REGULATION OF SYNTHESIS AND SECRETION OF

PINEAL HORMONAL PRINCIPLES

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CHAPTER I

INTRODUCTION

The pineal gland came under the scrutiny of the scientific community in the 17th century. Rene Descartes had proposed that the pineal gland functioned as the "Seat of the Soul." This idea was discarded and the pineal largely ignored until the latter part of the 19th century. Clinical observations began to link the pineal gland to reproduction. Since that time, the pineal gland has been thought to secrete an antigonadotropin and a progonadotropin as well as substances affecting the thyroid and adrenal glands.

Pinealectomy resulted in increased ovarian and uterine weights and produced a precocious vaginal opening which is indicative of puberty (Kitay, 1954; Wurtman et al., 1959). Conversely, administration of pineal extracts to normal rats decreased ovarian weight and delayed vaginal opening time (Wurtman et al., 1961). Thus, two of the requirements for endocrine gland status had been met. That is, removal of the pineal produced a series of changes and a pineal extract had the opposite effects. The only thing preventing the pineal from being classified as an endocrine gland was the determination of the material responsible for the effects described.

Lerner et al. (1959) found an indoleamine, melatonin, in large concentrations in the pineal gland. Studies by Wurtman and Axelrod (1965) found that melatonin mimicked the effect of crude pineal

extracts, and its synthesis was increased during conditions which stimulated pineal metabolism. Subsequent studies have suggested that melatonin acts on the hypothalamo-hypophyseal axis to inhibit luteinizing hormone release (Motta et al., 1967). However, recent studies indicate that melatonin may counter the antigonadotropic effects of the pineal (Reiter et al., 1974; Reiter et al., 1975). This provides a reasonable measure of doubt as to the efficacy of melatonin as the pineal antigonadotropin.

A most interesting relationship has been found between photoperiod, reproduction and the pineal gland. Prolongation of daily photoperiods, as well as constant light exposure, induce an increase in ovarian weight in rats (Fiske, 1941; Fiske et al., 1962). Under these conditions, the pinealocytes (the major pineal cell type) were observed to decline in size, to have a decreased RNA content, and a decrease in the activity of hydroxyindole-O-methyl transferase (an enzyme leading to melatonin synthesis) (Quay, 1961; Roth et al., 1962). Constant light was thus shown to inhibit the pineal gland and to stimulate reproduction. As would be expected, constant darkness produces the opposite effects. Constant darkness will delay the onset of the estrous cycle of young female rats (Fiske, 1941) and decrease testicular weight in male hamsters (Hoffman and Reiter, 1965). The pineal glands and pinealocytes are enlarged and the pinealocytes are reported to contain more RNA than control glands. Further studies have demonstrated that removal of the pineal gland will prevent constant dark and constant light from having an effect on reproduction.

Cook (N.Y.J. Obstet. Gynecol., editorial, 1894) reported that the lack of light during winter months in the north latitudes caused a

complete cessation of menstruation in Eskimo women for 4 months. Evaluation of birth records in Finland found a high frequency of conception during the summer months (Timonen and Carpen, 1968). The above studies indicate that the pineal gland is involved in some fashion in the regulation of reproductive patterns in mammals.

Although there is much evidence in support of melatonin as the pineal antigonadotropin, earlier studies had suggested another class of pineal compounds might be responsible for the observed antigonadotropic effects of pineal extracts (Thieblot and Blaise, 1963). Moszkowska (1967) isolated peptide fractions from the pineal gland which appeared to prevent the secretion of hypothalamic releasing factors. Milcu et al. (1963) suggested that the pineal contained a peptide with chemical properties similar to arginine vasotocin. Cheesman (1970) chemically identified arginine vasotocin (AVT) in the pineal gland.

Although the biological effects of AVT have not been fully investigated, some of its in vivo and in vitro effects have been documented. AVT was shown to inhibit compensatory ovarian hypertrophy or COH (removal of one ovary results in a qualifible increase in the weight of the contralateral ovary) and to inhibit gonadotropin release (Pavel et al., 1973). Vaughan et al. (1974, 1976a) found that AVT inhibited growth of accessory sex organs in male hamsters and mice and would prolong the estrous cycle in female mice. Vaughan et al. (1975) also found that AVT released prolactin in vitro.

AVT has been demonstrated to be released from fetal pineals in vitro (Pavel et al., 1973a). Further, hypertonic saline or melatonin injected into the third ventricle will stimulate AVT release into the

third ventricle (Pavel, 1973; Pavel and Coculescu, 1972). And finally, pineal gland content of AVT is elevated after 24 hours constant darkness and reduced after 24 hours constant light (Caleb et al., 1977).

Reiter and Vaughan (1977) have suggested that indoleamines and peptides act in concert to obtain the antigonadotropic effects ascribed to the pineal gland. Pavel (1973) has shown that melatonin will stimulate the secretion of AVT while Binkley et al. (1976) have shown that AVT inhibits pineal N-acetyltransferase, an enzyme leading to melatonin synthesis. Aside from these two studies, little work has been performed on the possible interrelationships between pineal indoles and AVT.

It is apparent that, either through the action of indoleamines or AVT, the pineal gland is capable of influencing the secretions of other endocrine glands in the maintenance of physiological homeostasis. This study was designed to determine some of the factors influencing AVT synthesis and secretion.

CHAPTER II

LITERATURE REVIEW

The epiphysis cerebri or pineal gland was first described by Herophilus and Erasistratus (325-280 B.C.). The pineal was to become a subject of religious, philosophical, and scientific controversy in the ensuing centuries. Descartes' hypothesis that the pineal was the seat of the soul sparked outcrys from men such as Emmanuel Kant who argued against Descartes on the grounds that no single organ housed the soul. Reports by Theophile Bonet in 1686 proposed a correlation between mental illness and a "petrified" pineal. Pineal stones were associated with insanity for more than a century until it was found that there was no difference in the presence of a calcified pineal in the insane versus normal populations. Marburg, in 1913 and 1930 reported on a condition of premature puberty caused by pineal degeneration. Marburg also proposed that the pineal gland was an endocrine gland which caused a reproductive quiescence in the young (historical overview from Kitay and Altschule, 1954).

Histology

The primary cell type found in the pineal gland is referred to as a pinealocyte. Pinealocytes have long processes which have reported to terminate near capillaries in the pineal (Romijn, 1973). Leonhardt

(1967) has reported the presence of dense core granules and mitochondria, suggestive of a secretory function. The pineal also contains glial cells, fibrocytes, mast cells, plasma cells, and acervuli. The acervuli are calcium deposits which are present with advancing age or neurophathologic symptoms (Quay, 1971). These acervuli contain calcium, magnesium, phosphorus, iron, and hydroxyapatite. The pineal also contains synaptic ribbons close to cell membranes. The synaptic ribbons are normally associated with retinal photoreceptors and are thought to be components of rudimentary pineal photoreceptors in lower vertebrates.

The blood supply to the pineal is relatively great and the proportion of cardiac output received by the pineal is reported as one of the largest in the body (Kappers, 1976). The arterial blood is from the posterior cerebral artery and the veinous drainage is by way of the internal cranial veins. There exist no portal vessels for transport of secretory products to other areas of the brain (Smith, 1971). Instead, the pineal veinous supply promptly enters systemic circulation.

The primary nerve supply is via sympathetic autonomic nerves originating from the superior cervical ganglion (Cajal, 1911). Kappers (1962, 1965) has described two nervi conarii which penetrate the caudal aspect of the gland. The autonomic nerve supply is intact and ready to modulate pineal metabolic activity by the fifth week of embryonic live (Hulseman, 1971). There is some evidence for parasympathetic autonomic innervation in rabbits (Kenny, 1961; Romijn, 1975). These cholinergic fibers reach the pineal as branches of the conary nerve and terminate between pinealocyte processes. Wartman (1969) has reported cholinergic activity in the rat pineal and suggested the presence of

parasympathetic nerves. Recently, Dafny (1977) has demonstrated by electrophysiological techniques, the presence of reciprocal nerves from the hypothalamus to the pineal. The pharmacologic classification of these nerve fibers has not yet been attempted.

Effects of Pinealectomy

Classically, removal of a suspected endocrine organ and observation of the resultant changes in normal physiology have provided clues as to the nature and function of the gland. Pinealectomy in rats was found to increase the number of rats ovulating in response to pregnant mares serum (PMS) and increased the mean number of eggs shed (Dunaway and O'Stein, 1967). Pinealectomy has also been reported to reverse blinding-induced gonadal atrophy (Hoffman and Reiter, 1965; Sorrentino and Benson, 1970). Similarly, the gonadotropic hormones are also altered to some degree by pinealectomy. Fraschini (1969) reported that removal of the pineal gland from adult rats elevated plasma LH and FSH. Dickson et al. (1971) later confirmed the effect on elevating FSH. Scemama (1976) found that pinealectomy produced no effects on LH or FSH levels in immature rats. Interestingly, in adult rats, the elevation in LH was shown to be preceded by a transient reduction in LH for seven days following the surgery (Bronswijk et al., 1975). These and other endocrine ablation experiments indicated that removal of the pineal provided a stimulus to reproduction. This supports the hypothesis that the pineal synthesizes and secretes an antigonadotropic or anti-reproductive compound(s) (Engel, 1936).

Constant Light

Constant light has effects similar to those observed with pinealectomy. Constant light caused uterine hypertrophy and a greater frequency of estrus (Browman, 1937). Constant light was also shown to increase ovarian weight (Fiske, 1941; Wurtman et al., 1961). Jochle (1956) found that constant light produced a state of constant estrus. Fiske et al. (1960, 1962) found that constant light decreased pineal weight while Roth et al. (1962) demonstrated a decrease in the size of pineal parenchymal cells. These effects of constant light were then found to be reversed by the administration of pineal extracts to constant light exposed rats (Ifft, 1962). These studies led Wurtman et al. (1961) to the conclusion that constant light inhibited the release of an antireproductive compound from the pineal. Subsequent studies have revealed that vaginal opening time occurred earlier in constant light exposed rats (Ramaley and Bartosik, 1975). Also, ovulation induced by gonadotropin releasing hormone (GnRH) in immature female rats occurred earlier when exposed to constant light (Steger et al., 1975). Constant light was found to increase pituitary prolactin and decrease plasma prolactin (Relkin, 1972) and to have no effect on LH and FSH in immature rats (Piacsek and Streur, 1975). And finally, FSH stimulated ovulation in rats could be blocked by constant light (Meyer and McCormack, 1967). Thus constant light stimulates reproduction, probably by inhibiting pineal synthesis and secretion of an anti-gonadal substance.

Effects of Blinding and Constant Darkness

Blinding or constant darkness is thought to stimulate the pineal gland to synthesize and release an antigonadotropin. Blinding for 4 weeks reduced testis and seminal vesicle weights in male hamsters (Hoffman and Reiter, 1965; Bush and Seibel, 1977). Reproductive organ weights in both male and female rats were also reduced by blinding (Sorrentino and Benson, 1970). Blinding of female rats has been shown to decrease serum LH and to reduce pituitary prolactin levels (Blask and Reiter, 1975). Blask et al. (1975) also found an increased hypothalamic LH-RH activity in blinded-anosmic hamsters. Other studies have found that the initial effect of blinding was to increase plasma LH and decrease FSH (Rønnekliev and McCann, 1975). However, with time the LH values returned to baseline while the FSH levels continued to decline. Blinding will reduce testicular synthesis and testicular vein concentrations of testosterone (Peat and Kinson, 1971; Kinson and Liu, 1973, 1974). Constant darkness or prolonged dark phases of the photoperiod produce similar effects to those observed with blinding (Reiter and Hester, 1966).

Physiology of Pineal Indoleamines

Pineal gland indoleamine compounds were once thought to be the pineal hormones and have consequently been studies intensively in the past few years. They have been demonstrated to modify sleep, locomotor activity rhythms, the electroencephalogram, and to inhibit pituitary gonadotropin secretion (Wurtman et al., 1968).

The pineal concentrations of indoleamines are 10-100 times greater than that found in other tissues (Garrantini and Valzelli, 1965; Wurtman et al., 1968). Exogenous administration of serotonin produces antireproductive effects. Serotonin was shown to cause degeneration of the testes of male rats (Boccabella et al., 1962). Although a vasodilator could block these effects, long term degeneratory effects have been produced from a single injection of serotonin which could not have been due to local degeneratory effects (O'Steen, 1963). Subcutaneous injections of serotonin could also block LH release (O'Steen, 1965). Corbin and Schottelius (1961) found that intraventricular injections of serotonin at 5-day intervals could delay vaginal opening time in immature female rats. Owman (1971) pointed out that only small quantities of serotonin were converted to melatonin and that the remainder might find entry to the third ventricle. Since serotonin is the inhibitory neurotransmitter for LHRH in the hypothalamus, entry of serotonin through the tanycytes lining the third ventricle (Card and Rofols, 1978) might alter gonadotropin release.

Melatonin was first isolated by Lerner et al. (1959) and subsequently found to have many antireproductive effects. The magnitude of the melatonin effect appears to be dependent upon dosage, time of administration, and age of the animal. The more frequently utilized measures of melatonin activity have been gonad and accessory sex organ weights, vaginal opening time, frequency of estrous smears, and plasma hormone concentrations. Daily injections of melatonin for 45 days decreased ovarian and uterine weights (Franchimont, 1964). There have been reports that melatonin (Chu et al., 1964) or serotonin (Albertazzi et al., 1966) will prevent the increased incidence of

estrous following pinealectomy. Also, melatonin administered to pinealectomized hamsters will result in marked gonadal atrophy (Turek, 1977; Tamarkin et al., 1977). Studies on compensatory ovarian hypertrophy (COH) indicated that a single intraperitoneal (IP) injection of melatonin reduced COH after 10 days (Vaughan et al., 1971). Norris et al. (1970) found that there was no difference between IP injections or injections into the ovarian capsule. However, smaller injections made into the third ventricle produced a more significant blockade of COH.

Measurement of pituitary LH levels demonstrated that both melatonin (Adams et al., 1965) and serotonin (Endersby et al., 1970) were capable of blocking LH release. Melatonin has also been shown to block LH release in PMS-primed immature rats (Reiter and Sorrentino, 1971) and following castration in male rats (Franschini et al., 1971). Melatonin and serotonin have been shown to inhibit pituitary LH secretion in vitro (Martin et al., 1977).

The anti-reproductive effects of melatonin on FSH are evidenced by the COH data of Sorrentino (1968). Melatonin injections into the third ventricle decreased basal FSH and prolactin but not when melatonin is introduced via the hypothalmo-hypophysial portal system (Kamberri et al., 1970, 1971). This effect also was seen with serotonin and tends to indicate that the indoleamines act at the hypothalamus rather than the pituitary. Since pharmacologic elevation of brain tissue serotonin was shown to inhibit PMS-induced ovulation in immature rats (Kordon et al., 1968), it has been suggested that indoleamines act via serotonergic mechanisms (Reiter et al., 1975b).

Melatonin exhibits those effects of blinding or constant dark. Endocrine ablation experiments have indicated that melatonin replacement therapy will reverse the pinealectomy effects on reproduction. From these studies it was concluded that the pineal was an endocrine gland and that melatonin was the pineal hormone.

Indoleamine Synthesis

The amino acid tryptophan is converted through two steps to form 5-hydroxytryptamine or serotonin. Serotonin (5-HT) is converted by an enzyme N-acetyltransferase to N-acetylserotonin (NAS) which in turn is converted by the enzyme hydroxyindole-O-methyltransferase to melatonin. This cycle occurs at night. During the daylight periods 5-HT is converted to hydroxytryptophol (HTOL) by the enzyme monoamine oxidase. HIAA is then converted to methoxyindoleacetic acid (MIAA). Alternatively 5-HT may be converted to hydroxytryptophol (HTOL) and then to methoxytryptophol (MTOL) (Quay, 1974). The synthesis of melatonin is under the regulation of the sympathetic autonomic nervous system via the neurotransmitter norepinephrine (NE) (Axelrod et al., 1969). Stimulation with NE in vitro has no effect on serotonin or hydroxyindole-O-methyltransferase (HIOMT) but rather stimulates N-acetyltransferase activity (Klein and Weller, 1971O; Klein et al., 1971a, 1971b). The NE stimulation of N-acetyltransferase activity appears to be mediated through cyclic AMP accumulation (Weiss and Costa, 1968).

The concentration of tryptophan in human and rat plasma varies markedly on a 24 hour cycle (Fernstrom and Wurtman, 1972). Tryptophan levels are low in the early morning and peak in the early afternoon.

This tryptophan rhythm ceases with fasting (Marliss et al., 1970). Fernstrom and Wurtman (1972) indicate that plasma tryptophan levels determine the rates of uptake of tryptophan by the brain and could easily influence the diurnal variation in other indoles.

Serotonin exists in high concentrations in the pineal. The pinealocyte cytoplasma has been shown to be the primary location of serotonin (Owman, 1965) and probably the site of its synthesis. The level of pineal serotonin has been found to fluctuate in a circadian rhythm (Quay, 1963, 1964) from a nadir of 10-20 ng to a peak of 60-90 ng per pineal gland. Pineal serotonin increases during the daylight hours and decreases at night (Quay, 1963, 1964a,b). A constant light environment causes a slight decrease in the pineal serotonin content in rats. Long term continuous light led to a decrease in cyclic AMP content (Ebadi et al., 1970) and a decline in general indoleamine production. This circadian rhythm was also shown to persist in blinded animals except when they wore a black hood (Snyder, 1968).

There is a prominent circadian rhythm in pineal N-acetyltransferase activity (Klein and Weller, 1970) which is mediated through the sympathetic nervous system (Weiss and Costa, 1968; Volkman and Heller, 1971; Yuwiler et al., 1977). During daylight hours the enzyme activity is lowered (Morgan et al., 1976) and at night the activity is elevated (Klein and Weller, 1970).

The second enzyme in the conversion of serotonin to melatonin is HIOMT. HIOMT has been identified in only 3 tissues, the pineal gland (Axelrod and Weissbach, 1961), the retina (Cardinali and Rosner, 1971), and the harderian gland (Vlahakes and Wurtman, 1972). HIOMT was inhibited by S-adenosylmethionine but not by thiol compounds

(Karahasanoglu and Ozard, 1972; Deguchi and Barchas, 1971). With darkness, HIOMT activity was found to increase significantly (Klein and Weller, 1973). Usually HIOMT activity indicates melatonin synthesis but Lynch and Ralph (1970) found that melatonin content decreases as the estrous cycle progresses to diestrus while HIOMT activity is elevated.

A prominent circadian rhythm in peripheral melatonin concentration has also been shown. Melatonin production is elevated with darkness (Lynch, 1971) and exhibits a duirnal rhythm in both the cerebrospinal fluid (CSF) and the blood in calves.

Hormone Effects on Indoleamines

The circadian rhythm in rat pineal serotonin was found to be unaffected by hypophysectomy, thyroidectomy, adrenalectomy or oöphorectomy (Snyder et al., 1965). Although adrenalectomy or administration of cortisol has no effect on pineal serotonin concentrations (McDennee et al., 1966), the metabolic products from serotonin and the content of HIOMT show changes associated with the estrous cycle or with the administration of estrogens. Small changes occur in the adult female rat pineal serotonin and 5-HIAA concentration related to the day or estrus (Quay, 1963; Quay, 1964b). Estrogens increase the pineal content of 5-HIAA and depress HIOMT activity while progesterone reverses the situation. Serotonin levels show higher night-time level on the first day of proestrous. Night-time levels of melatonin were found to decrease progressively through the cycle (Quay, 1964b) whereas HIOMT increased through the cycle (Wurtman et al., 1965). Studies have shown that ovariectomy of the 28 day old rat will lead to increased

HIOMT activity 25 days later (Alexander et al., 1970) which could be prevented by administration of estradiol (10 ug) (Wurtman et al., 1975). Further, ovarian secretion of estradiol inhibited norepinephrine stimulated adenylate cyclase (Weiss and Crayton, 1970). Norepinephrine stimulates adenylate cyclase activity during each stage of the estrous cycle except proestrous where the estrogen levels are highest. Progesterone or testosterone failed to affect norepinephrine stimulated adenylate cyclase activity.

Melatonin as a Progonadotropin

Although there have been many studies supporting melatonin as the antigonadotropic hormone of the pineal, recent opposing evidence exists. Some investigators found that melatonin increased gonadal weights (Thieblot and Blaise, 1966). Others have found no effect of melatonin on reducing gonadal or accessory organ weights (Ebels and Prop, 1965; Kunkel, 1969). While some studies have found uptake of ^{3}H-melatonin by the cat ovary (Wurtman et al., 1964) other researchers have shown that melatonin stimulated ^{32}P uptake by the ovary (Norris, 1971). Melatonin treatment was found to block dark-induced gonadal degeneration in hamsters (Hoffman, 1974; Reiter et al., 1974) and rats (Reiter et al., 1975a). It has also been shown that melatonin can prevent the involution of sexual organs of male and female hamsters that normally follows constant darkness or blinding (Reiter et al., 1977).

