CIRCADIAN VARIATIONS IN BRAIN SEROTONIN CONCENTRATION IN THE LIZARD *Anolis carolinensis*

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by
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ABSTRACT

Whole brain serotonin content and concentration was determined in male and female rats using a modification of the extraction procedure outlined by Quay (1963). The mean concentration in male rats was found to be 0.61 μg/g ± 0.222 (S.D.) and in female rats the mean was significantly higher, i.e. 0.89 μg/g ± 0.228 (S.D.)
The mean concentration of 5-HT in the whole brain of male lizards was found to be as follows: 5.01 μg/g ± 1.63 at 7:00 AM, 1.57 μg/g ± 0.935 at 12 noon, and 5.06 μg/g ± 1.33 at 4:00 PM. This indicates a definite circadian rhythm, with the minimum concentration occurring during the middle of the day.

INTRODUCTION

For the past hundred years it has been known that a vasoconstrictor substance is present in the blood serum. This compound was isolated and given the name serotonin by investigators at the Cleveland Clinic in 1948 (Rapport et al.). In 1949 Rapport determined that the active principle of this compound was 5-hydroxytryptamine (5-HT), an indoleamine derived from the amino acid tryptophan, and having the empirical formula $C_{10}H_{12}ON_2$: he recommended that the name serotonin be reserved for this indoleamine rather than for the complex. Serotonin has subsequently been shown to occur in the tissues of plants (Bowden et al., 1954), animals (Bogdanski et al. 1963), and in unicellular organisms (Welsh et al. 1960) indicating that it appeared early in evolution. The development of the spectrophotofluorometer (Bowman et al. 1955) has made possible the identification and assay of this substance.

In 1955 Bowman and his colleagues devised a method for the spectrophotofluorometric assay of a number of organic compounds including serotonin. Later that year, members of the same group further developed the technique so that it was possible to distinguish between 5-hydroxyindoles and other compounds such as tryptamine and tryptophan, which are also found in brain tissue. Members of this group then went on to develop a specific method for determination of 5-HT in brain (Bogdanski et al., 1956) which involved extraction with butanol at pH 10 and then making the extract 3N with HCl for fluorometric examination. Quay (1963) modified the method of Bogdanski by adding four steps of diethyl ether extraction, the last two being at pH 10. This removes interfering indole compounds such as N-acetyl serotonin, 5-methoxy tryptamine, bufotenine, and 5-hydroxytryptophan.

Lucasiewicz and Fitzgerald later (1973) developed a method in which fluorescence sensitivity is improved by increasing the intensity of the exciting radiation. A single source of polychromatic ultraviolet radiation is used to excite fluorescence and to initiate photochemical reaction. In dilute solution both fluorescence intensity and rate of photochemical reaction are dependent on the incident intensity, and pseudo first-order decay curves of fluorescence signal vs time are obtained. Initial fluorophor concentration can be measured by digital integration of the fluorescence signal for a fixed amount of time. Chemical pretreatment of the sample is not required, and native fluorescence of the fluorophor is used. The high precision and increased sensitivity make it possible to distinguish between very small concentration differences at parts per billion levels. This method was used in this present study to assay samples treated by a modification of Quay's procedure, as will be described under "Materials and Methods".

The finding that 5-HT existed in considerable quantities in the brain led to studies which established its role as a neurotransmitter. Gaddum (1953) showed that the molecular structure of lysergic acid diethylamide (LSD) is similar to that of serotonin, and that LSD inhibits the action of serotonin, but not that of oxytocin, on the rat uterus, possibly indicating that these two substances interact with each other within the brain. Welsh (1954) demonstrated that blocking the action of acetylcholine on the heart of a gastropod did not affect sensitivity to 5-HT, and he also showed that the response of the heart to acetylcholine was not affected by LSD or ergot alkaloids which block the action of 5-HT, suggesting different receptors for

acetylcholine and serotonin, and also that serotonin might be a neurotransmitter. Studies by Brodie et al. (1955) on the sedative reserpine, produced evidence that this drug acted through the liberation of 5-HT in the brain, and further supported the suggestion that 5-HT acts as a neurotransmitter and plays an important role in brain activity.

Many studies have been done to determine the role of serotonin in the central nervous system, and some of these have concerned themselves with various environmental factors such as stress, temperature, and light regimen on the 5-HT content of the brain, pineal, serum, etc.

Aprison et al. (1962) showed that the decreased pecking activity in pigeons produced by an increase in 5-HT in the body was a central nervous system rather than a peripheral effect. The 5-HT concentration in the telencephalon and diencephalon were directly related to changes in behavior, with the concentration of 5-HT at its maximum at the time of the depressed behavior and returning to normal at the same time behavior returned to normal. It was suggested that 5-HT might exert its effect on the limbic system and its associated nuclear structures.

Jouvet (1968) showed that 80-90% destruction of the raphe system of cats caused permanent wakefulness together with a significant decrease in brain 5-HT which correlated well with percent destruction of the raphé system and degree of insomnia. Pujol and his associates (1971) found that cats whose raphe system had been coagulated exhibited chronic insomnia in conjunction with a very much decreased synthesis of 5-HT.

Decreased sleep times have been reported in cats (13), rats (16), and monkeys (29) following the administration of para-chlorophenylalanine (pCPA), a specific 5-HT synthesis inhibitor, and it has been suggested (11) that 5-HT is necessary for the production of sleep, particularly slow-wave sleep. Johnson and his colleagues, however, (1972) contrasted the effects of pCPA with another drug, fenfluramine, which selectively depletes brain 5-HT. It was found, moreover, that the latter drug produced a sedative effect, increasing sleep time, and that 5-HT was not necessary for the sedative action of fenfluramine.

