Planta (1984) 161:381–384

Planta

A rapid circadian rhythm of carbon-dioxide metabolism in *Bryophyllum fedtschenkoi*

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Abstract. Leaves of *Bryophyllum fedtschenkoi* Hamet et Perrier maintained in a stream of normal air and at 15° C exhibit a circadian rhythm of CO_2 uptake in continuous light but not in continuous darkness. The rhythm is unusual in that it persists for at least 10 d, and has a short period of approximately 18 h. The mechanism by which this rhythm is generated is discussed.

Key words: *Bryophyllum* (circadian rhythm) – Circadian rhythm – Carbon dioxide fixation – Photosynthesis (circadian rhythm).

Introduction

The circadian rhythm of CO_2 output in detached leaves of *Bryophyllum fedtschenkoi* kept in darkness and an initially CO_2-free air stream is well documented. It persists for about 4 d at 15° C, has a period of 23.8 ± 0.3 h and is inhibited by light (Wilkins 1959, 1960, 1962, 1973, 1983a, b; Bollig and Wilkins 1979). Recently, Buchanan-Bollig (1984) reported the occurrence of a marked rhythm of CO_2 uptake in leaves of *Bryophyllum daigremontianum* kept in a stream of normal air and continuous illumination. This paper reports on the behaviour of leaves of *B. fedtschenkoi* in normal air in both continuous light and darkness. The leaves exhibit a rhythm in CO_2 metabolism only in light, the rhythm being remarkable for its persistence and period. The origin of the rhythm and its unusual features are discussed in relation to the hypotheses proposed for the generation of the rhythm in leaves in a CO_2-free air stream and darkness by Bollig and Wilkins (1979), and for the induction of phase shifts by Wilkins (1983a, b).

Abbreviation: PEPCase = phosphoenolpyruvate carboxylase

Material and Methods

Plants of *Bryophyllum (Kalanchoë) fedtschenkoi* Hamet et Perrier were derived vegetatively from the original stock (Wilkins 1959, 1960) used in previous studies. Cuttings were grown in a glass-house under a 16-h photoperiod maintained throughout the year with mercury-vapour lamps. Four weeks before being used, the plants were transferred to a controlled-environment room in which the 8-h photoperiod extending from 08.00 to 16.00 h was provided by warm-white and white fluorescent lamps giving a radiant fluence rate of about 20 W m^{-2} at the level of the plants.

The method of measuring the rate of CO_2 exchange by the leaves has been described in earlier papers (Wilkins 1973, 1983a). Three samples of leaves were used in each experiment; two were kept in continuous light of fluence rate 15 W m^{-2} provided by white fluorescent tubes, and the third in darkness. The leaves were illuminated only from above. Each leaf sample was kept in a specially constructed, gas-tight, brass container surrounded by a water jacket, through which water at 15 ± 0.1° C was circulated from a water bath. A stream of normal air from a cylinder passed over the leaves at a flow-rate of 1.55 l h^{-1}. The effluent from each leaf chamber was directed sequentially into an infra-red gas analyser (ADC 225 Mark 3; Analytical Development Co. Ltd., Hoddesdon Herts., UK) for 15 min in each hour by means of a series of Bürkert three-way solenoid valves, and for the remaining 45 min, to waste. The air stream from a blank leaf chamber was also passed through the analyser for 15 min in each hour to provide a check of the zero stability of the instrument.

Activation of the Bürkert (Bürkert Contromatic Ltd., Stroud, Glos., UK) microsolenoid valves was controlled by means of a BBC microcomputer model B (Commscot, Glasgow, UK) with an appropriate interface. The output signals of the gas analyser were plotted on a Servoscribe chart recorder (Belmont Instruments Ltd., Glasgow, UK), but were also collected and processed by the computer. The blank reading was first deducted from the raw signal and the resultant figure multiplied by the calibration value for the analyser and then divided by the fresh weight of the leaves so that the computer printed out every 15 min the CO_2 uptake or emission of a leaf sample in μg CO_2 h^{-1} g^{-1} fresh weight. The computer also stored these data so that they could be plotted automatically against time by the printer (Radioshack TRS80 color graphic; Tandy Corporation, Glasgow, UK) if required.

