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# Toward a Chronotherapy of Neoplasia: Tolerance of Treatment Depends upon Host Rhythms*

by F. HALBERG, E. HAUS, S. S. CARDOSO, L. E. SCHEVING, J. F. W. KÜHL, R. SHIOTSUKA, G. ROSENE, J. E. PAULY, W. RUNGE, J. F. SPALDING, J. K. LEE and R. A. GOOD

Chronobiology Laboratories, University of Minnesota, 380 Lyon Laboratories, Minneapolis (Minnesota 55455, USA) Department of Anatomic and Clinical Pathology, St. Paul-Ramsey Hospital, St. Paul, Minnesota; Department of Pharmacology, University of Tennessee, Memphis, Tennessee; Department of Anatomy, University of Arkansas, Little Rock, Arkansas; Department of Physiology and Health Science, Ball State University, Muncie, Indiana; Los Alamos Scientific Laboratory of the University of California, Los Alamos, New Mexico; and Sloan-Kettering Institute for Cancer Research, New York (New York, USA).

## I. *Abstract*

Circadian rhythms drastically influence the effect of drugs and other agents in experimental animals[1–9] and in man[10–12]; they even tip the scale between life and death following a mammal's exposure to bacterial endotoxins, noise, ethanol, ouabain, librium, acetylcholine, pentobarbital and a host of other agents, including whole-body or partial-body X-ray irradiation[13–18]. The exploitation of information about these rhythms has already begun in the clinic; thus, when a given prominent hormonal rhythm is absent, as in the case of Addison's disease, substitution treatment can be timed not only to reproduce the periodicity but also to locate optimal performance, such as grip strength, according to the requirements (e.g., work times) of the patient[12].

In the laboratory and in the clinic, timing can gravely influence the effects of medication. The evidence is overwhelming for the probability that ignoring rhythms can lead to spurious interpretation and hence questionable diagnosis and treatment[3]. By contrast, evaluation of rhythmic variability yields new and useful information about the characteristics of rhythms as the basis for a kind of chronotherapy aimed eventually at correcting any pathogenetically altered rhythms.

What already has been documented is that as a minimum drug testing is rendered more efficient when undue variability is reduced by evaluating rhythms and carrying out work in defined stages of predictable cycles. As the optimum achieved thus far, rhythm assessment allows timing according to these predictable changes that improve tolerance of a widely used carcinostatic drug. Any organismic response quite generally depends upon the dose of the stimulus applied. The evaluation of rhythms in response to drugs when tested at several dose levels, reveals various kinds of effects:

The slope of a linear dose-response relation may remain constant, but the dose required to produce a given response (i.e., drug potency) may change along the 24-hour scale. This kind of circadian rhythm was demonstrated for the susceptibility of mice to ouabain[7] or endotoxin[1]. The slope of the dose-response relation, itself, also may change during a 24-hour span, as observed for the in vitro response of murine adrenal

* Dedicated on the occasion of his 60th birthday to J. ASCHOFF who has eloquently emphasized the survival value and significance of rhythms and thus has visualized the results herein[78].

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glands to ACTH[3]. On the other hand, the *shape* of the dose-response relation may vary. Thus, the characteristics of a susceptibility rhythm, including timing, may differ with dose, as recently demonstrated for pentobarbital-induced sleep in the mouse[19]. Mortality from ara-C as well is a function not only of the magnitude of a single dose, but also dependent on whether single or multiple doses are being injected. The change in a susceptibility rhythm with increases or repeated applications of a given dose may well be due to phase-shifts of underlying rhythms, here illustrated. Indeed, it is shown herein that carcinochemotherapy must be given with chronobiologic considerations in mind.

Thus, a carcinostatic drug extensively used by clinicians is tolerated better when timed according to the host's circadian rhythms than when administered on a conventional schedule: tolerance for the treatment of an experimental murine leukemia by arabinosyl cytosine (ara-C) was markedly increased when a circadian-rhythm-adjusted (sinusoidal) schedule of gradually increasing and decreasing doses was used instead of a conventional schedule (same total dosage) putatively adjusted to a leukemic cell cycle[8]. In a follow-up on an earlier series of three studies[20] that apply exclusively to toxicity, now evaluated at 70, 54 or 42 days after start by leukemia inoculation[20], the gain in tolerance for the drug tested averaged 15.59

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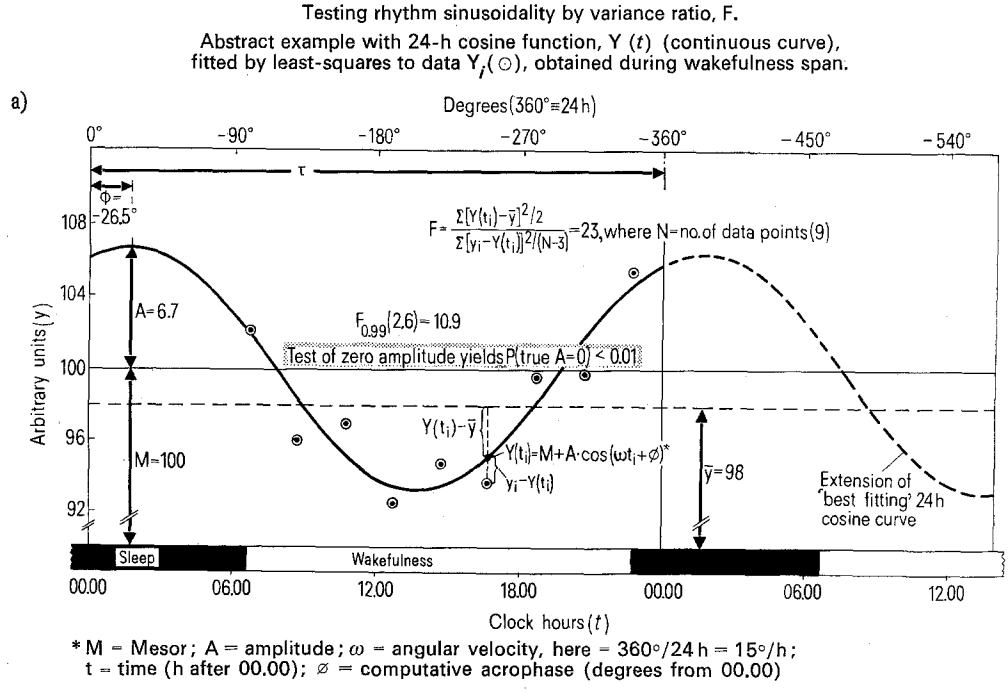


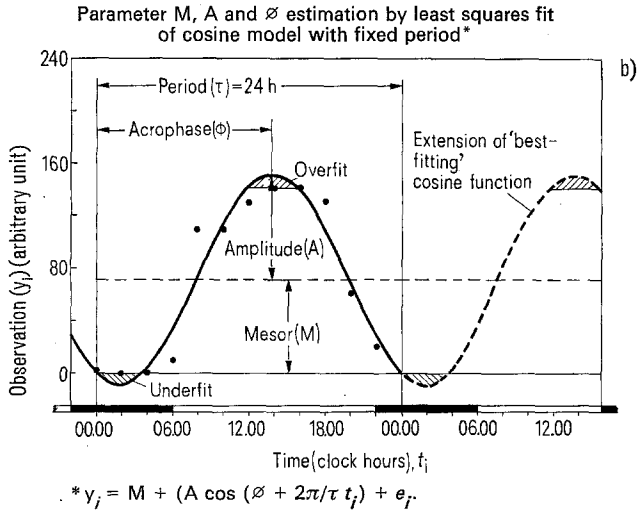


Fig. 1. a) Testing rhythm sinusoidality by variance ratio, F. Abstract example with 24-h cosine function, Y (*t*), continuous curve, fitted by least squares to data *y* (○), obtained during wakefulness span. M, mesor; A, amplitude; $\omega$, angular velocity, here = 360°/24 h = 15°/h; *t*, time (h after 00.00); $\varnothing$, computative acrophase (degrees from 00.00). b) Parameter M, A and $\varnothing$ estimation by least squares fit of cosine model with fixed period: $y_i$, M + A cos ($\varnothing$ + $2\pi/\tau t_i$) + $e_i$. $t_i$, time; $y_i$, observation at $t_i$; $e_i$, error occurred at $t_i$, having the same independent normal distribution with mean zero an unknown variance $\sigma^2$. Computer programs facilitate inferential statistical rhythm detection in noisy data such as those usually collected in the clinic (a) and the estimation of the rhythm's properties (b). $\omega = 2\pi/\tau$.

mouse days. If murine experience in this case can be extrapolated to man, the gain in murine tolerance conceivably represents the equivalent of 545 days of human life, as survival time increment.

Herein we further document the fact that sinusoidal schedules with a given temporal placement of high and low doses along the 24-hour scale are superior to others involving the same total dose but with a drastically different placement. The overall sequence of the high and low doses used is the same for the several sinusoidal treatment schemes. Each sequence is initiated at the same circadian time for all groups; only the initial dose (amount) in the sequence is varied for different groups in order to achieve at different circadian times a differing temporal placement of high and low doses. It seems likely that this placement – a circadian susceptibility rhythm – contributes importantly to the improved tolerance of certain sinusoidal ara-C administration schedules, in conjunction, probably, with an effect of the particular sequence of high and low doses. To the extent that a susceptibility rhythm characterizes the host, the tools of modern chronobiology for resolving the characteristics of rhythms (Figure 1) – the rhythm-adjusted mean (mesor), the measure of extent of change (amplitude) and a location of timing (acrophase) – become critically pertinent to an eventual clinical chronotherapy.

## II. *Current concepts governing the chemotherapy of neoplasia*

A considerable body of cytokinetic, pharmacologic and biochemical information has successfully served as base for the currently employed schedules of drug administration both at the preclinical and clinical levels[21–24]. On the other hand, rather limited attention has been focused on the extensive and well documented information pertaining to biological rhythms, such as circadian rhythms in the synthesis of DNA and mitosis and their role in schedules of administration of cancer chemotherapeutic agents[8, 20, 25].

Indeed, effective therapeutic schemes have been based on the assumption that cell populations in the host grow asynchronously, 'so that at any given time cells are distributed throughout the cycle... there are twice as many cells starting the cycle as there are cells about to divide[26].' Nothing would be lost, and much might well be gained, if evidence contrary to the foregoing views also were to be considered. Indeed, rhythms characterize human skin[27] (Figures 2 and 3), growing (or regenerating) mouse liver (Figure 4)[28], mouse pancreas (Figure 5), healthy rat bone marrow (Figure 6)[27], many other tissues of mammalian hosts (Figure 7), and even human breast cancer, multiple myeloma and an Ehrlich ascites tumor in the mouse (Figure 8[29], cf also [30–34]).

To cite another example, for a number of years a controversy has existed concerning whether epithelial cells in the gut of rodents divide with a circadian frequency. Although the actual mitotic counts published in an early negative report demonstrated such variation, there was a rather widespread notion that the gut

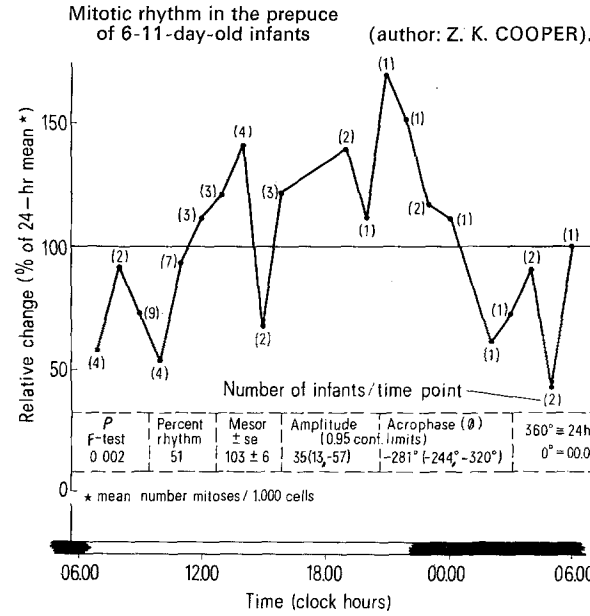


Fig. 2. Mitotic rhythm in the prepuce of 6–11-day-old infants. Author: Z. K. COOPER. Variability (noise) in mitotic counts of foreskins removed at circumcision from different children along the 24-hour scale, in the absence of a standardized routine; the fit of a 24-hour cosine curve to such data (not shown) demonstrates a statistically significant rhythm.


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epithelium does not exhibit circadian variation, and a number of studies have been designed with this misconception in mind.

A recent study offers additional data to support a circadian variation in the mitotic index of gut epithelium and also in the rate of $^3$H-thymidine uptake into the gut epithelial cells of the mouse[35]. Another interesting feature of this same report is that rhythms in the mitotic index and $^3$H-thymidine uptake (DNA synthesis) – based on autoradiographic analysis – are similarly timed. This could indicate that one population of cells is synthesizing while another is dividing. A similar finding for corneal epithelium in the rat was reported earlier[36] and recently discussed[27].

A later paper confirmed the mitotic index and $^3$H-thymidine rhythms in the duodenum of the rat and also reported that the phasing of the two was similar (Figure 9)[37]. A comparable overall timing was supported further by the fit of a 24-hour cosine curve to each series of data; the acrophase (and its 95% confidence arc) for the mitotic index rhythm was −196° (−181° to −206°), and for the $^3$H-thymidine rhythm −181° (−161° to −202°), with respect to the same phase reference.

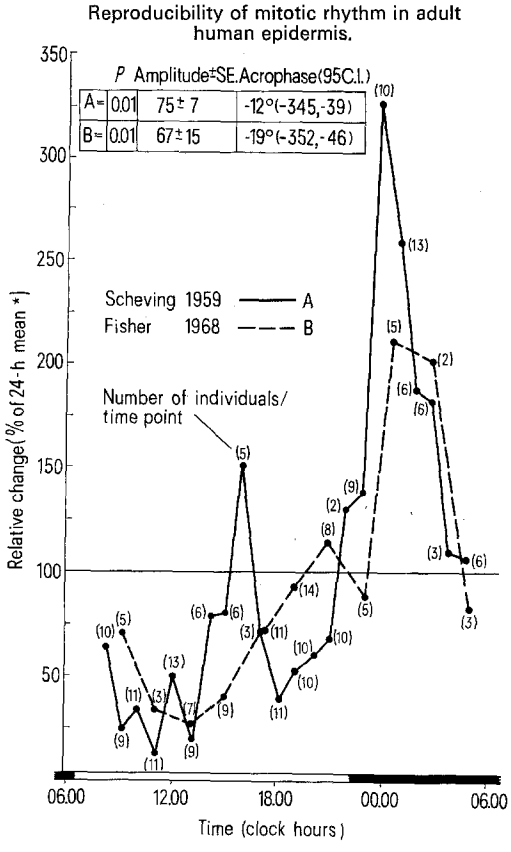


Fig. 3. Reproducibility of mitotic rhythm in adult human epidermis. Fischer, mean number mitoses/1,000 cells. Scheving, mean number mitoses/1,000 cells (2,500–3,000 cells/specimen). Circadian rhythm in number of human epidermal mitoses. A majority of cell divisions in epithelial cells occur at a defined and predictable circadian system phase. Remarkable reproducibility of results obtained many years and miles apart (cf. also[39]).

This latter paper[37] also discusses some of the reasons for the controversy mentioned above; the methodology responsible for seemingly negative results on periodicity in gut epithelium may be contributing to some of the present conflicting results regarding the nature of periodicity in cell proliferation in tumors. Indeed, much of the past work on periodicity of tumors needs to be reevaluated. Some sources of conflicting results include 1. carrying out group studies on animals kept in continuous illumination, 2. using colchicine over extended spans of time, i.e., allowing a span of many hours between colchicine injection and killing and 3. sampling at too infrequent intervals.

Interestingly, it also has recently been reported for gut epithelium of the mouse that the 'cell cycle time' is circadian phase-dependent[38]. Circadian kinetics of cell proliferation, although complex, are pertinent to chronoradiotherapy, to chronochemotherapy and to the ultimate understanding of the mechanism of cell division and its control.

