to my wife,Maureen

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ABSTRACT

The object of the present study was to evaluate both the endocrine and the gametogenic functions of the rat testis in response to treatment with pineal indoles. Two experimental designs were utilized; one,a chronic in vivo investigation where indoles (1μmole) were administered subcutaneously and weekly to adult rats (evaluation monthly for 3 months),and an in vitro investigation where indoles were added directly to testicular homogenates incubated with pregneneolone.

In vivo,the influence of indoles (1μmole/week) on the reproductive functions of the adult male rat were very modest. Indoles had no effect on the gametogenic function of the testis,and influenced the endocrine function of the testis to a minor degree. Indoles induced only slight alterations in testosterone secretion,but apparently affected androstenedione secretion to a greater extent. Indoles administered at 1μmole/week affected the metabolism of testosterone from the blood stream. The regulation of the metabolic (mainly hepatic) clearance of testosterone appears to be the important feature of indolic function at this dose level.

Indoles significantly inhibited the in vitro biotransformation of pregneneolone into androgens. Melatonin was

by far the most potent inhibitor in this respect. The mechanism of indolic action may be attributed to a non-competitive type of inhibition of the enzymes of androgenesis. In this fashion, indole interaction at the testicular level appears related to structural similarities at the 5 position of the indane ring system. Interaction with the two component 17β-hydroxysteroid dehydrogenase system appears to be indole specific. Each indolic compound induced a specific pattern of influences on the activities of the 17β-hydroxysteroid dehydrogenase and the 17-keto-reductase enzymes. Consequently, melatonin and 5-methoxytryptophol diminished both testosterone and androstenedione formation while serotonin and 5-hydroxytryptophol diminished testosterone formation but did not alter the formation of androstenedione.

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CHAPTER I

REVIEW OF THE LITERATURE

I. THE TESTIS

The testis in the adult rat serves two distinct functions which maintain the reproductive status of the animal, namely, the gametogenic function of providing sex cells, and the endocrine function of secreting hormones, principally androgens. Divorced from the influence of higher centers (pituitary and hypothalamus), however, fulfillment of these functions would be impossible. The lifelines connecting the higher centers with the main works (testis) are the gonadotrophins ie; follicle stimulating hormone (FSH), and interstitial cell stimulating hormone (ICSH): the indispensible couriers of the complex system.

(A) THE ENDOCRINE FUNCTION OF THE TESTIS

Historical Introduction

In some respects, knowledge of the endocrinology of the testis has advanced more rapidly than that of other endocrine organs (Hall, 1970). The role of the testis in normal male development and the effects of castration were known in the time of Aristotle. In 1849, Berthold demonstrated that regression of the cock's comb followed castration and that this regression was prevented by transplanting the testis to a new site. This experiment demonstrated that the testis controlled growth of the cock's comb and that the influence

was endocrine in nature,since transplantation of the testis prevented regression of the comb. In 1889 Brown-Sequard reported an increased vigour after self treatment with testicular extracts. According to Tepperman (1968),this experiment was one of many that prompted an early lack of respect for endocrinology. More than 40 years later in 1931,Butenandt crystalized androsterone from urine. This allowed characterization of androgenic structure. In 1935 testosterone was isolated in crystalline form and in the same year Butenandt synthesized the hormone. Soon relatively cheap preparations of testosterone were available for experimental use. These advances paved the way for a detailed study of the nature of androgenic function. Recently, specific and accurate methods for measuring androgens in body fluids have been developed. Finally,considerable progress has been made in elucidating the mechanism of action of androgens. However, progress has been slow in some aspects of male sex gland function ie;the action of FSH on the testis is still not understood.

Biosynthesis of Androgens

The testis secretes three main androgens: testosterone, androstenedione,and dehydroepiandrosterone (Eik-Nes and Hall, 1965). Testosterone and androstenedione are considerably more androgenically potent than dehydroepiandrosterone (Dorfmann and Shipley,1956).

Androgenesis has been known for some time to occur in the Leydig or interstitial cells of the testis (Hall,1970). Christensen and Mason (1965) succeeded in separating tubules from Leydig cells and were able to show that androgenesis also occured in the tubules. Under the assumption that cholesterol is an obligatory intermediate in steroid hormone biosynthesis, Hall (1970) questioned the seminiferous tubules as a further site of androgen synthesis when he was unable to demonstrate androgen function from radio-labelled cholesterol with tubular preparations *in vitro*. Recently, Lacy (1973) has shown that tubular preparations can in fact, produce androgens from cholesterol, and a microsomal fraction of tubules possessed greater activity in metabolizing progesterone than a similar interstitial microsomal fraction. At the present time the physiological significance of tubular androgenesis is uncertain.

It has been clear for some time that cholesterol is a precursor of steroid hormones (Samuels,1960). However, side chain cleavage at C-17 must precede formation of steroid hormones.Side chain cleavage is accomplished by an important pathway whereby cholesterol is converted to pregneneolone and isocapraldehyde. This conversion takes place in mitochondria of the testis cells (Toren *et al*,1964) and has been shown to be the rate limiting feature of steroidogenesis.

In the rat, further transformation of pregneneolone

to androgens takes place in the microsomes; the supernatant fluid (105,000 x G) is essentially devoid of enzymatic activity (Tamaoki,1965). However, the supernatant fluid is capable of stimulating the conversion of pregneneolone to testosterone by a NADPH independent mechanism.

Two pathways* for the production of androgens from pregneneolone have been described in the rat (Slaunwhite and Samuels, 1956,Slaunwhite and Burgett, 1965). The first consists of the formation of testosterone via progesterone, 17α-hydroxyprogesterone, and androstenedione: the progesterone or delta-4 pathway. The second involves 17α-hydroxypregneneolone,dehydroepiandrosterone,5-androstenediol, and testosterone: the dehydroepiandrosterone or delta-5 pathway.

Slaunwhite and Samuels (1956) incubated homogenates of rat testis with progesterone-C^{14}. Whenever androstenedione-C^{14} and testosterone-C^{14} were obtained, and progesterone-C^{14} was in excess, 17α-hydroxyprogesterone-C^{14} was isolated. With 17α-hydroxyprogesterone-C^{14} as substrate both labelled androgens (testosterone and androstenedione) were rapidly formed.

Workers sought to determine whether or not the delta-4 pathway was the predominant one. Shikita et al (1964) showed that in the conversion of pregneneolone-H^3 to testosterone-H^3 by rat testicular microsomes, progesterone-H^3, 17α-hydroxyprogesterone-H^3 and androstenedione-H^3 were found

* - Figure 17, page 110 illustrates the pathways to androgen formation

as intermediates. Labelled 17α-hydroxypregneneolone was present only in negligible amounts. On incubation with a mixture of pregneneolone-H^3 and progesterone-C^{14}, progesterone-C^{14} was more rapidly converted to testosterone than was pregneneolone-H^3. Androstenedione was produced from both substrates. Incubation with progesterone-C^{14} and 17α-hydroxypregneneolone-H^3 showed that the former steroid was more readily converted to testosterone. Slaunwhite and Burgett (1965) derived similar results and attempted to quantify the extent of conversion from pregneneolone. They showed that the progesterone pathway normally predominates; 44% of pregneneolone was converted to progesterone while only 19% of pregneneolone was converted to 17α-hydroxypregneneolone.

The delta-5 pathway has been described for the rat testis by Slaunwhite and Burgett (1965). They concluded that dehydroepiandrosterone was not an important intermediate in testosterone biosynthesis,being readily converted to androstenedione.

Slaunwhite and Burgett (1965) proposed that androstenedione and testosterone are formed via independent pathways, the former by the delta-4 pathway and the latter by a delta-5 route and that these pathways are not readily interconvertible.

Hormonal Control of Androgenesis

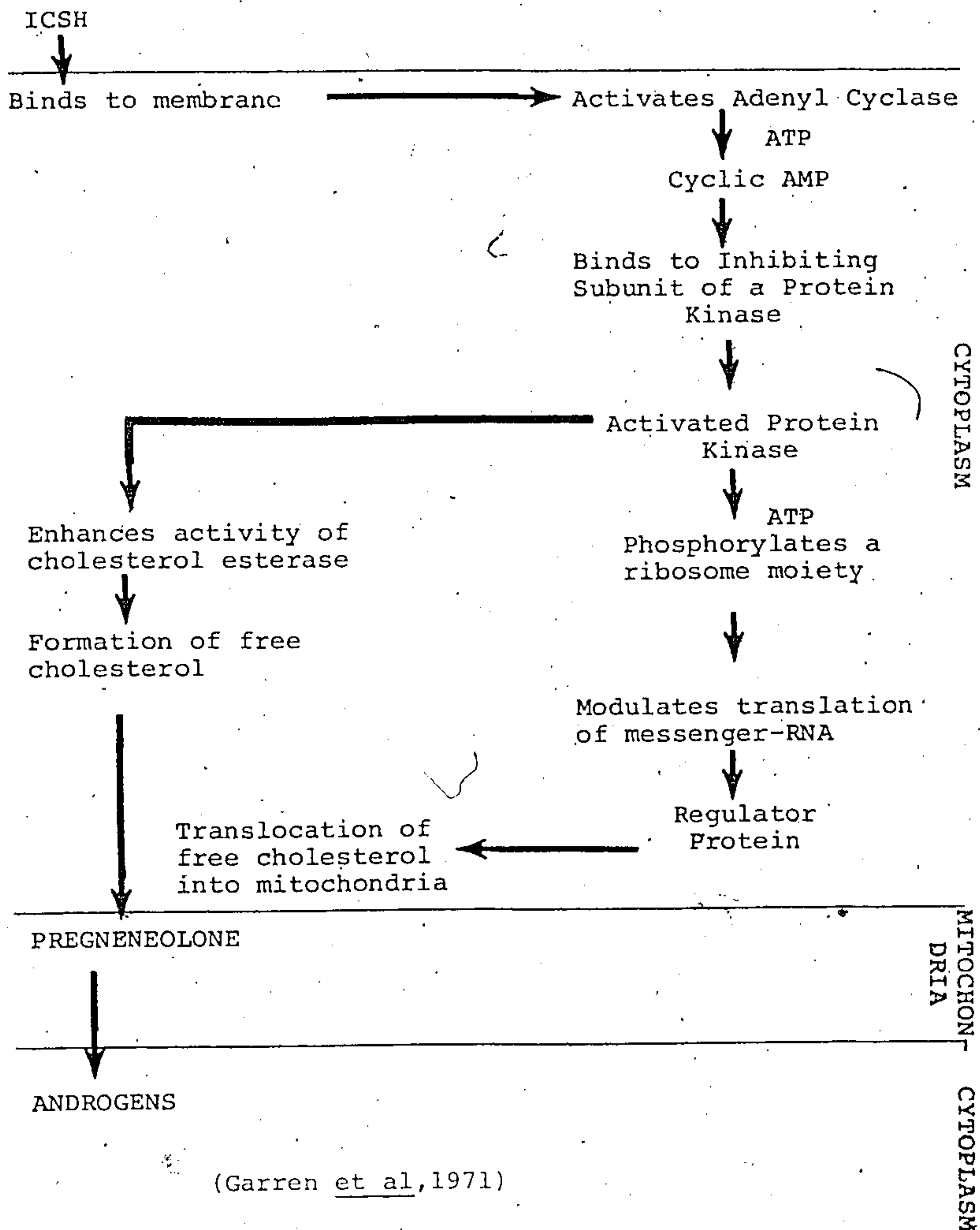
For some years it has been known that a hormone from the anterior pituitary stimulates the synthesis of androgens

by the testis (Hall,1970). This hormone has been called interstitial cell stimulating hormone (ICSH). Trophic stimulation of the testis by ICSH results in enhancement of testosterone, androstenedione and dehydroepiandrosterone synthesis both in vivo and in vitro (Brinck-Johnsen and Eik-Nes,1957). Moreover, the increase in synthesis of androgens is accompanied by increased secretion of these steroids into the blood stream (Van der Molen et al,1973).

ICSH has been shown to interact with the testis, specifically with the Leydig cells (Greep,1973). According to Van der Molen et al (1973) the mechanism of ICSH action is believed to correlate well with the general scheme, based mainly on evidence from the action of ACTH on the adrenal cortex.

The proposed mechanism of action of ICSH is presented in Figure 1. ICSH binds specifically to receptor proteins on the surface of the Leydig cell membrane (Moudgal et al, 1971). ICSH activates adenyl cyclase in the membrane to produce the second messenger, cyclic AMP (Sandler and Hall, 1966). The cyclic adenosine monophosphate that is formed activates a series of kinases by binding with an inhibiting subunit of a protein kinase, thus releasing the active kinase (Garren et al,1971). This process initiates the activation of cholesterol esterase (Behrman and Armstrong,1969)

FIGURE I. Proposed mechanism of action of interstitial cell stimulating hormone (ICSH).



(Garren <u>et al</u>,1971)

which hastens the transformation of cholesterol esters to free cholesterol,a precursor of androgens. The activated protein kinase catalyses the phosphorylation of a ribosome moiety,thereby modulating the translation of a messenger RNA (Garren et al,1971). This results in the induction of a regulator protein which facilitates the translocation of cholesterol into the mitochondria.

ICSH appears to stimulate androgenesis by a mechanism involving RNA and or protein synthesis. Reel and Gorski (1968) observed that actinomysin D and cyclohexamide inhibited the action of ICSH. Cyclohexamide administration had no influence on the conversion of acetate to cholesterol,but inhibited the ACTH stimulated fall in free cholesterol and the ACTH stimulated conversion to corticosteroids (Davis,1969).Results of this nature ie; stimulation of the rate limiting step of steroidogenesis by trophic hormones are in agreement with those reported by Van der Molen et al(1973). These workers infused labelled pregneneolone into the spermatic artery of the testes concomitantly with human chorionic gonadotrophin (HCG) or cyclic AMP. They discovered that the specific activities of all intermediates between pregneneolone and testosterone in the spermatic venous blood were decreased when compared to the unstimulated controls. They concluded that this decrease confirms that gonadotrophins do stimulate steroid biosynthesis prior to pregneneolone formation. Since

a large mass of pregneneolone is formed during gonadotrophin administration,the specific activities of other steroids that can be synthesized from pregneneolone are then decreased when compared to the controls.

ICSH interaction at a site other than the conversion of cholesterol to pregneneolone has also been suggested. Bardin and Peterson (1967) administered HCG to rats and examined the concentrations of testosterone and androstenedione in testicular venous blood. They observed that rats with less apparent gonadotrophin stimulation (0.01 IU HCG) had testosterone to androstenedione (T/A) ratios of about 4. Strong gonadotrophin stimulation (1.0 IU HCG) maintained T/A ratios in the order of 8 . The authors concluded that the gonadotrophin was in part responsible for the high secretion of testosterone relative to androstenedione. These results suggest that perhaps ICSH acts to stimulate the conversion of androstenedione to testosterone.

Recently,the involvement of prolactin in androgenic process has been suggested. Hafiez et al(1972b),using hypophysectomized rats,made injections twice daily with saline,prolactin,LH,prolactin + LH for 3.5 days,beginning 18 days post-surgery. These investigators incubated minced testes *in vitro* with 1-C^{14}-acetate for three hours at 37°C, and calculated the conversion of acetate into testosterone. Conversion of acetate to testosterone was not changed by

prolactin treatment,was significantly increased by LH,and further increased by prolactin + LH to approximately the level observed in the intact controls. These results demonstrate a synergistic action of prolactin and LH on the synthesis of testosterone *in vitro*. Hafiez *et al*(1972a) demonstrated that testosterone plasma levels were significantly greater in the prolactin + LH-treated group than in the LH-treated group. These results indicate that prolactin potentiated the effects of LH on both the synthesis and the release by the testis.

(B) THE GAMETOGENIC FUNCTION OF THE TESTIS

The gametogenic function of the testis is of prime importance for the continuity of the species. Male germ cells or spermatozoa are produced in the seminiferous tubules by a process termed spermatogenesis.

In the adult rat,the seminiferous epithelium is composed of two categories of cells:supporting or Sertoli cells,and germ cells at different stages of development(Patt and Patt,1969).

Sertoli cells are the supportive cells of the seminiferous epithelium which lie very close to the basement membrane. The cytoplasm of Sertoli cells exhibits an elaborate system of thin processes surrounding all germ cells except the spermatogonial stem cells(Monesi,1972). The function of the Sertoli cells is not clear. They certainly play a role in the mechanical support of the germ cells,the release of mature

spermatozoa from the tubules,and the resorption of residual bodies(Monesi,1972). Sertoli cells never divide in the mature testis and they are very resistant to X-rays and other ionizing radiation,and other toxic agents that destroy the germ cells(Monesi,1972).

Spermatogenesis can be divided into three phases (Clermont,1972).The first phase involves the spermatogonia which proliferate and simultaneously maintain their numbers by renewal.In the rat,type A,Intermediate and type B spermatogonia are present near the basement membrane.The type A spermatogonia give rise to other type A spermatogonia (A_1-A_4); of this progeny,some cells divide to form Intermediate spermatogonia,some degenerate and some form new type A_1 spermatogonia (Clermont and Bustos-Obregon,1968).Another class of type A spermatogonia,designated as A_o were discovered after irradiation of the testis (Dym and Clermont,1970).These investigators discovered that type A_o spermatogonia were the most resistant germ cell type and served as reserve stem cells, which rarely divided. Intermediate spermatogonia divide by mitosis to produce type B spermatogonia which consequently mitose to form primary spermatocytes.

The second phase of spermatogenesis involves the primary and secondary spermatocytes which divide by meiosis to produce haploid cells,the spermatids.The third phase concerns the spermatids.The spermatids undergo a complex series of

cytological transformations (spermiogenesis) which produce spermatozoa.No cell division occurs during this process(Patt and Patt,1969).At the completion of spermiogenesis most of the excess cytoplasm containing ribosomes,lipid droplets,Golgi membranes and degenerating mitochondria is cast off (Monesi, 1972).This mass,termed the residual bodies is phagocytosed by the Sertoli cells.

The seminiferous epithelium of the adult rat is composed of,in addition to the Sertoli cells,one or two generations of spermatogonia seen along the basement membrane,one or two generations of spermatocytes,and one or two generations of spermatids bordering the lumen of the tubule(Leblond and Clermont,1952).According to the authors a generation represents a group of cells at the same stage of development,produced at the same time and evolved synchronously through the spermatogenic process. The various generations of germ cells form cellular associations of fixed composition,and only limited numbers of such cell associations can be observed in various cross sections of seminiferous tubules (Clermont,1972). The cell associations appear and follow one another in a given and fixed sequence,which is repeated indefinately in a cyclic manner.The complete series of cell associations is termed the cycle of the seminiferous epithelium and each cell association is a stage of the cycle (Clermont,1972).Clermont and Perey (1957) demonstrated that in the rat the cycle of the

seminiferous epithelium has 14 stages (Figure 2).

Spermatogenesis commences when a type A spermatogonium initiates mitosis (Monesi,1972). In the seminiferous epithelium mitoses are initiated at regular intervals corresponding to the completion of a cycle of the seminiferous epithelium. Clermont and Harvey (1965) discovered that 12.9 days were required to complete one cycle of the seminiferous epithelium in the Sprague-Dawley rat. These investigators observed that during the interval necessary for a type A spermatogonium to differentiate into a spermatozoon, 4 cycles of the seminiferous epithelium had proceeded. Thus the spermatogenic process in the Sprague-Dawley rat was calculated to require 51.6 days.

The Hormonal Control of Spermatogenesis

Until recently, the status of the knowledge regarding hormonal control of spermatogenesis relied heavily on the assumption that FSH was the prime control factor. However, recent advances have cast serious doubts on the role of FSH in the spermatogenic process. Workers in the field have utilized various experimental approaches to more clearly define the role of FSH, as well as the functions of ICSH and androgens in the spermatogenic process.

