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PHARMACOLOGICAL IMPLICATIONS

OF CIRCADIAN RHYTHMS

THE RELATIONSHIP OF CIRCADIAN RHYTHMS TO SPECIFIC CNS

AND PERIPHERAL BIOLOGICAL SUBSTANCES,

PHYSIOLOGICAL FUNCTION AND DRUG RESPONSIVENESS

By

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A Dissertation Submitted to the Faculty of the Graduate School

of Loyola University in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

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1969

BIOGRAPHY

Charles Andrew Walker was born on December 14, 1935 in Arkansas, where he received his primary, secondary and undergraduate college education. In 1953 he graduated from the Lincoln High School in Forrest City, Arkansas. In September of 1953, he entered Arkansas State College where he majored in animal science and minored in biology. In June of 1957, he was awarded the degree of Bachelor of Science. The following September Mr. Walker was granted a research assistantship and admitted for graduate study at Washington State University. In June of 1959, he received the Master of Science degree in animal nutrition and biochemistry. From 1959 to 1963 he served on the faculty at The Fort Valley State College as an assistant professor of biology. From 1963 to 1965, Mr. Walker taught pharmacology and physiology in the School of Veterinary Medicine at the Tuskegee Institute. In June of 1965, he was admitted to Loyola University's Graduate School and awarded a trainee grant by the Department of Pharmacology.

BIOGRAPHY (Continued)

MEMBERSHIP IN PROFESSIONAL SOCIETIES

The American Association for the Advancement of Science

The New York Academy of Sciences

The Alabama Academy of Sciences

The American Association of Clinical Chemists

HONORS AND AWARDS

One of fifteen graduate students chosen for an award by the

American Society of Pharmacology and Experimental Therapeutics for

research (on indoleaklylamines) conducted while a graduate student.

LIST OF PUBLICATIONS

1. Siddique, I. A. and Walker, C. A. (1967) Effects of Listeria mono-
     cytogenes hemolysin on isolated ileum of rabbit. Am. J. Vet.
     Res., 28:1843-1849.

2. Walker, C. A. and Friedman, A. H. (1967) Circadian rhythms of
     biogenic amine levels in the brain stem and caudate nucleus of the
     rat. Pharmacologist, 9:236. (Abstract)

3. Friedman, A. H. and Walker, C. A. (1968) Circadian rhythms in rat
     midbrain and caudate nucleus biogenic amine levels. J. Physiol.
     (London), 197:77-85.

4. Walker, C. A. and Friedman, A. H. (1968) Circadian rhythms in the
     toxicity of cholinomimetics in mice. Fed. Proc., 27:600.
     (Abstract)

5. Walker, C. A., Speciale, S. G. and Friedman, A. H. (1968) Circadian
     changes in pentobarbital-induced sleeping time and their relation-
     ship to biogenic amine levels in the rat. Pharmacologist, 10:105.
     (Abstract)

6. Friedman, A. H. and Walker, C. A. (1968) Rat brain amines, blood
     histamine and glucose levels in relationship to circadian changes
     in sleep induced by pentobarbital sodium. J. Physiol. (London),
     202:133-146.

7. Friedman, A. H. and Walker, C. A. (1969) Circadian rhythms in
     central acetylcholine and the toxicity of cholinergic drugs.
     Fed. Proc., 28:251. (Symposium)

8. Walker, C. A. and Friedman, A. H. (1969) Circadian rhythms in hypo-
     thalamic and caudate nucleus ultrastructure of untreated rats and
     those pretreated with reserpine of dl-DOPA and an MAO inhibitor.
     Fed. Proc., 28:444. (Abstract)

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ABSTRACT

PHARMACOLOGICAL IMPLICATIONS OF CIRCADIAN RHYTHMS

Circadian patterns for rectal temperature and biogenic amine levels in the midbrain and caudate nucleus were established in rodents maintained under stable laboratory conditions for at least three weeks on an automatically-timed light program in which the light phase lasted from 0800 to 2000 hours daily. Levels of norepinephrine (NE), histamine (H) and acetylcholine (ACh) in the rat were maximal during the dark phase of the illumination cycle coinciding with maxima for rectal temperature and motor activity and were minimal during the light phase when these nocturnal animals are quiescent. The circadian pattern for serotonin (5-HT) is twelve hours out of phase with the other parameters measured. Bilateral adrenalectomy did not alter these patterns qualitatively, although quantitative changes were observed, e.g., peak rectal temperature was significantly lowered. Pentobarbital sodium reversed the rectal temperature pattern significantly. Minima occurred during the dark phase of the illumination cycle. Significant increases in NE maxima in midbrain and caudate nucleus and in caudate nucleus H were observed after pentobarbital sodium. The 5-HT pattern however was depressed. The onset and duration of sleep induced by pentobarbital sodium exhibited a circadian pattern. Onset time

was significantly longer and duration significantly shorter during the dark phase coinciding with ACh, NE, H, rectal temperature and motor activity maxima. Peak blood H levels were noted at the end of the light phase and the circadian patterns were unaltered by pentobarbital sodium. Blood glucose patterns were bimodal in nature. A primary peak occurred at the end of the dark phase toward the end of the normal feeding period, while a secondary peak occurred at the end of the light phase. These patterns were unaltered by pentobarbital sodium.

Norepinephrine levels in rat hypothalamus were significantly higher at 0300 hours than at 1500 hours, reflecting similar changes observed in midbrain and caudate nucleus. Significant reductions and elevations were obtained by pretreatment with reserpine or dl-DOPA and pargyline (an MAO inhibitor), respectively.

An examination of the ultrastructure of the hypothalamus and caudate nucleus showed that circadian changes were demonstrable when synaptic vesicles in tissue removed at 0300 and 1500 hours were counted. The number of granular vesicles (presumably containing NE) were significantly higher at 0300 hours in the anterior hypothalamus and caudate nucleus, but not in the posterior hypothalamus. The number of agranular vesicles (presumably containing ACh) was higher at 0300 hours, but only in

the posterior hypothalamus was this increase statistically significant. Granular vesicle counts from tissues of rats pretreated with reserpine or dl-DOPA and pargyline were similar to those of the control study in that they were higher at 0300 hours than at 1500 hours. A more variable pattern was obtained with the agranular vesicle counts. The most significant finding was the greater number at 1500 hours in the tissues from reserpinized animals. A similar finding was obtained in the anterior hypothalamus after dl-DOPA and pargyline administration.

Circadian patterns for the toxicity of cholinergic compounds were demonstrated in mice. LD50's for ACh, pilocarpine and oxotremorine were minimal (greatest toxicity) during the dark phase of the illumination cycle. The circadian pattern for atropine toxicity was essentially a mirror image with maximum toxicity (minimal LD50) during the light phase. A quaternary anticholinesterase, neostigmine, is most toxic during the late dark phase, while a tertiary anticholinesterase, physostigmine, was least toxic at that time and became progressively more toxic during the late light and early dark phase of the illumination cycle. Atropine methyl nitrate decreased toxicity of acetylcholine and physostigmine but the circadian pattern was essentially unchanged. An ultradian toxicity pattern having two peaks, one at midday, the second at midnight, was obtained with carbachol.

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LIST OF ABBREVIATIONS

Serotonin, 5-Hydroxytryptamine 5-HT

Norepinephrine, Noradrenaline .... NE, NA

Acetylcholine .... ACh

Acetylcholinesterase .... AChE

Hydroxyindole-o-methyl transferase .... HIOMT

Monoamine Oxidase .... MAO

Monoamine Oxidase Inhibitor .... MAOI

Deoxyriboneucleic Acid .... DNA

Riboneucleic Acid .... RNA

5-Hydroxytryptophane 5-HTP

O-Phthalaldehyde .... OPT

Standard Deviation .... S.D.

Probability .... P

Lethal Dose 50 .... LD50

Effective Dose 50 .... ED50

Central Nervous System .... CNS

Alpha Methyl Meta Tyrosine .... AMMT

Dihydroxyphenylalanine .... DOPA

Electron Microscope .... E. M.

Intravenous .... I. V.

Intraperitoneal .... I. P.

Subcutaneous .... S. C.

CHAPTER I

INTRODUCTION

The influence of biological rhythms on physiological phenomena and body structure is illustrated in the works of art of ancient Egypt. According to Fuchs (1964) many of the harpists shown on the bas reliefs in the tombs of Pharaohs and high dignitaries of Egypt were blind and exhibited physical characteristics quite different from persons with sight. The blind harpists were depicted with puffy faces and wrinkles on the neck and abdomen whereas persons with sight (open-eyed) were shown with slim figures and smooth skin. Fuchs (1964) indicated that endocrine disturbances and alterations in metabolism result from blindness. Today we would have described this condition by stating that visual pathways alter biological rhythms concerned with endocrine metabolism. While alterations in biological rhythms have been implied by an examination of Egyptian art at the time of the Pharaohs, this aspect of science has received concentrated and specialized impetus within recent years. We are now beginning to develop an interest in the role and significance of biological rhythms in scientific experimentation, clinical application and in the therapeutic effectiveness of drugs. An understanding of various biological patterns for different species and classes of animals might help to explain some of the factors that influence the psychology, physiology and physical characteristics peculiar to different types of animals.

Since the early nineteenth century, the terminology describing biological rhythms in animals has undergone numerous changes. "Biological clock" is a common term applied to such rhythms. This term was used primarily in describing the rhythmic activity of marine animals. Rhythms in terrestrial animals were mainly referred to as physiological or biological rhythms. The term "circadian" was introduced by Halberg (1959) and means "about 24 hours". The term, as now generally used, includes nocturnal (night) and diurnal (day) rhythms when both or each occur within a 24-hour day. Halberg (1962) described cycles longer than 24 hours as supradian and cycles shorter than 24 hours as infradian. Halberg and Reinberg (1967) subdivided the broad spectrum of physiological rhythms and described the terminology mathematically as follows: "I. A high frequency domain of rhythms with periods (τ) shorter than 0.5 hours; II. A medial frequency domain of rhythms with τ ranging in length from 0.5 hours to 2.5 days, including the regions of ultradian (0.5 hours $\leqslant \tau <$ 20 hours), circadian (20 hours $\leqslant \tau \leqslant$ 28 hours) and infradian (28 hours $< \tau \leqslant$ 2.5 days) rhythms; III. A low frequency domain of rhythms with τ longer than 2.5 days: inter alia, circaseptan ($\tau \sim$ 7 days), circavigintan ($\tau \sim$ 20 days), circatrignintan ($\tau \sim$ 30 days), circannual ($\tau \sim$ 1 year) rhythms."

The improper use of the term "circadian" has met with some criticism. Wurtman (1967) noted that unless a consistant pattern is determined for several days within the same animal for a specific biological substance, one cannot accurately state that the cycle is approximately 24 hours. In addition to the so-called "circadian" rhythm, some animals display yearly, seasonal, monthly and even hourly rhythms. Halberg and Reinberg (1967), previously mentioned, suggested terminology for these rhythms but the scientific usage of these terms is not wide spread.

CHAPTER II

REVIEW OF LITERATURE AND OBJECTIVES

A. PARAMETERS AFFECTING BIOLOGICAL RHYTHMS

There are a number of factors that directly or indirectly influence biological rhythms. Aschoff (1963) is of the opinion that there is no convincing evidence in favor of the hypothesis that circadian rhythms under constant conditions are controlled by unknown periodic variables in the environment although external parameters do to some extent influence physiological mechanisms and endogenous substances. He further states that all facts are in agreement with the idea that circadian rhythms are autonomous and largely self-sustained oscillations. It is of value to consider some of the factors that modify biological rhythms (vide infra).

1. Temperature (Ambient and Rectal)

Harker (1958) noted that biological rhythms are not sensitive to moderate ambient temperature changes (fluctuations of 5 to 10°F). Brown and Webb (1948) found that by lowering the environmental temperature to 0°C the diurnal color changes of Uca pugnax (a species of crab) could be delayed for a period equivalent to the duration of the decreased temperature. These findings suggest the possibility that two biological clocks exist, one which is insensitive to moderate ambient temperature changes and another for emergency or extreme changes that is temperature sensitive.

Kleitman (1949) found that rectal temperature of rabbits exhibited a rhythm but that the rhythm was linked to the feeding habit of the species. When food was restricted to a few hours (0900 hours to 1600 hours) the rectal temperature peaks shifted from mid-evening to mid-afternoon. Halberg (1959) showed that the rectal temperature rhythm is exactly in phase with the motor activity rhythm. He showed that maxima and minima fell about midnight and noon, respectively, even when body temperature is artificially raised and lowered and the activity rhythm remains constant. Brahmachary (1967) concluded that in nocturnal animals the activity rhythm is apparently a primary rhythm while body temperature is a function of motor activity and metabolism. Kleitman (1949) indicated that the daily diurnal body temperature rhythm in man is developed by acculturation and maintained by and through adherence to the socially prevalent routine of living. He further concluded that, in man, body temperature reaches its peak during the wakeful phase of the 24-hour period and troughs during sleep. This relationship for body temperature circadian patterns is reversed in nocturnal animals such as the rat. Kleitman and Ramsaroop (1948) associated the circadian rhythm in body temperature to circadian patterns of heart and pulse rates. They noted that following a 14-day period of thyroid hormone administration, body temperature paralled

pulse rate. The pulse rate changed 26 counts/minute for each degree (F)

change. Mills (1966) states that neither temperature nor pulse rates show

rhythmic patterns at birth but these are initiated at about four - six weeks

of age and increase in amplitude to approximately two years.

Recently investigators have shown that body temperature changes

are associated with central nervous system biogenic amine changes.

Feldberg, et al. (1954, 1966) noted that brain amines were directly involved

in the hypothalamic control of body temperature. In cats and dogs, serotonin

injected intraventricularly produced an increase in body temperature while

administered norepinephrine caused a fall. Sheard and Aghajanian (1967)

stimulated the caudal midbrain raphé region of rats and increased body

temperature. They also noted a fall in serotonin and a rise in 5-hydroxy-

indole acetic acid. Reserpine and p-chlorophenylalanine which deplete

serotonin, nonspecifically and specifically blocked the rise in body

temperature indicating an apparent involvement of serotonin in the CNS

control of body temperature.

In summary, these studies indicate that moderate ambient

temperature changes do not influence endogenous biological rhythms to a

great extent when compared to the effects of extreme temperature changes.

Rectal temperature exhibits a rhythmic pattern but the rhythm is apparently

a function of physical activity and metabolism. Body temperature changes also reflect or contribute to changes noted in certain CNS biogenic amines.

2. Illumination

Light has been shown to affect biological systems in numerous ways. Probably the major environmental parameter influencing biological rhythms is illumination. Ott (1964) used time-lapse photography to demonstrate that many different animal cells in tissue culture varied in growth pattern when different colored filters were placed in the photographic light source. He also showed that the duration and intensity of light influenced reproduction in fish (guppies). Fish under cool white light produced offspring in ratio's of 4:1 female to male, but the male fish were abnormally retarded in the development of some secondary sex characteristics. Apparently no reproduction occurred in fish maintained under daylight white bluish fluorescent light. He noted that hens subjected to deep pink fluorescent light produced many unfertile eggs while the eggs produced under daylight white fluorescent light were largely fertile. Ott (1964) also reported that mice under daylight produced equal numbers of female to male offspring, while animals under daylight white fluorescent bulbs produced approximately 70% female and 30% male. Under pink fluorescent light the percentages were approximately reversed.

Light has been shown to influence a number of hormonal and enzymatic systems. Axelrod, et al. (1966) noted that the activity of hydroxy-O-methyl transferase in the rat pineal gland is several times greater in rats kept in continuous darkness than for those kept in continuous light. They suggested that the medial forebrain bundle may participate in the control of the enzymatic response to environmental lighting. Wurtman, et al. (1967-a) noted that lesions in the medial hypothalamus or caudate nucleus do not interfere with these responses to light. Rodeick and Smith (1966) observed that cat retinal ganglion cells fired in a slow rhythmic manner in the dark. The rates recorded varied from 2/minute to 2/hour. These slow rhythms were abolished after moderate illumination but were still present following sectioning of the optic nerve. Snyder, et al. (1965) suggested that pineal serotonin and melatonin might be controlled by environmental lighting mediated by the sympathetic nervous system and photoreceptors other than the retinae. Wurtman, et al. (1964) stated that light acts by generating or inhibiting nerve impulses which travel along specific pathways in the brain to centers concerned with the control of endocrine function. Harker (1958) concluded that continuous light may inhibit movement and other functions in animals and may cause drifting of rhythms so that peaks consistenly appear either earlier or later.

Continuous darkness can affect animals similarly. Harker (1958) further states that "the shifting of rhythms in dark and light phases ceases when peaks are adjusted to the time in the cycle in which they normally appear for that particular light or dark phase, also 24-hour rhythms can probably be altered for a number of biological substances by using alternating light and darkness".

In summary, these investigations concerning light and its effect on rhythms indicate that both the quantity and quality of light influences physiological and behavioral responses in animals. Apparently light receptors receive impulses and affect brain levels of biological substances thereby altering and controlling normal physiological rhythmic patterns.

3. Barometric Pressure

Barometric pressure has been shown by some workers to affect 24-hour rhythms. Brown, et al. (1956-a) showed a positive correlation between the activity of oysters and quahogs and the rate of pressure change. Brown (1954) suggested that the respiratory rates of two species of Uca pugnax and of the salamander Triturus showed a significant correlation with the rate of pressure changes. Guyselman (1957) investigated the locomotor activity of the crayfish Cambarus virilis and found a high positive correlation between motor activity and pressure changes. He

indicated that the correlation might be only coincidental since a constant barometric pressure did not alter the 24-hour rhythm. Harker (1958) concluded that barometric pressure fluctuations can influence rhythms in the same manner as light and darkness or temperature do, but that barometric pressure functions as a contributing factor and not as a controlling factor.

4. Feeding Habits

Feeding methods and habits have been found to influence biological rhythms in several different animal species. Von Stein-Beling (1935) found that bees could be trained to collect food during the night but that they were not quite as accurate as during light periods. When cockroaches were fed during their inactive period there was a slight rise in motor activity at the time of feeding but no persistant rhythm was induced that would continue in the absence of food (Harker, 1955). Stein (1951) reported that feeding rhythms in birds will persist in constant light or darkness even after the sleeping rhythm has broken down. Harker (1958) observed that oxygen consumption rhythms in young chicks continue in the absence of food and apparently are more persistant than the feeding rhythm. Calhoun (1945) found that the peak motor activity in the rodent Sigmadon hispidus appears earlier each day when on a meager diet. Rats were observed to lose part

of their motor activity rhythm when on a reduced diet. Shirley (1928) noted
that when mice were fed a high fat diet there were increased periods of
activity but indications of the 24-hour activity rhythm persisted. Wurtman,
et al. (1967-b) reported that persistent low protein diets did not alter the
tyrosine rhythm in humans. Honova, et al. (1968) observed a tyrosine
transaminase rhythm in rats and indicated that a similar type reverse
rhythm that occurs in humans may be controlled by both feeding patterns and
protein diets. Harker (1958) concluded that hunger, digestion and feeding
are likely to effect biological rhythms concerned with locomotion, metabo-
lism and motor activity in animals.

5. Population (Isolation vs Aggregation)

The rhythmic activity of some insects has been shown to vary with
the size of the population. Park and Sejba (1935) found that in the beetle
the position of the peak of activity in the 24-hour cycle, Megalodacne heros,
is a function of the number of animals per group. It was also noted that the
time of emergence of the Drosophila is later each day depending on and in
proportion to the increase in population (Harker, 1958).

In rats it has been shown that a complete reversal of the motor
activity rhythm develops for animals which are low in the pecking order
within the group (Harker, 1958). Allee (1942) and Scott (1959) noted that

prolonged isolation in mice produces an aggressive behavior that can be evaluated in a quantitative manner. The development of aggressiveness in mice has been shown to depend upon strain, sex and duration of isolation (Valzelli, 1967). He further reported that 5-hydroxyindole acetic acid in the brain of isolated animals is lowered when compared to grouped animals. Garattini and Valzelli (1965) noted that brain serotonin turnover rate was decreased and that turnover time was increased in isolated mice as compared to grouped mice. The change in turnover rate did not parallel the onset of aggressiveness.

In summary, isolation and/or aggregation does influence certain biological rhythms. The differences in the rhythms of isolated and aggregated animals are a function of the size of the population and duration of isolation. Recently, differences in specific brain biogenic amines have been noted in grouped vs isolated animals which in turn may be related to the differences noted in social behavior, habits, metabolism, etc.

6. Sex

Certain circadian patterns have been shown to correspond with the sexual cycle in animals. Slonaker (1912), Richter (1927) and Browman (1937) demonstrated that motor activity of the female albino rat increased with successive phases of the estrus cycle and maximum activity was noted

at the peak of the cycle and coincided with the period of sexual receptivity.

Slonaker (1912) observed a marked increase in the motor activity of rats during early pregnancy but a decrease during late pregnancy. Curtis (1937) noted that the sow will double its walking activity during estrus. The activity is circadian, the peaks occur at night but when the sow was in heat 2,243 steps were taken at night as compared to 346 when the animal was not in heat. Colvin, et al. (1968) chronically implanted cerebral electrodes in five female rats throughout their 4-day or 5-day estrus cycles. The results revealed rapid shifts from alertness to sleep after the lights came on in the morning. The highest level of alertness occurred during the early evening while paradoxical sleep (PS) occurred during the afternoon, including the afternoon of proestrous. Hormonal changes during the day of proestrous increased alertness that evening followed by an increase in PS sleep the following day. Garattini and Valzelli (1965) found that female mice do not become aggressive during isolation and they do not show a difference in the rate of brain serotonin increase following MAO blockade as do male mice. These authors also noted that in two different strains of mice the serotonin turnover rate is higher and the turnover time is longer in females than in males.

In summary, these investigations suggest that various sex hormones influence biogenic amine levels specifically and biological rhythmic activity in general.

7. Species

Some differences in rhythmic patterns have been attributed to variation in species. Aschoff (1963) noted that circadian phase-shifting varied among different classes and species of animals. Normally when light cycles or other factors that influence rhythms are shifted, the resynchronization of the activity rhythm takes from within only two days in the spider to as much as three weeks in the rat and mouse. There are also indications that the rate of resynchronization is not constant for all species of rats (Kleitman, 1949). Browman (1937) observed that there are variations in the motor activity of the female albino rat during the estrus cycles but this has been found not to be true in the female wood rat. Scudder, et al. (1966) studied six genera and three strains of mice and noted differences in habitats, behavioral profiles and brain levels of DOPA, dopamine, norepinephrine, epinephrine and serotonin. It was found that the brain of the Reithrodontomy strain contained the greatest total amount of biogenic amines while Mus musculus "Missouri", the least. The Mus musculus "Missouri" also demonstrated the shortest convulsive latency

period. Valzelli and Garattini (1968) reported that isolation produced aggression in several strains of mice including the Swiss strain but that this process did not produce aggression in the Wistar strain.

In summary, the variations in biological rhythms as described by the above investigators reflect the specificity in the biological system for different phyla, classes and species of animals.

8. Geographic Factors and Travel

Only a limited number of investigations have been conducted concerning the influence of different time zones and space travel on circadian rhythms. In terrestrial and space travel the biological system can cross several time zones and experience many different environmental factors within a matter of a few days and sometimes within a few hours. Strughold (1962) recognized that when man flies at supersonic speeds across many time zones he experiences day - night cycles different from the point of departure and different from the physiological cycles to which the body is adjusted. This means that the body must phase shift in an effort to adjust to the new cycle. In long distance flights, 70% of the people are sensitive to this shift and experience some physiological discomfort. This condition has been termed asynchronosis and it has been noted that actors, chess-players, athletes and even race horses suffer from this condition

immediately after arriving from regions four or more time zones away. Strughold (1962) also suggested three possible ways to prevent asynchronosis following long distance travel; "(1) arrive at one's destination several days in advance and allow the biological system to adjust rhythmically to the environment (2) pre-adaptation or pre-flight synchronization by initiating a sleep-wake schedule identical to that at the point of destination several days before travel or (3) the use of mild drugs to accelerate the physiological adjustment". He also noted that chronic travelers suffer most from asynchronosis which sometimes causes nervous stress and may lead to more acute diseases or physical conditions involving the cardiovascular and gastrointestinal systems. Parkinson (1957) noted that one way of encouraging an executive to retire was to fly him all over the globe without time for adequate readjustment to local rhythms.

The orbital flights of man present problems similar to that of air travel. The spacemen encounter extra-atmospheric erratic photic environments in near and deep space, with short irregular and contrast-rich photic periodicities or no photic periodicities at all (Strughold, 1962). Hauty (1962) discussed the periodic desynchronization in humans under outer space conditions. He reported the following observations when humans were placed in one-man space cabins and all conditions were maintained in an

identical manner to an actual space flight.   In the first trial the subject did not adjust sufficiently and showed progressive fatigue.   As fatigue accumulated, proficiency deteriorated in the execution of those tasks requiring high levels of vigilance and judgement.   During the second flight the subject suffered almost no loss in functional adequacy.   Strughold (1962) also indicated that weightlessness and the resulting hypodynamic conditions might lead to certain patho-physiological effects.   In order to control these patho-physiological effects, the spacemen must perform exercises and space calisthenics.   This activity can alter or shift biological rhythms.

In summary, another important aspect to be considered in space travel as it influences circadian rhythms is the fact that various celestial bodies present different environmental problems relating to biological rhythms.

9. Biological Rhythms with Periodicity Greater than One Day

Many investigators have noted that the duration of biologically rhythmic periods may vary from a few days to several months.   Kleitman (1949) discovered six-day feeding cycles in bed-bugs and also indicated that regular cyclic fluctuation occurred in the sleep characteristic of three psychotic patients with intervals ranging from four to six days.   He also

mentioned that weekly periodicities have been reported for body temperature and heart rate in man, with low values for these parameters occurring during week ends corresponding to periods of relative inactivity for a group of adult male triplets. Reimann (1951) reported periodicity in certain types of diseases. He reported on three cases of fever recurring at intervals of 7 to 22 days, 7 and 14 to 28 days for from three to eleven years, and one case each of neutropenia, arthalgia and myasthenia, with periods of 20 to 22, and 14 to 21 days, respectively. This process was observed from two to three years. Slonaker (1912) observed cycles of increased spontaneous motor activity of from two to six months duration in albino rats. Kleitman (1949) concluded that this spontaneous activity cycle in rats is common for both sexes and even extends into old age. He noted that during its lifetime the albino rat has at least three waves of increased motor activity of around 200 or more days. This activity in the rat is superimposed upon the daily motor activity rhythms previously mentioned.

Seasonal variations have been noted in the basal metabolic rate of humans (Gustafson and Benedict, 1928; Hitchock and Wardell, 1929) and in body weight, skin color and certain aspects of hematology in hibernating frogs (Holzapfel, 1937). Recent data indicate seasonal variations for specific biogenic amines. Donoso and Segura (1965) reported that in the

plasma of toads, epinephrine decreased during the mating season while significant increases were noted during autumn and winter and coincided with the premating period. Norepinephrine was found to decrease during autumn while no changes were observed during other seasons. Valzelli (1967) studied brain serotonin levels in the rat on a monthly basis for four years and observed peak levels occurring during the months of December and January while troughs occurred during the summer months of June, July and August.

10. Lunar and Solar Influence

Biological response to lunar and sun-spot (solar) activity have been observed. Kleitman (1949) reported that in humans many periodicities are reflections of lunar variations. When statistical data on scarlet fever was arranged on a lunar axis 14 days following a full moon, it was noted that severity of the fever was related to blood pH and that pH rhythm was positively correlated with fullness of the moon. He also stated that epileptic attacks have been correlated with fullness of the moon. A search of the vital statistics indicates that in general more male and fewer female deaths occur during the full moon while deaths from cardiovascular conditions and tuberculosis occur in greater number after, rather than before, the fullness of the moon. Brown, et al. (1956-b) reported that male

rats held in continuous dim illumination displayed greater motor activity when the moon was below the horizon than when the moon was above the horizon.

Kleitman (1949) noted that sun-spot periods which occur each eleven and one-half years were linked either directly or through their effects on temperature and weather conditions to a variety of rhythms and fluctuations. An association between sun-spots and various human psychological and social events was indicated. For example, totalitarian revolutions in Italy (1922) and Germany (1933) occurred during periods of maximum sun-spot activity.

In summary, rhythms other than those of a circadian nature can affect the physiological system. The circadian or daily rhythms are superimposed upon these weekly, seasonal, lunar and solar rhythms thereby adding to the already confusing picture associated with these patterns and their relationships.

B. RHYTHMS EXHIBITED BY PLANTS AND LOWER ANIMALS

1. Plants

Biological rhythms have been observed in plants. Yarwood and Cohen (1949) discovered a rhythm in the germination of certain types of fungi. Altman and Dittmer (1966) reported biological rhythms in cell division, photosynthesis activity and spore ejection for different types of fungi and algae. Brown, et al. (1955) found that oxygen consumption of carrot, potato and fucus tissue follow both a circadian and a lunar rhythm. Rhythmic patterns have been noted in the closing and opening of leaves of Mimosa and in the growth rates of Avena coleoptile (Ball and Dyke, 1954). These rhythms were correlated with the time of day. Ambient temperatures between 16° and $28^{\circ}C$ have no effect on these rhythms. Altman and Dittmer (1966) noted rhythmic responses in carbon dioxide output, flowering, leaf movement, growth rates and germination for several different type angiosperms. Rhythms that run for periods longer than 24 hours can be imposed on some plants, but under constant conditions these rhythms can be changed to a spontaneous 24-hour cycle (Harker, 1958).

2. Lower Animals

Rhythms in unicellular animals have been reported by several investigators. Hodge and Aikens (1893) and Gibbs and Dillinger (1908)

reported activity rhythms in the free-living protozoa (amoeba). Harker (1958) reported that cell division in the Paramecium and Strombidium is periodic and apparently exogenous, but that the phototactic sensitivity rhythm of the Euglena gracilis is apparently endogenous in that the rhythm is unaffected by temperatures between 17° and 33^OC. He also noted that a tidal rhythm (in which the animal burrows in the mud at high tide) has been demonstrated in the Euglena limosa. Barnett (1966) conducted several investigations on the Paramecium multimicronucleatum. It was noted that the mating type reversal rhythm is circadian and even in continuous dark-ness or light of six days duration each, the period is somewhat less than 24 hours. The reversal of mating type ceases in continuous light of high intensities. Feldman (1968) reported daily rhythms in the incorporation of amino acids in non-dividing cultures of Euglena gracilis. This may indicate rhythmicity in the rate of protein synthesis or peptide bond formation.

Information on circadian rhythms involving the phylum Coelenterata was reported by Mori (1943) who conducted several studies on the sea-pen Cavernularia obesa. He noted that colonies expanded after sunset and that contractions were observed during the light phase. It was also observed that identical rhythms could be induced by alternating periods of light of different intensities. A tidal rhythm related to body expansion has been

observed in <u>Actinia</u> equina and 24-hour rhythm in swimming movements have been noted in <u>Aurelia</u> (Harker, 1958).

Rhythms have been investigated in multicellular lower animal forms. Bohn and Drzewcina (1928) indicated a tidal rhythm for burrowing habits in a class of Platyhelminthes (<u>Convoluta roscoffensis</u>). Baldwin (1917) worked with the phylum <u>Annelida</u> and observed biological rhythms in the activity of the earthworm (<u>Lumbricus</u>).

In addition to circadian rhythm, many classes in the phylum <u>Mollusca</u> appear to respond to a lunar and solar rhythm. Brown, <u>et al.</u> (1956-a) observed an apparent rhythm associated with the shell opening of oysters. These rhythms have been associated with 24-hour rhythms, lunar rhythms and tidal rhythms. Brown, <u>et al.</u> (1956-a) further indicates that in oysters there is a 27-day cycle which appears to be associated with variations in radiation intensity resulting from the 27-day cycle of the sun's rotation on its axis.

Research on the Holothurian, <u>Thyone briareus</u> (phylum <u>Echino-dermata</u>) reveals 24-hour rhythms in locomotor wave and the feeding movements of the tentacles (Stier, 1933). These rhythms were present in continuous darkness and there seems to be some superimposition of seasonal variations on these daily rhythms.

Many investigations concerning circadian rhythms have been
conducted in Arthropoda. Brown and Webb (1949-a, b) studied the chroma-
tophore rhythms in the crab (Uca pugnax). It was observed that three
pigments are involved, red, white and black. The color rhythm is
determined by the color background, light, darkness, and temperature
(Brown, Webb and Guyselman, 1949). Brown, et al. (1949) indicated that
the dispersion of the black chromatophores is influenced by the length of
the light period. Brown and Stephens (1951) showed that the degree of
expansion of the melanophores was also related to the photoperiod. They
concluded that there are two centers concerned with the rhythmicity of
color changes in crabs. One center is concerned with the duration of the
photoperiod and the other is concerned with the production of hormones that
control the expansion and contraction of the melanophores. These hormones
are affected by light, darkness and the intensity of light. There are
indications that tidal and lunar rhythms also influence color change in Uca
pugnax and Callinecter sapidus (Brown, Sandeen and Fingerman, 1952 and
Brown, Webb and Graves, 1952).

Other rhythmic cycles noted in various species of Crustacea include
tidal rhythms of phototactic response in the hermit crab, Clibanarius,
24-hour rhythms of oxygen consumption in the Uca pugnax and daily activity

rhythms involving locomotion in the crayfish Cambarus virilis (Harker, 1958). Recently Enright and Hamner (1967) studied the mixed populations of marine zooplanktons and showed that internal rhythms which are synchronized by a light-dark cycle are of dominant importance for the vertical migration of these species.

In addition to the class Crustacea numerous studies concerning biological rhythms have been conducted on various species of insects. Motor activity rhythms have been observed in the beetle Cotinis, the cricket Gryllus, the stick insects Dixippus, the dragonfly Aeschnes and the cockroach Periplaneta americana (Harker, 1958). McCluskey (1965) observed a circadian motor rhythm in the activity of five species of male ants. Rhythms have also been reported which involve the movement of the retinal pigment in the Lepidoptera and oxygen consumption of the mealworms (Harker, 1958).

The emergence of many developing insects through the various stages of metamorphosis follows periodic patterns (Lewis and Bletchly, 1943; Pittendrige, 1954 and Harker, 1958). Roberts (1962) reported that 24-hour cycles of either light or temperature can control the circadian locomotor activity rhythm in the cockroach and that light cycles of four or eight hours duration entrain the rhythm to precise 24-hour periods.

