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There is an increasing body of evidence that the pineal gland acts as a neuroendocrine transducer, translating neural information about environmental lighting condition into hormonal information. In the rat, light controls the production of melatonin which acts via the hypothalamic-hypophyseal pathway to control the function of the gonads. A similar mechanism seems to operate in other laboratory animals. The effect of photoperiod on the onset of the breeding season in large animals such as sheep has been studied, but the mechanism by which light affects their reproduction has not been elucidated. To test the hypothesis that the mechanism by which light controls reproduction in sheep is similar to the mechanism that operates in rats, an experiment was conducted to determine the effects of melatonin on reproductive phenomena in sheep. A solid-phase radioimmunoassay was

developed as a means of studying the effects of melatonin on serum interstitial cell stimulating hormone (ICSH).

Twenty-eight ewe, ram and wether lambs were divided into a control and a treatment group. Treatment consisted of daily subcutaneous injections of 0.1 mg. melatonin per kilogram of body weight administered in propylene glycol. Treatment was initiated on the third day after birth and continued until autopsy just before the suspected onset of puberty.

Melatonin in the doses employed caused a significant increase ($P < .05$) in ovarian weight in ewe lambs and a depression ($P < .10$) in castration-hypersecretion of ICSH in wether lambs. No other significant effects were observed.

The role of melatonin in controlling reproductive phenomena in sheep is still not clear. The known effects of melatonin in rats could not be duplicated in sheep. It appears that the mechanism by which light controls reproduction in sheep is not similar to that which operates in rats.

The solid phase radioimmunoassay developed for this experiment proved to be a rapid, sensitive and highly reliable technique for the determination of serum ICSH.

The Effects of Exogenous Melatonin on the Plasma Levels of Interstitial Cell Stimulating Hormone in Sheep

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THE EFFECTS OF EXOGENOUS MELATONIN ON THE PLASMA LEVELS OF INTERSTITIAL CELL STIMULATING HORMONE IN SHEEP

I. INTRODUCTION

The existence of the pineal gland has been known for over 2000 years (Herophilos of Alexandria, 325-280 B. C.). Up until a few years ago the function of this gland remained a mystery. There is, however, an increasing body of evidence that the pineal functions as a neuroendocrine transducer, receiving neural information about environmental lighting conditions and translating it into hormonal information. This in turn affects the growth and development of the reproductive system.

The mechanism by which light affects gonadal development in rats is reasonably well understood. Light controls the production of melatonin, a hormone produced exclusively in the pineal gland, which in turn acts via the hypothalamic-hypophyseal pathway to retard the growth and development of the gonads.

Although the effect of photoperiod on the onset of the estrous season has been studied in sheep, the mechanism by which light affects their reproduction has not been elucidated. Since the mechanism that operates in rats seems to operate in other laboratory animals, it is possible that melatonin could also control certain aspects of reproduction in sheep.

An understanding of the mechanism by which light affects reproduction in seasonal breeders such as sheep will enable man to intervene for his own benefit. The advantages of such manipulation are clearly evident.

This experiment was carried out to determine if melatonin affects reproductive phenomena in sheep. An equally important part of this study was the development of a rapid and simple solid phase radioimmunoassay for ovine interstitial cell stimulating hormone (ICSH).

II. REVIEW OF LITERATURE

Otto Heubner, a German physician, was probably the first person to associate the pineal organ with the endocrine system. In 1898 he reported a case of precocious puberty in a four year old boy. A pineal tumor was found at autopsy. Later a comprehensive review of pineal tumors revealed two distinct types (Kitay, 1954a). True pineal tumors which resulted in hyperfunction of the gland were associated with delayed puberty while a tumor located near but not in the pineal led to hypofunction and precocious puberty.

The Effects of Pinealectomy and Pineal Extracts

Many data have been accumulated about the possible functions of the pineal gland. In their review of the literature, however, Kitay and Altschule (1954a) found that only a few of the 1800 references they examined could be analyzed statistically. Many of the papers prior to 1954 suggest a pineal-pituitary-gonadal interrelationship but little credence can be given to this early work because of an insufficient number of experimental animals.

Kitay's review on pineal tumors indicates that lesion of the gland in young boys is often associated with precocious puberty. Pinealectomy in 26 to 30 days old intact female rats resulted in ovarian hypertrophy at 50 to 60 days of age (Izawa, 1926; Kitay, 1954b;

Wurtman, Altschule and Holmgren, 1959; Wurtman et al., 1960:1961). Female rats pinealectomized at 21 days of age or earlier did not show the characteristic ovarian hypertrophy indicating that the mechanism involved is age-dependent (Kitay, 1954b; Wragg, 1967). Removal of the pineal in mature female rats also led to an increase in pituitary weight (Wurtman et al., 1959). Pinealectomy in female hamsters of any age, however, did not appear to alter ovarian weight (Hoffman and Reiter, 1966).

Ablation of the pineal gland in immature male rats increased the weights of the seminal vesicles and prostates as compared to the sham-operated controls (Izawa, 1926; Thiéblot and Blaise, 1963; and Roth, 1965). Pinealectomy in sexually mature male rats significantly increased the pituitary weight (Motta, Fraschini and Martini, 1967; Fraschini, Mess and Martini, 1968). Ablation of the pineal resulted in a significant increase in pituitary levels of ICSH and follicle stimulating hormone (FSH) (Motta et al., 1967; Fraschini et al., 1968). Some workers have reported testicular hypertrophy after pinealectomy (Izawa, 1926; Thiéblot and Blaise, 1963). However, Roth (1965), Motta et al. (1967) and Fraschini et al. (1968) have all concluded that pinealectomy does not affect testicular weight. Pinealectomy in the ewe did not appear to affect seasonal breeding or the length of the estrous cycle (Roche et al., 1969a). Pinealectomy was

also without effect on the serum ICSH levels as determined by radioimmunoassay (Roche et al., 1969b).

The administration of a protein-free bovine pineal extract (BPE) to intact immature female rats caused a decrease in ovarian weight which was proportional to the administered dose (Kitay and Altschule, 1954b; Wurtman et al., 1959). Administration of BPE also decreased the ovarian weight in adult females, caused anestrus to occur in middle-aged rats in persistent estrus (Meyer et al., 1961) and depressed pineal cell activity (Holmgren, Altschule and Wurtman, 1960). Subcutaneous administration of BPE reversed the ovarian hypertrophy due to pinealectomy, decreased pituitary weight in intact females, but failed to reverse pituitary hypertrophy caused by pinealectomy (Wurtman et al., 1959).

Isolation of Melatonin and Enzymes Involved in its Synthesis in the Pineal Gland

Lerner and his co-workers isolated a new compound from bovine pineal glands which was 5000 times more potent than any other known substance in its ability to lighten frog melanocytes (Lerner et al., 1958). Because of its melanocyte-lightening ability the compound was called melatonin. In the following year the chemical structure of melatonin was found to be N-acetyl-5-methoxytryptamine (Lerner, Case and Heinzelman, 1959b). Melatonin was found to be

highly localized in the pineals of cattle, monkeys and man (Lerner et al., 1959c), rats (Prop and Ariens Kappers, 1961), domestic fowl (Axelrod, Wurtman and Winget, 1964), cats (Wurtman, Axelrod and Potter, 1964) and lambs (Van de Veerdonk, 1965). The formation of melatonin starts with the decarboxylation of 5-hydroxytryptophan to form serotonin which is then acylated to form N-acetyl serotonin, the immediate precursor of melatonin (Weissbach and Axelrod, 1960). The final step in the biosynthesis of melatonin involves the 0-methylation of N-acetyl-serotonin by the enzyme hydroxyindole-0-methyl transferase (HIOMT) with S-adenosyl methionine participating as a methyl donor (Axelrod and Weissbach, 1960; Wurtman and Axelrod, 1965a, b). The key enzyme in melatonin synthesis, HIOMT, is found only in the pineal gland (Axelrod and Weissbach, 1961; Barchas and Lerner, 1964).

