Journal of Biological Rhythms, Vol. 5, No. 1, 1990, pp. 35–46

Food-Anticipatory Rhythms under 24-Hour Schedules of Limited Access to Single Macronutrients

R. E. Mistlberger,*[1] *T. A. Houpt, and M. C. Moore-Ede
Department of Physiology and Biophysics, Harvard Medical School and Institute for Circadian Physiology, Boston, Massachusetts 02115

Abstract Food-restricted rats anticipate a fixed daily mealtime by entrainment of a circadian timekeeping mechanism separate from that which generates daily light-entrainable activity rhythms. The entrainment pathways and rhythm-generating substrates for food-anticipatory rhythms are unknown. In this study, we attempted to define minimal food-related stimuli necessary or sufficient for food anticipation by employing schedules of restricted macronutrient availability, with or without free access to a complementary diet. Rats did not anticipate a daily meal of protein, carbohydrate, or fat, as measured by tilt-cage, running-wheel, or food-bin activity, when they had free access to other nutrients. However, rats did anticipate single-macronutrient meals when they were limited to only two, larger, complementary meals each day (protein–fat, protein–carbohydrate) providing a reduced total number of calories. Previous work has shown that caloric restriction per se is not a prerequisite for food anticipation. In combination with that study, the present results indicate that the size of a nutrient meal, in absolute terms or relative to total daily nutrient intake, is of pre-eminent importance in determining its value as a synchronizer of anticipatory rhythms. The results further suggest that physiological responses unique to the ingestion and absorption of any particular macronutrient are not necessary components of the entrainment pathway.

The physiological system timing daily rhythms of activity in the rat consists of at least two separable rhythm-generating substrates, entrained by periodic stimuli (zeitgebers) of different modalities. One substrate includes the suprachiasmatic nuclei (SCN), which function as a pacemaker entrained by daily light–dark (LD) cycles (Rosenwasser and Adler, 1986). A second substrate, located outside of the SCN (Stephan, 1981, 1983; Stephan et al., 1979), is responsible for the generation of circadian rhythms entrained by restricted food availability (e.g., one meal per day) (Boulos and Terman, 1980; Rosenwasser and Adler, 1986). This nonphotic entrainment is manifest in rats as a bout of activity that anticipates the daily mealtime by up to several hours. Although independent of LD-entrained rhythms, food-anticipatory rhythms exhibit similar oscillatory properties, including circadian limits to entrainment (Stephan, 1981) and persistence in constant conditions (i.e., during food deprivation) (Stephan, 1981; Coleman et al., 1982; Rosenwasser et al., 1984).

The entrainment pathway conveying photic information to the SCN pacemaker is well described both anatomically (Pickard, 1982) and electrophysiologically (Meijer et al., 1986). In contrast, the entrainment pathways and rhythm-generating substrates for food-anticipatory

1. To whom all correspondence should be addressed, at Department of Psychology, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada.

rhythms are completely unknown (Mistlberger and Rechtschaffen, 1984; Mistlberger and Rusak, 1988), as indeed is the very nature of the entraining stimuli. In previous studies, it was observed (1) that periodic availability of water, unlike food, was not effective as a zeitgeber for anticipatory activity rhythms (Mistlberger and Rechtschaffen, 1985); and (2) that timed daily access to a supplemental sweetened meal in free-feeding rats was an effective zeitgeber for anticipatory rhythms, but only if the sweetened meal contained nutrients (Mistlberger and Rusak, 1987). These findings suggest a working hypothesis that the circadian mechanism responsible for food anticipation, rather than being entrainable by any motivationally significant, nonphotic stimuli, might be sensitive primarily to stimuli associated with nutrient ingestion. According to this hypothesis, isolating nutrient-related stimuli necessary or sufficient for food anticipatory rhythms might greatly facilitate the identification of entrainment pathways and rhythm-generating substrates.

In this study, we examined the ability of rats to anticipate one or two temporally fixed daily meals consisting of a single macronutrient (protein, carbohydrate, or fat) otherwise missing from their diet. The object was to expand our knowledge of meal characteristics necessary or sufficient for entrainment. If certain macronutrients were found to be uniquely effective zeitgebers for food anticipation, then nutrient-specific physiological responses—such as stimulation of visceral or central chemoreceptors for amino acids or glucose, release of hormones such as cholecystokinin (to fat or protein) or insulin (to carbohydrates), or changes in central neurotransmitters sensitive to substrate availability—would be implicated as possible components of the entrainment pathway.

EXPERIMENT 1

In Experiment 1, we asked whether rats could anticipate a daily meal that provided a single macronutrient (protein, carbohydrate, or fat) absent from their freely available diet. Locomotor activity measured by a tilt floor was used to assay meal anticipation.

