses of intestine to 1,25-dihydroxyvitamin D₃. Work will therefore continue in this general direction in an attempt to delineate the component of the calcium transport system especially at the brush border surface. It would be our intention to isolate the calcium binding protein, its precursor protein and the large molecular weight brush border protein as well as its precursor protein in pure form, prepare antibodies including fluorescent antibodies of them so that we may study their cellular location and in addition set up immunodiffusion assays for each of the proteins. We will also continue to search for the appearance of other proteins in various cell fractions in response to the 1,25-dihydroxyvitamin D₃. Once the proteins are obtained in pure form, attempts to detect calcium binding properties, ATPase activities and alkaline phosphatase activities will be made. Attempts to incorporate these proteins into synthetic membranes will be made in an attempt to obtain evidence for their function in transport if possible.

Because of our continuing investigation of vitamin D metabolism and analogs of vitamin D, an important area of investigation is the testing of these agents on the intestinal calcium transport system. Of special interest may be the synthesis of an analog of vitamin D which may prove to be an antivitamin for the 1,25-dihydroxyvitamin D₃ and its function in the intestine. For this reason, as has been described for the trans vitamin D compounds we would like to continue to test these analogs in the intestinal calcium transport system with the hope of obtaining both an antivitamin D and a form of vitamin D which would stimulate only intestine and not bone or kidney. Our NIH grant will be used primarily to support work on the metabolism of vitamin D and synthesis of the vitamin D analogs.

If sufficient funding is possible we would like to continue work on our vascularly perfused intestine, which has been developed by the work of Dr. E. B. Olson, Jr. under the current AEC contract. At the present time we have no one to carry out these studies since the current AEC contract monies are being relegated to a study of the mechanism of intestinal calcium transport and factors which regulate 1,25-dihydroxyvitamin D₃ synthesis. The vascularly perfused intestine provides a unique opportunity to study the direct action of hormones, antivitamins and analogs of vitamin D on intestine. With the use of this tool we have been able to demonstrate that calcitonin inhibits directly intestinal calcium absorption (59). We have also provided some evidence that parathyroid hormone can directly stimulate intestinal calcium absorption. Furthermore, using this system we have been able to demonstrate directly the effect of less than 6.5 pmoles of the 1,25-dihydroxyvitamin D₃ on the intestine without participation of other organs. We would like to use the vascularly perfused intestine to continue studying the direct effect of the metabolites and analogs of vitamin D on the intestinal calcium transport system again with the hope of using it to identify antimetabolites of vitamin D and biologically active synthetic analogs of the vitamin. In addition,

we would like to continue studying the effect of various hormones directly on the intestinal calcium transport. Of special interest are the effects of cortisol, cortisone, estrogens and thyroxin. We would like also to examine the question of whether the intestine will respond to variation in various ions including ions known to produce vitamin D-resistant rickets such as barium, beryllium and strontium. Although the evidence suggests that these agents act by inhibiting the production of $1\alpha,25$ -dihydroxyvitamin D_3 it would be of great interest to study whether they have in addition a direct effect on intestine itself. Vascularly perfused intestine has the great advantage over the intestinal sac technique of allowing the intestine to be viable for up to 8 hours. In addition it by-passes the important criticism of the gut sac system that calcium must after being absorbed by the absorption cell diffuse through many layers of submucosa, lamina propria, and muscle before it appears in the serosal fluid. Thus the vascularly perfused intestine, although an in vitro system, more closely approaches the in vivo situation. We would like to apply filipin and perhaps Amphotericin B, the polyene antibiotic used by many investigators for the study of sodium and potassium transport across toad bladder in the vascularly perfused intestine.

One of the overall aims of the proposed AEC contract for the next three years is to help delineate insofar as possible those factors which affect intestinal calcium absorption by affecting the synthesis of $1\alpha,25$ -dihydroxyvitamin D_3 in the kidney. Those factors which affect calcium transport directly can therefore be separated from those which affect calcium transport in the intestine by such a systemic mechanism. This will be coupled with the vascularly perfused intestinal system which will help describe more completely the site at which these agents act. Furthermore, the vascularly perfused intestine will allow a more controlled in vitro system which more closely approaches the in vivo situation than does the intestinal sac technique.