Experiments were set up between 15.30 h and 16.00 h and the detached leaves were placed in continuous light (15 W m^{-2})

at 16.00 h. The rates of CO_2 metabolism of the leaves shown in Fig. 1 A, B were plotted at hourly intervals against time of day.

Results

The results of two independent experiments carried out on different occasions are shown in Fig. 1 A, B. In each case leaves kept in a stream of normal air at 15° C and continuous darkness did not exhibit a rhythm in their rate of CO_2 metabolism. There was an initial, very marked, period of CO_2 fixation with a maximum occurring at about 01.00 h, but after 15 h this net uptake of CO_2 was replaced by a net output of CO_2 which increased to a maximum at about 16.00 h and then gradually declined to a constant rate of 10–15 μg CO_2 h^{-1} g^{-1} fresh weight which was maintained until at least the eighth day.

In contrast, leaves kept under white light at 15° C exhibited a persistent rhythm of CO_2 uptake which showed a very gradual decrease in amplitude with time. The most striking features of this rhythm are the length of time for which it persists under constant conditions, and the length of the period which for the four curves shown in Fig. 1 A, B is 18.09 ± 0.12 h. In some, but not all, experiments the rhythms in the two samples of leaves showed a difference in phase after several days in constant conditions (compare Fig. 1 B with Fig. 1 A). This difference developed gradually over the duration of the experiment in Fig. 1 B due to the period of the rhythm in one sample being slightly longer than that of the rhythm in the other sample.

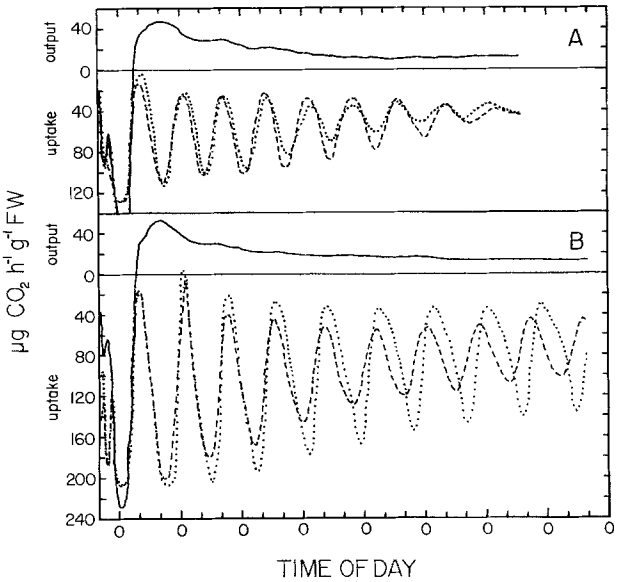

Fig. 1 A, B. The circadian rhythm of CO_2 uptake by leaves of *B. fedtschenkoi* kept in a stream of normal air at 15° C and in continuous white light of fluence rate 15 W m^{-2} (*broken lines*). The non-rhythmic CO_2 uptake and output of leaves at 15° C in darkness is shown by the *continuous lines*. **A** and **B** show independent experiments carried out on different occasions, and the two broken lines duplicate samples within each experiment

Discussion

The lack of a rhythm of CO_2 metabolism in leaves of *B. fedtschenkoi* kept in normal air at 15° C and continuous darkness is in accord with an earlier observation made in leaves under similar conditions but at 26° C (Wilkins 1959). The occurrence of a most marked rhythm of CO_2 uptake in leaves in continuous white light confirms the recent report of Buchanan-Bollig (1984) of such a rhythm in detached leaves of *B. daigremontianum* in which there was also no rhythm in darkness.

The most unusual features of this rhythm are the large amplitude, the remarkable degree of persistence, and the shortness of the period. All three features distinguish this rhythm from that of CO_2 output in the leaves when they are kept in an initially CO_2-free air stream and continuous darkness (Wilkins 1960, 1962, 1973, 1983 a, b). In the latter case the amplitude is much less, the rhythm damps out after about 4 d and the period at 15° C is 23.8 ± 0.3 h.