Hanks (1967) studied the relationship between serotonin and the circadian rhythms in some nocturnal moths and concluded that light activation rhythm is mediated through the nervous system and that dark activation rhythm is either mediated or enhanced by the secretion of serotonin from what he termed the "A" cells of the brain. Saunders (1967) observed that by chilling one could reverse the photoperiodic control of sexual reproduction (diapause) in Nasonia vitripennis.

The retinal pigment in normal and hypophysectomized catfish have been shown to follow a rhythmic pattern (Welsh and Osborne, 1937). Rahn and Rosendale (1941) noted color change in the lizard Anolis occurs in the light, ceases in darkness and when the pars intermedia was destroyed or removed indicating the possibility that the pars intermedia controls the color changes. Motor activity rhythms as a function of light cycles have also been observed in axolotls, toads (Higginbotham, 1939 and Kalmus, 1940) and in lizards (Barden, 1942).

Simpson and Galbraith (1905) investigated the body temperature rhythms in nocturnal and other birds and found that the temperature rhythm continues after the feed and motor activity rhythm breaks down. Shaw (1933) observed cyclic activity in the occurrence of various types of leukocytes in pigeons. Barrott, et al. (1938) indicated that oxygen consumption rhythm

of hatched chicks follows their motor activity rhythm. Fraps (1954) studied the periodicity of ovulation in hens and noted that estradiol benzoate suppressed ovulation for several hours, but on subsequent days without injections, ovulation occurred on schedule. His investigation showed that ovulation would not occur out of phase or at other periods of the day, indicating that ovulation follows a rhythm in response to other excitatory hormones. Matthews (1955) discovered an internal chronometer in birds that is used in celestial navigation. This chronometer factor is dependent on the light-dark cycle. Wahlstrom (1964) studied the circadian rhythms of rest and activity in the canary. Gaston and Menaker (1968) noted that the pineal gland of the house sparrow Passer domesticus is essential for the persistence of the rhythm of locomotion under stable conditions.

In summary, the results of this review is a varification by many scientists that a large number of biological rhythms exist for several different species of plants and lower animals.

C. RHYTHMIC PATTERNS IN MAMMALS

1. Physical Activity Rhythms in Nocturnal and Non-nocturnal Animals

The literature is extensive on the motor activity rhythms in mammals. Davis (1933), Johnson (1939) and Park and Woods (1940) have all studied motor activity rhythms in the mouse and found them to be circadian. Slonaker (1912), Shirley (1928) and Browman (1937) reported similar activity rhythms in the rat. It was also noted that in the rat the biological rhythms became more marked over the first six months of life but after two years the rhythms decrease (Richter, 1927). Menaker (1961) reported that free-running circadian rhythms persist in the activity and body temperature of hibernating bats. Holmquest, et al. (1966) studied locomotor activity in experimental rats and its relationship to increases in body weight and adrenal cortical function. It was noted that random light for forty days abolished activity rhythms but had no influence on increases in body weight or on the weight of endocrine organs.

In addition to the nocturnal activity rhythms noted in the mouse and rat, Curtis (1937) reported that the sheep and pig also possessed motor activity rhythms but that their rhythms were diurnal. He noted that the motor activity rhythm of the pig was greater than the sheep but both animals showed maximum responses between 0600 hours and 1800 hours,

during the light phase. Lindsley, et al. (1964) found that when infant monkeys were raised in total darkness for three years a diurnal motor activity rhythm could be initiated by light stimulation and that three to five weeks were required to stabilize the activity period following the shift in the light period. Mills (1966) reported that man also exhibits a diurnal rhythm and the motor activity is closely associated with the rhythmic responses noted in metabolism, body temperature and other body organs.

2. Leukocyte Rhythms in Mammals

Harker (1958) discovered that the number of eosinophil leukocytes in mice follows a 24-hour rhythm which continues in the absence of external fluctuations, but can be altered by light and dark cycles. Brown (1962) noted that the number of blood leukocytes in the mouse are subject to at least two separate periodicities. The first is related to the circadian cycle of light and darkness and is present in both male and female. The second is related to the female sexual cycle and is superimposed upon the daily dark and light cycle.

Bartter, et al. (1962) reported that in humans the eosinophil count falls during the first few hours after waking. A similar fall in lymphocytes and a rise in the neutrophil count has been described. Doe, et al. (1956) indicated that the rhythms in white blood cells are dependent upon the

rhythms of adrenocortical activity. Mills (1966) reported that the regular morning eosinopenia is absent in Addison's disease, following adrenalectomy (bilateral) and in hypopituitarism. Mills (1966) further stated that the social habits seemingly play an important role in determining the timing of the eosinophil rhythm. In subjects habituated to early waking, the fall in the eosinophil count starts somewhat earlier in the morning. Light and season of the year also are factors related to morning eosinopenia in that it occurs earlier in summer months (Mills, 1966). Landau and Feldman (1954) reported that the eosinophil rhythm is absent or diminished in blind subjects. Renbourn (1947) observed diurnal variations over a long period of time in blood hemoglobin levels, hematocrits, plasma protein levels, sedimentation rates and blood chloride levels. Plasma iron has also been shown to follow a circadian rhythm with peaks occurring at or around waking time and troughs being observed at early night or initial darkness (Hamilton, et al. 1950).

3. Blood and Urinary Hormone Rhythms

Bartter, et al. (1962) noted daily rhythms in urinary levels of aldosterone, 17-ketosteroids and 17-hydroxycorticoids, with low values at night and high values at mid-day. He indicated that changes in sodium and potassium excretion and the circulating levels of white blood cells depended

upon steroid levels. It was also noted that ACTH secretion probably controlled circadian fluctuations in aldosterone secretion, and hydrogen ion excretion reflected excretion levels of steroids. Fiske and Leeman (1964) discovered that continuous light abolished diurnal glucocorticoid rhythmicity which in turn may account for the reduction in adrenal weight observed in intact female rats when they are exposed to continuous illumination. The rate at which corticosterone is degraded by the liver is unaffected by the exposure of female rats to continuous light. Diurnal corticoid rhythmicity is maintained in spayed animals. The neurosecretory activity of the supraoptic and paraventricular nuclei is markedly increased between the 14th and 22nd day in the neonatal rat and may be related functionally to the onset of diurnal corticoid rhythmicity which is established at the same age. Ungar (1967) studied the relationship of the adrenals to pituitary circadian rhythms and reported that hypothalamic stimulation of the pituitary adrenocortico-tropic activity occurs in the mouse toward the end of the light cycle. Boyd and McLeod (1964) investigated the circadian rhythms of plasma corticoids and intraocular pressure and found a positive correlation in cases of glaucoma. Intraocular pressure rhythms have a four hour lag behind the plasma corticoid rhythms. When the rise in plasma corticoids is blocked with hydroxylase inhibition, four hours later there is an interruption in the rising phase of intraocular pressure. It is believed that the circadian fluctuation

of intraocular pressure is controlled by the plasma corticoids. The

circadian rhythm of drainage is inversely related to intraocular pressure

in both normal and glaucomatous eyes.

Mills (1966) reported that plasma corticosteroid rhythms have

been demonstrated in several subjects and that plasma corticosteroid

rhythms are absent in Cushing's disease in which pituitary production of

ACTH may be deranged. This raises the question whether the plasma

corticosteroid rhythm is due to varying secretions of ACTH or to varying

adrenal sensitivity to this hormone (Mills, 1966). He stated that since

ACTH is believed to be controlled by the corticotrophin releasing factor

(CRF) many investigators have attempted to relate the periodic release of

CRF to the circadian rhythm of ACTH. When a corticotrophin releasing

peptide was administered to rats a rise in corticosteroids was noted but the

peak was out of phase with the normal biological rhythms of these hormones.

This may indicate again that a sensitivity factor is involved with the periodic

variations of the anterior pituitary. Migeon, et al. (1956) observed that

the peak urinary excretion of corticosteroids lags behind the peak plasma

concentrations by some three or four hours, but the type and concentration

of plasma and urinary corticosteroids are similar. Connolly and Wills

(1967) noted a relationship between cortisol circadian rhythms and patients

suffering from right and left ventricular failure. In thirty-one patients with right ventricular failure the peak cortisol level was increased at midnight with an abolition of the normal circadian rhythm. In patients with left ventricular failure the midnight levels were normal in two patients and greatly elevated in two terminal patients. Apparently the changes are due to stress responses and the stress mechanism is specific to right ventricular failure. Lohrenz, et al. (1967) observed circadian variations in urinary 17-hydroxycorticoids, 17-ketosteroids and plasma 17-hyroxycorticosteroids in ten depressed patients before they were treated. In five of seven men and in one of three women no trough or decreased nocturnal levels in 17-ketosteroids were noted, suggesting possibly that the circadian rhythm of the hypothalamic-pituitary adrenal axis is altered in some depressed patients.

The effects of various sex hormones on biological rhythms have been studied. Groot (1967) discovered that the ovaries of pseudopregnant immature rats contain less ascorbic acid and more progesterone at 1600 hours than at from 1000 to 1200 hours and 1400 hours. Two days following hypophysectomy when prolactin injections were administered to maintain pseudopregnancy, the ascorbic acid content was still decreased while the progesterone content failed to increase. The increase in progesterone and

part of the ascorbic acid decrease is explained by a rhythmic increase of endogenous luteinizing hormone (LH) during the afternoon. Colvin, et al. (1968) studied the effects of circadian and estrus cycles on different types of sleep. It was noted that hormonal changes during the day resulting from proestrus caused an increase in alertness the following night and an increase in paradoxical sleep the following day.

In addition to the rhythmicity noted in the hormones described above, Mills (1966) reported that urine flow has a circadian pattern mainly in response to the liberation of posterior pituitary anti-diuretic hormone. The serum content of ADH was higher at night than during the day in normal subjects.

4. Periodicity in Blood and Urinary Electrolytes

Simpson (1929) demonstrated a circadian rhythm for the excretion of phosphate in humans. He reported that phosphate levels decline during the night to minimal values immediately before waking. Longson and Mills (1953) noted that phosphate excretion rhythms depend upon plasma concentration and glomerular filtration rates. Mills and Stanbury (1954) reported that in normal human subjects, sodium, potassium and chloride excretion follow a periodic pattern with low values occurring during the night and peak values occurring between 0900 and 1200 hours (during the day). Acid and

ammonium excretion is high while the excretion of creatinine and uric acid is low at night. Calcium excretion has been reported to rise during the day and fall at night and magnesium excretion follows the same course as sodium and potassium in human subjects (Mills, 1966).

Min, et al. (1966) noted that in normal human subjects who are fed at constant intervals and are inactive, peak excretions of magnesium and calcium occurred early while peak excretions of sodium and potassium occurred late in the morning. Phosphorus followed a pattern about 180° out of phase with the patterns for magnesium and calcium. They also noted that potassium and phosphorus rhythms disappeared during fasting. Apparently magnesium and calcium are handled in a similar manner by the kidney. Bahorsky and Bernardis (1967), working with rats, observed rhythms similar to the urinary rhythms for serum potassium, sodium, phosphorus and urea and other electrolytes already mentioned above, but these were reversed for these nocturnal animals.

5. Periodicity in Carbohydrate Metabolism

A glycogen rhythm in the liver of rats and rabbits was discovered by Agren, et al. (1931). The rhythm was shown to be independent of feeding time but was destroyed when the adrenals were removed. Sollberger (1964) studied liver glycogen rhythms for several species of animals (humans,

rats, mice, rabbits, guinea pigs). In humans the peak was observed at between 1500 and 1600 hours, for rats and mice the main peak occurred at between 0600 and 0800 hours with secondary peaks at between 0200 and 0400 hours. In rabbits primary peaks were noted between 1500 and 1700 hours with secondary peaks at 0500 and 0600 hours. In guinea pigs the main peak was observed between 1300 and 1500 hours with a secondary peak noted at 0100 hours. Sollberger (1964) contends that the two parameters that serve best to alter glycogen rhythms are food and light. Bahorsky and Bernardis (1967) also observed a rhythm in blood glucose levels in fasted and fed weanling female rats. The primary peak was observed at 2000 hours with a secondary peak noted at 0800 hours and the trough observed at 1400 hours. Mills (1966) concluded that the rhythm of ACTH might also influence the tissue and blood carbohydrate rhythms.

6. Biological Rhythms in Enzyme Systems

Recently a few experiments have been conducted on the circadian patterns of specific enzyme systems in animals. Van Pilsum and Halberg (1964) noted a transmidinase activity in the kidney of the mouse. The enzyme levels were observed to be higher during the light period as compared to the dark phase. Rapoport, et al. (1966) observed that in the mouse hepatic tryptophan pyrrolase and its circulating blood substrate, tryptophan

exhibits a circadian rhythm. Intact adrenocortical function is required for the normal rhythmicity in both enzyme and substrate. A quantitatively altered and less apparent rhythm was noted in adrenalectomized animals. Circadian rhythmicity in enzymes concerned with biogenic amine synthesis has also been observed. Axelrod, et al. (1966) noted that the activity of hydroxyindole-O-methyl transferase, the melatonin-forming enzyme in the pineal gland is greater in rats kept in darkness than in rats kept in continuous light. Lesions transecting the forebrain bundle indicate the participation of neural pathways in the control of this enzymatic response to environmental lighting. McGeer and McGeer (1966) measured the activity of tyrosine hydroxylase (the rate limiting enzyme in catecholamine synthesis) in the rat pineal gland at 4-hour intervals over a daily cycle of 12 hours of light and 12 hours of darkness (0700 to 1900 hours). The results showed a circadian rhythm with maximum activity at from 2300 to 0300 hours during the dark phase. The maximum values were triple the low values which were noted at 1500 hours.

7. Biological Rhythms Involving Biogenic Amines

As in enzyme studies, only a few investigations have been conducted on the biological rhythms involving biogenic amines. Wilson (1965) studied the excretion of histamine and noted a circadian pattern in the rat. The

minimum value of 60% of the mean was noted at 1300 hours (mid-light phase), the maximum value of 150% was noted at 2130 (early dark phase). The urine volume maxima were out of phase with histamine peaks by about four hours, i. e., peak urine volume occurred at 0130 hours (mid-dark phase). The peak urinary volume was in phase with the circadian peak motor activity. Snyder, et al. (1965) noted that pineal serotonin follows a circadian pattern with maximum levels during the day and minimum levels at night. They demonstrated that removal of the superior cervical ganglion abolished these rhythms while blinding the animals did not affect them. They also demonstrated that the serotonin rhythm was a reverse of the melatonin rhythm in the rat pineal gland. Dixit and Buckley (1967) observed rhythms in serotonin levels in whole rat brain with peaks occurring at the mid-point of the light period. Wurtman, et al. (1967-a) noted circadian rhythms for norepinephrine in the pineal gland of the rat; the peaks occurred during the dark phase of the cycle. Garattini and Valzelli (1965) observed daily rhythmicity and seasonal variations in the levels of serotonin and 5-hydroxyindole acetic acid in rat and mouse brain. The daily rhythm peaks were observed during the day while the seasonal variation peaks were observed during the summer months of June, July and August or during periods of extended day-light hours. Scheving, et al. (1968) studied the circadian rhythms of serotonin, dopamine and norepinephrine of whole brain

obtained from different subgroups of adult male rats killed at hourly intervals. Serotonin peaks were noted during the light phase but circadian rhythms were not detected for dopamine and norepinephrine. Reproducible high frequency (ultradian) rhythms were observed for these amines.

Wurtman, et al. (1968-a) studied the circadian rhythms of some sixteen amino acids in the blood of twenty-three male subjects who received either 0.71 or 1.5 grams of protein per kilogram of body weight. It was noted that tyrosine, phenylalanine and tryptophan tended to be lowest at 0200 hours and highest at 1030 hours. All amino acids studied showed some variation in level with the time of day. Tyrosine, tryptophan, phenylalanine, methionine, cysteine and isoleucine underwent the greatest per cent change, but the amino acids with the highest plasma concentrations (alanine, glycine and glutamic acids) showed the least tendency to vary. Manshardt and Wurtman (1968) also showed circadian variations in the norepinephrine content of hypothalamus, midbrain, striatum and thalamus of the rat. In all tissues, except the striatum, peaks were noted during the dark phase. The striatal peak was observed toward the mid-light period.

8. Circadian Rhythms in Synaptic Vesicles and Their Possible Significance

Practically no research has been conducted to date on circadian patterns in certain subcellular structure involving granular and agranular

vesicles which are considered as possible synthetic and/or storage sites for biogenic amines in the CNS. However, there have been a number of investigations of their structure and possible function using the electron microscope. DeRobertis, et al. (1961) studied the ultra structure of peripheral nerve endings and observed a specific granulated vesicle at adrenergic endings which was between 500 and 1000 $\overset{o}{A}$ in diameter and whose density could be reduced with reserpine and increased with DOPA and MAO inhibitors. Another type of vesicle observed at the preganglionic site and at the neuromuscular junction was 300 to 500 $\overset{o}{A}$ in diameter and agranular. DeRobertis characterized these vesicles as norepinephrine and acetylcholine-containing vesicles, respectively. Hashimoto, et al. (1965) studied the ultra structural localization of norepinephrine and dopamine in the hypothalamus and caudate nucleus of reserpinized and dopa treated rabbits. They observed a reappearance of the large granulated vesicles with dense cores in the hypothalamus following the administration of DOPA. Fuxe, et al. (1965), using the electron microscope, studied the synaptic terminals in the nucleus caudatus, putamen, the nucleus of the tractus solatarius and the substantia grisea periventricularis of the 4th ventricle. They confirmed the presence of numerous small agranular vesicles (300 to 600 $\overset{o}{A}$) and a few large granular vesicles (800 to 1000 $\overset{o}{A}$) in the boutons. They reported no

observable difference in either type of vesicle following treatment with large

doses of reserpine. In a subsequent study, Fuxe, et al. (1967) suggested

the possibility that the granular vesicles contained catecholamines since

these were numerous in the perinuclear area where the concentration of

amines is highest. Wolfe, et al. (1962) using electron microscopic radio-

autography of the rat pineal gland reported an association of labelled norepi-

nephrine grains with the dense core of granulated vesicles in unmyelinated

axons. Lenn (1967) used electron microscopic autoradiography to localize

^{3}H norepinephrine uptake in vitro and in vivo after injection via the lateral

ventricle. In both preparations the labelled norepinephrine was found pre-

dominately in small unmyelinated axons and nerve endings which contained

a large number of granular vesicles and were located in areas of the brain

biochemically demonstrated to contain large amounts of norepinephrine.

Reivich and Glowinski (1967) partly confirmed the work of Lenn (1967) and

Wolfe, et al. (1962) in an autoradiography study of the distribution of

C_{14}-norepinephrine in the brain of the rat. They observed that intra-

ventricularly administered norepinephrine was taken up by the limbic

system and other areas which have been shown biochemically to contain

large amounts of catecholamines.

DeRobertis (1965) extended his studies of the bouton terminal. In addition to granular vesicles, he studied small agranular vesicles which might be involved in cholinergic transmission. By means of cell fractionation he was able to localize acetylcholine, choline acetylase and cholinesterase in regions containing small agranular vesicles. Pfeifer, et al. (1968) reported that the monoamine oxidase inhibitor, nialamid, increased the number of granular vesicles but had no influence on the number of agranular vesicles in the rat pineal gland.

Vesicles other than those associated with norepinephrine and acetylcholine have also been identified in nervous tissue. Richardson (1962) described a vesicle in the autonomic nerve ending of smooth muscle which was about 900 $\overset{o}{A}$ in diameter with a 500 $\overset{o}{A}$ inner dense core. Palay (1957) studied the fine structure of rat neurohypophysis and observed vesicles that ranged from 1200 to 1500 $\overset{o}{A}$ in diameter. These structures contained large dense droplets that filled the entire vesicle. Palay concluded that these vesicles contained vasopressin and oxytocin. Spherical and elongated small diameter agranular vesicles have been observed in spinal motoneurons of cats (Bodian, 1966). These investigations indicate that many different types of vesicles are located in nervous tissue.

D. DRUG INFLUENCES ON BIOLOGICAL RHYTHMS

1. Barbiturates

Davis (1962) studied the day-night periodicity in pentobarbital response of mice and the influence of sociopsychological conditions. He observed that both grouped and isolated animals showed identical trough values, but he also noted that the grouped animals showed longer sleep duration during the day than did the isolated animals. Wahlstrom (1964) investigated the effect of sodium pentobarbital on activity and rest rhythms in the canary after 20 mg/kg orally of sodium pentobarbital. In some cases the birds were placid and indications of coordinated movements could be seen but pentobarbital sodium had no effect on the motor activity rhythm. When 25 mg/kg of sodium pentobarbital was administered, anesthesia was sometimes noted but in the majority of cases severe intoxication was observed with no effect on the circadian activity rhythm. Millichap and Millichap (1966) studied the effects of pentobarbital on locomotor activity in mice for 24 hours and in hamsters for one - eight days. A 5 mg/kg dose of pentobarbital (s. c.) caused a suppression of activity, 100 to 150 mg/kg (s. c.) caused stimulation while 200 mg/kg (s. c.) caused a biphasic suppressive-stimulant effect. Artificial light failed to abolish the circadian increase in activity at night in either the treated or untreated animals.

Radzialowski and Bousquet (1968) investigated the daily rhythmic variation in hepatic drug metabolism in the rat and mouse and noted that hexobarbital metabolism and the metabolites of certain other drugs followed a circadian rhythm with peaks at 0200 hours and troughs at 1400 hours. Adrenalectomy was found to abolish the circadian rhythm in metabolism of hexobarbital. In rats pretreated for four days with pentobarbital sodium the activity of some enzymes was abolished thereby eliminating the circadian pattern. Kalyanpur, et al. (1968) pretreated rats with thiamine and nicotinic acid and noted a prolongation in the sleeping time for both pentobarbital and barbital. Biotin pretreatment also prolonged pentobarbital sleeping time. They also noted that nicotinamide and pyridoxine slightly shortened pentobarbital sleeping time. Pretreatment of female rats with specific hormones (progesterone, stilbestrol and aldosterone) considerably shortened the sleeping time of pentobarbital. This is somewhat unexpected in view of the work of Selye (1949) who showed that certain steroid hormones produced anesthesia. He demonstrated that in 70 gm female rats intraperitoneal administration of 10 μg of progesterone in 0.5 cc of oil produced complete muscle relaxation.

2. Cholinomimetics

Spoor and Jackson (1966) noted a circadian rhythm in the response of the isolated right atria of the rat to two concentrations of acetylcholine. The beating rate decreased more in response to the drug in atria isolated at 1100 than at 2300 hours. Krieger and Krieger (1967) observed that when atropine was administered to rats between 1600 and 2000 hours prior to the expected rise in blood levels of 17-hydroxycorticosteroid, the rise was blocked. When atropine was administered at other points in the cycle the circadian levels of 17-hydroxycorticosteroid was not altered. Dibenzyline did not block the circadian rise in 17-hydroxycorticosteroid when administered at the time when atropine was effective.

3. Convulsants

Webb and Russell (1966) studied the diurnal influence on latency intervals between convulsive response thresholds of rats to hexafluorodiethyl ether. It was noted that myoclonic jerks, clonic and tonic convulsions were shorter in duration (terms of minutes) at 2200 hours (dark phase) than at 1000 hours (light phase). The interval for tonic convulsions and onset of post-ictal depression was longer at 2200 hours (dark phase) and shorter at 1000 hours (light phase). A circadian rhythm was also observed in the number of animals exhibiting two clonic convulsions from a single exposure

of hexafluorodiethyl ether. Fifty per cent responded twice at 1000 hours while only 10% responded twice at 2200 hours. Lutsch and Morris (1967) adapted mice to alternating 12-hour light and 12-hour dark (0605 to 1805 hours) regimen and treated groups of these animals with lidocaine hydrochloride every three hours for a 24-hour period. It was noted that the maximum or peak convulsant activity occurred at 2100 hours while the minimum convulsant activity occurred at 1500 hours. The maximum represented a fourteen fold increase over minimum.

4. Monoamine Oxidase Inhibitors

Wahlstrom (1964) studied the effects of certain MAO inhibitors (pheniprazine, pargyline and nialamide) on the circadian motor activity, circadian roosting time and circadian resting time in the canary. It was observed that MAO inhibitors for the first few days caused a decrease in the duration of motor activity rhythm followed by an increase. The converse was true for the length of the circadian resting period. It was also noted that the increase in the duration of motor activity was more pronounced if the drugs were given in the late P.M. (1900 to 2100 hours) rather than in the early A.M. (0200 to 0300 hours).

5. Central Nervous System Depressants

Wahlstrom (1964) studied the influence of a single oral dose of reserpine (0.5 to 1.5 mg/kg) on the duration of the motor activity in the canary. Reserpine decreased the duration of the motor activity rhythm, but had no influence on the circadian "waking up" time in the canary. Wurtman, et al. (1968-b) investigated the effects of brain norepinephrine on the daily rhythms observed in hepatic tyrosine transaminase activity in the rat. He used reserpine as a depletor of brain norepinephrine and found that it abolished the rhythm for adrenal corticosterone but did not affect the tyrosine transaminase rhythm in the liver.

6. Antipyretics

The diurnal excretion of orally administered salicylates in six human subjects adapted to a routine sleep cycle (2300 to 0700 hours) was investigated by Reinberg, et al. (1967). The drug was administered four different times per day with equal intervals between doses. The duration of the urinary salicylate excretion was longest (22 hours) when the drug was administered at 0700 hours and shortest (17 hours) when the drug was administered at 1900 hours. Radzialowski and Bousquet (1968) noted that aminopyrine metabolism follows a circadian rhythm with maximum metabolism occurring at 0200 hours and minimum metabolism occurring

at 1400 hours in both male and female rats. Adrenalectomy abolished the circadian rhythm in the metabolism of this drug.

7. Antihistaminics

The circadian pattern in the inhibitory effects of an antihistaminic drug in humans was studied by Reinberg and Sidi (1965). They used six male subjects adapted for one week to a routine circadian cycle (2300 to 0700 hours). Intradermal injections of 10 μg of histamine in the flexor surface of the forearm were made at four hour intervals and observed for erythema and wheal responses for periods of from 15 to 20 minutes. The skin reaction to histamine showed peak responses in terms of erythema and wheal at 2300 hours while the antihistamine, cyproheptadine (periactin, which also has antiserotonin properties) showed an effective circadian duration, when administered at 0700 hours, of 15.1 hours (erythema) and 17.5 hours (wheal). When this compound was administered at 1900 hours, the effectiveness of duration against erythema was 5.9 hours and for wheal it was 8.6 hours.

8. Antibiotics

No one per se has investigated the circadian effectiveness of anti- biotics, but some indirect effects have received attention. Wilson (1965)

noted that chloramphenicol shifted the peak rat histamine urinary excretory curve from the beginning of the dark phase to the center of the dark period, coinciding with the peak motor activity rhythm and the peak urinary volume rhythm. At the same time it was observed that the total amount of histamine excreted was reduced when compared to that of the normal rhythm. Cohen (1965) found that puromycin and actinomycin blocked the mating rhythms observed in the Paramecium bursaria. Peak mating ability was observed at about noon but it decreased in the evening.

Recently, studies on the control of biological rhythms have been centered around investigations at the molecular level. Brahmachary (1967) reported that a circadian rhythm in mRNA (messenger RNA) and DNA was noted in mouse and rat cells. The peaks were observed in the evening. DNA peaks were occasionally two or three times the minimum values. He also reported that peaks in cellular DNA and RNA have been correlated with peaks in mitosis and protein synthesis. Brahmachary (1967) further states that puromycin, actinomycin, mitomycin C and novobiocin stop cell mitosis and growth. Mitomycin abolishes the DNA rhythm and the dependent RNA rhythm as well but when chloramphenicol was tested after infections, increases in phage mRNA were observed. Flexner and Flexner (1968) noted that puromycin causes a loss in memory in mice but these mice can relearn.

An hypothesis concerning the influence of cholinesterase in learning as influenced by protein synthesis has been expounded by A. G. Karczmar (1967). Agranoff, et al. (1967) reported that actinomycin D blocked the formation of long-term memory in goldfish immediately following a training session.

In summary, only a few drugs have been investigated with regard to their influence on/or in connection with various types of biological rhythms. In some cases it is noted that these drugs are effective in altering or completely abolishing these rhythms. In others they are without effect. These few studies indicate the need for additional pharmacological investigations of circadian influences on drug action and efficacy.

E. OBJECTIVES

Patterns of rhythmicity are extant in the biological world and such rhythms apparently modify the responsiveness of organisms to the chemical stimuli we designate as drugs. It is the purpose of this study;

I. To investigate under controlled lighting conditions the nature of some of these rhythms, especially those involving biogenic amines,

II. To examine the phase relationship of these amine patterns and other parameters such as body temperature and motor activity,

III. To determine whether such patterns can be correlated with ultrastructural changes at the submicroscopic level. These studies will then serve as reference points for subsequent investigations involving drug effects and drug efficacy on a circadian basis.

CHAPTER III

METHODS AND PROCEDURES

A. EXPERIMENTAL CONDITIONS AND PROCEDURES

1. Environmental Conditions

Male albino rats of the Sprague-Dawley strain weighing approximately 250 grams each and supplied by Abrams Small Stock Breeders, Inc. of Chicago, Illinois were used in these experiments. All animals were subjected to a three week adaptation period in a concrete 9 x 12 x 13.5 foot room with no external light source. The light cycle was automatically timed (Sears Kenmore 24-hour timer, 15 amps capacity) and lasted from 0800 to 2000 hours daily. The light was supplied by two General Electric 150 watt white light bulbs that were spaced at an average equal distance of 3.5 feet from the experimental animals. The amount of light furnished by these bulbs, as measured by a Goosen Luna-Pro CdS Light Meter, ranged from 13 - 66 foot candles for all animals.

During the adaptation period, all animals were maintained under conditions of ambient barometric pressure that ranged from 742 - 772 mm Hg with an average of 757 mm Hg. Relative humidity ranged from 30 to 55% with an average of 40%. The laboratory temperature was stable at $23 \pm 1^{o}C$ for the duration of the adjustment period. Barometric pressure, humidity and temperature were recorded daily on a 24-hour Wilh Lambrecht KG Göttingen recorder (Type 235).

2. Housing and Feeding Methods

All rats were housed in groups of four and/or six animals per cage (dimensions 18 x 11 x 9 inches) and fed Purina Rat Chow ad libitum. The food was added to the cages at different times of the day in order to avoid initiating a feed dependent rhythm. The Purina Chow nutrient composition consisted of 24% crude protein, not less than 4% fat, 5% crude fiber and 9% ash, with minerals and vitamins added. All animals were maintained on raised wire screens and pelted cellulose (Pel-E-Cel, Paxton Processing Co., New Jersey) was used for bedding which was changed once a week. Tap water was supplied for drinking ad libitum.

3. Adrenalectomy

Bilateral adrenalectomy was performed on one group of animals anesthetized with 50 mg/kg of sodium pentobarbital I.P. Each adrenal gland was removed by a dorsal approach under sterile conditions. Following adrenalectomy and after recovery from anesthesia these animals were placed in individual cages for from 3 to 5 days until the incisions had healed. The animals were then regrouped (4 to 6 animals/group). These animals received food ad libitum and were adapted to the three week circadian cycle as previously described. Physiological saline (0.9%) was substituted for the drinking water.

4. Rectal Temperature

Rectal temperature was measured in groups of twelve normal or adrenalectomized rats every three hours for a 24-hour period with a Yellow Springs Telethermometer (Model No. 44TA) and a stainless steel thermister probe (Type 405). The probe was lubricated with Mazola corn oil (Corn Products Co., New York) and inserted approximately 5 cm into the colon. The probe was maintained in situ for approximately three minutes before the temperature was recorded. All animals were handled in a gentle manner in order to minimize excitement.

5. Brain Dissection and Homogenization

Groups of four normal and four adrenalectomized rats were killed by a Harvard Decapitator (Model No. 130 R. M.) at intervals of six hours for a total period of 24 hours. The skull was opened, dura incised, the brain removed and placed on a block of cold aluminum. The midbrain and both caudate nuclei were dissected out within two minutes following decapitation (Figures 1 and 2). The dissected portion was immediately weighed on a Roller-Smith Precision Balance (Model G, 500 mg range) and then placed in a reagent appropriate for the specific amine being analyzed. For norepi-nephrine and serotonin analysis, approximately 500 mg of tissue was placed in 2 ml of cold 0.01 N HCl. For histamine analysis, one gram of tissue

FIGURE 1

DISSECTION DIAGRAM FOR RAT MIDBRAIN

DORSAL VIEW
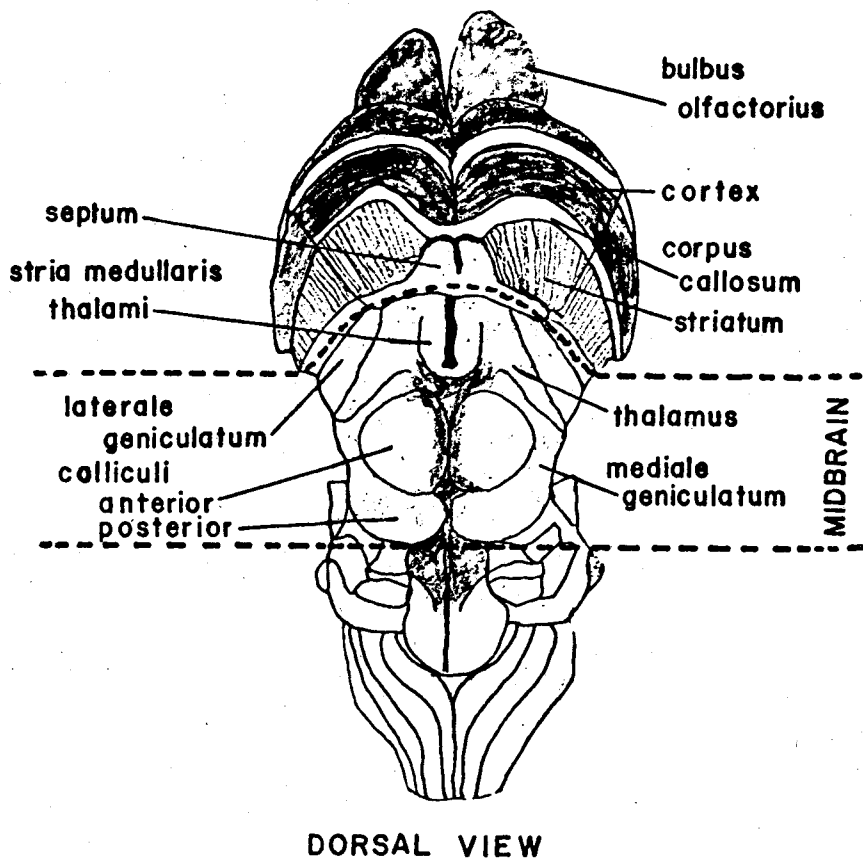
Reference: Zeman and Innes, Craigie's Neuroanatomy of the Rat, 1963, p. 24.

