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Chronobiology in laboratory medicine

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Summary. - A critical amount of information has accumulated over the last decades to allow the application of chronobiology to clinical and laboratory medicine. The tasks faced in laboratory medicine include the quantitative measurement of the multifrequency human time structure in health and disease. For this purpose, it is essential to choose an adequate sample size in order to obtain meaningful results and quantitative endpoints which can be interpreted by inferential statistical techniques. No statistical technique is applicable for all purposes and it is essential that the assumptions underlying each technique and its limitation are well known to the investigator. The multifrequency nature of the human time structure has to be kept in mind in order to avoid erroneous results. Time qualified reference ranges have to be established for high amplitude rhythms. Circadian and/or circannual rhythm alterations have been described as group phenomenon in subjects with epidemiologically determined risk states for common diseases, but will require much further studies for the application to individual subjects. Rhythm parameters are new endpoints in the evaluation of the human time structure in health. Alterations of these parameters may occur as cause or as consequence of disease. Recognition of rhythm abnormalities in disease are critical for a meaningful application of chronopharmacology. Time dependent changes in pharmacokinetics and pharmacodynamics have to be taken into account in the interpretation of drug level determinations. A considerable degree of individuality of timing has been documented in some frequencies. This individuality and the rhythm abnormalities found in disease require the study of reference or marker rhythms. If the complexity of the human time structure is clearly understood and its study pursued in a critical manner with quantitative endpoints, chronobiology opens a new dimension in laboratory and clinical medicine.

Key words: time qualified reference values, circadian, circannual, marker rhythms, clinical chronobiology.

Riassunto *(Cronobiologia nella medicina di laboratorio)*. - Una notevole quantità di informazioni sono state raccolte negli ultimi decenni tali da consentire una applicazione della cronobiologia alla medicina clinica e di laboratorio. I compiti che la medicina di laboratorio deve affrontare includono le misure quantitative della struttura temporale multifrequenziale dell'uomo in condizioni di salute e di malattia. Per questa finalità è essenziale scegliere un campione numericamente adeguato onde ottenere risultati validi e stime quantitative che possano essere interpretate dalle tecniche di statistica inferenziale. Nessuna tecnica statistica è valida per tutti gli scopi, ed è essenziale che i presupposti e le limitazioni di ogni tecnica siano ben note all'investigatore. La natura multifrequenziale della struttura temporale dell'uomo deve essere ben presente al fine di evitare risultati erronei. Gli intervalli di riferimento qualificati in funzione del tempo debbono essere stabiliti per i ritmi con evidente ampiezza. Sono state descritte alterazioni dei ritmi circadiani e circannuali come fenomeno di gruppo in soggetti in stato di rischio epidemiologicamente determinato per le comuni malattie. Occorrono però molti più studi per poter arrivare alla applicazione su base individuale. I parametri ritmometrici sono nuovi punti di riferimento nella valutazione della struttura temporale umana in salute. Si possono verificare alterazioni di questi parametri come causa o conseguenza di malattia. Il riconoscimento delle anomalie dei ritmi in malattia è critico ai fini di una applicazione logica della cronofarmacologia. Le variazioni tempo-dipendenti dalla farmacocinetica e dalla farmacodinamica debbono essere tenute presenti nella interpretazione della determinazione dei tassi di farmaco. Un considerevole grado di individualità nella temporalità è stato descritto in ciascuna frequenza. Queste individualità, insieme alle anomalie dei ritmi riscontrate in malattia, richiedono lo studio di ritmi di riferimento o "marker". Se la complessità della struttura temporale umana viene chiaramente capita ed il suo studio viene perseguito in maniera critica con finalità quantitative, allora si può dire che la cronobiologia apre una nuova dimensione nella medicina clinica e di laboratorio.

Parole chiave: valori di riferimento temporali, ritmi circadiani, ritmi circannuali, ritmi di riferimento, cronobiologia clinica.

Introduction

All living matter from unicellular organisms to human subjects shows variations in time consisting of trends, and superimposed upon these a variety of rhythms in several frequencies. These time dependent events form an intricate time structure, which is essential for the normal function of the organism and for the adaptation to its surrounding. Many of the rhythms observed appear to be genetically fixed [1]. Specific genes and gene products have been identified in various forms of life which determine

rhythmicity and rhythm parameters [2-5]. A genetic component in the determination of rhythm parameters in several frequencies is evident in several mammalian species [6, 7] and becomes apparent also in human twin studies [8, 9]. The genetically fixed (endogenous) rhythm components are continuously modified and adjusted in their timing, and in some instances in their amplitude by environmental stimuli. Rhythm alterations and desynchronization of rhythms, which usually show a certain phase relation to each other and/or to the organisms periodic surrounding, may lead to dysfunction of the organism as evidenced by the clinical syndrome of jet lag, and by loss of performance, increased number of accidents, and gastrointestinal and sleep disorders in shift workers [10-13]. Rhythm alterations may be the expression of disease and it appears likely that rhythm alterations may play a role in the etiology and/or pathogenesis of some common disorders [14-16].

The complex multifrequency human time structure represents a challenge but also an opportunity for the application of laboratory diagnosis in the description of health [14, 17, 18] and in the recognition of risk states for a variety of chronic disorders [19-23] and in the study of disease [14, 24-26]. Any single laboratory measurement of a biologic variable has to be understood as a spotcheck determined by the interaction of multiple endogenous rhythms, the timing of some of which is adjusted by environmental periodic factors (so-called synchronizers, entraining agents or Zeitgebers) and may be altered by other periodic and nonperiodic environmental input. These numerous factors acting upon a given variable at any one time together with technical and analytical sources of variation may lead to a very substantial overall variability, which in the past has very often limited the diagnostic value of laboratory measurements. With decrease in the analytical variations in the clinical laboratory, due to improved accuracy and precision, and rigid quality control, a more refined exploration of the biologic sources of variation has become feasible. This has been greatly facilitated during the last three decades by the development of automated and relatively inexpensive techniques of measurement of physiologic functions and laboratory variables. The study of the human time structure has been made more meaningful by the development of inferential statistical procedures to detect rhythms and quantify their parameters in the very often noisy time series of biologic measurements which can be applied easily with the help of electronic computers which are now widely available in the clinical laboratory [27-30]. Single measurements can now be interpreted against the background of the rhythmic and thus largely predictable biologic variations studied previously in the same subject or in comparable peer groups. In addition, the quantitative measurement of rhythmic variations provides the statistically verified parameters of biologic rhythms like rhythm adjusted mean (MESOR), phase, and amplitude as new endpoints in the study of physiologic functions and their disturbances. These developments and a considerable amount of material accumulated during the last decades has allowed to delineate specific tasks for the incorporation of chronobiology in laboratory research and its clinical application [31, 32].

The exploration, measurement, and mapping of the human time structure

Although much information on the human time structure has accumulated over the last decades, laboratory medicine still faces the task to explore the broad range of rhythms, near rhythms, and trends in health and detect, measure, and interpret changes related to malfunction and disease. The application of chronobiology to laboratory and clinical medicine depends critically upon the objective and quantitative evaluation of data collected as a function of time. The problems encountered in clinical chronobiology are varied and complex and require a variety of methods for data collection and analysis. The amount of data which can be collected on a patient is often very limited (e.g. blood drawings) and the data obtained have to be examined critically for the information they can or cannot provide. In the analysis of time dependent variations no single procedure is applicable to all situations and the choice of procedure will depend both on the data available and the information desired.

Measurement and evaluation of rhythms

The first step in the evaluation of data obtained as a function of time is the inspection of the raw data plotted as a function of time (the chronogram) Fig. 1 (left). Obvious rhythmicity may easily be recognized and an impression concerning the shape of the waveform can be gained which, in part, may determine the statistical method to be used for its quantitation. If a rhythm is relatively regular and prominent and enough data are available, even the computation of means and standard errors of data obtained at different sampling times can give reproducible curves on a chronogram and allow tentative rhythm detection. The data can then be evaluated by conventional statistical procedures like a t-test or an analysis of variance (ANOVA). The t-test can determine the statistical significance of peak-trough differences. In the analysis of the data by one factor (time) ANOVA the procedure indicates whether the variance between timepoints is significantly greater than the variation within them. The times of measurements do not have to be equally spaced and the shape of the rhythm does not affect the statistical outcome. Although the ANOVA may establish that a time-dependent variation exists it

gives no information as to its period, extent (amplitude), or timing (phase).

Biologic data obtained in healthy subjects on their usual living routine and in patients are often rather "noisy" and it has to be determined whether variations found are truly biologic rather than analytic variations, whether they occur at random or whether they do show rhythmicity. A variety of mathematical-statistical techniques has been developed or adapted to measure biologic rhythms. These include different applications of Fourier or harmonic analysis [33], the periodogram [34], and related techniques [35-37], power (variance) spectra [38-40] and curve fitting procedures by least squares techniques [27, 41] including the different variants of the cosinor procedure [28, 42]. Each of these techniques have certain differences in their assumptions, data requirements and information provided (for review see [29, 30, 43, 44]) a discussion of which would go beyond the scope of this review. In order

