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CIRCADIAN CHRONOPHARMACOLOGY[1]

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CHRONOBIOLOGY: A BASIS FOR CHRONOPHARMACOLOGY

INTRODUCTORY EXAMPLES

Biologic responses to various agents, including chemical substances such as drugs or poisons, are neither constant as a function of time nor are they subject solely to random variations (1–11). A rapidly growing number of circadian (about-24-hour) (1–11), circannual (about-1-year) (12) and other (13, 14) rhythms (Table 1) of susceptibility are being reported for various life forms including human beings.

As a first example, let us consider the effects of ACTH upon adrenal secretion in the mouse (Haus & Halberg 15, 16). For at least one week before the study and during sampling, male and female mice of the Bagg albino (C) stock were maintained in individual cages on a regimen standardized for periodicity analysis (17)—12 hours of light (from 06^{00} to 18^{00}) alternating with 12 hours of darkness (from 18^{00} to 06^{00})[2]—with room temperature being kept at $24 \pm .5°C$ and food and water being freely available at all times. In each of two experiments, two groups of animals, comparable as to genetic background, sex, and past history, were divided into three subgroups. At 4-hour intervals, from 08^{00} on one day to 08^{00} on the next, 10 animals from each subgroup were killed: Subgroup 1, immediately; Subgroup 2, 15 minutes after an injection of saline (0.2 ml/20 gm body weight, i.p.); and Subgroup 3, 15 minutes after an injection of ACTH (0.4 I.U./2 ml/20 gm b.wt., i.p.). Determinations of corticosterone concentration were made on pooled sera (5 mice) and on adrenal gland pairs from individuals.

Untreated animals showed a circadian rhythm of corticosterone concentration in serum and gland with a crest at about 16^{00} and a trough at

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[2] Clock hours are given according to international nomenclature in hours followed by minutes as superscripts. Thus, 6 A.M. = 06^{00}; 37 minutes past 6 P.M. = 18^{37}; noon = 12^{00}; midnight = 00^{00} (of the following day). Alternation of 12 hours (h) of darkness (D), and 12 h of light (L) can be written: $LD_{12:12}$; constant light = LL; and constant darkness = DD. In the given experimental example, we could write: $L06^{00}$-18^{00} $D18^{00}$-06^{00}.

TABLE 1.

ILLUSTRATIVE SPECTRUM OF SOME HUMAN RHYTHMS IN HEALTH

DOMAIN:*	HIGH FREQUENCY $\tau \leq 0.5h$	MEDIAL FREQUENCY $0.5h \leq \tau \leq 6d$	LOW FREQUENCY $\tau \geq 6d$
REGIONS:	$\tau \sim 0.1$ s $\tau \sim 1$ s et cetera	ULTRADIAN $(0.5 \leq \tau < 20h)$ CIRCADIAN $(20 \leq \tau \leq 28h)$ INFRADIAN $28 \leq \tau < 6d)$	CIRCASEPTAN $(\tau \sim 7d)$ CIRCAVIGINTAN $(\tau \sim 20d)$ CIRCATRIGINTAN $(\tau \sim 30d)$ CIRCANNUAL $\tau \sim 1$ yr.
RHYTHMS in:	Electroencephalogram Electrocardiogram Respiration Peristalsis	REM, Rest-Activity Sleep-wakefulness Responses to drugs Blood constituents Urinary variables Metabolic processes, generally	Menstruation 17-Ketosteroid excretion with spectral components in all regions indicated above and in other domains

*Domains and regions [named according to frequency (f) criteria] delineated according to reciprocal f, i.e., period (τ) of function approximating rhythm. s = second, h = hour, d = day.

Several variables examined thus far exhibit statistically significant components in several spectral domains.

about 04^{00}. The animals injected either with saline or ACTH in constant dose and at fixed times also showed a circadian rhythm. However, the response assessed as corticosterone level elevation varied as a function of the phase of the adrenal cycle in which the treatment was introduced. In serum, as well as in adrenal, the greatest response to the saline injection occurred 4–8 hours before the circadian corticosterone crest, while the greatest response to ACTH was found about 8 hours after this crest (15, 16).

In further experiments Ungar & Halberg (18) demonstrated a circadian rhythm in the in vitro response of mouse adrenals to graded doses of ACTH (Figure 1, on the right). Corticosterone secretion from adrenals incubated with fixed doses of ACTH in the medium depended upon the circadian phase of the gland at the clock hour at which the adrenals were removed. When mice are standardized with $LD_{12:12}$(L from 06^{00} to 18^{00}) the circadian crest of adrenal susceptibility to ACTH occurs at about 04^{00} while the circadian trough in response occurs at about 16^{00}.

Of particular interest was the occurrence of a statistically highly significant predictable change in the slope of this in vitro response (steroid production) of murine adrenals to ACTH. Such determinations of dose-response relations at different stages of a circadian (or other) rhythm have also been applied to the response (mortality) of mice to endotoxin by Halberg et al (19) and to whole body irradiation by Haus et al (20). This approach, complementing single-dose chronobiologic studies, does not invariably reveal a change in the slope of the dose response (Figure 1, on the left). A parallel shift of the dose-response relation may suggest that the mechanism of action is similar at the times tested and that other factors account for the change in

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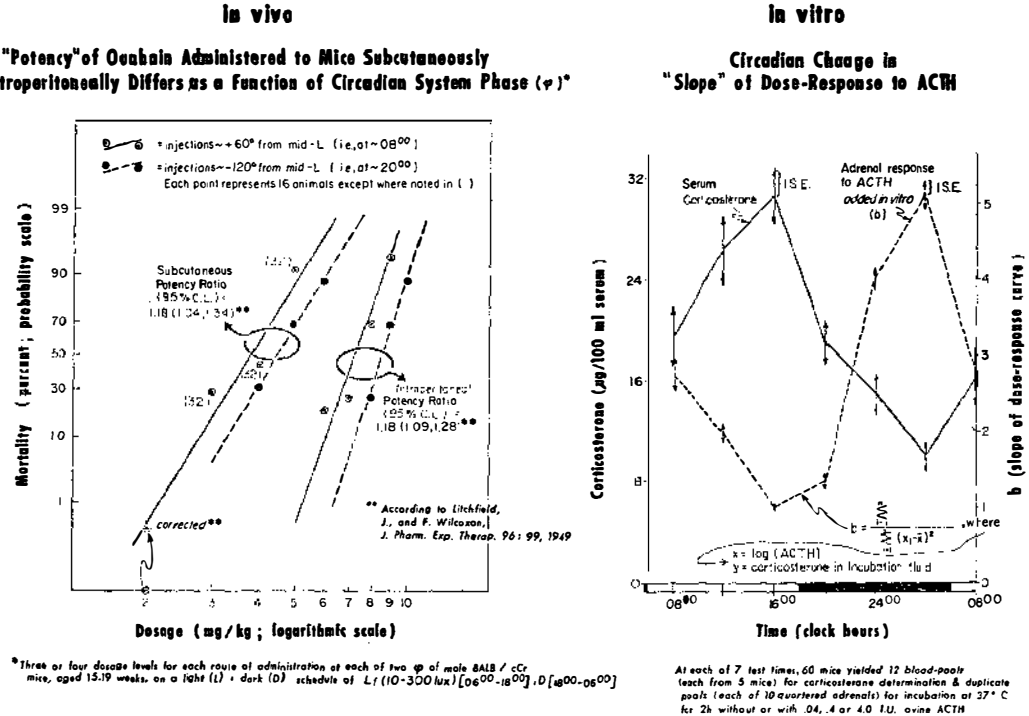

FIG. 1. The slope of a dose response relationship is usually assumed to remain the same under different conditions, even if the slope's position along the dose scale changes. Earlier chronotoxicologic data in keeping with this assumption (18) have recently been complemented by Nelson et al (21), as shown on the left in this figure. However, on the right in this same figure a drastic change in slope is seen for a dose response relationship tested in vitro in the absence of interacting neural controls and humoral agents other than those present in the quartered gland at the time of its removal for incubation (18 cf 210).

susceptibility, such as changes in sensitivity at the site of action or differences in absorption, distribution, metabolism, or excretion. By contrast, a change in slope may indicate a change in the agent's mechanisms of action, thereby providing a more meaningful measure of the factors underlying a change in susceptibility. These points have recently been discussed in a dose-response (mortality) evaluation of a circadian rhythmic change in susceptibility of mice to ouabain by Nelson et al (21) (Figure 1, on the left).

Whatever the underlying mechanisms, the fact that part of the variability underlying responses to drugs is rhythmic rather than random compels us to dispense with the still all-too-common view that—apart from changes with development, growth, and aging—organisms behave in a more or less constant fashion along the scales of a day, a month, or a year. In abandoning this misconception, one may not only wish to control rhythms as a source of variability [Figure 2 (53)], but also may endeavor to exploit information on rhythms by focusing upon recent advances in chronobiology as they relate to biology and medicine (8, 9, 23–27). While a summary of specific advances in biorhythm research remains beyond the scope of this review, the reader interested in biological rhythms can be referred to reviews and books which reveal the background of the field (6, 8, 28–52, 211).

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Definitions and Characteristics

Biological rhythms can be defined as statistically validated physiologic changes recurring with a reproducible waveform in several frequency domains (Table 1). From a "macroscopic" or gross point of view, rhythm denotes a reliably periodic aspect of data displayed as a function of time. With the help of electronic computers and special programs developed for this purpose at the University of Minnesota (54–57) it is now possible to obtain "microscopic" as well as gross displays serving for the detection and quantification of any rhythms characterizing the data. Once one or several rhythms have been objectively detected in a biologic time series, each of them can be characterized by the inferential statistical estimation of several parameters: *period*, τ, and/or *acrophase*, ϕ, φ, or Φ, Table 2, (crest time of the function used to approximate the rhythm), *amplitude*, C, and *rhythm-adjusted level*, C_0. Endpoint and confidence interval estimates for these rhythm parameters are obtained by the use of approximating functions—among others, by the least squares fit of a cosine curve (or of several of them) of the form: $y = C_0 + C\,\mathrm{Cos}(\omega t + \phi)$, where ω = angular frequency and t = time, as sketched in Figure 3.