FIGURE 2

DISSECTION DIAGRAM FOR RAT CAUDATE NUCLEI
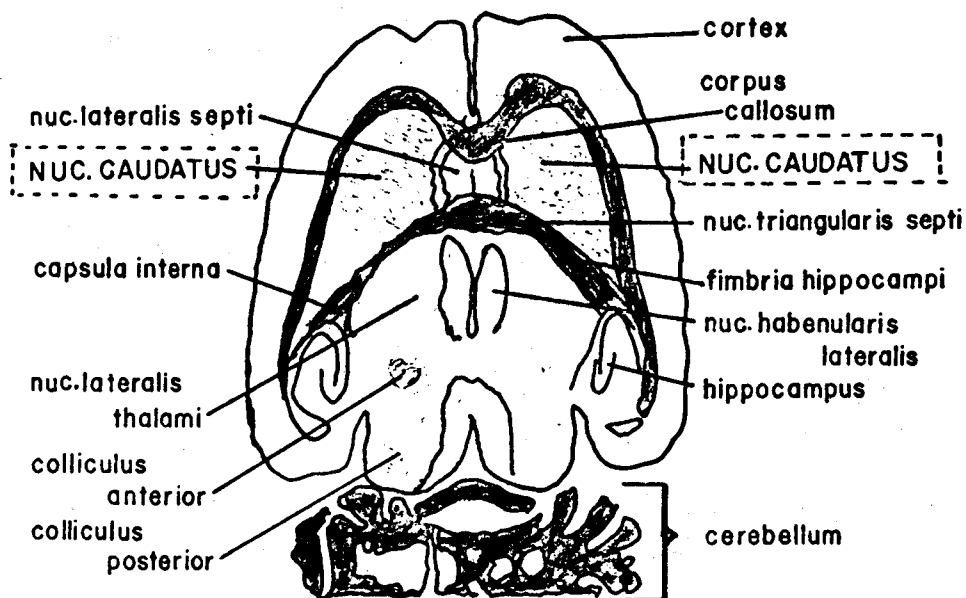
HORIZONTAL SECTION

Reference: Zeman and Innes, Craigie's Neuroanatomy of the Rat,
1963, p. 219.

was added to 9 volumes of cold 0. 4 N perchloric acid. For acetylcholine
analysis, the tissue was rapidly frozen in liquid nitrogen and the equivalent
of one gram of tissue was placed in 5 ml of cold acid-ethanol (75% ethanol,
0. 15% glacial acetic acid). All tissues were homogenized for one minute
and thirty seconds using glass pestles in glass homogenizing vessels
(Erway, 30 x 120 mm) immersed in an ice bath. A stirring motor (Eber-
bach) attached to the pestle was used to homogenize all tissues.

6. Procedures for Specific Biogenic Amines

a. Norepinephrine and Serotonin

The method of Mead and Finger (1961) was used for the
determination of norepinephrine and serotonin. This method is a modifi-
cation of the Shore and Olin (1958) method for catecholamines. (The
advantage of the Mead and Finger (1961) method is that both norepinephrine
and serotonin can be extracted into n-butanol from acid homogenates.) In
this procedure, 2 ml of homogenate are transferred to a 35 ml (115 mm long)
reaction vessel containing 2 gm of NaCl and 20. 0 ml of purified n-butanol.
The mixture is shaken at moderate speed for 30 minutes on an Eberbach
Variable Speed Shaker to extract the norepinephrine and serotonin from the
tissue into the butanol. The mixture was centrifuged on a Model V Inter-
national Centrifuge for 10 minutes at 1500 r.p.m. For norepinephrine

analysis, 12 ml of the butanol extract was transferred to an identical reaction vessel containing 15 ml of purified heptane and 2 ml of 0.01 N HCl. The extract was shaken for 15 minutes as previously described. The heptane corrects for fat interference while 0.01 N HCl is the necessary acid medium for stabilizing extracted norepinephrine. One and one-half milliliters of the 0.01 N HCl and norepinephrine mixture were treated with 0.05 ml of 0.1 N iodine reagent to activate the fluorescent properties of the norepinephrine molecule. After exactly six minutes, the iodine activity was destroyed by the addition of 0.1 ml of 0.05 N sodium thiosulfate. The tissue norepinephrine was then incubated with 0.5 ml of alkaline ascorbate for 45 minutes. An aliquot was placed in a quartz cuvette having a one centimeter light path and read in an Aminco-Bowman Spectrophotofluorometer (Model SPF) using an activating wavelength of 400 millimicrons and a fluorescent wavelength of 520 millimicrons.

For serotonin analysis, 6 ml of n-butanol extract was placed into 18 ml of heptane and 1.5 ml of 0.1 N HCl. After shaking for 15 minutes and centrifuging for 5 minutes, 1 ml of the aqueous layer was transferred to 0.3 ml of concentrated HCl. Serotonin in the acid medium was read in the Aminco-Bowman Spectrophotofluorometer at an activation wavelength of 300 millimicrons and a fluorescent wavelength of 540 millimicrons. In

this procedure norepinephrine is determined after its oxidation at pH 5 into a highly fluorescent trihydroxyindole compound (Figure 3). The native fluorescence of serotonin in concentrated HCl (Figure 4) is the basis for its quantification. For both norepinephrine and serotonin analyses, concentrations of unknowns (tissue extracts) were determined by using standard known concentrations of the specific amines and relating these readings to those of the unknowns.

According to Mead and Finger (1961), tryptamine, metanephrine, normetanephrine and 3, 4-dihydroxybenzylamine are also extracted into n-butanol but these compounds do not interfere with the fluorescence of NE and serotonin. 5-Hydroxytryptophane (5-HTP), which has fluorescence characteristic of serotonin, is partially extracted at acid pH but this does not create a problem because 5-HTP is not present in detectable amounts in tissue. Some limitations of this method were noted by Udenfriend (1962) who reported that problems exist with fluorophore stability in that progressive changes occur in blank values (increases with time). He also noted that differences were observed among the fluorophores of trihydroxyindoles, ethylenediamine condensation products and enzymatic procedures. The catecholamine 0. 01 N HCl mixture, upon standing, is converted to a pink compound which can interfere with quantification. It is possible to estimate

FIGURE 3

CONVERSION OF NOREPINEPRINE TO A HIGHLY
FLUORESCENT TRIHYDROXYINDOLE

NOREPINEPHRINE

NOREPINEPHRINE-QUINONE

NORADRENOLUTIN
(3, 5, 6-trihydroxyindole)

NORADRENOCHROME
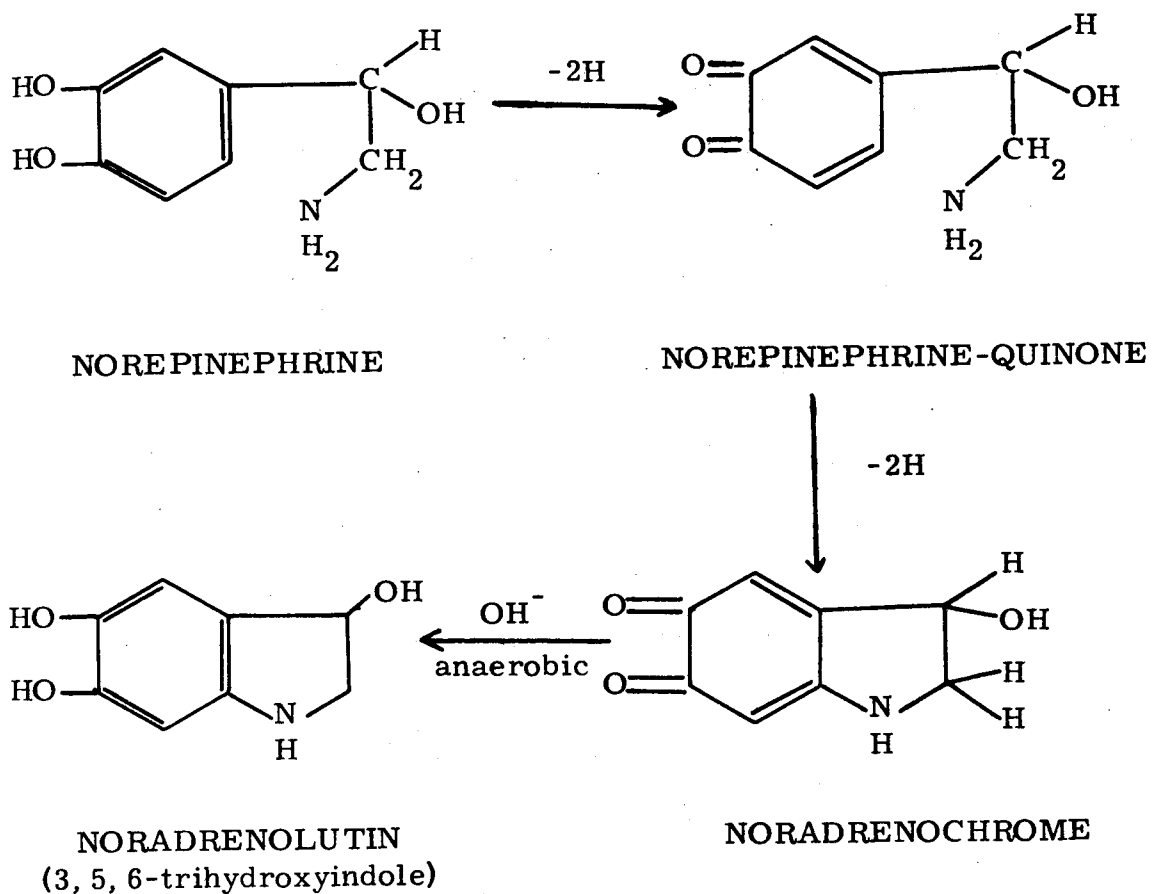
The compounds above indicate the conversion scheme of norepinephrine
to a fluorescent trihydroxyindole derivative (Udenfriend, 1962).

FIGURE 4

NATURAL FLUORESCENT PROPERTIES OF
5-HYDROXYTRYPTAMINE
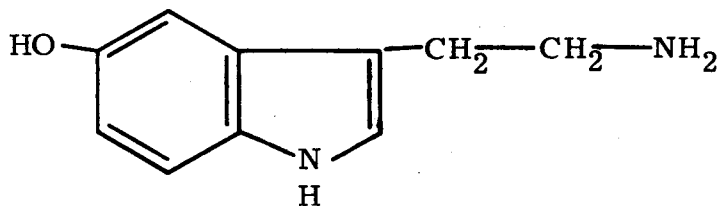
5-HYDROXYTRYPTAMINE (Serotonin)

5-Hydroxyindoles duch as the compound shown above are naturally
fluorescent in aqueous solutions.   Acid shifts the fluorescence from the
ultraviolet to the visible range and increases fluorescence to a maximum
(Udenfriend, 1962).

the original concentration of catecholamines by means of a conversion

constant (Walker and Friedman, unpublished).

b. Histamine

Histamine was analyzed by the methods of Shore, et al.

(1959) and Anton and Sayre (1968). These methods involve the extraction of

histamine into n-butanol from alkalinized perchloric acid tissue extracts.

The histamine was then returned to an aqueous solution and condensed with

o-phthal-aldehyde (OPT) to yield a product which is stable and strongly

fluorescent (Figure 5). Shore, et al. (1959) also stated that histidine and

histidyl-histidine produce fluorophores spectrally similar to that of

histamine when treated with OPT, but do not interfere since they are not

extracted by butanol from an alkaline solution. They also indicated that

ammonium can interfere with the fluorescence of histamine when the concen-

trations in tissue are greater than 4 μg/gm, but ammonia is also poorly

extracted into butanol. Kremzner and Pfeiffer (1966) found spermidine to

be a major contaminant of brain histamine when analyzed by the histamine

procedure of Shore, et al. (1959). Michaelson (1967) discovered that

histamine could be separated from the contaminant (spermidine) by exposing

the tissue extract to phosphorylated cellulose, Cellex-P. Apparently the

spectral displacement of the OPT-spermidine complex is greater in the

FIGURE 5

CONDENSATION OF HISTAMINE WITH O-PHTHALALDEHYDE (OPT) TO YIELD A FLUORESCENT PRODUCT

OPT   HISTAMINE

OPT-HISTAMINE COMPLEX
(Fluorescent)
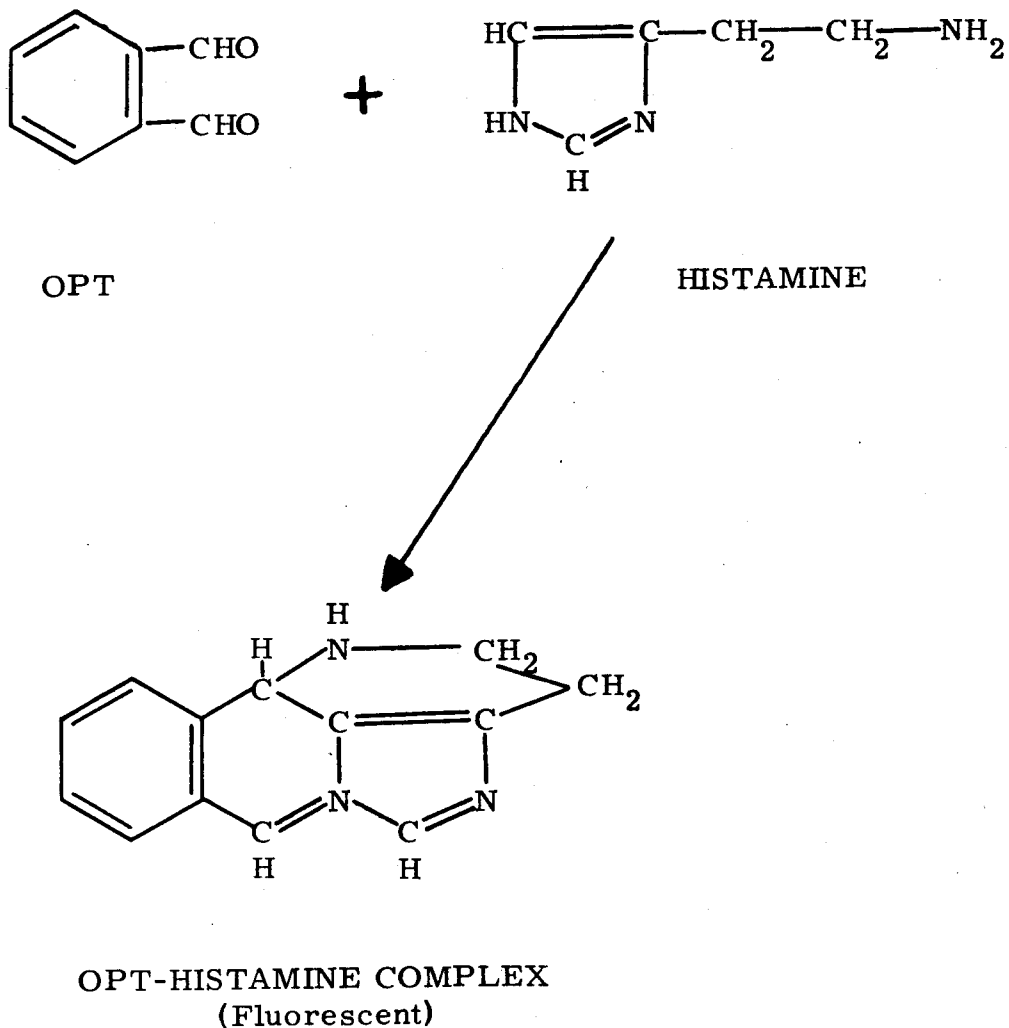
The above is the suggested fluorescent product formed by the OPT-histamine reaction (Udenfriend, 1962).

presence of phosphoric acid. The method of Anton and Sayre (1968), a modification of the Shore, et al. (1959) procedure, includes additional purification steps that correct for spermidine and other contaminants. Histamine was determined from readings on the Aminco-Bowman Spectrophotofluorometer with an activating wavelength of 350 millimicrons and a fluorescent wavelength of 450 millimicrons. The spermidine-OPT complex shows optimum fluorescence at an activating wavelength of 350 millimicrons and a fluorescent wavelength of 400 millimicrons.

c. Acetylcholine

Midbrain and caudate nucleus acetylcholine were extracted and analyzed according to the procedures of Crossland (1961) and Toru and Aprison (1966). Tissue frozen with liquid nitrogen was placed in chilled 15% formic acid-acetone solution, homogenized and centrifuged for five minutes as described previously for NE and serotonin. The supernatants were separated and saved while the residue was resuspended twice in 1 ml of 70% formic acid-acetone solution. All supernatants were combined and evaporated to dryness with a stream of dry air in a 35°C water bath to 1 ml. The final volume was diluted to 6 ml with triple distilled water and frozen until ready for use in the bioassay.

Crossland (1961) noted that the original acetylcholine extraction medium, trichloroacetic acid (TCA) destroyed acetylcholine. Bennett, et al. (1960) found that acid-alcohol was a better extraction medium. Toru and Aprison (1966) developed a new extraction procedure using acid-acetone (described above).

Isolated guinea pig ileum was used for the bioassay. The Tyrode solution contained tryptamine HCl (2×10^{-4}M) and mepyramine maleate (2.5×10^{-6}M) to block the effects of serotonin and histamine respectively. The final brain extract was incubated for ten minutes prior to the bioassay with alpha-chymotrypsin, 400 μg/ml at 38°C and a pH of 7.0. According to Toru and Aprison (1966), alpha-chymotrypsin corrects for substance P, while the pH adjustment to neutrality is important in preventing possible effects of formic acid on tissue contractility.

7. Statistical Analysis (Latin Square Procedure)

a. Calculation of Potency (Formula)

Data Terminology

Standard Solution = S
Test Solution = T
Low Concentration of Standard Solution = S_1
High Concentration of Standard Solution = S_2
Low Concentration of Test Solution = T_1
High Concentration of Test Solution = T_2

Latin Square Design (Randomized)

X =	S_1	S_2	T_1	T_2
	A	B	C	D
	B	A	D	C
	C	D	A	B
	D	C	B	A
Σ	$4\,(S_1)$	$4\,(S_2)$	$4\,(T_1)$	$4\,(T_2)$
X	S_1	S_2	T_1	T_2

Graphic Solution Formula

1. X milliliters of standard solution = Y milliliters of test solution; standard = micrograms per milliliter.

2. X times standard equals Y times test.

3. $T = \dfrac{X \text{ times standard}}{Y}$ = concentration (μg/ml).

4. Concentration (μg/ml) x dilution factor = total concentration (μg/ml).

5. Concentration (μg/ml) x total ml of original extract = total micrograms in extract.

6. Total micrograms in extract x tissue factor = micrograms per gram of tissue.

Mean Effects

Preparation Difference $F = 1/2\,(T_1 + T_2 - S_1 + S_2)$

Dose Difference $E = 1/2\,(T_2 - T_1 + S_2 - S_1)$

Estimate of Potency

Log Ratio of Doses = I

Slope = B = E/I

Log Potency Ratio = (T/S) = M = F/B

R = Potency Ratio = The antilog of the Log Potency Ratio

Potency Ratio = Concentration of test solution in relation to the known concentration of the standard solution ($\mu g/ml$).

Test solution concentrations times the dilution factor equals the total concentration of the test solution ($\mu g/ml$).

Test solution concentrations treated with above graphic formula (4-6) = the concentration of the test tissue in $\mu g/gm$ of tissue.
For identical tissue samples, both methods should produce identical values.

b. Calculation of Errors

Variance of each mean = $V = S^2/N$

Calculation of S; $S^2 = (D^2)/ (N-1)$

Variance of Preparation Difference F = A = V

Variance of Slope B = B = V/I^2

Index of Significance of B = $G = \dfrac{BT^2}{B^2}$

c. Calculation of T

$$T = (T_2 - T_1) - (S_2 - S_1)/2 \quad V$$

d. Statistics for Limits of Error

Fieller's Theorem
$$g = \frac{BT^2}{b^2}$$

Fiducial Limits (range of potency of test solution as per cent of the estimate)

$$\text{Log (fiducial limits \%)} = 2 + \frac{gM}{1-g} \pm \frac{T}{b\,(1-g)} \sqrt{A\,(1-g)\,B\,M^2}$$

When g is greater than 1, the assay is invalid.

When g is less than 0.1, it can be neglected and the equation is as follows:

$$\text{Log (fiducial limits \%)} = 2 \pm \frac{T}{b} \sqrt{A + B\,M^2}$$

The antilog values for the final numbers of the above equation are the limits of error for the particular sample in terms of percentages.

B. CIRCADIAN TOXICITY STUDIES IN MICE

1. Environmental Conditions

Male mice of the Swiss Webster strain, weighing 25 ± 2.5 gm each were used in these experiments. All animals were housed in groups of 9 or 11 corresponding to the number of animals used for each dose level. All groups of mice were conditioned for a three week period prior to experimentation (as previously described for rats). The animals received food and water ad libitum and were on a programmed illumination cycle. The light cycle was automatically timed and lasted from 0800 to 2000 hours daily. The amount of light received by these animals during the illumination periods was estimated as described previously for rats and ranged from between 13 to 66 foot candles. These animals were also maintained under conditions of ambient barometric pressure and humidity and a laboratory temperature of $23 \pm 1.0^{\circ}C$.

Drugs were administered I.P. and/or I.V. (tail vein). Each animal was observed for approximately two hours following drug administration until it either died or recovered. The number of animals dying at each dose level was used to calculate the LD50 for specific drugs at the designated circadian periods. Two groups of animals received atropine methyl nitrate (25 mg/kg I.P.) thirty minutes prior to the LD50 determination of acetylcholine and physostigmine.

2. Statistical Analysis

The LD50 and the statistical analysis of these values were determined according to the method described by Litchfield and Wilcoxon (1949). The method is described below:

a. Median Lethal Dose (LD50) and Standard Deviation (S. D.)

(1) An odd number of animals was always used, e. g. 9 or 11, in order to interpolate the median lethal dose.

(2) The number of deaths for each dose level was converted to per cent total animals used at that dose. This was then converted to probits in order to rectify dose response data.

(3) A probit value of 5.00 represents the median lethal dose.

(4) X_1 = high dose (mg/kg), X_2 = low dose (mg/kg).

(5) Y_1 = mortality rate from X_1, Y_2 = mortality rate from X_2.

(6) Slope b $= \dfrac{Y_2 - Y_1 \text{ (probit)}}{X_1 - X_2 \text{ (log)}}$

(7) S. D. $= \dfrac{1}{b \sqrt{N/2}}$

(8) Log Dose $= \dfrac{X_1 + (5.00 - Y_2)}{b}$

(9) Antilog of log dose = LD50 (mg/kg)

(10) LD50 ± S. D. =

$$\frac{\text{Antilog (log LD50)} + \text{S. D.} + \text{Antilog (log LD50)} - \text{S. D.}}{2}$$

b. A Specific Example (Litchfield and Wilcoxon, 1949, Procedure)

(The example is from course material presented by Dr. A. H. Friedman in 1966 which enables one to determine LD50 and S. D. quantal data by calculation.)

Dose	mg/kg	Log Dose	Mortality	Per Cent	Probit
X_1	100	2.00000	Y_1 7/9	77.8	5.765
X_2	75	1.87506	Y_2 2/9	22.2	4.235

$$ b = \frac{Y_2 - Y_1}{X_2 - X_1} = \frac{5.765 - 4.235}{2.00000 - 1.87506} = 12.2458 $$

$$ S.D. = \frac{1}{b\sqrt{n/2}} = \frac{1}{12.2458\sqrt{9/2}} = \frac{1}{(12.2458)\,2.1212} = \frac{1}{25.9758} = 0.03849 $$

$$ \text{Log Dose} = X_1 + \frac{5.000 - Y_1}{b} = 2.00000 + \frac{(5.000 - 5.065)}{12.2459} $$

$$ = 2.00000 - 0.06247 = 1.93753 $$

$$ \text{Antilog of } 1.93753 = 86.60 $$

1.93753		1.93753
+ 0.03849		− 0.03849
1.97602		1.89904

The Antilog of 1.97602 = 90.63

The Antilog of 1.89904 = 79.26

Difference = 15.37

15.37/2 = 7.685 or S.D. = 7.69 mg/kg

Therefore: LD50 ± S.D. = 86.60 ± 7.69

C. CIRCADIAN BARBITURATE SLEEPING TIME IN RATS

1. Environmental Conditions

Male albino rats of the Sprague-Dawley strain weighing approxi-
mately 300 gm each were used in this experiment. Barometric pressure,
humidity, laboratory temperature, light programming, preconditioning,
feeding and housing were the same as previously described for rats on
circadian biogenic amine experiments. In the experimental procedure,
groups of animals were divided as follows; (1) untreated control animals,
(2) animals anesthetized with pentobarbital sodium (50 mg/kg I. P.) and
observed for onset and duration of sleep and (3) animals treated with pento-
barbital sodium (50 mg/kg I. P.) and sacrificed at the approximate mid-
point of the specific sleeping period in the circadian cycle (mid-points were
estimated after preliminary determination of sleep duration for a particular
hour of the circadian cycle).

At six hour intervals for a total period of 24 hours, groups
containing four controls and four pentobarbital treated rats were killed by
decapitation. The midbrain and caudate nuclei were removed and analyzed
according to the methods previously described. Immediately following
decapitation, blood samples were collected from the jugular vein of each
animal for blood glucose and blood histamine analysis (see methods below).

The sleep onset time was determined as that period between pento-barbital sodium injection and the loss of the righting reflex. The duration of sleep was determined by recording the interval from the loss to the recovery of the righting reflex. Rectal temperature was measured in all animals in each group as previously described. When decapitation was performed, all rats were isolated from other test animals in an effort to prevent subsequent test animals from becoming excited.

2. Determination of Blood Glucose and Blood Histamine

a. Blood Glucose

Blood glucose was determined by the colorimetric method of Hugget and Nixon (1957). The procedure is based on the following coupled enzymatic reactions.

(1) $Glucose + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} Gluconic\ Acid + H_2O_2$

(2) $H_2O_2 + o\text{-Dianisidine} \xrightarrow{\text{perioxidase}} Oxidized\ o\text{-Dianisidine}$
(colorless) (brown)

The color intensity produced by the perioxidase is proportional to the amount of glucose present. A known glucose standard (100 mg%) is run with the unknown or test sample. The glucose concentration in the test sample is calculated according to the formula;

$$\text{Glucose in Test Sample} = \frac{\text{O. D. of Test Sample}}{\text{O. D. of Standard}} = 100 \text{ mg\%}$$

The optical density (O. D.) was read at a wavelength of 540 mu on a Coleman Junior Colorimeter (Model II).

b. Blood Histamine

Blood histamine was analyzed according to the fluorometric method of Anton and Sayre (1968) which is a modification of the Shore, Burkhalter and Cohn (1959) procedure. In this method 6 ml of whole blood was collected in concentrated perchloric acid. The blood and acid were shaken for 5 minutes after which the mixture was centrifuged for 15 minutes at 1500 r.p.m. The supernatant was extracted in butanol and heptane. From this point the procedure was identical to that used in analyzing for brain histamine (described previously).

D. ELECTRON MICROSCOPIC EXAMINATION OF
SYNAPTIC VESICLES IN BRAIN TISSUE

1. Environmental Conditions and Drug Treatment

Male albino Sprague-Dawley rats weighing approximately 300 gm each were used for this part of the experiment. Animals were housed in groups of six and were adapted to a programmed light - dark cycle with feeding, handling, watering and sacrificing as previously described.

Adapted animals were sacrificed by decapitation during the late-light phase (1500 hours) and during the late-dark phase (0300 hours) according to the following schema; (1) one group of animals was treated with dl-DOPA (500 mg/kg I. P.) and with MAO inhibitor, pargyline (6 mg/kg I. P.) one hour and thirty minutes, respectively, before sacrificing, (2) a second group was treated with 5 mg/kg of reserpine (I. P.) four hours before sacrificing, and (3) a third untreated group served as a control for the study.

2. Brain Areas Selected for Dissection

The anterior parts of both caudate nuclei (Figure 2) and the hypo-thalamus (Figure 6) were dissected from each of four animals in the three experimental groups. The tissues were analyzed as previously described for norepinephrine. The anterior regions of both caudate nuclei (Figure 2)

FIGURE 6

DISSECTION DIAGRAM FOR RAT HYPOTHALAMUS

Ventral View

Horizontal View

Medial Sagittal View
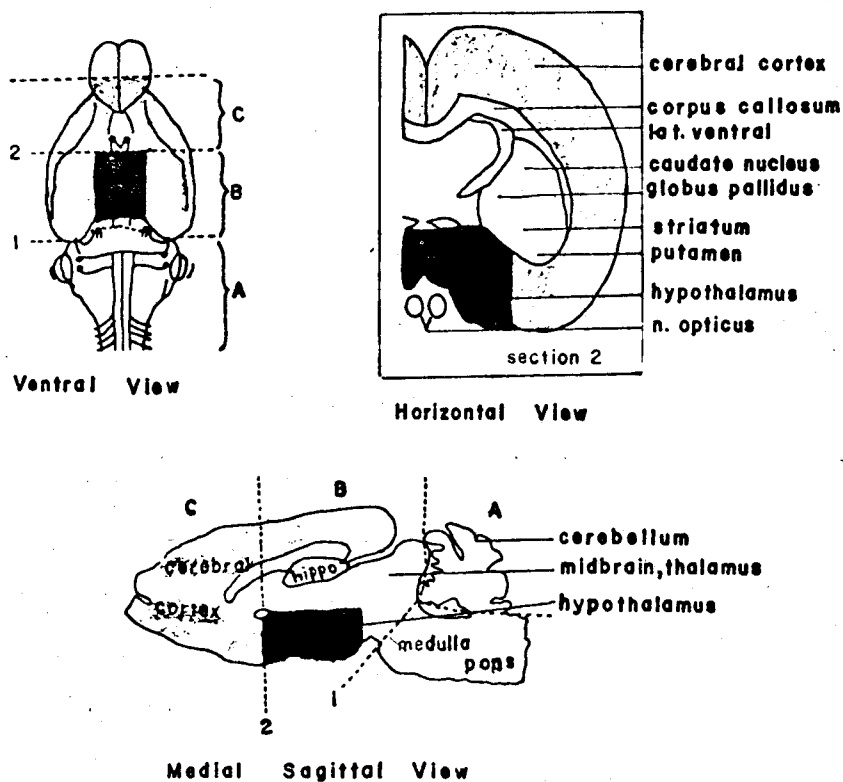
The diagram shows vertical, horizontal and sagittal views of the rat hypothalamus. The dark blocks represent the portion of hypothalamus dissected for analysis. Specific areas for electron microscopy were further identified by cell morphology using light microscopy.
Reference: Glowinski and Iversen (1966) J. Neurochem. 13:655-699.

together with the anterior and posterior hypothalamus (Figure 6) were removed from each of two animals of the three groups and prepared for electron microscopic viewing according to the following procedure.

3. Preparation of Tissue for Ultrastructural Examination

a. Fixing and Embedding

Immediately after sacrifice and within 1.0 to 1.5 minutes after the removal of the brain from the skull, the tissue was fixed in 6.0% gluteraldehyde, then washed in cold (0-4°C) phosphate buffer (pH 6.4) for approximately 15 minutes, and placed in cold (0-4°C) 1.0% phosphate buffered osmium tetroxide solution (pH 7.4) for 15 minutes. It was returned to fresh osmium tetroxide solution at 4°C for two hours and maintained at room temperature for 30 minutes. The tissue was rinsed twice in a phosphate buffer (pH 7.4) and dehydrated by exposing to a series of ethyl alcohol concentrations for short periods of time (50% for 2 minutes, 70% for 2 minutes, 90% for 2 minutes and 100% twice for 15 minute intervals each), followed by 30 minutes in 1:1 propylene oxide-epon mixture.*

Prior to final embedding, the tissue was placed in a 1:2 mixture of propylene oxide-epon overnight. Single pieces of tissue

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* According to Kay (1965), epon mixtures consist of different ratios of epon A and B. Mixture A = Epon 812 (62 ml) and DDSA (dodecenyl succinic anhydride) 100 ml. Mixture B = Epon 812 (100 ml) and MNA (methyl-nadic anhydride) 89 ml. The Epon mixture used in this experiment was 1:1 A-B.

approximately one-half millimeter in diameter were placed in a gelatin capsule, covered with epon mixture plus a 2% catalyst (2, 4, 6-dimethyl-aminomethylphenol) and incubated overnight at $60^{\circ}C$.

b. Sectioning and Staining

The polymerized tissue blocks were placed on a tissue holder and the embedded material was trimmed with a razor blade to form a pyramid with an angle of 60° until the tip was less than 1 square mm in area. Sections between 700 and 800 $\overset{\circ}{A}$ were cut on a Porter-Blum Ultramicrotome (MT-2) with a Dupont Diamond Knife (Model No. 3002062). The sections were floated off the knife edge onto the surface of a water trough, from which they were picked up on a carbon coated copper grid. In order to enhance electron density, the sections were subsequently stained with 15% uranyl acetate (in absolute methanol) and aqueous 0.35% lead citrate, for two minutes each, with washing in distilled water after each stain.

c. Viewing and Photographing

Sections were viewed on an RCA EMU-3H electron micro-scope. Photographs were taken on 2" x 10" Kodak Projection Slide Plates. Prints were made with an Omega D-2 enlarger to final magnifications of 32,400; 36,000; 47,600; 70,000 and 93,000 using Kodak Kodabromide F-3 paper (7 1/2" x 7 1/2" prints on 8" x 10").

