



The Goodwin Model: Simulating the Effect of Cycloheximide and Heat Shock on the Sporulation Rhythm of *Neurospora crassa*

PETER RUOFF†*, MERETE VINSJEVIK†, SAADAT MOHSENZADEH‡
AND LUDGER RENSING‡

†School of Technology and Science, Stavanger College, P.O. Box 2557 Ullandhaug, N-4004 Stavanger, Norway and ‡Department of Biology, University of Bremen, P.O. Box 33 04 40, D-28334 Bremen, Germany

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The Goodwin model is a negative feedback oscillator which describes rather closely the putative molecular mechanism of the circadian clock of *Neurospora* and *Drosophila*. An essential feature is that one or two clock proteins are synthesized and degraded in a rhythmic fashion. When protein synthesis in *N. crassa* (wild-type *frq*⁺ and long-period mutant *frq*⁷) was inhibited by continuous incubation with increasing concentrations of cycloheximide (CHX) the period of the circadian sporulation rhythmicity is only slightly increased. The explanation of this effect may be seen in the inhibition of protein synthesis and protein degradation. In the model, increasing inhibition of both processes led to very similar results with respect to period length. That protein degradation is, in fact, inhibited by CHX is shown by determining protein degradation in *N. crassa* by means of pulse chase experiments. Phase response curves (PRCs) of the *N. crassa* sporulation rhythm toward CHX which were reported in the literature and investigated in this paper revealed significant differences between *frq*⁺ and the long period mutants *frq*⁷ and *csp-1 frq*⁷. These PRCs were also convincingly simulated by the model, if a transient inhibition of protein degradation by CHX is assumed as well as a lower constitutive degradation rate of FRQ-protein in the *frq*⁷/*csp-1 frq*⁷ mutants. The lower sensitivities of *frq*⁷ and *csp-1 frq*⁷ towards CHX may thus be explained by a lower degradation rate of clock protein FRQ⁷. The phase shifting by moderate temperature pulses (from 25 to 30°C) can also be simulated by the Goodwin model and shows large phase advances at about CT 16–20 as observed in experiments. In case of higher temperature pulses (from 35 to 42 or 45°C = heat shock) the phase position and form of the PRC changes as protein synthesis is increasingly inhibited. It is known from earlier experiments that heat shock not only inhibits the synthesis of many proteins but also inhibits protein degradation. Taking this into account, the Goodwin model also simulates the PRCs of high temperature (heat shock) pulses.

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Introduction

More than 30 years ago Goodwin (1963, 1965) proposed an oscillatory reaction scheme that

now appears to be, in its essence, a correct description of central elements in the circadian oscillators of *Neurospora*, *Drosophila* and mammals (Dunlap, 1998). The model is based on the transcription and translation of a “clock-gene” [in *Neurospora* the *frequency (frq)*-gene] to

*Author to whom correspondence should be addressed.

(*frq*)mRNA and (FRQ)protein and the subsequent formation of a transcriptional inhibitor that blocks further expression (Aronson *et al.*, 1994). This negative feedback oscillator not only shows the correct phasing of the *frq*-mRNA and FRQ-protein levels compared with what is found experimentally (Garceau *et al.*, 1997), but also phase response curves to temperature-steps and -pulses are in qualitative agreement with experimental phase response curves from both *Drosophila* and *Neurospora* (Ruoff & Rensing, 1996).

The important elements in the (*Neurospora*) negative feedback *frq*/FRQ oscillator concept (and analysed here by the Goodwin model, Fig. 1) is the formation of *frq*-mRNA (variable X) from the *frq*-gene. This process, i.e. transcription of the gene and transport of *frq*-mRNA into the cytosol is represented by reaction R1 (Fig. 1). In the cytosol the *frq*-mRNA is translated into FRQ-protein (reaction R2, variable Y). From the cytosol the FRQ-protein is transported back into the nucleus where FRQ or a modified (perhaps phosphorylated) form of it (variable Z) is assumed to inhibit transcription of *frq*. The entry of FRQ into the nucleus, modification of FRQ into an inhibitory factor, and inhibition of *frq*-transcription are in the Goodwin model all

summarized as reaction R3. Reactions R4, R5, and R6 are degradation reactions of *frq*-mRNA (X), FRQ-protein (Y), and the inhibitory factor (Z), respectively. In mathematical terms, the (non-inhibited) transcription rate is assumed to be constant and described by the single rate constant k_1 . The rate of translation is dependent on the amount of mRNA (X) and is written as k_2X . Likewise, the rate of formation of the inhibitory factor Z is represented by the term k_3Y . Inhibition of transcription is introduced by the factor $1/1 + Z^9$ in relation to the uninhibited rate of transcription (k_1). All degradation reactions are assumed to be first-order with respect to the degraded species. A critical discussion of the experimental basis for the *frq*/FRQ oscillator concept has been given by Lakin-Thomas (1998).

A characteristic feature of the Goodwin model is that the degradation reactions R4, R5, and R6 determine the oscillatory period, while the synthesis reactions R1, R2, and R3 have little influence on the period (Ruoff & Rensing, 1996; Ruoff *et al.*, 1996). If these properties of the model were also true for the biological clock it can be predicted that clock protein or clock-mRNA stabilities are the main factors determining the circadian period both in *Neurospora* and *Drosophila*. In fact, when simulating the

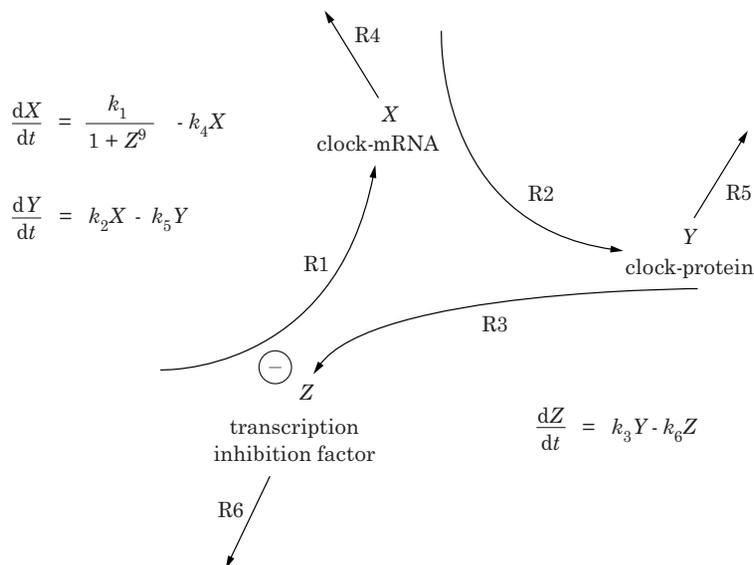


FIG. 1. The Goodwin model. Reaction R1 is the formation of clock mRNA (X); reaction R2 is the synthesis of clock protein (Y) and R3 is the production of a transcription inhibitor (Z). R4, R5 and R6 represent degradation reactions.

