VARIATIONS IN URINARY MELATONIN CONCENTRATIONS IN RATS SUBJECTED TO ENVIRONMENTAL STRESS

by

JOSEPH A. RUKEYSER

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Date

J. Golubow
Chairman of Examining Committee
Professor J. Golubow

May 20, 1974
Date

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_____ — _____
Institution

The City University of New York

Abstract

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Joseph A. Rukeyser

Adviser: Professor Julius Golubow

The role of the pineal gland as a senso-neuroendocrine transducer capable of converting environmental stimuli into an endocrine response has been further defined by this study. Urinary melatonin concentrations, indicative of pineal secretory activity have been found to vary with changes in specific, stressful, environmental stimuli. The quantitative measurement of urinary melatonin concentrations has been made possible by the development of a sensitive, specific bioassay procedure. The gel column chromatography of urine samples simultaneously accomplished the recovery of 92-96% of added melatonin and the separation of melatonin from biogenic amines and small polypeptides. The melatonin concentration of chromatographed urine samples was determined by a specific tadpole tail bioassay which is sensitive to melatonin concen-

trations in the range of 0.1-100ng/ml. The recovery of 83.5% of melatonin added to urine has been found following chromatography and bioassay procedures.

The stresses of cold exposure, immobilization, and combined cold and immobilization, have been found to cause significant increases in the urinary melatonin concentrations in intact rats. Immobilization stress was found to cause a greater stimulation of melatonin secretion than does cold stress.

Superior cervical ganglionectomized animals in stress released more melatonin than did intact animals. This finding provides further evidence of a supersensitivity of the pineal beta-adrenergic receptors to catecholamines when the gland is denervated. Supersensitivity results in a superinduction of the melatonin biosynthetic enzymes in response to the stress-induced liberation of catecholamines.

A comparison of urinary corticosteroid and melatonin concentrations has revealed an inhibitory control by melatonin over corticosteroid secretion in certain stress conditions. No clear relationship between either melatonin or corticosteroid concentrations and stress-induced gastric ulceration has been found. The physiological effects of increased melatonin concentrations during stress are apparently not involved in the ulceration process but would appear to be more involved with the modulation of the adrenal and thyroid responses to stress.

In relation to the well-established relationship

between the pineal gland and mammalian reproductive function, the evidence presented here provide further insight into the mechanism of stress-induced modifications in reproductive physiology.

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INTRODUCTION

The mammalian pineal gland is now recognized as a neuroendocrine transducer capable of translating a neural impulse into an endocrine response (Wurtman, 1969). Such neural impulses are generally thought to be the result of photic stimulation of the eyes. The impulses generated pass through the midbrain and a sympathetic ganglion to the pineal parenchymal cells where they cause alterations in the activity of at least one biosynthetic pathway, Alterations are seen in the level of activity of enzymes responsible for the synthesis of a unique pineal hormone, melatonin (5-methoxy-N-acetyltryptamine).

Evidence is now accumulating which may permit an extension of the characterization of mammalian pineal function to that of a senso-neuroendocrine organ (Miline, 1971) in that the pineal might respond to other sensory inputs in addition to photic stimuli (Lynch et al., 1973a).

Following immobilization and cold stress, it has been shown that the mammalian pinealocytes undergo histological and biochemical modifications including: increased synaptic ribbon formation, an increase in the extent of the golgi zone, an increase in the number of secretory granules, and an increase in the activity of the enzymes succinic dehydrogenase, monoamine oxidase, alkaline phosphatase, acid

phosphatase, and ATPase (Miline, 1971).

It has been shown that plasma corticosterone levels are increased following histamine-induced stress. This increase is significantly diminished if melatonin is administered with the histamine (Motta et al., 1971).

Pinealectomy has been found to cause hyperplasia of the adrenal zona fasiculata and zona reticularis, a decrease in adrenal ascorbic acid and an increase in adrenal weight and corticosterone secretion (Miline, 1971; Motta et al., 1971).

Acute stress caused by immobilization, cold and fasting, has been shown to have various effects on endocrine as well as non-endocrine structures (Pfeiffer, 1967). Restraint stress, whether in combination with cold or not, is characteristically followed by gastric mucosal ulceration (Brodie and Hanson, 1960) and increased adrenal function (Silberstein et al., 1972) as well as changes in thyroid, pituitary (Mateef, et al., 1972) and thymic activity (Pfeiffer, 1967). Increases in epinephrine and norepinephrine synthesis and release are seen (Kvetnansky and Mikulaj, 1970) as well as increased norepinephrine and serotonin turnover in the brain. The latter has been associated with changes in melatonin concentration (Anton-Tay, 1971).

In relation to the above evidence, Motta et al. (1971) have suggested that "...it would be important to know whether stressful stimulation modifies the biosynthesis

and release of pineal indoles and methoxyindoles." In this pursuit, a specific, sensitive, quantitative assay for the presence of melatonin in biological fluids is required. Wurtman (1971) has suggested that an assay system which utilizes "a chromatography method followed by bioassay is the most hopeful." No such method, applicable to urine, has as yet, been described.

The research herein described was undertaken to develop a method of melatonin assay, capable of quantitatively estimating melatonin concentrations in urine and to use such an assay system to determine the existence of variations in urinary melatonin content in animals exposed to a variety of stressful conditions. Further, experiments were designed to provide information concerning the existence of an endocrine relationship between environmental stress, melatonin excretion, corticosteroid excretion, gastric ulceration and the weight of various endocrine organs.

In light of the known relationship that exists between pineal activity and reproductive function, the effect of stress upon pineal function and, therefore, its possible effect on reproductive function is of considerable interest. With further information concerning the non-photic factors modulating pineal gland secretory activity, a deeper understanding of the role of the pineal gland in both normal and abnormal mammalian physiology will be possible.

LITERATURE REVIEW

Pineal physiology

The mammalian pineal gland is an organ of very high biochemical activity with the unique capability of synthesizing the hormone melatonin (N-acetyl-5-methoxytryptamine) (Axelrod, 1970). Following its isolation from bovine pineal glands (Lerner et al., 1958), the biochemical pathway leading to its synthesis from tryptophan was elucidated. Through the systematic isolation of the various enzymes involved in its biosynthesis, the sequence of events was found to be as follows: tryptophan ——→ 5-hydroxytryptophan ——→ serotonin ——→ N-acetylserotonin ——→ melatonin (Axelrod, 1970). The enzyme responsible for the conversion of N-acetylserotonin to melatonin, hydroxyindole-O-methyltransferase (HIOMT), has recently been found to exist in small quantities in the mammalian retina (Nagle et al., 1972) and Harderian gland (Cardinali et al., 1972a). An enzyme capable of forming melatonin from N-acetylserotonin has also been found in human erythrocytes (Rosengarten et al., 1972). Although capable of similar synthetic activities, these enzymes are apparently not identical (Cardinali et al., 1972a). Despite the presence of such methyltransferase enzymes in tissues other than pineal gland, melatonin is highly localized in pineal tissue (Axelrod, 1970).

The fate of melatonin, once secreted from the pineal gland, has been studied. ^{3}H-melatonin administered to rats is rapidly metabolized and taken up by various tissues (Kopin et al., 1961). There is a rapid decrease in whole body ^{3}H-melatonin (biological half-life approximately 2 min) immediately following its intravenous administration. The rate of its disappearance diminishes soon thereafter (biological half-life approximately 35 min). ^{3}H-melatonin was found in liver, kidney, brain, fat and skin. Unchanged melatonin could not be found in the urine or feces. Sixty to seventy per cent of administered radioactivity was found in urine, primarily in the form of 6-hydroxymelatonin sulfate and the glucuronic acid conjugate of hydroxy-melatonin. Hydroxylation is accomplished by a liver microsomal enzyme. In a similar study, Wurtman et al. (1963) found ^{3}H-melatonin to be concentrated in the pineal gland, iris-choroid, peripheral nervous structures, ovary, and other endocrine organs. The finding of native melatonin in peripheral nervous tissue has been reported (Barchas and Lerner, 1964). Tritiated melatonin administered to rats intraperitoneally was found to be concentrated in the midbrain and hypothalamus (Anton-Tay et al., 1968).

The treatment of rats with chlorpromazine has been shown to markedly inhibit the disappearance of melatonin from the blood and tissues, perhaps by inhibiting its metabolism (Wurtman and Axelrod, 1966b). In a recent study, it was found that ^{3}H-melatonin was not metabolized

when incubated with human and rat venous blood *in vitro*. The protection of 72-82% of added ^{3}H-melatonin from metabolism was found to be due to the binding of melatonin to a native, specific, high capacity, binding albumin (Cardinali *et al.*, 1972b).

A variety of surgical procedures and environmental modifications have been found to affect the activity of the pineal gland and, through it, the activity of other organs, particularly the reproductive organs. The most often studied environmental modifications are those in relation to photic stimulation. Modification of mammalian sexual cycles by adjusting the photoperiod were reported in the ferret (Bissonette, 1932) and albino rat (Browman, 1937), before the role of the pineal gland in the control of reproductive function was proposed. The effect of light on the reproductive organs was believed to be mediated by the photostimulation of the retina and the transmission of the impulse directly to the anterior pituitary gland resulting in a release of hypophyseal gonadotrophins (Browman, 1940).

The effect of light on the pineal gland was first observed by Fiske *et al.* (1960) who demonstrated that the pineal gland weight of animals maintained in constant light was significantly lower than controls maintained in a natural light schedule. This finding was confirmed in further studies (Fiske *et al.*, 1962). In addition, the observation was made that animals maintained in constant light for 9

weeks or more contained ovaries that became increasingly follicular. Thus, through the use of a constant light environment, a relationship between the pineal gland and reproductive function was proposed.

Further studies both confirmed and expanded the understanding of that relationship. Exposure of rats to constant light for 80 days resulted in animals with lighter pineal glands, larger ovaries and uterine hypertrophy (Wurtman et al., 1961). The incidence of estrus in animals maintained under constant illumination was found to be significantly increased (Wurtman et al., 1964a).

The finding that visible light can penetrate the organs and tissues of the eye-window and reach the hypothalamus in the duck (Benoit, 1964) suggested a possible direct effect of light on the hypothalamus, if not the pineal, in other organisms. A light-sensitive photovoltaic capsule implanted in the brain of several mammals demonstrated the penetration of small amounts of light into the brain of all species tested (Van Brunt et al., 1964). Further evidence, however, focused more attention on the relationship of light to pineal and reproductive function. Animals maintained in constant light and treated with varying concentrations of melatonin were found to have decreased seminal vesicle and anterior pituitary weights (Debeljuk, 1969), indicating that the release of melatonin, a possible antigonadotrophic substance, may be inhibited by constant light.

An increase in electrophysiological nerve activity at nerve terminals in the pineal gland of rats was found in darkness and a four-fold depression in this activity was measured immediately upon exposure of the animals to room light (Taylor and Wilson, 1969). The inhibitory effect of light on pineal function was found to be related to the light spectrum (Wurtman and Weisel, 1969), cool-white illumination being more effective than Vita-lite (a fluorescent source closer to daylight).

In a study of the effect of exogenous stimuli on the synthesis and release of hypothalamic releasing factors in rats (Meites, 1970), it was found that constant light caused an increased release of FSH from the pituitary gland and increased growth of ovarian follicles. Similarly, an increased release of pituitary LH and a subsequent state of constant estrus was found. In a separate study (Brown-Grant et al., 1973), constant light was found to cause an inhibition of ovulation in 95% of the rats studied while 90% of the animals were found to be sexually receptive, a state of constant estrus.

Recently, continuous illumination for 64 days was found to increase the number of synaptic ribbons in the pineal glands of guinea pigs (Vollrath and Huss, 1973), indicating increased intercellular communication.

The results of studies in which animals were exposed to continuous illumination have served to indicate that light plays an inhibitory role in the control of

physiological activities in the pineal and, therefore, permits a release of the inhibitory control of the pineal over reproductive function. In an attempt to further elucidate this relationship, studies were undertaken to determine the effects of constant darkness on pineal-gonadal functions. Histological changes were reported (Roth et al., 1962) in the pineal glands of rats exposed to constant darkness. Pineal parenchymal cells were found to be enlarged, highly basophilic, more granular, and had nuclei which were irregular with very prominent nucleoli. An increased synthesis of proteins in the pineal glands of these animals was indicated.

Constant dark (LD 1:23) was found to cause atrophy of the gonads in male hamsters (Hoffman and Reiter, 1965). Female rats maintained in constant darkness for 8 months, beginning at 21 days of age, began showing prolonged estrus cycles at 2-3 months and, at autopsy, showed significantly lower uterine, ovarian, adrenal and pituitary weights than controls (Hoffman, 1967). It was found, however, that if animals were kept in constant darkness from birth, the degree of pineal activity, as evidenced by the degree of sexual maturation, was the same as that found in animals exposed to constant illumination (Relkin, 1967). It was suggested that the pineal gland requires a period of light in order to "prime" it to function in the dark (Relkin, 1968).

Further understanding of pineal function in constant

darkness was provided by a study which demonstrated that while constant darkness caused testicular atrophy in hamsters, it also was found to cause a 2-fold increase in pineal HIOMT activity (Anton-Tay and Wurtman, 1968). In addition, Kinson and Robinson (1970) reported that the inhibitory effects of constant darkness on male rat gonadal and accessory organ activity could be mimicked by the administration of melatonin. While the circadian variation of both N-acetyltransferase (Klein and Weller, 1970) and melatonin (Ralph et al., 1971) in the pineal glands of animals maintained in constant dark appeared to be unchanged, that of melatonin in the pineal glands of animals maintained in continuous light was found to be significantly depressed (Ralph et al., 1971).

In order to determine whether photic effects on the pineal gland were mediated through the eyes, a series of experiments were conducted in which animals were blinded by bilateral orbital enucleation. Male albino rats bilaterally enucleated on the first day of life were found to weigh less than normal-eyed controls and were found to have retarded testicular and seminal vesicle development at 35-40 days (Browman, 1940). In mature female rats, orbital enucleation eliminated the light-induced hypertrophy of the ovaries and uterus and prevented the associated decreases in pineal weight and HIOMT activity (Wurtman et al., 1964b). In this study it was also demonstrated that the bilateral removal of the

superior cervical ganglia resulted in effects identical to those caused by blinding. Bilateral enucleation and/or ganglionectomy were also shown to abolish the response of pineal HIOMT to lighting changes (Wurtman et al., 1964c). Blinding of hamsters was found to double the activity of pineal HIOMT (Anton-Tay and Wurtman, 1968).

The effects of bilateral enucleation in causing gonadal atrophy in male hamsters was found to be prevented by pinealectomy (Hoffman and Reiter, 1965). This report was confirmed in both male and female rats and hamsters (Reiter, 1968; Reiter et al., 1968b). However, Reiter (1969) demonstrated that 22-27 weeks after blinding, the gonads regenerated, even if the pineal was intact. The transplantation of the pineal gland to under the renal capsule in blinded-pinealectomized animals was found to result in a failure of blinding and pinealectomy to cause gonadal involution (Reiter, 1967).

The disruption of estrus cycles in animals maintained in constant dark was duplicated in blinded rats (Hoffman, 1967) and hamsters (Sorrentino and Reiter, 1970).

The pathway through which nervous information concerning lighting changes is transmitted to the rat pineal gland was studied by Moore et al. (1967). While a normal diurnal variation in pineal HIOMT activity could be demonstrated in control animals, this diurnal

variation was abolished by transection of the inferior accessory optic tracts in the medial forebrain bundle. Transection of the optic tracts did not alter the normal rhythm. The involvement of the inferior accessory optic tracts in mediating photic effects on the pineal gland was also shown in the rhesus monkey (Moore, 1969).

The combination of blinding and anosmia (olfactory bulb removal) in male rats was found to exaggerate the effects of blinding alone on reproductive organ weights but did not similarly increase the effects of blinding on pineal HIOMT activity. The entire effect was reversed by pinealectomy (Reiter et al., 1969). A similar study, using female rats, showed that blinding and anosmia caused hypertrophy of sex organs but not significant alterations in pineal HIOMT activity (Reiter et al., 1970). Blinded, young, male rats show a significant inhibition of growth hormone production and release by the pituitary gland. This effect, like the response of the reproductive organs, is enhanced when rats are also olfactory-deprived. The total effect is abolished by pinealectomy (Sorrentino et al., 1971b and c). A similar inhibitory effect of blinding and anosmia was shown on pituitary prolactin levels (Donofrio and Reiter, 1972). In this study it was also shown that the effects of blinding and anosmia were abolished by pinealectomy.

The effects of blinding on pituitary growth hormone content were also found to be exaggerated by reducing to

one half the normal intake of food. This effect was found to be abolished by the removal of the pineal gland (Sorrentino et al., 1971a).

The effects of blinding on reproductive function in humans has also been studied. In 56 girls with varying degrees of legal blindness it was found that there was a significantly earlier age of onset of menarche in the girls with minimal or no light perception (Magee et al., 1970). This report compares favorably with the studies of light deprivation in the rat.

In a study of seasonal variation in growth rates in pre-adolescent normal and blind children, it was found that, although both groups showed similar amplitude and variation, there was a significant difference in the season during which maximum growth rates were observed (Marshall and Swan, 1971).

The studies described above, in which organisms were visually deprived, provide substantial evidence that the mammalian pineal gland responds to changes in at least one environmental factor by altering the level of its biosynthetic activities. These alterations are then reflected in modifications in the functional state of various organs, with particular reference to the reproductive organs.

Some reference has been made above to the pathway through which visual stimuli affect biochemical changes in the pineal and to the extent of those biochemical

modifications. Further consideration and clarification of both can now be presented.

The observations of Moore and co-workers (Moore et al., 1967; Moore, 1969) established that the visual pathway mediating pineal responses to environmental lighting involves the inferior accessory optic tract in both albino rats and the rhesus monkey. Further evidence concerning the visual pathway to the pineal gland has been reviewed by Reiter and Fraschini (1969), Axelrod (1970; 1971), and Reiter et al.,(1971), and can be summarized as follows: retina ——→ inferior accessory optic tract (passing through the medial forebrain bundle in the lateral hypothalamus) ——→ rostral midbrain tegmentum center ——→ intermediolateral nucleus in the upper thoracic cord ——→ preganglionic sympathetic fibers ——→ superior cervical ganglion ——→ postganglionic sympathetic fibers (nervi conarii) ——→ pineal parenchymal cells. Interruption of the pathway at any point by optical enucleation, transection of the inferior accessory optic tract or medial forebrain bundle, or removal or decentralization of the superior cervical ganglion, has been shown to abolish the normal levels of pineal enzymes. The normal diurnal rhythm of both HIOMT and N-acetyltransferase is supressed by any of the above procedures (Moore et al., 1967; Axelrod et al., 1969; Moore, 1969; Reiter et al., 1971; Moore and Rapport, 1971; Klein et al., 1971).

Recently, additional sympathetic and cholinergic

fibers have been described innervating the pineal gland in several mammalian species. A rich peripheral sympathetic innervation in the medial habenular nucleus of the rat brain has been found (Bjorklund et al., 1972). There is apparent continuity between the sympathetic innervation of the pineal gland, the lamina intercalaris, and the medial habenular nucleus, suggesting some functional interconnection or coordination between these structures. Acetylcholinesterase activity has been found in bovine pineal glands suggesting a role of cholinergic fibers in the darkness-induced increase of pineal HIOMT activity (Aro et al., 1973). Ariëns Kappers (1971) has described cholinergic fibers in both primates and ferrets. In a recent study, the innervation of the human fetal pineal gland was found to include: postganglionic sympathetic fibers in the nervi conarii, possible parasympathetic fibers from the Marburg and Pastori ganglia impinging upon pineal blood vessels, and a nerve considered to carry impulses from receptors in the pineal gland to a nerve cell cluster in the rostral part of the subcommissural organ (Møllgaard and Møller, 1973).

The study of pineal innervation has been oriented toward an elucidation of the mechanisms of the sympathetic control of pineal function.

Intact sympathetic innervation was found to be required for the maintenance of the normal circadian rhythm of serotonin content in the rat pineal gland

(Snyder et al., 1964). A 3-fold variation in the content of norepinephrine (NE) in the rat pineal gland was also found to exist during each 24-hour period (Wurtman and Axelrod, 1966a). Transection of the medial forebrain bundle was found to abolish both rhythms and at the same time to eliminate the variation in HIOMT levels known to be caused by changes in environmental lighting (Axelrod et al., 1966). HIOMT was, at the time of this study, believed to be the rate limiting enzyme in the synthesis of melatonin. Thus, on the basis of this evidence, a correlation between norepinephrine content, serotonin content and melatonin synthesis could be made. This relationship was further expanded by the finding that the rate of formation of cyclic 3',5'-adenosine monophosphate (cAMP) in pineal homogenates was enhanced by the addition of NE while denervation of the pineal by superior cervical ganglionectomy did not significantly reduce that rate (Weiss and Costa, 1967). This suggested that the cAMP-forming enzyme (adenylate cyclase) is linked to a receptor at the post-synaptic membrane.

The development of the technique of pineal gland organ culture was most significant in the further elucidation of mechanism of the control of indolamine metabolism in the pineal. The results of the use of this technique have been described in detail in a recent review (Shein, 1971).

The synthesis of ^{14}C-melatonin from ^{14}C-tryptophan

was found to be significantly stimulated by the addition of NE to the pineal organ culture medium (Axelrod et al., 1969). Later, it was found that treatment of cultured rat pineal glands with NE causes a 6- to 10-fold stimulation of the enzyme N-acetyltransferase, the enzyme which converts serotonin to N-acetylserotonin, the immediate precursor of melatonin (Klein et al., 1970). Intact sympathetic innervation of the pineal gland was found to be essential to maintain the 24 hour rhythm of pineal N-acetyltransferase activity (Klein et al., 1971).

In a recent report by Berg and Klein (1972), a further effect of NE on pineal glands in organ culture was described. They reported that the treatment of cultured pineal glands with NE stimulated the incorporation of ^{32}P into a phospholipid fraction of membranes and particulates containing phosphatidyl serine and phosphatidyl inositol. This effect was suggested to be associated with the interaction of NE with a membrane-bound post-synaptic receptor. It was also reported that this effect is not known to occur in a tissue in which cholinergic fibers are absent.

Using microelectrodes to monitor cell membrane potentials, Sakai and Marks (1972) have shown that both NE and isoproterenol (a sympathomimetic) caused a dose-dependent hyperpolarization of the pinealocyte membrane. These changes were inhibited by propranolol (a beta-adrenergic blocker). Estradiol also was found to inhibit the effects of NE.

Lynch and co-workers (1973a) have recently reported that factors, other than environmental lighting, which are known to increase sympathetic nervous activity elsewhere in the body, can alter pineal indole metabolism. They have shown increased pineal N-acetyltransferase activity and melatonin content as a result of either insulin-induced hypoglycemia or immobilization for 3 hours. In further studies, subcutaneous injection of L-DOPA (L-dihydroxy-phenylalanine) was found to cause a 3 to 6-fold increase in pineal melatonin content (Lynch et al., 1973b).

Having established the requirement of an intact sympathetic innervation for the maintenance of normal pineal function, further questions were raised. The mechanisms mediating the control by NE of indole metabolism remained to be clarified.

NE was found to increase adenylate cyclase activity and cAMP levels in the pineal (Weiss and Costa, 1967). This effect was closely related to the effect of environmental lighting on the pineal content of cAMP and adenylate cyclase (Ebadi et al., 1970) and could be duplicated in vitro by both NE and NaF (Weiss, 1969). Sodium fluoride was not found to be effective in stimulating adenylate cyclase in vivo. In a review of the neuroendocrine control of the cyclic AMP system in the pineal gland, Weiss and Strada (1972) reported that while many factors affect adenylate cyclase activity, they do not seem to affect actual levels of enzyme present.

While the role of increased activity of adenylate cyclase is that of increasing the synthesis of intracellular cAMP from ATP, the role of increased cAMP in the control of pineal indole metabolism is less well defined. In organ culture of rat pineal glands, dibutyryl adenosine 3',5'-monophosphate, an analog of cAMP, was found to stimulate the conversion of tritiated tryptophan to tritiated melatonin (Klein et al., 1969). Dibutyryl cAMP was also found to stimulate the conversion of ^{14}C-serotonin to ^{14}C-melatonin without altering HIOMT activity. In a separate study, dibutyryl cAMP was found not to enhance the accumulation of labeled tryptophan or protein within the pineal (Shein and Wurtman, 1969). It was suggested that cAMP mediates some, but not all, of the effects of NE.

The finding that ^{14}C-melatonin synthesis was enhanced by treatment of cultured pineal glands with dibutyryl cAMP while (Klein et al., 1969) HIOMT activity was found to be unchanged was explained by a further study by Klein et al. (1970). It was found that the treatment of cultured rat pineal glands with NE or dibutyryl cAMP causes a 6- to 10-fold stimulation of N-acetyltransferase activity. It was thus concluded that N-acetyltransferase was more important in controlling the rate of melatonin synthesis than was HIOMT and that N-acetyltransferase was under the control of cAMP.

In pineal organ culture studies, Strada and coworkers (1972) have established a temporal relationship

between NE addition and the increase in endogenous cAMP. This increase was evident within 5 min and the concentration of cAMP was found to remain elevated for more than 2 hr.

Recently, further clarification of the role of cAMP in indole metabolism in the pineal was presented by Fontana and Lovenberg (1973). Their work was based upon the assumption that an increase in cAMP leads to an increase in pineal protein kinase which in turn increases the phosphorylation of certain regions of chromatin, causing a relaxation of the tight association between DNA and histones. Since the effects of cAMP can be blocked by cycloheximide and actinomycin D, this indicates that cAMP increases the transcription of certain regions of DNA. They have shown that pineal protein kinase does phosphorylate chromatin and does in fact allow more transcription to take place.

The question presented by this evidence deals with the nature of the protein coded for by the section of DNA uncovered by the phosphorylation reaction. On the basis of the evidence presented above, the protein suggested may be the enzyme N-acetyltransferase.

The two enzymes implicated in control of melatonin synthesis have been isolated and purified by Weissbach and co-workers (1960; 1961). HIOMT was purified by Axelrod and Weissbach (1961). The role of HIOMT in the control of indoleamine metabolism in the pineal gland has been implicated by a decrease in HIOMT activity in response to constant light (Wurtman et al., 1963) and medial forebrain

bundle transection (Axelrod et al., 1966), and its increase in activity in response to darkness or blinding (Brownstein and Heller, 1968) and blinding combined with anosmia (Reiter et al., 1969). The finding that HIOMT activity is unchanged by treatment of cultured pineal glands with dibutyryl cAMP (Klein et al., 1969) while the same treatment causes an increase in N-acetyltransferase activity (Klein et al., 1970) has diminished the consideration of the former and heightened the consideration of the latter as the primary control of melatonin synthesis in response to sympathetic innervation. This view is supported by studies showing the control of N-acetyltransferase activity by postganglionic sympathetic fibers from the superior cervical ganglion (Volkman and Heller, 1971; Klein et al., 1971). and the rapid decline in its activity upon exposure to light (Klein and Weller, 1972). The further study of the role of N-acetyltransferase was aided by the development of a sensitive assay for the determination of its activity in the rat pineal (Deguchi and Axelrod, 1972c). The direction of further research was toward the relationship of N-acetyltransferase activity and sympathetic innervation of the pineal gland. Pellegrino de Iraldi and Zieher (1966) observed that denervation of the pineal resulted in a decrease in pineal NE content, since pineal NE is contained primarily in the sympathetic nerve endings. An increase in the responsiveness of adenylate cyclase to the stimulatory effects of exogenous NE was found in homogenates

of pineal glands following denervation. The factors causing a change in the sensitivity of organs to sympathomimetic amines was discussed by Trendelenburg (1971). The two possible mechanisms proposed involve either (a) the impairment of a site of loss of neurotransmitter as would be the case if re-uptake of the amine was eliminated or (b) an increase in effector organ responsiveness by a change in the nature of the receptor. The control of the activity of serotonin N-acetyltransferase in the rat pineal gland by the beta-adrenergic receptor was established by Deguchi and Axelrod (1972a). The observation that the nocturnal rise in N-acetyltransferase activity is prevented by removal of the superior cervical ganglion or treatment with l-propranolol, reserpine (a catecholamine depleting agent) or cycloheximide (a protein synthesis inhibitor), indicated that N-acetyltransferase activity is induced by the release of NE. The NE activates the beta-adrenergic receptor of the pineal parenchyma cell which then stimulates the synthesis of enzyme molecules. The synthesis of enzyme is presumed to be under control of the adenylate cyclase system. The rapid decline in enzyme activity upon photostimulation was suggested to be due to the conversion of enzyme from an active form to an inactive form or to the disaggregation of enzyme subunits.

