DISSERTATION

BIOCHEMICAL DIFFERENTIATION AND HORMONAL REGULATION OF THE DEVELOPING TESTES IN *TENEBRIO MOLITOR*

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer, 1980

COLORADO STATE UNIVERSITY

Summer, 1980

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY HUSSAIN FADHIL ALRUBEAI ENTITLED BIOCHEMICAL DIFFERENTIATION AND HORMONAL REGULATION OF THE DEVELOPING TESTES IN TENEBRIO MOLITOR BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

[redacted]

[redacted]

Adviser

ABSTRACT OF DISSERTATION

BIOCHEMICAL DIFFERENTIATION AND HORMONAL REGULATION OF THE DEVELOPING TESTES IN TENEBRIO MOLITOR

During differentiation, the testes of Tenebrio molitor have been found to exhibit increases in biosynthetic capacity reflected in alterations in testicular protein and RNA. This biochemical differentiation was influenced by endogenous and/or exogenous hormones.

The testes underwent dramatic increases in size and weight during the prepupal stage that were continued through later developmental stages. Histological analysis revealed that the maturation process of the germ cells to produce spermatozoa proceeded from the distal end of the follicles and toward the basal region to form a "differentiation wave." Spermatozoa were found in the prepupal testes.

The underlying biochemical machinery of the developmental process was found to be accelerated in manufacturing different elements for germ cell differentiation at certain stages and particularly when the endogenous level of ecdysterone rose during the late prepupal and at mid-pupal stages. Gradual increases in testicular protein and RNA content were observed during the prepupal stage.

The observed increases were more dramatic for both protein and RNA content in the pupal stage. The testicular protein and RNA content reached their maximum levels between days 4 and 7 of the pupal stage as did the rate of ^{3}H-leucine incorporation. During the adult stage, the biosynthetic processes for producing protein and RNA were apparently reduced following the first few days after adult emergence.

The protein products of the mealworm testes were shown by gel electrophoresis to be many and diverse. The 27 protein products were of various molecular weights, ranging from 12,000 to 127,000 daltons. These products were present at different ages of development and persisted for various times indicating that some of these proteins may be necessary for the formation of specific germ cell types. In addition, a variety of these testicular protein components incorporated leucine at measurable levels throughout development, particularly during the pupal stage.

It was ascertained that the rate of incorporation of radioactive leucine into TCA-precipitable testicular protein was not affected by the administration of exogenous juvenile hormone alone (JHI, 1 μg/animal) during the pupal stage. However, the administration of exogenous ecdysterone (0.5 μg/animal) to pupal Tenebrio resulted in an increase in the rate of radioactive leucine incorporation into TCA-precipitable testicular proteins, particularly during the first

six days after pupal ecdysis. The amount of ecdysterone injected appeared to stimulate the production of the same testicular protein components that were present during normal pupal development. Injection of a higher dose of ecdysterone (1.5 μg/animal) during some of the pupal ages appeared to alter the testicular differentiation program by enhancing the incorporation of leucine into not only the age-specific testicular protein components but also into new protein components which did not normally appear at these specific ages.

Simultaneous administration of both JH and ecdysterone on mealworm pupae at specific ages indicated that there was no apparent interaction, synergistic nor antagonistic, between these two hormones. Furthermore, the incorporation rate of leucine closely resembled that rate obtained following injection of ecdysterone alone in all the pupal ages studied.

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ACKNOWLEDGEMENTS

I would like to express my deep gratitude to Dr. Thomas A. Gorell for supervising this research and for his support, guidance, and assistance during the course of my graduate program. I am also grateful to Dr. George M. Happ for his valuable advice through the early part of this research.

I would like to thank Dr. P. Elaine Roberts, Dr. John L. Capinera, and Dr. George E. Seidel, Jr., who served as members of my graduate committee.

My loyalty and devotion are pledged to my country for allowing me the freedom of choice and the means to engage myself in pursuit of my graduate studies. I also gratefully acknowledge the support from the Ministry of Higher Education and Scientific Research of the Republic of Iraq.

My heartfelt gratitude forever belongs to my wife for her patience, understanding, and love that I received during this difficult period.

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INTRODUCTION

Insects constitute 90% of the phylum Arthropoda and nearly three-quarters of all animal species. In spite of their abundance, relatively little is known of the endocrine control of reproductive maturation and physiology in male insects. Over the past decade, the bulk of the experimentation has been devoted to studies of the control of reproductive maturation in the females of many different insect species. Reproductive maturation involves not only the gonads but also a variety of secondary structures including the transport system (e.g., oviducts), the storage areas (e.g., egg sacs and spermatheca), and the associated accessory glands (Leopold, 1976).

In the mealworm, Tenebrio molitor, the regulation of ovarian development and oocyte production have been investigated by Laverdure (1967, 1971, 1975a and b). Differentiation of young pupal Tenebrio ovaries required ecdysone whether the ovary remained in situ or was cultured in vivo or in vitro. Further growth of the oocyte by vitellogenesis in the older pupae also required ecdysone, but the ecdysone concentration was higher than that required for differentiation of the young pupal ovaries (Laverdure, 1971). Addition of juvenile hormone (JH) analogues in vitro

prevented the ovary's response to ecdysone (Laverdure, 1971). It was subsequently shown that JH was not required for in vitro vitellogenesis, although the ovaries did require macromolecular compounds (chick embryo extract, muscle extract, ovarian extract) in order to further differentiate (Laverdure, 1975). Mordue (1965a and b) showed in ablation experiments that corpora allata enhanced oocyte development in adult Tenebrio. However, Trautmann (1972) was not able to detect JH activity in the first few days after adult ecdysis. Gerber (1976) demonstrated histogenic changes in the internal genitalia of the female mealworm during sexual maturation. The ultrastructure and histochemistry of the spermathecal gland in the mealworm beetle and the protein and RNA content of the female adult spermathecal accessory glands have been reported (Happ and Happ, 1970, 1975; Happ and Yuncker, 1978). In addition, numerous studies centering on the endocrine control of egg growth, ovarian protein and RNA synthesis, and vitellogenesis in other insect species have been reported (Engleman, 1970; Stay and Tobe, 1977; Handler and Postlethwait, 1978; Herman and Bennett, 1975).

Although the cytodifferentiation of the insect male reproductive system has been extensively studied in several insect species, no in-depth studies on the control of reproductive maturation in any single species have been reported. The possibility of controlling various insect pests by manipulating insect reproductive maturation

has resulted in a renewed interest in factors affecting spermatogenesis as well as the physiology of male reproductive organs.

General reviews of testicular morphology in insects have been written by Deegener in 1928 and by Depdolla in 1928 (Roosen-Runge, 1977). A highly competent and condensed summary of the structure of the male gonads may be found in Snodgrass (1935), in addition to the recent articles concerning testes differentiation, spermatogenesis and reproductive maturation in male insects written by Davey (1965), Doane (1973), Engelmann (1970), Leopold (1976) and Smith (1968).

Spermatogenesis is a process which generates haploid cells that are essentially motile nuclei designed to deliver genetic material to eggs during fertilization. The testes of insects are usually paired organs consisting of a series of tubes, tubules, or follicles emptying into a common vas deferens through narrow vasa efferentia. Differentiation of the testes and the process of spermatogenesis are usually completed before adult ecdysis although Dumser and Davey (1974) reported that spermatogenesis may continue into the adult stage in some species. Nowock (1972) reported that during metamorphosis the testes of Ephestia kuhnielia undergo two differentiation steps including fusion of the paired organs in the pharate pupa and torsion in the young pupae. Garbina et al. (1977) described spermatogenesis in the same lepidopteran species. The spermatids differentiated from the apical cells surrounded by predefinitive gonia, then associated with nurse cells

to form the spermatocytes where mitotic and meiotic divisions produce the spermatids. Plasmic interactions and the canal system between the spermatocytes of insects belonging to the Diptera and Lepidoptera have been demonstrated in electron micrographs (Nakanishi et al., 1965; King and Akai, 1971). Testes development and spermatogenesis have been studied in some other lepidopteran species (Holt and North, 1970; Salama, 1976; Laviatan and Friedlander, 1979; Shen and Berryman, 1967).

The dynamics of spermatogenesis in Drosophila (particularly D. melanogaster) have been extensively studied in vitro and in vivo and well described at the cytological level with electron and light microscopy (Stanley et al., 1972; Rungger-Brandle, 1976; Hardy et al., 1979; Fowler, 1973; Gould-Somers and Holland, 1974). The spermatogonia in the apical end of the testis divide mitotically and ultimately give rise to groups of primary spermatocytes encased in a cyst formed by two cyst cells. The spermatocytes in the cyst then undergo a synchronous meitoic division forming a group of immature spermatids. Following their differentiation, these spermatids ultimately are individualized into active sperm.

Although the effects of endocrine hormones on reproductive maturation in male insects have been reviewed (Engelmann, 1970; Doane, 1973; DeWilds and DeLoof, 1973), the hormonal control of

insect testicular development has not been studied as extensively as that of ovarian development (Davey, 1965).

A sex hormone involved in the development of male reproductive organs of insects has not been identified. Three hormones which may be involved are the activation hormone secreted by the neurosecretory cells of the brain, the juvenile hormone secreted from the corpora allata, and the molting hormone secreted from the prothoracic glands. However, in the male firefly, Lampyris noctiluca, Naisse (1966, 1969) reported an unusual androgenic hormone, produced apparently by apical cells of the testes that acts to promote differentiation of primary and secondary sexual characters in this highly dimorphic species. Meanwhile, the neurosecretory brain cells showed an endocrine role in the function of apical tissue in the testes of this insect.

The influence of the corpora allata on the activity of the reproductive organs has been studied in several insect species. Most authors agreed that the corpora allata are necessary for the normal function of the ovary, but not of the testes (Wigglesworth, 1936; Day, 1943; Thomsen, 1940; Scharrer, 1946; Engelmann, 1970; DeWilde, 1964). However, Sehnal (1968) observed that after implanting corpora allata into last instar Galleria larvae, the testes remained in a larval form until after the next ecdysis. Blaine and Dixon (1970) reported that corpora allata from a nymphal instar of Periplaneta americana

were responsible for maintaining the testes in a juvenile state by retarding their development. Leviatan and Friedlander (1979) demonstrated that the elongation of eupyrene spermatid was inhibited by a high titer of the JH mimic, while the apyrene spermatogenesis was found to be unrelated to the decline in the JH titer toward pupation of the carob moth Ectomyelois ceratoniae. Takeuchi (1969) has verified the acceleratory effect of ecdysone on germ cell numbers and differentiation and the inhibitory effect of juvenile hormone on spermatozoal differentiation in Bombyx mori. Ecdysone stimulated and juvenile hormone inhbited testicular differentiation and spermatogenesis was reported also in Rhodnius prolixus as well as in several other species (Dumser and Davey, 1974). Schmidt and Williams (1953) studied cultures containing germinal cysts from pupae of diapausing cecropia moths and found that spermatogenesis depended on the presence of the molting hormone.

The effect of ecdysone has been studied on the testicular development of the rice stem borer, Chilo suppressalis, by Yagi et al. (1969). Testes taken from diapausing larvae were cultivated in a medium free of insect hemolymph and subsequently treated with ecdysterone. A large increase in the size of the testes, in muscle contraction, and in the differentiation of the spermatocytes into spermatids were noted after a few days of hormone exposure; however, only 5% of the specimens showed any comparable

development in the untreated cultures. Rungger-Brandle (1976) suggested that both nutritive conditions and the level of ecdysone play a role in the testes development of Drosophila hydei cultured in vivo.

One of the unique characteristics observed in the testes of Calliphora vicina and Dystercus intermedius was the ability to sequester a considerable amount of ecdysteroid, assumed to be involved in the morphogenesis of the reproductive system and in reproduction itself (Koolman et al., 1979). The involvement of ecdysteroids in the imaginal cell differentiation in the spermiduct of Samia cynthia has been clearly demonstrated by Szollosi and Landureau (1977).

In some insects, other factors may control spermatogenesis. Ketchel and Williams (1955) further investigated the in vitro development of sperm of the cecropia silkworm and postulated the presence of a "volatile factor" that regulated development. Bowers (1961) and Laufer and Berman (1961) pointed out the importance of the role of the osmolarity of the nutrient medium in controlling developmental processes. Meanwhile, Williams and Kambysellis (1969) confirmed the original findings of Schmidt and Williams (1953) by demonstrating that "naked" spermatocytes taken from diapausing pupae of the silkworm, Samia cynthia, would differentiate in vitro in the absence of ecdysone when a "macromolecular factor" (MF)

isolated from the blood was added. However, cells within intact tests responded only when both the MF and α-ecdysone or endocrinologically competent prothoracic glands were present (Kambysellis and Williams, 1972). The same authors suggested that ecdysone alters the permeability properties of the testicular sheath (in vitro) and thus allowed the MF to enter and to act upon the cysts (Kambysellis and Williams, 1971a and b). A variety of exogenous proteins can be substituted for endogenous MF and consequently, the physiological significance of MF is unclear at present. Takeda (1972) has demonstrated the successful cultivation of testes obtained from diapausing slug moth pupae, Monema flavescens, in the synthetic medium of CSM-2F. These results indicated that the spermatocytes developed into spermatids with the addition of ecdysterone to the medium and that the "naked" spermatocytes were more sensitive to the hormone than were the intact spermatocytes. In addition, Takeda speculated that the MF corresponded to a factor contained in the fetal bovine serum of the synthetic medium, CSM-2F, suggesting that ecdysone may activate the masked MF and the ability of the MF to enter the testes and the cyst wall.

Ephestia kuhniella testes have two distinctly definable morphogenetic processes: Fusion of the paired testes and torsion (Nowock, 1971 and 1972). The results of in vitro experiments have demonstrated that ecdysone was a necessary stimulus to the initiation of both of these

processes. While ecdysone alone was sufficient for fusion of the testes, an additional morphogenic factor (or factors) mediated by the hemolymph was necessary for torsion to occur. It was assumed by Nowock (1973) that this morphogenic factor was either a labile substance or was present in minute amounts in the animal, and that the process of torsion required a continuous supply of this factor.

In summary, the mechanisms of spermatogenesis, including sperm differentiation, are rather similar in most animals (invertebrates and vertebrates). In insects, testis follicle differentiation proceeds from the formation of the primary spermatogonia to the primary spermatocysts, with subsequent first and second meiotic divisions giving rise to spermatids which then differentiate to become spermatozoa. In the ongoing process of testes differentiation, cell phenotypes appear to change at times of discrete developmental transitions. Certain of these transitions are preprogrammed and autonomous while others are dependent upon direct or indirect hormonal stimuli.

Evidence for the hormonal control of spermatogenesis analogous to the control mechanisms for oogenesis in females has been sought by some investigators. In the vast number of species examined, ecdysone stimulates and juvenile hormone inhibits testicular differentiation and spermatogenesis. In addition to ecdysone, a variety of exogenous proteins and MF were found to be required for spermatogenesis in

some insect species. As stated previously, most techniques involve histochemical studies, the culturing of testes in vivo or in vitro, and ligation as a means of following testes differentiation and its endocrine control. Thus, for careful validation of the endocrine control of testes differentiation and spermatogenesis it is important to clarify the biosynthetic capacity during different stages of differentiation (e.g., the pattern of DNA, RNA, and protein synthesis) and how these changes are correlated with the endocrine content. Since the testes undergo organogenesis in early stages of the animal's development and pass through phases of cell proliferation and cell specialization, they must exhibit changes in their biosynthetic capacity at the molecular level.

The present investigation utilized the testes of the mealworm, Tenebrio molitor, as an experimental model to study the biochemical and morphological indices by which the extent of differentiation can be scored. Additional studies were conducted to evaluate the role of hormonal controls on these indices during different developmental stages. The reasons for using Tenebrio testes as an experimental model were: (1) the beetles can be easily maintained as a stock culture in the laboratory; (2) developmental stages are readily distinguishable because Tenebrio is a holometabolous insect; (3) the ocular method for aging prepupal and pupal stage has been established (Stellwaag-Kittler, 1954; Delbecque et al., 1978); (4) the tests of Tenebrio

have a rapid development and pass through different stages of cell proliferation and specialization during spermatogenesis; and (5) the insect can be hormonally manipulated.

Furthermore, considerable data are available on the hormonal milieu of Tenebrio within which the testes develop. The role of the corpora allata in maintaining larval characteristics of developing Tenebrio has been demonstrated by Radtke (1942). The activity of corpora allata appear to decline in the last instar larva and there is, presumably, little or no JH in the pupal stage or in the first week of adult life (Caveney, 1970; Reddy and Krishnakumaran, 1973; Trautmann et al., 1974a, b; Judy et al., 1975). The ecdysteroid levels in last instar larvae (prepupae) and in pupae have been determined by mass spectroscopy and radioimmunoassay (Delbecque et al., 1975, 1978a, b). In the prepupal stage, the maximum molting hormone titer was reached at ocular stage 12 (1200-1600 ng/ml); in the pupae the molting hormone peak occurred at day 4. In addition, Glitho et al. (1979) demonstrated the relations between cytological cyclic activity of prothoracic glands and ecdysteroid levels.

The purpose of this study was: (1) to examine the biochemical pattern of the testes differentiation, including measurement of the total testicular protein and RNA; (2) to determine the testicular protein components throughout different developmental stages; (3) to investigate the incorporation of specific radioactive precursors into

testicular proteins during differentiation; and (4) to examine the effect of juvenile hormone and ecdysterone on the biochemical parameters of testes differentiation.

MATERIALS AND METHODS

Animal Rearing

Larval mealworms, Tenebrio molitor L., were purchased from Sure-live Mealworms Co. (Paramount, CA) and were maintained at room temperature. The insects were kept on a diet of chicken feed (Purina Startena) supplemented with either a piece of potato or wet sponge as a source of moisture. All animals used for experimental purposes were acclimated to laboratory conditions.

While the sex of insects at pupal ecdysis was easily established, the sex of larvae was more difficult to determine due to the lack of defined external sexual characters. Thus, in experiments involving the last larval instar (prepupae), a large number was used to ensure an adequate number of males.

Since there is no strict clock timing of physiological age in last instar larvae, dating of this instar was carried out using Stellwaag-Kittler's method (1954), which defines 14 steps in the differentiation of the eye. The developmental ages of the pupal stage were determined by using the original observational techniques of Wigglesworth (1948) as subsequently confirmed and modified by Delbecque (1978a). Nine physiological pupal stages were distinguished. Each stage corresponded to approximately one day at 26°C. Structural characteristics used in the dating method

mentioned are the appearance of ommatidia and darkening of the cuticle. Newly ecdysed male adults were placed in 20 ml glass scintillation vials (six per vial) and maintained at 26°C.

Tissue Preparation

In all experiments the mealworm testes were exposed by dissecting the animals in cold Tenebrio saline (Butz, 1957) or in Ringer's solution under a dissecting microscope. The adhering fat body and external trachea were extruded and the testes were separated from the remainder of the reproductive tract using microtweezers and scissors. The testes of early prepupae could not be removed successfully since they were minute and not distinguishable from the mass of fat body present at this time.

The average wet weight of the testes during the pupal stage and adult life was determined from the total net weight of five to ten pooled testes pairs.

Excised testes were homogenized immediately for biochemical analysis as described below (Analytical Procedures). In some experiments, testes were kept in Tenebrio saline up to one hour prior to gross morphological observations or fixation and subsequent histological examination.

Histology

Testes from different developmental stages were fixed in aqueous Bouin's fluid, embedded in a paraffin wax block, and sectioned serially at 5 μm. The sections were then processed through a normal course of dehydration using a graded series of increasing alcohol concentrations. The sections were stained with Mayer's acid haemalum and eosin.

Analytical Procedures

All subsequent procedures were carried out using pooled homogenate of five to ten testes pairs and at least five determinations were made at each developmental age, except where specified otherwise. The results obtained were expressed per testes pair in all experiments.

A. - Protein and RNA Content. The total protein content of the prepupal, pupal and adult testes was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Testicular RNA content was measured by an adaptation of the procedure of Raikow and Fristrom (1971) as modified by Happ et al. (1977). Testes were homogenized in 1 ml Ringer's solution and protein was precipitated with 10% TCA (Trichloroacetic acid). After a brief centrifugation in a refrigerated Sorval centrifuge (Model RC2-B) using an SS-34 rotor at 1,200 xg for 10 minutes, the RNA

in the supernatant was precipitated with 95% ethanol. The RNA precipitate was washed again with 95% ethanol and subsequently with diethylether. The dried precipitate was subjected to hydrolysis at 37°C in 0.3 N NaOH for one hour. The DNA and soluble protein were precipitated with cold 1 N $HClO_4$ and removed by centrifugation (1,200 xg, 10 min.). The remaining supernatant was brought to pH 8.0 with tris buffer (2.9 M), and the absorption was measured at 260 and 280 nm in Beckman (Model 24) spectrophotometer. Two standards (yeast RNA) were run in parallel with each set of experiments with a recovery of 85%. The RNA concentrations determined by the absorption method were confirmed by using the orcinol method (Ceriotti, 1955).

B. - Gel Electrophoresis. The proteins of prepupal, pupal, and adult testes were separated according to their molecular weight by polyacrylamide gel electrophoresis. The method used was a modification of the Davis (1964) method with tris-glycine buffer pH 8.9, but omitted both the spacer and the sample gels. Six microliters of fresh testes homogenate were placed directly on the 7.5% acrylamide separating gel (7.5 x 0.5 cm), and run at 5 mAmp per tube. The gels were run until the bromophenol blue marker dye had migrated 7.0 cm. The gels were removed from the gel tubes and stained with Coomassie brilliant blue overnight. The gels were destained in 10% acetic acid and 30% methanol (Bertolini et al., 1976) in order to

visualize the protein bands. Sodium dodecylsulfate (SDS) polyacrylamide gels (7.5%, Weber and Osborne, 1969) were also used to separate testes proteins. Samples were heated in a solubilizing buffer for 10 minutes before application to the SDS jels (Paul et al., 1972). The following marker proteins were obtained from Sigma Chemical Co. (St. Louis, MO): catalase (60,000 d), bovine serum albumin (65,000 d), pepsin (35,000 d), trypsin (23,000 d), ribonuclease (13,700 d), and cytochrome C (11,700 d).

C. - Incorporation of [^{3}H]-Leucine into Testicular Proteins. Experiments were conducted to determine the incorporation of radioactive leucine (3,4,5 ^{3}H; specific activity:110 Ci/mM; New England Nuclear, Boston, MA) into TCA-precipitable testicular proteins of pupae and adults.

