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PHYSIOLOGIC CIRCADIAN SYSTEMS

(Differences in Period of Circadian Rhythms or in their Component Frequencies; Some Methodologic Implications to Biology and Medicine *

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Introduction: Physiological rhythms with periods of about 24 hours may be called circadian rhythms (*circa*=about, *dies*=day) (1). In biology, these rhythms characterize phenomena ranging from the well-known alternation of sleep and wakefulness in man (2) to the luminescence of a photosynthetic dinoflagellate (3).

Biophysical, biochemical, morphological, and other variables of interest to medical science are also known to exhibit circadian rhythms (Fig. 1) (4-6). Illustrative examples include body temperature readings, serum chemistries, cell or parasite counts on blood (7-9), observations on tissue culture (10), as well as data obtained by combining several research techniques, as for instance differential centrifugation and radioactive tracer methods with histology, on comparable organ samples (11-13).

Reliable observations on circadian periodicity over this wide range of phenomena have steadily risen in number, with the current trend toward studies based upon statistical designs and analyses. Information already available seems to warrant the concept of biologic circadian systems. The synchronization and desynchronization of such systems will be considered herein and a few implications as to methods of biomedical research are offered.

Circadian Systems: A morphologic entity may be viewed as a circadian system, if it exhibits one or more functions with a frequency of about one cycle per day. This view applies, for instance, to a

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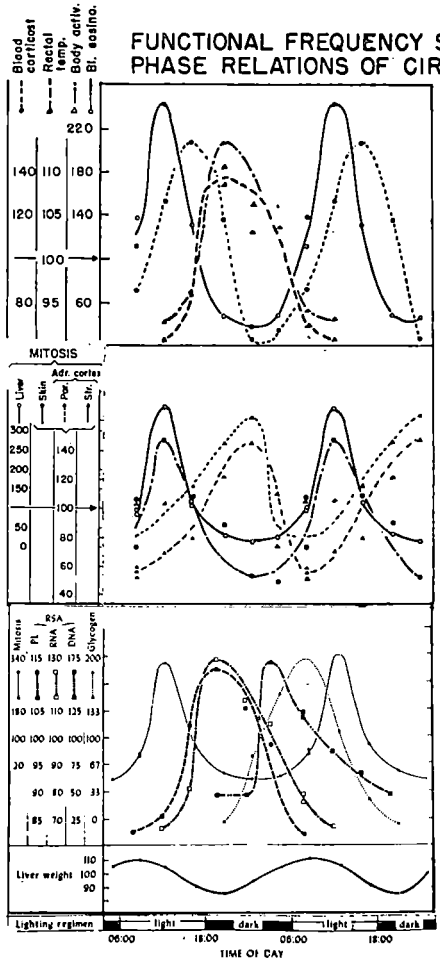

Figure 1: Circadian system in the growing mouse: synchronization—with difference in phase.

single cell, tissue or organ as well as to an organ system or a multicellular organism. Groups of any one of these entities also can be analyzed as circadian systems; indeed, many circadian functions are studied as group phenomena (14), whether one is dealing with groups of cells (1) and/or groups of individuals (15).

Biologic circadian functions, more often than not, are actually interacting among themselves and with external factors. But their physiologic periodicity, as such—or, to be sure, the ability of a given function under appropriate conditions to show a frequency of about one cycle/day—is as much, or more, characteristic of organisms or of their subdivisions as it is a given system's response to factors

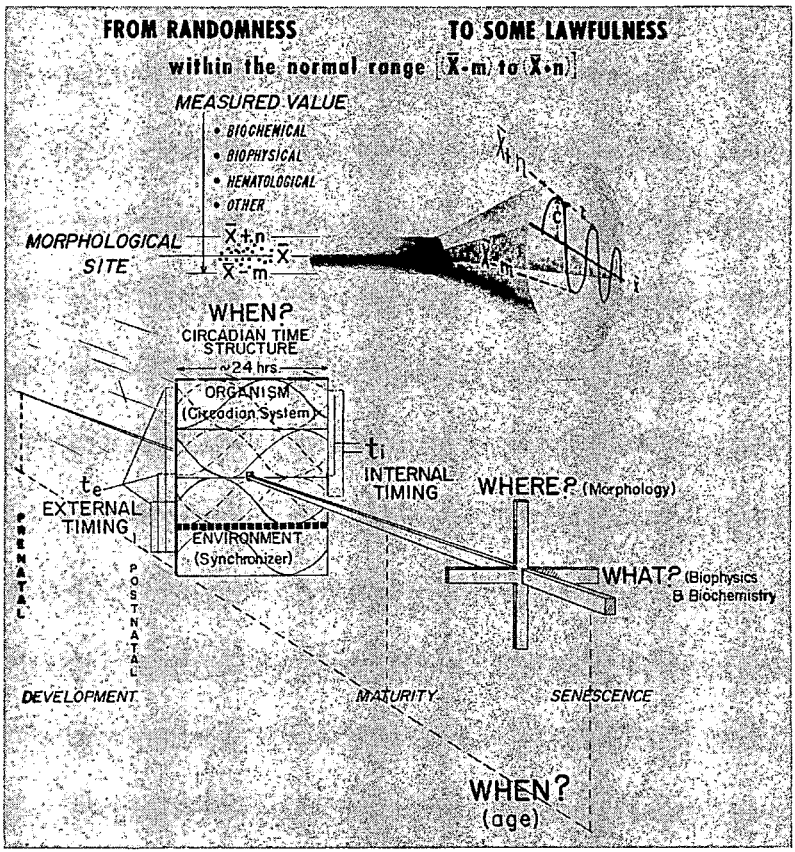

Figure 2: Parameters of circadian systems: period (τ), amplitude (C), level ($\bar{X}$) and time relations (t_e and t_i, see text). Circadian aspects of external and internal timing are schematically aligned with other temporal facets of the body, such as age. The physico-chemistry of an organism, as well as its overall behavior, varies with statistical predictability from one hour to the next (Figs. 1 and 3), just as it differs among tissues or organs. In addition to spatial organization in morphological entities, and apart from changes with age, there is a temporal organization along the 24-hour scale. This time-varying circadian behavior is often viewed as a physiologic "superstructure", or "another factor" (cf.1); herein it will be explored as a temporal basis of integrated and adapted physico-chemistry in organisms or in their parts.

topographically external to it. In other words, the integrative periodicity, e.g., of circadian organisms, is to a significant extent the system's own "action"; while this is intimately related to those many external factors which can elicit "reaction," the system's circadian behavior is not entirely determined by outside factors (5, 16, 17). The important suggestion of Hildebrandt (18) that psysiologic functional reaction, as well as action, can be essentially periodic deserves serious consideration.

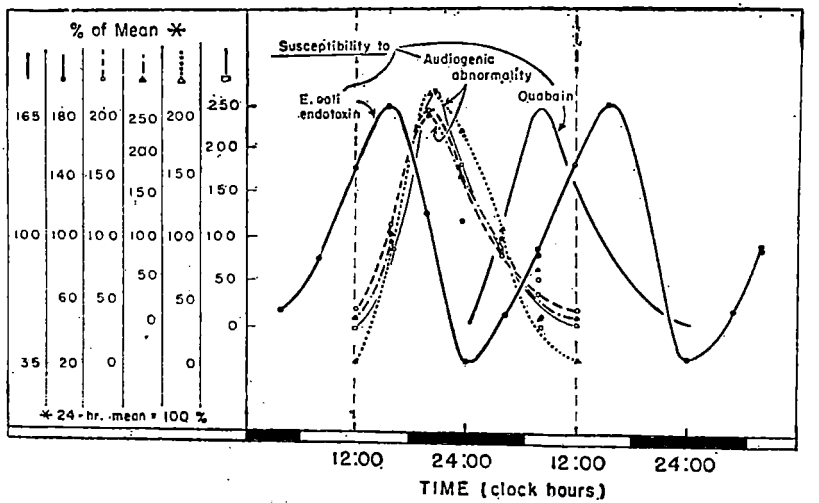

Figure 3: Hours of changing resistance. Circadian synchronization of susceptibility rhythms—with differences in phase.

