Light-Induced Phase Shifts of Circadian Leaf Movements of *Phaseolus*: Comparison with the Effects of Potassium and of Ethyl Alcohol

(circadian rhythms/membranes/alcohol)

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ABSTRACT   Leaves of *Phaseolus coccineus* were exposed to high concentrations of KCl (0.1–0.3 M) for periods of 4–5 hr, or to ethyl alcohol (5–30%) for periods of 2 hr. The treatment started at various phases of the free-running circadian rhythm in continuous light. Whereas the K^+ pulses induced only advance phase shifts, i.e., shortening of the periods, the alcohol pulses caused stronger advances and also slight delays, i.e., lengthening of the periods. These delays became stronger with lower concentrations of alcohol (1.7%) applied for a longer time. The lengthening effect by alcohol supplied continuously depends on the intensity of light. The rather high intensity of 3500 lux, resulting in very long free-running periods of about 29 hr, inhibits a further strong lengthening by alcohol. The phases reacting with advances to K^+ and to alcohol are about the same phases that respond to light pulses with advances. The advances are reached at earlier phases of the circadian cycle when the concentration of alcohol or the strength of the light pulse is increased. The phases that respond with advances are those which, according to earlier publications, are within the energy-independent part of the cycle. The phases responding with delays belong to the energy-requiring part of the cycle. Consequently, it is suggested that advances are due to accelerated membrane depolarization and delays are due to stabilizing effects on membranes.

Light-dark cycles usually are the most effective means to entrain the circadian rhythms of plants and animals to exactly 24 hr. This entrainment is possible by phase shifts which are either advances (shortening of the periods) or delays (lengthening of the periods). Certain phases of the circadian cycle react with advances, other phases with delays, to light perturbations offered during continuous darkness (DD). Experiments testing the several phases of the whole circadian cycle result in well-known phase-response curves. The phase-response curves are similar when pulses of high-intensity light are offered during continuous light (LL) of lower light intensity. These phase shifts are connected with influences on K^+ fluxes (1). Consequently, high K^+ pulses (2), as well as influences of valinomycin on the K^+ fluxes (3), can result in phase shifts, which to a certain extent resemble the light-induced phase shifts. Valinomycin, on the other hand, does not influence the final K^+ concentration, but only accelerates the equilibration (4). Thus the process underlying light-induced phase shifts must be more complex. Pulses of valinomycin can induce strong advances of the circadian rhythm in *Phaseolus*, but the possible delaying effect of valinomycin is much smaller than the possible delaying effect of light. Con-

Abbreviations: LL, continuous light; DD, continuous darkness.

sequently, valinomycin proved to have no lengthening effect on the periods when supplied continuously over several periods. But continuous white or red light results in an increase in the periods of *Phaseolus* by about 2 hr, as compared with the DD controls.

In the experiments described here, we attempt to further elucidate these phenomena by a systematic investigation of the influences of pulses of K^+ and of ethyl alcohol. The continuous presence of alcohol is known to have a strong lengthening influence on the periods in plants (5) and in animals (6). This influence is qualitatively and quantitatively similar to the above-mentioned influence of LL in the case of *Phaseolus*.

MATERIALS AND METHODS

The experiments were performed with detached plants of *Phaseolus coccineus* in growth chambers at 21° and with white LL of 3500 lux, from fluorescent tubes. The periods of the free-running rhythm are about 29 hr under these conditions. Pulses of KCl or of ethyl alcohol reached the leaves by way of the transpiration stream. All the values given in the text and in the figures are mean values from 5 to 11 experiments. Standard errors were always around ±1 hr.

RESULTS

Pulses of high K^+ concentration can induce advances, i.e., a shortening of the periods (Fig. 1). The effects were stronger than those of other tested substances with the same molar concentration. The phases of maximum response are around 8 hr after the preceding maximum night position, designated "zero," of the leaf. None of the phases reacts with significant delays. Thus, in *Phaseolus*, in contrast to *Aplysia* (2), only light-induced advances and not light-induced delays can be simulated by K^+ pulses.

