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Investigative Methodology for Chronobiology

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Progress in the developing science of chronobiology has closely paralleled the emergence of new and improved research methodologies. Methodological requirements for biological rhythm study have been reviewed previously (Halberg et al. 1972, 1977; Reinberg 1971, 1974; Smolensky et al. 1974). This chapter outlines a minimum set of conditions and procedures necessary for conducting sound chronobiologic investigations. The recommendations which are put forward should be regarded as proposals and/or suggestions rather than rules or criteria for judging the quality of experimentation. It is obvious that each study dictates a specific methodology depending upon, among other things, the goals of the investigation and the state of knowledge. The contents of this chapter provide necessary information for designing chronobiologic research protocols and for minimizing the occurrence of those types of mistakes typically experienced in earlier chronobiologic investigations.

Types of Synchronizers and the Synchronization of Biological Rhythms

Common to all biological research, particulars related to species, sex, age, weight, height, food intake, state of health or disease, etc., must be stated. These general requirements are mentioned here as a reminder, only, despite the fact they are critical.

With respect to chronobiologic methods, when experimenting on laboratory animals, it is mandatory that the timing of the natural or artificial light (L)–dark (D) cycle be monitored, recorded, and reported since the LD cycle is recognized as a primary synchronizer of circadian and possibly other rhythms. The LD cycle influences the period length and peak time of rhythms of many species, such as birds, rodents, and monkeys.* Other potential synchronizers,

* The term synchronizer refers to an environmental periodicity capable of determining the temporal staging, with respect to clock hour or calendar date, of a given endogenous rhythmicity.

such as cyclic changes in temperature, noise, odors, humidity, and food availability, should be maintained at more or less constant levels. In so doing, only *one* (known) synchronizer is operational, whether or not it is manipulated. The concurrent influence of several synchronizers may lead to a complex situation which may be difficult to analyze. Even with one synchronizer, such as the LD alternation, the duration, intensity, and quality (wavelength) of the light as well as the abruptness of change from L to D can influence the findings (Aschoff 1960; Boissin and Assenmacher 1971; Halberg et al. 1959). Therefore each must be well defined.

Many authors prefer using the LD:12/12 lighting regimen in which 12 hr of light alternate with 12 hr of darkness.* With regard to animal models involving typically nocturnally active rodents (rats and mice), some authors (Halberg 1973; von Mayersbach 1978) recommend a LD:8/16 schedule, since it seems to better simulate human synchronization, i.e., 16 hr of activity alternating with 8 hr of rest.

Selection of appropriate LD schedules constitutes one of the most fundamentally important steps in conducting animal research, whether or not one is involved in chronobiologic studies, for many reasons as discussed below. Some experimenters are unaware of, or choose to ignore, the significance of the LD schedule as a synchronizer of animal rhythms. Oftentimes, animals are maintained under constant illumination. The assumption made in housing rodents and other species under such conditions is that the absence of alternating LD cyclicity attenuates or obliterates biological rhythmicity. Some investigators, even if a LD schedule is provided, attempt to "control" for rhythm effects by restricting research procedures to one or two particular clock hours. The experimenter by adhering to this type of sampling schedule does a disservice to his research and to science for several reasons. With respect to research on rodents, a vast number of chronobiologic investigations conducted during the past two to three decades utilizing comparable LD synchronizer schedules (usually LD:12/12) have enabled the "mapping" of a multitude of circadian rhythmicities. The term mapping refers to aspects of the rhythm's form over time, for example, the peak and trough times, with respect to the stated LD schedule. When the LD schedule is known, it is possible to predict rather precisely when either high, low, or average levels of a constituent of tissue, blood, urine, etc., will occur. Thus, the maximum of the circadian rhythm in serum corticosterone (the major adrenal corticosteroid hormone in mice) is expected to coincide with the timing of the transition of light to darkness in the animal room. It is also predictable that reduced levels of corticosterone will occur approximately 12 hr earlier or later, around the transition of darkness to light (Fig. 1) (Smolensky et al. 1978). In other words, different—early, middle, or late—stages of the light or dark span are coincident with particular features, such as the peak values (also referred to as the acrophase, discussed later in this chapter) of various circadian rhythmicities (Fig. 2). Relating the LD schedule to local time enables the determination of the clock hour of the aforementioned rhythmic features, since animals synchronized to fixed LD cycles display periodicities of almost precisely 24.0 hr. Under such highly standardized research conditions, clock hour thus is representative of circadian stage.

When animals are housed under constant light or darkness, i.e., without a LD synchronizer, the phenomenon of "free-running" often results—the occurrence of differing non-24-hr circadian cycles which vary in periodicity (τ) between biological functions in the same animal and/or be-

* Chronobiologists rely upon certain abbreviations to convey pertinent information about synchronizer type and schedule. LD:12/12 designates the synchronizer to be the Light (L)-dark (D) cycle with L and D each having a 12-hr duration. LD:8/16 conveys that the synchronizer is the light–dark cycle with the duration of the former being 8 hr and the latter 16 hr. Time or clock hour is expressed using the international designation, e.g., 1:00 P.M. is referred to as 1300.

tween animals for the same function. Under free-running conditions, it is possible, for example, that a given variable in one rodent might exhibit a 23.8-hr rhythm, while in a litter mate in another cage in the same animal room it may exhibit a 23.4-hr rhythm (Apfelbaum et al. 1969). The existence of free-running rhythms in the absence of synchronizers results in the clock hour being predictive of neither a particular circadian phase in one nor an identical circadian phase in all of the animals in the colony. For example, for the 0.4-hr difference in period duration for the theoretical condition raised above (one animal had a period of 23.8 hr and the other 23.4 hr), there would exist after 10 days a 4-hr (0.4 hr/day × 10 day) difference in phasing of this rhythmic function between the two animals. One can envision a large colony of animals, hundreds for example, under constant light or darkness with each animal being desynchronized at least to some extent with respect to the others. Under such experimental conditions, a clock hour would not correspond to or predict a given circadian stage in any one animal, nor in the colony as a whole (Scheving et al. 1977).

Research restricted only to certain clock hours in an assumed attempt either to "take into account" or "control for" rhythms should also be considered. Even when the animal colony is LD synchronized, the quantity and quality of data obtained are likely to be compromised when single-time-point samplings are done, since the responsiveness of animals is typically circadian-stage-dependent. For example, when conducting bioassays for potency, the injection of methylprednisolone (MP), a synthetic corticosteroid widely used to treat various human inflammatory disorders, is expected to produce a dose-dependent increase in liver glycogen deposition, as has been found so often in single-time-point experiments with this and many other synthetic corticosteroids. Yet as Fig. 3 shows, in singly caged, 5-week-old male Balb-C mice housed with food and water available *ad libitum*, ambient conditions of

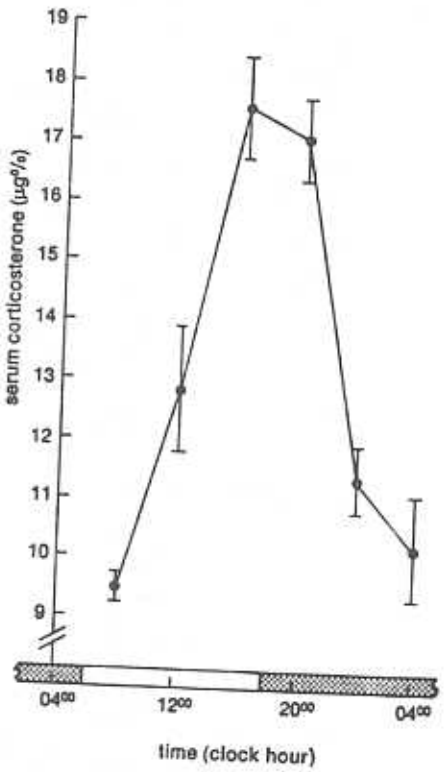

Fig. 1. Circadian rhythm of serum corticosterone in more than 240 ~12-week-old male Balb-C mice (40–44 mice killed at each of the 6 indicated time points over the 24-hr scale). For at least 2 weeks prior to investigation, animals were standardized for chronobiologic study of this adrenocortical rhythm by housing 1 per cage with food and water available *ad libitum*, in rooms constructed of sound-retarding materials with temperature 23° ± 1°C, humidity ≡ 50%, and light (L) from 0600–1800 alternating with darkness (D) from 1800–0600. The peak in adrenal corticosterone occurs between 1600 and 2000, or around the transition from inactivity to activity and as the environmental conditions change from light to dark. (From Smolensky et al. 1978.)