Yet irrespective of the foregoing considerations, which are supported by considerable evidence (reference 5, among many others in the same volume), the view of certain biologic entities as circadian systems presupposes neither that any or all of their functions with a frequency of about one cycle per day are necessarily and entirely "spontaneous" rather than "induced," nor does it imply that any or all such functions are necessarily and critically interdependent.

The concept of a given biologic entity as a circadian system, rather than presuming the foregoing qualifications, provides instead a basis for assessing such problems. Apart from this, the view of organisms as circadian systems underlines the need for circadian charts, which are the basis for the analysis-in-time of physiologic behavior, since it is from such charts that experimental and clinical zero-time may best be chosen, whenever one evaluates a function or system that is circadian rhythmic.

Reliable circadian charts are a requisite for resolution in time of functional interactions, but they are not, in themselves, the ultimate aim of periodicity analysis. The charts themselves provide information on external and internal timing (Figs. 1-3). Circadian analysis also involves additional procedures, the results of which must be evaluated among others as eventual modifications of the timing provided from the charts. Thus, in addition to mapping, circadian analysis involves the manipulation of a synchronizer (19, 20), e.g., for phase shifting the system or the manipulation of the system itself, e.g., in order to effect desynchronization. (See below and Fig. 4) (21-23).

External and Internal Timing: Circadian rhythms can be studied, first, as to their external timing (Fig. 2; cf. also Figs. 1 and 3). For

this purpose there is mapping of the timing of their peaks or troughs on a fixed environmental schedule, such as a regimen of light and darkness, or a routine of activity and rest (13-16).

An environmental cycle or routine may be called the synchronizer of a given circadian physiological rhythm, if two requirements are met (19, 20): First, the rhythm can adapt its circadian period to the particular environmental cycle length. Second, the rhythm can be pulled by phase-shifts of the environmental cycle; the peak of rhythm may thus be made to coincide with any desired clock hour. Therefore, indications of external timing are meaningful only if the synchronizer schedule is also indicated.

Internal timing (Fig. 1) is another aspect of systems that show circadian periodic functional integration (4, 5). The internal timing of a circadian system describes the time relations among certain of its circadian rhythms themselves; it may be expressed as a difference in time units, e.g., in hours, *inter alia*, between peaks, troughs or slopes of two rhythms. Study of internal timing, by definition, calls for the concomitant evaluation of two or more physiologic functions: the multivariate approach.

External timing is largely adaptive and entirely flexible as to clock hour—so long as the synchronizer's cycle length is acceptable to the system. Internal timing, subservient to functional integration as well, shows considerable although not unlimited plasticity (cf. 5). Circadian charts, descriptive of external and internal timing (Figs. 1 and 3) reveal the organism's adapted time structure along a 24-hour scale (Fig. 2).

Functional Integration and Adaptation: By analyzing the circadian rhythms of an organism one explores indeed two of its temporal characteristics: how diverse functions are integrated in time with each other and how they are adapted to time-varying environmental influences.

For studying environmental adaptation, one may focus, as a start, exclusively upon the behavior of an easily measured physiologic function, e.g., in the presence *and* absence of a known synchronizer.

Today, this univariate approach, qualified as the study of a single physiologic function, is the procedure most commonly used. Underlying such work is the tacit assumption that all circadian rhythms of an organism will behave similarly. Apparently justified in principle, this assumption cannot be extended to detail. To cite a pertinent example: in a mouse a variety of rhythms, including cellular and total-body functions, all can be shifted by manipulating the lighting cycle; yet, the shift-times of different rhythms can vary from one physiologic function to the next or even for the same physiologic function (e.g., mitosis), in different tissues of the same individual (6, 13).

For a more complete analysis of adaptation the behavior of different functions of the same organism is explored, e.g., following the manipulation of environmental factors (1, 4). For this end dependence is mostly upon the multivariate approach, while it is realized,

of course, that a given biologic time series usually reflects the operation of diverse physiological mechanisms.

As soon as one's interest shifts to functional integration, the measurement of more than one variable of the organism seems highly desirable; one is then dealing with a field of the multivariate approach, *par excellence* (5).

At first and on the surface, circadian aspects of integrative and adaptive mechanisms may seem trivial. Yet, such time-varying behavior can tip the balance toward death or survival in the outcome of our exposure to a variety of environmental agents – other things being largely controlled (Fig. 3) (4).

Control: It can be suggested that circadian periodicity analysis has an important bearing upon experimental biology and clinical medicine, whether or not one is interested in rhythms as such (5).

Empirically, in an era of natural science, the use of controls needs no emphasis. As discussed at the outset, many physiological functions studied in the laboratory, field or clinic, as controls as well as experimentals, show circadian rhythms. Interpretation of results, however, depends upon evaluation of controls; the attempt is made to show the advantages derived from the analysis of the time-varying aspect of the many "control" functions exhibiting circadian rhythms.

It is commonly believed or implied, or stated, that the circadian rhythm of a given function can be controlled by provisions for observations at a fixed clock hour. However, this is not necessarily so. Moreover, experimental designs providing for observations at a fixed time of day can be greatly improved if the timing of observations is based upon pertinence rather than convenience. (cf. 1). For this purpose, one may allude to circadian synchronization and desynchronization in living things (5) with reference to some of their consequences to experimental design and analysis.

Circadian Synchronization: In the circadian context, the term synchronization (or desynchronization – see below) can be used to describe the period (τ) of one, or more, functions by comparison to a reference τ – without necessarily implying causal interactions.

More often than not, circadian systems are indeed synchronized with some environmental change such as a light-dark cycle or a social routine. The synchronizing environmental change has been referred to as "cue" (24), time-giver (16), "clue" (25), or entraining agent (17), as well as synchronizer (19, 20).

All of these terms refer to some environmental factor (routine or single stimulus (17)) which affects an organism's periodic behavior; in the case of a routine with acceptable cycle length, its period is imparted to the physiological system. Thus, the circadian system of the mouse as a whole, at its different levels of physiologic organization, can apparently be synchronized by experimental 24-hour light-dark routines (13, 26). A primary synchronizer (or routine) may interact, however, with secondary factors (1, 20).

The synchronizer most frequently encountered in the ecologic habitat niche is the natural lighting regimen. In socially oriented man the mode of life, governed by local time, assumes the same role. In

most of these instances, the environmental synchronizer is of 24-hour cycle length. Since, moreover, circadian systems can be phase-shifted by the manipulation of 24-hour routines, it was convenient to provide for a similar change in illumination in the laboratory (by a clock-controlled electric switch) (1). For a general reference standard, subservient to the analysis of circadian synchronization (or desynchronization), the $\tau = 24$ hrs. is a satisfactory choice, but in specific cases non-24-hour τ's can also be chosen for reference.

Whatever the reference τ, two or more functions with the same frequency can be described as synchronized functions, and one may do so whether or not there are phase differences among these functions. Actually, one may speak generally of "synchronization – with differences in phase," since a phase difference can be zero, as well as positive or negative; when this difference is zero one may specify "in-phase synchronization."

