JUVENILE HORMONE REGULATION OF VITELLOGENIN GENES IN THE AFRICAN MIGRATORY LOCUST

by

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This thesis is dedicated to my late mother, Malvinder K. Dhadialla.

ABSTRACT

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In Locusta migratoria, vitellogenin (Vg) synthesis is normally induced by juvenile hormone (JH) only in adult females. Larval and adult female and male locusts were tested for inducibility of Vg synthesis by the synthetic JH analog, methoprene. While fourth and fifth larval stages of both sexes could be induced to synthesize Vg, adult males could not. Quantitative assays showed the relative response in the order: adult female > fifth instar female > fifth instar male. During the fifth stadium, maximal vitellogenic response was obtained in mid-instar; in the female, this was preceded by stimulated DNA synthesis. Experiments involving female-male fat body tranplantations and precociously induced adults indicated that sex-limited gene programming is determined in the fat body cells early in development and is partially expressed by the fifth instar but fully manifest only after the metamorphic molt.

Fat bodies from adult females were cultured for up to 72 hours in synthetic medium without loss of viability. With high levels of methoprene in vitro, successful secondary and a very weak, variable primary stimulation of Vg synthesis was obtained.

Total RNA was prepared from fat bodies in different states and the content of $mRNA_{Vg}$ assayed by dot hybridization with a cloned Vg gene probe. During primary stimulation of adult and fifth instar females, $mRNA_{Vg}$ was first detectable after 24 hours, then increased slowly for about 12 hours, and then much more rapidly. In secondary stimulation of allatectomized normal vitellogenic females, the lag was reduced to about 12 hours and initial $mRNA_{Vg}$ accumulation was more rapid. By 48 hours, the $mRNA_{Vg}$ level was only about 50% higher in secondary than in primary stimulation, but the rates of Vg synthesis, assayed in the same fat bodies, was about 3-fold higher. This suggests increased translational efficiency in the secondary response, possibly due to availability of ribosomes. After withdrawal of JH by allatectomy of vitellogenic females, $mRNA_{Vg}$ decayed with a half-life of about 24 hours, but the initial decline in Vg synthesis was much more rapid. These results suggest that JH-dependent Vg synthesis is regulated at the translational as well as the transcriptional level.

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$A_{\_}$	absorbance at _
BSA	bovine serum albumin
c-	centi-, 10^{-2}
^{o}C	degrees centrigrade
Ci	Curie
Con	conalbumin
cpm	counts per minute
DEP	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
cDNA	complementary DNA
dCTP	deoxycytosine triphosphate
EDTA	ethylenediamine tetraacetic acid
g	gram
xg	acceleration due to gravity
h	hour
^{3}H	tritium
HEPES	N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid
JH	juvenile hormone
Kb	kilo base
l	litre
M	molar
min	minute
μ	micro-, 10^{-6}

m-	milli-, 10^{-3}
n-	nano-, 10^{-9}
N	normal
nt	nucleotide
Ov	ovalbumin
p-	pico-, 10^{-12}
^{32}P	phosphorus - 32
PCA	perchloric acid
PEG	polyethylene glycol
poly(A)	polyadenylated
RNA	ribonucleic acid
RNAase	ribonuclease
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TCA	trichloracetic acid
TES	N-tris[Hydroxymethyl]methyl-2-aminomethane sulfonic acid
Tris	tris(hydroxymethyl) aminomethane
v	volume
Vg	vitellogenin
VLDL	very-light-density lipoprotein
Vn	vitellin
Yp	yolk polypeptide

I. INTRODUCTION

The fundamental problem in developmental biology is how a multitude of specialized cells and tissues are derived from the fertilized egg cell. At the molecular level, this becomes a question of the way in which different genes are expressed in different cells. Each gene in a developmental pathway must be strictly regulated so that it can be expressed at the proper time, in coordination with other genes. An understanding of the mechanisms involved in the regulation of eukaryotic gene expression is thus basic to the understanding of development.

A number of systems have been used to study this question. Among the most fruitful has been the exploitation of hormonally regulated genes. The study of this important group of genes is facilitated by the availability of hormones that can be used to regulate activity at will in the responsive cell population. The specificity of the response depends on the hormone and the target cell. While different responses can be evoked in different cells by one hormone, different hormones can induce different responses in a single cell type. The ability of a cell to respond to a hormone is a form of differentiation which depends on the expression of regulatory genes involved in patterns of development.

Among hormones, the steroids — androgens, estrogens, progesterone, adrenal corticoids and ecdysteroids — are of special interest because of their multiple effects in development. Steroid

hormones evoke two types of response in target tissues, a generalized and a specific response. A generalized response involves the synthesis of many proteins required for tissue growth. In the specific response the synthesis of a limited number of proteins characteristic of the target tissue is induced or repressed to a considerably greater extent than the synthesis of the vast majority of cellular proteins. This property of the steroids and the existence of certain model steroid-responsive systems has allowed considerable progress in unravelling the molecular basis of steroid hormone action. The results of many recent investigations with such steroid-responsive systems are in agreement that an important part of their action is at the level of gene transcription, resulting in production and accumulation of mRNA with a high metabolic stability.

These concepts, derived from recent research chiefly with vertebrate animals, provide the background for our studies on the action of the insect developmental hormone, juvenile hormone. In spite of the non-steroidal nature of juvenile hormone, there is increasing evidence that at the molecular level it may act like a steroid hormone. The fact that juvenile hormone is not a steroid increases the interest in examining its molecular mode of action.

Regulation of Egg-White Protein Synthesis in the Chick Oviduct

The most-studied hormonally controlled gene is without doubt the ovalbumin gene in chickens. In the chicken oviduct, steroid hormones, mainly estrogen and progesterone, stimulate the growth and

differentiation of the tubular gland cells which produce mainly four egg-white proteins, including ovalbumin, conalbumin, lysozyme and ovomucoid (O'Malley et al., 1975; Schimke et al., 1977). The four proteins are, however, synthesized at different rates, with ovalbumin alone accounting for 50-60 % of total protein synthesis (Palmiter, 1972). The common control, yet the different extent of their accumulation, has made the coordinated synthesis of these proteins attractive for the study of differential gene expression (Rosen and O'Malley, 1975; Schimke et al., 1975).

Initial exposure of the chick oviduct to estrogen or to progesterone produces different effects. Estrogen administration to immature chicks stimulates proliferation and differentiation of tubular gland cells, which then synthesize the egg white proteins (Palmiter, 1972; Schimke et al., 1977). When estrogen administration is discontinued (withdrawal), the synthesis of egg-white proteins declines to undetectable levels, although some of the differentiated tubular gland cells remain, and readministration of estrogen stimulates the capacity for egg-white protein synthesis in the pre-existing cells. Progesterone by itself induces limited cell proliferation and differentiation of surface epithelium, and in the absence of estrogen it prevents the cytodifferentiation of tubular gland cells. Remarkably, however, estrogen modifies the response to other steroid hormones, so that after estrogen treatment, progesterone, glucocorticoids and androgens can all stimulate egg-white protein synthesis (Palmiter, 1972). What follows is an account of steroid regulation of ovalbumin and conalbumin synthesis,

since by far the most attention has been given to these two proteins.

Induction in vivo

Primary stimulation of immature chick oviducts with estrogen induces ovalbumin synthesis with a lag of about 18 h. In the continued presence of estrogen, the rate of synthesis increases, reaching a plateau after about 10 days (Palmiter, 1972). On withdrawal of the hormone, ovalbumin synthesis falls rapidly and is undetectable after 20-25 days. Readministration of estrogen or progesterone induces a rapid increase in ovalbumin synthesis with a lag of about 3 h and maximal rates are achieved after only 4 days (Palmiter, 1972). A similar pattern of induction is observed when total RNA isolated from chick oviduct tissue after various times of hormone administration is translated in a rabbit reticulocyte cell-free translation system. The amount of translatable ovalbumin mRNA ($mRNA_{Ov}$) corresponds to the rate of ovalbumin synthesized *in vitro* in the same tissue (Palmiter *et al.* 1977).

Although these types of experiments showed mRNA and specific protein synthesis to be correlated, they did not differentiate between modulation of mRNA levels by increased synthesis or reduced degradation. This question and the problem of a lag period raised the possibility of steroid hormone control at the transcriptional, post-transcriptional or translational levels. Hence, to examine the molecular mechanisms of steroid hormone action studies were directed at the isolation and identification of the specific mRNA and the structural and regulatory sequences of the ovalbumin gene (Schimke *et*

al., 1975; Rosen and O'Malley, 1975; O'Malley et al., 1979).

As a first step, $mRNA_{Ov}$ was purified by techniques based on immunoprecipitation of ovalbumin-synthesizing polysomes (Palacios et al., 1973; Shapiro et al., 1974) and separation of poly(A)-containing RNA and fractionation by size (Rosenfeld et al., 1972; Rosen et al., 1975; Buell et al., 1978). Buell et al. (1978) also isolated mRNAs for conalbumin, lysozyme and ovomucoid. The size of $mRNA_{Ov}$ was determined by sucrose density centrifugation, by nucleic acid hybridization (Schimke et al., 1975) and by electron microscopy (Rosen and O'Malley, 1975) to be 2050 nucleotides, corresponding to a theoretical maximum protein molecular weight of 75,500. DNAs complementary to purified ovalbumin, conalbumin, ovomucoid and lysozyme mRNAs have been synthesized (Sullivan et al., 1973; Harris et al., 1975; Buell et al., 1978). Sequences complementary to $mRNA_{Ov}$ and $mRNA_{Con}$ ($cDNA_{Ov}$, $cDNA_{Con}$) have been used as probes to study the accumulation of their respective mRNAs in primary induced (Harris et al., 1975; Moen and Palmiter, 1980), withdrawn and secondary induced chick oviduct (Palmiter, 1973; Palmiter et al., 1976; Hynes et al., 1977; Schutz et al., 1977; Roop et al., 1978; Shepherd et al., 1980).

Much of the earlier work on the actions of estrogen or progesterone in the chick oviduct examined effects after withdrawal of estrogen following primary stimulation. Primary stimulation was thought to be qualitatively different from secondary stimulation, since during secondary induction the initial events in ovalbumin and conalbumin synthesis are not complicated by cell division and cytodifferentiation of tubular gland cells. However, more recent

work by Moen and Palmiter (1980) showed that primary stimulation of oviduct with estrogen is qualitatively similar to secondary stimulation.

Contrary to the findings of Harris et al. (1975), who could not detect $mRNA_{Ov}$ in unstimulated oviducts, Moen and Palmiter (1980) using $cDNA_{Ov}$ and $cDNA_{Con}$ and quantitative hybridization techniques detected about 4 $mRNA_{Ov}$ and 30 $mRNA_{Con}$ molecules/cell. One day after stimulation with estradiol benzoate, these low levels increased to about 250 to 300 molecules/cell, respectively. While the rates of accumulation for the two mRNAs 8-24 h after estrogen stimulation were similar (13-14 mol/h/cell), $mRNA_{Ov}$ increased only after a lag of 6-8 h and $mRNA_{Con}$ increased sooner. These rates were an order of magnitude lower than those observed during secondary stimulation. This sub-maximal response during primary stimulation was not due to a difference in the number of responsive cells or nuclear receptor levels. In situ hybridization with $cDNA_{Ov}$ revealed that nearly all of the epithelial cells of the oviduct accumulated $mRNA_{Ov}$ within a day of estrogen treatment and that the percent of responsive cells was about the same as in withdrawn oviducts. Moreover, within 3 h of estrogen administration in vivo, the number of nuclear estrogen receptors in the oviduct was in fact 3 times higher than the number of nuclear receptors measured during early secondary stimulation. DNA synthesis was also found to be unnecessary, since $mRNA_{Con}$ was induced in vitro by 17β-estradiol in the presence of hydroxyurea.

The early kinetics of ovalbumin and conalbumin mRNA synthesis during secondary induction have been studied in at least 3

laboratories with similar results. The main features of induction kinetics of the two proteins and their mRNAs after readministration of estrogen are as follows. (1) For ovalbumin, there is a lag phase during which there was little or no change in $mRNA_{Ov}$ level (Mcknight et al., 1975; Palmiter,et al., 1976; Schutz et al., 1977; Roop et al., 1978; Seaver et al., 1980), followed by a short transition phase during which $mRNA_{Ov}$ begin to accumulate, and after about 3 h the rate of $mRNA_{Ov}$ accumulation became constant at 18-22 molecules/min/tubular gland cell (Palmiter 1973; Palmiter et al., 1976). In contrast, there was no discernible lag before $mRNA_{Con}$ level increased and there was a significant increase in the rate of conalbumin synthesis as early as 30 min following estrogen stimulation. (2) Conalbumin synthesis generally represents about 6-8% of total protein synthesis after 8 h and plateaus at about 10% after 2 days. Ovalbumin is synthesized initially at a lower rate than conalbumin but this continues to rise for 4-5 days, finally reaching a value of 50-60% of total protein synthesis (Palmiter, 1973). The rate of ovalbumin induction during the accumulation phase is inversely related to the duration of the preceding withdrawal (Palmiter et al., 1981), probably due to the gradual loss of responsive tubular gland cells during extended withdrawal. (3) Ovalbumin synthesis is barely detectable in magnum from withdrawn birds (<0.1% of total protein synthesis), though some $mRNA_{Ov}$ is still present. Conalbumin synthesis, on the other hand, falls during withdrawal to an average value of 1.3% of total protein synthesis and this is maintained for up to 30 days. (4) Neither concomitant protein nor DNA synthesis is required for

induction of mRNA synthesis. However, cycloheximide was found to inhibit $mRNA_{Ov}$ accumulation whether given with estrogen or 3 or 6 h after estrogen when $mRNA_{Ov}$ has already been induced (Palmiter et al., 1976). (5) The duration of the lag and the subsequent rate of $mRNA_{Ov}$ accumulation are independently controlled. The lag can be varied with progesterone or hydroxyurea without affecting the rate of $mRNA_{Ov}$ accumulation (Palmiter, 1973; Palmiter et al., 1976). The rate of $mRNA_{Ov}$ accumulation is related to the concentration of nuclear receptors and can be altered by varying the dose of estrogen without affecting the duration of the lag (Palmiter et al., 1976,1977).

The existence of a lag has important implications because it suggests that there are as yet undefined, rate-limiting nuclear events interposed between receptor binding in the nucleus and the acceleration of mRNA production that occurs a few hours later. This lag phenomenon is prevalent in steroid responsive systems (refer Palmiter et al., 1976 for examples) but is not universal, even within one tissue (e.g. induction of conalbumin versus ovalbumin with estrogen). However, the lag is not due to slow accumulation of estrogen receptors, because within 15-20 min estrogen receptors reach a maximal level, elevated about seven fold (to 8,000 molecules/tubular gland cell), which is maintained for the next 6 h (Palmiter et al., 1977; Schutz et al., 1977). This point is emphasized by the primary induction, where the lag is longer in spite of a 3 fold higher number of receptors/cell than for secondary stimulation (Moen and Palmiter, 1980). Moreover, with suboptimal doses of estrogen, lower levels of estrogen receptors are attained without affecting the lag. The

receptor-dependent response is even more complicated when both $mRNA_{Con}$ and $mRNA_{Ov}$ are considered. While $mRNA_{Con}$ and conalbumin synthesis are directly related to nuclear estrogen receptor level in a dose-dependent way, much lower doses of estrogen and nuclear receptor levels are needed than for ovalbumin synthesis (Palmiter et al., 1977).

The results presented above, obtained in vivo, have been duplicated in vitro (McKnight, 1978). Oviduct explants from estrogen pretreated chicks could be restimulated in vitro by physiological concentrations of estrogen and progesterone. Although there were overall similarities in the induction of ovalbumin and conalbumin by both estrogen and progesterone in vitro and in vivo, there were minor differences in the lag periods and rate of ovalbumin synthesis which may be the result of sub-optimal culture conditions. However, once again these results showed that lag periods and induction of specific mRNA are both a function of the direct interaction of steroids with oviduct tissue. This in vitro system allowed the use of protein synthesis inhibitors without the added complications of their use in vivo. That all the protein synthesis inhibitors blocked both $mRNA_{Ov}$ and $mRNA_{Con}$ induction and rapidly stopped an ongoing synthesis of $mRNA_{Con}$ implicated an important role of protein synthesis in the steroid induction of egg-white mRNAs.

In the above studies the accumulation of mRNAs in response to estrogen or progesterone was measured. Rates of accumulation depend on both synthesis and degradation. By labelling the RNA in vivo in secondary induced chicks followed by subsequent purification and

determination of its specific activity a half-life of 24 h for $mRNA_{Ov}$ was shown to decrease to 2-3 h upon withdrawal of hormone (Palmiter, 1973; Palmiter and Carey, 1974). To find whether the primary effect of estrogen was upon transcription or upon mRNA degradation, rates of $mRNA_{Ov}$ and $mRNA_{Con}$ synthesis have been studied in isolated nuclei (Schutz et al., 1977; Roop et al., 1978; McKnight and Palmiter, 1979; Swaneck et al., 1979; Palmiter et al., 1981). The results were in general agreement in demonstrating transcriptional activation of the respective genes.

Additional evidence was obtained after the ovalbumin gene had been shown to contain seven non-coding sequences (introns) interrupting the structural sequences of the ovalbumin gene (Breathnach et al., 1977; Dugaiczyk et al., 1978; Royal et al., 1979). When nuclear RNA separated by gel electrophoresis was transferred onto diazotized paper and probed with cloned $cDNA_{Ov}$, multiple discrete bands containing ovalbumin structural sequences were detected ranging from the size of mature mRNA up to more than 4 times this size (Roop et al., 1978). The high molecular weight $mRNA_{Ov}$ species, detected only in oviduct tissue after estrogen administration, were presumably $mRNA_{Ov}$ precursors. Withdrawal of the hormone depleted the nucleus of these precursors as well as mature $mRNA_{Ov}$, and readministration of estrogen induced the accumulation of both species. These results ruled out the possibility that the rapid accumulation of mature $mRNA_{Ov}$ after secondary stimulation might result from processing of $mRNA_{Ov}$ precursors stored in the withdrawn oviduct (Roop et al., 1978; Swaneck et al., 1979).

Interaction of estrogen with other steroid hormones

In addition to sensitizing cells to its own response, estrogen also expands their responsiveness to two other steroid hormones, progesterone and glucocorticoids. This phenomenon resembles cell determination or commitment in that estrogen irreversibly alters the potential of the oviduct cells. The altered responsiveness of oviduct to progesterone and glucocorticoids after exposure to estrogen is maintained in the absence of estrogen (Palmiter et al., 1976; Hager et al., 1980), but at least 36 h of estrogen exposure is required to bring about this change in the cells (Moen and Palmiter, 1980). During this 36 h period progesterone receptor levels increased several fold.

During secondary stimulation, progesterone and estrogen had opposite effects on the duration of the lag phase for transcription of the two genes. The lag period for $mRNA_{Ov}$ was shorter after progesterone treatment (1.5-2 h) than after estrogen (McKnight et al., 1975; Palmiter et al., 1976). In contrast to its effect on ovalbumin synthesis, progesterone supressed estrogen-induced conalbumin synthesis and introduced a lag of about 2 h (Palmiter et al., 1977). The effect of progesterone was shown to be dominant over that of estrogen, when it was given 2 or 4 h after estrogen administration; conalbumin synthesis was temporarily inhibited and ovalbumin synthesis was enhanced but always after a lag of 2 h (Palmiter et al., 1977). Progesterone, like estrogen, was shown to regulate the rate of transcription.

