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Subcellular Location of Vitamin D and Its Metabolites in Intestinal Mucosa after a 10-IU Dose*

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ABSTRACT: The intracellular localization of ^3H from 10 IU of [^3H]vitamin D in rat or chick intestinal mucosa indicates that a major site of accumulation is the nuclear membrane. When the outer membrane of the nucleus is fragmented or stripped off with citric acid or Triton X-100, 60–80% of the nuclear radioactivity from [^3H]vitamin D can be removed. Of the ^3H from [^3H]-vitamin D associated with the nuclei, 80% is in the form of a polar metabolite(s) which is known to be biologically active.

The nuclear receptor sites for vitamin D and/or its metabolites can be saturated by the administration of large doses of the vitamin, dihydrotachysterol-2, but not 7-dehydrocholesterol.

There is little doubt that a lag exists between the time of vitamin D administration and the subsequent increase in the intestinal transport of calcium or the mobilization of mineral from bone (DeLuca, 1967). At least a portion of this time lag can be explained by some induction process which mediates the action of the vitamin (DeLuca, 1967). Thus actinomycin D, which inhibits DNA-directed synthesis of RNA,[1] has repeatedly been shown to block completely the physiologic responses to vitamin D (Eisenstein and Passavoy, 1964; Zull *et al.*, 1965, 1966a; Schachter and Kowarski, 1965; Norman, 1965, 1966; Harrison and Harrison, 1966). Other RNA and protein synthesis inhibitors such as puromycin and 5-fluororotic acid have given partial inhibition of vitamin D responses (Zull *et al.*, 1966a). It has also recently been demonstrated that vitamin D, when administered to vitamin D deficient rats, induces a two- to threefold stimulation of [^3H]orotic acid incorporation into nuclear ribonucleic acid (nRNA) of intestinal mucosa. This stimulation is completely blocked by actinomycin D (Zull *et al.*, 1966b; Stohs *et al.*, 1967), indicating the formation of mRNA (Reich, 1964). Therefore, the present knowledge of the mechanism of vitamin D action suggests that the physiologic expression of the vitamin and/or its metabolite(s) involves the transcription of specific genetic information into mRNA. The subsequent translation of this mRNA into functional protein components results in the maintenance of the $[Ca^{2+}]\cdot[HPO_4^{2-}]$ product of the blood at an appropriate level ensuring deposition of mineral into newly formed bone collagen (DeLuca, 1967).

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[1] Abbreviation used: TMK, 0.05 M Tris-Cl (pH 7.4), 0.005 M $MgCl_2$, and 0.025 M KCl.

How and where vitamin D exerts its initial action involving RNA and subsequent protein synthesis is of great interest in the further elucidation of the vitamin's mechanism of action. The biochemical responses induced in the small intestine and bone by vitamin D or its metabolite(s) must result from an initial interaction with the cells of that tissue. A knowledge of the cellular component(s) involved as well as the nature of such an interaction is essential for a mechanistic understanding of vitamin D function. Thus, it is of basic importance to determine the subcellular location of physiological levels of vitamin D in intestinal mucosa.

Previous experiments in this laboratory designed to examine the subcellular location of [^{3}H]vitamin D_3 in the intestinal mucosa of vitamin D deficient rats utilized vitamin D doses of 500 IU/rat (Norman and DeLuca, 1964). Kodicek (1960) studied the subcellular location of [^{14}C]vitamin D_2 in liver following doses as large as 40,000 IU. The intracellular distribution of unlabeled vitamin D in the liver of normal (vitamin D fed) rats has also been reported, employing doses of 8000–80,000 IU (Nair and Bucana, 1966). Wilson *et al.* (1967) have even more recently published results of their intracellular distribution of [^{3}H]vitamin D_3 in the intestinal mucosa of vitamin D deficient and supplemented rats. They administered 100–20,000 IU of a vitamin D preparation having a specific activity of approximately 1000 dpm/IU. They concluded that the vitamin in the nuclear-debris fraction and a portion, at least, in the microsomal fraction was of physiological significance. All of the above experiments suffer from the criticism that the doses of the vitamin given greatly exceed the 10-IU daily dose/rat deemed as physiological (Carlsson and Lindquist, 1955). It is not clear whether the distribution observed in these experiments reflects function or merely storage of the vitamin. Furthermore, there is no evidence at the present time that the liver is a target of vitamin D action.

Studies on the subcellular distribution of 5–50-IU doses of [^{3}H]vitamin D in rachitic chicks have recently been published (Haussler and Norman, 1967). These authors report that as much as 40% of the ^{3}H localizes in the small intestine 24 hr after the administration of 5 IU of [^{3}H]vitamin D_3 (sp act. 1000 dpm/IU). That no more than 3% of a 10-IU dose of [^{3}H]vitamin D_3 (sp act. 26,000 dpm/IU) accumulates in the small intestine of rat (Neville and DeLuca, 1966) or chick (Imrie *et al.*, 1967) has been reported. This discrepancy casts doubt on the value of their subcellular distribution data.

The synthesis of specifically labeled [1,2-^{3}H]vitamin D_3 (Neville and DeLuca, 1966) and [22,23-^{3}H]vitamin D_4 (H. F. DeLuca and H. M. Weller, in preparation) of high specific activities (26,000 and 53,000 dpm/IU, respectively) has made possible extensive investigations on the subcellular distribution of physiological levels of vitamin D and its metabolites in rat and chick intestinal mucosa. The results herein presented indicate that the nuclear membrane provides the major site for the accumulation of vitamin D and its metabolites in mucosa of the small intestine. Significant amounts also accumulate in the cytoplasm and deoxyribonucleoprotein. The saturation or swamping of the nuclear sites can be achieved by administration of large doses of nonradioactive vitamin D or dihydrotachysterol-2, but not by 7-dehydrocholesterol. In addition, 80% of the ^{3}H from [^{3}H]vitamin D is present in the nuclei in the form of a polar metabolite considered to be the metabolically active form (Lund and DeLuca, 1966).

Experimental Procedure

Preparation of Rats. Male weanling rats (Holtzman Co., Madison, Wis.), weighing 50–60 g, were housed in individual hanging wire cages and given food and distilled water *ad libitum*. They were fed a diet adequate in calcium (0.47%) and phosphorus (0.3%) as previously described (Steenbock and Herting, 1955). Fat-soluble vitamins A, E, and K were given orally three times a week in cottonseed (Wesson) oil. Lowered serum calcium levels and retarded growth after 3–4 weeks indicated the animals were vitamin D deficient, at which time they were used for experimental purposes.

Preparation of Chicks. One-day-old white Leghorn cockerals were obtained from Sunnyside Hatcheries, Oregon, Wis., and housed in an incubator maintained at 37°. They were fed a purified rachitogenic diet as previously described (Imrie *et al.*, 1967). On this diet the chicks became severely rachitic and were used after 3–4 weeks.

Isotopes. [1,2-^{3}H]Vitamin D_3 with a specific activity of 26,000 dpm/IU (Neville and DeLuca, 1966), [22,23-^{3}H]vitamin D_4 having a specific activity of 53,000 dpm/IU (H. F. DeLuca and H. M. Weller, in preparation), or generally labeled [^{3}H]vitamin D_3 with a specific activity of 1600 dpm/IU (P. F. Neville and H. F. DeLuca, unpublished data) were synthesized in this laboratory.