Pineal Gland Peptides

Peptide Extracts

Crude extracts of bovine or ovine pineals have yielded peptide fractions which exhibit both inhibitory and stimulatory effects upon reproduction (Milcu et al., 1963; Ebels et al., 1975; Benson et al., 1971; Orts et al., 1975). Some of these compounds have been shown to be melatonin free (Orts et al., 1974) and to be more potent than melatonin in blocking compensatory ovarian hypertrophy (COH) in mice (Benson et al., 1972). Besides blockage of COH, these peptides have been shown to delay vaginal opening time and reduce the incidence of persistent estrus (Orts et al., 1974). Hormone data indicate that a peptide fraction causes inhibition of LH secretion in castrate males (Orts et al., 1973, 1974) as well as increased prolactin secretion (Orts, unpublished data). Other studies have indicated that pineal peptide fractions decrease serum testosterone in male rats (Milcu et al., 1975; Orts, 1977). Further, some of these fractions have been shown to exhibit antifertility properties in female rats (Orts et al., 1977). One of these compounds inhibiting reproduction was found to be the pineal peptide arginine vasotocin (Cheesman, 1970), a compound structurally similar to arginine vasopressin and oxytocin (Figure 2).

Physiology of Arginine Vasotocin

Pavel and Petrescu (1966) introduced evidence that AVT might be the pineal antigonadotropin by demonstrating that injections of AVT into the third ventricle inhibited pregnant mares serum (PMS) stimulated hypertrophy of ovaries and uteri of immature female mice.

Johnson et al. (1978) subsequently found that AVT blocks PMS-induced ovulation in immature female rats. AVT injected into the third ventricle inhibited COH in mice (Pavel et al., 1973). Vaughan et al. (1974) have demonstrated a decreased growth of gonadal-dependent structures in male mice and hamsters by injection of AVT. Further, studies revealed that AVT inhibits human chorionic gonadotropin (HCG) induced hypertrophy of ovaries and uteri (Vaughan et al., 1976). Conversely, AVT administered at postnatal days 1-5 caused an increase in testis weights at 30 and 60 days when compared to saline injected controls (Vaughan et al., 1976), but by 90 days of age there was no difference between control and treated animals. If AVT injections were provided daily through pregnancy, the females would not deliver viable pups (Vaughan et al., 1976).

Moszkowska et al. (1958) incubated rat pituitary glands with and without AVT. Bioassay of the incubation media revealed that AVT decreased FSH release. Moszkowska suggested the pituitary as the site of action for AVT. Incubation of rat pituitary glands with AVT (Vaughan et al., 1976) revealed that AVT would enhance the in vitro effects of LHRH stimulated LH secretion. In a similar study Vaughan et al. (1975) found that AVT released prolactin. IP injection of AVT has been shown to produce this same prolactin releasing activity as was found in vitro (Vaughan et al., 1976). Propranalol, a β-receptor blocker, inhibits AVT release of prolactin (Blask et al., 1977). This indicates the possibility that AVT stimulates a β-receptor in the pituitary to release prolactin. Bruot et al. (1977) have shown that AVT will block LH release in vitro in estrogen-progesterone primed castrate rats but not in

intact rats, suggesting the necessity of a proper steroid environment for the biological activity of AVT.

In contrast to Vaughan's experiments were those of Demoulin (1977). These experimenters found that AVT had no effect upon the release of gonadotropins by cultured anterior pituitary cells. They further suggest that if AVT were to alter the hypothalamo-hypophysial-gonadal axis, that the site of action would be the hypothalamus. In vivo studies by Cheesman et al. (1977a) and Osland et al. (1977) have shown no effect of AVT on basal LH secretion. Instead, they found that intraventricular administration of AVT would block the preovulatory surge of LH. These authors suggest centers in the hypothalamus other than the median eminence as the site of action of AVT. Cheesman et al. (1977b), contrary to Vaughan's data, found that AVT blocks the prolactin surge in proestrous rats. No effect was found on FSH.

AVT also appears to have some inhibitory influences upon the hypothalamo-hypophysial-adrenal axis. Pavel et al. (1973c) found that intraventricular administration of AVT inhibited compensatory adrenal hypertrophy. Subsequently, Pavel et al. (1977a) have shown that AVT introduced to the third ventricle will inhibit cortisol release from the adrenal glands of anesthetized cats. Pavel interpreted this to indicate the AVT will inhibit corticotropin-releasing factor (CRF) secretion which will in turn reduce adrenocorticotropin (ACTH) secretion. Further, this experiment provided evidence that the AVT receptors in the hypothalamus may be serotonin containing neurons.

Identification and Localization of Arginine Vasotocin

Milcu et al. (1963) measured the presence of AVT in pineals by use of a differential bioassay. Subsequent experiments (Cheesman, 1970; Cheesman and Fariss, 1970) utilized both amino acid analysis and mass spectrometry to provide definitive proof that AVT was found in bovine pineal glands. Radioimmunoassay techniques have since determined that partially purified extracts of rat pineal glands contain significant quantities of AVT (Rosenbloom and Fischer, 1975).

AVP is shown to be bound to a specific neurophysin and stored in storage granules. It was thus not unexpected that Reinharz et al. (1974) succeeded in isolating neurophysin from bovine pineal glands. Legros et al. (1976) also have isolated both neurophysins and AVT from human fetal pineal glands while Reinharz and Valloton (1977) have isolated vasotocin and two neurophysins from adult human pineal glands.

Localization of AVT within the pineal has provided an area of controversy. Pavel (1971) found that in the adult rat, bioassayable AVT was located only in the pineal stalk. This data was later confirmed by Benson et al. (1976) also using a bioassay technique. Pavel (1971) and later Benson et al. (1976) have speculated that AVT is synthesized, stored and secreted from ependymal cells adjacent to the third ventricle. However, contrary data has been presented by Bowie and Herbert (1976) who found immunocytochemical evidence for AVT within the body of the pineal as well as the stalk. They further described a specific population of cells which were identified by the AVT antibody.

Regulation of Arginine Vasotocin Secretion

Pavel et al. (1971) have suggested that AVT is synthesized and secreted into the CSF by specialized ependymal cells lining the stalk of the pineal and exposed to the third ventricle. Kappers (1976) contends that this type of direct secretion into the third ventricle does not occur in the rat. Quay (1973) indicated that entry of pineal materials into the ventricular system is by retorgrade flow of blood through the vena cerbri magna into the pineal sac.

The pineal gland content of AVT was found to be the highest in fetal animals, decreasing following birth (Pavel, 1973b). Pavel has shown that AVT is released from human (Pavel, 1973) and rat (Pavel et al., 1977b) fetal pineal glands in vitro. In vivo secretion of AVT into the third ventricle is augmented by hypertonic saline injections to the third ventricle (Pavel and Coculescu, 1972). Calb et al. (1977) have demonstrated that 24 hours constant light decreases pineal AVT content and 24 hours constant darkness increases pineal gland AVT. Calb also found that pineal AVT content was highest at midnight and lowest at noon. As expected, this data correlates well with pineal metabolic activity (and melatonin synthesis) but does not indicate either synthesis or secretion. Interestingly, Pavel (1973) has shown that intraventricular (third ventricle) administration of melatonin stimulates the release of bioassayable AVT into the third ventricle. Recently Goldstein et al. (1978) have indicated that gonadotropin releasing hormone, thyrotropin releasing hormone, and growth hormone release-inhibiting hormone will also stimulate AVT release into the third ventricle in cats. These hormones also promote AVP secretion.

Cusack et al. (1978) have found that intraperitoneal injections of norepinephrine will increase blood levels of AVT. This combined with CSF release and *in vitro* data provides strong evidence for the secretion of AVT.

At the present time there is little data available concerning hormone and neurotransmitter regulation of arginine vasotocin synthesis and secretion. In the past, the lack of a sensitive and specific assay for AVT has produced many questions concerning this issue which have been difficult to answer. This thesis attempts to examine some of the factors regulating AVT secretion and whether, as suggested by Pavel (1973) and Reiter et al. (1976), that there is an interaction between indoleamines and peptides.

CHAPTER III

MATERIALS AND METHODS

This investigation was divided into two phases. Phase I was a study of the factors which altered *in vivo* pineal synthesis and secretion of arginine vasotocin. Phase II was designed to study the effects of neurotransmitters and indoleamines upon arginine vasotocin secretion in vitro. This phase was also designed to investigate the effects of AVT on indoleamine production and release *in vitro*.

In Vivo Experiments

Animals

Male rats (80 days of age) of the Charles Rivers cd strain were utilized in the blinding, constant light, and castration studies. Male rats (Charles River) aged 15, 30, and 60 days were utilized for the puberty study. Animals were maintained in the Department of Physiological Sciences animal facility under standard conditions ($24 \pm 1^{o}C$; light: dark cycle of 14:10 hours). Food and water were provided ad libitum.

Experimental Protocol

Thirty rats were blinded by orbital enulceation while another group of thirty rats were placed under constant illumination. Six

rats were not subjected to any treatment and served as controls. At 2,4,6, and 8 weeks after treatments were initiated, 6 blinded and 6 constant light exposed rats were sacrificed by decapitation and the trunk blood collected in centrifuge tubes. Pineal and pituitary glands were collected, weighed, placed in storage vials containing normal saline, and frozen with acetone-dry ice. The blood was centrifuged and serum removed to storage vials and frozen. All samples were stored at -20°C until assayed.

Ten rats were castrated and maintained in the animal facility for 3 weeks to ensure complete metabolism of circulating testosterone. Animals were sacrificed as described above. Only the pineal glands from these castrate rats were removed. Glands were rapidly weighed, placed in storage vials containing normal saline, and frozen with acetone-dry ice.

Animals for the puberty study were obtained by matings of (Charles River cd) rats in the departmental animal facility. At 15,30, and 60 days after birth, animals were sacrificed by decapitation. Pineal glands were rapidly removed, weighed, placed into storage vials containing normal saline and frozen with acetone-dry ice.

Surgical Procedures

Blinding was accomplished by orbital enucleation. The rats were anesthetized with ether prior to surgery. A hemostat was placed so that one jaw of the hemostat was above the eye and one below the eye. Pressure was applied to the hemostat until the eye protruded between the jaws of the hemostat. The jaws of the hemostat were closed and the eye removed by severing the optic nerve behind the eye (lateral to the

hemostat). The eye socket was treated with 10% ethanol. The same procedure was applied to the other eye, and the animals returned to the animal room for recovery.

Castration was also performed under ether anesthesia. An incision was made through the scrotum, each testis elevated from the scrotum, and a ligature applied around the vas deferens and spermatic artery. A scissor cut was made between the testis and the ligature. The procedure was repeated for the other testis. The scrotum was closed with autoclips and treated with 10% ethanol.

In Vitro Experiments

Experiments were performed in vitro to determine whether de novo synthesis of AVT occurs in the pineal and what effects the various neurotransmitters and indoleamines have upon AVT synthesis and release. Another group of experiments were performed to determine what effect AVT has on the metabolism of melatonin and the other indoleamines.

Animals

Male rats (80 days of age) of the Charles River cd strain were utilized in this study. Animals were maintained in the departmental animal facility (as described previously) until sacrificed.

Incubation Procedures

In vitro production of AVT by rat pineals was studied using ^{14}C-isoleucine (Ile; New England Nuclear; 283 mCi/mMol) as the precursor to AVT production (AVT and AVP are not chemically separable by

conventional techniques but Ile is not found in ADH). Pineal glands were removed, weighed, and placed into incubation vials containing Krebs Ringers bicarbonate (KRBC) plus 1 mg/ml bovine serum albumen (BSA) and 2 mg/ml glucose (Appendix A). The incubation media were as described above or with the addition of amino acid precursors. Glands were incubated for 10 hrs in a metabolic shaking incubator (37°C; 60 cycles/min.). Glands were incubated with 95% O_2 every 30 min. The incubation media and AVT standards were passed over a 2 x 10 cm column containing G-25 Sephadex. Five drop samples were collected and read on a spectrometer. Aliquots were then counted for ^{14}C-isoleucine labeled AVT. To ensure that the ^{14}C label was AVT, pooled incubation media were subjected to amino acid analysis. Other samples of the incubation media were subjected to antibody precipitation to aid in verifying the presence of ^{14}C-Ile-AVT.

Animals for the neurotransmitter study were lightly etherized and decapitated. The pineal glands were rapidly removed, weighed, and placed into stoppered incubation flasks containing 1 ml KRBC plus 2 mg/ml glucose and 1 mg/ml bovine serum albumen (BSA:Sigma). The incubation media was fortified with 10^{-6}M or 10^{-8}M norepinephrine (Sigma), serotonin (Calbiochem), acetylcholine (Sigma), dopamine (Sigma) or melatonin (Regis). Glands incubated with KRBC plus glucose and BSA or KRBC plus glucose, BSA, and 0.5% alcohol served as controls. The incubations were performed in a metabolic shaking incubator (37°C, 60 cycles/min.) for 8 hours. The flasks were gassed each 30 minutes with 95% O_2-5% CO_2. At the termination of the experiment, pineal glands were removed to stoppered vials containing 500ul phosphate buffer (0.05M; pH 7.4) and frozen with acetone-dry ice. Incubation media were

frozen in the incubation flasks using acetone-dry ice. Samples were stored at -20°C until assayed.

Rats for the study of the effects of AVT upon indoleamine metabolism were sacrificed as described above. The pineal glands were placed in stoppered incubation flasks containing 1 ml KRBC plus 2 mg/ml glucose, 1 mg/ml bovine serum albumen, 0.1 uCi DL ^{14}C-Tryptophan (New England Nuclear; 33.19 mCi/mMol), 1 x 10^{-6}M norepinephrine (Sigma), and graded concentrations of AVT (Calbiochem; 225 IU/mg). Glands incubated without norepinephrine were utilized to determine the effects of non-stimulated indole metabolism. The incubations were performed as described above except the time of incubation was expanded to 10 hours. At the termination of the experiment, pineal glands were removed to storage vials and frozen with acetone-dry ice and stored at -20°C until assayed.

Viability of Incubated Pineals

A separate experiment was performed to assess the viability of pineal glands during a 10 hour incubation. Light microscopic examination was performed on pineals incubated for 0,1,2,4,6,8, and 10 hours. Glands incubated without O_2 for 10 hours were also examined for a comparison to tissue undergoing cell death. The incubation media were analyzed for the enzyme glutamic oxalacetic acid transaminase (GOT; E.C. 2.6.1.1) as another indicator of cell death (enzyme kits obtained from Dow Diagnostics).

Assay Techniques

Gland Extractions

Anterior pituitary glands were homogenized in ground glass tissue grinders with 1 ml 0.05M phosphate buffer (pH 7.4). The homogenate was decanted to centrifuge tubes and the tissue grinder washed with a second ml of phosphate buffer. The combined homogenate and rinse were centrifuged (5000 rpm) and the supernatant decanted to storage vials. The supernatant was diluted 1:200 with 0.05M phosphate buffer (pH 7.4) and radioimmunoassayed for LH, FSH, and prolactin.

Pineal glands were homogenized in 0.5 ml of .05M phosphate buffer. The homogenate was removed to centrifuge tubes and placed in a boiling water bath for 3 minutes, and centrifuged. Following centrifugation, the supernatant was decanted to storage vials. Duplicate 200 µl samples were removed for the radioimmunoassay of AVT.

Processing of Blood

After collection, the blood was centrifuged at 5000 rpm (radius of the centrifuge head is 10.8 cm) for 15 minutes. The plasma was removed to storage vials and stored at -20°C until assay. Blood was assayed by radioimmunoassay for LH, FSH, prolactin, and testosterone. For the testosterone assay, 400 µl blood was removed and extracted first with ether and then with methanol. The dried extract was radioimmunoassayed for testosterone. LH, FSH, and prolactin were assayed from unextracted serum samples.

Radioimmunoassay

The LH RIA procedure was that of Niswender et al. (1968). The LH antibody (anti-ovine LH) was obtained from Dr. Gordon Niswender and the purified LH for iodination obtained from Dr. Leo Riechert. The LH concentrations are expressed in terms of NIAMDD rat LH RP-1 having a biological potency of 0.03 x NIH LH-S1 ovarian ascorbic acid depletion assay.

The prolactin RIA was performed using a kit supplied by NIAMDD. Purified prolactin for iodination was NIAMDD-rat prolactin-I-2. The rat prolactin reference preparation was NIAMDD-rat prolactin-RP-1 with a biological potency of approximately 11 IU/mg (mouse deciduoma assay). The antibody supplied by Dr. Parlow was NIAMDD-anti-rat prolactin serum-4.

The FSH RIA was performed using a kit supplied by NIAMDD. Purified FSH for iodination was NIAMDD-rat FSH I-3. The rat FSH reference preparation was NIAMDD-rat FSH-RP-1 with a biological potency of approximately 2.1 x NIH-FSH-S1 (HCG augmentation assay). The antibody supplied by Dr. Parlow was NIAMDD-anti-rat FSH-serum-7.

The AVT antibody was provided by Dr. R. Weitzman (Appendix B). The AVT for iodination and standards was purchased from Calbiochem having a potency of 225 IU/mg (rat pressor bioassay). The antibody has a 100 percent cross reactivity with arginine vasopressin (AVP). Although AVP may be present in the pineal, it is not detectable by radioimmunoassay (Rosenbloom and Fisher, 1975), bioassay (Milcu et al., 1963), nor by mass spectrometry or amino acid analysis (Cheesman, 1970; Cheesman and Farris, 1970).

The antibody for testosterone radioimmunoassay was obtained from Dr. Guy Abraham. This antibody exhibits 70% cross-reactivity with 5α-dihydrotestosterone (Coyotupa et al., 1972). The values reported herein include both dihydrotestosterone and testosterone.

Indoleamine Extraction

The incubation media from glands treated with AVT alone (Table I) were extracted for melatonin and serotonin after techniques described elsewhere (Axelrod et al., 1969). Briefly, for the melatonin extractions, a 100 ul aliquot of the incubation media was added to extraction tubes containing 2 ml of 0.2M borate buffer (pH 10.0) and extracted into 6 ml chloroform. Following centrifugation, 1 ml of the aqueous phase was removed for serotonin extraction. The remaining organic phase was washed with borate buffer and 4 ml of the organic phase was removed for counting in a Packard Tri-Carb Counter. The cocktail was toluene plus PPO (2,5-diphenyloxazole), and the counting efficiency was 86%.

The aqueous aliquot removed for serotonin analysis was extracted into 6 ml chloroform:butanol (3:1). The organic layer was washed with 2 ml borate buffer (pH 10.0) and 4 ml of the organic phase were removed for counting.

Following the removal of 4 ml of the 6 ml organic phase the remaining organic layers (2 ml per extraction vial) were combined and concentrated in such a manner as to yield separate samples of both indoles. The indoleamines were verified by descending paper chromatography, against the appropriate standard, using a chloroform:butanol: acetic acid (8:2:2) solvent.

The separation of other indole metabolites from the incubation media was accomplished by thin-layer chromatography (Klein and Notides, 1969). Standard indoleamines, 5-hydroxytryptophol (HTOL), 5-hydroxyindoleacetic acid (HIAA), N-acetylserotonin (NAS), 5-methoxyindoleacetic acid (MIAA), 5-methoxytryptophol (MTOL) (all from Sigma), and melatonin (Regis) were dissolved in a solution of ethanol (50%), 0.1 N HCL (50%), and ascorbic acid (0.5%), to give a final indoleamine concentration of 1 mM.

Twenty-microliter samples containing 10 ul of the standard indole solution and 10 ul of pineal incubation media were applied to precoated thin-layer plates (Brinkman Instruments: 20 x 20 cm, 0.25 mm silica gel, fluorescent indicator UV_{254}) and the spots dried under a stream of nitrogen. The thin-layer plates were first run in a chloroform:methanol:acetic acid (93:7:1) solvent system. When developed, the plates were immediately redeveloped in the same direction. After developing the second time, the plates were chromatographed in a second direction in ethyl acetate. Areas corresponding to the appropriate indoles (Figure 18) were scraped from the plates, pulverized, and placed into scintillation vials. The pulverized material was then treated with 0.5 ml of Soluene (Packard) followed by the scintillation cocktail. The material was then counted on a Packard Tri-Carb. Counting efficiency is 53% with Soluene present.

Data were expressed as percentages of the ^{14}C-tryptophan converted to the specific indoleamine per mg gland weight (Axelrod et al., 1969).