METHODS

ANIMALS

Thirty-seven young adult, female, CD rats (Charles River) were used. All rats were naive to experimentation and were group-housed with *ad libitum* access to food and water prior to implementation of restricted feeding schedules.

DATA COLLECTION

Locomotor activity was monitored using modified, opaque plastic rodent-holding cages (45 × 25 × 20 cm) with metal grid tilting floors balanced on a central horizontal beam. The cages were housed in pairs within isolation chambers with incandescent lighting; fans provided air flow and white noise. Floor tilts were detected by microswitches, which were monitored continuously by computer. Tilt counts for groups 1 and 4 below were summed and stored at 15-min intervals using an Esterline–Angus microprocessor and DEC MINC. All other groups were recorded using an Apple 2e microcomputer with a data collection interval of 10 min. An Atari 1040st computer was used for generating visual plots and numerical analyses of the data off-line.

PROCEDURES

The rats were tested in four groups under different nutrient restriction schedules. Each schedule consisted of four sequential conditions lasting a variable number of days or months:

a. Free access to a diet missing a specific macronutrient.

b. Access to a supplementary meal of the restricted macronutrient for 1 or 2 hr each day at a fixed time, with free access to the complementary diet. Food-anticipatory rhythms are usually clearly apparent within 3–7 days in rats restricted to a single daily meal, but can take 1–3 weeks to become manifest in free-feeding rats with daily access to a 1-hr meal of a highly palatable chow (Mistlberger and Rusak, 1987). Consequently, the rats in the present study were maintained in the limited-access condition for at least 25 days and in most cases 2–3 months.

c. A second period of specific macronutrient deprivation. If meal anticipation were to be evident in condition b, then condition c would test whether the anticipation rhythm could continue (i.e., be self-sustaining) for at least two cycles in the absence of daily nutrient meal cues. If no meal anticipation were to be apparent in condition b, then condition c would serve to test whether the rats could entrain with a zero phase angle difference—that is, with the onset of food-entrained activity occurring spontaneously at expected mealtime, but not before (independent of actual food presentation).

d. Total food deprivation. If no anticipation were to become apparent in condition b, then this final condition would serve to test the possibility that "latent" food entrainment could be revealed by caloric deprivation—in other words, that the influence of food-entrained clocks on behavior would be more prominent within a motivational context (energy deprivation) that favored locomotor activity.

Group 1: Protein Restriction. Twelve rats were maintained on a protein-free diet for 10 days while housed in the colony room under LD 14:10. The diet consisted of corn oil (34% by weight), sucrose (29%), starch (29%), cellulose (4%), mineral mix (3%), and vitamin mix (1%). The rats were then provided each day with a 5-g protein meal of casein (33%) in mineral water with vanilla extract (0.2%, no alcohol) and saccharin (0.01%). The protein was freshly mixed and available for 2 hr beginning 8 hr after lights-on.

After 1 month, six rats were transferred into the tilt cages. Four of the rats were recorded under the same LD and feeding schedules for 21 more days, followed by 2 days during which no protein was provided. The other two rats were recorded under LD 12:12, with the protein meal ensuing 5 hr after lights-on. The remaining six rats were then recorded in the tilt cages for 14 days of food restriction under LD 14:10. The rats of the first group were returned to the colony, protein-deprived for 5 days, and then provided with a supplemental 10-g protein meal each day 8 hr after lights-on. These rats were recorded a second time in the tilt cages for 28 days of protein restriction, 5 days of protein deprivation, and 3 days of total food deprivation.

Group 2: Carbohydrate Restriction. Eleven rats were maintained on a carbohydrate-free diet for 14 days. The diet contained corn oil (45%), casein (36%), cellulose (15%), mineral mix (3%), and vitamin mix (1%). A supplemental 5-g carbohydrate meal containing sucrose (56%), starch (21%), and artificial chocolate syrup (23%, generic) was then provided each day for 2 hr beginning 5 hr after lights-on (LD 12:12) for 2 months. The rats were then transferred to the tilt cages and recorded for 19–42 days under these lighting and feeding schedules, for 2 days of carbohydrate deprivation, and a final 4 days of total food deprivation.

Group 3: Fat Restriction. Eight rats were placed in the tilt cages and maintained on a fat-free diet containing casein (28%), sucrose (14%), cellulose (7%), minerals (3%), vitamins (1%), and water for 25 days. A supplemental 3-g meal of corn oil (66%) and cellulose (33%) was provided each day for 1 hr beginning 5 hr after lights-on (LD 12:12). The rats were subsequently maintained on the fat-free diet alone for 7 days and then food-deprived for 4 days.