For the following discussion, the standard of reference for a circadian system will be its quantified behavior under conditions of synchronization with a 24-hour routine. Effects of manipulating experimentally the circadian system itself or some (but not all) aspects of its environment will be evaluated against this reference standard.

Circadian Differences in Phase: To examine a synchronized circadian system with its multitude of rhythms at different levels of physiologic organization (Figs. 1, 3) (4, 5), one may now view phenomena ranging from the effect of environmental agents upon the body as a whole to the metabolism of some cytoplasmic cell fractions. The rhythms uncovered show similar periods, but their peaks or troughs do not all occur at the same time.

The circadian system of the mouse, illustrated in Figs. 1 and 3, reveals a physiologic division of labor in time. One may examine, first, at the bottom of Fig. 1 the external and internal timing of some periodic cellular processes during growth. Cell division in mouse liver parenchyma reaches its peak during the middle of the daily light period. One can describe its external timing by indicating that peak mitosis occurs $\approx$ 12, on a lighting cycle providing for light (L) from 06-18 and darkness (D) from 18-06.

Two of the curves in the bottom row of Fig. 1 seem to peak at the same time; it would appear that in mouse liver the incorporation of radio-phosphorus into RNA on one hand, and phospholipid on the other, is synchronized in-phase, i.e., with a zero phase difference. The internal timing of the two functions can be described, in part, by the statement that their peaks are separated by less than 4 hours (if at all).

The detection of the correct phase difference, in this instance and others, is obviously a function of the interval between successive observations. The shorter this interval, the more likely it is that one will detect the right timing. Accordingly, findings of differences in phase, whether they are positive, zero, or negative, must be qualified by reference to the interval between observations; in circadian analysis, just as in microscopy, limits to resolving power must be recognized.

With already available resolution, it seems established that in

intact growing mouse liver the internal timing of rhythms in (a) the incorporation of P_{32} into DNA and in (b) mitosis, involves a lag of $\approx$ 8 hours ($\pm$ 4 hours) of (b) behind (a) (Fig. 1, bottom) (11-13). Similar considerations also hold for the internal timing of mitotic rhythms in liver parenchyma and pinnal epidermis on the one hand and adrenal cortex, on the other. This is shown in the middle row of Fig. 1, which compares the mitotic "time zones" in different tissues of a growing organism.

In turning from the bottom of Fig. 2 to its middle, one moves from synchronization at the intracellular level to that at the tissue or organ level. The top of this figure indicates functions of the total organism. Considerations of internal timing are germane to rhythms at any one of these levels of integration; they also apply to two or more functions at different levels of organization (Fig. 1, as a whole), as well as to the way in which circadian system-phase affects reactivity to external agents (Fig. 3).

Certain circadian rhythms are dependent upon known internal factors, while they are timed by external synchronizers. The rhythmic incorporation of P_{32} into hepatic phospholipid, the circadian rhythms in mitotic activity of pinnal epidermis and in blood eosinophils (27) depend thus upon the adrenal pacemaker (1, 4). But functional interrelations have to be checked, apart from mapping, by other procedures (13). It cannot be overemphasized that circadian charts such as those in Figs. 1 and 3, in themselves, do not imply functional relationships.

Circadian Desynchronization: Two or more previously synchronized functions can cease to vary with the same frequency: desynchronization has occurred. As evaluated in relation to some circadian reference function, the behavior of a given desynchronized physiological function falls into one of the following theoretical categories:

Case 1: cessation of all recognizable biologic variation (hardly before death, cf. Fig. 10).

Case 2: cessation of recognizable circadian-periodic variation; continuation of non-circadian-periodic and/or non-periodic biologic variation (encountered or implied mostly in homeostatic theory, a useful first approximation).

Case 3: continuation of recognizable circadian periodic variation, with a new frequency; the new τ differs from the reference τ by minutes or a few hours. In this case, documented by Fig. 4 data, the function under study may be said to be free-running from the reference function (but not necessarily from any and all external influences). If 24 hours are chosen as reference τ, a case 3 desynchronized function will free-run from the 24-hour clock; its period will be shorter or longer than 24 hours, by minutes or a few hours.

Demonstrated cases of circadian desynchronization all suggest that an environmental regimen which had previously pulled the system as a whole or some of its constituent units does so no more. Desynchronization can occur wherever synchronization had prevailed. Circadian desynchronization thus may be qualified as to integration

level(s); e.g., it may apply to rhythms in an individual cell or in an organism, or in a population of either.

Desynchronization of a circadian rhythm from the lighting cycle has been demonstrated in the acutely blinded mouse (Fig. 4) (1, 21-23); immediately following blinding, the rhythm in rectal temperature of most individuals assumed a period that was consistently shorter than 24 hours, by minutes or a few hours.

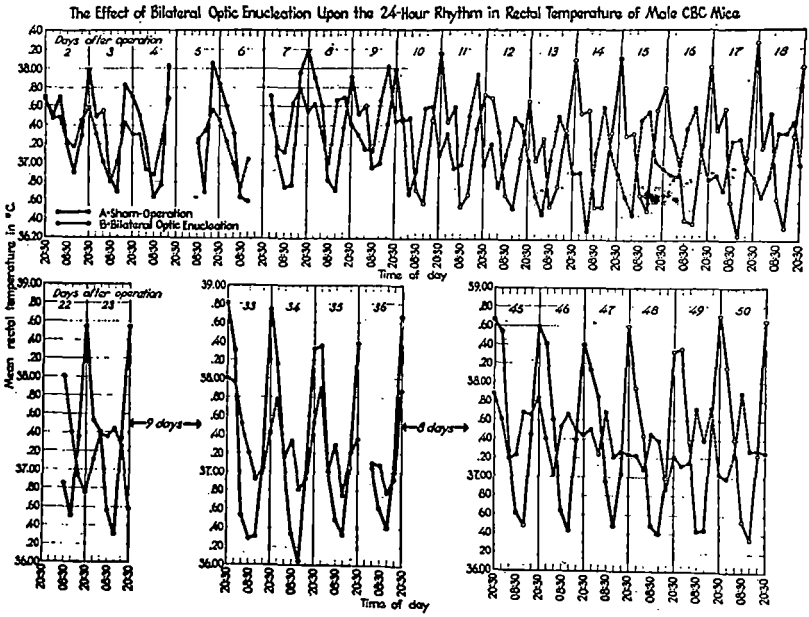

Figure 4: Bilateral optic enucleation uncoupled the rectal temperature rhythm of male hybrid mice. Note lead in phase of temperature rhythm in blinded mice; by day 22, this rhythm has passed through temporary antiphase, in relation to that of controls. The average circadian period in rectal temperature of the group of blinded mice was slightly but consistently shorter than 24 hours; desynchronization of rhythm from the 24-hour clock-regulated lighting cycle.

Moreover, rectal temperature series of over 20 blinded mice studied concomitantly (21-23) showed periods differing from one individual to the next. Extrinsic cycles of corresponding frequency are not known; it would appear that such non-24-hour circadian periods characterize primarily the physiological system rather than directly or exclusively its environment.

Desynchronized rhythms with circadian periods of non-24-hour length are rhythms uncoupled from their synchronizer—if the latter continues with a τ of exactly 24 hours. It is possible, of course, that *some* rhythms, uncoupled from their synchronizer, will continue with periods of exactly 24-hour length. From available evidence, however, the chance that *most* uncoupled rhythms in a population of

individuals will have rhythms of exactly 24-hour length seems to be exceedingly small.