A marked induction of pineal N-acetyltransferase in denervated pineal glands was found to be caused by treatment with catecholamines, MAO (monoamine oxidase) inhibitors or

theophylline (a phosphodiesterase inhibitor) (Deguchi and Axelrod, 1972b). This effect was blocked by propranalol or cycloheximide treatment. Super-induction of N-acetyltransferase activity was also observed with L-DOPA treatment.

The results of the studies by Deguchi (1973) and Deguchi and Axelrod (1973) indicate that denervation induces a rapid supersensitivity in the postsynaptic beta-adrenergic receptor to catecholamines and subsequently an enhancement of the cAMP level which results in the superinduction of N-acetyltransferase. Further evidence concerning the relationship between N-acetyltransferase activity, pineal serotonin content and the beta-adrenergic receptor was established by Brownstein et al. (1973). Normally, in diurnal lighting, N-acetyltransferase activity in the rat is low during the light period while serotonin levels during the same period are high. The reverse is true in darkness. Isoproterenol, given during the light period, causes an increase in N-acetyltransferase activity and a decrease in serotonin content in the pineal. Propranalol blocks this effect while phentolamine (an alpha blocker) did not.

Lynch et al. (1973b) have recently shown that while L-DOPA treatment causes an increase in pineal melatonin content, this effect is potentiated when postganglionic sympathetic neurons are damaged by pre-treatment with 6-hydroxydopamine.

From the results of the studies described above, it can be seen that the biosynthetic activity of the pineal

gland can be modulated by variations in the degree of sympathetic stimulation of pineal parenchymal cells. In the absence of intact sympathetic innervation, the presence of sympathomimetic compounds impinging upon the beta-adrenergic receptor sites will also cause a stimulation of pineal biosynthetic activity.

While it has been established that the pineal gland produces a hormone, probably melatonin, which plays a modulatory role in the reproductive activity of certain mammalian species, little research has been undertaken to determine the existence of feedback mechanisms. It has been found, however, that ovariectomy causes a slight increase in the stimulatory effect of NE on the pineal adenylate cyclase activity (Weiss and Crayton, 1970). In this same study it was shown that the administration of estradiol to ovariectomized rats for 2 days inhibited the NE-induced activation of adenylate cyclase. There was no inhibition observed if estradiol was given 1 hr before sacrifice or if it was added *in vitro* to pineal homogenates.

On the assumption that estradiol administration causes a decrease in pineal weight and a depression of pinealocyte cytoplasmic RNA, Nir *et al.* (1970) injected 17-beta-estradiol, subcutaneously, into immature female rats and, at various intervals during the following 24 hr, sacrificed them and measured pineal DNA, RNA and protein. They found a significant increase in pineal protein levels in estradiol treated rats as well as increases in both RNA and DNA. It

was also found that ovariectomy caused an increase in pineal function and volume, an apparently contradictory finding. As a result of their findings Nir et al. (1970) suggested that there exists a possible positive feedback of gonadotrophin (LH) released by the pituitary on the pineal gland. In a later study, Nir et al. (1972a) reported that the phospholipid content and HIOMT activity of the rat pineal are at their highest during diestrus, pointing towards a maximum pineal activity when ovarian function is at a minimum. In the same study, no changes in pineal protein or nucleic acid levels were found during the entire estrus cycle but there was a tendency towards high protein and RNA content during diestrus and low levels during estrus.

Certain enzymes involved in glucose metabolism were found to be depressed in the pineal gland in pregnant rats as compared to non-pregnant rats (Devečerski, 1972). However, while a diurnal rhythm of pineal glycogen content has been found, no significant differences could be correlated with phases of the estrus cycle (Kachi et al., 1973).

Houssay and Barcelo (1972a), while reporting that previous findings indicated that injections of estradiol benzoate caused a decrease in HIOMT activity, found that estrogen administration caused an increase in HIOMT activity and progesterone caused a decrease. Testosterone injections were found to decrease HIOMT activity while castration had no effect (Houssay and Barcelo, 1972b). While no explanation has been found for the contradictory

results of gonadal hormone administration on HIOMT activity, perhaps the thrust of future research will be directed towards the effect of such hormones on the activity of N-acetyltransferase, the enzyme thought now to be rate limiting in the production of melatonin.

Recently, Trentini et al. (1973) have demonstrated that chronic treatment with bretylium or guanethidine prevent the weight increases and the histomorphological and histoenzymatic changes in the rat pineal which normally follow ovariectomy and gonadotrophin treatment. Since both drugs block NE release from sympathetic nerve endings, it is suggested that circulating gonadotrophins stimulate the pineal through its sympathetic innervation.

The possibility that gonadal hormones have direct effects on the pineal gland is supported by the finding that the pineal gland has a much greater content of estradiol binding protein that other brain regions (Marks et al., 1972). The values found approached the level found in the adenohypophysis. Superior cervical ganglionectomy and 6-hydroxydopamine treatment reduced the level of this binding protein in pineal glands.

Some study has been directed towards the effect of other hormones on pineal function. Cady and Dillman (1971) have noted that previous studies have shown that the pineal has a high uptake of radioactive iodine, triiodothyronine and thyroxine and that thyroxine stimulates pineal lipid metabolism and Embden-Myerhoff pathways. They have

reported that thyroxine uptake by bovine pineal slices is by a mediated diffusion mechanism. Thyroxine metabolism by pineal slices has also been studied (Dillman and Cady, 1971). Bovine pineal tissue slices rapidly take up thyroxine. Radioactive thyroxine taken up by such slices can be found, following cell fractionation, primarily in the nuclear fraction and the microsomal fractions. Precipitation of the supernatant solution indicates the presence of iodinated material other than thyroxine. Such results indicate that thyroxine is deiodinated by the pineal cell and that perhaps the site of deiodination is the nucleus, where thyroxine may have an hormonal action.

Radioactive melanocyte stimulating hormone release-inhibitory factor (MIF) was found to be concentrated in the anterior pituitary, posterior pituitary, kidney, liver and, to the greatest extent, in the pineal gland (Redding et al., 1972). No accumulation was found in the cerebral cortex, hypothalamus, or muscle.

In an attempt to determine the possibility of feedback mechanisms between the pineal gland and other glands which are not known to be under pituitary control, Csaba and Bernád (1972) incubated rat pineal glands in the presence of various hormones and measured changes in serotonin content in the pineal. Incubation with thyroxine resulted in the disappearance of serotonin fluorescence while epinephrine, norepinephrine and insulin led to an increase in serotonin fluorescence. The increase in fluorescence

indicated that serotonin is either not being excreted or converted to melatonin and therefore may represent a possible negative feedback mechanism between the pineal and the source of those hormones.

Hyppä *et al.* (1973) have recently shown that male rats postnatally injected with 6-hydroxydopamine exhibited a significantly increased pineal HIOMT activity on the 30th day of life when compared to uninjected controls. Females did not show a change. Neonatal castration abolished this response while concurrent treatment with testosterone in females caused HIOMT activity to increase as in males. This suggests that androgens act upon catecholaminergic neurons in the brain to cause long term changes in pineal function.

Since the observation by Kitay and Altschule (1954) that a bovine pineal extract caused an inhibition of reproductive function and the subsequent isolation of melatonin from such an extract (Lerner *et al.*, 1958), the concentration of pineal research has been centered around the general assumption that melatonin is the pineal hormone responsible for mediating the effects of the pineal gland on other organs. Research, however, has been undertaken to determine the presence of and possible actions of other pineal hormones. In the pineal gland, melatonin is only one of the products of serotonin metabolism. Serotonin may be converted to 5-hydroxyindole acetaldehyde which in turn may be converted into either 5-hydroxytrypto-

phol (5-HTPOL) or to 5-hydroxyindoleacetic acid (5-HIAA). The 5-HTPOL may be methylated by HIOMT to 5-methoxytryptophol (5-MTPOL). Many of these compounds have been considered as putative pineal hormones. Daily subcutaneous injections of 5-MTPOL in microgram quantities in rats was found to decrease the incidence of estrus and to reduce ovarian weights in maturing rats (McIssac et al., 1964). In the same study, 5-HTPOL was found to be without effect. Vaughan et al. (1971b; 1972a) have reported antigonadotrophic effects of various pineal constituents. Following the removal of one ovary, the remaining ovary undergoes compensatory ovarian hypertrophy (COH). Vaughan and coworkers (1971a) have shown that intraperitoneal injection of N-acetylserotonin, melatonin, 5-HTPOL and 5-MTPOL were effective in inhibiting COH in mice while other indoles (6-hydroxymelatonin, serotonin, 5-hydroxytryptophan, 5-MIAA and 5-HIAA) were not. Voles (Microtus agrestis) given melatonin or Altschule's extract (Kitay and Altschule, 1954) and vasopressin also significantly restricted COH in the mouse but oxytocin did not. Vaughan et al. (1972b) concluded that the "pineal gland produces more than one antigonadotrophic factor."

A polypeptide with pressor and oxytocic activities was isolated from bovine pineal glands (Milcu et al., 1963). This polypeptide was found to differ from arginine and lysine vasopressin and oxytocin but was found to be similar to arginine vasotocin. Milcu et al. (1963)

suggested that this substance was produced in the habenula and stored in and released from the pineal gland. A similar polypeptide was identified in porcine pineal glands (Pavel, 1965). This polypeptide was found to have biological and chromatographic characteristics not significantly different from those of synthetic lysine vasotocin (Pavel and Petrescu, 1966). Cheesman and Fariss (1970) have also found a cyclic nonapeptide, characterized as 8-arginine vasotocin, in bovine pineal glands.

Benson et al. (1971) have isolated a substance of a molecular weight of 500-1000 in a melatonin-free filtrate of bovine pineal glands. This substance collected by filtration through Sephadex G-25 was found to inhibit COH in mice. A similar gonadotrophin inhibiting substance was found in homogenates of human pineal glands. On a Sephadex G-25 column this substance was collected in the non-melatonin containing fraction (F3) (Benson et al., 1972) of pineal extracts and was found to be 60-70 times more effective than the melatonin fraction in inhibiting COH and may or may not be identical with arginine vasotocin.

Fraction F3 was further filtered on Amicon membranes and found by Citharel et al. (1973) to have the capacity to inhibit the secretion of hypophysiotrophic hormones in rats and mice and to have a molecular weight of 1000. Moszkowska et al. (1973) found pineal fraction F3 to

inhibit follicle stimulating hormone releasing factor (FSH-RF) secretion. They also found serotonin and melatonin to be equally as effective in inhibiting FSH-RF secretion. LH (luteinizing hormone) synthesis and release by the pituitary was found to be inhibited by a similar non-melatonin-containing pineal extract (Orts and Benson, 1973). The activity of the non-melatonin pineal anti-gonadotrophin was found to be abolished by incubation with trypsin or chymotrypsin (Matthews and Benson, 1973).

A peptide with approximately 19 residues and a molecular weight of about 2000 was found in bovine pineal extracts to have both lipolytic and melanotropic (darkening) activities (Rudman et al., 1972).

A substance, thought to be a carboline (1-methyl-6-methoxy-1,2,3,4-tetrahydro-2-carboline) was purified from beef pineal glands and was found to have an adrenoglomerulotropic actvity in that it stimulated the secretion of aldosterone in midcollicular brain removed-hypophysectomized dogs (Farell and McIssac, 1961). However Jouan and Samperez (1964) discounted the presence of a pineal adrenoglomerulotropic hormone and attributed such effects to serotonin in the extracts.

A glycoprotein has been found to be synthesized in cultured pineal glands (Lott et al., 1972) and it has been speculated that this may serve as a carrier for secreted melatonin, though other functions have not been eliminated.

The effect of the mammalian pineal gland on the

activities of other organs has been the subject of considerable study. The primary effects of the pineal gland appear to be mediated through the brain (Cardinali et al., 1973). The nature of the pineal-brain relationship has recently been described in detail by Anton-Tay (1971) and will be briefly reviewed here.

Fiske et al. (1962), having removed endocrine organs (gonads, adrenals, pituitary and thyroid) through which light may mediate its effects on the pineal gland, concluded that the pathway must involve the central nervous system. They also reported that the supraoptic nuclei of the hypothalamus were active during continuous exposure to light, a condition known to effect the pineal gland. The central nervous system involvement in the control of pineal function has been described above (Axelrod, 1970; 1971; Reiter and Fraschini, 1969; Reiter et al., 1971). Of interest here is the effect of pineal products on the brain. The effect of a pineal substance on the hypothalamus was indicated by the finding that neuroglandular cells of the paraventricular nucleus showed compensatory hypertrophy when rats were treated with a pineal extract (Miline, 1963). Pinealectomy was found to have an opposite effect on these cells. Pinealectomy was also found to result in an increased activity of the supraoptic nucleus (Miline, 1971). The neurosecretory activity of the supraoptic nucleus, as measured by the level of thiamine diphosphate-phosphohydrolase was found to decrease following pinealectomy (de Vries

and Ariëns Kappers, 1971). Though no specific region was cited, it was reported that melatonin, administered intraperitoneally, caused an increase in brain pyridoxal kinase activity (Anton-Tay, 1971). Pyridoxal kinase catalyses the synthesis of brain pyridoxal phosphate which, in turn, is a coenzyme of aromatic-L-amino acid decarboxylase, an enzyme essential for the synthesis of dopamine, GABA and serotonin. These findings prompted the statement that "the hypothalamus is the strategic point for the action of pineal hormones" (Miline, 1971).

Support for the hypothesis that the site of action is the central nervous system can be found in studies relating the presence and uptake of melatonin by nervous structures. Endogenous melatonin has been found in human peripheral nerves (Barchas and Lerner, 1964). Peripheral nervous structures have been found to take up ^{3}H-melatonin *in vivo* in the rat (Wurtman *et al.*, 1964d). Intraperitoneally administered melatonin was found to increase brain serotonin concentrations (Anton-Tay *et al.*, 1968) especially in the midbrain and hypothalamus, where ^{3}H-melatonin is concentrated to the greatest extent. The fate of intracisternally injected melatonin in the rat brain has recently been reported (Cardinali *et al.*, 1973). While it was found that ^{3}H-melatonin was metabolized rapidly by the brain (only 6% of the administered dose was present unchanged in 5 min), the concentration of melatonin in the hypothalamus was 3.8-5.1 times that in the rest of the brain.

Reiter and Sorrentino (1972) reported that the effects of darkness and the pineal gland on the pituitary-gonadal axis were completely prevented by total or anterior hypothalamic disconnections. It was suggested that pineal-afferent fibers and neurons entering the hypothalamus, which themselves have been stimulated by pineal factors, may have been cut.

Citharel et al. (1972) have shown that, in the case of the Sephadex G-25 fraction F3, the action of the pineal gland on the hypothalamic hypophysiotropic activity is one of inhibition of secretion of hypophysiotropic hormones. Similar results have been shown by studies involving other pineal products. The release of LH was found to be increased by pinealectomy, an effect presumably mediated through an effect on hypothalamic releasing factor (Vaughan and Reiter, 1971). Moszkowska et al. (1973) have reported an inhibition of hypothalamic FSH-RF synthesis and release by various pineal fractions (F3, serotonin and melatonin), confirming an earlier report by Fraschini et al. (1971) in which FSH release was found to be under the control of serotonin and 5-MTPOL and LH release to be under the control of melatonin and 5-HTPOL.

Several mechanisms by which pineal factors modify hypothalamic releasing factor content have been suggested (Fuxe et al., 1970; Tima et al., 1973). Melatonin may cause an increase in hypothalamic serotoninergic activity which in turn inhibits the release of LH-RF. Alternatively,

pineal products may directly stimulate dopaminergic neurons which are known to inhibit LH-RF release or they may stimulate prolactin release which in turn would increase dopaminergic activity, subsequently causing the inhibition of LH-RF release.

Pineal control of the hypothalamic release of MSH release-inhibitory factor (MIF) has also been reported (Kastin et al., 1972) and attributed to melatonin.

Of considerable concern has been the route of secretion of pineal products. The arguments supporting systemic circulation release and cerebrospinal fluid release have been discussed without resolution (Wurtman, 1971). Recently, Quay (1973) has proposed a direct, non-systemic vascular pathway which may carry pineal hormone to the brain. This pathway assumes the reversibility of direction of blood flow in the vena cerebri magna and the choroid plexuses of the suprapineal recess.

The effects of the administration of melatonin on brain function have been studied. Within 15-30 min, melatonin caused a slowing of brain electrical activity and the inducement of sleep in cats (Marczynski et al., 1964), indicating a role of melatonin "in modulating the state of wakefulness and sleep." Barchas et al. (1967) could find no acute pharmacological or toxic effects of melatonin in mice or 4 day old chicks. Nir et al. (1969) have demonstrated modifications in the EEG patterns of rats following pinealectomy.

The effect of melatonin, administered orally, on the human brain has also been studied (Anton-Tay et al., 1971). Melatonin administration was followed by a general electrophysiological deactivation without a deterioration of brain function. EEG rhythms were slowed; sleep was induced; and an increase in REM sleep was seen. A rise in the convulsive threshold was seen in epileptics. It was suggested that melatonin caused an increased neuronal availability of pyridoxal phosphate with subsequent changes in the metabolism of biogenic amines.

Drew and Batt (1972) have suggested that the pineal, through its ability to influence the REM state and its enhancement of arousal mechanisms, plays a major role in species and specimen protection.

The effect of the pineal gland on the brain is manifested in modifications of pituitary function. The mechanisms involved in the control, by pineal constituents, of the pituitary gland release of gonadotrophins (Fraschini et al., 1971; Moszkowska et al., 1971) and ACTH (Motta et al., 1971) have recently been reviewed. The implantation of melatonin or 5-HTPOL in the median eminence or the reticular substance has been shown to inhibit the synthesis and release of pituitary LH (Fraschini et al., 1971; Fraschini et al., 1968a; 1968b) while pinealectomy results in an LH increase (Fraschini et al., 1968a). Implantation of serotonin or 5-MTPOL has been shown to inhibit FSH synthesis and release (Fraschini et al., 1971).

The intracardiac administration of melatonin inhibits the release of pineal LH stores (Ying and Greep, 1973).

The release of pituitary stores of prolactin can be stimulated by intraventricular administration of melatonin (Lu and Meites, 1973) or N-acetylserotonin (Porter et al., 1971/72) or serotonin (Kamberi et al., 1971). The effect of melatonin on the release of prolactin has been suggested to involve both the inhibition of a prolactin release-inhibiting factor (Relkin et al., 1972) and the stimulation of a prolactin releasing factor (Lu and Meites, 1973).

An inhibitory effect of the pineal gland on the synthesis and release of pituitary growth hormone has been reported by Sorrentino and co-workers (Sorrentino et al., 1971a; 1971b; 1971c). These reports have been supported by recent studies. Extremes of lighting (constant dark or constant light) have been found to inhibit tibial cartilage growth in rats only in the presence of an intact pineal gland (Nir et al., 1972b). Recently, Smythe and Lazarus (1973a; 1973b) have shown that the serotonin-induced release of growth hormone can be completely blocked by intraperitoneal injections of melatonin.

The effect of the pineal gland on pituitary MSH levels has been investigated by Kastin and co-workers. The intravenous injection of melatonin was found to cause a decrease in pituitary MSH content (Kastin and Schally, 1967; Piezzi and Wurtman, 1970), indicating an increased release of the hormone. Pinealectomy results in an

increased pituitary MSH level (Kastin et al., 1969). Support for a pineal-pituitary MSH relationship is given by the finding that radioactive synthetic MIF as well as radioactive MSH are markedly accumulated by the pineal gland (Kastin et al., 1972).

Pituitary thyroid stimulating hormone (TSH) content has been found to decrease while plasma TSH and protein bound iodide increase in pinealectomized rats (Relkin, 1972a) indicating an inhibitory effect of the pineal gland on TSH release.

An inhibitory effect of the pineal gland on pituitary release of ACTH has been described by Motta et al. (1971) in a recent review and supported by others (Dickson and Hasty, 1972; Vaughan et al., 1972b).

The effects of the pineal gland on the hypothalamo-hypophyseal system are translated into effects on the target organs of the hypophysiotropic hormones.

Pinealectomy in the rat has been reported to cause a significant increase in arterial blood pressure (Zanaboni and Zanaboni-Muciaccia, 1967). This may result from a release of the pineal inhibition of aldosterone secretion, as this procedure has been shown to increase aldosterone secretion (Kinson and Singer, 1967). Gromova et al. (1967) have found that melatonin administration in intact rats has differential effects on adrenal corticoids. Aldosterone production is reportedly increased. After 30 days following pinealectomy, the secretion of both aldosterone

and corticosterone was found to be elevated (Kinson et al., 1968). Pinealectomy has also been shown to decrease adrenal ascorbic acid concentrations while elevating corticosterone secretion and increasing adrenal weight, although the latter effect is inconsistent (Motta et al., 1971). Hyperplasia of the zona fasiculata and reticularis have also been reported (Miline, 1971). Melatonin injection was found to inhibit adrenal hypertrophy following unilateral adrenalectomy and to reduce plasma corticosterone levels (Vaughan et al., 1972b). Similar results were reported for the effect of a bovine pineal extract on adrenal function (Dickson and Hasty, 1972). Melatonin administration has also been shown to reduce adrenal transcriptional activity as measured by RNA/DNA ratio (De Fronzo and Roth, 1972).

A polypeptide has been found in the pineal gland of the rat which, when injected, causes hyperkaliemia. This effect may or may not be mediated by the adrenal glands (Chazov et al., 1972).

Subcutaneous injection of melatonin has been found to decrease thyroid weight, thyroid hyperplasia and ^{131}I uptake (Baschieri et al., 1963) as well as thyroid hormone secretion rate (Ishibashi et al., 1966; Narang et al., 1967; Singh and Turner, 1972). Rowe et al. (1970), while reporting previous findings of increased thyroid function following pinealectomy, found the effects of constant light and dark on the thyroid to be independent of the

pineal gland.

Contradictory effects of the pineal gland on the parathyroid gland have been reported. A decrease in parathyroid activity has been demonstrated following both administration of a pineal extract (Krstic, 1968) and pinealectomy (Kiss et al., 1969).

The best documented aspect of pineal function is its role in the modulation of mammalian reproductive function. The effects of variations in lighting conditions and various surgical procedures on the pineal-gonadal relationship as well as the effect of the pineal gland on the gonadotrophin secreting activity of the pituitary, have been described above. The effect of the administration of pineal extracts or pineal products on gonadal function has been the subject of many studies. The administration of melatonin has been found to decrease testicular, seminal vesicle, and ventral prostatic weights in rats (Debeljuk, 1969; Debeljuk et al., 1970a; 1970b; Motta et al., 1967; Reiss et al., 1963). A decrease in testicular metabolic activity has also been reported following melatonin administration (Kinson and Robinson, 1970). Both melatonin and serotonin have been found to cause a decrease in testicular androgen synthesis (Ellis, 1969) and release (Kinson et al., 1973) and testicular tubular activity (Liu and Kinson, 1973).

Melatonin administration has been shown to decrease the incidence of estrus in the rat (Chu et al., 1964;

Wurtman et al., 1964a) as well as to delay spontaneous vaginal opening time (Osman et al., 1972; Wurtman et al., 1963). Reflex ovulation in the rabbit (Farrell et al., 1968) and pregnant mare serum-induced ovulation in the rat (Pomerantz and Sorrentino, 1973) are both inhibited by melatonin treatment.

Bovine pineal extract administration has been reported to decrease ovarian weight in rats (Kitay and Altschule, 1954; Wurtman et al., 1959). Melatonin has been found to duplicate this effect (Sorrentino, 1968; Wurtman et al., 1963) and to inhibit the compensatory hypertrophy of the ovary which generally follows unilateral ovariectomy (Sorrentino, 1968; Vaughan et al., 1971a; 1971b; 1972a). Contradictory findings have also been reported in which no effect of melatonin administration or of the pineal on ovarian weight or the estrus cycle has been seen (Alleva et al., 1970; Ebels and Prop, 1965; Ota and Hsieh, 1968; Reiter, 1973a).

The pineal gland, through the mediation of melatonin, has been shown to have a variety of effects in mammals which are not related to gonadal function. The subcutaneous injection of melatonin has been shown to cause the growth of white hair in the coat of brown, male, short-tailed weasels (Rust and Meyer, 1969). Melatonin-induced hypoglycemia, increased glucose tolerance and increased hepatic and muscular glycogen synthesis have been reported (Milcou et al., 1963). Recently, melatonin has been reported to

inhibit glucose-induced insulin secretion from the islets of Langerhans of normal and obese mice as well as from pieces of rat pancreas (Bailey et al., 1974).

An effect of melatonin on smooth muscle contraction has also been shown. Melatonin induces, in vitro, a marked inhibition of the amplitude of spontaneous contractions and motile response of the rat duodenum to serotonin (Quastel and Rahamimoff, 1965). The stimulatory effect of pitocin on the estrus mouse uterus was effectively blocked by melatonin (Davis et al., 1971).

Melatonin administered to women with advanced breast carcinoma was found to decrease urinary estrogen levels (Burns, 1973).

Pinealectomy

Pinealectomy has been shown to produce significant effects on the function of various mammalian organs. The effects of pinealectomy on the reversal of the effects of constant darkness or blinding have been described above. Pinealectomy alone has been shown to result in ovarian hypertrophy in the rat (Kitay, 1954; Wurtman et al., 1961) and to prevent the winter decline in the reproductive activity of male golden hamsters (Reiter, 1973b). An increase in testicular incorporation of ^{3}H-thymidine (Kinson and Robinson, 1970) and tubular enzymatic activity (Timmermans and Devečerski, 1972) has been demonstrated in pinealectomized rats. Pinealectomy results in hypertrophy

of testes and accessory organs in the rat (Motta et al., 1967) and mouse (Vaughan and Reiter, 1971). The anovulatory syndrome produced by lesion of preoptic and suprachiasmatic areas of the rat brain has been abolished by pinealectomy (Tima et al., 1973). Pinealectomy has recently been shown to significantly decrease the number of live fetuses carried by pregnant rats (Guerra et al., 1973).

The effects of pinealectomy on the adrenal glands and the related effects on mineral metabolism and hypertension have been studied. Pinealectomy has been shown to result in adrenal hypertrophy in female hamsters (Hoffman and Reiter, 1966), male and female rats (Motta et al., 1971; Relkin, 1972b). An increase in aldosterone secretion has been reported to result from pinealectomy (Kinson and Singer, 1967; Kinson et al., 1968; 1967; Motta et al., 1971). The removal of the pineal gland in rats has been reported to result in an increase in arterial blood pressure to hypertensive levels (Karppanen and Vapaatalo, 1971; Karppanen, et al., 1970; Zanaboni and Zanaboni-Muciaccia, 1967), a result possibly mediated by elevations in circulating aldosterone levels. While an increase in circulating aldosterone levels would lead to an increased retention of sodium, pinealectomy does not result in modifications in sodium intake or drinking rhythms (Bliss and Bates, 1972; Donovick et al., 1973; Stephan and Zucker, 1972b). Nocturnal and circadian

rhythms in drinking behavior are, however, eliminated by bilateral lesions in the suprachiasmatic nuclei (Stephan and Zucker, 1972a). Pinealectomy has resulted in modifications in the activities of the adenohypophysis and pars intermedia. Pineal gland removal is, reportedly, followed by an increase in adenohypophyseal mitotic activity (Bindoni and Raffaele, 1968) and an increase in pituitary prolactin levels (Donofrio and Reiter, 1972; Relkin et al., 1972; Ronnekleiv et al., 1973). The latter finding indicates a decrease in secretion in both male and female rats. A decrease in MSH secretion following pinealectomy has also been shown (Kastin et al., 1967; 1969). Conversely, pinealectomy has been found to increase the secretion of TSH (Relkin, 1972a) and growth hormone (Nir et al., 1972b).

