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pH HOMEOSTASIS OF THE CIRCADIAN SPORULATION RHYTHM IN CLOCK MUTANTS OF *NEUROSPORA CRASSA*

Peter Ruoff,* Amrollah Behzadi, Marianne Hauglid, Merete Vinsjevik, and Harald Havås
School of Science and Technology, Stavanger University College, P.O. Box 2557 Ullandhaug, N-4091 Stavanger, Norway

ABSTRACT

The influence of environmental (extracellular) pH on the sporulation rhythm in *Neurospora crassa* was investigated for wild-type (frq^+) and the mutants *chr*, frq^1, frq^7, and frq^8. In all mutants, including wild type, the growth rate was found to be influenced strongly by extracellular pH in the range 4–9. On the other hand, for the same pH range, the period length of the sporulation rhythm is little influenced in wild type, *chr*, and frq^1. A loss of pH homeostasis of the period, however, was observed in the mutants frq^7 and frq^8, which also are known to have lost temperature compensation. Concerning the influence of extracellular pH on growth rates, a clear correspondence between growth rates and the concentration of available $H_2PO_4^-$ ion has been found, indicating that the uptake of $H_2PO_4^-$ may be a limiting factor for growth under our experimental conditions. The loss of pH compensation in the frq^7 and frq^8 mutants may be related to less easily degradable $FRQ^{7,8}$ proteins when compared with wild-type FRQ. Results from recent model considerations and experimental results predict that, with increasing extra- and intracellular pH, the FRQ^7 protein degradation increases and should lead to shorter period lengths. (*Chronobiology International*, *17*(*6*)*, 733–750, 2000*)

Key Words: Chrono—Circadian rhythms—Clock mutants—Frequency—Goodwin model—Homeostasis of period—*Neurospora crassa*—pH—Temperature compensation.

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*To whom correspondence should be addressed at: School of Science and Technology, Stavanger University College, P.O. Box 2557 Ullandhaug, N-4091 Stavanger, Norway. E-mail: peter.ruoff@tn.his.no

Circadian rhythms reflect the adaptation of organisms to their environments. It is now generally believed that circadian rhythms have a clock function for timing important physiological processes with respect to daily and seasonal environmental changes. Typical examples are the flowering of plants, the migrations of birds or butterflies, the sleep/wake cycle, hibernation, or the space-time memory in bees (Edmunds 1988). To work as useful chronometers, circadian oscillators are expected to compensate for environmental fluctuations (e.g., changes in temperature, salinity, pH, or nutrient supply) that may otherwise influence the oscillator's period and clock function and thus the temporal organization of the organism (Pittendrigh and Caldarola 1973; Pittendrigh 1993).

By use of clock mutants and molecular analyses, important insights into the molecular mechanisms of circadian rhythms have been obtained (Pittendrigh 1993; Dunlap 1998, 1999; Dunlap et al. 1999). The basic mechanism of circadian organization in cyanobacteria, *Drosophila, Neurospora*, and mammals consists of a negative-feedback loop of transcription and translation of clock genes, by which the negative feedback is achieved by an inhibition of the clock mRNA production at the transcriptional or post-transcriptional level by the clock proteins and/or their derivatives (Dunlap 1998, 1999; Kondo and Ishiura 1999; Iwasaki and Dunlap 2000). A minimal model of such negative-feedback loops is the Goodwin oscillator. This three-variable model was proposed more than 30 years ago (Goodwin 1965) and has served recently as a basis to model essential circadian properties in *Neurospora* and *Drosophila* (Ruoff and Rensing 1996; Ruoff et al. 1996, 1997; Hong and Tyson 1997; Leloup and Goldbeter 1997, 1998, 2000; Ruoff, Vinsjevik, Mohsenzadeh, et al. 1999; Tyson et al. 1999; Gonze et al. 2000). One of the predictions of the Goodwin model is that degradation of clock proteins may be an important determinant of the circadian period (Ruoff et al. 1996; Ruoff, Vinsjevik, Monnerjahn, et al. 1999). Experimental results on phosphorylation and degradation of the FRQ protein in *Neurospora* support this view (Liu et al. 2000).

In *Neurospora crassa*, the *frequency* (*frq*) gene (Gardner and Feldman 1980) has been shown to play an important role in the "circadian timing" of the sporulation rhythm (Iwasaki and Dunlap 2000). Although rhythms with variable period lengths have been observed in *Neurospora* mutants without any *frq* gene (Merrow et al. 1999; Lakin-Thomas 2000; Lakin-Thomas and Brody 2000), these *frq*-deficient mutants seem to lack properties like temperature compensation (Iwasaki and Dunlap 2000) and do not seem to have functional clocks. Temperature compensation, an important property of circadian oscillators (Winfree 1980; Edmunds 1988; Lakin-Thomas et al. 1990; Ruoff 1997), means that the circadian period remains practically unchanged for different (but constant) temperatures within a certain physiological range. According to Pittendrigh and Caldarola (1973), temperature compensation is only one aspect of a general homeostatic mechanism, which compensates the clock's period for environmental influences like nutrition, pH, salinity, and the like. Several *Neurospora* circadian rhythm mutants are now available that show altered period lengths and lost temperature compensation. Especially, the short and long period alleles of the *frq* gene (frq^1 and frq^7, respectively) are interesting study objects because they represent point mutations (Aronson, Johnson, and Dunlap 1994). While the frq^1 mutant, like the wild type (frq^+), is still temperature compensated, the period of frq^7 decreases significantly with increasing temperature (Gardner and Feldman 1981).

The effect of pH on circadian rhythms is less well studied. West (1975) investigated the influence of pH and biotin on the *Neurospora* sporulation rhythm and found that conidiation is enhanced in cultures grown on medium that is neutral and biotin deficient.

However, no explicit determinations of growth rates or period lengths as a function of extracellular pH were performed. Kruse (1984) studied the influence of pH changes in *Neurospora* by phase-response curves and found that acetic acid, a weak acid, produced larger phase shifts than the strong acids HCl or H_2SO_4.

The goal of our research was to investigate the influence of extracellular pH on *Neurospora* clock mutants and see whether *Neurospora* has the ability to compensate for changes in environmental pH over an extended pH range and whether strains that have lost temperature compensation also have lost the ability to compensate for external pH variations. In this paper, we show that, for the *frq* mutants, there is a clear-cut correlation between the existence and loss of temperature compensation and a corresponding existence and loss in pH homeostasis of the period.

