



1972

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Anthony V. Fasano
Loyola University Chicago

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HISTOCHEMICAL AND GRAVIMETRIC ANALYSES OF THE PINEAL
GLAND OF THE ALBINO RAT DURING THE ESTROUS
CYCLE, PREGNANCY AND PSEUDOPREGNANCY

by

Anthony V. Fasano

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy

February

1972

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LIFE

Anthony Vincent Fasano was born in Montclair, New Jersey on December 28, 1936, the son of Mr. and Mrs. Benjamin V. Fasano.

He attended elementary and high school in Newark, New Jersey, and was graduated from Colorado State College, Greeley, Colorado in June, 1964. During the ensuing three years, Mr. Fasano was engaged as an analytical chemist for Fisher Scientific Company, Fair Lawn, New Jersey and as a research chemist for the John L. Smith Memorial for Cancer Research in Maywood, New Jersey.

In January, 1967, Mr. Fasano began his graduate study in the Department of Physiology of Fairleigh Dickinson University, Teaneck, New Jersey. He remained there until he entered the Department of Anatomy, Loyola University of Chicago, Stritch School of Medicine, Maywood, Illinois, September, 1967. Here he continued his scientific investigations for the doctorate of philosophy degree in anatomy.

From 1968 to 1971, Mr. Fasano has had a National Defense Education Act fellowship. He is a member of the American Association for the Advancement of Science, American Society of Zoologists, and the New York Museum of Natural History.

During the period from 1954 to 1962, Mr. Fasano was an active member of the United States Naval Reserve from which he received an Honorable Discharge in August, 1962. In 1965, he married Linda Jacobsen and is the father of a boy, Steven Michael, now seventeen months old, and a girl, Dana Nicole, one month old.

ACKNOWLEDGEMENTS

No research endeavor is ever the work of a lone investigator; it is the culmination of the talents, guidance and assistance of a multitude of people. The author of this dissertation wishes to thank a few of his fellow workers for freely rendering their talents. First and foremost, an especial thanks to my adviser, Professor Joseph T. Velardo for his painstaking help and guidance; to Mrs. Velardo for her understanding and always constant encouragement; and to Dr. Barbara Kaspro for her help, instruction and guidance. A warm thanks to Dr. Leslie Emmert, Francis Kovarik and Grover Ericson for their assistance with both the animal work and the preparatory written work for this dissertation; to my committee for their ever friendly and patient guidance; and to Mrs. Canuti, Mrs. Schultz, Mrs. Smelte, and Mr. and Mrs. Kovarik for their pleasant support. Last, but not least, a very grateful thanks to my wife, Linda, who gave up so much and did so much so that this investigator could reach his goal.

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CHAPTER I

INTRODUCTION

The epiphysis cerebri, commonly known as the pineal gland, has come to the forefront, in recent years, in the search, through laboratory investigations, for more knowledge regarding the physical makeup of biological systems. This organ, which acts or reacts partially as an endocrine organ and as neural tissue, has been associated with the timing of ovulation in mammals as well as with the onset of puberty.

Since much is known of the estrous cycle of the albino rat, and since this cycle in some ways resembles that of the human female, the rat appeared to be an excellent animal for the study of the interrelationships of different enzyme activities of the pineal gland and the cycling reproductive organs.

Perusal of the literature reveals a paucity of knowledge concerning the enzyme biochemistry and histochemistry of the pineal gland. It has been shown histochemically that the pineal gland of the rat contains aminopeptidase (Niemi and Ikoken, 1960), which could denote secretions of compounds with a protein base; phospholipids (Zweens, 1963), which are a possible indication of

lipid metabolism and stores; and lipids (Quay, 1965) which have been identified as a possible source of compounds to supply energy necessary for protein synthesis, and basic metabolic activity of the parenchymal cells of the epiphysis cerebri. Interestingly enough, the report by Zweens (1963) is the only study which attempted to correlate the phospholipids of the pineal gland with the different stages of the estrous cycle of the rat. He demonstrated that phospholipids were lowest just before ovulation and commenced rising to a peak during mid-cycle (diestrus).

Since it has been demonstrated that there is a shift in the phospholipid concentration in the pineal gland during the estrous cycle, the thought emerged that other specific substances might, to one degree or another, also vary. Thus it seemed of paramount interest to ascertain the roles, relationships and possible interrelationships of a number of key compounds with a view toward relating them between reproductive mechanisms and levels of concentrations of these substances in the pineal gland.

The purpose of this investigation, therefore, is to assess a series of important enzymes of the pineal gland in the albino rat during the seven distinctive stages of the estrous cycle, and to determine whether or not there are associative changes in the pineal gland during the superimposed events of pseudopregnancy and pregnancy.

Alkaline phosphatase and acid phosphatase were studied in an attempt to determine possible sources of high energy phosphate bonds; lactic dehydrogenase was examined as it is an enzyme which converts lactate to pyruvate, the latter in turn enters the citric acid cycle; and succinic dehydrogenase was analyzed as it is a tricarboxylic acid cycle enzyme which converts succinate to fumarate.

Realizing both alkaline and acid phosphatase enzymes denote sources of high energy phosphate bonds, and lactic dehydrogenase and succinic dehydrogenase demonstrate metabolic activity, the two phosphatases being necessary for the dehydrogenases to function efficiently, this study would then denote a distinct interrelationship. Since this investigation is being performed within the critical stages of the estrous cycle, it thus becomes possible to establish other correlations between the enzymes studied in the pineal gland and reproductive variations in the albino rat.

With these base-line studies established, new avenues of investigations can then attempt to demonstrate possible neural effects on reproductive organs and conversely reproductive effects on neural structures, studies worthy of future investigative attempts.

Thus in the main, this study will attempt to assess specific (control) enzymatic activities which occur in the

pineal gland of the albino rat throughout the estrous cycle as well as the activity which occurs when the animal is pregnant or pseudopregnant.

CHAPTER II

REVIEW OF SIGNIFICANT LITERATURE AND ANALYSIS

The pineal gland was first described before 200 A. D. by Galen who stated that the pineal was probably a gland similar to lymph glands. This idea continued until the seventeenth century when Descartes designated the pineal as the seat of the rational soul which received information via the eyes and produced animal humors which controlled the response by muscles. This idea gained prominence regardless of the fact that some investigators like Bartholin, a Galenist physician of the era, contended that the pineal was a gland sphincter which served to filter lymph from the veins (Kitay, 1954).

The idea that the pineal might be an endocrine organ was not formulated until the nineteenth century when a physician, Pellizzi, described two cases of pubertas parecox and declared that they were due to pineal tumors. Similar case reports led to the formulation of theories concerning the hormonal function of this organ. These were: (1) that the glands stimulated bodily and sexual development, (2) that it inhibits bodily and sexual development, and (3) that it had no effect at all. A landmark discovery of pineal function was made by Mc Cord and Alan who in

1917 ascertained that extracts of cattle pineal glands when added to water containing tadpoles blanched the skin of the tadpoles (Kitay, 1954).

This led Lerner, et al. (1958), of Yale University, to attempt to isolate the blanching agent from the pineal of cattle in an attempt to control the dermatological condition known as vitiligo. These investigators isolated this blanching agent, but unfortunately found it had no effect on human pigmentation. They, however, named the blanching agent melatonin for its ability to blanch the melanophores of the frog skin at concentrations of 10^{-13} gm/ml. As a blanching agent, this compound, on a weight basis, was found to be one hundred times as active as adrenalin or noradrenalin, two hundred times as active as triiodothyronine, and five hundred times as active as serotonin (5-hydroxytryptamine).

Two years later, Axelrod and Weissbach (1961) working at the National Institutes of Health, purified and subsequently characterized the properties of hydroxyindole-O-methyltransferase (HIOMT), the pineal specific enzyme which converts N-acetyl-serotonin to melatonin. In this work they ascertained that melatonin formation occurred more rapidly with N-acetylserotonin than with serotonin. They also demonstrated that S-adenosyl-methionine, which acts as a methyl donor, and hydroxyindole-O-methyltransferase are necessary for the reaction to go to completion.

In this country, until the monograph of Kitay in 1954, most investigators considered the pineal gland a vestigial organ, but the isolation of pineal specific compounds, which could react or act as hormones, stimulated new interest in this gland. Investigations of the pineal gland now not only progressed in biochemical laboratories but also in the anatomical and neuro-anatomical laboratories.

In 1960, Kappers traced the nerve pathways to the pineal gland and found only sympathetic fibers from the superior cervical ganglia innervated this gland. He stated that the few fibers which enter the pineal stalk from the brain turned backward toward the midbrain. There then appears to be an evolutionary change in the pineal from other neural structures in that it is innervated by motor endings rather than by the brain itself. The postganglionic sympathetic fibers which enter the pineal gland from the superior cervical ganglia enter via the nervi conarii and the blood vessels. A large number of sympathetic nerves which enter the gland terminate among the parenchymal cells.

Wolfe, et al. (1962) have shown electron microscopically, using tritiated norepinephrine, that the pineal granular vesicles of the rat represent storage sites for norepinephrine in the sympathetic nerve endings. Pelegrino de Iraldi and Zieher (1966) went on to show that the pineal gland of the rat also

contains dopamine as well as norepinephrine. Sympathetic denervation of the pineal gland was found to decrease the norepinephrine concentration to non-detectable levels.

Quay (1957) demonstrated that in the epiphysis cerebri there are two types of parenchymal cells. The first is characterized by abundant lipid droplets composed primarily of ethanol soluble carbonyl lipids coated with phospholipids and a second type distinguishable by its content of phospholipid cytoplasmic matrix containing few lipid droplets or vacuoles. The author stated that the lipids were seen to be frequently associated with the pineal capillaries and this frequency of association possibly suggests endocrine activity.

Trentini and Silva (1965) have shown two distinct areas in the pineal gland of the rat after superior cervical ganglionectomy. These authors described a central part, medulla, consisting predominantly of dark cells and a peripheral part, cortex, consisting predominantly of the clear variety of cells.

It was only after Quay realized that the pineal gland had much fascination for an in-depth study did he turn his attention to the basic biology of the pineal including its embryology. Quay (1965) demonstrated that the parenchymal cells develop from neural ectoderm and are usually arranged in solid clusters, cords, or as incompletely separated lobules. Mesodermal derivatives enter the pineal via the vascular system

which passes through the pineal meningeal covering to penetrate the interior of this gland.

Milofsky (1957) demonstrated, electron microscopically, that the sympathetic nerves not terminating among the parenchymal cells end either in or on the blood vessels of the epiphysis cerebri. This same author has shown that the endoplasmic reticular membrane is mostly smooth, but some are present which are studded with ribose nucleoprotein, which confirms the light microscopic findings of Wislocki and his co-workers (1948).

Jordan, in 1921, demonstrated by means of light microscopy that in the cytoplasm of the parenchymal cells there are organelles suggestive of active metabolism and protein synthesis. Wolfe (1965) affirmed the results of Jordan by demonstrating electron microscopically an abundance of ribose nucleoprotein studded endoplasmic reticular membrane, thus suggesting protein synthesis within the pineal gland.

Das Gupta (1962) working with the hamster pineal also demonstrated two types of cells within the pineal gland. One type he designated as glial cells, and characterized them as having irregularly shaped nuclei without prominent nucleoli and a slightly dark staining cytoplasm. The second type, the light cell or parenchymal cell, was characterized as having a light staining cytoplasm and a round nucleus with a prominent nucleolus.

In an extension of his earlier studies, Quay (1963 a) undertook a number of experiments in an attempt to characterize

some of the basic biologic properties of the pineal gland. In so doing, he was able to show that the pineal is a gland which has circadian rhythms. He derived highly quantifiable evidence for this concept by studying the 5-hydroxytryptamine (serotonin) concentrations in male rats. Quay ascertained that from a nocturnal minimum of approximately 10 ng/pineal, the concentrations gradually increased to a mid-day maximum of 90 ng/ pineal. Immediately following the onset of darkness, the pineal 5-hydroxytryptamine (5-HT) concentration decreased at the rate of 25 ng/ hour to the nocturnal level of 10 ng around midnight. He also reported that there are modifications in the concentration of 5-hydroxytryptamine in the pineal that can be correlated with certain phases of the estrous cycle.

Early morning levels of 5-hydroxytryptamine were found higher on the day in which the animals showed a cornified vaginal smear (estrus) than on the following day. The late evening minimum of 5-hydroxytryptamine concentration was significantly higher on the day the animals were in the proestrus phase of the cycle than on the day the animals demonstrated a diestrus smear.

O'Steen (1970) demonstrated, in the retina of adult female rats, a possible relationship between 5-hydroxytryptamine and photoperiods after intraocular injections of tritiated 5-hydroxytryptophan, the precursor of serotonin. This author demonstrated that serotonin is influenced by photoperiod

fluctuations and suggests that this amine may be related to light-induced changes in neuroendocrine function.

Quay, also in 1963 (1963b), devised a number of experiments showing that in male rats kept in constant light, there is a definite metabolic inhibition manifested by decreases in glycogen content, succinic dehydrogenase activity, and respiratory activity. Pineal ATP content, P^{32} -phosphate uptake, and 5-hydroxyindoleacetic acid (HIAA) content did not appear to be modified. He suggested pineal inhibition by continuous light primarily involves the citric acid cycle, accumulation of metabolites and lipids, and the synthesis of protein. This investigation suggested a possible influence of environmental lighting on pineal metabolism.

In 1965, Axelrod, Wurtman and Snyder showed that hydroxyindole-O-methyltransferase (HIOMT) activity varied with environmental lighting. Female rats which were exposed to continuous darkness showed an increase in hydroxyindole-O-methyltransferase concentrations up to a plateau which is from two to ten times greater than normal values. Conversely, hydroxyindole-O-methyltransferase concentrations decrease to about one-third of the normal value in rats kept in continuous light. By subjecting rats to alternating twelve hour periods of light and dark, these investigators demonstrated a twenty-four hour circadian rhythm of hydroxyindole-O-methyltransferase

activity of approximately three-fold. No such diurnal rhythm has yet been demonstrated for any other enzyme in the pineal.

Hoffmann (1968) exposed female rats to twelve hours, fourteen hours, and sixteen hours of light per day beginning at sixty days of age. Rats exposed to twelve hours of light per day were predominantly four day cycling animals (70%) with 20% being of irregular cyclicity and 10% being of five day cycling phenomena. At sixteen hours of light per day, 21% of the rats observed manifested four day cycles, 33% were irregular and 46% had five day cycles. Thus, Hoffmann suggested the possibility that longer daily photoperiods raise the threshold at which steroid secretions trigger the ovulatory luteinizing hormone (LH) release.

Since it was now demonstrated that the pineal gland is affected by environmental lighting and it is innervated by the superior cervical ganglia, it now seemed mandatory to find out the pathway or a possible pathway by which environmental lighting affects pineal function. Moore and his co-workers (1967) demonstrated that cutting of certain fibers within the optic tract abolishes the pineal response to light without causing blindness. Normal visual response occurs via the retina, optic tract, lateral geniculate body, and optic radiations to the primary visual cortex. Moore, et al. demonstrated that fibers regulating pineal function leave the optic tract before the lateral geniculate body, pass through the hypothalamus via the medial

forebrain bundle down through the brain stem to the upper thoracic spinal cord levels, out the preganglionic sympathetic fibers to the superior cervical ganglia where they synapse, and then pass to the pineal gland as postganglionic fibers. Cutting of the medial forebrain bundle will abolish the pineal response to light while leaving the animal with complete vision (Wurtman, et al., 1967). Sectioning of the optic tract after the fibers to the median forebrain bundle have left blinds the animal, but does not alter the pineal response to light.

Wurtman, et al. (1964) have also shown that the pineal response to light is lost by bilateral superior cervical ganglionectomy or destruction of their preganglionic roots. These same authors also demonstrated that the hydroxyindole-O-methyltransferase response to environmental light is lost by bilateral enucleation which indicates the locus of photic input is in the retina and not in the pineal.

Since it has been shown that environmental lighting does, in fact, affect the pineal gland, it seems important at this time to state some of the findings that show the effect of environmental lighting on the pineal. Quay (1961) demonstrated that both male and female rats housed in continuous light or with long daily photoperiods have decreased pineal weights and stores of lipids. In 1962, Roth, et al. demonstrated, under the same conditions, decreases in the size of the parenchymal

cells. In 1965 and 1966, Hoffman and Reiter showed that rats housed under continuous light have the gonadal inhibitory influence of the pineal blocked. Wurtman, Axelrod and Phillips (1963) and Axelrod, et al. (1965) showed that animals housed under constant light conditions demonstrated decreased amounts of melatonin synthesis and decreased hydroxyindole-O-methyltransferase activity. Darkness generally has the reverse effect. Pineal monoamine oxidase is unaffected by environmental lighting. Increased hydroxyindole-O-methyltransferase activity in animals in constant darkness is probably representative of increased synthesis of the enzyme protein.

Reiter (1968) has shown that blinding of male hamsters leads to decreased testicular and accessory organ weights of approximately 10% and 33% respectively within eight weeks. The atrophic testes exhibited a complete loss of spermatogenesis and an apparent reduction of androgenic secretion. Hoffman (1967) blinded female rats at twenty-one days of age and subsequently observed, up to eight months, normal uterine weights, and decreased ovarian and pituitary weights in these animals. Rats blinded at ninety days of age, after estrous cycling had been established, began showing prolonged vaginal cycles and many of them showed significantly decreased uterine, adrenal, ovarian, and pituitary weights. Chu (1965) observed increased incidences of phases of heat (estrus) in rats and mice kept in constant light. Darkness had the opposite effect. The author

also states that there was good correlation between the vaginal smear and vaginal epithelial histology.

Motta, et al. (1967) have shown that pinealectomy in male rats resulted in no change in pituitary weights, little change in testicular weights, and a significant increase in the weights of the prostate and seminal vesicles, the effect of which was reversed by two hundred ug/day injections of melatonin. Prepubertal female rats injected with melatonin resulted in retardation of vaginal canalization and decreased uterine and pituitary weights.

Although it is known that light does affect the pineal, essentially nothing is known about the relation between the physical characteristic of light sources and their ability to modify pineal function, e.g. degree of light, wavelength, etc.

In order to understand the affect of melatonin on other organs in the body, it is important to first understand the biosynthesis of this compound. The biosynthesis of melatonin is first initiated by the uptake of circulating tryptophan into the parenchymal cells. This amino acid is then hydroxylated at the number five position by tryptophan hydroxylase and dihydro-nicotinamide adenine dinucleotide phosphate (NADPH_2) (Wurtman, et al., 1968). Tryptophan hydroxylase has been shown by Lovenberg, et al. (1967) that, in the rat, the activity of this enzyme is higher per unit weight of pineal tissue than any other tissue or

organ in the body. The resulting 5-hydroxytryptophan is rapidly decarboxylated by 5-hydroxytryptophan decarboxylase, which is affected by light (Hernandez and Illnerova, 1970), and pyridoxyl phosphate to form 5-hydroxytryptamine (Buzzard and Nytech, 1957). Quay and Halevey in 1962 demonstrated that the concentration of 5-hydroxytryptamine in the pineal gland is several times greater than in any other organ in the rat. In the pineal, serotonin can be stored in place of norepinephrine within the local sympathetic nerve endings or within the parenchymal cells (Bertler, et al., 1964).

Klein and Weller (1970) have shown that serotonin within the pineal gland will either be metabolized by monoamine oxidase or it can be acetylated with the action of N-acetyltransferase and acetylcoenzyme A to form N-acetylserotonin, the immediate precursor to melatonin (Owman, 1965). N-acetylserotonin is converted to melatonin (5-methoxy-N-acetylserotonin) by the action of hydroxyindole-O-methyltransferase. This final step in the biosynthesis of melatonin occurs by the incorporation of a methoxyl group at the number five position. The methyl group is donated by S-adenosylmethionine.

Neff, et al. (1969) demonstrated, by fluorescent histochemical procedures, that approximately 30% of the 5-hydroxytryptamine stores of the pineal gland of the rat are found in the sympathetic nerve endings. Since this serotonin store almost completely disappears after treatment with desipramine, a drug

which blocks amine transport in neurons, it was concluded by the authors, that 5-hydroxytryptamine is synthesized in the parenchymal cells and subsequently taken into the axons. Zweig and Axelrod (1969) have shown that blockage of norepinephrine synthesis results in an elevation in pineal serotonin content. Bernad and Csaba (1970) have shown both serotonin and histamine in the intracytoplasmic granules of the pinealocyte through tissue culture preparations and fluorescence microscopy of the pineal gland of the rat. These granules also contained mucopolysaccharides and protein, but no catecholamines could be demonstrated.

Klein and Rowe (1970) observed that inhibition of the oxidation of C¹⁴-serotonin in organ culture of pineal glands of rats with harmine resulted in enhanced N-acetylation. The resulting high levels of N-acetylserotonin caused increased melatonin production. These investigators also reported decreased production of hydroxyindoleacetic acid, hydroxytryptophol, and methoxytryptophol. The hydroxyindole-O-methyltransferase activity in the glands of harmine-treated rats was no different than in non-treated controls. This would indicate that there was an increase in melatonin production by a mechanism not dependent upon increased production of hydroxyindole-O-methyltransferase.

As previously mentioned, melatonin is effective at

very low concentrations as a blanching agent and is found in very low concentrations in the pineal gland of all animals studied as well as in the retinas of amphibians, fishes, reptiles and some birds (Quay, 1965). At this time however, there is no truly effective way of assaying for melatonin; therefore, demonstration of hydroxyindole-O-methyltransferase activity or changes of hydroxyindole-O-methyltransferase activity is considered sufficient to establish the melatonin forming ability of this tissue (Van de Veerdonk, 1965). Current methods for possible assaying of melatonin include incubation of homogenized tissue with N-acetylserotonin and a tagged methyl donor (C^{14} -methyl-S-adenosylmethionine) and radioimmunoassay for the tagged melatonin; or by melanophore response of the larvae of amphibians (Ralph and Lynch, 1970). Hydroxyindole-O-methyltransferase is the rate controlling enzyme in these reactions.

Melatonin has been suggested to be a pineal hormone which has an inhibitory effect on the reproductive organs in mammals. In 1963, Wurtman, Axelrod and Chu studied melatonin to determine the effect of this compound on the ovary of the rat. They injected one to twenty μ g melatonin/day into twenty-eight day old female rats and found delayed vaginal opening, decreased ovarian weights and decreased incidence of cornified vaginal smears (estrus). They showed that circulating melatonin was selectively taken up and retained by the pineal and the ovary,

the effect of which was reduced when the animals were similarly treated but exposed to constant light. In a companion study from this same laboratory, Chu, et al. (1964), confirmed the previous work using twenty μg melatonin/day and went on to ascertain the sensitivity of melatonin in animals prior to gonadal maturation. Thus, they demonstrated that the immature rat was exceedingly sensitive to minute (one-tenth) doses of melatonin.

Kappers (1962) injected five hundred μg melatonin/day into male rats and observed decreased weights of the seminal vesicles. Ebels and Prob (1965), however, injected thirty μg melatonin/day into twenty-eight day old female rats and did not observe any appreciable differences on vaginal openings, vaginal smears, and ovarian weights, i.e. the vagina and ovary resembled those of non-melatonized animals. Chessman (1970) and Chessman and Fariss (1970) determined the gonadotropin inhibiting substance of the pineal gland, by mass spectrometry and amino acid analysis to be 8-arginine vasotocin. Thus, there then seems to be a disparity in our knowledge of the function of melatonin, and a disagreement as to whether or not it is an inhibitor secreted by the pineal gland.

Debeljuk (1969) injected one hundred μg , three hundred μg , and five hundred μg of melatonin into prepubertal male rats housed under constant illumination. He noted decreased anterior pituitary and seminal vesicle weights. Only the injection of

five hundred μg of melatonin significantly affected testicular weight.

In 1965, Mc Issac and his co-workers isolated and described other compounds found in the mammalian pineal. These were 5-hydroxytryptophol and 5-methoxytryptophol which arise by deamination and reduction of 5-hydroxytryptamine followed by O-methylation.

Three years later, Fraschini, Mess and Martini (1968) pinealectomized male rats and found testicular hypertrophy, increased ventral prostate and seminal vesicles weights, and stores of increased amounts of pituitary luteinizing hormone. These investigators then stereotaxically placed pineal fragments or crystalline forms of melatonin into the median eminence or reticular formation of the midbrain. Five days later, they noted a significant reduction of adenohipophyseal luteinizing hormone. The luteinizing hormone content was not affected when melatonin was implanted into the pituitary gland. This would suggest that the indole compounds of the pineal modify pituitary function by acting on receptors located in the median eminence and/or the reticular formation of the midbrain. In the same year, Fraschini, Mess, Piva and Martini (1968) placed melatonin and 5-hydroxytryptophol into the median eminence and reticular formation of the midbrain of castrated male rats. This was followed five days later by a significant decrease in the pituitary luteinizing

hormone content. One year later, Fraschini and his co-workers (1969) implanted melatonin, 5-hydroxytryptamine, 5-methoxytryptophol, and 5-hydroxytryptophol in the midbrain and reticular formation of rats. They noted five days later that luteinizing hormone within the adenohipophysis was decreased in those animals in which melatonin or 5-hydroxytryptophol was implanted and a decrease in follicle stimulating hormone pituitary stores within the adenohipophysis of those animals containing implants of 5-hydroxytryptamine or 5-methoxytryptophol. The authors suggested that the pineal influences pituitary secretion of gonadotropins through two different humoral channels. Debeljuk (1969), however, observed no change in the gonadotropic levels of follicle stimulating hormone or luteinizing hormone in the pituitary following melatonin injection.

While the biogenic amines were being investigated in regard to their affect on reproductive phenomena, other compounds were also being studied in an attempt to ascertain the gonadotropic inhibiting substance. Pavel (1965) identified an extract of the pineal gland of the pig and found its biological, enzymatic and chromatographic characteristics similar to those of synthetic lysine vasotocin (8-lysine oxytocin). Three years later, Moszkowska and Ebels (1968) stated that synthetic arginine vasoctocin acts in the gonads or on the gonadotropic hormones and not on the secretions of the adenohipophysis in vitro.

In 1947, Borell and Örström (1947a) determined that the P^{32} turnover rate in the pineal gland of the rat is three to four times higher than that of the pituitary gland or choroid plexus. This turnover occurs in two principle ways: the first is a rapid turnover which enters chiefly into the carbohydrate phosphate esters, and the second is a slow turnover which enters the phospholipid and nucleic acids. In the same year, Borell and Örström (1947b) examined forty-two different organs of pineal-ectomized rats and observed that only the P^{32} turnover rate of the ovary was decreased to a statistically probable extent. There was a considerable increase in P^{32} turnover in the anterior and posterior aspects of the pituitary gland and in the tuber cinerium in female rats. They also noted an increased turnover in the pineal and anterior pituitary after castration and found this to be most pronounced in the female rat. The authors stated that there appears to be a reciprocal effect of a similar nature between the ovary and the pineal as that which exists between the adenohipophysis and the ovaries.

In 1961, Hellman and Larsson (1961) demonstrated in goats by radio-paper chromatographic assay that the pineal gland has an oxygen consumption approximately equal to that of the posterior pituitary. They also ascertained that the carbon dioxide and lactic acid formation from glucose decreases with age as do the other amino acids formed from this carbohydrate. Gluta-

mic acid was found to be in the greatest concentration of the amino acids formed in this way in the younger goats. Appreciable amounts of arginine, glutamine, gamma-aminobutyric acid, and aspartic acid were also noted. Microscopic observations revealed progressive degeneration and decreased numbers of parenchymal cells. The authors suggested that the high rate of amino acid formation found in the pineal gland of young goats can be regarded as a mechanism for supplying the raw material for a secretory product of protein nature.

Nir and his co-workers (1970) injected ten μg of estradiol-17- β subcutaneously into immature female rats (twenty-one to thirty-one days of age) and determined the pineal ribose-nucleic acid, deoxyribonucleic acid, and protein levels which were measured at fifteen, eighteen, and twenty-four hours after injection. The authors noted increased protein content of the pineal occurred twenty-four hours after the injection and this was accompanied by a prior elevation of pineal ribosenucleic acid and deoxyribonucleic acid content thus indicating accelerated protein metabolism.

Mitchell and Yochim (1970) indicated that prolonged daily illumination (twenty-two hours light; two hours dark) led to an apparent twelve hour increase in the duration of gestation. The time of parturition also was photoperiod-dependent as the animals exhibited a marked tendency to deliver during the light

phase of the light:dark cycle. These data indicated that the effect of light on the prolongation of pregnancy in part was due to an action of delayed implantation. Pinealectomy, however, did not significantly alter the effect of photoperiod on the duration of gestation or the time of day, which would then indicate light mediates control of the duration of gestation other than via the pineal gland. Huang and Everitt (1965) observed changes in the pineal weight during the latter stages of pregnancy. The change in weight was found to be inversely proportional to the number of fetuses carried.

Prop and Kappers (1961) demonstrated the presence of lipids, aromatic amines (catecholamines), ascorbic acid, and indole amines by histochemical and paper chromatographic procedures. Wight and Mackenzie (1971) demonstrated in the domestic fowl, by histochemical means, that there is an abundance of lipids in the form of triglycerides, phospholipids, and cholesterol and its esters. The authors did not observe any lipofuchsin. The authors also determined that there is considerable enzyme activity in the pineal gland. They histochemically identified alkaline phosphatase, acid phosphatase, adenosine tri-phosphate, lipase, non-specific esterase, nicotinamide adenine dinucleotide diaphorase, cytochrome oxidase, beta-glucuronidase, and amino peptidase. The pineal was found, by these investigators, to be rich in ribosenucleic acid, but

observed no glycogen, intracytoplasmic mucopolysaccharides, or Gomori-positive neurosecretory substances. Machado, et al. (1967, 1968) observed no 5-hydroxytryptamine fluorescence in nineteen day old rat fetuses. Two hours after birth the first fluorescence to appear in the sympathetic nerves was green (probably catecholamines). By the twenty-first day, the pineal gland of immature rats appears similar to the adult gland with a yellow fluorescence for serotonin.

Niemi and Ikoken (1960) histochemically demonstrated the presence of aminopeptidase activity in the pineal gland of the rat. It is their opinion that the activity was the result of production and secretion of certain agents of a protein nature. Zweens (1963) histochemically and biochemically demonstrated a significant decrease in the phospholipid concentration during proestrus with a peak observed during the diestrous phase of the estrous cycle. Quay (1959) biochemically demonstrated succinic dehydrogenase activity in the rat pineal with respect to age. This enzyme was found highest during the first six weeks of postnatal life with a decline at approximately one year concomitant with increased incidences of variation among the animals studied.

Quay (1956) histochemically demonstrated that the pineal gland of the rat displays an intense and specific reaction with chrom-alum hematoxylin and phloxine technique of Gomori

(1941) for staining pancreatic islets. The author states that the parenchymal cells of the pineal gland in the rat appeared to take part in a secretory process which is in agreement with Owman (1960-1961) who studied parenchymal cell secretory substances in the fetal rat.

The literature is replete with numerous studies of the pineal gland, but is most unfortunately devoid of any pertinent and critical information of the pineal gland during specific reproductive mechanisms, i.e. from the estrous cycle throughout its entirety to pregnancy and to pseudopregnancy. The major aim of this dissertation is therefore involved in an elucidation of the pineal gland of the rat (a) during the estrous cycle, (b) during pseudopregnancy and prolonged pseudopregnancy and (c) during pregnancy.

CHAPTER III

MATERIALS AND METHODS

The investigations carried out for this dissertation utilized 506 adult, Charles River, Caesarean-Originated, Barrier-sustained Sprague-Dawley, derived female albino rats. Of this number, 362 rats, sixty-nine to ninety-one days of age, were utilized for the studies involving the pineal gland and the estrous cycle. A total of fifty-two rats, ninety to 130 days of age, were investigated for the possible modulating roles of pregnancy on the pineal gland, and a total of ninety-two rats, ages ninety to 130 days, were likewise pursued for the possible influences of pseudopregnancy and prolonged pseudopregnancy (i.e. rats containing decidual reactions and manifesting extended luteal life beyond the normal range of pseudopregnancy, 13.0 days) on the pineal gland. Of the ninety-two on the pseudopregnancy studies, thirty were utilized as pseudopregnancy controls (i.e. without endometrial traumatization) and sixty-two were pursued with experimentally-induced decidual reactions.

Food (Purina Rat Chow) and water were supplied ad libitum and the animals were housed in a 72°F room with a cool white fluorescent light source (3000-5000 Å) on a 7:00 A.M. to

7:00 P.M. light cycle. Rats were numbered and housed six in a cage. Due to the importance of light on the pineal gland, the cages were oriented parallel to the light source so as to assure complete penetration of light into the cages.

In an effort to obtain as complete a reproductive history as possible, the animals were staged according to estrous cycle phases. Vaginal smears were taken twice a day at 8:00 A.M. and 5:00 P.M. for three weeks, subsequently stained with Giemsa solution, and were read and recorded. To determine the onset of estrus, animals were smeared at 9:00 P.M. (or at 12:00 A.M. if an estrus smear was not observed at 9:00 P.M.) on the evening following a proestrous smear, and the first indication of a predominance of vaginal cornified cells with degenerative nuclei was taken as the zero hour of estrus.

