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FORTY YEARS OF PRCs—
WHAT HAVE WE LEARNED?

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ABSTRACT

What are phase-response curves (PRCs)? How can they be measured? How should they be plotted? These questions and many other fascinating facets of PRCs are addressed in this review, including research topics in which phase-resetting data have provided crucial insights: entrainment, phototransduction, pacemaker mechanism, phase markers of the pacemaker, and gauges of oscillator amplitude. PRCs have enlightened us and will continue to be a valuable tool in clock research. (Chronobiology International, 16(6), 711–743, 1999)

INTRODUCTION/HISTORY

Bünning recognized in the 1930s that light can set the phase of circadian clocks, and that there was a daily cycle of responsiveness to light pulses given at different phases of the circadian cycle. Bünning, Rawson, and DeCoursey discovered the phase-dependent resetting by light serendipitously as they attempted to find the best protocol for taking care of their experimental organisms—Bünning wanted to water his plants, and Rawson and DeCoursey wanted to take care of their rodents (feeding and cleaning) without disturbing their clocks (Bünning 1970; P. DeCoursey, personal communication, 1999).

In the 1950s, a number of circadian researchers developed these ideas further and began to map the daily patterns of light responsiveness (e.g., Rawson 1956; Pittendrigh and Bruce 1957; Bruce and Pittendrigh 1958). Just a little over 40 years ago, the first journal publication of phase-shifting data that was plotted into the now-familiar form of a phase-response curve (PRC) appeared, namely, light-induced phase resetting of the clock of that scintillating model system, Gonyaulax (Hastings and Sweeney 1958). PRCs of rodents were reported in Ph.D. theses by Burchard (1958) and DeCoursey (1959). Journal publications by Pittendrigh, Bruce, and DeCoursey quickly followed that defined the PRCs of fruit flies (Pittendrigh and Bruce 1959) and flying squirrels (DeCoursey 1960).

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Compiling the PRC Atlas induced me to study all the PRCs that had been published up to 1990 (Johnson 1990). That project and several publications since 1990 led to some generalizations that I believe are worthwhile to summarize here. This paper is a modified and updated version of a paper that was originally published in *Circadian Clocks from Cell to Human* (Johnson 1992). That paper was modified and is published here by permission of Drs. Ken-ichi and Sato Honma.

Data that come from the PRC Atlas are quoted extensively here. For data that have been published, the primary publication is usually referenced. For those cases of data that have not been published other than in the atlas, the reference is in the atlas format (e.g., “H/Ma-9” for the hamster PRC measured after entrainment to LD 18:6). The reference for the atlas is the 1990 citation by Johnson.

**PHASE-RESPONSE CURVES AND ENTRAINMENT**

Two major classes of models have been proposed to explain the mechanism(s) by which circadian clocks are entrained to environmental cycles: the discrete model (also called nonparametric or phasic) and the continuous model (also called parametric or tonic) (Daan 1977). The continuous entrainment model has been based on the observation that the free-running period (FRP) is dependent on light intensity and suggests that light has a continuous action on the clock to entrain it to the light-dark (LD) cycle. One mechanism that has been suggested is that the acceleration and deceleration of the FRP (i.e., angular velocity) by daily changes in light intensity could allow the circadian pacemaker to adjust its cycle length continuously to that of the environment (Aschoff 1960). The potential involvement of continuous effects in circadian entrainment is addressed below.

The discrete model has been the most successful model to date in predicting the entrainment of some organisms, most notably *Drosophila* and nocturnal rodents. Its basic premise is that an entrained circadian pacemaker is in equilibrium with an LD cycle consisting of repetitive light pulses (the zeitgeber) when each pulse falls at that phase in which the light pulse elicits a phase shift that is equal to the difference between the FRP and the period of the entraining cycle. In nature, the zeitgebers are the dawn and dusk transitions, which are mimicked in the laboratory by light pulses. Because the effective action of light is considered to be due merely to discrete time cues in nature (e.g., at dawn and/or dusk), this mechanism of entrainment has been called the discrete or nonparametric model. The elegant simplicity of this model lies in its excellent predictive properties based on only two pieces of information: the FRP and the map of phase-dependent resetting called the PRC. The discrete model has been described in detail elsewhere (Pittendrigh and Minis 1964; Pittendrigh and Daan 1976; Pittendrigh 1981; Johnson et al. 1999).

**Phase-Response Curves and Phase-Transition Curves**

The discrete model posits that, to entrain to the daily light-dark cycle, the circadian oscillator must respond differently to light at different phases of its cycle. PRCs are useful descriptions of this phase-dependent response. A PRC is a plot of phase shifts of a circadian rhythm as a function of the circadian phase that a stimulus, or zeitgeber, is given. Stimuli include light pulses, temperature pulses, or pulses of drugs or chemicals.
As shown in Fig. 1, representative PRCs of circadian oscillators for light pulses exhibit delay phase shifts in the early subjective night and advance phase shifts in the late subjective night, with little phase shifting occurring during the subjective day (hence, the subjective day portion of the PRC is often referred to as the "dead zone"). As discussed below, some of these topological features of light PRCs are crucial in determining the ability of circadian pacemakers to entrain to the daily light-dark cycle.

As illustrated in Fig. 1, there are two so-called types of PRCs—type 1 and type 0 (Winfree 1980). Type 1 displays relatively small phase shifts (e.g., usually less than 6h phase shifts) and has a continuous transition between delays and advances, whereas type 0 PRCs show large phase shifts. Whether type 1 or type 0 resetting is exhibited often depends on the strength of the stimulus. For example, increasing the light fluence or drug dosage can convert type 1 into type 0 resetting. Other factors, however, can also cause the conversion from type 1 to type 0, such as genetic mutation (as in the per' mutant of Drosophila; Saunders et al. 1994) and background light quality and/or intensity (as in Gonyaulax; Christianson and Sweeney 1973). These other factors probably affect the responsiveness of the clock to stimuli to modulate the perceived stimulus strength.

The terms type 0 and type 1 refer to the average slope of the curve when plotted as "new phase" versus "initial phase"—a so-called phase-transition curve (PTC) (whereas a PRC plots phase-shift versus initial phase). As compared in Figs. 2A and 2B, type 1 PRC resetting can be visualized as a PTC with an average slope of 1 (45° angle), whereas Fig. 2E depicts a type 0 PTC, which has an average slope of 0 (0° angle). A "limit-cycle" interpretation of type 1 versus type 0 resetting is compared in Figs. 2C and 2F. Phase-shifting stimuli are posited to change the state variables from the limit cycle (the circle of Figs. 2C, 2F, 2I) to another area of the phase plane labeled the "resetting contour" (the heavy dashed line of Figs. 2C, 2F, 2I). If this change moves the state variables to another isochron on the phase plane, a steady-state phase shift will be observed (iso-

![Type 1 PRC](image1.png)

![Type 0 PRC](image2.png)

**Initial circadian phase (phase of the pulse onset)**

**FIGURE 1.** Types of phase-response curves. Advance phase shifts are plotted as positive values; delay phase shifts are plotted as negative values. Subjective day is circadian phases 0–12; subjective night is circadian phases 12–24. The "dead zone" is the area in the subjective day in which the phase shift is zero. The type 0 PRC is plotted in both the usual format (middle panel) and also monotonically, by which all phase shifts are plotted as delays. Advance phase shift points are open circles for easier comparison.
Circadian phase of pulse onset ("initial phase")

FIGURE 2. Representative PRCs, PTCs, and limit-cycle diagrams for type 1 resetting versus type 0 resetting. Figures 2A–2C depict type 1 resetting from the mosquito *Culex* (Peterson 1980). Figures 2D–2F depict type 0 resetting from the fruit fly *Drosophila pseudoobscura* (F/Dp-2 from the atlas). Figures 2G–2I depict "critical stimulus" resetting from the unicellular alga *Gonyaulax* (Johnson and Hastings 1989). Figures 2A, 2D, and 2G are PRCs plotted as phase shift (ordinate: advances positive, delays negative for 2A and 2G; 2D is plotted monotonically) versus circadian time of stimulus onset (abscissa). Figures 2B, 2E, and 2H are PTCs plotted as "new" phase (ordinate: new phase = phase of clock after the phase shift) versus circadian time of stimulus onset (abscissa). Figures 2C, 2F, and 2I are limit-cycle diagrams in which the limit cycle is the circle with isochrons radiating from the central "singularity" point. The heavy dashed line is the resetting contour, that is, the points on the phase plane to which state variables are changed by resetting stimuli. The resetting of state variables is depicted by the arrows from points on the limit cycle to points on the resetting contour.
chron: *iso* = same; *chronos* = time). Type 1 resetting results if the resetting contour is not moved beyond the singularity (resetting contour on “near side” of singularity), whereas type 0 resetting occurs if the stimulus is strong enough to move the variables beyond the singular region (resetting contour on “far side” of singularity) (Peterson 1980; Winfree 1980; Lakin-Thomas 1995; Johnson et al. 1999).