Insects were removed from the incubator, affixed to microscope slides with wax, and briefly cooled to 3^{o}C on ice. The [^{3}H]-leucine was taken up with a 10 µl Hamilton syringe, dissolved in 2 to 4 µl of Ringer's solution and injected into the dorsal surface of the abdomen. The injection site was sealed with wax. The control beetles received 2 to 4 µl of Ringer's solution in order to determine whether alterations in the total precipitable testicular proteins occurred as a result of the trauma of injection. All animals were incubated at room temperature prior to sacrifice. The appropriate time and dose of [^{3}H]-leucine necessary for the sufficient incorporation

of radioactive precursor into the TCA-precipitable protein fraction was determined by using different incubation times (1 to 8 hrs) and different doses of [^{3}H]-leucine (1-6 μl; 0.125-0.75 μCi). Following incubation, TCA-precipitable resticular proteins were extracted using the procedure of Kennell (1967). The pupal testes at each developmental age and adult testes from 0 to 10 days of age were removed and homogenized in 1 ml of distilled water. The testicular homogenate was mixed with an equal volume of 20% TCA and then 5% TCA was added to bring the volume to 3 ml. Nucleic acids were solubilized from the tissue homogenate by incubating at 80°C for 30 minutes. The acid-precipitated proteins were collected on glass microfiber paper (Whatman FG/A, 2.5 cm) presoaked in 10% TCA at 0°C. Collection and washing of the precipitated proteins were accomplished by setting a vacuum under the filter holder. The filters were washed three times with 10% TCA at 0°C and successively washed with 70% ethanol at 0°C and 40°C and finally washed with 95% ethanol at 40°C. All TCA washes contained 10 mM of cold leucine. The filters were then air dried and prepared for estimation of radioactivity.

Some of the samples were counted with and without a specific amount of [^{3}H] water (NEN) in order to detect sample quenching caused by traces of TCA which were not removed during the ethanol washings.

The incorporation of radioactive leucine into specific testicular protein bands, as defined by electrophoretic mobility, was analyzed from pupae at each developmental age and adults from 0 to 10 days of age. The insects were injected with [^{3}H]-leucine (3 μCi) and incubated at room temperature for six hours. Following incubation, the testes were removed, homogenized, and duplicate samples of each homogenate subjected to separation by polyacrylamide gel electrophoresis. Following electrophoresis, one gel of each duplicate was stained with Coomassie blue to determine the positions of testicular protein bands. The non-stained duplicate gels were sliced into 1 mm segments using a BioRad Model 190 Gel Slicer and were placed on the bottom of separate 10 ml glass scintillation vials. Gel slices were solubilized in 0.5 ml of a 9:1 aqueous solution of protosol (NEN) at 50°C for 2 to 3 hours and subsequently radioassayed.

In vitro experiments were conducted as described above to detect the incorporation pattern of [^{3}H]-leucine into testicular protein bands of 6 day old pupae. Following incubation the testes were removed, homogenized in distilled water, and each homogenate subjected to separation by polyacrylamide gel electrophoresis and radioassayed.

Juvenile Hormone and Ecdysterone Administration

The following experiments were designed to test the effects of juvenile hormone I (JH-I), ecdysterone (both purchased from

Calbiochem-Behring Corp., CA), and mixtures of the two hormones on the incorporation patterns of [^{3}H]-leucine into testicular proteins during pupal development.

Male pupae at 8 days of age were treated with 0.01, 0.1, or 1.0 μg of JH-I (George M. Happ, personal communication) in mineral oil by topical application to the dorsal abdominal surface. Male puape (1 day old) were injected intraabdominally with 0.05, 0.1, or 0.5 μg of ecdysterone in water. Pupae at each age were treated with: JH-I alone (1 μg applied topically to the dorsal abdominal surface); ecdysterone alone (0.5 μg injected intraabdominally); or both JH-I and ecdysterone. Simultaneously with the hormonal treatment, 0.5 μl of [^{3}H]-leucine was injected and its incorporation into the TCA-precipitable protein fraction of the testes was determined.

The effect of ecdysterone injections on the incorporation of [^{3}H]-leucine into specific testicular proteins, as defined by electrophoretic mobility, was determined. Male pupae at each day of development up to day six and male pupae at 1 and 3 days of age were injected intraabdominally with 0.5 μg or 1.5 μg ecdysterone, respectively, along with 3 μCi of [^{3}H]-leucine. After six hours, the testes were removed, homogenized in distilled water, and duplicate samples analyzed.

Measurement of Radioactivity

All radioactive samples were analyzed in a Beckman (Model LS7000) liquid scintillation spectrometer with an efficiency of 37% for tritium. Radioactive samples were placed into scintillation vials containing 10 ml of scintillation cocktail (toluene 750 ml/l, triton X-100 250 ml/l, μg/l PPO (2,5-Diphenyl-oxazolyl), and H_2O 60 ml/l) and counted for 10 minutes. All results were expressed either as DPM/testes pair or DPM/μg testicular protein.

RESULTS

Tenebrio molitor develops and matures by cycles of apolysis and ecdysis and as a holometabolous insect passes through three distinct stages: larval, pupal, and adult (Figure 1).

The reproductive system of male Tenebrio (Figure 2) consists of paired testes, seminal vesicles, and two types of accessory glands (tubular and bean-shaped). The accessory glands along with the seminal vesicles lead into the proximal end of the ejaculatory duct. The testis consists of a rounded aggregate of six follicles surrounded by a basement membrane and connective tissue sheath cells (Figure 3). During developmental stages of the mealworm beetle, the testes grew in size and were more apparent. In the prepupal stage, the testes were minute and barely distinguishable from the mass of fat body present at this time while pupal and adult testes were larger and easily distinguishable from the fat body (Figure 4).

Figure 5 illustrates the average wet weight of the testes during the pupal stage of development. The average wet weight of the testes during this stage gradually increased up to the last day of pupal life at which time the testes pair weighed 7.5 mg. During the adult stage, no sharp modification in testes weight occurred although the testes

Figure 1. The development stages of Tenebrio molitor. L, larva; P, pupa; A, adult. (Scale in cm).

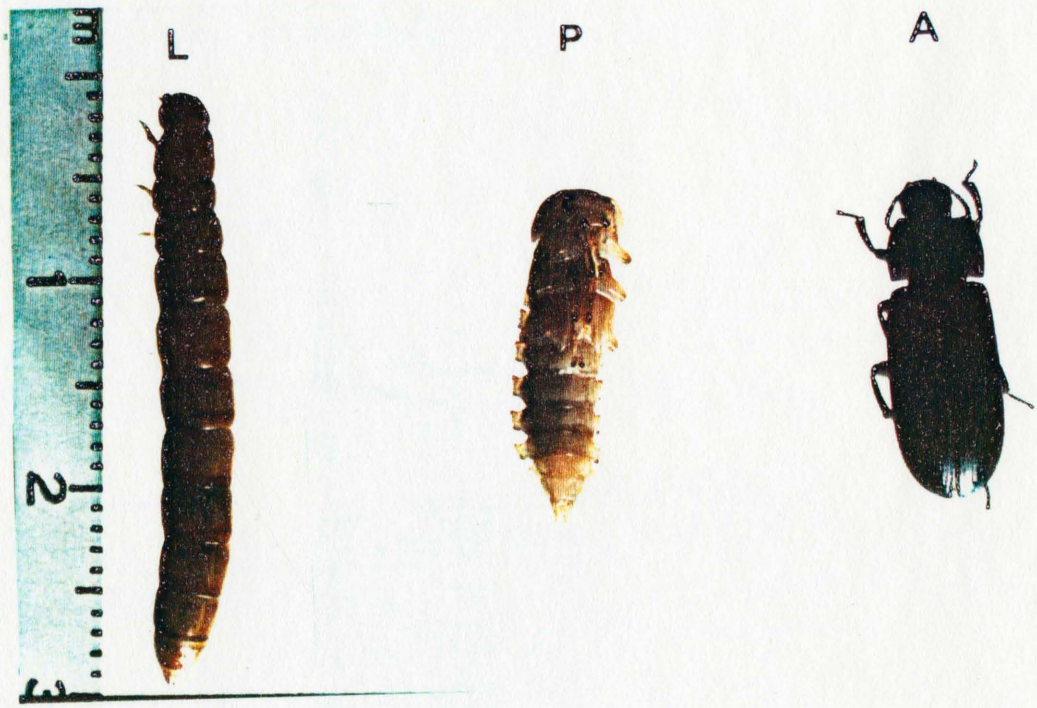

L
P
A

Figure 2. Dorsal aspect of the reproductive system of the male mealworm beetle. TAG, tubular accessory gland; BAG, bean-shaped accessory gland; SV, seminal vesicle; Ts, testis; ED, ejaculatory duct. Fresh preparation. (X90).

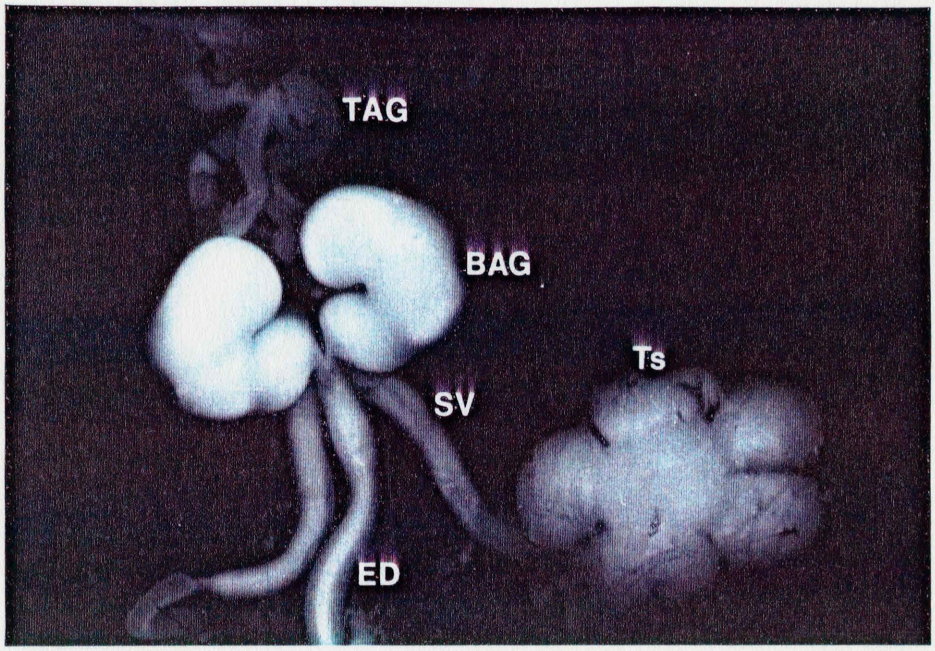
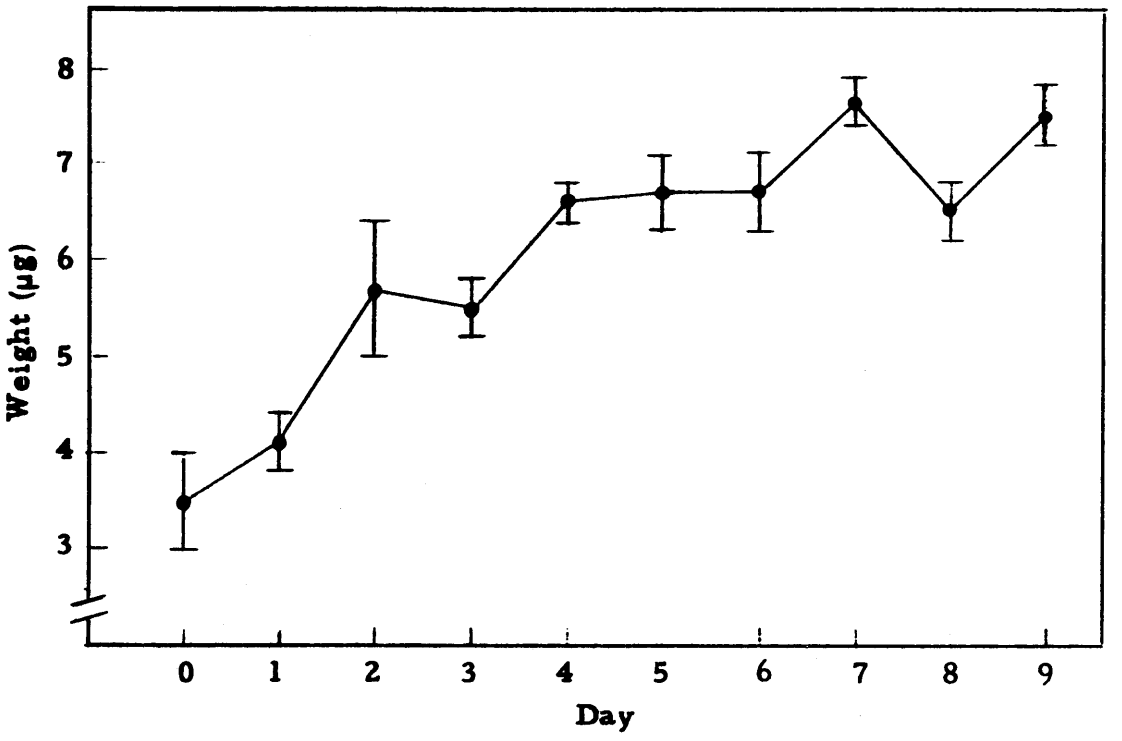

TAG
BAG
Ts
SV
ED

Figure 3. Fully developed adult testis showing the follicles. (X150).

Figure 4. Testes from different developmental stages. PP, prepupa; P, pupa; A, adult. (X70).

PP P A

Figure 5. The average wet weight of the testes during the pupal stage. Each point represents the mean of eight pairs of testes; bars indicate standard errors. Data are expressed per testes pair.


Weight (µg)
8
7
6
5
4
3
0
1
2
3
4
5
6
7
8
9
Day

weight increased slightly through day 7 reaching a maximum of 9.5 mg, then apparently declined gradually through day 10 of adult life (Figure 6).

Testicular Histology

Tissue sections of the mealworm testes indicated that spermatogenesis began during the prepupal stage and continued through both pupal and adult stages. Observations of sections of the testes obtained from prepupae at various ocular ages indicated that the testes had already developed spermatids and sperms. The spermatogonial and spermatocyst compartments comprised most of the follicles of the prepupal testes while the sperm compartment occupied only a small portion of the follicle (Figure 7). Early in pupal development, the spermatogonial, spermatocyst, and sperm compartments of the testicular follicle are roughly equal while later in pupal development, the sperm compartment predominates (Figure 8). The dominance of the sperm compartment in terms of comprising the largest portion of the testicular follicle is maintained throughout adult life while other cell type populations have localized along the boundary of the follicle (Figure 9).

Testicular Protein and RNA Content

The testicular protein content of Tenebrio at various ocular ages of the prepupal stage is shown in Figure 10. The protein content

Figure 6. The average wet weight of the testes during the first 10 days after adult emergence. Each point represents the mean of eight pairs of testes; bars indicate standard errors. Data are expressed per testes pair.

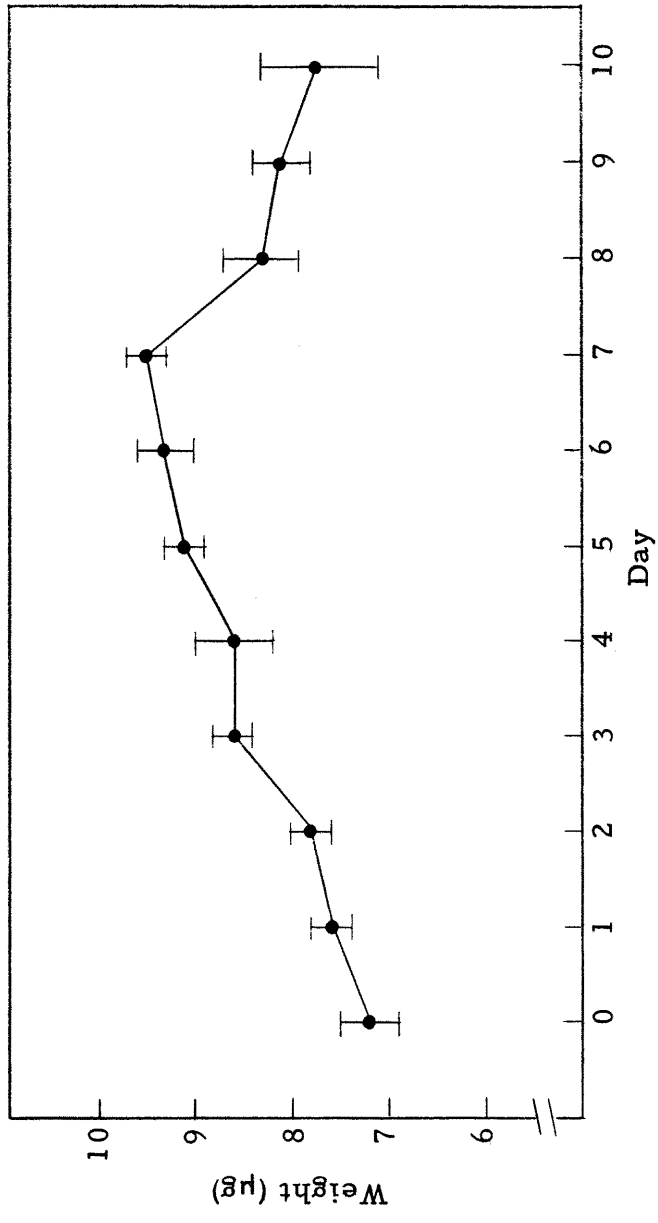

Weight (µg)
10
9
8
7
6
0
1
2
3
4
5
6
7
8
9
10
Day

Figure 7. Longitudinal section of a testis from Tenebrio in the prepupal stage. CT, connective tissue; BM, basement membrane; SG, spermatogonia; SC, spermatocytes; SZ, spermatozoa. (X300).

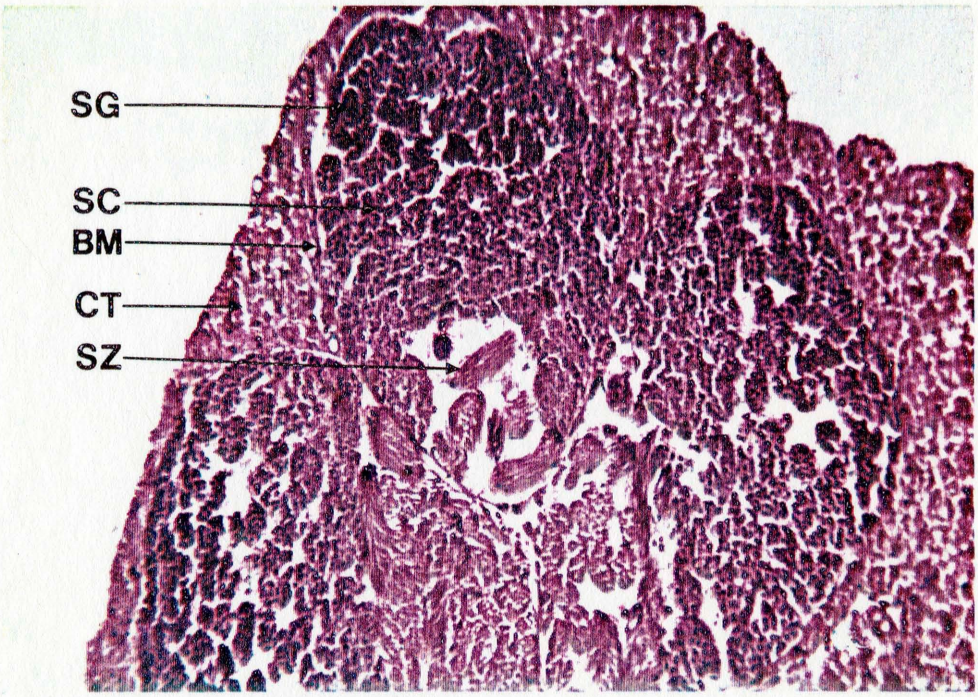

SG
SC
BM
CT
SZ

Figure 8. Longitudinal section of a testis from Tenebrio in the pupal stage. SG, spermatogonia; SC, spermatocytes; SZ, spermatozoa. (X300).

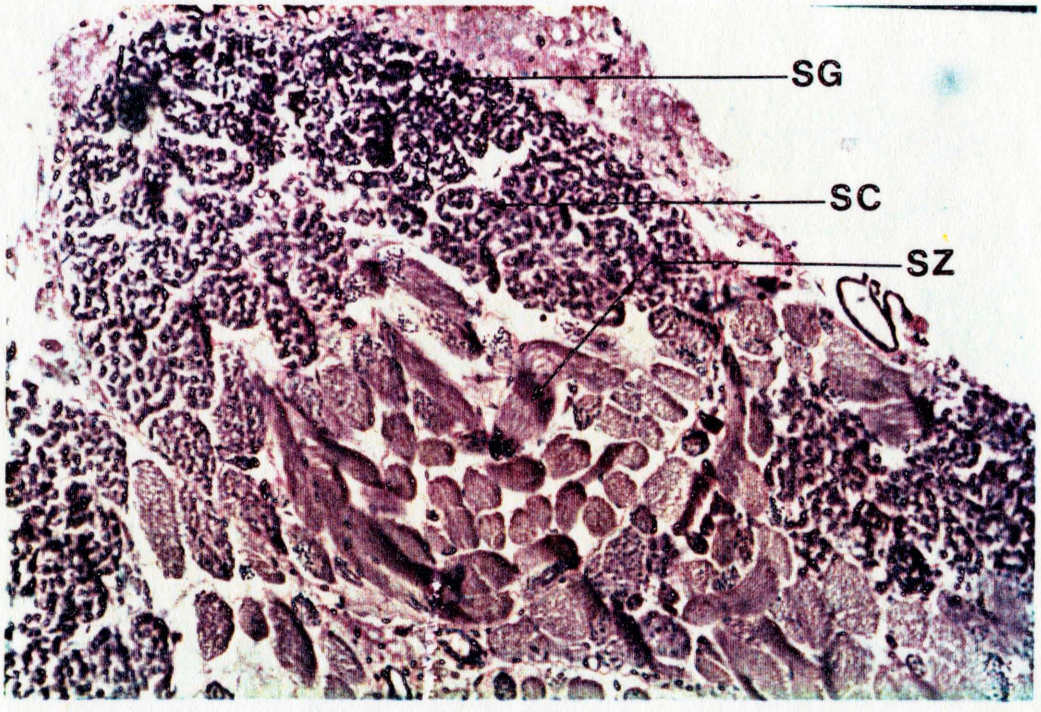

SG
SC
SZ

Figure 9. Longitudinal section of a testis from Tenebrio in the adult stage. SG, spermatogonia; SC, spermatocytes; SZ, spermatozoa. (X300).

