Vitamin D Stimulation of [^{3}H]Orotic Acid Incorporation into Ribonucleic Acid of Rat Intestinal Mucosa*

Sidney J. Stohs, J. Elwood Zull, and H. F. DeLuca

ABSTRACT: Within 3 hr after vitamin D_3 administration to vitamin D deficient rats fed a diet normal in calcium and phosphorous, the incorporation of [^{3}H]orotic acid into the ribonucleic acid of intestinal mucosa is stimulated two- to threefold. This stimulation is completely blocked by actinomycin D, and is also observed in rats fed a high calcium rachitogenic diet where growth stimulation by vitamin D does not occur. Little or no effect of vitamin D_3 could be observed in liver and kidney.

At the physiologic level, it is now generally accepted that vitamin D plays an essential role in the absorption of calcium from the small intestine (Nicolaysen and Eeg-Larsen, 1953) and in the mobilization of mineral from bone (Carlsson, 1952). These actions are believed to be responsible for the well-known over-all action of the vitamin in elevating calcium and phosphorus of the blood serum which is in turn essential to normal bone mineralization (Rasmussen and DeLuca, 1963). Schachter and co-workers (Schachter and Rosen, 1959; Dowdle *et al.*, 1960) first established that vitamin D stimulates an active transport of calcium across intestinal wall. This was then confirmed by Harrison and Harrison (1960) and by Sallis and Holdsworth (1962). It is also well known that there is a 4–24-hr lag between the administration of vitamin D and the initiation of intestinal transport of calcium or bone mineral mobilization depending on the dosage and mode of administration (Carlsson and Hollunger, 1954; Sallis and Holdsworth, 1962; Schachter *et al.*, 1961; Zull *et al.*, 1966a,b).

With the advent of RNA and protein synthesis inhibitors, a new approach to the physiologic actions of vitamin D was possible. It has recently been demonstrated that actinomycin D, which is known to inhibit DNA-directed RNA synthesis, blocks completely the physiologic responses to vitamin D (Eisenstein and Passavoy, 1964; Zull *et al.*, 1965, 1966a,b; Schachter and Kowarski, 1965; Norman, 1965; Harrison and Harrison, 1966). Doses of actinomycin D ranging from 0.66 to 1.0 μg/g have been employed in these studies. At least partial inhibition could also be obtained with 5-fluoroorotic acid and puromycin. Evidence was further presented that the actinomycin block was not due to general toxicity or inhibition of vitamin D absorption, transport, and localization (Zull *et al.*, 1966a,b). It was also shown that actinomycin administered 3–4 hr after vitamin D was no longer able to block the physiologic responses (Zull *et al.*, 1966a,b). Thus it appeared that the physiologic actions of vitamin D must be mediated by DNA transcription into RNA and subsequent protein synthesis. It would be expected therefore that, in the target tissues, vitamin D would stimulate the synthesis and thus pulse labeling of RNA by its precursors. This was in fact demonstrated (Zull *et al.*, 1966b), and it is the purpose of the present communication to report this important finding in detail.

Experimental Procedure

Preparation of Rats. Male weanling rats (Holtzman Co., Madison, Wis.) weighing 50–60 g were housed in individual hanging wire cages and fed a diet adequate in calcium (0.47%) and phosphorus (0.3%) as previously described by Steenbock and Herting (1955). Fat-soluble vitamins were given orally in cottonseed (Wesson) oil three times a week. The animals were vitamin D deficient after 3–4 weeks, as was evidenced by reduced growth and lowered serum calcium levels. In those experiments in which rachitic rats were used, male weanlings were maintained for at least 3 weeks on diet 2965 of Steenbock and Black (1925) which has a high Ca : P ratio.

Isotopes. Either [^{3}H]orotic acid (generally labeled) obtained from International Chemical and Nuclear Corp., City of Industry, Calif., or [5-^{3}H]orotic acid obtained from New England Nuclear Corp., Boston, Mass., was employed.