Because it is highly concentrated in the pineal and the enzyme HIOMT necessary for its production is found exclusively there, melatonin is considered to be a pineal hormone. Although melatonin has not yet been isolated from blood or urine, its presence there can be inferred from the fact that it has been found in minute quantities in peripheral nerves. These nerves cannot synthesize melatonin and presumably must concentrate it from the blood (Lerner et al., 1959c). It must be remembered that there are a host of other biologically

active amines found in the pineal gland and melatonin may only be one of a group of hormones produced in this structure.

Effects of Exogenous Melatonin on Reproduction

When immature female rats were injected with 20 ug. of melatonin daily for four weeks and kept in normal lighting the ovarian weights and the incidence of estrus were both significantly decreased (Wurtman, Axelrod and Chu, 1963a). In a subsequent experiment it was shown that as little as 1 ug. of melatonin given subcutaneously daily for four weeks could significantly decrease ovarian weight (Wurtman et al., 1963a). Melatonin treatment in prepuberal rats was also associated with a delay in the canalization of the vagina (Wurtman et al., 1963a; Motta et al., 1967) and a decrease in pituitary and uterine weights (Motta et al., 1967).

Administration of 20 ug. of melatonin per day to immature female rats decreased the incidence of proestrus, estrus and metestrus to 37 percent from a control value of 52 percent (Chu, Wurtman and Axelrod, 1964). In a similar experiment as little as 2 ug. melatonin administered daily to immature females resulted in a 20 percent incidence of estrus as compared to 45 percent for the control group (Chu et al., 1964). Within three days after the cessation of melatonin treatment the incidence of estrus of the treatment group rose to a

value equal to the control value of 45 percent and continued to rise to a peak value of 62 percent after which it again returned to a level comparable to the controls. Serotonin under the same conditions did not elicit a similar response (Chu et al., 1964).

The daily injection of 10 or more ug. of melatonin into mature females also resulted in a decreased incidence of estrus while dose levels below 10 ug. were ineffective (Chu et al., 1964). In a typical experiment 160 gram rats were given 10 ug. of melatonin daily for three weeks. At the end of this time the incidence of estrus had fallen to 27 percent. The same rats were then given only the vehicle for another three-week period, at the end of which the incidence of estrus had risen to 52 percent. When melatonin was again administered the incidence of estrus fell to 38 percent. Neither N-acetyl serotonin, the immediate precursor of melatonin, nor 6-hydroxymelatonin, the main metabolite, could evoke this response under the same conditions (Chu et al., 1964).

As mentioned, pinealectomy in female rats is associated with an increased incidence of estrus and ovarian hypertrophy. This increase in the incidence of estrus and ovarian weight can be inhibited by melatonin (Wurtman et al., 1959; Chu et al., 1964).

Injection of 200 ug. of melatonin per day into adult male rats significantly reduced the weights of the prostates and the seminal vesicles but failed to change the weight of the pituitary or the testes

(Motta et al., 1967; Fraschini et al., 1968). The fact that pinealectomy enhanced testicular as well as prostate and seminal vesicle weights indicates that the release of both FSH and ICSH from the pituitary were affected by the treatment. Pinealectomy resulted in an increased rate of release of ICSH as well as a significant increase in pituitary levels of this hormone (Frashcini, et al., 1968). It has also been demonstrated that a crude pineal extract was capable of inhibiting FSH release in vitro (Moszkowska, 1965) and in vivo (Thiéblot and Blaise, 1963). These data help to substantiate the theory that the inhibitory effect that the pineal exerts on the gonads is a secondary effect involving pituitary gonadotrophins rather than a direct peripheral inhibition of the gonads (Motta et al., 1967). The injection of very large doses of melatonin was unable to reverse all of the effects of pinealectomy as indicated by the failure of the treatment to affect testicular weight (Motta et al., 1967). The failure of melatonin to modify the FSH-dependent phenomena such as testicular weight indicates that there is possibly a separate factor in the pineal gland that controls the release of FSH (Motta et al., 1967). According to this hypothesis melatonin is capable of modifying ICSH secretion and therby affecting the weights of the testosterone-dependent structures while the FSH-dependent structures are unaffected (Motta et al., 1967; Fraschini et al., 1968).

The action of melatonin in delaying puberty can also be explained by its inhibitory effect of ICSH release and synthesis (Motta et al., 1967). It has been demonstrated that there is a drop in pituitary levels and a rise in plasma levels of ICSH during the normal onset of puberty (Ramirez and Sawyer, 1965a, 1966; Goldman and Mahesh, 1969). The increase in plasma ICSH levels is closely correlated to that period of growth in which vaginal opening and ovarian hypertrophy normally occur (Ramirez and Sawyer, 1965b, 1966; Corbin and Daniels, 1969). Since injection of melatonin delays the normal onset of puberty (Wurtman et al., 1963a, b; Motta et al., 1967) and inhibits ICSH release (Motta et al., 1967; Fraschini et al., 1968), the puberty delaying action of melatonin could be due to the inhibition of the normal rise in plasma ICSH during puberty. This has been demonstrated in rats by Adams, Wan and Sohler (1965) who found that the pituitary levels of ICSH decreased during days 35 to 39 in the controls, but in animals receiving 100 ug. of melatonin daily pituitary levels of ICSH did not decrease, the ovaries did not show normal hypertrophy, and the opening of the vagina was delayed.

Effect of Light on Reproduction

Fiske (1941) demonstrated that female rats maintained under conditions of constant illumination displayed a higher incidence of estrus than animals under normal light-dark conditions. The persistent estrus resulting from treatment with constant light was accompanied

by an anovulatory state (Lawton and Schwarz, 1965). The ovaries of such rats kept in constant light were characterized by well developed follicles and a few small corpora lutea (Negro-Vilar, Dickerman and Meites, 1968). Constant estrus could be induced in hamsters only after a six-month period in constant light (Kent, Ridgway and Strobel, 1968). The persistent estrus in hamsters was accompanied by an anovulatory state with no corpora lutea present in the ovaries (Kent et al., 1968). Exposure of immature female rats or hamsters to continuous illumination also resulted in ovarian and uterine hypertrophy (Fiske, 1941; Wurtman et al., 1960; Wurtman et al., 1961; Negro-Vilar et al., 1968; Kent et al., 1968). The effects of constant illumination on rat ovarian and uterine weights are transitory, being demonstrable after 56 days of light but not after 79 days (Wurtman et al., 1960). The presence of large ovarian follicles reported by Kent and his coworkers (1968) suggests that stimulatory levels of FSH are still present in the blood even after six months of constant light. The anovulatory state and the lack of corpora lutea resulting from constant lighting suggest that the release of ICSH from the pituitary has been inhibited. This is consistent with the theory of Critchlow (1963) who suggests that the cyclic release of ICSH essential for ovulation is inhibited in rats under constant lighting conditions. The retained follicles are responsible for the continuous estrogen production and the resulting constant vaginal estrus. Further

verification of this theory was provided by Maric, Matsuyama and Lloyd (1965) who found that the pituitary ICSH levels of animals in constant estrus were significantly lower than the controls and by Negro-Vilar et al. (1968) who demonstrated that the uteri of animals in constant light were characteristic of those due to prolonged estrogen action. No comparable work has been done on the effects of constant light on reproduction in sheep. The effect of photoperiod on the seasonal onset of estrus in the ewe is well known, but the mechanism involved has not been elucidated (Hafez, 1952).

