J. Comp. Physiol. 130, 359–362 (1979)

Journal of Comparative Physiology · B

Aldehydes Phase Shift the *Gonyaulax* Clock*

Walter Taylor and J.W. Hastings

Division of Biological Sciences, University of Michigan, Ann Arbor, Michigan 48109, USA, and
The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138, USA

Accepted January 13, 1979

Summary. Aliphatic aldehydes ranging in chain length from one to four carbon atoms have a significant phase shifting effect upon the circadian rhythm of bioluminescence (glow) in the dinoflagellate (*Gonyaulax polyedra*. Cells exposed for two hours to 18 mM acetaldehyde starting at about circadian time 12 experience a permanent phase delay of up to about 12 h. The phase response curve relationship with acetaldehyde is presented, as well as the relationship between concentration and phase delay for the four aldehydes studied. Reactions of aldehydes which may be implicated are discussed. The possibility that sulfhydryl reagents generally may perturb circadian systems is suggested.

Introduction

Knowledge of biological systems has been aided in many instances by the use of mutants and specific inhibitors, where interference with normal functioning permits analysis and understanding of the normal. Studies of this type concerning the mechanism of circadian rhythms must be viewed somewhat differently, since the "end product" of the system is an oscillation in time, for which changes in period or phase represent the relevant measurement.

Sweeney (1976) studied the effects of 4 h treatments with alcohols of different chain lengths upon the rhythm of stimulated luminescence in *Gonyaulax*. She found that the effectiveness in phase shifting decreased with increasing chain length, suggesting that the effect may not be upon membranes, as had previously been considered likely. At about the same time, Brinkmann (1976) came to a similar conclusion in experiments with *Euglena*, showing that the alcohol effect (period lengthening) occurred only if the alcohol was metabolized. In recent experiments we have shown that the period of the *Gonyaulax* glow rhythm is significantly shortened in cells exposed continously to 0.1% ethanol (Taylor et al., 1979). In addition ethanol pulses also cause phase shifts in the glow rhythm, with little or no after-effects on the period.

An immediate product of ethanol metabolism is acetaldehyde. We therefore tested acetaldehyde for its ability to shift the circadian glow rhythm in *Gonyaulax* and found it to be highly effective, indeed far more so than ethanol. The results suggest that alcohols may exert their effects by being metabolized to the corresponding aldehyde, which then functions as the effective compound in vivo.

Materials and Methods

All experiments were performed using *Gonyaulax polyedra*, strain 70, isolated in 1970 by B.M. Sweeney. Cells were grown in f/2, an enriched sea water medium, supplemented with soil extract (Guillard and Ryther, 1962). In any given experiment the glow rhythm could be measured from 29 vials, each containing 10 ml of cell suspension at a density of 6,000 to 10,000 cells/ml. The device for this purpose, designed by Dr. Van Gooch, enabled measurement of the rhythm without disturbing the cells, as previous devices used for this purpose (modified scintillation counters) had done. The vials are mounted in water-jacketed holders (19 ± 0.2 °C) in a circle on a platform with openings underneath to allow constant exposure to dim light (22 microeinsteins m^{-2} s^{-1}) from a circular fluorescent lamp (cool white). For 45 s every 20 min a fiber optics light pipe is automatically rotated directly under the vial, blocking the light from the fluorescent lamp and allowing measurement of the luminescence. The fiber optics conduct the light to a photomultiplier tube located in the center of the platform. The output of the photomultiplier is amplified and recorded on an Esterline Angus chart recorder.

* This work has been supported in part by a grant from the National Institutes of Health GM-19536 to J.W. Hastings, and by a Rackham Dissertation/Thesis grant to W. Taylor

Abbreviation: CT, circadian time

0340-7616/79/0130/0359/$01.00

Cells were grown at 19 °C and LD 12:12 at a light intensity of 120–180 microeinsteins $m^{-2} s^{-1}$ (bright light). Midway through a given light period 10 ml aliquots of cells were transferred to vials. At the beginning of the next light period (circadian time 0), the vials were transferred to the instrument platform and measurement of the glow rhythm begun. Treatments were initiated 12 to 36 h after transfer to the instrument.