Methods. Two vitamin D deficient rats, fasted 12–14 hr, were given either 2000 IU of vitamin D_3 intraperitoneally in 0.5 ml of aqueous solution containing 0.9% NaCl and 0.1% Tween 40 or 10 IU intrajugularly in 0.04 ml of 95% ethanol. Two deficient animals were used as controls and were given the appropriate solution without the vitamin. All animals were given 50 μc of tritiated orotic acid 1 hr prior to sacrificing. They were killed by a sharp blow on the head followed by

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exsanguination either 2, 3, 4, 5, 8, or 12 hr after having received vitamin D (or the blank solution). Approximately 45–50 cm of the duodenal end of the small intestine was quickly removed and chilled in an ice-cold solution containing 0.25 M sucrose and 0.05 M Tris-chloride (pH 7.4). The intestinal segment was removed, quickly slit open, washed in the sucrose solution, blotted on filter paper, and laid on a glass or aluminum plate which rested on ice. The mucosa was removed by scraping with a microscope slide (Crane and Mandelstam, 1960) and weighed on a torsion balance. A 10% homogenate of the combined mucosa from the two rats was prepared in the sucrose solution with a Potter-Elvehjem homogenizer fitted with a Teflon pestle (A. H. Thomas Co.). The homogenate was centrifuged for 10 min at 600*g* in a Lourdes refrigerated centrifuge equipped with a 9-RA rotor. The resulting pellet, containing primarily nuclei, cell debris, and unbroken cells, was used as such for RNA extraction and will be referred to as the crude nuclear pellet. The nuclear-rich fraction of kidney and liver was prepared in a manner similar to that described for intestinal mucosa. Mitochondria were obtained by centrifuging the 600*g* supernatant at 7000*g* for 10 min. Microsomes were prepared from the 7000*g* supernatant by centrifuging at 85,000*g* for 90 min in a Spinco Model L-2 equipped with a No. 40 rotor. All of the pellets obtained by centrifugation were washed once with the 0.25 M sucrose solution. Animals receiving actinomycin D were given 100 μg intraperitoneally in 0.5 ml of freshly prepared water–ethanol (9:1) solution 2 hr before administration of vitamin D_3 or control solution and were sacrificed 5 hr later.

All pellets were maintained at −20° until needed. RNA was extracted from the crude pellets by a hot phenol method modified after the procedure of Gierer and Schramm (1956). The frozen pellets were thawed and resuspended in cold 0.01 M acetate buffer (pH 5.1). Sodium dodecyl sulfate (1 ml of 10%) and approximately 20 mg of bentonite/g of tissue were added. The suspension was adjusted to a final volume of 15 ml with the acetate buffer and the stoppered tubes were placed in a 65° water bath for 5 min. The nuclear suspension was emulsified with an equal volume of 80% phenol at 65° and the mixture was shaken for 3 min at this temperature. The mixture was rapidly cooled and the emulsion was broken by centrifuging at 10,000*g* for 5–10 min. The aqueous phase was removed by pipet, and the phenol phase, pelleted material, and interphase were discarded. The aqueous phase was extracted twice more with hot phenol with additions of bentonite and sodium dodecyl sulfate. After the last phenol extraction, traces of phenol were removed by extracting twice with 10 ml of ether, and residual ether was removed by bubbling N_2 through the solution.

For the extraction of RNA from the 85,000*g* supernatant (cytoplasmic) solution, the supernatant was adjusted to pH 5.1 with 2 N HCl. Addition of sodium dodecyl sulfate and bentonite was made, and the extraction of RNA from 15 ml of this solution was performed by the phenol method as described above.

