# CONTROL OF SPERMATOGENESIS IN RHODNIUS PROLIXUS.

by

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# ABSTRACT

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This study involves essentially two phases: firstly, the description of testis structure and germ cell dynamics in *Rhodnius*, leading to the formulation of a kinetic hypothesis of germ cell development, which predicts division rate enhancement by ecdysone, and the suppression of this enhancement by juvenile hormone. The second phase involves a critical evaluation of the validity of this hypothesis, by experimental investigation of three major aspects: a demonstration of appropriate mitotic index response to hormonal presence; hormonally instituted changes in moult cycle duration; and hormonally independent sequential germ cell differentiation. A fourth major control mechanism, an ecdysone-related, non-kinetic autolysis of the most differentiated germ cell compartment during each larval diapause period is also described.

# ABREGE

Ph.D. J. B. DUMSER PARASITOLOGY

## CONTROL OF SPERMATOGENESIS IN RHODNIUS PROLIXUS.

Deux étapes sont impliquées dans cette étude: premièrement, une description de la structure des testicules et de la dynamique des cellules germinatives chez Rhodnius. Cette description permets l'avancement d'une hypothèse cynétique sur le développement des cellules germinatives laquelle, prévoit un taux de division augmenté par ecdysone ainsi que la suppression de cette augmentation par l'hormone juvénile. La seconde étape est une évaluation critique de la validité de cette hypothèse présentée par une investigation de trois aspects majeurs: une démonstration de la réponse de l'index mitotique approprié à la présence de l'hormone; les changements du cycle de la moue sous l'influence hormonale; et une différentiation des cellules germinatives indépendamment de la séquence des hormones. Un quatrième mécanisme de contrôle majeur en relation avec ecdysone est l'autolyse non-cynétique des composantes des cellules germinatives très différenciées durant chacune des périodes de diapouse larvaire est aussi décrit.

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## STATEMENT OF ORIGINALITY AND ACKNOWLEDGEMENTS

While aspects of the gross morphology of the Rhodnius testis have been described elsewhere, as referenced within the text, the structural and cytogenetic accounts provided in the latter half of Chapter Three are the author's original contributions. Other original work is represented by the demonstration of specific germ cell autolysis and its hormonal involvement (Chapter Four); and the analysis of the timing of germ cell development and dynamics (Chapters Four and Five) which led to the formulation of the kinetic hypothesis (Chapter Eight). Much of this last has been accepted for publication (Dumser and Davey, Can.J.Zool., 1974).

Experiments conducted to investigate the validity of the kinetic hypothesis, including: the effect of hormones on mitotic index (Chapter Nine); on the duration of the moult cycle (Chapter Eleven); and on the process of sequential differentiation of the germ cell (Chapter Ten) are original contributions of the author.

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# PREFACE

One very biased way of viewing the larval/ adult transformation would be to consider it analogous to vertebrate puberty. It is biased because structural and physiological changes not directly related to reproduction are enormous at this time, and to the developmental biologist the changes observed in the gonad are really no more striking than those observed in metamorphosis of external structures, and the abrupt shift in lifestyle these often make possible. On the other hand, the author's unseemly preoccupation with sex, revealed in the opening sentence, can be justified on both theoretical and practical grounds. Theoretical, in that given the dogma of continuity of the germ line, we may view an insect in all of its stages as a disposable shell, a kind of culture vessel designed to propagate the species' germ cells. Practical, in that man has always had an economic, social, and medical interest in the number and kind of his insect neighbours, and hopes to exercise some control over these numbers by a selective attack on the insect's reproductive faculties.

Historically, a very large number of studies have been carried out on the physiology of egg production by the female insect. The literature on the male is minute by comparison, and one justification of this disparity is that the male in the wild population is often redundant, sometimes dispensed with entirely as in parthenogenetically reproducing forms. The female is entrusted with forming the egg, the protective shell which nurtures all subsequent progeny through embryonic development. The male provides genetic diversity, the basis for evolutionary selection vital to the maintenance of population fitness, but of small impact on next season's population levels. Development of broad spectrum insecticides in the mid 1940's enabled us to deal very effectively with agriculturally and medically important insects through any of their life stages. Increasing resistance in these rapidly evolving populations, largely a result of the extraordinary selection pressures inherent in our methods of control, has resulted in a constant increase in the amounts of the chemicals used. The accumulation of larger and larger levels of toxic residues by both biological and physical amplifications is beginning to be felt as population instabilities in life forms often quite remote from the original target populations. The limitations of the broad-spectrum attack are now clear. Attention thus has recently been focused on the male as one potentially specific agent for control. As carrier of genetic information, he can also be manipulated to carry misinformation through the mechanism of the semi-sterile translocation heterozygote (Laven et al 1971), a Machiavellian Mendelism derived from the sterile male technique (Knipling, 1955).

However, we have as yet no clear picture of the development of the male germ cell system, its maturation at metamorphosis, and internal control exercised by the insect over this development. 'Reaching puberty', the attainment of function at metamorphosis, can be experimentally manipulated; thus, as we shall see, controls do certainly exist. In a recent and thoroughly excellent review of comparative endocrinology, (Highnam and Hill, 1969), twenty-eight pages are devoted to the endocrinology of insect oögenesis, and precisely one hundred and twenty-five words to the endocrine control of reproduction in the male. Their conclusions are, " ... it is likely, therefore, that it is controlled by the same combination of hormones that effects metamorphosis." In a very broad sense, this thesis is intended to investigate the validity of this statement.

Following a very brief review of the endocrinology of metamorphosis, a comprehensive review of the literature specifically dealing with testicular growth and endocrinology in insects will be attempted. In this way, the question can be refined to what this author chooses to call the 'larval-adult paradox': the maintenance of spermatogenesis in vastly differing endocrinological milieu. The necessity for isolating parameters of growth from those of differentiation should also become apparent, justifying a review of testicular morphology in the 'generalized' insect, and an intensive analysis of structure in Rhodnius.

Certain control points can be isolated from this analysis, and a limited series of endocrinological investigations will be described. In an attempt to gain a more precise picture of germ cell differentiation and production, a study is described defining the dynamic relationships between differentiated germ cell compartments, and some effects of hormonal manipulation on these relationships are reported. From these results, and from correlations with the body of endocrinological information already present for Rhodnius, a kinetic hypothesis is proposed which satisfies the conditions dictated by the larval-adult spermatogenesis paradox.

This kinetic hypothesis depends in large part on two unsupported assumptions: That germ cell division rate is subject to endocrine regulation; and that spermatogonial differentiation proceeds in a series of steps related to the number of divisions experienced, but not directly related to time per se. The two succeeding chapters will therefore present more precise definitions of these questions, and describe experiments carried out to investigate the validity of the concepts and hence the validity of the kinetic hypothesis.

These experimental procedures reveal that the onset of certain events is non-randomly distributed in time. A further analysis of this effect reveals the existence of a fourth significant endocrinological intervention in the developmental schema of the testis, control over the availability of time for development.

Following these experimental procedures, the discussion is presented. This section is divided into three parts: The first contains a brief review of major findings, and an assessment of their validity. The kinetic hypothesis is then restated, and applied to the observations on testis development from the literature; the second section comprises a more detailed and theoretical account of division rate and its potential control, and concludes with a somewhat modified and speculative version of the kinetic hypothesis; the final section presents a comparison of this speculative hypothesis with a control system which has been proposed for certain vertebrate tissues.

The organization of this thesis thus departs from the standard format of historical review, experimental observations, and discussion in a fairly radical manner, and the author regards this departure as necessary to preserve the continuity of the logic presented. This structural complexity arises largely from the lengthy observations on structure and dynamics necessary to isolate and identify possible control points in testis development prior to the experimental investigation of these control phenomena. The thesis thus corresponds in organization to two such studies: the one to define the problem and suggest solutions; the second to provide an experimental investigation of the validity of these solutions. The organization adopted, while somewhat unusual, can thus be seen to reflect an orderly and logical progression in our understanding of the control of testicular development.

# Chapter One: Metamorphosis

An insect's life is commonly, and efficiently divided into a number of stages, or stadia. Each may be considered to end, and the next begin with the shedding of the old cuticular exoskeleton, a process termed ecdysis. In its earlier stadia, the insect's activities are directed toward an accumulation of nutrients, and growth toward the size which natural selection dictates. While the young or larval insect shows a steady increase in mass, a volumetric constraint is imposed by the relatively inflexible cuticle, hence its periodic removal. This ecdysis is a very visible process, and provides the insect physiologist with a precise and unmistakeable marker. The entire catalogue of events by which the insect replaces this cuticle is cyclical and continuous, and is referred to as the moulting process. Briefly, it consists of the separation, or apolysis of underlying epidermal cells from the old cuticle, the production of a new cuticle by these cells, digestion and resorption of valuable, largely proteinaceous materials from the old cuticle, and finally the shedding or ecdysis. Additional internal and external layers are added to the cuticle following its expansion, hardening and darkening. Apolysis then begins the next moulting cycle.

Eventually this cycle is halted in the two major groups of insects, exopterygota and endopterygota, by the production of the final reproductive stage, the adult or imago. This stage is responsible for dispersal, and the production of the succeeding generation. Obvious specializations for these purposes are found in all insects. Wings, and sensory structures for locating the mate appear, and external reproductive structures are developed. Patterns of pigmentation of the cuticle may be drastically altered. Changes also occur in the internal organs and tissues, including the maturation of the gonads, and there is a general rearrangement of structure and function consistent with the new role assumed by the insect. Taken as a whole, these changes are termed metamorphosis. In the exopterygota, the adult and larval stages are recognizably similar, the adult specializations being added to a common body plan at the final or metamorphic moult. In the endopterygota, an entirely new body plan is realized in the adult. The massive reorganization required is carried out during a stage intervening between larva and adult, termed the pupa, and this type of metamorphosis is called complete metamorphosis, in contrast to the incomplete metamorphosis of the exopterygota.

In the exopterygota, and to some extent in the endopterygota as well, the same ectodermal cells produce the larval and adult cuticles, and this extraordinary differentiation has been the focus of concerted examination for forty years. The volume of literature has doubled and redoubled in the past decade, and as Willis (1974) points out, it sometimes seems that little or no progress has been made. This, of course, is not really so, the questions asked today are increasingly more sophisticated, and one problem certainly is the collation of so much available data. However, the brilliance and vision of the early research in insect endocrinology cannot be lightly dismissed. This thesis concerns the differentiation and development of the testis, an internal organ, and how this development relates to metamorphosis. A general, and very brief review of the endocrinology of metamorphosis is presented here. Most of this information has resulted from studies of the metamorphic process affecting cuticular differentiation, much of it stated some time ago. Virtually every point is disputable, and a careful analysis would take us very far from testis development, the matter under primary consideration. Thus, what follows is a simple statement of what is sometimes termed the Classic Theory of insect endocrinology. Contentious issues are avoided, and it is hoped the non-specialist will be provided in this way with a simple framework on which to interpret the experimental and descriptive work to follow. Where a greater depth of understanding is required, for the interpretation of an observation or appreciation of a question posed, a short review has been included in the relevant section. The author recognizes that this technique introduces an inconvenient fragmentation of the information presented, but hopes that in this manner a vast and intractable literature can be made available to a wider audience than would otherwise be possible. As the information presented here represents the contributions of very many workers over a long period of time, internal referencing in this section has been avoided except where the information is unlikely to occur in a standard review. For the rest, the material has been taken from, and the reader is recommended, the following reviews on insect endocrinology, in order of increasing complexity, and completeness: Highnam and Hill, 1969; Wigglesworth, 1964 and 1970; Gilbert and King, 1973. The aforementioned review by Willis (1974) is unique in that it deals almost entirely with what we do not know, being a discussion of current points of contention. Finally, areas of physiology, not specifically endocrinological in nature, are described largely from Wigglesworth (1965).

Each cycle of the epidermal cell activity responsible for the moulting process earlier described is brought about by the steroid hormone termed moulting hormone. The structure of three similar steroids, termed $\alpha$-ecdysone, $\beta$-ecdysone (crustecdysone or ecdysterone, the 20- OH form of $\alpha$-ecdysone), and 20-26 dihydroxyecdysone, all possessing some activity, is now known, as well as a large number of similar molecules of plant origin, the phytoecdysones. The precise role of these products is the current subject of intensive research, complicated by apparent species differences (Marks, 1971, 1973). Moulting hormone has long been thought to be produced in a somewhat variable, loosely organized anterior mass of tissue termed the prothoracic gland, and this has been very recently convincingly demonstrated in vitro by Chino et al (1974). It is possible that other tissues may have the capacity to synthesize ecdysone as well, and very many tissues can effect the required hydroxylation reactions (King, 1973). The prothoracic glands are certainly the chief source of ecdysone in the larval insect, however.

The specific effect of ecdysone on the epidermal cell is similarly an area of some speculation. Observations include both the initiation of DNA and RNA synthesis, cytoplasmic events such as protein synthesis, and membrane related events, such as permeability alterations. In general terms, it may be stated that ecdysone activates cells to perform a pre-programmed series of activities, which may or may not include some or all of the above. In the complete absence of ecdysone many tissues of the larva enter a stage of developmental arrest termed diapause, which is often characterized by a much reduced level of oxidative metabolism.

The type of cuticle produced by ecdysone activated epidermal cells is determined by the titer of another circulating hormone, juvenile hormone. Juvenile hormone is produced by the paired, or sometimes fused corpora allata located in the head capsule. The structure of three naturally occurring juvenile hormone molecules has been elucidated, and the synthesis also has been recently carried out in vitro (Judy et al, 1973a & b; Pratt & Tobe, 1973). Over twenty five hundred active analogues of juvenile hormone have been synthesized, including farnesoic acid esters, terpenoid ethers, p-substituted benzoates, peptide analogues of polyisoprenoids, and chrysanthemic acid ethers. Most of these compounds possess a terminal carboxy alkyl or similar group conjugated with a double bond in trans configuration with a long alkyl chain (Punja et al, 1973). In contrast to ecdysone, juvenile hormone mimics appear to be more species-specific, although most have considerable activity in other insects (Schneiderman, 1965, 1972). Once again, the precise mode of action is obscure; however, in affecting the direction of cuticular development, the action of juvenile hormone on epidermal cells seems to require the prior activation of these cells by ecdysone. In the presence of juvenile hormone, ecdysone activated cells secrete a larval cuticle, whereas, in its absence, an adult cuticle is produced. The intervening pupal cuticle of endopterygote insects results from activation in a low titer of juvenile hormone presence. These and related observations on the polytene chromosomes of some dipteran salivary gland cells, have led to a concept of 'gene-switching' for juvenile hormone. It is postulated that each epidermal cell contains the genetic information for production of the two, or three types of cuticle. Ecdysone provides that some set of genetic information will be read; juvenile hormone decides which set. Juvenile hormone production in the early stadia maintains the larval character of each moult. A lower titer in the pre-pupal stadium results in the 'reading' of genes for pupal cuticle production, and the absence of juvenile hormone in the pupal or final larval metamorphic moult results in the production of adult cuticle by the ecdysone activated epidermal cells.

Initiation of this process, the production and secretion of ecdysone by the prothoracic gland, results from the release of an as yet uncharacterized secretion produced in modified neurons of the brain. Axons of these neurons terminate in a neurohaenal organ, the corpus cardiacum, situated in close proximity to the corpus allatum. This spatial separation of synthesis and storage sites for this brain hormone introduces some difficulty in analysis of the events culminating in brain hormone release. Indeed, species-specificity in this step reaches its highest development, and environmental and physiological information is clearly being fed into a neural network with requirements peculiar to each species under consideration.

Activation of the prothoracic gland by the neurosecretory brain hormone, in the absence of juvenile hormone, leads to the subsequent degeneration of these glands; the adult thus lacks the capacity for ecdysone production, or further moulting activity. The allatum, by contrast, resumes its activity and secretion of juvenile hormone at even greater levels than in the larva. Juvenile hormone now acts as a gonadotropin, involved in the synthesis and transport of yolk proteins for the developing eggs, and accessory gland secretions for production of the spermatophore or sperm-sac in the male. Further roles concerning changes in pigmentation and behaviour are also indicated.

Rhodnius, our experimental subject, served as a model for much of the early elucidation of the above story. The physiology and life-history of this bug, as in all insects, imposes certain specializations on the endocrine pattern, and it is useful to briefly review these now.

Rhodnius prolixus Stål is a Reduviidid Hemipteran, and an obligate micropredator. Rhodnius in the wild exhibits a complex ecology involving blood feeding on both birds and rodents (Baldwin and Cowper, 1969). However, like other members of this group this bug readily becomes domesticated, inhabitating native dwellings in South and Central America, and feeding on household pets and human inhabitants. Rhodnius may harbor the causative agent of Chagas' disease, Trypanosoma cruzi; it transmits by posterior station infection. (Cheng, 1964).

Rhodnius exhibits a variable span of time in the egg. On eclosion the first larval instar resembles a diminutive adult, lacking, however, wings, ocelli, and external genitalia, and differing from the adult in details of cuticular structure and pigmentation. (Wigglesworth, 1933). The bug will accept its first blood meal a few days after eclosion, or ecdysis in succeeding stadia. However, if isolated from a source of food, the animal remains active but undergoes little or no development. This period may be extremely long. Under some conditions, later instars may be maintained for a year or more. Presented with a source of blood, the bug gorges, taking several times its own weight in blood. Distension of the abdomen activates stretch receptors which trigger the release of brain hormone from the corpus cardiacum, hence activating the prothoracic glands to produce ecdysone, and setting into action events culminating in ecdysis a specific number of days following the feed. Five larval instars are produced, each characterized by a period of developmental stasis of variable duration, a single blood meal, and subsequent moult. A certain degree of cuticular morphogenesis proceeds during each moulting cycle. The insects, for instance, can be easily differentiated as to sex by variations of the IXth and Xth sternites during the fifth stadia, but final adult form is attained at the fifth ecdysis.

The developmental stasis encountered in each stadium results from a lack of ecdysone, and is hence functionally equivalent to larval diapause (Wigglesworth, 1970) in the endocrine scheme first described by Williams (1952). The ability to manipulate this important parameter through feeding is one of Rhodnius' great assets as an experimental animal. As well, the neurosecretory cells, corpus cardiacum, and corpus allatum are all admirably positioned and constituted for efficient and relatively non-damaging surgical removal. Unfortunately, this is not true of the prothoracic gland, a structure which can effectively be removed from very few species of insect.

## Chapter Two: The Endocrinology of Testis Development

Early in the history of the investigation of insect endocrinology, it was shown that a headless, larval *Rhodnius* could be caused to moult if connected by parabiosis to another headless bug, decapitated after the critical period for the release of brain hormone (Wigglesworth, 1934). In effect, the parabiosed individual is exposed to ecdysone and, under certain circumstances, juvenile hormone normally present at each larval moult can be avoided. Under these conditions, many external structures of the larva at the succeeding ecdysis show an advanced, adultoid development. Internal structures, such as the ovary, were also seen to be more advanced developmentally than would have been the case following a normal larval moult (Wigglesworth, 1936). The ovary of a third instar larvae parabiosed to a fed, decapitated fifth instar bug, displayed recognizable oöcytes, follicular cells, and nurse cells, although it did not reach the level of differentiation of a normal adult. Wigglesworth concluded from these experiments that the corpus allatum secretions, which were necessary to maintain the larval cuticular structure at each moult, also inhibited development in the larval gonads. These results were also obtained for *Bombyx mori* by Fukada (1944). In this case, adultoids produced by allatectomy of the third, the penultimate larval instar, contained ovaries or testes which were functionally adult. Females laid a small number of eggs which could be fertilized, and the male organs contained mature spermatozoa. Fukada's conclusion, like those of Wigglesworth, were that gonad maturation is under the control of the same hormones which control imaginal differentiation.

Piepho (1939, 1940, 1942), performed the converse experiment, implantation of active corpora allata into the final larval instar of <u>Galleria mellonella</u>, resulting in a supernumerary larval moult, and in some cases, larval-pupal intermediates as defined by external morphology. Sehnal (1968), has repeated these experiments and demonstrated by volumetric estimation an inhibitory effect of corpora allata on the growth of the gonad. The degree of inhibition depends on the time of implantation of the allatum within the stadium, suggesting a continuous development of the gonad (Sehnal, 1968). An inhibitory effect of the corpus allatum or its secretions on gonad development is further reported: Zdárek & Sláma (1968) utilized applications of 'paper factor' and Law's mixture (Law et al, 1966) on <u>Pyrrhochoris apterus</u>; by Takeuchi (1969), in <u>Bombyx mori</u> through gonad and allatal transplants; by Blaine and Dixon (1970) through allatectomy of <u>Periplaneta americana</u>; by Madhavan (1973) through applications of a variety of juvenile hormone analogues on <u>Drosophila melanogaster</u>; by Riddiford (1972) through application of juvenile hormone analogues to

*Hyalophora cecropia* larvae and pupae; finally, Nowock (1973), through the feeding of farnesyl methyl ether (FME), a juvenile hormone analogue to larval *Ephestia kuhniella*, obtained similar inhibitory effects. Criteria for development vary somewhat from study to study, but generally involve either organ size, or the appearance of spermatozoa. At the fine structural level, Cantacuzène and Seureau (1970) have demonstrated degenerative changes in the parietal cells and associated spermatids of *Locusta migratoria migratorioides* in response to implantation of corpora allata. In contrast to these above findings, Economopoulos and Gordon (1971) report an absence of effect of juvenile hormone analogue applications on testis development, despite considerable juvenilization of external characters in *Oncopeltus fasciatus*. Similarly, Bhaskaran (1972) reports fully differentiated sperm in the testes of *Sarcophaga bullata* intermediates following juvenile hormone analogue treatment. In this case, however, testis ducts were absent.

In many species of insect, larval or pupal diapause results in a cessation of growth of the testes. (Williams, 1948, Church, 1955, Beck and Hanec, 1960). Williams (1951) attributed pupal diapause in the wild silkworm *Hyalophora cecropia* to a lack of ecdysone, and was able to provoke spermatozoal development in testes, or isolated spermatocysts *in vitro* by adding the blood of a non-diapausing pupa, believed to contain a high titer of ecdysone. These results were successfully repeated by other workers (Laufer and Berman, 1961, Bowers, 1961) in Hyalophora and in the closely related Samia cynthia. Yagi et al (1969) demonstrated that the diapausing Hyalophora spermatocysts could be made to undergo development in insect haemolymph free media by the addition of $\beta$-ecdysone, commercially derived from plant sources. The in vitro system has been further refined (Williams and Kambysellis, 1969; Kambysellis and Williams, 1971a & b) defining a permissive role for ecdysone in the transport across the testis sheath of a blood-borne factor, macromolecular factor (MF), necessary for spermatozoal production. The authors demonstrated a limited capacity of various bovine sera to duplicate the activity of MF; essentially similar results have been recently reported for the slug moth, Monema flavescens (Takeda, 1972).

On the other hand, in vitro cultivation of larval testes of the non-diapausing wax moth Galleria mellonella in the absence of any insect hormones was performed by Lender and Duveau-Hagege (1963), both germarial mitoses and spermiogenesis being reported. The same results have been obtained in a variety of Dipterans (Stern, 1940; Leloup, 1964; Kuroda, 1974; see review by Marks, 1970).

Evidence favoring the hypothesis that ecdysone stimulates testicular development has been obtained by *in vivo* experimentation. Testes transplanted from diapausing to non-diapausing type larvae in *Papilio xuthus* showed advanced differentiation, as did those in larvae receiving supernumerary brain-prothoracic gland complexes (Nishiitsutsuji-uwo, 1961). Takeuchi (1969), in a series of transplant experiments, showed maximum development of embryonic testes in last instar larvae of *Bombyx mori*, slight development in those implanted in newly pupated hosts, and no development in the remainder of the hosts which were believed low in ecdysone titer. On the distaff side, Blaine and Dixon (1970) were unable to show accelerated development by transplantation in *Periplaneta americana*. Economopoulos and Gordon (1971) also transplanted fourth, and last instar testes into adult *Oncopeltus fasciatus*, and obtained mature spermatozoa, indicating development unrelated at least to the last, metamorphic moult.

On balance, the experimental evidence appears to favor the hypothesis that testicular development is enhanced by ecdysone, and inhibited by juvenile hormone. Insofar as negative reports involving juvenile hormone are concerned, the work by Economopoulos and Gordon (1971) and Bhaskaran (1972) might be interpreted as indicating a lack of inhibition of spermiation, rather than earlier stages of spermatogenesis. Certainly, both preparations contain differentiated spermatids at the time of hormone application, and neither work assessed the relative number of spermatozoa produced.

Objections to the contention that ecdysone accelerates testis development are equally suspect. In all cases so far reported of anhormonal in vitro development, the immediate history of the explant includes stages likely to have involved exposure to some titer of ecdysone. The importance of this fact is underscored by the demonstration of 'covert' ecdysone effects in flies (Žďárek and Frankel, 1970), which indicate that the ecdysone 'message' can be retained for some time in its absence. Economopoulos and Gordon's (1971) results in vivo indicate that normal development can occur in the absence of the metamorphic ecdysis, but the authors point out that a low titer of ecdysone is present in the adult host during the time of this experiment (Feir and Winkler, 1969). Similar ecdysone levels have been found in the adults of another species (Bombyx mori), and it is not known how general this phenomenon might be. The observations by Blaine and Dixon (1970), of normal spermatogenesis in Periplaneta lacking a source of brain hormone are more puzzling. One complicating factor in the interpretation of these results is the report of continued growth, metamorphosis, and reproduction in three species of cockroach, including Periplaneta, deprived of prothoracic glands (Nutting, 1955; Chadwick, 1955, 1956), indicating ecdysone synthesis outside the classical schema in this group of insects.

An overall view of the role of these morphogenetic hormones in spermatogenesis was proposed by Takeuchi (1969) for Bombyx. He suggests that in larval instars, germ cells differentiate to form a large pool of secondary spermatocytes. On release from juvenile hormone inhibition, and in the presence of high titer of ecdysone, a "... simultaneous production of large numbers of spermatozoa in the late larval period" ensues. Such an hypothesis is consistent with much of the experimental evidence so far compiled, and admirably fits the endocrine patterns believed responsible for metamorphic development. However, in an impressive number of insect species, spermatogenesis continues during the adult stage (Bonhag and Wick, 1953; Giles, 1961; Hannah-Alava, 1965; Amerson and Hayes, 1967; McLaughlin and Lusk, 1967; Shen and Berryman, 1967; Jones, 1967; Phillips, 1970). In these cases, a contradiction arises; the endocrine picture of the adult, with its very high titer of juvenile hormone (Wigglesworth, 1936; Gilbert and Schneiderman, 1961) and absence of ecdysone (Wigglesworth, 1934) would appear extremely unfavorable to spermatogenesis. The adult, in fact, carries out this spermatogenesis in an endocrine milieu which is precisely the reverse of the late larval/pupal period of maximum germ cell differentiation.

As we have seen, Rhodnius served as the first example of juvenile hormone inhibition of gonad development (Wigglesworth, 1934). Moreover, each larval instar of Rhodnius enters an endocrinological diapause, terminated by feeding, during which no appreciable developmental growth occurs (Wigglesworth, 1934). The germarial area of the adult Rhodnius is mitotically active (Buck, 1967) and in fact all elements of spermatogenesis are present until the death of the insect, six months or more after the metamorphic moult. Thus, Rhodnius would appear to be a particularly apt experimental system in which to attempt to resolve this larval/adult spermatogenesis paradox.

# Chapter Three: Testis Structure

It should be clear from the foregoing review of the experimental investigation of testicular development and its potential endocrine control that the design of such experiments, and the interpretation of results obtained requires a better understanding of the changes which occur throughout the normal larval span than is currently available in standard texts. Therefore, a series of histological investigations on Rhodnius at various stages of larval development was undertaken, and the information obtained presented in this chapter. By way of introduction, a review of structure in the 'generalized insect' is presented. The initial part of this introduction deals with the embryology of the testis. As in the information presented in Chapter One, this work represents the efforts of a great number of workers over many years, and the account presented here is taken largely from the classic works of Imms (1957) and Snodgrass (1933, 1935). Cumbersome internal referencing is thus avoided, but it should be understood that none of the work regarding embryology reported here has been carried out by the present author. The remainder of the chapter includes the author's original observations on Rhodnius, and comparisons with other insects which are referenced in the standard manner.

Formation of the two-layered band in insects occurs by one of several processes, none of them closely resembling that of other animals on which classical embryology is based. From the assumption that these processes are all highly modified forms of gastrulation, we may tentatively define the outer cell layer of the germ band as ectoderm, and the inner, mesoderm. This longitudinal mesoderm forms two bands, which are connected across the midline by a single layer of cells. Transverse constrictions result in the formation of mesoblastic somites, and each comes to contain a coelomic sac through the appearance of a cleft, or through folding of the lateral margin. The inner layer can now be termed splanchnic mesoderm, the outer, somatic. Somatic mesoderm will give rise to body muscles, dorsal diaphragm, and pericardial cells. The splanchnic layer will form visceral muscles, the bulk of the fat body, and the genital ridges.

The primitive germ cells, which will give rise to the sex cells, have been shown to arise directly from certain posterior pole cells in the eggs of several insects (Snodgrass, 1935). These cells migrate to the splanchnic mesoblast and come to lie in the walls of the coelomic sac in abdominal segments I or II to VI or VIII. While this has not been observed in other insects, it probably is the rule, rather than differentiation of sex cells from splanchnic mesoderm in situ as is occasionally suggested. Thus, at least initially, the gonads are segmental and metameric in origin. Multiplication of both sex cells and mesodermal tissue soon results in the fusion of adjacent groups to form a continuous genital ridge on each side of the embryo. The enclosing sheet of splanchnic mesoderm becomes thickened dorsally to form a dorsal suspensorium, and ventrally to form a ventral strand. The mesoderm ridges in posterior segments not containing germ cells also fuse with the ventral strand, eventually giving rise to a continuous duct terminating at the Xth abdominal segment. The gonads thereby become paired asegmental structures.

At this time, columns of mesodermal cells form in the dorsal suspensorium over regions of germ cells, and form the terminal filaments of presumptive ovaries in the case of the female. Grooves form between the terminal filaments, eventually reaching the ventral strand, thus defining the ovariole by secondary segementation. Mesodermal cells of the ventral strand differentiate to produce pedicle, calyx and lateral oviduct. Essentially the same process is believed to occur in the testis, resulting in the formation of sperm tubes, though terminal filaments are generally absent. Unlike the ovary, the testis is contained in a mesodermal sac. The ventral strand gives rise to vasa efferentia, and the paired vasa deferentia in posterior segments, which terminate in ampullae on the Xth segment. The single median ejaculatory duct arises post-embryonically from an ectodermal evagination, the anterior of which provides also for the formation of various accessory glands. It should be noted that Seidel (1924) has described, in the bug Pyrrhocoris apterus, an alternate scheme. He claims that incomplete fusion of the mesodermal/germ cell groups initially directly defines the ovarioles and sperm tubes, the mature gonad thus being primarily segmented. Further work would be required to determine if this may not be an hemipteran specialization generally. In either case, the result is the production of nests of sex cells within an internally segmented, wholly mesodermal supporting structure.

In the generalized insect thus the testis comes to be composed of a number of sperm tubes, the number being constant in any species. The number varies widely: from one in the Diptera to several hundred in many Orthoptera (Imms, 1957). These tubes are sometimes termed testicular follicles (Imms, 1957; Phillips, 1970) but, being developmentally equivalent to ovarioles rather than ovarian follicles, I shall henceforth refer to them as testicular tubules (Snodgrass, 1935). The walls of the tubule are delineated by an epithelial or internal sheath, and several tubules enclosed in a peritoneal sheath of varying complexity. The tubules are free apically in the sense that they lack the terminal filament typical of ovarioles, and continue basally as short vasa efferentia, simple epithelial ducts. The vas efferentia join to form the vas deferens, characterized by the addition of a muscular layer, and outer peritoneal cell coat (Imms, 1957). No dilation at the junction of these ducts is found equivalent to the female calyx, but a dilation in the vas deferens generally serves as a seminal vesicle. The vasa deferentia continue basally and, in the adult, terminate in the single ventral ductus ejaculatorius, formed post-embryonically as an invagination of the ectoderm from the IXth abdominal segment. Various accessory glands may be present, those derived from mesodermal ducts being referred to as mesadenes, those derived from the ejaculatory duct as ectadenes (Imms, 1957).

The contents of the testicular tubule may be divided into a number of functional zones (Depdolla, 1928). The apical portion contains the unencysted predefinitive spermatogonia (Hannah-Alava, 1965), often surrounding a trophocytic apical cell or apical complex (Carson, 1945) and is referred to as the germarium. Some of the progeny of these pre-definitive spermatogonia become surrounded by a cyst cell of mesodermal origin (Zick, 1911) to form the definitive spermatogonial cyst. Generally, no intervening indefinitive divisions occur between pre-definitive stem-cell division and encystment.(Hannah-Alava, 1965). The encysted spermatogonia undergo a species-specific number of mitotic divisions (White, 1955; King and Akai, 1971) with incomplete cytokinesis (Lavallette, 1865; Smith, 1916; Phillips, 1970) and the area occupied by this activity is termed the zone of growth.

Following the ultimate mitotic division, the spermatocyte is recognized. Cysts containing these spermatocytes in interphase and prophase make up the area of the tubule called the zone of spermatocytes. The active components of the mitotic divisions provide the zone of maturation and reduction. Spermiation, the development process by which spermatids become mature spermatozoa, takes place in the basal zone of transformation.

In addition to the apical cell and cyst wall cells, trophocytic interstitial cells may be present (Cooper, 1950). At the junction of the tubule epithelium and the vas efferens, the tubule is closed, at least during larval development, by cells variously termed plug cells.(Davey, 1958, 1965), neck cells (Lusis et al, 1970) or the basilar membrane (Omura, 1936; Edwards, 1961).

The ontogeny of the Hemiptera presents a confusing picture of apparently primitive characteristics which on investigation are seen in fact to be rather specialized, and this seems to hold for the reproductive system (Seidel, 1924). Thus it is no surprize to find that in our experimental animal, *Rhodnius prolixus*, the structure of the testis, while closely resembling that of the 'generalized' insect described, possesses a number of interesting modifications. What follows here is a fairly detailed catalogue of *Rhodnius*' testicular structure. The reader should note, however, that techniques used are such as provide morphological detail only, and that characterization of function generally must await precise histochemical and cytochemical analyses.

At the moment of eclosion, both testes and ovaries are roughly similar in size (100 x 300 microns). Diffetantiation of the gonads has progressed, however, to such a stage that the ovaries may be clearly distinguished by the thickening of the suspensorial sheet which is designed to become the terminal filaments (Snodgrass, 1933) (fig. 1, 3). In the testis this event does not occur and the seven tubules can be visualized 'free' in the enclosing membrane (fig. 2).

