Ann. Ist. Super. Sanità, vol. 29, n. 4 (1993), pp. 521-537

Historical comments on chronobiology with emphasis on cell proliferation, the concept of free running, cancer chemotherapy and experimental design

Lawrence E. SCHEVING (*a*), Lawrence A. SCHEVING (*b*), Ritchie J. FEUERS (*c*), Tien H. TSAI (*c*), Edgar A. LUCAS (*d*) and Frederick O. COPE (*e*)

(a) Department of Anatomy, University of Arkansas for Medical Sciences and John L. McClellan Memorial Veterans Hospital, Little Rock, Arkansas, USA
(b) Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA
(c) National Center for Toxicological Research, Food and Drug Administration, US Department of Health and Human Services, Jefferson, Arkansas, USA
(d) The Sleep Laboratory, All Saints Episcopal Hospital, Fort Worth, Texas, USA
(e) Ross Laboratories, Columbus, Ohio, USA

Summary. - A brief historical summary is presented regarding the emergence, over the past several decades, of chronobiology as the newest of the integrating discipline of biology. The emphasis is on the circadian system which normally is synchronized to the 24 h environmental light-dark cycle. In the absence of a suitable synchronizer, the system free runs on its own endogenous genetically determined frequency, which usually only approximates 24 h. Since the metabolic system changes rhythmically in time it follows that an organism such as man is biochemically and physiologically a different entity at different circadian stages; therefore it reacts differently to an identical stimulus given at different times. Different stimuli such as anticancer agents are examples considered clearly timed treatment has been shown to significantly improve therapeutic efficacy, data will be presented using the L1210 mouse leukemic model. Moreover data is presented showing that to ignore such rhythmic fluctuation when designing experiments that such can bring about experimental error and false interpretation. The common "same time of day" sampling does not take care of the rhythmic problem!

Key words: circadian rhythm, free running, homeostasis, man, rodent, chemotherapy-cancer.

Riassunto *(Commenti storici sulla cronobiologia con riguardo alla proliferazione cellulare, al concetto di libera corsa, alla chemioterapia del cancro e al disegno sperimentale).* - Viene presentato un breve sommario storico della emergenza, nelle ultime decadi, della cronobiologia come la più nuova delle discipline integranti la biologia. Vengono enfatizzati i ritmi circadiani che sono di norma sincronizzati dal ciclo ambientale di 24 ore della luce e del buio. In mancanza di un valido sincronizzatore il sistema diviene "free running" con una frequenza che è determinata dal proprio sistema endogeno genetico approssimativamente nelle 24 ore. Poiché il sistema metabolico cambia ritmicamente nel tempo ne consegue che un organismo quale l'uomo, è biochimicamente e fisiologicamente una entità differente a differenti stadi circadiani; pertanto egli reagisce differentemente ad un identico stimolo apportato a differenti tempi. Stimoli diversi come gli agenti antineoplastici sono esempi da considerare in quanto il loro trattamento effettuato in funzione del tempo è stato visto migliorare significativamente l'efficacia terapeutica. I dati saranno presentati con riferimento al modello di topo leucemico L1210. Inoltre sono presentati dati che mostrano come l'ignorare questa fluttazione ritmica quando si disegna un esperimento può portare a errori sperimentali e false interpretazioni. Il cosiddetto campionamento alla "stessa ora del giorno" di comune uso non tiene conto del problema ritmico!

Parole chiave: ritmo circadiano, libera corsa, omeostasi, uomo, roditori, chemioterapia oncologica.

Introduction

Although the concept of chronobiology goes back into antiquity, it has not been a subject widely taught in science courses in either high school, college or medical school. One reason is the fact that physiology courses for many years emphasized the concept of *milieu interieur constant* which was put forth by Claude Bernard [1] and later was emphasized by Walter B. Cannon [2], which he called homeostasis. They both believed that metabolic and physiological functions, in the healthy state, maintained relative constancy in body functions (steady state) and that regulatory mechanisms maintained such a state. Curiously, that dogma still prevails and today is taught in many physiology courses. Only recently has it been recognized by some textbook writers of physiology that chrono-

biology is contrary to the once prevailing view of "steady state" [3]. Halberg [4] and others have written extensively on this topic.

It should be realized that, although homeostasis was taught, a few scientists had recognized, even thirty years ago, that some metabolic events did not maintain a near steady state over a 24 h time scale. For example, 50 years ago it had been reported that the 17-Keto steroids circulating in the urine of man did so with a three- to five-fold variation along a 24 h time scale [5]. Scheving and Pauly [6] reviewed the early literature related to urine and plasma steroid rhythms in rodents and man. Such became known as circadian variation [4]. The adjective diurnal often has been used to describe a rhythm whose period was one 24 h span. It is an ambiguous term since it was/is properly used to distinguish day (diurnal) from night (nocturnal). Such misuse of the adjective indicates a lack of knowledge of the meaning of such words and of modern day chronobiology.

Only a few scientists realized that a circadian response in susceptibility to therapeutic drug effects also varied; although isolated reports existed 30 years ago, little attention was given to them [7].

Background

I (Lawrence E. Scheving) began working in this field over 30 years ago and once I presented data showing such variations during the Chicago Pharmacology Society Meeting in the Fall of 1965. Illustrated in a poster session at that time were data showing some dramatic time-dependent variation along the 24 h time scale on the effect of pentobarbital sodium on the sleep duration time in male rats. These data will be shown later in this paper. The participants of that meeting were intrigued, although some were skeptical. Such is mentioned only to point out that variation in response, on the scale that we were showing it in the experimental animal, was very hard for pharmacologists to accept. In fact, pharmacologists and pharmacists have continued - at least up until recent years - to be quite skeptical of the importance of such variation as it relates to man. The phenomenon was frequently explained away as being due to exogenous forces such as cyclic variation in activity and rest (sleep), or that it was caused by "meal timing" etc. The point emphasized thus far is that it took many years to accumulate the critical mass of data necessary to bring about the doubt which now seriously discredits the concept of homeostasis as it once was widely taught. Today we must think of an endogenous *rhythmic* homeostasis [8, 9].

It has been recognized for a long time that such events as heartbeat and respiration were characterized by high frequency ultradian rhythms. Later it was learned that these were superimposed on the circadian frequency which shall be emphasized in this paper. Why some of the high frequency rhythms were recognized much earlier was not difficult to understand since all one had to do was to monitor his/her own pulse to realize that it beats along clock time with a rhythm. Moreover, as technology developed it was further discovered that a particular wave-form of the rhythm characterized the normal state. The high frequency rhythm of breathing which animates mammalian life is another example. Only recently this rhythm has been hypothesized to be generated by rhythmically active neurons located in the pre-Bötzinger complex of the rat brainstem [10]. The respiration rhythm is *overt* but it took years of advancement in technology to identify the above proposed *covert* neuronal pacemaker. On the contrary, most of the lowerfrequency biochemical and metabolic rhythms which we allude to in this paper were covert; that is, one had to obtain data on components of blood, urine, or different tissues at frequent intervals of time to demonstrate in the plotted data the rhythmic nature, of the biochemical or metabolic variables being studied to render them overt. Thus, it took many years to gather the critical mass of data now available which clearly documents that there is a strong *temporal organization with multiple frequencies which is fundamental to all plant and animal life, and that this property of life has important implications for medicine and research in general.*

Most of the data to be presented in this paper were gathered in our own laboratory, but it should be emphasized that much work by many others also contributed to the overall development of the field. I (L.E.S.) have had many collaborators, so when using the first person, I am also including them.

Objectives

Data showing rhythmic variations in metabolic events occurring in serum, urine, and in various tissues, will be presented.

Some *properties* of rhythms will be described, especially the ability of rhythms to be *synchronized* by either geophysical or social cycles. We shall discuss an additional important fundamental concept, that of *free running*. This property will be emphasized, and it is what happens to these rhythms when an organism is isolated from all possible synchronizing forces or when the synchronizing force changes, as in shift work or from travel through time zones. When the word organism is used, we imply that the same property characterizes both plants and animals.

We shall further consider, using cancer as a model, why it is important to recognize variation in susceptibility to drugs or physical agents such as X-irradiation. This will be followed by a demonstration of how knowledge of these rhythms can be utilized to advantage in the experimental treatment of cancer in rodents. Following this, a rationale for achieving therapeutic advantage will be presented.

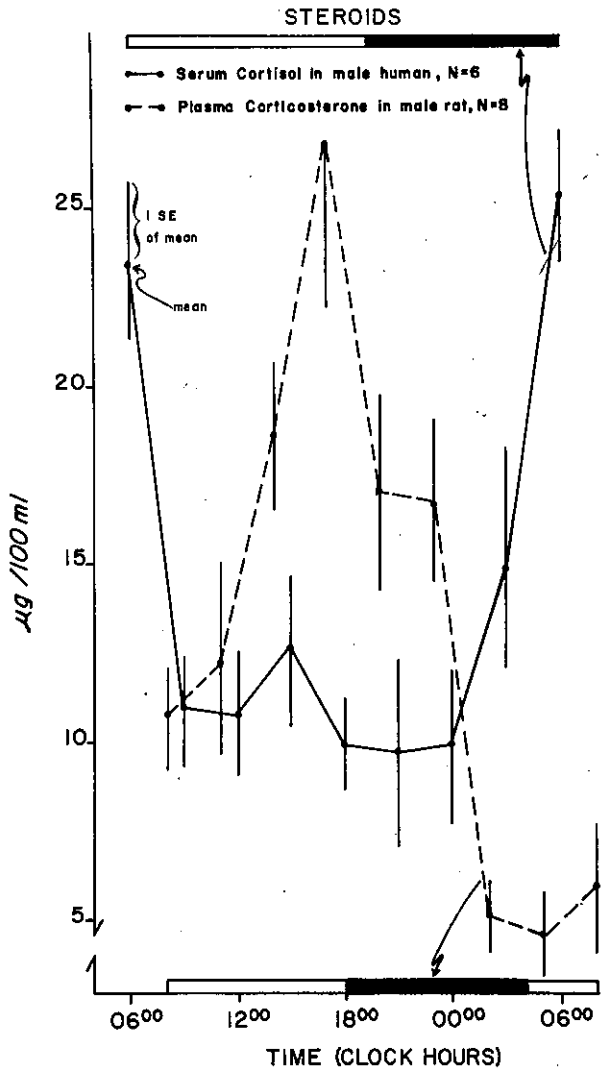

Fig. 1. - Circadian fluctuation of the predominant serum steroids of rat (corticosterone) and man (cortisol). The rats were standardized to a light-dark cycle (14 h of light alternating with 10 h of darkness) and fed *ad libitum* for two weeks prior to the study. For man, the meal times were 07.00, 12.45 and 16.45 h; rest (recumbency) or sleep time was 21.00-06.00; the human subjects were awakened for sampling at 24.00 and 03.00. [7, 51].