For example, data on adrenal cortical reactivity in vitro were reanalyzed (8) according to the cosinor method (56–58). The 24-hour cosine function fitted by least squares had a period = 24 hr = 360°; hence, 1 hr = 15°. The middle of the daily L-span (mid-L), a point on the synchronizing environmental $LD_{12:12}$ cycle, was taken as phase reference for these night-active rodents. For mice synchronized in L $06^{00}-18^{00}D18^{00}-06^{00}$, mid-$L$ is 12^{00} (noon), at $-180°$ from 00^{00} (local midnight). The acrophase is then usually

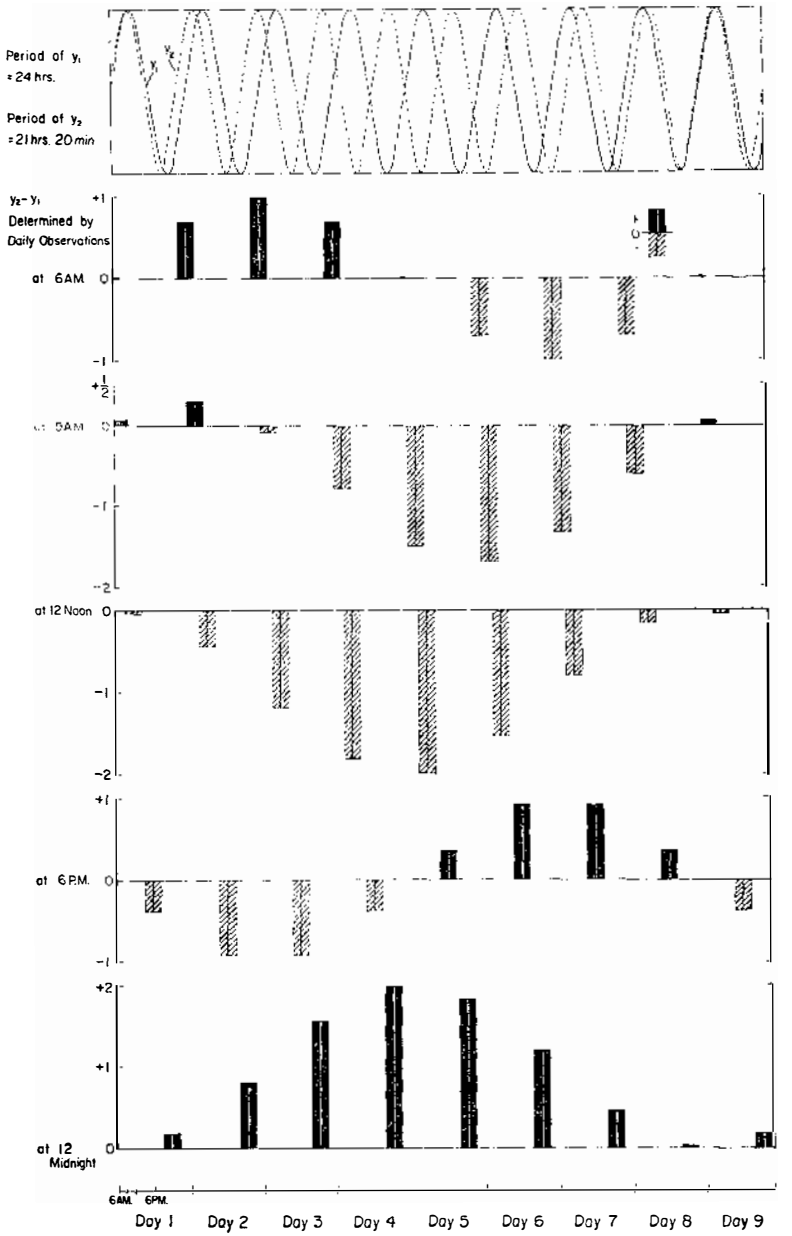

FIG. 2. Circadian system physiology is not merely a study of clock-hour effects. Work at a fixed time of day does not forestall disastrous pitfalls possibly invalidating much research on rhythmic functions. This is seen from a study of the time-course of an inter-group difference between synchronized controls and desynchronized ex-

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perimentals—when comparisons are made 24 hours apart, at one or the other clock hour (53).

In this figure, 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 minutes in time. On the plot, y_1 and y_2 start out in phase at 06^{00} in each case. Note that the difference between the two groups being compared will change drastically with time, as a function of the particular clock hour chosen for observation.

This figure is intended for authors (and referees!) who, believing that chronobiology is 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 a 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 of course vary with the circadian systems of the investigators and their social schedules. Thus, the abstract Figure 2 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 0, 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 result in free-running of y_2 but not of y_1. This is shown at the top of Figure 2.

An early-rising student will compare y_1 and y_2 daily at 06^{00} (second row in Figure 2). His post-operative "finding" is an initial rise of the physiologic function above the control level and a subsequent fall below that level. An equally skilled person working at 09^{00} confirms his observation but with some differences in the time course and extent of change (third row in Figure 2). Both presume that "effects of rhythms" are eliminated since each made his observations on both controls and experimentals at the same clock hour the same day of the week, or the same time of the year. They are skeptical of course when a third equally "competent" investigator of the same functions, y_1 and y_2, working at 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. Yet another "biphasic response" will be recorded by a student working at 18^{00} and it will be rather opposite to that reported by his fellow 06^{00} worker. The "monophasic response" of the man on a midnight shift (bottom of Figure 2), in its turn, will be nearly the reverse of that found during the customary lunch hour.

Results such as those considered in this figure are often complicated by yet other factors. Such "noise" renders most biologic data more complex. Whether or not our research interest includes circadian and other rhythms as such, understanding of circadian or circannual systems seems essential to interpreting one or the other "monophasic" or "biphasic" response to the identical treatment.

The cases here discussed are germane to pharmacology and therapeutics, insofar as animal experiments and clinical trials are concerned. In such endeavors we may record even from healthy subjects responses such as those presented in this figure, notably if we attempt to "control" conditions by instituting, say, constancy in a number of environmental factors such as the lighting regimen for an experimental animal

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given as delay from the phase reference. The delay is indicated by a negative sign whereas an advance is shown as positive. Ninety-five percent confidence limits (CL) are usually given in parentheses. Under the above-defined experimental conditions ($LD_{12:12}$), the φ for the circadian rhythm of in vitro adrenal reactivity to ACTH is at $-235°$ from mid-L ($-210°$ to $-258°$), while that for serum corticosterone is at $-66°$ ($-42°$ to $-89°$) and that for adrenal corticosterone at $-79°$ ($-47°$ to $-112°$).

With reference to the period, τ (or rather to its reciprocal, $1/\tau = f$ or frequency), rhythms can be analyzed in terms of a *spectrum* with statistically significant components in several spectral domains (6, 8, 22, 53, 56). These domains shown in Table 1 correspond to *high frequency rhythms* ($\tau < 0.5$ hour), *medial frequency rhythms* ($0.5\ h \leq \tau \leq 2.5$ days) and *low frequency rhythms* ($\tau > 2.5$ days). *Circadian rhythms* ($20\ h \leq \tau \leq 28\ h$) fall in the medial frequency domain and include most of the cyclic changes in susceptibility of animals to toxic or pharmacologic agents, or both, described in recent years. We intend to focus this review on such cyclic changes. It should be noted, however, that intensive work continues to explore drug effects not

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group. In the event that we synchronize our animals, like a human being is synchronized by his social routine, we must also ascertain that the period or phase of a rhythm is the same in both the presence and absence of drug administration or disease. If this is not the case, "responses" such as those in the figure may mislead us to wishing to replace what is superficially believed to be missing (yet is not missing) or to remove what superficially (and wrongly) appears to be excessive. Let us consider more specifically against the background of the figure what may be gained for therapeutic action when the responses shown are used as a basis for judgment and an unevaluated rhythm confounds the results.

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, 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 this figure, 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, these responses will be the same in principle but will "occur" more slowly—after 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 and short-term "phenomena" alike—are amenable to more meaningful resolution after scrutiny by circadian analysis. Whether or not this be so, the trivial response spectrum of this figure approximates factual observations, as may indirectly become apparent from many studies cited or reported at the 1960 Cold Spring Harbor Symposium on Quantitative Biology (e.g., 1) (53).

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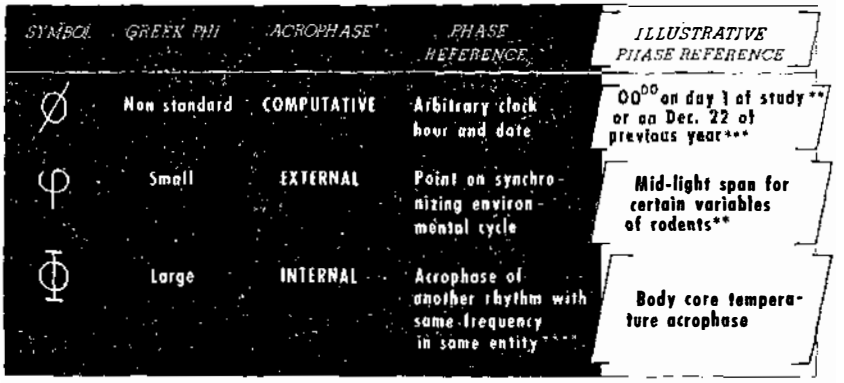
TABLE 2.

Symbols and Terms for Different Acrophases*



SYMBOL	GREEK PHI	ACROPHASE	PHASE REFERENCE	ILLUSTRATIVE PHASE REFERENCE
∅	Non standard	COMPUTATIVE	Arbitrary clock hour and date	00^{00} on day 1 of study** or on Dec. 22 of previous year***
φ	Small	EXTERNAL	Point on synchronizing environmental cycle	Mid-light span for certain variables of rodents**
Φ	Large	INTERNAL	Acrophase of another rhythm with same frequency in same entity****	Body core temperature acrophase

* *As estimates of a rhythm's timing in relation to different phase references*

** *For circadian rhythm(s)*

*** *For circannual rhythm(s)*

**** *Entities exhibiting rhythms include (spatially) cells, tissues, organs, organ-systems, organisms and environments, or sets of any one of these, or (temporally) processes ranging from intracellular to ecologic.*

only upon classical high-frequency cycles (Table 1), but also upon low-frequency rhythms. Studies of interest to pharmacologists, some of them reviewed by Haus & Halberg (12), include those by Beauvallet et al (59, 60), Aron et al (61), Miller et al (62–64), Kayser et al (65, 66), Hayashi (67), Petkov (68) and Matsuno (69).

Within certain limits the period, amplitude, acrophase, or waveform of circadian rhythms can be influenced by the cyclic variations of certain environmental factors—the alternation of light and darkness, heat and cold, noise and silence (1, 17, 28–33)—and possibly also by electro-magnetic fields (70–72). Thus Wever examined the circadian periodicity of human subjects in two insulated underground bunkers, one of which was shielded against the effects of static magnetic field. Individuals or groups were isolated in the two bunkers for periods from 3–6 weeks. During the span of isolation, activities as well as temperature, urine samples, physiological data, and psychological data were explored. A gross tendency toward internal desynchronization was reported and thought to affect optimal performance (72).

Most important, however, for modern man, are his artificial schedules of work and rest and the associated exigencies of social life (1, 6, 8, 73, 74, 79). These factors are called synchronizers (31, 37, 75, 76), Zeitgebers (77) or entraining-agents (78), the three terms being synonymous.

