CIRCADIAN RHYTHM IN PLASMA INORGANIC PHOSPHORUS AND SULFUR OF THE RAT: ALSO IN SUSCEPTIBILITY TO STRYCHNINE

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INTRODUCTION

There is unequivocal evidence that most behavioral and physiological properties of plants and animals show rhythmic variation with a daily repetition. Two recent articles, one dealing with plants and the other with animals, have reviewed the literature. By rhythmic is meant that at certain times within a 24-hr period there are maximum and minimum values of the measured function which do not occur at random but rather are regulated along the time scale. These variations may be from a few percent as in the case of body temperature to over a ten-fold range; one example of the extreme range is the cell division rate in the corneal epithelium of the rat.

The period of the cycle usually is precisely 24-hrs when the organism is subjected to the natural geophysical light-dark cycle or to the artificial one of the laboratory. In the absence of the environmental light-dark cycle the oscillation may persist, but the period may deviate more or less from that of the earth's rotation. Such a rhythm, that persists in the absence of an environmental periodic synchronizer, is endogenous and is called a circadian rhythm.

Once determined, the occurrence of any phase of the rhythm such as the beginning or the end of an event (only if continuously monitored) or the "crest" or "trough" can be predicted in relation to local clock time, providing all environmental conditions remain exactly the same.

As a consequence of the time structure, the organism is characterized by a periodically changing sensitivity to stimuli from the environment. The effect of a potentially noxious agent such as a drug depends, at least partly,
on the phase at which it encounters the circadian system. The present report further documents the periodically changing sensitivity in the rat to strychnine and also to the traumatic procedure of doing a cardiac tap to obtain blood subsequent to giving a therapeutic dose of sodium pentobarbital. In each case mortality was the end-point measured.

MATERIALS AND METHODS

Male Sprague-Dawley rats averaging 250 g were used in the first phase of the experiment. Four weeks prior to the study, the animals were housed, two to a cage, in a light-tight room maintained at 23 ± 2°C. The room was illuminated artificially from 0600 to 1800 (CST) and completely darkened from 1800 to 0600 (the abbreviation LD used in this report indicates this specific type of light-dark cycle). Only in the case of starved animals were the lights gradually dimmed from 1700 until 1800 at which time they were completely off. The intensity of light gradually began to increase from zero at 0500 and reached full illumination (about 600 lux) at 0600. In all other aspects of this study the "lights on" and "lights off" was the result of abrupt flick of a switch. Rockland rat chow and water were available ad lib. The room was entered three times per week on Monday, Wednesday, and Friday at exactly 1400 for cage cleaning and replenishing of food and water.

A record of the daily motor activity of the colony shown in FIGS. 1, 5 and 7 was obtained by placing a sensitive microphone in the room. Noises emanating from the colony through feeding, scratching on wire cages, running, vocalizing or fighting were picked up by the microphone, amplified, and fed into a capacitor which, in turn, discharged every 2 sec. This discharge drove a galvanometer in a strip-chart recorder which operated 24-hr/day.

Every hour during one 24-hr period, a subgroup of four rats was anesthesized with sodium pentobarbital (35 mg/kg), and cardiac taps were performed. The blood was centrifuged, and the plasma was removed and frozen. An identical procedure was repeated 14 days later on the same colony. The subgroup representing one particular hour on the 1st experimental day did not represent the same hour on the 2nd sampling day, because both times the animals were selected at random. Estimation of inorganic phosphorus was carried out by the method of Fiske and Subbarow, and inorganic sulfur by the method of Henry.

In the second phase, the effect of subjecting the colony to continuous illumination (about 600 lux) on the inorganic phosphorus and inorganic sulfur rhythm over a 24-hr period was examined (the abbreviation LL signifies this type of environmental control). All procedures described above for the first phase were identical except for lighting. The LL rats were slightly older (340 g) and were subjected to this environment for 19 days prior to sampling.

In the third phase animals were blinded by bilateral enucleation; and 25 days subsequent to the operation (4–26–66), blood samples were obtained as previously described during a 24-hr period. These samples were analyzed for inorganic phosphorus and sulfur. These rats averaged 390 g. Approximately five months later (9–8–66) a second sampling was obtained similarly from the same colony of rats during another 24-hr period. These rats averaged 495 g. In the latter group only inorganic phosphorus was determined, and only 4 samples at bihourly rather than hourly intervals were collected.