Uncoupled systems with periods of exactly 24 hours cannot be distinguished from 24-hour synchronized rhythms when period-length is the sole criterion. Non-24-hour circadian functions, in turn, almost certainly are uncoupled, as long as synchronizer(s) continue to be applied with a schedule of exactly 24 hours (Fig. 4).

The extent to which non-24-hour circadian rhythms are influenced by extrinsic factors remains an interesting problem, particularly for environmental physiologists (1, 28). Multiple factors probably underlie the now well-established non-24-hour circadian behavior of organisms. Consideration of complex interactions among such factors probably involves the whole of an organism's antecedents, including its genetic make-up. Yet, irrespective of interpretations (1, 5), one can be operational: For the purpose of methodology one may view the free-running case of desynchronization (case 3 above) simply as the statistically ascertained free-running from the 24-hour clock.

The occurrence of free-running circadian periods, e.g., in one of two experimental groups beings compared, has a bearing upon biomedical research. Moreover, the added possibility exists that two or more circadian components contribute to the same data; for instance, an exactly 24-hour component may be superimposed upon a free-running one.

Time series containing different, although sometimes close, frequencies can exhibit beats on their envelope. This aspect of physiologic circadian rhythms is hardly trivial yet seldom considered.

Beats remain unrecognized when, conventionally, a mean amplitude or period is computed, e.g., by periodograms (29) or when the evaluation of data remains restricted to only one prominent point of the rhythm such as the onset of some activity. When detected, in turn, beat frequency yields information on the interacting periods.

Therefore, it is important to recognize the operation of different, although close frequencies, in two time series being compared, or in the same time series being analyzed.

A. *Comparison of Functions with a Slight Difference in Period: General Considerations.* For many biologic phenomena a circadian rhythm synchronized with a 24-hour environmental routine serves readily as control. Therefore, one may consider the following case: One function, y_1, is assigned a period of exactly 24 hours, corresponding to the period of many environmental cycles. Other functions, y_2 (y_3....y_n) can be compared with y_1; e.g., y_2 can be chosen so that its period is shorter than 24 hours by minutes or a few hours. Similar, although not identical, considerations also will apply when y_2 has a period longer than 24 hours.

y_2 can illustrate a physiologic function. One may think of y_1, in turn as the 24-hour clock used for setting social routines or those of experimental laboratories.

For other considerations a 24-hour function y_1, can also represent a physiological variable; this physiological y_1 can be compared, e.g., with other phy-

siological rhythms (y_2, y_3 . . . y_n) in the same individual or with the same physiological rhythm in different individuals.

Finally, whether it characterizes a function of the organism or of the environment, y_1 can be assigned periods different from 24-hours, but different also from y_2.

Fig. 5 shows how certain periodic functions with unequal periods will alter their phase relations with time. If they start out in phase, they will gradually glide into temporary anti-phase, then back into phase and so forth. Phase relations will depend upon time and the difference in period of the two functions.

Fig. 5 actually summarizes two simple periodic functions: $y_1 = C_1 \sin \varphi$ and $y_2 = C_2 \sin \left[\frac{360}{360\text{-}k}\right] \varphi$; where φ and k are in degrees (360° correspond, e.g., to 24 hours). For simplification, both functions can be assigned unit amplitude ($C_1 = C_2 = 1$). Therefore, the solid curve: $y_1 = \sin \varphi$; and the dotted curve, $y_2 = \sin \left[\frac{360}{360\text{-}k}\right] \varphi$.

In Figs. 5a, 5b, and 5c, k is equated to 10°, 20°, or 40°, respectively. The constant, k, expressed in degrees, and describing events in time, relates to the difference in the periods (τ) of y_1 and y_2.

Thus, if y_1 is assigned a τ of exactly 24-hours, in our examples, k will relate to a difference in τ of 40 minutes, 80 minutes, or 160 minutes, respectively. Accordingly, y_2 can be taken to represent a function with a τ of 23 hours and 20 minutes, 22 hours and 40 minutes, or 21 hours and 20 minutes, in Figures 5a, 5b, or 5c, respectively.

Fig. 5 thus can be used to visualize the time relations of circadian curves differing in their periods by 40 (top of figure), 80 (middle) or 160 (bottom) minutes in time. y_2, with the shorter period, will lead y_1; this lead in phase increases by k degrees every 24 hours.

To start out with the two functions in phase in all cases, y_1 and y_2 will then be in temporary antiphase (180° or 12 hours out-of-phase) after a time interval which depends upon k. When k = 10° (corresponding to a difference in τ of 40 minutes in time), antiphase is reached in 17½ days and the functions are back in phase in 35 days (Fig. 5a). When k = 20° (Fig. 5b), temporary antiphase is reached in 8½ days; the functions are again in-phase in 17 days. In Figure 5c, k = 40° (corresponding to a difference in τ of 160 minutes); antiphase is reached 4 days after a start in-phase.

Observations on a circadian variable having a period differing from

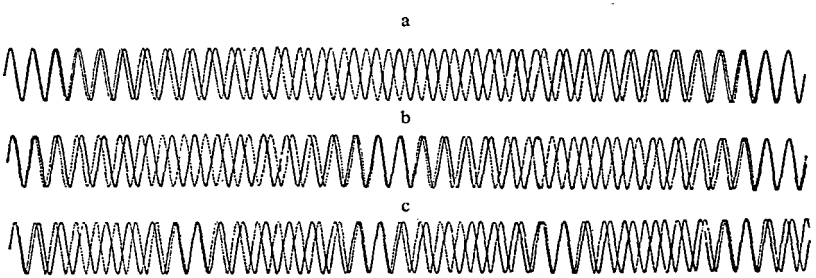

Figure 5: Changes in time relations of two circadian rhythms with different periods [see text and Fig. 4 (21-23)].

24 hours by a few minutes or a few hours, may be made on successive days at the same clock time, i.e., 24 hours apart. y_1 now corresponds to the 24-hour clock, its peak corresponds e.g., to noon. Examination of the dotted curve in Figure 5 reveals that these successive observations on y_2 made at noon (peak of y_1) will vary around the mean level in a predictable manner. Unless the desynchronization (e.g., free-running from the 24-hour clock) is recognized, a systematic variability may readily be ignored and added to the random variability. Alternatively, such desynchronization, following some experimental treatment but not a placebo, may be misinterpreted as a controlled response (see discussion of Fig. 7).

B. *Intergroup-Differences Observed at a Conveniently Fixed Clock Hour:* For many circadian functions there is a huge list of so-called biologic responses. These responses include, often, rather diverse effects, that are reported for one and the same treatment or are attributed to one and the same process. What seems particularly unsatisfactory, however, is the fact that contradictory and, in themselves, trivial responses can be obtained under conditions that usually are accepted as being controlled studies.

For instance, an intergroup-difference can change with time, in a systematic fashion. If such a temporal change follows a given treatment or operation, one may remain skeptical. There can readily be invoked the fallacies of *post hoc ergo propter hoc* reasoning. When the same change is not recorded, however, following the administration of placebos, or after a sham-operation, caution is left by the wayside and the results of the treatment or operation are commonly accepted as a "controlled response."

Many of these controlled responses are nonetheless controversial. Whether circadian desynchronization has contributed to their unreliability may be explored by spotchecks at two times of day, as shown in the abstract Fig. 6. The failure to do so, in turn, may be costly, as can be seen from the abstract Fig. 7. This figure is intended for those who, in believing that the physiology and pathology of circadian systems are too complex, have "done something about rhythms" by repeating their observations at a conveniently fixed clock hour. These students may be helped by the recognition that the time-course *and sign* (!) of an intergroup difference can be rather critically dependent upon the particular clock hour chosen for study.

