Final Progress Report for National Aeronautics &
Space Administration Contract NAS 9-11011
"The Control of Calcium Metabolism by Parathyroid
Hormone, Calcitonin and Vitamin D"

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Harvard Medical School
Endocrine Unit
Massachusetts General Hospital
Boston, Massachusetts

Principal Investigator: John T. Potts, Jr., M.D.

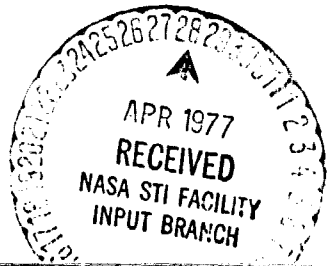
The NASA-supported research efforts of the Endocrine Unit Massachusetts General Hospital are based on fundamental advances in analysis of chemistry and physiology of parathyroid hormone, calcitonin and Vitamin D as well as in evolution of newer techniques in radioassay methods, in order to develop assays to permit assessment of normal and abnormal patterns of secretion of these hormones in specific relation to the physiological adaptations of weightlessness and space flight as well as in related diseases that involve perturbations in normal skeletal and calcium homeostasis.

This progress report lists: I) a description of basic advances in chemistry, especially synthesis, of parathyroid hormone; II) studies of biosynthesis, secretion and metabolism of parathyroid hormone and calcitonin with special emphasis on pertinent information concerning the complex, heterogeneous nature of circulating forms of hormone in blood which interfere with measurement of active hormone and newer assay methods developed to circumvent these difficulties; III) studies in the photobiology, metabolism and assay of vitamin D and its metabolites essential to understanding the consequences of environmental light exposure and the regulation of vitamin D action on skeletal mineral.

I. Chemical Studies of the Polypeptide Hormones

A. Sequence Analysis

Newer methods permit accurate sequence analysis to be carried out on extremely small amounts of material, an essential requirement for work with, for example, human parathyroid hormone which is available in only very limited quantities. Use of radioisotopes to increase sensitivity has proven effective; a) labeling of histidine and tyrosine residues with radioactive iodine. This permits identification of these residues in picomole quantities of peptide. The procedure has proven useful in the characterization, by Edman degradation, of parathyroid peptide fragments in plasma using the labeled tyrosine residue at position 43 as a marker; b) internal labeling of parathyroid peptides by incubation with tissue slices <u>in vitro</u>. The appropriate ^{14}C and/or ^{3}H-labeled amino acids are introduced into the tissue incubation medium, after which the peptides are purified by standard techniques in presence of carrier hormone to improve recoveries of labeled hormone. The labeled amino acids are identified during sequence analysis by detection of radioactivity in the phenylthiohydantoin derivative. This method was employed in the sequence analysis of human parathyroid hormone, recently completed in our laboratory, and in the structural analysis of the ribosomal precursor form (pre-proparathyroid hormone). Figure 1.



Previous work in this laboratory has established the amino-acid sequence of parathyroid hormone from the bovine and the porcine species. More recent work has been directed toward completion of the sequence of the human hormone. Since the hormone must be extracted from pooled adenoma tissue removed at surgery, the quantities of material available for structural work is extremely small, placing a particular premium on the high sensitivity methodology just described. To date we have established 80 out of the 84 sequence positions in the molecule. Figure 2.

B. Synthesis

Polypeptides varying in length from six to forty amino acids have been synthesized in our laboratory employing the solid-phase technique. Rigorous monitoring of syntheses as they progress, as well as the sequential application of several purification modalities has made solid-phase synthesis an extremely valuable and reliable tool for endocrinological investigation.

Two broad areas have been the subject of this laboratory's research: (1) the synthesis of fragments of the natural sequence of parathyroid hormone, and (2) the synthesis of analogues of parathyroid hormone containing structural alterations, such as amino acid substitutions with naturally, modified, and non-naturally occurring amino acids. The latter category has made possible extensive structure-function studies as well as providing information about the structural requirements for inhibition or antagonism of parathyroid hormone.

Previous research by this laboratory defined the minimum sequence length of parathyroid hormone, an 84-amino-acid polypeptide, necessary for biological activity (as demonstrated in the in vitro renal adenylyl cyclase assay). This region comprised the NH_2-terminal amino acids 2 through 27. The amino-terminal sequence 1-34 contains all the requirements for biological activity equal to that of the native hormone. Because the synthesis of the 34-amino-acid, fully-active fragment of parathyroid hormone can be readily performed by solid-phase procedure, it may have important clinical and therapeutic uses. Current studies performed in collaboration with Dr. John Parsons and colleagues at the Medical Research Council in Great Britain suggest a possible beneficial role of parathyroid hormone in the treatment of osteoporosis. Because native human parathyroid hormone is available in only minute quantities, these studies must rely on synthetic hormone. Obviously, the design and synthesis of super-potent analogues of parathyroid hormone would be of great advantage.

An analogue of bovine parathyroid hormone containing two structural alterations at the carboxy terminus of the 1-34 fragment was synthesized. Tyrosine was substituted for phenylalanine at position 34, and the carboxy-terminal carboxylic acid group was changed to the amide function to yield {Tyr34}bPTH(1-34 amide). The activity of the analogue is nearly twice that of the natural sequence, bPTH (1-34). Whether the increased potency is due to facilitated receptor binding or increased resistance to degradation remains to be established.

Previous studies in this laboratory have also defined some of the structural requirements for inhibition of parathyroid hormone. The sequence 3-34 lacks the amino-terminal residues required for biological activity in the renal adenylyl cyclase assay, yet it acts as a competitive inhibitor of hormone action. Hence, a dichotomy between receptor binding and adenylyl cyclase activation was demonstrated by deletion of only two NH_2-terminal amino acids. Suspecting that super-

potent analogues of parathyroid hormone might provide the structural features necessary for "super-potent" antagonists of hormone action, an analogue containing the C-terminal tyrosine-amide substitution as well as the NH_2-terminal amino-acid deletions, {Tyr^{34}}bPTH (3-34 amide), was synthesized. Similar analogues lacking methionines at positions 8 and 18, and hence sulfur-free, {Nle^{8}, Nle^{18}, Tyr^{34}}bPTH(3-34)and {Nle^{8}, Nle^{18}, Tyr^{34}}bPTH (3-34 amide) were also prepared. These analogues are currently being evaluated. Powerful hormone antagonist properties of these compounds have been confirmed in the in vitro adenylyl cyclase assay.

Studies have been undertaken toward the development of a membrane-receptor binding assay, based on the binding of radiolabeled parathyroid hormone to receptor sites in renal cortical membranes. This approach necessitated the design of an analogue of parathyroid hormone resistant to oxidation and containing a radioiodine attachment site outside the sequence region 2-27 known to be critical for biological activity. Bovine parathyroid hormone contains two methionine residues, one at position 8 and one at position 18. Oxidation of the 84-amino-acid parathyroid hormone molecule or the active fragment results in dramatic loss of biological activity because of the formation of sulfoxide and sulfone groups at the methionine residues, which presumably interferes with receptor binding. Since oxidation is inherent in conventional iodination techniques, radioiodination of parathyroid hormone to high specific activity to produce a biologically active tracer suitable for receptor binding studies is virtually impossible. An analogue of the 1-34 active fragment of the molecule was designed containing isosteric substitution of methionine with norleucine at positions 8 and 18, and with tyrosine substitution for phenylalanine at position 34, in order to direct iodine attachment to the C terminus. The analogue {Nle^{8}, Nle^{18},Tyr^{34}}bPTH(1-34) was found to be 76% as potent as the fully active fragment bPTH(1-34). After iodination of 90% of the tyrosine residues, there was no decline in biological activity. Besides providing a suitable analogue to serve as a tracer for membrane binding studies, this derivative demonstrates that methionine is not essential for biological activity, although oxidation of methionine drastically impairs biological activity.

