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Title: The Effects of Melatonin and Isoproterenol on Estrous Cyclicity in Golden Hamsters

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ABSTRACT

The effects of melatonin and isoproterenol injections on estrous cyclicity, the ovarian, adrenal and pineal gland weights, and on locomotor activity were examined in Syrian hamsters maintained in constant light (LL). Isoproterenol effects on the same parameters also were studied by injections 15 minutes before lights-out in hamsters maintained on a 14 hour photoperiod (14L:10D). Melatonin (25 ug), isoproterenol (0.2mg), or oil-control (0.1 ml) injections were administered daily, between 1930-2100 EST, except in two groups which also received daily, morning injections of melatonin or oil at 0700 EST.

Single-melatonin injections, and single- or double-oil injections had no significant effects on estrous cyclicity, organ weights or locomotor free-running activity. Double injections of melatonin, however, induced estrous acyclicity in 66.7% of those hamsters and severely depressed uterine weights, as well. Double-melatonin injections did not affect the locomotor activity since the free-running rhythm was near 24 hours. Isoproterenol injections in LL-exposed hamsters, in contrast, induced persistent estrus in two groups of animals. Conversely, isoproterenol injections in animals maintained in 14L:10D induced acyclicity and marked uterine atrophy.

The present findings suggest that LL affects the hamster in a manner similar to pinealectomy since single-melatonin injections neither induced estrous acyclicity nor entrained locomotor activity. Injections of isoproterenol in LL-exposed animals are postulated to affect the luteinizing-hormone cyclic center in the anterior hypothalamus, because of the induction of persistent estrus. In contrast, isoproterenol treatment in 14L:10D-exposed animals appears

to suppress gonadal function by inducing melatonin synthesis. The daily double-melatonin injections are theorized to be antigonadal due to the "melatonin rhythm" established by the protocol employed in the hamsters to LL. The results of this study further support the hypothesis that melatonin is the antigonadal hormone of the pineal gland.

THE EFFECTS OF MELATONIN AND ISOPROTERENOL ON ESTROUS CYCLICITY IN GOLDEN HAMSTERS

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by

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INTRODUCTION

The rodent pineal gland (epiphysis ceribri), which lies between the posterior lobes of the cerebral hemispheres in a supra-callosal position, is embryologically derived from ectodermal tissue of the neural crest. Shortly after birth the rodent pineal loses all direct innervation from the brain and the pineal parenchymal cells appear to be innervated solely by sympathetic fibers arising from the superior cervical sympathetic ganglia (Kappers, 1960).

In 1958, Lerner et al., isolated a substance from bovine pineal glands that lightened the skin of larval amphibians and inhibited melanophore stimulating hormone. They suggested the compound be named melatonin and characterized its structure the following year (Lerner et al., 1959).

The biosynthetic pathway for pineal melatonin production is delineated in figure 1. The amino acid tryptophan is taken up from the circulation by the pineal parenchymal cells (Axelrod et al., 1969) and is either incorporated into protein or converted to 5-hydroxytryptophan (Wurtman et al., 1969) by the enzyme tryptophan hydroxylase (Lovenberg et al., 1967). The 5-hydroxytryptophan is decarboxylated to form 5-hydroxytryptamine (serotonin). This reaction is catalyzed by aromatic-L-amino acid decarboxylase (Snyder and Axelrod, 1964). Most of the serotonin produced is deaminated by monoamine oxidase to form 5-hydroxyindoleacetic acid (Wurtman, 1967). Part of the non-deaminated serotonin leaves the pinealocytes and is taken up by the sympathetic nerve terminals to the gland where it is stored with norepinephrine (Axelrod, 1974), while the remainder of

Figure 1. Biosynthesis of melatonin.

Legend:

1 = Tryptophan hydroxylase
2 = L-amino acid decarboxylase
3 = N-acetyl transferase
4 = Hydroxyindole-O-methyltransferase

AcCoA, acetyl coenzyme A
CoA, coenzyme A
SAM, S-adenosyl methionine
SAH, S-adenosyl homocysteine

serotonin is converted to melatonin in the pinealocytes by two enzymatic reactions. The first reaction is catalyzed by the enzyme serotonin N-acetyltransferase (NAT) (Weissbach et al., 1960) and involves the addition of an acetyl group to serotonin, forming N-acetyl serotonin. In the second reaction, a methyl group is transfered from S-adenosylmethionine to the 5-hydroxy group of N-acetyl serotonin to form melatonin. This reaction is catalyzed by hydroxyindole-O-methyl transferase (HIOMT) (Axelrod and Weissbach, 1961). Melatonin can be degraded by melatonin hydroxylase in the liver to form 6-hydroxymelatonin which is conjugated with sulfate and excreted in the urine (Kopin et al., 1961).

Evolutionarily, the pineal apparently functioned as a median eye in early primitive vertebrates. Modern amphibian pineal glands retain some photoreceptor capabilities (Eakin, 1961), although they may function as endocrine glands as well (Bagnara, 1960), since amphibian pineal glands contain melatonin and the biosynthetic machinery necessary to produce it (Axelrod et al., 1965). Reptilian pineal glands, in contrast, have a reduced number of sensory nerve cells and sensory fibers passing into the pineal tract (Kappers, 1971). While the pineal in some birds may retain a rudimentary photosensory function (Kappers, 1971), the avian pineal is probably innervated solely by sympathetic nerves (Hedlund, 1970).

The mammalian pineal has lost its photoreceptor function and has become a solid parenchymatous secretory organ (Wolfe, 1969). The current theory of mammalian pineal function holds that the gland acts as a neuroendocrine transducer, receiving nerve impulses from the sympathetic nerves and translating the message into a hormonal

output, probably melatonin (Wurtman, 1969; Axelrod, 1974; Cardinali, 1974).

Although the mammalian pineal no longer functions as a direct photoreceptive organ, its action appears to be stimulated by light input which serves as the factor controlling activity of the sympathetic nerves. The pathway of photic stimulation of the rat's pineal is initiated by light stimulation of the retina which signals the release of neural impulses to the inferior accessory tract (Moore et al., 1967), which transmits the impulses through the median forebrain bundle to the medial terminal nucleus of the accessory optic system (Moore et al., 1968). The impulses then are transmitted to the superior cervical ganglia via the preganglionic sympathetic tract of the spinal cord (Moore et al., 1968). Finally, the impulses are carried by the postganglionic sympathetic fibers to the parenchymal cells of the pineal (Kappers, 1960). A recent report, however, indicates that the retinal impulses may travel to the suprachiasmatic nucleus via retinal-hypothalmic projections rather than to the inferior accessory optic tract before reaching the median forebrain bundle (Klein, 1978).

That the photic input to the pineal is important to pineal function was demonstrated by many investigations of the effects of photoperiod on the pineal-gonadal axis. Fiske, et al. (1960) reported that rats became persistently estrus when continuously exposed to light. Rats exposed to constant light (LL) also exhibited a significant increase in ovarian weight. Pinealectomized rats exposed to a normal 14L:10D (Light:Dark) lighting regime showed comparable increases in ovarian weight. When pinealectomized rats were exposed

to LL the ovarian weights were found to be similar to those of the intact rats rather than exhibiting an additive effect (Wurtman et al., 1961). Those results suggested that both exposure to LL and pinealectomy exerted their effects through melatonin suppression. Wurtman et al., (1963) subsequently found that daily melatonin injections could reverse the inhibitory effects of LL on estrous cyclicity in the rat.

The finding that differing lengths of environmental photoperiods greatly affected the reproductive system of rats led to investigations of the rhythmicity of pineal biochemical activity. In 1963, Quay reported a daily rhythm of serotonin in the pineal of rats exposed to a normal lighting schedule. Rats killed during the light period had pineal serotonin levels 2-3 times greater than animals killed during the dark period. Subsequently, Fiske and Huppert (1968) reported that maximal concentrations of serotonin in the rat were reached after 8 hours of light, after which the levels rapidly decreased at the onset of darkness, reaching a minimum level four hours after the onset of darkness. Snyder et al. (1965) found that the serotonin rhythm was maintained in blinded rats, as well as in those exposed to constant darkness. However, exposure of rats to constant light resulted in high, constant levels of pineal serotonin with a loss of the serotonin rhythm. The cycle was also abolished by removal of both superior cervical ganglia (Snyder et al., 1965). The serotonin rhythm could be reversed 180^{o} by reversing the light regimen, indicating synchronization of the rhythm with environmental light (Snyder et al., 1967). Circadian rhythms also have been found in pineal NAT (Lynch, 1971), and most recently, tryptophan hydroxylase (Shibuya et al., 1978).

Klein and Weller (1970) found that the pineal NAT activity increases 15-40 times in the dark. The NAT rhythm is almost conpletely abolished by ganglionectomy or decentralization of the superior cervical ganglia (Klein et al., 1971). The NAT rhythm probably free-runs in blinded rats and in rats exposed to constant darkness, increasing during free-running locomotor activity (Ralph, 1976). Exposure to constant light, however, suppresses the NAT rhythm and results in low levels of NAT (Klein et al., 1971). The circadian rhythm of NAT activity is 180^{o} out of phase with that of serotonin (Brownstein et al., 1973). This suggests that NAT may serve as a regulatory step in the synthesis of melatonin. In a recent review, Brownstein (1975) stated that the cyclic changes of NAT activity are apparently responsible for the cyclic changes in serotonin, N-acetyl serotonin and melatonin.

Brownstein and Axelrod (1974) reported a 24 hour turnover in the release of norepinephrine by the sympathetic nerves innervating the pineal. In the same investigation it was found that the rate of norepinephrine turnover in blinded and intact rats was identical during the day and night. Exposure to constant light destroyed the rhythm. Organ culture studies of the rat's pineal gland have been employed in order to assess the effects of norepinephrine on melatonin synthesis. In 1969, Axelrod, Shein and Wurtman added L-norepinephrine to pineal organ cultures of the rat containing ^{14}C tryptophan and found a three-fold increase in the amount of ^{14}C melatonin formed, when compared to a control. Other compounds tested, including serotonin, melatonin and 5-hydroxyindole acetic acid had no stimulatory effect on ^{14}C melatonin sythesis. Klein

et al. (1971) showed that pineal gland NAT activity is dependent on the release of norepinephrine from the sympathetic neurons innervating the pineal. When cycloheximide, an inhibitor of protein synthesis, was added to the organ culture ^{14}C melatonin synthesis was completely blocked, indicating that the synthesis of a new enzyme was obligatory for the formation of melatonin (Axelrod et al., 1969). The requirement for new protein synthesis was confirmed in 1975, when Romero, Zatz and Axelrod showed that NAT induction by norepinephrine release was mediated by 3' - 5'cyclic adenosine monophosphate (cAMP) and had a lag period which was dependent on RNA synthesis.

Once norepinephrine is released from the sympathetic neurons it stimulates beta-adrenergic receptors in the pinealocytes (Delguchi and Axelrod, 1972). The beta-adrenergic response appears to be mediated by cAMP since the addition of dibutyryl cAMP to pineal organ cultures of the rat resulted in a 6-10 fold increase in NAT activity (Klein et al., 1970). The increase found in NAT activity was not blocked by propranolol, a beta-adrenergic blocking agent, indicating that the action of dibutyryl cAMP occurred at some point after the stimulation of the beta-adrenergic receptor (Delguchi and Axelrod, 1973). In 1970, Ebaldi et al. found that rats killed at the end of the light period had 6 times more cAMP in the pineal gland than those killed at the end of the dark period. Blinded rats, in contrast, showed no increase in pineal concentrations of cAMP. Minnerman and Iverson (1976) reported a diurnal cycle of phosphodiesterase, the cAMP hydroxylating enzyme, in the rat's pineal gland, indicating that phosphodiesterase activity may serve to regulate changes in pineal cAMP and, therefore, NAT concentrations. The

addition of theophylline, a compound that inhibits phosphodiesterase, to pineal organ cultures of the rat increased the induction of NAT by norepinephrine and isoproterenol, a beta-adrenergic agonist (Stroda et al., 1972). The normal nocturnal rise in NAT activity in rats was abolished by l-propranolol, cycloheximide and reserpine, a compound that depletes norepinephrine from the sympathetic nerves (Delguchi and Axelrod, 1972). Treatment of rats with the beta-agonist, l-isoproterenol, during the latter part of the day initiated a 60-fold rise in NAT activity. L-isoproterenol administered to animals maintained in LL also produced a 25- to 60-fold increase in NAT activity after a 60 minute lag period (Delguchi and Axelrod, 1972b). Superinduction of NAT activity was demonstrated in ganglionectomized rats which exhibited a 100-fold increase in pineal NAT activity within 24 hours of the operation when treated with l-DOPA, as compared to a 40-fold increase in NAT activity in similarly-treated intact rats. Isoproterenol injections into rats that were either pineal-denervated or treated with reserpine produced a large induction in NAT when compared to the NAT induction found in control rats with innervated pineals (Delguchi and Axelrod, 1973). When the pineals of rats treated the isoproterenol were placed in organ cultures, the addition of low doses of isoproterenol resulted in reduced NAT induction, causing the glands to become subsensitive (Romero and Axelrod, 1974). Those investigators also postulated that the diurnal changes in pineal sensitivity to NAT induction were due to the degree of stimulation received by the adrenergic receptor. The decrease in sympathetic activity, which occurred during the day, resulted in increased pineal sensitivity to NAT induction after treatment with isoproterenol (Romero and Axelrod, 1975).

