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Oscillatory Behavior of Control-Systems of Calcium Homeostasis in Chickens

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Abstract Computer simulation of calcium homeostasis in chicks predicted an oscillatory behavior of bone calcium flow and kidney 25-hydroxyvitamin D_3-1-hydroxylase with a periodicity of 56 h and a 9 h phase difference between the two signals. In growing chickens subjected to a light: dark cycle of 22:2 h, and intravenously dosed with ^{45}Ca, the temporal changes in plasma ^{45}Ca could be described by an exponential decline with superimposed diurnal oscillations. The activity of the renal 25-hydroxyvitamin D_3-1-hydroxylase in chicks subjected to a 12:12 h light: dark cycle ALSO followed diurnal oscillations, with a nadir at the beginning of the light period and a peak 12 h later. The production of 1,25-dihydroxyvitamin D_3 by primary cultures of chicken kidney cells oscillated with a periodicity of 5.6 h or shorter. It is suggested that despite the differences in phase and periodicity between the simulation predictions and actual results, the oscillations in both 1-hydroxylase and bone calcium flow could be coupled through the hormonal systems involved in regulation of plasma calcium. © 1994 Wiley-Liss, Inc.

Key words: diurnal cycles, Zeitgeber, vitamin D, bone, hydroxylase, renal cells

In some regulated biological systems, a periodic behavior is considered the normal mode of operation conferring a positive functional advantage for the organism [Rapp, 1987]. Until recently, plasma calcium concentration had been viewed as one of nature's constants although evidence has been accumulating on the existence of temporal oscillations in plasma calcium concentration and some of its controlling systems in growing rats [Staub et al., 1988], dogs [Wong and Klein, 1984], humans [Carruthers et al., 1964; Halloran et al., 1985; Jubitz et al., 1972; Markowitz et al., 1981, 1988], and chickens [Miller and Norman, 1979, 1982]. The significance of such oscillations in calcium homeostasis has been reviewed by Parfitt [1987].

In mature female chickens, diurnal patterns due to rhythmic perturbations introduced by the reproductive cycle have been described for plasma calcium [Taylor and Hertelendy, 1961] and some of the calcium regulatory systems such as calcium absorption [Hurwitz and Bar, 1965; Hurwitz et al., 1973] and uterine calbindin gene expression [Bar et al., 1992]. These patterns are associated with the periodic egg shell calcification process and the accompanying high calcium needs.

An algorithm that simulated the system of calcium homeostasis in the growing chicken [Hurwitz et al., 1987a] suggested the existence of spontaneous oscillations in the controlled-plasma Ca^{2+} concentration, as well as in the various controlling systems which constitute the regulatory machinery. The appearance of oscillations in the calcium control system was found to be dependent on the process of growth. Furthermore, in agreement with actual observations in dogs [Wong and Klein, 1984], the model predicted that oscillations in the control system would be abolished by the feeding of a very low calcium diet [Hurwitz et al., 1987b]. Also in agreement with the study with dogs, but in contrast with studies with rats [Staub et al., 1988], the model predicted the essentiality of a normal parathyroid function in the generation of the oscillations. In effect, an analysis of the simulation results suggested that the spontaneous oscillations resulted from lag periods among the responses of the various calcium control systems, providing the components essential for generation of oscillations [Friesen and Block,

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1984]. Specifically, the spontaneous oscillations in the calcium control systems resulted from the dual action of parathyroid hormone [PTH] in promotion of bone resorption, on the one hand, and the stimulation of the activity of the renal 25-hydroxyvitamin D_3-1-hydroxylase system [1-hydroxylase], on the other, with a phase of approximately 9 h between them. Experimental evidence for such spontaneous oscillations in the calcium control systems in growing chickens was previously provided for 1-hydroxylase activity by Miller and Norman [1979, 1982]. In the present study, oscillations predicted by model-simulation-1-hydroxylase activity and Ca flow from bone to blood were studied by model simulation and experimentally in chicks in an effort to validate model predictions and to elucidate the mechanisms involved.