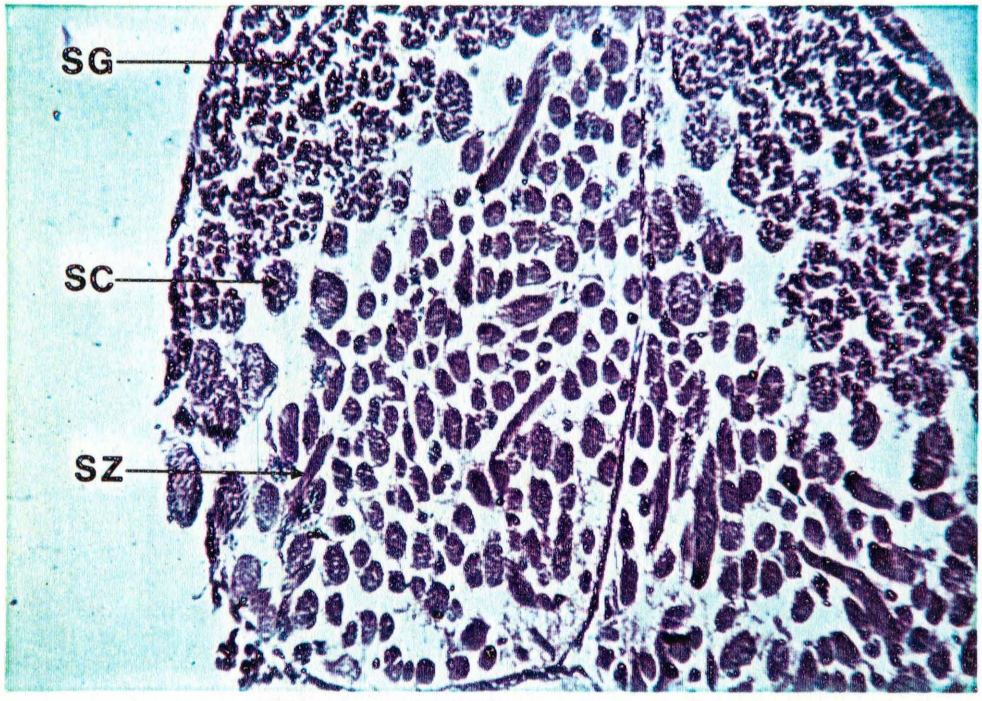
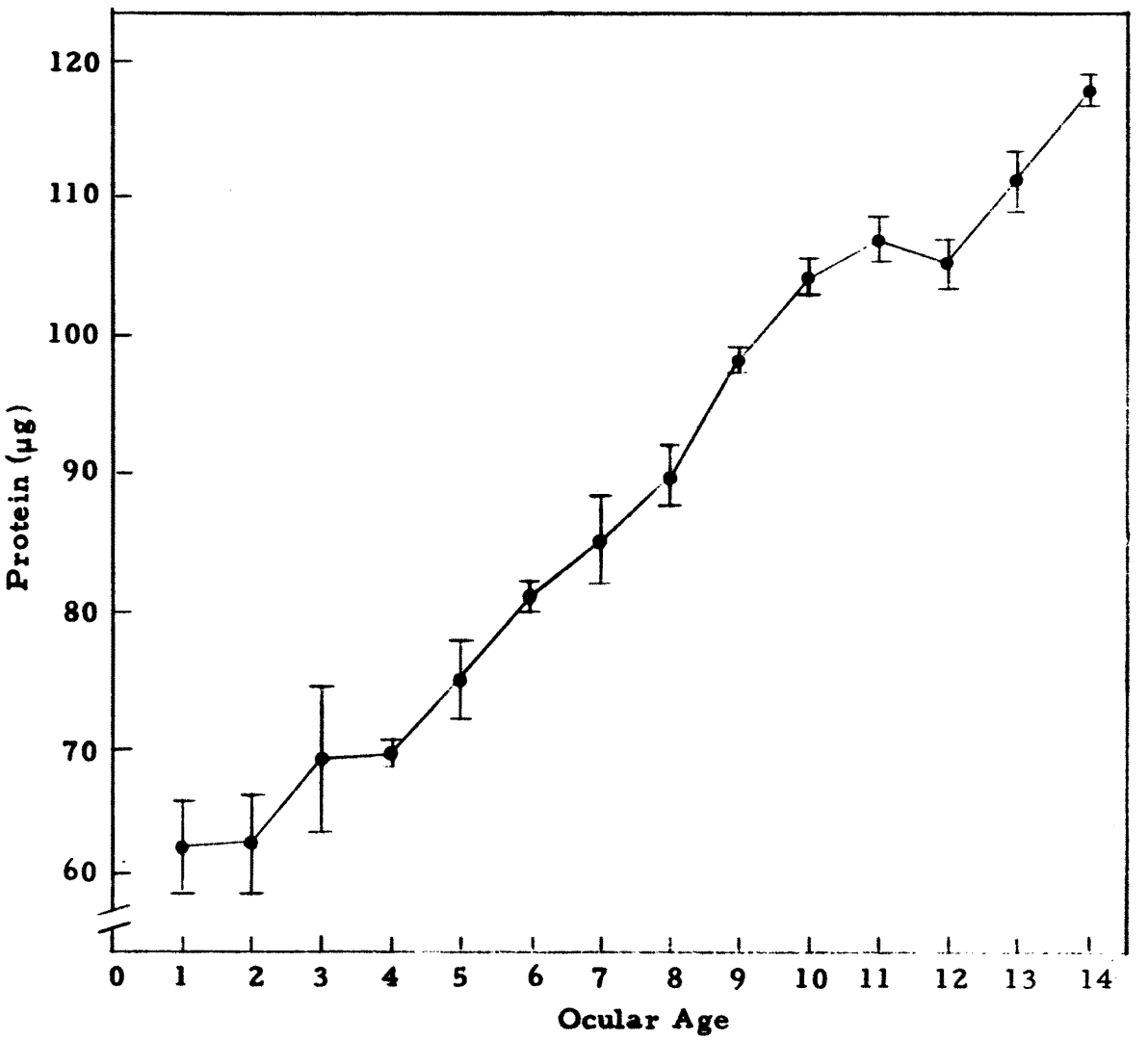

SG
SC
SZ

Figure 10. The testicular protein content during the prepupal stage. Each point represents the mean of at least seven determinations at each ocular age; bars indicate standard errors. The data are expressed per testes pair.


120
110
100
90
80
70
60
Protein (µg)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Ocular Age

increased from approximately 60 μg per testes pair at prepupal ocular age 1 to 118 μg per testes pair at the end of this stage (ocular age 14). The increase in testicular protein content from ocular age 1 to 11 was essentially linear while a slight decline occurred at ocular age 12 followed by an increase at ocular ages 13 and 14. Figure 11 illustrates that during the pupal stage testicular protein content increased substantially following pupal ecdysis and reached a maximum of 192 μg per testes pair at day 5 of pupal life. From the fifth through the ninth day of pupal life the average testicular protein content apparently declined slightly to the end of this developmental stage. Figure 12 illustrates the total protein content of the testes following emergence through day 10 of adult life. The protein content increased immediately following adult emergence and reached a maximum at day 2, then fluctuated and declined through day 10 of adult life.

The total RNA content of the testes at various ocular ages of the prepupal stage is shown in Figure 13. RNA content increased gradually from less than 1 μg RNA per testes pair at ocular age 1 to 6 μg RNA per testes pair at ocular age 14. After pupal ecdysis and through day 2 of pupal life (Figure 14) there was a slight increase in testicular RNA content followed by a more dramatic increase beginning at day 3 and reaching a maximum on day 4 of 11 μg RNA per testes pair. The RNA content declined gradually through the last day of pupal

Figure 11. The testicular protein content during the pupal stage. Each point represents the mean of at least seven determinations at each age; bars indicate standard errors. The data are expressed per testes pair.

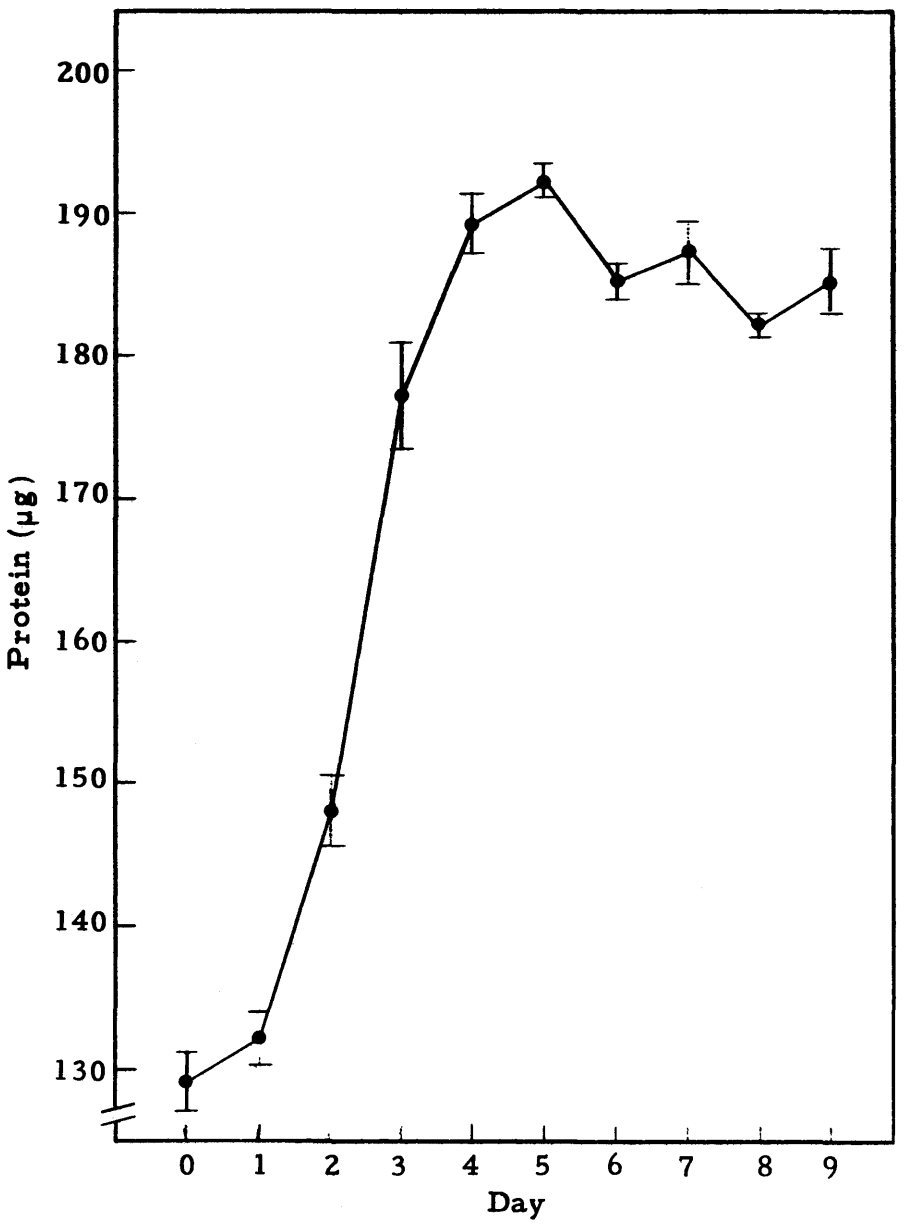

200
190
180
170
160
150
140
130
Protein (µg)
0
1
2
3
4
5
6
7
8
9
Day

Figure 12. The testicular protein content during the first 10 days after adult emergence. Each point represents the mean of at least seven determinations at each age; bars indicate standard errors. The data are expressed per testes pair.

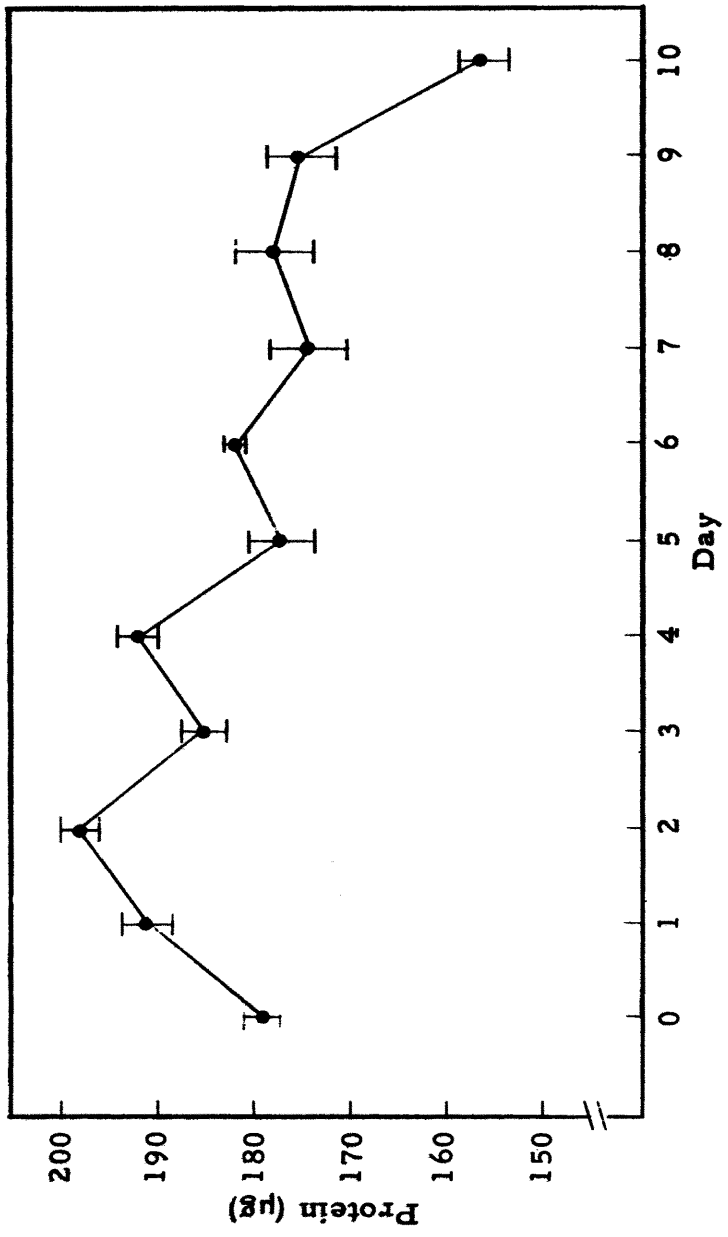

Protein (µg)
200
190
180
170
160
150
0
1
2
3
4
5
6
7
8
9
10
Day

Figure 13. The testicular RNA content during the prepupal stage. Each point represents the mean of at least five determinations at each ocular age; bars indicate standard errors. The data are expressed per testes pair.

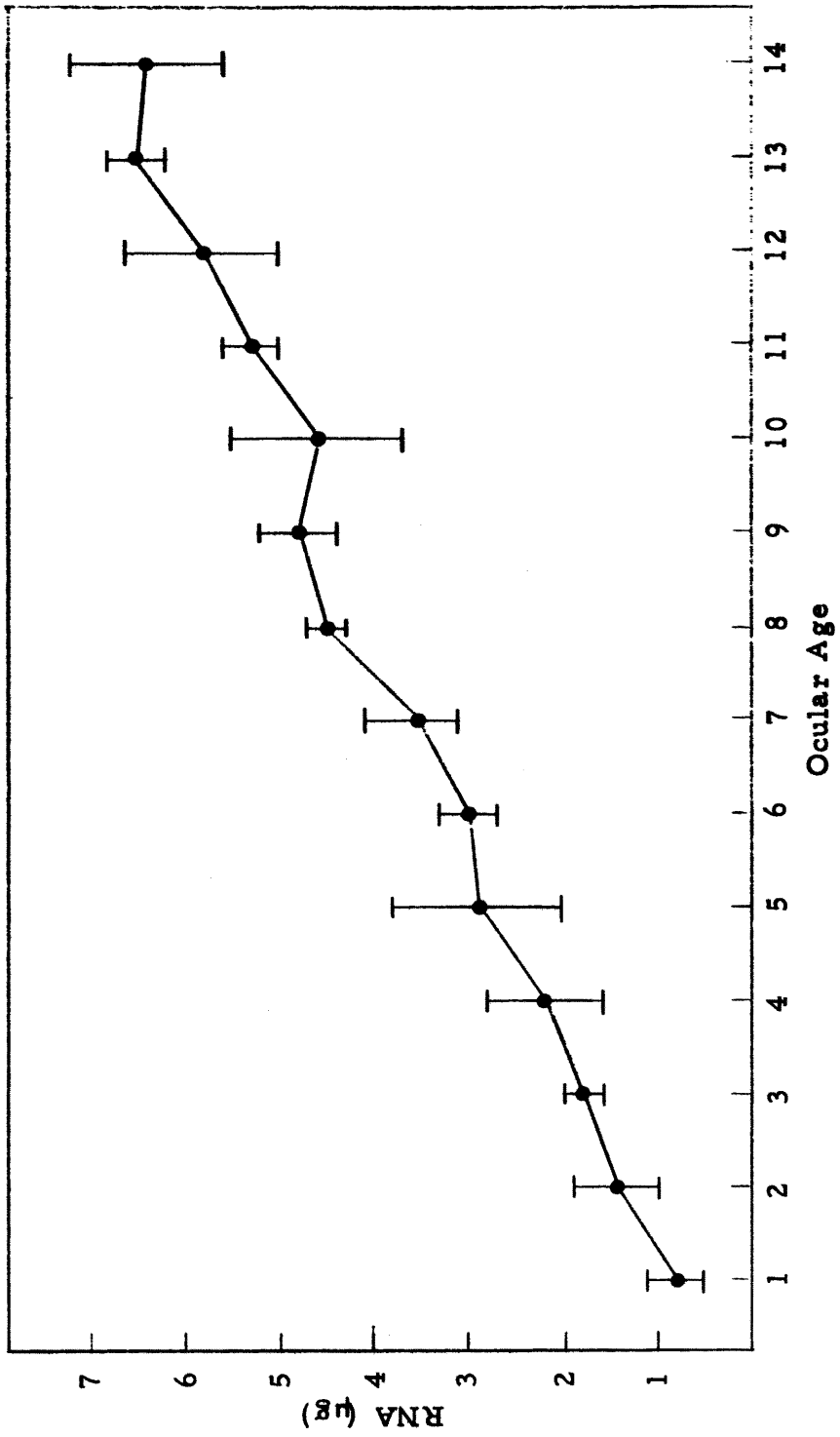
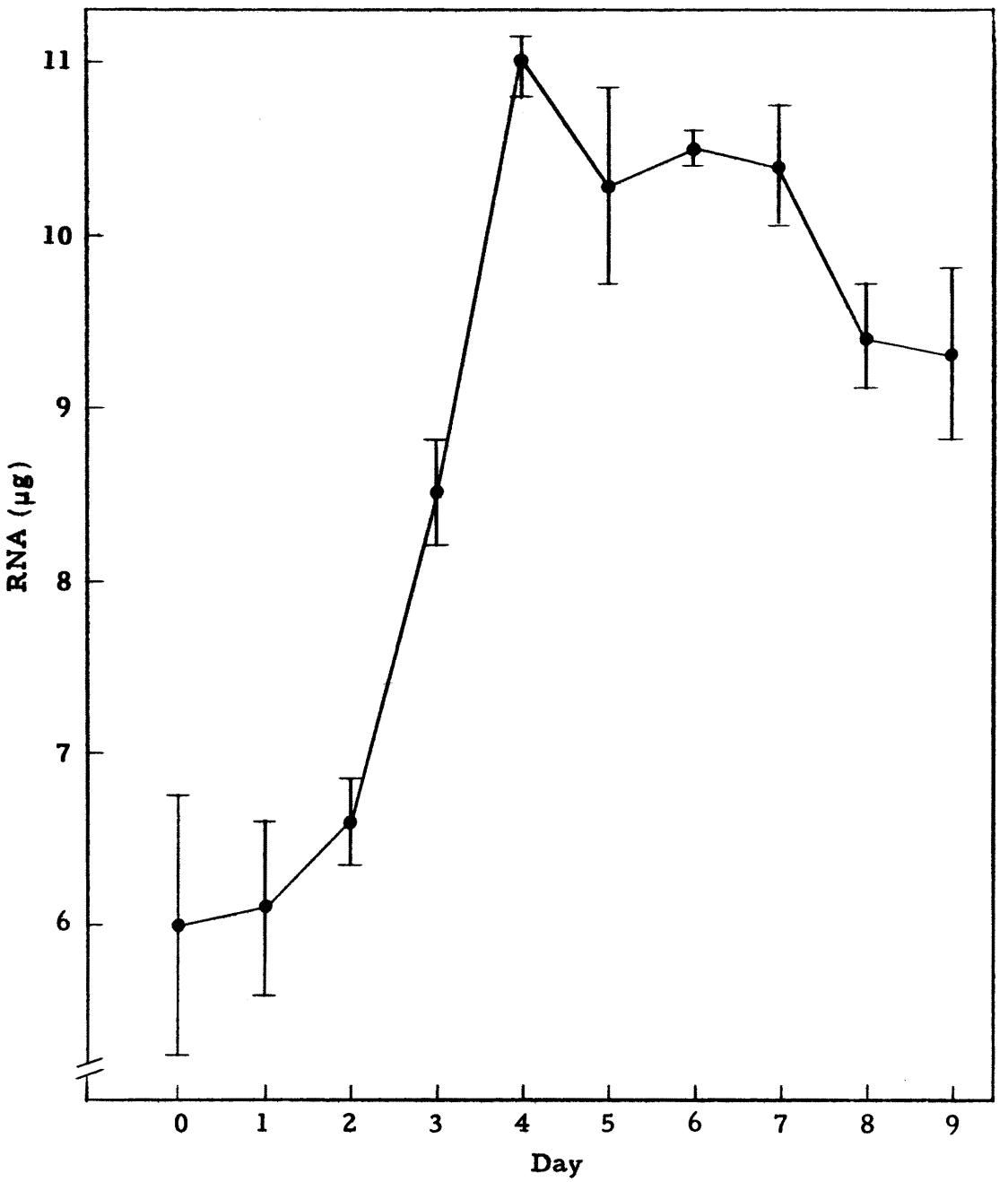

RNA (μg)
1
2
3
4
5
6
7
Ocular Age
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Figure 14. The testicular RNA content during the pupal stage. Each point represents the mean of at least five determinations at each age; bars indicate standard errors. The data are expressed per testes pair.


11
10
9
8
7
6
RNA (µg)
0
1
2
3
4
5
6
7
8
9
Day

development. The testicular RNA content increased sharply following adult emergence, reaching a value of about 10 μg RNA per testes pair at day 1 (Figure 15). A slight decline in testicular RNA content occurred at day 2 but then remained relatively constant through day 9 of adult life until it declined at day 10.