Perhaps the most central aim of this proposal is to try to determine the molecular events which are involved in the transfer of calcium from the lumen of intestine to the serosal fluid in response to vitamin D. This will be greatly aided by the availability of chemically synthesized $1\alpha,25$ -dihydroxyvitamin D_3 . Calcium binding protein and a proposed precursor protein will be isolated in pure form from both rats and chicks, studied physically and used to prepare antibodies for immunoassay and for study of possible conversion under the influence of $1,25$ -dihydroxyvitamin D_3 to the calcium binding protein. The new high molecular weight protein just discovered in the brush borders of animals given $1,25$ -dihydroxyvitamin D_3 will be isolated in pure form as will the protein which appears to be its precursor protein. If it can be obtained in an undenatured form, its molecular weight and other physical characteristics will be determined. Its ability to bind calcium, its lipid content, its phosphatase and calcium ATPase activities will be measured. Finally, fluorescent antibodies will be prepared of this protein and its precursor protein and attempts will be made to delineate its cellular site of formation and its cellular location during its potential function as a calcium transport agent. Attempts will be made to correlate its appearance and its quantity with the appearance of calcium absorption in response to vitamin D.

Methods of Procedure

1. Regulation of 1,25-Dihydroxycholecalciferol Synthesis

We have already established that the parathyroid hormone is responsible for regulating the synthesis of 1,25-dihydroxyvitamin D₃ (60). Since it is known from the work of Chase and Aurbach (61, 62) and Wells and Lloyd (63) that the parathyroid hormone activates adenyl cyclase of the kidney to produce cyclic AMP, it is generally believed that this second messenger then initiates the known function of the parathyroid hormone. Rasmussen and his colleagues have reported that cyclic AMP added to renal tubules mimics the effect of parathyroid hormone in stimulating 1,25-dihydroxyvitamin D₃ synthesis (64). Infusion of dibutyl cyclic AMP has on preliminary attempt in our hands been unsuccessful in terms of stimulating synthesis of 1,25-dihydroxyvitamin D₃. We would, therefore, like to give vitamin D-deficient animals a daily injection of 65 pmoles of 1,25-dihydroxyvitamin D₃, thyroparathyroidectomize them and 48 hours later, when their ability to make 1,25-dihydroxyvitamin D₃ has decreased to essentially zero, we would like to infuse dibutyl cyclic AMP every 6 hours. At the end of 12 hours of this procedure the animals will receive an intravenous injection of radioactive 25-hydroxyvitamin D₃. Twelve hours later with continued treatment with cyclic AMP the animals will be killed ^{and} the tissues which will include blood, kidney and intestine will be extracted and chromatographed on Sephadex LH-20 to reveal the in vivo synthesis of the 1,25-dihydroxyvitamin D₃. Control animals will receive only the vehicle in which the cyclic AMP is injected. This will demonstrate whether cyclic AMP is responsible for the parathyroid hormone's action.

It has been demonstrated in this laboratory that animals on a high calcium low phosphorus diet have a hypercalcemia and supposedly suppressed parathyroid secretion. However, these animals in the absence of parathyroid hormone still synthesize large quantities of 1,25-dihydroxyvitamin D₃. Furthermore, thyroparathyroidectomy of such animals does not decrease the animals ability to make 1,25-dihydroxyvitamin D₃. We believe that low plasma phosphorus which results from this diet is actually the trigger for 1-hydroxylation mechanism. It is also possible that the parathyroid hormone may bring about increased 1,25-dihydroxyvitamin D₃ synthesis by merely depleting cellular phosphate of the kidneys providing essentially a low tissue level of phosphorus. Thus low cellular phosphorus either by high calcium-low phosphorus diets or by parathyroid hormone injection may be the basic cause for production of 1,25-dihydroxyvitamin D₃. In the thyrparathyroidectomized rat on a high calcium diet and by varying dietary phosphorus or by injecting phosphorus we would like to regulate the plasma level of phosphorus and then determine these animal's ability to make 1,25-dihydroxyvitamin D₃. We would also like to measure kidney tissue inorganic phosphate by the method of Chen et al. (65) in animals given parathyroid hormone or by other manipulations to determine if we can correlate cellular inorganic phosphorus and the ability of the animal to produce 1,25-dihydroxyvitamin D₃. Finally, we would like to isolate tubules by the method of Berg and Orloff (66) and determine if by changing the phosphorus concentration of the medium we can alter the ability of the tubule cells to make 1,25-dihydroxyvitamin D₃. We would like also to determine if parathyroid hormone added to tubule suspension will decrease the cellular level of phosphate which could account for the increased 1,25-dihydroxyvitamin D₃ synthesis in that system.