Group 4: Total Food Restriction. Six rats from the protein restriction group were fed standard Purina rat chow *ad lib.* for 1 month, transferred to the tilt cages, food-deprived for 24 hr, and then limited to a 2-hr daily meal of chow pellets provided 5 hr after lights-on (LD 12:12). After 23 days, the rats were food-deprived for 2 days and then recorded for 2 weeks with *ad lib.* food access.

RESULTS AND DISCUSSION

The rats on selective protein, fat, and carbohydrate restriction schedules showed no evidence of anticipating their daily supplemental nutrient meals. Some rats showed bouts of activity during the light period, but in no case were these concentrated before the daily nutrient meal. In contrast, the rats on total food restriction showed diurnal activity that was clearly concentrated within the 1 or 2 hr immediately preceding mealtime and that accelerated as mealtime approached. Histograms of activity within each 15- or 10-min interval averaged over the last 7 days of the feeding schedules are presented in Figure 1 for a typical rat in each group.

All of the rats in groups 1–3 were recorded over at least 2 days during which the daily nutrient meal was withheld. The rats showed no changes in their activity levels to suggest that they expected the supplemental meal to be available at the usual time. Each group was also subjected to 3–4 days of total food deprivation. This procedure has previously been used to reveal persisting rhythms of activity associated with prior daily mealtimes (Coleman

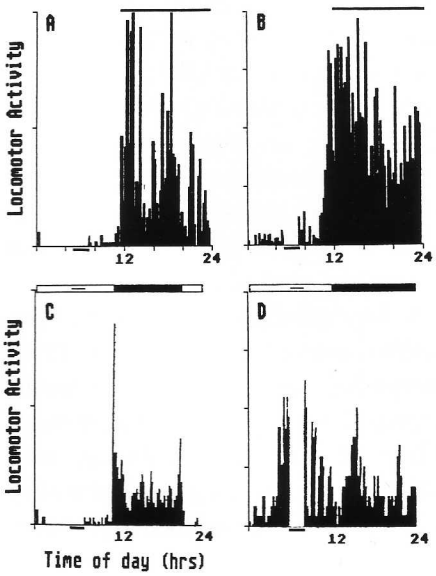

FIGURE 1. Histograms of tilt-cage measured activity within each 10-min (A, B) or 15-min (C, D) time bin, averaged over the last 7 days of nutrient restriction. (A) A carbohydrate-restricted rat. (B) A fat-restricted rat. (C) A protein-restricted rat. (D) A totally food-restricted rat. Lights-off hours are indicated by the solid horizontal bar at the top of each chart. Nutrient meals were provided in the middle of the light period, as indicated by the small horizontal bar below the abscissa of each chart. Vertical data bars for the nutrient meals hours have been omitted.

et al., 1982; Rosenwasser et al., 1984). However, the activity patterns evident during the food deprivation days were strongly nocturnal, and provided no evidence for preferential activity at the time of day formerly associated with nutrient meals.

Ten of the 12 rats in the protein restriction condition were recorded under LD 14:10, with protein access beginning 8 hr after lights-on; all of the other rats were recorded under LD 12:12, with nutrient access beginning 5 or 6 hr after lights-on. Many previous studies indicate that food anticipatory rhythms can be observed at any phase of the LD cycle, so it is unlikely that this procedural difference had any effect on the outcomes. The results of Experiment 2, below, suggest that this factor was of no consequence.

Despite failing to behaviorally anticipate their daily nutrient meals, all of the rats showed substantial intake of the supplemental nutrients each day. The latency and rate of ingestion were not formally measured, but most rats began eating the supplemental meals immediately and appeared to consume their daily allotment within 30 min. The calories provided by these meals, as a percentage of the observed total daily caloric intake, averaged 8.9% ± 2.9% of 64.4 ± 10.8 kcal for the 5-g protein group; 24.8% ± 3.2% of 51.3 ± 5.2 kcal for the 10-g protein group; 22.5% ± 5.7% of 56.6 ± 8.7 kcal for the carbohydrate group; and 18.5% ± 3.5% of 60.6 ± 6.3 kcal for the fat group. Body weights were not systematically measured in all groups, but the protein-restricted rats were visibly smaller than the other rats. This was due mostly to weight losses ranging from 2.9% to 9.4% during the 10 days of protein deprivation preceding the implementation of the daily protein access schedule. The carbohydrate- and fat-restricted groups, in constrast, did not sustain weight losses during the initial deprivation period and, with one exception, exhibited apparently normal growth rates over the course of the feeding schedules. The exceptional case was a rat in the carbohydrate-restricted group that was strikingly obese by the end of the study.