Fig. 2 shows the phase shifts after 2-hr pulses of ethyl alcohol. The lowest concentration tested (5%) resulted in a maximum advance when the treatment started about 8 hr after "zero." This advance corresponds to the degree of responsiveness toward light pulses (Fig. 3) or toward K^+ pulses.

Increasing the concentration of alcohol results in shifting the maximum advances to earlier phases. With concentrations higher than 20%, the strongest advance was reached when the application started about 2 hr after "zero." This shift is not surprising since it corresponds to the effect of light pulses. The phases up to about 10–12 hr after "zero" have features of a relative refractory period (7), comparable to
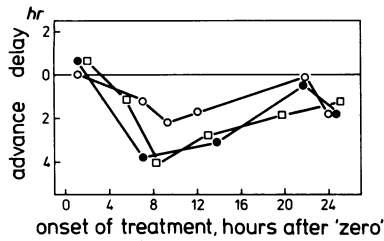
FIG. 1. *Phaseolus coccineus.* Phase shifts (*ordinate*) of the free-running circadian rhythm in LL due to 5-hr (0.1 M, O) or 4-hr (0.2, ●, and 0.3 M, □) pulses of KCl. *"Zero"* indicates the last subjective night peak before treatment. The data refer to the phase shift in the fourth cycle after treatment.

well-known refractory periods in short-period biological oscillations. During this refractory period, the threshold for inducing a new action gradually decreases, leading to a value which allows a spontaneous new action, i.e., starting a new period at about 10–12 hr after "zero." An external stimulus, such as a light pulse, allows an earlier breaking of the relative refractory period which may be compared with an extra systole in heart beat. The stronger the stimulus, the greater will be the possibility for the "extra systole" to be shifted toward "zero." This relationship is shown in Fig. 2 for the alcohol effect. A similar influence of the strength of light pulses on the shape of the phase-response curves is known (7). Fig. 3 shows that strong light pulses (light breaks during DD, instead of high-intensity light pulses during LL) result in a phase-response curve that resembles the phase-response curve for 30% alcohol pulses, as far as advances are concerned.

The possible delaying effects of brief exposures to alcohol are much smaller than the possible delaying effects of light pulses. But the delaying effects become very clear in the presence of lower concentrations of alcohol over longer periods of time. The lengthening effect of continuously supplied alcohol (5, 6) might be explained by assuming a preponderance of delays as opposed to advances under these conditions. This assumption is supported by further experiments. Transition from water to 1.7% alcohol strongly increased the first
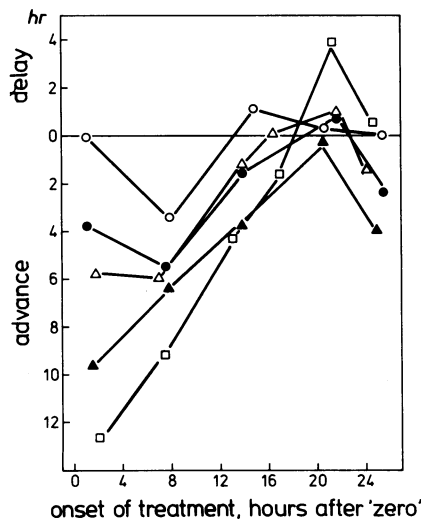

FIG. 2. Phase shifts (*ordinate*) in the fourth cycle after 2-hr pulses of ethyl alcohol. Concentrations in % (v/v). Other conditions as in Fig. 1. (O) 5% alcohol; (●) 15%; (△) 20%; (▲) 25%; (□) 30%.
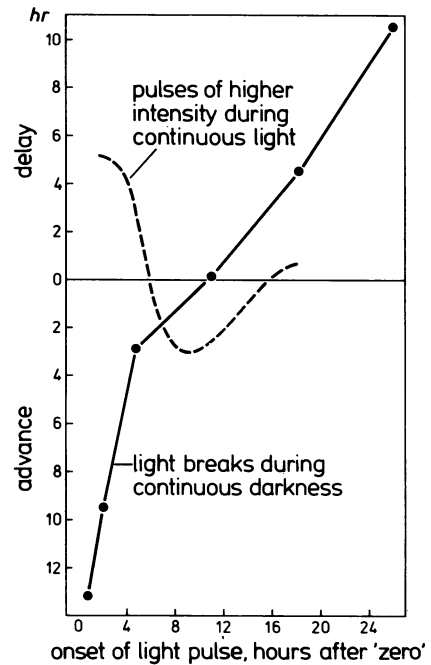