Protein Analysis

Pineal glands were homogenized in ground glass tissue grinders (Kimble; 2 ml) in 1.0 ml of 10% trichloroacetic acid (TCA). The extract was removed to centrifuge tubes and the tissue grinder rinsed with a second ml of 10% TCA which was added to the centrifuge tubes. The dentrifuge tubes were placed in a boiling water bath for 3 minutes, removed, and centrifuged. The supernatant was decanted to scintillation vials and the remaining TCA insoluble material was washed twice with 4% TCA. Following centrifugation, the supernatants were combined in scintillation vials for counting purposes. This material represented the ^{14}C-tryptophan accumulated in the pineal gland (Wurtman et al., 1969).

Five hundred microliters of distilled water were added to the TCA-insoluble material. Duplicate 100 µl aliquots were removed for counting purposes (^{14}C-protein). Results were expressed as counts per minute per mg gland weight (CPM/mg).

Statistical Analysis

The data from the constant light and blinding data were analyzed by analysis of variance and Students t-test. All other data were analyzed by use of Students t-test.

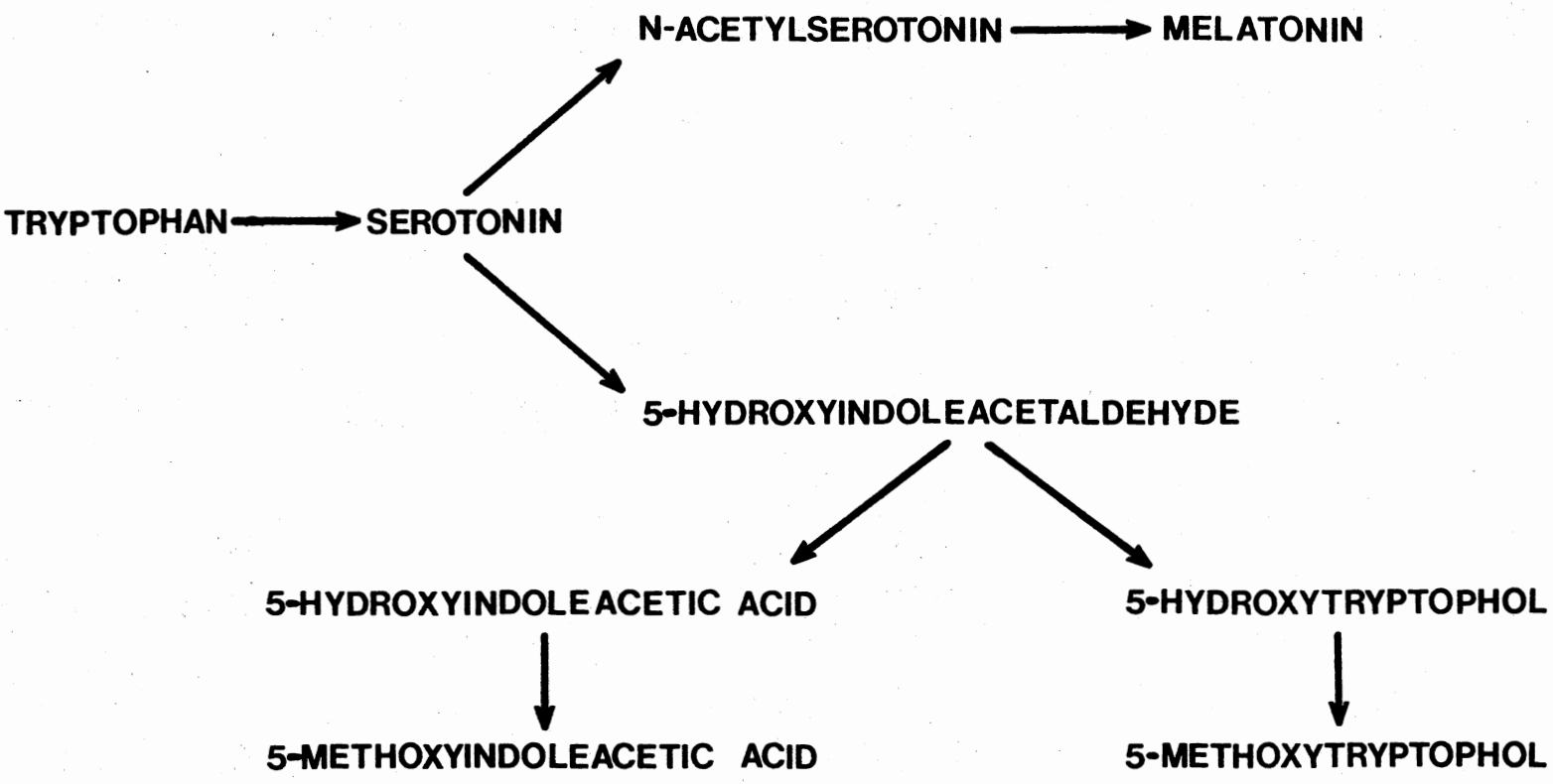

Figure 1. Outline of the Metabolism of the Major Pineal Indoleamines

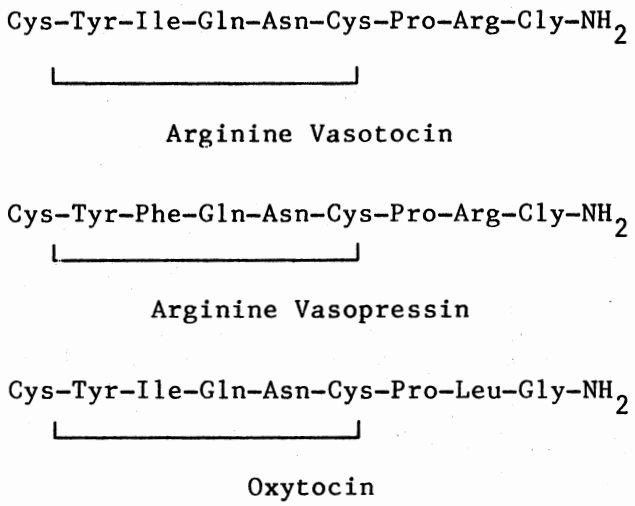

Figure 2. The Structures of Arginine Vasotocin, Arginine Vasopressin, and Oxytocin

CHAPTER IV

RESULTS

In Vivo Experiments

The pineal gland weight was found to increase with the body weight as the animal developed through puberty (see Table I). The pineal gland AVT concentration during this period (Figure 3) decreased from 15 days of age to its lowest concentration in the sexually mature 60 day old rat ($p < .05$).

Castration

Castration of mature male rats decreased pineal gland weight from 1.5 to 0.80 mg ($p < .05$). The concentration of AVT in the pineal gland was observed to decrease ($p < .05$) following castration (Figure 4).

Blinding and Constant Light

Neither blinding nor constant light had an effect on pineal gland weight (Table II). Blinding was found to lower the pineal gland concentration of AVT at 2 and 4 weeks following the initiation of treatment (Figure 5), however the differences were not statistically significant. By 6 weeks following blinding, there was a significant depletion ($p < .05$) of the pineal concentration of AVT. Two weeks later (week 8), the pineal gland AVT concentration returned to control levels.

TABLE I

THE INFLUENCE OF SEXUAL MATURATION ON BODY AND PINEAL GLAND WEIGHTS

Age	n	Body Weight (gm.)	Pineal Weight (mg.)
15[a]	6	30 ± 0.6^{bc}	0.7 ± 0.07^{bef}
30	6	76 ± 2.1^{c}	0.99 ± 0.01^{e}
60	6	199 ± 8.8^{d}	1.45 ± 0.12^{f}

a) Age in days

b) Mean $\pm$ standard error of the mean; like letters indicate significant differences

d-g) $p < .05$

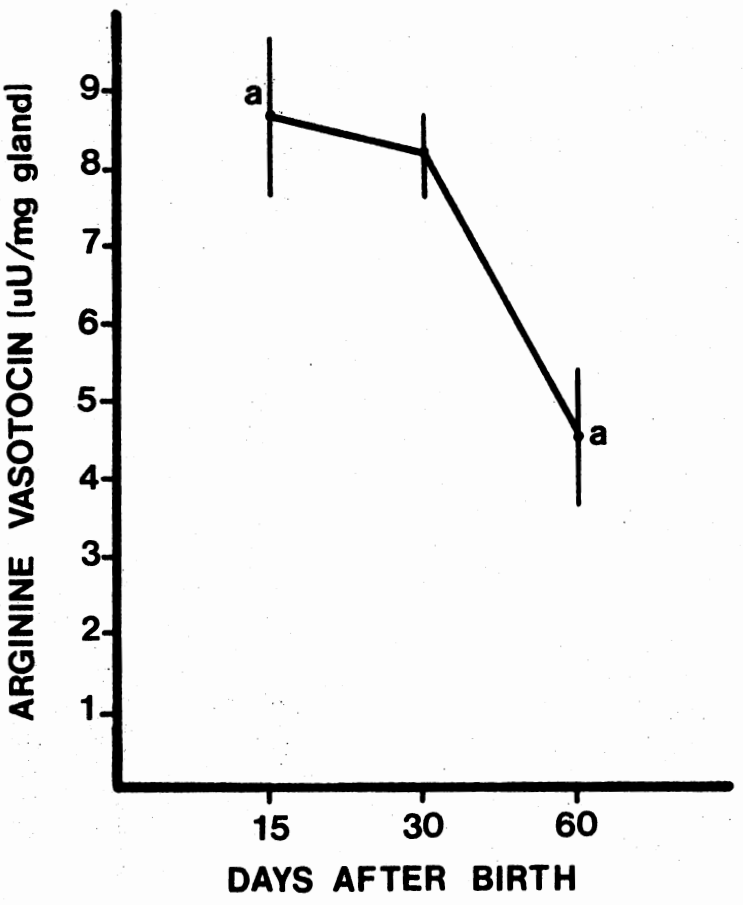

Figure 3. The Influence of Sexual Maturation on Pineal Gland Arginine Vasotocon Concentration. Like letters indicate statistical differences: a=$p < .05$; n= 6 pineals per treatment group.

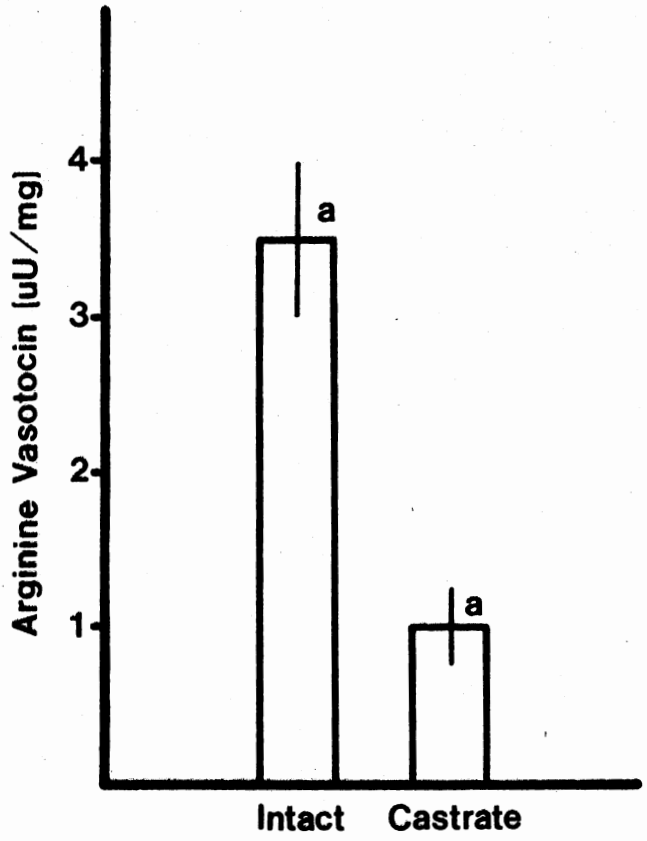

Figure 4. The Effect of Castration on Pineal Gland Arginine Vasotocin Concentration. Like letters indicate statistical differences: a=p < .05; n=10 pineals per treatment group.

TABLE II

THE EFFECT OF BLINDING AND CONSTANT LIGHT
ON PINEAL GLAND WEIGHTS

Weeks	n	Blinded	Constant Light
0	6	1.5 ± 0.1^{ab}	1.5 ± 0.1^{abc}
2	6	1.5 ± 0.2	0.9 ± 0.1^{c}
4	6	1.6 ± 0.1	1.4 ± 0.2
6	6	1.7 ± 0.2	1.3 ± 0.1
8	6	1.3 ± 0.1	1.5 ± 0.1

a) Mean $\pm$ standard error of the mean

b) Weights are in mg

c) $p < .01$

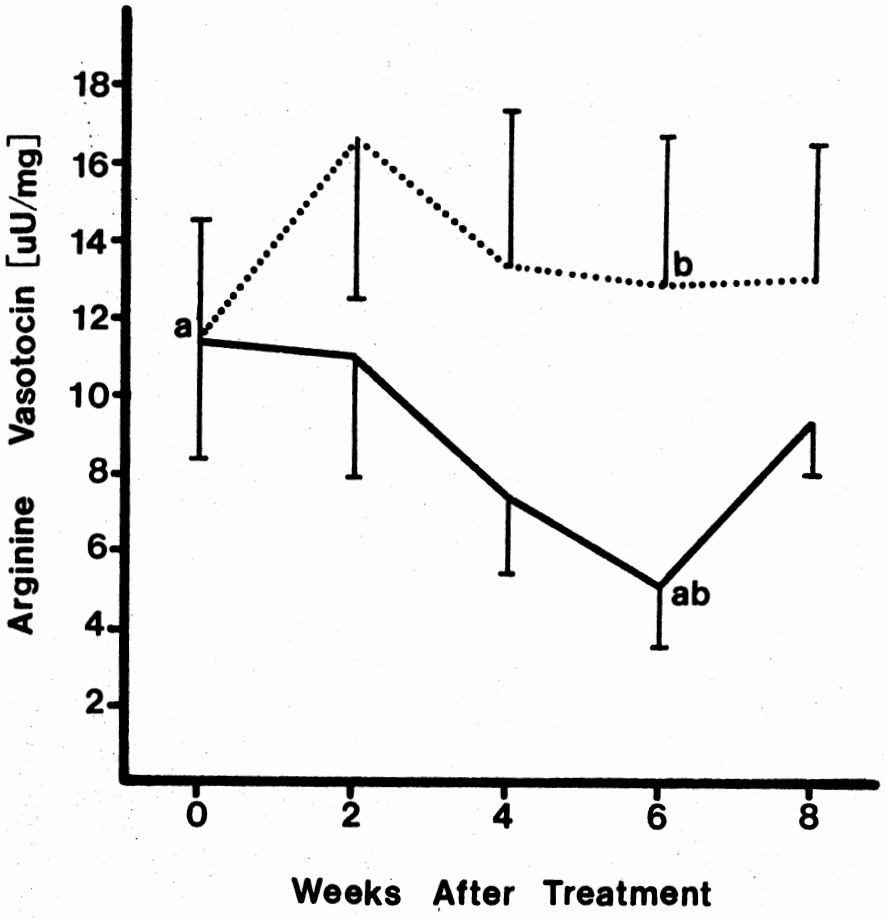

Figure 5. Blinding and Constant Light Induced Alterations in Pineal Gland Arginine Vasotocin. Treatments were blinding (solid line) and constant light (dotted line). Like letters indicate statistical differences: a= $p < .05$; b=$p < .05$; n=6 pineals per treatment group.

Exposure to constant illumination (Figure 5) for 2 weeks produced a slight but nonsignificant elevation in pineal AVT. From 2-8 weeks there was no change in pineal AVT concentration.

Evaluation of blinding effects on other hormones reveals that plasma FSH (Figure 6) content was lowered by 6 weeks following surgery ($p < .05$). Likewise plasma testosterone (Figure 7) was lowered by 6 weeks ($p < .05$). Plasma prolactin (Figure 8) was apparently elevated, however, the differences were not statistically significant. Blinding (Figure 9) significantly elevated plasma LH at 4 and 6 weeks after blinding ($p < .05$).

Constant light was found to have no effect on plasma FSH levels (Figure 6). On the other hand, exposure to constant light produced a nonsignificant elevation in plasma testosterone (Figure 7) and reduced plasma prolactin (Figure 8) to a low at 8 weeks ($p < .05$). Plasma LH (Figure 9) was elevated a 2 weeks and remained significantly greater than controls throughout the experiment ($p < .05$).

Pituitary gland FSH concentrations (Table III) were elevated by both constant light and blinding but there were no statistically significant difference. Blinding was found to lower pituitary gland prolactin concentration (Table IV) at 2 weeks through 8 weeks ($p < .01$) but constant light produced no statistically discernible alterations in pituitary prolactin concentrations. Both blinding and constant light (Table V) increased the pituitary concentration of LH ($p < .01$).

In Vitro Experiments

Preliminary incubations were performed to assess the viability of pineal glands incubated for 1-10 hours. Light microscopic

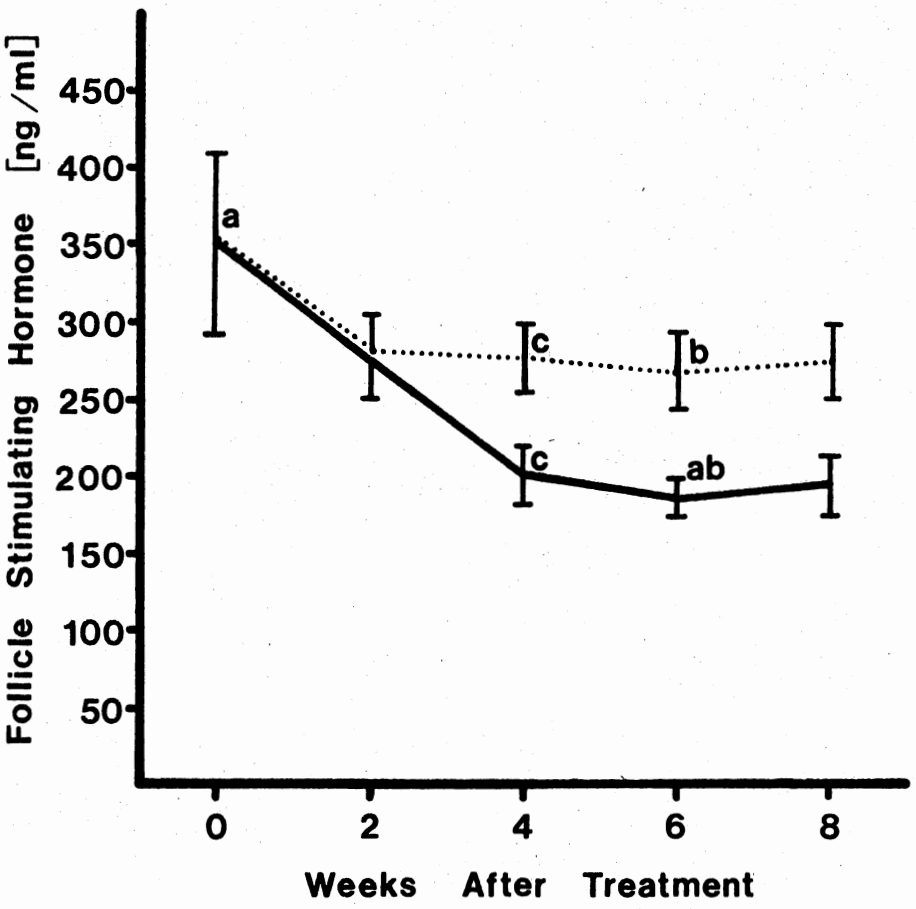

Figure 6. Blinding and Constant Light Induced Alterations in Plasma Follicle Stimulating Hormone. Treatments were blinding (solid line) and constant light (dotted line). Like letters indicate statistical differences: a=$p < .05$, b=$p < .05$, c=$p < .08$; n=6 animals per treatment group.