However, a suitable preparation of membrane-receptors has not yet been satisfactorily accomplished. Rat renal cortical membranes contain large numbers of low affinity, high capacity sites, as well as high degradative capabilities. Further work with membranes of other species and other purification techniques for isolation of receptors is underway. Particularly promising are our initial studies with high purified bovine renal cortical membranes.

Synthetic polypeptides representing specific regions of the hormone have been prepared by the solid-phase procedure. The C-terminal fragment of bovine parathyroid hormone, bPTH(53-84) has been synthesized. This peptide will also be evaluated for bioactivity or antagonist activity in multiple systems, and will be used for immunization purposes. The synthesis of a hexapeptide representing the unique sequence of proparathyroid hormone (-6-(-)1) has been achieved. The role of this highly basic hexapeptide and its degradative metabolites in the regulation of parathyroid hormone biosynthesis and secretion is under study. Recently, we have also completed the synthesis of the preproparathyroid hormone specific peptide of 25 amino acids. The generation of antibodies specific to the preproparathyroid hormone, prohormone, NH_2-terminal, and C-terminal regions of the molecule

have now been accomplished or are in progress. These newer assays based on antibodies directed against selected portions of the hormone and its precursors and their application to improved methods of detection of normal and abnormal states of parathyroid hormone secretion are discussed in Section II below.

II. Biosynthesis, Metabolism, Control of Secretion of Parathyroid Hormone and Calcitonin: Application of the Radioimmunoassay to Clinical Disorders of Parathyroid and C-cell Function

During the past year we have made substantial progress in our studies of the biochemical mechanisms and the controlling factors involved in the biosynthesis, secretion and metabolism of parathyroid hormone. Further studies of the pre-proparathyroid hormone, the major product that we have identified, of the translation of parathyroid messenger RNA in cell-free systems, indicate that the pre-hormone is the initial protein-product of the gene for parathyroid hormone. Proparathyroid hormone, the biosynthetic precursor identified by our group previously, is now recognized to be an intermediate between pre-proparathyroid hormone and parathyroid hormone. Investigations have continued on the nature and origin of the circulating peptide fragments of parathyroid hormone, and new insight has been gained regarding the role of the kidney in the metabolism of the hormone. Further refinements and developments have been made in the radioimmunoassays for the parathyroid hormone and calcitonin. A sensitive assay has been developed for the detection of human proparathyroid hormone, for the prohormone-specific hexapeptide, and also for the biologically active amino-terminal sequence of human parathyroid hormone. The immunoassay for calcitonin has been improved and applied to the measurement of calcitonin in the blood of patients with thyroid and nonthyroid (lung) malignancies. These studies have led to a deeper understanding of the processes responsible for and the diagnosis of clinical disorders of parathyroid and C-cell hyperfunction in man through more critical interpretation of radioimmunoassay measurements.

A. Radioimmunoassay of Human Proparathyroid Hormone: Analysis of Hormone Content in Tissue Extracts and in Plasma

We have developed a radioimmunoassay for human proparathyroid hormone (hProPTH) and have applied it to an evaluation of prohormone content in parathyroid tissues and in plasma. Antisera were produced in rabbits by immunization with a synthetic octadecapeptide fragment of bovine ProPTH (bProPTH). A synthetic analogue of the prohormone peptide fragment containing tyrosine was used as the radioiodinated tracer, and a synthetic prohormone peptide consisting of the first 40 amino acids of hProPTH was used as the assay standard. The immunological activity of synthetic hProPTH peptide was unstable in plasma and buffers containing plasma or serum, but degradation was prevented by using heat-inactivated serum and EDTA in the assay solutions. The sensitivity of the assay for detection of hProPTH peptides (0.2 ng) was 5,000-times greater than for the detection of cross-reacting parathyroid hormone (PTH) peptides (1 µg), and the prohormone-specific hexapeptide did not react in the assay. The amounts of ProPTH in extracts of freshly-collected human parathyroid adenomas and in extracts of bovine parathyroid glands were 0.35 $\pm$ 0.13 and 0.38 $\pm$ 0.13 ug/mg protein, respectively, whereas the amounts of ProPTH in extracts of adenomas stored for up to a year before extraction and in extracts of human parathyroid glands obtained at autopsy were approximately ten-times less than in freshly-collected and extracted tissues. PTH in parathyroid adenomas (0.32 $\pm$ 0.14 ug/mg tissue protein) was much less than it was in bovine parathyroid glands (4.96 $\pm$ 1.34); in parathyroid adenomas, ProPTH contributes 50% of total immunoreactive glandular hormone compared with 7% in bovine parathyroids. No prohormone was detected in

parathyroid venous effluent blood (<2.0 ng/ml) under conditions in which ProPTH was shown to remain immunologically stable. PTH levels in effluents were estimated separately and found to be as high as 190 ng/ml. Thus, ProPTH, if released, can only comprise less than 1-2% of the secreted immunoreactive hormone. Although studies of prohormone content in parathyroid tissue under various circumstances may provide important information concerning biosynthetic mechanisms, it does not appear likely that assays for proparathyroid hormone will be helpful as markers of parathyroid-gland secretory activity.

B. Radioimmunoassay of Preproparathyroid Hormone

With the recent successful synthesis of the 25 amino acid peptide specific for the preproparathyroid hormone (figure 1) intensive efforts are now underway to develop by methods used successfully with our other synthetic peptides, an immunoassay for the initial glandular form of the hormone. Certain evidence, particularly that relating to unusual immunoreactivity of circulating hormone in patients with parathyroid-hormone-like humoral hypercalcemia secondary to malignant tumors of various types, suggests that under certain conditions the preproparathyroid hormone may be secreted into the circulation. If this is true, assays for this precursor form may provide an alternate approach to more accurate delineation of abnormal gland functions.

C. Radioimmunoassay of Parathyroid Secretory Protein

The high molecular weight polypeptide, parathyroid secretory protein (PSP), identified earlier by one group as a molecular entity of unknown function but under absolutely parallel control with respect to parathyroid hormone regarding calcium effects on glandular secretion in vitro represents an alternate approach to monitoring of parathyroid gland function.

Recently, sufficient of this polypeptide has been isolated to develop a radioimmunoassay for its detection. Preliminary studies indicate that PSP is secreted in vivo and hence clearly represents yet another alternate to detection of glandular secretion activity.