Accordingly, one may anticipate that any substantial gains in the therapeutic index of agents used in cancer chemotherapy will be harder to achieve on empirically designed therapeutic schedules that fail to consider: a) Periodic and thus predictable changes in cell populations, which render them either more sensitive or more resistant to a given agent, i.e., the circadian kinetics of normal and neoplastic cell populations; b) The mechanisms governing and maintaining circadian mitotic rhythms in normal and neoplastic cells: hormonal as well as environmental effects upon cell division[28,39–42]; c) Circadian variations in the rates of drug metabolism: changes in blood and tissue half-lives of cancer chemotherapeutic agents as influenced by circadian system phases.

## III. *Problem*

Various administration schedules for ara-C, now used in the clinic, have been exhaustively tested in the treatment of an experimental murine leukemia[22].

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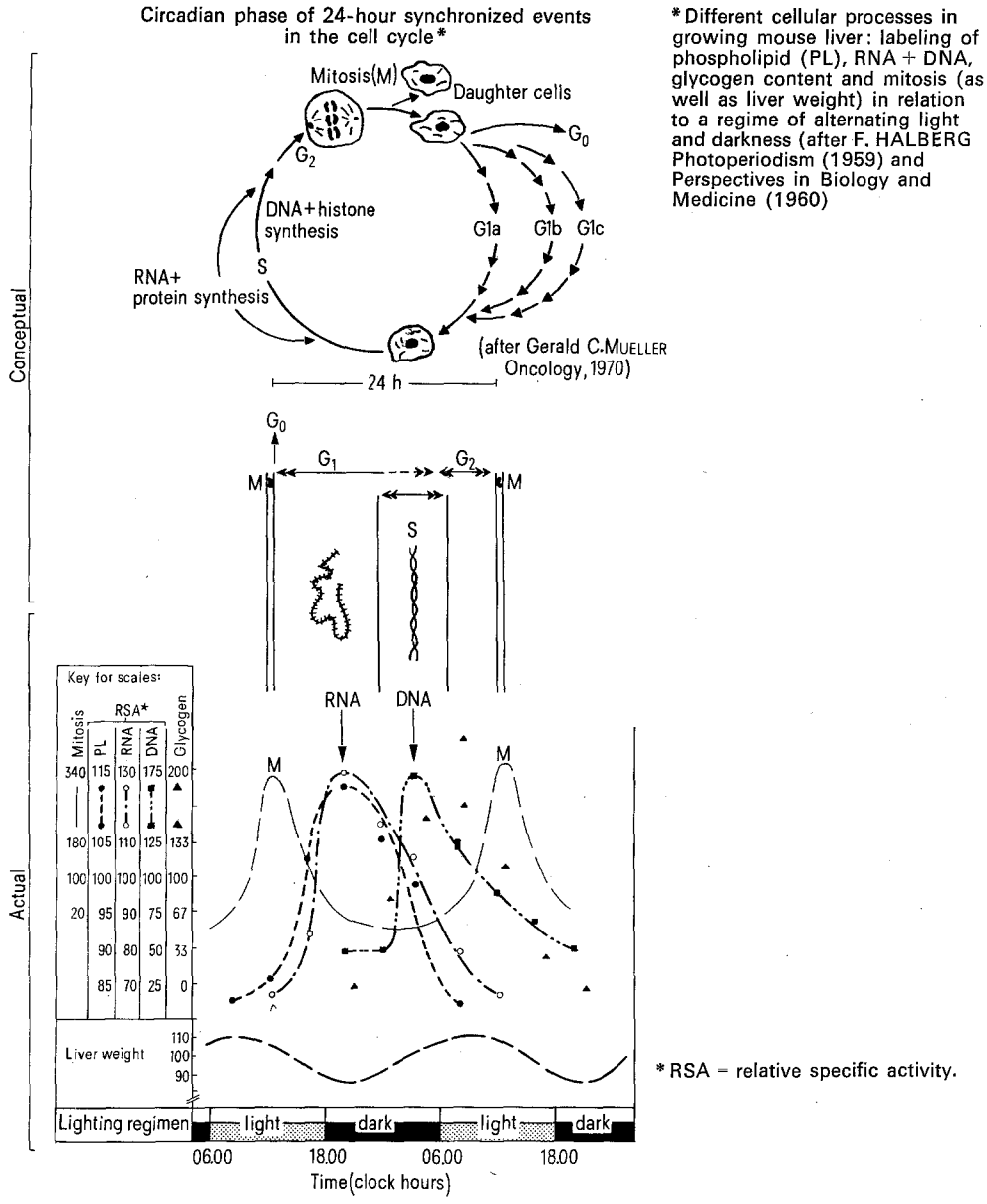


*Different cellular processes in growing mouse liver: labeling of phospholipid (PL), RNA + DNA, glycogen content and mitosis (as well as liver weight) in relation to a regime of alternating light and darkness (after F. HALBERG Photoperiodism (1959) and Perspectives in Biology and Medicine (1960)

*RSA = relative specific activity.

Fig. 4. Circadian phase of 24-h synchronized events in the cell cycle. Different cellular processes in growing mouse liver: Labeling of phospholipid (PL), RNA and DNA, glycogen content and mitosis (as well as liver weight) in relation to a regime of alternating light and darkness. RSA, relative specific activity. Sensitive stages of the cell cycle may become directly amenable to therapeutic exploitation by timing carcinostatic treatment according to rhythms. The classical cell cycle, as sketched inter alios, by MUELLER[63], may well be aligned for this purpose with the actual cellular circadian system here demonstrated for immature growing mouse liver[28]. Similar circadian and other rhythmicity (with definable though not the same timing) characterizes many tissues of the mammalian host with neoplasia[29] and should be exploited for timed treatment.

A schedule of 4 treatment courses, each consisting of 8 equal doses given at 3-hour intervals, has been recommended[22]. Figure 10 shows in the right upper corner such a series, consisting of 8 equal doses at 3-hour intervals: this treatment can be denoted as Reference (R) treatment[20]. It has been compared in terms of tolerance – though not in terms of cure of the underlying disease – with a schedule of gradually increasing and decreasing doses[20] so designed that even the smallest dose given when the host is most susceptible still exerts therapeutic activity, perferably with little or no toxicity[22]. This schedule, denoted as 'sinusoidal' and illustrated for the case of multiple daily doses in Figure 10, is generalized also in the abstract Figure 11. Figure 12, in turn, elaborates on this concept, and Figures 13 and 14 on the hours of changing resistance established by data on the response to a single stimulus[1–20, 43, 44].

Figure 12 sketches the two patterns for drug administration in single daily doses (for review see[1–3])

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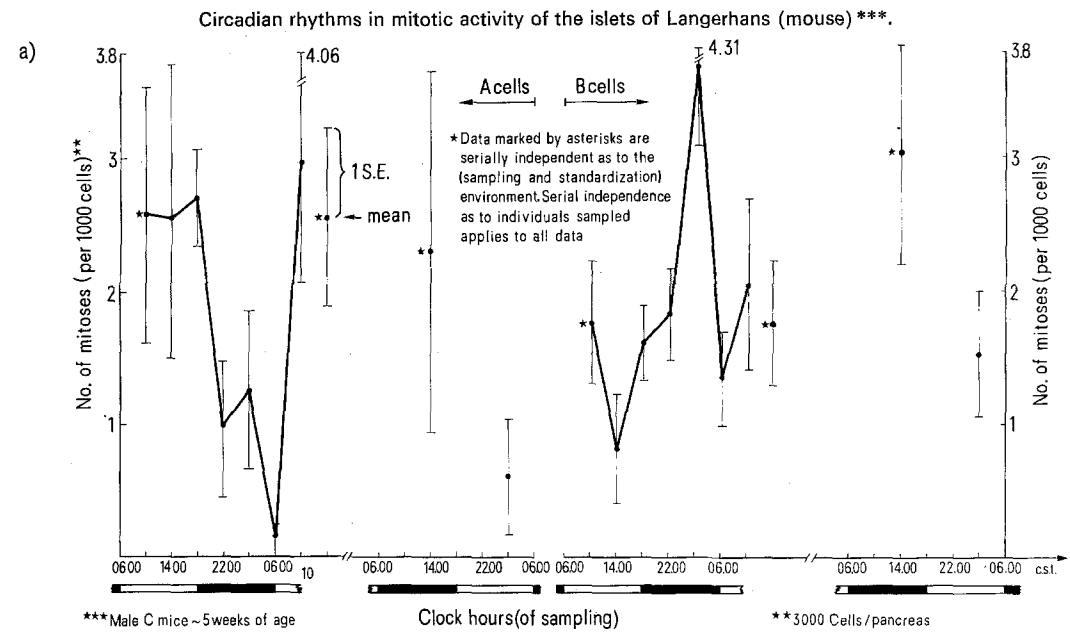


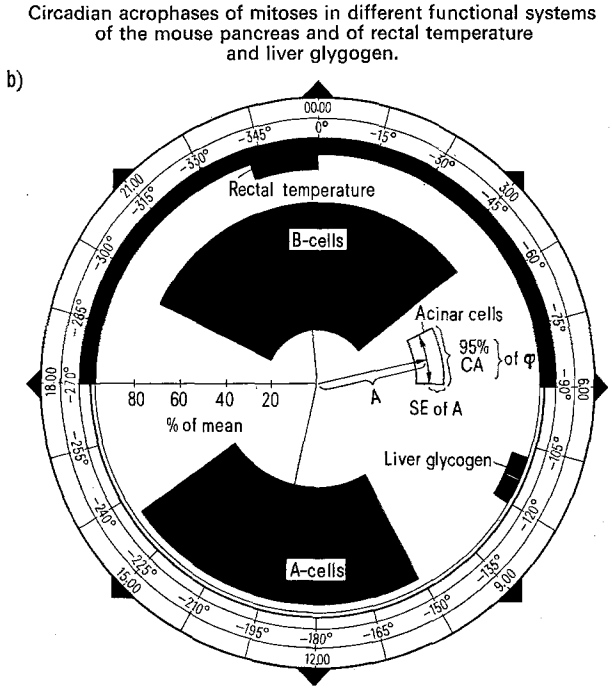


Fig. 5. a) Circadian rhythm in mitotic activity of the islets of Langerhans (mouse). b) Circadian acrophases of mitoses in 3 different functional systems of mouse pancreas as well as of rectal temperature and liver glycogen. Rhythms with widely differing timing are found for the same variable – cell division – in the pancreas (in glucagon-producing A-cells, insulin-producing B-cells and acinar cells of the same organ) by curve-fitting procedures (discussed in Figure 1) (b) as well as by time plots (a).

and their theoretical results. Figure 11 refers to multiple daily doses, modeling the real case here discussed – namely ara-C treatment of hybrid $BDF_1$ mice, tested as shown in Figures 10 and 16. The schemes in the abstract Figures 11 and 12 show two ways of administering a drug as one of the many potential loads to which an organism may be exposed. At the left in Figure 11 and on top in Figure 12, a time-invariant pattern of administration results in a time-varying response. To obtain a more or less time-invariant response, one needs to use a time-variant drug test pattern by one of several possible approaches, as illustrated at the right in Figure 11 and the bottom of Figure 12, in the abstract schemes.

## IV. *Background*

It has been shown, first, that the toxicity of single daily doses of ara-C varies as a function of circadian phase[8]. For instance, two of the earlier studies[8, 25, 38] shown in Figure 15 were begun after standardization on a regimen of artificial light from 06.00 to 18.00 h alternating with darkness for 7 days, on male $BDF_1$

mice weighing on the average 16 g at the start of study, on January 23, 1971 and February 23, 1971, respectively. At 08.30 h on the first day marking the beginning of each experiment 15 animals were injected i.p. with 400 mg/kg of ara-C. This prodecure was repeated on different subgroups of 15 mice each at 13.00, 18.30, 23.00 and 03.00 h. Thus, 5 subgroups received ara-C at defined circadian system phases. Beginning on the second day at 08.30 h, the subgroup injected on the previous day at the same time was given one-half the initial dosage (200 mg/kg) for 4 more consecutive days. Exactly the same procedure was followed for the other subgroups of mice injected consistently at 13.00, 18.30, 23.00 and 03.00. Thus, the animals received a total of 1200 mg/kg divided into 5 injections over a 5-day span.

The mice were checked and any deaths recorded each day for 13 days after the last injection, at which time all survivors looked healthy and had regained their initial weight. (No animals had died after the 9th day following the initial injection).

Figure 15 shows results on mortality from ara-C as a function of circadian time. 74% or 67% of the animals died from ara-C injected at one time as compared to 15% of comparable animals given the identical doses of the same drug at the same intervals yet at a different circadian time. Mortality rhythms are highly reproducible in these two studies done 1 month apart in January and February, 1971. However, in studies done at greater intervals or with different doses, the effect of low-frequency changes, including about-yearly or circannual rhythms, may modify circadian rhythms so that an overall summary of changes in susceptibility (gauged, e.g., by mortality) requires techniques that can identify the interacting contributions of several rhythmic mechanisms.

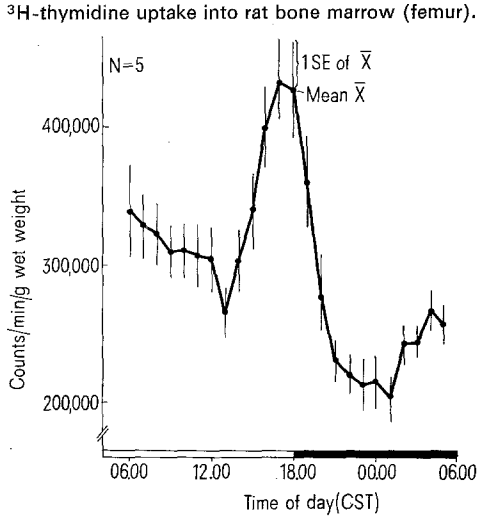


Fig. 6. $^3$H-thymidine uptake into rat bone marrow (femur). Circadian rhythm in healthy rat bone marrow activity revealed by changes in $^3$H-thymidine uptake. ($^3$H-thymidine given at hourly intervals to separate subgroups of $BDF_1$ mice. Times of killing on abscissa[27].

As the Table (p. 932) indicates, by a combination of linear least squares analyses introduced by J. K. Lee, rhythms with a period of about 2, 3, and over 5 months have been found for weekly data on hemoglobin and mean corpuscular volume of mice[45]. Whether such low-frequency changes in blood are accompanied by changes with corresponding frequency in host resistance can not be decided without further study. It can be pointed out, however, that yet more powerful computer techniques such as a combination of linear and nonlinear least squares analyses[46] now allow the resolution of multiple rhythms that are not readily apparent to the naked eye.

Just as a cell has to be resolved by combining histological techniques with the use of a microscope, by the same token rhythms have to be quantified if not resolved by computer techniques – even when they stand out in clear view of the naked eye, as they do in Figure 15. Although the conditions are controlled here, the results of the analysis must not be generalized without reference to interacting circannual and other sources of variation. Awaiting resolution are the factors underlying these multiple mortality rhythms, including deaths from cancers[47].

The bioperiodicity of the bone marrow revealed by labeling studies demonstrated in Figure 6 and discussed elsewhere[27] may be one such mechanism. The determination of $^3$H-thymidine incorporation into tissues of the rat has been previously described in detail[36]. Isotope injections were made 1 h prior to sacrifice. The time points given as the abscissa of Figure 6 are kill times.

As a followup on work suggesting the occurrence of a susceptibility rhythm, reflected by changes in the location along the 24-hour scale of times associated with a high (or low) mortality from the drug given in *single* daily doses repeated for several days, a first study of circadian-phase-dependent effects of *multiple* daily doses of ara-C was carried out with a scheme such as that shown in the upper right hand corner of Figure 10[20]. It was presumed that the optimal timing – from the viewpoint of host resistance – of multiple daily doses will require that more of the drug be given at the (circadian) time when single daily doses are best tolerated, the dosage decreasing as one encounters times along the 24-hour scale when susceptibility to single doses has been shown to increase. Quite clearly, however, possible cumulation and/or rhythm desynchronization could not be assessed in this first series of studies.