MATERIALS AND METHODS

Strains

All investigated strains carry the *band* (*bd*) mutation, which reduces inhibition of conidiation by accumulating CO_2 (Sargent and Kaltenborn 1972). The wild-type (frq^+) strain, the frequency mutants frq^1 and frq^8, as well as the *chr* (*chrono*) strain were obtained from Fungal Genetics Stock Center (FGSC; University of Kansas Medical Center, Department of Microbiology, Kansas City). The FGSC stock numbers were as follows: wild type (frq^+), 1858; frq^1, 2670; frq^8, 4900; *chr*, 4908. The frq^1 strain is a short period mutant (period ≈ 16h), while frq^7 and frq^8 have long periods (≈29h). Both frq^7 and frq^8 have lost temperature compensation, but frq^1 is temperature compensated (Gardner and Feldman 1981). Compared with wild type (frq^+, period 21.5h), *chr* has an altered period (23h–24h at 25°C), and temperature compensation is good above 30°C (Gardner and Feldman 1981). The *csp*-1 mutation prevents that conidia separate and become airborne. The *csp* mutation is known to shorten periods about 1h (Dharmanda and Feldman 1979; Lakin-Thomas and Brody 1985). Samples of a *bd csp*-1 frq^7 mutant, which was used by us in an earlier study (Ruoff, Vinsjevik, Mohsenzadeh, et al. 1999), were a gift from Dr. Patricia Lakin-Thomas (University of Cambridge, UK). Descriptions and references of the *Neurospora crassa* mutants can be found at the FGSC web site at http://www.fgsc.net/.

Culture Conditions and Assays

Cultures were grown in constant light (LL) in liquid Vogel's medium (Vogel 1956; Davis and De Serres 1970) with 2% sucrose (LL medium). After inoculation of approximately 6×10^7 conidia L^{-1} in petri dishes (90 mm diameter) containing 20 mL LL medium, the medium was exposed to white fluorescent light (25 μmol $seconds^{-1}$ m^{-2}) for approximately 40h at 25°C. Mycel disks were then cut out (1 cm diameter) by a cork borer and were placed in Vogel's medium with low sucrose (0.4%) at constant darkness and at 25°C.

After 12h in darkness, mycelial disks were transferred to race tubes, which contained agar and nutrients at different pH at 25°C. After another 12h of growth at 25°C, growing fronts were marked at the bottom of the race tube. The marking was repeated every 24h for 4 days. An exception was the frq^1 mutant, which was the only strain grown in race tubes at 30°C to get better separation of conidial bands. All growing strains

showed linear growth during these 96h. In the final determination of the growth rate and the period length, however, strains were allowed to grow in race tubes undisturbed for a 96h period. For each pH and each strain, at least three repetitive experiments always containing a set of 6 parallel race tubes were tested. The growth rate (i.e., growth length per 24h) was determined as the average of the 96h growth period; period lengths were calculated as the average of the first 3–4 bands that appeared.

Densitometric analysis of digital images of banding was done by the public domain program NIH Image (version 1.61; available at http://rsb.info.nih.gov/nih-image/).

Preparation of Growth Medium and Race Tubes

The extracellular pH ranged between 4 and 9 with an increment of approximately 0.5 units, which gives rise to the 11 nominal pH values: 4.0, 4.5, . . . , 8.5, 9.0. For each pH, an agar solution with a final volume of 200 mL was prepared as follows. To approximately 150 mL double-distilled water (Erlenmeyer flask), 0.8 g sucrose and 4 mL 50× Vogel solution were added. The pH of this solution was adjusted by means of 2 M HCl or 2 M KOH according to Table 1. Then, 2 g agar (agar-agar, ultrapure, Merck, Darmstadt, Germany) was added. The medium was diluted to 200 mL and heated under continuous stirring until the solution became completely clear. The hot agar was transferred into 6 race tubes (15 mL each) and the rest agar was kept. Race tubes together with the rest agar were autoclaved for approximately 7 minutes. Part of the hot and fluid rest agar was transferred into four plastic disposable cuvettes, and a suitable indicator was added to three of them. The agar was then allowed to stiffen. Then, the pH was determined spectrophotometrically (see below). Later in the study, when a special pH electrode became available, the hot autoclaved rest agar was poured into a petri dish. After cooling, the pH of the stiff agar was determined directly using the electrode.

Determination of pH in Agar

Since normal pH glass electrodes are generally not designed to measure pH in agar or other gels, we developed a spectrophotometric method to make precise pH measure-

Table 1. pH Adjustment of Growth Medium Before Agar Addition

Nominal pH	pH before agar addition
4.0	4.1
4.5	4.7
5.0	5.3
5.5	5.5
6.0	6.1
6.5	6.5
7.0	7.3
7.5	8.1
8.0	Add 4.5 mL 2 M KOH
8.5	Add 5.0 mL 2 M KOH
9.0	Add 5.5 mL 2 M KOH

ments in the range 2–9. Later, a special pH electrode (Metrohm, Herisau, Switzerland, model 6.0217.00) became available; it enabled us to measure pH directly in the gel and served as a control for our method.

To cover the pH range between 2 and 9, we chose the following indicators: bromphenol blue (Riedel-de Haën, Seelze, Germany), bromcresol green (Darmstadt, Merck), methyl red (Riedel-de Haën), bromthymol blue (Riedel-de Haën), and thymol blue (Merck). The method is based on the determination of the ratio between the acid and basic absorption peaks of the indicator. From the Henderson-Hasselbalch equation for the indicator, it can easily be shown that the logarithm of this ratio determines the pH in a solution or gel. As an example, Fig. 1a shows the acid and basic absorption peaks of bromphenol blue calibration at various pH. Figure 1b shows the corresponding calibration curve for the pH range 2–4. From the linear regression of the calibration points

$$\log_{10}(A_{acid}/A_{base}) = \mathbf{a} \times \mathrm{pH} + \mathbf{b} \tag{1}$$

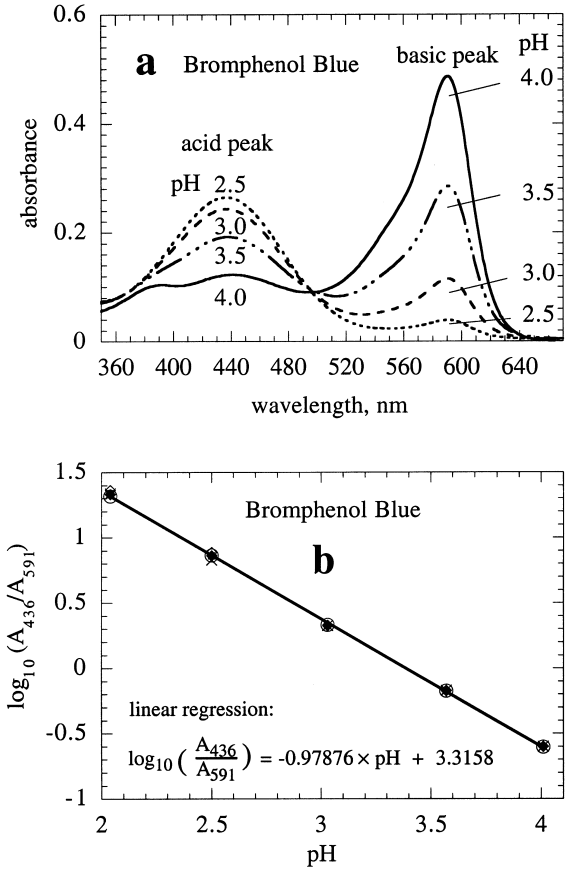

FIGURE 1. (a) Absorption spectrum of bromphenol blue calibration solutions (50 µL indicator solution in 3.0 mL 0.1 M Tris of various pH). The acid peak is at 436 nm and the basic peak at 591 nm. (b) Calibration curve of bromphenol blue indicator ($n = 4$, $r = 99984$). For calibration values of the other indicators, see Table 2.