Figures 2G, 2H, and 2I also illustrate an interesting case of “critical stimulus” resetting in *Gonyaulax* (Johnson and Hastings 1989). Light pulses given early in the subjective night provoke type 1 resetting, while light pulses given later yield type 0 resetting, yielding a highly asymmetrical PRC and resetting contour.

It might be assumed that stimuli presented during the dead zone (e.g., circadian time [CT] 2–10) do not modify the state variables. While this can be true for some specific models, it is not a necessity for a limit-cycle model. The other, equally plausible, alternative is that stimuli presented during the dead zone induce changes of the state variables, but these altered values do not move the variables to a different isochron (see Fig. 2F). Therefore, no phase shift results. Consequently, state variables of the pacemaker are not necessarily insensitive to the stimulus during the dead zone—in fact, the stimulus could induce large changes of the state variables, but these changes do not move the pacemaker to a different isochron. This view has important implications for identifying molecular correlates of state variables. In particular, we should not make it a criterion for a state variable of the clockwork that its responsiveness to phase-resetting stimuli correlates directly with the magnitude of phase shifting at every phase—especially at phases in the dead zone.

If the phase shifts of a type 0 PRC are plotted as advances and delays, a discontinuity (the “break point”—see Fig. 1) often appears at the transition between delay and advance phase shifts. The break point discontinuity of PRCs is, in some cases, merely a plotting convention of arbitrarily assigning phase shifts in one half-cycle (12h) as delays and in the other half-cycle as advances. To avoid these arbitrary distinctions, sometimes type 0 PRCs are plotted monotonically—that is, all phase shifts are plotted as delays from 0h to 24h (Fig. 1). When plotting those type 0 PRCs that happen to be asymmetric (e.g., Fig. 2G), the break point is not an arbitrary convention because the PRC has a discontinuity no matter how the PRC is plotted.

An advantage of plotting phase shifts monotonically (or as a PTC) is that such plots do not lead to an assumption that advance resetting versus delay resetting is mechanistically different (e.g., that advance phase shifts result from a pacemaker’s state variable being changed in one direction), while delay phase shifts change the variable in the opposite direction. In fact, limit-cycle models usually suggest no mechanistic difference between advances and delays in type 0 resetting. Moreover, the limit-cycle models usually interpret the transition from type 1 to type 0 resetting to be merely dependent on whether the magnitude of the stimulus is sufficient to shift the resetting contour beyond the “singularity.”

**Measurement of a Phase-Response Curve**

In principle, PRCs can be determined by a number of different protocols, as described by Aschoff (1965). Four of the most commonly used protocols are described below:

1. The stimulus (pulse) is applied while the oscillator is free running (e.g., a light pulse to an organism free running in constant darkness [DD]). In this case, the individual organism serves as its own control, and accurate assignment of the
circadian time of the stimulus depends on knowledge of the circadian time of
the phase reference point in a free run. Usually, the circadian time of the phase
reference point is assessed by its phase in an LD 12:12 cycle, but it is impor-
tant to make sure that no “masking” of the phase reference point occurs in LD.
The possibility of masking can be evaluated by releasing the organism from
LD to a free run and confirming that the phase reference point in the free run
extrapolates back to the phase reference point in LD.

2. The stimulus (pulse) is applied in a free run shortly after release from entrain-
ing conditions (e.g., a light pulse to an organism in DD within a few cycles
after release from LD 12:12). This is a good method when a population of
organisms is being tested; it requires a few control organisms (cultures) that
do not receive a stimulus with which to compare the treated ones. This is
probably the best method for estimating entrainment behavior as the PRC
shape soon after release from entrainment should be more reflective of its
shape during entrainment than its shape will after a long exposure to free-
running conditions (also see Mrosovsky 1996b for other advantages of this
protocol).

3. The stimulus is a “step” from one continuous condition to another (e.g., DD
to LL).

4. The PRC can be estimated from the phase angle assumed by the rhythm to
different T cycles (periods of a zeitgeber) of the stimulus (e.g., T cycles of light
pulses). An example of this method is that of Eskin (1971), who compared the
PRC derived by method 1 above with that derived from method 4 and found
them to be equivalent. Method 4 cannot give a complete PRC because the
phase angle will not be stable around the break point region of the PRC during
entrainment to T cycles.

Phase-Response Curve Plotting

The PRCs in the atlas were plotted in a standardized format so that different PRCs
could be compared easily. The comments below refer to the format used in the PRC
Atlas.

General Information

The abscissa is the circadian time (CT) of the stimulus, from CT 0 to CT 24. The
time between CT 0 and CT 24 is the duration of the endogenous FRP. Because LD 12:12
is taken to be standard entrainment conditions, CT 0 is defined as the beginning of the
subjective daytime (therefore, subjective dawn or “lights on”) and CT 12 as the beginning
of the subjective night (therefore subjective dusk). The ordinate is the magnitude of the
phase shift in circadian hours. Advances are plotted above the abscissa as positive values,
while delays are plotted below as negative values. For type 0 resetting, all the phase
shifts may be plotted monotonically, as in Figs. 1 and 2D.

Circadian Time

Because circadian pacemakers have different endogenous frequencies, the PRCs
among different organisms cannot be compared directly unless their timescales are stan-
dardized to circadian time. The first aspect of circadian time is that the scales for both
the circadian time of stimulus (abscissa) and the magnitude of phase shift (ordinate) are expressed in “circadian hours.” PRCs are scaled in circadian hours so that both the horizontal and vertical axes of PRCs from different organisms may be compared directly. A circadian hour is equal to 1/24 of the FRP (therefore, a circadian hour = FRP/24 [in hours]). To convert “real” hours to “circadian” hours, the number of real hours (e.g., of the phase shift) is multiplied by 24/FRP.

Definition of Circadian Time Zero

The second aspect of circadian time is that PRCs must be plotted along the abscissa relative to some defined time, that is, circadian time zero (CT 0). In general, the definition of CT 0 has been the least standardized variable of PRCs in the literature, and yet, it is crucial for the comparison of the phase-shifting responses among organisms.

The standard definition of CT 0 is that it is the phase in the free run that extrapolates back to the last “dawn” (i.e., lights on of the last-seen LD 12:12 cycle prior to release into constant conditions—DD or LL). In many cases, however, alternative definitions are necessary. PRCs that are measured in LL often use the beginning of LL as the extrapolated dawn rather than lights on of the final light cycle. In addition, many PRCs have been measured from organisms that have been in constant conditions for a long time so that it is inaccurate or inconvenient to extrapolate to the final lights-on signal. For these PRCs, circadian time is usually defined from the phase reference point. First, the phase angle in LD 12:12 of the phase reference point of the rhythm is measured. Then, this phase reference point is assumed to define the same phase of the oscillator in free-running conditions, and CT 0 becomes a certain number of circadian hours before or after the phase reference point in the free run. For example, activity onset of nocturnal rodents occurs at dusk in LD 12:12 and is consequently defined as occurring at CT 12. Therefore, CT 0 becomes the time that is 12 circadian hours before or after activity onset in a free run.

As discussed above (method 1 of PRC measurement), determining the phase reference point in LD can be complicated by the problem of masking. Consequently, in all cases, the circadian time of the phase reference point in LD should be determined by releasing the organism into free-running conditions and extrapolating the phase reference point back to its phase angle in the last cycle of LD.

Estimation of the Magnitude and Direction of the Phase Shift

There are two major problems to be taken into account when estimating the phase shift: (1) frequently, the period (FRP) changes after a stimulus (“aftereffects”); and (2) often—especially in the case of advance phase shifts—there can be transient cycles of little or no phase resetting before the steady-state phase shift is established. The best way to avoid both of these problems is to extrapolate the phase reference point for many cycles before and after the stimulus, preferably by a least-squares linear regression. Obvious transient cycles should be excluded from this regression. Then, the phase shift is calculated by the difference on the day of the stimulus between the extrapolated phase reference point before and after the stimulus. In the case of method 2 of PRC measurement, the phase reference points of control organisms are extrapolated back to the day of the stimulus and used to compare with the extrapolations from experimental organisms.

For type 1 resetting, it is usually easy to determine whether the phase shift should be plotted as an advance or delay on a PRC, but when the large phase shifts of type 0
resetting are encountered, it is often difficult to assign the direction of the phase shift unambiguously. One approach is merely to plot the PRC monotonically (as in Fig. 1)—from a limit-cycle perspective, the distinction between advances and delays in type 0 resetting is arbitrary.