In a recent series of studies by Reiter and coworkers, pinealectomy, when following parathyro- or thyroparathyroidectomy, was shown to result in muscular spasms, convulsive seizures and death in rats (Reiter et al., 1973; Reiter et al., 1972; Pomerantz and Reiter, 1973). These results could not be related to any changes in serum Na^{+}, Mg^{++}, Ca^{++} or K^{+}.

Modifications in various aspects of brain function have been attributed to pinealectomy. Pineal gland removal has been reported to delay rat brain maturation by inhibiting myelin formation (Relkin et al., 1973), to modify brain electrical activity in the rat (Nir et al.,

1969), and to reduce the cataleptic effects of ketamine administration (Winters et al., 1973).

Findings contradictory to those reported above have also been described. Wragg (1967) found no effect of pinealectomy on any aspect of reproductive function in the newborn female rat while Herbert (1969) reported no effect of pinealectomy on the ovary or estrus cycle of the ferret. Following pinealectomy, no alteration in the metabolism of 3,4-benzpyrene or aminopyrene, or the concentration of cytochrome P-450, was found by Heikkinen et al. (1973).

Superior cervical ganglionectomy

The bilateral removal of the superior cervical ganglia, the source of pineal sympathetic innervation in mammals, has been shown to result in both predictable and unexpected effects. Cervical sympathectomy was found to abolish the effects of constant light on the estrus cycle in ferrets (Abrams et al., 1954) and on the gonads in rats (Wurtman et al., 1964b). Bilateral superior cervical ganglionectomy has been associated with a decrease in pineal weight and HIOMT activity (Nagle et al., 1973; Wurtman et al., 1964b) as well as a nearly total loss of the N-acetyltransferase rhythm (Klein et al., 1971). The administration of NE has been found to significantly increase both HIOMT activity (Nagle et al., 1973) and N-acetyltransferase activity (Klein et al., 1971) in ganglionectomized rat pineal glands. The phenomenon of

supersensitivity of the pineal beta-adrenergic receptor to catecholamines and, subsequently, the superinduction of pineal N-acetyltransferase activity following ganglionectomy has been studied in detail by Deguchi and Axelrod (1972a; 1972b; 1973).

Fendler and co-workers have examined the relationship between superior cervical ganglionectomy and pituitary and pituitary-adrenal function. Ganglionectomy has been shown to reduce compensatory adrenal hypersecretion in unilateral adrenalectomized rats (Fendler and Endroczi, 1965), indicating an influence on the vasomotor control of the hypothalamo-hypophyseal portal system. Ganglionectomy has been shown to increase the oxytocic activity in the posterior pituitary of stressed rats but to decrease that activity in non-stressed rats (Fendler et al., 1965). Cervical sympathectomy prevented the development of a "miniature neurohypophysis" which normally occurs following pituitary stalk transection (Fendler et al., 1970) but did not change water uptake or diuresis in similarly treated rats (Fendler et al., 1972). Ganglionectomy was also found to have no effect on the activity of hydroxymethyl-glutaryl-coenzyme A reductase in rat liver (Huber et al., 1973) or on blood flow to the anterior and posterior pituitary in the rat (Goldman, 1968).

Stress

The effects of non-specific stress on living

organisms were first observed by Selye (1936) and have subsequently been the subject of intense investigations. The physiological, biochemical and behavioral effects of environmental stressors have recently been reviewed (Buckley, 1972). Stress apparently contributes to the development of disease of the central nervous system, cardiovascular and gastro-intestinal systems as well as other chronic diseases (Buckley, 1972).

Non-sexual stressful stimuli have been shown to inhibit ovulation (Brown-Grant et al., 1973) and delay the onset of ovulation and mating (Euker and Reigle, 1973) in rats. Exposure of pregnant rats to intensive sound, light and electrical stimulation has resulted in an increase in the incidence of developmental disorders in the newborns (Buckley, 1972).

A variety of stressful conditions cause an increased systolic blood pressure (Buckley, 1972) and excretion of catecholamines, indicating an increased adrenal medullary activity in rodents (von Euler, 1971) and man (Slob et al., 1973). An increase in circulating levels of catecholamines results in the increased mobilization of free fatty acids observed in stressful situations (von Euler, 1972).

Patients with a previous history of myocardial infarction show an increased serum cholesterol level during stressful life experiences, a condition associated with an increased incidence of atherosclerosis (Rothfeld et al., 1973).

Stressful stimuli have also been reported to affect the release of hormones by the hypothalamo-hypophyseal system. Stress has been found to increase prolactin secretion (Ajika et al., 1973; Buckley, 1972) as well as the secretion of radioimmunoassayable growth hormone (Collu et al., 1973), and TSH (Langer and Lichardus, 1969). Stress-induced increases in ACTH release are abolished by transection of the medial forebrain bundle (Feldman et al., 1971; 1972).

The relationship between stress and the brain has been studied. Experimental stress in rabbits has resulted in an increase in the nuclear size of cells in the hypothalamic locus ceruleus (Bubenik and Monnier, 1972), indicating its possible role in the maintenance of alertness. Acute and chronic stresses in mice and rats have been shown to increase the brain concentration of 5-HIAA (Bliss et al., 1972). An increase in the turnover of brain NE has been reported in rats following a variety of stressful treatments (Stone, 1970; 1971; 1973).

The pineal gland has been reported to show both histologic and enzymatic changes following exposure to various environmental stressors. Prolonged exposure to olfactory stimuli results in the atrophy of pinealocytes and a decrease in nuclear and nucleolar volumes (Miline et al., 1963). The following modifications in the pineal gland cells were observed in rats following 24 hours of immobilization stress (Miline et al., 1968): hypertrophy

of nuclei, nucleoli, mitochondria and the golgi zone; an increase in alkaline and acid phosphatases and non-specific esterase activities; degranulation of the endoplasmic reticulum and sympathetic synaptic vesicles. Hyperplasia of pinealocytes, an increase in the endoplasmic reticulum and the number of lamellar bodies, and mitochondrial polymorphism, are conditions which have been shown to result from chronic immobilization stress (Miline et al., 1968). Miline (1971) has also reported changes in the rat pineal gland following cold exposure for 2 weeks. Increases in the activities of succinic dehydrogenase, monoamine oxidase, alkaline and acid phosphatases, and ATPase were seen. Dilation of the golgi zone and the appearance of secretory granules and synaptic ribbons were also observed.

The bat shows an involutive histophysiological reaction to auditory stress (Miline et al., 1969). Lynch et al. (1973a) have recently reported an increase in pineal N-acetyltransferase activity following insulin-induced hypoglycemic stress. Increases in pineal melatonin content have been reported following insulin injection and immobilization stress (Lynch et al., 1973a).

Immobilization, cold and a combination of cold and immobilization stresses have been shown to have marked effects on a variety of physiological activities.

Immobilization stress characteristically results in adrenal hypertrophy, thymic involution and testicular

atrophy (Pfeiffer, 1967; Selye, 1936; 1956). Chronic immobilization has also been reported to result in decreased thyroid weight, decreases in spermatogenesis, urinary gonadotrophin levels, and secretory structures in the pituitary (Mateef et al., 1972). That immobilization causes peripheral sympathetic nerve stimulation is evidenced by increases in both serum dopamine-beta-hydroxylase levels and arterial blood pressure (Lamprecht et al., 1973). Significant increases in serotonin levels in the rat spleen have been demonstrated (Scheving et al., 1972) as have serotonin levels in the brain (Curzon et al.,1972). Increases in brain tryptophan and 5-HIAA concentrations following a 3 hr period of restraint have been demonstrated (Curzon et al., 1972; Knott et al., 1973). Increased concentrations of NE and dopamine have been reported following restraint (Corrodi et al., 1968; Welch and Welch, 1968a; 1968b) and have been attributed, in part, to a decrease in brain monoamine oxidase activity (Welch and Welch, 1968b).

Studies employing immobilization stress have concentrated on its effects on adrenal and peripheral sympathetic nervous activities and its role in the production of gastric erosions.

Adrenal hypertrophy is an almost invariable result of stress (Woods, 1957b). Immobilization results in an increase in adrenal medullary and cortical weights (Kvetnansky and Mikulaj, 1970) and nuclear volume and

optical permeability of the cells of the adrenal zona fasiculata (Tsacheva and Kiliovska, 1972). Increases in adrenal function have been associated with increased ACTH secretion in stress (Buckley, 1972), ACTH release is stimulated by corticotrophin releasing factor (CRF) which is believed to be under the control of serotoninergic neurons in the hypophysiotropic region of the hypothalamus (Popova et al., 1972). Immobilization stress has been shown to increase ACTH release (Buckley, 1972; Ducommun et al., 1966; Reklewska et al., 1972; Sandman et al., 1973). Adrenal cyclic AMP levels have been found to increase in response to both immobilization stress and ACTH administration (Paul et al., 1971). ACTH administration has also been found to deplete the ascorbate content of rat adrenal cortex (Chayen et al., 1971), a response believed to be linked to corticosterone secretion (Motta et al., 1971). Plasma corticosterone concentrations are found to increase following restraint (Buckley, 1972; Dallman and Jones, 1973; Dunn et al., 1972b; Perhack and Barry, 1970; Riegle, 1973). Increases are found to vary according to the phase of the corticosterone rhythm at the time the immobilization is performed (Ader and Friedman, 1968). Chronic immobilization has been found to reduce the adrenocortical release of corticosteroids in a subsequent acute restraint stress (Riegle, 1973). This decrease in stress responsiveness was greater in young as compared to old rats (Riegle, 1973). Injections of

corticosterone or ACTH were also found to inhibit subsequent stress responses (Dallman and Jones, 1973).

ACTH has been found to participate in the regulation of enzymes in adrenal medullary synthesis of catecholamines. Tyrosine hydroxylase (TH), dopamine beta-hydroxylase and phenylethanolamine methyltransferase (PNMT) activities have been reported to show large increases following repeated immobilization (Ciaranello et al., 1972; Paul et al., 1971; Silberstein et al., 1972). Hypophysectomy or adrenal denervation blocks the increase in these enzymes following immobilization (Paul et al., 1971) as will inhibitors of protein synthesis (Ciaranello et al., 1972).

While an increase in total adrenal catecholamines was found after repeated immobilization (Kvetnansky and Mikulaj, 1970), after a single short term (90 min) period of immobilization both adrenal epinephrine and NE were found to be decreased. Urinary epinephrine and NE were found to be increased after 1 hr of restraint and epinephrine excretion levels remained elevated for as long as restraint was maintained (Graham, 1966; Kvetnansky and Mikulaj, 1970; Tomaszewska et al., 1971). The excretion of catecholamines has been suggested to be partially under the control of adrenocortical glucocorticoids (Parvez and Parvez, 1972).

Immobilization stress has been closely associated with gastric ulceration since the first observation of that effect by Selye (1936). Erosions of the gastric

mucosa resulting from varying periods of stress are characteristically found in the glandular portion of the stomach. While gastric erosions in rats have been reported following 4 hr (Guth and Mendick, 1964) and 6 hr of restraint (Ader, 1964), longer periods (24 hr) have generally been employed (Brodie and Hanson, 1960; Caul and Buchanan, 1971; Lovibond, 1969; Mikhail and Holland, 1966a; 1966b; Senay and Levine, 1967; Weinstein and Driscoll, 1972). Immobilization has been most effective in producing gastric ulceration when begun during the active phase of the rat activity cycle (Ader, 1964), and when preceded by a period of starvation (Caul and Buchanan, 1971; Guth and Mendick, 1964; Mikhail and Holland, 1966a; 1966b; Weinstein and Driscoll, 1972). In a comparison between laboratory and wild rats, it was found that laboratory rats were significantly more susceptible to ulceration due to restraint (Weinstein and Driscoll, 1972). The average weight of the rat used was found to have no effect on the incidence of ulceration due to immobilization (Brodie and Hanson, 1960). In the same study it was shown that bilateral adrenalectomy markedly increases both susceptibility to restraint ulceration and mortality rates during the restraint period.

Physical immobilization was found to produce greater ulceration in diestrus female rats than in males or in estrus females (Herner and Caul, 1972). Pregnancy was found to aggravate restraint ulceration in the rat when

restraint was performed during the last 7 days (Luther et al., 1969).

The response of organisms to cold stress differs somewhat from that obtained with other types of stressors (Buckley, 1972). Leduc (1961) showed marked increases in the urinary excretion of catecholamines during the exposure of rats to cold (3°C), indicating increased activity of the sympatho-adrenal system. Von Euler (1971) has suggested that during exposure to cold, the main response of the organism is a greatly increased secretion of catecholamines. Confirmatory studies have been reported using rats in environmental temperatures as low as -20°C (Chin et al., 1973), golden hamsters (Feist, 1972) and humans (Lamke et al., 1972). Plasma corticosteroid levels have also been found to be elevated during cold stress (Chin et al., 1973). Circulating glucocorticoids are apparently necessary for the catecholamines to exert their calorigenic and free fatty acid mobilizing action (von Euler, 1972).

Cold exposure has been found to affect catecholamine turnover in various organs. In initial cold exposure, significant but variable, increases in ^{3}H-NE turnover were found in rat heart, skeletal muscle, lung, kidney and spleen (Bralet et al., 1972). A decrease in brain NE was found in shaved rats exposed to cold (Maynert and Klingman, 1962). Cold adaptation has been reported to sensitize animals to the metabolic effects of NE. Since isoproterenol duplicates this sensitizing effect, it has

been suggested that beta-receptors primarily are the metabolic receptors (Le Blanc et al., 1972).

The release of NE in the central nervous system has been implicated in the de-activation of heat producing systems while peripherally the reverse is true (Breese et al., 1972). Serotonin release in the anterior hypothalamus is thought to activate heat producing systems.

Cold exposure has been found to result in adrenal hypertrophy in rats (Woods, 1957b). Decreases in adrenal ascorbate are found following initial cold exposure but control levels are regained following cold exposure lasting longer than 24 hr (Woods, 1957a; 1957b). Adrenal epinephrine has been shown to decrease in 24 hr of cold exposure in rats but to increase in concentration which persists for as long as the animal is exposed to cold (Leduc, 1961). Variations in adrenal catecholamine levels during cold exposure have been associated with corresponding variations in the activity of adrenal catecholamine synthetic enzymes, TH and PNMT (Gordon et al., 1966; Roffi and Maziere, 1971; Silberstein et al., 1972). The stimulatory effects of ACTH, glucocorticoids and extracellular potassium have been associated with increases in TH and PNMT activities (Silberstein et al., 1972; Wurtman and Axelrod, 1963). An increase in adrenal medullary cyclic AMP during cold exposure has been associated with initiating the chain of biochemical events modulating the synthesis of TH (Guidotti et al., 1973).

Increases in ACTH release have been found to follow cold exposure (Buckley, 1972; Chowers et al., 1972; Dupont et al., 1972; Jobin and Fortier, 1965). The increased release of ACTH has been associated with a stimulation of cutaneous cold receptors and impulse transmission through the lateral spinothalamic tract, reticular formation and posterior hypothalamus, resulting in a stimulation of CRF release (Chowers et al., 1972).

The sympatho-adrenal system has been found to be absolutely essential for the mammalian organism to maintain thermal homeostasis when exposed to low temperatures (Buckley, 1972). Intact rats exposed to low environmental temperatures maintain body temperature by piloerection, vasoconstriction, shivering and mobilization of glucose and free fatty acids (Maikel et al., 1967b). Adrenalectomized (Maikel et al., 1967a), immunosympathectomized (Tarpy and Van-Toller, 1972), and alpha-methyl tyrosine treated (Legrand and Weil-Fugazza, 1972) animals are unable to withstand cold exposure. However, adrenalectomized rats maintained on 1% saline or treated with corticosteroids have been found to survive by utilizing all usual heat conserving and heat generating mechanisms (Maikel et al., 1967b). These authors have suggested that the corticoids are required to maintain a proper electrolyte balance in cells so that they are responsive to the action of the catecholamines which are directly responsible for causing heat production and conservation.

A variety of other physiological modifications have been observed in organisms exposed to cold. Thyroidal activity has been found to be increased in cold exposure. Thyrotropic hormone releasing factor release is stimulated by cold exposure (Lichtensteiger, 1970) following an activation of tubero-infundibular neurons and the increased release of dopamine in the median eminence. Cold exposure has been found to cause increased plasma TSH values in rats (Dupont et al., 1972; Jobin and Fortier, 1965; Langer and Lichardus, 1969) and in humans (Woolf et al., 1972). Increased thyroidal function, as indicated by increased intracellular colloid droplet formation (Kajihara et al., 1972; Kotani et al., 1973), and increased thyroid hormone secretion (Héroux and Brauer, 1965; Melander et al., 1972), has been reported in many mammalian species. A direct action of sympathetic stimulation on alpha adrenergic receptors in the thyroid follicle cells has been found to induce thyroid hormone secretion in the cold (Kotani et al., 1973; Melander et al., 1972).

Cold exposure has been found to induce an increased release of pituitary growth hormone in rats (Ankermann et al., 1972; Yamamoto and Ieiri, 1972), and in man (Woolf et al., 1972) and pituitary prolactin in rats (Yamamoto and Ieiri, 1972). Serum free fatty acid levels in rats (Pirioux-Guyonneau and Buchel, 1972; Yamamoto and Ieiri, 1972) and dogs (Paul and Holmes, 1973), and oxygen uptake in man (Lamke et al., 1972) are seen to increase in cold

exposure. Cold-induced increases in diuresis have also been observed (Berberich et al., 1971; Lamke et al., 1972).

Modifications in carbohydrate metabolism have been reported in rats during cold exposure. Decreases in serum glucose and serum insulin (Beck et al., 1967) and glucose tolerance (Joseph and Brahmacheri, 1972) have been shown. Cold has been found to decrease brain synthesis and utilization of NE (Stone, 1970) and to increase the permeability of the blood-brain barrier below a critical body temperature (24°C) (Wells, 1973) in rats.

Cold stress was found to cause an increase in the effectiveness of rat liver microsomal drug metabolizing enzymes (Fuller et al., 1972; Inscoe et al., 1965).

Reiter (1973b) has suggested that a decrease in environmental temperature may potentiate the brain's sensitivity to pineal gland control.

The synergistic effects of cold and restraint stress in producing gastric ulceration were first demonstrated in the rat by Senay and Levine (1967). While simple physical restraint has been found to require 12 to 24 hr in order to produce gastric lesions, immobilization of fasted animals at 4°C required only 2 hr to produce lesions in 67% of the stressed animals (Senay and Levine, 1967). Djahanguiri et al. (1973) have shown that combined immobilization and cold (4°C) produced ulcerations in 96.8% of the animals stressed for 2 hr. In the same study,

it was found that immobilization alone resulted in acute gastric mucosal ulcerations in 83% of rats restrained for 10 hr.

The comparative effects of cold and restraint on different rat strains have been studied (Goldenberg, 1973). The incidence of gastric lesions was greatest in the Sprague-Dawley rat following stress. The Wistar rat showed the most resistence to lesion formation. A recently developed strain of Wistar rats that develop hypertension spontaneously several months after birth (spontaneously hypertensive) was apparently more affected by stress than the Wistar strain. The spontaneously hypertensive (SH) rats produce more adrenal catecholamines in stress than the Wistar. This is indicated by the finding that TH and dopamine-beta-hydroxylase activities in the SH rat were double those in normal Wistar rats (Goldenberg, 1973).

Mice immobilized at 2^{o}C for 9 hr were shown to have increased hepatic microsomal protein content, increased activity of the drug metabolising enzymes, p-nitrobenzoic acid reductase, aminopyrine N-demethylase, and aniline p-hydroxylase (Shysh and Norijaim, 1972).

Gastric ulceration

Experimental stress ulcers are acute, multiple, superficial fundic gastric lesions which bleed but do not perforate, and occur in a linear fashion along the crests

of gastric rugae (Goodman and Osborne, 1973). Several factors have been implicated as essential in the genesis of stress ulcers. Hydrochloric acid and pepsin concentrations were found to be elevated in the fundic region of the stomach of patients with gastric ulcers (Stadelmann et al., 1972). Gastric pepsin secretion has been shown to be stimulated by increased adrenal corticosteroid secretion (Nagamachi, 1973). Pentagastrin and histamine have been shown to increase gastric mucosal cyclic AMP which stimulates protein kinase activation, leading to an increase in carbonic anhydrase activity resulting in an increased acid secretion into the gastric lumen (Narumi and Maki, 1973). A decrease in the pH of arterial blood was induced in rats by the administration of 1.6% ammonium chloride in the place of drinking water and an increase in the number of gastric lesions produced by restraint stress was observed (Mullane et al., 1973b). The presence of acid in contact with the gastric mucosa has been considered as necessary for the development of stress induced mucosal defects (Safaie-Shirazi and Zike, 1973). However, all acute mucosal lesions are not unequivocally associated with acid back-diffusion (Gerety and Guth, 1972; Guth, 1971). Mikhail (1969) has suggested that forms of stress which involve an anxiety component may in fact cause a decrease in gastric acid secretion. Goodman and Osborne (1973) have found no diminution in ulceration induced by shock stress in piglets previously surgically treated to reduce

gastric acid secretion. Nevertheless, gastric acid back-diffusion is considered to be an agressive factor in the production of gastric ulceration due to environmental stress (Fenster, 1973).

Glucocorticoids have been causally related to gastric ulceration. The subcutaneous injection of cortisol has been shown to induce gastric ulcers in rats which are identical to those caused by stressful procedures (Loeb and Sternschein, 1973; Robert and Nezamis, 1958). Plasma corticosterone levels were found to be elevated in rats subjected to stressful stimuli (Ader and Friedman, 1968). The concentration of cortisol administered to rats was found to be proportionally related to peptic ulceration severity (Kilmore et al., 1972). Endogenous corticosteroids appear to be necessary for normal acid production, as patients with Addison's disease are usually hypochlorhydric. Steroid therapy is a common post-surgery treatment of neurosurgical patients but has been found to aggravate gastric ulcerations (Valergakis et al., 1972). Steroid administration has been associated with a decrease in gastric mucus production and a decrease in mucosal cell renewal rates (Fenster, 1973), both of which have been associated with the genesis of gastric ulceration.

The role of catecholamines in the production of gastric ulcers has been studied. The NE turnover rate, which reflects the level of sympathetic activity, was found to be significantly increased in the stomach of rats

subjected to restraint (Djahanguiri et al., 1973). Norepinephrine has been associated with inducing the release of ACTH which results in the release of adrenal corticosteroids and increases in gastric pepsin secretion (Nagamachi, 1973). Norepinephrine has also been found to decrease gastric vascular flow and cell renewal rates (Djahanguiri et al., 1973).

Various other factors have been related to experimental gastric ulceration. Injection of varying concentrations of sodium salicylate was found to be proportional to the development of gastric ulcers (Kilmore et al., 1972). The interaction of aspirin and acid in the production of gastrointestinal erosions and hemorrhage has been studied by Cooke (1973). An excess of gastric acid in combination with aspirin resulted in erosions and overt blood loss. Aspirin has been found to reduce gastric mucosal blood flow and to increase acid back-diffusion as has sodium taurocholate (bile salt) perfusion (O'Brien and Silen, 1973). Methylxanthines (caffeine, theophylline) are stimulators of gastric secretion in man (Bieck et al., 1973). They also potentiate the effects of histamine on HCl and pepsinogen secretion.

Daily intravenous injections of lysine vasopressin in rabbits was found to produce gastric ulceration in all animals studied (Laumonier et al., 1972). Ischemia was suggested as a possible result of vasopressin administration in causing hemorrhagic lesions. Hypoxia has been

considered as contributory in the production of gastric ulcers with stress (Mullane et al., 1973a). Intravenous injection of E. coli endotoxin caused ischemia in the corpus of piglet stomachs and also massive ulcers in the same area (Richardson et al., 1973).

Food restriction has been associated with the production of gastric ulceration (Barboriak and Knoblock, 1972; Barboriak et al., 1971; Pare and Temple, 1973).

Variations in gastric acid secretion have been related to stages of the estrus cycle in rats (Omole, 1972). Diestrus females showed greater gastric acid secretion in response to exogenous gastrin than females in pro-estrus or estrus. Males and ovariectomized females showed higher acid secretion than unoperated females at any stage of estrus.

Recently, the administration of salmon calcitonin has been found to effectively prevent restraint-induced gastric ulceration through an unknown mechanism (Noda et al., 1973). Its effect as an anti-gastrin agent similar to that of 2-phenyl-2-(2-pyridyl)-thioacetamide (Cook and Bianchi, 1967) was suggested. Vagal stimuli have been found to increase gastric juice secretion (Dragstedt, 1969). Vagotomy has been shown to protect against restraint-induced ulceration (Ritchie et al., 1972) and is a recommended treatment for excessive ulceration and bleeding (Byrne and Guardione, 1973).

In assessing the role of each of the above factors

in the production of gastric ulcers, several authors have proposed mechanisms of stress-induced ulceration. Hypersecretion is not always found in gastric ulceration. Other humoral abnormalities, such as excess adrenal corticosteroid secretion might be implicated, but not on the basis of causing excess acid-pepsin secretion (Bynum et al., 1972). These steroids have antiphogistic properties and could also conceivably lyse fibrous tissue in healed ulcers. Normally, the epithelial lining of the stomach presents an impermeable barrier to the passage of acid from the lumen into the tissue. A unified concept of stress-induced ulceration has been presented by Guth and Hall (1966) and modified by Guth (1972) and Bynum et al. (1972). It has been proposed that stress, following an early protective response phase in which there is an increased corpus mucosal blood flow and a decrease in HCl secretion, causes certain pathogenic changes in the gastric mucosa. Gastric mucosal blood flow is decreased under the influence of hypothalamic stimulation (Gilsdorf et al., 1973). Gastric mucosal mast cell degranulation occurs with the release of histamine. Histamine causes local microvascular adjustments leading to venular stasis and superficial mucosal vascular engorgement. Decreased mucosal resistance to acid-pepsin digestion results. Glucocorticoids decrease mucus secretion (Menguy, 1969) and a decrease in epithelial cell renewal rate is seen. This leads to acute gastric mucosal lesions which increases

the back-diffusion of acid which stimulates further pepsin and mast cell histamine release. This leads to further vascular stasis and acid release. The result is an increasing gastric erosion which eventually leads to frank bleeding. A late effect of stress has been found to be an increased cardiac output and an increased gastric blood flow, in an attempt to reverse some damage.

The etiological factors in gastric ulceration have been discussed by Cowley and Baron (1972) and Fenster (1973). Heredity, physicochemical trauma, smoking, drugs, nutrition, acid, pepsin and blood flow have been associated as aggressive factors while defensive factors include the normal state of the mucosal barrier and adequate blood flow.

The clinical implications of stress-induced gastric ulceration have been widely discussed (Roth, 1971). Ulceration during recovery from operation or major illness is frequently lethal, with an incidence and mortality rate proportional to the degree of stress during convalescence (Douglas and Le Veen, 1970). Klein et al. (1973) have found acute mucosal erosions to be contributory in the mortality of patients with malignant diseases. In a recent series of studies of 701 patients with benign gastric ulceration, it was found that surgical treatment, including vagotomy, antrectomy and gastric resection and pyloroplasty was favored over non-surgical medical treatment (Christiansen et al., 1973; Fenger et al., 1973;

Nielsen et al., 1973). Goodman and Osborne (1973) found these procedures to be without effect in preventing stress ulcer lesions induced by blood loss and reinfusion.