4. Analysis of Data

Photographs were examined for synaptic elements. The vesicles, both granular and agranular, were identified within nerve terminals (boutons) and a system of quantification was devised as follows:

Vesicles fell into two distinct ranges of size; relatively large granular and small agranular. Granular vesicles are often larger in diameter than 650 Å and can measure 1100 - 1200 Å. The total number of granular vesicles per photographic print was counted (this is approximately an area of 23 square microns at a magnification of 47, 600).

Agranular vesicles range in size from 250 - 600 Å but average 400 ± 100 Å. Squares of one centimeter were drawn on each photographic print within the boutons and the vesicles contained within them counted. A technique similar to that employed for RBC and WBC counts was used (only vesicles touching half of the periphery of the drawn squares, i.e. two sides, were counted). The number of vesicles for four (4) of these centimeter squares were counted and arbitrarily considered to represent the relative number of vesicles for that particular print. Since each millimeter (at a magnification of 47, 600) equals 215 Å, the actual tissue area examined was equal to 0. 46 square microns.

CHAPTER IV

RESULTS

A. <u>CIRCADIAN RECTAL TEMPERATURE, BIOGENIC AMINE AND ACETYLCHOLINE RHYTHMS IN THE RAT</u>

1. <u>Rectal Temperature</u>

The circadian pattern for rectal temperature characteristically reaches a peak during the dark phase of the illumination cycle and falls to its lowest value late in the light phase of the illumination cycle (Tables 1, 2 and 8; Figures 7 and 19). The pattern in the adrenalectomized animal is not qualitatively altered, although all values for rectal temperature in these animals are below those for normal rats. In this experiment, the greatest difference seen between peak and trough values in untreated rats was 1.5^{o}C. In the concomitantly treated group of adrenalectomized rats, the temperature difference was only 1.0^{o}C (Table 2; Figure 7). Both differences were highly significant statistically. The rectal temperature for rats before and following pentobarbital treatment was also determined (Table 8; Figure 19). The results for the controls in the pentobarbital experiment were similar to the results for controls in the adrenalectomized experiment. In both cases the steepest rise in rectal temperature of normal animals occurred from 1800 to 2400 hours, during the transition from the light to the dark phase of the illumination cycle (Tables 1 and 8; Figures 7 and 19), when the motor activity of these nocturnal animals was approaching a maximum. In both

FIGURE 7

RAT CIRCADIAN RECTAL TEMPERATURE VALUES
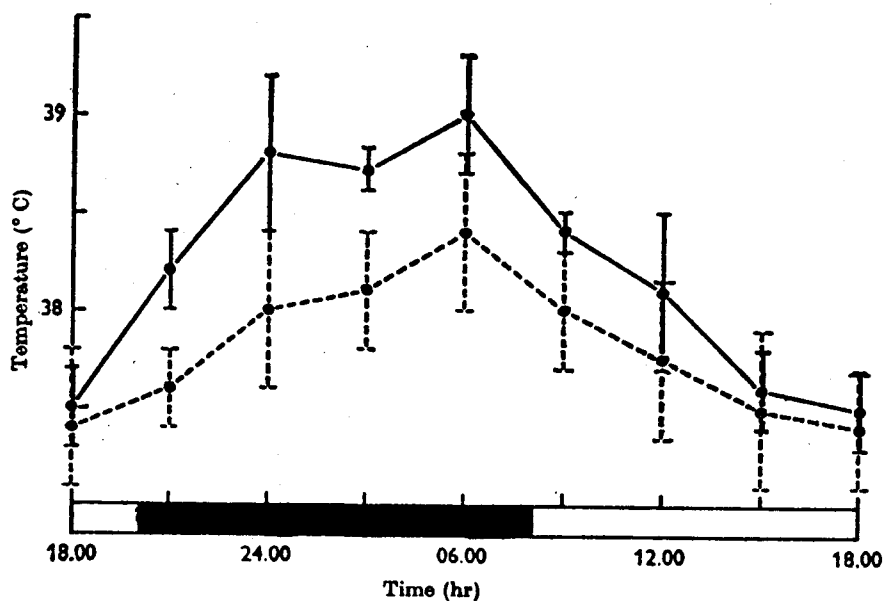
Circadian rhythm for rectal temperature in normal (continuous line) and adrenalectomized (dashed line) rats. The clear bar indicates the light phase and the black bar indicates the dark phase of the illumination cycle. Verticle brackets indicate standard deviations.

TABLE 1. Mean concentrations of histamine, noradrenaline (NA), and serotonin (5-HT), μg/g wet brain tissue, ±s.d.

Time of day	Caudate nucleus			Mid-brain			Rectal temperature (°C)
	Histamine	NA	5-HT	Histamine	NA	5-HT	
03.00	—	—	—	—	—	—	38.7 ± 0.1
06.00	2.29 ± 0.35	1.25 ± 0.05	0.26 ± 0.03	0.063 ± 0.007	0.94 ± 0.01	0.17 ± 0.02	39.0 ± 0.3
09.00	—	—	—	—	—	—	38.4 ± 0.1
12.00	1.78 ± 0.24	0.96 ± 0.09	0.35 ± 0.05	0.041 ± 0.005	0.83 ± 0.03	0.18 ± 0.02	38.1 ± 0.4
15.00	—	—	—	—	—	—	37.6 ± 0.2
18.00	1.17 ± 0.09**	0.85 ± 0.11***	0.43 ± 0.05***	0.033 ± 0.005***	0.58 ± 0.13**	0.21 ± 0.08†	37.5 ± 0.2***
21.00	—	—	—	—	—	—	38.2 ± 0.2
24.00	2.22 ± 0.11	0.93 ± 0.14	0.34 ± 0.02	0.032 ± 0.008	0.80 ± 0.09	0.20 ± 0.02	38.8 ± 0.4

** Difference between maximum and minimum values significant at $P < 0.01$.

*** Difference between maximum and minimum values significant at $P < 0.001$.

† Significance not proven.

TABLE 2. Adrenalectomized rats. Mean concentrations of histamine, noradrenaline (NA), and serotonin (5-HT), μg/g wet brain tissue, ±s.d.

Time of day	Caudate nucleus: Histamine	Mid-brain		Rectal temperature (°C)
		NA	5-HT	
03.00	—	—	—	38.1 ± 0.3
06.00	2.66 ± 0.70	0.81 ± 0.04	0.16 ± 0.03	38.4 ± 0.4
09.00	—	—	—	38.0 ± 0.3
12.00	1.63 ± 0.12	0.78 ± 0.07	0.20 ± 0.02	37.8 ± 0.4
15.00	—	—	—	37.5 ± 0.4
18.00	0.69 ± 0.18***	0.69 ± 0.14†	0.21 ± 0.02*	37.4 ± 0.3**
21.00	—	—	—	37.6 ± 0.2
24.00	2.20 ± 0.17	0.78 ± 0.02	0.20 ± 0.04	38.0 ± 0.4

* Difference between maximum and minimum values significant at $P < 0.05$.

** Difference between maximum and minimum values significant at $P < 0.01$.

*** Difference between maximum and minimum values significant at $P < 0.001$.

† Significance not proven.

cases the mean rectal temperature rose only slightly from 2400 to 0600 hours, then fell gradually to its lowest level from 1200 to 1800 hours. The circadian pattern for the adrenalectomized rat was similar to both controls (Table 2; Figure 7) but the mean rectal temperature during the dark phase was significantly lower. In the pentobarbital sodium treated rats (Table 8; Figure 19), the rectal temperature decreased during the dark phase and was actually a mirror image of the controls. The differences in temperature between treated and control animals at 1200, 2400 and 0600 hours were significant statistically ($P < 0.01$).

2. Central Biogenic Amines

In the transition from the light to the dark phase of illumination, caudate nucleus histamine levels in the normal rat rose sharply, approximately doubling by 2400 hours and reaching a peak at 0600 hours (Figure 8; Table 1). They then fell significantly during the transition from the dark to the light phase, reaching their lowest level at 1800 hours ($P < 0.01$). The mid-brain circadian pattern was similar (Table 1). In adrenalectomized animals, the peak histamine levels in the caudate nucleus increased by 228% over minimum values (Figure 8; Table 2). Minimal histamine values in the caudate nucleus of these animals differed significantly from those of normal rats although the peak values did not.

FIGURE 8

RAT CIRCADIAN CAUDATE NUCLEUS HISTAMINE LEVELS
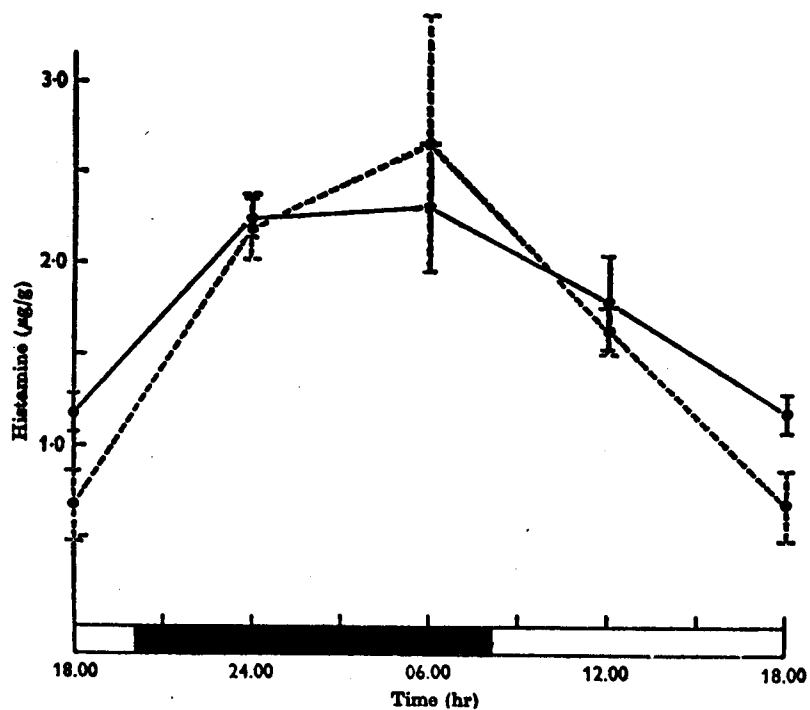
Circadian pattern for rat caudate nucleus histamine levels in normal (continuous line) and adrenalectomized (dashed line) animals.

Circadian patterns for noradrenaline in midbrain and caudate nucleus were similar to those of histamine, but the percentage change was less pronounced. The rise over minimal values in midbrain of normal animals was 62% and significant, as was the 47% rise in the caudate nucleus (Table 1). The 17% rise in the midbrain of adrenalectomized rats, however, was not significant (Table 2).

Peak serotonin values in normal animals were reached in the caudate nucleus and midbrain at 1800 hours at the conclusion of the light phase when the rats were inactive or asleep (Figure 9) and when mean rectal temperature was at its lowest level (Table 1). The change in the caudate nucleus level was significant when compared with its minimal value. A significant rise (31%) occurred in the midbrain serotonin levels of adrenalectomized rats (Table 2).

The noradrenaline and histamine content of caudate nucleus and midbrain follow a time course similar to that of rectal temperature, with maximal values at 0600 hours and minimal values at 1800 or 2400 hours (Figures 7, 8 and 9; Tables 1 and 2). Midbrain and caudate nucleus serotonin values were maximal at 1800 hours and minimal at 0600 hours. These trends were noted in normal and adrenalectomized rats, except that midbrain histamine and caudate nucleus norepinephrine and serotonin was not determined in adrenalectomized animals.

FIGURE 9

CIRCADIAN CAUDATE NUCLEUS BIOGENIC AMINE AND RECTAL TEMPERATURE LEVELS (THE NORMAL RAT)
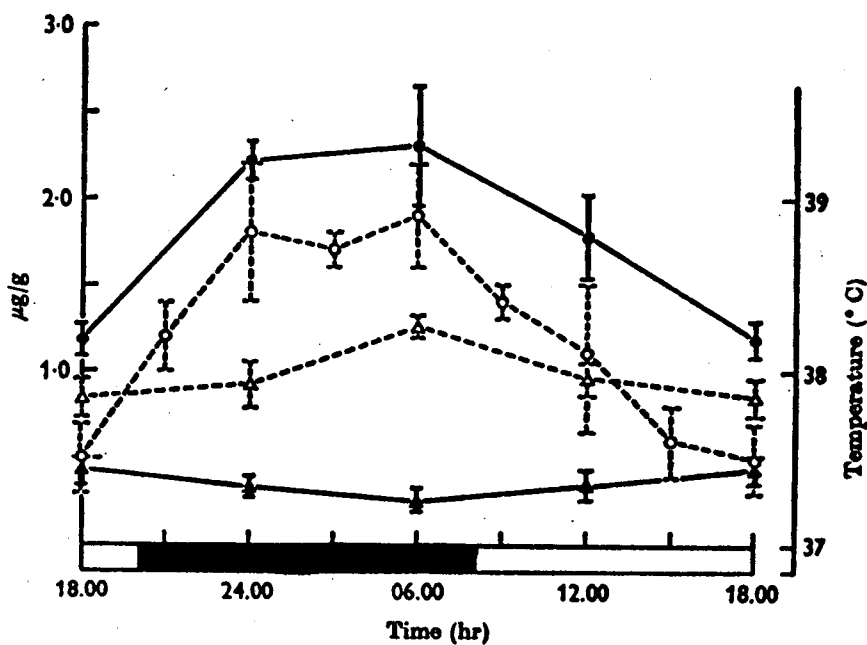
Circadian pattern for caudate nucleus histamine, norepinephrine and serotonin levels and rectal temperature in the normal rat.
Rectal temperature O----O ; Histamine ●——● ;
Norepinephrine △----△ ; Serotonin ▲——▲ .

In the pentobarbital sodium treated rats, midbrain NE and 5-HT showed an inverse relationship during the dark and light phase of the circadian cycle (Figure 17, Table 7). Norepinephrine levels for both normal and pentobarbital-treated animals were maximal at 2400 hours and minimal at 1800 hours. The difference between the peak and trough values was highly significant statistically with $P < 0.005$. The maximum 5-HT levels for control and pentobarbital-treated animals occurred during the light phase of illumination cycle at 1200 and 1800 hours, respectively. The minimum 5-HT levels for both treated and untreated groups was observed during the dark phase at 0600 hours. These values, however, did not differ significantly from those observed at 2400 hours. The difference between 5-HT peaks and troughs were significant at the 1% level.

Noradrenaline levels were significantly higher ($P < 0.05$) in the pentobarbital-treated animals at 2400, 0600 and 1200 hours. Serotonin concentration was lower in the treated than in the control animals during the light phase of the illumination cycle (1200 and 1800 hours), with $P < 0.05$. Following pentobarbital treatment the normally lower 5-HT levels of the dark phase extended into the light phase.

Figure 18 and Table 7 show that the caudate histamine circadian pattern is similar to that of midbrain norepinephrine with peak levels

occurring at 2400 hours and trough levels occurring during the light phase for both control and pentobarbital-treated animals. The difference between these values is significant at the 1% level. A significant increase over controls at both peaks and troughs was also noted for animals treated with pentobarbital ($P < 0.01$). By comparison, the caudate histamine values noted for this experiment (Figure 18; Table 7) are considerably below the values shown in Figure 8 and Table 1. The previous caudate histamine values were determined by the method of Shore, Burkhalter and Cohn (1959). The latter values were determined by using the technique of Anton and Sayre (1968). The most recent procedure eliminates the contaminant, spermidine, found in n-butanol extracts of some brain tissues (Michaelson, 1967). Spermidine has a fluorescence spectrum similar to that of histamine. It is noted in these results that the circadian patterns obtained by both methods are similar. Values for midbrain histamine was the same by both techniques (Tables 1 and 7), indicating the probable absence of spermidine from the midbrain. The role of spermidine in the caudate nucleus and its possible function in the central nervous system in general is under investigation.

3. Acetylcholine

Midbrain and caudate nucleus acetylcholine exhibited a circadian pattern in normal animals with maximum values occurring at 2400 hours for both tissues. Trough or minimal values occurred at 1200 hours in the midbrain and at 1800 hours in the caudate nucleus (Figure 10 and Table 3). A 32.3% difference was noted between the midbrain acetylcholine maximum and minimum values. The difference is highly significant statistically with a $P < 0.001$. A 21.5% difference was noted between maximum and minimum caudate nucleus values. Significance, although borderline, was not proven in the case of the caudate nucleus. Midbrain and caudate acetylcholine follow a circadian pattern similar to circadian rectal temperature, histamine and norepinephrine which also peak during the dark phase of the illumination cycle.

FIGURE 10

RAT BRAIN ACETYLCHOLINE LEVELS
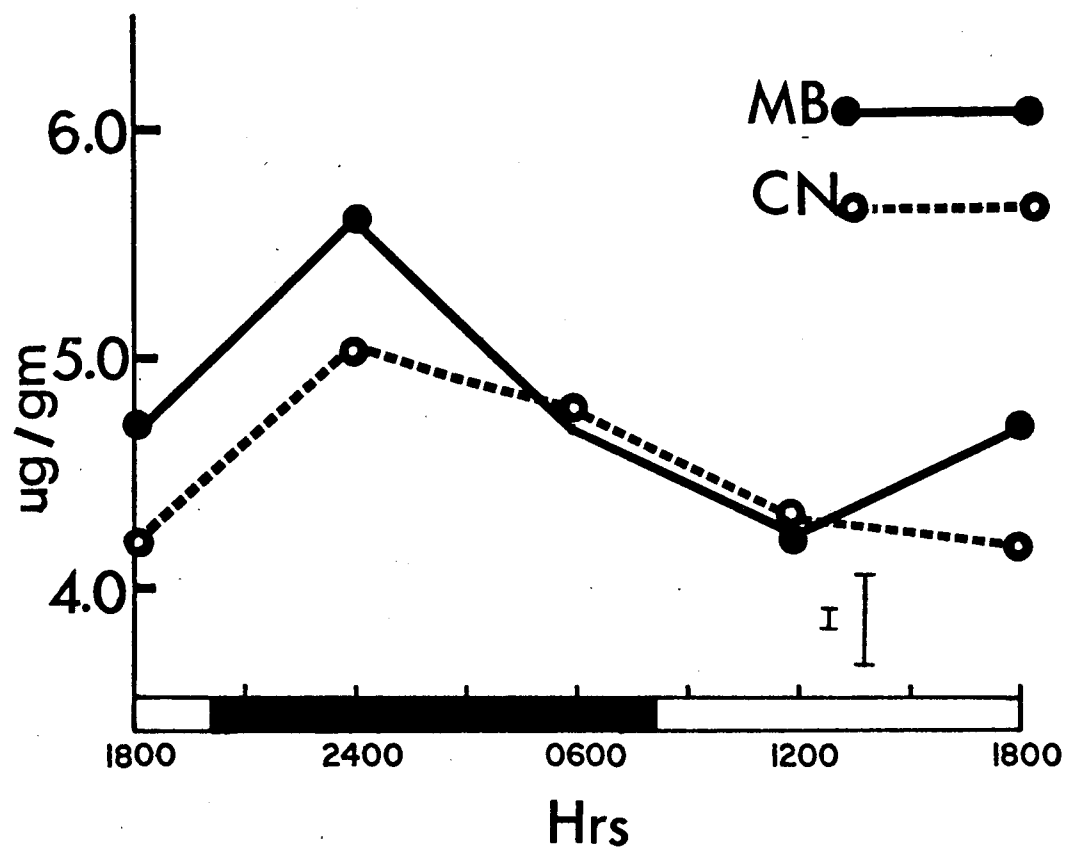
Acetylcholine levels for the midbrain and caudate nucleus of the rat. Vertical brackets indicate range of standard deviation.

TABLE 3

ACETYLCHOLINE LEVELS IN RAT MIDBRAIN
AND CAUDATE NUCLEUS AS A FUNCTION OF TIME OF DAY

	Time of Day (Hours)			
	0600	1200	1800	2400
Midbrain				
Acetylcholine	4.688 ± 0.114	4.328 ± 0.289*	4.707 ± 0.169	5.607 ± 0.149*
% Change	110.6	100.0	111.0	132.3
Caudate Nucleus				
Acetylcholine	4.784 ± 0.660	4.273 ± 0.770	4.172 ± 0.470	5.067 ± 0.710
% Change	114.7	102.4	100.0	121.5

All values represent the mean ± standard deviation.
% Change equals the per cent increase over 100 (minimum value).
* Difference between maximum and minimum value, significant at $P < 0.001$.

B. CIRCADIAN RHYTHMS IN THE TOXICITY OF CHOLINOMIMETICS IN MICE

Figure 11, Tables 4 and 5 show the circadian toxicity of acetylcholine in mice by I.V. and I.P. routes of administration. For both routes, peak LD50 levels (i.e., least toxic doses) were noted during the light phase and troughs during the dark phase of the illumination cycle. The maximum value for I.P. administered acetylcholine occurred at 1200 hours while the minimum value was noted at 2400 hours. The 17.2% difference between maxima and minima is highly significant ($P < 0.001$). The maximum toxicity value for I.V. administered acetylcholine also occurred at 1200 hours and the minimum value was noted at 0400 hours. The 50.7% difference between the peak and trough value is also significant at the 1% level. The relative toxicity of I.V. vs I.P. routes of administration ranged from 15.1 times during minima to 11.6 times during maxima.

The circadian toxicity of the cholinergic compounds acetylcholine, pilocarpine and oxotremorine are shown in Figure 12, Tables 4 and 5. Oxotremorine and acetylcholine show lowest LD50 values or greatest toxicity levels at 2400 hours. Pilocarpine's greatest toxicity is at 0600 hours but the change is not significantly different from the 2400 hour value. Acetylcholine, pilocarpine and oxotremorine are least toxic during the light

FIGURE 11

ACETYLCHOLINE TOXICITY LEVELS
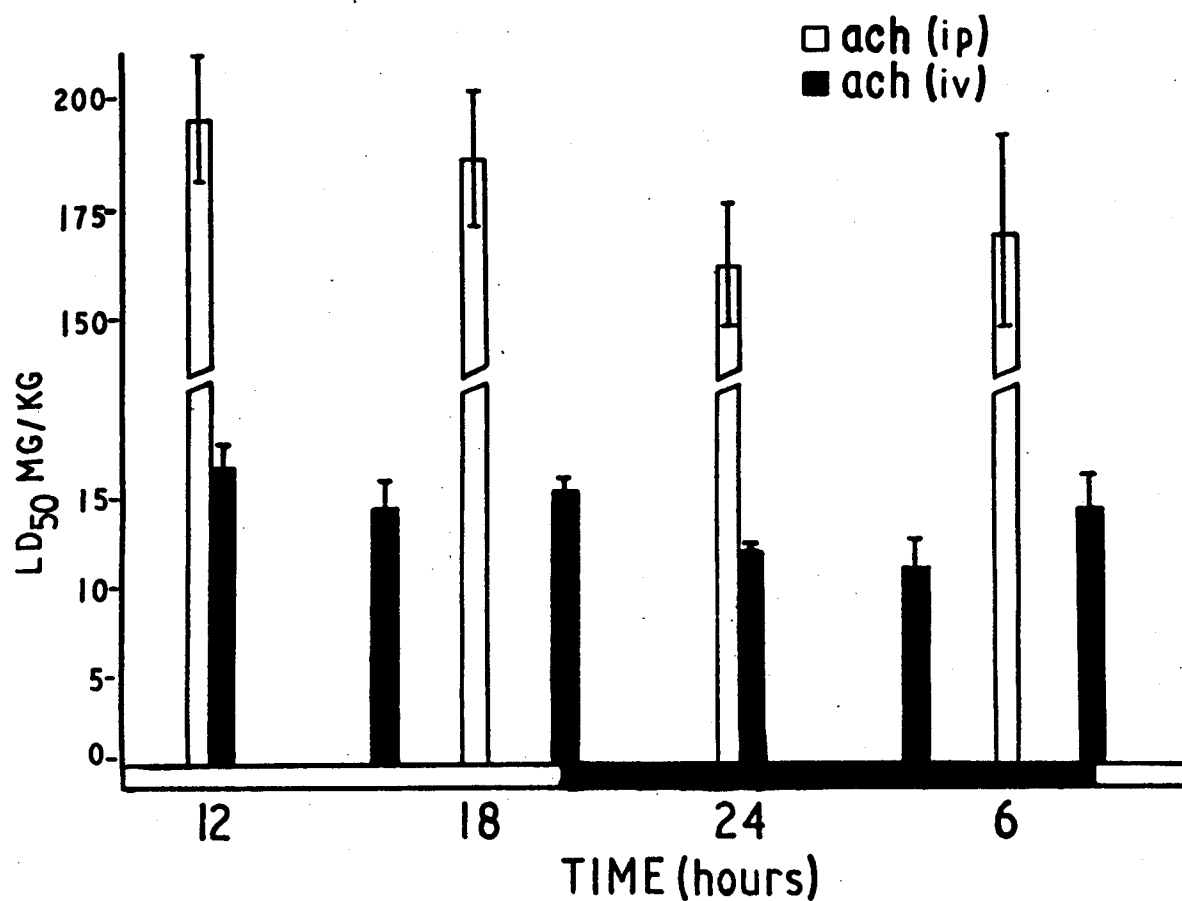
Circadian rhythms for acetylcholine LD50 levels in mice by i. v. and i. p. routes of administration. Verticle brackets indicate standard deviations.

FIGURE 12

TOXICITY OF CHOLINERGIC DRUGS
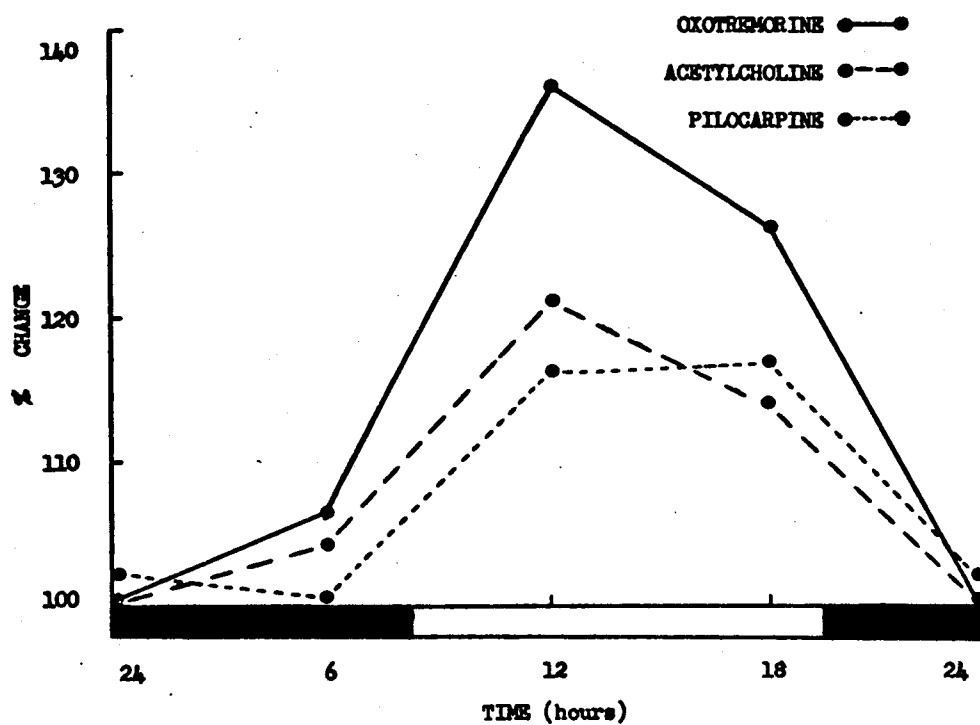
Circadian patterns for the LD50 of oxotremorine, acetylcholine, and pilocarpine in the mouse (I. P.)

FIGURE 13

TOXICITY PATTERNS FOR PHYSOSTIGMINE AND NEOSTIGMINE
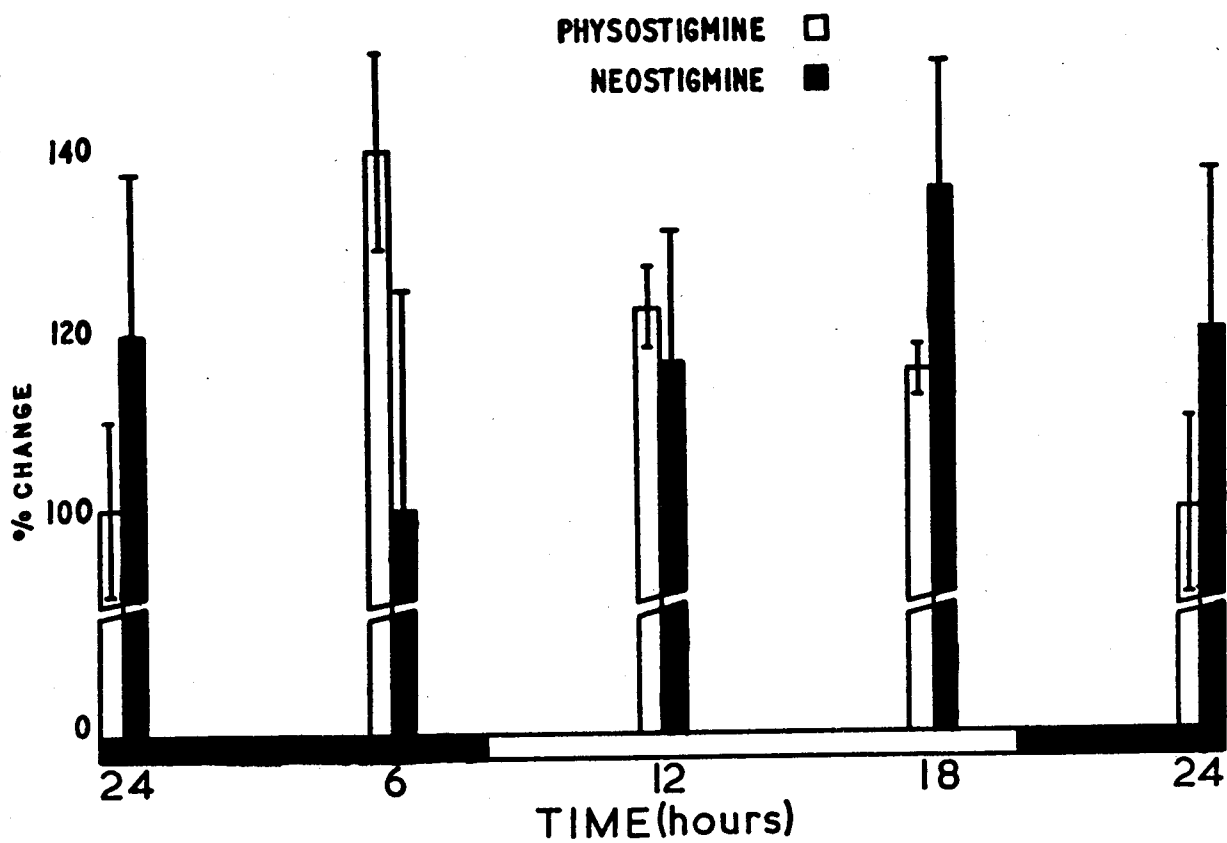
Circadian rhythms for LD50 levels of neostigmine and physostigmine in the mouse (I.P.).

FIGURE 14

TOXICITY OF ATROPINE SULFATE AND ACETYLCHOLINE
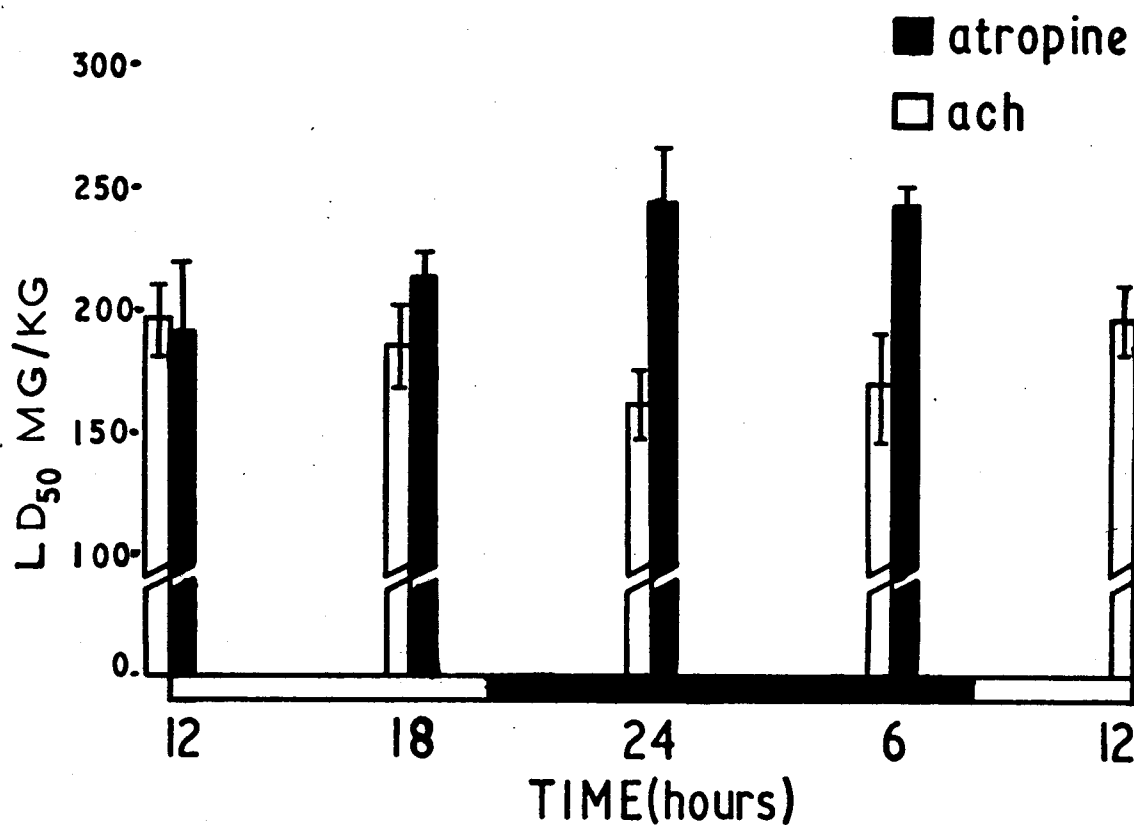
Circadian rhythms for (I. P.) LD50 levels of atropine sulfate and acetylcholine in the mouse.