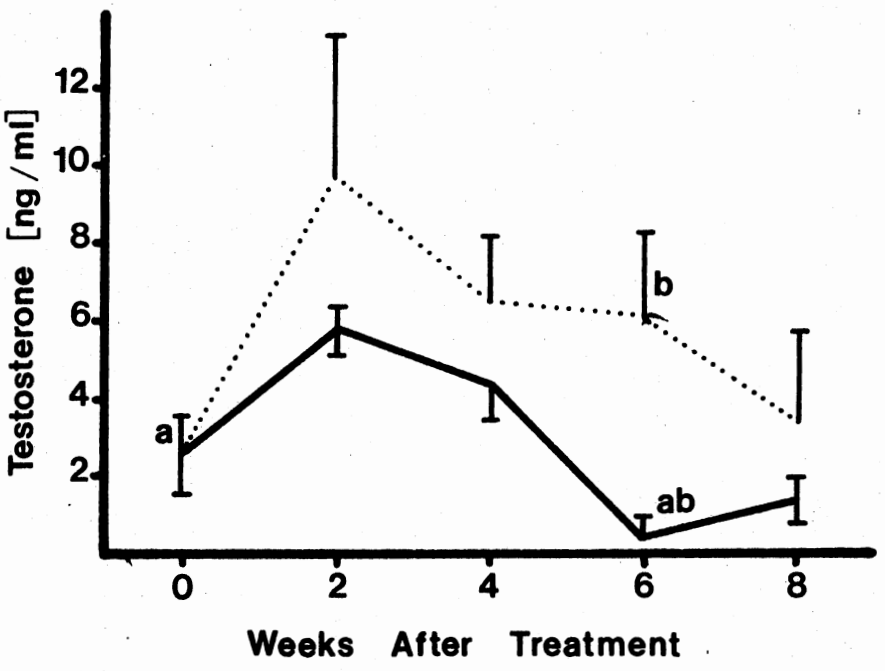

Figure 7. Blinding and Constant Light Induced Alterations in Plasma Testosterone. Like letters indicate statistically significant differences: a=$p < .05$, b=$p < .05$; n=6 animals per treatment group.

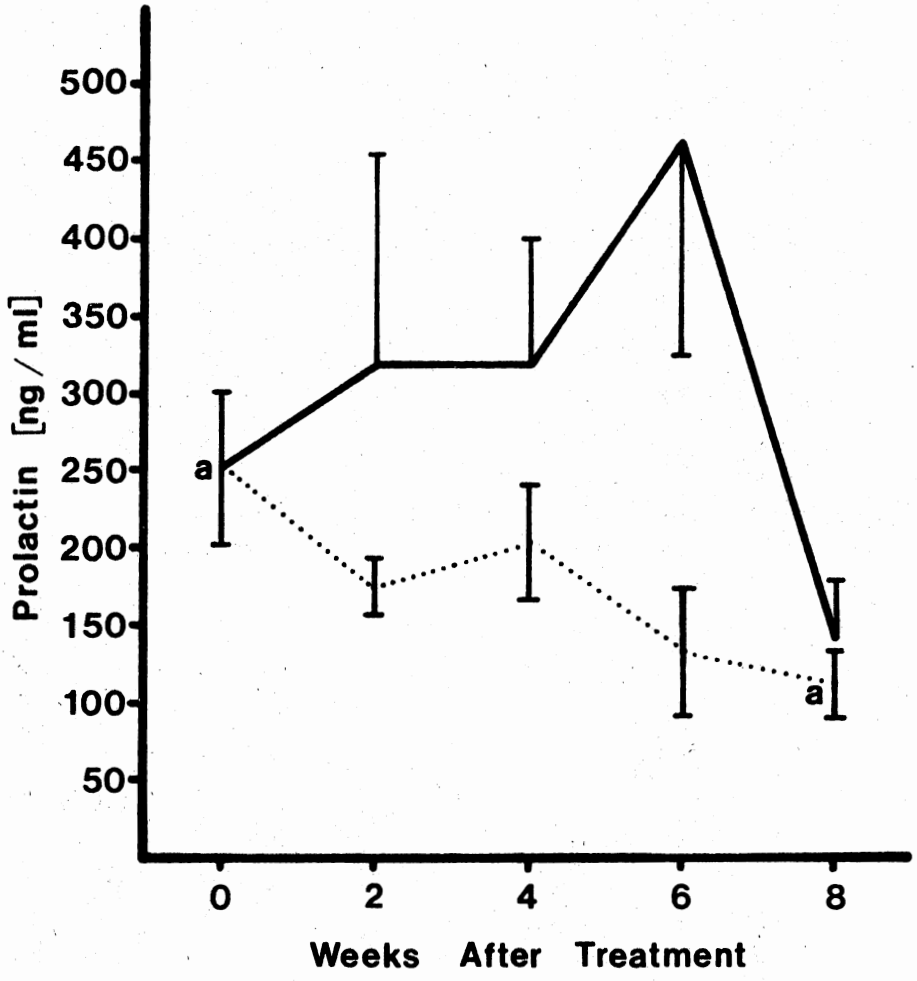

Figure 8. Blinding and Constant Light Induced Alterations in Plasma Prolactin. Like letters indicate statistically significant differences: a=$p < .05$; n=6 animals per treatment group.

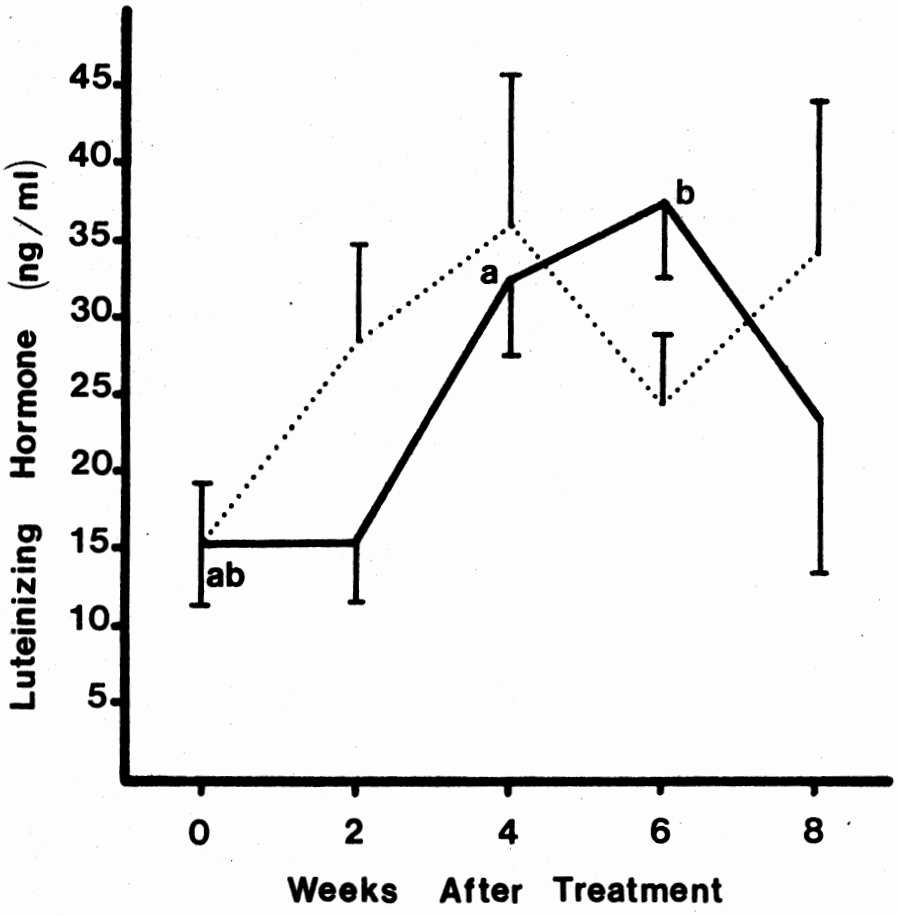

Figure 9. Blinding and Constant Light Induced Alterations in Plasma Luteinizing Hormone. Like letters indicate statistically significant differences: a=$p < .05$, b=$p < .05$; n=6 animals per treatment group.

TABLE III

THE EFFECT OF BLINDING AND CONSTANT LIGHT
ON PITUITARY GLAND FSH CONCENTRATION

Weeks	n	Pituitary FSH (ug/mg)	
		Blinded	Constant Light
0	6	53.9 ± 9.1[a]	53.9 ± 9.1[a]
2	6	55.4 ± 13.5	52.7 ± 7.4
4	6	68.9 ± 9.8	63.1 ± 22.7
6	6	61.1 ± 11.6	48.1 ± 7.3
8	6	51.5 ± 14.4	25.7 ± 8.7

a) Mean ± standard error

TABLE IV

THE EFFECT OF BLINDING AND CONSTANT LIGHT ON PITUITARY GLAND PROLACTIN CONCENTRATION

Weeks	n	Pituitary Prolactin (ng/mg)	
		Blinded	Constant Light
0	6	943 ± 113[abcde]	943 ± 113[ab]
2	6	303 ± 138[c]	580 ± 150
4	6	462 ± 100[d]	2000 ± 510
6	6	555 ± 218	850 ± 240
8	6	176 ± 15[e]	570 ± 260

a) Mean ± standard error

b) Like letters indicate statistical differences

c) $p < .01$

d) $p < .01$

e) $p < .001$

TABLE V

THE EFFECT OF BLINDING AND CONSTANT LIGHT
ON PITUITARY GLAND LH CONCENTRATION

Weeks	n	Pituitary LH (ug/mg)	
		Blinded	Constant Light
0	6	8.7 ± 2.1[abc]	8.7 ± 2.1[abde]
2	6	14.6 ± 4.1	18.0 ± 2.2[d]
4	6	24.0 ± 3.6[c]	18.1 ± 2.1[e]
6	6	16.3 ± 4.4	12.9 ± 2.1
8	6	15.5 ± 3.3	8.9 ± 2.4

a) Mean ± Standard Error

b) Like letters indicate statistical differences

c) $p < .01$

d) $p < .01$

e) $p < .01$

examination revealed little evidence of cellular death. The enzyme glutamate oxalacetate transminase were assayed as indicators of cellular death (Figure 10). GOT levels were constant for 6-8 hours. After this time there was a slight increase in enzyme activity but at 10 hours the values were still 5 fold less than a corresponding gland incubated under anoxic conditions.

AVT Synthesis

Fractionation of the incubation media was accomplished by column chromatography on a 0.5 x 10 cm column of G-25 sephadex. The separation yielded a peak of radioactivity which had the same elution volume as the AVT standard (Figure 11). Pooled samples of this radioactive peak from 4 incubations were subjected to amino acid analysis. The amino acid analysis did not confirm the presence of AVT (Table VI) in the incubation media. An antibody separation technique was also employed (Figure 12) but there was no difference between the nonspecific binding and the total binding tubes.

Neurotransmitter Experiments

An investigation of the effects of various neurotransmitter substances on in vitro AVT secretion was studied next. The data revealed that Ach expressed a dose-related stimulation of pineal AVT secretion in vitro (Figure 13). The 10^{-8}M Ach concentration elevated the AVT content of the media from 0.88 μU/mg gland weight to 1.33 μU/mg while 10^{-6}M Ach produced a total of 1.84 μU/mg of AVT secreted ($p < .01$).

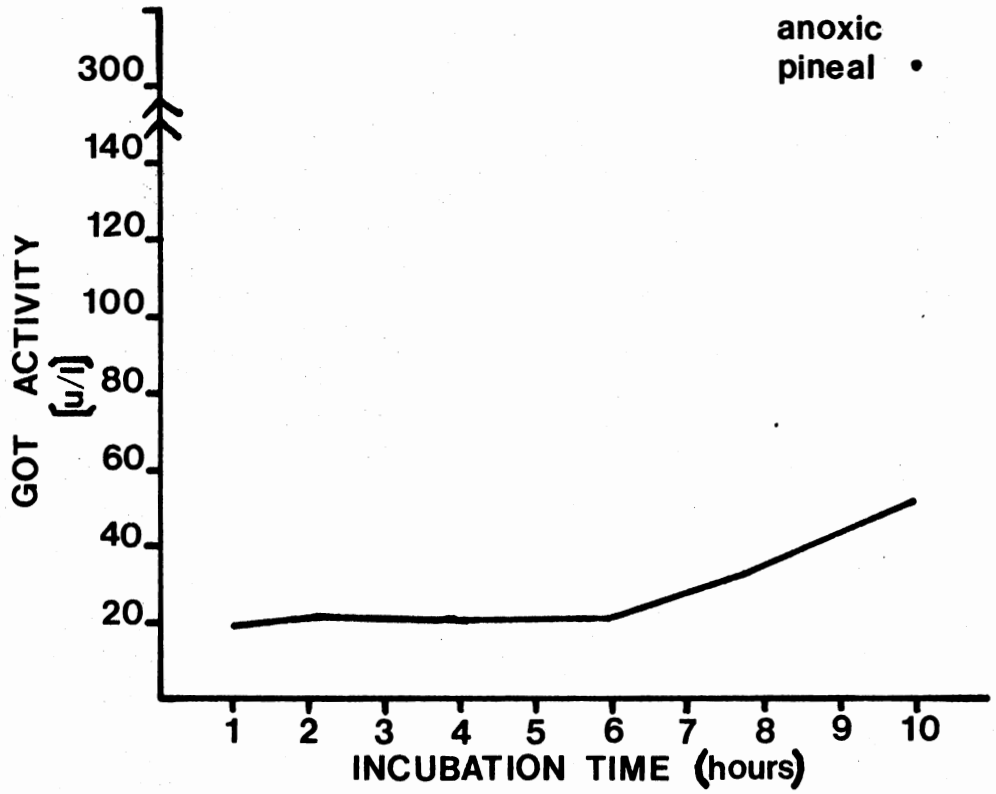

Figure 10. Enzyme Activity in Pineal Indubation Media

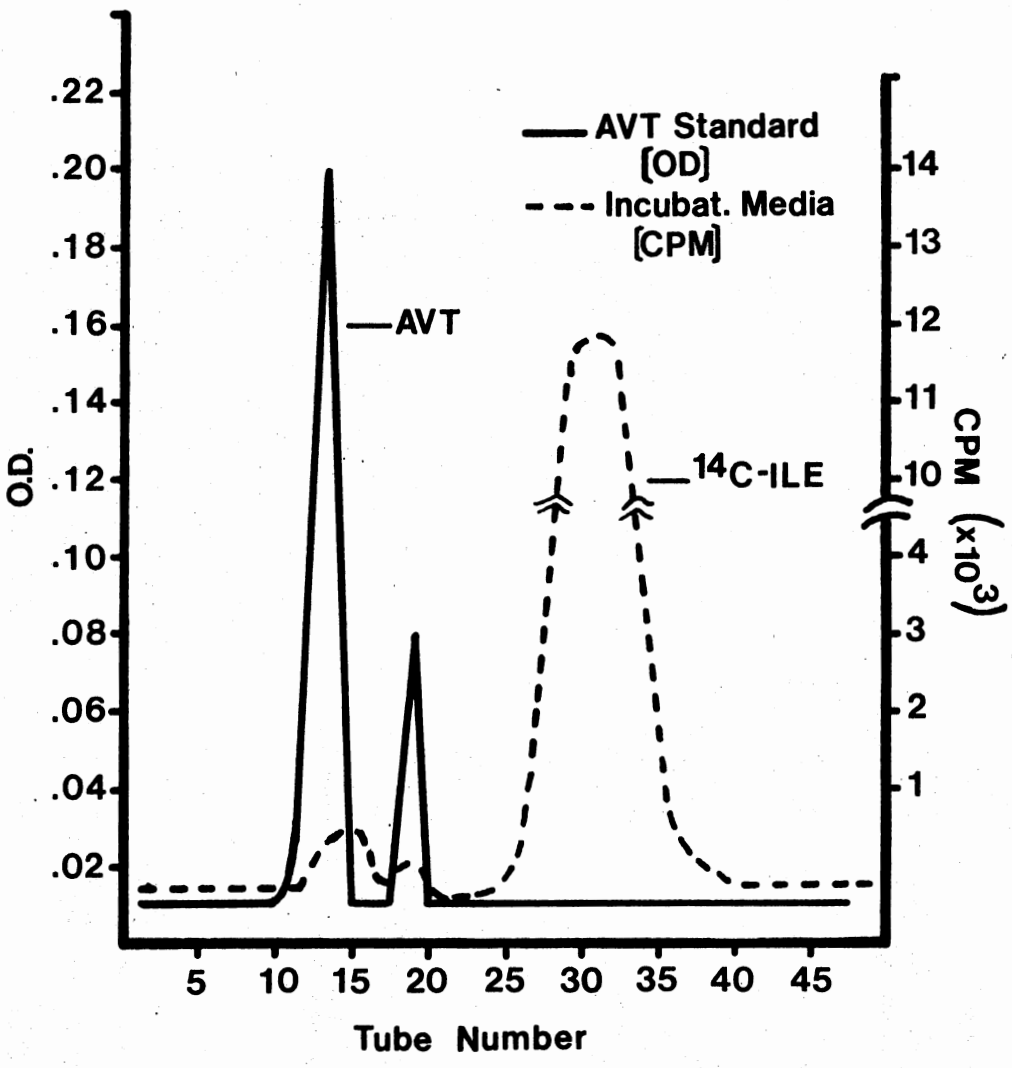

Figure 11. Column Chromatographic Separation of Pineal Incubation Media. Arginine vasotocin (AVT) standard (solid line) and ^{14}C-isoleucine (dotted lines) profiles following perfusion through a sephadex G-25 column.

TABLE VI

AMINO ACID ANALYSIS PROFILE OF PINEAL INCUBATION MEDIA PARTIALLY PURIFIED BY COLUMN CHROMATOGRAPHY

Amino Acid	Content (nm)[a]
Threonine	2.9
Serine	16.4
Glutamic Acid	17.8
Proline	5.1
Glycine	33.5
Alanine	10.6
Valine	5.4
Isoleucine	4.1
Leucine	5.1
Phenylalanine	1.6
Histidine	1.1
Lysine	3.7
Arginine	1.9

a) Amino acid content in nanomoles

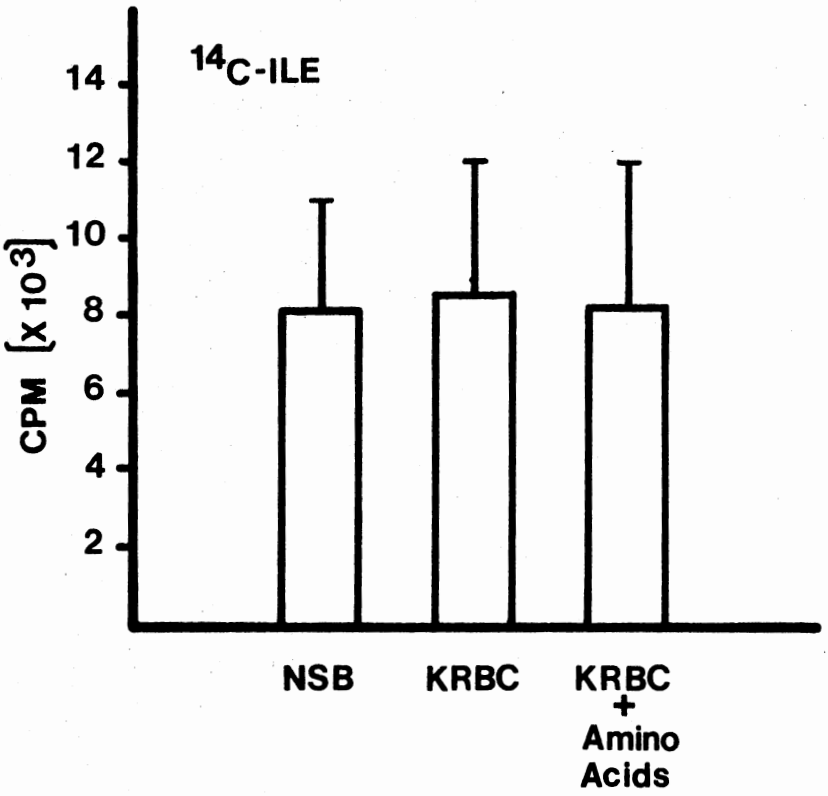

Figure 12. Antibody Precipitation of Pineal Incubation Media. NSB is non-specific binding and KRBC is Krebs Ringers incubation media. Each of the incubated groups represents 10 pineals.