Rudeen and Reiter (1977) studied the effects of shortened photoperiods on NAT activity in rats and found that light pulses as short as 15 minutes, during the dark phase of the day, depressed NAT activity to minimum daytime levels. Following the exposure to the light pulse there was an 8 hour refractory period before NAT activity increased again. NAT activity then rose 110-130 times greater than minimum levels. Those authors postulated that the greater induction of NAT activity in the pineal of rats exposed to short photoperiods may be due to conformational changes in the pineal receptors rather than to supersensitivity, as postulated by Delguchi and Axelrod (1972b). In contrast to the dramatic increase in NAT activity found in the pineal of the rat during the dark period, pineal NAT activity in the golden hamster was found to exhibit only a 3-fold elevation at night. However, both species reached maximal NAT activities 8 hours after the onset of darkness (Rudeen et al., 1975).

The dark induction of NAT activity has been reported to be regulated by an endogenously generated rhythm, since rats exposed to 4 hours of darkness, during 36 hours of LL, exhibited a 10-fold increase in NAT activity only when the dark pulse corresponded to the dark period of the animals' prior customary lighting schedule. The rhythm also could be reversed 180^{o} by reversing the light-dark cycle, indicating that the NAT rhythm is endogenously entrained by the animals' lighting schedule and can be shifted by a change in the lighting pattern (Alphs and Heller, 1978).

Axelrod and Weissbach purified and characterized HIOMT in 1961. They found that HIOMT had an absolute requirement for S-adenosylmethionine and was found only in the pineal gland. Subsequent studies

with rats have established the presence of HIOMT in the Harderian gland and retina (Cardinali and Wurtman, 1972).

Wurtman et al. (1963) found that LL decreased HIOMT activity in the rat's pineal to about half the enzyme activity found in darkness. Bilateral removal of the superior cervical ganglia eliminated night-day differences in HIOMT activity, while blinded rats continued to show an HIOMT rhythm (Wurtman et al., 1974).

In contrast to the male, female rats showed fluctuations in HIOMT activity which were dependent upon the estrous cycle, since exogenous estradiol inhibited HIOMT activity (Wurtman et al., 1965). In 1974, Wallen and Yochim found that HIOMT activity peaked and began to decline during the dark phase of the day in female rats. The greatest change in HIOMT amplitude occurred between diestrus and proestrus, however, the HIOMT activity only was significantly depressed between estrus and metestrus. They postulated two component hypothetical rhythms in HIOMT activity. The first rhythm was the diurnal, 24-hour change in HIOMT activity. The second rhythm was dependent on the estrous cycle since rats with a four day estrous cycle had a 19.2 hour HIOMT rhythm, while rats with a five day estrous cycle had a 20 hour rhythm. The HIOMT rhythm was abolished in female rats placed in LL, but maintained in rats exposed to constant darkness, indicating that the rhythm was endogenous (Yochim and Wallen, 1974a). The two component rhythm hypothesis of HIOMT activity was further supported by ovariectomy and hormone replacement studies in the rat. Although the rhythm of the cycle was the same in ovariectomized rats as found in intact controls, the amplitude was significantly depressed (Wallen and Yochim, 1974b). Injections of

estrogen into oophorectomized rats restored the amplitude of HIOMT activity to values comparable to those present in control animals, but resulted in a distorted HIOMT rhythm. The results indicated that the ovaries influence the amplitude of HIOMT activity, but not the endogenous pineal HIOMT rhythm. The mixed wave hypothesis subsequently was found to be capable of predicting the effects of photoperiod on estrous cyclicity (Wallen and Yochim, 1974), and HIOMT activity (Yochim and Wallen 1974b), since rats have a five-day cycle became acyclic when exposed to artificial days of 20 hours in length, with a 1.4 to 1 (light/dark) ratio.

The Syrian hamster, Mesocricetus auratus, is a winter hibernator. In their natural habitat hamsters are exposed to progressively shorter days and spend a greater amount of time in burrows with the approach of winter. Experiments testing a variety of photoperiods demonstrated that adult male hamsters needed at least 12.5 hours of light to maintain normal testes weights and spermatogenesis (Gaston and Menaker, 1967). A decrease in light exposure and heightened pineal antigonadal activity in the hamster results in gonadal regression (Reiter, 1975). The dramatic gonadal regression that occurs in the hamster deprived of light or placed in short-day photoperiods makes it an ideal animal for studies investigating pineal-gonadal interactions (Reiter, 1975).

Hoffman and Reiter (1965) reported that blinding or maintaining male hamsters in constant darkness resulted in gonadal involution. Pinealectomy, however, prevented testicular atrophy in hamsters exposed to a light dark (LD) cycle of 1:23 hours.

Elliot et al. (1972) exposed groups of male hamsters to photo-

periods having six hours of light coupled with varying periods of darkness, including 18, 30, 42 and 54 hours to produce 24, 36, 48 and 60 hour photoperiod lengths. Animals exposed to 6L:18D and 6L:42D interpreted the light stimulus as short days and exhibited testicular regression, while animals maintained in 6L:30D and 6L:54D interpreted the light information as long days and had normal testes and seminal vesicles. Recrudescence of reproductive structures occurred when hamsters with involuted testes were maintained in 6L:30D and 6L:54D photoperiods (Stetson et al., 1975). Those results indicated the presence of a circadian rhythm of photosensitivity in hamsters.

In 1975, Turek et al. found that subcutaneous implants of melatonin in polydemethysiloxane capsules had a dose-dependent action on the reproductive system of male hamsters. Animals which received implants releasing 50 ug of melatonin per day maintained their testes in 6L:18D, while animals exposed to 14L:10D, with capsules that released 75-100 ug of melatonin per day, exhibited marked gonadal regression.

Tamarkin et al. (1976) found that injections of 10-25 ug of melatonin were progressively more effective in causing gonadal quiescence in male and female hamsters exposed to 14L:10D as the time of injection approached the onset of darkness; the most rapid inhibition of the reproductive system occurred when the animals were injected 15 minutes before darkness. Melatonin injections three hours after the onset of light had no effect on the reproductive system after seven weeks of treatment, while females injected 15 minutes before dark became anovulatory within three weeks. In contrast, melatonin injections

(25 ug) administered 15 minutes before the onset of darkness had no effect on male hamsters after pinealectomy, superior cervical ganglionectomy, decentralization of the ganglia or anterior hypothalamic decentralization (Reiter et al., 1976).

Blinding of female hamsters causes involution of the uteri (Reiter and Hester, 1966) and cessation of the estrous cycle (Seegal and Goldman, 1975). The ovaries enlarged but the ovarian follicles did not attain maturity. Pinealectomy or superior cervical ganglionectomy of female hamsters prevented gonadal suppression (Reiter and Johnson, 1974). Tamakin et al. (1977b) showed that 10 ug-injections of melatonin during the middle of the dark period did not depress gonadal function in male and female hamsters. However, injections administered at two hours before lights-on induced testicular regression in males and acyclicity in females after 6 weeks of treatment. Andre and Parrish (1978) reported that quartran injections of 25 ug of melatonin 15 minutes before the onset of darkness on the day of proestrus induced acyclicity within two to three weeks in hamsters maintained in 14L:10D photoperiod, indicating a sensitivity to melatonin only on the night before ovulation.

Pinealectomized female hamsters receiving three injections of 25 ug of melatonin at three-hour intervals became acyclic after 7 weeks of treatment in a 14L:10D photoperiod. Pinealectomized males, receiving triple 25 ug melatonin-injections also became reproductively quiescent, and had significantly reduced testes weights when compared to similarly treated controls. A single daily injection of 75 ug of melatonin did not cause gonadal regression in pinealectomized male hamsters when injected at the onset of darkness, however,

pinealectomized males receiving a similar injection four hours after the onset of light exhibited testicular atrophy (Tamarkin et al., 1977a). Those data support the hypothesis that melatonin may be the antigonadal hormone of the pineal, and suggest that because the time and number of injections determine the antigonadal effectiveness of the injected melatonin, the exogeneous melatonin may interact with the photoperiod-controlled endogenous melatonin rhythm.

The acceptance of melatonin as the antigonadotrophic principle of the pineal gland is not universal. Recent experimental evidence indicates that melatonin action may be progonadal in the hamster. In 1974, Hoffman, using beeswax implants releasing 34 ug of melatonin per day, failed to induce gonadal regression in male Djungarian hamsters (Phodopus sungorus) exposed to a 14L:10D photoperiod, and prevented gonadal regression in animals exposed to short days. Reiter et al. (1974) prevented gonadal regression in male Syrian hamsters maintained in 1L:23D by melatonin administrated in weekly, subcutaneous beeswax implants. Similar treatment of blinded female hamsters prevented uterine atrophy (Reiter et al., 1975).

In addition to melatonin, several pineal peptides have been found that inhibit reproductive function in mammals. In 1976, Benson et al. purified an antigonadotrophic peptide from bovine pineals. Arginine vasotosin, another pineal peptide which has antigonadotrophic activity (Vaugham et al., 1974) was found in the mammalian pineal stalk (Pavel, 1971). The findings of those investigators, coupled with the progonadal effects observed in hamsters treated with melatonin implants, has led to speculation that pineal function may depend upon low molecular weight polypeptides for expression

of antigonadotrophic effects (Reiter et al., 1976).

The present study was initiated to determine the effects of daily injections of melatonin and isoproterenol on the estrous cycle and free-running activity of hamsters maintained in LL, and to elucidate the reason for the insensitivity of female hamsters to melatonin injections during the non-photosensitive part of the day.

Although previous studies have shown that daily, single injections of melatonin induce uterine atrophy and estrous acyclicity in hamsters maintained in a 14L:10D photoperiod, pinealectomized female hamsters are not responsive to similar treatment. In contrast, thrice-daily melatonin injections are effective in the inhibition of estrous cyclicity in pinealectomized hamsters (Tamarkin et al., 1977). The present study was designed to test the hypothesis that the exposure of female hamsters to LL, in effect, simulates pinealectomy. The published reports that pineal NAT shows a diurnal rhythm in sensitivity to induction by the beta-agonist isoproterenol, prompted an investigation to determine if isoproterenol injections, 15 minutes before the onset of activity, or dark onset, in LL- or 14L:10D- exposed animals, could abolish the estrous cycle when the gland is supersensitive.

MATERIALS AND METHODS

Adult female Syrian hamsters (Mesocricetus auratus) were purchased from Mid-Continent Research, Incorporated (Shawnee, Kansas). Initially, they were housed eight to a cage at room temperature and maintained on a 14L:10D photoperiod (lights on 08:00-22:00 CST) for two weeks prior to exposure to constant illumination (LL). Beginning on April 13, 1978, the hamster weighing 90-144g were divided into five experimental groups of four to seven animals each, weighted and placed in an environmental chamber in individual 54 x 17 x 25 cm stainless steel wire bottoned cages (Hoeltge Brothers Incorporated, Harrison, New Jersey). Constant illumination (LL), which ranged from 50-100 Lux in intensity at the front of the cages, was supplied by two cool-white fluorescent lights placed in opposite ends of the chamber. Temperature in the chamber was thermostatically maintained at $23^{o} \pm 2^{o}$ C. The remaining hamsters were housed in groups of five or six per cage in plastic cages (50 x 40 x 20 cm) and maintained in the same 14L:10D photoperiod for use in later experiments. Purina Lab Chow and water were continuously available to all of the animals throughout the study. Feeding and cleaning were routinely performed at night in order to minimize extraneous stimuli.

