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A Practical Approach to Study Circadian Biochemical Rhythms in Animals

KEYWORDS: Circadian biochemical rhythms, Circadian clocks, Free running period, Release cycle, Synthetic cycle.

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ABSTRACT *The biochemical events of organisms manifest in the form of endogenous and self-sustaining cyclic changes called circadian biochemical rhythms. Like other circadian rhythms, they oscillate on a 24 hr scale under the influence of light and are monitored by endogenous biological clocks. Though, the circadian biology has been extensively studied with reference to its molecular and genetic bases, no effort has since been made to trace their biochemical and physiological reflections. Such correlative studies are expected to open new vistas for research in chronobiology. Keeping in view the emerging practical impediments, a pragmatic analytical approach has been suggested for their study. The approach is based on the analysis of phase response curve of the rhythm, in terms of peaks and troughs and intervals between them. It is expected to provide meaningful insights into circadian biochemical data in terms of synthetic and release cycles of biochemical constituents that could establish correct genotype-phenotype correlations.*

INTRODUCTION

Ever since the origin of life, the cyclic nature has become an essential feature of all living organisms; from microbes to protists, from amoeba to man and from algae to angiosperms. The cyclic nature is reflected in their actions and activities in the form of circannual, circalunar and circadian rhythms under the influence of light. The circadian rhythms (CRs), which express on 24 hr scale (Latin: Circa = about; dien = day) are of special interest for biologists as they modulate all molecular, behavioural and physiological events of organisms by synchronizing them with the external environment (Lamont and Amir, 2010; Sivaprasad, 2014). The daily recurring behavioural and physiological activities of animals such as eating, sleeping, mating, hibernation, migration, regeneration, digestion, blood pressure, temperature, rate of cell division, blood cell count, alertness, urine composition, metabolic rate, sex drive etc continue to oscillate in a circadian fashion (Sharma, 2003; Campbell and Reece, 2005). Strictly monitored by self-sustaining endogenous devices called circadian clocks (or pacemakers) such activities are reflected as measurable parameters; detectable internally as changes in the levels of biochemical constituents and externally as observable behavioural responses Of late the studies on chronobiology have assumed significance in biological research. Investigations on circadian biology have been taken-up more extensively than ever before. Broadly, the research focussed on elucidating the molecular and genetic bases of circadian rhythms in a variety of organisms such as Drosophila, Bombyx mori (Sauman and Reppert,1996; Sehadova et al., 2004; Williams and Sehgal, 2001; Hall, 2003; Reppert, 2006), Zebrafish (Pando and Sassone-Corsi, 2002) and mouse (Reppert and Weaver, 2000, 2001). However, no significant work has been done with reference to the biochemical and physiological reflections of CRs, except for some preliminary studies on Bombyx mori from our laboratory (Sailaja and Sivaprasad, 2010a, 2010b, 2011; Sailaja et al., 2011; Sivaprasad and Sailaja, 2011; Bhuvanewari and Sivaprasad, 2012a, 2012b, 2013; Bhuvanewari et al., 2013a, 2013b). It is pertinent that the molecular and genetic mechanisms underlying the circadian biology need correlates from animal biochemistry and physiology. Such studies are expected to open new vistas for further research in the emerging field of chronobiology. We experienced impediments while analyzing the circadian data on various biochemical parameters due the lack of sound practical approach. Essentially, a pragmatic analytical approach that could provide insights into circadian biochemical data is necessary not only for drawing meaningful conclusions but also for establishing genotype-phenotype correlations in all such studies. The methodological approach suggested in the present paper intends to achieve this objective.

EXPERIMENTAL DESIGN

1. Selection of test species and its maintenance: The selection of an appropriate animal model is the foremost step in all circadian biochemical studies. Keeping in view the past literature, a mammal (eg. mouse, squirrel etc) or an insect (Drosophila, Bombyx mori etc) could be selected as the test species. An ideal test species is one which should have emanated from the same age group and litter and should have same body weight. Since, the light acts as the principal zeitgeber (time giver) and plays pivotal role in entraining the circadian clock mechanism, the test species should be allowed to grow under different photoperiodic conditions, viz., 12 h light and 12 h dark cycle (LD), continuous light (LL) continuous dark (DD), 6 h light and 18 h dark (6 L: 18 D) and 18 h light and 6 hr dark (18 L: 6 D) simultaneously in different experimental batches. The test species should be maintained in the desired light condition at least one week prior to the commencement of the experiment so as to enable it to acclimatize to altered photoperiods. Needless to say that the other environmental conditions (eg. temperature, diet, relative humidity, barometric pressure etc) should be invariably maintained at constant levels throughout the study in order to check their interference with the rhythm. Similarly, the role of other environmental factors could be similarly examined by altering one factor at a time, while keeping the others constant.

2. Methodology: Appropriate and well planned experimental design is indispensible for any investigation, which obviously includes the selection of tissues and suitable methods for the assay of various biochemical constituents. While selecting tissues, care should be taken to choose most active tissues and the circulating fluid. For instance, in case of mammal tissues such as the brain, liver, muscle, blood could be selected, while in insects tissues such as the fat body, central nervous system, muscle and haemolymph could be selected. The desired tissues should be isolated by dissecting the animal in respective Ringers. The levels of biochemical constituents in tissues should be estimated by preparing standardised tissue homogenates in the suitable media. The following methods may be used in biochemical assays.

- Proteins (total, soluble and structural): Lowry et al, 1951.
- Protease activity: Davis and Smith, 1955,
- Free Amino Acid levels: Moore and Stein,1954.

