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STUDIES ON THE METABOLISM OF CALCIFEROL XIV: EVIDENCE OF A CIRCADIAN RHYTHM IN THE ACTIVITY OF THE 25-HYDROXYVITAMIN D_3-1-HYDROXYLASE#

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SUMMARY: The regulated production of 1α,25-dihydroxyvitamin D_3 by the renal enzyme 25-hydroxyvitamin D_3-1α-hydroxylase is known to be positively related to the calcium needs of the chick. The activity of this enzyme is now shown to exhibit a circadian-like rhythmicity with peak periods occurring every 20-26 hours. This rhythmicity in activity appears to be affected by the external light/dark cycle to which the birds are exposed.

Introduction

Vitamin D_3 is known to undergo a two-step metabolic conversion to a hormonally active form, 1,25-dihydroxyvitamin D_3 (1,2). This seco-steroid is responsible for stimulating intestinal calcium transport (3) and bone calcium mobilization (4). $1,25(OH)_2D_3$* is produced exclusively by a renal mitochondrially localized, cytochrome P450 containing enzyme, the 25-hydroxyvitamin D_3-1α-hydroxylase (5,6). The activity of this enzyme has been shown to be regulated by changes in both parathyroid hormone and $1,25(OH)_2D_3$ status (6), and plasma levels of calcium and phosphate (7,8).

We have studied in the vitamin D_3-replete chick, the time course of activation of the $25(OH)D_3$-1-hydroxylase* which was initiated as a consequence of dietary calcium deprivation. It is known that the activity of this enzyme is inversely related to the calcium content of the diet (8,9). During this study it was observed that the activity levels of the $25(OH)D_3$-1-hydroxylase increased as serum calcium levels fell; however, this increased activity was

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* Abbreviations: $25(OH)D_3$ = 25 hydroxyvitamin D_3; $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3; $24,25(OH)_2D_3$ = 24,25-dihydroxyvitamin D_3; $25(OH)D_3$-1-hydroxylase = 25 hydroxyvitamin D_3-1α-hydroxylase.

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not constant but displayed rhythmic high and low periods (9). This report describes in detail an experiment which demonstrates a circadian rhythm in the activity of the renal 25(OH)D_3-1-hydroxylase.

Material and Methods

Animals. One-day-old White Leghorn cockerels (obtained from Pace/Setter Hatchery, Ontario, California) were raised on a high calcium (2.5%), normal phosphate (0.8%) diet (10) for a period of 3 weeks. The diet was then changed to one containing low calcium (0.2%), normal phosphate (0.8%). Both the high and low calcium diets were supplemented with vitamin D_3. The chicks consumed their diet ad libitum and accordingly (based on weekly grain consumption records for several chicks) each bird received approximately 1.3 nmol (20 IU) vitamin D_3 per day. As a control group, 25 chicks were raised on a non-vitamin D_3-supplemented diet (10); these birds received 1.3 nmol (20 IU) vitamin D_3 per day administered orally with a syringe between 10:00 a.m. and 12:00 p.m.

Seventy-five chicks were maintained in a room providing 24 hours of continuous light (group A). Another 75 chicks (group B) plus the 25 control chicks (group C) were maintained in a room providing 18 hours light (lights on at 6:00 a.m.), and 6 hours dark. Four birds from each of groups A and B were killed at the following time intervals: 6-hour intervals beginning 18 hours prior to the dietary calcium change and continuing for 6 hours after the change, 4-hour intervals for the next 36 hours, and 6-hour intervals for the remaining 24 hours of the experiment. The 25 control birds (group C) were killed at 4-hour intervals beginning 14 hours after the dietary change.

25-Hydroxyvitamin D_3-1 -hydroxylase activity determination

Upon sacrifice, kidneys were removed from each bird, washed in ice cold 0.15 M NaCl and then homogenized in 0.25 M sucrose to yield a 10% whole kidney homogenate. The assay for enzyme activity was by the method of Henry et al. (6). This involves the conversion of 25(OH)-[26,27-^{3}H]-D_3* into 1,25(OH)$_2$-[^{3}H]-D_3 or 24,25(OH)$_2$-[^{3}H]-D_3*. Aliquots, removed from each incubation at 0, 4, 8, and 12 minutes, were extracted by the method of Bligh and Dyer (11). The chloroform layer was evaporated under N_2 and the sample was redissolved in approximately 0.5 ml chloroform-petroleum ether (65:35) and applied to a Sephadex LH-20 (0.8 x 23 cm) column. The substrate, 25(OH)D_3, and the products, 1,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$, are separated completely on this size column. The eluting solvent was chloroform-petroleum ether (65:35). Fractions were collected in the following manner: 18 ml containing the 25(OH)D_3, 3 fractions of 2 ml each, a 10 ml fraction containing 24,25(OH)$_2D_3$, 3 fractions of 2 ml each, a 20 ml fraction containing the 1,25(OH)$_2D_3$, one fraction of 2 ml and 2 washes of 20 ml each. All fractions were transferred to liquid scintillation vials and the solvent was evaporated under air. Eight ml of liquid scintillation mixture (5 g of phenylbiphenyloxadiazole-1,3,4 per liter toluene) was added to each vial and the radioactivity measured in a Beckman LS-200 or LS-233 liquid scintillation counter. The percent of radioactivity recovered as 1,25(OH)$_2D_3$ was plotted as a function of incubation time. The rate of production of 1,25(OH)$_2D_3$ was determined from the slope of this line. Picomoles of product produced per minute were calculated and protein concentrations were determined by the Biuret method (12).