RNA was precipitated from the final clear aqueous solution by the addition of sodium acetate to a final concentration of about 0.2 M and two volumes of cold 95% ethanol. After 12–18 hr at −20° the RNA was recovered by centrifugation, dissolved in 0.01 M acetate buffer (pH 5.1), and subsequently reprecipitated twice as described above. The RNA was dissolved in 0.01 M acetate buffer and the absorbancy determined at 260 mμ with either a Beckman DU or a Cary Model 15 spectrophotometer. Aliquots were placed in liquid scintillation counting vials and dried under an infrared lamp. NCS solubilizing reagent (0.5 ml) (Nuclear Chicago Corp., Chicago, Ill.) was added, followed by the addition of toluene counting solution several hours later. The samples were counted in a Packard Tri-Carb liquid scintillation counter no. 3000 (Packard Instrument Corp., LaGrange, Ill.), employing an external standard, and the data were expressed as specific activity in disintegrations per minute per optical density.

[^{3}H]RNA Hydrolysis and Paper Chromatography. To show that [^{3}H]orotic acid was indeed being incorporated into RNA, [^{3}H]RNA, which had been isolated from the crude nuclear pellets of rat intestinal mucosa during the course of the various experiments, was pooled. The RNA was hydrolyzed to the mononucleotides by heating at 95° in a water bath for 60 min in 0.05 M KOH (Dr. R. M. Bock, personal communication). Separation of the mononucleotides was performed by the use of two paper chromatographic systems (Chargaff and Davidson, 1955). Whatman No. 1 paper was employed for both ascending systems. Solvent system A was composed of isopropyl alcohol–concentrated HCl–water (65:16.7:18.3), while system B contained saturated ammonium sulfate–0.1 M sodium phosphate buffer (pH 6.0)–isopropyl alcohol (79:19:2). Development was carried out at 29° with a 60-min equilibration time. Standard solutions of the mononucleotides (AMP,[1] CMP, GMP, and UMP) were employed for the identification of the components in the [^{3}H]RNA hydrolysate. The components were located on the paper chromatograms using ultraviolet light. The separated spots were cut out and eluted with 0.01 M acetate buffer (pH 5.1). The absorbancies at 260 mμ as well as the ultraviolet spectra were determined with a Model 15 Cary recording spectrophotometer. The samples were dried and counted as previously described.

Results

Nuclear Ribonucleic Acid (nRNA) in Rat Intestinal Mucosa. Table I shows that a two- to threefold stimulation of [^{3}H]orotic acid incorporation in nRNA results following intraperitoneal injection of 2000 IU of vitamin D_3. A maximum response occurs at 3–5 hr

[1] Abbreviations used: AMP, CMP, GMP, and UMP, adenosine, cytidine, guanosine, and uridine monophosphates.

TABLE I: Influence of 2000 IU of Vitamin D_3 on the Incorporation of [^{3}H]Orotic Acid into nRNA in Rat Intestinal Mucosa.[a]

Time (hr)	Specific Activity of [^{3}H]RNA (dpm/OD)	
	Vitamin D	Control
2	258 ± 8 (4)	166 ± 13 (4)
3	376 ± 58 (10)	134 ± 22 (10)
5	323 ± 23 (8)	178 ± 21 (8)
8	207 ± 83 (8)	233 ± 99 (8)

[a] Vitamin D deficient rats maintained on a diet of normal calcium and phosphorus were sacrificed 2, 3, 5, and 8 hr after having received 2000 IU of vitamin D intraperitoneally. All rats received 50 μc of [^{3}H]orotic acid 1 hr before being killed. Values in parentheses represent number of animals.

TABLE III: Effect of Actinomycin D on Vitamin D Stimulated Incorporation of [^{3}H]Orotic Acid into nRNA of Rat Intestinal Mucosa.[a]

Treatment		Sp Act. of [^{3}H]-RNA (dpm/OD)
Actinomycin D	Vitamin D	
+	0	76 ± 5
+	+	67 ± 9
0	+	137 ± 11
0	0	80 ± 6

[a] Vitamin D deficient rats maintained on a diet of normal calcium and phosphorus were given 100 μg of actinomycin D or saline. Two hours later they were given 2000 IU of vitamin D_3 or a blank solution. Each animal received 50 μc of [^{3}H]orotic acid 2 hr after vitamin D or blank solution and all were sacrificed 1 hr later. Each value represents the average of four animals.

after administration of the vitamin, using an *in vivo* 1-hr pulse labeling of RNA with [^{3}H]orotic acid. By 8 hr after vitamin D administration, no effect of the vitamin is observed on RNA synthesis. In experiments not shown here, a vitamin D stimulation of ^{32}P incorporation into RNA could also be demonstrated 3 hr after administration of the vitamin.