Experimental Protocol

In the first experiment, one group of LL-exposed hamsters were treated with single daily 25 ug doses of melatonin in 0.1 ml of an alcoholic Planter's peanut-oil vehicle (LL-melatonin: 1x), approx-

imately one hour before the onset of activity until the last few weeks of the experiment, when some animals became arrhythmic or exhibited such greatly reduced activity periods that the single injection was given near of slightly after activity onset. A double-melatonin injected (LL-melatonin: 2x) group was given daily injections of melatonin between 1930-2100 Central Standard Time (CST) and at 0700 (CST). A third group of hamsters was treated with single, daily injections of an emulsion of 0.2 mg of isoproterenol in 0.1 ml of peanut oil, initially at 2100 CST and later, at 1930 CST (LL-isoproterenol: 1x, A). Two groups of controls were injected with 0.1 ml of the peanut oil vehicle at the same time periods as above (LL-oil: 1x and LL-oil: 2x, respectively).

In the second experiment, three groups of five to six hamsters weighing 86-138g were housed in plastic cages and maintained in either a 14L:10D or an LL photoperiod. The first two groups of hamsters were maintained on a 14L:10D photoperiod starting on May 19. One of these groups was injected daily with 0.2 mg of isoproterenol 15 minutes before the onset of activity (LD-isoproterenol: 1x). The second group of hamsters served as uninjected controls. The remaining hamsters were maintained in LL beginning on June 1, and were injected with 0.2 mg of isoproterenol one hour before the onset of activity every day (LL-isoproterenol: 1x, B).

Throughout the study all of the injections were administered subcutaneously either in the dorsum of the neck or in the ventral surface of the thigh. The injections initially were administered one hour before the onset of activity. When the animals were

transferred from the 14L:10D photoperiod (lights on 08:00-22:00 CST) to LL, the pre-activity injections were administered at 21:00 hours, while the double injections were given at 07:00 and 21:00 hours for the first 16 days. Thereafter, the pre-activity injections were administered at 19:30 hours until the conslusion of the experiment.

Melatonin and L-isoproterenol-d-bitartrate were purchased from the Sigma Chemical Company (Saint Louis, Missouri). Melatonin was recrystallized in aqueous glass-distilled methanol (Burdick and Jackson Laboratories, Inc., Muskegon, Michigan) before use. Purity was confirmed by thin layer chromatography on silica gel G, in the dark, using a glass-distilled chloroform: methanol (9:1) solvent system.

Activity Records

Individual animals were transferred between cages that lacked or possessed a running wheel (Hartz Mountain Corporation, Harrison, New Jersey) in 2-6 day shifts in order to monitor locomotor activity (Plate 1). Electromagnetic reed switches (Burstein-Applebee Company, number 77A20-6) were glued to the activity-wheel support stands (Plate 2) and connected through a common 72 ohm resistor to a model A620X Esterline-Angus event recorder (Plate 3). The reed switches were activated by small ceramic magnets which were attached to the cross bars of the running wheels, so that each time a wheel completed a revolution, the reed switch closed and caused a deflection of a pen on the recorder. The chart paper (type 1720X) speed was set at three-fourths of an inch per hour or 18 inches

Plate 1. General design of cages for monitoring locomotor activity.

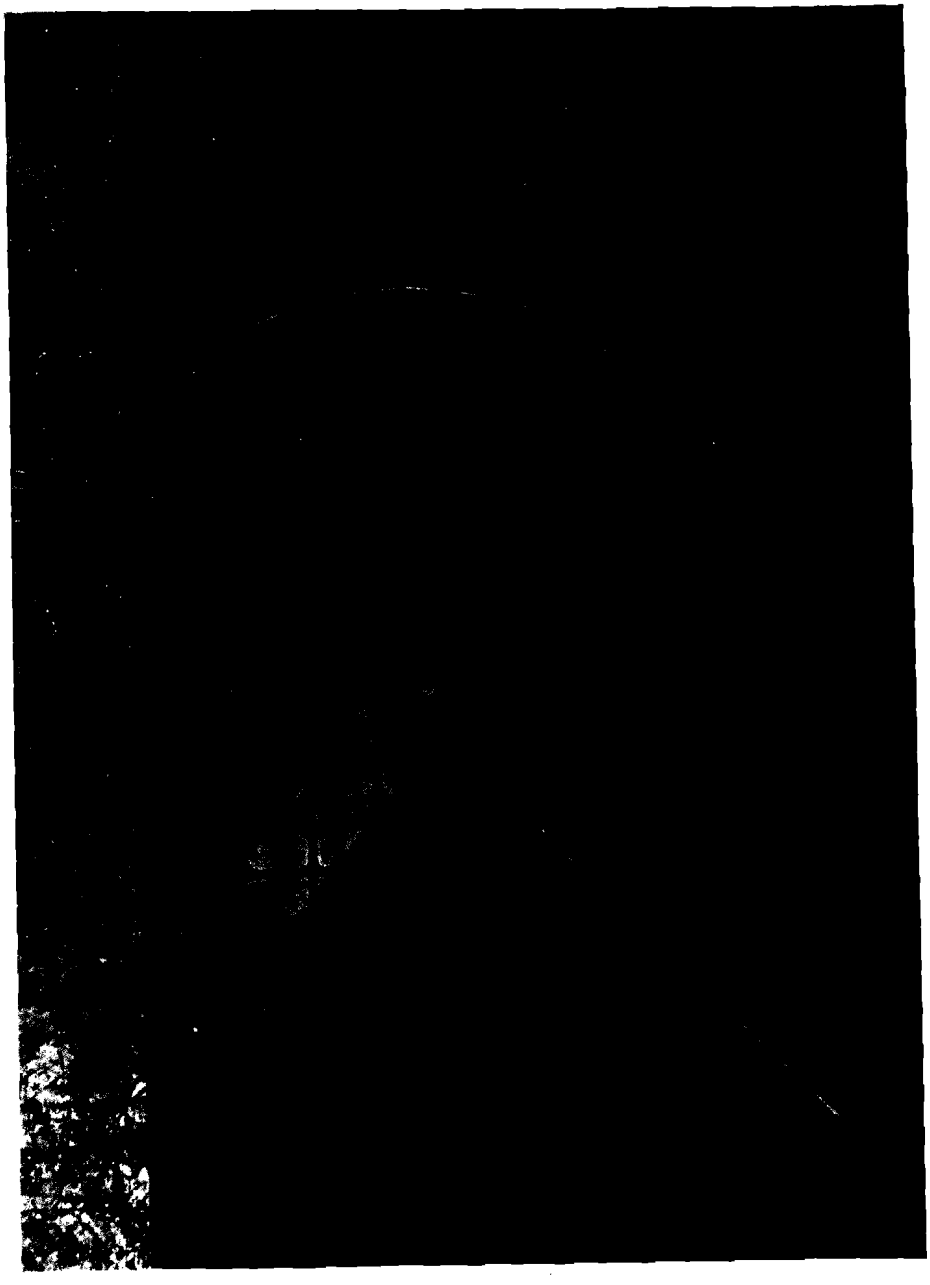


Plate 2. Close-up illustrating the activity wheel, reed switch and magnet arrangement.

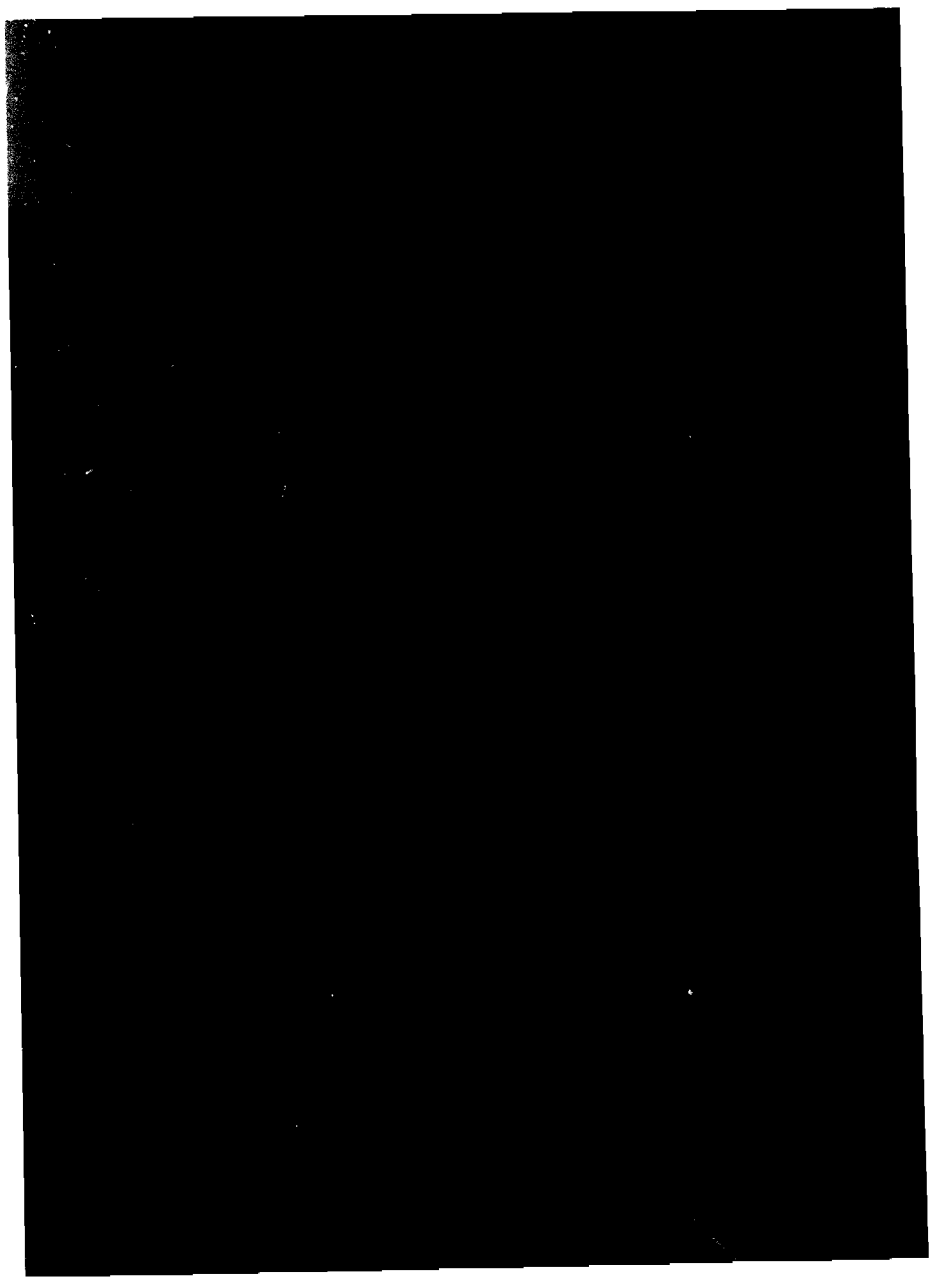
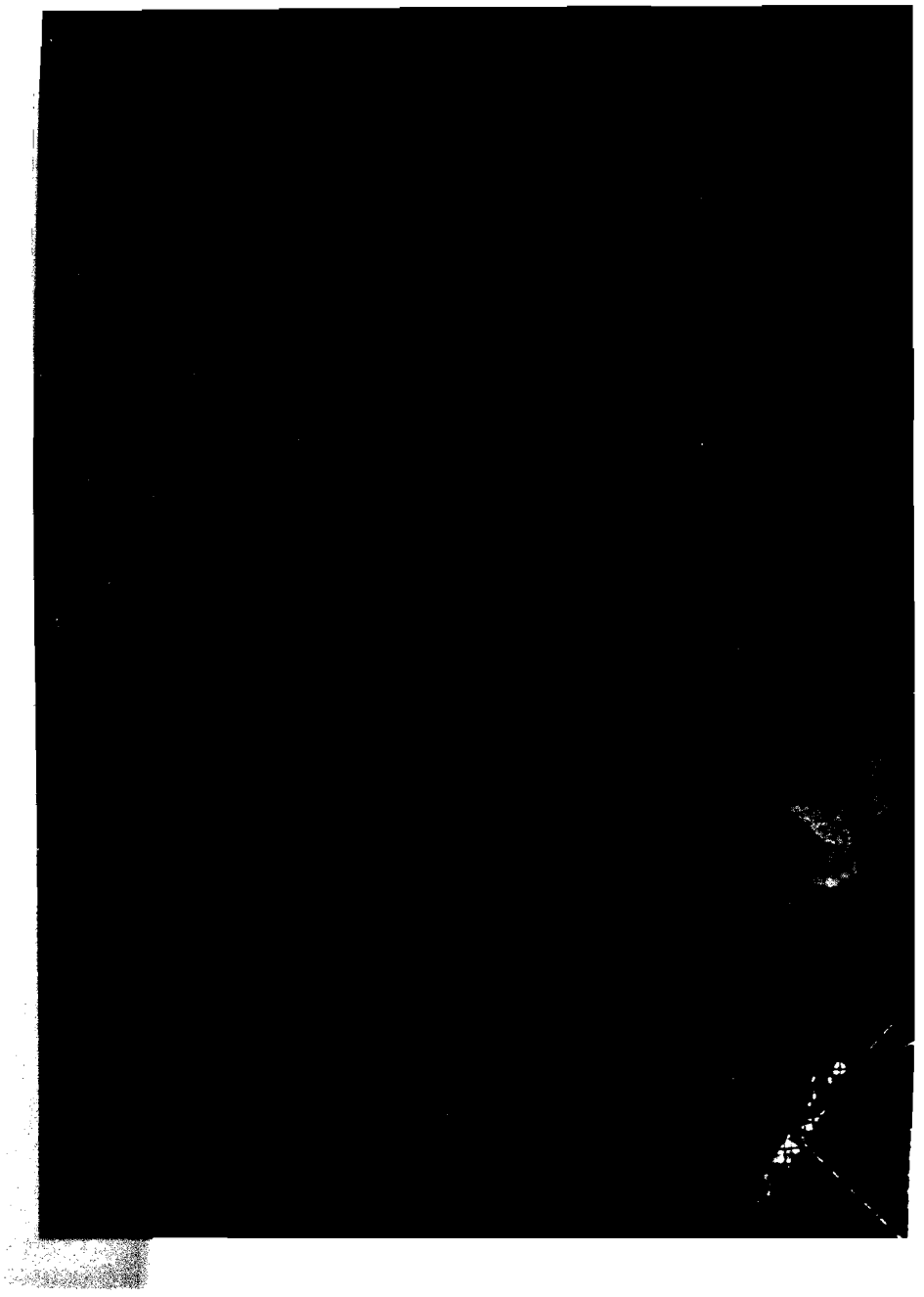