Vitamin D Administration and Tissue Preparation. Vitamin D deficient rats and rachitic chicks were given 10 or 5000 IU of [^{3}H]vitamin D_3 or D_4 intrajugularly in 0.025–0.04 ml of ethanol. All animals were fasted 12–14 hr prior to sacrificing. The animals that received 10 IU were killed at 4, 8, 12, or 24 hr after dosing, while those receiving 5000 IU were killed after 8 or 12 hr. Approximately 40–50 cm of the small intestine from the duodenal end was quickly removed and chilled in ice-cold 0.25 M sucrose and 0.05 M Tris-Cl solution (pH 7.4). The intestinal segment was removed, slit open, washed with the solution, blotted on filter paper, and laid on a stainless-steel plate which rested on ice. The mucosa was removed by scraping with a microscope slide (Crane and Mandelstam, 1960) and weighed.

Subcellular Distribution of [^{3}H]Vitamin D_3 in Vitamin D Fed Rats. Vitamin D deficient rats were given 50 IU of unlabeled vitamin D_3 daily by mouth in cottonseed (Wesson) oil for 7 days. Twelve hours after the last administration each animal received 10 IU of [^{3}H]-vitamin D_3. The animals were sacrificed 8 hr later and the intestinal mucosa was subjected to subcellular

fractionation in the 0.25 M sucrose solution by differential centrifugation.

Influence of Actinomycin D on the Subcellular Distribution of [^{3}H]Vitamin D. Vitamin D deficient rats were given 100 μg of actinomycin D intraperitoneally in a 0.5-ml solution of water–ethanol (9:1). Five hours later each animal received 10 IU of [^{3}H]vitamin D_3, and was killed 8 hr after the administration of the vitamin. The intestinal mucosa was subcellularly fractionated in the 0.25 M sucrose solution.

Prevention of ^{3}H Accumulation from 10 IU of [^{3}H]-Vitamin D_3 in Chick Mucosal Nuclei. Rachitic chicks were given 62.5 μg of nonradioactive vitamin D_3, 7-dehydrocholesterol, or dihydrotachysterol-2 at 36 and 24 hr prior to sacrifice followed by 10 IU of [^{3}H]-vitamin D_3 12 hr before sacrificing. Nuclei were isolated from the intestinal mucosa employing either 2.2 or 2.3 M sucrose (see "Sucrose" Nuclei). Rachitic chicks which had been given only 10 IU of [1,2-^{3}H]vitamin D_3 or 5000 IU of generally labeled [^{3}H]vitamin D_3 for 12 hr served as controls.

Subcellular Fractionation. The preparation of subcellular fractions was accomplished by the conventional method of Schneider and Hogeboom (1950). A 10% homogenate of the mucosa from one or two rats or chicks was prepared in 0.25 M sucrose plus 0.05 M Tris-Cl (pH 7.4) employing a Potter-Elvehjem homogenizer fitted with a Teflon pestle (A. H. Thomas Co.). The crude nuclear-debris fraction was sedimented by centrifuging at 700*g* for 10 min in a refrigerated International PR-2 centrifuge. The resulting supernatant fraction was centrifuged for 10 min at 7000*g* yielding the mitochondrial fraction. The 7000*g* supernatant fraction was centrifuged in a Spinco Model L-2 ultracentrifuge either at 85,000*g* for 90 min in a no. 40 rotor or at 105,000*g* for 60 min in a no. 50 titanium rotor. The resulting microsomal pellet was washed once, while the nuclear and mitochondrial pellets were washed twice in the sucrose solution. An alternative and more drastic method of tissue homogenization was employed in one series of experiments to ensure complete cell rupture. This method was the procedure of Waldorf as described by Norman and DeLuca (1964) and entailed the preparation of a 10% tissue homogenate in a 0.25 M mannitol solution by sand grinding in a mortar with a pestle. Fractionation was carried out as described above.

"Citric Acid" Nuclei. The method of Mirsky and Pollister (1946) was employed to isolate nuclei from intestinal mucosa which were free of debris and adhering cytoplasm. The resulting white nuclear pellet was found to consist of nuclei which were observed to be relatively free of cytoplasmic adherences as viewed through the light microscope at a magnification of 1000×. However, electron micrographs revealed that the nuclei had not retained their double membrane. The outer membrane was shown to be badly fragmented and in some cases absent.

"Sucrose" Nuclei. Nuclei with the double membrane intact were prepared by two methods. A modification of the Chaveau (1952) technique was employed. Mucosal homogenates (5%) were prepared in 2.2 M sucrose solution containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 2 mM K_2HPO_4 (pH 6.5). The homogenates were centrifuged for 3 hr at 50,000*g* in a SW 25.1 rotor using a Spinco Model L-2 ultracentrifuge. A discontinuous sucrose gradient method (Blobel and Potter, 1966) was also utilized for the isolation of nuclei from rat liver and chick mucosa. A 2.3 M sucrose solution was layered beneath at 10% tissue homogenate in 1.61 M sucrose solution. All solutions contained 0.05 M Tris-Cl (pH 7.4), 0.005 M $MgCl_2$, and 0.025 M KCl. Isolation was achieved by centrifuging 30 min at 124,000*g*, using a SW 50L rotor in a Spinco Model L-2 ultracentrifuge. All isolated nuclei were washed twice. In some cases 1% citric acid was added to 2.2 M sucrose and nuclei isolated by the Chaveau (1952) method. These isolated nuclei were then washed two times with filtration using 0.2% citric acid (see "Citric Acid" Nuclei). To remove the outer membrane of nuclei as reported by Blobel and Potter (1966), isolated nuclei were washed three times in 0.22 M sucrose solution containing 1% Triton X-100.

Deoxyribonucleoprotein Preparation. To determine the amounts of ^{3}H from [^{3}H]vitamin D associated with the DNA of intestinal mucosa, deoxyribonucleoprotein was prepared from the nuclear fraction (Zubay and Doty, 1959). Each rat received 10 IU of [^{3}H]-vitamin D_3 for 8 hr. The per cent of the total ^{3}H present in association with the DNA was based on a determination of the fraction of total mucosal DNA isolated as the deoxyribonucleohistone.

Ribosome Preparation. Ribosomes were isolated from the 7000*g* supernatant fractions of tissue homogenates prepared in 0.25 M sucrose solution. To these supernatant fractions was added one-ninth volume of 5% sodium deoxycholate (pH 7.5), and the resulting solutions were centrifuged at 85,000*g* for 90 min in an ultracentrifuge equipped with a no. 40 rotor. The ribosomes were washed once.

Brush Border Preparation. The isolation of brush borders (microvilli) from rat intestinal mucosa used essentially the differential centrifugation method of Miller and Crane (1961) employing 0.005 M EDTA (pH 7.4) for homogenization and washing.

Vitamin D–Metabolite Extraction and Chromatography. The radioactivity present in the nuclei was extracted by a previously described chloroform–methanol procedure (Lund and DeLuca, 1966). The silicic acid column chromatography of the extracted vitamin D and its metabolites was carried out essentially as described elsewhere (Lund and DeLuca, 1966), but omitting the 100% diethyl ether elution which elutes no metabolites of vitamin D.