Figure 1. Whole mount of the ovary at eclosion. Ovarioles (O) well defined, terminal filament (t) recognizable in dorsal suspensorium (D). Trioxyhaematin, X 620

Figure 2. Whole mount of the testis at eclosion. Tubules (T) well defined, lacking anterior terminal filament. Vasa efferentia (VE) join to form vas deferens (VD), exiting as the gonoduct (G). Trioxyhaematin, X 700

Figure 3. Detail of the first instar ovary. Germinal cells of ovariole (O), and terminal filament cells (t). Epon, Sato's polychrome, X 650

Figure 4. Detail of the first instar testis. Continuous mesodermal sheath (S), tubular epithelium (E), spermatogonia (SG) and presumptive mesodermal cells (MS) of undefined function. Epon, Sato's polychrome X 650

Figure 5. Desheathed, mounted testes from fourth, fifth instar and adult insects immediately after ecdysis. Note highly coiled spermatocyte region of adult testis. (arrow). X 10

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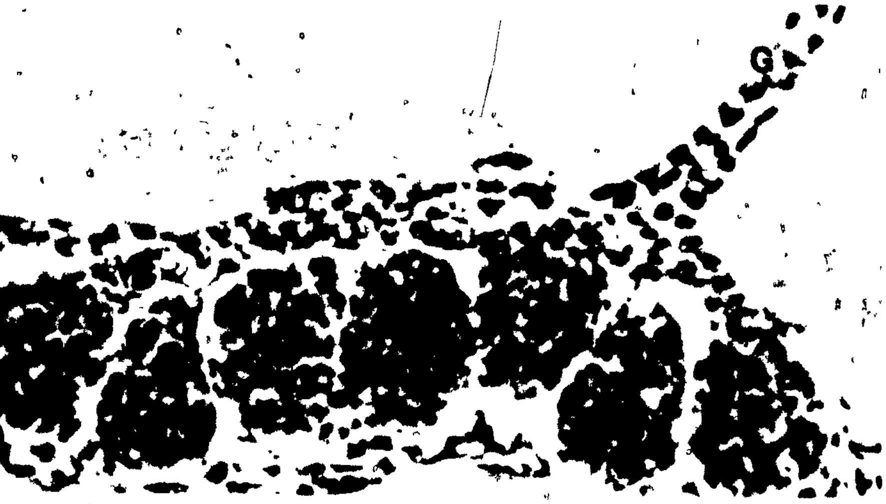

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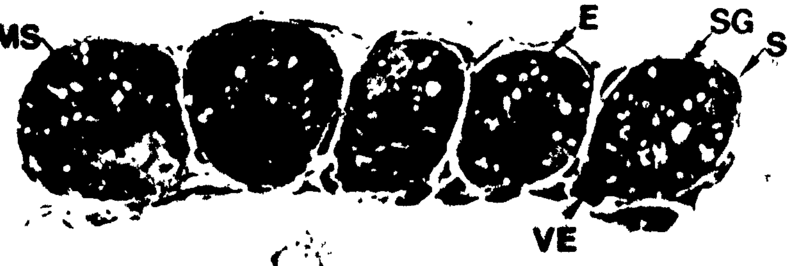

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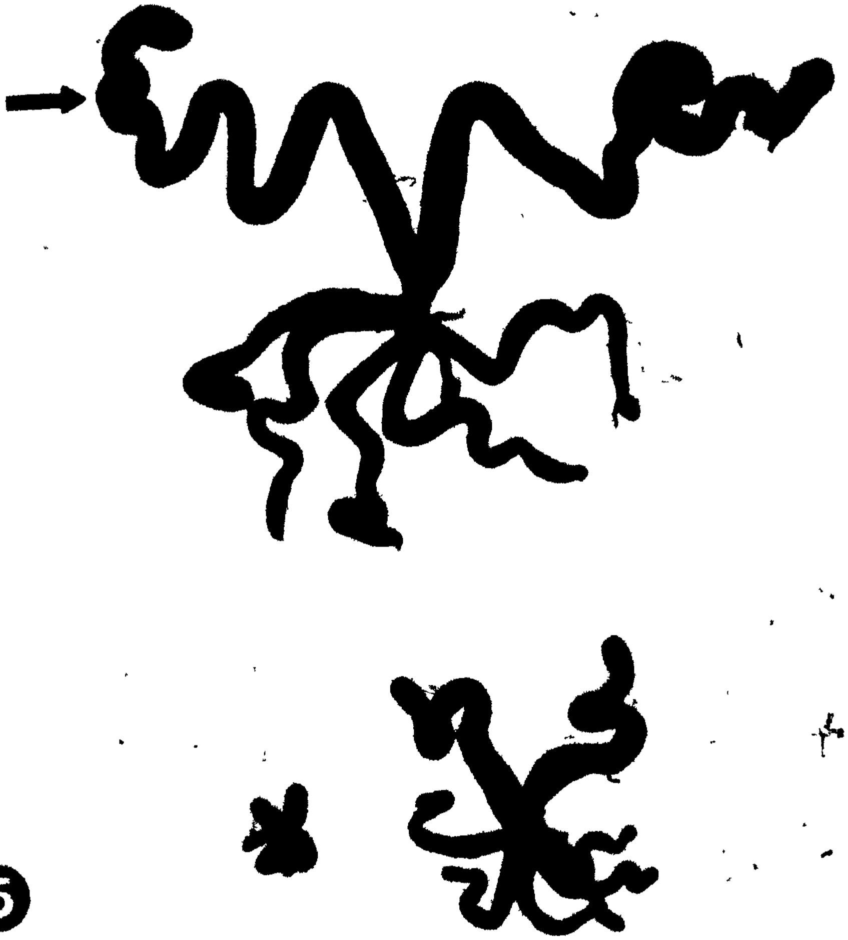

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In the gonads of both sexes, the tubules are fully delineated by their presumably mesodermal sheaths, including the ducts (fig. 2, 4), and at least for the testis, the sheath organization, spatially and in terms of elements represented, has already reached its final form. The bulk of the tubule in both cases consists of indefinitive germ cells, and possibly some scattered non-germinal elements, (fig. 4) and a tubular epithelium which, by convention, must be considered mesodermal (fig. 4). In dissection, both terminal filament and gonoduct are found present, the 'terminal filament' being attached to the external layer of the peritoneal sheath (fig. 11) rather than individual tubules. Lobes of fat body are also found attached to the testes in characteristic positions by a fine hyaline envelope, the cellularity of which could not be determined. These connections are maintained throughout the life of the insect.

Verson's cell now known as the apical cell, was first described by Spichardt (1886) in the Lepidopteran <u>Smerinthus populi</u>. Controversy as to the origin and function of the cell ensued, and this stage is admirably reviewed by Carson (1945). He reports definite identification of this cell or cell complex in the testes of Lepidoptera, Diptera, Orthoptera and, possibly, some Heteroptera. Noting the capriciousness of appearance, even in closely related insects, he concludes a trophocytic function possibly related to mitochondrial respiration of the young spermatogonia, and not a matter of fundamental significance. The matter has been more recently reviewed by Hannah-Alava (1965) in a monograph directed toward the question of comparative stem-cell renewal. Noting reports of the absence of division in the cell, she rejects any stem-cell function. In sum, she appears to favor the hypothesis first presented by Nelson (1931) that indefinitive spermatogonia in close association with the apical cell or complex are nourished by its secretions, and those which become displaced by division differentiate as cyst wall cells, and are thus not mesodermal in origin.

Interest in the apical cells has been rekindled by the demonstration of an androgenic capacity by these cells in the glow worm Lampyris noctiluca by Naisse (1966) analgous to the androgen gland of the amphipod Orchestia gammarella (Charniaux-Cotton, 1954), which determines both primary and secondary sexual characteristics. Extensive electron microscopal investigations by Menon ( 1969 ) have established the existence of apical cell complexes in the related Coleopterans Tenebrio molitor and Zophobas rugipes, and show them structurally similar to crustacean androgenic cells.

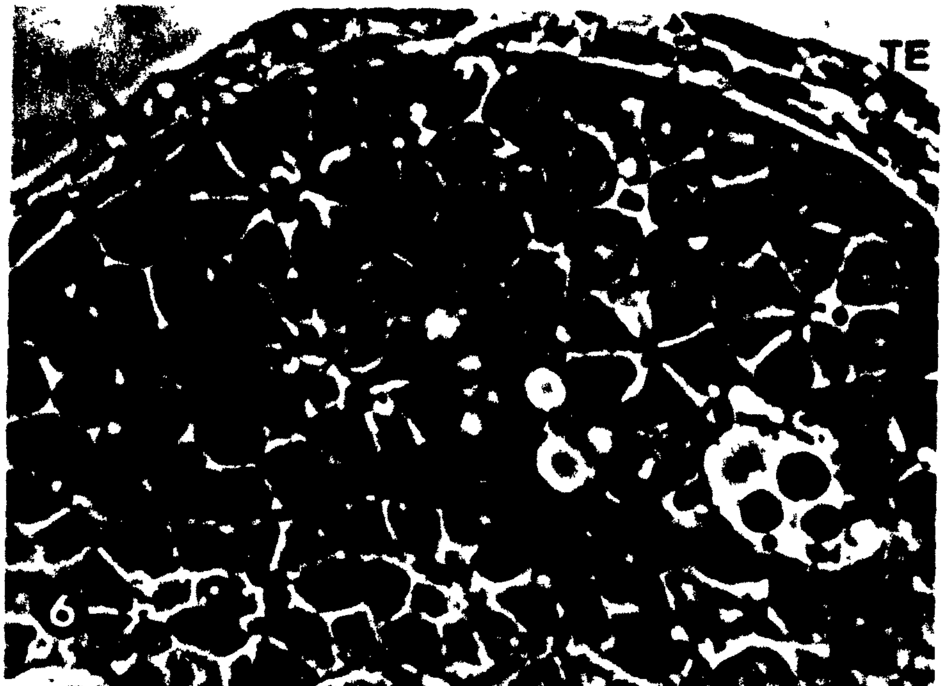
However, attempts at sex-reversal were negative, perhaps because in these insects as in Rhodnius and most other insects (Wigglesworth, 1970), sexual determination is already well-established by the time of eclosion.

Bonhag and Wick (1953) describe an 'apical complex' in the Hemipteran Oncopeltus fasciatus, containing 9 to 11 nuclei, and surrounded by spermatogonia in a partial state of dissolution.

In Rhodnius, no clear evidence of an apical cell or complex has been observed, although, immediately following eclosion, the germ cells may give the impression of surrounding a somewhat more heterogeneous mass which contains some cells of apparently mesodermal origin (fig.4 ). The germarial area in general presents a rather confused appearance, and not every cell can be reliably characterized. Single, unencysted cells are quite rare, however, with the exception of these cells comprising the basement epithelium (fig.6 ). Occasional single mitoses may be seen in close apposition to the tubule epithelium, which may represent a quasidichotomous stem cell division (fig.7,8 ). It is possible that this mesodermal epithelium carries out the trophocytic function of the mesodermal apical cell system of other insects. Another point worth mentioning is that in

Figure 6. Anterior ( germarial ) segment of fifth instar tubule. Note two-layered tunica externa (TE), cells of the tubular epithelium (E) (= tunica interna). Typical spermatogonial cysts (SG) evident, as well as pycnotic nuclei (P) of degenerating cyst. Osmium ethyl gallate, X 1600

Figure 7,8. As above, illustrating single mitotic cells in close apposition to tubular epithelium (arrows). X 1600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Rhodnius, as in other germ cell systems, (Roosen-Runge, 1973) nuclear pycnosis and degeneration are common occurrences. On close investigation, it can be determined that these foci of degeneration can occur over a wide range of cyst sizes, and an intact cyst wall is generally present (fig. 6 ). The description of the apical cell complex, and micrographs presented both by Bonhag and Wick (1953) and Economopolous and Gordon (1971) also on Oncopeltus, bear a close resemblance to this widespread degenerative process.

The sheaths surrounding Rhodnius' testis appear similar to, but slightly more complex than those of many other insects (Snodgrass, 1935). The overall organization can best be appreciated perhaps from a semi-thin (1µ ) section in epon of a freshly moulted third instar (fig. 9). Here, one can recognize first, a cellular sheath surrounding the entire testis proper, in the manner of a scrotal sac. A very similar sheath can be seen to surround each tubule individually, and numerous profiles of tracheoles are visible between the two, but not penetrating the inner sheath. This complex likely represents the tunica externa of earlier authors (Keuchenius, 1913).

The nuclei are extremely stellate or more likely discoid in shape, have one or very few nucleoli, are relatively basophilic, and fix rather poorly. This sheath complex can be easily removed from the testis,

Figure 9. Third instar testis, two weeks post ecdysis. One major tubule (far right) damaged in processing. Note variation in basophilia in developing spermatogonial cysts (SG), and the continuity of the sheath membranes. Epon, Sato's polychrome, X 300

Figure 10. Detail from above preparation. Apical tubular area, showing delamination of inner and outer tunica externa layers (TE). Cyst wall cell (C). Epon, Toluidine Blue, X 1250

Figure 11. Same , showing continuity of 'terminal filament' (F) with outer tunica externa layer (TE). Delamination of TE layers produces a potential tunica externa lumen (extratubular lumen, TEL). Epon, Sato's polychrome, X 1250

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The nuclei are extremely stellate or more likely discoid in shape, have one or very few nucleoli, are relatively basophilic, and fix rather poorly. This sheath complex can be easily removed from the testis, stretched on glass, fixed and stained. The preparation obtained consists nearly exclusively of a single cell type (fig.12 ). In the fed insect, normal appearing mitoses are prominent (fig.14 ), and late prophase appearance shows a chromosome count which is essentially diploid (fig.13 ).

The testis consists of seven tubules, two of which are about twice the size of the remaining five. The structure of both major and minor tubules appears identical. None of the tubules show the bizarre specializations, such as harlequin lobes, found in some Pentatomidae (Fain-Maurel, 1966; Schrader, 1960). The tubule proper is bounded by a sparsely distributed layer of cells which are triangular in cross sections, and possess large, multinucleolar nuclei (figs. 15,17 ). At higher magnification, a thin ( 0.6$\mu$ ) basement membrane can be seen between these cells and the inner layer of the tunica externa (fig. 16 ). This cellular layer, with its external basement membrane probably is the equivalent of Keuchenius' (1916) tunica interna. Fig. 17 demonstrates that the epithelial tunica interna cell is distinct from the generally more basophilic and osmiophilic cyst wall cells, which will be described shortly. In passing, it might be worthwhile to point out the homogenous, granular material collected in the middle layer of the tunica in this fifth instar insect five days after the blood meal. This phenomenon is typical of fed insects, and the material

Figure 12. Whole mount of tunica externa sheath, affixed to slide. Variations in the shape of the nuclei due to stretching of cytoplasmic components. Trioxyhaematin, X 675

Figure 13. Detail from above, illustrating a late prophase nucleus twenty four hours following the blood meal. N approximates 22, the normal diploid chromosome number. X 1550

Figure 14. Same, showing metaphase plates. X 1550

Figure 15. Fifth instar testis, junction of two tubules. Four days post feed. Note accumulation of amorphous material in lumen of tunica externa (TE). Tubular epithelium illustrated distinct from cyst wall cells (C). Spermatocyte cyst (SC). Osmium Ethyl Gallate, X 700

Figure 16. Similar area in unfed fifth instar insect. Illustrates basal lamina (BL), tubular epithelium (E) which together comprise the tunica interna. Tunica externa lumen (TEL) defined at this level as the area between TE cell layers of adjacent tubules. Note mitotic figures at lower left. Osmium Ethyl Gallate, X 1350

Figure 17. Same, illustrating cyst wall cells (C) adjacent to tubular epithelium (E). X 1350


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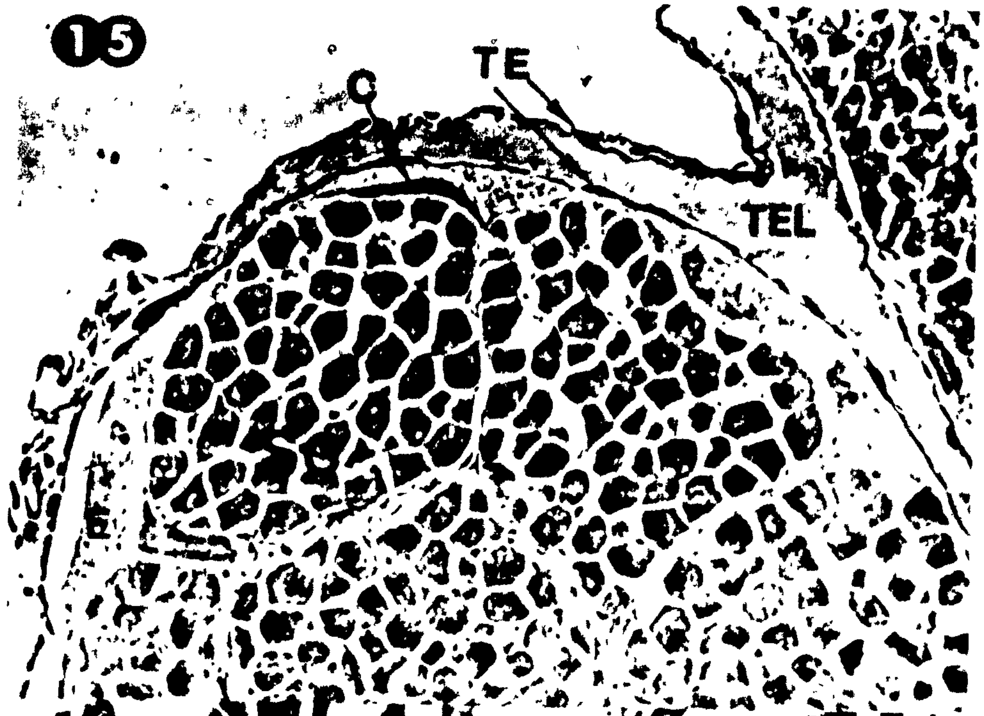

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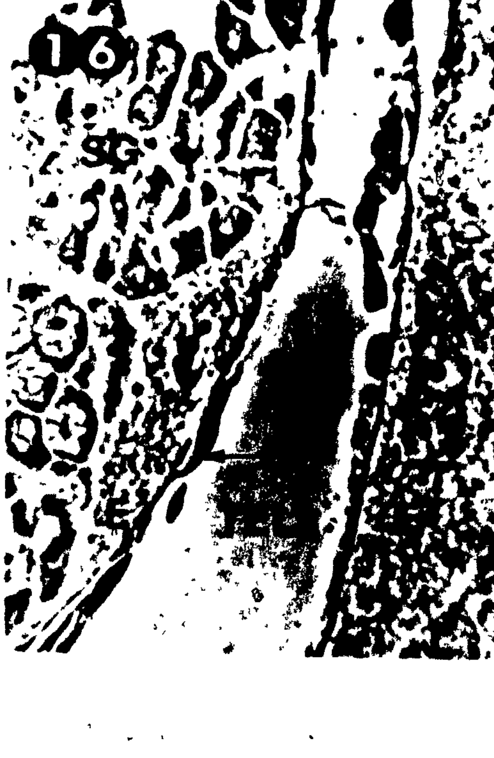

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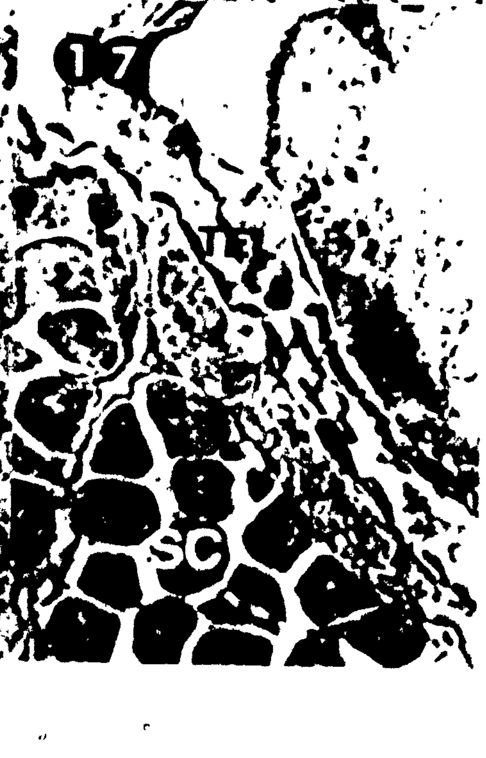

is never found inside the inner layer of the tunica externa, in spite of the all too common separation of layers due to fixation and histological processing (fig.15 ). The author has never observed division in these interna cells, in several hundred preparations at all stages of development.

Both the tunica externa and the tunica interna extend to cover the anterior of the vas deferens (fig.18,19) The elements of the tunica interna, following the contours of the tubule, meet and 'join' at the junctions of the vasa efferentia, which in section gives the impression of a blind pocket. The overall topology might better be represented by the analogy of a hand in a glove, enclosed to the wrist in a bag. The fingers of the hand represent the tubules, bounded by the tunica interna. The glove represents the inner cellular sheath of the tunica externa, and the bag the outer cellular layer.

Despite considerable coiling during the later development of the testis, the germarial areas of all the tubules always occupy an externally directed position (fig.92 ), which might provide maximum efficacy of exchange with the haemolymph. No pigmentation is found in the Rhodnius' testicular sheath, unlike the condition in many insects (Blum, 1970).

Nor are fat body cells found within the tunica externa, as is reported for Ips confusus (Bhakthan et al,

Figure 18. Fifth instar tubule, at junction of tubular epithelium and plug cell layer (P). Continuity of tunica externa (TE). Davidsons' Fluid, Biebrich Scarlet., X 1500

Figure 19. Cross section of vas efferens (VE). Both layers of the tunica externa still recognizable, defining lumen (TEL). Osmium Ethyl Gallate, X 1750

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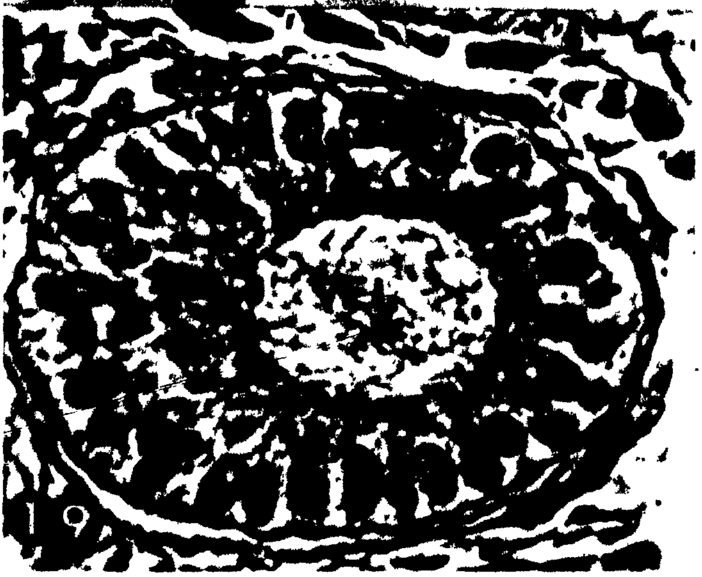

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1969).

The basal end of the tubule is closed by a ring of cells which extend down from the tunica interna, and merge imperceptibly with the epithelial cells of the vas efferens (fig.24 ). These cells, termed plug cells by Davey (1958), resemble the epithelial cells of the vas efferens except that they are larger, lack the hairlike processes which characterize the luminal border of the vas efferens epithelium, and possess several nucleoli in contrast to the single nucleolus of the vas efferens cell. The nucleus becomes folded and ruffled (fig.23 ), and division figures indicate a high degree of polypoidy. The cells nevertheless form metaphase plates, though sometimes in more than one plane (figs. 20,21 ), possess visible spindles (fig.22 ), and undergo anaphase segregation. Cytokinesis has not been observed. The nuclei of these plug cells are situated around the periphery of the tubule, the central area being occupied largely by a thin interdigitated cytoplasmic sheet. Thus, these cells may easily be missed in a true longitudinal section of the tubule. More tangential sections show similar cells extending for a short distance up the tubule. As these cells have not been seen to divide, it is suspected that these are 'en face' profiles of the tunica interna epidermal cells. Neck cells have been described

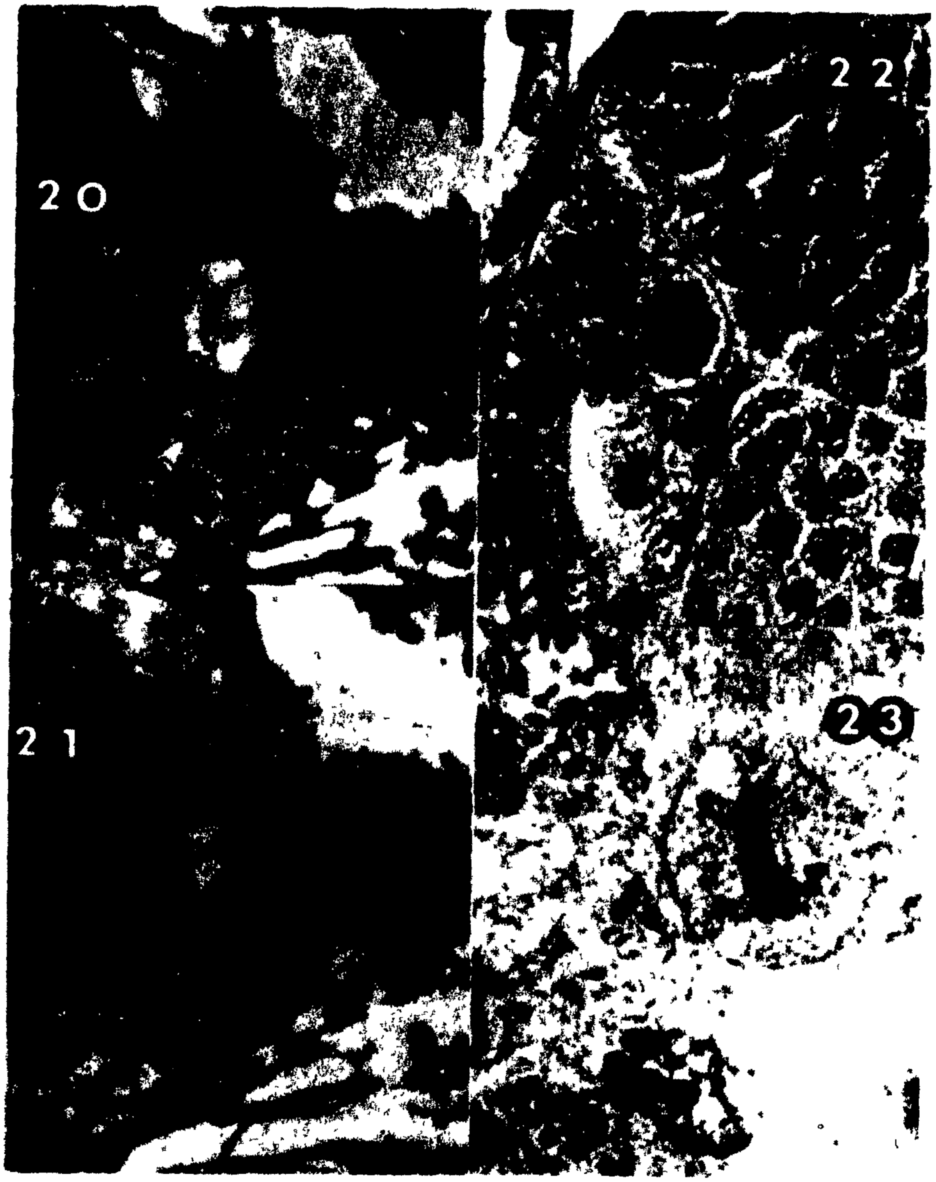
Figure 20. Base of the tubule, tangential to the longitudinal axis, in a recently ecdysed fifth instar insect. Illustrates plug cell layer (P), including lateral metaphase plate. Trioxyhaematin, X 560

Figure 21. Adjacent section in series. Polar metaphase plate visible in same cell. X 560

Figure 22. Anaphase in plug cell. Note continuity of vas efferens epithelia, plug cells, and tubular epithelia. Trioxyhaematin, X 800

Figure 23. Interphase, prophase, and metaphase in plug cells. Note lobulated nuclear membrane of interphase plug cell. Trioxyhaematin, X 1450

Figure 24. Basal area of tubule and vas efferens of fifth instar insect, four days following the blood meal. Note elongating spermatids (SP), mitosis in plug cells (P), hairlike processes of vas efferens (H). Osmium Ethyl Gallate, X 1050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in this area of the tubules of the roaches Gromphadorhina portentosa and Byrsothria fumigata by Lusis et al (1970), and a secretory function postulated on the basis of histochemical investigation. Since these neck cells do not form an effective barrier between the tubule and the vas efferens lumen, and since the plug cells of Rhodnius performed rather differently under limited histochemical investigation (were negative when stained with Orange G, negative for disulphide groups, and negative for acid phosphatase activity), they are not likely analogous. As described originally by Davey (1958), the plug cells remain intact in the adult, each sperm bundle passing through the central cytoplasmic area.

The vas efferens, immediately beneath the plug cell sheet, is a single celled tube during larval life. It is surrounded by the tunica externa as previously described (fig.18 ) and possesses numerous hairlike processes on its luminal surface, described previously by Davey (1958). Omura (1936) has described a similar specialization in Bombyx mori. Danilova (1971) suggests a mechanical role in assisting sperm through the basilar membrane which seals the tubule in this insect. A more likely but equally unproven hypothesis, that these hairs provide an amplification of surface area for fluid absorption, will be advanced in a later section.

The process of encystment of the spermatogonia was described in the early part of this century. Zick (1911), concluded that the cells which become the cyst wall were mesodermal in origin, arising from the epithelium of the sperm tubule. Other authors have advanced a germinal origin (Nelson, 1931). No conclusion on this matter will be attempted here. The encystment of an apparent single spermatogonium is illustrated in figure 25, in the testis of a newly moulted third instar larva. One to a few of these cyst wall cells can be seen around every cyst, in every stage of development (figs. 26,27 ), including cysts containing mature spermatozoa, unlike the situation in some insects which lack cysts surrounding mature germ cells.

The cyst wall cells are presumably stellate and have a very reduced cytoplasm. The nuclei, while presenting a variable appearance in cross section, are quite large and possess several nucleoli. The nucleus is generally somewhat more osmiophilic than that of the epithelial cells or the plug cells. On only one occasion has a mitotic figure been observed in these cells, and even in that case identification of the cell as a cyst wall cell was not positive. The nuclear size appears to increase basally, suggesting endoploidy without spindle formation and chromosome segregation, unlike the events occurring in the plug cells.

Figure 25. Third instar testis, apical area of tubule. Cyst wall cells (C) surrounding what appears in this, and in subsequent sections of the series, to be a single spermatogonium. Epon, Toluidine Blue X 1700

Figure 26. Multinucleolar cyst wall cell (C) surrounding spermatocyte cyst (SC). Trioxyhaematin, X 1700

Figure 27. Cyst wall cell (C) between adjacent cysts of advanced spermatozoa (SZ). Trioxyhaematin, X 1700

Figure 28. Junction of three spermatocyte cysts, illustrating independence of cyst wall cells, and the lack of cellular continuity between cysts. Biebrich Scarlet, X 1700


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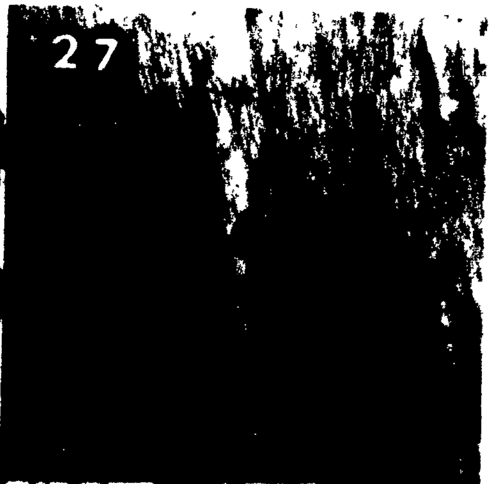
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Synchronous development within each cyst was reported early (Smith, 1916) and this synchrony is presumed to result from cytoplasmic continuity, a result of incomplete cytokinesis (LaValette St. George, 1865; reviews by Fawcett et al., 1959; Dym and Fawcett, 1971 and Phillips, 1970). Characterization of the intercellular bridge at the fine structural level, and the suggestion that such bridges within the clonal cell population might direct differentiation of the oocyte (Koch and King, 1967) and spermatocyte (King and Akai, 1971) will be dealt with more fully in a succeeding section (Chapt. 10 ). Ring canals stain with iron trioxyhaematin, and particularly are highly osmiophilic (fig 29,30). With increasing cyst size, more than one centre of ring canal fusion may exist (fig. 31 ). Since the groups formed are roughly equal in size, they are presumably formed by an extension of the ring canal centre itself rather than by displacement of one cell through spindle orientation or crowding. Slight differences in division phase are noted between these subdivisions within the cyst (fig. 32 ). Due to crowding, the existence of the inter-connections cannot be visualized in late spermatogonial or spermatocyte cysts. However, the phase variations persist, suggesting retention of the bridges through to the spermatid stage as in mammals (Dym and Fawcett, 1971 ).

Figure 29. Spermatogonial cyst (SG) at prophase of approximately 32 cell stage. Note central fusion of ring canals (R). Trioxyhaematin, X 1600

Figure 30. Same, 16 cell stage, Osmium Ethyl Gallate, X 1600

Figure 31. Spermatogonial cyst, approximately 128 cell stage. Two centers of ring canal (R) fusion apparent in same cyst. Note typical circumnuclear accumulation of mitochondria (m). Osmium Ethyl Gallate, X 1600

Figure 32. Approximately the same size of cyst, demonstrating partial asynchrony of development: Half the cells are in prophase, half in metaphase. Trioxyhaematin, X 500


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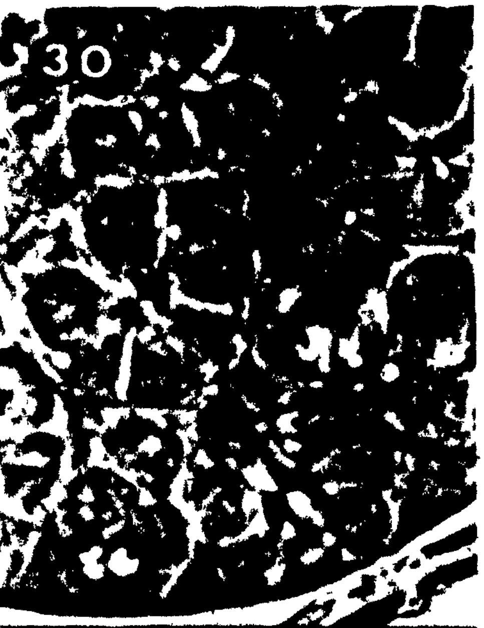
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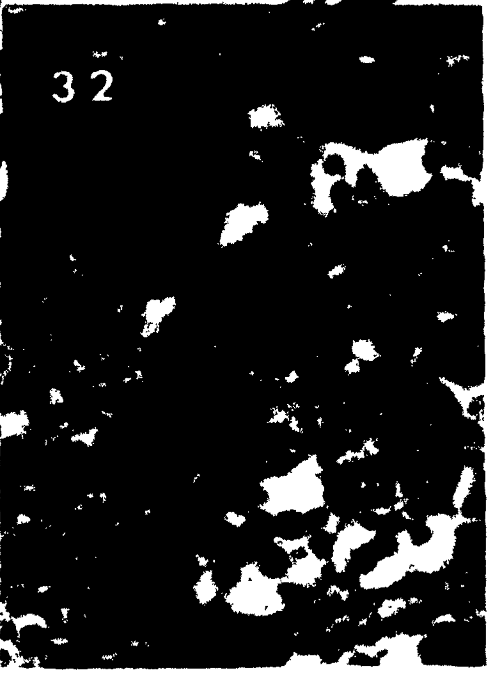
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The Rhodnius germ cell shows a cytological development very similar to the typical hemipteran cytogenetic plan exhaustively described by Wilson (1912). Accurate staging of the degree of germ cell differentiation forms the basis for many of the assay techniques subsequently used in experimentation, thus a series of squashed and air-dried preparations of tubules, and portions of tubules were prepared and compared to flat-mounted sections of tubules stained with trioxyhaematin (Appendix: Materials & Methods). The long spermatocyte prophase provided the best means of quantification in the Rhodnius tubule, and attention was focused on that compartment.