The wide deviations were found to occur in part from pooling results from animals from different experiments. Such deviations may be due in part to minor variations in size and age of the animals since for some experiments results from different experimental groups were pooled. One must also bear in mind that these are whole animal experiments. Within each experimental group of animals the results showed much smaller standard deviations. In any case, statistical significance is clearly established.

Table II indicates that a stimulation of [^{3}H]orotic acid into nRNA from rat intestinal mucosa occurs following a 10-IU intrajugular injection of vitamin D_3. With this smaller dose a much longer lag period is observed. As previously stated, it is well known that there is a lag of 4–24 hr between the time of administration of vitamin D and the initiation of intestinal transport of calcium depending on the dosage and mode of administration. By 12 hr after administration, little effect of the vitamin is observed on RNA labeling, a phenomenon also observed with the 2000-IU dose.

Table III shows that the vitamin D stimulation of

TABLE II: Influence of 10 IU of Vitamin D_3 on the Incorporation of [^{3}H]Orotic Acid into nRNA in Rat Intestinal Mucosa.[a]

Time (hr)	Specific Activity of [^{3}H]RNA (dpm/OD)	
	Vitamin D	Control
5	69 ± 2 (4)	70 ± 10 (4)
8	119 ± 8 (4)	77 ± 6 (4)
12	97 ± 3 (4)	73 ± 2 (4)

[a] Vitamin D deficient rats maintained on a diet normal in calcium and phosphorus were sacrificed 5, 8, and 12 hr after having received 10 IU of vitamin D intrajugularly in 0.04 ml of ethanol. All rats received 50 μc of [^{3}H]orotic acid 1 hr prior to sacrificing. Values in parentheses represent number of animals.

TABLE IV: Influence of Vitamin D_3 on the Incorporation of [^{3}H]Orotic Acid into Microsomal, Mitochondrial, and cRNA in Rat Intestinal Mucosa.[a]

Fraction	Specific Activity of [^{3}H]RNA (dpm/OD)	
	Vitamin D	Control
Microsomes	31 ± 5 (8)	18 ± 3 (8)
Mitochondria	57 ± 4 (4)	59 ± 8 (4)
Cytoplasm	268 ± 17 (8)	200 ± 21 (8)

[a] Vitamin D deficient animals were sacrificed 3 hr after having received 2000 IU vitamin D_3 intraperitoneally or a control solution. All rats received 50 μc of [^{3}H]orotic acid 1 hr before sacrificing. The values in parentheses are the number of rats.

TABLE V: Influence of Vitamin D_3 on nRNA Labeling in Intestinal Mucosa of Rats on a High Calcium–Low Phosphate Diet.[a]

Time after Vitamin D (hr)	Specific Activity of [^{3}H]RNA (dpm/OD)	
	Vitamin D	Control
3	793 ± 205	307 ± 172

[a] Vitamin D deficient animals maintained on a diet with high Ca:P ratio (Steenbock and Black, 1925) received 2000 IU of vitamin D_3 or blank solution 3 hr prior to sacrifice. All rats received 50 μc of [^{3}H]orotic acid 1 hr before being killed. Each value represents the average of eight rats.

nRNA synthesis at 3 hr is completely blocked by a dose of 100 μg of actinomycin D/animal. This is in harmony with the earlier reports that this dose of actinomycin D blocks the *in vivo* action of vitamin D on intestinal transport of calcium, and provides some insight pertaining to this inhibition.