In Vitro Incubations of Nuclei with Enzymes. To obtain information pertaining to the nature of the binding of vitamin D and its metabolites to nuclei of intestinal mucosa, isolated nuclei were incubated with RNase, DNase, and trypsin. Rachitic chicks were given 10 IU of [1,2-^{3}H]vitamin D_3 intrajugularly in 0.025–0.04 ml of ethanol for 12 hr. The crude nuclear 700*g* fraction was isolated from a 10% homogenate

of the intestinal mucosa in 0.25 M sucrose plus TMK.[1] The crude nuclei were suspended and resedimented at 700*g* two times in 0.25 M sucrose plus TMK, suspended in 0.88 M sucrose plus TMK and sedimented at 1800*g*, and finally washed again in the 0.25 M sucrose plus TMK solution. The nuclei obtained from 6.5 to 7.0 g of mucosa were dispersed in 12.0 ml of 0.25 M sucrose plus TMK. Aliquots (3 ml) of the nuclear suspension were mixed with 3.0 ml of 0.25 M sucrose plus TMK containing the enzyme. Concentrations of enzymes in the final incubation mixtures were RNase (Nutritional Biochemicals Corp., Cleveland, Ohio), 500 μg/ml; DNase (Sigma Chemical Co., St. Louis, Mo.), 500 μg/ml; and trypsin (DIFCO Laboratories, Inc., Detroit, Mich.), 25 μg/ml. The nuclease samples were incubated at 31° in a water bath shaker oscillating at 120 cpm. The trypsin samples were incubated at 26°. At various times 0.50-ml aliquots were removed, diluted to 10.5 ml with 0.25 M sucrose plus TMK, and centrifuged at 700*g* for 15 min at 4°. The resulting nuclear pellets were suspended in 2.0 ml of water, transferred to counting vials, and lysed by the addition of 1.0 ml of 0.1% sodium lauryl sulfate plus 0.04 M NaOH solution. The samples were evaporated almost to dryness and subsequently prepared and counted as described under the methods for liquid scintillation counting.

In addition, nuclei were incubated with trypsin (25 μg/ml) for 2 hr at 26°. This incubation mixture was then centrifuged at 85,000*g* for 1 hr in a Spinco L-2 ultracentrifuge equipped with a no. 40 rotor. The resulting pellets were solubilized and the ^3H was counted.

Electron Microscopy.[2] The nuclei and the nuclear membranes were examined by electron microscopy. Isolated nuclei were fixed in 3.5% glutaraldehyde, rinsed in 0.1 M cacodylate buffer (pH 7.4), containing 7.5% sucrose, postfixed in 1% osmium tetroxide, and embedded in Epon–araldite.

General Methods. Each pellet obtained by fractionation or isolation was dissolved in a final volume of 10 ml of water which contained 0.10 ml of 10% sodium lauryl sulfate and 0.10 ml of 2 N NaOH. Aliquots were used for tritium, protein, DNA, and phospholipid determinations.

Protein determinations were made utilizing the Folin phenol method (Lowry *et al.*, 1951), with bovine serum albumin used as the standard.

To determine what fraction of the nuclei in the original homogenates were represented by the purified nuclear preparations, it was assumed that all the DNA resided in the nuclei. DNA assays were made on aliquots of the homogenates and the purified nuclei by either the indole method (Ceriotti, 1952) or the diphenylamine method (Burton, 1956). Yeast DNA was used as the standard.

Lipids were extracted from aliquots of the "citrate" nuclei by the procedure of Bligh and Dyer (1959), and the organic phosphate in the aqueous methanol phase was determined by the Martin and Doty method (1949).

Tritium was counted in a Packard Tri-Carb liquid scintillation counter, Model no. 3000 (Packard Instrument Corp., LaGrange, Ill.), employing an external standard for counting efficiency determination. Samples were prepared for counting by placing aliquots of the homogenates or subcellular fractions in liquid scintillation counting vials and warmed under an infrared lamp almost to dryness. To each sample was added 0.5–1.0 ml of NCS solubilizing reagent (Nuclear-Chicago Corp., Chicago, Ill.). For the homogenates in 2.2 or 2.3 M sucrose solution, 1.5 ml of NCS was added to 0.5 ml of homogenate, followed by 1.0 ml of 2-ethoxyethanol. All samples were allowed to dissolve by standing overnight, and on occasion complete solution was facilitated by warming at 37° on a water bath shaker. Toluene counting solution (Herberg, 1960) was subsequently added.

Results

The subcellular location of ^3H from [^3H]vitamin D_3 in intestinal mucosa of vitamin D deficient, vitamin D fed, and vitamin D deficient actinomycin D treated rats is given in Table I. All animals received 10 IU of [^3H]vitamin D_3 intrajugularly for 8 hr. The mucosa was fractionated in 0.25 M sucrose plus 0.05 M Tris-Cl (pH 7.4), and the ^3H associated with each fraction is expressed as per cent of the total ^3H present in the mucosa. The crude nuclei contain 50–60% of the radioactivity in all cases. No significant differences are noted between the distribution in the vitamin D deficient and vitamin D fed groups. In these two groups approximately 20% of the ^3H present in the mucosa is associated with the cytoplasmic fraction, and 15–20% with the mitochondria plus microsomes. Very little radioactivity is present in the ribosomes. In those animals given actinomycin D, there is a significant increase in the amount of ^3H present in the cytoplasm, with lesser amounts present in all membrane fractions.

In Table II the intracellular distribution of 10 IU of 1,2[^3H]vitamin D_3 in intestinal mucosa after 4 and 8 hr in vitamin D deficient rats and after 12 hr in rachitic chicks is expressed as dpm/100 mg of protein. At 4 hr in the rat there is a significantly greater amount of ^3H associated with the supernatant (cytoplasmic) fraction than with the other fractions. By 8 hr no highly significant differences were noted among the various subcellular fractions, although the specific activity of the total homogenate is lower than for the other fractions. Of greatest importance is the observation that the highly purified "sucrose" nuclei have a specific activity four to five times that of the total homogenate in the chick at 12 hr after administration of the radioactive vitamin. One notes that the brush borders (microvilli) did not concentrate ^3H from [^3H]vitamin D_3 above that observed for the intracellular fractions.

In Table III the data for the subcellular location of ^3H from 10 IU of [^3H]vitamin D_3 in rat intestinal mucosa 4 and 8 hr after administration are expressed as total disintegrations per minute of ^3H in the mucosa.

[2] The authors are indebted to Drs. Nelson Westmoreland and Irving B. Sachs for their preparation of the electron micrographs.

TABLE I: Subcellular Distribution of ^3H in Rat Intestinal Mucosa 8 hr after a 10-IU Intrajugular Dose of [^3H]Vitamin D_3.[a]

Fraction	% Total Radioactivity in Mucosa		
	Vitamin D Deficient Rats	Vitamin D Fed Rats	Actinomycin D Treated Rats
Citric acid nuclei	12.1 ± 3.8	13.3 ± 3.3	8.4 ± 0.4
Crude nuclei	57.6 ± 3.4	63.0 ± 2.3	53.3 ± 2.5
Mitochondria	6.7 ± 0.4	7.8 ± 0.6	4.2 ± 0.1
Microsomes	10.3 ± 1.2	11.5 ± 0.1	6.9 ± 0.3
Ribosomes	0.6 ± 0.2		
Supernatant	22.8 ± 6.2	17.3 ± 8.9	40.5 ± 1.3

[a] All animals received 10 IU of [1,2-^3H]vitamin D_3 intrajugularly for 8 hr. The vitamin D fed rats received 50 IU of unlabeled vitamin D_3 daily for 7 days followed by 10 IU of the labeled vitamin. The actinomycin D treated rats were vitamin D deficient animals which received 100 μg of actinomycin D intraperitoneally 5 hr before being given 10 IU of the labeled vitamin. Fractionation by conventional differential centrifugation was performed in 0.25 M sucrose plus 0.05 M Tris-Cl (pH 7.4). The values represent the average of six to ten animals with the standard deviations.