EXPERIMENT 2

The failure to observe anticipation of daily nutrient meals in Experiment 1 may have been due to the nonspecific measure of activity employed. Although tilt cages were adequate for detecting behavioral anticipation of mealtime in rats with access to a single daily meal, they may be less useful for animals that are not subjected to caloric restriction. In a previous study, prominent anticipation of a daily sweetened meal was observed in nondeprived rats recorded in running wheels (Mistlberger and Rusak, 1987). Also, food-restricted rats with hypothalamic lesions have been shown to exhibit anticipatory food-bin-associated activity without showing anticipation in activity measured using a tilt floor (Mistlberger and Rusak, 1988). Consequently, the 10-g protein restriction test was repeated in separate groups of rats recorded in running wheels and in cages equipped with food-bin monitors.

METHODS

Five rats were recorded in Wahmann running wheels housed within individual isolation chambers. The rats were maintained on the protein-free diet for 5 days and then provided with a 10-g protein meal each day for 26 days beginning 5 hr after lights-on (LD 12:12). Wheel revolutions were detected by microswitches and monitored by Apple 2e computers.

In a second group, 11 rats were recorded in standard opaque plastic cages, each equipped with a feeding bin accessible through a small opening on the side of the cage. An infrared

photobeam and photosensitive resistor were positioned to detect movements of a rat's head over the feeding bin. The protein-free diet was continuously available in the feeding bin for 10 days and then was replaced each day by the protein meal for 2 hr beginning 6 hr after lights-on. This schedule was maintained for 26 days. On days 27 and 28, the protein meals were provided at the usual time, but the animals were kept in constant dark (DD). The LD cycle was then reinstated, and the protein meal was withheld for 2 days. The rats were then completely food-deprived for a final 3 days.

RESULTS AND DISCUSSION

Histograms of running-wheel activity within each 10-min interval averaged over the last 7 days of the protein restriction schedule are illustrated in Figures 2A and 2B for 2 representative rats. The rats showed no evidence of wheel running in anticipation of the protein meal. A third histogram (Fig. 2C) is provided from another study to illustrate typical anticipatory wheel running in a rat restricted to a single meal of balanced rat chow each day. Although no meal anticipation was evident among protein-restricted rats, the rats ate all or most of the protein each day.

The rats recorded in food-bin cages were (with two exceptions) similar to the rats in running-wheel cages, in that they consumed all or most of the daily protein meal but did not display behavioral anticipation of the meal. Diurnal activity was sporadically evident in most rats but was never concentrated before mealtime. The activity record of a typical rat, plotted in standard "actogram" format, is provided in Figure 3A. Two of the rats (P3, P4), however, deviated from this pattern in showing persistent food-bin activity throughout the lights-on period. Actograms for these two rats are illustrated in Figures 3B and 3C. Both rats showed a gradual increase of diurnal activity over the first few days of daily protein access, until activity was fairly continuous during the 6 hr of light preceding the protein meal.

This activity may reflect entrainment of a circadian oscillator. However, there are two reasons to doubt such an interpretation. First, the diurnal activity lacked temporal specificity; it was relatively constant and did not accelerate as the protein mealtime approached. This can be appreciated by comparing the actograms of rats P4 (Fig. 3B) and P3 (Fig. 3C) with that of a food-entrained rat recorded in the same apparatus but limited to a single daily meal of balanced chow (Fig. 3D). This is further illustrated in Figure 4: Rats P4 (Fig. 4A) and

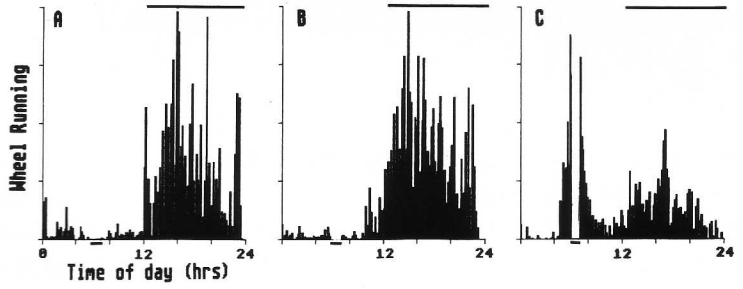

FIGURE 2. Histograms of average wheel-running activity in each 10-min bin during nutrient restriction in two protein-restricted rats (A, B) and one totally food-restricted rat (C). Conventions as in Figure 1.

