The Occurrence of an Endogenous Circadian Rhythm in a Plant Tissue Culture [1, 2]

Malcolm B. Wilkins[3] and Andrew W. Holowinsky
The Biological Laboratory, Harvard University, Cambridge, Massachusetts

Leaves of the succulent plants *Bryophyllum fedtschenkoi* and *B. daigremontianum* show an endogenous circadian rhythm in their rate of CO_2 metabolism (9, 10, 11, 12). The extent to which the occurrence of the rhythm depends upon the organization of the leaf has been investigated with callus cultures of *Bryophyllum* leaf tissue.

There has been but one previous report of a persistent rhythm in a plant tissue culture. Enderle (3) observed rhythmic fluctuations in growth and turgidity of cultures of *Daucus carota* and found the rhythmicity to persist in prolonged darkness. Investigation of the rhythm necessarily involved microscopic examination of the cultures and this was done with red light at intervals of approximately 4 hours. Since red light is particularly active in affecting plant rhythms (4, 5, 7, 11), and some organisms (e.g. *Euglena*) are known to have the capacity for frequency demultiplication (1, 2), the diurnal periodicity in growth and turgidity might have arisen from the periodic stimulation with red light. Enderle (3) does not appear to have established whether or not the phase of the rhythm can be set at different times of day. Unequivocal evidence of the occurrence of endogenous rhythms in plant tissue cultures is therefore lacking. The endogenous nature of rhythmicity in animal tissue cultures (6, 8) has also yet to be firmly established.

Material and Method

Two types of tissue of *Bryophyllum daigremontianum* Bgr. have been used: segments of mature leaves and cultured, unorganized leaf callus. The leaf segments were excised from plants grown under natural illumination in a heated greenhouse. The leaf callus was derived from lamina explants set out on a medium of the following composition: Ca $(NO_3)_2 \cdot 4\ H_2O$, 1.02 mM; $MgSO_4$, 0.17 mM; KNO_3, 0.84 mM; KCl, 0.81 mM; KH_2PO_4, 0.14 mM; $FeCl_3$, 9.16 μM; sucrose, 117 mM; Difco yeast extract, 1.0 g per liter; 2, 4-dichlorophenoxyacetic acid, 6.0 μM; kinetin (6-furfurylamino purine), 5 μM; agar, 7.0 g per liter. The pH of the medium was adjusted to 5.5 before autoclaving. The frequency of successful callus growth from lamina explants on this medium was 1 to 2 %. Callus from 1 successful explant has been maintained for more than 18 months on the above medium with the addition of 8 % coco-nut milk. The cultures were grown in a controlled environment at 23° with a 24-hour cycle of light and darkness. The 12-hour photofraction extended from 0900 hours to 2100 hours, and the radiant flux was 150 ft-c from fluorescent sources. The callus was subcultured at intervals of 6 weeks and proliferated rapidly as unorganized tissue, doubling in volume every 3 weeks. The cells possessed numerous green chloroplasts and had the capacity for photosynthesis (fig 1).

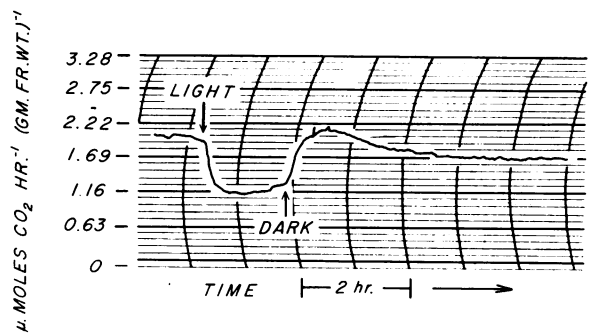

FIG. 1. The photosynthetic capacity of the *Bryophyllum* leaf callus cultures. Exposure to light (approx. 1000 ft-c) began at light and ended at dark. Ordinate: rate of CO_2 emission of the tissue. Abscissa: time in hours.

The rate of CO_2 metabolism of the tissues was measured with an infrared gas analyser (Grubb, Parsons and Company, Newcastle-Upon-Tyne, England, single box model). Air from a commercial tank was passed first through several CO_2 absorption towers containing 10 % potassium hydroxide and then in sequence through a flowmeter, the comparison tube of the gas analyzer, the culture tube and finally the sample tube of the analyser. A uniform flow-rate of about 2 liters per hour was main-

[1] Received March 31, 1965.
[2] The work was supported by NSF Grant No. G-21799 to Professor K. V. Thimann and by National Institute of Health, PHS Grant No. GM-08145 to Professor J. G. Torrey.
[3] Present address: School of Biological Sciences, University of East Anglia, Norwich, Norfolk, NOR 77H, Great Britain.

tained throughout each experiment and a continuous record of the rate of CO_2 output of the tissue was plotted automatically against time of day.