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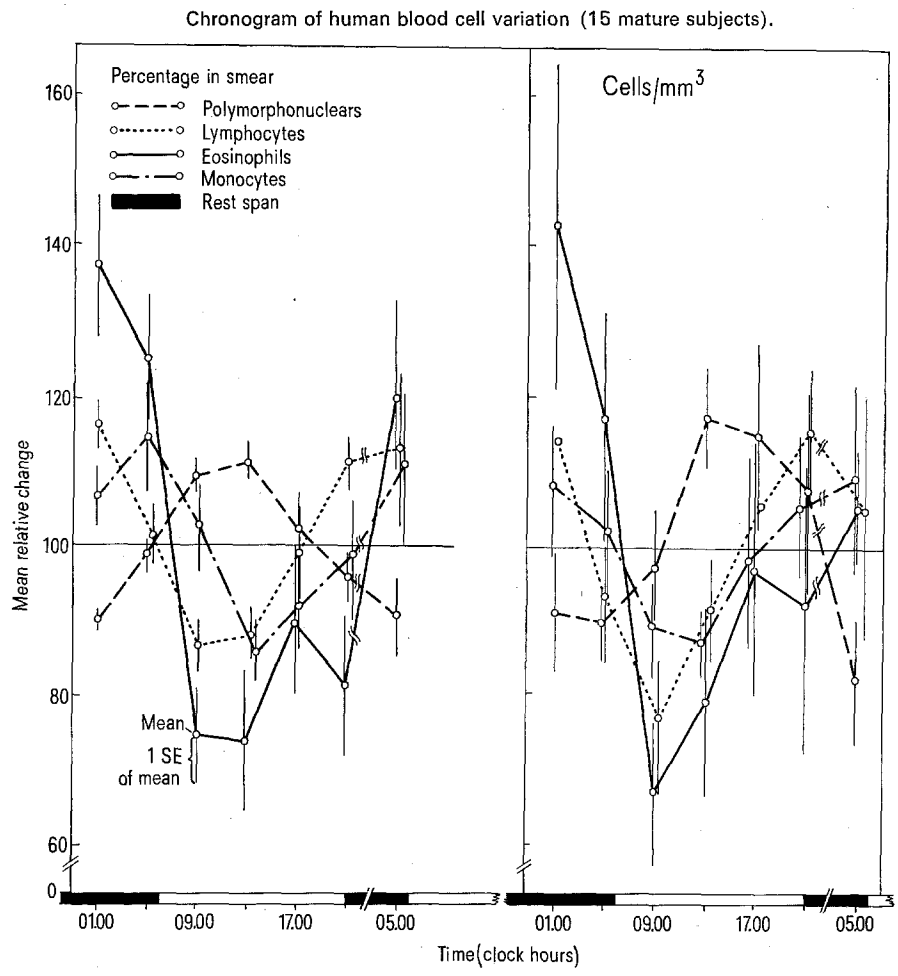



Fig. 7. Chronogram of human blood cell variation (15 mature subjects). Circadian rhythms of leucocyte count in circulating human blood. Left: Percentage of cells in smear. Right: Calculated absolute numbers of different cell types.

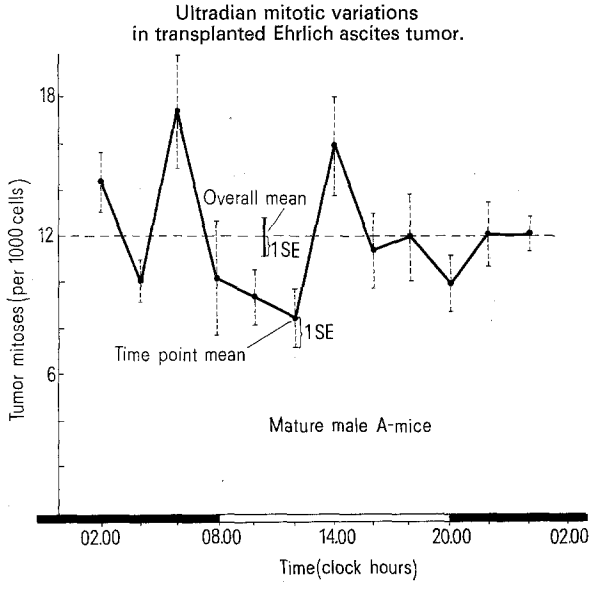



Fig. 8. Ultradian mitotic variations in transplanted Ehrlich ascites tumor. More generally, mitotic variations in neoplasia may assume frequencies higher than circadian[32].

Moreover, in view of the earlier experience[25], it was anticipated that the multiple-daily-dose ara-C effect would further depend upon circannual variations, perhaps altering along the 1-year scale not only the mesor (rhythm-adjusted mean) of a given function studied, but also the circadian acrophase, as has been demonstrated for the adrenocortical cycle[47–49]. Accordingly, the optimal tolerance of the animals for ara-C was expected to occur at times of high resistance in the circadian susceptibility cycle that are not immutably fixed, for it was recognized that the circadian rhythm characteristics may vary with the stages of rhythms with other frequencies. This fact will be considered for a hypothetical case at the end of this presentation.

This time of high circadian resistance to ara-C, adjusted for the stage of the previously explored

[48] F. HALBERG, M. ENGELI, C. HAMBURGER and D. HILLMAN, Acta Endocr. Suppl. *103*, 54 (1965).
[49] E. HAUS and F. HALBERG, Envir. Res. *3*, 81 (1970).

circannual variation in circadian acrophase, was selected as the high point of a multiple-daily-dose sinusoidal drug-administration pattern, namely one involving ara-C administration in increasing and then decreasing doses every 3 h for 24 h every 4th day[20]. The highest doses of this so-called (somewhat asymmetrical) sinusoid were administered at the antiphase of the mortality acrophase for the given month of the year – extrapolated from results obtained earlier by single-dose ara-C administration[25]. Three different total daily doses of ara-C, namely 120, 180 and 240 mg/kg, were used for treating mice inoculated with $L_{1210}$ leukemia cells ($10^6$ or $10^7$) suspended in Eagle Minimum Essential Medium, ara-C being given according to either a reference schedule or a sinusoidal schedule. The 180 mg/kg total daily dose, again given in both a reference and a sinusoidal pattern, was used for added toxicity studies on animals that were not inoculated with leukemia. It should be emphasized that there were studies of tolerance and the doses were advisedly chosen from a range previously demonstrated to be toxic[22].

A first set of multiple-daily-dose experiments was done, taking into account only changes in host resistance[20] yet with the realization that additional studies will be needed for better pinpointing and then exploiting 1. the known multiplicity of now readily assessed host rhythms – circadian[9,50,51], circaseptan[48], circannual[48,49] and others[45] – and 2. the rhythms of the tumor[29–34,52,53], quite apart from considering, as noted 3. diverse possible cumulative effects, namely cumulation from a sequence of consecutive doses within a 24-hour treatment series and perhaps also from one series to the next, as well as the choice of large or small doses at the start of a given sequence and, finally, 4. desynchronization of circadian rhythms as a function of treatment[54], a circumstance that requires the monitoring of variables that are indicators of appropriate timing.

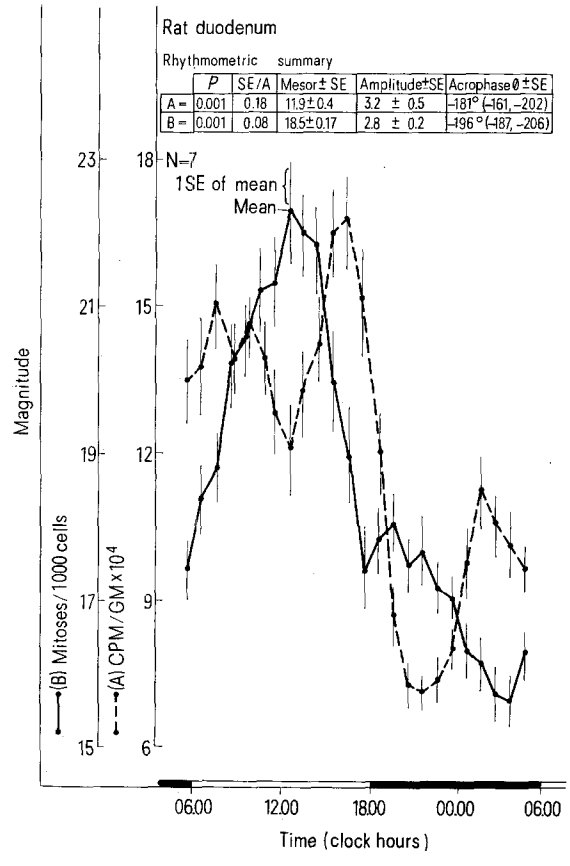



Fig. 9. Circadian rhythm characterizes cell division in duodenum, as demonstrated in this figure for the rat (by L. E. S. and J. E. P.) and in the Chronobiology Laboratories at the University of Minnesota (unpublished) for mouse duodenum as well.

## V. *New results*

It remained to be shown that sinusoids with a timing adjusted to a particular rhythm such as the circadian (and eventually to each additional rhythm demonstrated to be significantly influencing the outcome of a given therapy) are superior to a 'sinusoid control'. Hence, sinusoidal (S) treatment patterns placed in such a fashion that the highest doses are given at the times of the expected highest resistance of the animals were compared with sinusoids so placed that the highest doses are scheduled at the time of anticipated lowest resistance (Figure 16). In such experiments a reference treatment also was included and was found to compare unfavorably with the best S-schedules tested. It is true that one must keep in mind a probable effect of sinusoidal (rather than fixed-dose) sequencing per se – apart from the temporal placement of the sinusoid along the 24-hour scale. Nevertheless, the latter possibility does not account fully or even primarily for the merits of chronochemotherapy: some S-series are dramatically and statistically significantly ($P < 0.01$ for time effect in analysis of variance) superior to others used in the same experiment on mice receiving the same total dosage but with a different timing of the high and low doses along the 24-hour scale (Figure 16).

[50] L. A. Scheving, L. E. Scheving and F. Halberg, *Chronology* (Eds. L. E. Scheving, F. Halberg and J. E. Pauly; Int. Soc. for Study of Biological Rhythms, Little Rock, Arkansas; Igaku Shoin Ltd., Tokyo), in press.

[51] F. Halberg, Lancet *73*, 20 (1953).

[52] H. H. Zinneman, M. Thompson, F. Halberg, M. E. Kaplan and E. Haus, Clin. Res. *20*, 798 (1972).

[53] N. B. Kachergene, I. V. Koshel and R. P. Nartsissov, Pedriatica *51*, 81 (1972).

[54] K. Charyulu, F. Halberg, E. Recker, E. Haus and H. Buchwald, *Chronology* (Eds. L. E. Scheving, F. Halberg and J. E. Pauly; Int. Soc. for Study of Biological Rhythms, Little Rock, Arkansas; Igaku Shoin Ltd., Tokyo), in press.

Survival of mice as a function of time after leukemia inoculation and schedule of cytosine arabinoside (ara-C) treatment ($R_x$).

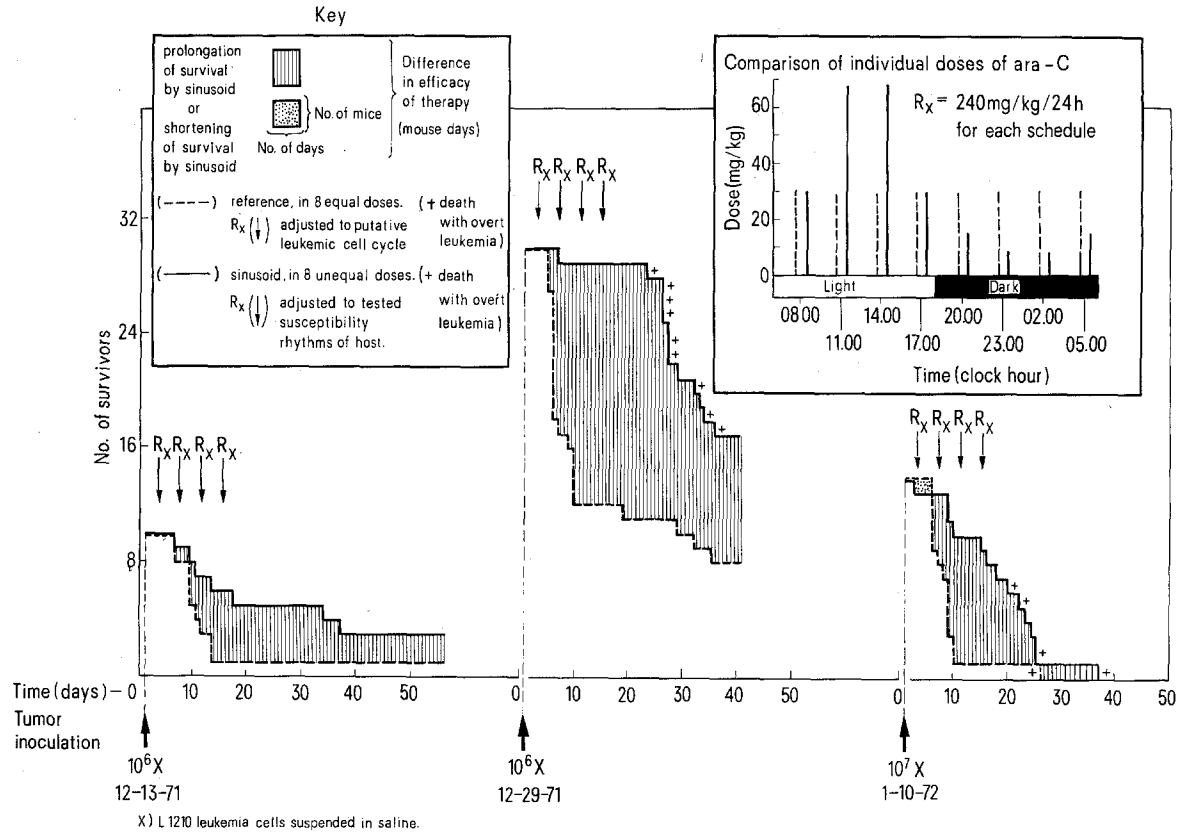



Fig. 10. Survival of mice as a function of time after leukemia inoculation and schedule of cytosine arabinosyl (ara-C) treatment ($R_x$). X = L 1210 leukemia cells suspended in saline. Prolongation of average survival time (corresponding to shaded areas) of mice inoculated with L 1210 leukemia when antimetabolite ara-C is given with a sinusoidal (rather than a conventional fixed-dose) drug-administration schedule.

Relations of external loads to internal cost in rhythmic systems.

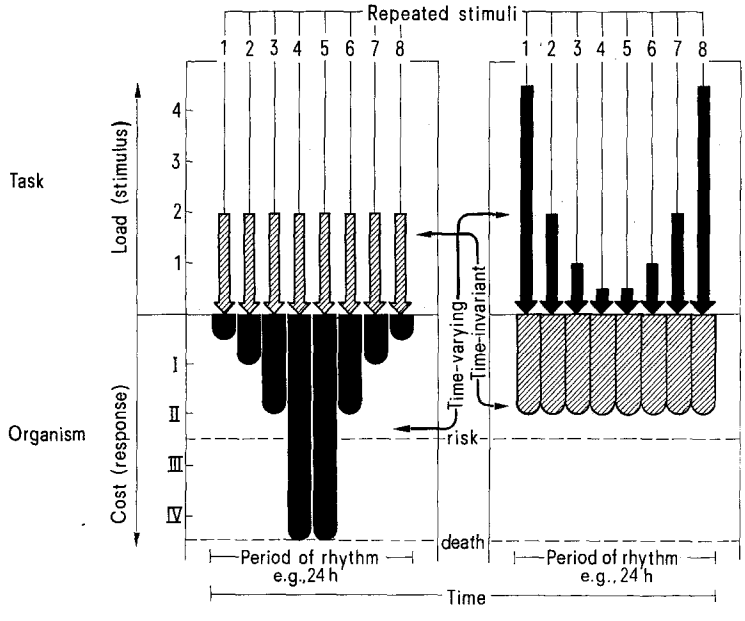



Fig. 11. Relations of external loads to internal cost in rhythmic systems. The effects of repeated stimuli – such as a course of drug administration (Figure 11) – can be time-dependent [see also hours of changing resistance (Figures 8 and 9)]; if so, a series of doses given in a time-invariant fashion might involve risk or result in death, whereas the same total dosage will be better tolerated when given in a fashion varying with due regard for the body's rhythms.