Electrophoretic Analysis of Testicular Protein

Proteins of the mealworm testes were readily separated by polyacrylamide gel electrophoresis with or without SDS. The number of testicular protein components varied from 3 to 27 different protein bands. The protein banding pattern of testicular homogenates obtained from animals in the prepupal, pupal, and adult stages of development are depicted in Figures 16, 17, and 18, respectively. Due to varying staining intensities of the protein bands, the photographs of the gels did not illustrate the total number of bands that can be discerned upon inspection of the actual gels. Consequently, the testicular protein banding pattern for each developmental stage is depicted diagrammatically in Figures 19, 20, and 21. For convenience, the protein bands were numbered sequentially starting from the top of the gel (origin) and continuing to the anodal end. The assigned numbers were based on the testicular protein banding pattern obtained from six day old pupae since this testes homogenate contained the highest number of protein bands. The protein banding patterns of testes homogenates from different developmental stages

Figure 15. The testicular RNA content during the first 10 days after adult emergence. Each point represents the mean of at least five determinations at each age; bars indicate standard errors. The data are expressed per testes pair.

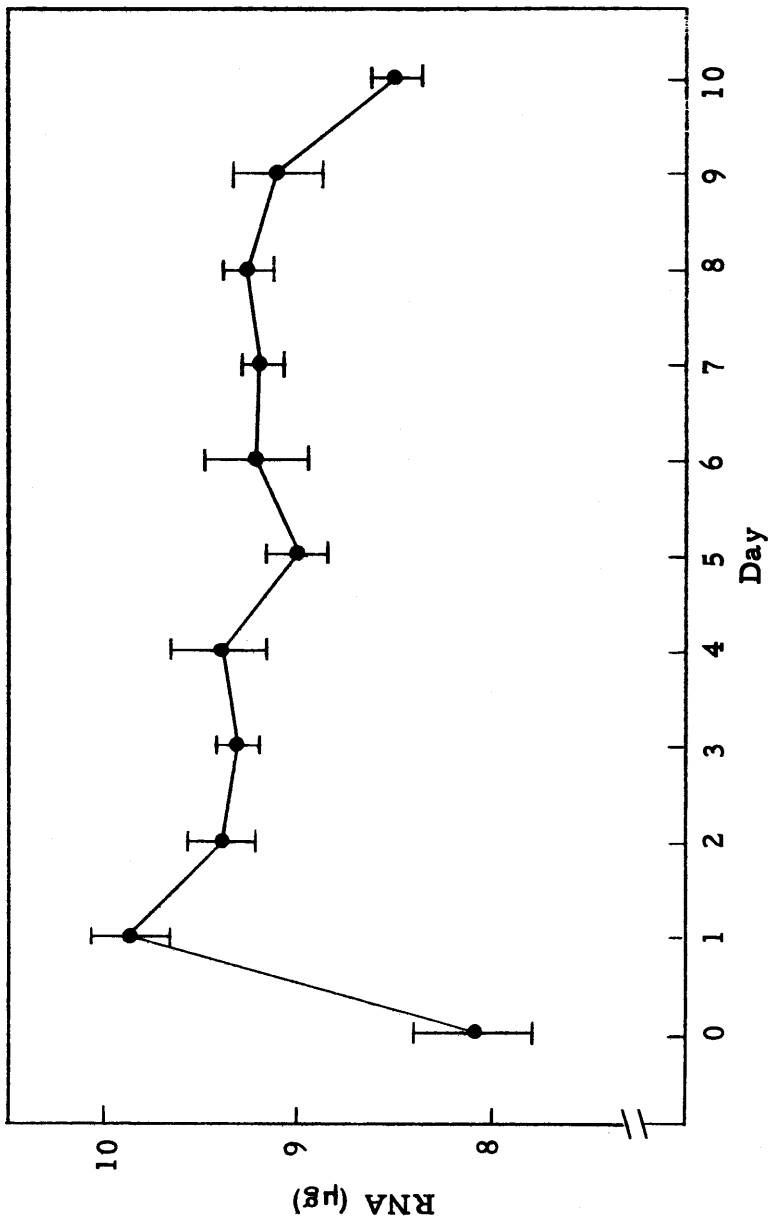
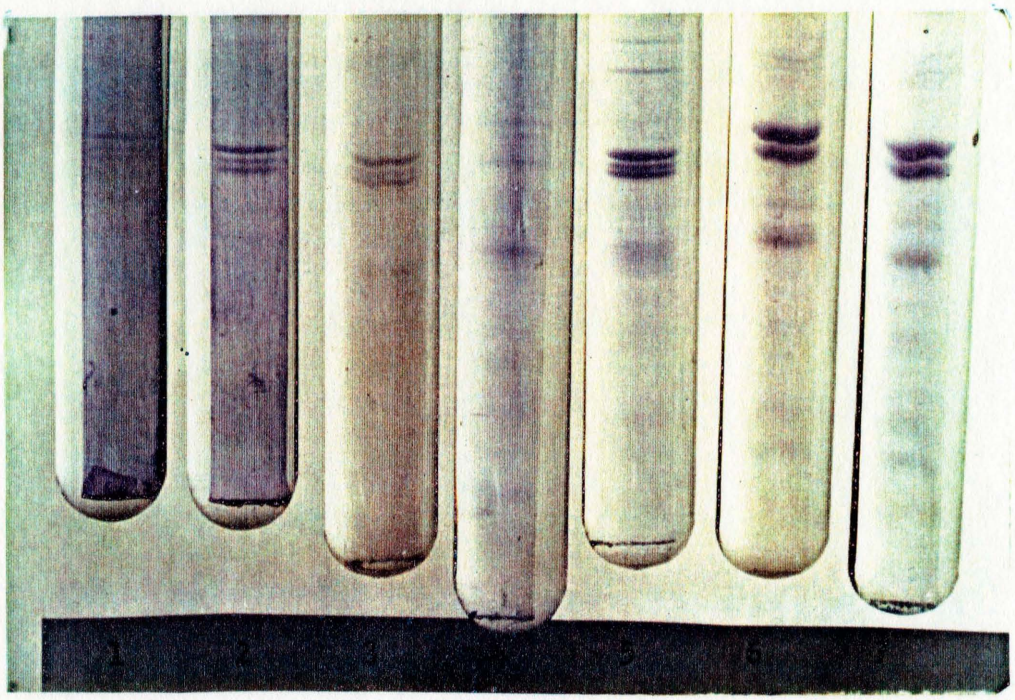

RNA (μg)
10
9
8
0
1
2
3
4
5
6
7
8
9
10
Day

Figure 16. Polyacrylamide gel electrophoretic pattern of testicular proteins during the prepupal stage. Six microliters of total testes homogenate were used per gel. Orientation of gels - origin at top, anode at bottom. The number on the bottom of each gel tube indicates the prepupal ocular age.


1
2
5
6

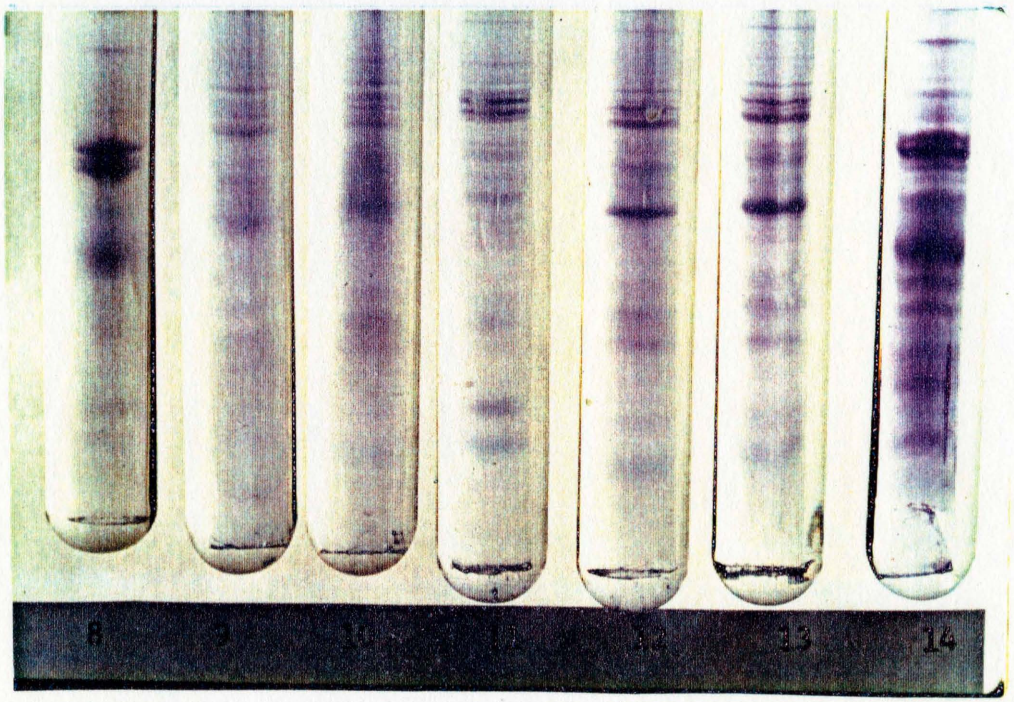

8
13
14

Figure 17. Polyacrylamide gel electrophoretic pattern of testicular proteins during the pupal stage. Six microliters of total testes homogenate were used per gel. Orientation of gels - origin at top, anode at bottom. The number on the bottom of each gel tube indicates the pupal developmental age.

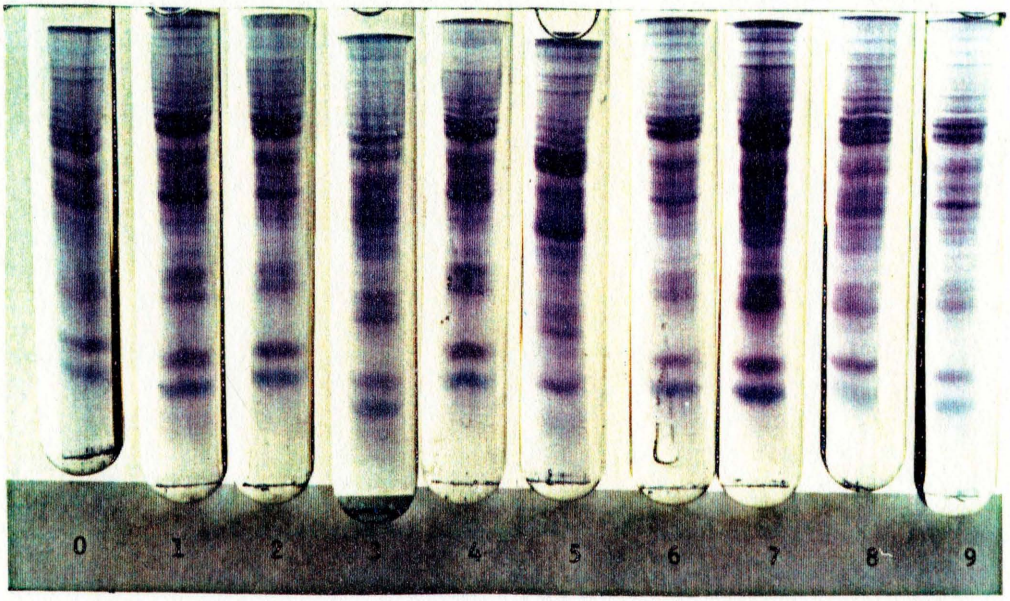

0
1
2
3
4
5
6
7
8
9

Figure 18. Polyacrylamide gel electrophoretic pattern of testicular proteins during the first 10 days after adult emergence. Six microliters of total testes homogenate were used per gel. Orientation of gels - origin at top, anode at bottom. The number on the bottom of each gel tube indicates the adult developmental age.

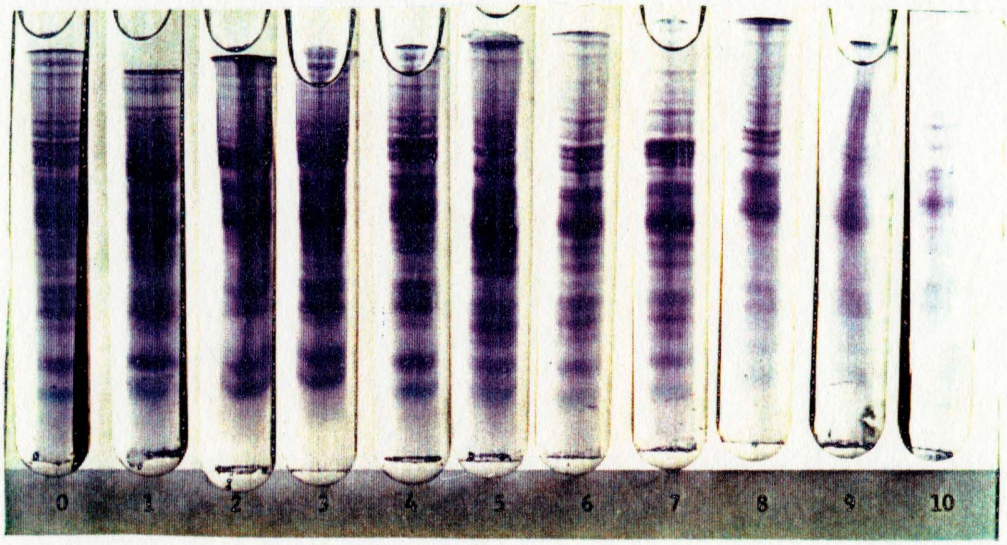
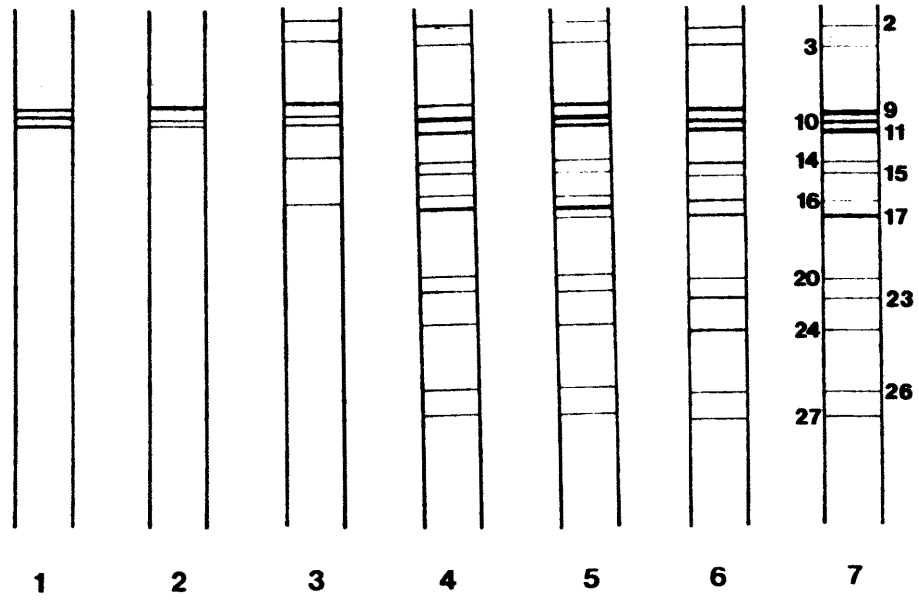

0
1
2
3
4
5
6
7
8
9
10

Figure 19. Diagrammatic representation of testicular protein banding patterns obtained by gel electrophoresis during prepupal ocular ages 1 through 14. Numbers were assigned for each protein band from ocular ages 7 and 14 prepupal testes.


1
2
3
4
5
6
7
2
3
9
10
11
14
15
16
17
20
23
24
26
27

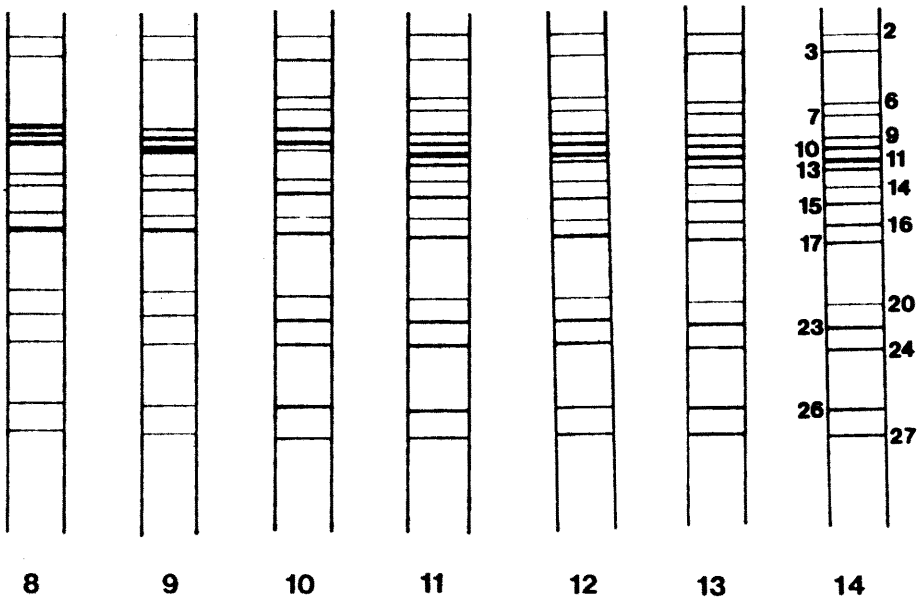

8
9
10
11
12
13
14
2
3
6
7
9
10
11
13
14
15
16
17
20
23
24
26
27

Figure 20. Diagrammatic representation of testicular protein banding patterns obtained by gel electrophoresis during pupal developmental ages. Numbers were assigned for each protein band from 6 day old pupal testes. The day 6 diagram represents the same protein banding pattern obtained in 7, 8, and 9 day old pupae.

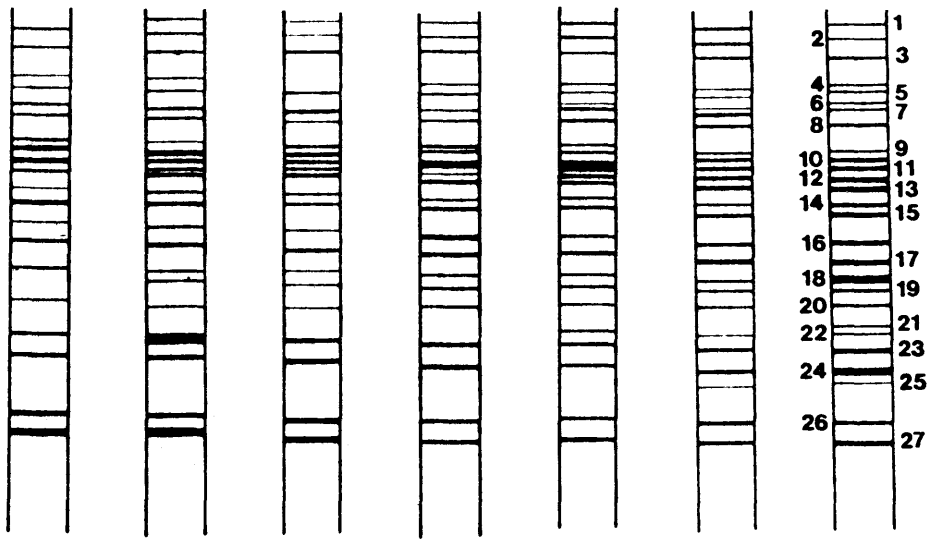
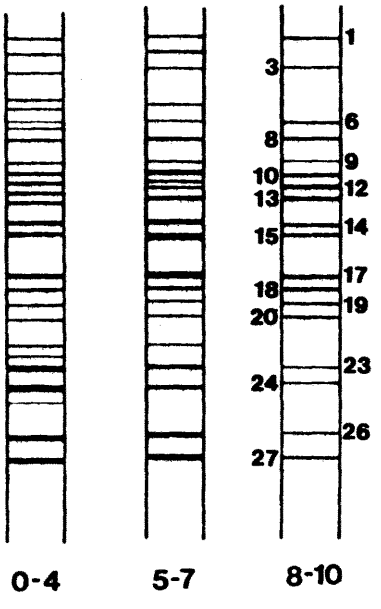

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
0
1
2
3
4
5
6-9

Figure 21. Diagrammatic representation of testicular protein banding patterns obtained by gel electrophoresis during the first 10 days after adult emergence. The three gels represent the patterns obtained from adult animals grouped as follows: 0 to 4, 5 to 7, and 8 to 10 days of age, respectively. Animals at any age within each group had the same testicular protein banding pattern as shown. Numbers were assigned for each protein band from 8 day old adult testes.


1
3
6
8
9
10
12
13
14
15
17
18
19
20
23
24
26
27
0-4
5-7
8-10

were subsequently compared with the banding pattern obtained from six day old pupal testes homogenates. The number of the bands and the relative amount of each protein constituent were resolved densitometrically for all the gels and depicted in Figures 22 through 28. A summary of analysis of gel patterns and densitometer tracings is shown in Table 1. The molecular weights of the testicular protein components were resolved in a range from 12,000 to 127,000 daltons depending on the stage of development. In the early prepupal ocular ages 1, 2, and 3 only a few protein components were observed in the testes. The earliest proteins to appear at ocular ages 1 and 2 were those numbered 9, 10, and 11 and were the most constant protein components observed throughout the developmental process, although their relative concentrations varied dramatically. Additional protein bands were detected as prepupal development continued with a maximum of 17 different protein components present at ocular age 14.

During pupal development, additional testicular protein bands appeared with the maximum number of bands (27) detected during days 6 through 9 of pupal life (Figure 20).

During the adult stage, the number of testicular protein bands decreased from 26 bands observed during days 0 to 4 following emergence, to 22 bands present on days 5 through 7 and, subsequently, to 18 bands on days 8 through 10 of adult life.

Figure 22. Densitometric tracing of the testicular protein banding patterns obtained by gel electrophoresis during the prepupal stage. PP1 through PP4 refer to prepupal ocular age. The numbers of the peaks refer to those specific protein bands detected in 6-day old pupal testes (see Figure 20). Ordinate, absorbance at 525 nm; abscissa, migration distance (cm).