Another approach to distinguish operationally between advances and delays while using a PRC-type presentation is to perform dose-response experiments, thereby generating dose-response curves (DRCs). DRCs assay the response at a given circadian phase of the clock to varying intensities/concentrations of the stimulus. Reducing the stimulus intensity will switch type 0 resetting to type 1 resetting, at which point the distinction between advances and delays becomes more obvious.

Definition of Stimulus Phase

No matter what type of stimulus is considered, be it a light, temperature, or chemical pulse, the onset of the stimulus pulse was plotted as the “stimulus phase” on the abscissas of the PRCs in the atlas. In their original papers, many authors plotted PRCs using other conventions for stimulus time—often the midpoint was used, and sometimes even the end of the pulse. There is no a priori reasoning that favors any of these criteria as the stimulus phase. All are arbitrary. Aschoff (1965) urged that the stimulus midpoint be used as the stimulus time. His argument—that PRCs plotted that way “line up” better—makes sense for many light PRCs. When one considers all the types of stimuli and PRC shapes, however, such a plotting convention can cause problems. In particular, I will argue later that, for many chemical/drug stimuli, the effective duration of the pulse is unknown since the time of recovery from a drug does not always coincide with the time of washout. If the effective duration is unknown, the midpoint is unknown.

The beginning of the pulse was therefore chosen as the standard marker for stimulus phase in the PRC Atlas. The phase-shifting response of an oscillator is likely to be a characteristic of the phase when the stimulus begins, that is, of the first unperturbed phase to be presented with a stimulus. If one uses the midpoint or end of the stimulus as the marker, then one is choosing a phase that has already been perturbed. Moreover, using the onset as the marker for the stimulus phase circumvents the complications of recovery time.

Single or Multiple Stimuli?

What about cases of PRCs for which the stimulus is not a single pulse, but a series of pulses? For example, one “PRC” that appears in the PRC Atlas is that of Czeisler et al. (1989) in which 5h pulses of light were presented to human subjects on three consecutive days. This study became well known because it claimed to have achieved type 0 resetting in humans, a conclusion that was controversial (see Beersma and Daan 1993; Kronauer et al. 1993; Stokes et al. 1999). The issue of type 1 versus type 0 resetting of the human pacemaker need not be answered here, but it should be noted that PRCs have traditionally been considered to be the response of a circadian oscillator to a single stimulus. From that perspective, the three-pulse study of Czeisler et al. (1989) would not, strictly speaking, be called a PRC. Nevertheless, because one main use of PRCs is to explain entrainment (see below), and the Czeisler et al. (1989) protocol is essentially a brief entrainment experiment, it was included in the PRC Atlas. To have excluded it might have been a case of excessive rigor resulting in unenlightening rigor mortis.
Phase-Response Curves and Entrainment

The most extensive use of PRCs to light and temperature stimuli has been to help understand how circadian pacemakers are entrained to the daily cycles of light and temperature (Pittendrigh 1981). Briefly, phase resetting compensates for the fact that the FRP of circadian oscillators is not equal to 24h—therefore, entraining stimuli (e.g., light) reset the clock to equalize the period of the entrained oscillator (the circadian clock) to the period of the entraining oscillator (the daily rotation of the earth). It is important to keep in mind two key assumptions of the model: (1) that the FRP measured in constant conditions accurately reflects the circadian period functioning under entrainment conditions, and (2) that the stimuli used in entrainment and to derive the PRC are effectively the same. The basic principle of the discrete model of entrainment is summarized by the following equation for a circadian oscillator under steady-state entrainment. For one light pulse per cycle, the circadian pacemaker is entrained solely by stimuli that fall at that phase of the pacemaker specified by the PRC such that a phase shift is evoked that is equal in magnitude to the difference between the FRP and the period of the entraining cycle (i.e., T). In other words,

\[ \text{Phase Shift} = \text{FRP} - T \]

For example, if the FRP is 23h, then the pacemaker must experience a net delay phase shift of 1h (−1h phase shift) to entrain to a 24h LD cycle. For an FRP of 21h, the phase shift in steady state must be a delay of 3h, which will be accomplished by the light pulse striking the PRC in the early subjective night. Conversely, for an FRP of 27h, the steady-state phase shift must be an advance of 3h, so that the light pulse will strike the PRC in the late subjective night. Because the light pulse must strike a different phase of the pacemaker (as gauged by the PRC) for FRP = 21h versus FRP = 27h to achieve steady-state entrainment to a LD cycle of 24h, the phase angle (or phase relationship) between the entraining light pulse and a given pacemaker will be different for different FRPs (Johnson et al. 1999).

Light Pulse Resetting

Light is usually the most important zeitgeber for entraining circadian oscillators. Therefore, PRCs for light stimuli have special interest and, indeed, have been studied most extensively. As mentioned above, light pulse PRCs usually have similar characteristics: delay phase shifts in the early subjective night, advance phase shifts in the late subjective night, and little phase shifting during the subjective daytime. This generalization holds true whether or not the overt rhythm peaks in the day or night or at twilight (Pittendrigh 1981; Johnson et al. 1999). Therefore, the PRCs of nocturnal organisms versus those of diurnal organisms are similarly phased to the light-dark cycle, although their rhythms are not.

The magnitude of phase shifting exhibited by the clock is a gauge to its “limits of entrainment.” Obviously, PRCs with large phase shifts can permit synchronization to light-dark T cycles of a broader range compared with low-amplitude PRCs. The magnitude of phase shifting by light is dependent on the intensity and duration of the stimulus (among other factors). As the intensity and/or duration is increased, light PRCs of limit-cycle pacemakers go through two transitions. As mentioned above, the phase shifting first changes from type 1 to type 0 resetting (Figs. 1 and 3), so the magnitude of phase shifting increases, but the circadian time of the transition between delay and advance
FIGURE 3. Changes in the PRC to light pulses as the duration of the light pulses is increased in Sarcophaga (Saunders 1978). Durations of light pulses are 1h, 3h, 14h, and 20h. The PRC to 1h pulses is type 1, but the longer duration light pulse PRCs are type 0. As the duration of the pulse is increased from 3h to 14h to 20h, the PRC shifts leftward (or downward, depending on your perspective). The type 0 PRCs are plotted monotonically.

shifts remains fixed. There are several good examples of this type of PRC transition (type 1 to type 0) with increasing stimulus strength that appear in the PRC Atlas from the algae Chlamydomonas and Euglena, the plant Kalanchoe, insects (cockroaches, mosquitoes, Nauphoeta, Sarcophaga, and Drosophila), and rats (all listed in Johnson 1990).

As the duration (and possibly intensity) of the light pulse is increased further, the second transition occurs: the PRC shifts leftward (Fig. 3). This second transition has been interpreted as the clock “stopping” at CT 12 until the light pulse is terminated, but other data suggest that, during the light pulse, the clock continues to oscillate on another limit cycle that is near an isochron of CT 12 of the limit cycle in DD (Peterson 1980; Johnson et al. 1999). Fewer examples of the second type of PRC transition exist for light PRCs—-the best are Drosophila (Pittendrigh 1960) and Sarcophaga (Saunders 1978; see Fig. 3).

Stable entrainment does not necessarily require a PRC that has essentially the same symmetrical advance-versus-delay topology as do the PRCs depicted in Figs. 2A and 2D. In fact, for entrainment to occur, a circadian oscillator’s PRC need only have (1) a region of negative slope (between 0 and −2) and (2) a point on the PRC at which the phase shift equals FRP − T. In particular, it is not necessary to have a PRC with both delays and advances. If the free-running period is longer than 24h, a PRC that exhibits only advance resetting could allow stable entrainment. A specific example is Gonyaulax cells under red light illumination (Fig. 2G): The period is 25h, and the PRC for blue or white light pulses is essentially all advances (up to 12h advance). In this case, Gonyaulax will entrain to a light-dark cycle (or white/red light cycle), with the onset of the light pulse (dawn) occurring at the circadian time that results in a 1h phase advance (Johnson and Hastings 1989). Therefore, highly asymmetric PRCs can allow stable entrainment.
Dark Pulse Resetting

The apparently opposite stimulus of a light pulse is to give a dark pulse to an organism in LL. The most simplistic model would predict that dark-pulse PRCs will be the mirror image of light-pulse PRCs, which is nearly true for Paramecium (Fig. 4; Johnson et al. 1989). Although this is an approximately valid description of some dark-pulse resetting, dark-pulse PRCs are usually not the exact mirror image of light-pulse PRCs. One rationale for explaining why the two types of PRCs may not be mirror images is that the duration of the dark-pulse stimulus usually needs to be longer than the corresponding light-pulse stimulus to get comparable phase shifts, and the discrepancy between the durations of light versus dark pulses could alter the shape of the resulting PRCs. The PRC Atlas includes dark-pulse PRCs for Acetabularia, Euglena, Gonyaulax, Paramecium, Lemna, chicken pineals, hamsters, sparrows, and bats (Taphozous).