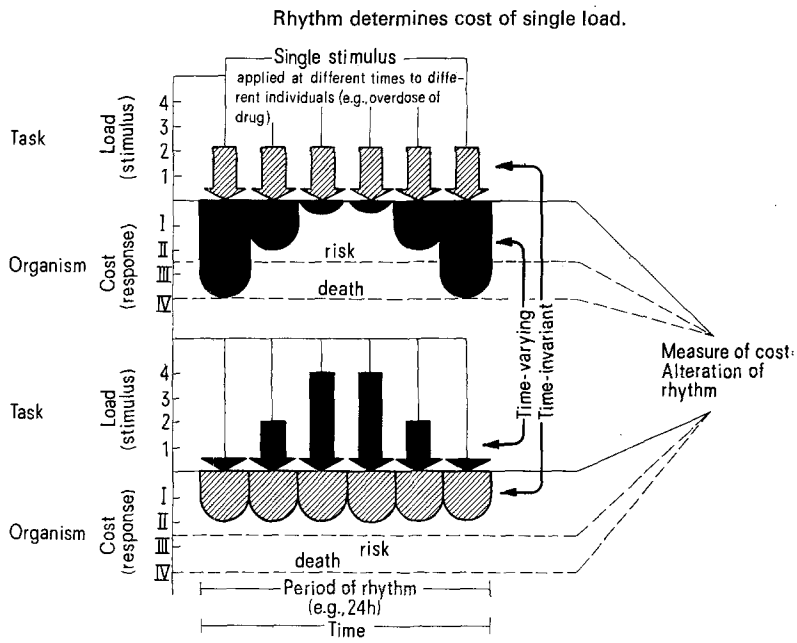



Fig. 12. Rhythm determines cost of single load. Hours of changing resistance. As a function of the body's rhythms a potentially harmful stimulus, such as a drug given in the same dose at different times to different yet comparable individuals (top), will have drastically different effects; not even risk is incurred at one time from a stimulus that is deadly at another time.

## VI. *Implications*

For the clinic the possibility should be kept in mind that circadian, circannual, and other variations in host physiology (Figure 17)[48], will affect resistance and thus the outcome of treatment. And if full advantage is to be derived from a drug administration timed according to rhythms, the probable periodicities of the human tumor itself will also have to be mapped at several frequencies.

Ideally, in considering the extent of work and the difficulties involved in mapping all the pertinent variables, synchronizing the frequencies of both normal and neoplastic cells in such a way as to cause their respective mitotic crests to occupy distinctly separate positions along a temporal dimension would represent a major step toward further improvement of chronotherapy. Partial success has been obtained in shifting, with the use of dexamethasone, the usual mitotic crests in healthy but not in neoplastic tissues of rats and mice[55–57]. In any event, in the experimental animal and eventually in the clinic, it remains to be seen whether pertinent organismic and neoplastic reference functions can be found and treatment timed

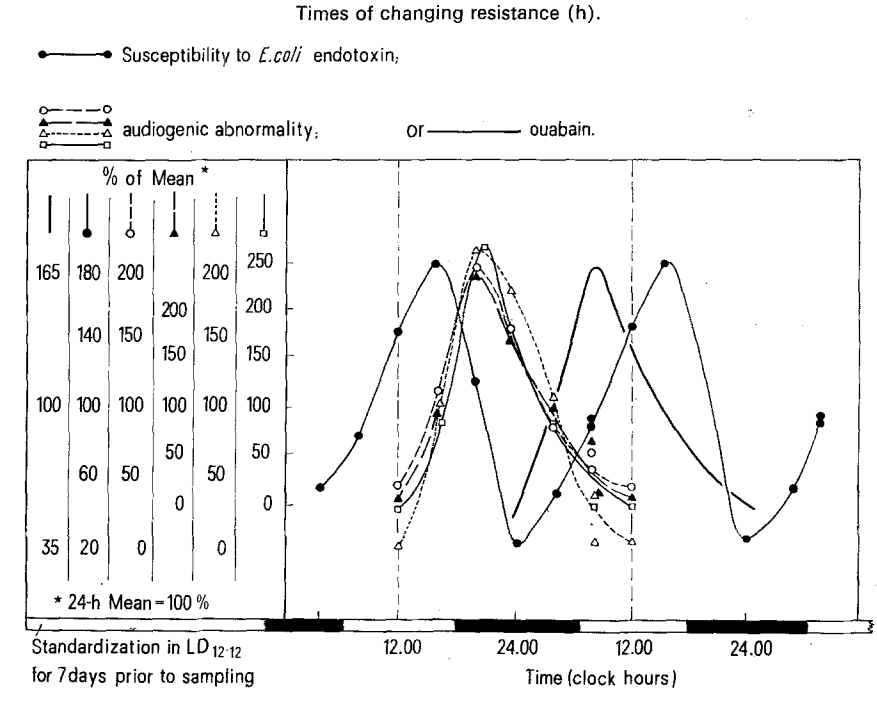



Fig. 13. Times of changing resistance (h): Hours of changing resistance in mice kept on $LD_{12:12}$ lighting regimen. Mortality from identical insult varies as function of circadian system phase at time of exposure. Timing of susceptibility-resistance cycles is characteristic for each noxious agent examined (cf. also[77]).

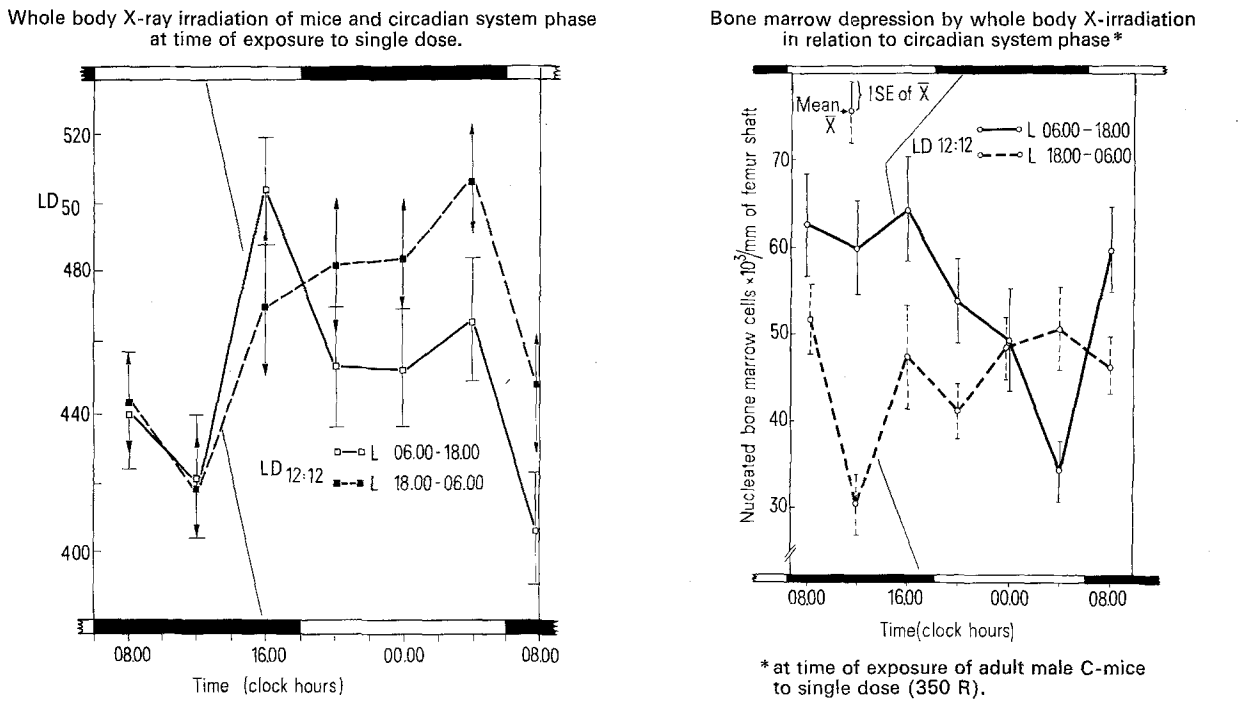



Fig. 14. a) Whole body X-ray irradiation of mice and circadian system phase at time of exposure to single dose. b) Bone marrow depression by whole body X-irradiation in relation to circadian system phase at time of exposure of adult male C-mice to single dose (350 r). Circadian radiosensitivity rhythm of mice after standardization for 14 days on 2 lighting regimens differing 180° in phase. $LD_{50}$ in roentgens. Data obtained in 1958 in the Chronobiology Laboratories, University of Minnesota. Circadian rhythm in $LD_{50}$ (a) and bone marrow depression (b). A remarkable circadian change in radiosensitivity also characterizes the Chinese hamster[81].

in keeping with the rhythms of a given host and those of his tumor. As monitor or reference variables, host hematology and body core temperature as well as tumor radiotracer uptake[30] and/or tumor temperature[31,58] may be explored.

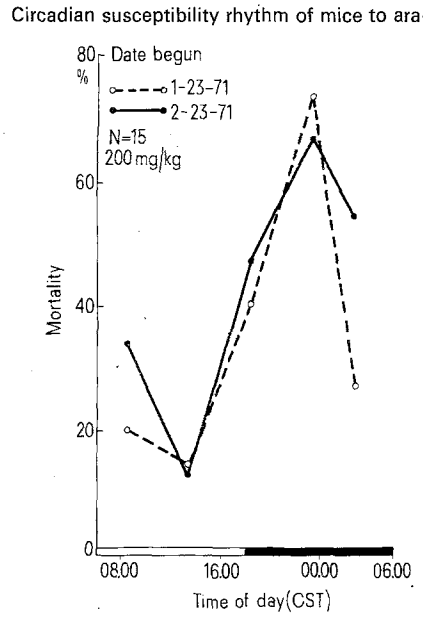



Fig. 15. Circadian susceptibility rhythm of mice to ara-C. Circadian susceptibility rhythm of $BDF_1$ mice to arabinosyl cytosine, given on 5 consecutive days at single defined circadian system phase ((see text).

It is encouraging that, taking into account only host resistance to the drug's toxicity (but as yet ignoring timing according to any probable periodicity of the tumor), findings in current studies[20], and the Figure 16 results here noted for the first time, show a clear advantage from rhythm-adjusted sinusoidal therapy (cf. also Figure 18). Eventually, when any periodicities of the tumor have been mapped and fully exploited, the advantage already known to be derived from information on periodic host factors will be yet further augmented for the patient's benefit.

This line of thought may apply to all drugs or agents which, when given in the same single dose at different times to different (groups of) individuals, yield a time-varying response[3].

## VII. *Follow-up observations on earlier study*[20]

Apart from the new results in Figures 16 and 18, the follow-up of an earlier study[20] indeed is encouraging if it can be generalized.

[55] S. S. CARDOSO, J. G. SOWELL, P. J. GOODRUM and K. FUSTE, Proc. Soc. exp. Biol. Med. *140*, 1235 (1972).

[56] S. S. CARDOSO, C. M. BLATTEIS, F. G. FUSTE and H. P. MORRIS, Abstracts of the 5th Int. Congress on Pharmacology, San Francisco, July 1972, in press.

[57] S. S. CARDOSO, Abstracts of the 4th Int. Congress on Pharmacology, Basel, Switzerland 1969, p. 487.

[58] C. M. MANSFIELD, G. D. DODD, J. D. WALLACE, S. KRAMER and R. F. CURLEY, Radiology *91*, 673 (1968).

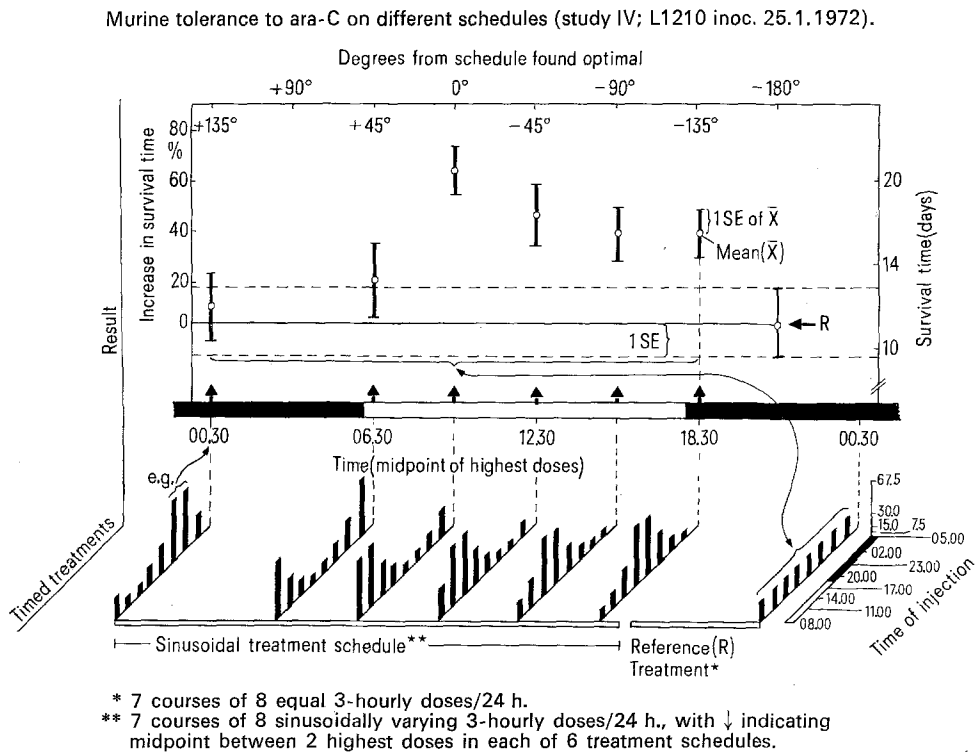



Fig. 16. Murine tolerance to ara-C on different schedules. Study IV (L 1210; inoc. 1.26.72). * 7 courses of 8 equal 3-h doses/24 h. ** 7 courses of 8 sinosoidally varying 3-h doses/24 h with ↑ indicating midpoint between 2 highest doses in each of 6 treatment chedules. Survival time of leukemic $BDF_1$ mice on different drug administration schedules (top) and timing of doses of ara-C (bottom) in sinusoidal and reference schedule consisting of 4 courses of 240 mg/kg/24 h each. When the same total dose of ara-C is given, certain sinusoidal drug administration schedules are definitely better tolerated by the mice than are other sinusoids or a currently conventional reference treatment of eight equal doses over a 24-hour span.

Arrows in Figures 10 and 18 denote daily courses (i.e., a series of 8 doses at 3-hour intervals). In each $R_x$ series of Figure 10, the sinusoidal doses are increased and then decreased at the 8 consecutive time points, as modeled more generally in Figure 11. The $R_x$ series as a whole is repeated every 4th day on 4 occasions, as shown by the arrows in Figure 10. The total sinusoidal dose/day is so chosen as to be equivalent in terms of total load (Figure 11), or in the case of ara-C in terms of total dose (Figure 10), to the total dose per day of the time-invariant schedule (240 mg/kg/24 h in both treatment schedules). Thus, in each of 3 separate experiments, the time course of each series of 8 injections at the 30 mg/kg average dose level, as shown in Figure 10, was repeated every 4 days for a total of 4 consecutive $R_x$ series. Pooling all results, one finds statistically significant differences in survival time in the anticipated direction, i.e., in favor of the sinusoid, Figure 10. The prediction from earlier work[8] according to the Figure 11 model thus holds to a reasonable extent.