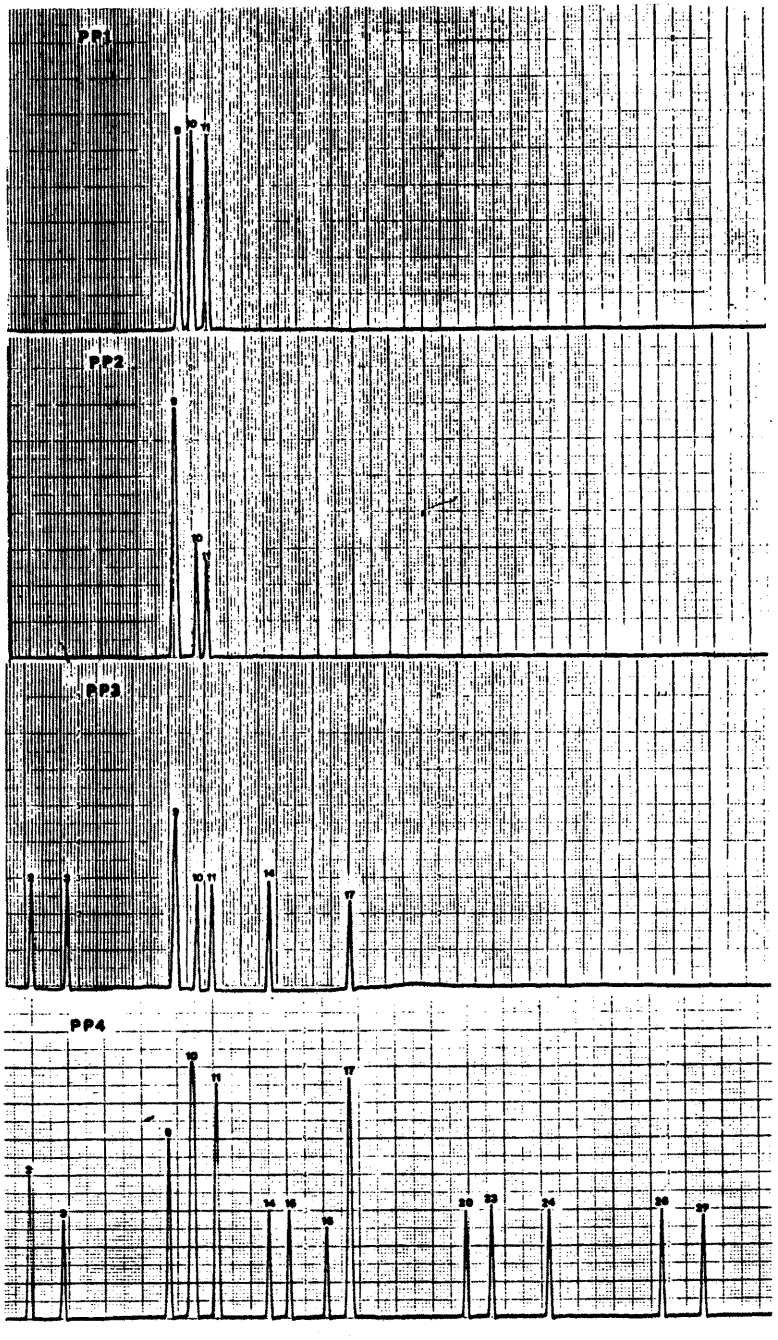

PP1
10
11
PP2
10
11
PP3
10
11
14
17
PP4
10
11
17
14
16
18
20
23
24
26
27

Figure 23. Densitometric tracing of the testicular protein banding patterns obtained by gel electrophoresis during the prepupal stage. PP5 through PP8 refer to prepupal ocular age. The numbers of the peaks refer to those specific protein bands detected in 6-day old pupal testes (see Figure 20). Ordinate, absorbance at 525 nm; abscissa, migration distance (cm).

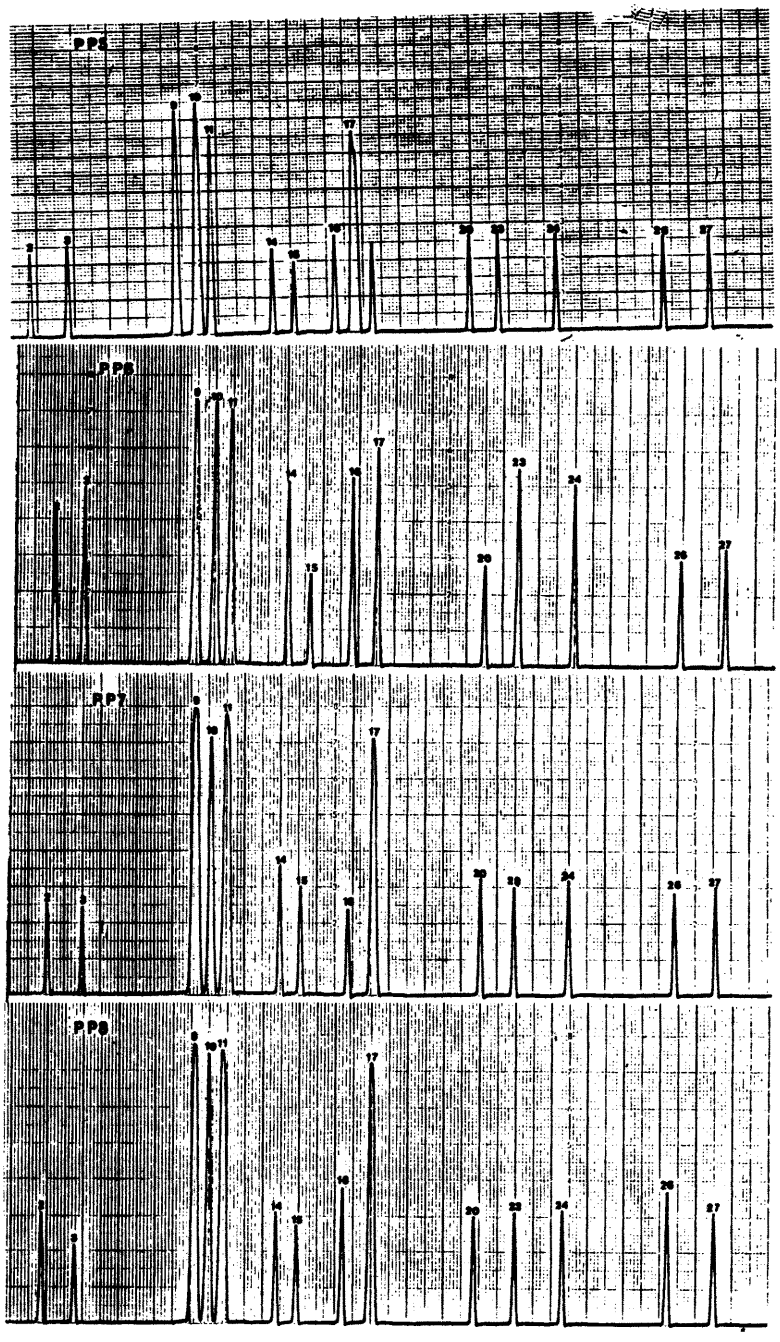

PPS
PPS
PPY
PPS

Figure 24. Densitometric tracing of the testicular protein banding patterns obtained by gel electrophoresis during the prepupal stage. PP9 through PP12 refer to prepupal ocular age. The numbers of the peaks refer to those specific protein bands detected in 6-day old pupal testes (see Figure 20). Ordinate, absorbance at 525 nm; abscissa, migration distance (cm).

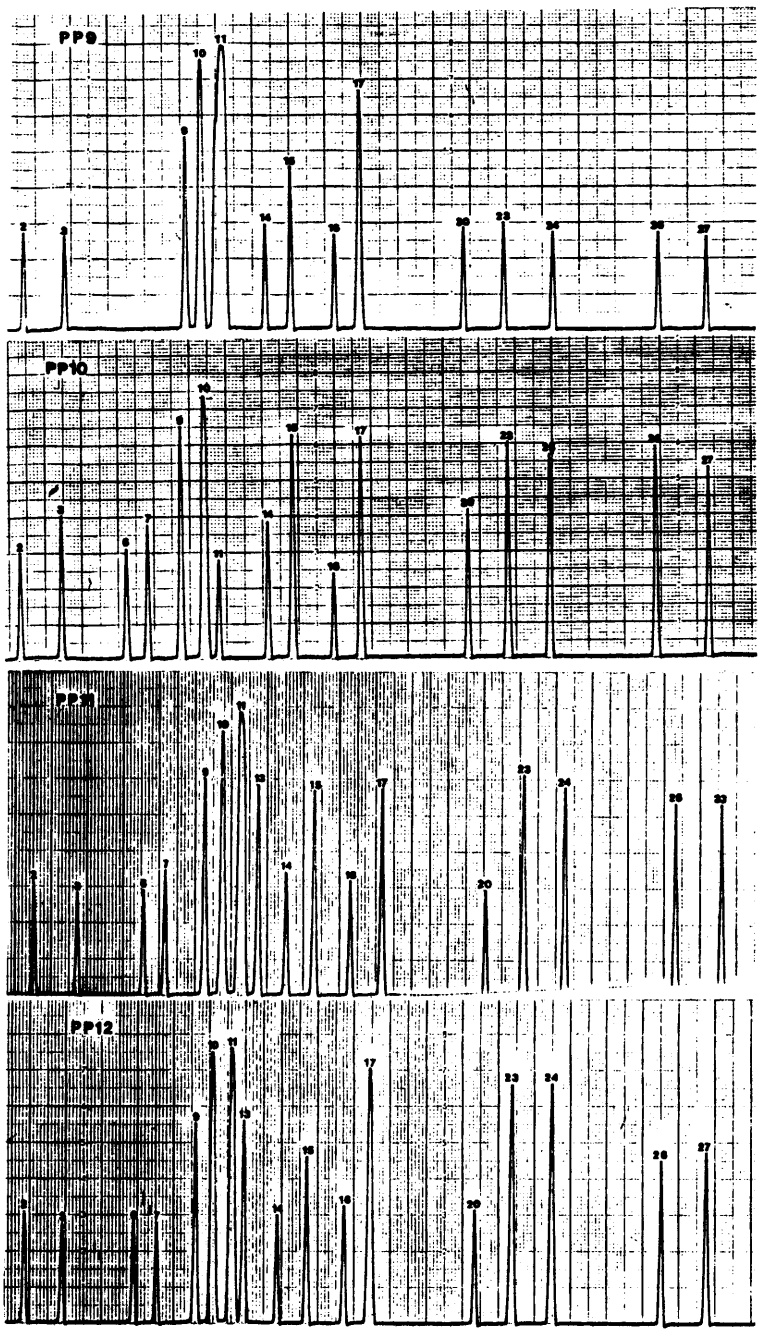

PP9
PP10
PP11
PP12

Figure 25. Densitometric tracing of the testicular protein banding patterns obtained by gel electrophoresis during the prepupal stage. PP13 through PP14 refer to prepupal ocular age. The numbers of the peaks refer to those specific protein bands detected in 6-day old pupal testes (see Figure 20). Ordinate, absorbance at 525 nm; abscissa, migration distance (cm).

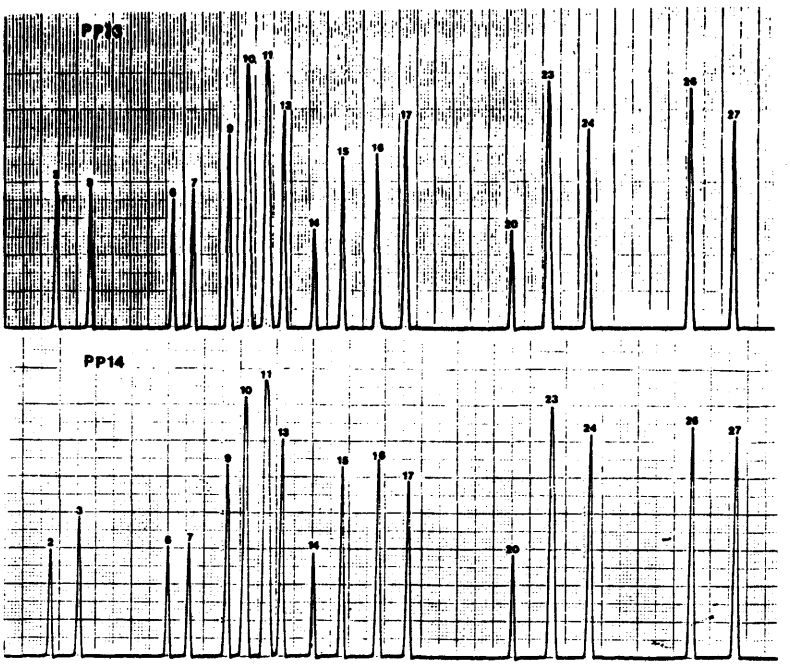

PP13
PP14

Figure 26. Densitometric tracing of the testicular protein banding patterns obtained by gel electrophoresis during the pupal stage. P0 through P3 refer to pupal age in days. The numbers of the peaks refer to those specific protein bands detected in 6-day old pupal testes (see Figure 20). Ordinate, absorbance at 525 nm; abscissa, migration distance (cm).

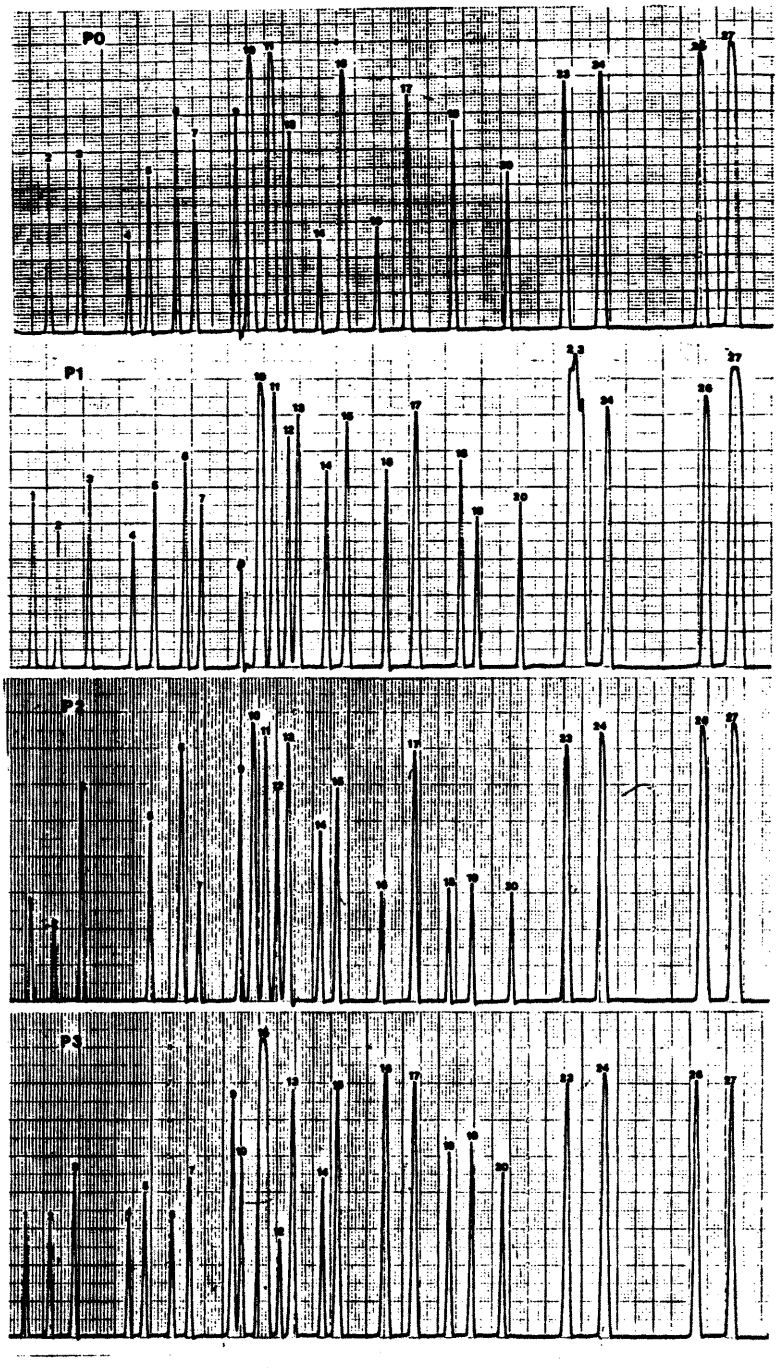

P0
P1
P2
P3

Figure 27. Densitometric tracing of the testicular protein banding patterns obtained by gel electrophoresis during the prepupal stage. P4 through P6 refer to pupal age in days. The numbers of the peaks refer to those specific protein bands detected in 6-day old pupal testes (see Figure 20). Ordinate, absorbance at 525 nm; abscissa, migration distance (cm).

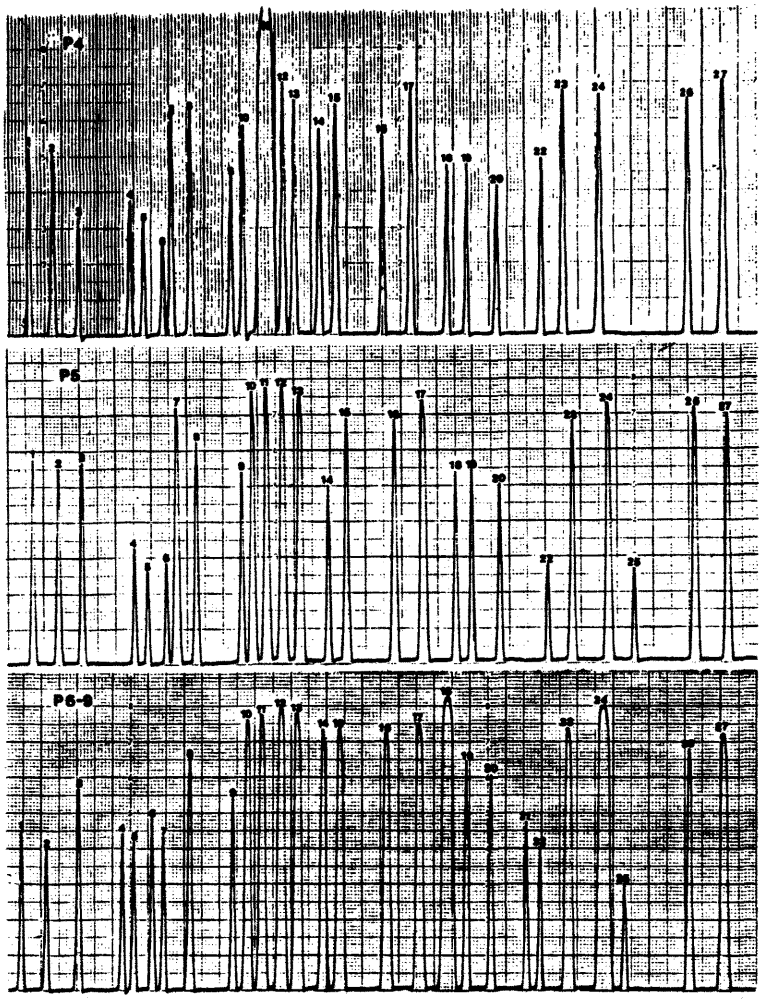

P4
P5
P6-8

Figure 28. Densitometric tracing of the testicular protein banding patterns obtained by gel electrophoresis during the adult stage. A0-4, A5-7, and A8-10 refer to adult age in days. The numbers of the peaks refer to those specific protein bands detected in 6-day old pupal testes (see Figure 20). Ordinate, absorbance at 525 nm; abscissa, migration distance (cm).

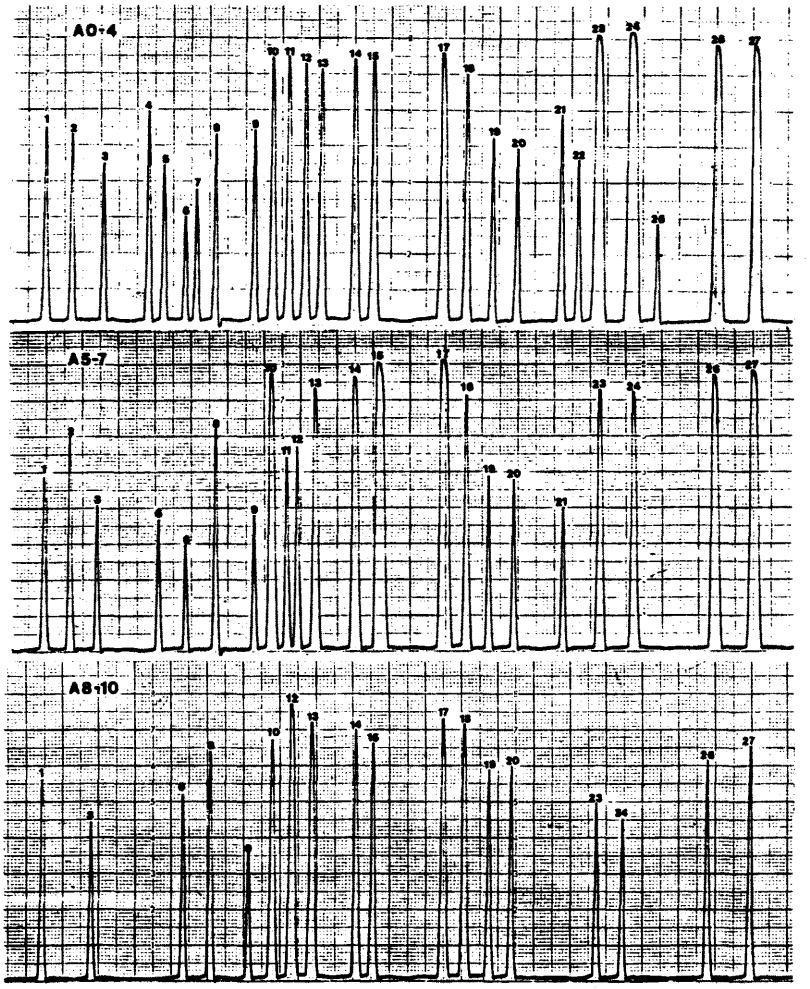

A0-4
A5-7
A8-10

Table 1. Summary of qualitative changes in the Tenebrio testicular protein banding pattern resolved by gel electrophoresis during developmental stages.

Protein Band Number	Molecular Weight (dalton X 10^4)	Prepupae (ocular age)														Pupal Stage (days)										Adult Stage (days)										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	10
1	12.7															*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	12.4			—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	12.0			—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	11.6															—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				
5	11.2															—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
6	11.1										—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	10.9										—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				
8	10.5																			—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	10.1		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	9.9		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	9.4		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
12	9.0																—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
13	8.1											—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	7.2			—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	6.8				—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16	5.4				—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—										
17	4.7			—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	4.2															—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
19	3.7																—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	3.3				—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
21	2.8																					—	—	—	—	—	—	—	—	—	—	—	—			
22	2.7																			—	—	—	—	—	—	—	—	—	—	—	—	—				
23	2.4				—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
24	2.0				—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
25	1.9																			—	—	—	—	—	—	—	—	—	—	—	—	—				
26	1.4				—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
27	1.2				—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

*Line indicates presence of proteins.

The relative concentration of the testicular protein bands 9 through 17, 23-24, and 26-27 varied dramatically throughout all developmental stages.

Incorporation of [^{3}H]-Leucine

The incorporation of ^{3}H-leucine into the TCA-precipitable testicular protein fraction was determined from animals during the pupal and adult stages. Preliminary experiments were conducted to ascertain the appropriate conditions with respect to dose and time of exposure of the radioactive precursor. The incorporation of ^{3}H-leucine into the testicular proteins of 1 day old pupae was essentially linear up to eight hours following injection of the radioactive precursor (Figure 29). The effect of increasing the amount of ^{3}H-leucine injected into one day old pupae on the incorporation of the radioactive precursor into testicular proteins is illustrated in Figure 30. The incorporation rate was essentially linear with doses of ^{3}H-leucine ranging from 0.125 μCi up to 0.5 μCi while larger doses did not substantially increase the incorporation rate. In all subsequent experiments, 0.5 μCi of ^{3}H-leucine were injected into pupae and adults 6 hours prior to analysis of the incorporation of the radioactive precursor into the testicular proteins.

