Protein Synthesis during Endogenous Rhythmic Leaflet Movement in *Albizzia*

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ABSTRACT   A rhythm was found in protein synthesis accompanying rhythmic leaflet opening and closing in the dark in the plant *Albizzia*. More protein was synthesized during the opening of the leaflets than during the closing. Furthermore, an inhibitor of protein synthesis, cycloheximide, prevented rhythmic opening of leaflets but had no effect on rhythmic closing. It is suggested that protein synthesis is involved in the movement across membranes of K^+ ions that cause turgor changes and leaflet movement.

INTRODUCTION

Circadian rhythms have been found in both rates of total protein synthesis in *Euglena* (Feldman, 1968), rat liver (Sestan, 1964; Lebouton and Handler, 1970), and neurosecretory cells of a cricket (Cymborowski and Dutkowski, 1970), and synthesis or activity of specific proteins such as glycogen synthetase in rat liver (McVerry and Kim, 1972), adenyl cyclase in rat adrenals (Moore and Qari, 1971), phosphatase in cactus (Sanwal and Krishman, 1960), and luciferase in *Gonyaulax* (McMurry and Hastings, 1972). With the exception of the last study, these experiments were performed under alternating conditions of light and dark and were not therefore shown to be endogenous rhythms. Inhibitors of protein synthesis alter the amplitude of rhythm in bioluminescent glow in *Gonyaulax* (Karakashian and Hastings, 1963) and the period length of phototaxis in *Euglena* (Feldman, 1967), but not swimming activity in an isopod (Enright, 1971) nor photosynthesis in *Acetabularia* (Sweeney et al., 1967). As is often pointed out, protein synthesis rhythms are not necessarily a part of the circadian clock but more likely are either components of the behavior being measured or events controlled by the clock.

We undertook similar studies in the higher plant *Albizzia julibrissin*, whose anatomy, growth characteristics (Satter et al., 1970), and rhythm (Satter and Galston, 1971 *a* and *b*) of leaflet opening and closing in constant darkness have already been described.

MATERIALS AND METHODS

For all experiments, leaflet pairs were excised from intact plants and floated in 10 ml of distilled water in petri dishes. A leaflet pair consists of two opposite leaflets, each with its pulvinule (the motor organ at the base of the lamina with associated motor cells) attached to the rachilla. When the leaflets are separated by an angle of 180° they are considered fully open; 0° represents complete closure.

The plants were grown with 16-h light, 8-h dark cycles. In the rhythmic experiments, leaflets were excised from a plant in the dark part of its cycle and kept in darkness at 24°C for the experimental period. Under these conditions, leaflets continue to open and close for several days, with each phase lasting 11–12 h (Satter and Galston, 1971 b). To determine whether there are rhythmic oscillations in the rates of protein synthesis accompanying these leaflet movements, 20 μl of [^{3}H]leucine (specific activity 60 Ci/mmol, 2 μg/ml) was added to a petri dish during the closed (a.m.) phase on days 1, 2, and 3 and during the open (p.m.) phase on days 1 and 2. After 1 h of incubation, the leaflets were removed, washed in water to remove surface radioactivity, homogenized in 1 ml of distilled water in a Potter-Elvehjem grinder, and centrifuged (500 g) to remove the largest particles. A 100-μl aliquot of the supernatant homogenate was placed on a filter paper disk, dried for 5 min and then subjected to TCA extraction (Byfield and Scherbaum, 1966). Disks were run in duplicate and counted for 4 min each. To determine whether a rhythm in protein synthesis was related to a rhythm in uptake of leucine, other sets of disks were run with the TCA extraction step omitted. Radioactivity of these disks consisted of unmetabolized leucine, intermediate substances formed from leucine, plus leucine incorporated into protein. Counts on these disks, minus counts on the TCA-treated disks (protein) give an indication of relative precursor pool size, and hence of uptake of leucine.