TABLE 1
 frq^+ and frq^7 parametrizations*

Type	k_4	k_5	k_6	Period	Start values ($t = 0$)
frq^+	0.2	0.2	0.1	22.3	$X_0 = 3.931 \times 10^{-2}$ $Y_0 = 1.811 \times 10^{-1}$ $Z_0 = 1.712$
frq^7	0.2	0.1	0.1	28.2	$X_0 = 2.214 \times 10^{-2}$ $Y_0 = 1.918 \times 10^{-1}$ $Z_0 = 1.826$

* $k_1 = k_2 = k_3 = 1.0$ in all cases.

effect of different constant temperatures on the protein degradation of long-period and short-period mutants of *Neurospora* and *Drosophila*, quantitative agreement between calculations and experiments were obtained (Ruoff *et al.*, 1996).

Here we studied the effects of a constant inhibition of protein synthesis (and degradation) on the period lengths of *Neurospora*'s circadian sporulation rhythm (Lakin-Thomas *et al.*, 1990), both in the wild-type (frq^+) and the long-period (frq^7) mutant and compared these with the effects of such inhibition in the model. We further studied phase shifting effects of inhibitory pulses on protein synthesis and degradation in the Goodwin model and compared them with experimental phase response curves for pulses of cycloheximide (CHX), moderate temperature changes, and heat shock (HS) on the sporulation rhythm of *Neurospora crassa* wild-type strain and frq mutants. Computations with the Goodwin model show that, although the rates of clock gene transcription or clock protein synthesis have little influence on the period length, the phase is considerably influenced by changes in both transcription or translation rates. Interestingly, parametrizations of the Goodwin model reflecting the wild-type (frq^+) and the long-period frq^7 mutant show, as observed experimentally, that the frq^7 parametrization (Table 1) is considerably less sensitive towards inhibitory pulses in R2 and R5 than the frq^+ parametrization. In a similar way, the effects of HS pulses on the *Neurospora* rhythm can be considered as a result of temporary inhibition of both transcription and translation.

Materials and Methods

STRAINS AND CULTURE METHODS

The strains used were *band* (*bd*) with the wild-type *frequency* gene (frq^+) or the frq^7 mutation. The HS and the CHX PRCs were performed with the *bd conidial separation* (*csp-1*) frq^7 mutant. The $bd frq^+$ and the $bd frq^7$ mutants were obtained from Fungal Genetics Stock Center (FGSC), The University of Kansas Medical Center, Department of Microbiology, 3901 Rainbow Blvd., Kansas City, Kansas 66160-7420, USA. FGSC Stock No.: *bd*, 1858; frq^7 , 4898 (Internet: <http://www.kumc.edu/research/fgsc/main.html>). Patricia Lakin-Thomas, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, U.K. kindly sent us samples of the *bd csp-1 frq^7* mutant. In the *csp-1* mutant conidia fail to separate and become airborne. The *csp* mutation is known to shorten periods about 1 hr (Dharmanda & Feldman, 1979; Lakin-Thomas & Brody, 1985). In our experiments free-running controls of the *bd csp-1 frq^7* strain showed an average period of 27.9 hr in agreement with the work by Lakin-Thomas & Brody (1985).

Cultures were grown in liquid Vogel's medium (Vogel, 1956) with 2% sucrose (LL-medium). After inoculation of approximately 6×10^7 conidia l^{-1} in Petri dishes (90 mm diameter) they were exposed to white fluorescent light ($80 \mu\text{mol s}^{-1} \text{m}^{-2}$ in the range of 400–700 nm; blue light intensity $24 \mu\text{mol s}^{-1} \text{m}^{-2}$ in the range of 400–500 nm) for 36 hr at 25°C. Then discs were cut out (1 cm in diameter) by means of a cork borer and placed in Vogel's medium with low sucrose (0.4%) at constant darkness, which corresponds to circadian time (CT) 12.

In phase response curve (PRC) experiments mycelia were exposed to 1 hr heat shock (45°C), elevated temperatures (30, 35, 40°C), or 3 hr CHX pulses ($1 \mu\text{g ml}^{-1}$), starting 12 CT units after the transfer to constant darkness (CT 12).

PHASE SHIFTING ASSAY

After treatment with CHX, the discs were washed in fresh medium and placed in race tubes (25°C) on solid agar containing $1 \times$ Vogel's medium with 0.4% sucrose and 1.0% agar.

Temperature-treated discs were placed in race tubes (25°C) immediately after the perturbation.

CONTINUOUS CHX INFLUENCE

To study the effect of CHX on growth rate and sporulation, mycelial discs were transferred to race tubes (25°C) with different CHX concentration in the agar. The CHX was added to the agar before casting the gel into the race tubes.

DETERMINATION OF PROTEIN DEGRADATION

Protein degradation *in vivo* was determined according to a modified pulse-chase method (Cornelius *et al.*, 1985). ³⁵S-methionine was incorporated into mycelial discs during a 2 or 3 hr incubation period. Part of the mycelial discs were frozen thereafter in liquid nitrogen, stored at -20°C, and maximum incorporated radioactivity and protein content were determined. The other part of the discs were washed in LL-medium and either treated with CHX in LL-medium (with an excess of non-radioactive L-methionine) or were kept in the same medium at 20, 30 and 42°C without CHX. At certain time intervals mycelial samples were taken and protein content and the remaining radioactivity determined. Protein degradation was expressed as radioactivity μg^{-1} protein in relation to maximum incorporated radioactivity μg^{-1} protein (in %).

COMPUTATIONS

We have previously studied the three-dimensional Goodwin model with respect to temperature behavior of short- and long-period mutants in *Neurospora crassa* (Ruoff *et al.*, 1996). In these studies sets of rate constant values have been used to describe temperature-compensation of frq^+ , frq^1 , and frq^7 . In the present paper we have used the same sets of rate constants for frq^+ and frq^7 . The sets will be described as frq^+ or frq^7 parametrizations (Table 1).