Reebs and Mrosovsky discovered an interesting “artifact” in hamsters with regard to dark pulses and pulses of some drugs. They noticed that dark pulses and some antidepressant drugs (e.g., the benzodiazepine triazolam) stimulate wheel-running activity in hamsters. They subsequently tested whether the stimulation of locomotor activity would alone mimic the phase-shifting action of dark pulses (Reebs and Mrosovsky 1989; Mrosovsky 1996a). It did. Furthermore, van Reeth and Turek (1989) found that stimulation of activity was likely to be the means by which phase shifting by the drug triazolam was accomplished. Therefore, in hamsters, dark pulses appear to reset by feedback of the overt rhythm onto the pacemaker (Reebs et al. 1989). As Mrosovsky is the first to admit, however, dark-pulse stimulation of activity cannot explain phase shifting by dark pulses in all cases, such as dark-pulse phase resetting of organ cultures (Mrosovsky 1996a). Nevertheless, it will be interesting to find other examples of feedback of overt rhythms onto pacemakers.

Temperature Pulse Resetting

Temperature-pulse PRCs have been measured in a variety of organisms; those included in the atlas test the clock response in Euglena, Gonyaulax, Oedogonium, Neurospora, Bryophyllum, Kalanchoe, Lemna, Phaseolus, Hemideina, Leucophaea, Uca, Per-

![Graph](image)

**FIGURE 4.** Light-pulse PRCs versus dark-pulse PRCs in Paramecium bursaria (Johnson et al. 1989). Figure 4A is the PRC for 4h light pulses; Fig. 4B is the PRC for 6h dark pulses.
ognathus, and hamsters (in this final case, pulses of hypothermia). Although temperature can undoubtedly function as a zeitgeber, it apparently plays a supporting role to the light-dark cycle. In entrainment studies of conflicting light and temperature cycles, the light-dark cycle predominates in *Euglena* (Bruce 1960); cyanobacteria (Lin et al. 1999); *Drosophila* and cockroaches (Pittendrigh 1960); and *Pectinophora* (Pittendrigh and Minis 1971). Comparisons of the amplitudes of the light PRCs and the temperature PRCs were not done in these studies. It would be interesting to repeat these types of experiments using light and temperature stimuli that elicit PRCs of equivalent amplitude and then determine whether light still predominates. In *Neurospora*, temperature cycles with amplitudes that are ecologically reasonable appear to take precedence over light-dark cycles with a saturating light pulse in entrainment (Liu et al. 1998; Merrow et al. 1999). More studies of this type are warranted.

**Lability of Phase-Response Curve Shape**

The shape and amplitude of PRCs are subject to many influences. As discussed above, perhaps the most obvious parameter that alters PRC shape and amplitude is the strength of the stimulus, in terms of either intensity/concentration or duration (Figs. 1–3). But, there are many factors that can influence PRC shape, some of which are described below.

**Environment**

Environmental conditions can have major effects on PRC shape. For example, the shape of PRCs can change after entrainment to different photoperiods or different *T* cycles (Binkley and Mosher 1986; H/Ma-7–11). Even a single stimulus can alter the shape of the subsequent PRC (H/Ma-12, 13). Background illumination conditions can have profound influence on the magnitude of phase shifting and PRC shape. For example, there are many examples of stronger responses to light pulses for organisms in DD compared with organisms in LL (e.g., Christianson and Sweeney 1973; Kondo 1982, 1983; Johnson et al. 1989). Background illumination can even change the spectral sensitivity of light-induced phase resetting. For example, in *Chlamydomonas*, the action spectra for cells in DD versus LL are different. The clocks of *Chlamydomonas* cells in LL respond to red and blue light, and photosynthetic inhibitors prevent light-induced phase resetting, suggesting that components of photosynthesis are involved in clock resetting of cells in LL (Johnson et al. 1991). On the other hand, green and red light reset the *Chlamydomonas* clock in DD, and photosynthetic inhibitors are ineffectual (Kondo et al. 1991).

Similarly, different colors of background illumination can affect light-induced phase resetting. For example, the amplitude and shape of the *Gonyaulax* PRC to white light pulses are different in a background of constant red versus constant white light (Johnson and Hastings 1989). Another component of the environment that in some cases affects the phase response to light is temperature. In particular, the response of *Neurospora* to light is a function of temperature (Nakashima and Feldman 1980; also see below). In contrast, the response of the *Drosophila* oscillator to light pulses is not affected by different ambient temperatures (F/Dp-2, 7, 9, 10).
Developmental History

Although most organisms that have been tested have not displayed developmental plasticity of PRC shape, cockroach larvae are an exception (Page and Barrett 1989; Page 1991). Raising roach larvae in various illumination regimes (LD, LL, DD, and T cycles) modifies the FRP and transforms the light PRCs of the mature cockroaches—that is, the resulting PRCs become mostly advance, mostly delay, or symmetric PRCs, depending on the developmental conditions. This unexpected result demonstrates that PRCs are not developmentally immutable.

Strains

Some different strains/mutants have different PRC shapes. There are a number of mutants that were isolated on the basis of an altered FRP that were found subsequently to have altered PRCs as well, for example, *Drosophila per* mutants (Konopka 1979; Saunders et al. 1994), *Neurospora frq* mutants (Dharmananda 1980), and the hamster *tau* mutant (Shimomura and Menaker 1994). Another interesting example is that of strains of *Drosophila auraria* collected from different latitudes in Japan; the strains have significantly different sensitivities to light that are reflected in varying PRC shape (Pittendrigh and Takamura 1989). These differences in PRC shape and amplitude may be a key to understanding the differing photoperiodic responsivities of this fly along the latitudinal gradient.

Ecological Strategies of Phase-Response Curve Shape

Another kind of PRC shape lability is that of the possible functional significance of having a PRC that is asymmetric. These are PRCs that exhibit both delays and advances, but in which the area under either the advance or the delay portion of the PRC predominates (referred to below as the ratio of advance area to delay area, or A/D). Figure 5 illustrates PRCs with various A/D shapes. Pittendrigh and Daan have pointed out that an appropriate combination of FRP values and asymmetric PRC shapes can give

![Figure 5. PRCs exemplifying different A/D shapes. Panel A has a large advance: delay ratio (sparrows after LD 12:12; Binkley and Mosher 1986). Panel B shows a PRC that is approximately symmetrical (sparrows after LD 8:16; Binkley and Mosher 1986), while panel C depicts a PRC of small A/D from rats (Honma et al. 1985).](image)
stable entrainment of pacemakers to various photoperiods such that a specific phase of the oscillator will always occur at a given phase angle to either dawn or dusk of the various light-dark cycles (Pittendrigh and Daan 1976; Pittendrigh 1980, 1981). This phase angle will be independent of the length of the photoperiod, so that it is compensated for seasonal changes in the photoperiod. The consequence of this observation is that it could account for the ability of organisms to maintain a relevant phase relationship throughout the year; a “clock for all seasons.”

For example, an FRP of less than 24h in combination with a PRC that has relatively more delay area than advance area (= small A/D) will allow CT 12 of the pacemaker’s cycle to coincide with dusk on light-dark cycles that have a variety of ecologically relevant day lengths (e.g., photoperiods from 6h to 18h). This is a strategy that may be adaptive for a nocturnal animal (e.g., a mouse) that needs to begin its activity at dusk. The converse example—long FRP and large advance/delay PRC—yields an oscillator with a CT 0 phase that will coincide with dawn irrespective of the photoperiod’s duration, hence, an optimal strategy for a diurnal organism like an “early bird that wants to catch the worm” at dawn (Pittendrigh and Daan 1976; Pittendrigh 1980, 1981).

Related to the above is whether the organism is exposed to the complete photoperiod during the day under natural conditions. (This “daylight exposure” criterion is not the same as “day activity”; for example, nocturnal predators such as cats may be exposed to natural sunlight throughout the day, but hunt at night.) Whether or not an organism is exposed to a more-or-less complete photoperiod (PP) is important from the perspective of entrainment. Clocks that are exposed to the full photoperiod could afford to be less light sensitive than clocks that see only brief light pulses at dawn and/or dusk.

The hypothesis that PRC shape versus FRP are adjusted to allow seasonally relevant phase angles is elegant. Is it really true that organisms do it this way? To determine if PRC shape, FRP, and sensitivity are correlated with PP, exposure and/or activity patterns, information from the light PRCs in the atlas was compared (Johnson 1992). The first conclusion was that the data in the atlas are inadequate to determine whether there are clear differences between the relative sensitivities of clocks that are PP, exposed versus those that are not PP, exposed. Whether one defines “sensitivity” on the basis of (1) threshold intensity, (2) threshold duration, and/or (3) amplitude of PRC, few PRCs can be compared directly.