Experiments were performed testing the effect of cycloheximide (CHI), an inhibitor of protein synthesis, on rhythmic movement during a long dark period. Leaflets were excised during the closed phase of the rhythm and floated on H$_2$O. In one experiment (rhythmic opening), they were transferred to CHI approximately 1 h before the beginning of the open phase; in another experiment (rhythmic closing), they remained on H$_2$O during the open phase and were transferred to CHI just as they were beginning to close. Leaflet angles were measured at intervals while the leaflets were exposed to CHI.

It has been shown for *Albizzia* that short red (R) and far red (FR) light treatments have differential effects upon rhythmic leaflet movement due to absorption by the protein pigment phytochrome (Satter and Galston, 1971 a). To determine if CHI altered the phytochrome effect on rhythms, half of the leaflets received R irradiation (660 nm, 1,200 ergs·cm^{-2}·s^{-1}, 3 min) and half received FR (720 nm, 9,000 ergs·cm^{-2}·s^{-1}, 1.5 min) (Satter et al., 1970) immediately after they were excised. Leaflets also close if moved from the light to the dark (nyctinastic closure) and this movement is also regulated by phytochrome. Nyctinastic closure is different from rhythmic closure and consequently allows the analysis of closure under different conditions. To determine whether CHI affected nyctinastic closure, leaflets were excised at the 2nd h of the photoperiod, floated on CHI or water, then exposed to R or FR followed by

darkness. Leaflet angles were measured at indicated intervals during the next few hours. Protein synthesis during nyctinastic closure was also investigated. Leaflets were exposed to R or FR light followed by darkness as in the previous experiments, and a control group remained in the light. 20 μl of [^{3}H]leucine was added to each petri dish for 1 h of incubation, and proteins were extracted as described above. The sample size was 5–7 petri dishes, 10 leaflets pairs per dish, for each treatment in each of the above experiments.

RESULTS

The results of the protein synthesis rhythm experiment are presented in Fig. 1. A rhythm in protein synthesis does exist as there is significantly less synthesis
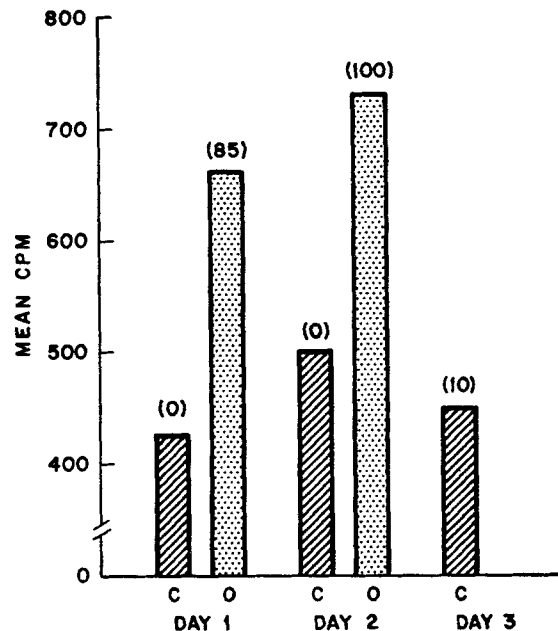

FIGURE 1.   Rhythm in rate of protein synthesis. Mean number of counts per minute, background subtracted, of radioactive leucine (1 h incubation time) incorporated into protein for closed (C) versus open (O) state. Mean leaflet angles (degrees) in parenthesis.

during the closed than during the open phase ($P < 0.01$, two-tailed Wilcoxon test). Values during the open phase are not significantly different from each other ($P > 0.1$) nor are the values during the closed phase ($P > 0.1$) by the same test. The mean of the total radioactivity for the closed state is 639 (457 cpm protein plus 182 pool) and for the open, 753 (695 protein plus 58 pool). Thus the ratio of protein:pool is 2.5 during the closed state and 12 during the open phase. The increased rate of protein synthesis in the open phase may be due in part to increased uptake of leucine since the total counts for open and closed are unequal.