Pineal Gland as a Transducer of Environmental Lighting

Wurtman and his coworkers (1960, 1961) noticed the similarities between the effects of constant lighting and pinealectomy. Both treatments resulted in ovarian hypertrophy and an increased incidence of estrus, but when given simultaneously the effects of the two treatments were not additive, indicating that the treatments were probably affecting a common physiological pathway (Wurtman et al., 1960; 1961). To determine if melatonin was involved in the effects of light on the rat gonad, Wurtman and his associates placed female rats in constant light for a period of four weeks after which the incidence of estrus was 85 percent. A single injection of 10 ug. of melatonin reduced the incidence of estrus to 38 percent. Furthermore, it was found that constant lighting decreased pineal weight (Wurtman et al.,

1960, 1961; Fiske, Bryant and Putnam, 1960) and inhibited pineal cell activity (Roth, Wurtman and Altschule, 1962). Gonadectomy, adrenalectomy, hypophysectomy and thiouracil feeding were all without effect on the increase in the weight of the pineal following exposure to constant light (Fiske, Pound and Putnam, 1962). These experiments provided the initial evidence that the mechanism by which photo-period affects the gonads involves the pineal gland.

Since it has already been demonstrated that the pineal contained large stores of serotonin (Giarman, 1959), a precursor of melatonin, the limiting factor in melatonin production must be the level of the enzyme HIOMT (Wurtman, Axelrod and Phillips, 1963b). The results of Roth and his coworkers (1962) indicated that protein systhesis in the pineal is inhibited when an animal is exposed to constant light. The inhibition of melatonin production by light is possibly the result of a decreased synthesis of HIOMT. Many data support the theory that light affects the production of HIOMT. Wurtman and his associates (1963b) placed 21 day old rats in constant light for six days and found that the levels of HIOMT and melatonin in the pineal gland were significantly reduced. The HIOMT activity of rats maintained in constant light was only one-half that of animals in normal lighting and one-tenth that of the animals maintained in constant darkness (Wurtman et al., 1963b). The effect of light on the pineal enzymes is specific for HIOMT with other enzymes such as

monoamine oxidase not being affected (Wurtman et al., 1963b). Furthermore, the decrease in HIOMT activity accompanying constant lighting is unaffected by removal of the ovaries or the pituitary (Wurtman, Axelrod and Fischer, 1964b). The level of HIOMT in the pineal follows a circadian rhythm being highest at night and lowest during the day (Axelrod, Wurtman and Snyder, 1965). This circadian rhythm is light-dependent in that it is abolished by the absence of diurnal photoperiod or by blinding (Wurtman et al., 1965). Superimposed on this circadian rhythm is an estrous rhythm in which the level of HIOMT and melatonin is highest at diestrus and falls during proestrus and estrus (Wurtman, et al., 1965).

The HIOMT and melatonin rhythms correspond to the rhythm of stalk median eminence (SME) ICSH-releasing factor during the estrous cycle (Ramirez and Sawyer, 1965b; Chowers and McCann, 1965). These investigators have shown a significant decrease in the SME level of ICSH-releasing factor during late proestrus and continuing through estrus. The decrease in SME ICSH-releasing factor during late proestrus and early estrus indicates an increase in the release of this hormone. Supporting evidence for this was provided by Lawton and Schwartz (1965) and Goldman and Mahesh (1969) who demonstrated that there was a 50 percent decrease in the pituitary levels of ICSH between proestrus and estrus. A similar pattern of ICSH secretion has been reported in sheep. Geschwind and Dewey

(1968) found that the plasma levels of ICSH in the cycling ewe were low throughout the cycle except for a very sharp peak at the beginning of estrus. The pituitary levels of ICSH showed a drop at the onset of estrus that corresponded to the rise in plasma levels (Robertson and Rakha, 1966).

The innervation of the pineal is largely sympathetic and originates from the superior cervical ganglia. It has been found that severing these nerves, removing both ganglia or removing the eyes abolishes the effect of environmental lighting conditions on HIOMT levels (Wurtman et al., 1964b). Severing the optic tracts, however, had no effect under the same conditions (Critchlow, 1963).

Blinding 25 day old rats or hamsters resulted in decreased weights of the seminal vesicles at 72 days of age and 150 days, respectively. This decrease was prevented if the animals were also pinealectomized (Reiter, Hoffman and Rubin, 1968). Blinding also eliminated the light-induced ovarian and uterine hypertrophy, the increase in the incidence of estrus, and the decrease in pineal weight associated with constant lighting (Wurtman et al., 1964b). Information about environmental lighting is received by the eye and transmitted to the pineal via the superior cervical ganglia (Wurtman et al., 1964b). In the pineal this information controls the production of melatonin by altering the synthesis of HIOMT (Roth et al., 1962; Wurtman et al., 1963a, b). Melatonin in turn exerts its inhibitory

effect on ICSH synthesis and release presumably by acting on the hypothalamic releasing factor (Fiske and Greep, 1959). This has been substantiated by Mess and Martini (1968) who implanted melatonin in the median eminence of rats and found that the plasma and pituitary levels of ICSH were significantly reduced. Placing melatonin directly in the pituitary or in the cerebral cortex did not show the same effect.

III. MATERIALS AND METHODS

An experiment was designed to test the effects of exogenous melatonin on reproductive phenomena in lambs. The end points considered were endocrine gland weights and serum ICSH levels.

Experimental Treatment

Twenty-eight Targhee X Columbia lambs were made available to study the effects of exogenous melatonin on the reproductive tracts and on the levels of serum ICSH. The lambs were divided at random into a control and a treatment group. The treatment group contained six ewe, three ram and six wether lambs while the control group consisted of five ewe, four ram and four wether lambs.

The experimental treatment was initiated on the third day after birth. The treatment consisted of a daily subcutaneous injection of 0.1 mg. of melatonin[1] per kilogram of body weight, administered in propylene glycol. The control group received an equivalent volume of propylene glycol without melatonin. The treatment was maintained throughout the experiment which lasted from July 1968 to January 1969. Both the treatment and the control groups were kept under identical environmental conditions.

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[1] Sigma Chemical Company, St. Louis, Missouri.

Two hundred ml. of blood were obtained from each lamb on the day before slaughter. The blood was obtained by jugular puncture between the hours of 9 a.m. and 12 noon. The blood samples were allowed to coagulate at 4^{o} C. The samples were then centrifuged and stored at -20^{o} C until used. The age at slaughter ranged from 139 to 161 days with a mean of 147 days.

The blood levels of ICSH were assayed by a solid phase radioimmunoassay based on the principles outlined by Catt, Niall and Tregear (1966). This technique is based on the binding of antibodies to polystyrene surfaces. The solid phase supports used in this assay were 15 mm. by 70 mm. plastic tubes. The ovine ICSH antibody used was developed in rabbits by Thompson (1970). This antibody was very potent and highly specific in that it did not cross-react with other ovine pituitary hormones.