One hour after a dose of [^{3}H]orotic acid and 3 hr after 2000 IU of vitamin D_3, a stimulation in RNA labeling of the microsomes and cytoplasm, but not mitochondria, can be observed (Table IV). Unpublished results indicate that there is no stimulatory effect on labeling of rRNA. The cytoplasmic RNA (cRNA) would be composed of sRNA, mRNA, and precursor rRNA.

Table V contains the results of the effect of vitamin D_3 on the synthesis of nRNA in the intestinal mucosa of rats on a diet with a high Ca:P ratio. The stimulation of RNA labeling is obtained in rats on a diet containing other than normal calcium and phosphorus levels. The higher specific activities of the RNA may be due to the fact that these rachitic rats weighed 80–100 g as compared to 150 g for rats fed the adequate calcium and phosphorus diet used in the case of the data shown in Tables I–IV and VI–VIII. All rats in each case received 50 μc of [^{3}H]orotic acid. It should be noted that the growth of rats fed a high calcium diet such as the one used in this experiment is actually inhibited by vitamin D (Steenbock and Herting, 1955). This contrasts with an actual growth stimulation by the vitamin in cases of either low calcium or normal calcium and phosphorus diets such as those used in the experiments described in Tables I–III.

The data in Table VI express the specific activity of the [^{3}H]RNA as a function of the ^{3}H pool size in the rat intestinal mucosa from which the RNA was isolated. A two- to threefold stimulation of RNA labeling is still obtained under the influence of vitamin D_3 when the data are expressed in this manner.

Figure 1 indicates that a pulse of [^{3}H]orotic acid of 1 hr prior to sacrificing the animal provides maximal incorporation of ^{3}H into the RNA of intestinal mucosa, thus confirming preliminary experiments from which the arbitrary 1-hr pulse labeling period was selected.

Location of Incorporated ^{3}H in Pyrimidine Moieties of RNA. Table VII verifies that the administered [^{3}H]orotic acid is incorporated into the pyrimidine moieties of RNA, as would be expected. Solvent system A provided good paper chromatographic separation of UMP, but with overlapping of the AMP, CMP, and GMP. Solvent system B separated the purine mononucleotides from the pyrimidine mononucleotides, but gave the two pyrimidine mononucleotides as a single spot on the paper chromatogram.

Further evidence that we were indeed working with RNA is given in Figure 2 by the ultraviolet absorption spectra of the isolated RNA and the UMP isolated from the hydrolyzed RNA. The spectrum of the isolated UMP coincides with that of the standard UMP having a maximum absorption at 262 mμ.

RNA Labeling in Kidney and Liver. Table VIII indicates that RNA labeling is also stimulated by

TABLE VI: Influence of ^{3}H Pool Size on the Incorporation of [^{3}H]Orotic Acid into RNA of Rat Intestinal Mucosa 3 Hr after Vitamin D_3 Administration.[a]

Expt	Dpm/OD/Total ^{3}H in Mucosal Homogenate		Stimulation Factor (+D/−D)
	+ Vitamin D_3 (%)	− Vitamin D_3 (%)	
1	0.146	0.045	3.25
2	0.106	0.055	1.93
3	0.150	0.053	2.81
4	0.167	0.054	3.09
Mean	0.142 ± 0.026%	0.052 ± 0.004%	2.77 ± 0.57

[a] Vitamin D deficient rats were given 2000 IU of vitamin D_3 or a blank solution intraperitoneally. Each animal received 50 μc of [^{3}H]orotic acid 2 hr later, and was sacrificed 1 hr after administration of the isotope. Aliquots of the mucosal homogenate were removed prior to preparation of the crude nuclear pellet and the radioactivity was counted. Each value represents the results from the combined mucosa of two rats.