In a scientific community at large the timing of observations will vary, of course, with the circadian systems of the investigators and their social schedules. Thus, the abstract Fig. 7 may not be unrealistic if it compares the results obtained by several investigators, each working at a fixed time of day, with the clock hour for daily observations differing, however, from one student to the next.

At the identical time, on day O, each of five investigators performs the same operation or treatment on a group of experimentals, and thereafter observes some physiologic function y_2. Concomitantly, a sham-operation or treatment is done for the study of the same physiologic function, y_1, on a control group. The operations may

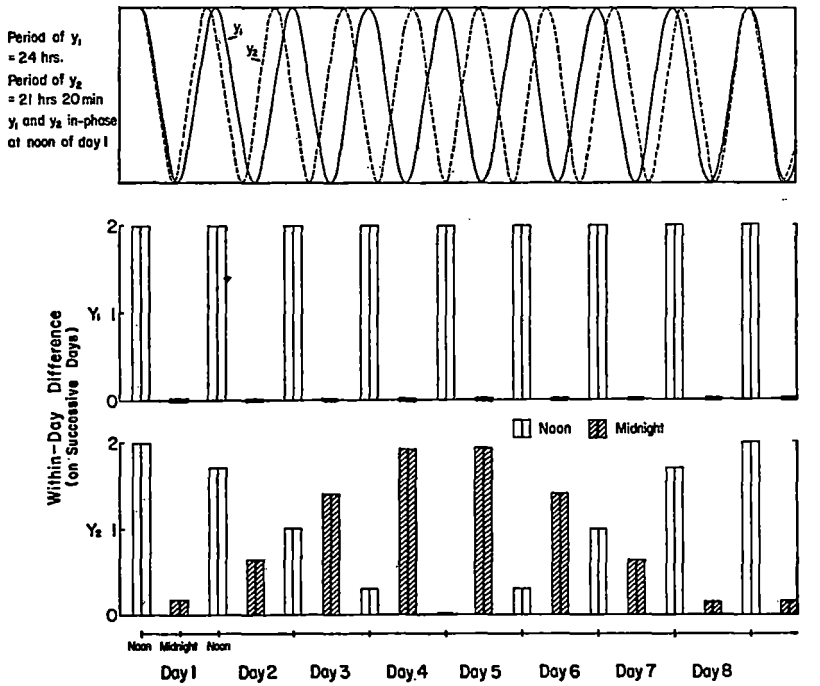

Figure 6: Time-varying behavior of "within-day difference" from observations made 12 hours apart, in a group of desynchronized (case 3, see text) experimentals (bottom) by comparison to the same difference in synchronized controls (middle). A physiological function, y, evaluated in each of these groups, is assumed to be circadian periodic; the periods of these functions, however, differ by 160 minutes in time (top). This example could represent a 24-hour synchronized function, y_1, being compared with the same function, y_2, free-running in another group. Note that y_2 shows predictable changes in within-day difference, as a function of its particular period.

$$[y_2 = 1 + \cos \frac{360}{360\text{-}40} \varphi \text{ and}$$
$$y_1 = 1 + \cos \varphi].$$

result in free-running of y_2 but not of y_1. This is shown on top of Fig. 7. Fig. 4 (21-23), in its turn, presents corresponding factual material, except that the periods of y_1 and y_2 in Fig. 4 differ by about 40 minutes rather than 160 minutes as in Fig. 7. Drawing Figure 7 with $k = 40°$ rather than 10° was easier.

An early-rising student will compare y_1 and y_2 daily at 6 a.m. (Fig. 7). His post-operative "finding" is an initial rise of his physiologic function above the control level and a subsequent fall below that level. An equally skilled person, working at 9 a.m. confirms him, although with some differences in the time course and extent of change (Fig. 7). Both presume that "effects of rhythms are eliminated since observations on controls and experimentals were done at the same clock hour (sic)." They are skeptical of course when a

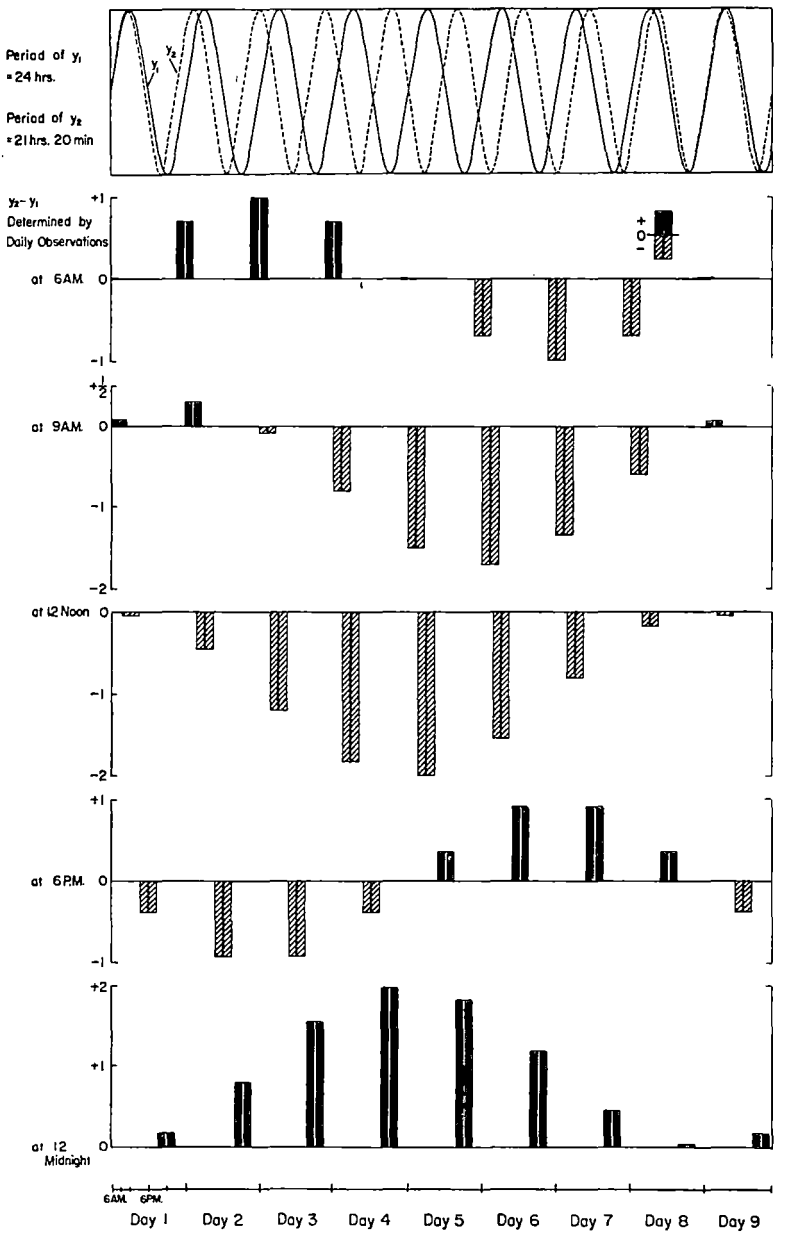

Figure 7: Time-course of an inter-group difference between synchronized controls and desynchronized experimentals—when comparisons are made 24 hours apart, at one *or* the other clock hour. A given physiologic function, y, is assumed to be circadian periodic in both groups compared. y_1 could represent a 24-hour synchronized case, while y_2 could differ in period from y_1 by 160

competent investigator of the same functions, y_1 and y_2, working each noon, describes as the result of the same operation, an initial fall (not rise!) of the physiologic function in experimentals and a subsequent return to control values.