Rats were staged as carefully as possible so as to assure the critical selection of a sufficiently, quantifiable number for each of the six distinctively different estrous cycle stages. For each of the critical phases, a special effort was made to obtain animals prior to, during, and immediately after the sought after phases. It was, therefore, possible to obtain good diagnostic criteria for each of the stages under study. For each rat, therefore, the time of the onset of estrus, the hours after the onset of estrus and the hours within each stage were known prior to and at the time of necropsy.

The six distinctive stages of the estrous cycle of the albino rat were further subdivided into a seventh stage. This was done by dividing the phase of estrus of the cycle into two parts, the first encompassing hours zero through six and the second, hours six through twelve of the stage of estrus. Diestrus was ordinarily divided into early diestrus, zero through twenty-five hours within the stage, and late diestrus, twenty-five to fifty hours within the stage. In this study, the histochemical and biochemical tests were performed on the following stages of the cycle with the hours after onset of estrus as indicated in brackets: Early Estrus (E_1 , zero to six hours); Late Estrus (E_2 , six to twelve hours); Metestrus (ME, twelve to eighteen hours); Early Diestrus (D_1 , eighteen to fifty hours); Late Diestrus (D_2 , fifty-one to seventy-six hours); Preproestrus (PPE, seventy-seven to ninety hours); and Proestrus (PE, ninety to ninety-seven hours).

At the selected stage of the estrous cycle, the rats were weighed on a Torbal Torsion Balance to the nearest gram, necropsied by decapitation, and the following tissues were removed: the pineal, hypothalamus, hypophysis, thyroids, adrenals, ovaries, uterus, and vagina. These tissues were rapidly removed, cleaned, weighed on a FPE Precision balance to the nearest tenth of a milligram and either fixed in Bouins' fixative or frozen on dry ice for subsequent histomorphological

studies. Tissues fixed in Bouins' for eighteen hours were washed in tap water, dehydrated through graded alcohols, and embedded in Tissuemat (M. P. = $53 \pm 0.5^{\circ}\text{C}$).

Pineal glands of the rats were exposed by cutting first through the sutura fontalis and secondly along the os temporalis on both sides. The calvarium was then lifted dorsally to expose the cerebrum and cerebellum. The pineal, which lies in the triangular space formed at the junction of the cerebral hemispheres and the cerebellum, was rapidly removed, cleaned of connecting dura mater and blood vessels, weighed on a Roller Smith torsion balance to the nearest tenth of a milligram and either frozen or placed in Bouins' fixative for histochemical and biochemical assessments and histological characterization, respectively.

Gravimetric Data

Weights taken for each organ removed at necropsy, with the exception of the vagina and hypothalamus, were analyzed for the arithmetical mean, standard deviation, and standard error; these data were then applied to the Student's "t" test for significance using the Olivetti-Underwood Programma 101. This was done in an attempt to gravimetrically ascertain if a correlation exists between the pineal gland and changes of the other endocrine organs as influenced by the estrous cycle.

Histomorphology

Bouins' fixed tissues were dehydrated and embedded in paraffin, sectioned at five μ and stained with hematoxylin-eosin (Harris, 1900) for observing general morphology and chrom-alum hematoxylin (Bargmann, 1949) for demonstrating neurosecretory substances.

Paraffin sections were deparaffinized and placed in Bouins' fixative containing 3.5% chrom-alum for twelve to fourteen hours at 37°C. Sections were then washed in running tap water until colorless and oxidized for two minutes in one part 2.5% potassium permanganate, one part 5% sulfuric acid, and six to eight parts distilled water. Sections were washed in distilled water, bleached in 1% oxalic acid solutions for one minute, re-washed in distilled water, and stained for ten minutes. The stain used in this method consisted of a mixture of 50 ml 1% aqueous hematoxylin, 50 ml 3% aqueous chrom-alum $[\text{Cr}_2(\text{SO}_4)_3(\text{NH}_4)_2\text{SO}_4 \cdot 24 \text{H}_2\text{O}]$, 2 ml 5% aqueous potassium dichromate, and 1 ml 5% aqueous sulfuric acid. The stain was allowed to ripen for forty-eight hours at 0-4°C and filtered before use. The slides were differentiated in acid alcohol and/or ammonium hydroxide to develop nuclear color to a sharp contrast. Sections were then washed in running tap water for three minutes, counterstained with 0.5% aqueous phloxine solution for three minutes and rinsed in 5% aqueous solution of phosphotungstic acid for two minutes.

Following counterstaining, the sections were washed in running tap water for five minutes, dehydrated, and mounted with Permount.

Deep purple staining of chrom-alum hematoxylin positive substances indicated sites of the neurosecretory material. The nuclei stained with a slightly less purple intensity and the cytoplasm stained a pale pinkish red.

Enzyme Histochemistry

A minimum of three frozen pineal glands for each stage of the estrous cycle, pseudopregnancy, prolonged pseudopregnancy and pregnancy were sectioned at ten μ on an International cryostat and subjected to the following histochemical tests: alkaline phosphatase (naphthol phosphate method, Gomori, 1951); acid phosphatase (azo-coupling method, Barka, 1960); succinic dehydrogenase, a Krebs cycle enzyme which converts succinate to fumarate (Rosa and Velardo, 1954); and lactic dehydrogenase, which converts pyruvate to lactate (Manocha and Bourne, 1968).

Succinic Dehydrogenase Histochemistry

The method of Rosa and Velardo (1954) with a minor modification was used for the histochemical demonstration of succinic dehydrogenase (SDH 1.3.99.1.). Pineal glands removed at necropsy were frozen on dry ice and stored at -70°C until ready for use.

In preparation for sectioning, the tissues were mounted

by freezing on the stage of an International cryostat. Sections were cut at ten μ and mounted on coverslips in spaced serial sections and air-dried for one-half hour.

Sections were then incubated for two hours at 37°C in a media composed of 30 ml 0.1 M phosphate buffer containing 0.1% sodium cyanide at pH 8.2, 4 ml 0.5 M sodium succinate, and 30 mg nitro blue tetrazolium. The buffer was prepared by dissolving 1 gm sodium cyanide in 500 ml 0.1 M disodium phosphate solution, adjusting the solution to pH 8.2 by adding, with constant stirring, 0.1 M monosodium phosphate, then adding pH 8.2 0.1 M phosphate buffer to a final volume of one liter. Following incubation, the slides were rinsed in distilled water and fixed for three hours in 10% neutral formalin.

A control slide was also run with the same chemicals in the incubation media with the exception of the substrate, sodium succinate.

Criteria used for evaluating the histochemical reaction were as follows: (a) heavy deposits of violet to black diformazan granules were indicative of high succinic dehydrogenase activity sites; (b) areas staining pink were considered to have lower activity, their color probably indicative of an intermediate state of formazan production (Eadie, 1970).

The theory concerning the formation of the formazan crystals indicates that a hydrogen atom is removed from the

number two and three carbon atom; a hydrogen atom reacts with nicotinamide adenine dinucleotide (NAD) which is reduced to form dihydronicotinamide adenine dinucleotide (NADH). Since this coenzyme operates by virtue of reversible oxidation and reduction reactions, the dihydronicotinamide adenine dinucleotide is oxidized to again form nicotinamide adenine dinucleotide and a hydrogen atom is incorporated into the nitro blue tetrazolium molecule to form the insoluble formazan. Nicotinamide adenine dinucleotide operates as a hydrogen and electron transfer agent. One of the two hydrogens lost when succinate is oxidized, is incorporated into the nicotinamide adenine dinucleotide molecule, the reduction occurring in the para position, while the second hydrogen atom enters the media.

Since oxaloacetic acid is an inhibitor to succinic dehydrogenase by virtue of its similar chemical structure to succinate, sodium cyanide was used in the incubation media in order to trap by cyanhydrin formation any oxaloacetic acid possibly formed in the tissue during processing and/or incubation.

Lactic Dehydrogenase Histochemistry

The histochemical determination of lactic dehydrogenase (LDH 1.1.1.27) was demonstrated according to the slightly modified procedure of Manocha and Bourne (1968). The media used in this procedure was composed of 112.07 mg sodium lactate (lactic acid, sodium salt), 66.34 mg nicotinamide adenine

dinucleotide, 4.9 mg sodium cyanide, 10.5 mg magnesium chloride, 2.5 mg nitro blue tetrazolium, 750 mg polyvinyl pyrrolidone, 2.5 ml 0.06 M pH 7.4 phosphate buffer, and 10 ml distilled water.

Cryostat sections were allowed to dry one-half hour and then incubated in the above media for one hour at 37°C. On completion of incubation, the tissue was washed in distilled water, post-fixed in 10% neutral formalin for three hours, removed from the neutral formalin, washed in distilled water, and fixed to a slide with glycerol-gelatin. Slides were stored in the freezer until evaluation and photographs could be made.

A control slide was run along with all experimental slides. The media used was as above except lacked the substrate (sodium lactate).

Alkaline Phosphatase Histochemistry

Alkaline phosphatase (Alk. P'tase 3.1.3.1) acts on monoesters of ortho-phosphate and has little effect on phosphoric acid, meta phosphates, or pyrophosphates. The activity appears to depend on the presence of free hydroxyl (-OH) groups of tyrosine in the enzyme. This enzyme is activated by metal ions, particularly magnesium, and shows optimal activity in the alkaline range from pH 7.6-9.9.

Alkaline phosphatase can catalyze two types of reactions that of glycerol-1-phosphate and water to glycerol and inorganic phosphate and glycerol-1-phosphate and glucose to glycerol and

glucose-6-phosphate. It has been implicated in the maintenance of the intracellular concentration of phosphate and histochemically is frequently localized in cell membranes where active transport occurs (Danelli, 1953).

A modification of the naphthol phosphate method of Gomori (1952) was used in this study to demonstrate alkaline phosphatase. In this procedure, the naphthol of the phosphomonoesterase is trapped by simultaneous coupling with the diazonium salt, Fast Blue RR.

Magnesium ions are used as they activate alkaline phosphatase which splits the substrate (α -naphthol acid phosphate) with the release of naphthol AS-MX which combines with the diazonium salt to form the azo dye.

The procedure used is as follows: fresh cryostat sections were allowed to air-dry, then incubated in freshly prepared and filtered incubation media for one hour at 37°C. Media was prepared by combining in the following order, a 2% solution of barbitol sodium (sodium-5,5-diethylbarbituate), 10 mg naphthol acid phosphate (sodium salt), 0.2 ml 10% magnesium chloride and 25 mg Fast Blue RR Salt (the final pH being 9.2). The solution was then vigorously shaken, filtered, and used immediately. After one hour, the slides were washed in distilled water and placed in a 1% acetic acid solution, mounted with glycerol, and stored in the dark at -4°C until analyzed. A purple to purple-

black precipitate denoted sites of alkaline phosphatase activity. Controls minus the α -naphthol acid phosphate were run with each experimental section.

Acid Phosphatase Histochemistry

Acid phosphatase (Acid P'tase 3.1.3.2) catalyzes the hydrolysis of most phosphomonoesters, of creatine phosphate and of amino-phosphate. It is activated by manganese and has an optimal pH range of 4.5-5.2. Dimethyl formamide was used to dissolve the substrate. The reaction sequence is the same as for alkaline phosphatase.

A modification of Burnstone's (1959) method for the histochemical demonstration of acid phosphate was used. The procedure involved use of fresh frozen cryostat sections which were air-dried on cover slips. The incubation media consisted of 5 mg naphthol acid phosphate (naphthol AS-MX phosphate), 0.25 ml dimethyl formamide, 25 ml 0.2 M pH 5.2 acetate buffer, 0.1 ml 10% manganese chloride, 30 mg Fast Red Violet LB (diazonium salt), and 25 ml distilled water to a final volume of 50 ml.

Experimental and control (minus α -naphthol acid phosphate) tissue sections were concurrently processed.

Counterstaining

Counterstaining of the histochemical procedure was

accomplished by using hematoxylin alone or hematoxylin-eosin in order to generally localize sites of activity. All such counterstaining for each stain was as follows: hematoxylin, forty-five seconds; eosin, fifteen seconds. Counterstained sections were mounted as previously indicated.

Biochemical Determinations

Frozen pineal glands were subjected to colorimetric biochemical analyses. These were succinic dehydrogenase (Quay, 1959), lactic dehydrogenase (Berger and Broida, 1969), alkaline phosphatase and acid phosphatase (Bessey, et al., 1946), glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase (Reitman and Frankel, 1957).

All biochemical determinations were performed on the Beckman DU Spectrophotometer with a ten mm cell. The instrument was used exclusively between 9:00 P.M. and 5:00 A.M. as electrical current was found to fluctuate during the day thus giving false readings.

Four samples were run for each of the stages of the estrous cycle. Two samples were run on one night and two on a succeeding night in an effort to minimize possible sources of error on any one night, whether it be chemical, instrumental, or human. Calibration curves were run each night along with each procedure.

Succinic Dehydrogenase Biochemistry

Pineal succinic dehydrogenase (SDH) activity was determined according to a modification of the procedure of Quay (1959). Pineal glands were individually homogenized in 0.50 ml 0.025 M sodium phosphate buffer containing 0.1% sodium cyanide; 0.50 ml 5.4% sodium succinate containing M/100 aluminum chloride and M/1000 calcium chloride; and 0.50 ml 1.0% tetrazolium 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride. The above solution, with homogenized pineal, was incubated in a 37°C water bath for two hours with constant shaking.

After completion of this incubation, 10% neutral formalin was added to terminate the formazan production and the solution taken to dryness in a 68°C oven. The colored formazan was extracted with A.C.S. (American Chemical Society) grade ethyl acetate and the optical density was determined at 490 m μ on a Beckman DU Spectrophotometer. Ethyl acetate was used as a reference blank.

The micromoles of tetrazolium reduced per pineal was calculated according to the method of Shelton and Rice (1957). The formula used is $D=KCL$ where C is the molar concentration of substance in solution, L is the length of the light path in centimeters (in this case, one), D is the optical density of the sample, and K is a constant found to be 5.34 at O.D.490 by Shelton and Rice. The resulting molar concentration of the

substance in solution was divided by the wet weight of the pineal gland used which resulted in the micromoles of tetrazolium reduced or the micromoles of formazan formed per milligram pineal tissue.

Lactic Dehydrogenase Calibration Curve

The calibration curve for the colorimetric determination of lactic dehydrogenase (LDH) was performed according to the following procedure (Sigma Technical Bulletin #500). Six tubes were prepared as follows:

(1) Tube #	(2) Pyruvate Substrate (ml)	(3) H ₂ O (ml)	(4) Berger-Broida LDH Units/ Pineal
1	1.0	0.1	0
2	0.8	0.3	280
3	0.6	0.5	640
4	0.4	0.7	1040
5	0.2	0.9	1530
6	0.1	1.0	2000

Into each of the six tubes, 1.0 ml color reagent (2,4-dinitrophenylhydrazine) was added, mixed, and left at room temperature ($25 \pm 5^{\circ}\text{C}$). Exactly twenty minutes later, 10.0 ml 0.40 N sodium hydroxide was pipetted into the tubes and they were mixed by inversion. Five minutes later, the optical densities of the solutions were read at $525 \text{ m}\mu$ with carbon dioxide free distilled water as a reference.

The calibration curve was obtained by plotting optical

density readings against the corresponding units of lactic dehydrogenase from column four above.

Lactic dehydrogenase in this procedure catalyzes the conversion of pyruvic acid to lactic acid according to the reaction:



The speed of the reaction is proportional to the amount of lactic dehydrogenase present. Pyruvic acid reacts with 2,4-dinitrophenylhydrazine to form an intensely colored hydrazone. Lactic acid, dihydronicotinamide adenine dinucleotide phosphate and nicotinamide adenine dinucleotide do not add a significant amount of optical density to the solution; therefore, the standardized pyruvate substrate which yields the same hydrazone optical density will be accurate and reproducible. The amount of pyruvate remaining after the incubation is inversely proportional to the amount of lactic dehydrogenase present in the reaction. One unit of lactic dehydrogenase activity will reduce $4.8 \times 10^{-4} \mu\text{M}$ pyruvate/minute/mg pineal.

Total Lactate Dehydrogenase Biochemistry

Frozen pineal glands were homogenized with 0.50 ml carbon dioxide free distilled water and diluted one part sample plus four parts distilled water (1:4). This was added to 1.0 ml Sigma standardized pyruvate substrate and 1.0 mg dihydronicotinamide adenine dinucleotide and placed in a water bath at 37°C.

Exactly thirty minutes later, the sample was removed from the water bath and 1.0 ml of Sigma standardized 2,4-dinitrophenylhydrazine was added to the sample (stops the reaction and starts the color development), mixed by swirling, and left at room temperature ($25 \pm 5^{\circ}\text{C}$).

Twenty minutes after the addition of the color reagent, 10.0 ml 0.40 N sodium hydroxide was added to each sample and mixed by inversion. Fifteen minutes later, the optical density of the sample was read at $525 \text{ m}\mu$ using carbon dioxide free distilled water as a reference blank. Lactic dehydrogenase activity was then determined from the calibration curve; the units per milligram of pineal gland were determined by dividing the units of lactic dehydrogenase activity by the weight of the pineal gland.

Acid Phosphatase and Alkaline Phosphatase Calibration Curves

The alkaline phosphatase (Alk. P'tase) and acid phosphatase (Acid P'tase) colorimetric procedures used were a modification of the method of Bessey, et al. (1946) and were taken from the Sigma Technical Bulletin #104 to ascertain the units of activity of this enzyme within the pineal gland.

A calibration curve for both alkaline and acid phosphatases was first determined according to the following procedure:

- (1) A standard solution was prepared by pipetting 0.50

ml p-nitrophenol standard solution (Sigma Stock Number 104-1) into a 100 ml volumetric flask, diluting to 100 ml with 0.02 N sodium hydroxide and mixing by inversion.

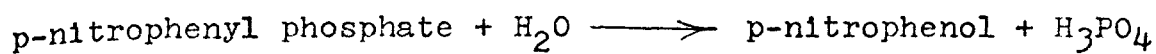
(2) The standard solution and 0.02 N sodium hydroxide were pipetted according to the following chart:

(1) Tube #	(2) Standard Solution (ml)	(3) 0.02 <u>N</u> NaOH (ml)	(4) Equivalent to the following: Sigma Units Alkaline	(5) Sigma Units Acid
1	1.0	10.0	1.0	0.28
2	2.0	9.0	2.0	0.56
3	4.0	7.0	4.0	1.12
4	6.0	5.0	6.0	1.67
5	8.0	3.0	8.0	2.23
6	10.0	1.0	10.0	2.80

(3) The optical density of the above mixtures was determined at 410 m μ using 0.02 N sodium hydroxide as the reference.

(4) The calibration curves for both acid and alkaline phosphatase were prepared by plotting the six optical density readings against their respective units as listed in columns four and five.

The reagent, p-nitrophenyl phosphate, is colorless. Upon hydrolysis of the phosphate group, p-nitrophenol, a yellow salt is liberated. In so doing, the substrate itself acts as an indicator of the amount of splitting and hence the measure of phosphatase activity. The following is the overall reaction:



p-nitrophenol is colorless in an acidic solution and yellow in an alkaline solution. One unit of alkaline and acid phosphatase will liberate 1.0 μM p-nitrophenol/hour/mg pineal.

Alkaline Phosphatase Biochemistry

Alkaline phosphatase activity was determined by pipetting into each of three tubes 0.50 ml alkaline buffer solution (p-nitrophenol) and 0.50 ml stock substrate. The tubes were placed in a 38°C water bath and allowed to warm for a few minutes, and 0.10 ml water was pipetted into one tube (blank) and the pineal glands were homogenated and added to the other tubes. These were placed in a 38°C water bath for exactly thirty minutes and removed. To each tube, 10.0 ml 0.020 N sodium hydroxide was added and mixed by inversion. The addition of the sodium hydroxide terminated the reaction and developed the color which is stable for several hours.

Two optical density readings were then made as follows at 410 m μ on the Beckman DU Spectrophotometer with the blank as reference: (a) initial optical density readings were determined after addition of sodium hydroxide; (b) two drops of concentrated hydrochloric acid was then added to remove color due to the p-nitrophenol and the second optical density reading made.

Alkaline phosphatase units of activity was then determined on both readings from the calibration curve and the

second reading subtracted from the first to give actual (or corrected) alkaline phosphatase activity. The corrected alkaline phosphatase activity of the sample was then divided by the milligram weight of the pineal gland used to determine the units of activity per milligram pineal.

Acid Phosphatase Biochemistry

Three tubes were used for each stage of the estrous cycle on each of the nights the determination was run. Into each tube was pipetted 0.50 ml substrate (40 mg p-nitrophenyl phosphate in 10 ml distilled water) and 0.50 ml citric acid buffer (pH 4.8). Into one of the tubes 0.20 ml distilled water was pipetted (blank) and into each of the other two tubes, a pineal gland was placed and homogenized. All three tubes were then placed in a shaker water bath at 38°C. Exactly thirty minutes later, the tubes were removed from the water bath and 5.0 ml 0.10 N sodium hydroxide was added to each tube to stop the reaction and develop the color. The optical density of the solutions were read at 410 m μ . Units of acid phosphatase activity were determined from the calibration curve; the units per milligram of pineal gland were determined by dividing the units of acid phosphatase activity by the weight of the pineal gland.

Glutamic-Oxaloacetic Transaminase and Glutamic-Pyruvic
Transaminase Calibration Curve

A preliminary study was performed on the units of activity of glutamic-oxaloacetic transaminase (GOT 2.6.1.1) and glutamic-pyruvic transaminase (GPT 2.6.1.2) in an attempt to learn and understand more about the function of the pineal gland with respect to reproductive phenomena.

The determination of the aforementioned two enzymes was performed according to the Sigma Technical Bulletin #505 (a modification of Reitman and Frankel, 1957). A calibration curve was also run for these two enzymes according to the following protocol:

(1) Into six test tubes, the solutions indicated in columns (2), (3), and (4) were pipetted:

(1) Tube #	(2) 505-10 Std. Sol. (ml)	(3) Sigma Prepared Substrate (ml)	(4) H ₂ O (ml)	(5) Sigma Frankel Units GOT	(6) Sigma Frankel Units GPT
1	0	1.0	0.20	0	0
2	0.10	0.9	0.20	20	23
3	0.20	0.8	0.20	55	50
4	0.30	0.7	0.20	95	83
5	0.40	0.6	0.20	148	125
6	0.50	0.5	0.20	216	---

(2) 1.0 ml Sigma color reagent (2,4-dinitrophenyl hydrazine and hydrochloric acid) was added to each tube. The tubes were shaken gently and allowed to stand at room temperature

(25 ± 5°C) for twenty minutes.

(3) 10.0 ml 0.40 N sodium hydroxide was added and mixed by inversion.

(4) After five minutes, the optical density was determined at 505 m μ using distilled water as a reference.

(5) A calibration curve was then constructed plotting the optical density readings against the corresponding units of glutamic-oxaloacetic transaminase found in column five.

(6) A second curve was also set up plotting the optical density readings against the corresponding units of glutamic-pyruvic transaminase found in column six.

Glutamic-oxaloacetic transaminase is an enzyme which catalyzes the conversion of aspartic acid and α -ketoglutaric acid to oxaloacetic acid and glutamic acid according to the equation:



Glutamic-pyruvic transaminase is an enzyme which catalyzes the conversion of alanine and α -ketoglutaric acid to pyruvic acid and glutamic acid according to the equation:
 alanine + α -ketoglutaric acid $\xrightarrow{\text{GPT}}$ pyruvic acid + glutamic acid
 In the procedure used, the amount of oxaloacetic acid or pyruvate formed in one hour is determined colorimetrically by the formation of a hydrazone which is highly colored. Theoretically,

one unit of activity of either glutamic-oxaloacetic transaminase or glutamic-pyruvic transaminase will form 4.82×10^{-4} μM of glutamate per minute at pH 7.5.

Glutamic-Oxaloacetic Transaminase Biochemistry

Pineal glands were homogenized in 1.0 ml of Sigma standardized aspartate- α -ketoglutarate^(T_m) substrate (pH 7.5) and placed in a shaker water bath at 37°C. Exactly one hour later, the samples were removed from the bath and 1.0 ml of Sigma standardized 2,4-dinitrophenyl hydrazine was added to each tube (which stops the enzymatic reaction and starts the color reaction). The samples were then mixed by inversion and allowed to stand at room temperature ($25 \pm 5^\circ\text{C}$). Exactly twenty minutes later, 10.0 ml 0.40 N sodium hydroxide was added and each tube mixed. Five minutes later, the optical densities of the solutions were read at 505 m μ , utilizing distilled water as a reference blank. The units of glutamic-oxaloacetic transaminase activity were determined from the calibration curve; the units per milligram of pineal gland were determined by dividing the units of glutamic-oxaloacetic acid activity by the weight of the pineal gland.

Glutamic-Pyruvic Transaminase Biochemistry

Pineal glands were homogenized in 1.0 ml Sigma standardized aspartate- α -ketoglutarate^(T_m) substrate (pH 7.5) and placed

in a shaker water bath at 37°C. Exactly thirty minutes later, 1.0 ml of Sigma standardized 2,4-dinitrophenyl hydrazine (which stops the enzymatic activity and starts the color reaction) was added to the samples, shaken, and left at room temperature. Twenty minutes after the addition of the color reagent 10.0 ml 0.40 N sodium hydroxide was added and each tube was mixed by inversion. The optical densities were read after five minutes at 505 m μ with distilled water as a reference blank. The units of glutamic-pyruvic transaminase activity were determined from the calibration curve; the units per milligram of pineal gland were determined by dividing the units of glutamic-pyruvic transaminase activity by the weight of the pineal gland.

Reproductive Studies

Pseudopregnancy was induced by vibration of the cervix during estrus. A group of pseudopregnant rats were endometrially traumatized by scratching the antimesometrial aspect of the endometrium on the fifth day after vibration of the cervix to induce a decidual reaction of the uterus according to the procedure of Velardo, et al., 1955. Both groups of pseudopregnant rats were necropsied on days six, eight, ten, and thirteen. The pineal glands were removed as previously detailed, and either fixed in Bouins' or frozen and subjected to the previously mentioned histo- and cytochemical analyses.

Pregnant rats were necropsied on six, eight, ten, thirteen, fifteen, eighteen, twenty, and twenty-one days of pregnancy. Pineal glands were removed and studied gravimetrically and histochemically as previously stated.

Photomicrographs

Photomicrographs of representative histochemical reactions were taken using an American Optical microscope, equipped with an oil immersion objective with a 5X adapter, Kodak 35 millimeter camera loaded with Kodak Panatomic X Film (FX-402 35 millimeter), and utilizing a Waatten Number 58 filter. Exposure time and shutter speed were employed as per the American Optical Photomicrographic Equipment Manual. Developing and printing were performed by the investigator.

CHAPTER IV

RESULTS

A. Prefatory Remarks

1. Gross Anatomical Aspects of Pineal Gland of the Adult Albino Rat.

In situ, the epiphysis cerebri (pineal gland) in the adult mammal is a readily observable spherical body in the triangular space formed by the occipital poles of the cerebral hemispheres and the tentorium cerebelli which forms the roof of the cerebellum. In man, this gland is seen to lie between the two superior colliculi. The pineal gland is connected to the third ventricle, from whence it originates, by the pineal stalk which is a relatively long, thin and solid appendage except at its base where there is a very short recessus pinealis. The recess is bordered dorsally by the habenular commissure and ventrally by the posterior commissure.

Surrounding the pineal gland is a thick covering of dura mater and an encapsulating layer of pia mater. Meningeal mesenchyme surrounds the pineal gland, thus forming a thin sheath of connective tissue which invades the organ as connective tissue septa containing blood vessels (Crosby, et al., 1962).

Arterial blood to the pineal gland in adult mammals is supplied from medial branches of the posterior choroidal artery which enter first the tentorium cerebelli. At this point, these blood vessels become intimately associated with the nervi conarii in which are carried the postganglionic sympathetic nerves from the superior cervical ganglia. Both the blood vessels and nerves enter the pineal body together. The nerves ramify throughout the entire structure and the arterial branches terminate as small capillaries deep within the body of the pineal gland (Le Gros Clark, 1939-1940).

Venous drainage from the pineal gland is via small capillaries from the body of the pineal. These venous tributaries empty into larger veins located in the pia mater. The larger veins eventually empty into either the superior sagittal sinus or the sinus confluens. Sexual differences in the blood content of the pineal gland of the rat have not been observed (Quay, 1958).

The pineal gland of the rat is a small, conical shaped structure measuring approximately one millimeter in length and one-half millimeter in width. The actual weight range is from 0.9-1.9 mg with a mean weight of approximately 1.4-1.5 mg. The weights of the pineal varies with the different stages of the estrous cycle cf. Table I, p. 57.

2. Normal Pineal Gland During Estrous Cycle of the Rat as Seen with Hematoxylin and Eosin and Bargmann's Chrom-Alum Hematoxylin.

Microscopic examination of tissue sections of the pineal gland of the rat, after staining with hematoxylin-eosin or Bargmann's chrom-alum hematoxylin, reveals neurosecretory material and two types of cells. The first type of cell is round and contains a round nucleus and prominent nucleolus. The so-called round cell has a pale staining cytoplasm. The second type of cell is irregular in shape and contains an irregularly shaped nucleus and seemingly does not contain an apparent nucleolus. This so-called irregular cell has a darker staining cytoplasm (Plate I, figure 20). Many of these cells are usually found in close association with pineal capillaries (Plate I, figure 21; Plate II, figures 22 and 23).

An abundance of neurosecretory material has also been observed in the pineal stalk (Plate III, figures 24 and 25) which is believed to contain aberrant fibers from the posterior commissure. These fibers course into and out of the stalk with seemingly no readily identifiable connection with the pineal gland itself

B. Gravimetric, Enzymorphological and Biochemical Studies on the Pineal Gland During the Estrous Cycle of the Adult Rat.

1. Gravimetric Data

In an effort to ascertain as complete a picture as

possible as to the possible influences of the different stages of the estrous cycle on the pineal gland and vice versa, it appeared of specific interest to determine the possible gravimetric differences throughout the cycle. Inasmuch as it has been roundly claimed in the literature that the pineal gland is somehow related to reproductive phenomena, it appeared of further interest to develop companion studies on the hypophyseal, ovarian, uterine, and adrenal weights of these same animals.

a. The Pineal Gland

Interestingly enough, of the seven different stages of the estrous cycle, the pineal gland was heaviest (0.647 mg%) during metestrus and lightest (0.467 mg%) during late estrus. Pineal gland weights throughout the estrous cycle are summarized in Table I, and for direct comparisons, stage-wise, the pineal of rats in metestrus > late diestrus > preproestrus > proestrus > early diestrus > early estrus > late estrus (Tables I and II; Figure 1).

b. The Hypophysis

In a companion study of the pituitary bodies of the rats, in which the pineal glands were studied, it was determined that the hypophysis was heaviest (5.876 mg%) during metestrus, as were the pineals, but were lightest during late diestrus (4.737 mg%) in contrast to late estrus for the pineals. Comparatively, stage-wise, the hypophyses of rats in metestrus > early and late

estrus phases (almost identical, 5.408 and 5.390 mg% respectively) > early diestrus and preproestrus (again almost identical, 4.855 and 4.842 mg% respectively) > proestrus > late diestrus (Tables I and III; Figure 1).

c. The Ovaries

Companion gravimetric studies on the ovaries revealed that those of rats in late estrus were heaviest (54.776 mg%) while those in proestrus and late diestrus were lightest, the latter two being almost comparable (i.e. 47.640 and 47.683 mg% respectively). Comparatively, stage-wise, the ovaries of rats in late estrus > early estrus > metestrus > early diestrus > preproestrus > proestrus and late diestrus (Tables I and IV; Figure 2).

d. The Uteri

Studies on a much investigated reproductive tract, target-organ, the uterus, identified additional points of interest. Of the seven different stages studied, the uteri of early estrus were the heaviest (227.217 mg%), whereas those of early diestrus were the lightest (138.903 mg%). Comparatively, stage-wise, the uteri of rats in early estrus > proestrus > late estrus > late diestrus > metestrus > preproestrus > early diestrus (Tables I and V; Figure 3).

e. The Adrenal Glands

Inasmuch as the adrenal glands have been importantly related to reproductive function, it thus became of essential

significance to include similar studies on the gravimetrics of these glands. Interestingly enough, as with the ovaries, the adrenal glands of rats in late estrus were heaviest (31.075 mg%) and those of animals in proestrus were lightest (26.167 mg%). Comparatively, stage-wise, late estrus > late diestrus > metestrus > early diestrus > early estrus and preproestrus > proestrus (Tables I and VI; Figure 2).