L(0600–1800):D(1800–0600), and constant temperature and relative humidity, the response to MP varies according to the circadian stage of treatment. Intraperitoneal injections of MP—4 mg daily or 8 mg on alternate days per 20 g body weight—only at 1600 (around the validated crest of the

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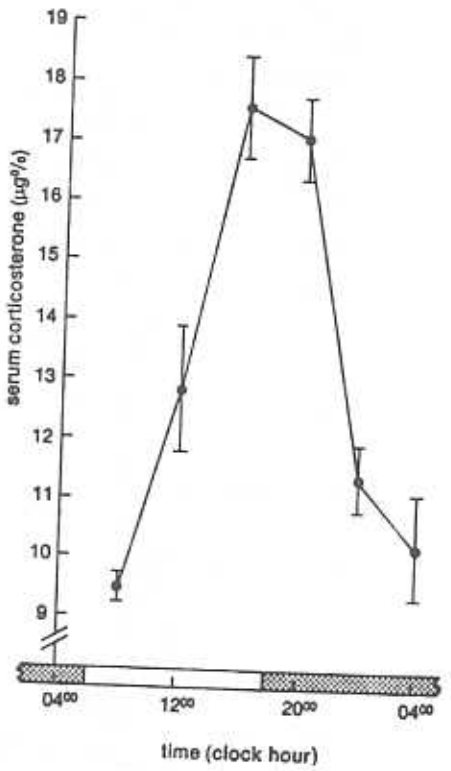

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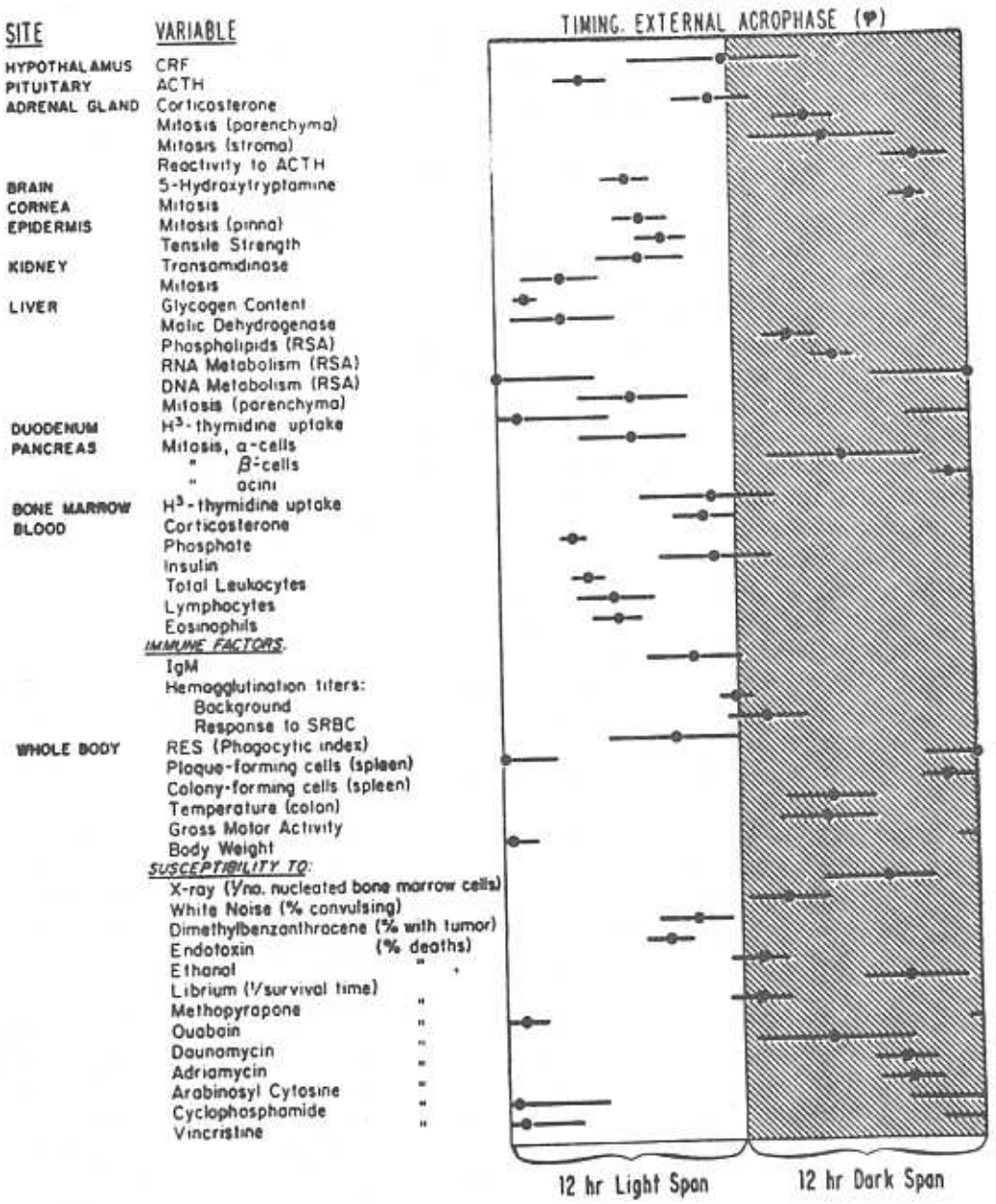


Fig. 2. The circadian temporal structure of the mouse is shown by a so-called acrophase map—a display by graphic means of the circadian acrophase (designated by φ for a phase reference corresponding to midlight), the time of the crest, of different biological functions. The acrophase, *black dot*, and the 95% confidence limits, *lateral extensions*, depict the timing of highest expected values for the designated function. By knowing the LD schedule, one can determine with confidence the biological time structure at a given clock hour of sampling or experimentation. (From Halberg and Nelson 1978, reproduced with permission.)

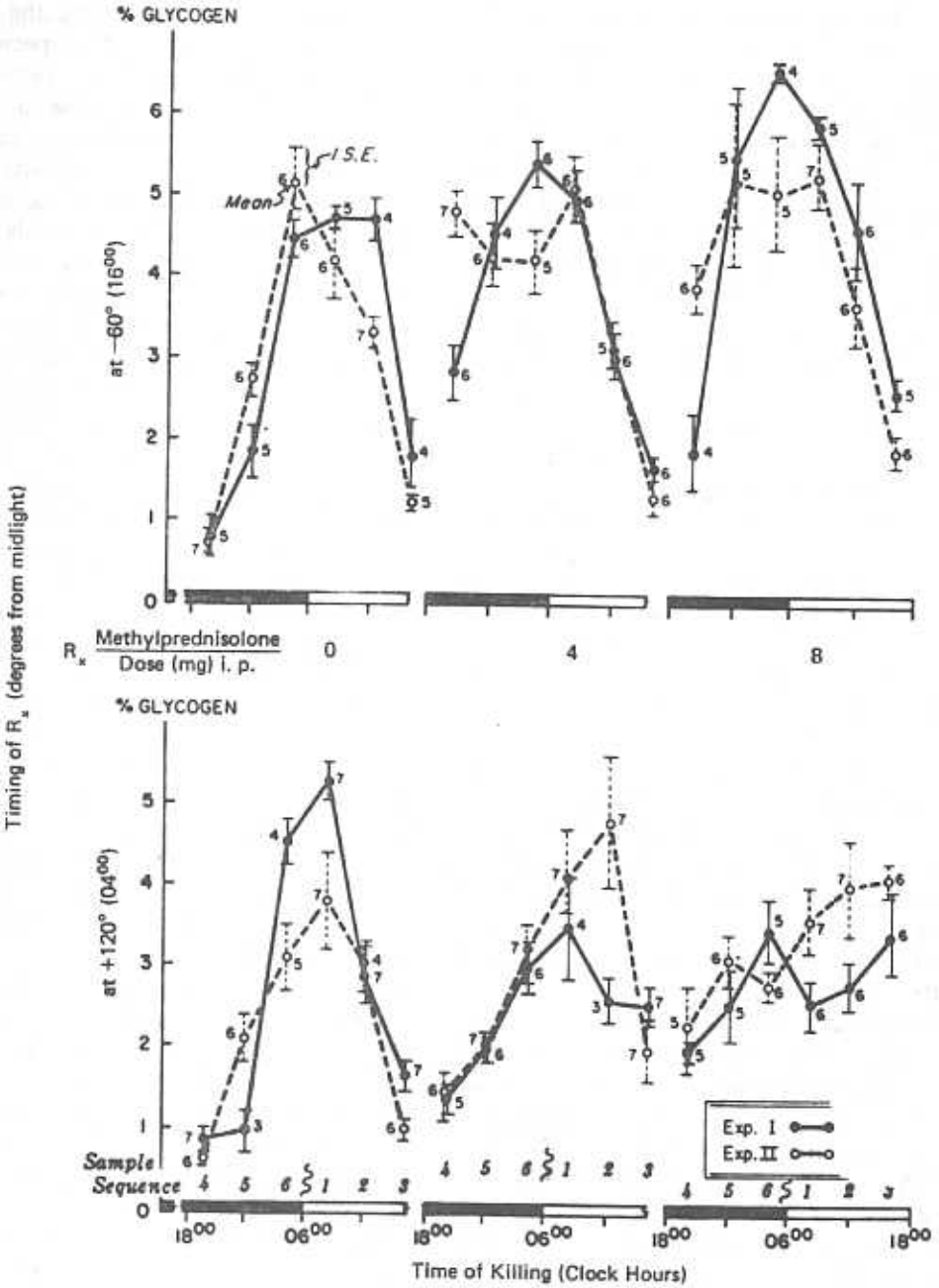


Fig. 3. Temporal changes in liver glycogen based on data from 2 experiments of 7 and 8 days duration, respectively. For groups of mice given the vehicle for MP, greatest glycogen content occurred during the commencement of the diurnal span of inactivity, around 0400–0800. In general, MP given daily or on alternate days (AD) did not appreciably alter the waveform of this rhythm except for the "1600–4 mg" daily and "0400–8 mg" AD groups of Experiment II. For mice given MP at 1600, glycogen content increased with dosage Although the "0400–8 mg" glycogen content increased over control values, the change was very slight and less than that observed for groups administered MP at 1600 on alternate days. (From Smolensky et al. 1980a.)