Preparation of Antibody for Coating

The ovine ICSH antiserum was dialyzed against 2M ammonium sulphate for 12 hours. The dialysis bag was then washed free of precipitate with 2M ammonium sulphate, and the mixture was centrifuged at 500 times gravity for twenty minutes. The supernatent was aspirated off and discarded. The precipitate was dissolved in .05M phosphate buffer solution (PBS) pH 7.5 and dialyzed against this same buffer for an additional 12 hours. The resulting partially purified

γ-globulin was diluted 1:1000 in .05M PBS (pH 7.5) on the basis of the volume of original antiserum.

Coating Tubes with Antibody

Previous workers have specified that the pH of the antibody should be from 9.0 to 10.0 for good coating (Catt et al., 1966). This requirement was not necessary as a pH of 7.5 yielded excellent results. This pH was deemed more desirable because of the possibility of damage to the antibody at higher pH's.

The tubes to be coated were placed in specially designed racks and the antibody added through a long hypodermic needle so that only the desired surface area would be coated. An automatic syringe was used to deliver 0.7 ml. of a 1:1000 dilution of purified antiovine γ-globulin to each tube. The tubes were allowed to stand 30 minutes at room temperature after which the antibody was aspirated off and saved for reuse. The tubes were then completely coated with one percent bovine serum albumin (BSA) to occupy all the remaining binding sights. One ml. of one percent BSA solution was added and the tubes stored at 4^{o} C until they were used. The storage time for this assay was only two hours. At the start of the assay the one percent BSA was aspirated from the tubes. Each tube was then washed two times with 3 ml. of .05M PBS (pH 7.5). After the second wash the

tubes were allowed to stand 10 minutes and all of the remaining liquid in the tubes was removed by aspiration.

Iodination of Hormone

The labeled hormone was prepared by a modification of the method of Greenwood and Hunter (1963). This technique uses chloramine-T as an oxidant allowing free iodine to attach to the tyrosine residues of the hormone. The stepwise procedure for the iodination of ovine ICSH was as follows:

1. To a vial containing 2.5 ug. of hormone, add 25 ul. of 0.5M phosphate buffer, pH 7.5.

2. Add 1.0 millicurie of I^{131} and mix by tapping.

3. Add 30 ug. chloramine-T in 15 ul. distilled water and mix thoroughly.

4. After 1.5 minutes add 50 ul. of a solution containing 2.4 ug. sodium metabisulfite per microliter.

5. Add 100 ul. of a 16 percent sucrose solution containing 1 mg. of potassium iodide and 10 ug. bromophenol blue per 100 ul.

6. Remove mixture from the reaction vial a layer beneath .05M PBS on a 1 x 15 cm. disposable column of Biogel-P-60.[2]

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[2] Bio Rad Laboratories, distributed by Calbiochem, Los Angeles, California.

7. Wash the reaction vial with 75 ul. of the 16 percent sucrose solution and add it to the layered material on the column.

The labeled hormone was separated by collecting 8 drop fractions in tubes containing 8 drops of BSA. The peak tube of the protein fraction was diluted with .05M PBS containing 0.1 percent egg white until 0.1 ml. of the solution gave 50,000 to 55,000 counts per minute on an autogamma counter.[3]

Preparation of Standard Curve and Unknowns

Standards ranging from 0.2 to 25 nanograms were run in triplicate to establish a standard curve. The blood samples to be assayed were run in quadruplicate using 0.5 ml. of serum per tube. Standards and unknowns were allowed to react with the coated tubes for eight hours with continuous agitation on an automatic shaker at room temperature. At this time 100 ul. of the labeled hormone (approximately 55,000 cpm) were added to each tube. The tubes were then vortexed and placed on the shaker for an additional 12 hours.

Six tubes containing only the labeled hormone were used to determine the total number of counts added. These tubes were designated as total count tubes (TCT). Five tubes were coated with bovine γ-globulin instead of antiovine γ-globulin. The bovine γ-globulin does

[3] Model #3002, Packard Instrument Company, La Grange, Illinois.

not bind ovine ICSH specifically but does bind to the active sites on the tube surface. The bovine δ-globulin tubes are used to simultaneously determine the counts nonspecifically bound and the background count. The average of these tubes was automatically subtracted from all other tubes during the counting procedure. Nine antibody coated tubes were run containing 0.1 percent BSA in place of a standard or unknown. These tubes were designated BSA tubes. The average of these nine tubes gives the number of counts specifically bound in the absence of unlabeled hormone. Thus, these tubes establish the 100 percent point. All subsequent count rate were expressed as a percentage of the BSA count rate. In the presence of any unlabeled hormone the number of counts is decreased from the BSA count. Over a certain range the decrease in count rate is linearly related to the log of the amount of unlabeled hormone present.

In addition to the standard curve, a series of dilutions of a standard sheep serum known to contain ICSH was run. This determined if the large amount of blood added (0.5ml) seriously affected the performance of the assay.

Preparation of Tubes for Counting

After the twelve-hour incubation with the labeled hormone the assay tubes were aspirated and washed twice with 3 ml. of .05M PBS (pH 7.5). The assay tubes were then inserted into autogamma tubes

for counting. The five bovine γ-globulin tubes were counted first and the average count rate set into the automatic substraction unit so that all preceding tubes were corrected for background and counts nonspecifically bound. All tubes were counted for one minute. Because the counting took only three hours the count rates were not corrected for decay. The average number of counts for each level of standard and unknown was then expressed as a percent of the BSA tubes.

IV. RESULTS

Autopsy

The autopsy data for the control and treated ewe lambs are presented in Table 1. The means and standard deviations for each character measured are presented along with the t values comparing the difference between the two groups for each individual character. A two-tailed t test was used to compare significance at the .05 level (Steel and Torrie, 1960).

Table 1. Autopsy Data for Melatonin-Treated and Control Ewe Lambs

Character Measured	Melatonin-treated	Control	t Value
Age, days	136±14.4[a]	135.80±9.5[b]	
Body wt, lb.	101.16±2.5[a]	97.20±6.4[b]	1.46
Pituitary wt., mg.	539.77±57.3[a]	474.00±81.9[b]	1.57
Pineal gland wt., mg.	62.85±29.2[a]	77.90±40.6[c]	0.69
Adrenal wt., g.	2.73±0.70[a]	2.09±0.74[c]	1.39
Ovarian wt., g.	1.36±0.32[a]	0.77±0.21[c]	3.21[d]

[a] Mean based on 6 animals

[b] Mean based on 5 animals

[c] Mean based on 4 animals

[d] $P < .05$

The mean body weight for the melatonin-treated group was slightly higher than the control group. The mean pituitary weight for

the melatonin-treated group was approximately 65 mg. higher than the mean of the control group, but this difference was not significant. There was a great deal of variation between individual pituitary glands within the control and treatment groups. The pineal gland weight in the melatonin-treated group was lower than in the control group, but here again the difference was not significant. Since the melatonin-treated group had heavier pituitaries and lighter pineals than the control group, a negative correlation between pituitary weight and pineal weight was suspected. The correlation coefficient, however, was only -0.56. The ovaries of the melatonin-treated group were significantly ($P < .05$) heavier than the ovaries of the control group. There were no gross indications that any of the ewe lambs had reached puberty at the time of slaughter; the ovaries and reproductive tracts were infantile in appearance.

The autopsy data for the wether lambs are presented in Table 2. None of the characters measured for the treatment group was significantly different from the control values.

The data for the ram lambs are presented in Table 3. Unfortunately, two of the three ram lambs assigned to the treatment group died during the experiment from causes unrelated to treatment. For this reason no comparisons could be made.