The differential equations of the Goodwin model (Fig. 1) were solved numerically on a HP9000 computer using the double-precision FORTRAN subroutine LSODE (Hindmarsh, 1980). Phase response curves were calculated relative to the maximum of X (mRNA). The phase shift $\Delta\Phi$ was calculated, due to the

“softness” of the oscillations, 800 time units after the perturbation, i.e.

$$\Delta\Phi = \Phi_{unper}^m - \Phi_{per}^m \quad (1)$$

where Φ_{unper}^m is the phase of the unperturbed X maximum, while Φ_{per}^m is the corresponding X maximum of the perturbed oscillator (Ruoff & Rensing, 1996). According to the definition of eqn (1), *phase delays* result in *negative* $\Delta\Phi$ s, while *phase advances* are described by *positive* $\Delta\Phi$ s.

The perturbations applied were inhibitory pulses of 1 time unit duration. During a pulse, one or more rate constants were lowered from k_j to k'_j by multiplying each k_j with a constant inhibition factor $f_{j,inhib}$ ($0 < f_{j,inhib} < 1$), i.e.

$$k'_j = k_j \times f_{j,inhib} \quad (2)$$

The percentage of inhibition for process R_j is calculated as $(1 - f_{j,inhib}) \times 100\%$. After the inhibitory pulse, the original value of k_j is restored, and the oscillator proceeds with the original parametrization.

Results

INFLUENCE OF CONTINUOUS CHX APPLICATION

We have investigated the effect of different CHX concentrations in the growth medium (agar) of racetubes on the growth rate and period length of the sporulation rhythm of frq^+ and frq^7 mutants. Figure 2(a) shows that the growth rate decreases with increasing CHX concentrations both in frq^+ and frq^7 . Figure 2(b) shows that increasing CHX concentrations result in a slight increase of the period length in both mutants. Calculations simulating the continuous effect of CHX reveal a close agreement between calculations and experiments obtained when both the synthesis (R2) and the degradation (R5) of the clock protein (Y -species) are inhibited by CHX [Fig. 2(c)].

INHIBITION OF PROTEIN DEGRADATION BY CHX AND HEAT SHOCK

Apart from the primary effect of CHX to inhibit protein synthesis, CHX also inhibits protein degradation. Figure 3(a) shows the effect of a 3 hr CHX pulse ($5.7 \mu\text{M} = 1.6 \mu\text{g ml}^{-1}$) on the degradation rate of frq^+ *N. crassa* cyto-

plasmic proteins. CHX resulted in a clear reduction of proteolysis of the treated mycelia.

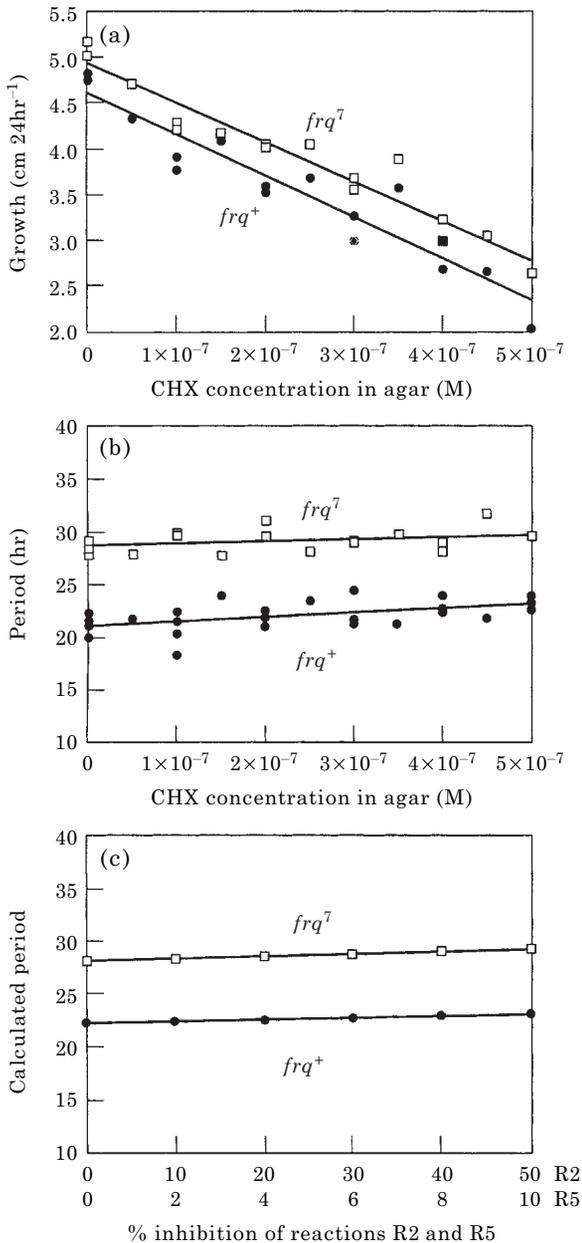


FIG. 2. Growth rates of *frq*⁺ and *frq*⁷ strains in racetubes as a function of CHX concentration in the agar of the tubes. No growth has been observed for a CHX concentration above 5×10^{-7} M; (b) periods of the sporulation rhythm of *frq*⁺ and *frq*⁷ strains in the same racetubes as in (a); (c) simulating the effect of the continuous presence of CHX on the period length of Goodwin oscillator by assuming simultaneous inhibition of clock protein synthesis and degradation (reactions R2 and R5). Upper curve: period of *frq*⁷ parametrization; lower curve: period of *frq*⁺ parametrization. Upper row of abscissa shows % inhibition of R2, while lower row shows % inhibition of R5.