TABLE I

COMPARISON OF PINEAL, HYPOPHYSEAL, OVARIAN, ADRENAL, AND
UTERINE WEIGHTS OF ADULT ALBINO RATS IN MG% DURING
DIFFERENT PHASES OF THE ESTROUS CYCLE

	Pineal	Hypophysis	Ovary	Uterus	Adrenal
Pro- estrus	0.554 ±0.000* (56)	4.798 ±0.085 (56)	47.640 ±0.851 (57)	212.108 ±4.17 (58)	26.167 ±0.557 (58)
Early Estrus	0.487 ±0.044 (28)	5.408 ±0.320 (28)	53.921 ±2.992 (28)	228.217 ±2.123 (28)	27.034 ±2.024 (28)
Late Estrus	0.467 ±0.061 (28)	5.390 ±0.349 (28)	54.776 ±2.532 (28)	194.870 ±2.357 (28)	31.075 ±1.042 (28)
Met- estrus	0.647 ±0.014 (56)	5.876 ±0.653 (56)	53.076 ±1.150 (56)	157.017 ±3.380 (56)	29.035 ±0.570 (56)
Early Diestrus	0.504 ±0.000 (27)	4.855 ±0.104 (27)	50.367 ±0.864 (27)	138.903 ±3.473 (27)	28.216 ±0.554 (27)
Late Diestrus	0.613 ±0.083 (27)	4.737 ±0.176 (27)	47.683 ±1.000 (27)	169.017 ±6.017 (27)	29.313 ±0.914 (27)
Prepro- estrus	0.583 ±0.031 (50)	4.842 ±0.104 (55)	47.884 ±0.734 (56)	156.670 ±4.030 (56)	27.092 ±0.480 (54)

* plus-minus designations are standard error.

** figures in brackets are numbers of animals in each group.

TABLE II

SIGNIFICANCES OF DIFFERENCES OF PINEAL GLAND WEIGHTS
DURING SEVEN DIFFERENT STAGES OF THE ESTROUS
CYCLE OF THE RAT, P VALUES*

	Pro- estrus	Early Estrus	Late Estrus	Met- estrus	Early Diestrus	Late Diestrus	Prepro- estrus
Pro- estrus (56)		<0.20	<0.20	<0.001		<0.50	<0.30
Early Estrus (28)	<0.20		<0.80	<0.001	<0.60	<0.20	<0.10
Late Estrus (28)	<0.20	<0.80		<0.01	<0.60	<0.20	<0.10
Met- estrus (56)	<0.001	<0.001	<0.001		<0.001	<0.70	<0.05
Early Diestrus (27)		<0.60	<0.60	<0.001		<0.20	<0.02
Late Diestrus (27)	<0.50	<0.20	<0.20	<0.70	<0.20		<0.80
Prepro- estrus (50)	<0.30	<0.10	<0.10	<0.05	<0.02	<0.80	

*P= The probability that the difference observed in mg% being compared would occur only by chance.

TABLE III

SIGNIFICANCES OF DIFFERENCES OF HYPOPHYSEAL WEIGHTS
DURING SEVEN DIFFERENT STAGES OF THE ESTROUS
CYCLE OF THE RAT, P VALUES*

	Pro- estrus	Early Estrus	Late Estrus	Met- estrus	Early Diestrus	Late Diestrus	Prepro- estrus
Pro- estrus (56)		<0.20	<0.20	<0.20	<0.70	<0.80	<0.80
Early Estrus (28)	<0.10		<0.80	<0.70	<0.20	<0.10	<0.20
Late Estrus (28)	<0.20	<0.80		<0.60	<0.20	<0.20	<0.20
Met- estrus (56)	<0.20	<0.70	<0.60		<0.20	<0.10	<0.20
Early Diestrus (27)	<0.70	<0.20	<0.20	<0.20		<0.60	<0.40
Late Diestrus (27)	<0.80	<0.10	<0.20	<0.10	<0.60		<0.70
Prepro- estrus (55)	<0.80	<0.20	<0.20	<0.20	<0.40	<0.70	

* P = The probability that the difference observed in mg% being compared would occur only by chance.

TABLE IV

SIGNIFICANCES OF DIFFERENCES OF OVARIAN WEIGHTS
DURING SEVEN DIFFERENT STAGES OF THE ESTROUS
CYCLE OF THE RAT, P VALUES*

	Pro- estrus	Early Estrus	Late Estrus	Met- estrus	Early Diestrus	Late Diestrus	Prepro- estrus
Pro- estrus (57)		<0.05	<0.02	<0.001	<0.05	<0.975	<0.90
Early Estrus (28)	<0.05		<0.99	<0.80	<0.30	<0.10	<0.10
Late Estrus (28)	<0.02	<0.99		<0.60	<0.30	<0.02	<0.02
Met- estrus (56)	<0.001	<0.80	<0.60		<0.10	<0.001	<0.001
Early Diestrus (27)	<0.05	<0.30	<0.30	<0.10		<0.05	<0.05
Late Diestrus (27)	<0.975	<0.10	<0.02	<0.001	<0.05		<0.90
Prepro- estrus (56)	<0.90	<0.10	<0.02	<0.001	<0.05	<0.90	

* P = The probability that the difference observed in mg% being compared would occur only by chance.

TABLE V

SIGNIFICANCES OF DIFFERENCES OF UTERINE WEIGHTS
DURING SEVEN DIFFERENT STAGES OF THE ESTROUS
CYCLE OF THE RAT, P VALUES*

	Pro- estrus	Early Estrus	Late Estrus	Met- estrus	Early Diestrus	Late Diestrus	Prepro- estrus
Pro- estrus (58)		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Early Estrus (28)	<0.001		<0.001	<0.001	<0.001	<0.001	<0.001
Late Estrus (28)	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001
Met- estrus (56)	<0.001	<0.001	<0.001		<0.001	<0.10	<0.95
Early Diestrus (27)	<0.001	<0.001	<0.001	<0.001		<0.001	<0.005
Late Diestrus (27)	<0.001	<0.001	<0.001	<0.10	<0.001		<0.10
Prepro- estrus (56)	<0.001	<0.001	<0.001	<0.95	<0.005	<0.10	

* P = The probability that the difference observed in mg% being compared would occur only by chance.

TABLE VI

SIGNIFICANCES OF DIFFERENCES OF ADRENAL WEIGHTS
DURING SEVEN DIFFERENT STAGES OF THE ESTROUS
CYCLE OF THE RAT, P VALUES*

	Pro- estrus	Early Estrus	Late Estrus	Met- estrus	Early Diestrus	Late Diestrus	Prepro- estrus
Pro- estrus (58)		<0.70	<0.001	<0.15	<0.02	<0.005	<0.30
Early Estrus (28)	<0.70		<0.10	<0.40	<0.60	<0.40	<0.98
Late Estrus (28)	<0.001	<0.10		<0.999	<0.02	<0.30	<0.001
Met- estrus (60)	<0.15	<0.40	<0.999		<0.40	<0.80	<0.02
Early Diestrus (27)	<0.02	<0.60	<0.02	<0.40		<0.40	<0.10
Late Diestrus (27)	<0.005	<0.40	<0.30	<0.80	<0.40		<0.05
Prepro- estrus (54)	<0.30	<0.98	<0.001	<0.02	<0.10	<0.005	

* P = The probability that the difference observed in mg% being compared would occur only by chance.

FIGURE 1

GRAPH SHOWING WEIGHT RELATIONSHIPS OF THE HYPOPHYSIS AND PINEAL GLAND DURING SEVEN DIFFERENT STAGES OF THE ESTROUS CYCLE OF THE RAT

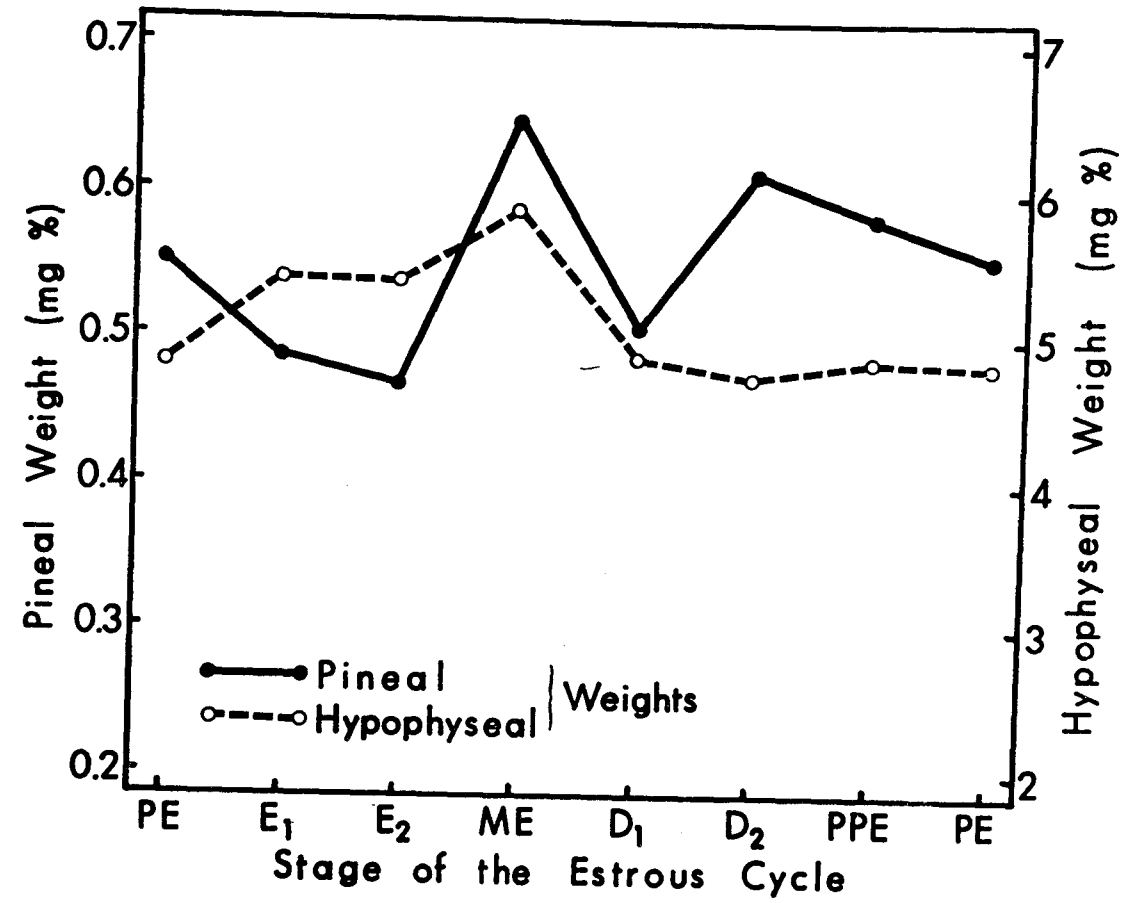


FIGURE 2

GRAPH SHOWING WEIGHT RELATIONSHIPS OF THE OVARY, ADRENAL AND PINEAL GLAND DURING SEVEN DIFFERENT STAGES OF THE ESTROUS CYCLE OF THE RAT

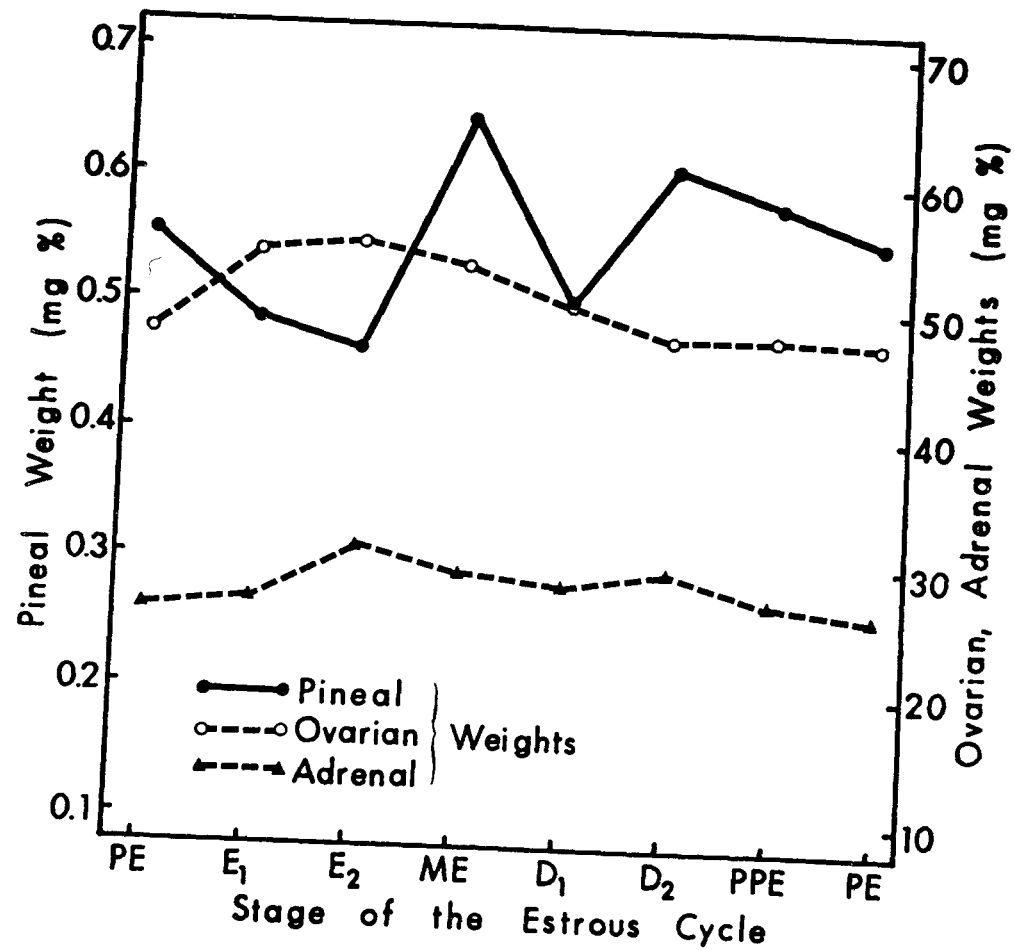
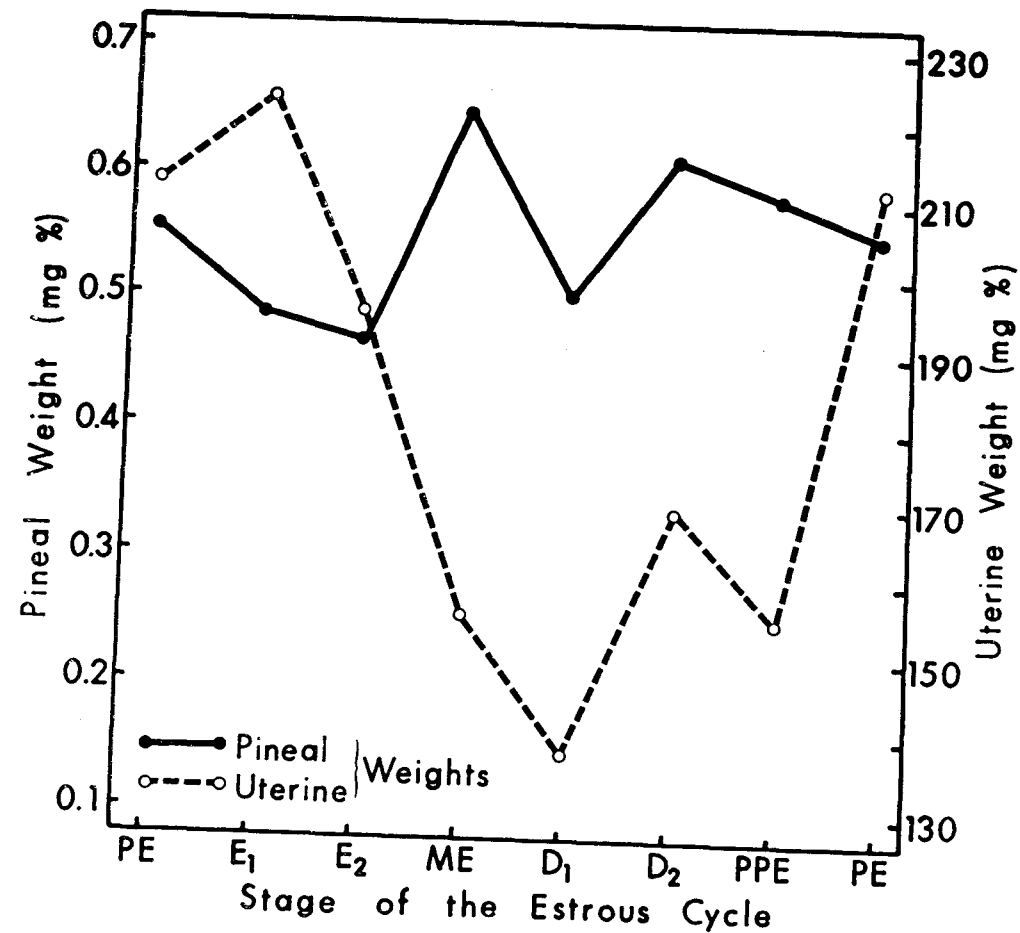


FIGURE 3

GRAPH SHOWING WEIGHT RELATIONSHIPS OF THE UTERUS AND PINEAL GLAND DURING SEVEN DIFFERENT STAGES OF THE ESTROUS CYCLE OF THE RAT



2. Enzymorphological Assessments of the Pineal Gland During the Estrous Cycle

The semi-quantitative histochemical evaluations of the enzymatic activity of the pineal gland are based on a scale ranging from plus-minus (trace histochemical enzymatic activity), to a plus four (maximal histochemical enzymatic activity). Phase contrast microscopy and counterstaining with hematoxylin or hematoxylin-eosin revealed that the enzymatic activities, for the four enzymes studied, succinic dehydrogenase (SDH), lactic dehydrogenase (LDH), alkaline phosphatase (Alk. P'tase) and acid phosphatase (Acid P'tase) are located within the cytoplasm of the pinealocytes (Plates IV and V, figures 26-29).

a. Succinic Dehydrogenase (SDH)

Comparative histochemical studies of the enzymatic activity revealed the following for succinic dehydrogenase in pinealocytes:

(1.) Proestrus. During the proestrous stage of the estrous cycle, it was noted that there is a distinctive array of diformazan granules scattered throughout the pineal gland, thus indicating a scattering of active areas of metabolic activity. Of equal importance was the finding of an inappreciable intensity of monoformazan granules, thus indicating minimal background areas of metabolically active sites (Velardo, 1971). Histochemically, an overall evaluation of +1 characterizes this reaction (Plate VI, figure 30).

(2.) Early Estrus (zero to six hours after the initial appearance of fully cornified vaginal epithelial cells). With the onset of the stage of estrous, a small scatter of diformazan granules became evident. The monoformazan material, however, was quite marked, in contrast, and appeared almost maximal in concentration (Plate VI, figure 31). As a matter of fact, its intensity was so marked as to make it almost impossible to differentiate the cellular elements. This reaction has been semi-quantitatively estimated at +3 or approximately one-quarter less than the maximally observed reaction.

(3.) Late Estrus (six to twelve hours after the initial appearance of fully cornified vaginal epithelial cells). As estrus fully developed, the succinic dehydrogenase activity became quite evident. Abundant deposition of diformazan granules were found throughout the pineal gland during late estrus. These diformazan granules were small in size. There was also a considerable intensity of monoformazan throughout the gland. Histochemically, the gland during late estrus displays maximal succinic dehydrogenase activity a +4 reaction (Plate VI, figure 32; cf. figures 30-37).

(4.) Metestrus. Throughout the metestrum a small scattering of diformazan granules was observable. Only trace intensities of monoformazan material was detected, a \pm reaction (Plate VI, figure 33).

(5.) Early Diestrus (zero to twenty-five hours within the stage of diestrus). During this phase of the estrous cycle, an increased deposition of large diformazan granules was observed in the pineal gland. A moderate intensity (+2.5) of material was present (Plate VI, figure 34).

(6.) Late Diestrus (twenty-five to fifty hours within the stage of diestrus). As the diestrus fully developed, an increased intensity of diformazan granules was found in a scattered array throughout the pineal gland. Most of the markedly intense succinic dehydrogenase activity was found in perinuclear areas of the pinealocytes. Also noted was increased intensity of the monoformazan background, a +2.5 composite reaction (Plate VI, figure 35).

(7.) Preproestrus. During this phase of the estrous cycle, a marked reduction in the intensity of diformazan granules was quite evident. Also, there was an inappreciable intensity of monoformazan material approximating a +1.5 overall reaction (Plate VI, figure 36).

For the purpose of having this study rigidly controlled, it appeared quite advisable to include parallel control sections, i.e. those incubated without the substrate (sodium succinate) for every pineal gland studied. Thus, for each determination of the seven different stages of the estrous cycle, parallel controls were incorporated. Such controls were uniformly devoid of

mono- and diformazan materials in each of the seven stages of the estrous cycle. A typical control section is depicted in Plate VI, figure 37. For immediate comparisons, both a summary table and an illustrative graph are presented (Table VI; Figure 4).

b. Lactic Dehydrogenase (LDH)

The semi-quantitative histochemical analysis of lactic dehydrogenase activity revealed the following for this enzyme in pinealocytes:

(1.) Proestrus. During this phase of the estrous cycle, there is evidence of a maximally intense diformazan granule reaction throughout the pineal gland. Likewise, there was a maximally intense monoformazan reaction. The semi-quantitative histochemical assessment of this stage of the estrous cycle was the maximal designation, +4 (Plate VII, figure 38).

(2.) Early Estrus (zero to six hours). Coincident with the onset of heat, i.e. early estrus, a marked decrease in intensity of lactic dehydrogenase became quite noticeable. Both the mono- and diformazan material had overall intensities of +1 (Plate VII, figure 39).

(3.) Late Estrus (six to twelve hours). With the full development of estrus, an almost maximally intense diformazan reaction was observable (+3.5); likewise, a near maximal monoformazan reaction was equally evident, thus giving an overall

+3.5 reaction (Plate VII, figure 40).

(4.) Metestrus. During this phase, following late estrus, only a slight reduction in diformazan granules and intensity was observed, a +3 reaction. The monoformazan, although present, was less pronounced. The intensity of the +3 reaction was for the most part due primarily to the diformazan (Plate VII, figure 41).

(5.) Early Diestrus (zero to twenty-five hours). With the onset of the diestrus, a further reduction in overall intensity was observed. Scattered diformazan granules of moderate intensities were widespread. The monoformazan distribution and intensity, however, was slightly elevated over that observed during the previous phase of the metestrus, thus giving an overall +2.5 reaction (Plate VII, figure 42).

(6.) Late Diestrus (twenty-five to fifty hours). As the diestrus progressed, the intensity of lactic dehydrogenase in the pinealocytes, as assessed by the intensities of mono- and diformazan, became markedly reduced from the previous estimate of +2.5 down to +0.5 (Plate VII, figure 43).

(7.) Preproestrus. As the diestrus waned, and the initial appearance of estrus emerged, there was an overall increase in the number of diformazan granules. There was also a marked increase in the intensity of monoformazan material, giving the overall reaction of a +1 (Plate VII, figure 44).

In parallel control sections, wherein the sodium lactate substrate was omitted, the reaction proved uniformly negative in each of the seven stages of the estrous cycle (Plate VII, figure 45). The semi-quantitative histochemical evaluation of lactic dehydrogenase is summarized on Table VII and Figure 5.

c. Alkaline Phosphatase (Alk. P'tase)

Comparative histochemical analyses of the enzymatic activity revealed the following for alkaline phosphatase in pinealocytes:

(1.) Proestrus. During this time of the cycle just prior to estrus, only a scattered intensity of diazonium reactive materials are detectable. The background observed is the result of tissue thickness; most likely, at this stage histochemical sites are not readily identifiable. The semi-quantitative histochemical assessment of this stage of the estrous cycle is definitely low, a +1 (Plate VIII, figure 46).

(2.) Early Estrus (zero to six hours). With the onset of the early aspects of estrus, an abundance of small diazonium granules are quite well formed, and are readily discernible throughout the pinealocytes, a maximal reaction of +4 appearing quite prominently (Plate VIII, figure 47).

(3.) Late Estrus (six to twelve hours). As estrus becomes fully developed, a scattered number of diazonium granules are in large measure the only reactive indicators of alkaline

phosphatase, a \pm reaction or trace intensities (Plate VIII, figure 48).

(4.) Metestrus. With the development of the subsequent phase of metestrus, the activity of this enzyme in the pinealocytes is further reduced, and only a slightly increased reaction is obtainable, a +0.5 (one-half), i.e. intermediate between a +1 and trace (\pm) intensities (Plate VIII, figure 49).

(5.) Early Diestrus (zero to twenty-five hours). During the pause of the periods of estrus, one observes a reaccumulation of activity of this enzyme. Interestingly enough, one observes a comparatively strong reaction, climbing to a +2.5 (Plate VIII, figure 50).

(6.) Late Diestrus (twenty-five to fifty hours). With the further development of the diestrus, one detects a minimal reduction of alkaline phosphatase activity. Specifically, a decrease in diazonium granules is observable, and a +2 reaction is seemingly widespread throughout the pineal gland (Plate VIII, figure 51).

(7.) Preproestrus. As the animal recommences its cycle towards the initial phases of heat (estrus), the alkaline phosphatase activity shows heightened activity, thus reaching +3 intensities (Plate VIII, figure 52).

Again, control studies of slides of the pineal gland incubated without the substrate (α -naphthol acid phosphate)

clearly indicated that the pineal tissue sections so treated were devoid of activity in each of the seven stages of the estrous cycle. (Plate VIII, figure 53). Tabular and graphic representations of these data are presented in Table VII and Figure 6.

d. Acid Phosphatase (Acid P'tase)

Comparative histochemical analyses of the enzymatic activity revealed the following for acid phosphatase in pinealocytes:

(1.) Proestrus. During this phase of the estrous cycle, just prior to estrus, large scattered diformazan granules, indicating acid phosphatase activity, begin to become prominent. The granules, however, are of low intensity, a +1.5 (Plate IX, figure 54).

(2.) Early Estrus (zero to six hours). As estrus develops, both the number and intensity of the diazonium granules become maximally developed and intensified, thus giving a +4 reaction for acid phosphatase (Plate IX, figure 55).

(3.) Late Estrus (six to twelve hours). With the full development of the phase of heat, late estrus, the activity of acid phosphatase becomes markedly reduced, and appears only of a +1 intensity (Plate IX, figure 56).

(4.) Metestrus. Interestingly enough, the activity of acid phosphatase during the metestrus is quite comparable to that observed during the previous stage of late estrus (Plate IX,

figures 56 and 57).

(5.) Early Diestrus (zero to twenty-five hours). With the onset and development of the diestrus, the diazonium granules show large coalescing clusters, several cell layers deep, and of heightened intensity, thus giving a +3 reaction (Plate IX, figure 58).

(6.) Late Diestrus (twenty-five to fifty hours). Clearly, the activity of acid phosphatase shows a marked rise during the further development of the diestrus, a +3.5 (Plate IX, figure 59).

(7.) Preproestrus. With the reappearance of the initial estrous phases, the acid phosphatase activity shows a marked reduction to a +2 (Plate IX, figure 60).

The control studies, omitting the α -naphthol acid phosphate substrate, was uniformly negative throughout the seven stages of the estrous cycle. A representative control section of the pineal gland is shown in Plate IX, figure 61. The semi-quantitative histochemical evaluation for acid phosphatase is summarized on Table VII and Figure 7.

3. Comparative Histochemical and Biochemical Assessments of the Pineal Gland of the Rat During the Estrous Cycle.

a. Succinic Dehydrogenase (SDH).

Utilizing similar techniques for the histochemical and biochemical assessments for succinic dehydrogenase, it was determined that similar maxima and minima were obtained. The

highest intensities and concentrations were found during late estrus and late diestrus. The lowest intensities and concentrations of succinic dehydrogenase were found at metestrus and proestrus (Table VIII; Figure 4). It is noteworthy to point out the fact of the similarity of curves obtained for these studies, thus giving added significance to these data (Figure 4).

b. Lactic Dehydrogenase (LDH)

Interestingly enough, both of the in vitro techniques, performed for the histochemical and biochemical detection of lactic dehydrogenase, revealed maxima during proestrus and late estrus and minima during early estrus and late diestrus (Table VIII; Figure 5).

c. Alkaline Phosphatase (Alk. P'tase)

As with the techniques for two aforementioned dehydrogenases, the techniques utilized for the detection and measurement of alkaline phosphatase, histochemically and biochemically, revealed maxima at early estrus, early diestrus and proestrus. Minima for alkaline phosphatase were obtained at late estrus and metestrus (Table VIII; Figure 6). The curves based on data from each of the techniques follow one another in parallel, and are seemingly superimposed one upon the other, thus reinforcing the data obtained from each technique respectively (Figure 6).

d. Acid Phosphatase (Acid P'tase)

Utilizing comparable histochemical and biochemical techniques as were used for the estrous cycle studies on alkaline phosphatase, except for the difference in pH, these studies revealed acid phosphatase maxima at early estrus and late diestrus. Minima for acid phosphatase were found at late estrus and metestrus. It is of added interest to point out here the fact of similarity of curves obtained for the histo- and biochemical studies, thus showing remarkable agreements throughout the studies of acid phosphatase throughout the seven stages of the estrous cycle of the rat (Table VIII; Figure 7).

4. Preliminary Biochemical Data of the Pineal Gland During the Estrous Cycle of the Rat.

In a continuation of the biochemical studies, it appeared of added interest to ascertain the biochemical concentrations of two transaminases, glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase, with the idea of obtaining some preliminary data for future experimentation. Furthermore, inasmuch as these two transaminases are importantly concerned with glutamic acid metabolism and the central nervous system, it seemed of interest to attempt to ascertain what role(s) such might have in the pineal gland.

a. Glutamic-Oxaloacetic Transaminase (GOT)

Utilizing a colorimetric test for glutamic-oxaloacetic

transaminase on the pineal gland of the rat during seven different stages of the estrous cycle, it was ascertained that the peak, maximal reaction was obtained at early diestrus, having ascended during the stage of the metestrus. Thereafter, the peak descends in two phases: first, from the peak of early diestrus down 7% to late diestrus; secondly, almost 25% to the minimal reaction obtained during preproestrus (Table IX; Figure 8).

b. Glutamic-Pyruvic Transaminase (GPT)

Utilizing a similar colorimetric test for glutamic-pyruvic transaminase on the pineal gland of the rat during the seven different stages of the estrous cycle, an initial early cyclical rise of glutamic-pyruvic transaminase activity was obtained during late estrus, followed by a steady decline through metestrus and early diestrus, after which the enzymatic activity ascended to its maximal peak during late diestrus, thereafter falling precipitously at the initial phase of estrus, i.e. at preproestrus. Succinctly, the dominant and maximal peak activity occurred at late diestrus whereas the lowest was clearly associated with preproestrus (Table IX; Figure 9).

TABLE VII

SEMI-QUANTITATIVE HISTOCHEMICAL ESTIMATES OF SUCCINIC DEHYDROGENASE (SDH), LACTIC DEHYDROGENASE (LDH), ALKALINE PHOSPHATASE (ALK. P'TASE), AND ACID PHOSPHATASE (ACID P'TASE) OF THE PINEAL GLAND OF THE RAT DURING THE ESTROUS CYCLE

	SDH	LDH	Alk. P'tase	Acid P'tase
Early Estrus	+3	+1	+4	+4
Late Estrus	+4	+3.5	±	+1
Met-estrus	±	+3	+0.5	+1
Early Diestrus	+2	+2.5	+2.5	+3
Late Diestrus	+2.5	+0.5	+2	+3.5
Prepro-estrus	+1.5	+2	+3.2	+2
Pro-estrus	+1	+4	+1	+1.5

TABLE VIII

QUANTITATIVE BIOCHEMICAL ANALYSIS OF SUCCINIC DEHYDROGENASE (SDH), LACTIC DEHYDROGENASE (LDH), ALKALINE PHOSPHATASE (ALK. P'TASE), AND ACID PHOSPHATASE (ACID P'TASE) OF THE PINEAL GLAND OF THE RAT DURING THE ESTROUS CYCLE

	SDH*	LDH**	Alk. P'tase***	Acid P'tase***
Early Estrus	0.10±0.006	33.2±0.111	7.02±0.463	1.16±0.015
Late Estrus	0.13±0.006	117.2±0.312	3.57±0.197	0.71±0.013
Met-estrus	0.034±0.003	67.9±0.070	4.00±0.190	0.76±0.016
Early Diestrus	0.080±0.003	62.5±0.100	6.45±0.775	1.04±0.016
Late Diestrus	0.089±0.009	32.6±0.052	5.85±0.849	1.13±0.011
Prepro-estrus	0.055±0.004	60.5±0.172	6.59±1.123	0.88±0.010
Pro-estrus	0.054±0.006	123.7±0.877	4.93±0.570	0.86±0.008

* 1 unit of SDH activity equals the number of μM of formazan formed/mg pineal gland.