FIGURE 15

TOXICITY OF ACETYLCHOLINE AND PHYSOSTIGMINE
BEFORE AND AFTER ATROPINE METHYL NITRATE
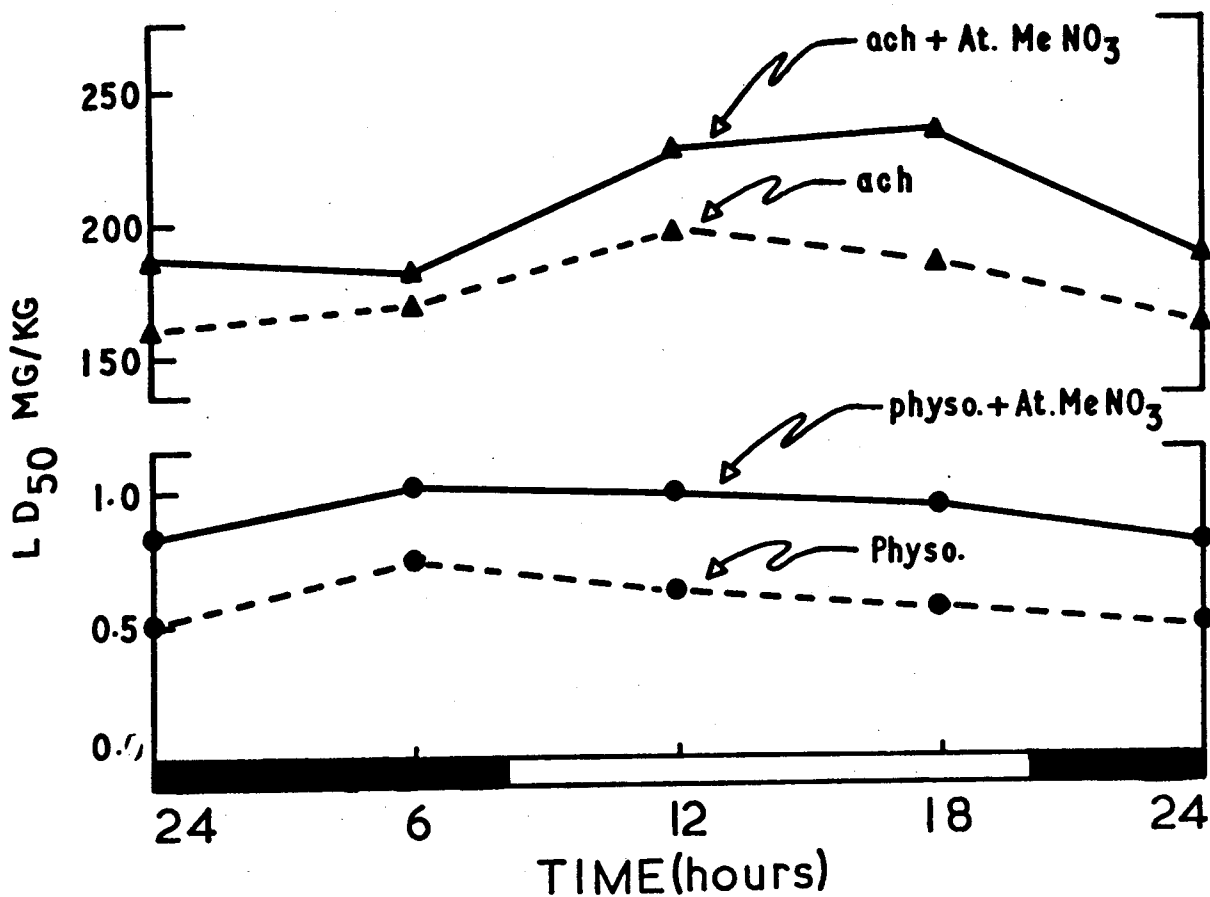
Circadian rhythm LD50 levels for acetylcholine before (▲----▲) and after (▲———▲) atropine methyl nitrate and for physostigmine before (●----●) and after (●———●) atropine methyl nitrate.

TABLE 4

CIRCADIAN LD50 VALUES FOR CHOLINOMIMETICS IN MICE

Compounds	LD50 Values - mg/kg (I. P.)			
	Time of Day (Hours)			
	0600	1200	1800	2400
Cholinergic				
Acetylcholine·Cl	168.70 ± 21.40	195.90 ± 13.60**	185.80 ± 15.20	162.20 ± 13.70
Carbachol·Cl	0.37 ± 0.06	0.56 ± 0.08**	0.40 ± 0.10	0.56 ± 0.07
Pilocarpine·HCl	154.50 ± 15.30	179.40 ± 8.20	181.10 ± 7.10*	158.50 ± 9.70
Oxotremorine Sesquifumerate	3.20 ± 0.20	4.10 ± 0.97*	3.80 ± 0.10	3.00 ± 0.20
Anticholinesterase				
Physostigmine·SO$_4$	0.71 ± 0.05**	0.63 ± 0.03	0.59 ± 0.01	0.51 ± 0.05
Prostigmine·CH$_3$SO$_4$	0.23 ± 0.06	0.28 ± 0.03	0.36 ± 0.05**	0.29 ± 0.03
Anticholinergic				
Atropine·SO$_4$	243.30 ± 7.60	191.50 ± 28.80	223.90 ± 9.50	243.90 ± 21.40**
Cholinomimetic (After 25 mg/kg Atropine CH$_3$NO$_3$)				
Acetylcholine·Cl	180.70 ± 19.80	223.90 ± 15.50	230.70 ± 9.20**	187.90 ± 40.00
Physostigmine·SO$_4$	1.00 ± 0.03	1.01 ± 0.02*	0.97 ± 0.03	0.86 ± 0.09

All values represent the mean ± standard deviation.
* Difference between maximum and minimum values significant at $P < 0.05$.
** Difference between maximum and minimum values significant at $P < 0.01$.

TABLE 5

CIRCADIAN LD50 VALUES FOR CHOLINOMIMETICS
IN MICE AS PER CENT OF MINIMUM VALUE

Compounds	Time of Day (Hours)			
	0600	1200	1800	2400
Cholinergic				
Acetylcholine·Cl	104.00	120.80	114.50	100.00
Carbachol·Cl	100.00	151.40	108.10	151.40
Pilocarpine·HCl	100.00	116.10	117.20	102.50
Oxotremorine Sesquifumerate	106.60	136.60	126.70	100.00
Anticholinesterase				
Physostigmine·SO_4	139.20	123.50	115.70	100.00
Prostigmine·CH_3SO_4	100.00	121.70	156.50	126.00
Anticholinergic				
Atropine·SO_4	127.00	100.00	116.90	127.30
Cholinomimetic (After 25 mg/kg Atropine CH_3NO_3)				
Acetylcholine·Cl	100.00	123.90	127.70	104.00
Physostigmine·SO_4	116.30	117.40	112.80	100.00

TABLE 6

ACETYLCHOLINE TOXICITY
(I. V.)

Time of Day (Hours)	LD50 Values (mg/kg)
0400	11.17 ± 1.40*
0800	14.55 ± 1.73
1200	16.83 ± 1.36*
1600	14.62 ± 1.47
2000	15.41 ± 1.55
2400	11.86 ± 1.14

All values represent the mean $\pm$ standard deviation.
* Difference between maximum and minimum, $P < 0.01$.

phase of the illumination cycle (from 1200 to 1800 hours), i.e., higher doses

must be given during the animal's quiescent period to produce lethal effects.

The quaternary compound, neostigmine is most toxic at 0600 hours

(Figure 13; Tables 4 and 5) but less toxic during the light phase of the

illumination cycle. The tertiary anticholinesterase, physostigmine on the

other hand is least toxic at 0600 hours (Figure 13; Tables 4 and 5), and

becomes progressively more toxic during the late light and early dark phase

of the illumination cycle.

The circadian toxicity pattern of atropine sulfate is essentially a

mirror image of that produced by acetylcholine, oxotremorine and pilo-

carpine. Atropine is more toxic during the light phase and least toxic from

2400 to 0600 hours (Figure 14; Tables 4 and 5) when the animal's general

motor activity and metabolic activity is maximal.

When mice were treated with 25 mg/kg I. P. of atropine methyl

nitrate 30 minutes prior to the toxicity studies of acetylcholine and physo-

stigmine, the circadian pattern was relatively unchanged but the toxicity of

these compounds was decreased (Figure 15; Tables 4 and 5). Acetylcholine

toxicity decreased from 6.7 to 19.9%. Maximal decreases were observed

between 1800 and 2400 hours. The toxicity of physostigmine was also

decreased but to a greater degree, from 29 to 41% with the maximum

decrease noted at 2400 hours. Carbachol showed equal peaks at 1200 and

2400 hours while the minimum value was noted at 0600 hours. The

differences between peaks and troughs are highly significant.

C. <u>CIRCADIAN CHANGES IN PENTOBARBITAL TREATED RATS</u>

1. <u>Sleeping Time</u>

Figure 16 and Table 7 show the circadian patterns for onset and duration of sleep. The shortest duration and longest onset times occurred during the dark phase of the cycle, while a reverse relationship was observed during the light phase. The maximum sleeping time was noted at 1800 hours while the minimum time occurred at 0600 hours. The difference between the minimum value and each value recorded during the light phase for sleep duration is highly significant with $P < 0.005$. The maximum time for sleep onset occurred at 0600 hours and the minimum time was noted at 1800 hours. The difference between these two values are significant at the 5% level.

2. <u>Blood Histamine and Glucose Levels for Treated and Untreated Rats</u>

The maximum blood serum histamine concentration occurred at 1800 hours (Figure 20, Table 8), while the minimum concentration was observed at 1200 hours. The difference between the peak and trough values for both treated and untreated groups was significant at the 1% level. There was also a significant difference between treated and untreated groups at 2400 hours.

Figure 21 and Table 8 show the bimodal nature of blood glucose levels in control and pentobarbital-treated rats. Maximal blood glucose

concentration occurred late in the feeding period (dark phase). A secondary

peak occurred at the conclusion of the light phase. The minimum concen-

tration was noted at 2400 hours, for both control and pentobarbital-treated

animals. The differences between the two peaks and the trough glucose

levels for the control and treated animals were highly significant statisti-

cally ($P < 0.005$). The differences between the bimodal peaks for control

animals are significant at the 5% level as are the differences between the

maximum glucose levels of treated and control animals at 0600 hours.

FIGURE 16

PENTOBARBITAL SODIUM SLEEP AND ONSET TO SLEEP TIMES
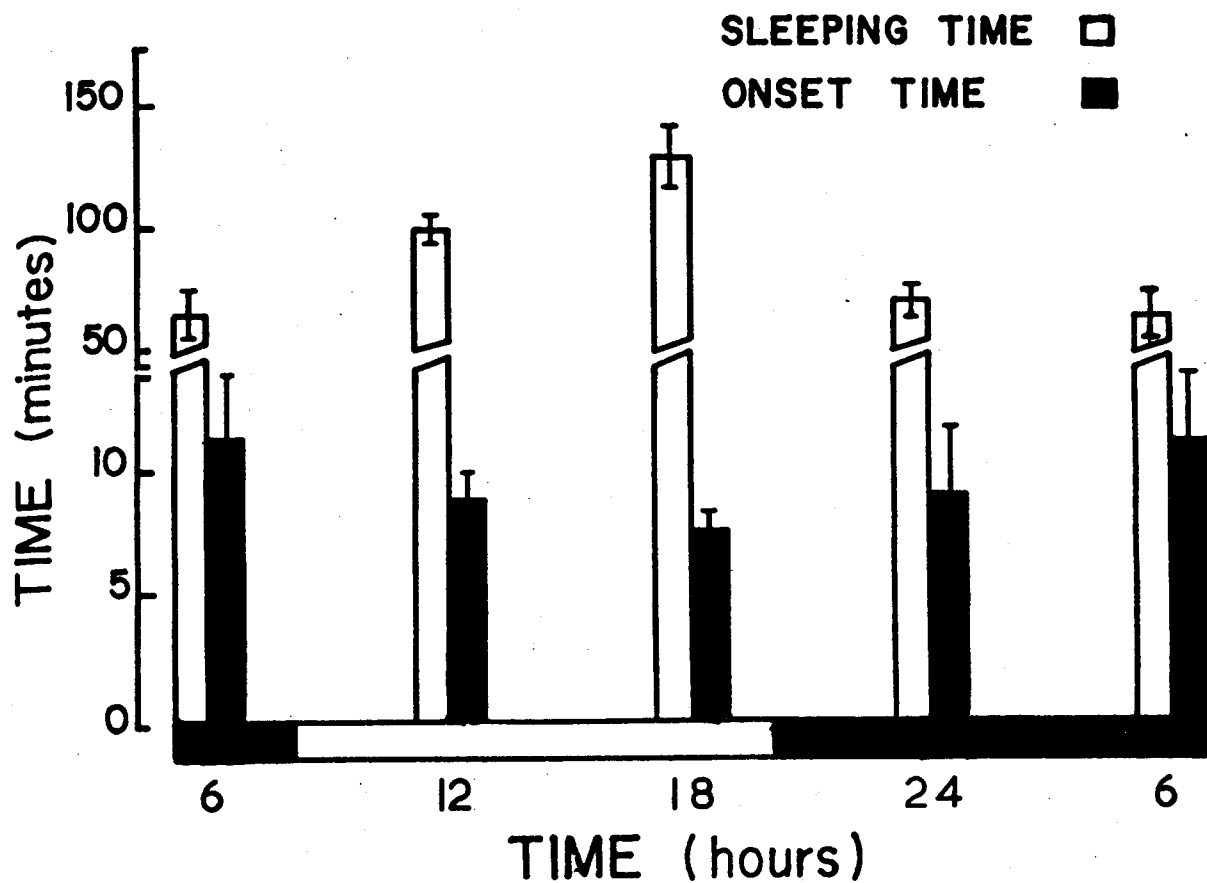
Circadian pattern for pentobarbital sodium (50 mg/kg) induced
sleeping time and sleep onset time in the rat.

FIGURE 17

BIOGENIC AMINE LEVELS BEFORE AND AFTER
BARBITURATE TREATMENT
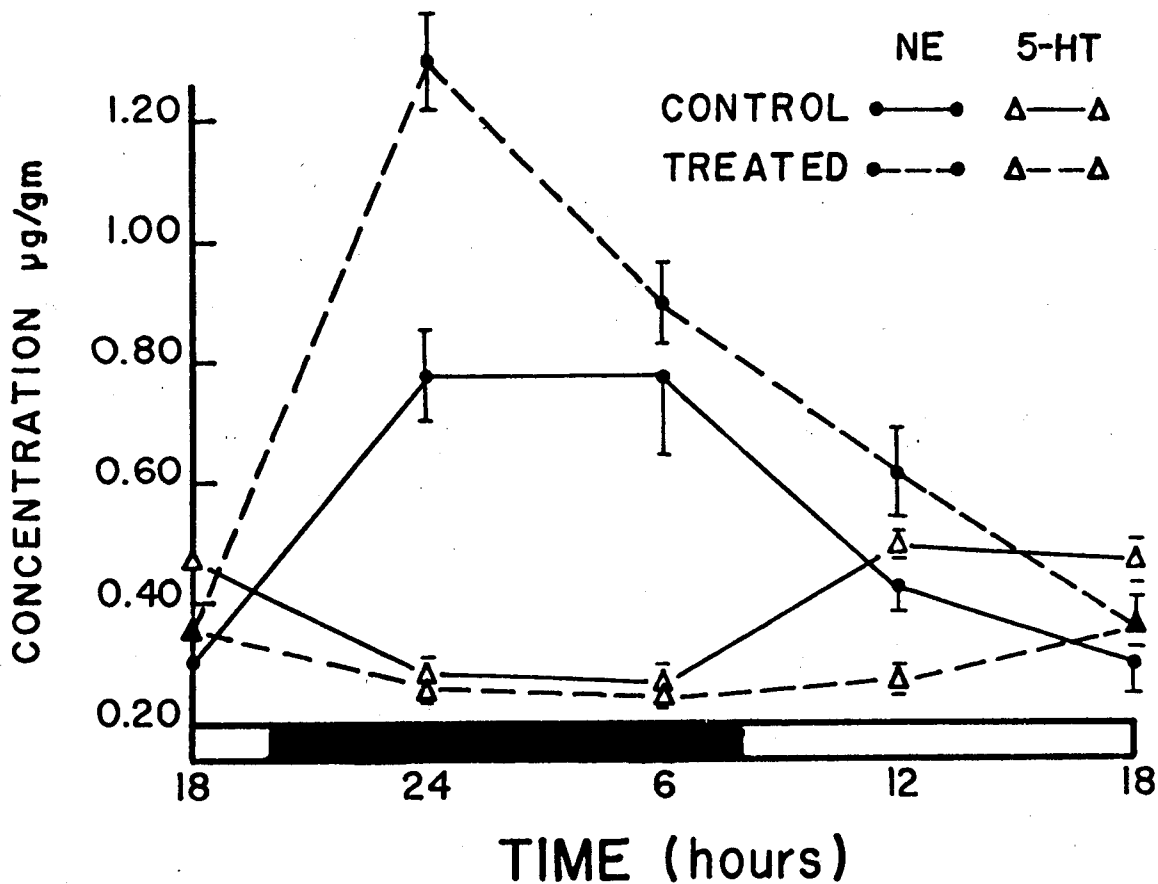
Circadian patterns for midbrain noradrenaline (circles) and serotonin
(triangles) for control (solid line) and pentobarbital sodium treated
rats (dashed line).

FIGURE 18

HISTAMINE LEVELS BEFORE AND AFTER
BARBITURATE TREATMENT
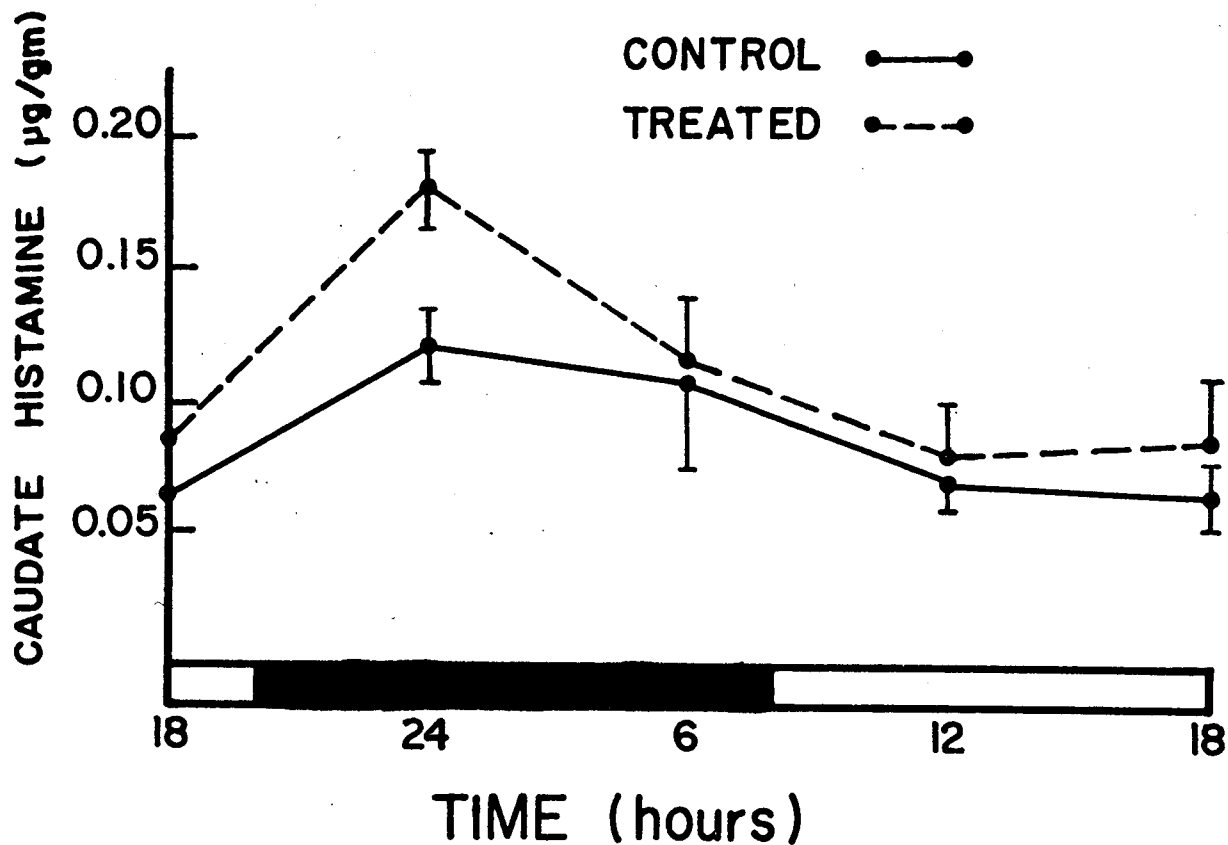
Circadian pattern for rat caudate nucleus histamine levels in
control and pentobarbital sodium treated animals.

FIGURE 19

RECTAL TEMPERATURE VALUES BEFORE AND AFTER
BARBITURATE TREATMENT
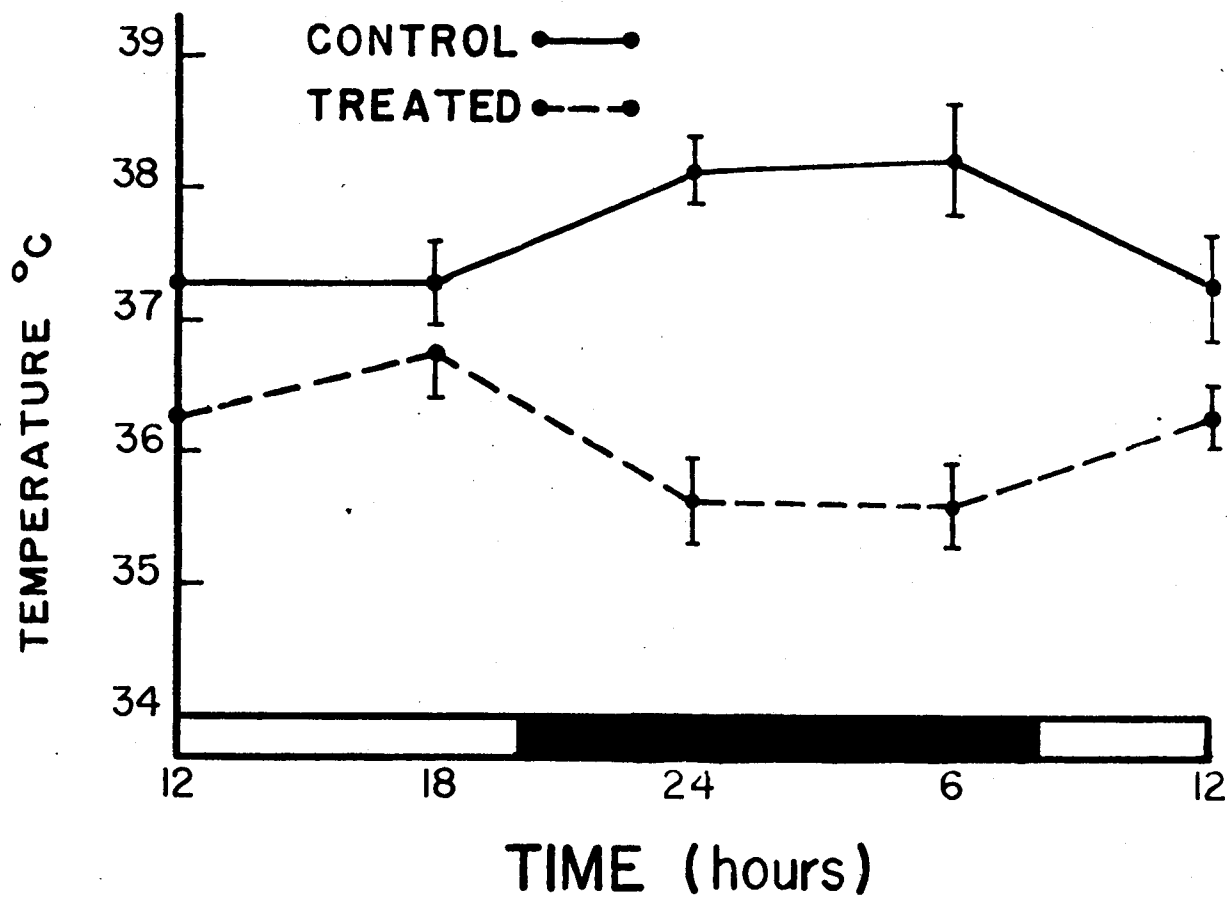
Circadian pattern for rectal temperature in control and pentobarbital
sodium treated rats.

FIGURE 20

BLOOD HISTAMINE LEVELS BEFORE AND AFTER
BARBITURATE TREATMENT
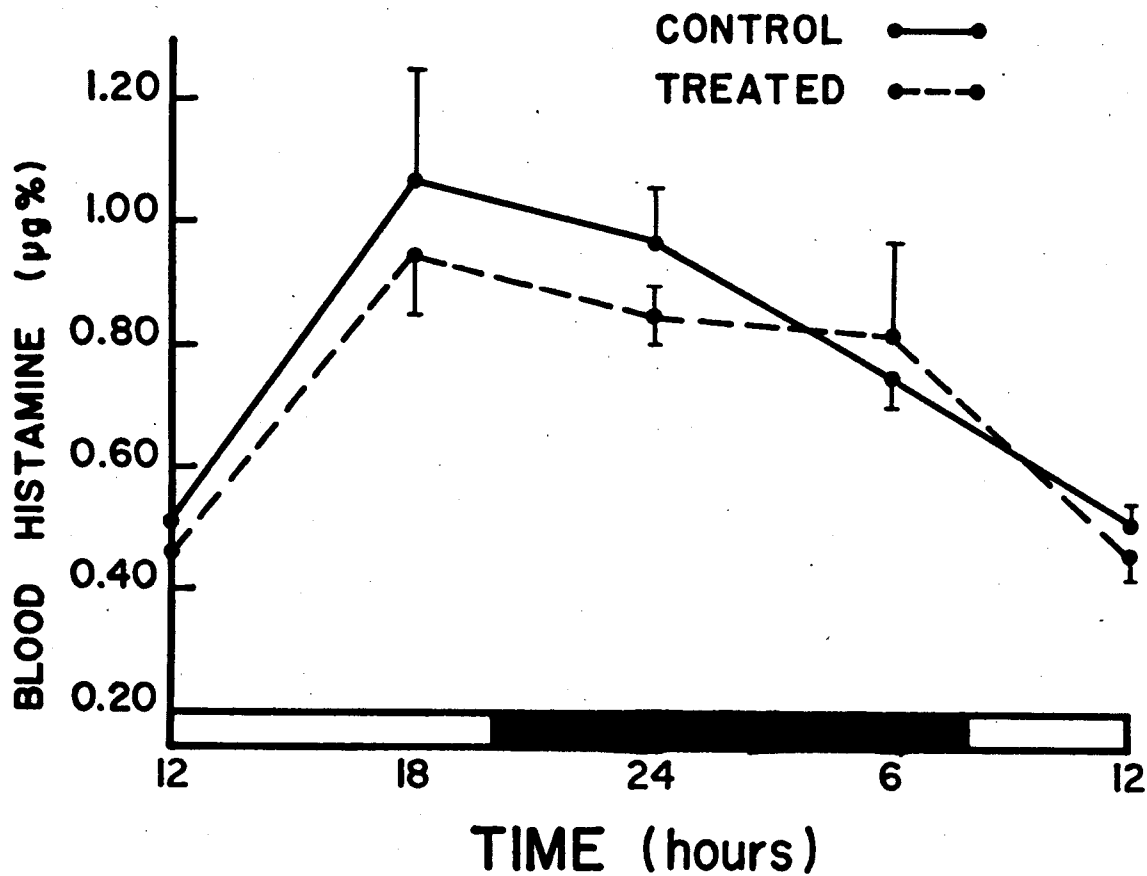
Circadian pattern for blood serum histamine levels in control rats
and in pentobarbital sodium treated animals.

FIGURE 21

BLOOD GLUCOSE LEVELS BEFORE AND AFTER
BARBITURATE TREATMENT
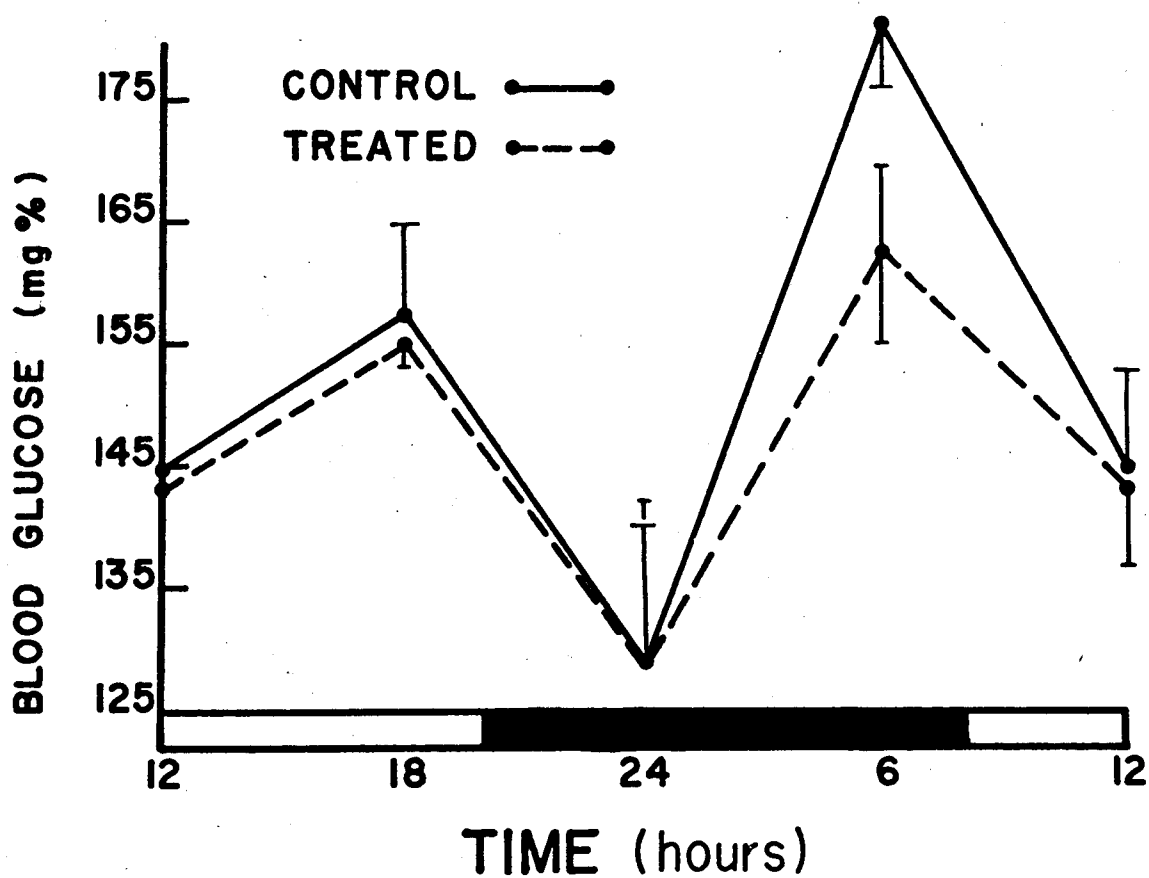
Circadian pattern for blood serum glucose levels in control and
pentobarbital sodium treated rats.

TABLE 7

CIRCADIAN PATTERNS OF SLEEP ONSET TIME AND DURATION
AND BIOGENIC AMINE LEVELS

|		Time of Day (Hours)			
		0600	1200	1800	2400
Sleep Duration (minutes)		65.30 ± 9.85**	101.30 ± 5.74	130.30 ± 11.60**	71.25 ± 7.40
Sleep Onset (minutes)		11.50 ± 2.79*	9.00 ± 0.95	7.82 ± 0.02	9.20 ± 2.72
		Biogenic Amine Levels (µg/g)			
Norepinephrine (rat midbrain)	Control	0.77 ± 0.13	0.42 ± 0.04†	0.30 ± 0.06**	0.77 ± 0.07**†
	Treated	0.90 ± 0.07	0.61 ± 0.08†	0.36 ± 0.05**	1.29 ± 0.09**†
5-Hydroxytryptamine (rat midbrain)	Control	0.27 ± 0.03*	0.49 ± 0.02*†	0.47 ± 0.03 †	0.28 ± 0.02
	Treated	0.25 ± 0.02*	0.27 ± 0.02 †	0.36 ± 0.03*†	0.27 ± 0.01
Histamine (caudate nuclei)	Control	0.11 ± 0.06	0.07 ± 0.02*†	0.07 ± 0.03†	0.12 ± 0.03*†
	Treated	0.12 ± 0.04	0.08 ± 0.04*†	0.09 ± 0.04	0.18 ± 0.03*†

All values represent the mean ± standard deviation.
  * Difference between maximum and minimum values, significant at $P < 0.01$.
 ** Difference between maximum and minimum values, significant at $P < 0.005$.
  † Difference between treated and control values, significant at $P < 0.01$.

TABLE 8

CIRCADIAN PATTERNS OF RECTAL TEMPERATURE, BLOOD HISTAMINE AND BLOOD GLUCOSE LEVELS

	Time of Day (Hours)			
	0600	1200	1800	2400
Rectal Temperature (0°C)				
Control	38.21 ± 0.42*††	37.27 ± 0.37*†	37.28 ± 0.30	38.13 ± 0.26*††
Treated	35.63 ± 0.32*††	36.25 ± 0.20*†	36.75 ± 0.35	35.63 ± 0.32*††
Difference	2.58	1.02	0.53	2.50
Blood Histamine (μg/100 ml)				
Control	0.75 ± 0.10	0.51 ± 0.06*	1.07 ± 0.36*	0.97 ± 0.17†
Treated	0.82 ± 0.29	0.46 ± 0.08*	0.95 ± 0.19*	0.85 ± 0.10†
Blood Glucose (mg/100 ml)				
Control	181.10 ± 9.84 ††	144.64 ± 15.80	157.14 ± 15.40**	128.84 ± 22.10**
Treated	162.21 ± 14.73**††	142.85 ± 12.70	154.75 ± 3.60**	128.80 ± 26.20**

All values represent the mean ± standard deviation.