Plate 3. General cage arrangement and Esterline-Angus recorder for monitoring hamster locomotor activity.



per day.

The activity records were cut into 24-hour sections and each individual animal's 24-hour record of activity was mounted on a white paper backing directly below the preceding 24-hour's record. Representative records from each group were photographed to make a permanent record.

Estrous Cycle

The hamsters were checked daily for a postovulatory discharge using the method described by Orsini (1961). Near the end of the experiment, vaginal smears were made to insure the proper timing of sacrifice.

Collection of Tissue and Plasma Samples

The first groups of hamsters were sacrificed between June 23 and June 27, 1978, at mid-activity and mid-rest, as determined from the activity records. Animals that were still exhibiting estrous cycles were sacrificed either during mid-activity when they were in the proestrus stage of the estrous cycle, or at mid-rest when they were in metestrus. The remaining groups were sacrificed July 27-28, 1978, according to the same protocol.

The hamsters were weighed just prior to being killed by decapitation. Trunk blood samples were collected in test tubes containing 32 units of heparin in 0.1 ml of distilled water. The blood was centrifuged to separate the plasma from the formed elements and the plasma was frozen at -60^{o}C for future hormone assays. The uterus and vagina, ovaries, and adrenal glands were trimmed of

extraneous fatty and connective tissues under a dissecting microscope and weighed to the nearest 0.1 mg on a Model AG Sartorious Analytical balance. The pineal gland was rapidly dissected, rapidly frozen on dry ice, weighed and stored at -60°C for future hormone assays.

Statistical Treatments

The data among groups were statistically analyzed by analysis of variance or Student's "t" test after Sokal and Rohlf (1974), using a Monroe programmable calculator (Model 1785-WI). Calculated "t" or "F" values were considered significant if they equaled or exceeded two-tailed table values for the 0.05 level of probability.

RESULTS

Melatonin or L-Isoproterenol Treatment in LL-Exposed Hamsters

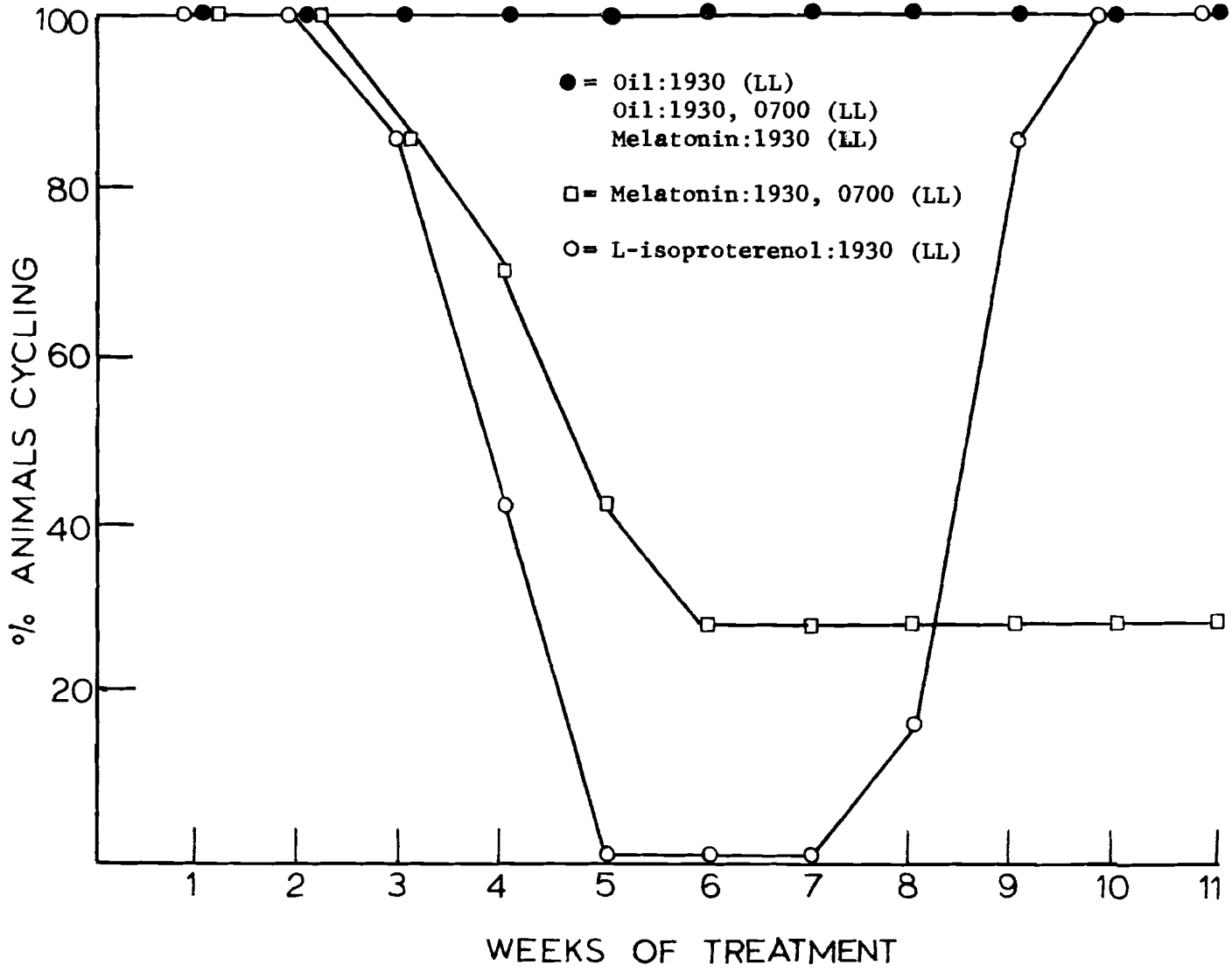
Effects on estrous cyclicity

All of the hamsters treated with either single or double injections of the peanut oil vehicle continued to cycle regularly throughout the duration of the experiment. The hamsters which received single injections of melatonin also showed normal estrous cycles while 66.6% of the double-melatonin injected animals were acyclic by the end of the study. In contrast, 100% of the hamsters injected with isoproterenol became acyclic after five weeks of treatment and remained acyclic for the next three weeks (Figure 2). However, the estrous cycle was re-established in the isoproterenol-injected animals when the injections, by that time, were being administered after activity onset. A second group of hamsters, which were initially group-housed for one month and injected with isoproterenol became acyclic by seven weeks (three weeks after being indivdually-housed) confirming the results observed in the first group. Vaginal smears of the second group of isoproterenol-treated hamsters, taken several days before sacrifice, exhibited cornified-epithelial cells and nucleated-epithelial cells which indicated that they were in a state of persistent estrus.

Effects on tissue and body weights

Daily injections of oil, in both single- and double-injected groups did not statistically affect the uterine, ovarian, adrenal and pineal gland weights in hamsters maintained in LL when compared to uninjected animals maintained in 14L:10D (Fig. 3, Table 1). Isoproterenol and single, daily injections of melatonin also did not

Figure 2. The effects of daily injections of 25 ug melatonin, 0.2 mg isoproterenol, 0.1 ml peanut oil vehicle, and double injections of melatonin or oil on the estrous cyclicity of hamsters maintained in constant light. The estrous cycle was monitered daily and the percentage of hamsters cycling was determined at the end of each week of treatment for 11 weeks. Single injections were administered between standard time 1930-2100 (CST), while double injections were given at CST 0700 and between 1930-2100 CST.


100
80
60
40
20
% ANIMALS CYCLING
● = Oil:1930 (LL)
Oil:1930, 0700 (LL)
Melatonin:1930 (LL)
□= Melatonin:1930, 0700 (LL)
○= L-isoproterenol:1930 (LL)
1
2
3
4
5
6
7
8
9
10
11
WEEKS OF TREATMENT

significantly affect any of the organ weights. However, a slight, although nonstatistical increase (about 40%) in pineal weights per 100g body weight was observed in hamsters treated with single injections of melatonin. In contrast, daily double injections of melatonin effected a dramatic 2.7-fold increase in pineal gland weight/100g body weight (Table 1). Double injections of melatonin also produced a significant increase in adrenal weights (mean = 25.55 mg/100g body weight) when compared to the controls which received two injections of oil (mean = 14.46 mg/100g body weight). Absolute adrenal weights were not significantly different among any of the groups, however (Table 1). Double-melatonin injections caused a decrease in the uterine weights ($p < 0.05$) (Fig. 3). All groups exhibited increased body weights during the course of the experiment. The significant difference in body weight ($p < 0.05$) found between single- and double-injected oil controls was probably due to the lower average weight of the double-injected oil controls (106.2g) when compared to the 118.7g average body weight found in single-injected oil controls at the beginning of the experiment. One single-injected control animal failed to gain weight during the experiment, which further contributed to the difference observed between these groups.

Effects on locomotor activity

Female hamsters which received single injections of oil, melatonin or isoproterenol exhibited periods of free-running locomotor activity (tau or T) that were shortened or lengthened when maintained in LL (Fig. 5, 6 and 7) The t values for those groups ranged from -0.81 to 3.38 minutes per day. However, only the hamsters treated with isoproterenol showed a reduction in intensity and duration of

Table I. Effect of melatonin and isoproterenol on the ovarian, adrenal and pineal weights (mg/100g BW) of hamsters exposed to constant light and a 14L:10D photoperiod.