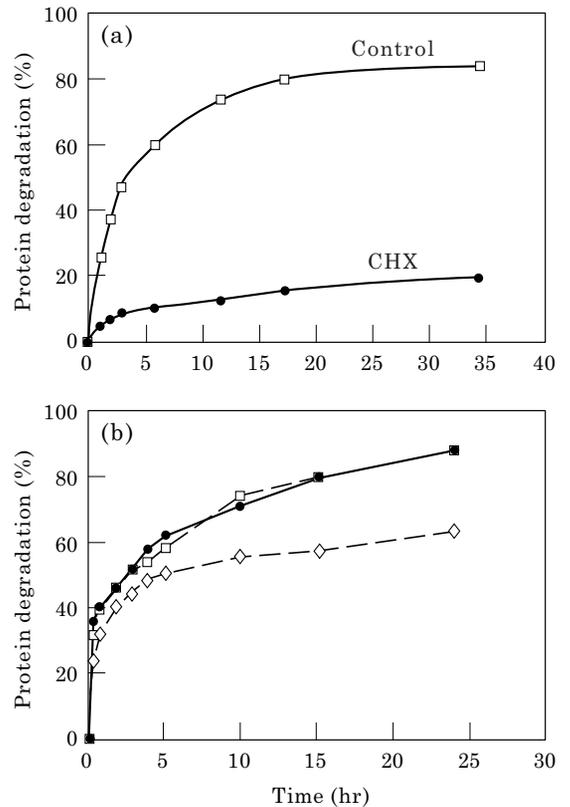


FIG. 3. (a) Influence of $5.7 \mu\text{M}$ CHX on the *in vivo* degradation of cytoplasmic proteins in *frq*⁺ *N. crassa*. The degradation was determined after a 2 hr incubation with ³⁵S-methionine (defining 100% of incorporated radioactivity μg^{-1} protein) and is expressed as % of degradation-dependent decrease of ³⁵S-methionine radioactivity per μg protein; (b) influence of temperature on *in vivo* degradation of cytoplasmic proteins in *frq*⁺ *N. crassa*. The degradation was determined after a 3 hr incubation with ³⁵S-methionine. Otherwise identical to (a). (●) 20°C; (□) 30°C; (◇) 42°C.

Heat shock (HS) leads not only to a reduction in overall protein synthesis, but also to a reduction in protein degradation [Fig. 3(b); Cornelius & Rensing, 1986; Mohsenzadeh *et al.*, 1994; Rensing *et al.*, 1995].

In the model calculations HS has therefore been simulated by a temporary inhibition (1 time unit duration) of protein synthesis and protein degradation, i.e. a temporary decrease in the rate constants k_2 and k_5 ; see eqn (2).

PHASE RESPONSE CURVES (PRCS) OF CHX AND TEMPERATURE/HS PULSES

The phase response of CHX pulses on *Neurospora's* sporulation rhythm have been studied by several investigators (Nakashima *et*

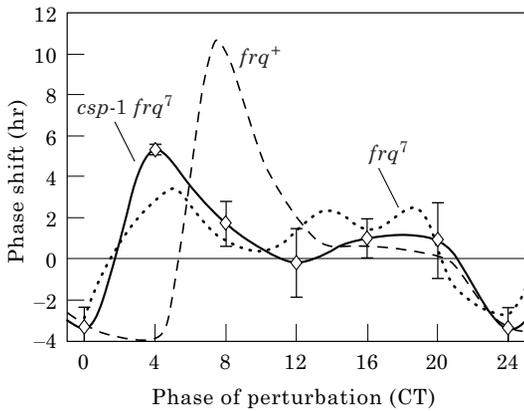


FIG. 4. Overview of CHX-PRCs with *frq*⁺ (Nakashima, 1984), *frq*⁷ (Dunlap & Feldman, 1988), and *csp-1 frq*⁷ (this work). In all three cases 1 $\mu\text{g ml}^{-1}$ CHX was applied as a 3 hr pulse. The *csp-1 frq*⁷ PRC represents the mean of three independent experiments with indicated standard deviations.

al., 1981; Nakashima, 1984; Schulz *et al.*, 1985; Dunlap & Feldman, 1988). Typically, large positive phase shifts are observed at about CT 4–8.

Interesting is the behavior of the *frq*⁷ mutant compared with the wild-type (*frq*⁺). Dunlap & Feldman (1988) reported that *frq*⁷ is considerably less sensitive towards CHX pulses compared with *frq*⁺. When clock protein synthesis reaction R2 was strongly pulse-inhibited initial calculations with the Goodwin model indicated that the resulting phase response curves for *frq*⁺ and *frq*⁷ parametrization show similar large phase advances at about CT 4–6 (Lakin-Thomas, pers. comm.). This prompted us to repeat the experimental CHX-PRC for the *frq*⁷ mutant and to investigate the Goodwin model more closely. In our experiments we used the *bd csp-1 frq*⁷ mutant, and kept the other experimental conditions equal to those of the work by Dunlap & Feldman (1988). Figure 4 summarizes the PRCs of a 3 hr CHX pulse (1 $\mu\text{g ml}^{-1}$) for *frq*⁺ (Nakashima, 1984), *frq*⁷ (Dunlap & Feldman, 1988) and the PRC of this study for *csp-1 frq*⁷. The *frq*⁷ and *csp-1 frq*⁷ strains show considerably

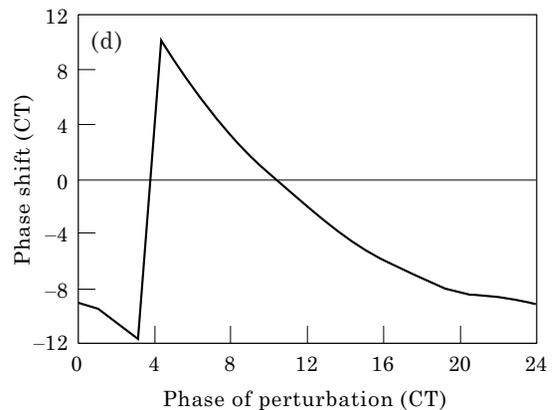
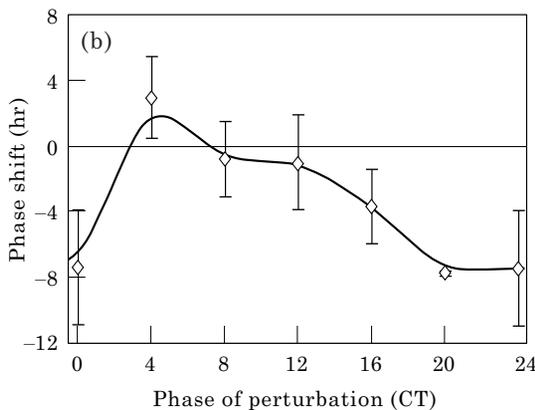
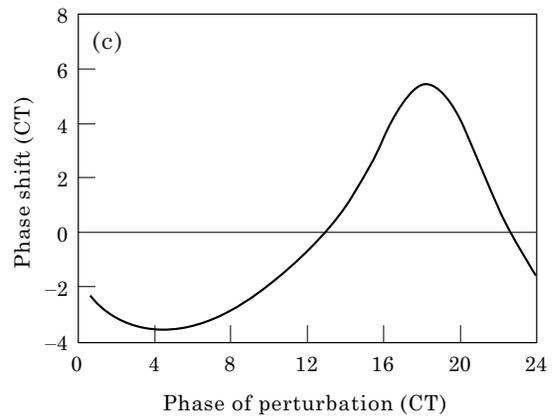
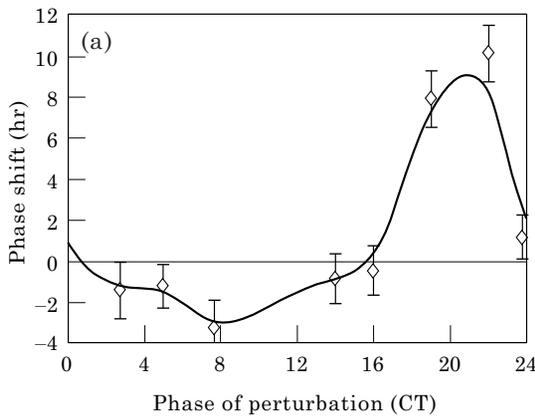