Each experiment was carried out with a culture of about 5 to 7 g fresh weight during the sixth week after the last subculture. The inlet and exhaust tubes of the culture tube were fitted with cotton wool filters to prevent infection of the callus while CO_2 output was being recorded. During the experiments the cultures were maintained in continuous darkness and at 23° in a water bath.

For some experiments leaf segments were prepared under aseptic conditions and placed on moist filter paper in tubes similar to those used for the callus cultures.

The method of estimating the time at which peaks occurred has been described in previous papers (12, 13).

Results

The emission of CO_2 from leaf callus cultures of *B. daigremontianum* is shown in figure 2. The curves in figures 2a and 2c were derived from cultures transferred to prolonged darkness at 2000h and 1600h respectively, and the one in figure 2b from a culture which was exposed to 2 reversed cycles of light and darkness before being placed in continuous darkness at 1000h. The 3 cultures exhibited persistent rhythms in CO_2 output under uniform environmental conditions. The phase of the rhythm is clearly independent of time of day and determined by the time at which prolonged darkness begins. In each culture the first peak of the rhythm occurred 20 hours after the onset of darkness. The mean period of the rhythm at 23° is 25.5 ± 0.33 hours.

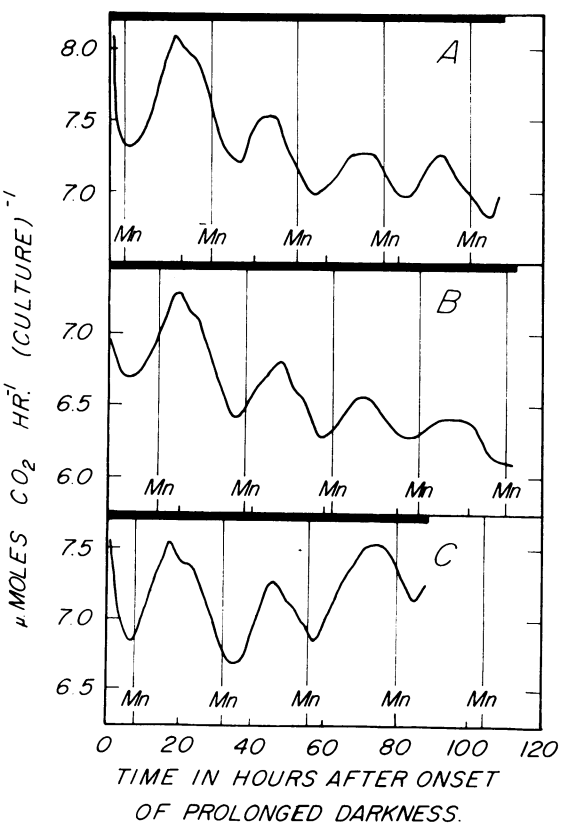

FIG. 2. The rhythm of CO_2 emission in leaf callus cultures of *Bryophyllum* darkened at 2000h (A) and 1600h (C), or exposed to 2 reversed cycles of light and darkness before being transferred to prolonged darkness at 1000h (B). Bar above each curve indicates darkness. Mn = midnight.

Leaf segments of *B. daigremontianum* exhibited a closely similar rhythm to that shown by the callus cultures except that the amplitude was greater during the first 24 to 36 hours of darkness. The curves were closely similar to those already published for whole leaves of *B. daigremontianum* (10). The first peak occurred about 23 hours after the onset of darkness at 23° and subsequent peaks at intervals of approximately 24 hours.

Discussion

The endogenous nature of the rhythm of CO_2 metabolism in *Bryophyllum* callus cultures is strongly suggested by the facts that A) the rhythm persists under uniform environmental conditions of temperature and darkness, B) the phase can be set at any time of the solar day, and C) the period is significantly longer than 24 hours. The occurrence of the rhythm in whole leaves (10), leaf segments and leaf callus cultures shows that the rhythmicity is a general cellular property and is not dependent upon the structural organization of the leaf.