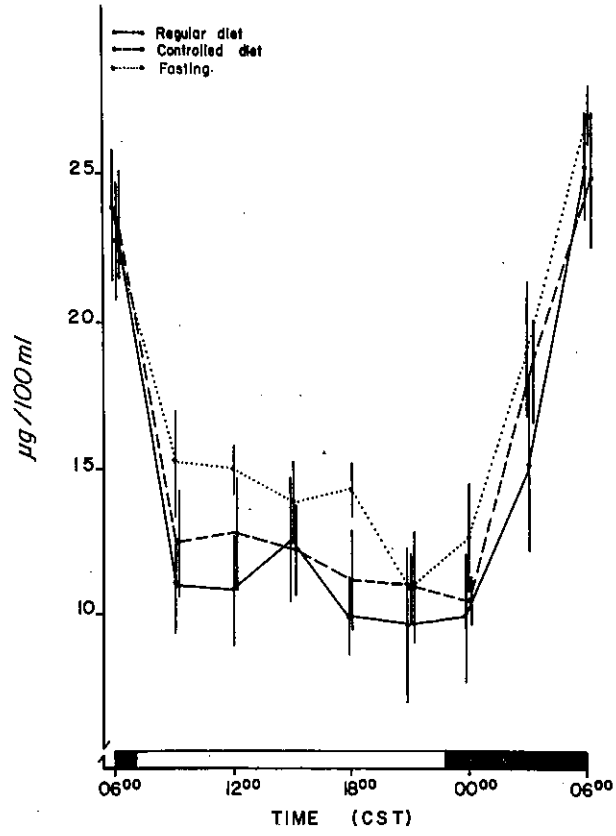

Fig. 2. - Circadian variation in serum cortisol in presumably healthy young men over a 24 h span. The *regular diet* consisted of each person eating the same menu at 06.15, 12.15, and 16.30 (the quantity eaten was not controlled). In the *controlled diet* a fixed quantity and same type of food was eaten by each individual at each sampling time. The *fasting group* received no food (only water) from 16.30 of the day prior to the first sampling time (06.00). The phasing of the rhythm in each case remained remarkably similar, and this was verified by statistical analysis. Rest or sleep time was from 21.00 to 06.00; however, the subjects were awakened for sampling at 24.00 and 03.00. Each point represents the mean ± S.E. of six individuals. [13].

Data will then be presented showing how failure to consider temporal organization can give rise to *false interpretation* of experimental data.

Examples of rhythms

The first example (Fig. 1) of rhythmic fluctuation is in the previously mentioned steroid levels circulating in the blood [7, 11, 12]. Here we see from the plotted data that the predominant steroid, cortisol, in the diurnally active man starts to rise in the serum during what is normally the time of late sleep. It rises to a peak about the time one awakens in the morning, then falls throughout the day, only to fall again the next night and re-occur the following day. The broken line shows the same thing in the predominant steroid (corticosterone) in the plasma of the nocturnally active rodent, but it is 180° out of phase with that of man. As is readily seen, these very important steroids fluctuate with a high amplitude rhythm; the change from the lowest to the highest value can be *four or five fold.* Such variation can hardly be considered "steady state" or to represent only minor fluctuation around a 24 h mean which may be of little

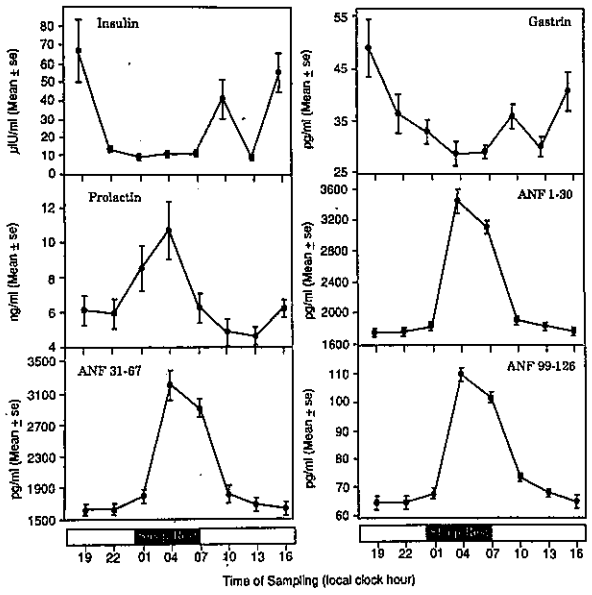

Fig. 3. - Time point means and standard errors for several serum components of the 9 men studied. Note the dramatic increases at 19.00 and 16.00 in insulin and gastrin. Each of the three prohormone atrial natriuretic factors (ANF) displayed a prominent peak during mid-sleep (04.00), as does prolactin. [14, 15].

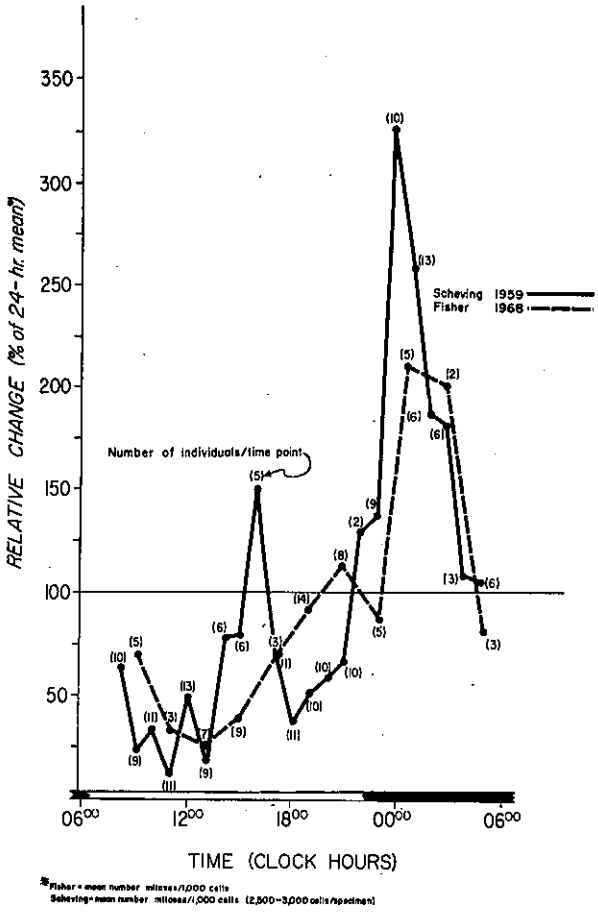

Fig. 4. - Reproducibility of the mitotic index rhythm in the adult human epidermis. This data is best explained in the text. Standard errors have been omitted to avoid a cluttered graph. For details see Scheving [17] and Fischer [24].

consequence! It is important to recognize that the rhythmic variation is generated by more than being awake during the day and in bed during the night. Food intake also is not the single cause of most rhythms, and the data which is plotted on Fig. 2 demonstrates that rhythmicity is not lost during fasting [13].

Fig. 3 shows data from diverse hormonal variables found in the serum of man which also undergo very robust rhythmic variation, among them are insulin, prolactin, and the recently discovered atrial natriuretic prohormone factors (ANF) [14-16].

Still another example of a rhythmic variable is the mitotic index in the skin of man which represents one of the first rhythmic variables on which I published data obtained on the skin of the shoulder of a man over thirty years ago (Fig. 4) [17, 18]. Of interest was the reason for investigating this particular rhythm. It arose from the fact that about 40 years ago histologists and pathologists were very puzzled by why they encountered so few mitotic figures in histological preparations of skin, when samples normally were taken by routine biopsy for clinical examination or from cadavers. Samples were typically obtained during the morning working hours. In fact, the paucity of mitotic figures had become so confusing when I began rhythm research that a new hypothesis was being advanced by some scientists. One such person was Warren Andrew, a well known medical histologist. He was among the first to popularize the idea that since there was so little evidence of mitotic figures present in the skin there had to be another replacement mechanism [19].

Andrew postulated that lymphocytes migrated into and through the skin and morphologically transformed into skin cells. When I first heard him speak on this subject, I was teaching botany in a small college and had become aware from the literature that cell division in many plant tissues were very rhythmic along a 24 h time scale. For example, cells of *Zea mays* (corn) were known to divide mostly at night, and many other plants also had been analyzed for 24 h variation in mitotic rates [20]. See Scheving *et al.* [9] for a more extensive review of cell proliferation rhythms in plants. Two claims, based on very limited data, had been reported that in the infant prepuce of man the same time-dependent mitosis in epithial cells occurred [21-23].

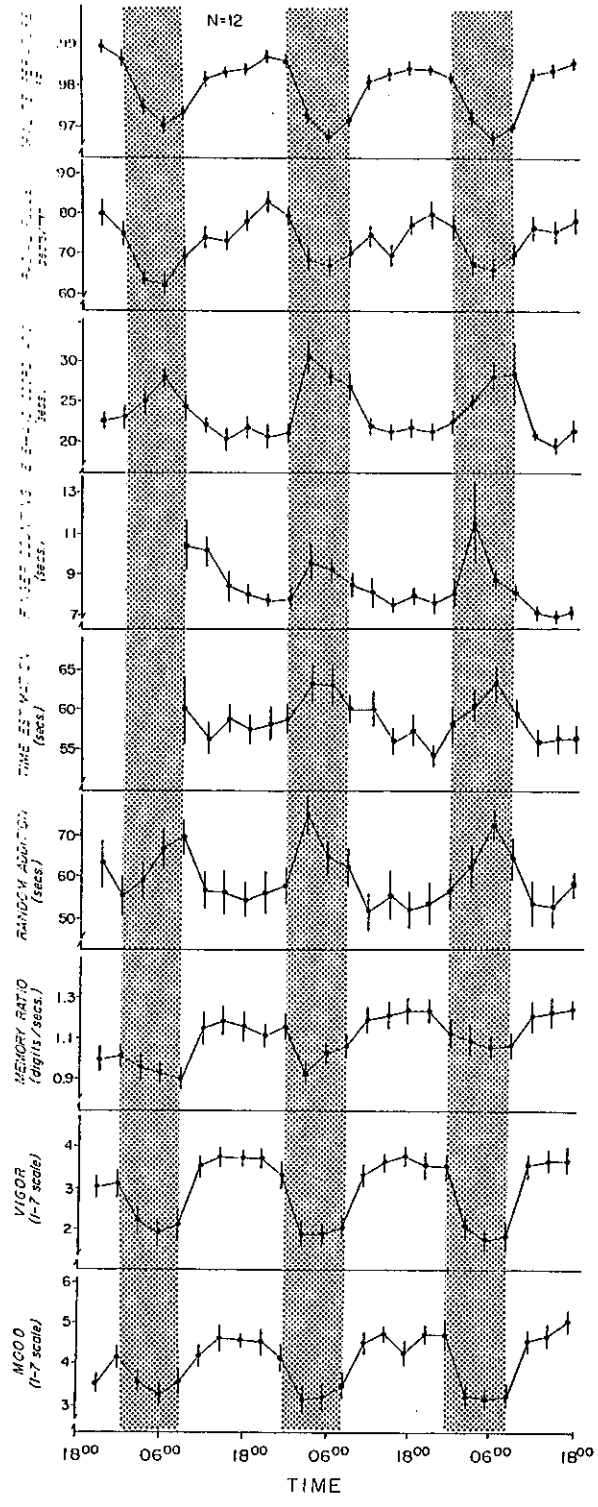

Fig. 5. - Rhythmic variation in 9 diverse variables in a group of 12 presumably healthy young men over a 72 h span (sampled at 3 h intervals). Note that the time of poorest performance *represents the crest* of the rhythm. The three vertical bars represent rest and/or sleep spans. Meals were eaten at 06.15, 12.15 and 16.30 h; rest or sleep time was from 21.00-06.00 h; however, the subjects were awakened for sampling at 24.00 and 03.00 [25].