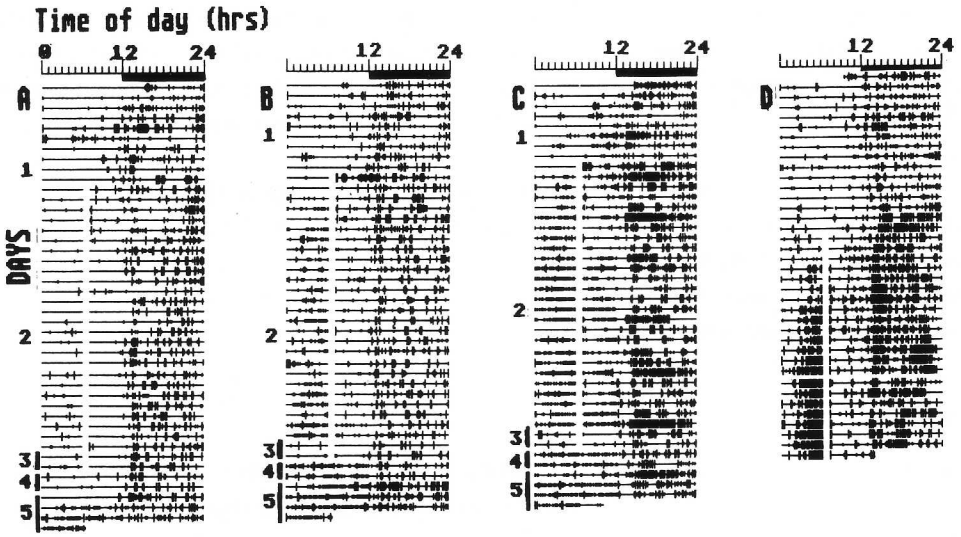

FIGURE 3. Actograms of food-bin activity from three protein-restricted rats (A, B, C) and one totally food-restricted rat (D). Each line represents a day and consists of 144 time bins of 10 min each, plotted left to right. The intensity of activity in each bin is reflected in the height of the vertical deflections from the zero-activity baseline for each day. Charts A, B, and C have five feeding conditions indicated by the small numbers on their left margins: (1) *Ad libitum* access to a protein-free diet; (2) restricted access to 10 g protein for 1 hr each day, indicated by the blank section in each line; (3) DD for 2 days; (4) no protein meal for 2 days in LD; (5) no food for the last 3.25 days. Chart D is from a rat fed a balanced chow *ad lib.* and then maintained on a single daily meal on the days indicated by the 1-hr blank section in each line. The lights-off period is indicated by the horizontal bar at the top right of each chart.

P3 (Fig. 4B) showed a flat, low-level distribution of activity throughout the lights-on period during the 26 days on the protein schedule, whereas the rat maintained on a single daily meal (Fig. 4C) showed a marked acceleration of food-bin activity over the 2–3 hr preceding mealtime.

The second reason for doubting an entrainment interpretation for these two rats is that their premeal activity was markedly reduced during the 2 days of recording in DD (see Fig. 3). A few short diurnal activity bouts were evident, but these were not closely associated

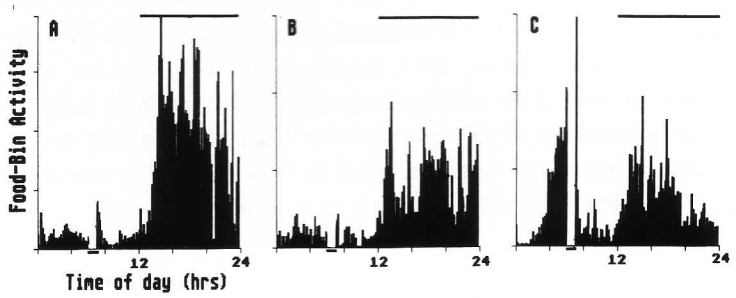

FIGURE 4. Histograms of average food-bin activity in each 10-min interval in two protein-restricted rats (A and B, which correspond to Figs. 3B and 3C, respectively) and one totally food-restricted rat (C, corresponding to Fig. 3D). Conventions as in Figure 1.

with mealtime. The rats may have been using lights-on as a conditioned cue to predict protein availability. Exteroceptive cues can modulate the expression of food anticipatory activity in rats (Terman et al., 1984), but there is no precedent in the food restriction literature for the effect observed here. In any case, when the LD cycle was reinstated, both rats were once again active throughout the day.

When protein was withheld for 2 days, all of the rats displayed their usual patterns of activity; that is, rats P3 and P4 were constantly active, and the other rats were largely inactive. When all food was withheld for 3 days, most of the rats became active throughout the day. However, there was little evidence for a concentration of activity during the hours previously associated with protein meals.