** 1 unit of LDH activity will reduce 4.8×10^{-4} μM pyruvate/minute/mg pineal gland.

*** 1 unit of Alk. and Acid P'tase activity will liberate 1 μM p-nitrophenol/hour/mg pineal gland (1 μM = 0.1391 mg).

TABLE IX

QUANTITATIVE BIOCHEMICAL ANALYSIS OF GLUTAMIC-OXALOACETIC (GOT)
AND GLUTAMIC-PYRUVIC TRANSAMINASE (GPT) ACTIVITY OF THE
PINEAL GLAND OF THE RAT DURING THE ESTROUS CYCLE

	GOT*	GPT*
Pro- estrus	84.27±2.44	13.93±0.497
Early Estrus	78.46±4.38	17.95±1.019
Late Estrus	76.37±3.10	18.83±1.136
Met- estrus	80.00±1.12	14.00±0.843
Early Diestrus	98.17±0.600	11.93±1.376
Late Diestrus	91.39±0.734	20.85±1.184
Prepro- estrus	73.77±1.29	8.02±0.843

* 1 unit of GOT or GPT activity will form
4.82 X 10⁻⁴ μM glutamate/minute/mg
pineal gland.

FIGURE 4
 SEMI-QUANTITATIVE HISTOCHEMICAL AND QUANTITATIVE
 BIOCHEMICAL ANALYSIS OF SUCCINIC DEHYDROGENASE
 (SDH) ACTIVITY OF THE PINEAL GLAND
 DURING THE ESTROUS CYCLE

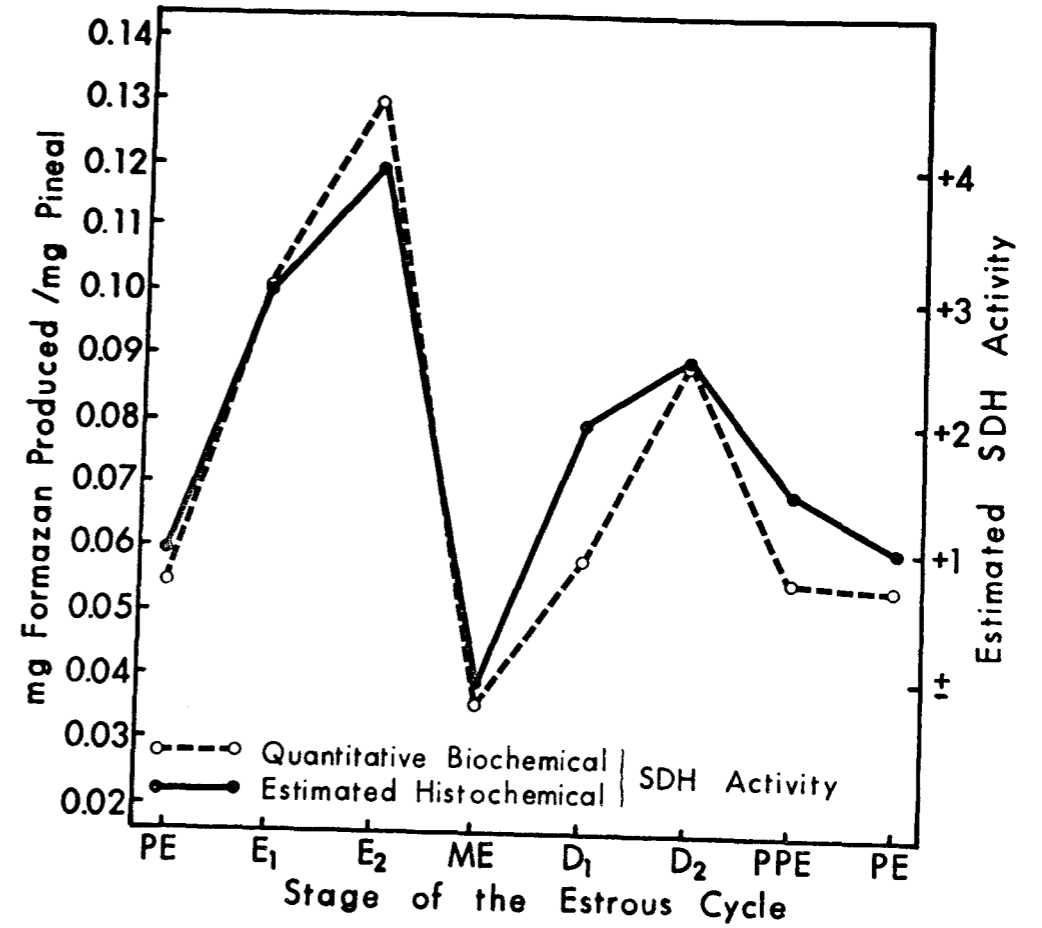


FIGURE 5
SEMI-QUANTITATIVE HISTOCHEMICAL AND QUANTITATIVE
BIOCHEMICAL ANALYSIS OF LACTIC DEHYDROGENASE
(LDH) ACTIVITY OF THE PINEAL GLAND
DURING THE ESTROUS CYCLE

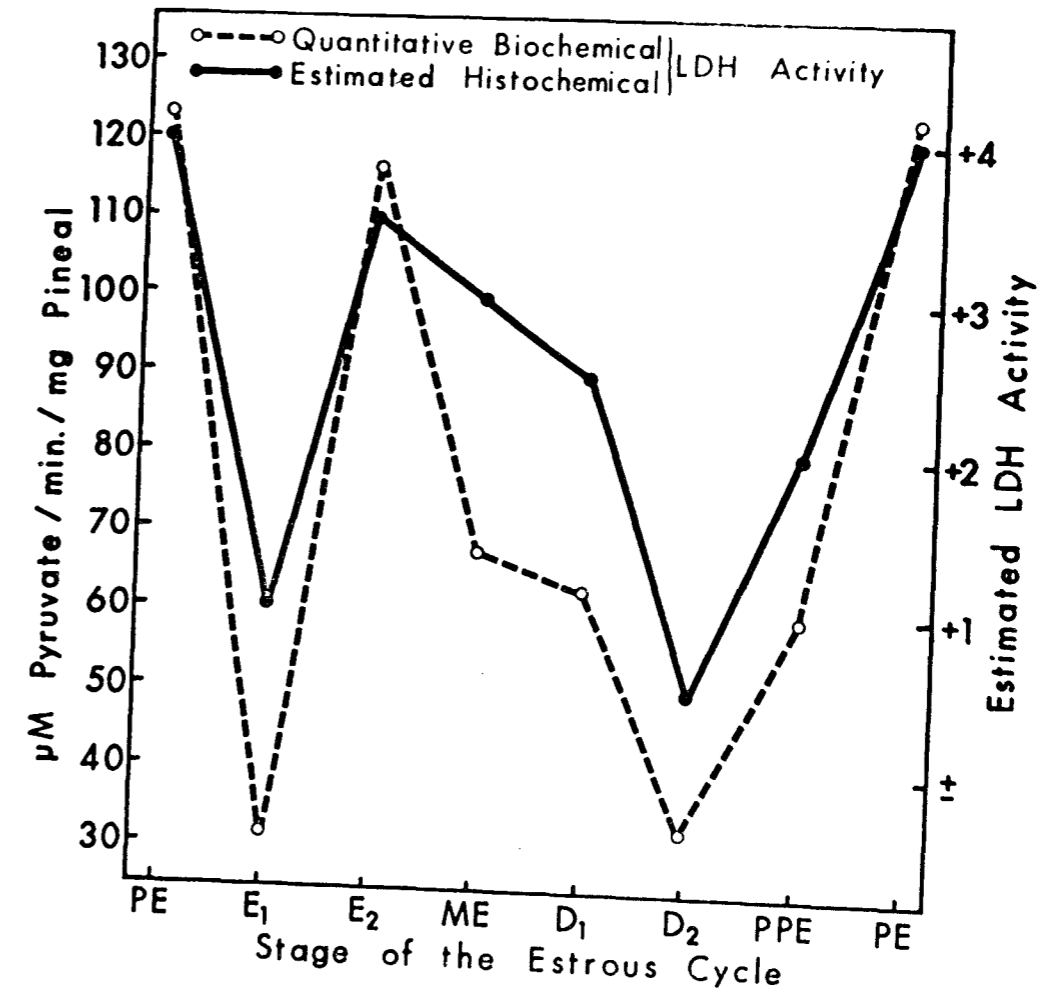


FIGURE 6
 SEMI-QUANTITATIVE HISTOCHEMICAL AND QUANTITATIVE
 BIOCHEMICAL ANALYSIS OF ALKALINE PHOSPHATASE
 (ALK. P'TASE) ACTIVITY OF THE PINEAL GLAND
 DURING THE ESTROUS CYCLE

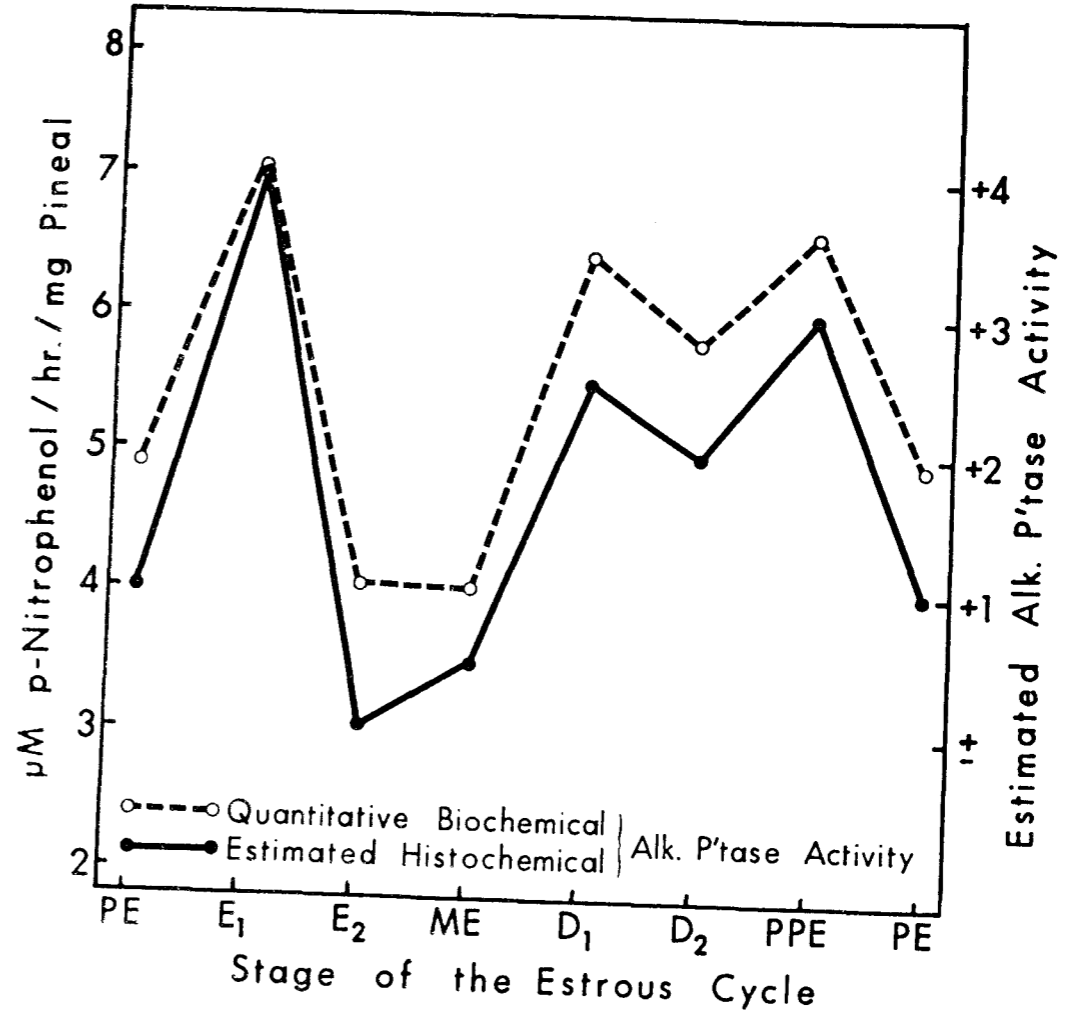


FIGURE 7
SEMI-QUANTITATIVE HISTOCHEMICAL AND QUANTITATIVE
BIOCHEMICAL ANALYSIS OF ACID PHOSPHATASE (ACID
P'TASE) ACTIVITY OF THE PINEAL GLAND
DURING THE ESTROUS CYCLE

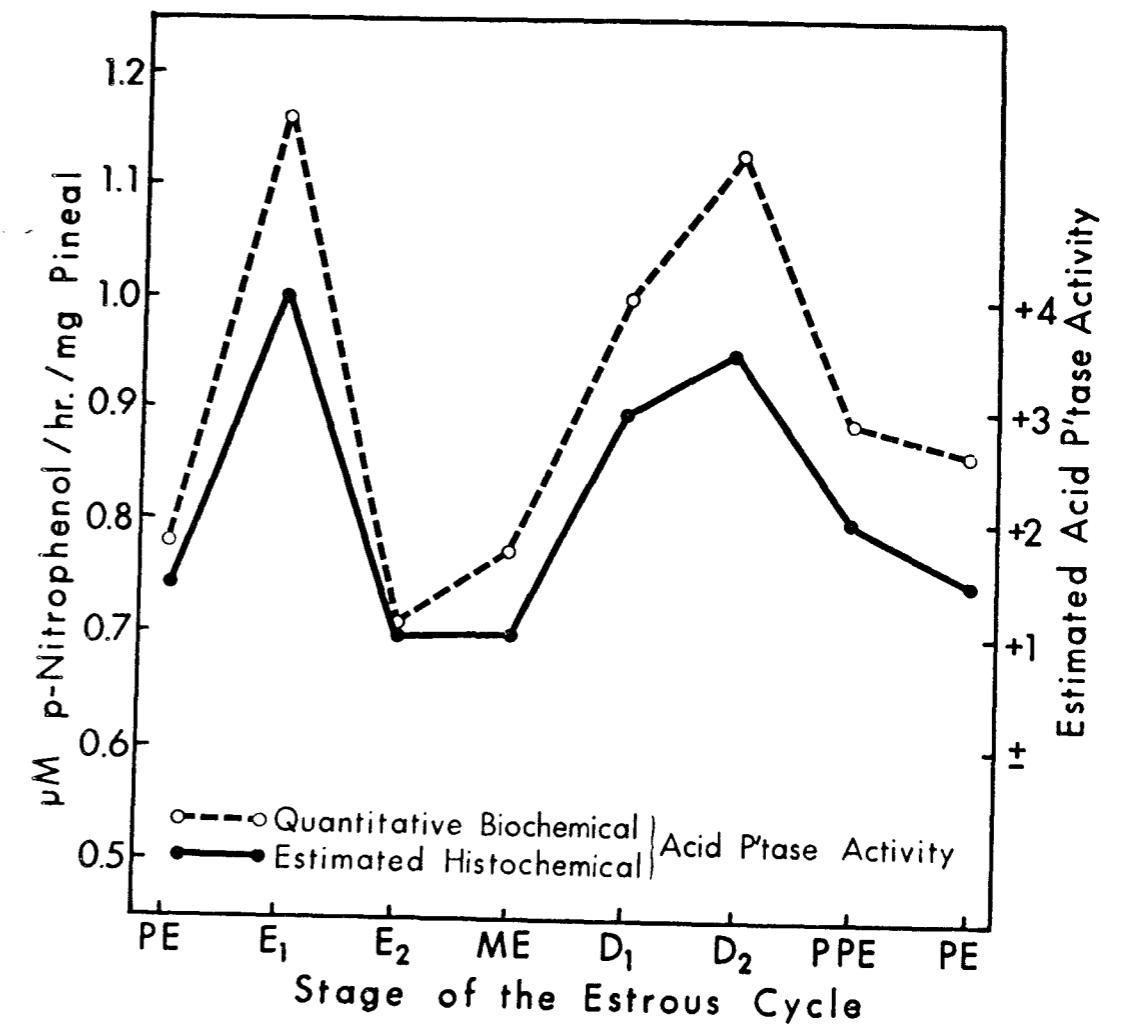


FIGURE 8
QUANTITATIVE BIOCHEMICAL ANALYSIS OF GLUTAMIC-
OXALOACETIC TRANSAMINASE ACTIVITY OF THE
PINEAL GLAND DURING THE ESTROUS CYCLE

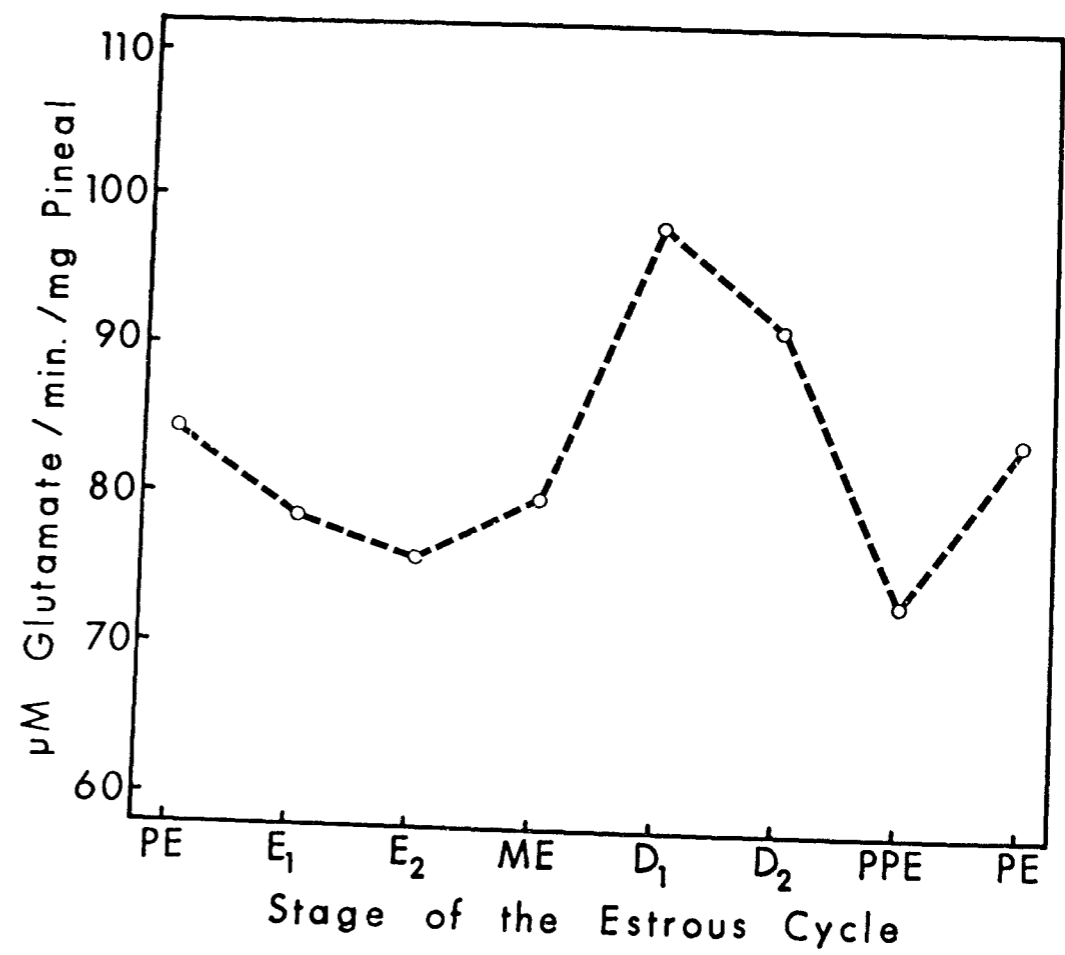
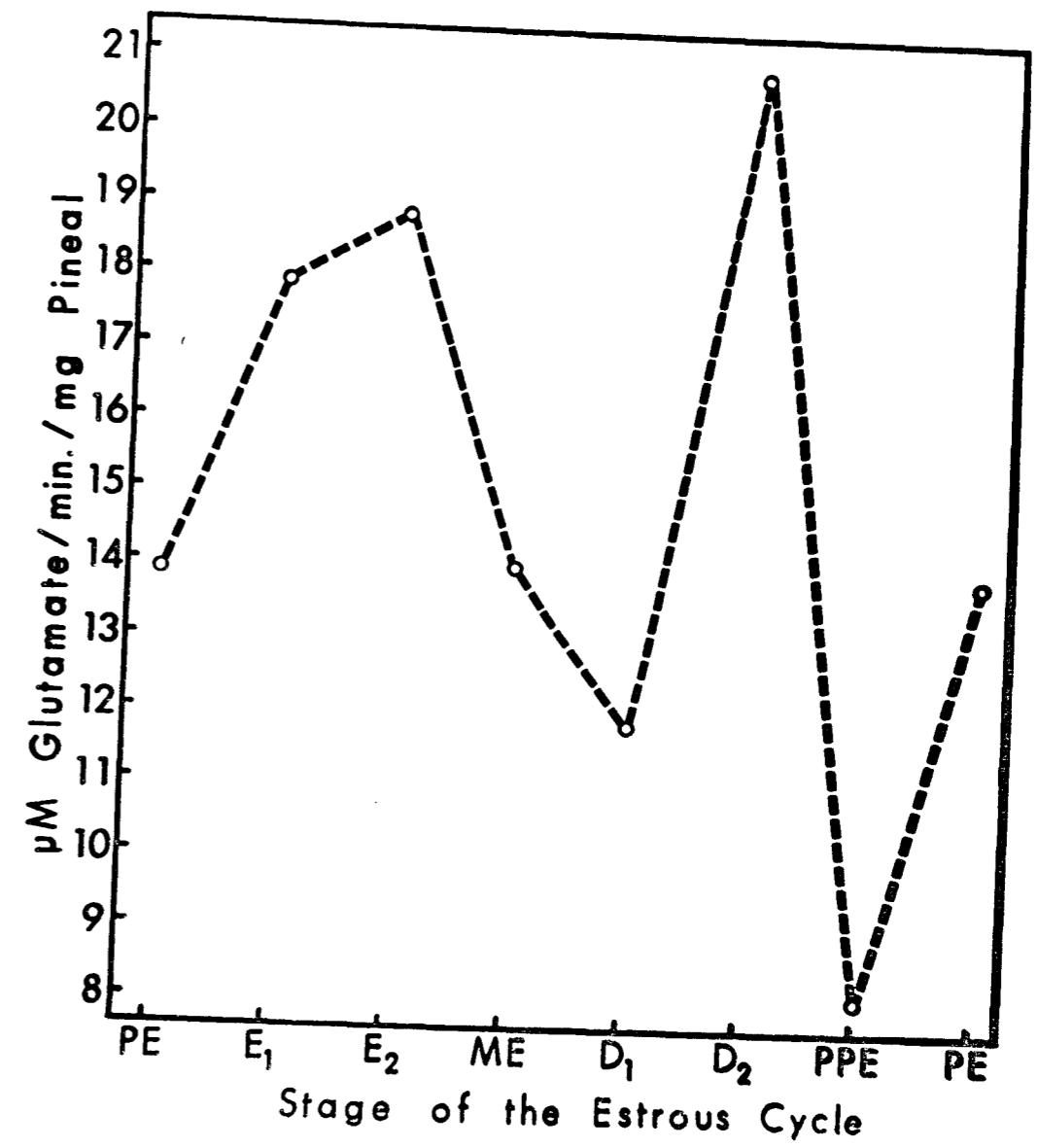


FIGURE 9

QUANTITATIVE BIOCHEMICAL ANALYSIS OF GLUTAMIC-PYRUVIC TRANSAMINASE ACTIVITY OF THE PINEAL GLAND DURING THE ESTROUS CYCLE



C. Gravimetric and Enzymorphological Studies of the Pineal Gland During Three Stages of Pseudopregnancy in the Rat.

Inasmuch as the normal duration of pseudopregnancy lasts for approximately thirteen days, it seemed desirable to study three phases: (a) early, six days; (b) mid-point, at ten days; and (c) the terminal day, at thirteen days. In this way, one could obtain a rather informative index of activity during the major aspects of pseudopregnancy in the rat.

1. Gravimetric Data of Pineal Glands of Rats During Pseudopregnancy.

Contrasting the weights of the pineal glands of pseudopregnant rats with those of the estrous cycle, one finds two interesting phenomena: first, the pineal glands of early pseudopregnancy (day six) and those of the terminal day of pseudopregnancy (day thirteen) closely approximate the weights of the pineal glands during proestrus (cf. Tables I and X); secondly, the weights of the pineal glands of rats of advanced pseudopregnancy (day ten) are quite variable, and closely resemble the arithmetic mean of the pineal glands of rats in the late phase of estrus (cf. Tables I and X).

TABLE X

THE WEIGHTS OF PINEAL GLANDS OF RATS DURING
THREE STAGES OF PSEUDOPREGNANCY

Days of Pseudo- pregnancy	Number of Animals	Weights of Pineal Glands (mg% \pm S.E.)
Day 6	8	0.540 \pm 0.027
Day 10	7	0.461 \pm 0.253
Day 13	13	0.546 \pm 0.028

2. Enzymorphological Assessments of the Pineal Gland During Pseudopregnancy.

a. Succinic Dehydrogenase (SDH)

During the early phase of pseudopregnancy, the succinic dehydrogenase activity in the pinealocyte appears almost maximally (+3), and achieves maximal and sustained activity at the mid-point through the terminal day of pseudopregnancy (Table X; Figure 10; Plate X, figures 62-64).

For each period of pseudopregnancy, controls without substrate were incorporated. The results of the controls, i.e. without the sodium succinate substrate, were uniformly negative.

b. Lactic Dehydrogenase (LDH)

A maximal reaction for lactic dehydrogenase activity (+4) is found in the pinealocytes throughout the gland during the early phase of pseudopregnancy, i.e. day six (Plate XI, figure 65). As pseudopregnancy continues, the reaction for lactic dehydrogenase becomes much less intense, the mono- and diformazan granules manifesting only +2 reactions (Plate XI, figure 66). With the nearing of the cessation of pseudopregnancy, at thirteen days, the reactive mono- and diformazan granules show heightened activity and are typically +3 in intensity (Table 10; Figure 10; Plate XI, figure 67).

As previously stated, controls were studied along with each period of pseudopregnancy. The pineal sections incubated without the sodium lactate substrate were uniformly negative.

c. Alkaline Phosphatase (Alk. P'tase)

The pineal gland during these three periods of pseudopregnancy contains pinealocytes that are heavily laden with alkaline phosphatase (Plate XII, figures 68-70). The alkaline phosphatase activity is maximum in intensity (+4) on day ten, and is only about 25% reduced during the early and terminal stages of pseudopregnancy, +3 reactions (Table X; Figure 11).

Control sections incubated without the α -naphthol acid phosphate substrate manifested negative reactions.

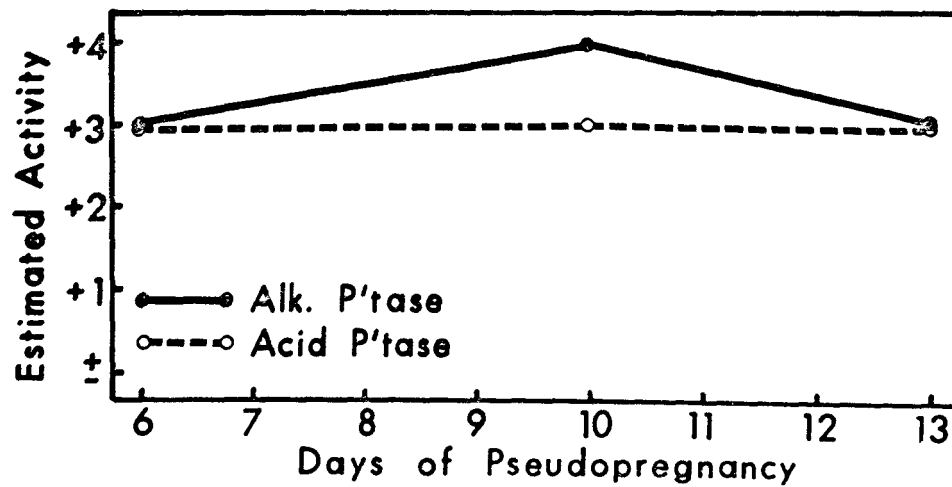
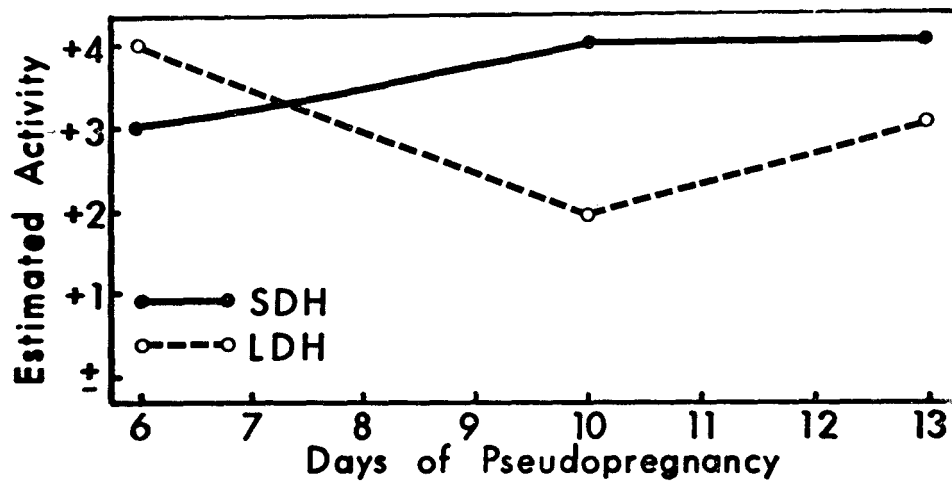
d. Acid Phosphatase (Acid P'tase)

Throughout the three periods of pseudopregnancy, days six, ten, and thirteen, the deposition and intensity of acid phosphatase was of high moderate characterization, +3 intensities. The control sections, without the α -naphthol acid phosphate, were uniformly negative (Table X; Figure 11; Plate XIII, figures 71-73).

TABLE XI

SEMI-QUANTITATIVE HISTOCHEMICAL ESTIMATES OF SUCCINIC DEHYDROGENASE (SDH), LACTIC DEHYDROGENASE (LDH), ALKALINE PHOSPHATSE (ALK. P'TASE), AND ACID PHOSPHATASE (ACID P'TASE) OF THE PINEAL GLAND OF THE RAT DURING PSEUDOPREGNANCY

	SDH	LDH	Alk. P'tase	Acid P'tase
Day 6	+3	+4	+3	+3
Day 10	+4	+2	+4	+3
Day 13	+4	+3	+3	+3



D. Gravimetric and Enzymorphological Studies of the Pineal Glands in Rats with Decidual Reactions.

1. Gravimetric Data of the Pineal Gland of Rats Bearing Decidual Reactions.

It is of interest to point up the fact of similarities between pseudopregnant rats with and without decidual reactions and with non-pseudopregnant, normally cycling albino rats. As stated in the previous section (Cl.), rats on the terminal day of pseudopregnancy have pineal gland weights that closely approximate those of rats during the proestrous phase. Likewise, the pineal glands of rats with decidual reactions on day thirteen and during the terminal phase of pseudopregnancy (day twenty-one) resemble in weight those of rats in proestrus (cf. Tables I, X, and XII). As pseudopregnancy advances to prolonged pseudopregnancy (i.e. extending beyond the normal phase-length of pseudopregnancy, 13.0 days), it appears from these data that the weights of the pineal glands resemble those of rats in early diestrus and early and late estrus, i.e. rats of fifteen and eighteen days of prolonged pseudopregnancy status, respectively (Tables I and XII). Although only three to five animals are utilized per point, the trend of the gravimetrics is both interesting and informative.

Specifically, it should be pointed out that the weights of the pineal glands of rats with decidual reactions for the most part resemble those of rats during preproestrus, proestrus, and estrus (cf. Tables I and XII).

TABLE XII

THE WEIGHTS OF PINEAL GLANDS OF RATS BEARING DECIDUAL REACTIONS

Days of Pseudo-pregnancy	Number of Animals	Weights of Pineal Glands (mg% \pm S. E.)
Day 10	4	0.580 \pm 0.010
Day 13	5	0.548 \pm 0.003
Day 15	3	0.511 \pm 0.020
Day 18	4	0.480 \pm 0.003
Day 21	4	0.542 \pm 0.013

2. Enzymorphological Assessments of the Pineal Glands of Pseudopregnant Rats Bearing Decidual Reactions.

a. Succinic Dehydrogenase (SDH)

The pineal glands of rats bearing decidual reactions show for the most part moderate to maximal succinic dehydrogenase intensities in pinealocytes, a notable exception being those of day fifteen of prolonged pseudopregnancy wherein the reaction is slightly less than moderate, a +1.5 (Figure 12; Plate XIV, figures 74-80). Initially, during the development of the antimesometrial reaction (AMR) of the decidual reaction, the succinic dehydrogenase reaction within the pinealocytes appears as a moderate reaction, a +2. On day ten during which time the antimesometrial reaction has reached its peak with some areas showing initial regression, and the mesometrial reaction (MR) becoming quite enlarged, the pinealocytes reach almost maximal intensity (+3.5) for succinic dehydrogenase (Plate XIV, figure 75).