circadian rhythm of serum corticosterone in rodents kept under the aforementioned synchronization) for either 7 or 8 days produces the expected increased liver glycogen deposition relative to vehicle-injected (0 mg MP) controls. Litter mates of these same mice injected with MP 12 hr earlier, at 0400, fail to respond with a statistically significant elevation in liver glycogen in comparison to vehicle-injected controls. Obviously, the circadian (biological) timing of MP has an effect on the bioassay. The extent to which failure to utilize appropriate chronobiologic research protocols, including animal synchronization and multiple-time-point samplings, has led to incorrect characterizations of potentially useful pharmacologic agents in the past and present is unknown. Similarly, the extent to which failure to use chronobiologic methods had led to different findings among alledgedly competent investigators working in the same or different laboratories because of an inadvertent selection of different circadian stages for experimentation, even in standardized animals, also is not known.

With respect to methodological procedures for chronobiologic research on rodents, it is pertinent to discuss here some of the ramifications of the all too common practice of conducting studies on mice or rats only during the light span of the LD synchronizer schedule. Quite often the investigator finds it least troublesome to conduct research during the daytime, when the laboratory is well lighted. Even though work during the light instead of the dark span is more convenient, let us point out that rodents, being nocturnally active animals, are physiologically at rest when studied during their light span. *A priori* it seems illogical to predict human responses to different agents—chemical, bacterial, or physical—utilizing data obtained from animals awakened from rest rather than utilizing data obtained from animals during their usual activity span, since human exposure to potentially hazardous materials is most likely during the span of activity. Actually, data from both the rest and activity spans are required to best describe the overall susceptibility/resistance of a species to a given agent. To facilitate the gathering of data from rodents, it is possible to alter the LD schedule of an animal colony in such a manner that the rodents' activity (dark) span coincides with the experimenter's activity span. By carefully manipulating the LD synchronizer schedule in the animal colony rooms so that in some it is opposite that of the ambient surroundings, animals removed from the different colonies at the same clock hour will differ in circadian staging. This type of experimental methodology enables the study of biological rhythms during the normal diurnal activity span of the researcher. However, it should be recognized that a sufficiently long standardization span (at least 2 to 3 weeks) is required for the animals' biological rhythms to become synchronized to the altered LD schedule (see below).

Also related to laboratory animal synchronization is the important problem of biological samplings and data collections during the experimenter's usual rest span, i.e., when sampling cannot be automated or involves the removal of organs, tissues, or blood or necessitates carrying out manmade measurements. To better standardize an experiment as well as to avoid night work, it has been proposed that two to six isolation facilities be utilized in which comparable subsets of animals, usually rats and mice, can be maintained under the same fixed duration of light and dark. In each of the isolation facilities all conditions are maintained identically except for the particular clock hours of light-on and light-off, which varies between the isolation facilities. With this methodology diurnal samplings during the usual working hours of the laboratory personnel are sufficient to evaluate responses at several different circadian stages. (This is the methodology used in the chronobiology laboratories in L'Aquila, Italy, and Little Rock, Arkansas, among other places.) Thus, one can detect and characterize circadian rhythms, at least for chronopharmacologic studies. However, it

is necessary that a set of control experiments be performed to demonstrate that the animals are actually synchronized to each one of the programed LD cycles. Some circadian rhythms—for example, body core temperature and locomotor activity—can be adjusted to a new schedule rather rapidly, in about 1 week; others—for example, the mitotic rhythm of the digestive tract (Scheving, Chap. 4)—require more time and may not be completely synchronized to the new LD schedule even 1 month following the LD change. This explains why many chronobiologists still prefer experimental protocols involving data gathering at equal intervals, every 2 or 4 hr around the clock, even if work must be done at night and sleep foregone.

Research on human beings in both the laboratory and field requires special attention to the subjects' synchronization. The suppression of known synchronizers, as demonstrated by isolation experiments conducted in caves where time clues and cues are absent, raises a set of practical considerations which sometimes are not fully understood (Apfelbaum et al. 1969; Halberg 1973; Wever 1979). Subtle synchronizers such as changes in the magnetic field (Wever 1979) as well as other possible influences, such as cosmic radiation, must be taken into account. As suggested by Brown (1965), the influence of the lunar day with a period of 24.8 hr must be examined when biological rhythms of such periods are detected in so-called free-running experiments (Apfelbaum et al. 1969).

In most research involving human subjects, socioecologic synchronizers are present even if they vary, for example, because of shift work or rapid travel across several time zones by transmeridian flight. Circadian changes in the clock hour of the sleep–wake schedule reveal the timing of the rest and activity cycle to be the most powerful synchronizer for man (Apfelbaum et al. 1969; Aschoff et al. 1971; Halberg et al. 1959). However, several components of man's socioecologic niche vary over 24 hr: light and darkness, heat and cold, noise and silence, and changes in psychological affect and stimulation resulting from social constraints related to work, activity, and interhuman relationships (Czeisler et al. 1981). Under certain circumstances these may have direct or indirect synchronizer action. Nonetheless, under usual situations, it seems that the timing of environmental factors associated with sleep and activity is the primary synchronizer of human circadian rhythms. Thus, in human research it is critical to ascertain and report the respective mean duration and timing of sleep and wakefulness or, at least, rest and activity during each 24 hr for either an individual or group under study. This information should be stated in terms of the clock hour of light-on and light-off with appropriate data from self-maintained diaries confirming the regularity and duration in days, weeks, and months of adherence to the given schedule.

Strict attention to synchronizer schedule is stressed for good reason. In chronobiologic investigations the timing, with regard to clock hour, of the synchronizer determines when the rhythm's crest, termed the acrophase, will occur. To locate the acrophase, ϕ, of a given circadian rhythm, for example, the phase reference, ϕ_0, must be known. The ϕ_0 may be midnight (0000) or another local time; both of these are obviously associated with the information related to light-on/light-off. Halberg and Simpson (1967) proposed that the midsleep (or midrest) span be used as the ϕ_0. When using this ϕ_0, comparison of acrophase values is possible between various populations adhering to different activity–rest routines or between those living at different geographic locations as well as between males and females, etc. Reference to the midrest span for ϕ_0 in animal experiments is recommended rather than midnight, since the former represents an index of the organism's internal time as synchronized by the imposed LD schedule. In certain experiments, the ϕ of one biological rhythm constitutes the best reference (ϕ_0) for the acrophase of another rhythmic function

studied concomitantly. This is the case, for example, in the chronoradiotherapy of solid tumors of the oral cavity investigated by Gupta and Deka (1972). X-ray therapy appears to be more rapidly efficient when given in phase with the ϕ of the tumor temperature. It is, therefore, of interest to consider this latter ϕ as the ϕ_0 for future research with this type of chronotherapy.

Besides synchronizer schedule, two additional pieces of information—*geographic location* and *time of year* (even if the research involves only circadian rhythms) are of concern. With regard to the latter, research on mice (Haus and Halberg 1970), rats (von Mayersbach 1978), frogs (Dupont et al. 1979), and man (Reinberg 1974; Reinberg et al. 1975, 1978) reveals circannual alterations of parameters characterizing a set of circadian rhythms, such as the 24-hr time series mean M (referred to as the mesor), amplitude A, and acrophase ϕ. Not only can the 24-hr M vary as a function of the time of the year, but so can the ϕ with reference to clock hour on the 24-hr scale. Usually, but not necessarily, small variation in the circadian M, A, and ϕ may result because of differences between the time of year experiments are conducted. Even the ability to detect circadian rhythms may vary according to the time of year the research is done. Certain rhythms may be undetectable during certain months, whereas they may be easily detectable and found to be statistically highly significant (with a large amplitude) during other months (Dupont et al. 1979; Reinberg et al. 1975). The finding that the M, A, and ϕ of circadian rhythms may be modulated over the year is not totally unexpected since rhythms of approximately one year represent an important facet of one's biological time structure. Similarly for women and female rodents, as well as perhaps females of other species, there appears to exist a circamensual (about monthly) or circaestrual modulation of circadian rhythm characteristics—M, A, and ϕ (McGovern et al. 1977; Procacci et al. 1972; Simpson and Halberg 1974; Simpson and Bohlen 1973; Smolensky et al. 1974). The other variable, geographic location, implicitly conveys certain types of information, such as the extent of change and timing of annual synchronizers that differ as a function of latitude (Aschoff 1981; Batchelet et al. 1973; Ghata et al. 1977). Yet geographic location appears to have greater significance than that directly ascribed to latitude, alone, since it is closely related to the social and eating habits, type of population, and way of life of the studied sample (DuRuisseau 1965; Ghata et al. 1977; Reinberg et al. 1975). With respect to geographic location, certain chronobiologic questions remain to be answered. For example, what is the nature of circannual synchronizers and does adjustment of circannual rhythms occur when moving from the Northern to the Southern Hemisphere, or in the reverse direction (Halberg et al, 1983)?