Bliss et al. (1972) subjected mice and rats to various stressful situations and found no change in 5-HT levels, but did find increases in 5-HIAA, a metabolite of 5-HT, indicating a higher turnover rate for serotonin.

Segura et al. (1967) showed that monoamine levels in the brain of the toad paralleled changes in EEG and body activities throughout the yearly cycle. Quay (1967) found no significant change in brain serotonin concentration over a 24 hour period in turtles kept at a constant temperature with an artificial daily photoperiod. Sheving and his associates, on the other hand, found a daily fluctuation in 5-HT content in the rat brain, it being highest during sleep and lowest during the period of peak activity, during the early part of the night. In 1972, Sheving and his colleagues also found that blood serum 5-HT levels fluctuate in a manner exactly opposite to that of brain levels, being highest during the early part of the evening when activity is highest and lowest during sleep. He included a study of several different environmental conditions which showed that exposure to ether and immobilization had a damping effect on the 5-HT rhythm, but that exposure to a novel situation had no effect.

Illnerova (1971) reported that 5-HT concentration in rat pineals shows a diurnal rhythm, reaching a maximum about 8 hours after the onset of light, and a minimum about 4 hours after the onset of darkness, and that it was possible to change this rhythm by artificial environmental lighting. It was also found that a stress agent, cold, did not cause any change in the rhythm.

In several papers, data on the serotonin content of reptile brains have been reported, but no data have been found for Anolis carolinensis, the common anole of wide distribution in the southern United States. Bogdanski, Bonomi, and Brodie (1963) reported brain 5-HT concentrations in many different animals such as mammals, birds, amphibians, fish, and one reptile species, the lizard S. cyanogens, in which it was found to be 3.1 µg/g of tissue. In 1964, Quay and Wilhoft reported an extensive study on serotonin concentration in the brains of 14 species of reptiles, with averages of 2.10-2.73 µg/g for members of the Order Chelonia, 2.44-2.73 µg/g for Ophidia, and 1.70-3.53 µg/g for the Order Squamata. These are similar to the value found in the earlier study by Bogdanski et al. A single specimen of alligator yielded a value of 0.25 µg/g.

A number of different authors have analyzed mammalian brains for both total and regional 5-HT concentration, and it appears that the 5-HT content of the reptilian brain is several times that of mammals. This finding is not surprising given the fact that most of the serotonin-producing neurons of the mammalian brain are localized in the raphe nuclei of the lower midbrain and upper pons. These are an old group of structures, phylogenetically, and therefore constitute a larger percentage of the brain weight in reptiles than in mammals. (27, 33)

It was decided that it would be interesting to determine whether there was a daily rhythm in brain serotonin concentration in a reptile, and the lizard, *Anolis carolinensis*, was chosen because of availability, and also because of its known responsiveness to light.

Since the amount of brain tissue obtainable from the lizards was very small, the extraction technique was first tested on rats in order to be sure that the values obtained were comparable to those indicated in previous studies.

MATERIALS AND METHODS

Anolis carolinensis is one of the more common lizard species found in the southeastern United States, and the only species commonly found in cities. They are generally found on trees, in low vegetation, and on buildings, and are capable of climbing on vertical surfaces because of specially adapted pads on the feet. These animals are capable of changing color, appearing brown when the chromatophores are expanded due to direct light or other factors such as low temperature, while a green color is generally induced by such factors as darkness, excitement, exposure to pure green light, and at death. The males possess a prominent red throat fan which they display during courtship or fighting. Eggs, usually 2 in number, are laid in June or July and hatch in 6-7 weeks. The lizards are insectivores, serving a useful function in the control of mosquitoes and garden pests. (26)

Anoles possess a parapineal body which arises as an outgrowth of the pineal, and it is well developed to form a small, median eyelike structure, the "pineal eye". (32). This organ probably helps to mediate various responses to light and shadow. These animals are diurnal, beginning their movements when the temperature has risen in late morning and are most active during the middle of the day.

The lizards for this study were collected from their natural habitat and kept in a glass aquarium in a sunny window, the sides facing the room being covered to prevent accidental exposure to artificial lighting during the dark period. They were fed live insects and given water which they drank from drops adhering to leaves of plants placed in the aquarium.

Male lizards were sacrificed by decapitation at three different times of day; at about sunrise (7:00 AM DST), at noon (DST), and in mid-afternoon (4:00 PM DST). The heads were then frozen at -15°C to preserve them until used. On the day of the assay, the brains were removed from the heads as quickly as possible, while they were still frozen. Brains for each time period were assayed separately. Each of the three groups contained 12 animals with an average body weight of 4.7g and average brain weight of 0.046g. The samples were treated as described below and the final aqueous phase was adjusted to 100 ml by the addition of distilled water. This was divided into 4 samples of 25 ml each, which were read separately in the spectrophotofluorometer.

The rats used to test the extraction technique were also killed by decapitation, during the afternoon, and the heads frozen until used.