* Difference between maximum and minimum values, significant at $P < 0.01$.

** Difference between maximum and minimum values, significant at $P < 0.005$.

† Difference between treated and control values, significant at $P < 0.01$.

†† Difference between treated and control values, significant at $P < 0.005$.

D. THE ULTRASTRUCTURE OF RAT HYPOTHALAMUS
AND CAUDATE NUCLEUS

Figure 22 and Table 9 show norepinephrine levels for rat hypo-
thalamus and caudate nucleus in DOPA-MAO inhibitor and reserpine treated
groups. As anticipated, the higher concentration of norepinephrine in the
hypothalamus and caudate nucleus occurred in the DOPA-MAO inhibitor
treated groups, while lower concentrations were noted in the reserpinized
groups. At both 0300 and 1500 hours these differences were statistically
significant from controls in identical tissues. In control and treated groups
significant increases were noted in the hypothalamus and caudate nucleus of
aniamls sacrificed at 0300 hours when compared to animals sacrificed at
1500 hours. These results verify the fact that catecholamine precursors
and depletors can modify tissue levels of norepinephrine and that greater
concentrations are noted for both normal and treated animals during the
dark period (when the motor activity of the rat is maximal).

Isolated nerve boutons are shown in Figure 23 in order to demon-
strate the morphology and internal structure of such terminals. One can
easily observe the differences between the large granular and small
agranular vesicles. Figure 24 shows two boutons connecting to form a
characteristic synaptic junction. Mitochondria, granular and agranular

FIGURE 22

HYPOTHALAMUS AND CAUDATE NUCLEUS NOREPINEPHRINE
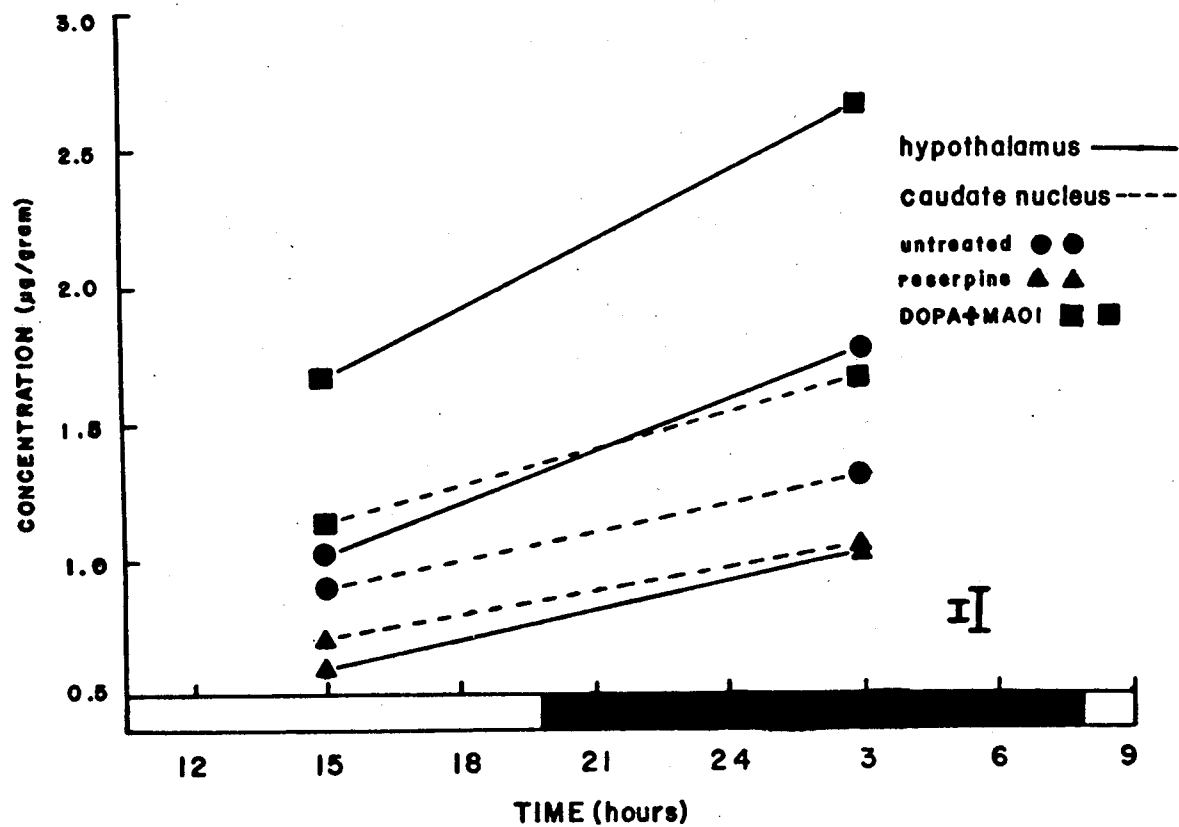
Hypothalamus and caudate nucleus norepinephrine levels for untreated, reserpine treated and DOPA plus MAOI treated rats at 0300 and 1500 hours.

TABLE 9

NOREPINEPHRINE LEVELS IN THE HYPOTHALAMUS AND CAUDATE NUCLEUS

Treatment[a]	Norepinephrine Levels (μg/g)	
	Time of Day (Hours)	
	0300	1500
Hypothalamus		
Control	1.78 ± 0.110**	1.06 ± 0.028**
Reserpine	1.13 ± 0.022**	0.69 ± 0.024**
dl-DOPA + pargyline	2.62 ± 0.121**	1.69 ± 0.062**
Caudate Nucleus		
Control	1.27 ± 0.104	0.896 ± 0.014*
Reserpine	1.02 ± 0.090	0.716 ± 0.030*
dl-DOPA + pargyline	1.43 ± 0.070	1.160 ± 0.024**

All values represent the mean ± standard deviation.

a Drug treatments (Reserpine 5 mg/kg; pargyline 6 mg/kg; dl-DOPA 500 mg/kg) were I. P.

* Difference between maximum and minimum values significant at $P < 0.05$.

** Difference between maximum and minimum values significant at $P < 0.01$.

FIGURE 23

ISOLATED NERVE BOUTONS

This figure shows the characteristic ultrastructure of a nerve bouton.
Note: granular and agranular vesicles located within the terminals.
X 47,600.

FIGURE 24

AN ISOLATED SYNAPTIC OR DESMOSOMAL JUNCTION
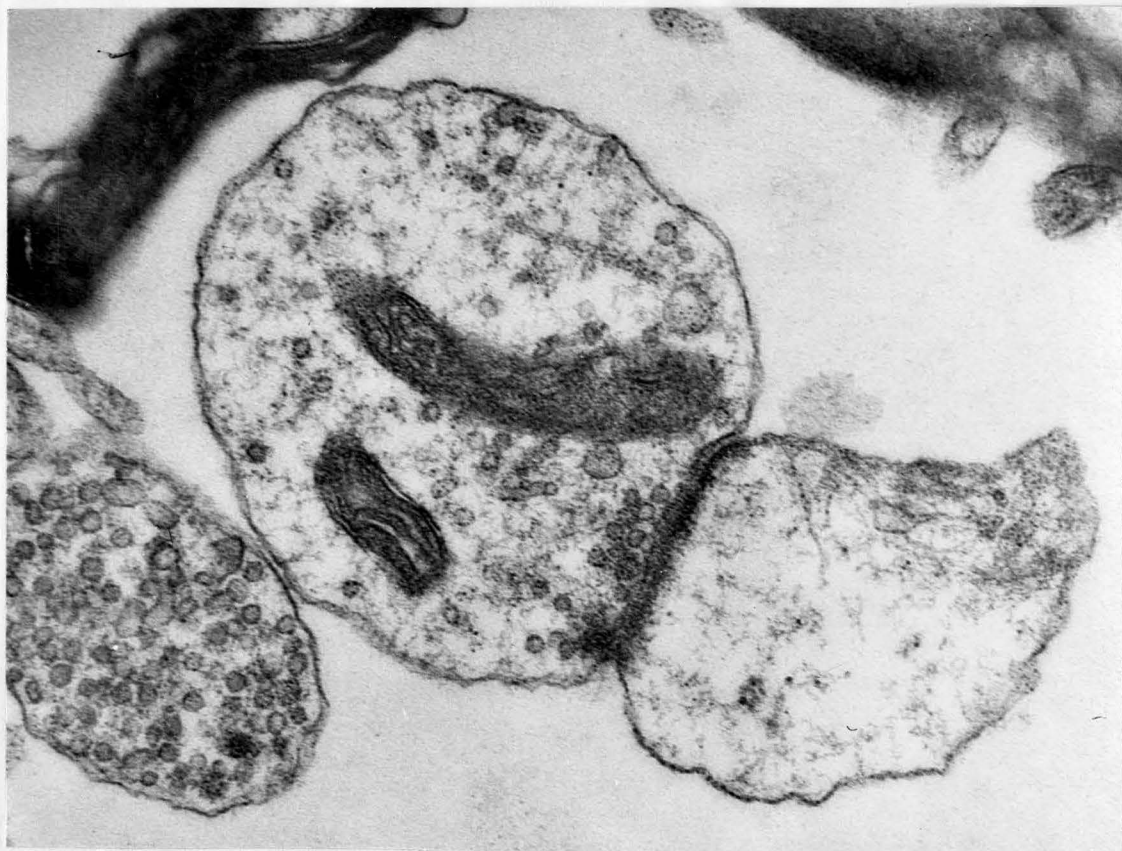
This figure shows the ultrastructure of an isolated synaptic junction. Granular and agranular vesicles plus mitochondria are shown within the boutons. In the larger bouton near the synaptic cleft a cluster of agranular vesicles (an active point) is noted. X 47, 600.

vesicles can be distinguished within these boutons. The synaptic cleft and a small cluster of agranular vesicles near the cleft are apparent.

Figures 25 through 27 show the ultrastructure of the anterior hypothalamus, posterior hypothalamus and caudate nucleus for untreated controls, DOPA-MAO inhibitor and reserpine treated groups at 0300 and at 1500 hours. The solid arrows point to the large granular vesicles while the short clear arrows point to the small agranular vesicles, both are localized within boutons. The diameters of the granular vesicles range from 600 to 1100 $\overset{\circ}{A}$ while the diameters of the agranular vesicles range from 200 to 500 $\overset{\circ}{A}$. The boutons are delineated by their double membrane structures. In addition to synaptic vesicles, mitochondria are also seen. In area 1 of Figure 25 a group of dense core vesicles similar to those described by Grillo and Palay (1962) can be seen.

Tables 10 and 11 and Figures 28 and 29 show the number of granular vesicles per 23 square microns in reserpine and DOPA-MAO inhibitor treated rats. In all treated animals the number of granular vesicles was greater at 0300 hours than at 1500 hours. These differences are statistically significant for the posterior hypothalamus and caudate nucleus of DOPA-MAO inhibitor treated animals and for the posterior hypothalamus of the reserpine treated animals. Granular vesicles for all

FIGURE 25

ULTRASTRUCTURE
ANTERIOR HYPOTHALAMUS
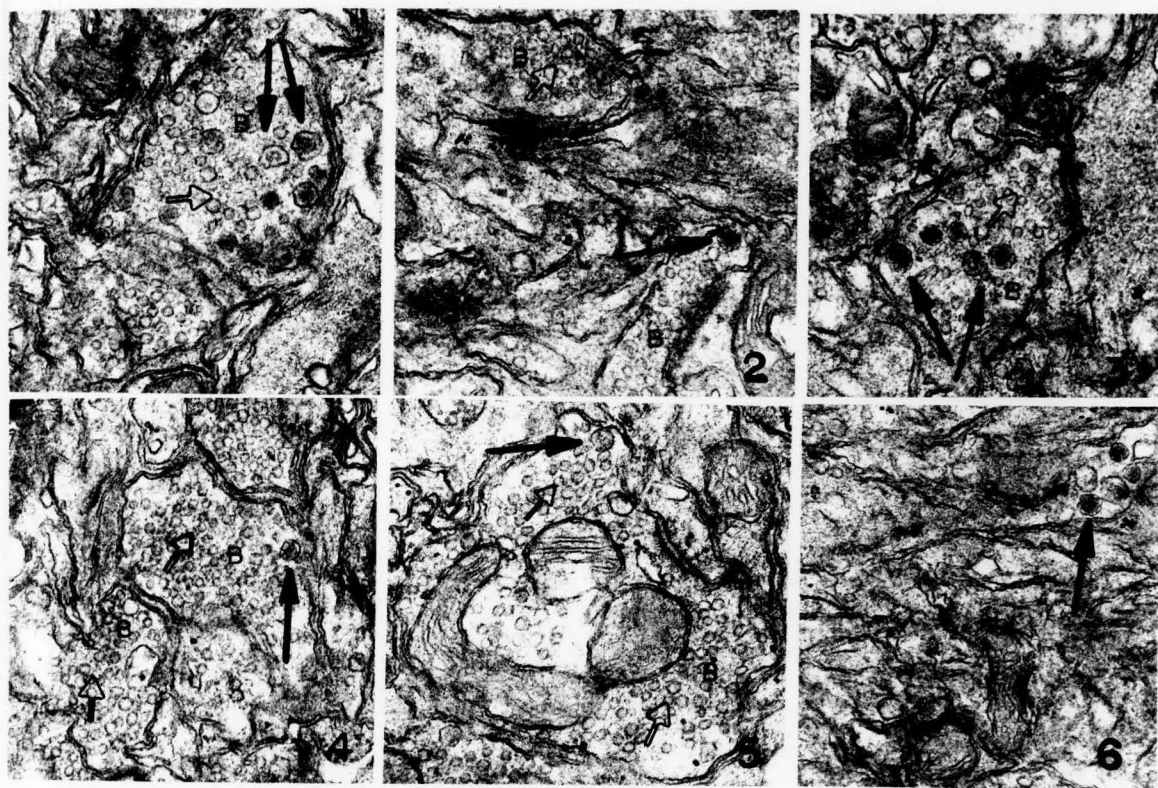
Anterior hypothalamus of rats sacrificed at 0300 (1,2 and 3) and at
1500 hours (4,5 and 6). Tissues from untreated controls (1 and 4),
reserpine treated (2 and 5) and dl-DOPA and pargyline (3 and 6)
are shown. Granular (———▶) and agranular (—▷) vesicles are
located within boutons (B). X 47,600.

FIGURE 26

ULTRASTRUCTURE
CAUDATE NUCLEUS
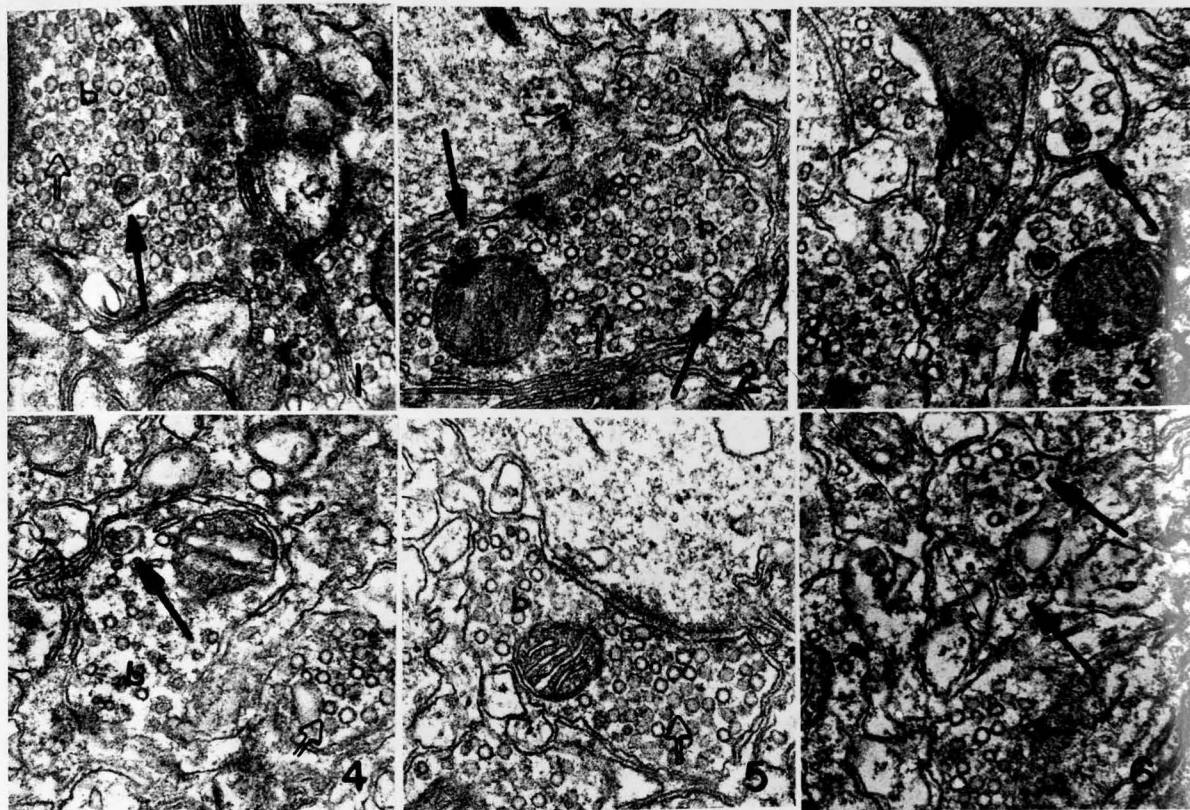
Caudate nucleus of rats sacrificed at 0300 (1,2 and 3) and at 1500
hours (4,5 and 6). Tissues from untreated controls (1 and 4),
reserpine treated (2 and 5) and dl-DOPA and pargyline (3 and 6) are
shown. Granular (⟶) and agranular (⟶▷) vesicles are
located within boutons (b). X 47,600.

FIGURE 27

ULTRASTRUCTURE
POSTERIOR HYPOTHALAMUS
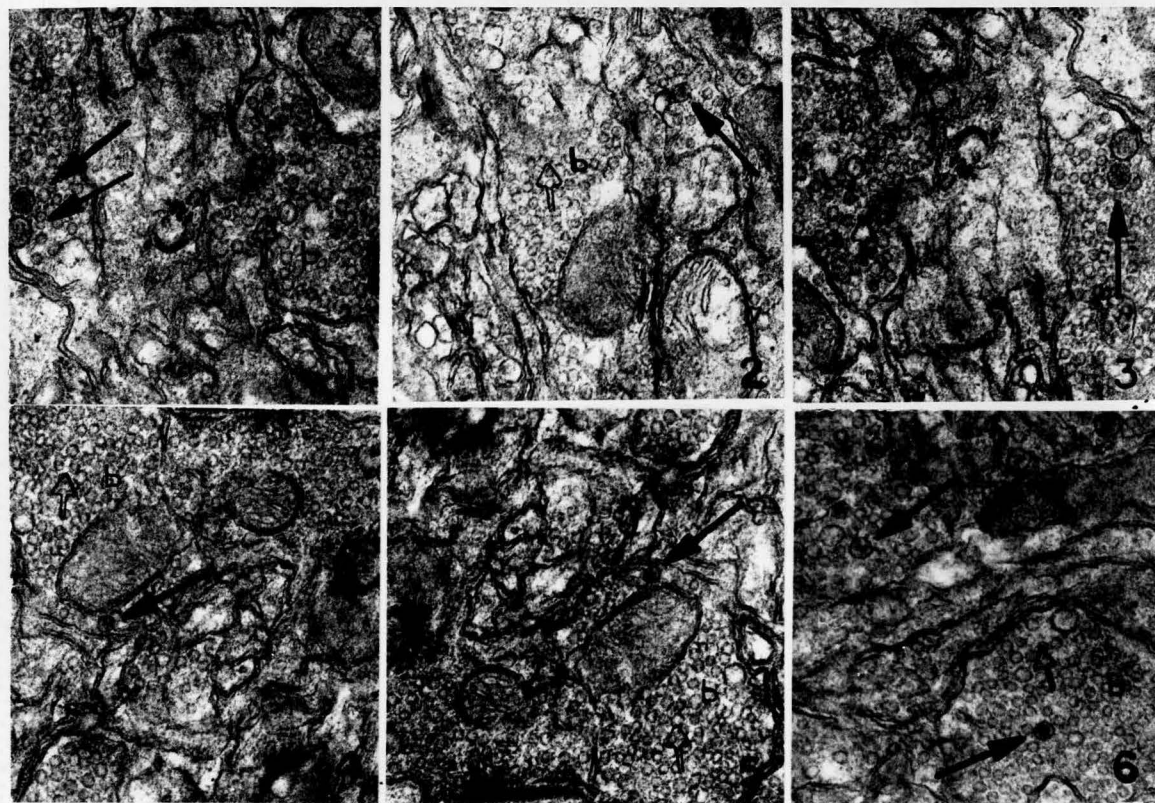
Posterior hypothalamus of rats sacrificed at 0300 (1,2 and 3) and at 1500 hours (4,5 and 6). Tissues from untreated controls (1 and 4), reserpine treated (2 and 5) and dl-DOPA and pargyline (3 and 6) are shown. Granular (——▶) and agranular (—▷) vesicles are located within boutons (ᑲ). X 47,600.

TABLE 10

PER CENT CHANGE OF HYPOTHALAMIC AND CAUDATE
NUCLEI GRANULAR VESICLES AT 0300 AND 1500 HOURS

Treatment[a]	Tissues		
	Anterior Hypothalamus	Posterior Hypothalamus	Caudate Nucleus
Control	153. 58	84. 00	169. 60
dl-DOPA + pargyline	108. 51	145. 92	127. 22
Reserpine	118. 66	135. 37	107. 48

[a] Drug treatments (Reserpine 5 mg/kg; dl-DOPA 500 mg/kg;
pargyline 6 mg/kg) were I. P.

The per cent changes is represented by the increase of maximum at
0300 over the minimum at 1500 hours.   The value for each minimum
is set at 100 per cent.

TABLE 11

NUMBER OF SYNAPTIC VESICLES IN THE ANTERIOR HYPOTHALAMUS, POSTERIOR HYPOTHALAMUS AND CAUDATE NUCLEUS

Type of Tissue	Time of Day (Hours)			
	0300	1500	0300	1500
	Granular Vesicles (per 23 square microns)		Agranular Vesicles (per 0.46 square microns)	
Control				
Anterior Hypothalamus	12.15 ± 1.78***	5.61 ± 1.21***	40.03 ± 3.13	39.39 ± 3.71
Posterior Hypothalamus	2.16 ± 0.53	2.59 ± 0.04	41.20 ± 4.97***	21.13 ± 4.40***
Caudate Nucleus	7.03 ± 0.66***	2.17 ± 0.64***	30.62 ± 2.50	28.18 ± 3.06
MAOI-dl DOPA				
Anterior Hypothalamus	13.83 ± 3.55	12.79 ± 3.22	34.74 ± 5.79***	45.65 ± 3.98***
Posterior Hypothalamus	9.65 ± 3.45**	5.80 ± 1.47**	45.00 ± 7.24**	39.78 ± 4.78**
Caudate Nucleus	7.25 ± 2.38*	5.35 ± 2.09*	52.05 ± 6.45**	38.55 ± 4.96**
Reserpine				
Anterior Hypothalamus	10.08 ± 4.12	8.20 ± 4.19	39.78 ± 4.78***	55.30 ± 6.72***
Posterior Hypothalamus	6.90 ± 2.81*	4.47 ± 1.13*	38.55 ± 4.96***	59.73 ± 4.95***
Caudate Nucleus	4.55 ± 1.69	4.21 ± 1.50	43.81 ± 3.25**	50.29 ± 5.14**

All values represent the mean ± standard deviation.

* Difference between 0300 and 1500 hours for identical tissue in similarly treated groups, $P < 0.1$.

** Difference between 0300 and 1500 hours for identical tissue in similarly treated groups, $P < 0.01$.

*** Difference between 0300 and 1500 hours for identical tissue in similarly treated groups, $P < 0.001$.

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FIGURE 28

GRANULAR VESICLES FOR UNTREATED TISSUES
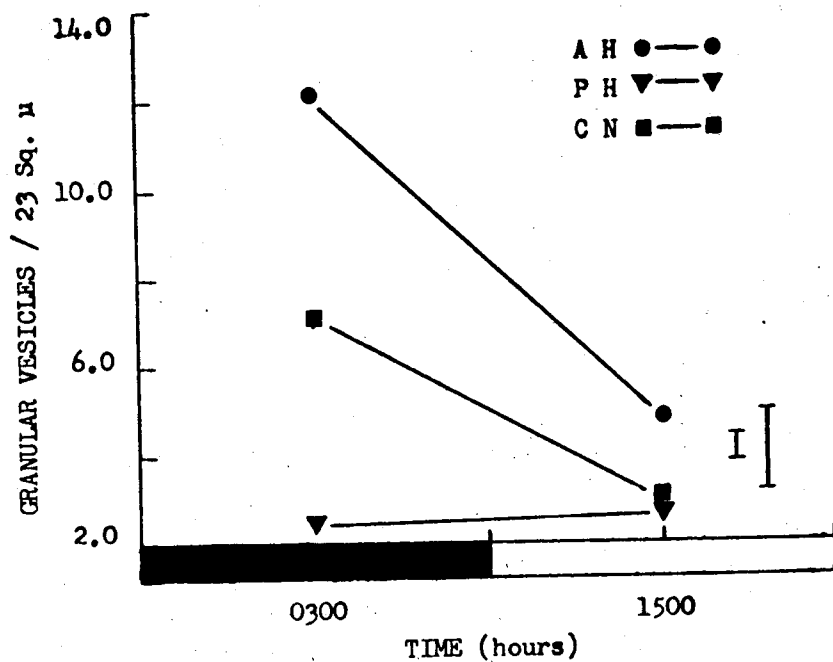
This figure shows the number of granular vesicles per 23 square microns of anterior hypothalamus (AH), posterior hypothalamus (PH) and caudate nucleus (CN) of untreated control rats.

FIGURE 29

GRANULAR VESICLES FOR TREATED TISSUES
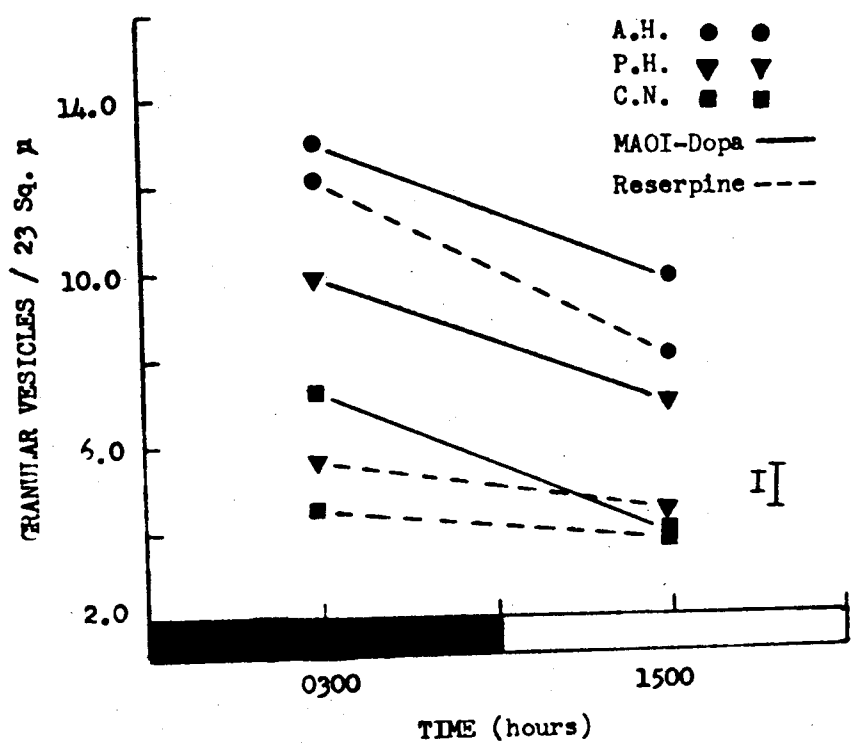
This figure shows the number of granular vesicles per 23 square microns in the anterior hypothalamus (AH), posterior hypothalamus (PH) and caudate nucleus (CN) of MAOI-DOPA and reserpine treated rats.

tissues were greater in number in the DOPA-MAO inhibitor treated groups
at 0300 hours when compared to reserpine groups at 0300 hours. These
findings correlate with the fluorometric analysis previously described and
are in keeping with the results of Pfeifer, et al. (1968) who showed that the
number of granular vesicles could be increased following pre-treatment
with the monoamine oxidase inhibitor, nialamide.

In control animals at 0300 hours, a two fold increase was noted in
the number of granular vesicles of the anterior hypothalamus when compared
to 1500 hours while a three fold increase was observed for the caudate
nucleus during the same time period. These differences are highly signifi-
cant with P values of less than 0.01. The number of granular vesicles in
the posterior hypothalamus of untreated rats was statistically unchanged
between 0300 and 1500 hours.

Table 11 also shows an analysis of the agranular vesicles (number
per 0.46 square microns). In control animals a highly significant increase
was noted for the posterior hypothalamus at 0300 hours when compared to
1500 hours ($P < 0.001$). No significant changes were noted for the anterior
hypothalamus or caudate nucleus of controls during the same time period.
In the DOPA-MAO inhibitor treated groups, significant increases were
noted at 0300 hours for the posterior hypothalamus and caudate nucleus

when compared to 1500 hours, while a highly significant decrease was noted during the same period for the anterior hypothalamus ($P < 0.001$).

In the reserpine treated groups, all three tissues showed highly significant increases at 1500 hours when compared to 0300 hours ($P < 0.01$).

Figure 30 shows the per cent change and relationship of granular to agranular vesicles in the anterior hypothalamus, posterior hypothalamus and caudate nucleus at 0300 hours over 1500 hours. As previously mentioned, highly significant increases in the number of granular vesicles were noted for the caudate nucleus and anterior hypothalamus, while significant increases in the number of agranular vesicles were noted for the posterior hypothalamus. In all cases the P value was less than 0.001.

FIGURE 30

GRANULAR AND AGRANULAR VESICLES
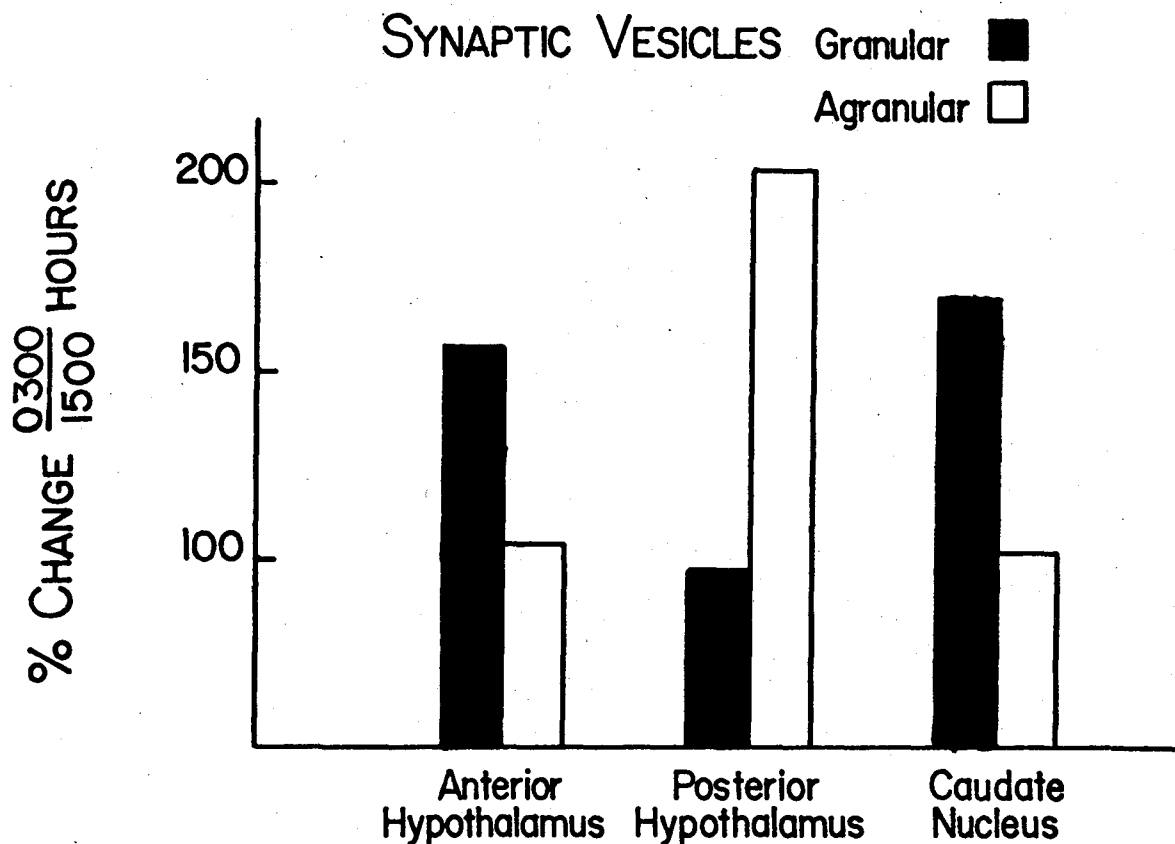
This figure shows the per cent change in the number of granular
and agranular vesicles at 0300 hours over 1500 hours in the
tissues identified.   The minimum value at 1500 hours is set at
100 per cent.

CHAPTER V

GENERAL DISCUSSION

A. CIRCADIAN BIOGENIC AMINES IN THE CENTRAL NERVOUS SYSTEM

In the rat, a rise in caudate nucleus and midbrain histamine occurs during the dark phase of the illumination cycle and coincides with a period of elevated rectal temperature (Figures 7, 8 and 9; Tables 1 and 2) and augmented motor activity. The maximum histamine level, surprisingly, is about double that of the minimum values found during decreased motor activity and sleep. Recently, Monnier and co-workers (1967) reported that the intravenous infusion of histamine in the rabbit sharply decreased the delta activity of the cortex, i.e., high voltage 0.5 - 3.5 cycles per second sleep spindles, and increased the desynchronization of the EEG. This waking action of histamine occurred independently of minor changes in blood pressure and respiration. Based on the above findings and in keeping with the results of the present investigation, it appears that histamine plays some role in wakefulness, and that the sedative action of antihistamines might be due to their ability to block this action.