D. Metabolism of Amino- and Carboxyl-Sequence Immunoreactive Parathyroid Hormone in the Bovine: Evidence for Peripheral Cleavage of Hormone

Several studies have shown that the immunoreactive heterogeneity of parathyroid hormone (PTH) in blood, first described by Berson and Yalow, is due to the presence in blood of one or more immunoreactive fragments of PTH. The use of sequence-specific radioimmunoassays for PTH indicate that the predominant form of PTH in the circulation of patients with hyperparathyroidism and in dogs after intravenous injection of bovine PTH is a large fragment of the hormone that consists of the middle and carboxyl two-thirds of the hormonal polypeptide and lacks the amino-terminal sequence required for biological activity. Some, but not all, investigators have found, in addition to the large C-fragment of the hormone, smaller N- and C-sequence fragments in blood.

Disagreement exists concerning the physiological processes leading to the appearance of hormonal fragments in the circulation. Earlier work indicated that the principal, if not sole, secreted form of hormone was intact, suggesting that fragments must originate by cleavage of intact hormone at peripheral sites in the circulation. It has been reported, however, that, in certain patients with

hyperparathyroidism, fragments of the hormone may also be secreted from the parathyroids. In a patient with renal failure, it was reported that, after secretion, the fragments had a greatly prolonged existence in the circulation, with half-times of disappearance on the order of days. These important findings led to the introduction of the thesis that fragments found in peripheral blood might arise largely from the gland, even though present in parathyroid effluent at low concentrations.

To ascertain directly whether PTH undergoes cleavage to fragments at peripheral sites in the circulation, and to further determine the clearance rate of fragments formed, the concentrations of immunoreactive PTH in the circulation of calves after the intravenous injection and infusion of intact bPTH were analyzed by gel filtration and sequence-specific radioimmunoassays.

We have utilized radioimmunoassays that detect specifically peptide sequences with either the biologically active amino region (N-assay) or inactive carboxyl region (C-assay) or parathyroid hormone (PTH) to evaluate the metabolism of PTH during and after infusion and injection of homogenous (containing less than 0.1% hormonal fragments) intact bovine PTH (bPTH) into calves. During continuous infusions of hormone, when constant blood levels of immunoreactive PTH were reached, a dissociation between the concentrations of amino versus carboxyl immunoreactivity was observed; concentrations of hormone measured by the C-assay rose to a level of approximately three-times higher than that measured by N-assay. Analysis by gel filtration of immunoreactive PTH in plasma samples from calves after injection of hormone showed the rapid disappearance of intact hormone (N and C assays) and the appearance of a large fragment detected by the C-assay but not by the N-assay. The hormonal fragment lacked antigenic determinants within the amino peptide sequence required for biological activity. No additional fragments of PTH were detected by gel filtration using the N and C assays. No detectable conversion of intact PTH to hormonal fragments occurred during incubation in vitro in bovine serum. The results are consistent with the concept that intact PTH is metabolized after entry into the circulation at peripheral sites located outside the vascular space, resulting in the rapid disappearance from blood of intact hormone and the appearance of a biologically inactive hormonal fragment(s). These studies done in calves agree with earlier studies done in dogs and man and point to the existence in mammals of common pathways for the peripheral metabolism of PTH.

E. Metabolism of Parathyroid Hormone in vivo

Recent findings in parathyroid research have led not only to improved understanding of parathyroid physiology but also to confusion and uncertainty in parathyroid-related physiological and clinical research due to recognition of complexity in hormone metabolism. Multiple forms of PTH have been detected in the circulation. Present evidence suggests that some, but not necessarily all, of these multiple forms of circulating PTH represent peripheral cleavage of the intact PTH once the hormonal polypeptide is secreted. Since studies of the synthesis of amino-terminal fragments of the PTH polypeptide have suggested that there are biologically active fragments containing

only 27-34 amino acids out of the total 84 residues present in the native hormone, some of the circulating fragments of hormone might be biologically active. Conflicting reports have appeared from various laboratories concerning the absolute concentration and the rate of metabolic turnover of PTH in the circulation, particularly in primary and secondary hyperparathyroidism. These findings, which have clouded interpretation of immunoassays in clinical studies, undoubtedly relate to the complexity of forms of circulating endogenous hormone. We have concluded, therefore, that despite the great advances made in chemical analysis of parathyroid hormone, there is considerable uncertainty as to the precise chemical nature of the chemical species of hormone active at receptors in bone and kidney, and the hormonal species actually measured as immunoreactive parathyroid hormone.

Since last year's report, considerable progress has been made in understanding the nature of this heterogeneity of circulating parathyroid hormone and in ascertaining the significance of metabolism of parathyroid hormone.

Information from several laboratories concerning the heterogeneity and metabolism of parathyroid hormone contain many areas of agreement and several areas of disagreement. Our studies of exogenous hormone administered to cows, dogs, rats and man have demonstrated that parathyroid hormone undergoes metabolism after secretion. However, other reports suggest that fragments are also secreted from the gland.

A second issue relates to the number and nature of the circulating fragments. All laboratories agree that the dominant circulating immunoreactive form of the hormone is fragments which comprise the middle and carboxyl portions of the sequence and, therefore, must be biologically inert. Intact hormone contributes no more than 25% of the circulating immunoreactivity.

In addition, evidence presented by other groups has indicated that there may be other, still smaller immunoreactive forms of parathyroid hormone in the circulation. One of these, with an estimated molecular weight of approximately 2000, has been reported in the plasma of some patients with chronic renal failure. Another, with an estimated molecular weight of 4500, has been recovered from concentrates of human plasma and has been reported to be biologically active in the rat renal cortical adenylyl cyclase assay. This latter finding, if confirmed, is obviously of considerable significance.

Thus far, however, in studies in our laboratory, we have not detected the presence of these two smaller forms of the hormone, despite the use of multiple antisera in the radioimmunoassay of fractions from gel filtration of endogenous hormone from man and cow and of exogenously-administered bovine PTH in dogs. The reasons for this discrepancy are not clear. Our studies have led to the conclusion that cleavage of the intact hormone after secretion may result in the generation of a biologically-active NH_2-terminal fragment. Such a fragment, our studies indicate, must circulate at very low concentrations, if at all. Therefore, it would seem possible that the concentration process used by Canterbury and Reiss may have resulted in their ability to detect an NH_2-terminal fragment.

We have used two approachs to further explore whether fragments other than the large middle-carboxyl terminal fragment circulate--particularly amino-terminal fragments.

In one approach, we have concentrated circulating hormone by affinity chromatography and carefully examined the concentrate for fragments, including amino-terminal fragments. Anti-bovine PTH and anti-bovine PTH (1-34) antisera have been coupled to Sepharose after it has been activated by cyanogen bromide. After exposure of the ligand-resin to large volumes of patient plasma, approximately 50% of the total immunoreactivity in 240 ml of plasma can be recovered in approximately 3 ml of eluate, resulting in a 40-fold increase in concentration. Gel filtration and analysis using sequence-specific radioimmunoassays has not demonstrated the presence of any circulating fragments other than those seen prior to concentration. Specifically, no amino-terminal fragments were detected.

In a second approach, we have attempted to circumvent the difficulties intrinsic to immunoassays by using internally-labeled hormone containing specific radioactive amino acids to identify different portions of the parathyroid hormone sequence. Sufficient quantities of highly-purified, internally-labeled bovine PTH have been prepared to permit a series of in vivo experiments in rats. The approach looks especially promising but has not yet definitively settled the issue of circulating amino-terminal fragments.