By now a "monophasic" and two "biphasic" responses are available to describe the same post-operative phenomenon (Fig. 7). However, the situation can gain further in variety. Yet another "biphasic response" will be recorded by a student working at 6 p.m. and it will be rather opposite to that reported by his fellow 6 a.m. worker. The "monophasic response" of the man on a midnight shift, in its turn, will be nearly the reverse of that found during the customary lunch hour (Fig. 7).

Cases such as those considered in Fig. 7 are often obscured by other factors. Such "noise" renders most biologic data more complex. The phase relations of y_1 and y_2 do not always change so clearly as in Fig. 4. But even when the responses mentioned above are prominent, as in Fig. 7, their heuristic value may justly be questioned, as long as their circadian aspect is ignored.

Whether or not our research interest includes circadian periodicity as such, understanding of circadian systems (1) seems essential to interpreting one and/or the other of their "monophasic" and/or "biphasic" responses, *to the identical treatment.*

The cases discussed in Fig. 7 are germane to medicine. An important reason for the recording of responses in health and disease is the motivation to replace what is found missing and to remove what seems to be excessive. One may consider, therefore, what is gained for therapeutic action when judgment rests on responses such as those in Fig. 7.

The student working daily at noon, who recorded a "drop", e.g., in a biochemical value of his patient, will advocate replacement therapy. This would be disputed (and should be) by his colleague working at midnight, who recorded a "rise" and recommends the opposite treatment.

Were it not that the more prominent and thus more influential investigators of circadian systems are themselves synchronized by rather similar social schedules, such disputes would be much more frequent. However, whether or not a "response" is contested matters little; actually, the undisputed "result" is the more dangerous one, since it forms the basis of unwarranted clinical action.

We have noted earlier that y_1 and y_2, as computed and drawn for Fig. 7, differ in their circadian period by 160 minutes. The monophasic and biphasic responses thus "occurred" within a few days. Obviously, if the difference in the periods of y_1 and y_2 is smaller,

minutes in time (case 3 desynchronization, see text). On the plot, y_1 and y_2 start out in phase at 06, in each case. Note nonetheless that the difference between the two groups being compared will undergo drastically different changes with time, simply as a function of the particular clock hour chosen for observation. Similar patterns may be found in a plethora of publications on functions previously demonstrated as circadian periodic, but their value, as such, is questioned (see text).

these responses will be the same in principle but will "occur" more slowly—during weeks, months, or years. It may be worthwhile, in the future, to see whether any of our "controlled responses" in biology and medicine—long-term or short-term "phenomena" alike—are amenable to more meaningful resolution after scrutiny by circadian analysis. Whether or not this is so, the trivial response spectrum of Fig. 7 approximates factual observations (Fig. 4). Fig. 4, in turn, is hardly a unique curiosity, as may become apparent indirectly from many studies cited or reported at the 1960 Cold Spring Harbor Symposium on Quantitative Biology (cf. 5, 21-23).

Two Frequencies Underlying a Rhythmic Variable: Certain physiologic time series may result from two distinct periodic components that are additive functions. If y is a numerical-valued observation associated with the phenomenon of interest, and y_1 and y_2 are the corresponding values associated with the components, then $y = y_1 + y_2$. If y_1 and y_2 are not of the same period, y will exhibit beats.

The two components will reinforce or cancel each other in a periodic manner, depending upon the difference in their periods. Maximum reinforcement occurs when the phase difference is smallest and maximum cancellation occurs when the phase difference is greatest (Fig. 8).

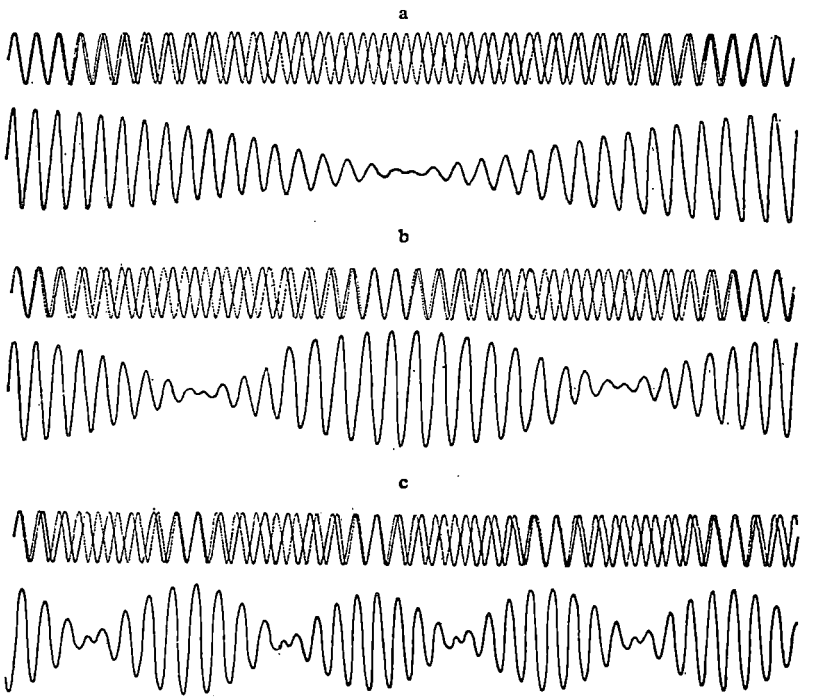

Figure 8: Beat frequency as a function of the difference in period of two component frequencies underlying a numerical-valued observation.

Using the functions defined above, for addition rather than for comparison, $y = y_1 + y_2 = \sin \varphi + \sin \left[\frac{360}{360\text{-}k} \varphi\right]$. Beat frequency then varies with k[1], related to the difference in period of the component frequencies. Beat frequencies corresponding to a k of 10°, 20°, or 40° are summarized in Fig. 8.

Figs. 8a, b, and c thus can be used to depict the beat frequency of circadian rhythms with periods differing by 40 minutes, 80 minutes, or 160 minutes, respectively; it can be seen that minimum and maximum amplitude will alternate in a predictable fashion, in these particular cases at intervals of 17½ days, 8½ days and 4 days, respectively.

Are some aspects of Figure 9 (30, 31) a physiologic counterpart of our abstract Fig. 8c? The latter was computed and drawn without reference to the former. Water excretion data, particularly those of subject D in Fig. 9, suggest that beats on physiological rhythms can indeed result from the interaction of two circadian components, that are additive functions.

Similar but not identical considerations also apply, of course, for the interaction of circadian components on the one hand with periodic components of grossly different periods on the other (32).

The data of Fig 9 (30, 31) were obtained in an isolated community in Spitzbergen, under conditions of relatively far-reaching environmental control. Most unusual and valuable is another aspect of the same study: the subjects lived on a 21-hour routine; they adhered strictly to a schedule dictated by specially adjusted wrist watches showing 12 hours during 10½ ordinary hours. If these water excretion data are interpreted as resulting from the interference of two periodic components, it seems most plausible that the artificial routine of the "21-hour day" was one such component.

The other component(s) could be the transiently continuing effect of the 24-hour schedule on which the subjects had previously lived, it may be a free-running function with a period close to 24 hours, and/or synchronizer interaction, discussed elsewhere (1, 5, 13). The factors underlying this beating circadian function are beyond the scope of this analysis, which consists solely of illustrating the pertinence of beats to the analysis of circadian rhythms.