At present, the underlying mechanism of this waking action of histamine is unclear. Histamine might act by virtue of its ability to alter blood flow (Virno, et al. 1956) or by its capacity to modulate synaptic transmission (Gertner and Kohn, 1959; Konzett, 1952). It is also possible

that histamine accumulates because of increased motor activity or under-
utilization. These speculations require further investigation.

A relationship between alerting behavior and an increase in central
nervous system catechol amines has been demonstrated by several investi-
gators (Monnier, 1960; Everett, 1961; Mantegezza, 1961). The role of
epinephrine and norepinephrine in mobilizing energy via cyclic-3'-5' AMP
(Sutherland and Rall, 1960) has recently been established. The increase in
norepinephrine levels in the present study was found to be associated with
increased motor activity and occurred during the dark period of the
illumination cycle, coinciding with elevated histamine levels. Essentially
identical time courses for circadian patterns in the rat for pineal norepi-
nephrine and hydroxyindole-o-methyl transferase (HIOMT) activity have
been reported by Wurtman, et al. (1967-a). These rhythms are abolished
by blindness. Pineal norepinephrine, according to these workers, is
influenced by light on the retina which sends impulses via the inferior
accessory optic tract to the midbrain tegmentum. This would suggest that
midbrain norepinephrine might similarly be influenced by light impinging
on the retina. Rodeick and Smith (1966) suggest that impulses traveling in
the optic tract might originate from cellular elements other than those
arising in the retina, so that retina itself might not contain the trigger

regulating CNS biogenic amine rhythms. It is also possible that substances circulating in the blood initiate optic tract impulses which in turn control the diurnal pattern for norepinephrine.

Serotonin levels in the brain reached a peak at a time when the rats were inactive or asleep and when rectal temperature was lowest. They were also twelve hours out of phase with histamine, norepinephrine and rectal temperature peaks (Figure 9). These results are in keeping with the work of Koella and Czicman (1966) who associated serotonin with sleep. They postulated that serotonin produces occular signs of synchronized sleep by its action on receptor sites in the area postrema. Feldberg and Sherwood (1954) observed that when serotonin was injected into the lateral cerebral ventricles of cats, they displayed muscular weakness and a swaying gait and adopted a sleeping posture. These studies together with the present one indicate the possible involvement of CNS serotonin with a depressor or sleep mechanism.

The possibility that rhythmicity in CNS biogenic amine levels might in some way be related to or influenced by an inhibitory feedback system involving the adrenal gland is not strongly supported by the present study in that bilateral adrenalectomy did not qualitatively alter the circadian

rhythms studied (Tables 1 and 2), although in some instances quantitative differences were noted.

It is difficult to ascertain whether there is a causal relationship among the various amines measured. The levels of histamine and norepinephrine appear to be directly in phase with body temperature and motor activity, while serotonin shows the reverse relationship. The possibility that these inter-relationships are causal might be investigated by means of drugs which separately deplete or augment these amines.

B. THE CIRCADIAN RHYTHMS OF CHOLINOMIMETICS
IN RATS AND MICE

1. Endogenous Circadian Acetylcholine Levels

Figure 10 and Table 3 show that midbrain and caudate nucleus acetylcholine exhibit a circadian rhythm. The peak levels for both tissues occurred during the mid-dark period and coincided with the time of day for peak circadian norepinephrine, rectal temperature, histamine and motor activity rhythms previously reported. The maximum over minimum increase for midbrain acetylcholine was 32. 3% and 21. 5% for caudate nucleus levels. Cholinergic influences on the central nervous system have been studied by Bowers, et al. (1966) who showed that rats deprived of D-state sleep (and to some extent slow wave sleep) for 96 hours show a significant fall in brain acetylcholine (35% decrease) in the telencephalon. Killam (1962) discussed the cholinergic mechanism relative to the function of the reticular formation and noted that following the administration of acetylcholine and certain anticholinesterases (DFP, TEPP and physostig-mine) the EEG observed in unanesthetized immobilized animals was that of a "waking EEG", i. e. , low-voltage fast activity. When atropine, trihexy-phenidyl, or benztropine methane sulfonate was given a "sleep-like" or high-voltage, slow-wave EEG was noted as well as a blockade of the cholinergic effects.

Herz and Zieglgansberger (1968) studied the influence of micro-electrophoretically applied acetylcholine on the rabbit corpus striatum and found some excitatory and some inhibitory neuronal effects. Aprison, et al. (1968) noted changes in acetylcholine levels in the telencephalon of the rat which corresponded to increased behavioural excitation in the nocturnal animals.

These findings are in agreement with our results relative to peak endogenous acetylcholine levels and support the theory that acetylcholine and possibly cholinergic transmission are involved in wakefulness and elevated motor activity.

2. Circadian Toxicity of Cholinomimetics in Mice

The toxicity of acetylcholine, pilocarpine and oxotremorine was less during the light phase and greater during the dark phase of the diurnal illumination cycle, i.e., the amount of exogenous drugs (mg/kg) needed to kill 50% of the mice was greater during the light than during the dark phase. A similar pattern was observed for I.V. and I.P. administered acetyl-choline with the I.V. route showing a significantly greater toxicity during both dark and light phases. These studies indicate that the toxicity of cholinergic compounds undergo daily variations that attain significant magnitudes.

The toxicity of atropine is also circadian and is almost a mirror image of that produced with acetylcholine, oxotremorine and pilocarpine. Atropine is least toxic at a time when acetylcholine is most toxic (2400 hours). The maximum toxicity for atropine occurs at noon (1200 hours) during the light phase of the illumination cycle when general metabolism and motor activity rhythms are reduced. Apparently during the dark phase, when endogenous acetylcholine levels are elevated, there is a greater antagonism between acetylcholine and atropine resulting in a decrease in atropine potency and/or toxicity. It has been shown by Berry and Starz (1966) and Giarman and Pepeu (1962, 1964) that scopolamine and atropine decreased total brain acetylcholine. Beani, et al. (1964) demonstrated a decrease in acetylcholine levels in some cortical and subcortical structures minutes after a dose of scopolamine (25 mg/kg). Some areas were not affected. This suggests that acetylcholine depletion and scopolamine tissue distribution proceed at different rates. It has been suggested that atropine competatively blocks acetylcholine by occupying muscarinic receptor sites. Mitchell (1963) indicated that atropine also interfers with acetylcholine release. Schuberth and Sundwall (1967) reported that atropine prevents the uptake of acetylcholine in the mouse cortex by blocking the acetylcholine transport mechanism. As mentioned previously, we believe that the

toxicity of exogenous acetylcholine increases during the dark period because it adds algebraically to rising levels of endogenous acetylcholine during the dark phase, while at the same time atropine toxicity decreases because it competes for receptor sites with acetylcholine. The similar results noted for oxotremorine and pilocarpine can possibly be explained by the work of Holmstedt and Lundgren (1966) who showed that atropine is capable of preventing the mobilization of acetylcholine by oxotremorine. Pilocarpine acts directly on muscarinic receptors and is easily blocked by atropine methyl bromide.

Carbachol exhibits a pattern which is not circadian but apparently ultradian with two minimum toxicity levels within a 24-hour period; one at 1200 hours and the other at 2400 hours. It is well established that carbachol is not destroyed by cholinesterase. McKinstry and Koelle (1967-a, b) showed that carbachol released acetylcholine from the perfused superior cervical ganglion by what is believed to be a simple ion exchange mechanism. This type of release unlike that produced by preganglionic stimulation can be blocked by calcium ions. This does not explain the different pattern presented by carbachol from acetylcholine, pilocarpine and oxotremorine. It is possible that the enzymes or mechanisms associated with carbachol destruction and/or degradation have a different phase relationship to this substrate than do the cholinesterases.

The toxicity of a quarternary anticholinesterase, neostigmine, and a tertiary anticholinesterase, physostigmine, which presumably can penetrate the blood brain barrier was also studied. Neostigmine's greatest toxicity was noted at the end of the dark phase while the least toxicity occurred at the end of the light phase. Physostigmine's greatest toxicity occurred at 2400 hours during the mid-dark phase and coincided with the peak toxicity levels for acetylcholine and pilocarpine. The least toxicity occurred near the beginning of the light phase and the end of the dark phase at 0600 hours. Since physostigmine has the ability to cross the blood brain barrier it might be interacting with the rising phase of endogenous acetyl-choline at 2400 hours. Probably the sudden decrease in toxicity of physo-stigmine reflects a circadian change in central cholinesterase levels which may be increasing in response to increased acetylcholine levels.

The toxicity of acetylcholine and physostigmine was significantly decreased following pretreatment with a peripheral cholinergic muscarinic blocking drug (atropine methyl nitrate). While the toxicity was decreased, the circadian pattern for both drugs remained unchanged.

The toxicity of anticholinergic drugs and cholinomimetics are somewhat dependent upon endogenous levels of transmitter material, especially acetylcholine. When endogenous levels of acetylcholine are high,

lower doses of cholinomimetic drugs are required to produce lethal effects, conversely higher doses of anticholinergic drugs are needed to manifest toxicity when endogenous levels of acetylcholine are elevated. The interaction between cholinergic blockers, cholinomimetics and of acetylcholine is exemplified by the study of Rinaldi and Himwich (1955) who demonstrated that atropine raises the threshold to EEG arousal responses evoked by stimulation of the reticular formation; Bradley and Key (1959) showed that physostigmine antagonizes atropine at this site, and by White and Rudolph (1968) who showed that physostigmine had a facilitatory effect in rabbit midbrain. Villarreal and Domino (1964) postulated the presence of both nicotinic and muscarinic cholinergic receptor sites in the midbrain of cats and suggested that both sites are involved in EEG desynchronization. On the other hand, Nickander and Yim (1964) studied the effects of tremorine and cholinergic drugs on the isolated cerebral cortex and indicated the possible absence of the nicotinic receptor site from this location.

The present study indicates that the circadian patterns of toxicity of cholinomimetics and cholinergic blocking compounds are determined by both central and peripheral mechanisms, in that muscarinic blockade of the peripheral influences decreases toxicity but does not alter the circadian pattern or abolish toxicity.

C. THE INFLUENCE OF PENTOBARBITAL SODIUM ON SLEEPING TIME AND CENTRAL AND PERIPHERAL BIOGENIC AMINES IN THE RAT

The onset and duration of sleep induced by pentobarbital sodium varies in a circadian manner (Figure 16; Table 7). The sleep onset time increases and the sleep duration time decreases when the rat's motor activity is at its peak. The converse is true for the rat's quiescent period.

Vesell (1968) associated the diurnal variation observed in hexobarbital sleeping time in mice to an increased hexobarbital oxidase activity which was noted during the active phase of the circadian cycle.

Bahorsky and Bernardis (1967) observed peak circadian fluctuations for several key metabolic substances during the dark phase. The above findings, and the results of this investigation, indicate the possibility that increased barbiturate metabolism at a time when the physical activity of these nocturnal animals is increasing contributes to a decrease in the hypnotic potency of pentobarbital. Biogenic amine levels (NA, 5-HT, and histamine) in brain tissues display circadian rhythms (Figures 17 and 18; Table 7). In control animals, midbrain NA levels are high at a time when the duration of sleep is shortest and when the sleep onset time is longest. Sato, et al. (1967) noted that, in Molluscs, pentobarbital sodium blocks

synaptic transmission by hyperpolarizing the post-synaptic membrane.
Since NA serves as a neurotransmitter, the short duration and long onset
times for pentobarbital-induced sleep at a time when NA levels are normally
high might indicate a physiological antagonism between pentobarbital and
interneuronal NA on membrane permeability at post-synaptic sites.

Eakins, et al. (1968) reported that pentobarbital anesthesia pro-
duced a four-fold decrease in the NA turnover rate in cats, plus a significant
decrease in body temperature. They noted that when body temperature was
maintained, the decreased turnover rate of NA was abolished. A slow
turnover rate following pentobarbital treatment together with a decreased
uptake of NA by the nerve terminal (vide supra) might account for a net
increase in extra-neuronal NA which is reflected in these results. It has
been shown that barbiturates as a drug class can release catecholamines
(Friedman, unpublished data) both in the brain and in myocardial tissue.

Peak 5-HT levels in control animals were noted during motor
inactivity. These results support the hypothesis of 5-HT involvement in
the mechanism of sleep (Snyder, et al. 1965; Quay, 1966; Koella and
Czicman, 1966).

Biogenic amine synthesis, release, utilization and/or destruction
are affected by pentobarbital pretreatment. The significant decrease in

5-HT levels during the light phase of the illumination cycle following pre-treatment with pentobarbital sodium suggests either an increase in util-ization of 5-HT during barbiturate sleep and/or a net decrease in 5-HT synthesis. The latter might be due to a decrease in body temperature resulting in a slowing of the circulatory processes and enzymatic activity (Friedman and Everett, 1964). Rectal temperature decreased significantly in animals treated with pentobarbital sodium (Figure 19; Table 8). The average difference in rectal temperature between control and treated rats observed during the dark phase of the illumination cycle was $2.54^{\circ}C$, while the average difference noted during the light phase was only $0.78^{\circ}C$. The decrease in body temperature in the pentobarbital-treated animal is in part the result of decreased motor activity and depressed metabolic activity. Sheard and Aghajanian (1967) linked 5-HT to the hypothalamic control of body temperature (see also Feldberg, Hellon and Myers, 1966). They found that stimulation of the midbrain raphé of rats resulted in an increase in body temperature, resistance to the hypothermia of anesthetics, a fall in midbrain 5-hydroxytryptamine levels and an increase in 5-hydroxyindole-acetic acid. Similar effects were obtained in animals pretreated with 5-hydroxytryptophan (cf. review by Mantegazzini, 1966), although this varies according to the species studied and the dose administered.

Parenterally administered 5-HT does not readily penetrate the blood barrier and produces hypothermia by peripheral vasodilation (Mantegazzini, 1966).

Midbrain 5-HT levels in control animals are lowest during the period of peak body temperature. In pentobarbital sodium treated animals 5-HT levels are always below those of the control and this reduction extends even beyond the period of increased motor activity. These findings suggest that after pentobarbital sodium administration the utilization or turnover of 5-HT increases to counteract a reduction in body temperature (vide supra). 5-Hydroxytryptamine has been shown to potentiate the action of hypnotics and they in turn have been implicated in its mobilization, although this is still a matter of some controversy (Garattini and Valzelli, 1965). Further-more, there is considerable evidence showing that 5-HT and catecholamines have antagonistic actions and compete for some of the same synthetic and degradative enzymes (Garattini and Valzelli, 1965). This interaction is clearly suggested by the inverse circadian patterns observed between 5-HT and NA both before and after pentobarbital sodium treatment (Figure 17).

Brain histamine levels increased significantly after barbiturate administration (Figure 18; Table 7). Green and Erickson (1967) found that chlorpromazine and diphenhydramine produced increased histamine levels of from 37 to 44% in guinea pig cerebrum but no significant changes in brain

stem histamine. These findings and those obtained with pentobarbital sodium suggest that these drugs might release histamine from tissue stores or depress turnover or degradation. A decrease in membrane permeability would prevent neuronal uptake, resulting in a net histamine increase during a period of increased motor activity. In addition, degradative enzymes might be less effective when body temperature is lowered.

Blood histamine peaks during the transition from the light to the dark phase of the illumination cycle (Figure 20; Table 8), and these high levels are maintained throughout the dark phase. Wilson (1965) observing the circadian rhythmicity of urinary histamine noted that levels peaked at the initiation of the dark phase, but following treatment with chloramphen-icol the peak shifted to the mid-point of the dark phase. The results of the present study parallel the circadian patterns for urinary histamine levels observed by Wilson (1965). Peak blood histamine levels were noted at the end of the light phase. According to Wilson, bacterial synthesis of histidine to histamine in the gastrointestinal tract accounts for urinary histamine peaks at this period (the beginning of the active phase in the circadian cycle). The maintenance of blood histamine levels throughout the dark phase of the illumination cycle in this investigation and the shifting of peak urinary levels following treatment with antibiotics indicate a relationship between

peak motor activity, CNS histamine levels and increased blood histamine levels during the dark phase.

Blood glucose levels in the rat exhibit bimodal circadian peaks (Figure 21; Table 8). The greater and more significant peak occurs at the end of the dark phase while a secondary and less significant peak is found at the end of the light phase. The relationship of CNS catecholamines to cyclic 3'-5'-AMP and blood glucose levels was discussed previously. The secondary blood glucose peak at the end of the light phase quite possibly is related to the blood histamine peak which occurs at the same time. Fearn, Karady and West (1968) observed that blood histamine can stimulate the adrenal medulla to release relatively large amounts of catecholamines resulting in increased blood levels. Gershbein (1968) reported that histamine also produced hyperglycemia in bilaterally adrenalectomized rats, indicating a direct histamine-insulin type of antagonism. In the present study, the primary or more significant peak observed towards the end of the dark phase is associated with peak noradrenaline and histamine in the CNS and maintained high levels of blood histamine. It also coincides with the period of feeding and increased motor activity.

D. THE ULTRASTRUCTURE OF THE RAT HYPOTHALAMUS AND CAUDATE NUCLEUS AT 0300 AND 1500 HOURS

The results of electron microscopic examination of ultrastructural elements in the anterior hypothalamus, posterior hypothalamus and caudate nucleus are represented in Figures 23 through 27. In all tissues the basic elements, mitochondria, boutons and synaptic vesicles are shown. At nerve endings, the functional relationships of these structures are not clear. It has been suggested by DeRobertis, et al. (1965) that the mitochondria might function by contributing to the development of the vesicular membrane structures. An interesting finding that somewhat relates the mitochondria to the vesicles functionally was introduced by Bodian (1967) who reported that in the nerve terminals of newly regenerated fibers the threshold for excitation is decreased, resulting in increased excitability following stimulation of the axon. In addition to this observation, he noted changes in the cytoplasmic structure of the newly-formed terminals. These include increases in mitochondria, protein synthesis, synaptic vesicles and an increase in active ion transport. Apparently these structures contribute in some way to this hyperexcitability which suggests an association of these elements in neurotransmission. However, one ought to keep in mind previous reports relating granular and agranular vesicles and neurotransmitter materials.

Palay (1967) showed that mitochondria are packed to a greater extent in the terminal part of the axon than in any other part of the nerve cell. This does not necessarily mean an association of mitochondria with synaptic vesicles and neurotransmission. However, Palay (1967) further observed that other cytoplasmic structures such as lysosomes and multi-vesicular bodies are rarely found at nerve endings. Glycogen particles on the other hand have been found to concentrate in the terminals. Lehninger (1967) reported that the mitochondria do function to provide energy utilizing glycogen in the process. He also noted that in the nerve terminals the mitochondria are also involved in protein synthesis and possibly the manu-facturing of membrane structures.

These observations point to a need for additional studies relative to the specific role of mitochondria at the nerve terminal and its possible association with synaptic vesicles.

In the tissues examined, a variety of synaptic vesicles were observed. One type of vesicle is characterized as granular and range in diameter from 500 to 1100 $\overset{o}{A}$. Similar types of vesicles were first observed in the adrenal medulla by Sjöstrand and Watzstein (1956). Recently several investigators have studied these granular vesicles. Pellegrino de Iraldi and DeRobertis (1961, 1963) have shown that granular vesicles completely

disappeared within a few minutes following reserpine treatment and that
normal vesicles did not reappear for several days. Wolf, et al. (1962) and
Van Orden, et al. (1966) observed granular vesicles at nerve endings. Bak
(1965) observed that in the mouse brain the depletion of granular vesicles
was prevented by monoamine oxidase inhibitors. Hashimoto, et al. (1965)
studied the ultrastructure of the so called adrenergic synaptic vesicle in the
rabbit hypothalamus and reported that decreases in density were observed
for the reserpine pretreated groups, while increases in density were
observed for groups pretreated with dl-DOPA. Hökfelt (1967), however,
attempted to study the dense core found in granular synaptic vesicles. He
stated that when the small granular vesicles were examined by incubating
slices from reserpine-treated animals in an α-methyl norepinephrine free
medium the number of granular vesicles decreased. Pfeifer, et al. (1968)
showed that the number of granular vesicles could be increased in the rat
pineal gland following pretreatment with DOPA, dopamine and nialamid.
On the other hand, Fuxe, et al. (1965) used fluorescence and electron
microscopy in studies on certain brain regions rich in monoamine terminals.
It was noted that when animals were pretreated with reserpine or nialamid
no observable changes were noted in vesicular type. Bloom, et al. (1966)
studied the ultrastructure of nerve endings in the anterior hypothalamus

and noted that when the hypothalamus was examined in rats 14 to 18 hours following treatment with 100 mg/kg of AMMT (alpha meta methyl tyrosine) I.V., there was no reduction in the core content of large vesicles. There was also no increase in density following oxidation with dichromate salt or osmium tetroxide.

In the present study, increases in granular vesicles of control animals were noted in the anterior hypothalamus and caudate nucleus at 0300 hours when compared to the results at 1500 hours. The increase in granular vesicles occurred during the dark phase coinciding with increases in motor activity and norepinephrine levels found during this same period. These findings might also indicate a functional relationship between the number of granular vesicles and increased motor activity. DeRobertis, et al. (1965) demonstrated that when nerve axons were stimulated electrically at a frequency of 100 per second an increase in the number of granular vesicles was noted, but when the frequency was increased to 400 per second a decrease in the number of granular vesicles was noted at the nerve terminals. The motor activity of these nocturnal animals during the dark period might simulate moderate stimulation and could possibly account for the increase in granular vesicles at 0300 hours.

Small agranular vesicles approximately 300 $\overset{o}{A}$ in diameter were

also observed in these tissues. Figures 23 and 24 show isolated boutons

with mostly agranular vesicles and a small number of granular vesicles.

In Figure 24, a cluster of agranular vesicles is noted near the synaptic

junction. This structure is a classical example of what DeRobertis, et al.

(1965) described as the "active point" of the synapse. This area is located

opposite the intersynaptic filaments and the synaptic cleft. This cluster of

agranular vesicles is almost resting on the inner membrane of the bouton.

It is possible that such "active points" provide the direct link between

electrical conductance and chemical transmission. Depolarization of the

nerve axon causes neurotransmitter material to be released at the synapse

resulting in a continuation of the impulse. The mechanism which causes

the chemical transmitter to be released from the cell is not clear. Hubbard,

et al. (1967) reported that ions that affect depolarization of the nerve

membrane such as calcium and potassium can also cause increases in the

quantal release of acetylcholine. Apparently the active sites are also

affected by membrane depolarization or ions that increase depolarization.

It is noted in these results that both granular and agranular vesicles are

located within the same boutons. Holmstedt (1967) showed that cholinergic

drugs and anticholinesterases could cause an increase in the mobilization

of brain acetylcholine from homogenates. These findings might also be associated with the "active point" in that in addition to acetylcholine diffusing across the nerve membrane and the synapse, it is possible that acetylcholine can diffuse within the bouton depolarizing the membranes of the synaptic vesicles (including the granular vesicles) and release norepinephrine. This theory might be used to support the Burn and Rand hypothesis (1962). Kopin (1967-a, b) reported that bretylium tosylate blocks the release of norepinephrine by nerve stimulation but does not block the release of norepinephrine by acetylcholine.

Vesicles other than those associated with norepinephrine and acetylcholine have been identified in nerve tissue. Richardson (1962) described a group of vesicles in the autonomic nerve endings of smooth muscle which was about 900 $\overset{o}{A}$ in diameter with a 500 $\overset{o}{A}$ inner dense core. The function of these vesicles is not clear. An interesting possibility might be that they contain both norepinephrine and acetylcholine. Another possibility could be that these are fragments of norepinephrine containing granular vesicles and the clear peripheral area represents a partial release of norepinephrine from a mobile pool, while the inner dense core represents an immobile pool.

Palay (1967) studied the fine structure of the rat adenohypophysis and observed vesicles about 1200 to 1500 $\overset{o}{A}$ in diameter that contained

large dense droplets that almost entirely fill the vesicle. Palay concluded

that these vesicles contained vasopressin and oxytocin.

Spherical and elongated small diameter agranular vesicles have

been observed in spinal motorneurons (Bodian, 1966). These vesicles might

be related to acetylcholine but this relationship has not been studied.

Figures 24 through 27 show the ultrastructure of the anterior and

posterior hypothalamus and caudate nucleus of treated and untreated rats.

Significant increases were observed in the number of granular vesicles at

0300 hours when compared to 1500 hours in the anterior hypothalamus and

caudate nucleus. A significant increase was noted in the agranular vesicles

of the posterior hypothalamus at 0300 hours. This is difficult to explain

but it is interesting that at a time when the granular vesicles increased in

the anterior hypothalamus and caudate nucleus the number of agranular

vesicles was relatively unchanged in these same tissues. However, a

significant increase in the number of agranular vesicles was observed in

the posterior hypothalamus at a time when the number of granular vesicles

was unchanged. These results indicate that while the number of granular

and agranular vesicles vary with the time of day, differences are also

noted if one compares specific brain areas.

While these studies of ultrastructure on a circadian basis are interesting, they are only preliminary and raise numerous questions and speculations. Such studies are not conclusive for designating norepinephrine or acetylcholine storage areas. In order to test this hypothesis and to relate circadian rhythms in synaptic vesicles with corresponding fluorometric findings, one could utilize electron microscopic autoradiography to verify these results.

CHAPTER VI

SUMMARY

1. The circadian patterns for rectal temperature and biogenic amine levels in midbrain and caudate nucleus have been measured in normal, adrenalectomized and pentobarbital sodium treated rats adapted to controlled lighting conditions over a three week period.

2. Rectal temperature reached peak values between 2400 hours and 0600 hours during the dark phase of the illumination cycle at a time when motor activity is maximal. In adrenalectomized rats the pattern is similar but the peak is significantly lower. In pentobarbital sodium treated rats, during the dark phase, the rectal temperature significantly decreased and produced a mirror image of the control pattern.

3. Histamine levels peaked in the caudate nucleus and midbrain of normal and adrenalectomized rats at a time when body temperature and motor activity are maximal. In the caudate nucleus of pentobarbital sodium treated rats, histamine levels were greater than in controls.

4. Caudate nucleus and midbrain norepinephrine levels reached their peaks during the dark phase of the illumination cycle. No significant differences were noted in the patterns obtained with adrenal-

ectomized rats. Pentobarbital sodium treatment significantly elevated midbrain and caudate nucleus norepinephrine levels during the dark phase of the illumination cycle.

5. Peak serotonin levels in the caudate nucleus and midbrain of normal and adrenalectomized rats were twelve hours out of phase with peak values obtained for the other parameters. Pentobarbital treatment did not significantly alter serotonin levels although the circadian pattern was depressed.

6. Peak acetylcholine levels in rat caudate nucleus and midbrain coincided with those of norepinephrine and histamine as well as with maxima of rectal temperature and motor activity. Acetylcholine rhythms were associated with the circadian toxicity rhythms observed for cholinergic drugs (acetylcholine, pilocarpine and oxotremorine), i. e., peak circadian toxicity was related to peak acetylcholine levels while trough toxicity or increased LD50 levels were related to lower levels of acetylcholine. An ultradian toxicity pattern was obtained with carbachol with a peak occurring at midday and a second peak at midnight.

7. The circadian pattern for atropine toxicity was essentially a mirror image with maximum toxicity (minimal LD50) during the light phase. A quaternary anticholinesterase, neostigmine, is most toxic during the late dark phase, while a tertiary anticholinesterase, physostigmine, was least toxic at that time and became progressively more toxic during the late light and early dark phase of the illumination cycle. Atropine methyl nitrate decreased toxicity of acetylcholine and physostigmine but the circadian pattern was essentially unchanged.

8. Sleeping time and onset to sleep time of pentobarbital sodium were studied and found to exhibit a circadian rhythm. The longest onset and shortest duration of sleep occurred during the dark phase while a reverse type relationship was observed during the light phase.

9. Circadian patterns for blood histamine and blood glucose was determined before and after pentobarbital sodium treatment. Peak blood histamine levels were noted at the end of the light phase while blood glucose showed bimodal peaks. A primary blood glucose peak occurred at the end of the dark phase while a secondary peak was noted at the end of the light phase. Pentobarbital sodium treatment did not alter the circadian patterns for blood histamine or blood glucose.

10. Norepinephrine levels in rat hypothalamus were significantly lower at 0300 hours than at 1500 hours, reflecting similar changes observed in midbrain and caudate nucleus. Significant reductions and elevations were obtained by pretreatment with reserpine or dl-DOPA and pargyline (an MAO inhibitor), respectively.

11. An examination of the ultrastructure of the hypothalamus and caudate nucleus showed that circadian changes were demonstrable when synaptic vesicles in tissue removed at 0300 and 1500 hours were counted. The number of granular vesicles (presumably containing NE) were significantly higher at 0300 hours in the anterior hypothalamus and caudate nucleus, but not in the posterior hypothalamus. The number of agranular vesicles (presumably containing ACh) was higher at 0300 hours, but only in the posterior hypothalamus was this increase statistically significant. Granular vesicle counts from tissues of rats pretreated with reserpine or dl-DOPA and pargyline were similar to those of the control study in that they were higher at 0300 hours than at 1500 hours.

A more variable pattern was obtained with the agranular vesicle counts. The most significant finding was the greater number at

1500 hours in the tissues from reserpinized animals. A similar

finding was obtained in the anterior hypothalamus after dl-DOPA

and pargyline administration.

CHAPTER VII

BIBLIOGRAPHY

1. Agranoff, B.W., Davis, R.E., Casola, L. and Lim, R. (1967) Actinomycin D blocks formation of memory of shock-avoidance in goldfish. Science, 158:1600-1601.

2. Agren, G., Wilander, O. and Jorpes, E. (1931) Cyclic changes in glycogen content of the liver and the muscles on rats and mice. Biochem. J., 25:777-785.

3. Allee, W.C. (1942) Group organization among vertebrates. Science, 95:289-293.

4. Altman, P.L. and Dittmer, D.S. (1966) Environmental Biology. FASEB, Bethesda, 570.

5. Anton, A.H. and Sayre, D.F. (1968) A modified fluorometric procedure for the determination of tissue histamine. Fed. Proc., 27:243.

6. Aprison, M.H., Kariya, T., Hingtgen, J.N. and Toru, M. (1968). Neurochemical correlates of behaviour. J. Neurochem., 15:1131-1139.

7. Aschoff, J. (1963) Comparative physiology: Diurnal rhythms. An. Rev. Physiol., 25:581-600.

8. Axelrod, J., Snyder, S.H., Moore, R.Y. and Heller, A. (1966) Abolition of the circadian serotonin rhythm and light controlled changes in hydroxy-indole-o-methyl transferase (HIOMT) in the pineal gland by lesions in the medial forebrain bundle. Science, 154:898-899.

9. Bahorsky, M.S. and Bernardis, L.L. (1967) Circadian rhythmicity of some key metabolites in the fasting and fed weanling female rat. Experientia, 23:634-635.

10. Bak, I.J. (1965) Electron microscopic observations in the substantia nigra of mouse during reserpine administration. Experientia, 21:568.

11. Baldwin, F. M. (1917) Diurnal activity of the earthworm. J. Anim. Behavior, 7:187-191.

12. Ball, N. G. and Dyke, I. J. (1954) An endogenous 24-hour rhythm in the growth rate of Avena coleoptile. J. exp. Bot., 5:421-433.

13. Barden, A. (1942) Activity of the lizard Cnemidophorus sexlineatus. Ecology, 23:336.

14. Barnett, A. (1966) A circadian rhythm of mating type reversals in Paramecium multimicronucleatum, Syngen 2, and its genetic control. J. cell. comp. Physiol., 67:239-242.

15. Barrott, H. G., Fritz, J. C., Pringle, E. M. and Titus, H. W. (1938) Heat production and gaseous metabolism of young male chicks. J. Nutr., 15:145-150.

16. Bartter, F. C., Delea, C. S. and Halberg, F. (1962) A map of blood and urinary changes related to circadian variations in adrenal cortical function in normal subjects. Ann. N. Y. Acad. Sci., 98:969-983.

17. Beani, L., Blanchi, C. and Mazzini, P. (1964) Regional changes of acetylcholine and choline acetylase activity in the guinea pig's brain after scopolamine. Experientia, 20:667-668.

18. Bennett, E. L., Crossland, J., Krech, D. and Rosenzweig, M. R. (1960) Strain differences in acetylcholine concentration in the brain of the rat. Nature, 187:787-788.

19. Berry, J. F. and Starz, E. (1966) Acetylcholine and acetone in brain during acetylaldehyde intoxication. Quart. J. Stud. Alcohol, 17:190-194.

20. Bloom, F. E., Aghajanian, G. A. and Barnett, R. J. (1966) Regional brain amines and the fine structural identification of norepinephrine. Anat. Rec., 154:318-319.

21. Bodian, D. (1966) Electron microscopy: Two major synaptic types on spinal motorneurons. Science, 151:1093-1094.

22. Bodian, D. (1967) Neurons, circuits and neuroglia. The Neurosciences, The Rockefeller Univ. Press, New York, pp. 6-23.

23. Bohn, G. and Drzewina, A. (1928) Les 'Convoluta'. Ann. Nat. Acad. Sci., 11:299-301.

24. Bowers, M.B., Hartmann, E.L. and Freedman, D.X. (1966) Sleep deprivation and brain acetylcholine. Science, 153:1416-1417.

25. Boyd, T.A.S. and McLeod, L.E. (1964) Circadian rhythms of plasma corticoid levels, intraocular pressure and aqueous outflow facility in normal and glaucomatous eyes. Ann. N.Y. Acad. Sci., 117: 597-613.

26. Bradley, P.B. and Key, B.J. (1958) The effect of drugs on arousal responses produced by electrical stimulation of reticular formation of the brain. Electroenceph. clin. Neurophysiol., 10:97-110.

27. Bradley, P.B. and Key, B.J. (1959) A comparative study of the effects of drugs on the arousal system of the brain. Brit. J. Pharmacol., 14:340-349.

28. Brahmachary, R.L. (1967) Physiological clocks. Int. Rev. Cytol. 21:1-39.

29. Browman, L.G. (1937) Light in relation to activity and estrus rhythm in albino rats and mice. J. exp. Zool., 75:375-380.

30. Brown, F.A. (1954) Biological clocks and the fiddler crab. Sci. Amer., 190:34-37.

31. Brown, F.A. (1963) The biological rhythm problem and its bearing on space biology. Advanc. Astronautical Sci., 17:29-39.