Hemipteran chromosomes, holocentric, small, and very similar in size to one another are extremely unfavorable material for cytologic investigation. Very little could be determined from spermatogonial or somatic cell squashes, save the diploid chromosome number, twenty-two in both sexes. With regard to the highly endoploid mesodermal cells, examples were seen as high as sixteen-ploid (fig.55 ). Large interphase endoploid nuclei were also identified, with numerous heteropycnotic sex elements (fig.54 ). The degree of ploidy could not be ascertained in these cells, however, due to the small size of the sex chromosomes, and a complication in their number shortly to be described. Similar findings are reported in the testis 'sheath' of related species (Geitler, 1937, 1938, 1939a and b, 1941).

As previously mentioned, the stem cell could not be located spatially, and thus cannot be characterized cytologically. In mammalian systems, cytological identification of spermatogonial types is well advanced (Clermont, 1972). Very simply, ignoring the many subdivisions now recognized, these spermatogonia may be divided into type A or 'dustlike' spermatogonia, being stem cells or predefinitive, uncommitted spermatogonia, and type B or 'crustlike' spermatogonia, which are destined to become spermatocytes through a species-specific number of divisions. Hannah-Alava (1965) suggests that similar cytological distinctions may be made in insects. However, distribution of the chromatin required for this technique is most favorable with Zenker's fixative, and is completely eliminated by the Lebrun's Carnoy found most suitable for the preservation of spatial orientation and spermatocyte identification in <u>Rhodnius</u>. Thus, only one basic spermatogonial cell may be recognized (figs. 33-37 ). Some distinction can be made on the basis of position in the tubule, and cyst size, but these criteria are not entirely reliable and become quite hopeless in the germarial region.

By contrast, the cytological events during the spermatocyte prophase result in distinctive and easily

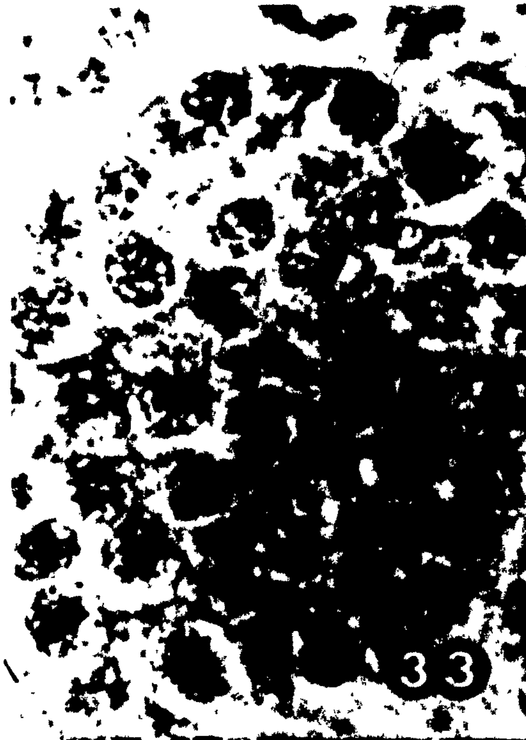
Figure 33. Final spermatogonial cyst, as identified by position in the tubule. Interphase; compare with figure 38. Trioxyhaematin, X 1800

Figure 34. Late spermatogonial prophase; Aceto-Carmine squash, X 1800

Figure 35. Polar and lateral spermatogonial metaphase plates. Trioxyhaematin, X 1800

Figure 36. Male somatic chromosome spread, Aceto Orcein squash preparation of accessory gland. X 1800

Figure 37. Female somatic chromosome spread, Aceto Orcein squash preparation, oviduct. X 1800



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characterized stages. The initial stages, interphase and leptotene, closely resemble the last spermatogonial stage both cytologically and in terms of cyst size (figs.38,39 ). Typically, the hemipteran spermatocyte nucleus then enters a phase of maximal coiling of the chromosomes to form the synizetic knot of the zygotene stage, or synizesis. This is a particularly well-represented stage in Rhodnius (figs. 40,41 ). During the succeeding pachytene stage, the chromosomes gradually uncoil, revealing for the first time the sex vesicle, but no well-characterized bouquet formation (figs. 42-44 ). Complete decondensation of the nuclear material, with the exception of the heteropycnotic sex complement, provides a well-marked diffuse diplotene stage (figs. 45, 46 ). Diakinesis is evident (figs. 47-49 ), and the first meiotic metaphase, anaphase, and telophase follow in order, unremarkable save for the late segregation of the sex chromosomes (figs.50-53,56). No second interphase or prophase is recognized, and the spermatocytes likely pass directly to the second metaphase, which is indistinguishable in sections from the first meiotic division figures.

This progression represents a simplified version of the twelve stages described by Wilson (1912) for Lygaeus and Oncopeltus, and differs largely in the exaggerated synizesis and diffuse diplotene stages displayed

Figure 38. Interphase of spermatocyte cyst, defined by position in tubule. Trioxyhaematin, X 1800

Figure 39. Chromosome spread from same stage, some pairing evident. Note heteropycnotic sex elements (arrow). Aceto Orcein, X 1800

Figure 40. Characteristic zygotene spermatocytes, showing highly condensed, tangled chromosome mass (synizetic knot) with clear area surrounding. Trioxyhaematin, X 1800

Figure 41. Possibly same stage in squash preparation. Note small and large (double) sex elements (arrows). Aceto Carmine, X 1800

Figure 42. Pachytene spermatocytes, poorly characterized bouquet configuration around sex vesicle (arrow). Trioxyhaematin. X 1800

Figure 43. Same stage, Aceto Orcein squash preparation X 1800

Figure 44. Slightly later in pachytene. Trioxyhaematin, X 1800

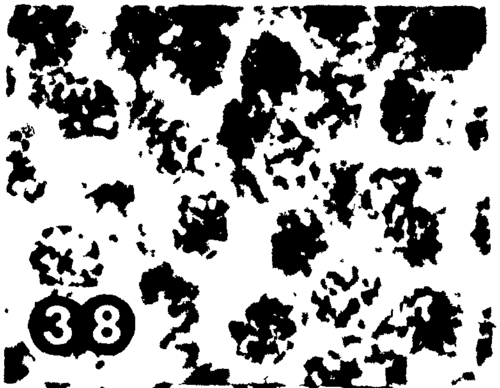
Figure 45. Diffuse diplotene stage, Aceto Orcein squash preparation. Three doubled heteropycnotic sex elements, no condensed autosomal chromatin. X 1800

Figure 46. Early diffuse diplotene, Trioxyhaematin, X 1800

Figure 47. Early diakinesis, Aceto Orcein squash preparation. Note pairing of two of the sex elements. X 1800

Figure 48. Diakinesis. Distribution of chromosomes on the nuclear membrane. Trioxyhaematin, X 1800

Figure 49. Aceto Orcein squash preparation at same stage as figure 48. Note sex chromatin now nearly isopycnotic with autosomes. X 1800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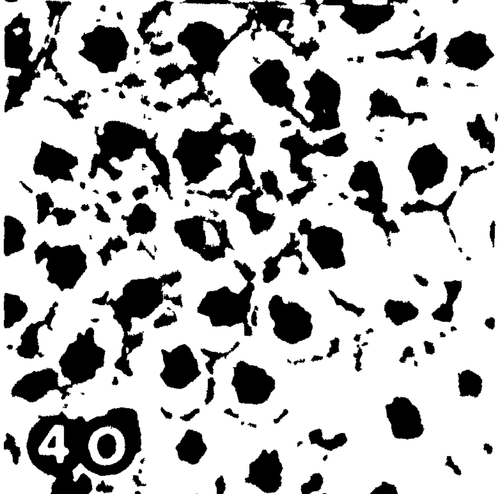
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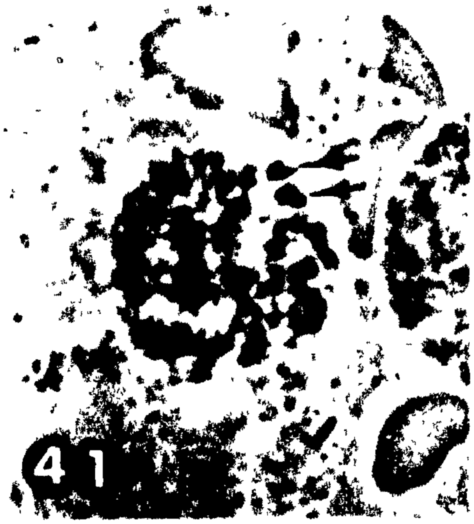
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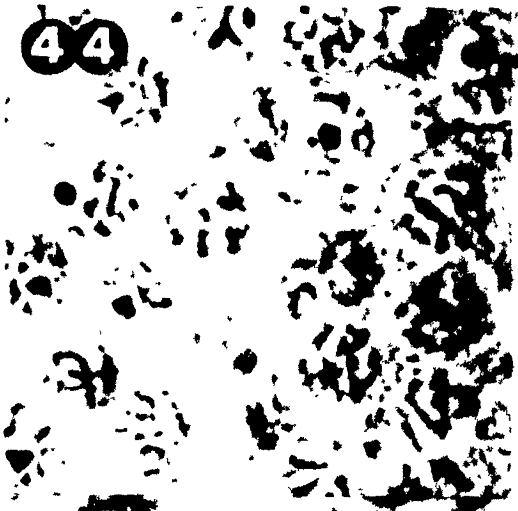

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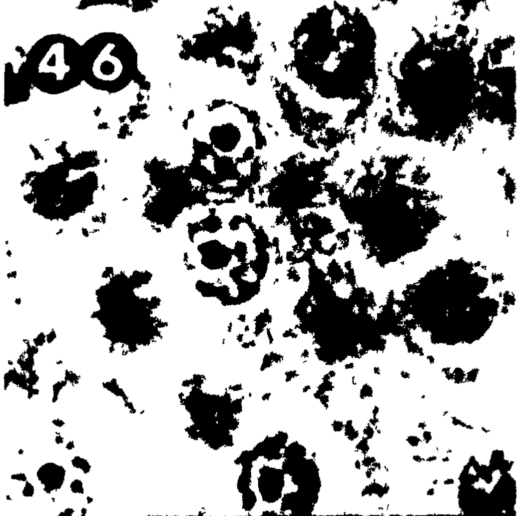

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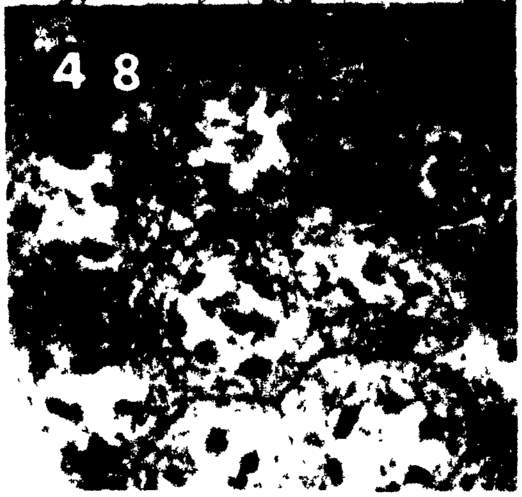

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by the Rhodnius spermatocyte. The sex chromosome complement in Rhodnius follows the hereropycnotic behaviour pattern defined by Wilson; however, in Rhodnius, an interesting variation arises. Three doubled sex elements can be seen in early prophase (figs. 45,47 ). The two larger of these pair to form a bivalent at first metaphase, and segregate apparently in the same manner as the autosomes (figs. 50,52 ). In the second metaphase, the unlike sex chromsomes apparently join by 'touch and go' pairing, common to hemiptera (White, 1973), resulting in a reduction in chromosome number (ten + two; ten + one). In this case, the sex complement at the second metaphase is tripartite, and might be assumed to represent an $X_1X_2Y$ group, also quite common to the hemiptera (White, 1973). However, the equality of chromosome number between male and female somatic cells (Twenty + two) would suggest that this sex complement is $X_1Y_1Y_2$ in Rhodnius. In view of the rarity of such a phenomenon among the hemiptera, this observation would need to be verified by an investigation of the oocyte chromosome complement, a technique which was not carried out.

In any case, following the meiotic divisions, the spermatids so formed are small ( 5.0µ ) cells, without much apparent internal structure (figs. 57 ). Turning to other histological techniques, spermiation

Figure 50. Primary spermatocyte metaphase, polar view. Ten autosomes, two sex elements. Aceto Orcein, X 1700

Figure 51. Secondary spermatocyte, metaphase, polar view. Ten autosomes, one sex element. Aceto Orcein, X 1700

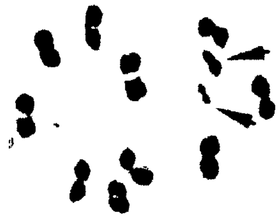
Figure 52. Primary spermatocyte metaphase, lateral view. Note that paired sex element (left arrow) has apparent bivalent structure, unlike single sex element (right arrow). Aceto Orcein, X 1700

Figure 53. Secondary spermatocyte metaphase, lateral view, illustrating 'touch and go' pairing of sex elements. Aceto Orcein, X 1700

Figure 54. Heteropycnotic elements in interphase nucleus of polyploid cell. Aceto Orcein, X 1700

Figure 55. Polyploid chromosome spread, approximately 8N. Aceto Orcein, X 1700

Figure 56. Late segregation of the presumed sex element in meiotic telophase. Aceto Carmine, X 1700



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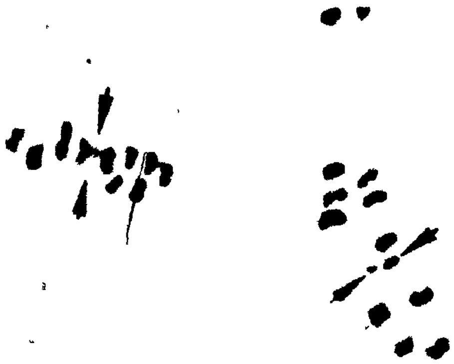

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could be followed with particular reference to the development of the acrosome, centriolar reorganization, and coalescence of the mitochondria to form the nebenkern (figs. 58-60 ). Mature and maturing sperm of <u>Rhodnius</u> are not well-characterized at the level of the light microscope (fig.27 ). As Davey (1965) points out, they are long and filamentous in character, presumably so that the micropylar opening in the egg may be as small as possible thus minimizing water loss through evaporation. The heads are maintained well in register with one another, typical of insects (Phillips, 1970), and no specific sperm cap cell or association with any particular area of the cyst wall is evident.

Figure 57. Early spermatids, showing nucleus (N) and forming acrosomal granule (A).

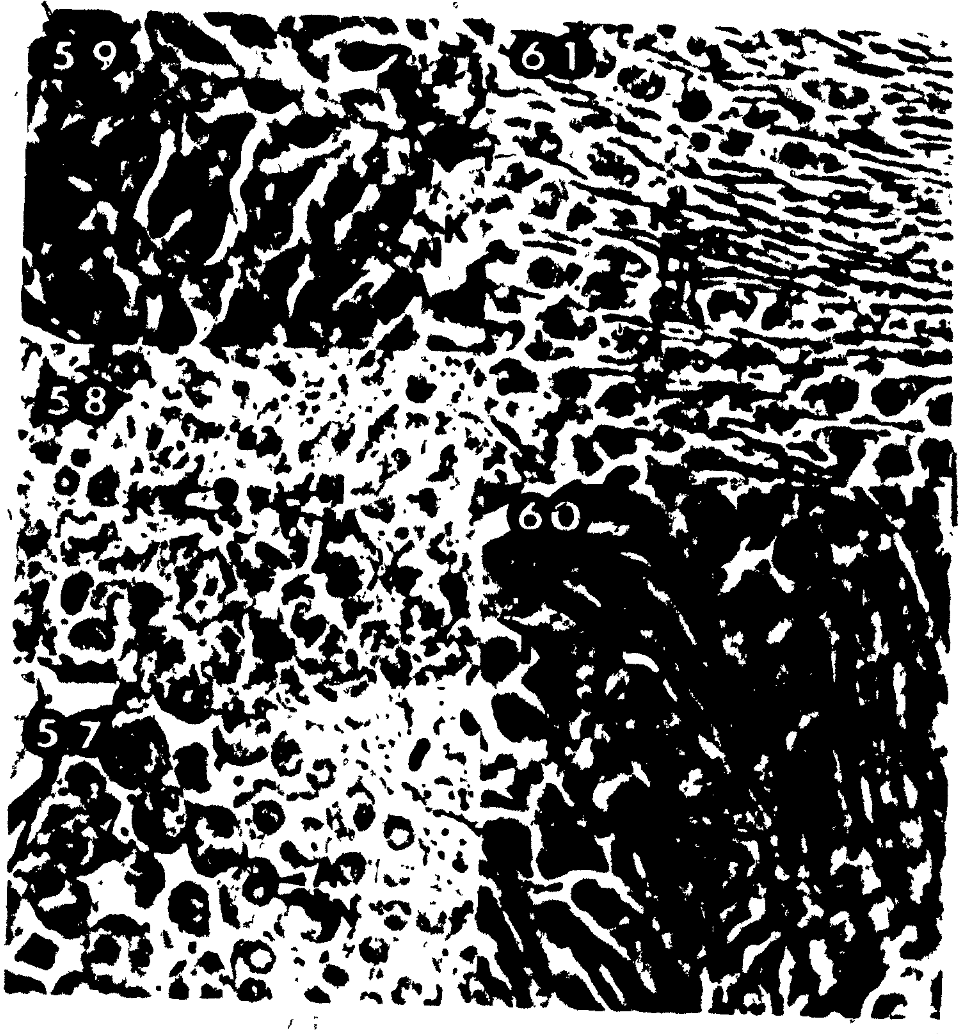
Figure 58. Later stage. Mitochondria have coalesced to form two nebenkern (N) bodies. Acrosome (A) in characteristic position lateral to nucleus (N).

Figure 59. Elongation of nebenkern.

Figure 60. At later stage, centrosomes (CN) seen at base of nucleus, 'head' of flagellum produced by nebenkern.

Figure 61. Filiform spermatids. Acrosome remains lateral, centrosomes still double.

All micrographs Osmium Ethyl Gallate, X1500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## Chapter Four: Developmental Events; Autolysis

One criterion of development attended to by previous workers in other species has been the change in size of the testis through larval development. A common pattern observed is that of slow growth through early instars, supplanted by a more rapid increase in the last larval instar and pupa, as in Bombyx (Takeuchi, 1969) and Aedes stimulans (Horsfall and Ronquillo, 1970). McLaughlin and Lusk (1967), on the other hand define a stable growth rate for Anthonomus grandis, similar to other larval structures. Economopoulos and Gordon report a slightly declining growth rate through to the young adult in Oncopeltus (1971). Both studies begin with late larval development, however, and are thus not comparable to the above work. A declining growth rate is also defined for Drosophila, apparently influenced in part by the spiralization of the testis directed by the vas efferens (Kerkis, 1931). The testis size of the adult in some insects is considerably less than that in a mature larva or pupa (Chironomous plumosus, Wensler and Rempel, 1962; Hyolaphora cecropia, Riddiford, 1972), a consequence of migration of spermatozoa to the seminal vesicle.

The testes of Rhodnius are quite small in earlier instars, making direct volumetric measurements by displacement or estimates from weight impossible. As well, considerable variation between insects of the same age is noted, as often reported in other species (Economopoulos and Gordon 1971; McLaughlin and Lusk, 1967). As estimates based on surface area were considered imprecise, no detailed analysis was carried out, but casual inspection of available preparations indicates an approximate doubling of surface dimensions of the testis between eclosion (100$\mu$ x 300$\mu$) and mid third instar (200$\mu$ x 600$\mu$ ). Practically all growth during this period is by cell division. Similarly, the early fourth instar testis has dimensions of about 260$\mu$ x 780$\mu$ . A line drawn through these points is linear (fig.62 ). Changes in size between the fourth and fifth instar, and the fifth instar and young adult are considerably greater, as is illustrated in fig. 5 . These quantitative changes are accompanied by qualitative changes in the state of differentiation of the germ cell cysts. Thus, the fourth instar testis prior to the blood meal contains only spermatogonial cysts, each cyst containing up to about $2^6$ cells (fig.98 ). In the fifth instar; prior to the feed, the dominant cellular elements are not spermatogonia but spermatocytes (fig.20 ). Sections cut of testes at daily intervals indicate the initial appearance of spermatocytes at about the eighth day following the feed in the fourth instar. By approxi-

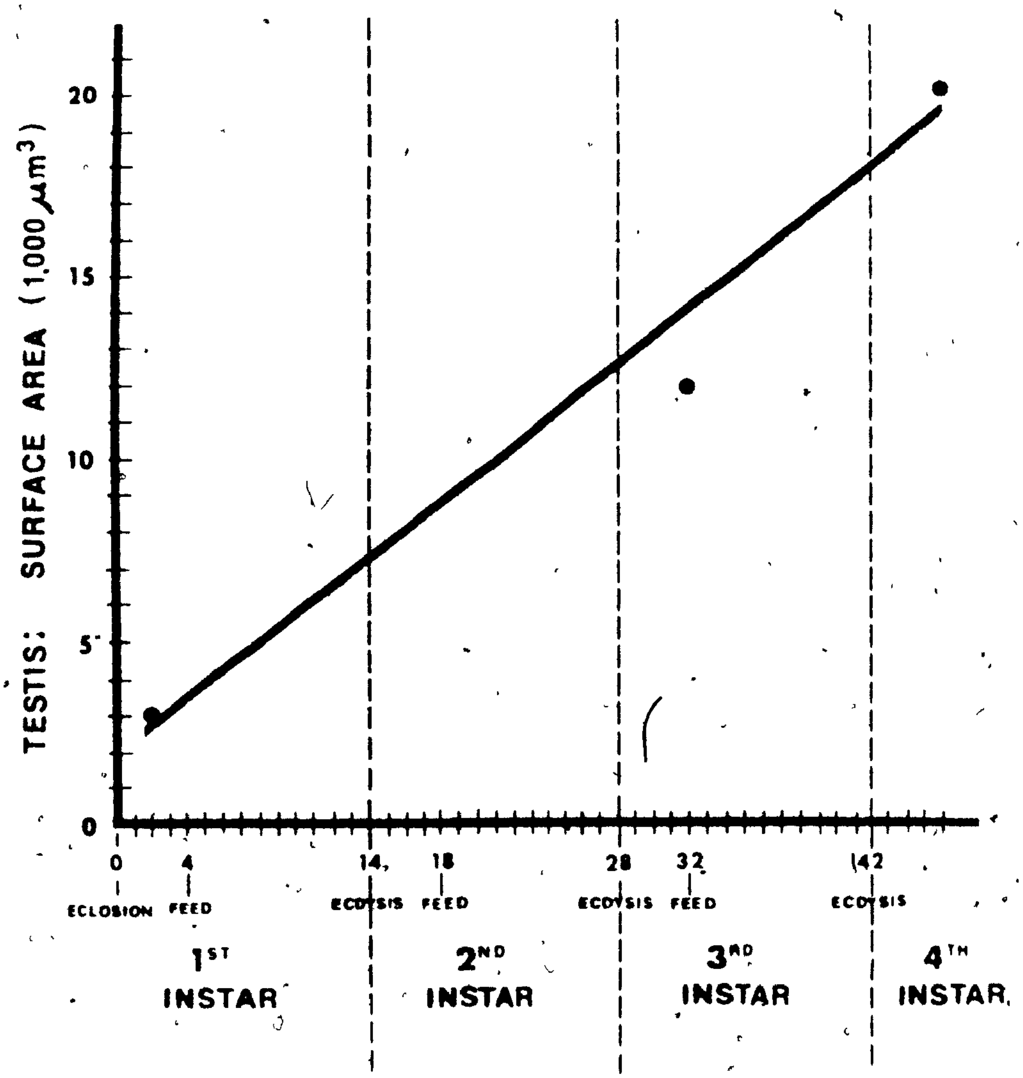

FIGURE 62. Surface area of cross sections of testes at various stages in larval development. Points represent single individuals. A four day period is assumed following each ecdysis prior to the blood meal. X axis represents days following eclosion.

mately the fourth day following the moult to the fifth instar, a period of about eight days from first appearance, the testis has been largely colonized by spermatocytes. The increase in testis size between the fourth and fifth instar this involves not only cell division, but the accumulation and subsequent increase in individual cell size of a large pool of primary spermatocytes. After the feed in the fifth instar, spermiation begins. In the adult, most of the testicular volume is taken up by spermatozoa and developing spermatids. These developments are illustrated in fig.63 .

It is important to note that when a given stage of germ cell differentiation is reached, earlier stages do not decline or disappear. Spermatogonial mitoses continue during the period of spermiation and, indeed, both processes occur throughout the life of the adult. The germarial region of an actively breeding adult, while occupying a very small part of the total testicular volume, does not appear to differ in absolute size markedly from that found, for instance, in a fed fourth instar.

Histological investigations carried out during the diapause and post-feed periods revealed a surprizing fact. Metaphase figures in the germarial and growth areas of the tubule, and meiotic metaphase plates in the appropriate instar were present in the

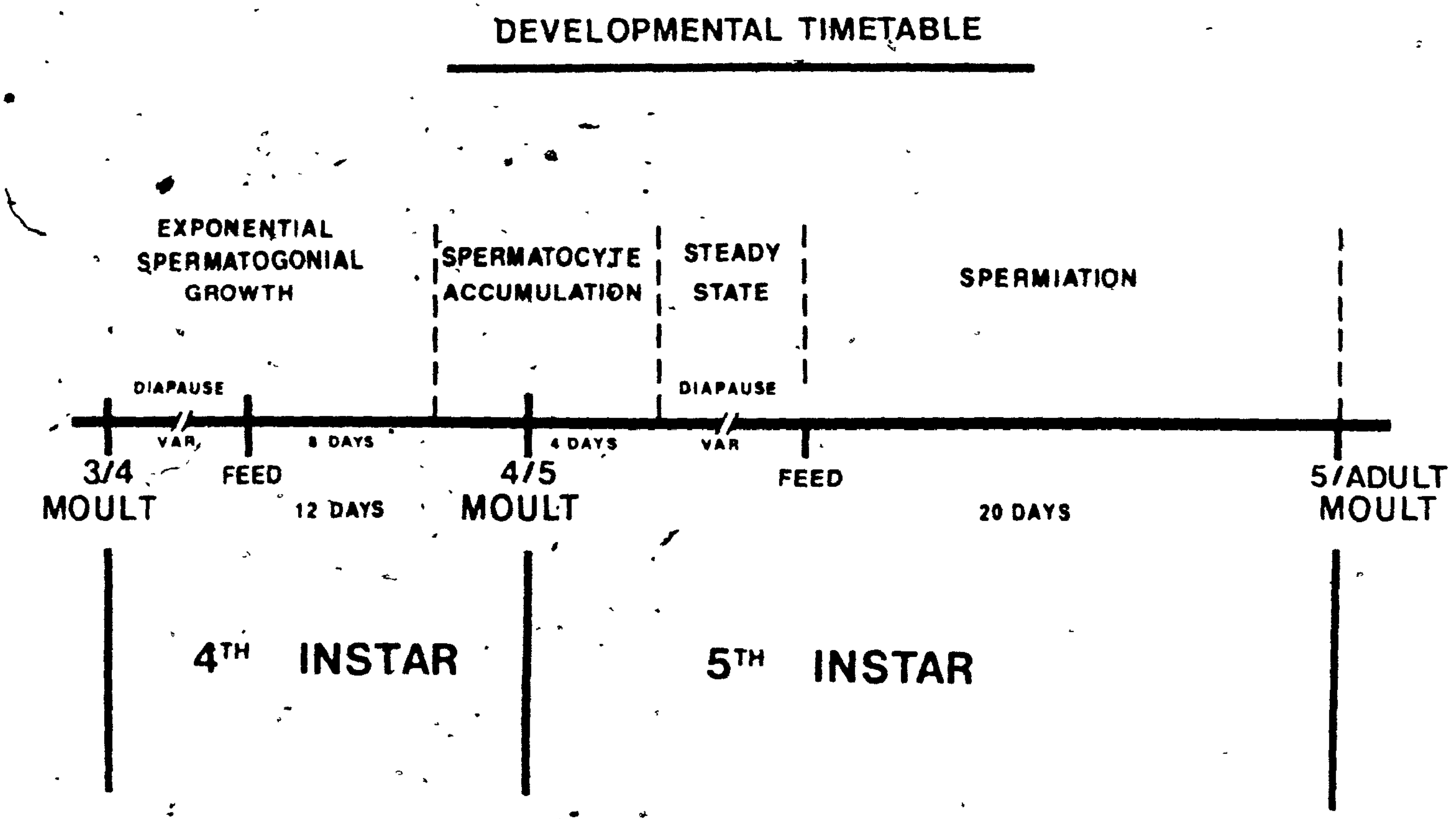

FIGURE 63. A timetable of testis development in larval Rhodnius, interpreting volumetric changes (see figs.5, 62) in terms of germ cell differentiation. Major points of interest: appearance of ( synizetic ) spermatocytes at day 8 post feed in the 4th instar, and their subsequent accumulation; appearance of meiotic figures at approximately day 4 of the 5th instar; specific autolysis of spermatids resulting in a 'steady state' condition; successful spermiation following the 5th instar blood meal.

diapausing as well as the fed insect, in spite of the fact that no increase in testis volume or cell numbers occurred during the diapause. In earlier instars, the mechanism of this stasis was puzzling. The size and linearity of the fifth instar tubule, however, permitted a reconstruction of events suggesting the solution. Meiotic divisions began early: approximately day two to five days post-moult in the unfed fifth instar. No spermiation occurred in these unfed, diapausing insects, however. Instead, the spermatid cysts so formed underwent degeneration (fig.64 ), and all cysts so produced were subjected to this fate until the fifth instar feed. Spermatid cysts produced by meiotic division subsequent to the blood meal underwent spermiation and maturation to spermatozoa.

It is often impossible, of course, to identify accurately the source or stage of differentiation of degenerating cells, since the process itself results in a progressive and total morphological and cytological disruption. In this case, however, differentiation of the spermatid results in the formation of specific, highly distinctive organelles: the acrosome or pro-acrosomal granule, and the nebenkern (figs. 58-65 ) which are not found in any other cell state. Conclusive

Figure 64. Testis tubule of a fifth instar insect 10 days following ecdysis. Note spermatocyte cyst (SC), meiosis (M), spermatid cyst (SD), and degenerating spermatid cyst (DG) in basal progression. Osmium Ethyl Gallate, X 775

Figure 65. Detail of degenerating spermatid cyst. Illustrating acrosomal granule (A), nucleus (N) and nebenkern (K) in cells. Osmium Ethyl Gallate, X 1950

Figure 66. Accumulation of cyst wall cell nuclei (arrows) in plug cell region of unfed insect tubule. Biebrich Scarlet, X 1550


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identification of these structures in cysts early in the degenerative process (fig. 65) and position of the degenerating cysts basal to meiotic metaphase figures, and early, apparently normal spermatid cysts (fig. 64) provided a means of easy identification. It thus seemed that the spermatid, inappropriate to the developmental schema during the variable length of time spent in the fifth instar diapause, was selectively and totally removed from the system. Evidence that the process was continuous was found in the accumulation of cyst cell nuclei in the basal area of the tubule, adjacent to the cytoplasmic barrier formed by the plug cells (fig. 66). This accumulation did not occur indefinitely. Disappearance of the remnants of the degenerating cysts from the basal area of the tubule occurred by about four days post-feed, providing a rough estimate of the transit time for the degenerating cells.

The appearance of the degenerating spermatids is quite distinct from that of degenerating spermatogonial cells, alluded to in an earlier section (see Chapter Three). These earlier examples of degeneration infrequently and apparently randomly distributed throughout the tubule, are characterized by extreme shrinkage and pycnosis of the nuclei, which come to be clumped centrally in a much reduced cyst (fig. 6 ). The specific spermatid autolysis in its early stages, shows a dramatic swelling of both cell and nucleus, from approximately 5, 3.5µ in diameter in the spermatid, to 20 µ and 5 µ respectively in the degenerating cell (fig. 65). Lysis of the cell occurs, releasing nuclei and nebenkern into the cyst interior. Individual components become difficult to follow in subsequent stages, but pycnosis of nuclei does not appear to be part of the process.

As the events always occurred in the basal region of the tubule in close proximity to the plug cells, it was suspected that these cells might be instrumental in the process. There are reports, scattered throughout the literature of acid phosphatase activity in similar cells: neck cells in Gromphadorhina and Byrsothria (Lusis et al, 1969); supportive cells of the testis of Artemia salina (Wolfe, 1971); nutritive phagocytes of the testis of the sea urchin Stylocidaris, (Holland, 1967). This enzyme is generally associated with lysosomes (DeDuve, 1963), and while the role of acid phosphatase activity at least in the neck cells above is not clear, two possibilities might be projected for a similar activity in the plug cells. First, resulting from lysosomes produced and packaged for export to the cyst to initiate the histolysis reported, or, second, as lysosomal acid phosphatase for the digestion of phagocytosed remnants of the spermatid autolysis.

To investigate these possibilities, a population of sibling Rhodnius males were selected at the moult from fourth to fifth instar, held ten days, and then fed. Two to four insects were removed from the population at daily intervals from the moult, dissected, and the testes investigated for the presence and distribution of acid phosphatase. The technique employed was that of Barka and Anderson (1965), using 8 μ serial sections obtained by cryotomy. (Appendix Two: Histology). The experiment was continued for fifteen days following the blood meal.

The results obtained indicated that acid phosphate activity was present in the testis but localized strictly within the degenerating cyst. The activity was so pronounced, in fact, that it was clearly visualized in fresh, intact testes incubated in the histochemical medium. This activity was demonstrated at those times when degenerating cysts were present in the testis: that is from four days post-moult to four days post-feed. In the intact testes, the position of the reaction product and timing of its appearance coincided precisely with the appearance of a yellowish, refractile mass. This was similar in appearance to that resulting from oöcyte resorption and degradation of postovulationary follicles, and referred to as 'yellow bodies' (Cone and Eschenberg, 1966). Relatively late in the post-feed period, acid phosphatase activity could be seen in scattered mesodermal cells, but the precise identification of these cells was not possible. At the same time, a slight amount of activity was found free in the vas efferens and vas deferens lumen. At no time, however, was activity demonstrable in the plug cells. This finding might be explained by the relatively high, and hence non-optimal pH range used in incubation of the tissue, which was necessary to control diffusion of the reaction product from the centre of activity. This interpreation is argued against by the vigorous reaction of the mesodermal cells, and visualization of free activity in the duct system. The plug cells may or may not be involved in the initiation of spermatid autolysis, but, if so, it would seem not be through the production and export of acid phosphatase. As well, there would seem not to be a role for these cells in the phagocytosis of cyst remnants, though the confusing mix of cell detritus and plug cells in this region of the tubule does not permit any firm conclusion.