Interestingly enough, during the next forty-eight to seventy-two hours, while the mesometrial reaction reaches its peak in development and the metrial gland (MG) becomes organized, the pinealocytes begin to show decreasing intensities of this enzyme (Plate XIV, figure 76). With the full development of the metrial gland on day fifteen of prolonged pseudopregnancy, the succinic dehydrogenase activity within the pinealocytes reaches its lowest intensity throughout the superimposed event, a +1.5

(Plate XIV, figure 77). Thereafter, on day eighteen, when both the antimesometrial reaction and metrial reaction are almost totally necrotic and during which time the metrial gland is the most prominent, the pinealocytes show maximal intensities (+4) for succinic dehydrogenase (Plate XIV, figure 78). As the metrial gland undergoes necrosis, dissolution and liquefaction, the pinealocytes show a marked drop in succinic dehydrogenase, from a peak reaction of a +4 to a +2.5 on day twenty (Plate XIV, figure 79) and finally at the time of metrial gland involution, i.e. day 21, the pinealocytes reveal a further decrease to a +2 (moderate) reaction (Table XIII; Figure 12; Plate XIV, figure 80).

It is of further interest to point out here that the very strong (+3.5) and maximal succinic dehydrogenase reactions (+4) obtained on days ten and eighteen in the pinealocytes are truly typical of those seen during early and late estrus phases of the estrous cycle.

Control sections of pineal glands incubated without sodium succinate were entirely devoid of any detectable succinic dehydrogenase activity.

b. Lactic Dehydrogenase (LDH)

Interestingly enough, whereas the maximal reaction (+4) for succinic dehydrogenase in the pinealocytes was on day eighteen, the maximal intensity (+4) for lactic dehydrogenase in the pineal parenchymal cells appears on day thirteen

(cf. Figures 12 and 13; Plate XIV, figure 78 and Plate XV, figure 83). As with succinic dehydrogenase, the lactic dehydrogenase intensities in these cells reveal a picture of increasing intensity from days six through ten (cf. Figures 12 and 13; Plate XV, figures 81 and 82). Thereafter, the lactic dehydrogenase picture shows slight variation from that of succinic dehydrogenase. In the lactic dehydrogenase series, on day thirteen the peak is reached (Figure 13; Plate XV, figure 83), but in the succinic dehydrogenase series a slight decrease is observed (Figure 12). On day fifteen, however, a precipitous decrease in intensity of both succinic dehydrogenase and lactic dehydrogenase is observed (cf. Figures 12 and 13; Plates XIV and XV, figures 77 and 84). Both enzymes show a rise in intensity on day eighteen, being maximal (+4) for succinic dehydrogenase, and very strong (+3) for lactic dehydrogenase, but approximately 25% less than the maximal for succinic dehydrogenase (cf. Figures 12 and 13; Plates XIV and XV, figures 78 and 85). Thereafter, the intensities for succinic dehydrogenase and lactic dehydrogenase decrease in steps to a +2.5 on day twenty (cf. Figures 12 and 13; Plates XIV and XV, figures 80 and 87; Table XIII).

For each pineal gland, a control without the sodium lactate substrate was incubated, and as previously noted, resulted in a negative reaction.

c. Alkaline Phosphatase (Alk. P'tase)

The pinealocytes throughout the superimposed physiological event of pseudopregnancy, with the exception of the terminal day i.e. day twenty-one, reveal a moderate to maximal intensity for alkaline phosphatase. The reaction is maximal on day fifteen, falls precipitously to a moderate intensity on day eighteen (i.e. a +2), plateaus at same through day twenty, and becomes slightly less intense on day twenty-one, scoring the lowest reaction for alkaline phosphatase at this time, a +1.5 (cf. Table XIII; Figure 14; Plate XVI, figures 88-94).

Control pineal sections incubated without the α -naphthol acid phosphate were totally devoid of any alkaline phosphatase activity.

d. Acid Phosphatase (Acid P'tase)

Histochemical studies for acid phosphatase in the pineal glands of pseudopregnant rats bearing decidual reactions clearly indicate that the pinealocytes react most strongly for this enzyme. From day six through thirteen, very strong to near maximal reactions are obvious (+3.5, day six; +3.0, day ten; +3.5, day thirteen). A maximal peak reaction of a +4 becomes quite prominent on day fifteen, after which linear, precipitous decreases become quite noticeable on days eighteen and twenty, a +3 and +2, respectively. On day twenty-one, the terminal day of prolonged pseudopregnancy, a strong, moderate acid phosphatase

reaction in the pinealocytes emerges from the previous days minimal reaction, thus increasing from +2 to +2.5 (Table XIII; Figure 15; Plate XVII, figures 95-101). Control pineal gland sections incubated without α -naphthol acid phosphate proved uniformly negative.

TABLE XIII

SEMI-QUANTITATIVE HISTOCHEMICAL ESTIMATES OF SUCCINIC DEHYDROGENASE (SDH), LACTIC DEHYDROGENASE (LDH), ALKALINE PHOSPHATASE (ALK. P'TASE), AND ACID PHOSPHATASE (ACID P'TASE) OF THE PINEAL GLAND OF THE RAT WITH DECIDUAL REACTIONS

	SDH	LDH	Alk. P'tase	Acid P'tase
Day 6	+2	+3.2	+3	+3.5
Day 10	+3.5	+3.5	+2.5	+3
Day 13	+3	+4	+3	+3.5
Day 15	+1.5	+2	+4	+4
Day 18	+4	+3	+2	+3
Day 20	+2.5	+2.5	+2	+2
Day 21	+2	+2	+1.5	+2.5

FIGURE 12

SEMI-QUANTITATIVE HISTOCHEMICAL EVALUATION OF SUCCINIC
DEHYDROGENASE (SDH) ACTIVITY OF THE PINEAL GLAND
IN RATS BEARING DECIDUAL REACTIONS

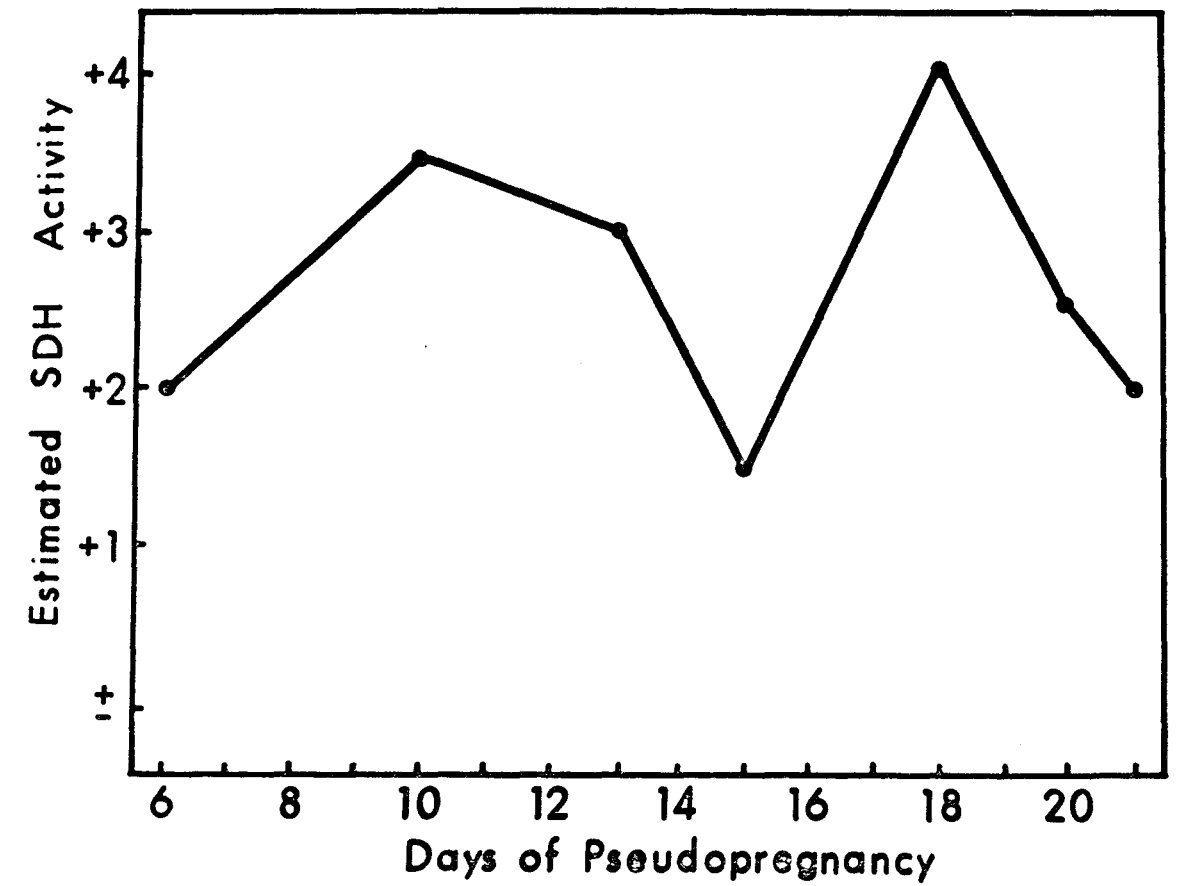


FIGURE 13

SEMI-QUANTITATIVE HISTOCHEMICAL EVALUATION OF LACTIC DEHYDROGENASE (LDH) ACTIVITY OF THE PINEAL GLAND IN RATS BEARING DECIDUAL REACTIONS

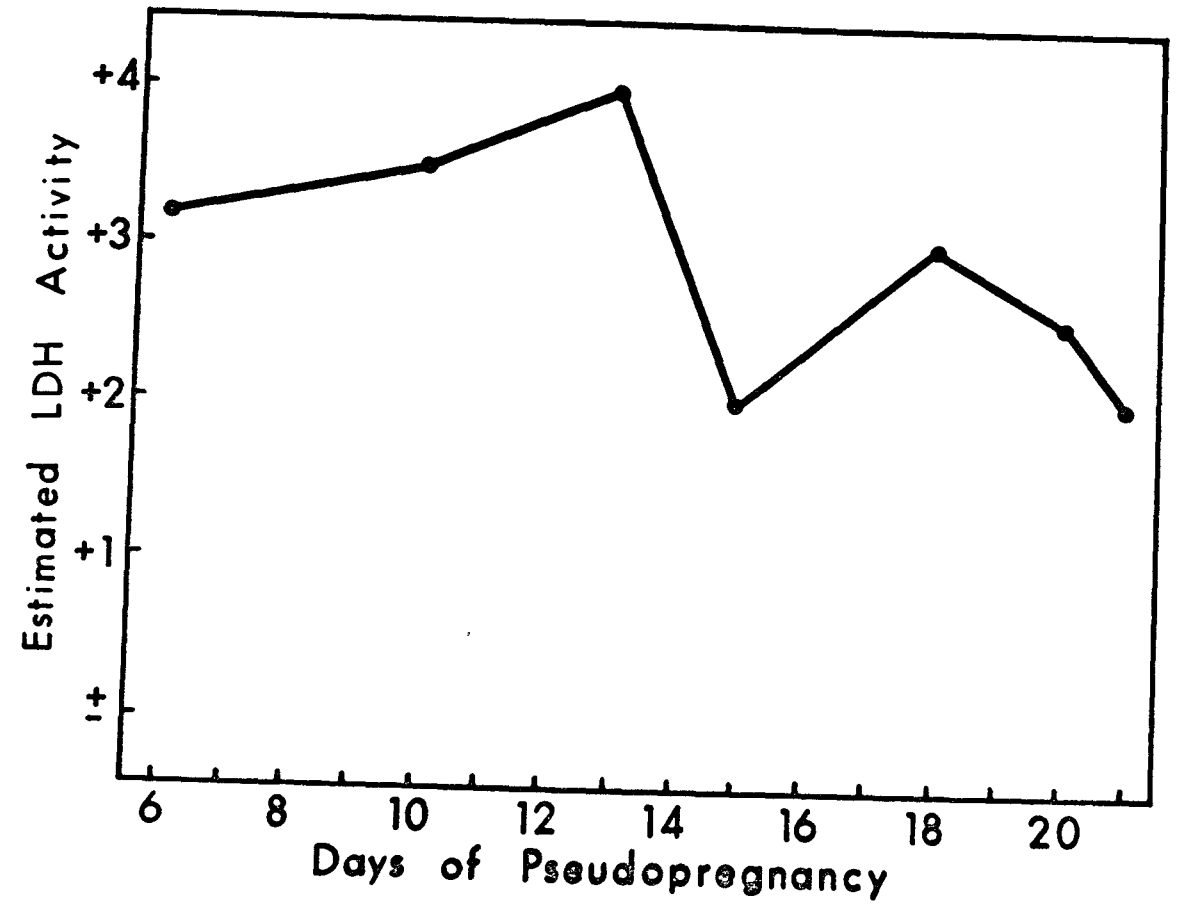


FIGURE 14

SEMI-QUANTITATIVE HISTOCHEMICAL EVALUATION OF ALKALINE PHOSPHATASE (ALK. P'TASE) ACTIVITY OF THE PINEAL GLAND IN RATS BEARING DECIDUAL REACTIONS

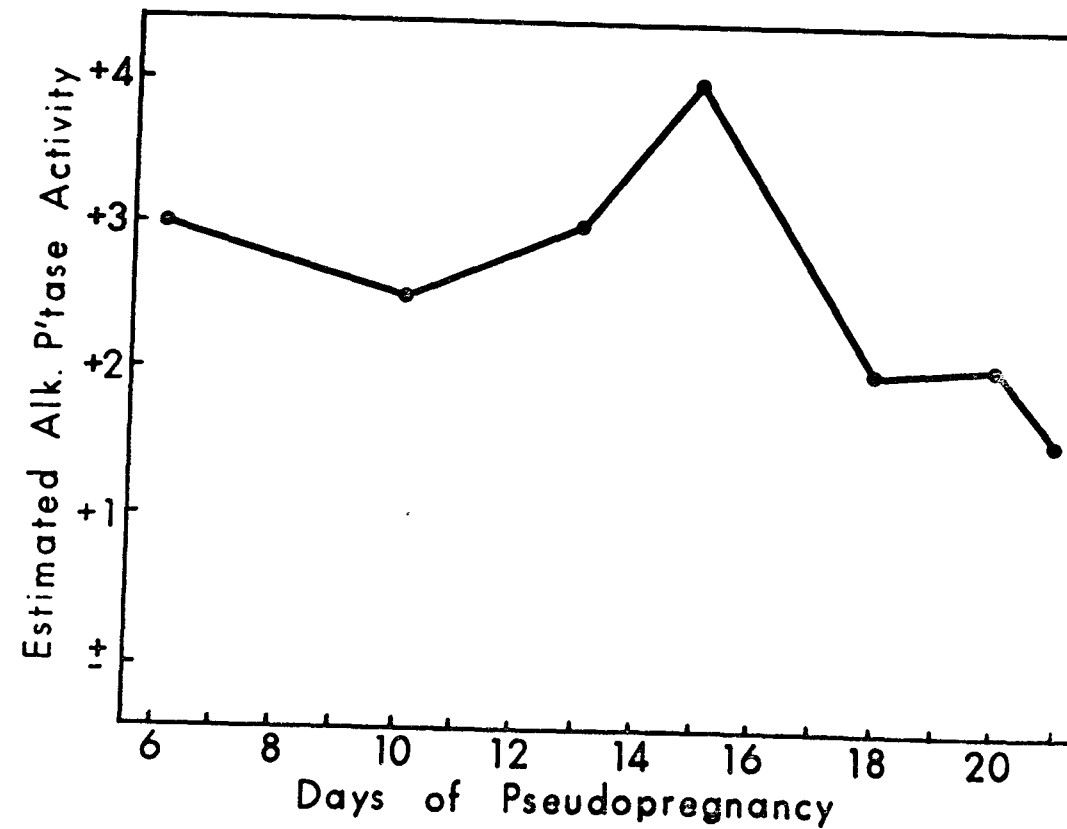
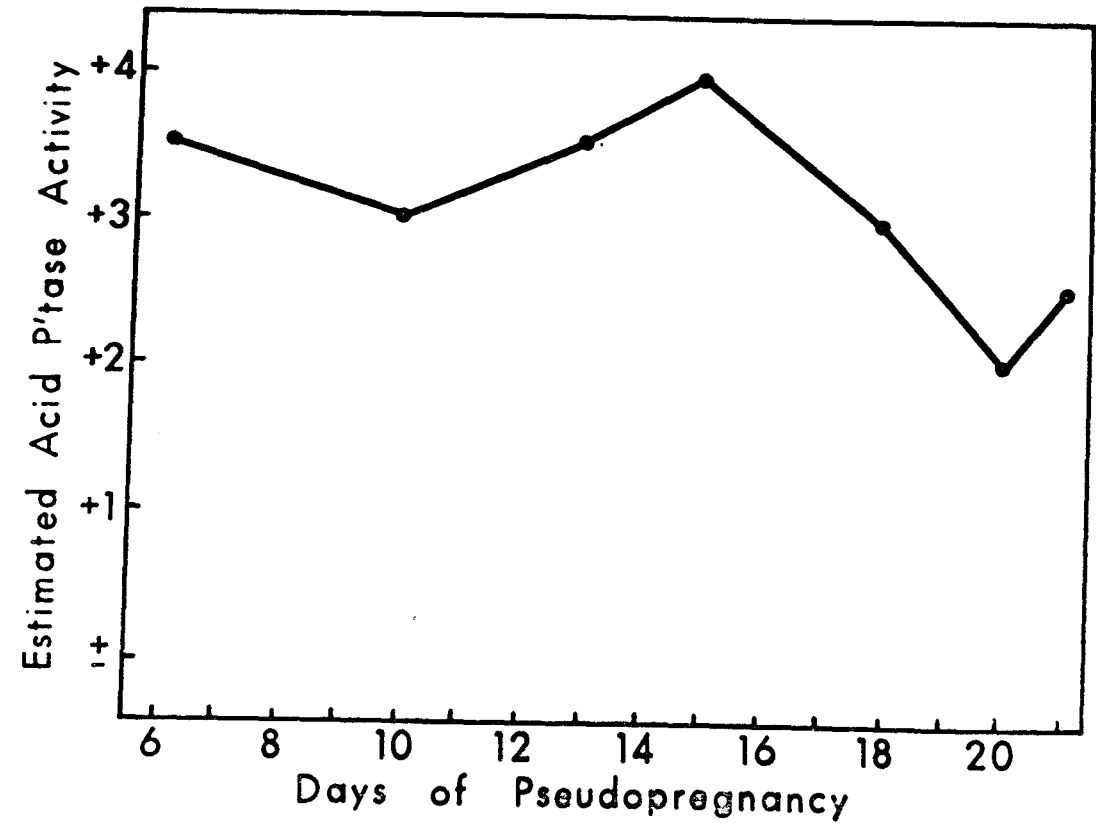


FIGURE 15

SEMI-QUANTITATIVE HISTOCHEMICAL EVALUATION OF ACID PHOSPHATASE (ACID P'TASE) ACTIVITY OF THE PINEAL GLAND IN RATS BEARING DECIDUAL REACTIONS



E. Gravimetric and Enzymorphological Studies of the Pineal Gland of Pregnant Rats.

Prefatory Remarks

The major aim of this dissertation was focused on the pineal gland of the rat during the estrous cycle, and to a much lesser extent on the influence of pseudopregnancy and decidual tissue on the pineal gland. Following the studies on pseudopregnancy and decidual tissue, however, it became of interest to attempt a small, pilot study on the influence of pregnancy on the pineal gland, owing primarily to the fact that the observations on pseudopregnancy and prolonged pseudopregnancy with decidual tissue proved of remarkable significance. Immediately noteworthy are the facts of increased intensities of succinic dehydrogenase, lactic dehydrogenase, alkaline phosphatase, and acid phosphatase during prolonged pseudopregnancy. The question naturally prompted the investigation of the pineal gland of rats during the biological life span of pregnancy. Specifically, it appeared of great interest to ascertain whether or not the presence of viable fetuses in addition to decidua could in some way further modify the enzymatic intensities of each of the enzymes studied.

1. Gravimetric Data of Pineal Glands of Pregnant Rats

The gravimetric studies on the pineal glands of pregnant rats reveal that there is a marked rise in the weights during the early aspects of pregnancy. Notably, a marked rise in pineal weights is seen over that of estrus, during which time

fertilization occurs. On days six and eight of pregnancy, the pineal gland weights are 0.630 and 0.732 mg%, respectively (Table XIV). Thereafter, weights of the pineal glands are truly representative of those observed during metestrus, particularly day thirteen of pregnancy, and of proestrus (days ten and fifteen) and early estrus (days eighteen, twenty, and twenty-one).

Comparatively, these data are best viewed by examining Tables X, XII, and XIV).

TABLE XIV
WEIGHTS OF PINEAL GLANDS OF RATS DURING PREGNANCY*

Days of Pregnancy**	Number of Animals	Number of Fetuses	Weights of Pineal Glands (mg% \pm S. E.)
Day 6	5	9	0.630 \pm 0.018
Day 8	8	8	0.732 \pm 0.043
Day 10	4	13	0.528 \pm 0.045
Day 13	5	8	0.676 \pm 0.055
Day 15	6	9	0.549 \pm 0.022
Day 18	7	10	0.490 \pm 0.036
Day 20	5	10	0.492 \pm 0.027
Day 21	6	11	0.484 \pm 0.020

* Obtained from Charles River Breeding Laboratories, North Wilmington, Massachusetts.

** Verified as to duration of pregnancy according to the criteria of Christie, 1964, cf. Appendix A.

pinealocytes in the gland of the pregnant rat is of comparatively low succinic dehydrogenase activity, a +1.5. Following this to day eight of pregnancy, during which time the development of differentiated germ layers is nearing completion (totally on day nine), the succinic dehydrogenase picture in the pinealocytes becomes almost maximally intense, a +3.8). During the early development of the central nervous system (CNS), starting from approximately day ten, one observes an increase in intensity in almost linear fashion through day eighteen, at which time much of the central nervous system is complete. The succinic dehydrogenase pattern goes from a +1 to a maximal +4 reaction during this time (Plate XVIII, figures 102-107). During the time when the fetus is normally achieving its definitive form, eighteen to eighteen and one-half days, and the time of parturition (days twenty-one to twenty-two), the succinic dehydrogenase reaction decreases, v.s. to an all-time low of a +1 (Table XV; Figure 16; Plate XVIII, figures 102-109).

b. Lactic Dehydrogenase (LDH)

The pinealocytes of the rat from day six through day twenty-one of pregnancy show moderate to maximal intensities for lactic dehydrogenase. The semi-quantitative histochemical estimates for lactic dehydrogenase reveal a strongly moderate intensity on day six (+2.5), a decrease on day eight (+1), and a steady increase in intensities in three steps to the maximal

reaction day fifteen (+4), cf. Table XV; Figure 17; Plate XIX, figures 110-114. Whereas the minimal reaction (+1) appears on day twenty-one for succinic dehydrogenase, the minimal lactic dehydrogenase intensities (+2) appear on days eight and eighteen (cf. Table XV). Interestingly enough, the reaction for lactic dehydrogenase shows another upswing after the second minima, ascending to a +3.5 on day twenty and slightly less to a +3 on day twenty-one (Table XV; Figure 17; Plate XIX, figures 115-117). The lactic dehydrogenase intensities in the pinealocytes seem to rebound in deep intensities immediately after each of the minima (cf. Plate XIX, figures 110-117). Control pineal gland sections incubated without the substrate, sodium lactate, proved entirely negative.

The maximal reactions for succinic dehydrogenase and lactic dehydrogenase appear in the pregnant animal after much of the central nervous system has been formed in the embryo, a rather interesting finding, especially so when one attempts a correlation between the pineal gland and the events of reproduction. Another interesting finding is that of the maxima for succinic dehydrogenase and lactic dehydrogenase when the corpora lutea vera are the largest in the pregnant rat (Velardo, 1958). Thus, a rather interesting series of events, making this aspect a worthy point of departure for future studies.

c. Alkaline Phosphatase (Alk. P'tase)

The alkaline phosphatase activity of the pineal gland of the pregnant rat shows a most remarkable picture, starting with trace (\pm) intensities on day six, becoming almost moderately intense (+1.5) on days eight and ten, thereafter becoming more intense on day thirteen (+3) and subsequently becoming maximally intense (+4) on day fifteen. The alkaline phosphatase reaction decreases almost 50% on day eighteen (+2) and rises only meagerly thereafter, i.e. a +2.5 on days twenty and twenty-one (Table XV; Figure 18; Plate XX, figures 118-125). Control pineal gland sections incubated without the substrate, α -naphthol acid phosphate, were entirely negative for alkaline phosphatase.

The general observations for the dehydrogenases again pertain here, and one begins to see quite interesting relationships among succinic dehydrogenase, lactic dehydrogenase, and alkaline phosphatase in the pinealocytes during pregnancy.

d. Acid Phosphatase (Acid P'tase)

The results for acid phosphatase quite closely parallel those obtained for lactic dehydrogenase (cf. Table XV; Figures 17 and 19). The acid phosphatase reaction in the pinealocytes on day six appears moderately intense (+2), becomes slightly more intense on day eight (+2.5), remains similarly intense on day ten, thereafter becoming substantially more intense on days thirteen (+3), fifteen (+3.5) and maximally so on

day eighteen (+4). The pinealocytes are heavily laden with acid phosphatase (diazonium granules) on day eighteen, and appear only slightly less so on days twenty and twenty-one (+3), cf. Table XV; Figure 19: Plate XXI, figures 126-133. Control pineal gland sections incubated without the substrate, α -naphthol acid phosphate, proved devoid of any acid phosphatase activity.

It is of interest to point up here the remarkable finding of the near uniformity of intensities of the four enzymes studied, at the time of maximum corpus luteum size during pregnancy, and at the time of near completion of foetal internal development and definitive foetal form (Christie, 1964).

These studies point the way for an extensive analysis of the pineal gland during the whole biological career of pregnancy, with an attempt to ascertain and possibly correlate the several neuroendocrinal relationships (and interrelationships) among the pineal gland, the hypothalamus, the ovaries, the uterus, and the products of conception as well.

TABLE XV

SEMI-QUANTITATIVE HISTOCHEMICAL ESTIMATES OF SUCCINIC DEHYDROGENASE (SDH), LACTIC DEHYDROGENASE (LDH) ALKALINE PHOSPHATASE (ALK. P'TASE), AND ACID PHOSPHATASE (ACID P'TASE) OF THE PINEAL GLAND OF THE RAT DURING PREGNANCY

	SDH	LDH	Alk. P'tase	Acid P'tase
Day 6	+1.5	+2.5	±	+2
Day 8	+3.8	+2	+1.5	+2.5
Day 10	+2	+3	+1.5	+2.5
Day 13	+2.5	+3.5	+3	+3
Day 15	+2	+4	+4	+3.5
Day 18	+4	+2	+2	+4
Day 20	+3.5	+3.5	+2.5	+3
Day 21	+1	+3	+2.5	+3

FIGURE 16
SEMI-QUANTITATIVE HISTOCHEMICAL EVALUATION OF SUCCINIC
DEHYDROGENASE (SDH) ACTIVITY OF THE PINEAL GLAND
OF PREGNANT RATS

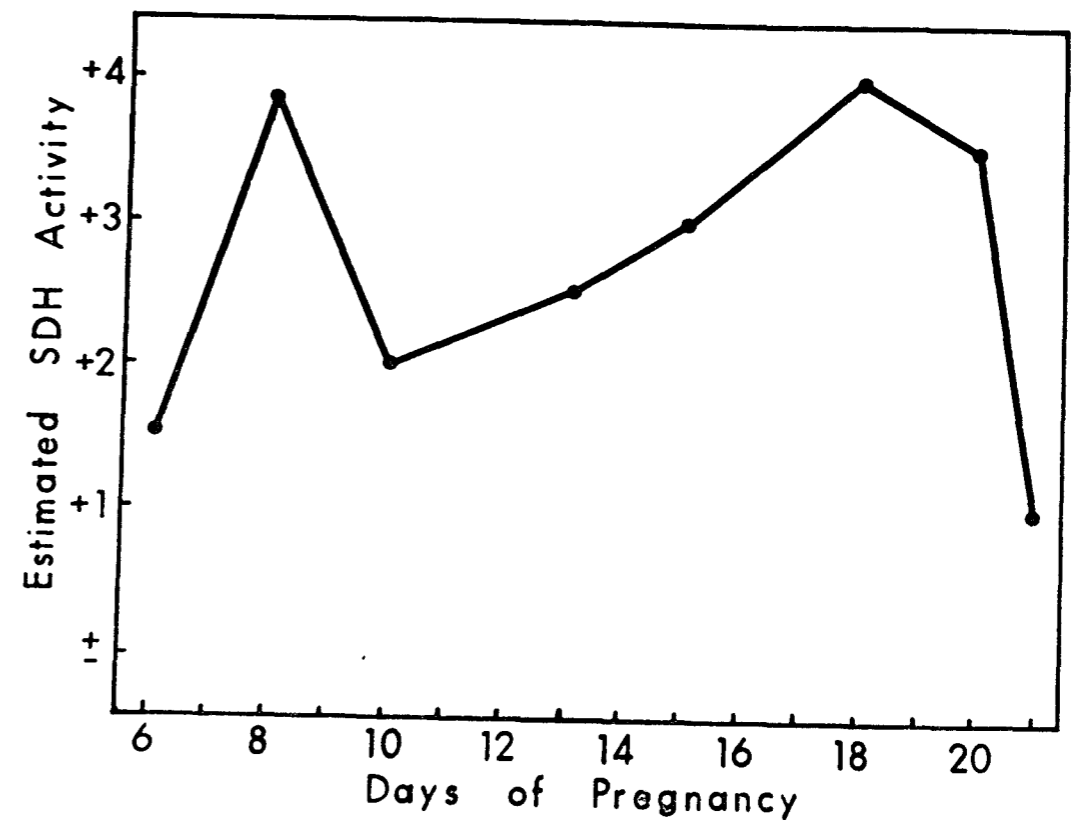


FIGURE 17
SEMI-QUANTITATIVE HISTOCHEMICAL EVALUATION OF LACTIC
DEHYDROGENASE (LDH) ACTIVITY OF THE PINEAL GLAND
OF PREGNANT RATS

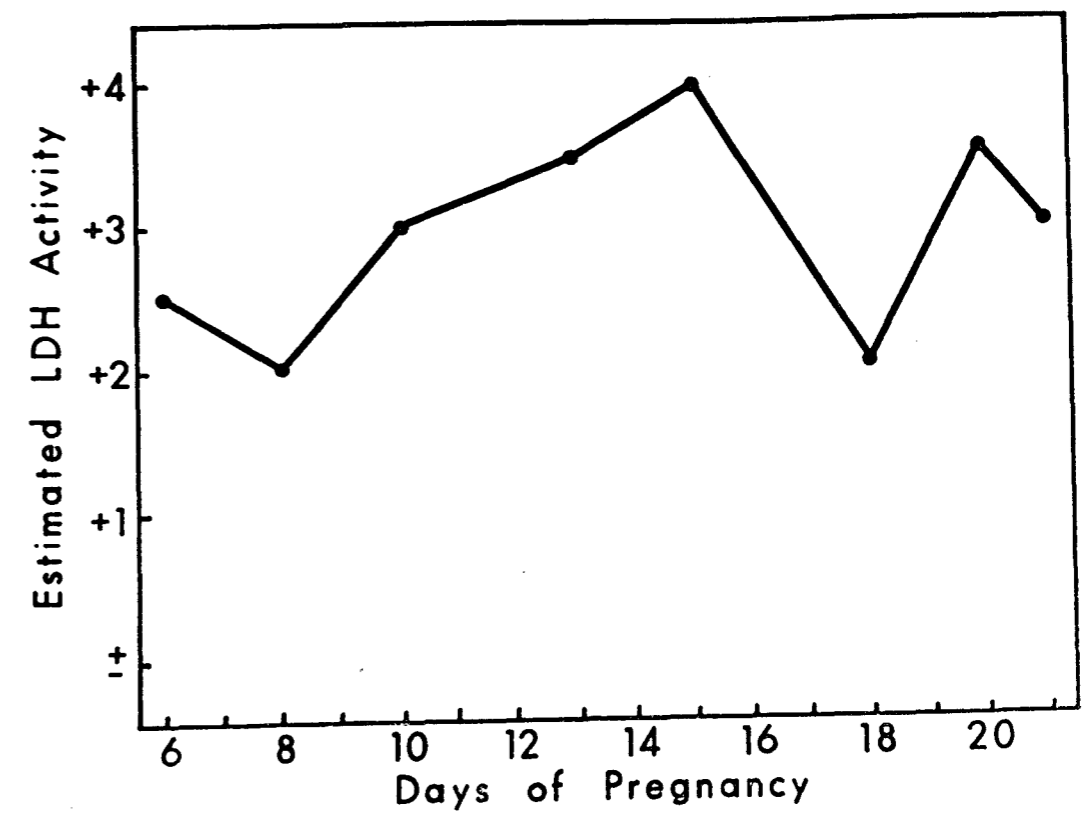


FIGURE 18

SEMI-QUANTITATIVE HISTOCHEMICAL EVALUATION OF ALKALINE
PHOSPHATASE (ALK. P'TASE) ACTIVITY OF THE PINEAL
GLAND OF PREGNANT RATS