Materials used for extraction of serotonin from brain tissue were as follows: HCl, EDTA, NaCl, diethyl ether, n-butanol, heptane, and pH 10 buffer prepared from boric acid, NaOH, and water as described by Quay (18). The method used was based upon Quay's modification of the procedure originally outlined by Bogdanski et al. (3) for assay of serotonin. It was necessary to treat the brain homogenates with diethyl ether to remove several of the tryptophan derivatives which would otherwise have interferred with serotonin assay. It was found that when a portion of the pooled brain homogenates from one male and one female rat were not treated with ehter but were put through the remainder of the extraction process, they showed an increased fluorescence equivalent to 1.18 μg/g of serotonin as compared to the ether treated portion. Since the extraction removes at least 60% of the interfering substances, results were corrected accordingly.

(Figure 2). It was also found that during the extraction procedure significant amounts of a fluorescing compound were extracted from heptane into the aqueous phase along with serotonin. (Figure 1). Again, results were corrected accordingly. It was found that to obtain the best extractions of serotonin it was necessary to shake the two phases together in an automatic shaker for at least 5 minutes (Figure 3).

Fluorescence was measured using a new digital integration method developed by J.M. Fitzgerald of the University of Houston Chemistry Department, and R.J. Lukasiewicz (14). Since the sensitivity of this instrument is far greater than that of the conventional spectrophotofluorometer, it was possible to extract several times for serotonin using much larger volumes than those given in the Quay procedure. As a consequence, efficiency of the extraction was very high, being more than 95%.

After removal of the brains from the frozen heads of the animals, the tissue was weighed and homogenized in a glass homogenizer in twice its weight of 0.1N HCl. The homogenate was then centrifuged, and the supernatant removed and washed with 3 ml. ether to remove N-acetyl serotonin. To remove 5-methoxy tryptamine, 5-hydroxytryptophan and bufotenine the aqueous phase was then treated as follows:

1. Add 1 ml. EDTA, 500 mg. NaCl and 3 ml. ether, shake 1 minute and discard ether.
2. Add another 3 ml. ether, shake 1 minute and discard ether phase.
3. Add 1 ml. pH 10 buffer and 3 ml. ether, shake 1 minute and discard ether phase.
4. Add 5 ml. n-butanol, shake 5 minutes, take aqueous phase, extract again with another 5 ml. of butanol, then discard aqueous phase.

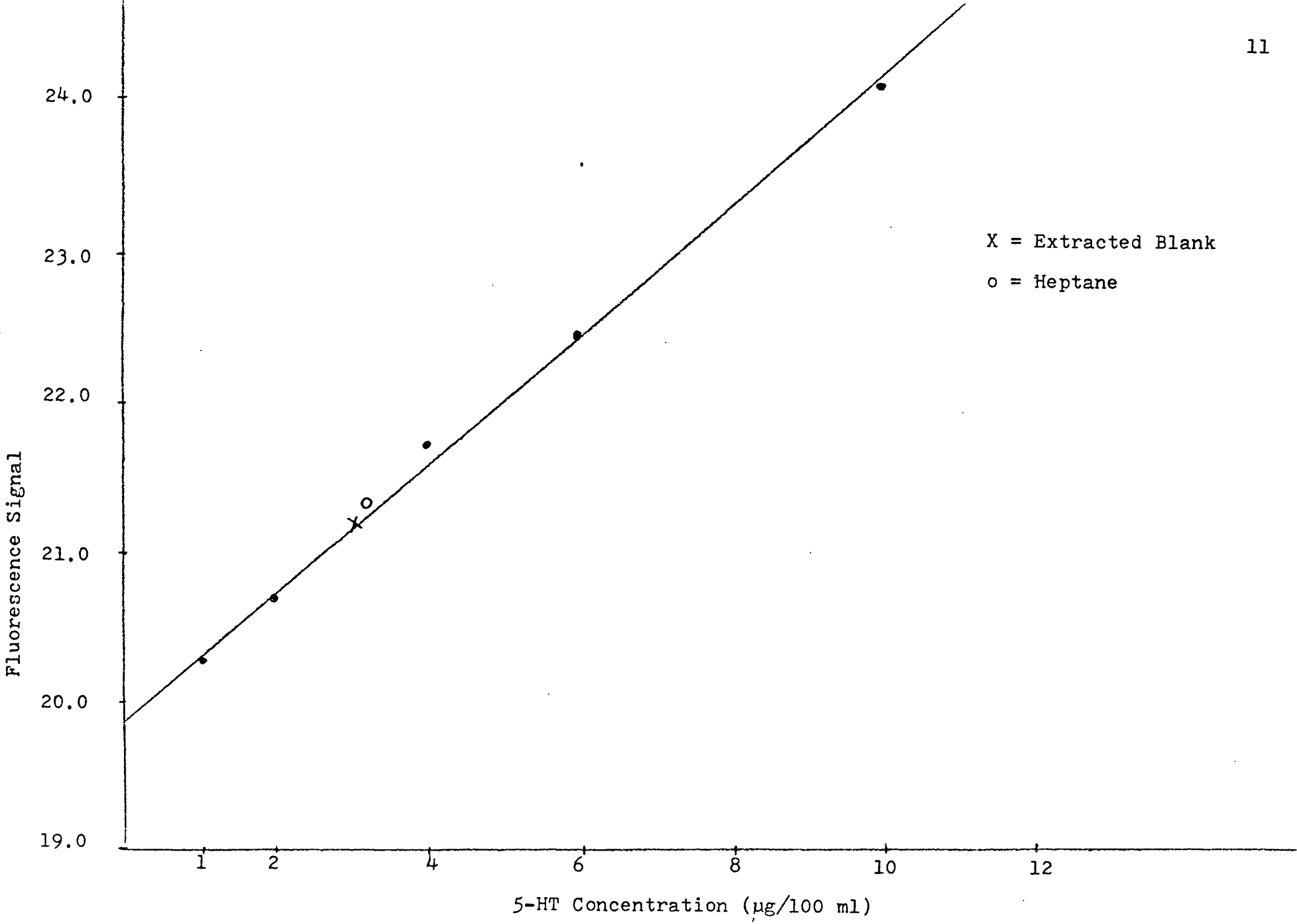

24.0
23.0
22.0
21.0
20.0
19.0
Fluorescence Signal
X = Extracted Blank
o = Heptane
1
2
4
6
8
10
12
5-HT Concentration (µg/100 ml)