As the cessation of autolysis coincides with activation of the endocrine system in preparation for to that resulting from oöcyte resorption and degradation of postovulationary follicles, and referred to as, 'yellow bodies' (Cone and Eschenberg, 1966). Relatively late in the post-feed period, acid phosphatase activity could be seen in scattered mesodermal cells, but the precise identification of these cells was not possible. At the same time, a slight amount of activity was found free in the vas efferens and vas deferens lumen. At no time, however, was activity demonstrable in the plug cells. This finding might be explained by the relatively high, and hence non-optimal pH range used in incubation of the tissue, which was necessary to control diffusion of the reaction product from the centre of activity. This interpreation is argued against by the vigorous reaction of the mesodermal cells, and visualization of free activity in the duct system. The plug cells may or may not be involved in the initiation of spermatid autolysis, but, if so, it would seem not be through the production and export of acid phosphatase. As well, there would seem not to be a role for these cells in the phagocytosis of cyst remnants, though the confusing mix of cell detritus and plug cells in this region of the tubule does not permit any firm conclusion.

As the cessation of autolysis coincides with activation of the endocrine system in preparation for the oncoming moult, a limited series of experiments was performed to clarify the role of hormones in this event. Ten appropriate young fifth instar insects were subjected to removal of the neurosecretory cells of the pars intercerebralis, held for ten days, and then fed. Similarly, ten insects were fed, and decapitated twenty-four hours later. As expected, no moulting activity was evinced by either of these preparations. Tissue was taken at five and ten days from the feed, and no cessation of autolysis was found in either preparation.

A regimen intended to provide a normal endocrine environment for morphogenetic development, in the absence of nutritional and osmotic influence of the blood meal was then attempted. Young fifth instar larvae were immobilised with tape and an incision was made in the dorsum of the abdomen. A sterilized human hair was looped around the junction of the crop and midgut and pulled tight. The free ends of the hair were trimmed and the wound sealed with wax in the usual manner. As above, these insects were held for a ten day recovery period, though during this time, half the operated insects died. The survivors were fed and four bugs sacrificed at six days following the feed. No cessation of autolysis was found in any preparation. Four remaining insects which had fed were held to assess moulting activity. None of these insects ever showed signs of apolysis or new cuticle production. Survival was variable. One insect which apparently received some limited flow of material into the midgut survived through three feedings, fully gorging each time over a period of five months. At death, even this insect showed no cessation of autolysis, nor testicular development beyond that typical of a diapaused fifth instar larva.

The approach next tried was parabiosis, using a variety of larval or adult insects with unfed fifth instar Rhodnius. As further controls, fifth instar bugs were implanted with sealed capillary tubes. In the normal insect it regularly but infrequently happens that one or two cysts in a tubule will be found in a fairly advanced state of spermiation, or advanced cysts will be seen degenerating. Many of the parabiotic preparations showed this phenomenon, including controls. None showed evidence of moulting, probably due to the severity of the operation as performed by this author. Thus, in all the experiments so far performed, no evidence of a normal production of ecdysone was obtained. All that can be concluded was that, in the presence of markedly unusual physiological, but not necessarily endocrinological conditions, no functional spermiation occurred.

Finally, the problem was approached with exogenous supply of commercial insect hormones. Three separate experiments were performed. The initial experiment was carried out to investigate the feasibility of injecting $\beta$- ecdysone, and whether or not any response could be obtained in the intact insect. Six unfed fifth instar insects were injected, three times each at daily intervals from the third day post-moult, with $3.0\mu l.$ of a $0.1\mu g/\mu l.$ solution of $\beta$-ecdysone in distilled water. An additional six siblings were similarly injected with water only. The hormone-treated insects thus received $0.9\mu g.$ ecdysone in $9\mu l.$ distilled water and the controls water only. The right testis was removed from two of each group at two, five and twelve days from the onset of the injection series, fixed in Bouin's, sectioned and stained. The sampling times were chosen to indicate development commencing immediately, (2 days), to approximate the normal transit time for degenerating cysts (5 days), and to sample development potentially realized by an induction of the insects' own endocrine system (12 days). However, the results showed elongating spermatids or spermatozoa in all the insects receiving $\beta$-ecdysone, and no such development in any of the control insects.

Experiments carried out in the intact insect do not permit a very precise analysis of hormonal action. Ecdysone has been reported, for instance, to induce RNA synthesis in the corpus allatum, suggesting activation (Siew and Gilbert, 1971), and excessive handling and injection might conceivably trigger neurosecretory release (Highnam, 1961). A group of fifth instar male siblings were thus isolated two days following the moult, decapitated in such a manner as to remove the brain, allatum-cardiacum complex, and suboesophageal ganglion, and returned to the incubator for two days. Kambvsellis and Williams' experiments (1971a & b) indicated that spermiation could be induced <u>in vitro</u> by appropriate concentrations of short chain alcohols, presumably through a disruptive effect on the testicular sheath. To investigate this possibility, the insects were injected with 2 $\mu$l of 2%, and ten with 6% ethanol in distilled water, each day for three consecutive days. Controls were injected with water only, and a fourth group received three injections of 2.0 $\mu$l of 0.15 $\mu$g./$\mu$l. β- ecdysone in distilled water, giving a total dose of 0.9 $\mu$g. per insect. The groups were sampled as above, at two and five days from the onset of the injection series. Results were consistent with those obtained in intact insects. β-ecdysone injections resulted in spermiation in all cases, and water produced no development. Injections of ethanol produced considerable lethality at the higher dose, only three of seven potential samples surviving to five days. At neither dose, however, was any spermatid development noted.

A final series was conducted to investigate the relation of β-ecdysone dose to spermatid development. A group of fifth instar siblings were decapitated as previously, but on the day of the moult. These insects were held in the incubator for ten days, after which the population was once again separated into four groups of ten insects each, and exposed to three consecutive daily injections, in this case of 2.0, 1.5 and 1.5 μl. respectively. The solutions employed, serial dilutions of the same stock, provided eventual exposures of 5.0, 0.5, 0.05 and 0.005 μg. β-ecdysone per insect. Three insects from each group were dissected and the testes prepared and investigated as above on days two and four from the beginning of the injection series, and usually three, but, in some cases, two survivors sampled on day eight.

On day two, at which time the insects have received seventy percent of the projected total dose of β-ecdysone, spermatids could be found in two of three testes in both the high (5.0 μg.) and 'physiological' (0.5μg.) dose level samples. No spermatids were seen at the 0.05μg. dose, but one insect in the group receiving 0.005μg. contained a tubule with several very advanced elongating spermatids. The degree of development in these cysts was such that it could not have been instituted by the injections begun only two days previously, and thus can be considered an example of the occasional development noted in normal insects referred to earlier. No development of spermatids or accessory glands, and no mitosis in the vas efferens occurred in any other insect at this dose level (0.005μg.) on either four or eight days.

At the 0.05μg. level, no spermatids were noted in any preparation. However, a slight level of mitosis was noted in the vasa efferentia at day two, and in the accessory gland complex at day four. At eight days, the accessory glands had reached about half the size seen in preparations from higher dose levels, and mitotic activity had ceased.

At 0.5μg., spermatids were present in two of three samples at day two, one of three at day four, and all of the eight day samples. At the highest dose level (5.0μg.), developing spermatids were found in two of three testes sampled at day two, and in all the remaining samples. Accessory gland development was quite prominent.

As these testes were fixed without removing the sheaths and flat embedding quantitative analysis of spermatid survival is impossible. However, the data clearly show a cessation of autolysis and subsequent spermiation in the presence of β-ecdysone in what may be considered roughly physiological amounts (Wigglesworth, 1970). Accessory gland development would appear to be induced at even lower levels of β-ecdysone exposure.

## Chapter Five: Developmental Events; Dynamics

The timing of onset of differentiated states within the germ cell complement of the testis having been accomplished (fig. 63), attention was turned to an analysis of the kinetic relationship of these differentiated states to one another. As previously described, the primary germ cells arise in the apex of the tubule. As they divide, they become surrounded by a single layer of squamous cells, forming a cluster of spermatogonial cells termed a cyst. Divisions are approximately synchronous within each cyst, and cytoplasmic continuity retained by incomplete cytokinesis. This synchrony of division results in the cell number per cyst being $2^n$, where n equals the number of divisions experienced by the clone of cells comprising the cyst. The cysts increase in size as they move down the tubule, and the testis presents a linear display of advancing stages of differentiation, the zonation referred to earlier (Depdolla, 1928). The most mature cysts come to lie at the junction of the tubule and the vas efferens, which is blocked by the plug cells until the moult from the fifth instar to the adult.

The various spermatogonial cysts, as previously noted, are cytologically indistinguishable and their apparent diameter and cell number are dependent on the plane of section. These circumstances render it impossible, using the present technique, to interpret the development of the spermatogonial cysts in terms of the number of divisions which they have experienced. The linear arrangement of successive stages of the meiotic prophase, however, provides a basis for estimation of the size of the spermatocyte compartment.

The term 'compartment' is used to indicate all those cysts in a given tubule which are at a specific level of differentiation. Such cysts will occupy a continuous segment of the tubule. The use of this term, however, is not intended to imply a structural or mechanical separation from adjacent cysts. For the purpose of the experiments to be described in this section, an accurate picture of changes in the differentiated compartments rather than the total number of cysts or cells, was required. Counting techniques were therefore directed toward sampling a constant proportion of each compartment throughout the time span of the study. Measurements obtained are thus an index of compartment size, but are not intended to represent the entire population of cells in any given compartment.

As described previously, the hemipteran spermatocyte prophase is characterized by a number of cytologically distinctive stages. Following interphase or pre-leptotene DNA synthesis, leptotene, synizesis, pachytene, diffuse diplotene, diakinesis, and metaphase follow in order. The second metaphase occurs without intervention of a second interphase or prophase. Those spermatocytes which are in the first meiotic interphase and leptotene stages strongly resemble the final spermatogonia, both cytologically and in terms of cyst size. A preliminary investigation was carried out in favorable sections to estimate the size of this early meiotic period, defined as extending from the last spermatogonial mitotic figures to the onset of synizesis, and no accumulation was noted which did not also affect the remainder of the spermatocyte compartment. As these early meiotic stages could not be defined in that majority of preparations which lacked mitotic figures in the final spermatogonial cysts, they were not included in the estimates of cyst numbers used to determine the spermatocyte compartment index.

A median longitudinal section through a testis tubule would permit a reasonably precise sampling of the total population of cysts within the tubule. However, because the tubule is very long with respect to its diameter, it is impossible to achieve precise planar orientation of the sections. Therefore, each prophase sub-division with the exception of leptotene, that is, synizesis, pachytene, diffuse diplotene, and diakinesis, plus any division figures, were counted separately in several sections of the most favorable area. The maximum number obtained for each sub-division was added to obtain the final spermatocyte compartment index. This number thus approximates the total of the cysts in an ideal section through the longitudinal axis of the tubule.

The term 'spermatid compartment' is used here to refer to all of the cysts produced by the meiotic divisions, that is, those cysts which contain spermatids and developing and mature spermatozoa. The cysts of spermatids are cytologically distinct and no problems of recognition occur. During the course of spermiation, however, the cyst assumes a corkscrew shape, and may be represented by several profiles in a single section. Therefore, only cysts showing nuclei or sperm heads were counted. The compartment was divided into newly-formed circular spermatids, and those which had begun elongation, and the results were summed as above to determine the spermatid compartment index.

Histological preparation of tubules for flat embedding and sectioning is described in the Appendix (Appendix Two: Histology). In order to demonstrate changes in the spermatocyte and spermatid compartments during the fifth instar, one hundred insects with identical developmental histories were fed as fourth instars. These were sexed at the moult from fourth to fifth instar, and the males fed again ten days later. Two insects were sacrificed daily until the stock was exhausted, eight days after the feed. From approximately fifty fifth instar males, thirty successful preparations of major tubules were obtained; the chief sources of loss were damage to the testis during de-sheathing, unacceptable plane of section, excessive coiling of the testis, and failure of the insect to feed.

Because of these losses during the preparation, the points on the graph (fig. 67 ) represent the results from individual insects. Thus, for most days during the course of the experiment, data from only one insect is available, an admittedly small sample. However, variation on those days for which two points are available is not extreme, and a line drawn to the points shows a relatively smooth progression with certain inflections of considerable magnitude. Since only these major changes will be interpreted, the graph can be considered a sufficiently accurate representation of changes in the spermatocyte and spermatid compartment sizes from the moult from the fourth to the fifth instar,

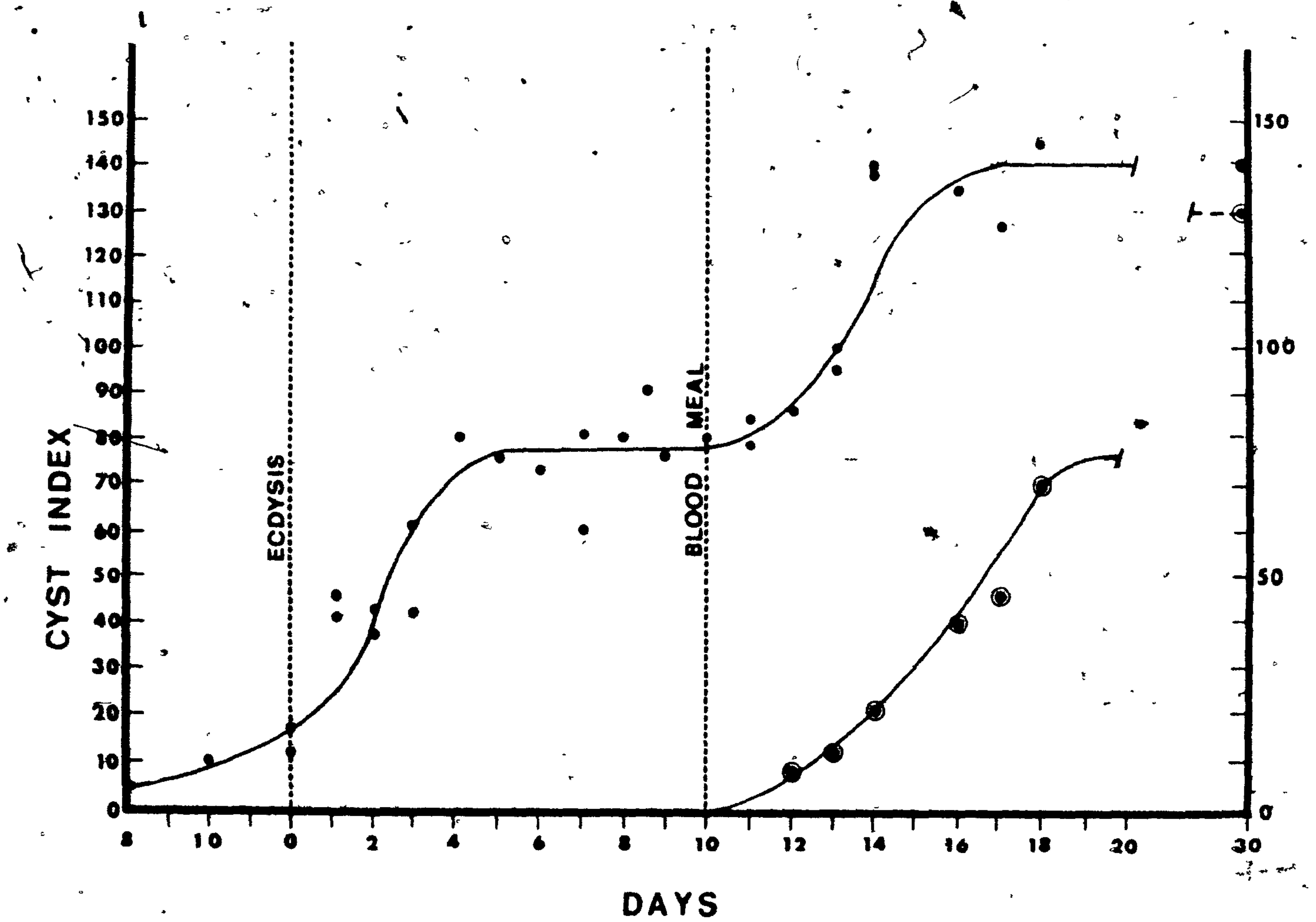


FIGURE 67 Graph of the spermatocyte ( solid dots ) and spermatid ( circled dots) cyst indices in major tubules of testes during the late 4th and 5th instar through the critical post-feed period. Points represent results from single insects. See text ( Chapter 5 ) for detailed explanation.

through the diapause period in the fifth instar, and the critical period of the post-feed fifth instar.

Spermatocytes are first recognized at approximately day eight post-feed in the fourth instar, four days prior to the moult from fourth to fifth. Their number increases slowly through the late fourth instar, then much more rapidly during the early fifth instar, reaching a plateau at about four days after the moult to the fifth instar. This level is maintained during the remainder of the fifth instar until the blood meal. Subsequent investigation of fifth instar larvae which had been kept unfed for as long as six months show that the level declines only slightly from this plateau. It is important to recall at this time that the spermatocyte compartment receives a continuous input from spermatogonial mitoses and, since the histological observations show that meiosis begins about day four, it follows that there is also a continuous output due to spermatocyte meiosis. This steady state condition of the compartment is thus a dynamic rather than a static phenomenon and reflects an equal mitotic and meiotic activity.

Immediately following the blood meal in the fifth instar, the spermatocyte compartment shows a dramatic increase. Numerous meiotic and mitotic figures can be demonstrated during this period, and an increase in compartment size indicates that input exceeds output, that is, that total mitotic activity exceeds total meiotic activity.

In contrast, the level of the spermatid compartment size index is zero to five for the entire period of the diapause. Since these few spermatid cysts present are always in a state of advancing autolysis, they are not included in the graph. After the feed, this compartment also increases in size, though perhaps not so rapidly as the spermatocyte compartment. Since no output exists for the spermatids until after the moult to the adult, the compartment size index shows a continued increase during the remainder of the fifth instar, albeit at a reduced rate.

An interesting inference which may be drawn from these results is that, in the fifth instar, when juvenile hormone is absent (Wigglesworth, 1934), mitotic activity exceeds meiotic activity for at least the four days following the blood meal. Such an observation is inconsistent with any hypothesis which involves the selective inhibition of meiosis by juvenile hormone, for, in the absence of juvenile hormone, we would expect meiosis to be greatly enhanced. Therefore, it was decided to investigate the role of juvenile hormone directly through the use of juvenile hormone analogues on this preparation.

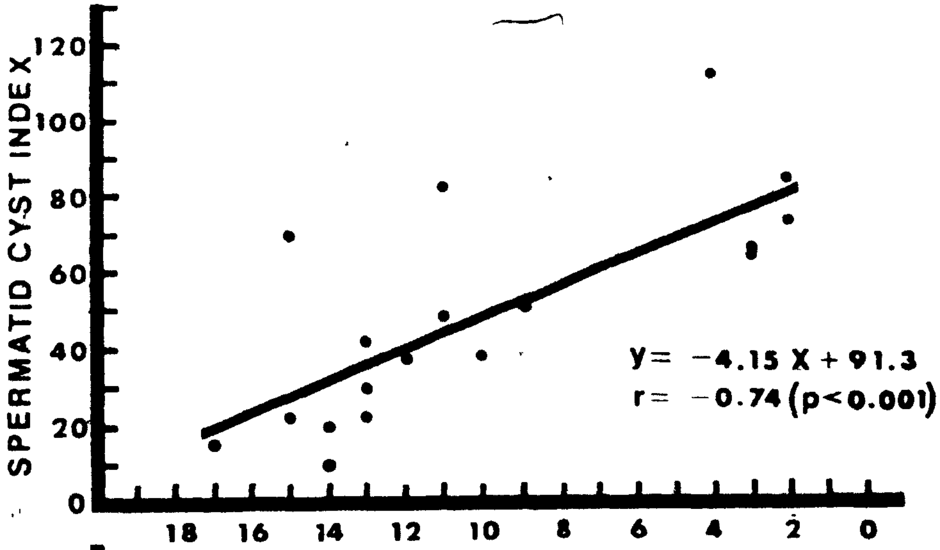
Application of the potent juvenile hormone analogue, farnesyl methyl ether (FME) to recently fed fifth instar Rhodnius leads to the production of a range of larval/adult intermediates in the succeeding moult (Wigglesworth, 1969; Barrett, 1974); the wide range in effect on the population after a single uniform application is believed to be the result of variations in the rate of penetration of the hormone analogue through the cuticle, and hence its availability and catabolism in the individual insect (Patterson, 1973; Wigglesworth, 1973). Wigglesworth (1969), has devised a scale for gauging the effect of the analogue on morphogenesis of the resultant cuticle. This scale thus represents a measure of effective dose of the analogue on any given individual within the population.

In this study FME was dissolved in iso-octane in ratios of 0.1, 1.0, and 2.5 nl. FME to 1.0 µl. iso-octane. One microliter applications of the resultant solutions were made to the ventral abdominal cuticle of fifth instar Rhodnius siblings, twenty-four hours after the blood meal. Controls were treated in the same fashion, but with iso-octane only. Of fifty insects receiving 1.0 or 2.5 nanoliters of FME, forty moulted to intermediates with a score of 2 (highly adult) to 17 (highly larval) on the Wigglesworth scale. Controls, and insects receiving 0.1 nanoliter FME, moulted to normal adults. Scoring, and histological procedures as in the previous experiment, were carried out within eight hours of the moult.

Counts were made of the spermatocyte and spermatid compartments as before. Losses were again encountered, particularly in the highly coiled spermatocyte region of the major tubules (fig.69 ). Since it was not possible to recognize any trend in that compartment due to the small number of points obtained, data from the minor tubules was also computed, and presented here (figs.70,71 ).

The index of spermatid compartment size for both minor and major tubules (figs.68,70 ) displays a linear decrease with effective dose of FME. As no output for this compartment exists at this time, the decrease in compartment size index must be interpreted as a dose related depression in the output of the products of meiotic division.

The data for the spermatocyte of the minor tubules (fig.71 ), on the other hand, shows no significant change with the degree of larvalization. If the inhibition described above were specific for the meiotic division, a rise in the index of this compartment would result. The constant level of the spermatocyte compart-



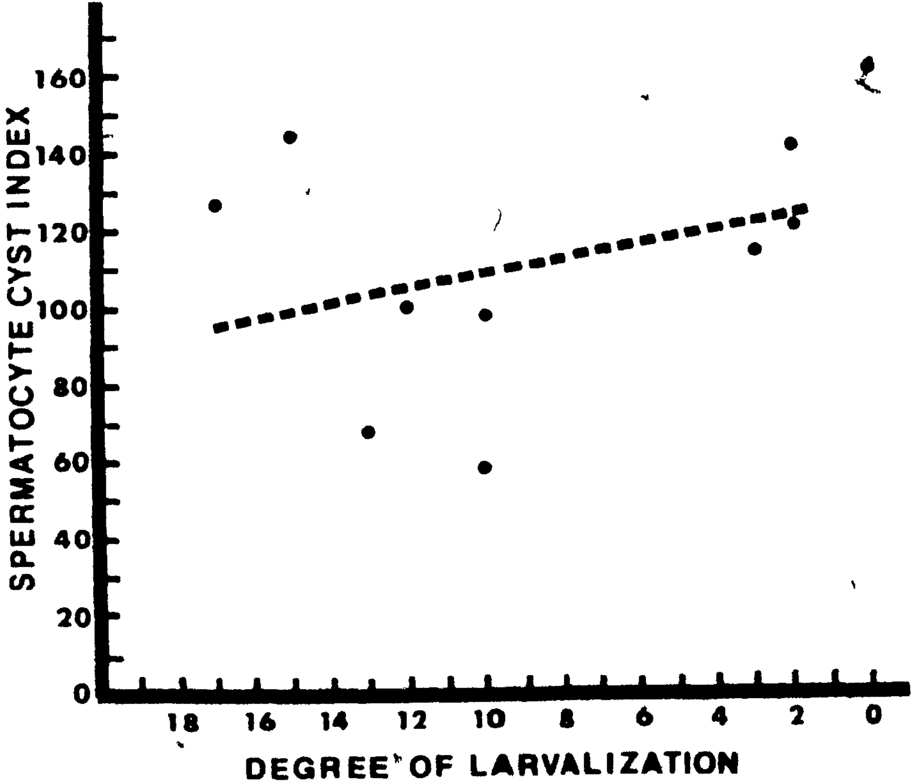


FIGURE 68 Effect of Farnesyl Methyl Ether, a Juvenile Hormone analogue, on spermatid cyst index. X axis represents scale devised by Wigglesworth (1969). Data compiled for major tubule, points represent individual samples.

FIGURE 69 Spermatocyte cyst index, major tubule, as described above and in text (Chapter 5). Widely scattered points precludes fitting of valid line.

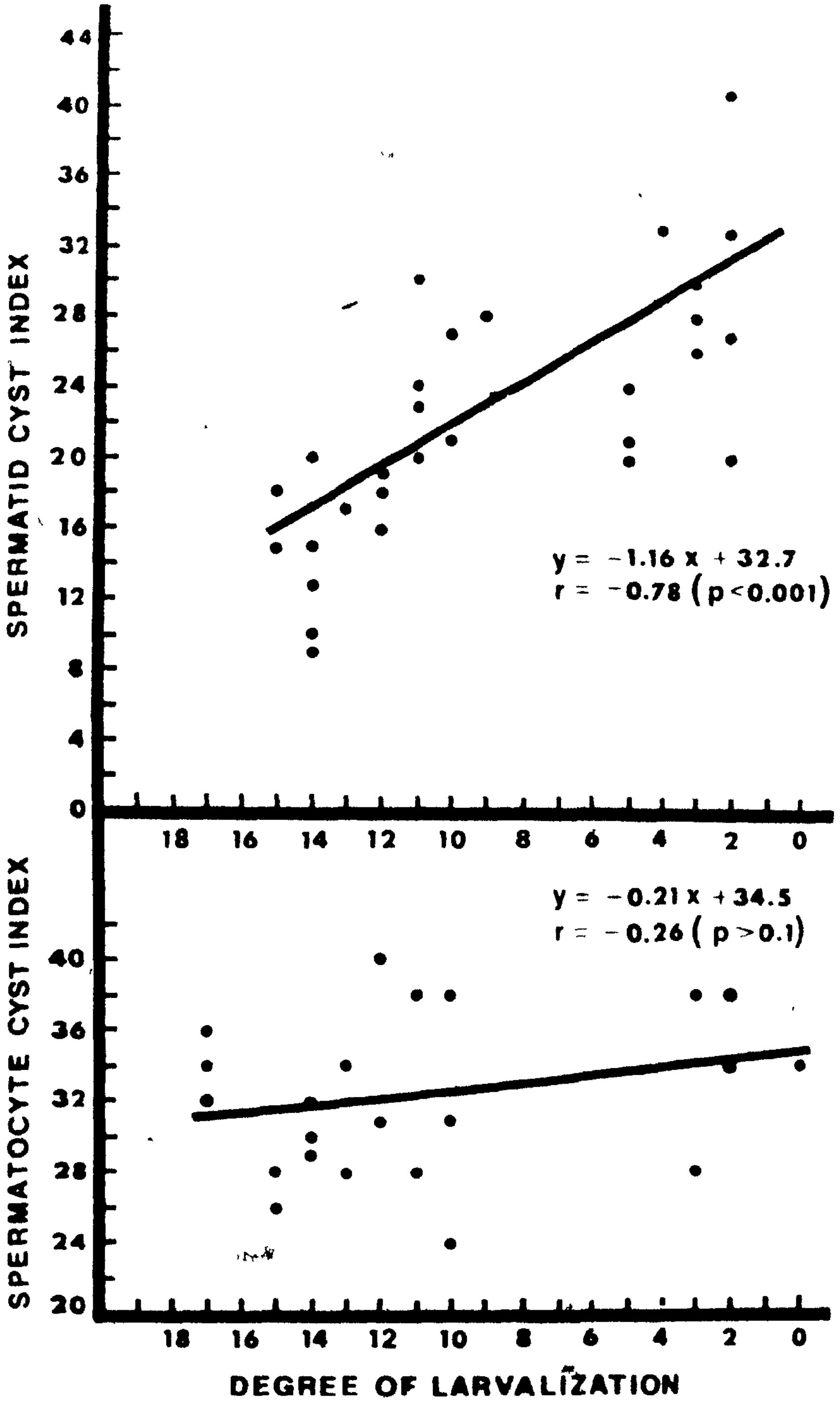


FIGURE 70 Data for spermatid compartment index of minor tubules of same preparations as in two previous figures. Essentially the same results as in fig. 68

FIGURE 71 Data for spermatocyte compartment cyst index of minor tubules, same preparations as above. Note that Y axis has not been extended to the baseline.

ment thus demonstrated an equal inhibitory effect of FME on both the meiotic and mitotic divisions.

# Chapter Six: Interim Review; Autolysis

Thus far, the investigations have been directed toward unravelling testicular development into its separate aspects of growth and differentiation and identifying possible control points within these developmental processes. The author's plan of attack now calls for a 'stock taking', an interim review of results so far collected, and a correlation with the main body of endocrinological information in Rhodnius and other insects. In this way, a working hypothesis consistent with the observations and with the larval/adult paradox earlier defined, can be put forward for classical endocrinological investigation.

The most striking and significant control mechanism available to the insect lies in the ability to selectively and totally destroy the most differentiated element of the germ cell line. A dynamic stasis is thus achieved, resulting in a condition of no growth in the testis during diapause whether or not additional controls are in force.

Degeneration of germ cell elements is by no means an unusual phenomenon. Roosen-Runge (1973), in a recent and comprehensive review of the subject, states that "... one gains the impression that degenerative phenomenon have been found wherever the spermatogenic process of a species has been thoroughly investigated." The degree to which this occurs varies considerably with species and also with the stage of differentiation under investigation. Quantitative data are scarce. One well-characterized mammalian species and variety, the Sprague-Dawley rat, gives the following percentage losses: A1 (predefinitive or stem cell) to Intermediate type spermatogonia, 60% loss (Clermont, 1967); Late spermatogonial to advanced spermatid, 22%, with only 2% directly involved with the meiotic divisions (RoOsen-Runge, 1955). In other species, the loss associated with the meiotic divisions in rabbits is 24% (Swierstra and Foote, 1963), and 35% in humans (Barr et al., 1971).

With the difficulty of establishing the progression of events, and subsequent lack of quantitation, the significance of degeneration in the insect testis has not often been appreciated. Reports which suggest specific autolysis of germ cells can be found for Hyalophora cecropia (Schmidt and Williams, 1953), Cephus cinctus (Church, 1955), and Papilio xuthus (Nishiitsutsuji-uwo, 1961). Particularly interesting is that different elements are involved in each case, respectively spermatids, spermatocytes, and both these elements in Papilio. These events have been reported to occur only during diapause, and always at the level of the most differentiated germinal element. In Rhodnius, due to the linearity of the system and ease of recognition of at least later stages of differentiation, a clear distinction can be drawn between spermatogonial pycnoses and spermatid or spermatogonial autolysis. The earlier events appear not only histologically distinct, but are randomly dispersed through successive generations of clone size and are relatively infrequent in relation to apparently normal cysts. The basal autolysis in the fifth instar diapause is spermatid specific, and inexorably erases the entire population. A re-investigation of earlier instars armed with the knowledge of this dichotomy led to the demonstration of both pycnosis and the basal autolytic degeneration in a diapaused third instar testis (fig. 72,73) suggesting a position specificity rather than a specificity for level of differentiation for the phenomenon.

In an earlier paper, Roosen-Runge and Leik (1968) examined in considerable detail the evidence for the various types of germ cell degeneration in the rat. Conclusions reached are that the pycnotic nucleus type of cell death is a firmly established phenomenon. In the later review, Roosen-Runge (1973), puts forth the hypothesis that this phenomenon is division-related, and possibly a summation of small

Figure 72. Extra-luminally directed vacuolization (arrow) in vas efferens epithelium of unfed fifth instar insect. Osmium Ethyl Gallate, X 600

Figure 73. Same. This specimen has been recently fed,but has not yet cleared all products of autolysis from the tubule. Osmium Ethyl Gallate X 600

Figure 74. Apparent specific autolysis at the plug cell (P) / vas efferens (E) area of the unfed third instar testis tubule. Epon, Sato's polychrome, X 1250

Figure 75. Pycnotic degeneration in apical region of same tubule. Epon, Sato's polychrome, X 1250

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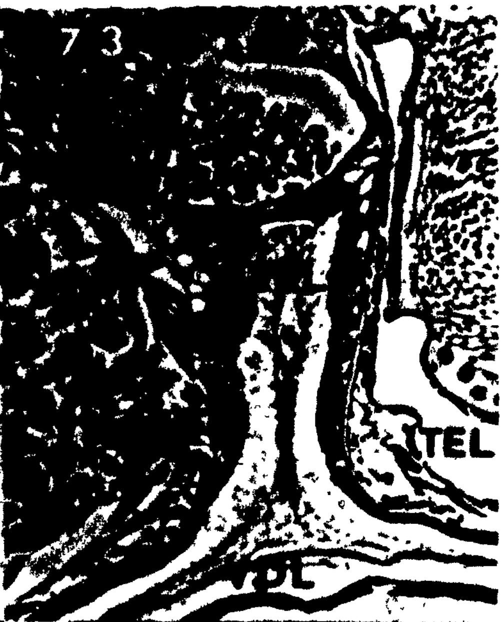

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errors or catastrophes occurring during division, eventually resulting in an abortive mitosis or meiosis. These authors regard the evidence for the lytic type of cell death, described in rats by Franchi and Mandl (1964), as less conclusive, pointing out a resemblance between the events described and pre-mitotic or pre-meiotic events as visualized with certain conditions of fixation. The non-dividing nature of the spermatid and complete lack of survival negate such an argument for the results reported in this thesis on Rhodnius testis. A third type of degeneration, characterized by bizarre multinucleate forms and described by Huckins (1965), in fact could apply to either of the two types described in the insect.

The occurrence of this division unrelated, apparently position specific autolysis during the diapause period, immediately suggests a role for ecdysone in the process. This observation was also made by Nishiitsutsuji-uwo (1961) for Papilio. She further reports that experiments designed to investigate the problem were inconclusive, as the mechanical shock of transplantation induced degeneration in many cysts. The present author echoes and amplifies this complaint. Surgical injury quite removed from the testis proper seems capable of inducing degenerative events, or, conversely, partially alleviating degeneration. Possibly, some component of the complex system of events concerned with wound metabolism (Harvey, 1962; Wyatt, 1971), is involved.

Ecdysone has been experimentally demonstrated to be effective in preventing the onset of degeneration in vivo in Rhodnius, in the absence of nutritional and biophysical influences of the blood meal, and in the absence of other obvious sources of hormones. It is impossible to predict how many intervening steps might be involved in the process. However, Kiss, in Williams' laboratory (Williams, personal communication, 1973), has succeeded in demonstrating a necessity for the continued presence of macromolecular factor fractions of insect haemolymph for the survival of isolated spermatidal cysts in vitro. In an earlier work, Kambysellis and Williams (1971a) also reported a peak of MF activity present in the haemolymph of isolated abdomens subjected to cuticular wounding. These results would suggest a role for a similar factor in Rhodnius as well. The limited experiments with injected alcohol, an effective activator of MF induced spermiation in in vitro studies on Cecropia testes, possibly were ineffective in Rhodnius due to a rapid clearance of this metabolizable molecule from the in vivo preparation.