MATERIALS AND METHODS

The Simulation Algorithm

A computer algorithm was used to simulate kidney 1-hydroxylase and bone-to-blood calcium movement together with other control signals of the plasma calcium homeostasis system of a 1–3 week old chick. The model is shown schematically in Figure 1. As detailed previously [Hurwitz et al., 1987a,b], the model includes a series of differential equations describing the contributions of intestinal absorption, kidney, and bone to plasma calcium. These major control systems are regulated by PTH and $1,25[OH]_2D_3$, the concentration of which is the integral of their formation and decay rates. The formation of the hormones is in turn feedback-related either directly or indirectly to plasma calcium, and their decay follows first order kinetics. Chick growth is described in the model by a Gompertz growth equation. Calcium intake is calculated using simulated feed intake based on the dietary energy requirements which vary with body size and growth rate.

The model equations were included in a Stella® simulation program [Richmond and Peterson, 1992] operating on a Macintosh computer.

Bet Dagan Experiment

Day-old male Cobb broiler chicks were obtained from a commercial hatchery [Hafetz-Haim] and raised in battery brooders situated in

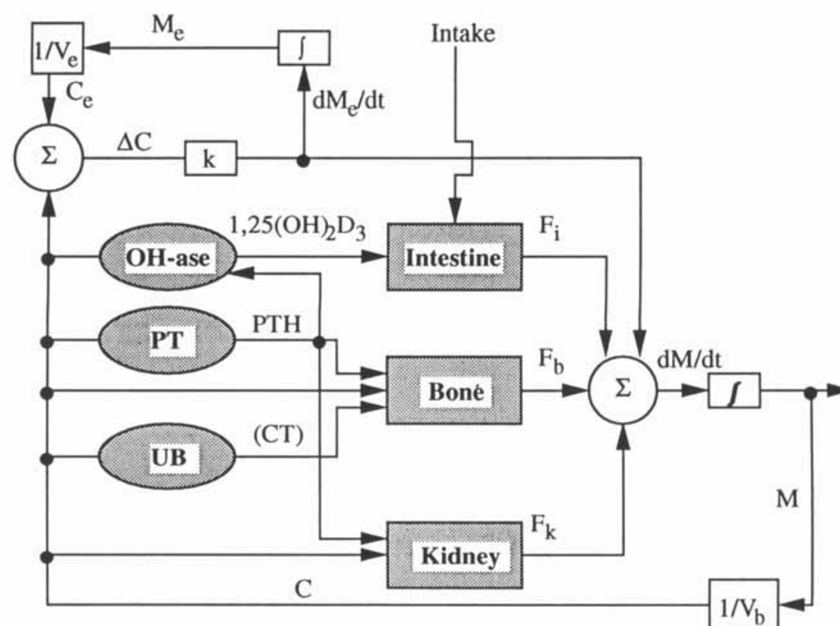

Fig. 1. Feedback regulation of plasma calcium concentration [C]. The sum [Σ] of the net calcium flows from intestine [F_i], bone [F_b], and kidney [F_k] is the change in total plasma calcium [dM/dt] which when added [∫] to existing plasma calcium and divided by the blood volume [V_b], yields the plasma calcium concentration [C]. Plasma calcium then undergoes a rapid exchange with extracellular calcium [M_e], which upon division by the extracellular volume [V_e] yields extracellular calcium concentration [C_e]. Plasma calcium concentration determines kidney calcium excretion [F_k], bone calcium flow [F_b], calcitonin [CT] secretion by the ultimobranchial gland [UB], parathyroid hormone [PTH] from the parathyroid gland [PT], and production of $1,25[OH]_2D$ by the kidney 25-hydroxycholecalciferol-1-hydroxylase system [OH-ase]. Bone flow and $1,25[OH]_2D$ production are also controlled by PTH.