Chronobiologic Studies of Individuals

Many of the published findings and analyses from chronobiologic research deal with data from groups of subjects. For the most part, little attention has been given to the existence of interindividual variations in the rhythm characteristics between participants. Thus, although the definition of chronobiology is the elucidation of *organismic* temporal structure based upon the study of individuals, surprisingly few investigations pertain specifically to individual rather than group rhythmic phenomena. Yet interindividual differences of various magnitude are known to chronobiologists through their own research. The implication of these interindividual differences requires examination with at least the following two goals: (1) to obtain a realistic and generally meaningful quantification of rhythm parameters as a group phenomenon for a given species, strain, or sample and (2) to demarcate the range of features and alterations of rhythms which are usual and representative of health as opposed to disease. Examination of interindividual differ

ences in rhythmic phenomena provides a broader and deeper insight into conventional concepts such as the "homeostatically" derived one of "individual variability," which completely ignores the temporal dimension of biological organization. For the purpose of discussing the individuality of temporal structure, three illustrative examples are presented.

The first example deals with a comparison of circadian rhythmic patterns of monozygotic (MZ) and dizygotic (DZ) twins (Barcal et al. 1968). Since in this case the healthy MZ and DZ sibling reside in more or less the same physical and sociocultural environment interindividual differences in rhythms imply a genetic origin. The findings indicate what might be expected. The patterns and waveforms of the circadian variables of core temperature, heart rate, and systolic blood pressure were quite similar for MZ siblings. There were greater qualitative and quantitative differences between DZ siblings

The second example is intended not only to elucidate the existence of interindividual variability in rhythmic phenomena, but to show also the need for specific chrono biologic methods to detect and quantify such. Wever in 1979, analyzing results of many human experiments conducted under temporal isolation, demonstrated that, inter alia, the period of free-running circadian rhythms varied within certain limits from subject to subject In addition if in most subjects all the measured variables had the same period, some subjects exhibited an internal desynchronization, that is different variables oscillated with differing periods. The span of time needed to re-entrain the rhythms or to resynchronize the organism to an exact 24.0-hr periodicity when effective synchronizers were reinstituted, varied also from subject to subject These findings suggest the existence of interindividual differences in the tolerance to alterations in synchronizer schedule, such as that encountered in transmeridian flight ("jet lag") and shift work (Reinberg et al 1979) There now exists sufficient evidence to consider certain persons, because of interindividual differences in their temporal anatomy, to be more fit for shift work than others. A question currently of interest is whether interindividual differences, for example, in the amplitude of the circadian rhythm of core temperature, is associated with interindividual differences in the ability of employees to tolerate shift work (Reinberg et al 1979) To completely explore this possibility, autorhythmometry (self-monitoring of rhythms) is required to obtain sufficiently long time series both on large samples of employees who are tolerant and intolerant of shift work as well as large samples of other workers upon whom this hypothesis can be further explored.

The third example comes from studies by Bicakova-Rocher et al (1980) Her results show that some subjects are prone to rhythmometric alterations, such as a reduction in amplitude or loss of rhythmicity during control spans of research studies and also when confronted with certain atypical situations, such as when taking a placebo believed to be a tranquilizer In her study, one-half of the subjects (six of twelve) exhibited such alterations

From a methodological point of view these examples show the need for control(s) in both laboratory animal and clinical experiments. One of the issues as far as man is concerned, is the design of protocols so that the subject serves as his own control. Rhythm patterns obtained for a set of variables during the control span may be used as a reference for a given individual in order to study changes resulting from various other situations, such as those observed in investigations on nutrition transmeridian flight, physical training, aging and disease. Methodological procedures for studying interindividual differences using longitudinal samplings as well as those for studying group phenomena are presented in detail in the next section. This section, referring to differences, should be kept in mind when considering the goals and limitations of the methods available for chrono biologic investigations.

Data Sampling and Gathering

Before initiating chronobiologic research and accumulating a time series several decisions concerning sampling and design must be made: these are outlined below

Δt: time interval between each datum—physical measurement, chemical determination, or other. Δt can be either fixed or varying, even random (unequal Δt's).

T: total duration of the sampling span.

Nos: number of samples during T.

A relationship exists between Δt, T, and Nos, as far as circadian, circamensual, and circannual rhythms are concerned. For both biological and statistical reasons, it is necessary to have, for example, as a minimum for circadian rhythms, $T \geq 24$ hr, $\Delta t \leq 4$ hr, and Nos ≥ 36. This means that for a selected variable, measurements must be done every 4 hr (preferably at fixed clock times) either during *at least* 24 hr in 6 subjects or during at least 6 days in 1 subject.

Longitudinal sampling, for example, of one subject when $T > 6$ days and $\Delta t \leq 4$ hr is preferred for documenting rhythms in individual subjects. On the other hand, transverse sampling, for example of 6 subjects when $T \geq 24$ hr and $\Delta t \leq 4$ hr, is appropriate for documenting rhythms of small groups. Longitudinal and transverse sampling usually leads to similar results when comparable experimental conditions are maintained with respect to circadian and circannual synchronization as well as other criteria of experimental standardization (Halberg 1973).

In chronobiologic studies it must be decided whether data are to be acquired by serially dependent samplings provided by the same subject(s) over time, or by serially independent samplings provided by different but supposedly comparable subjects over time. When feasible, serially dependent data gathering is preferable. For example, similar circannual changes in urinary catecholamine excretion were found in persons residing in Paris with ϕ in January (T = 14 months; Δt = 1 month; serially dependent data of 5 subjects) and in those dwelling in Minneapolis with ϕ in December (T = 5 years; Δt = 1 day; serially dependent data of 1 subject). On the other hand, ϕ was found to be different in Milan (Descovich et al. 1974), occurring in June (serially independent data from various subjects). In certain circumstances, it is impossible to obtain more than a single datum from an organism. This is the case in chronotoxicology studies on mice when death is the monitored response. Obviously, only serially independent sampling is feasible. In this case, rigid standardization of experiments is required to "construct" the circadian or circannual curve (plexogram) (Halberg et al. 1977).

The choice of Δt, T, and Nos cannot be arbitrary. T must be equal to or preferably exceed the period (τ) for ultradian, circadian, circamensual, or circannual rhythms. If several τ's are evaluated simultaneously, Δt must be carefully selected. For example, the plasma cortisol circadian rhythm can be documented with $\Delta t = 4$ hr when $T \geq 24$ hr. However, some ultradian rhythms ($\tau < 4$ hr) cannot be examined. A Δt of 5–20 min makes possible the study of both circadian rhythms as well as so-called pulsatile secretory variations (Fig. 4) which may exhibit a specific "macroscopic" pattern over 24 hr (Weitzman et al. 1971).

When investigating circannual rhythms, transverse 24-hr samplings seem to offer several advantages, including the evaluation of both circadian and circannual periodicities (Reinberg 1974). At fixed time intervals, every month or every second or third month, transverse samplings with $\Delta t = 4$ hr and $T \geq 24$ hr should be conducted during a span of at least 1 year. Since the circadian M, A, and ϕ of a variable may differ circannually, study of circannual rhythms by sampling only once daily, even if the clock hour is fixed and subjects are rigidly synchronized, may provide erroneous information (Dupont et al. 1979; Haus and Halberg 1970; Reinberg et al. 1975, 1978). Similarly, the possibly significant influence of 28- to 30-day hormonal variations in women sug-

