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Light-Dark and Feeding Regimens Affect Circadian Phasing of Blood-Ethanol Decay Rates

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STURTEVANT, R. P. AND S. L. GARBER. *Light-dark and feeding regimens affect circadian phasing of blood-ethanol decay rates.* PHARMAC. BIOCHEM. BEHAV. 13(5) 637–642, 1980.—Rats maintained on a 12 hour:12 hour light-dark cycle, with food continuously available, exhibited a prominent and reproducible circadian rhythm in the slope of the linear blood-ethanol clearance curve. Peak values fell near the end of the dark period and trough values late in the light period. These phase relationships persisted with 4, 8 and 12 hour phase shifts of the illumination schedule. However, when food availability was restricted to 4 hours per day the feeding regimen became the dominant synchronizer for the rhythms in blood-ethanol decay rate and body core temperature. With resumption of the ad lib feeding regimen, the L-D cycle again entrained these rhythms. Ethanol injections (1.5 g/kg, IP) did not alter the expected excursion of the circadian temperature curve, as measured 4 hours after dosing.

Chronopharmacokinetics Circadian rhythms Ethanol clearance rates Feeding restriction Pharmacokinetics Phase shifts

THE concept of a time-dependent component of pharmacological response is slowly gaining recognition among investigators who formerly and traditionally had focused upon dose-dependent effects to define pharmacokinetic parameters. Temporal variation in the rate of blood-ethanol clearance has been reported in human subjects following oral dosing [5, 7, 14, 17, 18, 24] and in the rat after intraperitoneal injection [13, 16, 22].

Our earlier studies [20], in which constant-rate ethanol infusions were maintained for several hours in anesthetized rats, suggested circadian variation in the blood-ethanol decay rate. The present study was undertaken to extend those observations to unanesthetized animals receiving single intraperitoneal ethanol injections first, after acclimatization to a defined L-D cycle, then after abrupt phase-shifts of the illumination cycle and, finally, after restricted daily feeding. In addition, we sought to examine the effects of these environmental manipulations on the rhythm of body core temperature.

METHOD

Animals

Forty-eight male Charles River rats, received at 1 month of age, were caged in pairs within environmental chambers equipped with individual air-exchange blowers and light-control timers. Two Duro-test fluorescent lamps provided an illumination intensity of 400 lux at cage floor level. A 12 hour:12 hour L-D cycle was maintained during the 3 studies reported here, with the light phase beginning at 0900 (CDT) during Studies 1 and 2-A. During Studies 2-B and 3 the onset of the daily light phase was staggered by 4-hour intervals for the various experimental groups, as described below. All animals were weighed at weekly intervals.

Temperature Measurements

After a 4-week acclimatization to each new L-D or feeding regimen, body core temperatures were measured using a thermistor thermometer (Yellow Springs Instrument, model 43 TA) by inserting a lubricated probe approximately 3 cm into the anal opening. Readings were made at 4-hour intervals for a total of 7 readings per animal, thus repeating the initial time point 24 hours later. For those rats receiving ethanol at the time of temperature measurement, the temperature determination always preceded the ethanol injection because of the known hypothermic effect of ethanol.

Ethanol Dosage and Analysis

Ethanol was administered as an 11.7% (w/v) solution, diluted with 0.9% NaCl. All doses were 1.5 g ethanol/kg body weight and were administered intraperitoneally. The 48 rats were divided into 6 equal groups, and each group was injected successively at 4-hour intervals (serially-independent injections). Injection times for the different groups were 0900, 1300, 1700, 2100, 0100 and 0500 with each treatment group being housed within a single environmental chamber. Our standard ethanol concentration and the optimal dose protocol had been established in earlier studies [23].