windowless constant-temperature rooms at 24°C. Fluorescent lighting provided the required daylight of 22 h light–2 h dark cycle. This cycle was chosen in order to maintain synchronization of diurnal rhythms. The short dark phase of 2 h only, just shorter than the passage time through the chick gastrointestinal tract [Sklan et al., 1975], avoided an interrupted supply of nutrients and calcium expected during longer dark periods [Wong and Klein, 1984]. The chicks were fed a diet designed to satisfy the recommendations of the National Research Council [1984] and composed primarily of corn, sorghum grain, and soybean oil meal. The diet contained 1% calcium and 0.7% phosphorus.

The experiments were started at the age of 24 days, when the birds weighed 922 ± 69 g [average ± SD]. Six groups of eight birds was given an intravenous [brachial vein] dose of 79 μc/bird ^{45}Ca. A sample of 1 ml blood was obtained from the opposite brachial vein at different time intervals after dosing, so that each group was bled only once daily. The order of bleeding was alternated every day in order to minimize introduction of any artifact by the experimental procedure. Furthermore, samples were taken only during the light hours, leaving the birds undisturbed during darkness.

Heparin was used as an anticoagulant. The collected blood was immediately centrifuged and an aliquot of the separated plasma was placed in scintillation liquid and counted in a liquid scintillation detector equipped with a computerized correction for quenching and chemical luminescence. Aliquots of the injected dose and blanks were counted at the same time with the same amount of nonradioactive plasma, thus keeping counting conditions constant. Plasma calcium was determined by an automatic fluorimetric titration.

For calculation, the radioactivity of the injected dose was corrected for body weight at the time of injection. Plasma specific radioactivity could then be expressed as percentage dose [corrected] per gram of calcium, by dividing by the plasma calcium concentration.

Riverside Experiments

In vivo 25 hydroxyvitamin D_3-1-hydroxylase. Day-old male White Leghorn chicks were obtained from Pace-Setter Hatcheries [Anaheim, CA]. They received ad libitum a moderately low calcium (0.6%) diet [Norman and Wong, 1972] and were maintained in a room providing constant temperature and a 12:12 h light-dark cycle, representing a "normal" 24 h periodicity, with the aid of incandescent illumination. When 3–4 weeks old, the birds were killed by decapitation in groups of 4 chicks at 0.5–1 h intervals over a 26 h period. At the time of sacrifice, care was taken not to interrupt the established light-dark cycle.

Kidneys were removed into ice-cold 0.15 M NaCl and immediately homogenized in 0.25 M sucrose to yield a 10% whole kidney homogenate. Aliquots were then taken for the 25-hydroxyvitamin D_3-1-hydroxylase assay.

25-hydroxyvitamin D_3-1-hydroxylase assay. The activity of the 1-hydroxylase enzyme was determined as described by Henry and Norman [1974]. The homogenate was incubated with 25-[26,27-^{3}H]-[OH]D_3 [specific activity 75 Ci/mole] at a final concentration of 2.5 μmole/l, in 0.05 M Tris-HCl (pH 7.4), 2 mM $MgCl_2$, and 10 mM malate. Aliquots were removed at 0, 4, 8, and 12 min of incubation and their lipid was extracted by a modification of the method of Bligh and Dyer [1959]. The lipid fraction was dried under N_2, dissolved in 5% isopropanol in hexane, filtered through 0.5 μ millipore filter, and redried. The fraction was then taken up with the same solvent and subjected to high-performance liquid chromatography. The solvent of the collected fractions was then evaporated and the samples were counted in a scintillation counter. The rate of production of 1,25[OH]$_2D_3$ was estimated from the slope of the function of the tritium-labeled metabolite recovered, over time of incubation.