Table 2. Autopsy Data for Melatonin-Treated and Control Wethers

Character Measured	Melatonin-treated[a]	Control[b]	t Value
Age, days	145.8±4.9	147.8±9.4	
Body wt., lb.	109.33±14.5	113.00±11.8	0.45
Pituitary wt., mg.	571.43±103.9	583.30±67.6	0.07
Pineal gland wt., mg.	65.93±39.4	62.85±19.8	0.18
Adrenal wt., g.	2.90±1.1	2.44±1.3	0.77

[a]Mean based on 6 animals

[b]Mean based on 4 animals

Table 3. Autopsy Data for Melatonin-Treated and Control, Intact Ram Lambs

Character Measured	Melatonin-treated[a]	Control[b]
Age, days	153	149.0±2.7
Body wt., lb.	133	116.50±14.5
Pituitary wt., mg.	599.6	575.50±215
Pineal wt., mg.	---	83.90±19.3
Adrenal wt., mg.	2.50	2.64±0.25
Testes wt., g.	442	433.75±121.00

[a]Mean based on one animal

[b]Mean based on four animals

Radioimmunoassay

The tabulated data for the standard curve are presented in Table 4. The amount of labeled hormone added to each tube was approximately 55,000 cpm, while the number of counts nonspecifically

bound averaged 183. The number of counts bound in the absence of any unlabeled hormone averaged 43,821, which represented 79 percent of total activity added. The percent of the counts bound for the standards ranged from 76.6 percent for 0.2 mug. to 11.4 percent for 25 mμg.

Table 4. Results for the Standard Curve Determination

Sample	No. of Determinations	Average No. of Counts/min.	Percent of BSA
Total count	3	55,422	
Bovine γ-globulin	5	183	
Bovine serum albumin tubes	9	43,821	100
mμg. of Ovine ICSH			
.2	3	33,574	76.6
.3	3	32,615	74.4
.4	3	32,783	74.8
.7	3	32,329	73.8
1.0	3	31,813	72.6
1.5	3	26,908	61.4
2.5	3	24,207	55.2
4.0	3	18,952	43.3
6.0	3	14,335	32.7
9.0	3	12,005	27.4
15.0	3	6,874	15.7
25.0	3	4,987	11.4
Standard serum in μl.			
100	3	33,029	75.4
200	3	30,946	70.6
300	3	28,751	65.6
500	3	22,798	52.0

The percentage of the counts bound was plotted against the log of the nanograms of ovine ICSH as illustrated in Graph 1. The percentage of the counts bound was linearly related to the log of mug. of ICSH from 1.0 to 15 mug. A regression line was fitted for this linear portion of the curve which represents a range in percent bound from 74 percent to 15 percent. Above 74 percent and below 15 percent binding the curve flattens out and is irregular.

The estimates of the levels of serum ICSH for the ewe lambs are presented in Table 5.

Table 5. Serum ICSH Levels for Melatonin-treated and Control Ewe Lambs

Animal No.	No. of Determinations	Average Counts/min.	Percent of BSA	mμg. ICSH/ml.
		Melatonin-treated animals		
352	4	31,037	70.8	2.36
362	4	28,810	65.7	2.98
218	4	31,163	71.1	2.30
				Mean = 2.55±0.39
		Control		
358	4	30,615	69.9	2.54
360	4	29,290	66.8	2.98
366	4	29,211	66.7	3.00
				Mean = 2.84±0.27

The serum level of ICSH was very low in both the melatonin-treated and control groups. These low levels are very near the limit of sensitivity for this particular assay.

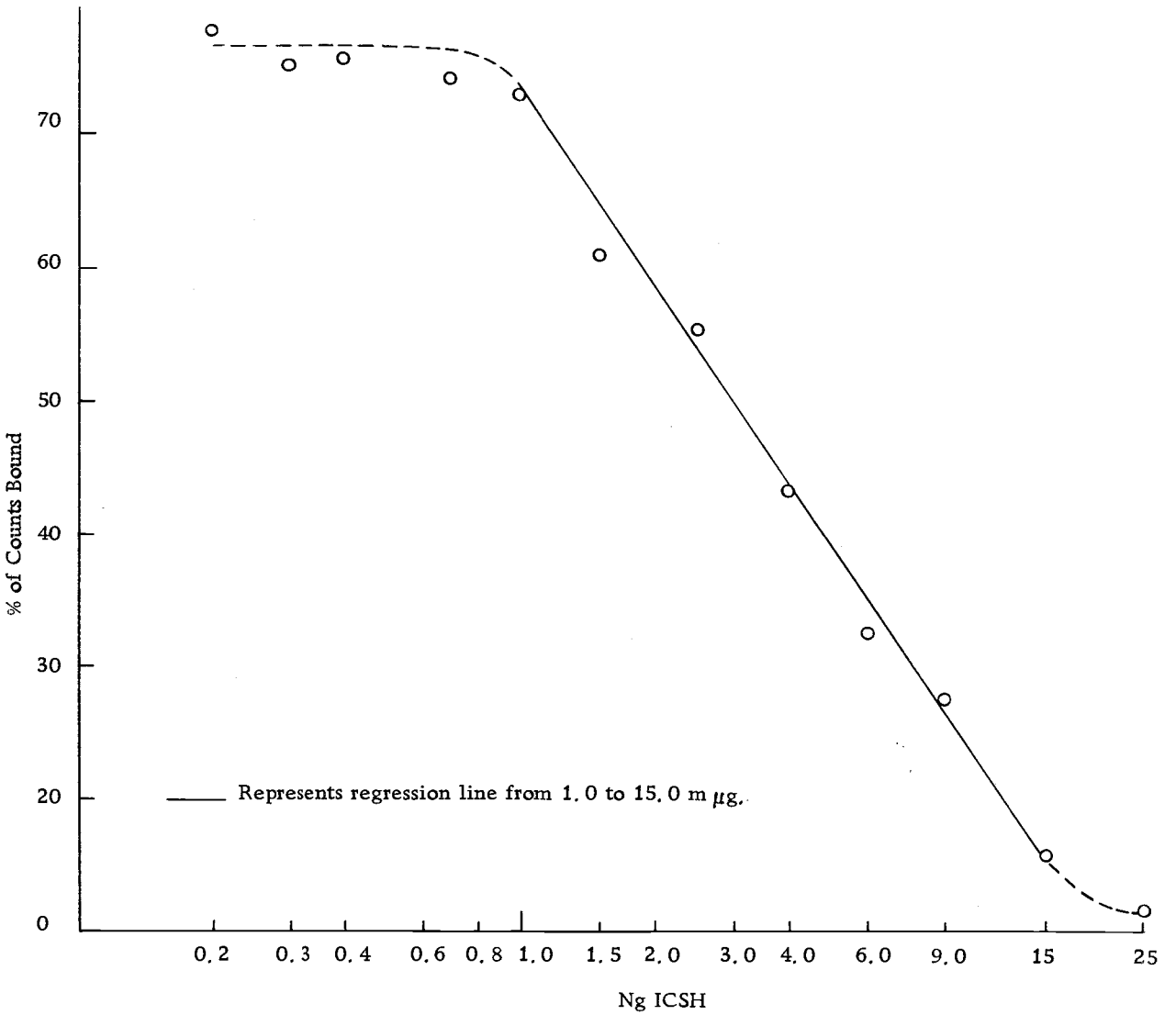

Figure 1. Percent of Counts vs. Nanograms of ICSH

The serum levels of ICSH for the melatonin-treated, control wethers, and intact ram lambs are presented in Table 6.