In the fourth phase (2–27–69) animals were deprived of food but not water for 24 hours prior to the initial cardiac taps at 0900. From 0900 on, subgroups of 8 rats were
sampled over a single 24-hr period; the 0700 subgroup had been starved for 46 hours. As usual some animals died as a result of the cardiac tap; in this particular study we recorded the per cent mortality at each time point.

The individual hourly (not bihourly) data obtained are expressed as 3-hr moving averages±SE. Reporting the data in such a manner eliminates some of the hourly fluctuations that may be of little significance in the over-all daily pattern, and the basic trends over the 24-hr period are better illustrated.

Statistical analyses were made by calculating the standard error of the difference between the means to be compared and the resulting t values. The probability then was determined by referring to standard t tables.

RESULTS

Fluctuations in inorganic phosphorus

LD 12-15-65. FIG. 1 demonstrates that a crest in inorganic phosphorus occurred between 1400 and 1600; an hourly peak mean value of 7.7 mg/100 ml of plasma was recorded for both peak hours. Subsequent to the crest hours there initially was a rather abrupt decrease which gradually leveled off until the lowest hourly mean of 6.4 mg/100 ml of plasma was recorded at 0600. The overall 24-hr mean value was 7.0 mg/100 ml of plasma.

LD 12-29-65. In the second study (12-15-65, FIG. 1), the pattern in phasing was strikingly similar to one for total protein which was reported previously by Scheving, Pauly and Tsai, 1968. The highest recorded value of 7.8 mg/100 ml of plasma occurred toward the end of the crest at 1600. The lowest recorded value of 6.0 mg/100 ml of plasma occurred at 0300, and this trough continued until 0500 when levels began increasing. The overall 24-hr mean was 6.3 mg/100 ml of plasma.

When the highest recorded hourly mean values of each of the above experiments were compared with their respective 24-hr mean values, the differences were statistically significant (P<.01).

Effects of continuous illumination on inorganic phosphorus rhythm

LL. FIG. 2 demonstrates the effect of continuous illumination on the circadian rhythm of inorganic phosphorus. The highest recorded mean value was 7.2 mg/100 ml of plasma at 2100. The lowest recorded mean value (5.6 mg/100 ml of plasma) occurred at 0500. The overall 24-hr mean was 6.5 mg/100 ml of plasma. When the highest and lowest hourly mean values were compared with the overall 24-hr mean (6.5 mg/100 ml of plasma), there was statistical significance (P<.001).

Effects of blinding on inorganic phosphorus rhythm

Blinded. FIG. 3 demonstrates the effect of blinding on the circadian rhythm of inorganic phosphorus. The sampling done on April 26 and 27, 1966 demonstrates a rhythm with the peak value (5.7 mg/100 ml plasma)
FIG. 1. The individual plotted values, calculated as 3-hr moving averages, are the means (with standard error in one case) which indicate the plasma inorganic phosphorus pattern in a colony of rats over a 24-hr period. Both studies were performed on the same animals. Also, activity pattern of a colony of rats over a 24-hr period based on noise emitted by the colony. Animals were maintained under a light-dark cycle (light from 0600 to 1800).

occurring at 2000; the lowest mean value (4.4 mg/100 ml plasma) was recorded at 0300. When both the highest and lowest recorded mean values were compared to the overall 24-hr mean (5.1 mg/100 ml plasma), they were significant (P<.05). On the second experimental day begun on 9-8-66, the peak hour (5.5 mg/100 ml plasma) had shifted to 2200. The lowest recorded mean value was 4.7 mg/100 ml of plasma and occurred at 1800. The overall 24-hr mean was 5.1 mg/100 ml of plasma. When the highest recorded mean value was compared to the overall 24-hr mean, their differences were statistically
FIG. 2. Comparison of 24-hr patterns in plasma inorganic phosphorus in rats maintained under LD 12:12 (light 0600-1800) with animals subjected to constant illumination (LL).