However, these results of an actual test situation are less than optimal; in the first experiments comparing effects of a reference treatment with those of a sinusoidal drug administration, summarized in Figure 10, there are heavy losses from both treatment regimens. Moreover, statistically significant differences in number of survivors also are transient. The survival-time difference is the only long-term index to be taken at face value (see experiment on the right in Figure 10). These results suggest that much work remains to be done before we realize the goals shown at the right in Figure 11 and at the bottom in Figure 12. There one is led to expect – as a result of optimally timed drug administration – a response that does not approach the lethal effect and preferably does not even constitute a risk. Whereas both $ED_{50}$-and $LD_{50}$-work remains to be done, the possibility of a time-dose interaction must be considered in the light of a recent precedent ([19] and unpublished data by J. KÜHL et al. on ara-C).

In the data on hand, one can turn only to an inferential statistical evaluation of mortality. The difference in actual percentage of survivors tested by FISHER's exact test is not significant at the 5% level in Study 1 (Figure 10) based upon only 10 mice per group. The average survival time in this same study, however, is much longer with the sinusoidal than with the reference treatment: it differs at the 2% level of statistical significance and corresponds to an average prolongation of life by over 12 'leukemic mouse-days'. Although we are dealing herein with measures of lesser toxicity rather than of cure, this circumstance should not lead to an undervaluation of the results. Toxicity is the major problem in treating cancer patients today.

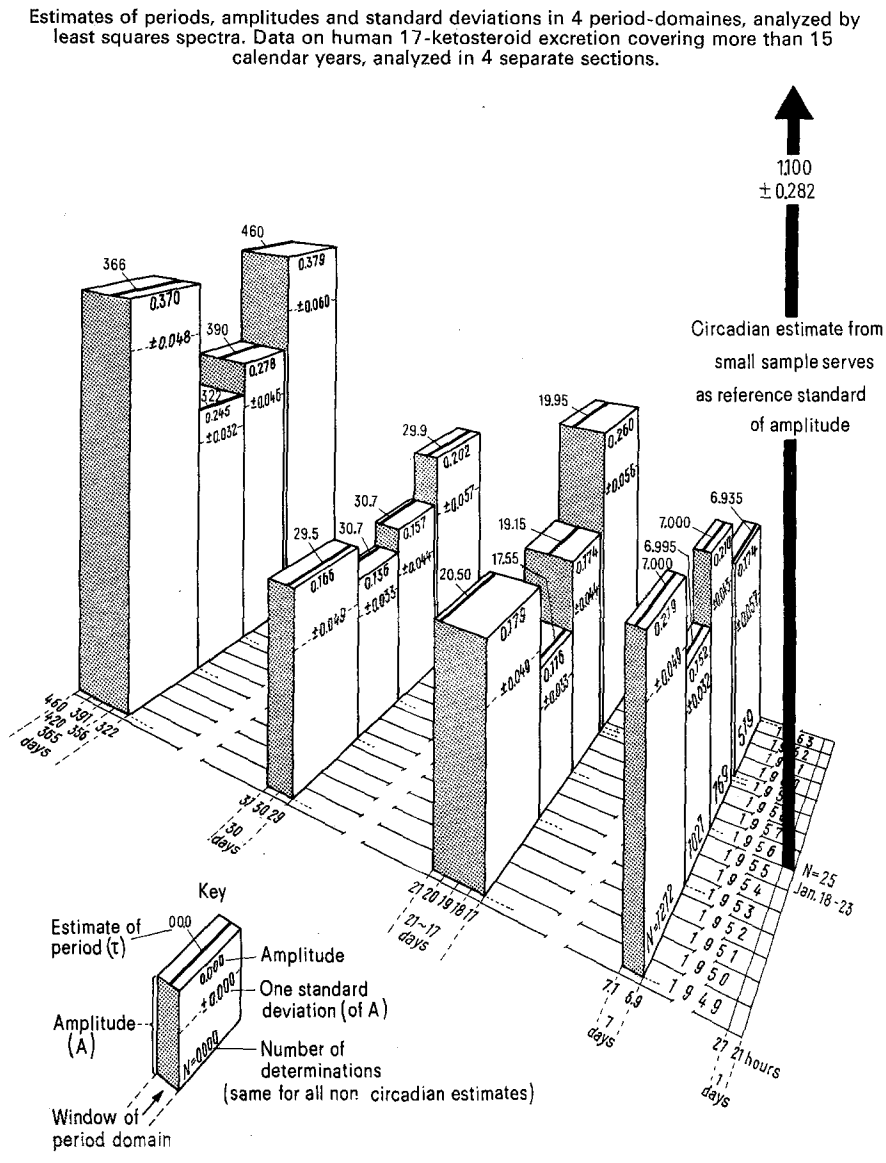
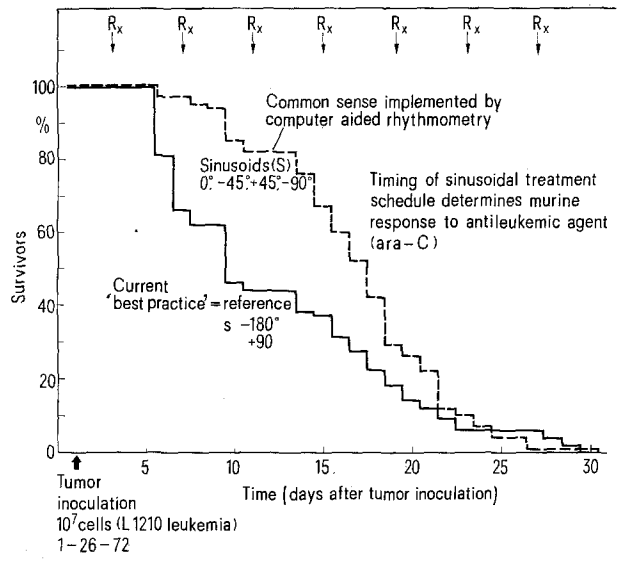



Fig. 17. Estimates of periods, amplitudes and standard deviations in 4 period domains, analyzed by least squares spectra. Data on human 17-ketosteroid excretion covering more than 15 calendar years, analyzed in 4 separate sections. Frequency spectrum of urinary 17-ketosteroid excretion in a clinically healthy man measured over 15 calendar years. A number of low-frequency changes characterize reproducibly this one-and the-same biologic variable (and other body functions) (cf.[78,79]).

Fig. 18. a) Survival of leukemic mice on different schedules of treatment with arabinosyl cytosine. Pooled results from differently timed sinusoidal schedules vs. results from reference schedule (study IV). Timing of sinusoidal treatment schedule determines murine response to antileukemic agent (ara-C). $R_x$, treatment day with reference schedule (8 equal 3-h doses/24 h) or sinusoidal schedule (8 sinusoidally-varying 3-h doses/24 h). b) Survival of leukemic mice on different schedules of treatment with ara-C. Reference schedule and differently-timed sinusoidal schedules (S). With respect to anticancer treatment timing of sinusoid determines murine tolerance to antileukemic agent (ara-C). * 0°S, anticipated 'optimal' sinusoid adjusted to tested rhythm in susceptibility of mice to ara-C. Timing of other S indicated in relation to 0°S, with 360° = 24 h. $R_x$, treatment-day with reference schedule (8 equal 3-h doses/24 h) or S (8 sinusoidally-varying 3-h doses/24 h). Prolongation of survival of leukemic $BDF_1$ mice by ara-C treatment timed according to susceptibility-resistance cycle as compared with currently recommended schedule ([22] disregarding circadian changes in host resistance). Time course of results summarized in Figure 16 [showing on top degrees for designating sinusoids as a function of their time relation to the optimal sinusoid (= 0°), with 15° = 1 h (since 24 h = 360°)]. Note that around days 9–11, when all of untreated controls are dead (not shown), less than half of the mice treated as is now conventional and mice given the 'badly timed' sinusoids survive; by contrast, fewer than 20% of the mice receiving the appropriately timed sinusoids have died by days 9 to 11 (a). Detailed data for each group also are given (b).

a) Pool

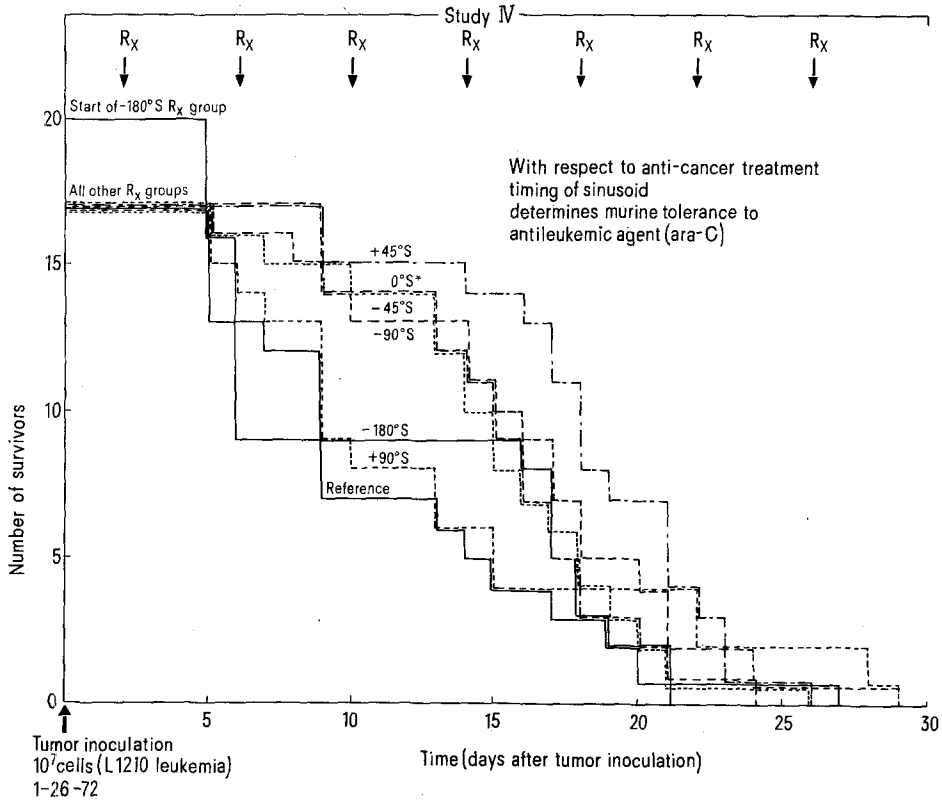
Survival of leukemic mice on different schedules of treatment with arabinosyl cytosine. Pooled results from differently-timed sinusoidal schedules vs. results from reference schedule (study IV).




$R_x$ = treatment-day with reference schedule (8 equal 3-hourly doses/24 h) or sinusoidal schedule (8 sinusoidally-varying 3-hourly doses/24 h).

b) Separate $R_x$ groups (pooled above)

Survival of leukemic mice on different schedules of treatment with ara-C. Reference schedule and differently-timed sinuoidal schedules (S).




* O°S = anticipated 'optimal'-sinusoid (S) adjusted to tested rhythm in susceptibility of mice to ara-C. Timing of other S indicated in relation to 0 °S, with 360 ° = 24 h.

$R_x$ = treatment-day with reference schedule (8 equal 3-h doses/24 h) or S (8 sinusoidally-varying 3-h doses/24h).

Phase-shift of circadian rhythm in mitoses of mouse cornea by arabinosyl cytosine (ara-C).

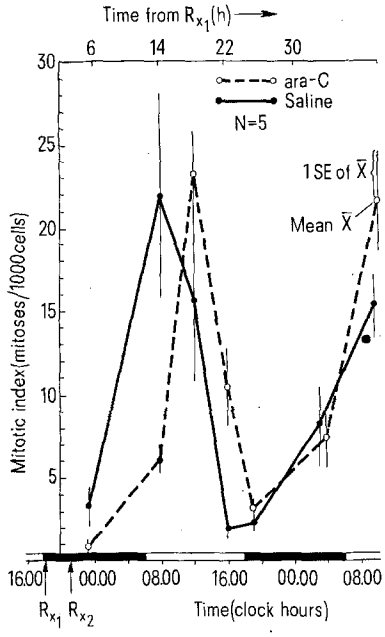



Fig. 19. Phase-shift of circadian rhythm in murine corneal mitoses by arabinosyl cytosine (ara-C). Arabinosyl-cytosine-induced phase-shift of circadian rhythm in corneal mitoses of the mouse. 2.6 mg/kg ara-C given at 18.00 and 21.00 h. Begin of sampling 2 h after latter injection[27].

Phase-shift of circadian rhythm in mitosis of male rat cornea by arabinosyl cytosine (ara-C).

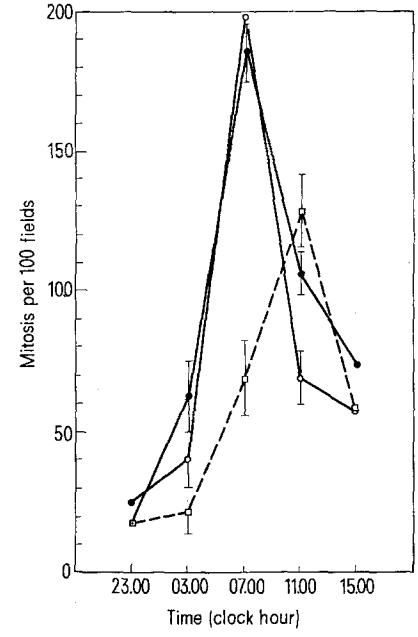



Fig. 20. Phase-shift of circadian rhythm in mitosis of male rat cornea by arabinosyl cytosine (ara-C). Arabinosyl-cytosine-induced phase-shift of circadian rhythm in corneal mitoses of the rat[42].

It is difficult and admittedly dangerous to extrapolate from the life span of a mouse to that of a man. If the life span of a mouse be 2 years and that of a man 70, a ratio of 1 to 35 results (for comparing results in mice to those in man). In a summary of all available data collected at Minnesota to date, the gain in tolerance from an S-schedule equals 15.59 mouse days. This suggests – tentatively, to be sure – a gain of 543 human days of tolerance from a 'sinusoidal' schedule as compared to the conventional one.