The state of phytochrome (R versus FR) does not affect the rate of protein synthesis during nyctinastic closure, since there was no significant difference in [³H]leucine incorporation into protein in R-treated (112 cpm) compared to FR-treated (119 cpm) leaflets. However control leaflets in the light incorporated almost twice as much (216 cpm) leucine in the same interval.

CHI (10⁻⁴ M) inhibited protein synthesis 95% as indicated by a comparison of counts per minute after TCA extraction of leaflets exposed to CHI with water controls. The same rate of protein synthesis inhibition was found in the pulvini alone, devoid of rachillae and laminae. This same concentration prevented rhythmic opening; both R- and FR-irradiated, CHI-treated leaflets failed to open during 3 h incubation (mean angle 5°) compared to 100° for the FR and 50° for R water controls. CHI was not toxic since treated leaflets that were washed and placed in water for 27 h had nearly the same angles (105°) as water controls (95°). Threshold effects on protein synthesis and rhythmic opening were noted when the CHI concentration was near 10⁻⁶ M. By contrast, CHI has no effect upon rhythmic closure as Fig. 2 demonstrates ($P > 0.2$, Wilcoxon). Fig. 3 shows that CHI promotes nyctinastic closure, both CHI curves being lower than the water controls ($P < 0.01$ Wilcoxon), and eliminates the differential effect of preirradiation with R and FR. Because of the different effect of CHI on rhythmic and nyctinastic closure, the con-
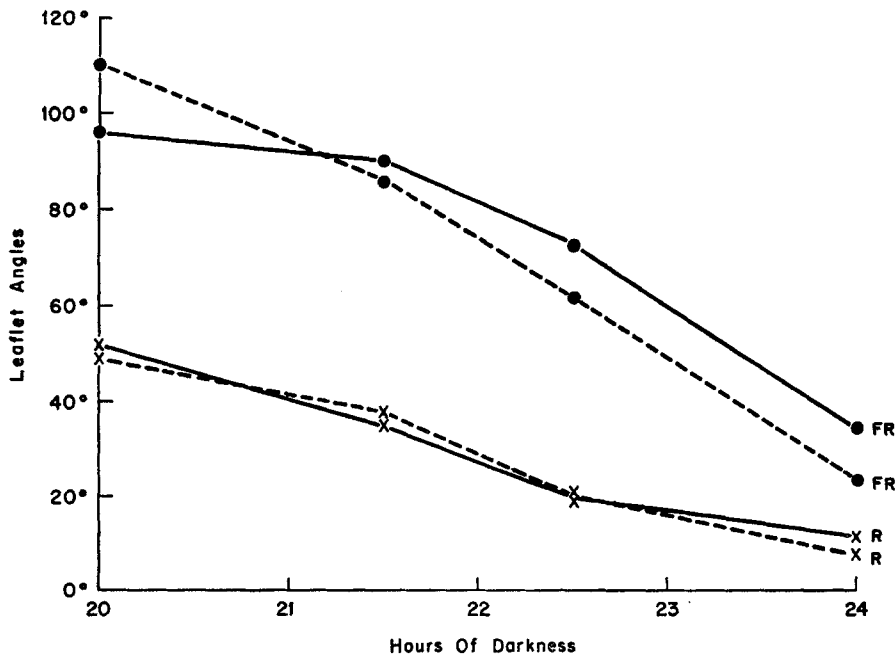