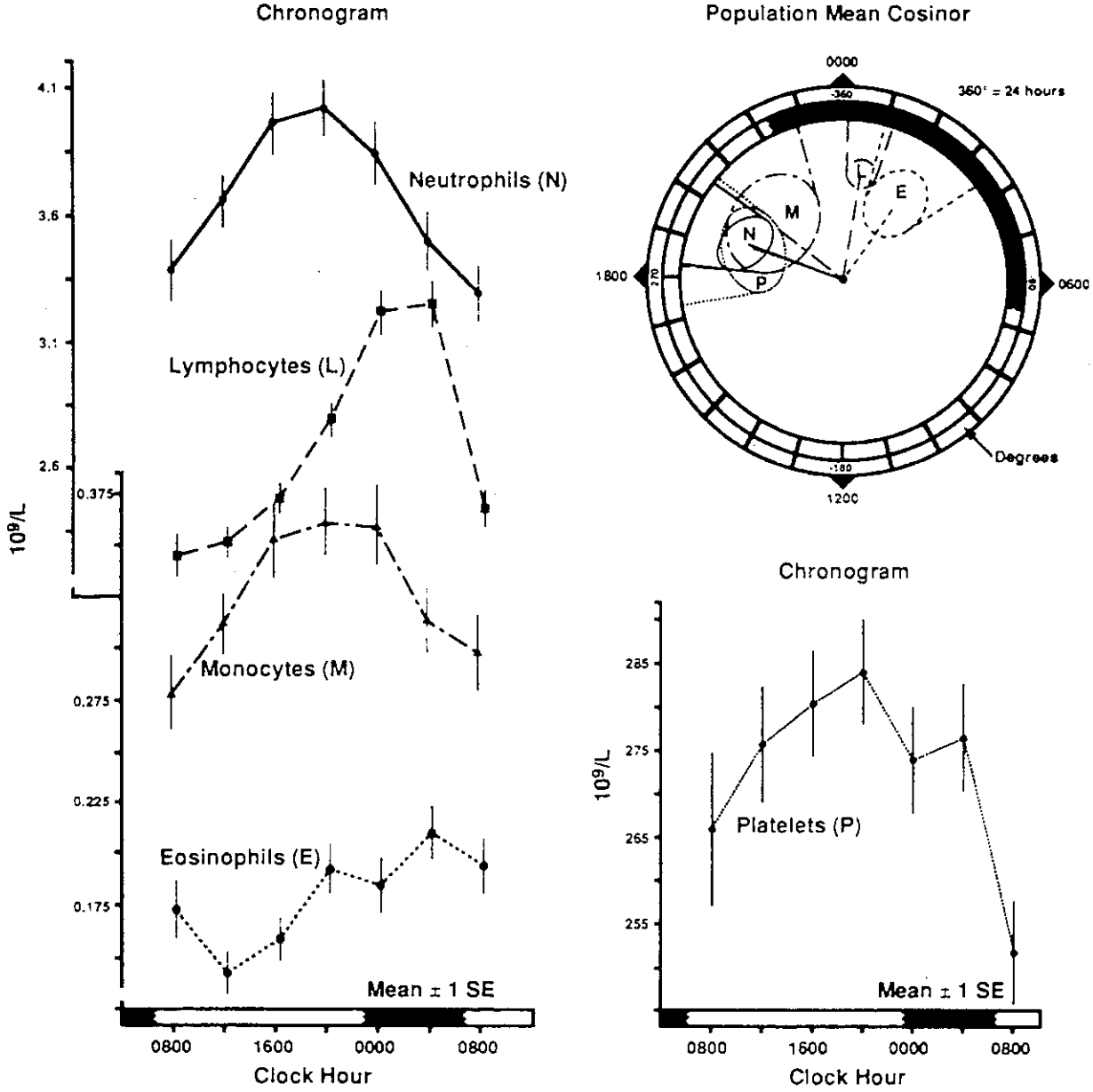


Fig. 1. - Chronogram (left and right bottom) and circular cosinor plot (right top) of circulating leukocytes and platelets in the peripheral blood of 150 clinically healthy subjects. In the polar cosinor plot, the period is shown as circle (360°) with the synchronizer phase (e.g. sleep span) shown (e.g. as dark circle segment) in the outer part of the circle. The phase reference (e.g. local midnight) is on top of the circle (0°). The direction of the vector (phase angle) indicates the timing of the rhythm (its acrophase) and the length of the vector its extent (the amplitude). The ellipse around the tip of the vector indicates the 95% confidence interval for acrophase and amplitude.

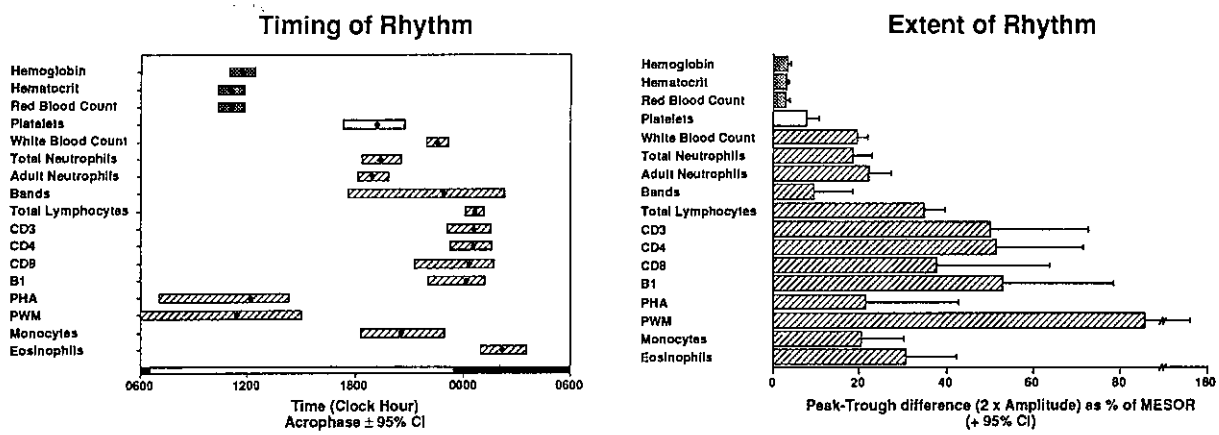


Fig. 2. - Timing and extent of circadian rhythms in circulating formed elements in peripheral blood and lymphocyte response to stimulation with phytohemagglutinin (PHA) and pokeweed mitogen (PWM) evaluated by population mean cosinor. Circadian acrophase chart of hematologic variables at the left and extent of the circadian variation at the right (expressed as double amplitude in percent of the MESOR). The circadian acrophase is shown as a solid circle bracketed by a horizontal bar which represents the 95% confidence interval of the acrophase.

to apply the study of biologic rhythms of laboratory variables to medical practice, statistically meaningful endpoints have to be reached in single subjects. The rhythmometric inferential statistical methods used have to be applicable to relatively short time series of measurements which may have to be obtained in unequal intervals and should provide as much quantitative information on a rhythm as the available data permit. The information needed to describe a rhythm is the rhythm adjusted mean (or MESOR: Midline Estimating Statistic of Rhythm), a measure of the extent of the rhythmic variation (the amplitude) and as indication of the rhythm's timing, the phase. Since biologic rhythms are not as precise as their counterparts in physics each of these rhythm parameters has to be presented with a variance estimate which will also help in the recognition of statistically significant differences between the rhythm parameters.

The description of rhythm parameters by the fitting of mathematical models to the data is frequently used, and in many instances yields valuable information. The fitting of sinusoidal models by least squares techniques has been widely applied, e.g., in the form of the different cosinor procedures [27-29, 41, 42].

In the single cosinor procedure [28, 42] the cosine curve best fitting to the data is determined by the method of least squares. The period to be examined is chosen by the investigator. If the period is not known a series of cosine curves covering multiple periods (a "spectral window") can be fitted to the data and the "goodness of fit" determined for each period. In order to test the presence of a rhythm in the data the total sum of squares is partitioned into the sum of squares due to the regression model and the residual sum of squares. The sum of squares due to the regression is the amount of variability accounted for by the fit of the model to the data. The residual sum of squares indicates the difference between the data and the fitted model. "Rhythm detection" is sought by testing the zero amplitude hypothesis for the best fitting cosine curve with an F-test.

For rhythms "detected", the cosinor procedures yields the rhythm adjusted mean (MESOR), the amplitude (the distance from the MESOR to the peak or the trough of the cosine curve best fitting to the data), and the acrophase (the peak time of the cosine curve best fitting to the data) each with their variance estimate (Fig. 2). The method also provides a confidence region for phase and amplitude which in the graphic representation of this procedure, the "polar cosinor plot" (Fig.1, right top) is usually presented in the form of an ellipse.

The rejection of the zero amplitude hypothesis ("rhythm detection") by single cosinor refers to a single data set and does not allow extrapolation to the population as a whole. In order to summarize results obtained for different individuals belonging to the same population, the rhythm characteristics obtained by the single cosinor can be further analyzed by the population mean cosinor [41, 42]. The rhythm characteristics obtained by the single cosinor are then considered as imputations or first order statistic. The population mean cosinor, in turn, contributes a second order statistic applied to derive confidence regions relating to the whole population. Differences in rhythm parameters between subjects or within a subject occurring e.g. as manifestation of disease should be ascertained by inferential statistical procedures (parameter tests) [29].

However, all these methods have certain limitations and assumptions which have to be met to avoid pitfalls which may lead to erroneous interpretations. The cosinor analysis requires as a major

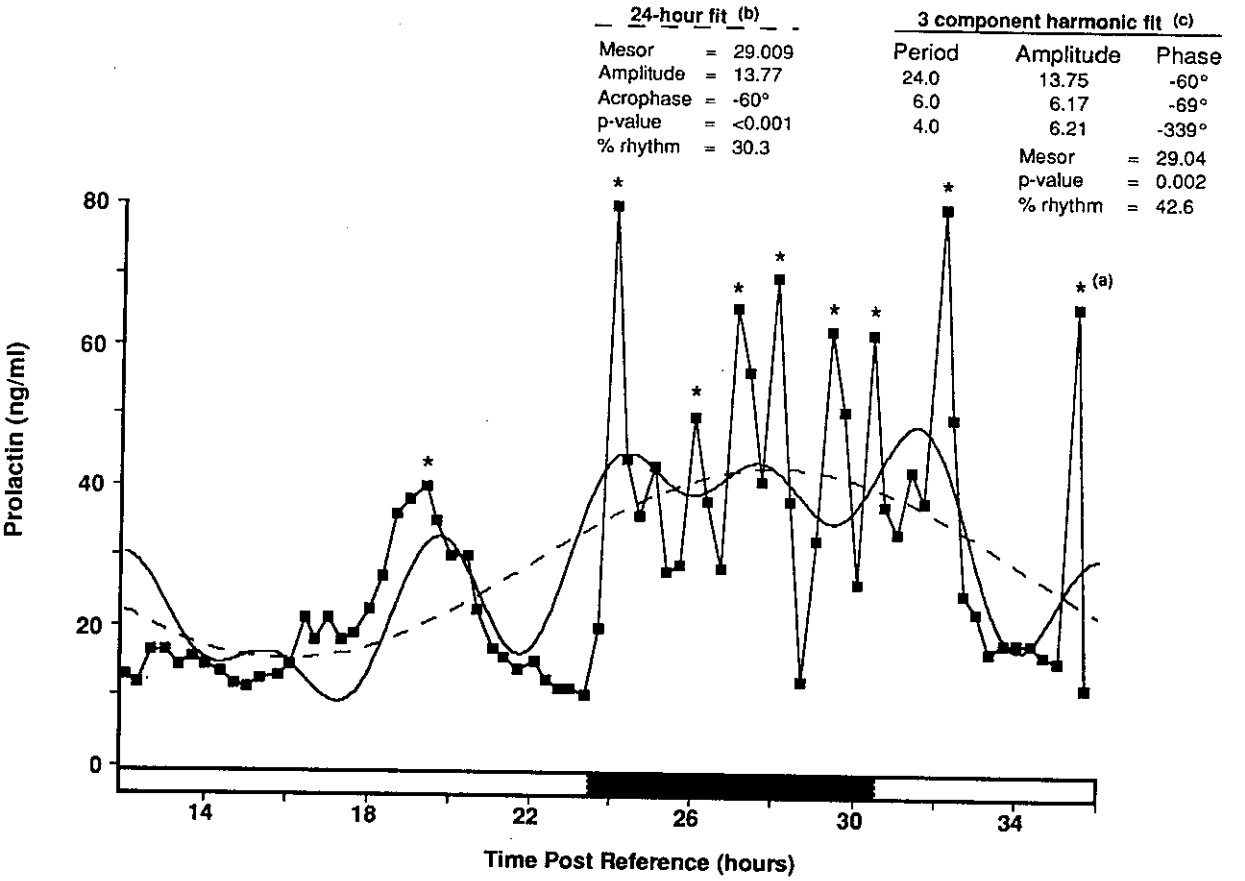


Fig. 3. - Plasma prolactin in a 19 year old woman determined at 20 min intervals over a 24 h span:
(*a*) Pulsatile secretion analyzed by Cluster Program of Veldhuis and Johnson [47]. «Significant» pulses recognized by program indicated by asterisk.
(*b*) 24 h cosine fit (phase reference: midnight of the first day of sampling). «Rhythm detection» by single cosinor. The circadian acrophase is not the highest value measured and the circadian amplitude is much smaller than most pulse amplitudes. Longer sampling intervals in a variable with pulsatile variations may lead to aliasing.
(*c*) Fit of a multi-component model consisting of a 24 h, a 6 h and a 4 h frequency best approximates the data (phase reference: midnight of the first day of sampling). Most pulse amplitudes are substantially larger than the amplitudes of any of the three components.