Beginning precisely 60 min after dosing, a 20 μl tail-vein blood sample was withdrawn using a calibrated capillary pipet. The sample was mixed with l-propanol (G.C. internal standard) plus preservatives (NaF and $NaNO_2$) in a 100×125 mm Vacutainer and then immediately chilled. All blood samples

TABLE 1
EXPERIMENTAL REGIMENS

Study	n	Hrs light	Feeding	Ethanol (1.5 g/kg) IP
1	48	0900–2100	Ad lib	4-hr intervals 6 subgroups (n=8)
2				
Group A	24	0900–2100	Ad lib	4-hr intervals 6 subgroups (n=4)
Group B	24	Staggered	4 hr/day	4-hr intervals 6 subgroups (n=4)
3	48	Staggered	Ad lib	4-hr intervals 6 subgroups (n=8)

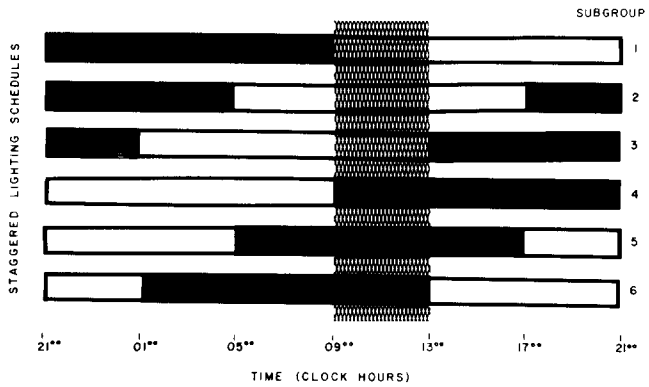

FIG. 1. Schedule of staggered 12 hour:12 hour light and dark periods for subgroups 1–6 in Study 2-B. The black bars represent the hours of darkness. The hatched vertical bar indicates the 4 hrs of daily food availability. For each of the subgroups (n=4), the feeding period encompassed a different segment of the light or dark phase.

were frozen within 1 hour of collection and were stored at −70°C until analyzed. Seven additional blood samples per animal were drawn at 20-min intervals, the last sample being taken 200 min after dosing. Blood-ethanol concentrations were determined by gas chromatography using a modification of the micromethod developed in our laboratory [19].

Light-Dark Phase-Shifts and Feeding Regimens

The L-D schedules and feeding regimens in the 3 studies are outlined in Table 1. For Study 1, the rats were maintained on an L-D schedule of light 0900–2100 (CDT) and were allowed food (Purina Rat Chow) ad lib. After the initial series of ethanol injections on 12 July, 1978, these 48 animals were divided into 2 equal groups, A and B, for Study 2.

Group A of Study 2 was continued on the same illumination and feeding regimen as before. For Group B, 20 of the 24 rats had the hours of light and dark abruptly advanced or delayed for 4, 8 or 12 hours in order to effect 6 different 12 hour:12 hour L-D schedules, with the time of lights-on beginning successively 4 hours later for each subgroup of 4 animals. No phase shifting was performed for the remaining rats of Group B. Each subgroup of 4 rats in Group B was housed in an individual environmental chamber.

Food and water continued to be available ad lib for all Group B animals for 7 days, after which food was presented manually at 0900 and the uneaten pellets removed at 1300 each day. Thus, as shown in Fig. 1, subgroup 1 ate their single daily meal during the first 4 hours of the light period, subgroup 2 during the middle 4 hours and subgroup 3 during the last 4 hours of the light period. The rats in subgroups 4–6 were fed during the first, middle or final 4 hours of the dark period.

All rats had water continuously available up to the time of ethanol dosing and again after the last blood sampling. Food, where otherwise available, was similarly removed during the period from dosing to final blood sampling.

Following the ethanol injections and blood sampling of Study 2 on 18 August, 1978, food was once again made continuously available to Group B rats, who remained on the staggered L-D schedules. For Study 3 the rats from Group A in Study 2 were subjected to the same staggered L-D schedules to which Group B rats had been subjected a month earlier. At all times Group A rats had had food continuously available. On 6 September, 1978 all rats were again injected with ethanol, by which time the rats from Group A had been maintained on staggered L-D schedules for 3 weeks and those from Group B for 8 weeks. All rats were allowed food ad lib for Study 3.