The rhythm in *B. daigremontianum* callus cultures contrasts with that in whole leaves (10) and leaf segments in that the amplitude is much less during the first 1 or 2 days in darkness. The damping of the oscillation observed in whole leaves is much less marked in callus cultures. The presence of the epidermis and movement of stomatal guard cells may be responsible in part for the large amplitude of the rhythm in whole leaves and leaf segments during the first 24 to 36 hours of darkness. Removal of the epidermis from small segments of leaves of *B. fedtschenkoi* greatly reduces the initial amplitude of the rhythm (10). The damping of the rhythm in detached whole leaves and in leaf segments may also be accentuated by the gradual depletion of nutrients whereas a continued supply of nutrients is available to the callus cultures.

As in the case of whole leaves and leaf segments, the phase of the rhythm in *Bryophyllum* callus is determined by the time darkness begins. A similar situation may exist in cultures of *Daucus carota*, but the experiments described by Enderle (3) do

not include any in which the time of onset of darkness has been varied. Even in *Daucus* cultures darkened at the same time, however, there is variation in the time of occurrence of the first peak and trough of the rhythm.

The *Bryophyllum* leaf callus cultures possess a rhythm which is easily monitored under uniform environmental conditions and in which the phase can be readily manipulated to suit experimental requirements.

Summary

Leaf callus cultures of the succulent plant *Bryophyllum daigremontianum* have been shown to possess an endogenous circadian rhythm of carbon dioxide metabolism. The rhythm persists in total darkness and at a uniform temperature, the phase being determined by the time at which prolonged darkness begins.

Acknowledgments

The authors are greatly indebted to Professors Kenneth V. Thimann and John G. Torrey for the laboratory facilities placed at their disposal, and in particular to Professor Torrey for his guidance in establishing the callus cultures. One author (M.B.W.) was a Research Fellow of Harvard University, supported by N.S.F. Grant number G-21799 to Professor K. V. Thimann, and the other (A.W.H.) was a United States Public Health Service Post-Doctoral Fellow. The research was supported in part by N.I.H., P.H.S. Grant number GM-08145 to Professor J. G. Torrey.

Literature Cited

1. Bruce, V. G. and C. S. Pittendrigh. 1956. Temperature independence in a unicellular clock. P. N.A.S. 42: 676–82.
2. Bruce, V. G. 1960. Environmental entrainment of circadian rhythms. Cold Spring Harbor Symp. 25: 29–48.
3. Enderle, W. 1951. Tagesperiodische Wachstums- und-Turgor-schwankungen an Gewebekulturen. Planta 39: 570–88.
4. Hastings, J. W. and B. W. Sweeney. 1960. The action spectrum for shifting the phase of the rhythm of luminescence in *Gonyaulax polyedra*. J. Gen. Physiol. 43: 697–706.
5. Holdsworth, M. B. 1960. The spectral sensitivity of light-induced leaf movements. J. Exptl. Botany 11: 40–44.
6. Hupe, K. and A. Gropp. 1957. Über den zeitlichen Verlauf der Mitoseaktivität in Gewebekulturen. Z. Zellforsch 46: 67–70.
7. Lörcher, L. 1958. Die Wirkung verschiedener Lichtqualitäten auf die Tagesrhythmik von *Phaseolus*. Z. Botany 46: 209–41.
8. Voutilainen, A. 1953. Über die 24-Std-Rhythmik der Mitosenfrequenz in malignen Tumoren. Acta Pathol. Scand. Suppl. 89: 104.
9. Warren, D. M. and M. B. Wilkins. 1961. An endogenous rhythm in the rate of dark fixation of carbon dioxide in leaves of *Bryophyllum fedtchenkoi*. Nature 191: 686–88.
10. Wilkins, M. B. 1959. An endogenous rhythm in the rate of carbon dioxide output of *Bryophyllum*. I. Some preliminary experiments. J. Expl. Botany 10: 377–90.
11. Wilkins, M. B. 1960. An endogenous rhythm in the rate of carbon dioxide output of *Bryophyllum*. II. The effects of light and darkness on the phase and period of the rhythm. J. Expl. Botany 11: 269–88.
12. Wilkins, M. B. 1962. An endogenous rhythm in the rate of carbon dioxide output of *Bryophyllum*. III. The effects of temperature changes on the phase and period of the rhythm. Proc. Royal Soc. London Ser. B 156: 220–41.
13. Wilkins, M. B. and D. M. Warren. 1963. The influence of low partial pressures of oxygen on the rhythm in the growth rate of the *Avena* coleoptile. Planta 60: 261–73.