But, are there correlations between PRC shape/FRP on the one hand and PP, exposure and/or activity patterns on the other hand? Tables 1 and 2 summarize the data from my 1992 analyses. Table 1 shows the correlations between PRC shape, FRP, and activity

<table>
<thead>
<tr>
<th>Active interval</th>
<th>A/D</th>
<th>FRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>&gt;1</td>
<td>≤1</td>
</tr>
<tr>
<td>Night</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 1. Activity Patterns, Phase-Response Curve (PRC) Shape, and Free-Running Period (FRP) in Animals

Data from PRC Atlas (Johnson 1990) and reported in Johnson 1992. A/D = advance/delay phase shift area ratio in a PRC.
patterns in animals. Pittendrigh's prediction that night-active animals might prefer A/D < 1 and FRP < 24 is weakly supported by the data. The prediction that day-active animals might prefer A/D 1 and FRP > 24 is not supported by the data; there are no significant trends for day-active animals (Table 1).

Table 2 summarizes PRC shapes and FRP data for all representative organisms on the basis of PP, exposure. PP, exposed organisms exhibit A/D shapes of all kinds, although there are slightly more symmetrical PRCs than either A/D < 1 or A/D > 1 PRCs. PP, exposed organisms seem to avoid FRP values close to 24h. Organisms not exposed to PP, favor A/D < 1 and type 1 PRCs. When the data of Table 2 are reanalyzed on the basis of plants versus animals or whether the PRC was measured in the presence of background illumination (DD vs. LL), no new correlations emerge. (Note, however, that Aschoff's extensive analysis of FRP vs. intensity of LL does indicate significant differences for the response of FRP to LL between day-active and night-active organisms; see Aschoff 1979. Unfortunately, PRC shape is not known for most of those organisms.) The data of Table 2 demonstrate that some correlations between PRC shape and photoperiod exposure may exist.

My intuition is that the Pittendrigh and Daan hypothesis may prove to be an excellent model in the case of nocturnal animals, which have daily exposures to light in nature that are behaviorally modulated to be essentially the same as skeleton photoperiods (Decourcy 1986), but for organisms exposed to the complete photoperiod each day, other factors will need to be taken into account, as suggested in the next section.

Continuous Entrainment

The data shown in Tables 1 and 2 should prompt some revision of our concepts of entrainment mechanisms. The discrete mechanism successfully models entrainment of clocks not exposed to PP, with the exception of the "phase-angle jump" observed in skeleton photoperiods of long duration; the discrete model cannot explicitly explain why the phase-angle jump does not occur under natural conditions (Pittendrigh and Daan 1976; Pittendrigh 1981).

The organisms in which the discrete model of entrainment is most firmly entrenched are animals not exposed to PP, such as nocturnal rodents and fly pupae, all of which may effectively limit their light exposure in nature to skeleton photoperiods. Therefore, their entrainment properties and sensitivity to light may be optimized to those conditions in a way that is not representative of organisms that are exposed to a complete
photoperiod every day. It seems intuitively obvious that the entrainment of organisms that are exposed to complete photoperiods (e.g., plants and many animals) are likely to be a composite of continuous and discrete mechanisms.

One example of a case for which continuous entrainment must be an important factor in determining the phase relationship of a rhythm to a light-dark cycle is that of Arabidopsis, for which the FRP of the promoter activity of the cab gene in LL versus DD is distinctly different (FRP_{LL} = 25h and FRP_{DD} ≈ 30h; Millar et al. 1995). For example, in an LD 12:12 cycle, the dark interval is 12h long, but the subjective dark phases of the Arabidopsis circadian cycle will be over 15 hours in darkness (i.e., 50% of 30h). This means that, during the night, the clock will freely run through only 80% (12 + 15) of its subjective night phases before the dawn signal causes an advance phase shift. If its period in darkness were the same as its period in LL, then the clock would have free run through 96% (12 + 12.5) of its subjective night phases before dawn occurred. The circadian phase of the PRC at which the dawn signal will occur will be significantly different in these two cases, and therefore the phase relationship of the rhythm to the light-dark cycle will also differ. By the same reasoning, because daylight shortens the FRP during the day to a value closer to 24h, the Arabidopsis clock entrained to LD 12:12 will nearly reach CT 12 (subjective dusk) by the time of true dusk. If its FRP during the day were 30h, the clock would reach only about CT 9.6 by the time of sunset. The basic point is that, because light so profoundly modulates the FRP of Arabidopsis cab gene expression, the phase relationship of this rhythm under entrainment should be strongly influenced by the continuous action of light during the day.

Another example that cannot be explained by the discrete model is the activity pattern of the diurnal European ground squirrel (Hut et al. 1999). This rodent emerges from its burrow several hours after dawn and returns several hours before dusk every day. Therefore, it does not see either the dawn or dusk light-dark transitions, and yet it entrains with a stable phase angle. While the precise mechanism by which these squirrels accomplish this feat is unknown, the animals might be generating their own pattern of exposure to sunlight by modulating both phase and period (Hut et al. 1999).

Cases like these of Arabidopsis and ground squirrels demand that continuous entrainment be considered as an important factor in determining the phase relationship under entrained conditions. Past discussions of discrete versus continuous entrainment mechanisms have sometimes indicated that one or the other mechanism is operative in entraining circadian clocks. Rather, it is likely that many organisms utilize both mechanisms to some degree (Aschoff 1999).

LIGHT PHASE-RESPONSE CURVES AND PHOTOTRANSDUCTION PATHWAYS

The circadian clock became linked early in its evolution with (or had as an integral component) a photosensitive process that allowed the entrainment of the clock to the light-dark cycle of the sun. It might be supposed that this linkage could lead to valuable clues about the conservation or diversity of the oscillator's biochemical mechanism; if the circadian pacemaker originated once during evolution and its mechanism was subsequently conserved, then one scenario would predict that the pigment(s) involved in the photosensitive process might also be conserved. Recent experiments suggest that blue light photopigments called cryptochromes may play a role in the phototransduction path-

ways of organisms as diverse as the plant *Arabidopsis* (Somers et al. 1998), the arthropod *Drosophila* (Stanewsky et al. 1998), and the mouse (Thresher et al. 1998). In the mouse, it might be that the role of cryptochrome is integral to the clock, and that its ancient role as a clock photopigment has been lost (Kume et al. 1999; van der Horst et al. 1999). Cryptochrome is not the only clock photopigment in these organisms; in plants, phytochrome plays a major role (Somers et al. 1998), and in mammals, the primary clock photopigment(s) is as yet undiscovered (Freedman et al. 1999; Lucas and Foster 1999).

Phase setting by light pulses or by light-dark cycles has been the tool to discover clock phototransduction pathways. As such, PRCs have been an integral part of the hunt for clock photopigments. The approaches to identify photopigments have primarily been combinations of genetics (especially mutants and knockouts) and/or action spectroscopy. Because some aspects of the interpretation of action spectra have close ties to interpretations of PRC phenomena, these are briefly discussed here.

Action spectroscopy is dosimetry with light. To measure an action spectrum, one measures the photoresponse (in this case, the phase shift) at different wavelengths. For each wavelength, a range of fluences are used (fluence is number of photons per unit area; fluence rate is fluence per unit time, and it is equivalent to the less-precise term intensity). In the ideal case, for which "univariance" holds, the slope of the fluence response curve (plotted as phase shift vs. log fluence) is the same for each wavelength, and the sensitivity at each wavelength is evaluated as the fluence necessary to achieve a given arbitrary photoresponse. On the other hand, if the shape or linearity of the fluence response is different at various wavelengths, it can mean that a screening pigment is interfering with the spectral response.

It is beyond the scope of this paper to discuss action spectroscopy in detail (see Galland 1987 for a review). But, the special limit-cycle properties of circadian clocks have implications for action spectra that warrant discussion here. For example, in type 1 resetting, the resetting curve does not cross the singularity (Fig. 2C), and so the fluence response could match that of an ideal univariant case. This is in fact the result obtained by Takahashi et al. (1984) for phase resetting of the hamster clock.

For type 0 resetting, however, the expected response can be quite different even with an ideal univariant photoreceptor. At the phase(s) at which light pulses yield maximal phase resetting, increasing the pulse fluence can cause the clock to be reset to regions close to or beyond the singularity. This can cause a discontinuity in the fluence response curve. This type of response to varying fluence has been observed in *Gonyaulax* (Johnson and Hastings 1989), *Neurospora* (Fig. 32 in Dharmarana 1980), and *Chlamydomonas* (Johnson and Kondo 1992). These discontinuous fluence response curves are reminiscent of the dose response of the *Gonyaulax* clock to anisomycin, which is another case of singular behavior (Taylor et al. 1982).