Removal of the pituitary gland in the rat has been shown to produce an arrest of spermatogenesis at the stage of the primary spermatocytes (Mess,1952). The rate of spermatogenesis

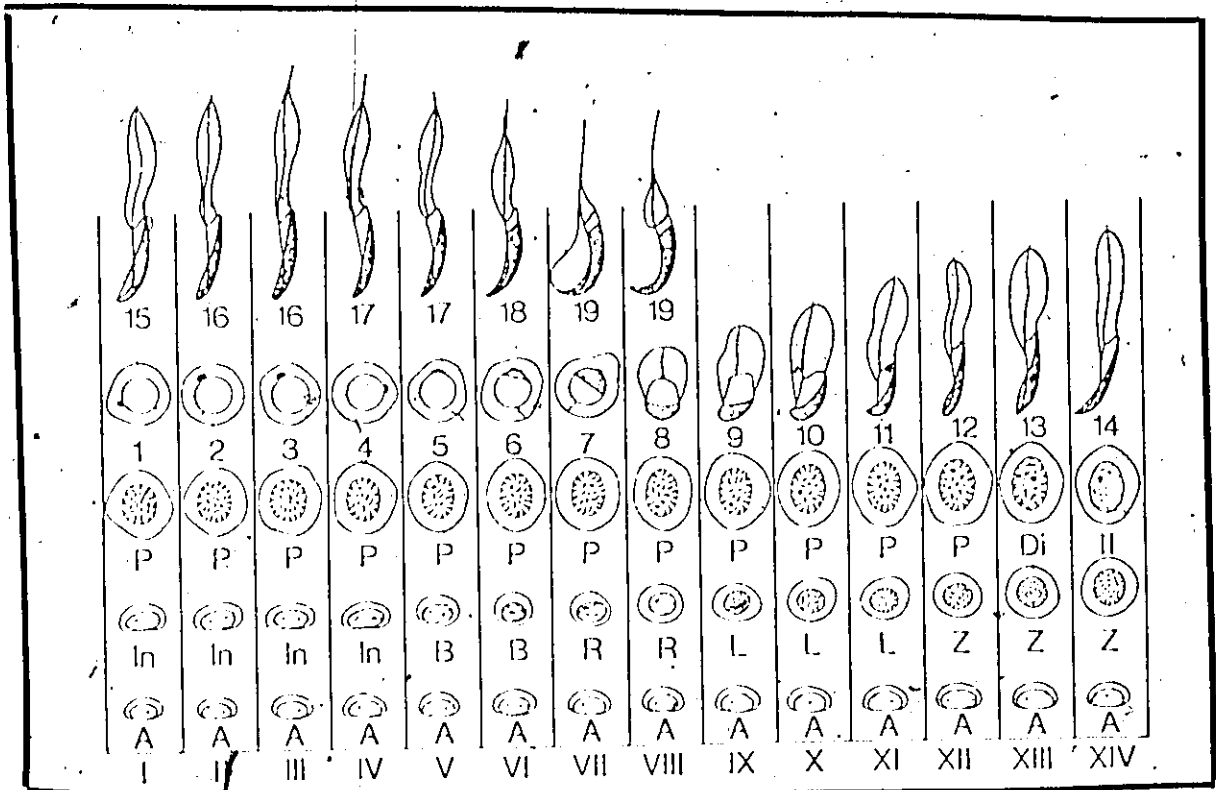


FIGURE 2. Composition of the fourteen cellular associations observed in the seminiferous epithelium of the rat. Each column consists of the various cell types composing a stage of the cycle(identified by roman numerals. Abbreviations: A,In,B: TypeA, Intermediate and type B spermatogonia;R,L,Z,P, Di: primary spermatocytes at pre-leptotene, leptotene,zygotene,pachytene,and diakinesis respectively;II:secondary spermatocytes;1-19 stages of spermiogenesis. From Perey et al(1961).

during the premeiotic stages was shown to be unaltered,but the quantitative aspects of germinal cell differentiation was affected (Clermont and Morgentaler, 1955). These investigators observed that only one-half of the type A spermatogonia complete mitotic divisions to form type B spermatogonia, and that spermatocytes tended to degenerate as evidenced by a high resting spermatocyte to pachytene spermatocyte ratio.

Hormone replacement therapy after hypophysectomy has been employed as an experimental approach to clarify gonadotrophin involvement in the spermatogenic process. Clermont and Harvey (1967) administered either ICSH,FSH, testosterone or a combination of these hormones to hypophysectomized rats and found that spermatogenesis was not quantitatively maintained. Although all stages of spermatogenesis were present in the ICSH-treated animals, only about two-thirds of the normal numbers of germ cells were present. The authors concluded that only ICSH was effective in the maintenance of spermatogenesis; FSH had no effect on the process.

Lostroh (1963), using relatively pure hormone, found that neither FSH nor ICSH alone was able to restore complete spermatogenesis in hypophysectomized rats and concluded that both FSH and ICSH must be available if spermatid formation is to be achieved.

Steinberger and Duckett (1967) suggested that FSH and testosterone act upon different germinal cells in a consecutive

fashion; a potentiating effect on the same cell type was implied. In immature rats, the gonadotrophins were suppressed with either testosterone or estrogen. Testosterone was revealed to be essential for the completion of the meiotic divisions and for the formation of spermatids up to stage 15 of spermiogenesis. Formation of more mature spermatids occurred only after FSH administration.

Lostroh (1969) observed an improvement of testicular morphology if growth hormone was concomitantly administered with testosterone to hypophysectomized rats. This study suggested that hormones, other than gonadotrophins may play a significant role in the quantitative aspects of spermatogenesis.

In vitro studies have confirmed many of the *in vivo* results. Steinberger and Steinberger (1965) demonstrated that the premeiotic stages of spermatogenesis in cell culture proceeded totally in the abscence of hormones. Utilizing radioautography these authors showed that differentiation of germ cells during the premeiotic stages proceeds in culture at normal rates. Steinberger and Steinberger (1973) observed that attempts to employ hormone therapy in cell culture had been disappointing. These investigators suggested that the hormones necessary for sprematogenesis may have to be modified in some way before their activity can be exerted on the target organ or cell.

Steinberger (1971) suggested that the hormonal control of spermatogenesis involved numerous hormones, at various stages along the spermatogenic process. He proposed that although the premiotic stages had been shown not to require gonadotrophins,perhaps growth hormone is required to stimulate the quantitative aspects. The author implied that hormonal control seemed to be exerted at two levels; (1) testosterone at the level of the meiotic divisions and spermatids to stage 15 of spermiogenesis (2) FSH at the later stages of spermatogenesis.

Lacy et al (1969) subjected rats to sterilizing doses of heat treatment to facilitate an increase in the ratio of Sertoli cells to germ cells. Seminiferous tubules were incubated with progesterone-C^{14} and the yields of endogenous and labelled testosterone were determined. There was no significant difference in the yields of either endogenous or labelled testosterone when compared to incubations carried out on normal tissue. These results suggest that the Sertoli cells are sites of androgen production. Recently, Castro et al (1970)demonstrated that FSH binds specifically to the Sertoli cells. These results imply that FSH acts upon the Sertoli cells to increase tubular androgen production. Thus the Sertoli cells may have an important role with regards to the hormonal control of spermatogenesis.

II. THE PINEAL AND MALE REPRODUCTION

Historical Introduction

The pineal is a small white structure shaped somewhat like a pinecone which is located near the center of the mammalian brain. Existence of such a body has been realized for at least 2000 years. Galen, in the second century A.D., quoted studies of early Greek anatomists who were impressed because of its unique perch on the aqueduct of the cerebrum.

Descartes, writing in the 17th century, formulated that the pineal body housed the seat of the rational soul. Philosophically, he proceeded to hypothesize the mode of pineal action. Descartes assumed that the eyes perceived the events of the world and that this information was transmitted by means of strings to the pineal. The pineal responded by releasing humors down hollow tubes to the muscles where the appropriate responses were initiated.

As it turns out, Descartes was not far from the truth in a sense, for the pineal does convert photic information which is neurally transmitted from the eyes to the gland, to elaborate a hormone type output which, via the blood stream then affects glandular performances (Kinson,1972). Such an organ which can respond to neural information in such a way as to synthesize a hormonal type output into the blood stream, has been designated a neuroendocrine transducer. The pineal is not unique in this respect, for the adrenal medulla,

posterior pituitary, and the pituitary releasing factor system operates in this fashion.

In 1889 Heubner published a case report of a boy who showed precocious puberty and was also found to have a pineal tumour. Over the next 50 years many other such cases were documented, as well as a smaller number of patients whose pineal tumours were associated with delayed sexual development. Kitay and Altschule (1954) reviewed the entire world literature concerning pineal tumours. They discovered that most of the tumours associated with precocious puberty were not really pineal in origin, but were tumours of the supporting tissues. The tumours associated with delayed puberty were, in most cases, true pineal tumours. From these reviews the relation of the pineal to reproduction became established ie; one of an inhibition of reproductive status.

Biochemistry of the Pineal

The pineal gland possesses complex biochemical machinery capable of translating neural influences into endocrine responses.A unique product of pineal function, melatonin, was isolated by Lerner et al (1958). It is a methoxyindole, the methoxy group being attached to the 5 position of the indane ring system.

The ultimate precursor of melatonin is tryptophan which is hydroxylated by the enzyme tryptophan hydroxylase to 5-hydroxytryptophan (Lovenberg et al,1967). This substrate

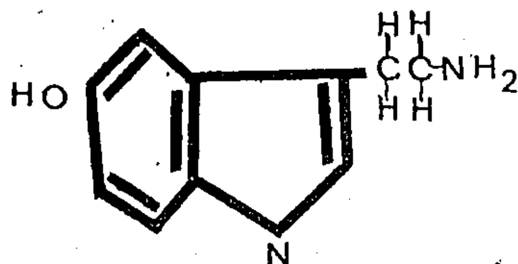
is catalysed by aromatic amino acid decarboxylase to form serotonin (Snyder and Axelrod,1954). Part of the serotonin is then acetylated by an acetylating enzyme,N-acetyl transferase (Weissbach et al,1960). The final step involves O-methylation to melatonin by hydroxy indole-O-methyl transferase (HIOMT) (Axelrod,1971). The enzymes catalysing the formation of melatonin are highly concentrated in the pineal (Axelrod,1971). Serotonin is the precursor of other indoles, not just melatonin; part of the serotonin is deaminated by monoamine oxidase to 5-hydroxyindole acetic acid and 5-hydroxytryptophol (Axelrod,1971). The enzyme HIOMT O-methylates 5-hydroxytryptophol to form 5-methoxytryptophol (Wurtman et al,1968). Figure 3 illustrates the stuctures of several of the indoles present in the pineal gland.

Relatively large amounts of amino acids were reported to be formed from glucose in the pineals of young goats (Hellman and Larsson,1961). The rapid formation of amino acids in the pineal may supply the material for producing a protein or polypeptide secretion (Wurtman et al,1968).

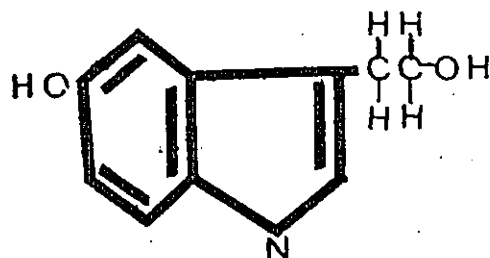
Anatomy and Innervation of the Pineal

In lower vertebrates the pineal was shown to contain photoreceptor elements not unlike the rods and cones of the retina (Kappers,1971). However,workers soon discovered,using electron microscopy,that the pineal of mammals had lost such photoreceptor elements (Wurtman et al,1968). Instead, the

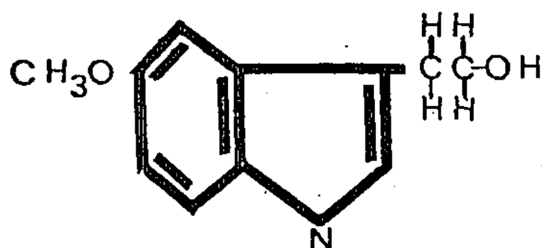
FIGURE 3. Structures of several pineal indoles.



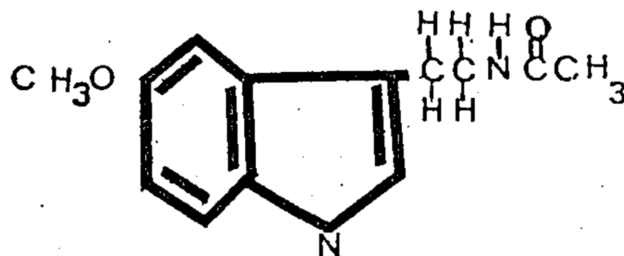
SEROTONIN



5-HYDROXYTRYTOPHOL



5-METHOXYTRYPTOPHOL



MELATONIN

mammalian pineal has become specialized during evolution to assume a secretory role (Wurtman et al, 1968). Response to light was altered from a direct photreception to an indirect photo-neurohumoral machanism. The nature of this indirect influence was discovered by Kappers (1960), who demonstrated that the mammalian pineal loses all nervous connections with the brain soon after birth and becomes extensively penetrated by sympathetic nerves of the autonomic nervous system. Electron microscope studies revealed that these nerves terminate directly on the pineal cells, instead of on blood vessels or smooth muscle cells, as in most other organs.

The exact innervation of the pineal gland (Wiener, 1968) clearly demonstrates the indirect influence of light on the pineal. Light energy is translated into nervous impulses in the retina. Nerve impulses are transmitted via the optic nerves to the inferior accessory optic tract. Via the medial forebrain bundle, and the tegmentum, the nerve impulses are relayed to the superior cervical ganglion. Sympathetic nerves innervating the pineal, thus transmit external photic information; this information is indirect since light as such does not stimulate the pineal directly.

Light and the Pineal

The first demonstration that environmental lighting influences the structure and function of the mammalian pineal was made by Fiske et al (1962). These workers

housed male and female rats in continuous darkness or light for 6 to 25 weeks. At the end of this time the weight of the pineal gland had decreased by about 25% in the light treated animals. Darkness had no effect on pineal weight. This result was rapidly confirmed in other laboratories. Moreover, when a large enough population of animals was studied, it was possible to demonstrate that pineal weight normally varied about a 24 hour cycle (Axelrod et al,(1965): the weight of the pineal was lowest at the end of the daily light period. These results demonstrated the inhibitory influence of light illumination on the pineal.

Wurtman et al (1963) subjected rats to an environment of either continuous light or darkness for 6 to 55 days. The pineals were then assayed for HIOMT activity. Continuous light produced a marked decline in the activity of this enzyme; animals maintained under light produced only one-third to one-tenth as much melatonin as rats kept in darkness. The effect of light on HIOMT appeared specific, inasmuch the activity of monoamine oxidase was unaltered.

Wurtman et al (1964a) demonstrated that the depression of HIOMT activity depended upon information carried to the pineal by its sympathetic nerves. These investigators removed the superior cervical ganglia bilaterally and subjected these surgically operated rats to an environment of constant light or darkness. The removal of the superior cervical ganglia

abolished the effects of environmental light on the pineal. To further examine the pathway involved in the transmission of light messages from the retina to the pineal,the inferior accessory optic tract was transected (Moore et al,1967). The lesion abolished the fall in HIOMT activity caused by continuous light. These observations conclusively demonstrated the indirect photo-neurohumoral influence of light upon pineal function.

Rhythms and the Pineal

Wurtman and Axelrod (1965) showed that HIOMT activity is very dependent on the status of the diurnal lighting regime,and oscillates closely about 24 hours in a circadian rhythm. HIOMT activity has been clearly demonstrated to be greatest in the dark phase and lowest during the light phase. However,the HIOMT activity rhythm is different from other biological rhythms in that light completely dictates the cyclicity. Exposure of animals to continuous dark maintains peak HIOMT activity while continuous light maintains minimal HIOMT activity. This exogenous rhythm is therefore completely dependent on environmental cues.

Serotonin content also cycles about a diurnal light pattern (Quay,1963). However,the rhythm is out of phase with the HIOMT rhythm. During darkness serotonin content is min-

imal and under light exposure peak serotonin levels are maintained. The significance of the out of phase relationship becomes apparent when one considers that serotonin is the precursor to melatonin. The serotonin content rhythm also differs with the HIOMT activity rhythm in that it is endogenous ie; a rhythm controlled by higher centers. This is clearly demonstrated when animals are exposed to continuous darkness. The rhythm persists, illustrating that environmental light acts only as an external synchronizer.

N-acetyl transferase, the enzyme that catalyzes the conversion of serotonin to N-acetylserotonin has also been shown to exhibit marked diurnal changes of activity in the rat pineal (Axelrod,1971). The enzyme exhibits greatest activity at night; thus more serotonin is acetylated at night. Since N-acetylserotonin is a precursor of melatonin the significance of the rhythm is evident.

Light, Reproduction and the Pineal

The influence of light or the lack of it on reproduction has been quite clearly demonstrated in a seasonal breeder, the hamster. Hoffman and Reiter (1965) found that if sexually mature hamsters were placed in an environment which allowed one hour of light per day, the testes suffered severe atrophic changes within 4 to 6 weeks. Proof that this alteration in growth was a consequence of a hyperactive pineal was demonstrated by the fact that the dark maintained ham-

sters,that had been pinealectomized failed to exhibit reproductive regression. This study and one by Wurtman et al (1961) clearly demonstated that the pineal secretes a gonad-inhibiting compound whose secretion is stimulated in darkness.

Effects of Pinealectomy on Male Reproduction

Pineal removal in experimental animals has been shown to be very inconsistent. Reiter and Sorrentino (1970) suggest that the absence of a response seen by some workers may be due to a multitude of factors; such as the age of animals at pinealectomy,ambient lighting conditions,nutritional status,difference in strain and species,and methods of caging the animals.

Motta and his colleagues(1967) observed 12 days after pineal removal an extraordinary 82 and 44% hypertrophy of the seminal vesicles and prostate glands respectively, compared to similar organs of the sham-operated controls: there was also an insignificant enhancement of testes weight. Kinson and Peat (1971) observed significant increases in prostate weight after pineal removal when compared to the sham-operated controls. However,this response at 4 weeks post-pinealectomy was nothing like the 44% hypertrophy observed by Motta et al (1967). The same study revealed a dramatic three fold rise in testicular venous testosterone, in the pinealectomized animals when compared to the sham-operated controls. These studies suggest that the pineal is

normally an inhibitory influence on male reproduction: its removal accelerates accessory sex gland weight and testosterone secretion.

Effects of Indole Treatment on Male Reproduction

The administration of melatonin and serotonin to experimental animals has been extensively utilized to gain insight to the influence of the pineal on male reproduction.

Boccabella et al (1962) studied the effects of high doses of serotonin (20mg/rat/day) on testicular function of the rat. These investigators reported dramatic impairment of spermatogenesis after 35 days of treatment. They attributed the massive damage to serotonin-induced testicular ischemia since simultaneous injections of serotonin with Apresoline(a vasodilator) produced no evident changes in testicular morphology. Kormano et al(1968) studied the long term influence of serotonin treatment on the testicular function of the rat. They reported dramatic impairment of spermatogenesis,similar to those induced by testicular ischemia. Kormano (1970) studied the influence of serotonin on testicular blood vessels. He observed that a single dose of serotonin(25 mg/Kg) could completely occlude the testicular artery and its branches. A smaller dose of serotonin (10 mg/Kg),though causing clear cut vasoconstriction,did not completely occlude the blood vessels.

Liu and Kinson (1973) administered melatonin and

serotonin in pellet form to mature male rats (10 mg indole/ rat), initially and again after 4 weeks. Administration of indoles in pellet form was employed to ensure a slow sustained release of indoles. Animals were sacrificed at 4,8, and 12 weeks after the initial implantation. Serotonin administration severely affected testis weight which was significantly depressed from the controls at 8 and 12 weeks and drastically impaired spermatogenesis; at 12 weeks over 85% of the tubules were damaged. The effect of serotonin upon spermatogenesis can not be attributed to testicular ischemia, since the dose level employed was not excessive.