Figure 1

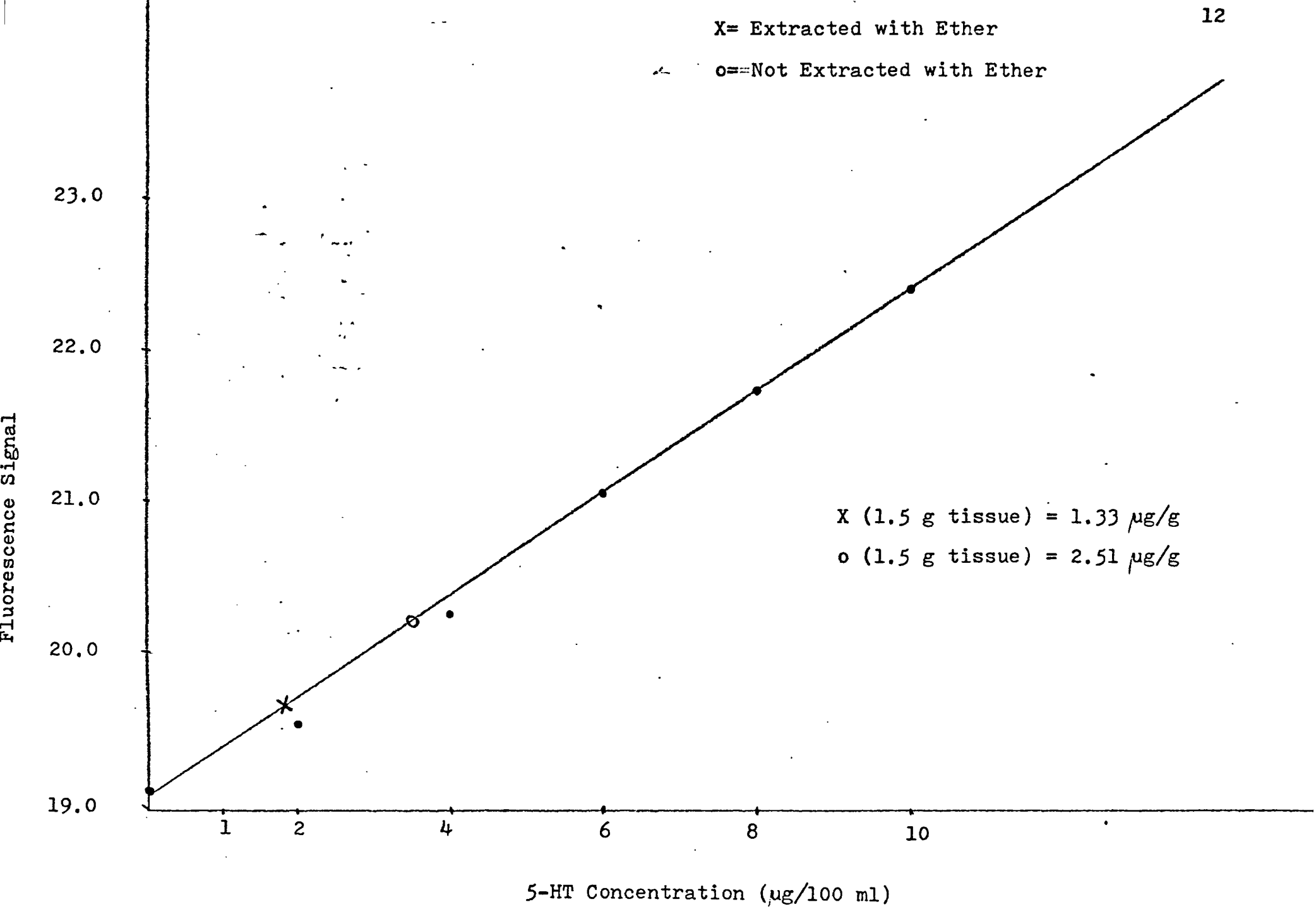
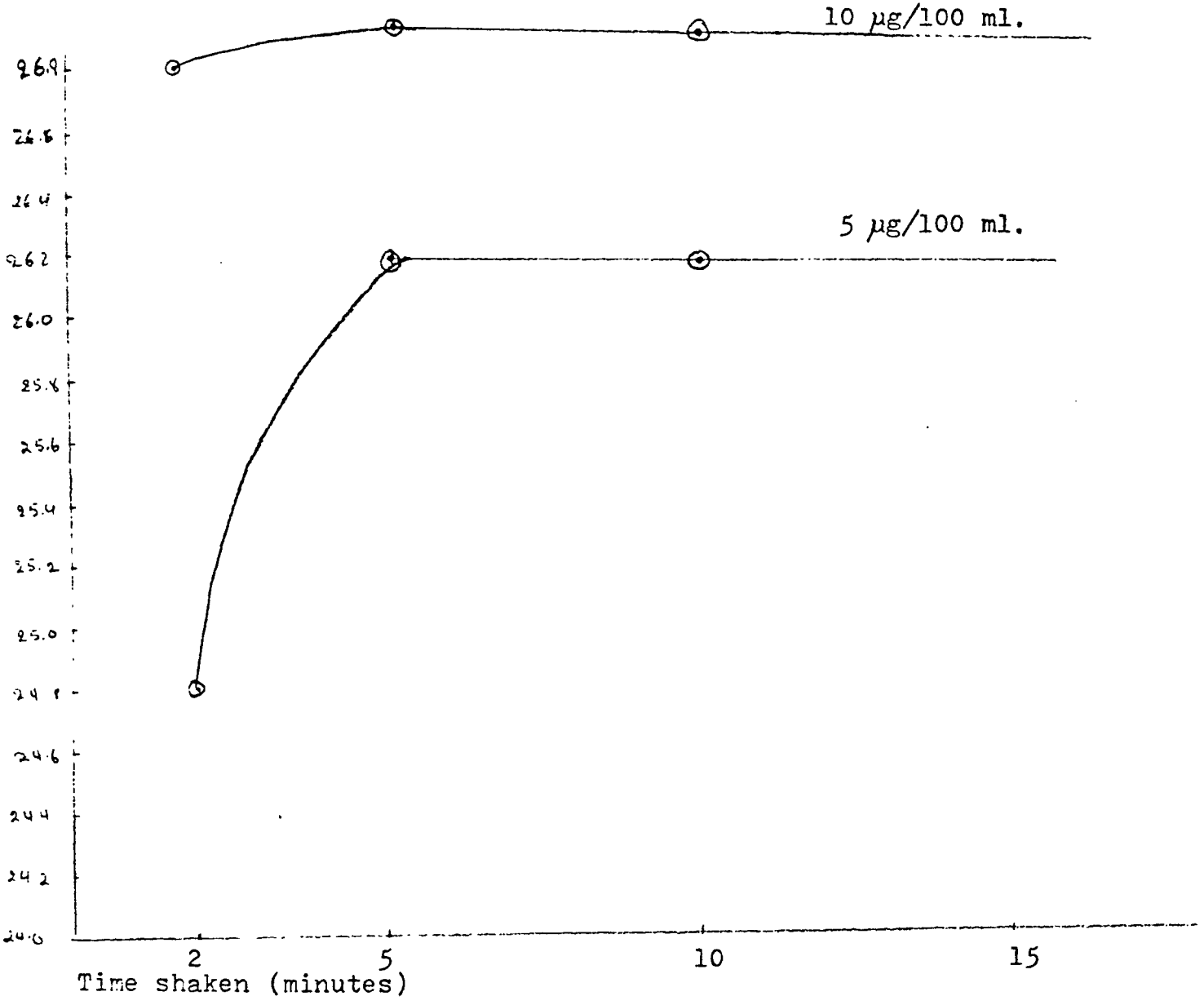