Timing of Ethanol Injections

For Study 2, dosing times for the food-restricted rats were selected to approximate the feeding phase of the animals at the times of injection for Study 1. This was done to assess the effectiveness of the restricted feeding regimen to entrain the blood-ethanol clearance rhythm. Sample population size precluded injection times also coinciding with clock hours of injection in both Studies 1 and 2-B. A preliminary study done in our laboratory had indicated that our rats consumed most of their food during the middle or late hours of the dark phase of the L-D cycle, as also reported by many other investigators for nocturnally active rodents. The rats injected during the latter part of the dark cycle in Study 1, presumed to be well-fed animals, were injected at the end of the mealtime (1300) for Study 2-B. Similarly, those rats injected at 1700, late in the light phase in Study 1, were injected at 0900 in Study 2-B, 20 hours after their last meal. The other 4 food-restricted groups were assigned to the remaining dosing intervals accordingly. Group A rats, having experienced neither food restriction nor L-D cycle phase changes, were injected at the same clock times in both Studies 1 and 2.

For Study 3, the rats injected at each of the 6 equispaced dosage intervals included representatives from each of the 6 staggered L-D schedules. The remaining rats were randomly distributed among the various injection periods. Furthermore, each injection group was composed equally of animals previously food restricted and those always fed ad lib.

RESULTS

Ad Lib-Fed Rats

In Study 1, a prominent circadian variation in the slope of the blood-ethanol clearance curve was observed (Fig. 2). The maximal slopes fell late in the dark phase and the minimal slopes late in the light phase. This was confirmed in Study 2-A, which showed a similar timing of the peak and trough values; however, the cosine-derived [10] mesor and amplitude were less (Table 2). In Study 3 those rats experiencing the same circadian phase at the time of injection,

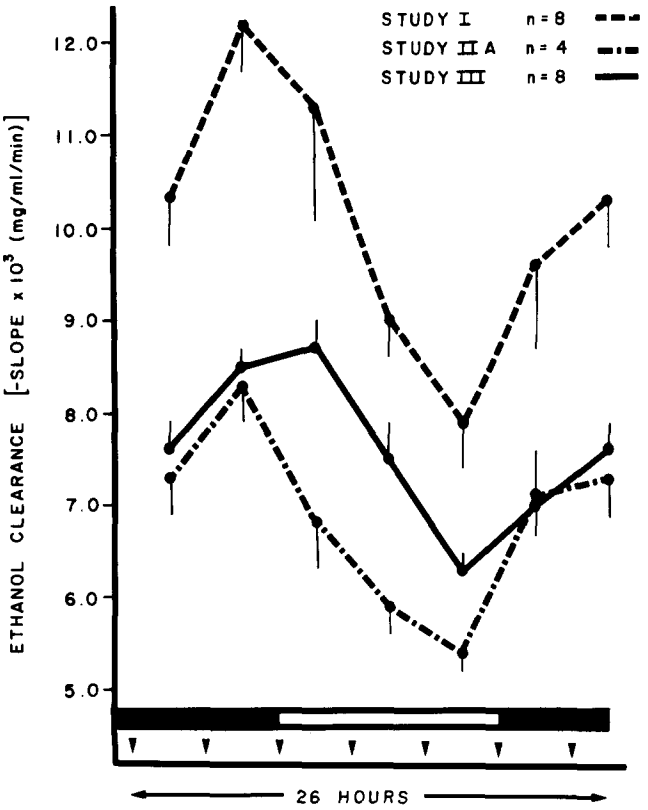

FIG. 2. Circadian variation of the mean estimated slopes of ethanol disappearance curves with the phase of the light-dark cycle for Studies 1, 2-A and 3. Arrowheads indicate 4-hr intervals of (serially-independent) ethanol injections (1.5 g/kg, IP) to groups of 4 or 8 rats fed ad lib. For Studies 1 and 2-A the 12 hr light phase began at 0900. For Study 3 the rats were on staggered 12 hr:12 hr L-D cycles. Note that the first and last points repeat. Slopes ± SE are shown at the midpoint of sampling.