assumption that the data obtained can be reasonably well represented by a cosine curve and non-sinusoidality limits the applicability of the method. Also, it has to be clearly understood that of the rhythm parameters obtained by the fitting of a cosine curve to a set of data, the acrophase (the peak of the cosine curve best fitting to the data) does not necessarily represent the highest measured value. In variables showing irregular curve forms, e.g., due to superimposed higher frequencies or episodic variations, the acrophase may actually be found to correspond to a trough (e.g. may occur between two peaks of a superimposed higher frequency or pulsatile variation) (Fig. 3). Also the amplitude represents the interval from the MESOR to the peak or trough of the best fitting cosine curve, and not the interval between the highest and lowest actual measurements in the time series. In variables with high amplitude pulsatile or episodic variations (e.g. ACTH, LH, prolactin, etc.) the circadian amplitude may be only a fraction of the extent of the pulses observed (Fig. 3) which may lead to substantial aliasing if only a limited number of samples is available for the evaluation of the circadian rhythm. The application of cosinor procedures requires examination of the sinusoidality of the data to document to what extent a set of data can actually be approximated by a cosine curve. If a lack of sinusoidality is indicated by the large amount of residuals, which cannot be accounted for by the fitted model, different procedures have to be used. Some impression on the degrees of sinusoidality can often already be gained from the observation of the chronogram. Superimposed higher frequency rhythms may have to be documented by fitting of multiple frequencies to establish the optimal fit and by characterization of the rhythm parameters of each of the rhythms

involved (Fig. 3). Rhythmometric procedures are now available for the use with small computers as they are found in most clinical laboratories. However as with any new methodology, the clinical investigator has to be aware not only of the possibilities of those methods but also of their limitations. In the use of rhythm parameters as new clinical endpoints an adequate sampling density over an adequate length of time is essential to obtain meaningful results. Inadequate data may lead to erroneous conclusions which are not the fault of the statistical procedure.

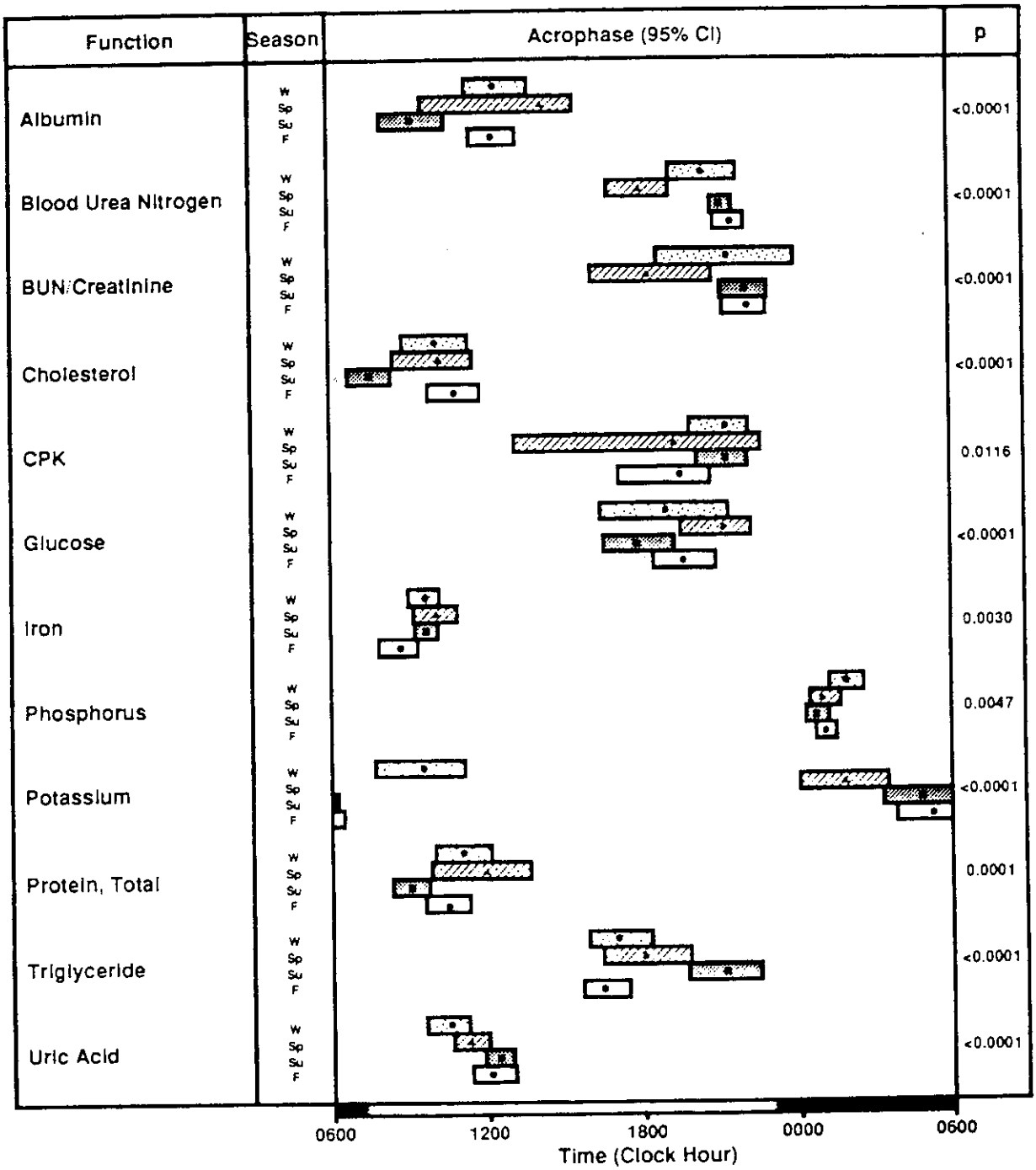


Fig. 4. - Seasonal variation of circadian timing of laboratory variables in 194 children 11 ± 1.5 years of age (comparable groups of children studied during different seasons; each child sampled 6 times over a 24 h span). Statistical significance of seasonal differences in acrophase as indicated by the Bingham parameter test [29] indicated at the right. Circadian rhythms are detectable during all seasons. The observed seasonal changes in acrophase may be the expression of circannual rhythms.

Measurement of episodic (pulsatile) events

In contrast to the evaluation of rhythms which are regularly recurring events, and thus predictable in their timing and detectable even in very noisy time series by rhythmometric procedures, the evaluation of the pulsatile, and in some instances, apparently randomly distributed episodic variations observed in many biologic variables, requires different methods of detection of the (unexpectedly occurring) pulses which have to be distinguished from the analytical sources of variation inherent in the methods of measurement including the chemical analysis used. Pulsatile variations of considerable amplitude are encountered, in the plasma concentration of most pituitary, adrenal, gonadal, pancreatic, and other hormones and neurotransmitters, and are of considerable physiologic importance for their action. For the recognition of a true pulse, e.g., of the secretion of a hormone, it is necessary to distinguish in each instance the variations due to methodologic factors from the secretory pulse. Methods developed for this purpose were introduced, and in their simplest form, were based on the comparison of the measured peak against multiples of the coefficients of variation of the method at one or at different levels of concentration in comparison to the previous and the following value [45, 46]. More recent programs take also the width of the pulse into consideration. In compounds with longer half lifetimes and higher frequency of pulses, we are faced with the problem of their superimposition. These problems have led to the development of a number of more elaborate computer programs, like the cluster program by Veldhuis's and Johnson [47] and others [48-51], and most recently to the development of methods using deconvolution analysis which are able to describe the dynamic processes, e.g., of hormone secretion and of removal of the solute measured from the circulation [52, 53].

The measurement of pulsatile secretions has led to a better understanding of numerous endocrine functions especially in the field of reproduction, where the response of the target organ depends critically on the pulsatility of the messenger substance from its superimposed control [53, 54]. Very essential information about the physiologic behavior of the hypothalamic-pituitary system and its target organs and tissues has been gained and is being applied, e.g., in the treatment of male and female infertility. However, the process of sample collection for the study of high frequency pulsatile variations of variables in blood is more complex and requires longer time series with short sampling intervals (e.g., 10 min for LH). Also the large number of costly and time consuming chemical assays is at this time still an obstacle to a more frequent practical application in endocrinology. Ongoing new developments in clinical chemistry with new automated instrumentation, however, may dramatically reduce the cost of the chemical determinations in the near future and make the rapid determination of large numbers of samples feasible.

Interaction of rhythms

The measurement and mapping of biologic rhythms in multiple frequency ranges and of trends like development and aging by quantitative techniques will have to include the relation between trends and rhythms, and between rhythms of the same and of different frequencies. The superimposition of different frequencies can lead to changes in the rhythm parameters of one or of several of the rhythms involved. Changes in circadian rhythm parameters have been observed as function of the season when a circadian rhythm was studied (Fig. 4). The changes in the circadian acrophase observed as a function of season imply, that in the description of a circannual rhythm, complete circadian profiles will have to be compared and that measurements, e.g., at a fixed clock hour during different seasons, may suggest a circannual difference in «level» when in reality a circannual difference in acrophase has occurred. The description of the human time structure in its complexity (beyond the description of individual rhythms) is still in its early infancy and will in part depend on the development and use of automatic measurement devices, allowing to quantitate biologic variables by noninvasive methods with as little as possible interference with the subject to be measured, and at relatively low cost. Preferably, these devices should be equipped with computer compatible output allowing rapid analysis, which is essential if chronobiologic techniques should have relevance for current patient management.