The fact that melatonin counteracts the effect on ACTH secretion exerted by histamine, a potent stressor, has suggested a possible role of the pineal gland in modifying the ulcerogenic action of the pituitary-adrenal axis under stressful conditions (Motta et al., 1971).

Ulcer assay procedures

The quantitative assessment of gastric ulceration can be accomplished reliably by calculating the number of lesions, disregarding size and shape, per animal (Mikhail and Holland, 1966b). Additional quantitative description of ulceration is provided by procedures which account for size, shape, depth and number of lesions per stomach. Techniques which account for size have been used by Mikhail and Holland (1966b), Guth (1972), and Desiderato et al. (1974). An assay in which each variable listed above is included was developed by Kilmore et al.,(1972).

Corticosteroid assay procedures

There appears to be complete agreement that corticosteroid secretion, as a result of pituitary-adrenal axis stimulation, is an effective indicator of the severity of environmental stress (Buckley, 1972). The bioassay for ACTH is a sensitive technique but is technically complex

and time consuming, with accurate results obtained only if large numbers of animals and large volumes of blood are used (Landon and Greenwood, 1968). Its use has been restricted to a few specialized centers (Ratcliffe, 1972). A radioimmunoassay for plasma ACTH has been developed which can detect circulating ACTH concentrations of 10pg or more per ml (Landon and Greenwood, 1968). The fragmentation of the ACTH molecule, leading to a separation of radioimmunoassayable activity from biological activity, has lead to the criticism of the radioimmunoassay (Besser, 1972; Chard, 1972; Fehm et al., 1972; Landon and Greenwood, 1968; Ratcliffe, 1972). The assay of adrenal ascorbate concentration has been used widely as a measure of ACTH activity and has been adapted to organ culture studies (Chayen et al., 1971).

The determination of urinary and plasma levels of corticosteroids may be accomplished by several chemical assays utilizing spectrophotometry and spectrophotofluorometry. Following hydrolysis with beta-glucuronidase, urinary corticosteroids can be measured spectrophotometrically (Glenn and Nelson, 1953). Spectrophotofluorometric assays of plasma corticosteroids have been described (Frankel et al., 1967; Guillemin et al., 1958; Silber et al., 1958). Urinary corticoids have been assayed by spectrophotofluorometric techniques (Appleby et al., 1955; Mattingly et al., 1964).and by gas-liquid chromatography (Bailey et al., 1973). Recently, competitive-protein binding assays for plasma, urinary (Ficher et al., 1973)

and tissue corticosteroids (Fazekas et al., 1972) have been developed. A double isotope derivative assay of urinary aldosterone has been developed (Kliman and Peterson, 1960).

Melatonin assay procedures

The accurate estimation of melatonin in biological materials is hampered by its presence in extremely low concentrations in blood, urine and the pineal gland. Chemical, chromatographic, spectrophotofluorometric and bioassay techniques have been developed with varying degrees of specificity and sensitivity. A complete review of all assay techniques has recently been presented by Cole and Crank (1972). Melatonin may be visualized, following paper or thin layer chromatography by a color reaction with Ehrlich's reagent. The paper chromatographic properties of pineal constituents have been reported by Prop and Ariens Kappers (1961). The paper and thin-layer chromatographic properties of melatonin have been described (Cole and Crank, 1972; Pelham et al., 1971; Rosengarten et al., 1972; Smith, 1969). The sensitivity of melatonin detection on paper chromatograms is approximately 0.5ug (Cole and Crank, 1972).

Spectrophotofluorometric assays of melatonin following chloroform extraction (Axelrod and Weissbach, 1961), p-cymene extraction (Quay, 1963) or ether extraction (Crank and Cole, 1973) have been reported with

sensitivities as low as 1-0.06ug. Following the reaction of melatonin with o-phthalaldehyde, pineal melatonin levels can be estimated fluorometrically in the range of 10-20ng (Maickel and Miller, 1968) with a recovery of approximately 84% of added melatonin (Miller and Maickel, 1970).

Combined gas-liquid chromatography and spectrophotofluorometry techniques have been reported (Greer and Williams, 1967; Pennington, 1968). A more sensitive procedure requires the conversion of melatonin to heptafluorobutyryl derivative before gas-liquid chromatography and mass spectrometry (Degen et al., 1972).

The mass spectral identification of melatonin, following thin layer chromatography has been reported (Hutzinger et al., 1972; Pelham et al., 1972) to have sensitivity in the microgram range.

Column chromatography of melatonin on Sephadex G-25 has been shown to separate melatonin from other, less potent, melanophore-contracting agents (Benson et al., 1972; van de Veerdonk, 1965) and has been used to isolate melatonin from biological materials and verify a melanophore-contracting agent as actually melatonin (Ralph and Lynch, 1970).

According to Lerner and Case (1958) the ability of melatonin to cause the aggregation of melanin granules around the cell nucleus of amphibian melanophores is its most striking physiological effect and has been used in the development of several bioassay techniques. Such

techniques have been found to be more specific and sensitive than the fluorimetric techniques (Cole and Crank, 1972). The blanching reaction of adult frog skin *in vitro* (Lerner and Wright, 1960; Mori and Lerner, 1960) or *in vivo* (Kastin and Schally, 1966) has been used in several assay procedures. The blanching reaction of dermal and epidermal melanophores of intact amphibian larvae has also served as the basis of bioassay procedures (Quay and Bagnara, 1964; Ralph *et al.*, 1967; Ralph and Lynch, 1970; van de Veerdonk, 1965) of varying degrees of quantitative accuracy. The use of *Xenopus laevis* larvae has been recommended over the use of *Rana pipiens* larvae for reasons of greater sensitivity and ease of handling (Ebels *et al.*, 1972; Lynch *et al.*, 1971).

The binding of melatonin to plasma proteins has been found not to interfere with the sensitivity or specificity of the bioassay procedure (Cardinali *et al.*, 1972b).

The mechanism of melanin granule aggregation can only be inferred from the results of studies on the melanin-dispersion response of melanophores to MSH (Brouwer, 1972; Magun, 1973; Taylor and Bagnara, 1972; van de Veerdonk, and Brouwer, 1973). Melanin dispersion has been shown to follow the interaction of MSH with a membrane receptor which causes the conversion of polyunsaturated fatty acids into prostaglandin PGE_1. PGE_1 causes an increased sodium uptake by the melanophore which stimulates adenylate cyclase and results in the increased

synthesis of intracellular cyclic AMP. Cyclic AMP has been found to activate a protein kinase which causes the conversion of microfilaments from an inactive to an active state. The active microfilaments mediate the dispersion reaction.

Melatonin has been shown to interact with a membrane receptor linked to adenylate cyclase (Abe et al., 1969) and to inhibit the MSH-induced response. It has been suggested that, by an unknown mechanism, a protein kinase activated by the melatonin-receptor interaction causes the inactivation of intracellular microtubules, causing the aggregation response (Magun, 1973).

While the hanging drop tissue culture of melanophores has been applied to the assay of MSH (Lyerla and Novales, 1972), no such procedure has, as yet, been applied to the assay of melatonin.

MATERIALS AND METHODS

Experimental procedure

In all experiments, adult, male, Sprague-Dawley rats (Carworth, New City, New York; Zivic-Miller Laboratories Inc., Allison Park, Pennsylvania), weighing 190-240g, were used. The animals, weighing 150-180g upon receipt, were housed in pairs in galvanized steel metabolic cages. A stainless steel funnel was affixed to the lower grid of each cage and was suspended above a 15ml graduated cylinder. A sheath of parafilm was used to form a connection between the funnel outlet and the cylinder mouth in order to reduce the evaporation of urine collected.

The animals were permitted to acclimate to laboratory conditions for at least one week prior to any operative or experimental procedures. All animals were provided with Purina lab chow and tap water ad lib. and were housed in an air conditioned room maintained at 23°C. Lighting was provided by ceiling mounted cool white fluorescent lamps for 12 hr per day (6AM-6PM).

Cages were cleaned, urine was collected and food and water were replenished at approximately 5PM each day. At this time, each animal was handled gently for a short period of time in order to reduce the potential stressful nature of human contact. No attempt was made to eliminate

ambient noise, neither were the animals exposed to sudden or loud noises.

Cage mate pairing was maintained unchanged for as long as the animals were housed in pairs. When, due to experimental requirement, cage mates were separated and housed individually, urine samples of former cage mates were combined.

Five groups of animals were used in this study. Each group contained 25-30 individuals that were subjected to identical surgical treatment. The five surgical treatment groups included pinealectomized, bilateral adrenalectomized, bilateral superior cervical ganglionectomized, sham-operated, and intact controls.

Within each surgical treatment group, the animals were further divided into 5 groups, of 4-6 animals each. Each of these groups was exposed to a different set of experimental conditions. One group was maintained at 23°C in their home cages (non-stressed controls). Two groups of animals were maintained at 4°±2°C for 24 hr; the individuals of one of these groups were unrestrained (cold stress) while those of the other were immobilized (cold-immobilized). A fourth group was maintained at 23°C for 24 hr while being subjected to immobilization (immobilized). The animals of the fifth group were subjected to immobilization at 4°C for 4 hr per day (4PM-8PM) for 4 consecutive days (chronic stress). Immobilization was accomplished by lightly etherizing the animal and then

placing the animal into a metabolic cage and securing a galvanized wire mesh around the animal. The wire mesh was fastened to the bottom of the cage on either side of the animal with copper wire. A tunnel of wire was thus formed which prevented movement of the animal but did not restrict its breathing. The 4°C environment was provided by a walk-in cold room. The lighting schedule was maintained throughout all experimental procedures.

Each group of animals with the exception of the chronic stress group was fasted for 24 hr prior to the initiation of stress procedures. All animals were permitted water ad lib, at all times. The chronic stress group was fasted for 18 hr prior to the 4 hr stress period and then, following the stress period, was permitted to feed for 2 hr. The fast, stress, feed procedure was then repeated twice. On the fourth day, following the fast and stress periods, the animals were sacrificed.

Animals of all groups, other than chronic stress, were stressed from 3PM one day to 3PM on the next day, at which time they were sacrificed. All animals were sacrificed by ether overdose.

Operative procedures

All operative procedures, with the exception of pinealectomy, were performed following the receipt of the animals. Pinealectomized animals were obtained from Zivic-Miller Laboratories and were operated on 3 days

prior to delivery.

All operations were performed under ether anesthesia. Bilateral adrenalectomy was performed by the dorsal approach. Each animal was shaved on the dorsal side. A single incision was made through the skin along the midline from the level of the last thoracic vertebrae extending caudally for approximately 2.5cm. Two separate incisions through the muscle wall were made on either side of the vertebral column. The muscle incision was held open with a spreader and each adrenal gland was held in its fat pad with a hemostat which occluded the adrenal vascular supply. The gland was then cut away, with surrounding fat, distal to the hemostat. Each muscle incision was closed with suture, as was the skin incision. The body surface was swabbed with a tincture of merthiolate solution and the animal was placed on a bed of soft paper toweling under an incandescent lamp for approximately 30 min. The animal was then placed back in its home cage. When possible, cage mates were operated on on the same day. Adrenalectomized animals were maintained on lab chow and 1% NaCl instead of tap water. The completeness of adrenalectomy was determined by examination at autopsy. Sham adrenalectomy was performed in an identical manner with the exception of adrenal gland removal. All animals survived the operative procedure.

Bilateral superior cervical ganglionectomy was performed by a modification of the procedure of Comsa

(1963). The body was shaved on the ventral surface from the tip of the jaw to the mid sternal area. A midline skin incision was made from the tip of the jaw to the manubrium sternum. The salivary glands and fascia were clamped and held aside. The biventer and subhyoid muscles were teased apart and a spreader was placed between them to hold the subhyoid against the trachea and the biventer to the distal side. The vagus nerve and carotid artery were then held to the side with a closed, curved, blunt-end forceps. Under a magnifying lens, the sympathetic ganglion was found below the carotid artery at its fork. The ganglion was separated from the artery with a bent-end disecting needle and then grasped at its cephalic end with a fine forceps. By a gentle but firm medial pulling action the ganglion was removed. The same procedure was repeated on the opposite side. The skin incision was then sutured and swabbed with tincture of merthiolate. Four animals (the first four operated on) died during the operation, one due to ether overdose and three apparently because of blood loss caused by puncture of the carotid artery. Sham ganglionectomy was performed by the same procedure with the exception of the removal of the ganglia. The effectiveness of the operative procedure was determined immediately by observing ptosis (sinking of the eye) and by inspection at autopsy.

All experimental procedures were begun one week following surgical treatment.

Corticosteroid assay

Urines were collected during the 24 hr period of stress for the acute stress and control groups and during the four 24 hr periods of chronic stress. The volume of each sample was recorded and the urine was transferred to a stoppered, 4 dram, glass bottle. The urine bottles were labelled and then frozen until assay. The assay for 11-hydroxylated corticosteroids in each urine sample was performed on the same day that the urine was chromatographed for melatonin bioassay. Each urine sample was thawed and a 10ml sample was pipetted into a 15ml Corex centrifuge tube and centrifuged for 5 min at 1000xg in a clinical centrifuge (International Equipment Co., Needham Heights, Mass.).

Corticosteroid assays were performed according to the method of Mattingly et al. (1964). All reagents used in this and following procedures were obtained from Fisher Scientific Co., Fair Lawn, N.J., unless otherwise specified. Two ml samples of urine were extracted by shaking by hand with 15ml of methylene chloride in a 20ml glass-stoppered tube. The upper aqueous layer was removed and discarded and replaced with 2.0ml of 1N NaOH. Following shaking for 20 seconds the tubes were allowed to stand at room temperature for 10 min. The upper layer, containing NaOH, was then removed. Ten ml of the methylene chloride extract was then added to a reagent composed of 7 volumes of concentrated H_2SO_4 and 3 volumes of absolute ethanol.

Following shaking for 20 sec, the mixture was permitted to separate into 2 layers. The supernatant methylene chloride layer was removed and discarded and the lower acid layer was transferred to a cuvette for fluorometry.

An Aminco-Bowman Spectrophotoflurometer was used. Excitation wavelength was set at 430nm and the emitted light setting was at 540nm.

A reagent blank of 2ml distilled water and a standard of 2ug cortisol in 2ml distilled water were both carried through the procedure and were used to calibrate the fluorometer. With the photomultiplier set for a reading of 100 with the cortisol standard, the reading for an unknown on the same range represents cortisol equivalents of 11-hydroxycorticosteroids in µg/100ml urine. Added cortisol results in over 97% recovery. The corticoids measured by this procedure are cortisol, 20-hydroxycortisol and corticosterone (Mattingly et al., 1964).

Bioassay of melatonin

A small population of male and female adult Xenopus laevis individuals (Charles W. Fletcher co., Glen Burnie, Md.) was maintained in tap water which had been aged for at least one week prior to use. The water temperature was maintained at 18°C in a controlled environment tank (Instant Ocean culture system M250B; Aquarium Systems Inc., Wycliffe, Ohio) which also constantly circulated, aerated, and filtered the tank water. Fecal material was removed

daily and 2/3 of the tank water was changed monthly.

The animals were fed once a week with fresh chicken liver cut into pieces which could be taken whole by even the smallest individual in the tank. Enough food was provided so that each animal injested two pieces.

Diets consisting of ground beef or ground beef heart mixed with a small amount of cod liver oil had been used but resulted in a great deal of debris left in the tank after feeding and therefore had to be discontinued.

One day prior to mating, a male was removed from the environmental tank, at approximately 12 noon, and was injected with 0.5ml of a solution of human chorionic gonadotrophin (500I.U./ml) in 0.9% sodium chloride (Antuitrin "S"; Parke-Davis, Detroit, Mich.) into the dorsal lymph sac. This animal was then placed in a separate glass tank filled with constantly aerated aged tap water maintained overnight at approximately 23°C.

On the day of mating, the male was again similarly injected. A female was then removed from the community tank and injected with 1.0ml of the Antuitrin "S" solution in the same manner.

To facilitate the handling and injection of the normally slippery animals, each was caught in a fine mesh net and then wrapped in a wet paper towel. A small tear in the towel was made above the dorsal lymph sac and the injection was made through the opening. This procedure allows for firm holding of the animal and restricts

possible movement during the injection procedure.

Both animals were then placed together in a stainless steel cage (24cm x 18cm x 18cm) which has a wide mesh grid on both the bottom and on one side of the cage. The cage was supported approximately 3cm above the bottom of a white polypropylene tub (32cm x 28cm x 13cm) filled with 8 liters of culture medium (aged tap water to which had been added 1ml/l of a solution of 1% disodium versenate (EDTA) and 1% $NaHCO_3$ and 1ml/l of a 2% calcium chloride solution, each prepared in deionized water). This solution is identified as the culture medium and will be referred to as such throughout the body of this text. The culture medium was found to be necessary for the survival of the larvae by eliminating divalent cations which were found to vary between toxic and non-toxic levels in the New York City water supply. The culture medium in the tub was constantly aerated. The cage was covered with a galvanized wire mesh, which did not contact the culture medium, in order to prevent escape of the animals.

The tub containing the mating cage was then placed in a controlled environment chamber (Biotronette Mark III Environmental Chamber, Lab Line Instruments Inc.) maintained at 23°C and under controlled diurnal lighting (lights on at 7 AM and off at 7 PM) under cool white fluorescent bulbs.

Spawning occured during the early morning hours of the following day and the fertilized eggs fell through the

grid into the space below, preventing the adults from eating the eggs. The adults, the mating cage, and any fecal material were then removed. This mating procedure was routinely carried out bi-monthly with an individual being used in a mating not more often than once every four weeks.

The larvae hatched two days following fertilization and generally numbered from five hundred to one thousand individuals. They remained in the polypropylene tub for seven days and were then removed with a fine mesh net and distributed evenly among several other tubs containing 4 liters of the culture medium. Each tub contained approximately 100 larvae and was maintained under the same conditions as the original mating tank.

The larvae were fed every other day, beginning on the fifth day following fertilization, with a 7% suspension of ground nettle leaves (S. B. Penick and Co., New York, N.Y.) in deionized water. This suspension was poured into each tub clouding the water and within two hours it settled to the bottom forming a uniform dark green layer on the lower surface of the tub. The larvae were thus exposed to an environment consisting of a dark lower background, white sides and diurnal fluorescent illumination from above. Tadpoles maintained under these conditions were ready for use in the bioassay for melatonin on the twelfth day following fertilization. At this point they had reached stage 49 in development, according to the normal table prepared

by Nieukoop and Faber (1967), and could be used in the assay procedure at any time during the next 14 days.

The bioassay procedure utilized the melanin aggregation response of dermal melanophores in a tail section of the Xenopus laevis larvae to solutions of various concentrations of melatonin. The tadpoles were responsive to melatonin following a period of light adaptation during which the melanophores became fully expanded, causing a marked darkening of the skin. In the light regimen used, full expansion was achieved by approximately 11:30 AM and was maintained until approximately 5:30 PM.

Tadpoles were removed from the culture tanks with a blunt pipette and were placed in a clean glass Petri dish with a small amount of the tank medium. These animals were immediately examined under a dissecting microscope at 20x magnification. Only those animals whose dermal melanophores in the tail region were fully expanded were used further in the assay procedure. The others were returned to the culture tanks. In this way, five fully light-adapted tadpoles were selected for use.

Within 30 sec following examination the tail sections of all tadpoles were severed from the head portion by holding the head gently in a pair of blunt forceps and swiftly slicing through the body at the base of the tail with a scalpel.

With a pair of fine jewelers forceps grasping only the tip of the tail, each of the tail sections was then

transferred to the same 4 dram, stoppered, glass bottle containing 2ml of the solution to be assayed. The bottle was then placed under a cool white fluorescent lamp on a white background for 9 minutes and 45 seconds.

The tails were then removed, with fine forceps, by the tip of the tail and placed into separate troughs containing a small sample of test solution. Each trough was cut out of hardened white parafin layered in the bottom section of a disposable plastic Petri dish. The 5 troughs used for the assay of a particular test solution were used only once. However, as many as 30 troughs could be cut out of a single dish.

The tadpoles were then examined immediately under 40x magnification using a dissecting microscope. The melanophores found on the dorsal surface of the tail along the midline of the body and no closer than 1mm nor farther than 4mm from the cut surface were examined. A number was assigned to each tail based upon the average stage of contraction of the melanophores examined. This number was subjectively derived from a comparison with the stages of melanophore contraction represented by the Hogben-Slome melanocyte index (Hogben and Slome, 1931). The Hogben-Slome index allows for the assignment of five stage numbers with 1 corresponding to complete contraction and 5 corresponding to complete expansion (see Figure 1). In any one test animal, all of the melanophores examined may not be found to be at the same stage of contraction, though cells

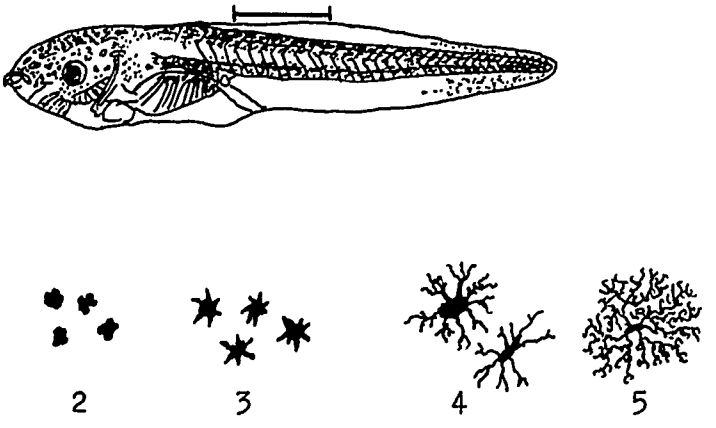

Figure 1. The stage 49 Xenopus laevis larva (re-drawn after Nieukoop and Faber, 1967) and the Hogben-Slome melanophore stage index. The dermal melanophores on dorsal side below the bracket are examined and compared to the melanophore index shown schematically here.

never vary in one animal by more than one stage. In such a case where cells of one stage were not predominant, a number, intermediate to the two stages seen, was assigned (ie. 1.5, 2.5, 3.5, 4.5). It was, therefore, possible to differentiate between 9 separate stage values rather than the 5 of the Hogben-Slome index. This was an attempt to reduce the admittedly high subjectivity of such an assay.

Each solution was assayed at least in duplicate and often in triplicate, providing 10-15 separate tadpole responses to each test solution. The result of each assay was recorded as the mean of the melanophore stages of all of the larvae exposed to the same solution.

The samples of solution to be assayed were coded in a manner which gave no indication of their contents, to eliminate bias in the determination of melanophore stages.

A standard curve was prepared by plotting the log of the concentration of a series of solutions of known concentration of authentic melatonin against the resultant melanocyte index, determined be assay, on linear graph paper. The relationship between the log of the melatonin concentration and melanocyte index was found to be linear between 0.10 and 100 nanograms/ml of sample. The log of the concentrations was determined and plotted on linear graph paper because of the ease of reading of the linear paper in contrast to the less easily read scale of semi-log paper.

The concentration of melatonin in the assayed

solutions was determined by comparison with the standard curve, as both known and unknown samples were prepared for assay in an identical manner.

The solutions of known melatonin concentration were prepared by dissolving 10 mg of authentic melatonin (Sigma Chemical Co., St. Louis, Mo.) in 5 drops of absolute ethanol and then diluting to 10ml with the culture medium resulting in a stock solution of $1x10^{-3}$g/ml melatonin. Appropriate dilutions of this stock solution were made with culture medium. Fresh stock solution was prepared for each standard curve preparation.

All solutions were prepared for bioassay by the same procedure. Urine samples, often containing solid material, were clarified by centrifugation at 1000xg in a clinical centrifuge for 5 minutes.

Solutions of known melatonin concentration as well as clarified urine samples were then subjected to gel filtration to isolate melatonin from other known melanophore concentrating agents. The gel filtration procedure used is a modification of a procedure described for the isolation of a melatonin-containing fraction from an aqueous extract of pineal glands (van de Veerdonk, 1965).

The gel column was prepared by swelling 15gm of Sephadex G-25 Fine (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) in 175ml of culture medium for 3 hours at room temperature with frequent stirring. The gel was poured into a glass column with an inside diameter of

2.8cm resulting in a column height of 21cm. The lower end of the column was fitted with a sintered glass disc filter above which was placed a layer of sterilized glass wool, approximately 1cm in thickness. Following packing and overnight equilibration of the column with culture medium, a disc of Whatman No. 1 filter paper, 2.8cm in diameter, was placed upon the upper gel surface to protect the surface from disturbance by the application of samples.

Two ml of sample were pipetted onto the protected gel surface and allowed to pass into the gel after opening the outlet valve at the lower end of the column. As soon as all of the sample passed into the gel, the outlet was shut and the column was filled with the eluant (culture medium). All air was removed from the system and eluant was permitted to flow through the column at a rate of 1ml/min.

A 40ml fraction of eluate which includes the 111^{th} through 150^{th}ml passing from the column was collected. This elution volume is known to contain authentic melatonin applied to the column (see below) and to be free of biogenic amines and small polypeptides known to be active in causing melanophore contraction (norepinephrine, serotonin, epinephrine, 8-arginine vasotocin) to varying degrees (see below; van de Veerdonk, 1965). This 40ml fraction was mixed well and a 10ml portion was transferred to and stored in a 4 dram, stoppered, glass bottle which was then refrigerated until the solution was bioassayed. The length of

time a solution was refrigerated before assay at no time exceeded 48 hours and routinely was less than 24 hr. No difference in the activity of melatonin solutions either assayed immediately or refrigerated for 24-48 hr could be detected.

As many as 5 separate columns were in use concurrently.

Following the collection of the fraction to be assayed, each column was washed by passing a solution of 0.3M formic acid through the column for a minimum of 1 hr and then reequilibrated with the culture medium until the pH of the eluate has returned to neutrality. The column was then ready for the application of another sample.

Paper and thin layer chromatography of melatonin

The purity of the melatonin used in the preparation of melatonin stock solutions was determined through the use of paper and thin-layer-cellulose chromatographic techniques. A 20ul sample of a solution of $1x10^{-3}$g/ml melatonin, prepared as described above, was pipetted at the origin of either a thin-layer cellulose plate or a sheet of Whatman No. 1 chromatography paper. Ascending paper chromatography was run in five different solvent systems: butanol:acetic acid:water (3:1:1 and 12:3:5); methanol:n-butanol:benzene:water (4:3:2:1); phenol:HCl (4:1); and n-butanol saturated with 1N HCl. Thin layer chromatograms were developed in 5 solvent systems: methanol:n-butanol:

benzene:water (4:3:2:1); n-butanol:acetic acid:water (4:1:5 and 3:1:1); n-butanol saturated with 3N HCl; and chloroform:methanol (9:1). When dry, each chromatogram was examined under a short wave ultraviolet lamp and then sprayed with freshly prepared Ehrlich's reagent. Ehrlich's reagent was prepared by combining 2.5g p-dimethylaminobenzaldehyde with 5ml concentrated HCl and 20ml acetone. The presence of melatonin (as well as other indoles) is indicated by the development of a purple spot. The Rf of the spot on each chromatogram was compared to the Rf reported for authentic melatonin. Samples of urine and urine samples to which authentic melatonin ($0.5x10^{-3}$g/ml final concentration) was added were also chromatographed in the same manner to determine the presence of existing urinary constituents on the migration of known melatonin.