A discontinuous fluence response curve means that some process "downstream" from the photopigment's absorption of light is converting the initially continuous photochemical response into a discontinuous biological response. In the case of clock photoreceptors, it is the limit-cycle organization of the circadian oscillator that is responsible for converting the initially monotonic response into a discontinuous response as the light pulse moves the pacemaker past the singular region.

How, then, should the spectral sensitivity of clock photoreceptors be measured? The following three tactics should be valid procedures to construct accurate action spectra for clock photopigments (see Johnson and Kondo 1992 for more discussion and experimental examples). The first tactic is to use fluences and/or durations that elicit only
type 1 resetting (e.g., as in Takahashi et al. 1984). In this case, the critical response is defined as an arbitrary "percentage response" along a continuous fluence response curve. If type 0 resetting is involved in the experiment, the second tactic is to measure the action spectrum at a circadian phase that is not close to the PRC's break point. If this is done, the likelihood that the pacemaker will be moved through the singularity is reduced, and therefore the fluence response will probably be continuous. As in the first tactic, the critical response will be selected by the experimenter as an arbitrary percentage response.

Finally, in the case of type 0 resetting, one can plot on the ordinate of the action spectrum the fluence at which singular behavior is elicited (i.e., arhythmicity or a discontinuity in the fluence response curve). This third tactic depends on the pacemaker itself to set a critical threshold from which the action spectrum is derived in lieu of the experimenter selecting a percentage response level from a continuous function (Johnson and Kondo 1992). In Fig. 6, tactics 2 versus 3 are compared for describing the spectral response of resetting the circadian phototaxis rhythm of *Chlamydomonas* by light. The action spectra derived from the different tactics are similar (Johnson and Kondo 1992).

**PHASE-RESPONSE CURVES AS PROBES OF THE OSCILLATOR**

The PRCs are maps of the phase-dependent responsiveness of circadian pacemakers. As such, they can be used as probes of the phase, period, and amplitude of the central oscillator. Until recent advances in the identification of molecules that we believe may function as components of the circadian clockwork, PRCs have been our only gauges of the mysterious inner workings of this biological timepiece and remain an important tool.

![Diagram of action spectra for resetting the circadian phototaxis rhythm of *Chlamydomonas* calculated from two different critical response criteria. The "initial slope" (open circles) is the traditional method, calculated from the fluence required to elicit a 4h phase-delay shift (tactic 2 in the text). The "singular behavior" is calculated from the range of fluences that evoke (1) a change in direction of phase resetting (i.e., delays to advances) and (2) reduced amplitude or arhythmicity (tactic 3 in the text). The singular behavior data are plotted as bars that are the range of 1/(fluence in which singular behavior is stimulated. (From Johnson and Kondo 1992.)
Phase-Response Curves as Maps of Pacemaker Phase

PRCs have been used frequently to probe the phase of the pacemaker underlying rhythmic behavior. This procedure has led to several insights into essential features of circadian organization in multicellular organisms. For example, Daan and Pittendrigh tested whether the FRP-lengthening effect of deuterium oxide was exerted equally throughout the circadian cycle of the mouse by measuring the light-pulse PRC of mice drinking deuterium oxide (Daan and Pittendrigh 1976). Another example has been to determine whether the PRC has been deformed by prior exposure to various photoperiods or T cycles (H/Ma-6–11; Binkley and Mosher 1986).

PRC analyses led to the conclusion that the pacemaker of many multicellular organisms resets rapidly to light stimuli, whereas the overt rhythms display “transient” cycles after a light pulse that is given in the late subjective night (advance phase shift). On the other hand, light pulses given in the early subjective night (delay phase shifts) usually provoke few, if any, transients (Pittendrigh 1981). This observation prompted the hypothesis of multiple, hierarchically coupled oscillators within multicellular organisms: Overt rhythms are directly controlled by “slave” (B) oscillators that are synchronized to a “master” (A) circadian oscillator. This master oscillator, in turn, is entrained to the solar cycle by the resetting characteristics described by the PRC (Pittendrigh 1981). The evidence that the pacemaker resets rapidly relies on a special kind of PRC experiment, the elegant two-pulse PRC, which uses a second pulse to probe the phase of the pacemaker. These results have been interpreted to mean that the transient cycles observed after an advance phase shift are a reflection of the resynchronization of the intermediary slave (B) oscillator, not of the master (A) oscillator (Pittendrigh 1981). Two-pulse light PRC experiments have been performed in Drosophila (Pittendrigh 1981), hamsters (H/Ma-12–13), and sparrows (Binkley and Mosher 1987). At the present time, transient cycles after phase resetting by light have not been documented for unicellular organisms. This may mean that rhythms in single cells are controlled directly by light-sensitive pacemakers without any intermediary slave oscillators.

In addition, Hobohm et al. (1984) have used the two-pulse PRC paradigm to answer a different question: Is the oscillator of Gonyaulax precessing under some conditions in which the overt rhythm is not expressed? In particular, pulses of the protein synthesis inhibitor anisomycin can result in several days of arrhythmicity. By using a second stimulus, the authors could show that the Gonyaulax clock was still ticking when no rhythm was expressed (Hobohm et al. 1984).

Another class of stimuli that has been used to map phases of the circadian pacemaker is pulses of chemicals or drugs. This information can then be used to determine whether mutations or pharmacological treatments that affect the FRP exert their effects throughout the cycle or during only a fraction of the pacemaker’s time course. Nakashima (1985) has used light, temperature, and chemical/drug PRCs to map the phases affected by FRP mutations in Neurospora, and Kondo (1989) has used pulses of amino acid analogs as resetting stimuli to map pacemaker phases in the duckweed Lemna. The use of PRCs to these various stimuli that have break points spread throughout the pacemaker’s cycle has allowed a finer mapping of the circadian phases than a single PRC would have permitted.

Mapping the pacemaker with PRCs can be problematic, however, for the same reason that various concentrations of anisomycin modulate the position of the PRC along the abscissa in Gonyaulax. As shown in Fig. 7, 1h pulses of anisomycin at increasing concentrations to Gonyaulax cells at first cause a transition from type 1 to type 0, then a progressive shift of the break point to the left, or a displacement of the PRC downward,
depending on your perspective (Taylor et al. 1982). A possible mechanistic explanation for this effect is discussed below, but in the context of using PRCs for mapping, imagine for a moment using anisomycin to map the pacemaker in *Gonyaulax* under two different conditions (e.g., strain, mutation, temperature, background illumination). If the sensitivity of the cells to anisomycin (or recovery from anisomycin) is altered by the differing conditions, then cells may respond differently to the same concentration of anisomycin. This could create an effect similar to that seen in Fig. 7. In that case, the clocks of cells under two different conditions might be perfectly in phase, but the phase of the PRCs elicited by type 0 resetting by anisomycin could be quite different. While this example is for a chemical stimulus, the same problem of altering sensitivity could be true for any type of stimulus, including light, and illustrates a potential pitfall of using PRCs to map any pacemaker.

Can this problem be circumvented? Yes. First, use a stimulus strength (fluence or dose) that elicits only type 1 resetting. If the larger magnitude of type 0 resetting is desirable, then the stimulus strength should be adjusted to be just above threshold for type 0 resetting to avoid the "sliding" PRC problem (Figs. 3 and 7). This transition from type 1 to type 0 must be independently measured for each experimental condition. Ultimately, phase resetting to various stimulus strengths must be measured to ensure that a PRC can be used to map the pacemaker under type 0 resetting conditions.
Phase-Response Curves as Maps of Pacemaker Amplitude

Nakashima and Feldman's (1980) light PRCs at different ambient temperatures provide an example of how PRC data can be used to estimate pacemaker amplitude. The data, depicted in Fig. 8, show that the amplitude of the PRC to light decreases as the ambient temperature is increased. This might simply mean that the phototransduction mechanism becomes less efficient at higher temperatures. On the other hand, if one assumes that the light pulses alter the state variables to a similar extent at all temperatures, an alternative model emerges. Lakin-Thomas et al. (1990) hypothesized that these data indicate that the amplitude (= diameter) of the limit cycle may increase as the temperature is increased. Thus, the same stimulus strength could provoke type 1 resetting at

![Graphs and diagrams showing phase response curves at different temperatures.](image)

**FIGURE 8.** A limit-cycle interpretation of the reduction of PRC amplitude with increasing ambient temperature. PRCs to 5-minute light pulses in *Neurospora* at 25°C (panel A), 30°C (panel B), and 34°C (panel C) (Nakashima and Feldman 1980). Panel D depicts a limit-cycle model in which the larger diameter limit cycle is at a higher temperature than the smaller limit cycle. Light stimuli provoke changes of the state variables, depicted by the horizontal arrows. Although the changes of state variables are equivalent at both temperatures, the stimuli move the state variables beyond the singularity at the lower temperature (therefore, type 0 resetting), whereas only type 1 resetting is elicited at the higher temperature (larger limit cycle).
high temperature (large diameter limit cycle) or type 0 resetting at lower temperature (smaller diameter limit cycle; Fig. 8D). This interpretation has led to an interesting limit-cycle interpretation for temperature compensation of the FRP (Lakin-Thomas et al. 1990; Johnson 1992).