Melatonin treatment in this study resulted in a significant depression of prostate weight at 12 weeks when compared to the controls. Melatonin treatment significantly depressed testicular venous levels of testosterone at 4 weeks. The effect persisted throughout the study, nonetheless, was not significantly different from the controls at 8 and 12 weeks. Melatonin administration had no effect on the gametogenic function of the testis throughout the study. This study clearly demonstrated that the locus of serotonin action is at the tubular level while melatonin predominantly affects the endocrine function of the testis.

Kinson _et al_ (1973) demonstrated that the fractional blood flow to the testis was not significantly altered during melatonin or serotonin administration. These investigators

concluded that the vascular effects of indoles are of secondary importance.

The effects of other indoles, 5-hydroxytryptophol and 5-methoxytryptophol, have not been extensively examined. Vilchez-Martinez and Debeljuk (1972) administered 5-methoxy-tryptophol to immature rats and observed that the indole had no significant effects on testis, ventral prostate and seminal vesicle weight.

The Effects of the Pineal: Levels of Interaction

The pineal is believed to exert its influence at two main levels; (1) hypothalamo-pituitary level (2) testicular level (Kinson,1972).

The influence of pineal indoles at the hypothalamo-pituitary level is widely documented. Nevertheless, there is little evidence that the pineal gland or its principles normally determine the synthesis or release of specific pituitary gonadotrophins (Reiter,1973).

Fraschini et al (1968) castrated adult male rats to stimulate the production and storage of LH in the anterior pituitary. By means of cannulae, various pineal-derived indoles were then placed in several central nervous system areas. When deposited into the median eminence both melatonin and 5-hydroxytryptophol significantly retarded the accumulation of LH in the pituitary. Serotonin and 5-methoxytryptophol were ineffective at this site. These results indicated that either the release of LH had been activated or the synthesis

had been inhibited under melatonin or 5-hydroxytryptophol treatment. Fraschini et al(1970) observed low pituitary and plasma levels of LH after implantation of these indoles,and therefore proposed that melatonin and 5-hydroxytryptophol act by inhibiting the synthesis of LH.

Fraschini et al(1970),using similar experimental methods,demonstrated the effects of pineal indoles on FSH. Serotonin and 5-methoxytryptophol implanted into the median eminence were most effective in reducing pituitary FSH content.

Kamberi et al(1970) infused melatonin (2μg/minute) directly into a cannulated stalk portal vessel,the blood of which drains into the sinusoids of the anterior pituitary; did not affect the concentration of LH in the peripheral serum. Its administration,via the ventricular route,caused a marked but transitory reduction in serum LH. From these results,it appears that melatonin has the ability to inhibit the release of the luteinizing hormone releasing factor into the primary portal plexus.

The results seem to indicate that pineal indoles influence the secretion of pituitary gonadotrophins through at least two different channels by means of inhibition of the releasing factors from the median eminence. Since methoxyindoles can be synthesized only by the pineal gland,melatonin and 5-methoxytryptophol are probably more important than

serotonin and 5-hydroxytryptophol as pineal regulators of pituitary activity (Fraschini et al(1970).

The major problem with the physiological interpretation of these results rests with the fact that workers are still not clear whether pineal indoles are secreted into the cerebral spinal fluid or enter the brain through the blood brain barrier. If indoles are secreted directly into the cerebral spinal fluid then perhaps the results reported by Fraschini and his colleagues may duplicate what happens normally (Reiter,1973).

The direct interaction of pineal indoles with the testis has been demonstrated recently. Ellis (1969) incubated testicular homogenates with pregneneolone-H^3 and progesterone-C^{14} and utilized serotonin (2 μmoles/ml) and melatonin (4 mμ moles/ml) as inhibitors. Both melatonin and serotonin inhibited testosterone and androstenedione formation,although the former was more effective. Inhibition of the enzymes of androgenesis appeared to be the site of action of these indoles.

Peat and Kinson (1971) incubated testicular homogenates with pregneneolone-H^3 and employed melatonin as the inhibitor. Addition of 0.2 μmoles of melatonin to the incubation mixture,significantly inhibited androstenedione formation only whereas,the addition of 20 μmoles was inhibitory towards the biosynthesis of both androstenedione and testos-

terone.

Ellis (1972) revealed the details of his previous experiment. Serotonin and melatonin inhibited the activity of 17α-hydroxylase and 17α-hydroxypregneneolone-C_{17}-C_{20} lyase. The author observed a differential action of serotonin and melatonin on the 17β-hydroxysteroid dehydrogenase system. He concluded that this was consistent with the hypothesis that this system is comprised of two components: one, a 17-ketoreductase and the other a 17β-hydroxysteroid dehydrogenase. Melatonin inhibited the 17β-hydroxysteroid dehydrogenase with no effect on the 17-ketoreductase. Serotonin increased the activity of the former enzyme and inhibited the activity of the latter enzyme. These results clearly demonstrate that pineal indoles exert a direct effect on androgen biosynthesis.

Debeljuk et al (1971) demonstrated a direct effect of melatonin on testis and or ventral prostate weight. These investigators showed that in hypophysectomized rats, melatonin is able to further lower the weights of the testes and or ventral prostates, and is able to modify the responses of these structures to HCG administration.

CHAPTER II

AIMS OF THIS STUDY

Previous investigations have demonstrated the inhibitory nature of melatonin and serotonin upon male reproduction. Melatonin administration primarily compromises testicular androgen synthesis while serotonin drastically impairs spermatogenisis. The influences of 5-hydroxytryptophol and 5-methoxytryptophol upon male reproduction have not been studied in any detail, except at the hypothalamo-pituitary level.

It seemed appropriate therefore, to investigate the effects of 5-hydroxytryptophol and 5-methoxytryptophol upon male reproductive functions and to examine the nature of responses in comparison with melatonin and serotonin. A chronic in vivo approach was taken whereby the influence of indoles upon the endocrine and gametogenic function of the testis was examined. Since gonadotrophins were not measured, it seems logical that such a study would not yield any information concerning the mode or level of pineal interaction, but would facilitate the evaluation of pineal indoles as possible agents for male fertility control. Information to date would suggest that one or more of the known pineal compounds might sustain the endocrine function of the testis, while the gametogenic function would be impaired. Such an action would be a highly desirable feature

of future male contraceptive chemicals.

The pineal appears to exert influences at two levels: (1) the hypothalamo-pituitary level (2) the testicular level. The relative physiological importance of either mode of pineal regulation on male gonadal functions has yet to be clarified. Any effects of indole administration might presumably result from influences at both levels, particularly when large pharmacological doses were employed.

The influence of indoles at the testicular level seemed the logical step for investigation both from an uncomplicated and mechanistic standpoint. Direct testicular actions have been demonstrated for both melatonin and serotonin,but such an interaction has not been examined with 5-hydroxytryptophol and 5-methoxytryptophol.

Therefore experiments were carried out to test the effects of 5-hydroxytroptophol and 5-methoxytryptophol at the testicular level and to compare their actions with those of melatonin and serotonin. The direct influences of these compounds were studied quantitively by measuring androgen formation from radio-labelled pregneneolone by rat testicular preparation <u>in vitro</u>, with and without added indole. In similar fashion their effects on the terminal step to testosterone ie; androstenedione- testosterone interconversion was also examined.

In conclusion, *in vivo* and *in vitro* experimental approaches were utilized to examine the effects of pineal indoles upon reproductive function of the adult male rat, in an effort to further clarify pineal-gonadal interrelationships. Continued efforts along these lines could eventually lead to the development of an effective method of male fertility control.

CHAPTER III

MATERIALS AND METHODS

Animals

The animals used in these experiments were adult male Sprague-Dawley rats purchased from Biobreeders of Ottawa. The rats were caged individually in a controlled environment, with a daily light cycle of 12 hours light to 12 hours darkness (lights on at 0600 hours and off at 1800 hours) and an ambient temperature of 20±1°C. Food and water were administered *ad lib* and the animals were allowed to acclimatize for several days prior to their experimental use.

Indoles

Indoles were utilized as purchased from the Sigma Chemical Co. and included : serotonin creatine sulphate(S),melatonin (M),5-methoxytryptophol (Me),and 5-hydroxytryptophol (Ho).

(A) *IN VIVO* EXPERIMENTATION

The animals were divided into five groups,ie; four experimental (corresponding to the indoles employed) and one control groups,and were injected,beginning when the rats were 63 days of age. Homogeneous suspensions of indoles in peanut oil (1μmole/0.1 ml) were prepared and thoroughly mixed by mechanical shaking for at least one hour prior to injection. Rats were injected subcutaneously,once a week,with 0.1 ml of the respective indole (1 μmole) suspension and the control

animals similarly,received 0.1 ml of vehicle alone. Six rats from each group were removed for study at intervals of 4,8, and 12 weeks following the initial injections (Figure 4). Body weights were recorded and the animals were anaesthetized with sodium pentobarbitol (25 mg/Kg body weight).

Blood Collection

Animals were heparinized (1000 USP units/0.1 ml isotonic saline) via the tail vein and all blood samples were collected between 0900 and 1200 hours in an effort to minimize variance due to circadian fluctuation (Kinson and Liu, 1973a).

The right testis was exposed and testicular venous blood was collected from a prominent branch of the testicular vein for five minutes as outlined by Kinson and Peat (1971). Following immediate centrifugation at 900 X G for 20 minutes,plasma was collected for the estimation of testosterone and androstenedione levels in testicular venous plasma(TVP). The estimation of hormone levels in TVP should provide a good index of testicular endocrine function,androstenedione and testosterone being regarded as the major secretory products of the testis (Hall,1970).

Mixed venous blood was collected from the right ventricle of the heart. The plasma was utilized for the estimation of testosterone levels in mixed venous plasma (MVP). Circulating testosterone was measured as an index of androgen

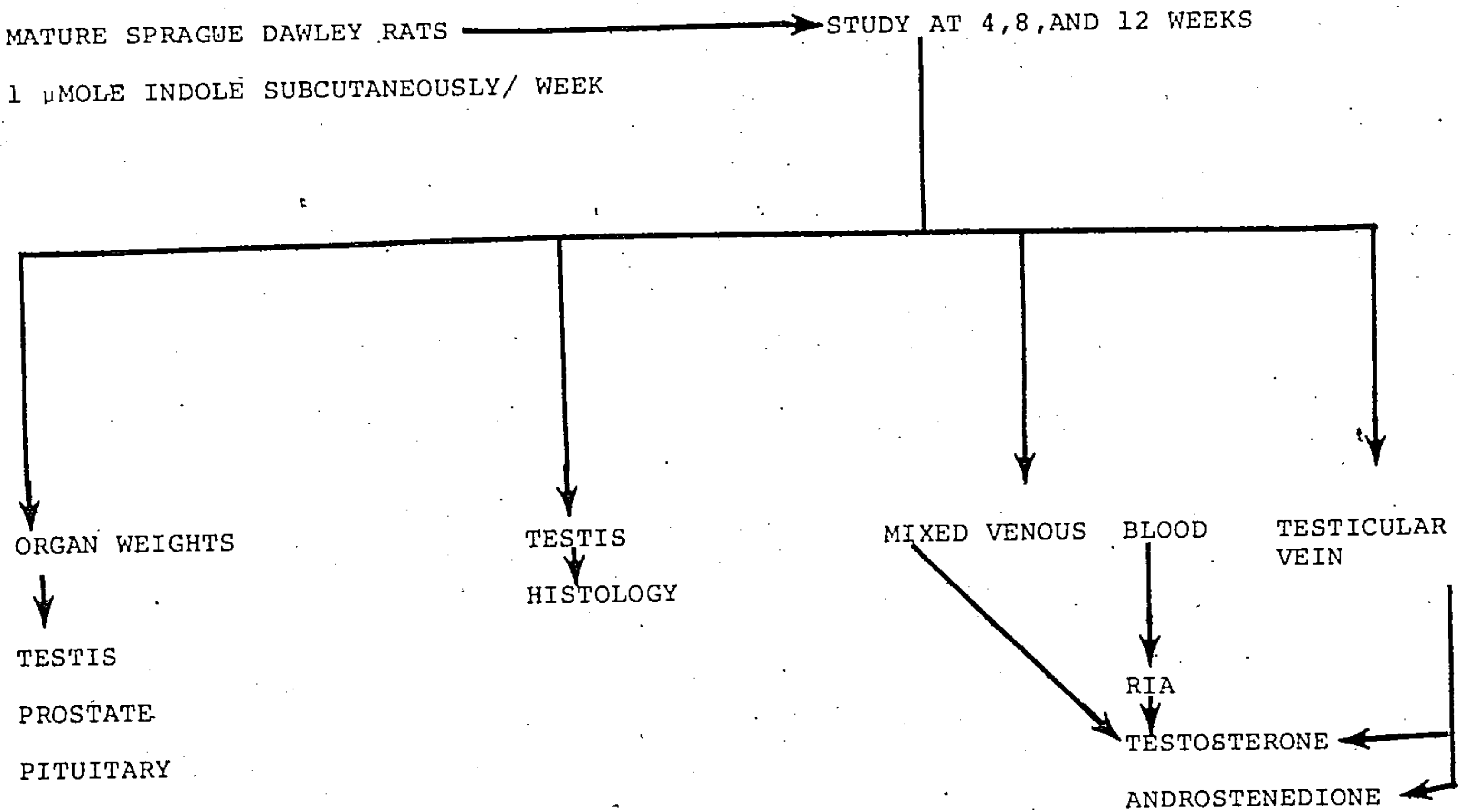


FIGURE 4. FLOWSHEET OF EXPERIMENTAL PROCEDURES EMPLOYED <u>IN VIVO</u>.

status.Furthermore,any possible change in circulating hormone in the absence of alterations in TVP levels would tend to suggest effects on the metabolic clearance of the hormone.

Examination of Organs

Immediately following blood collection,the contralateral testis was removed,weighed,and placed in Helly's fixative for subsequent histological examination.The accessory sex complex was removed and the androgen-dependent ventral prostate carefully separated and weighed.Finally,the whole pituitary was excised and the weight recorded.

Hormone Assays

Both testosterone and androstenedione levels were estimated by a modification of the method outlined by Furuyama et al(1970)(Figures 5&6).Essentially,the procedure can be divided into three stages ie; (1) extraction of hormones from plasma (2) purification of hormone (3) radioimmunoassay (RIA).

(1) Extraction of Hormones from the Plasma

(a) Testosterone

Aliquots of 0.005 ml of TVP and 0.05 ml of MVP were utilized for the estimation of testosterone levels. These volumes of plasma were used because testosterone levels in TVP are approximately ten fold higher than those of testosterone in MVP.Consequently,this enabled the simultaneous estimation of testosterone in both TVP and MVP,employing one

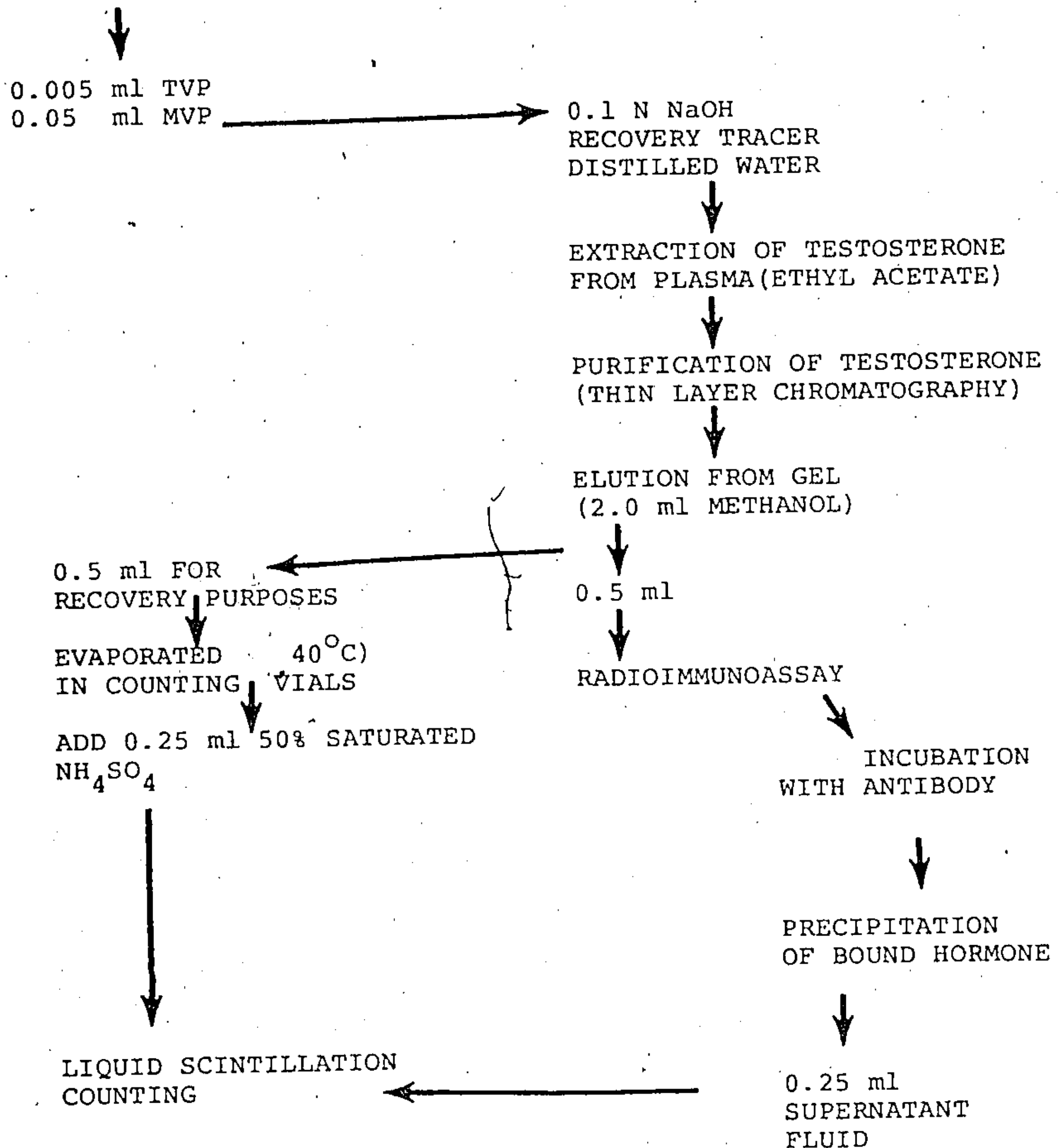


FIGURE 5. FLOW SHEET ILLUSTRATING EXTRACTION AND RADIOIMMUNOASSAY PROCEDURES FOR THE ESTIMATION OF TESTOSTERONE.

BLOOD SAMPLES
↓
0.025 ml TVP → 0.1 N NaOH
DISTILLED WATER
↓
RECOVERY BLANKS
↓
RECOVERY TRACER
DISTILLED WATER → EXTRACTION OF ANDROSTENEDIONE
(HEXANE:ETHYL ACETATE, 100:1)

0.5 ml
↓
EVAPORATED(40°C)
IN COUNTING VIALS
↓
ADD 0.25 ml 50% SATURATED
NH_4SO_4
↓
LIQUID SCINTILLATION
COUNTING

0.5 ml
↓
RADIOIMMUNOASSAY
↓
INCUBATION WITH
ANTIBODY
↓
PRECIPITATION
OF BOUND HORMONE
↓
0.25 ml SUPERNATANT
FLUID → LIQUID SCINTILLATION COUNTING

FIGURE 6. FLOWSHEET ILLUSTRATING EXTRACTION AND RADIOIMMUNO-ASSAY PROCEDURES FOR THE ESTIMATION OF ANDROSTENEDIONE.

range of standards. 1,2-H^3-testosterone (5000 DPM) was added to each sample for recovery purposes and the plasma sample was made alkaline by the addition of 0.1 N NaOH, in order to render estrogen extraction negligible. An aliquot of distilled water (0.5 ml) was added to wash the recovery tracer down the sides of the tubes. Extraction of hormone from plasma was achieved by adding 6.0 ml ethyl acetate and mechanical shaking for one hour. The aqueous layer was aspirated and discarded and the organic phase dried by standing over anhydrous sodium sulphate for 1.5 hours. The dried organic layers were carefully decanted into tubes and evaporated under a stream of N_2 at 40°C.