Butyraldehyde and propionaldehyde were obtained from Aldrich, acetaldehyde from Eastman, and formaldehyde from Mallinckrodt as a 37% solution. If the acetaldehyde was older than a month, it was redistilled before use, since polymerization occurs with time. The formaldehyde contained 10–15% methanol as a preservative to prevent polymerization. The final concentrations of methanol that resulted from the use of this formaldehyde solution were never high enough to have significant effect on the glow rhythm. Aldehydes were added directly to the cultures, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and oligomycin were added dissolved in dimethylsulfoxide, which itself caused no phase shifts at the concentrations used. Added compounds were removed by pelleting the cells by gentle centrifugation in a clinical centrifuge, aspirating off the supernatant and adding back fresh medium.

Respiration of *Gonyaulax* was measured with a Clark type oxygen electrode, using a chamber temperature controlled at 25 ± 0.1 °C.

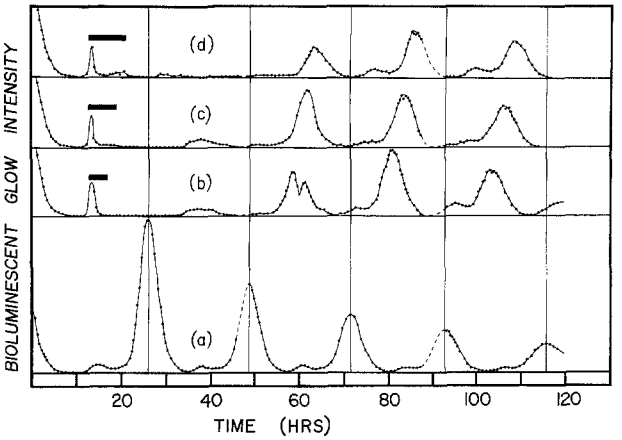

Fig. 1 a–d. The effect of pulses of acetaldehyde on the *Gonyaulax* glow rhythm. (**a**) Control (**b**), (**c**), and (**d**), 18 mM pulses of acetaldehyde for 4.4 h, 6.5 h, and 8.4 h, respectively (black bars). Time 0 is the time at which the cells were transferred to the rhythm measuring device, and corresponds to CT 0

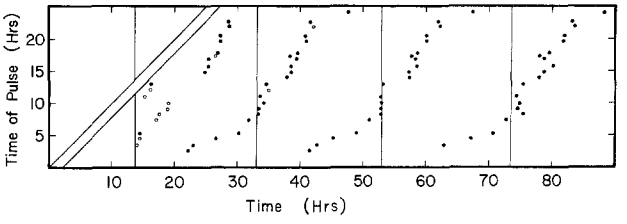

Fig. 2. The effect of two hour pulses of 18 mM acetaldehyde applied at different times of the cycle on the *Gonyaulax* glow rhythm. Time at which the pulses were administred is indicated by the diagonal bar, and subsequent peaks of the glow rhythm are indicated by the dots. Open circles indicate peaks of very low amplitude. Vertical lines indicate times at which control peaks occurred; these occur at about CT 23. The behavior of an individual culture with time can be seen by proceeding horizontally across the graph

Results and Discussion

Compared to ethanol (Taylor et al., 1979), acetaldehyde was found to cause far larger phase shifts when given as 2 to 8 h pulses in the range of 5 to 18 mM. Figure 1 shows the effect on the glow rhythm of 18 mM pulses of varying duration of acetaldehyde beginning 12 h after transfer to constant conditions. Large phase changes were obtained with these treatments, with the apparent phase delay increasing as the duration of the pulse increased. Curiously, pulses shorter than about 2 h were not very effective, even with concentrations of aldehyde 10 times higher. Thus, a 180 mM pulse of 1 h duration caused only a 2 h phase delay, while a 2 h pulse of 18 mM acetaldehyde given at the same time (starting at hour 12) can cause an 11.5 h phase shift (Fig. 2).