PHASE-RESPONSE CURVES AND THE MECHANISM(S) OF CIRCADIAN PACEMAKERS

In addition to light and temperature, chemical and drug stimuli have also been extensively tested for phase-resetting action. Early studies (e.g., Hastings 1960) suggested that the circadian clock was relatively resistant to drugs and chemicals, but now many pharmacological treatments have been discovered that reset the clock.

In general, the motivation for studying the phase response of circadian pacemakers to light or temperature stimuli has been to understand (1) how the entrainment of the pacemaker to the solar day is accomplished and (2) other formal properties of circadian oscillators. The motive for studying the pacemaker's response to chemicals and drugs is different. The hope is to unveil the biochemical mechanism of the pacemaker by assessing its pharmacological sensitivity. The impact of chemicals on the pacemaker has been assayed by their effect on both period and phase.

What can chemical/drug PRCs tell us about the pacemaker? PRCs for pulses of chemicals/drugs are usually interpreted to mean that the presumed biochemical target(s) affected by the chemical is either a state variable or a state parameter of the pacemaker. In what follows, I discuss chemical-induced clock resetting in the context of changes of state variables. In a very simple oscillator composed of only a single biochemical component with two state variables (e.g., concentration and rate of change of concentration), chemicals that increase versus those that decrease the level of the component should evoke PRCs that are 180° apart and should have a predictable phase relationship to the phase of the oscillation of the component (see Rensing and Hardeland 1990, for an example).

The situation is considerably more complicated for an oscillator that is composed of multiple components with multiple state variables, which will probably be true for circadian oscillators. For multidimensional oscillators, the PRCs for perturbation of state variables cannot be predicted by the oscillation of any single state variable. Can PRCs still then be used to test whether biochemical entities are potential state variables? Yes—but accurate prediction of PRC shape depends on modeling of all or most of the specific state variables and parameters in the oscillator and the interactions between these components (as in Leloup and Goldbeter 1998). In the absence of such a specific model, the only unassailable prediction that can be made is that perturbation of the level of a state variable should provoke phase resetting. The shape or phase angle of the resulting PRC is not diagnostic in the absence of a specific multicomponent model.

If this is true, is there any value in measuring PRCs for drugs/chemicals? In other words, does measuring phase responsiveness at phases throughout the circadian cycle tell us anything more than data from a single phase point? I think the answer is yes, for several reasons. First, observing phase shifts at various phases reassures us that the result is not an artifact. Second, knowing the phase responsiveness throughout the cycle will be useful for later modeling of the pacemaker or for designing future experiments. Finally, responsiveness must be measured at many phases to detect discontinuities or to distin-
guish type 1 from type 0 resetting; this information will undoubtedly be crucial when we model a pacemaker's biochemistry.

Furthermore, note that the state variables of a limit-cycle oscillator may be changed by phase-resetting stimuli, even if the stimuli are presented at phases of the "dead zone." As discussed previously in this article, the pacemaker is not necessarily insensitive to resetting stimuli presented during the dead zone; the state variables might be changed at these phases, but this change does not move the pacemaker to a different isochron. This phenomenon is relevant to methods of testing whether a specific biochemical substance is a state variable: Phase-resetting stimuli presented during dead zone phases may modify state variables, even though no phase shift is elicited.

**Trends in Chemical/Drug Phase-Response Curves**

Do different organisms show similar responses to pharmacologic treatments? The class of drugs with phase-shifting action that has been best characterized in a variety of organisms is that of protein synthesis inhibitors on 80S ribosomes: cycloheximide, puromycin, anisomycin, and streptimidine. These drugs reset the clocks in a wide range of organisms, including algae (*Acetabularia* and *Gonyaulax*), fungi (*Neurospora*), angiosperms (*Phaseolus* and *Lemma*), the eyes of mollusks (*Aplysia*), chicken pinealocytes, and hamsters (references from Johnson 1990 and Inouye et al. 1988). Figure 9 illustrates that most of these PRCs show strong type 0 resetting. Cycloheximide is also known to lengthen the period of *Euglena* (Feldman 1967) and *Chlamydomonas* (Goodenough et al. 1981), but PRCs have not been reported for these organisms. Amino acid analogs—which may ultimately affect the clockwork in a fashion similar to protein synthesis inhibitors—also have potent resetting effects in *Lemma* (Kondo 1989). Finally, it is interesting

![Graph showing PRCs for protein synthesis inhibitors](image)

**FIGURE 9.** PRCs for protein synthesis inhibitors. Cycloheximide phase shifts the clocks of (A) *Gonyaulax*, (B) *Neurospora*, (D) *Phaseolus*, and (E) *Lemma*. Also shown are PRCs to anisomycin in (C) isolated *Aplysia* eyes and (F) chick pineal cells in vitro. (Primary references for each of these studies appear in the atlas.)
to note that inhibitors of protein synthesis on 70S ribosomes (e.g., chloramphenicol) have little or no effect on circadian rhythms in eukaryotes. Therefore, pulsatile interruption of protein synthesis on mitochondrial or chloroplast ribosomes has little impact on circadian precession in eukaryotic cells.

Many other chemicals and drugs have been tested for phase-shifting efficacy. These include (1) metabolic inhibitors such as cyanide, azide, dinitrophenol; (2) drugs that affect cyclic nucleotide levels such as theophylline and forskolin; (3) drugs that affect intracellular Ca\(^{2+}\) or calmodulin such as calcium ionophores (e.g., A23187), chlorpromazine, EGTA, and verapamil; and (4) drugs that affect membrane properties such as ion fluxes (e.g., of K\(^{+}\), Li\(^{+}\)), strophanthidin, valinomycin, and CCCP. In general, the responses to most of these chemicals/drugs are more variable among different species than are the responses to protein synthesis inhibitors.

**Using Chemicals/Drugs to Block Resetting by Other Stimuli**

Not only can chemicals and drugs be used to elicit phase shifts, they also can be used to dissect the mechanism by which other stimuli affect the clock mechanism. For example, if a drug that inhibits a specific process can block light-induced phase resetting, then that process may be involved in the phototransduction pathway. This approach was used by Johnson and Nakashima (1989) to implicate new protein synthesis in clock phototransduction. We found that the translational inhibitor cycloheximide prevented light-induced phase shifts in a dose-dependent manner. As a control, we showed that phase shifting by light was not inhibited by the drug in mutants with a protein synthesis mechanism that was resistant to cycloheximide.

That study exemplifies other ways in which chemical/drug stimuli can be used to study circadian systems. In the case of blocking treatments that do not themselves cause phase resetting, the interpretation of the results is relatively straightforward—if the blocking treatment inhibits the phase shift by the tested stimulus (e.g., light), then the process affected by the blocking treatment may be involved in the transduction/transmission of the tested stimulus. The approach of using chemicals/drugs to block phase shifting by another stimulus can, however, be difficult to interpret if the blocking treatment also causes phase resetting. This complication and a strategy to circumvent it is discussed in Johnson and Nakashima (1989).

**Interpretive Problems with Chemical/Drug Resetting**

Of course, there are many caveats for the interpretation of chemical/drug PRCs. Most obvious is that one must be cautious about assigning the site of action of the chemical/drug. Side effects of pharmacological treatments abound and are notorious for misleading conclusions from researchers. Four kinds of controls have been used as evidence for specificity of drug action. The first control is to measure the concentration dependence of the drug's effect on the presumed site of action (e.g., protein synthesis) and compare it with the concentration dependence of the drug's phase-resetting efficacy. If the two do not correlate closely, then the drug's impact on phase is likely to be via a different site of action. The second way to assess the possibility of side effects is to test mutants with a presumed site of action that is resistant to the drug. If phase shifting is concomitantly reduced in these mutants, one may be more confident that side effects are not responsible for phase-resetting action (Nakashima et al. 1981). The third method is
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to check whether derivatives of a drug that are inactive at the presumed biochemical target are also impotent for clock resetting (Jacklet 1980). Finally, a fourth control is to test various drugs that inhibit the same overall biochemical process, but by different mechanisms. A prime example of this control is the testing of various drugs that inhibit protein synthesis by different mechanisms: cycloheximide, anisomycin, puromycin, streptomidone, and so on (see Fig. 9).