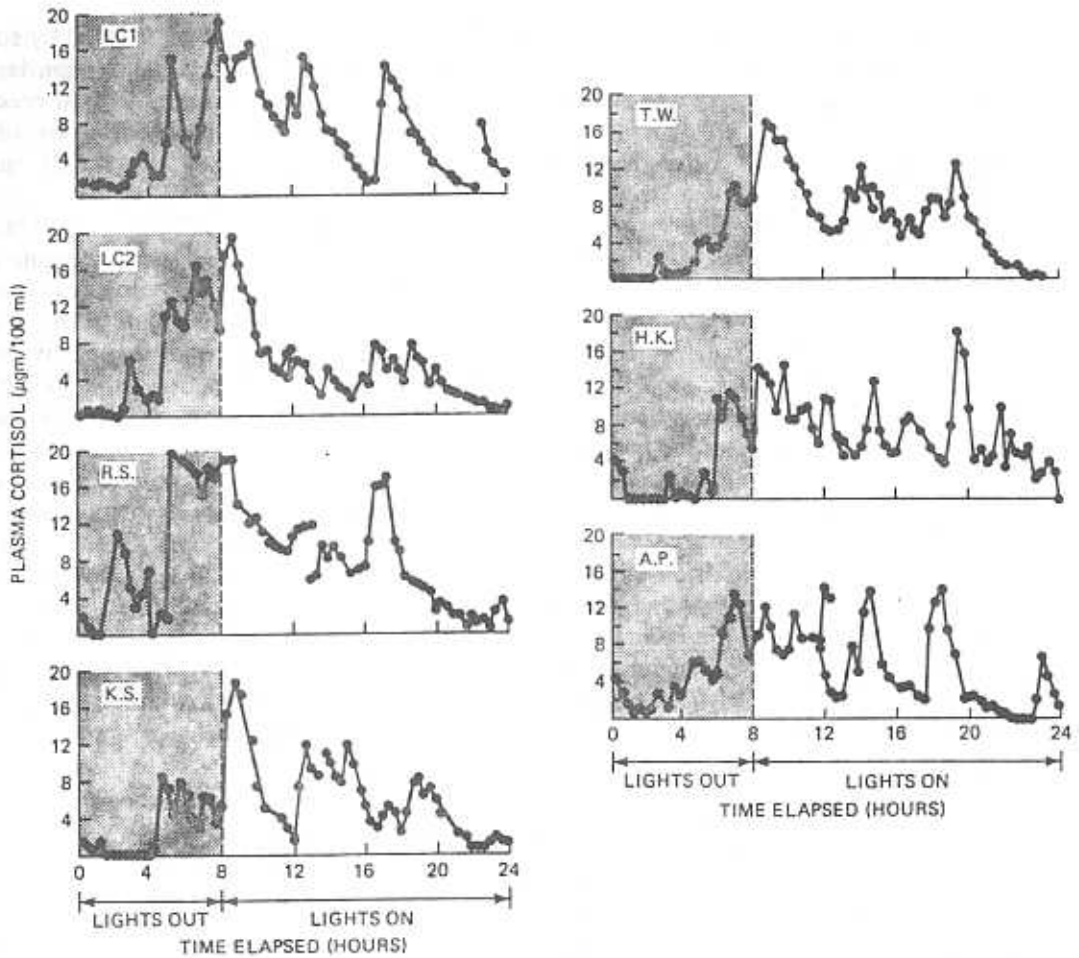


Fig. 4. Plasma cortisol values of normal subjects for 24-hr periods of study. Samples were obtained every 20 min. Period of time of lights out corresponds to sleep span. (From Weitzman et al. 1971, reproduced with permission.)

gests the transverse 24-hr sampling protocol to be the most efficient one for quantifying circadian as well as circamensual periodicities. In this case, a series of transverse samplings, each of 24-hr duration, is recommended at intervals of ≦7 days throughout one or more menstrual cycles (Smolensky et al. 1974).

Quality of Data

Not only the quantity but also the quality—the precision, specificity, and reproducibility with regard to methodology, instrumentation, and techniques—of data collection are critical. This is true for each investigation in biology. Chronobiologists require instrumentation which enables precise measurements, yet just as importantly, the instrumentation must be small, portable, and lightweight. A set of tools for measuring and recording data on body temperature, airway patency, blood pressure, heart rate, EKG, EEG, and wrist movement is currently available for use in investigations on ambulatory rodents, monkeys, and man. However, the need for precision and specificity in data collection is even more important than it appears. Two examples illustrate this point.

It is necessary for both practical and theoretical reasons to measure the bronchial patency (bronchial diameter) of human subjects. Features of the circadian rhythm in bronchial patency are critical for evaluating, from a clinical point of view, the patient suffering from pulmonary disease, such as asthma, as well as for evaluating the effect of air pollutants on asthmatic and other types of patients (Gervais and Reinberg 1978; Prevost et al. 1980; Reinberg et al. 1971; Smolensky 1976; Smolensky et al. 1980a). The bronchial patency can be accurately measured in the laboratory with bulky, fragile, sophisticated, and expensive instruments. Although each datum obtained in this manner is very precise, it is ambiguous if the measurement is done without regard to time-qualified references (chronodesm, discussed later in this chapter) pertaining to time of day, week, month, and year (Gaultier et al. 1977; Tammeling et al. 1977). The bronchial patency can be measured adequately, although less accurately, by lightweight, portable, easy to carry, easy to check, and inexpensive instruments such as peak flow meters (Wright) or spirometers (Hildebrandt). The peak expiratory flow (PEF) thus measured is considered less precise and informative than that obtained by laboratory instruments, such as spirometry (for $FEV_{1.0}$) and plethysmography (for dynamic compliance and airways resistance), since PEF is representative only of the patency of bronchi up to the eighth dichotomy of the respiratory tree. In addition, PEF (and $FEV_{1.0}$) is an indicator of not only the bronchial diameter, but also the strength of the musculature of the chest (Brody et al. 1969). Despite these qualifications, with PEF measurements circadian and circannual changes in bronchial patency can be examined by the subject himself in different situations—at home, at work, or in environments having different levels of air pollution as well as before, during, and after transmeridian flights (Gervais and Reinberg 1976; Prevost et al. 1977; Reinberg et al. 1971; Smolensky 1976; Smolensky et al. 1980b). The high precision of one datum provided by sophisticated laboratory instrumentation is sufficiently compensated by the high precision resulting from a large series of meaningful PEF measurements appropriately quantified by time series analyses.

The significance of the quality of data also is exemplified by studies relying upon so-called "continuous" determination of blood variables. To examine both ultradian and circadian human hormonal rhythms, a large number of blood samples, each of 2 ml, is required. Using a venous catheter, it is possible to withdraw the 2 ml blood samples at 6-min intervals; however, over a single 24-hr span, a sizeable blood withdrawal of 480 ml would be necessary. To avoid the deleterious effects of such a large blood loss on the studied variables, new devices have been developed by Weitzman et al. (1971), among others, to replace the exact amount of blood withdrawn by saline via the same catheter.

Ethical Requirements

Obviously, for both healthy human beings and patients, experimental methods must fulfill certain ethical criteria. They must be safe, non-painful, non-disturbing, and preferably non-invasive. Moreover, the research must address meaningful hypotheses. These same considerations hold true for all patient and animal research.

Time Series Analysis

General Considerations

The problem of objectively evaluating collected time series data is among the most critical for chronobiology. The development of appropriate statistical procedures for analyzing time series was a major goal for chronobiologists in the Sixties. Despite great progress [spectral analyses and Cosinor methods (Halberg et al. 1965, 1967, 1972, 1977)], difficulties resulting from certain experimental circumstances have yet to be overcome.

Time series obtained by data gathering techniques serve as the basis for the *detection, description,* and *quantification* of rhythmicity. Quantitative methods for analysis of periodic phenomena were a primary concern of mathematicians and physicists during the nineteenth century. Chronobiologists, in developing more specific techniques of time series data analysis to fit their unique needs, nonetheless, use the same parameters and terminologies as physicists and mathematicians. A considerable amount of work has been devoted (Aschoff 1960; Aschoff et al. 1965; Halberg et al. 1977; Wever 1965; Winfree 1980) to the quantitative characterization of rhythms. Four major parameters are commonly utilized to achieve this objective. They are the period (τ), the time of the crest or acrophase (ϕ), the amplitude (A), and the rhythm-adjusted mean or mesor (M)—all determined through curve fitting techniques by the method of least squares (Halberg 1973; Halberg and Simpson 1967; Halberg et al. 1965, 1967, 1972, 1977; Nelson et al. 1979).

The period τ is the duration of one complete cycle of a rhythmic variation. It is customarily expressed in units of time, e.g. sec, min, hr, day, or year.

The acrophase ϕ is the estimated span of time to reach the crest of the validated rhythm for the τ under consideration. When using the Cosinor method (discussed later in this chapter), ϕ represents the crest time of the best-fitting mathematical function approximating the data. It is expressed as an interval from a designated phase reference (ϕ_0).

The amplitude A is the amount of variability due to a given rhythm. When the Cosinor method is used, it is numerically equal to one-half of the extent of rhythmic change for the considered τ. In other words, 2A is the crest-to-trough difference.

The mesor M is the rhythm-adjusted mean. When the interval of time between data sampling (Δt) is constant, M equals the arithmetic mean ($\bar{x}$)

Cosinor Methods

Halberg and coauthors (Halberg 1973; Halberg and Simpson 1967; Halberg et al. 1959, 1965, 1972, 1977; Nelson et al. 1979; Cornelissen et al. 1980) developed a computerized technique, the Cosinor, for the analysis of time series data. The least squares method serves to determine the best fitting function (usually a cosine) for approximating the data (Fig. 5). For this purpose one uses the following function:

$$y(t_i) = M + A \cos(\omega t_i + \phi)$$

in which: t_i = time; A = amplitude; ϕ = acrophase, ω = angular frequency ($\omega = 2\pi/\tau$ where τ = period and $1/\tau$ = frequency).