Table 6. Serum ICSH Levels for Melatonin and Control Wethers and Intact Control Ram Lambs

Animal No.	No. of Determinations	Average Counts/min.	Percent of BSA	mμg. ICSH/ml.
		Melatonin-treated Wethers		
215	3	18,970	43.3	8.10
223	4	22,664	51.7	5.60
353	4	24,824	56.7	4.48
363	4	17,410	39.7	9.62
365	4	23,925	54.6	4.90
371	4	23,870	54.5	4.94
				Mean = 6.27±2.09
		Control Wethers		
209	4	13,833	31.6	13.70
361	4	17,069	39.0	10.00
369	4	22,654	51.7	5.60
373	4	16,587	37.8	10.44
				Mean = 9.94±3.33
		Intact Controls		
219	4	30,288	69.1	2.56
225	4	29,733	67.9	2.68
357	4	30,570	69.6	2.48
				Mean = 2.57±0.10

The serum ICSH levels for the intact control ram lambs were very low. As expected, castration produced a marked rise in serum ICSH ($P < .01$). This castration-hypersecretion appears to be partially blocked by melatonin treatment. Although the difference between

the melatonin-treated and control wethers was not significant at the .05 level, it was significant at the .10 level.

Table 7 summarizes the results for the ICSH determinations.

Table 7. Summary of ICSH determinations

Group	Melatonin-treated		
Mean, mμg.	ewe lambs	Control ewe lambs	t=1.07
	2.55±0.39	2.84±0.27	
Group	Melatonin-treated		
Mean, mμg.	wethers	Control wethers	
	6.27±2.09	9.94±3.33	t=2.17[a]
Group	Control ram lambs	Control wethers	
Mean, mμg.	2.57±0.10	9.94±3.33	t=4.41[b]

[a] $P < .10 > .05$

[b] $P < .01$

V. DISCUSSION and CONCLUSIONS

Although melatonin is a very important hormone in controlling reproduction in rats and other small laboratory animals, its possible role in controlling reproductive phenomena in sheep is still not clear. None of the known effects of melatonin in rats could be duplicated in sheep. The only significant effects of melatonin in this experiment were an increase in ovarian weight in ewe lambs and a suppressed castration-hypersecretion in wether lambs. It appears that the mechanism by which environmental lighting affects reproduction in sheep is not similar to the mechanism that operates in small laboratory animals. The conclusions of Roche et al. (1969a, b), that pinealectomy does not affect cyclic reproduction or serum ICSH levels, would indicate that the pineal gland plays little, if any, role in controlling reproduction in mature sheep. There is no information concerning the physiological levels of melatonin in sheep, but the doses used were assumed to be large. The effects of melatonin observed in this experiment may only be due to the dose level and may reflect a pharmacological rather than a physiological response.

It can be concluded, however, that at least under the conditions employed in this experiment, melatonin can modify some aspects of reproduction in sheep. The ovaries of the melatonin-treated ewe lambs were significantly heavier ($P < .05$) than those of the controls.

This finding is in direct opposition to the reported effects of melatonin treatment in prepuberal rats. When immature rats were given 20 ug. of melatonin daily for four weeks, their ovarian weights were significantly decreased (Wurtman et al., 1963a). These melatonin-treated rats, however, were postpuberal at the time of autopsy, whereas the sheep in this experiment were prepuberal. The levels of ICSH for these ewe lambs were very low and did not differ from the control group. An increase in serum FSH could be a possible explanation, but this seems unlikely in view of the fact that clinical examination of the ovaries did not reveal any evidence of FSH stimulation. In addition, pinealectomy in mature ewes does not affect cyclic sexual activity (Roche et al., 1969a). It appears unlikely that the increase in ovarian weight in the melatonin-treated ewe lambs can be attributed to changes in gonadotrophin levels. Since tritiated melatonin is selectively concentrated in the ovary, a direct effect of melatonin on the ovary is possible (Wurtman, Axelrod and Phillips, 1963b). However, all existing evidence indicates that in rats melatonin decreases rather than increases ovarian weight. Further work will be necessary to elucidate the significance of this finding.

As expected, castration of the ram lambs caused a significant increase ($P < .01$) in serum ICSH levels. This indicates that the steroid feedback mechanism is functional in the prepuberal ram lamb and that castration results in gonadotrophin hypersecretion. This castration-hypersecretion appears to be inhibited by melatonin

treatment. The difference in ICSH levels between the melatonin-treated and control wethers was not significant at the .05 level, but it was significant at the .10 level. This decrease in serum ICSH is consistent with the observations that melatonin decreases seminal vesicle and ventral prostate weights in rats (Motta et al., 1967; Fraschini et al., 1968). The weights of these structures are known to decrease as ICSH titers decrease.

The second objective of this study, the development of a solid phase radioimmunoassay, was highly successful. The assay used has many advantages over traditional double antibody techniques. The solid phase assay can be carried out in 24 hours or less compared to 120 hours for the traditional double antibody technique. The solid phase assay can be run at room temperature, while the double antibody technique is run at 4^{o} C. A second antibody is not required, and there are no precipitation or centrifugation steps. The nonspecific binding is very low in the solid phase assay as compared to the double antibody technique. This increases sensitivity and allows a wider range of hormone values to be determined.

The solid phase assay is much more versatile than other immunoassay techniques and easily lends itself to modifications for special purposes. For instance, antibody coated tubes may be prepared in large batches at the convenience of the experimenter and stored for future use. The assay may also be run in two parts. The

first half of the assay, the reaction with standards and unknowns, can be completed. The tubes can then be stored and the second half, the reaction with the labeled hormone, can be carried out at a later date. Since I^{131} has a short halflife, the labeled hormone is only useful for approximately two weeks. By storing the tubes, the experimenter need not prepare the labeled hormone as often, resulting in considerable saving of time and money.

The solid phase assay seems to be more versatile in the amount of serum that can be used in a determination. It is a general practice in the double antibody technique to use only 0.2 ml. of serum per tube in running an assay to avoid nonspecific binding of labeled hormone to serum proteins. Because of the very low levels of gonadotrophins in some serums, hormone determinations using only 0.2 ml. of serum are not possible. Using 0.5 ml. of serum, however, did not prove detrimental in the solid phase assay. When the ICSH in a standard serum was determined for 0.1, 0.2, 0.3 and 0.5 ml. of serum per tube and the amount of ICSH per ml. estimated for each level, the agreement was nearly perfect.

The antibody used for the solid phase assay need not be as specific as for other assays. Undesirable cross-reactivity may be removed by pre-incubating the tubes with very high levels of the hormones that cross-react.

Besides its great speed and versatility, the sensitivity of the solid phase assay is equal to or greater than other antibody techniques. Although the lower limit of detection of this particular standard curve was 2×10^{-9} g/ml. of serum, preliminary experiments indicate that as little as 5×10^{-10} g. of ovine ICSH per ml. can be detected.

VI. SUMMARY

An experiment was conducted to determine the effects of melatonin, a pineal gland hormone, on reproductive phenomena in sheep. A solid phase radioimmunoassay was developed as a means of studying the effect of melatonin on serum levels of ICSH.

Twenty-eight ewe, ram and wether lambs were randomly divided into a control and a treatment group. Treatment consisted of daily subcutaneous injections of 0.1 mg. of melatonin per kilogram of body weight administered in propylene glycol. Treatment was initiated on the third day after birth and continued until autopsy just before the expected onset of puberty.