The establishment of time qualified reference ranges (chronodesms)

The mapping of the human time structure in clinically apparently healthy peer populations leads to the establishment of time qualified reference ranges. These reference ranges will have to include trends and rhythms and their many interactions. This is a complex task and will have to be accomplished by the collaboration of numerous laboratories at different geographic locations using as far as feasible comparable techniques of data collection, data analysis, and rhythmometric statistical evaluation. Information available is, thus far, limited to some populations in certain geographic locations and often to groups with small numbers of subjects. The preliminary data, thus far available, indicate in many variables, temporal differences with age, sex, ethnic-geographic background and differences due to the interaction of rhythms of different frequencies like, e.g., changes in circadian rhythm parameters as function of circannual rhythms. Different kinds of chronobiologic reference ranges can be established [17, 18, 31, 55-58]. E.g., an individual reference

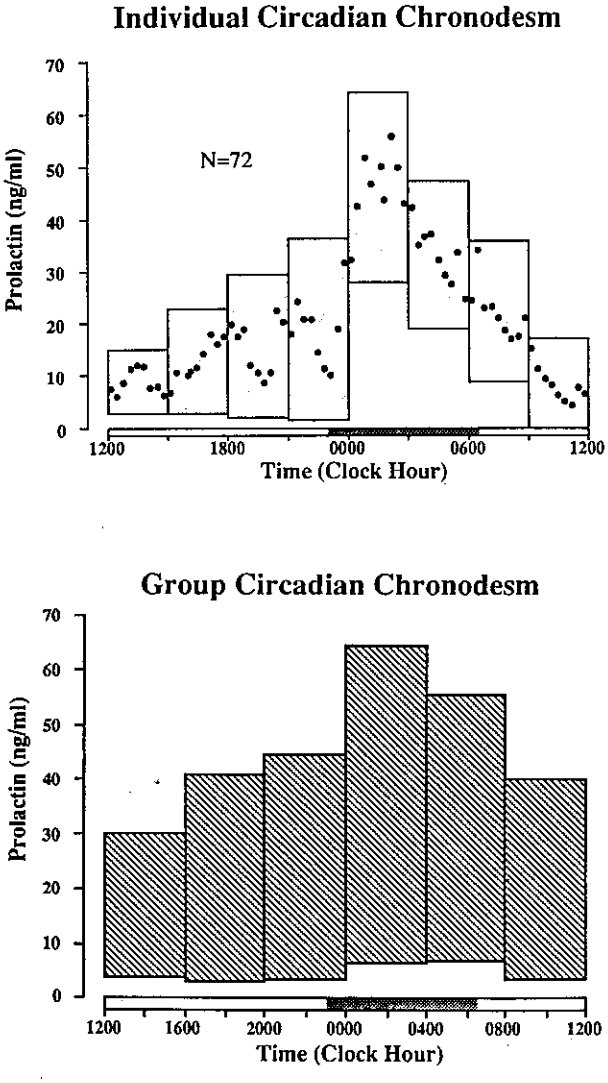


Fig. 5. - Individual circadian chronodesm and group circadian chronodesm for plasma prolactin concentration in clinical healthy diurnally active women.
(Top) Individual chronodesm in a 46 year old woman, 72 determinations over a 24 h span. Tolerance intervals determined separately for each 3 h span indicate limits within which 90% of measurements would be expected to fall with 90% confidence.
(Bottom) Group circadian chronodesm. Reference range delineated by 2.5 and 97.5 percentile of 816 to 1025 data points per 4 h span. The nongaussian distribution of the serum prolactin concentration requires use of a nonparametric statistic to delineate the usual range. The use of percentiles is simple and practical but requires an adequate number of measurements per time span to be meaningful.

range established by longitudinal observation of a single individual may allow the early recognition of changes which may indicate a risk state or may be the harbinger or the expression of disease. Individual reference ranges are usually relatively narrow and if the data base is adequate, may be most sensitive in recognizing change and potentially early pathology (Fig. 5, top).

In contrast, the time qualified reference ranges provided by peer groups will usually be broader (Fig. 5, bottom; Fig. 6), and in variables with large interindividual differences may be so broad as to add little value to reference ranges established without regard to time in the evaluation of single measurements (Fig. 6, left). The more uniform the peer group and the more comparable it is to the subject under study in ethnic-geographic and social background, age, sex, sleep-wakefulness schedule, life style, etc., and the less it is exposed to a variety of environmental stimuli which may be different from the subject to be studied, the more pertinent and sensitive a reference range will be. Reference ranges obtained by peer groups will be useful especially in variables with high amplitude rhythms. In time qualified reference ranges of such rhythms, the same measured value can in an individual be below, within, or above the usual range, depending on the time of sample collection [31, 59] (Fig. 5, top). In the establishment of time qualified usual ranges the distribution of the measurements in the different stages of the rhythm has to be considered. Non-gaussian distribution of the data requires e.g. logarithmic transformation or the use of a non-parametric statistic. Delineation of the usual range by e.g. the 2.5th and 97.5th percentile is commonly used in our laboratory whenever adequate data are available (more than 120 measurements per timepoint).

A more advanced chronobiologic application to laboratory and clinical medicine than the evaluation of a single or of a few measurements against a chronobiologic reference range is the comparison of the mathematically defined rhythm parameters of a subject, with those of a peer group (Fig. 7) with statistical validation of differences by rhythmometric techniques, including a parameter test [29]. The establishment of statistically valid rhythm parameters requires an adequate data base. In many clinical investigations, data collection, especially if it involves invasive techniques like blood drawing, has to be limited (e.g., 6 samples at 4 h intervals over a 24 h span). With such limited sampling, statistically meaningful rhythm parameters will often not be obtainable by rhythmometric techniques in a single subject, or if obtainable, may vary considerably from one sampling span in the same subject to the next, and/or from one subject to the other and thus may be of limited value (Fig. 8). The data requirement for obtaining reliable rhythm parameters will vary from one biologic variable to the other, and depends on numerous factors like the regularity of the rhythm, superimposed higher frequencies and/or pulsatile variations, the susceptibility of the rhythm under study to environmental stimuli (e.g. in catecholamines) and other factors [31, 32, 60].

Characteristic patterns of pulsatile or episodic secretions have been described for clinically healthy subjects and changes in these patterns have been

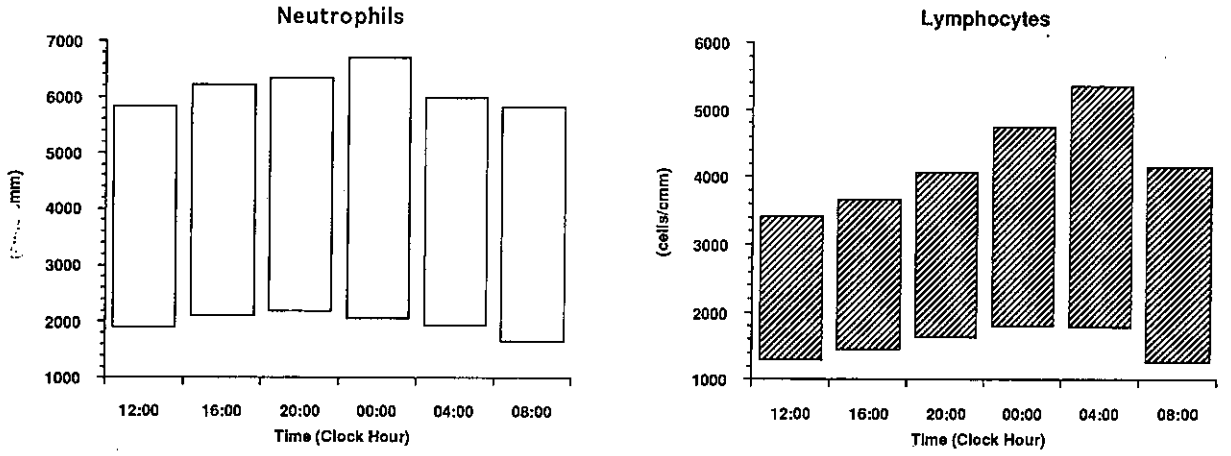


Fig. 6. - Time qualified reference ranges (chronodesms) for number of circulating neutrophils and lymphocytes in 150 clinically healthy young adult and adult subjects (mean age 24, range 11 to 57 years of age). The usual range for neutrophils is wide due to individual variability in MESOR and acrophase. Although the changes of most subjects within a 24 h span are quite considerable (and may be of clinical importance). The interindividual differences in numbers and in timing of the circadian rhythm make the circadian peer group chronodesm of the number of circulating neutrophils so large as to be of little advantage above non-time qualified reference ranges in the evaluation of single measurements.

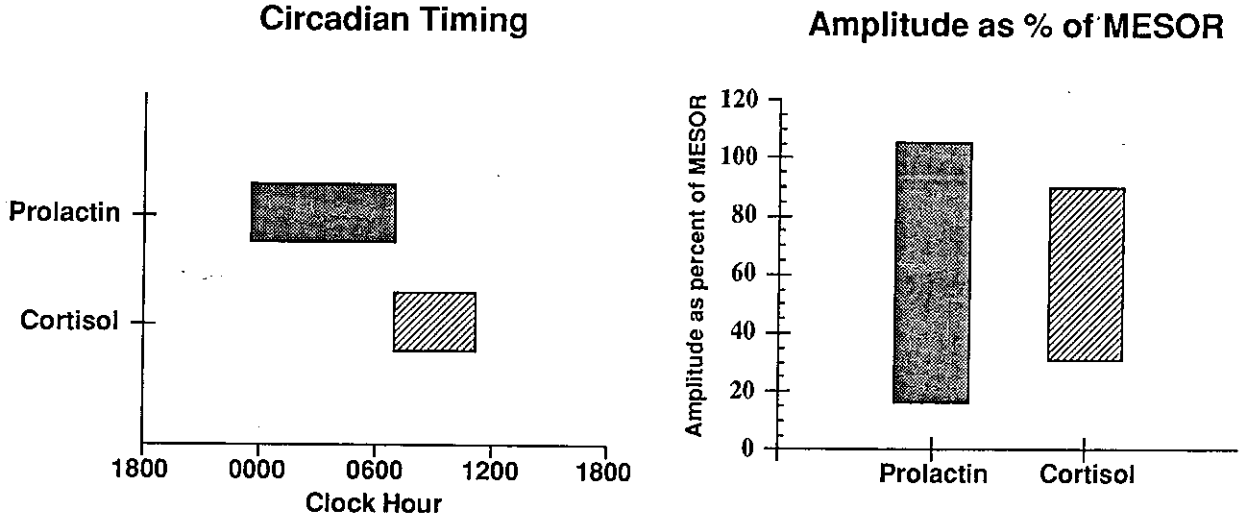


Fig. 7. - Usual range of circadian acrophase and amplitude in plasma cortisol and prolactin concentration in 140 clinically healthy women (15 to 58 years of age), each measured 72 times over one 24 h span. Usual range delineated by 2.5 and 97.5 percentile of acrophase and amplitude respectively. The acrophase in prolactin precedes that in cortisol ($p < 0.001$, by Bingham parameter test). Rhythm parameters are new endpoints in defining «normalcy».