FIG. 3. Phase shifts (*ordinate*) in the fourth cycle after 3-hr pulses of 3500 lux of white light, interrupting DD. Compared with previously published effects of high-intensity pulses of light interrupting a lower-intensity LL.

cycle to be affected when the treatment started at a time that excludes the phases reacting to alcohol pulses with advances, which means excluding the phases from "zero" until 8 hr after "zero." The first affected cycle increased to 33.4 hr when the transition to 1.7% alcohol started 12–13 hr after "zero." But there was no significant increase of this cycle when the transition started 6–7 hr after "zero." After they reached a new steady state in the fourth cycle (still in alcohol), the periods had about the same length in both cases (29.5–29.6 hr). But there was no compensation for the possible lengthening effect in the first cycle. When the transition to alcohol was started about 12 hr after "zero," the 4-hr delay persisted during the following 4 cycles.

Under the conditions of the present experiments, the lengthening effect of continuously supplied alcohol is not very strong and, except for the first cycle, not significant. The earlier experiments in our laboratory (5), with a somewhat higher concentration of alcohol (2%), showed a lengthening up to 32.8 hr. In those experiments the intensity of the LL was only 400–900 lux, resulting in periods of 27.4 hr for the controls. Thus the increase of the intensity to 3500 lux in the present experiments, resulting in control periods of nearly 29.0 hr, seems to inhibit a further strong lengthening by alcohol.

DISCUSSION

About half of the circadian cycle shows features of tension (energy-requiring) processes, whereas the other part of the cycle shows characteristics of relaxation processes. This separation becomes evident by experiments in which the metabolism was suppressed at various phases of the circadian cycle. In the cockroach, *Periplaneta,* certain phases respond with no delay or with only a slight delay in the running of the clock after the organism is chilled to temperatures between −2 and +5°, whereas other phases respond with strong de-

lays (8). Experiments in which plants (*Kalanchoe, Phaseolus*) were exposed to transient chilling, to transient treatment with DNP or KCN, or to transient withdrawal of O_2, allowed similar conclusions (7, 9). More recent results with *Drosophila* (10) and with *Albizzia* (11) confirm these conclusions.

In *Albizzia*, the energy-dependent phases are characterized by active transport of K^+, whereas the energy-independent phases show diffusion as the main pathway of K^+ movement (12). A reduction of the metabolism by poisons or by anoxia may not only fail to inhibit the running of circadian clocks; it may even cause an acceleration (9, 10). This is in accordance with the mentioned conclusions. Semipermeability of biological membranes and resting potentials are well known to depend on the presence of oxygen. Therefore, a reduction of the respiration can result in an acceleration of the efflux of potassium by diffusion (13, 14).

For these reasons, light pulses, as well as K^+ or alcohol pulses, meet the oscillator at qualitatively different conditions when offered at different phases. Thus, it is not surprising to find a diversity of primary processes in light-induced advances and in light-induced delays. Such a diversity is indicated by several facts. The minimum intensities required for advances and for delays are different in *Phaseolus* (15) and *Drosophila* (16). Even the action spectra for advances and for delays can be different (17).

As was mentioned, advances mean a premature induction of a new circadian period. Membrane depolarization, which has often been suggested as a consequence of light pulses and of high K^+ pulses, might be responsible for these advances. This explanation agrees with the fact that the advance responses are characteristic for the "nonenergetic" phases of the cycle. It should also be mentioned that large advances in *Phaseolus* can also be induced by pulses of far-red light, which is known to convert phytochrome into the physiological inactive form, whereas delays by far-red are not possible, or at least only very slight as compared with the strong delaying (period-lengthening) effect of red light, bringing phytochrome into its physiological active form (15).