In addition to possessing receptors for estrogen, progesterone and

androgens, oviduct tubular gland cells also possess glucocorticoid receptors which can mediate induction of egg-white proteins *in vivo* and *in vitro* (Hager *et al.*, 1980). However, unlike progesterone, glucocorticoid stimulated estrogen-mediated $mRNA_{Con}$ synthesis. This also indicated that glucocorticoids did not act through progesterone receptors.

Androgens can also modulate the induction of egg-white proteins. Androgens act synergistically with estradiol to stimulate growth and protein synthesis of the immature chick oviduct (Palmiter, 1972). Unlike progesterone and glucocorticoids, neither testosterone nor dihydrotestosterone acting alone can induce egg-white proteins in chicks withdrawn from estrogen treatment for 2-4 weeks (Palmiter, 1972). However, both testosterone and dihydrotestosterone could stimulate magnum growth and maintain $mRNA_{Ov}$ levels as long as androgen receptors remained elevated in estrogen pretreated chicks (Tokarz *et al.*, 1979). These results and those of Moen and Palmiter (1980) indicate how estrogen treatment may broaden the responsiveness of oviduct tubular gland cells to other classes of steroids.

Regulation of Vitellogenin Synthesis in Oviparous Vertebrates

In egg-laying vertebrates, the liver under the influence of estradiol synthesizes and secretes massive amounts of the yolk protein precursor, vitellogenin (Vg), into the plasma (Wallace *et al.*, 1969; Bergink and Wallace, 1974; Deeley *et al.*, 1975; Tata and Smith, 1979 Shapiro, 1982). The Vg is taken up from the blood by the developing

oocytes where it is cleaved into lipovitellin and phosvitin, a highly phosphorylated serine-rich protein (Bergink and Wallace, 1974).

Vitellogenins are encoded by at least 4 genes in Xenopus (Wahli et al., 1979) and possibly two in the chicken (Wang and Williams, 1980), although in the latter conclusive data is not available. The mRNAs (6,300-6,500 nts; Wahli et al., 1976; Shapiro and Baker, 1977) and the monomeric protein subunit (M_r = 180,000-197,000; Wiley and Wallace, 1978) are almost identical to those of the locust (Chinzei et al., 1982; Wyatt and Belland, unpublished data). Before being secreted, the vertebrate vitellogenins undergo glycosylation and phosphorylation.

Induction of vitellogenin mRNA and vitellogenin in vivo

In contrast to the ovalbumin system, Vg synthesis in the liver of oviparous vertebrates takes place without cell proliferation and has an absolute and specific requirement for estrogen. Vitellogenin is normally found in females but not in males. However, the liver of roosters or male Xenopus can be induced by estrogen to synthesize and secrete large amounts of this protein. These factors have made Vg synthesis in oviparous vertebrates a good model system for study of the early effects of estrogen at the molecular level, uncomplicated by the need for cell proliferation.

Administration of estradiol-17β to male X. laevis causes ultrastructural changes in hepatocytes typical of the activation of cells for synthesis and secretion of large amounts of proteins (Tata, 1976). Accumulation of $mRNA_{Vg}$ after estrogen administration to

cockerels and male X. laevis has been measured. Vitellogenin mRNA can account for as much as 70% of liver cell mRNA in fully-stimulated Xenopus (Shapiro et al., 1976) and 10-40% of liver cell mRNA in different strains of rooster (Deeley et al., 1977; Jost et al., 1978). A characteristic feature of estrogen-induced Vg synthesis in Xenopus is a lag period of 4 1/2 h after hormone administration before appearance of Vg in the plasma. When withdrawn animals (avian or amphibian) are given a second stimulation with estrogen, weeks or months after the first (by which time Vg synthesis has dropped to an undetectable level) the vitellogenic response takes place with greater rapidity and magnitude (Tata, 1976; Green and Tata, 1979; Goldberger and Deeley, 1980; Shapiro, 1982) indicating the persistence of a "memory" of the primary stimulation.

The rate of accumulation of $mRNA_{Vg}$ following hormone administration has been measured and compared with the synthesis of Vg. The earlier work depended on assaying the ability of extracted RNA to direct synthesis of immunoprecipitable Vg in a cell-free protein synthesizing system from rabbit reticulocytes (Shapiro et al., 1976; Farmer et al., 1978) or wheat germ (Mullinix et al., 1976). More recently, $mRNA_{Vg}$ was purified, aided by its large size, high concentration and poly(A) content, and used to make cDNA hybridization probes for more accurate and sensitive assay of mRNA (Baker and Shapiro, 1977, 1978; Deeley et al., 1977a, b; Burns et al., 1978; Jost et al., 1978).

In the liver of unstimulated roosters or male Xenopus, $mRNA_{Vg}$ is present at 0-5 molecules/cell (Deeley et al., 1977 a, b) or less than

1 molecule/cell (Baker and Shapiro, 1977), respectively. During primary stimulation with estrogen, the accumulation of $mRNA_{Vg}$ was first detectable after 30 min in the rooster and 4.5 h in Xenopus. This lag period was followed by a biphasic curve of mRNA accumulation. After a slow rate of accumulation during the first 4 h in the rooster (Deeley et al., 1977 a, b) and 8 h in Xenopus (Baker and Shapiro, 1977), the $mRNA_{Vg}$ accumulated at a higher rate. Following withdrawal of estrogen, the cellular level of $mRNA_{Vg}$ declined to an undetectable level by 60 days in Xenopus and 17-20 days in the rooster.

During secondary stimulation in both Xenopus (Baker and Shapiro, 1977, 1978) and the rooster (Deeley et al., 1977b; Jost et al., 1978), administration of estrogen elicited a rapid appearance of new $mRNA_{Vg}$. In Xenopus significant levels of $mRNA_{Vg}$ were found after 1-2 h. In the rooster, $mRNA_{Vg}$ accumulated during the first 4 h at a rate similar to the second, more rapid phase observed in primary stimulation (Deeley et al., 1977b). In general, the induction of $mRNA_{Vg}$ in the rooster during primary and secondary stimulation follows a similar but a more rapid time course than in Xenopus.

Induction of vitellogenin and vitellogenin mRNA in vitro

Studies in vivo present serious limitations to the analysis of the early events leading to the activation of genes by hormones. The complex dynamics of interactions between tissues, and distribution and metabolism of hormones in the intact animal, lead to uncertainty about the rate and level of hormone delivery to the target cells. To overcome these problems in the vertebrate vitellogenin system and to

study the kinetics of the induction process, methods have been developed for the culture of liver tissue *in vitro*.

Direct *in vitro* induction of vitellogenin has been achieved in organ cultures of both adult and immature *Xenopus* liver (Wangh and Knowland, 1975; Green and Tata, 1976; Knowland, 1978). Successful induction has also been obtained in primary cultures of hepatocytes from embryonic chicken liver (Traniello *et al.*, 1978), adult *Xenopus* (Wangh *et al.*, 1979; Searle and Tata, 1981) and the frog, *Rana catesbiana* (Stanchfield and Yager, 1978, 1980). The kinetics and specificity of the response appear to be the same as *in vivo* though the dose of estradiol required to initiate vitellogenin synthesis can be as low as 10^{-8}M - 10^{-9}M (Stanchfield and Yager, 1978; Wangh *et al.*, 1979). There have been only two reports on the induction of vitellogenin mRNA *in vitro* (Felber *et al.*, 1978; Searle and Tata, 1981). Felber *et al.* (1978) highlighted the problem of progressive necrosis in organ culture and showed that the response *in vitro* improved in liver tissues of pre-stimulated animals and by adaptation of liver explants to the culture medium before hormone treatment. Searle and Tata (1981) used the *Xenopus* liver parenchymal cell culture methods developed by Wangh *et al.*, (1979) to obtain both the primary and secondary *in vitro* responses to estrogen with similar kinetics but lower intensity than those obtained *in vivo* (Baker and Shapiro, 1978). Using a quantitative dot-hybridization assay, they were able to monitor the accumulation of $mRNA_{Vg}$. During primary induction *in vitro*, they detected complementary $mRNA_{Vg}$ sequences after 2 h as opposed to 4.5 h observed *in vivo* by Baker and Shapiro (1978), perhaps

due to immediate availability of estradiol *in vitro*. An interesting aspect brought out in this study was the rapidity with which 17β-estradiol was metabolized *in vitro*.

Recent studies of induction of vitellogenin synthesis *in vitro* in *Xenopus* liver cell cultures have questioned the role of estradiol as the only hormonal requirement (Wangh and Schneider, 1982, Wangh, 1982). When fully perfused liver tissue or isolated parenchymal cells were maintained in culture for 1-2 weeks before the addition of estrogen, little or no primary induction of Vg was observed with physiological doses of 17β-estradiol. When thyroid hormones were added along with estradiol 17-β, however, Vg was produced. Thyroid hormones were also shown to potentiate the *in vitro* effects of dexamethasone on albumin and fibrinogen synthesis in liver cell cultures (Wangh, 1982). The results of these studies implicate the co-requirement for thyroid hormones in induction of Vg synthesis by estrogen and reaffirm the need for tissue or cell culture systems to reveal such relationships. Unfortunately, attempts to culture adult avian liver *in vitro* have been much less successful. However, primary suspension cultures of hepatocytes from adult rooster have been maintained viable for longer than 12 h (Garber and Brasch, 1982). Although these authors could not obtain a secondary induction of Vg with estradiol 17-β added *in vitro*, the hepatocyte cultures from *in vivo* induced livers continued to produce Vg at levels comparable to those observed *in vivo*.

Ontogeny of the vitellogenic response to estrogen

Another approach to elucidating the mechanisms involved in the estrogenic control of gene expression is to determine the developmental stage before which the liver will not synthesize Vg in response to exogenous estrogen. Then the mechanisms underlying the transition from the uninducible to the inducible state can be explored. Such approaches have been attempted for both Xenopus and chicken.

In Xenopus, Vg was first inducible at the metamorphic stage 62, and the transition to the inducible state did not occur in larvae developmentally arrested in prometamorphosis for up to 6 months (May and Knowland, 1980). However, estrogen treatment during stages 53-66 affected protein synthesis in the liver, which suggests that the transition of Vg genes to the inducible state is controlled by specific rather than general factors (May and Knowland, 1981). Different responses to estrogen do not appear synchronously. Larval stages which could not be induced to synthesize Vg with exogenously administered estrogen, but which responded by synthesizing other proteins, contained an estrogen receptor with the same affinity and specificity as the adult receptor (May and Knowland, 1981). The nuclear estrogen receptors increased in response to estrogen only in the Vg-inducible stage, which is consistent with the hypothesis that increased levels of receptors are required for induction of Vg synthesis. Since metamorphosis is dependent upon the action of thyroxine, the transition from the uninducible to the inducible state of the Vg gene may be due to this hormone. Experiments in vivo with thyrostatic tadpoles (May and Knowland, 1980) and in vitro with livers

from thyrostatic larvae (Wangh, 1982), however, have demonstrated that thyroxine does not directly confer inducibility on the Vg gene and that the lack of vitellogenic competence in pre-metamorphic tadpole is intrinsic to the immature cell.

Differential ontogeny of estrogen responsiveness in the chick embryonic liver has also been investigated (Lazier, 1978; Elbrecht et al., 1981). Vitellogenin synthesis was first inducible with exogenously administered estrogen on days 13-15 of embryonic development. When estrogen was injected into 10 day old chick embryos, however, an increase in estrogen binding activity was observed 48 h later, and an increase in the constitutively expressed VLDL-apoprotein B in response to estrogen was observed in 11 day old chick embryonic liver. Hence, unlike the situation in Xenopus, the acquisition of ability to increase the concentrations of soluble nuclear receptors in response to estrogen precedes the development of the ability of chick liver to synthesize Vg.

Growth and Developmental Hormones in Insects

Characteristic features of postembryonic development common to all insects are molting and metamorphosis. These are brought about by two lipoidal hormones, ecdysone and juvenile hormone, under the control of neurosecretion from the brain. After metamorphosis into the adult, the same two hormones play important roles in reproduction.

The first evidence that insects use hormones to control their growth and development came from experiments done by Kopec in 1917.

However, it was much later through the cumulative efforts of Wigglesworth, Fukuda and Williams that an integrated scheme of the endocrine control of molting and metamorphosis was established. According to this "classical scheme", upon receiving appropriate stimuli the neurosecretory cells of the brain release a "brain hormone" (now known as the prothoracicotropic hormone) that stimulates the prothoracic glands to release into the hemolymph the molting hormone (ecdysone). The latter, upon contact with the insect's epidermal cells, initiates molting. The character of the molt, whether it is larval-larval, larval-pupal, or pupal-adult, is determined by the titer of a third hormone, juvenile hormone, secreted by the corpus allatum, at or before the molt. It was hypothesized that ecdysone in the presence of a high titer of JH elicits a larval molt and presence of an intermediate titer of JH evokes a pupal molt. Metamorphosis to the adult occurs in the absence of JH. Details of experiments leading to this scheme and the wealth of information that has added much to it since its initial formulation have been summarized in a number of excellent reviews (Doane, 1972; Wyatt, 1972; Riddiford and Truman, 1978; Richards, 1981; Granger and Bollenbacher, 1981).

The "classical scheme" has been accepted for a number of years. However, some aspects of the model have lacked direct experimental support; for example, the exact meaning of "intermediate or low titers of JH" was not defined. Recently, it has become possible to measure precisely the titers of ecdysteroids and JH (reviewed by Gilbert et al., 1980; de Kort and Granger, 1981; Gilbert and

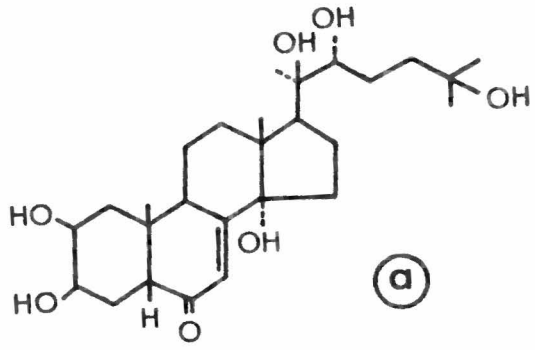
Goodman, 1981) and a modified model has gained support (Nijhout and Wheeler, 1982). In this model it is the timing of the presence (or absence) of JH rather than its concentration that determines what character will be expressed during the next molt. The hypothesis is that an insect's cells experience one or more periods of sensitivity to JH during each instar. Such critical periods are temporal "windows" during which a gene or group of genes is susceptible to repression or derepression, the choice depending on whether JH is present or absent. A substantial amount of data that lends support to this model comes from the careful work of Lynn Riddiford and colleagues with the tobacco hornworm, Manduca sexta (Riddiford, 1976; also reviews by Riddiford and Truman, 1978; Nijhout and Wheeler, 1982).

The chemical characterization (figure 1), transport and metabolism of the ecdysteroids and juvenile hormones have also been studied and recently reviewed (Richards, 1981; Gilbert and Goodman, 1981). Once the potential of these hormones as "insect growth regulators" for practical applications in control of undesired species was realized, efforts were directed at synthesizing superactive analogs of JH. Of over 5,000 JH analogs prepared, one (methoprene) has been rewarding not only for the control of floodwater mosquitoes, hornflies and fleas, but also in studying the JH control of physiological processes. The structures of methoprene and another active JH analog (hydroprene) are shown in figure 1.

Another class of chemicals, that have anti-juvenile hormone activity, was discovered and isolated from the common bedding plant

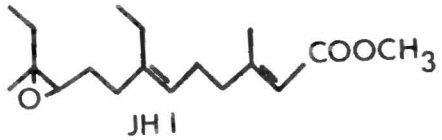
Figure 1

The chemical structures of ecdysterone (a), juvenile hormones 0, I, II and III (b), the JH analogs methoprene and hydroprene (c), and ethoxyprecocene (d).

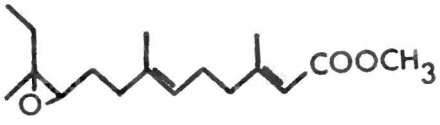


(a)

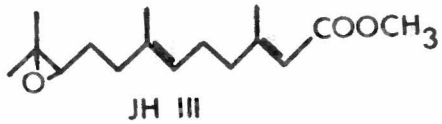
JH 0



JH I

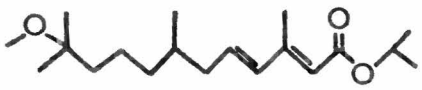


JH II



JH III

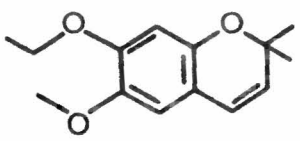
(b)



methoprene

hydroprene

(c)



(d)

Ageratum houstonianum (Bowers et al., 1976). These compounds caused precocious metamorphosis when given to early larvae of certain bugs (hence they were named Precocenes). Precocenes were later shown to be specifically cytotoxic for the corpora allata of hemimetabolous insects (Unnithan et al., 1977) and have been used by insect physiologists as an alternative to surgical allatectomy. In figure 1 is also shown the structure of ethoxyprecocene, a highly active synthetic analog.

Gonadotropic Hormones in Insects

After fulfilling their roles as growth and developmental hormones, JH and, in a few insects, ecdysone serve new functions as reproductive hormones in the adult stage. In most insects, the various steps of oocyte maturation are under the control of JH. In the mosquito, Aedes aegypti, the ovaries and the fat body become competent to respond to an egg development neurosecretory hormone and ecdysone, respectively, only after a prior exposure to JH (Flanagan and Hagedorn, 1977; Shapiro and Hagedorn, 1982). The regulation of Vg synthesis in the fat body of most insects is under the control of JH (reviewed by Hagedorn and Kunkel, 1979; Engelmann, 1979), although ecdysone has also been shown to play an important role in mosquitoes (reviewed by Fuchs and Kang, 1981), Drosophila (reviewed by Postlethwait and Jowett, 1981; Bownes, 1982) and the fleshfly, Sarcophaga bullata (de Loof et al., 1981). Once synthesized, the uptake of Vg into the oocytes may also be under the control of JH. The first evidence of

this came from experiments with Periplaneta where injected Vg was not incorporated into the oocytes unless JH was also made available (Bell, 1969). Later, it was shown that although sterile apterous 4 Drosophila females had yolk proteins (YPs) in their hemolymph, they accumulated YPs in their ovaries only after treatment with methoprene (Postlethwait and Weiser, 1974). A more direct evidence for this role for JH was provided by utilizing an in vitro system for Drosophila (Giorgi, 1979) and Locusta migratoria ovaries (Ferenz et al., 1981). The uptake of Vg into the oocytes is probably facilitated by an increase in the intercellular spaces in the follicular epithelium, a process termed "patency" in Rhodnius (Abu-Hakima and Davey, 1975). It was shown for Rhodnius that "patency" was under the direct control of JH and independent of the availability of Vg for uptake by the oocytes (Abu-Hakima and Davey, 1977).

In the adult female of some insects JH is also necessary for reproductive processes other than oocyte maturation. It has been shown to regulate the maturation of female accessory reproductive glands in the desert locust, Schistocerca gregaria (Szopa, 1981), the left colleterial glands of cockroaches (Adiyodi and Adiyodi, 1974; Weaver, 1981; Pau, 1981), synthesis of the sex pheromone in the cockroach, Byrsotria fumigata (Barth, 1962 cited in Adiyodi and Adiyodi, 1974), and sexual maturation in male S. gregaria (Amersinghe, 1978).

In the adult male S. gregaria, development of the accessory reproductive glands, which provide material for spermatophore formation, has also been shown to be under the influence of JH

(Odhiambo, 1966).