(Fig. 1b), the pH for a given $\log_{10}(A_{acid}/A_{base})$ ratio can be determined. A_{acid} and A_{basic} denote the absorption of the acid and basic forms of the indicator, respectively. It should be noted that, when applying this method for samples containing high salt concentrations, the ionic strength of samples and calibration solutions should be approximately equal. In our case, we used 0.1 M Tris solutions for calibration.

The procedure to determine pH was as follows: 3 mL of the sample solution (note that the agar still has to be hot and fluid) were taken into four 1-cm plastic disposable cuvettes. Into three of the cuvettes, 50 µL of the indicator solution was added. Table 2 contains the suitable pH range for each indicator and shows how indicator solutions were prepared. Cuvettes (using parafilm) were shaken several times to ensure homogeneous distribution of indicator and agar. After stiffening of the agar, the cuvette without indicator was taken, and the background correction function of the spectrophotometer for the wavelength range 300–750 nm was used. After the completed background correction, absorptions A_{acid} at wavelength λ_{acid} and A_{base} at λ_{base} (Table 2) with a precision of 4 digits for the remaining cuvettes were measured. $\text{Log}_{10}(A_{acid}/A_{base})$ was calculated, and the average pH from the calibration curve was determined (Eq. 1, Table 2).

Later in our study, we were able to measure the pH directly in the agar by a special gel electrode. Comparisons between these two methods showed excellent agreement (Fig. 2).

Calculation of $\alpha_{H_2PO_4^-}$

Dissociation constant values $K_1 = 7.52 \times 10^{-3}$, $K_2 = 6.23 \times 10^{-8}$, and $K_3 = 2.2 \times 10^{-13}$ of H_3PO_4 at 25°C were taken from Weast (1969). If c_0 denotes the total concentration of

Table 2. Spectroscopic Data of Indicators and Determined Values of Calibration Curves (Eq. 1)

pH range	Indicator	λ_{acid}, nm	λ_{base}, nm	**a** (Eq.1)	**b** (Eq.1)	*n*,*r* (Eq.1)
2.0–4.0	Bromphenol blue[a] 0.1 g in 14.9 mL 0.01 M NaOH + 235.1 mL H_2O	436	591	–0.97876	3.3158	$n = 4$ $r = 0.99984$
2.5–4.5	Bromcresol green: 0.1 g in 14.3 mL 0.01 M NaOH + 235.7 mL H_2O	446	616	–0.89316	3.8169	$n = 4$ $r = 0.99956$
4.5–6.0	Methyl red: 0.02 g in 60.0 mL ethanol + 40.0 mL H_2O	520	423	–0.77732	4.1357	$n = 4$ $r = 0.99979$
5.5–7.5	Bromthymol blue: 50 mg in 8.0 mL 0.01 M NaOH + 117.0 mL H_2O	432	615	–0.93181	6.3521	$n = 3$ $r = 0.9953$
7.0–9.0	Thymol blue: 0.1 g in 21.5 mL 0.01 M NaOH + 229.5 mL H_2O	435	594	–0.87487	7.4832	$n = 4$ $r = 0.99977$

[a]Bromphenol blue was not used in the determination of pH in this paper. For the sake of completeness, however, we include its properties.

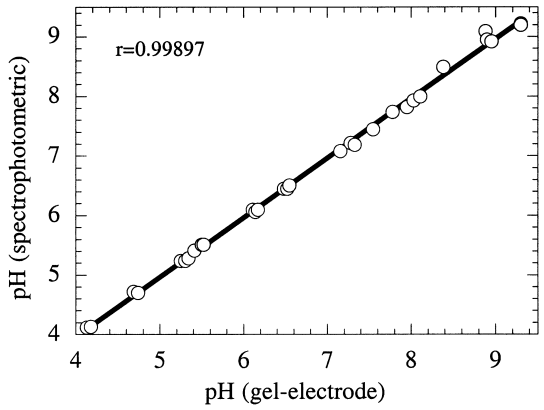

FIGURE 2. Comparison of the spectrophotometric method and the Metrohm 6.0217.000 gel-electrode determination of pH values in agar race tube medium.

phosphate, that is, $c_0 = [H_3PO_4] + [H_2PO_4^-] + [HPO_4^{2-}] + [PO_4^{3-}]$, then the concentration ratio between $H_2PO_4^-$ and c_0, $\alpha_{H_2PO_4^-}$, is defined as (Skoog et al. 1996)

$$\alpha_{H_2PO_4^-} = \frac{[H_2PO_4^-]}{c_0} \tag{2}$$

and can be described as a function of $[H^+]$ by

$$\alpha_{H_2PO_4^-} = \frac{K_1[H^+]^2}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3} \tag{3}$$

Since $[H^+] = 10^{-pH}$, $\alpha_{H_2PO_4^-}$ can be calculated as a function of pH. $\alpha_{H_2PO_4^-}$ has its maximum value (0.9925) at pH $4.7 = (1/2)(pK_1 + pK_2)$.

RESULTS

Influence of pH on Growth and pH Compensation of the Circadian Period

All strains that have been investigated show a considerable and very similar pH dependence of the growth rate. A typical example is presented in Fig. 3a for the *chr* strain. At pH 4, the growth rate is high, and it starts to drop off at about pH 5. When the pH in agar is further increased, the growth rate decreases almost linearly with increasing pH. However, despite these large changes in growth, pH has practically no influence on the period (Fig. 3b), which indicates pH compensation for the *chr* strain.

frq Mutants: Correlation Between Temperature and pH Compensation

The *frq* gene has been shown to play an important role in the regulation of the circadian sporulation rhythm in *Neurospora crassa* (Gardner and Feldman 1980; Aronson, Johnson, and Dunlap 1994; Aronson, Johnson, Loros, et al. 1994; Dunlap 1998, 1999; Luo et al. 1998; Iwasaki and Dunlap 2000). frq^1 or frq^7 represent point mutations