FIG. 5. (a) PRC of a 3 hr 30°C temperature pulse on the *frq*⁺ strain (Rensing *et al.*, 1987); (b) PRC of a 1 hr 45°C temperature pulse on the *frq*⁺ strain; (c) calculated PRC for moderate temperature pulse perturbations. The PRC is a replot of Fig. 5(a), curve 4 of Ruoff & Rensing (1996); (d) calculated PRC for high temperature pulses (HS) assuming 98% inhibition of reaction R2 using *frq*⁺-parametrization (Table 1).

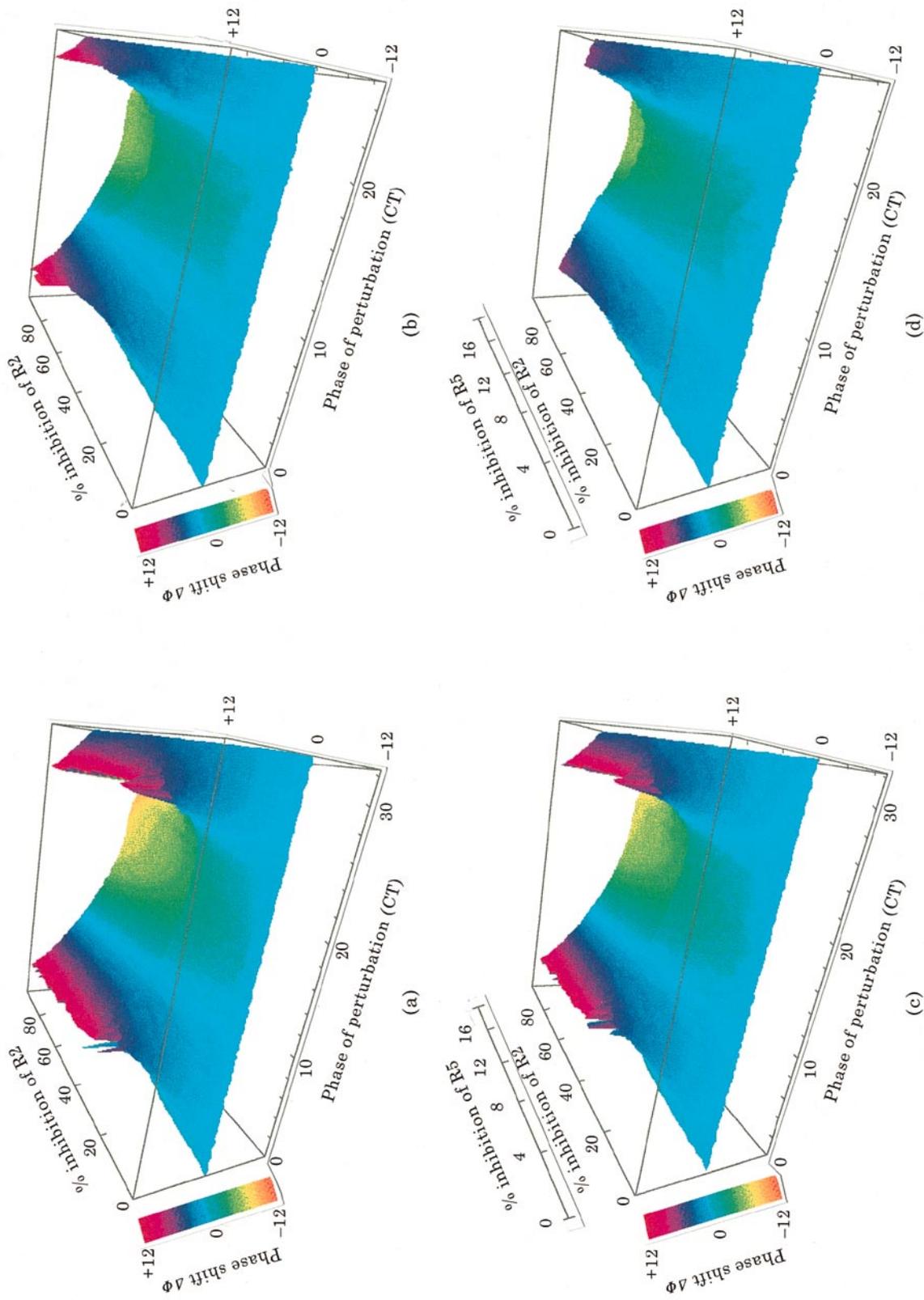


FIG. 6. (a) Calculated PRCs of increasing inhibitory strength of pulses (1 time unit duration) on reaction R2 using $freq^+$ -parametrization (Table 1); (b) Same as (a), but using $freq^-$ parametrization instead; (c) calculated PRCs of increasing inhibitory strength of pulses (1 time unit duration) acting simultaneously both on reaction R2 and R5 using the $freq^+$ parametrization. The inhibition of R5 is always 1/5 of the inhibition of R2; (d) same as (c), but $freq^-$ parametrization has been used instead.

smaller phase advances compared with frq^+ with the maximal advance shift at CT4 instead of CT8 as in frq^+ . Whether the approximately 2 hr larger maximum phase advance of $csp-1\ frq^7$ as compared with frq^7 is a significant difference, remains unclear. A possible correlation between period length and the sensitivity towards CHX is discussed below.