The studies necessary to analyze for such rhythmic variation in the skin of adult man were carried out over a two-year span (Fig. 4) [17, 18, 24]. Evidence of dividing cells was found by counting mitotic figures at frequent intervals along the 24 h day; the finding was that most adult skin cells divide at night when samples usually were not obtained. About a decade later a similar investigation was repeated in London by another scientist Fisher [24]; the data are shown in the second curve on Fig. 4 and demonstrate essentially the same finding. Today, few persons quote any of these studies, as it is now an accepted fact. Interestingly, I presented some of these data in 1962 at an Anatomical Society meeting just after a major presentation by Andrew on his hypothesis. Subsequently he never

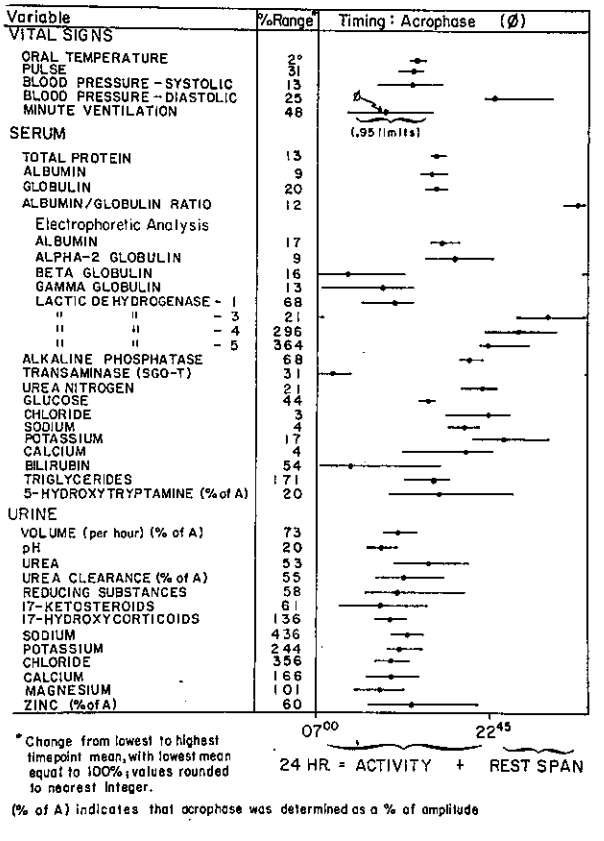

Fig. 6. - Acrophase map showing data obtained from studies on man. The map illustrates 41 different rhythmic variables in vital signs and in constituents of serum and of urine. Mealtimes were 08.30, 14.30 and 16.30 h; rest and sleep time was between 22.45-07.00 h. The Dot represents the time when the crest of the rhythm occurs in relation to the rest-activity cycle. The horizontal bars represent the confidence intervals. The numbers in the center column givers the average 24 h range of change for the group, that is, the percent difference between the highest recorded means. [26].

pursued, with data, in the literature the "*lymphocyte theory*", but still by 1957 he had gathered a group of followers. This is mentioned only to emphasize that it took the better part of two years to complete a 24 h span of all the tissue samples and to make the mitotic counts that were necessary to render this rhythm overt. It may not appear like much work when one simply views the data plotted in Fig. 4, but it was the result of much tedious and painstaking effort. Incidentally, in this same study no circadian variation was found in cell division in the infant prepuce which was mentioned above, see Scheving *et al.* [23] and Fisher [24] for further details.

Fig. 5 shows a composite of the mean and S.E. of several diverse rhythms, including oral temperature with data plotted over a 72 h span in a group of presumably healthy young men, these curves chronobiologists refer to as chronograms [25].

Fig. 6 shows the crest of the high points (acrophase) represented by dots of a series of many rhythmic variables collected over a 24 h time scale on a group of 13 healthy young men. The horizontal lines represent the 95% confidence limits of the mean (dot) as determined by fitting the data to a 24 h cosine curve [27]. We emphasize mean values because, the range of change for a particular variable in one individual may be greater than in another person, this type of presentation of the data seen in Fig. 6 dampens the extremes of variation that can be seen in the raw data obtained from individual subjects. In other words, there are certain individuals who have rhythmic variables such as systolic blood pressure that may fluctuate 30 or more degrees over the same time span, but another person may fluctuate much less over the same span of time (Fig. 7). Thus, when you plot the data of the individuals as mean values you flatten out total variation. The variation you see for the individual may be higher or lower than the group. It is important to keep this under consideration when viewing such variation in data [27].

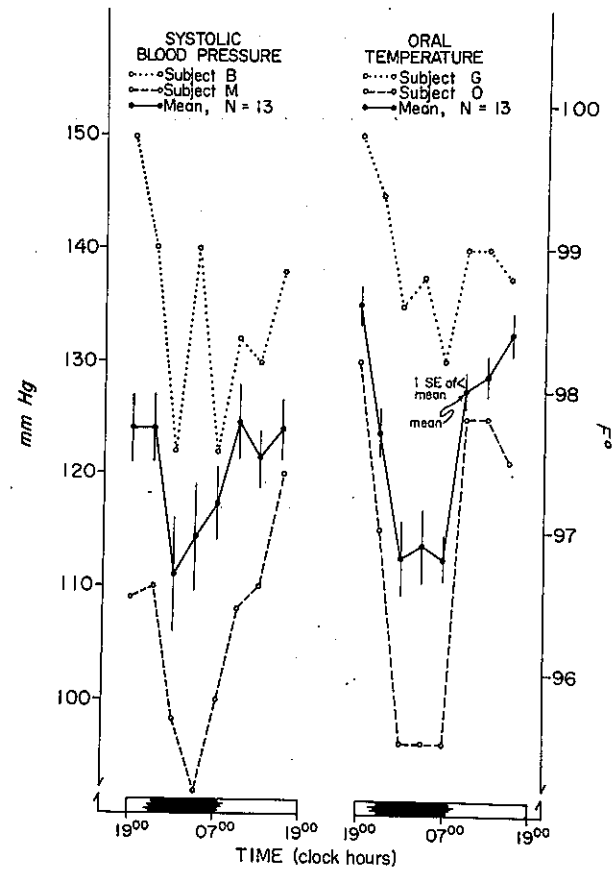

Fig. 7. - The data in this figure is best explained by referring to the text. This is a necessary concept to understand if group data is being considered. [27].

Properties and generalization about rhythms

Over the years, many things about rhythmic behavior have been learned which can only briefly be mentioned in the interest of space.

To summarize, rhythms in general can now be considered: 1) *innate*, 2) *endogenous*, 3) *ubiquitous in living systems*, 4) *they are found at all levels of organization* (i.e., from enzyme activity associated with mitochondria function to gross motor activity), 5) they can be *synchronized* to the light-dark cycle (artificial or natural) or, in the case of man, even to his social cycle, 6) in the absence of a synchronizing force, rhythms will *free run* [28]. Understanding *synchronization* and *free running* are essential if we are to understand the necessity for such properties.

The ability of the rodent to synchronize to the light-dark cycle is important, because an investigator can determine the profile of a rhythm under a certain light-dark cycle for a particular variable in which he is interested, and then learn where within the light-dark cycle any particular phase of the rhythm (peak, trough, ascending limb or descending limb) occurs in relation to local clock time. Moreover, one can predict, under the conditions of the standardization used, the approximate range of change along the 24 h time span that can be expected. Monitoring of metabolic rhythms usually is accomplished by sampling at a fixed interval along the 24 h time span, a 3 or 4 h interval frequently has been the custom, especially if one is interested only in the circadian frequency. However, with intervals of sampling at one hour or less, there usually is evidence of what can be suspected as representing an underlying higher frequency rhythm (ultradian) superimposed on the circadian. We will not consider these higher frequency rhythms herein, but they are very important to a better understanding of temporal organization.

Free-running rhythms. - If a synchronization agent, such as the light-dark cycle, is taken away the rhythm continues, but it persists with a frequency which usually is different from 24 h; this is referred to as a free-running rhythm.

As mentioned above, a prevalent attitude held by many for a long time was that periodic biological behavior represented nothing more than a passive response to feeding or to some periodicity in the environment, such as to light-dark or temperature cycles, etc. De Mairan [29], an astronomer and botanist, while studying rhythms in the movement of plant leaves, reported that the leaves would continue their periodic movement when kept in a darkened cave away from sunlight or open air; this suggested to him that some endogenous factor within the plant was responsible for this behavior and was not necessarily caused by the environment [30].

It is known that if we remove the rodent (or other animals) from the influence of the light-dark cycle mediated by their eyes (by blinding), or if they are subjected to continuous light in the absence of all environmental or social cues, rhythmic variables as diverse as serum corticosterone and motor activity will continue to oscillate. It is rare that the frequencies of such rhythms will average precisely 24 h, but usually they are about 24 h long; consequently, their timing, such as peak or trough, change predictably each day in relation to local clock time. Such free-running rhythms are described as *circadian*. While the term was first coined by Halberg [4] to describe only the free-running state in the first publication on this topic he suggested it could also be used to describe the about 24 h rhythms, whether or not they were 24 h synchronized, this has become a common practice. Two examples of free-running rhythms are illustrated in Fig. 8. One depicts the perch-hopping activity pattern of a bird, which first was maintained in alternating 12 h spans of light and darkness (LD 12:12); then it was subjected to constant dim light (LL 0.4 lux). Thus when kept under constant conditions, without time cues from the environment, its activity pattern free ran (drifted in time). Under conditions of constant dim light, its activity began a little later each day, which is evidence that the periodicity was slightly longer than 24 h. After about 26 days it can be seen that the bird started its activity at about the same clock time that it had started its rest span at the beginning of the experiment. In the third phase of the study, the bird once again was subjected to alternating spans of light and darkness and that immediately synchronized its activity to the light span. In the final experimental phase, the bird was subjected to constant brighter light (120 lux) and its rhythm deviated from the 24 h day by a fixed amount and began to free run. This time, however, under the conditions of bright light, the period of the rhythm was shorter than 24 h; that is, the bird began its activity a little earlier each day. Thus we