characterized in a number of endocrinopathies [52, 61]. The establishment of quantitatively defined usual ranges for pulse characteristics and their possible interaction with other frequencies (ultradian and/or circadian) will still require further study with the use of comparable clinical, biochemical and statistical methods. The establishment of usual ranges for pulse parameters has become more complex since recent observation from our laboratory showed statistically significant differences between ethnically-geographically different populations [62]. Japanese and American women of different ages studied under as far as feasible comparable conditions showed in their pulse characteristics statistically significant differences in cortisol as well as in prolactin, which in view of the epidemiologically determined differences of the two populations in their risk to develop malignancies in endocrine target organs (i.e. the breast and the prostate) may be of some clinical importance. These observations suggest

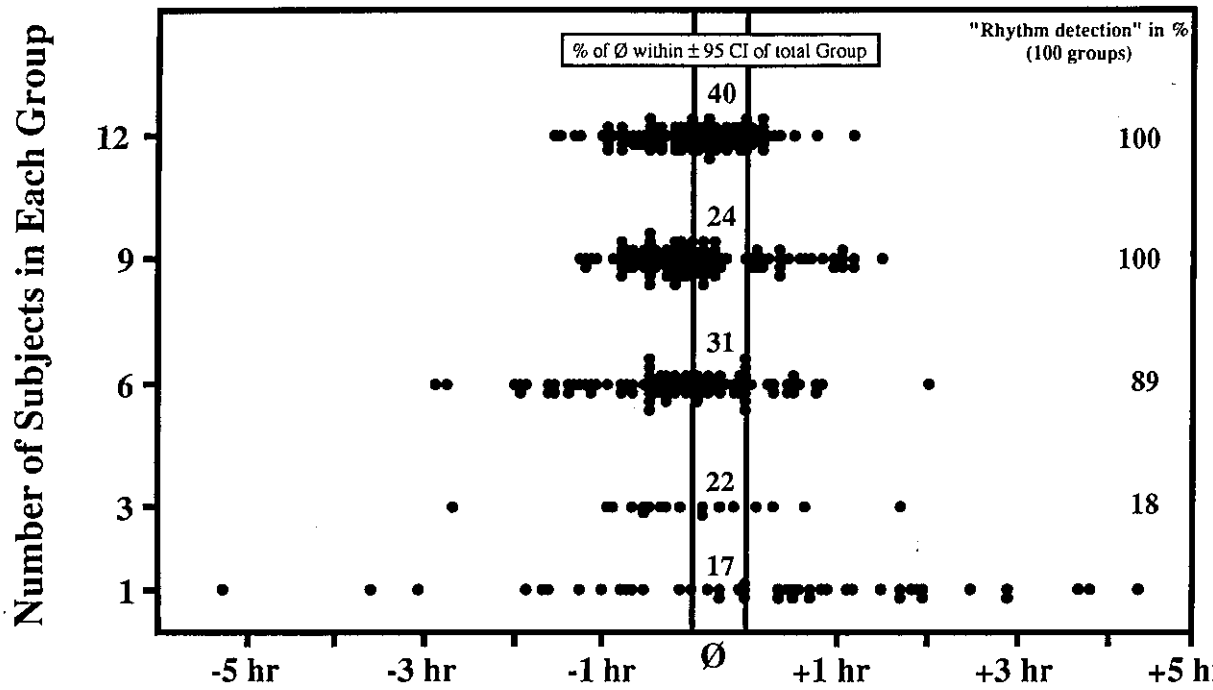


Fig. 8. - Circadian acrophase of plasma cortisol concentration in 278 elderly subjects (77 ± 8 years of age) measured 6 times (t = 4 h) over a 24 h span. Acrophase of entire group with its 95% CI is bracketed and the deviation from the group acrophase (plus or minus) is indicated on abscissa.
The acrophases obtained in individual subjects (single cosinor) is shown in bottom line. Only in 17% of subjects was «rhythm detection» obtained and only 17% of those were within the 95% CI of the acrophase of the entire group of 278 subjects.
Above bottom line are the acrophases of randomly selected groups of 3, 6, 9, and 12 subjects showing «rhythm detection» by population mean cosinor. The percentage of groups with «rhythm detection» by cosinor is indicated at the right, the percentage of acrophases falling within the group 95% CI of the population mean cosinor for the entire group of 278 is shown in center.
With this sampling length and density (6 samples/24 h) only 46 individual subjects show statistical «rhythm detection» which is not much improved by a group size of 3. A group size of over six subjects is needed to allow «rhythm detection» in 90% or more of the groups.

that in the study of pulsatile secretions, the results obtained in one population cannot necessarily be generalized and applied to others.

Detection of risk states for common diseases

The quantitative measurement of rhythm parameters in multiple frequencies has led to the detection of rhythm alterations in apparently clinically healthy subjects, who on the basis of epidemiologic or other information, are at risk to develop some common disease states like hypertension [21, 63], eclampsia [64], breast cancer [19, 20, 59], the tendency to develop alcohol and drug addiction [19, 65], and others. The detection of a risk state may be of considerable importance for an attempt to prevent disease before it is fully established. In some of these risk states, the endocrine or other rhythm alterations detected may play a role in the development and pathogenesis of the disease. The early detection of a risk state may lead to rational prevention either by, e.g., general measures of hygiene, early endocrine manipulation, or perhaps, by adjustment of the temporal alteration observed. The problem we are facing in risk state detection by the study of temporal abnormalities are the data requirements. These may not be insurmountable when a variable can be measured by non-invasive biophysical means, e.g., by automated monitoring as has been shown, e.g., in the detection of the risk state to develop hypertension by blood pressure monitoring even in the newborn [66, 67]. If, however, the risk state detection is based upon plasma hormone measurements at a limited specified circadian time, at a certain stage of the circannual cycle of this hormone [19-21] or upon changes in the circannual amplitude of a hormone [59], the finding is certainly very interesting from a pathophysiologic viewpoint. However, in view of the multiple variables determining a certain hormone concentration at a certain time and/or the data requirements to obtain representative and statistically vaild endpoints like a circannual amplitude, the detection of a risk state by such time-specified measurements may, with to-

day's technology, not be realistically obtainable in an individual patient. Greatly facilitated measurement techniques (e.g. dipsticks) in material obtained by noninvasive techniques (e.g. in saliva or urine) may make risk state detection through the study of biochemical endpoints by chronobiologic techniques in the future more feasible.

Detection of temporal pathology in clinical disease

Optimal function («health») of the human organism depends upon time dependent changes like the pulsatile secretion of hormones and rhythms in numerous frequency ranges, their sequence, internal and external phase relations, and interactions. Suboptimal function and outright pathology may be caused by or lead to changes in the human time structure. Chronobiology adds to the description of a pathologic process in morphologic terms (anatomic pathology), and to the biochemical description of the process (chemical pathology), a pathology in time (chronopathology) which in some instances may be the first or even the predominant change observed.

Abnormalities in pulsatile (episodic) hormone secretions characterize numerous endocrine related disease states. This has been recognized originally by observation of the pulse pattern on the chronogram and more recently by mathematically defined and statistically verified abnormalities in frequency, width and/or amplitude of pulses indicating differences in secretory dynamics of endocrine functions (for reviews see [52-54, 61, 68].

The study of rhythm alterations in disease requires adequate data to arrive at a statistically significant quantitative description of the rhythm parameters in order to recognize a deviation from their usual range including, in some instances, the absence of a detectable rhythm. The environmental synchronization of the patient has to be known to avoid pitfalls due to changes in synchronization like night and shiftwork with differences in activity-rest schedule, a recent history of transmeridian flights or changes due to transient rhythm alterations (masking) by environmental factors (like a hospital admission or the data collection procedures themselves). If a rhythm alteration is suspected on the basis of rhythmometric techniques a validation of the difference by a parameter test [29] or by comparable procedures is essential.

In the study of rhythm alterations in disease, a change in rhythm parameters may be caused by interference with synchronizer perception, with the pacemaker(s), with signal transmission or with alterations in target tissue response. Blind subjects who have no light perception at the retinal photoreceptor level may be synchronized by their social surroundings, rest-activity pattern, and time of food uptake but often show low amplitudes in their circadian rhythms and/or a tendency for free running of circadian rhythms [69, 70]. Circadian rhythm alterations have been reported with destruction of the hypothalamic pacemaker in patients with hypothalamic lesions and pituitary adenomas [71].

However, free running of circadian rhythms from every known environmental synchronizer can be observed occasionally in apparently healthy subjects, and/or can be the consequence of "unacceptable" environmental schedules (e.g. 8 h "days"), but can also develop during treatment of a patient e.g. with radiotherapy for cancer [72]. The observation that some circadian rhythms may follow their usual synchronizer while others develop a different frequency with resulting internal desynchronization [72] may be of importance in timed treatment with potentially toxic compounds and may require continuous monitoring of the patient's circadian system and the choice of a "correct" marker rhythm remaining in phase with the susceptibility-resistance cycle being followed.

Abnormalities in both phase and amplitude of several circadian rhythms are found in affective disorders [73-77]. The pulsatile secretion of ACTH and cortisol shows in depression numerous high pulses alternating with deep valleys and low values between pulses leading to no clinical hypercorticism although the 24 h mean values may be at a similar level as seen in patients developing Cushing's disease [78]. A phase and response alteration in the pituitary-adrenocortical system underlies the dexamethazone suppression test as used in the diagnosis of affective disorders [79]. Circadian as well as circannual rhythms may be involved in the pathophysiology of affective disorders in a complex manner which still requires further investigation [74].

In hematology, the large amplitude rhythms in white blood cell counts may be of diagnostic importance and have to be considered in the evaluation of consecutive leukocyte and differential counts. Although the time qualified usual ranges of peer populations especially of granulocytes (Fig. 6) are rather broad, the extent of the circadian change in individuals in number of circulating granulocytes and lymphocytes will be on the average 80-100% of the lowest value encountered [80, 81].

Infection with the human immunodeficiency virus (HIV) leads to an alteration in the circadian rhythm not only of circulating lymphocytes and lymphocyte subtypes but also of neutrophils as an early event in the disease process [82-85]. The circadian rhythm alterations of the circulating neutrophils and lymphocyte subtypes were found irrespective of whether these cells in a given patient were within, below or in the case of CD8 positive ("T-suppressor") cells even above the usual range [85].