Melatonin in the doses employed caused a significant increase ($P < .05$) in ovarian weight in ewe lambs and a depression ($P < .10$) in castration hypersecretion of ICSH in wether lambs. No other significant effects were observed.

The role of melatonin in controlling reproductive phenomena in sheep is still not clear. The known effects of melatonin in rats could not be duplicated in sheep. It appears that the mechanism by which light controls reproduction in sheep is not similar to that which operates in rats.

The solid phase radioimmunoassay developed for this experiment proved to be a rapid, sensitive and highly reliable technique for the determination of serum ICSH.

VII. BIBLIOGRAPHY

Adams, W. C., Lillian Wan and Arthur Sohler. 1964. Effect of melatonin on anterior pituitary luteinizing hormone. Journal of Endocrinology 31:295-296.

Ariëns Kappers, J. 1963. Recent advances in our knowledge of the structure and function of the pineal organ. Proceedings of XVI International Congress of Zoologists 3:238-241.

Ariëns Kappers, J. 1965. Survey of the innervation of the epiphysis cerebri and the accessory pineal organs of vertebrates. In: Progress in brain research, ed. by J. Ariëns Kappers and J. P. Schade. Vol. 10, Amsterdam, Elsevier, p. 87-154.

Axelrod, Julius and Herbert Weissbach. 1960. Enzymatic 0-methylation of N-acetyl serotonin to melatonin. Science 131:1312.

Axelrod, Julius and Herbert Weissbach. 1961. Purification and properties of hydroxyindole-0-methyl transferase. Journal of Biological Chemistry 236:211-213.

Axelrod, Julius, R. J. Wurtman and E. W. Chu. 1963. Effects of melatonin, a pineal gland substance, on the rat ovary. Science 140:378.

Axelrod, Julius, R. J. Wurtman and S. H. Snyder. 1965. Control of hydroxyindole-0-methyl transferase activity in the rat pineal gland by environmental lighting. Journal of Biological Chemistry 240:949-954.

Axelrod, Julius, R. J. Wurtman and C. M. Winget. 1964. Melatonin synthesis in the hen pineal gland and its control by light. Nature 201:1134-1135.

Barchas, J. D. and A. B. Lerner. 1964. Localization of melatonin in the nervous system. Journal of Neurochemistry 11:489-491.

Barraclough, C. A. and R. A. Gorski. 1961. Evidence that the hypothalamus is responsible for androgen-induced sterility in the female rat. Endocrinology 68:68-79.

Catt, K. J., H. D. Niall and G. W. Tregear. 1966. Solid-phase radioimmunoassay of human growth hormone. Biochemical Journal 100:31-33c.

Corbin, Alan and E. L. Daniels. 1969. Induction of puberty in immature female rat: effect of estrogen on pituitary FSH and stalk-median eminence FSH-releasing factor. Neuroendocrinology 4(2):65-74.

Chowers, Israel and S. M. McCann. 1965. Content of luteinizing hormone-releasing factor and luteinizing hormone during the estrous cycle and after changes in gonadal steroid titers. Endocrinology 76:700-708.

Chu, E. W., R. J. Wurtman and Julius Axelrod. 1964. An inhibitory effect of melatonin on the estrus phase of the estrous cycle of the rodent. Endocrinology 75:238-242.

Critchlow, Vaughn. 1963. The role of light in the neuroendocrine system. In: Advances in neuroendocrinology, ed. by A. V. Nalbandov, Urbana, University of Illinois Press, p. 377-396.

Fiske, V. M. 1941. Effect of light of sexual maturation, estrous cycles and anterior pituitary of the rat. Endocrinology 29:187-196.

Fiske, V. M., G. K. Bryant and Janet Putnam. 1960. Effect of light on the weight of the pineal body of the rat. Endocrinology 16:489-491.

Fiske, V. M. and R. O. Greep. 1959. Neurosecretory activity in rats under conditions of continuous light or darkness. Endocrinology 64:175-185.

Fiske, V. M., Judith Pound and Janet Putnam. 1962. Effect of light on the pineal organ in hypophysectomized, gonadectomized, adrenalectomized or thiouracil-fed rats. Endocrinology 71:130-133.

Fraschini, Franco, Bela Mess and Luciano Martini. 1968. Pineal gland, melatonin and the control of luteinizing hormone secretion. Endocrinology 82:919-924.

Geschwind, I. I. and Robert Dewey. 1968. Dynamics of luteinizing hormone secretion in the cycling ewe: a radioimmunoassay study. Proceedings of the Society for Experimental Biology and Medicine 129:451-455.

Giarman, N. J. 1959. Neurohumors in the brain. Yale Journal of Biology and Medicine 32:73-92.

Gittes, R. F. and E. W. Chu. 1965. Reversal of the effect of pinealectomy in female rats by multiple isogenic transplants. Endocrinology 77:1061-1067.

Goldman, R. D. and V. B. Mahesh. 1969. A possible role of acute FSH-release in ovulation in the hamster, as demonstrated by utilization of antibodies to LH and FSH. Endocrinology 84:236-243.

Greenwood, F. C., W. M. Hunter and J. S. Glover. 1963. The preparation of 131I-labelled human growth hormone of high specific radioactivity. Biochemical Journal 89:114-123.

Hafez, E. S. E. 1952. Studies on the breeding season and reproduction in the ewe. Journal of Agricultural Sciences 42:189-265.

Halasz, Bela and R. A. Gorski. 1967. Gonadotrophic hormone secretion in female rats after partial or total interruption of neural afferents to the medial basal hypothalamus. Endocrinology 80:608-622.

Halasz, Bela, L. Pupp and S. Uhlorik. 1962. Hypophysiotrophic area in the hypothalamus. Journal of Endocrinology 25:147-154.

Herophilos and Alexandria (325-280 B. C.). Cited in: Progress in brain research, ed. by J. Ariëns Kappers and J. P. Schade. Vol. 10, Amsterdam, Elsevier, p. ix.

Hoffman, J. C. 1968. Effect of photoperiod on estrous cycle length in the rat. Endocrinology 83:1355-1356.

Hoffman, R. A. and R. J. Reiter. 1966. Responses of some endocrine organs of female hamsters to pinealectomy and light. Life Sciences 5:1147-1151.

Holmgren, Uno, M. D. Altschule and R. J. Wurtman. 1960. Effects of injection of bovine pineal extract on the nuclei of rat pineal parenchymal cells. Nature 186:393-394.

Izawa, Yoshitome. 1926. On some anatomical changes which follow removal of the pineal body from both sexes of the immature albino rat. American Journal of Physiology 77:126-139.

Kent, G. C., P. M. Ridgway and E. F. Strobel. 1968. Continual light and constant estrus in hamsters. Endocrinology 82:699-703.

Kitay, J. I. 1954a. Pineal lesions and precocious puberty: a review. Journal of Clinical Endocrinology 14:622-625.

Kitay, J. I. 1954b. Effect of pinealectomy on ovary weight in immature rats. Endocrinology 54:114-116.

Kitay, J. I. and M. D. Altschule. 1954a. The pineal gland. Cambridge, Harvard University Press, 280 p.

Kitay, J. I. and M. D. Altschule. 1954b. Effects of pineal extract administration on ovary weight in rats. Endocrinology 55:782-784.

Lawton, I. E. and N. B. Schwartz. 1965. Pituitary LH content in rats exposed to continuous illumination. Endocrinology 77:1140-1142.