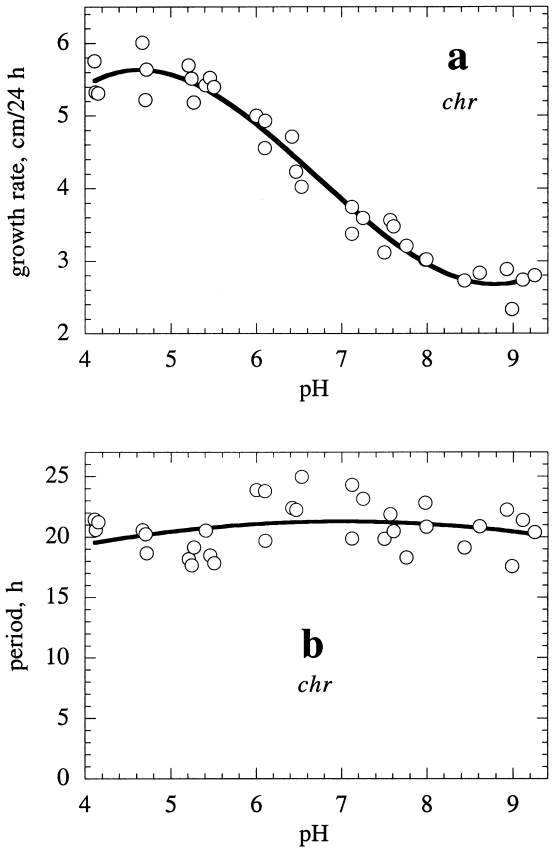

FIGURE 3. (a) Growth rate of *chr* as a function of extracellular pH. The solid line represents a curve fit to a fourth-order polynomial. (b) Circadian period as a function of pH. The solid line shows a quadratic regression. Each point in Figs. 3a and 3b represents an average of six racetubes.

(Aronson, Johnson, and Dunlap 1994) and lead to short or long periods, respectively. While the *frq*^{1} mutant still shows temperature compensation similar to *frq*$^{+}$, the *frq*^{7} mutant has lost temperature compensation and shows, very similar to a purely chemical oscillator (Ruoff 1995), a decrease in period with increasing temperature (Gardner and Feldman 1981). However, for completeness, it should be mentioned here that the first purely chemical oscillator showing temperature compensation was recently described (Rábai and Hanazaki 1999).

To see whether there is a correlation between pH and temperature compensation, we investigated the influence of extracellular pH on the sporulation rhythm for the long- and short-period *frq* mutants and compared their behavior with that of *frq*$^{+}$. Figure 4a shows the dependencies of the circadian period in *csp-1*; *frq*^{7}, *frq*$^{+}$, and *frq*^{1} as a function of the pH. Clearly, *frq*$^{+}$ is pH compensated; *frq*^{1} shows only a small variation of the period, while the period of *frq*^{7} decreases significantly with increasing pH and approaches the wild-type period at high pH values. Growth rates for *csp-1*; *frq*^{7}, *frq*$^{+}$, and *frq*^{1} are very similar to those for *chr* (data not shown, but see discussion below). This analogous influence of pH (Fig. 4a) and temperature (Fig. 4b) on the period in these mutants is

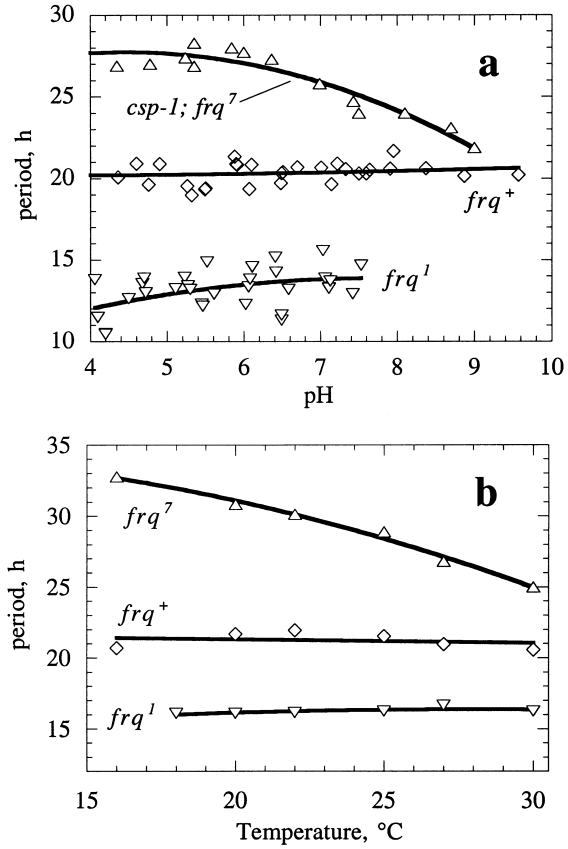

FIGURE 4. (a) Dependence of circadian period in *csp-1*; frq^7, frq^+, and frq^1 mutants as a function of pH. Each point represents the average of six racetubes. As in the case of temperature (Fig. 4b), frq^+ and frq^1 show homeostasis of the period, while the frq^7 mutant does not. (b) Dependence of circadian period in frq^7, frq^+, and frq^1 mutants as a function of temperature. Data replotted from the work by Gardner and Feldman (1981).

indeed astonishing. However, there are also fundamental differences: While both increasing temperature and pH lead to a decrease in the period for frq^7, growth rates are oppositely affected, that is, increasing temperature and pH lead to increases and decreases of the growth rate, respectively.

To obtain more distinguishable banding in frq^1, this strain was grown at 30°C instead of 25°C. However, in contrast to what has been observed in the other *frq* mutants at 25°C, no clear banding in frq^1 is found for pH values of about 8 and higher. When banding is observed, however, conidia become more dense during growth, and banding eventually disappears (Fig. 5).

The frq^8 mutant shows behavior similar to frq^7, that is, the period depends on pH, but the maximum period length occurs at about pH 6–6.5 (Fig. 6). Period lengths in frq^8 were found to be subject to considerable variation, while growth rates were very similar to those of the other tested strains (Fig. 7). The reason for this variability in period is not understood.

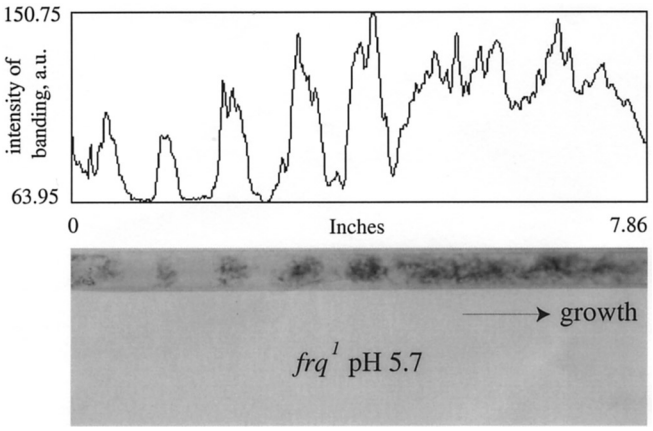

FIGURE 5. Banding behavior observed in *frq*[1] at 30°C. Lower part of figure shows photograph of race tube, while upper part shows the density of the conidial bands taken along the center of the tube. Typically, after the fourth or fifth cycle, the rhythmic banding disappears.