Since TCA was used to precipitate the testicular protein fraction it was important to determine if TCA caused quenching during the radioassay procedure. Therefore, all filters were treated with a final

Figure 29. Time course of incorporation of ^{3}H-leucine into TCA-precipitable testicular protein of one day old pupae. Animals were sacrificed at several times following injection of 0.5 μCi. Each point represents the average incorporation rate of 3 animals.

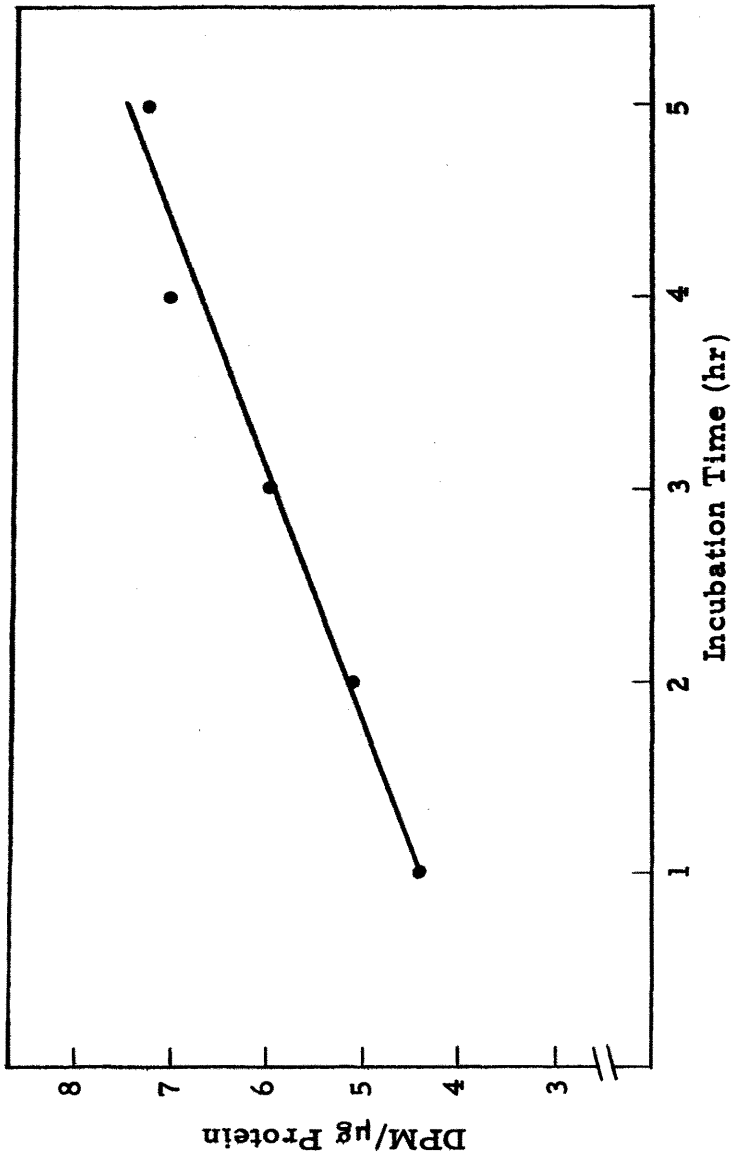
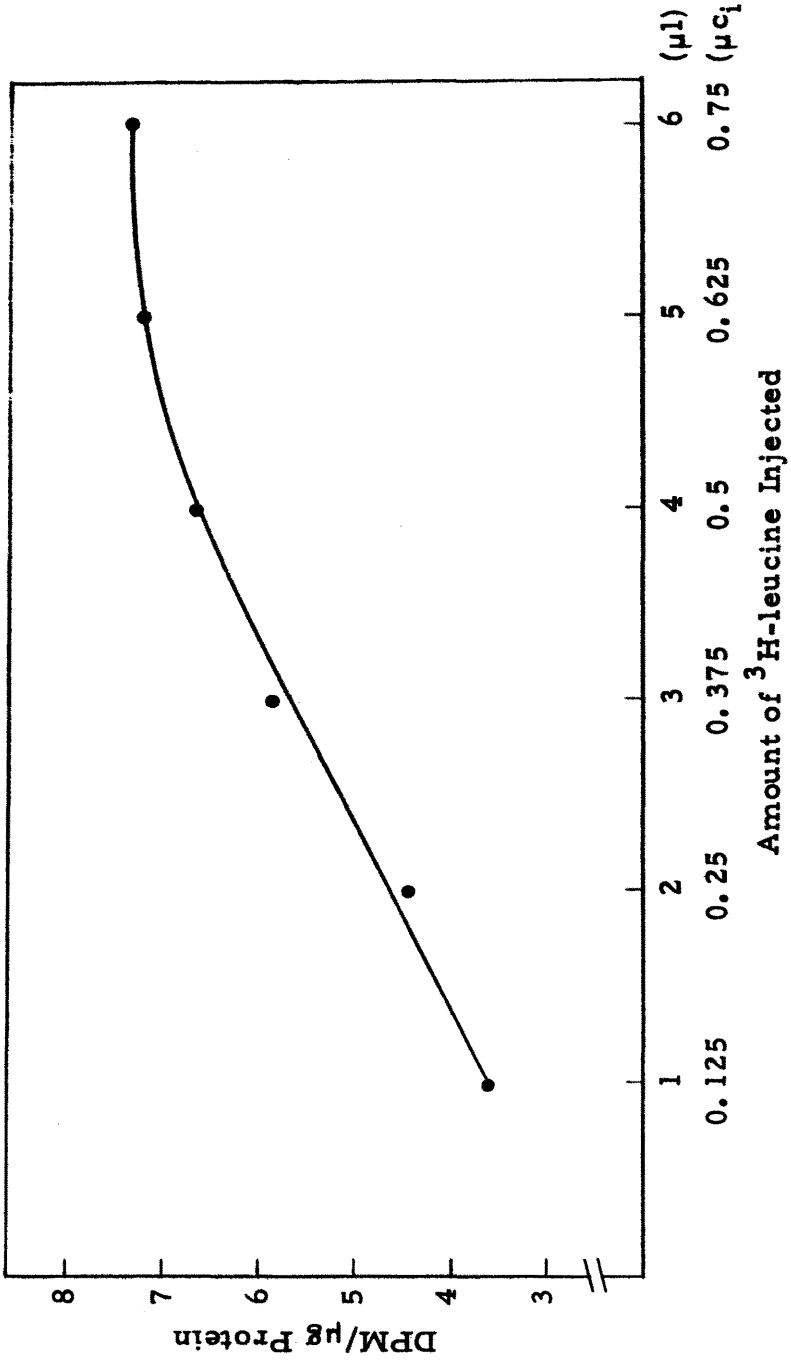

DPM/μg Protein
8
7
6
5
4
3
1
2
3
4
5
Incubation Time (hr)

Figure 30. Incorporation of ^{3}H-leucine into the TCA-precipitable testicular protein of one day old pupae injected with increasing amounts of ^{3}H-leucine. Animals were sacrificed after 6 hours of incubation. Each point represents the average incorporation rate of 3 animals.


DPM/μg Protein
8
7
6
5
4
3
1
2
3
4
5
6 (μl)
0.125
0.25
0.375
0.5
0.625
0.75 (μci)
Amount of 3H-leucine Injected

wash of ether in order to remove traces of TCA prior to air-drying the filters and subsequent radioassay. The results (not shown) demonstrated that no detectable quenching was observed in random samples washed with ether.

Figure 31 illustrates the rate of ^{3}H-leucine incorporation into TCA-precipitable testicular proteins during the pupal stage. The results demonstrated a substantial increase in the incorporation of ^{3}H-leucine into testicular protein at day 2 of pupal development, a slight decrease on day 3, and a subsequent increase at day 4. The rate of incorporation reached a maximum at day 5 and then declined again through last day of pupal life.

Figure 32 demonstrates the incorporation rate of ^{3}H-leucine into testicular protein during the first 10 days of adult life. The incorporation rate of ^{3}H-leucine into testicular protein increased on day 1 following emergence, decreased during the following two days, apparently increased once more at day 4, and remained relatively unchanged until day 6. The incorporation rate declined through day 10 of adult life and reached the lowest average value during this stage.

The incorporation of ^{3}H-leucine into specific testicular protein components during the pupal and adult stages was analyzed by gel electrophoresis and gel slicing methods described in the Materials and Methods. The resulting radioactive slices were correlated with the testicular proteins based on relative mobilities using the

Figure 31. The rate of ^{3}H-leucine (0.5 μCi) incorporation into the TCA-precipitable testicular protein during each day of pupal development. Each point represents the mean of at least seven testes pairs; bars indicate standard errors.

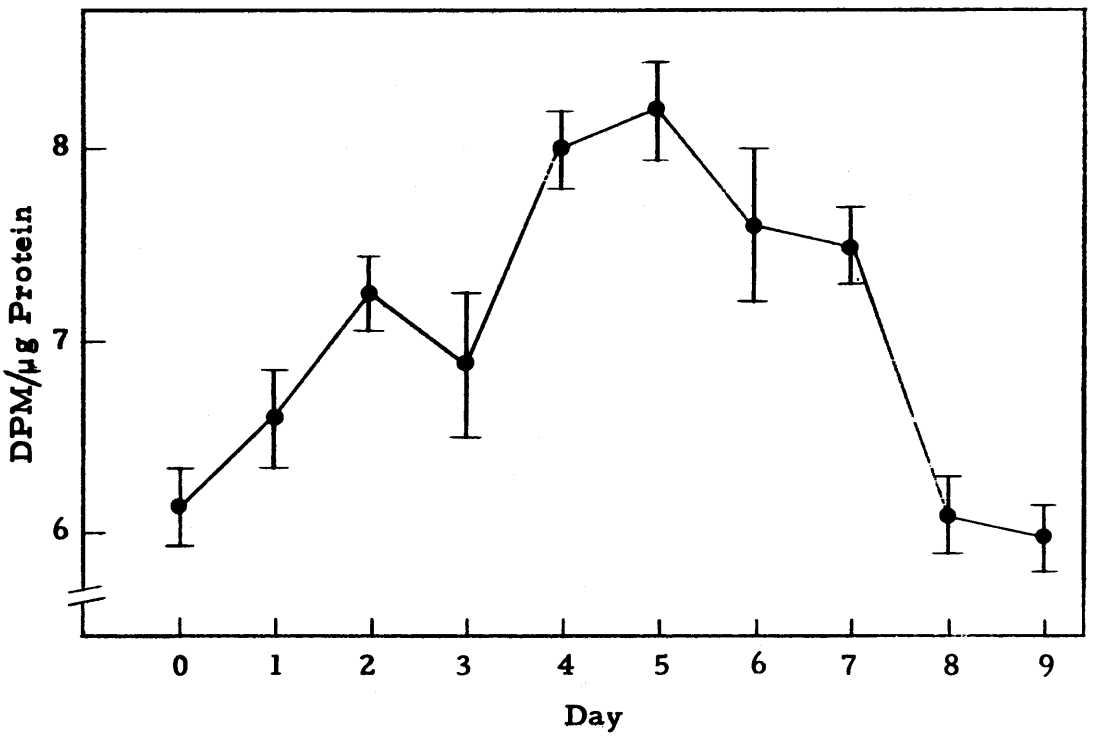
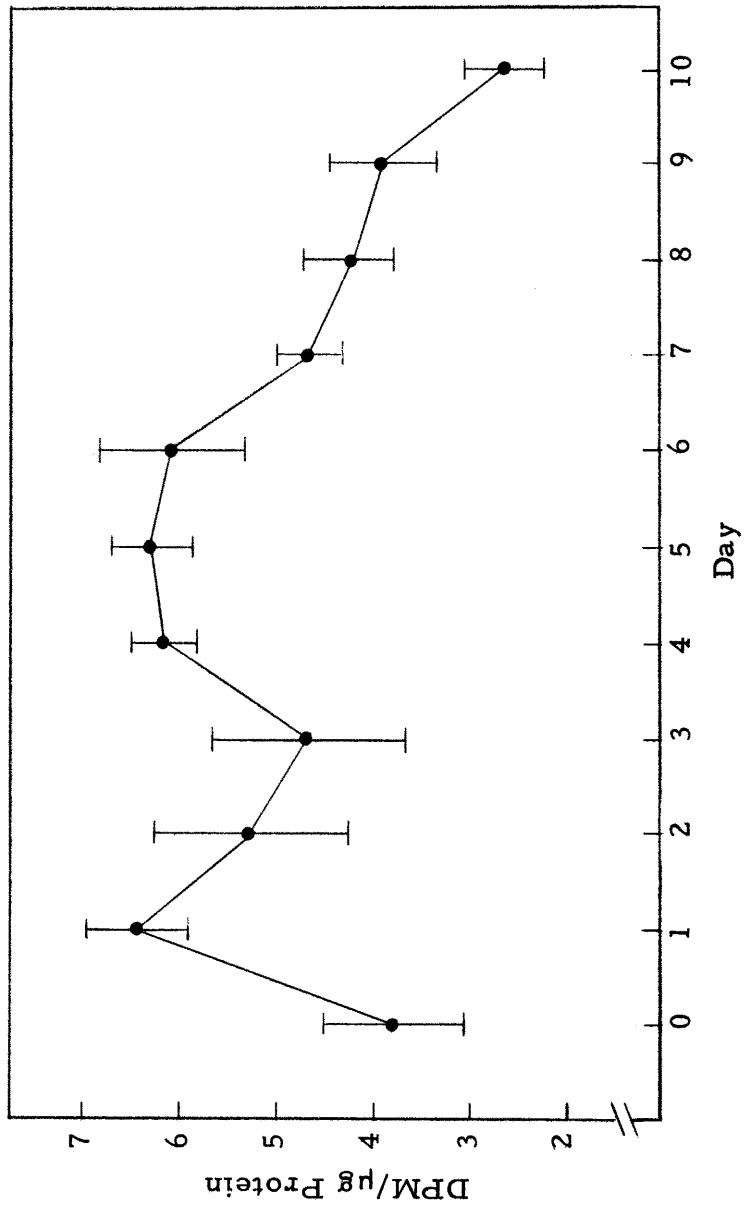

DPM/µg Protein
8
7
6
0
1
2
3
4
5
6
7
8
9
Day

Figure 32. The rate of ^{3}H-leucine (0.5 μCi) incorporation into the TCA-precipitable testicular protein during the first 10 days after adult emergence. Each point represents the mean of at least seven testes pairs; bars indicate standard errors.


DPM/μg Protein
7
6
5
4
3
2
0
1
2
3
4
5
6
7
8
9
10
Day

previously described protein numbering system. Figure 33 depicts the incorporation pattern of ^{3}H-leucine into various testicular protein components during the pupal stage of development. Not all of the 27 protein bands identified during pupal development were shown to have an increased incorporation of the radioactive precursor. The fewest number of testicular protein components into which ^{3}H-leucine was incorporated, as well as the lowest amount of incorporation of the radioactive precursor, was observed on days 0, 1, and 9 of pupal life. The largest number of testicular protein components containing ^{3}H-leucine was observed on days 6 and 7 and corresponded with the highest amount of incorporated radioactivity observed on those days.

Figure 34 demonstrates that little incorporation of ^{3}H-leucine into testicular protein components occurred during adult life. Concomitantly, low incorporation levels were associated with only a few protein bands indicating that synthesis of specific testicular proteins was lower during the adult stage than during pupal development.

Testes from 6 day old pupae were incubated in vitro in the presence of ^{3}H-leucine and the incorporation pattern of the radioactive precursor into testicular protein components determined. Figure 35 illustrates that essentially the same incorporation pattern occurred whether ^{3}H-leucine was administered in vitro or in vivo

Figure 33. The incorporation pattern of ^{3}H-leucine into testicular protein components resolved by gel electrophoresis during the pupal stage. P0 through P9 represent the pupal age in days. The numbers on each major peak refer to the specific testicular protein bands obtained from 6 day old pupal testes (see Figure 20). Ordinate, DPM x 10^{-2}; abscissa, gel slice number.

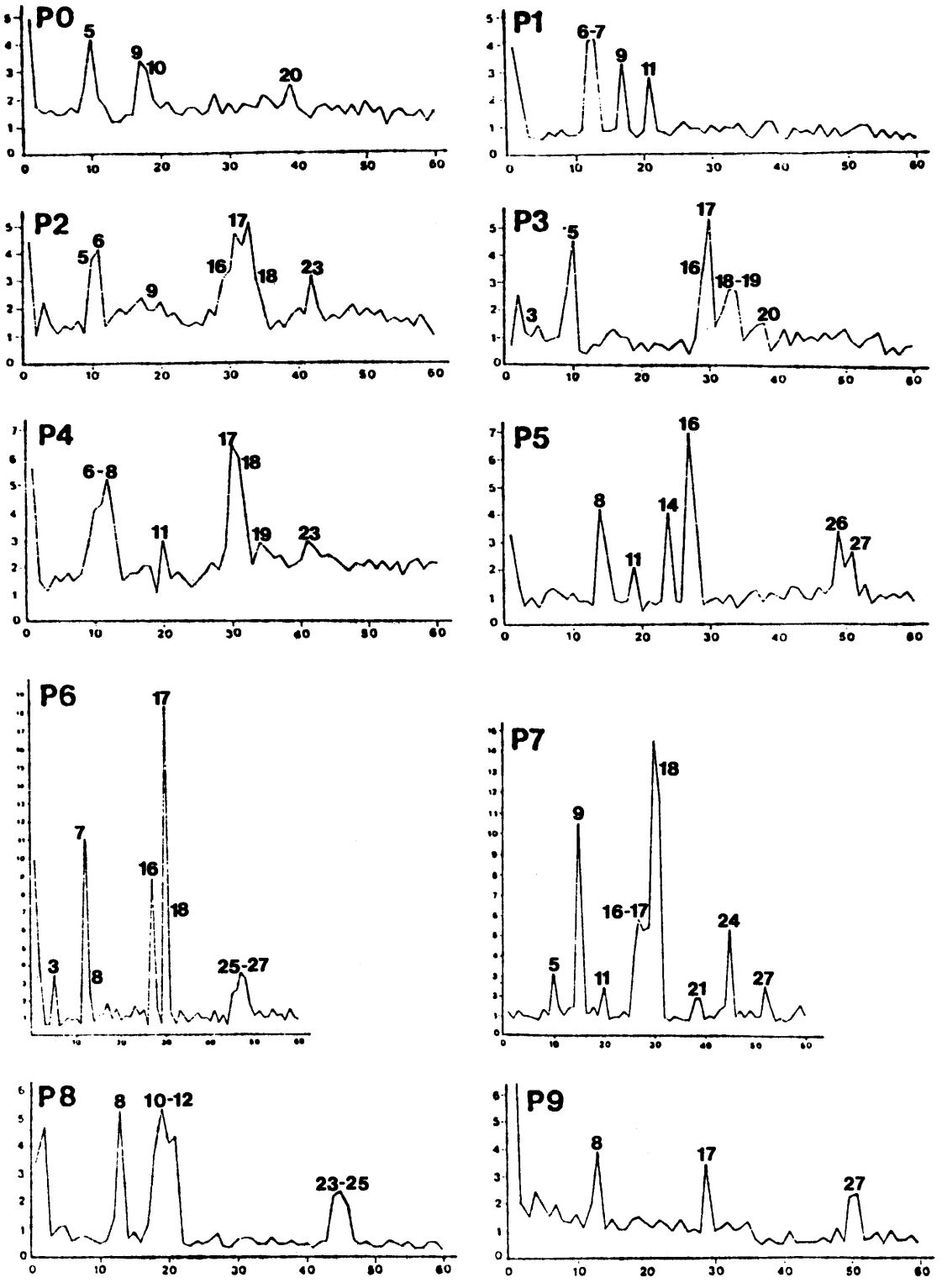

P0
5
9
10
20
P1
6-7
9
11
P2
5
6
9
16
17
18
23
P3
3
5
16
17
18-19
20
P4
6-8
11
17
18
19
23
P5
8
11
14
16
26
27
P6
3
7
8
16
17
18
25-27
P7
5
9
11
16-17
18
21
24
27
P8
8
10-12
23-25
P9
8
17
27

Figure 34. The incorporation pattern of ^{3}H-leucine into testicular protein components resolved by gel electrophoresis during the first 10 days after adult emergence. A0 through A10 represent the adult age in days. The numbers on each major peak refer to the specific testicular protein bands (see Figure 20). Ordinate, DPM x 10^{-2}; abscissa, gel slice number.