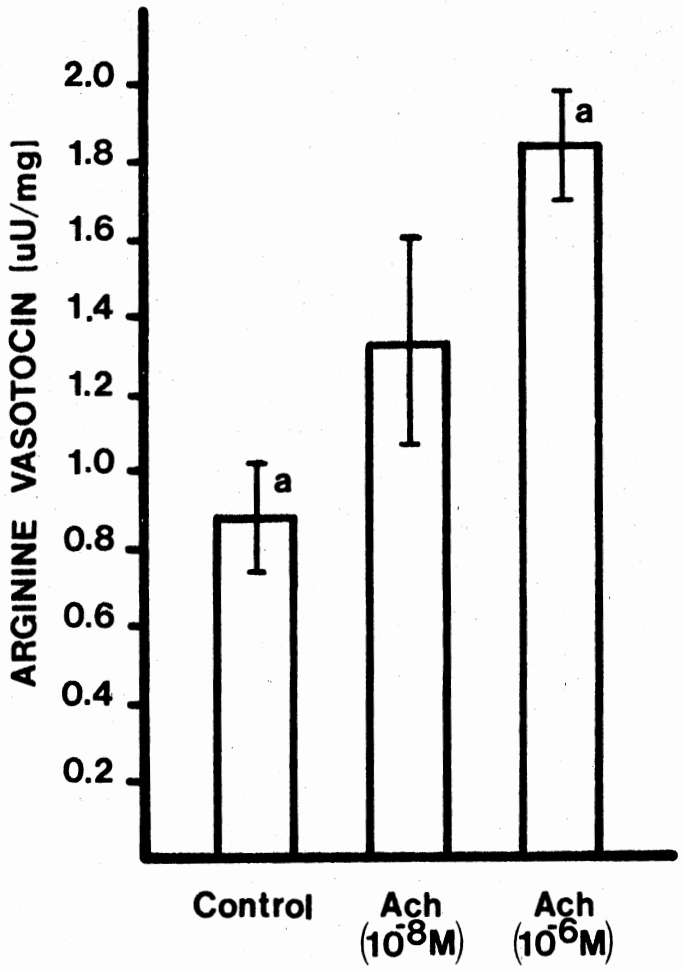

Figure 13. The Effect of Acetylcholine on Arginine Vasotocin Secretion In Vitro. Like letters indicate statistically significant differences: a=$p < .01$; n= 6 pineals per treatment group.

NE had no effect on AVT secretion (Figure 14) at either concentration utilized. In a repeat study, NE appeared to partially inhibit ($p < .06$) AVT secretion (Figure 15). DA produced a slight dose related stimulation of AVT release with 10^{-6}M DA having the most prominent effect ($p < .06$). A second corroborative study (Figure 16) indicated that DA (10^{-6}M) had no statistically significant effect on AVT secretion.

Serotonin (Figures 16, 17) also had no effect on AVT secretion at either concentration for both studies. A second study likewise produced no significant effects on AVT secretion.

Melatonin produced a nonsignificant dose-related stimulation of AVT secretion. Melatonin (10^{-6}M) elevated the media content from 0.88 μU/mg to 1.72 μU, just below the 1.84 μU/mg stimulation produced by Ach.

Indoleamine Experiments

The effects of AVT upon melatonin and serotonin synthesis (Table VII) were first investigated. The data indicated that AVT had no effect upon melatonin nor serotonin synthesis.

The effects of AVT upon NE stimulated indoleamine synthesis were also studied. NE (Table VIII) stimulated NAS ($p < .001$) and melatonin ($p < .001$) production and decreased HIAA ($p < .025$). The other indoleamines (MIAA, HTOL, MTOL, and serotonin) were unaffected by NE. The addition of 0.1 ug AVT (Figure 19) slightly depressed the NE stimulated conversion of ^{14}C-tryptophan to ^{14}C-serotonin ($p < .01$) but the differences were not significant. The larger doses of AVT had no effect. NAS synthesis (Figure 20) from serotonin was inhibited (58%) by

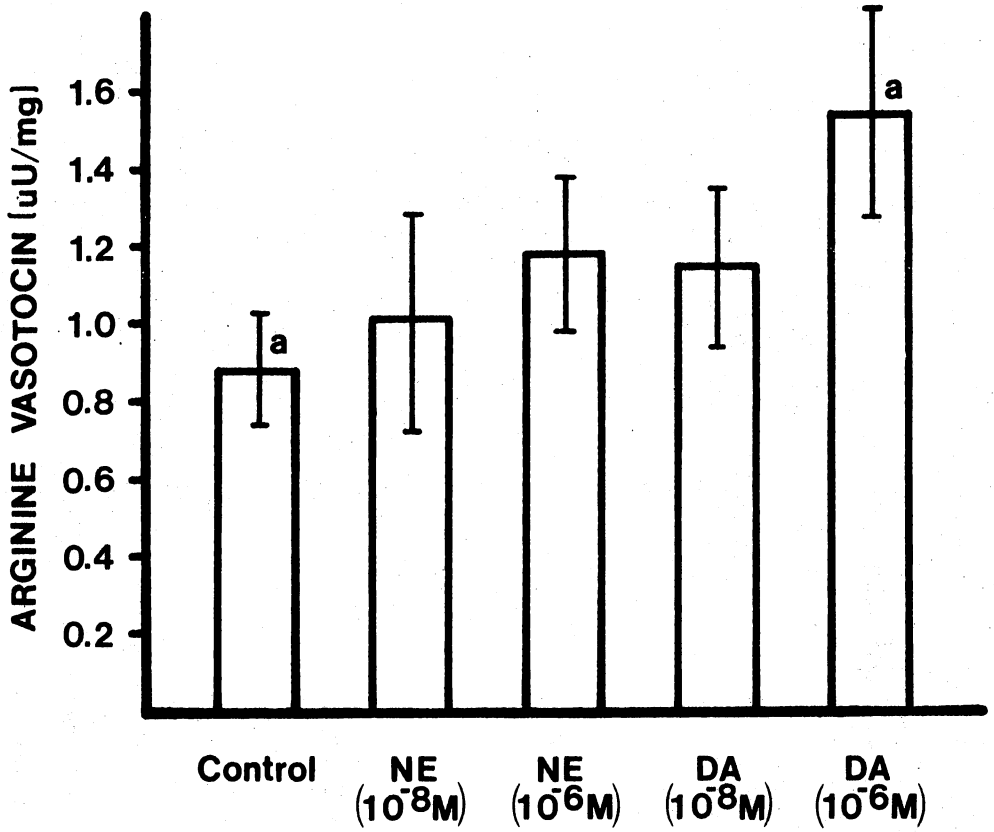

Figure 14. The Effect of Norepinephrine and Dopamine on Arginine Vasotocin Secretion In Vitro. Like letters indicate statistically significant differences: a=$p < .06$; n=6 pineals per treatment group.

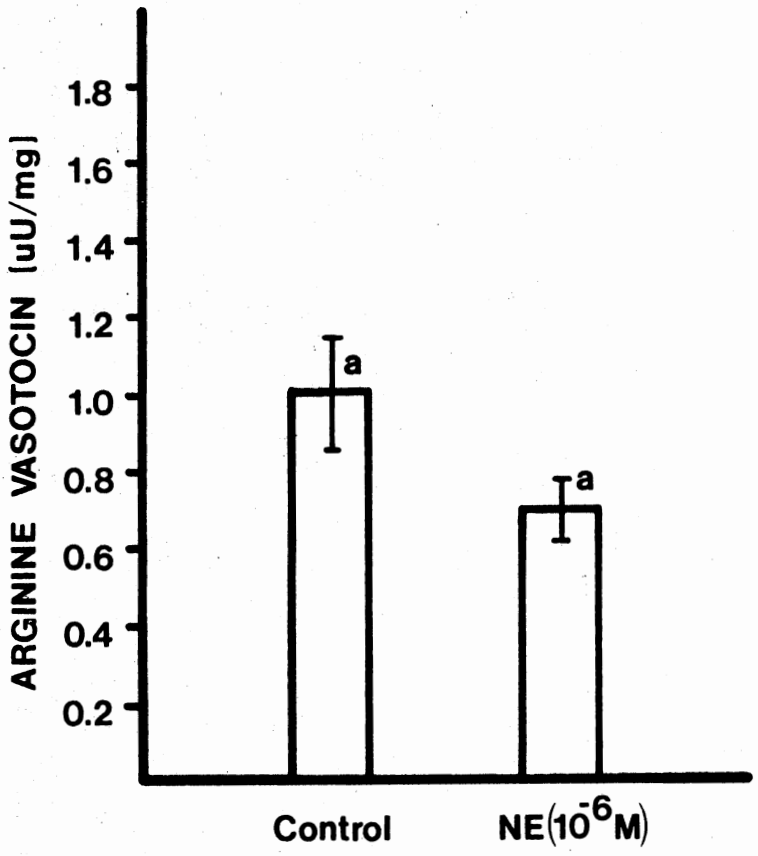

Figure 15. The Effect of Norepinephrine and Ascorbic Acid on Arginine Vasotocin Secretion In Vitro. Like letters indicate statistically significant differences: a=$p < .06$; n=6 pineals per treatment group.

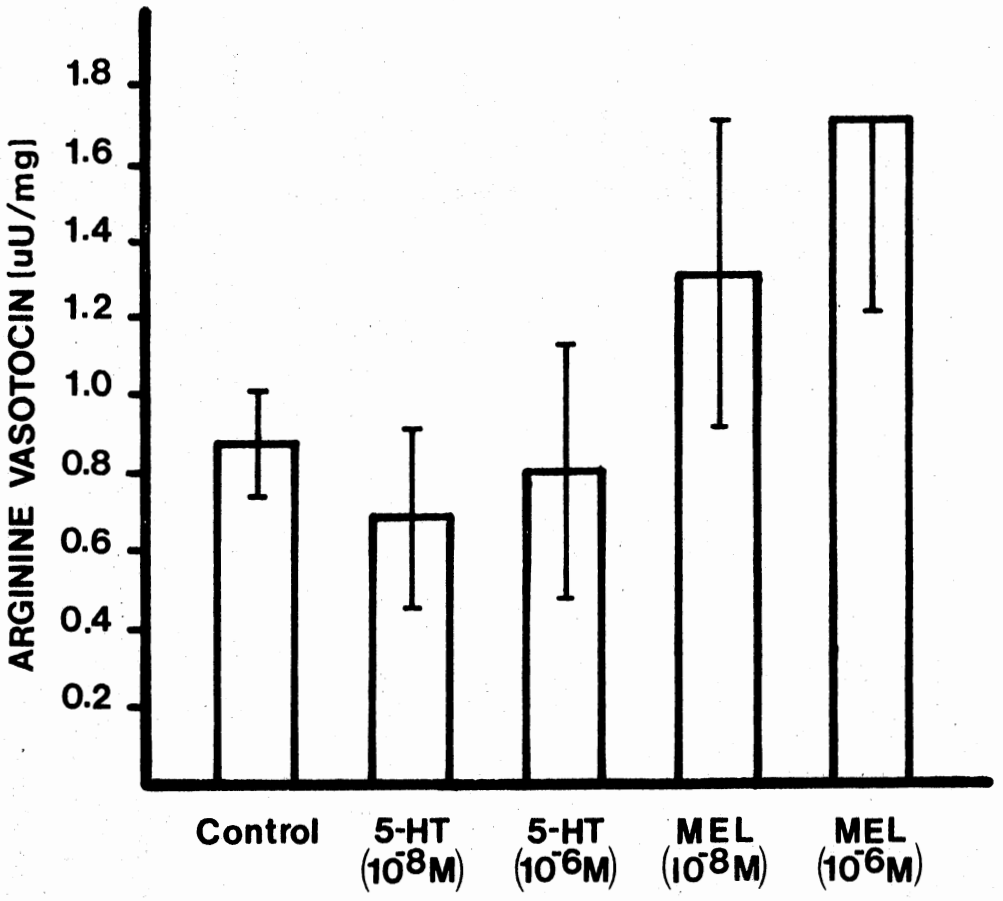

Figure 16. The Effect of Serotonin and Melatonin on Arginine Vasotocin Secretion In Vitro. n=6 pineals per treatment group.

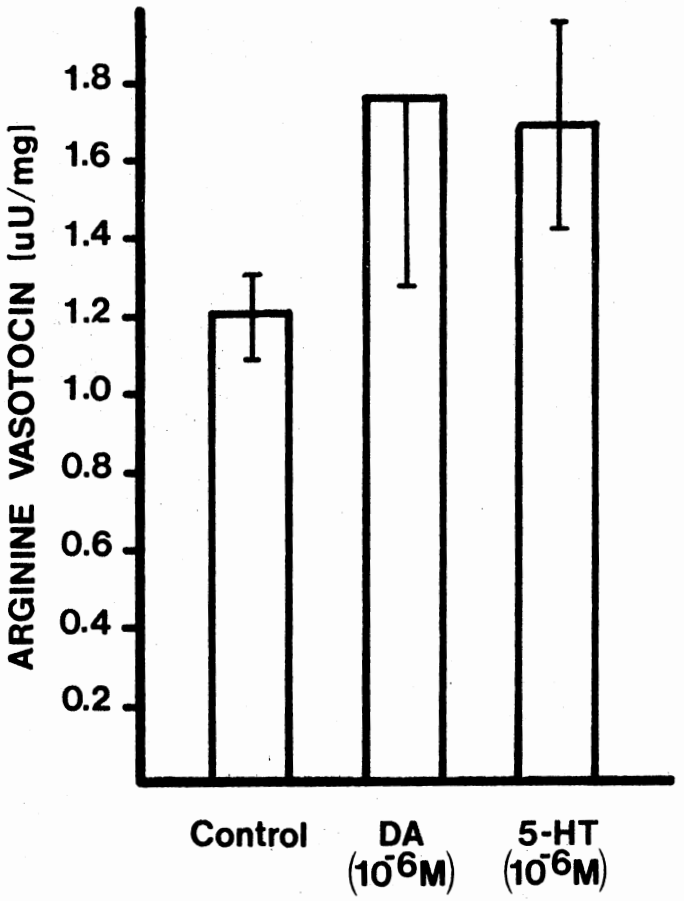

Figure 17. The Effect of Dopamine and Serotonin on Arginine Vasotocin In Vitro. n=6 pineals per treatment group.

TABLE VII

THE EFFECT OF AVT UPON MELATONIN AND SEROTONIN SYNTHESIS

Treatment	n	^{14}C-Melatonin	^{14}C-Serotonin
Control	6	1.84 ± .54[abc]	10.52 ± 2.31[abc]
AVT (0.1 ug)	6	2.34 ± .59	10.56 ± 1.78
AVT (1.0 ug)	6	1.86 ± .35	8.39 ± 1.41
AVT (10 ug)	6	1.49 ± .23	8.68 ± 1.23

a) Mean ± standard error

b) Data expressed as % conversion from ^{14}C-tryptophan

c) No treatment produced significant differences from controls

TABLE VIII

EFFECT OF NOREPINEPHRINE UPON
INDOLEAMINE SYNTHESIS

Indoleamines	n	Control[a]	Norepinephrine[a] (10^{-6}M)
Hydroxytryptophol	6	2.17 ± .38	1.55 ± .12
Hydroxyindoleacetic acid	6	8.11 ± 1.46[b]	3.65 ± .83[b]
Melatonin	6	1.19 ± .32[c]	8.38 ± .67[c]
Methoxyindoleacetic acid	6	0.65 ± .11	0.70 ± .06
Methoxytryptophol	6	0.40 ± .10	0.44 ± .11
N-Acetylserotonin	6	0.63 ± .23[d]	6.68 ± .59[d]
Serotonin	6	10.45 ± 2.36	8.95 ± .92

a) Mean ± standard error; data expressed as % conversion from ^{14}C-tryptophan to the specific indoleamine.

Like letters between treatment groups indicate statistically significant differences:

b) = $p < .025$; c) = $p < .001$; d) = $p < .001$

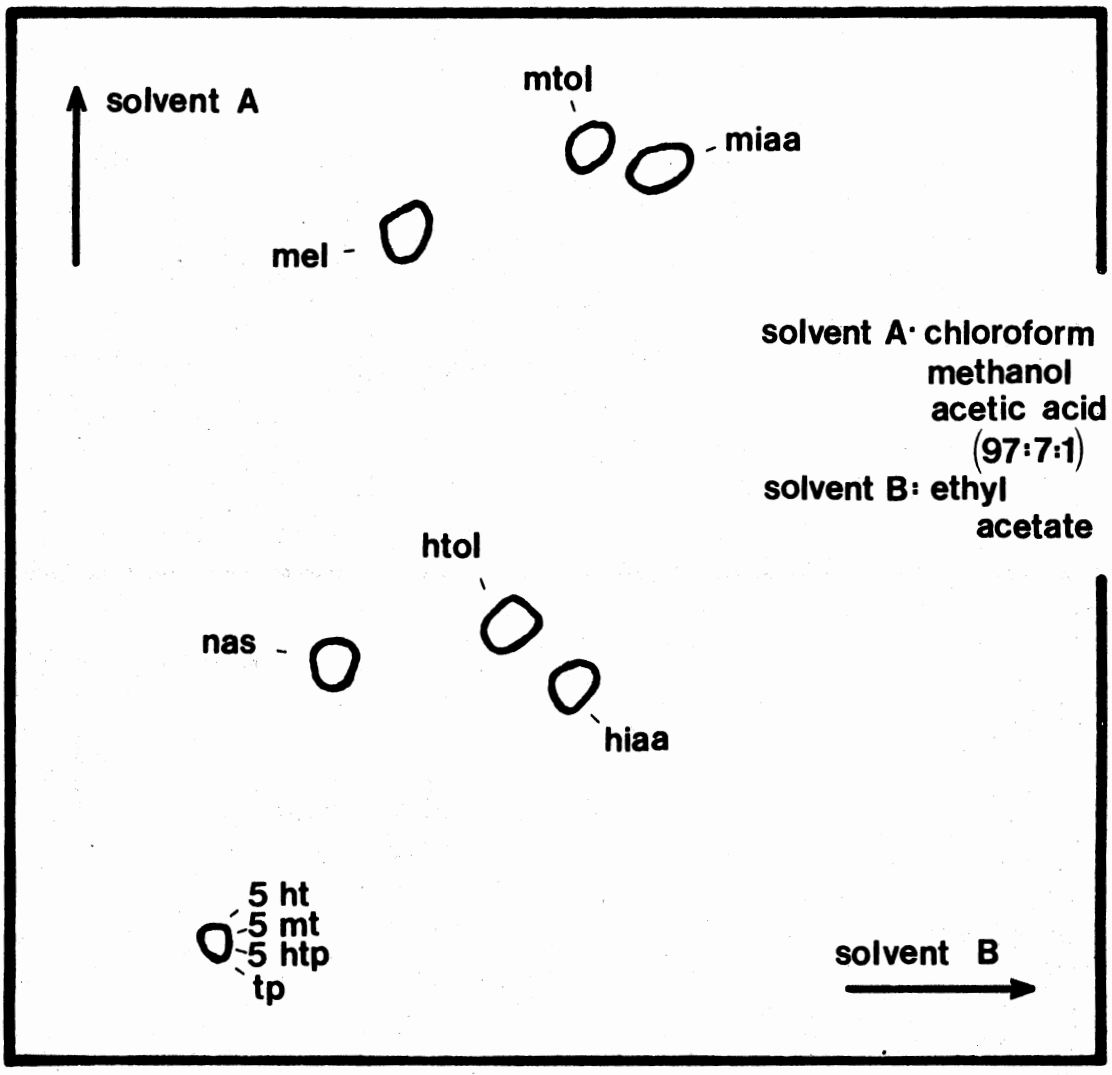

Figure 18. Thin-layer Chromatographic Separation of Pineal Indoleamines

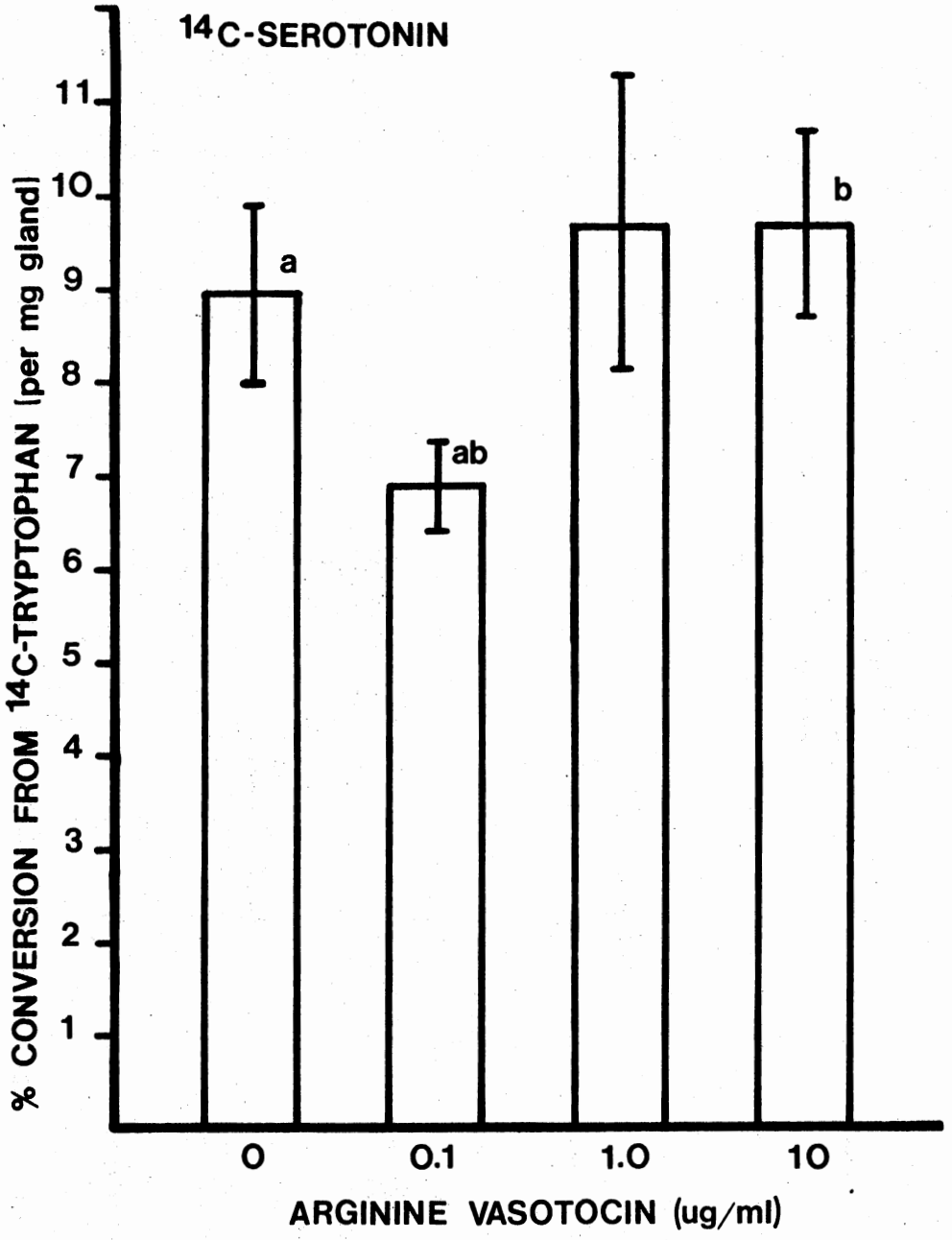

Figure 19. The Effect of Arginine Vasotocin (AVT) Upon Norepinephrine Stimulated Serotonin Synthesis. All glands were treated with NE (10^{-6}M) and the indicated dose of AVT. Like letters indicate statistically significant differences: a=$p < 0.1$; b=$p < 0.05$; n=6 pineals per treatment group.