EXPERIMENT 3

The failure of rats to anticipate the opportunity to consume restricted macronutrients in the first two experiments suggests that the ingestion, digestion, and absorption of individual macronutrients in the amounts permitted do not provide sensory cues that are alone sufficient to entrain the circadian clocks responsible for food anticipation. The conditions under which food anticipation is reliably observed differ from the nutrient restriction paradigm in several ways. First, the rats are calorically deprived; second, the daily meal provides all of the rats' calorie intake; and, third, the food is usually nutritionally complex. In a previous study, strong anticipation of a large, nutritionally heterogeneous sweetened meal was observed in rats that had *ad lib.* access to regular chow and thus were not calorically deprived (Mistlberger and Rusak, 1987). We estimate that the calories provided by the sweetened meal in that study accounted for 40–90% of the rats' total daily energy intake. Combined with the present results, this implies that for a meal to be an effective zeitgeber for the food-entrainable circadian mechanism, it either must be nutritionally complex or must provide a larger proportion of the animals' daily energy intake than was provided by the nutrient meals in Experiments 1 and 2.

To test the latter possibility, it would be desirable to increase the size of the restricted meals without significantly altering the duration of the meal or the total daily caloric intake. However, pilot observations indicated that only two of these three objectives could be accomplished concurrently. We opted for a feeding schedule that permitted two meals each day, separated by 7 hr, which together provided an estimated 90% of a typical rat's daily caloric intake. This design had the advantage of allowing two different nutrients to be tested simultaneously in each rat. Previous studies have shown that calorically restricted rats will reliably anticipate two daily meals of a balanced diet separated within the lights-on period by as little as 6 hr (Bolles and Moot, 1973; Stephan et al., 1979; see also Stephan, 1983). If calorically restricted rats did not anticipate two daily meals of single macronutrients, this would point to the importance of diet composition. If the rats did anticipate these meals, this would underscore meal size (either relative or absolute) as an important variable in determining the value of a single macronutrient as a circadian zeitgeber.

METHODS

Eleven rats were recorded in the food-bin cages under LD 12:12 for 5 days with *ad lib.* access to the protein-free diet. The rats were then provided with two 1-hr meals each day,

one 3 hr after lights-on and the second 7 hr later. All of the rats received 15 g of the protein diet for the first meal. For the second meal, five rats received 9 g of a modified version of the carbohydrate diet (mineral oil was substituted for artificial chocolate syrup), and the other six rats received 6 g of the fat diet. The fat and carbohydrate meals were designed to be isocaloric. This feeding schedule was enforced for 14 days, the last 2 of which were conducted in DD. The LD cycle was then reinstated for 2 days, during which the rats were left undisturbed with no food.

RESULTS AND DISCUSSION

All of the rats ate all or most of both macronutrient meals each day. Average calorie intake by the protein–carbohydrate group was 42.8 ± 2.9 kcal, 33.5% ± 4.4% of which was provided by the protein meal. The protein–fat group averaged 47.6 ± 2.8 kcal daily, 29.4% ± 2.1% of which was provided by protein intake. All of the rats showed considerable diurnal activity preceding both meals. However, unlike the protein-restricted rats in Experiment 2, the intensity of food-bin activity increased as each mealtime approached. This is clearly illustrated in Figures 5-1A and 5-2A for two representative rats. Most significantly, when the LD cycle was replaced by DD, the premeal activity was still evident and appeared to be more concentrated within the 1–2 hr immediately preceding mealtime (Figs. 5-1B, 5-2B). When food was withheld for the last 2 days of LD, the rats showed food-bin activity

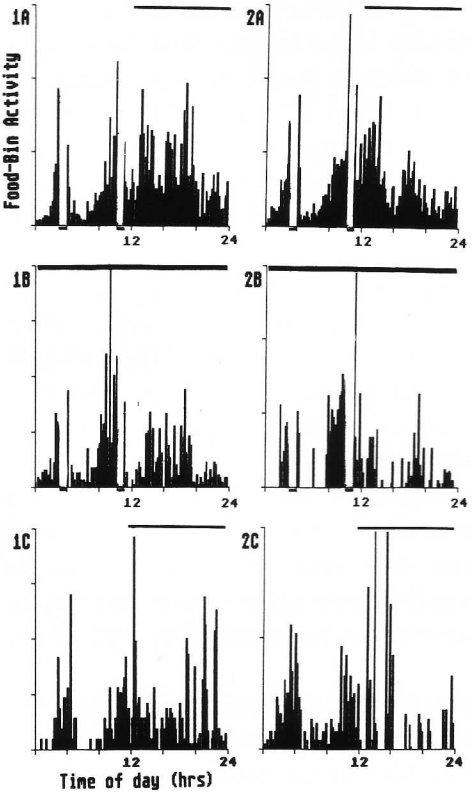

FIGURE 5. Histograms of average food-bin activity in two rats fed two pure-macronutrient meals each day. (1A, 1B, 1C) Protein–carbohydrate rat. (2A, 2B, 2C) Protein–fat rat. (1A, 2A) First 12 days on schedule under LD 12:12. (1B, 2B) Two days in DD. (1C, 2C) Two days undisturbed with no food in LD 12:12. Conventions as in Figure 1.

throughout the day, with the highest levels usually occurring close to the previously scheduled mealtime (Figs. 5-1C, 5-2C). The mealtime-associated activity in this group thus displayed three primary characteristics of food entrainment: anticipation, independence of ambient LD cycles, and persistence for at least two circadian cycles in the absence of food cues.