regardless of the clock hour of injection, were grouped together to assess a weighted mean blood-ethanol clearance rate (Fig. 2). Although the rats had been subjected to a 4, 8 or 12-hr L-D phase shift prior to their third ethanol injection, a similar circadian-phased pattern was again noted. Therefore, the L-D schedule synchronized the rhythm under conditions of both standard and phase-shifted illumination regimens when food was continuously available.

A statistical analysis of the 48 rats injected in Study 1 and again in Study 3 was performed. Analysis of covariance [15] tested the null hypothesis that the slopes of the individual regression lines could have come from the same population. For Study 1, $F(5,285)=2.81$, $p<0.05$. For Study 3, $F(5,218)=3.18$, $p<0.01$.

Food-Restricted Rats

The rhythm of the 24-hr blood-ethanol clearance pattern persisted during restriction of food availability to 4 hours/day. As seen in Table 2, this feed-starve regimen effected only minimal alterations in those rhythm characteristics noted during the ad lib feeding regimens. The mean slopes for each of the 6 injection-sampling periods are displayed in Fig. 3. On the left of the figure (A), the slope values are shown as a function of the hours of food deprivation. No significant difference was noted among slopes of those rats injected 0, 4, 8 or 12 hours after feeding; however, a Student's *t*-test indicated a difference at the 0.05 level between the groups injected 16 and 20 hours after feeding and the 4 groups more recently fed.

Figure 3,B depicts the same data as a function of the phase of the L-D cycle at the time of dosing. Although the first 2 data points reflect the clearance rates at about the time of the dark-to-light change, they differ significantly. The same is true of the last 2 data points, which occur 8 hours after the start of the light phase. The 2 central points, at 4–5 hours into the light phase, do not quite reach a level of statistical significance.

Although an analysis of covariance of the slopes in Study 2-B did not result in a statistically significant finding ($p>0.05$), this could have been a result of the small number of animals involved. Thus, when the data are depicted as in Fig. 3,C, it is evident that the feeding phase, rather than the L-D regimen, has entrained the rhythm in blood-ethanol clearance rate. Maximal values occurred during the mid- to late-dark hours with minimal values during the mid- to late hours of the light phase of the L-D cycle.

No single group consistently gained more or less weight than the others among the 6 food-restricted groups. These groups had gained 23.5 g (range 11.8–29.5 g) after 11 days of food restriction, an additional 24.8 g (range 10.5–26.8 g) 7 days later, and 18.6 g (range 8.5–18.8) another 8 days later. The ad lib-fed group had gained 33.7, 30.6 and 16.0 g during the same intervals, giving weight gain ratios of 0.70, 0.81 and 1.16 for the former rats compared to the latter.

Body Core Temperature

When the rats fed ad lib were subjected to the staggered

TABLE 2

CHARACTERISTICS OF BLOOD-ETHANOL DECAY RATE RHYTHMS

Study	Mesor (95% C.L.)*	Amplitude (95% C.L.)*	Light-dark phase Peak†	Trough†
1	10.1 (0.73)	1.9 (1.4)	MD-EL	LL
2-A	6.8 (0.57)	1.3 (1.1)	MD	LL
2-B	6.3 (0.55)	1.2 (1.1)	LD	ML
3	7.5 (0.49)	1.0 (0.94)	LD	LL

*From single cosinor analysis with 24-hr period [10] as ($-\text{slope}\times 10^3$) in mg/ml/min.

†EL=early light; ML=mid-light; LL=late light; ED=early dark; MD=mid-dark; LD=late dark.