Since under the conditions of daily life in developed areas socio-ecologic synchronizers (79) prevail for man, the average period, τ, of our circadian

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SCHEME OF QUANTITATIVE RHYTHM CHARACTERISTICS

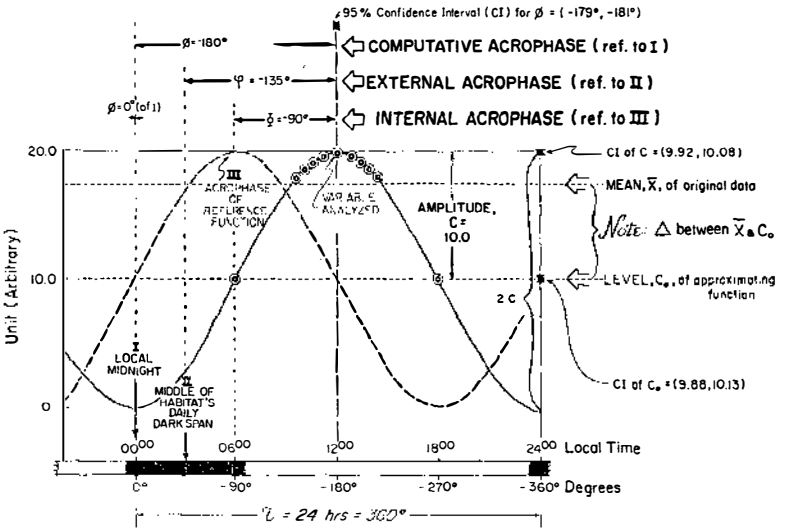

FIG. 3. Rhythm characteristics obtained by the least squares fit of a single cosine curve: The level, which can be different from the mean in the case of unequidistant data: The amplitude and the extent of total change predictable by the fit of a single cosine curve, namely the double amplitude; The several types of phase estimate (representing the timing of the cosine curve approximating all data best): (*a*) computative acrophase referred to some computationally convenient reference such as midnight local time, (*b*) The external acrophase referred to a point on some environmental cycle such as the midpoint of the dark-span (in one's bedroom or habitat niche) when living on a 24-hour cycle of light and darkness, or (*c*) the internal acrophase referred to the peak of another cosine curve approximating another physiologic series used as the reference series.

rhythms is, on the average, 24 hours in length, in keeping with the alternation of activity (in light) and rest (in darkness), tied to relatively regular hourly exigencies. The τ will be the same for experimental animals kept in $LD_{12:12}$, even for rhythms at the cellular level (79–87). Following certain manipulations of synchronizers, modulators, or influencers (8) (or of the organism), changes in period or in other parameters can be observed. However, although extrinsic factors can influence the τ or ϕ, C, and C_0 of the rhythms investigated thus far, these factors do not necessarily bring about rhythmicity as such. The organism's time structure, revealed by several of its biorhythms (8), can be considered as being at least partly genetically determined; the major promise of modern methods for microscopic rhythmometry lies in their potential to quantify concomitantly the relative contributions of nature and nurture to a given rhythm, as has been done by Simpson et al (88).

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The study of bioperiodicity, including circadian susceptibility rhythms, is best performed under several special standardized conditions (17). Thus, one may manipulate the rest-activity schedules of human beings (88) and thus, as far as possible, sleep-wakefulness as well. To begin with, 24-hour synchronizer cycles "acceptable" in terms of phase as well as period can be instituted. Effects of synchronizer phase-shifting, a most powerful tool for the study of mechanisms, also are important to study once a fixed synchronized state is appropriately described in both biologic and inferential statistical terms. Other valuable study conditions are the removal of known synchronizers or their imposition on cycles of lengths other than 24 hours, or both.

By the same token, for certain experimental animals the lighting regimen to which they are exposed may be manipulated according to special test sequences (89) while one collects longitudinal data, e.g., on gross motor as well as feeding activity or on body temperature, or both. Such monitoring by telemetry from experimental animals is particularly valuable before the withdrawal of single samples (89) or the exposure to stimuli or drugs tested in specific circadian system phases. A similar indirect definition of circadian phase may be achieved with self-measurements by human beings. The specification of study conditions, notably the kind, duration, and achieved stability of synchronization prior to study as well as during sampling, leads to data that, after microscopic analyses, lend themselves to the exploration of the several subspecialties in the fledgling field of chronobiology.

Chronobiology is the study of the temporal characteristics of biologic phenomena, leading to an objective description of biologic time structure (8). Biologic time structure, in turn, can be defined as the sum of nonrandom, and thus predictable, temporal aspects of organismic behavior including among others bioperiodicity and developmental changes; it characterizes species, groups of organisms, and individuals, as well as their sub-divisions: organ systems, organs, tissues, cells, and intracellular elements (including ultramicroscopic structures). Rhythmic changes can be demonstrated at all these levels of organization and can be considered objectively as a fundamental property of living matter (6, 8, 22, 28).

Chronobiology includes the following "subspecialties," among others:

1. *Chronopharmacology*: investigation of drug effects upon rhythm characteristics, on the one hand, and as a function of biologic timing on the other hand.

2. *Chronotoxicology*: investigation of undesired or harmful effects from chemical, physical, or other agents including poisons, pollutants, and overdoses of drugs upon rhythm characteristics and as a function of biologic timing.

3. *Chronophysiology*: investigation of temporal features in physiologic behavior and of physiologic factors underlying biologic temporal characteristics.

4. *Chronopathology*: investigation of alterations in biologic temporal characteristics as a function of disease and as determinants of disease. Ex-

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perimental reports relating to fields 1 and 2 above have been selected for this review on the following bases: (*a*) appropriate methodology for the control synchronizers and for data gathering; (*b*) accurate technical procedures for determinations, measures, etc. on each biologic variable selected as an index of periodicity; and, what is most important, (*c*) statistical analyses of the time series with preference for "microscopic" as compared to exclusively "macroscopic" methods of parameter estimation. At our stage of knowledge in chronobiology, priority has to be given to the quantitative and inferential statistical description of rhythmic phenomena as a *sine qua non* in the approach to other problems (8).

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THE HOURS OF CHANGING RESPONSIVENESS OR SUSCEPTIBILITY

This new concept has been experimentally demonstrated in animals (1–3, 10, 11) as well as in man (5–7). The study of the hours of changing responsiveness has been leading to concepts of circadian chronotoxicology and circadian chronopharmacology. As a matter of fact, regular and predictable circadian changes in biologic susceptibility can now be viewed as a rather common phenomenon. The following review of chronotoxicology may serve to introduce the topic of temporal aspects in drugs effects.

Experiments were carried out with mice of the inbred D_8 and C (Bagg albino) stocks. During a standardization span of at least one week and throughout sampling, mice were kept singly housed at 24 ± 0.5°C, in $L06^{00}$ -18^{00}, with food and water freely available. Separate groups of mice, comparable as to body weight, age, and sex, were injected or submitted to stimulation at 4-hour intervals, starting at 08^{00} of one day for a first group and ending 24 hours, 48 hours, or yet later for the last one.

Several types of agents were tested:

E. coli Endotoxin (*90, 91*).—In several experiments, seven groups, each composed of 15–20 C-mice were given intraperitoneal injections of *E. coli* lipopolysaccharide (Difco) in a dose of 100 mg/0.2 ml per 20 mg of body weight. Mortality was recorded at 4-hour intervals for 2 days after injection.

Ouabain (*90, 93*).—0.5 or 0.15 mg of ouabain (Lilly) per 20 g of body weight in 0.2 ml of saline was given intraperitoneally to separate groups of D_8 and C mice, respectively. The number of deaths was recorded after each of the 4-hour tests—at 10 minutes post-injection (by that time most deaths had occurred) and at several later time points until one week post-injection.

White Noise (*10, 11*).—At several test-times along the 24-hour scale D_8 mice were transferred individually from their cage to a stimulator, yielding white-noise ($\sim$104 db above 0.0002 dynes/square centimeter r.m.s. pressure). Stimulation was of 60-second duration. Audiogenic convulsions and deaths were recorded for each group.

Librium (*94*).—Intraperitoneal injections of this neuroleptic drug were given at 4-hour intervals to D_8 mice (5.4 mg/20 g body wt). Survival time was recorded.

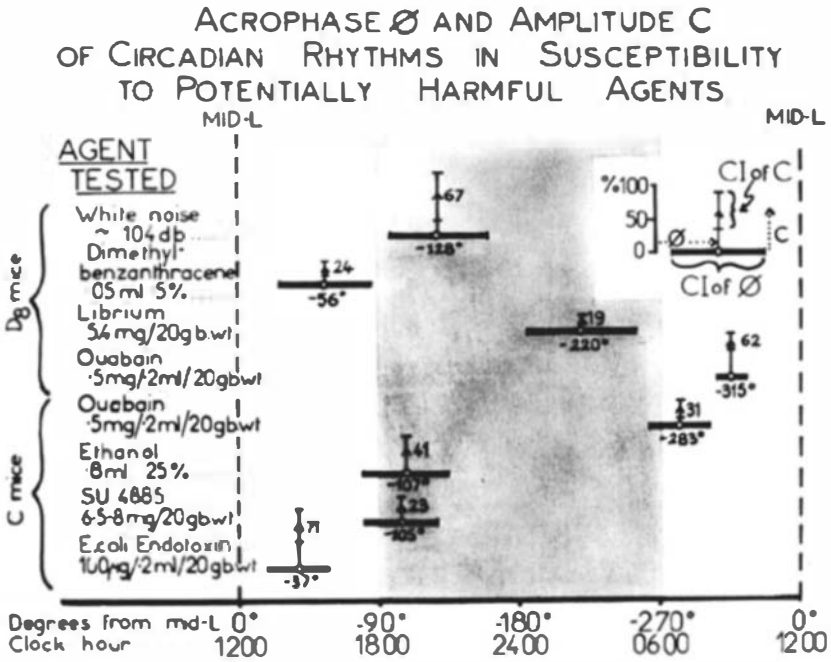

FIG. 4. Combined circadian acrophase and amplitude chart for two strains of inbred mice (original data summarized in Ref. 2).

Ethanol (*95*).—*C* mice were injected intraperitoneally with 25 percent ethanol solution (0.8 ml/20 g body wt). Mortality was recorded.

Methopyrapone or Su 4885 (*96*).—Groups of *C*-mice were given this compound (6.5–8 mg/20 g body wt), which is a specific inhibitor of the 11-beta steroid hydroxylase. Percent of death in each group was recorded.

Dimethylbenzanthracene (*97*).—This agent was used in doses of 0.05 ml of 0.5 percent solution to induce breast cancer in D_8 mice. Percent of animals with tumor was recorded under standardized experimental conditions.