Treatment	Ovary Wt.	Adrenal Wt.	Pineal Wt.
Constant light			
Control 1x	55.27 ± 6.86*	15.53 ± 0.98*	0.786 ± 0.043*
Control 2x	69.32 ± 9.24	14.46 ± 1.14	0.708 ± 0.058
Melatonin 1x	47.01 ± 5.73	14.97 ± 1.74	1.100 ± 0.113
Melatonin 2x	62.66 ± 7.96	25.55 ± 2.35**	1.953 ± 0.309**
Isoproterenol exp. 1	56.08 ± 5.29	14.47 ± 1.02	0.890 ± 0.149
Isoproterenol exp. 2	47.03 ± 1.47	13.11 ± 0.57	0.746 ± 0.014
14L:10D			
Control	47.34 ± 2.90	14.34 ± 0.67	0.870 ± 0.038
Isoproterenol	55.14 ± 6.10	13.09 ± 0.67	0.756 ± 0.031**

* Mean ± SEM
** $P < 0.05$ when compared to oil control group

Figure 3. The effect of daily injections of 0.2 mg isoproterenol on estrous cyclicity of hamsters maintained in constant light or in a 14L:10D photoperiod. The percentage of hamsters cycling was determined at the end of each week. The closed circles represent uninjected controls maintained in LD. The animals treated with isoproterenol and maintained in LL were injected for 8 weeks. The single daily injections were administered at 1930 CST in LL and 15 minutes before the lights out in LD.

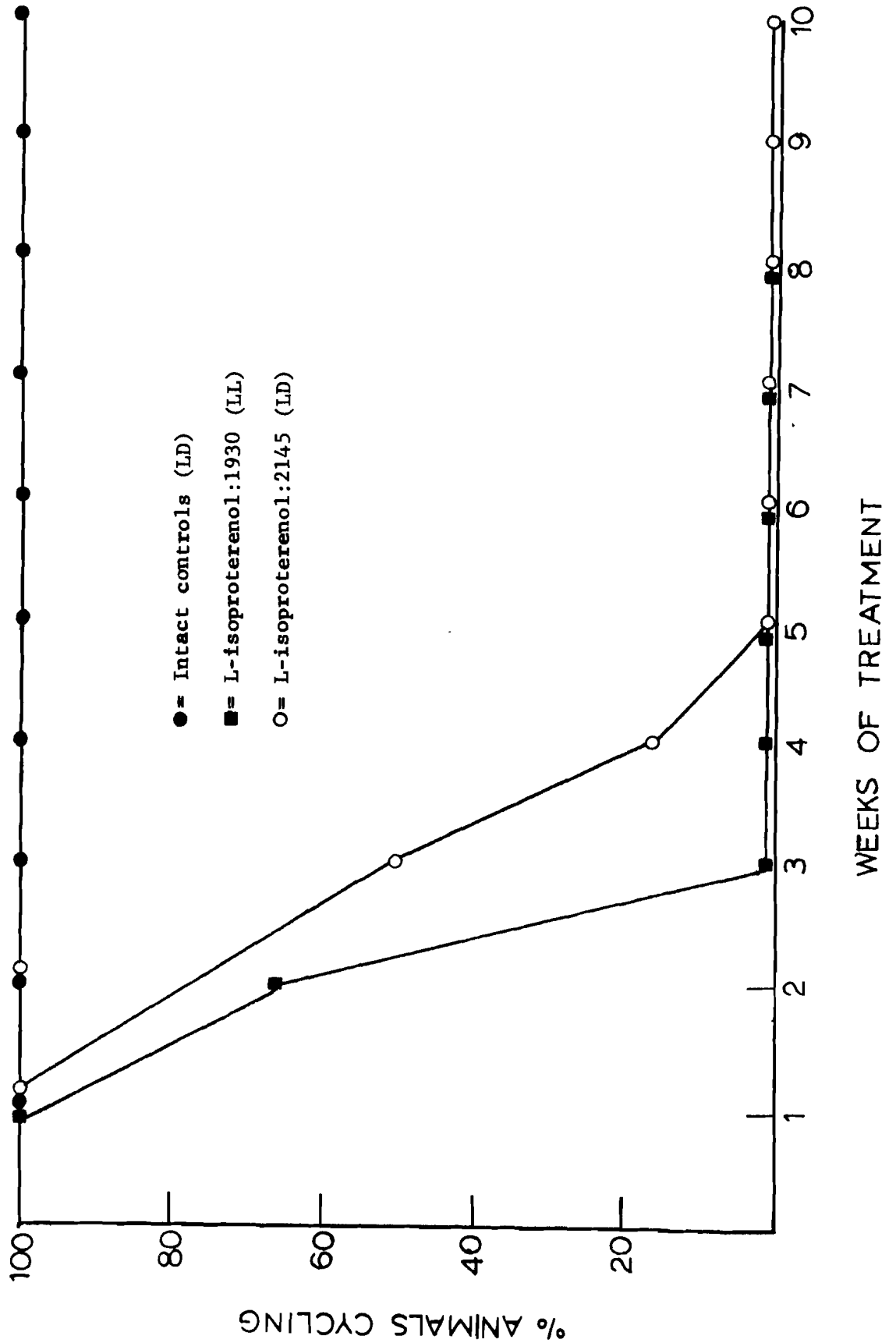

● = Intact controls (LD)
■ = L-isoproterenol:1930 (LL)
○ = L-isoproterenol:2145 (LD)
% ANIMALS CYCLING
100
80
60
40
20
1
2
3
4
5
6
7
8
9
10
WEEKS OF TREATMENT

Figure 4. The effect of daily injections of 25 ug melatonin, 0.2 mg isoproterenol, 0.1 ml of the peanut oil vehicle and double injections of melatonin and oil on uterine weights of hamsters maintained in constant light and a 14L:10D photoperiod. The uterine weights are expressed in mg/100 g BW. The bars represent the mean weight of each group. The single, verticle lines indicate the standard error of the mean. The number in parenthesis shows the number of animals in each group.

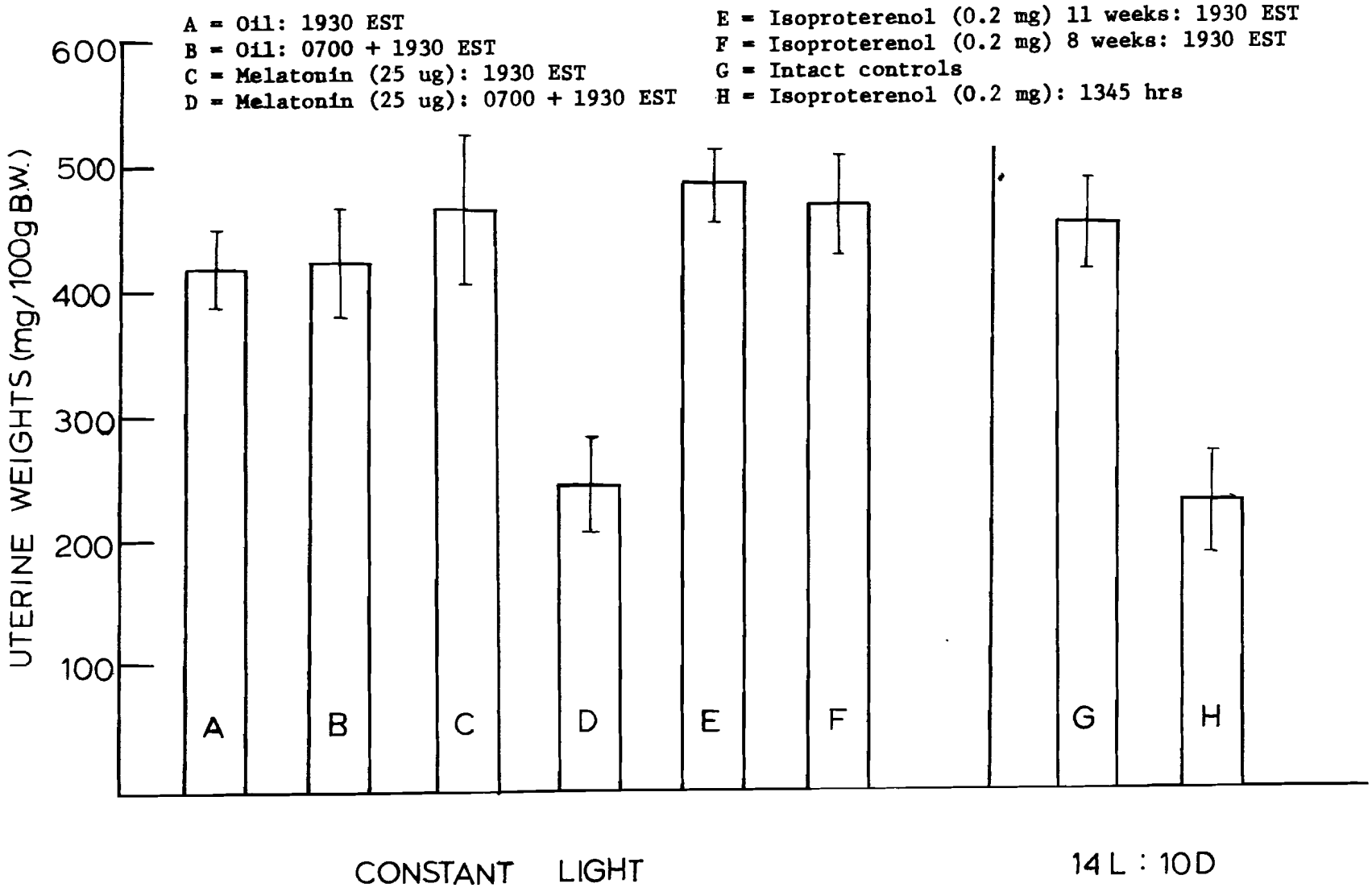

A = Oil: 1930 EST
B = Oil: 0700 + 1930 EST
C = Melatonin (25 ug): 1930 EST
D = Melatonin (25 ug): 0700 + 1930 EST
E = Isoproterenol (0.2 mg) 11 weeks: 1930 EST
F = Isoproterenol (0.2 mg) 8 weeks: 1930 EST
G = Intact controls
H = Isoproterenol (0.2 mg): 1345 hrs
UTERINE WEIGHTS (mg/100g B.W.)
600
500
400
300
200
100
A
B
C
D
E
F
G
H
CONSTANT LIGHT
14 L : 10D

Figure 5. Representative locomotor activity record showing free-running activity for a hamster treated with single daily oil injections and maintained in constant light. The 0 and 24 hour marks show the beginning and end of the standard day. The arrows mark the time of injection.