TABLE II: Subcellular Distribution of ^3H in Intestinal Mucosa after a 10-IU Intrajugular Dose of [^3H]Vitamin D_3.[a]

Fraction	Time after Administration (hr)		
	4 (in rat)	8 (in rat)	12 (in chick)
Total homogenate	1490 ± 446	1265 ± 205	1145 ± 283
"Crude" nuclei		1930 ± 414	2065 ± 283
Sucrose nuclei			5150 ± 444
Citric acid nuclei	1537 ± 405	1680 ± 280	
Mitochondria	1570 ± 340	2300 ± 185	
Microsomes	1665 ± 140	3160 ± 1050	
Brush borders		2120 ± 40	
Supernatant	3180 ± 380	2200 ± 35	

[a] All animals received 10 IU of [1,2-^3H]vitamin D_3 intrajugularly for 4, 8, or 12 hr. Citric acid nuclei were isolated by the method of Mirsky and Pollister (1946). Brush borders (microvilli) were isolated by the method of Miller and Crane (1961). Sucrose nuclei were isolated by the method of Chaveau (1952). Other fractions were prepared by conventional differential centrifugation in 0.25 M sucrose plus 0.05 M Tris-Cl (pH 7.4). The values given represent the average of six to ten rats. Protein was determined by the Folin phenol method (Lowry *et al.*, 1951). Data are expressed as dpm/100 mg of protein.

Each value represents the average of pooled fractions from four to five pairs of animals. The results are consistent with those given in Table II for which the data are expressed as dpm/100 mg of protein.

From Table I it is apparent that 50–60% of the radioactivity from [^3H]vitamin D_3 is associated with the initial crude nuclear-debris fraction. To verify, in addition to microscopic examination, that we were obtaining essentially complete cell breakage by the use of the Potter-Elvehjem homogenizer and not accumulating a significant number of unbroken cells in the crude nuclear fraction, an alternative method of cell rupture was employed. The method used was a procedure described by Norman and DeLuca (1964) involving the preparation of a tissue homogenate in a 0.25 M mannitol solution with the aid of sand grinding in a mortar with a pestle. The resulting homogenates were microscopically observed to contain less than 1% unbroken cells. However, an increase in the number of damaged nuclei was noted. The localization of the radioactivity from 10 IU of [^3H]vitamin D_3 in the intestinal mucosa of vitamin D deficient rats homogenized by sand grinding in 0.25 M mannitol is given in Table IV. No significant differences are observed between the distribution of ^3H when the mucosal tissue is homogenized in 0.25 M sucrose by the use of the Potter-Elvehjem homogenizer (Table I) or in 0.25 M mannitol by sand grinding.

TABLE III: Subcellular Distribution of ^3H in Rat Intestinal Mucosa Following a 10-IU Intrajugular Dose of [^3H]Vitamin D_3.[a]

Fraction	Time after Administration (hr)	
	4	8
Homogenate	7670 ± 800	7950 ± 965
"Crude" nuclei	3840 ± 596	4385 ± 430
Mitochondria	350 ± 110	533 ± 54
Microsomes	582 ± 55	905 ± 171
Supernatant	2850 ± 710	1880 ± 426

[a] All animals received 10 IU of [1,2-^3H]vitamin D_3 intrajugularly for 4 or 8 hr. Fractions were prepared by conventional differential centrifugation in 0.25 M sucrose plus 0.05 M Tris-Cl (pH 7.4). Each value represents the average of pooled fractions from four to five pairs of animals. Data expressed as total disintegrations per minute of ^3H in mucosa.

TABLE V: Subcellular Distribution of ^3H from 10 IU of [22,23-^3H]Vitamin D_4 in Intestinal Mucosa of Vitamin D Deficient Rats 8 hr after Intrajugular Administration.[a]

Fraction	% Total ^3H in Mucosa
Citric acid nuclei	7.6 ± 1.4
Crude nuclei	55.0 ± 6.4
Mitochondria	8.7 ± 1.7
Microsomes	9.9 ± 0.5
Supernatant	23.6 ± 2.7

[a] Four rats were given 10 IU of [22,23-^3H]vitamin D_4 intrajugularly for 8 hr. The mucosa was homogenized and fractionated by conventional differential centrifugation in 0.25 M sucrose plus 0.05 M Tris-Cl (pH 7.4). The average values with the standard deviations are given for each fraction.

Since [22,23-^3H]vitamin D_4 was available and was used on occasion due to its higher specific activity (53,000 dpm/IU as compared to 26,000 dpm/IU for the [1,2-^3H]vitamin D_3), it was desirable to compare the subcellular distribution of [^3H]vitamin D_4 (Table V) with [^3H]vitamin D_3 (Table I) in the intestinal mucosa of vitamin D deficient rats. Although the biological activity of vitamin D_4 is reported to be only 50–75% that of vitamin D_3 (Windaus and Trautmann, 1937; Grab, 1936; H. F. DeLuca, unpublished data), no highly significant differences were noted in the subcellular localizations of the two vitamins.

The data in Table VI reveal that approximately 70% of the ^3H from 5000 IU of generally labeled [^3H]-vitamin D_3 (sp act. 1600 dpm/IU) is associated with the crude nuclear-debris fraction in intestinal mucosa. Only 50–60% of the ^3H from a 10-IU dose of [^3H]-vitamin D_3 is associated with the nuclear-debris fraction 8 hr after administration. This increased association with a higher dose of [^3H]vitamin D_3 may be attributed to nonspecific binding. Similar results have been observed by Wilson *et al.* (1967).

As shown in Table VII, "citric acid" nuclei were isolated from vitamin D deficient rats which had received 10 IU of [^3H]vitamin D_3. The ^3H associated with these nuclei is expressed as per cent of total ^3H in the mucosa, dpm/100 mg of protein, or dpm/100 μmoles of organic phosphate. No differences were observed over the 4–24-hr time period when the data are expressed using these three parameters. Approximately 12% of the ^3H present in the mucosa is associated with the citric acid nuclei.

Since 50–60% of the ^3H from a 10-IU dose of [^3H]-

TABLE IV: Localization of ^3H from 10 IU of [^3H]-Vitamin D_3 in Rat Intestinal Mucosa Homogenized by Sand Grinding in 0.25 M Mannitol.[a]

Fraction	% Total ^3H in Mucosa
Crude nuclei debris	53.5 ± 4.6
Mitochondria	5.4 ± 1.1
Microsomes	8.8 ± 0.7
Supernatant	30.1 ± 3.0

[a] Each animal received 10 IU of [^3H]vitamin D_3 intrajugularly for 8 hr. The muscoa was homogenized in a 0.25 M mannitol solution as described by Norman and DeLuca (1964) and fractionated by conventional differential centrifugation (see Methods). The values and their standard deviations were obtained from four animals.