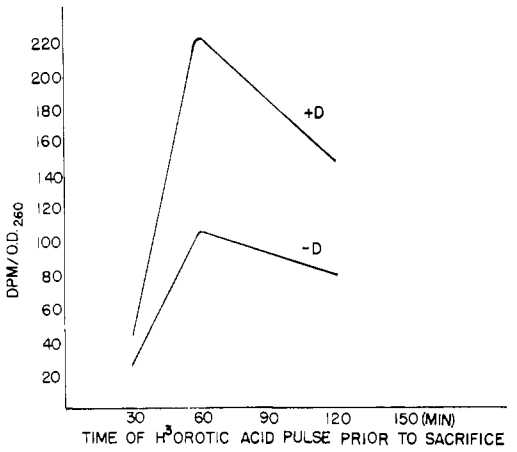

FIGURE 1: Vitamin D deficient rats were given 2000 IU of vitamin D_3 or a blank solution intraperitoneally. The animals received 50 μc of [^{3}H]orotic acid either 30, 60, or 120 min prior to sacrificing. Each point represents the average of two to four animals.

vitamin D in liver and kidneys, but to a much smaller extent. These tissues are not known to be affected by vitamin D. The high specific activities perhaps reflect either the greater metabolic activity of these tissues as compared to the intestinal mucosa or the greater uptake of [^{3}H]orotic acid from the blood.

Discussion

There is increasing evidence to suggest that the physiologic expression of vitamin D action may involve the synthesis of one or more key proteins. One of the first pieces of evidence centers around the fact that there is a time lag between vitamin D administration and its earliest recorded physiologic response (Schachter *et al.*, 1961; Carlsson and Hollunger, 1954; Zull *et al.*, 1966a,b). In addition, the small amounts of the vitamin required for a response, as well as congenital defects such as vitamin D resistant rickets, are consistent with a mechanism involving the translation of genetic information into the synthesis of a material which makes the expression of vitamin D action possible. Recently, strong support for such a hypothesis came as the result of experiments involving actinomycin D. This metabolic inhibitor is believed to exert its effect by combining with the guanine moieties of DNA (Hamilton *et al.*, 1963). The accepted concept for its mode of action is that it blocks DNA-directed synthesis of mRNA (Reich, 1964). It has been clearly demonstrated that this agent blocks the expression of vitamin D action. That the actinomycin D block is consistent with the hypothesis that vitamin D in some way initiated events involving an RNA–protein synthesis mechanism was clearly shown (Zull *et al.*, 1966a,b). Thus it would be expected that vitamin D might stimulate the rapid labeling of intestinal nRNA by a precursor such as [^{3}H]orotic acid. This has now been demonstrated.

From Table I it appears that an early event in vitamin D action is an increased synthesis of RNA in intestinal mucosa. A maximal response is obtained approximately 3–5 hr after intraperitoneal administration of the vitamin. Several explanations are possible for the lack of stimulation in RNA labeling 8 hr after the administration of 2000 IU of vitamin D to

TABLE VII: Specific Activities of [^{3}H]RNA and Its Hydrolysis Products Obtained from Rat Intestinal Mucosa 1 Hr after the Administration of 50 μc of [^{3}H]Orotic Acid.[a]

Sample	Sp Act. (dpm/OD)	Solvent System for Paper Chromatography
Hydrolyzed [^{3}H]RNA	73	–
Pyrimidine mononucleotides	176	B
Purine mononucleotides	1.1	B
Uridine mononucleotide	165	A

[a] [^{3}H]RNA was isolated from the intestinal mucosa of rats which had received 50 μc of [^{3}H]orotic acid intraperitoneally and were sacrificed 1 hr later. The RNA was hydrolyzed with 0.05 M KOH at 95° for 60 min, and separated by ascending paper chromatography on Whatman No. 1 paper. Solvent system A was composed of isopropyl alcohol–concentrated HCl–water (65:16.7:18.3) while solvent system B contained saturated ammonium sulfate–0.1 M sodium phosphate buffer (pH 6.0)–isopropyl alcohol (79:19:2).