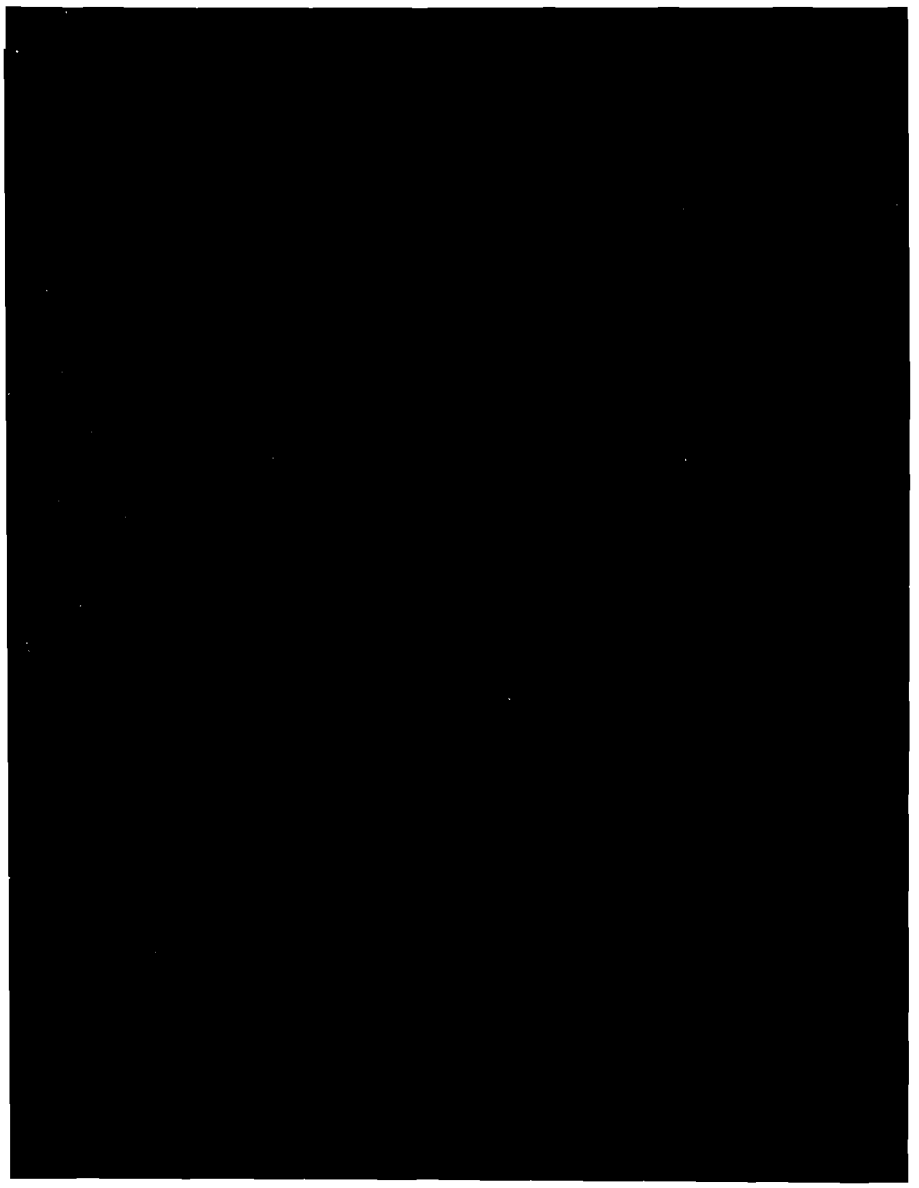
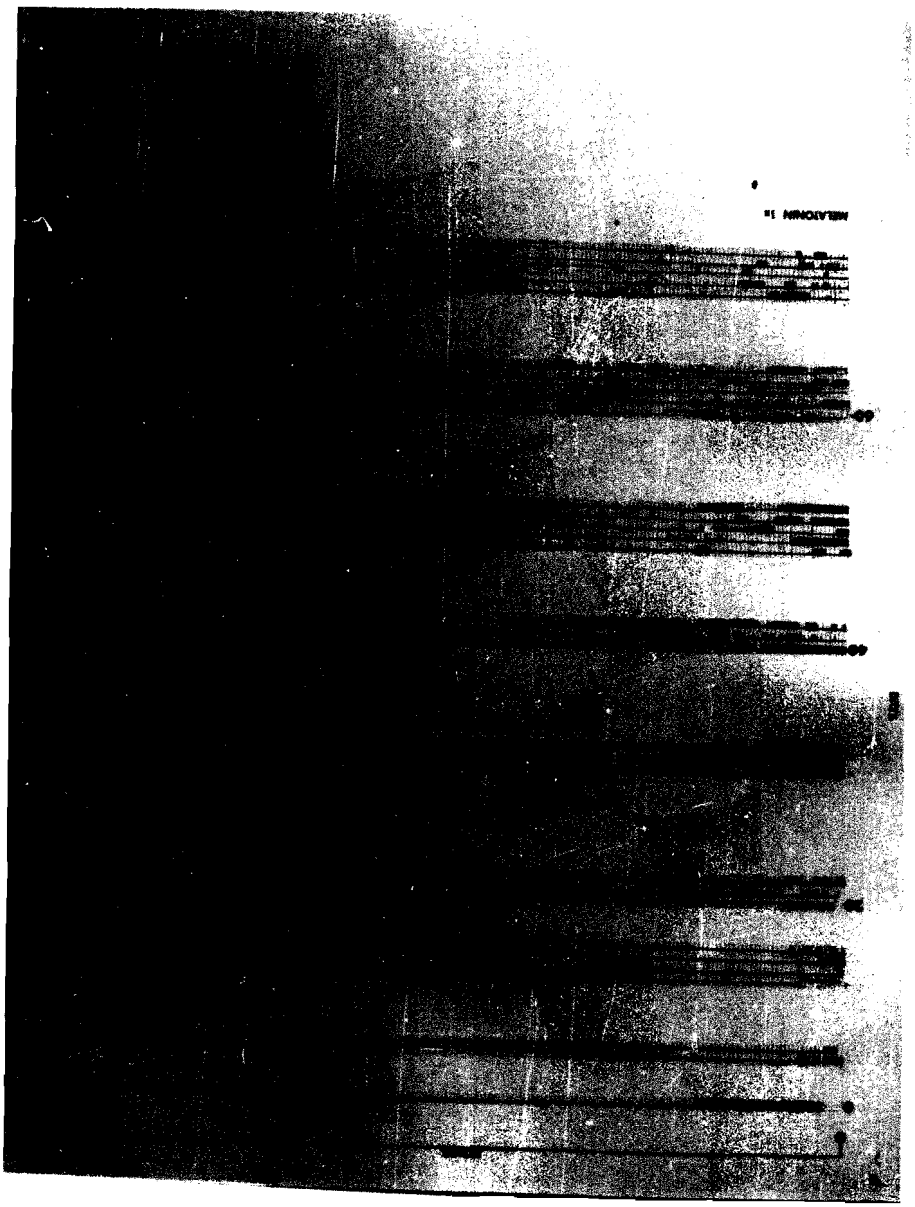


Figure 6. Representative locomotor activity record showing free-running activity from a hamster treated with single, daily melatonin injections and maintained in constant light. The 0 and 24 hour marks show the beginning and end of the standard day. The arrows mark the time of injection.


MELATONIN 1a

locomotor activity (Fig. 7). The LL-oil 2x group exhibited atypical activity records. A representative animal for that group showed increased T for 5 weeks, then shifted locomotor activity 180^{o} for 5 days, after which the animals returned to the initial activity pattern (Fig. 8). The T values found in the group treated with double injections of melatonin were small, ranging from -1.04 to 2.6 minutes per day. A few animals in the other groups exhibited arrhythmic activity patterns following six or more weeks of exposure to constant light (Fig. 10).

Isoproterenol Injections in 14L:10D-Exposed Hamsters

Effects on the estrous cycle

All of the uninjected control animals maintained in 14L:10D had regular estrous cycles throughout the experiments, while 100% of the hamsters that received isoproterenol 15 minutes before the onset of darkness became acyclic after eight weeks of treatment (Fig. 4). Vaginal smears, taken over a few days before sacrifice showed a predominance of leukocytes and ovoid epithelial cells which indicated that the animals were in a diestrus-like stage of the estrous cycle.

Effects of body and tissue weights

Control hamsters maintained in 14L:10D had organ weights similar to those found in control animals maintained in LL. However, hamsters treated with evening injections of isoproterenol while in the 14L:10D photoperiod showed a marked 49% depression in uterine weight/100g body weight when compared to the control group ($p < 0.01$). The ovarian weights in the LD-isoproterenol group were slightly elevated, while pineal weights per 100g body weight were significantly de-

Figure 7. Representative locomotor activity record showing free-running activity and decreased activity in a hamster treated with single daily injections of isoproterenol and maintained in constant light. The 0 and 24 hour marks indicate the beginning and end of a standard day. The arrows indicate the time of injection.

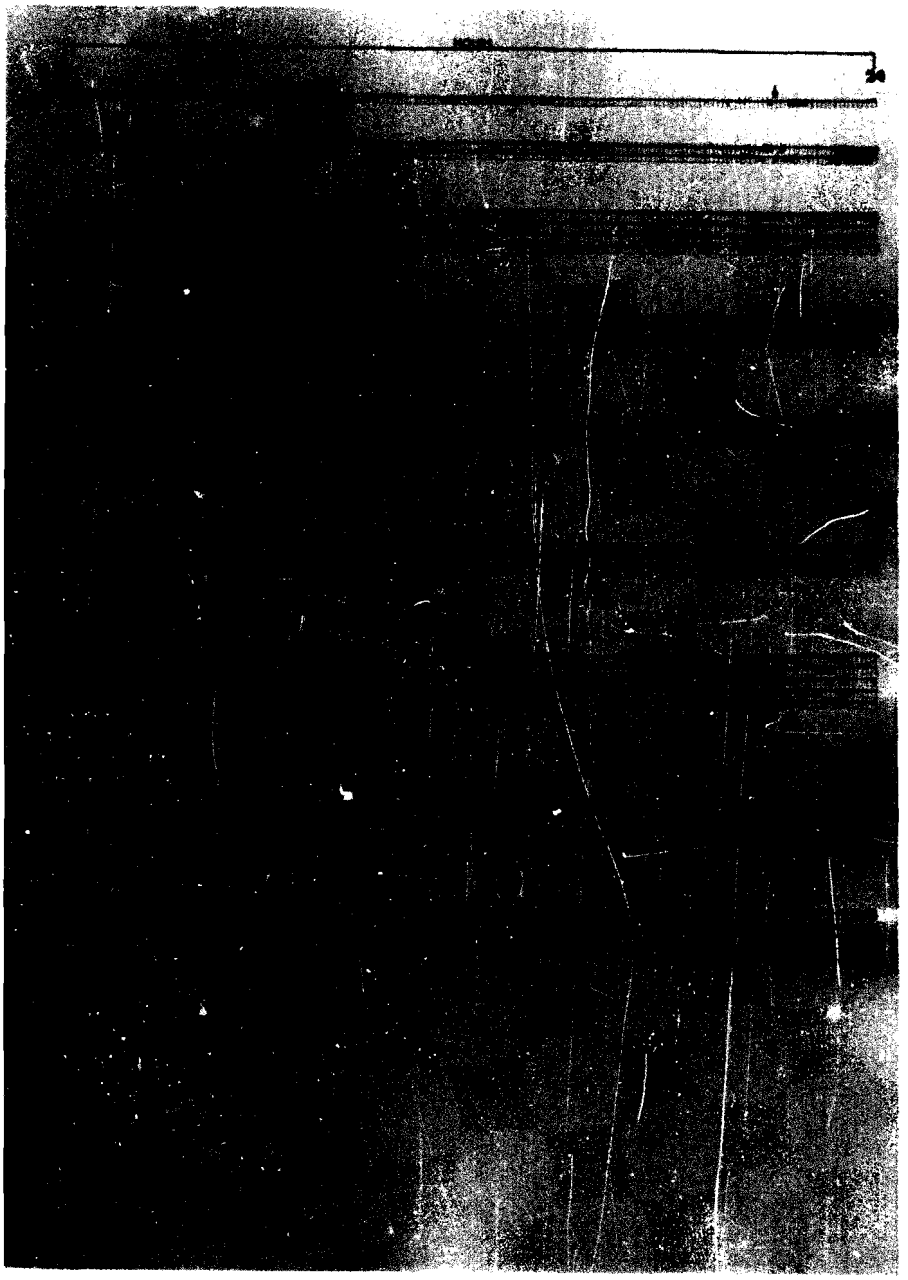


Figure 8. Representative locomotor activity record from a female hamster treated with double injections of oil and maintained in constant light. The 0 and 24 hour marks show the beginning and end of the standar day. During days 35 through 39 the animal exhibited a 120° shift in locomotor activity, then returned to its normal free-running pattern by day 43.


HOURS
24

Figure 9. Representative locomotor activity record from a female hamster treated with double injections of melatonin and maintained in constant light. The 0 and 24 hour marks show the beginning and end of the standard day. The arrows indicate the times of injection.