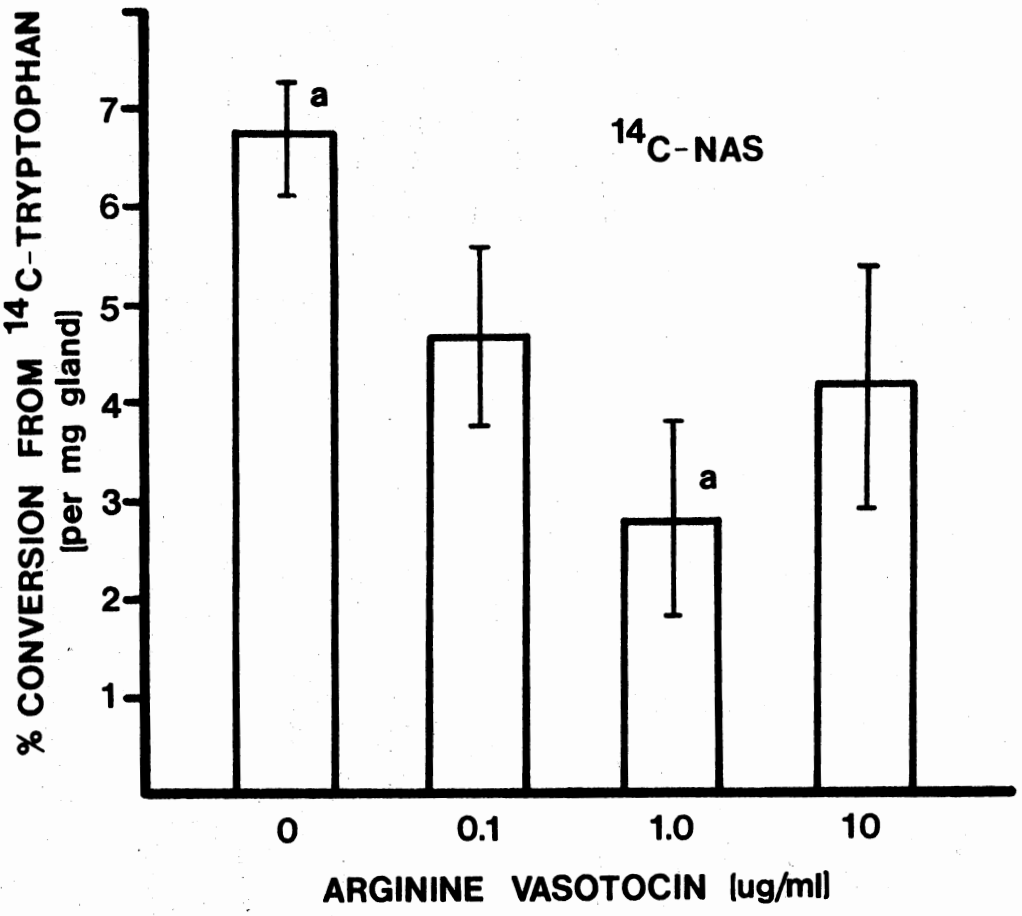

Figure 20. The Effect of Arginine Vasotocin (AVT) Upon Norepinephrine Stimulated N-acetylserotonin Synthesis. All glands were treated with NE (10^{-6}M) and the indicated dose of AVT. Like letters indicate statistically significant differences: a=$p < 0.01$; n=6 pineals per treatment group.

1.0 ug AVT ($p < .01$). Although the other concentrations of AVT decreased NAS production by 30%, the differences were not statistically significant. NAS conversion to melatonin (Figure 21) was also inhibited. The 0.1 ug (22.5 mU) concentration of AVT reduced the synthesis of melatonin by 49% ($p < .005$) while 1.0 ug AVT (225 mU) decreased melatonin by 53% ($p < .001$). These results with melatonin were repeated using a differential organic extraction (rather than thin-layer separation) and the results were similar. As with NAS, the higher dose of AVT produced a slight (27%) but statistically insignificant decline in production of melatonin.

The metabolism of ^{14}C-serotonin to HIAA (Figure 22) appeared to be reduced but the differences were not significant. Although percent conversion of HIAA to MIAA (Figure 22) was altered by AVT, the accumulation of counts was not enough to be considered significant.

Serotonin metabolism to HTOL (Figure 23) was reduced 41% by 0.1 ug AVT (p .1) and 32% by 1.0 ug AVT ($p < .01$). The subsequent conversion of ^{14}C-tryptophan to MTOL (Figure 25) was not statistically affected by AVT at the concentrations utilized.

The amount of inhibition produced by AVT was altered by the concentration of NE utilized (Figure 26). An increase in NE from 10^{-6}M to 10^{-4}M reduced the inhibition obtained with AVT. NE at 10^{-6}M plus 1.0 ug AVT produced 53% decrease in melatonin production. NE at 10^{-4}M plus 1.0 ug AVT produced only 38% inhibition of melatonin synthesis.

The glandular content of ^{14}C-tryptophan and its incorporation into protein were also investigated (Table IX). Although AVT appeared to reduce the amount of ^{14}C-tryptophan incorporation to protein the

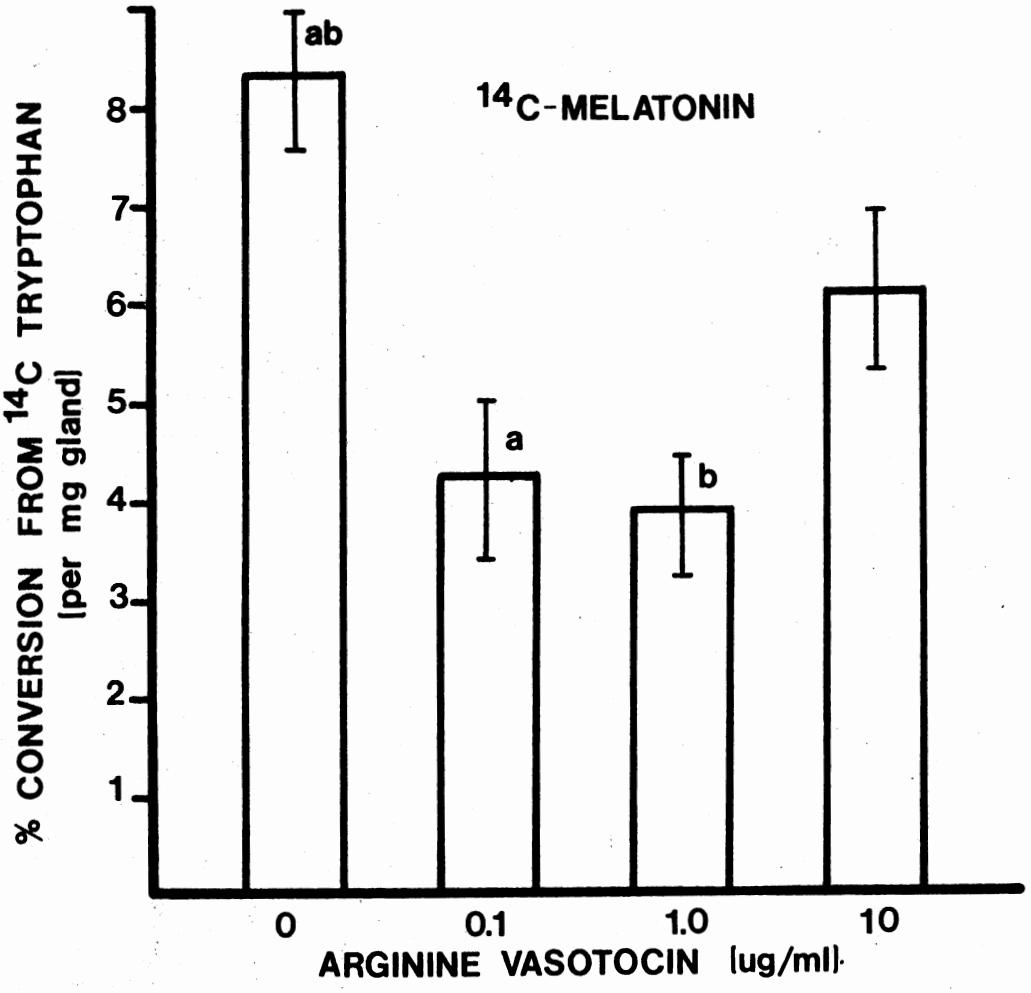

Figure 21. The Effect of Arginine Vasotocin (AVT) Upon Norepinephrine Stimulated Melatonin Synthesis. All glands were treated with NE (10^{-6}M) and the indicated dose of AVT. Like letters indicate statistically significant differences: a= $p < 0.005$, b=$p < 0.005$; n=6 pineal glands per treatment group.

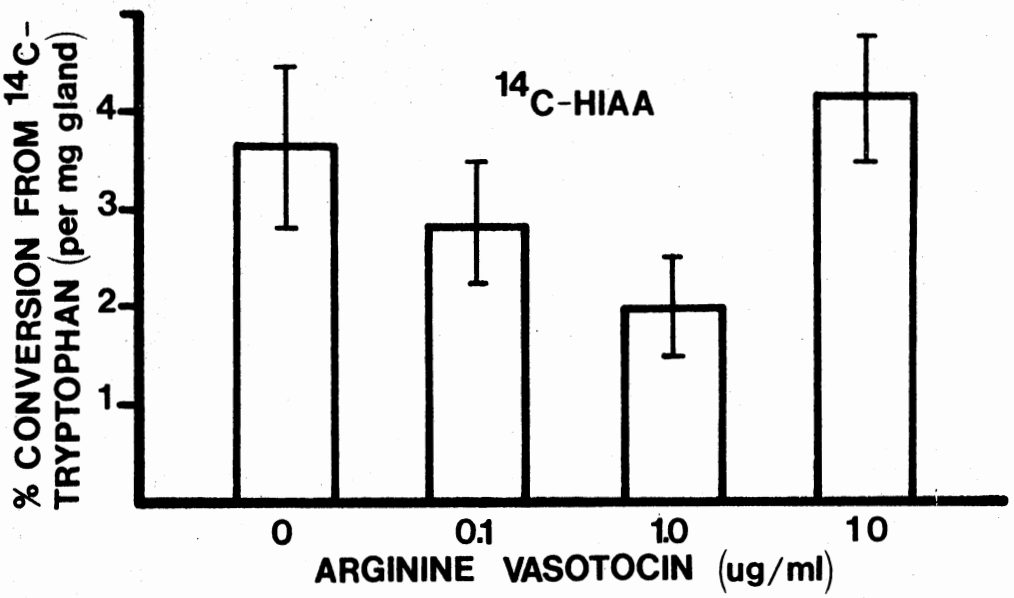

Figure 22. The Effect of Arginine Vasotocin (AVT) Upon Norepinephrine Stimulated Hydroxyindoleacetic Acid Synthesis. All glands were treated with NE (10^{-6}M) and the indicated dose of AVT. n=6 pineals per treatment group.

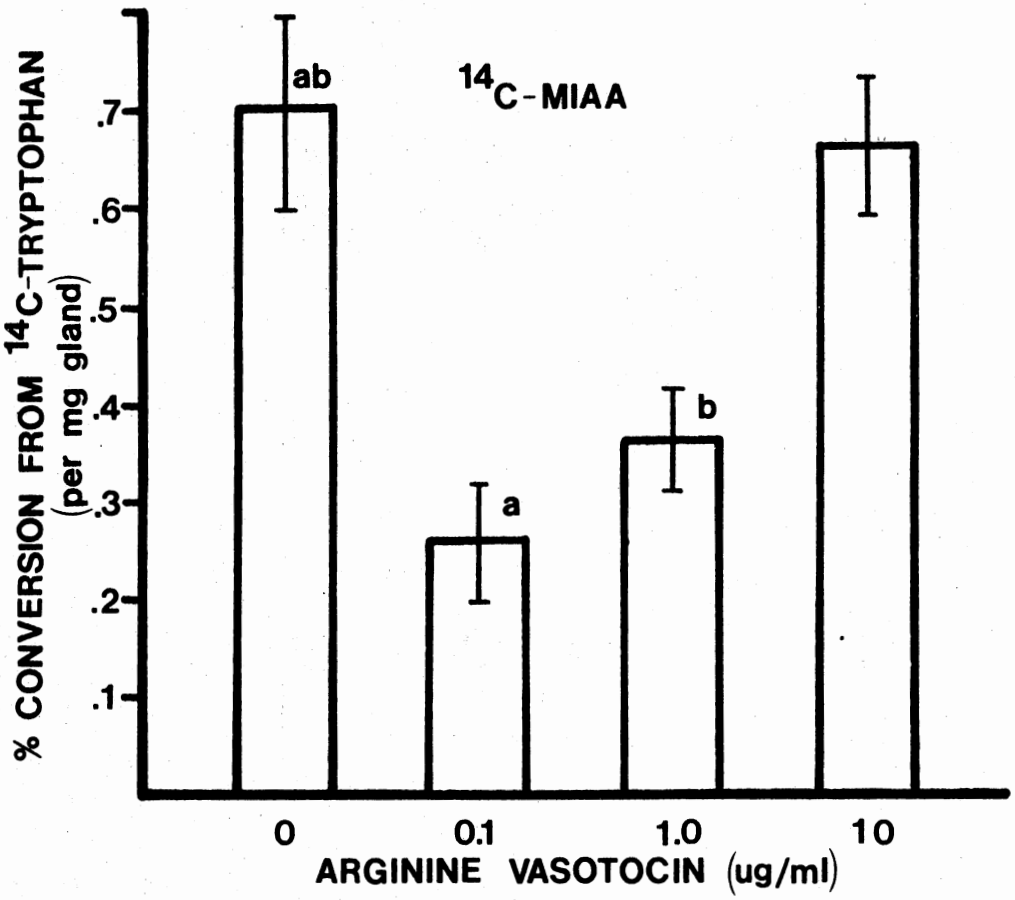

Figure 23. The Effect of Arginine Vasotocin (AVT) Upon Norepinephrine Stimulated Methoxyindoleacetic Acid Synthesis. All glands were treated with NE (10^{-6}M) and the indicated dose of AVT. Like letters indicate statistically significant differences: a=$p < 0.01$, b=$p < 0.025$; n=6 pineals per treatment group. Counts for methoxyindoleacetic acid were not large enough for significant comparisons.

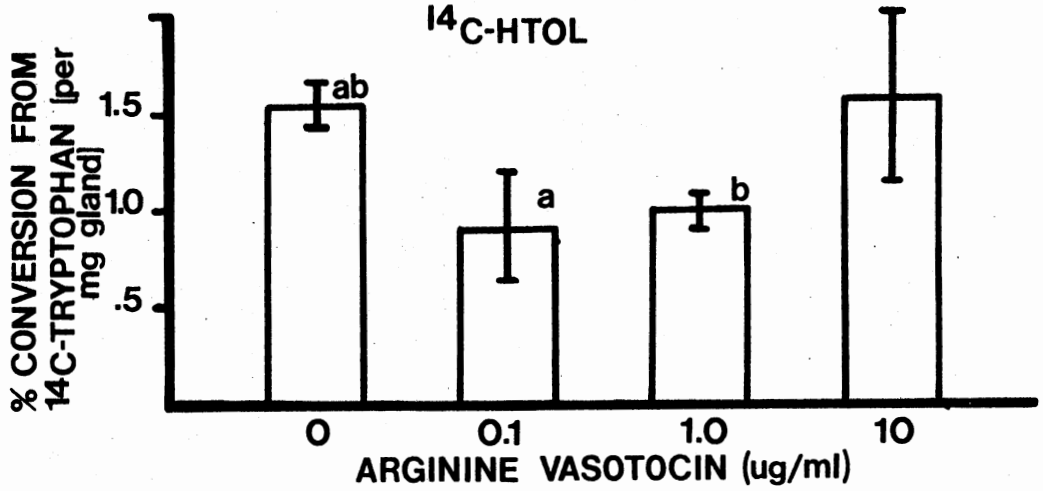

Figure 24. The Effect of Arginine Vasotocin (AVT) Upon Norepinephrine Stimulated Hydroxytryptophol Synthesis. All glands were treated with NE (10^{-6}M) and the indicated dose of AVT. Like letters indicate statistically significant differences: a=$p < 0.1$, b=$p < 0.01$; n=6 pineals per treatment group.

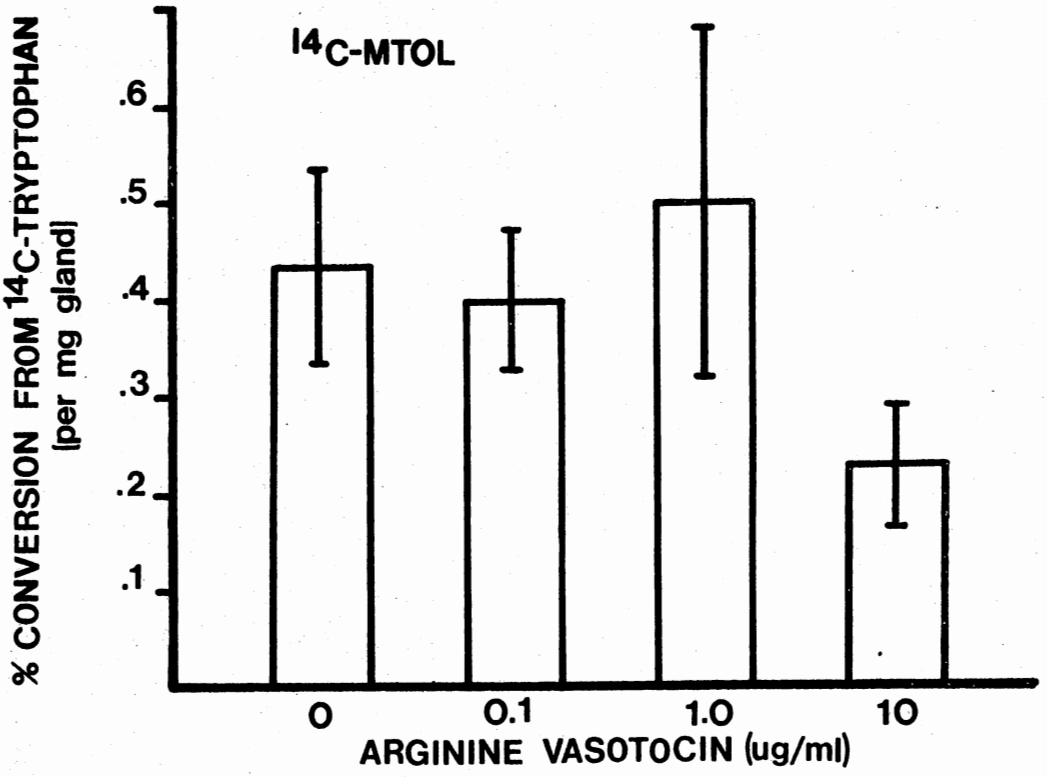

Figure 25. The Effect of Arginine Vasotocin (AVT) Upon Norepinephrine (NE) Stimulated Methoxytryptophol Synthesis. All glands were treated with NE (10^{-6}M) and the indicated dose of AVT. Counts were not large enough for significant comparison; n=6 pineals per treatment group.