The regulatory functions of JH and ecdysone in the growth and development of insects and in their reproductive processes clearly warrant research directed at understanding the mode and molecular basis of action. Analysis at the molecular level is facilitated if a cell can be induced by a hormone or another stimulus to produce products of a few genes in large amounts. Some reproductive processes, such as vitellogenin synthesis, fulfill this criterion better than premetamorphic development.

Vitellogenins in Insects

During a study of hemolymph antigens in the saturniid moth, Hyalophora cecropia, Telfer (1954) observed a female-specific protein which declined in titer during oogenesis, apparently as a result of uptake by the developing oocytes. This protein was later shown to be made in the fat body and named vitellogenin (Vg) on the basis of its function as a precursor to the yolk (Pan et al., 1969). Subsequent purification and biochemical characterization of Vgs has revealed that they are glycolipoproteins generally of high native molecular weight. Upon denaturation, a varying number of different sized polypeptides are released, some of which, at least, are products of post-translational cleavage (reviewed by Hagedorn and Kunkel, 1979; Engelmann, 1979; Wyatt and Pan, 1978; also Izumi et al., 1980; Harnish and White, 1982a; Giorgi et al., 1982). Harnish and White (1982b) have proposed an evolutionary model of Vg structure based on

the polypeptide size of vitellins of a number of insect species (Harnish and White, 1982a) as well as the early products of translation (Harnish et al., 1982). According to this model, all known insect vitellogenins are assumed to be derived from an ancestral gene with two coding regions which are reflected in the two polypeptide domains of the Vg of most present day insects.

Endocrine Control of Vitellogenin Synthesis

It was more than 10 years after Telfer's (1954) discovery of the female-specific protein in Hyalophora that research on the endocrine regulation of insect Vg synthesis began. Convincing evidence for the involvement of JH in Vg synthesis was first obtained by Engelmann and Penny (1966) in the woodroach, Leucophaea maderae. They showed that an antigen characteristic of reproductively mature females, which was a prominent yolk protein, failed to appear after allatectomy. The effect of allatectomy could be reversed by implantation of corpora allata or application of JH (Engelmann, 1969). Engelmann (1971) also demonstrated that when allatectomized females were injected with any of the three then known JHs, de novo synthesis of Vg took place in a dose-related manner. Since then, similar dose responses have been obtained for JH-induced Vg synthesis in some other insects (Pan and Wyatt, 1976; Bühlmann, 1976; Chen et al., 1979).

Although these results demonstrated a role of JH in regulation of Vg synthesis, they did not rule out the possibility of an indirect effect. A direct effect of JH on the fat body for inducing Vg

synthesis can be shown in a completely *in vitro* system. However, this has been difficult to achieve and only two reports have appeared so far (Wyatt *et al.*, 1976; Abu-Hakima, 1981).

The transcriptional inhibitors, actinomycin D and α-amanitin, have been used to probe into the molecular level of JH action (Engelmann, 1971, 1976). The results argued in favour of JH control of vitellogenin mRNA transcription. This was further supported by isolation, from egg maturing females, of a class of polysomes containing 40 ribosomes and nascent Vg polypeptide chains. The heavy polysome fraction was not present in polysome profiles from non-vitellogenic females or males (Engelmann, 1977). When the RNAs of the Vg containing polysomes were fractionated on oligo(dT)-cellulose and the poly(A)+ fractions translated *in vitro*, a product immunoprecipitable by anti-Vg serum was obtained (Engelmann, 1978; 1980). Recently, more direct supporting data have come from the work on *Locusta migratoria* by actually monitoring the levels of $mRNA_{Vg}$ in fat bodies exposed to methoprene (see below and Chapter 5).

In *Oncopeltus fasciatus* a different regulatory role for JH has been suggested in vitellogenesis. Immunoelectrophoretic analysis of the blood of immature and precocious (by treatment with precocene in early fourth instar) adult females of *O. fasciatus* showed the presence of the A form of Vg but not the AB form, which is present in mature adult female hemolymph along with the A form (Kelly and Hunt, 1982). Rankin and Jackle (1980) suggested that Vg A may be a precursor of the Vg AB (their Vg B) since it had a M_r of 200,000 , approximately three times that of Vg AB (M_r 68,000) and analysis by limited proteolysis

showed the two forms to be nearly identical. Kelly and Hunt (1982) observed that in immature and precocious adult females, the appearance of the AB form could be induced with JH, thus supporting the hypothesis of Kelly and Telfer (1977) that JH induced the conversion of Vg A to Vg AB, perhaps enzymatically. However, this interesting hypothesis needs to be substantiated with more experimental data.

Vitellogenin synthesis is not entirely JH-dependent in all insects. In a few insects, the steroid hormone, ecdysone plays an important role in the control of Vg synthesis (Hagedorn and Kunkel, 1979; Engelmann, 1979; de Loof et al., 1981; Fuchs and Kang, 1981: Postlethwait and Jowett, 1981; Bownes, 1982) although JH is also involved directly or indirectly in the process.

That ecdysone is involved in the initiation of egg maturation in the anautogenous mosquito, A. aegypti, was first demonstrated by Spielman et al. (1971). Since then, mainly through the efforts of Hagedorn and collaborators and also Lea, the scheme that has emerged for the endocrine regulation of Vg synthesis in A. aegypti is as follows: Soon after eclosion and before a blood meal is taken by the female, JH stimulates the previtellogenic development of the ovary (Gwadz and Spielman, 1973). After a blood meal, an ovarian factor (Borovsky, 1982; Lea and Van Handel, 1982) triggers the release of the egg development neurosecretory hormone (EDNH) stored in the corpus cardiacum (Lea, 1972). In response to EDNH, the ovary secretes ecdysone which, after conversion to ecdysterone, initiated Vg synthesis in the fat body (Hagedorn et al., 1975; Hagedorn et al., 1979; Hanaoka and Hagedorn, 1980). However, a previous exposure to

JH is needed before the ovary can respond to EDNH (Shapiro and Hagedorn, 1980) and the fat body to ecdysterone (Flanagan and Hagedorn, 1977).

While much of the above scheme is accepted, and additional support for it has come from recent work on the endocrine control of Vg synthesis in the autogenous mosquito, A. atropalpus (Fuchs et al., 1980; Kelly and Fuchs, 1980; Masler et al., 1980; Kelly et al., 1981), the postulated Vg-inducing role of ecdysone in A. aegypti is debated. After an unsuccessful attempt by Borovsky and Van Handel (1979) to stimulate Vg synthesis in fat bodies of unfed mosquitoes cultured with ecdysterone added in vitro, Borovsky (1981) admitted success in obtaining induction of Vg synthesis in vitro. However, the response was very weak, in contrast to a strong response reported earlier (Fallon et al., 1974). More recently, Lea (1982) failed to observe yolk deposition in sugar-fed A. aegypti females with physiological doses of ecdysterone or implantation of ecdysone secreting ovaries. To complicate the story further, Borovsky (1981) reported that ovarian development and Vg synthesis in decapitated blood-fed A. aegypti could be restored with doses of JH closer to physiological levels than the required doses of ecdysterone. Similarly, JH has been shown to quantitatively potentiate the effect of ecdysterone in inducing Vg synthesis in A. atropalpus (Kelly and Fuchs, 1980). Hence, although the endocrine control of vitellogenesis in mosquitoes is not yet clear, there is evidence for roles of both ecdysterone and JH in this process.

The model originally proposed for the endocrine regulation of Vg

synthesis in A. aegypti has been used as the working hypothesis for the same process in Drosophila. In Drosophila, in contrast to the situation in most insects, both the fat body and the ovary have been shown to be involved in yolk polypeptide (YP) synthesis (Bownes and Hames, 1978; Hames and Bownes, 1978; Postlethwait and Kaschnitz, 1978; Bownes, 1980; Postlethwait et al., 1980a; Brennan et al., 1982). The hormonal regulation of YP synthesis in Drosophila has been investigated by surgical manipulations such as decapitation and abdomen isolation, hormone replacement therapy, ovary transplantation and organ culture experiments. In this respect, mutants that are defective in JH or ecdysone production or in various steps of vitellogenesis have been very useful. As a result, it was suggested that the brain, under the influence of photoperiodic cues, stimulates the corpora allata to produce JH which then initiates vitellogenesis (Handler and Postlethwait, 1977, 1978). Since YP synthesis in isolated abdomens could be restored with either methoprene or ecdysterone (Postlethwait and Handler, 1979; Jowett and Postlethwait, 1980) and males could be stimulated to produce YPs only with ecdysterone (Postlethwait et al., 1980a; Jowett and Postlethwait, 1980; Bownes, 1982), the question of which hormone acted on which tissue was resolved by Jowett and Postlethwait (1980). They demonstrated that the fat body responded to either hormone in producing YPs, whereas the ovary responded only to methoprene. This led to the speculation that JH acted on some female-specific tissue in the abdomen to produce ecdysteroids which in turn stimulated the fat body to produce YPs. However, the ovaries were shown not to play such

a role in initiating YP synthesis in the fat body (Bownes, 1980: Postlethwait et al., 1980b). The failure to observe an increase in ecdysteroid titers in isolated abdomens injected with methoprene also argues against the above hypothesis (Handler, 1982). Hence, while it is clear from experiments in vitro that both JH and ecdysterone are involved in regulating Vg synthesis in Drosophila, more experiments are needed before their precise roles are understood. In this direction, it is worth mentioning that recent experiments using an in vitro system have failed to demonstrate a direct effect of ecdysterone to stimulate YP synthesis in fat bodies of either sex (Bownes, 1982).

At the cytogenetic and molecular level, the genes for the 3 YPs have been shown to be sex-linked (Postlethwait and Jowett, 1980; Barnett et al., 1980; Riddell et al., 1981; Hovemann and Galer, 1982; Hung et al., 1982). The cloned YP-genes have been used to study the molecular level of ecdysterone action in the fat body of Drosophila (Shirk et al., 1983). After exposure to ecdysterone, co-ordinate increases in YP synthesis, translatable YP mRNA and YP1 and YP3 mRNA transcripts were observed in both sexes, but the relative response in males was always less than that in females. While methoprene did not evoke any measurable increase in YP mRNA transcripts in adult males, its effect on female fat body was not tested. These differences in gene expression in response to ecdysterone and methoprene in males and females may indicate that the fat body or the YP genes are responding to each hormone with a distinctive mechanism which is sex-specifically established.

Vitellogenin Synthesis in the Migratory Locust, Locusta migratoria

As an experimental insect for the study of molecular aspects of JH-induced Vg synthesis, L. migratoria has certain advantages: large size, rapid growth and development, established methods of mass rearing and the absence of bacterial symbiotes from the fat body. Moreover, there is already an extensive background of physiological and endocrinological research on this species (Uvarov, 1966). Amongst insects in which Vg synthesis is under the control of JH, L. migratoria is perhaps the most intensively studied. Both Vg and Vn have been purified and biochemically characterized. Cytological and molecular changes in the fat body during normal development and hormonally induced states have also been examined (Chen et al., 1976; Chen and Wyatt, 1981).

Electrophoretic and immunochemical analysis of the hemolymph of L. migratoria showed the presence of an adult female-specific protein (Vg) which was also present in egg extracts (Vn: Chen et al., 1976, 1978; Gellissen and Emmerich, 1978; Chinzei et al., 1980). Purified Vg and Vn have a native molecular weight of 550,000 (Gellissen et al., 1976; Chen et al., 1978; Chinzei et al., 1980). However, pulse-chase experiments and translation of $mRNA_{Vg}$ in Xenopus oocytes showed that the early products of amino acid labelling have molecular weights of 235,000 and 225,000 (Chen et al., 1978; Chen, 1980). Tryptic mapping and proteolytic digestion of these two polypeptides showed them to be different, suggesting that they were the products of two different structural genes (Chen, 1980). It was hypothesized

that, within the fat body, these polypeptides were enzymatically cleaved at sensitive sites, but remained together due to weak chemical interactions amongst the processed polypeptides. Electrophoresis of these "processed" polypeptides in the presence of SDS separated them into two sets ranging in molecular weight from 126,000 to 54,000 (Gellissen et al., 1976; Chen et al., 1978; Chen, 1980; Chinzei et al., 1980). It was thought that before secretion into the hemolymph, the polypeptides underwent glycosylation, lipidation, dimerization and "processing". Recent work (Wyatt and Belland, unpublished) has provided evidence for glycosylation of the primary polypeptides and shown that: (i) pulse-labelling of fat body with [^{3}H]leucine in the presence of tunicamycin abolished incorporation at the 235,000 and 225,000 M_r polypeptides and instead incorporation was observed into two products with molecular weights of 205,000 and 180,000, (ii) the products of cell-free translation of mature, adult female fat body RNA were similar in size to those observed in cells under tunicamycin inhibition, and (iii) fat bodies cultured in the presence of [^{3}H]mannose showed incorporation of the label into the 235,000 and 225,000 M_r products. From these results, it is concluded that the primary products of translation have molecular weights of 205,000 and 180,000, and the 235,000 and 225,000 M_r products are derived from them by glycosylation (but which comes from which is not yet known).

Endocrine control and vitellogenin synthesis during a normal vitellogenic cycle

Earlier work on L. migratoria had demonstrated that both the

neurosecretory system and the corpora allata are required for the production of mature oocytes (McCaffery, 1976; Minks, 1967). The median neurosecretory cells were implicated in activating and maintaining the activity of the corpora allata during egg development (McCaffery, 1976). These results have been further supported by measuring the JH biosynthetic rates of corpora allata from females whose median neurosecretory cells had been either electrostimulated or electrocoagulated (Tobe et al., 1982; Pratt and Pener, 1983). The fluctuations in the hemolymph JH titers and the corpus allatum activity in vitro have been correlated with hemolymph Vg titers and maturation of ooctyes (Johnson and Hill, 1975; Ferenz and Kaufner, 1981; Girardie et al., 1981; Rembold, 1981).

If the corpora allata of newly ecdysed adult females are removed surgically or chemically with ethoxyprecocene, fat bodies from these insects do not synthesize Vg and oocytes do not mature (Chen et al., 1976; Lazarovici and Pener, 1977; Chen et al., 1979). Vitellogenin synthesis, however, can be restored in the allatectomized females by either implantation of active corpora allata or by injection or topical application of JH-I, JH-II or its active analog, methoprene (Lazarovici and Pener, 1977; Chen et al., 1979). The dose-response relationships after topical application of JH-I and methoprene in inducing Vg synthesis in fat bodies of previously allatectomized adult females have been determined (Chen et al., 1979). Results obtained after injection of the analog will be reported below (Chapter 3; Dhadialla and Wyatt, 1983). Normal sigmoid curves were obtained for the relationship to the logarithm of JH-I or methoprene dose. It is

interesting to note that although JH-III has been identified as the principal JH in adult L. migratoria (Huibregtse-Minderhoud et al., 1980; Bergot et al., 1981; Rembold, 1981), JH-I and II were more effective in inducing oocyte maturation in allatectomized females (Lazarovici and Pener, 1977).

The above observations demonstrate JH control of Vg synthesis in L. migratoria. However, proof of direct induction of Vg synthesis in the fat body must come from experiments with fat bodies cultured in the presence of JH. This has been difficult to accomplish and initially only weak irreproducible responses were reported for Locusta (Wyatt et al., 1976). Recently, much stronger responses to JH in vitro have been reported (Abu-Hakima, 1981), but difficulty in reproducing some of these results will be described below (Chapter 4). Difficulties have also been encountered for in vitro induction of Vg synthesis in fat bodies from mosquitoes (Borovsky, 1981) and Drosophila (Bownes, 1982), which suggests that the limited success may be due to technical problems.

In normal adult females, Vg first appeared in the hemolymph around eight days after eclosion, rose to a maximum and then fluctuated with successive gonadotropic cycles (Gellissen and Emmerich, 1978). Chen et al. (1979) obtained a similar pattern for Vg synthetic capacity of adult female fat bodies by incubating them with [^{3}H]leucine followed by specific immunoprecipitation of secreted Vg from the medium. The normal variation in the onset of Vg synthesis in a locust colony can be circumvented by correlating the vitellogenic capacity of the fat body to the length of terminal oocytes during a gonotrophic cycle.

The rate of Vg output during a vitellogenic cycle rose from zero to a maximum at 4-5 mm terminal oocyte length and then fell back almost to zero when the oocytes were mature (Gellissen and Wyatt, 1981). However, a similar sharp rise and fall was not observed by Harry and Applebaum (1982). At the peak of its synthesis about 60% of the labelled protein secreted by the fat body was Vg (Gellissen and Wyatt, 1981).

Vitellogenin is detectable only in the hemolymph of adult females, although JH is present in the hemolymph of both sexes of all developmental stages (Johnson and Hill, 1973, 1975). Earlier attempts to induce Vg synthesis in adult males by implantation of adult female corpora allata or by injection of high doses of methoprene were unsuccessful (Chen and Wyatt, 1981). However, after it was shown by Kunkel (1981) that in the cockroach, Blattella germanica, both larval males and females could be induced to synthesize Vg, experiments on L. migratoria were re-initiated (Chapter III).

Cytological and molecular changes associated with normal development of vitellogenin synthesis

After adult ecdysis, the female fat body prepares itself for Vg synthesis in response to JH by undergoing cytological changes that are typical of a cell becoming active in protein synthesis (Lauverjat, 1977; Couble et al., 1979; Irvine and Brasch, 1981). Fat body cells of previtellogenic females were packed with lipid and glycogen and the rough endoplasmic reticulum and Golgi complexes were scarce in the cytoplasm. The nuclei, compressed between lipid globules, were

irregular in shape. As the fat body matured into a vitellogenic condition, the amount of lipid and glycogen decreased while the rough endoplasmic reticulum and Golgi increased. Moreover, the nuclei became round, enlarged greatly and exhibited intense basophilic staining. The latter changes suggested an increase in nuclear DNA, which was confirmed by biochemical analysis (Chen et al., 1979; Irvine and Brasch, 1981). The fat body cells did not undergo mitosis, but became polyploid (Nair et al., 1981; Irvine and Brasch, 1981).

By means of different techniques to determine the ploidy levels in the fat bodies of adult females, Irvine and Brasch (1981) and Nair et al. (1981) found that at adult ecdysis the fat body cells were largely tetraploid. By the onset of Vg synthesis octaploid fat body cells were predominant, and higher ploidy levels were subsequently attained. The fat body cells in adult males also underwent polyploidization, but had a lower average ploidy level than in females.

As a step to study the level of gene expression at which JH acted, studies to monitor changes in the amount of $mRNA_{Vg}$ during a gonadotropic cycle were initiated (Chinzei et al., 1982). Electrophoresis of fat body total RNA on agarose-methyl mercuric hydroxide gels showed a sharp band of about 6,300 nt, which was not present in total RNA samples from males or from pre-vitellogenic and allatectomized females (Chinzei et al.,1982). This RNA component was purified by oligo(dT)-cellulose chromatography and sucrose density gradient centrifugation (Chinzei et al., 1982) and identified as $mRNA_{Vg}$ by translation in Xenopus oocytes (Chen, 1980; Chinzei et al., 1982).

In the absence of a specific cloned hybridization probe at the time, Chinzei et al. (1982) used photometric scanning of photographs of electrophoretically separated total RNA samples to monitor changes in $mRNA_{Vg}$ in fat bodies during the gonadotropic cycle. They found that total RNA (chiefly rRNA) started to accumulate at about the same time as $mRNA_{Vg}$ did, both reaching maximum levels at 4-5 mm oocyte length, corresponding to a stage when Vg output was also found to be at its peak. Vitellogenin-specific polysomes were identified as a fraction containing about 40-50 ribosome monomers, present in vitellogenic female fat body, which was precipitated by Vn antiserum (Reid and Chen, 1981). On comparing the results for Vg output (Gellissen and Wyatt, 1981), Vg-containing polysomes (Reid and Chen, 1981) and $mRNA_{Vg}$ levels (Chinzei et al., 1982) good correlations could be drawn during the rising phase of the vitellogenic cycle. After that, the correlation seemed to break down. While Vg production decreased to zero level and specific Vg-polysomes disappeared completely at 7 mm oocyte length, $mRNA_{Vg}$ levels decreased only slightly. These data suggested some form of a translational control at the end of a gonadotropic cycle.