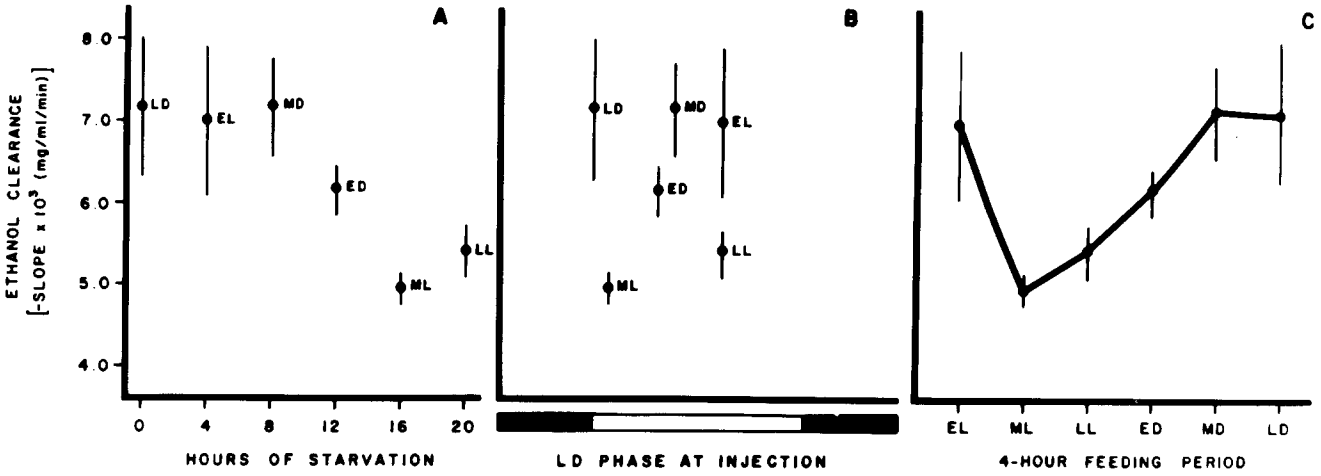

FIG. 3. Mean blood-ethanol decay slopes ± SE for groups of rats on 6 different 4-hr feeding schedules (LD = fed during late dark; EL = early light; MD=mid-dark; ED=early dark; ML=mid-light; LL=late light). The blood-ethanol clearance slopes are shown as a function of 3 variables; on the left (A) as the time lapse (hours) between the last meal and ethanol administration, in middle (B) as the illumination phase at the time of injection and on the right (C) as the feeding phase and its relationship to the L-D cycle.

L-D cycles in Study 3, the temperature rhythm was phase-shifted accordingly, with the maximal values falling in the dark phase and the minimal values observed approximately 12 hours later, as expected (Fig. 4).

In Study 2-B, 7 days after the imposition of the staggered L-D schedules, the body temperature rhythms revealed a shift in accordance with the L-D phase changes, with maximal values being attained during the dark phase (Figs. 5 and 6, dashed lines). At this time the rats were still being fed ad lib. Food restriction regimens were then imposed as described earlier, and 25 days later temperature rhythms were once again assessed. A ca. 4-hour phase advance was seen in those rats fed early in the light phase (Fig. 5, solid line). The greatest phase change was noted in those rats fed during the middle of the light phase (Fig. 6). The peak of one curve and the trough of the other occurred at 0100 hr. Lesser phase shifts, or none at all, were evident in the other groups after food restriction. The cosinor-derived [10] mesor (M) and amplitude (A) of the rhythmic patterns were somewhat greater during the restricted feeding regimens than during the ad lib regimens in most cases. In no case did the ethanol injection alter the expected excursion of the circadian temperature curve, as measured 4 hours after dosing, regardless of whether the curve was on a rising or falling slope (Figs. 5 and 6, arrows).