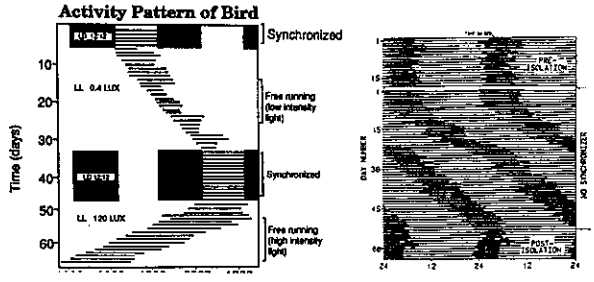

Fig. 8. - On the left is a free-running rhythm in the perch-hopping activity of a chaffinch bird subjected first to a light-dark synchronized schedule, then to a dim light and later to a much brighter light [32]. See text for further information. On the right is an example of a free running rhythm in the rest/or sleep activity pattern of a presumably healthy 47-year-old male who was isolated from most time cues beginning day 1. Prior to the second day 1 (isolation) he was in the chamber for the 18 days, but he had a clock and was subjected to occasional social interaction (labeled on Fig. as pre-isolation). Once the clock was removed and social and other synchronizers were discontinued, he began to free-run with a period of greater than 24 h, as evidenced by the black horizontal bar which indicates the times he was actually in bed. On day 53 he left the chamber and became synchronized to local time (post-isolation). The ability to free run is a basic property of plant and animal life and is strong evidence of an endogenous mechanism of control. [32, 34].

see that not only is the light-dark cycle a dominant synchronizer of most plants and animals, but the intensity of the light can influence the period of the rhythm under free running conditions. Johnson, Aschoff and Halberg [31, 32, 4] were among the first to show free-running in rodents. Later, in the early 1960's, the same was found for man. The data on the bird was furnished by Aschoff [32].

The panel on the right (Fig. 8) depicts the rest-activity cycle for a 47 year-old, healthy man studied in our isolation chamber. Note that at first the rhythm of the subject's sleep-wake cycle was synchronized when living in an isolation chamber with a clock and some social interaction from time to time (pre isolation). On the second day 1 (isolation) the clock was removed from the chamber and for 53 days the subject was completely isolated from any time cues (no synchronizer). It is evident that he free ran in a manner similar to the bird. It is unlikely, but not proven from this study, that man would respond, at least to some degree, to intensity of light as did the bird; nevertheless, intensity of light can be important to man [33]. The specific data on man are previously unpublished from our laboratory; for more detail of our isolation studies see Lucas *et al.* [34]. Fig. 9 is an example of the systolic and diastolic blood pressure of the same 47 year-old individual isolated in the experi-

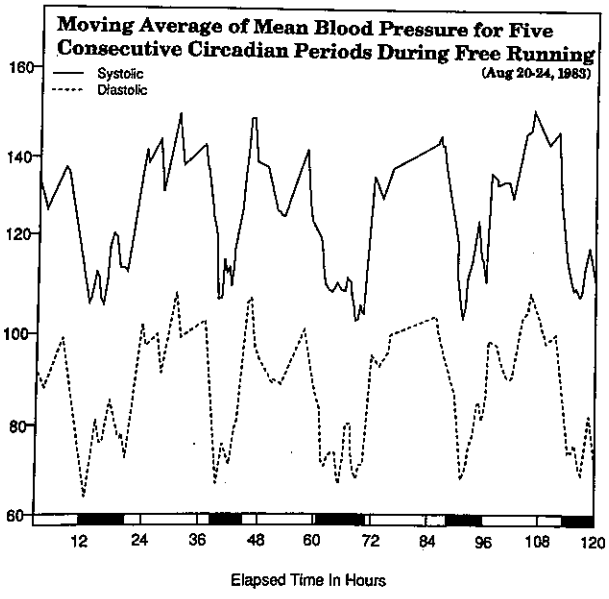

Fig. 9. - Note the dramatic circadian variation in blood pressure which also was monitored on the same 47-year-old man who was isolated as described above in Fig. 8. The sleep-wake data shown in Fig. 8 and blood pressure data on the same 47-year-old man (Fig. 9) are being published for the first time (see Lucas *et al.* [34] for further details of the design of the studies in general). As mentioned in the text the intervals of sampling were very dense as data was obtained every 15 min, except when subject was walking around.

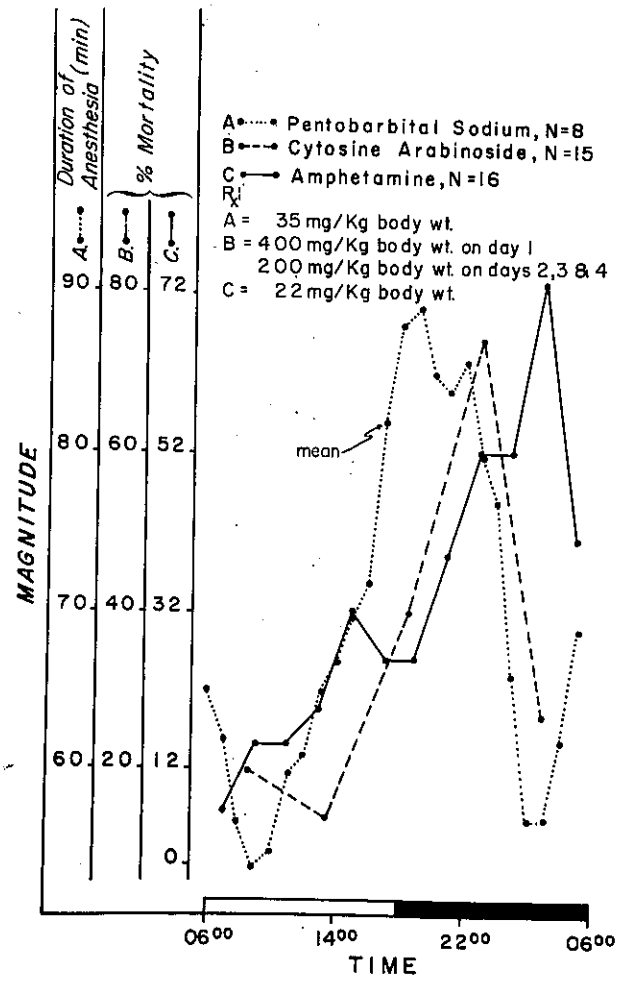

Fig. 10. - Circadian susceptibility rhythms of rats to pentobarbital sodium, as measured by duration of sleep [35], cytosine arabinoside (ara-C), and amphetamine, as measured by mortality for both. This is a clear demonstration that the time a stimulus is applied can *tip the scale between life or death.* [7, 36, 37].

ment design described in Fig. 8. The intervals of sampling were very dense, being approximately every 15 min during much of the subject's inactive time over the 5 day span, thus moving averages of the mean blood pressure data were determined for only five consecutive circadian spans of the total isolation span.

Free running and the ability to synchronize are among the *fundamental properties* of all plant and animal life. It is this endogenous mechanism that permits animals or plants to adjust to changes in the environment. Well known examples of such change in the case of man would be adjustment to displacement in time by jet travel or by shift in work schedules. This same elegant mechanism assists the animal or plant in adjusting to day length or seasonal changes.

Chronopharmacological implications

Since the metabolic system is rhythmically changing, it follows that the organism is biochemically and physiologically a different entity at different circadian stages; therefore, it reacts differently to an identical stimulus at different times.

Among some of the many categories of agents reported to elicit differing responses when administered at different circadian stages are: 1) carcinogens; 2) tumor cells; 3) viruses; 4) bacteria; 5) antigens, such as sheep red blood cells; 6) physical agents such as X-rays and 7) a host of poisons, chemicals and drugs. The endpoints measured also have been diverse and have included: 1) mortality rate; 2) duration of sleep subsequent to a fixed dose of an anesthetic agent; 3) duration of time required for the onset of tremor subsequent to giving a drug capable of inducing them; and 4) survival time and "cure" rate such as can be demonstrated subsequent to treating L1210 leukemic mice on a therapeutic protocol designed to exploit the circadian system [7].

Three diverse examples of such responses are illustrated in Fig. 10. It is evident that such re-

sponses vary dramatically as a function of time of day or night. For example, the mean duration of sleep resulting from an identical dose of pentobarbital sodium given to different groups of similarly standardized rats at different times during the rat's circadian cycle averaged *91 min* when the agent was given at one phase of the cycle and only *53 min* when the same dose was given at another time [35]. We alluded to these sleep data at the beginning of the paper. This same figure illustrates data showing that whether or not an animal survives a potentially lethal, fixed dose of amphetamine is circadian-stage dependent; for at one stage the mortality was *76.6%*, and at another only *6.6%* [36]. The third example similarly demonstrates that a carcinostatic drug, cytosine arabinoside (ara-C), was far more toxic at one stage of the mouse circadian system than at other stages [37]. This latter cancer agent (ara-C) shall be discussed later in this paper.

Most, if not all, carcinogenic agents are capable of eliciting equally dramatic circadian variation in response as those shown above. Clearly the pharmacological response is circadian-stage dependent. Sufficient evidence now exists which challenges any pharmacokinetic study done without consideration of circadian variation. For example, ethanolemia studies suggest that pharmacokinetic rates and rate-constants cannot be considered *a priori* to be unchanging along the entire 24 h span [38, 39]. Obviously, the possibility of such influence should be investigated if pharmacokinetic results are intended to be used in calculating therapeutic doses of a drug. It may be inappropriate to administer a drug repeatedly over a 24 h span on the basis of a single pharmacokinetic study performed at only one circadian time; today the latter is a common practice in spite of the data available to show that this practice may not be good science.

The above mentioned variation in toxicity suggests that such could have a profound effect on clinical therapy. To date, little has been done in the clinics on a large scale. Among those drugs whose effectiveness has been shown to depend on the phase of the circadian system when administered are the corticosteroids and anti-tumor agents.