There was no significant difference when the lowest hourly mean was compared with the overall 24-hr mean. The general trend of the rhythm in blinded animals is to flatten and become more irregular.

There is a decrease in the overall 24-hr mean in the plasma inorganic phosphorus level in blinded animals when compared to LD or LL animals. The average 24-hr mean value for LD animals was 6.6 mg/100 ml of plasma; for LL animals it was 6.5 mg/100 ml of plasma; the same average for blinded animals was 5.1 mg/100 ml of plasma. The differences were statistically significant (P<.01).
**Effects of starvation on inorganic phosphorus rhythm**

*Starved 2-27-69.* Fig. 4 demonstrates that a rhythm persists for inorganic phosphorus in the starved animal. The highest recorded mean hourly value was 7.0 mg/100 ml of plasma at 1100; the lowest recorded hourly mean (5.6 mg/100 ml of plasma) occurred at 1900. When the highest and lowest recorded hourly means were compared with the overall 24-hr mean value (6.5 mg/100 ml of plasma), the resulting P values were <.05 and <.001 respectively.

**Fluctuations in inorganic sulfur**

*LD 12-15-65.* Fig. 5 demonstrates an overt rhythm for inorganic sulfur
Comparison of the 24-hr patterns in plasma inorganic phosphorus in rats maintained on ad libitum and starvation diets. All animals were maintained under LD 12:12 (light from 0600-1800).

with the crest (3.6 mg/100 ml of plasma) occurring at 1300 and 1400; the lowest recorded hourly mean (1.85 mg/100 ml of plasma) occurred at 2200. The overall 24-hr mean was 2.6 mg/100 ml of plasma. When the maximum and minimum 24-hr means were compared to the 24-hr mean, the difference were statistically significant (P<.001).

Effects of continuous illumination on inorganic sulfur rhythm

LL. Fig. 6 demonstrates the effect of continuous illumination on the synchronized circadian rhythm of inorganic sulfur. The highest hourly mean was 2.9 mg/100 ml of plasma and occurred 0800; the lowest (2.4 mg/100 ml of plasma) was recorded at 2200. When the highest and lowest recorded hourly means were compared with the overall 24-hr mean value (2.7 mg/100 ml of
The individual plotted values, calculated as 3-hr moving averages, are the means (with standard errors) which indicate the plasma inorganic sulfur pattern in a colony of rats over a 24-hr period. Also, activity pattern of a colony of rats a 24-hr period based on noise emitted by the colony. Animals were maintained under a light-dark cycle (light from 0600 to 1800).

plasma), there was some degree of significance (P<.05). The general trend was an overall flattening of the rhythm.

**Effect of blinding on the plasma inorganic sulfur rhythm**

4-26-66. The maximum hourly value occurred at 1500 with 2.5 mg/100 ml of plasma; the minimum hourly value (1.8 mg/100 ml of plasma) was recorded at 1000. When both the highest and lowest recorded means were compared with the overall 24-hr mean, the differences were statistically significant (P<.01).

The data indicate a relationship between the blinded state and the absolute levels of inorganic sulfur. The 24-hr mean for the blinded animals was 2.2 mg/100 ml of plasma; whereas the same was 2.6 and 2.7 for LD and LL animals respectively. This difference is highly significant (P<.001).
DISCUSSION

LD The results clearly indicate that inorganic phosphorus and sulfur levels in the plasma of rats vary with a circadian period. The percentage increase for inorganic phosphorus between the minimum and maximum mean hourly values was 21% in the first and 32% in the second analysis. Nelson reported a similar rhythm for inorganic phosphorus in mice.