Although our data suggests that, at least using pharmacological doses of hormone in the rat, amino-terminal fragments can be detected in circulation, it is still uncertain whether amino-terminal fragments circulate under physiologic conditions.

These results have also somewhat hindered the development of new assays to measure intact hormone, exclusively (see below). It is still not clear whether such assays will require only assay systems to measure the amino-terminal portion of the sequence or whether techniques such as the "sandwich assay" will be necessary. Methods have been developed for iodination and purification of labeled antisera and for lessening and non-specific adsorption of the labeled antisera to the solid-phase support used in the"sandwich assay". However, the priorty of this project is uncertain. Our hypothesis is that measurements using antisera that recognize amino-terminal determinants will accurately reflect the circulating concentration of intact hormone. Accordingly, emphasis has shifted toward attempts to develop suitable antisera by immunization, and to improving and streamlining the application of affinity chromatographic concentration of the various immunoreactive species. Nevertheless, the sandwich approach will serve as a valuable alternative assay method which, at minimum, will be available to confirm the results obtained using other methods.

An additional issue relates to the question of whether hormonal metabolism is of importance in establishing the circulating concentration of biologically active hormone. Accordingly, studies have also been initiated to examine whether physiologic perturbations of the state of the animal alter the pattern of metabolism. In preliminary studies, circulating calcium concentrations were acutely altered by calcium or EDTA-infusions after steady-state concentrations of immunoreactive hormone had been achieved during infusion of bovine PTH. These acute perturbations did not alter the concentrations of immunoreactive hormone in circulation.

Studies have been initiated with both radioiodinated and cold bovine PTH, in order to explore not only the effects of acute perturbations in the serum calcium on metabolism of the hormone, but also hormone metabolism in a number of chronically perturbated models. These models include parathyroidectomized-hypocalcemic-dogs maintained with vitamin D, parathyroid-intact, -vitamin D-deficient, hypocalcemic dogs, parathyroidectomized, -vitamin D intoxicated, -hypercalcemic dogs, and parathyroid-hormone-infused, -vitamin D-intact, -hypercalcemic dogs.

In addition we have studied the nature of the iodinated and internally-labeled hormone fragments that are present in the liver and kidney after injection of the hormone has been studied in the intact rat, at time intervals of from 2 to 96 minutes after injection.

Identification of fragments in kidney and liver by sequence analysis of the radioiodinated fragments at early time intervals has shown that whereas the radioactive fragments in the liver are identical to those in circulation, those in the kidney are different. Particularly at the earliest time intervals, the kidney contains only the fragment cleaved between residues 38-39, a fragment not seen in circulation. The significance of this observation is not clear, but suggests that the liver may be the principal organ contributing to the circulating fragments.

F. Summary- Present Achievements and Future Directions in the Development of Improved Radioimmunoassays for Parathyroid Hormone and Application to Man

Figure 3 illustrates the known or possible products of the parathyroid gland secreted into the blood in vivo. As described above, assays are being developed for each of the potential secretory products of the gland. These assays may provide direct approaches to assessment of the secretory activity of the parathyroid, independent of the confusion presently introduced by variable detections of fragments of the hormone itself. The results with the assay for PSP already shows great promise; others are being evaluated.

Alternate approaches are our present efforts to develop radioimmunoassays that will detect intact hormone, bPTH-(1-84), exclusively. The major effort is directed toward the development of assays with the extraordinarily high sensitivities required to measure the small concentrations of circulating hormone with amino-terminal reactivity. If our results are correct, such assays will measure intact hormone, exclusively. Alternatively, if we find amino-terminal circulating fragments, the antisera developed which recognize determinants in the amino-terminal portion of the sequence will be used in conjunction with antisera that recognize only carboxyl-terminal determinants in a "sandwich assay".

Attempts to produce assays which measure only amino-terminal determinants by using tracers of synthetic fragments (bovine PTH 1-34 and 1-33 [tyr^{34}]) have thus far yielded assays that are not sensitive enough to measure circulating concentrations in man. Efforts using a number of synthetic fragment analogs (i.e. - with "spacers" between the tyrosine residue and the remainder of the sequence) will be initiated later this year.

In addition, immunizations have recently been initiated using the synthetically-prepared peptide of human PTH--which extends from residue 53-84. The antigen developed will be useful in measuring the carboxyl portion of the sequence and, of course, will be essential in the development of the "sandwich assay".

These assays will be particularly useful for measuring intact hormone and therefore should detect the immunoreactive hormone in circulation which more accurately reflects parathyroid secretory activity than the present assays which all measure the dominant circulating form of the hormone, the large, biologically-inactive fragment which results from metabolism of the originally secreted hormone.

III. Vitamin D Hormone Metabolism and Assay of Active Metabolites

Studies on vitamin D production in skin, its circulating concentration in plasma, its hydroxylation to 25-hydroxyvitamin D by the liver and improved assays for active metabolites are all under study.

A. Production of Vitamin D in the Skin

Vitamin D is produced in the skin by the action of ultraviolet radiation on a precursor 7-dehydrocholesterol. The vitamin is also present, as a supplement to the endogenous source, in food. Vitamin D intoxication has been shown to occur with oral consumption, but not with U.V. exposure, thus leading to the postulation that some natural mechanism must exist in the skin to prevent intoxication by U.V. radiation.

In an effort to explore this theory, several preliminary experiments have been carried out. In the first, skin biopsies were taken from pigs given U.V. irradiation. In these samples, an increase in skin content of vitamin D seemed to occur as the length of exposure to the radiation increased, and this increase in the vitamin concentration seemed to occur mainly in the subcutaneous fat layer. However, the technical problems which arose in the extraction of the small amounts of vitamin D from the other neutral lipid in the skin samples have made this method of studying vitamin D production in skin less immediately useful, and another approach has been made.

Work using radioactive precursors of vitamin D, specifically 7-dehydrocholesterol, has led to systematic analysis of the products resulting from U V irradiation! (See attached preprint). This work permits dissection of the controlling principles believed to operate in skin to foster vitamin D productions, yet prevent D intoxication.

B. Studies on Tissue Receptors for Vitamin D Metabolites

It has been shown that the rat intestinal cytoplasmic vitamin D receptor is a single, monomeric, 5.5S protein with M.W. 80,000, which is negatively charged at physiologic pH and is clearly separable from the corresponding serum-binding protein by sucrose-gradient ultracentrifugation and by ion-exchange chromatography. Rapid loss of binding activity of 4°C can be prevented by protease inhibitors, and pH, temperature and ionic-strength binding optima have been defined. While results to date suggest a three-fold greater avidity for 25-HCC than for 1,25-HCC, accurate definition of binding specificity awaits further improvement in the assay technique. Notably, most of these results are quite unlike those obtained from study of the avian intestinal receptor by Haussler et al.

Examination of cytosols from a wide variety of rat tissues, including many not presently considered "target organs" of vitamin D action, disclosed the presence in each of a single receptor which is indistinguishable from the intestinal receptor by ultracentrigugation or disc-gel electrophoresis and which, like the intestinal receptor, is labeled after in vivo administration of H^3-25-HCC as well as in in vitro studies with H^3-25-HCC or H^3-1,25-DHCC. These results confirm and extend those of Haddad et al., and suggest that vitamin D

may participate more fundamentally in cellular metabolism than has previously been inferred from its known effects on calcium and phosphate homeostasis.