FIGURE 2.   CHI and rhythmic closure. Mean leaflet angles versus time for R (×) and FR (●) groups, CHI 10⁻⁴ M, incubated for 4 h, (---) or water controls (—).
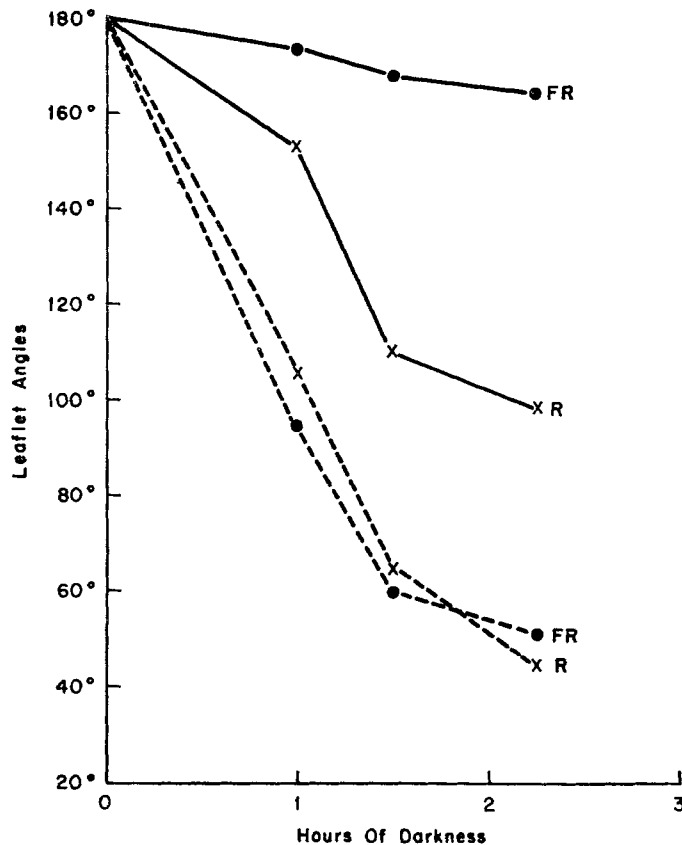
FIGURE 3. CHI and nyctinastic closure. Mean leaflet angles versus time for R (X) and FR (●) groups, CHI 10^{-4} M, incubated for 2.5 h, (---) or water controls (—).

clusion is that they are different processes. Actinomycin D (10^{-3} M), chloramphenicol (10^{-2} M), and puromycin (10^{-2} M) had no effect on leaflet movement but we do not know if uptake occurred for these inhibitors.

DISCUSSION

The rhythmic protein synthesis and cycloheximide studies on rhythmic movement complement each other; there is more protein synthesis in the open than the closed phase and an inhibitor of protein synthesis prevents rhythmic opening but has no effect upon rhythmic closing. Our results support a model proposed to describe this rhythm in *Albizzia* (Satter and Galston, 1973). The model is based on studies showing that leaflets move in response to changes in the turgidity of motor cells on dorsal and ventral sides of the pulvinus, with turgor changes resulting from K^+ flux through motor cell membranes followed by water movement in the direction of K^+ flow. The rhythmic opening phase is temperature sensitive (Satter and Galston, 1973; and Satter et al., 1973),

subject to inhibition by uncouplers of oxidative phosphorylation, suggestive of active transport, and dependent upon the flow of K^+ ions into ventral and out of dorsal motor cells. The higher rate of protein synthesis described here may be necessary to maintain membrane active transport and other energy-requiring processes. In rhythmic closing, K^+ ions move in the opposite direction (into dorsal and out of ventral motor cells) by an energy and temperature independent process, diffusion. The insensitivity of rhythmic closing to CHI is consistent with a nonenergy-requiring process and the hypothesized loss of membrane integrity (resulting in diffusion) in this phase. The model further proposes a combination of active and passive transport of K^+ through motor cell membranes when leaflets are transferred from light to darkness (nyctinastic closure). If protein synthesis is necessary for the active transport part as suggested above, then the promotive effect of CHI on nyctinastic closure would be explained. Protein synthesis in *Albizzia* is endogenously rhythmic and is probably required for rhythmic leaflet movement and K^+ flux. This suggests protein synthesis may precede permeability changes and ion flux in the chain of events controlled by the biological clock. While it is clear that protein synthesis is important for leaflet movement we cannot claim it is a property of the circadian clock. It has been shown for some plants that chloramphenicol prevents the uptake from solution of certain ions, suggesting active transport may be linked to membrane protein synthesis (Sutcliffe, 1962). Investigation of membrane properties during different stages of the rhythm is a logical next step.

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