The reproducibility in phasing seen in Fig. 1 clearly demonstrates that in relation to local clock time the phasing of the inorganic phosphorus rhythm, once determined, can be predicted as long as the animals used from study are subjected to the same rigidly standardized conditions. The tendency for absolute values of inorganic phosphorus in the second analysis (Fig. 1, 12-29-65) to be slightly lower than those seen in the first analysis (Fig. 1, 12-15-65) at specific hours along the 24-hr time scale cannot be explained at
this time. When compared to the inorganic phosphorus, a higher amplitude rhythm exists for inorganic sulfur; the difference between the minimum and maximum mean values was 93%. Both inorganic phosphorus and sulfur essentially have identical phasing (cf., Figs. 1 and 5). The peak levels of these two metabolic end products occur at a time when animals first begin to show signs of arousal. Examination of the data relating to daily motor activity of the entire colony in Figs. 1 and 5 demonstrates that rats commence to be active at about 1500; this is a time of day when they first begin to move around. The activity is a low level type and remains so until the lights "go off" at 1800 at which time there is a burst of activity for the colony. Up to the time of the enhanced sympathetic activity, the two metabolic end-products under discussion tend to increase in amount in the blood and then fall precipitously until the lights "go off." Once the animals are in the dark and are active, their inorganic sulfur levels gradually begin to increase; whereas the inorganic phosphorus merely levels off and begin to increase several hours later.

Many other components of blood or plasma in rats fluctuate with the same phasing as inorganic phosphorus or sulfur; one which is documented well is plasma corticosterone levels in rats and mice. In recent work levels of serotonin in whole brains of rats were shown to have essentially the same phasing as the plasma inorganic phosphorus and sulfur in this study; norepinephrine in these same brains was at its lowest level when serotonin was at its highest level. Taken collectively we interpret the pattern in the phasing of the above mentioned physiological variables as evidence that 1400 to 1600 represents a turning point in time for metabolic machinery, in this case the net result being a switch from inactivity or sleep to a generalized enhanced activity. We predict that if the environmental light-dark cycle were shifted, there gradually would follow a corresponding shift in the time when this enhanced activity would begin. Further evidence to support this impression of a switch in metabolism can be obtained by analyzing the fluctuation seen in other physiological variables such as the cell division rate in corneal epithelium.

Since the main source of sulfur in the body comes from the protein diet, specifically from the amino acids, cystine, cysteine and methionine, it was interesting to compare the phasing of the inorganic sulfur rhythm with the rhythm of total protein which had previously been determined on the same plasma. Fig. 7 shows that the phasing of the total protein rhythm and inorganic sulfur rhythm essentially are reversed. Such is what one would expect; since at least some of the protein metabolism must be reflected in the inorganic sulfur rhythm. The same reasoning can be applied to the inorganic phosphorus rhythm inasmuch as its production depended, in part, on protein metabolism.
FIG. 7. Comparison of the 24-hr patterns in plasma inorganic sulfur and total protein in the same plasma. Also, activity pattern of a colony of rats over a 24-hr period based on noise emitted by the colony. Animals were maintained under LD 12:12 (light from 0600 to 1800).

Some early investigators attributed metabolic rhythms to a "feeding phenomenon"; therefore, ostensibly to avoid these fluctuations, they employed fasting animals in their studies. It is evident from Fig. 4 that the levels of nutrition do affect the absolute values of inorganic phosphorus but not the rhythmic pattern; although a slight phase shift in this particular case persists. The shift in phasing could be due to the fact that the starved animals of this investigation were on a somewhat different light-dark cycle. The difference involved the gradual dimming of the lights rather than the abrupt "on or off" type of switching. The dimming program used is discussed in the
Fig. 8. The plotted values in one case are means which indicate the percentage of mortality when subgroups of 15 rats were injected with a potentially lethal dose of strychnine at different time points along a 24-hr scale. The second set of plotted values also are means; they indicate the percentage mortality in rats (8 per time point) that were injected with a therapeutic dose of sodium pentobarbital and subsequently subjected to a cardiac tap, at which time about four ml of blood were removed. In both cases the rats had been subjected to LD 12:12 (light from 0600 to 1800).

Section on MATERIALS AND METHODS. This finding of a persisting rhythm in starvation is similar to a paper by Pauly and Scheving (1967), which reported that the plasma glucose rhythm persisted in rats during starvation. Also, ChiaKulas and Scheving (1965) reported that a liver glycogen rhythm persisted in urodele larvae deprived of food for as long as three weeks. Extensive studies by Halberg, et al. (1965) show that circadian rhythms in such diverse functions as pituitary adrenocorticotropic activity, pineal mitosis and rectal
temperature, as well as in the corticosterone content of serum and adrenal persist in mice deprived of both food and water.