C. Development of an Assay for 1,25-$(OH)_2$-D

The development of an assay for the active metabolite of vitamin D, i.e., 1,25 $(OH)_2D_3$, has been a subject of much interest. Both the intestinal receptor and the plasma vitamin D-binding protein have high affinities for the metabolites, but in each case the affinity for 25-OH-D metabolite is as great if not greater. Therefore, separation of these two metabolites is a necessary step in any assay. This separation was envisaged as being possible on the high pressure liquid chromatography system, but in the original experiments the lipid content of the plasma extracts was too great for the chromatography columns in this system. However, a method of separating this neutral lipid from the polar metabolites in serum is available, and may allow the application to this effective separation system of polar fat-soluble serum components alone and not the overload of neutral lipid. This approach will make the assay of the active metabolite 1,25-$(OH)_2D$ available as a routine, specific, rapid assay.

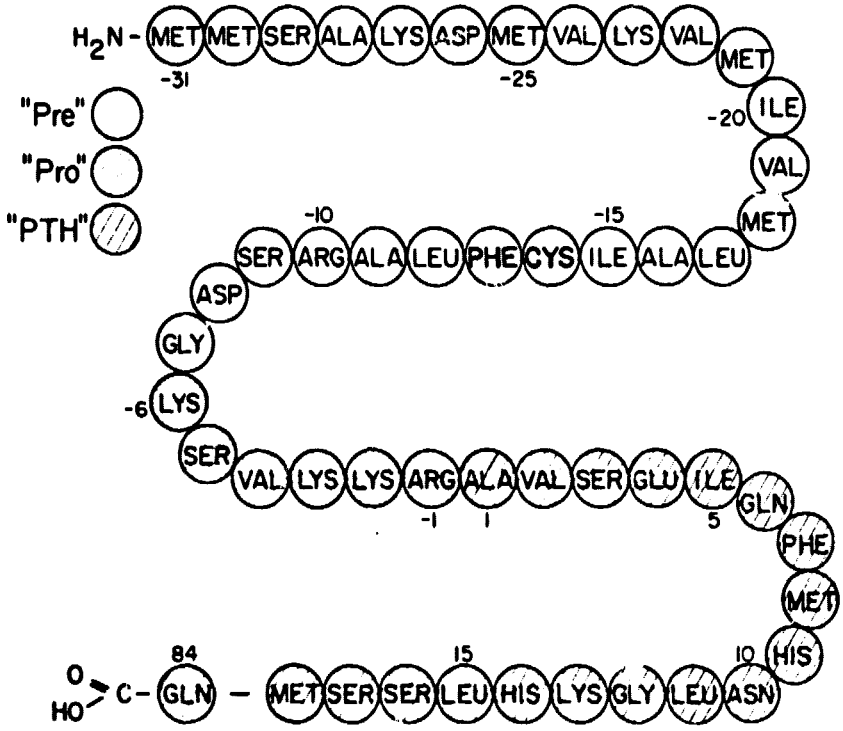

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FIGURE 1

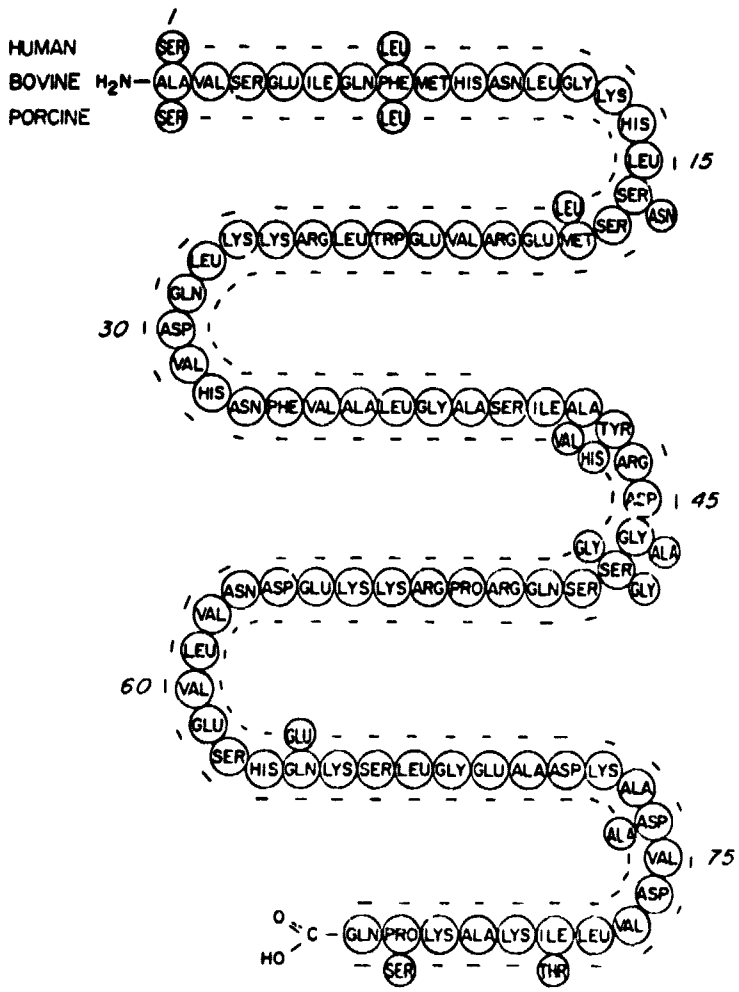
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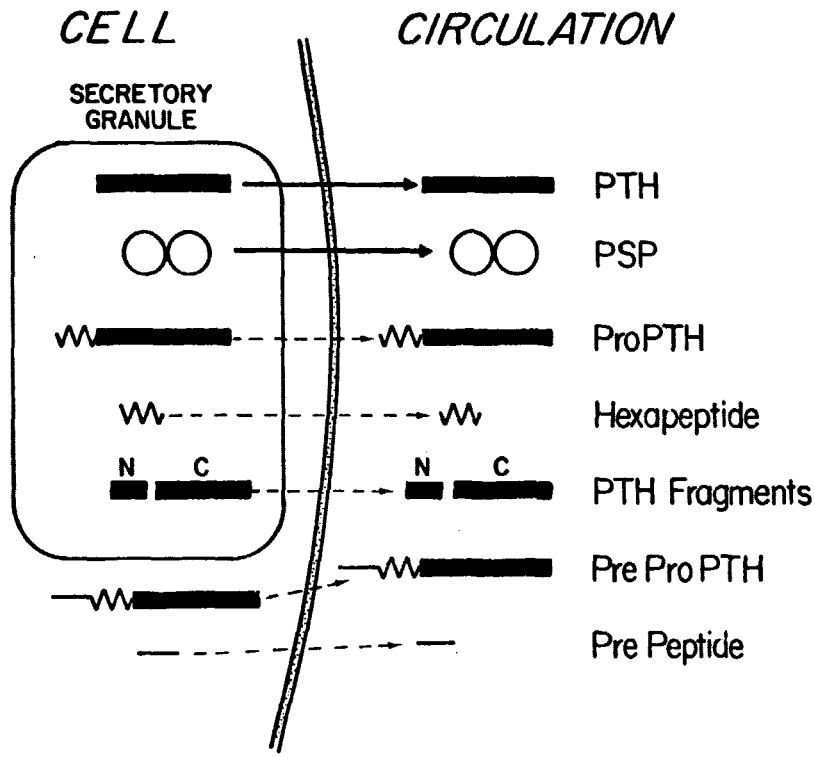
FIGURE 2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PTH Fragments
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Pre Peptide

FIGURE 3

Endocrine Unit Publications Related to NASA Mission and Support (1975-1977)

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PHOTOMETABOLISM OF 7-DEHYDROCHOLESTEROL TO PREVITAMIN D_3 IN SKIN

M.F. Holick, J.E. Frommer, S.C. McNeill, N.M. Richtand,
J.W. Henley, and J.T. Potts, Jr.