(b) Androstenedione

Aliquots of 0.025 ml of TVP were utilized for the estimation of androstenedione. Distilled water was added to make up the volume to 0.5 ml. Extraction of the hormone from TVP utilized a solvent mixture of hexane:ethyl acetate (100:1), and after mechanical shaking, the aqueous layer was aspirated and discarded. Aliquots of 0.5 ml of the organic layers were pipetted into 2.0 ml conical tubes and evaporated under a stream of N_2 at 40°C. Recovery was determined for each set of samples by adding 20,000 DPM of 1,2-H^3-androstenedione to two extraction tubes and two counting vials. The volume in the extraction tubes was made

up to 0.5 ml with distilled water and the hormone extracted similarly to the samples. Duplicate aliquots of 0.5 mls organic layers were pipetted into counting vials after mechanical shaking, and evaporated to dryness under a stream of N_2 at $40^{o}C$.

(2) Purification of the Hormones

(a) Testosterone

Thin layer chromatographic techniques were employed in the purification of testosterone. Silica gel F_{254}plates purchased from the E.M.Merck Co., containing a fluorescent indicator, were pre-washed at least two times in a 50:50 methanol:acetone mixture. An inert testosterone standard was spotted along one side of each plate to which two samples had also been applied. After development in a 17:3 benzene:methanol solvent mixture,sample areas corresponding to the height of migration of the inert standard, which were located under UV light, were scraped off the plates. Elution of testosterone from the gel was achieved by mixing with 2.0 ml methanol. The gel was sedimented by centrifugation at 1000 x G for 20 minutes and 0.5 ml eluate were pipetted into counting vials for recovery purposes and equivalent amounts were used for the RIA.

(b) Androstenedione

Androstenedione was assayed without preliminary chromatographic purification. The high specificity of the antibody used in the RIA was considered sufficient to allow determination of androstenedione in direct extract, rendering purification unnecessary. This was further substantiated by exhaustive analysis of plasma samples with and without prior chromatographic purification, which gave almost identical results (Chandler et al, in press).

(3) Radioimmunoassay*

The radioimmunoassay procedures employed antisera to bovine serum albumin (BSA) complexes of testosterone-3-oxime and androstenedione-6-thioter (Endocrine Sciences), to which tritium labelled testosterone or androstenedione respectively were added as competitors in the antigen-antibody reaction. Dilute working solutions of testosterone antiserum was freshly prepared by adding 0.1 ml bovine serum albumin (BSA), 0.2 ml bovine gamma globulin (BGG), 0.02 ml stock antiserum, and 0.1 ml borate buffer pH 8.3. Fresh working solutions of androstenedione antiserum were similarly prepared by adding 0.15 ml BSA, 0.2 ml BGG, 0.02 ml stock antiserum, and 0.02 ml stock 1,2-H^3-androstenedione (260,000 DPM) to 10 ml borate buffer, pH 8.3. Aliquots of 0.25 ml dilute antisera were pipetted into each sample and into counting vials, as well as into tubes containing known

* for composition of buffers refer to the Appendix

amounts of the hormone. The steroids were incubated with the antibody for three hours at room temperature, and 0.25 ml saturated ammonium sulphate solution was added to precipitate the bound form of the hormones. The precipitated complex was sedimented by centrifugation at 1000 x G for 30 minutes and 0.25 ml supernatant was pipetted into counting vials for the estimation of unbound labelled hormone by liquid scintillation counting. Aliquots of 50% saturated ammonium sulphate were added to the blanks.

The samples were counted in a Nuclear Chicago Mark I liquid scintillation spectrophotometer following the addition of 10 ml scintillator solution which contained 4 g PPO, and 2% methanol per liter of toluene. Samples were mechanically shaken for 1 hour prior to counting to effect extraction into the saturated medium. Counting techniques employed the channels ratios method to correct for possible quenching.

A Standard curve was prepared for each hormone assay (Figs, 7&8). Known amounts of hormones were assayed and the results were plotted against % free label. The relationship obtained was utilized to determine the concentration of hormone in the samples. Following correction for procedural losses based on tritium recovery in the chromatographic elute, results were expressed as ug hormone/100 ml plasma.

The methods employed were considered to be sensitive to 10 pg steroid per sample. Accuracy was previously tested

FIGURE 7

RADIOIMMUNOASSAY OF TESTOSTERONE : STANDARD PLOT.

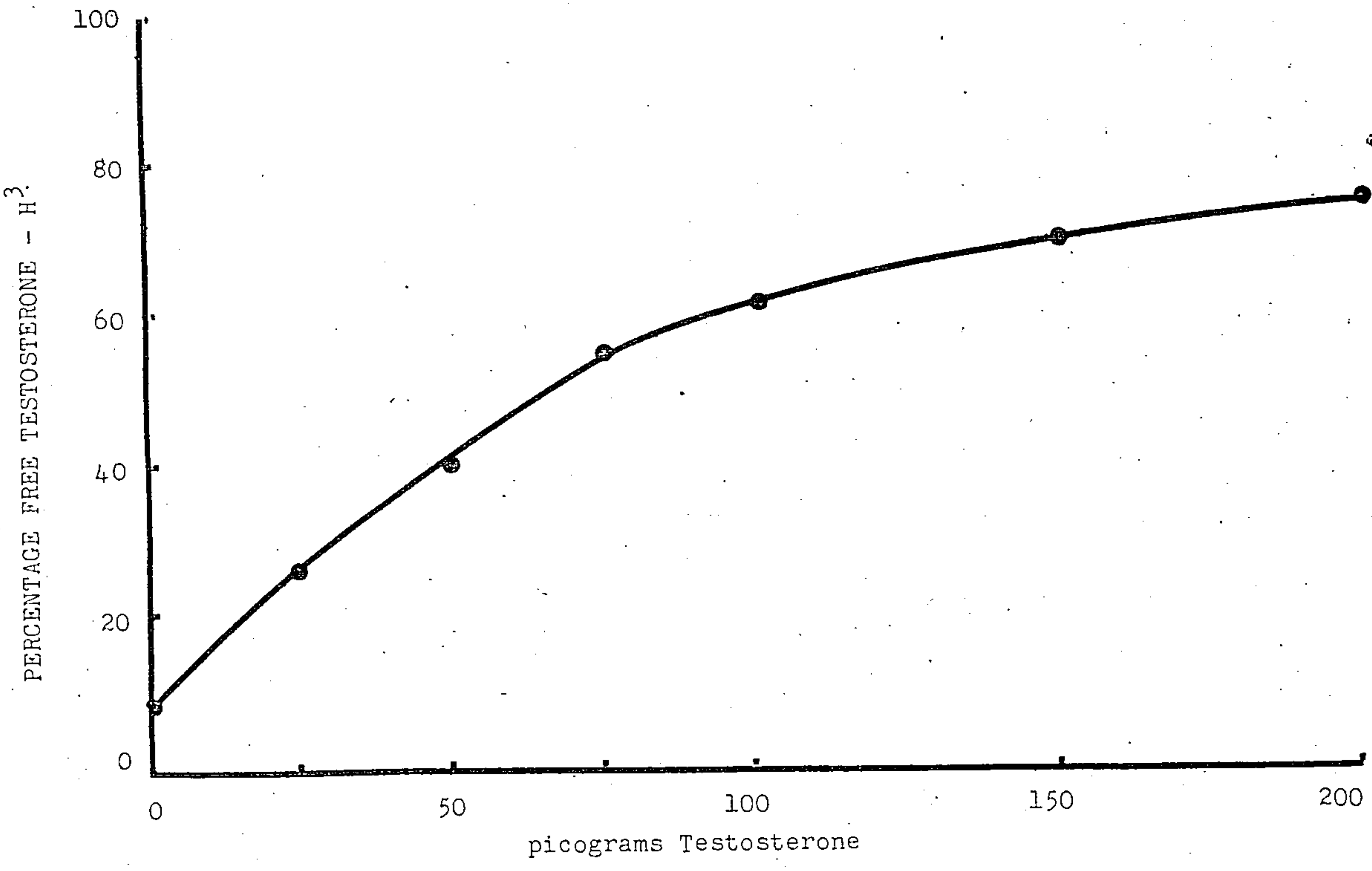
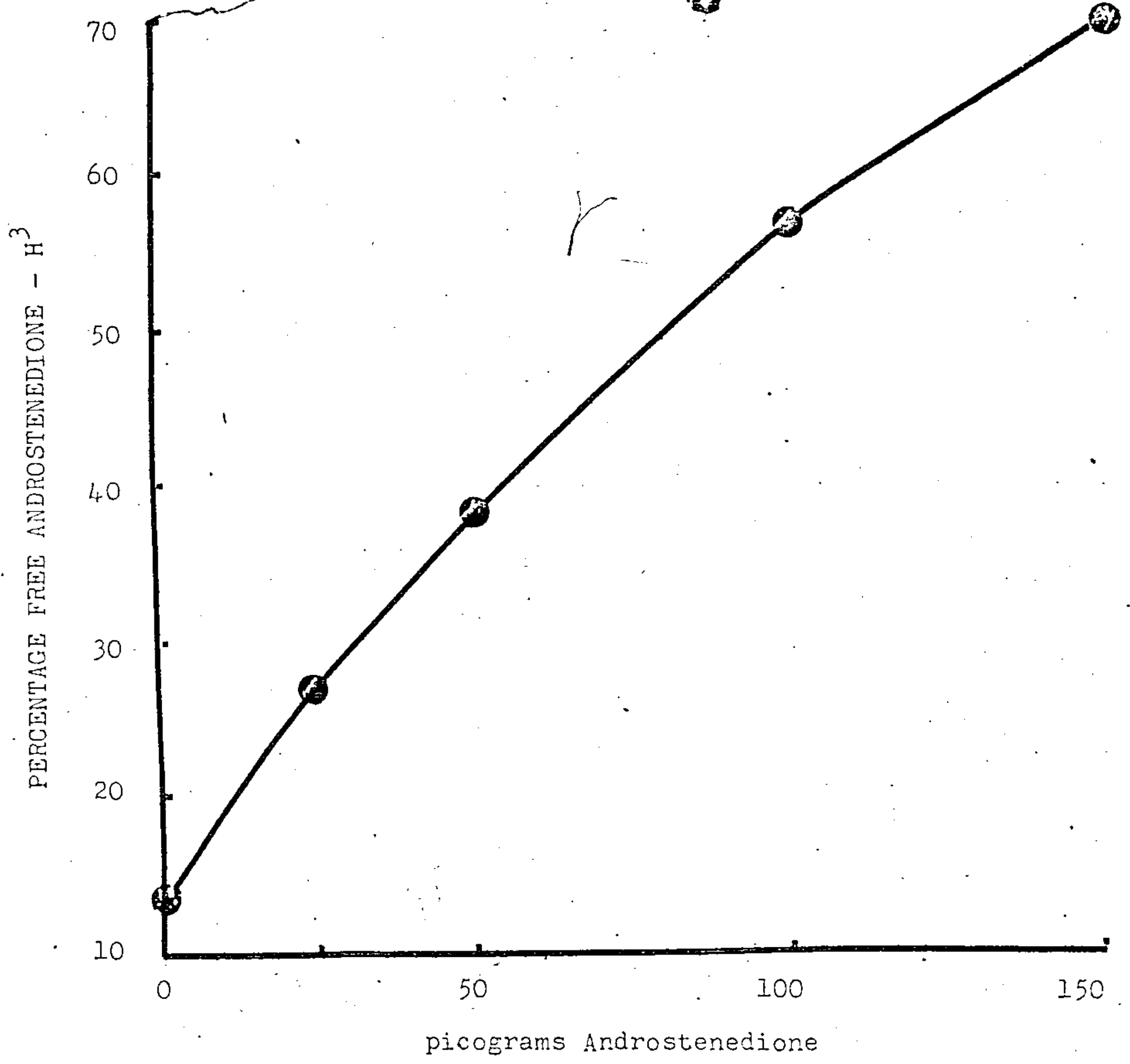


FIGURE 8 RADIOIMMUNOASSAY OF ANDROSTENEDIONE : STANDARD PLOT.



over the range of 10-200 pg steroid, amounts being added to ether extracted samples of TVP of the rat. Determined values were within 97-101.3% of the added amounts of steroid. The mean blank value for the testosterone assay was 7 pg and for androstenedione, 16 pg. The latter was appreciably greater by virtue of the lack of chromatographic purification in the androstenedione assay procedure.

These proceudres applied to rat plasma samples were found to yield values quite comparable with those derived by the double isotope derivative assay of Bardin and Peterson (1967). The latter method afforded 4.2±0.56 μg testosterone and 0.56±0.10 μg androstenedione per 100 ml TVP while values of 4.3±0.61 and 0.72±0.09 respectively were obtained by RIA.

Histological Techniques*

Conventional histological techniques were employed to quantitatively evaluate spermatogenesis (Fig. 9).

The major aims of the histological manipulations were: (1) to prevent postmortem changes to the tissue (2) to section the tissue to such a diaphaneity as to render the tissue microscopically observable without damage and (3) to differentiate portions of the tissue so as to make characterization possible.

Fixation should satisfy the initial aim. Helly's fixative was employed for this purpose and the testes were immersed

* for composition of histological solutions refer to the Appendix

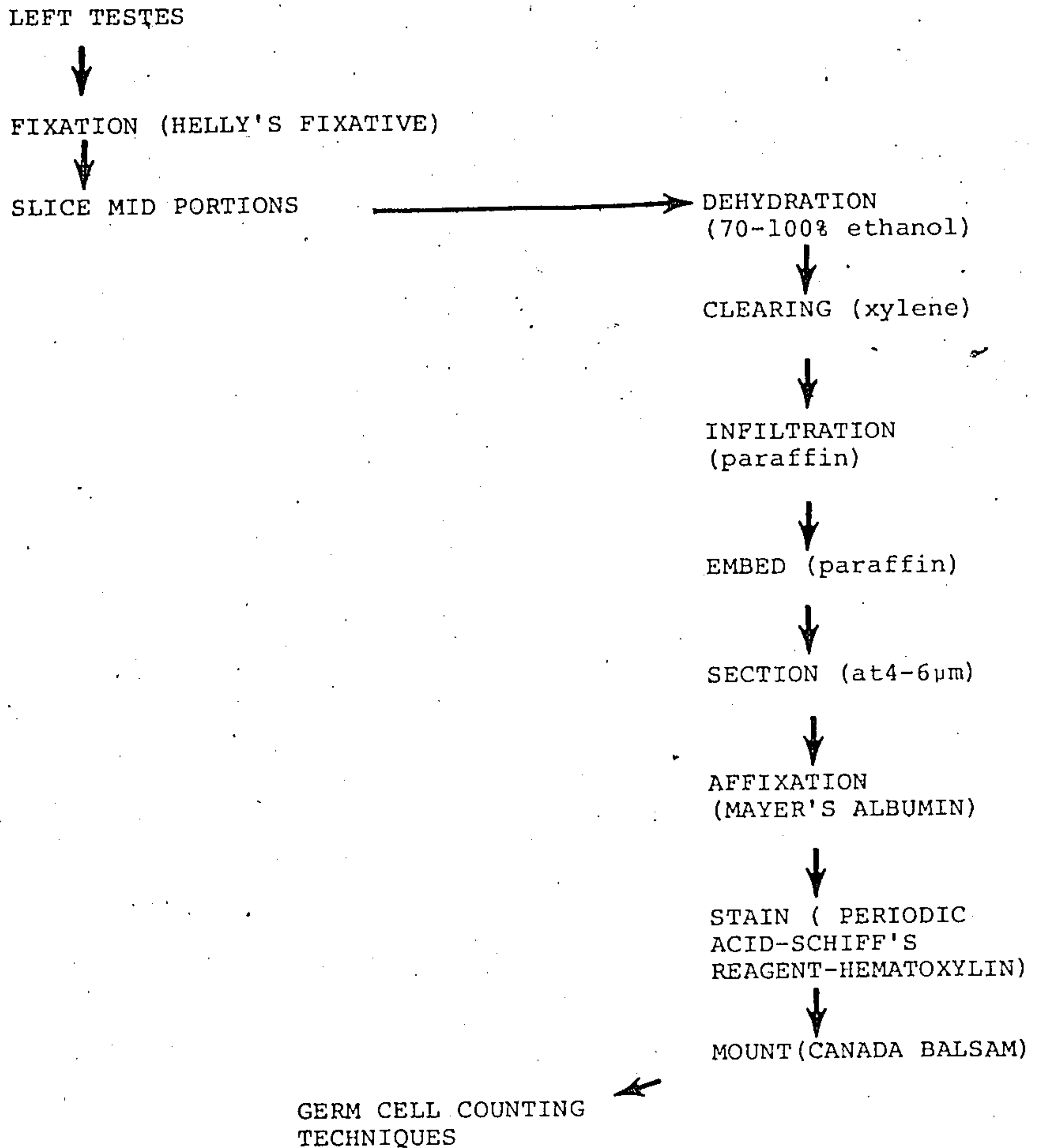


FIGURE 9. FLOWSHEET ILLUSTRATING HISTOLOGICAL PROCEDURES INVOLVED IN THE QUANTIFICATION OF SPERMATOGENESIS.

immediately after removal from the animals. After treatment for 18 hours, a mid portion of each testis was sliced and returned to the fixation process for a further 6 hours. Post-fixation involved washing for a period of 24 hours prior to further phases of manipulation.

Sectioning of the tissue can only be employed when the tissue has been supported in a paraffin block and itself infiltrated with paraffin. Omission of these treatments would yield a preparation too soft and fragile for sectioning techniques. Several phases usually precede infiltration and embedding; a dehydration step is employed since paraffin is not miscible with water. Dehydration involves the subjection of tissue to a series of alcohols (70%-100% ethanol) to remove all traces of water from the tissue. Ethanol is also not miscible with paraffin, and consequently an intermediate clearing procedure is next employed. Clearing removes opacity from dehydrated tissues making them transparent (Humason,1972). Xylene was used for this purpose and is miscible with both ethanol and paraffin. Therefore, the tissue was subjected to a series of phases ie;(1) 1:1 xylene:absolute ethanol (v/v) (2) pure xylene (3) 1:1 xylene:paraffin (v/v).

Infiltration with "Tissuemat" paraffin (melting point 52.5°C, Fisher Scientific Co.) was next employed and involved three successive changes of paraffin in three hours. Embedding of the tissue in "Tissueprep" paraffin (melting point 56.5°C,

Fisher Scientific Co.) followed infiltration, rendering sectioning possible. The tissue was sectioned on a rotary microtome (Leitz-Wetzlar) to yield sections of 4-6 μm thickness and these were affixed to glass slides with Mayer's albumin. The glass slides were allowed to dry for a period of 48 hours so that affixation would be complete.

The remaining manipulations served to satisfy the final aims, ie;that of differentiation. The tissue sections need to be deparaffinized in xylene and rehydrated in a series of alcohols since staining occurs in an aqueous state. Complexes of $HgCl_2$ must be removed and this was achieved with an iodine solution (Lugol in 70 % ethanol). The stain used was the Periodic acid-Schiff-Hematoxylin stain as outlined by Leblond and Clermont (1952). Initially,the tissue sections were immersed in periodic acid for ten minutes,followed by immersion in Schiff's reagent for 30 minutes. Hematoxylin was used as a counterstain and the sections forwarded to a dehydration series of alcohols to remove all traces of water. The tissue sections were finally mounted with cover slips using Canada Balsam.

The tissue sections were examined under a microscope and ten stage V and stage VII tubules were examined for germ cells. From the stage VII tubules,the number of type A spermatogonia,resting and pachytene spermatocytes,spermatids,and Sertoli cells were noted. Stage V tubules were examined for

type B spermatogonia. The numbers of these germ cells were recorded as crude counts of germ cells. However,these crude counts do not compensate for differences in thickness of section or shortening of tubular diameters (Clermont and Morgentaler,1955). Therefore,the number of germ cells was corrected for these factors. Using an ocular micrometer,the mean diameter of ten of each respective germ cell type was noted. True germ cell counts were calculated utilizing these diameters as well as thickness of section by means of Abercrombie's formula (Clermont and Morgentaler,1955). Since in the adult rat, Sertoli cells do not divide (Monesi,1972), an increase in the number of Sertoli cells would indicate that shortening of tubules had occured in the histological manipulations. Therefore,the number of germ cells was once more corrected by a Sertoli correction factor (Clermont and Morgentaler,1955).