Vitellogenin induction in vivo with methoprene

The cytological changes along with the increase in ploidy level in the fat bodies from previtellogenic to vitellogenic stages could be prevented by allatectomy of newly ecdysed adult females and subsequently restored with methoprene (Couble et al., 1979; Irvine and Brasch, 1981; Nair et al., 1981). Likewise, the molecular events

at the RNA and protein level could be prevented by allatectomy and induced with methoprene. Experimental stimulation by hormone analog allowed changes to be observed with greater time precision than was possible during normal development.

When allatectomized females were topically treated with 250 µg methoprene (primary response) and the rate of Vg secretion from fat bodies was followed after various time intervals, Vg was produced at a slow rate for the first 48 h (Chen et al., 1979). The rate of synthesis then increased steeply to a maximum by 72 h and then declined sharply. A second exposure to the hormone (secondary response), after the effect of the first exposure had decayed, resulted in a rapid production of Vg, peak levels being attained at 48 h. This enhanced response for secondary Vg synthesis suggested the presence of a "cellular memory" effect after primary stimulation, which could be due to accelerated $mRNA_{Vg}$ synthesis or increased efficiency of translation due to retention of a population of ribosomes.

Changes in the levels of $mRNA_{Vg}$ in fat bodies from previously allatectomized locusts during primary and secondary stimulation by methoprene were followed by photometric scanning of electrophoresed total RNA on stained gels (Chinzei et al., 1982). Upon primary hormonal stimulation, $mRNA_{Vg}$ was first detected at 48 h and accumulated rapidly only after a lag of about 4 days. Total RNA increased rapidly beginning about 24 h. The secondary response was induced 5 weeks after the first, since this time was required for Vg in the hemolymph to reach immunochemically undetectable levels after

the primary stimulation by injection of 150 µg methoprene/locust (Chinzei and Wyatt, unpublished results). A second exposure to the hormone induced rapid accumulation of $mRNA_{Vg}$ in fat bodies with a reduced lag accompanied by only a small increase in total RNA.

Similar differences in rates of accumulation during primary and secondary responses have been observed for Vg-specific polysomes (Reid and Chen, 1981). Even with high doses of methoprene injected (300 µg/locust), Vg-specific polysomes were first detected at 48 h during primary stimulation and reached maximum levels at 72 h. Curiously enough, 96 h after primary stimulation, the heavy Vg-synthesizing polysomes were absent, although with only 150 µg methoprene, Chinzei and Wyatt (unpublished results) could immunochemically detect Vg in the hemolymph for at least four weeks after hormone treatment. Chen and Reid (1981) also observed that 14 days after primary stimulation, a second treatment with 300 µg methoprene/locust resulted in an increase in Vg-specific polysomes by 24 h. Unlike the situation in primary hormonal stimulation, the increase in Vg-specific polysomes during secondary stimulation occurred without prior increase in monomers and light weight polysomes. Once again, a comparison of data (Chen and Reid, 1981; Chinzei et al., 1982) reveals the selective nature of the methoprene effect on Vg gene expression during secondary stimulation, since it did not involve enhanced ribosomal synthesis or general protein synthesis. Vitellogenin mRNA accumulated for several days past the time of maximal Vg-polysome level and cellular Vg production, again implicating some form of translational control.

Juvenile hormone receptor protein in fat body

The actions of hormones on tissues are generally mediated by specific receptor proteins (Vedeckis et al., 1978; Yund et al., 1978; Westely, 1979). The classical scheme that has developed for vertebrate steroid hormone action is that a steroid, after passive diffusion into the cell, binds to cytoplasmic receptors which function as vehicles in translocating it into the nucleus (Yamomoto and Alberts, 1976; Gorski and Gannon, 1976). In the nucleus the steroid-receptor complexes bind to specific acceptor sites to initiate transcriptional activity (Spelsberg, 1982).

Although JH is not a steroid, there is increasing evidence that it may act in a manner analogous to the mode of action of steroid hormones (Engelmann, 1980; Chen and Wyatt, 1981). In the fat body from L. migratoria, a cytosol component with a binding specificity for JH-I has been characterized (Roberts and Wyatt, 1983). This presumptive JH-receptor protein had a binding affinity (K_D) of 1.7×10^{-8} M for JH-I and exhibited similar affinity for JH-III, which is the predominant JH in L. migratoria. The relative binding specificity of the receptor protein for juvenile hormones and their analogs, shown by competition assay, was JH-I $\approx$ JH-III > methoprene >> hydroprene $\approx$ kinoprene.

The presence of high affinity JH-binding sites has also been detected in the fat body and ovarian tissue of L. maderae (Engelmann, 1981; Koeppe et al., 1981), in cytosol from D. hydei epidermis (Klages et al., 1980) and D. melanogaster K_c cells (Chang et al., 1980). These investigations are promising and future work on the

changes in cytosol and nuclear hormone receptor complexes in hormonally induced tissues should contribute to elucidation of the mode of JH action.

Molecular cloning of vitellogenin genes

In order to find the number of Vg genes, identify the 5' end regulatory sequences necessary for gene expression and investigate the molecular basis of JH action, it was necessary to clone the Vg genes. The pulse-labelling experiments with L. migratoria fat body and the translation of $mRNA_{Vg}$, described above, showed the presence of two primary polypeptides which appeared to be the products of two structural genes. Vitellogenin mRNA, however, has not been electrophoretically resolved into more than one band on agarose - methyl mercuric hydroxide gels. In Xenopus, the 6,300 nt long $mRNA_{Vg}$ also appears as single stained band on gels, but a family of Vg genes have been discovered (Wahli et al., 1979; Tata et al., 1980). Four of these genes were shown to be expressed and to code for 4 different $mRNA_{Vg}$ of identical electrophoretic mobility (Wahli et al., 1979; Felber et al., 1980).

The molecular cloning of L. migratoria Vg gene sequences commenced with the construction and characterization of clones with cDNA to locust $mRNA_{Vg}$ (Wyatt et al., 1981). Four female-specific cDNA clones (pcLmVg 1-4) were isolated which, on northern blots of electrophoresed male and female total RNA, hybridized specifically to the $mRNA_{Vg}$ band in female RNA preparations. Southern blots of restriction digest of each of the cDNA clones, probed with each clone, showed that the four

fell into two groups. This suggested that these groups represented either two different genes or sequences which were not at the 3′ termini of $mRNA_{Vg}$ (oligo(dT) had been used as the primer for cDNA synthesis).

The next step was construction of a L. migratoria genomic DNA library by partial cleavage with the restriction enzyme EcoRI and cloning in the λ vector Charon 4 (Wyatt et al., 1981). cDNAs to female fat body RNA and the cloned cDNA sequences were used to screen this genomic library and two clones were isolated which were female-specific and carried sequences complementary to $mRNA_{Vg}$. Restriction site mapping, heteroduplex and cross hybridization analysis showed that these clones (λLmVg128 and 144) carried apparently identical sequences of locust DNA, except that one of them extended to an additional EcoRI site. They include the probable 3′-end of the coding region of a Vg gene (gene A: J. Locke, unpublished).

Sequences in one of these genomic clones were sub-cloned, and one sub-clone, pLmVg144-4.6, has been used to demonstrate the chromosomal occurrence of this gene (Bradfield and Wyatt, 1983). This gene is present at one copy per haploid genome in females and at only one-half the number in males. Similar results for Vg gene copy number in females were reported by using cloned DNA copied from Locusta $mRNA_{Vg}$ (James et al., 1982). Together with data on the occurrence of a restriction fragment length polymorphism, these findings localized L. migratoria Vg gene A to the X-chromosome (Bradfield and Wyatt, 1983). The sub-clone, pLmVg144-4.6 has also been used in the present work for

assay of $mRNA_{Vg}$ (Chapter 5).

More recently, two additional L. migratoria genomic libraries have been constructed using different restriction enzymes, and genomic clones with restriction sites and sequences different from λLmVg144 have been isolated. These represent a second L. migratoria Vg gene (gene B; J. Locke, unpublished results). In the near future, interesting structural and regulatory information on Vg genes should be forthcoming.

Research Objectives

When this project was started, there was already a fair amount of information on various aspects of JH controlled Vg synthesis in L. migratoria. However, the experimental procedures and techniques, mainly in the route and dose of hormone application, had evolved, so that correlations and interpretations had to be made on the basis of results which were not strictly comparable. A first objective, therefore, was to obtain a dose-relationship for the action of methoprene (injected) in vivo in inducing Vg synthesis in fat bodies of previously allatectomized adult female locusts.

In relation to this, it was important to know when during the adult stage is the fat body competent to respond to methoprene in producing Vg. If we could find a transition stage when Vg genes become expressible this might also indicate when to look for structural changes in the regulatory region of the Vg genes or their arrangement in the chromatin that allow their expression in the presence of JH.

After we learned that Dr. J.G. Kunkel (personal communication) had been able to induce Vg synthesis in both female and male penultimate larval instars of the cockroach, B. germanica, it was clearly worth determining whether a similar result might be obtained in L. migratoria. If larval males could be induced to synthesize Vg, this could identify a transition from an inducible to an uninducible stage, since earlier attempts at inducing Vg in adult males had been unsuccessful. These ideas were the genesis of the work presented in Chapter III.

The need to show direct stimulation of Vg synthesis in fat bodies with JH (or ecdysterone) or an analog in vitro has always been in the minds of researchers in the field, since results obtained in vivo cannot be conclusive about the direct action of a hormone, and because of toxicity problems in using inhibitors of biosynthetic steps in vivo. In Chapter IV, I report on some success in obtaining primary and secondary induction of Vg synthesis in vitro, and on my attempts and frustrations in establishing a long term fat body culture system.

Transcriptional activity of genes can be manifested in the accumulation of specific RNA transcripts. Chinzei et al. (1982) had been able to quantify $mRNA_{Vg}$ in fat bodies over extended periods of time after primary and secondary exposure of L. migratoria to methoprene in vivo. However, due to the relative insensitivity of the photometric technique used, $mRNA_{Vg}$ could be detected in primary induced fat bodies no earlier that 48 h. This seemed like a long lag period for JH to initiate transcription, especially in light of the results obtained with estradiol in oviparous vertebrates. Hence, as

soon as the first cloned Vg probe was available (thanks to J. Locke), I turned to the final objectives of this research, namely, to study simultaneously the kinetics of Vg synthesis and $mRNA_{Vg}$ accumulation in fat bodies after primary and secondary induction with methoprene *in vivo*. Because of the developmentally changing competence for Vg synthesis, it was also of interest to see how the fifth instar females responded in this assay. Equally interesting was to find the relative levels of $mRNA_{Vg}$ expression in both sexes of fifth larval instars and adults 72 h after the administration of methoprene. Finally, with the techniques developed, it was of interest to study the decay kinetics of $mRNA_{Vg}$ and Vg in the adult female fat body after removal of the JH source, the corpora allata. The results of these experiments have been presented in Chapter V.

II. MATERIALS AND METHODS

Materials

Locusts

Stocks of the African migratory locust, Locusta migratoria migratorioides, were obtained from Dr. B.G. Loughton (York University, Toronto) and the Centre for Overseas Pest Research, London.

Isotopes

All radiochemicals were purchased from New England Nuclear Corp. L-[4,5-^{3}H(N)]leucine was at a specific activity of 59.8 Ci/mmole and [methyl-^{3}H]thymidine had a specific activity of 22 Ci/mmole. α-[^{32}P]-dCTP was at a specific activity of 3200 Ci/mmole.

Chemicals

Omnifluor and Protosol were purchased from New England Nuclear Corp., Decon from British Drug Houses (BDH), oligo(dT)-cellulose from Collaborative Research and methylmercuric hydroxide from Alfa-Ventron Chemicals. Reagent grade phenol from Fisher was redistilled before use. Ultrapure sucrose (DNase and RNase free) for sucrose density gradient centrifugation was purchased from Bethesda Research Laboratories (BRL). Amino acids (L-form), vitamins and sugars for tissue culture media were obtained from Sigma Chemical Co. Rimocidin sulfate and Amphotericin B, methyl ester, were free samples from Pfizer Inc., and Squibb Canada Inc., respectively.

Nitrocellulose membrane (BA 85, pore size 0.45 μm) was obtained from Schleicher and Schuell and Gene Screen from New England Nuclear Corp.

Hormones

Precocene III (6-methoxy-7-ethoxy-2,2-dimethyl chromene) was purchased from Aldrich Chemical Company, Inc. The JH analogs, methoprene (ZR 515: isopropyl-11-methoxy-3,7,11-trimethyl dodeca-2, 4-dienoate) and hydroprene (ZR 512: 3,7,11-trimethyl-trans-2, trans-4-dodecadienoate) were generous gifts from Dr. G.B. Staal of Zoecon Corporation (Palo Alto, California).

Tissue Culture Plasticware

Tissue culture petri-dishes (35x10 mm, style #3001) and Multiwell tissue culture plates (#3047) were purchased from Falcon (Maryland).

Methods

Locusts

Locusta migratoria migratorioides were reared by the method of Hunter-Jones (1966). Locusts were kept under crowded conditions (about 50-75 adults of each sex in a cage 40 x 40 x 60 cm) maintained at about 34°C by a 60-watt light bulb for 16 h each day and falling at night to room temperature, which was maintained at 25°C. A mixture of bran, powdered skim milk and brewer's yeast (10:10:1) was available ad libitum, and wheat seedlings were provided daily. Under these

conditions the females first oviposited in the third week of adult life. Eggs were deposited in cups filled with moist sand, which were then covered and incubated at 30°C until they hatched (about 10 days).

Experimental locusts were kept in smaller cages under similar photoperiod and temperature conditions as for the colony. Wheat seedlings, cut free of the roots, were also provided daily.

Allatectomy and Hormone Administration

Adult locusts were allatectomized either surgically (Strong, 1963) or by treatment with ethoxyprecocene (Pener et al., 1978: Bowers and Feldlaufer, 1982; Pratt and Pener, 1983). The corpora allata were destroyed chemically by topical application of 500 µg ethoxyprecocene in 5 µl acetone on the neck membrane of adult locusts within 12 h after final ecdysis, as described by Chinzei et al. (1982). Adult females were surgically allatectomized when 2 or 14-16 days of age. All operations were carried out under CO_2 anaesthesia. The neck membrane was slit open dorsally, the head pulled down and the corpora allata pulled out with a fine pair of forceps. Care was taken to disturb or remove as little as possible of other tissue. A few crystals of penicillin G and streptomycin sulfate were dropped into the hemocoel before sealing the wound with a mixture of melted paraffin and bee's wax. A survival rate of 70-80% of the operated locusts was obtained. Two weeks later, the success of surgical or chemical allatectomy was confirmed by the absence of Vg as determined by rocket immunoelectrophoresis of a hemolymph sample taken from each individual.

The JH analogs, methoprene or hydroprene, were injected in 5 µl mineral oil, ventrally between the first and the second abdominal segments.

Fat Body Implantations

Fat bodies from fifth instar male or larval or adult female locusts were dissected in locust Ringer solution (Wyatt et al., 1976) and implanted into males on day 2 of the fifth instar, which allowed time for healing of the wound before the metamorphic molt. Recipient locusts were anaesthetized with CO_2, wiped with 70% ethanol, and an incision was made laterally in the third and fourth abdominal segments. Generally, the testes were removed and most of the fat body from a female was implanted in their place, but in some individuals fat body was implanted without testis removal, with no difference in results. The wound was first sealed with melted paraffin wax and then coated with a thin layer of rubber cement.

Preparation of Ringers, Media and Treatment of Glassware for Tissue Culture

Glass distilled and autoclaved water was used to prepare Ringers and tissue culture media. Locust Ringer and incorporation medium were prepared as described by Wyatt et al. (1976; Appendix I for recipes). Culture medium, S-20, for long term maintenance of fat bodies was prepared as described by Landureau (1976; Appendix I for recipe) with slight modification. Phosphorous acid as the buffering agent was replaced with 10 mM HEPES and 2.5 mM Na_2HPO_4. Also, in addition to

the suggested antibiotics, Rimocidin sulfate was added at a concentration of 120 µg/ml for its anti-fungal action.

The prepared Ringer and fat body culture media were filtered in a laminar flow tissue culture hood using a Nalgene sterilization filter unit (membrane pore size - 0.45 µm). The filtered solutions were stored in sterile 100 ml glass bottles.

All glassware for fat body culture was soaked in chromic acid solution for 15 min, thoroughly rinsed with distilled water, covered with aluminium foil and autoclaved before use. Sterile glass petri dishes for tissue culture, when needed for hormone treatment of cultured fat bodies in vitro, were coated with polyethylene glycol (PEG) by immersing in sterile 5% aqueous solution of PEG ($\overline{M}_t$ 17,000-20,000) for 5 h, rinsed with sterile water and dried at 100°C (Giese et al., 1977).

Fat Body Culture

Long-term: In order to induce vitellogenin synthesis in fat bodies in vitro with methoprene, it was necessary to maintain the tissues viable in culture medium for up to 72 h. All tissue culture procedures were carried out in a sterile laminar flow hood and dissecting equipment was rinsed in 70% ethanol and lightly flamed before use. Locusts were surface sterilized by washing successively with 0.1% Decon, 0.1% sodium hypochlorite, 0.1% hyamine hydroxide and 3 times with sterile distilled water. Fat bodies were dissected and transferred into locust Ringer. Large tracheal sacs and tracheae were gently separated from the fat body and fat bodies were rinsed 3 times

in S-20 before being transferred into 35 mm diameter glass petri dishes containing 2 ml S-20 medium. Cultures were incubated in a humidified box at 30°C with gentle rotary shaking.

Fat body cultures were examined every 24 h and discarded if contaminated with bacteria or fungi. At various times after the initiation of fat body cultures, tissue viability was determined by measuring incorporation of [^{3}H]leucine into secreted total proteins during 3 h culture in incorporation medium (Wyatt et al., 1976). Similarly, incorporation of [^{3}H]leucine into secreted Vg was measured by immunoprecipitation.

Short term: When fat bodies from hormone or mineral oil treated (control) locusts were to be cultured for the 3 h incorporation of [^{3}H]leucine or [^{3}H]thymidine, it was not necessary to disinfect locusts as extensively as for long term fat body cultures. Locusts were disinfected by rinsing with water, 70% ethanol and water. Fat bodies were dissected out, rinsed in locust Ringer and incorporation medium before being transferred into medium containing [^{3}H]leucine or [^{3}H]thymidine. Fat bodies were cultured either in Multiwell tissue culture plates or glass scintillation vials.

Measurement of Protein Synthesis in Vitro

Synthesis of vitellogenin and other secreted proteins was assayed by fat body cultured for 3 h as described before. Whole fat bodies or portions were cultured in 500 µl of incorporation medium containing [^{3}H]leucine (4 µCi/ml) and 30 µM L-leucine (final specific activity, 133 mCi/mmole) in Multiwell tissue culture plates. For experiments

involving simultaneous determination of secreted proteins and RNA content, fat bodies were incubated in liquid scintillation glass vials containing 1 ml of medium with labelled precursors. The cultures were shaken at 110 rpm on a rotary shaker at 30°C for 3 h.