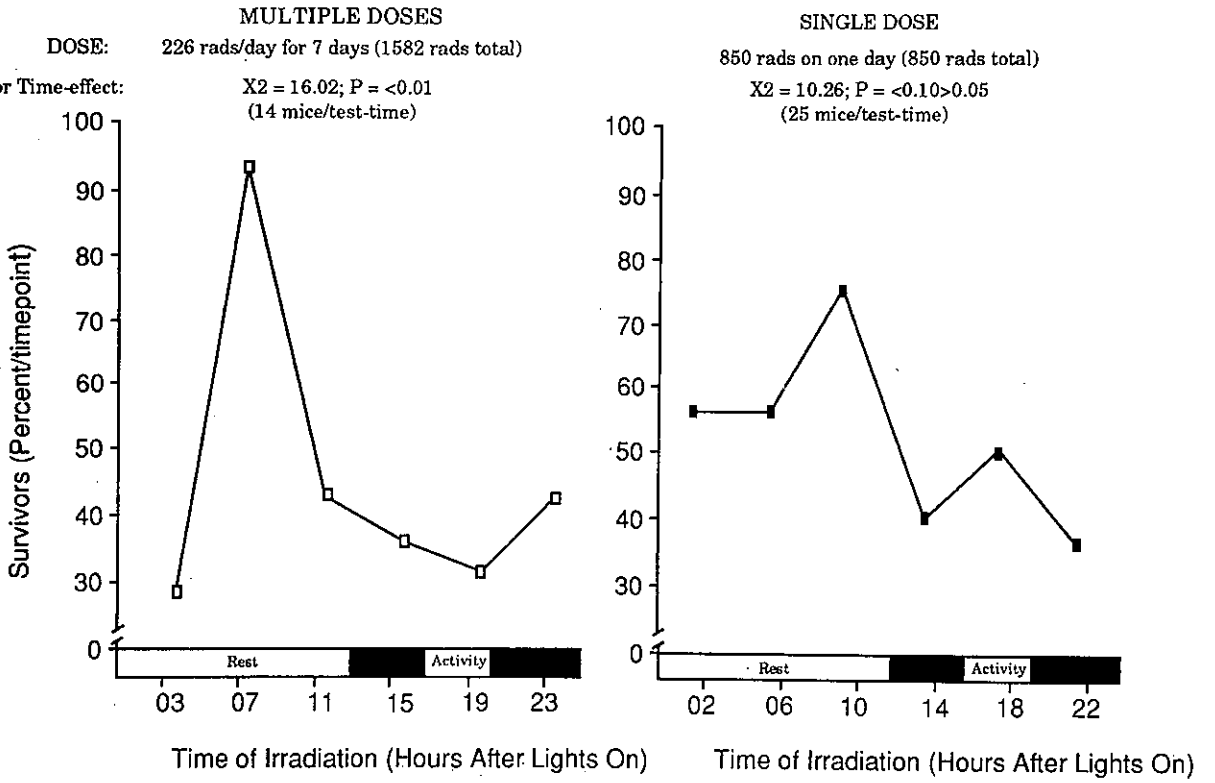

Fig. 11. - (*a*) Variation in susceptibility to irradiation in mice x-irradiated at six different circadian stages for 7 days. More than 90% of the animals irradiated at one circadian stage survived, whereas fewer than 30% irradiated at another circadian stage survived. Mortality was monitored for 30 days, after which none of the surviving animals appeared sick [23]. (*b*) Effect of a single dose of 850 rad on one day. [40].

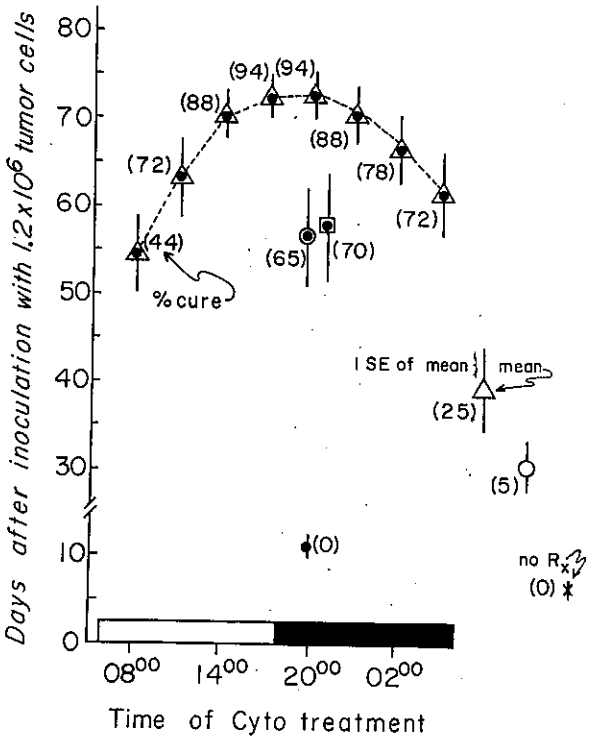

Fig. 12. - Survival time and percentage of cures in L1210 leukemic mice treated with ara-C plus cyclophosphamide (CTX). The △ implies the best sinusoidal ara-C treatment schedule. The □ and O implies the reference treatment schedules (no chronobiological consideration). The ● implies that CTX was administered, in combination with ara-C, once per course to each mouse; however, different groups received it at different circadian stage, ○. Refer to the text for further details of the treatment protocol. The percentage of cures (percentage alive 75 days after tumor inoculation) is shown in parentheses. The mice alive after 60 days customarily are considered cured in this model, and they are not likely to die from leukemia. Time of the CTX administration is indicated on the horizontal scale [11, 12, 51]. The groups that did not receive CTX are shown just to the right of the time scale. N = 20 for each group. This investigation was far more extensive than indicated on this figure. [12, 51].

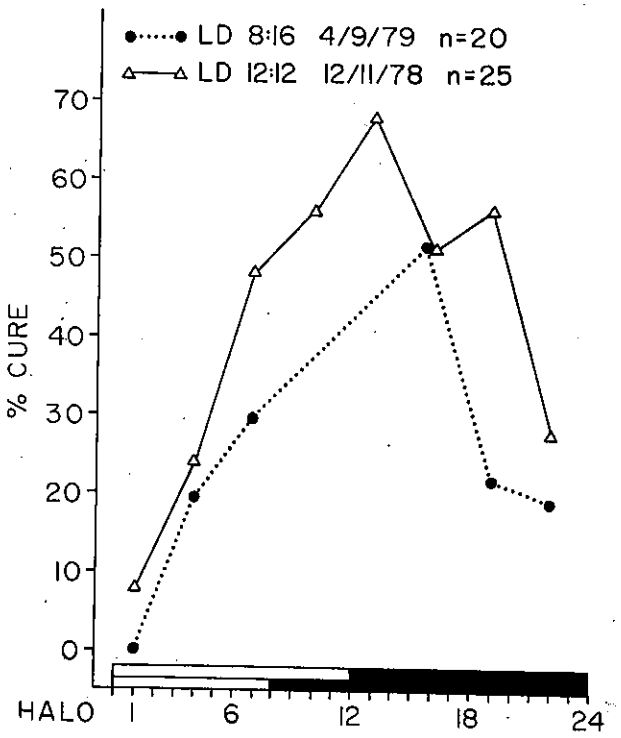

Fig. 13. - Variation in cure rate of leukemic mice depending on the timing of combined treatment with cyclophosphamide (CTX) and adriamycin (ADR). Study 1 (solid line) on male mice in LD 12:12; study 2 (dotted line) on female mice in LD 8:16. HALO = h after lights on. [41, 52].

Moreover, physical stimuli such as whole body radiation bring about variation in response which is best illustrated in the data plotted in Fig. 11 [40].

Experimental therapeutics using the leukemic mouse model

It has been demonstrated experimentally in rodents that timed treatment according to body rhythms, can increase survival time as well as "cure" rate. We shall give a couple of examples (among the many available) of what has been accomplished in this field with the hope that this might serve to stimulate attempts to design protocols for the clinical treatment of cancer. Thus, in Fig. 12, the data show the optimization of the treatment of mouse leukemia (L-1210) by a chronobiological approach [12]. Each mouse was injected with 1.2×10^6 leukemia cells 44 h prior to the initiation of treatment. The triangles on Fig. 12 indicate that each mouse in those groups received 4 courses of cytosine arabinoside (ara-C) with a 3-day rest span between courses; each course consisted of a total of 150 mg/kg of ara-C administered in 8 injections, at 3 h intervals over a single 24 h span. The dosage administered at each of the 8 time points varied so that the greatest amount of the drug was administered at a time when the mouse was known to be most resistant to it, and the smallest dosage at a time when the host was known to be least resistant. In addition to the ara-C, each of these mice received 50 mg/kg of cyclophosphamide (Cyto) once per course, which is indicated by the dot within the triangle. Other groups of mice, the controls, received the same total amount of ara-C, but it was administered in 8 equaldose injections; these mice, indicated by the square, also received the 50 mg/kg of cyclophosphamide.

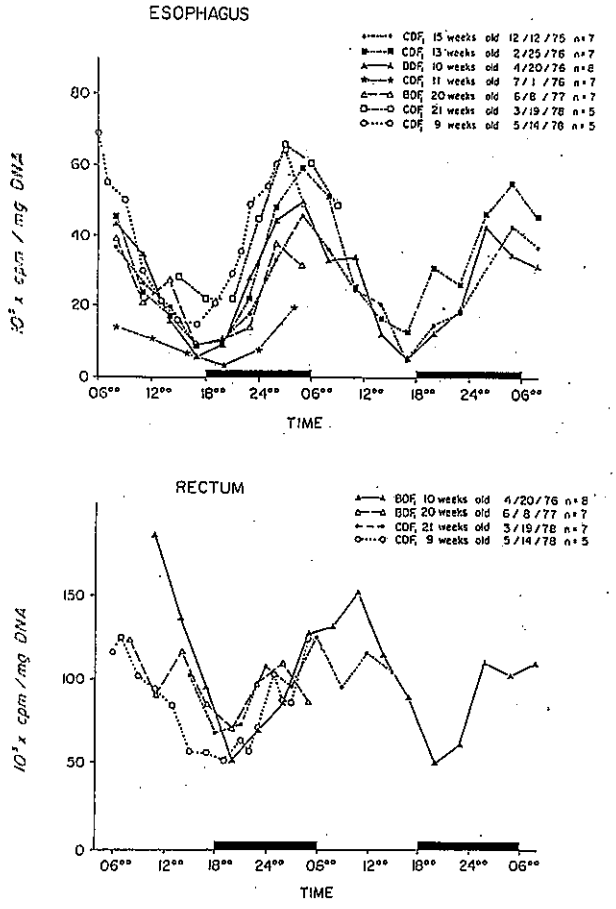

Fig. 14. - Remarkable reproducibility of the phasing of the rhythm in DNA synthesis in the mouse esophagus and rectum in relation to the light-dark cycle from one study to another. It is important to bear in mind that this represents a *retrospective* approach in that data were obtained from animals that had served as controls for various experiments over a 5-year span. The differences seen could be due to many factors, including the specific activity of the isotope, and the season. We believe from multiple studies the rectum follows in phase by about 2 h. The trough of the rhythm has less scatter than the peak or acrophase. Interestingly, the same phenomenon has been reported for the rectum of *ad libitum* fed and fasting man [45]. Standard errors were purposely omitted to avoid an overly cluttered graph; however, analysis of variance for each series of data showed highly statistically significant changes ($P < 0.05$). [23].

An important observation was that all groups receiving the chronobiological treatment schedule suffered only 1 death out of the 140 animals involved, which could even have been accidental or from acute drug toxicity. On the other hand, those animals receiving the non-chronobiological schedule suffered a mortality rate of 30% which was clearly due to acute drug toxicity, thus making it a non-satisfactory treatment protocol. Attempts to lower the conventional dosage of either or both drugs to avoid the 30% toxicity resulted in an increase in animals dying from leukemia. Thus the data clearly indicate that there is a right and a wrong chronobiological time to administer the drug. All animals receiving no treatment died within 7.1 ± 0.4 days; those receiving ara-C alone (open triangle) had a mean survival time of 28.2 ± 0.6 days; and those receiving cyclophosphamide alone (solid circle) had a mean survival time of 11 ± 0.8 days. The data in Fig. 13 show another example of leukemic mice whose treatment was dependent upon the timing of combined treatment using cyclophosphamide (CTX) and adriamycin (ADR) [41].