The cosine function was selected since the cosine of zero, being zero, is a handy phase reference, and since it provides a clockwise presentation (zero being midnight, 0000) when a polar plot is used to summarize ϕ and A estimates with confidence limits (Figs. 6 and 7). The Cosinor has been programed for use by large computers as well as for hand and pocket calculators. Estimation of τ with its confidence limits also can be achieved by this method. The Cosinor method can be used iteratively with different trial periods (e.g., τ = 12 hr, τ = 8 hr). Other techniques, such as fast Fourier transform and power spectrum, are also of great use for validating and quantifying prominent period(s) in time series. Thus, a spectral analysis can be obtained when values of T and Nos are large enough (Halberg et al. 1965; van Cauter 1974), making possible the detection of prominent periods in the ultradian, circadian, and infradian spectral domains.

When τ is known, either from data analysis or from the experimental conditions (as when subjects are standardized with a synchronizing period of ≅24 hr), other parameters such as ϕ, A, and M can be estimated. According to the type of data gathered, the Mean Cosinor or the Single Cosinor should be used (Halberg et al. 1977). The Mean Cosinor is the original procedure applicable to parameter (A, ϕ) estimation when deal-

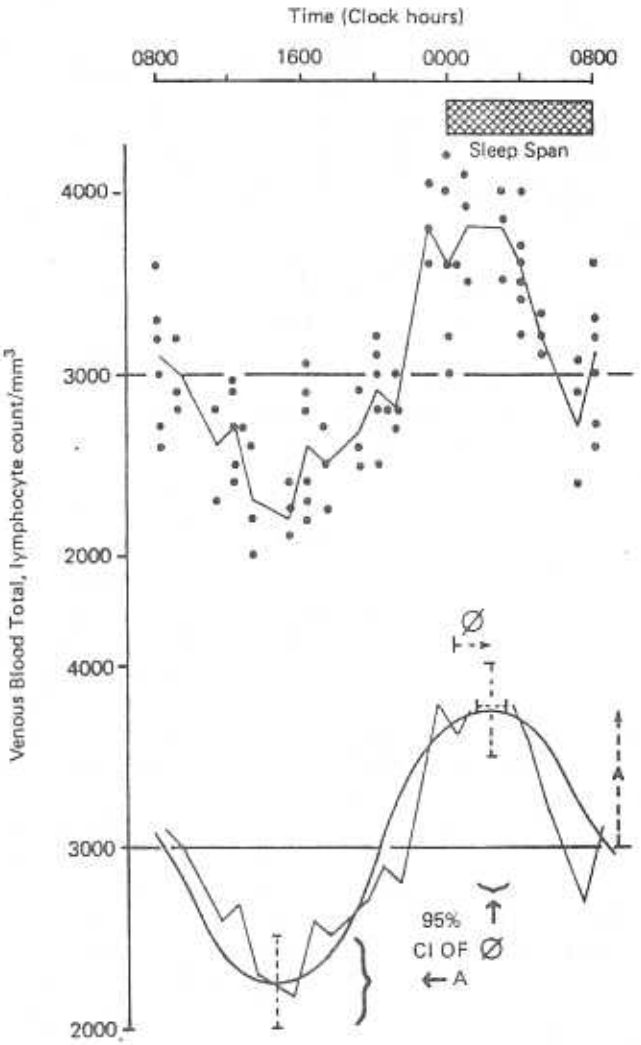


Fig. 5. A circadian rhythm in the total lymphocyte count/mm³ of venous blood was substantiated in a sample of 12 healthy young adults (6 men, 20–28 years of age, and 6 women, 23–24 years of age) during December, 1974 in Paris, France. Subjects were synchronized with diurnal activity from 0800 to 0000 (midnight) and nocturnal rest (sleep). Blood samples were obtained every 4 hr (Δt = 4 hr) at fixed clock hours with the exact times of sampling differing between the three subgroups of subjects. **Top:** Chronogram. Raw data are displayed as a function of time (clock hours). The arithmetic mean of each of the time points (thin line) when connected appears to resemble a sine wave with small swings. **Bottom:** Single Cosinor analysis. The best-fitting cosine function approximating all data is presented. The least squares method is used; parameters characterizing the rhythm are given with their 95% confidence interval (CI). The acrophase ϕ, crest time with midnight as phase reference (ϕ_0) given in hours and minutes, is 0208 (95% CI = 0128–0248). ϕ also can be expressed in degrees (τ = 24 hr = 360°) as a delay from the ϕ_0 (as a negative value with ϕ_0 being the midsleep span = −60°); in this case, $-\phi$ = −332° [or +28°] (95% CI, −304° to −350°). The amplitude A, equal to one-half of the total variability, is 756 lymphocytes/mm³ (CI = 503–1009 lymphocytes/mm³). The mesor M, the 24-hr rhythm-adjusted mean, is 3002 ± 39 (SE) lymphocytes/mm³. A differs from zero with $P < 0.0001$. (Unpublished data from A. Reinberg and J. Clench.)

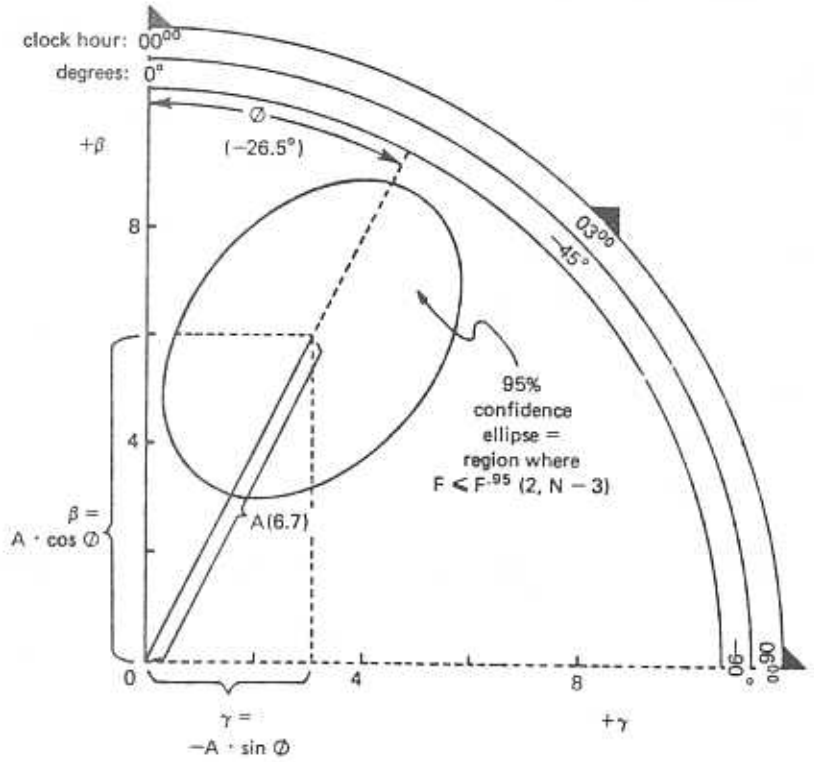


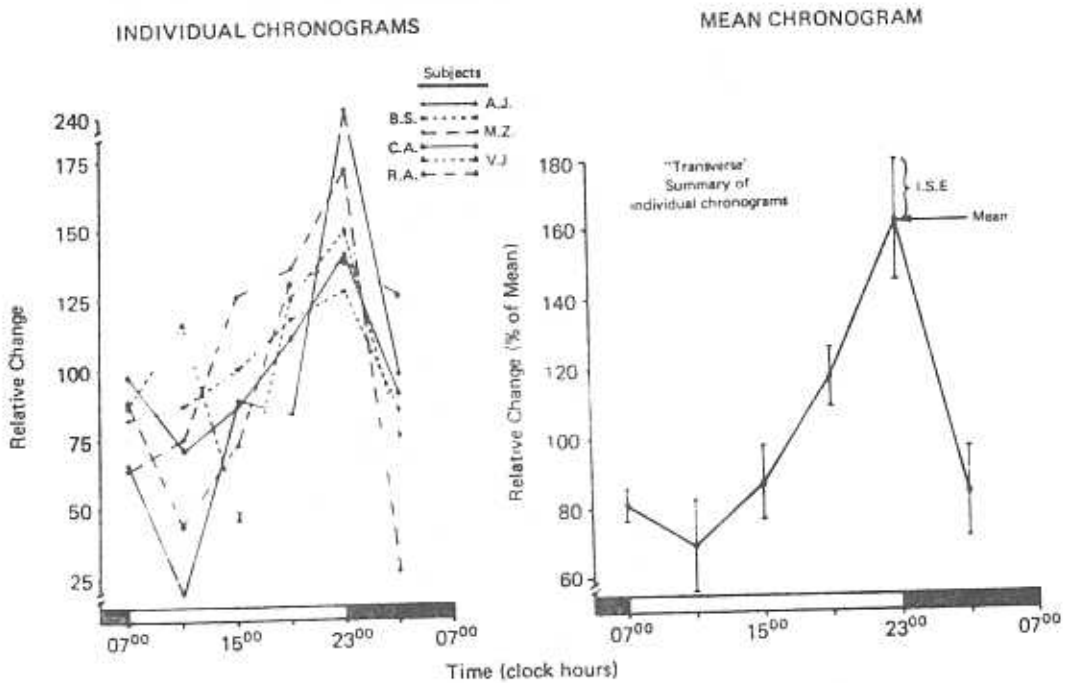
Fig. 6. Rectangular (β, γ) and polar (A, ϕ) representation of circadian rhythm parameter estimates with joint confidence region (single series). Estimates based on an abstract example with 24-hr cosine function. $(Y_{ti}) = M + A \cos(\omega t_i + \phi)$, fitted by least-squares to data collected at 2-hr intervals during the activity span. In this example, A = amplitude; ϕ = acrophase (degrees from 0000); M = mesor; ω = 15°/hr; t = hours after 0000. (From Halberg et al. 1977, reproduced with permission.)