TABLE VIII: Influence of Vitamin D_3 on [^{3}H]Orotic Acid Incorporation into nRNA of Rat Liver and Kidneys.[a]

3-Hr Vitamin D_3 Treatment	Specific Activity of [^{3}H]RNA (dpm/OD)	
	Liver	Kidney
+	20640 ± 1050	5830 ± 2930
0	15830 ± 1880	3830 ± 1630

[a] Vitamin D deficient rats maintained on a diet of normal calcium and phosphate for 4 weeks were sacrificed 3 hr after having received 2000 IU of vitamin D_3. All rats received 50 μc of [^{3}H]orotic acid 1 hr before being killed. All values represent the average of six animals.

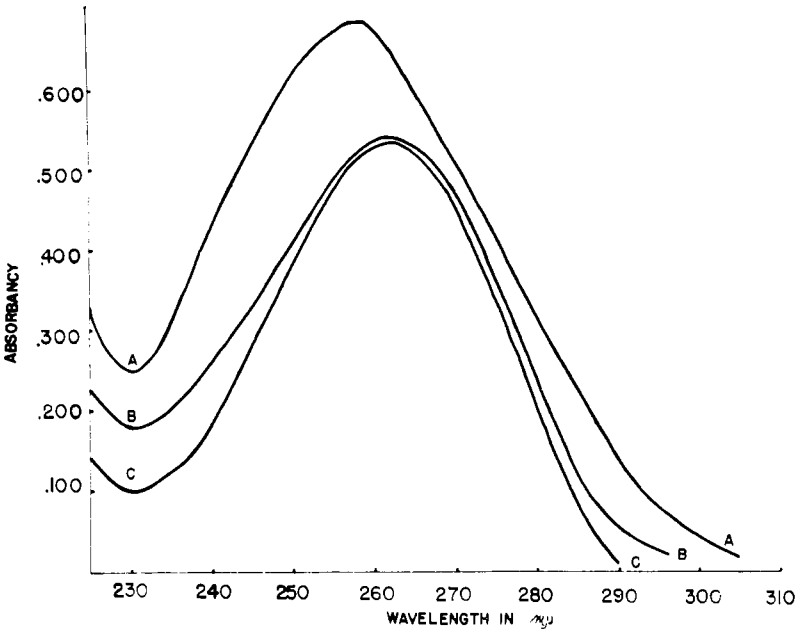

FIGURE 2: (A) Spectrum of RNA isolated from intestinal mucosa of rats, (B) spectrum of UMP obtained by paper chromotographic separation of alkaline-hydrolyzed [^{3}H]RNA obtained from rat intestinal mucosa, and (C) spectrum of standard UMP obtained from Pabst Laboratories, Division of Pabst Brewing Co., Milwaukee, Wis. (B) The [^{3}H]RNA was hydrolyzed with 0.05 M KOH at 95° for 60 min and separated on Whatman No. 1 paper using an ascending solvent system of isopropyl alcohol–concentrated HCl–water (65:16.7:18.3).

vitamin D deficient rats. In everted gut sacs, calcium transport may be maximal at 8 hr after a dose of 2000 IU (Zull *et al.*, 1966a). Thus intracellular calcium may be acting as a feedback inhibitor, blocking the synthesis of more RNA following the initial burst. This initial burst of RNA may then be sufficient to return the mucosa to a state of normal calcium transport, with subsequent small amounts of stimulated mRNA production masked by the mean RNA production and turnover. A less likely possibility is the depletion of RNA precursors following the two- to threefold increase in RNA production.

In Table II we observe that a 10-IU dose of vitamin D_3 elicits an RNA-labeling stimulation, although a longer lag is observed. A daily dose of 10 IU/rat is deemed as physiological (Carlsson and Lindquist, 1955). The longer lag is consistent with variations in vitamin D response depending on size of the dose and mode of administration. For example, using everted gut sacs and following calcium transport, a maximal response may be seen 8 hr after an oral dose of 2000 IU (Zull *et al.*, 1966a). A response is not seen until approximately 20 hr following a 10-IU oral dose, but may be seen at approximately 12 hr following a 10-IU intrajugular dose of vitamin D (Morii and DeLuca, 1967).