Short, or moderate temperature pulses ($\approx 30\text{--}40^\circ\text{C}$) lead to PRCs with maximum phase shifts at about CT 16–20 [Fig. 5(a), Francis & Sargent, 1979; Rensing *et al.*, 1987; Nakashima, 1987; Goto *et al.*, 1994], while long, or high temperature pulses [heat shock (HS), 45°C] lead to PRCs with maximum phase shifts at about CT4 [Fig. 5(b)] similar to the PRC of CHX. No differences in phase response curves between frq^7 and frq^+ were observed when moderate (positive) temperature pulses were applied (Nakashima, 1987). When we studied the HS (1 hr 45°C) PRC for $csp-1\ frq^7$, also no significant differences to frq^+ were observed (not shown). Computations with the Goodwin model show the same behavior: when all rate constants were allowed to increase by means of a moderate temperature pulse, the PRC showed its maximum phase shift between CT 16–20 [Fig. 5(c)]. However, when the protein synthesis reaction R2 was inhibited by a HS or a CHX pulse, the maximum phase shift occurred between CT 4 and 8 [Fig. 5(d)], as it was in the experiment [Fig. 5(b)].

Because of the different responses towards CHX between the frq^+ and frq^7 strains (Dunlap & Feldman, 1988; Fig. 4), we studied the effects of inhibitory pulses on protein synthesis (reaction R2) and protein degradation (reaction R5) in more detail. Figure 6(a,b) show three-dimensional representations of the phase response curve with increasing inhibition of reaction R2 for frq^+ and frq^7 parametrizations. In these calculations the inhibition was gradually increased from 0% ($f_{2,\text{inhib}} = 1.00$) to 98% ($f_{2,\text{inhib}} = 0.02$). When comparing Fig. 6(b) with Fig. 6(a) it is evident that the frq^7 parametrization is less sensitive towards inhibition than frq^+ , because in frq^7 a higher inhibition of R2 is necessary to get large phase shifts of “type 0” (Winfree, 1980). Only when very strong inhibitory pulses act on R2 ($>80\%$) frq^+ and frq^7

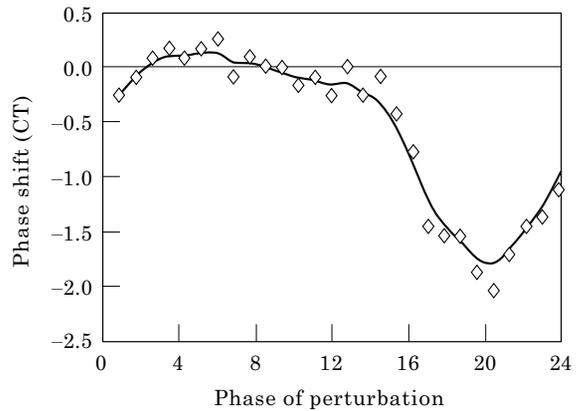


FIG. 7. Phase shifts in the Goodwin model become mostly negative when the inhibition of reaction R5 becomes high in addition to a high inhibition of reaction R2. The figure shows a calculated PRC for the frq^7 parametrization when reactions R2 and R5 are inhibited by 98 and 82%, respectively.

parametrizations behave similarly and generate both large advances and delays.

When protein degradation reaction R5 becomes inhibited in addition (as the experiments in Fig. 3 show), then the transition to “type 0” PRCs occurs both for frq^+ and frq^7 parametrizations at a higher degree of inhibition. At such conditions the frq^7 parametrization may become less sensitive towards simultaneous R2 and R5 inhibitions as compared with the frq^+ parametrization. Figure 6(c) and Fig. 6(d) illustrate the phase responses when the inhibition of clock protein degradation is one-fifth of the inhibition of clock protein synthesis.

By increasing the inhibition factor $f_{5,\text{inhib}}$ further, the resulting phase shifts become mostly negative (Fig. 7), similar to what is observed in the *Neurospora* HS PRC. However, the calculated phase shifts are smaller than in the experiment [Fig. 5(b)].

Discussion

The Goodwin model is considered as a *minimum* model, i.e. it is assumed to contain the most important parts of the circadian pacemaker (without making more assumptions than minimally needed). However, in dealing with a minimum model certain sacrifices have to be made. Here, one of them is the unrealistically

high exponent in the inhibition factor $1/1 + Z^9$. Another seems to be the slow ("soft") resetting of the oscillations, describing better the slower *Drosophila* resetting behavior (Zimmerman *et al.*, 1968) than the more rapid *Neurospora* resetting (Lakin-Thomas *et al.*, 1990). However, from the Goodwin model some remarkable predictions about the role of degradation processes in the circadian oscillator can be made. As discussed below, some of these predictions will need still further experimental investigations, while others fit very well with experiments.

CONTINUOUS CHX PERTURBATION IN RACETUBES

When CHX is present in the agar of racetubes, growth and sporulation can be observed up to a CHX concentration of 5×10^{-7} M ($0.14 \mu\text{g ml}^{-1}$). Above this concentration no growth has been observed. Earlier studies (Dunlap & Feldman, 1988) showed that 5×10^{-7} M CHX inhibited total protein synthesis by approximately 50%. Assuming that this value may also apply to the inhibition of FRQ protein synthesis and that the inhibition of the FRQ protein degradation rate is as low as assumed in the calculations of Fig. 6(c) and Fig. 6(d), computed period lengths with increasing inhibition of processes R2 and R5 [Fig. 2(c)] are indeed consistent with the corresponding experimental results [Fig. 2(b)]. With increasing inhibition of FRQ protein synthesis (and degradation) by increasing CHX concentrations a slight increase of the period length is observed. Although the observed experimental increase of period lengths with increasing CHX concentrations is relatively small, a similar increase of period length caused by protein synthesis inhibitors has been observed in *Euglena* (Feldman, 1967) and *Bulla* (Khalsa *et al.*, 1992). Our earlier findings (Ruoff *et al.*, 1996) that the degradation reactions of the (oscillatory) intermediates in the Goodwin model have a major influence on its period may apply to the experiments with *Euglena* and *Bulla*. We hypothesize that influences on the degradation of protein-components of the central pacemaker (for example by protein synthesis inhibitors) play an important role in the control of the circadian period length.