Influence of pH on Phosphate Uptake and Correlation to Growth Rates

The change of environmental pH to higher values has, like temperature, a profound (but opposite) effect on the growth rate. Like temperature, changes in environmental pH probably have quite complex influences on physiological processes. Since the growth of all investigated strains showed a very similar dependency on pH, an increase of pH may be considered as a decrease in the availability of certain nutrients or ions.

For example, Versaw and Metzenberg (1995) studied the phosphate uptake by two high-affinity phosphate permeases, PHO-4 and PHO-5. They found that uptake by PHO-5, which appears to be a phosphate-H^+ symporter, is considerably dependent on pH, while the uptake velocity of PHO-4 (which is considered a Na^+-phosphate symporter) has little dependence on extracellular pH. At neutral conditions, phosphate uptake rates of PHO-4 are considerably lower than for PHO-5. Because *N. crassa* is often found growing on burnt vegetation, an environment that is likely to be both alkaline and low in phosphate, Versaw and Metzenberg (1995) suggested that the primary role of PHO-4 is to provide phosphate uptake under these demanding conditions. Because within the extracellular pH range of 4–7.5 phosphate uptake is regulated by PHO-5, for which $H_2PO_4^-$ is apparently the main ionic form, we wondered whether the pH dependence of growth rates might be correlated with the pH dependence of the amount of available $H_2PO_4^-$. For this purpose, we first selected the strains *csp-1*; *frq*[7], *frq*[8], and *chr* because they were very similar in their pH dependence of growth. Growth rates for *frq*[8] and *chr* were changed by adding 0.422447 cm/24h and 0.2080 cm/24h to the experimental growth rates of *frq*[8] and *chr*, respectively. In this way, the root-mean-square deviations between the fitted fourth-order polynomials of *chr* and *frq*[8] (Figs. 3a, 6a) on one hand and the fitted fourth-order polynomial of the unchanged *csp-1*; *frq*[7] growth rate on the other hand (Fig. 7) became as low as possible. In this way, the overlap between all three experimental growth rate data sets became as large as possible (Fig. 7). To test a correlation between pH dependencies of growth rates and the amount of available $H_2PO_4^-$, the maxi-

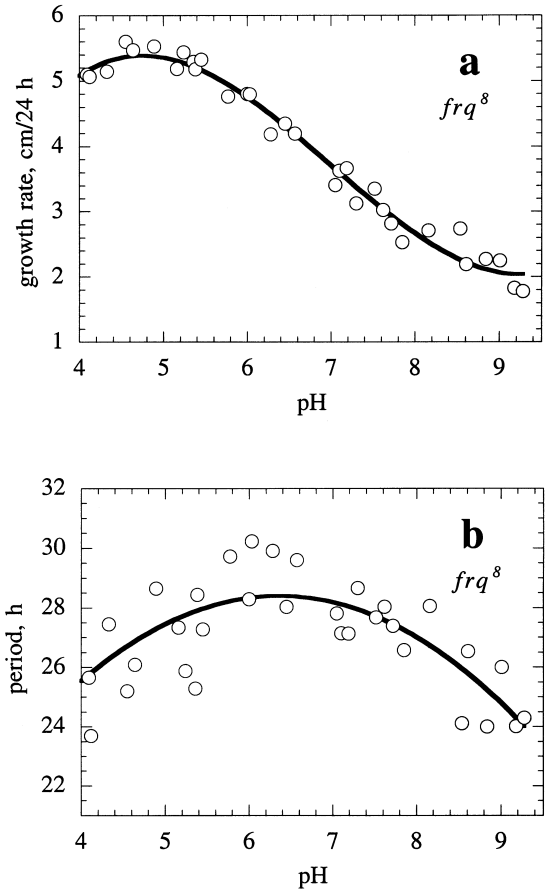

FIGURE 6. (a) Growth rate of *frq*^{8} as a function of pH. The solid line represents a curve fit to a fourth-order polynomial. (b) Circadian period as a function of extracellular pH. The solid line shows a quadratic regression. Each point in Figs. 6a and 6b represents an average of six racetubes.

mum value of $\alpha_{H_2PO_4^-}$ (Eq. 3) is assigned to maximum growth rates, while the minimum value of $\alpha_{H_2PO_4^-}$ at pH 9 is assigned to the growth rates at this pH. In this way, the $\alpha_{H_2PO_4^-}$ function shows a remarkably good correlation with the growth rates, indicating that phosphate uptake may be due mainly to the $H_2PO_4^-$ ionic form and that the growth rate appears to be regulated (limited) by phosphate uptake. As a consequence, the effect of temperature on growth rate at constant pH may then be due to the increased uptake of $H_2PO_4^-$ at increased temperatures.

Although Fig. 7 shows evidence that phosphate may be the growth-limiting nutrient when the extracellular pH is changed, ions as nitrate (Blatt et al. 1997) and potassium (Rodriguez-Navarro et al. 1986; Blatt et al. 1987) have also been reported to be cotransported with H^+. Membrane potential and pH control the kinetics of their uptake, such that these ions may also be growth limiting.

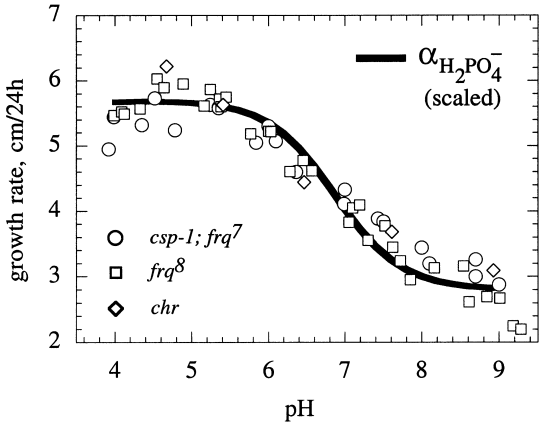

FIGURE 7. Open circles represent the (unchanged) experimental growth rates of *csp-1*; *frq*^{7} (each point represents the average of six race tubes). Other symbols represent changed growth rates for *frq*^{8} and *chr*. Growth rates for *frq*^{8} (Fig. 6) and *chr* (Fig. 3) were increased by 0.422447 cm/24h and 0.2080 cm/24h, respectively. As a result, the two root-mean-square deviations between the fitted fourth-order polynomials of *frq*^{8} and *csp-1*; *frq*^{7} (note that the fitted fourth-order polynomial of *csp-1*; *frq*^{7} growth rate data is not shown in this figure) and *chr* and *csp-1*; *frq*^{7} are at a minimum, which leads to a maximum overlap of experimental data points. Solid line is a scaled $\alpha_{H_2PO_4^-}$ function (Eq. 3) to fit maximum and minimum growth rates. The relationship between scaled and unscaled $\alpha_{H_2PO_4^-}$ is as follows: $\alpha_{H_2PO_4^-}$ (scaled) = $2.8 + 2.9 \times \alpha_{H_2PO_4^-}$.