Lerner, A. B., J. D. Case and R. V. Heinzelman. 1959b. Structure of melatonin. Journal of the American Chemical Society 81: 6084-6085.

Lerner, A. B., J. D. Case and Yoshiyata Takahashi. 1960. Isolation of melatonin and 5-methoxyindole-3-acetic acid from bovine pineal glands. Journal of Biological Chemistry 235:1992-1997.

Lerner, A. B., et al. 1958. Isolation of melatonin, the pineal gland factor that lightens melanocytes. Journal of the American Chemical Society 80:2587.

Lerner, A. B., et al. 1959a. Isolation of 5-methoxyindole-3-acetic acid from bovine pineal glands. Journal of the American Chemical Society 81:5264-5265.

Lerner, A. B., et al. 1959c. Melatonin in peripheral nerve. Nature 183(4678):1821.

Maric, D. K., Eikichi Matsuyama and C. W. Lloyd. 1965. Gonadotropin content of pituitaries of rats in constant estrus induced by continuous light. Endocrinology 77:529-536.

Mess, Bela and Luciano Martini. 1968. Brain receptors sensitive to indole compounds: function in control of luteinizing hormone. Science 159:1104-1105.

Meyer, C. J., et al. 1961. The arrest of prolonged estrus in middle-aged rats by pineal gland extract. Endocrinology 68:795-800.

Moore, R. Y., et al. 1967. Visual pathway mediating pineal response to environmental light. Science 155(3759):220-223.

Motta, Marcella, Franco Fraschini and Luciano Martini. 1967. Endocrine effects of pineal gland and of melatonin. Proceedings of the Society for Experimental Biology and Medicine 126:431-435.

Mozkowska, A. 1965. Contribution à l'étude du mécanisme de l'antagonisme épiphyso-hypophysaire. In: Progress in brain research, ed. by J. Ariëns Kappers and J. P. Schade. Vol. 10, Amsterdam, Elsevier, p. 564-576.

Negro-Vilar, Andres, Elias Dickerman and Joseph Meites. 1968. Effects of continuous light on hypothalamic FSH-releasing factor and pituitary FSH levels in rats. Proceedings of the Society for Experimental Biology and Medicine 127:751-755.

Prop, N. and J. Ariëns Kappers. 1961. Demonstration of some compounds present in the pineal organ of the albino rat by histochemical methods and paper chromatography. Acta Anatomica 45:90-109.

Ramirez, V. D. and C. H. Sawyer. 1965a. Advancement of puberty in the female rat by estrogen. Endocrinology 76:1158-1168.

Ramirez, V. D. and C. H. Sawyer. 1965b. Fluctuations in hypothalamic LH-RF (luteinizing hormone-releasing factor) during the rat estrous cycle. Endocrinology 76:282-289.

Ramirez, V. D. and C. H. Sawyer. 1966. Changes in hypothalamic luteinizing hormone during puberty. Endocrinology 78:958-964.

Reiter, R. J. and R. J. Hester. 1966. Interrelationships of the pineal gland, the superior cervical ganglia and the photoperiod in the regulation of the endocrine systems of hamsters. Endocrinology 79:1168-1169.

Reiter, R. J., J. C. Hoffman and P. H. Rubin. 1968. Pineal gland: influence on gonads of male rats treated with androgen three days after birth. Science 160:420-421.

Robertson, H. A. and A. M. Rakha. 1966. The sequence, time, and duration of the release of follicle-stimulating hormone and luteinizing hormone in relation to oestrus and to ovulation in the sheep. Journal of Endocrinology 35:177-184.

Roche, J. F., et al. 1969a. Effect of pinealectomy on estrus and ovulation in ewes. (Abstract) Journal of Animal Science 29:197-198.

Roche, J. F., et al. 1969b. LH in serum of pinealectomized and castrated ewes. (Abstract) Journal of Animal Science 29:197.

Roth, W. D. 1965. Metabolic and morphologic studies on the rat pineal organ during puberty. IN: Progress in brain research, ed. by J. Ariëns Kappers and J. P. Schade. Vol. 10, Amsterdam, Elsevier, p. 552-564.

Roth, W. D., R. J. Wurtman and M. D. Altschule. 1962. Morphologic changes in the pineal parenchyma cells of rats exposed to continuous light or darkness. Endocrinology 71:888-892.

Steel, R. G. D. and J. H. Torrie. 1960. Principles and procedures of statistics. New York, McGraw-Hill, 481 p.

Thiéblot, L. P. 1965. Physiology of the pineal body. In: Progress in brain research, ed. by J. Ariëns Kappers and J. P. Schade. Vol. 10, Amsterdam, Elsevier, p. 479-489.

Thiéblot, L. P. and S. Blaise. 1963. Influence de la glande pineal sur les gonades. Annales d'Endocrinologie (Paris) 24:270-286.

Thompson, K. W. Feb. 1970. Professor, University of Wisconsin, Department of Obstetrics and Gynecology. Personal communication. Madison, Wisconsin.

Van de Veerdonk, F. C. G. 1965. Separation method for melatonin in pineal extracts. Nature 208(5017):1324-1325.

Weissbach, Herbert and Julius Axelrod. 1960. The enzymatic biosynthesis of melatonin. Federation Proceedings 19:50.

Wheatly, I. S. and H. M. Radfor. 1969. Luteinizing hormone secretion during the estrous cycle of the ewe as determined by radioimmunoassay. Journal of Reproduction and Fertility 19:211-214.

Wragg, L. E. 1967. Effects of pinealectomy in the newborn female rat. American Journal of Anatomy 120:391-402.

Wurtman, R. J., M. D. Altschule and Uno Holmgren. 1959. Effects of pinealectomy and of a bovine pineal extract in rats. American Journal of Physiology 197:108-110.

Wurtman, R. J. and Julius Axelrod. 1965a. The formation, metabolism, and physiologic effects of melatonin in mammals. In: Progress in brain research, ed. by J. Ariëns Kappers and J. P. Schade. Vol. 10, Amsterdam, Elsevier, p. 520-529.

Wurtman, R. J. and Julius Axelrod. 1965b. The pineal gland. Scientific American 213(1):50-60.

Wurtman, R. J., Julius Axelrod and J. D. Barchas. 1964a. Age and enzyme activity in the human pineal. Journal of Clinical Endocrinology and Metabolism 24:299-301.

Wurtman, R. J., Julius Axelrod and E. W. Chu. 1963a. Melatonin, a pineal gland substance: effect on the rat ovary. Science 141:277-278.

Wurtman, R. J., Julius Axelrod and J. C. Fischer. 1964b. Melatonin synthesis in the pineal gland: effect of light mediated by the sympathetic nervous system. Science 143:1329-1330.

Wurtman, R. J., Julius Axelrod and L. S. Phillips. 1963b. Melatonin systhesis in the pineal gland: control by light. Science 142:1071-1073.

Wurtman, R. J., Julius Axelrod and L. T. Potter. 1964c. The uptake of H^3-melatonin in endocrine and nervous tissues and the effects of constant light exposure. Journal of Pharmacology and Experimental Therapeutics 143:314-318.

Wurtman, R. J., et al. 1960. Interaction of effects of pinealectomy and constant light in the female rat. Federation Proceedings 19:53.

Wurtman, R. J., et al. 1961. Interactions of the pineal and exposure to continuous light on organ weights of female rats. Acta Encocrinologica 36:617-624.

Wurtman, R. J., et al. 1965. Changes in the enzymatic synthesis of melatonin in the pineal during the estrous cycle. Endocrinology 76:798-800.