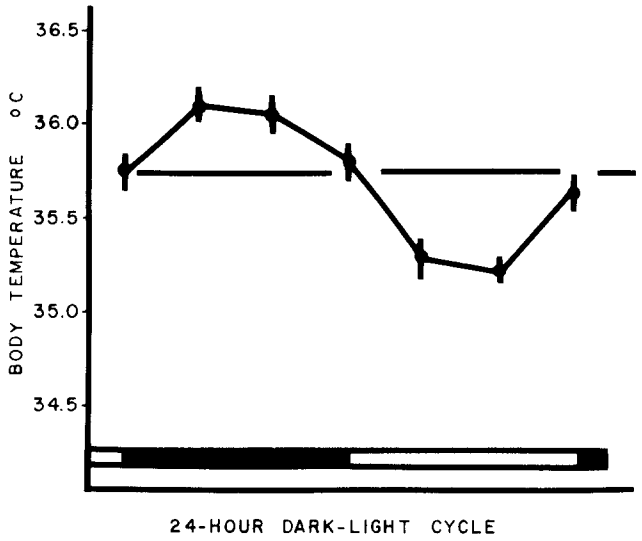

FIG. 4. Circadian variation in body core temperature of rats on 6 different 12 hr:12 hr light-dark schedules. Sample population was 48 rats, fed ad lib. Sampling interval was 4 hours, with a total of 7 measurements per animal. The horizontal line indicates the cosinor-derived [10] mesor (M ± 95% confidence limits) = 35.7°C ± 0.10; amplitude (A ± C.L.) = 0.5°C ± 0.20; acrophase (ϕ ± C.L.) = −180° ± 24 referenced to mid-light span.

DISCUSSION

These results confirm our earlier findings [20, 22, 23] of a circadian variability in the blood-ethanol clearance rate in rats fed ad lib. The timing of the maximal and minimal rates of ethanol disappearance continued to be synchronized by the L-D cycle and, as demonstrated in Study 3, responded to phase advances and delays of the clock hour at which lights were turned on and off. On the other hand, when food availability was restricted to only 4 hours/day, the rhythm was synchronized by the feeding schedule. However, when the rats were returned to an ad lib feeding regimen (Study 3), the L-D cycle once again became the dominant synchronizer of the rhythm. In fact, in Study 3 no difference in the rates of drug clearance was noted between the group with food always available (Study 2-A) and the group formerly food-restricted (2-B).

The rhythm of blood-ethanol decay rate also persisted with repeated dosing; however, both the amplitude and mesor were decreased with subsequent drug exposure (Table 2). We assume this is not an age-related effect, because the rats were still "young adults" (2, 3 and 4 months of age) at the time of injection in Studies 1, 2 and 3, respectively. The 3 doses of 1.5 g/kg ethanol were well below those expected to cause liver damage and were separated by 4-week intervals, a drug exposure considerably less than that normally associated with development of tolerance. One cannot discount