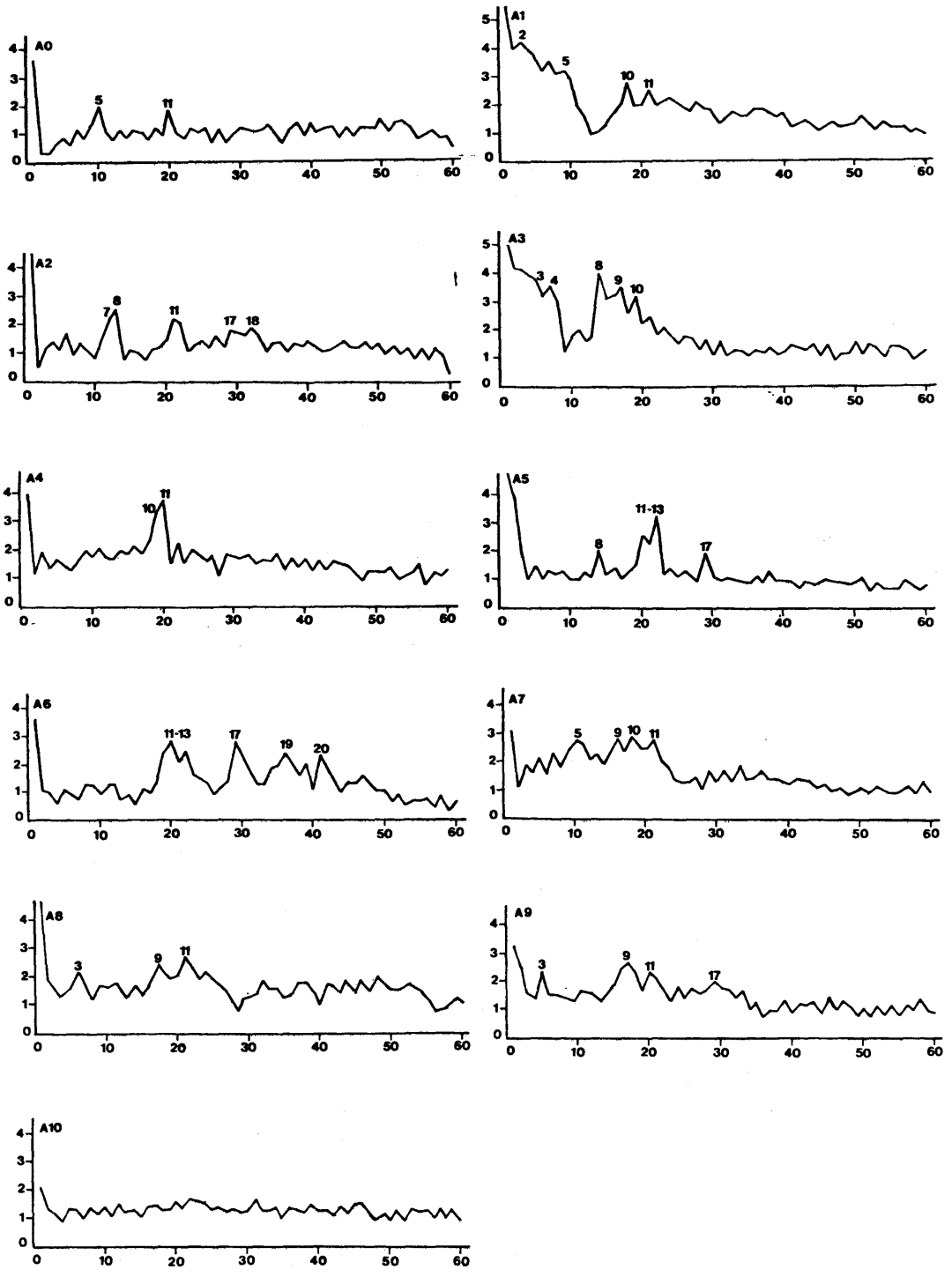
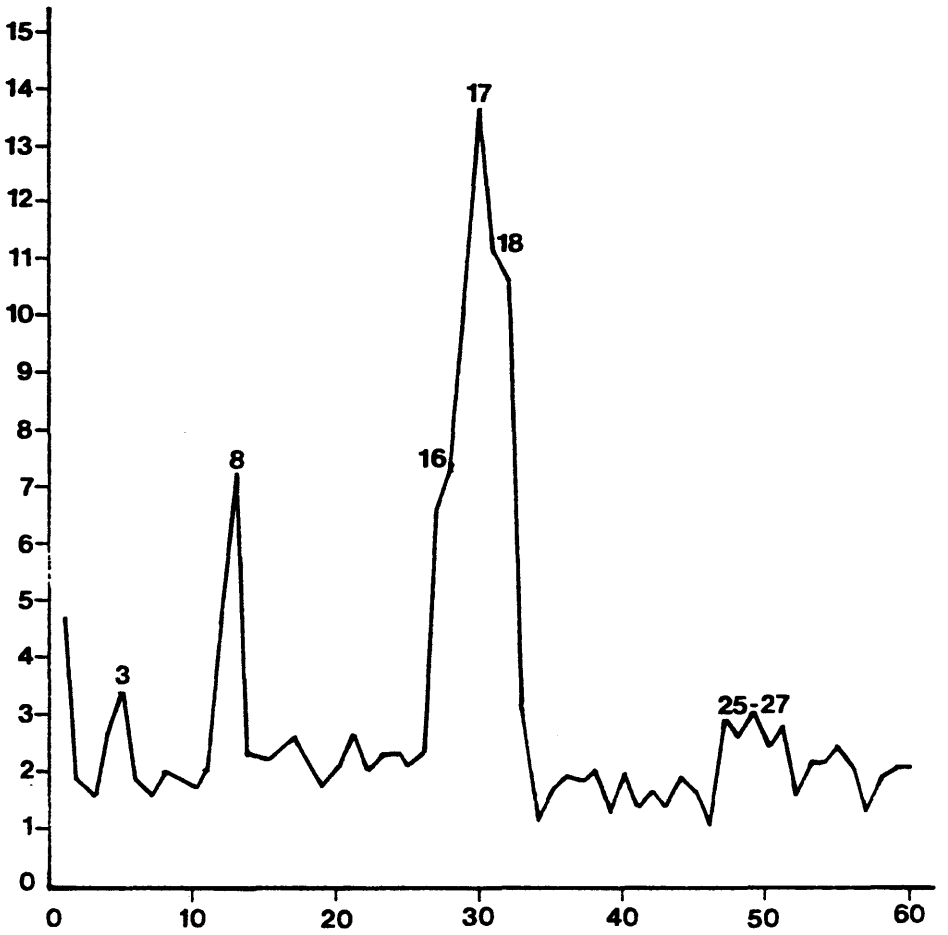

A0
5
11
A1
2
5
10
11
A2
7
8
11
17
18
A3
3
4
8
9
10
A4
10
11
A5
8
11-13
17
A6
11-13
17
19
20
A7
5
9
10
11
A8
3
9
11
A9
3
9
11
17
A10

Figure 35. In vitro incorporation of ^{3}H-leucine into testicular protein components resolved by gel electrophoresis from 6-day old pupae. The numbers on each major peak refer to the specific testicular protein bands (see Figure 20). Ordinate, DPM x 10^{-2}; abscissa, gel slice number.


15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
3
8
16
17
18
25-27

(Figure 33) although a slightly reduced incorporation was observed in vitro.

Hormonal Effects

The effect of juvenile hormone I on the incorporation of ^{3}H-leucine into TCA-precipitable testicular proteins during pupal development is shown in Figure 36. The dose of juvenile hormone (1 μg per animal) was selected because of its ability to enhance the 6 hour incorporation rate of ^{3}H-leucine into testicular proteins of 8 day old pupa when compared with lower doses of juvenile hormone (data not shown). Eight day old pupae were selected because the titer of juvenile hormone was assumed to be low near the end of pupal development. Juvenile hormone did not significantly alter the amount of ^{3}H-leucine incorporated into testicular proteins at any pupal age when compared with non-treated control animals at the same stage of development.

The effect of ecdysterone on the incorporation of ^{3}H-leucine into TCA-precipitable testicular protein during pupal development is shown in Figure 37. The dose of ecdysterone used (0.5 μg per animal) was selected because it did not cause precocious development while it enhanced the six hour incorporation rate of ^{3}H-leucine into testicular proteins of one day old pupae when compared to lower doses of ecdysterone (data not shown). One day old pupae were used because the titer of molting hormone was presumed to be low following

Figure 36. The effect of juvenile hormone (1 μg/animal) on the incorporation rate of ^{3}H-leucine into the TCA-precipitable testicular protein during the pupal stage. The values obtained represent the mean ± standard error from seven testes pairs. JH-I: juvenile hormone I.

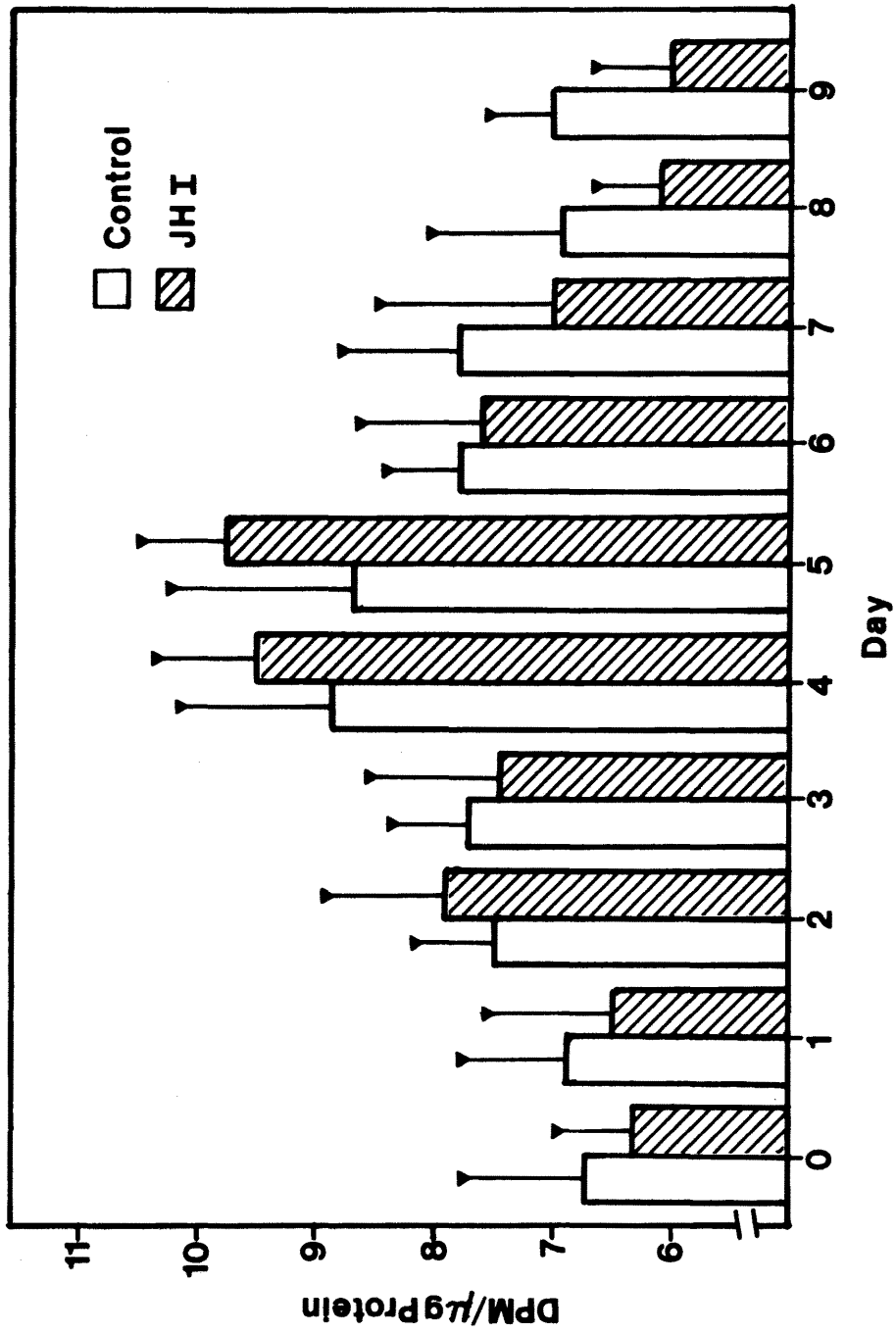
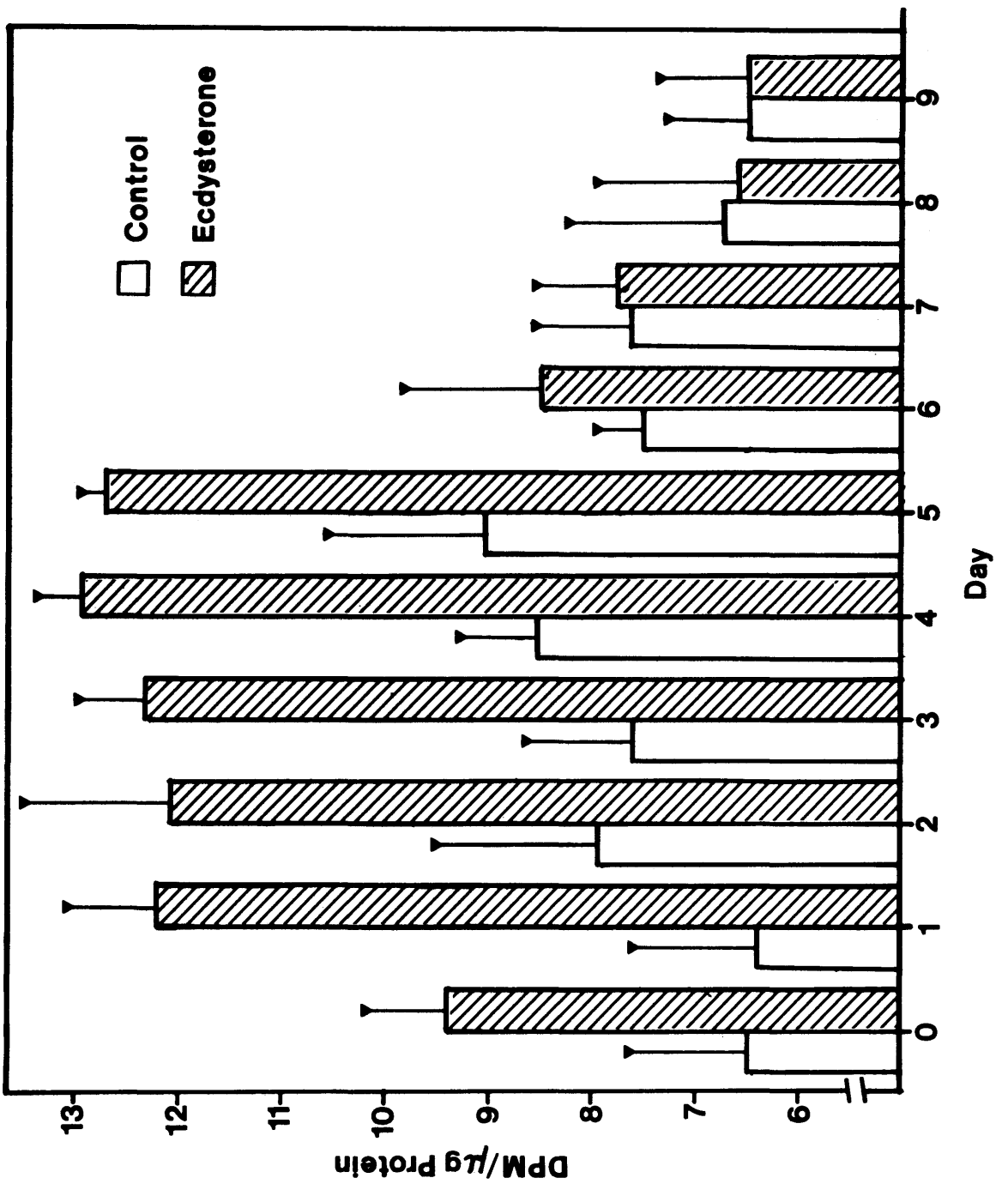

Control
JH I
DPM/μg Protein
11
10
9
8
7
6
0
1
2
3
4
5
6
7
8
9
Day

Figure 37. The effect of ecdysterone (0.5 μg/animal) on the incorporation rate of ^{3}H-leucine into the TCA-precipitable testicular protein during the pupal stage. The values obtained represent the mean ± standard error from seven testes pairs.


Control
Ecdysterone
DPM/μg Protein
13
12
11
10
9
8
7
6
0
1
2
3
4
5
6
7
8
9
Day

emergence. Ecdysterone caused a significant increase in the incorporation of ^{3}H-leucine into testicular proteins through day 5 of pupal development when compared to non-injected control animals. No apparent differences in incorporation rates between control and ecdysterone-treated animals were observed from day 6 through day 9 of pupal development.

The effect of a combination of ecdysterone and juvenile hormone administered to animals at 0, 1, and 8 days of pupal development on the incorporation of ^{3}H-leucine into testicular proteins is demonstrated in Figure 38. The administration of both hormones resulted in an enhancement of ^{3}H-leucine incorporation into testicular proteins on day 0 and day 1 of pupal development, when compared to the incorporation rate of control and juvenile hormone treated animals, however, the combined hormone treatment was not appreciably different from animals treated with ecdysterone alone. No apparent differences in ^{3}H-leucine incorporation were detected in 8 day old pupae regardless of the hormonal treatment when compared to the non-treated control animals.

The effect of ecdysterone (0.5 μg per animal) on the incorporation rate of ^{3}H-leucine into specific testicular protein components of pupae 0 to 6 days old was determined using polyacrylamide gel electrophoresis and gel slicing methods described in the Material and Methods section. The results, illustrated in Figure 39, indicated that the

Figure 38. The effect of combined ecdysterone and juvenile hormone administration on the incorporation rate of ^{3}H-leucine into the TCA-precipitable testicular protein from 0, 1, and 8 day old pupae. The values obtained represent the mean $\pm$ standard error from seven testes pairs. JH-I: juvenile hormone I.

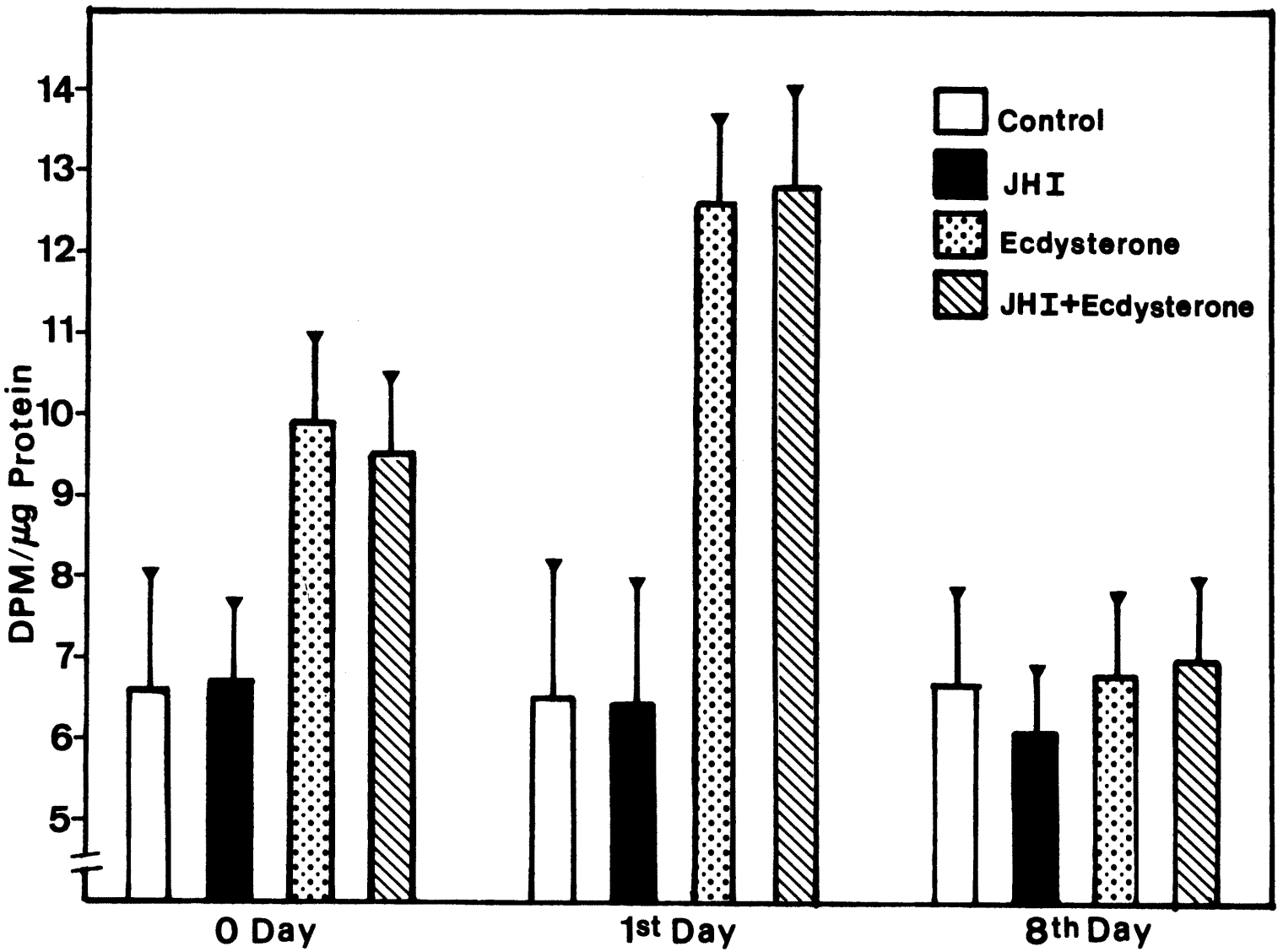
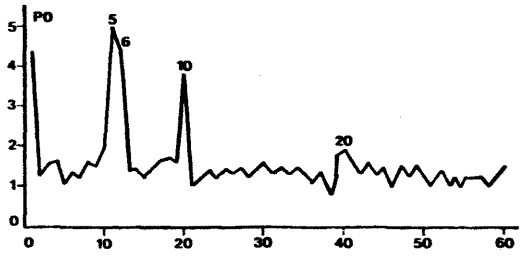
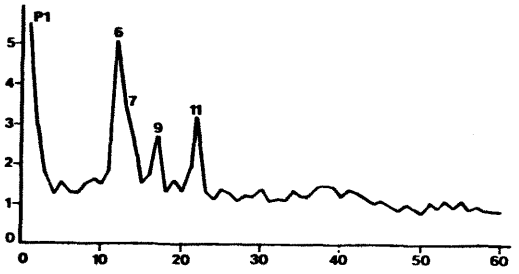
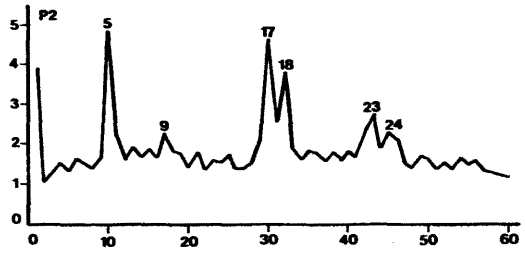
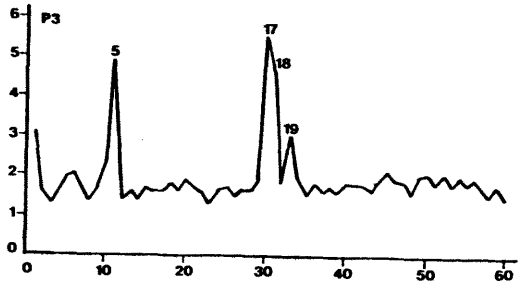

DPM/μg Protein
14
13
12
11
10
9
8
7
6
5
Control
JHI
Ecdysterone
JHI+Ecdysterone
0 Day
1st Day
8th Day

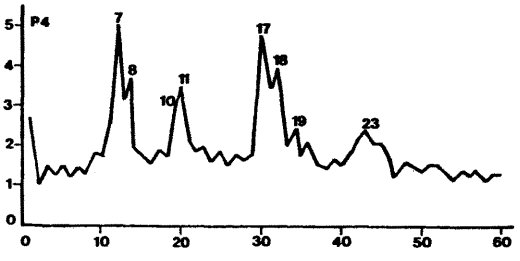
Figure 39. The effect of ecdysterone (0.5 μg/animal) administration on the incorporation pattern of ^{3}H-leucine into testicular protein components resolved by gel electrophoresis during the pupal stage. P0 through P6 represent the pupal age in days. The numbers on each major peak refer to the specific testicular protein bands obtained from 6 day old pupal testes (see Figure 20). Ordinate, DPM x 10^{-2}; abscissa, gel slice number.