X= Extracted with Ether
o=Not Extracted with Ether
X (1.5 g tissue) = 1.33 µg/g
o (1.5 g tissue) = 2.51 µg/g
Fluorescence Signal
23.0
22.0
21.0
20.0
19.0
1
2
4
6
8
10
5-HT Concentration (µg/100 ml)

Figure 2



EFFECT OF SHAKING TIME ON EXTRACTION OF SEROTONIN

Figure 3

5. Add butanol to a 125 ml. flask containing 10 ml. heptane and 30 ml. 0.1N HCl, shake 5 minutes, extract butanol phase twice more with 30 ml. 0.1N HCl and discard butanol.
6. Add distilled water to aqueous phase to make 100 ml. and read fluorescence at 355 nm.

RESULTS

The 5-HT concentration in male rats was found to average 0.61 ± 0.222 µg/g wet weight of tissue, and in female rats the average was found to be 0.89 ± 0.228 µg/g. A pooled sample containing one male and one female brain was found to contain 0.59 µg/g (See Table 1).

The brain samples from male lizards were found to have the following average total serotonin contents (See Table 2): At 7:00 AM, a pooled sample weighing 0.56g contained 2.81 µg; at 12 noon, a sample weighing 0.55g contained 0.87µg; and at 4:00 PM, a sample weighing 0.55g contained 2.78 µg. Therefore, the average serotonin concentration in ug/g wet weight of brain tissue was as follows: 5.01 µg/g at 7:00 AM, 1.57 µg/g at 12 noon, and 5.06 µg/g at 4:00 PM. (See Figure 4 and Table 3).