Department of Medicine, Harvard Medical School, and Endocrine
Unit, Massachusetts General Hospital, Boston, Mass. 02114

SUMMARY: The photometabolism of [3α-^{3}H]-7-dehydrocholesterol in skin was studied in groups of rats exposed to ultraviolet irradiation. The major photolytic product was identified as previtamin D_3 by its identical migration with authentic previtamin D_3 on high-pressure liquid chromatography. Furthermore, this photometabolite was isolated in pure form from endogenous precursors in skins of rats exposed to ultraviolet irradiation; identification as previtamin D_3 was based on its ultraviolet absorption spectrum, mass spectrum and its thermal conversion to vitamin D_3.

INTRODUCTION: Vitamin D, a fat-soluble factor that acts to heal rachitic lesions, was discovered in the early 1900's (1,2). Subsequently, Steenbock (3) and Hess et al. (4) independently demonstrated that this factor could be generated simply by irradiating either rachitic animals or their food. Further work led to the isolation and structural identification of vitamins D_2, D_3, and their precursors, ergosterol and 7-dehydrocholesterol, respectively (5—7).

Photolysis of ergosterol or 7-dehydrocholesterol *in vitro* yields, at equilibrium, at least three isomeric products: tachysterol, lumisterol, and a 9,10-seco-steroid with a 5,6-cis-triene conformation. This latter compound was shown to have antirachitic activity and, depending on the starting material, ergosterol or 7-dehydrocholesterol, was named vitamin D_2 or vitamin D_3, respectively. At first it was believed that 7-dehydrocholesterol and ergosterol were converted directly to vitamin D_3 and vitamin D_2 by ultraviolet photolysis (8). In 1949, however, Velluz et al. (9) demonstrated that a 6,7-cis isomer, which they named previtamin D, was the initial irradiation product and that, at varying rates as a function of temperature, previtamin D converted to vitamin D with the equilibrium position in favor of vitamin D. Thus, under controlled conditions, ultraviolet

Abbreviations: HPLC, High-Pressure Liquid Chromatography; TLC, Thin Layer Chromatography

irradiation of 7-dehydrocholesterol generates three major products: tachysterol, lumisterol, and previtamin D_3 (Fig. 1). Previtamin D_3 is then converted to the thermodynamically favored 5,6-cis isomer, vitamin D_3, by thermal equilibration.

Although it has been generally accepted that vitamin D_3 is generated in vivo by ultraviolet irradiation of the 7-dehydrocholesterol in the skin, the mechanism by which this occurs has not been investigated. We have examined this mechanism and clearly demonstrated that 7-dehydrocholesterol is initially converted to previtamin D_3 in the skin in vivo, as it is in the test-tube model, by the action of ultraviolet light.

METHODS:

Synthesis of [3α-^{3}H]-7-dehydrocholesterol. Synthesis of this isotope was carried out as described for [3α-^{3}H]-ergosterol (10). Crystalline 7-dehydrocholesterol was oxidized to 4,7-cholestadiene-3-one (λ_{max} 240 nm) by the method of Shepherd et al. (11). This α-β unsaturated ketone was acetylated with acetic anhydride and pyridine, and the isolated product exhibited λ_{max} 331, 316, and 303 nm, which is characteristic for the $\Delta^{5,7}$-enol acetate. The enol acetate (10 mg) was dissolved in 10 ml isopropanol containing 100 µl H_2O and to this solution was added 1 mg [^{3}H]-sodium borohydride (S.A. 8.0 Ci/mM) purchased from New England Nuclear, Boston, Massachusetts. The mixture was stirred at 25°C for 2 h and then extracted with ether:water. The product was dried under N_2 and applied to preparative thin-layer chromatographic plates (Uniplate, Analtech Inc., Delaware) and developed in n-hexane: ethylacetate (8:2 v/v). The compound with an R_f of 0.6 was eluted and exhibited an ultraviolet spectrum with λ_{max} 295, 282, and 271 nm, which is identical to the ultraviolet spectrum for 7-dehydrocholesterol (10). Furthermore, this product migrated identically with authentic 7-dehydrocholesterol on thin-layer chromatography (TLC) and on high-pressure liquid chromatography (HPLC) using a 0.4-mmx30-cm µ-Porasil column developed with 1% isopropanol in n-hexane. An additional product with an identical ultraviolet-absorption spectrum but with an R_f of 0.8 was also recovered; the latter did not migrate with 7-dehydrocholesterol on HPLC or TLC. This additional compound was presumed to be [3β-^{3}H]-3-epi-7-dehydrocholesterol, a predicted side-product of the reduction reaction.

Topical application and irradiation of [3α-^{3}H]-7-dehydrocholesterol. Areas (3 X 3 cm) of the backs of two groups of vitamin-D-deficient rats were shaved. Two µCi of [3α-^{3}H]-7-dehydrocholesterol dissolved in 0.1 ml of Wesson oil was applied to this exposed skin 24 h after shaving. The control group was kept in the dark; the treatment group, after an additional 24-h delay, was placed under an ultraviolet light of wave-length 250—350 nm for 3 h (0.23 joules/cm^2 at $\lambda_{280\ nm}$). Immediately after irradiation of the treatment group, all animals were killed by exsanguination, and the 3x3-cm area of skin was removed and frozen on Dry Ice.

Isolation and identification of radioactive photolysis products. Individual skins were homogenized in 10 ml 0.9% saline with a Polytron homogenizer for 30 sec and extracted with 40 ml cholorform:methanol (1:1). The cholorform phase was collected, and the aqueous phase was re-extracted twice with 10 ml chloroform. The chloroform phases were combined, dried under N_2 at 4°C, dissolved in n-hexane:chloroform (19:1), and applied to a 1x60-cm glass column packed with 15 grams of a Neodox-1518 alkoxyl derivative of Sephadex LH-20 prepared according to the procedure of Ellingboe et al. (12). The column was eluted with n-hexane: chloroform (19:1). Fractions (2 ml) were collected, dried under air, and dissolved in Instagel (Packard) prior to counting of their tritium content in the Packard Tricarb 3375 scintillation counter.

The various peaks of radioactivity were identified by taking individual peak fractions from the Neodox column and subjecting them to chromatography simultaneously with standard compounds on HPLC equipped with a 0.4-cmx30-cm μ-Porasil column using 1% isopropanol in n-hexane as solvent. The peak assigned as previtamin D_3 was mixed with 100 μg authentic previtamin D_3, chromatographed on HPLC as described above, and collected in 1-ml fractions. Half of each fraction was dried and counted for tritium. The remaining fractions containing the previtamin-D_3 peak were pooled and warmed at 60°C in methanol for 3 h before re-chromatography on the same column under identical conditions.