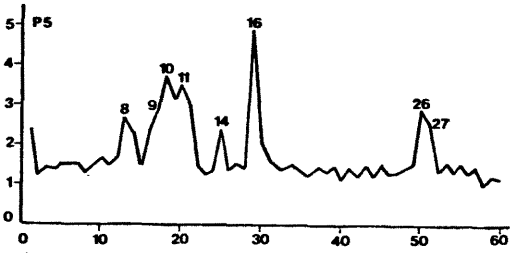

P0
5
6
10
20

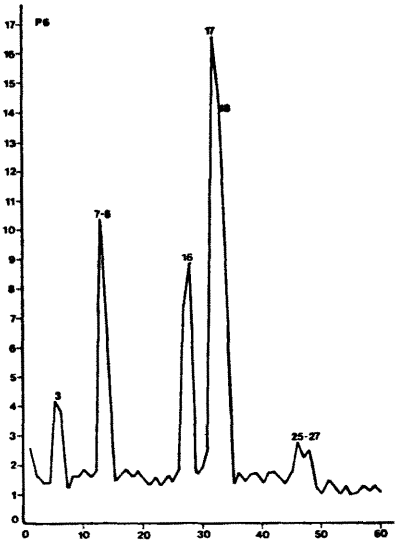

P1
6
7
9
11


P2
5
9
17
18
23
24


P3
5
17
18
19


P4
7
8
10
11
17
18
19
23

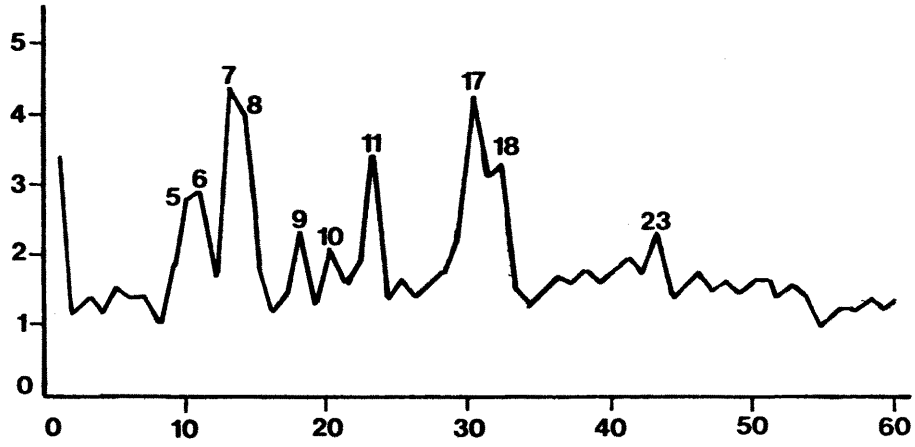

P5
8
9
10
11
14
16
26
27

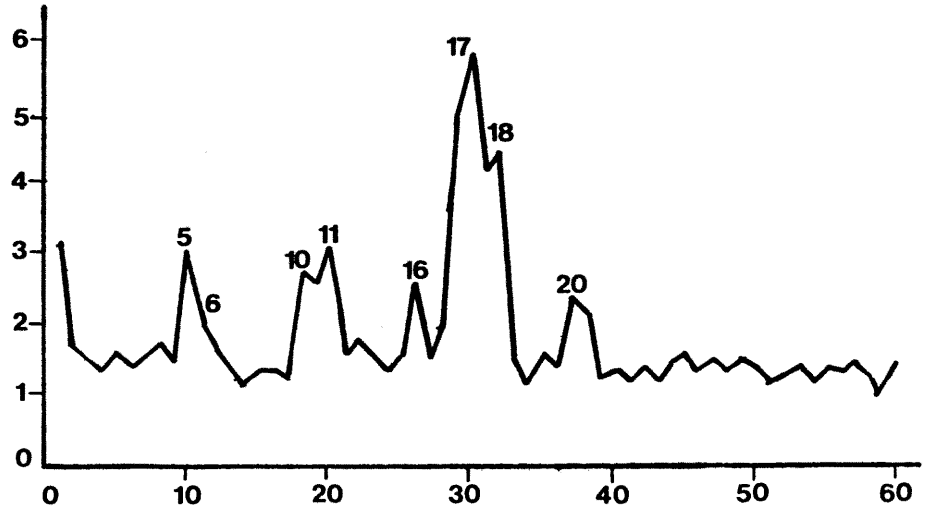

P6
3
7-8
16
17
25-27

pattern and rate of ^{3}H-leucine incorporation into specific testicular protein bands after ecdysterone administration during day 0 through day 6 resembled the pattern of ^{3}H-leucine incorporation observed in non-treated animals (Figure 33).

The results illustrated in Figure 40 were obtained when a higher dose of ecdysterone (1.5 μg per animal, a dose sufficient to accelerate pupal development) was administered to pupa at 1 and 3 days of age and the incorporation of ^{3}H-leucine into testicular protein components determined. Ecdysterone administration stimulated the incorporation of ^{3}H-leucine into testicular protein components (protein bands number 5, 8, 10, 17, 18, and 23 at day 1 old pupae and protein bands number 10 and 11 at day 3 old pupae) that were not labeled when testes from non-treated animals at the same stage of development were analyzed.

Figure 40. The effect of ecdysterone (1.5 μg/animal) administration on the incorporation pattern of ^{3}H-leucine into testicular protein components resolved by gel electrophoresis for 1 day and 3 day old pupae. The numbers on each major peak refer to the specific testicular protein bands obtained from 6 day old pupal testes (see Figure 20). Ordinate, DPM x 10^{-2}; abscissa, gel slice number.


5
4
3
2
1
0
0
10
20
30
40
50
60
5
6
7
8
9
10
11
17
18
23


6
5
4
3
2
1
0
0
10
20
30
40
50
60
5
6
10
11
16
17
18
20

DISCUSSION

Because developing secretory glands often pass through subsequent stages of progressive biosynthetic capacity of different essential elements, they have proven to be of practical value for the study of numerous structures and their properties. These model systems range from the rat pancreas (Rutter et al., 1968) and chick oviduct (Palmiter et al., 1971) to the insect ovarian follicle (Paul and Kafatos, 1975). The insect testes offer equally favorable opportunities for resolving questions concerning insect endocrinology, reproductive physiology, and biochemistry. As an approach to the resolution of the endocrinological paradox which may govern the underlying biochemical shift at the molecular level of insect reproductive system differentiation, this investigation described aspects of biochemical differentiation in Tenebrio molitor testes in addition to the influence of ecdysterone and juvenile hormone (JH) upon these biochemical indices.

The results obtained in this study demonstrated that the testes of Tenebrio underwent organogenesis during the prepupal stage and continued differentiation through the adult stage by passing through phases of cell proliferation and cell specialization. During differentiation, the testes exhibited increases in biosynthetic capacity reflected

in alterations in testicular protein and RNA. The biochemical differentiation was found to be correlated and influenced by the endogenous and/or exogenous presence of hormones.

The testes of Tenebrio underwent dramatic increases in size which were reflected in increased testes weight. This increase began at the prepupal stage and continued through pupal life and the first week of adult life. The noticeable growth of the testes during developmental stages can be attributed largely to the acceleration of the mitotic and meiotic machinery and subsequently to the accumulation of more spermatozoa. This may all be affected by the hormonal milieu of the insect, especially ecdysterone. After adult eclosion, testicular organogenesis has already been completed and the testes are occupied by fully differentiated sperm. The adult becomes sexually mature by the fifth day of adult life after the maturation of the secondary sex organs (Gerber, 1973; 1975).

The maturation process of the germ cells in mealworm beetles proceeded from the distal end of the testis follicle, where the apical cells are located (Menon, 1969), and toward the basal region, where the vasa efferentia are located, to form a "differentiation wave." This zone of maturation (spermatogonia and spermatocytes) was followed by a zone of spermatids and then by the accumulated mass of mature spermatozoa. In the adult stage, this gradient of developmental stages was less obvious as sperm became the predominant

cell type. Silkworm testes were found to contain the same sequence of differentiation and maturation of the germ cells as found in Tenebrio testes (Toshimori et al., 1979). This type of sequencing in mealworm testes may reflect some differentiative mechanism which controls the transformation and development of each germ cell type to the subsequent type, in addition to the accumulation of one call type at a specific age of the animal as occurred with spermatocytes in the late prepupal and early pupal testes. Takeuchi (1969), working with Bombyx mori testes, regarded the accumulation of spermatocytes at specific ages as a result of a greater inhibitory effect on meiotic than mitotic division caused by JH. Economopolous and Gordon (1971), in a similar study on Oncopeltus fasciatus, concluded that the accumulation of spermatocytes occurred as a result of the mitotic proliferation of spermatogonia and the failure of the resting spermatocytes to undergo meiosis. In Rhodnius prolixus, Dumser and Davey (1974) reported that accumulation in some of the germ cell types occurred as a result of the lengthy period of meiotic phase maturation.

An interesting histological observation in the late prepupal testis is the presence of differentiated spermatozoa at the basal region of the follicle. Such observations have been reported in some holometabolus insects. The germ cells in Aedes aegypti may begin the process of differentiation of spermatids into spermatozoa within

the terminal cysts just before pupation occurs (Jones, 1969). Garcia-Bellido (1964a) and Hardy et al. (1979) observed that spermiogenesis began in the larval testis of Drosophila melanogaster and continued through the adult stage. Although Economopoulos and Gordon (1971) stated that meiosis and the formation of differentiated spermatozoa were not common occurrences in the larval stage of holometabolus insects, no references were cited substantiating their claim. The results obtained in this study concerning the presence of spermatozoa in the late prepupal stage can be attributed to the rapid rate of cell division (meiotic) in the spermatocytes and the process of sperm formation. This acceleration may be triggered by the increasing level of endogenous ecdysterone at this time of insect life (Delbecque et al., 1978a) thus preparing the larva to enter the pupal stage by initiating pupal ecdysis. In addition, the decreasing titer of JH during the last larval stage previously reported in many insect species may contribute to the maturation of sperms during the prepupal stage of Tenebrio. It appeared that the time of commitment or reprogramming of the testes occurred late in the prepupal stage when JH levels are presumably minimal and when there was a significant increase in the ecdysterone titer. Meanwhile, cellular growth and germ cell differentiation, which were most likely completed before adult emergence, ensured ample supplies of spermatozoa that were released from the testes. In Drosophila melanogaster, spermiogenesis begins in the

larval testis and the differentiation of older cysts continues suggesting a competition between proliferating spermatogonia and differentiating spermatids for a common, necessary substrate (Garcia-Bellido, 1964a).

As the morphological differentiation of the testes proceeded during developmental stages, the underlying biochemical machinery was already established to manufacture different essential elements for germ cell differentiation. Gradual increases in protein and RNA content were observed during the prepupal stage. The increases were more dramatic for both protein and RNA content in the pupal stage. Between days 4 and 7 after pupal ecdysis, a rapid change occurred in certain quantitative aspects of differentiation. Thus, the testicular protein and RNA content reached their maximum levels, as did the rate of leucine incorporation, presumably caused by the stimulation in the activity of the biochemical machinery which may have been affected by the changing hormonal milieu during insect life. It appeared that the ecdysterone peaks slightly preceeded the increases in the testicular protein and RNA content in prepupae and pupae. The level of ecdysterone was shown to be high during prepupal ocular ages 10-14 and from day 3 up to day 7 of the pupal stage (Delbecque et al., 1978a). Ecdysterone has been shown to play a role in the morphogenesis and differentiation of the reproductive system in many other insect species (Koolman et al., 1979; Nowock,

1972; Kambysellis and Williams, 1971b). In addition, the correlation between ecdysone levels and the increased synthesis of macromolecules has been demonstrated in a variety of insect tissues (Berry et al., 1967; Kress, 1979; Arking and Shaaya, 1969). It should be noted that the production of ecdysterone in adult Tenebrio occurs in the abdomen and in the oenocytes (Romer et al., 1974; Delbecque et al., 1978b) and not from the prothoracic glands which disappeared during the late prepupal stage (Srivastava, 1960). Thus, ecdysone could sustain the continuous production of sperm in insects with a relatively long adult life (e.g. many beetles) in which meiotic and even premeiotic stages are found in the adult testis (Roosen-Runge, 1977). This phenomenon may also be true for the Tenebrio adult, although this point was beyond the scope of this investigation. During the adult stage, the testes contained mostly spermatozoa, thus the biosynthetic processes for producing protein and RNA were apparently reduced following the first few days after adult emergence in contrast to the high amount of protein and RNA present in the pupal stage. The protein and RNA content of the testes appeared to increase similarly in all stages studied. RNA content increased at least one day prior to the observed protein increase. The increase in testicular protein and RNA content suggested an increased synthesis of these elements during morphological maturation.

The protein products of the mealworm testes were shown to be many and diverse. Polyacrylamide gel electrophoresis demonstrated products of various molecular weights (27) ranging from 12,000 to 127,000 daltons during the different developmental stages. These protein components differed not only in their molecular weights and charge properties, but were present at different ages and persisted for various times. Although few of these testicular protein components were present in the early ocular ages of prepupae, more protein products were evident during later stages of development until the maximum number of proteins was present in day 6 pupal testes. Approximately half of the protein components were present in the testes of the early prepupal stage and remained through both pupal and adult stages. These testicular protein components may constitute main sources of material for the formation of the spermatogonial cells. The remainder of the testicular protein components appeared in late prepupal testes and/or early pupal testes. These protein products may be necessary for the production of the secondary spermatocytes and subsequent spermatozoa. Since the production and presence of these testicular proteins coincided with the increasing ecdysterone level and the probable lower titer of JH, it was presumed that their production was stimulated by ecdysterone. Additional protein components (testicular protein bands 21, 22, and 25) were resolved by gel electrophoresis during the pupal stage. These

protein products were present for a short duration at a time when the testes had already produced sperm and, thus, may represent by-products of some of the major components of the testes produced during proliferation and transformation of the testicular cells. The relative intensity of each of the testicular protein components and their duration during each developmental stage varied greatly. Testicular proteins numbered 9 through 17, 23-24, and 26-27 varied in their relative concentrations. Their relative concentrations may be more sensitive to the hormonal milieu of the insect as was obvious at day 4 of pupal life after which both protein concentration and the endogenous titer of ecdysterone increased.

The rate of incorporation of radioactive leucine into TCA-precipitable testicular proteins was measured during the pupal and adult stages. The rate of incorporation was found to increase significantly in the mid-pupal stage when compared to the incorporation during the early days of pupal life. The rate of incorporation decreased through the last two days of the pupal stage. The increased ^{3}H-leucine incorporation correlated with the increasing endogenous titer of ecdysterone found in the pupal stage. A similar observation was reported for the imaginal wing disc proteins of Samia cynthia ricini (Patel and Madhavan, 1969).

The testicular proteins of the Tenebrio adult showed a generally lower rate of incorporation than was observed in the pupal stage. The

rate of incorporation decreased steadily throughout the first 10 days after adult emergence. This observation may be explained by the accumulation of sperm during earlier developmental stages (e.g., in pupae). Additionally, the adult does not demonstrate sexual behavior until day 2 of adult life although the maturation of the secondary sex organs continues until the fifth day (Gerber, 1973; 1975).

A variety of protein bands incorporated leucine at measurable levels throughout development, particularly during the pupal stage. These testicular protein bands fall essentially into three groups: 1) those proteins that incorporate leucine at a relatively high level when compared to the other protein bands at the same age. These include testicular protein bands 16, 17, and 18 which have high rates of incorporation occurring during most of the pupal ages; 2) those proteins that incorporate leucine at a somewhat intermediate level during fewer days of pupal ages. These include testicular protein bands 5, 6, 7, 8, 9, 11, 23, and 27; and 3) those proteins comprising the remaining testicular protein bands that incorporate leucine at a relatively low level and at one or two times during the pupal stage. These include testicular protein bands 10, 12, 14, 19, 20, 24, 25, and 26. The testicular proteins from group 1 were present at the second day after pupal ecdysis and demonstrated a high level of incorporation that continued through day 7 of the pupal

stage. It may be of interest to note that the level of incorporation was extremely high at day 6 and 7. The results from *in vitro* incorporation experiments using day 6 pupal testes compared favorably with the results obtained from incorporation experiments performed *in situ*. The increased level of incorporation into the testicular protein bands of group 1 was concomitant with the increasing level of endogenous ecdysterone. In general, the level of ^{3}H-leucine incorporation into specific testicular protein bands was very low during the adult stage indicating a reduction in the activity of the biosynthetic machinery of the testes during this stage.

The effects of JH and/or ecdysterone on testes differentiation in general, and on testicular protein production, specifically, were determined following administration of these hormonal agents to pupal *Tenebrio*. It was ascertained that the rate of incorporation of radioactive leucine into TCA-precipitable testicular protein was not affected by the administration of JH-I alone during the pupal stage. In addition, the amount of incorporation into specific testicular proteins was not significantly different following administration of JH-I when compared to the topically applied saline control animals. This observation suggests that the absence of endogenous JH may not be responsible for triggering testes differentiation toward maturation. Germ cell differentiation was not affected by treatment of the last nymphal instar of *Oncopeltus fasciatus* with a synthetic JH analog

although in every instance JH acted during the "critical period" early within the instar (Economopoulos and Gordon, 1971). If similar mechanisms occur within the Tenebrio testes, JH should exert its effects early in the pupal stage by influencing the biochemical machinery to produce products that may contribute to subsequent testes differentiation. However, the results of the present study demonstrated no effect of JH on testicular protein even at the earliest ages of the pupal stage. However, given a particular concentration of JH, 18 hr old Tenebrio pupae showed a higher morphogenetic response than younger pupae (Krishnakumaran, 1974). Furthermore, no effect of JH on the basal anhormonal rate of cell division during spermatogenesis in unfed Rhodnius prolixus was observed (Dumser and Davey, 1975). The application of JH or its mimics was proven to act as an inhibitor of cell growth and macromolecule synthesis of an insect cell line (Himeno et al., 1979). Thus, it appears that JH may act to keep the mealworm testes in the status quo, while the presence of ecdysterone at the prepupal and pupal stage to initiate the molt may stimulate the biochemical machinery of the mealworm testes. The corpora allata were found to be responsible for maintaining the testes in a juvenile state in other insect species (Sehnal, 1968; Blaine and Dixon, 1970).

Ecdysterone administration to pupal Tenebrio resulted in an increase in the rate of radioactive leucine into TCA-precipitable

testicular protein. The rate of testicular incorporation increased in response to ecdysterone during the first six days after pupal ecdysis when compared with results obtained from control insects. After the sixth day, the rate of incorporation was similar to that of the control insects. Thus, the sensitivity of the testicular cells to ecdysterone was much greater in early pupal development than during the later days of this stage, although this sensitivity preceded and continued during the rising of endogenous molting hormone levels. Neufeld et al. (1968) found that protein and RNA synthesis in Calliphora larval body wall and fat body were most sensitive to exogenous 20-hydroxyecdysone somewhat before the level of endogenous molting hormone rises. Ecdysterone was found to have a stimulatory effect on the incorporation of precursors into the imaginal disc RNA of Drosophila melanogaster (Raikow and Fristrom, 1971). In the Rhodinus testes, the presence of ecdysone increased the rate of cell division during spermatogenesis (Dumser and Davey, 1974). This acceleration of cell division by ecdysone is an expression of the direct enhancement of the biosynthetic level of testicular macromolecules.

The acceleration in the biochemical machinery for manufacturing testicular protein in response to acdysterone resulted in precocious synthesis of the same age-specific testicular protein found during the first six days of pupal stage without ecdysterone. Thus, ecdysterone injection (0.5 μl/animal) appeared to stimulate the production of the

same testicular proteins that were present during normal development (see Figure 33). Injection of a higher dose of ecdysterone (1.5 μl/animal) to day 1 and day 3 pupae appeared to alter the testicular differentiation program by enhancing the incorporation of radioactive leucine into not only the age-specific testicular proteins but also into new testicular proteins. These new testicular proteins did not normally appear at these ages (1 and 3 day old pupae). These results indicated that ecdysterone, by its influence on testicular protein synthesis and morphological maturation, can be considered a regulatory factor for the growth and differentiation of the mealworm testes by its ability to manipulate the program of testes differentiation. Such a relation between the endocrine hormone and germ cell multiplication, particularly with regard to protein synthesis, was also demonstrated by Garcia-Bellido (1964a).

Mediation of similar maturation processes by other steroid hormones involves receptors that have been identified in both the nuclei and cytoplasm of many higher animal target tissues and it would not be surprising to discover that binding-proteins for ecdysone also have a wide distribution within insect tissues or organs (e.g., the testes). This conept is especially intriguing with the observation that blowfly testes sequestered a high level of ecdysteriod (Koolman et al., 1979). Furthermore, ecdysone seems to differ from mammalian steroid hormones in that it has no single target tissue

and is highly soluble in water in comparison with most mammalian steroids. Our conclusions about the effect of ecdysterone on testicular differentiation are consistent with increasing body of evidence that ecdysone promotes the growth and differentiation of different cell types in the insect and appears to be more than just a molting hormones (Patel and Madhavan, 1969; Natori, 1976; Nishiura and Fristrom, 1975; Nakano and Natori, 1978; Berger et al., 1978). The suggestion that ecdysterone may work at the cellular level by altering the program of these cells with regard to their biosynthetic capacity in order to satisfy the new environmental requirements created by the high titer of ecdysterone seems appropriate. This type of cellular reprogramming was observed after applying ecdysone to Tenebrio pupae. Reddy and Krishnakumaran (1972) and Socha and Sehnal (1972) reported that some doses of ecdysone (mostly higher than 1 μg/pupae) had juvenilizing effects on the pupae and shortened the duration of adult development. While the process of cuticle secretion and sclerotization in insects appears to depend on the prior synthesis of macromolecules that are induced by ecdysone (Karlson and Sekeris, 1966), we conclude that most of the effects elicited by a high dose of ecdysterone in the mealworm testes were brought about by abnormal transcription of some RNA's not related to that specific age. The derepression of these genes may depend on

the kind of program established in the testes cells and on the progress of development according to this program.

One final result of this investigation supports the conclusions and suggestions contained in this discussion. This result was obtained following the simultaneous administration of both JH and ecdysterone and the subsequent measurement of their effects on the incorporation of leucine into TCA-precipitable testicular protein. The results indicated that there was no apparent interaction, synergistic nor antagonistic, between these hormones. Furthermore, the incorporation rate of ^{3}H-leucine closely resembled that rate obtained following injection of ecdysterone alone in all the pupal ages studied. This supports Richards' (1978) suggestion that the role of JH appears to lie in modifying the acquisition of competence of polytene chromosomes to respond to ecdysone rather than in a direct antagonism between the two hormones.

In summary, the results of this investigation suggest that the process of testicular differentiation continues through different developmental stages; the first steps of development occur through cell divisions and morphogenetic movements, and ultimately through the manufacture of differentiative proteins. Certain of these transitional developmental steps are autonomous and others dependent upon hormonal signals from the endocrine centers.

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