Primary cultures of chick kidney cells. Three-week-old rachitic White Leghorn male chicks were decapitated and thoroughly rinsed with 70% ethanol. Their kidneys were perfused with 0.15 M NaCl under sterile conditions and then removed. The tissue was teased with forceps until fragments of approximately 1 mm were obtained. These were then incubated for 15 min with 2 mg/ml collagenase and 1.5 mg/ml hyaluronidase. Trypsin was added and the incubation was continued for 5 min. The cells were centrifuged and washed in Gibco Minimal Essential Medium (MEM). The pellet was then suspended to 5.5 × 10^5 cells/ml in MEM, and 10% fetal calf serum, and plated in 30 mm culture dishes. After 24 h of incubation at 37°C, 5% CO_2, the medium was changed to fresh MEM, with 10% fetal calf serum, and the cultures were incubated for 4 days when they reached conflu-

ency. The medium was then replaced with serum-free medium 4 h prior to initiation of each experimental incubation. The reaction was initiated by the addition of 25[OH]-[26,27-^{3}H]D_3 (specific activity, 75 Ci/mole) in ethanol to a final concentration of 2.5×10^{-8} M. After 30 min of incubation, the reaction was stopped by the addition of chloroform: methanol (1:2), and the cells were scrapped off the bottom of the culture dish. A lipid extract was then prepared for HPLC measurement of tritiated-1,25[OH]$_2$D$_3$, as described above.

Kinetic Models

Oscillations in 1-hydroxylase activity were described by

$$Y = \alpha \sin\left(2\pi \frac{(t - \tau)}{P}\right) + \beta, \qquad (1)$$

where, α is the amplitude (pmol/min/mg protein), t is the time of observation, τ is the phase, and P the periodicity, in hours. β is the average value, in pmol/min/mg protein.

To the best of our knowledge, except for two studies previously conducted by us in laying hens [Hurwitz, 1964], plasma ^{45}Ca kinetics has not been evaluated in birds in general and in the growing chicken, in particular. In the rat, ^{45}Ca kinetics could be described by a two compartment model within the time framework of 2 h to 3 days [Bronner, 1967; Hurwitz et al., 1969]. An 8-compartment non-linear model, capable of describing oscillations, was used by Staub et al. [1988]. Wong and Klein [1984] used repeated labeling with ^{45}Ca in dogs to overcome the complication due to the exponential temporal behavior, in order to demonstrate diurnal oscillations. The extremely rapid growth of chicks which results in a non-linear temporal expansion of the various calcium pools impedes the application of classical models of compartmental analysis. Therefore, a non-mechanistic model has been used in the present study. Since no effort was made towards compartmental analysis, no assumption was necessary with regard to a steady state distribution of ^{45}Ca. The problem of fast early exponentials was avoided by including in the analysis data collected only after 24 h of ^{45}Ca dosing. Plasma ^{45}Ca specific activity following the single injection could be described by diurnal oscillations superimposed on an exponential decline. The amplitude of the diurnal oscillations was assumed to obey the same rate of decline as specific activity itself,

$$Y = e^{-\gamma t}\left[\alpha + \beta \sin\left(2\pi \frac{(t - \tau)}{P}\right)\right], \qquad (2)$$

where, α is the initial specific activity and β is the initial amplitude of oscillation [% dose/g Ca]; γ is the exponent of decay; and t and τ and P are the time, phase, and periodicity in hours, respectively.

Since bone contains over 99% of body calcium and since no slowly calcium exchanging compartments (relative to mixing in the extracellular pool) have been identified outside the skeleton, any increase in plasma ^{45}Ca during the exponential decline following the single dose may be assumed to have been the result of a bone-to-circulation isotope flow.

Numerical Analysis

Fitting of the kinetic model to the results and parameter estimation, were computed with the aid of SHAZAM [White and Horsman, 1986]. The program also computed the standard error and *t*-value for each of the estimated parameters, the correlation coefficient between the predicted and observed value, and the Durbin-Watson coefficient [Theil, 1971], which tests against an autocorrelation of the deviations.