Isolation and identification of a sterol formed from photolysis of endogenous 7-dehydrocholesterol. 50 rats previously maintained on a vitamin-D-deficient diet for 3 weeks were shaven as before. 24-h later they were exposed to 0.23 joules/cm^2 at λ_{280} nm of ultraviolet light over a period of 2 h and then immediately killed by exsanguination. Topical doses of [3α-^{3}H]-7-dehydrocholesterol were applied to 3 rats as described above, before irradiation, to act as a radio-active marker. The shaved skin from all animals was collected, homogenized and extracted as previously described. The extract was dried under N_2 in the cold room and the lipid residue was re-extracted in 800 ml water:n-hexane (1:1). The n-hexane phase was collected, and the aqueous phase was re-extracted twice with 100 ml n-hexane. The n-hexane phases were combined and dried under N_2 at 4°C, and the residue was dissolved in n-hexane:chloroform (19:1) and applied to a 3x40-cm glass column packed with 60 g Neodox-1518 derivative of Sephadex LH-20 in the same solvent. The previtamin-D_3 peak was collected, dried under N_2, redissolved in 10 ml methanol, and warmed at 60°C for 3 h. The product was applied to the HPLC, and 50 μl from the resulting 1-ml fractions was counted for tritium. Ultraviolet-absorption spectra were made on the previtamin-D_3 peak before and after heating, on a Beckman DB-G spectrophotometer.

RESULTS: The chromatographic profiles of the skin extracts from animals given topical applications of [3α-^{3}H]-7-dehydrocholesterol and then exposed to 0.23 joules/cm^2 ultraviolet radiation (A) or confined in the dark (B) are shown in Fig. 2. The chromatogram from the confined animals shows three peaks. The earliest peak (fractions no. 10—20) is presumed to be an ester(s) of the starting material. The two additional peaks were tentatively identified as 7-dehydrocholesterol and its Δ^7 reduction product, cholesterol. This

identification was confirmed by cochromatography with authentic compounds on HPLC. The chromatogram from animals that received ultraviolet irradiation demonstrated one additional major peak labeled as previtamin D_3 (Fig. 2B). The identification of this radioactive peak as previtamin D_3 was established by (i) identical mobility with authentic previtamin D_3 on the high-resolution HPLC system and (ii), after warming, identical mobility with vitamin D_3 formed by thermal conversion of authentic previtamin D_3 (Fig. 3).

DISCUSSION: Although it has been generally accepted that vitamin D_3 is made in the skin by the action of solar ultraviolet irradiation on 7-dehydrocholesterol, the mechanism by which this occurs has never been investigated. In this paper we present evidence that 7-dehydrocholesterol in the skin is converted to previtamin D_3 and not vitamin D_3, a result similar to that seen *in vitro*. This was demonstrated by two methods. In the first, [3α-^{3}H]-7-dehydrocholesterol applied topically was converted to a compound that migrated identically with authentic previtamin D_3 on high-resolution separation systems. The concern that topically applied 7-dehydrocholesterol might not enter epithelial cells where the photolytic reaction naturally occurs was lessened when it was demonstrated that cholesterol was generated from its precursor 7-dehydrocholesterol. Therefore, at least some of the 7-dehydrocholesterol had to enter viable epithelial cells for the 7-dehydrocholesterol-reductase to work.

It could be argued, however, that the photolytic products recovered were similar to those that would be found if we applied 7-dehydrocholesterol to a piece of glass and irradiated it. Therefore, the previtamin D_3 that we observed in our isotope experiments might in fact have been an artefact generated on the surface of the skin and may not have entered the cells or be a physiological product.

To eliminate this possibility an alternate approach was used. We irradiated 50 vitamin-D-deficient rats and isolated a product with an

ultraviolet absorption spectrum λ_{max}257 nm and λ_{min}230 nm that was similar to previtamin D_3 (λ_{max}260 nm, λ_{min}230 nm). This product, formed from endogenous 7-dehydrocholesterol, migrated identically with authentic previtamin D_3 and, when warmed at 60°, chromatographed identically with vitamin D_3. Furthermore, the product after heating had an ultraviolet absorption spectrum λ_{max}265 nm, λ_{min}228 nm, which is identical to that of the 5,6-cis-triene system for vitamin D_3 (Fig. 4). To confirm that the isolated 5,6-cis-triene was vitamin D_3, a mass spectrum of the isolated product was analyzed. The compound was shown to have a molecular ion 384 (m/e) and fragments 369 (M^+-CH_3), 366 (M^+-H_2O), 271 (M^+-side chain), 253 (M^+-side chain-H_2O), 136 (A ring + C_6 and C_7), and 118 (136-H_2O), which are identical to authentic vitamin D_3 (13).

In this paper we report the isolation of a photolytic product from skin and its identification as previtamin D_3. Therefore we suggest that the mechanism for the photoconversion of 7-dehydrocholesterol to vitamin D_3 in the skin occurs through an intermediate product, previtamin D_3, which is further converted to vitamin D_3 presumably by the thermal action of body heat. Qualitative or quantitative differences, if any, in the overall process of photolytic activation of 7-dehydrocholesterol <u>in vitro</u> versus <u>in vivo</u> await further study, especially the issue of biological control of the <u>in vivo</u> process.

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Fig. 1. Photometabolism of 7-dehydrocholesterol to lumisterol, tachysterol, and previtamin D_3, which is converted to vitamin D_3 by thermal energy.

Fig. 2. Neodox 1518 Sephadex column (1 × 60 cm packed in 19:1 n-hexane: chloroform) profiles of skin lipid extracts from animals treated with topical application of 2 μCi [3α-^{3}H]-7-dehydrocholesterol and then killed 24 h later (A) or irradiated with 0.23 joules/cm^2 and then killed (B).

Fig. 3. Chromatography of fractions 35—45 from Fig. 2B with 100 μg of authentic previtamin D_3 on HPLC (A). Peak 1 was collected and warmed at 65°C for 3 h and rechromatographed on the same column (B), which demonstrated conversion to vitamin D_3.

Fig. 4. Ultraviolet absorption spectrum of the isolated product from the skin that was warmed at 65° for 3 h and chromatographed on HPLC as shown in Fig. 3.