High-amplitude circadian rhythms have been reported in healthy rodents for the synthesis of DNA ongoing in the liver, bone marrow, gut, thymus and spleen of normal rodents. Fig. 14 and 15 illustrate such rhythms in DNA synthesis in the esophagus, rectum, bone marrow and thymus and spleen. These are all high amplitude rhythms, and it is our conclusion that what we are experiencing is a *shielding in time* of these orfans from the harmful effects of the carcinogenic agents which are known to have very toxic effects on dividing cells. In our opinion tumor cells in most cases (but not all) have escaped *from circadian control* [11, 12, 23, 42].

Importance of considering chronobiology in experimental design

Based on data, we now must abandon, when designing experiments, the erroneous concept that somehow sampling at the *same time of day* takes care of the rhythmic problem and the Fig. 16, 17, and 18 illustrate why one can make such a statement. Specific reasons are explained best in the legends of these three figures.

Conclusions

We predict that in the near future more attention will be given to timed treatment in the management of cancer and to understanding the mechanism of rhythmic normal and abnormal cell proliferation. We still do not fully understand the *in vivo* mechanism of *cell proliferation*!

The major shortcoming of classic non-circadian cytokinetics has been the inability to translate *in vitro* advances directly from the Petri dish to the cancer patient. This has been due to the fact that it was thought that the *in vitro* synchronized cell populations behaved differently from the *in vitro* populations which were classically assumed to be non-rhythmic with regard to cytokinetic parameters. The realization that there is relative synchrony along the circadian time scale within all normal *in vivo* cell populations is the only solution

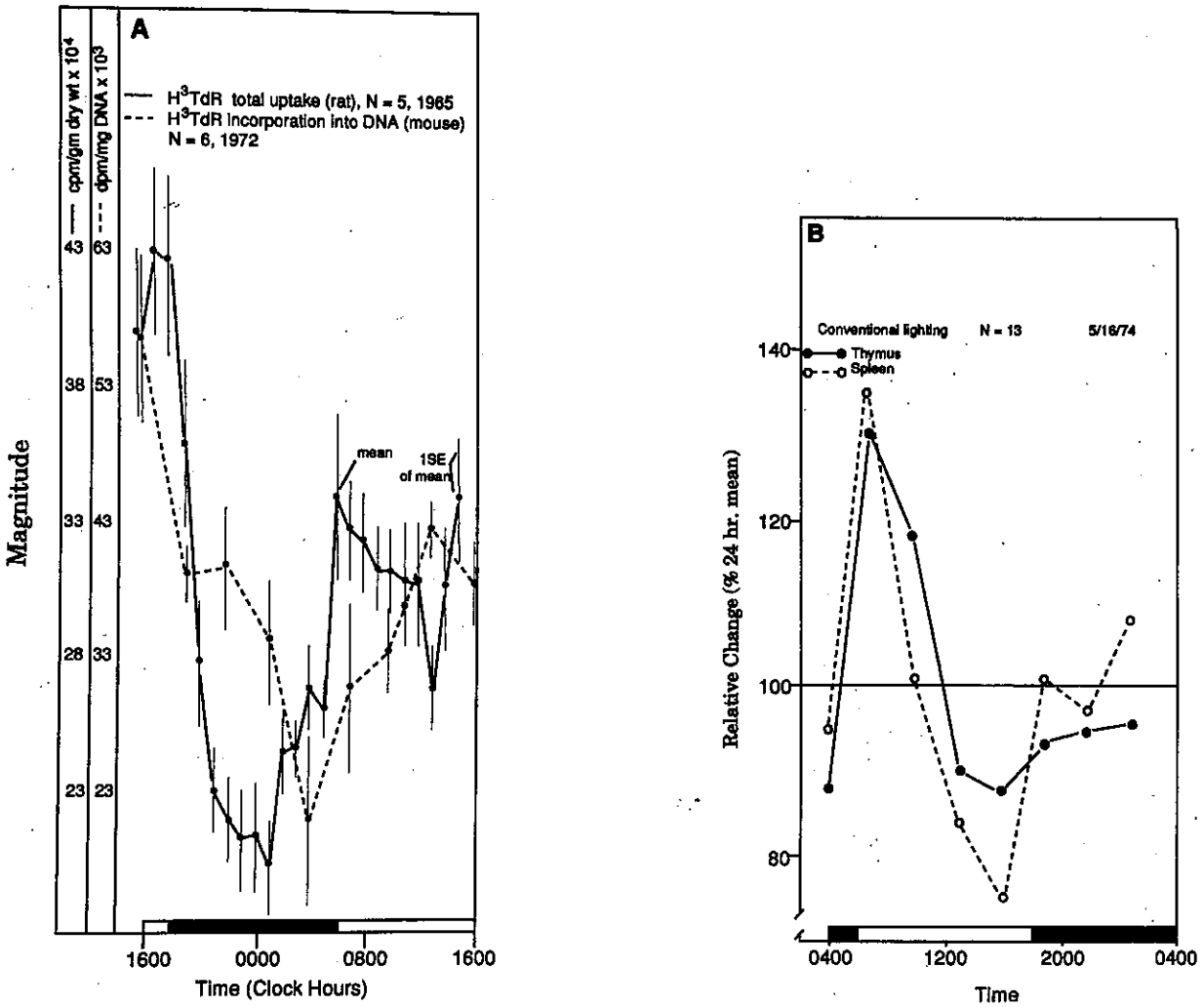

Fig. 15. - Reproducibility of the rhythm in studies done in 1965 and 1972 in the uptake of [³H]TdR in the bone marrow of rodents (A). The rhythms were determined by injecting subgroups of animals with [³H]TdR during a single 24 h span at the time intervals shown on the chronograms. The animals were killed 1 h after injection and the tissues were collected and analyzed by scintillation-counting techniques. The two chronograms (B) demonstrate the similarity in the phasing of the rhythm in DNA synthesis in the thymus and spleen of $CD2F_1$ mice. The two rhythmic variables do have a tendency to peak in the light span when compared to the same rhythm in the intestinal tract (Fig. 14). The standard errors were intentionally left off to avoid an overly cluttered figure. The data are highly statistically significant. [53].

a: •——• [³H]TdR total uptake (rat), n = 5, 1965; •– –• [³H]TdR incorporation into DNA (mouse), n = 6, 1972; *b:* conventional lighting, n = 13, 5/16/74; •——• thymus; o– –o spleen.

for using cytokinetics as a practical clinically relevant concept for treating cancer patients. If the basic rhythmicity in cell proliferation inherent in the organism is not ignored, we believe the cancer therapy will benefit. Perhaps ultimately we can gain a better understanding of abnormal as well as normal cell division.

Fortunately, at this time clinical human studies are beginning and they appear promising. Presently, human data exists that show survival time can be significantly extended [43-48]. Moreover, many of the toxic effects known to accompany the administration of anti-cancer agents can also be reduced. Hopefully, the duration of time for these fledgling studies to be recognized as important in the management of the cancer patient will not take over 30 years! To avoid this, policymakers in cancer chemotherapy must become familiar with the potential usefulness of such data and *encourage* future research; unfortunately, this has not been the case to date [48, 49].

As positive data on human beings accumulates, as it unequivocally has in many animal studies, it may ultimately become an ethical issue as to whether the cancer patient will be treated with or without chronobiological consideration.

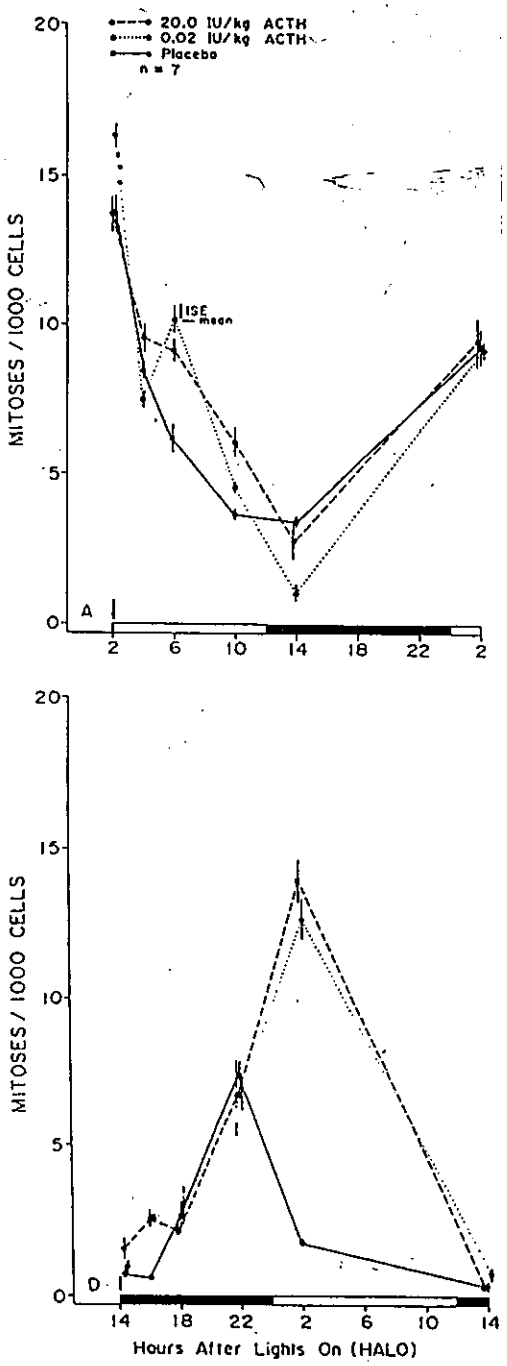

Fig. 16. - Plotted data from the corneal epithelium mitotic index represent response 12 h after treatment. Note the remarkable *decrease* in the mitotic activity when ACTH 1-17 was administered at 2 h into the dark span (inverted error) and animals were killed 12 h later. No such response was found when ACTH 1-17 was administered 2 h into the light span and killed 12 h later (14 h after lights on (HALO). [54, 55].