Column chromatography of known melatonin

To determine the elution profile for known melatonin on the Sephadex G-25 Fine column, prepared as described above, and to compare this elution profile with previously reported results under similar, but not identical, conditions, the following methods were used. A 2ml sample of a solution of $1x10^{-3}$g/ml of melatonin was applied to the column and was permitted to run into the gel. Without permitting the surface to dry, the column space above the gel surface was filled with the culture medium described above, which served as the eluant in all column chromato-

graphic procedures. The column was connected to the eluant reservoir and air was removed from the system. The rate of elution was adjusted to 1ml/min. Thirty-five consecutive 5ml fractions were collected in 16x150mm culture tubes (Bellco Biological Glassware, Vineland, N.J.) on a Gilson fraction collector set for volume collection mode. Two drops of each fraction were pipetted and allowed to dry on a sheet of Whatman No. 1 chromatography paper. When dry, the paper was sprayed with freshly prepared Ehrlich's reagent. In this way the fractions containing known melatonin could be identified, the elution volume of known melatonin calculated, and then compared with the elution volume of known melatonin reported in the literature.

Column chromatographic separation of melatonin, norepinephrine, and epinephrine

To determine the ability of Sephadex G-25 Fine to separate melatonin from other substances known to be found in urine which also posess a certain degree of melanophore-containing activity, the following procedures were employed.

A solution containing melatonin, norepinephrine, and epinephrine was prepared in the following manner. Ten mg of melatonin (Sigma Chemical Co., St. Louis, Mo.), 10mg of norepinephrine (Winthrop Laboratories, New York, N.Y.) and 1mg epinephrine (Parke-Davis Co. Detroit, Mich.) were dissolved in 5 drops of absolute ethanol and 4 drops of

glacial acetic acid. Culture medium was added to bring the volume to 10ml thereby giving a solution containing 1mg/ml melatonin, 1mg/ml norepinephrine and 0.1mg/ml epinephrine. Two ml of this solution were applied to the Sephadex G-25 Fine column described above and 97 consecutive 5 ml fractions were collected as described above. Two drops of each fraction were pipetted and allowed to dry on each of 2 pieces of Whatman No. 1 chromatography paper. When dry the papers were sprayed with freshly prepared Ehrlich's reagent and freshly prepared potassium ferricyanide in sodium hydroxide solution (0.6g $K_3Fe(CN)_6$ in 100ml 1.5% NaOH), respectively. Ehrlich's reagent permits the visualization of the catecholamines. While the specific results of this fractionation will be discussed separately below, it is of importance to note here that neither epinephrine nor norepinephrine were detected in any fraction collected although 485ml had been eluted.

Detection of ^{14}C-norepinephrine eluted through a Sephadex G-25 Fine column

A solution was prepared consisting of 0.05ml of a 5μc/ml ^{14}C-norepinephrine (D,L-norepinephrine,carbinol-^{14}C; Amershaw-Searle, Nuclear-Chicago) and 10mg of norepinephrine (Winthrop Laboratories, New York, N.Y.) in 10ml of 0.1N HCl. A 2ml sample of this solution was applied to a Sephadex G-25 Fine column as described above and eluted with culture

medium. Sixty-nine consecutive 5ml fractions were collected.

A 1ml sample of each fraction collected was pipetted into a planchet and dried under a planchet drying lamp. The planchet was then placed in a gas-flow counter (Nuclear-Chicago; Model 181B Scaler) and a 1 minute count for ^{14}C-activity was made. A 0.5ml sample of the solution applied to the gel column was similarly counted for an estimate of the total activity applied to the column. One drop of each fraction collected was spotted on Whatman No. 1 chromatography paper which, when dry, was sprayed with the potassium ferricyanide-sodium hydroxide solution to test for the presence of color reactive catecholamines. One ml of each fraction collected was transferred to a disposable polypropylene tube and a pH reading was made on a Radiometer Copenhagen pH meter 25.

A 2ml sample of a solution containing ^{14}C-norepinephrine (5μc/ml) in a carrier of unlabeled norepinephrine, prepared as described above, was applied to a Sephadex G-25 Fine column equilibrated with a 0.3M formic acid solution. Elution of the sample was with 0.3M formic acid. Thirty-five consecutive 5ml samples were collected.

One drop of each fraction was spotted on chromatography paper, as was one drop of the original sample, and, when dry, the paper was sprayed with the potassium-ferricyanide-sodium hydroxide reagent to reveal those spots containing color reactive catecholamines. A 0.5ml sample

of the solution applied to the column and 1ml samples of each fraction eluted were counted for ^{14}C activity as described above.

Qualitative correlation of color reactive melatonin, isolated by chromatography, with biological activity

Two 20ul samples of a $1x10^{-3}$g/ml melatonin solution, prepared as described above, were spotted on the same sheet of Whatman No. 1 chromatography paper along the origin 15mm above the bottom of the paper and approximately 15cm apart. The applied solutions were allowed to dry and then developed by ascending chromatography in butanol:acetic acid:water (3:1:1). The chromatogram was removed from the solvent after three hours and permitted to dry in air at room temperature. When dry one half of the paper was sprayed with Ehrlich's reagent to determine the Rf of the chromatographed melatonin. The other half of the chromatogram was cut into five horizontal strips, only one of which included the area surrounding the Rf of the melatonin as determined by the sprayed portion. Each strip was then cut into many smaller pieces and placed in one of five test tubes containing 10ml of culture medium. The tubes were refrigerated overnight and the next day. After the solutions had returned to room temperature, a 2ml sample taken from each tube was assayed, as described above, with the tadpole tail bioassay procedure. In this way, the melanophorotropic activity of all regions of the

chromatogram were determined and compared with the regions containing color-reactive melatonin.

In a similar procedure, 2ml of a $1x10^{-3}$g/ml melatonin solution, prepared as described above, were applied to a Sephadex G-25 Fine column equilibrated with culture medium, as described above. Thirty-five consecutive 5ml fractions were collected using a Buchler fractomette 200 automatic fraction collector (Buchler Instrument Co. Fort Lee, N.J.) set for drop mode collection. From each fraction a 1 drop sample was removed and spotted on chromatography paper and, when dry, sprayed with Ehrlich's reagent. Those fractions demonstrating the presence of color-reactive melatonin were pooled and bioassayed for melanophorotropic activity as described above. Several fractions showing the presence of no color-reactive melatonin were also assayed for melanophorotropic activity.

Quantitative determination of the recovery of ^{3}H-melatonin chromatographed on Sephadex G-25 Fine

^{3}H-melatonin (N-acetyl-H^{3}melatonin; lot no. 762-018; specific activity 55.8mc/mM) was obtained from New England Nuclear Corporation (Boston, Mass.). The sample, containing 250uc, was dissolved in 0.25ml of acetonitrile. The entire sample was, upon receipt, diluted to 10ml with deionized water resulting in a solution containing 0.025mc/ml. Of this solution, 0.1ml was then diluted to 10ml resulting in a solution containing 0.25uc/ml and a separate

0.1ml sample was diluted to 100ml resulting in a solution containing 0.025uc/ml.

^{3}H-melatonin was used in several procedures, as will be described below. All samples to be analyzed for ^{3}H-activity were handled in the same way. ^{3}H-activity was measured in a Beckman liquid scintillation counter (Beckman LS-150 Liquid Scintillation System; fixed window: H^{3} below C^{14}). The scintillation cocktail used was a modification of the Beckman DXB formula. It was prepared by combining 100g napthalene (scintillation grade), 7g PPO (2,5-Diphenyloxazole; ICN Chemical and Radioisotope Division, Irvine, California), and 0.3g POPOP (1,4-bis- 2-(5phenyloxazolyl) benzene; Packard Instrument Co., Downer's Grove, Ill.) and then dissolving these in 1,4-dioxane (scintillation grade) brought to a final volume of 1 liter. In all cases, counting vials were filled with 15ml of this cocktail and 1ml of sample. Counting of ^{3}H activity was routinely done overnight with each sample counted for 10 minutes.

In order to determine the elution profile of ^{3}H-melatonin passing through a Sephadex G-25 Fine column and, in so doing, determine the percent recovery of melatonin from such a column, the following procedures were used.

A 2ml sample of 0.25uc/ml ^{3}H-melatonin, in which was dissolved 2mg of unlabeled melatonin (with 2 drops of absolute ethanol) was applied to a Sephadex G-25 Fine column, prepared as described above, and eluted with

culture medium at a rate of 1ml/min. Forty-two consecutive 5ml fractions were collected. A 1 drop sample of each fraction was removed and spotted on chromatography paper for a determination of the presence of color-reactive melatonin. The contents of each successive pair of fractions were combined (ie. 1+2, 3+4, ... 41+42) to conserve scintillation cocktail and a 1ml sample of each combined fraction was added to a counting vial. Each counting vial had previously been filled with a 15ml portion of the scintillation cocktail and counted for background activity. The 21 fraction samples were then counted for ^{3}H-activity along with 3 vials containing a 1ml sample of 0.25uc/ml ^{3}H-melatonin solution, a 1ml sample of 0.025uc/ml ^{3}H-melatonin solution, and a 1ml sample of culture medium, respectively. In addition to duplicate chromatography of 2ml of the labeled and unlabeled melatonin, 1ml samples were applied to the column to determine the effects of sample volume on the elution profile of melatonin. Elution and ^{3}H-activity assays were performed in a manner identical to that described immediately above.

A qualitative comparison was made between the presence of color-reactive melatonin and radioactive melatonin in each fraction collected as described above. A quantitative comparison was also made between the total activity applied to the Sephadex columns and the total activity recovered from the column with special reference to the activity in those fractions showing color-reactive

melatonin.

Column chromatography of urine and the recovery of bioassayable melatonin added to urine following chromatography

To determine the applicability of the column chromatographic procedures described above to the isolation of a melatonin-containing fraction from urine and to determine the degree of recovery of melatonin added to urine from the chromatographic procedures, the following procedures were employed.

A 1.5ml sample of a solution of $1x10^{-7}$g/ml melatonin (prepared from a $1x10^{-3}$g/ml stock solution as described above) was combined with each of 2 separate 1.5ml samples of rodent urine. The 2 rodent urine samples were chosen because of an anticipated marked difference in their native melatonin concentration. One animal had been pinealectomized and 1 animal had been superior cervical ganglionectomized, following the procedures described above. A 2ml sample of each urine-melatonin mixture was applied to a separate Sephadex G-25 Fine column, prepared as described above, and eluted with culture medium. At the same time, on separate columns, a 2ml sample of each urine and a 2ml sample of the $1x10^{-7}$g/ml melatonin solution were individually chromatographed in the same manner.

The eluate from each column corresponding to the elution volume of 111 to 150 ml was collected and refrigerated in stoppered glass bottles. (As a result of the

procedures described above, it was determined that melatonin is eluted from a Sephadex G-25 Fine column, prepared as described above, in the 40ml volume corresponding to the 111th through the 150th ml passing through the column.)

The refrigerated samples were, within 48 hr, brought to room temperature and bioassayed for melanophorotropic activity in the manner described above. In this way, the melanophorotropic activities of urine and a melatonin solution were determined separately and a theoretical value for the combined solution was determined. The theoretical value was then compared to the actual value measured and a percent recovery of melatonin from the chromatographic and bioassay procedures was determined.

Autopsy procedure

Prior to sacrifice, the weight of each animal was recorded. All animals were sacrificed with an ether overdose. All of the animals of a particular surgical and experimental group were sacrificed at the same time.

A ventral incision through the skin and body wall was made along the midline from the penis to the xyphisternum. Two lateral cuts were then made at the ends of this incision forming two flaps which, when laid aside, exposed the viscera. The testes were removed through this opening and placed on a piece of moist paper toweling placed in the bottom of a disposable petri dish and then were covered. All other organs removed from the same

organism were placed in the same petri dish.

The stomach was removed and prepared for ulcer assay according to the method of Mikhail and Holland (1966b). The esophagus was ligated and cut anterior to the ligation. The stomach was freed from nearby viscera and the duodenum was sectioned approximately 1 inch from the pyloric end of the stomach. Through the duodenal end, 7ml of 0.9% NaCl was injected into the stomach to inflate it. Before the removal of the syringe the duodenum was ligated. The inflated stomach was put aside and 15 min later it was opened along the greater curvature. Any residual contents were gently flushed with saline. The stomach was then placed upon a glass slide which was positioned above an upturned microscope lamp and below a magnifying glass. This transillumination made ulcers appear darker and denser than the surrounding tissue. The number and size of erosions were recorded. An ulcer score was calculated for each stomach according to the method of Desiderato et al. (1974).

Following the removal of the stomach, the adrenal glands (if present) were removed, cleaned and placed in the petri dish. The thyroid gland was then removed through a midline incision extending from the sternum to the tip of the jaw. At this time the absence of the superior cervical ganglia in ganglionectomized animals was verified. The thyroid gland was cleaned of adhering muscle tissue and the parathyroid tissue at the superior border and then placed in the petri dish.

Using a pair of scissors, a flap of bone was removed from the cranium by removing the skin from the dorsal surface of the skull and cutting across the bone between the eyes and then extending the cut backwards bilaterally to the base of the skull. The bone flap was gently reflected posteriorly and the pineal gland was exposed by placing a pair of fine forceps at the cerebral-cerebellar border and spreading the cerebral hemispheres laterally. The pineal gland was then removed with forceps, cleaned of any adhering membranes and stored.

The cerebrum was then reflected posteriorly and the pituitary gland was exposed on the floor of the cranial cavity. The pituitary gland was removed with a pair of forceps under a magnifying lens and placed in the petri dish.

All organs were then weighed and the weights recorded. Organ weights for each animal were then converted to mg/100g body weight, with the exception of testicular weights which were converted to g/100g body weight.

Where mean values $\pm$ the standard error of the means are statistically compared for significant differences, the method of analysis used was the Students t test.

RESULTS

Paper and thin-layer chromatography of melatonin

Paper and thin-layer chromatography of melatonin confirmed the authenticity of the compund used in the standardization and validation of the bioassay procedure. In all solvent systems used, the Rf of the melatonin preparation chromatographed was identical to the Rf of authentic melatonin (see Table 1) (Cole and Crank, 1972; Smith, 1969). The presence of urine, epinephrine, or norepinephrine in the solution of melatonin spotted at the origin of the chromatogram had no effect on the Rf of the melatonin. The Rf of melatonin in all cases remained at approximately 0.90. The only exception to this was found in the thin-layer chromatogram run in chloroform:methanol, in which the Rf was found to be 0.50. No other melanophorotropic substances are known to have Rf values, in the solvent systems used, of greater than 0.50 (Prop and Ariëns Kappers, 1961).

Column chromatography of melatonin

A 2 drop sample of each 5ml fraction eluted from the Sephadex column, to which a solution of $1x10^{-3}$g/ml melatonin had been applied, was spotted on chromatography paper. When dry, the paper was sprayed with freshly prepared

Table 1. Paper and thin-layer cellulose chromatography of melatonin.

Solvent systems	Proportions	Rf for melatonin	Published Rf[1]
Paper			
1. n-butanol-acetic acid-water	3:1:1	0.90	0.89
	12:3:5	0.90	0.90
2. methanol-n-butanol-benzene-water	4:3:2:1	0.93	
3. phenol-HCl	4:1	0.92	
4. n-butanol-HCl	saturated	0.96	
Thin-layer			
1. methanol-n-butanol benzene-water	4:3:2:1	0.92	
2. chloroform-methanol	9:1	0.50	0.34
3. n-butanol-acetic acid-water	3:1:1	0.91	
	4:1:5	0.92	0.90
4. n-butanol-HCl	saturated	0.93	

[1]Cole and Crank (1972)

Ehrlich's reagent. The spots containing samples of fractions 22 through 30 rapidly developed a purple color, indicative of the presence of an indole nucleus. As each fraction collected contained 5ml, the elution of melatonin, an indole, through a Sephadex column under the conditions specified, is accomplished in the elution volume containing the 111^{th} through the 150^{th} ml passing from the column. The most intense color reaction was found in the spots sampled from fractions 25 (125 ml) through 28 (140 ml); fractions 22 (110 ml) through 24 (120 ml) and fractions 29 (145 ml) and 30 (150 ml) all showed less color reactive indole. The color intensity of each spot was subjectively compared to the color intensity of a series of similarly treated spots taken from solutions of decreasing melatonin concentration. The color reaction of the more intense spots corresponded to the reaction of spots containing $1x10^{-5}$g/ml of melatonin. The spots with the least intensity corresponded to a solution of $1x10^{-7}$g/ml melatonin. Solutions containing concentrations lower than $1x10^{-7}$g/ml did not show color reactivity.

The elution volume of melatonin under the conditions described above (111-150 ml) coincides with the elution volume reported elsewhere for melatonin under similar conditions (van de Veerdonk, 1965).

The elution volume of melatonin chromatographed in combination with norepinephrine and epinephrine was identical to that of melatonin chromatographed alone.

The recovery of color reactive catecholamines applied to the Sephadex column and eluted in culture medium was not accomplished although 97 consecutive 5ml fractions were collected and sampled.

To examine the possibility that the reason no color reaction was obtained in any fraction was due to the elution of catecholamine in a diffuse, rather than a discrete, band, radioactive norepinephrine was applied to the column. The sensitivity of detection of radioactive catecholamine is greater than the sensitivity of the color reaction.

The beta-activity of each 98 consecutive 5ml fractions was determined and compared to the total beta-activity applied to the column in the form of a 2ml solution of 5uc/ml ^{14}C-norepinephrine. In addition, a 2 drop sample of each fraction was spotted on chromatography paper and sprayed to determine the presence of color-reactive catecholamine. The pH of each fraction was also determined because of the known increased solubility of catecholamines in acid solution.

No fraction contained color-reactive norepinephrine. The total activity of each fraction was determined by adjusting the counts per minute (CPM) measured for each sample for background radiation and then multiplying the adjusted figure by 5 since the volume collected was 5ml and the volume assayed was 1ml.

The pH of fractions 1 through 10 was found to be at

approximately 7.0 (range 7.3-6.9) and these fractions contained only background levels of radioactivity. Fractions numbered 11 through 15 ranged in pH from 2.1 to 3.1 and contained a total of 19,245 CPM or 5% of the total activity applied to the column (398,976 CPM). Each subsequent fraction collected, through fraction 69 (345 ml), had a pH above 6.0 (range 6.3-7.3) and an average activity of 465 CPM/5ml for a total of 25,124 CPM or 6.3% of the total activity applied.

The column was left standing overnight and then the eluant (culture medium) was removed and replaced with a solution of 0.3M formic acid. The pH of fractions 70 through 77 ranged from 6.7 to 5.8. The pH of fractions 78 through 98 ranged from 4.8 to 2.3 with an average of 2.7. The total recovery of activity in fractions 70 through 98 was 188,230 CPM or 47% of the total applied.

The elution of a sample of ^{14}C-norepinephrine entirely with 0.3M formic acid resulted in the complete recovery of all activity applied. A total of 163,916 CPM were applied to the column and 230,265 CPM were recovered in 35 consecutive 5ml fractions, following background and volume adjustments (see Table 2). The recovery was 140% of that applied. One hundred per cent of the activity applied was recovered in fractions 14 through 18, an elution volume of 25ml (the 70th through 90th ml). It is believed that 40% more activity was eluted from the column than was applied in this experiment because residual

Table 2. Recovery of ^{14}C-norepinephrine from a Sephadex G-25 Fine column by elution with 0.3M formic acid.

Fraction	elution volume	CPM[1]	Fraction	elution volume	CPM
1	5	0	20	100	2710
2	10	0	21	105	1640
3	15	0	22	110	990
4	20	0	23	115	765
5	25	0	24	120	565
6	30	0	25	125	440
7	35	0	26	130	370
8	40	0	27	135	320
9	45	0	28	140	275
10	50	380	29	145	255
11	55	1350	30	150	240
12	60	4640	31	155	210
13	65	13900	32	160	195
14	70	33185	33	165	265
15	75	44475	34	170	170
16	80	48200	35	175	100
17	85	43280			
18	90	23095	Total	175	230,265
19	95	8250			

[1]CPM, counts per minute.

activity from the previous run (42% of that applied) probably was brought down with the formic acid in this run.

Qualitative determination of biological activity of melatonin isolated by paper and column chromatography

A sample of melatonin solution was pipetted on chromatography paper and the chromatogram was developed in a butanol:acetic acid:water solvent as described above. The chromatogram was cut into sections and eluted in culture medium. A 2ml sample of each elution was then bioassayed for the presence of melatonin. Each sample showed a very slight degree of background lightening in the tadpole tail assay, probably due to the acetic acid residue in the paper. Only the sample taken from the elution of the section of the chromatogram corresponding to the Rf of melatonin (approximately 0.90) showed marked melanophore-contracting activity.

Similarly, the fractions collected from a Sephadex column corresponding to the elution volume of melatonin were the only fractions collected which showed any melanophore-contracting activity. The elution volume showing melanophore-contracting activity (111-150 ml) was, in addition, the only volume of eluant in which the presence of color-reactive melatonin could be detected.

Quantitative recovery of ^{3}H-melatonin from column chromatography

A 2ml sample of ^{3}H-melatonin applied to a Sephadex column as described above was found to contain 519,820 CPM of beta-activity. The elution profile of this sample of melatonin was found to coincide with the elution of unlabeled color-reactive melatonin. A total of 548,067 CPM were recovered from the column in 210 ml eluted in 42 consecutive 5ml fractions. This figure represents a recovery of 105% of the activity applied. It was found that 92% of the total applied activity (475,767 CPM) was recovered in the elution volume of 111-150 ml, the elution volume showing the presence of color-reactive melatonin (see Table 3). This is also the only elution volume showing melanophorotropic activity in the tadpole bioassay.

In a duplicate chromatography-activity study the elution volume of 111-150 ml showed the recovery of 473,240 CPM of a total of 518,036 CPM applied. This represents a recovery of 91% of the applied activity. The total recovery in an elution of 215 ml was 543,450 CPM which represents a recovery of 105%.

These results are typical of several similar chromatographic runs using 2ml of application sample. Similar results were obtained with 1ml of application sample. A sample containing 25,092 CPM was applied to the column and a total of 24,170 CPM were recovered in a volume of 220ml. All 24,170 CPM were found to be within the elution volume

Table 3. Recovery of ^{3}H-melatonin from a Sephadex G-25 Fine column by elution with culture medium.

Vial no.	Elution volume	CPM[1]	Vial no.	Elution volume	CPM
1	standard[2]	519,820	16	111-120	2090
3	blank	0	17	121-130	80357
5	1-10	0	18	131-140	255750
6	11-20	0	19	141-150	137510
7	21-30	0	20	151-160	47890
8	31-40	0	21	161-170	6010
9	41-50	0	22	171-180	1410
10	51-60	0	23	181-190	1640
11	61-70	300	24	191-200	870
12	71-80	6830	25	201-210	590
13	81-90	4540	TOTAL	210	548,067
14	91-100	1380			
15	101-110	840			

[1]CPM, counts per minute.
[2]A solution containing 0.25μc/ml

111-150 ml. This is a 96% recovery of applied activity.

Column chromatography and recovery of bioassayable melatonin added to urine

A series of dilutions of a melatonin stock solution ($1x10^{-3}$g/ml) were made. The dilutions ranged from 0.1 to 100 nanograms of melatonin/ml. The standard dilutions were chromatographed on Sephadex G-25 Fine as described above and then bioassayed with the tadpole tail melanophore assay described above. The results of these assays are represented in Figure 2 as a typical standard curve demonstrating the linear relationship between the log of the melatonin concentration and melanocyte index. The standard curve is reproducible and is repeated with each assay of unknown samples. In comparing 15 different standard curve preparations it was found that the mean and range of the melanophore responses to each of the four major melatonin concentrations was as follows: 100ng/ml, 2.26 (range 1.8-2.6); 10ng/ml, 3.05 (range 2.8-3.1); 1ng/ml, 3.60 (range 3.4-3.8); 0.1ng/ml, 4.35 (range 4.1-4.7).

Melatonin was added to urine samples of differing melanophorotropic activities. Two samples of a rodent urine were chromatographed as described above. At the same time, on separate columns, 2 samples of the same urines to which a solution of melatonin ($1x10^{-7}$g/ml) had been added, were similarly chromatographed. In addition, a sample of the $1x10^{-7}$g/ml melatonin solution was also similarly

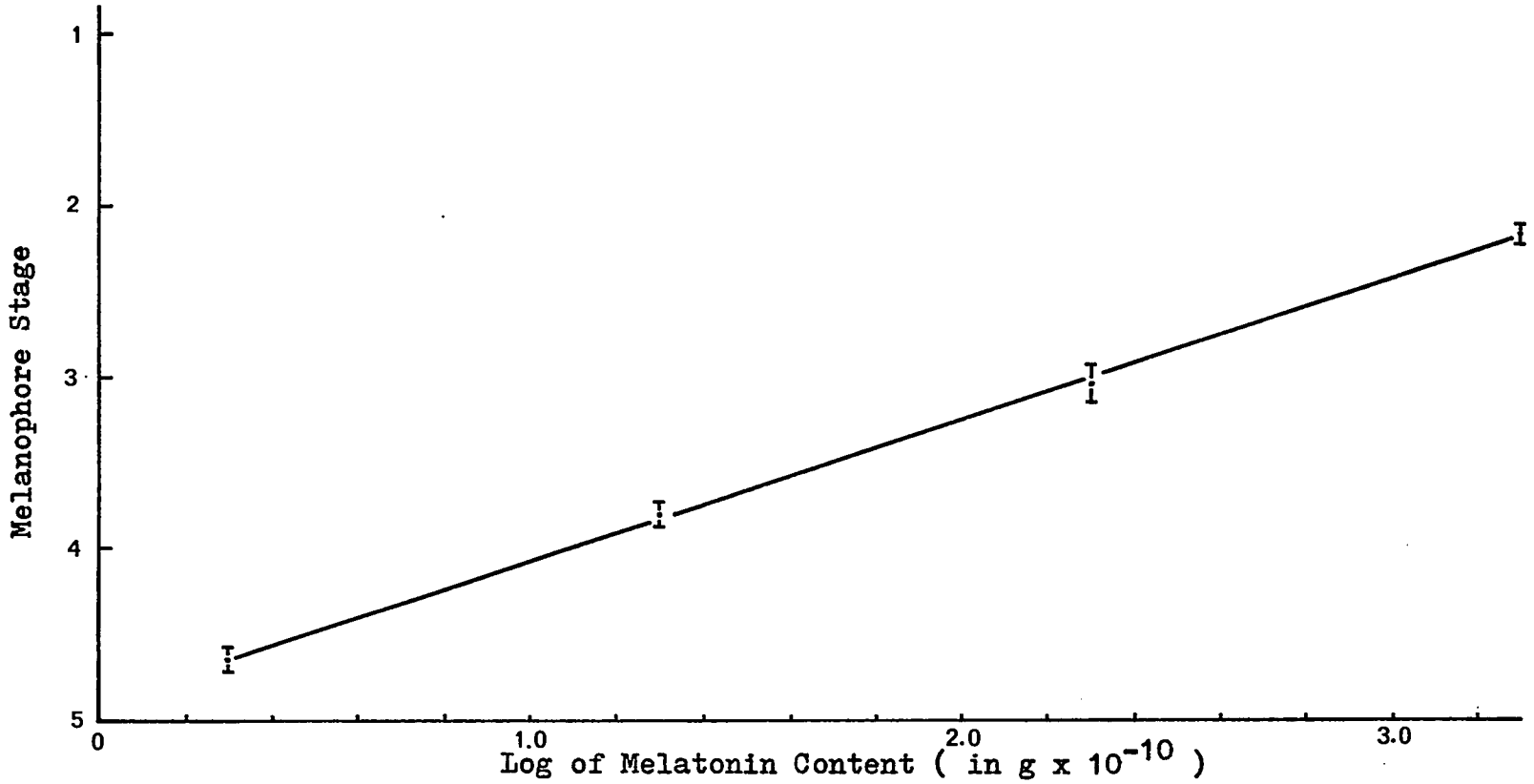

Figure 2. Response of Xenopus laevis tadpole tail melanophores to known concentrations of authentic melatonin. Each point represents the mean of from 10 to 15 estimates of melanophore response. The vertical line through each point indicates ± one S.E.M.

chromatographed. A 40ml fraction corresponding to the elution volume of 111-150ml was collected from each column and bioassayed as described above.