However, the demonstration of autolytic

activity in the testes of a diapausing third instar larvae, and the variation in stage specificity of an apparently similar process reported by other workers, suggests a specificity for site in the tubule rather than stage of germ cell differentiation, and thus suggests the notion that autolysis is an active process mediated by site specific elements in the tubular system. Once underway, spermiation soon results in the colonization of the tubule by elongating spermatids. This lack of site specificity for spermiation, the converse of autolysis, argues against a simple hypothesis of control over metabolites necessary for spermiation, or further development of spermatogonia.

A final observation on this system is warranted here, but the reader is cautioned that these results are highly tentative. During such time as degenerative cysts are present in the tubule basal area, vacuolization is often noted in the vas efferens of the affected tubule, and these vacuoles are always extra-luminal in position in the vas efferens epithelia. Vacuolization occurs at this time in the plug cells as well, but appears to be randomly directed (figs.72, 73 ). This phenomenon is extremely rare in younger, non-degenerative tubules, or in fed insects (figs.20,21, 24 ). If the vacuolization denotes a secretive process and the testis sheath is selectively permeable, as Kambysellis and Williams' (1971a & b) work indicates, the architecture of the system as previously described, would result in a recycling of material back into the extra-tubular lumen of the testis. Such a process has direct parallels in the mammalian testis (Waites and Setchell, 1969). A similar vacuolization has been described for guinea-pig vasa efferentia and epididymis (Ladman and Young, 1958). Setchell and Sirinathsinghji (1972) suggest a role of this process in the recycling not only of trophic materials, but bioactive materials as well, a topic which will be explored more fully in the final chapter. One possibility is that the cytoplasm of the plug cells, rather than acting as phagocytic elements, act as a macromolecular sieve for the autolyzed cyst products. Valuable metabolites are then routed back into the testis through the vas efferens epithelial cells, either by an active transport process, or simply through a kind of solvent drag phenomenon associated with the uptake and transfer of large fluid volumes. However, although these results have been noted in preparations utilizing three entirely different fixation procedures, vacuolization of epithelial cells is a very common histological artifact, and further evidence would certainly be required before much confidence could be placed in this testicular recycling hypothesis.

## Chapter Seven: Interim Review; Dynamics

A second significant conclusion which can be drawn from this study concerns the accumulation of spermatocytes which occurs early in the last larval instar. Takeuchi (1969), working with Bombyx mori, regards this accumulation as resulting from a greater inhibitory effect on meiotic than mitotic division by juvenile hormone. Economopoulos and Gordon (1971), in a similar study on Oncopeltus fasciatus, conclude that the accumulation of spermatocytes occurs as a result of mitotic proliferation of spermatogonia. Failure of these resting spermatocytes to undergo meiosis in the fourth instar, and their high level of meiotic activity in the last interval instar, is regarded by the authors as being due to the absence of an "adultoid biochemical milieu." They suggest that this alteration in blood composition is unrelated to juvenile hormone.

The results reported in this work indicate that spermatocyte accumulation, at least in Rhodnius, is a direct effect of the difference in time spent in the spermatogonial and spermatocyte compartments by the developing cyst. It can be argued that the difference in the duration of meiosis and mitosis is a consequence of a long obligatory prophase during meiosis. In the simplest case, where the spermatogonia and spermatocytes have approximately equal cell cycle times, with the exception of the prophase in meiosis, spermatocyte accumulation must inevitably occur until saturation of this prophase provides an outlet for the spermatocytal compartment.

The degree of accumulation which is achieved is illustrated in the following example: In such a simple case as outlined above, the percentage of germ cells which are spermatocytes at the onset of meiosis can be predicted by the following relationship:

$$\frac{\frac{M}{m} \cdot 2^n}{\left(\frac{M}{m} \cdot 2^n\right) + 2^n - 1} \times 100$$

Melanoplus differentialis is one of the few insects for which the cell cycle duration is known. If it is assumed that Melanoplus fits the simple conditions outlined above, the figures of Muckenthaler (1964), yield an accumulation of spermatocytes of approximately 90% of the total germ cell complement.

$M$ = duration of the meiotic cell cycle.
20 days.

$m$ = duration of the mitotic cell cycle.
28 hours.

$2^n$ = size of the spermatocyte cyst, $2^7 = 256$

This is not to suggest that specific inhibition of meiosis does not also occur, simply that the initial spermatocyte accumulation is a kinetic event rather than the result of a block on differentiation or division. However, if hormonal or biochemical conditions favored the inhibition of meiotic division specifically, spermatocyte accumulation would occur indefinitely, reflecting a condition of blocked output and continuous input. It can be seen from the data on Rhodnius that this does not occur, and a static compartment size is reached and maintained even in the presence of exogenously applied FME (fig.71 ). In the case of Oncopeltus and Bombyx, in both of which the duration of larval instars is rather closely defined, it is questionable whether such inhibition is even possible. The time from onset of the appearance of spermatocytes to meiosis in Takeuchi's study (1969) is approximately eleven days, agreeing well with the duration of the meiotic stage in Bombyx determined from repopulation of the testes following acute x-irradiation (Sado, 1961, 1963a & b). Exact figures are not available for Oncopeltus, but Economopoulos and Gordon (1971) indicate that spermatocyte accumulation begins in the fourth instar and meiosis starts in the early fifth instar, an interval of only three or four days. The onset of meiosis in insects showing such rigid temporal development might simply reflect the time required for passage through the meiotic prophase. It is quite clear that juvenile hormone does affect testicular development in many insects, but the precise nature of the effect is still in some doubt. The results of these experiments suggest that this role may also be kinetic in nature; that juvenile hormone acts as a division rate inhibitor affecting the entire germ cell population equally. The results demonstrated by Sehnal (1968), indicating an inhibition of testicular development through the larval instar by implanted corpora allata, would also argue in favor of an effect of juvenile hormone on a continuous process such as cell division.

Having established that the appearance of meiotic figures in Rhodnius is a time-dependent phenomenon reflecting the maturation of the meiocyte, and that 'inappropriate' spermatids are removed by autolysis during the diapause period, there would seem to be no requirement for control over division in such a system. Nonetheless, as previously noted, division figures are far more prevalent in sections of testes fixed immediately after the fourth/fifth moult, and for several days following the fifth instar blood meal, than at any other time during the instar.

Similar discontinuities in division frequency have been noted in mosquito larvae (Jones, 1967; Horsfall and Ronquillo, 1970). Because of the small sample size in the present study, no meaningful comparison of the slopes of the compartment size index curves is possible. However, certain trends are seen which might be interpreted as reflecting changes in division frequency which are noted histologically. In particular, the rapid attainment of plateau level by the spermatocyte compartment in the four days following the moult from fourth to fifth instar is indicative of considerable input due to mitotic activity.

The changes in this compartment following the blood meal are more complex in nature since, at this time, both input to and output from the compartment occur. If meiosis necessary for spermatid production were favored at this time, a fall in spermatocyte compartment would be expected. Instead, a quite considerable rise is observed, indicating a rate of mitotic input which exceeds meiotic output. Such a result is in fact predicted by the principle of dynamic accumulation earlier outlined. If the duration of the meiotic prophase is relatively inflexible and thus changes in division rate affect largely the remainder of the cell cycle, mitotic and meiotic cells equally, the meiocyte is not in as favorable a position to react to these changes by division as is the spermatogonium. A lag in output would result in a form influenced by complex factors not here determined, such as the degree of flexibility of the meiotic prophase, the exact position in the cell cycle of inhibition or stimulation, and the rate of change of the mitotic and meiotic cell cycle durations. The net result would be the temporary attainment of a new level of dynamic accumulation in the spermatocyte compartment, which should eventually be redressed with saturation of the prophase.

The data presented here do not extend far enough into the period between the attainment of maximum compartment size at the fourth day after feeding and the moult to the adult stage to recognize any possible trend. In any case, the architecture of the major tubule at this stage of development is unfavorable for the counting of cysts, largely as a result of the excessive coiling of the tubule (fig. 5 ). Thus, the compartment size index after four days post feed is variable as determined by the methods used here, and a downward trend would be difficult or impossible to recognize.

# Chapter Eight: Interim Review; Division Rate and The Kinetic Hypothesis

The period following the blood meal in Rhodnius larvae has been demonstrated to be a time of prothoracic gland activity and ecdysone production (Wigglesworth, 1934). The post-feed rise in division activity thus very simply coincides with a time of known ecdysone presence in Rhodnius, suggesting a stimulatory role for this hormone in accordance with the hypothesis of Takeuchi (1969).

Correlation of post-moult division activity with hormonal events is more problematical. Williams and Kambysellis (1969) and Kambysellis and Williams (1971a & b) have shown that ecdysone exerts its effects on spermiation at least through a second biologically active factor, MF, rather than directly. In such a complex system, the effect of ecdysone might be mimicked by other substances which could, for instance, cause changes in MF titer or availability. On the other hand, both Bowers and Williams (1964) and Greenstein (1972) have described events occurring during the pre-diapause and very early diapause in developing wings and cuticle of Hyalophora cecropia pupae which suggest low levels of ecdysone production immediately following the moult from larva to pupa. No evidence exists for such activity at the larval/larval moult in Rhodnius; however, Steele and Harmsen (1971) have described a neurosecretory release during the larval/adult ecdysis in Rhodnius. Should such an event also occur during the larval/larval ecdyses, low levels of pre-diapause ecdysone secretion might be predicted.

The ubiquity of presence of juvenile hormone during the earlier larval stadia, and the qualitative difference in testis development in the last larval or pupal stadium in the absence of juvenile hormone, has given rise to a speculative role for this hormone in the differentiation of the meiocyte or spermatid. The results reported in this thesis, in contrast, show no specific inhibition of meiosis or differentiation. The suggestion of specific inhbition of the meiocyte, perhaps in analogy to the diplotene oocyte, has been demonstrated to be unlikely on the basis of kinetics of the germ cell population, and will be dealt with further in a chapter on differentiation to follow. The excellent electron microscopical evidence for juvenile hormone induced degeneration in Locusta (Cantacuzène and Seureau, 1969) is complicated by the involvement of highly specialized, trophic, parietal cells in this insect, and thus cannot be generalized.

While volumetric analyses cannot provide much information regarding the parameter of germ cell development affected, the dose-related depression (Nowock, 1973) and depression of development related to the time of implantation of supernumerary corpora allata (Sehnal, 1968), indicate that the affected process is ongoing throughout the stadium. This can be taken as further evidence against the notion of a hormone-linked simultaneous differentiation of spermatids, and in favor of an effect mediated through a continuous level of cell division.

It is clear that in many insect species, the size of the spermatocyte cyst in terms of cells per cyst, is specific and invariant (White, 1955; Phillips, 1970; King and Akai, 1971). This fact would indicate that meiocyte differentiation is a sequential rather than an abrupt process. The rigorous correlation of cyst size (cells per cyst) with stage of differentiation would suggest that division rate control alone is a sufficient mechanism for explanation of the temporal pattern of sperm production. Although dynamic accumulation accounts for the eventual size of the spermatocyte compartment, the rate of division in the last-larval instar is much higher than that encountered in earlier instars. The role of juvenile hormone in these earlier stadia might be in the suppression of a maximal division rate, allowing the germ cell compartment to build slowly toward a level of differentiation consistent with the pre-imaginal state.

Such a kinetic hypothesis of germ cell development requires an endogenous level of division in the absence of endocrine activity, as is observed during the fifth instar diapause in <u>Rhodnius</u>. If juvenile hormone inhibition affects only ecdysone accelerated and not endogenous division activity, several conflicting observations may be reconciled. Some level of division and, thus, differentiation would occur in the absence of ecdysone as reported <u>in vivo</u> (Blaine and Dixon, 1970; Economopoulos and Gordon, 1971), and <u>in vitro</u> (see review by Marks, 1970). Similarly, adult spermatogenesis, not requiring the explosive cell division of the pre-imaginal instar, would continue at the endogenous level in the absence of ecdysone and in the presence of a high juvenile hormone titer.

## Chapter Nine: Division Rate; Colchicine Accumulation

Evidence of changes in division rate with respect to hormonal influence both in the results so far presented and in the work of other authors, has been largely correlative and unquantified. The kinetic hypothesis put forward in the previous chapter would require an increase in division rate in the presence of ecdysone, and the absence of this increase in the simultaneous presence of juvenile hormone. This hypothesis was put to the classical endocrinologist's test of measurement of the relevant parameter following removal of the hormone source, and resupply of hormone.

From the data earlier presented on spermatocyte accumulation, it could be deduced that no spermatocytes are present in the fourth instar testis for at least six days post-feed (fig. 67 ). One measure of division activity is the mitotic index (MI): the percentage of cells in metaphase with respect to the total cell population. Spermatocytes, due to the long prophase, would not be expected to divide within the time course of the following experiments, and thus could be considered operationally non-proliferative cells. The mitotic index, or any similar technique which compares a visible division state to total cell population, is only valid as long as the non-proliferative segment of the total is a reasonably constant proportion. Accumulation of prophase spermatocytes would result in an increase in the non-proliferative segment of the cells relative to the total cell complement, and, in this case, to an artificial decrease in MI or a 'false-negative' effect. The use of fourth instar testes, assessed within a few days of feeding or hormone treatment of the insect, avoids this complication. Exposure of dividing cells to the plant alkaloid, colchicine, results in a disruption of spindle fibres and thus an accumulation of cells in C-mitosis, anaphase segregation being impossible until the concentration of colchicine becomes sufficiently low to allow spindle organization. This technique is often employed to provide a larger sample of metaphase chromosome preparations for cytological purposes. It is particularly important in the measurement of MI, however, since it permits the sampling of all division activity within a twenty-four hour exposure period, thus negating any variation produced by potential circadian influences on division rate.

An initial experiment was designed to investigate the proposed endocrine effects on division rate as well as the feasibility of the technique. Sixty-six fourth instar insects of similar size and history were isolated twenty days after the moult from the third to the fourth instar. All were subjected to removal of the corpus allatum, corpus cardiacum, and the neurosecretory cells of the pars intercerebralis. During post-operative recovery (Appendix One), thirty percent of the sample died. Following this ten day period, the remaining bugs were permitted to feed. Eighty percent, that is fifty-six percent of the original group, gorged to repletion, the remainder either partially feeding, refusing entirely, or gorging until the crop and the integument burst.

The gorged insects were held for twenty-four hours in the incubator to permit diuresis, and randomly segregated into four groups of nine insects each. Two groups were then injected with 1.0μl. of a mixture containing 1.0μg. of β-ecdysone in a $2.5 \times 10^{-4}$M colchicine solution, and two groups were injected with the colchicine solution alone. One hour following this treatment, one group receiving β-ecdysone, and one control group, received 0.5 nanoliters of FME dissolved in 0.5μl iso-octane, applied topically to the ventral abdomen. The remaining β-ecdysone group and control group received control applications of 0.5μl. iso-octane alone. The insects were returned to the incubator, and twenty-four hours later removed and held at 4°C. Each insect was then dissected in Ringer's solution, and the right testis removed and squashed in hot aceto-carmine (Appendix Two).

The slides were counted for metaphase plates and total cell numbers, and the MI computed for the twenty-four hour exposure. Results (fig.76 ), clearly indicate a doubling of the MI in the presence of ecdysone, which is entirely abolished by the simultaneous application of the juvenile hormone analogue. FME had no effect on the endogenous divison rate demonstrated by MI in the absence of ecdysone.

In spite of the rather clear results produced, these preparations were regarded as promising rather than definitive. Sample numbers eventually obtained were very small in relation to the amount of tedious surgical, cytological, and microscopical work performed; so small in fact that statistical analysis was not worthwhile. Many preparations had to be discarded due to an inability to identify reliably the typical condensed ball mitoses, and differentiate them from simple germ cell pycnosis.

To improve and simplify the above experimental preparations, a number of changes were introduced in the procedure (Appendix Two). Greatly improved visualization of chromosomes was obtained by pre-treatment of the testes in 0.8% sodium citrate, and squashing in

FIGURE 76
4th INSTAR: $NSC^-$, $CA^-$, FED
EFFECT OF ECDYSONE AND FME

| SAMPLE | TREATMENT | | MITOTIC INDEX: 24 HOURS |
|---|---|---|---|
| 5 | WATER | ISO-OCTANE | 8.9 ± 1.2 |
| 3 | WATER | FME | 8.4 ± 1.4 |
| 3 | ECDYSONE | ISO-OCTANE | 19.8 ± 4.1 |
| 2 | ECDYSONE | FME | 8.7 ± 1.0 |

FIGURE 77
4th INSTAR: FED, DECAPITATED
EFFECT OF ECDYSONE

| SAMPLE | TREATMENT | MITOTIC INDEX: NO COLCHICINE |
|---|---|---|
| 7 | WATER | 1.0 ± 0.3 |
| 7 | ECDYSONE | 2.9 ± 1.5 |
| 6 | UNTREATED UNFED | 1.0 ± 0.6 |

ECDYSONE vs WATER: $0.01 > P > 0.001$
ECDYSONE vs UNFED: $P = 0.01$
WATER vs UNFED: NOT SIGNIFICANT

cold aceto-orcein. These, and all the following preparations, were blinded prior to counting, and exhaustive counting techniques introduced. To avoid the necessity of time-consuming surgical techniques, a feasibility study was done with fourth instar insects decapitated twenty-four hours after feeding. No colchicine was used in this experiment, to ascertain if the MI was sufficiently high to avoid this step, with its somewhat abnormal cytological effects. Since these preparations lacked both brain and sub-oesophageal ganglion, the persistence of circadian rhythms was considered unlikely. Results (fig.77 ) indicated that the control injected insects did not differ significantly from unfed intact fourth instar insects in terms of testis MI and that β-ecdysone was quite effective in such preparations; however, further improvements were judged desirable.

Decapitates were prepared as in the previous experiment. As the unmanipulated MI was judged too low for reliable measurement, colchicine was again introduced into the procedure. To avoid 'breakthrough' anaphase noticed in some preparations in the initial experiment, the dose was increased to $2\mu l$ of $10^{-3}$M solution per bug. On the assumption that alterations in MI resulted from changes in the duration of the $G_1$ phase of the cell cycle, β-ecdysone or distilled water injections were carried out twelve hours prior to colchicine injections. Further, to decrease abnormal effects due to hyperecdysonism, the dose of β-ecdysone administered was reduced to 0.5μg per insect. Not bound by circadian inputs, the colchicine exposure time was reduced to ten hours.

This experiment involved two groups only, those receiving β-ecdysone solution, and those receiving water alone. Each group initially was comprised of thirty insects, yielding an average of eight countable samples. The experiment was repeated as precisely as possible, two weeks later. The results (figs.78, 79 ) of these two runs, while showing considerable statistical significance, and qualitatively agreeing with earlier experiments, are quantitatively different not only from earlier experiments but from one another as well. A potential source of this variation may be in well-documented but little understood phenomenon of hyperecdysonism (Williams, 1968; Mouze ~~et~~ al, 1974). In support of this explanation is the observation that all of the ecdysone-treated individuals in both runs of this experiment had completed apolysis within the twenty-four hours between injection and dissection, a response considerably advanced over that produced by activation of the endocrine system through feeding. Therefore, a final investigation was designed to assess division activity under somewhat more physiological

FIGURE 78
4th INSTAR: FED, DECAPITATED
EFFECT OF ECDYSONE: TRIAL ONE

| SAMPLE | TREATMENT | MITOTIC INDEX: 10 HOURS |
|---|---|---|
| 8 | ECDYSONE | 7.2 ± 2.6 |
| 8 | WATER | 4.6 ± 1.7 |

$0.05 > P > 0.02$

FIGURE 79
4th INSTAR: FED, DECAPITATED
EFFECT OF ECDYSONE: TRIAL TWO

| SAMPLE | TREATMENT | MITOTIC INDEX: 10 HOURS |
|---|---|---|
| 9 | ECDYSONE | 4.3 ± 1.2 |
| 7 | WATER | 3.5 ± 1.1 |

$0.1 > P > 0.05$

FIGURE 80
4th INSTAR: $CA^-$, FED
EFFECTS OF ENDOGENOUS AND EXOGENOUS JUVENILE HORMONE

| SAMPLE | TREATMENT | | MITOTIC INDEX: 24 HOURS |
|---|---|---|---|
| 9 | SHAM OP. | ISO-OCTANE | 9.2 ± 2.6 |
| 7 | $CA^-$ | ISO-OCTANE | 16.0 ± 3.7 |
| 11 | $CA^-$ | FME | 9.2 ± 2.3 |

SHAM *vs* $CA^-$/ISO-OCTANE: NOT SIGNIFICANT

SHAM *vs* $CA^-$/ISO-OCTANE; $CA^-$/ISO-OCTANE *vs* $CA^-$/FME: $P<0.001$

conditions.

Fourth instar siblings, approximately one month after the moult from third to fourth instar, were subjected to allatectomy. Particular care was taken to avoid damage to the corpus cardiacum, and to avoid damage through overheating when sealing the wound with molten tackiwax. Such emphasis, designed to favor normal feeding responses, brain hormone release and prothoracic gland activation, might be expected to produce a higher level of mortality due to dessication from inadequate sealing of the cuticle. However, mortality was a relatively standard twenty-five percent in the allatectomy series during the ten day post-operative period. Sham operated controls, in which a similar incision was made and one salivary gland reservoir removed, suffered a forty percent mortality.

The insects were fed to repletion ten days following the surgery, and returned to the incubator. Twenty-four hours after the blood meal, thirty allatectomized individuals received an application of 0.7 nanoliters of FME in iso-octane, and thirty received iso-octane only. Sham operated controls similarly received iso-octane. At sixty hours post-feed, all the insects were injected with 2$\mu$l of 2 x $10^{-3}$M colchicine. Twenty-four hours later, all insects were removed from the incubator to 4°C for subsequent dissection and preparation of right testis squash.

Counts received from this experiment yielded consistent and highly significant results (fig. 80 ). Activation of the bugs own brain hormone and prothoracic glands with subsequent ecdysone production, in the absence of juvenile hormone, resulted in a seventy-five percent increase in MI over the sham operated animals activiated in the presence of naturally occurring juvenile hormone. Moreover, application of the synthetic analogue FME, totally abolished this increase. There was no FME-related depression of MI below the endogenous level demonstrated by the controls however, in spite of the rather arbitrary dose of the analogue. The results obtained are also strikingly similar quantitatively to those obtained by injection of $\beta$-ecdysone in the original experiment performed (fig.76).

## Chapter Ten: Sequential Differentiation

Having presented experimental evidence in support of hormonally affected changes in division activity consistent with the kinetic hypothesis, the validity of the sequential differentiation hypothesis becomes a matter of tantamount importance. The encysted, clonal nature of the insect spermatocyte provides a simple means of determining the past history of division of the cyst. In the great number of species so far investigated, a precise relationship between the onset of appearance of the meiocyte and number of cells per cyst can be established. (White, 1955; Phillips, 1970; King and Akai, 1971). Exceptions to this rule are found only in strain differences in silkworms (Murakami, 1971).

Cytochemical evidence favoring a sequential differentiation of the meiocyte is provided by Ansley's study (1958) which demonstrates a progressive change in histone character of the spermatogonial nucleus of the Pentatomid bug, <u>Loxa</u> <u>flavicolis</u>. Similarly, meiotic precocity in chromosomal pairing is well-established in some Dipteran spermatogonia (White, 1973). Finally, following the early work of Regaud (1901), workers have defined a sequential and predictable lineage in cytological appearance of various spermatogonial stages in a number of mammals (Clermont, 1972).

However, the classic experiments of Fukada (1944) demonstrated that the removal of the corpus allatum from a third or fourth instar Bombyx resulted in the production of a precocious adult, which contained fully mature sperm in the testis. This could be interpreted as the removal of a juvenile hormone inhibition on differentiation of the meiocyte or spermatid as it would seem unrelated to the age of the larvae so manipulated. That is, the possibility exists that, in the absence of juvenile hormone, meiocytes could be recruited from any stage in the spermatogonial division sequence.

Wigglesworth's (1934, 1936) early studies on corpora allata and precocious adult formation in Rhodnius, were limited to ovarian development. However, he states that development, while advanced in such preparations, does not approach that realized in the normal adult. Previous histological examination provides a rough estimate of the maximum cyst size in third (approximately $2^5$) and fourth (approximately $2^6$) instars. The question posed was this: given the same conditions of allatectomy and precocious adult formation, would the third and fourth instar bugs achieve the same level of spermatocyte or spermatozoal differentiation, as would be predicted if a differentiation block were involved, or would they maintain the discrepancy imposed by the necessity of one to one and a half extra divisions in the third instar as predicted by the sequential differentiation hypothesis?

To answer this question allatectomy was carried out on a number of third and fourth instar insects early in the diapause period. The animals were held for ten days, fed and returned to the incubator. The results of this initial experiment supported the sequential differentiation hypothesis in that adultoids resulting from third instar allatectomies never showed spermatozoal development, and those produced by allatectomy of fourth instar bugs always did. However, rather few survived to the metamorphic moult, and an extremely large variation in the duration of the succeeding moult resulted in no valid comparison between the conditions imposed on any pair of third and fourth instar insects.

A more extensive series of allatectomies was thus performed to provide adultoid individuals from third and fourth instars. A detailed analysis of the duration of the moult period in this sample will be provided in the following chapter. However, from thirty-two successful third/adultoid and seventeen fourth/adultoid insects, considerable pairing of individuals of equal duration of the moulting process is possible.

None of the third/adultoid individuals contained spermatozoa at the time of the moult. In fact, those individuals which moulted prior to twenty-two days post-feed contained only spermatogonia. Recognizable spermatocytes appear at twenty-two to twenty-five days. By contrast, 'perfect' adultoid cuticular morphogenesis, as evidenced by ocellar and external genitalia development (fig.83,88) and internal genitalia development(fig.94-96) occurs at about eighteen days after the blood meal.

In the fourth/adultoid series, spermatocytes and spermatids are evident in all preparations. In two insects which moulted to highly larval intermediates in twelve and fifteen days, very little development of the duct system characteristic of adultoids was noted, and a specific spermatid autolysis was observed in these specimens. At eighteen days, full duct and accessory gland growth is achieved. The testis contains spermatids in early stages of elongation. By twenty-three days post-feed, filiform spermatozoa are recognizable. In the sample of longest moult period duration, thirty-two days, further differentiation of the most advanced spermatozoa is evident. The maximum differentiation of external(fig.84,89) and internal (fig.93,97) structures occurs by contrast at about twenty

Figure 81. Female adultoid produced by allatectomy of third instar larva. Insect completed ecdysis, except for head capsule and inflation of wings. Note adult cuticular pigmentation. X 12

Figure 82.. Adult male, and adultoid male produced by allatectomy of third instar larva. Illustrating size difference. X 3

Figure 83, 84, 85. Extension of aedegus (a) produced by injection of ringer solution. Note also lateral pleat development (L). Third and fourth instar allatectomy/adultoids, adult. X 12

Figure 86. Ocellus (OC) of normal adult. X 7

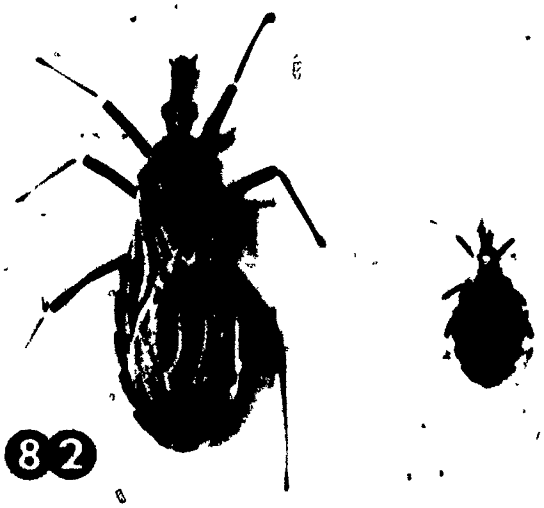
Figure 87. Slight ocellar pigmentation (OC) of normal fifth instar nymph, visualized in teneral nymph. X 10

Figure 88. Ocellus of adultoid produced from third instar nymph. X 10

Figure 89. Same, adultoid produced from fourth instar nymph. X 10

Figure 90. Derangement of ocellar development as a result of the incision during allatectomy. X 10

81



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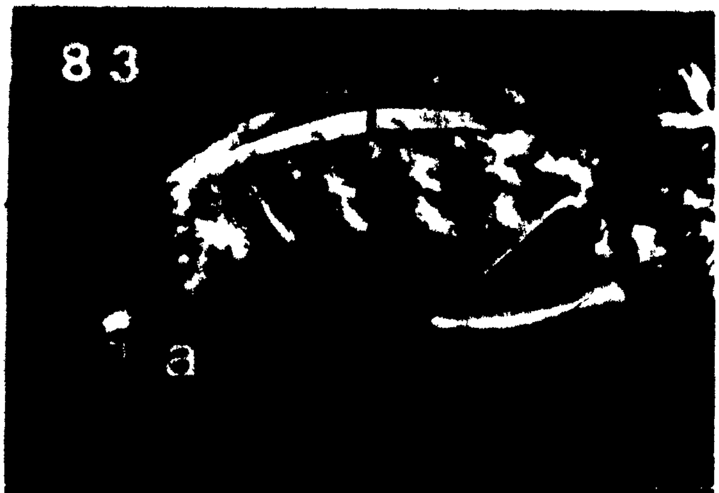

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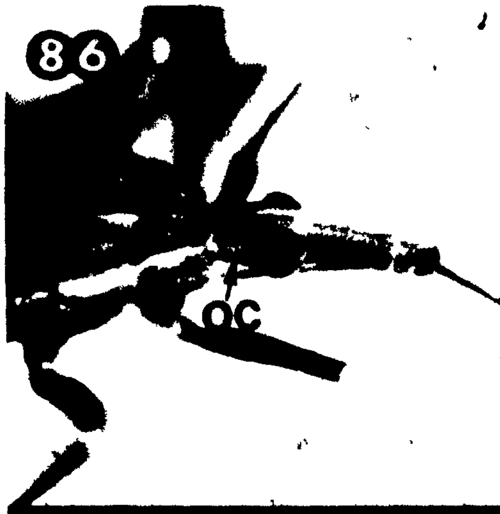

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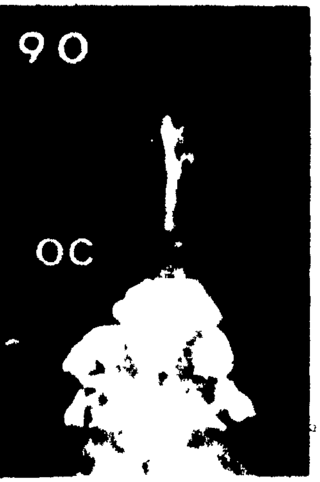


days.

It can be concluded from this study that the disparity in level of germ cell differentiation between third and fourth instar insects cannot be redressed solely by the removal of juvenile hormone. The level of differentiation attained by the adultoid testis appears inflexibly tied to the completion of a species specific division sequence. The advancing stages of development with increase in duration of the moulting period, indicates that the process of differentiation continues during times which are not necessarily under the direct influence of ecdysone. These effects are in contrast to the effects of the morphogenetic hormones on vasa deferentia and accessory glands. These structures, even in the third instar preparations, show considerable mitotic growth and individual cellular differentiation when exposed to ecdysone in the absence of juvenile hormone (figs. 93,94 ). Similarly, morphogenesis of the cuticular structures follows the classical endocrine scheme, and can thus be 'dissassembled' from the sequentially differentiating germ cell compartment.

Figure 91. Internal genitalia of a freshly ecdysed adult. Right complex removed to permit visualization. Accessory gland/seminal vesicle complex (ag), vas deferens (vd), and genital ampulla (ga). X 10

Figure 92. Same, freshly ecdysed fifth instar nymph. Vas deferens (vd) faintly visible (arrows), minute genital ampullae. Note externally directed apices of major tubules (large arrows). X 10

Figure 93. Same, adultoid produced by allatectomy of fourth instar nymph. Testes (T)not markedly larger than normal fifth instar nymph, but extreme differentiation of vas deferens, accessory gland and genital ampulla. Ventral fat body sheet not removed from this specimen. X 10

Figure 94. Same, from third instar allatectomized nymph. Small testes, considerable ectodermal and mesodermal duct development. X 10

Figure 95. Adultoid produced by allatectomy of third instar nymph. Vas efferens, and basal area of the tubule. Vas efferens epithelium columnar, secretory. Cysts contain spermatogonia. Trioxyhaematin, X 550

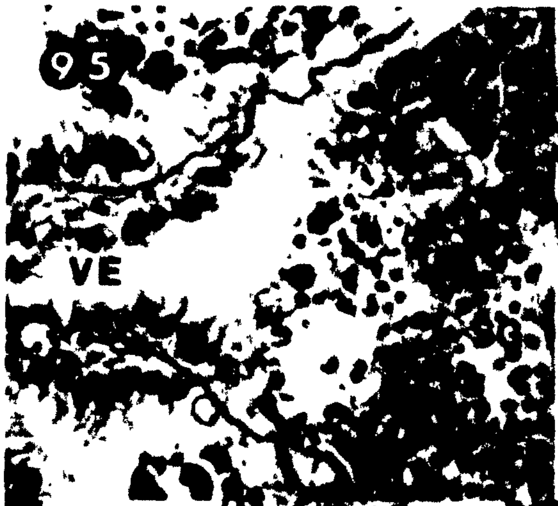
Figure 96. Same, major tubules , vas deferens. Trioxyhaematin, X 225

Figure 97. Similar to figure 95, but adultoid produced by allatectomy of fourth instar nymph. Note spermatozoa (SZ), secretory epithelium of vasa efferentia and vas deferens. Trioxyhaematin, X 225

Figure 98. Same area in the testis of a freshly ecdysed fourth instar nymph. Vas efferens low cuboidal, cysts contain spermatogonia. Trioxyhaematin, X 550


vd
T
ag
9 1
vd
vd
9 3

95



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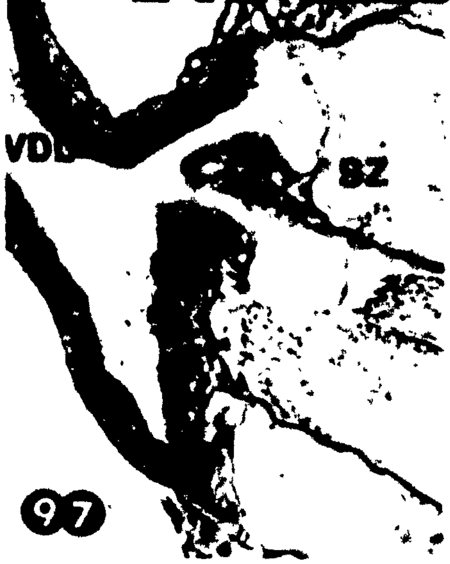

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## Chapter Eleven: Moult Duration

Following allatectomy, the duration of the succeeding development to the metamorphic moult is characteristically considerably longer than that experienced by an unoperated larva (Wigglesworth, 1934; Fukada, 1944). Certainly, some of this delay might be imposed by wounding, and regenerating tissue (see review by Harvey, 1962; Wyatt, 1971). However, experiments demonstrating larvalization of Rhodnius by applications of FME and other analogues of juvenile hormone have indicated a positive correlation between the degree of juvenilization attained by the intermediate, and shortening of the moult period (Wigglesworth, 1940; Barrett, 1974). Somewhat similar results have been obtained for a variety of hemipterans (Sláma, 1971), for Tenebrio molitor, (Socha and Sehnal, 1973), and for the dragonfly Aeshna cyanea Müll. (Schaller and Defossez, 1974). In this last case, the authors have interpreted the effect as due to a prothoracotropic action of juvenile hormone, which has repeatedly been demonstrated in diapausing pupae of Hyalophora (Williams, 1959; Gilbert and Schneiderman, 1959). Evidence for a prothoracotropic function of juvenile hormone is not entirely conclusive, and this rather complex question will be dealt with more fully in the discussion.