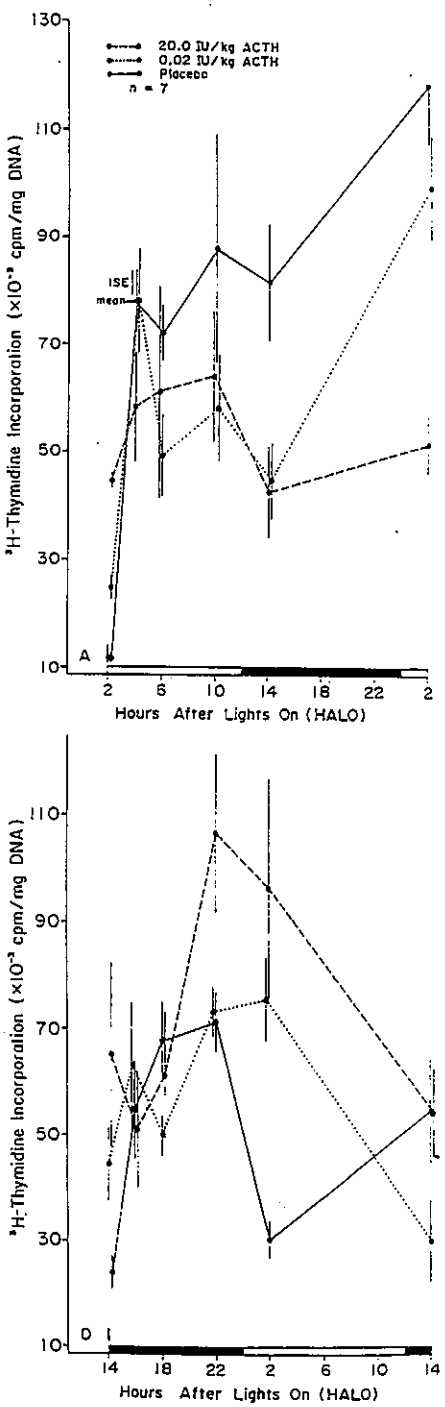

Fig. 17. - The data show, among other things, that when two different doses of ACTH 1-17 were administered intraperitoneally (inverted arrow) at 2 *h* after *l*ights *o*n (HALO) (A) there were rather dramatic decreases in DNA synthesis in the epithelium of the mouse rectum compared to the placebo at 12 h post treatment. Note inverted arrow. The opposite effect was recorded 12 h later if treatment was administered at 2 h into the dark (D). Note inverted arrow. [56].

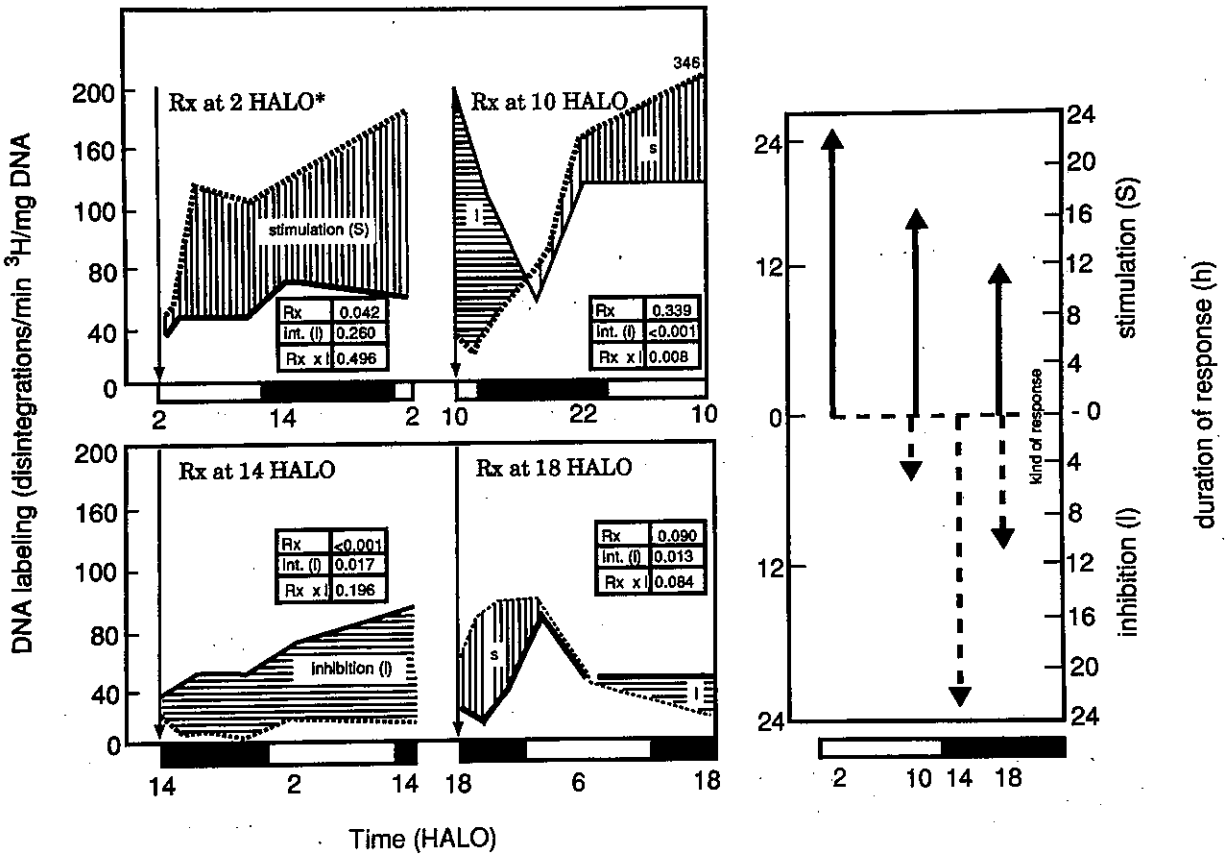

Fig. 18. - DNA labelling** in metaphyseal bone of $CD2F_1$ mice during a 24 h span after treatment with either a placebo (saline) or ACTH 1-17 on the (left) and duration of response, based on the same data (right). The statistical significance of the findings was established by three-way ANOVA of all data and by two-way ANOVA for data at each treatment time. The latter yielded the p values inserted in the 4 left hand sections of the graph for the effect of treatment (R_x) those of the time interval elapsed since treatment (Int) and for interaction. – placebo; – 20 IU/kg ACTH 1-17; * hours after light onset for mice ($CD2F_1$); ** results of 2-way analysis of variance at each circadian stage in boxes: main effects are kind of R_x (placebo vs ACTH 1-17) and Rx-to-kill interval (2, 4, 8, 12, 24 h); O outlier (that would raise mean to 338) removed. See reference for further explanation. Timing does make a difference! [57].

With the rapid development of chronobiological pumps for the delivery of multiple drugs, many of the complex methodologic and logistical problems associated with timed drug delivery have been solved. There needs to be a concentrated effort by those involved in clinical timed treatment to coordinate their efforts on an international scale [49].

The time has now arrived when scientists must accept, based on a critical mass of data, the existence of and the potential importance of such rhythms, even though it still remains a mystery why some still ignore them in their experimental design. Those charged with regulatory control of toxicity and drug therapeutic effectiveness must become more sensitive to such data than they have been! We predict that the 1991 conference on "Temporal Control of Drug Delivery" [46] will prove to be a milestone in encouraging the utilization of experimental data now available to application in the clinics. A report dealing with *Chronobiology in human medicine and beyond* recently appeared [50].

Acknowledgment

The authors are grateful to Ms. Kathy Ivy for typing and editorial assistance.

Submitted on invitation.
Accepted on 18 February 1993.

REFERENCES

1. BERNARD, C. 1885. *Secons sur phenomenes de la vie communs aux animaux et aux végétans*. J.B. Bailliere, Paris.

2. CANNON, W.B. 1929. Organization for physiological homeostasis. *Physiological Rev.* **9**: 399-431.

3. GIESE, A.C. 1973. *Cell Physiology* 4th ed., W.B. Saunders, Philadelphia. pp. 157-177.

4. HALBERG, F. 1959. Physiological 24 hour periodicity; general and procedural considerations with reference to the adrenal cycle. *Z. Vitamin, hormon-h, Fermentforsch.* **10**: 225-296.

5. PINCUS, G. 1943. A diurnal rhythm in the excretion of urinary ketosteroids by young men. *J. Clin. Endocrin.* **3**: 195-199.

6. SCHEVING, L.E. & PAULY, J.E. 1966. Effect of light on corticosteroid levels of rats. *Am. J. Physiol.* **210**: 1112-1117.

7. SCHEVING, L.E., von MAYERSBACH, H. & PAULY, J.E. 1974. An overview of chronopharmacology (a general review). *J. Eur. Toxicol.* **7**: 203-227.

8. SCHEVING, L.A., YEH, Y.C., TSAI, T.H. & SCHEVING, L.E. 1980. Circadian-phase dependent stimulatory effects of epidermal growth factor on DNA synthesis in the duodenum, jejunum, ileum, caecum, colon and rectum of the adult male mouse. *Endocrinology* **106**: 1498-1503.

9. SCHEVING, L.E., TSAI, T.H., SCHEVING, L.A. & FEUERS, R. 1991. The potential of using natural rhythmicity of cell proliferation in improving cancer chemotherapy in rodents. In: *Temporal control of drug delivery*. W.J.M. Hrushesky, R. Sanger & F. Theeuwes (Eds). *Ann. Acad. Sci.* **618**: 182-227.

10. SMITH, J.C., ELLENBERGER, H.H., BALLANYI, K., RICHTER, D.W. & FELDMAN, J.L. 1991. Pre-Bötzinger complex: a brainstem region that may generate respiratory rhythm in mammals. *Science* **254**: 726-729.

11. SCHEVING, L.E., BURNS, E.R. & PAULY, J.E. 1977. Can chronobiology be ignored when considering the cancer problem? In: *Prevention and detection of cancer, Pt. 1, prevention*. Nieburgs H.E. (Ed.). Vol. 1, Etiology. Marcel Dekker, New York. pp. 1063-1069.

12. SCHEVING, L.E., BURNS, E.R., PAULY, J.E., HALBERG, F. & HAUS, E. 1977. Survival and cure of leukemic mice after optimization of cancer treatment with cyclophosphamide and arabinosyl cytosine. *Cancer Res.* **37**: 3648.

13. SCHEVING, L.E. 1981. Chronobiological aspects of comparative experimental chemotherapy of neoplasms. In: *Neoplasms-comparative pathology of growth in animals, plants, and man*. H.E. Kaiser (Ed.). Williams and Wilkins, Baltimore. pp. 317-334.

14. VESELY, D.L., KANABROCKI, E.L., SOTHERN, R.B., SCHEVING, L.E., TSAI, T.H., GRECO, J., BUSHNELL, D.L., KAPLAN, E., RUMBYRT, J., STURTEVANT, R.P., STURTEVANT, F.M., BREMNER, W.F., THIRD, J.L.H.C., HRUSHESKY, W.J.M. & OLWIN, J.H. 1990. The circadian rhythm of the N-terminus and C-terminus of the atrial natriuretic factor prohormone. *Chronobiol. Int.* **7**: 51-57.

15. KANABROCKI, E.L., SOTHERN, R.B., SCHEVING, L.E., HALBERG, F., PAULY, J.E., GRECO, J., NEMCHAUSKY, B.A., DEBARTOLO, M., KAPLAN, E., MCCORMICK, J.B., OLWIN, J.H., MARKS, G.E., BIRD, T., REDMOND, D.P., GRABER, R.C., FERRARA, A. & HRUSHESKY, W.J.M. 1988. Ten-year replicated circadian profiles for 36 physiological, serological and urinary variables in healthy men. *Chronobiol. Int.* **5**: 237-284.

