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Period Shortening and Phase Shifting Effects of Ethanol on the *Gonyaulax* Glow Rhythm*

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Summary. In a number of organisms which exhibit circadian rhythmicity, a continuous exposure to ethanol at moderate (0.1%) concentrations is known to cause period lengthening. In studies of the effects of ethanol on the circadian luminescence glow rhythm of the marine dinoflagellate *Gonyaulax*, we observed that 0.1% ethanol causes instead a period shortening. We have also found that ethanol pulses cause phase shifts, with little or no after-effects on the period of the circadian rhythm which continues thereafter.

Introduction

Ethanol was first reported to cause period lengthening in the circadian rhythm of leaf movement in *Phaseolus* (Keller, 1960), and has since been shown to have significant effects in several other systems (Bünning and Baltes, 1962; Enright, 1971; Bünning and Moser, 1973; Sweeney, 1974; Brinkmann, 1976). In all of these cases, with continuous exposure to ethanol, a period lengthening effect has been reported. However, in studies of the effects of ethanol on the glow rhythm of *Gonyaulax*, we observed that instead there is a period shortening: the oscillation is apparently speeded up.

Materials and Methods

Gonyaulax polyedra, strain GP 52, isolated by B.M. Sweeney in 1952, was used for most experiments. In certain cases, another strain isolated by Sweeney in 1970 was used. Cells were grown in 2.8 liter Fernbach flasks under a LD 12:12 lighting regime, at an intensity that varied between 120 and 180 microeinsteins m^{-2} s^{-1}, depending on the position of the flask on the light rack. Medium "31" was used for growing strain GP 52 (Fogel and Hastings, 1971) and f/2, enriched with soil extract was used for growing strain GP 70 (Guillard and Ryther, 1962). The glow rhythm of 50 vials could be followed in a given experiment using a modified Packard Scintillation Counter, or by using a device designed by Dr. Van Gooch for this purpose. The device consists of a polyvinylchloride platform on which vials, each containing 10 ml of a cell suspension at a density of 5,000 to 10,000 cells/ml, are mounted in holders in a circle. The cells are exposed to dim illumination [about 20 microeinsteins m^{-2} s^{-1}, measured with a Licor (Lincoln, Nebraska) LI-1952 underwater quantum sensor, which measures photosynthetically active radiation] from beneath the platform. A water bath around the vial holders maintains the vials at any desired temperature, 19 ± 0.2 °C in the experiments reported here. For 45 s every 20 min a fiber optics light pipe is automatically rotated under the vial, blocking the illumination from outside and allowing measurement of the bioluminescence being emitted. The fiber optics conduct the light to a photomultiplier tube located in the center of the platform, whose output is amplified and recorded graphically.

All experiments were carried out with unialgal, but not bacteria-free cultures. In general, bacterial growth was not stimulated by added ethanol. This became a problem only with continuous ethanol treatments of greater than 2 days, and even then contamination was not observed in all cases. In instances where pulses of ethanol were used, the treatment was terminated by pelleting the cells in a clinical centrifuge, aspirating off the medium, and resuspending the cells in the same volume of fresh medium without ethanol.

Results

Figure 1 illustrates the immediate effects of ethanol at several different concentrations upon the bioluminescent glow, added at a time during the circadian cycle just after the onset of the glow. At low concentrations of ethanol (0.17 to 4.3 mM), the glow is only slightly inhibited during the first hour of exposure and the position of the "glow peak" is still evident. At higher concentrations (17 mM) the glow is more strongly inhibited, such that a glow peak cannot always be easily discerned. The bioluminescent flash-

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Abbreviation: CT, circadian time

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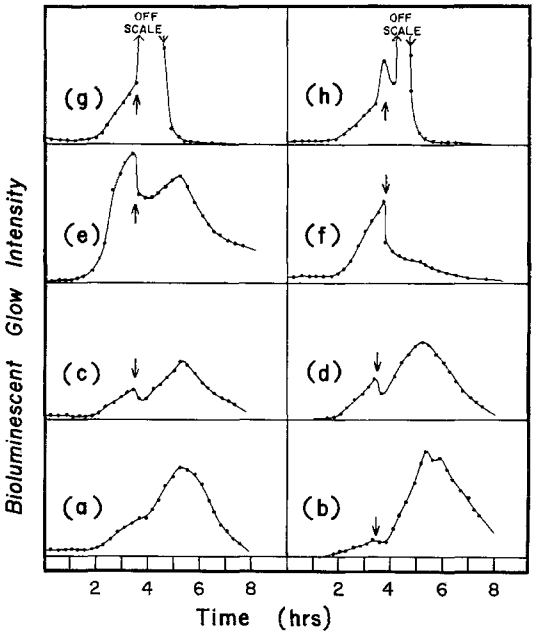