FIG. 1

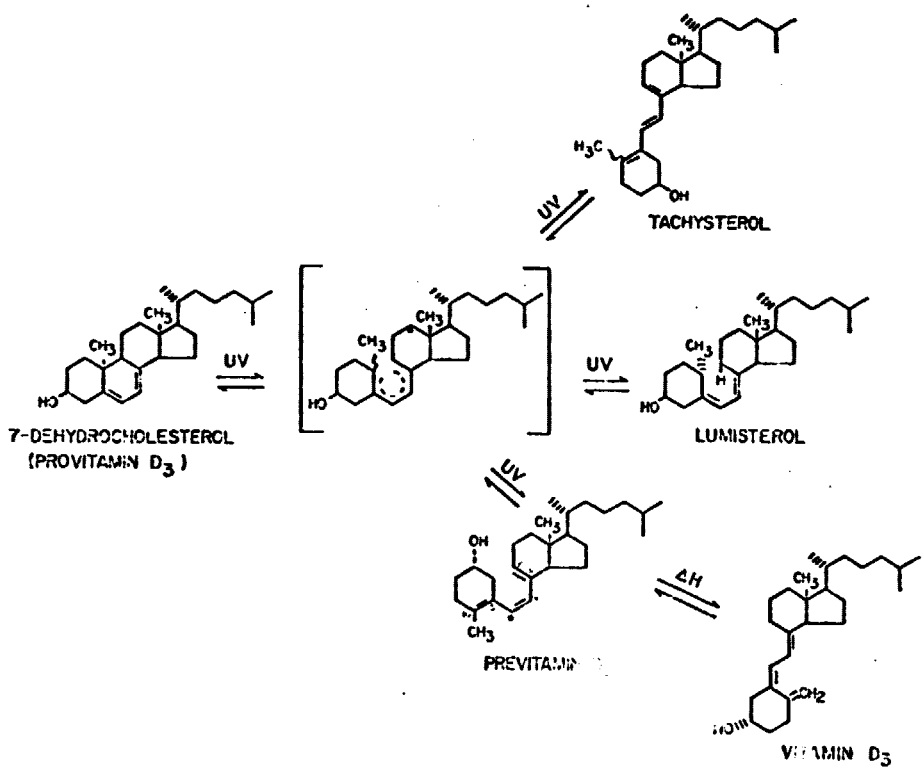
TACHYSTEROL
UV
7-DEHYDROCHOLESTEROL
(PROVITAMIN D_3)
UV
UV
LUMISTEROL
UV
PREVITAMIN
ΔH
VITAMIN D_3

FIG. 2

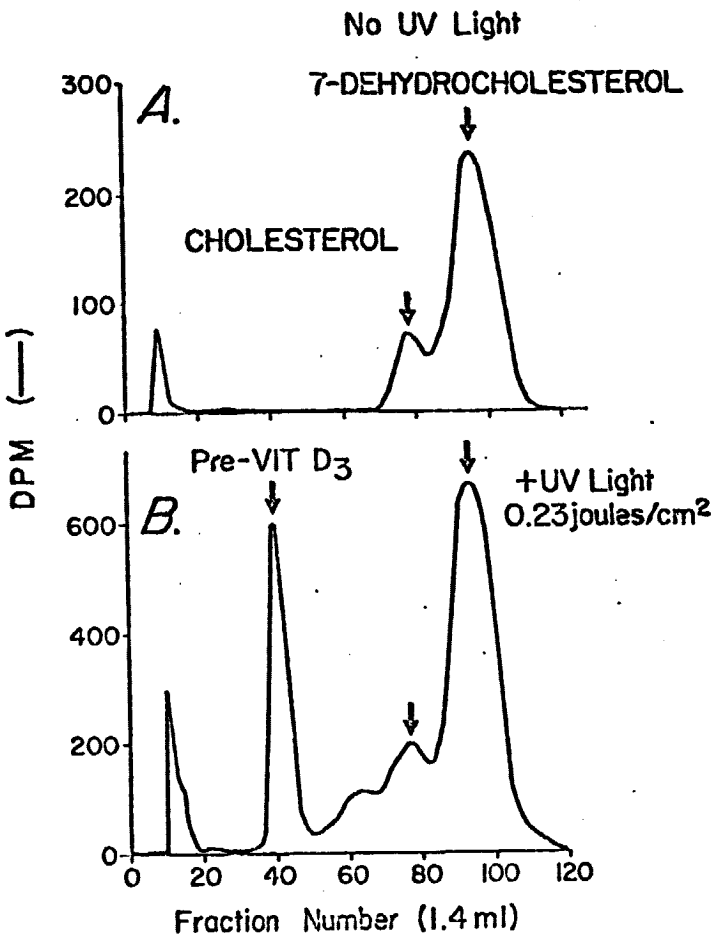
No UV Light
7-DEHYDROCHOLESTEROL
A.
CHOLESTEROL
300
200
100
0
DPM (—)
Pre-VIT D3
+UV Light
0.23joules/cm2
B.
600
400
200
0
0
20
40
60
80
100
120
Fraction Number (1.4 ml)

FIG. 3

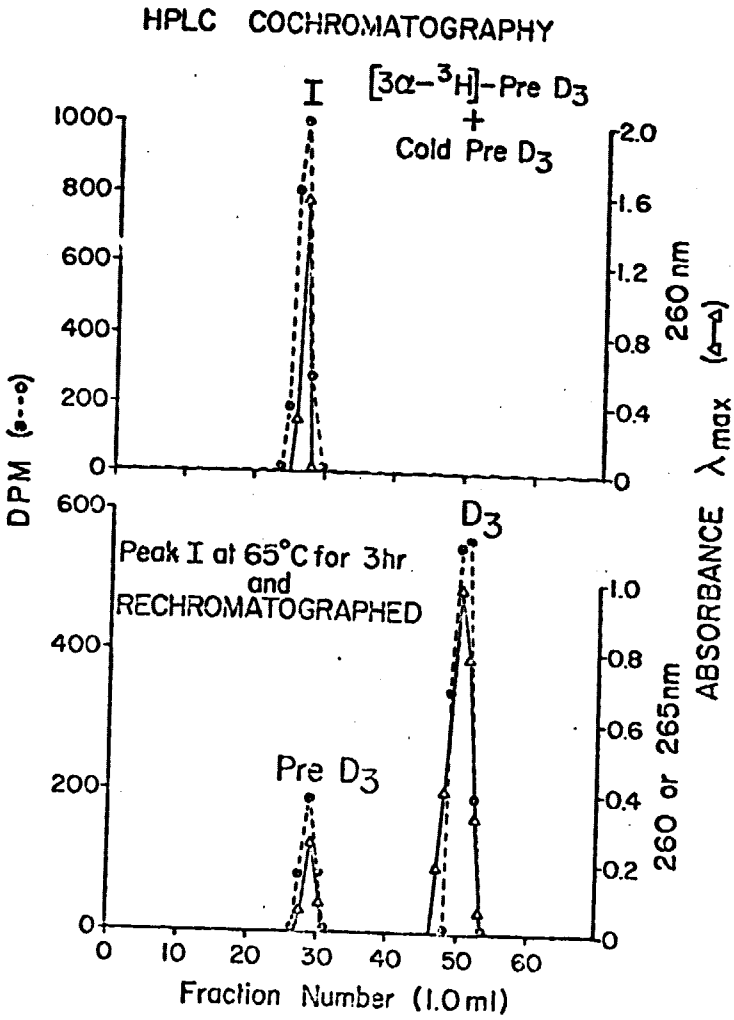
HPLC COCHROMATOGRAPHY
I
[3α-³H]-Pre D3
+
Cold Pre D3
DPM (o--o)
260 nm λ max (Δ—Δ)
ABSORBANCE
1000
800
600
400
200
0
2.0
1.6
1.2
0.8
0.4
0
600
400
200
0
1.0
0.8
0.6
0.4
0.2
0
D3
Peak I at 65°C for 3hr
and
RECHROMATOGRAPHED
Pre D3
260 or 265nm
0 10 20 30 40 50 60
Fraction Number (1.0 ml)

Fig. 4