Another problem with pulse application of chemicals/drugs is that the organism may not recover quickly from the inhibition or stimulation (whereas the recovery from light pulses is usually considered to be rapid relative to the circadian timescale). The times at which (1) a drug/chemical has begun to affect a targeted biochemical process significantly and (2) the targeted process has significantly recovered from the effects of the drug/chemical may be quite different from the times of drug/chemical (1) addition and (2) washout. These are important considerations in comparing PRCs for different drugs/chemicals with penetration/recovery kinetics that may differ or even in comparing PRCs for the same drug/chemical in different organisms with permeability characteristics that may differ. Although this problem may seem obvious, it has been scarcely discussed in the literature and seems to be little appreciated.

The best solution would be to measure the penetration/recovery times for each drug/chemical and for each organism. One of the very few examples for which such measurements have been made is that of the inhibition of protein synthesis by anisomycin in *Gonyaulax*. In this cell, protein synthesis is significantly inhibited by anisomycin within 5 minutes after the cells are exposed to the drug. But, the recovery of protein synthesis after the drug is removed can require much more time, depending on the concentration used. In particular, recovery of *Gonyaulax* cells from anisomycin can require hours (perhaps even days), and this recovery is a function of the concentration of the inhibitor that was administered to the cells: the higher the concentration, the longer the effective duration of the pulse (Olesiak et al. 1987). This is significant because increasing the duration of phase-shifting stimuli usually results in an increase of the magnitude of phase resetting and, for prolonged stimuli, often shifts the circadian time of the break point of the PRC (Fig. 3).

This effect may help to explain the shifting break points of PRCs to various doses of anisomycin in *Gonyaulax* (Taylor et al. 1982). As shown in Fig. 7, 1h pulses of anisomycin to *Gonyaulax* cells yields a transition from type 1 to type 0 when the dose is increased from 0.1 to 0.3 micromolar. As the concentration is increased further, there is a progressive shift of the break point to the left. At 2.0 micromolar anisomycin, the break point is seen to have come through almost a full cycle. Presumably, the state variables are being pushed farther and farther out on the phase plane as the concentration is progressively increased. The effects of anisomycin persist after washout, and the duration of that persistence is determined by the dose originally presented; as the dose of the anisomycin pulse is increased, the amount of time required for full recovery increases (Olesiak et al. 1987). Thus, a DRC for anisomycin in *Gonyaulax* is simultaneously a duration response curve. Consequently, PRCs for various doses of a drug/chemical can exhibit results analogous to that of PRCs for various durations of light stimuli, which exhibit shifting break points if the duration of the light pulse is long enough, as mentioned above for the series of *Sarcophaga* PRCs (Fig. 3).

Furthermore, the ambient temperature might also affect recovery kinetics and may account for the shift in the PRC to cycloheximide at 20°C versus 25°C that was observed in *Acetabularia* (Karakashian and Schweiger 1976; Broda et al. 1989). Thus, one can
never be completely certain when a chemical/drug pulse ends unless the recovery of the biochemical process is measured directly. This is one reason that I favor the use of stimulus onset as the standard stimulus marker for PRCs. (In truth, the effective onset may also be uncertain since the kinetics of drug penetration can vary. In general, however, recovery usually depends on more factors than penetration and is therefore likely to be more variable.)

Additional problems in interpreting the effects of drugs/chemicals on circadian rhythms that specifically address the problems for intact animal studies have been discussed by Mrosovsky (1997).

**New Tools Provided by Molecular Genetics**

The major problem with dissecting circadian systems pharmacologically is that of specificity. There are few chemicals or drugs that have only one effect on living systems. It is true that standard pharmacological controls such as testing dose-response relationships as described above can be very helpful in assessing whether drug-induced effects are due to a primary site of action as opposed to side effects. Nevertheless, no such control is absolute. Moreover, many drugs do not have a primary site of action that is specific enough to focus on single biochemical components that might be parts of the clockwork. For example, there is strong support for the conclusion that protein synthesis inhibitors reset the clock by the primary site of action, but knowing that global protein synthetic rates are important is not very helpful in determining which molecules are the key players.

In recent years, genetic analyses of circadian systems have identified genes that are essential for clock function. Testing whether the products encoded by these genes are acting as biochemical correlates of state variables or parameters is crucial to our understanding of the role these genes play. A key test is to alter the levels of the gene products within the physiological range and determine if these alterations will affect clock function, especially phase and/or period. Using protein synthesis inhibitors or other standard drugs does not allow us the specificity needed to perform such a test. Fortunately, the newfound "power of molecular genetics" has provided us with a novel tool—using inducible promoter-induced expression of specific genes. This approach was first applied to induced expression of the *frq* gene in *Neurospora* and the *per* gene in *Drosophila* (Aronson et al. 1994; Edery et al. 1994). Aronson et al. (1994) found that step changes in the level of *frq* expression could set the phase of the circadian clock in *Neurospora*, and Edery et al. (1994) found that pulsed expression of *per* could cause phase shifts in *Drosophila*. More recently, Ishiura et al. (1998) found that pulsed expression of the *kaiC* gene could phase shift the clock of prokaryotic cyanobacteria.

Inducible expression of candidate genes is a tremendous tool because it allows specificity of action at the first step in the process (of course, it could be that later steps in the action of these candidate gene products might have a very indirect effect on the clockwork). These experiments have set a new standard by which hypotheses about the clockwork's mechanism must be tested.

However, let us not be carried away by the power of these new tools to forget the hard-earned lessons of pharmacology. In particular, we want to test whether alterations of the levels of "clock gene" products within the physiological range will alter clock properties. So far, none of the studies using inducible promoters have done the simple dose-response comparisons to be certain that the induction of the candidate genes results
in physiologically relevant levels. Both the *Neurospora* (Aronson et al. 1994) and cyanobacterial (Ishiiura et al. 1998) studies test whether constant levels of induction within the physiological range cause arrhythmicity, but neither study shows that the concentration of the inducer for the phase-setting experiments elicits a physiological level of gene product. This is an important point: If the concentration of inducer required to elicit phase setting raises the gene product's level far beyond the physiological range, it is likely to be an artifact.

Other simple controls need to be used in inducible promoter studies. For example, the promoter should be fused to a housekeeping gene and tested to make sure that induction of any ordinary gene does not affect the clockwork. Also, the interpretation of the results is much clearer if the inducing agent does not itself elicit phase shifts in wild-type organisms. This was the case for quinic acid in the *Neurospora* study (Aronson et al. 1994) and for IPTG in the cyanobacterial study (Ishiiura et al. 1998), but the *Drosophila* study used a high-temperature-induced promoter to drive *per* expression (Edery et al. 1994). Temperature stimuli phase shift circadian clocks, and so the study of *per* gene induction has the complication that the final phase shift is a function of both temperature shifts and *per* gene expression. The authors fully acknowledge this point and attempt to correct their *per*-induction PRC for the temperature effects (Edery et al. 1994). Nevertheless, in nonlinear limit-cycle oscillators such as circadian pacemakers, such corrections can only approximate the true condition. While it is clear from the Edery et al. (1994) study that *per* gene induction can reset the *Drosophila* clock, the corrected PRC might not be an accurate portrayal of the precise phase-dependent action of *per* gene overexpression.

Therefore, future studies using inducible gene expression to test for the phase-shifting efficacy should include the following controls:

1. Perform dose-response comparisons to be sure that the induction is within the physiological range.
2. Use the inducible promoter to drive a housekeeping gene as a control.
3. If possible, use a promoter that is induced by an agent that does not itself cause a phase shift in the organism.

These caveats aside, the inducible promoter experiment promises to be a key diagnostic criterion for future dissections of circadian clocks.

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As of this writing, no more hardcopies of the PRC Atlas are available, and another copying run is not contemplated. Fear not, however—there are computer versions of the PRC Atlas for both the Macintosh (using HyperCard) and PC platforms. The programs can be downloaded from my laboratory’s Web site (http://johnsonlab.biology.vanderbilt.edu) or obtained directly:

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GLOSSARY

A/D ratio of area of advance phase shifts to area of delay phase shifts in a PRC
DRC dose-response curve
FRP free-running period of a biological rhythm
pacemaker oscillator; clock; master (A) oscillator
phase angle phase relationship
$PP_c$ “complete” photoperiod
$PP_s$ “skeleton” photoperiod
PRC phase-response curve; a plot of circadian time of stimulus (“old phase”) versus phase shift
PTC phase-transition curve; a plot of circadian time of stimulus (“old phase”) versus “new phase” (i.e., phase of rhythm after phase shift has occurred)
rhythm overt rhythm; “hand”
$T$ period of a zeitgeber
zeitgeber “time giver”; an environmental signal that can entrain a biological rhythm

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