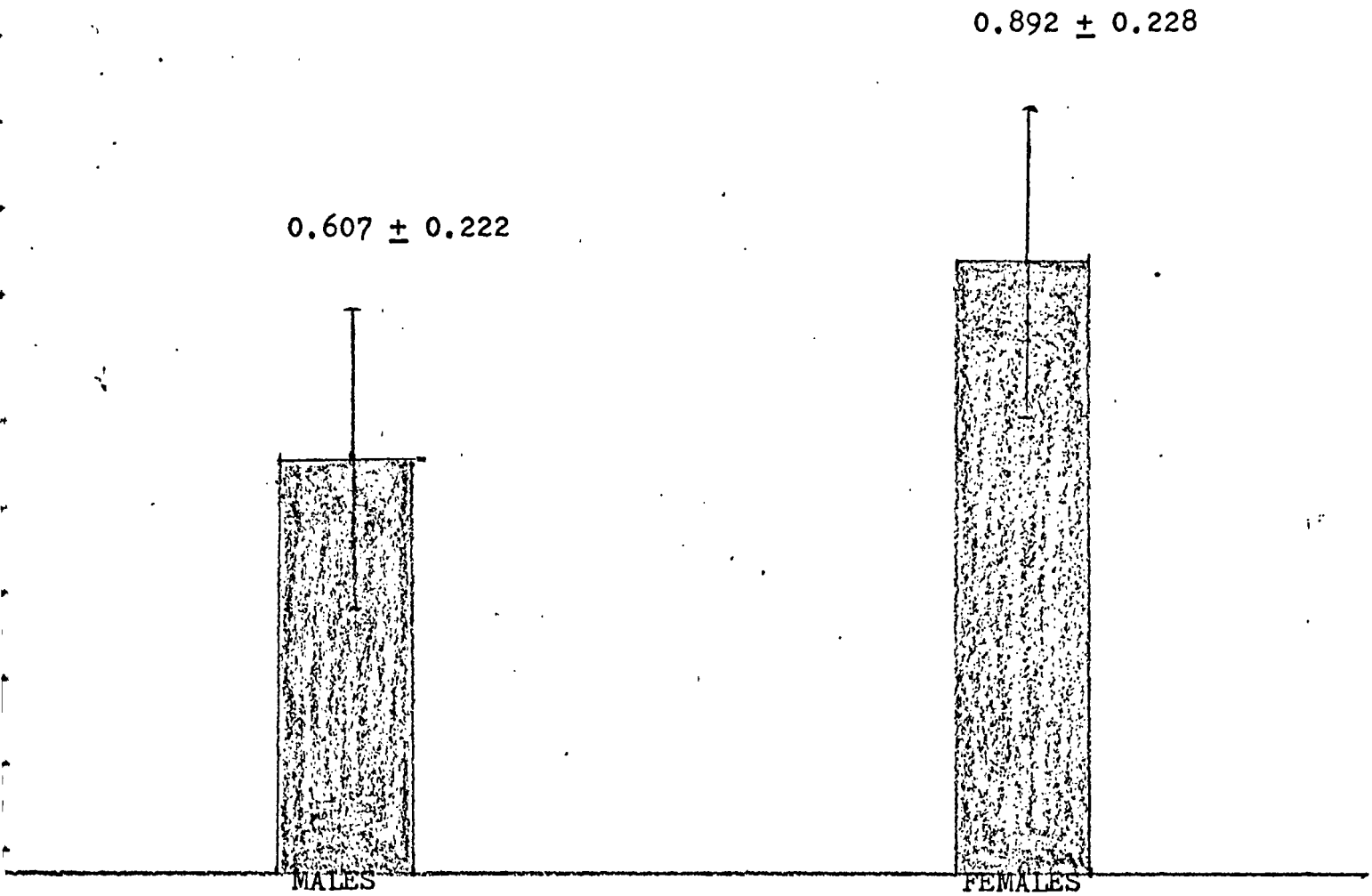
TABLE 1

CONCENTRATION OF SEROTONIN IN WHOLE BRAIN OF RATS ($\mu g/g$ wet weight)

MALE RATS (8)	FEMALE RATS (12)
0.70	1.36
1.11	1.02
0.43	0.80
0.60	0.85
0.42	0.47
0.50	0.70
0.60	0.85
0.50	0.80
	0.86
	1.20
	0.95
	0.84
Average = 0.607 $\pm$ 0.222	Average = 0.892 $\pm$ 0.228

Pooled sample containing brains from 1 male and 1 female rat = 0.59

Significance of differences calculated using student's t test = .02



AVERAGE SEROTONIN CONCENTRATION IN THE WHOLE BRAIN OF MALE AND FEMALE RATS
(μg/g)

FIGURE 4

TABLE 2

TOTAL SEROTONIN CONTENT OF WHOLE BRAIN SAMPLES FROM THE LIZARD *Anolis carolinensis* KILLED AT DIFFERENT TIMES OF DAY (12 males per sample) GIVEN IN μg.

Time	7:00 AM (DST)	12:00 Noon (DST)	4:00 PM (DST)
Weight of Tissue	0.56 g	0.55 g	0.55 g
	2.00	1.40	2.20
	3.25	0.40	3.05
	3.80	1.25	3.75
	2.10	0.45	2.25