## VIII. *Discussion.*

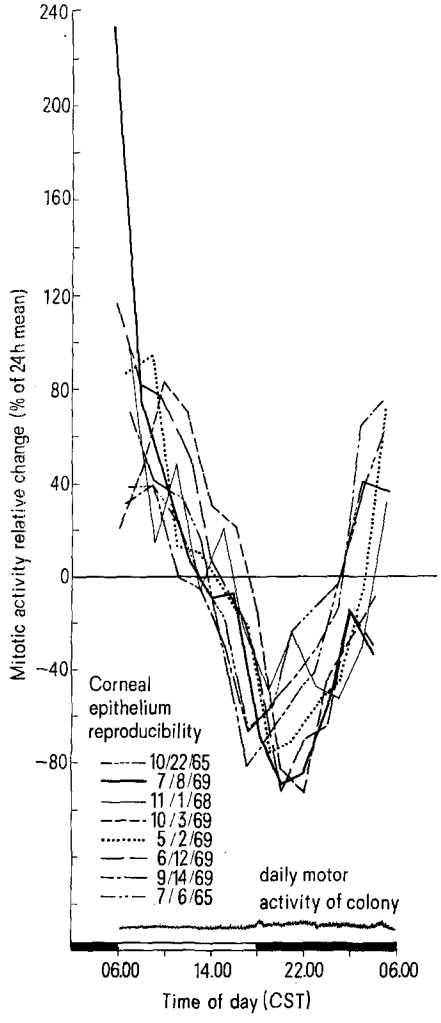
With respect to the schemes in the abstract Figures 11 and 12, the question may be raised whether the organism's rhythms are obliterated in the case of a time-invariant response. Certainly, this is *not* implied by the equal height of a set of downward-pointing columns in Figures 11 and 12. The purpose of these abstract schemes is simply to indicate that if the impact of a drug or other load is to be similar at

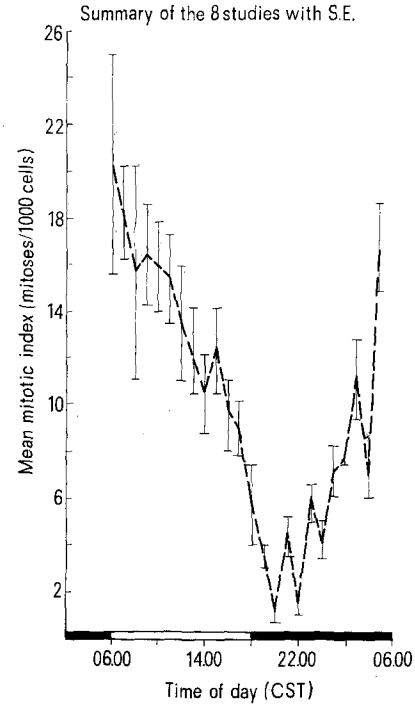
→

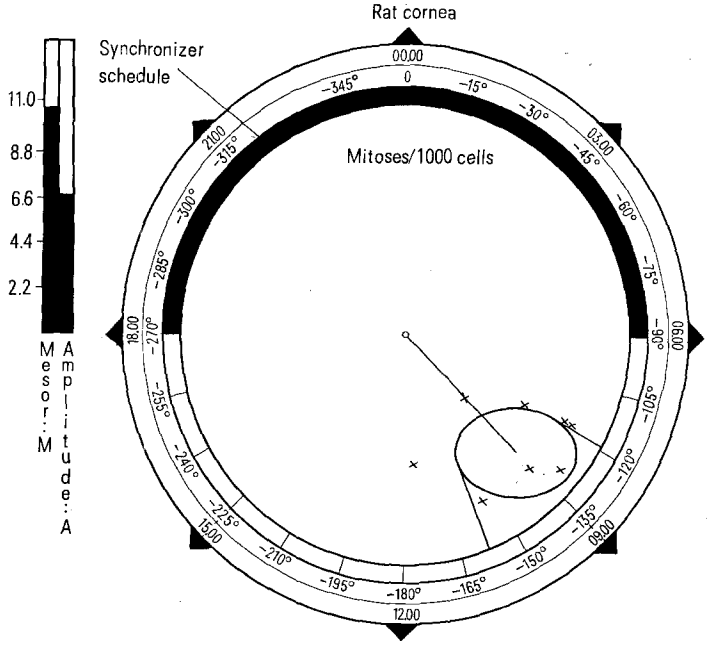
| Date | N | P | SE/A | M ± SE | A ± SE | ø (0.95 CI) |
|---|---|---|---|---|---|---|
| L06-18D18-06 | | | | | | |
| 07-06-65 | 10 | 0.01 | 0.09 | 14.90 ± 0.63 | 9.81 ± 0.092 | −138 (−128 to −149) |
| 09-14-65 | 14 | 0.01 | 0.10 | 12.48 ± 0.55 | 7.72 ± 0.101 | −119 (−108 to −131) |
| 10-22-65 | 8 | 0.01 | 0.15 | 11.61 ± 0.63 | 5.64 ± 0.158 | −179 (−161 to −197) |
| 11-01-68 | 7 | 0.01 | 0.26 | 7.56 ± 0.68 | 3.79 ± 0.252 | −140 (−111 to −168) |
| 05-02-69 | 8 | 0.01 | 0.12 | 7.46 ± 0.51 | 5.58 ± 0.129 | −125 (−110 to −140) |
| 06-12-69 | 8 | 0.01 | 0.14 | 9.13 ± 0.75 | 7.61 ± 0.139 | −135 (−119 to −151) |
| 07-08-69 | 8 | 0.02 | 0.28 | 8.59 ± 1.55 | 7.96 ± 0.276 | −120 (− 89 to −151) |
| 10-03-69 | 8 | 0.01 | 0.10 | 10.41 ± 0.52 | 7.57 ± 0.096 | −155 (−146 to −166) |

Acrophase reference = local midnight.

Fig. 21. Reproducibility of circadian rhythm in corneal mitoses of rats.



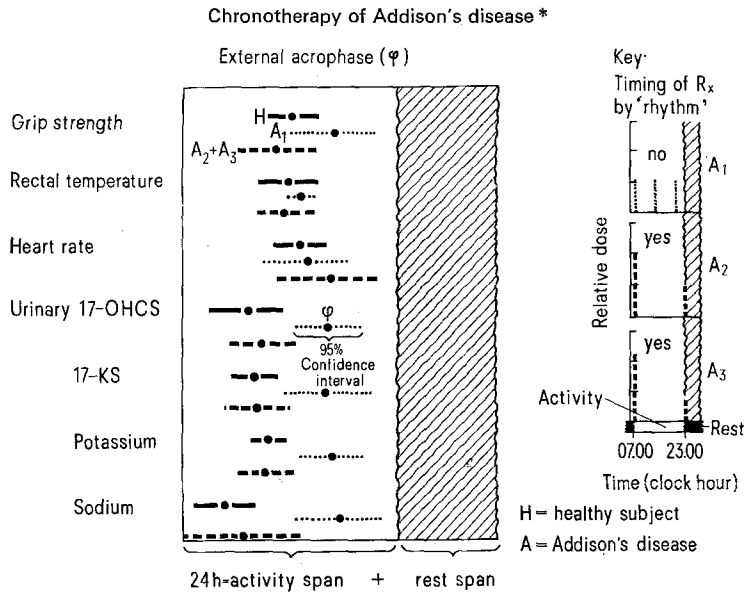




$P < 0.01$

No. of series in set = 9.
Set acrophase (0.95CI) = 136° (−119°, −157°).
Set amplitude (0.95CI) = 6.82 (4.81, 8.83).
Set mesor (0.95CI) = 10.56 (5.47, 15.66).

CI, confidence interval.




* Treatment timed according to 'rhythm' ($A_2$ or $A_3$) renders certain acrophases in patients coincident with those of healthy subjects. Treatment ($R_x$): Cortisol 25–35 mg/24 h, with or without 9α-fluorocortisol, 50 μg/24 h.

Fig. 22. Chronotherapy of Addison's disease. Treatment timed according to rhythm ($A_2$ or $A_3$) renders certain acrophases in patients coincident with those of healthy subjects. Treatment ($R_x$): Cortisol, 25–35 mg/24 h, with or without 9α-fluorocortisol, 50 μg/24 h. In the case of Addison's disease, substitution treatment timed according to rhythms influences the circadian acrophase of grip strength among other functions and serves to bring about best performance when it is most needed – e.g., optimal grip strength during the working hours.

different times, the impacting agent should be given in a time-varying fashion. It is a well-documented fact that various impacts exerted upon an organism affect a rhythmic system rather than an imaginary horizontal baseline[59]. Thus, a similar impact by an identical load repeatedly administered to influence a periodic function will be compatible with the continuance of a rhythmic change; if the impact affects only the rhythm-determined average or mesor, the organism will exhibit changes similar to those taking place in the absence of impacts except for the different mesor (and perhaps, a change in amplitude proportional to that in mesor).

For instance, an agent, therapeutic or other, may lower body temperature[44,60], deplete the bone marrow[17,18], or more generally, remove cells or other agents essential to defense: in different circadian system phases, this effect upon body temperature[61] or upon some other variable may differ drastically in kind or at least in extent. Nonetheless, the host rhythms could continue unchanged around a lower mesor, if appropriately differing (rather than identical) doses of a load can be imposed in different circadian phases. The situation becomes more complex when the drug or other load affects amplitude, acrophase, and/or period, as well as mesor, each time it is given – and yet more so if these effects upon rhythm parameters also are time-varying. Cumulative effects, as noted earlier, constitute a further complication, particularly the cumulation of effects within each given series of loads. Cumulation of effects from one 24-hour $R_x$ series to the next becomes apparent when one considers only the effect of the drug itself. Moreover, the first course of drug may phase-shift the circadian system even at the cellular level and this effect may cumulate as well.

In Figures 19 and 20 we demonstrate, for mice and rats respectively, such an effect of ara-C on the timing of mitotic activity. The cornea is chosen as host tissue to be studied in view of its accessibility and its prominent as well as reproducible rhythm (Figure 21). Figure 21 shows on the left how the average curves for mitotic activity agree from one study to the next, whether they are presented as such for each experiment or summarized in an overall grand mean as indicated in the middle of Figure 21. A microscopic analysis of such data is given on the upper left of the figure, with a cosinor summary on the right. The extent of reproducibility indeed warrants the choice of the tissue, but its pertinence as a guide of host behavior in chronotherapy will have to await correlations with the behavior of bone marrow and intestine as well as other tissues, under a variety of clinically relevant conditions.

To turn back to Figure 19, revealing the ara-C induced phase-shift of corneal mitoses, 40 $BDF_1$ mice, standardized in light from 06.00–18.00 h alternating with darkness, were injected i.p. with 2.6 mg of ara-C in 0.2 ml of saline. Another 40 mice were injected only with 0.2 ml of saline. Injection of both ara-C and saline was made twice on the same day, at 18.00 and 21.00 h. Two h after the latter injection, subgroups of 5 mice

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from the control and experimental groups were killed at the intervals indicated on the graph. Corneas were removed and evaluated for mitotic rate. The mitotic rate of the corneal epithelium in saline-injected animals showed the expected circadian rhythm, with a timing corresponding to that found previously in similarly standardized animals. In the ara-C injected animals, however, a phase shift of about 5 h becomes apparent on the 1st day after the administration of the drug. On the following day (the 2nd day after treatment), the usual phase relationship between the mitotic rhythm in the ara-C treated animals (as well as the saline-injected mice) and the synchronizer cycle of alternating light-darkness seems to have been restored.

The rationale for performing the experiment dealing with the effect of ara-C on mitotic rate in the corneal epithelium stemmed from a series of earlier experiments by two of us (L.E.S. and J.E.P.)[62]. It had been found that if ara-C was injected during the circadian system phase in which the number of corneal mitoses usually is highest, no statistically significant decrease in mitotic count could be detected and on the next day the mitotic peak occurred at the expected time. However, if the same dose of ara-C was injected at the circadian system phase when the corneal mitotic rate usually is low, there was a highly significant decrease in mitotic rate the next day at the time of the expected circadian high.

The question asked was whether this apparent decrease represented a phase shift in the rhythm, or an actual depression in mitotic rate? From an examination of Figures 19 and 20 it seems that the results obtained were due to a phase shift by about 5 h in the temporal placement of the mitotic rhythm along the 24-hour scale; consequently, single-time-point sampling was misleading because it suggested a depression of mitosis when actually a phase-shift had taken place. We recognize, of course, that ara-C can depress mitosis in the corneal epithelium if it is given in sufficiently high doses. However, the results presented in the composite figure above do clearly point out the complexity of evaluating the effect of a drug on mitotic activity and demonstrate as well the pitfalls awaiting those investigators who ignore the organism's time structure in circadian and other frequency ranges. These results from ongoing chronobiologic studies relate to the cure of leukemia in mice by enabling us to improve the therapeutic ratio: their relation to chrono-chemotherapy in general can also be suggested.

In any event, when we deal with a comparison of survival times rather than mitoses, we can affirm that a given series of doses kills many animals rapidly when given in equal amounts at fixed intervals, while the same total daily amount is associated with lower risk (longer survival) when the schedule of drug administration is adjusted to the body's rhythms (Figure 18).

The 3 experiments summarized in Figure 10 as a follow-up on an evaluation made before completion of study[20], and the new results in Figure 16, show unambiguously that a periodicity-adjusted treatment does increase tolerance to high doses of ara-C; they emphasize for the basic scientist the dire need to complement information on the classical cell cycle of the leukemia (Figure 4 above)[63] – considered usually only in terms of the length of its stages – with information on the actual timing along the 24-hour scale of a circadian cell cycle that becomes usable only when it is well-mapped. For the clinician as well, a circadian rhythm-adjusted treatment may compare most favorably with the now-conventional 'optimal' schedules of treatment.

In many other experimental situations, rhythmometry as a basis for chronotherapy promises to serve the maintenance of human health. Chronotherapy already has been advocated in the case of Addison's disease[12] (Figure 22); our results show promise that it may well be extended to the treatment of cancer, among other conditions. Modern rhythmometry could add substantially to the success of cancer radiotherapy[13–18] as well as chemotherapy. Indeed, without rhythmometry, a valuable drug may be considered to have an unacceptable toxic-therapeutic ratio because features of periodicity other than the overall operating mesor of the circadian cell cycle were not taken into account.

Recently, on the presumption that at all clock hours some cells may be found dividing, i.e., tacitly assuming that rhythms can be ignored without harm to the patient, others have chosen for their treatment-mode continuous infusions of 24 or 48 hours duration (64, cf.[29]). This practice represents a complete lack of concern for physiologic timing (Figure 17), insofar as it ignores a system of rhythms with different frequencies that characterize agents known to influence mitoses, such as epinephrine and norepinephrine[65]. Physiologic timing requires attention to the time span during which an agent continues to act; irrespective of clock hour, one endeavors to expose continuously to ara-C those cells that are actively proliferating and might be more sensitive to the therapeutic agent at the stage of DNA turnover (S) or in the stage of mitosis (M). However, if there be circadian periodicity, one should provide a higher drug concentration when host cells are in the resistant stages and tumor cells in the sensitive stages of their circadian cycle, instead of giving a fixed and high drug concentration continuously without

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considering rhythms in either the desired and undesired effect.

Continuous drug administration by infusion is desirable only if throughout the day the S and M stages in both host and tumor are continuously and randomly represented. While this may be so for many tumors, certain human breast cancers[32] and 2 multiple myelomas[52] are now known to constitute exceptions in that they exhibit circadian mitotic or protein excretory rhythms, respectively. Moreover, low-frequency rhythms, as well as circadian ones[53], also have been reported for human leukemia[66], and ultradian rhythms for a mouse Ehrlich ascites tumor[29].

The host-tolerance rhythms reported herein present further experimental evidence for the merits of chronotherapy. The time thus has come to consider timing in ways that take into account the important difference between single doses and infusions. The latter consideration was the subject of a clinical 'dose schedule and antitumor' study on 88 patients with metastatic cancer[64]. When single doses of a potential chemotherapeutic agent were tested in this large scale cooperative study, anti-tumor effects were not noted. By contrast, continuous infusion of the same agent for spans of 24 or 48 to 96 h reportedly resulted in tumor regression in 10 or in 18 to 38% of the cases, respectively. Since scheduling brought about some therapeutic effect yet not a 'particularly impressive' response rate, the authors condensed two extensions of their study: a) the use of 24-hour infusions at intervals shorter than 2 weeks, such infusions at 4-day intervals being 'optimal in experimental systems...'; and b) 'the use of continuous infusion for 8 to 10 days, particularly in patients with adenocarcinoma of the gastrointestinal tract'.

With regard to infusion or related modes of treatment, a third possibility, mentioned earlier[29], also should be considered. It seems reasonable that one might further improve a therapeutic index by taking advantage of the possible occurrence of circadian and/or ultradian rhythms in the susceptibility of the tumor, in the desired effect, and/or in the host's susceptibility to side effects such as bone marrow depression. For the patient treated by continuous infusion, the lengths of infusion spans and the intervals between consecutive spans may well be adjusted to physiopathologic periodicities. Thus, when an infusion span is of one or several days' duration, the chemotherapeutic agent might be infused according to circadian rhythms, ultradian rhythms, and/or rhythms with lower frequency[48], rather than at a steady rate.

It was suggested earlier that a tumor's response to irradiation might be enhanced by gauging its uptake of $P_{32}$ and then timing treatment according to the ultradian rhythm thus identified[30,67]. By the same token, any circadian rhythm that occurs in the tumor itself may form the basis for timed treatment[29,58]. Such considerations should now be combined with close attention to circadian changes in the sensitivity of structures that underlie undesirable effects such as bone marrow suppression, in order to gain further benefit for the patient.