TABLE IX

EFFECT OF ARGININE VASOTOCIN UPON NOREPINEPHRINE STIMULATED INCORPORATION OF ^{14}C-TRYPTOPHAN TO PROTEIN

Treatment	n	^{14}C-Tryptophan (CPM/mg Gland)	^{14}C-Protein (CPM/mg Gland)
NE (10^{-6}M)	6	1485 ± 144[ab]	4044 ± 571[ab]
AVT (0.1 ug)+NE	6	1218 ± 235	3849 ± 454
AVT (1.0 ug)+NE	6	1108 ± 286	3347 ± 456
AVT (10 ug) +NE	6	1218 ± 241	3916 ± 303

a) Mean ± standard error

b) No statistical difference between treatment groups

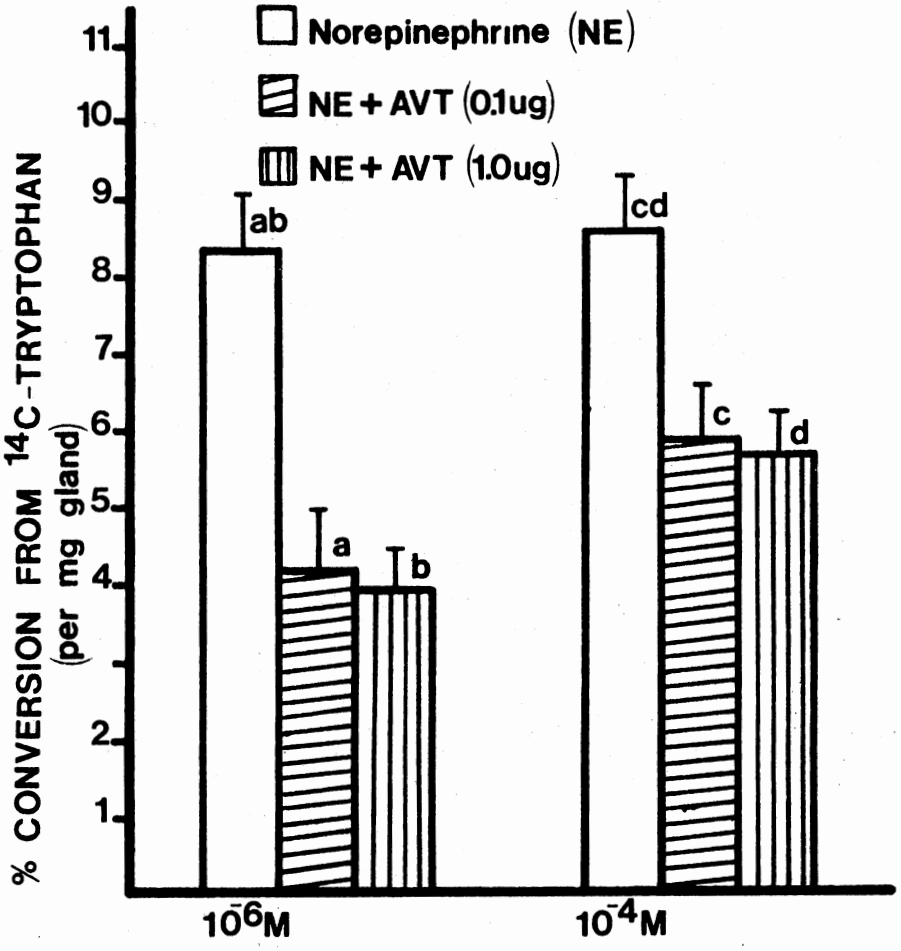

Figure 26. Changes in the Magnitude of Arginine Vasotocin Inhibition of Melatonin Synthesis in the Presence of Different Concentrations of Norepinephrine. Like letters indicate statistically significant differences: a=$p < 0.005$, b=$p < 0.005$, c=$p < 0.01$, d=$p < 0.01$; n=6 pineals per treatment group.

difference from the NE stimulated control was not significant. At those doses where AVT decreased protein synthesis, there was a corresponding (though also nonsignificant) decline in ^{14}C-tryptophan in the pineal glands.

This experiment was repeated twice with similar results for indoleamines, protein, and ^{14}C-tryptophan uptake.

CHAPTER V

DISCUSSION

In Vivo Experiments

The primary stimuli affecting pineal metabolism are constant darkness (blinding) and constant light. The effects of these and other factors on the pineal gland concentration of AVT were studied in vivo. Neither plasma levels nor cerebrospinal fluid (CSF) levels of AVT could be determined by use of the antibody for AVT due to cross reactivity of this antibody with AVP.

Age Effects

As the rats approached sexual maturity there was a significant decline in pineal AVT (Figure 3). There are two ways to interpret this data. Either AVT synthesis was inhibited or AVT release was augmented thus depleting the pineal levels of AVT. Since AVT is thought of as an antireproductive compound, it may be that AVT is involved in the onset of puberty. If AVT synthesis and secretion decline as the animal ages, then an antireproductive agent is removed and with its removal comes the increase in plasma gonadotropins and sex steroids found in the mature animal. More data needs to be developed as to whether AVT secretion as well as gland content decreases with puberty. Interestingly, cyclic AMP production has been shown to decrease from birth to 90

days of age in male rats (Weiss and Costa, 1970). This decline in cyclic AMP would produce a decline in melatonin synthesis. Therefore, a precedent for decreased pineal synthetic activity with age has been set.

The other possible event at puberty could be an increase in AVT secretion. Since AVT has been shown to inhibit adrenal corticosterone secretion via hypothalamic mechanisms (Pavel et al., 1977a), and since the adrenal has been implicated in the onset of puberty (Ramaley and Bartosik, 1965) in the rat, perhaps AVT could signal a rise in gonadotropin secretion by its presence, rather than its absence. However, the biological effects of AVT are contradictory at present and without more detailed knowledge of AVT actions and secretions, the relationship between AVT and puberty remains conjecture.

Castration

Castration provides a situation in the male rat where hypothalamic and pituitary reproductive hormones are elevated but where testosterone is absent. In this study, castration reduced the pineal content of AVT (Figure 4) either by release or decreased synthesis. Because testosterone is necessary for pineal protein synthesis (Cardinali et al., 1976), pineal AVT production in this study was probably depressed. The other possibility cannot, however, be ignored. It may be that AVT secretion was elevated. If AVT functions as an antigonadotropin, an elevation in the hypothalamic releasing factors or pituitary FSH and LH may provide a stimulus for AVT secretion. Recently, Goldstein and Pavel (1978) have indicated that LRF will stimulate AVT secretion into the cerebrospinal fluid of the third ventricle.

Blinding and Constant Light Effects

Blinding and constant light are widely utilized treatments which increase or decrease pineal metabolism, respectively. There were no apparent effects of blinding on pineal weights (Table II). However, exposure to constant illumination significantly lowered pineal weight ($p < .01$) at 2 weeks after the onset of treatment. Constant light will lower RNA synthesis (Quay, 1974) and presumably protein synthesis which might account for the transient effects on pineal gland weight. Another possible effect could be a superimposed stress response to constant light exposure. As the animal adapted to the stress the gland weight could return towards normal.

Analysis of AVT concentration in the pineal revealed that the constant light paradigm produced no major alterations (Figure 5). This might well be expected if there was a reduction in both synthesis and secretion of AVT. Pineal synthesis and secretion of melatonin declines during prolonged light exposure. Thus the gland levels of melatonin would not be expected to change much.

Blinding produced dramatic effects upon the pineal concentration of AVT (Figure 5). The pineal concentration of AVT was observed to decline between 2 and 4 weeks following the initiation of treatment. Six weeks into the experiment the pineal concentration of AVT was significantly depressed ($p < .05$) and by the eighth week, AVT levels were returned toward the initial control concentration. Blindness or light deprivation stimulates pineal RNA production (Quay, 1974) and stimulates the synthesis and secretion of pineal gland melatonin (Reiter et al., 1971). Thus, there exists a strong probability that AVT

secretion may also be enhanced. The data on AVT gland content suggests this possibility. Further, AVT is an antireproductive compound and blinding produces antireproductive effects in rodents (Sorrentino and Benson, 1970; Reiter et al., 1971). By serendipity it follows that AVT would also be secreted with blinding albeit the data presented herein are only circumstantial.

Due to the antireproductive nature of AVT, it might be possible to compare the effects of blinding on pineal AVT concentration to the effects of blinding on hormones essential to normal reproductive function in rodents. Plasma FSH (Figure 6) and testosterone (Figure 7) were found to decrease with blinding (Dickson et al., 1971; Rønnekliev and McCann, 1976; Kinson and Liu, 1973). The lowest plasma levels of these hormones were found at 6 weeks corresponding to the presumed time of maximal release of AVT (Figure 5). Although prolactin (Figure 8) was not significantly elevated, both this hormone and LH (Figure 9) were elevated to a maximum at 6 weeks, which were comparable to changes in pineal AVT. These effects of blinding on prolactin and LH have been observed before (Blask and Reiter, 1975; Vaughan et al., 1976a). AVT has been reported to elevate LH and prolactin (Vaughan et al., 1976a; Vaughan et al., 1976c), and possibly to decrease FSH (Vaughan et al., 1976c) as observed by a reduction in compensatory ovarian hypertrophy. These effects of AVT are consistent with the data presented. However, Cheesman et al. (1977a, 1977b) has shown that AVT does not alter FSH, inhibits the proestrus surge of prolactin and inhibits LH. The data obtained by Cheesman tends to indicate that some substance other than AVT could be responsible for the effects of blinding as observed herein. Since melatonin will inhibit gonadotropon secretion, and stimulate

prolactin secretion (Kamberi et al., 1970) and inhibit testosterone (Peat and Kinson, 1971), perhaps melatonin could be responsible for these blinding effects. Melatonin would not directly stimulate LH release but may inhibit testosterone synthesis and secretion which would stimulate LH release by removal of negative feedback inhibition. However, there is evidence that melatonin may not mediate the blinding-induced regression in gonadal weights (Niles et al., 1977). Since other pineal fractions may also inhibit testosterone (Milcu et al., 1975; Orts, 1977) or other reproductive hormones, one of these (melatonin and AVT) or a combination of antireproductive compounds may be necessary to account for these observations, but these questions are outside of the scope of this investigation.

Pituitary LH, FSH, and prolactin were analyzed in constant light and blinded rats (Tables IV, V, VI). The data indicate that the pituitary FSH concentration was not statistically altered by blinding. However when compared with plasma FSH, the pituitary FSH concentration is probably reduced reflecting a reduction of synthesis and release of FSH. Blinding elevated the plasma prolactin concentration which was reflected by a drop in the pituitary prolactin concentration. Under these conditions prolactin release was stimulated but prolactin synthesis was probably unaffected. The LH concentration in the pituitary was elevated along with plasma LH, probably due to an increase in synthesis and secretion of LH. These data are not unlike that found by Rønnekliev and McCann (1975) and Blask and Reiter (1975).

Examination of constant lighting effects revealed an increase in plasma LH (Figure 9) and testosterone (Figure 7) with no change in FSH (Figure 6), and a decline in prolactin (Figure 8). Gland content

of FSH was elevated with constant light as was LH. Prolactin concentration in the pituitary glands was unchanged. These data indicate that LH synthesis and secretion were elevated; FSH synthesis and secretion were not appreciably altered; and that prolactin synthesis and secretion were both decreasing. The in vivo experiments do not conclusively prove that AVT is a secretory product of the pineal. These studies as a group do indicate that pineal concentrations of AVT are altered by stimuli which affect pineal metabolism.

In Vitro Experiments

Incubation procedures were checked to determine the viability of incubated pineal glands for up to 10 hours. Analysis of the glands for glutamate oxalacetate transaminase indicated that GOT activity began to increase between 6 and 8 hours. Although this increase became apparent at 6-8 hours, the GOT activity at 10 hours was not much higher than at 8 hours and was 5-fold lower than glands incubated without oxygen. Inspection of incubated tissue by light microscopy indicated little evidence of cellular death though some piknotic cell nuclei were visible following incubations for 10 hours.

Synthesis of AVT

AVT synthesis from ^{14}C-isoleucine was studied in vitro. Chromatography revealed a small peak of radioactive material corresponding to unlabeled AVT (Figure 11). However, amino acid analysis revealed no evidence that this peak was indeed AVT (Table VI). Attempts to use a vasotocin antibody to precipitate labeled AVT were inconclusive (Figure 12). Although it would appear that ^{14}C-Ile was incoporated by the

pineals and converted into AVT, there is not at present firm evidence to support this possibility.

Neurotransmitter Effects

The system for regulating general pineal metabolism is a neural mechanism. The pineal has a rich adrenergic innervation (Cajal, 1911) and melatonin synthesis and secretion is regulated primarily by the neurotransmitter NE (Axelrod et al., 1969). Although the pineal nerve supply is primarily from the superior cervical ganglion, some species have parasympathetic innervation (Kenny, 1961; Romijn, 1975). Parasympathetic fibers in the rat have not yet been demonstrated but the pineal does contain significant quantities of acetylcholinesterase (Eranko et al., 1970; Machado and Lemos, 1971). Further, Wartman (1969) has shown that atropine (a cholinergic-muscarinic receptor blocking agent) will inhibit melatonin synthesis by decreasing the activity of HIOMT (the enzyme converting N-acetylserotonin to melatonin). The presence of cholinergic receptors in the pineal led Wartman (1969) to propose that the rat pineal did indeed contain parasympathetic innervation. Since the pineal contains several neurotransmitters besides NE (Quay, 1974), this study was initiated to determine which neurotransmitters might regulate AVT secretion.

The data indicated that Ach could stimulate AVT secretion in vitro and that the effect was dose-related (Figure 13). In vitro studies of hypothalamo-hypophyseal explants have shown that Ach will stimulate AVP secretion in a dose-related manner with the lease sensitivity at 10^{-6}M Ach (Sladek and Knigge, 1977). Intracarotid injections of Ach will also rease AVP (Bridges and Thorn, 1970). Thus it was not

surprising to find this similarity between the release of 2 similar compounds. The effect of Ach on AVT secretion however, implies the existence of a cholinergic nerve supply to the pineal gland. Although no parasympathetic fibers have been found, Wartman (1969) has proposed their existence. Further, Dafny (1977) has demonstrated the presence of heretofore unknown nerve tracts from the hypothalamus to the pineal, but their pharmacologic characterization has not yet been attempted. The possibility exists that these nerve tracts are cholinergic or have a cholinergic component whose receptors must be located at the pineal gland.

A second possible explanation for the presence of cholinergic receptors exists. Since acetylcholinesterase has been localized to amine containing neurons in the pineal (Eranko et al., 1970; Machado and Lemos, 1971), the possibility arises that the sympathetic neurons contain Ach. According to the Burn-Rand Hypothesis (Burn and Rand, 1965) Ach is required for the release of NE from the presynaptic membrane. Further, the postsynaptic membrane contains receptors for both Ach and NE (Jacobowitz and Koelle, 1965) so Ach may have an effect on the postsynaptic membrane that acts in concert or independent of the NE receptors.

If AVT release is accomplished by postsynaptic Ach receptors of the sympathetic nervous system, then AVT release should parallel that observed for melatonin. In other words, constant darkness and blinding should release AVT while constant light should have no effect or reduce AVT release. Circumstantial evidence has been provided by this study (Figure 5) and by Calb et al. (1977) to support these possibilities.

A recent study (Contestabile et al., 1978) found that blinding reduced acetylcholinesterase (AChE) activity in the optic tectum of reptiles. If blinding in mammals will decrease pineal AChE activity this would increase the effects of pineal AChE and should account for changes in pineal AVT following blinding (Figure 5). Interestingly, contestabile and co-workers found that AChE activity decreased to 4 weeks and then began to increase again. This is similar to the effects of blinding on pineal AVT reported herein.

According to the Burn-Rand Hypothesis, Ach should release NE which should then produce the sympathetic nervous system effects. If the administration of exogenous Ach released NE which subsequently released AVT, then the addition of exogenous NE should also enhance AVT secretion. The results of this study indicated that NE had no effect upon AVT secretion in vitro (Figures 4, 15). This indicates that there is a separate action of NE and Ach on AVT secretion in vitro. Either Ach comes from a distinct population of parasympathetic fibers (Wartman, 1969) or Ach is released from sympathetic neurons but acts independently of the adrenergic nervous system receptors.

In contrast to this study, Cusack et al. (1978) found that NE would release AVT in vivo. Mathis and Lederis (1977) have shown that cyclic AMP depresses AVP release from the neural lobe in vitro. NE stimulates the production of cyclic AMP in the pineal gland. With the apparent similarity between AVP and AVT, NE in vitro should inhibit AVT secretion. Since there are some neurons entering the pineal from the hypothalamus (Dafny, 1977), the NE receptor responsible for AVT secretion in the study by Cusack may be located in the hypothalamus or some other extra-pineal site. Removal of the pineal for incubation

purposes could remove this adrenergic receptor. Bridges et al. (1976) have shown that in vivo NE administration will release ADH but in vitro hypothalmic incubations with NE has virtually no effect. These authors also suggest adrenergic receptors not present in vitro but responsive in vivo. Bridges et al. (1976) further suggest that in vivo effects of NE are on dopaminergic neurons not noradrenergic neurons, thus suggesting that dopamine not NE is involved in AVP and possibly in AVT (Figure 14) secretion.

Another explanation is also possible. Carrol and Buterbaugh (1975) have suggested that NE stimulates high affinity choline transport in the guinea pig brain. Since the rate of high affinity choline uptake is rate limiting for Ach synthesis (Haubrich and Chippendale, 1977), NE could modify the production and possible release of Ach but require more than 8 hrs to be detected. This possibility is the most remote for explaining the NE effect seen by Cusack et al. (1978). This does however indicate a possible area of research to explore in determining the factors regulating pineal metabolism.

The neurotransmitter DA exists within the pineal gland (Quay, 1974) and has been shown to stimulate melatonin production in vitro (Axelrod et al., 1969). DA produces a pronounced increase in AVP secretion (Bridges et al., 1976) from the posterior pituitary gland. In this experiment (Figure 14), DA (10^{-6}M) produced a slight stimulation ($p < .06$) of AVT secretion in vitro. The lower dose (10^{-8}M) of DA released more AVT than did controls but the differences were not significant. A second experiment with ascorbic acid to prevent oxidation of DA yielded similar results (Figure 17). Although DA may play some role in regulating pineal AVT secretion, the physiological importance

of DA must await further studies.

The neurotransmitter 5-HT also exists in the pineal in high concentrations (Quay, 1974) and also produces a slight stimulation of AVP secretion (Jones et al., 1977) from the pituitary. The data from Jones et al. (1977) revealed that 5-HT had no effect on AVT (Figures 16, 17). 5-HT probably plays no role in regulation of AVT secretion in vitro.

The pineal gland is also known to accumulate quantities of melatonin (Quay, 1974). Since most peptide secreting endocrine tissues produce indoleamines which regulate peptide secretion (Owman et al., 1973), Reiter and Vaughan (1977a) have suggested a similar function in the pineal. Pavel (1973) has shown that intraventricular administration of melatonin will release AVT. Studies by Benson and Krasovich (1977) have demonstrated an effect of melatonin on dense core storage vessicles and have suggested that melatonin releases an antigonadotropic substance from the pineal gland. Comparisons of pineal gland AVT (Calb et al., 1977) to melatonin suggest that melatonin synthesis and secretion correlates to AVT secretion.

Incubation of pineal glands with melatonin (Figure 16) elevated AVT secretion but the differences were not significant. Thus these data suggest that in vitro, melatonin has no effect on AVT production and secretion. There are several explanations for the data by Pavel (1973) data showing release. Melatonin may release AVT by a secondary mechanism rather than a direct action on the pineal. Alternatively, melatonin may release AVT from the subcommissural organ rather than from the pineal. Only Pavel (1973) has directly named the release product from melatonin stimulation. Benson and Krasovich (1977) and Reiter et al. (1976) only state that an antigonadotropin is released

by melatonin. The data presented herein tends to indicate that AVT is not that antigonadotropin or that melatonin does not act directly on the pineal.