Beats will be likely encountered in many other physiologic time series, as are usually obtained in clinic or laboratory. 24-hour periodic components usually are imposed upon living things by the 24-hour routines of activity and feeding, among other sociologic or ecologic factors. In disease, in turn, a free-running component also may come to the fore, by synchronization failure of the organism and this is of particular interest to medicine. The situation may resemble that encountered in the blinded mouse (Fig. 4), but there may be important differences as well.

After receptor removal, some blinded mice were readily uncoupled from the continued cyclic alternation of light and darkness, their

[1] The general relationship describing the beat frequency is $\frac{360° - k}{k}$ daily cycles. As above, k is the difference (in degrees) between the periods of the two functions, y_1 and y_2; and 360° corresponds to one 24-hour period. Thus, in the example, when k = 10° the time between peaks in amplitude is 35 days; when k = 20° it is 17 days; and when k = 40° the peaks are 8 days apart.

normally-dominant synchronizer. These experimental animals, however, were able to follow their own rhythms of activity and feeding. In human beings, in turn, conceivable desynchronization from social or other synchronizing schedules probably will be complicated by the

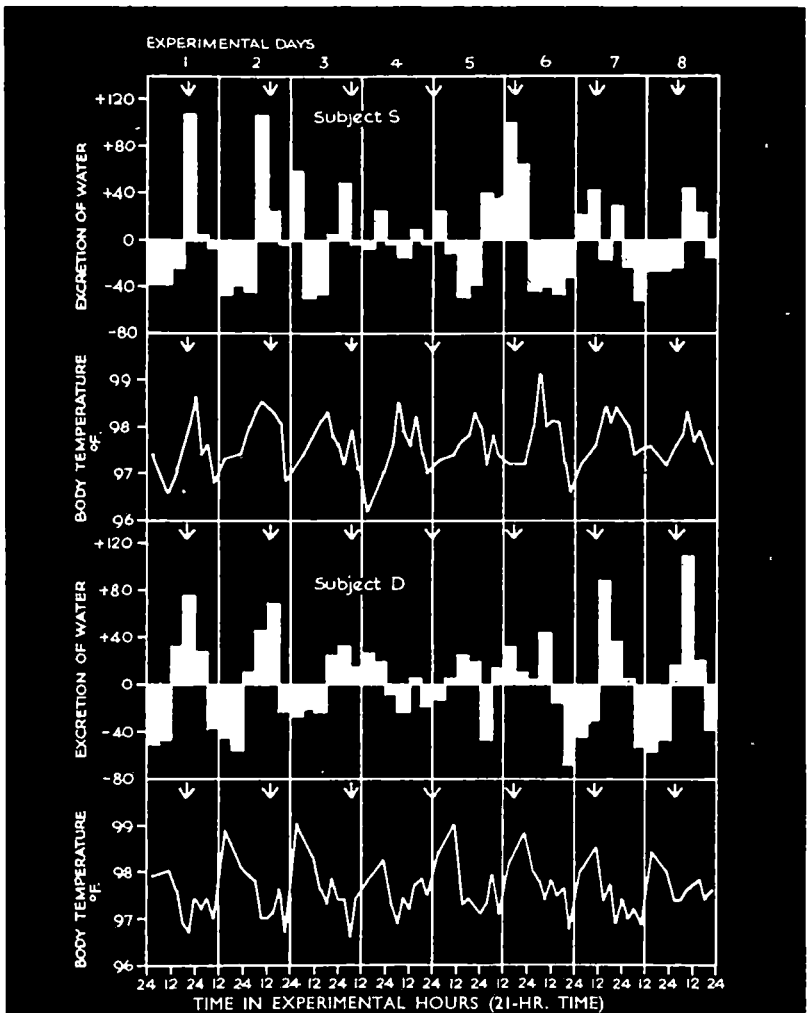

FIG. 2.—Comparison of water excretion and body temperature rhythms for 2 subjects during the first recording cycle on 21-hr. time. The rates of water excretion for successive periods of 4 *hr.* are plotted as percentages about the mean rate for each *day*. Arrows indicate the occurrence of midday by B.S.T. throughout the cycle.

Figure 9: Beats in data on water excretion [Lewis and Lobban (30, 31)]. Note particularly the findings for subject D, and compare them with the abstract Figure 8. Subjects on unusual schedules, studied during the arctic summer (see text).

added effect of a roughly 24-hour periodic schedule of activity and feeding. Along the lines of the foregoing discussion, we shall then have to analyze $y = y_1 + y_2$.

In other words, the task of evaluating a hypothesized free-running component, y_2, in the presence of y_1 (a 24-hour routine of activity and feeding) is often faced. Moreover, in clinical time series, many other components, over and above $y_1 + y_2$, will further contribute to the data. Therefore, certain circadian rhythms studied in usual life situations may end up as beats on relatively weak rhythmic signals, in rather noisy channels (5). Computational procedures for noise removal (33) have now become available as electronic computer programs; despite their availability (5), however, the need for experimental precautions to keep noise out of our time series, as far as this is economically and otherwise feasible, should not be forgotten. The standardization of conditions of observation for circadian analysis remains desirable, if not indispensable (1, 5).

Summary and Conclusions: The mere recording of clock hours in a given experimental or clinical protocol—as was done, *inter alios*, by great physiologists (cf. e.g., 34)—does not necessarily yield information on the timing of body functions (internal or external timing, Fig. 2). Unqualified clock-hour-effects, the so-called diurnal variations, are not always meaningful temporal parameters of physiologic function.

Methodologically and heuristically more promising is the concept of circadian systems (5), Fig. 2, applicable to biological entities exhibiting one or more functions with a frequency of about one cycle per day. This concept, its temporal parameters, and some of its implications to biologic methodology, are discussed and illustrated herein in some detail, Figs. 1 and 3, while the physiological mechanisms of circadian mammalian systems are discussed elsewhere (1, 13).

Circadian systems can exhibit beats on one or more of their observed functions, Fig. 9, as the result of two distinct but additive and close frequencies, underlying the same numerical-valued observation, Fig. 8.

Circadian systems are synchronized when their physiologic periods correspond to the period of a reference function, internal or external to the system. Circadian systems are desynchronized when one or more of their functions have periods different from that of a reference function. The 24-hour period is a convenient general standard of reference in circadian physiology and pathology, but it is not the sole such standard.

In comparing the circadian periodic functions of a synchronized control group with those of an unwittingly desynchronized group—or with those of a phase-shifted group—at different arbitrarily fixed clock hours, contradictory intergroup-differences are readily obtained. In repeating such comparisons, e.g., daily or weekly, at some convenient yet fixed time of day, controversial and, as such, meaningless biological "responses" are usually recorded, Fig. 7.

The problem whether or not a circadian system is desynchronized,

or shifted in its phase—in these cases, in particular, physiologic time relations are of methodologic interest—can be explored by relatively few spotchecks, e.g., at the time of the peak and trough of a synchronized control group, Fig. 6.

Circadian desynchronization can be the sole objective finding which characterizes a given physiological function observed in frank abnormality, e.g., immediately following blinding, Fig. 4. Thus one can ask whether changes of the (external or internal) timing of circadian systems are detrimental—particularly if they are long-continued. Functional alterations in disease, in terms of the *too early* or *too late* along a circadian scale, are of interest, since in the long run, the effects of an early rise in a biochemical function, for instance, may be equated, perhaps, to *too much*, while *too late* an increase of the same biochemical quantity may ultimately be *not enough*.