The shortness of the period, and the fact that samples being simultaneously monitored show slightly different periods under identical conditions, thus leading to different phases after 7 or 8 d (Fig. 1 B) leave no room for doubt that the rhythm is of endogenous origin.

The period of this circadian rhythm is one of the shortest that has been recorded, especially at 15° C (Bünning 1973; Altman and Dittmer 1966; Wilkins 1965). The value recorded here in leaves of *B. fedtschenkoi* contrasts with that of 23–24 h reported by Buchanan-Bollig (1984) in leaves of *B. daigremontianum* at 23.3° C under a radiant fluence rate of white fluorescent light of 10 W m^{-2}.

When leaves of *B. fedtschenkoi* are kept in a stream of CO_2-free air and continuous darkness, the circadian rhythm of CO_2 output is generated by the periodic activity of phosphoenolpyruvic carboxylase (PEPCase) attributable to the sequential accumulation of malate in the cytoplasm and its removal to the vacuole (Warren and Wilkins 1961; Bollig and Wilkins 1979). Furthermore, the induction of phase shifts in that rhythm by light and high-temperature (35° C) signals has been ascribed to these stimuli opening "gates" in the tonoplast

thus allowing malate leakage to take place along a concentration gradient from the vacuole to the cytoplasm across the tonoplast (Wilkins 1983a). The origin of the rhythm in leaves kept in normal air and continuous light has not been established, but Buchanan-Bollig et al. (1984) have shown that fixation of CO_2 into malate occurs periodically in *B. daigremontianum* leaves.

It is necessary, therefore, to consider how a rhythm in PEPCase activity can be generated in continuous light in terms which are compatible with the proposals for the origin of the rhythm in CO_2-free air and darkness (Bollig and Wilkins 1979), with the tonoplast "gate" hypothesis for the induction of phase shifts (Wilkins 1983a, b), and with the absence of a rhythm in normal air in darkness. In addition, the much longer persistence of the rhythm and its short period in normal air and light require explanation.

It is therefore proposed that the rhythm in PEPCase activity, and hence in CO_2 fixation, in leaves in normal air and continuous illumination is generated by the following mechanism. At the end of the normal 8-h day when the experiments are set up, the leaves are relatively free of malate or contain only insignificant levels (Osmond 1978). The onset of the rhythm therefore involves PEPCase becoming active with a major period of CO_2 fixation leading to the accumulation of substantial levels of malate in the cytoplasm and the total inhibition of PEPCase. This corresponds with the first peak (minimum of CO_2 uptake) in the rhythms shown in Fig. 1A, B. No further fixation of CO_2 by PEPCase can occur until the malate is removed from the cytoplasm. In the rhythm in leaves in a CO_2-free atmosphere and darkness this is achieved by malate being pumped from the cytoplasm into the vacuole (Bollig and Wilkins 1979), but since Wilkins (1983a, b) has proposed that in light "gates" in the tonoplast will be open, it is clearly impossible for leaf cells in normal air and light to free the cytoplasm of malate by pumping it to the vacuole because it would immediately leak back along a concentration gradient. Because the cytoplasm must be freed of malate if a further period of PEPCase activity is to occur, it appears that this must be accomplished by the metabolic breakdown of malate. Such a metabolic breakdown of malate might be achieved by the activity of malic enzyme producing pyruvate and CO_2 (Osmond 1978). Since the CO_2 and much of the pyruvate would be removed by the photosynthetic mechanism which will be operating in the light, the breakdown of malate could proceed virtually to completion, and PEPCase could become active once again because the end products of photosynthesis would not inhibit PEPCase. The rhythm in leaves in normal air and continuous light would, therefore, be generated by the periodic activity of PEPCase brought about by the periodic accumulation of malate in the cytoplasm, and the removal of malate in the absence of PEPCase activity by metabolic breakdown involving malic enzyme and photosynthesis. In contrast, the rhythm in leaves in CO_2-free air and darkness depends upon the removal of the cytoplasmic malate by its transfer to the vacuole since photosynthetic removal of CO_2 produced by the action of malic enzyme would be impossible.