PULSED PROTEIN SYNTHESIS AND PROTEIN DEGRADATION PERTURBATIONS

Although the anabolic rate constants of reactions R1, R2, and R3 in the Goodwin model have little influence on the period (Ruoff *et al.*, 1996), sudden changes of these rate constants have a marked influence on the phase of the oscillations. A pulsed inhibition of clock protein synthesis (reaction R2) shows positive phase shifts at about CT 4–8 [Figs 5(d), 6(a,b)] similar to CHX pulses (Nakashima *et al.*, 1981; Nakashima, 1984; Schulz *et al.*, 1985; Dunlap & Feldman, 1988) or HS pulses [Fig. 5(b)]. The agreement between experiment [Fig. 5(b)] and calculation [Fig. 5(d)] is only qualitative, probably reflecting the simplicity of the model. When, in addition, also clock protein degradation (reaction R5) becomes pulse-inhibited large phase shifts ("type 0" PRCs) occur only at a higher strength of inhibition of reaction R5. The reason why the *frq*⁷ parametrization is less sensitive towards pulse inhibition of R2 compared with the *frq*⁺ parametrization, is because of the lower degradation rate of FRQ⁷ (*Y*) in relation to FRQ⁺. In the *frq*⁺-oscillator an inhibition of R2 leads, during the perturbation, to a decrease in *Y* twice as fast as in the *frq*⁷ parametrization, because for *frq*⁺ *k*₅ is twice as large as for *frq*⁷. Due to this faster decrease, the *frq*⁺-trajectory spans a portion of the phase space twice as large as the *frq*⁷ trajectory during the pulse. Figures 8(a,b) illustrate this situation for a small (20%) inhibitory pulse (1 time unit duration) on the projection of the trajectory in the *X*–*Y* phase plane. An additional simultaneous inhibition of R5 lowers the net change of *Y* during the pulse which for a certain ratio of R2/R5 inhibition does not differ significantly from the change in *Y* of the unperturbed oscillator [Fig. 8(c)]. This is apparently the reason for the even greater insensitivity of the *frq*⁷ parametrization when both protein synthesis and protein degradation become inhibited [Fig. 6(d)]. Of course, as indicated by Fig. 6(c), also the *frq*⁺ parametrization will become less sensitive towards an inhibitory pulse when both reactions R2 and R5 are inhibited during the perturbation.

When comparing the *csp-1 frq*⁷ CHX PRC with the *frq*⁷ and *frq*⁺ PRC (Fig. 4) one may

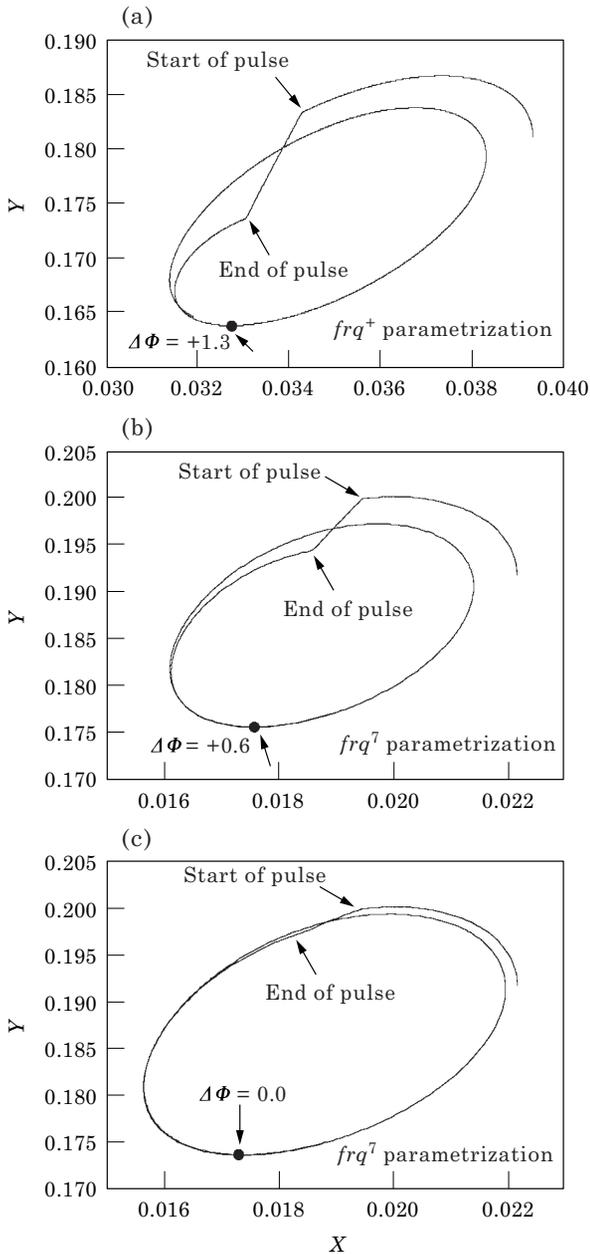


FIG. 8. (a) Projection of trajectory in the XY phase plane using frq^+ parametrization. Arrows indicate a pulse of 20% inhibition on R2 (duration of 1 time unit). $\Delta\Phi$ shows the induced phase advance due to the decrease in Y during the pulse. It should be noted that the oscillator does reset on a trajectory with somewhat smaller X - Y amplitudes, because under these conditions (Table 1) the model does not show limit cycle oscillations. Despite the slight damping of the unperturbed oscillator, the period of both frq^7 and frq^+ parametrizations remain unchanged (± 0.1 time units) during a time interval of 3000 time units; (b) Same trajectory as in (a) but frq^7 parametrization used instead. The gain in phase advance due to the perturbation is now half of the advance of the frq^+ parametrization; (c) same trajectory as in (b) but in addition to the 20% R2 inhibition a simultaneous 15% inhibition on reaction R5 is introduced. No gain in phase advance due to the inhibitory pulse is observed.

wonder whether the amplitude differences in the maximum advance may be related to the different periods of these mutants. In fact, the Goodwin model predicts such a behaviour for which the stabilities of the FRQ-protein or the frq -mRNA appear to be the determining factors.