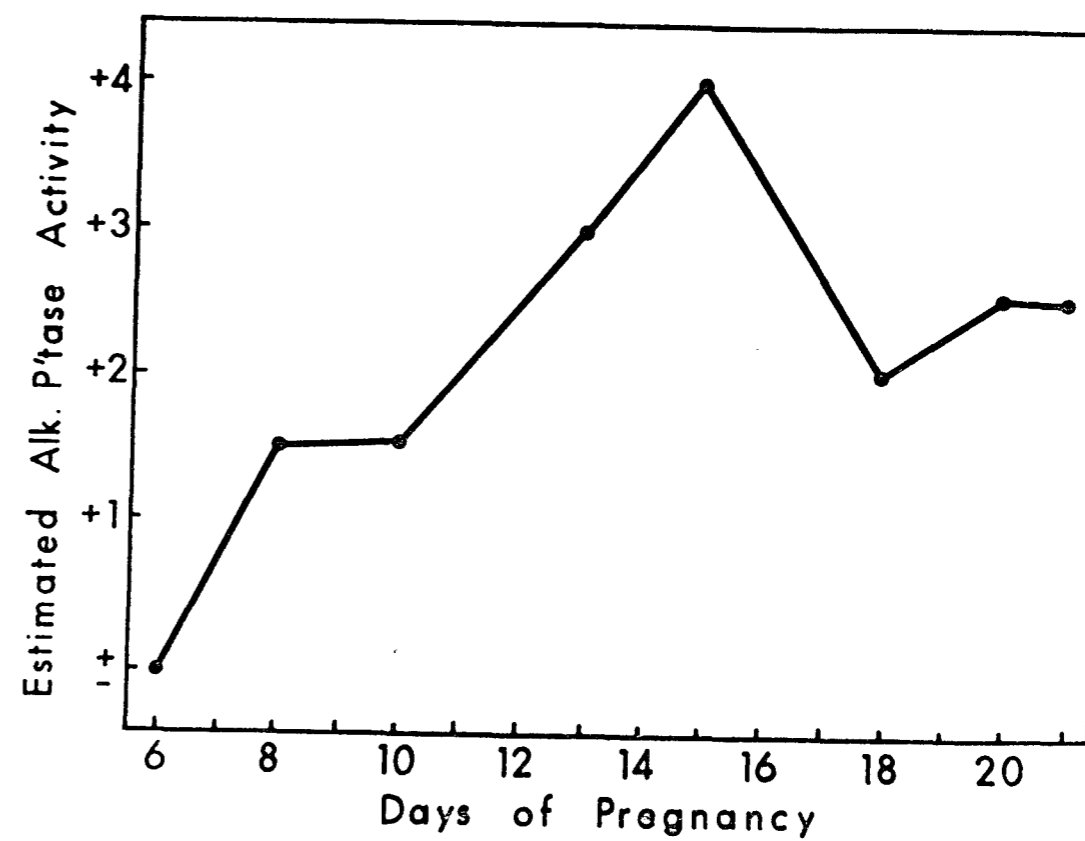
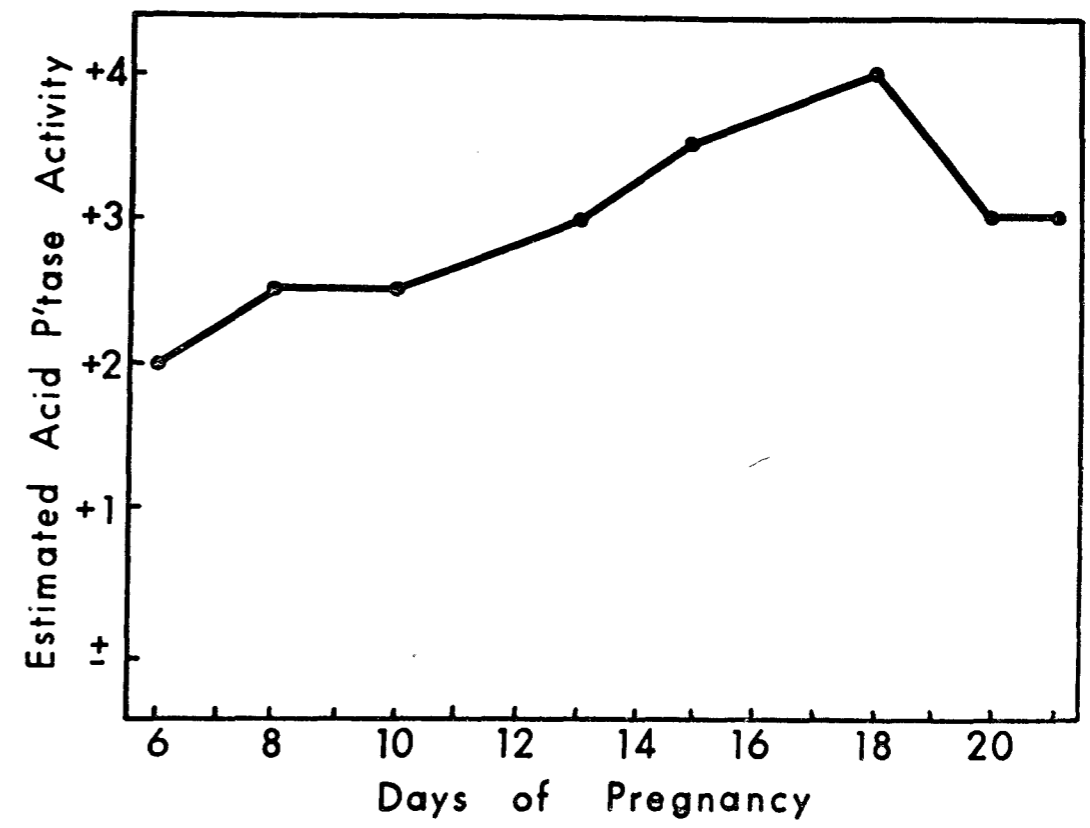


FIGURE 19
SEMI-QUANTITATIVE HISTOCHEMICAL EVALUATION OF ACID
PHOSPHATASE (ACID P'TASE) ACTIVITY OF THE
PINEAL GLAND OF PREGNANT RATS



CHAPTER V

DISCUSSION

As an organ of uncertain endocrine function, the pineal gland has been at the forefront long before the study of endocrinology became a reality. The suspicions that the pineal gland might in some way be associated with gonadal function emerged from two pieces of evidence: first, true parenchymatous pineal tumors are usually associated with depressed gonadal function; secondly, pineal tumors which eventually become widespread throughout the gland, so as to cause enormous destruction of it, are usually associated with precocious puberty (Kitay, 1954). Thus the concept emerged that the pineal gland is the source of a hormone which could affect the gonads. That the pineal gland is enshrouded in rather complex- or at least hard to elucidate-physiological phenomena is well documented (Quay, 1969; Sommers, 1958; Albert, et al., 1954; Kitay and Altschule, 1954; Kappers and Shade, 1965; Cohen et al., 1964; Rilkin, 1966; and Wurtman and Axelrod, 1965).

However complex the pineal may be, it is not surprising to note that endocrinologists and general medical scientists have been in agreement for the past two thousand years regarding the

obscurity of the function(s) of this gland. The stark facts point up one basic concept: the physiological functions of the pineal gland in mammals remain to be elucidated. This is not to convey the thought that practically nothing is known; to the contrary, some major pieces of knowledge exist. It is for exactly the reason of the obscurity of knowledge pertaining to the pineal that this dissertation came into being. The facts that were available made it all the more challenging, for like all new basic sciences in general "Endocrinology is not truly beyond the pioneering stages" (Velardo, 1958).

In a careful appraisal of the information extant, several challenging pieces of information are available:

First, embryologically, it is well known that the pineal gland develops from an evagination of the roof of the diencephalon and that the pineal parenchymal cell is most likely derived from the primitive ependymal lining of the third ventricle. Neuroglia is its chief supporting structure. The surprising fact, however, emerges from the concept that while it is developed from neural tissue, the pineal gland has no direct innervation from the brain in mammals.

Secondly, although nerve fibers from the epithalamic region (habenular) penetrate the pineal, it has been shown that these fibers are exceptional (for their straying characteristics) and emerge from the habenular commissure, loop through the pineal

without synapsing, and return to the opposite side of the brain. While habenular fibers do not in fact innervate the pineal, it is known that postganglionic sympathetic nerve fibers, which have cellular origins in the superior cervical ganglia, reach the pineal gland in association with an abundant blood supply. The sympathetic nerves terminate chiefly in the interstitial tissue spaces of the pineal, and not uncommonly in direct contact with a pinealocyte, thus making a substantial arrangement for allowing neurohumoral substances released from sympathetic neuronal endings in these spaces to diffuse into the pinealocytes. Giving the picture of a true endocrine gland is the fact that the pinealocytes are in close association with a basement membrane in contact with the interstitial space, and this basement membrane in turn is in contact with a capillary basement membrane and a fenestrated capillary endothelium. This cytomorphological architecture allows for control of the pinealocytes by the autonomic nervous system (Wolfe, 1965).

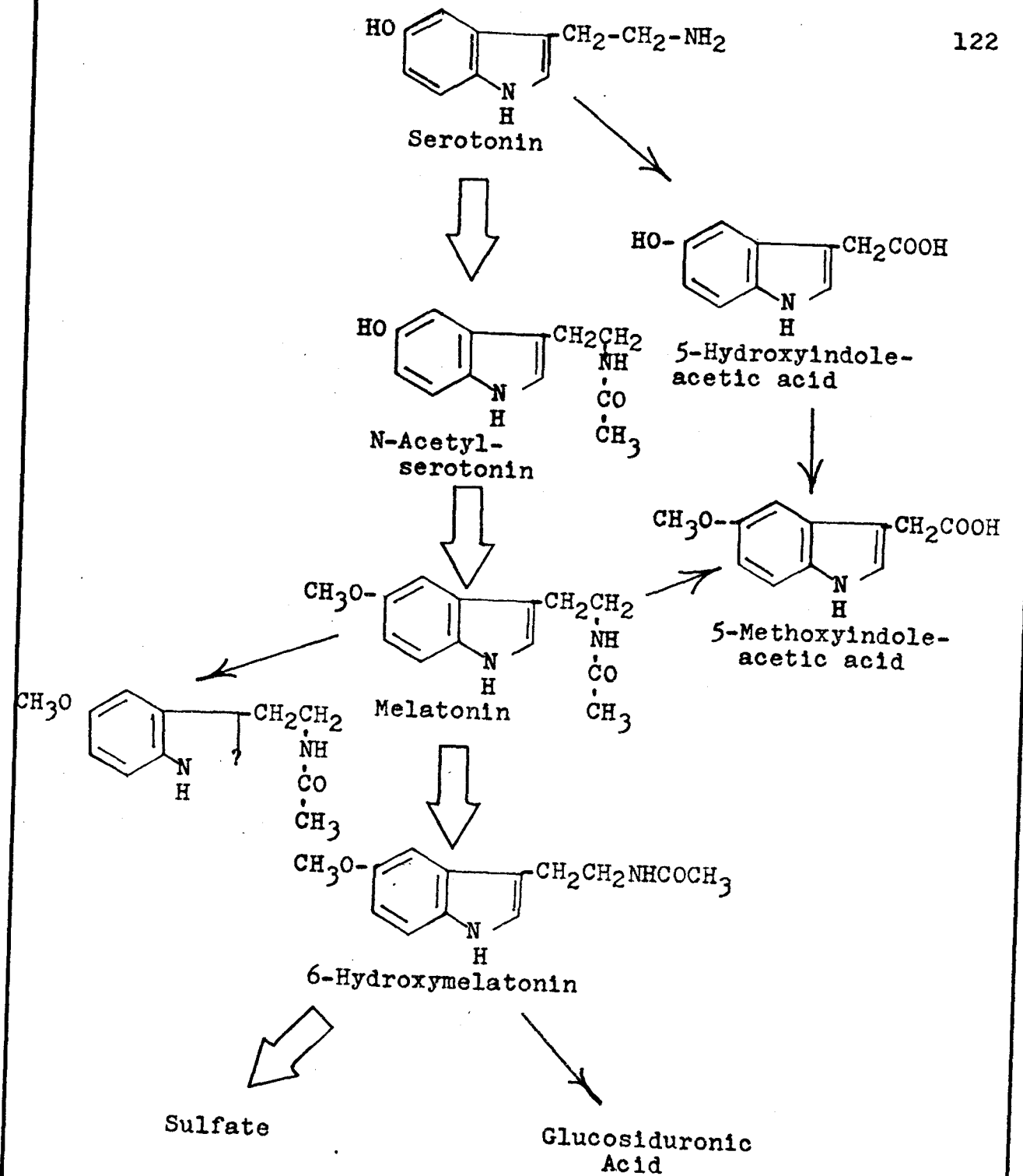
Thirdly, at least three active substances have been isolated from the pineal gland: melatonin, norepinephrine and serotonin. It has been roundly demonstrated by histochemical and pharmacological techniques that the sympathetic nerve fiber endings of the pineal gland contain norepinephrine and serotonin. In the pineal gland, however, serotonin occurs in sympathetic nerve endings. It has also been roundly established that

pinealocytes synthesize rather copious quantities of serotonin which diffuse into the interstitial space, the latter being bound by the nerve endings.

It should be emphasized that serotonin and norepinephrine are not unique to the pineal gland. Specifically, it should be emphasized that melatonin is synthesized only in the pinealocytes. As was stated earlier, Lerner and his associates, in the Department of Dermatology at Yale University School of Medicine, attempting to isolate the active principle in the pineal gland which caused blanching of the skin of frogs, came upon such an amine and thus discovered melatonin. Of especial significance is the fact that melatonin, a most active melanophore contracting principle, acts on cells to cause their granules to cluster with a dramatic lightening of the skin. This important aspect, serving as a biological assay, gave rise to a number of significant observations which ultimately led to the chemical isolation and synthesis of melatonin (Lerner, et al., 1959; Cohen, et al., 1961).

Fourthly, melatonin is formed from N-acetylserotonin, having been derived in turn from serotonin, cf. accompanying diagram.

Biosynthesis of Melatonin. Thick arrows indicate major metabolic pathways in the biosynthesis; thin arrows indicate metabolites of serotonin and melatonin. Illustration adapted from Cohen, *et al.*, 1964, *Ann Int. Med.* 61: 1144.



The enzyme which methylates N-acetylserotonin, hydroxyindole-O-methyltransferase (HIOMT), is found only in the pineal gland. Inasmuch as hydroxyindole-O-methyltransferase can be made to vary under specific experimental conditions, it provides bona fide clues indicating certain of the factors which regulate pineal activity. It is well established that hydroxyindole-O-methyltransferase is found within the pinealocytes. The concentration of this enzyme depends upon the physiological activity of the sympathetic nerves which are in association with the pineal gland, for it is known that sectioning of these nerves markedly diminishes the hydroxyindole-O-methyltransferase activity. Likewise, transplantation of the gland to an ectopic location remote from the nerve supply to the pineal gland results in markedly reduced hydroxyindole-O-methyltransferase activity.

Fifthly, the endocrine influence of the mammalian pineal gland seemingly depends upon alterations in the photoperiod and possible other environmental factors (Quay, 1970).

Sixthly, the reproductive systems of mammalian species are morphologically and physiologically altered by the pineal gland. It is now well known that removal of the pineal gland accelerates gonadal growth in immature animals, and causes a transient, but less well pronounced enlargement of the reproductive organs when the pinealectomy is performed after adulthood. Evidence is also at hand showing that shortened light cycles tend

to make the pineal remarkably antigonadotropic. It has also been shown that chronic administration of pineal extracts or pineal substances elicits effects which are opposite to those of pinealectomy. Specifically, such extracts restrict gonadal development as well as gonadal growth (Reiter and Sorrentino, 1970).

To be sure, one could add a number of other associated facts, and one could present commentaries on some controversial and discredited concepts concerning the pineal gland, e.g. adrenoglomerulotropic, cardiovascular, thymic, hypophyseal, thyroïdal effects, etc. Suffice it to say that the six categories above are in the main some of the most basically discussed and in varying degrees yet tenable concepts regarding the pineal. For added details on the pineal, including numerous non-tenable points, the reader's attention is invited to the review of the literature herein presented, wherein a number of additional points, facts and contentions are also presented. In the main, the above six concepts present a balanced foundation upon which to erect further exploratory studies including those of the present dissertation.

The aforementioned facts are quite revealing of the paucity of basic information pertaining to the status of knowledge of the pineal gland. It, therefore, became of paramount interest to initiate a series of experiments that would in fact beam new

informative insights into the pineal gland. In the process of attempting to set up some new experiments, much helpful guidance was derived from (a) the status of published knowledge on the subject, and (b) some specific questions that are important to our further elucidation of pineal histophysiology.

Specifically, it appeared of interest to ascertain something of the "form and function" relationships of the pineal gland during specific reproductive mechanisms in the rat. First, it appeared of interest to answer certain basic questions regarding the role of the pineal gland during the estrous cycle. Is the pineal gland in any way modified as a result of the estrous cycle changes in the reproductive tract? Can one detect histomorphological changes? Are there some basic and critical weight changes within the pineal gland? Can one demonstrate specific changes in certain groups of enzymes, e.g. dehydrogenases and phosphatases, during estrous cyclicity? Are there any detectable biochemical changes? Thus, the aforementioned questions set the pace. Almost concurrently, a second and a third group of questions evolved.

Of no less importance than the planned estrous cycle studies, the thought of investigating differences in pineal weights and histomorphology during pseudopregnancy became quite important. Of added significance, the whole idea of pursuing studies of the pineal gland during pseudopregnancy in rats bearing

decidual reactions soon became of a realistic nature to stimulate another set of questions.

With the original goals set forth to attempt to uncover some basic, and quantifiable data on the pineal gland of the rat during the estrous cycle and in pseudopregnant rats with and without decidual reactions, it sequentially appeared of interest to pursue a number of studies on the pineal gland during pregnancy. The one basic question which was quite stimulating pressed forward: Can basic changes be demonstrated in the pineal gland throughout the biological career of pregnancy? The challenging concept lurking behind such thinking was to initially examine the pineal gland during a time when the life of the corpus luteum is seemingly quiescent, as it is during the estrous cycle when luteal function cannot be demonstrated, to a time when the rat is reproductively dependent upon the corpus luteum as it is during pseudopregnancy, decidual development and much of pregnancy.

In a series of experiments, commencing with the estrous cycle, two major thrusts were aimed at elucidating basic data pertinent and associated with estrous cycle phenomena. Specifically, it was determined that there are statistically significant changes in the weight of the pineal gland in each of the seven stages of the estrous cycle. Gravimetric data at hand reveals that the pineal glands of rats in metestrus are

heaviest while those in late estrus are lightest, weighing about one-third less.

Examination of the gravimetric data suggests rather convincingly that the pineal gland is quite cyclical in weight relationships in regard to the estrous cycle, and shows most noteworthy correlation with changes in the cycle. During that time (estrus) when the pineal gland is lightest in weight, the ovaries are undergoing an enormous amount of endocrine activity, just prior to ovulation. The ovaries are responding to pituitary gonadotropins, and pineal gland secretion of melatonin can, in fact, control the level of at least one pituitary gonadotropin, luteinizing hormone(LH), as borne out in the researches of Fraschini, Piva and Martini (1969). These workers showed quite convincingly that melatonin can in fact reduce the adeno-hypophyseal stores of luteinizing hormone.

Recently, Kamberi, Mical and Porter (1971) reported that melatonin, as well as serotonin, when injected into the third ventricle of the brain of male rats, appears to stimulate the release of prolactin and inhibit the release of follicle stimulating hormone (FSH) as judged by the changes in the concentrations of these hormones in the plasma of recipient animals. Careful analysis of their radioimmunoassays suggests that melatonin or serotonin "may have suppressed" the discharge of prolactin inhibiting factor (PIF) and follicle releasing

hormone (FRH) and thereby indirectly affected the release of prolactin and follicle stimulating hormone.

In pseudopregnant rats without decidual reactions, in initial and terminal phases of pseudopregnancy, the weights of the pineal gland are typical of the weights of pineals of proestrous rats, whereas those of the mid-aspect (day ten of pseudopregnancy) are more typical of late estrus, i.e. during the time one would normally expect the secretion of melatonin to be at a level to physiologically control luteinizing hormone.

The pineal glands of rats with decidual reactions are equally of interest. Previously, it was shown and discussed that the pineal gland of the rat on the terminal day of pseudopregnancy (day thirteen) has a pineal gland weight typical of that of a proestrous rat; likewise, the pineal gland of the pseudopregnant rat bearing a decidual reaction on day thirteen is of a similar weight relationship. Moreover, for the most part, the pineal gland weight relationships throughout prolonged pseudopregnancy (i.e. of pseudopregnant rats bearing decidual reactions) are typical of preproestrus, proestrus and early estrus; one notable exception being on day fifteen of prolonged pseudopregnancy, at which time the weight relationship resembles that of early diestrus. Again, the point can be made that during luteal functional activity and during the time of decidual development, the pineal gland is comparatively smallest.

The pineal gland weights during pregnancy present a most noteworthy condition, namely that there is a truly marked rise initially. During both initial times, days six and eight, pineal weights appear somewhat typical of metestrus; the pineal glands are the heaviest at these times, respectively. Thereafter, at day ten, the weight drops to that seen during proestrus, but again rises to the metestrus pattern on day thirteen, after which the weight pattern drops to proestrous (day fifteen) and estrous patterns (days eighteen to twenty-one). The noteworthy concept here is that during pregnancy, the weights of the pineal gland are truly typical of those obtained during the three shortest phases of the estrous cycle.

An additional commentary on the gravimetric data concerns the hypophyseal, ovarian, uterine, and adrenal weights. On one hand, one observes that when the pineal gland weight is the heaviest, weights for the pituitary body are also highest; but, on the other hand, at the time of highest pineal weights, the weights of the ovaries and adrenals fluctuate about the mid-point ranges, while the uterine weights are almost at their lowest point. Conversely, when one observes that the weight of the pineal gland is lightest, one finds in association the heaviest ovarian and adrenal weights, and at this time the pituitary body and uterine weights fluctuate about the mid-point ranges. A future arbeit will focus on the microanatomy and

histochemistry of these removed organs so as to look for additional relationships.

Subsequent investigations could develop major inroads into the study of the pineal gland with detailed biostatistical analyses. Also, in a future study, precise wet and dry pineal weight measurements could be determined so as to get another index of activity of this gland. This would facilitate a much more accurate assessment of the changes which occur throughout the estrous cycle, pseudopregnancy, prolonged pseudopregnancy and pregnancy.

Although the gravimetric data forms a small but interesting part of this dissertation, it does point up areas that can in fact provide take-off points for correlated form and function studies, especially at the times when the pineal is heaviest and lightest, and perhaps when the pineal can be shown to be changing in predictable increments and decrements. The present attempt, although in minor proportion to the major histochemical theme, does provide worthy and quantifiable data, and even more important, avenues for future research in this area.

The histochemical and biochemical studies provide a major inroad into some of the hitherto, non-explained concepts regarding the pineal gland during reproductive mechanisms. Especially important and noteworthy are the estrous cycle studies. It is of clear significance that both the histochemical and

biochemical studies pertaining to succinic dehydrogenase (SDH) reveal that of the seven stages investigated, the highest intensities and concentrations within the pinealocytes were found to occur at the time of estrus. This is the time during which an enormous amount of metabolic energy is required for the great crescendo of endocrine activity associated with the period of heat and the events of ovulation (Velardo, 1951; Bever, et al., 1954; Rosa and Velardo, 1954a and b, 1955; 1958, 1959; Velardo and Rosa, 1963). It is also of notable significance that the lowest intensities and concentrations of this dehydrogenase were determined to occur immediately following ovulation, i.e. in the estrous cycle stages designated as metestrus (cf. preceding references of Rosa and Velardo, and Velardo and Rosa).

Realizing the significance of the finding pertaining to succinic dehydrogenase, it naturally appeared of interest to ascertain the pattern of lactic dehydrogenase (LDH) activity during the estrous cycle. Histochemical and biochemical determinations for lactic dehydrogenase proved to be quite rewarding. Whereas the maximal peak for succinic dehydrogenase occurred at estrus and the minimal value at metestrus, the maximal and minimal points for lactic dehydrogenase occurred at proestrus and late diestrus, respectively. Here, one should emphasize that the metabolic energy cycle leading to lactate production precedes that of the tricarboxylic acid-succinate energy yielding reaction.

Again, realizing the enormous amount of metabolic work and energy required for the accomplishment of endocrine expression associated with the periods of heat and ovulation, it is indeed quite significant to point up the findings suggesting a biological order of priorities: first, during proestrus, lactic dehydrogenase appears maximally; secondly, as proestrus advances to estrus, succinic dehydrogenase appears in maximal designations, in both histochemically demonstrable intensities and biochemically determined concentrations. Such has also been found in the reproductive tracts of estrous, intact and estrogenized, ovariectomized rats (Bever, Velardo and Hisaw, 1953 a, b; Rosa and Velardo, 1953, 1954 a, b; 1959; and Velardo and Rosa, 1963).

Observations on alkaline and acid phosphatase revealed that the pineal gland was maximal for both of these enzymes during early estrus. The high intensities and concentrations of these two phosphatases markedly decrease after early estrus, decreasing to minimal reactions during late estrus and metestrus. The data on the phosphatases are of exciting interest, particularly since it has been repeatedly shown that (a) alkaline phosphatase is associated with rapidly growing tissues in the reproductive tracts of animals and man, especially during periods of estrus or in ovariectomized animals given estrogens (Velardo, 1954; Mc Kay, et al., 1966 a, b; and Velardo and Rosa, 1963); (b) acid phosphatase is also associated with rapidly growing

tissues in health and disease, and it has been shown to occur in near peak concentrations and intensities in the reproductive tracts during estrous phases and after estrogenization in a number of species including the human female (cf. Velardo and Rosa, 1963); and (c) the results for alkaline and acid phosphatase obtained in the pineal gland are in essential agreement with those found in the reproductive tract.

Of added interest are the results of the preliminary colorimetric determination of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT). Both of these enzymes are known to catalyze the reaction resulting in the formation of glutamic acid. This amino acid, in turn, can either be metabolized to glutamine or to gamma-aminobutyric acid. Current investigations on gamma-aminobutyric acid (GABA) have recently become of great interest stemming from the hypothesis that this acid probably plays an important functional role in the modulation (or inhibition) of neurotransmitter substances in the mammalian central nervous system (Bloom and Iverson, 1970; Sze and Lovell, 1970).

The high concentration of glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase in the pineal gland during the diestrus could possibly be indicative of increased gamma-aminobutyric acid production. If this is indeed true, the increased concentration of gamma-aminobutyric acid may be an

indicator of pineal inhibition other than via melatonin and the biogenic amines as has been suggested and refuted. (Wurtman, et al., 1963; Chu, et al., 1964; Kappers, 1962; Ebels and Prop, 1965; Chessman, 1970; Chessman and Farriss, 1970; Debeljuk, 1969; Moszkowska and Ebels, 1968; etc.).

During pseudopregnancy, as with the estrous phases of the cycle, the semi-quantitative histochemical estimates for succinic dehydrogenase, lactic dehydrogenase, alkaline phosphatase, and acid phosphatase in the pineal gland are very strong to maximal, the notable exception being lactic dehydrogenase on day ten of pseudopregnancy at which time only a moderate intensity (+2) is obvious. Previously, it was noted that during the estrous cycle the maximum for lactic dehydrogenase in the pineal gland appears before that of succinic dehydrogenase, and the same situation occurs during pseudopregnancy. Specifically, lactic dehydrogenase is maximal on day six whereas succinic dehydrogenase appears to be maximal on day ten. It is at this time that the corpus luteum of pseudopregnancy is nearly maximum in size, as has been reported by Dawson and Velardo (1955). The phosphatases are likewise of interest for they are of very strong intensities (all +3 reactions with a maximum +4 for alkaline phosphatase occurring only on day ten). This correlates quite nicely with the high concentrations of these phosphatases in the reproductive tract when the corpus luteum is functional (cf. Velardo and Rosa,

1963; Dawson and Velardo, 1955).

Inasmuch as it appeared quite important to correlate some data with the pineal studies in pseudopregnant rats bearing decidual reactions, it will perhaps be sufficient to preface those remarks here, and then provide a commentary on the possible correlation of the pineal with the physiological expression of prolonged pseudopregnancy. It was quickly seen that the succinic dehydrogenase reaction (+2) and those of lactic dehydrogenase, alkaline phosphatase and acid phosphatase (+3 to +3.5 reactions) are moderate to strong at the time of the initial developing stages of the antimesometrial reaction, the latter normally seen on day six of pseudopregnancy in rats with decidual reactions (cf. Velardo, Dawson, Olson and Hisaw, 1953 for decidual development). It was also pointed up that on day thirteen the intensities for all four of these enzymes are near maximal intensities, and it is at this time that the prolonged pseudopregnancy study of Velardo, et al. (1953) indicated that the mesometrial reaction reaches peak development. It is also at this time that the metrial gland becomes organized and proceeds at a fast developmental pace (Velardo, Dawson, Olson and Hisaw, 1953). The present study clearly identifies this as the time during which the enzymatic intensities in the pineal gland show variable but yet substantial intensities; (a) succinic dehydrogenase varies from a +3 on day thirteen, thereafter declines to a +1.5 on day

fifteen, rises to a +4 on day eighteen, and declines to a +2.5 and a +2 on days twenty and twenty-one; (b) the lactic dehydrogenase pattern is almost exact, with the notable exception of a +3 rather than a +4 on day eighteen; (c) the alkaline phosphatase intensities increase from a +3 (on day thirteen) to a +4 on day fifteen, thereafter plateau at a +2 on days eighteen and twenty and slightly decrease on day twenty-one to a +1.5; and lastly (d) acid phosphatase shows a near maximal (+3.5) reaction (on day thirteen), increases to a maximal reaction day fifteen, thereafter decreases in intensity from a +3 to a +2 to a +2.5 on days eighteen, twenty, and twenty-one, respectively.

The cogency for repeating the data from the thirteenth day of pseudopregnancy resides in the fact of the coincidence of time of the initial aspect of prolonged pseudopregnancy, for it is at this time that if large decidual reactions are induced, the animals do not return to estrus, and reappearance of estrus is delayed until days twenty-one to twenty-two, the normal duration of prolonged pseudopregnancy. Specifically, some rather interesting histochemical data from the pineal gland studies pose some remarkable thoughts regarding some of the non-explored areas of reproductive endocrinology. Numerous thoughts emerge: (a) What accounts for the sudden, remarkable decrease in intensity of succinic dehydrogenase at the time of the metrial gland development, and the noteworthy rise in intensity to maximal

strength within seventy-two hours following the minimal reaction observed throughout the course of prolonged pseudopregnancy?

(b) Can one ascribe such to the elusive, but present hormone called relaxin, a hormone secreted by the reproductive tract primarily during luteal activity? (cf. Velardo, et al., 1963; Wislocki, et al., 1957; Dixon and Bulmer, 1971); (c) Can the variations in enzymatic activity as observed in the pineal gland during pseudopregnancy be duplicated with injections of relaxin, estrogens and progestins alone or in dual combinations, or in concert?; and (d) Can one show even more dramatic changes in the pineal gland during pregnancy?

To be sure, the thought emerged to pursue these studies in pregnant animals so as to determine the pineal:reproductive interrelationships during the time when reproductive activity and luteal life are seemingly dominant. The results obtained were much more intriguing than any of the data obtained from pineal glands of rats during pseudopregnancy, with and without decidual reactions. Whereas the maximal reactions of each of these enzymes during pseudopregnancy was between days six and ten, and whereas the maximal reactions for these enzymes during prolonged pseudopregnancy (pseudopregnant animals bearing decidual reactions) appeared as early as day thirteen for lactic dehydrogenase, followed by equally maximally intense reactions for alkaline and acid phosphatase on day fifteen, and thence followed seventy-two

hours later, on day eighteen, by maximal reactions for succinic dehydrogenase, the histochemical reactions during pregnancy showed most remarkable and intriguing patterns for maxima: on day fifteen both lactic dehydrogenase and alkaline phosphatase are maximal; on day eighteen, both succinic dehydrogenase and acid phosphatase are maximal. Even more intriguing is the fact that none of the maxima are actually observed until after the onset of the second half of pregnancy, specifically the mid- to near-terminal aspects of pregnancy. These data make it rather obvious that the maximal reactions for lactic dehydrogenase and alkaline phosphatase not only precede those of succinic dehydrogenase and acid phosphatase but are importantly prominent at the time of full metrial gland development. Note well: the maxima for alkaline phosphatase, +4 reaction, occurs on day fifteen in both pregnancy and prolonged pseudopregnancy states. The question reappears: Is there a correlation between relaxin of the metrial gland and the pineal gland - specifically between relaxin and melatonin? Is the correlation in any way governed by the high intensities of lactic dehydrogenase and alkaline phosphatase within the pineal gland?