In patients who had received autologous bone marrow transplantation for acute leukemia or non-Hodgkins lymphoma with the marrow treated *in vitro* with cyclophosphamide, Martini

et al. [86, 87] reported with sampling limited to 0800 and 0000 rhythm alterations in the number of CD4 positive ("T-helper") cells even when the total number of cells was within the usual range.

More impressive than the circadian change in the number of circulating lymphocytes was the change in response to lectin mitogens like phytohemagglutinin (PHA) and pokeweed mitogen (PWM) with mitogen induced cell proliferation measured by ^{3}H-thymidine uptake showing a circadian rhythm with an extent of around 400% over the lowest value [80]. Also the changes in platelet function are more important than those in platelet numbers. Platelet aggregability to stimulation with adenosine diphosphate (ADP) or epinephrine shows a circadian rhythm usually with peak during the late night and morning hours [88-91] and in platelet adhesiveness measured by retention on a glass bead column with a peak in the early morning hours [90]. Together with the circadian rhythm in coagulation factor activity showing a peak in the morning [88, 90, 92] and a minimum in fibrinolytic activity occurring at the same time [93, 94] and the morning rise in plasma catecholamines [89], the circadian rhythm in platelet function is a likely contributor to the transient circadian risk state for the occurrence of sudden cardiac death [26, 95, 96], myocardial infarction [25, 97], and cerebral infarction [97, 98] which all occur most frequently in the early and mid-morning hours.

Other than circadian rhythms are encountered in hematology. Cyclic hematopoiesis with a frequency of 18-22 days had been reported in clinically healthy subjects [99-101], but could not be found by other investigators [102, 103]. Cyclic hematopoiesis occurs in a number of distinctive disease entities, i.e., cyclic neutropenia and cyclic thrombocytopenia characterized by about 21 day fluctuations in the number of circulating blood neutrophils, monocytes, eosinophils, lymphocytes, platelets, and reticulocytes [104, 105]. The numbers of monocytes, platelets and reticulocytes oscillate in cyclic neutropenia frequently with the same period as the neutrophil count but out of phase. The basic defect in these disease entities appears to be a stem cell disorder [106, 107]. The symptomology varies with the cell line primarily involved and the severity of the condition.

Cyclic oscillations in the white blood cell count were reported in a subpopulation of 10 to 20 percent of patients with Phl positive or Phl negative chronic myelogenous leukemia [108-110]. It was suggested that a closer longitudinal study might lead to the detection of even larger numbers of patients with more or less pronounced cycling of the leukemic elements. The periods reported varied between 30 and 120 days, most often around 70 days. In the course of progression of the disease or of lowering in the white cell count by treatment, the cycling continues following the upward or downward trend of the white blood count.

The immune system in its different components shows a multifrequency time structure with circadian, circaseptan and lower frequency rhythms (for reviews see [80, 111, 112]). Many of the cells involved in immune reactions like the T-cell subsets, B cells, macrophages and natural killer cells show circadian variations in number and activity [80, 81, 113-115]. Circulating immune complexes in lupus and rheumatoid arthritis patients undergo circadian variations [116]. Alteration in the circadian acrophase of the circulating eosinophils in the active form of rheumatoid arthritis have been reported [117]. The initiation of episodes of kidney homograft rejection appears to be circadian periodic [118, 119]. Of interest is the infradian (predominantly circaseptan) response pattern of the immune system in the production of antibodies [120], in the regeneration of immunologically competent cells after treatment with immunosuppressants [121] and in homograft rejection in experimental animals as well as in human subjects [122, 123]. These rhythms represent the expression of an infradian response pattern of the immune system triggered by the introduction of an antigen irrespective of the day of the week or other known environmental synchronizers. Due to the importance of the immune system for the organism's defense and for self recognition and/or autoimmunity and the related autoimmune diseases, a closer attention to the infradian (e.g. circaseptan, circatrigintan and circannual) variations in its activity may be of importance in future investigations of this system.

Circaseptan response patterns are not limited to the immune system but come to the fore in other variables, e.g., when experimental animals have to adapt to an environmental load like e.g. a high salt diet [124]. In human subjects in this context, the circaseptan pattern of sudden cardiac death may be of importance which was observed also under rather uniform environmental conditions [26].

In gastrointestinal pathology circadian, as well as seasonal rhythms, have been shown to be of importance and their disturbance by shiftwork and unusual schedules especially with alteration in the usual relation between activity and food uptake may play a role in ulcerogenesis [125-127].

Desynchronization of circadian rhythmicity has been reported in cancer patients [128-130] and requires verification and/or monitoring of host rhythms during timed chronotherapy.

Of recent interest in laboratory medicine are rhythms potentially related to growth and metabolism of tumors. In peritoneal washings in patients with ovarian carcinoma, Klevecz and Braly [131] found ultradian as well as circadian rhythms in tumor cell proliferation. These and studies involving repeated tumor biopsies e.g. in breast or in metastatic squamous cell carcinoma showed circadian rhythms in tumor as well as in host cells [132, 133, and Sothern *et al.* 1992, unpublished data]. How-

ever, studies on tumor rhythms based on invasive procedures are not easily applicable to the every-day clinical situation. Less invasive or noninvasive means for the study of tumor periodicity may be the measurement of metabolic products of the tumor in the form of diverse chemical or antigenic substances, growth factors, receptors, etc., serving as tumor markers. Ultradian, circadian and infradian rhythms in tumor markers have been found and need to be explored further [129]. Circadian variations have been shown to occur in carcino-embryonic antigen in patients with advanced ovarian or bladder cancer [129, 134] and in the concentration of alpha fetoprotein [129, 135]. Circadian variations in prostate specific antigen have been reported in patients with prostate cancer [136]. Circadian rhythms in CA125 and CA130 have been found also in a patient with ovarian cancer in serum and saliva. If the changes found in saliva would reflect rhythms in tumor growth and metabolism, this would allow the monitoring of the tumor by non-invasive means [137, 138]. Ultradian rhythms in CA125 and CA130 [139], in macrophage colony stimulating factor (M-CSF) [140] and in urinary chorionic gonadotropin protein [141] and circaseptan rhythms in CA125 [142] and M-CSF [140] have been found in the same patient. The about 14 h ultradian rhythms in this patient may be comparable to the ultradian rhythms described by Klevecz and Braly [131] in proliferating tumor cells in the peritoneal lavage. It will have to be determined if these variations in tumor markers actually reflect tumor rhythms in metabolism and/or in cell proliferation which may provide an opportunity to treat at certain stages of a rhythmic variation in an attempt to achieve an optimal effect on the tumor, and, if possible, minimize the undesirable side effects upon critical cell systems of the host. The timing of treatment with bone marrow toxic agents may then take into account both the tumor rhythms and the circadian rhythm in cell proliferation of the human bone marrow originally described by Mauer [143] and more recently, more extensively documented by Smaaland *et al.*, [144, 145]. In patients with malignancies, the study of bone marrow mitotic activity suggested a slightly different acrophase [146] which may have to be taken into consideration in the timing of cancer chemotherapy. If this promising approach is successful it will be the task of the clinical laboratory to provide timely and cost-effective laboratory support including the statistical analyses to monitor both host and tumor rhythms during treatment.

"Therapeutic drug level monitoring" (TDM), chronopharmacokinetics, and chronopharmacodynamics

Drug resorption, drug binding, metabolism, disposition, and excretion determine the drug levels regarded as therapeutic and measured in the clinical laboratory but the patient's response is essential for their interpretation. All of these factors have been shown to vary rhythmically in one or more frequencies with the circadian one being the best explored. The rhythmicity of pharmacokinetic as well as pharmacodynamic factors plays a role in the expression of the often very substantial time dependent differences in drug effects (for reviews see [15, 147, 148]) and contributes to the susceptibility resistance cycles towards numerous potentially noxious agents shown so dramatically in animal experiments [149-154] and experienced also in human subjects [15, 147, 148, 155].

In the application of chronobiology to therapeutic drug level monitoring, it has become obvious that a drug level obtained at a certain time has to be interpreted not only in relation to the time after administration, but also in relation to the time of administration. The pharmacokinetics of many drugs changes as a function of the stage of the circadian system of the patients at the time of dosing. However, the differences in pharmacokinetics do not necessarily determine a corresponding response of the patient. The effect of a certain drug level obtained at one time is not necessarily biologically equivalent to the same level at a different stage of a circadian (or other frequency) rhythm.

The pharmacokinetics of a large number of drugs has been shown to be different after dosing at different circadian stages (for review see [148]). For example, the time to reach maximum drug concentration after oral ingestion of ethanol (T_{max}) was found to be shorter, the maximal concentration (C_{max}) higher and the area under the time concentration curve (AUC) larger and elimination faster after ingestion in the morning as compared to the evening [156, 157] (Fig. 9 and 10). Similar circadian stage dependent differences with shorter T_{max}, and higher C_{max} in the morning hours are found irrespective of food uptake for a variety of non-steroidal anti-inflammatory agents [158-160], a beta blocker (propanolol) [161], aspirin, theophyllin, and others [15, 127, 148, 162]. Circadian periodic gastrointestinal factors like time of gastric emptying, intestinal propulsion and metabolic factors of resorption may play a role (for review see [127]). In this context, it is likely that although the rhythms in food and drug absorption may not be caused by food uptake at the day of the study, they may be entrained in their timing by the habitual time of food uptake over a prolonged time span, as it has been shown in animal experiments [14, 163-165]. Substantial differences in the circadian periodicity of the resorptive behavior of water soluble and lipid soluble agents were observed [161, 166].

Once resorbed, protein binding may vary with circadian periodic changes in the plasma concentrations in total plasma protein, albumin, globulin [17, 167] and specific carrier proteins like thyroxin

binding globulin [17, 168], sex hormone binding globulin [169] and others. Also interactions with other circadian periodic metabolites which interfere with a drug's protein binding may play a role in the distribution between the protein bound and the biologically active free form of the drug as has been shown, e.g., for valproate and free fatty acids [170].