Urine 1 chromatographed alone was bioassayed and resulted, as can be seen in Figure 3, in a melanocyte index (MI) of 2.80. An MI of 2.80 corresponds to a melatonin concentration of 16.95×10^{-9}g/ml. The theoretical mixture of urine 1 and an equal volume of 1×10^{-7}g/ml melatonin should theoretically result in a solution with a concentration of 58.48×10^{-9}g/ml.

The MI of the actual mixture following chromatography was 2.43 which corresponds to a melatonin concentration of 48.8×10^{-9}g/ml. When comparing the theoretical value of the concentration of the mixture, 58.48ng/ml, the recovery is found to be 83.46%. These results are illustrated in Figure 3.

As can be seen in Figure 4, urine 2 had a native MI of 4.40 and a concentration of 0.19×10^{-9}g/ml melatonin. The theoretical mixture of this urine with a solution of 1×10^{-7}g/ml melatonin, in equal volumes, should have resulted in a solution with a melatonin concentration of 50.09ng/ml. The actual mixture had, as a result of bioassay, an MI of 2.50. This corresponds in concentration to 39.75ng/ml. By comparison, it is seen that there was a recovery of 79.4% of the added melatonin.

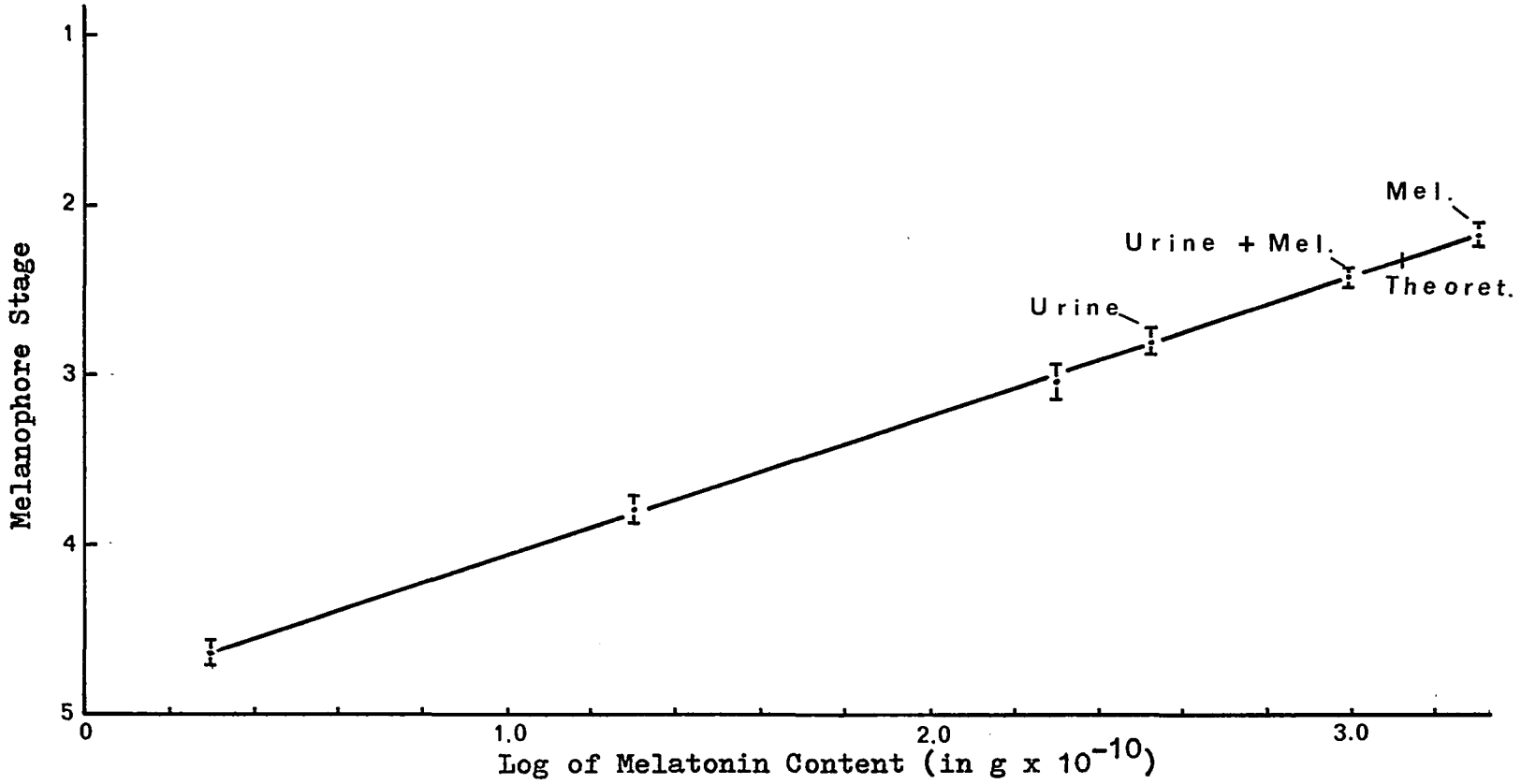

Figure 3. The recovery of melatonin added to a rat urine of high native melatonin content. The results are superimposed on the standard curve developed for this assay. Each point represents the mean of from 10 to 15 estimates of melanophore response. The vertical line through each point indicates $\pm$ one S.E.M.

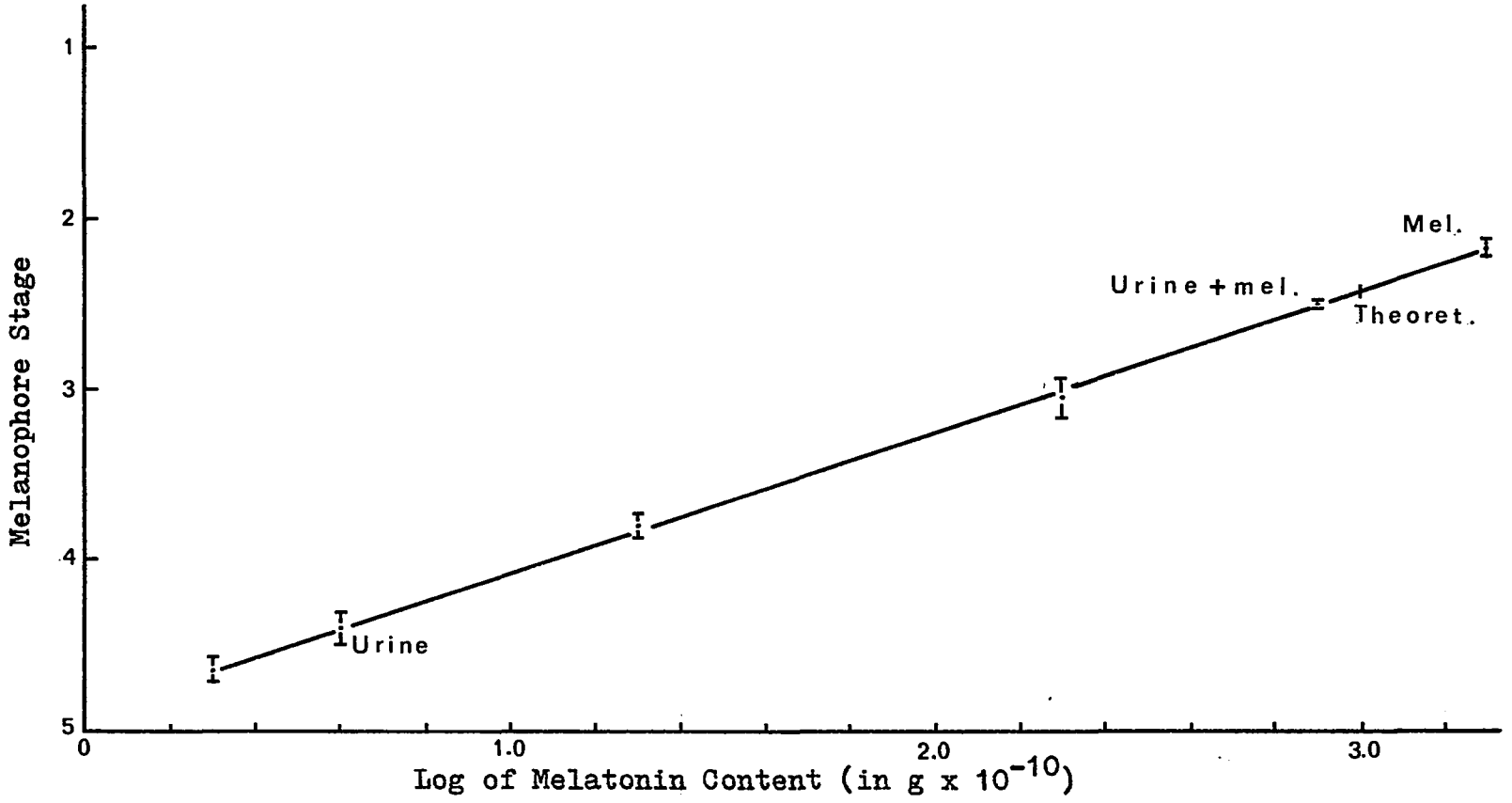

Figure 4. The recovery of melatonin added to a rat urine of low native melatonin content. The results are superimposed on the standard curve developed for this assay. Each point represents the mean of from 10 to 15 estimates of melanophore response. The vertical line through each point indicates ± one S.E.M.

Body weight

All animals exhibited a decrease in body weight during the 24 hr fasting period and the 24 hr stress period. The decrease in weight was approximately 10% with no differences seen between different surgical treatment groups.

Organ weights

The organ weights of intact animals subjected to different environmental conditions can be seen in Table 4. There was no significant difference in adrenal or pineal organ weights in the stressed groups when compared with the 23°C control group. The pineal gland weights of intact animals in each of the acute stress conditions showed a tendency toward increased values although these were not found to be statistically significant. Thyroid weight was found to increase significantly in the cold with a tendency for a similar change in the cold-immobilized group. Following chronic cold-immobilization stress, thyroid weights were significantly lower than the non-stressed controls. Testicular weights showed no significant changes in the acute stress conditions but were significantly decreased following chronic stress.

In Table 5 it can be seen that in the pinealectomized group neither acute nor chronic stress affected a change in thyroid, pituitary or testicular weights. Adrenal weights were found to be significantly increased in the cold and in

Table 4. Effect of environmental stress on selected endocrine organ weights in intact rats. Values are expressed as mean weight in mg/100g body wt ± S.E.M. except for testicular weights, which are in g/100g body wt ± S.E.M. Values in parentheses are the number of rats sampled.

Treatment	paired adrenal wt	thyroid wt	paired testicular wt	pineal wt
23°C	22.61±0.39 (6)	6.56±0.41 (5)	1.29±0.07 (6)	0.66±0.04 (6)
23°C Imm.	24.35±1.68 (6)	8.50±0.54 (6)[b]	1.31±0.06 (6)	0.74±0.08 (6)
4°C	20.82±1.21 (6)	8.00±0.30 (6)[b]	1.39±0.04 (6)	0.73±0.07 (6)
4°C Imm.	23.55±1.39 (6)	7.46±0.31 (6)	1.29±0.04 (6)	0.82±0.09 (6)
Chronic	23.69±1.64 (6)	4.35±0.35 (6)[c]	1.12±0.03 (6)[a]	0.52±0.06 (5)

[a]$P<0.05$, [b]$P<0.025$, [c]$P<0.005$, compared to 23°C control group.

Table 5. Effect of environmental stress on selected endocrine organ weights in pinealectomized rats. Values are expressed as mean weight in mg/100g body wt ± S.E.M. except for testicular weights, which are in g/100g body wt ± S.E.M. Values in parentheses are the number of rats sampled.

Treatment	paired adrenal wt	thyroid wt	paired testicular wt	pituitary wt
23°C	22.14±0.86 (6)	6.90±0.56 (5)	1.24±0.04 (6)	3.52±0.12 (5)
23°C Imm.	21.06±0.53 (6)	6.21±0.63 (6)	1.23±0.04 (6)	3.77±0.20 (6)
4°C	29.21±2.39 (6)[b]	7.18±0.49 (6)	1.35±0.05 (6)	3.95±0.18 (6)
4°C Imm.	29.03±1.72 (6)[c]	6.14±0.39 (5)	1.19±0.05 (6)	3.62±0.20 (6)
Chronic	25.30±0.90 (6)[a]	7.36±0.93 (6)	1.25±0.05 (6)	3.79±0.13 (6)
Sham	16.00±0.88 (5)[d]	5.20±0.68 (5)	1.04±0.04 (5)[c]	3.23±0.05 (5)

[a]$P<0.05$, [b]$P<0.025$, [c]$P<0.005$, [d]$P<0.001$, compared to 23°C control group.

both acute and chronic cold-immobilization stress, indicating the effect of cold on adrenal weight.

The pinealectomy-sham group at 23°C, when compared to the pinealectomized group at the same temperature was found to have significantly different adrenal and testicular weights. In both cases the weights were lower in the sham operated group. The pinealectomy-sham operations were not performed at the same time, nor by the same individual, as were the pinealectomy operations since an incomplete shipment of animals was sent by the supplier on a date different from the request date.

As can be seen in Table 6, adrenalectomized animals were found to have significantly decreased pituitary, thyroid and pineal gland weights in those environments which included immobilization. No significant change was seen in testicular weights in any stress condition when compared with non-stressed controls.

Superior cervical ganglionectomy resulted in no significant difference in organ weights when compared with non-stressed sham operated animals. In Table 7 it can be seen that neither testicular nor pituitary weights were affected by stress conditions in ganglionectomized animals. The adrenal glands showed a slightly significant weight increase in the cold. Thyroid and pineal gland weights were both significantly increased in the chronic stress condition.

In comparing adrenal weights in groups of animals

Table 6. Effect of environmental stress on selected endocrine organ weights in adrenalectomized rats. Values are expressed as mean weight in mg/100g body wt ± S.E.M. except for testicular weights, which are in g/100g body wt ± S.E.M. Values in parentheses are the number of rats sampled.

Treatment	pituitary wt	thyroid wt	paired testicular wt	pineal wt
23°C	4.17±0.10 (4)	6.54±0.08 (4)	1.37±0.13 (4)	0.60±0.05 (4)
23°C Imm.	2.81±0.16 (5)[a]	4.35±0.35 (5)[a]	1.12±0.07 (5)	0.33±0.04 (5)[b]
4°C	3.69±0.34 (5)	5.82±0.48 (5)	1.10±0.10 (5)	0.58±0.04 (5)
4°C Imm.	2.69±0.27 (5)[a]	4.79±0.22 (5)[a]	1.11±0.08 (5)	0.37±0.03 (5)[c]
Chronic	3.23±0.02 (4)[a]	4.20±0.33 (4)[a]	1.30±0.04 (4)	0.43±0.04 (3)[d]
Sham	3.90±0.20 (3)	5.72±0.75 (3)	1.07±0.07 (3)	0.59±0.08 (3)

[a]$P<0.001$. [b]$P<0.005$, [c]$P<0.01$, [d]$P<0.05$, compared to 23°C control group.

Table 7. Effect of environmental stress on selected endocrine organ weights in superior cervical ganglionectomized rats. Values are expressed as mean weight in mg/100g body wt ± S.E.M. except for testicular weights, which are in g/100g body wt ± S.E.M. Values in parentheses are the number of rats sampled.

Treatment	paired adrenal wt	thyroid wt	paired testicular wt	pituitary wt	pineal wt
23°C	22.68±0.99 (5)	5.06±0.39 (5)	1.24±0.05 (5)	3.78±0.37 (5)	0.49±0.04 (5)
23°C Imm.	21.99±0.90 (5)	5.64±0.37 (5)	1.31±0.03 (5)	4.19±0.15 (5)	0.60±0.03 (4)
4°C	27.09±1.37 (5)[a]	5.58±0.72 (5)	1.24±0.04 (5)	4.10±0.06 (5)	0.40±0.03 (5)
4°C Imm.	20.92±1.83 (5)	5.82±0.58 (5)	1.19±0.06 (5)	3.89±0.24 (5)	0.58±0.12 (5)
Chronic	26.00±1.64 (5)	6.41±0.39 (5)[a]	1.32±0.05 (5)	4.19±0.26 (5)	0.69±0.04 (5)[b]
Sham	25.17±2.66 (4)	6.35±0.39 (4)	1.35±0.09 (4)	3.62±0.19 (4)	0.44±0.09 (4)

[a]$P<0.05$, [b]$P<0.005$, compared to 23°C control group.

subjected to different surgical treatments (Table 8), it can be seen that there are significantly higher adrenal weights in pinealectomized and ganglionectomized animals subjected to the cold. Adrenal weights of animals subjected to both cold and immobilization were found to be significantly increased only in the pinealectomized group.

Thyroidal weights were found to be significantly decreased in ganglionectomized animals at 23°C when compared to non-stressed, unoperated, controls (Table 9). Significant decreases in thyroid weights were found in all surgical treatment groups in both acutely immobilized groups. Thyroid weights were also found to be significantly lower in both adrenalectomized and ganglionectomized groups exposed to cold. Chronic stress caused an increased thyroid weight in both pinealectomized and ganglionectomized animals.

In Table 10 it can be seen that no significant change in testicular weights could be associated with surgical treatment in non-stressed or in either of the acutely immobilized groups. There was a tendency, though not statistically significant, towards a decrease in testicular weights in acute stress. In chronic stress, adrenalectomy and ganglionectomy resulted in significantly higher testicular weights.

In both non-stress and cold exposure conditions ganglionectomy was found to result in a decrease in pineal gland weights while chronic stress caused a significant

Table 8. Effect of surgical treatment on paired adrenal weight in rats exposed to environmental stress. Values are expressed as mean weight in mg/100g body wt ± S.E.M. Values in parentheses are the number of rats sampled.

Treatment	23°C	23°C Imm.	4°C	4°C Imm.	Chronic
Intact (6)	22.61±0.93	24.35±1.69	20.82±1.21	23.55±1.39	23.69±1.64
PX (6)	22.14±0.86	21.06±0.53	29.21±2.39[a]	29.03±1.72[b]	25.30±0.90
SCGX (5)	22.68±0.99	21.99±0.90	27.09±1.37[c]	20.92±1.83	26.00±1.64

[a]P<0.025, [b]P<0.05, [c]P<0.01, compared to intact group. PX, pinealectomy; SCGX, superior cervical ganglionectomy.

Table 9. Effect of surgical treatment on thyroid weight in rats exposed to environmental stress. Values are expressed as mean weight in mg/100g body wt ± S.E.M. Values in parentheses are the number of rats sampled.

Treatment	23°C	23°C Imm.	4°C	4°C Imm.	Chronic
Intact	6.56±0.41 (5)	8.50±0.54 (6)	8.00±0.30 (6)	7.46±0.31 (6)	4.35±0.35 (6)
PX	6.90±0.56 (5)	6.21±0.63 (6)[a]	7.18±0.49 (6)	6.14±0.39 (5)[b]	7.36±0.93 (6)[a]
ADRX	6.54±0.08 (4)	4.35±0.35 (5)[c]	5.82±0.48 (5)[d]	4.79±0.22 (5)[c]	4.20±0.33 (4)
SCGX	5.06±0.39 (5)[b]	5.64±0.37 (5)[d]	5.58±0.72 (5)[d]	5.82±0.58 (5)[b]	6.14±0.39 (5)[e]

[a]$P<0.025$, [b]$P<0.05$, [c]$P<0.001$, [d]$P<0.005$, [e]$P<0.01$, compared to intact group. PX, pinealectomy; ADRX, adrenalectomy; SCGX, superior cervical ganglionectomy.

Table 10. Effect of surgical treatment on paired testicular weight in rats exposed to environmental stress. Values are expressed as mean weight in g/100g body wt ± S.E.M. Values in parentheses are the number of rats sampled.

Treatment	23°C	23°C Imm.	4°C	4°C Imm.	Chronic
Intact	1.29±0.07 (6)	1.31±0.06 (6)	1.39±0.04 (6)	1.29±0.04 (6)	1.12±0.03 (6)
PX	1.24±0.04 (6)	1.23±0.04 (6)	1.35±0.05 (6)	1.19±0.05 (6)	1.25±0.05 (6)
ADRX	1.37±0.13 (4)	1.12±0.07 (5)	1.10±0.10 (5)[a]	1.11±0.08 (5)	1.30±0.04 (4)[b]
SCGX	1.24±0.05 (5)	1.31±0.03 (5)	1.24±0.04 (5)[c]	1.19±0.06 (5)	1.32±0.05 (5)[b]

[a]$P<0.025$, [b]$P<0.01$, [c]$P<0.05$, compared to intact group. PX, pinealectomy; ADRX, adrenalectomy; SCGX, superior cervical ganglionectomy.

increase in pineal weights in similarly operated animals (Table 11). Acute immobilization was found to result in significantly lowered pineal gland weights in adrenalectomized animals.

A comparison of pituitary gland weights between surgical treatment groups in similar environmental conditions could not be made because no pituitary gland weights were recorded for unoperated animals.

Gastric ulceration

In intact animals at 23°C or immobilized at 23°C only one individual from each group developed gastric mucosal ulcerations (Table 12). Cold alone or cold in combination with restraint resulted in marked increases in the incidence and severity of ulceration. Immobilization in the cold was found to cause significantly higher levels of ulceration than were caused by cold alone.

As can be seen in Table 13, one animal subjected to adrenalectomy and kept at 23°C developed a single gastric erosion. Adrenalectomy was found to result in significant increases in both ulcer incidence and severity in all stress groups.

Pinealectomized animals were found to develop no erosions in either non-stress or immobilization (23°C) conditions. When exposed to cold, pinealectomized animals were found to have ulcers in 67% of the animals examined. Cold and restraint in combination was found

Table 11. Effect of surgical treatment on pineal gland weight in rats exposed to environmental stress. Values are expressed as mean weight in mg/100g body wt ± S.E.M. Values in parentheses are the number of rats sampled.

Treatment	23°C	23°C Imm.	4°C	4°C Imm.	Chronic
Intact	0.66±0.04 (6)	0.74±0.08 (6)	0.73±0.07 (6)	0.82±0.09 (6)	0.52±0.06 (5)
ADRX	0.60±0.05 (4)	0.33±0.04 (5)[a]	0.58±0.04 (5)	0.37±0.03 (5)	0.43±0.04 (3)
SCGX	0.49±0.04 (5)[b]	0.60±0.03 (4)	0.40±0.03 (5)[a]	0.58±0.12 (5)	0.69±0.04 (5)[c]

[a]$P<0.005$, [b]$P<0.025$, [c]$P<0.05$, compared to intact group. ADRX, adrenalectomy; SCGX, superior cervical ganglionectomy.

Table 12. Gastric ulcer formation in intact rats exposed to environmental stress. Mean values are expressed as mean ± S.E.M. See text for ulcer score description.

Treatment	n	Ulcer incidence	Mean no. ulcers	Mean ulcer score
23°C	6	17%	0.50±0.50	0.83±0.83
23°C Imm.	6	17	0.17±0.17	0.33±0.33
4°C	6	67	1.50±0.57	2.17±0.86
4°C Imm.	6	100	13.17±2.89[a]	18.17±2.52[b]

[a]P<0.005, [b]P<0.001, compared to all other treatment groups.

Table 13. Gastric ulcer formation in adrenalectomized rats exposed to environmental stress. Mean values are expressed as mean ± S.E.M. See text for ulcer score description.

Treatment	n	Ulcer incidence	Mean no. ulcers	Mean ulcer score
23°C	4	25%	0.25±0.25	0.50±0.50
23°C Imm.	5	80	2.60±1.43	3.60±2.40
4°C	5	100	9.40±3.96	21.20±10.10
4°C Imm.	5	100	16.00±3.73[a]	31.80±7.09[a]

[a] $P<0.01$ compared to 23°C and 23°C Imm. groups.

to result in a 100% incidence of ulceration in such animals (Table 14).

In superior cervical ganglionectomized animals only cold and cold-restraint stress was found to cause gastric ulceration (Table 15). Cold-restraint stress was significantly more effective in causing gastric ulceration than was cold alone.

At 23°C neither pinealectomized nor ganglionectomized animals were found to develop gastric erosions while both intact and adrenalectomized animals showed 17% and 25% ulceration respectively (Table 16).

Immobilization at 23°C was found to result in 17% ulceration in intact animals and 80% ulceration in adrenalectomized animals while neither pinealectomized nor ganglionectomized animals were found to develop any ulcers (Table 17).

At 4°C all surgically treated, as well as intact, animals showed the development of gastric ulceration. Ganglionectomy resulted in 100% ulceration (Table 18). No statistically significant differences could be detected in either the mean number of erosions or mean ulcer scores among any of the surgical treatment groups.

Immobilization at 4°C resulted in 100% ulceration in all surgical treatment groups (Table 19). No significant differences in mean number of erosions or mean ulcer scores could be found among any of the surgical treatment groups.

Adrenalectomy was found to result in increased

Table 14. Gastric ulcer formation in pinealectomized rats exposed to environmental stress. Mean values are expressed as mean ± S.E.M. See text for ulcer score description.

Treatment	n	Ulcer incidence	Mean no. ulcers	Mean ulcer score
23°C	6	0%	0	0
23°C Imm.	6	0	0	0
4°C	6	67	1.33±0.49	3.17± 1.51
4°C Imm.	6	100	15.83±7.16[a]	28.00±11.90[a]

[a]$P<0.05$ compared to all other treatment groups.

Table 15. Gastric ulcer formation in superior cervical ganglionectomized rats exposed to environmental stress. Mean values are expressed as mean ± S.E.M. See text for ulcer score description.

Treatment	n	Ulcer incidence	Mean no. ulcers	Mean ulcer score
23°C	5	0%	0	0
23°C Imm.	5	0	0	0
4°C	5	40	1.33±1.37	2.83±2.94
4°C Imm.	5	100	17.17±4.59[a]	31.17±9.25[a]

[a] $P<0.05$ compared to all other treatment groups.

Table 16. Effect of surgical treatment on gastric ulcer formation in rats maintained at 23°C. Mean values are expressed as mean ± S.E.M. See text for ulcer score description.

Treatment	n	Ulcer incidence	Mean no. ulcers	Mean ulcer score
Intact	6	17%	0.50±0.50	0.83±0.83
ADRX	4	25	0.25±0.25	0.50±0.50
PX	6	0	0	0
SCGX	5	0	0	0

ADRX, adrenalectomy; PX, pinealectomy; SCGX, superior cervical ganglionectomy.

Table 17. Effect of surgical treatment on gastric ulcer formation in rats immobilized at 23°C. Mean values are expressed as mean ± S.E.M. See text for ulcer score description.

Treatment	n	Ulcer incidence	Mean no. ulcers	Mean ulcer score
Intact	6	17%	0.17±0.17	0.33±0.33
ADRX	5	80	2.60±1.43	3.60±2.40
PX	6	0	0	0
SCGX	5	0	0	0

ADRX, adrenalectomy; PX, pinealectomy; SCGX, superior cervical ganglionectomy.

Table 18. Effect of surgical treatment on gastric ulcer formation in rats maintained at 4°C. Mean values are expressed as mean ± S.E.M. See text for ulcer score description.

Treatment	n	Ulcer incidence	Mean no. ulcers	Mean ulcer score
Intact	6	67%	1.50±0.57	2.17±00.86
ADRX	5	100	9.40±3.96	21.20±10.10
PX	6	67	1.33±0.49	3.17± 1.51
SCGX	5	40	1.33±1.37	2.83± 2.94

ADRX, adrenalectomy; PX, pinealectomy; SCGX, superior cervical ganglionectomy.

Table 19. Effect of surgical treatment on gastric ulcer formation in rats immobilized at 4°C. Mean values are expressed as mean ± S.E.M. See text for ulcer score description.