Circadian rhythms in susceptibility to these potentially noxious agents were first macroscopically analyzed by procedures designed to locate peaks in time series (98). Results revealed two points: (*a*) agents as different as a bacterial endotoxin *E. coli* or Brucella melitensis (99), a plant alkaloid (ouabain), a neuroleptic (Librium), an enzyme inhibitor (Su-4885), a carcinogen, or noise (acoustic stimulation) can be used to uncover hours of changing responsiveness; and *b*) the timing of crests and troughs in susceptibility can vary from one agent to the other.

The same time series were reanalyzed by the fit of 24-hour cosine curves (57), and results thus obtained are summarized in Figure 4. These analyses

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reinforce conclusions (*a*) and (*b*) above with complementary information: apart from an added inferential statistical validation of the circadian rhythms themselves, this "microscopic" approach allows parameter estimations, providing the C and φ with their 95% confidence limits—also drawn in Figure 4.

Such facts should lead one to test the probability that circadian rhythms of responsiveness or susceptibility probably characterize many more agents and species. This assumption is supported thus far by macroscopic and microscopic studies on plants, several species of insects, other strains of mice and rats, and on a wide variety of chemical agents, as can be seen in Tables 3 and 4. In all these various experimental circumstances circadian rhythms in susceptibility to toxins were reported.

The occurrence of circadian rhythms in susceptibility to several potentially harmful physical agents has been reported: leaf resistance of a plant (*Kalanchoe*) to heat (Schwemmle et al, 130), recovery rate of *Drosophila* from heat (Pittendrigh, 51), mortality of *Drosophila* from x-rays (Rensing, 131), mortality of mice from x-rays, assessed as LD_{50} (Halberg 1, Haus et al, 20) or by other endpoints (Pizzarello et al, 132, Nelson, 133), mortality of rats and of CBA mice from gamma irradiation (Grigoryev et al, 134) or body weight loss from partial body x-rays (Garcia-Sainz et al, 135).

This coherent body of knowledge leads one to question the validity of evaluating acute, subacute, or chronic toxicity of a given drug by a time-unqualified median lethal dose (LD_{50}), corresponding to the quantity of drug which has to be administered to individuals of a presumably homogenous group of animals in order to kill 50% of them (2, 3, 6, 7). Definition of an LD_{50} test in terms of its timing has to be added to other conventional references such as species, sex, and age of test animal, among others (1, 132). Thus one could refer to a maximal LD_{50} at the circadian resistance crest for a given drug or agent and to a minimal LD_{50} (located in time) with a specified phase difference from the resistance crest (e.g., 180°).

CIRCADIAN CHRONOPHARMACOLOGIC EFFECTS CAN BE DEMONSTRATED AT ALL LEVELS OF ORGANIZATION AND FOR AN ALREADY WIDE VARIETY OF CHEMICAL AGENTS AND ANIMAL SPECIES INCLUDING MAN

Tables 3 to 7 document the statement made in the above subtitle with data from animal experiments using mice, rats, cats, and monkeys as well as human adults or children, healthy or sick (136–146).

In some experiments, the biologic variable investigated is susceptibility of the body as a whole. Thus Scheving, Vedral & Pauly (104, 124) demonstrated that the duration of sleep induced by pentobarbital sodium injection (35 mg/kg) in the white adult rat is a function of the hour of administration or, in other words, is circadian-system-phase dependent (Figure 5). This is also the case for the length of time required for the onset of constant tremor in all members of experimental groups of white rats injected at fixed times

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TABLE 3. Circadian Susceptibility Rhythms to Chemical Agents, Including Overdoses of Drugs or Poisons[a]

Species	Chemical Agent (Fixed Doses and Multiple Test Times)	Synchronizer Schedule	Series Average $\bar{X}$	Amplitude C	Acrophase—or Crest—from Different Origin	References
Hamster[b]	Dimethylbenzanthracene	*L* [0600–1800] *D* [1800–0600]	=100%	[25%]	Mid-*L*: ~ 0° *L*-off: ~– 18 hr	Halberg 100
White rat	D-amphetamine sulfate Nicotine	*L* [0600–1800] *D* [1800–0600]	=100	21.5 (15.7–27.3)	Mid-*L*: –182° (–152 to –213) *L*-off: 0 hr	Scheving, Vedral 101, Scheving 102
White rat	Pentobarbital sodium	*L* [0600–1800] *D* [1800–0600]	48	[25]	Mid-*L*: ~–150° *L*-off: ~– 4 hr	Pauly, Scheving 103
White rat	Tremorine	*L* [0600–1800] *D* [1800–0600]	58	[30]	Mid-*L*: ~–180° *L*-off: ~– 6 hr	Scheving, Vedral, Pauly 104
White rat	Strychnine	*L* [0600–1800] *D* [1800–0600]	=100	[25%]	Mid-*L*: ~–135° *L*-off: ~– 3 hr	Tsai, Scheving, Pauly 105
C mouse	Brucella somatic antigen	—	—	—	—	Halberg, Spink et al 99
C mouse	*E. Coli* lipopolysaccharide	*L* [0600–1800] *D* [1800–0600]	=100	71% (47–95)	Mid-*L*: – 37° (–18 to –57) *L*-off: –20.5 hr	Halberg, Stephens 90 Halberg et al 91
C mouse	Ethanol	*L* [0600–1800] *D* [1800–0600]	39	16 (9–23)	Mid-*L*: –107° (–80 to –134) *L*-off: –1.1 hr	Haus, Halberg 95
C mouse	Methopyrapone (SU-4885)	*L* [0600–1800] *D* [1800–0600]	=100	23% (11–35)	Mid-*L*: –105° (–82 to –128) *L*-off: – 1 hr	Ertel, Halberg Ungar et al 96
B_8 mouse	Acetylcholine	*L* [0600–1800] *D* [1800–0600]	~76	[14]	Mid-*L*. ~–120° *L*-off. ~– 2 hr	Jones et al 106
B_1 and C mouse	Fluothane	*L* [0600–1800]			Mid-*L*: ~–180°	Matthews et al 107

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D_1 mouse	Ouabain	*L* [0600–1800] *D* [1800–0600]	=100	(57–67)	(−305 to −324) *L*-off: − 15 hr	Haus 92, 93
D_2 Mouse[b]	Dimethyl benzanthracene	*L* [0600–1800] *D* [1800–0600]	~36	[9]	Mid-*L*: ~− 60° *L*-off: ~− 22 hr	Haus, Halberg 97 (cf. also 100)
D_3 mouse[c]	Librium	*L* [0600–1800] *D* [1800–0600]	=100	19% (6–24)	Mid-*L*: −220° (−184 to −263) *L*-off: −8.6 hr	Marte, Halberg 94
Swiss mouse ♀	Pentobarbital sodium	*L* [0730–1930] *D* [1930–0730]	~108	—	Mid-*L*: ~− 7° *L*-off: ~−17.5 hr	Lindsay et al 108
Mouse	Aurothioglucose	—	—	—	—	Wiepkema 109
CF, Mouse ♀	Lidocaine hydrochloride	*L* [0605–1805] *D* [1805–0605]	16 to 20	[38%]	Mid-*L*: ~−135° *L*-off: ~− 3 hr	Lutsch, Morris 110
House fly[d]	Pyrethrum	*L* [0500–2000] *D* [2000–0500]	=100	18% (15–21)	Mid-*L*: − 40° (−19 to −61) *L*-off: − 19 hr	Sullivan et al 111
Madeira cockroach[d]	Pyrethrum	*L* [0500–2000] *D* [2000–0500]	=100	19% (15–23)	Mid-*L*: − 76° (−54 to −96) *L*-off: −21.5 hr	
Two-spotted spider mite	DDVP (Insecticide)	—	—	—	—	Pollick et al 112
Boll-weevil (insect)	Methylparathion (insecticide)	*L* [1000–2400] *D* [0000–1000]	~20	[40%]	Crest of resistance at *L*-on	Cole, Adkisson 113, 114

[a] SYNCHRONIZER: lighting regimen. SERIES AVERAGE: mean over all sampling times and subjects; "$\bar{X}$ =100%" indicates data were reported at different points only as percentage deviations from the overall mean value. CIRCADIAN AMPLITUDE: difference between the highest (or lowest) value and mean in a sinusoidal oscillation, determined by harmonic analysis. Values in parentheses are confidence limits. Values in brackets give one-half the range of group means over the circadian period (included as an approximation of circadian amplitude, when only group means at different clock hours were available). ACROPHASE OR CREST OF RHYTHM FROM DIFFERENT ORIGINS is given redundantly in several units: in degrees from "Mid-*D*" or "Mid-*L*" (with 360° =period of rhythm—e.g., 24 h); in hours from "light on" (*L*-on) or "light off" (*L*-off); and in local clock time (only for the 24-hour-synchronized rhythm of man). A minus value denotes that the phase marker on the rhythm occurred later (by the unit or span specified, e.g., in degrees) than the temporal reference point of time origin. Values in parentheses are approximate 95% confidence limits.

[b] Gauged by percentage of tumors.

[c] Gauged by percentage of death or survival time.

[d] Knockdown as well as mortality used as index.

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Table 4. Biologic Effects of Chemical Agents, Including Drugs, as a Function of Circadian System Phase

Species	Chemical Agent (Fixed Doses & Multiple Test Times)	Variable Investigated	Synchronizer Schedule	Series Average	Amplitude C	Acrophase ϕ from Different Origin	References
Cotton (plant)	Herbicides (dicryl, etc.)	Inhibition of growth of 12-day-old cotton seedlings	*L* [0600–1800] *D* [1800–0600]	—	—	Mid-*L*: −270° *L*-off: ~− 12 hr	Gosselink, Standifer 115
House cricket (insect)	Ether, chloroform, carbon tetrachloride	Recovery time of 50% of subjects	*L* [0800–2000] *D* [2000–0800]	~40	[3]	Mid-*L*: ~−135° *L*-off: ~− 3 hr	Nowosielski et al 116
Lower vertebrates (Fish, Frog, Lizard). White-throated sparrow (bird)	Prolactin	Fattening response	*LD* 16: 8	—	—	Mid-*L*: ~ 0° *L*-off: ~− 20 hr	Meier et al 117–119
C mouse	ACTH	Corticosterone response; standardized groups of animals	*L* [0600–1800] *D* [1800–0600]	~210	[110]	Mid-*L*: ~−180° *L*-off: ~− 6 hr	Haus, Halberg 15, 16
C mouse	ACTH	Incubated adrenal removed from group of animals at fixed hours. Corticosterone response	*L* [0600–1800] *D* [1800–0600]	$\bar{X}$ = 100%	[75%]	Mid-*L*: −235° (−210 to −258) *L*-off: −9. 6 hr	Ungar, Halberg 18
Mouse (immature ♀)	Gonadotropin	Uterine & ovarian weights	*L* [0600–1800] *D* [1800–0600]	—	—	Mid-*L*: ~− 75° *L*-off: ~− 17 hr	Lamond Braden 120
$C_{57}B_1$ mouse	Pentobarbital Sodium	Duration of anesthesia	*L* [0800–2000] *D* [2000–0800]	$\bar{X}$ = 100%	[30%]	Mid-*L*: ~ 0° *L*-off: ~− 18 hr	Davis 121