TABLE VI: Subcellular Distribution of 5000 IU of [^3H]-Vitamin D_3 in Intestinal Mucosa of Vitamin D Deficient Rats 8 hr after Intrajugular Administration.

Fraction	% Total ^3H in Mucosa
Crude nuclei	71.0 ± 6.6
Mitochondria	7.1 ± 0.7
Microsomes	8.8 ± 1.1
Supernatant	11.3 ± 1.2

[a] Each rat received 5000 IU generally labeled [^3H]-vitamin D_3 intrajugularly for 8 hr. Fractionation was accomplished by differential centrifugation in 0.25 M sucrose plus 0.05 M Tris-Cl (pH 7.4). Each value represents the average of four animals.

TABLE VII: Distribution of 10 IU of [^{3}H]Vitamin D_3 in Citric Acid Nuclei from Rat Intestinal Mucosa.[a]

Data Expressed as	Time after Administration (hr)			
	4	8	12	24
Per cent total ^{3}H in mucosa	12.1 ± 0.2	12.2 ± 3.8	10.0 ± 1.2	13.1 ± 0.4
Dpm/100 mg of protein	1458 ± 198	1680 ± 280	1452 ± 168	1683 ± 0.4
Dpm/100 μmoles of organic phosphate	8010 ± 660		8090 ± 350	9075 ± 110

[a] Rats were given 10 IU of [1,2-^{3}H]vitamin D_3 intrajugularly for 4, 8, 12, or 24 hr. Citric acid nuclei were isolated by a modification of the Mirsky and Pollister (1946) method. Protein was determined by the Folin phenol method (Lowry *et al.*, 1951), and organic phosphate was measured by the method of Martin and Doty (1949).

TABLE VIII: ^{3}H in Nuclei after a 10-IU Intravenous Dose of [^{3}H]Vitamin D.

Nuclear Source	Method of Preparation	% of Total ^{3}H in Tissues[a]
Rat mucosa	1% citric acid	12.2 ± 3.8
Rat mucosa	2.2 M sucrose	46.4 ± 11.3
Rat liver	2.3 M sucrose	2.52 ± 0.57
Rat mucosal deoxyribonucleoprotein	EDTA + NaCl	9.6 ± 0.7
Chick mucosa	1% citric acid	14.8 ± 6.9
Chick mucosa	2.3 M sucrose	52.1 ± 8.4
Chick mucosa	2.2 M sucrose + 1% citric acid	15.1 ± 6.7
Chick mucosa	2.2 M sucrose, washed three times with 1% Triton X-100	22.0 ± 7.4

[a] Each value represents the average of four to eight animals. All animals were given 10 IU of [1,2-^{3}H]vitamin D_3 or [22,23-^{3}H]vitamin D_4. Rats were sacrificed after 8 hr and chicks after 12 hr.

vitamin D_3 was associated with the crude nuclear pellet isolated in 0.25 M sucrose solution, while only 12% was associated with the more highly purified citric acid nuclei, it was considered desirable to isolate nuclei by other methods. The results of various isolation methods are given in Table VIII. When nuclei are isolated from rat or chick intestinal mucosa using either 2.2 M sucrose solution (Chaveau, 1952) or a discontinuous sucrose gradient underlaying with 2.3 M sucrose (Blobel and Potter, 1966), approximately 50% of the ^{3}H in the mucosa is associated with these highly purified nuclei. This agrees well with the crude nuclei isolated from 0.25 M sucrose solution. When nuclei are isolated by the citric acid method (Mirsky and Pollister, 1946), or from 2.2 M sucrose to which has been added 1% citric acid, only 12% of the ^{3}H is associated with the nuclei from rat mucosa and 15% of the ^{3}H with chick mucosal nuclei. If nuclei from chick intestinal mucosa are isolated *via* the Chaveau method employing 2.2 M sucrose and subsequently washed three times with a 0.22 M sucrose solution containing 1% Triton X-100, the resulting nuclei contain only 22% of the total radioactivity present in the mucosa. Thus, approximately 60% of the ^{3}H originally associated with the nuclei has been removed by washing with 1% Triton X-100, lowering the level almost to that produced by the citric acid procedures. Deoxyribonucleoprotein (histone) was isolated from the nuclei of rat intestinal mucosa (Zubay and Doty, 1959). Approximately 10% of the ^{3}H was associated with the deoxyribonucleoprotein, indicating that nearly all of the ^{3}H from [^{3}H]-vitamin D found in citric acid nuclei is associated with this intranuclear component. As a point of comparison, nuclei were isolated by the discontinuous sucrose gradient method from the liver of vitamin D deficient rats which had received 10 IU of [^{3}H]vitamin D_3 for 8 hr. Only 2.5% of the total ^{3}H present in the liver was found in the nuclei as compared to 50% present in nuclei of intestinal mucosa.

In Figure 1 are shown electron micrographs of nuclei from intestinal mucosa of chicks isolated (A) from 2.2 M sucrose, and (B) from 2.2 M sucrose plus 1% citric acid. The outer nuclear membrane can be seen in A (arrow) but is badly fragmented or absent in B. A similar picture was obtained in nuclei isolated in 2.2 M sucrose but washed with 0.22 M sucrose plus 1% Triton X-100. Blobel and Potter (1966) have shown that Triton X-100 removes the outer membrane of liver nuclei, and similar results have been shown by Gurr *et al.* (1963) employing citric acid.

The data in Table VIII and Figure 1 suggest that a major site of accumulation of vitamin D in intestinal