It has been demonstrated in this thesis that spermatogenesis proceeds in a series of closely defined and obligatory steps. The rate of passage through this sequence may be modified by alterations in the rate of cell division. Equally important, at least in the interpretation of adultoid development, and by extension in the interpretation of spermatogenesis in those insects lacking additional diapause-related controls, is the amount of time available for cell division and thus germ cell differentiation within each stadium.

The question posed then, without regard to mode of action for the moment at least is, do the metamorphic hormones exert a regular and predictable influence on the developmental period within each stadium in <u>Rhodnius</u>; that is, the duration of time from the blood meal to the moult?

The author previously conducted an experiment designed to investigate the question of the time of sensitivity to FME during the fifth instar. This experiment is thus complicated by the factor of the time of application of the analogue. That question involves essentially cuticular morphogenesis, and does not directly concern the questions posed in this thesis, and a full compilation of results and graphic representations has been included in an appendix (Appendix Three) for the curious. However, the sample size of the experiment was quite large, and an analysis of results concerning the correlation of degree of larvalization and duration of the developmental period preceding the moult is thus highly valid.

A large population of fifth instar siblings were isolated at the moult from fourth to fifth instar, held for ten days, and fed. Applications of 2.5 nanoliters or 1.0 nanoliter of FME in 1.0 µl. iso-octane were made to the ventral cuticle at one hour post-feed, and every twenty-four hours for the succeeding fourteen days. This procedure was carried out on ten bugs per dose level each day, and the insects returned to the incubator. No iso-octane controls were carried out, as previous experience indicated that many insects, particularly in the lower dose range, would be unaffected by the treatment. The insects were observed daily, moulting individuals removed, and scored by the technique devised by Wigglesworth (1969).

The histogram (fig.99 ) shows that nearly half the sample showed little or no larvalization (score of 0-3), and the majority of these insects moulted in the normal time period of approximately twenty days. The developmental time can be observed

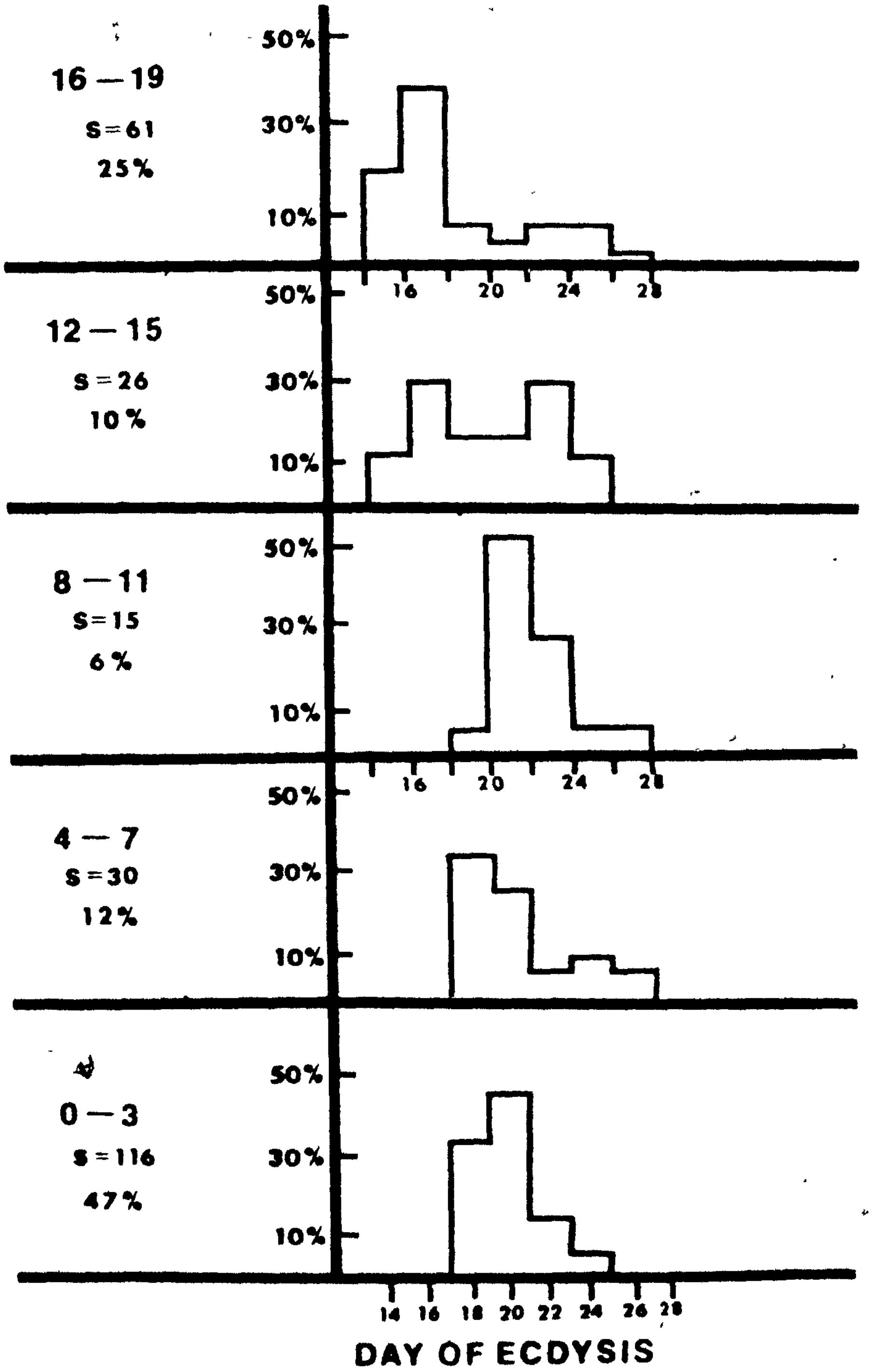

FIGURE 99 Effect of FME on duration of moult cycle. Insects are grouped (0-3, highly adult; 16-19, highly larval) according to scale devised by Wigglesworth (1969). S = sample size, and percent of total sample in given group. Intervals on X axis represent total insects moulting within a 2 day span.

to shorten progressively with advances in the degree of larvalization, arriving eventually at a peak of fifteen to sixteen days for perfect and near perfect sixth instar larvae.

Similarly, a more modest experiment carried out by application of the analogue at twenty-four hours following the feed only, provides almost precisely the same figures (fig.100). The perfect sixth instar larvae moult at day fourteen to fifteen in this experiment. In fact, this represents only two insects, one on each day. Day thirteen is the earliest occurrence that this author has noted in these, and several related experiments using FME on the fifth instar.

The fifth instar *Rhodnius* larva is not considered to possess an active corpus allatum during at least the early 'critical' period of the developmental span (Wigglesworth, 1964). Not surprisingly, therefore, allatectomy had very little effect on moult duration in this insect (fig.101). As noted above, this is not the case with earlier instars. Initial experiments involving allatectomy of third and fourth instar bugs resulted in widely varying results. However, more careful surgical procedures, and a larger sample of insects (Chapter Eleven) permitted the demonstration of moulting durations of both third and fourth instar insects with a definite peak (fig. 102 ). In both

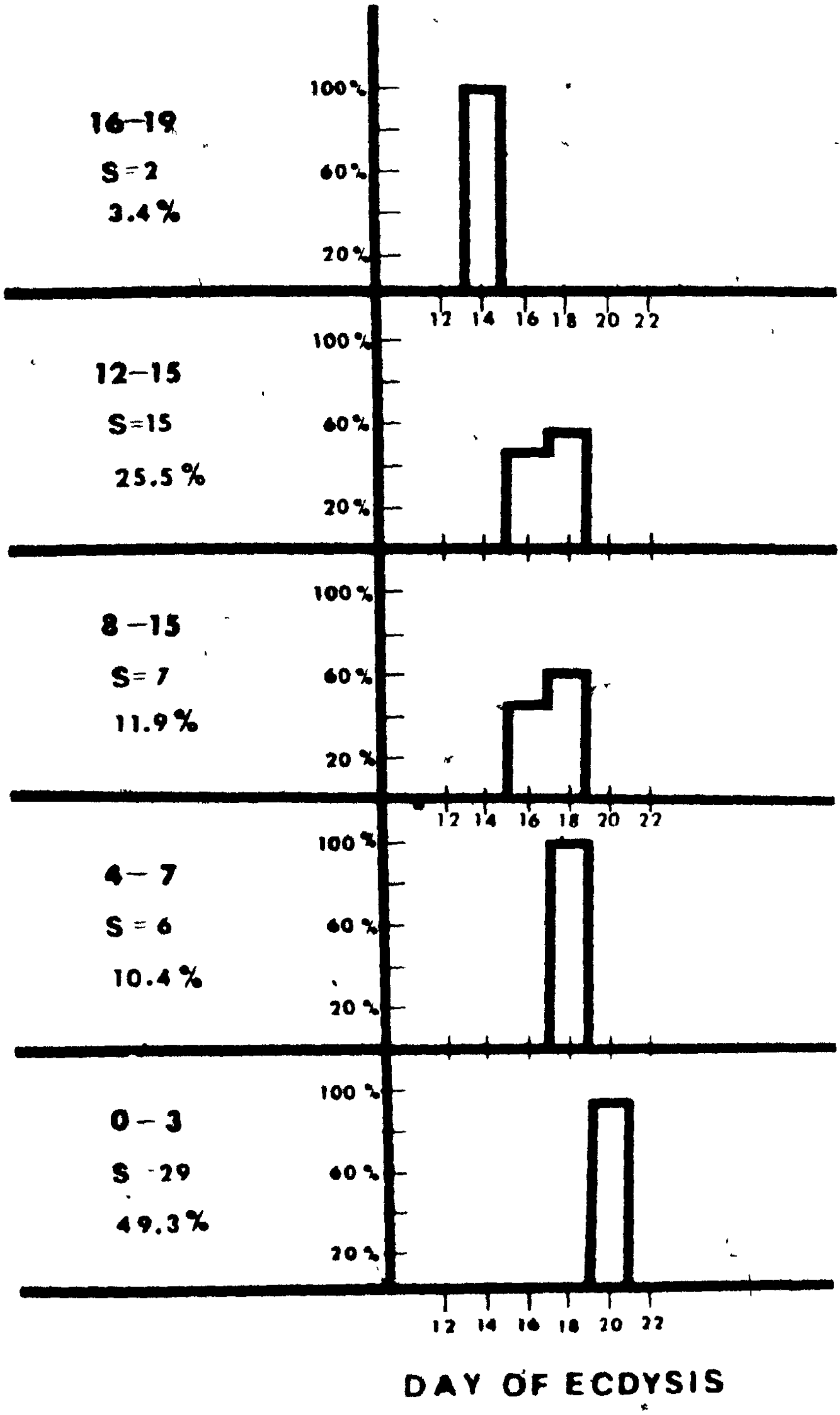

FIGURE 100 Effect of FME on duration of the moult cycle. FME applied 24 hours post-feed. Presentation as in fig. 99.

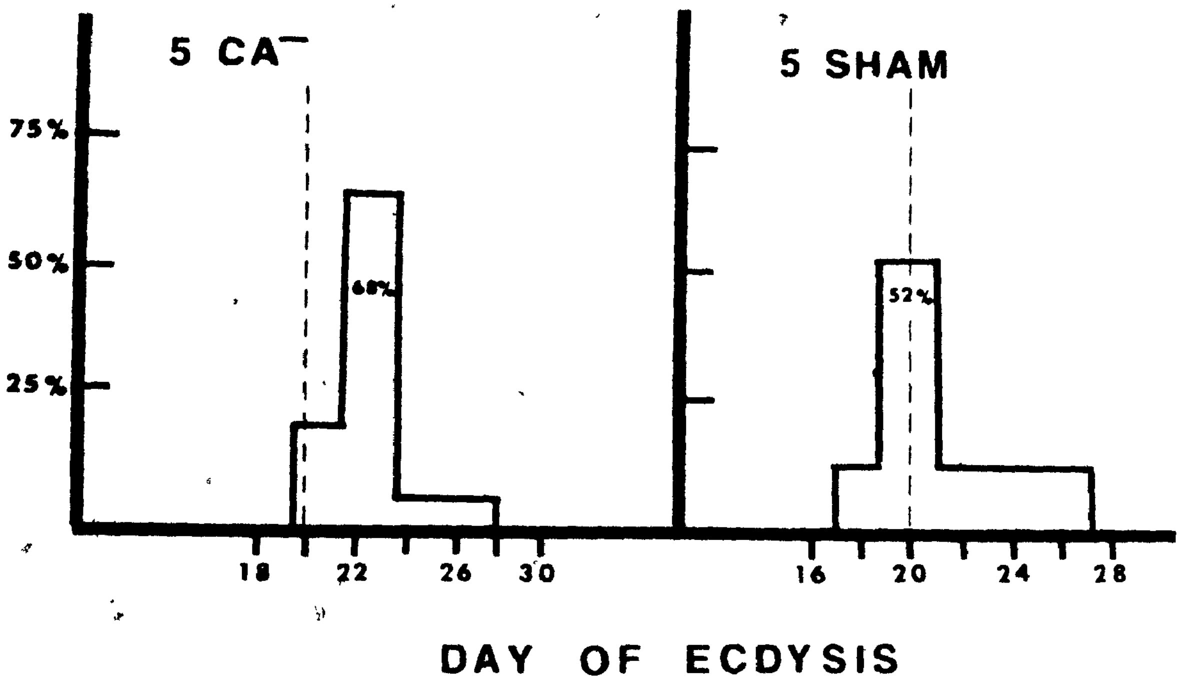

FIGURE 101 Effect of allatectomy on moult duration in the 5th instar. Sample sizes: Allatectomized insects- 17, sham operated controls- 22. Approximately 10% difference in results not regarded as significant.

cases, approximately fifty percent of the sample moulted within a three day span. Particularly interesting is that the span, though slightly different for each instar, closely approximated the normal duration of the fifth instar metamorphic moult (twenty ± one day).

To investigate the role of juvenile hormone in this phenomenon, fourth instar bugs were isolated, allatectomized as above, held for ten days and permitted to gorge. Insects which fed to repletion were held an additional twenty-four hours for diuresis to take place, and 0.7 nanoliters FME in 0.5 l. iso-octane topically applied to the ventral abdomen. It may be seen from the histogram of the results (fig.102) that over sixty percent of these insects moulted within the three span comprising a moulting cycle duration of twelve to fifteen days. The normal duration of the fourth instar developmental period is twelve ± one day; hence, this sample may be regarded as closely approximating a normal larval moult in duration.

These experiments, while providing no information on the means by which juvenile hormone accomplishes this end, do conclusively demonstrate that analogues of the hormone can tailor the duration of the developmental period. Close approximation of this duration with that observed in the normal insect under differing con-

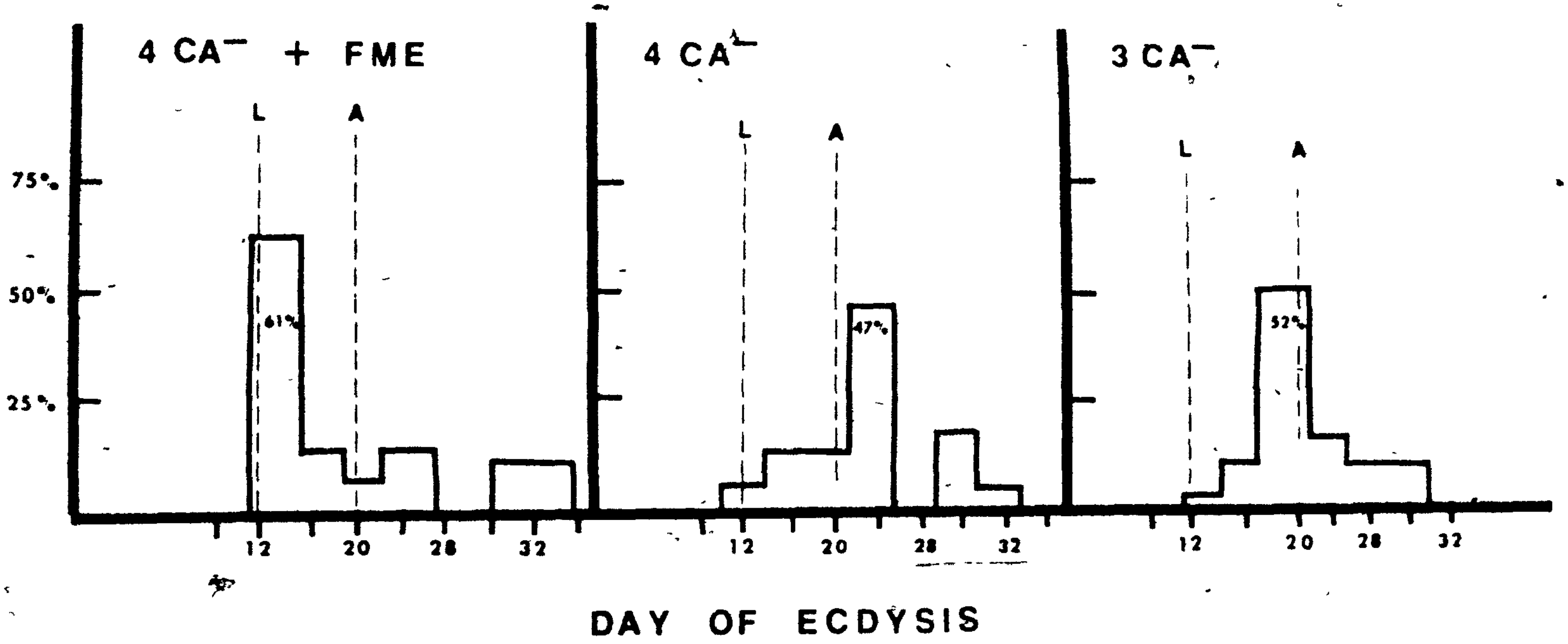

FIGURE 102 Effect of allatectomy, and juvenile hormone analogue replacement on earlier larval instars. Intervals on the X axis represent the total of insects which moulted in a 3 day span. Y axis represents percentage of total sample. Normal day of larval and adult (morphogenetic) ecdyses represented by dashed lines L and A. Dose of FME topically applied; 0.7 nanoliters, 24 hours post-feed. Sample sizes: 4th $CA^-$ + FME- 18; 4th $CA^-$ - 17; 3rd $CA^-$ - 32.

ditions of natural hormone titer strongly suggest that juvenile hormone exerts these effects in the normal as well as the experimental situation.

## Discussion: Introduction

This discussion has been organized into three sections, the first of which deals with what we may now be said to know about the control of testicular development. That is, this section includes a brief general review of structure and function in the developing testis which may be considered to be built on firm observational and experimental evidence. The limitations of this evidence will be discussed in relation to the several factors impinging on testicular development, and a conservative restatement of the kinetic hypothesis made. The section will conclude with an application of this hypothesis to the observations on testicular development existing in the literature.

One important concept, central to the kinetic hypothesis, is that the rate of cell division in the germ line is under hormonal influence. This cannot be unequivocally concluded from the evidence presented in this thesis. The importance of the concept requires a thorough analysis of the experimental evidence obtained through this study, and ancillary evidence from the literature. This section will necessarily be more speculative and argumentative than the preceding section, and a tentative conclusion will be presented.

In the final section, an attempt will be made to compare the systems operating in testicular development to an hypothetical construct of the control of proliferative tissue which has been defined in vertebrate systems. In this manner, on the basis of fragmentary evidence and analogy to systems phylogenetically far removed from the Insecta, an hypothetical schema of testicular developmental control will be developed.

## Chapter Twelve: Discussion; Kinetic Hypothesis

The question initially posed in this thesis was: do the hormones of morphogenesis, ecdysone and juvenile hormone, exert a regulatory effect on testis development? A review of the literature indicated that they do indeed, but the precise nature of their effects was obscured by a mass of conflicting data. Certainly, some of this conflict has been generated by species-specific hormonal responses, and also much of the information consists of valuable but cursory observations on gonad development extracted from studies conducted for quite different purposes. The major source of confusion, however, was the lack of any real understanding of the many complex cellular events which comprise the development of the testis, or even simply the germ line. As a result, even the most carefully conceived experiments often produce results which, lacking a suitable framework of normal development, are cryptic and uninterpretable. Similarly, with no developmental timetable as a guide, the choice of experimental system has often been completely unsuitable for the investigation proposed. Contention and confusion were the result, borne of misinterpretation and the comparison of unlike data. Nonetheless, certain studies stand out as milestones in the history of testicular and gonadal endocrinology. Williams' (1952) demonstration of a role for ecdysone in lifting diapause inhibition is one of these, though the assay employed is nearly as obscure today as it was then. Demonstrations by Wigglesworth (1934, 1936) and Fukada (1944) that the corpus allatum, hence juvenile hormone played an inhibitory role in some way on gonad development are equally important in the history of this topic. Finally, the imaginative and courageous attempt by Takeuchi (1969) to consolidate these findings into a comprehensive hypothesis of gonadal endocrine control must be noted.

Takeuchi's (1969) explanation of events impinging on spermatogenesis is suitable only for those insects which do not mature sperm during the adult stage. Many do, however, and indications in the literature suggested that the pattern of larval development displayed by these insects was not unlike that in <u>Bombyx</u>, Takeuchi's experimental model. <u>Rhodnius</u> possesses many admirable traits as an experimental animal, and carries out both larval and adult spermatogenesis. In view of the confusion alluded to in the preceding paragraph, it was decided to attack the problem not by posing a question for experimental analysis initially, but by attempting an understanding of the timetable of normal larval and metamorphic gonadal development, and, particularly, to isolate parameters of growth from those of differentiation.

Some success was achieved by this approach, though certainly major areas, such as stem cell division, were avoided entirely. However, an analysis of the cytology and histology of the testis and, particularly, a semi-quantitative analysis of germ cell kinetics during the last larval moult, provided handsome rewards. As these observations are exhaustively catalogued in the relevant sections of the thesis, only a brief statement of findings of major significance will be presented here.

Sexual differentiation of the Rhodnius testis has been accomplished prior to eclosion. Spermatogonia arise in the tubule apex, become encysted, and pass through a series of seven disivions. Following the eighth division, the cells may be recognized as spermatocytes. Following a prophase period of approximately ten days, the spermatids are produced by meiotic divisions. Spermiation immediately ensues in those insects which have received a blood meal. In contrast, in those insects which have not fed all spermatids produced are autolyzed in an histologically distinct fashion. A similar fate befalls the most differentiated spermatogonial cysts in earlier instars.

The author has demonstrated that this specific autolysis can be halted by injections of $\beta$-ecdysone, in physiological amounts, in preparations lacking brains, corpora cardiaca, corpora allata, and sub-oesophageal ganglia, and further deprived of any potential nutritional input or osmotic influence from a blood meal. The process thus resembles Williams' (Kambysellis and Williams, 1971a & b) demonstrations of in vitro spermiation stimulated by ecdysone. As the process does not occur in the adult, which lacks a source of ecdysone, the precise significance of this finding remains obscure. It can be concluded that juvenile hormone does not play a direct role in the phenomenon since the diapause-related autolysis and its alleviation occur in each instar and in allatectomized last larval instars, hence in both the presence or absence of juvenile hormone. It is not possible, of course, to assess the significance of macro-molecular factors in an in vivo system, but the resemblance to the system defined for Hyalophora and Samia (Kambysellis and Williams, 1971a & b), invites the speculation that ecdysone acts in a similar manner in Rhodnius testis, permitting the access of bioactive haemocyte products to the developing system rather than exerting a direct effect. Regardless of the mode of action, the importance of the phenomenon should not be underestimated.

This is the system which permits Rhodnius to maintain a constant testis volume, and a static level of differentiation during each larval diapause. However, similar observations exist for only three other insects (Hyalophora cecropia, Bowers and Williams, 1964; Cephus cinctus, Church, 1955; Papilio xuthus, Nishiitsutsuji-uwo, 1961), in each case only during the period of diapause. It is thus not known how general the phenomenon may be. If non-diapause species lack the mechanism for this specific autolysis, then there is no obvious reason why spermatogenesis could not proceed in vitro in the absence of insect hormones (see review by Marks, 1970).

As more fully described and illustrated in the text (Chapter Five), spermatocytes accumulate in the tubule and reach a plateau level of accumulation over a time course equivalent to the duration of the meiotic prophase. Of course, this fact should be self-evident, however, the significance is underscored by a simplified mathematical relationship which predicts an accumulation of germ cells in the spermatocyte compartment which reaches and sustains a level of over ninety percent of the total germ cell population. This mechanism explains the accumulation of spermatocytes typically observed in late larval insects without recourse to a specific inhibitory role of juvenile hormone on the meiotic divisions, as suggested by Takeuchi (1969). Experiments conducted by the author with applications of a juvenile hormone analogue argue strongly against the existence of a specific meiotic inhibitor. The products of meiosis, the spermatid cysts, decline in number with effective dose, as is also suggested by the results obtained by Nowock (1973) in Ephestia kuhniella. However, an analysis of the size of the spermatocyte compartment at this time shows no change at all with hormone exposure. If output from this compartment were specifically inhibited, a substantial rise in number of cysts would occur. This does not happen, and the conclusion can be drawn that the final spermatogonial mitosis, input to the spermatocyte compartment, is inhibited equally. While the figures presented show a maximum decline to twenty-five to thirty percent of the normal spermatid output in the most highly larvalized insects, subsequent experiments have demonstrated that the duration of the moulting process must also be taken into account. As these most highly larvalized insects moulted in approximately seventy percent of the normal duration of the metamorphic moult, the corrected figure for maximum inhibition of spermatocyte meiosis approaches fifty percent, and is thus very similar to the figure obtained experimentally for inhibition of spermatogonial mitosis as measured by colchicine metaphase accumulation.

Experiments conducted by the author (ChapterTen), demonstrate that the sequential differentiation observed in Rhodnius, which occurs also in other insects (White, 1955; Phillips, 1970; King and Akai, 1971), is unaffected by alterations in the titer of juvenile hormone. Thus the discrepancy of approximately one and a half divisions between third and fourth instar testes could not be corrected by the allatectomized insect, and an adultoid was produced with juvenile gonads. The mesodermal and ectodermal components of the reproductive system, while attaining a size reflecting the stadium fron which they were derived, underwent extensive metamorphosis typical of the final moult. Curiously, in both the third and fourth instar preparations, little evidence of an accelerated division rate could be found. For instance, the normal third instar might be expected to spend ten days in development to the fourth instar, perhaps four days continued growth before diapause, and produce spermatocytes eight days following the blood meal. The total developmental time in that case would be twenty-two days, approximately the time of appearance of spermatocytes in the allatectomized third instar/adultoid. However, many important parameters are yet unknown in the system, such as the duration of exposure to ecdysone, the role of macromolecular factors, the amount of juvenile hormone required to inhibit the mitotic index increase, and the degree of inhibition experienced in any given instar. These factors, together with the difficulty in recognizing early spermatocytes, render this puzzling observation difficult to interpret but perhaps not as important as it might seem.

The nature of the cellular events resulting in this sequential differentiation is not known. Koch and King (1967) have carried out extensive research in the related area of oöcyte differentiation in Drosophila and have proposed that the distribution of ring canals, organelles encircling the cytoplasmic interconnections in germ cell cysts, act in some undetermined manner as a differentiator. King and Akai (1971) have suggested a similar mechanism functioning in the male germ cell cyst. However, even in the oöcyte, the final stages of differentiation are decided by other influences since two potential oöcytes per cyst are possible on the basis of ring canal distribution per se. (Koch and King, 1967). Recently, Mindek and Nöthiger (1974) have conclusively demonstrated that competence to undergo metamorphosis and begin cuticle deposition in Drosophila wing discs is attained through a succession of cell divisions. How this relates to the embryonically, structurally, and functionally distinct germ cell is not at all clear, but it does indicate that sequential differentiation does not necessarily require the influence of incomplete cytokinesis and ring canal presence.

The first half of the thesis was thus largely directed toward a critical observation of normal testicular growth and differentiation, and culminated in the proposal of a kinetic hypothesis for the maintenance of germ cell development in phase with normal larval development and metamorphosis. As is true of most such hypotheses, the work is not really original to this author, but rather an extension of theories provided largely by the authors mentioned in the introduction to this chapter. However, a continuation of this logical series, integrating the fact of adult spermatogenesis and anhormonal _in vitro_ spermatogenesis, leads to the postulation that an endogenous division rate must exist which is not subject to further decline in the absence of insect hormones, nor in the presence of inhibitory hormones. Further, this endogenous rate must be raised in the presence of ecdysone; it is this increase in rate which is affected by juvenile hormone, the endogenous rate being unaffected. These conditions are indeed satisfied for the mitotic index (MI) using a variety of experimental preparations, and with a very high degree of statistical validity. The MI is an adequate estimate of the number of cysts which, in effect, are changing differentiated compartments through division, but it is unfortunately not an adequate estimate of the rate at which they do so. The kinetic hypothesis can be considered greatly strengthened by these results, but it is not proven.

It was earlier pointed out that the MI, as a measure of division state compared to total cell population, is affected by any changes in that cell population. This principle has its greatest impact in tissues containing a mixture of cells committed to division and cells, which for one reason or another, have ceased dividing. This non-proliferative fraction compromises any such technique using total population size as a reference. In some cases, major sources of non-proliferative cells can be determined or avoided, as in the case of the choice of instar for the tests reported in this thesis. The complication of 'non-proliferative' spermatocytes was thus avoided. However, at least one other potentially serious source of non-proliferative cells exists in the system, the random degeneration of spermatogonial cells characterized by nuclear pycnosis. The specific autolysis need not be considered, as it has been shown to be juvenile hormone insensitive. The manner in which these pycnotic cells may effect the MI is succinctly stated in Houck's Laws, being first; that dead cells don't divide, and second, that dying cells divide very slowly (Houck, 1973). There is at present no valid technique to identify which cells are on this non-proliferative pathway (Roosen-Runge and Leik, 1968), and attempts at quantifying the degree of pycnosis in any preparation have not met with success. There is no firm evidence that the percentage of the population engaged in this activity varies with normal alterations in the physiological milieu, but neither is there evidence that it does not.

The author considers that any influence of this phenomenon is likely to be negligible on the basis that, first; if Roosen-Runge (1973) is correct in assuming the phenomenon to be a result of an accumulation of genetic deficiencies resulting in an abortive division, the frequency of such an accumulation is unlikely to be influenced by hormonal conditions, nor functioning through catastrophic division, would there be an interphase cell lingering in the non-proliferative compartment. Second, the degree of increase in MI would require a pool of cryptic pycnotic cells considerably larger than estimated to exist in the population at any given time. Of course, such an estimation depends in part on the length of the cell cycle compared to the length of time required for the complete degeneration of a cyst, and this information is not available. However, cysts with actively pycnotic nuclei occur at the rate of about five to ten percent, and often less, of the total cell population. Furthermore, such cysts can often be found in areas where the surrounding cysts are more advanced in terms of number of cells per cyst. This indicates that even the actively degenerating phase of the phenomenon is longer than a normal cell cycle, thus the frequency of occurrence is less than the five to ten percent figure estimated. Taking such observations into account, it would seem likely that the increase in mitotic index represents a real shortening of the germ cell cycle duration. However, any more concrete statement must await a direct measurement of this parameter under the appropriate hormonal conditions.

The final potentially significant effect of morphogenetic hormones on testicular development, concerns the ability of juvenile hormone to alter the duration of the moult. That FME, a powerful juvenile hormone analogue, has this ability has been conclusively demonstrated in allatectomized fourth instar and unoperated fifth instar Rhodnius. That it does so in the normal larval span is not as clear, but the increase in duration of the moulting process noted by other authors (Wigglesworth, 1940; Fukada, 1944) as a result of removal of the source of juvenile hormone, and the shortening of this process by application of analogues (Sláma, 1971; Socha and Sehnal, 1973; Barrett, 1974), subsequently quantified in this thesis, argues strongly that it does. Juvenile hormone is unlikely to be the only significant input to this parameter, of course, and the variation in duration between third and fourth instar insects is retained even in the allatectomized preparations (10/12 days; CA$^-$, 20/24 days). However, the difference between late larval and metamorphic moult durations can be imposed or abolished by removal or resupply of the hormone, and the influence of this phenomenon on the spermatid production and spermiation during the final moult period is obvious.

The mode of action of juvenile hormone in affecting moult duration is thought to be a consequence of a direct effect on the prothoracic glands, with a subsequent stimulation of ecdysone production (Krishnakumaran and Schneiderman, 1965). Thus, moulting can be induced in some diapaused Lepidopteran pupae and brainless daüer pupae by the implantation of corpora allata, or the injection of natural <u>Cecropia</u>

oil, or juvenile hormone analogues. The response does not occur in isolated pupal abdomens, which lack prothoracic glands, arguing against a direct effect of juvenile hormone on the epidermal cells (Oberlander and Schneiderman, 1966). Furthermore, juvenile hormone analogues can be shown to promote an increase in RNA synthesis in prothoracic glands (Siew and Gilbert, 1971). These results, while very leading, cannot be considered conclusive. Pupae of some, though not all, diapausing insects are sensitive to various types of manipulation and injury-induced activation. Similarly, RNA synthesis in the prothoracic glands can be induced by a variety of techniques not related to juvenile hormone (Berry et al, 1964) and, furthermore, cannot be reliably linked to ecdysone production. Finally, none of the above effects have been demonstrated in other than the Lepidopteran pupae, although attempts have been made with other groups (Fraenkel and Hsiao, 1968).

Rhodnius larvae, in the absence of a functioning neural and retrocerebral endocrine axis, can be induced to moult by the injection of ecdysone (Wigglesworth, 1970). Doses of $\beta$-ecdysone employed in various experiments reported in this thesis were selected from a series of experiments correlating $\beta$-ecdysone dose with duration of the moult period (Appendix One). The acceleratory effect of ecdysone on apolysis, new cuticle deposition, and ecdysis, referred to as hyperecdysonism (Williams, 1968) is thus readily demonstrable in *Rhodnius*. It is conceivable that juvenile hormone exerts a physiological prothoracotropic effect of ecdysone at the initiation of each larval moult. Davey (1971) has also reported a juvenile hormone-induced discharge of neurosecretory material in *Phocanema decipiens*, and here too, the physiological significance is simply not known. Haemocyte factors have been shown to be implicated in the response of epidermal cells of various species to ecdysone (Wigglesworth, 1955; Hoffmann, 1971), and it is known that juvenile hormone can repress RNA synthesis in haemocytes (Berry et al, 1964). Finally, it has been shown that *in vivo* ecdysone stimulated RNA synthesis in the wing discs of *Samia* (Patel and Madhavan, 1969) and *Calliphora* fat body *in vitro* (Congote et al, 1969), is abolished by simultaneous application of juvenile hormone analogues, and protein synthesis also being greatly reduced, indicating a direct effect on the epidermal cells themselves.