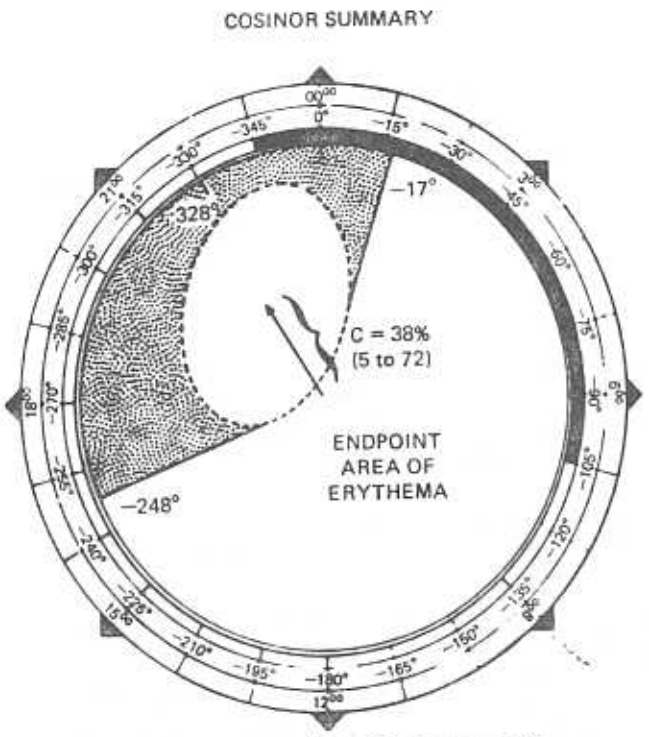
ing with three or more biological time series; this method is most often used for assessing the rhythm characteristics of a group or population. The Single Cosinor is applicable to a single biological time series composed of data from one individual (serially dependent data) or a group of individuals (serially independent data). When dealing with data from groups of subjects, individual time series are placed end-to-end with the option of adjusting the data into relative values first. For serially dependent data, both the Mean and Single Cosinor can be used. On the other hand, for serially independent data only the Single Cosinor is appropriate.

When rhythms are substantiated, estimates of A and ϕ are given with their respective 95% confidence limits. When utilizing the Cosinor, it is assumed that the data are normally distributed around each of the sampled time points. This can be tested by examining the residuals resulting from the fitting of the approximating function (De Prins et al. 1976).

One of the major advantages of the Cosinor is that it permits objective testing of the hypothesis that the rhythm's amplitude differs from zero, in other words that a rhythm is validated for a considered τ. Usually evidence for rhythm detection is accepted when the probability (P) of A being zero is equal to or less than 0.05.

Estimates and end points from Cosinor analyses ordinarily are summarized in tables or by figures allowing the visualization





No Pole Overlap if Confidence Coefficient < .968

of either the best-fitting cosine function along with plotted data (Fig. 5) or by means of a polar plot (Fig. 6). In the latter, confidence limits for A and ϕ are represented by an ellipse of error (Figs. 6 and 7). Estimates and end points of ϕ are depicted on the circular plot with regard to a phase reference, $\phi_0 = 0$. In this presentation, when $\tau = 24$ hr = 360°, then 1 hr = 15° and 1° = 4 min. The projection of a vector on the circle indicates the ϕ location; the tangents to the ellipse of error, drawn from the center of the polar plot, indicate the 95% confidence interval of ϕ. The length of the vector from the pole is proportional to the amplitude. Intersections of this vector with the error ellipse give the 95% confidence interval of A.

The Cosinor method also provides a "goodness of fit" approximation, i.e., the so-called percent of rhythm (PR). Most biological rhythms do not exactly resemble a cosine function. It is, therefore, important to determine the percentage of data included in the 95% confidence limits of the best-fitting cosine function. For example, a PR equal to 80% indicates that the proportion of variance accounted for by the approximation used, a single cosine curve, is rather good.

There are several major reasons why the Cosinor method is currently used widely by chronobiologists. It is useful for validating a rhythm and for quantifying the parameters of rhythms—τ, ϕ, A, and M. It is important to obtain these parameters as estimates and their respective confidence limits. The method can be used even if one deals with short time series as in the case when T = 24 hr, Δt = 4 hr, number of subjects = 6, and the total number of data (Nos) = 36. Moreover with the Cosinor, Δt need not be fixed nor constant; this means that missing data as well as unequal sampling intervals are well tolerated. In other words, Cosinor methods are useful tools for validating, quantifying, and describing a rhythm. The Cosinor method can be used several times, utilizing different trial periods to detect harmonics. For example, in Figure 8 a better approximation of the overall time series is obtained when using $\tau = 12$ hr (1st harmonic) in addition to $\tau = 24$ hr, than when using only $\tau = 24$ hr for the fitting of the data. In other cases, several validated harmonics may be needed for the complete description of a rhythm's waveform.

Although the Cosinor method has proven valuable, there are some situations in which it is inadequate for rhythm description and quantification. This is the case when periodic phenomena are asymmetrical and therefore not amenable to fitting by a cosine function, even with the use of harmonics. In a case such as this, the computed acrophase (crest of the best-fitting cosine function) may not correspond well to the actual crest time (De Prins 1975; De Prins et al. 1976). To solve this problem, several other methods have been proposed, such as the computation of the *orthophase* or *paraphase* (De Prins et al. in press). These latter methods maintain the advantage of a rhythm detection and quantification while improving rhythm description relative to the "basic" Cosinor.

Fig. 7. Individual and mean chronograms and parameter estimations (Cosinor summary) for a circadian susceptibility rhythm of the skin (intradermal injections) to house dust extract: 6 allergic adult patients (2 women, 4 men). The direction of the arrow in the polar plot of the Cosinor and the adjacent shaded area depict the peak and 95% confidence range of greatest susceptibility, respectively; the length of the same arrow represents the extent of the predictable periodic change, i.e., the rhythm's amplitude. That a rhythm does indeed occur is indicated by the Cosinor method when the error ellipse, the white space within the shaded area, does not cover the middle of the center pole of the plot. (From Reinberg et al. 1969.)

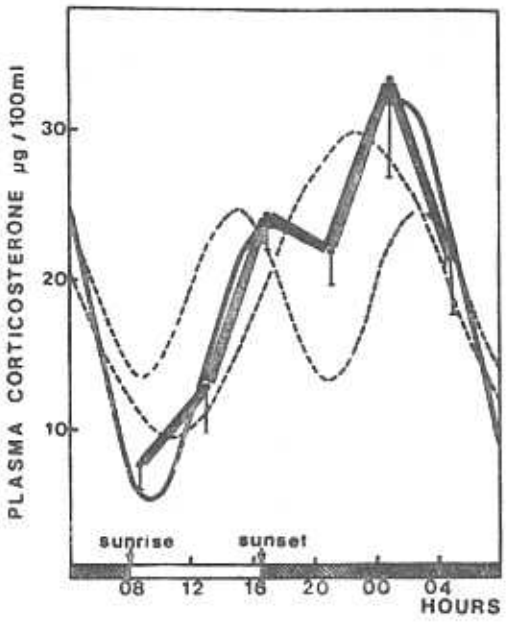


Fig. 8. Circadian rhythm in plasma corticosterone of intact male mature rats, synchronized with natural daylight (November), room temperature of 22° ± 1°C, and food and water available *ad libitum*. Sampling interval ≅ 4 hr (8–10 animals sampled at each time point). Raw data (chronogram) given with time point $\bar{x} \pm 1$ SE in µg/100 ml (*heavy line*). Cosinor analyses by best-fitting cosine functions with τ's = 24 and 12 hr, respectively (*dashed lines*). Rhythm approximation with both component periods (24 and 12 hr) (*solid line*). The crest time of the latter is located at approximately 0015. The resultant curve thus obtained is closer to the chronogram form than that of the cosine function with τ = 24 hr. (From Guillemant et al. 1979, reproduced with permission.)