TABLE 3

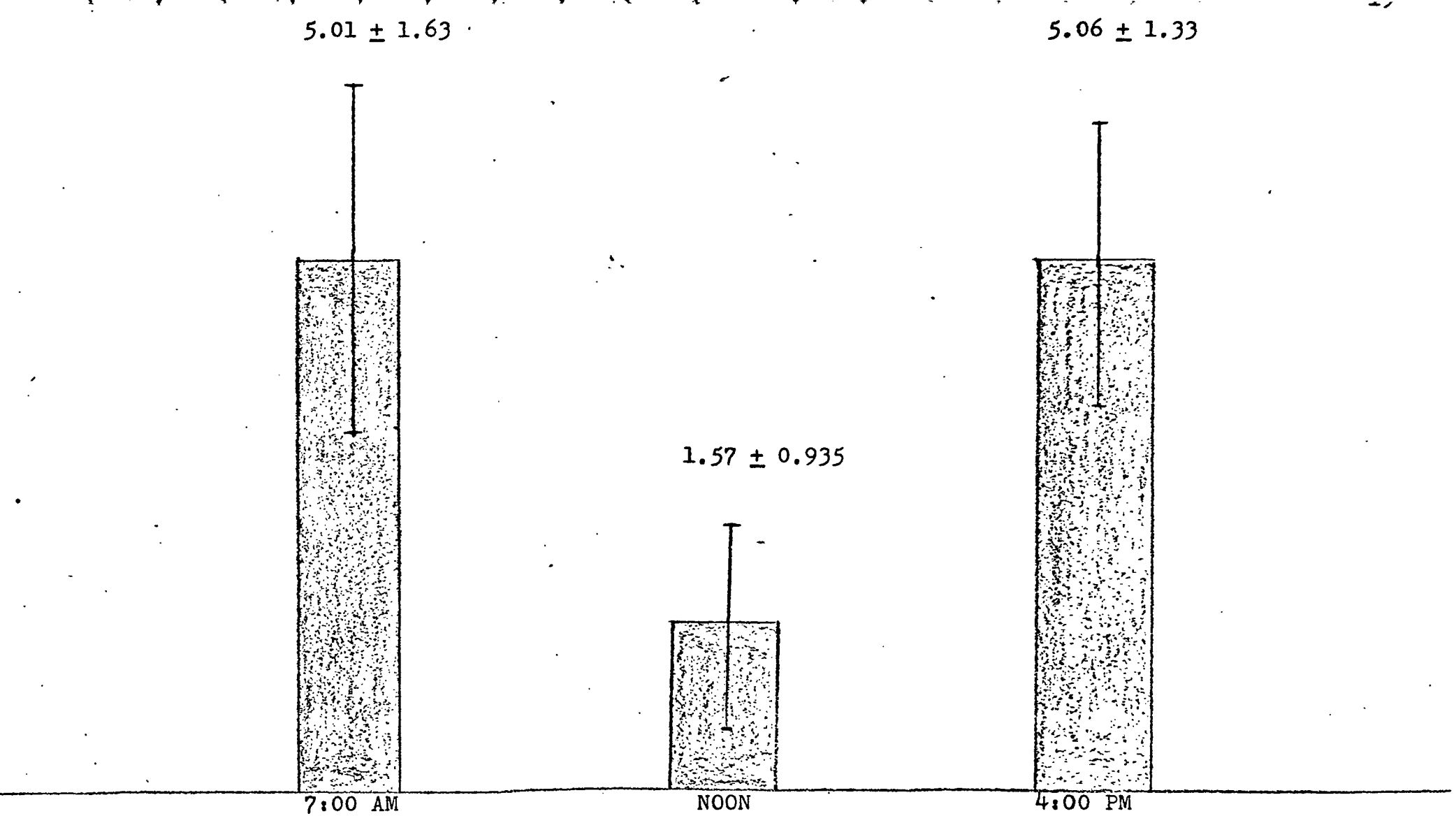
SEROTONIN CONCENTRATION GIVEN IN μg/g WET WEIGHT OF TISSUE

Time	7:00 AM (DST)	12 Noon (DST)	4:00 PM (DST
	3.60	2.52	3.96
	5.85	0.72	5.49
	6.84	2.23	6.75
	3.78	0.81	4.05
Average	5.01 ± 1.63	1.57 ± 0.935	5.06 ± 1.33

Significance of difference between 7:00 AM and Noon = .02

Significance of difference between 4:00 PM and Noon = .02

(Calculated using student's t test)



CONCENTRATION OF SEROTONIN IN THE WHOLE BRAIN OF THE MALE LIZARD (Anolis carolinensis)

AT THREE DIFFERENT TIMES OF DAY

(μg/g)

FIGURE 5

DISCUSSION

In rats, the values found for both sexes were close to those cited in the literature, being 0.61 μg/g for the males and 0.89 μg/g for the females. The mean concentration for the females was significantly higher than that for the males, which agrees with the findings of Ladosky and Gaziri (1970). Their study also reported that in rats under 8 days old there were no sex differences in brain 5-HT concentration, but that after that the female level was higher than that of the male. They suggest that a possible explanation for these sex differences in brain 5-HT concentration might be an increased ability of the female to synthesize or conserve 5-HT in the brain, and they have proposed that testosterone may act on the serotonin metabolism to lower its concentration in the brain. They found that neonatal castration of males resulted in higher serotonin levels, comparable to those of the females.

Another possible cause of the sex differences might conceivably be the fact that the average body weight of the female rats was slightly less than that of the males. It has been shown that smaller animals have a higher brain serotonin concentration than medium and large sized animals of the same species (Baker & Hoff, 1972). In this case, this explanation would seem unlikely because of the rather great individual variation and overlap between body weights of male and female animals, and also because the differences in average weight are too small to account for the marked differences in brain 5-HT content.

A pooled sample of one male and one female rat brain yielded a value of 0.59 μg/g, and the fact that this is lower than the average concentration for either male or female animals assayed separately may be

attributable to the fact that this assay was carried out at a later date than the others, and the brain tissue had been frozen for nearly two months at that time. It is possible that some loss of serotonin content had occurred. Thyon Rujirekagulwat, however, in his thesis entitled "Brain Serotonin Content and Concentration in the Siamese Crocodile, Crocodylus siamensis" (1973) states that it is possible to keep whole frozen heads of animals for relatively long periods of time without any appreciable loss of serotonin content. Thus, a more likely explanation is individual variation and chance selection within the population: that is the range of values for the females varied from 0.48 μg/g to 1.36 μg/g and that for the males varied from 0.42-1.1 μg/g.