RESULTS

Results of simulation presented in Figure 2 show a constant pattern of oscillations both in bone calcium flow and in 1-hydroxylase activity along with a decrease with age. The phase difference between these two signals of the plasma calcium homeostasis system is clearly visible, and estimated at approximately 9 h, in agreement with the previous simulation, conducted under conditions of a slightly lower weight gain [Hurwitz et al., 1987a]. Also in similarity to the previous study, periodicity of the oscillations in either signal was approximately 57 h.

^{45}Ca Kinetics

During the experiment, the birds gained weight at approximately 60 g/day. This rate of weight gain is normal for the respective age and suggests that the welfare of the birds was not affected appreciably by the experimental procedure. Analysis of the plasma calcium data failed to show any significant diurnal pattern, hence not presented.

^{45}Ca specific activity is presented in Figure 3, together with the functions fitted to the model described by Eq. 2. The estimated parameters are given in Table I. By visual inspection, the calculated functions appeared to have described the experimental data well. From the statistical viewpoint, t-values indicated high significance [$P < 0.01$] for each of the calculated parameters. Furthermore, the correlation coefficients between the observed and predicted values were highly significant [$P < 0.01$]. The Durbin-Watson coefficient was in the range indicative of no autocorrelation between the deviations, suggesting that the residuals are randomly distributed around the function. The amplitude of the oscillations was 7% of the initial intercept while the rate of exponential decay was approximately 1%/h. The calculated phase cannot be taken as an actual parameter since it is dependent on the time of start of analysis.

The specific activity declined starting from the maximum daily point 1 h after "sunrise"; nadir was reached 12 h later and was followed by an increase towards the dark period.

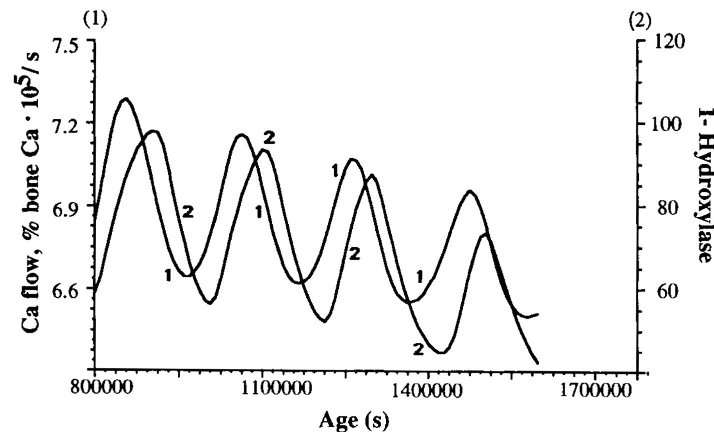

Fig. 2. Computer simulated values of bone calcium flow (% bone Ca × 10^5), and kidney 1-hydroxylase (μmolel 1,25[OH]$_2$D$_3$ produced/s/g body weight). Periodicity was determined at 56.6 and 56.7 h for bone Ca flow and 1-hydroxylase, respectively. The phase difference between the two signals was estimated at 8.64 h.