DISCUSSION

Formulation of pH Homeostasis in *Neurospora crassa*

Despite the marked dependence of growth rate on the extracellular pH, the circadian period of the sporulation rhythm of the studied strains, with the exception of *csp-1*; *frq*^{7} and *frq*^{8}, have been found to be influenced little by pH. Especially, the correlation between temperature compensation of the period and influence of pH on the period, as seen in the *frq*$^+$, *frq*^{1}, and *csp-1*; *frq*^{7} strains (Fig. 4), suggests the existence of a homeostatic mechanism that keeps the period constant even under various environmental conditions. How can such pH homeostasis be explained?

The correlation between temperature compensation and pH homeostasis in the investigated *frq* strains suggests that similar factors may be responsible for the loss of temperature compensation and pH homeostasis. The *frq* locus is considered to play an important role in the *Neurospora* circadian clock (Gardner and Feldman 1980) and its temperature compensation (Aronson, Johnson, and Dunlap 1994). FRQ proteins of different lengths were described (Garceau et al. 1997). Their phosphorylated forms are transported rapidly into the nucleus, where they apparently function as inhibitors at the transcriptional or posttranscriptional level. In fact, the nuclear localization of FRQ is necessary for a functional sporulation rhythm (Luo et al. 1998). FRQ is phosphorylated rapidly and probably also is degraded rapidly by the ATP-dependent ubiquitin-proteasome pathway.

In this way, the *frq* locus and its products define an oscillatory negative-feedback loop. Temperature compensation of this feedback loop can be understood as a balance

between period-increasing and period-decreasing reactions within a certain (physiological) temperature range (Ruoff and Rensing 1996). On the other hand, loss of temperature compensation (for example, in the frq^7 mutant) can be described semiquantitatively by a slower degradation rate of FRQ7 protein together with a higher activation energy of the corresponding degradation reaction (Ruoff et al. 1996; Ruoff, Vinsjevik, Monnerjahn, et al. 1999). Similar conclusions have been reached by Liu et al. (2000). These authors removed some of the phosphorylation sites of the FRQ protein by site-directed mutagenesis. Especially, mutation at Ser 513 was found to lead to a dramatic reduction of the rate of FRQ degradation and to a very long period length (31h).

Like temperature compensation (Ruoff 1992), pH homeostasis of the period may be formulated in an analogous way. Let us assume that all biochemical processes i within the cell are known together with their rate constants k_i. Then, the period P will be a complicated function f of the rate constants k_i, which themselves will depend on pH, temperature, and so on:

$$P = f[k_i(\mathrm{T}, \mathrm{pH}, \ldots)] \tag{4}$$

A change in extracellular pH (temperature and other parameters being constant) may affect the pH within the cell and thus change the k_i's in different ways. The influence of intracellular pH on P is given by the derivative $\partial P/\partial \mathrm{pH}$, that is,

$$\frac{\partial P}{\partial \mathrm{pH}} = \sum_{\mathrm{i}} \left(\frac{\partial f}{\partial k_i} \right) \left(\frac{\partial k_i}{\partial \mathrm{pH}} \right) \tag{5}$$

To analyze Eq. 5 further, the dependencies of all k_i on pH should be known. Since all biochemical processes i are catalyzed by enzymes E_i, we need to know the influence of pH for each E_i. In general, enzymes show a "bell-shaped" dependence of their activity on pH, with maximum activity at a certain $\mathrm{pH}_i^{\max}$. The diprotic model (Tipton and Dixon 1979) provides a basic description of how enzyme-catalyzed reactions will depend on pH. An alternative mathematical description of the bell-shaped activity-pH profile in the diprotic model can be given by a Gaussian relationship:

$$k_i = k_i^0 \exp[-\sigma_i (\mathrm{pH} - \mathrm{pH}_i^{\max})^2] \tag{6}$$

which very closely can describe a symmetric Michaelian type of activity-pH profile. The k_i^0, σ_i, and $\mathrm{pH}_i^{\max}$ may be considered pH independent. Equation 6 describes the rate constant for process i, which in general is due to the pH-dependent ionizations of both the enzyme and its substrates (Murray et al. 1996). From Eq. 6, $\partial k_i/\partial \mathrm{pH}$ can be calculated as

$$\frac{\partial k_i}{\partial \mathrm{pH}} = -2\sigma_i(\mathrm{pH} - \mathrm{pH}_i^{\max})k_i = -2\sigma_i(\Delta \mathrm{pH}_i)k_i \tag{7}$$

where $\Delta \mathrm{pH}_i$ denotes the difference in pH between the actual cellular environment and the pH leading to a maximum velocity of reaction i. The pH homeostasis of the period requires that $\partial P/\partial \mathrm{pH} = 0$, which leads to the expression

$$\sum_{\mathrm{i}} \left(\frac{\partial \ln f}{\partial \ln k_i} \right) [(\Delta \mathrm{pH}_i)\sigma_i] = 0 \tag{8}$$

It may be noted that Eq. 8 is analogous to the expression derived for temperature compensation (Ruoff 1992; Ruoff, Vinsjevik, Monnerjahn, et al. 1999):

$$\sum_{i}\left(\frac{\partial \ln f}{\partial \ln k_i}\right)\times E_i^a = 0 \qquad (9)$$

and can be considered a balance between period-increasing and period-decreasing reactions. However, there are fundamental differences between Eqs. 8 and 9.

While temperature for single-cell organisms and fungi may be assumed to be the same throughout the whole organism, this is certainly not the case for intracellular pH. Different cell organelles are known to have different pH, with enzymes adapted to these environments. In this way, pH is not, as temperature, a global variable, but a local one. As the extracellular pH varies, the intracellular pH will change, and ΔpH_i will change accordingly.

How large are such changes? By use of microelectrodes, Sanders and Slayman (1982) found that the (wild-type) *Neurospora crassa* internal pH varies between 7.0 and 7.5 when the extracellular pH varies in the range between 4 and 9, respectively. These results are basically in agreement with ^{15}N- and ^{31}P-NMR (nuclear magnetic resonance) studies (Legerton et al. 1983; Pilatus and Techel 1991; Yang et al. 1993) and a radioactive method based on the distribution of ^{14}C-labeled DMO (5,5-dimethyl-2,4-oxazolidinedione) (Johnson 1983). While Johnson (1983) found fluctuations of cytosolic pH in the range 6.5–7.1, Pilatus and Techel (1991) did not observe circadian changes of cytoplasmatic or vacuolar pH. Thus, it appears that there is already sufficient homeostasis of the internal pH, and that enzymes may indeed work at their optimum (or near optimum) internal pH independently of the pH of the extracellular medium. A consequence of this, then, is that the ΔpH_i's remain practically constant (zero) such that, even for large variations in extracellular pH, Eq.8 is satisfied, and the oscillator is pH compensated. The individual σ_i and ΔpH_i values can be understood as the result of an evolutionary process leading to general homeostasis of the period in the organism by satisfying Eqs. 8 and 9. Even in the case the ΔpH_i's are not constant, Eq. 8 may still be satisfied due—through evolution—to an acquired dynamic balance between positive and negative contributions. Any changes that disturb the balances of Eq. 8 or 9 will lead to a loss of pH homeostasis of the period or a loss of temperature compensation.