Display of Time Series as Chronograms and Plexograms

The description of a biological rhythm should first start with a simple display of the raw data as a function of time (Figs. 5 and 7). This elementary step provides an initial impression about the shape of the waveform of the time series and gives some idea of the already available method(s) that can be used to analyze the data (De Prins 1975; De Prins et al. 1976; Halberg et al. 1977; Reinberg 1971). A simple plot of the data usually provides information not available from more sophisticated methods. For example, plotting the raw data of plasma cortisol obtained by frequent blood sampling with Δt = 5 or 10 min throughout 24 hr reveals, according to Weitzman et al. (1971), that the waveform of this circadian rhythm in many subjects is asymmetrical. In diurnally active persons the curve (Fig. 4) shows a large peak in the morning and a total absence of hormone between 2100 and 0300. (Due to ultradian rhythms, averaging the data of several subjects may not reveal this important fact.) Yet generalization from a simple plot of raw data alone is not sufficiently informative in most cases to adequately describe the periodicity; nor does it lead to an unbiased, objective description or quantification of the time series.

Chronograms (and plexograms) constitute a second step in the analysis of time series data (Halberg et al. 1977). A *chronogram* is defined as an individual or averaged display of data as a function of time. A *plexogram* is defined as a display of data which were collected during a span longer than the period of the rhythm investigated ($T > \tau$) along the abscissa of a chosen single period (τ), only. Such a display may be presented irrespective of the (time) order of data collection, for example, as a function of a single conventional or other time unit such as day, without regard for calendar date and/or subject. Since conventional statistical methods are used (means, variance analyses, t-test, etc.), parameters such as the mean (e.g., 24-hr mean), peak–trough difference, peak time location, and period of the rhythm can be estimated. In addition, the waveform of the curve can be visualized. If this latter appears to resemble a cosine function, it is of interest to use one of the Cosinor methods for the evaluation and quantification of rhythmicity. The chronogram, a plot of the data over time, proves to be very important in many circumstances. For example, in studies of shift-workers (Rutenfranz 1978) or of passengers or employees on transmeridian

flights (Klein and Wegmann 1979), the chronogram indicates that the crest time of the body temperature rhythm shifts earlier (and faster) than the trough when the socioecologic (rest–activity) synchronizer is manipulated.

In many chronobiologic publications, both the raw data, in the form of chronograms, and the results of other data analyses (such as the Cosinor) are included. In so doing, several methods for the validation, description, and quantification of rhythms are presented.

Other Methods of Data Analysis

Many methods have been proposed for analyzing time series. Each offers certain advantages and disadvantages depending upon the type of experiment and the manner of data collection. All fulfill a specified purpose. The periodogram (van Cauter 1974), linear and nonlinear procedures (Batschelet et al. 1973), as well as methods such as those proposed by Wever (1965), Sollberger (1970), Del Pozo et al. (1979), Marotte (1979), De Prins (1975), De Prins et al. (1977), Martin (1981), and Winfree (1980) are available. The reader is encouraged to review the referenced works to obtain details about these complementary and/or alternate approaches.

It is not uncommon that nonmathematically inclined or inexperienced students and even accomplished scientists are confused when initially confronted with the practical problems of time series analysis. A large choice of methods, each having a set of advantages and limitations, compounds the problem. Perhaps the best approach in solving the problems of data analysis is to follow the advice of J. De Prins (De Prins 1975; De Prins et al. 1976, 1977, 1978): first consider the raw data displayed in the form of a chronogram or plexogram; then select several relevant methods of data analysis to determine which provides appropriate quantification of the considered time series. Today, the latter can be accomplished using a small computer equipped with a set of programs for the statistical analysis of time series. This approach allows one to visualize and screen the results to evaluate the respective advantages provided by the methods considered, including the display of data and the Cosinor.

The Chronodesm and the Problem of a Single Datum

Not only chronobiologists, but all biologists are troubled when trying to interpret the significance of a single datum. In the first chapter, it was mentioned that it is no longer acceptable to include as "noise" variations in biological values due to *predictable* circadian, circannual, and other rhythms. Inclusion of such rhythmic variability contributes to a widening of the limits or "range" of reference values. The influence of bioperiodic variabilities, especially ones of high amplitude, cannot be ignored. A special method for establishing and interpreting so-called physiochemical reference values has been suggested by Halberg et al. (1977). Conventionally (with the homeostatic approach), reference values are given as a mean ($\bar{x}$) ± confidence limits (SD or SE) ranging from 90% to 95%. A better and more appropriate representation is obtainable if time-qualified reference values are available for biological functions known to exhibit circadian and/or other rhythms. The time-qualified ranges for reference values can be presented in the form of a cosine function with confidence limits. Obviously the information must be defined with respect to sex, age, geographic location, subject's synchronization, etc. For circadian, circamensual, circannual, and other rhythms, such chronobiologically considered reference systems have been termed *chronodesms* (from the Greek, meaning "linked to time") by Halberg et al. (1977). For example, in considering circadian rhythms with only one datum, if both the time of sampling and the subject synchronization (e.g., light-on/light-off) are known it is possible to determine from the

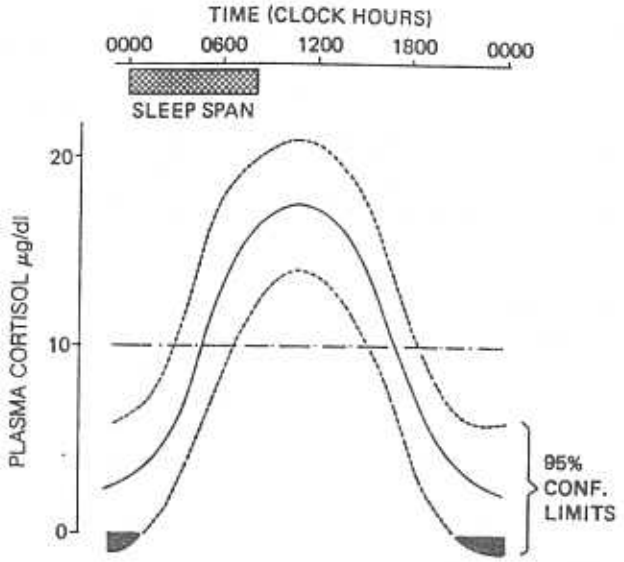


Fig. 9. Chronodesm of plasma cortisol of 8 healthy males, 20–30 years of age. Synchronization with light-on at 0800, light-off at 0000. Sampling interval is $\Delta t = 1$ hr; sampling span is $T = 24$ hr. Despite the fact that circadian changes in plasma cortisol do not exactly represent a cosine function, the latter can be used to derive a chronodesm. The conventional statistical analysis (without chronobiologic consideration) gives $\bar{x} = 10.0 \pm 9.2$ μg/100 ml (95% confidence limits). The chronodesm reveals that around 1100 in diurnally active persons plasma cortisol values of between 13.9 and 20.5 μg/100 ml (95% confidence limits) are within normal; while 12 hr later at 2300, plasma cortisol values between 0 and 5.7 μg/100 ml are within normal. If the subject's synchronization is known, the chronodesm allows the interpretation of one datum given with its sampling time. For example, 13 μg/100 ml at 0800 can be considered "normal," while at 2000 it would not be so for an adult subject with diurnal activity and nocturnal rest. (Unpublished data gathered by M. Guignard, M. Lagoguey, and A. Reinberg.)

chronodesm whether or not the value in question is "normal." For a healthy adult with diurnal activity and nocturnal rest, a plasma cortisol level of zero is within the "normal range" if the blood sample is drawn at midnight (0000), while it is abnormal if obtained at 0800. On the contrary, a plasma cortisol of 20 μg/100 ml may well be considered evidence of pathology if obtained at 0000; it would definitively be considered normal if obtained at 0800 (Fig. 9).

The presentation of reference values as chronodesms constitutes a step forward in the appreciation and diagnosis of health and disease, specifically when dealing with a single datum. A single piece of data provided from the clinical laboratory through the use of sophisticated and precise instrumention has greater diagnostic value when related appropriately to the pertinent chronodesm made available by time series studies. In other words, a chronobiologic approach adds precision and avoids misinterpretation of clinical and laboratory data.

Summary

Various necessities of chronobiologic research have been presented. Subject standardization and synchronization as well as special considerations for data sampling, collection, and analysis using specific methodologies are indispensable for research of bioperiodic phenomena. Different types of investigative protocols are necessary depending on the nature of the research. The results of studies utilizing various kinds of chronobiologic protocols

such as those used in detecting and quantifying rhythms in cell morphology (Chap. 3) and mitosis (Chap. 4), pathological processes and symptoms of human diseases (Chap. 5), and metabolism and effects of pharmaceutical agents (Chap. 6) and nutrients (Chap. 7) are presented later. Although the major focus of each of these chapters is the findings, appropriate detail about research procedures also is provided.

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