Besides the above studies which will be aimed at determining the true endogenous factors that are responsible for regulating or stimulating the synthesis of 1,25-dihydroxyvitamin D₃ we would like also to determine whether such agents as barium, beryllium, strontium, cortisone, and estrogens which are known to affect intestinal calcium absorption do so by affecting the synthesis of 1,25-dihydroxyvitamin D₃. These experiments will essentially be carried out in the following manner. In either adrenalectomized, vitamin D-deficient animals maintained on saline water or the appropriate animal in which the endocrine organ is removed, the appropriate hormone will be replaced in part of the group by an exogenous supply. Thus in a typical experiment we would have adrenalectomized animals, adrenalectomized animals who are given cortisol, intact animals, and intact animals given cortisol. Some of the animals will be used to measure intestinal calcium absorption or transport by the everted sac technique to establish the inhibitory action of cortisol as has been reported by Harrison and Harrison (25) and by Kimberg (26). The remaining animals will be given an injection of radioactive 25-hydroxyvitamin D₃ and 6-12 hours later they will be killed, their tissues removed, extracted and chromatographed on Sephadex LH-20 columns to reveal the production of 1,25-dihydroxyvitamin D₃. Finally if changes are found, animals will be fed or treated in the same way except some of the animals will be given exogenous 1,25-dihydroxyvitamin D₃ to determine if the inhibitory action of the particular agent on intestinal calcium absorption can be overcome by an exogenous supply of 1,25-dihydroxyvitamin D₃. These experiments would be repeated for ovariectomized animals in the case of estrogens. In the case of barium and beryllium the experiments will be carried out similar to those we have described for strontium (22, 23).

In addition to the possible block at the 1-hydroxylation stage we would like to examine the question of whether some of the agents which might inhibit intestinal calcium absorption might block hydroxylation of vitamin D at the 25 position which is the initial functional metabolic step involving vitamin D. This would especially be the case for aflatoxin, a toxin which is known to destroy certain liver cells. It is likely that the cells that are destroyed by this toxin are those which synthesize 25-hydroxyvitamin D₃. Furthermore, there is evidence that the dilantin or the phenobarbital anticonvulsant drugs interfere with vitamin D metabolism in the liver. In each case we would like to give animals a small amount of vitamin D plus their respective diet to which is added either the barium, the strontium, the beryllium, aflatoxin, barbiturates, or dilantin. The treatment will continue in half the animals while the other half will receive diets without the compound in question. At various times after the feeding has begun the animals will be sacrificed in both groups to determine their intestinal calcium absorption. At the time the intestinal calcium absorption becomes depressed the following experiments will be done. One-half the animals will be used for intestinal calcium absorption tests, serum calcium measurements and serum phosphorus measurements. The other half of the animals will receive intravenously an injection of radioactive 25-hydroxyvitamin D₃ and 12 hours later they will be killed, and their tissues extracted and chromatographed on Sephadex LH-20 to reveal the amount of 1,25-dihydroxyvitamin D₃ made. Again the block in intestinal calcium absorption which may result from the feeding of one of these agents will be examined for their responsiveness to 1,25-dihydroxyvitamin D₃ given intravenously or 25-hydroxyvitamin D₃ given intravenously. In the case of the liver toxins it is likely that the 25-hydroxyvitamin D₃ will overcome the repressed intestinal calcium absorption.