32. Brown, F.A., Bennett, M.F., Webb, H.M. and Ralph, C.L. (1956-a) Persistent daily, monthly and 27-day cycles of activity in the oyster and quahog. J. exp. Zool., 131:235-262.

33. Brown, F.A., Freeland, R.O. and Ralph, C.L. (1955) Persistent rhythm of oxygen consumption in potatoes, carrots, and a seaweed Fucus. Plant Physiol., 30:280-284.

34. Brown, F.A., Sandeen, M.I. and Fingerman, M. (1952) Modification of the tidal rhythms of Uca by tidal differences and by illumination. Biol. Bull., 103:297-299.

35. Brown, F.A., Shriner, J. and Ralph, C.L. (1956-b) Solar and lunar rhythmicity in the rat in constant conditions and the mechanisms of physiological time measurement. Amer. J. Physiol., 184:491-495.

36. Brown, F.A. and Stephens, G.C. (1951) Studies on the daily rhythmicity of the fiddler crab Uca modifications by photoperiod. Biol. Bull., 101:71-82.

37. Brown, F.A. and Webb, H.M. (1948) Temperature relations of an endogenous daily rhythmicity in the fiddler crab, Uca. Physiol. Zool., 21:371-381.

38. Brown, F.A. and Webb, H.M. (1949-a) Analysis of the mechanism of diurnal rhythmicity in crustacea. Fed. Proc., 8:18.

39. Brown, F.A. and Webb, H.M. (1949-b) Studies of the daily rhythmicity of the fiddler crab, Uca. Physiol. Zool., 22:136-139.

40. Brown, F.A., Webb, H.M. and Graves, R.C. (1952) A persistent tidal rhythm in the fiddler crab Uca pugnax. Biol. Bull., 103:299-300.

41. Brown, F.A., Webb, H.M. and Guyselman, J.B. (1949) A persistent rhythmicity in Uca red chromatophores. Anat. Rec., 105:286-290.

42. Brown, H.E. (1962) Periodicity of leukocytes in normal and adrenalectomized mice. Ann. N.Y. Acad. Sci., 98:995-1006.

43. Burn, J.H. and Rand, M.J. (1962) A new interpretation of the adrenergic nerve fiber. Advanc. Pharmacol., 1:2-29.

44. Calhoun, J.B. (1945) Diel activity of rodents. Ecology, 26:251-253.

45. Civen, M., Ulrich, R., Trimmer, B.M. and Brown, C.B. (1967) Circadian rhythms of liver enzymes and their relationship to enzyme induction. Science, 157:1563-1564.

46. Cohen, L.W. (1965) The effects of puromycin and actinomycin D on the mating rhythm of the Paramecium bursaria. Exp. Cell Res., 37:360-365.

47. Colvin, G.B., Whitmoyer, D.L., Lisk, R.D., Walter, D.O. and Sawyer, C.H. (1968) Changes in sleep-wakefulness in female rats during circadian and estrous cycles. Brain Res., 7:173-181.

48. Connolly, C.K. and Wills, M.R. (1967) Cortisol circadian rhythm. Brit. med. J., 2:640.

49. Crossland, J. (1961) Biologic estimation of acetylcholine. Methods in Medical Research. 9:125-129.

50. Curtis, Q.F. (1937) Dirunal variations in the free activity of sheep and pig. Proc. Soc. exp. Biol., 35:566-567.

51. Davis, D.H.S. (1933) Rhythmic activity in the short-tailed vole Microtus. J. Anim. Ecol., 2:232-235.

52. Davis, W.M. (1962) Day-night periodicity in pentobarbital response of mice and the influence of socio-psychological conditions. Experientia, 18:235-236.

53. DeRobertis, E., Pellegrino De Iraldi, A., Rodriguez De Loren Arnaiz, G. and Gomez, C.J. (1961) The isolation of nerve endings and synaptic veiscles. J. biophys. biochem. Cytol. 9:229-233.

54. DeRobertis, E.D.P., Nowinski, W.W. and Saez, F.A. (1965) Cell Biology. W.B. Saunders Co., pp. 411-425.

55. Dixit, B.N. and Buckley, J.P. (1967) Circadian changes in brain 5-hydroxytryptamine and plasma corticosterone in the rat. Life Sci., 6:755-758.

56. Doe, R.P., Flink, E.B. and Goodsell, M.G. (1956) Relationship of diurnal variations in 17-hydroxycorticosteroid levels in blood and urine to eosinophils and electrolyte excretion. J. clin. Endocrin. Metab., 16:196-206.

57. Donoso, A.O. and Sequra, E.T. (1965) Seasonal variations of plasma adrenaline and noradrenaline in toads. Gen. Comp. Endocrin. 5:440-443.

58. Eakins, K.E., Costa, E., Katz, R.L. and Reyes, C.L. (1968) Effect of pentobarbital anesthesia on the turnover of ^{3}H noradrenaline in peripheral tissues of cats. Life Sci., 7:71-76.

59. Enright, J.T. and Hamner, W.M. (1967) Vertical diurnal migration and endogenous rhythmicity. Science, 157:937-941.

60. Everett, G.M. (1961) Some electrophysiological and biochemical correlates of motor activity and aggressive behavior. Neuropsychopharmacology, 2:479-484.

61. Fearn, H.J., Karady, S. and West, G.B. (1966) Histamine and rat blood pressure. J. Pharm., London, 18:407-408.

62. Feldberg, W., Hellon, R.F. and Myers, R.D. (1966) Effects on temperature of monoamines injected into cerebral ventricles of anesthetized dogs. J. Physiol., 186:416-423.

63. Feldberg, W. and Sherwood, S. L. (1954) Injection of drugs into the lateral ventricle of the cat. J. Physiol., 123:246-255.

64. Feldman, J. F. (1968) Circadian rhythmicity in amino acid incorporation in Euglena gracilis. Science, 160:1454-1456.

65. Fiske, V. M. and Leeman, S. E. (1964) Observations on adrenal rhythmicity and associated phenomena in the rat: Effect of light on adrenalcortical function; maturation of the hypothalamic system in relation to adrenal secretion. Ann. N. Y. Acad. Sci., 117:231-243.

66. Flexner, L. B. and Flexner, J. B. (1968) Intracerebral saline: Effect on memory of trained mice treated with puromycin. Science, 159:330-331.

67. Fraps, R. M. (1954) Neural basis of diurnal periodicity in release of ovule-inducing hormone in fowl. Proc. nat. Acad. Sci., 40:348-356.

68. Friedman, A. H. and Everett, G. M. (1964) Pharmacological aspects of Parkinsonism. Advanc. Pharmacol., 3:83-127.

69. Friedman, A. H. and Kulkarni, A. S. (1961) Analeptic action of adrenergic neuron blockers in barbiturate depression. Pharmacologist, 3:78.

70. Fuchs, J. (1964) Physical alterations which occur in the blind are illustrated in ancient Egyption works of art. Ann. N. Y. Acad. Sci., 117:618-621.

71. Fuxe, K., Hokfelt, T. and Nilsson, O. (1965) A fluorescence and electronmicroscopic study on certain brain regions rich in monoamine terminals. Amer. J. Anat., 117:33-46.

72. Fuxe, K., Hokfelt, T., Nilsson, O. and Reinius, S. (1967) A fluorescence and electron microscopic study on central monoamine nerve cells. Anat. Rec., 155:33-40.

73. Garattini, S. and Valzelli, L. (1965) Serotonin. Elsevier Pub. Co., Amsterdam.

74. Gaston, S. and Menaker, M. (1968) Pineal function: The biological clock in the sparrow. Science, 160:1125-1127.

75. Gershbein, L.L. (1968) Histamine-insulin antagonism in relation to rat liver and muscle glycogen turnover. Arch. int. Pharmacodyn., 173:16-26.

76. Gertner, S.B. and Kohn, R. (1959) Effect of histamine on ganglionic transmission. Brit. J. Pharmacol., 14:179-182.

77. Giarman, N.J. and Pepeu, G. (1962) Drug-induced changes in brain acetylcholine. Brit. J. Pharmacol., 19:226-234.

78. Giarman, N.J. and Pepeu, G. (1964) The influence of centrally acting cholinolytic drugs on brain acetylcholine levels. Brit. J. Pharmacol., 23:223-230.

79. Gibbs, D. and Dillinger, O.P. (1908) The daily life of amoeba proteus. Amer. J. Psychol., 19:232-235.

80. Glowinski, J. and Iversen, L.L. (1966) Regional studies of catecholamines in the rat brain-1. J. Neurochem., 13:655-669.

81. Green, H. and Erickson, R.W. (1967) Effect of some drugs upon the histamine concentration of guinea pig brain. Arch. int. Pharmacodyn., 166:121-126.

82. Grillo, M.A. and Palay, S.L. (1962) Granule-containing vesicles in the autonomic nervous system. Proceedings of the Fifth International Congress on Electron Microscopy. Ed. S.S. Breese, Academic Press, New York, p. u-2.

83. Groot, C.A. (1967) The diurnal fluctuation of ascorbic acid and progesterone contents in the ovaries of intact and hypophysectomized immature pseudopregnant rats. Acta endocr., 56:459-468.

84. Gustafson, G.L. and Benedict, F.G. (1928) The seasonal variation in basal metabolism. Amer. J. Physiol., 85:43-58.

85. Guyselman, J.B. (1957) Solar and lunar rhythms of locomotor activity in the crayfish Cambarus virilis. Physiol. Zool., 30:70-87.

86. Halberg, F. (1959) Physiologic 24-hour periodicity; general and procedural considerations with reference to the adrenal cycle. Z.f. Vitamin-Hormon-und Fermentforschung, 10:225-296.

87. Halberg, F. (1962) Circadian (about twenty-four hour) rhythms in experimental medicine. Proc. R. Soc. Med., 56:253-260.

88. Halberg, F. and Reinberg, A. (1967) Rythmes circadiens et basses frequences en physiologic humaine. J. Physiol., Paris, 59:117-200.

89. Hamilton, L.D., Gubler, C.J., Cartwright, G.E. and Wintrobe, M.M. (1950) Diurnal variation in the plasma iron level of man. Proc. Soc. exp. Biol., 75:65-68.

90. Hanks, C.F. (1967) Relationship between serotonin and the circadian rhythm in some nocturnal moths. Nature, 214:386-387.

91. Harker, J.E. (1955) Control of diurnal rhythms of activity in Periplaneta americana. Nature, 175:773.

92. Harker, J.E. (1958) Diurnal rhythms in the animal kingdom. Biol. Rev., 33:1-52.

93. Hashimoto, Y., Ishii, S., Ohi, Y., Shimizu, N. and Imaizum, R. (1965) Effect of DOPA on the norepinephrine and dopamine contents and on the granulated vesicles of the hypothalamus or reserpinized rabbits. Jap. J. Pharmacol., 15:395-400.

94. Hauty, G.T. (1962) Periodic desynchronization in humans under outer space conditions. Ann. N.Y. Acad. Sci., 98:1116-1125.

95. Herz, A. and Zieglgansberger, W. (1968) The influence of microelectrophretically applied biogenic amines, cholinomimetics and procains on sympathetic excitation in the corpus striatum. Int. J. Neuropharmacol., 7:221-230.

96. Higginbotham, A. C. (1939) Studies on amphibian activity. I. Preliminary report on the rhythmicity activity of Bufo a. americanus holbrook and Bufo fowleri hinckley. Ecology, 20:58-70.

97. Hitchcock, F. A. and Wardwell, F. R. (1929) Cyclic variations in the basal metabolic rate of women. J. Nutr. 2:203-215.

98. Hodge, C. F. and Aikens, H. A. (1893) The daily life of a protozoan. Amer. J. Psychol., 6:524-526.

99. Hökfelt, T. (1967) Electron microscopic studies on brain slices from regions rich in catecholamine nerve terminals. Acta physiol. scand., 69:119-120.

100. Holmquest, D. L., Retiene, K. and Lipscomb, H. S. (1966) Circadian rhythms in rats: Effects of random lighting. Science, 152:662-664.

101. Holmstedt, B. (1967) Mobilization of acetylcholine by cholinergic agents. Ann. N. Y. Acad. Sci., 144:433-458.

102. Holmstedt, B. and Lundgren, G. (1966) Tremorgenic agents and brain acetylcholine. Mechanisms of Release of Biogenic Amines (Symposium). Stockholm, Pergamon Press, pp. 439-468.

103. Holzapfel, R. A. (1937) Seasonal variations in the hematology of hibernating frogs. Quart. Rev. Biol., 12:65-67.

104. Honova, E., Miller, R. A. and Ehrenkranz, A. W. (1968) Tyrosine transaminase: Development of daily rhythms in liver of neonatal rat. Science, 162:999-1000.

105. Hubbard, J. I., Jones, S. F. and Landau, E. M. (1967) The relationship between the state of nerve terminal polarization and liberation of acetylcholine. Ann. N. Y. Acad. Sci., 144:459-470.

106. Hugget, A. and Nixon, D.A. (1967) Use of glucose oxidase, perioxidase and o-dianisidine in the determination of blood and urinary glucose. Lancet, 2:368-370.

107. Iversen, L.L. and Glowinski, J. (1966) Regional studies of catecholamines in the rat brain. J. Neurochem., 13:671-682.

108. Johnson, M.S. (1939) Effects of continuous light on periodic spontaneous activity of white-footed mice (Peromyscus). J. exp. Zool., 82:315-328.

109. Kalmus, H. (1940) New research in the diurnal periodicity of animals. Acta med. scand., 108:227-231.

110. Kalyanpur, S.G., Naik, S.R. and Sheth, U.K. (1968) Study of some factors affecting pentobarbital sleeping time. Arch. int. Pharmacodyn., 173:1-10.

111. Karczmar, A.G. (1967) Lecture in central nervous system pharmacology, relative to learning and memory and cholinesterase. Department of Pharmacology, Loyola University, Hines, Ill.

112. Kay, D.H. (1965) Techniques for Electron Microscopy. Second Ed., Davis Co., Philadelphia, pp. 153-310.

113. Kessler, E. and Czygan, F.C. (1963) Seasonal changes in the nitrate-reducing activity of green algae. Experientia, 19:89-90.

114. Keston, A.S. (1956) Specific colorimetric enzymatic analytical reagents for glucose. Proc. 129th Meeting ACS, Dallas, p. 310.

115. Killam, E.K. (1962) Drug action on the brain-stem reticular formation. Pharmacol. Rev., 14:175-210.

116. Kleitman, N. (1949) Biological rhythms and cycles. Physiol. Rev. 29:1-30.

117. Kleitman, N. and Ramsaroop, A. (1948) Periodicity in body temperature and heart rate. Endocrinology, 43:1-6.

118. Kobayashi, T., Kato, J. and Minagughi, H. (1964) Fluctuations in
monoamine oxidase activity in the rat hypothalamus during the
estrous cycle and after castration. Endocrinol. Japon.,
11:283-290.

119. Koella, W.P. and Czicman, J. (1966) Mechanism of EEG synchro-
nizing action of serotonin. Amer. J. Physiol., 211:926-934.

120. Konzett, H. (1952) The effect of histamine on isolated sympathetic
ganglion. J. Mt. Siani Hosp., New York, 19:149-153.

121. Kopin, I.J. (1967-a) Acetylcholine, bretylium and release of norepi-
nephrine from sympathetic nerve endings. Ann. N.Y. Acad. Sci.,
144:558-562.

122. Kopin, I.J. (1967-b) The adrenergic synapse. The Neurosciences,
The Rocefeller Univ. Press, New York. pp. 427-432.

123. Kremzner, L.T. and Pfeiffer, C.C. (1966) Identification of sub-
stances interfering with the fluorometric determination of brain
histamine. Biochem. Pharmacol., 15:197-200.

124. Krieger, D.T. and Krieger, H.P. (1967) Circadian pattern of plasma
17-hydroxycorticosteroid: Alteration by anticholinergic agents.
Science, 155:1421-1422.

125. Landau, J. and Feldman, S. (1954) Diminished endogenous morning
eosinopenia in blind subjects. Acta Endocrin., 15:53-60.

126. Lehninger, A.L. (1967) Cell organelles: The mitochondrion.
The Neurosciences, The Rockefeller Univ. Press, New York.
pp. 91-100.

127. Lenn, N.J. (1967) Localization of uptake of tritiated norepinephrine
by rat brain in vivo and in vitro using electron microscopic
autoradiography. Amer. J. Anat., 120:377-390.

128. Lewis, C.B. and Bletchly, J.D. (1943) The emergence rhythm of the
dungfly Scopeuma stercoraria L. J. Anim. Ecol., 12:11-13.

129. Lindsley, D. B., Wendt, R. H., Lindsey, D. F., Fox, S. S., Howell, J., and Adey, W. R. (1964) Diurnal activity, behavior and EEG responses in visually deprived monkeys. Ann. N. Y. Acad. Sci., 117:564-588.

130. Litchfield, J. T., Jr. and Wilcoxon, F. (1949) A simplified method of evaluating dose-effect experiments. J. Pharmacol., 96:99-113.

131. Lohrenz, F. N., Fullerton, D. T., Fahs, H. and Wenzel, F. J. (1967) Adrenal cortical function in depressive states - study of circadian variation in plasma and urinary steroids. Int. J. Neuropsych., 3:21-25.

132. Longson, D. and Mills, J. N. (1953) The failure of the kidney to respond to respiratory acidosis. J. Physiol. (London), 122:81-92.

133. Lutsch, E. F. and Morris, R. W. (1967) Circadian periodicity in susceptibility to lidocaine hydrochloride. Science, 155:1421-1422.

134. McCluskey, E. S. (1965) Circadian rhythms in male ants of five diverse species. Science, 150:1037-1038.

135. McGeer, E. G. and McGeer, P. L. (1966) Circadian rhythms in pineal tyrosine hydroxylase. Science, 152:73-74.

136. McKinstry, D. N. and Koelle, G. B. (1967-a) Acetylcholine release from the cat superior cervical ganglion by carbachol. J. Pharmacol., 157:319-327.

137. McKinstry, D. N. and Koelle, G. B. (1967-b) Effects of drugs on acetylcholine release from the cat superior cervical ganglion by carbachol and by preganglionic stimulation. J. Pharmacol. 157:328-336.

138. Manshardt, J. and Wurtman, R. J. (1968) Daily rhythms in the nor-adrenaline content of rat hypothalamus. Nature, 217:574-575.

139. Mantegazzini, P. (1966) Pharmacological actions of indolealkylamines and precursor amino acids on the central nervous system. 5-Hydroxytryptamine and Related Indolealkylamines, Springer-Verlag, pp. 425-470.

140. Mantegezza, P. and Riva, M. (1961) Anorexigenic activity of L (-) DOPA in animals pretreated with monoamine oxidase inhibitor. Med. Experimentalis, 4:367-373.

141. Matthews, G. V. T. (1955) Investigation of chronometer factor in bird navigation. J. exp. Biol., 32:39-52.

142. Mead, J. A. R. and Finger, K. F. (1961) A single extraction method for the determination of both norepinephrine and serotonin in brain. Biochem. Pharmacol., 6:52-53.

143. Menaker, M. (1959) Endogenous rhythms of body temperature in hibernating bats. Nature, 184:1251-1252.

144. Menaker, M. (1961) The free running period of the bat clock; seasonal variations at low body temperature. J. comp. cell. Physiol., 57:81-86.

145. Michaelson, I. A. (1967) Spermidine: a contaminant in the n-butanol extraction of brain in the fluorometric assay of histamine. Europ. J. Pharmacol., 1:378-382.

146. Migeon, C. E., Tyler, F. H., Mahoney, J. P., Flourentin, A. A., Castle, H., Bliss, E. L. and Samuels, L. T. (1956) The diurnal variation of plasma levels and urinary excretion of 17-hydroxy-corticosteroids in normal subjects, night workers, and blind subjects. J. clin. endocrin. Metab., 16:622-633.

147. Millichap, J. G. and Millichap, P. A. (1966) Circadian analysis of pentobarbital-induced hyperkinesia in mice and hamsters. Fed. Proc., 121:752-754.

148. Mills, J. N. (1966) Human circadian rhythms. Physiol. Rev., 46:128-171.

149. Mills, J.N. and Stanbury, S.W. (1954) A reciprocal relationship between potassium and hydrogen ion excretion in the diurnal excretory rhythm in man. Clin. Sci., 13:177-186.

150. Min, H.K., Jones, J.E. and Flink, E.B. (1966) Circadian variations in renal excretion of magnesium, calcium, phosphorus, sodium, and potassium during frequent feeding. Fed. Proc., 25:917-921.

151. Mitchell, J.F. (1963) The spontaneous and evoked release of acetylcholine from the cerebral cortex. J. Physiol., 165:98-116.

152. Monnier, M. (1960) Actions electro-physiologiques des stimulants du systeme nerveux central. Arch. int. Pharmacodyn., 124:281-301.

153. Monnier, M., Fallert, M. and Bhattacharya, I.C. (1967) The waking action of histamine. Experientia, 23:21-22.

154. Mori, S. (1943) Daily rhythmical activities of Cavernularia obesa valenciennes. I. The activities and the natural conditions. Zool. Mag., 55:247-250.

155. Nickander, R.C. and Yim, G.K.W. (1964) Effects of tremorine and cholinergic drugs on the isolated cerebral cortex. Int. J. Neuropharmacol., 3:571-578.

156. Ott, J.N. (1964) Some responses of plants and animals to variations in wavelengths of light energy. Ann. N.Y. Acad. Sci., 117:624-635.

157. Palay, S.L. (1957) The fine structure of the neurohypophysis. Progr. Neurobiol., 2:31-44.

158. Palay, S.L. (1967) Principles of cellular organization in the nervous system. The Neurosciences, The Rockefeller Univ. Press, New York, pp. 24-34.

159. Park, O. and Sejba, O. (1935) Studies in nocturnal ecology. IV. Megalodacne heros. Ecology, 16:164-172.

160. Park, O. and Woods, L.P. (1940) A modified hemmingsen-krarup mammalian activity recorder. Proc. Soc. exp. Biol., 43:366-369.

161. Parkinson, C.N. (1957) Parkinson's Law and Other Studies in Administration. Houghton Mifflin Co., Boston, pp. 107-109.

162. Pellegrino de Iraldi, A. and DeRobertis, E. (1961) Action of reserpine on the submicroscopic morphology of the pineal gland. Experientia, 17:122.

163. Pellegrino de Iraldi, A. and DeRobertis, E. (1963) Action of reserpine iproniazid and pyrogallol on nerve endings of the pineal gland. Int. J. Neuropharmacol., 2:231-238.

164. Pfeifer, A.K., Szabo, D., Palkovits, M. and Okros, I. (1968) Correlation between norepinephrine content of the brain and the number of granular vesicles in rat hypothalamus during nialamid administration. Exp. Brain Res., 5:79-84.

165. Pittendrige, C.S. (1954) On the temperature independence in the clock system controlling emergence time in Drosophila. Proc. nat. Acad. Sci., 40:1018.

166. Quay, W.B. (1966) Twenty-four hour rhythms in pineal 5-hydroxy-tryptamine and hydroxyindole-o-methyl transferase in the macaque. Proc. Soc. exp. Biol., 121:946-948.

167. Radzialowski, F.M. and Bousquet, W.F. (1968) Daily rhythmic variation in hepatic drug metabolism in the rat and mouse. J. Pharmacol., 163:229-238.

168. Rahn, H. and Rosendale, F. (1941) Diurnal rhythms of the melanophore hormone secretion in the Anolis pituitary. Proc. Soc. exp. Biol., 48:100.

169. Rapoport, M.I., Feigin, R.D., Bruton, J. and Biesel, W.R. (1966) Circadian rhythm for tryptophan pyrrolase activity and its circulating substrate. Science, 153:1642-1644.

170. Reimann, H. A. (1951) Periodic diseases. Medicine, 30:219-225.

171. Reinberg, A. and Sidi, E. (1965) Circadian changes in the inhibitory effects of an antihistaminic drug in man. J. invest. Derm., 46:415-419.

172. Reinberg, A., Zagula-Mally, Z. W., Ghata, J. and Halberg, F. (1967) Circadian rhythm in the duration of salicylate excretion referred to phase of excretory rhythms and routine. Proc. exp. Biol., 124:826-832.

173. Reivich, M. and Glowinski, J. (1967) An autoradiographic study of the distribution of carbon-14-norepinephrine in the brain of the rat. Brain, 90:633-646.

174. Renbourn, E. T. (1947) Variation, diurnal and over longer periods of time, in blood hemoglobin, hematocrit, plasma protein, erythrocyte sedimentation rate and blood chloride. J. Hyg., 45:455-467.

175. Richardson, K. C. (1962) The fine structure of autonomic nerve endings in smooth muscle of the rat vas deferens. J. Anat., 96:427-431.

176. Richter, C. P. (1927) Animal behavior and internal drives. Quart. Rev. Biol., 2:307-312.

177. Rinaldi, F. and Himwich, H. E. (1955) Alerting responses and actions of atropine and cholinergic drugs. Arch. Neurol. Psychiat., 73:396-402.

178. Roberts, S. K. (1962) Circadian activity rhythms in cockroaches. II. Entrainment and phase shifting. J. cell. comp. Physiol., 59:175-186.

179. Rodeick, R. W. and Smith, P. S. (1966) Slow dark discharge rhythms of cat retinal ganglion cells. J. Neurophysiol., 29:942-953.

180. Sato, M., Austin, G. M. and Yai, H. (1967) Increase in permeability of the post-synaptic membrane to potassium produced by nembutal. Nature, 215:1506-1508.

181. Saunders, D. S. (1967) Time measurements in insect photoperiodism reversal of a photoperiotic effect by chilling. Science, 156:1126-1127.

182. Scheving, L. E., Harrison, W. H., Gordon, P. and Pauly, J. E. (1968) Daily fluctuation (circadian and ultradian) in biogenic amines of the rat brain. Amer. J. Physiol., 214:166-173.

183. Schuberth, J. and Sundwall, A. (1967) Effects of some drugs on the uptake of acetylcholine in cortex slices of mouse brain. J. Neurochem., 14:807-812.

184. Scott, J. P. (1959) Aggression, Univ. of Chicago Press, Chicago, Ill.

185. Scudder, C. L., Karczmar, A. G., Everett, G. M., Gibson, J. E. and Rifkin, M. (1966) Brain catecholamine and serotonin levels in various strains and genera of mice and a possible interpretation for the correlations of amine levels with electrashock latency and behavior. Int. J. Neurochem., 5:343-351.

186. Seyle, H. (1949) Textbook of Endocrinology, Second Ed., Acta Endocrinology Inc., Montreal, Canada.

187. Shaw, A. F. B. (1933) The leucocytes of the pigeon with special reference to a diurnal rhythm. J. Path. Bact., 37:411-414.

188. Sheard, M. H. and Aghajanian, G. K. (1967) Neural release of brain serotonin and body temperature. Nature, 216:495-496.

189. Shirley, M. (1928) Studies in activity. II. Activity rhythms, age and activity, activity after rest. J. comp. physiol. Psychol. 8:159-162.

190. Shore, P. A., Burkhalter, A. and Cohn, V. H. (1959) A method for the fluormetric assay of histamine in tissues. J. Pharmacol. 127:182-185.

191. Shore, P. A. and Olin, J. S. (1958) Identification and chemical assay of norepinephrine in brain and other tissues. J. Pharmacol. 122:295-300.

192. Simpson, G. E. (1929) Changes in the composition of urine brought about by sleep and other factors. J. biol. Chem., 84:393-411.

193. Simpson, S. and Galbraith, J. J. (1905) An investigation into the diurnal variation of the body temperature of nocturnal and other birds and a few mammals. J. Physiol., 33:225-229.

194. Sjostrand, F. S. and Watzstein, R. (1956) Elektronmikroskopische untersuchungen der phaechromen (chromaffinen) Granula in der Nebenniere. Experientia, 12:196.

195. Slonaker, J. H. (1912) The normal activity of the albino rat from birth to natural death, its rate of growth and duration of life. J. anim. Behav., 2:20-23.

196. Snyder, S. H. and Axelrod, J. (1964) Circadian rhythm in pineal serotonin: Effect on monoamine oxidase inhibition and reserpine. Science, 149:542-544.

197. Snyder, S. H., Axelrod, J., Wurtman, R. J. and Fischer, J. E. (1965) Control of 5-hydroxytryptophan decarboxylase activity in the rat pineal gland by sympathetic nerves. J. Pharmacol., 147:371-375.

198. Sollberger, A. (1964) The control of circadian glycogen rhythms. Ann. N. Y. Acad. Sci., 117:519-554.

199. Spoor, R. P. and Jackson, D. B. (1966) Circadian rhythms: Variations in sensitivity of isolated rat atria to acetylcholine. Science, 154:782.

200. Stein, H. (1951) Untersuchungen uber den Zeitsinn bei Vogeln. Z. vergl. Physiol., 33:387.

201. Stier, T.J.B. (1933) Dirunal changes in activity and getropism in Thyone briareus. Biol. Bull., 64:326-329.

202. Strughold, H. (1962) Day-night cycling in atmospheric flight, space flight, and on other celestial bodies. Ann. N.Y. Acad. Sci., 98:1109-1115.

203. Sutherland, E.W. and Rall, T.W. (1960) The relation of adenosine 3'-5'-phosphate and phosphorylase to the action of catecholamines and other hormones. Pharmacol. Rev., 12:265-300.

204. Toru, M. and Aprison, M.H. (1966) Brain acetylcholine studies: A new procedure. J. Neurochem., 13:1533-1544.

205. Udenfriend, S. (1962) Fluorescence Assay in Biology and Medicine, Academic Press, New York. pp. 140-182.

206. Ungar, F. (1964) In vitro studies of adrenal-pituitary circadian rhythms in the mouse. Ann. N.Y. Acad. Sci., 117:374-385.

207. Valzelli, L. (1967) Drugs and Aggressiveness. Advances in Pharmacology, Ed. Garattini and Shore, Academic Press, New York. 5:79-108.

208. Valzelli, L. and Garattini, S. (1968) Behavioral changes in 5-HT turnover in animals. Advanc. Pharmacol., 6-B:249-260.

209. Van Orden, L.S., Bloom, F.E., Barrnett, R.J. and Giarman, N.J. (1966) Histochemical and functional relationships of catecholamines in adrenergic nerve endings. Participation of granular vesicles. J. Pharmacol., 154:185-191.

210. Van Pilsum, J.F. and Halberg, F. (1964) Transamidinase activity in mouse kidney - an aspect of circadian periodic enzyme activity. Ann. N.Y. Acad. Sci., 117:337-353.

211. Vesell, E.S. (1968) Genetic and environmental factors affecting hexobarbital induced sleeping time (ST) in mice. Fed. Proc. 27:349.

212. Villarreal, J.E. and Domino, E.F. (1964) Evidence for two types of cholinergic receptors involved in EEG desynchronization. Pharmacologist, 6:192.

213. Virno, M., Gertner, S.B. and Bovet, D. (1956) Action of histamine on the jugular, venous pressure and cerebral circulation of the dog: Effects of antihistaminic drugs (pyrilamine and chlorpheniramine) and a histamine-liberating agent (48/80 B.W.). J. Pharmacol., 118:63-76.

214. von Mayersbach, H. (1967) Cellular Aspects of Biorhythms. Springer-Verlag, New York, p. 167.

215. von Stein-Beling, I. (1935) Uber das Zeitgedachtnis bei Tieren. Biol. Rev., 10:18-28.

216. Wahlstrom, G. (1964) Influence of psychopharmacological agents on circadian rhythms in the canary studies by self selection of light and darkness. Ann. N.Y. Acad. Sci., 117:320-336.

217. Webb, M.H. and Brown, Frank A. (1965) Interactions of diurnal and tidal rhythms in the fiddler crab, Uca pugnax. Biol. Bull., 129:582-591.

218. Webb, O.L. and Russell, O.L. (1966) Diurnal chemoconvulsive responses and central inhibition. Arch. int. Pharmacodyn., 159:471-476.

219. Welsh, J.H. and Osborne, C.M. (1937) Diurnal changes in the retina of the catfish, Ameiurus nebulosus. J. comp. Neurol., 66:349-359.

220. White, R.P. and Rudolph, A.S. (1968) Neuropharmacological comparison of subcortical actions of anticholinergic compounds. Progr. Brain Res., 28:14-26.

221. Wilson, C.W.M. (1965) The occurrence of circadian histamine rhythms in the rat. Int. Arch. Allergy, 28:32-34.

222. Wolf, D.E., Potter, T.L., Richardson, K.C. and Axelrod, J. (1962) Localized tritiated norepinephrine in sympathetic axons by electron microscopic autoradiography. Science, 138:440-442.

223. Wurtman, R.J. (1967) Ambiguities in the use of the term "Circadian". Science, 156:104.

224. Wurtman, R.J., Axelrod, J. and Chu, E.W. (1964) The relation between melatonin, a pineal substance and the effects of light on the rat gonad. Ann. N.Y. Acad. Sci., 117:228-230.

225. Wurtman, R.J., Axelrod, J., Sedvall, G. and Moore, R.Y. (1967-a) Photic and neural control of the 24-hour norepinephrine rhythm in the rat pineal gland. J. Pharmacol., 157:487-492.

226. Wurtman, R.J., Chou, C. and Rose, C.M. (1967-b) Daily rhythm in tyrosine concentration in human plasma: Persistence on low-protein diets. Science, 158:660-662.

227. Wurtman, R.J., Rose, C.M., Chuan Chou, M.S. and Larin, F.F. (1968-a) Daily rhythms in the concentrations of various amino acids in human plasma. New Engl. J. Med., 279:171-175.

228. Wurtman, R.J., Shoemaker, W.J., Larin, F. and Zigmond, M. (1968-b) Failure of brain norepinephrine depletion to extinguish the daily rhythm in hepatic tyrosine transaminase activity. Nature, 219:1049-1050.

229. Yarwood, C.E. and Cohen, M. (1949) Diurnal responsiveness of Erysipha graminisis to nutrients. Science, 110:477-479.

230. Zeman, W. and Innes, J.R.M. (1963) Craigie's Neuroanatomy of the Rat. Academic Press, New York.

APPROVAL SHEET

The dissertation submitted by Charles Andrew Walker has been read and approved by five members of the faculty of the Graduate School of Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

15 June 1969
Date

Signature of Advisor
A. H. Friedman, Ph. D.