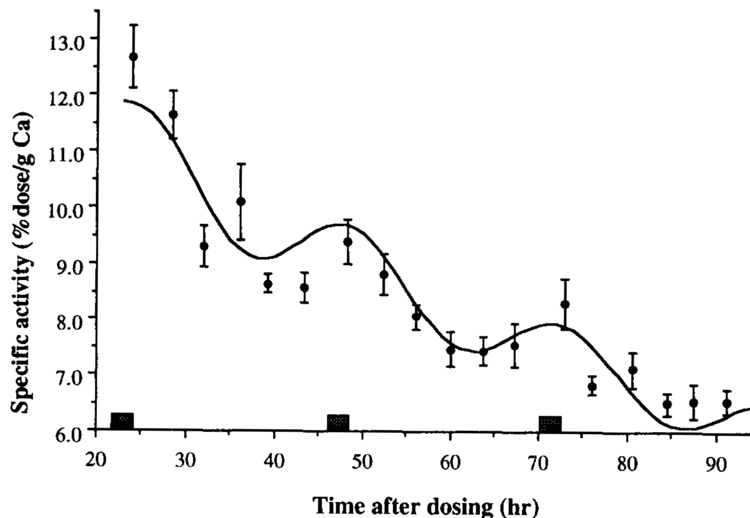

Fig. 3. Pattern of plasma ^{45}Ca activity in chicks following a single dose of the isotope. The fitted function is given by Equation 2 with P = 24 h. The dark periods are shown by the filled bars. No samples were taken during the dark periods. Vertical bars represent the standard error for each time point (n = 8). Estimated parameters are given in Table I.

TABLE I. Parameters and Statistical Analysis of the Oscillations in Specific Activity of ^{45}Ca in Plasma of ^{45}Ca-Injected Chicks*

Symbol	Description	Value	SE	*t* value
β	Amplitude, % dose/g Ca	0.7988	0.0224	3.572
τ	Phase, h	−1.841	0.292	6.317
ϵ	Intercept, % dose/g Ca	13.345	0.568	23.868
γ	Decay	0.0084	0.00077	10.904

*Parameters estimated from results given in Figure 3 using a model given in Equation 2. The periodicity, was taken as P = 24 h. Durbin-Watson coefficient, 2.151; R (correlation coefficient between predicted and observed), 0.949.

TABLE II. Parameters and Statistical Analysis of the Oscillations in the Renal 25-Hydroxy-Vitamin D_3-1-Hydroxylase Activity of Chicks*

Symbol	Description	Value	SE	*t* value
α	Amplitude, pmole/min/mg protein	0.1177	0.0255	4.609
τ	Phase, h	7.45	0.19	38.975
β	Average, pmole/min/mg protein	0.3063	0.0172	17.786

*Parameters estimated from results given in Figure 4 using a model given in Eq. 1. Periodicity was taken as 24 h. Durbin-Watson, 1.413; R (predicted vs. observed), 0.640.

In Vivo 25-Hydroxyvitamin D_3-1-Hydroxylase

Visual inspection of the results [Fig. 4] suggested that the activity of the 1-hydroxylase enzyme followed a diurnal cyclic pattern. The model fitting procedure resulted in highly significant three parameters (Table II) when periodicity was set at 24 h. The correlation coefficient between the observed and predicted values was highly significant [$P < 0.01$], but the Durbin-Watson coefficient left the possibility of an autocorrelation among the deviations. The peak and nadir of the oscillations were computed at 13.5 h or 1.5 h prior to the end of the light (peak) and dark period (nadir). The determined amplitude of the oscillation was about one third of the mean value.

In Vitro 1-Hydroxylase in Cells

Results of one experiment are shown in Figure 5. The validity of the apparent rhythmicity was supported by the statistical analysis of the model fit as given in Table III. The calculated parameters were highly significant as was the correlation coefficient between the predicted and observed values. Furthermore, the Durbin-Watson coefficient indicated no autocorrelation among the deviations. In contrast to the in vivo temporal behavior which was consistent with a 24 h periodicity, the cells in this experiment showed a 5.6 h periodicity. The amplitude was, however only slightly greater than 10% of the average, whereas in vivo the amplitude exceeded 30% of the asymptotic value.

A similar periodicity was observed in another experiment (not presented here) but in a third, periodicity of the in vitro 1-hydroxylase activity was approximately 4 h.