The related phenomenon of a delay in ecdysis has long been linked with wound healing (Harvey, 1962; Wyatt, 1971), the aforementioned disruption of haemocyte function, the presence of regenerating tissues (O'Farrell and Stock, 1953) and, recently, with high doses of juvenile hormone analogues (Hangartner and Masner, 1973). It should be noted that many of these phenomena are highly specific with regard to the age of the larval stage investigated. For instance, wounding, or autotomy may result in an accelerated, supernumerary ecdysis if carried out early in the instar, or a normal but delayed moult if performed at a later date (Krishnakumaran, 1972).

A report of correlation of moult duration and juvenilization in Aeshna cyanea, similar to that described for Rhodnius and reported by Barrett (1974), has appeared and the authors have attempted to explain the observations on the basis of prothoracotropic and morphogenetic functions of juvenile hormone (Schaller and Defossez, 1974). Such attempts are laudable and necessary to the development of a knowledge of these complex interactions. However, the present author, in light of the above reported multiplicity of potential parameters involved, and the essentially intact or nearly intact specimens used, concludes that an interpretation of the effects on Rhodnius would be a futile exercise at this time.

The kinetic hypothesis can be succinctly stated as follows: the germ cells differentiate to spermatocytes and spermatids sequentially through a species-specific number of divisions. The rate at which these divisions are carried out is sensitive to the morphogenetic hormones. Ecdysone markedly increases the rate, and juvenile hormone inhibits that increase in a dose related fashion. Juvenile hormone has no effect on the endogenous division rate maintained in the absence of ecdysone. The division and hence the differentiation of the germ cells is a continuous process and thus hormonal effects on the duration of any stadium, the 'availability of developmental time', will have a noticeable impact. Finally, a brutal, non-kinetic check on differentiation is imposed in systems exhibiting diapause, through the autolysis of the most differentiated germ cell element.

This hypothesis can account for virtually all the observations currently in the literature regarding hormonal effects on insect spermatogenesis and spermiation, providing that sufficient information concerning the normal developmental timing is available. The case for differentiation of germ cells in adultoids produced from third and fourth instar <u>Rhodnius</u> has been presented. In <u>Bombyx</u>, where third instar larvae as well as fourth instar larvae may mature spermatozoa subsequent to allatectomy, sufficient developmental time to adultoid emergence must exist in both cases.

This indeed is found to be so. Takeuchi (1969) reports recognizable spermatocytes in the second instar larvae in this species. In fact, this is predictable from his observation of spermiogenesis, hence meiosis, on the second day of the fifth instar and Sado's (1961, 1963a & b) approximation of ten to twelve days for the duration of the spermatocyte stage. Fukada's (1944) figures for pupation and subsequent emergence of the diminutive third instar/adultoid, fourteen days, thus provides a developmental time of at least sixteen days and probably eighteen days for spermatocyte maturation, meiosis, and spermiation.

In regard to the application of juvenile hormone analogues, or the implantation of corpora allata and subsequent gonad development, several conflicting observations can be reconciled. The appearance of spermatozoa has been reported in *Oncopeltus* and *Sarcophaga* following juvenile hormone applications which resulted in considerable juvenilization of internal and external structures. Precise data are not available with regard to the kinetics of these preparations. In *Oncopeltus*, applications of Bower's compound VII (Bowers, 1969) to the penultimate larval instar had no apparent effect on testis development as assessed in the last larval stadium, consistent with the demonstrated inability of juvenile hormone to repress the basal or endogenous level of cell division. Application just prior to the fourth/fifth ecdysis resulted in the analogue being lost with the exuvium, and thus was not very enlightening. Treatment during the early fifth instar was judged ineffective in preventing testis development. However, meiosis and spermiation had begun by this time, and no quantification of the level of spermatozoal output was attempted. Bhaskaran's (1973) results on Sarcophaga similarly are produced by late application of the analogue, and no quantification of the data presented.

Nowock (1973) and Sehnal (1969) provide volumetric quantification, but no analysis as to the cytological parameters involved. Feeding of the juvenile hormone analogue FME to Ephestia kuhniella larvae resulted in a dose-linked depression in testis volume. Implantation of active corpora allata into last instar larva of Galleria mellonella similarly reduced testis volume in what may be considered a dose/time fashion. Both results are likely directly comparable to the dose related decline in spermatid output demonstrated in Rhodnius, a consequence of juvenile hormone inhibition of ecdysone-stimulated cell division.

Ždárek and Sláma (1968) produced a series of adultoids, of varying level of juvenilization, by rearing Pyrrhocoris apterus on filter paper containing Law's mixture (Law et al, 1966) or by applying purified paper factor, a mixture of juvenile hormone analogues specific for <u>Pyrrhocoris</u> (Sláma and Williams, 1966). Spermatozoa were found in the testes of adult-larval intermediates which showed little external adult structure, although such internal non-germinal elements as seminal vesicles and accessory glands were well-developed. In morphologically perfect supernumerary larvae, testes were described as 'undifferentiated'. No data are presented on the duration of the moult, known to vary with juvenilization in this group (Sláma, 1971). These results would seem to parallel those reported above, indicating division rate inhibition.

The most interesting and complex report on juvenile hormone effects on testis development is in regard to the Cecropia silkmoth (Riddiford, 1972). Application of juvenile hormone analogues prior to the spinning of the pupal cocoon has no effect on internal or external development, nor on the incidence of pupal diapause, normally obligatory in this species. Application subsequent to spinning, or during the pre-pupal period, resulted in papae which failed to diapause, developing instead toward the adult ecdysis. The resultant pharate adult moths exhibited larval testes. If the juvenile hormone analogue is applied during this sensitive period, diapause induced by removal of the brain, and adult development subsequently initiated after a normal three month diapause period by an injection of ecdysone, the gonads are once more found to be adult in character. Conversely, if diapause is averted in pupae which have not been exposed to juvenile hormone analogues, by ecdysone injection, the gonads are still found to be adult. Therefore, the retarded development found in moths exposed to juvenile hormone analogues and averting diapause represented a real inhibition, and not one imposed by precocious adult development. This developmental inhibition is redressed during diapause, whether naturally occurring or artificially induced.

Such a result is quite consistent with the kinetic hypothesis. The retardation of development produced by juvenile hormone analogue application is a consequence of general germ cell division rate inhibition. During the diapause period, regardless of how it is induced, the endogenous division rate will redress this inhibition. That is, the level of differentiation attained will be that 'permitted' by the specific autolysis reported in this species (Bowers and Williams, 1964), and any effect of juvenile hormone acting through division rate inhibition and sequential differentiation will gradually disappear through the long diapause period. Adults from any diapausing pupa will thus always have adult gonads. In the case of forced development of the normal, and juvenile hormone analogue-treated pupae in equal developmental time, the kinetic effects of the analogue on sequential differentiation cannot be redressed, since no non-kinetic specific autolysis is encountered.

An interesting observation in this case is that, since the normal pupa has reached a level of differentiation of the germ line sufficiently advanced to allow normal development of spermatozoa during the induced adult developmental time span, diapause is truly 'imposed' on this insect as far as gonadal development is concerned. The Williams' ecdysone assay, (Kambysellis and Williams, 1971a & b), based on an alleviation of spermatid autolysis, has been criticized by Gilbert and King (1973) on the grounds that in one insect the assay responds to rubrasterone, a generally inactive phyto-ecdysone (Takeda, 1972). Such anomalies in specificity would be expected in a diapause limited system, which certainly must have arisen later and quite independently of ecdysone's role in epidermal cell activation.

Finally, all <u>in vitro</u> work carried out on non-diapause species should show a complete catalogue of spermatogenic events and thus need not be considered separately. Conditions of culture will certainly affect the rate at which events occur but the testis, in the absence of specific autolysis, is truly an autodifferential organ. In the adult a similar condition pertains. In the adults of insects which show a larval or pupal diapause, however, the situation is not as clear. Specific autolysis would be expected in the absence of a source of ecdysone, but it is not observed. Possibly, some component of this complex function has been 'dismantled' during the metamorphic moult, analogous to the dissolution of the prothoracic gland itself at this time (Wigglesworth, 1955a).

## Chapter Thirteen: Discussion; Division Rate and Hormonal Action

On the basis of the evidence presented in this thesis, the testis can be seen to be an autodifferential organ, whose state of differentiation is maintained in phase with the total developmental pattern of the insect by mechanisms affecting both the rate and the available time of development, and specific events within the sequence. The importance of any one of these mechanisms is determined in large part by the precise characteristics of the species under consideration. In all species, however, the rate of passage of the germ cells through their predetermined developmental sequence will be a matter of underlying significance. The control exercised over the rate of cell division can thus be considered a very basic, perhaps primitive, component of the overall control system in all insects, a fact which justifies the more thorough analysis of this function presented in this section. As very little experimental information exists directly concerning rate changes in cell division in the testes, or any other insect tissue for that matter, supportive evidence will occasionally be drawn from sources quite far removed from the subject at hand. Similarly, an investigation of each of several facets of this problem will generally result in the determination of likely or potential mechanisms, the validity of which must remain speculative until established or rejected by direct experimentation. Nonetheless, an hypothetical model can be formulated in this manner which describes the mode of action of the hormonal control of germ cell division at a more nearly molecular level. The reader is cautioned that the model so produced is highly speculative, but does not contradict any observations or knowledge currently held and, consistent with the definition of an hypothesis, is amenable to experimental investigation.

The initial question vital to this analysis is whether the hormonally responsive MI does in fact represent changes in the rate of cell division. This question was considered in part in an earlier chapter (Chapter Nine) with the tentative conclusion reached that at least major shifts in the proportion of proliferative to non-proliferative cells did not seem able to produce the observed changes in MI. As was pointed out at that time, an actual change in cell cycle duration cannot be regarded as proven until directly measured, and pitfalls in the measurement of division activity are many. Largely due to the precise characterization of events possible in 'pure' culture systems, certain radiolabelling procedures are now available which would be adequate in the heterogenous, exponentially increasing organ systems dealt with here (Baserga and Nemeroff, 1962; Lala, 1968). However, the spermatogonial cell cycle has been demonstrated to be particularly susceptible to direct alteration by incorporated isotopes (Johnson and Cronkite, 1959) and hence requires extremely low levels of label for maximum validity of estimation by these methods. With the current level of technology, this procedure would take between one and two years to carry out and is thus not now practical. Using such techniques, Löbbecke (1969) has demonstrated that the duration of the S or DNA synthetic period of _Ephestia_ wing discs is shortened by half during the pre-pupal period, thought to be a time of high ecdysone titer. No direct hormonal manipulation was attempted, however, nor was the role of juvenile hormone investigated.

On the basis of the argument earlier proposed against the major source of artifact, proliferative/non-proliferative cell population shifts, and the above correlation of Löbbecke, it will be tentatively concluded here that the observed changes in MI represent alterations in cell cycle duration. Such an assumption is necessary for the remainder of this discussion, and is indeed not unlikely. However, in view of the notorious complexities of the measurement of division rate (Harriss and Hoelzer, 1972; Houck, 1973), confirmation can only be provided by a direct and cautious experimental program.

The second question of significance to be asked is whether this cell cycle alteration is affected in a specific and physiological manner by the circulating hormones, or is simply a manifestation of a general metabolic shift in the insect. Mitosis is, of course, affected by a very great number of factors, and the question normally asked is whether or not the induced changes are part of a normal biological pattern. In contrast, Ortavant (1958) demonstrated that the duration of the cycle of the seminiferous epithelium, and thus the cell cycle of the ram was remarkably constant. This finding has been confirmed for a number of mammalian species, including rats (Clermont and Harvey, 1965) and humans (Heller and Clermont, 1964). However, recent results indicate that earlier stages of spermatogenesis can be manipulated in a predictable manner (Clermont and Mauger, 1974), which will be considered in greater detail in the succeeding chapter.

The situation, of course, is not as well understood in insects. The moulting hormone, ecdysone, commonly institutes a pattern of activity in the epidermal cells which includes DNA synthesis and division, but it has been conclusively demonstrated that later events in the activated pattern, such as the secretion of new cuticle, will occur whether or not this division has taken place (Wigglesworth, 1970 ; Madhavan and Scheiderman, 1968) and that injury will induce these divisions in the absence of ecdysone (Wigglesworth, 1937). Similarly, injections of large doses of $\beta$-ecdysone will bring about apolysis and cuticular deposition without epidermal mitoses (Williams, 1968; Mouze et al, 1973) and in wing discs of some species cultured <u>in vitro</u>, $\beta$-ecdysone may actually inhibit division (Oberlander, 1972). Juvenile hormone, during larval development, does not seem to express its activity except through cells activated by ecdysone. In its presence, some areas of the epidermis carry out greater division, but some, such as those responsible for the external genitalia and wings, undergo greater cell division in its absence (Wigglesworth, 1963). As previously discussed, the current consensus is that ecdysone will activate epidermal cells to carry out a specific pattern, and the nature of the pattern is selected by juvenile hormone. Hence, no direct effect on DNA synthesis or division is postulated for either of the morphogenetic hormones.

The normal pattern of activity of the germ line is division, of course, and, unlike the epidermal cells, they do not require activation, being continuously engaged in proliferation. In the presence of a natural or artificial source of ecdysone, these cells increase the rate of their normal activity. This would seem to imply a different mechanism than that involved in epidermal cell activation. The role of juvenile hormone is somewhat more complex. As in the epidermal cell response, juvenile hormone is ineffective in the absence of a simultaneous ecdysone presence. However, juvenile hormone cannot alter the pathway of development, as this is a fixed, sequential differentiation, and is not amenable to redirection by any known hormonal presence. Instead, juvenile hormone completely extinguishes the ecdysone rate-stimulating effect. Such a role for juvenile hormone is not unique; juvenile hormone abolishes the ecdysone-stimulated RNA synthesis in *Samia cynthia* wing disc (Patel and Madhaven, 1969) and *Calliphora erythrocephala* fat body (Congote et al, 1969), as previously mentioned. Growth of *Tenebrio molitor* ovaries *in vitro* is also enhanced by ecdysone, an effect which is abolished in the presence of farnesol (Laverdure, 1969). Such results are uncommon, however, and no satisfactory explanation for them has yet been found.

It would thus seem that hormonal effects on the germ line are qualitatively distinct from those on ectodermal systems. A second possibility is that the effects noted are simply manifestations of an overall alteration in the rate of metabolism. This explanation has some experimental support. During the diapause period when ecdysone is absent in *Rhodnius*, oxygen utilization is some four to five times less than that measured after the activating blood meal (Zwicky and Wigglesworth, 1957). Similarly, while both stimulatory and inhibitory influences are reported in various species (DeWilde and Stegwee, 1958; Roussel, 1963), an important observation is the demonstration of an inhibition of NADH linked oxidation by juvenile hormone in vitro ( Firstenberg and Silhacek, 1973) the effects of division noted could be due to generalized metabolic events. However, Zwicky and Wigglesworth (1957) note that no unusual demands seem to be made on oxidative metabolism during the proscribed period of epidermal cell mitosis in Rhodnius, rather the curve of oxygen utilization seems to reflect increasing demands of protein synthesis. A comparison of oxidative requirements in the fourth and fifth instar, that is in the presence and absence of juvenile hormone, exactly parallels the increase in mass of the individual insect:

Ratio of fourth to fifth instar

| | | |
|---|---|---|
| $O_2$ utilization, $mm^3$ $O_2$/hr. | 1 : 2.3 | (Zwicky and Wigglesworth, 1957) |
| Wet weight, one day post feed | 1 : 2.4 | (Appendix One). |

This would indicate that, at least in Rhodnius, the effect of juvenile hormone on the normal physiological oxidative metabolism is negligible. As well, the endogenous division rate is maintained in the absence or presence of juvenile hormone, suggesting a specificity requirement not met by general metabolic considerations.

A final possibility of a non-specific effect on division rests in the demonstration that liposoluble materials may affect the rate of cell division directly in vitro, in a predictable manner related to their degree of hydrophilic activity (Ingram and Fisher, 1973). Substances highly insoluble in water, such as juvenile hormone, could be viewed as 'stabilizing' membranes, and thus inhibiting cytokinesis. The more water soluble ecdysone perhaps could be considered to act in the opposite manner. This interpretation does not explain the interaction of the hormones but, in any case, the concentrations required to produce these physico-chemical effects are approximately 10 mM, several orders of magnitude removed from the maximum effective concentrations employed in the studies conducted by the author. (Appendix One).

Williams and Kambysellis (1969; Kambysellis and Williams, 1971a & b) have conclusively demonstrated that a blood borne factor, probably produced in the haemocytes, is the active agent permitting spermiation in the Hyalophora in vitro testis preparation. Ecdysone acts to permit access of this molecule to the germ cells, but exerts no direct action on the germ cells in its own right. These authors do not restrict the effect of MF to a maintenance of spermiation; the present author has chosen that interpretation in the main body of this thesis since none of the techniques employed in the assay permit any conclusions concerning the possible role of MF in cell division.

The existence of a similar macromolecular factor has not been demonstrated in <u>Rhodnius</u>. Such an undertaking would not likely be successful in an <u>in vivo</u> preparation of the type used in the experimentation reported in this thesis. Circumstantial evidence suggests that MF may also be the active component in spermatid survival in <u>Rhodnius</u>. Techniques which involve cuticular wounding in <u>Rhodnius</u> are apparently felt in the testis as a temporary derangement of the process of spermatid autolysis. Kambysellis and Williams (1971b) have demonstrated that similar cuticular injury in diapausing <u>Hyalophora</u> pupae provokes a marked increase in the titer of available MF in the blood of this insect, reaching a peak four days after the injury and then declining gradually.

A useful, though entirely theoretical construct at least in the insect testis, is that the endogenous division rate measured at times of maximum testicular isolation from circulating hormonal activity represents a level which is inhibited rather than a strictly autonomous basal level of germ cell division. The increase in division rate hormonally instituted is thus seen as drifting upwards to reach a 'normal' physiological division rate prescribed, perhaps, by genetic constitution. The simplest way of viewing the situation is, of course, the reverse, that the hormonal effects noted represent an active stimulation. However, there is some justification for the viewpoint that the maximum division rate, under any defined conditions of substrate availability, cellular integrity, temperature, etc. is at least the mechanically 'normal' condition. Any reduction in rate concomitant with further differentiation or tissue formation must be imposed in some way. In this view, the molecular syntheses and reorganization underlying division must proceed at optimal rate under optimal conditions. Inhibition is possible, and a cessation of inhibition, but not stimulation.

Such an inhibition can be imposed in myriads of ways, including regulation of the availability of substrates, repression of the genome, or alterations in the structure and function of the membrane systems. One clear demonstration of such an inhibition in invertebrate gonadal systems exists. The lugworm, *Arenicola marina*, exhibits a low rate of spermatogonial mitosis in the presence of its own spermatocytes. If these are removed experimentally, or through periodic gamete shedding, the spermatogonial mitotic index increases considerably. Similarly, if the testes are cultured <u>in vitro</u>, dilution of this endogenous inhibitor results in an increased MI. The more differentiated elements in the cell lineage are thus producing an inhibitor which limits the rate of cell division and hence the rate of growth of the spermatocyte compartment; a classic example of negative feedback (Olive, 1972a & b).

An hypothetical schema can therefore be envisaged for the insect testis which, incorporating these last two highly speculative components, postulates the action of ecdysone as permitting the access of an anti-inhibitor, MF, to a self-regulated internal hormonal milieu. Juvenile hormone, as a repressor of RNA and protein synthesis in the haemocyte (Berry et al, 1964) might be involved in this system as a specific inhibitor of MF synthesis. The complex, selectively permeable testis sheath and the potential recycling current earlier described, might function to retain adequate concentrations of the endogenous inhibitor as well as limiting access of the anti-inhibitor.

The only thoroughly unfamiliar and experimentally unsupported concept in this schema is the endogenous inhibitor, which may seem a needless complication. However, the existence of such a molecule would confer a self-regulatory function on the developing gonad, already known to be self-differentiating. The role of the morphogenetic hormones could be construed as providing information regarding the total developmental pattern of the insect to the autonomous testis; advance warning of oncoming imaginal differentiation. The somewhat anomolous, non-classical effects of these hormones as measured by changes in the germ cells, could thus be seen due to these germ cells not being the target tissue directly: the mesodermal support structures which provide testicular isolation, and haemolymph components synthesizing mitogenic messengers being the true target of morphogenetic hormone activity. On the basis of the evidence presented such a schema must be regarded as yet somewhat fanciful, but it is a compelling fantasy, and moreover one for which considerable evidence has recently been compiled in vertebrate proliferative tissue systems, the topic of the next section.

## Chapter Fourteen: Discussion; Chalones

The principle of negative feedback inhibition is not new, nor is the concept of local tissue induction, or inhibitive interaction. Numerous examples of these phenomena continually appear and re-appear in the biological literature. The specific form of one such system governing mammalian epidermal growth and repair began to take shape in the early nineteen sixties, and was termed the chalone concept (see review by Bullough, 1967).

This concept proposes the existence of a mitotic inhibitor within the organized tissue, produced by the more differentiated cellular elements and exerting its major effect on the less differentiated or stem cell elements in the cell lineage. The inhibitor is not cytotoxic, is reversible in action, and accomplishes a limitation in rate rather than a complete block in division. The chalone is highly specific to the tissue, but not to the species of origin (Bullough, 1967).

Over the succeeding ten years, endogenous mitotic inhibitors fitting this description have been described for a large number of tissues, chiefly mammalian but including codfish epithelium, (Bullough et al., 1967), and *Xenopus* kidney (Chopra and Simnett, 1969). Very recently, a spermatogonial chalone has been described, affecting early, uncommitted spermatogonia in the rat seminiferous epithelium (Clermont and Mauger, 1974). Attempts at purification have been somewhat less successful. Results so far have demonstrated activity in two proteinaceous fractions of approximately 3 to 5 x $10^4$ and 1 to 3 x $10^3$ daltons respectively (Marks, 1971; Maugh, 1972). Speculation exists whether these classes of molecules might not represent the observed inhibition at different points in the cell cycle.

A very significant finding of direct relevance to the chalone concept is the anti-chalone, or mitogen. Recently, Houck and Cheng (1973) have demonstrated such a mitogen in mammalian sera from four species, and shown it necessary for continued mitoses in human fibroblast culture. This factor has been purified, and identified as a sialoprotein of molecular weight of 12 x $10^4$ daltons. All the mitogenic activity of mammalian sera on fibroblast culture is contained in this fraction. If mixtures of partially purified fibroblast chalone and the sialoprotein mitogen are subjected to ultrafiltration, essentially all of the chalone is recovered. The authors conclude that the demonstrated competitive effect between chalone and anti-chalone in the fibroblast culture system likely resides in a reactive site competition on the fibroblast membrane (Houck, Sharma, and Cheng, 1973). Houck further speculates, on the basis of as yet incomplete evidence, that the initial action of chalones is in reducing $Ca^{++}$ permeability with subsequent effects on c-AMP mediated inhibition of DNA synthesis (Houck, 1973). Laurence et al (1972) and Moreau and Bullough (1972) define a somewhat similar anti-chalone operating in the epidermal cell system, but point out additional complexities such as highly site-specific activities in this organized tissue.

In addition, Laurence et al (1972) postulate a mechanism for the anomalous effects of the hormones adrenalin and hydrocortisone in the in vitro system. It is known that the hormones are inactive by themselves in mitotic inhibition (Bullough and Laurence, 1968). Such hormones considerably strengthen the inhibitory activities of chalones, however, and the authors suggest, from experimental analyses, that adrenalin blocks or destroys anti-chalone, and that hydrocortisone inhibits its synthesis. An interesting correlation which is presented in this work is that the diurnal rhythm of epidermal mitosis is thus due to the fluctuating adrenalin concentration, accounting for the absence of such a rhythm in adrenalectomized animals (Bullough and Laurence, 1966).

The net effect of a chalone in any given complex cellular system is imperfectly understood at present. The precise point of inhibition within the cell cycle will determine in part the nature of measured effects. A view which is gaining support is that the several defined points of inhibition of chalones, $G_1$ - S transition, but not S duration; $G_2$ - M transition, hence $G_2$ duration, may lead to premature differentiation of cells from $G_2$ at least, and possibly from $G_1$ (Frankfürt, 1971). Moreau and Laurence further identify a dichophase, immediately following division at which the 'decision' to differentiate and mature, or re-enter the proliferative pool is taken by the cell, and suggest a chalone influence here as well. These authors thus suggest that the observed changes in cell cycle duration are secondary to more subtle alterations in the direction of differentiation (Bullough and Rytömaa, 1965; Kiviaakso and Rytömaa, 1971).

While these concepts are not universally accepted (Voorhees, 1974), the case presented is constantly gaining ground. Parallels with the previously described hypothetical testicular system in insects are many, though perhaps the most important, a demonstration of an endogenous mitotic inhibitor in that system, is lacking at present. Close similarities between MF and anti-chalone, and between the inhibition potentiation of adrenalin and hydrocortisone, and the inhibitory effect of juvenile hormone are particularly striking. As mentioned previously, the apparent recycling current in the insect vas efferens is well-characterized in mammals (Waites and Setchell, 1969) and is suggested to act to concentrate chalone (inhibin) (Setchell and Sirinathsinghji, 1972). A final point of interest is the suggestion that rapidly proliferating cells may respond to chalone blockage by death (Frankfürt, 1971), perhaps as a result of the premature differentiation effects described above. Either or both of the insect testis cell death phenomena, pycnosis or site-specific autolysis, might have a basis in this as yet poorly-characterized chalone function.

# Appendix One: Experimental Stock, Surgical and Hormonal Procedures

## Experimental Stock

The insects used in this study were taken from the colony of Rhodnius prolixus which has been maintained at the Institute of Parasitology for approximately ten years. Very little maintenance is required in the breeding and raising of Rhodnius. The bugs are kept on folded filter paper, thirty to fifty insects per one pound mason jar, fitted with screened lids. These jars are kept in an incubator at twenty-eight degrees C. and a high relative humidity is provided by placing an open jar of water in front of the heater/fan of the incubator. These incubators do not have lighting units, and thus the bugs are kept in continuous darkness except when the door to the incubator is opened. No particular emphasis has been placed on the regularity or timing of these light exposures and the insects might be expected to lack any strong circadian rhymicity. Nonetheless, certain events, such as ecdysis, seem to occur with much greater frequency from about six to eight o'clock in the morning. Several possible explanations for this apparent rhymicity can be suggested. The insects are often first exposed to light at about eight in the morning, at the start of each working day. Daily temperature cycles probably exist within the incubator. Finally, the evening and early morning hours generally represent a period of minimum disturbance for the insects, and the apparent circadian activity peak might thus be simply induced by the experimentor each day.

Feeding is carried out on immobilized rabbits. The procedure takes approximately twenty minutes per jar, and the filter paper and jar are changed shortly after each feed. This is necessary to prevent the insects drowning in the copious urine produced immediately post-feed and to prevent the growth of fungus on the filter paper. No rigorous record was kept on any specific group of insects; however, feeding and moulting success is very high, certainly over ninety-five percent in any stadium. Under these conditions of culture, the morphogenetic moult from fifth instar to adult takes twenty ± one day from the blood meal, with no evidence of difference in duration between the sexes. The moult from fourth to fifth instar takes twelve ± one day from the blood meal, and earlier moults are accomplished in ten or eleven days.

Breeding adults were selected from large and vigorous members of the colony, and fed at about two week intervals. At this time, the eggs are removed to clean mason jars for hatching. First instar insects which have undergone eclosion over a period of two to three days were isolated and carried through to maturity together, with blood meals approximately three weeks apart. For experimental purposes, a group would be selected which had ecdysed within an eight hour period at the last moult. These insects will have had identical feeding and moulting histories. Very small, and very large individuals were removed from such a population prior to experimentation. These groups are referred to in the text as sibling populations to emphasize their uniformity; they are not, however, necessarily true siblings.

## Surgical Procedures

The various surgical procedures performed were carried out without anaesthesia. Such techniques may result in, for instance, activation of the corpora allata (Cassier and Fain-Maurel, 1970), possibly as a result of irreversible damage to inhibitory neurons (Hafemann, 1969). Etherization and nitrous oxide also cause irreversible nerve damage (Krishnakumaran, 1972). Instead, the insects were fastened to the bench top with strips of cellulose tape. Surgical equipment consisting entirely of jeweler's forceps, electrolytically sharpened tungsten probes, and microscalpels made of razor blade shards were rinsed as necessary in seventy percent ethanol. No antibiotic preparations were employed. Aside from the case where injury to the gut liberates <u>Nocardia</u> <u>rhodnii</u>, the normal actinomyete gut symbiont of Rhodnius, no incidence of infection occurred. Except in the case of ligature of the midgut, more fully described in the text (Chapter Four), no ringer or saline solutions were employed. All procedures were carried out under the binocular microscope.

The majority of surgical procedures involved the removal of endocrine organs from the head capsule. In these cases, the insect was taped down with the head extended over a voke made from a capillary tube. The tip of the rostrum was taped down as well; in this manner the relevant area of the head capsule was well exposed by the slight flexing and extension of the head, and damage to the antennae and mouthparts was avoided. A small cap of cuticle was removed with the razor blade, and retained. In the case of removal of the corpus allatum or the corpus cardiacum, this cut would extend from immediately anterior to the cervical membrane, to the area of ocellar pigmentation. For removal of the neurosecretory cells of the pars intercerebralis, a more anterior incision was employed. The organ involved was most effectively removed with two hooked probes. Allatectomy was largely complete, with some cardiacum inevitably removed as well; total removal requires a bisection of the corpus cardiacum. Removal of the neurosecretory cells was always partial, and, indeed, some of these insects would moult after five or six blood meals over a period of many months. In no case was any development noted in these insects after one blood meal, however.

The cuticular cap was replaced following removal of the endocrine organ and the wound sealed with moulten Tackiwax (Cenco Ltd.). This procedure was apparently the most damaging aspect of the surgical technique and great care was required to avoid over-heating of the insect's nervous system. Touching the wax-melting probe to various areas of the insect's undamaged cuticle indicated that the quivering and motor collapse characteristic of this damage was probably a result of neural damage to the important locomotor systems of the suboesophageal ganglion rather than to the 'brain', the supraoesophageal ganglion. Best results in this procedure were thus obtained on days when the room temperature was relatively low, or when a breeze could be induced to flow over the preparation.

Control procedures in the case of allatectomy involved removing one bright red salivary gland reservoir through the same type of incision as in the allatectomized individuals. This process occurs accidentally in a small proportion of the operations, presumably as a result of haemocoele pressure and, surprisingly, does not seem to interfere with gorging even when both reservoirs were removed. In the case of neurosecretory cell removal, controls were produced by tearing the membrane overlying the brain. Following the surgical procedures, the insects in all cases were placed in an incubator at thirteen degrees C. for forty-eight hours. Such a technique is thought to prevent massive diuresis and dessication brought about by a stress-induced release of neurosecretion. The insects were then removed to the incubator at twenty-eight degrees C. for a minimum of eight days to permit the decay of wound-induced metabolic stimulation (Okasha, 1970) and the repair and regeneration of neurohaemal tissue. Insects subjected to these techniques behaved in a relatively normal manner, with the exception that allatectomized animals, although capable of vigorous locomotor activity, tended to show reduced spontaneous movement. The normal insect will attempt to avoid capture, for instance, but the allatectomized insect would usually remain motionless until actually touched or prodded. It would thereafter respond in a normal and well co-ordinated manner.

Similar procedures were employed for parabiosis and decapitation. In the case of fed, decapitated insects, only those insects which could be decapitated between twenty-three and twenty-five hours were used.

In this way, a reasonably consistent exposure to endogenous ecdysone secretion was approximated.

## Hormonal Procedures

Information regarding effective doses of juvenile hormone analogues and ecdysone for some Rhodnius larvae are available in the literature (Wigglesworth, 1969, 1970). To provide information permitting similar doses in several instars, and to permit comparison of dose levels with other insect preparations, three procedures were carried out: Fed and unfed wet weights; Inulin space (blood volume); and response to β -ecdysone injection.

## Wet Weight

Groups of ten fourth instar, fifth instar males, and fifth instar females were weighed individually prior to feeding, within one half hour post-feed and twenty-four hours post-feed. The following average values were obtained:

| INSTAR | UNFED | 30 MIN.P.F. | 24 HRS.P.F. |
|---|---|---|---|
| Fourth | 15 ± 3 mg. | 116 ±24 mg. | 75 ±13 mg. |
| Fifth Male | 33 ± 6 mg. | 293 ±45 mg. | 173 ±20 mg. |
| Fifth Female | 39 ± 8 mg. | 329 ±69 mg. | 186 ±39 mg. |

It can be seen that both fourth and fifth instar bugs take a blood meal which, following diuresis, amounts to four times the previous body weight. The size disparity between fourth and fifth instar insects, both fed and unfed, is 1 : 2.4. Figures obtained at thirty minutes post-feed are more variable, probably because some individuals have not yet completed the initial diuresis.

Response to Ecdysone

Wigglesworth states that the dose of ecdysone necessary to induce moulting in the fourth instar insect is about one microgram (Wigglesworth, 1970). A dose at this level produced very rapid apolysis in the insects from our colony, and since the phenomenon of hyperecdysonism was a potentially serious problem in the colchicine mitotic accumulation experiments, an injection series was prepared to define the dose more closely.

A population of fourth instar Rhodnius larvae were subjected to allatectomy, and removal of the corpus cardiacum and neurosecretory cells of the pars intercerebralis, as previously described. On feeding, thirty-five fully gorged individuals were obtained and these were randomly assigned to one of six groups. Three of the groups received topical applications of farnesyl methyl ether (FME), 0.5 nanoliters in .0.5 microliters of iso-octane. The remaining three received applications of iso-octane only. The groups were then injected through the last thoracic leg with 0.1, 0.5, or 1.0 micrograms of $\beta$-ecdysone (Rohto Pharmaceuticals, Osaka, Japan) in 0.5 microliters of distilled water. Injections were carried out with a five microliter Hamilton syringe fitted with a thirty-one gauge needle, and the injection site was sealed with molten tackiwax. The insects were returned to the incubator at twenty-eight degrees C., and observed daily to assess the onset of ecdysis. Results can be best appreciated from the histogram (fig.103) of the percentage of each sample versus the day of ecdysis. It can be seen that a hyperecdysone effect is well-marked at one microgram $\beta$-ecdysone dose level. The 0.5 microgram dose level results in a slight decrease in moult cycle duration over that of a normal fed fourth instar (twelve ± one day). 0.1 microgram resulted in a lengthy moulting span, and many insects in this sample died without moulting. Applications of FME were carried out since this procedure would be used in the colchicine-squash technique, and a possibility existed that a direct prothoracotropic stimulation would result. No such trend is noted in the histogram, possibly due to the small sample, or to the overriding significance of the simultaneous ecdysone injection. On the basis of this experiment, a rough physiological dose of 0.5 micrograms of $\beta$-ecdysone was estimated for a fed ourth instar bug. This is about 6.7 micrograms per gram wet weight of bug, but it should be kept in mind that four-fifths of this weight is undigested blood.