When one analyzes the rhythm of inorganic phosphorus in a colony of animals subjected to LL, it is evident that there still is significant fluctuation even though the profile of the rhythm has radically changed; the percentage increase between the lowest and highest recorded hourly mean is 29% (P < .001). The fluctuation in inorganic sulfur differs in that it is very much flattened, and the variation seen between any two points along the 24 hour time scale is not statistically significant. In the past we have found that there is an extreme flattening or even a complete loss of the characteristic LD rhythm in the colony for a number of different variables when animals are subjected to LL. The possible explanation we have offered is that individual rats freerun on their own frequencies, because the animals no longer are synchronized to a light-dark cycle; the net result is a mathematical flattening or loss of the LD rhythm in the colony. This explanation would satisfy the loss of the inorganic sulfur rhythm in LL. We are at a loss to explain why inorganic phosphorus behaves differently. Only one other physiological variable which we have tested so far continues to show an overt rhythm in LL, and that is the rhythm seen in blood clotting time in rats. In both inorganic phosphorus and sulfur, the rhythms persist but are modified very much. Such is in agreement with every other physiological function we have tested to date. The data on Fig. 3 demonstrates that the phasing of the rhythm that does persist in inorganic phosphorus changes from time to time. Again a suggested explanation is that the blinded animals are freerunning both as individuals and as socially synchronized subgroups on different frequencies. This also could account for the multiple peaks characterizing the rhythm in the blinded rats and the ultimate flattening which was seen in the second experiment on blinded animals (Fig. 3, 9–8–66).

We stress that the explanation offered here to account for the changes seen in the typical LD pattern when animals are subjected to LL or are blinded is speculative; the data presented only suggest these changes might be due to the phenomenon of freerunning.

The data indicate a relationship between the blinded state and the absolute level of inorganic phosphorus and sulfur; these substances are significantly lower in the blinded animals when compared either with the LD or LL animals. We can offer no explanation for this observation.

Some investigators have the erroneous impression that as long as they sample experimental and control animals at the same time, they do not have to worry that rhythms might affect the results. This is not the case; for Fig. 2 demonstrates what could happen to such an investigator interested in determining the effect of continuous illumination on inorganic phosphorus levels in the plasma of rats. If he used only one time point for sampling the
controls and experimental animals, he might arrive at different conclusions depending on the time he selected. If sampling occurred between 0600 and 1800 (the normal light hours), the result might be anywhere from slight to significant increase in inorganic phosphorus as a result of the stimuli of constant light; if sampling was performed from 1800 to 0600 (the normal dark hours), the result could be anywhere from slight to significant decrease in inorganic phosphorus as a result of the stimulus.

Similarly if a researcher were interested in the effect of blinding on inorganic phosphorus levels, he might conclude that the procedure significantly decreased the levels in plasma, as our results demonstrate. However, if he sampled at night (as graduate students frequently do), the differences would not be as significant; because this is a time when the controls have the lowest levels of inorganic phosphorus, and these low levels approximate the highest levels of blinded animals. Fig. 6 demonstrates more dramatically that the methodological pitfall described for experiments on inorganic phosphorus also awaits those concerned with the assay of inorganic sulfur in normal and experimental situations.

These studies like ones we have reported for other metabolic parameters indicate that information about the lighting schedule to which animals were exposed, and times of day they were sacrificed probably should be provided in the reports of all investigations dealing with inorganic phosphorus and sulfur. Sampling at the same time of day is not the solution one should use to avoid the effect of rhythms. The best such a practice can do is to assure one that he is sampling either in the trough, on the incline, the crest or on the slope of the rhythm; and he only can be assured of this if the rhythm is first mapped, and his animals then are maintained under the same light-dark cycle. In the light-dark cycle of nature, the phasing of the rhythm continually changes with the progression of the seasons.

During the past two decades the idea that the capacity for circadian time-keeping is found in all forms of life from the eukarotic microorganism to man has become widely accepted (EHRET and WILLE, in the press). We support the view that such is a ubiquitous, innate, property that is a fundamental requisite for normal functioning of most organisms of higher order than the prokaryotes.