2. Mechanism of Calcium Transport in the Intestine

Using 1,25-dihydroxyvitamin D₃, the time course of appearance of the calcium dependent ATPase, calcium binding protein (by immunoassay), alkaline phosphatase of the brush border and the new high molecular weight brush border protein will be studied. In addition, the decrease of these parameters in relation to the decrease in intestinal calcium absorption after a single pulse dose of 1,25-dihydroxyvitamin D₃ will be examined. These experiments will be carried out in both rats and chicks. Furthermore, the calcium uptake by brush borders as described by Martin and DeLuca (41) will be examined in these animals given 1,25-dihydroxyvitamin D₃. This study may allow a decision to be made regarding which component or components which appear in response to 1,25-dihydroxyvitamin D₃ is responsible for calcium transport.

To determine if vitamin D plays a role in the extrusion of calcium from the basal lateral membrane, intestines from both vitamin D-deficient animals and animals given 25-hydroxyvitamin D₃ will be incubated in a medium deprived of sodium. This prevents the extrusion of calcium into the serosal fluid. We could then measure the calcium uptake from the brush border surface and demonstrate presumably the effect of the 1,25-dihydroxyvitamin D₃ thereon. After about 1/2 hour of incubation the cells should be loaded with calcium 45 and then sodium ions will be added to the incubation medium of the intestinal tissue from both groups. The rate of extrusion of calcium from the intestinal cells to the serosal medium will be measured to determine if vitamin D has an effect on the extrusion of calcium across the lateral-basal membrane.

Calcium binding protein from both chick and rats will be isolated in pure form by procedures described either by Wasserman and his colleagues or in the case of rats by our own research group. We have begun attempting to make antibodies to both proteins so that we can set up an immunoassay for these proteins to aid our study of the time course of their appearance in response to the 1,25-dihydroxyvitamin D₃. We are also isolating in each case a possible precursor protein which decreases in amount in response to the 1,25-dihydroxyvitamin D₃ at the same time that the calcium binding protein is increased. We plan to isolate these precursor proteins in pure form, measure their molecular weight both by ultracentrifugation and gel filtration. We plan also to carry out an amino acid residue analysis as compared to the respective calcium binding protein. We also plan to prepare fluorescent antibodies to them to determine their cellular location. We will attempt with the precursor proteins by means of incubating them with tissue homogenates in the presence of 1,25-dihydroxyvitamin D₃ to bring about the conversion to the calcium binding protein.

In addition to the calcium binding protein and their precursor proteins, we would like to continue our work on the isolation of a large molecular weight protein found in the brush border of chicks in response to the 1,25-dihydroxyvitamin D₃. This protein is about 200,000 molecular weight, is distinguishable on disc gel

electrophoresis carried out in sodium dodecyl sulfate. We are currently isolating this protein in quantity so that we may carry out the usual physical measurements to determine molecular weight and molecular architecture. We would like to determine its lipid content, whether it contains $1\alpha,25$ -dihydroxyvitamin D_3 , whether it contains calcium binding protein and whether it has calcium ATPase activity. If we can maintain it in an undenatured state we would like to add it to synthetic membranes to determine whether it can function in a transfer capacity.

3. Continued Evaluation of Vitamin D Metabolites, Analogs and Antivitamins for Their Effect in Intestinal Calcium Transport

We are currently synthesizing a wide variety of analogs to the $1,25$ -dihydroxyvitamin D_3 . A number of isomers of $1\alpha,25$ -dihydroxyvitamin D_3 such as the 3α isomer, and 1β isomer are currently undergoing synthesis. Furthermore, we have plans to make the 3-deoxy-1-hydroxy vitamin D analog. We have plans to make a 1-methoxy-25-hydroxy vitamin D_3 , which we feel might be an antivitamin. We would like to evaluate each of these by the intestinal calcium transport measurement in the rat as described previously and by means of the intestinal calcium absorption measurement in chickens as described previously. In addition we would like to test the inactive compounds for their potential use as an antivitamin D. We would like to administer it prior to and at the same time as the $1,25$ -dihydroxyvitamin D_3 to intact animals and in the vascularly perfused intestine to determine if these compounds can prevent the action of $1,25$ -dihydroxyvitamin D_3 on the intestine. If such a compound is found by such a test, it will be tested more systemically to determine what functions of vitamin D can be blocked by such an antimetabolite.