The phase response curve for 2 h pulses of 18 mM acetaldehyde, plotted monotonically, is given in Fig. 2. Casual inspection of this Figure leads one to the conclusion that both phase advances and delays have occurred. However, acetaldehyde, like ethanol, inhibits bioluminescent glow itself, but even more strongly than ethanol. Recovery of luminescence after a pulse of acetaldehyde often takes more than 15 h, such that the first luminescent glow expected to occur after an 18 mM pulse is often not seen. This makes it difficult to ascertain whether a given acetaldehyde-induced phase shift is an advance or a delay. We have attempted to resolve this question by varying the concentration of acetaldehyde pulses and looking to see whether the shifts become larger in one direction or the other as related to the strength of the pulse (Sweeney, 1963). This data is summarized in Table 1. In all cases, only delays appear to occur, since increasing acetaldehyde concentration always causes peaks to occur later. These data obviously present a problem, since the upper nine vials of Fig. 2 (approx. CT 3–13) are perforce advances. We are unable to explain this paradox at this time.

Experiments concerned with effectiveness of several other straight chain aliphatic aldehydes on the glow rhythm are summarized in Fig. 3, showing that the effect is greater the shorter the carbon length. A similar relationship was also reported for alcohols (Sweeney, 1976). The shorter chain length aldehydes also appear to be more toxic. In our experiments it was thus not possible to obtain data with formaldehyde concentrations higher than 2.75 mM; in fact, its toxicity is such that one cannot obtain phase shifts as large as those obtained with acetaldehyde. Indeed, with the possible exception of butyraldehyde, which

Table 1. Phase shifts of the *Gonyaulax* glow rhythm produced by various concentrations and durations of acetaldehyde. Phase shifts are indicated as delays

Time of pulse[a]	Concentration (mM)	Phase change (h)
CT 8–10	8.9	4.07
CT 8–10	4.5	2.68
CT 9–11	8.9	4.30
CT 9–11	4.5	3.39
CT 10–12	18.0	4.82
CT 10–12	13.3	4.44
CT 10–12	8.9	3.46
CT 10–12	4.5	1.76
CT 10–13	14.2	8.61
CT 10–13	10.7	8.19
CT 10–13	7.1	8.19
CT 10–13	3.5	0.85
CT 10–13	18.0	6.62
CT 10–13	13.3	6.23
CT 10–13	4.5	4.26
CT 10–13	8.9	10.33
CT 12–16	18.0	11.58
CT 12–16	10.7	4.62
CT 12–16	3.5	0.0
CT 12–15	10.7	7.35
CT 12–15	7.1	3.57
CT 12–15	3.5	1.26
CT 12–15	17.8	9.43
CT 12–15	8.9	5.87
CT 22–2	18.0	4.58
CT 22–2	10.7	1.86
CT 22–2	3.5	0.0

[a] Approximate values

may exhibit saturation at about 5 to 10 mM, none of the aldehydes appear to exhibit saturation in the phase shifting effect. With the longer chain length aldehydes this may be attributable in part to their lower solubility.

If alcohols exert their effects by being metabolized to the corresponding aldehyde, then the rhythm of cells exposed continuously to aldehydes should also show an altered (shorter) period. This prediction could not be evaluated adequately. As mentioned above, added aldehydes strongly inhibit the bioluminescent glow and are also quite toxic. At the maximum acetaldehyde concentration (0.2 mM) which still allowed survival and a detectable glow in cells exposed continuously to it, there was no effect on the period of the glow rhythm. Thus it is possible that the effect of alcohol on the period of the circadian rhythm may involve a mechanism which differs from that which is responsible for phase shifting.