Fig. 1a–h. The effects of varying concentrations of ethanol on the *Gonyaulax* glow. Cells were transferred to the turnable at CT 0, first glow peak occurs at about CT 23. Ethanol addition, indicated by arrows, is during the rising phase of the first glow peak after transfer to recording device. **a** control; **b** 0.17 mM ethanol; **c** 1.7 mM ethanol; **d** 4.3 mM ethanol, **e, f** 17 mM ethanol, illustrating the extremes in the observed response; **g** 170 mM ethanol; **h** 340 mM ethanol

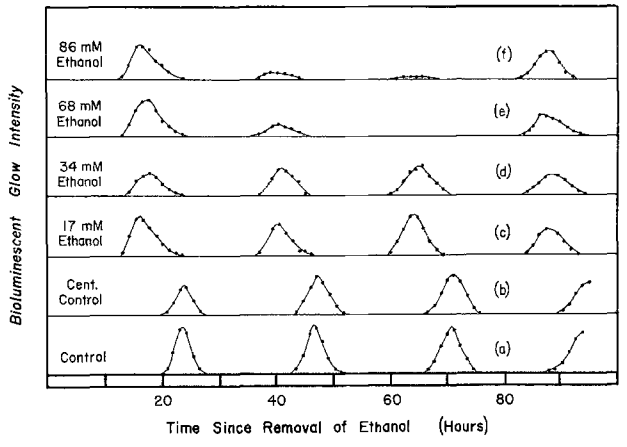

Fig. 2a–f. The effects of 12 h pulses of ethanol, given from CT 12 to CT 24 in the first cycle after transfer to continuous conditions (dim light), on the *Gonyaulax* glow rhythm. **a** Control; **b** centrifuged control; **c** 17 mM ethanol; **d** 34 mM ethanol; **e** 68.5 mM ethanol; **f** 86 mM ethanol. The cause of the recovery of luminescence after an initial suppression, seen in **e** and **f**, is unkonwn. Phase changes are assumed to be advances

ing of cells (Hastings and Sweeney, 1975a; Hastings, 1960), which is also substantially stimulated by the above concentrations of ethanol, is not illustrated. At the higher concentrations of ethanol (85–107 mM), the induced flashing activity is so strong that it may

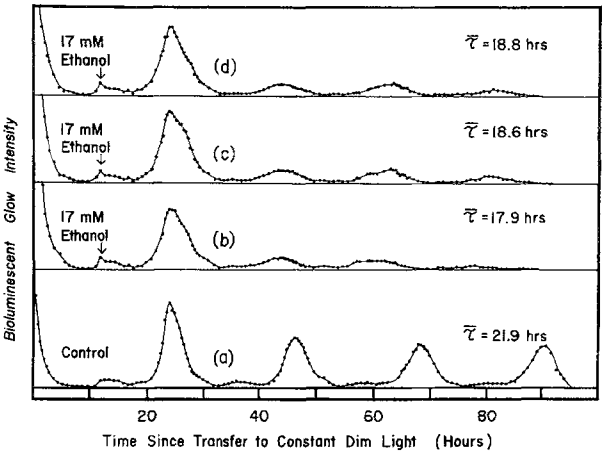

Fig. 3a–d. The effect of ethanol on the free-running period of the *Gonyaulax* glow rhythm. Time 0 is the time of transfer to continuous low intensity light (100 ft-c). **a** Control; **b**, **c**, and **d** 17 mM ethanol added at time indicated by arrows. Note that the average period (τ) is significantly (by about 16%, or 3.5 h) shortened when ethanol is present