The amount of label present in Vg in the medium after 3 h has been shown to reflect rates of Vg synthesis (Chen et al., 1979). After the culture period, the medium was spun at full speed (≈ 12,000 x g) in a Beckman microfuge for 5 min. Duplicate 100 µl portions of the culture medium were measured into 6 x 50 mm glass culture tubes, and Vg and total proteins were precipitated by anti-vitellin serum and TCA, respectively (Chen et al., 1979). Each immunoprecipitation contained 100 µl medium, 50 µg vitellin as carrier, 1% of each Triton-X and sodium deoxycholate and 50-75 µl anti-vitellin serum (the equivalence point determined in a separate experiment). Total protein was precipitated by making the medium 10% TCA in the presence of 100 µg BSA.

The immunoprecipitates were washed 3 times with washing buffer (0.1 M sodium phosphate, pH 7.4, 0.9% NaCl, 1% Triton-X, 1% sodium deoxycholate, 0.3% BSA and 0.1% L-leucine) by suspending and centrifuging (Gellissen and Wyatt, 1981). Acid-insoluble precipitates were centrifuged, suspended and washed three times in 5% TCA. Both types of precipitates were then solubilized in 50 µl Protosol overnight at room temperature. The solubilized samples were mixed with 0.8 ml Omnifluor and counted in the same 6 x 50 mm tube supported in standard scintillation vials in a Packard liquid scintillation counter at 25% efficiency for [^{3}H]. Blank values obtained from

uninduced male fat body (usually 100-300 cpm) were subtracted from the radioactivity of immunoprecipitates.

Determination of Fat Body Protein Content

When results were to be expressed relative to total soluble tissue protein, fat bodies were homogenized in cold homogenization buffer (0.05 M Tris-HCl pH 8.1, 0.4 M NaCl, 1% Triton-X, 1% sodium deoxycholate, 1 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride) in 0.5 ml Eppendorf tubes fitted with pestles (Bodnaryk, 1979). The homogenate was centrifuged in a Beckman microfuge at about 12,000 x g for 5 min and the supernatant (without the lipid layer) used for the estimation of soluble proteins by the method of Bramhall et al. (1969). Five and 10 µl portions of each sample were spotted on 2 cm square filter papers (Whatman 42) and dried in air. The protein was fixed by immersion in cold 7.5% TCA for at least 2 h and then heated at 80°C for 30 min. The acid solution was discarded and the filters washed in ethanol:ether (1:1 v/v) followed by ether alone for 30 min each. The papers were then stained in Coomassie brilliant blue G (10 mg/ml in 7% acetic acid) with frequent stirring at 50°C for 15 min, and the excess dye was removed with repeated changes of hot (50-60°C) 7% acetic acid, until the background was almost white. The papers were drained, dried and put in individual 10 ml glass tubes containing 5 ml destaining solution (66 ml methanol, 34 ml water and 1 ml concentrated ammonia). The dye released into the solution was measured at 610 nm. For each set of tissue protein estimations, a standard curve was generated with 0-35 µg BSA (1 mg/ml in 0.9% NaCl).

[^{3}H]Thymidine Incorporation and Determination of Fat Body DNA Content

For measurement of DNA synthesis, fat bodies of individual locusts or two fifth larval instars were incubated for 3 h in the presence of [^{3}H]thymidine (4 µCi/ml), under conditions for short term fat body culture described before. DNA from fat bodies was extracted as described by Dyer et al. (1981). Fat body was homogenized in 1 ml ice-cold 0.5 N PCA and kept on ice for 15 min and then centrifuged at 9,000 x g at 4°C. The pellet was washed 3 times with 2.5 ml cold 0.3 N PCA and hydrolyzed in 1 ml 0.3 N KOH for one hour at room temperature to remove RNA. DNA and protein were precipitated with 0.6 ml 1.2 N PCA in an ice bath and the precipitate was washed once with cold 0.3 N PCA. The DNA was extracted by 1 ml 0.5 N PCA at 70°C for 30 min. A 100 µl sample of the hydrolysate was counted in a Beckman liquid scintillation counter with 50% counting efficiency for ^{3}H.

When necessary, DNA in the hydrolysate was determined by the diphenylamine assay as described by Giles and Myers (1965). 0.3 ml of hydrolysed sample was added to 0.8 ml diphenylamine reagent (1.5% diphenylamine, 0.008% acetaldehyde in 1.5% sulphuric acid/98.5% acetic acid) and mixed immediately. All samples were processed in duplicate and allowed to sit for 12-16 h (covered with aluminium foil in the dark) at room temperature, before $A_{600\ nm}$ was read. Herring sperm DNA (1 mg/ml) samples in 0.5 N PCA (0-100 µg DNA) were hydrolysed and processed as for unknowns.

Preparation Of Vitellin

Vitellin was purified from ovaries as described by Chinzei et al. (1981). Ovaries, dissected from mature adult females, were rinsed in locust Ringer and homogenized in 10 volumes of cold 0.5 M KCl-0.5 mM EDTA-0.2 M sodium phosphate buffer (pH 7.5) and 1 mM PMSF. The homogenate was centrifuged at 12,000 x g at 4°C for 15 min and protein was precipitated from the supernatant with 70% saturated ammonium sulfate. The precipitate was dissolved in water and dialyzed against distilled water at 4°C. Dialysis was stopped when the first turbidity appeared, and the solution was adjusted to pH 6.0 with 0.2 M NaH_2PO_4. The precipitate was removed by centrifugation and the vitellin precipitated by dilution with chilled water. The precipitate was dissolved in 0.2 M sodium phosphate (pH 6.3) and the ionic strength lowered to 0.13-0.15 M phosphate with water at 20°C. Any precipitate that formed was removed by centrifugation and the supernatant was loaded on a DEAE-cellulose column equilibrated with 0.15 M sodium phosphate buffer (pH 6.3). The column was washed with the same buffer until the effluent gave A_{280} <0.01, and the Vn was eluted with 0.25 M KCl-0.1 M sodium phosphate buffer (pH 6.3). The fractions containing Vn were pooled and dialyzed against 0.05 M Tris-citrate pH 8.2. The dialysate containing Vn was lyophilized and stored at -20°C. When needed, Vn was redissolved in water and its protein content determined by the method of Bramhall et al. (1969).

Preparation of Anti-Vitellin Serum

One to two mg of partially purified Vn was mixed with Freund's complete adjuvant (1:1 by volume). The emulsion in about 1 ml was injected at multiple intramuscular sites into 6 month old rabbits weighing about 1 kg. Two booster injections of the same amount without adjuvant were given intravenously (ears) at 4 week intervals. After the second booster 2 mg Vn in 1 ml was again injected intravenously and a week later the rabbits were bled by intra-cardiac puncture. The blood collected in 30 ml Corex tubes was allowed to clot at 37°C for an hour, and the clot shrunk at 4°C overnight. The serum containing antibodies to Vn was collected with a pasteur pipette and centrifuged to remove red blood cells. The clear serum was made 0.05% with sodium azide and stored at -70°C in 4 ml aliquots.

The serum was rendered female-specific by absorbing it exhaustively with male locust hemolymph. Vitellin and Vg have been shown to be immunochemically identical (Chinzei et al., 1981).

Immunochemical Techniques

Ouchterlony immunodiffusion and rocket immunoelectrophoresis techinques were as described by Chinzei and Wyatt (in preparation). Plates for Ouchterlony immunodiffusion were prepared by pouring 1% agarose in 0.05 M sodium phosphate pH 7.2, containing 0.15 M NaCl and 0.05% sodium azide into 35 mm diameter petri dishes.

Rocket immunoelectrophoresis plates of uniform thickness were poured by pipetting, at 56°C, 1% agarose in 0.05 M Tris-citrate buffer (pH 8.2) containing female specific anti-Vn serum (usually 0.5%),

between two 10 cm square glass plates separated at two edges by 1 mm thick glass strips. After the agarose had solidified, the upper glass plate and the spacers were removed. Plates prepared in bulk were stored at 4°C in a humidified box containing 0.05% sodium azide. One plate from every batch was used to construct a curve with purified vitellin (0.25 to 1.25 µg). A linear relationship was obtained when rocket height was plotted against µg Vn applied. Along with hemolymph samples of unknown Vg content, a known quantity of Vn was routinely run as a standard.

Extraction of Fat Body RNA

All glassware was soaked in chromic acid solution for 15-30 min, washed extensively in distilled water, treated with 0.5% diethylpyrocarbonate (DEP) solution and autoclaved. Solutions (excluding Tris-containing buffers) and plasticware were treated with DEP to a final concentration of 0.5% before being autoclaved.

The sodium-dodecyl sulphate-phenol-chloroform method for RNA extraction was adapted from the procedure described by Rosen and Monahan (1981). Fat bodies were suspended in 10 volumes (w/v) of homogenization buffer (0.1 M sodium acetate pH 5.0, 25 mM NaCl, 5 mM $MgCl_2$, 25 µg/ml polyvinyl sulphate, 35 µg/ml spermidine and 0.5% SDS) and an equal volume of buffer-saturated phenol. The suspension was homogenized on ice for 2 x 1 min with a Polytron at setting 8. An equal volume of chloroform was added to the homogenate, and the resulting suspension shaken gently and centrifuged at 1,000 rpm at 4°C to separate the phases. The lower organic phase was removed by

aspiration, taking care not to disturb the interphase. The aqueous and interphases were re-extracted with an equal volume of chloroform (about 3 times) until the interphase disappeared entirely or ceased to diminish. The aqueous phase was precipitated by the addition of 2.5 volumes of cold ethanol overnight at -20°C.

The precipitate was collected by centrifugation at 9,500 x g for 10 min at -10°C, and dried under vacuum. The precipitate was dissolved at approximately 5 mg/ml in water and made 0.1% in SDS. The solution, in polyallomer tubes, was spun at 4°C for 36 min in the SB-283 rotor of the IEC B60 ultracentrifuge at 30,000 rpm to remove glycogen. The supernatant was made 10 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 5 mM EDTA, 0.5% SDS and 500 g/ml proteinase K was added (Gordon et al., 1978). The solution was incubated at 37°C for 2 h, phenol:chloroform extracted and precipitated again by the addition of 2.5 volume cold ethanol overnight at -20°C.

The precipitate was collected as above, dissolved in water, adjusted to 3 M sodium acetate (pH 5.8) and placed at -20°C for 30-60 min. The high salt precipitate was collected by centrifugation at 9,000 rpm at 4°C. The 3 M sodium acetate precipitations were repeated twice more. The precipitate was washed twice with cold 80% ethanol and dried under vacuum. The precipitate was dissolved in water and either stored at -20°C or passed over an oligo(dT)-cellulose column to enrich for poly(A)-containing RNA species.

RNA from individual fat bodies was extracted essentially as described above. Each fat body was homogenized using a micro-attachment to the Omnimixer (Sorvall, Connecticut). Glycogen

and DNA were removed with 3 M sodium acetate washes.

Oligo(dT)-Cellulose Column Chromatography

The method of Aviv and Leder (1972) was followed to enrich for poly(A)$^+$ sequences by oligo(dT)-cellulose column chromatography. The column and tubing were washed with 0.1 N NaOH to destroy any ribonucleases and then neutralized to pH 7.0. The column was equilibrated with binding buffer (10 mM Tris-HCl pH 7.5, 0.5 M NaCl). RNA dissolved at a concentration of 1 mg/ml in binding buffer was denatured at 65°C for 15 min and rapidly cooled before being recirculated 3 times over the oligo(dT)-column at 4°C at 20 ml/h. The column was washed with 150 ml binding buffer at 60-80 ml/h until the absorbance at 260 nm dropped to 0.01. This was followed by washing with 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl at about 80 ml/h until the A_{260} reading was again 0.01. The bound RNA was eluted at 20 ml/h with 10 mM Tris-HCl (pH 7.5) and the peak fractions (first poly(A)$^+$) were pooled.

The poly(A)-enriched fractions from above were made 0.5 M NaCl, heat denatured and loaded on the column for the second time and the bound poly(A)-containing RNA was eluted with water at 20 ml/h. The peak fractions were pooled, made 0.3 M sodium acetate and precipitated with 2.5 volume cold ethanol overnight at -20°C. About 3% of the total RNA was isolated as second pass poly(A)$^+$ RNA.

Sucrose Density Gradient Centrifugation

Vitellogenin mRNA ($mRNA_{Vg}$) was purified from poly(A)$^+$ RNA as

described by Chinzei et al. (1981). The gradient former (model 230, Biorad) and the tubing were washed with 0.5 M NaOH. The two were then extensively washed with sterile-DEP-treated water until the washings were neutral. Isokinetic 10-37.8% sucrose gradients in 12 ml of 25 mM Tris-HCl (pH 7.5), 1% SDS and 2 mM EDTA were pumped with a 4 channel pump (adjusted to equal flow) into the bottoms of polyallomer tubes. Poly(A)-containing RNA (100 µg) in the gradient buffer was denatured at 65°C for 5-7 min, rapidly cooled on ice and layered on top of the sucrose gradients. The gradients were centrifuged in the SB-283 rotor of the IEC B60 ultracentrifuge at 32,000 rpm at 20°C for 15 h and eluted through an ISCO ultraviolet absorbance monitor. Fractions under different peaks were separately pooled, brought to 0.3 M sodium acetate and precipitated with cold ethanol at -20°C overnight. The precipitates were centrifuged, washed with cold 80% ethanol, dried under vacuum and dissolved in an appropriate amount of sterile DEP-treated water. The purified fractions were stored at -70°C. Of the total poly(A)$^+$ RNA centrifuged, about 10% was recovered as enriched $mRNA_{Vg}$.

Electrophoresis of RNA

Electrophoretic analysis of RNA was achieved as described by Bailey and Davidson (1976). Agarose (1.2%) in 50 mM H_3BO_3, 5 mM sodium borate (pH 8.19), 10 mM sodium sulfate, 1 mM EDTA and 6 mM methyl mercuric hydroxide was poured on a horizontal slab to obtain a thickness of 6 mm. RNA was applied to the gel in borate buffer containing 3 mM methyl mercuric hydroxide, 2.5% glycerol and 0.5%

bromophenol blue. Electrophoresis was in a horizontal "submarine" apparatus at 70 volts for 5 h. The buffer was circulated between the electrode vessels throughout the electrophoresis period. Gels were stained for 20-30 min in 0.5 M ammonium acetate containing 2 µg/ml ethidium bromide. Pictures of the separated RNA were taken by fluorescence from ultraviolet illumination passed through a Corning 754 filter, on 400 ASA film with exposure ranging from 1-2 min.

Treatment and Northern Blotting of Gels

After the gels containing RNA were photographed, they were treated at room temperature with 50 mM NaOH, 5 mM 2-mercaptoethanol for 60 min with shaking followed by 2 x 10 min washes in 0.2 M potassium phosphate (pH 6.5), 7 mM iodoacetic acid. The gel was finally washed at room temperature 2 x 5 min in 25 mM sodium phosphate pH 6.5 for transfer onto Gene Screen. Gene Screen, blotting paper (Whatman 3MM) and paper towels used in the transfer were all cut to the exact size of the gel. Transfer of RNA from the gel to paper was done in the same buffer as used for the last gel wash.

Six sheets of Whatman 3MM soaked in buffer were placed over an elevated glass plate so that the ends formed wicks. The gel was placed on top of filter papers and Gene Screen was placed directly on the gel. The RNA binding paper was then covered with 4 layers of dry 3MM paper, several layers of absorbant paper towels, and finally a weight. The buffer was allowed to blot through for 15-18 h.

After the blotting period Gene Screen was carefully lifted off the gel and soaked in the reservoir buffer. It was carefully washed with

buffer by gentle rubbing with a gloved hand to remove residual agarose. The membrane was damp dried and RNA baked onto it at 80-100°C for 2-4 h in a vacuum oven. After this, the Gene Screen blot was either stored or soaked in prehybridization buffer.

Treatment of Gene Screen

The use of Gene Screen for northern blotting was found much easier and less tedious than preparing and using diazobenzyloxymethyl-paper (Alwine et al., 1977). Instructions in the New England Nuclear manual for the transfer and hybridization of RNA to Gene Screen were followed. Gene Screen pre-cut to the size of the gel was soaked in 25 mM sodium phosphate buffer (pH 6.5) for 20 min before placing on the gel.

Pre-Hybridization and Hybridizing Conditions for Northern Blots

Gene Screen blots were pre-hybridized under similar conditions adapted from Alwine et al. (1977). Blots with bound RNA were placed directly into 250 ml of pre-hybridization buffer containing 50% formamide, 0.9 M NaCl, 5 mM EDTA, 0.1% SDS, 10 x Denhardt's solution (Denhardt, 1966), 50 mM sodium phosphate (pH 7.0), 500 µg/ml sheared, salmon sperm DNA and 1% glycine and incubated at 42°C with shaking for at least 15 h.

The hybridization buffer was the same as the pre-hybridization solution minus glycine. Dextran sulfate (10%), as recommended by Wahl et al. (1979) was included in the hybridization solution. Nick-translated probe (specific activity of 1-2 x 10^8 cpm/µg DNA) was

added to 5-10 ml of hybridization solution, boiled for 10 min, quenched on ice and added to a sealable plastic bag containing the blot. The plastic bag was sealed and incubated at 42°C with constant agitation for 15-18 h.

After hybridization, the protocol for washing Gene Screen blots as described in the New England Nuclear manual was followed. The blots were washed once in 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate), 2 mM EDTA and 0.5% SDS for 15 min at room temperature and twice for 30 min in the same solution at 60°C. The blots were rinsed in the same solution at room temperature and washed three times 20 min in 3 mM Tris base at room temperature with constant agitation. The blots were damp dried, put in a freezer bag and exposed at -70°C using Dupont Cronex 4 film and a Dupont Lightning Plus intensifying screen.

Hybridization Probe and Nick Translation

Two genomic clones, containing sequences complementary to the 3' end of vitellogenin mRNA, were isolated by Mr. John Locke from a partial EcoRl *L. migratoria* genomic DNA library constructed by Dr. B.N. White with the bacteriophage lambda Charon 4 as the vector (Wyatt *et al.*, 1981). The insert in one of the clones, λLmVg144, was digested with EcoRl and HindIII and the digests were subcloned into the plasmid pAT153. A sub-clone, p144-4.6, containing the entire 4.6 kb of the coding region in λLmVg144 was isolated (Locke, unpublished data). DNA from this sub-clone was used for probing Northern blots and dot-blots.

DNA isolated from p144-4.6 was labelled by the nick-translation

technique of Rigby et al. (1977) using a limited DNase I digestion to generate 3′ hydroxyl groups for the polymerase reaction. The DNase I (1 mg/ml in 10 mM HCl) was stored at -20°C in 50 µl aliquot portions. Aliquots of DNase I were activated by dilution to 100 µg/ml in 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 1 mg/ml BSA followed by a 2 h incubation at 4°C. The activated DNase was stored frozen in small portions for use when needed. Immediately before use, the activated DNase I was diluted to 133 ng/ml in the same dilution buffer.

The standard labelling reaction of 20 µl contained 200 ng DNA to be labelled, 15 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 15 µM of each unlabelled dNTP and 3.6 µM α-[^{32}P]-dCTP. Diluted, activated DNase was added to a final concentration of 11.3 ng/ml and the reaction was allowed to proceed for 2 min at room temperature before 20 units of DNA polymerase I were added. The reaction was carried out at 14°C for 4 h and terminated by adjusting to 10 mM EDTA, 0.2% SDS, 5% glycerol and the mixture was then passed over a Sephadex G-50 column equilibrated with TNE_2 (10 mM Tris, pH 8.0, 10 mM NaCl and 2 mM EDTA), 0.1% SDS. Routinely, the DNA obtained from this reaction had a specific activity of 1-2 x 10^8 cpm/µg.