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Dawley rat ♂	(p-nitroanisol)	cadian reactivity rhythm of hepatic drug-metabolizing enzymes	*D* [1800–0600]			*L*-off: ~– 4 hr	
Sprague-Dawley rat ♂	Hexobarbital	Duration of sleep as a function of the hour of drug administration.	*L* [0600–1800] *D* [1800–0600]	~54 min	[10 min.]	Mid-*L*: ~– 30° *L*-off: ~– 20 hr	Nair, Casper 123
White rat	Pentobarbital sodium	Duration of sleep as function of the hour of drug administration	*L* [0600–1800] *D* [1800–0600]	~67 min	[24 min.]	Mid-*L*: ~–105° *L*-off: ~– 1 hr	Scheving, Vedral, Pauly 103, 104, 124
White rat	Tremorine (1,3-pyrrolidino-2-butyne)	Time required for the onset of constant tremor in all members of experimental group	*L* [0600–1800] *D* [1800–0600]	~90 min.	[60 min.]	Mid-*L*: ~– 30° *L*-off: ~– 20 hr	
White rat	Colchicine	Rhythms of cell division in the cornea	—	—	—	—	Scheving, Pauly 125
White rat	Acetylcholine	Right atrium beat frequency in vitro.	*L* [0600–1800] *D* [1800–0600]	—	—	Mid-*L*: ~–345° *L*-off: ~– 17 hr	Spoor, Jackson, 126 Jackson 127
Peromyscus	Pentobarbital sodium	Rate of recovery	*LD*: natural	—	Recovery is more rapid during the active (in *D*) span of the cycle		Emlen, Kem 128
Cat	Atropine Short acting barbiturate	Inhibition of circadian rise in plasma 17-OHCS level	*LD*: natural	—	Injection just prior the rise of 17-OHCS in plasma is more effective than at any other tested clock hour		Krieger (DT & HP) 129

with a fixed dose (64 mg/kg) of tremorine (1,3-pyrrolidino-2-butyne), according to Pauly, Scheving & Vedral (103, 124). With $L\ 06^{00}$-$18^{10}D18^{00}$-06^{00}, at about 14^{00} 150 minutes were required to produce tremors, whereas at about 22^{00} only 35 minutes were required.

The susceptibility of certain organ systems also was explored from a chronopharmacologic point of view. Circadian variations of uterine and ovarian weights following timed injections of gonadotropin to immature female mice are cases in point (120) as is the inhibition rate of the physiologic

TABLE 5. Chemical Agent Effects upon Circadian Rhythms of Experimental Animals

Species	Chemical Agent (Fixed Dose, Single or Multiple Test Times)	Effect	References
Canary	Barbiturates, reserpine, MAO inhibitors, etc.	Behavior (circadian rhythm of self-selected rest and activity).	Wahlström 197
Mouse	Morphine chlorhydrate	Alteration in blood glucose and in liver glycogen circadian rhythms	Sable et al 198
Holtzman rat	Corticosterone, phenobarbital.	Alteration of circadian rhythm of hepatic drug-metabolizing enzyme activity	Radzialowski, Bousquet 122
Rat	Parachlorophenylalanine, Disulfiram, MAO inhibitors.	Alteration in sleep circadian rhythms (24 hrs EEG)	Mouret 136
Rat	Chloramphenicol	Changes in circadian rhythms of histamine excretion	Wilson 199
Rat	Cytosine arabinoside	Mitotic inhibition at acrophase in corneal epithelium	Cardoso et al 162
Monkey	Reserpine	Changes in circadian rhythms of circulating eosinophils and of urinary 5-hydroxyindol acetic acid	Anderson 137
Monkey	Anti-depressant (analog of thioridazine)	Experimentally induced neurosis and psychosis with alteration of physiological rhythms. Rapidity of the animal recovery.	Stroebel 200

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TABLE 6. Effects of Chemical Agents, Including Drugs, as a Function of Circadian System Phase in Healthy or Allergic (a) Human Beings

No. of subjects	No. of days [Δt, hr]	Chemical agent (fixed doses & multiple test times)	Variable investigated	Synchronizer schedule	Amplitude C as p. cent of 24-hr mean	Acrophase		References
						ϕ in hour & minute; origin 0000 hr	φ in degree; origin: mid-sleep =Mid-*D*	
12	1 [4]	Histamine	Skin reactions (erythema & wheal) to intradermal injection	*L* [0730–2330] *D* [2330–0730]	24% (19 to 29)	2308 (2144 to 0048)	−302° (−281 to −327)	Reinberg et al 138, 139
6	1 [4]	48/80 (Histamine liberator)	Skin reactions (erythema & wheal) to intradermal injection	*L* [0730–2330] *D* [2330–0730]	[38%]	2300	−293°	Reinberg et al 138
5[a]	1 [4]	Penicillin	Immediate skin reaction (erythema) to the antigen	*L* [0700–2300] *D* [2300–0700]	41% (10 to 71)	2032 (1848 to 0400)	−263° (−237 to −15)	Reinberg et al 139
6[a]	1 [4]	House dust extract	Immediate skin reaction (erythema) to the antigen	*L* [0700–2300] *D* [2300–0700]	38% (5 to 72)	2152 (1632 to 0108)	−283° (−203 to −332)	Reinberg et al 139
6	4 [6]	Sodium Salicylate	Total duration of salicylate excretion in urines (oral administration)	*L* [0700–2300] *D* [2300–0700]	12.3% (7.5 to 16.8)	0641 (0145 to 1052)	−55° (−341 to −118)	Reinberg et al. 141
7	1 [6]	Acetylcholine	Bronchial reaction	*L* [0700–2300] *D* [2300–0700]	29% (4 to 53)	1427 (1101 to 1753)	−172° (−121 to −224)	M. Morin, Reinberg et al (unpublished)

TABLE 7. Chemical Agent Effects upon Circadian Rhythms of Human Beings and as a Function of Circadian System Phase

Group	Chemical Agent (Fixed Dose, Multiple Test Times)	Effect	References
Mentally deficient adult	Reserpine	Alteration of body temperature circadian rhythm	Halberg 3
Healthy adults	Cyproheptadine (per os) (antihistaminic drug)	Circadian changes in the duration of the inhibitory effect on skin reaction to histamine	Reinberg, Sidi 140
Healthy adults	Dexamethasone (per os)	Alteration of circadian rhythms in plasma and urine 17-hydroxycorticosteroid.	Nichols et al 142, D'Agata et al 143, Bricaire et al 144
Asthmatic children	Prednisone (per os)	Phase shift in acrophase of circadian rhythms for peak expiratory flow and urinary K and Cl (No change when drug is given at the acrophase of the plasma 17-OHCS circadian rhythm)	Reindl et al 146, Halberg 22
Healthy adults	ACTH (I.V. infusion)	Phase shift in circadian rhythm of 17-OHCS excretion (No change when ACTH infusion corresponds to the time of the expected peak of 17-OHCS excretion)	Martin et al 207, 208
Adrenal insufficiency	Cortisol (per os)	Phase shift in acrophase of circadian rhythms for heart rate, dynamometry, and K, Na, 17-OHCS, 17-KS urinary excretions.	Reinberg, Ghata Halberg, Gervais, Apfelbaum et al (unpublished)
Healthy adults	Ethanol (per os)	Circadian changes in blood ethanol concentration. Alteration in psychophysiologic test circadian rhythms	Rutenfranz et al 145
Diabetic adults	Regular insulin	Alteration of cortisol circadian rhythm in plasma	Serio, Della Corte et al 209

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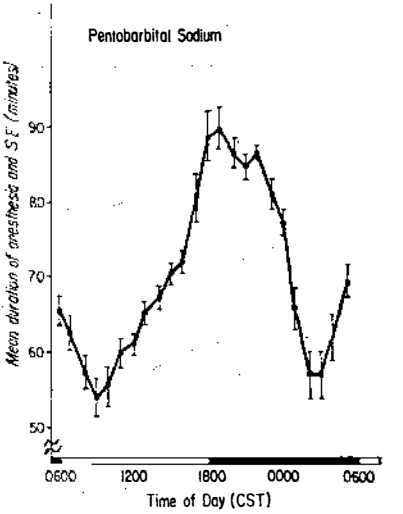

FIG. 5. Circadian rhythm in a "desired effect"—anesthesia duration—stands out grossly in plot of original data collected by Pauly & Scheving (103); for rhythmic response to other anesthetics see Ref. 107.

circadian rise in plasma 17-OHCS level following single timed injections of atropine, short-acting barbiturates, or dibenzyline, etc. in cats (129).

Grossly apparent and microscopically examined chronopharmacologic effects have also been demonstrated for human beings, as illustrated by the following studies (138–141).

Adults—apparently healthy subjects as well as allergic patients—were standardized for at least one week on a routine of diurnal activity and nocturnal rest (from 23^{00} to 07^{00}). Subject's profiles were obtained at 4-hour intervals during 24 hours on several functions, including among others the evaluation of an erythematous area measured 15 minutes after a standardized intradermal injection of histamine on the flexor surfaces of the forearms (10 μg in 0.1 ml of saline solution) of healthy subjects or of house dust extract (0.1 ml of a freshly prepared 1/50,000 dilution) for allergic subjects; standardized scratch tests were done with sodium benzyl-penicillin (0.4 ml of a freshly prepared solution of 25,000 μ/ml) on patients sensitized to this antibiotic.