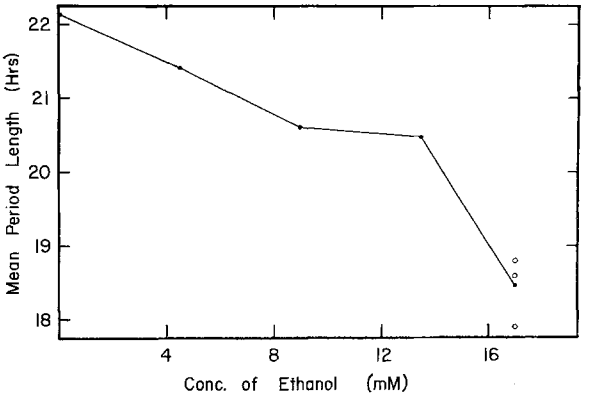

Fig. 4. Period length of the *Gonyaulax* glow rhythm as a function of ethanol concentration (continuous exposure). Open circles are data from Fig. 3; closed circles are data from a separate experiment

obscure the glow; it lasts for about 30 to 60 min and is then followed by extinction of luminescence emission. A similar "burst" of luminescence is commonly seen when toxic chemicals are added to *Gonyaulax*, possibly due to the non-specific disruption of cellular and subcellular integrity, presumably including the structures and molecular species that segregate luciferin and luciferase and prevent light emission and the unstimulated cell (Fogel and Hastings 1971; Hastings, 1979).

The experiments of Fig. 2 show that exposure to ethanol for a limited period, i.e. 12 h pulses, can cause large phase shifts in the ensuing glow rhythm. Note that the effect is not strongly concentration dependent over the ranges reported; Sweeney (1974) also found (with 4 h pulses) that increasing the ethanol concen-

tration above 17 mM did not result in larger phase changes. She also demonstrated that the response in the stimulated rhythm is dependent upon the time during the circadian cycle when the ethanol pulse was applied, and reported a phase response curve. We also exposed *Gonvaulax* to 1 h pulses of ethanol (171 mM and 343 mM at CT 12–13 and CT 14–15, 343 mM at CT 16–17). At these high concentrations, short duration pulses had very little effect on the phase of the rhythm, demonstrating that exposure to ethanol at low concentration for a relatively long period of time cannot be mimicked by exposure to high concentrations for a short period of time.

Figure 3 shows experiments in which ethanol was added but not removed. In such cells there was some variability in the response and circadian glow peaks were not always observed in cells placed and kept in 17 mM ethanol. In many cases however, it was possible to estimate the period of a circadian rhythmicity continuing under these conditions. In three replicate cultures (Fig. 3) the period was significantly less than in the controls. The average period (τ) of the control is 21.9 h over the 3 cycles shown (curve a). The average periods of the three vials of cells to which 17 mM ethanol had been added, were 17.9, 18.5, and 18.8 h respectively (curves b, c, and d), an average period shortening of about 16%. Figure 4 shows the effect of lower concentrations of ethanol on the period.

Discussion

The effect of ethanol reported here differs from that previously found in other systems in that during continuous exposure the period is shortened rather than lengthened. A similar effect has recently been reported for the rhythm of petal movement in *Kalanchoë* (Kastenmeier et al., 1977). It had been tempting to speculate that the period shortening with ethanol in *Gonyaulax* might be correlated with the fact that its circadian rhythmicity exhibits a Q_{10} of less than 1.0 (Hastings and Sweeney, 1957b; Hastings, 1959). However, *Kalanchoë* was reported to exhibit a Q_{10} of 1.1 for its circadian rhythm of petal movement (Oltmanns, 1960).

Since these experiments were done with cultures containing bacteria, growth and metabolism of the ethanol by these bacteria surely occurred to some extent. This nevertheless appeared to have little or no effect upon the circadian system. Moreover, Sweeney (1974) demonstrated that ethanol has phase shifting capability in bacteria-free cultures, so it is unlikely that the effects recorded here can be attributed solely to some bacterial metabolic product, derived from ethanol or otherwise. However, the immediate metabolic products of alcohols, the aldehydes, have been found to be effective in phase shifting (Taylor and Hastings, 1979) so we cannot exclude the possible cooperativity of bacterial metabolism in causing the effects recorded here.

