VITAMIN D MEDIATED SYNTHESIS OF RAPIDLY LABELED RNA FROM INTESTINAL MUCOSA*

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One of the characteristic features of the physiological expression of vitamin D activity has been a time lag of 5-8, 10-15 or 15-25 hours between the administration of 20,000, 100 or 10 international units[1] (IU), of vitamin D_3 respectively, and the enhancement of calcium absorption across the intestinal mucosa of the chick (Norman, 1966). This delay is likely not due to a necessity for metabolic conversion of vitamin D by the adrenals as proposed by Sallis and Holdsworth (1965), nor is it due to a slow absorption and transportation of the vitamin to active sites. Haussler and Norman (1966) have found when 15-50 IU of ^{3}H-vitamin D_3 was administered orally or intracardially to rachitic chicks that maximal levels of radioactivity were present in the intestinal mucosa within one hour of the dose. Furthermore, it was observed that 70% of the radioactivity bound by the intestinal mucosa was associated with the nuclear fraction. Also Norman, et al. (1964) found no metabolites of ^{3}H-vitamin D_3 in the rat kidney or intestine which had full biological activity in comparison to the parent ^{3}H-vitamin D_3.

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[1] One international unit of vitamin D_2 or D_3 is 0.025 μg. Vitamin D_2 and D_3 have an equal antirachitic potency in the rat; in the chick vitamin D_3 is approximately 10 times more active than vitamin D_2 on a weight basis, Chen and Bosmann (1964).

As a consequence of these findings it has been proposed that the delay in biological response to vitamin D was due to the necessity for vitamin D to induce the synthesis of the appropriate enzyme systems or the alteration of membrane structure necessary for calcium absorption. This has been tested by the use of actinomycin D. Norman (1965, 1966) and Zull et al. (1965, 1966) have recently shown that in both the chick and the rat, actinomycin D has the ability to inhibit the action of a subsequent dose of vitamin D in promoting calcium absorption from the intestine. Norman (1966) has also reported that actinomycin D treatment for 36-48 hours could effectively convert a pre-vitamin D treated chick to a vitamin D deficient chick. This suggested a turnover time of 24-36 hours for the biochemical machinery associated with vitamin D-mediated calcium absorption.

It is the purpose of this paper to further test the validity of the vitamin D induction hypothesis by measuring the effect of the vitamin on the pulse labeling of intestinal mucosa RNA by ^{3}H-uridine.

Methods

Vitamin D deficient chicks were raised on a diet which has previously been described (Norman, 1966). Vitamin D_2 or D_3 was injected intracardially in 0.20 ml of 1,3-propanediol. At varying time periods after vitamin D administration, 4-12 μcuries of ^{3}H-uridine (5.1 c/mM) was injected intracardially in 0.20 ml of 0.15 M NaCl. Twenty minutes later the chicks were sacrificed, the small intestine excised and washed in ice-cold 0.25 M mannitol. The mucosa was scraped from the serosa with the aid of microscope slides, and then wrapped and frozen immediately in a dry ice-acetone bath.

RNA was isolated by a procedure similar to that used by Hiatt (1962) or Scherrer and Darnell (1962). The frozen mucosa from two chicks (3-5 g) was homogenized in a Waring blendor with 30 ml of 0.5% Na-laurylsulfate for 30 seconds. Thirty ml of liquified phenol was

added and the solution shaken at room temperature for 1 hour. The resulting emulsion was centrifuged at 4°C for 20 minutes at 8500 rpm and the supernatant aqueous solution removed. The phenol layer was washed and centrifuged 3 times with 25 ml of H_2O; each aqueous supernatant layer was combined with the first. After addition of 1/10 volume of 20% Na acetate and 2 volumes of 95% ethanol to the aqueous supernatant fraction, the RNA was precipitated overnight at 4°C. The precipitate was recovered via centrifugation, washed successively with 95% ethanol, acetone, and ether and air dried to constant weight.

Twenty mg of dried nucleic acid were dissolved in 1.5 ml of 0.5 N NaOH (overnight at 37°C). Two 0.50 ml aliquots were removed for liquid scintillation counting. The yield of nucleic acid averaged 15.3 ± 1.1 mg/gram of mucosa (14 determinations). It is quite likely, as suggested orginally by Hiatt (1962), that there is some DNA coprecipitated with the RNA. However, this constant inclusion will not affect the interpretation of the results. The purity of the isolated nucleic acid was determined by absorbancy at 260 mμ.

Results and Discussion

In Table 1 is shown the effect of 1/2-5 hour *in vivo* pretreatment with 5000 IU of vitamin D_3 on the 20 minute labeling of RNA by ^{3}H-uridine in the rachitic chick. One-half hour after vitamin D_3 administration there is a three-fold increase in the rate of RNA labeling. This is the most rapid metabolic effect of vitamin D that has been reported to date. Five hours after 5000 IU of vitamin D_3 there is however, only a modest increase in the rate of RNA labeling. These data may provide the first direct evidence that the primary biochemical response to vitamin D is the initiation or stimulation of RNA synthesis.