Another interesting aspect regarding maxima is the fact that the maximal reactions for succinic dehydrogenase during prolonged pseudopregnancy and pregnancy occur on the same day, day eighteen. The acid phosphatase reaction maximizes on day

eighteen, seventy-two hours later than that seen during prolonged pseudopregnancy. The interesting aspects now emerge: seemingly exhaustion of the antimesometrial reaction, and the necrosis of the mesometrial reaction can in some way be correlated with the maximal reactions for lactic dehydrogenase and alkaline phosphatase. Moreover, the full development of the metrial gland, too, can in some way be correlated with the pineal gland peak reactions of lactic dehydrogenase, alkaline phosphatase and during the subsequent seventy-two hours with the rising to peak activities of succinic dehydrogenase and acid phosphatase. Dallenbach-Hillweg, Battista and Dallenbach (1965); Wislocki, Weiss, Burgos and Ellis (1957); and Dixon and Bulmer (1971) have independently shown by histochemical, immunofluorescent, and electron microscopical evidence that the metrial gland is heavily laden at this time, and present further evidence suggesting that relaxin is a hormone of the metrial gland. Thus the future experiments of choice must consider and incorporate relaxin with melatonin so as to get a more lucid understanding of reproductive mechanism in the rat.

No discussion of the pineal gland would be complete without first relating these aforementioned findings to the effect of light and the inherent capacity of the gland toward a circadian (diurnal) rhythm. It is true that many chemical substances within the pineal gland do demonstrate a rhythm, and this rhythm

has been shown to be regulated by environmental lighting. Naturally the question arises: Do the results obtained in this investigation demonstrate actual changes in the weights of the pineal and in the activity of the enzymes studied or are these variations a function of an epiphyseal circadian rhythm? To this investigator, the changes are actual changes which occur irregardless of the light:dark cycle. This has been demonstrated individually for the gravimetric study and the histochemical and/or biochemical determinations as reported herein.

Observations of the gravimetric results during the estrous cycle indicate a maximal weight for the pineal gland (in mg%) during metestrus with a second maximal peak during late diestrus. The animals were necropsied during the light phase of the light:dark cycle during metestrus and during both the light and dark phases of the light:dark cycle during late diestrus. Concerning the maximal peaks, there does not appear to be a correlation with a circadian rhythm in the pineal because during the early diestrus phase, the animals were also necropsied during the light and dark phases of the light cycle, and there is a statistically significant difference between metestrus and early diestrus. Also, there is a statistically significant difference between proestrus and metestrus, although both stages were obtained after necropsies of animals during the light phase (cf. Tables I, II; Figure 1).

Gravimetric analyses of the pineal glands of rats which were pseudopregnant, pseudopregnant bearing decidual reactions, and pregnant reveal both maximal and minimal weights. All of these animals, however, were sacrificed and the pineal glands collected and weighed during the light phase of the light:dark cycle (cf. Tables X, XI, XIV). It thus appears that the weight of the pineal gland is independent of the effect of light or darkness.

The histochemical and biochemical results obtained for succinic dehydrogenase, lactic dehydrogenase, alkaline phosphatase and acid phosphatase during the estrous cycle also prove the contention that the activity of these enzymes is independent of light. Succinic dehydrogenase demonstrates a major peak during the dark phase of the light:dark cycle (i.e. during late estrus) and a secondary peak in those animals which were sacrificed during both the light and dark phases (i.e. during late diestrus). Both the semi-quantitative histochemical and quantitative biochemical determinations of the activity of succinic dehydrogenase in those animals which were in the late diestrus stage of the estrous cycle were performed on an equal number of animals necropsied during the light and dark phases of the cycle. There was no observable differences in the histochemical evaluation and no appreciable difference in the biochemical determination (cf. Tables VII, VIII; Figure 4).

Lactic dehydrogenase proves the contention that the activity is independent of light even more emphatically. The maximal reaction occurs both histochemically and biochemically during proestrus at which time the animals were necropsied during the light phase of the light:dark cycle. The second maxima however occurs during late estrus, or in that stage when the animals are necropsied entirely during the dark phase of the light:dark cycle. It should also be pointed out at this time that the minimal reaction for lactic dehydrogenase occurs during early estrus, a stage in which the animals were sacrificed during the dark phase. If light was the regulating factor, and if a circadian rhythm does exist then one would not expect both the maximal and the secondary peak to be during different phases of the light:dark cycle. Also, one would not expect both the second maximal and the minimal reactions to occur during the same phase of the light:dark cycle nor in the same stage of the estrous cycle.

The thought that the diurnal variation, if present, may be a twelve hour rhythm is not born out by the subsequent twelve, twenty-four, thirty-six and forty-eight hours where this enzyme demonstrates a steady decline to a minimal reaction during late diestrus (cf. Tables VII, VIII; Figure 5).

Alkaline phosphatase activity displays a maximal reaction during early estrus and a minimal during late estrus.

Therefore, a maximal reaction is observed during the dark phase of the light:dark cycle (early estrus) and a minimal reaction during the dark phase (late estrus) of the light:dark cycle (cf. Tables VII, VIII; Figure 5).

Acid phosphatase, as alkaline phosphatase, also has a maximal reaction during the dark phase (early estrus) and a minimal reaction during the dark phase (late estrus). However, there is a second minimal reaction which occurs during metestrus and during this stage the animals were necropsied during the light phase of the light:dark cycle (cf. Tables VII, VIII; Figure 7).

As a result of these findings the changes which occur in the pineal gland weights and in the enzymes studied appear to occur without the mediating effect of light, at least in a 12:12 light:dark environment.

During pseudopregnancy, prolonged pseudopregnancy in rats bearing decidual reactions, and during pregnancy, the enzymes studied histochemically also demonstrated varying degrees of activity ranging from maximal to minimal. All of these animals were necropsied during the light phase of the light:dark cycle (cf. Tables XI, XII, XV; Figures 10-19). There, then, does not appear to be any strong, compelling reason to assume that these particular enzymes studied demonstrate a diurnal rhythm; rather, it appears that their activity is a function of the aforementioned

criteria.

Like most studies, the present dissertation unlocks the door to more questions than were present in the initial stages of these investigations. As with most works, and the present is certainly no exception, each investigation adds to the sum of our knowledge.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The main thrust of the dissertation involves a number of studies on the histochemistry of the pineal gland of the rat during seven stages of the estrous cycle: early estrus (A_1), late estrus (A_2), metestrus (B), early diestrus (C), late diestrus (D), preproestrus (E) and proestrus (F). An important offshoot of these studies on the pineal gland branched into three aspects: pseudopregnancy, prolonged pseudopregnancy (rats with decidual reactions), and pregnancy. Accompanying the first part of this dissertation are a number of biochemical observations on the pineal gland. Also, a large series of gravimetric data is included on weight relationships of the pineal gland, the pituitary body, adrenals, ovaries and uteri of the rat during the estrous cycle.

Specifically, this dissertation is concerned with a gravimetric analysis and a histochemical elucidation of the activity of succinic dehydrogenase (SDH), lactic dehydrogenase (LDH), alkaline phosphatase (Alk. P'tase), and acid phosphatase (Acid P'tase) activity in the pineal gland of the albino rat during seven different stages of the estrous cycle, pseudopreg-

nancy, prolonged pseudopregnancy in rats bearing decidual reactions, and in pregnancy.

1. Gravimetric analysis of the pineal gland revealed a maximal weight, in mg%, during metestrus with a minimal weight occurring during late estrus, the latter weighing one-third those of metestrus.

2. When the pineal gland is lightest in weight, the ovary and adrenal glands are heaviest and the hypophysis and uterine weights are approximately at the mid-point in their weight range. Conversely, when the pineal gland is heaviest, the hypophysis is heaviest, but the ovaries and adrenals are at the mid-ranges, whereas the uterus is at a low range.

3. The pineal gland is quite cyclical in weight changes during the estrous cycle.

4. During the three stages of pseudopregnancy, the weight of the pineal gland is typical of those of proestrus on the initial and terminal days of pseudopregnancy (days six and thirteen); on day ten, the weight of the pineal appears similar to that found during late estrus.

5. For the most part, the pineal gland weights during prolonged pseudopregnancy in rats bearing decidual reaction show weight relationships which are typical of those observed during preproestrus, proestrus and early estrus; the one exception being day fifteen of prolonged pseudopregnancy which appears to

have a mean weight typical of those of early diestrus.

6. The weights of the pineal gland of pregnant rats are highest on days six and eight (being somewhat typical of metestrus), lightest on days eighteen, twenty, and twenty-one (being somewhat typical of early estrus), and appear cyclic in nature during the period of gestation with the lowest weights occurring during the last four to five days of pregnancy.

7. The four enzymes studied are localized in the cytoplasm of the pinealocytes.

8. Histochemical and biochemical studies on the pineal gland of the rat during the estrous cycle revealed the following results:

- a. For SDH: A_2 (maximal) $>$ A_1 $>$ D $>$ C $>$ E $>$ F $>$ B (minimal);
- b. For LDH: $F > A_2 > B > C > E > A_1 > D$;
- c. For Alk. P'tase: $A_1 > E > C > D > F > B > A_2$;
- d. For Acid P'tase: $A_1 > D > C > E > F > A_2 > B$.

9. The biochemical studies on the pineal gland of the rat during the estrous cycle revealed the following results:

- a. For GOT: $C > D > F > B > A_1 > A_2 > E$;
- b. For GPT: $D > A_2 > A_1 > B > F > C > E$;

10. Histochemical analyses of the pineal gland of the rat during three stages of pseudopregnancy studied revealed the following on days:

	<u>6</u>	<u>10</u>	<u>13</u>
a. For SDH:	+3	+4	+4
b. For LDH:	+4	+2	+3
c. For Alk. P'tase:	+3	+4	+3
d. For Acid P'tase:	+3	+3	+3

11. Histochemical determinations of the pineal glands of pseudopregnant rats bearing decidual reactions displayed the following intensities on days:

	<u>6</u>	<u>10</u>	<u>13</u>	<u>15</u>	<u>18</u>	<u>20</u>	<u>21</u>
a. For SDH:	+2	+3.5	+3	+1.5	+4	+2.5	+2
b. For LDH:	+3.2	+3.5	+4	+2	+3	+2.5	+2
c. For Alk. P'tase:	+3	+2.5	+3	+4	+2	+2	+1.5
d. For Acid P'tase:	+3.5	+3	+3.5	+4	+3	+2	+2.5

12. Histochemical analyses of the pineal gland during pregnancy displayed the following intensities on days:

	<u>6</u>	<u>8</u>	<u>10</u>	<u>13</u>	<u>15</u>	<u>18</u>	<u>20</u>	<u>21</u>
a. For SDH:	+1.5	+3.8	+2	+2.5	+2	+4	+3.5	+1
b. For LDH:	+2.5	+2	+3	+3.5	+4	+2	+3.5	+3
c. For Alk. P'tase:	±	+1.5	+1.5	+3	+4	+2	+2.5	+2.5
d. For Acid P'tase:	+2	+2.5	+2.5	+2.5	+3.5	+4	+3	+3

13. High or maximal intensities of lactic dehydrogenase precede those of succinic dehydrogenase in the pineal glands of rats during the estrous cycle, pseudopregnancy, prolonged pseudopregnancy and during pregnancy.

14. Maximal phosphatase activities in the pineal gland either precede or are coincident with maximal intensities for succinic dehydrogenase.

15. During the estrous cycle, the maximal succinic dehydrogenase activity was coincident with the events of heat and ovulation in the rat, while during pseudopregnancy there appears to be a relationship between the activity of this enzyme and that of the corpus luteum of pseudopregnancy.

16. Succinic dehydrogenase maxima during prolonged pseudopregnancy and pregnancy indicate a possible relationship between this enzyme and the increased production and secretion of relaxin.

17. Experimentally-derived data clearly indicate that it is possible to demonstrate and compare the cyclical nature of the pineal gland, both gravimetrically and histochemically, the cyclicity of the estrous cycle in albino rats. Consequently, numerous correlative relationships and interrelationships were established, ranging from (a) high intensities of enzymes in the pineal gland with high concentrations of similar enzymes in reproductive tracts of rats during estrous and in estrogenized,

ovariectomized rats, to (b) specific concentrations of pineal-containing enzymes with specific aspects of adenohipophyseal-gonadal relationships.

18. Likewise, experimentally-derived data from this dissertation make it possible to establish numerous correlations between pineal enzymorphological and reproductive histomorphological observations during pseudopregnancy, prolonged pseudopregnancy and pregnancy. Notable examples include specific intensities of the dehydrogenases and phosphatases coinciding with the development, maturation and subsequent necrosis of the antimesometrial, mesometrial and metrial gland. Also, correlative relationships were established between pineal enzymatic intensities and the biological career of pregnancy.

19. Enzymorphological studies on the pineal gland reveal that lactic dehydrogenase, a glycolytic enzyme in a system which produces a small amount of ATP, always precedes succinic dehydrogenase, an enzyme of the tricarboxylic acid cycle known for its high production of ATP, in the metabolic scheme.

20. The results from these investigations clearly reveal that the pineal gland enzymology and gravimetrics can be related to the dynamics of reproductive mechanisms, both endocrinologically and neuroendocrinologically.

21. These data form a workable standard reference baseline for several important subsequent investigations which

can be performed on the pineal gland, the reproductive mechanism, and the interrelationship of one with the other.

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APPENDIX A

Stage	Length (mm)	Somite Number	Age (days)	Main Commencing Features
15		1-3	9 1/2- 9 3/4	Somites have appeared. Neural folds elevating.
17		4-6	9 3/4- 10	Delimited otic (4th) rhombomere and post-otic sulcus.
18		7-9	10- 10 1/2	Neural canal closed from the level of the 2nd to the 6th somite.
19		10-14	10 1/2- 10 3/4	Neural folds fused at dienceph- alic-mesencephalic junction. Otic pit forms.
20	1.6-3.0	15/16- 22	10 3/4- 11 1/2	Anterior neurophore and rhomben- cephalon closed.
21	3.0-4.1	23/24- 28	11 1/2- 12	Posterior neuropore and otic pit closed.
22(A)	4.1-4.6	29-32	12- 12 1/2	Endolymphatic sac appears "pinched off" from otic vesicle. Maxillary process has reached lateral nasal process.
22(B)	4.6-5.8	33-37	12 1/2- 13	Projection into roof of Rathke's pouch visible. Dorsum of posterior limb bud flattened.
23(A)	5.8-7.1	38/39- 41	13- 13 1/2	The lens vesicle is closed. Tubercles visible on contiguous sides of mandibular and hyoid arches.

Stage	Length (mm)	Somite Number	Age (days)	Main Commencing Features
23(B)	7.1-7.9	42-44	13 1/2- 13 3/4	Primitive posterior naris visible
23(C)	7.9-9.4	45-47	13 3/4- 14	Rathke's pouch closed.
24	9.4-10.3	48-	14- 14 1/4	First vibrissary papilla appears on maxillary process. First traces of digital condensations in fore-paw.
25	10.3-11.5		14 1/4- 14 3/4	Four rows of papillae visible, with invagination starting.
26	11.5-12.1		14 3/4- 15 1/2	Six rows of papillae present. Swelling of dorsal part of tongue visible.
27	12.1-12.7		15 1/2- 16	First trunk hair papillae present.
28	12.7-14.5		16- 16 1/2	First set of papillae on dorsum of tongue.
29	14.5-16.0		16 1/2- 17	Digits fully separated on forepaw. Ventral third of palate fused.
30	16.0-17.6		17- 17 1/2	Vibrissae appear from maxillary follicles. Middle thirds of palate fused.
31	17.6-19.1		17 1/2- 18	Digits separated on hind-paw. Claw bearing area differentiated on fore-paw.
32	19.1-22.0		18- 18 1/2	Umbilical hernia reduced. Eye and ear not yet fully closed.

PLATE I

PHOTOMICROGRAPHS OF THE NORMAL HISTOLOGY OF THE PINEAL GLAND
OF RAT. X 2000.

Figure 20 Pineal Gland. Hematoxylin-Eosin. Two types of cells are observed: 1) light cell (l) with a round nucleus, prominent nucleolus and a pale staining cytoplasm; and 2) a dark cell (d) with irregularly shaped nucleus, no apparent nucleolus and a darker staining cytoplasm. Note also lipid vacuoles surrounding capillaries (c).

Figure 21 Pineal Gland. Bargmann's chrom-alum hematoxylin. Longitudinal section of a pineal capillary (c), containing red blood cells and lined with neurosecretory material of moderate staining intensity.

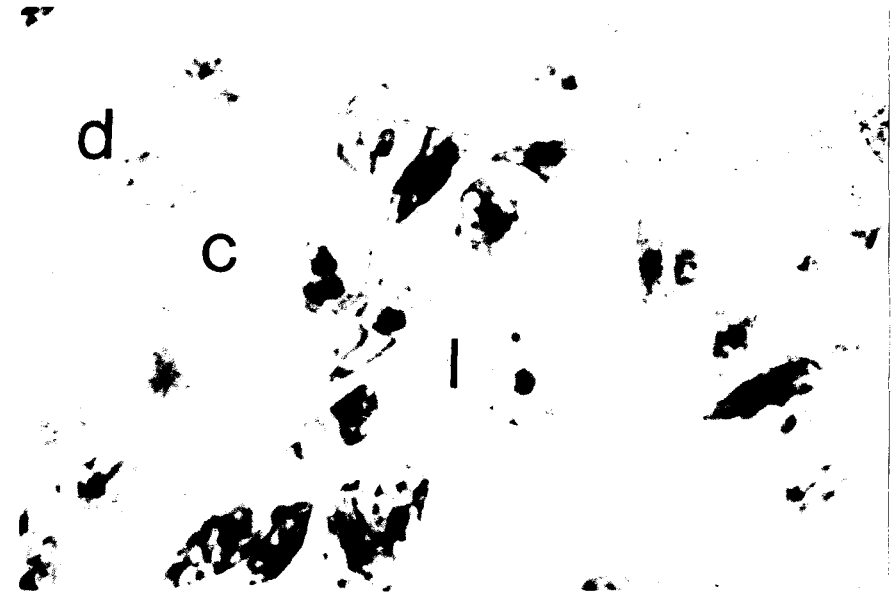


Figure 20

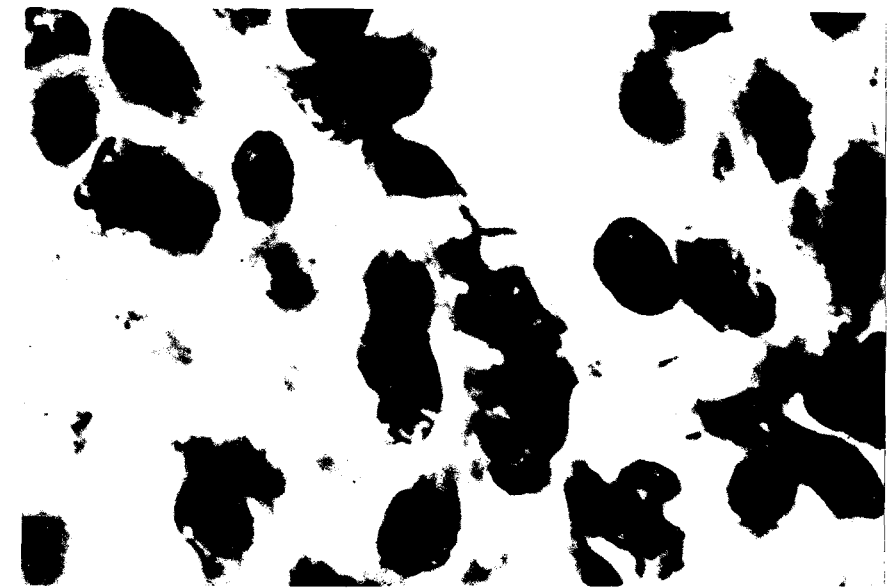


Figure 21

PLATE II

NORMAL HISTOLOGY OF THE PINEAL GLAND OF THE RAT: CAPILLARIES.
X 2000. Bargmann's chrom-alum hematoxylin.

Figure 22 Cross section of a pineal capillary (c) showing darkly-staining neurosecretory material surrounding the vessel.

Figure 23 Tangential section of a pineal capillary (c) from an animal different from that of figure 22. The typical dark staining neurosecretory material is again seen surrounding the capillary.

PLATE II

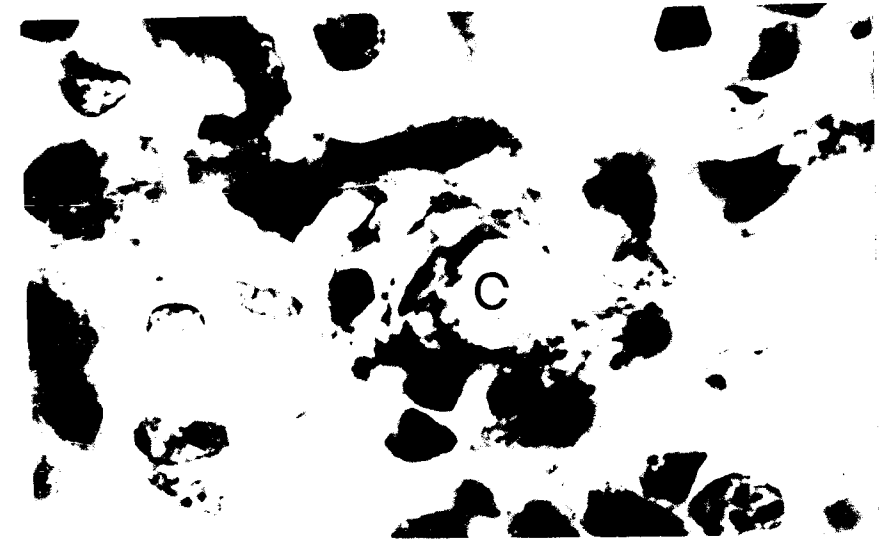


Figure 22



Figure 23

PLATE III

LONGITUDINAL SECTION OF THE PINEAL STALK OF THE RAT. X 2000.
Bargmann's chrom-alum hematoxylin.

Figure 24 Dark staining neurosecretory material is seen here
in an irregular pattern along this portion of the
pineal stalk.

Figure 25 In this section of the pineal stalk the darkly-
staining neurosecretory material is seen in discrete
lines.



Figure 24



Figure 25

PLATE IV

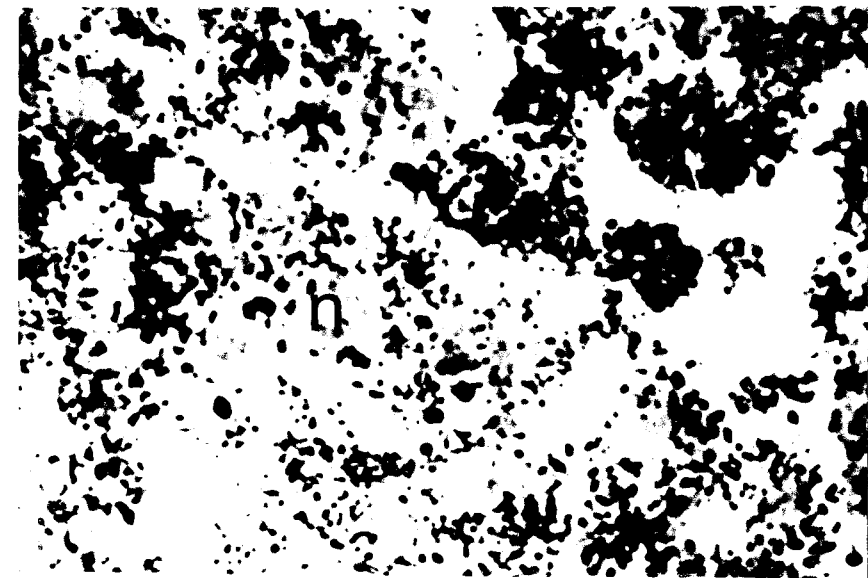


Figure 26

LOCALIZATION OF SUCCINIC DEHYDROGENASE AND LACTIC DEHYDROGENASE
ACTIVITY IN THE PINEALOCYTES OF THE RAT. X 2000.

Figure 26 Pineal gland. Succinic dehydrogenase reaction with hematoxylin counterstain demonstrating the nuclear area (n). Enzyme reaction sites are located in the cytoplasm.

Figure 27 Pineal gland. Lactic dehydrogenase reaction is localized in the cytoplasm. Hematoxylin is used as a counterstain to demonstrate the nuclear areas (n) of the cell.



Figure 27

PLATE V

LOCALIZATION OF ALKALINE AND ACID PHOSPHATASE ACTIVITY IN THE
PINEALOCYTES OF THE RAT. X 2000.

Figure 28 Pineal gland. Alkaline phosphatase reaction is localized in the cytoplasm of the pinealocytes. Hematoxylin is used as a counterstain to demonstrate the nuclear areas (n).

Figure 29 Pineal gland. Acid phosphatase activity within the pinealocytes is localized in the cytoplasm. Hematoxylin is used as a counterstain to demonstrate nuclear areas (n).

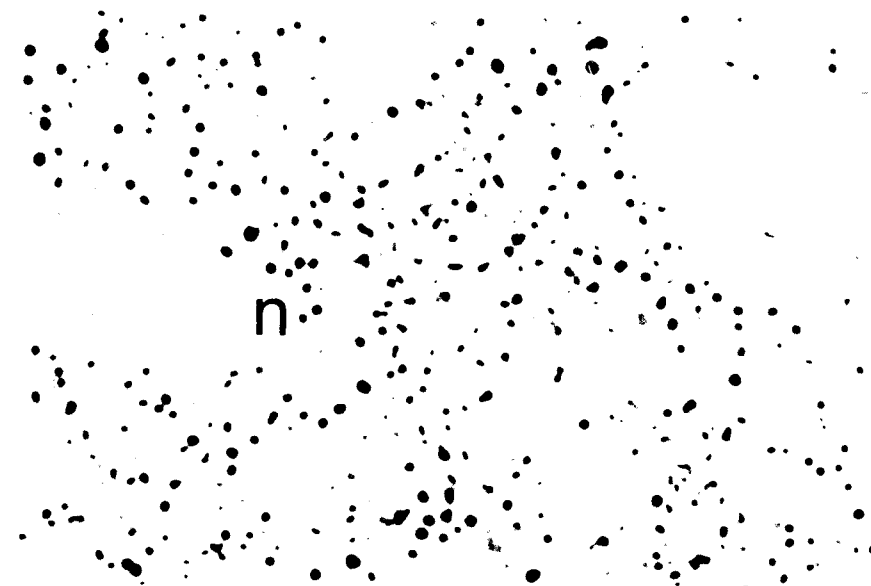


Figure 28

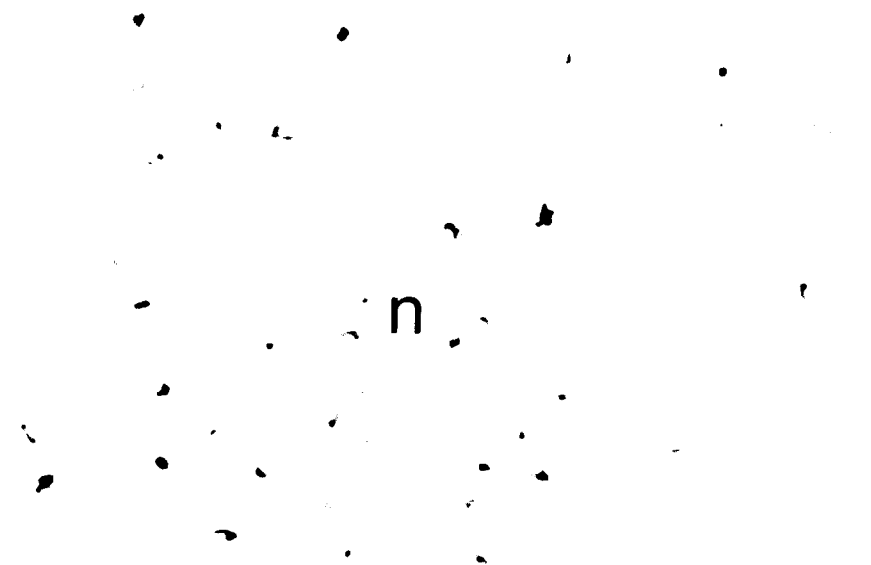


Figure 29

PLATE VI

SUCCINIC DEHYDROGENASE ACTIVITY OF THE PINEAL GLAND OF THE RAT DURING SEVEN DIFFERENT STAGES OF THE ESTROUS CYCLE. X 2000.

- Figure 30 Proestrus. This gland shows a distinctive array of diformazan granules with an inappreciable intensity of monoformazan material.
- Figure 31 Early Estrus. A small scatter of diformazan granules with an almost maximal intensity of monoformazan material is evident.
- Figure 32 Late Estrus. Maximal diformazan deposition with considerable intensities of monoformazan material readily observable in the background.
- Figure 33 Metestrus. Small scatter of diformazan granules with only trace intensities of monoformazan material is discernible.
- Figure 34 Early Diestrus. Increased intensity of both mono- and diformazan material is seen when comparing with metestrus.
- Figure 35 Late Diestrus. Intense diformazan granules in a scattered array are observed. Increased intensity of monoformazan material is visible in the perinuclear areas of the pinealocytes.
- Figure 36 Preproestrus. There is a marked reduction in the intensity of the diformazan granules. An appreciable intensity of only monoformazan material is evident.
- Figure 37 Control Section. Control sections are uniformly negative for each of the seven stages of the estrous cycle, also during pseudopregnancy, prolonged pseudopregnancy and pregnancy.

PLATE VI

Figure 30



Figure 34

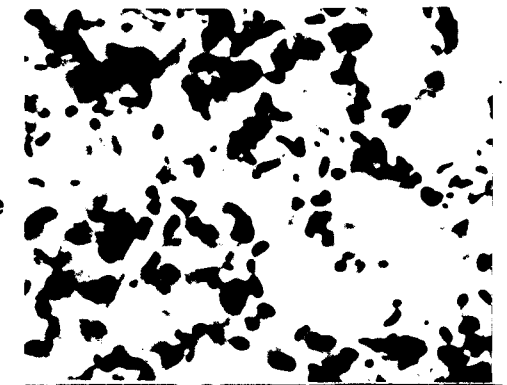


Figure 31



Figure 35



Figure 32

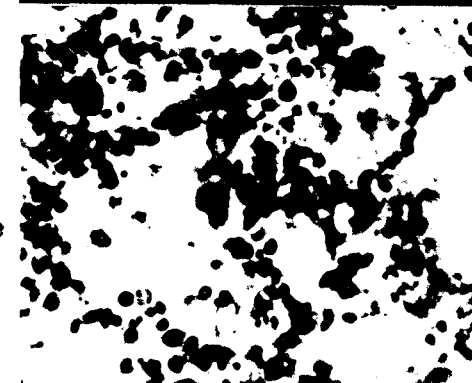


Figure 36



Figure 33



Figure 37

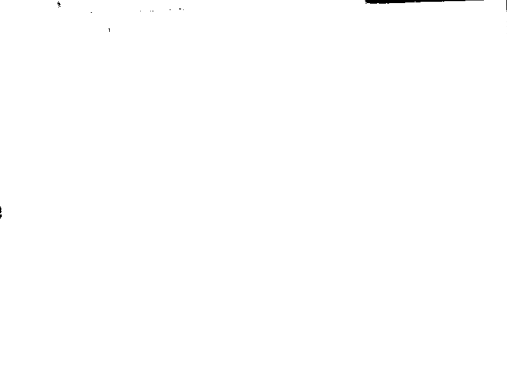


PLATE VII

LACTIC DEHYDROGENASE ACTIVITY OF THE PINEAL GLAND OF THE RAT
DURING SEVEN DIFFERENT STAGES OF THE ESTROUS CYCLE. X 2000.

Figure 38 Proestrus. A maximal intensity of both mono- and diformazan material is readily discernible.

Figure 39 Early Estrus. A marked decrease is observed in the intensity of both the mono- and diformazan reaction sites.

Figure 40 Late Estrus. A near maximal intensity is seen for both the mono- and diformazan material.

Figure 41 Metestrus. Slight reduction in intensity of the diformazan granules with a marked reduction in monoformazan material is demonstrated.

Figure 42 Early Diestrus. Further reduction in the overall reaction with scattered diformazan granules of moderated intensity and a slight elevation in the monoformazan distribution and intensity is shown.

Figure 43 Late Diestrus. Marked reduction in the intensities of both mono- and diformazan material is demonstrated.

Figure 44 Preproestrus. This reaction shows an overall increase in the number of diformazan granules with a markedly increased intensity of monoformazan material.

Figure 45 Control Sections. Control sections were uniformly negative for each of the seven stages of the estrous cycle, also during pseudopregnancy, prolonged pseudopregnancy and pregnancy.