DISCUSSION

In a comparison of the results of computer simulation and experimental observations, a previous study showed a close agreement between nutritional calcium requirement of growing chicks, estimated by the simulation algorithm and that determined by actual experimentation [Hurwitz et al., 1987b]. The decline in 1-hydroxylase with age as observed in the present computer simulation is also in agreement with several empirical studies, including that of Bar and Hurwitz [1981]. Most importantly, the existence of simulation-predicted oscillations in components of the calcium control systems-1-hydroxylase, and blood to bone calcium flow, has been validated experimentally. The times of peaks and nadirs exhibited by the experimental results in chickens correspond to those found in humans [Carruthers et al., 1964; Halloran et al., 1985; Jubitz et al., 1972; Markowitz et al., 1981, 1988; Nielsen et al., 1990] and dogs [Wong and Klein, 1984]. On the other hand, a reverse pattern was observed in rats which are nocturnal animals [Staub et al., 1988]. The present results further show that at least in the chicken an actual 12 h dark period is not required for the generation of the diurnal rhythms since the results were obtained with a dark period as short as 2 h.

The cause of oscillations in the calcium control system is a matter of speculation. It could be argued that the increased ^{45}Ca release from bone during the night, as observed by Wong and Klein [1984] in dogs and in the present study in chicks, may have been the direct result of the exhaustion of dietary calcium supply during the hours of noneating. However, the dark period in the present study was only 2 h long, shorter than a normal intestinal transit time in the chicken [Sklan et al., 1975]. However, since the pattern of the daily feed intake had not been monitored,

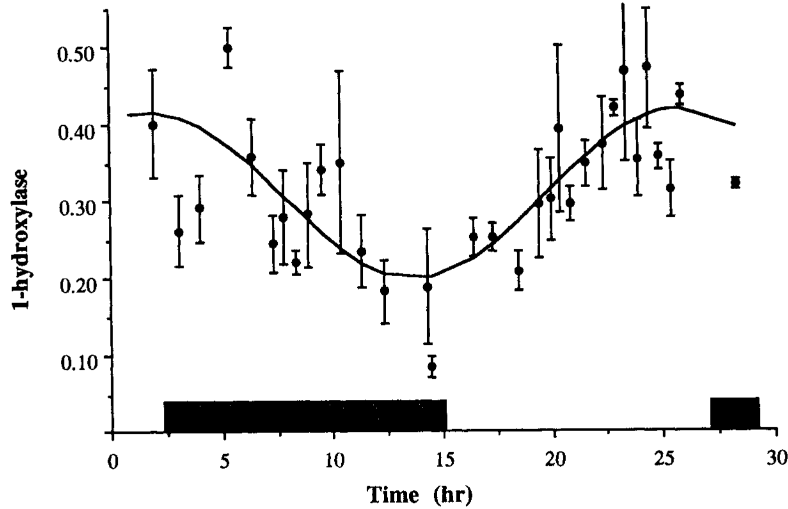

Fig. 4. Diurnal pattern in the renal 25-hydroxyvitamin D_3-1-hydroxylase activity in chicks. The fitted function is given by Eq. 1 with P = 24 h. The dark periods are shown by the filled bars. Vertical bars represent the standard error for each time point (n = 4). Estimated parameters are given in Table II.

