Circadian Rhythmicity in the Activities of Phenylalanine Ammonia-Lyase from *Lemna perpusilla* and *Spirodela polyrhiza* 1

Received for publication February 8, 1977 and in revised form June 12, 1978

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ABSTRACT

The oscillations in phenylalanine-ammonia-lyase activity from *Spirodela polyrhiza* and phenylalanine-ammonia-lyase and tyrosine ammonia-lyase activities from *Lemna perpusilla* displayed a circadian rhythm under continuous light. Rhythmicity in enzymic activity could not be detected in continuous darkness since under this condition phenylalanine ammonia-lyase activity remains at a fairly constantly low level. Results from our studies of the oscillatory pattern of the respective activities of phenylalanine and tyrosine ammonia-lyase support their "inseparability."

The study of biochemical oscillations, especially of enzyme systems, has become a very important area of research (11). Among the more extensively investigated examples of oscillatory or rhythmic behavior at the metabolic level are the enzymic reactions of glycolysis in yeasts (24, 26). There are examples of soluble enzyme systems in plants which exhibit rhythmicity (17, 19, 25), and a recent review of biological rhythms and physiological timing in plants (16) suggests that rhythms are common in plant metabolism.

In the Lennacea, rhythmic behavior of respiratory gas exchange has received considerable attention. Uptake of O2 by *Lemna gibba* entrained to a short day schedule displays circadian periodicity when transferred to continuous light (20). Also, a CO2 output rhythm has been identified with *Lemna perpusilla* (14, 15) that is associated with floral induction.

The enzyme l-phenylalanine ammonia-lyase (EC 4.3.1.5) catalyzes the deamination reactions in which l-phenylalanine and l-tyrosine are degraded to trans-cinnamic acid and trans-p-coumaric acid plus ammonia, respectively. These reactions represent an important metabolic sequence in plants which results in the diversion of the two amino acids from the pathways of protein synthesis into secondary metabolism with the subsequent production of metabolites such as lignins, flavonoids, tannins, and alkaloids (27). Thus, PAL* has a pivotal role in the reactions of secondary metabolism and in this manner, is thought to exert its influence on plant growth and development (3). Since oscillations and rhythms have been associated with processes of growth and development (5) and because the activity of PAL has been reported to display fluctuations in response to various environmental conditions (3), an investigation into the nature of fluctuations in PAL activity from members of the Lennaceae during growth and development seemed appropriate. In this paper, circadian rhythmicity in the activity of PAL is described in *L. perpusilla* 6746 and *Spirodela polyrhiza* L. (Schleiden).

MATERIALS AND METHODS

Cultures of *L. perpusilla* 6746 and *S. polyrhiza* L. (Schleiden) were maintained axenically in 125-ml Erlenmeyer flasks containing 50 ml of 0.5-strength Hutner's medium supplemented with 1% sucrose (13). Plants were entrained on a LD 16:8 cycle (16 hr of light followed by 8 hr of darkness) at 22 to 23 C before exposure to LL or DD. The light source used during entrainment for LL was three 15-w cool-white Sylvania fluorescent lamps which provided 235 ft-c (7.4 x 104 ergs cm-2 sec-1) at a distance of 48.4 cm from flasks.

PAL was extracted from 3-g fresh weight aliquots of 15-day-old plant tissue in 0.1 M Tris buffer (pH 7.5) containing 28 mM mercaptoethanol. PVP was added in the amount of 10% (w/w) of the tissue fresh weight during extraction to promote removal of phenols and tannins. A PAL-rich fraction was obtained from crude extracts by treatment of the same with 15% (w/v) (PEG)polyethylene glycol 6000 (7). PAL assays were conducted spectrophotometrically (30). The reaction mixture contained 80 μmol of borate buffer (pH 8.9), 30 μmol of l-phenylalanine, and 0.2 ml of enzyme preparation containing 79 to 160 μg of protein. Production of trans-cinnamic acid was followed by measuring increases in A at 290 nm against substrate blanks using a Beckman spectrophotometer equipped with a sipper-cell accessory. TAL assays were conducted similarly with 3 μmol of l-tyrosine replacing l-phenylalanine in the reaction mixture and production of p-coumaric acid was followed by measuring changes in A at 333 nm (12).