If funding permits we should like to acquire a postdoctoral level person who can continue work on the vascularly perfused intestine. We would like first of all to test the metabolites and analogs of vitamin D in the vascularly perfused intestine and determine their activities or possible antivitamin D activity. In addition, we would like to determine whether there is a direct effect on various hormones such as cortisol, estrogens, etc. on the intestinal calcium transport process. Once an action of the hormone is found the nature of its action can be studied more thoroughly.

Finally, in the vascularly perfused intestine we would like to determine whether ethaquinic acid, which Avioli and his associates (67) have shown in rats to inhibit sodium-potassium ATPase at the lateral basal membrane, can inhibit directly intestinal calcium transport. In addition, we would like to study the effect of the polyene antibiotics on intestinal calcium absorption of both vitamin D-deficient animals and animals given the $1,25$ -dihydroxyvitamin D_3 . We would, however, make the vascularly perfused intestine our lowest priority project.

Summary

The ultimate aim of this proposal is to determine insofar as techniques will allow the mechanism whereby a wide variety of agents affect intestinal calcium absorption. In particular we wish to segregate out those substances which affect intestinal calcium absorption by interfering with vitamin D metabolism from those which act directly on the intestine. To do this we wish to do both in vivo studies and vascularly perfused intestinal studies. We wish also to study the metabolism of both radioactive vitamin D₃ and radioactive 25-hydroxyvitamin D₃ under conditions where agents are known to inhibit intestinal calcium absorption.

Major emphasis will be placed on our continued investigation of the intestinal calcium transport mechanism with the aim of explaining the transfer of calcium across intestinal cells on a molecular basis. Of particular interest will be the brush border proteins and lipids which are believed to play a role in vitamin D-stimulated intestinal calcium absorption. Possible precursor proteins to the calcium binding protein will be isolated, their amino acid residues will be determined as well as their physical characteristics. Fluorescent antibodies to each will be prepared to determine their cellular location. They will be used in incubation experiments to determine if in the presence of 1,25-dihydroxyvitamin D₃ they can be converted to the calcium binding protein. A new protein of high molecular weight found in the brush border in response to vitamin D will be isolated in pure form and its catalytic activities as well as physical properties determined. The correlation of its appearance with the 1,25-dihydroxyvitamin D₃-induced calcium absorption will be studied. Attempts will be made to determine its role as well as the role of calcium binding protein and calcium dependent ATPase in the calcium transport system.

Work will continue on the evaluation of the effect of synthetic vitamin D analogs and possible antivitamin D's on the intestinal calcium transport system. Of special interest will be the detection of an antivitamin D metabolite which would prevent the action of 1 α ,25-dihydroxyvitamin D₃ on the intestine, both in vivo and in vascularly perfused intestine.

Significance

The need of all terrestrial animals for efficient intestinal calcium absorption is clear. They exist in a relatively calcium-poor environment, but require large amounts of this important cation for many functions throughout the body, including the structural role of bone, neuromuscular activity, clotting of blood, nerve transmission, muscle contraction, enzymatic activities, cell adhesion, and cell permeability. In addition the regulation of this important cation must be regarded as one of the most important and tightly controlled systems in biology. Certainly a clear understanding of the mechanisms of calcium absorption would contribute greatly to our understanding of these important functions. In addition, an understanding of the regulation of calcium metabolism is not only extremely important to biology but must be regarded as a major system in medicine.

The importance of these investigations to medicine are more easily defined. It is known for example that patients suffering from chronic renal disease develop a severe deficiency in calcium absorption, a secondary hyperparathyroidism and severe bone disease. The most recent AEC contract awarded our laboratory played some role in delineating the role of the kidney in the regulation of intestinal calcium absorption. It now appears likely that the $1\alpha,25$ -dihydroxy-vitamin D_3 is not made in chronic renal disease which results in a failure of intestinal calcium absorption, secondary hyperparathyroidism, osteomalacia, and osteitis fibrosa cystica. A number of vitamin D-resistant diseases are known and a number of diseases in which there is poor calcium absorption are also well recognized. Many of these patients cannot survive until calcium absorption is restored to reasonable levels. An understanding of the factors which regulate intestinal calcium absorption and which play an essential role in this important transfer would have great significance in the treatment of such diseases.

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