Aldehydes share with alcohols and D_2O the property of having multiple cellular sites of action, making

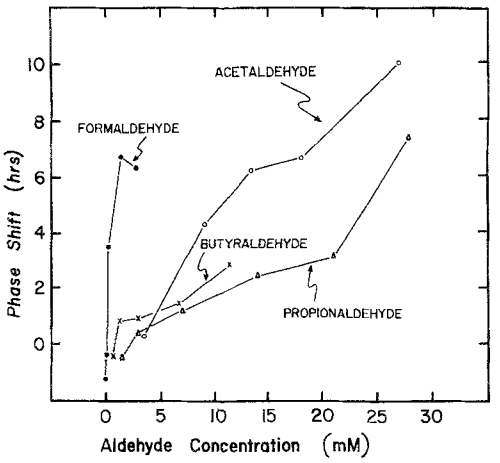

Fig. 3. Comparison of the phase-shifting effectiveness of several straight-chain aliphatic aldehydes. All pulses given at approximately CT 10–13 and all shifts are assumed to be delays. (●), formaldehyde; (○), acetaldehyde; (x), butyraldehyde; (△), propionaldehyde

it difficult to know which aldehyde-affected process in the cell is responsible for the effects on circadian rhythmicity. Among the well-documented and important effects of aliphatic aldehydes on cells are the inhibition of mitochondrial respiration, inhibition of protein synthesis, and reactions with the amino and sulfhydryl groups of proteins (Schauenstein et al., 1977). The extent to which any of these effects actually occur in *Gonyaulax*, and which may be responsible for the clock effects is unknown. However, as discussed below, there is evidence with *Gonyaulax* which suggests that reactions with thiols may be of key importance.

We have found that respiration in *Gonyaulax* is inhibited about 60% by 18 mM acetaldehyde. This occurs within 10 min and remains the same for at least two hours. It nevertheless seems probable that the blockage of respiratory activity by aldehyde is not responsible for the observed phase shifts, since several other well-known respiratory inhibitors do not have substantial effects on the clock in *Oedogonium*, *Gonyaulax*, and other systems (Bühnemann, 1955; Hastings, 1960; Bünning, 1973). From experiments carried out in the course of the present study oligomycin may also be placed in this category. It is without effect on the phase of the *Gonyaulax* glow rhythm in concentrations ranging up to 5 μgm ml^{-1}. However there are some reports of small but significant effects on circadian systems of respiratory inhibitors, notably 2,4-dinitrophenol and cyanide (Keller, 1960; Eskin and Corrent, 1977). Studies of such inhibitors should be pursued and extended to other systems.

Another possibility is that aldehydes affect the circadian oscillator by inhibiting protein synthesis. It is not known whether or not protein synthesis is

inhibited by aldehydes in *Gonyaulax*. However, the ability of cycloheximide, puromycin, and anisomycin to cause period changes and sometimes large phase shifts in several different circadian systems is well documented (Feldman, 1967; Jacklett, 1977; Rothman and Strumwasser, 1976, 1977; Karakashian and Schweiger, 1976a, b). In fact, of all the chemicals known to have effects on circadian systems, only the 80 s ribosome protein inhibitors seem to be generally able to cause phase shifts as great as the 10–12 h reported here for acetaldehyde. Aldehydes are capable of reaction with thiol compounds to form thiozolidine carboxylic acids (Schauenstein et al., 1977). Some authors believe that the inhibition of protein synthesis by alkanals is due to the removal of cysteine from the cell by such a reaction (Loreti et al., 1971). Recent experiments in our laboratory have shown that a specific inhibitor of eukaryotic ribosomal function, cycloheximide, causes phase shifts in the *Gonyaulax* glow rhythm. If it is found that aldehydes inhibit protein synthesis in *Gonyaulax*, then it may be considered possible that the aldehyde effects are at least partly due to this.