With the use of maximally tolerated toxic levels of carcinostatic agents – a 'dare-devil' type of approach – the therapeutic effectiveness of cancer chemotherapy becomes critically dependent on narrow differences in response between normal and neoplastic cells. Even slight gains in the therapeutic indices of these agents could conceivably lead to substantially improved treatment schedules and therapeutic results. Therefore, the role of drug metabolism as a determining factor in drug toxicity is of perhaps greater importance in cancer chemotherapy than in other areas of therapeutics. Rates of drug metabolism have received appropriately prime consideration in the design of drug-administration schedules[68,69]. Genetic and environmental factors are known to influence rates of drug metabolism[70]. Yet the influence on drug toxicity of circadian changes in rate of drug metabolism – likewise dependent upon both genetics and environment – has largely been neglected.

Considering the rather significant circadian differences in certain rates of drug metabolism[71], the time-independence of blood and tissue half-lives for carcinostatic agents awaits further scrutiny. Future preclinical and clinical studies should consider the occurrence of rhythmic variations with several frequencies in drug metabolism for the design of possibly more efficient drug-administration schedules[20].

The timing of treatment may perhaps be further improved by attempts to phase-shift the pertinent rhythms through scheduling as well as through chemotherapy. Such attempts may be particularly indicated when similar if not coincident maxima are apparent for circadian and/or ultradian rhythms in both desired and undesired effects of the drug upon tumor and host. In such instances the possibility remains that the underlying rhythms are only loosely coupled at the circadian and/or ultradian frequency; if so, the shift-times of these rhythms will differ and that difference can perhaps be exploited.

One may then wish to induce artificially a transient state of desynchronization among certain rhythmic variables of tumor and host that are related, perhaps

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critically, to the effects of the drug. Thus one may search for a synchronizer or set of synchronizers[28] whereby one can induce, inter alia, 1. a phase-shift of the host's rhythms without affecting those of the tumor, or 2. a shift of host rhythms in a direction, or at least at a rate, different from any change in tumor rhythm characteristics[55–57,72,73]. Such differential phase-shifting in man is no longer utopian: phase-shifts can be induced by rest or meal timing or drugs, in man as well as in the experimental animal[72,74]. The pertinent details of a phase-shift must be singled out and reliably quantified. Thereafter, one can institute phase-shifts in attempts to cut down the undesired effects as well as to enhance the desired ones whenever the ultradian and circadian peaks in desired and undesired effects coincide.

Circadian considerations seem pertinent for those interested in the mammalian cell cycle. In the introduction to a review BASERGA et al.[75] state that their discussion will be limited to 'the length of the cell cycle and its 4 phases in mammalian cells, either in vivo or in vitro'. These authors conclude the same review by the statement that 'the determination of the length of the cell cycle and its phases is now part of the basic information that one wishes to obtain on any tissue under investigation'. The authors go on to say that for practical reasons as well '... X-ray therapy and chemotherapy can gain considerable advantages from a knowledge of the cell cycle. This is not just a vain hope, but a belief based on the observation that both X-rays and chemotherapeutic agents are more effective on certain phases of the cell cycle than on others. The possibility of concentrating the therapeutic efforts on the more sensitive phases are notably of interest'[75].

The results here presented suggest that one can exploit the pertinent mammalian cell cycle(s) by

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Growth hormone (STH) effect on murine hepatic mitoses (pool of 2 experiments) depends on circadian phase of injection and observation.

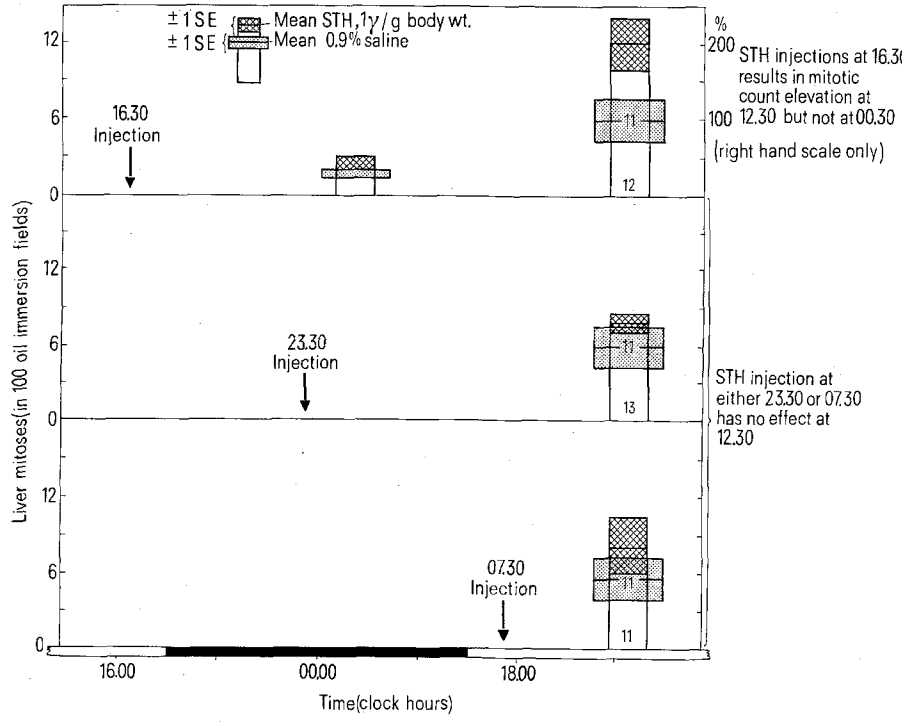


Fig. 23. Composite graph summarizing 2 separate experiments. Top row data demonstrate that the organism's circadian phase determines the detection of growth hormone stimulation of cell division in liver parenchyma of growing mice. The effect can be detected at the time of usual mitotic high (mid-light span on a regimen of 12 h of light alternating with 12 h of darkness) but not at the time of the usual low in mitotic count. This experiment involved a series of 3 injections at the fixed time shown by the arrow in the top row; results are graphed only as per cent change from values of saline-treated controls killed concomitantly (right-hand sclae only). In another study, the effect of injection at 3 different circadian times (with killing at the 'right time for detection of effect') is investigated (all 3 rows of graph; left-hand scale only). This study is ambiguous in that replicate injections will be needed at different circadian times before the possibility that time from injection may play a role has been ruled out or ruled in. Moreover, a single killing time does not suffice to resolve a phase-shift of mitotic rhythm, demonstrated for another agent in Figures 19 and 20. However, a phase-shift may not only simulate mitotic depression when none occurs but it also may spuriously indicate a lack of effect when such an effect does indeed take place. With such qualifying restrictions it may be pointed out (until proof is offered to the contrary) that the organism's circadian injection time may determine also the occurrence or non-occurrence of growth hormone stimulation of cell division in mouse liver parenchyma.

manipulating synchronizers of rhythms. In the case of a 24-hour environmental synchronizer, one hopes to assess the cell cycle's timing by a monitored pertinent function – possibly serum iron for the case of bone marrow cycles. Already we know that certain host rhythms such as the circadian change in serum iron persist in patients with multiple myeloma[50,76]. Patient monitoring is indispensable when circadian rhythms in host and/or tumor, including the cell cycle, are not of precise 24-hour duration. Unless such rhythms can be 24-hour-synchronized, they cannot be described in terms of events anticipated for the same fixed clock hours on any one day and all days. Yet more recent papers on the 'cell cycle'[75,77] continue to discuss cycle length and related problems without reference to predictable changes identified as to timing in the 24-hour period. From a practical viewpoint, however, it seems important to predict that certain cell-cycle events will occur at identifiable clock hours. The achievement of this essential goal seems to be possible by means of rhythmometry, as results here documented suggest; the approach is likely to be yet more successful to the extent that one can resolve before beginning treatment all of the pertinent cellular and superimposed cycles with several frequencies in host and tumor.

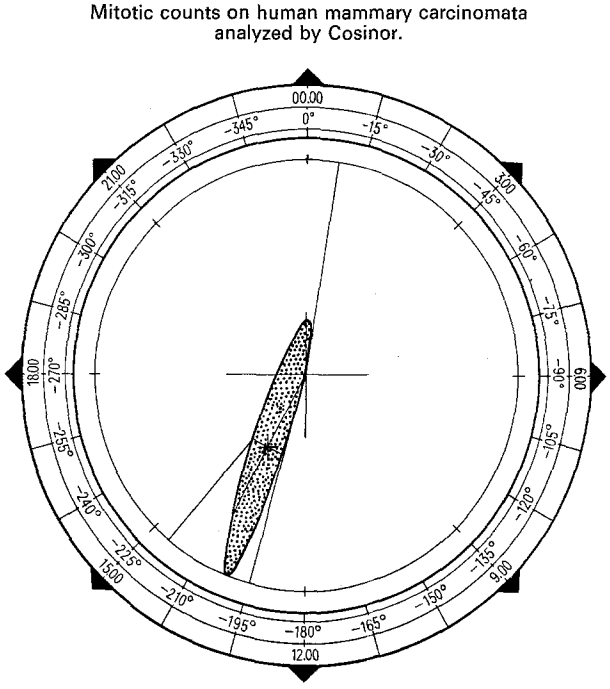


Fig. 24. Mitotic counts on human mammary carcinomata analyzed by Cosinor. Cosinor analyses of serial mitotic counts originally published by Dr. E. TÄHTI and Dr. A. VOUTILAINEN suggest that a circadian rhythm persists in human breast cancers.

## IX. *Outlook*

Chronobiology may benefit the patient by leading to drug administration timed according to rhythms in order to take advantage of predictable states having improved toxic-therapeutic ratios. In the treatment of malignancies[78], among other conditions[3,12], there are several aspects of a chronochemotherapy and chronoradiotherapy that need to be taken into account.

Chronobiologic computer techniques for the display of rhythm characteristics, including timing – originally

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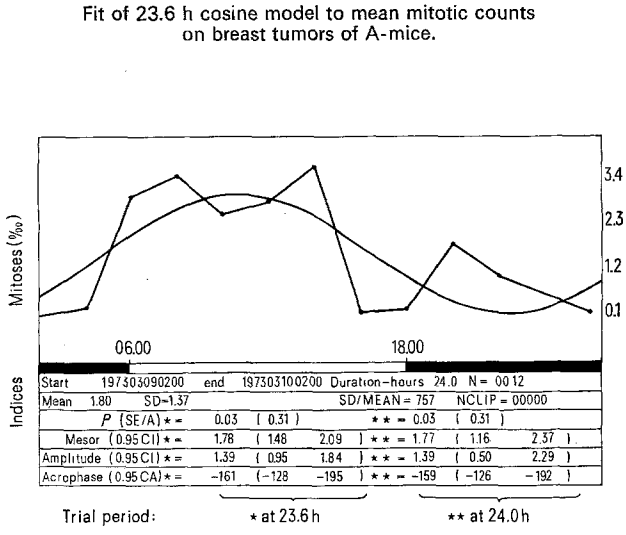


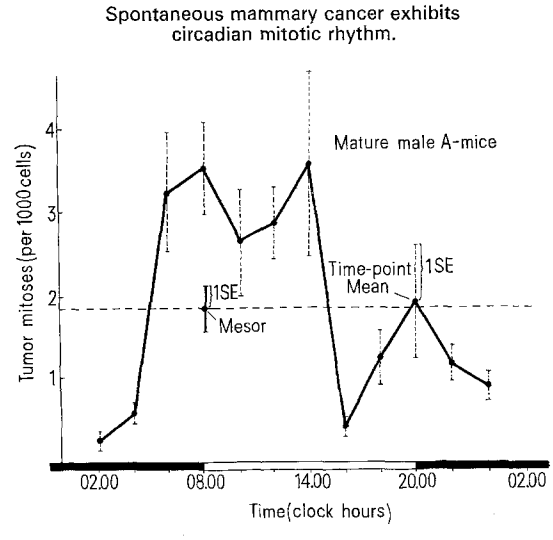


Fig. 25. a) Fit of 23.6-h Cosine model to mean mitotic counts on breast tumours of A-mice. b) Spontaneous mammary cancer exhibits circadian mitotic rhythm. Chronobiologic window summarizes circadian mitotic rhythm studied by serially independent sampling on 96 A-strain mice, carrying a spontaneous mammary carcinoma (a). Standard errors [of the means (shown also on top of Figure 25a)] in Figure 25b.

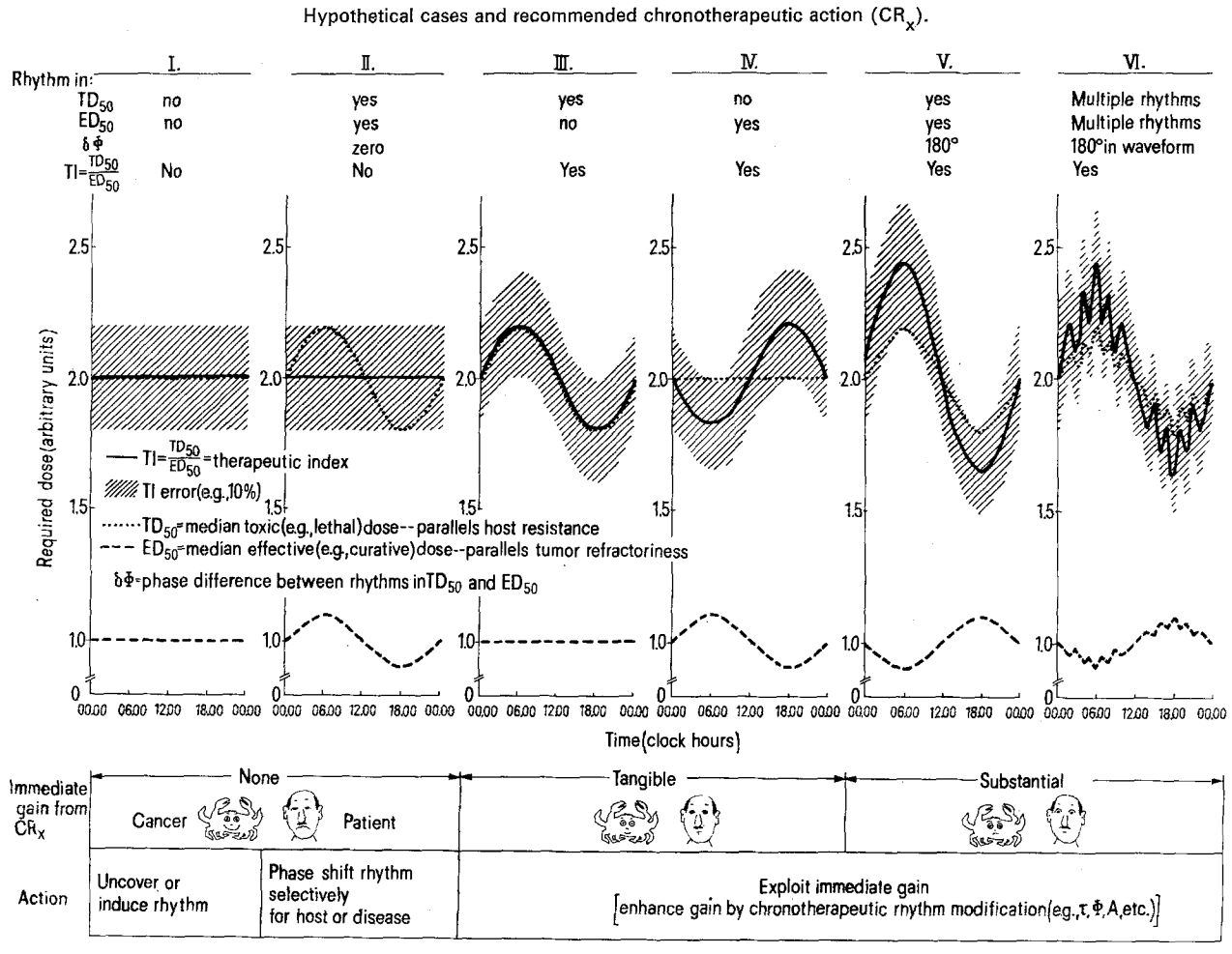


Fig. 26. Hypothetical cases and recommended chronotherapeutic action (CR). Reliance upon the susceptibility rhythms of either host or tumor promises to yield tangible improvement of cancer therapy, though reliance upon rhythms in both host and tumor is likely to yield more subtantial gains.