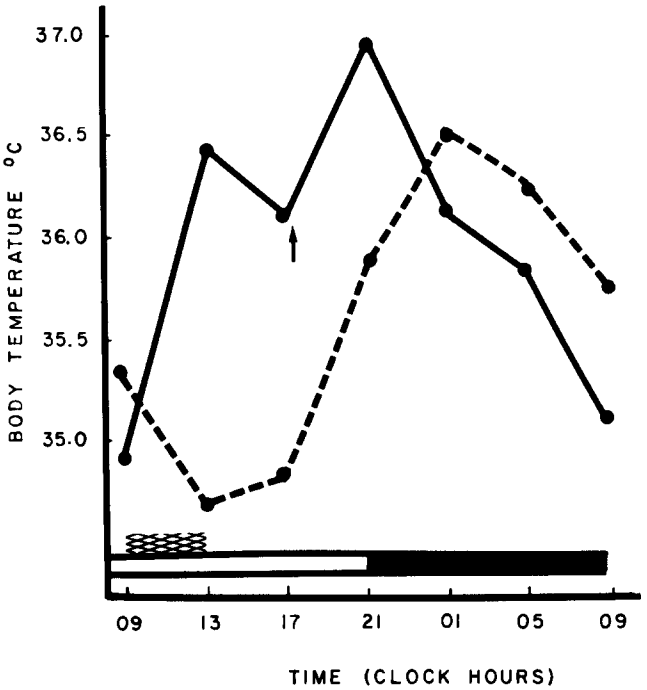

FIG. 5. Chronogram of body core temperature of 4 rats fed ad lib (dashed line) and after 26 days of feeding restricted to the early portion of the light phase (solid line) (feeding phase indicated by cross-hatched bar along abscissa). Note that the imposed feeding regimen effected a ca. 4-hr phase advance of the high and low 24-hr temperature values, the maximum value seen 4 hrs after ethanol injection (arrow). By cosinor analysis, for rats fed ad lib, M ± C.L. = 35.60°C ± 0.08, A ± C.L. = 1.0°C ± 0.15, ϕ = −254° ± 8.6 referenced to 0900, or 0120–0230 hr; for rats on restricted feeding, M ± C.L. = 36.2°C ± 0.51, A ± C.L. = 0.5°C ± 1.0, temperature maximum = 2100 hr.

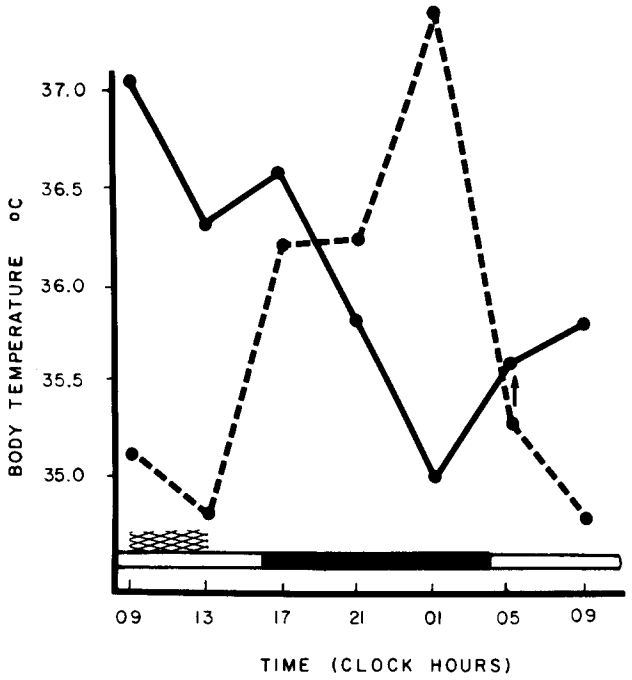

FIG. 6. Chronogram of body core temperature of 4 rats on 2 different feeding regimens; food continuously available (dashed line) and food available only during the mid-light phase (solid line). Feeding restricted to the hours shown by the cross-hatched bar along the abscissa resulted in a shifting of the temperature curve so that the trough of this curve fell at 0100 hr, where the peak occurred for the ad lib-fed rats. Arrow indicates ethanol injections (1.5 g/kg, IP). By cosinor analysis, for continuous feeding, M ± C.L. = 35.8°C ± 0.79, A ± C.L. = 1.2°C ± 1.53, temperature maximum = 0100 hr; for restricted feeding, M ± C.L. = 35.8°C ± 0.42, A ± C.L. = 0.6°C ± 0.82, temperature maximum = 0900 hr.

the possibility that the decrease in rhythm amplitude may be reflecting a circannual variation in ethanol metabolism, although we have observed similar results in previous studies begun during various seasons over the past 4 years. Since our rats have always grown at a very rapid rate during their first 6 months of age, we suggest that the observed attenuation of the rhythm may be a result of changes in body surface-to-mass ratio and that, since doses are given on a body weight basis, a dose of 1.5 g/kg body weight, given in one study, may not be equivalent to that same dose in a later study.