The influences of temperature pulses can be explained along similar lines. The effect of moderate temperature pulses (for example in the range up to $+30^\circ\text{C}$) appears to be due to a general increase of the anabolic and catabolic reaction rates because at this temperature no inhibitory effects on protein synthesis or degradation [Fig. 3(b)] are observed. For such systems the PRC shows a typical phase advance at about CT 16–20 [Fig. 5(a)]. This behavior has been found in a variety of *N. crassa* mutants including frq^7 (Francis & Sargent, 1979; Rensing *et al.*, 1987; Nakashima, 1987; Goto *et al.*, 1994). In fact, this behaviour can be modelled by the Goodwin oscillator, simply by increasing all reaction rates during the temperature pulse [Fig. 5(c); Ruoff & Rensing, 1996].

In case of a pulsed HS, both protein synthesis and protein degradation are drastically reduced, in which the PRC leads to a change of the positive phase shift peak from CT 16–20 to CT 4–8 [Fig. 5(b)] leading to a PRC similar to that of CHX. In the calculations the same change of positive phase shifts to earlier CT values is observed [Fig. 5(d)]. In case of a HS pulse, the rather low amplitude in the PRC [Fig. 5(b)] suggests that, in terms of the Goodwin oscillator (Fig. 7), the degradation of the clock protein may become inhibited. The fact that no significant differences in the HS PRCs have been observed between *csp-1 frq^7* and frq^+ indicates that a HS-pulse may lead to an equally strong inhibition of clock-protein degradation as well as clock-protein synthesis. At such conditions PRCs of frq^7 and frq^+ parametrizations become very similar (Fig. 7). On the other hand, HS may inhibit other processes of the clock (Rensing & Monnerjahn, 1996; Rensing *et al.*, 1995).

The observation that the maximum advance moves to lower CTs and decreases with increasing strength of protein synthesis inhibition has also been reported for the anisomycin PRC in *Gonyaulax* (review Cornelius & Rensing, 1982). That most (or even all) phase shifts in the

HS-PRC become negative has not only been observed in *Neurospora* [Rensing *et al.*, 1995; Fig. 5(b)], but also in *Bulla* eye preparations (see first comment by G. Block in Discussion of Rensing *et al.*, 1995, p. 41). In *Drosophila* even moderate temperature pulses lead to PRCs with mostly delays (Maier, 1973; Edery *et al.*, 1994; Sidote *et al.*, 1998). In the latter case, it appears interesting to explore the “*Drosophila* parametrization” of the Goodwin oscillator (Ruoff *et al.*, 1996) in more detail.

AMPLITUDE MODEL

Lakin-Thomas *et al.* (1991) suggested, on the basis of a two-dimensional model, that the lesser sensitivities of the *N. crassa* long period mutants towards CHX (or light) may be related to an increased amplitude in these oscillators. Although an assay for the amplitude in the *Neurospora* rhythm is still to be found (Lakin-Thomas, 1998), *frq*-mRNA or/and FRQ-protein might be possible candidates in such an assay. Indeed, Northern analysis (Aronson *et al.*, 1994) shows a considerably larger *frq*⁷-mRNA amplitude when compared with *frq*⁺. On the other hand, this is opposite to the behavior found in the Goodwin model where the mRNA amplitude in the *frq*⁷-parametrization is lower than in the *frq*⁺-parametrization (not shown). More experiments are necessary to test a possible relationship between *frq*-mRNA/FRQ-protein amplitudes and *Neurospora*'s phase resetting behavior.

PRCS OF RESPIRATORY INHIBITORS AND ALCOHOL

Interestingly, pulses of respiratory inhibitors such as potassium cyanide, sodium azide, antimycin A, and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) show PRCs in *Neurospora* that resemble the PRC for CHX (Nakashima, 1984). The effect of these inhibitors may be explained by an indirect inhibition of protein synthesis by blocking the synthesis of ATP and thereby the formation of necessary amino-acyl-AMP compounds. To the extent these compounds also inhibit the degradation of proteins (by inhibition of ATP-dependent proteases), they may also increase the period, as the calculations with the Goodwin model suggest.

When analysing PRCs toward ethanol lower concentrations led to PRCs similar to PRCs in response to moderate temperature pulses, whereas higher concentrations resulted in a PRC similar to the influence of protein synthesis inhibitors or HS. No tendency to more negative phase shifts, however, was observed (not shown). This indicates that ethanol at higher concentrations also inhibits protein synthesis, which was actually observed in *N. crassa* (Meyer *et al.*, 1995).

Conclusion

The temperature-compensated Goodwin model has been shown earlier (Ruoff & Rensing, 1996) to simulate many circadian clock properties, as, for example, PRCs of temperature steps or pulses. Here we have demonstrated that changes of PRC due to an assumed inhibition of clock protein synthesis and clock protein degradation by HS, and protein synthesis inhibitors, (or respiratory inhibitors) are in agreement with the changes of PRCs observed experimentally. The experimentally observed difference between *Neurospora frq*⁺ and *frq*⁷ mutants towards CHX can also be described by the model, stressing the importance of degradation of intermediates in the central pacemaker.

NOTE ADDED IN PROOF

While this paper was in press, we became aware of the work by Onai *et al.* (1998) who found that an apparent lower rate in protein synthesis in the *Neurospora crassa un*-18 mutant increased the period length by approximately 2 hours. We believe that this behavior is closely related to the here discussed period increases when a protein synthesis inhibitor is continuously applied.

ONAI, K., KATAGIRI, S., AKIYAMA, M. & NAKASHIMA, H. (1998). Mutation of the gene for the second-largest subunit of RNA polymerase I prolongs the period length of the circadian conidiation rhythm in *Neurospora crassa*. *Mol. Gen. Genet.* **259**, 264–271.

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APPENDIX

Abbreviations

- $\Delta\Phi$: phase shift [eqn (1)]
- Φ_{unper}^m : phase of X maximum of unperturbed oscillator [eqn (1)]
- Φ_{per}^m : phase of X maximum of perturbed oscillator [eqn (1)]

- bd*: band mutant strain of *N. crassa*
- CHX: cycloheximide
- csp-1*: conidial separation gene “1” in *N. crassa*
- CT*n*: circadian time *n*. CT*n* is the time/phase equal to $n/24$ of the circadian oscillators period length
- $f_{j,inhib}$: factor by which rate constant k_j is temporarily decreased leading to an inhibition of reaction R_j [eqn (2)]
- FRQ: protein of *frequency* (*freq*) gene
- freq*⁺: *frequency* gene in *N. crassa* wild type
- freq*⁷: mutated allele “7” of *frequency* gene in *N. crassa*
- HS: heat shock
- k_i : the rate constant of reaction R_i (Fig. 1)
- PRC: phase response curve