Light-induced delays mean the extension of an energy-requiring action beyond the normally reached maximum. The tension process is continued for a longer time, resulting in higher maximum (7). Membrane-stabilizing effects of light (18) and of alcohol (6) might be discussed as possible processes in the delaying effects of these agents when offered to the respective phases.

1. Satter, R. L. & Galston, A. W. (1971) "Potassium flux: a common feature of *Albizzia* leaflet movement controlled by phytochrome or endogenous rhythms," *Science* **174,** 518–519.
2. Eskin, A. (1972) "Phase shifting a circadian rhythm in the eye of *Aplysia* by high potassium fluxes," *J. Comp. Physiol.* **80,** 353–376.
3. Bünning, E. & Moser, I. (1972) "Influence of valinomycin on circadian leaf movements of *Phaseolus*," *Proc. Nat. Acad. Sci. USA* **69,** 2732–2733.
4. Rottenberg, H. (1973) "The mechanism of energy-dependent ion transport in mitochondria," *J. Membrane Biol.* **11,** 117–137.
5. Keller, S. (1960) "Über die Wirkung chemischer Faktoren auf die tagesperiodischen Blattbewegungen von *Phaseolus multiflorus*," *Z. Bot.* **48,** 32–57.
6. Enright, J. T. (1971) "The internal clock of drunken isopods," *Z. Vergl. Physiol.* **75,** 332–346.
7. Bünning, E. (1973) *"The Physiological Clock"* (Springer-Verlag, New York), rev. 3rd ed., pp. 118–132.
8. Bünning, E. (1959) "Zur Analyse des Zeitsinnes bei *Periplaneta americana*," *Z. Naturforsch. B* **14,** 1–4.
9. Bünning, E., Kurras, S. & Vielhaben, V. (1965) "Phasenverschiebungen der endogenen Tagesrhythmik durch Reduktion der Atmung," *Planta* **64,** 291–300.
10. Pittendrigh, C. S. (1973) "Circadian oscillations in cells and the circadian organization of multicellular systems," in *The Neurosciences; Third Study Program,* ed. Schmitt, F. O. (MIT Press), in press.
11. Satter, R. L. & Galston, A. W. (1971) "Phytochrome controlled nyctinasty in *Albizzia julibrissin.* Interaction between an endogenous rhythm and phytochrome in control of potassium flux and leaflet movement," *Plant Physiol.* **48,** 740–746.
12. Satter, R. L., Galston, A. W. & Applewhite, P. B. (1973) "Characterization of two distinct phases of rhythmic leaflet movement in *Albizzia julibrissin*," *Plant Physiol.* **51,** suppl. (Abstracts), 16.
13. Giese, A. C. (1968) *Cell Physiology* (W. B. Saunders Co., Philadelphia, London), 3rd ed., p. 526.
14. Bertrand, D., Perrelet, A. & Baumann, F. (1973) "Influence of anoxia on the membrane potential of reticula and pigment cells in the retina of the drone," *Exprientia* **29,** 740.
15. Bünning, E. & Moser, I. (1966) "Response-Kurven bei der circadianen Rhythmik von *Phaseolus*," *Planta* **69,** 101–110.
16. Chandrashekaran, M. K. & Engelmann, W. (1973) "The early and late night phases of the *Drosophila pseudoobscura* circadian rhythm require different energies of blue light for phase shifting," *Z. Naturforsch.*, in press.
17. Halaban, R. (1969) "Effect of light quality on the circadian rhythm of leaf movement of a short-day plant," *Plant Physiol.* **44,** 973–977.
18. Satter, R. L. & Galston, A. W. (1973) "Leaf movements: rosetta stones of plant behavior?" *Bio Sci.,* **23,** 407–416.