Such studies demonstrate below the 0.1% level of significance that a circadian rhythm characterizes the erythematous response of healthy subjects to a fixed dose of histamine, as shown in Figure 6. Under the conditions of the study this rhythm's amplitude is 24% of the rhythm-adjusted (cf Figure 3) level (equated to 100%), the 95% confidence interval of this C extending from 19 to 29%. The computative acrophase ϕ (of Table 2) is found at 23^{08}, with a 95% confidence arc from 21^{44} to 00^{48} when local midnight is taken as phase reference; the external acrophase φ (95% confidence

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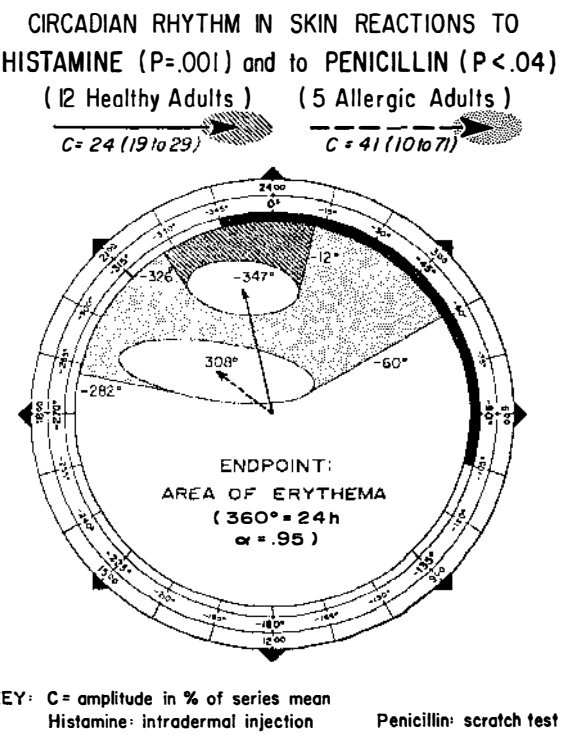

FIG. 6. Circadian susceptibility rhythms of mature human beings. Note remarkable agreement in timing of acrophases for susceptibility rhythms to histamine and penicillin, agreement apparent from the closeness of the arrows and the overlap of areas delineated as 95 percent confidence intervals by tangents drawn to the error ellipses (139).

arc) is found at $-292°$ (-271 to -317) when mid-D (03^{00} or $-45°$) is taken as phase reference.

Figure 6 also demonstrates a circadian rhythm in allergic cutaneous responses to penicillin ($P<0.04$). The amplitude is particularly large—C of 41% (CI from 10 to 71%); the ϕ is found at 20^{32} (CI from 18^{48} to 04^{00}), when the ϕ reference is 00^{00} local time. Figure 7, in turn, demonstrates a circadian rhythm for the erythematous response of allergic patients to fixed doses of house dust extract.

The duration of urinary salicylate excretion following oral administration of a fixed dose (1 gm of sodium salicylate) of the drug was studied on six human adults standardized on a routine of sleep daily from 23^{00} to 07^{00} (141). Urine was sampled from each subject at 4-hour intervals for 48 hours. Salicylate in each sample was determined by two closely related methods after extraction with dichloroethane. Average duration of drug excretion depended on the time of its administration. A circadian rhythm in this pharmacologic phenomenon was detected ($P<0.002$) by the cosinor method

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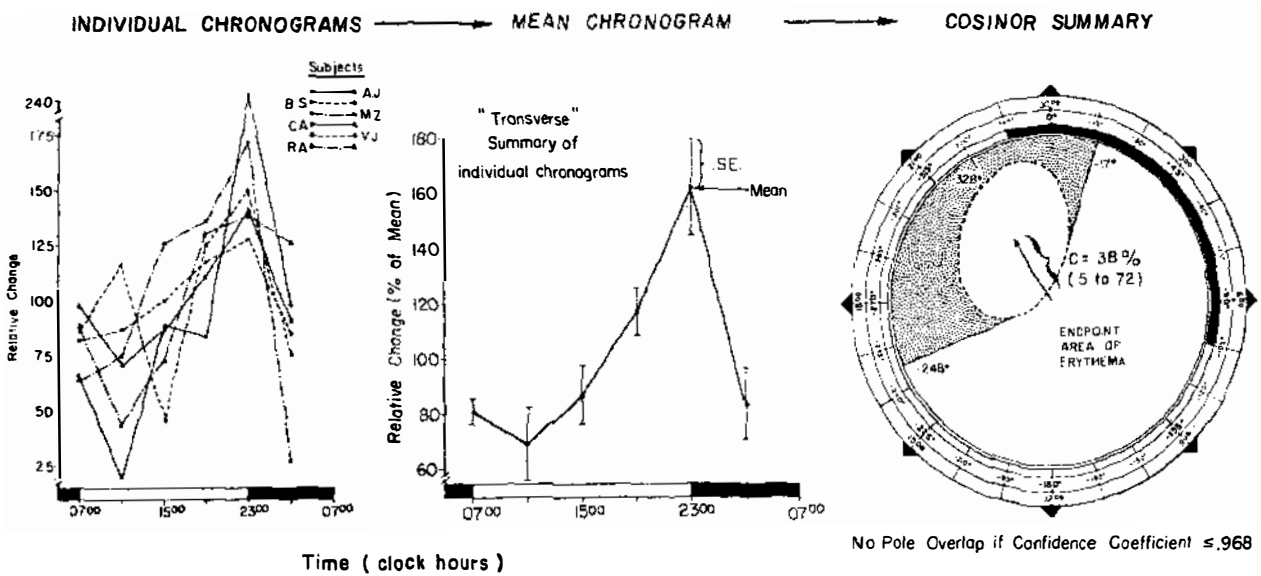

FIG. 7. Macroscopic chronograms (left and middle) and microscopic parameter estimations (right) on a circadian susceptibility rhythm to house dust: 6 mature human beings (2 women, 4 men). The direction of the arrow in the cosinor and the adjacent shaded area indicate the span of greater susceptibility; the length of the same arrow indicates the extent of the predictable periodic change, i.e., the rhythm's amplitude. That a rhythm does indeed occur is detected by the cosinor method when the error ellipse, the white space within the shaded area, does not cover the middle of the plot, the so-called pole (139).

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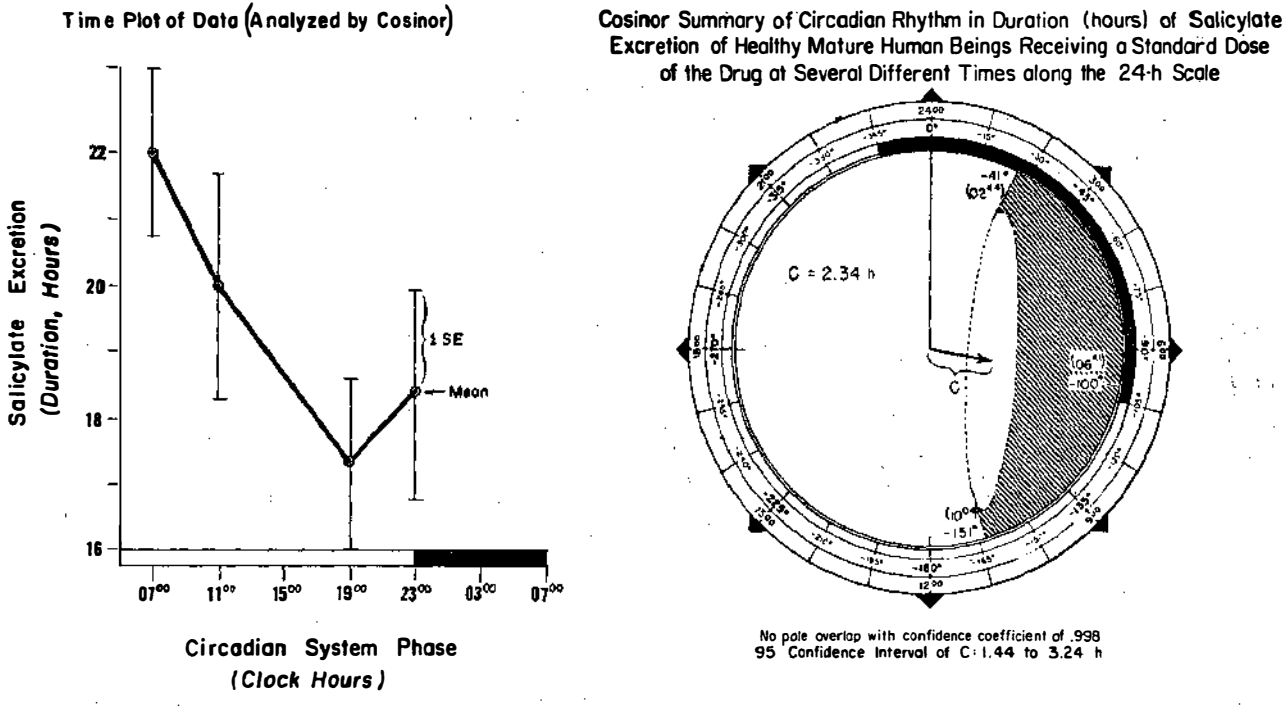

FIG. 8. Plots of time-varying average duration of salicylate excretion shown on the left, analyzed by cosinor on the right; cosinor demonstrates the detection of a pharmacologically interesting circadian rhythm—error ellipse away from the pole—and estimates rhythm parameters (amplitude and acrophase) (141).

and results on parameters (Figure 8 on the right) thus obtained were compared and agreed with impressions gained from a conventional time plot of the data (Figure 8 on the left). The longest duration of salicylate excretion corresponds to a drug administration at 06^{41}. The 95% confidence arc for this circadian acrophase ϕ extends clockwise from 02^{44} to 10^{04}. The φ is $-55°$ (from $-341°$ to $-118°$) when the middle of the daily span of darkness (in one's bedroom) is chosen as phase reference.

CIRCADIAN VARIATION IN PHARMACOLOGIC EFFECTS AT THE ORGAN OR TISSUE LEVEL

A first example introducing this report (Figure 1, on the right) was one describing circadian changes in the response of isolated adrenal glands to fixed constant doses of ACTH. Subsequent studies by Andrews & Folk, *inter alia*, on organ cultures of adrenals are of particular interest, providing as they do a tool to study circadian rhythms in vivo (148–153).

Jackson & Spoor (126, 127) studied circadian changes in sensitivity of isolated rat atria to acetylcholine (ACh). Animals were standardized with $L06^{00}$–$18^{00}D18^{00}$–06^{00}. At fixed times the heart was removed from each animal of several different groups and the in vitro effect of ACh tested. The percent decrease in rate at 1 and at 10 μg/ml in the medium was greater if the atria were isolated at 11^{00} than if isolated at 23^{00}. The 50% effective concentration, determined graphically, for the atria isolated at 23^{00} is 1.6 times greater than those isolated at 11^{00} (5, 7 μg/ml and 3.6 μg/ml respectively).

The clam *Mercenaria mercenaria* exhibits persistent circadian rhythms in shell growth (Pannella & MacClintock, 155) and shell opening and closing (Thompson & Barnwell, 156). These findings suggest that circadian rhythms may occur in other physiological processes of the clam, including the responsiveness to pharmacological agents. The school of F. A. Brown, more generally, while not primarily concerned with pharmacologic problems, continues to contribute stimulating data and thought to the broader field of chronobiology (175–179).

CIRCADIAN CHANGES AT CELLULAR AND SUBCELLULAR LEVELS

Rhythms of cell division in the white rat cornea can be influenced by timed injection of colchicine (Scheving & Pauly, 125). As reported for the case of murine liver (80), partial (~70%) hepatectomy increases the level of mitotic activity in the corneal epithelium of rats (160). This increased level of mitotic activity occurs ~48 hours after partial hepatectomy, with maintenance of the general circadian pattern—the hours of the day in which acrophase and trough occur—grossly the same in partially hepatectomized and controls. Adrenalectomy abolishes and dexamethasone administration to adrenalectomized rats reestablishes the above response to partial hepatectomy (160, 161).