GENERAL DISCUSSION

These studies indicate that entrainment cues for circadian rhythms of food-anticipatory behavior can be provided by the regular, timed ingestion of any single macronutrient. However, these cues are not effective under all conditions. Chronically protein-deprived rats with free access to a complementary diet were unable to anticipate a 5-g or 10-g daily meal of protein, despite showing a voracious appetite for the sweetened, essential nutrient. Sweetened and/or oily meals of carbohydrates or fats were similarly ineffective under conditions of *ad lib.* access to a complementary diet. The cues provided by these meals only became effective as circadian zeitgebers when the rats were limited to two daily meals providing a reduced number of total daily calories.

These results could be taken to suggest that for feeding-related stimuli to act as entrainment cues for anticipatory rhythms, they must recur each day within a motivational context of caloric deprivation. However, the results of a previous study, in which rats anticipated a sweetened, oily mixture of rat chow without being food-deprived, suggest that caloric deprivation per se is not necessary for entrainment (Mistlberger and Rusak, 1987). Rather, it may be the size of the meal that is critical, either in absolute terms or relative to the total amount of nutrient (or calorie) intake in a day. In the two-meal condition, the macronutrient meals were on average larger than in the single-meal condition. Also, the amount of food consumed in each meal provided a greater proportion of the animals' total daily caloric intake. Presumably, under these conditions, meal-associated stimuli conveying timing information necessary for entrainment would have a greater salience and would thus be more effective zeitgebers. This interpretation is congruent with the results of an earlier study, noted above, in which 4-g daily meals were observed to be largely ineffective as zeitgebers for food anticipation, whereas 10-g meals of the same food were very effective (Mistlberger and Rusak, 1987).

Although caloric deprivation may be unnecessary for food anticipation, it could interact with meal size to determine the value of a meal as a zeitgeber. For example, caloric deprivation could modulate the sensitivity of food-entrainable circadian oscillators to meal-associated entrainment cues: As the degree of deprivation is increased, the minimal meal size sufficient for entrainment may be lowered, both in absolute size and as a proportion of total daily intake. Thus, although rats in Experiment 2 did not anticipate 10-g protein meals providing 25% of daily calories, whereas rats in Experiment 3 did anticipate 15-g protein meals providing 30% of daily calories, the differences between the two groups in total daily caloric intake preclude any conclusions from these data about a threshold meal size for entrainment. Such a threshold and possible interactions with caloric deprivation may be revealed in a two-meal paradigm in which the relative size of the meals and the total calories available in a day are varied systematically.

Although the differences observed between rats in the three experiments may be explicable in terms of nutrient meal size, the results leave unanswered the questions as to what constitutes the entrainment signal for food-anticipatory rhythms. All three macronutrients were effective

under the conditions of Experiment 3, so the entrainment cues are not solely provided by physiological events uniquely associated with the ingestion of any particular macronutrient. This would include the release of such hormones as cholecystokinin and insulin, the stimulation of nutrient-specific duodenal chemoreceptors, and the absorption and utilization of specific food metabolites. If these stimuli do provide entrainment information, they must be doing so redundantly. We have recently observed robust behavioral food anticipation in rats bearing total subdiaphragmatic vagotomies, indicating that vagally mediated neural and endocrine responses to food ingestion, which include some of the responses listed above, are not necessary components of the entrainment pathway (Mistlberger, Houpt, and Moore-Ede, unpublished observations).

In a recently published study, Rosenwasser et al. (1988) provided evidence that a circadian mechanism in free-feeding rats might be entrainable by restricted access to an essential micronutrient. Rats with experimentally enhanced salt appetite exhibited anticipatory lever pressing (but not wheel running; Rosenwasser et al., 1985) to an unsignaled daily period of salt access. The authors also reported preliminary evidence that salt anticipation is absent if the onset of salt access is signaled by exteroceptive cues. The salt restriction condition is analogous to protein restriction, in that both involve elements essential to the rats' health and both engender specific, vigorous appetites for these elements. However, in the present study, rats did display clear anticipatory food-bin activity to protein meals that were signaled by a complex of exteroceptive cues—namely, the experimenter with the food dish. This is consistent with the suggestion that the manifestation of food anticipation is more robust and less susceptible to situational variables than is salt anticipation (Rosenwasser et al., 1988). At present, it remains to be determined whether unsignaled access procedures can be used to reveal behavioral anticipation under conditions that have previously not been associated with anticipatory rhythms, including restricted access to small, essential macronutrient meals (present study), sweetened non-nutritive meals (Mistlberger and Rusak, 1987), and water (Mistlberger and Rechtschaffen, 1985). It also remains to be determined whether exteroceptive cues affect the entrainment process per se or just the behavioral expression of oscillators that are not photically entrained. This issue could perhaps be resolved by measurements of other circadian variables, such as physiological processes functionally related to the restricted micro- or macronutrient (e.g., enteropancreatic enzyme or hormone rhythms, etc).