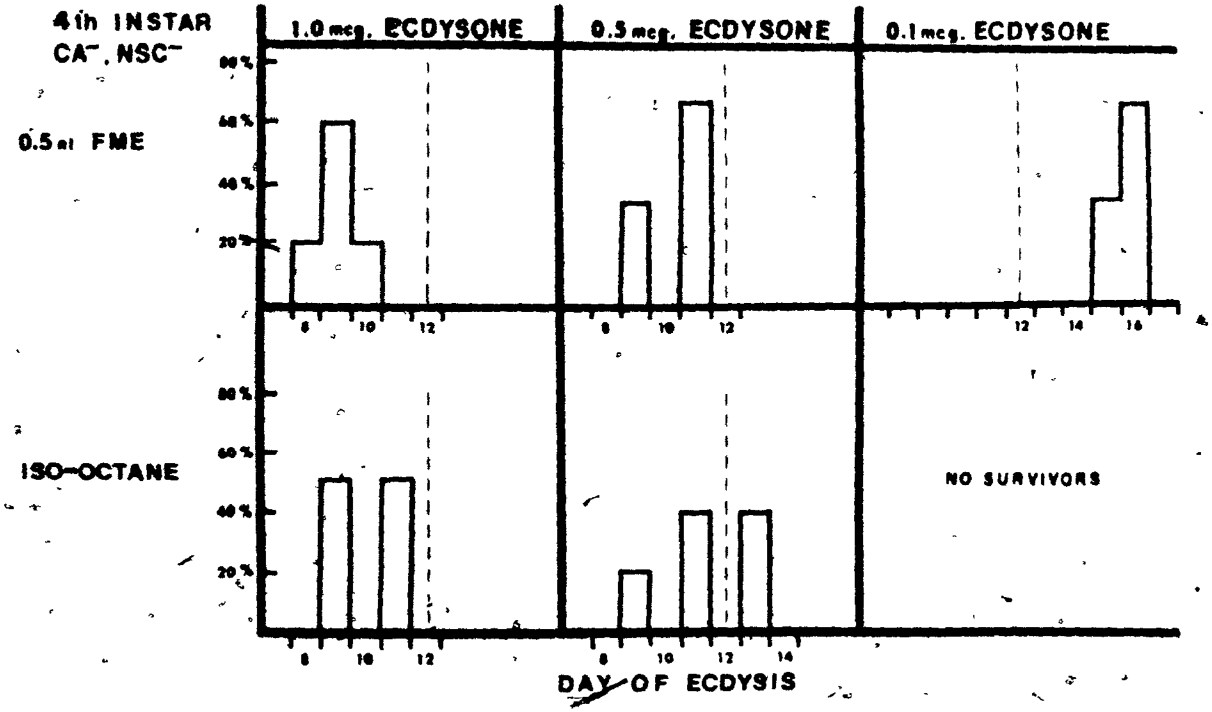

FIGURE 103 Effect of β-Ecdysone dose and FME on moult duration. X axis represents insects ecdysing on a single day. Sample sizes: FME, high dose ecdysone- 5; mid-dose-6; low dose- 3 . Iso-octane, high dose ecdysone- 4; mid dose - 5.

## Juvenile Hormone Analogue

The juvenile hormone analogue used in the above experiment and in all subsequent experiments was farnesyl methyl ether (FME) a gift of Hoffmann-LaRoche Pharmaceuticals, Basel. The naturally-occurring juvenile hormone of Rhodnius is not known. Choice of this particular analogue is partly simply a matter of availability; however, aside from species-specificity, the action of any given analogue seems remarkably similar to that of any other analogue (Schneiderman et al, 1965). FME, while not specific to Rhodnius, is highly active. FME differs from most analogues in that a single topical application provides maximal juvenilizing activity. It is thought that this effect results from a slow rate of penetration of the cuticle, and/or a slow rate of metabolism and excretion (Wigglesworth, 1973 ; Patterson, 1973). This molecule has been demonstrated to be non-cytotoxic in contrast to the extracted and concentrated natural Cecropia oil (Chihara et al, 1972). In the fed fifth instar, topical application of 1.0 and 2.5 nonoliters gives an approximate dose of 5.0 and 12.5 micrograms per gram wet weight. The actual amount of hormone reaching the target tissue is presumably considerably less than this figure. 0.5 nanoliters per fed fourth instar similarly corresponds to 6 micrograms per gram. All solutions were prepared in iso-octane, and stored at -20°C.

# Appendix Two: Histology

A variety of standard histological techniques were employed in this study. The nature and justification of the technique used for each purpose will be briefly mentioned here, with a more complete account of unusual or intricate techniques. Except as otherwise noted, the testis was dissected free of tracheal and fat body connections, and the terminal filament and vas deferens cut while bathed in insect ringer and then removed to fixative. The composition of this Ringer's solution (Davey, 1958) is:

| | |
|---|---|
| NaCl | 7.5 g |
| KCl | 0.1 g |
| $CaCl_2$ (anhyd.) | 0.2 g |
| $NaHCO_3$ | 0.2 g |
| Distilled Water | 1.0 l |

Initial morphological studies were carried out by fixation and staining with osmium-ethyl gallate, as described by Wigglesworth (1957). The tissue so prepared was infiltrated, oriented, and embedded in five percent agar. These blocks were dehydrated in an ethanol/cellosolve (=ethylene glycol monoethyl ether) series to pure cellosolve, and infiltrated and embedded in Ester wax (Wigglesworth, 1959). 0.5 to 1.0 micron sections were obtained from such preparations on the Cambridge Instrument Co. rocking microtome, and mounted in Farant's medium (Microtomist's Vade Mecum, 1937).

Using this technique, excellent micro-anatomical detail could be visualized under phase contrast optics and with objective and condenser oil immersion.

An appreciation of the progression of cytologic events in the linear tubule (Chapter Five) was provided by the following technique: The insect was dissected under Ringer's solution, and the testis removed together with a drop of Ringer's solution to a small petri dish. Under the binocular microscope, the external sheath, trachea and fat body were removed, and the testicular tubules arranged flat, and free of one another. The Ringer's solution was carefully removed from the preparation, and the testis tubules gently bathed in Lebrun's modification of Carnoy's fixative (Microtomist's Vade Mecum, 1937), for fifteen minutes. Owing, perhaps, to the anhydrous nature of this fixative, a firm bond was produced between the tissue and the glass, permitting washing in absolute ethanol, clearing and infiltration in 56.5°C. Tissuemat (Fisher Scientific Co., Montreal) to be carried out in the petri dish without disturbing the tissue. The final ethanol rinse contained 0.1 percent spirit soluble eosin, greatly aiding the subsequent orientation of the tissue in the wax block. Moulds for the production of wax blocks were prepared from rectangular iron bars, surrounded at the anterior by a cup of cellulose tape. Such moulds were heated on the hot plate, and molten wax poured into the tape cup. The infiltrated tubules were gently pried loose from the petri dish with a razor blade chip, transferred to the iron bar-cellulose tape mould, oriented under the binocular microscope, and chilled by plunging the base in ice water. Blocks were hardened for several days at room temperature, and serial sections cut on the rocking microtome at four to five microns in thickness. Wax was removed and the sections hydrated in the normal manner, and the series was stained for forty-five seconds in Hansen's Iron Trioxyhaematin (Pantin, 1946). The stain solution was kept frozen when not in use, and $H_2SO_4$ was not considered necessary to reduce cytoplasmic staining. The sections were 'blued' in tap water for twenty minutes, dehydrated, cleared and mounted in Permount (Fisher Scientific Co., Montreal). Standard transmission microscopy was then employed. This technique, while somewhat disruptive in fourth and earlier instars, provided excellent cytological detail of spermatocyte accumulation in the fifth instar.

More standard techniques were employed in some other experiments. In estimating the effects of ecdysone on autolysis (Chapter Four) and the degree of development of allatectomized precocious adults (Chapter Ten), the testes were removed in Ringer's solution, fixed in alcoholic Bouin's (= Duboscq-Brasil, Pantin, 1946), sectioned without prior dissection, and stained with Hansen's Iron Trioxyhaematin as before. This schedule allowed for the accumulation of testes in fixative from the somewhat randomly moulting precocious adults, and from variously timed ecdysone exposures. Minimum fixation time in any case was forty-eight hours. Several days' washing in seventy percent ethanol was required to remove the picric acid from these preparations.

The demonstration of whole, desheathed fourth instar, fifth instar, and adult testes was carried out as if for flat embedding, but on a cover glass rather than directly in the petri dish. Following over-staining with spirit soluble eosin, the preparations were cleared and mounted directly in Permount, and photographed on a lighted background.

Whole mounts of first instar testes and ovaries were prepared in the following manner: The gonad was dissected free of attachments in Ringer's solution, and transferred to a depression slide containing Karnovsky's glutaraldehyde fixative (Karnovsky, 1965) for fifteen minutes. The gonads were transferred through three changes of 0.1 M sodium cacodylate buffer, thence to a diluted (1 : 3) mixture of Hansen's Iron Trioxyhaematin in distilled water. The progress of staining was followed under the binocular microscope, and the organ removed at the appropriate time. Following 'blueing' in tap water, the gonads were mounted directly in Zeiss aqueous phase contrast medium (Carl Zeiss, Oberkochen), a cover slip applied, and the material viewed in the phase contrast microscope.

Semi-thin sections of testes from young males were prepared in the following manner: For the first instar, the gonad was dissected as before in Ringer's solution and then placed in the fixative. For the third instar testes, however, the insects were placed on cellulose tape over an ice-filled petri dish. The dorsum was removed, body cavity flooded with ice-cold Karnovsky's glutaraldehyde, and the testis quickly removed to fresh Karnovsky's fixative (1965) at 4°C. The recipe for this fixative is:

Solution A

| | |
|---|---|
| Distilled water, plus 2 or 3 drops 1N NaOH | 25.0 ml |
| Paraformaldehyde | 0.5 g |

heat to dissolve, then cool in refrigerator

Solution B

| | |
|---|---|
| Sodium cacodylate buffer, 0.2M | 22.0 ml |
| Glutaraldehyde, 50% | 3.0 ml |
| Acrolein | 0.5 ml |
| $CaCl_2$ | 25.0 mg |

Cool solution in the refrigerator

Add Solution A to Solution B, keeping cold. The solution is then shaken with activated charcoal, and filtered in the cold. This fixative should be used within one or two weeks. During filtration, the solutions should be packed in ice and kept in the fume hood, owing to the exceptionally noxious acrolein fumes.

Following one hour fixation at 4°C., the organs were washed in three hourly changes of 0.1M sodium cacodylate, and post-fixed for one hour in 0.1% osmium tetroxide in 0.2M Sorenson's phosphate at 4°C., adjusted to a pH of 7.4. The testes were then rapidly dehydrated through seventy, eighty, ninety, and ninety-five percent ethanol, and two fifteen minute changes of absolute ethanol, all at 4°C. The testes were then transferred to fresh absolute ethanol, allowed to reach room temperature, and the alcohol bath changed once more in the course of two hours. The tissue was taken through a fifteen minute change of propylene oxide/absolute ethanol (1:1) and two fifteen minute changes of propylene oxide. Next, the tissue was transferred to a 1:1 mixture of epon/araldite (Anderson and Ellis, 1965) and propylene Oxide, and kept uncapped in the refrigerator overnight. The mixture was brought to room temperature on the following day, and embedded in fresh epon/araldite. This fixation and embedding routine is that devised by Huebner and Anderson (1972).

Following polymerization of the blocks, semi-thin (one micron) sections were cut on the LKB ultramicrotome. Sections were stained in 1% Toluidine blue in 1% borax (Huebner and Anderson, 1972) or the basic fuchsin and methylene blue polychrome stain described by Sato and Shamoto (1973). Viewing was accomplished with phase contrast and oil immersion.

In an attempt to define optimal fixation and staining routines, a number of combinations were also attempted, including Baker's formol calcium (Pantin, 1946), Davidson's fluid,and standard Carnoy's fixative (Microtomist's Vade Mecum, 1937). Staining techniques employed include Hubschman's Azan (Hubschman, 1962), Heidenhain's Azan (Mallory, 1938), Paraldehyde fuchsin (Meola, 1970), Schiff's reagent (de Tomasi method, Pearse, 1960), Picric Acid Schiff/Methylene blue (Green, 1970), Methyl Green/Pyronin Y (Ahlqvist, 1972), and Biebrich's Scarlet/Fast Green (Beckert and Garher, 1966). Results obtained with Hansen's iron trioxyhaematin were generally superior to any of these more complex mixtures.

For the demonstration of acid phosphatase activity (Chapter Three), the basic technique employed was that of Barka (1960; Barka and Anderson, 1965). The incubation medium was composed of sodium alpha-napthyl acid phosphate in Michaelis' veronal acetate buffer. To this was added hexazonium salt freshly prepared from equal volumes of four percent pararosaniline hydrochloride (Brickman Chemicals, Montreal) in 2N HCl and four percent sodium nitrate. Following the advice of Cone and Eschenberg (1966), the medium was adjusted to a pH of 6.0 prior to filtration and incubation at room temperature. Controls were carried out in the absence of sodium alpha-napthyl acid phosphate (substrate) and in the presence of 0.01M sodium fluoride. Either technique resulted in a complete absence of the characteristic bright red reaction product.

A number of fixation schedules were tried to provide optimal morphological detail. Karnovsky's glutaraldehyde without acrolein was finally adopted for routine use, buffered with 0.2M sucrose. In the absence of sucrose, developing testes from fed insects would swell, and burst the testicular sheath. The amorphous material found between the tunica interna and externa of fed insects is likely the agent of this phenomenon. Testes were dissected free in fixative and transferred to fresh fixative at four degrees C. for one half hour. They were then washed in three half-hour rinses of 0.1M sodium cacodylate and sucrose at four degrees C., brought to room temperature, and infiltrated for one hour in gelatine at thirty-seven degrees C. (Pearse, 1960). The infiltration mixture was cooled, cut in blocks, and hardened in forty percent formalin at room temperature for one hour. Following one hour wash in running tap water, the blocks were oriented in Tissuetek (Ames Co., Elkhart, Indiana) frozen to -20°C., and eight micron serial sections were cut on the Ames Lab-Tek cryostat microtome. Sections were mounted on subbed slides (Humason, 1962), permitted to dry for one half hour, and incubated at room temperature for one half hour. The sections were rinsed briefly in distilled water, dehydrated rapidly in ethanol and mounted through xylene in Permount. Various counter-stains were tried, but results were undesirable.

## Cytological Techniques

Confirmation of cytology defined by longitudinal sectioning of the testis tubule, fixed in Lebrun's Carnoy and stained with Hansen's Iron Trioxyhaematin, and quantitative colchicine metaphase accumulations were carried out using the following techniques: Initially, the testis or relevant portion of the tubule was removed under Ringer's and placed in a drop of aceto-carmine (Microtonist's Vade Mecum, 1937), on a cleaned slide. The drop of stain and tubule were warmed slightly over a gas flame, a coverslip applied and the squash carried out by thumb pressure. The coverslip was then ringed with clear nail polish. Overheating led to the loss of many preparations, and insufficient pressure often resulted in ambiguous chromosomal spreads.

The following modifications were suggested by G. Fontana, Department of Entomology, MacDonald College. The testes were dissected in one percent sodium citrate solution, and transferred to fresH sodium citrate solution at room temperature for ten to fifteen minutes. The testes or tubules were then transferred to a clean microscope slide, drained, and a drop of aceto-orcein solution added. This mixture was permitted to remain for two to three minutes, a coverslip applied, and squash produced by tapping gently with a rubber-tipped iron rod. The coverslip was then ringed with clear nail polish, and the preparation heated overnight at 40°C. Aceto-orcein solution was prepared in the following manner:

| | |
|---|---|
| Distilled water | 20.0 ml |
| Glacial Acetic Acid | 20.0 ml |
| Mix and boil gently for a few minutes | |
| Add: Orcein (synthetic, Gurr) | 1.0 g |
| Boil gently for a few more minutes | |
| Add: Glacial Acetic Acid | 10.0 ml |
| Glycerine | 6 drops |
| Filter. May be diluted with additional distilled water. | |

## Colchicine Metaphase Accumulation

The techniques for investigating changes in the mitotic index subsequent to hormonal treatment evolved during the course of the experiments, as indicated in the text (Chapter Nine). The initial experiment was carried out using the hot aceto-carmine technique. Slides were not blinded, and some difficulties were experienced in distinguishing between insufficently spread 'ball' mitoses and pycnotic nuclei. As the changes in mitotic index were of considerable importance in the investigation of the kinetic hypothesis, modifications in the above procedure were introduced to ensure a high level of validity and confidence.

As previously discussed, (Chapter Nine), the testis at the selected time in the fourth instar contains only spermatogonial cells with an attendant sheath of various mesodermal cells. Prior to incubation in sodium citrate solution, the testis was divested of the tunica externa, thus reducing the presence of mesodermal cells to those lining the tubules, cyst wall cells, and a few vas efferens and plug cells. The proportion of mesodermal cells is thus very small in relation to the germ cells, and no effort was made to separate the cell types when counting cells or metaphase plates.

The germ cell cysts increase in cell number in an exponential fashion; hence the great proportion of cells will be those of the most advanced cysts. The final three categories of germ cell cysts would be expected to represent eighty-seven and a half percent of the total germ cell complement. This has advantages and disadvantages. It permits the experimenter to ignore the problem of a progressive and regular change in cell cycle duration during the course of germ cell differentiation. However, the statistical validity of the mitotic index within any given field in the squash may be reduced by the synchronous development of the large cysts. In fact, the resultant squash preparations are not at all homogeneous in frequency of division figures, reflecting this condition. Fortunately, the testis at this time is of such a size that the entire cellular complement is displayed on a single slide. The technique adopted to provide a complete and random sampling of the germ cell mitotic index was to focus at forty power on the top left margin of the squash, and proceed in short steps horizontally through the squash, then move vertically, and proceed horizontally once again but in the opposite direction. This process was continued until no more cells were encountered. Twenty-five to fifty samples were thus taken on each testis, comprising between two and four thousand cells. Since these preparations were not particularly homogeneous as to cell density either, all cells within the mm$^2$ ocular grid were counted for each sampling, and metaphase figures counted to provide, in effect, a separate mitotic index for each field. Total cells were compared to total metaphase plates to provide an average mitotic index for the entire testis. Although all preparations after the first experiment were carried out by the improved cold aceto-orcein method, it still occurred that insufficient pressure would result in ambiguous chromosome figures. Such preparations were discarded. All the slides were blinded with opaque white tape, shuffled and numbered randomly prior to counting. These techniques are extremely time-consuming; so much so that the sample size of the experiments are limited by the four to five day usable life of the squash preparations. Nonetheless, the author believes that the high level of validity of the individual mitotic indices so produced outweighs the disadvantages of small sample size.

Colchicine injections were carried out in the same fashion as the ecdysone injections, through the thoracic leg. Volume and concentration eventually adopted was two microliters of $10^{-3}$M solution in distilled water. An exposure time of twenty-four hours was used in several of the experiments. Concentrations and durations used in earlier experiments should not be considered invalid, merely non-optimal. Failure of the colchicine to block mitosis completely resulted in the appearance of anaphase and telophase figures, and such preparations were discarded. An interesting occurrence is the paralysis of insects which occurred some time after the injection. The onset of paralysis was much earlier, and the fraction of the population affected much greater in those groups receiving ecdysone injections or in normal fed insects.

The final cytological technique employed was the air-dried aceto-lactic orcein technique described by Crozier (1968). Excellent permanent preparations with a wealth of cytological detail were obtained in this manner. However, very considerable loss of cells occurs, and the preparations are thus not suited for quantitative studies.

## Appendix Three

Inulin Space:

| | BLOOD VOLUME IN µl | | | | |
|---|---|---|---|---|---|
| SAMPLES | 1 | 2 | 3 | 4 | 5 |
| 5th MALE UNFED | 11.0 | 8.8 | 14.2 | 5.0 | 12.6 |
| 5th MALE FED | 38.5 | 33.7 | 76.5 | 33.7 | |
| 5th FEMALE UNFED | 15.5 | 15.4 | 11.3 | 8.8 | 7.0 |
| 5th FEMALE FED | 42.0 | 38.0 | 38.5 | 49.5 | |
| ADULT MALE UNFED | 23.0 | 27.5 | 29.0 | 30.0 | |
| ADULT MALE FED | 29.5 | 29.5 | 29.5 | 46.5 | |
| ADULT FEMALE UNFED | 26.6 | 31.7 | 26.2 | 23.5 | 39.5 |
| ADULT FEMALE FED | 41.2 | 49.0 | 37.0 | 44.5 | 32.5 |

Figure 104. $^{14}$C-Inulin space (≃ haemolymph volume). All insects sampled (unfed) at 10 days post ecdysis, or fed at 10 days, sampled 48 hours post feed. 2 µl $^{14}$C-Inulin injected, 1 or 2 µl haemolymph taken at one hour. Volume calculated from the method adapted by Tobe (1972), from the modified relationship:

$$V_o = \left(\frac{A_i}{A_s} : V_s\right) - V_i$$

$A_i$ = amount Inulin injected (in dpm's)
$A_s$ = amount Inulin in sample ( in dpm's)
$V_o$ = haemolymph volume
$V_i$ = volume of injection
$V_s$ = volume of sample

## 'Critical Period' for Juvenile Hormone Action:

As more fully explained in the text (Chapter 11), an experiment was carried out to define the effective period of juvenile hormone analogue activity following the blood meal. In this experiment, groups of fifth instar insects were treated with a single application of FME at one to fourteen days post feed. The insects were permitted to ecdyse, and the duration of the moulting cycle and degree of larvalization were noted. These results are presented in the following:

Figure 105. Day of Application (2.5 µl FME) vs. Day of Ecdysis.

Figure 106. Day of Application (1.0 µl FME) vs. Day of Ecdysis.

Figure 107. Day of Ecdysis vs. Degree of Larvalization. (Histogram, Figure 99, taken from this data)

Figure 108. Graph of Degree of Larvalization vs. Day of Application (2.5 µl FME).

Figure 109. Same as Figure 108 but 1.0 µl FME

Results are essentially those reported by Barrett (1972), except that insects which required longer than a normal moulting cycle to complete ecdysis tended to be highly juvenilized. The average values of larvalization (solid circles) thus tended to remain high (4-8) at any given day of application. The data representing insects treated after the 5 day critical period can be resolved into two distinct groups, and the average of the highly larvalized group (starred circles) and the adult (open circles) seperately presented. The line chosen in each graph (Figs. 108, 109) follows the more adultoid group.

The more larvalized insects possibly reflect the direct inhibition of ecdysis reported by Hangartner and Masner (1973) after the application of high doses of FME.

Figure 105

| DAY OF APPLICATION (POST FEED) DOSE (2.5 nl FME) | DAY OF ECDYSIS (POST FEED) | | | | | | | | | | | | | | AVG. DEGREE LARVALIZ'TN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | |
| 0 | | | | | | 2 | 1 | 4 | 6 | | 2 | | | | 0 |
| 1 | 1 | 5 | 2 | | | | | | 1 | 1 | | | | | 18.8 |
| 2 | | 3 | 5 | 1 | | | 1 | | | | | | | | 16.6 |
| 3 | | 1 | 2 | 1 | | | 1 | | | | 1 | 1 | | | 15.7 |
| 4 | | | | 7 | 5 | 1 | | | | | | | | | 16.3 |
| 5 | | | | | 5 | | 2 | 1 | | | | | | 1 | 7.1 |
| 6 | | | | | 4 | 3 | 1 | | | | | | | | 4.4 |
| 7 | | | | | 6 | 2 | 2 | | | | | | | | 4.0 |
| 8 | | | | | | 4 | 3 | | | | | | | 1 | 3.5 |
| 9 | | | | | | | 5 | 2 | 1 | 2 | 1 | 1 | | | 6.0 |
| 10 | | | | | | 1 | 2 | 3 | | | | | | | 2.3 |
| 11 | | | | | | 2 | 2 | | | | | | | | 0.8 |
| 12 | | | | | | | 3 | | 3 | 2 | | 1 | | | 7.0 |
| 13 | | | | | | | | | 1 | 2 | 3 | 1 | | 1 | 2.8 |
| 14 | | | | | | | 1 | | 3 | 3 | | 1 | | 1 | 2.0 |

Figure 106

| DAY OF APPLICATION (POST FEED) DOSE (1.0 nl FME) | DAY OF ECDYSIS (POST FEED) 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | AVG. DEGREE LARVALIZ'TN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 |  |  |  |  | 2 | 7 | 2 |  | 5 |  | 1 |  |  |  | 0 |
| 1 |  | 2 | 2 | 2 | 1 | 1 | 2 |  |  |  |  |  |  |  | 14.4 |
| 2 |  | 1 | 7 | 2 |  |  |  |  |  |  |  |  |  |  | 15.6 |
| 3 |  |  | 1 | 2 |  | 2 | 2 |  |  |  |  | 1 |  |  | 15.1 |
| 4 |  |  |  | 5 | 2 | 1 |  |  |  |  |  |  |  |  | 13.6 |
| 5 |  |  |  |  | 5 | 3 | 1 |  |  |  |  |  |  |  | 6.4 |
| 6 |  |  |  |  | 6 | 2 | 1 | 1 |  |  |  |  |  |  | 3.7 |
| 7 |  |  |  |  | 3 | 4 | 1 |  | 1 |  |  |  |  |  | 5.2 |
| 8 |  |  |  |  |  | 1 | 1 |  | 4 | 1 | 1 | 1 |  |  | 3.8 |
| 9 |  |  |  |  |  |  | 2 | 2 |  | 3 | 1 |  |  |  | 7.5 |
| 10 |  |  |  |  |  |  | 1 | 2 | 2 | 1 |  | 1 |  |  | 5.7 |
| 11 |  |  |  |  |  | 1 | 2 |  | 4 |  |  |  |  |  | 8.3 |
| 12 |  |  |  |  |  |  | 1 |  | 3 |  |  |  |  |  | 0.3 |
| 13 |  |  |  |  |  |  | 3 | 2 |  | 1 | 1 |  |  | 1 | 0.8 |
| 14 |  |  |  |  |  |  | 1 |  | 3 |  | 1 | 3 |  |  | 1.3 |

| DAY OF ECDYSIS | DEGREE OF LARVALIZATION | | | | | | | | | | | | | | | | | | | | # IN SAMPLE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 19 | 18 | 17 | 16 | 15 | 14 | 13 | 12 | 11 | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 | 0 | |
| 13 | 1 | | | | | | | | | | | | | | | | | | | | 1 |
| 14 | 7 | 2 | | 3 | | | | | | | | | | | | | | | | | 12 |
| 15 | 5 | 4 | 1 | 5 | 3 | | | | | | | | | | | | | | | | 18 |
| 16 | 1 | 4 | 4 | 5 | 3 | 1 | | 1 | | | | | | | | | | | | | 19 |
| 17 | | | | | | 1 | 1 | 1 | | | | | 2 | | 2 | 6 | 8 | 7 | 1 | 2 | 31 |
| 18 | 2 | 1 | 2 | | | | | 1 | | | 1 | | | 2 | | 3 | 6 | 2 | 2 | 11 | 34 |
| 19 | | 2 | 1 | | | | 2 | 1 | 2 | | 1 | 3 | 1 | 1 | | 3 | 2 | 4 | 7 | 13 | 43 |
| 20 | | | | | | | | 1 | | 1 | 1 | | | 1 | 1 | 1 | 4 | | 5 | 17 | 32 |
| 21 | 1 | 2 | | | 1 | 2 | 4 | | 2 | 1 | | | | | | | 1 | 2 | 4 | 1 | 21 |
| 22 | 1 | | 1 | | 1 | | | | | 1 | | | | | 1 | 1 | 1 | 2 | 5 | 2 | 16 |
| 23 | 1 | 1 | | 2 | | | | | | | | | | | | | 1 | | 3 | | 8 |
| 24 | | 1 | | | | 1 | | 1 | | | 1 | | | 1 | 1 | 1 | 1 | 2 | | | 10 |
| 25 | | | | | | | | | | | | | | | | | | | | | 0 |
| 26 | | 1 | | | | | | | | 1 | | | | | 1 | 1 | | | | | 4 |
| # IN SAMPLE | 19 | 18 | 9 | 15 | 8 | 5 | 7 | 6 | 4 | 4 | 4 | 3 | 3 | 5 | 6 | 16 | 24 | 19 | 27 | 46 | 249 |
| | total 61 | | | | total 26 | | | | total 15 | | | | total 30 | | | | total 116 | | | | |

Figure 107

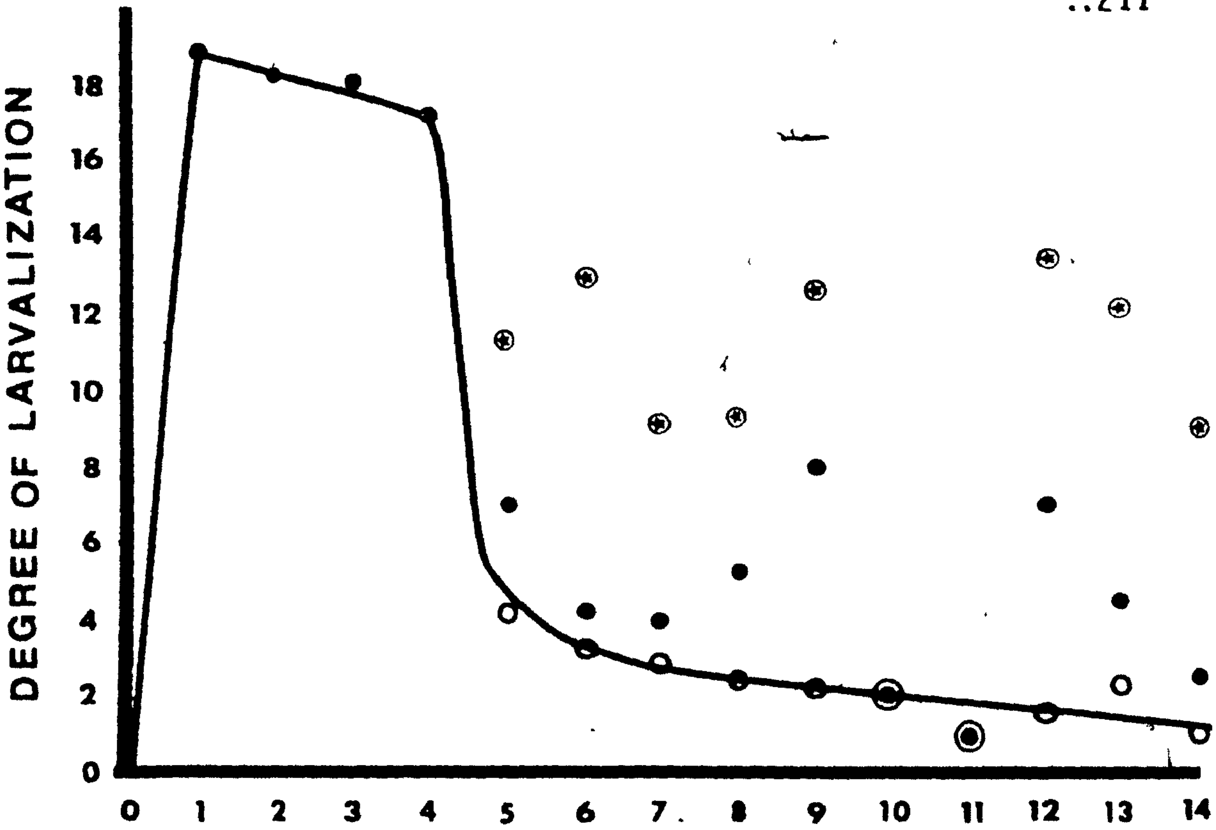

FIGURE 108 DAY OF APPLICATION 2.5 nl FME

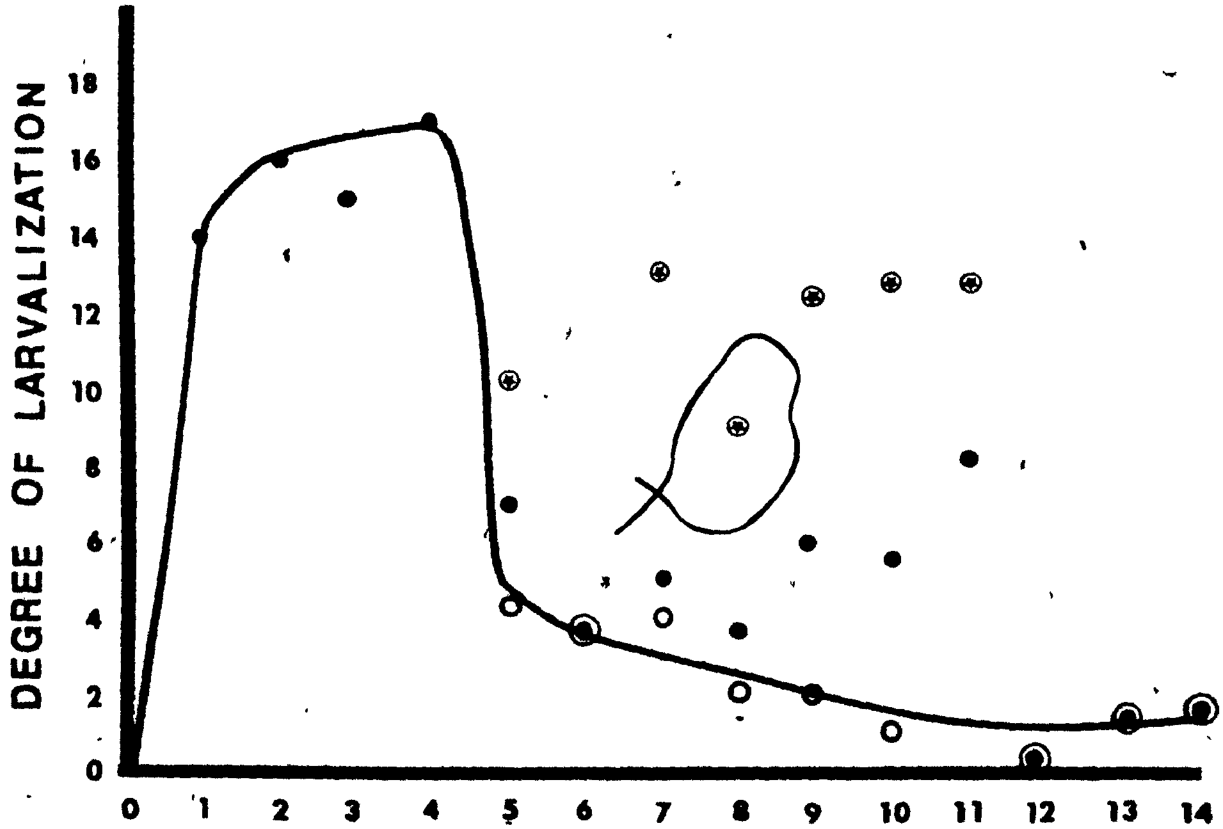

FIGURE 109 DAY OF APPLICATION 1.0 nl FME

## Statistical Methods:

All statistical methods used in the interpretation of data presented in this thesis; including the means, standard deviations, and probability analysis (Student's t test) mitotic indices (Chapter 10), and the analysis of variance, regression line correlation coefficients and probability analysis for the effect of FME on spermatid and spermatocyte output (Chapter 5), were carried out as prescribed in Basic Statistics: A Primer for the Biomedical Sciences, by O.J. Dunn, John Wiley and Sons Inc.. N.Y., 1967. In all cases, methods used included Bessel's correction for small sample sizes.

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