Quantification of Vitellogenin mRNA by Dot-Blot Hybridization

Vitellogenin mRNA in total RNA samples from individual fat bodies was quantified by spotting $mRNA_{Vg}$ on nitrocellulose membrane, hybridizing with ^{32}P-labelled p144-4.6, washing, cutting and counting the spots. The technique was modified from that described by Searle and Tata (1981).

Standard curves for each hybridization were constructed with appropriate dilutions of a purified $mRNA_{Vg}$ preparation used together with 2 µg adult male fat body total RNA in 50 µl 50 mM sodium phosphate buffer, pH 6.0. The standard curve covered a range from 10-8000 pg $mRNA_{Vg}$. Appropriate dilutions of total RNA of unknown $mRNA_{Vg}$ content were prepared in the same way as for the standard. Samples contained 2-15 µg total RNA. The larger amounts were used from RNA preparations expected to contain little or no $mRNA_{Vg}$. Each sample was prepared in duplicate and often two dilutions of one sample were used to monitor the variation due to pipetting and extent of hybridization. The RNA samples in the phosphate buffer were denatured by heating to 65°C for 2 min and cooled rapidly. To each sample 150 µl cold 20 x SSC was added.

A pre-cut nitrocellulose sheet (Schleicher and Schuell BA 45, 0.45 µm) was wetted with water and saturated with 20 x SSC. Samples (200 µl) were applied with a very low suction pressure to a 3 mm-diameter spot on the high salt saturated nitrocellulose sheet supported in a 96-hole Hybri-dot apparatus (Bethesda Research Laboratories). Any trapped bubbles at the bottom of the wells were removed by vigorous pipetting since they interfered with the flow of the sample solution. The wells were rinsed with additional 200 µl cold 20 x SSC. Three to five minutes after the sample buffer had been sucked through in all the wells, the nitrocellulose sheet was carefully removed, dried under a hot lamp and baked (80°C, 2 h) *in vacuo* to fix the RNA.

Prehybridization and hybridization conditions were the same as for Northern blots. However, 400 ng of ^{32}P-labelled p144-4.6 (specific

activity 1-2 x 10^8 cpm/μg) was mixed in 15 ml of hybridization solution. After 15-18 h hybridization period, the nitrocellulose sheet was washed with 250 ml 2 x SSC, 0.1% SDS, 4 x 5 min at room temperature and then two times with 250 ml of 0.1 x SSC, 0.1% SDS at 60°C for 30 min each (Wahl et al., 1979). The nitrocellulose sheet was damp dried, placed in a sealable bag and exposed for autoradiography as described earlier. Generally two autoradiographs of each blot were exposed, one for record and the other served as a template for cutting the individual spots from the nitrocellulose sheet. Each cut spot was dried, soaked in 5 ml of Scinti-verse and counted for 10 min in a Beckman liquid scintillation counter.

Analysis of the Dot-Blot Hybridization Data

The radioactivity non-specifically bound to spots loaded with adult male locust fat body RNA or rat liver RNA (80-250 cpm) was subtracted from the values obtained with known amounts of standard and samples. Linear regression analysis of the data showed a good correlation (correlation coefficient of 0.97 to 0.99) between the amount of $mRNA_{Vg}$ applied and the amount of radioactive probe hybridized, when plotted on a log-log scale. Vitellogenin mRNA amounts in unknown samples were estimated by reading from the standard curve. With this technique, the lower limit of detection (i.e. cpm significantly different from that for male locust fat body RNA or rat liver RNA) was 10 pg $mRNA_{Vg}$.

The numbers of $mRNA_{Vg}$ molecules per fat body cell were calculated as described by Chinzei et al. (1982). The calculation of average

number of fat body cells per individual was based on a DNA content of 40 µg and an average cellular ploidy level of 4.5 n for the newly molted adult female fat body (Chen et al., 1979; Nair et al., 1981; Irvine and Brasch, 1981) and assuming the diploid complement of DNA in L. migratoria to be 12.7 pg (Rees et al., 1978). Based on these values, the total number of fat body cells per individual was estimated as 1.4×10^6 (Chinzei et al., 1982). Knowing the molecular weight of $mRNA_{Vg}$ to be 6300 nucleotides ($M_r = 2.05 \times 10^6$), the number of molecules of $mRNA_{Vg}$ per microgram total RNA can be calculated and this value used to estimate the number of $mRNA_{Vg}$ molecules per fat body cell.

III. DEVELOPMENT OF SEX- AND STAGE-SPECIFIC COMPETENCY FOR JUVENILE HORMONE DEPENDENT VITELLOGENIN SYNTHESIS

Introduction

An important aspect of egg development in females of most insects and oviparous vertebrates is the hormonally regulated extra-ovarian synthesis of Vg. In oviparous vertebrates the female and male liver respond equally to estrogen, a sex-limited hormone, in synthesing Vg (Tata and Smith, 1979; Shapiro, 1982). However, in most insects, in spite of the presence of JH in all stages of development of both sexes, Vg is normally produced in adult females only (Engelmann, 1979). Hence, in contrast to oviparous vertebrates where the sex-limitation for Vg synthesis is dependent on the presence of the sex-hormone, estrogen, in insects it may reside in the competence for hormone-dependent expression of Vg genes.

In L. migratoria, Vg can be first detected in the hemolymph of sexually mature females around day 8 after the larval-adult ecdysis, correlated with and dependent upon the release of JH from the corpora allata (Johnson and Hill, 1973; Chen et al., 1976; Gellissen and Emmerich, 1978; Chen et al., 1979; Rembold, 1981). By the use of standard immunochemical techniques Vg cannot be detected in the hemolymph of larval stages or adult males, although JH is produced in larval and adult stages of both sexes (Johnson and Hill, 1973, 1975)

and, at least, in the adult male the fat body is a target tissue for JH action (Nair et al., 1981; Irvine and Brasch, 1981). Therefore, in order to understand the sex- and stage-specificity for Vg synthesis in L. migratoria, I have attempted to answer the following questions: (a) When, after the adult ecdysis, is the female fat body competent to respond to JH by synthesizing Vg? (b) Can the larval female fat body be induced to synthesize Vg? (c) Can fat body from any male stage be induced to synthesize Vg in response to JH or an analog? The rationale for posing these questions was to identify the transition of fat body from an uninducible stage to an inducible one or vice versa, since this would allow future investigations to be directed towards the molecular events associated with altered Vg gene expressibility.

Juvenile hormone, in addition to stimulating Vg synthesis in adult female, also controls DNA synthesis and nuclear ploidy levels in locust fat body throughout adult development in both sexes (Nair et al., 1981; Irvine and Brasch, 1981). In these studies it was reported that maximal DNA synthesis preceded maximal Vg synthesis. The significance of this relationship is not known but it presumably serves for the accelerated production of mRNA for Vg and other proteins. Hence, in an attempt to induce Vg synthesis in larval stages, the temporal relationship of DNA and Vg synthesis was also investigated.

Finally, experiments were conducted on the regional differentiation of the fat body at the biochemical level. Although these experiments are not within the main stream of the work, the results are interesting enough to be reported. Conflicting reports

have appeared in the literature on the morphological distinction between the deep (or paragonadal) fat body and the peripheral fat body (Lauverjat, 1977; Couble et al., 1979) with respect to differentiation for protein synthesis. In neither of these studies were the anatomical locations of the paragonadal and peripheral fat body clearly defined, which may have been a reason for disparity between results. The results in this study briefly describe the 3 regionally identifiable fat bodies in the locust and some experiments comparing the capacity of two types (paragonadal and perivisceral) to synthesize Vg.

Results

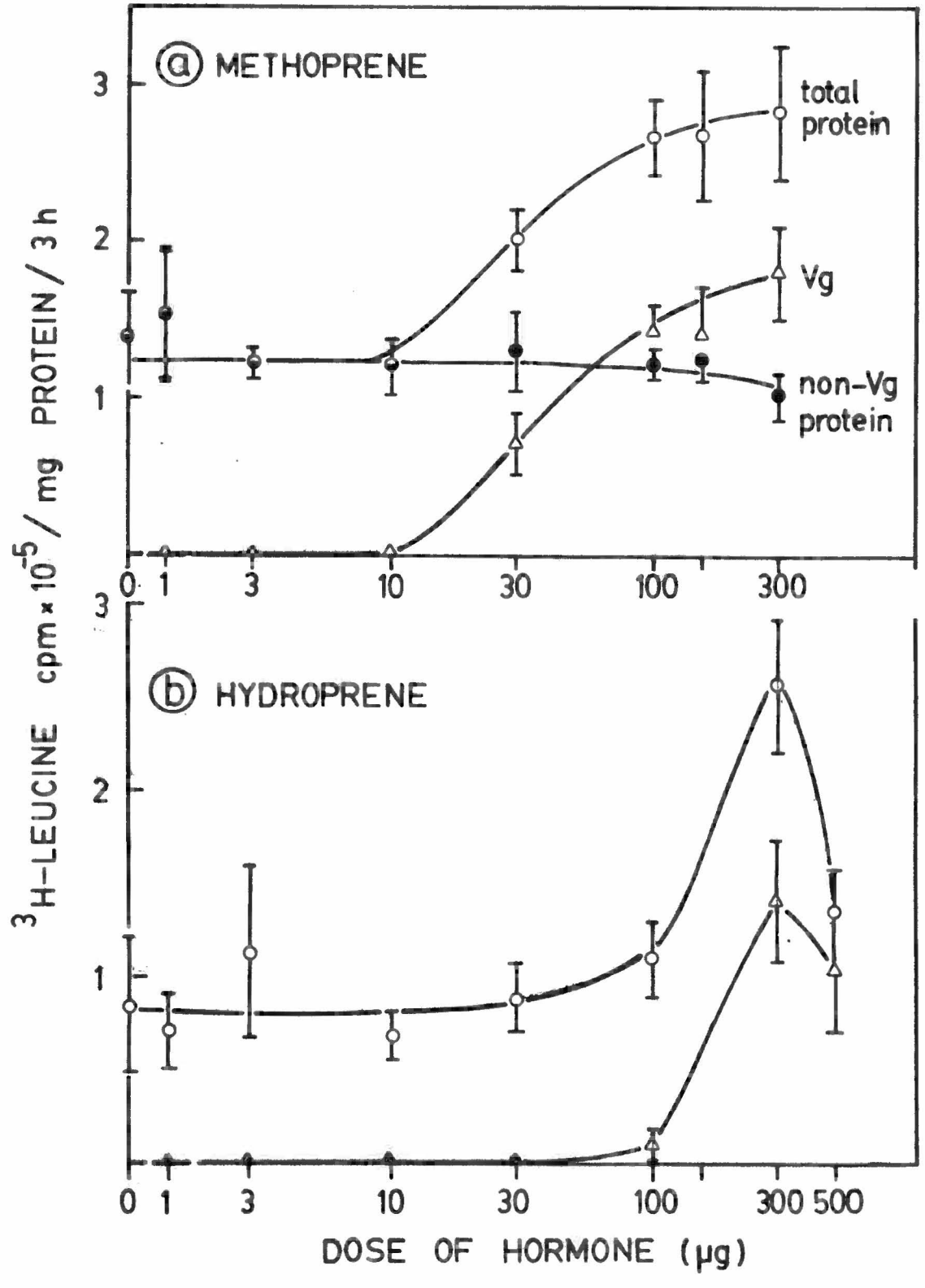
The Response of Adult Females to Methoprene

Dose-response relationship. The effects of various doses of methoprene when applied to fully competent 8 day old adult females and assayed in fat body 3 days later are shown in figure 2a. Synthesis of Vg was dose-related with a mid-point at about 40 µg/locust. Total secreted proteins also increased with the dose of methoprene, but there was no net increase in non-Vg proteins (calculated by difference) when results were expressed as [^{3}H]leucine incorporated/mg fat body protein. A tissue protein basis is used for calculation of data in order to adjust for differences in amounts of fat body cultured, but changes in total tissue protein are not revealed by this procedure. Data expressed per total locust fat body (not shown)

Figure 2

Dose response relationship for stimulation of protein synthesis in adult female locust fat body by injected methoprene (a) and hydroprene (b).

Eight days after chemical allatectomy with precocene, adult female locusts were injected with the indicated doses of either hormone analog in mineral oil. After 72 h, fat bodies were dissected out and incorporation of [^{3}H]leucine into secreted Vg and total proteins during 3 h culture was measured. For the methoprene dose-response experiment (2a) radioactivity in non-Vg proteins was calculated by difference. Each point shows the mean and S.E.M. from four individual fat bodies.


ⓐ METHOPRENE
total protein
Vg
non-Vg protein
ⓑ HYDROPRENE
^{3}H-LEUCINE cpm × 10^{-5} / mg PROTEIN / 3 h
DOSE OF HORMONE (µg)
0 1 3 10 30 100 300
0 1 3 10 30 100 300 500
1 2 3

indicate that the total tissue protein, and the rate of synthesis of non-Vg proteins per fat body, rise about 2-fold during 3 days of saturating methoprene stimulation. The latter results are in good agreement with those reported per fat body by Gellissen and Wyatt (1981). The proportion of Vg in secreted proteins was raised from zero in controls to about 60% at a saturating dose of 300 µg methoprene/locust.

Another JH analog, hydroprene (ZR-512) also induced the synthesis of Vg in adult female fat body in a dose-dependent manner (figure 2 b). However, it was about 4 times less effective than methoprene in inducing Vg (ED_{50} 175 µg/adult). When hydroprene was injected at a dose of 500 µg/locust, the synthesis of non-Vg proteins (calculated by difference) decreased.

Comparison of response to methoprene after surgical or chemical allatectomy. Since, during the course of this study, adult female locusts were allatectomized either surgically or chemically with ethoxyprecocene, it was necessary to compare the effects of methoprene in inducing Vg synthesis in fat bodies after each method. The results of such a comparison are shown in figure 3. Fat bodies from females allatectomized with either method responded to a similar extent in producing Vg (expressed as percent of total secreted proteins) up to 3 days after exposure to methoprene, except that after 24 h a measurable Vg response (3% of total proteins) was detectable only from fat bodies of surgically allatectomized females. The response at 48 and 72 h after methoprene treatment was not significantly different.

Development of competence for Vg synthesis in adult female fat body.

To determine how soon after adult ecdysis the female fat body is competent to synthesize Vg, adult females were treated with precocene on the day of ecdysis and injected on the same or subsequent days with a single dose of 150 µg methoprene. At different times after the hormone treatments, fat bodies were excised and assayed *in vitro* for Vg synthesizing activity (figure 4). There was great variation in individual responses, but some generalizations can be made. When the methoprene was injected on the day of adult ecdysis, Vg synthesis was first detected at day 5 and had declined by day 7. When the JH analog was injected on day 2 or later, Vg synthesis was always detected on the second day after treatment (no assay was done on the first day), and persisted for at least 5 days more. As the female fat body matured, the response increased, and fat bodies from 8-day old locusts were fully as competent as those from 10 to 14-day old females. Maximal responses from 8-14-day old fat bodies were exhibited 4-5 days after hormone treatment.

Responsiveness of Adult Males to Methoprene

Attempts were made to induce Vg synthesis in adult male *Locusta* with high doses of methoprene. Even when 500 µg was injected and locusts were kept as long as 10 days, Vg could not be detected in the hemolymph by immunochemical techniques. When fat bodies were assayed *in vitro* for biosynthetic activity 3 days after injection of high

Figure 3

Comparison of induction of Vitellogenin synthesis by adult female locust fat body after chemical or surgical allatectomy.

Adult females were allatectomized with either ethoxyprecocene (P-) or surgically (-CA-) within 12 h of ecdysis or when 1 day old, respectively, and then injected with 150 µg methoprene (M) on day 14 of adult life ($P\text{-}M_{14}$ or $-CA\text{-}M_{14}$). Fat bodies were dissected out 1, 2 and 3 days after hormone treatment and assayed in vitro for incorporation of [^{3}H]leucine into secreted Vg and total proteins as described under Methods. For purposes of comparison, the response for Vg synthesis has been expressed as a percentage of total proteins. Each point shows the mean ± S.E.M. from four individual fat bodies.

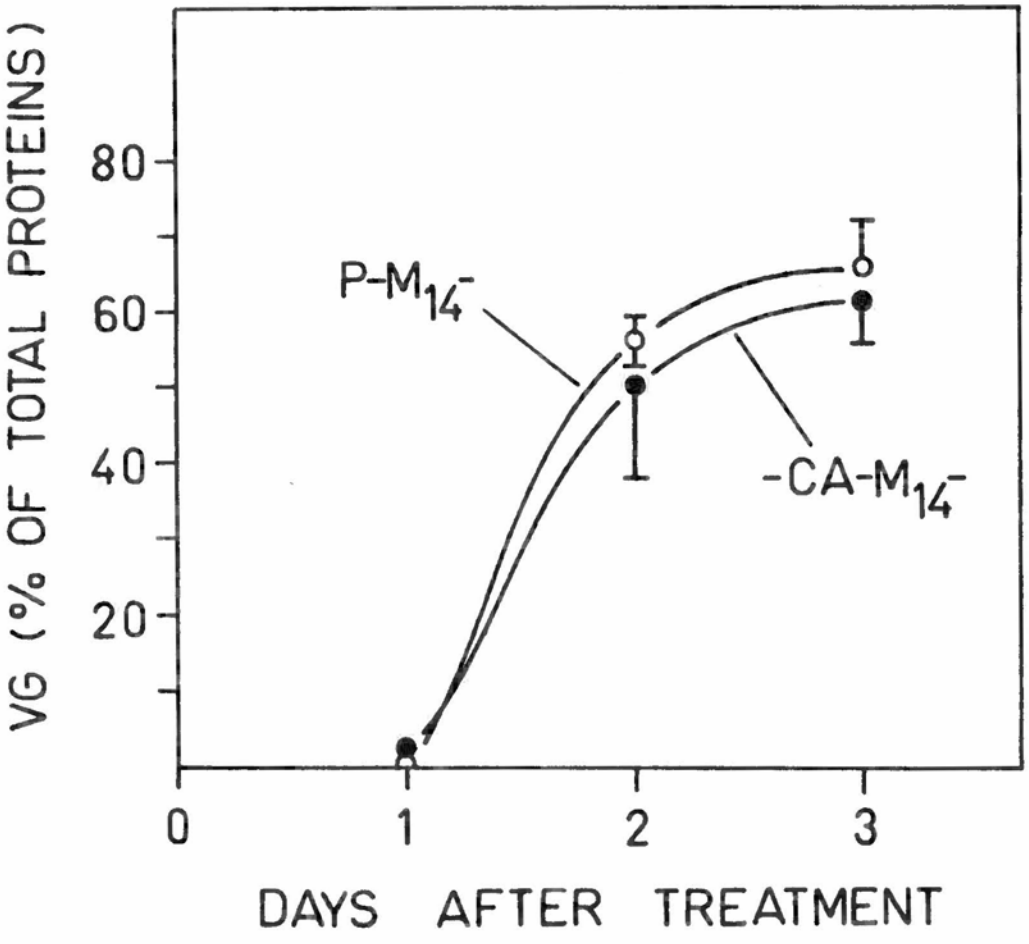
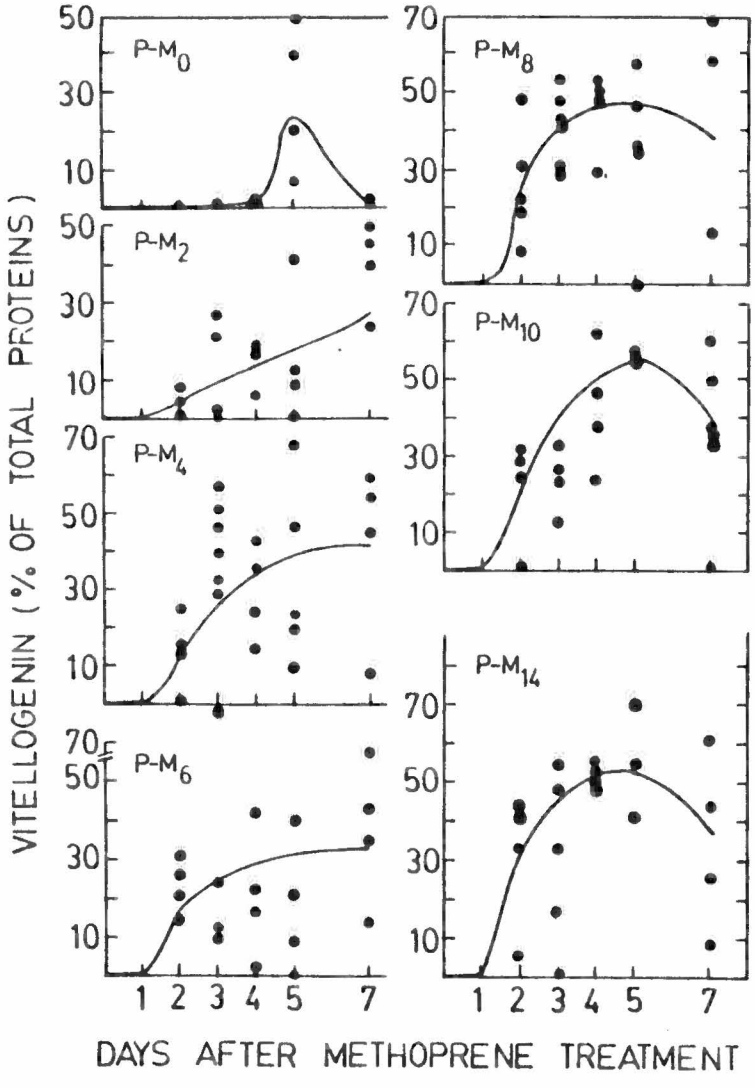

VG (% OF TOTAL PROTEINS)
80
60
40
20
0
1
2
3
DAYS AFTER TREATMENT
P-M14-
-CA-M14-

Figure 4

Development of competence to synthesize vitellogenin by adult female locust fat body.