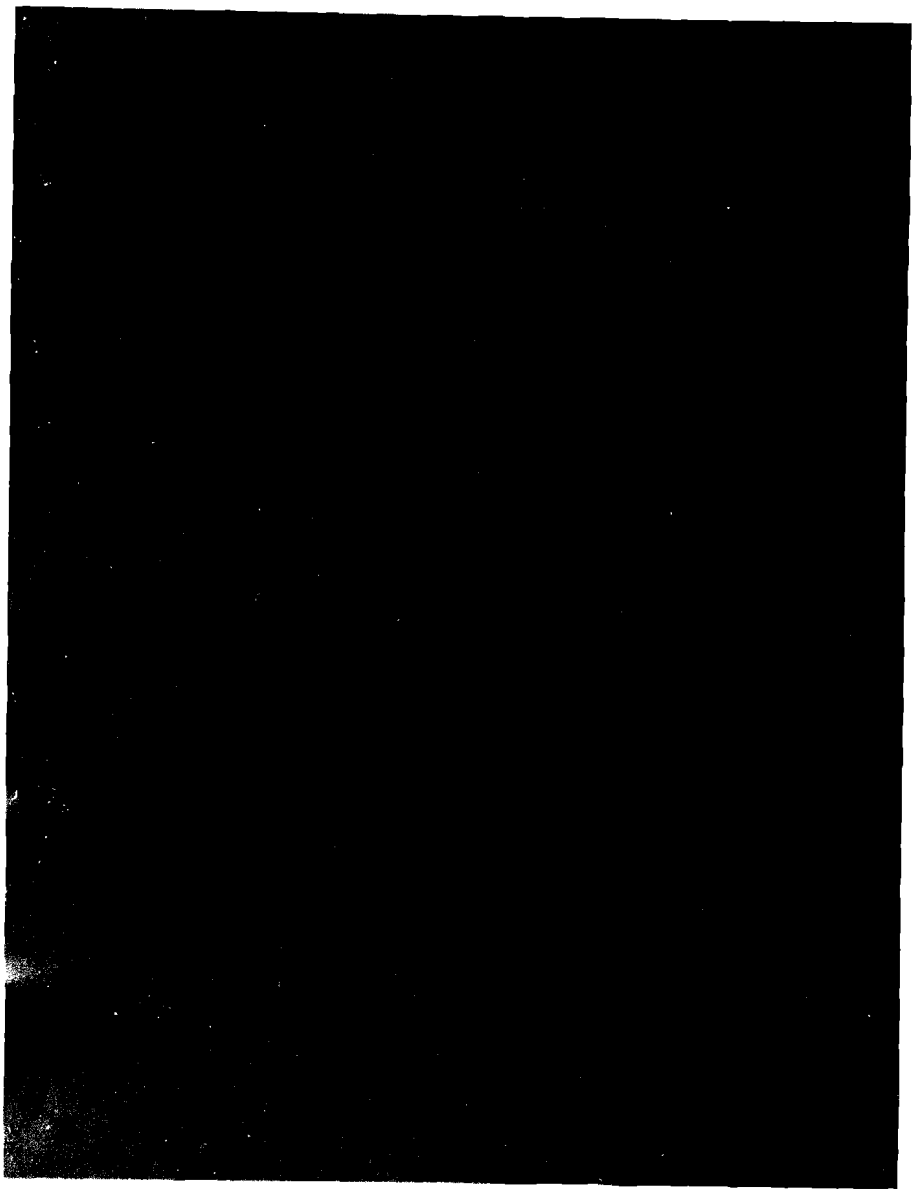
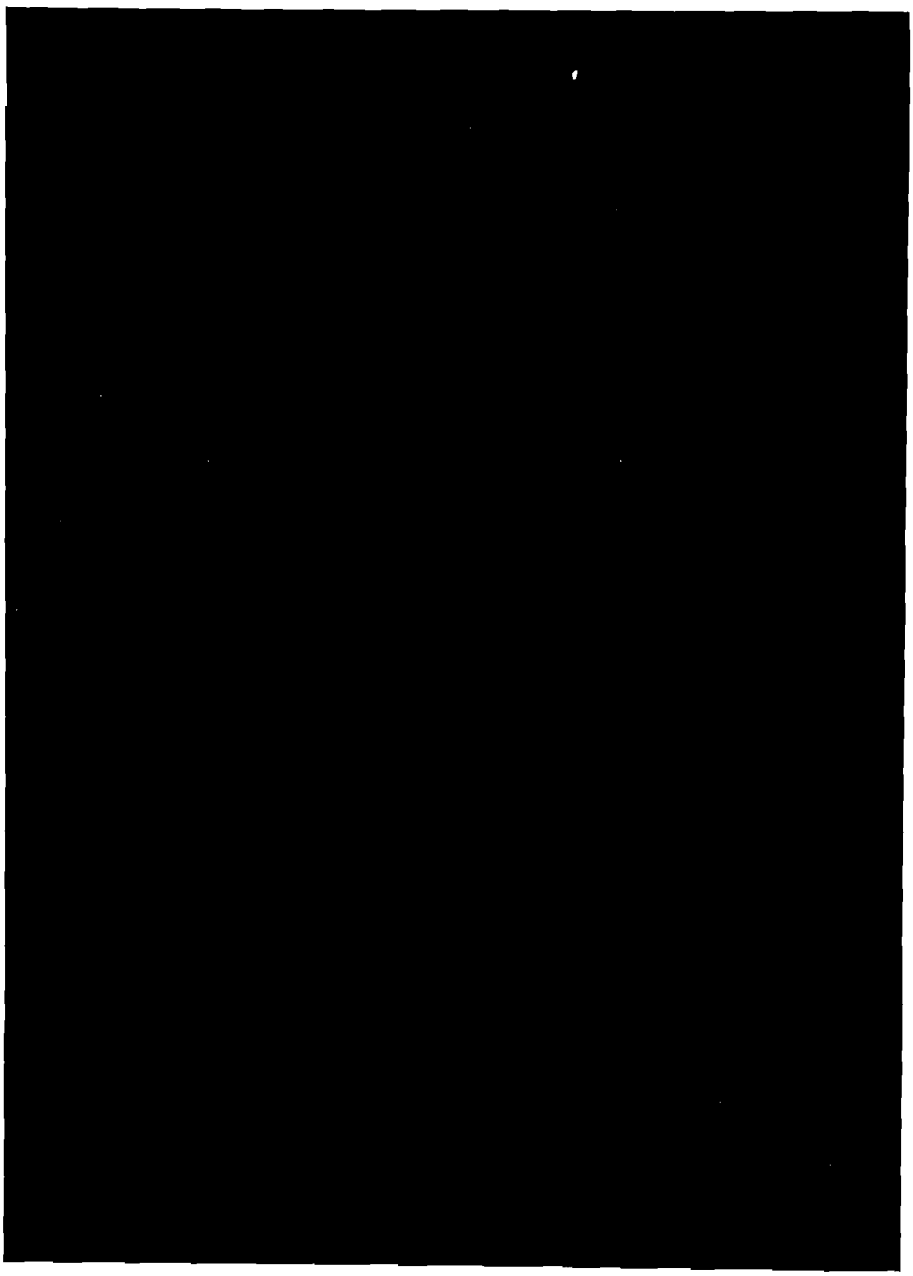


Figure 10. Representative locomotor activity of a female hamster demonstrating arrhythmic activity. The hamster was from the double-injected melatonin group.



pressed ($p < 0.05$) when compared to LD controls. However, absolute pineal weights were not significantly different in these two groups. The adrenal weights were not significantly affected by evening isoproterenol treatment (Table 1).

Body weights increased in both groups during the course of the experiment. The statistically significant difference found between the LL-oil control 1x and uninjected LD-controls was probably due to the much smaller initial average 91.6g weight of LD control animals when compared to the 118.7g average weight of LL-oil control 1x animals at the start of the experiment.

DISSCUSION

Estrous Cyclicity

Effects of Isoproterenol Treatment

The results from the present investigation clearly showed that isoproterenol injections administered shortly before the onset of darkness to hamsters maintained on a 14L:10D photoperiod abolished the estrous cycle (Fig. 4). Vaginal smears indicated that those animals were in a constant diestrous state, which is similar to the diestrous condition found in hamsters that have been shown to become acyclic after melatonin treatment (Goldman, 1978, personal communication) or exposure to short days (Seegal and Goldman, 1975). This constitutes the first report of the induction of estrous acyclicity by isoproterenol treatment in hamsters.

Isoproterenol is a beta-agonist which induces a large increase in N-acetyltransferase (NAT) activity in the pineal gland of the rat when injected during the daytime (Delguchi and Axelrod, 1972b), at which time the enzyme is normally at its minimal level (Klein and Weller, 1970). In 1974, Romero and Axelrod reported that isoproterenol induction of NAT activity in the rat was dependent upon the previous activity of the sympathetic nerve fibers innervating the pineal. The pineal gland was shown to be most sensitive to isoproterenol induction of NAT during the latter part of the light period when sympathetic nerve activity was depressed (Romero and Axelrod, 1975).

Binkley (1976) reported that isoproterenol treatment during the day did not produce a significant induction of pineal NAT in male and female hamsters. The results obtained in the present study suggest, however, that isoproterenol injections can induce NAT activity in the

pineal when administered shortly before the onset of darkness (i.e., when the pineal is most sensitive) as evidenced by the abolishment of the estrous cycle. The hypothesized induction of NAT activity by isoproterenol treatment should result in an increased synthesis of melatonin. This interpretation is supported by the recent demonstration that chronic melatonin treatment caused inhibition of the estrous cycle in 14L:10D-exposed hamsters (Tamarkin et al., 1976). It is possible that the apparent differences between the present study and that of Binkley's (1976) due to by a difference in the time of injection, but those data are unavailable. The time of isoproterenol administration may be especially critical in studies using the hamster since evidence indicates that the day-night differences in pineal NAT levels are far less dramatic in the hamster (Rudeen et al., 1975, Binkley, 1976) than those found in the rat (Klein and Weller, 1970). Further experiments are required to determine the effects of isoproterenol treatment on the estrous cycle and NAT levels during the subsensitive and supersensitive time periods.

Hamsters maintained in LL and treated daily with isoproterenol before the onset of activity became acyclic after five weeks of treatment (Fig. 2). However, the estrous cycle in those animals was reestablished three weeks later. During that time the injections were administered after the onset of activity because of the progressive decrease in T in those animals. The reappearance of estrous cyclicity was unexpected since exposure of rats to LL depresses the diurnal rise of NAT activity (Klein et al., 1971) and reduces sympathetic nerve activity (Axelrod, 1974) in the pineal. Those conditions should make the pineal gland sensitive to isoproterenol induction of NAT

activity regardless of the time of injection, provided enough time is allowed between treatments to prevent the subsensitivity induced by repeated isoproterenol administration (Axelrod and Romero, 1974). A second group of hamsters treated with isoproterenol in LL also became acyclic, confirming the results observed in the first isoproterenol treated group. The animals in the second group were sacrificed while they were acyclic, and vaginal smears taken several days before the hamsters were sacrificed showed that those animals were in a state of persistent estrus. It is likely that isoproterenol treatment in LL affects the estrous cycle via the cyclic center in the preoptic area of the hypothalamus since inhibition of that center induces high, constant levels of luteinizing hormone (LH) in persistently estrous rats (Donovan, 1970). Although isoproterenol injections may stimulate a surge in pineal melatonin content, this surge should not affect the estrous cycle in LL since daily, single melatonin-injected hamsters exhibited regular estrous cycles throughout the experiment (Fig. 2).

Effects of Melatonin Treatment

Hamsters exposed to constant light and treated with single injections of oil or melatonin continued to cycle regularly throughout the experiment. Those data support the contention that exposure of hamsters to constant light acts similarly to pinealectomy. Tamarkin et al. (1976) have shown the loss of a diurnal sensitivity of the reproductive system to single, daily melatonin injections in both male and female hamsters following pinealectomy. The observation that single injections of melatonin had no detectable effect on the estrous cycle (Fig. 2) when injected shortly before the onset of activity

(i.e., during the hamster's "night"), in hamsters exposed to LL, indicates that the animals may need a period of darkness to trigger the system's sensitivity to single injections of melatonin. Constant light apparently prevents the development of the sensitive period to single-melatonin injections reported by Tamarkin et al. (1976). Double-melatonin injections, in contrast, significantly reduced estrous cyclicity in LL-exposed hamsters. The finding that the double-melatonin injection scheme inhibited cyclicity, while single injections were without effect, implies that the double injections may have synchronized the diurnal sensitivity of those hamsters. It is not known why two of the animals in the LL-melatonin 2x group continued to exhibit regular estrous cycles through the duration of treatment. It should be emphasized that a few hamsters have been reported to continue to cycle even after exposure to short-day photoperiods for as long as 18 weeks (Seegal and Goldman, 1975).

Tissue and Body Weights

Effects of Isoproterenol Treatment on Uterine Weights

The uterine weights of hamsters maintained in 14L:10D were greatly depressed by isoproterenol treatment when compared to control animals (Fig. 3). Curiously, the persistently estrous uteri observed in LL-exposed hamsters treated with isoproterenol were slightly heavier than the uteri observed in the controls.

In nature, the uterus involutes in animals exposed to short days (Reiter, 1973). That effect has been attributed to increased pineal secretion of melatonin during the lengthening nights (Wurtman and

Moskowitz, 1977a, 1977b). The data obtained from isoproterenol treatment in hamsters exposed to a 14L:10D photoperiod supports this contention. The induction of increased amounts of NAT activity in the pineal by isoproterenol during the sensitive time period of the day stimulated an increase in pineal production of melatonin, which caused uterine atrophy. It appears that the melatonin produced by isoproterenol induction of pineal NAT activity interacts with the endogenous melatonin rhythm to produce gonadal atrophy in a similar manner to properly-timed melatonin injections, as postulated by Tamarkin et al. (1977a).