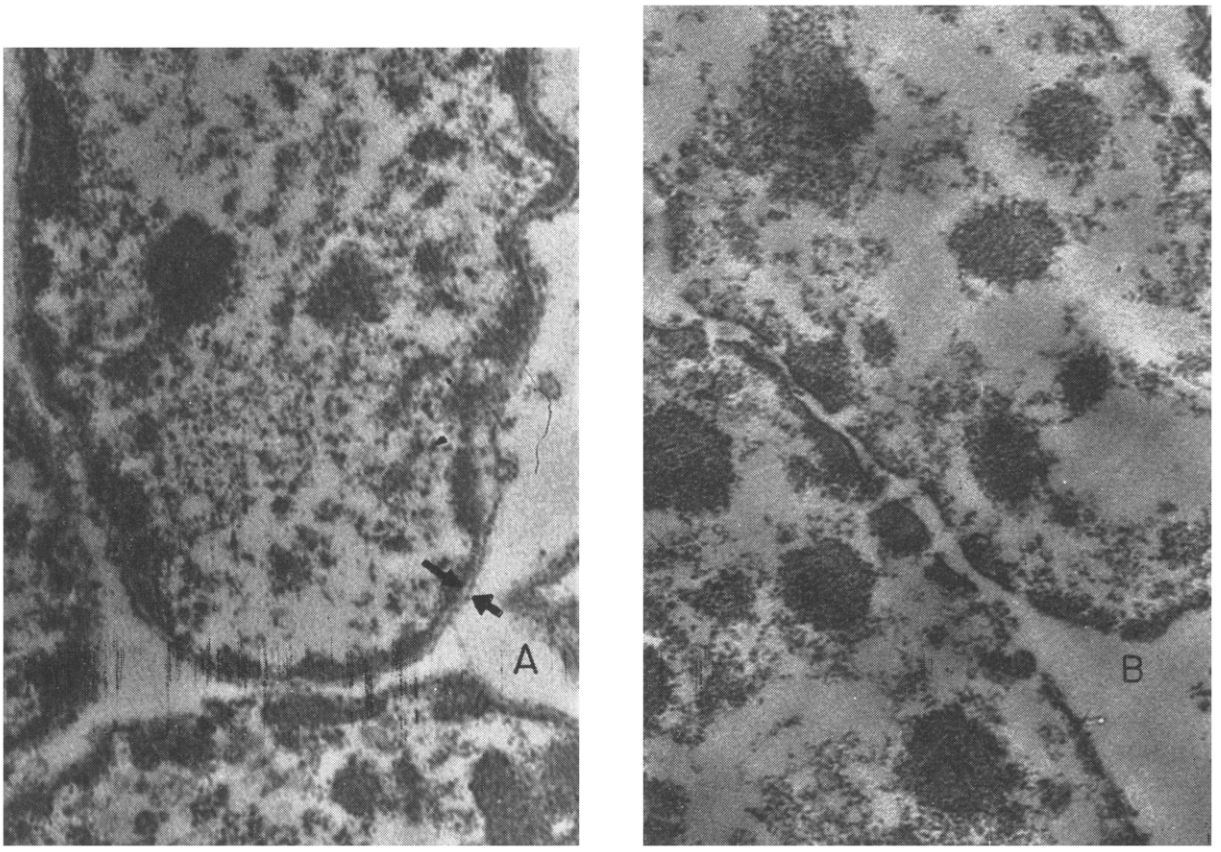

FIGURE 1: Nuclei from chick intestinal mucosa were isolated from (A) 2.2 M sucrose solution or (B) 2.2 M sucrose plus 1% citric acid. The outer nuclear membrane with ribosomes attached can be seen in A (arrow), but is badly fragmented or absent in B. Preparation for the electron microscopy is as described in the text. Magnification is approximately 30,000 ×.

mucosa of rat or chick is the nuclear membrane. Evidence that the nuclear receptor sites can be saturated is given in Table IX. The chicks receiving 10 IU of [^{3}H]vitamin D_3 served as the control, and the nuclei contained about 50% of total ^{3}H present in the mucosa. In chicks receiving excessively large doses (5000 IU) of [^{3}H]vitamin D_3, only 7.5% of the ^{3}H was associated with the nuclei. In vitamin D deficient chicks given 2500 IU (62.5 μg) of nonradioactive vitamin D_3 at 36 and 24 hr prior to sacrifice followed by 10 IU of [^{3}H]-vitamin D_3 12 hr before sacrificing, 18% of the ^{3}H was in the nuclei of the intestinal mucosa. This higher percentage when compared to the 7.5% obtained by using 5000 IU of [^{3}H]vitamin D_3 may be a reflection of vitamin D exchange or the experimental procedure. Chicks which received a total of 125 μg of 7-dehydrocholesterol prior to 10 IU of [^{3}H]vitamin D_3 contained almost 47% of the radioactivity present in the mucosa in association with the nuclei. The difference between this value and the 52% of the ^{3}H present in the nuclei of chicks which received only the 10 IU of [^{3}H]vitamin D_3 is not significant. In chicks which received a total of 125 μg of dihydrotachysterol-2 prior to 10 IU of [^{3}H]vitamin D_3, only 17.8% of the ^{3}H was associated with the nuclei. It is apparent that the nuclei of intestinal mucosa contain receptor sites capable of being saturated by vitamin D or its metabolite(s) as well as by dihydrotachysterol-2 or a metabolite(s) thereof, but not by 7-dehydrocholesterol.

The results of the *in vitro* incubation of nuclei (from the intestinal mucosa of chicks which had received 10 IU of [^{3}H]vitamin D_3 for 12 hr) with DNase, RNase, and trypsin are given in Figures 2 and 3 and Table X. The release of ^{3}H from the nuclei was followed. No loss of ^{3}H results on incubation of the nuclei with RNase (Figure 2). Trypsin is capable of liberating 35–40% of the ^{3}H, while DNase results in the rapid release of approximately 20% (Figure 3). The action of DNase followed by trypsin fails to release more of the ^{3}H than does trypsin alone. When nuclei were incubated for 2 hr with trypsin and subsequently centrifuged at 85,000*g* for 1 hr, all the ^{3}H from [^{3}H]-vitamin D_3 was recovered in the pellet (Table X), suggesting that the ^{3}H released by trypsin is in a bound and sedimentable form.

In Figure 4 is given the chromatographic profile of [^{3}H]vitamin D and its metabolites extracted from nuclei of chick intestinal mucosa. Peak I represents

TABLE IX: Prevention of ^3H Accumulation from [1,2-^3H]Vitamin D_3 in Chick Mucosal Nuclei.[a]

Treatment	% of Total Radioactivity in Mucosa
10 IU (0.25 μg) of [H^3]D_3	5.12 ± 8.4
5000 IU (125 μg) of [^3H]D_3	7.52 ± 0.3
5000[b] IU (125 μg) of D_3 + 10 IU of [^3H]D_3	18.0 ± 1.6
125[b] μg of 7-dehydrocholesterol + 10 IU of [^3H]D_3	46.6 ± 4.9
125[b] μg of dihydrotachysterol-2 + 10 IU of [^3H]D_3	17.8 ± 7.9

[a] All nuclei were prepared in 2.2 or 2.3 M sucrose. [b] 62 μg of nonradioactive vitamin D_3, 7-dehydrocholesterol, or dihydrotachysterol-2 was given 36 and 24 hr before sacrificing the chicks. The [1,2-^3H]vitamin D_3 was given 12 hr prior to sacrificing. Each value represents the average of four to six animals.

an ester of vitamin D, while peak III migrates identically with vitamin D_3, and peaks II and IV are as yet unidentified (Lund and DeLuca, 1966). As can be seen, approximately 80% of the ^3H is present as the peak IV metabolite(s) which has been shown to have a biopotency equivalent to that of vitamin D (Lund and DeLuca, 1966; Morii *et al.*, 1967), as assayed by the line test method (U. S. Pharmacopoeia, 1955), serum calcium response, and everted gut sac technique, and has been suggested to be the metabolically active form of the vitamin (Lund and DeLuca, 1966).

In Table XI is given the tissue distribution of radioactivity from 5 to 10 IU of [1,2-^3H]vitamin D_3 in propylene glycol (sp act. 26,000 dpm/IU) 24 hr after administration. Only 4.1% of a 5-IU dose given intraperitoneally and 2.2% of a 10-IU dose given intrajugularly were found to accumulate in the small intestine.

TABLE X: Incubation of Nuclei Containing ^3H from [^3H]Vitamin D_3 with Trypsin for 2 hr.[a]

	Dpm of ^3H in 85,000*g* Pellet
Control nuclei	788 ± 46
Trypsinized nuclei	791 ± 38

[a] Nuclei from rachitic chicks which had received 10 IU of [^3H]vitamin D_3 for 12 hr were isolated and incubated with trypsin (25 μg/ml) for 2 hr as described in the Methods. The resulting incubation mixtures were centrifuged for 1 hr at 85,000*g* at 4° in a Spinco L-2 ultracentrifuge. The pellets were solubilized and the ^3H was counted (see Methods). Each value represents the average of three determinations.