Adult females were allatectomized with ethoxyprecocene (P-) within 12 h of ecdysis and then injected with 150 g methoprene (M) on days 0, 2, 4, 6, 8, 10 and 14 ($P\text{-}M_0$.....$P\text{-}M_{14}$). Fat bodies were taken 2, 3, 4, 5 and 7 days after hormone treatment and assayed in vitro with [^{3}H]leucine for synthesis of Vg and TCA-precipitable proteins as described in the text. Labelling in Vg as % of that in total secreted proteins is shown. The curves are drawn to represent subjective interpretation of the data.


P-M0
P-M2
P-M4
P-M6
P-M8
P-M10
P-M14
VITELLOGENIN (% OF TOTAL PROTEINS)
DAYS AFTER METHOPRENE TREATMENT
1 2 3 4 5 7
10
30
50
70

doses of methoprene, the immunoprecipitates showed labelling significantly above controls (Table 1). This result suggests a very weak response (see Discussion, however).

Methoprene-Induced Changes in Female and Male Larvae.

Vitellogenin synthesis. In order to find whether locusts in any of the larval stages can be induced to synthesize Vg, fifth instar larvae of both sexes were injected with 150 µg methoprene on day 3 after the fourth larval molt. When hemolymph was assayed by an Ouchterlony test, Vg was readily detectable in the hormone treated individuals (figure 5). Quantitatively, males responded less strongly than females and, 4 days after treatment, had only one-tenth as much Vg in their hemolymph (Table 2). Controls injected with mineral oil made no detectable Vg.

After injection of 150 µg of methoprene in mid-fourth instar, about 8% of individuals of both sexes (4 out of 50) showed immunochemically detectable Vg in the hemolymph. The induced protein appeared identical in an Ouchterlony test with purified egg vitellin (figure 6). Because of the variability, the fourth instar response was not studied further.

Change in response during the fifth instar. The variation in response to methoprene during the fifth instar was investigated by injecting 300 µg methoprene/locust (see below) on days 1 to 5 and assaying fat bodies two days later for Vg-synthesizing activity *in vitro* (figure 7). With both females and males, the response was

Table 1

Effect of methoprene on immunoprecipitable and total proteins secreted by fat bodies of adult male Locusta migratoria

Treatment	N	[^{3}H]leucine cpm/fat body (mean ± S.E.M.)		% Vg
		Vg	Total proteins	
Mineral oil	4	0	44,648 ± 6,524	0.0
Methoprene				
300 µg	4	290 ± 122	58,684 ± 6,140	0.5
500 µg	4	1,230 ± 452	69,891 ± 11,240	1.8

Fourteen day old adult males were injected with 5 µl mineral oil or methoprene in 5 µl mineral oil. After 3 days fat bodies were assayed for [^{3}H]leucine incorporation in vitro.

Figure 5

Ouchterlony immunodiffusion showing identity of vitellogenin from hemolymph of methoprene-treated female adults and female and male larvae.

All locusts except controls were injected with 150 µg methoprene in mineral oil and hemolymph was collected 3 days later. Controls were injected with mineral oil alone. Adult females had been chemically allatectomized with precocene. Center well: specific anti-vitellin serum. Peripheral wells: a, purified vitellin; remaining wells, hemolymph from: b, male adults; c, female fifth instar larva control; d, female adult; e, female fifth instar; f, male fifth instar larva.

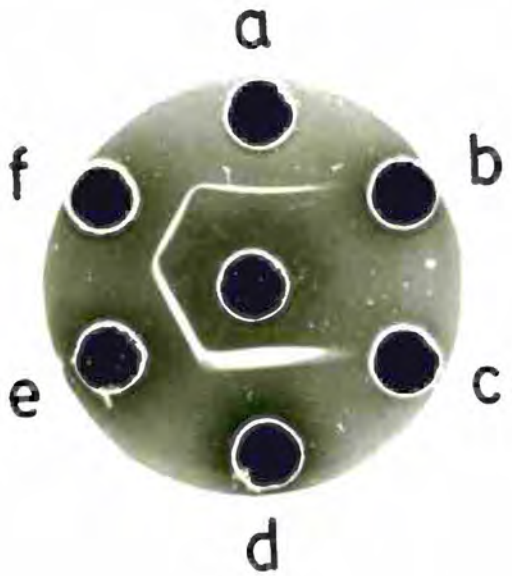

a
b
c
d
e
f

Table 2

Induction of vitellogenin in male and female fifth instar *L. migratoria*

Sex	Days after treatment		
	2	3	4
	(Hemolymph Vg titre, mg/ml)		
Female	0.6	10.3	31.8
Male	0	0.6	3.5

Individual fifth instar locusts were injected with 150 µg methoprene in 5 µl mineral oil on day 3 after the fourth molt and hemolymph was sampled on the days shown for assay by rocket immunoelectrophoresis. Results are means from groups of 5 animals. Controls injected with mineral oil alone gave no detectable Vg.

Figure 6

Ouchterlony immunodiffusion showing identity of vitellogenin from hemolymph of adult males containing implanted female fat body, and from induced male and female fourth instar larvae, with purified vitellin.

Portions of fat bodies from females were implanted into fifth instar males, as described in the text, and, 10 days after adult ecdysis, hemolymph from the recipient males was tested by immunodiffusion. Fourth instar males and females were injected with 300 µg methoprene in mineral oil on day 2 and hemolymph was collected 3 days later. Center well: specific anti-vitellin serum. Peripheral wells: a, purified vitellin; remaining wells, hemolymph from: b, male that received 3 day fifth instar female fat body; c, male that received 1-3 day adult female fat body; d, male that received 2 day fifth instar female fat body; e and f, induced fourth instar male and female, respectively.

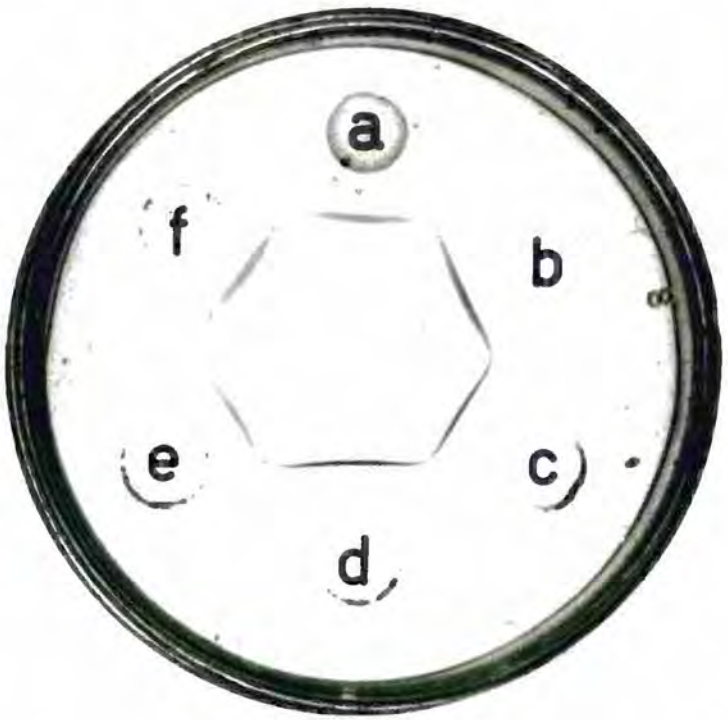
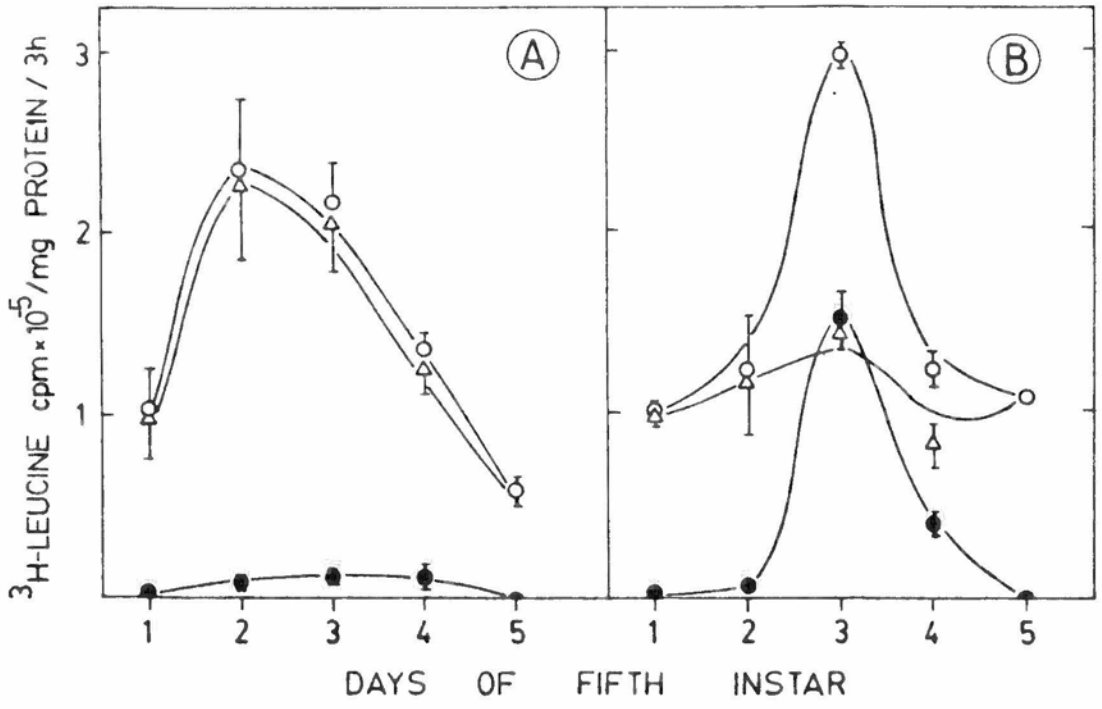

a
b
c
d
e
f

Figure 7

Competence to produce vitellogenin in response to methoprene by male (A) and female (B) fat bodies during the fifth instar.

Larval locusts were injected with 300 µg methoprene on days, 1, 2, 3, 4 and 5 of the fifth instar. After 48 hours, fat bodies were excised and their Vg (●), total protein (o) and non-Vg protein (Δ, by difference) synthetic activity assayed by incorporation of [^{3}H]leucine in vitro. Each point is a mean and S.E.M. of four separate fat bodies (except for females induced on day 5).


A
B
^{3}H-LEUCINE cpm × 10^{-5}/mg PROTEIN / 3h
1
2
3
1
2
3
4
5
1
2
3
4
5
DAYS OF FIFTH INSTAR

greatest in mid-instar (day 3) and declined to zero by day 5, two days before larval-adult ecdysis. The peak of responsiveness was very distinct in the female but broader and much lower in the male. Methoprene-treated males showed a pronounced mid-instar peak of non-Vg protein synthesis.

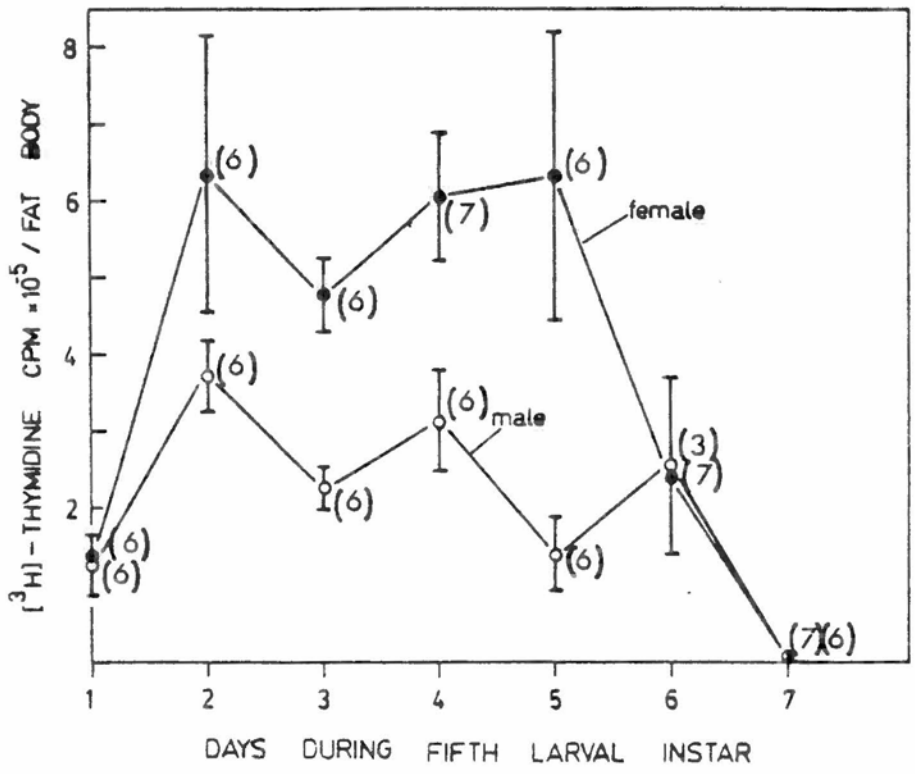
Incorporation of [^{3}H]thymidine into fat body DNA. Since it had been shown earlier that DNA synthesis and nuclear ploidy levels in adult locust fat body are regulated by JH (Nair et al., 1981; Irvine and Brasch, 1981), as is Vg synthesis in the adult females, it was important to find whether DNA synthesis was also influenced by JH in fat bodies of fifth larval instars. As an indicator of DNA synthesis, the incorporation of [^{3}H]thymidine into the DNA of fat body from both male and female fifth larval instars was measured during 3 h incubation in vitro.

Initially, to establish the basal level of DNA synthesis, the incorporation of [^{3}H]thymidine into fat body DNA of untreated larvae at various days during the fifth stadium was measured (figure 8). The incorporation of thymidine rose from a low value on day 1 to significantly higher values on day 2 in both sexes. On days 2 to 5, there were significantly higher rates of thymidine incorporation in female than in male fat body. The extent of incorporation in both sexes was similar on day 6 and dropped to almost zero level by day 7 (the normal day of ecdysis into adults). Because of the high scatter in the data, a distinct peak of incorporation on any particular day was not obvious. Similar variance in the uptake of thymidine into DNA of adult locust fat body has been observed previously (Chen et al.,

Figure 8

Incorporation of [^{3}H]thymidine into DNA of fat body from female and male fifth instar larvae.

Fat bodies were dissected from larvae on the indicated days during the fifth instar and incubated *in vitro*, two per dish, for 3 h with [^{3}H]thymidine. The amount of thymidine incorporated into DNA was determined as described under Methods and calculated per fat body. The numbers in parenthesis represent numbers of fat body pairs used per point and the values shown are means $\pm$ S.E.M.


8
6
4
2
[3H]-THYMIDINE CPM ×10^5 / FAT BODY
(6)
(6)
(6)
(6)
(7)
(6)
female
(6)
(6)
(6)
(6)
male
(6)
(3)
(7)
(7)
(6)
1
2
3
4
5
6
7
DAYS DURING FIFTH LARVAL INSTAR

1979; Nair et al., 1981).

The incorporation of [^{3}H]thymidine into DNA in fifth instar female fat body at various times after injection, on day 3, of either 300 µg methoprene in mineral oil or mineral oil alone is shown in figure 9. The control injection of mineral oil apparently caused elevated incorporation at 12 h. Significantly higher levels of thymidine were incorporated into fat bodies exposed to methoprene for 12 to 48 h, but after the maximum observed at 12 h after hormone treatment, incorporation decreased until at 72 h it was no greater than control values.

In another experiment, the DNA content as well as incorporation of [^{3}H]thymidine in fat bodies from male and female fifth instars was determined at 48 and 72 h after control or hormone treatment on day 3 (Table 3). A significant increase in the DNA content of both male and female fat bodies, greater in females than in males, was observed 72 h after methoprene treatment. In females, the DNA content was already significantly higher at 48 h after methoprene treatment.

Dose-response relationship. The response of fifth instar females to various doses of methoprene was assayed in terms of protein production by fat body in vitro (figure 10 a). Stimulation of Vg output was half-maximal at about 135 µg/individual. Unlike the results for adult females (figure 2 a), with larval fat body the hormone analog evoked no increase in total secreted TCA-precipitable proteins. The production of non-Vg proteins (by difference) decreased with increasing doses of methoprene, and at a saturating dose about 60% of the secreted protein was Vg. A similar relationship was

Figure 9

Effect of methoprene on the incorporation of [^{3}H]thymidine into DNA of fifth instar female fat body.

On day 3 of the fifth instar individual larvae were injected with either 300 µg methoprene in mineral oil or mineral oil alone. At the indicated times after the treatments, fat bodies were dissected out from pairs of females and assayed for [^{3}H]thymidine incorporation as described in figure 8. The numbers in parenthesis represent the numbers of fat body pairs used per point and the values shown are means $\pm$ S.E.M. For comparison, data from figure 8 for [^{3}H]thymidine incorporation into DNA of fat body from untreated female fifth instar for days 3 to 6 are also plotted. On the X-axis the equivalent days of development during the fifth instar are also shown.