This hypothesis, for the generation of the rhythm in leaves in normal air and continuous light, also provides an explanation for the fact that the rhythm persists for much longer than does the rhythm in CO_2-free air and darkness, and for the total absence of a rhythm in normal air and darkness. In leaves in CO_2-free air and darkness, the only CO_2 fixation and hence malate synthesis that can occur is by the fixation of the respiratory CO_2 produced by the leaves themselves (maximum rate at 15° C approx. 40 μg CO_2 h^{-1} g^{-1} fresh weight (Wilkins 1983a, b). Malate must therefore accumulate at a much lower rate than in normal air since the available CO_2 will be limiting. In CO_2-free air the amount of malate formed in the cytoplasm during the first period of fixation, whilst being adequate to inhibit PEPCase and hence halt CO_2 fixation (Warren and Wilkins 1964) for about 6–8 h, can evidently be totally removed by being pumped into the vacuole. After about four cycles of CO_2 fixation, however, the concentration of malate accumulated in the vacuole must become so high that any further removal of cytoplasmic malate to the vacuole is impossible with the result that CO_2 fixation by PEPCase is permanently inhibited and the rhythm disappears. In normal air and darkness, the very large amount of CO_2 fixation during the first 15 h (the maximum rate recorded being over 200 μg CO_2 h^{-1} g^{-1} fresh weight, Fig. 1B, would clearly lead to very high levels of malate in the cells, so high, in fact, that it would be impossible for the malate to be removed from the cytoplasm by being pumped into the vacuole across the tonoplast. This results in no further PEPCase activity being possible, and hence no rhythm is observed.

The remarkable persistence of the rhythm in normal air and continuous light finds ready explanation in the fact that, following the hypothesis for the generation of the rhythm proposed above, there will be no appreciable gradual accumulation of malate in the leaf cells with time as the result

of each period of PEPCase activity. After each period of CO_2 fixation and inhibition of PEPCase by malate accumulation, the cytoplasm (and cell) must be virtually freed of malate by the metabolic mechanism involving malic enzyme and photosynthesis so that the leaf cells, in effect, begin *each cycle* in virtually the same state with respect to the malate levels. No limitation on the persistence of the rhythm is thus imposed by the gradual accumulation of malate in the cells to totally inhibitory levels over a number of cycles, as occurs after about four or five cycles in leaves in CO_2-free air and darkness and after only one cycle in leaves in normal air and darkness.

It will be noted in Fig. 1 A, B that the first troughs (period of CO_2 fixation) of the rhythm in leaves in continuous light correspond in timing exactly with those in darkness up to the point just before the CO_2 uptake in the darkened leaves is replaced by CO_2 output. At this point, where the light and dark curves diverge, it is presumed that CO_2 fixation by PEPCase has virtually been stopped by malate accumulation. In the illuminated leaves, malate must be rapidly decarboxylated with the result that PEPCase activity again increases, leading to the onset of the next maximum of CO_2 uptake of the rhythm. The rate at which the malate is removed is presumably dependent, amongst other factors, upon the rate of photosynthesis and hence the light intensity. This possibility offers a possible explanation for the very short period of the rhythm under the conditions used in this investigation, since it would be predicted that the more rapid the removal of malate the higher would be the frequency of the rhythm. The radiant fluence rate used in this investigation may indeed lead to a much more rapid removal of the malate from the cytoplasm than does the trans-tonoplast pumping mechanism that operates in darkness and results in a period of 18.09 h in comparison with that of 23.8 h in leaves at the same temperature but in a CO_2-free atmosphere and darkness. The longer period reported by Buchanan-Bollig (1984) in leaves of *B. daigremontianum* at 23° C and in 10 W m^{-2} might be attributed, in part, to a somewhat lower photosynthetic rate. The exciting possibility that the period of the very persistent rhythm in normal air and continuous illumination is under the control of light intensity, and more specifically the quantum fluence rate of photosynthetically active radiation is now under investigation.

The author is indebted to Dr. A.M.M. Berrie, Dr. M.F. Hipkins and Mr. A. Anderson for help in establishing computer control of the apparatus and data processing, to Miss A. Halliday for technical assistance, and to Miss Clare Anderson for helpful discussion.

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Received 1 March; accepted 16 March 1984