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- Glutamate Dehydrogenase activity: Lee and Lardy, 1965.
- Total carbohydrates: Carroll et al, 1956.
- Alpha amylase activity: Bernfeld, 1955.
- Trehalose levels: Roe, 1955.
- Trehalase Activity: Dahlman, 1971.
- Sucrose levels: Plumer, 1978.
- Sucrase Activity: Ishaaya Swirski, 1970.
- Cellulose levels: Updegroff, 1969.
- Cellulase Activity: Miller,1959.

Circadian rhythmicity in the profiles of biochemical constituents should be monitored every hour during the period of 24 hrs. Choosing appropriate time schedule of 25 hrs starting from 6 AM on day-1 to 6 A.M on day-2 would be ideal. During this period, tissues should be isolated on hour-to-hour basis and stored in a deep freezer at -20 to -40^{0}C. Next couple of days could be ideally used for carrying out biochemical assays on the tissues, so stored. Thus, the experiment would continue for two consecutive days (from 6 A.M on day-1 to 6 A.M on day-2) and involves 3 to 5 batches of animals reared separately under different photoperiodic conditions, viz., 12: 12 hours of light and dark cycle (LD), continuous light (LL), continuous dark (DD), 18L: 6D or 6L:18D etc. The batch of animals reared under LD should be treated as the control and those reared under other light and dark conditions as the experimental samples.

3. Data analysis and interpretation: The data pertaining to circadian biochemical rhythms should be analysed using appropriate statistical tools such as the percentages, standard deviation, t-test and ANOVA etc. The biochemical rhythm recorded during the period of 24 hrs is designated the free running period or tau. This is represented graphically in the form of phase response curves (PRC) that show characteristic peaks (elevated points) and troughs (low points or depressions) as sown in the figure 1(which represents the circadian protein rhythm in the gut wall of the silkworm). Proper understanding and interpretation of the PRC of the circadian biochemical rhythm is indispensible for meaningful interpretations. One important clue is that the peaks in the PRC may be treated as active synthetic / uptake phases during which, a particular biochemical constituent is either synthesized de novo or taken-up from the surrounding fluid compartments (blood/ haemolymph/digestive juice etc.) and troughs as the low synthetic / up-take phases of biochemical constituents under study. For instance, the peaks in the PRC of the protein rhythm denote two things; first, higher levels of proteins / enzyme activities (Eg. protease, AAT, AlAT, GDH, SDH, trehalase, sucrase, amylase, cellulase) and second, a higher metabolic rate of the tissue concerned. Likewise, the troughs in such PRCs indicate low protein turnover and slow rate of protein/enzyme synthesis. The raising phase of the curve represents the corresponding raising phase of protein / enzyme synthesis and the falling phase the declining rate in protein / enzyme synthesis (Fig.2).

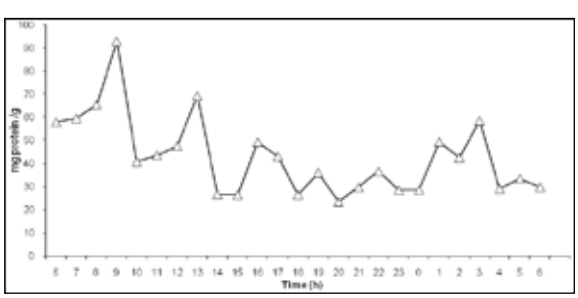

Fig. 1: Phase response curves (PRC) of the 24-hr- circadian protein rhythm (from 6AM on day 5 to 6 AM on day 6) in the larval gut wall of Bombyx mori during fifth instar development, under continuous (24 hr) light condition: Note characteristic higher peaks at 9.00 hr, 13.00 h, 16 hr and at 3.00 hr, and troughs at 10.00 hr, 14-24 hr in the curve.

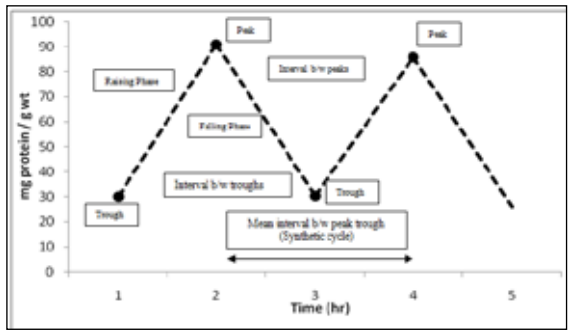

Fig. 2: Analysis and interpretation of PRC in terms of synthetic / release cycles of biochemical constituents. In this case the peaks represent higher synthetic/release rate and troughd low synthetic/release rate. The mean interval between peaks and troughs indicates the duration of synthetic/ release cycle of a biochemical constituent.

Given this fact, the timing of peaks and troughs denote two vital stages of gene expression, i.e., the translation (protein synthesis) and transcription (mRNA synthesis) respectively (Sailaja and Sivaprasad, 2010a, b). In this process, the mean interval between troughs is indicative of the timing of the translation process, during which the protein levels peak to heights, while the intervals between peaks represent the timing of transcription during which the cells prepare for the next phase of translation. The combined mean interval between peaks and troughs is thus viewed as the time required for one protein synthetic cycle. Similar interpretations could be made in respect of other biochemical constituents such as total carbohydrates, total lipids, free amino acids, glycogen, glucose, trehalose, cellulose sucrose etc, and the timing of their formation and utilisation could be inferred from the peaks and troughs respectively. Needless to say, the peaks in their levels indicate their active synthetic phases and troughs their low synthetic phases. However, in fluid tissues like the haemolymph of insects and blood of vertebrates and in their digestive juices, the PRC may be interpreted in terms of release cycles of biochemical constituents.

The proposed approach is a tentative model and requires further experimental validation. Nevertheless, it invites attention of the young researchers towards circadian biochemical studies.

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