Among the 6 food-restricted groups, no single group consistently gained more or less weight than the others. Thus, based on maximal weight gain, no one circadian feeding phase appeared to be more advantageous than another. This is in contrast to the findings of others [9,12] who reported that, as the timing of a single daily meal became progressively further displaced in time from the mid-dark phase, the average weight gain decreased in a parallel fashion. Although restriction of food to a single daily 4-hour period continued for about 26 days in both our study and in that of Philippens *et al.* [12], our rats were some 200 g heavier than theirs at the beginning of the study and were abruptly shifted from an ad lib feeding regimen to a restricted one, in contrast to a gradual decrease in hours of food availability. With both procedures, that is, with or without an acclimatization period, rats continue to gain weight, although initially at a lesser rate than ad lib-fed rats.

Ylikahri and Mäenpää [26] reported a 42% decrease in the elimination rate of blood-ethanol in rats starved for 48 hours as compared to those with food freely available. If the drug elimination rate were solely dependent upon the nutritional status of the animal at the time of dosing, we should have observed steadily decreasing slope values in those groups injected successively farther from the time of their last meal. However, this was not the case; the rate of blood-ethanol clearance in those rats injected 12 hours after feeding did not differ significantly from the group injected immediately after eating (Fig. 3,A).

On the other hand, if the rate of blood-ethanol decay were solely dependent upon the phase of the illumination cycle at the time of dosing, then the slope values for the 2 groups of animals injected 8 hours after the beginning of the light phase should have been similar; but this was not the case (Fig. 3,B).

Several years ago Halberg [3] pronounced that we are "not only what we eat, but when we eat" as well. Studies by a number of investigators have revealed statistically significant differences in effects from meal timing in human subjects [2], in squirrel monkeys [25] and in rodents [9,12]. If caloric intake at one circadian-system-phase is not the physiological equivalent of the same intake at another, it might be suggested that maximal nutritional value would result from food ingested during the most "natural" feeding phase for rats, that is, the mid-dark period. In Study 2-B, those rats allowed to feed during mid-dark metabolized ethanol at a

significantly greater rate than others fed at an "unnatural" feeding phase. Because the groups fed at the most "unnatural" circadian phases (mid- and late-light) had also been food-deprived for the longest times when injected (16 and 20 hours), it is not possible to separate the effects of feeding phase and time lapse since feeding in the present study.

When food availability is limited to a discrete portion of the light or dark period, modifications of the rhythmic pattern of a number of physiological variables have been reported [4, 8, 11]. Our findings that both the rhythm of body core temperature and blood-ethanol clearance rate are synchronized by limited hours of daily food availability confirm and augment the list of variables known to respond to environmental manipulation. However, not all physiological rhythms respond to light-dark phase shifts or to "meal" feeding at the same rate or to the same degree. Philippens *et al.* [12] reported that several hepatic enzymes as well as serum corticosteroid levels in rats appeared to reflect an interaction of both restricted feeding schedule and the light-dark cycle. On the other hand, Pauly *et al.* [11] reported that the rhythm of murine corneal mitosis was only minimally altered by food restriction.

Our observations of peak clearance rates during the late dark hours of the L-D cycle and lowest rates some 12 hours later (Fig. 2) complement the results of Soliman and Walker [16]. These investigators analyzed the ethanol content of various tissues in ad lib-fed male rats one hour after injection. They reported peak concentrations during the mid-dark hours in both brain and liver, near the circadian phase at which we found the greatest rate of blood-ethanol clearance.

We have found the Charles River rat to be suitable for studies of the chronopharmacokinetics of ethanol. The ethanolemia decay rate in the rat shows a temporal variation not unlike that reported in man [17, 18, 24]. The blood clearance curve for this drug is linear within the dose and blood concentration ranges studied here [21]. On the other hand, recent reports [1,6] suggest that the mouse is an inappropriate laboratory model for such investigations since these investigators found a time-invariant rate of ethanol disappearance from murine blood and brain. Therefore, it is suggested that the laboratory rat is a more suitable model species with which to pursue studies on this subject.

ACKNOWLEDGEMENT

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