The rapid effect of vitamin D_3 on RNA labeling correlates well with the lag in stimulation of calcium absorption. With the large dose of 5000 IU of vitamin D_3, suboptimal enhancement of calcium absorption has

Table 1

Effect of Vitamin D_3 on RNA Labeling by ^{3}H-uridine

Time between Vitamin D_3 and ^{3}H-uridine (hours)	DPM/20 mg of nucleic acid Experiment A	Experiment B
-D control	180 ± 56	1385 ± 260
1/2	557 ± 114*	2391 ± 510*
1	432 ± 186*	2210 ± 370*
3	297 ± 116	----
5	264 ± 173	1691 ± 750

The experimental protocol is described under Methods. The -D, control chicks received an intracardial injection of 0.20 ml of 1-3 propanediol 1/2 hour before ^{3}H-uridine injection. Each number is the average plus or minus the standard deviation for duplicate samples obtained from 4 pairs of chicks.

*Difference between ± D treatment is significant at $P \leq 0.01$.

occurred within 4-5 hours after vitamin D administration and maximal stimulation is reached within 5-8 hours, Norman (1966). This implies that 3-5 hours after vitamin D_3 administration, DNA transcription and resulting protein synthesis should be nearly completed and that there should be little effect on the labeling of RNA.

In Table 2, experiment C, is shown the results of varying the dose level of vitamin D_3, and the ineffectiveness of vitamin D_2 to stimulate RNA labeling. The 100 IU dose of vitamin D_3 is not as effective as the 5000 IU dose, however, there is still a measurable increase in RNA labeling. This smaller effect on RNA labeling after 1 hour pretreatment with 100 IU of vitamin D_3 also correlates well with the fact that the lag for this level dose is 10-15 hours. One might not anticipate as large nor as rapid a response to this lower level of vitamin D.

Table 2

The Effect of Vitamin D and Inhibitors on

RNA Labeling by ^{3}H-uridine

Treatment	DPM/20 mg Nucleic Acid
Experiment C	
-D control	280 ± 31
+ D_3 (5000 IU)	462 ± 71*
+ D_3 (100 IU)	332 ± 26
+ D_2 (100 IU)	262 ± 21
Experiment D	
-D control	1865 ± 380
+ D_3 (5000 IU)	2870 ± 210*
+ actinomycin D, +D_3 (5000 IU)	560 ± 120*
+ o,p'-DDD, +D_3 (5000 IU)	400 ± 50*

The experimental protocol is described under Methods. The -D control chicks received an intracardial injection of only 1,3-propanediol 1 hour before ^{3}H-uridine injection. Vitamin D_2 or D_3 was injected intracardially in 1,3-propanediol 1 hour before ^{3}H-uridine. Actinomycin D (10 μg/120 g chick) was injected intraperitoneally 2 hours before vitamin D. 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (o,p'-DDD) was fed orally, (40 mg/chick/day in capsule form) for two days preceding the day of the experiment and also 2 hours before vitamin D_3 injection. Each number is the average plus or minus the standard deviation for duplicate samples obtained from 4 pairs of chicks.

*Difference between ± D is significant at $P \leq 0.02$.

The absence of any stimulation of RNA labeling by vitamin D_2 is not surprising in view of its low antirachitic activity in the chick[1].

In Table 2, experiment D, is shown the marked inhibitory effect of actinomycin D and 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (o,p'-DDD) on the pulse labeling of RNA. This inhibition by actinomycin D of DNA directed RNA synthesis agrees with the observations of earlier workers (Fujioka et al., 1963). The rationale for testing o,p'-DDD stems from the report of Sallis and Holdsworth (1962) that long term treatment (2-3 days) with o,p'-DDD would eliminate the enhancement of calcium transport brought about by vitamin D_3 in the

rachitic chick. This compound was known (Newcomer, 1959) to cause atrophy of the adrenal cortex. Thus Sallis and Holdsworth (1962) postulated that vitamin D_3 was transformed into an active compound by the adrenal cortex and that as a consequence in the presence of o,p'-DDD no response to vitamin D would be attained. The results of Table 2 show an effect of o,p'-DDD on the inhibition of RNA labeling and suggest that in reality Sallis and Holdsworth were inhibiting vitamin D action by inhibiting RNA synthesis.

The data presented in this paper demonstrate a direct stimulatory effect of vitamin D on RNA labeling and strongly suggests that the primary biochemical response of intestinal mucosa cells to vitamin D is probably the initiation of RNA and protein synthesis. It is still quite unknown though as to how the vitamin might actually participate in this induction process.

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