The failure to see anticipation to single-nutrient meals under some conditions in this study could be explained by the fact that animals rarely if ever encounter such meals in nature. However, it is not unreasonable to suppose that animals may have evolved an anticipatory mechanism that is sensitive to only one or several constituent elements that are reliably found in their meals. Food restriction paradigms of any kind may seem artificial for some species, but many species studied so far do anticipate scheduled daily meals by entrainment of a circadian mechanism separate from the light-entrainable circadian clock (see Boulos and Terman, 1980, for a partial list). The challenge remains to elucidate the physiology of this timekeeping device.

REFERENCES

BOLLES, R. C., and S. A. MOOT (1973) The rat's anticipation of two meals a day. J. Comp. Physiol. Psychol. *83*: 510–514.

BOULOS, Z., and M. TERMAN (1980) Food availability and daily biological rhythms. Neurosci. Biobehav. Rev. *4*: 119–131.

COLEMAN, G. J., S. HARPER, J. D. CLARKE, and S. ARMSTRONG (1982) Evidence for a separate meal-associated oscillator in the rat. Physiol. Behav. *29*: 107–115.

MEIJER, J. H., G. A. GROOS, and B. RUSAK (1986) Luminance coding in a circadian pacemaker: The suprachiasmatic nucleus of the rat and the hamster. Brain Res. *382*: 109–118.

MISTLBERGER, R., and A. RECHTSCHAFFEN (1984) Recovery of anticipatory activity to restricted feeding in rats with ventromedial hypothalamic lesions. Physiol. Behav. *33*: 227–235.

MISTLBERGER, R., and A. RECHTSCHAFFEN (1985) Periodic water availability is not a potent zeitgeber for entrainment of circadian locomotor rhythms in rats. Physiol. Behav. *34*: 17–22.

MISTLBERGER, R., and B. RUSAK (1987) Palatable daily meals entrain anticipatory activity rhythms in free-feeding rats: Dependence on meal size and nutrient content. Physiol. Behav. *41*: 219–226.

MISTLBERGER, R., and B. RUSAK (1988) Food anticipatory circadian rhythms in rats with paraventricular and lateral hypothalamic lesions. J. Biol. Rhythms *3*: 277–291.

PICKARD, G. E. (1982) The afferent connections of the suprachiasmatic nucleus of the golden hamster with emphasis on the retinohypothalamic projection. J. Comp. Neurol. *211*: 65–83.

ROSENWASSER, A. M., and N. T. ADLER (1986) Structure and function in circadian timing systems: Evidence for multiple coupled circadian oscillators. Neurosci. Biobehav. Rev. *10*: 431–448.

ROSENWASSER, A. M., R. J. PELCHAT, and N. T. ADLER (1984) Memory for feeding time: Possible dependence on coupled circadian oscillators. Physiol. Behav. *32*: 25–30.

ROSENWASSER, A. M., J. SCHULKIN, and N. T. ADLER (1985) Circadian wheel-running activity of rats under schedules of limited daily access to salt. Chronobiol. Int. *2*: 115–119.

ROSENWASSER, A. M., J. SCHULKIN, and N. T. ADLER (1988) Anticipatory appetitive behaviour of adrenalectomized rats under circadian salt-access schedules. Animal Learn. Behav. *16*: 324–329.

STEPHAN, F. K. (1981) Limits to entrainment to periodic feeding in rats with suprachiasmatic lesions. J. Comp. Physiol. *143*: 401–4101.

STEPHAN, F. K. (1983) Circadian rhythm dissociation induced by periodic feeding in rats with suprachiasmatic lesions. Behav. Brain Res. *7*: 81–98.

STEPHAN, F. K., J. SWANN, and C. SISK (1979) Anticipation of 24 h feeding schedules in rats with lesions of the suprachiasmatic nucleus. Behav. Neural Biol. *25*: 346–363.

TERMAN, M., J. GIBBON, F. FAIRHURST, and A. WARING (1984) Daily meal anticipation: Interaction of circadian and interval timing. Ann. NY Acad. Sci. *423*: 470–4874.