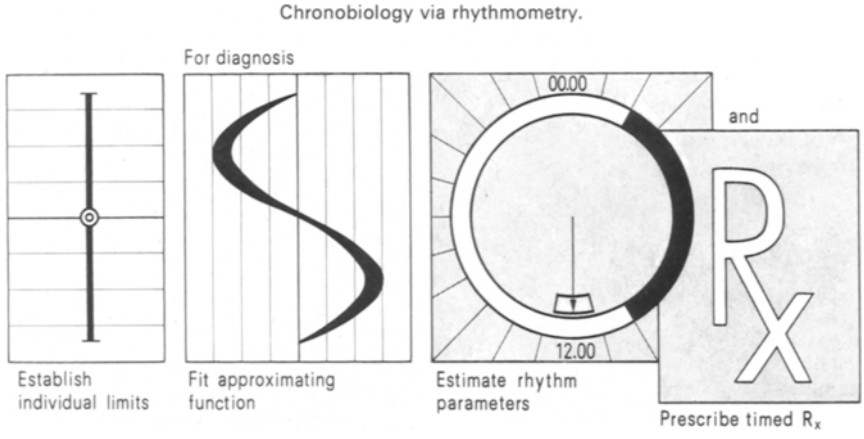


Fig. 27. Chronobiology via rhythmometry. On the path toward a chronotherapy we complement ranges of 'usual values' (left) by rhythm-qualified ranges (middle) and estimate rhythm parameters, notably timing, by cosinors or equivalent procedures (right).

developed for investigations in extraterrestrial space – are likely to serve the design of treatment schedules according to rhythms, as Figure 22 shows for the case of substitution therapy in Addison's disease.

Yet another related aspect of this discipline of chronobiology, sponsored in the USA primarily by the Space Agency, revolves around chronobiotics – drugs especially designed to manipulate rhythms and/or to treat rhythm alteration. For conventional drugs used in the treatment of cancer it can now be assumed that timing is critical; it will be yet more interesting if certain resting cells (in $G_0$) can be made to enter S and M – that is, to synthesize and divide – by hormones or drugs which are inactive unless properly timed (Figure 23).

## X. *Conclusion*

With respect to host tolerance, the implication that sinusoidal dosage schedules may markedly improve the therapeutic index [i.e., decrease the toxicity relative to the effectiveness (curative action)] of an antimetabolite, ara-C, and perhaps that of many other drugs, is inescapable. Furthermore, the increase in tolerance effectively achieved is not a trivial one; it could thus represent definitively the difference between success and failure in therapeutic application. Finally, focus upon the tumor's possible rhythms in addition to those of the host is warranted, as Figures 24 and 25 document for mammary cancer of a few women and 96 A-mice, respectively (cf. also Figure 26)[80,82].

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Multiple Rhythms in Mouse Blood Resolved in 31 Consecutive Weekly Samples with a Single-Double Cosine Model Fitted by Linear Least Squares (LLS)[a]

No. of cosine fitted: SINGLE $Y_t = M + A \cos(\varnothing + 2\pi/\tau\, t_i)$ DOUBLE $Y_t = M + A_1 \cos(\varnothing_1 + 2\pi/\tau_1\, t_i) + A_2 \cos(\varnothing_2 + 2\pi/\tau_2\, t_i)$

| Cosine model Fitted by LLS | Variable (units) | PR (%) | Mesors | Periods (h) | Amplitudes |
|---|---|---|---|---|---|
| Initial Information on Rhythms (SINGLE) | Hemoglobin (HGB) (gm/100 ml) | 20 | M = 15.98 | $\tau_1$ = 3630 [151][b] | $A_1$ = 3.40 (1.27)[c] |
| | | 25 | M = 15.98 | $\tau_2$ = 2094 [ 87] | $A_2$ = 3.87 (1.26) |
| | | 29 | M = 15.97 | $\tau_3$ = 1422 [ 59] | $A_3$ = 4.01 (1.22) |
| | Mean corpuscular volume (MCV) of Erythrocyte ($\mu cm^3$) | 27 | M = 51.73 | $\tau_1$ = 3630 [151] | $A_1$ = 9.21 (2.87) |
| | | 40 | M = 51.64 | $\tau_2$ = 1326 [ 55] | $A_2$ = 9.80 (2.32) |
| Best Resolved Rhythms (DOUBLE) | HGB Case I | 42 | M = 15.97 | $\tau_1$ = 3758 [157] | $A_1$ = 3.24 (1.16) |
| | | | | $\tau_2$ = 2057 [ 86] | $A_2$ = 3.65 (1.15) |
| | Case II | 51 | M = 15.97 | $\tau_1$ = 3681 [153] | $A_1$ = 3.55 (1.04) |
| | | | | $\tau_2$ = 1433 [ 60] | $A_2$ = 4.13 (1.15) |
| | Case III | 50 | M = 15.98 | $\tau_1$ = 2138 [ 89] | $A_1$ = 3.63 (1.08) |
| | | | | $\tau_2$ = 1393 [ 58] | $A_2$ = 3.88 (1.13) |
| | MCV | 67 | M = 51.70 | $\tau_1$ = 3681 [153] | $A_1$ = 9.20 (2.00) |
| | | | | $\tau_2$ = 1308 [ 55] | $A_2$ = 9.77 (1.75) |

When 3 cosine functions are concomitantly fitted, a PR of ~67% (from three periods near those detected by a single cosine fit) is obtained for HGB. For the same variable, a nonlinear least squares analysis has validated the finding of 3 periods.
[a] These results by J.F.S. are from controls in studies carried out to compare the effect of whole-body x-ray irradiation when it involves the exposure of mice either continually to a low-dose or to fractionated doses given in the same total dose over the same span (43).
[b] Period in days (rounded). [c] Standard error of estimate.

*Addendum in proof*

Recent results bearing on the chronotherapy of hypertension[83] were obtained on male young adult Minnesota Sprague Dawley rats, feeding ad libitum in a room with light from 06.00–18.00 alternating with darkness and at a rtmperature range of 77 ± 1°F. 6 groups of different yet comparable rats were injected 4 h apart with chlorothiazide or with a comparable volume of saline. The diuresis and saluresis in response to the drug were greater than those from saline at each of the circadian phases tested. Kaluresis similarly assessed displayed a chronotherapeutically pertinent response in that an increase associated with other injection times was not seen at 06.00 on the first day and at 02.00 and 06.00 on the second. A chronotherapeutic index (CTI) was formed from two ratios (after discussion with Dr. HEIN BESSELAAR, of Merck Co., West Point, Pennsylvania) the numerator being the ratio (relative to saline-injected controls) of chlorothiazide-induced saluresis and the denominator an equivalent ratio for potassium. The CTI's indicate the optimal compromise between tending to minimize hypokalemic side effects while tending to maximize the desired saluresis[83].

Yet more recently, the heretofore hypothetical rhythm in therapeutic index, Figure 26, has been experimentally validated by assessing Adriamycin-associated mouse breast-cancer-shrinkage and survival time augmentation[84]. 6 groups of inbred A-strain mice with spontaneous mammary cancer (and fo controls without palpable tumors, matched by strain, age and sex) feeding ad libitum in light from 06.00 to 18.00 alternating with darkness were given adriamycin i.p. at different circadian times. The numbers of survivors and caliper measurements of tumor size were recorded to find the best timing, if any, of Adriamycin in terms of reducing tumor volume and increasing survival time.

A chronotherapeutic index (CTI) was defined as CTI = PST × TVC, where PST = individual's percent survival time relative to overall mean (≡ 100%) of all treated individuals irrespective of treatment time, and TVC = individual's extrapolated 47-h post-treatment tumor volume change (a) relative to individual's extrapolated 1-h pre-treatment tumor volume (b) [expressed as ratio a/b)].

Results thus analyzed show the highest CTI (=122%) at 22.00, the second highest CTI (=104%) at 02.00 and the lowest CTI (=8%) at 18.00. A 24-h cosine model fitted by least squares showed statistical significance for the original mean survival times, for survival times expressed as percentage of over-all mean and for CTI.

*Zusammenfassung*

Bei Menschen und Versuchstieren beeinflussen periodische Veränderungen im physiologischen Zustand des Organismus die Resistenz gegenüber Nebenwirkungen von Arzneien und verschiedenen anderen, potentiell schädlichen, Umwelteinflüssen: Überleben ohne Behelligung einerseits *oder* Schaden und sogar Tod andererseits können experimentell als eine Funktion jener Phase des Zirkadiansystems dargestellt werden, in welcher ein Reiz gesetzt wird. Drastisch verschiedene Reaktionen, abhängig vom jeweiligen Stadium der Empfindlichkeitsrhythmen, kennzeichnen physikalische Reize [wie Röntgenbestrahlung oder Lärm (in hierfür empfindlichen ingezüchteten Mäusestämmen)]; ein bakterielles Endotoxin (*E. coli*); ein Herzglykosid (g-Strophanthin); ein Anästhetikum (Fluothane); andere das Nervensystem beeinflussende Mittel (wie Pentobarbital, Librium oder Acetylcholin), ein Diuretikum sowohl als auch Saluretikum (Chlorothiazid), ein Gift für pankreatische β-Zellen (Alloxan) und eine Reihe anderer Wirkstoffe[3].

Die Auswertung solcher Informationen hat eben begonnen. Schon kann in der Klinik beim Ausfall eines prominenten hormonalen Rhythmus, wie im Fall von Morbus Addison, die Substitutionsbehandlung zeitlich gezielt werden[12] – um die Periodik so zu reproduzieren, dass zu entsprechenden (etwa Arbeits-) Zeiten eine erhöhte Leistungsfähigkeit (wie beste Handkraft) erreicht wird.

Die Zeit beeinflusst auch Auswertungsverfahren im Laboratorium. Die Steigungsgerade (an und für sich) eines «Dosis-Reaktion-Verhältnisses» wird von klassischer Warte aus weitgehend als unter verschiedenen Bedingungen gleichbleibend angesehen – auch wenn sich die Lage dieser Steigungslinie entlang einer Dosis-Skala ändert. Einerseits zeigen frühere chronotoxikologische Ergebnisse, dass sich die Steigung des Dosis-Reaktion-Verhältnisses als Funktion der physiologischen Zeit entlang der Dosis-Skala verschiebt. Dies gilt für die Steigung der Reaktion zu einer ansteigenden Dosis von g-Strophantin und Endotoxin[1,3].

Andererseits kann sich als Funktion der Zirkadiansystemphase sogar die Steigung, per se, des Dosis-Reaktions-Verhältnisses ändern. Dies gilt etwa für die durch ACTH zu verschiedenen zirkadianen Zeiten sehr unterschiedlich stimulierte Corticosteronproduktion der Nebennieren, in vitro, also in der Abwesenheit neuraler und humoraler Agenten (diejenigen ausgenommen, welche zur Zeit des Aufsetzens einer Organinkubation schon im Gewebe vorhanden sind[39]). Schliesslich kann sich als Funktion der Dosis sogar die zeitliche Lage eines zirkadianen Empfindlichkeitsrhythmus ändern; dies lässt sich für die Dauer des Pentobarbital-Schlafes bei der Maus demonstrieren[53].

Solch eine Fülle von chronopharmakologischen Befunden konnte durch eine einwandfreie Versuchsanordnung erhoben werden: in all diesen Untersuchungen wurde jedes Versuchstier nur einer einmaligen Verabreichung von Testreizen und Dosen unterworfen. Als Ergänzung in der Richtung einer Chronotherapie

berichten wir hier über eine Serie von Studien an Individuen die multiple Dosen erhielten. In dieser Simulierung einer gesamten Behandlungsfolge kann gezeigt werden, dass auch ein gängiges Chemotherapieschema mit Hilfe chronobiologischer Erwägungen und Methoden noch erheblich verbessert werden kann: ein zur Zeit in der Klinik oft verschriebenes carcinostatisches Pharmakon wird viel besser vertragen wenn es zeitlich – den Rhythmen des Wirtes entsprechend – gezielt verabreicht wird.

Mäuse mit einer experimentellen Leukämie vertragen Arabinosyl Cytosin (ara-C) bei gleicher Gesamtdosis besser, wenn sie statt eines konventionellen Schemas (mit gleichen, im 3-Stunden-Intervall verabreichten, Dosen) ein den zirkadianen Rhythmen entsprechendes sinusoidales Schema (eine Folge von im 3-Stunden-Intervall graduell abfallenden und wieder ansteigenden Dosen) erhalten. Bei früher begonnenen Studien[20], die sich nach wie vor auf die Herabsetzung von Toxizität der Behandlung beschränken und die nunmehr 70, 54 and 42 Tage nach der Leukämieinokulation ausgewertet werden können, beträgt der Gewinn an Toleranz für das getestete Mittel (ara-C) im Durchschnitt 15.59 Mäusetage. Ergebnisse von Mäuseversuchen lassen sich natürlich nicht zwanglos auf die Humanmedizin übertragen. Eine jeweils noch zu erhärtende Extrapolation zum Menschen muss auch die entsprechenden durchschnittlichen Lebensdauern der beiden Spezies in Betracht ziehen. Mit solcher Einschränkung entspricht der Gewinn an Toleranz bei der Maus dem Äquivalent von 545 humanen Lebenstagen.

Hier belegen wir auch an Hand neuer Versuche den von Chronobiologen vorausgesagten Umstand, dass verschiedene sinusoidale Behandlungsarten nicht gleichwertig sind. Eine besondere zeitliche Lage der Folge von hohen und niedrigen Dosen entlang der 24-Stunden-Skala ist anderen, den Empfindlichkeitsrhythmen des Wirtes nicht angepassten Lagen des gleichen sinusoidalen Schemas (mit der gleichen Gesamtdosis) klar überlegen. Es erscheint unwahrscheinlich, dass wir es hier ausschliesslich mit einer Wirkung der Reihenfolge als solcher von hohen und niedrigen Dosen zu tun haben. Die Sequenz hoher und niedriger Dosen ist bei den verschiedenen gleichzeitig getesteten sinusoidalen Behandlungsarten die gleiche, Auch wird jede Sequenz zur gleichen zirkadianen Zeit für alle Gruppen begonnen. Nur die initiale Dosis in der sinusoidalen Sequenz wird variiert, um für verschiedene Gruppen von behandelten Tieren eine verschiedene zeitliche Lage hoher und niedriger Dosen in Bezug auf das zirkadiane System des Wirtes zu erreichen. Es erscheint wahrscheinlich, dass der letztere Faktor für die erhöhte Toleranz gewisser sinusoidaler ara-C Verabreichungssequenzen auch mit verantwortlich zeichnet.

# SPECIALIA

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## Deoxoscalarin, A Further Sesterterpene with the Unusual Tetracyclic Carbon Skeleton of Scalarin, from *Spongia officinalis*

Recently, HAFIZULLAH et al.[1] reported the occurrence in the fern, *Cheilanthes farinosa* Kaulf., of cheilanthatriol (I), which represents a new fundamental type of sesterterpene. It seems to be derived from geranylfarnesol by a cyclization initiated at the isopropylidene group, which is typical of triterpenes.

More recently, FATTORUSSO et al.[2] described scalarin (II) from the sponge, *Cacospongia scalaris*, a second sesterterpene with a carbon skeleton, which appears to be of the same origin as cheilanthatriol and formed by closely allied biosynthetic processes involving additional cyclizations.

Further studies of the extract from the sponge, *Spongia officinalis*, from which we have already described a number of furanoterpenes with linear C-25[3] and truncated C-21 chains[4,5], have now led us to the isolation of a new additional sesterterpene, present in small amounts, which proved to have the structure III, closely related to scalarin (II) and which we have named deoxoscalarin.

Extraction of fresh tissues (350 g, weighed dry) of *S. officinalis* and fractionation on silica gel column of solvent extracts were reported previously[4]. Fractions eluted with benzene-ether (7:3) were rechromatographed on preparative TLC (Merck precoated silica gel $F_{254}$ plates; benzene-ether, 7:3) to give crystalline deoxo-

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