16. KANABROCKI, E.L., SOTHERN, R.B., SCHEVING, L.E., VESELY, D.L. *et al.* 1990. Circadian reference data for men in fifth decade of life. In: *Chronobiology: its role in clinical medicine, general biology and agriculture, Part A*. D.K. Hayes, J.E. Pauly & F.J. Reiter (Eds). Proc. 19th Int. Conf. of Int. Soc. for Chronobiology, Bethesda, M.D. Wiley-Liss, New York. pp. 771-781.

17. SCHEVING, L.E. & GATZ, A.J. 1955. Mitotic activity in human epidermis. *Anat. Rec.* **121**: 363 (Abstract).

18. SCHEVING, L.E. 1959. Mitotic activity in human epidermis. *Anat. Rec.* **135**: 7-19.

19. ANDREW, W. & ANDREW, N.B. 1949. Lymphocytes in the normal epidermis of the rat and man. *Anat. Rec.* **104**: 217-241.

20. KARSTEN, G. 1918. Uber Tagesteriode der kern-und zell teilung. *Zeit. f Bot.* **10**: 1-20.

21. BRODERS, A.C. & DUBLIN, W.B. 1939. Rhythmicity of mitosis in the epidermis of human beings. *Proc. Staff Mtg. Mayo Clin.* **14**: 423-425.

22. COOPER, Z.K. & SCHIFF, A. 1938. Mitotic rhythms in the human epidermis. *Proc. Soc. Exp. Biol. Med.* **39**: 323-324.

23. SCHEVING, L.E., PAULY, J.E., TSAI, T.H. & SCHEVING, L.A. 1983. Chronobiology of cell proliferation: implications for cancer chemotherapy. In: *Biological rhythms in medicine*. A. Reinberg, & M.N. Smolensky (Eds). Springer-Verlag, Berlin. pp. 79-130.

24. FISHER, L.B. 1968. The diurnal mitotic rhythm in the human epidermis. *Br. J. Dermatol.* **80**: 75-80.

25. SCHEVING, L.E. 1976. The dimension of time in biology and medicine-chronobiology. *Endeavour* **35**: 66-72.

26. KANABROCKI, E.L., SCHEVING, L.E., HALBERG, F., BREWER, R.L. & BIRD, T.J. 1974. *Circadian variation in presumably healthy young soldiers*. US Dept. Commerce, Nat. Tech. Inf. Serv., Springfield, VA (Doc. No. PB228427).

27. SCHEVING, L.E., HALBERG, F. & KANABROCKI, E.L. 1977. Circadian rhythmometry on 42 variables of 13 presumably healthy young men. In: *12. International Conference Proceedings*. International Society for Chronobiology. Il Ponte, Milano. pp. 47-71.

28. EDMUNDS, Jr., L.N. 1983. Chronobiology at the cellular and molecular levels: models and mechanisms for circadian time keying. *Am. J. Anat.* **168**: 389-431.

29. De MAIRAN, M. 1729. Observation botanique. *Histoire de l'Académie Royal des Sciences.* Paris. p. 35.

30. BÜNNING, E. 1958. Das weiterlaufen der "physio logischen uhr" in sugerdarm ohne zentrale steuerung. *Naturwissenschaften* **45**: 68.

31. JOHNSON, M.S. 1939. Effect of continuous light on periodic spontaneous activity on white-fotted mice. *J. Exp. Zool.* **82**: 315.

32. ASCHOFF, J. 1960. Exogenous and endogenous components in circadian rhythms. In: Cold Spring Harbor Symposia on Quantitative Bio. Waverly Press. *Biological Clocks* **25**: 11-26.

33. KRIPKE, D.F., RISCHE, S.C. & JANOWSKY, D. 1983. Bright white light alleviates depression. *Psychiat. Res.* **10**: 105-112.

34. LUCAS, E.A., HALBERG, F., STRAUB, K.D., CARTER, J., REDMOND, D.P., SCRIMA, L. & SCHEVING, L.E. 1985. About 7-day (circaseptan) free-running rhythms in urinary norepinephrine and systolic and diastolic blood pressure during human social isolation studies. *Sleep Res.* **14**: 305.

35. SCHEVING, L.E., VEDRAL, D.F. & PAULY, J.E. 1968. A circadian susceptibility rhythm in rats to pentobarbital sodium. *Anat. Rec.* **160**: 741-750.

36. SCHEVING, L.E., VEDRAL, D.F. & PAULY, J.E. 1968. Daily circadian rhythm in rats to D-amphetamine sulphate: effect of blinding and continuous illumination on the rhythm. *Nature* **219**: 612-622.

37. SCHEVING, L.E., CARDOSO, S.S., PAULY, J.E., HALBERG, F. & HAUS, E. 1974. Variation in suscetibility of mice to the carcinostatic agent arabinosyl cytosine. In: *Chronobiology.* L.E. Scheving, F. Halberg & J.E. Pauly (Eds). Igaku Shoin, Tokyo, Japan. pp. 213-217.

38. STURTEVANT, R.P., STURTEVANT, F.M., PAULY, J.E. & SCHEVING, L.E. 1978. Chronopharmacokinetics of ethanol. III. Circadian variations in rate of ethanolemia decay in human subjects. *Int. J. Clin. Pharmacol. Biopharm.* **16**: 594-599.

39. STURTEVANT, R.P., STURTEVANT, F.M., SCHEVING, L.E. & PAULY, J.E. 1981. Chronobiologic aspect of blood cortisol levels and decay slopes following ethanol administration to adult human subjects. *Int. J. Clin. Pharmacol. Ther. Tox.* **19**: 432-439.

40. SCHEVING, L.D., TSAI, T.H., SOTHERN, R.B. & HRUSHESKY, W.J.M. 1988. Circadian susceptibility-resistance cycles to radiation and their manipulability by methylene blue. *Pharmacol. Ther.* **39**: 397-402.

41. SCHEVING, L.E., BURNS, E.R., HALBERG, F. & PAULY, J.E. 1980. Combined chronochemotherapy of L1210 leukemic mice using 1-beta-D-arabinofuranosyl-cytosine, cyclophosphamide, vincristine, methyl-prednisolone and cis-diaminedichloroplatinum. *Chronobiologia* **7**: 33-40.

42. SCHEVING, L.E., TSAI, T.H., SCHEVING, L.A., FEUERS, R.J. & KANABROCKI, E.L. 1992. Normal and abnormal cell proliferation in mice especially as it relates to cancer. In: *Biological rhythms in clinical and laboratory medicine.* Y. Touitou & F. Haus (Eds). Springer-Verlag, New York. 1990-1991.

43. HALBERG, F., GUPTA, B.D., HAUS, E., HALBERG, E., DEKA, A.C., NELSON, W., SOTHERN, R.B., CORNELISSEN, G., LEE, J.K., LAKATUA, D.J., SCHEVING, L.E. & BURNS, R. 1977. Steps toward a cancer chemopolytherapy. In: *Proceedings 14 international congress on therapeutics.* Montpellier, France. *L'Expansion Scientifique Française.* pp. 151-196.

44. HRUSHESKY, W.J.M. 1983. The clinical application of chronobiology to oncology. *Am. J. Anat.* **168**: 519-542.

45. HRUSHESKY, W.J.M. 1991. The multi-frequency (circadian, fertility cycle, and season) Balance between host and cancer. In: *Temporal control of drug delivery.* W.J.M. Hrushesky, R. Sanger & F. Theeuwes. *Ann. N.Y. Acad. Sci. USA* **618**: 247.

46. HRUSHESKY, W.J.M. 1991. Temporal optimizable delivery systems *Sine qua non* for molecular medicine. In: *Temporal control of drug delivery.* W.J.M. Hrushesky, R. Langer & F. Theeuwes (Eds). *Ann. N.Y. Acad. Sci. USA* **618**: XI.

47. LEVI, F., HRUSHESKY, W.J.M., HAUS, E., HALBERG, F., SCHEVING, L.E. & KENNEDY, B.J. 1980. *Experimental chrono-oncology. Principles and applications to shifts and schedules.* Alphen aan den Rijn, The Netherlands (NATO Advanced Study Inst. Ser. Sijthoff and Noordhoff) pp. 481-533.

48. CORNELISSEN, G., HALBERG, E., LONG III, H.J., PREM, BAKKEN, E. TOUITOU, F., HAUS, E. & HALBERG, F. 1991. Toward chronotherapy of ovarian cancer with Taxol Part I.: Basic Background. *Chronobiologia* **4**: 53-166.

49. HRUSHESKY, W.J.M., SANGER R. & THEEUWES F. 1991. Temporal control of drug delivery. *Ann. N.Y. Acad. Sci. USA* **618**: 641.

50. HALBERG, F., CORNELISON, G. & CARANDENTE, F. 1991. Chronobiology meets the need for integration in a reductionist climate of biology and medicine. *Chronobiologia* **18**: 93-103.

51. HRUSHESKY, W.J.M., LEVI, F., HALBERG, F., HAUS, E., SCHEVING, L.E., SANCHEZ, S., MEDINI, E., BROWN, H. & KENNEDY, B.J. 1980. Clinical chrono-oncology. In: *Chronobiology: principles and applications to shifts in schedules* L.E. Scheving & F. Halberg (Eds). Sijthoff and Noordhoff, Alphen aan den Rijn. The Netherlands. pp. 513-533.

SCHEVING, L.E., BURNS, E.R., PAULY, J.E. & HALBERG, F. 1980. Circadian bioperiodic response of mice bearing advanced L1210 leukemia to combination therapy with adriamycin and cyclophosphamide. *Cancer Res.* **40**: 1511-1515.

SCHEVING, L.E. & PAULY, J.E. 1974. Circadian rhythms: some examples and comments on clinical application. *Chronobiologia* **1**: 3-21.

SCHEVING, L.E., TSAI, T.H., PAULY, J.E. & HALBERG, F. 1983. Circadian effect of ACTH 1-17 on the mitotic index of the corneal epithelium of Balb/c mice. *Peptides* **4**: 183-190.

SCHEVING, L.E., TSAI, T.H., PAULY, J.E. & HALBERG, F. 1984. Effects of ACTH 1-17 at different circadian stages on ^{3}HTdR incorporation into DNA. *Peptides* **5**: 507-518.

SCHEVING, L.E., TSAI, T.H., PAULY, J.E. & HALBERG, F. 1984. Circadian-stage dependent ACTH 1-17 effect on DNA synthesis in murine duodenum, colon, and rectum. *Chronobiologia Int.* **1**: 251-266.

WALKER, W.V., RUSSELL, J.D., SIMMONS, D.J., SCHEVING, L.E., CORNELISON, G. & HALBERG, F. 1985. Effect of an adrenocorticotropin analogue 1-17 on DNA synthesis in murine metaphysiol bone. *Biochem. Pharm.* **34**: 11-19.