Cardoso has suggested the use of circadian mitotic rhythms as a guide

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for the administration of antimetabolites. Aracy (cytosine arabinoside) administered to rats (kept in $L06^{00}$–$17^{00}D18^{00}$–06^{00}) at 23^{00} hours profoundly inhibits the mitotic acrophase in corneal epithelium, whereas no inhibition of this acrophase is seen with the same dose of Aracy administered at 14^{00} of the same day (162).

In a recent study (163), cytosine arabinoside was administered to five different groups of BDF_1 mice (male, average weight of 16 grams). The mice, kept on $L06^{00}$–$18^{00}D18^{00}$–06^{00} were injected by the i.p. route with either 200 or 400 mg/Kg per single daily dose. The single daily doses were repeated for 5 consecutive days (same dose, same hour of administration). The hours in which the different groups received Aracy were: 03^{00}, 08^{00}, 13^{00}, and 18^{00}, and 23^{00}. The highest mortality was observed in the groups treated at either 13^{00} or 18^{00}. The lowest mortality was observed in the groups treated at 08^{00}. Information on circadian and other rhythms (mitotic ones in particular) of susceptibility to carcinostatic agents is likely to be pertinent in timing now conventional treatment. For instance, an inhibitor of nucleic acid synthesis could be given at times (circadian system phases) defined in relation to the rhythms in both tumor and host (13, 164). If a "hit-and-run" short-lived RNA inhibitor is found that is harmless to the host when administered at a given circadian system-phase, one could test added value from timing by searching for some ultradian rhythm stage when the drug best achieves its desired effect upon the tumor. A much more favorable therapeutic index might result from the search for such information, as well as from consideration of other rhythms (165, 166).

Radzialowski & Bousquet (122) have been studying circadian rhythmic activities of hepatic drug-metabolizing enzymes such as aminopyrine N-demethylase, 4-dimethyl-aminoazobenzene reductase, p-nitroanisole 0-demethylase, etc. in male and female Holtzman rats and male Swiss-Webster mice ($L06^{30}$–20^{00}) and ($D20^{00}$–06^{30}). Livers were removed from groups of animals at 2-hour intervals, and microsomal or 9000 g supernatant fractions were prepared and assayed. Under the conditions of study the activity of each of the enzyme systems examined showed a circadian rhythm with a peak at about 02^{00} and a trough at about 14^{00}. Adrenalectomy or maintenance of elevated plasma corticosterone levels, by administration of this steroid, reportedly altered some of these circadian rhythms. Such alteration was also observed in rats pretreated (for 4 days) with phenobarbital, although the circadian rhythm in plasma corticosterone level was not affected.

Vesell (167) and Colas et al (168) have extended work on rhythmic oxidative drug metabolism in the rat and mouse, and more recently longer-term variations in basal and phenobarbital-stimulated oxidative drug metabolism in the rat also have been reported (169).

Much early work on enzyme rhythms has been reviewed by Van Pilsum & Halberg (170), who also extensively studied a circadian rhythm in transamidinase of mouse kidney. Potter et al (171) discovered that hepatic transaminase activity in rat liver varies over an about-fourfold range each

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TABLE 8. Circadian Rhythm in Hepatic Tyrosine Transaminase Activity (μmoles/g liver/h) under Different Lighting Conditions in Rats

Original data of J. Axelrod

Lighting conditions	Amplitude, C C ± SE	SE/C	Acrophase, φ (.95 confidence arc)
*L*05–19:*D*19–05	41 ± 8	.18₄	−343° (−323 to − 3)
Constant darkness	37 ± 7	.19₃	−357° (−324 to − 19)
*L*19–05:*D*05–19	45 ± 14	.31₉	−182° (−128 to −236)

Acrophase reference = local midnight. 360° = 24 h; 15° = 1 h.

day. Pituitary or adrenal hormones (83, 172, 173) are not likely to constitute critical factors underlying the hepatic transaminase rhythm since this bioperiodicity (8) among others (79) reportedly persists in their absence. Controlling factors have been discussed by Axelrod (174); from his work and that of his group the behavioral characteristics of circadian rhythmicity (known to hold at the level of an organism as a whole), again emerge and are readily quantified by microscopic techniques.

Thus, Table 8 summarizes Axelrod's valuable data for the hepatic transaminase rhythm obtained at relatively few time points over a single cycle under several lighting conditions. The table demonstrates first of all the readily feasible quantification of the enzyme rhythm from the data on hand, under the varied circumstances; the amplitude of this rhythm is relatively large, as is that of several others in murine liver (71, 175, 176). Next, the rhythm's persistence in continuous darkness, and finally its amenability to phase-shifting upon manipulation of the lighting regimen, all are easily measured and presented as objective numerical endpoints in Table 8.

Since circadian rhythms occur at all levels of organization (1, 50, 177–179)—including subcellular metabolic activity such as that of DNA and enzymes—it will not be surprising that circadian rhythms in many pharmacologic effects eventually will be uncovered and then utilized in therapeutics based upon quantitative studies in chronopharmacology.

CHARACTERISTICS OF SUSCEPTIBILITY RHYTHMS AND CHANGES IN EXOGENOUS FACTORS

Changes in environmental factors and in particular the manipulation of a known synchronizer can influence circadian and other susceptibility rhythms, possibly modifying the parameters characterizing such rhythms. Experimental demonstrations of this fact have been extensively reviewed in symposium volumes and other books so that a few examples will suffice at this point.

Suppression of Known Synchronizers

Suppression of known synchronizers or of their transducers—mainte-

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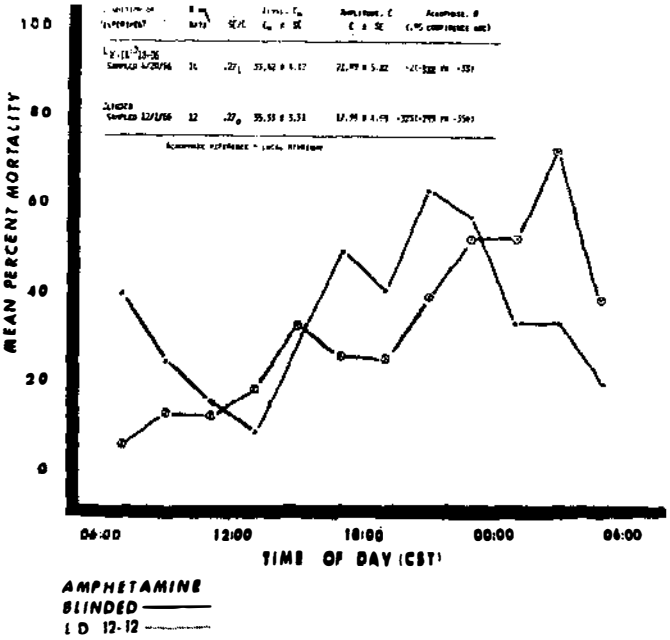

FIG. 9. (Courtesy of L. Schreving)

nance in continuous darkness (*DD*) or continuous light (*LL*), or again bilateral optic enucleation in experimental animals, or isolation of man underground without time cues (1–4, 6, 8, 26–33, 37–53, 73, 74, 88, 89, 180–184, 211)—leads to the following observations, among others: (*a*) circadian rhythms persist; (*b*) their periods differ with statistical significance from precisely 24 hrs.

In an experiment on the rhythm in susceptibility of *C*-mice to ethanol, Halberg studied the effect of constant darkness (185). Groups of mice were kept in *DD* for 5 days before starting the 4-hourly injections. A statistically significant susceptibility rhythm was detected under these experimental conditions.

Scheving & Vedral (101, 102) studied the circadian rhythm in susceptibility of white rats to D-amphetamine sulfate: (*a*) in $L06^{00}$–$18^{00}D18^{00}$–06^{00}, (*b*) in animals subjected to bilateral enucleation at 4 weeks before sampling, and (*c*) in *LL*. Both macroscopic and microscopic summaries—the latter based upon the fit of a 24-hour cosine curve to the data (cf 57)—are presented in Figure 9. Circadian susceptibility rhythms can be objectively detected in $LD_{12:12}$ as well as after bilateral optic enucleation. In another experiment on rats, Scheving & Vedral (124) observed that circadian changes in duration of sleep induced by pentobarbital injection persist in blinded animals. The major point to remember is that elimination of the synchronizer's major transducer (eye removal) or its suppression (*DD*) does not obliterate several

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circadian susceptibility rhythms; they persist with eventual changes in the parameters τ, φ, and C (cf 89).

Phase Shift of Synchronizer ($A\Phi$.)

A $\Delta\phi_s$ is followed by a phase shift of rhythms ($\Delta\phi_R$) with a delay of varying length, depending upon the function, species, variable, and other conditions of study (1, 49, 186).

Peak susceptibility of D_8 mice to ouabain (92) at about 08^{00} occurs only when the lighting conditions are $L06^{00}$–$18^{00}D18^{00}$–06^{00}. This peak may be placed at any desired clock hour by the appropriate shift in the lighting regimen and by allowing for a sufficient shift time. For instance, 17 days after light inversion (a $\Delta\phi_s$ of 12 hr or 180°) to $L18^{00}$–$06^{00}D06^{00}$–18^{00} peak susceptibility to ouabain in D_8 mice was indeed at 20^{00} while a trough was now found at 08^{00}, the within-day change being statistically significant.

From these facts several inferences can be drawn from a theoretical, as well as practical, viewpoint. First, it is now possible with relatively simple "microscopic" analyses to resolve critical aspects of temporal structure and to specify them reproducibly by taking as phase reference a characteristic point of the synchronizer [e.g., mid-L for a nocturnal animal or the middle of one's daily D-span (for diurnal man)] or, eventually, a characteristic point of the organism's circadian temporal structure such as a body temperature acrophase or 17-OHCS excretion acrophase (8). Second, let us insist once more therefore, on the necessity of specifying when possible and preferably controlling synchronizing factors in any biorhythm study (8), including biorhythms in susceptibility.

Biologic temporal quantification may be especially useful when one knows that the susceptibility acrophase may vary from agent to agent in a given species (Figure 4) and from species to species with a given agent (121, 124).

However, one must not interpret all statistically significant differences in the timing of susceptibility rhythms to a given agent as being the result of strain or species differences. Before the latter can be ruled in, one must rule out the effects of age, of circannual rhythms (12), and other factors. For instance, Figure 4 suggests a strain difference in acrophase of the susceptibility rhythm to ouabain between D_8 and C-mice. Even if the aforementioned effects of factors other than circadian rhythms can be ruled out, heritability studies remain indicated for the case of possible strain differences. Qualifications also apply to the reports of a circadian peak in duration of pentobarbital-induced anesthesia at about 7 hours after mid-L in white rats (124) and at about 1 hour from mid-L in C_{57} Black mice (121), both rats and mice being synchronized to $LD_{12:12}$.