A highly significant consequence of the oscillatory nature is that the biochemistry of an organism is changing continually. The changes resulting from rhythms do more than confound those concerned with bioassay; they even may determine whether or not an animal will have the capacity to survive a noxious stimulus. This latter point is demonstrated in Fig. 7. When 2 mg/kg of strychnine were administered to separate but comparable subgroups of 15 rats at 4-hr intervals over a single 24-hr period, it was observed that 35% died at 1500; whereas 85% died at 2100. Further evidence
that an organism will respond to the combined stimulus of anesthesia and experimental manipulation was seen in the experiment on starvation reported in this paper. Blood was obtained by cardiac tap from starved animals over a 24-hr period. The rats were anesthetized with a therapeutic dose (35 mg/kg) of sodium pentobarbital (Nembutal). Twice as many animals died during the dark hours of the period as did during the light; maximum mortality occurred at 0100 when 50% of the animals died. The early and middle portions of the dark hours previously have been shown to be times when rats are most susceptible to sodium pentobarbital. Such results were obtained by using an endpoint such as the duration time of narcrosis with therapeutic doses and also by determining mortality rate subsequent to injecting potentially lethal doses. It might be argued that the reduced efficiency on the part of the person doing the cardiac tap potentiated the mortality. It is our opinion that this was not the case; since the same experienced person performed all the cardiac taps, and also the same person gave all the injections. We believe that if technique alone were responsible for the deaths, the highest mortality should have been at 0900 when the experiment began; because this was a time when the technician was refamiliarizing himself with the delicate technique. We do emphasize that the mortality from cardiac taps, even in the hardy rat, can be very high when performed by inexperienced personnel.

What we have attempted to point out is that the rhythmic nature of any physiological variable such as inorganic phosphorus or sulfur would be of great importance to any quantitative study whether it be one to compare an experimental situation to a control or merely one to establish so-called "resting levels." To ignore this phenomenon in the experimental design of any such investigation could give rise to a great deal of variation and even to possibly conflicting results. Finally, it must be concluded that consideration of time structure in an organism as revealed by its rhythms may lead to the elucidation of many unexplained biological mechanisms. Understanding these rhythms may be necessary for the eventual explanation of why the time we administer a therapeutic or toxic substance may tip the scale between life or death, or between maximum or minimum effectiveness.

SUMMARY

In the first phase of this investigation adult, male Sprague-Dawley rats were maintained under rigidly standardized environmental conditions which included an artificial light-dark cycle (light from 0600 to 1800). During two separate 24-hr periods subgroups of rats were removed from the colony room every hour or the hour and a cardiac tap was performed on each rat subsequent to sodium pentobarbital anesthesia. Inorganic sulfur and phosphorus
determinations were made on the plasma obtained from these rats. The pattern of fluctuation in both compounds over the 24-hr period indicated that both had a circadian component with essentially the same phasing. The crest in the level of both compounds occurred between 1300 and 1600, the trough values for inorganic phosphorus occurred between 0300 and 0600, for the inorganic sulfur it occurred at about 2200.

Similar studies were made on starved, blinded, and also on rats subjected to prolonged periods of continuous illumination. The inorganic phosphorus rhythms persisted in starved animals, inorganic sulfur was not determined on starved animals.

The rhythms of both compounds persisted with altered phasing in blinded animals. The rhythms seen in blinded animals were somewhat flattened and had a tendency to be irregular when compared to animals kept in a light-dark cycle. Both the inorganic sulfur and phosphorus levels were significantly lower in blinded animals when compared to animals subjected to a light-dark cycle or to continuous illumination.

In those rats subjected to continuous illumination the general tendency was for the inorganic sulfur rhythm to flatten out. The inorganic phosphorus rhythm persisted under this environmental condition but the profile was radically altered.

Evidence is presented to indicate that the ability of the organism to respond to a potentially lethal dose of a drug depends on the time of day the drug is administered. When an identical dose, based on body weight, of strychnine was given to subgroups of rats at different time points along a single 24-hr time scale as many as 85% died at one time of day whereas only 35% died at another time of day.

Evidence also is presented to indicate that the ability of the rat to survive a cardiac tap while under a therapeutic dose of sodium pentobarbital may depend on the time of day the cardiac tap is performed. The importance of recognizing the time structure of the living organisms is discussed.

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REFERENCES