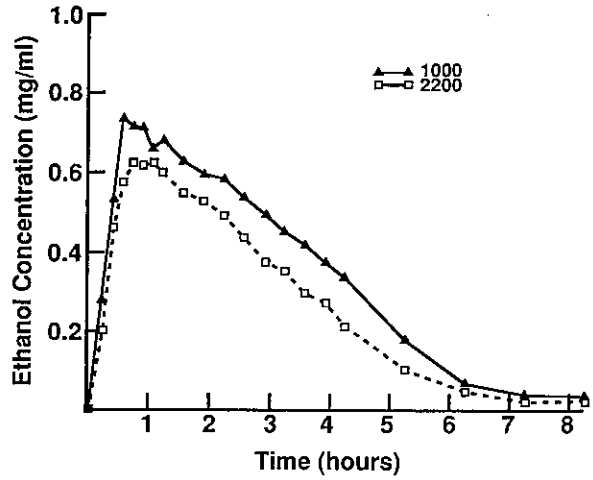
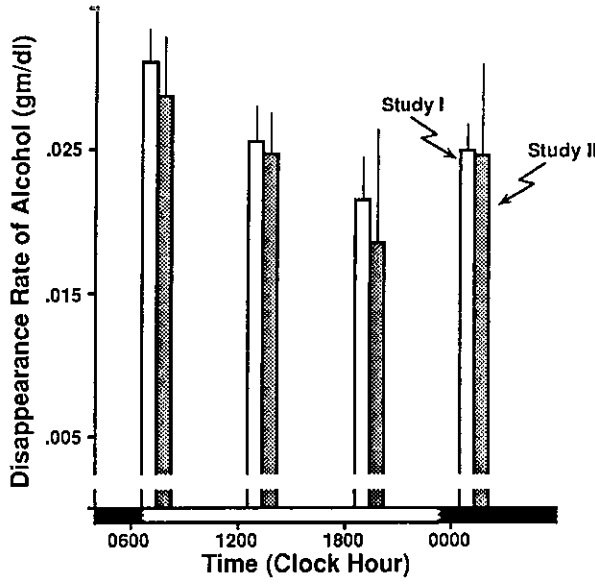


Fig. 9. - Concentration-time curve of blood ethanol in 11 clinically healthy young adult men and women after ingestion of 0.451 g/kg body weight of ethanol at 1000 and 2200 (in crossover design, 1 week apart). Statistically significant difference with higher maximum concentration (C_{max}), shorter time to maximal concentration (T_{max}) and larger area under the curve (AUC) after ingestion at 1000.

In drug metabolism, the large amplitude metabolic rhythms in the liver are of importance. These rhythms persist without the availability of food and water [171] and phase shift in rodents after a 12 h change in lighting regimen to reach target phase with similar or even somewhat slower (about 9 days) shift velocity than the circadian rhythm in plasma corticosterone (about 6-7 days) [172]. The phase adaptation of these metabolic rhythms is slower than the animal's circadian feeding behavior which follows rapidly the change in lighting regimen. In timed feeding patterns in animals (e.g. 4 h feeding spans per 24 h) and in similar studies in human subjects, lighting regimen and time of food uptake may act as competing synchronizers with, e.g., insulin, liver glycogen and serum thyroxin following predominantly the time of food uptake while other circadian rhythms like those of the circulating white blood cells or plasma cortisol either follow the lighting regimen or show some intermediate phase in relation to the two synchronizers [14, 17, 164, 173]. Also the blood flow to the liver shows a circadian rhythm which may play a role in drug metabolism [174, 175]. Differences in pharmacokinetics as a function of circadian rhythmicity in drug metabolism have been found, e.g., for intravenously administered prednisolone [176], with higher C_{max} and AUC after dosing at noon as compared to the evening. After oral administration of indomethacin-SR, not only the drug resorption showed a circadian rhythm but also the appearance of its main metabolite varied rhythmically and in contrast to the drug itself showed substantially higher serum concentrations and AUC after administration at 20.00 as com



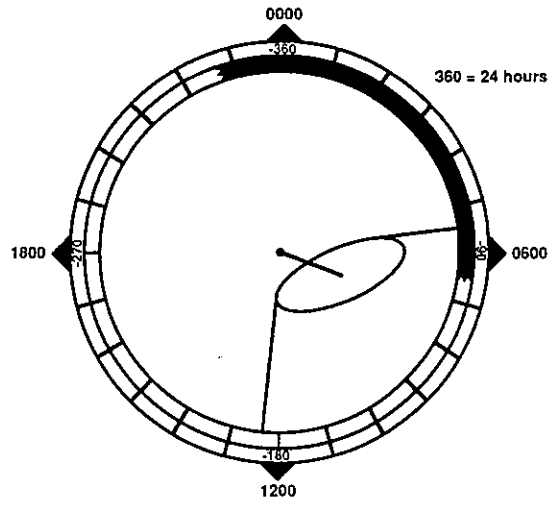


Fig. 10. - Circadian rhythm in ethanol disappearance rate from human plasma 30-120 min after oral ingestion. Two studies (Study I - 15 subjects: 8F, 7M and Study II - 4 subjects: 2F, 2M) with the same subjects tested at four different time points in latin square design 1 week apart.

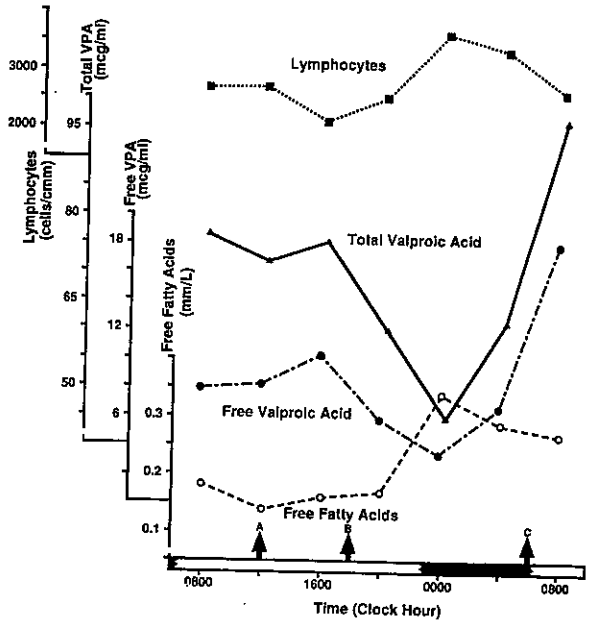


Fig. 11. - Circadian variations in plasma concentration of total valproic acid and free valproic acid. Free fatty acids and circulating lymphocytes as reference functions in a 50 year old epileptic patient. Although the drug was dosed to give what was thought to be a «steady state» with presumably «constant» blood level, the actual drug concentrations show marked circadian variations. Among the reference or marker rhythms, free fatty acids may show a causal relationship to the distribution between free and bound valproic acid [170] while the lymphocytes are purely a biologic time marker. Patient dosing of drugs was as follows on the chronogram: A = 250 mg Depakene, B = 50 mg Mebaral and 500 mg Depakote, and C = 500 mg Depakene.

pared with 08.00 or 12.00 [177]. This observation indicates that in substances with active metabolites, the plasma concentration of the mother drug may not be alone responsible for the therapeutic effect (or for undesirable side effects). Due to the rhythmic changes in drug handling in the body, the attempt to achieve a «steady state» with presumably constant drug levels over the 24 h span very often does not nearly lead to a constant plasma drug concentrations but to marked circadian variations (Fig. 11) which were found to occur even with continuous drug infusions as has been shown, e.g., for ketoprofen [178] or 5-fluorouracil [179]. On the other hand the circadian differences in heparin effects after constant rate infusion are more likely due to the circadian variation in coagulation activity, with a relative hypercoagulative state in the morning than due to a circadian variation in heparin plasma concentrations [89, 91, 180].

Although the pharmacokinetic behavior of a drug may at some rhythm stage correlate well with the observed effects, it may not do so at another [181]. At the level of the target tissue for drug action (but also in drug responsive non-target structures), there is often a multi-frequency periodic response which, in some instances, may be related to rhythmic changes in receptor concentrations in the circadian (Fig. 12) (modified from [182]), estral (Fig. 13) (modified from [183, 184]), menstrual [185] and circannual range [186-188].

In therapeutic drug monitoring we have to consider differences in the interpretation of a drug level, depending upon the time when it was obtained, both in regard to the desired therapeutic effect as well as in regard to undesirable side effects. In clinical chronotherapy the physician

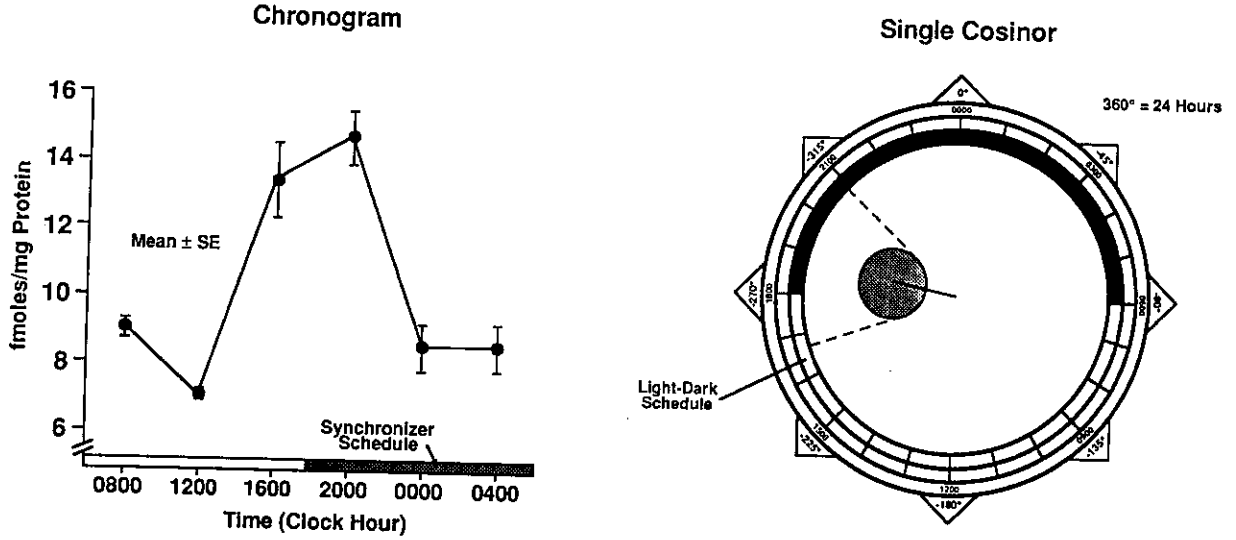


Fig. 12. - Circadian rhythm in cytoplasmic estrogen receptor concentration in the breast of young adult Balb/c female mice (20 mice per time point, tissues assayed in pools of 5 mice each). (modified from [182]).