TABLE XI: Tissue Distribution of ^3H from [1,2-^3H]-Vitamin D_3 in Rachitic Chicks 24 hr after Administration.[a]

Tissue	Dose (%)	
	5 IU Intraperitoneally	10 IU Intrajugularly
Small intestine	4.1	2.2
Liver	13.0	6.5
Bone	21.5	20.1
Muscle	17.9	21.6

[a] Chicks received either 5 IU of [1,2-^3H]vitamin D intraperitoneally in propylene glycol or 10 IU intrajugularly for 24 hr. All tissue fractions were assayed for radioactivity by combusting an aliquot of the dried sample and measuring the tritiated water produced.

Discussion

The evidence herein presented indicates that a major site of accumulation of vitamin D and/or its metabolite(s) in intestinal mucosa of rat or chick is the nuclei (Tables I–VI). Of the radioactivity from [^3H]vitamin D which is associated with nuclei, 60–80% can be removed when the nuclear membrane is fragmented or stripped off with citric acid or Triton X-100 (Figure 1 and Table VIII). These results suggest that the primary site of action of the active form of vitamin D may be the nuclear membrane, as was previously proposed without verification (Zull *et al.*, 1966a).

From the fact that the "clean" nuclei can be saturated by using large doses of vitamin D (Table IX) or dihydrotachysterol-2 but not by 7-dehydrocholesterol we may suggest that the nuclei contain specific or pseudo-specific receptor sites. No vitamin D (antirachitic) activity is associated with 7-dehydrocholesterol, and thus it has no effect on the association of vitamin D with the nuclei of intestinal mucosa. Bosmann and Chen (1966) have reported that on the basis of growth and bone weights, the peak antirachitic response to dihydrotachysterol-2 was obtained with a daily dose of 10 μg. Doses below this did not produce complete healing of the rachitic state in chicks. Chen and Bosmann (1964) earlier observed that the minimum daily requirement of vitamin D_3 was 0.15–0.25 μg (6–10 IU) for the chick. Although the daily antirachitic dose of dihydrotachysterol-2 is large compared with that of vitamin D_3, the 125-μg total dose given is sufficient to produce an antirachitic response and account for the prevention of the accumulation of ^3H in the nuclei of chick intestinal mucosa from a 10-IU (0.25 μg) dose of [1,2-^3H]-vitamin D_3 (Table IX).

In addition, the saturation of the vitamin D binding sites by dihydrotachysterol-2 but not by 7-dehydrocholesterol can be explained on the basis of their stereochemical configurations. Dihydrotachysterol-2

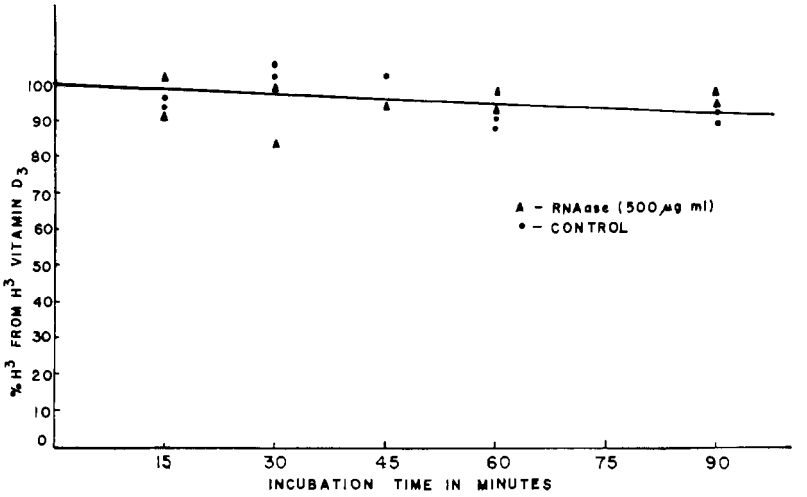

FIGURE 2: *In vitro* incubation of isolated nuclei from chick intestinal mucosa with RNase. Nuclei were isolated from the intestinal mucosa of rachitic chicks which had received 10 IU of [1,2-^{3}H]vitamin D_3 for 12 hr (see Methods). The nuclei were incubated at 31° with 500 µg/ml of RNase and the release of ^{3}H was followed.

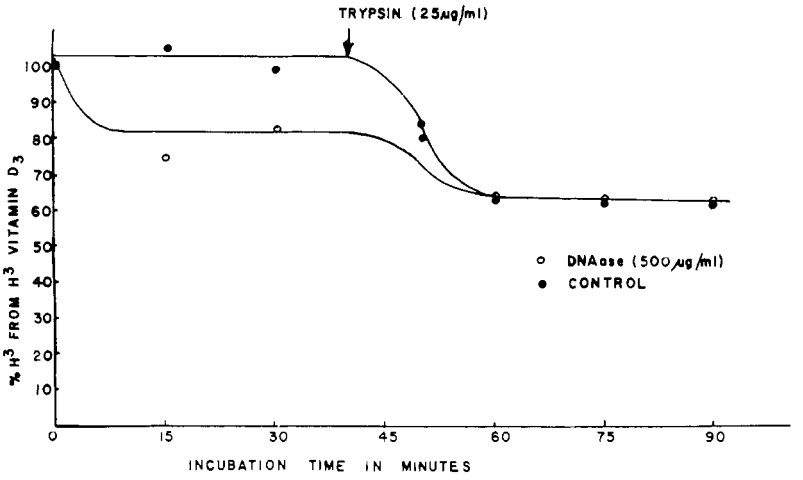

FIGURE 3: *In vitro* incubation of isolated nuclei from chick intestinal mucosa with DNase and trypsin. Nuclei were isolated from the intestinal mucosa of rachitic chicks which had received 10 IU of [1,2-^{3}H]vitamin D_3 for 12 hr (see Methods). The nuclei were incubated at 31° with 500 µg/ml of DNase for 40 min at which time 25 µg/ml of trypsin was added to both the control and the DNase containing mixtures and the incubation was continued at 26°. The rate of release of ^{3}H was followed.

and -3 are reduction products of vitamins D_2 and D_3, respectively, and possess nearly equal antirachitic potencies in the chick (Bosmann and Chen, 1966). The stereochemical configuration of the dihydrotachysterols much more closely resembles that of vitamin D than does 7-dehydrocholesterol, whose B ring is still intact.

The failure to observe a decrease in the amount of ^{3}H associated with the "crude" nuclei in vitamin D fed rats (Table I) or animals given large doses of labeled vitamin D (Table VI) may be attributed to nonspecific binding by these impure nuclear fractions. Such nuclear fractions contain much cellular debris, cell and cytoplasmic membranes, some unbroken cells, nuclei, as well as many nuclei with cytoplasmic adhesions (tabs). Similar results have been reported by Wilson *et al.* (1967). They observed that vitamin D fed rats accumulated a greater per cent of the isotopic label in the nuclear-debris fraction than did vitamin D deficient rats. In addition, increasing the dose of [^{3}H]vitamin D_3 by a factor of 200 (from 100 to 20,000 IU) resulted in an increased accumulation of ^{3}H in the nuclear-debris fraction in both vitamin D fed and deficient animals.