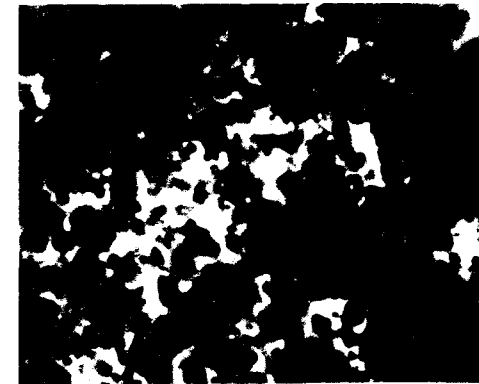
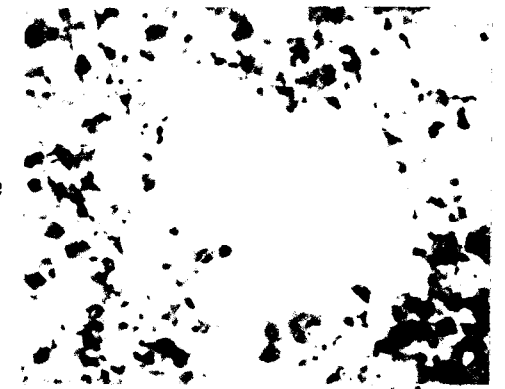
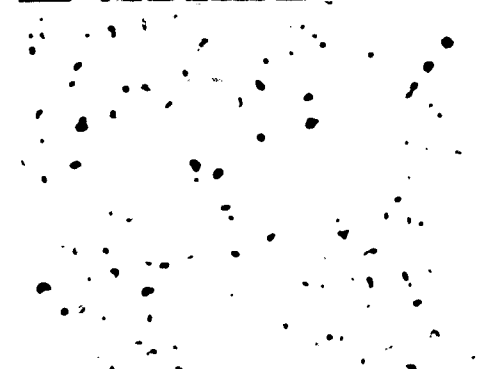
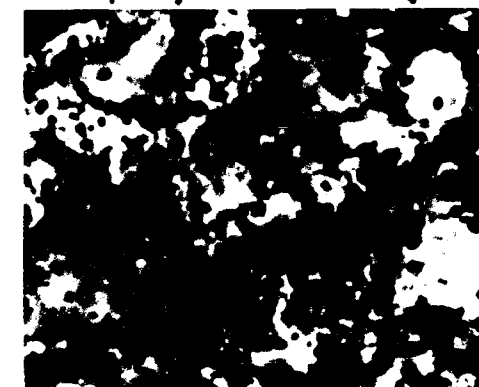
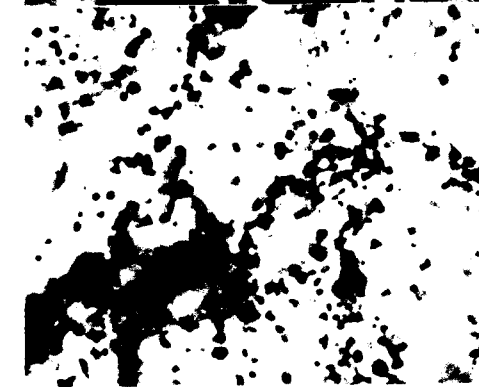
Figure
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45

PLATE VIII

ALKALINE PHOSPHATASE ACTIVITY OF THE PINEAL GLAND OF THE RAT DURING SEVEN DIFFERENT STAGES OF THE ESTROUS CYCLE. X 2000.

- Figure 46. Proestrus. Only a scatter of diazonium reactive material is detectable. Some background color is due to tissue thickness, but lightly appearing particles are out-of-focus diazonium granules.
- Figure 47. Early Estrus. A maximal density of diazonium granules is evident in the pineal gland during this stage of the estrous cycle.
- Figure 48. Late Estrus. A small scattered number of diazonium granules shows trace activity in the pineal gland.
- Figure 49. Metestrus. A slight increase in the number of diazonium reaction sites is seen.
- Figure 50. Early Diestrus. Reaccumulation of alkaline phosphatase reaction sites is seen. Some granules appear faint, being at a different level of focus.
- Figure 51. Late Diestrus. There is a very slight decrease in diazonium reactive sites.
- Figure 52. Preproestrus. Heightened activity is observed throughout the gland over the late diestrus reaction; there is, however, a lesser density of granules than seen in early estrus.
- Figure 53. Control Sections. Tissues are devoid of an apparent alkaline phosphatase activity in each of the seven stages of the estrous cycle, also during pseudopregnancy, prolonged pseudopregnancy, and pregnancy.

PLATE VIII

Figure 46

Figure 50

Figure 47

Figure 51

Figure 48

Figure 52

Figure 49

Figure 53

PLATE IX

ACID PHOSPHATASE ACTIVITY OF THE PINEAL GLAND OF THE RAT DURING SEVEN DIFFERENT STAGES OF THE ESTROUS CYCLE. X 2000.

Figure 54 Proestrus. The large diazonium granules are widely scattered.

Figure 55 Early Estrus. Both the number and intensity of the diazonium granules are increased to the maximum.

Figure 56 Late Estrus. There is a marked reduction in acid phosphatase activity compared with the reactivity in early estrus.

Figure 57 Metestrus. Comparable to Figure 56, Late Estrus.

Figure 58 Early Diestrus. Large coalescing clusters with heightened intensities, several cell layers thick, are present.

Figure 59 Late Diestrus. A slight overall increase in the number and intensity of diazonium reactive sites.

Figure 60 Preproestrus. There is a marked reduction in the acid phosphatase activity when compared with late diestrus.

Figure 61 Control Section. Enzymatic activity was uniformly negative for all seven stages of the estrous cycle, also during pseudopregnancy, prolonged pseudopregnancy and pregnancy.

PLATE IX

Figure 54

Figure 58

Figure 55

Figure 59

Figure 56

Figure 60

Figure 57

Figure 61

PLATE X

SUCCINIC DEHYDROGENASE ACTIVITY OF THE PINEAL GLAND OF THE RAT DURING THREE STAGES OF PSEUDOPREGNANCY. X 2000.

Figure 62 Day Six. Scatter of diformazan granules is observed in the perinuclear areas. Intense monoformazan material is readily observable in some areas.

Figure 63 Day Ten. Marked increase in intensity of the monoformazan material with a slight increase in diformazan deposition is demonstrated, giving the maximal overall reaction.

Figure 64 Day Thirteen. Increased intensity of diformazan granules with a slight decrease in intensity of the monoformazan material is shown.

PLATE X

174

Figure
62



Figure
64

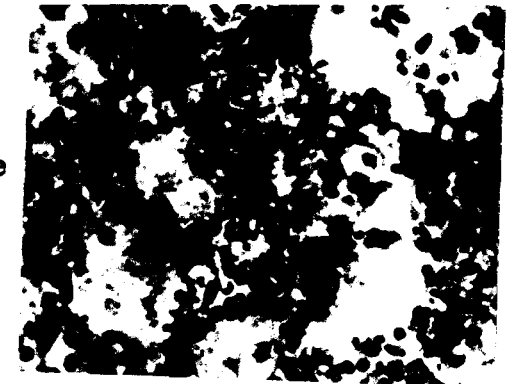


Figure
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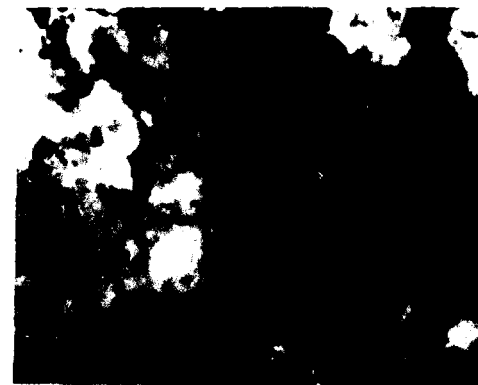


PLATE XI

PLATE XI

LACTIC DEHYDROGENASE ACTIVITY OF THE PINEAL GLAND OF THE RAT DURING THREE STAGES OF PSEUDOPREGNANCY. x 2000.

Figure 65 Day Six. Lactic dehydrogenase activity elicits maximal deposition of both mono- and diformazan material.

Figure 66 Day Ten. There is a marked reduction in the intensity of both mono- and diformazan material observed.

Figure 67 Day Thirteen. Heavy deposition of diformazan granules is seen in a scattered array throughout the gland.

Figure
65

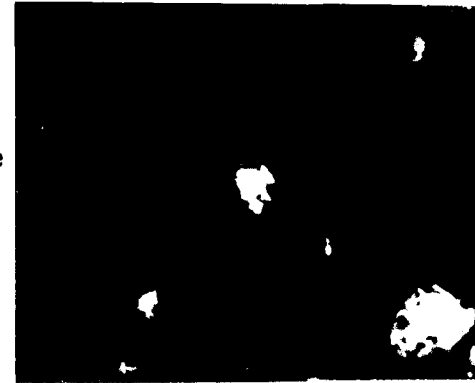


Figure
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Figure
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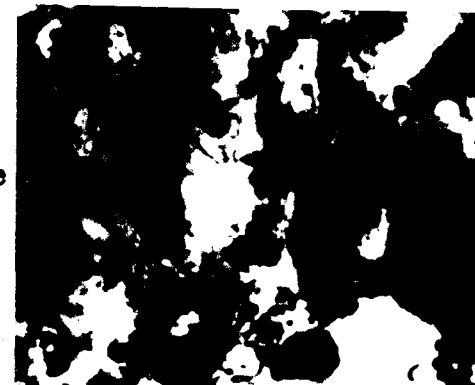


PLATE XII

ALKALINE PHOSPHATASE ACTIVITY OF THE PINEAL GLAND OF THE RAT
DURING THREE STAGES OF PSEUDOPREGNANCY. X 2000.

Figure 68 Day Six. Abundance of small diazonium granules
several cell layers deep is observed.

Figure 69 Day Ten. This figure shows maximal intensity for
an alkaline phosphatase reaction.

Figure 70 Day Thirteen. A slight decrease is observed in the
intensity of the diazonium granule deposition.

Figure
68

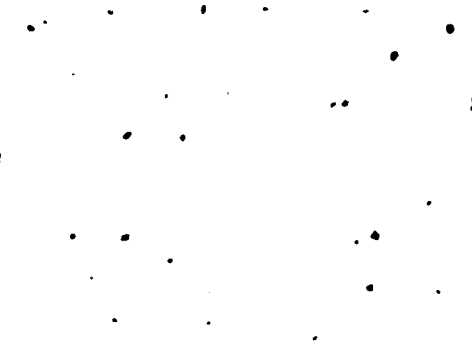


Figure
70



Figure
69



PLATE XIII

ACID PHOSPHATASE ACTIVITY OF THE PINEAL GLAND OF THE RAT
DURING THREE STAGES OF PSEUDOPREGNANCY. X 2000.

Figure 71 Day Six. A high intensity of diazonium granules is seen several cell layers thick.

Figure 72 Day Ten. Approximately the same intensity as days six and thirteen. Diazonium granules are not easily distinguishable as they are at different depths of this field.

Figure 73 Day Thirteen. The intensity is comparable to those of days six and ten. Acid phosphatase reactive sites are several cell layers thick. All the stages of acid phosphatase activity observed during pseudopregnancy were semi-quantitatively assigned a value of +3.

PLATE XIII

Figure
71



Figure
73



Figure
72



PLATE XIV

SUCCINIC DEHYDROGENASE ACTIVITY OF THE PINEAL GLAND IN PSEUDOPREGNANT RATS BEARING DECIDUAL REACTIONS. X 2000.

Figure 74 Day Six. Moderate diformazan granule intensity with trace amounts of monoformazan material is seen throughout the gland.

Figure 75 Day Ten. A near maximally intense reaction showing mono- and diformazan material is readily observable in the perinuclear areas.

Figure 76 Day Thirteen. Decreasing intensities of succinic dehydrogenase activity sites are demonstrated.

Figure 77 Day Fifteen. Minimal succinic dehydrogenase activity with a scatter of diformazan granules and trace monoformazan material.

Figure 78 Day Eighteen. Maximal intensities of both mono- and diformazan reaction sites are readily observable.

Figure 79 Day Twenty. A marked decrease in the intensities of both mono- and diformazan material is discernible.

Figure 80 Day Twenty-one. Continual decrease in the number of diformazan granules and negligible monoformazan material is visible.

Figure 74

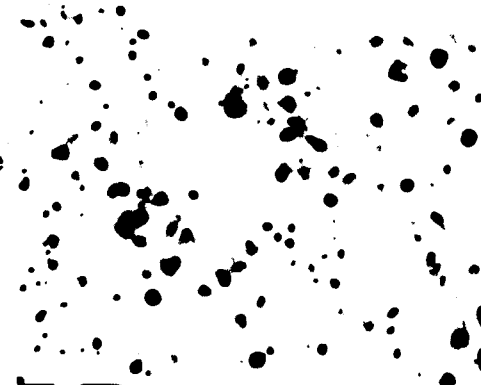


Figure 78

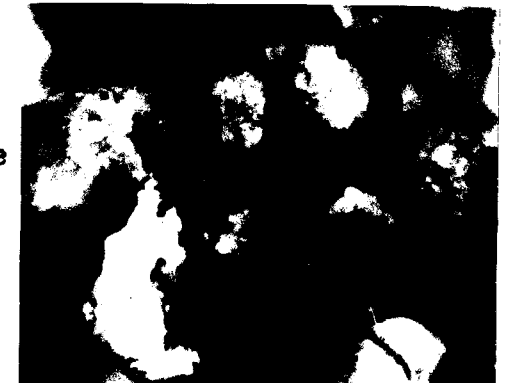


Figure 75



Figure 79

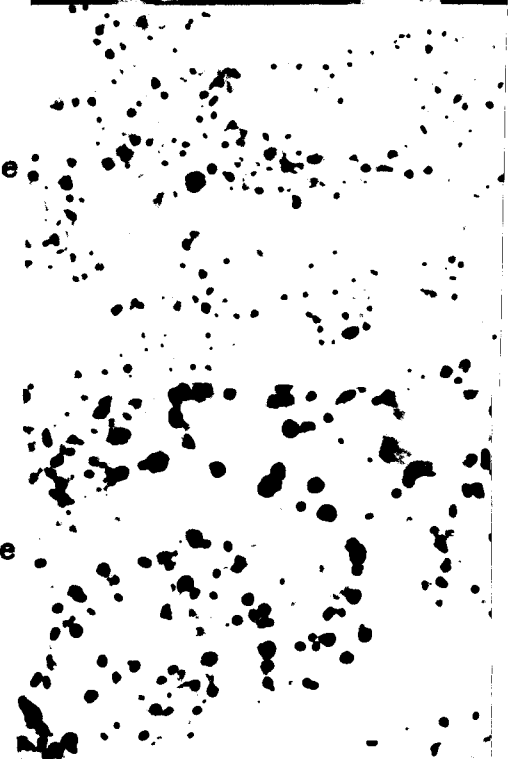


Figure 76

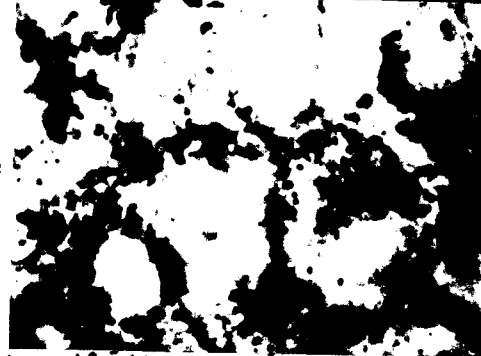


Figure 80

Figure 77



PLATE XV

LACTIC DEHYDROGENASE ACTIVITY OF THE PINEAL GLAND IN PSEUDO-PREGNANT RATS BEARING DECIDUAL REACTIONS. X 2000.

- Figure 81 Day Six. High diformazan granule intensity and moderate monoformazan intensity is observed.
- Figure 82 Day Ten. Increased intensities of both mono- and diformazan material is evident.
- Figure 83 Day Thirteen. Maximal lactic dehydrogenase intensities are observed in the pinealocytes.
- Figure 84 Day Fifteen. A precipitous decrease in both mono- and diformazan reaction sites is noticeable.
- Figure 85 Day Eighteen. A marked increase is seen in the intensity of diformazan granules with a moderate intensity of monoformazan material.
- Figure 86 Day Twenty. A decrease in the intensity of diformazan granules with trace monoformazan material is revealed.
- Figure 87 Day Twenty-one. A continual decrease of diformazan granule intensities and moderate monoformazan material are detectable. When compared to day twenty, it can be observed that there is a slight increase in the concentration of the monoformazan material on day twenty-one, but a decrease in the number of diformazan granules. On day twenty, the diformazan granules are small and evenly distributed throughout all of the pineal gland, while on day twenty-one, the diformazan granules are large with scattered coalescing clusters.

Figure 81

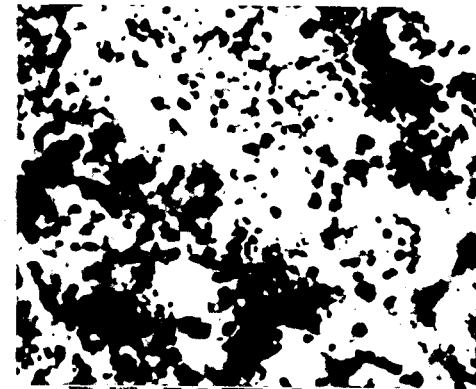


Figure 82

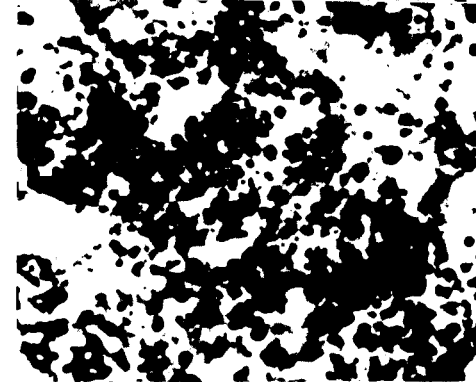


Figure 83

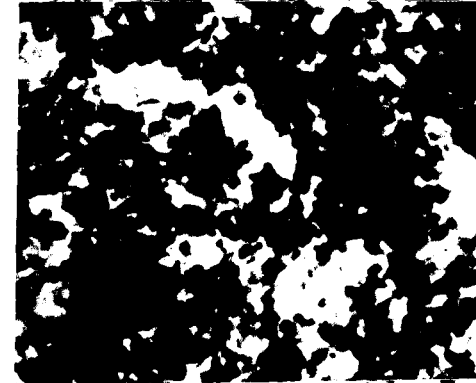


Figure 84

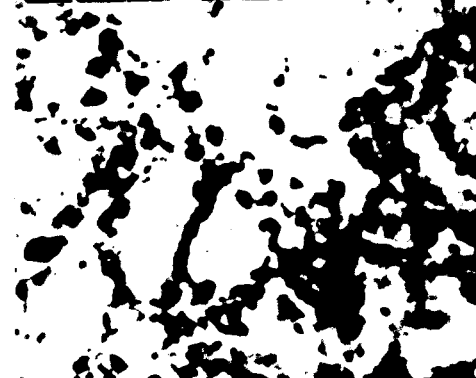


Figure 85

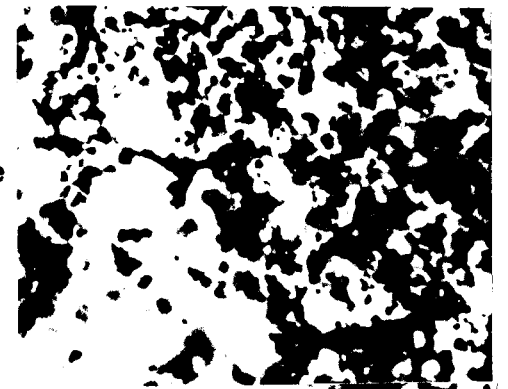


Figure 86

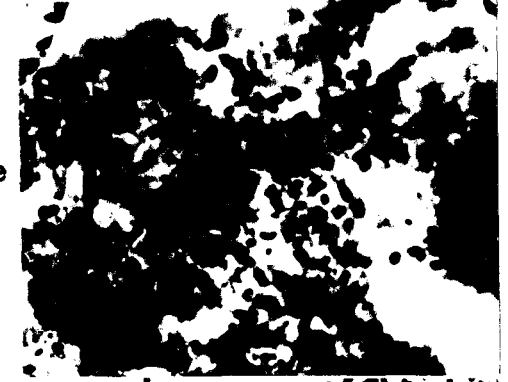


Figure 87



PLATE XVI

ALKALINE PHOSPHATASE ACTIVITY OF THE PINEAL GLAND IN PSEUDO-PREGNANT RATS BEARING DECIDUAL REACTIONS. X 2000.

Figure 88 Day Six. High alkaline phosphatase activity is seen throughout the pineal gland.

Figure 89 Day Ten. A marked decrease in the number and intensity of the diazonium reaction sites is evident.

Figure 90 Day Thirteen. The number of diazonium granules increases to a +3 reaction.

Figure 91 Day Fifteen. Maximal intensity of alkaline phosphatase activity is manifested.

Figure 92 Day Eighteen. Decrease in the number and intensity of the diazonium granules is observed.

Figure 93 Day Twenty. Intensity of the diazonium granules is of approximately the same intensity as on day eighteen.

Figure 94 Day Twenty-one. A slightly less intense reaction than on days eighteen and twenty is visible.

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PLATE XVII

PLATE XVII

ACID PHOSPHATASE ACTIVITY OF THE PINEAL GLAND IN PSEUDOPREGNANT RATS BEARING DECIDUAL REACTIONS. X 2000.

- Figure 95 Day Six. A very strong acid phosphatase reaction is shown.
- Figure 96 Day Ten. A slight decrease in the deposition of diazonium granules is detected.
- Figure 97 Day Thirteen. Reaccumulation in the number and intensity of the acid phosphatase reaction sites is observed.
- Figure 98 Day Fifteen. A maximal number of the diazonium granules is evident.
- Figure 99 Day Eighteen. A marked decrease is observed in the sites of action for acid phosphatase.
- Figure 100 Day Twenty. Continued decrease in the number of reactive sites is perceptible.
- Figure 101 Day Twenty-one. A moderate to strong reaction for acid phosphatase is detected.

Figure
95Figure
99Figure
96Figure
100Figure
97Figure
101Figure
98

PLATE XVIII

SUCCINIC DEHYDROGENASE ACTIVITY OF THE PINEAL GLAND OF PREGNANT RATS. X 2000.

Figure 102 Day Six. A less than moderate intensity of both mono- and diformazan material is readily seen in the perinuclear areas.

Figure 103 Day Eight. A near maximal intensity of both mono- and diformazan material is apparent.

Figure 104 Day Ten. There is a marked reduction in diformazan granules with a moderate intensity of monoformazan.

Figure 105 Day Thirteen. Reaccumulation of diformazan granules is observed with a slightly less than moderate intensity for monoformazan.

Figure 106 Day Fifteen. Moderate intensity of diformazan granules is seen in the perinuclear areas. Monoformazan material is intense in some areas.

Figure 107 Day Eighteen. A maximal reaction of small diformazan granules is demonstrated. Monoformazan background intensity is high.

Figure 108 Day Twenty. There is an intense monoformazan deposition throughout the pineal gland. Diformazan granules are slightly decreased in intensity.

Figure 109 Day Twenty-one. Marked reduction in monoformazan deposition is readily seen. Diformazan granules are scattered throughout the pineal gland.

Figure 102

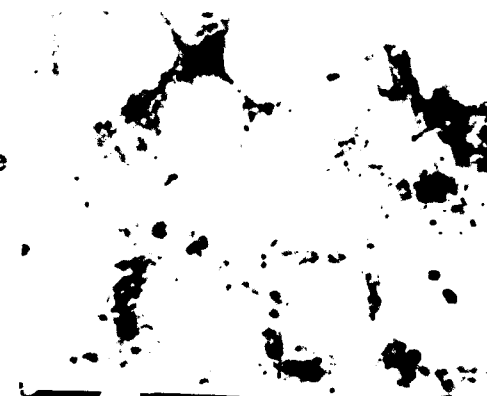


Figure 106

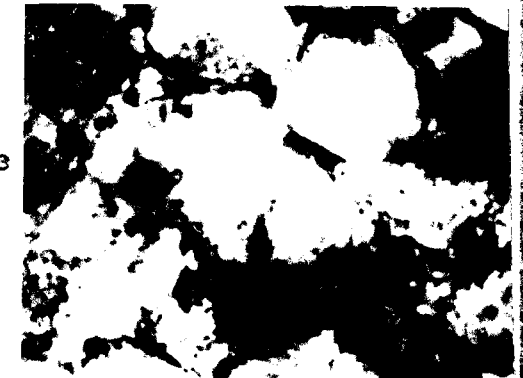


Figure 103



Figure 107



Figure 104



Figure 108



Figure 105



Figure 109



PLATE XIX

LACTIC DEHYDROGENASE ACTIVITY OF THE PINEAL GLAND OF PREGNANT RATS. X 2000.

Figure 110 Day Six. There is a high, moderate mono- and diformazan depositon throughout the pineal gland.

Figure 111 Day Eight. There is observed a moderate intensity of diformazan granules with trace amounts of monoformazan. Large, round black deposits were not taken into consideration when evaluating this section as they appear indicative of lipid droplets which have accumulated the indicator stain, Nitro BT.

Figure 112 Day Ten. A marked increase in the number and intensity of small diformazan granules with a moderate increase in monoformazan deposition is apparent.

Figure 113 Day Thirteen. An increase in diformazan granule intensity with a markedly increased monoformazan background is evident.

Figure 114 Day Fifteen. Maximal lactic dehydrogenase activity. High concentrations of diformazan granules with a strong background of monoformazan material are shown.

Figure 115 Day Eighteen. A moderate intensity of both mono- and diformazan material is visible throughout the pineal gland.

Figure 116 Day Twenty. An increase in number and intensity of both mono- and diformazan is perceptible.

Figure 117 Day Twenty-one. A slight increase in the number and intensity of diformazan with a moderate intensity of monoformazan is shown. A major portion of the gland appeared similar to that seen in the right side of the photomicrograph.

Figure 110

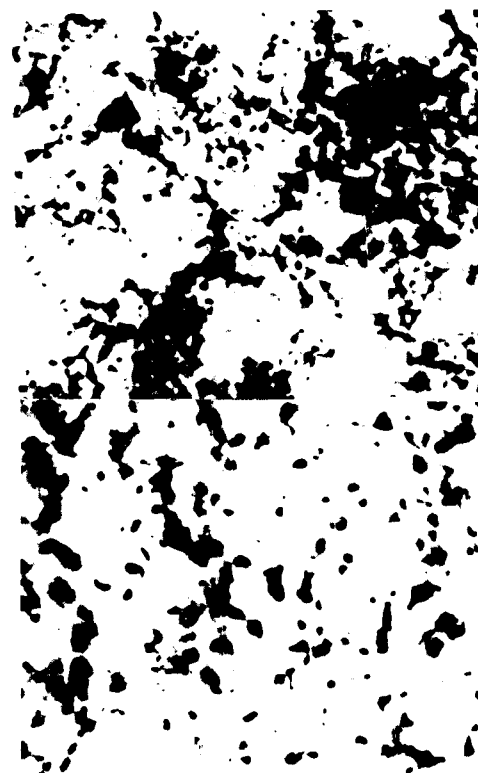


Figure 111

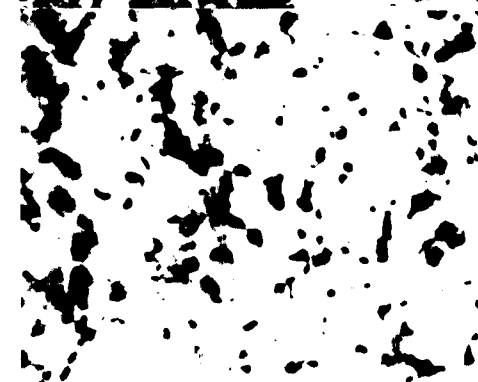


Figure 112

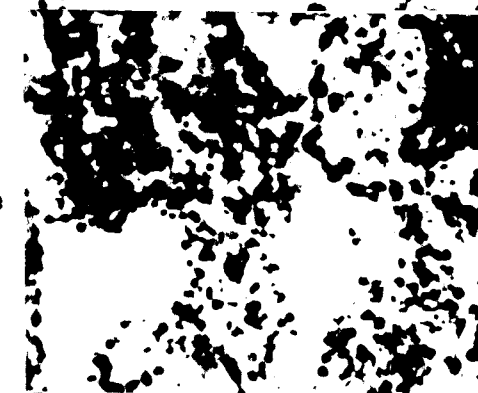


Figure 113

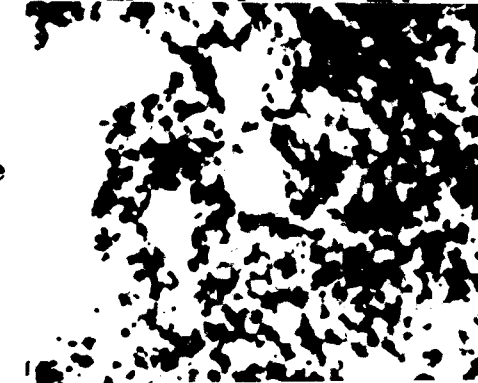


Figure 114



Figure 115

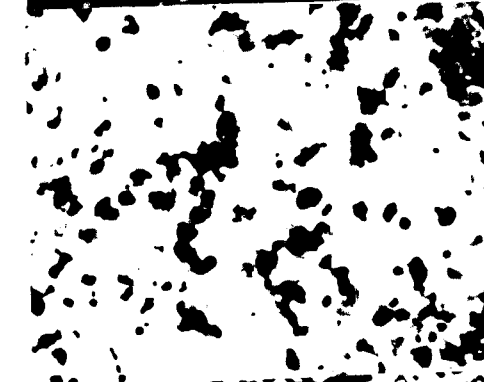


Figure 116

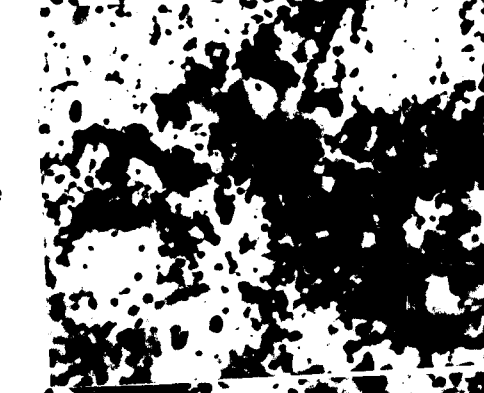


Figure 117

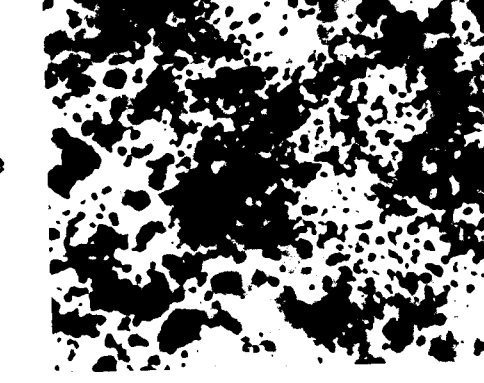


PLATE XX

ALKALINE PHOSPHATASE ACTIVITY OF THE PINEAL GLAND OF PREGNANT RATS. X 2000.

PLATE XX

Figure 118 Day Six. Trace alkaline phosphatase reaction is discernible.

Figure 119 Day Eight. An increase, but still weak, intensity of diazonium granules is seen scattered throughout the pineal cells.

Figure 120 Day Ten. This reaction is approximately equal in intensity to day eight.

Figure 121 Day Thirteen. High intensity of alkaline phosphatase reactive sites is evident.

Figure 122 Day Fifteen. Maximal intensity of diazonium granules is observed several cell layers deep.

Figure 123 Day Eighteen. A precipitous decrease in the intensity of the diazonium granules is manifested.

Figure 124 Day Twenty. A slight increase in the alkaline phosphatase reaction prevails throughout the pineal gland.

Figure 125 Day Twenty-one. This reaction is approximately equal in intensity to day twenty, a strong, moderate reaction.

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Figure
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Figure
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Figure
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Figure
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Figure
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Figure
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Figure
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PLATE XXI

ACID PHOSPHATASE ACTIVITY OF THE PINEAL GLAND OF PREGNANT RATS. X 2000.

- Figure 126 Day Six. A scatter of diazonium granules is observed. Background coloration is the result of tissue thickness and is not indicative of sites of acid phosphatase action.
- Figure 127 Day Eight. Large, intense but widely spaced diazonium granules are evident.
- Figure 128 Day Ten. Acid phosphatase reaction sites are observed more than one cell layer in thickness.
- Figure 129 Day Thirteen. Increase in the number and intensity of the diazonium granules is apparent.
- Figure 130 Day Fifteen. Near maximal intensity of diazonium deposition is visible.
- Figure 131 Day Eighteen. Maximal, intense diazonium granule deposition is observed several cell layers in thickness.
- Figure 132 Day Twenty. Strong acid phosphatase reaction is seen. Diazonium granules are intense but scattered.
- Figure 133 Day Twenty-one. Intense, but scattered, deposition of diazonium granules is demonstrated.

PLATE XXI

185

Figure 126

Figure 130

Figure 127

Figure 131

Figure 128

Figure 132

Figure 129

Figure 133

APPROVAL SHEET

The dissertation submitted by Anthony V. Fasano has been read and approved by six members of the faculty of the Graduate School of Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies that fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

January 14, 1972
Date

Joseph Thomas Peluso
Signature of Adviser