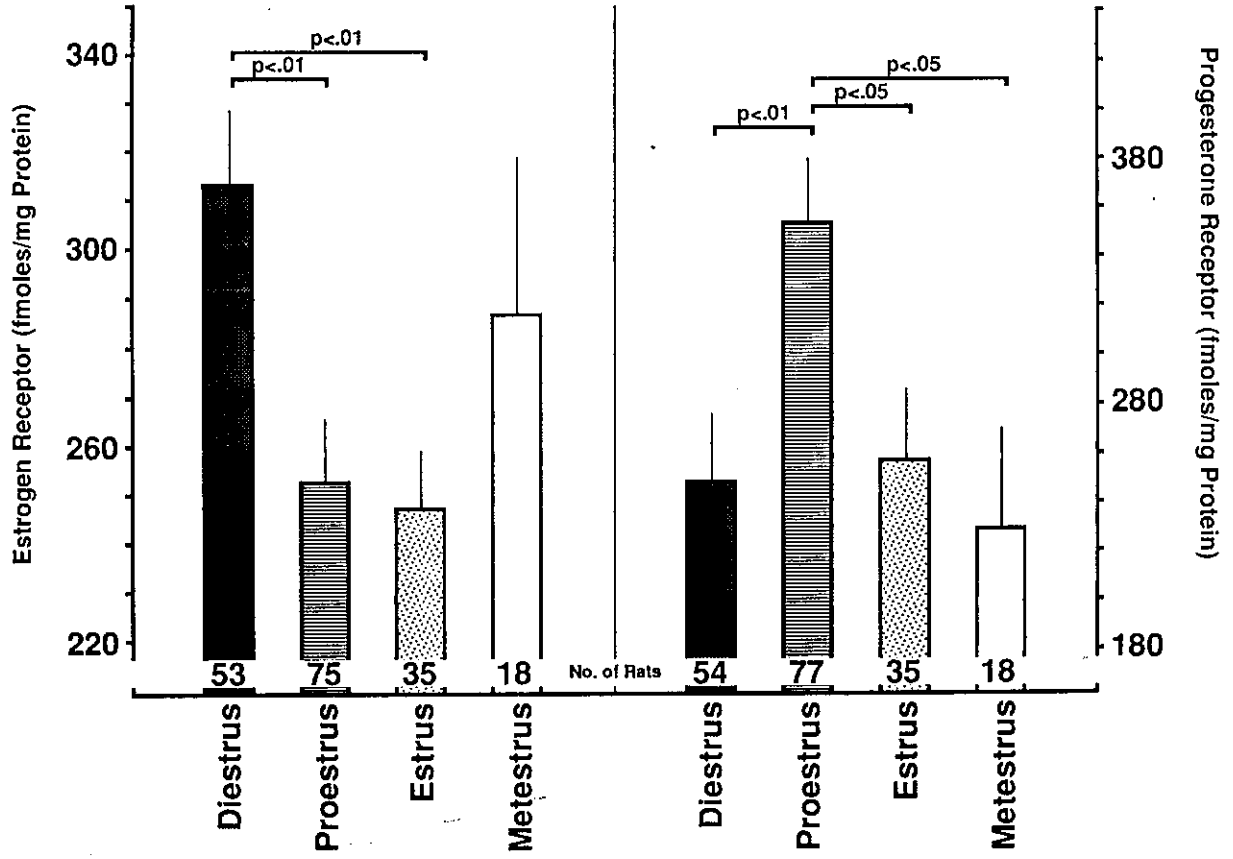


Fig. 13. - Estrous cycle dependent rhythm in uterine estrogen and progesterone receptor content in Wistar rats. Estrogen and progesterone receptors show a characteristic phase difference mediating hormone effects during the estral cycle. (modified from [184]).

should attempt to find in the design of dosing schedules, whenever possible, a temporal dissociation between drug toxicity and optimal drug effect. When the ideal situation of a dissociation of the time of desired effect and peak toxicity cannot be achieved, the choice of dosing time may have to be a compromise depending upon the clinical situation. In some instances different "therapeutic ranges" of plasma drug concentrations may have to be established for different stages of a biologic rhythm.

Drug concentrations measured in the clinical laboratory have to be understood against this background. Data providing clear guidelines for the chronopharmacologic evaluation of drug level determinations are thus far very limited [15, 148]. In view of the time-dependent large amplitude variations of pharmacokinetics and pharmacodynamics shown by some drugs it is expected that a chronopharmacologic approach to therapeutic drug monitoring (TDM) will alleviate many of the present problems and inconsistencies and improve the accuracy and the clinical value of the procedure.

Reference functions and marker rhythms

Free living human subjects show substantial individual differences in timing of circadian (and other frequency) rhythms (Fig. 8). A given clock hour, day of the week or month of the year is not necessarily representative of a certain biologic time of an organism. The same time of day may have an entirely different meaning for two individuals on two different working schedules. Individual differences in the timing of rhythms can be observed due to differences in the activity-rest schedule, personality traits like morning and evening types [189], differences in main meal-times [14, 17, 173], the effects of drugs and of smoking on some rhythmic variables [190, 191], and the intermodulation of rhythms of different frequencies (e.g., circadian acrophase changes as function of the stage of a circannual rhythm (Fig. 4)). Disease may be linked with differences in acrophase and other rhythm parameters [117,128,146]. Phase alterations and free running of some rhythms has been observed under treatment (e.g., radiotherapy for breast cancer [72]). In the latter situation, a "time of day" selected on the basis of independent information as most favorable

for treatment may every day coincide with a different circadian rhythm stage, including the most unfavorable one.

In the interpretation of test results the investigator has to be aware of these often very substantial individual differences in timing. Inquiry into some simple biologic time references like sleep-wakefulness pattern, working schedule and dietary habits, especially the timing of the main meals, will provide some information on the subject's synchronization and in variables with high amplitude circadian rhythms may be important if sampling has to be limited to a single or to a few time points. However, in situations in which the timing of a subject's circadian (or other) time structure is critical, like in chronotherapy with potent and potentially toxic agents, these simple environmental time markers may not be adequate. The same may be the case if a rhythm is *a priori* not environmentally synchronized, e.g., the non-24 h rhythms of a malignant tumor.

Often a rhythm of importance cannot easily be studied directly either because of the invasive nature of the procedure (e.g., repeated bone marrow aspirations) or because of the cost involved (e.g. chemical hormone determinations). In such a situation, it is desirable to follow in the individual under study a rhythmic variable which can serve as phase reference ("reference function") for the rhythm of interest. The rhythm of this variable is then followed as time marker ("marker rhythm") for the rhythm in which the investigator is primarily interested. To be suitable as marker rhythm, a variable has to be periodic in the same frequency, be easily measurable by non-invasive means, and has to maintain under the conditions of the study e.g. during treatment, a fixed phase relation to the rhythm of interest (e.g. the susceptibility cycle to a chemotherapeutic agent). A marker rhythm may occasionally have a direct causal relation to the rhythm under study (e.g. the serum concentration of a tumor marker). More often a rhythmic variable considered as reference function will not have such a relation and it will then have to be explored if the rhythm in question meets the conditions for a marker rhythm. After a potential marker rhythm has been found to be pertinent for the rhythm under study, one will have to establish the sampling requirements to obtain statistically valid quantitative results on the parameters of this rhythm in a single subject in an ongoing and timely manner. In the circadian domain, body temperature, urinary volume, some urinary and salivary variables like electrolytes and corticosteroids have been used. Physical or chemical endpoints that do not involve expensive and/or lengthy chemical analyses and which can be recorded automatically in a computer compatible form are particularly attractive. The development of instrumentation for telemetering and/or for inexpensive ambulatory self measurements of biochemical or physical reference functions will be of crucial importance for the clinical application of chronobiology and will - in the long run - greatly increase the potential and at the same time reduce the cost of chronobiologic studies and chronotherapeutic applications. The use of marker rhythms, especially with automated monitoring, will allow to limit invasive sample collection and/or costly procedures to a few well defined time points chosen for their pertinence rather than convenience.

Conclusions

Any single measurement of a clinical laboratory variable is a spotcheck of a dynamic biologic time structure consisting of trends and rhythms of multiple frequencies modified in many variables by pulsatile (episodic) events and by random and non-random environmental stimuli.

Many biologic rhythms show high amplitudes with the changes observed during one cycle (e.g. one 24 h span) being sometimes larger than those observed during a lifetime in the course of development and aging.

In variables with high amplitude rhythms each laboratory measurement has to be interpreted against time qualified reference ranges (chronodesms). Some data on chronobiologic reference values for absolute values as well as for rhythm parameters have become available from many laboratories [14, 17, 18, 31, 57, 60, 80, 85, 90, 167, 168, 192-208].

The interaction of rhythms of different frequencies encountered in some variables may require time qualified usual ranges in more than one frequency (e.g. [17, 44, 194, 198]).

Low amplitude rhythms may not require time qualified usual ranges but are nevertheless of interest as expression of the human time structure and because of possible relations to other rhythmic events including susceptibility-resistance cycles of importance, among others, for chronopharmacology.

Some rhythmic events in some frequencies (e.g. circaseptan) can be observed as response pattern to environmental loads (e.g. the introduction of an antigen, dietary loads, etc.) and may have to be considered in the interpretation of laboratory measurements.

Pulsatile (episodic) events characterize many physiologic variables and may in their amplitude predominate over the rhythmic variation upon which they are superimposed. Their recognition and quantitative measurement requires special methodology with techniques different from those used in rhythmometry (the measurement of regularly recurring events). Their occurence has to be kept in mind in the data requirement for the measurement of rhythms in the same variable to avoid erroneous information (aliasing).

Statistically quanitified rhythm parameters have become new endpoints in laboratory diagnosis. These include a rhythm's frequency or period, a rhythm adjusted mean (MESOR), the extent of a rhythm (its amplitude), and its timing (e.g. the

acrophase). In many variables which are less than ideally sinusoidal in their variation multicomponent models may have to be used to describe the variation encountered appropriately. In the ultradian range the description of rhythmic or pulsatile variations also includes number, height, width, and distribution of the episodic peaks and troughs (e.g. of polypeptide and steroid hormone concentrations). These new endpoints may not only serve to characterize normalcy but in their alteration may indicate risk states and disease.

In therapeutic drug level monitoring (TDM) chronopharmacokinetic and chronopharmacodynamic concepts and the observations presently available will have to be incorporated in the interpretation of the results of drug concentration measurements. It must be realized that not only the time after a drug has been administered but also the time when it has been given has to be considered. Also it has to be realized that a drug concentration measured at one time (at a certain stage of one or several rhythms) is not necessarily biologically equivalent to the same concentration measured at another. This field is in rapid development but many more data will be needed to allow specific recommendations.

The future application of chronobiology in laboratory medicine will depend critically on advances in the field of data collection and data analysis. The technology for automated sample collection including *in vivo* measurements of biochemical endpoints as such is available but has not been refined and miniaturized for routine chronobiologic sampling in biology and medicine. Development of such techniques will allow the longitudinal study of marker rhythms to monitor the human time structure for time specified (cost effective) sampling and chronotherapeutic interventions.

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