Indoleamine Synthesis

There is evidence that melatonin, by some unknown mechanism, can release AVT *in vivo* (Pavel, 1973). Further, Binkley et al. (1976) have found that AVT can inhibit N-acetyltransferase activity. Therefore the possibility exists that AVT may serve to regulate melatonin and indoleamine synthesis. These experiments were conducted to determine whether AVT might exert some regulatory action on pineal indoleamine synthesis *in vitro*.

AVT was found to have a potent inhibitory effect upon indoleamine synthesis and/or release from pineal glands during a short term (10 hours) incubation. Since most *in vitro* pineal studies have utilized tissue culture rather than incubations, it was necessary to compare the patterns of indole secretion between the two *in vitro* approaches. Data from the present study revealed that NE enhanced the conversion of ^{14}C-tryptophan to ^{14}C-melatonin and decreased the production of ^{14}C-HIAA (Table VIII). The same pineal response to NE has been described in tissue culture (Axelrod et al., 1969). Although the incubations utilized in this study were for 10 hours, Axelrod et al. (1969) have shown that significant NE stimulated melatonin production from ^{14}C-tryptophan is present within 4 hours. Further, the rate of melatonin production slowed at approximately 16 hours with little further increase to 48 hours. Thus the 10 hour incubation should produce relatively similar levels of melatonin to that found by

Axelrod et al. (1969). However, the percent conversion of tryptophan to melatonin was greater for both controls and for NE stimulation in this study. A possible explanation lies in the lesser quantity of ^{14}C-tryptophan used in this study (0.1 uCi per incubation flask versus 0.5 uCi for tissue culture). Therefore, percent conversion calculated using fewer total counts per minute could account for the apparent discrepancy in percent conversion figures.

Exposure of pineal glands to AVT in the absence of NE (Table VII) produced no effect upon either melatonin or serotonin metabolism. The expected results, based upon AVT inhibition of N-acetyltransferase, would have been an inhibition of ^{14}C-melatonin production and an accumulation of ^{14}C-serotonin. The absence of any effect of AVT on these indoles may have resulted from use of rats during the daylight hours when melatonin synthesis was already inhibited and serotonin production enhanced (Quay, 1964a, 1964b). Thus greater concentrations of AVT would have been necessary to facilitate the inhibition already present.

In the presence of both NE (10^{-6}M) and AVT (1 ug), there was a pronounced reduction of melatonin (Figure 21) and NAS (Figure 20) synthesis or release. The inhibition of melatonin was found to be related to the quantity of norepinephrine present (Figure 24). NE at 10^{-4}M partially reversed the inhibition of melatonin by AVT (when compared to 10^{-6}M NE). Since NE stimulates N-acetyltransferase activity and greater concentrations of NE partially reverse the effects of AVT, this provides more evidence for N-acetyltransferase as the site of AVT action. The inhibition of NAS production (Figure 20) indicates a decline in N-acetyltransferase activity as previously demonstrated by Binkley et al. (1976). Additionall, 0.1 ug AVT produced a greater

reduction in melatonin synthesis and release than was evident with NAS. This suggests an effect on AVT to decrease hydroxyindole-o-methyltransferase (an enzyme converting NAS to melatonin) activity. An inhibition of NE stimulation, a decreased accumulation of an enzyme leading to serotonin production (such as tryptophan hydroxylase) coupled with an inhibition of N-acetyltransferase might also explain the observed decrease in general indoleamine synthesis.

Another possibility must also be considered. AVT might not alter enzyme activity but may lower the specific activity and thus the percent conversion of ^{14}C-tryptophan to the various indoleamine metabolites. ^{14}C-tryptophan uptake (Table IX) and ^{14}C-serotonin production (Figure 19) were unaffected by AVT treatment. Thus a decrease in the specific activity of ^{14}C-serotonin metabolites due to AVT would be the result of a decreased utilization of ^{14}C-serotonin. This would in turn produce an accumulation of ^{14}C-serotonin which was not observed (Figure 19). In view of the study by Binkley et al. (1976) demonstrating an inhibition of N-acetyltransferase by AVT, the data from this study reflect primarily an inhibition of ^{14}C-melatonin production by AVT rather than merely a decrease in specific activity.

Finally, ^{14}C-tryptophan content of the pineals and its subsequent incorporation into protein was determined. Neither the pineal content of ^{14}C-tryptophan nor incorporation of tryptophan to protein were significantly altered. This suggests that AVT may have no effect upon ^{14}C-tryptophan uptake or pool size.

The observed inhibitory properties of AVT on indoleamine metabolism provide evidence for a potential control mechanism over the synthesis of melatonin and other pineal indoleamines. Alternatively, since Pavel

(1973) has shown that melatonin stimulates the release of AVT and our data reports an inhibition of melatonin production and/or release, the possibility arises for a feedback regulation of AVT. In order for this type of regulatory mechanism to exist, AVT synthesis and release should increase at night and decrease during the day, similar to that seen with melatonin. Indeed, a recent study (Calb et al., 1977) has indicated that AVT synthesis follows this predicted pattern.

The data indicated that 10 ug AVT was ineffective in reducing indoleamine metabolism though smaller concentrations were highly potent in that regard. Similar observations have been reported for AVT. Corticotrophin releasing factor (CRF) secretion is inhibited by small doses of AVT but the dose response relationship is lost with higher doses of AVT (Pavel et al., 1977). Further, Pavel reported that high concentrations of AVT may stimulate rather than inhibit CRF release. Since AVT with a reduced disulfide will stimulate N-acetyltransferase activity and since there is a contaminant in the AVT utilized (Figure 11), the higher (10 ug) doses of AVT may contain reduced AVT at a concentration sufficient to partially reverse the inhibitory effects described.

AVT has been found in the epiphyseal stalk (Benson et al., 1976) and within the body of the pineal (Bowie and Herbert, 1976) where indoleamine synthesis occurs. The presence of AVT within the gland plus the results of this study, raise the possibility that AVT might be involved in the regulation of melatonin and indoleamine synthesis. Whether the effects of AVT described reflect a primary control of melatonin and indoleamine synthesis or a regulation of its (AVT) own

synthesis remains to be determined. In either event, it is obvious that AVT can exert a potent effect on NE stimulated indoleamine synthesis and that the effect could be more involved than a simple inhibition of N-acetyltransferase activity.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The *in vivo* data indicates that sexual maturation has an influence on pineal production of AVT. Since the pineal concentration of AVT declines as the animal reaches maturity it may be the result of decreased synthesis or increased secretion. AVT may be involved in puberty onset or the decline in AVT may be associated with hormonal events which normally occur at puberty such as elevated plasma testosterone, LH, or LHRH.

Castration also decreases the pineal concentration of AVT. Since castration has the same effect on pineal gland AVT levels as sexual maturation, some inferences may be made. With sexual maturation or castration, serum LH and LHRH are elevated. Testosterone is elevated during sexual maturation but is depressed during castration. The possibility arises that either LH or LHRH may stimulate AVT secretion or inhibit AVT synthesis.

Constant light has no effect on the AVT concentration of the pineal. Blinding produces a decrease in pineal AVT concentration. Since pineal metabolism and melatonin secretion is elevated, the decrease in pineal AVT concentration probably reflects an increase in AVT secretion. However, the plasma gonadotropin secretion does not follow what would be expected from AVT. AVT should elevate prolactin, decrease LH, and have no effect on FSH. Since the results are at

variance with expected results, two conclusions may be reached. AVT is not secreted by blinding or, under conditions of blinding, AVT has a different set of effects from those found in other animal models. The critical observation is that blinding which increases pineal metabolic activity also will alter pineal AVT concentrations whereas constant light has no effect on AVT.

The in vitro data indicates that perhaps AVT may be synthesized by the pineal. Since the antibody precipitation and amino acid analysis data do not confirm in vitro synthesis, no firm conclusions can be made at this time.

The neurotransmitter data revealed that Ach and possibly DA stimulated AVT secretion while NE, 5-HT and melatonin had no significant effects in vitro. The pineal contains acetylcholinesterase activity and may contain cholinergic nerves or may have Ach within the sympathetic nerve terminus as stated in the Burn-Rand hypothesis. Ach, DA, and NE cause release of ADH but NE in vitro had no effect on AVT release. There is a possibility that there is an adrenergic nerve component within the nerves regulating AVT secretion. This adrenergic component may be removed when the pineal is placed in vitro. Thus Ach and DA are involved with regulation of AVT secretion in vitro. Ach, DA, and NE may all be involved with in vivo regulation of secretion.

Indoleamine synthesis was altered in vitro by AVT. AVT at 0.1 and 1.0 ug had no effect on serotonin but both concentrations inhibited both NAS and melatonin production. AVT also decreased HTOL production. Since AVT has been shown to reduce N-acetyltransferase activity, AVT may be involved in regulation of indole and melatonin synthesis. The effect of AVT may be to inhibit N-acetyltransferase or HIOMT activity

or AVT may interact at some other point in the metabolic pathway for indoleamine synthesis. AVT may be involved in regulation of melatonin and indole production or if melatonin actually stimulates AVT production, AVT may act in a negative feedback role to inhibit its (AVT) own production.

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APPENDICES

APPENDIX A

KREBS RINGERS BICARBONATE INCUBATION MEDIA

I. Incubation Media (Umbreit et al., 1964)

A. Stock solutions were prepared by the following methods and stores under refrigeration.

1. Sodium chloride (NaCl): 4.5 grams/500 ml distilled water
2. Potassium chloride (KCl): 1.73 grams/150 ml distilled water
3. Calcium chloride ($CaCl_2 \cdot 2$ HOH): 2.43 grams/150 ml distilled water
4. Potassium dihydrogen phosphate (KH_2PO_4): 3.17 grams/150 ml distilled water
5. Magnesium sulfate ($MgSO_4 \cdot 7$ HOH): 5.73 grams/150 ml distilled water
6. Sodium bicarbonate ($NaHCO_3$): 1.95 grams/150 ml distilled water

B. Preparations of the working solutions were made by removing aliquots of the stock solutions and mixed in the following proportions:

1. 100 parts solution 1
2. 4 parts solution 2
3. 3 parts solution 3*
4. 1 part solution 4
5. 1 part solution 5
6. 21 parts solution 6

C. Two hundred milligrams of glucose and bovine serum albumin (Cohn fraction V) were added per 100 ml of incubation media.

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*Add $CaCl_2$ last and very slowly to prevent formation of insoluble calcium salts.

APPENDIX B

VALIDATION OF AVT RADIOIMMUNOASSAY

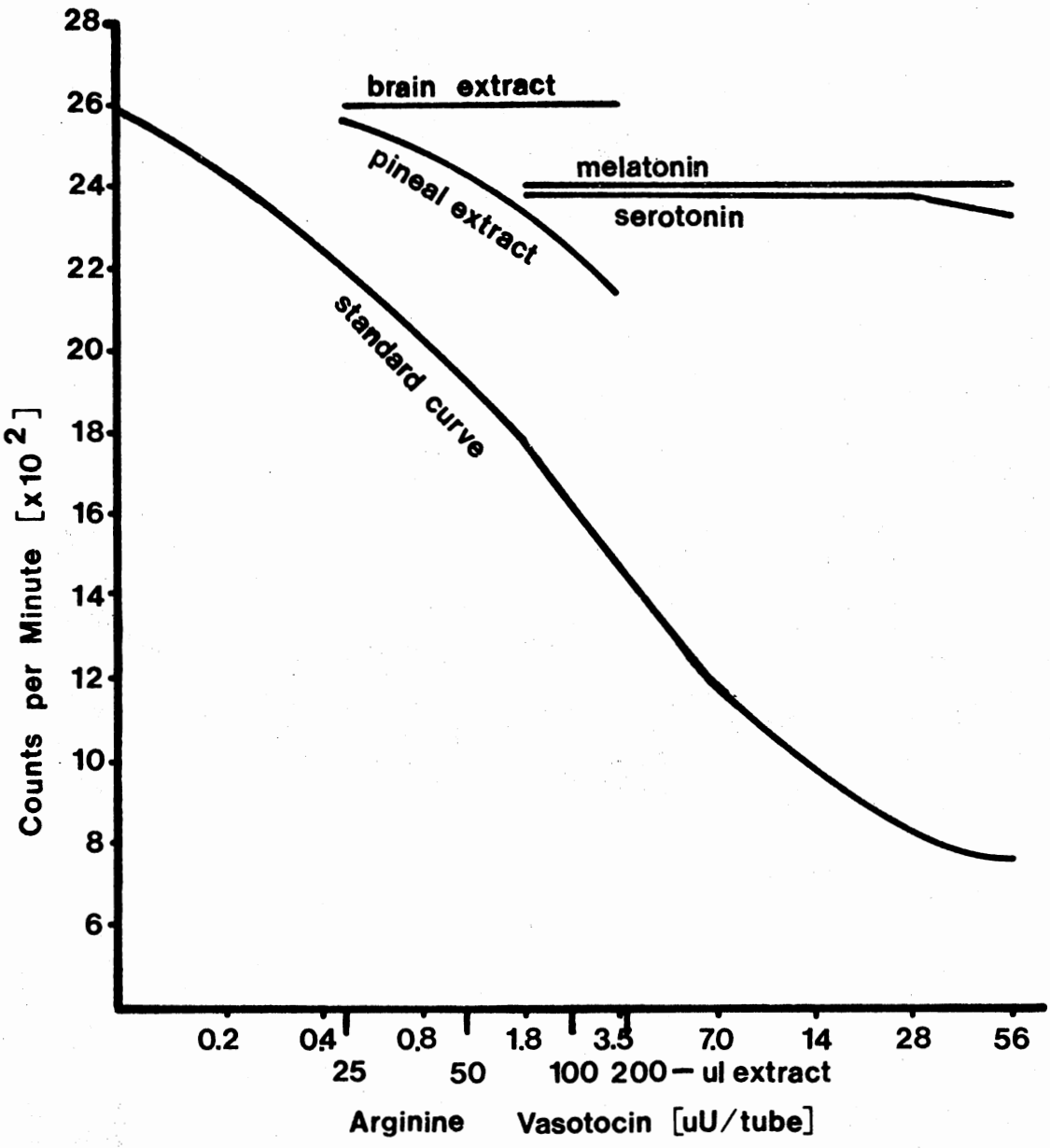

Figure 27. Arginine Vasotocin Standard Curves and Inhibition Curves for Pineal and Brain Extracts and Indoleamines

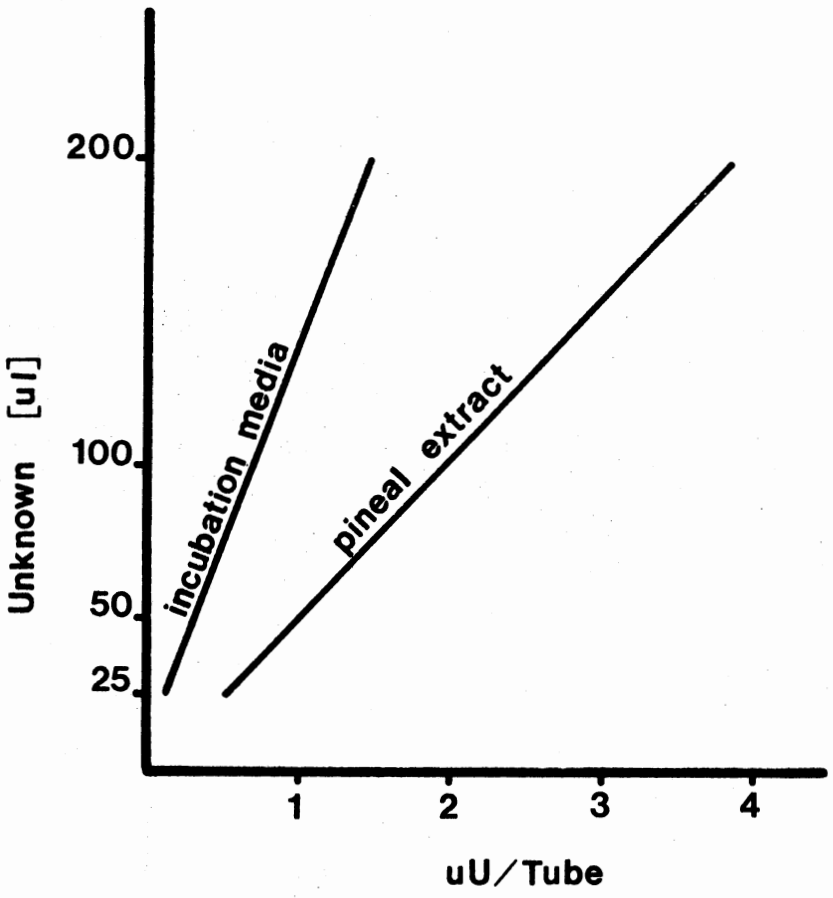

Figure 28. Comparison of Quantities of Pineal Extract and Incubation Media and the Values of AVT Following Quantification by Radioimmunoassay

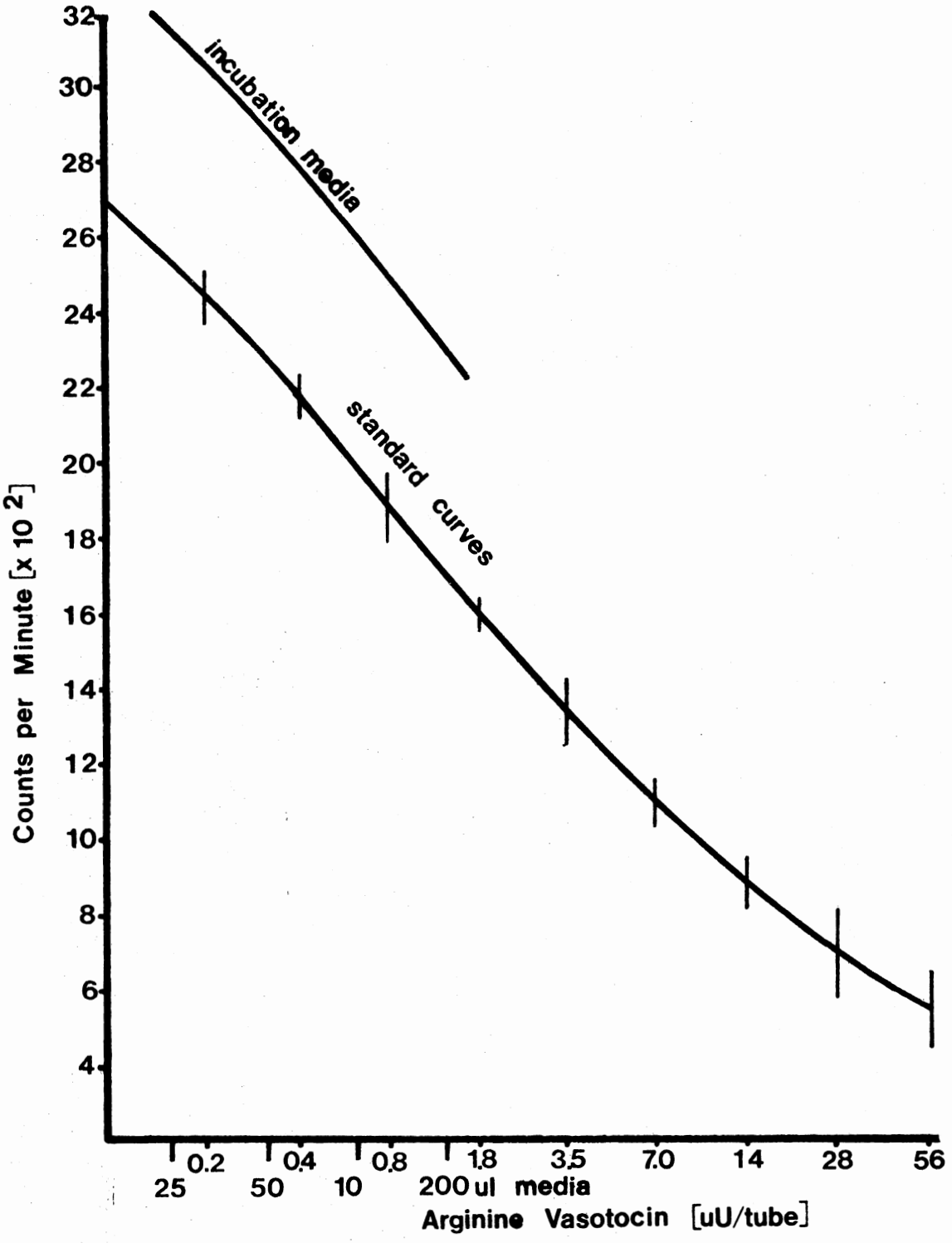

Figure 29. The Effect of Incubation Media, Neurotransmitters, and Indoleamines on an AVT Standard Curve. The standard curve represents 2 replicates of standard curves run in the presence of all neurotransmitters, indolemines, and Krebs Ringers incubation media.

VITA 2

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