From Table III we see that the stimulation of RNA labeling 3 hr after vitamin D administration is completely blocked by actinomycin D. This suggests that vitamin D is in some manner stimulating the *de novo* synthesis of RNA. Our evidence indicates that increased labeling of microsomal and cRNA also occurs in response to vitamin D. This may be similar to that seen in hormonal stimulation of RNA synthesis. Wicks *et al.* (1965) and Greenman *et al.* (1965) have described the rapid stimulation of RNA synthesis in the seminal vesicles of castrated rats after testosterone administration, and in the livers of adrenalectomized rats treated with hydrocortisone. In both organs RNA synthesis increased two- to threefold within 2 hr of hormone treatment. Characterization of the RNA after differential thermal extraction revealed an increase in DNA-like RNA, rRNA, as well as a *de novo* synthesis of transfer RNA. A dose of 100 μg of actinomycin was capable of completely abolishing the hormonal effect, blocking not only mRNA synthesis but all RNA synthesis.

Great care was taken to purify the isolated RNA before it was counted for ^{3}H content. Thus the RNA was precipitated three times after phenol extraction. That RNA was really isolated is shown first by the ultraviolet spectra routinely checked (Figure 2) and by hydrolysis of the RNA with subsequent isolation of the pyrimidine mononucleotides. The results in Table VII and Figure 2 show the radioactivity of isolated RNA was in fact found in the UMP and other pyrimidine mononucleotides but not in the purine nucleotides.

In an attempt to examine the possibility that vitamin D may have effected a change in the ^{3}H nucleotide

pool size, thereby giving an increased ^3H content of RNA, the total ^3H content of the intestinal homogenates after [^3H]orotic acid dosage was measured. The results in Table VI show that correction of the RNA incorporation data for any changes in total ^3H content of the homogenates did not modify the effect of vitamin D, thus making this possibility unlikely.

In Table V the stimulation of [^3H]orotic acid incorporation due to vitamin D is observed in rats fed a high calcium rachitogenic diet. It has been previously observed that vitamin D suppresses growth in animals fed a diet with a high Ca:P ratio (Steenbock and Herting, 1955). This demonstrates that the increase in RNA labeling due to vitamin D is not merely a reflection of increased growth or enhancement of general metabolism. The vitamin D response in RNA labeling observed with this high calcium diet is also of interest when considering the hypothesis that the nuclear calcium concentrations might play an important role in the formation of the vitamin D induced calcium transport system (Zull *et al.*, 1966b). The present observations in no way exclude this possibility since it may only be in the presence of vitamin D or an active form thereof that calcium can penetrate certain cellular compartments or systems. Thus vitamin D and calcium concentration may operate in concert to bring about the events resulting ultimately in the well-recognized physiologic effects.

Although kidney and liver are not considered to be primary target tissues of vitamin D or directly involved in calcium transport, Table V indicates that the vitamin may exert a small stimulatory effect on RNA labeling. The statistical significance of the results is questionable. However, in all experiments the values obtained for the animals receiving the vitamin were higher than the values for the control animals. These results are particularly interesting in view of the large accumulation of [^3H]vitamin D_3 in liver and kidney 3 hr after its administration (Neville and DeLuca, 1966; Norman and DeLuca, 1963).

Though the response of RNA synthesis to vitamin D administration may involve all major types of RNA, it cannot be concluded that the effect of the vitamin is indiscriminate. Any selective effects may not be detected by the methods employed. Conversely, the stimulation of all major types of RNA may be an integral part of vitamin D mechanism of action. It may suggest that the synthesis of mRNA necessitates the synthesis of other types of supporting RNA. At present it is difficult to discern the full importance of this stimulation or precisely place it in the sequence of events which occurs following the administration of vitamin D. It does seem possible, however, that the physiologic expression of vitamin D action involves translation of genetic information through RNA to functional components.

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