(B) IN *VITRO* EXPERIMENTATION

Experimentation entailed two phases ie; (1) the effects of added indoles on the conversion of 5-pregneneolone-H^3 to androgens and (2) the effects of added indoles on the interconversion of testosterone and androstenedione. Quantitative analysis was afforded through the use of radio-labelled substrates and thus 5-pregneneolone-7α-H^3 (specific activities 18.6 Ci and 11.3 Ci/m mole), testosterone-4-C^{14} and androstenedione-4-C^{14} (specific activities 46 mCi/m mole) were purchased (Amersham Searle Co.) and these were repurified by thin layer chromatography prior to use. The nicotinamide-adenine-dinucleotide coenzymes (NADP,NADPH) and indole compounds were obtained from the Sigma Chemical Co. and used without further purification.

Tissue Preparation

Adult rats were killed by swift decapitation. The testes were decapsulated and a homogenate in 9 volumes' ice cold 0.25 M sucrose solution was prepared, using homogenizers fitted with teflon pestles (TRI-R Instruments Inc.) which were kept cold by immersion in crushed ice. The homogenate was centrifuged on a high speed refrigerated (0^oC) centrifuge (Lourdes Instruments) at 10,000 x G for 20 minutes to remove debris, nuclei, unbroken cells and mitochondria. The supernatant fluid was pooled from all tubes, the total volume noted, and the volume corresponding to 0.25 g wet testis was calculated. A portion

of supernatant fluid was placed in a boiling water bath for 20 minutes and this preparation served as tissue controls.

Preparation of the Incubation Mixture*

Incubation tubes were prepared for six groups corresponding to the four indoles, control, and boiled-tissue control groups respectively, and were made up 24 hours prior to preparation of testicular homogenate.

The initial series of experiments, designed to test the influence of indoles on the conversion of 5-pregneneolone-H^3 to androgens, was executed at several different dose levels of indoles, ie; 90 nmoles, 450 nmoles, and 18 μmoles of indole per tube. Indole solutions were prepared in ethanol:0.05 M phosphate buffer, pH 7.4, (1:1), so that 0.1 ml of solution contained 90 nmoles, 450 nmoles, 900 nmoles, respectively. Control and boiled-tissue control tubes contained 0.1 ml ethanol:buffer (1:1) in lieu of indole solution. For study at the highest dose level, the appropriate amount of indole was weighed directly into the respective experimental tubes.

All tubes contained 0.1 ethanol solution of 5-pregneneolone-7α-H^3 (6.1×10^6 or 3.8×10^6 DPM, .152 mμmole), 1000 mμmoles NADPH and 100 mμmoles nicotinamide, dissolved in 0.1 ml of phosphate buffer.

For the study of the influence of indoles on the interconversion of testosterone and androstenedione, the basic

* for composition of buffers refer to Appendix

experimental approach was similar, with a few minor exceptions. Only two dose levels of indoles were studied, 900 nmoles, and 18 μmoles of indole per tube. Solutions of substrates utilized were such that 0.25 ml of ethanol solution contained 24,977 DPM testosterone-4-C^{14} (18.2 mμmole) or 0.2 ml ethanol solution contained 24,1000 DPM androstenedione-4-C^{14} (15.6 mμmole). For the metabolism of testosterone, 0.1 ml NADP (100 mμmoles) in buffer was present and for androstenedione metabolism, a corresponding amount of reduced coenzyme (NADPH) was added.

Incubation Procedures

Incubation mixtures were completed by the final addition of supernatant fluid, corresponding to 0.25 g wet testis and the final volume was adjusted to 5.0 ml with phosphate buffer. All incubations were carried out in air, at 32°C with constant shaking for two hours in a Dubnoff incubator. All incubations were terminated through rapid freezing to -50°C by placing the tubes in a dry ice:acetone mixture.

For the incubation of tritiated substrates, internal recovery standards of 10,000 DPM of testosterone-4-C^{14} and androstenedione-4-C^{14} were added to the incubations and thoroughly mixed.

Separation and Measurement of Testosterone and Androstenedione Formed from Pregneneolone-H^3

The androgens were extracted from the incubation mixtures

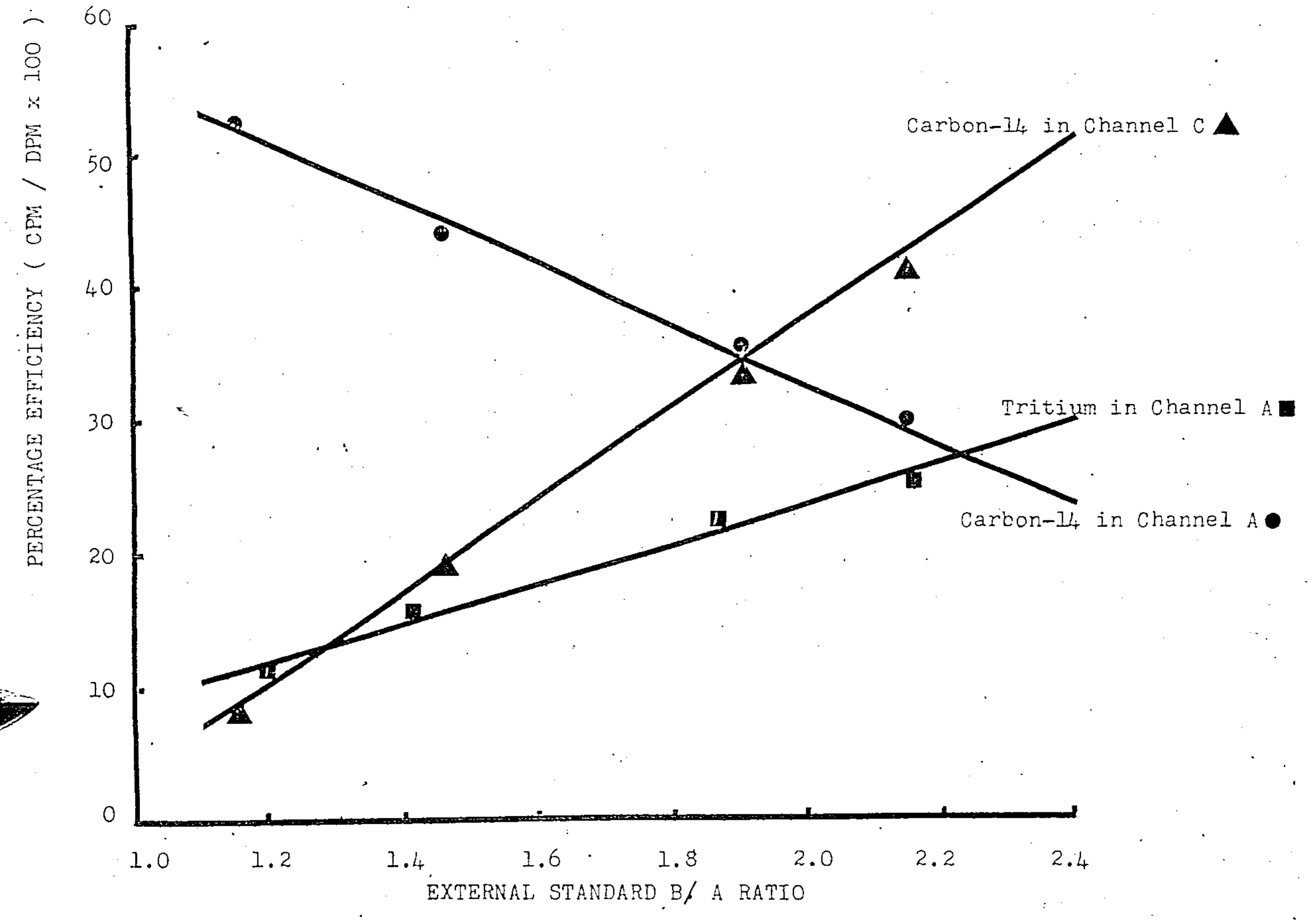
by mechanical shaking for 30 minutes with 10 ml of ethyl acetate. After standing to allow separation, aqueous layers were aspirated and discarded and the organic phases were evaporated under a stream of N_2 at 40°C. Inert chromatography markers (50 μg testosterone and 50 μg androstenedione) were applied to thin layer chromatography plates. The extracts were initially "defatted" on silica gel F_{254} plates, with chloroform as the running solvent. Androstenedione and testosterone were then separated by thin layer chromatography using a solvent mixture of benzene:ethyl acetate (3:2). Steroid zones were visualized under UV light (254 mμ) and these located with a pencil mark around the periphery. These areas of gel were scraped into tubes and eluted by shaking with 3.0 ml methanol:ethyl acetate (3:1). Following centrifugation, the solvent was decanted and evaporated under N_2 at 40°C. The separated steroid extracts were further purified by successive thin layer chromatography on silica gel F_{254} plates using the following solvent systems, benzene:hexane:ethanol (15:4:1), benzene:methanol (19:1), and benzene:methanol (17:3). This sequence of chromatographic procedures was found to yield pure products, as judged by the constancy of tritium: carbon^{14} ratios after the two final chromatography systems. Steroid areas on the last chromatogram were scraped directly into counting vials and shaken with 10 ml scintillator solution prior to counting. Doubly labelled products were assayed by

liquid scintillation counting using a Nuclear Chicago Mark I spectrophotometer set for double isotope analysis and providing efficiencies of 30% and 50% for tritium and carbon^{14}, respectively. Sample counting was carried out in 10 ml scintillator containing per liter of toluene; 4 g PPO, 0.1 g dimethyl POPOP, and 10 ml ethanol. A series of unquenched and quenched standards were prepared using n-hexadecane-C^{14} and n-hexadecane-H^3 (specific activities of 1.06 μCi/g and 2.00 μCi/g; Amersham/Searle Co.) and carbon tetrachloride, and were routinely used to determine quenching relations employing an external barium standard (Fig. 10). The tritium activity afforded estimation of the formation of testosterone and androstenedione and correction for procedural losses was derived from the carbon^{14} data. Results were expressed as per cent conversion of pregneneolone-H^3 to both androstenedione and testosterone.

Measurement of Interconversion of Testosterone and Androstenedione

The androgens were extracted from the incubation mixtures by shaking with 2 ml ethyl acetate. The extracts were evaporated to dryness under a stream of N_2 at 40°C. Inert chromatography markers (50 μg testosterone and 50 μg androstenedione) were added to the extracts and were spotted on silica gel F_{254} plates, which were developed with benzene: methanol (17:3). Following development, plates (5x20 cm) were scanned with a Nuclear Chicago Actigraph III instrument to

FIGURE 10. QUENCHING RELATIONS FOR n-HEXADECANE (C^{14}) AND (H^{3}) STANDARDS.



confirm the location of radioactive areas coincident with the UV-absorbing marker areas. No radioactive areas were detected routinely, other than those corresponding to labelled testosterone and androstenedione. Areas corresponding to testosterone and androstenedione were scraped directly into counting vials for liquid scintillation techniques in a Nuclear Chicago Mark I spectrophotometer. Samples were shaken with 10 ml scintillator solution containing per liter of toluene; 4 g PPO, 0.1 g dimethyl POPOP, and 10 ml ethanol, prior to counting. Unquenched and quenched standards employing n-hexadecane-C^{14} (specific activity of 1.00 μCi/g) were again routinely employed to establish quenching relations using an external barium standard. Results were expressed as per cent conversion of either testosterone to androstenedione or androstenedione to testosterone based on the total radioactivity recovered (50%-65%). The identity of steroid substrate end product was substantiated by chromatographic mobility in different solvent systems (Peat and Kinson,1971).

CHAPTER IV

RESULTS

(A) IN VIVO EXPERIMENTATION

Subcutaneous indole administration to adult male rats had only modest effects on testis,pituitary and body weights as shown in Table 1.

Alterations in testis weight as a result of indole treatment were observed only in the 5-hydroxytryptophol-treated animals; testis weight was significantly depressed at 4 weeks. Nonetheless,the influence of 5-hydroxytryptophol was transitory in nature since testis weight was not different from the controls during the remainder of the study.

Body and pituitary weight was not affected by indoles at 4 and 8 weeks. At 12 weeks,in the 5-methoxytryptophol-treated animals,a reduotion in body weight which was significantly different from the controls was observed. Serotonin treatment produced a depression in pituitary weight ($P < 0.025$) at this period of investigation. No other effects of indoles were observed on body and pituitary weight at the ultimate stage of investigation.

Ventral prostate weight has been shown to vary proportionally with the androgen status of the animal. Therefore, any change in ventral prostate weight might be expected to to coincide with alterations in the endocrine function of the testis. The indole-treated groups had ventral prostates

TABLE 1

EFFECTS OF INDOLES IN VIVO ON BODY AND ORGAN WEIGHTS AND ON CIRCULATING TESTOSTERONE

EXPERIMENTAL GROUP	BODY WEIGHT (g)	PITUITARY	TESTIS	VENTRAL PROSTATE	TESTOSTERONE (μg/100ml PLASMA)
		(mg / 100 GRAMS BODY WEIGHT)			
4 WEEKS OF TREATMENT					
CONTROL	419.0±12.9[a]	3.25±0.22	400.0±15.3	104.6± 8.1	1.80±0.37
MELATONIN	418.8± 9.6	2.90±0.15	357.5±15.6	109.4± 8.6	1.74±0.16
SEROTONIN	403.3±13.3	3.19±0.11	401.8±31.2	95.2± 5.9	1.29±0.20
5-METHOXYTRYPTOPHOL	441.5±11.4	3.43±0.27	382.2±16.9	83.7± 5.8	1.43±0.08
5-HYDROXYTRYPTOPHOL	414.2± 5.9	3.70±0.20	312.8±28.2**	99.7±11.2	1.55±0.15
8 WEEKS OF TREATMENT					
CONTROL	464.2±18.9	2.82±0.11	373.8±13.7	82.0± 4.6	0.40±0.09
MELATONIN	475.0±24.1	3.11±0.14	364.8±23.0	71.5± 5.3	0.41±0.06
SEROTONIN	479.2±17.1	2.68±0.06	349.4±11.0	73.9± 5.1	0.21±0.04
5-METHOXYTRYPTOPHOL	441.7±19.0	3.08±0.18	352.3±17.2	76.9± 6.5	0.32±0.06
5-HYDROXYTRYPTOPHOL	490.8±14.9	2.87±0.08	359.7±11.8	60.3± 3.3÷÷	0.37±0.07
12 WEEKS OF TREATMENT					
CONTROL	531.3±12.7	2.80±0.07	306.6±16.3	79.1± 5.0	1.06±0.18
MELATONIN	496.2±14.5	2.51±0.30	347.9±26.2	48.9± 2.9⊙	0.52±0.10**
SEROTONIN	548.7±19.6	2.24±0.16**	307.5±16.0	66.9± 4.6	0.54±0.09*
5-METHOXYTRYPTOPHOL	490.7± 2.5**	2.98±0.18	327.7±13.4	51.9± 3.6⊙	0.55±0.09*
5-HYDROXYTRYPTOPHOL	527.5±21.3	2.74±0.16	329.1±22.0	59.0± 6.2*	0.56±0.03*

(a- mean±S.E.)

1. Statistical significance using Student's t test as follows:

*- at the 0.05 level, **- at the 0.025 level, ÷- at the 0.01 level
÷÷- at the 0.005 level, ⊙- at the 0.001 level.

which persistently decreased in weight throughout the study. A significant decrease in ventral prostate weight was initially observed at 8 weeks in the 5-hydroxytryptophol-treated animals. However, at 12 weeks, all the indole-treated animals (except the serotonin-treated animals) had ventral prostates which were significantly lighter than the corresponding controls.

Circulating testosterone levels in mixed venous plasma (MVP), as anticipated, paralleled the alterations in ventral prostate weight very closely. A persistent decrease in testosterone as the study progressed, was observed in the indole-treated groups. Finally, at 12 weeks of investigation, circulating testosterone was significantly reduced in the indole-treated groups as compared to the controls.

Testosterone levels (TVP and MVP) interestingly enough, seemed to vary consistently from month to month, in a type of peak and trough relationship. As shown in Tables 1&2, a peak of testosterone was evident at 4 weeks, consequently followed by a fall in levels to a trough position at 8 weeks. At 12 weeks, the testosterone levels began to increase once more.

Effects of Indoles on Testicular Venous Plasma Androgens

In vivo, the effects of indoles (1 μmole/week) on testosterone secretion were apparently very modest. From Table 2, it is evident that indoles prompted a reduction in testosterone levels in TVP throughout the experimental period.

TABLE 2

EFFECT OF INDOLES ON TESTICULAR VENOUS PLASMA ANDROGENS

INTERVAL OF STUDY	EXPERIMENTAL GROUPS	TESTOSTERONE	ANDROSTENEDIONE
		(μg /100 ml TESTICULAR VENOUS PLASMA)	
4 WEEKS	CONTROL	19.14±3.32[a]	0.79±0.15
	MELATONIN	12.31±2.41	2.22±0.61*
	SEROTONIN	12.07±1.66	1.68±0.42
	5-METHOXYTRYPTOPHOL	20.75±2.68	2.63±0.41†
	5-HYDROXYTRYPTOPHOL	11.75±1.50	1.96±0.44*
8 WEEKS	CONTROL	4.82±0.86	1.86±0.50
	MELATONIN	3.98±0.47	1.30±0.59
	SEROTONIN	4.25±0.70	0.75±0.44
	5-METHOXYTRYPTOPHOL	4.98±0.68	0.41±0.24*
	5-HYDROXYTRYPTOPHOL	2.37±0.51*	0.42±0.24*
12 WEEKS	CONTROL	6.34±1.16	0.72±0.10
	MELATONIN	4.29±0.48	0.32±0.15*
	SEROTONIN	4.62±1.06	0.07±0.03θ
	5-METHOXYTRYPTOPHOL	6.95±1.41	0.90±0.49
	5-HYDROXYTRYPTOPHOL	6.51±1.36	0.68±0.33

1. Statistical significance using Student's t test as follows:
   - *- at the 0.05 level
   - **- at the 0.025 level
   - †- at the 0.01 level
   - ††- at the 0.005 level
   - θ- at the 0.001 level

(a - mean± S.E.)

5-methoxytryptophol was at variance with this pattern,for testosterone levels in TVP in the 5-methoxytryptophol-treated animals were slightly elevated at all intervals of investigation. Only,5-hydroxytryptophol prompted a significant reduction in testosterone levels at 8 weeks. The effect did not persist,in fact testosterone levels were slightly elevated at 12 weeks.

In contrast to the modest influence on TVP testosterone, indoles produced marked alterations in androstenedione levels during the course of the investigation. Initially,as shown in Table 2,a significant enhancement of androstenedione was observed in the indole-treated groups. Serotonin elevated androstenedione levels from the controls at 4 weeks,but the difference was insignificant. Investigation at 8 weeks revealed that the the enhancement of androstenedione,initially observed in the indole-treated groups,had disappeared and that the effect of indoles on androstenedione at this interval was of dramatic inhibition. However,only 5-hydroxytryptophol and 5-methoxytryptophol significantly reduced androstenedione levels when compared to the controls. At 12 weeks inhibition of androstenedione was still evident. However,only melatonin and serotonin significantly inhibited androstenedione. The latter indole prompted a severe depression of androstenedione secretion,since androstenedione levels in TVP were reduced to less than 10% of the controls. At this interval,5-hydroxy-

tryptophol and 5-methoxytryptophol had no effect on androstenedione.

Effects of Indoles on Total Testicular Venous Plasma Androgens

The influences of indole treatments on the testicular endocrine function of the adult rat, as revealed by examination of total androgens (testosterone and androstenedione) in TVP is presented in Figures 11-14. The indoles employed in these experiments induced various patterns of alterations in total androgenic status when compared to the controls.