There are several ways that the loss of pH compensation in the *frq*^{7}/*frq*^{8} mutants may be understood. One possibility is that, in these mutants, the intracellular pH (pH_i) is no longer regulated as in wild-type ("loss of pH_i homeostasis") mutants. As a consequence, extracellular pH variations in *frq*^{7}/*frq*^{8} may lead to larger pH_i changes, and physiological processes are no longer proceeding at their optimum (wild-type) conditions. However, so far, we have been unable to find any report suggesting that *frq*^{7} or *frq*^{8} mutants have larger pH_i variations due to altered uptake or transport properties. Another—perhaps more likely—explanation for the loss of pH compensation in *frq*^{7}/*frq*^{8} is that the mutated FRQ protein has changed its physical and chemical properties. This may lead to altered dynamics of the negative-feedback loop of the *frq* circadian pacemaker and thus to a loss in pH compensation. As indicated by site-directed mutagenesis experiments (Liu et al. 2000) and from predictions of the Goodwin model (Ruoff et al. 1996; Ruoff, Vinsjevik, Monnerjahn, et al. 1999), an important property of the (*frq*) negative-feedback loop is that degradation of the FRQ protein species determines the period of the *Neurospora* sporulation rhythm. The *frq*^{7} allele contains a point mutation (Aronson, Johnson, and Dunlap 1994), which according to the above-mentioned results, probably leads to a lower degradation rate of FRQ^7 protein compared to FRQ$^+$. One possibility to explain the influence of pH on *frq*^{7} period length (Fig. 4a) would be that,

due to altered ionization properties of FRQ^7, the degradation rate of FRQ^7 by certain proteases is increased as intracellular pH increases. The loss of pH compensation would then be analogous to the earlier proposed mechanism for the loss of temperature compensation in *frq*^{7} (Ruoff et al. 1996; Ruoff, Vinsjevik, Monnerjahn, et al. 1999). It will be important to measure the degradation rates of FRQ^+ and FRQ^7 at different pH and temperatures.

On the other hand, such a correspondence between pH and temperature compensation as found in the *frq* mutants is not clearly established in *chr.* In this mutant, pH compensation is good, but temperature compensation is only observed above 30°C (Gardner and Feldman 1981). Results obtained with the *cel* mutant (Ruoff unpublished, 1999) show similar behavior as for *chr* (i.e., pH compensation in the pH range 4–9), while temperature compensation is only observed above 22°C (Lakin-Thomas et al. 1997). However, in *cel*, certain fatty acids have considerable influence on temperature compensation (Mattern et al. 1982). Therefore, the influence of pH on *cel* in the presence of these fatty acids will be the subject of a later study. To investigate (and rule out) some of the above-discussed possibilities for understanding pH compensation, more knowledge about intracellular pH and its homeostasis in *Neurospora* clock mutants will be needed.

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REFERENCES

Aronson BD, Johnson KA, Dunlap JC. 1994. Circadian clock locus frequency: protein encoded by a single open reading frame defines period length and temperature compensation. Proc Natl Acad Sci USA. 91:7683–87.

Aronson BD, Johnson KA, Loros JJ, et al. 1994. Negative feedback defining a circadian clock: autoregulation of the clock gene frequency. Science. 263:1578–84.

Blatt MR, Maurousset L, Meharg AA. 1997. High-affinity NO_3^--H^+ cotransport in the fungus *Neurospora*: induction and control by pH and membrane voltage. J Membr Biol. 160:59–76.

Blatt MR, Rodriguez-Navarro A, Slayman CL. 1987. Potassium-proton symport in *Neurospora*: kinetic control by pH and membrane potential. J Membr Biol. 98:169–89.

Davis RH, De Serres FJ. 1970. Genetic and microbiological research techniques for *Neurospora crassa*. In: Tabor H, Tabor CW, editors. Methods in enzymology. Vol. 17A. New York: Academic, pp. 79–143.

Dharmanda S, Feldman JF. 1979. Spatial distribution of circadian clock phase in aging cultures of *Neurospora crassa*. Plant Physiol. 63:1049–54.

Dunlap J. 1998. Circadian rhythms. An end in the beginning. Science. 280:1548–49.

Dunlap JC. 1999. Molecular bases for circadian clocks. Cell. 96:271–90.

Dunlap JC, Loros JJ, Liu Y, et al. 1999. Eukaryotic circadian systems: cycles in common. Genes Cells. 4:1–10.

Edmunds LN. 1988. Cellular and molecular bases of biological clocks. New York: Springer Verlag.

Garceau NY, Liu Y, Loros JJ, et al. 1997. Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. Cell. 89:469–76.

Gardner GF, Feldman JF. 1980. The *frq* locus in *Neurospora crassa*: a key element in circadian clock organization. Genetics. 96:877–86.

Gardner GF, Feldman JF. 1981. Temperature compensation of circadian period length in clock mutants of *Neurospora crassa*. Plant Physiol. 68:1244–48.

Gonze D, Leloup J-C, Goldbeter A. 2000. Theoretical models for circadian rhythms in *Neurospora* and *Drosophila*. C R Acad Sci Paris, Life Sci. 323:57–67.

Goodwin BC. 1965. Oscillatory behavior in enzymatic control processes. In: Weber G, editor. Advances in enzyme regulation. Vol. 3. Oxford, England: Pergamon, pp. 425–38.

Hong CI, Tyson JJ. 1997. A proposal for temperature compensation of the circadian rhythm in *Drosophila* based on dimerization of the PER protein. Chronobiol Int. 14:521–30.

Iwasaki H, Dunlap JC. 2000. Microbial circadian oscillatory systems in *Neurospora* and *Synechococcus*: models for cellular clocks. Curr Opin Microbiol. 3:189–96.

Johnson CH. 1983. Changes in intracellular pH are not correlated with the circadian rhythm of *Neurospora*. Plant Physiol. 72:129–33.

Kondo T, Ishiura M. 1999. The circadian clocks of plants and cyanobacteria. Trends Plant Sci. 4:171–75.

Kruse M. 1984. Beeinflussung der Circadianen Rhythmik von *Neurospora crassa* durch verschiedene Protonenkonzentrationen im Medium. Diploma work, University of Bremen.