Although none of the above possibilities seems easy to prove or disprove, we believe it worth pointing out that sulfhydryl reagents generally appear to be effective in perturbing the biological clock in *Gonyaulax*. P-Chloromercuribenzoate and arsenite were found to be very effective in shifting the glow rhythm in *Gonyaulax* (Hastings, 1960). The uncoupler carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), which was found to cause phase shifts in *Gonyaulax* by Sweeney (1976), was reported to react in vivo with an aminothiol, this being involved in the production of the uncoupling effect (Heytler, 1963). We have found that CCCP also causes phase shifts (4 h phase delay with a 4 h pulse at CT 8 to 12 at a concentration of 0.05 μM) with the glow rhythm of *Gonyaulax*, as would be expected from Sweeney's data with the stimulated luminescence rhythm. Another sulfhydryl reagent, mersalyl, caused a 5% shortening (for as long as we measured, 3 cycles) of the glow rhythm after a 12 h pulse of 20 mM from CT 12 to CT 0 (Taylor, Gooch and Hastings, unpublished). Aldehydes may thus affect the clock by virtue of their reaction with free sulfhydryl groups. To our knowledge, sulfhydryl reagents have not been tested in many other circadian systems.

References

Brinkmann, K.: The influence of alcohols on the circadian rhythm and metabolism of *Euglena gracilis*. J. Interdiscip. Cycle Res. **7**, 149–170 (1979)

Bühnemann, F.: Das endodiurnale System der Oedogonium-Zelle II. Der Einfluß von Stoffwechselgiften und anderen Wirkstoffen. Biol. Zentralblatt **74**, 691–705 (1955)

Bünning, E.: The physiological clock. Third English edition. Berlin, Heidelberg, New York: Springer Verlag 1973

Eskin, A., Correnti, G.: Effects of divalent cations and metabolic poisons on the circadian rhythm from the *Aplasia* eye. J. Comp. Physiol. **117**, 1–21 (1977)

Feldman, J.: Lengthening the period of a biological clock in Euglena by cycloheximide, an inhibitor of protein synthesis. Proc. Natl. Acad. Sci. **57**, 1080–1087 (1967)

Guillard, R.R., 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.: Biochemical aspects of rhythms: Phase shifting by chemicals. Cold Spring Harbor Symp. Quant. Biol. **25**, 131–140 (1960)

Heytler, P.G.: Uncoupling of oxidative phosphorylation by carbonyl cyanide phenylhydrazones. I. Some characteristics of m-Cl-CCP action on mitochondria and chloroplasts. Biochemistry **2**, 357–361 (1963)

Jacklett, J.W.: Neuronal circadian rhythm: Phase shifting by a protein synthesis inhibitor. Science **198**, 69–71 (1977)

Karakashian, M., Schweiger, H.: Evidence for a cycloheximide-sensitive component in the biological clock of *Acetabularia*. Exp. Cell Res. **98**, 303–312 (1976a)

Karakashian, M., Schweiger, H.: Temperature dependence of cycloheximide-sensitive phase of circadian cycle in *Acetabularia mediterranea*. Proc. Natl. Acad. Sci. **73**, 3216–3219 (1976b)

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

Loreti, L, Ferioli, M.E., Gazzola, G.C., Guidotti, G.G.: Studies on the antitumour activity of aliphatic aldehydes III. Formation of thiozolidine carboxylic acid in tissues. Eur. J. Cancer **7**, 281 (1971)

Rothman, B., Strumwasser, F.: Phase shifting the circadian rhythm of neuronal activity in the isolated Aplysia eye with puromycin and cycloheximide. J. Gen. Physiol. **68**, 359–384 (1976)

Rothman, B., Strumwasser, F.: Manipulation of a neuronal circadian oscillator with inhibitors of macromolecular synthesis. Fed. Proc. **36**, 2050–2055 (1977)

Schauenstein, H., Esterbauer, H., Zollner, H.: Aldehydes in biological systems, pp. 9–24. London: Pion Ltd. 1977

Sweeney, B.M.: Resetting the biological clock in *Gonyaulax* with ultraviolet light. Plant Physiol. **38**, 704–708 (1963)

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., Gooch, V.D., Hastings, J.W.: Period shortening and phase shifting effects of ethanol on the *Gonyaulax* glow rhythm. J. Comp. Physiol. **130**, 355–358 (1979)