Serum calcium levels

Serum calcium levels were determined using a Perkin-Elmer 300 atomic absorption spectrophotometer on 0.2 ml serum mixed with 5.8 ml of 5% $LaCl_3$ in 25% HCl. A standard curve was prepared from the primary standard of calcium carbonate.

Chemicals

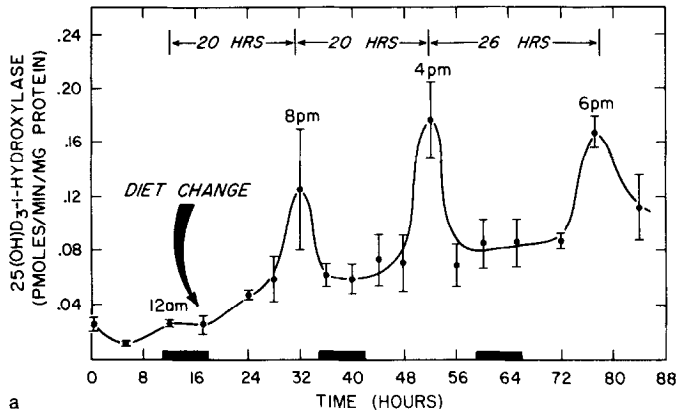
25-hydroxy-[26,27-^{3}H] vitamin D_3 (Amersham-Searle, Chicago) was diluted with nonradioactively labeled 25-hydroxyvitamin D_3 (kindly supplied by Hoffmann-LaRoche, Nutley, NJ) to yield a specific activity of 75 Ci/mole.

Results and Discussion

The 25 control birds (group C) that received daily oral doses of vitamin D_3 showed no difference in body weight, serum calcium levels, or in the $25(OH)D_3$-1-hydroxylase activity levels from those birds that had received the vitamin D_3-supplemented diets. Hence, in these experiments, providing the vitamin D_3 *ad libitum* in the diet was an acceptable method of supplying each bird with its daily requirements of the vitamin.

The $25(OH)D_3$-1-hydroxylase activity for birds maintained on the light/dark cycle (group B) and for those on the continuous light cycle (group A) is presented in Figures 1(a) and (b), respectively. For both groups of birds, an increase in enzyme activity occurred within 15-18 hours from the time of initial dietary calcium deprivation, and for each group the enzyme activity fluctuated in a rhythmic manner.

In comparing the enzyme rhythmicity for these two groups, it is apparent that the light/dark cycle birds (group B) exhibited longer periods of low enzymatic activity; these nadir points of activity correspond with the dark periods in the birds' cycle. This may indicate that periods of light and darkness alter the endogenous rhythmicity of the enzyme. Also it is apparent that the peak activity periods do not occur at the same time of day for the light/dark cycle birds (group B) as for the continuous light cycle birds (group A); nor is the time period between peaks of activity the same for both groups of birds. Peak activity periods were observed to occur every 20 to 26 hours for the group B birds. For the group A birds, two peak activity periods occurred at 12 hour intervals immediately following dietary calcium



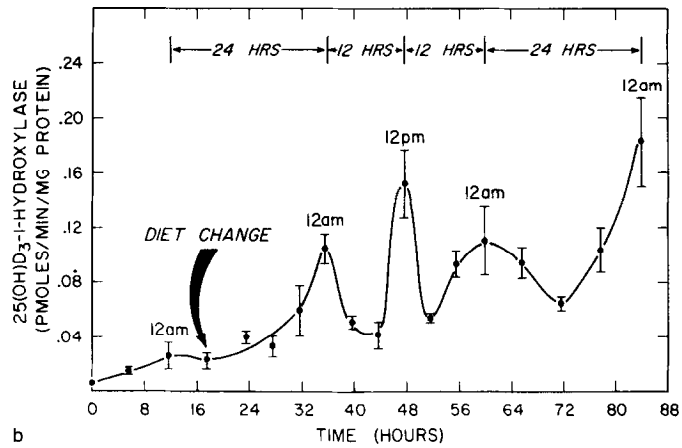


Fig. 1(a) and (b). Time course of changes in the activity of the $25(OH)D_3$-1-hydroxylase following dietary calcium deprivation. One day old male chicks were raised on a high calcium (2.5%), vitamin D_3-supplemented diet for 3 weeks. They were then placed on a low calcium (0.2%), vitamin D_3-supplemented diet. Enzyme activity measurements began prior to the dietary change and continued for 66 hours after the change. Each point represents the mean of 4 birds ± standard error. The time intervals between the activity peaks are shown at the top of each figure.

Figure 1(a) shows the change in the $25(OH)D_3$-1-hydroxylase activity for birds maintained on an 18 hour light and 6 hour dark cycle. The shaded bars on the abscissa correspond to periods of darkness.

Figure 1(b) shows the change in enzyme activity for birds maintained on the 24 hours continuous light regime.

change; the activity peaks then appeared to resume an apparent 24 hour periodicity. These differences in the length of the period may also be attributable to an effect of the external light/dark cycle on the rhythmicity of the enzyme as has been seen with other circadian enzymes (13).

Further studies are needed to determine the regulatory parameter(s) of this diurnal rhythm. One possibility is parathyroid hormone (PTH). The plasma concentration of PTH has been shown to exhibit a circadian rhythm in humans (14) and PTH is a known regulatory factor of the activity of the $25(OH)D_3$-1-hydroxylase (6).

In summary, these studies indicate that $25(OH)D_3$-1-hydroxylase activity fluctuates in a circadian manner and that these fluctuations in activity may be affected by visible light.

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