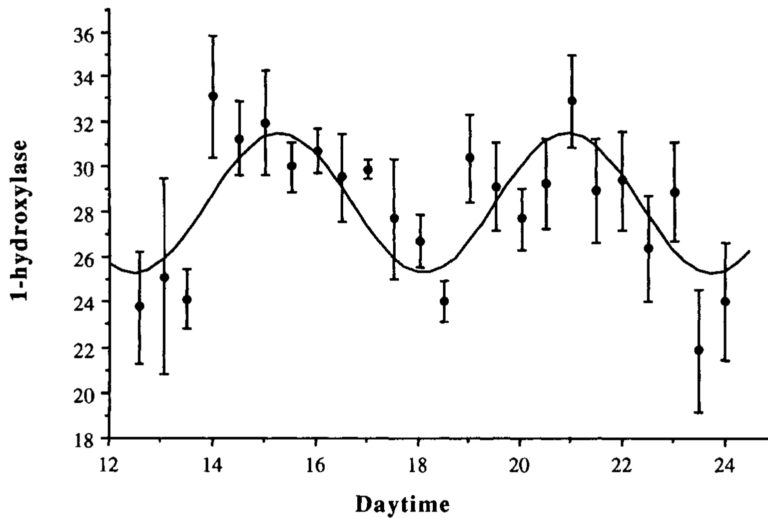

Fig. 5. Temporal pattern in the production of 1,25-dihydroxyvitamin D_3 of chick kidney cells in primary cell cultures. The fitted function is given by Eq. 1. Vertical bars represent the standard error for each time point (n = 4). Estimated parameters are given in Table III.

the possibility that feed intake was not discontinued prior to the onset of darkness cannot be ruled out. Results of computer simulation suggest that the calcium homeostatic machinery, as described by model equations, could be directly responsible for generating the observed oscillations in both signals. As previously discussed [Hurwitz et al., 1987a], oscillations originated from the difference in the response time of bone calcium release and 1-hydroxylase to stimulation by PTH, and the steady-state perturbation resulting from the process of growth. The essential role of the parathyroids in maintaining oscillations was demonstrated in dogs by Wong and Klein [1984].

Notwithstanding the differences in the experimental conditions of this study, some comparison can be made between the oscillatory behavior patterns of the calcium control systems. The 9 h difference in phase observed between bone

TABLE III. Parameters and Statistical Analysis of Oscillations in the Production of 1,25-Dihydroxy-Vitamin D_3 by Kidney Cells In Vitro*

Symbol	Description	Value	SE	*t* value
P	Periodicity, h	5.643	0.264	21.387
β	Amplitude, pmole/ min/mg protein	3.084	0.583	5.287
τ	Phase, h	−2.604	0.74	3.535
β	Average, pmole/ min/mg protein	28.35	0.44	65.138

*Parameters estimated from results given in Figure 5 using a model given in Equation 1. General statistics: Durbin-Watson, 2.228; R (predicted vs. observed), 0.739.

calcium flow and 1-hydroxylase activity in the simulation is similar to the 11–12 h difference observed when comparing the corresponding experimental results. However, the 56–57 h periodicity of the simulated oscillations is quite different from the 24 h periodicity observed in vivo, and the 5–6 h observed for 1-hydroxylase activity in kidney cells in vitro. A variable periodicity of 20–26 h was observed in 1-hydroxylase in chicks kept under a constant light regime [Miller and Norman, 1979] but exposure to a diurnal light-dark cycle stabilized the periodicity of oscillation at 24 h. The light-dark schedule may then be responsible for synchronizing the spontaneous oscillations [Zeitgeber] similarly to other systems of regulation in chicks [Takahashi et al., 1989]. It was formally shown that different oscillators are not necessarily mutually exclusive but can be synchronized by a single Zeitgeber [Gander et al., 1984]. Biological synchronization has been recently shown to be a general property of coupled oscillations [Strogatz and Stewart, 1993].

Some speculation may be offered as to the importance of the observed oscillations for maintenance of plasma calcium. A decrease in production of $1,25[OH]_2D_3$ was shown by us previously [Hurwitz et al., 1984] to be essential in avoiding hypercalcemia during ingestion of large amounts of calcium, by regulation of intestinal calcium absorption. The nadir in 1-hydroxylase towards the end of the dark hours may be the expression of anticipation of the large calcium intake expected at the beginning of the light hours. The increase in bone calcium release towards the period of darkness may be in anticipation of the reduced calcium supply from the intestine during the dark hours. Such an advantage for calcium homeostasis provided by the circadian rhythm in the two major controlling system is well in accord with the thesis presented by Moore-Ede [1986] of anticipatory regulation and by Rapp [1987].

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