Treatment	n	Ulcer incidence	Mean no. ulcers	Mean ulcer score
Intact	6.	100%	13.17±2.89	18.17± 2.52
ADRX	5	100	16.00±3.73	31.80± 7.09
PX	6	100	15.83±7.16	28.00±11.90
SCGX	5	100	17.17±4.59	31.17± 9.25

ADRX, adrenalectomy; PX, pinealectomy; SCGX, superior cervical ganglionectomy.

ulceration in all environmental conditions with the exception of acute cold-restraint stress. Pinealectomy and ganglionectomy reduced gastric ulceration at 23°C and in animals immobilized at 23°C.

In chronic stress conditions, unoperated animals showed an 83% incidence of ulceration. Adrenalectomized, chronically stressed animals showed severe ulceration in only one animal. This animal died during the second day of chronic stress treatment. One other similarly treated animal showed only one small circular erosion.

In the pinealectomized, chronically stressed animals there were no hemorrhagic erosions present at examination but each animal showed longitudinal bands of tissue which appeared to be areas of healed ulceration. These areas were lighter when transilluminated than surrounding tissue while ulcers are characteristically darker. The longitudinal orientation of these scar-like bands in the glandular stomach would indicate that mucosal ulceration had been so extensive along the crests of the gastric rugae that ulcers had run together and then had healed.

Superior cervical ganglionectomized animals exposed to chronic cold-restraint stress showed a similar tendency towards healing of ulceration while active hemorrhagic lesions were still present in 60% of the animals examined. In fact, ulceration was so severe that two stomachs perforated while being inflated prior to examination.

Urinary corticosteroid concentration

There was no significant difference in urinary corticosteroid measurements among operated and sham-operated animals under non-stress conditions.

In intact animals, both cold exposure and immobilization at 23°C were found to result in significant changes in urinary corticosteroid concentrations (Table 20) when compared with non-stressed controls. At 23°C, immobilization caused a significant decrease and at 4°C there was a significant increase in such concentrations.

Acute and chronic immobilization at 4°C were found to have no significant effect on urinary corticosteroid concentrations although a tendency towards increased levels was seen.

In adrenalectomized animals acute stress caused significant decreases in urinary 11-hydroxysteroids. Chronic stress caused a significant increase in 11-hydroxysteroid secretion in adrenalectomized animals.

Pinealectomy resulted in significant increases in urinary corticosteroids in acute cold exposure, with and without immobilization. No significant change in corticosteroid excretion was found in pinealectomized animals immobilized at 23°C or exposed to chronic cold-restraint stress. However, in both cases there was a tendency towards increased values.

The removal of the superior cervical ganglion resulted in significant changes in corticosteroid excretion

Table 20. Urinary corticosteroid concentrations in surgically treated rats exposed to environmental stress. Values are the mean of three urine samples and are expressed in µg/100ml urine ± S.E.M.

Treatment	Intact	PX	SCGX	ADRX
23°C	49.6±3.52[a]	75.6±7.64	84.3±2.60	50.0±0.10
23°C Imm.	21.6±3.32[b]	79.3±2.90	68.7±2.41[d]	33.0±5.76[e]
4°C	130.0±4.61[c]	149.3±19.3[d]	132.0±6.02[b]	25.0±0.10[c]
4°C Imm.	65.6±10.3	122.6±6.65[d]	53.5±3.00[d]	10.5±0.75[c]
Chronic	57.3±9.29	95.0±6.02	86.0±7.06	75.0±2.00[b]

[a]Not significantly different from all sham operated animals maintained at 23°C.
[b]$P<0.005$, [c]$P<0.001$, [d]$P<0.025$, [e]$P<0.05$, compared to 23°C group.
PX, pinealectomy; SCGX, superior cervical ganglionectomy; ADRX, adrenalectomy.

in acute stress. In both acutely immobilized groups there was a significant decrease and in the cold there was a significant increase. Chronic immobilization in the cold had no effect on urinary corticosteroid concentrations in ganglionectomized animals.

Comparison among different surgical treatment groups in similar environmental conditions (Table 21) reveals that at 23°C pinealectomized and ganglionectomized animals had elevated urinary corticosteroid concentrations. Similar results were seen in animals immobilized at 23°C. At 4°C no significant difference was seen in pinealectomized or ganglionectomized animals while adrenalectomy resulted in a significant decrease in corticosteroid excretion. While adrenalectomy resulted in significantly decreased levels, pinealectomy caused significantly increased corticosteroid levels in animals immobilized at 4°C. In chronic stress no significant differences in corticosteroid excretion levels were seen except in pinealectomized animals, in which they were increased significantly.

Urinary melatonin concentration

As can be seen in Table 22, intact animals were found to excrete higher levels of melatonin in all stress conditions when compared to non-stressed controls. Immobilization stress groups showed significantly higher levels than the groups subjected only to 4°C.

Pinealectomy was found to result in the expected

Table 21. Effect of surgical treatment on urinary corticosteroid concentrations in rats exposed to environmental stress. Values are the mean of three urine samples and are expressed in µg/100ml urine ± S.E.M.

Treatment	23°C	23°C Imm.	4°C	4°C Imm.	Chronic
Intact	49.6±3.52	21.6±3.32	130.0±4.61	65.6±10.3	57.3±9.29
PX	75.6±7.64[a]	79.3±2.90[c]	149.3±19.3	122.6±6.65[d]	95.0±6.02[a]
SCGX	84.3±2.60[b]	68.7±2.41[c]	132.0±6.02	53.5±3.10	86.0±7.06
ADRX	50.0±0.10	33.0±5.76	25.0±0.10[c]	10.5±0.75[e]	75.0±2.00
Sham	56.1±5.31	-	-	-	-

[a]P<0.05, [b]P<0.005, [c]P<0.001, [d]P<0.025, [e]P<0.01, compared to intact group.
PX, pinealectomy; SCGX, superior cervical ganglionectomy; ADRX, adrenalectomy.

Table 22. Urinary melatonin concentrations in surgically treated rats exposed to environmental stress. Values are the mean of three urine samples and are expressed in ng/ml urine ± S.E.M.

Treatment	Intact	PX	ADRX	SCGX
23°C	0.95±0.15	<0.05	2.23±0.32	1.35±0.15
23°C Imm.	23.95±5.03[a]	<0.05	28.75±4.31[a]	25.05±3.56[a]
4°C	1.77±0.26[b]	<0.05	2.88±0.32	7.05±1.06[a]
4°C Imm.	28.75±4.08[a]	<0.05	32.75±4.65[a]	57.50±8.62[c]
Chronic	22.30±4.68[a]	<0.05	25.05±5.26[a]	30.80±6.47[a]

[a]P<0.01, [b]P<0.05, [c]P<0.005, compared to 23°C group. PX, pinealectomized; ADRX, adrenalectomized; SCGX, superior cervical ganglionectomized.

marked decrease in urinary melatonin concentrations. Concentrations were below the scale of the standard curve for melatonin and were assigned values of ≤0.05 ng/ml but represent a lack of melatonin activity in the urine samples assayed.

Superior cervical ganglionectomy, in all stress conditions resulted in a statistically highly significant increase in urinary melatonin concentrations over non-stressed control levels. Stress conditions in which immobilization was present were found to cause higher melatonin excretion levels than in the stress condition in which animals were exposed only to cold as a stressor.

Adrenalectomized animals showed a significant rise in urinary melatonin activity in all stress conditions with the exception of cold.

There was no significant difference in urinary melatonin concentrations between intact and sham-operated animals at 23°C.

At 23°C, adrenalectomized animals showed a slightly significant increase in melatonin levels. Immobilization at 23°C resulted in no significant differences in urinary melatonin concentrations among any of the surgical treatment groups (Table 23).

At 4°C both adrenalectomy and superior cervical ganglionectomy resulted in elevated melatonin excretion. Ganglionectomy similarly resulted in increased melatonin levels in animals subjected to immobilization at 4°C.

Table 23. Effect of surgical treatment on urinary melatonin concentrations in rats exposed to environmental stress. Values are the mean of three urine samples and are expressed in ng/ml urine ± S.E.M.

Treatment	23°C	23°C Imm.	4°C	4°C Imm.	Chronic
Intact	0.95±0.15	23.95±5.03	1.77±0.26	28.75±4.08	22.30±4.68
PX	0.05	0.05	0.05	0.05	0.05
ADRX	2.23±0.32[a]	28.75±4.31	2.88±0.32[b]	32.75±4.65	25.05±5.26
SCGX	1.35±0.15	25.05±3.56	7.05±1.06[c]	57.50±8.62[b]	30.80±6.47
ADRX-sham	1.38±0.20	-	-	-	-
SCGX-sham	1.69±0.41	-	-	-	-
PX-sham	0.74±0.11	-	-	-	-

[a]$P<0.01$, [b]$P<0.025$, [c]$P<0.001$, compared to intact group. PX, pinealectomy; ADRX, adrenalectomy; SCGX, superior cervical ganglionectomy;

No significant differences could be detected among the melatonin excretion values in chronically stressed animals of any surgical treatment group.

DISCUSSION

Since the isolation of melatonin, 5-methoxy-N-acetyltryptamine, from bovine pineal extracts (Lerner et al., 1958), this methylated indole derivative has been implicated in a variety of physiological functions. The accurate measurement of melatonin in biological material is hampered by its presence in very small quantities in blood, urine, and particularly the pineal itself. The development of a specific, sensitive assay for the quantitative estimation of melatonin in biological samples is a necessary prerequisite for the further elucidation of the role of this compound in physiological functions.

The presence of an indole nucleus of the melatonin molecule permits its visualization, following paper or thin-layer chromatography, with Ehrlich's reagent. However, Prop and Ariëns Kappers (1961), in an extensive study of the paper chromatographic properties of pineal constituents, could demonstrate no color-reactive melatonin even though the preparation examined contained an extract of 79 adult albino rat pineals. While it is possible to prepare extracts of biological material which, when chromatographed on paper or thin-layer plates, show a color-reactive spot corresponding to the Rf of melatonin, procedures for the quantitation of the color reaction have not been developed

due to a lack of sensitivity and specificity (Cole and Crank, 1972). The sensitivity of detection on paper chromatograms is approximately 0.5ug (Cole and Crank, 1972).

Melatonin can be estimated indirectly by the assay of the activity of the melatonin-forming enzyme, hydroxyindole-O-methyltransferase (HIOMT), (Axelrod and Weissbach, 1961). This type of assay, at best, can be used to measure the potential of a tissue to form melatonin. The limitations of this assay are imposed by the restricted occurence of HIOMT in tissues and the assumptions that must be made concerning the *in vitro* activity of the enzyme in relation to its *in vivo* characteristics.

Various procedures for the spectrophotofluorometric assay of melatonin have been reported. Axelrod and Weissbach (1961) have reported the measurement of as little as 1ug of melatonin following its extraction into chloroform. The recovery of added melatonin in this procedure was 85-95%. In a similar procedure, Quay (1963) uses p-cymene extraction followed by spectrophotofluorometry. This procedure however, requires extensive purification of reagents and is, therefore, cumbersome. These spectrophotofluorometric methods are not sufficiently specific or sensitive to measure the concentrations of melatonin found in the pineal of most species (Wurtman *et al.*, 1968). Cole and Crank (1973) recently have developed a spectrophotofluorescent technique for the estimation of melatonin

in an ether extract of serum. They report 90% recovery but can estimate melatonin concentration only as low as 0.06 µg/ml.

Taking advantage of the formation of a highly fluorescent compound resulting from the reaction of indolealkylamines with O-phthalaldehyde, Maikel and Miller (1968) developed a fluorometric assay for melatonin with greater sensitivity. Following differential extraction of the various indolealkylamines present in pineal extracts, this system reportedly can measure melatonin in the 10-20ng range. Using this procedure, Miller and Maikel (1970) reportedly can recover 84±4ng of melatonin of a total of 100ng added to a pineal extract. The limitation of this assay procedure is in the narrow range of accuracy reported (10-20ng).

Several assay procedures utilizing gas-liquid chromatography followed by spectrophotofluorometry have been reported (Greer and Williams, 1967; Pennington, 1968). Recently, Degen et al. (1972) have reported the combination of gas-liquid chromatography with mass spectrometry in the assay of heptafluorobutyryl derivatives of various indoleamines including melatonin. While the conversion of pure melatonin to the pure heptafluorobutyryl derivative is only 55%, the extraction from pineal homogenates, using ^{3}H-melatonin, was found to be 80-82%. Using this procedure, the authors report the detection of melatonin in the range of 35-140pg, although no data on the resolution of individual

points on their dose response curve is presented.

The mass spectral analysis of melatonin has also been employed in quantitative assay procedures. Hutzinger et al. (1972) combine thin-layer chromatography and mass spectrometry following the application of an electron-acceptor reagent to the chromatogram. Indoles can be detected in the range of 0.5-1.0ug, depending upon the structure of the indole and the electron acceptor used. The mass spectral identification of melatonin in chicken blood, following an elaborate extraction procedure and thin-layer chromatography has been reported (Pelham et al., 1972).

The most striking physiological effect of melatonin is its ability to cause the aggregation of melanin granules around the cell nucleus of amphibian melanophores (Lerner and Case, 1958). This property has been utilized in the development of several bioassay procedures for the estimation of melatonin. The bioassay technique is highly specific and more sensitive than the fluorometric technique (Cole and Crank, 1972). Such procedures depend upon the blanching reaction of either isolated frog skin (Lerner and Wright, 1960; Mori and Lerner, 1960), or the skin of intact adult frogs (Kastin and Schally, 1966), or, alternatively, the skin of intact amphibian larvae (Quay and Bagnara, 1964; van de Veerdonk, 1965; Ralph et al., 1967; and Ralph and Lynch, 1970). The blanching response may be measured by the transmission or reflectance of transmitted light by frog skin or by the estimation of the degree of

contraction of melanophores by microscopic examination and comparison of the degree of contraction with the standard index of Hogben and Slome (1931). The latter method is preferred for practical considerations (Thring, 1952). It has been utilized variously as a general screening procedure for the presence of melatonin (Lerner and Wright, 1960; van de Veerdonk, 1965; Quay and Bagnara, 1964) or as an assay with varying degrees of quantitative reliability (Ralph et al., 1967; Ralph and Lynch, 1970).

The assay method described in detail above presents a quantitative bioassay for melatonin which incorporates those procedures considered (Wurtman, 1971) to be the most productive in terms of both specificity and sensitivity -- chromatography followed by bioassay.

The sensitivity of the body lightening reaction of Xenopus tadpoles has been proven to be 10 times as sensitive as the most sensitive spectrofluorometric procedure (Ebels et al., 1972). The choice of Xenopus over Rana larvae is based upon the greater ease of handling and the more sensitive response of Xenopus as compared to Rana larvae (Lynch et al., 1971).

The column chromatographic procedure utilized in the assay method described above has been reported to permit the "complete recovery" of added melatonin (van de Veerdonk, 1965) although no data supporting this contention was presented in the literature. The recovery of 92-96% of added ^{3}H-melatonin in a selected elution volume of 40ml and

105% in the entire elution volume is described here and compares favorably with an estimate of 95.6% recovery for a similar chromatographic procedure (Benson et al., 1972). Extraction of melatonin from serum with ether or ethyl acetate reportedly results in recovery of approximately 80% (Cole and Crank, 1972).

The incorporation of the column chromatographic isolation of melatonin on a Sephadex G-25 Fine column in the assay procedure, in addition, assures the specificity of the assay procedure as this technique has been used to establish that the melanophorotropic constituent of pineal homogenates was, indeed, melatonin (Ralph and Lynch, 1970).

In addition, it has been shown here that the chromatographic procedure employed successfully separates melatonin from other, though less potent, melanophorotropic agents. Quay and Bagnara (1964), in a study of the relative potencies of 46 indole and related compounds in the body lightening reaction of larval Xenopus found only 5 which caused significant melanophore contraction. The 5 active compounds with their minimal effective doses (ug/ml) are: melatonin ($1x10^{-4}$), 6-methoxyindole ($1x10^{-1}$), serotonin (10), N-acetylserotonin (10), and 5-methoxyindole (10). This is supported by another study (van de Veerdonk, 1965) in which it was shown that melatonin could be separated from serotonin, epinephrine and norepinephrine. Van de Veerdonk (1965) did not, however, account for any of

these biogenic amines applied to the column. It was merely stated that they were "retarded even more on the column." The formic acid wash used in the present procedure effectively eliminates these substances from the column and prevents any conflicting actions they might have on the bioassay of melatonin solutions subsequently chromatographed on the same column. Benson and co-workers (1971; 1972) have shown that using Sephadex G-25 Fine columns, melatonin is effectively separated from a small polypeptide-containing fraction (F3). It is therefore assumed that the 111-150 ml fraction used in this assay contains only one melanophore-contracting substance -- melatonin.

Further proof of the specificity of the assay procedure is given by the evidence that, following the paper chromatography of melatonin, the only portion of the chromatogram eliciting a melanophorotropic response in the bioassay procedure was that portion known to correspond to the Rf of authentic melatonin.

The specificity and sensitivity of the bioassay procedure described here is further demonstrated by the ability of the method to accurately measure quantities of melatonin added to urine of known melatonin content.

The sensitivity of the method described here may be due to one significant modification of the bioassay techniques described elsewhere. It had been found, during the developmental stages of the method, that although intact

larvae are highly sensitive to melatonin (responsive to concentrations as low as $1x10^{-15}$g/ml), changes in environmental conditions, some of which have yet to be identified, could significantly alter the responsiveness of the larvae to melatonin. On certain occasions, control tadpoles, immersed only in culture medium, would show unexplained, marked, melanophore contraction. This reduced the reliability of the melanophore contracting response of the larvae to the test solutions. This unexplained melanophorotropic response is eliminated by severing the tail section from the body of the tadpole. The response of the larvae to changes in the environment, which may be manifested in a release of endogenous melatonin, no longer can mitigate the effect of exogenous melatonin on the melanophores being examined.

The assay procedure described above is of sufficient specificity and sensitivity to assure the quantitative estimation of levels of melatonin present in urine, as evidenced by the experimental results herein described as well as in blood plasma and pineal gland homogenates.

The study of melatonin concentrations in the urine of rats subjected to various forms of environmental stress was prompted by several observations of stress-induced modifications of pineal gland function. The exposure of normal adult male rats to environmental temperatures of 3^{o}-10^{o}C for 1 week was found to result in enzymatic and morphological changes in pinealocytes (Miline, 1971)

indicative of increased biosynthetic activity. In the same study, pinealectomized rats were found to be "more sensitive to cold than normal rats." Similar evidences of hyperplasia and increased activity of pinealocytes were reported to follow a 24 hr period of immobilization in rats (Miline et al., 1968). The mammalian pineal gland apparently does not respond to all forms of stress in the same way. Miline and co-workers have found depressive changes in pinealocytes following prolonged olfactory stimulation in rats (Miline et al., 1963) and auditory stimulation in bats (Miline et al., 1969).

The stress effects of insulin-induced hypoglycemia and immobilization have recently been shown to result in marked increases in pineal N-acetyltransferase and melatonin concentrations (Lynch et al., 1973).

Although Kopin et al. (1961) could find no unchanged melatonin in the urine or feces of animals previously injected with melatonin-acetyl-H^3, subsequent studies have indicated the possibility that endogenous melatonin may be protected from a similar metabolic fate. A high-capacity binding protein (albumin) was found in rat and human plasma which when bound to melatonin inhibited its degradative metabolism. Neither the addition of the albumin nor whole plasma was found to modify the melanin-aggregating effect of melatonin on amphibian melanophores (Cardinali et al., 1972b). Lott et al. (1972) have suggested that pineal glands may use specific glycoproteins

as carriers for secreted melatonin and thus protect melatonin from hydroxylation and conjugation.

The present study of the effects of cold, immobilization and combined cold-immobilization stress on melatonin excretion and the exception of other stressors (noise, electric shock, tourniquet stress, traumatic stress, ether, chemical stress) is supported by the previous reports of the effects of those stressors on pineal function. Noise stress was used in preliminary studies with intact rats and was found to have no effect on melatonin excretion.

The terms cold and immobilization can both be taken to mean a variety of environmental conditions. Cold, as a stress, may involve the exposure of subjects to temperatures as low as -20°C or as high as 13°C (Buckley, 1972). The choice of 4°C was, in part, determined by the availability of cold room facilities at that temperature and the use of that temperature in previous studies in which increases in adrenal and thyroid function as well as ulceration were shown (Buckley, 1972). Immobilization or restraint stress may involve the taping of a rat's limbs to a board (Selye, 1936), or placing an animal in a plaster of Paris corset, a wire mesh cocoon or a variety of plastic restrainer devices (Brodie, 1972; Singh, 1972). Wire mesh was chosen in this study because of its simplicity of construction and the ability it affords of maintaining the same degree of restraint of animals of different sizes. Plastic immobilizing units (Arthur H. Thomas Co.) were found to be too

small to accomodate the size of rats used in this study and a larger size would have prohibited the provision of water for restrained animals.

Although diestrus female rats (Wistar) were found to develop greater ulceration due to immobilization than either estrus females or males (Herner and Caul, 1972), males were used in this study. Males were used in order to eliminate the stage of the estrus cycle as a variable to be contended with.

The collection of urine during the entire 24 hr period of stress was designed to further reduce the number of experimental variables, in that diurnal variations in urine volume, stress susceptibility, adrenal function and pineal synthetic activity were eliminated.

The finding that all forms of stress studied caused an increase in melatonin excretion in intact rats provides further evidence of increased pineal gland activity in certain stress conditions and supports the work of Miline and co-workers (1971; 1968) and Lynch et al. (1973b). The increased urinary melatonin concentrations, when viewed in relation to the findings of progressive histological and enzymatic modifications as well as increased pineal melatonin content in stress, can be attributed to increased secretory activity of the pineal. Although a decrease in the metabolic degradation of melatonin may contribute to a rise in urinary melatonin levels, this hypothesis can neither be supported nor refuted by this

study.

It has also been shown that immobilization is a more potent stimulator of melatonin release than is cold. The effects of cold and restraint do not appear to be additive as urinary melatonin concentrations during cold-restraint stress are not significantly different from those found during simple restraint. A tendency toward such an additive effect is, however, seen in both intact and adrenalectomized animals. An additive effect of cold and restraint is seen in ganglionectomized animals.

The absence of measurable melatonin levels in the urine of pinealectomized animals indicates the absence of significant sources of melatonin other than the pineal gland.

Superior cervical ganglionectomy has been shown to cause a decrease in pineal norepinephrine content (Pellegrino de Iraldi and Zieher, 1966). However, when catecholamine levels are raised *in vivo* or *in vitro*, following ganglionectomy, significant superinductive effects on pineal enzymes have been seen. Catecholamines have been shown to increase adenylate cyclase activity (Weiss and Costa, 1967), HIOMT activity (Nagle *et al.*, 1973), and N-acetyltransferase activity (Deguchi and Axelrod, 1972; 1973; Klein *et al.*, 1971). Chemical sympathectomy, induced by pre-treatment with 6-hydroxy dopamine, potentiates the effects of L-DOPA on increasing pineal melatonin content (Lynch *et al.*, 1973b). The role

of the beta-adrenergic receptor in the superinduction of N-acetyltransferase activity has been studied in detail by Deguchi (1973). The urinary melatonin concentrations following ganglionectomy and stress reported here provide further evidence that pineal catecholamine sensitivity is enhanced by ganglionectomy. The urinary melatonin concentrations in rats subjected to cold and cold-restraint stress are significantly higher in ganglionectomized rats than in intact rats. Both cold exposure (Leduc, 1961) and immobilization (Kvetnansky and Mikulaj, 1970) are known to elevate circulating catecholamine levels. The finding that urinary melatonin concentrations in unstressed rats were not significantly different from intact controls, and not significantly depressed as might be expected, can be explained by the evidence which indicates that underfeeding or starvation represents a minor form of stress (Meites, 1970; Sorrentino et al., 1971a) in rats. Urine collection from control rats at 23°C was performed during the second 24 hr period of a 48 hr fast.

The urinary melatonin concentrations in adrenalectomized animals follow the same general pattern of stress response as is observed in the intact animals. However, adrenalectomized animals at 23°C have significantly higher urinary melatonin levels than similarly treated intact animals. A similar comparison is found for the same groups of animals subjected to cold stress. There is a tendency towards higher melatonin levels in adrenalectomized animals

in all environmental conditions. These results indicate that there is a moderate inhibitory control exerted by the adrenal glands on pineal secretory activity. This inhibition is greatest in conditions causing greater adrenocortical activity.

The effects of chronic cold-restraint stress on melatonin excretion are quantitatively similar to those seen in animals subjected to a single immobilization stress period. The mechanism of the stress effect is seen in Figure 5.

The significance of the pineal response to cold and immobilization stress may, in part, be found in the relationship between stress and variations in biogenic amine concentrations in the brain. Significant increases in brain serotonin levels have been found in response to cold (Barchas and Freedman, 1963) and immobilization (Corrodi et al., 1968; Curzon et al., 1972) while brain NE levels have been found to decrease (Barchas and Freedman, 1963) in response to cold and to increase in response to immobilization (Corrodi et al., 1968). Such findings are consistent with the known effects of melatonin on biogenic amine metabolism in the brain (Anton-Tay, 1971).

As a consequence of increased melatonin secretion in cold and immobilization stress, reproductive function might be expected to be depressed. Such an inhibitory effect of stress upon reproductive function in rats has been demonstrated (Brown-Grant et al., 1973) although pineal mediation was neither implicated nor discounted.

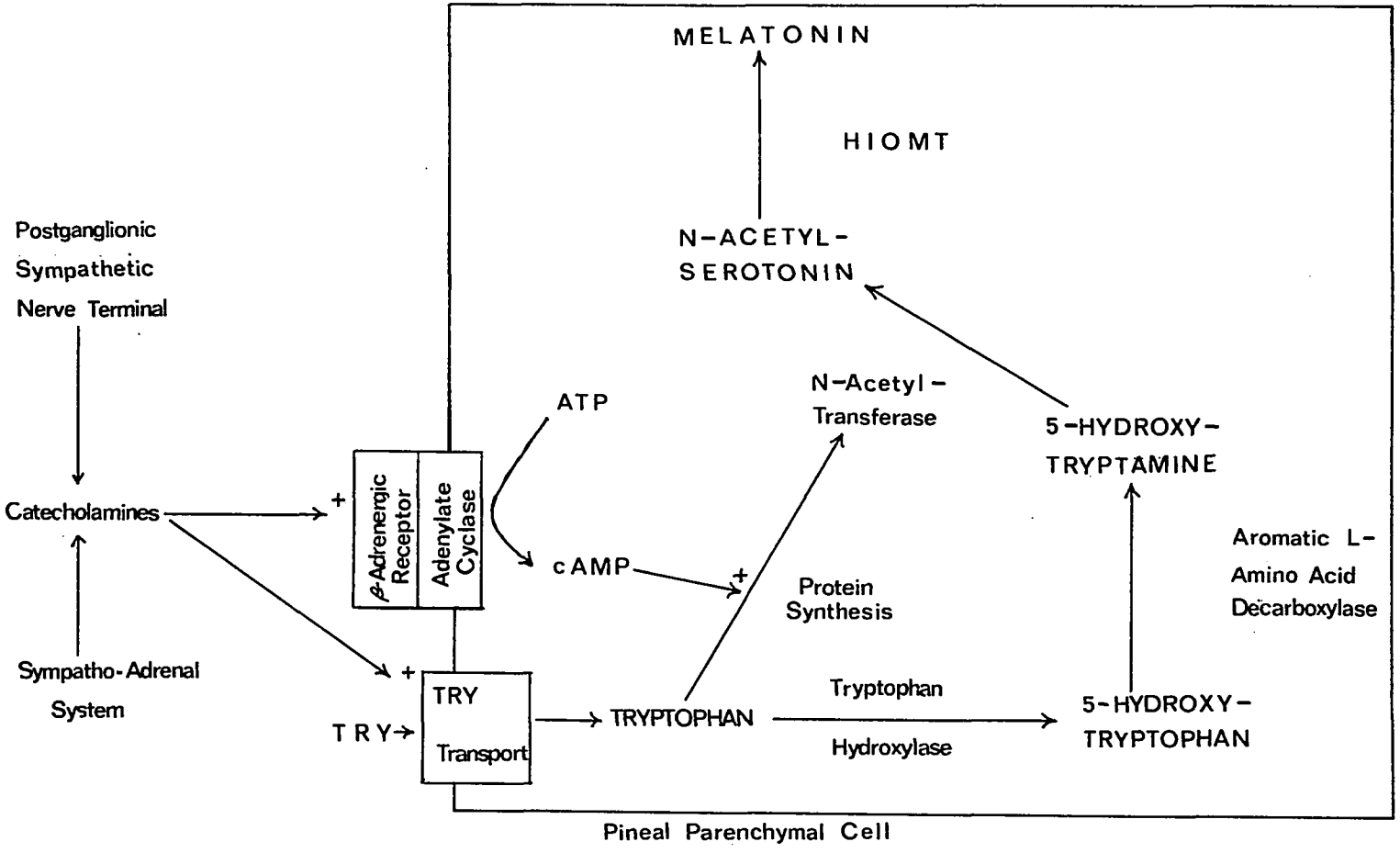

Figure 5. The mechanism of the stress-induced increase in melatonin production.