(a) Melatonin Treatment

Initially, melatonin prompted a reduction of the total TVP androgen status, even though androstenedione levels were significantly elevated from the controls. A coincident decrease of testosterone levels observed at this interval maintained a reduced total TVP androgenic status.

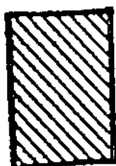
Study at 8 weeks revealed that androstenedione was slightly inhibited by melatonin. Since testosterone levels approached those of the controls, this feature of androstenedione suppression maintained the reduction in total TVP androgens when compared to the controls.

At 12 weeks, androstenedione levels were decreased ($P<0.05$) when compared to the controls. Testosterone levels were reduced, though not significantly. The additive effect of these androgens prompted a reduction in total androgen status.

LEGEND FOR FIGURES 11-14

(1) Control groups are represented by bars on the left at each interval of study

(2) Experimental groups are represented by bars on the right at each interval of study.



- corresponds to the level of androstenedione

- corresponds to the level of testosterone

S - statistical significance at 0.05 level, using Student's t test of the individual androgens.

FIGURE 11. The effects of melatonin treatment on total testicular venous plasma androgens.

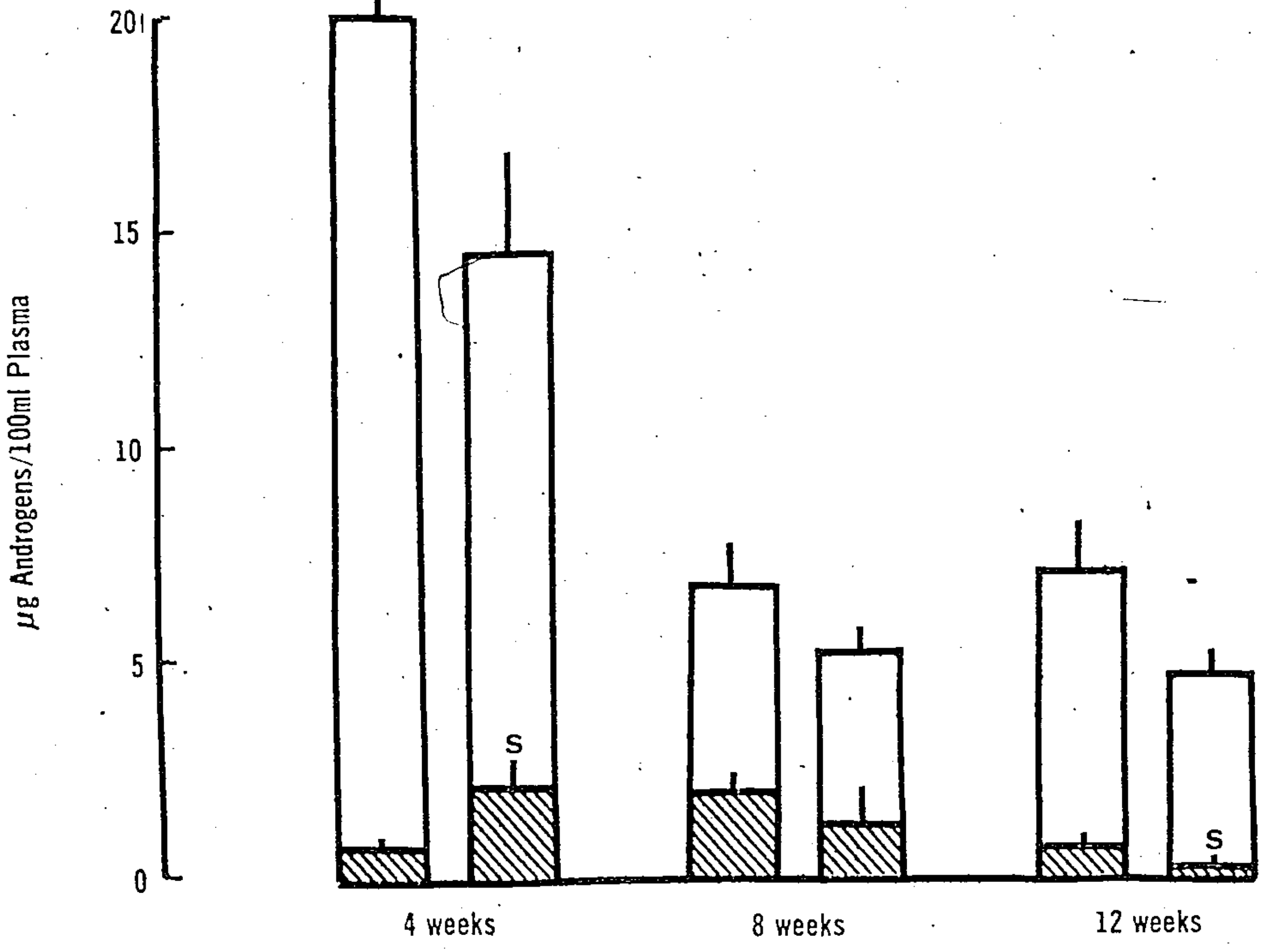
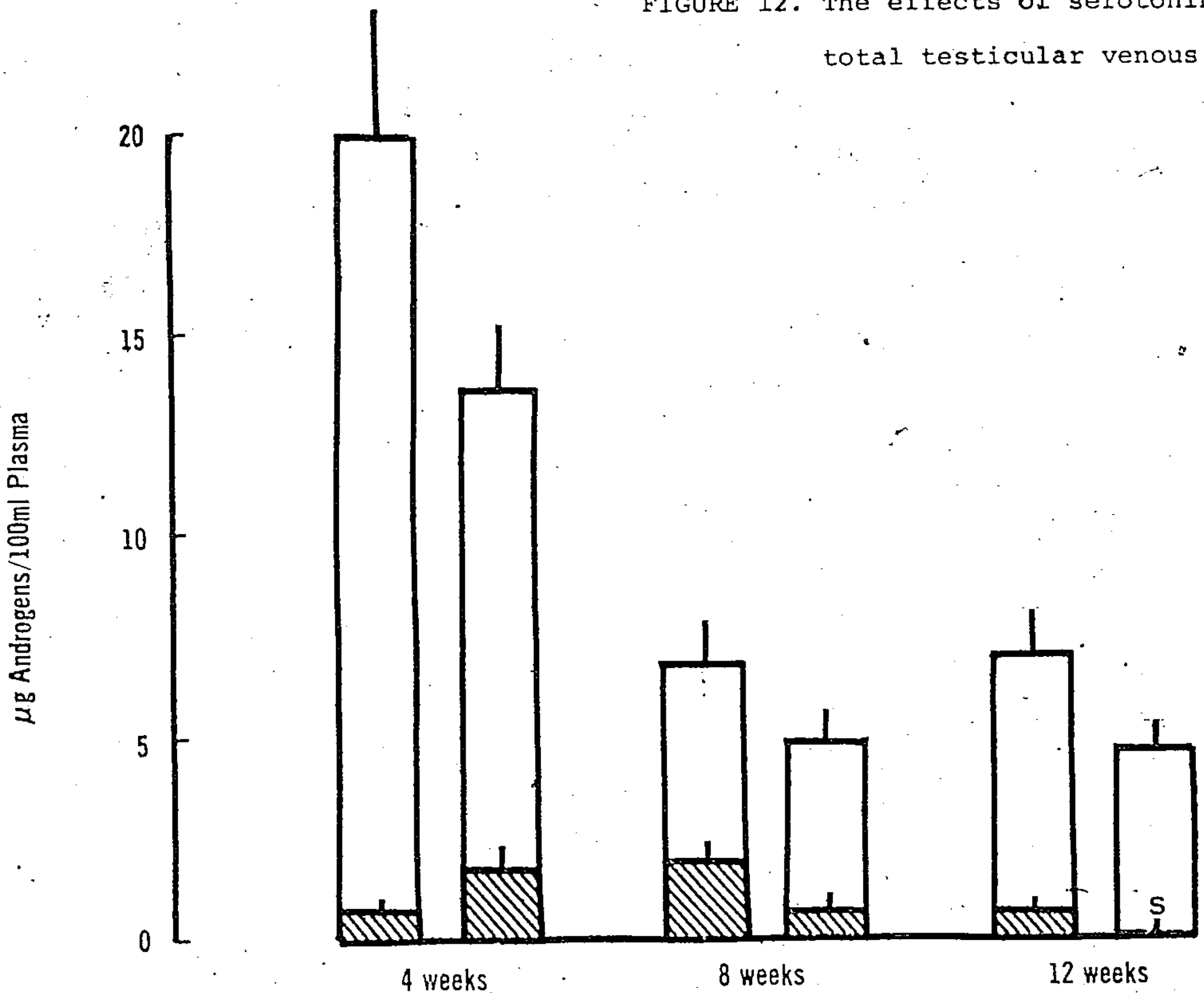


FIGURE 12. The effects of serotonin treatment on total testicular venous plasma androgens.



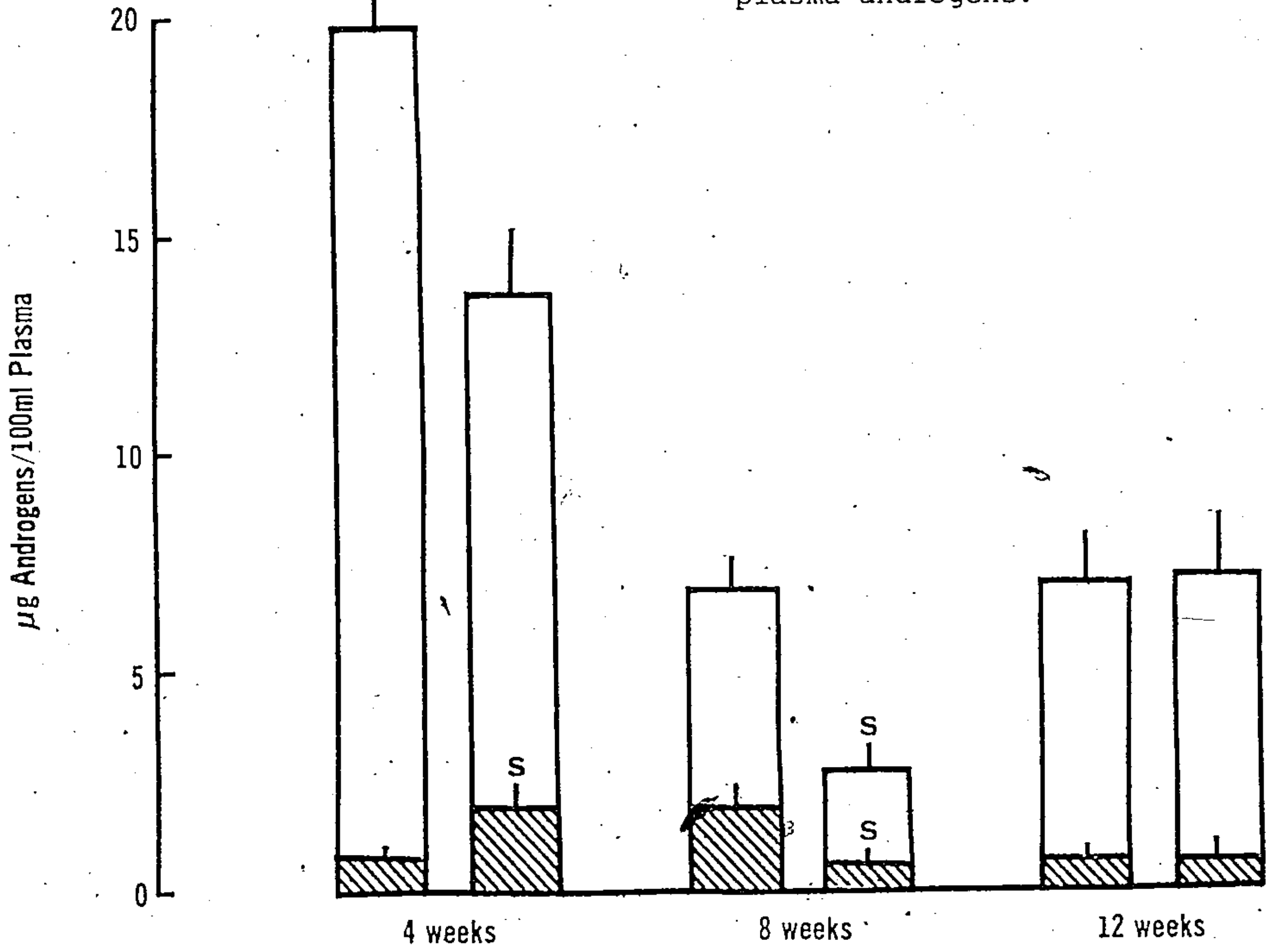


FIGURE 13. The effects of 5-hydroxytryptophol treatment on total testicular venous plasma androgens.

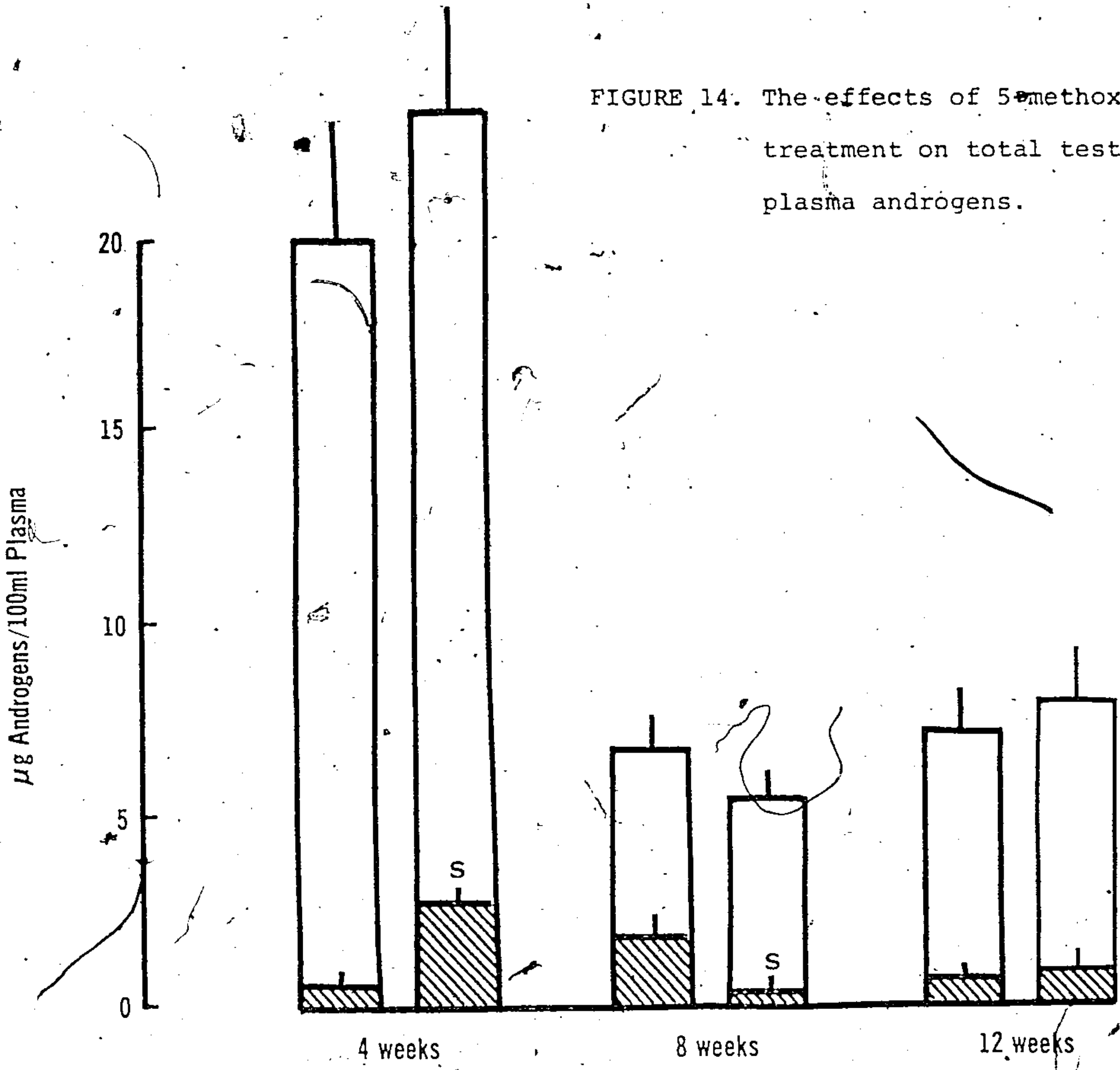


FIGURE 14. The effects of 5-methoxytryptophol treatment on total testicular venous plasma androgens.

(b) Serotonin Treatment

Serotonin administration was observed to produce a pattern of influences on total TVP androgens similar to the pattern revealed by melatonin treatment. Nevertheless, androstenedione levels were not significantly altered from the controls at 4 weeks of study.

(c) 5-Hydroxytryptophol Treatment

5-Hydroxytryptophol significantly enhanced androstenedione during the initial interval of study. A coincident insignificant decrease in testosterone levels maintained a reduced total TVP androgen status.

Study at 8 weeks revealed a marked inhibition of TVP androgens. Testosterone and androstenedione levels were significantly depressed when compared to the controls. Total TVP androgens as a result were reduced from the controls.

The dramatic inhibition of TVP androgens was not apparent at 12 weeks. Testosterone was slightly elevated and androstenedione was slightly reduced. In this fashion, total TVP androgens were slightly reduced from the controls.

(d) 5-Methoxytryptophol Treatment

At 4 weeks, total TVP androgens were elevated purely as a result of a significant enhancement of androstenedione.

At 8 weeks androstenedione levels were significantly depressed when compared to the controls. Therefore, total TVP androgens were reduced from the controls.

Study at 12 weeks revealed an enhancement of androstenedione and testosterone that was not significantly different from the controls. As a result,total TVP androgens were elevated over the controls.

Effects of Indoles on Spermatogenesis

The data concerning the effects of indoles on germ cell numbers is presented in Table 3.

Initially,indoles induced a significant increase in the number of type B spermatogonia when compared to the controls.

At 8 weeks,the numbers of type B spermatogonia was elevated,though not significantly. Indoles apparently reduced the number of resting spermatocytes,although statistical significance was revealed only by 5-methoxytryptophol. The numbers of pachytene spermatocytes were influenced by indoles. However,only 5-hydroxytryptophol and 5-methoxytryptophol were able to induce a significant reduction in the number of pachytene spermatocytes. Indoles affected spermatids in such a manner as to induce a significant reduction in numbers.

Examination at 12 weeks showed that the number of type B spermatogonia remained slightly elevated,except in the melatonin-treated group where a small reduction in numbers was observed. A reduction in the number of resting spermatocytes was evident,though only 5-hydroxytryptophol and 5-methoxytryptophol significantly reduced the numbers

TABLE 3

EVALUATION OF SPERMATOGENESIS :

A. EFFECT OF INDOLES ON GERM CELL NUMBERS[(1)]

INTERVAL OF STUDY	EXPERIMENTAL GROUPS	B-TYPE SPERMATAGONIA	RESTING SPERMATOCYTES	PACHYTENE SPERMATOCYTES	SPERMATIDS
4 WEEKS	CONTROL	4.27±0.18[a]	8.54±0.71	7.89±0.25	20.61±0.96
	MELATONIN	5.32±0.14††	8.80±0.35	8.10±0.34	21.19±0.75
	SEROTONIN	5.32±0.27†	8.59±0.43	7.84±0.28	21.50±1.13
	5-METHOXYTRYPTOPHOL	5.98±0.17⊙	9.62±0.38	8.26±0.33	19.93±0.46
	5-HYDROXYTRYPTOPHOL	6.05±0.26⊙	8.85±0.55	7.85±0.53	20.37±1.97
8 WEEKS	CONTROL	4.36±0.37	9.81±0.36	9.02±0.31	25.95±0.45
	MELATONIN	4.40±0.31	9.09±0.55	7.94±0.48†	19.58±1.35††
	SEROTONIN	5.25±0.26	8.83±0.36	7.68±0.22	20.92±0.97††
	5-METHOXYTRYPTOPHOL	4.41±0.23	8.60±0.34*	7.26±0.54*	19.03±0.82⊙
	5-HYDROXYTRYPTOPHOL	4.49±0.35	9.25±0.43	8.53±0.55	20.70±1.17††
12 WEEKS	CONTROL	4.82±0.40	8.63±0.44	7.84±0.26	18.54±0.91
	MELATONIN	4.65±0.20	8.20±0.54	8.57±0.82	19.55±0.65
	SEROTONIN	4.83±0.15	8.88±0.09	7.92±0.21	18.81±0.80
	5-METHOXYTRYPTOPHOL	5.15±0.36	6.95±0.42**	7.30±0.49	17.54±0.87
	5-HYDROXYTRYPTOPHOL	5.20±0.30	7.35±0.22*	7.72±0.38	17.22±0.50

(a - mean±S.E.)