Ovulatory surges of Luteinizing hormone (LH) occur once every four days during the proestrus stage of the cycle of rats (Daane and Parlow, 1971) and hamsters (Turgeon and Gilbert, 1972). In 1973, Ying and Greep showed that preovulatory melatonin administration blocked the ovulatory surge of LH in rats. It may be possible that isoproterenol treatment also blocked the ovulatory surge of LH in 14L:10D-exposed hamsters by stimulating melatonin production on the day of proestrus, in a manner similar to that suggested by Andre and Parrish (1978), who found that quartan (every four days) injections of melatonin on the day of proestrus induced estrous acyclicity in hamsters. It would be of interest to determine the effects of quartan injections of isoproterenol on uterine weights and estrous cyclicity when administered shortly before the onset of darkness during the proestrus stage of the estrous cycle in hamsters.

The higher uterine weights found in hamsters maintained in LL and treated with isoproterenol (Fig. 3) correlate with the presence of a persistent estrous condition. During estrus the uterus is large

and well-supplied with blood when compared to other stages of the estrous cycle.

Effects of Melatonin Treatment on Uterine Weight

The hamsters treated with single, daily injection of melatonin had uterine weights similar to those of the control group. Conversly, the hamsters treated with daily double-melatonin injections had greatly depressed uterine weights when compared to double-injected oil controls. Those results indicated that single, daily injections of melatonin in hamsters maintained in LL are ineffective in inducing uterine regression due to a lack of sensitivity to the indole because of the absence of an endogenous melatonin rhythm. Furthermore, the results from this experiment support the hypothesis that LL exposure causes functional pinealectomy in hamsters. Reiter et al. (1976) showed that melatonin injections in pinealectomized hamsters during the sensitive part of the day had no effect on uterine weights. Pinealectomized hamsters, like those maintained in LL, did not have an endogenous melatonin rhythm.

Although double injections of melatonin significantly reduced uterine weights in the hamster maintained in LL, two of the animals in that group, which continued to exhibit normal estrous cycles, had appreciably larger uteri. It is probable that the double injections would have caused acyclicity and complete uterine atrophy in all of the animals had the experiment been continued longer than 11 weeks. The presence of a regular estrous cycle in two of those hamsters is not readily explicable. Elliot (1978, Personal Communication) has shown a positive wheel-running effect on the testes of hamsters

maintained in nonstimulatory photoperiods. The animals that had running-wheels available exhibited considerably less testicular regression and higher blood levels of LH than animals that did not have running-wheels. It may be that the availability of running-wheels maintained cyclicity in two of the LL-melatonin 2x animals by promoting submaximal, but threshold LH secretion. It is possible that the double-melatonin injection created an endogenous rhythm of melatonin which induced significant uterine atrophy. This idea is supported by the discovery that triple injections of melatonin can cause gonadal regression in pinealectomized male and female hamsters (Tamarkin et al., 1977a) and the fact that uterine atrophy did not occur in control animals which received daily double injections of oil (Fig. 3). Further studies in progress indicate that uterine atrophy also may occur after daily triple-melatonin injections in intact hamsters maintained in constant light (Renner and Parrish, unpublished).

Effects of Injections on Ovarian, Adrenal, Pineal and Body Weights

The ovarian weights in all of the experimental hamsters were not significantly different from the ovarian weights observed in the controls (Table 1). Those results were expected since ovaries in hamsters do not regress but may enlarge slightly when the animals become acyclic (Reiter and Johnson, 1974).

The adrenal weights in the isoproterenol and single-melatonin treated hamsters were not significantly affected when compared to control animals (Table 1). A significant increase was observed in the relative weights (mg 100g/body wt.) of the adrenal glands from hamsters injected with double-doses of melatonin when compared to the adrenal weights found in double injected control animals (Table 1).

However, when absolute adrenal weights were compared in the two groups no significant difference was found. This can be accounted for by the fact that three out of the seven hamsters which received double-melatonin treatment lost weight during the course of the experiment, resulting in an inflation of the relative adrenal weights. Although, the adrenal weights were not depressed in any of the hamsters treated with oil or melatonin, as would be expected from a previous report (Reiter and Hesster, 1966), both exposure of female rats to constant light for 9.5 weeks (Reiter and Klein, 1972) or melatonin treatment (Motta et al., 1968) have been reported to raise adrenal weights of rats chronically treated with melatonin. Interactions between the adrenal gland and melatonin require further clarification.

The pineal weights reported in the present investigation were significantly different in animals treated with double-injections of melatonin when compared to the control animals. The decrease in body weight in some of the LL-melatonin 2x animals may have accounted for the increase in the gland's average relative weight, since the absolute pineal weights were not significantly different between the two groups. However, it is possible that the exogenous melatonin may have been taken up by the pineal glands of the hamsters maintained in LL. The hamsters treated with single, daily melatonin injections also showed a slight, but nonsignificant increase in pineal weight. In contrast, hamsters that received isoproterenol in the 14L:10D photoperiod had significantly reduced pineal weights when compared to the control group indicating that isoproterenol may not only induce melatonin synthesis by stimulating increases in NAT levels, but may also enhance the release of melatonin. The pineal weights of isoproterenol-

treated hamsters maintained in constant light did not differ significantly with pineal weights found in control animals (Table 1).

Locomotor Activity Records

Hamsters exposed to normal photoperiods are almost exclusively active during the dark phase of the day, while hamsters maintained in constant light exhibit free-running locomotor activity. In the present study, hamsters in the LL-melatonin 1x, LL-melatonin 2x, LL-oil 1x, LL-oil 2x and LL-isoproterenol 1x groups exhibited free-running activity (Fig. 5, 6, 7, 8, 9). In all of those animals, the initial period length of free-running activity varied from -1.84 minutes less than 24 hours to 3.83 minutes greater than 24 hours. The appreciable reduction in activity intensity exhibited by LL-isoproterenol 1x animals (Fig. 7) was attributed to the general effects of the drug. Those animals became inactive for approximately two hours after the isoproterenol treatment, indicating that the beta-agonist obviously affected sites other than the pineal gland.

The animals in the melatonin- and oil-injected groups maintained a regular estrous cycle, even when activity patterns indicated that the locomotor activity of several of the animals was arrhythmic. This contrasts with observations made by Stetson *et al.* (1976), who reported that arrhythmicity in locomotor activity was accompanied by acyclicity in non-injected hamsters maintained in LL. It may be that the daily injection regimes served as an environmental cue which stimulated the maintenance of the estrous cycle.

The double-injected oil group showed atypical locomotor activity patterns (Fig. 8). The injections may have generated an environ-

mental cue which prevented the normal free-running locomotor activity. The 180° shift in locomotor activity demonstrated by some of the animals in that group (Fig. 8) indicated that the animals may have vacillated between entraining on the morning and the evening oil injection.

Interations of Pineal Melatonin, the Estrous Cycle and Locomotor Activity

The demonstration of a diurnal sensitivity to daily, single injections of melatonin in 14L:10D ezposed hamsters and the absence of a similar response in pinealectomized (Tamarkin et al., 1977), as well as LL-exposed hamsters, as shown by the present study, suggests that an endogenous rhythm of melatonin synthesis is necessary for the induction of gonadal quiescence by exogeneous melatonin in female hamsters. This contention is further supported by the observation that isoproterenol completely suppressed ovarian and uterine function when administered to 14L:10D exposed hamsters (Table 1, Fig. 3). These results indicate that the hypothalamus-pituitary-gonadal axis is unresponsive to exogenous melatonin when there are no daily fluctuations in pineal melatonin content, as occurs in hamsters exposed to LL or 20L:4D photoperiods (Goldman, 1978, personal communication). The fact that isoproterenol injections induced a persistent estrous condition when the injections were administered before or near the onset of activity, while showing a loss of effectivemess when administration occurred one to two hours after the onset of activity (Fig. 2, 4) suggests that the endogenous estrus-cycle clock exhibits a time-dependent sensitivity to isoproterenol. Moreover, the theoretical diurnal sensitivity of

the estrous cycle to isoproterenol in LL does not appear to involve increased melatonin synthesis. While isoproterenol may induce NAT activity, and therefore, melatonin synthesis in LL conditions, the melatonin produced would not be expected to induce persistent estrus in view of the lack of a similar effect found in hamsters treated with single injections of melatonin. It appears that isoproterenol acted on other beta-receptors, possibly those of the LH cyclic center, located in the anterior hypothalamus, to stimulate a high constant level of LH, and thus, maintenance of persistent estrus. It would be of extreme interest to test this theory by treating pinealectomized hamsters maintained in LL with isoproterenol. The present finding that double injections of melatonin given 12.5-14 hours apart drastically inhibited estrous cyclicity (Fig. 2) and significantly reduced uterine weights, (Fig. 3) while double-oil injections were without similar effect, indicates that melatonin injections are inhibitory only when the hamster's biological clock is synchronized by long photoperiods or chronic melatonin injections spaced 12-14 hours apart, which may somehow simulate a long-day type photoperiod. It seems more than coincidental that melatonin injections two hours before the onset of darkness are as equally effective in the suppression of gonadal function in 14L:10D-exposed hamsters (Tamarkin et al., 1976), as injections of melatonin 14 hours earlier, two hours before the onset of light (Tamarkin et al., 1977a).

The data presented above seem to indicate that the diurnal sensitivity of the reproductive system to pineal melatonin is due to the presence of a sensitivity rhythm in the endogenous reproductive clock which in turn is caused by the diurnal rhythm of melatonin synthesis. The absence of an endogenous melatonin rhythm in pineal-

ectomized hamsters and in hamsters exposed to LL produces a nonsensitive endogenous clock. The double melatonin injection scheme employed in the present study produced an exogenous "clock" which may have induced a sensitivity rhythm in the endogenous reproductive clock. When the injections are interpreted as "long-days" as in the present study, the endogenous reproductive clock is sensitized and melatonin injections become inhibitory. Obviously, further investigations are required to support this hypothesis. Of paramount interest would be the response of LL-exposed hamsters to double injections of melatonin spaced at different time intervals during the inactive part of the hamster's day.

SUMMARY

Female Syrian hamsters were maintained in constant light (LL) or a 14L:10D photoperiod (LD). The animals were treated daily with melatonin (25 ug per injection), isoproterenol (0.2 mg per injection) or 0.1 ml of the peanut oil vehicle. The single, daily injections were administered prior to the onset of locomotor activity in LL or lights-off in LD. The double injections were administered prior to the onset of locomotor activity and 10-11.5 hours after the onset of locomotor activity. The locomotor activity and estrous cyclicity were monitered on a daily basis.

The animals treated with a single daily injection of melatonin, and single or double injections of oil continued to cycle throughout the experiment. The locomotor activity exhibited by the single-melatonin and -oil injected animals showed a free-running pattern in constant light. The uterine weights in those groups were not significantly different. The absence of an inhibitory effect of single melatonin injections on estrous cyclicity supports the hypothesis that constant light inhibits the diurnal rise in pineal melatonin production.

Hamsters treated with double injections of melatonin also exhibited free-running locomotor activity, however, the uterine weights in this group were depressed and 66.7% of the females became acyclic. These results suggest that properly timed double injections of melatonin are antigonadal because they produced a diurnal melatonin rhythm.

The animals treated with isoproterenol in LL became persistently estrus after 3-5 weeks of injections as evidenced by their uterine

weights which were similar to those found in the controls. The hamsters in this group showed reduced activity intensity. The reason for the persistent estrus condition in these animals was postulated to be due to the compound's effect on beta-adrenergic receptors in the cyclic center of the preoptic area of the hypothalamus. Although isoproterenol stimulates pineal NAT activity and thus, melatonin synthesis, the melatonin produced would not be expected to be antigonadal since single injections of melatonin were ineffective as an inhibitor of the reproductive function of female hamsters maintained in LL.

In contrast, isoproterenol injections in animals maintained in a 14L:10D lighting regimen were in a constant diestrous state after 5 weeks. Animals in this group showed dramatically depressed uterine weights similar to those found in hamsters treated with properly-timed double injections. These data support the hypothesis that isoproterenol induces melatonin synthesis by activation of NAT in the hamster. The data also supports the hypothesis that melatonin is the antigonadal hormone produced by the pineal gland.

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