Adrenal Cortical Cycle

An endogenous circadian cycle of the adrenal cortex can be considered as one of the mechanisms which contribute to changes in susceptibility to some agents but not to all of them. Pertinent are animal experiments (122, 124) including some studies on man (138, 139, 187; cf also 79). For instance, the

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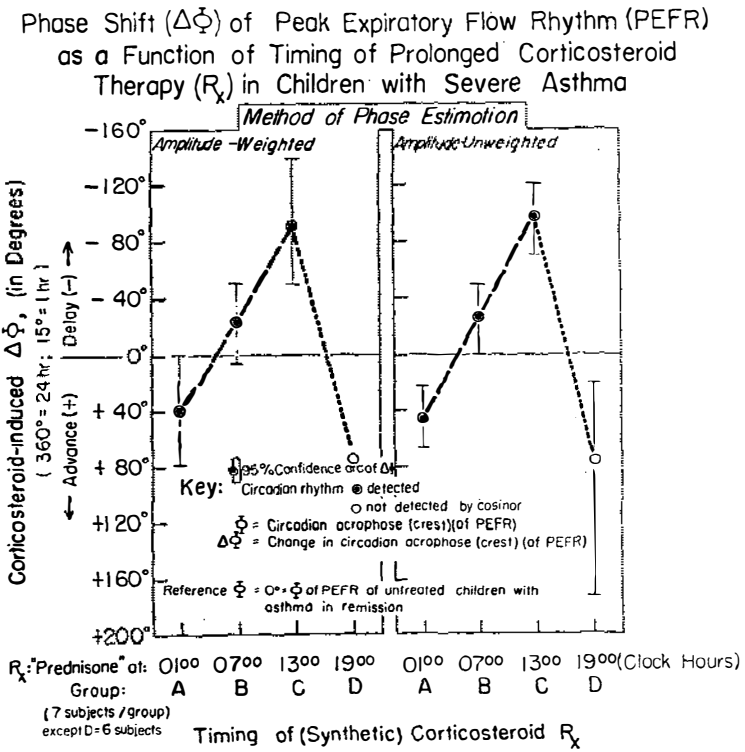

FIG. 10. Daily prednisone administration results in modification of circadian acrophase of rhythm in peak expiratory flow in children with asthma (146). Note drastic differences in extent or direction of Φ shift as a function of system phase at time of drug administration.

acrophases of skin susceptibility to histamine, to 48/80 (a histamine liberator) and, in allergic patients, to antigens (house dust extracts, penicillin) differ roughly by 180° (12 hours) from the acrophase of adrenal cortical secretion. The fact that blood corticosteroid levels were, on the average, lowest when the cutaneous response to histamine and other agents was highest does not necessarily imply direct causal relations but it should prompt follow-up studies, e.g., by phase-shifting.

DRUG-INDUCED ACROPHASE ALTERATION

The administration of certain drugs can influence some physiologic circadian rhythms, as do certain manipulations of the synchronizer. For example, a circadian rhythm in peak expiratory flow rate (PEFR) is detectable in healthy adults and in children, as well as in patients suffering from asthma. The PEFR acrophase can be shifted not only by changing the healthy subjects' synchronization, e.g., after intercontinental transmeridian flights (Reinberg & Halberg, unpublished data), but also by the timed administration of prednisone to asthmatic children [Figure 10 (22, 146)].

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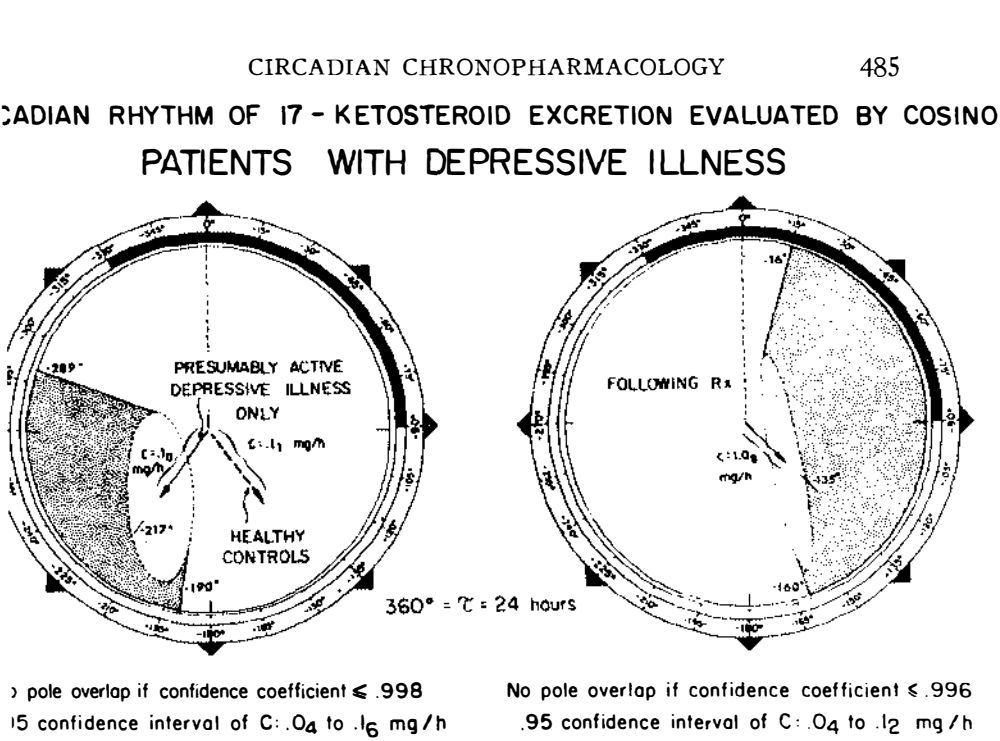

FIG. 11. Circadian dyschronism in patients with depressive illness (188).

Such pharmacologically-induced changes in physiologic rhythm characteristics can be desirable or undesirable. The timed administration of a drug might be desirable to treat a circadian desynchronization or other dyschronism occurring in certain diseases [e.g., in emotional illness such as depression Figure 11, (188)] or to treat the circadian dyschronism following a transmeridian or certain extraterrestrial flights (4, 8, 187–194) in order to shorten the duration of resynchronization or to avoid any performance deficit (25).

On the other hand, some other alterations of physiologic rhythms induced as side-effects of drugs, poisons, and even pollutants are undesirable. Pertinent are studies on cotton workers with byssinosis (195) and also on men with pneumoconiosis (196). In any event it seems obvious that "chronotoxicology" and "chronopharmacology" are in some respects two sides of the same coin and must be studied concomitantly for each drug.

PERSPECTIVES IN CHRONOPHARMACOLOGY

One of the aims of chronopharmacology is *better control and quantification*

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of drug effects. If circadian rhythms of susceptibility are not taken into consideration—as is usually the case with conventional testing procedures—predictable changes will be ignored and will enter the noise term, unnecessarily obscuring the results of analyses. Obviously, the confidence limits of a measured effect will be unduly enlarged. The timing of experiments according to physiologic desiderata and the synchronization of animals can be realized systematically to improve the accuracy of conventional methods in pharmacology.

Another aim is to investigate the circadian variations in the intensity or in the duration of pharmacologic effects as endpoints in their own right. Examples of drastic circadian changes in intensity of effect have already been given even for an in vitro (adrenal) response (to ACTH) (Figure 1 on the right). Let us consider, as another example of circadian changes in duration of a pharmacologic effect, the antihistaminic drug effect; it can be evaluated in man by studying the extent to which local skin reactions to intradermally injected histamine are inhibited. This test was applied with temporal consideration by Reinberg & Sidi (140).

Six apparently healthy subjects were standardized on a routine of diurnal activity and nocturnal (23^{00}–07^{00}) rest. For each subject a control curve was first obtained; it was a map of the circadian variation in skin reactions to histamine—in the absence of any antihistaminic drug administration. A second and a third group of profiles were obtained after a single (4 mg) dose of oral Periactine (cyproheptadine) an antihistaminic drug. Periactine was taken at 07^{00} for one of these last two profiles and at 19^{00} for the other one. For each of the subjects and each timing the changes in response after Periactine administration were expressed as percentage deviation from the control response.

The antihistaminic effect of the drug taken at 07^{00} lasted for 15–17 hours. This inhibitory effect lasted only 6–8 hours when Periactine was administered at 19^{00}. The difference in duration of this effect is statistically significant.

Usually, active synthetic drugs have several effects. Some of them can be interesting, the others being considered as vexing or incapacitating side effects. If circadian changes of desired and of undesired effects exhibit phase angle differences one can try, by the timing of drug administration, to maximize the useful action and to minimize the undesired one.

The eventual aim of studies on drug effects in relation to rhythms is chronotherapy. The possibilities of such an approach were almost certainly suspected earlier, *inter alios*, by Kisch (201), Volhard (202), Menzel (203), De Vries et al (212), and Carlsson & Serin (204), who provided the pioneering demonstration of what may be called a circadian susceptibility rhythm, even if time of day was thought to be the critical variable. The development of new instrumentation and techniques should lend even greater impetus to the newly emerging distinctive fields such as chronopharmacology, as reviewed by Haus (205) and Reinberg (7), inter alios (13)—among other aspects of chronobiology (147, 154, 157–159, 210, 213–223).

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CONCLUDING COMMENTS

"Further advances in chronopharmacology demand standardized studies on larger groups of human subjects for longer spans of time and the eventual collection of data by automatic sensors. Methods are being developed to evaluate not only the traditional parameters of rhythms but the waveform as well in data collected from different geographic locations as part of the cooperation within a large international biologic program (8, 206).

"Against this background, a first goal of chronopharmacology is the potentiation of desired effects while minimizing undesired ones by the timing of drug administration according to the phase of a reference rhythm rather than according to the local clock hour.

"A second as yet utopian aim of chronopharmacology involves preventive as well as curative measures against rhythm alteration or dyschronism.

"A preventive *therapia synchronisans*, i.e., a therapy to shorten the duration of dyschronism or even to prevent its onset, can be considered for individuals crossing several time zones by jet airplane or adjusting to a new routine of work.

"Curative chronobiotics may be visualized for diseases such as certain emotional disorders or rheumatoid arthritis—if, and only if, rhythm alteration can be recognized to be etiologically significant.

"The major disease in discussions of rhythms thus far has been a desynchronization of perspiration from inspiration, i.e., too much fancy and too little fact. The major emphasis of this review, therefore, must remain the documentation of rhythms by rigorously quantified observations as a basis (not as a substitute) for potential chronobiologic applications" (13).

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