Lakin-Thomas PL. 2000. Circadian rhythms: new functions for old clock genes. Trends Genet. 16:135–42.

Lakin-Thomas PL, Brody S. 1985. Circadian rhythms in *Neurospora crassa*: interactions between clock mutations. Genetics. 109:49–66.

Lakin-Thomas PL, Brody S. 2000. Circadian rhythms in *Neurospora crassa*: lipid deficiencies restore robust rhythmicity to null frequency and white-collar mutants. Proc Natl Acad Sci USA. 97:256–61.

Lakin-Thomas PL, Brody S, Cotè GG. 1997. Temperature compensation and membrane composition in *Neurospora crassa*. Chronobiol Int. 14:445–54.

Lakin-Thomas PL, Cotè GG, Brody S. 1990. Circadian rhythms in *Neurospora crassa*: biochemistry and genetics. Crit Rev Microbiol. 17:365–416.

Legerton TL, Kanamori K, Weiss RL, et al. 1983. Measurements of cytoplasmic and vacuolar pH in *Neurospora* using nitrogen-15 nuclear magnetic resonance spectroscopy. Biochemistry. 22:899–903.

Leloup J-C, Goldbeter A. 1997. Temperature compensation of circadian rhythms: control of the period in a model for circadian oscillations of the PER protein in *Drosophila*. Chronobiol Int. 14:511–20.

Leloup J-C, Goldbeter A. 1998. A model for circadian rhythms in *Drosophila* incorporating the formation of a complex between PER and TIM proteins. J Biol Rhythms. 13:70–87.

Leloup J-C, Goldbeter A. 2000. Modelling the molecular regulatory mechanism of circadian rhythms in *Drosophila*. BioEssays. 22:84–93.

Liu Y, Loros JJ, Dunlap JC. 2000. Phosphorylation of the *Neurospora* clock protein FREQUENCY determines its degradation rate and strongly influences the period length of the circadian clock. Proc Natl Acad Sci USA. 97:234–39.

Luo C, Loros JJ, Dunlap JC. 1998. Nuclear localization is required for function of the essential clock protein FRQ. EMBO J. 17:1228–35.

Mattern DL, Forman LR, Brody S. 1982. Circadian rhythms in *Neurospora crassa*: a mutation affecting temperature compensation. Proc Natl Acad Sci USA. 79: 825–29.

Merrow M, Brunner M, Roenneberg T. 1999. Assignment of circadian function for the *Neurospora* clock gene *frequency.* Nature. 399:584–86.

Murray RK, Granner DK, Mayes PA, et al. 1996. Harper's biochemistry. 24th ed. London: Prentice Hall.

Pilatus U, Techel D. 1991. ^{31}P-NMR-studies on intracellular pH and metabolite concentrations in relation to the circadian rhythm, temperature and nutrition in *Neurospora crassa*. Biochim Biophys Acta. 1091:349–55.

Pittendrigh CS. 1993. Temporal organization: reflections of a Darwinian clock-watcher. Annu Rev Physiol. 55:17–54.

Pittendrigh CS, Caldarola PC. 1973. General homeostasis of the frequency of circadian oscillations. Proc Natl Acad Sci USA. 70:2697–2701.

Rábai G, Hanazaki I. 1999. Temperature compensation in the oscillatory hydrogen peroxide-thiosulfate-sulfite flow system. Chem Commun. 1965–66.

Rodriguez-Navarro A, Blatt MR, Slayman CL. 1986. A potassium-proton symport in *Neurospora crassa*. J Gen Physiol. 87:649–74.

Ruoff P. 1992. Introducing temperature compensation in any reaction kinetic oscillator model. J Interdiscipl Cycle Res. 23:92–99.

Ruoff P. 1995. Antagonistic balance in the Oregonator: about the possibility of temperature compensation in the Belousov-Zhabotinsky reaction. Physica D. 84:204–11.

Ruoff P. 1997. Special issue: temperature-compensation of circadian and ultradian clocks. Chronobiol Int. 14(5).

Ruoff P, Mohsenzadeh S, Rensing L. 1996. Circadian rhythm and protein turnover: the influence of temperature on the period lengths of clock mutants simulated by the Goodwin oscillator. Naturwissenschaften. 83:514–17.

Ruoff P, Rensing L. 1996. The temperature compensated Goodwin model simulates many circadian clock properties. J Theor Biol. 179:275–85.

Ruoff P, Rensing L, Kommedal R, et al. 1997. Modelling temperature compensation in chemical and biological oscillators. Chronobiol Int. 14:499–511.

Ruoff P, Vinsjevik M, Mohsenzadeh S, et al. 1999. The Goodwin model: simulating the effect of cycloheximide and heat shock on the sporulation rhythm of *Neurospora crassa*. J Theor Biol. 196:483–94.

Ruoff P, Vinsjevik M, Monnerjahn C, et al. 1999. The Goodwin oscillator: on the importance of degradation reactions in the circadian clock. J Biol Rhythms. 14:37–47.

Sanders D, Slayman CL. 1982. Control of intracellular pH. Predominant role of oxidative metabolism, not proton transport, in the eukaryotic microorganism *Neurospora*. J Gen Physiol. 80:377–402.

Sargent ML, Kaltenborn SH. 1972. Effects of medium composition and carbon dioxide on circadian conidiation in *Neurospora*. Plant Physiol. 50:171–75.

Skoog DA, West DM, Holler FJ. 1996. Fundamentals of analytical chemistry. 7th ed. Fort Worth, Texas: Saunders College Publishing, chap. 11I.

Tipton KF, Dixon HBF. 1979. Effects of pH on enzymes. In: Purich DL, editor. Methods in enzymology. Vol. 63A. New York: Academic, pp. 183–234.

Tyson JJ, Hong CI, Thron CD, et al. 1999. A simple model of circadian rhythms based on dimerization and proteolysis of PER and TIM. Biophys J. 77:2411–17.

Versaw WK, Metzenberg RL. 1995. Repressible cation-phosphate symporters in *Neurospora crassa*. Proc Natl Acad Sci USA. 92:3884–87.

Vogel HA. 1956. A convenient growth medium for *Neurospora* (medium N). Microbiol Gen Bull. 15:42–43.

Weast RC. 1969. CRC handbook of chemistry and physics. 50th ed. Cleveland, Ohio: Chemical Rubber Company.

West DJ. 1975. Effect of pH and biotin on a circadian rhythm of conidiation in *Neurospora crassa*. J Bacteriol. 123:387–89.

Winfree AT. 1980. The geometry of biological time. New York: Springer Verlag.

Yang YC, Bastos M, Chen KY. 1993. Effects of osmotic stress and growth stage on cellular pH and polyphosphate metabolism in *Neurospora crassa* as studied by ^{31}P nuclear magnetic resonance spectroscopy. Biochim Biophys Acta. 1179:141–47.