It must be recognized, further, that in the case (3, see text) of desynchronization of a biochemical variable, its integrated 24-hour value can remain grossly the same as that of synchronized controls. Research on possible deviations from normal, in terms of too much or too little, employing an integrated 24-hour value as reference standard may thus be noncontributory in those very instances in which the circadian search for too early to too late may prove to be most rewarding.

To the extent to which these assumptions are correct, the introduction of circadian temporal parameters into studies of integrative and adaptive biologic functions constitutes a necessity, rather than a matter of choice. The concern for the "right circadian time" often seems just as indispensable for a controlled experiment as are the other important considerations of the "right place" for the "right amount" of the "right compound" i.e., the rules of morphology, biochemistry, pharmacology, etc. (cf. 4 and 5).

Whether or not one is interested in circadian systems as such, information on their synchronization or desynchronization, *inter alia*, should be available *before* considering undertaking an experiment or test—on any function that is known to exhibit significant time-varying behavior along the 24-hour scale. The decisions as to *when* to start observations and as to the intervals between successive observations also presuppose information on circadian system physiology, unless we wish to chance describing biologic responses alike to those in Fig. 7.

The degree of generality of these considerations seems clear if one realizes that there are many circadian physiologic functions, Figs. 1 and 3, and that a number of them show sizable peak-to-trough differences (5).

XII. *Acknowledgments and Comments*

Dr. Mary Lobban of London, England, kindly permitted the reproduction and discussion of her work in Fig. 9. Professor Otto Schmitt of the University of Minnesota, Minneapolis, gave valuable advice on physical models and nomenclature, and Professor Erwin Bünning, of the University of Tübingen, Germany, kindly referred

us to the work of Naylor (ref. 32). The term "circadian system" approximates Prof. Bünning's term "endo-diurnal system," and his authoritative book on the physiologic clock (*Die physiologische Uhr*. Berlin, Springer-Verlag, 1958, p. 105) offers valuable information on biological rhythms in general.

Circadian-system physiology, as discussed herein, *cannot* be fully identified, however, with the search for some biologic clock. The time-measurement in and by biologic systems remains an intriguing, but thus far apparently unsolved problem.

Outstanding students of biological clocks have themselves recognized that each rhythm is not a clock; they regard rhythms merely as the hands of a clock. Such interpretations, based on univariate observations, should not lead one, e.g., to discuss the menstrual and estrous cycles exclusively as the hands of a "clock," rather than as temporal interactions as well of the end-organs involved and the ovary, the pituitary, and the central nervous system. To carry this further, circadian parameters of the adrenal cycle have not infrequently been viewed by others, exclusively as the effect of some as yet mysterious "diurnal clock," and some students of rhythms continue indeed to do so. Whatever one's viewpoint, however, it may be remembered that important temporal parameters in biology are amenable to quantitative, reproducible, and physiologically meaningful study, whether or not one wishes to study time measurement *per se*.

As to biologic time measurement as such, it has two measurable facets, i.e., external and internal timing. External timing of physiological function depends heavily upon interactions among circadian rhythms, i.e., upon the plasticity of internal timing.

At the moment, external timing is studied mostly by the univariate approach and by modifications solely of the environment rather than by focusing upon internal timing within the system itself. The limits of this approach have led to discussions of timing mostly by analogy to physiologically unqualified "black boxes," clocks, or oscillators. Alternatively, physiologic models have been explored (cf. 13); more information is needed, among others, on circadian sequences of related cellular events, steroidal pacemakers, and juxtaposed as well as superimposed neural and humoral controls.

The system-phase, among other temporal aspects of circadian biological entities, is sufficiently critical to be analyzed in its own right—at a time when it is shown, e.g., to hold the balance between life or death from drugs or noxious agents (Fig. 3; 35). But irrespective of any interest in circadian-system physiology and pathology for its own sake, this methodologic paper was intended to sketch the bearing of circadian temporal parameters upon experimental method in biology and medicine. Circadian analysis might be incorporated in much seemingly unrelated research. An early example is a "mémoire" by Charles Chossat, M.D. (36), awarded the prize for experimental physiology by the French Academy of Sciences in 1841.

Some of Chossat's data, Figure 10, are pertinent to recent elegant studies on relatively temperature-independent biologic time-measurement. He was interested in mechanisms of circadian changes and alluded to the role played by the nervous system. More dramatically, Chossat reported highly significant physiologic changes along the 24-hr. scale in the body temperature of birds completely deprived of food and water until the day of their death, Figure 10 (36).

Body temperature rhythm of pigeons kept at different ambient temperatures—with and without food and water. (Analysis of Chossat's data published 1843)

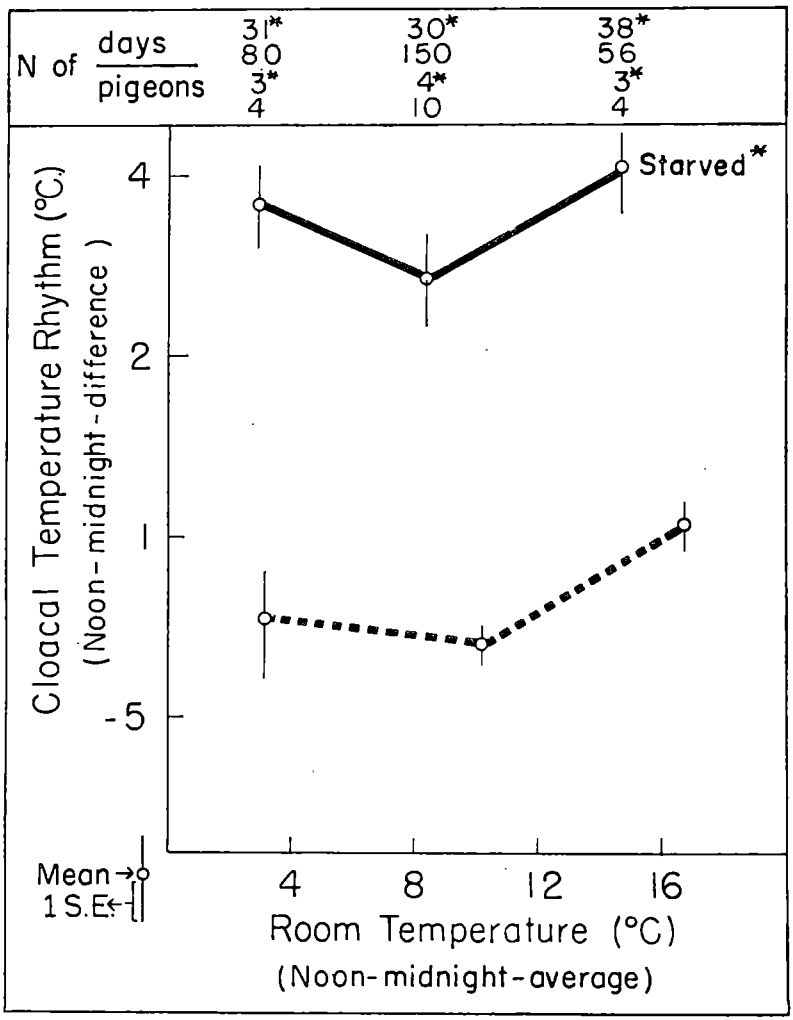

Figure 10: Significant within-day differences in cloacal temperature of birds feeding and drinking *ad libitum* (broken line) or completely deprived of food and water (continuous line). Computations based upon data in Tables 60 and 68 in reference 36. The within-day differences in body temperature are significant over a wide range of ambient temperatures. Lighting regimen unfortunately not stated. Chossat's data suggest that significant circadian periodicity persisted until the day of death from starvation and dehydration.

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