Little has been done concerning the mechanism by which ethanol affects the circadian oscillator. This may be due in part to an earlier tacit assumption that ethanol acts in a rather non-specific manner. In fact, it was suggested that the effectiveness of ethanol on circadian systems argues in favor of a membrane model for the basic oscillator (Njus et al., 1976). If this were true, alcohols that tend to partition themselves more into the lipid phase of cells should be more effective as phase shifters. But in *Gonyaulax*, the only organism in which the relationship between alcohol chain length and corresponding ability to cause phase shifts has been investigated, the opposite is true: methanol is the most effective alcohol and the effectiveness drops off as carbon chain length is increased (Sweeney, 1976). Thus, the postulate that alcohols exert their effect on the clock through a non-specific attack on membranes has been questioned. This was also suggested by Brinkmann's (1976) work with *Euglena*, in which he concluded that an alcohol must be metabolized in order to exert an effect on the circadian clock. In his experiments, no phase shifts were observed when ethanol was given to *Euglena* as pulses, and period changes were obtained only after the ethanol had been in the medium for 2–3 days. Although the precise way in which ethanol affects circadian rhythms may vary from organism to organism, if Brinkmann's reasoning applies to *Gonyaulax*, then it should be possible to obtain phase changes or period changes from ethanol metabolites, such as acetaldehyde and acetate, or even from metabolites of other alcohols. Experiments concerning this are reported in the accompanying publication (Taylor and Hastings, 1979).

References

Brinkmann, K.: The influence of alcohols on the circadian rhythm and metabolism of *Euglena gracilis*. J. Interdiscip. Cycle Res. **7**, 149–170 (1976)

Bünning, E., Baltes, J.: Wirkung von Äthylalkohol auf die physiologische Uhr. Naturwissenschaften **49**, 19–20 (1962)

Bünning, E., Moser, I.: Light-induced phase shifts of circadian leaf movements of *Phaseolus*: Comparison with the effects of potassium and ethyl alcohol. Proc. Natl. Acad. Sci. **70**, 3387–3389 (1973)

Enright, J.T.: The internal clock of drunken isopods. Z. vergl. Physiol. **75**, 332–346 (1971)

Fogel, M., Hastings, J.W.: A substrate binding protein in the *Gonyaulax* bioluminescence reaction. Arch. Biochem. Biophys. **142**, 310–321 (1971)

Guillard, R.R.L., Ryther, J.H.: Studies of marine plankton diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve Gran. Can. J. Microbiol. **8**, 229–239 (1962)

Hastings, J.W.: Unicellular clocks. Ann. Rev. Microbiol. **13**, 297–312 (1959)

Hastings, J.W.: Biochemical aspects of rhythms: Phase shifting by chemicals. Cold Spring Harbor Symp. Quant. Biol. **25**, 131–143 (1960)

Hastings, J.W.: The biochemical basis for dinoflagellate luminescence: A molecular model for the control of flashing. In: Bioluminescence today. Herring, P.J. (ed.). London: Academic Press (in press) 1979

Hastings, J.W., Sweeney, B.M.: The luminescent reaction in extracts of the marine dinoflagellate *Gonyaulax polyedra*. J. Cell. Comp. Physiol. **49**, 209–225 (1957a)

Hastings, J.W., Sweeney, B.M.: On the mechanism of temperature independence in a biological clock. Proc. Natl. Acad. Sci. **43**, 804–811 (1957b)

Kastenmeier, B., Reich, V., Engelmann, W.: Effects of alcohols on the circadian petal movement rhythm of *Kalanchoe* and the rhythmic movement of *Desmodium*. Chronobiologia **4**, 122 (1977)

Keller, S.: Über die Wirkung chemischer Faktoren auf die tagesperiodischen Blattbewegungen von *Phaseolus multiflorus*. Z. Bot. **48**, 32–57 (1960)

Njus, D., Sulzman, F.M., Hastings, J.W.: Membrane model for the circadian clock. Nature **248**, 116–120 (1974)

Oltmanns, O.: Über den Einfluß der Temperatur auf die endogene Tagesrhythmik und die Blühinduction bei der Kurztagpflanze *Kalanchoe blossfeldiana*. Planta **54**, 233–264 (1960)

Sweeney, B.M.: The potassium content of *Gonyaulax polyedra* and phase changes in the circadian rhythm of stimulated bioluminescence by short exposures to ethanol and valinomycin. Plant Physiol. **53**, 337–342 (1974)

Sweeney, B.M.: Evidence that membranes are components of circadian oscillators. In: The molecular basis of circadian rhythms Hastings, J.W., Schweiger, H.G. (eds.), pp. 267–281. Berlin: Abakon Verlagsgesellschaft 1976

Taylor, W., Hastings, J.W.: Aldehydes phase shift the *Gonyaulax* clock. J. Comp. Physiol. **130**, 359–362 (1979)