The whole brain 5-HT concentrations found in the lizard Anolis carolinensis varied from 1.57 μg/g at 12 noon to 5.06 μg/g at 4:00 PM. These values are about two to six times those found in the rat, and thus in agreement with the findings of other workers (4, 19). These studies show that animals lower in the phylogenetic scale have higher brain 5-HT concentrations, amphibians having the highest values followed by reptiles, birds, and mammals in order of decreasing concentration. Possible explanations for this phenomenon might include a higher capacity to synthesize or store 5-HT in the brain of lower vertebrates, or poikilothermal vs homiothermal factors. The fact that relatively low concentrations are found in birds as well as mammals would support the latter suggestion. In addition, Quay et al. (1970) suggested that the daily changes observed in 5-HT content of the brain represent, or contribute to, a central temperature compensating mechanism. The most obvious explanation, however, would be that in animals lower in the phylogenetic scale the brainstem constitutes a larger percentage of the total brain

weight. Since most of the serotonin-producing neurons are located in this region, and since this is where the greatest amount of serotonin is stored, this would account for the larger whole brain serotonin concentration of lower animals.

The results of the experiment carried out on the lizards indicate a circadian rhythm in the levels of serotonin in the whole brain. The fluctuation is relatively large, the values in the morning and in the afternoon being more than three times that found at noon.

Previous studies on rats (24, 25) have demonstrated that whole brain content of 5-HT shows a circadian cycle, being highest during the middle of the light period and lowest during the early part of the dark period. Since rats are nocturnal, the period of peak serotonin concentration corresponded to the time of sleeping, whereas the time of low concentration corresponded to the period of peak activity. Because the anoles are diurnal, it was not surprising to find that the concentration of 5-HT was relatively high in the early morning before the animals had begun their daily activities, and to find that it had dropped considerably by the middle of the day when activity was at its peak. The fact that the concentration at 4:00 PM was identical to that of the early morning seems to indicate that the serotonin level rises quickly after reaching a minimum value at about noon. This would be consistent with the hypothesis that brain 5-HT content is related to the sleep-activity cycle if the lizards were most active during the late morning and became less so as the afternoon progressed. Observation of behavior of these animals during the period in which the specimens for the study were being collected

did not consistently indicate that the anoles' activity decreased in the afternoon, as many specimens were found after 5:00 PM (DST), and as late as 7:00 PM (DST) during the month of March. Nonetheless, the majority of the lizards were found between 10:00 AM and 2:00 PM (DST) and this would seem to be the period during which the greatest number of animals are active and feeding. Thus there is evidence for a synchronization of brain serotonin content with both the light-dark cycle and also with the animals' daily activity cycle.

In 1970 Quay et al. observed that changes in brainstem and cerebral 5-HT and monoamine oxidase (MAO) in the lizard Sceloporus occidentalis appeared to be related both to time of day and to temperature, and to have different patterns of change on cold and warm days. On the cold days, the 5-HT concentration rose during the afternoon and fell towards the end of the afternoon, whereas on warm days 5-HT fell during the period from 11:00 AM to 1:00 PM and rose again thereafter. They also found peak concentrations to be different on warm and cold days. The lizards used in this study were kept at a fairly warm temperature (25°C or higher), and therefore the results obtained are consistent with those found by Quay and his associates on warm days. He suggested that these daily and temperature-related changes in lizard brain 5-HT content represent, or contribute to a central temperature compensating mechanism. In this mechanism it is hypothesized that during cooler days the changes in 5-HT may facilitate response by 5-HT receptor neurons, and during warmer days the changes in 5-HT may favor a relative inhibition of such neurons.

An interesting fact observed during the collection of the lizards for this study was that the number of males found was about twice that of females. It is possible that the males spent more time in the open than did the females, or else that there was simply a ratio of about two males for every female in the population.

SUMMARY

Whole brain serotonin content and concentration was determined in male and female rats using a modification of the extraction procedure outlined by Quay (18). The average concentration in male rats was found to be 0.61 µg/g and in female rats the average was 0.89 µg/g. The mean concentration for the females was significantly higher than that for the males. A possible explanation for this sex difference might be an increased ability of the female to synthesize or conserve 5-HT in the brain, and this may be due to the action of the male sex hormone testosterone on serotonin metabolism to reduce its concentration in the male brain.

Male lizards of the species Anolis carolinensis were collected from thei natural habitat and whole brain 5-HT content and concentration were determined by the same method as that used for the rats. The lizards were killed at three different times of day; 7:00 AM,(DST), 12 noon (DST) and 4:00 PM (DST). The values found were 5.01 µg/g at 7:00 AM, 1.57 µg/g at noon, and 5.01 µg/g at 4:00 PM. These values are about two to six times those found in the rat, and this is in agreement with th findings of many workers showing that animals lower in the phylogenetic scale have higher brain 5-HT concentrations. These results also indicate a circadian rhythm in the levels of serotonin in the whole brain with a relatively large fluctuation, the morning and afternoon values being more than three times that found at noon. This could be correlated with the animals' daily activity cycle and with the light-dark cycle. It has also been suggested by Quay et al. (1970) that these daily changes are also related to temperature and that they represent, or contribute to, a central temperature compensating mechanism.

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