A unit of activity was defined as the amount of enzyme which yields 1 μmol/min of product under standard assaying conditions (10). Protein was determined by the method of Potty (23) using BSA as a standard.

Enzyme activity measurements at 4 hr intervals were coded onto punch cards for computer analysis by the least squares method. Cosine curves were fitted to the data and rhythm parameters quantified according to methods previously published (18).

RESULTS

Changes in the activity of PAL from *L. perpusilla* plants maintained on a LD 16:8 schedule were similar to those observed in terrestrial plants (9, 28). Following a short lag period, PAL activity increased steadily, reaching a maximum after the 12th or 16th hr of light and then declined rapidly just prior to and during darkness (Fig. 1).

Extractable PAL activity from *S. polyrhiza* cultures which were

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1 This investigation was supported in part by a fellowship to W. R. G. from the National Fellowships Fund, Atlanta, Georgia 30308.
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4 Abbreviations: PAL: phenylalanine-ammonia-lyase; TAL: tyrosine ammonia-lyase; DD: continuous darkness; LL: continuous illumination; LD: light-dark.
transferred from LD to LL displayed rhythmic oscillations with periods of approximately 24 hr (Fig. 2). *L. perpusilla* plants grown under similar conditions also demonstrated this rhythmic pattern for both PAL and TAL activities (Fig. 3), with the free-running periods ranging from 22 to 25 hr (Table I). The difference in amplitudes observed between PAL and TAL activities was consistent with the reported magnitudes of the two activities, i.e., PAL activity is at least two to three times greater than TAL activity and often the level of PAL is more than 10 times greater than TAL (3). Following 48 hr in LL, extractable PAL activity from *L. perpusilla* continued to oscillate for at least two additional cycles in LL. Under conditions of reduced light intensity (1.6 × 10⁶ ergs cm⁻² sec⁻¹ compared to 7.4 × 10⁶ ergs cm⁻² sec⁻¹), the rhythm persisted for three to five cycles before damping out, and the amplitudes were much less than those of plants grown under the routine conditions of higher light intensity. Damping out was also observed when a low energy incandescent light supplement was given during entrainment and at the beginning of LL (Table I).

![Fig. 1](image1.png)

**Fig. 1.** Activity of PAL in *L. perpusilla* maintained under LD 16:8. Changes in PAL activity from 14-day-old cultures of *L. perpusilla*. Experiments were begun at the onset of the light span. Data points (●) represent the mean of three values.

![Fig. 2](image2.png)

**Fig. 2.** Oscillations of PAL from *S. polyrhiza* under LL. Assays were begun immediately after the end of the 8-hr dark span on the 16th day and thereafter repeated every 4 hr with the cultures remaining in LL for the duration of the experiment.

![Fig. 3](image3.png)

**Fig. 3.** Circadian rhythmicity of PAL and TAL activities from *L. perpusilla*. Plants were maintained under a LD 16:8 regimen for 12 days, thereafter they were placed under LL for 2 days before enzyme assays were started. PAL (●); TAL (○).

The results from several experiments showed that PAL activity from plants maintained in DD, in contrast to the activity of plants in LL, remained at a fairly constant low level throughout the duration of our experiments and did not show any indication of rhythmicity (Fig. 4).

### DISCUSSION

PAL activity extracted from *L. perpusilla* and *S. polyrhiza* cultures entrained in a LD 16:8 cycle displayed circadian rhythmicity in LL. Rhythm parameters, quantified by computer analysis of the data (18), indicated that the free running period of the PAL rhythm in both species was circadian (Table I). Although the results from the computer analysis showed that the free running period did deviate from exactly 24 hr in experiments 1, 2, and 3, more precise estimates were not possible due to the amount of time required in extraction and assays.