1. Germ cell numbers corrected for thickness of section only.

2. Statistical significance using Student's t test as follows:
   *- at the 0.05 level, **- at the 0.025 level
   †- at the 0.01 level, ††- at the 0.005 level
   ⊙- at the 0.001 level

from the controls. The numbers of pachytene spermatocytes and spermatids were not significantly altered by indoles at this interval of study. All pineal indoles induced a slight reduction in the numbers of these germ cells,except melatonin and serotonin which induced a small elevation of the numbers of these germ cells.

The influence of indoles upon spermatogenesis,as evaluated by germ cell ratios is shown in Table 4.

Initially,germ cell ratios were not altered by indole treatment,except 5-hydroxytryptophol which reduced the resting spermatocyte/type B spermatogonia ratio ($P < 0.05$).

Evaluation of spermatogenesis at 8 weeks demonstrated that indoles reduced the ratio of resting spermatocytes/type B spermatogonia,though only serotonin significantly reduced this ratio when compared to the controls. Indoles affected the resting spermatocyte/pachytene spermatocyte ratio by a small increase in magnitude. However, 5-hydroxytryptophol apparently decreased this ratio to a slight extent. All indoles reduced the ratio of spermatids/pachytene spermatocytes, although only melatonin and 5-hydroxytryptophol depressed this ratio significantly.

Upon investigation of the in vivo effects of indole treatment on spermatogenesis at 12 weeks,a decrease in the ratio of resting spermatocytes/type B spermatogonia was observed. Only 5-methoxytryptophol and 5-hydroxytryptophol significantly

TABLE 4

EVALUATION OF SPERMATOGENESIS :

B. EFFECT OF INDOLES ON GERM CELL RATIOS[1]

INTERVAL OF STUDY	EXPERIMENTAL GROUPS	RESTING/ TYPE B (SPERMATOCYTES/ SPERMATOGONIA)	RESTING/PACHYTENE (SPERMATOCYTES)	SPERMATID/ PACHYTENE / SPERMATOCYTES
4 WEEKS	CONTROL	2.03±0.21[a]	1.04±0.08	2.62±0.13
	MELATONIN	1.65±0.04	1.09±0.04	2.65±0.42
	SEROTONIN	1.62±0.05	1.09±0.03	2.75±0.14
	5-METHOXYTRYPTOPHOL	1.61±0.08	1.17±0.03	2.43±0.09
	5-HYDROXYTRYPTOPHOL	1.48±0.11*	1.14±0.07	2.57±0.13
8 WEEKS	CONTROL	2.31±0.19	1.09±0.02	2.89±0.09
	MELATONIN	2.10±0.17	1.15±0.06	2.47±0.09÷
	SEROTONIN	1.70±0.10*	1.15±0.05	2.73±0.01
	5-METHOXYTRYPTOPHOL	1.99±0.16	1.21±0.06	2.68±0.21
	5-HYDROXYTRYPTOPHOL	1.85±0.15	1.07±0.04	2.44±0.06÷÷
12 WEEKS	CONTROL	1.84±0.14	1.11±0.03	2.37±0.10
	MELATONIN	1.78±0.15	1.38±0.26	2.33±0.13
	SEROTONIN	1.84±0.05	1.13±0.03	2.37±0.09
	5-METHOXYTRYPTOPHOL	1.37±0.10*	0.96±0.03÷	2.43±0.13
	5-HYDROXYTRYPTOPHOL	1.43±0.08*	0.96±0.04**	2.24±0.06

(a - mean±S.E.)

1. Germ cell numbers corrected for thickness of section and Sertoli cell number.

2. Statistical significance using Student's t test as follows:
   *- at the 0.05 level , **- at the 0.025 level
   ÷- at the 0.01 level , ÷÷- at the 0.005 level
   ∋- at the 0.001 level

reduced the magnitude of this ratio when compared to the controls. All indoles except melatonin reduced the ratio of resting spermatocytes/pachytene spermatocytes, though 5-methoxytryptophol and 5-hydroxytryptophol significantly altered the ratio from the controls. No significant effect upon the ratio of spermatids/pachytene spermatocytes was observed at 12 weeks, though all indoles except 5-methoxytryptophol induced a slight reduction in magnitude.

(B) IN VITRO EXPERIMENTATION

The preparation of testicular homogenates involved the pooling of testes (weight±S.E, 1420±25 mg,range 1215 to 1851 mg) from mature animals whose body weight ranged from 296 g to 337 g and averaged 317.9±3.6 g.

From the results shown in Table 5 and Figures 15&16,it is evident that the in vitro biotransformation of pregneneolone into androstenedione and testosterone was inhibited by pineal-derived indoles. Melatonin is a more active inhibitor of androgen synthesis than other indoles. In this respect the addition of 18 μmoles(3.6 m molar) of serotonin significantly diminished testosterone formation; addition of 900n moles(180μmolar) of 5-methoxytryptophol or 5-hydroxytryptophol significantly diminished testosterone formation while the addition of 450 ηmoles(90μmolar) of melatonin significantly depressed both testosterone and androstenedione formation.

Melatonin affected the in vitro biotransformation of pregneneolone into androgens by consistently diminishing testosterone formation relative to androstenedione formation. However, after addition of 18 μmoles(3.6m molar) of melatonin inhibition was so severe that both androstenedione and testosterone were reduced to less than 5% of the controls.

The influence of serotonin upon androgen synthesis in vitro was quite different to that revealed by melatonin. Addition of 18μ moles(3.6mmolar) of serotonin to the

TABLE 5

DIRECT EFFECTS OF INDOLES ON CONVERSION

OF 5-PREGNENEOLONE-7α-H^3 TO TESTOSTERONE AND ANDROSTENEDIONE

DOSE LEVEL OF INDOLE	EXPERIMENTAL GROUPS		% FORMATION OF TESTOSTERONE		% FORMATION OF ANDROSTENEDIONE	TOTAL CONVERSION
	CONTROL	(5)	25.2±1.2 a	(5)	46.3±1.7	71.5±2.4
90 n moles	MELATONIN	(6)	22.3±1.4	(6)	51.3±2.5	73.7±3.3
	SEROTONIN	(6)	26.0±1.8	(6)	42.8±1.3	68.8±1.8
	5-METHOXYTRYPTOPHOL	(6)	25.4±1.4	(6)	44.6±1.4	70.0±0.8
	5-HYDROXYTRYPTOPHOL	(6)	24.9±1.6	(6)	47.7±2.3	72.5±3.3
	CONTROL	(6)	23.3±0.6	(5)	42.6±1.7	65.5±1.7
450 n moles	MELATONIN	(6)	13.7±0.4****	(6)	29.0±1.5****	42.7±1.6****
	SEROTONIN	(5)	27.2±0.1****	(5)	48.0±2.1	75.2±2.0***
	5-METHOXYTRYPTOPHOL	(6)	23.7±0.5	(6)	46.8±0.5*	70.5±0.6*
	5-HYDROXYTRYPTOPHOL	(5)	25.3±0.7	(5)	45.9±1.0	71.3±1.0*
	CONTROL	(6)	24.8±0.6	(6)	42.2±1.3	66.9±1.8
900 n moles	MELATONIN	(6)	8.5±0.3****	(6)	17.7±0.5****	26.2±0.5****
	SEROTONIN	(6)	23.8±0.4	(6)	44.8±0.9	68.7±1.1
	5-METHOXYTRYPTOPHOL	(6)	21.8±0.5***	(6)	42.7±1.1	64.5±1.4
	5-HYDROXYTRYPTOPHOL	(6)	23.2±0.3*	(6)	39.0±3.9	62.1±4.0
	CONTROL	(6)	22.3±0.6	(6)	37.7±1.1	60.0±1.4
18 μ moles	MELATONIN	(5)	1.2±0.3****	(6)	1.8±0.3****	2.9±0.4****
	SEROTONIN	(4)	9.8±0.9****	(5)	41.1±2.3	51.2±3.9
	5-METHOXYTRYPTOPHOL	(6)	8.2±0.9****	(6)	16.1±0.7****	24.3±1.2****
	5-HYDROXYTRYPTOPHOL	(5)	11.5±0.5****	(6)	35.8±2.2	48.9±1.8****

1. Numbers in parenthese refer to number of replicates (a - mean±S.E.)
2. Statistical significance using Student's t test as follows:

*- at the 0.05 level
**- at the 0.01 level
***- at the 0.005 level
****- at the 0.001 level

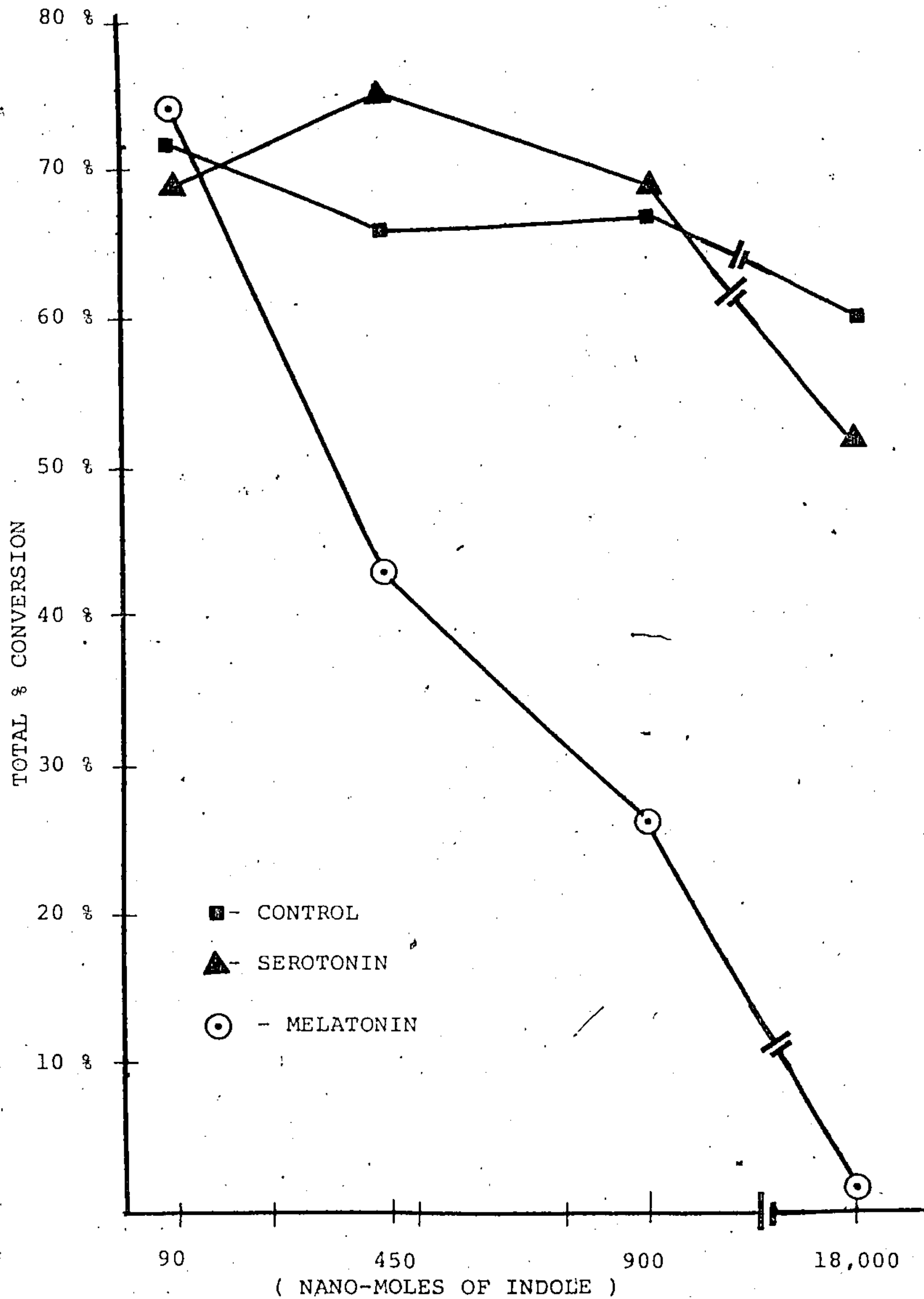


FIGURE 5. EFFECTS OF MELATONIN AND SEROTONIN ON TOTAL CONVERSION OF 5-PREGNENEOLONE-H^3 TO ANDROGENS.

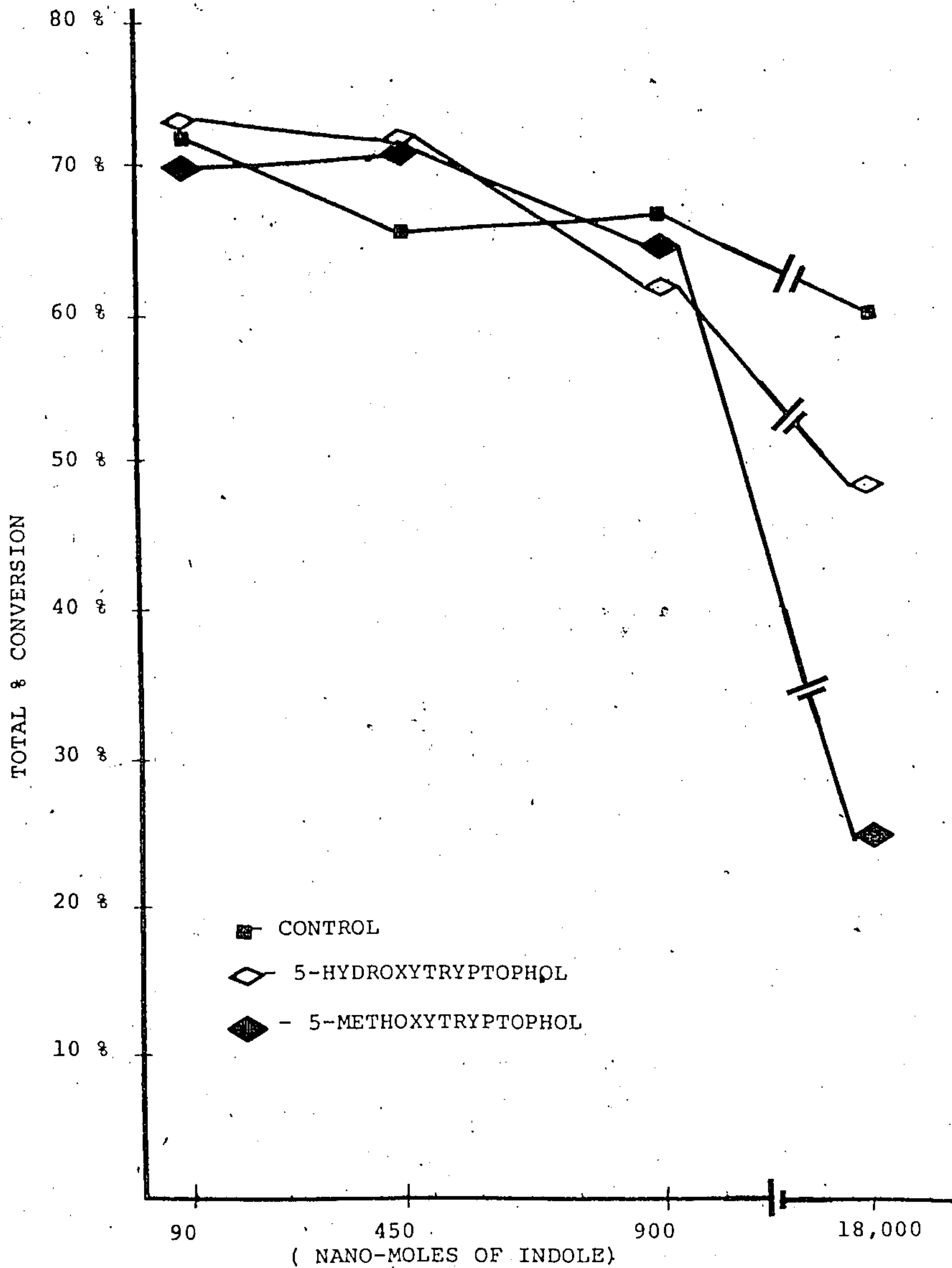


FIGURE 16 EFFECTS OF 5-METHOXYTRYPTOPHOL AND 5-HYDROXYTRYPTOPHOL ON TOTAL CONVERSION OF 5-PREGNENEOLONE-H^3 TO ANDROGENS.

mixture significantly depressed testosterone formation, but androstenedione formation was not different from the controls. Although testosterone formation was diminished relative to androstenedione formation, the fact that androstenedione formation was not affected by serotonin revealed a pattern of influence which differed markedly from the melatonin pattern.

Inhibition of androgen synthesis in vitro by 5-methoxytryptophol was demonstrated after the addition of 900n moles (180μ molar) of 5-methoxytryptophol to the incubation mixtures. At this dose level, only testosterone was significantly reduced from the controls. At the ultimate dose level of 5-methoxytryptophol tested, a pattern of influences on androgen synthesis quite similar to that revealed by melatonin, was observed. 5-Methoxytryptophol apparently diminished testosterone formation relative to androstenedione formation. Both androgens were significantly reduced from the controls but the magnitude of inhibition revealed by 5-methoxytryptophol was less than one-tenth of that observed by melatonin.

The influence of 5-hydroxytryptophol on the in vitro biotransformation of pregneneolone into androgens approximated the influence revealed by serotonin quite closely. 5-Hydroxytryptophol significantly diminished testosterone relative to androstenedione upon addition of either 900n moles (180μ molar) or 18μ moles (3.6m molar) of 5-hydroxytryptophol to the

incubation mixtures. Androstenedione was not altered from the controls. Apparently 5-hydroxytryptophol,like serotonin, did not influence androstenedione formation.

From the data shown in Table 6 ,it is evident that each pineal-derived indole induced a specific pattern of influences on the 17β-hydroxysteroid dehydrogenase enzyme system which catalyzes the interconversion of androstenedione and testosterone. According to Ellis (1972) , this enzyme system is composed of two components; a 17-keto reductase which hastens the conversion of androstenedione to testosterone, and a 17β-hydroxysteroid dehydrogenase which catalyzes the conversion of testosterone to androstenedione.

Addition of 18μmoles(3.6 m molar) of indoles to the incubation mixtures induced a significant alteration in the activity of the 17β-hydroxysteroid dehydrogenase system when compared to the controls.

Melatonin significantly depressed the activity of the 17-keto reductase and apparently did not influence the activity of the 17β-hydroxysteroid dehydrogenase. Serotonin did not alter the activity of the former enzyme but significantly depressed the activity of the latter enzyme. 5-Methoxytryptophol did not affect the 17-keto reductase but enhanced ($P<0.001$) the activity of the 17β-hydroxysteroid dehydrogenase. 5-Hydroxytryptophol significantly inhibited the former and significantly enhanced the latter enzyme activities.