When the subcellular localization of 10 IU of [1,2-^{3}H]vitamin D_3 and [22,23-^{3}H]vitamin D_4 in the intestinal mucosa of vitamin D deficient rats was compared

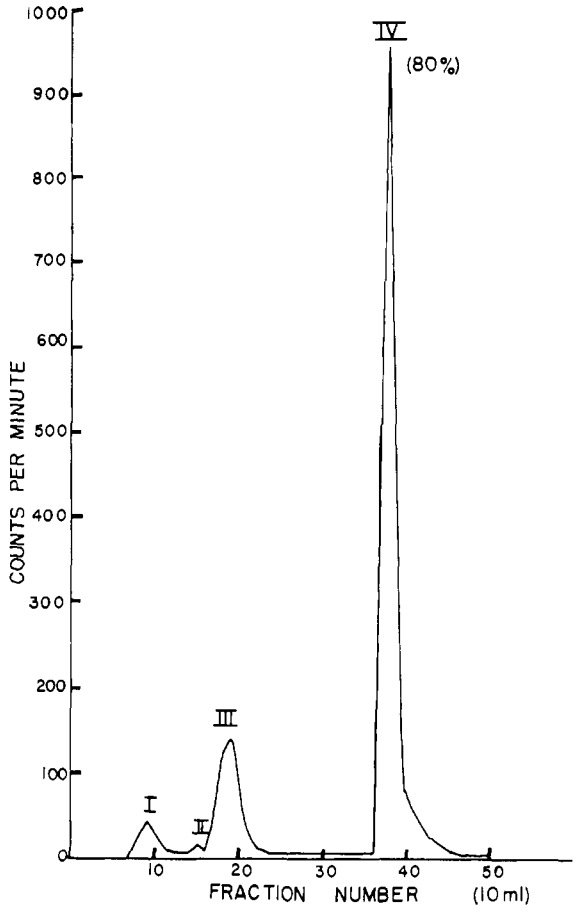

FIGURE 4: Radioactivity was extracted from chick mucosal nuclei by a previously described chloroform–methanol procedure (Lund and DeLuca, 1966). Silicic acid column chromatography of vitamin D and its metabolites was performed as previously reported from this laboratory (Lund and DeLuca, 1966).

(Tables I and V), no significant differences were noted. This suggests that both the side chain and the ring system remain intact, and that their modes of action are identical. Why then is vitamin D_4 only 50–75% as active as vitamin D_3? Tissue distribution studies have shown that the two forms of the vitamin are taken up equally as well by all tissues in the rat (DeLuca, 1967). However, a much greater loss of ^3H from [^3H]-vitamin D_4 results from blood, bone, and muscle than of the ^3H from [^3H]vitamin D_3. A corresponding increase in the ^3H present in the feces *via* the bile was observed. It is possible that some metabolite of the vitamin D_4 is discriminated against and excreted in large amounts resulting in a lowered effective lifetime of this vitamin or its metabolically active form in the target tissues (DeLuca, 1967).

Homogenization by sand grinding (Norman and DeLuca, 1964) provides an alternative and more drastic method of cell breakage than the Potter-Elvehjem homogenizer. We can conclude from the results given in Table IV as well as microscopic examination that the high percentage (50–60%) of radioactivity from [^3H]vitamin D_3 in the initial crude nuclear-debris fraction is not due to the presence of a significant number of unbroken cells.

Some information pertaining to the nature of the binding of [^3H]vitamin D and its metabolites to the nuclei of chick intestinal mucosa was obtained by incubating isolated nuclei with DNase, RNase, and trypsin, and following the release of the ^3H from [^3H]vitamin D_3. No appreciable loss of ^3H results on incubation of nuclei with RNase (Figure 2), suggesting that little if any vitamin D or its metabolites is bound to RNA within the nuclei or the ribosomes associated with the outer nuclear membrane. Alternatively the binding may be with ribosomal protein and not the RNA itself. DNase (Figure 3) digestion results in a very rapid release of approximately 20% of the ^3H. The association of 20% of [^3H]vitamin D or its metabolites with the DNA or deoxyribonucleoprotein is consistent with previous results. That trypsin does not solubilize the vitamin D and its metabolites, but rather released them in a particle (protein) bound form, can be seen in Table X.

Haussler and Norman (1967) studied the subcellular location of ^3H from [^3H]vitamin D_3. They concluded that the radioactivity was (a) associated with the "chromatin," and (b) in the form of vitamin D. Both of these are at variance with the present results, although there is agreement that the nuclei contain most of the radioactivity of the mucosa. They also reported that some 40% of an intraperitoneal dose of 5 IU of [^3H]-vitamin D_3 accumulated in the small intestine. These results again fail to agree with the findings of others (Neville and DeLuca, 1966; Imrie *et al.*, 1967) who have reported that not more than 2–4% of the ^3H from [^3H]vitamin D_3 given intrajugularly localizes in that tissue. Attempts to reproduce the distribution data of Haussler and Norman were unsuccessful (Table XI). Regardless of the route of administration, less than 5% of the dose was found in the small intestine. The discrepancy may be related to the low specific activity (1000 dpm/IU or a total of 5000 dpm/chick) of the [^3H]vitamin D preparations used in their studies.

The subcellular localization of physiological mounts of estrogens in rat uterus has been reported (Noteboom and Gorski, 1965) to be essentially that found for vitamin D_3 in rat intestinal mucosa (Table I). Of the ^3H from [^3H]17β-estradiol, 50–60% was nuclear bound. The nuclear membrane was also implicated. Digestion with DNase or RNase had little effect on the binding of estradiol which was rapidly released upon incubation with trypsin. Furthermore, pretreatment with diethylstilbestrol inhibited the incorporation of the isotope into all cellular fractions, while pretreatment with 17α-estradiol, testosterone, and cortisol had no inhibitory effect. In addition, hexestrol, another estrogenic compound, was also found to be competitive, suggesting the presence of specific (binding) receptor sites.

The presence in the nuclei of 80% of the ^3H from [^3H]vitamin D_3 in the form of a polar metabolite(s)

known as peak IV also implicates the nucleus as the subcellular component initially involved in vitamin D action. It has previously been suggested that this metabolite(s) may be the metabolically active form of the vitamin (Lund and DeLuca, 1966). Haussler and Norman (1967) and Wilson *et al.* (1967) reported the observations of no vitamin D metabolites in the intestinal mucosa. The existence and biopotency of such metabolites has been well documented (Norman *et al.*, 1964; Lund and DeLuca, 1966; DeLuca *et al.*, 1967; Morii *et al.*, 1967; P. F. Neville, H. F. DeLuca, and R. Prees, in preparation).

The induction process which mediates the action of vitamin D is not known. Zull *et al.* (1966a) have proposed that vitamin D may interact with the nuclear membrane, changing its permeability to calcium, and that calcium itself then acts as the controlling agent. The direct interaction of vitamin D or its metabolites with the genetic material is another possibility. Translational control of gene expression (Cline and Bock, 1966) provides us with another alternative model for vitamin D regulation of protein synthesis. Although no direct evidence exists for such a mechanism, present knowledge complies with this model. Large amounts of vitamin D and its metabolite(s) are associated with the nuclear membrane which can be seen to contain numerous ribosomes (Figure 1A). In addition, significant amounts of vitamin D and its metabolites are present in the cytoplasm and associated with the microsomes. Vitamin D, its metabolite(s), or an associated binding protein may provide the necessary conformational control, regulating the synthesis of a calcium transferase or permease enzyme(s). The actual mechanism by which vitamin D exerts its action awaits further subcellular characterization. The significance of the subcellular distribution of vitamin D cannot be immediately evaluated for even a minor fraction may be the most functionally important.

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