A recent review on the biochemistry and physiology of PAL (3) has discussed several properties of the enzyme which indicate that PAL and TAL activities from several plant and fungal sources are inseparable and may thus share a common active site. Results from our studies with *L. perpusilla* provide yet another property in support of this inseparability in that the rhythmic patterns of the two activities were very similar and essentially differed only in the levels of the two respective activities (Fig. 3 and Table I).

Prior to this investigation, studies of *Amaranthus caudatus* and *Beta vulgaris* demonstrated rhythmic behavior in PAL activity when measured in vitro during 72 hr in continuous light (2). However, a more recent report on the oscillatory behavior of PAL in black locust (22) suggests that the cyclic behavior of PAL (about 24 hr) could not be circadian because the oscillations did not persist in DD. Although persistence in DD has been suggested as one demonstration of endogeneity in the insect *Drosophila* (21), long persisting oscillations under LL in a number of rhythmic
systems support its classification as endogenous circadian rhythms as well (29). In contrast to PAL malate dehydrogenase from several sources exhibits rhythmic behavior in DD but remains at a relatively constant low level in LL due to photoactivation of the enzyme (4, 6). Yet in most plants studied, PAL activity is significantly higher in light than darkness, probably due to both increased inactivation (31) and production of an inactive form of the enzyme in darkness (1). The results of our experiments strongly support circadian rhythmicity in the PAL and TAL activities of PAL from <i>L. perpusilla</i> and <i>S. polyrhiza</i> and exhibit the free running period of the oscillations between 22 and 25 hr (Table I).

Both a CO₂ output rhythm and a light sensitivity rhythm related to flowering have been established by Hillman (15) in <i>L. perpusilla</i> which differ from the PAL rhythm in the same plant. These rhythms persist in darkness after continuous illumination or entrainment to a 0.25-hr daily red light treatment. This difference with respect to overt rhythms in the same plant is quite unexpected and suggests that either a single but complex regulatory mechanism is involved or that there is more than one mechanism.

In spite of the widely accepted importance of PAL in plant growth and development (3) and the correlation of rhythmicity with growth and developmental phenomena (5), few studies have been undertaken to examine the oscillatory behavior of the enzyme. Thus, devising a mechanistic explanation for rhythmic activity of PAL presents considerable difficulty. A recent investigation (1) supports the hypothesis that <i>de novo</i> PAL synthesis takes place primarily during darkness. Therefore, differential rates of enzyme synthesis should not contribute to rhythmic behavior in light. Because there is no evidence of cofactor requirements which might influence activity, the more plausible explanation for rhythmic behavior is the influence of accumulations of by-products of PAL and other enzymes of secondary metabolism. Support for this idea is to be found in the studies of Engelsma (8) on gherkin seedlings. These studies demonstrate that there is a significant correlation between accumulations of hydroxycinnamic acids and peaks of PAL activity in the hypocotyls of gherkin. Engelsma postulated that if these hydroxycinnamates were transferred to other parts of the plant instead of being stored in the cells where they are synthesized, PAL would experience periods of product inhibition followed by declines of the same. This would result in oscillations of PAL activity. Either a single light period of sufficient duration or continuous illumination should provide more than one PAL activity maximum. Inasmuch as synthesis of the enzyme is favored in darkness, continuous illuminations should be characterized by rhythmic activity with progressive dampening after each cycle (Fig. 4) due to protein turnover.

The foregoing discussion does not take into account two important properties of the enzyme which must be considered in developing an appropriate mechanistic model. First, there is no explanation of the role of light in PAL rhythmicity. Because light is necessary for the activation of the enzyme and persistence of its rhythmicity then activation and rhythmicity must be considered manifestations or related light effects. Second, the explanation does not consider the possible involvement of a specific PAL inactivator has been recently...
reported (1). Each of these properties seems crucial in developing a model for PAL rhythmicity and underlines the need for further study to promote greater understanding of rhythmicity in PAL.

Acknowledgement The authors wish to thank R. Sothorn, Department of Laboratory Medicine and Pathology, Chronobiology Laboratories, University of Minnesota, Minneapolis, for his valuable assistance in the computer analysis of data.

LITERATURE CITED