TABLE 6

EFFECT OF INDOLES ON THE INTERCONVERSION OF TESTOSTERONE AND ANDROSTENEDIONE

DOSE LEVEL OF INDOLE	EXPERIMENTAL GROUPS	% CONVERSION OF ANDROSTENEDIONE TO TESTOSTERONE (1)		% CONVERSION OF TESTOSTERONE TO ANDROSTENEDIONE (2)	
	CONTROL	(5)	32.3±0.4 [a]	(6)	72.4±0.7
900 n moles	MELATONIN	(6)	33.9±1.4	(5)	73.6±0.6
	SEROTONIN	(5)	29.2±1.6	(6)	72.9±0.9
	5-METHOXYTRYPTOPHOL	(6)	33.4±1.4	(6)	74.7±0.9
	5-HYDROXYTRYPTOPHOL	(6)	33.9±0.9	(4)	73.8±1.1
	CONTROL	(3)	42.3±0.6	(6)	68.0±0.7
18μ moles	MELATONIN	(4)	34.4±1.0****	(5)	69.4±0.9
	SEROTONIN	(4)	42.1±1.9	(6)	65.1±0.6**
	5-METHOXYTRYPTOPHOL	(4)	36.3±2.8	(6)	74.2±1.1*****
	5-HYDROXYTRYPTOPHOL	(4)	27.6±0.9*****	(5)	74.0±0.6*****

(a - mean±S.E.)

1. Substrate utilized was androstenedione-4-C^{14} (24,100 DPM, 15.6 mμmoles)
2. Substrate utilized was testosterone-4-C^{14} (24,977 DPM, 18.2 mμmoles)
3. Numbers in parentheses refer to the number of replicates.
4. Statistical significance using Student's t test as follows:
   - *- at the 0.05 level
   - **- at the 0.025 level
   - ***- at the 0.01 level
   - ****- at the 0.005 level
   - *****- at the 0.001 level

CHAPTER V

DISCUSSION

(A) IN VIVO EXPERIMENTATION

The data of this investigation show that the in vivo administration of indoles (1 μmole/week) had only modest effects on the testicular functions of the adult rat.

At 12 weeks, body weight in the 5-methoxytryptophol-treated animals was significantly reduced below the controls. Sorrentino et al (1971) observed that melatonin implantation in immature rats induced no alteration in growth. Collu and Fraschini (1972) reviewed the literature on this aspect of pineal influence and reported that the pineal does not seem to exert an effect on growth. From these reports, it is evident that the reduction in body weight observed in the 5-methoxytryptophol-treated animals was presumably not an effect due to indole treatment, but as a consequence of reduced food intake by these animals.

Pituitary weight was significantly reduced by serotonin treatment at 12 weeks of investigation. Fraschini et al (1970) reported that serotonin regulates FSH synthesis in the anterior pituitary. Reduction in the weight of the pituitary could occur as a result of less FSH in the pituitary.

Testis weight was significantly reduced by 5-hydroxytryptophol at 4 weeks of study. Nonetheless, the effect was transitory since testis weight was not significantly altered

during the remainder of the study. A reduction in testis weight suggests that the tubular function was affected since the bulk of testis tissue is comprised of seminiferous tubules. Interstitium occupies a minor proportion of testicular tissue. Hence,changes in the size of the latter would be slight in terms of the whole weight of the gland. Since 5-hydroxytrytophol presumably regulates LH(Fraschini et al, 1968),this indole would interfere with the endocrine function of the testis. However,the reduction of testis weight by 5-hydroxytrptophol at 4 weeks is not consistent with an interaction with the endocrine function of the testis. No logical explanation of this effect can be provided.

From the results,it is evident that ventral prostate weight was reduced throughout the study. At 8 weeks,5-hydroxytryptophol significantly reduced ventral prostate weight when compared to the controls,and at 12 weeks all indoles (except serotonin) significantly reduced ventral prostate weight. Ventral prostate weight closely paralleled the levels of circulating testosterone. Circulating testosterone was reduced throughout the study as a consequence of indole treatments. At 12 weeks,all the indoles significantly reduced circulating testosterone levels when compared to the controls. TVP testosterone was hardly affected by indole treatments during the investigation. Changes in circulating testosterone in the absence of changes in its secretion,suggest effects

on the metabolic (mainly hepatic) clearance of the hormone, Such actions for melatonin and serotonin have been reported upon (Kinson et al, 1973).

Examination of testosterone levels (TVP&MVP) throughout the study revealed that the base-line levels fluctuated from month to month. These findings are to be expected in view of reports attributing inherent testicular rhythms to the male laboratory rat. Gunn and Gould(1958) reported evidence for the existence of such rhythms. They reported that the uptake of radio-labelled zinc by the dorso-lateral prostate during the span of 1 year showed two distinct maxima; in February and in June. Kinson and Liu(1973b) examined testosterone levels (MVP&TVP) and discovered a peak of testosterone secretion in late May, accompanied by similar changes in the circulating levels of the hormone. A peak of testosterone would presumably precede any response of the androgen-dependent dorso-lateral prostate. These reports demonstrate that an inherent testicular rhythm exists in the male laboratory rat, and that testosterone levels are dependent on the status of this rhythm.

The data of this investigation suggest that indoles (1 μmole/week) had little influence on testosterone secretion. Although TVP testosterone was reduced during the study as a consequence of indole treatments (except 5-methoxytryptophol), only 5-hydroxytryptophol at 8 weeks, significantly depressed

TVP testosterone when compared to the controls. Liu and Kinson (1973), using doses that were five fold greater than those of this study, also reported insignificant influences on testosterone secretion in response to melatonin and serotonin administration. Only melatonin significantly reduced TVP testosterone at 4 weeks of investigation.

Indoles apparently influenced androstenedione secretion to a greater extent. At 4 weeks, indole treatments (except serotonin) significantly enhanced TVP androstenedione levels. At 8 weeks, indoles reduced androstenedione, though only 5-hydroxytryptophol and 5-methoxytryptophol significantly reduced androstenedione below the corresponding controls. At 12 weeks, only melatonin and serotonin significantly depressed androstenedione secretion.

Investigation at 4 weeks revealed that pineal indoles influenced TVP androgens in a differential manner ie; androstenedione was elevated relative to testosterone. A mechanism involving inhibition of gonadotrophins did not appear to be involved since inhibition of LH would presumably depress both testosterone and androstenedione secretion and synthesis below the controls. Indoles (except serotonin) induced a significant enhancement of androstenedione at this interval. This feature could not be explained by means of a mechanism involving LH inhibition. It is quite conceivable that indoles gave rise to a differential influence on TVP androgens by

a mechanism involving direct testicular interaction of androgen synthesis. Inhibition of the terminal step of androgenesis ie; the conversion of androstenedione to testosterone, could explain this phenomenom.

Both testosterone and androstenedione levels were depressed when compared to the controls, during the remainder of the study. Since gonadotrophins were not measured, it is impossible to attribute inhibition of TVP androgen secretion by indoles to gonadotrophin inhibition or to a direct testicular effect. Probably both were involved.

Indole treatments (1 μmole/week) apparently had little effect on the gametogenic function of the testis.

The numbers of type B spermatogonia were increased during the study, although significant alterations in these germ cell numbers were only expressed initially. An increase in type B spermatogonia could suggest a decrease in the frequency of mitotic events. However, the number of resting and pachytene spermatocytes should have reflected this trend. Some reduction in the numbers of these germ cells was observed but a consistent pattern was not revealed. At 8 weeks, the number of spermatids was significantly reduced from the controls. Steinberger and Steinberger (1973) reported that teatosterone is necessary for meiosis and up to stage 15 of spermiogenesis. Therefore, a reduction in stage 7 spermatids could reflect a decreased endocrine status. However, upon

closer examination, a definite correlation between testosterone and spermatid numbers is not warranted by the results of this investigation.

Examination of the effects of indoles on germ cell ratios are merely an extension of the germ cell number results. Generally, a significant decrease of a germ cell ratio was attributed to a decrease in the number of one cell type over an increase in another cell type. No detectable trends can be gathered from this data. Evaluation of these results suggests that indoles at this dose level produced no marked changes in the gametogenic function of the testis.

Liu and Kinson (1973), after implantation of pellets containing 10 mg serotonin or melatonin respectively, discovered that serotonin dramatically impaired spermatogenesis. At 12 weeks, examination revealed that over 85% of the tubules were damaged by serotonin treatment. Melatonin had no effect on gametogenesis. Since the dose level of indoles employed by these investigators was at least five fold greater than presently utilized, it is evident the effective dose was not approached in the present study.

The weekly administration of 1μmole of these indoles did not cause profound changes in the various parameters of male reproductive function of the adult rat, in the present study. However, strict interpretation of the results is difficult. The normal physiological levels of pineal indoles

in the plasma are currently unknown. These compounds are obviously present in minute amounts, since the methodology required for their routine estimation is, as yet, forthcoming.

The pharmacological potential of pineal indole derivatives have been recently demonstrated . Boris, DeMartino and Trmal (1974) reported that a single oral dose (150 mg/Kg) of pipecolinmethylhydroxyindane resulted in subnormal fertility during the 3rd and 4th week after administration. Sterility ensued from the 5th week and persisted to the termination of the experiment; 20 weeks after dosing. This compound possesses an indane ring system, upon which the structure of pineal indoles is based. These workers discovered that only testis weight was altered, no effects on the accessory sex glands were evident. They assumed that the endocrine function of the testis was not affected since accessory sex gland weight was not altered. However, the estimation of androgens in TVP and MVP was not attempted.

Further studies on the potential of this compound (Boris, Ng and Hurley, 1974) revealed that pipecolinmethylhydroxyindane was more effective in depressing testis weight and interfering with spermatogenesis than other anti-fertility drugs. Also, this compound reduced accessory sex gland weight to a lesser extent than other anti-fertility drugs. These findings suggest that this indole derivative is very effective in suppressing the gametogenic function of the testis, but the

data concerning the maintenance of the endocrine function of the testis is not conclusive. Only upon further examination will the influence of pipecolinmethylhydroxyindane on androgen secretion and metabolic clearance be clarified. If the endocrine function of the testis is not altered by this compound, then effective male fertility control may be viable, provided that toxic effects on other body organs are not present.

(B) IN VITRO EXPERIMENTATION

In the rat, the conversion of pregneneolone into testosterone is assumed to predominate via the progesterone or delta-4 pathway of androgenesis (Slaunwhite and Burgett,1965). The data of this present investigation show that the *in vitro* biotransformation of pregneneolone into testosterone is inhibited by the addition of pineal indoles to the incubation mixtures. This finding is in agreement with the results from this (Peat and Kinson,1971) and from other laboratories (Ellis, 1972).

Many of the specific features regarding the inhibiton of androgen biosynthesis by indoles revealed in this study, are different or in some cases, in opposition to findings reported elsewhere. Discrepancies are expected since incubation and steroid isolation techniques vary from study to study. Ellis (1972),using Holtzman rats of 12 weeks of age, incubated testicular homogenates corresponding to different weights of testicular tissue with progesterone at 37.5°C. This investigator made no correction for procedural losses in his experiments. In the present study, testicular homogenates corresponding to 0.25 g testicular tissue from 9 week old Sprague-Dawley rats were incubated with pregneneolone at 32°C. A correction for procedural losses utilized double isotope techniques (see methods).

Ellis (1972) confirmed the fact that melatonin is a

more active inhibitor of androgen biosynthesis in vitro than serotonin. Serotonin inhibited testosterone production at the 4,050 ηmolar concentration while melatonin inhibited testosterone and androstenedione formation at the 100 ηmolar concentration. The results of the present investigation revealed that serotonin inhibited testosterone production at the 3.6 m molar concentration while a 90 μmolar concentration of melatonin inhibited the production of both androgens. The differences in sensitivity towards melatonin and serotonin may be attributed to the variation in experimental protocol.

Ellis (1972) suggested a duality of effects when indoles were added to the incubation mixtures. He reported that melatonin diminished androstenedione production relative to testosterone production and that serotonin diminished testosterone formation relative to androstenedione formation. The results of the present study demonstrate that all indoles consistently diminished testosterone production relative to androstenedione. Nevertheless, a dual nature of influences on androgen synthesis in vitro is suggested. Melatonin and 5-methoxytryptophol significantly depressed both testosterone and androstenedione while serotonin and 5-hydroxytryptophol significantly depressed testosterone but did not alter androstenedione below the controls. Therefore, although a dual nature of effects of indoles to androgen biosynthesis is implied in this study, it is radically different from the concept proposed by Ellis (1972).

Examination of the terminal step of androgenesis ie; the interconversion of androstenedione and testosterone, revealed that each indolic compound affected the 17β-hydroxysteroid dehydrogenase system in a varied and specific manner.

Addition of 18 μmoles (3.6 m molar) of melatonin to the incubation mixture inhibited the activity of the 17-keto reductase but the activity of the 17β-hydroxysteroid dehydrogenase was not affected. Thus, while the conversion of androstenedione to testosterone was inhibited, the conversion of testosterone to androstenedione was not influenced. Peat and Kinson (1971) demonstrated that upon addition of a 9 m molar solution of melatonin to the incubation mixture, the conversion of androstenedione to testosterone was inhibited. However, these investigators also showed that the conversion of testosterone to androstenedione was enhanced. Presumably the effect of melatonin is first expressed upon the conversion of androstenedione to testosterone, and at higher doses the conversion of testosterone to androstenedione is influenced. Ellis (1972) demonstrated that melatonin (2 μmolar) did not influence the conversion of androstenedione to testosterone, but that melatonin (4 μmolar) inhibited the conversion of testosterone to androstenedione. Peat and Kinson (1971) showed that melatonin at a concentration of 90 μmolar did not influence the interconversion of androstenedione and testosterone. In the present

investigation a concentration of 180 μmolar melatonin failed to affect the interconversion of androstenedione and testosterone.

Serotonin (3.6 m molar) in this study did not influence the activity of the 17-keto reductase but the activity of the 17β-hydroxysteroid dehydrogenase was inhibited ($P < .025$). Thus, serotonin inhibited the conversion of testosterone to androstenedione but had no effect on the conversion of androstenedione to testosterone. Ellis (1972) demonstrated that serotonin (1 m molar) inhibited the conversion of androstenedione to testosterone, while the conversion of testosterone to androstenedione was significantly enhanced.

5-Methoxytryptophol (3.6 m molar) did not influence the activity of the 17-keto reductase but significantly enhanced the activity of the 17β-hydroxysteroid dehydrogenase. Thus, 5-methoxytryptophol enhanced the conversion of testosterone to androstenedione ($P < .001$), but had no effect on the conversion of androstenedione to testosterone. 5-Hydroxytryptophol (3.6 m molar) significantly inhibited the activity of the 17-keto reductase and significantly increased the activity of the latter enzyme. Thus the conversion of androstenedione to testosterone was inhibited and the conversion of testosterone to androstenedione was enhanced.

The magnitude of alterations in the activity of the 17β-hydroxysteroid dehydrogenase system was not great. Since

inhibition of testosterone formation was severe, inhibition cannot be explained solely as a result of indole interaction at this level. The results of this study suggest that inhibition of intermediates prior to androstenedione formation are involved. Ellis (1972) reported that upon incubation with progesterone, the formation of 17α-hydroxyprogesterone, androstenedione and testosterone was inhibited. He reported that inhibition of the activity of the 17α-hydroxylase and the 17α-hydroxypregnene-C_{17}-C_{20}lyase was involved. The results of this investigation suggest that the activities of these enzymes and the activities of the 3ß-hydroxysteroid dehydrogenase and the Δ^5-hydroxysteroid isomerase are inhibited by indoles since incubations utilized pregneneolone.

The data of this study suggest that methoxy-indoles influence the biotransformation of pregneneolone into testosterone *in vitro* in a similar fashion ie; both inhibit the formation of androstenedione and testosterone. Hydroxyindoles apparently influence *in vitro* androgen synthesis to a similar extent; both inhibit testosterone formation and do not affect androstenedione formation. At the testicular level, results of this nature were anticipated since indoles presumably affect androgen biosynthesis through a non-competitive type of enzyme inhibition (Ellis,1972). Lehninger (1970) reported that non-competitive inhibition involved the binding of an inhibitor at a locus other than the substrate binding

site. These findings suggest that inhibitors with similar chemical structures would exert similar effects. Melatonin and 5-methoxytryptophol possess,common to both compounds, a methoxy group at the 5 position of the indane ring system (Fig.3). Since both indoles induce similar effects on *in vitro* androgen synthesis it is implied that inhibition is induced after the 5 position of the indole binds to an enzyme site. In this fashion, serotonin and 5-hydroxytryptophol induce similar patterns of influence on the *in vitro* biotransformation of pregneneolone into androgens.

At the pituitary level of interaction, the effect of indoles upon gonadotrophins is markedly different. Fraschini *et al* (1970) reported that melatonin and 5-hydroxytryptophol regulate LH synthesis while serotonin and 5-methoxytryptophol regulate FSH synthesis. These findings suggest that indoles at this level either interact at specific sites receptive only to an individual indolic compound or that enzyme inhibition is not involved in the regulation of gonadotrophins.

The physiological significance of direct testicular interaction by indoles is not clear. Wurtman *et al* (1964b) demonstrated that radio-labelled melatonin is taken up by the testis. Recent studies from this laboratory have shown that melatonin is taken up specifically by the Leydig cells (Dr.G. A.Kinson-personal communication).

Inhibition of *in vitro* androgen synthesis by indoles

occurred at high dose levels. Presumably, these dose levels are pharmacological in nature, since 90 μmolar of melatonin was necessary to exert inhibition. Thus, such an interaction would be of little physiological significance in the control of reproductive function of the mature male rat. However, certain conditions may arise in the life of the rat where such interaction may be important ie; perhaps pre-puberty for one. Most likely, pineal interaction at the hypothalamo-pituitary level is of physiological value.

SUMMARY

1. *In vivo* administration of indoles (1 μmole/week) had only modest effects on the reproductive functions of the adult male rat. The influence of indoles upon the metabolism of testosterone seems to be the important aspect of indolic function, at this dose level.
2. *In vitro* studies have revealed supportive evidence of the direct inhibitory action of pineal indoles on androgen biosynthesis. The influence of indoles appears related to similarities in structure at the 5 position of the indane ring system.
3. The physiological role of indoles in the regulation of male reproductive functions of the adult rat is not understood. However, the pharmacological potential of male fertility control is evident.

CHAPTER VI

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CHAPTER VII

APPENDIX

(A) Hormone Assay Reagents

1. Borate buffer, 0.05 M, pH 8.3- dissolve 2 grams reagent grade boric acid crystals in 500 ml distilled H_2O, containing 0.40 ml of 10 N NaOH.
2. Bovine gamma globulin- dissolve 250 mg of powder in 10 ml normal saline, containing 0.1% sodium azide.
3. Bovine serum albumin- dissolve 1 gram in 10 ml distilled H_2O containing 0.1% sodium azide.

(B) Histological Reagents

1. Helly's fixative - dissolve 2.5 g potassium dichromate, 4.0-5.0 g mercuric chloride in 100 ml distilled H_2O. Just prior to use add 5.0 ml of concentrated formalin.
2. Mayer's albumin affixative- beat white of an egg until well broken up, but not stiff, with egg beater and pour into tall cylinder. Let stand until the air brings suspended material to the top (overnight). Pour off liquid from the bottom, and to it add an equal volume of glycerine. Add a bit of sodium salicylate to prevent growth of molds.
3. Lugol solution- dissolve 2 grams of potassium iodide and 1 gram of iodine crystals in 12 ml distilled H_2O.
4. Periodic acid- dissolve 0.6g periodic acid in 100 ml distilled H_2O. Add 0.3 ml concentrated nitric acid.

5. Schiff reagent(Fisher Scientific Co.,modified for aldehydes)- basic fuchsin,sodium meta bisulphate,hydrochloric acid.
6. Hematoxylin reagent (Fisher Scientific Co.,Harris-Lillie variation)- ammonium alum,hematoxylin,isopropanol,red mercuric oxide,and glacial acetic acid.

(C) Incubation Buffers

1. Phosphate buffer,0.05 M,pH 7.4(Fisher Scientific Co.)-potassium phosphate monobasic-sodium phosphate dibasic.

Figure 17. Flowsheet illustrating the pathways to androgen formation.