The direct inhibition effects of melatonin on reproductive function have been described in detail above.

A comparison between urinary melatonin concentrations and urinary corticosteroid concentrations found in this study indicates a close relationship between pineal and adrenal function. The corticosteroids measured by the method of Mattingly et al. (1964) are the 11-hydroxylated corticosteroids including cortisol, corticosterone and 20-hydroxycortisol. The 17-ketogenic steroids are not measured.

In the rat, the primary adrenal corticosteroid is corticosterone (Sawin, 1969). It is believed that ACTH affects cortisol biosynthesis by facilitating C-21 and C-11 hydroxylation (Molino et al., 1971).

In this study, it was found that cold exposure significantly increased corticosteroid excretion in intact, pinealectomized and superior cervical ganglionectomized animals. Cold exposure has previously been shown to stimulate the secretion of ACTH (Dupont et al., 1972; Ducommun et al., 1966; Langer and Lichardus, 1969) and to increase plasma corticosterone concentrations (Chin et al., 1973; Dixit and Buckley, 1969; Eisenberg et al., 1972; Jobin and Fortier, 1965; Maikel et al., 1967a) in the rat.

Immobilization stress in the rat has also been reported to cause increased plasma corticosterone concentrations (Dunn et al., 1972b; Perhack and Barry, 1970;

Paul et al., 1971; Riegle, 1973). It is of interest here, therefore, to note the inhibitory effect of immobilization stress on urinary corticosteroid concentrations in both intact and ganglionectomized animals. In pinealectomized animals, however, corticosteroid concentrations in immobilization did not differ significantly from controls. Pinealectomy has previously been reported to cause increased plasma corticosterone concentrations (Collu and Fraschini, 1972; Kinson and Singer, 1968; Kinson et al., 1967). Pinealectomy has also been shown to result in increased aldosterone secretion and hypertension (Karppanen and Vapaatalo, 1971; Kinson and Singer, 1967; Zanaboni and Zanaboni-Muciaccia, 1967).

It would appear, therefore, that the pineal gland produces a substance which is capable of inhibiting the release of adrenal corticoids and that pinealectomy removes such inhibition. Support for this hypothesis can be found in a report which demonstrated a reduction in the production of 11-hydroxycorticosteroids by a bovine pineal extract applied to slices of bovine adrenal cortex (Lommer, 1966). Motta et al. (1971) have demonstrated a significant decrease in plasma corticosteroids 60 min after the intraventricular injection of melatonin. Further support in the present study can be found by comparing the urinary corticosteroid concentrations found in this study with the concentration of melatonin found in the same urine samples. In both intact and ganglionecto-

mized animals, urinary corticosteroid concentrations were found to be lowest in those urine samples in which melatonin concentrations were greatest. In immobilization stress this effect is seen most clearly. The converse is also seen to be true. In the cold, melatonin levels are low and corticoid levels are at their highest. Further, in all surgical treatment groups, the combination of immobilization with cold stress was found to diminish significantly the corticosteroid response when compared with the response to cold alone. Significantly, the only exception is in the pinealectomized group, in which the stimulatory effects of cold stress on corticoid excretion were undiminished by combination with immobilization. It is suggested, as a result of this evidence, that melatonin is either capable of inhibiting the synthesis and/or release of corticosteroids or enhancing the catabolism of circulating corticoids. In light of the other reported data (Lommer, 1966), the former would appear to be a more likely explanation.

An alternative explanation for the observation of reciprocal urinary concentrations of corticosteroids and melatonin might be that corticoids are inhibiting the release of melatonin. This effect has been described above but only applies to the non-stress and cold treatment groups and is not found to be of significance in those stress conditions in which melatonin release is highly stimulated. If the adrenal corticoids do inhibit melatonin

release, the inhibition is easily overridden by catecholamine stimulation.

The finding of differential effects of different forms of stress on hormonal secretion is not without precedent. In swine, cold stress was found to have no effect on plasma ACTH levels while heat stress was found to increase those levels (Marple et al., 1972). Nevertheless, plasma corticoid levels were depressed in both stress conditions. A steady-state magnetic field of about 200 gauss has been found to increase urinary 17-hydroxycorticosteroids in monkeys (Friedman and Carey, 1972) while exposure to high pressure helium-oxygen environments were reported to increase free corticosterone excretion in man (Bitter and Nielsen, 1972). Traumatic stress by tibial fracture in the rat has been reported to increase plasma ACTH (Greer et al., 1970) and corticosterone levels (Guillemin et al., 1958) as does tourniquet stress (Greer et al., 1970) but both stressors stimulate such responses through separate pathways. Nemeth et al. (1973) have reported that traumatic tumbling stress for 5 min causes an increase in rat plasma corticosterone which then induces an increased liver tyrosine aminotransferase (TAT) activity. Noise stress not only does not cause the increase in corticosteroid levels but also partially inhibits the effects of exogenous corticosterone on the conduction of TAT. Noise stress was also found to have no effect on corticosteroid excretion in man (Slob et al., 1973).

The evidence presented above, in support of differential effects of stress on corticosteroid levels, does not, however, speak to the apparently contradictory findings, reported here, of decreased urinary corticosteroids during immobilization while others have reported increases in corticosteroids in plasma following immobilization stress. Several possible explanations may be offered for such findings. Sprague-Dawley rats were used in the present study while other studies have used Long-Evans (Riegle, 1973) and Wistar (Perhack and Barry, 1970) strains. Significant differences have been found between Long-Evans and Sprague-Dawley rats in relation to at least one physiological variable)liver microsomal enzyme induction) in a study by Jori et al.,(1972). In addition, in the same study, similar differences have been found between Long-Evans rats from two different sources. Significant differences have been found between Sprague-Dawley and Wistar rats in relation to resistance to stress-induced gastric ulceration (Goldenberg, 1973). In the two studies in which Sprague-Dawley rats were used, corticosteroid levels were not measured but only inferred from adrenal cAMP level changes in one study (Paul et al., 1971) and in the other the period of immobilization was only 3 minutes (Dunn et al., 1972b). The duration of immobilization stress used in the Riegle (1973) and Perhack and Barry (1970) studies was 2 hr while in this study restraint was maintained for 24 hr. Since it has been found that mere

handling of animals results in a rapid increase in plasma corticosteroids (Leppaluoto, 1972), it is possible that short periods of immobilization will result in increased corticosteroid secretion by the same mechanism. Longer periods of immobilization may be required to induce increased melatonin secretion which would then inhibit further corticosteroid production.

Grad and Rafizadeh (1971) have proposed that increased plasma corticosteroid due to severe cold stress in mice may be due in part to renal impairment. Their proposal is based upon increased plasma creatinine and blood urea nitrogen levels. These findings may not be applicable to the present study as the form of stress used by them was exposure to -10°C and is significantly different than that used in the study reported here.

The pathway through which melatonin may affect a change in adrenal corticoid production may involve serotoninergic fibers in the hypothalamic medial forebrain bundle as melatonin is known to affect changes in hypothalamic serotonin levels and the central nervous control over CRF and ACTH release resides there (Feldman et al., 1972; 1971; Motta et al., 1971; Popova et al., 1972; Scapagnini et al., 1971; Vaughan et al., 1972b).

The antistress effects of the corticosteroids have recently been described (Forsham, 1973; Selye, 1973). They include the expansion of blood volume, an increase in glomerular filtration rate, increased gluconeogenesis

and the mobilization of free fatty acids from fat deposits. They also cause a general stimulation of cerebral function and are anti-inflammatory and anti-allergenic. Their overall catabolic effect, however, decreases wound healing and defense against the spread of infection. In cold exposure the glucocorticoids are believed to be necessary for providing the proper physiological setting for the action of the catecholamines in maintaining body temperature (Euler, 1972; Maikel et al., 1967a; 1967b).

Cold exposure has been found to increase catecholamine concentrations in plasma (Buckley, 1972; Chin et al., 1973; Le Blanc et al., 1972) and urine (Euler, 1971; Feist, 1972; Leduc, 1961) as well as in the tissues (Gordon et al., 1966) in the rat but did not increase catecholamine secretion in the cat (Feurstein and Gutman, 1971). Sympathectomy has been found to upset the thermoregulatory mechanisms in rats exposed to cold (Legrand and Weil-Fugazza, 1972) and to lead to a rapid death (Krawczak and Brodie, 1970).

Restraint stress has also been found to increase the urinary concentrations of catecholamines (Graham, 1966; Kvetnansky and Mikulaj, 1970; Tomaszewska et al., 1971) in the rat.

Gastrointestinal damage is one of the several pathological changes that result from exposure to stressors, especially immobilization stress (Selye, 1956). Gastric ulceration is generally confined to the glandular portion

of the stomach and appears to be histologically similar, irrespective of the means by which the animal is immobilized (Buckley, 1972). The lesions are usually multiple and bleed but do not perforate. They occur in a linear pattern along the crests of the gastric rugae (Goodman and Osborne, 1973). Ulceration occurs in direct proportion to the degree of stress (Douglass and Le Veen, 1970).

Gastric ulceration was quantitatively determined in this study in order to assign degrees of severity to the various stress procedures used. The quantitative assessment of ulceration was based upon the ulcer incidence (% of animals showing ulceration), the mean number of erosions, and the mean ulcer score for the stomachs of each surgical treatment and stress group. The ulcer score used was that of Desiderato et al. (1974). In this assay, lesions under 1mm in length (or diameter) were given a value of 1; those between 1 and 2.9mm, 3 and 4.9mm, and those over 5mm, were given values of 2, 3, and 4, respectively.

The most severe stress studied was the combination of cold and immobilization for 24 hr. Cold exposure was found to be a more potent stressor than was simple immobilization. Starvation, which accompanied each of the other stress conditions was found to be a minimal stressor in that only one intact and one adrenalectomized animal developed gastric erosions when exposed only to starvation for 48 hr. Adrenalectomized animals were found to be more

susceptible to each of the stress conditions than the other surgical treatment groups. The only exception to this generalization was found in the most severe form of stress where all surgical treatment groups showed a 100% ulcer incidence.

No significant difference was found between intact, pinealectomized or ganglionectomized animals in their response to cold or cold-immobilization stress. However, in the less stressful conditions (23°C, 23°C immobilization) both pinealectomy and ganglionectomy were found to offer some protection from ulceration. Only in the chronic stress condition was there seen any difference between ulceration in pinealectomized and ganglionectomized animals. Pinealectomized animals had stomachs that were apparently, at one time, well ulcerated but had undergone a complete healing process. Ganglionectomized animals showed a similar repair process beginning but gastric erosions were still present. More importantly, ganglionectomized animals were the only animals in which stomachs perforated (2 of 5) when inflated with saline. No healing of erosions was seen in the intact group. One of the adrenalectomized animals showed the presence of healed erosions.

Immobilization stress has previously been reported to cause gastric erosions in rats (Ader, 1964; Brodie and Hanson, 1960; Buckley, 1972; Caul and Buchanan, 1971; Caul et al., 1972; Guth and Mendick, 1964; Herner and

Caul, 1972; Lovibond, 1969; Mikhail and Holland, 1966a; 1966b; Selye, 1936; Weinstein and Driscoll, 1972). Cold stress has not been reported to cause gastric erosions in rats although, in this study, cold stress was found to be more ulcerogenic than immobilization stress.

The combined ulcerogenic effects of cold and immobilization have been reported to surpass the effects of immobilization alone (Brodie and Hanson, 1960; Buckley, 1972; Djanhanguiri et al., 1973; Goldenberg, 1973; Senay and Levine, 1967). These findings have been confirmed in this study.

Food restriction has been associated with the production of gastric ulceration (Barboriak and Knobloch, 1972; Barboriak et al., 1971; Paré and Temple, 1973). Food restriction was not found to be a significant stressor in the present study.

The mechanism of ulceration has not been clearly defined, although several factors have been closely associated with ulcerogenesis. The hypersecretion of hydrochloric acid and pepsin is consistently implicated as a causative factor (Fenster, 1973; Mullane et al., 1973b; Safaie-Shirazi and Zike, 1973; Stadelmann et al., 1972). The stimulatory activity of the vagus nerve has been associated with increased acid secretion (Dragstedt, 1969) and corticosteroids have been related to increased gastric pepsin secretion (Bynum et al., 1972; Nagamachi, 1973). Pentagastrin and histamine have also been found to increase

acid secretion (Narumi and Maki, 1973).

Corticosteroid administration has been attributed to the production of gastric ulcers (Kilmore et al., 1972; Loeb and Sternschein, 1973; Robert and Nezamis, 1958) and the aggravation of already present ulceration (Valergakis et al., 1972). The role that corticosteroids play in ulcerogenesis may involve a decrease in gastric mucus secretion and a decrease in the cell renewal rate of the gastric mucosa (Fenster, 1973). Corticosteroids have also been found to have antiphlogistic properties and may lyse the fibrous tissue of healed ulcers (Bynum et al., 1972).

Norepinephrine has been found to increase ACTH secretion (Nagamachi, 1973) and to decrease gastric vascular flow as well as cell renewal rates (Djahanguiri et al., 1973).

A mechanism of gastric mucosal ulceration has been proposed (Guth, 1972; Guth and Hall, 1966) which accounts for many of the above factors. In the early response to stressful stimuli there is an increased gastric mucosal blood flow and a decreased HCl secretion. Following this protective phase, gastric mucosal blood flow is decreased, leading to gastric mucosal mast cell release of histamine, Histamine release causes venular stasis and superficial mucosal vascular engorgement resulting in decreased mucosal resistance to vagal- and corticosteroid-induced acid-pepsin digestion. The decrease in mucus secretion and decreased cell turnover due to increased glucocorticoid

concentrations allows further access of the acid-pepsin solution to mucosal cells. Damage to gastric mucosal cells permits an increase in acid back-diffusion which further stimulates pepsin and histamine release. Vascular stasis and acid secretion both increase. The result is a progressive erosion of the gastric mucosa which eventually leads to frank bleeding. A late effect of stress has been found to be an increased gastric blood flow which represents an attempt to reverse some damage.

Melatonin administration has been found to inhibit the stimulatory effects of histamine (a potent stressor) in causing ACTH secretion (Motta et al., 1971). This finding has suggested a possible role of the pineal gland in modifying the ulcerogenic action of the pituitary adrenal axis under stressful conditions (Motta et al., 1973). Similarly, the present findings of decreased corticosteroid levels in the presence of high melatonin concentrations, would indicate that melatonin may lessen the effects of corticosteroids in stress-induced ulcerogenesis. The ulceration data presented here do not, however, consistently corroborate such an hypothesis. In examining the immobilization stress response of pinealectomized, ganglionectomized and adrenalectomized animals, it can be seen that the most severe ulceration is found in the adrenalectomized animals, which have the lowest corticosteroid levels and which are also found to have high melatonin concentrations. The pinealectomized animals are found to have the lowest melatonin

levels while ganglionectomized animals have melatonin levels which are not significantly different from the adrenalectomized group. Pinealectomized and ganglionectomized animals have corticosteroid levels which are significantly different. The corticosteroid concentrations of both pinealectomized and ganglionectomized groups are significantly higher than the adrenalectomized animals but it is only the adrenalectomized group that shows stress-induced ulceration. The role, therefore, of the corticosteroids in the stress response is contradictory. While, at the same time adrenalectomized animals are found to be the least stress resistant, presumably due to a lack of circulating corticosteroids, they are found to develop the greatest degree of gastric ulceration, a result generally attributed to elevated corticosteroid concentrations. Further, all animals subjected to cold-immobilization stress were found to develop gastric erosions although corticosteroid concentrations varied from 10.50 ± 0.75 to 122.67 ± 6.65 µg/100ml.

The influence of the corticosteroids on the production of stress ulcers cannot, on the basis of the data presented here, be considered to be overriding.

The ulceration data presented here, when compared with the corticosteroid and melatonin concentration data, does not indicate a clear, causal relationship between melatonin, corticosteroids and ulceration, while such a relationship does appear to exist between melatonin and corticosteroid concentrations. However, the degree of

ulceration may be used as a general gauge of the severity of stressful environments, This was the intended purpose of the collection of ulceration profiles.

Various stress conditions and surgical procedures have been reported to induce significant changes in endocrine organ weights in the rat. Adrenal weights in intact animals have been reported to increase in response to cold exposure (Woods, 1957b) and to repeated immobilization (Kvetnansky and Mikulaj, 1970; Pfeiffer, 1967; Reklewska et al., 1972). In the present study no significant difference in adrenal weight in intact animals in any of the stress conditions was observed. Those studies in which adrenal weight changes were reported involved long term stress exposure (5 days to 5 weeks) and presumably one 24 hr period of stress is not sufficient to cause significant adrenal weight changes. As the adrenal gland does not store large quantities of hormone, adrenal weight change must involve cellular hyperplasia.

Pinealectomy has been reported to have variable effects on adrenal weight. Pineal gland removal has been shown to increase adrenal weight in female hamsters (Hoffman and Reiter, 1966), female rats (Wurtman et al., 1959), male rats (Relkin, 1972) and both male and female mice (Vaughan et al., 1972b). Contradictory effects have been reported (Karppanen et al., 1970; Roth, 1964; Wragg, 1967) in both male and female rats. Melatonin administration has also been found to have contradictory effects.

While Narang et al. (1967) have shown melatonin to increase adrenal weight, Vaughan et al. (1972b), in an extensive study, have shown melatonin to significantly reduce adrenal weight. In the present study pinealectomy was found to permit adrenal hypertrophy in the cold and cold-immobilization stress groups but not in unstressed controls.

While the thyroid gland has been found to increase its metabolic activity in response to cold stress (Heroux and Brauer, 1965; Kajihara et al., 1972; Kotani et al., 1973), no effect on thyroid weight has been reported in response to cold. Long term immobilization of rats (21-90 days) has been found to cause significant decreases in thyroid weights (Mateef et al., 1972; Reklewska et al., 1972). In the present study, 24 hr of cold or immobilization resulted in increased thyroid weights while chronic cold-restraint stress resulted in significant decreases in thyroid weight. Apparently short term stress stimulates thyroidal synthetic activity which results in increased hormone storage but prolonged stimulation results in an increased secretory rate not compensated for by hormone synthesis.

Pinealectomy has been reported to result in an increase in thyroid weight (De Fronzo and Roth, 1972) after six weeks, while melatonin administration resulted in decreased thyroid weight (Baschieri et al., 1963) following 10 days of treatment.

In the present study pinealectomy was found to

eliminate the response of the thyroid to environmental stress. The changes in thyroid weight observed in intact animals were not seen in animals without intact pineal glands. Superior cervical ganglionectomy resulted in significantly depressed thyroid weight. These data indicate that the thyroid gland is responsive to changes in the activity of the pineal gland and that melatonin may be the pineal hormone which exerts an inhibitory effect on thyroid activity. These findings confirm previous findings of depressed thyroid activity following melatonin administration (Cady and Dillman, 1971; Ishibashi et al., 1966; Narang et al., 1967). Relkin (1972) has suggested that the pineal inhibition of thyroid function is mediated through changes in the release of pituitary TSH.

Testicular weight has been reported to be slightly decreased as a result of chronic restraint stress in intact, adult, Sprague-Dawley rats (Pfeiffer, 1967). This finding has been confirmed in the present study.

Pinealectomy has been reported to increase testicular weight (Fraschini et al., 1968a; Motta et al., 1967; Wurtman, 1970) while melatonin administration has been found to decrease testicular weight (Debeljuk, 1969; Debeljuk et al., 1970a). Contradictory reports have also been published. Roth (1964) reported no change in testicular weight following pinealectomy and Liu and Kinson (1973) reported no effect of melatonin implants on testicular weights. Neither pinealectomy nor superior cervical

ganglionectomy were found to affect a change in testicular weights in the present study.

In the present study no effect on pituitary weight was seen as a function of environmental stress in either pinealectomized or ganglionectomized animals. However, adrenalectomized animals responded to those stress conditions which included immobilization by decreasing pituitary weights in relation to non-stressed adrenalectomized controls. Interpretation of the effects of stress on pineal function on pituitary weight is hindered by a lack of data concerning pituitary gland weights in intact animals.

In the above discussion, it can be seen that some effort has been made to account for those factors which may have contributed to the finding of experimental data which either fail to support or conflict with previous findings. The significance of any study can only be assessed in relation to other studies when the experimental conditions operating in each have been clearly defined. The effect of the animal strain on experimental results has been considered here and in the literature. Significant differences in stress responsiveness have been described between the Sprague-Dawley rat, the Wistar strain (Goldenberg, 1973) and the Long-Evans strain (Jori et al., 1972). Similarly, significant differences in the stress responses of wild and domesticated Norway rats (Weinstein and Driscoll, 1972; Woods, 1957a; 1957b) have been reported. In addition, Ebels and Prop (1965) have suggested the possibility "that

various rat colonies may possess a different sensitivity to melatonin treatment." Certainly, if inter-colony differences exist in relation to melatonin effects, differences may exist in regard to other experimental variables.

Significant differences in stress susceptibility have also been reported between animals housed individually or in groups (Hahn, 1965). Failure to include a description of housing conditions of experimental animals could easily result in misleading conclusions. However, until recently, such descriptions were not universally supplied.

As the purpose of experimentation is to provide understandings relevant to normal physiological function, it is of some importance to note that the lighting schedule used in this study was LD 12:12, which is considered to be "natural" lighting. However, the rat is a nocturnal animal and "continuous darkness would appear to be a more natural lighting environment for the rat" (Fishman and Roffwang, 1972). Significantly different lighting conditions were taken into consideration in comparing the results of this study with the results of previous studies.

Although no direct evidence has been provided by this study, certain of the reported effects of stress may now be attributed to the stress-induced activity of the pineal gland which has been shown here. Following ether or bleeding stress, marked elevations in pituitary prolactin release are seen (Ajika et al., 1973). Cold stress has also been shown to stimulate prolactin release (Yamamoto and Ieiri,

1972). Since it is known that melatonin administration will cause an increase in prolactin release (Lu and Meites, 1973; Ronnekliev et al., 1973) and it has been shown here that cold stress increases urinary melatonin concentrations, it may be inferred that the pineal release of melatonin may mediate the effects of stress on prolactin release. A similar mechanism may exist in the control of growth hormone release (Smythe and Lazarus, 1973a; 1973b; Sorrentino et al., 1971c; Yamamoto and Ieiri, 1972).

Immobilization stress has been reported to selectively increase brain tryptophan levels while causing no change in the levels of other amino acids (Knott et al., 1973). Immobilization stress has also been shown to induce changes in lipid and glucose metabolism (Piroux-Guyonneau and Buchel, 1972; Rothfeld et al., 1973). Future research may establish a relationship between the immobilization stress-induced increase in melatonin secretion and these findings.

With the understanding that certain forms of stress are stimulatory to melatonin release, consideration of the effects of stress on organs known to be affected by the pineal gland should provide the basis of further study. The thyro-parathyroid complex, the brain, and the reproductive organs are more than likely candidates.

CONCLUSIONS

The role of the pineal gland as a senso-neuroendocrine transducer capable of transforming environmental stimuli into an endocrine response has been further defined by this study. Urinary melatonin concentrations, indicative of pineal secretory activity have been found to vary with changes in specific, stressful, environmental stimuli. The quantitative measurement of urinary melatonin concentrations has been made possible by the development of a sensitive, specific bioassay system. Urine samples were chromatographed on gel columns which accomplished the recovery of 92-96% of added melatonin and at the same time separated melatonin from catecholamines, indoleamines and small polypeptides. The melatonin concentration of chromatographed urine samples was determined by a specific tadpole bioassay which is sensitive to melatonin concentrations in the range of 0.1-100ng/ml. Recovery of 83.5% of melatonin added to urine has been found following chromatography and bioassay procedures.

The stresses of cold exposure, immobilization, and combined cold and immobilization, have been found to cause significant increases in the urinary melatonin concentrations in intact rats. Immobilization stress was found to cause a greater stimulation of melatonin secretion than

does cold stress. The pineal response to stress is, therefore, specific and not merely the result of a general, "non-specific" sympathetic response to stress.

Superior cervical ganglionectomized animals in stress released more melatonin than did intact animals. This finding provides further evidence of a super-sensitivity of the pineal gland to catecholamines when the gland is denervated. Supersensitivity of the beta-adrenergic receptor results in a superinduction of the melatonin biosynthetic enzymes in response to catecholamine stimulation.

A comparison of urinary corticosteroid and melatonin concentrations has revealed an inhibitory control by melatonin over corticosteroid secretion. In stress conditions in which melatonin concentrations were found to be the greatest, corticosteroid concentrations were found to be the lowest. The reciprocity of this relationship was found to exist only in non-stress conditions as corticosteroid inhibition of melatonin secretion was ineffective in stress conditions.

No clear relationship between either melatonin concentration or corticosteroid concentration and stress-induced gastric ulceration has been found. Melatonin neither causes nor inhibits ulceration. The physiological effects of increased melatonin concentrations during stress are not apparently involved in the ulceration process but would appear to be more involved with the modulation of the adrenal and thyroid responses to stress.

The pineal gland appears to inhibit the activity of those organs stimulated by the hypothalamo-hypophyseal complex and would, therefore, be an integral, though not indispensible, part of homeostatic control mechanisms operating in both stress and non-stress conditions.

Because of the well-established relationship between the pineal gland and mammalian reproductive function, the evidence presented here provides further insights into the stress-induced modifications in reproductive physiology. The stress-induced increase in melatonin secretion may be effective in inhibiting the normal physiological release of gonadotrophins (Fuxe et al., 1970) which in turn might explain stress related modifications in ovulation (Brown-Grant et al., 1973; Farrell et al., 1968), menarche (Magee et al., 1970), pregnancy (Euker and Riegle, 1973), and fetal development (Buckley, 1972).

The bioassay method that has been described here may be applied to the study of variations in melatonin excretion in both physiological and pathological conditions. Its use may lead to a further understanding of the role of the pineal gland in the stabilization of circadian phase-shift changes during maturation (Quay, 1972a), the mechanisms of sleep induction and dreaming (Drew and Batt, 1972; Feldstein et al., 1970; Marczynski et al., 1964), the mechanisms of drug action on the brain (Winters et al., 1973; Wurtman and Axelrod, 1966b) and the control of electrolyte metabolism (Kiss et al., 1969; Pomerantz and Reiter, 1973; Reiter et

al., 1973; 1972).

On the basis of the ever-increasing body of pineal-related data, the pineal gland can now be seen as providing fine control modulation of certain homeostatic mechanisms. The role of the pineal gland as a senso-neuroendocrine transducer in the fine control of the body's response to stressful environmental stimuli may be but one aspect of its relation to the maintenance of homeostasis.

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