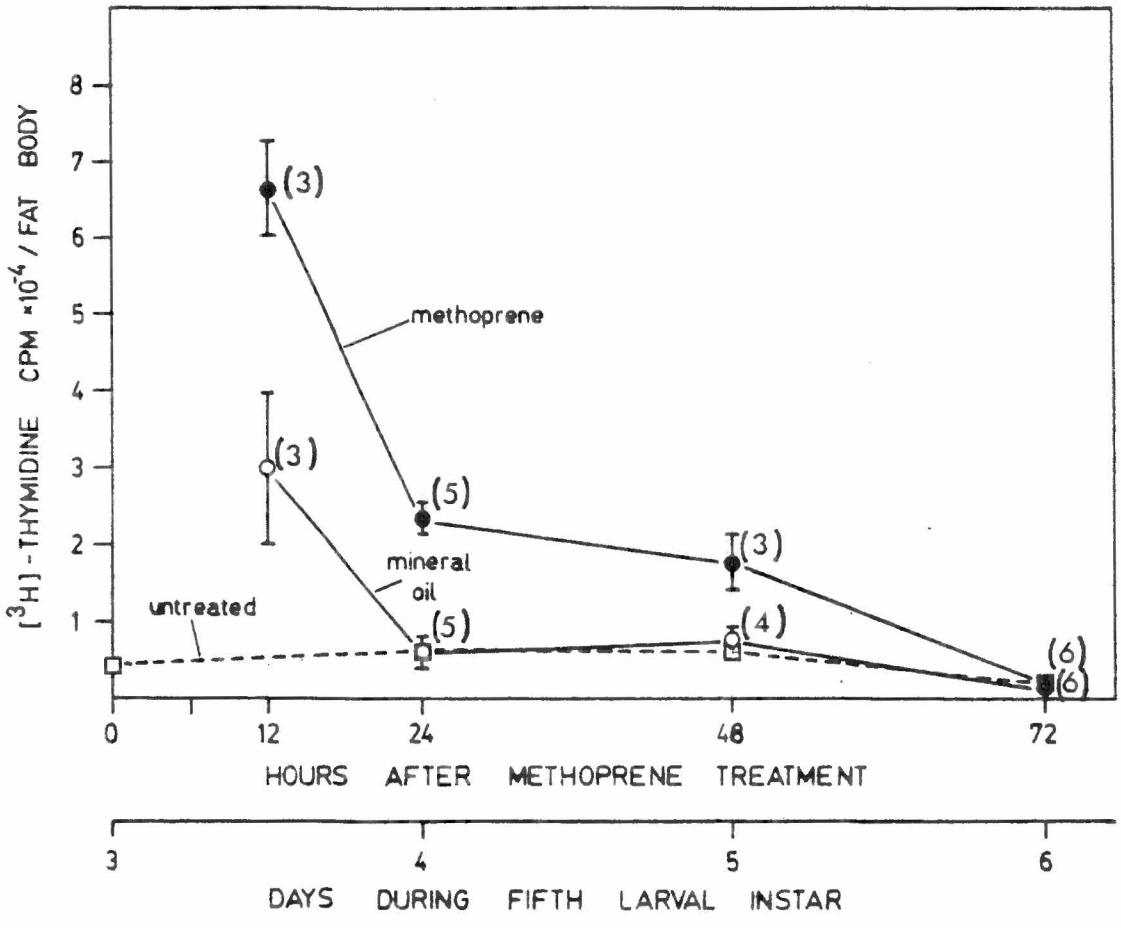

[3H]-THYMIDINE CPM ×10-4 / FAT BODY
8
7
6
5
4
3
2
1
(3)
methoprene
(5)
(3)
(3)
mineral
oil
untreated
(5)
(4)
(6)
(6)
0
12
24
48
72
HOURS AFTER METHOPRENE TREATMENT
3
4
5
6
DAYS DURING FIFTH LARVAL INSTAR

Table 3

Effect of methoprene on the DNA content and [^{3}H]thymidine incorporation of fat bodies from male and female fifth larval instar L. migratoria

Time after Treatment	TREATMENT			
	Control		Methoprene	
	[^{3}H]thymidine (cpm/fat body/3h)	DNA (µg/fat body)	[^{3}H]thymidine (cpm/fat body/3h)	DNA (µg/fat body)
Male				
48 h	4,726 ± 2,241	33.2 ± 3.6 (3)	6,681 ± 1,843	34.0 ± 2.8 (3)
72 h	808 ± 238	33.0 ± 4.4 (3)	1,608 ± 633	49.5 ± 6.5 (3)
Female				
48 h	7,841 ± 1,487	31.4 ± 4.4 (5)	17,965 ± 3,491	53.7 ± 9.7 (6)
72 h	1,060 ± 264	39.1 ± 16.3 (3)	2,418 ± 983	77.3 ± 2.3 (6)

Footnote, next page.

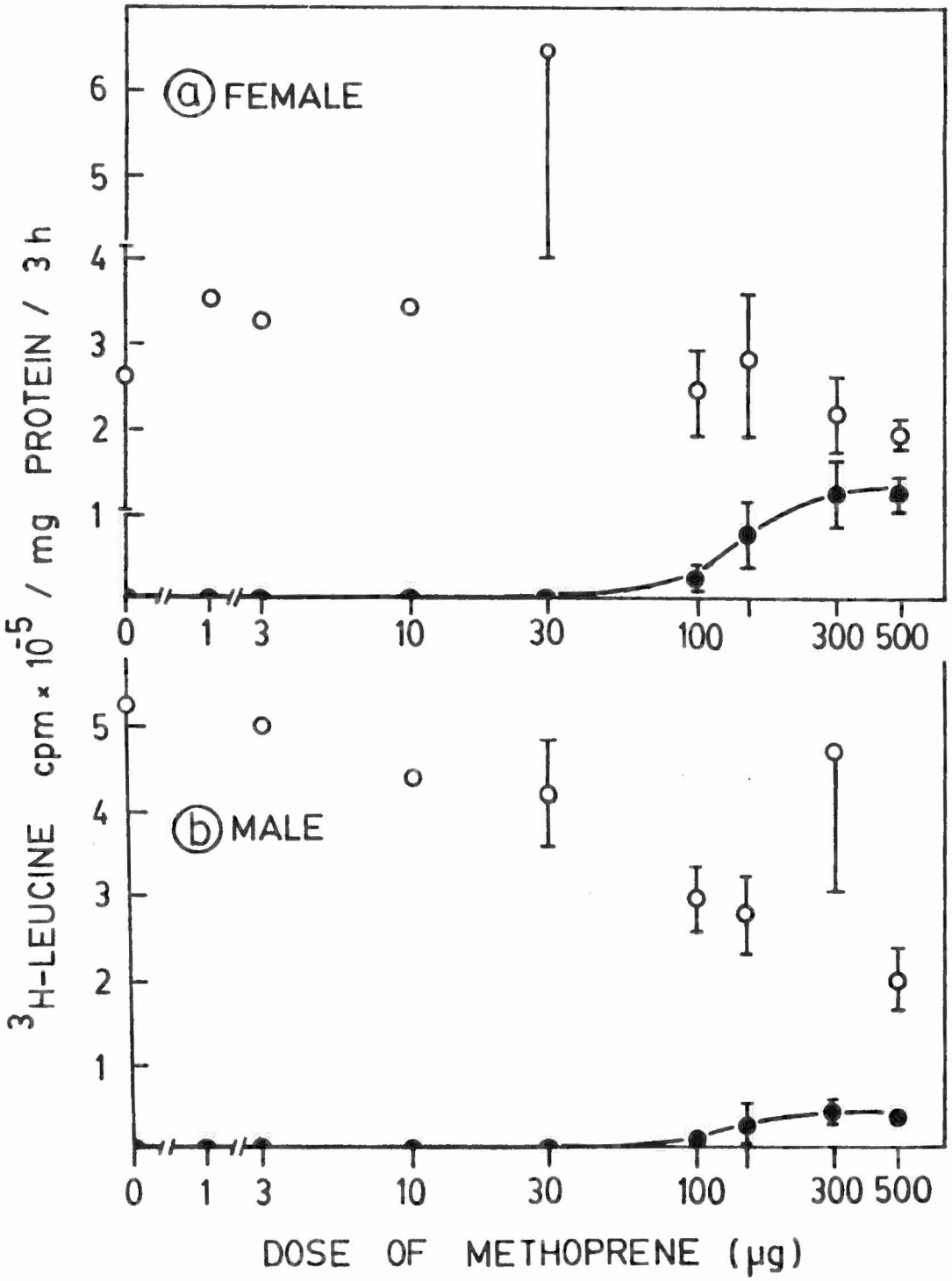
Table 3. (cont'd)

Individual fifth instar locusts were injected with 300 µg methoprene in 5 µl mineral oil or 5 µl mineral oil alone on day 3 after the fourth molt. After 48 and 72 h of treatment, fat bodies from pairs of individuals (represented in parenthesis) were assayed in vitro for the incorporation of [^{3}H]thymidine and for the DNA content as described under Methods. All values shown are means ± S.E.M.

Figure 10

Dose-response relationship for methoprene in stimulating synthesis of vitellogenin and total proteins in fat bodies from female (a) and male (b) fifth instar larvae.

On day 3 of the fifth instar, larvae of both sexes were injected with the indicated doses of methoprene. After 72 h, fat bodies were excised and incorporation of [^{3}H]leucine into secreted Vg (•) and total proteins (o) during 3 h culture was measured. Each point, except for 1-10 µg in (a) and 0-10 µg doses in (b), represents the mean ± S.E.M. from four individual fat bodies.


ⓐ FEMALE
ⓑ MALE
^{3}H-LEUCINE cpm × 10^{-5} / mg PROTEIN / 3 h
DOSE OF METHOPRENE (µg)
0
1
3
10
30
100
300
500
1
2
3
4
5
6

observed for immunoprecipitable and TCA-precipitable proteins from male fifth instar fat bodies. A half-maximal synthesis of Vg was achieved with 145 µg/individual, but even at the highest dose tested (500 µg/ locust) Vg was only 20% of total secreted protein (figure 10 b).

In figure 11 are compared the dose-response data for methoprene in inducing Vg synthesis as percentage of total protein synthesis in adults and fifth larval instars of both sexes. In adult females, the dose that produced half-maximal Vg synthesis as percentage of total protein synthesis (ED_{50}) was 30 µg. In adult males, only a slight response of doubtful specificity (see Discussion) was obtained with doses above 300 µg. Due to the decrease in non-Vg proteins with increasing doses of methoprene in fat bodies of fifth instar larvae (figure 10 a, b) the ED_{50} values obtained from figure 11 of 175 µg and 210 µg for female and males, respectively, are higher than when based on the incorporation of [^{3}H]leucine into immunoprecipitable Vg alone (see above). However, the results clearly demonstrate the marked sex- and stage-related differences in sensitivity to the JH analog.

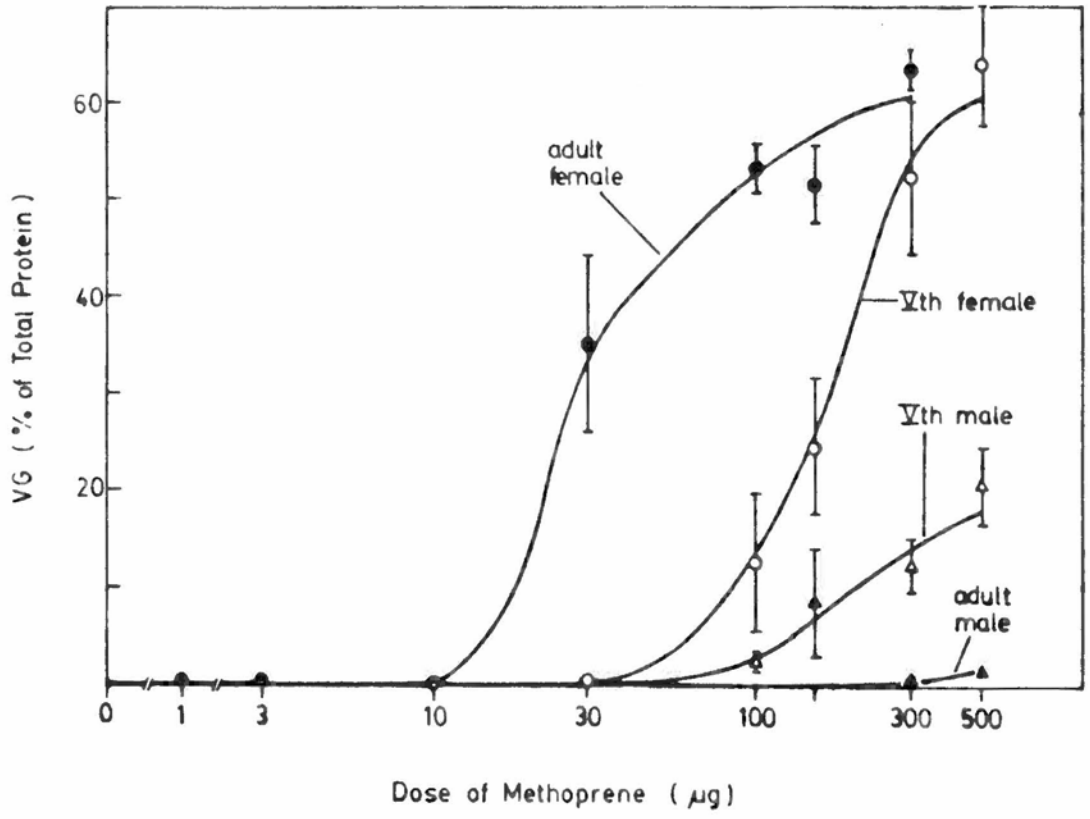
The Roles of Tissue and Hormonal Environment in the Sexual Differentiation of Fat Body

The observation that the fat body of males can be induced to Vg synthesis in the fifth instar and cannot in adults indicates that the Vg genes become repressed in the male during metamorphosis. This

Figure 11

Comparison of stimulation of vitellogenin synthesis by methoprene in fat bodies from adults and fifth instar larvae of both sexes.

Vitellogenin synthesis as percentage of total secreted protein synthesis has been calculated from the data of figures 2 a and 10 a, b and Table 1. Each point shows a mean and S.E.M. from four individual fat bodies.


VG (% of Total Protein)
60
40
20
adult female
Vth female
Vth male
adult male
0
1
3
10
30
100
300
500
Dose of Methoprene (µg)

raises the question whether repression is due to intrinsic characteristics of male fat body or the male milieu interieur. To test this, fat bodies from female fifth instars or adults were implanted into 2 day old fifth instar males. After ecdysis into adults, the recipient males were bled on day 10 and their hemolymph was checked by immunodiffusion for the presence of Vg (figure 6). It is evident that not only are female fat bodies, taken through metamorphosis in a male environment, competent to synthesize Vg, but that the adult male has a hormonal milieu adequate for expression of the Vg genes.

In another experiment, adult males having female fat body implanted before metamorphosis were injected with 150 µg methoprene per individual to boost the vitellogenic response and three days later their fat bodies were assayed for activity in vitro (Table 4). The results show production of Vg. The immunoprecipitable [^{3}H]leucine incorporated and the percent Vg in total proteins were low because the combined activites of the implanted female and host male tissues were measured. But it is clear that the male internal environment during metamorphosis did not cause repression of Vg genes in female fat body. The converse experiment, to test whether female milieu could make male fat body gene expressible, would require recognition and separation of implanted from endogenous fat body after the molt, which could not be done.

A further question is whether the effectiveness of the metamorphic molt in evoking sexual differentiation of Vg gene expressibility is due to molting (in response to ecdysone) in the

Table 4

Vitellogenic competence of female fat body after metamorphosis in male implanted *L. migratoria*.

Donor		N	[^{3}H]leucine cpm/fat body (mean ± S.E.M.)		% Vg
Stage	Age (days)		Vg	Total Proteins	
Adult	1-3	8	10,102 ± 2,629	104,630 ± 11,679	9.7
Vth	4	9	13,714 ± 4,607	120,974 ± 6,368	11.3
Vth	3	6	7,860 ± 1,208	139,825 ± 10,511	5.6
Vth	2	5	11,510 ± 5,404	144,720 ± 10,930	8
Vth	2	4	702 ± 140	101,837 ± 4,792	0.7

Fat bodies from adult and fifth instar females and fifth instar males were implanted into 2 day fifth instar males. Ten days after adult ecdysis, the recipient males were injected with 150 µg methoprene and 3 days later their fat bodies were used for assay of [^{3}H]leucine incorporation *in vitro*.

absence of JH or to the completion of a requisite number of molts. This was tested by treating newly molted fourth instars with ethoxyprecocene to produce precocious adults or "fifth instar adultiforms" (Pener et al., 1978; 1981). When 11-14-day old precocious adult males were injected with 300 µg methoprene, their fat bodies did not synthesize Vg (Table 5), even though this dose did induce Vg synthesis in normal fifth instar males (figure 11). When precocious adult females were treated with 30 or 150 µg methoprene, their fat bodies responded by active Vg synthesis (Table 5), even though 30 µg was insufficient to induce Vg synthesis in fifth instar larvae (figure 11). Thus, in both sexes, fat body from fifth instar precocious adults responded to methoprene similarly to fat body from normal adults, indicating that the sexual differentiation of the adult fat body is evoked by a molt in the absence of JH, rather than by molting a critical number of times.

Vitellogenin Synthetic Activity of Perivisceral and Paragonadal Fat Body

In the abdomen of adult female locusts, fat body in 3 distinctly different regions can be identified: (i) a thin sheet of fat body cells closely adhering to the abdominal wall (peripheral fat body); (ii) the bulk of the fat body, yellow in color and surrounding the gut and the ovaries (perivisceral fat body), and (iii) white in colour, sandwiched between the ovaries and the gut and invested along the mid-dorsal side of the ovaries (paragonadal fat body). A comparison

Table 5

Vitellogenin synthesis in fat bodies of precocious adult *L. migratoria*

Treatment	N	[^{3}H]leucine cpm/fat body (mean ± S.E.M.)		% Vg
		Vg	Total Protein	
Male				
Mineral oil	3	0	18,916 ± 7,999	0
300 µg methoprene	3	211 ± 33	26,026 ± 2,880	0.7
Female				
Mineral oil	3	0	46,296 ± 942	0
30 µg methoprene	5	11,030 ± 6,542	43,027 ± 7,452	22.0
150 µg methoprene	5	26,358 ± 8,272	48,386 ± 10,049	49.8

Footnote, next page.

Table 5. (cont'd)

Newly molted male and female fourth instars were topically treated with 130 µg ethoxyprecocene in 10 µl acetone. 11-14 days after the molt into precocious adultoids, they were injected with mineral oil or methoprene in mineral oil. Three days later, fat bodies were assayed for [^{3}H]leucine incorporation *in vitro*. From additional precocious adult males hemolymph was collected 3 and 10 days after treatment with methoprene, and was found to be negative for Vg by rocket immunoelectrophoresis.

Table 6

Vitellogenin synthesis in perivisceral and paragonadal fat bodies of mature adult female and methoprene treated female fifth larval instar L. migratoria.

Fat Body	N	[^{3}H]leucine cpm/fat body (mean ± S.E.M.)		% Vg
		Vg	Total proteins	
Adult				
Perivisceral	5	86,892 ± 13,300	132,195 ± 21,455	67.5 ± 8.7
Paragonadal*	5	23,967 ± 9,343	29,926 ± 11,879	81.0 ± 6.2
Fifth Instar				
Perivisceral	3	48,321 ± 3,727	71,411 ± 4,366	67.0 ± 2.6
Paragonadal†	3	12,657 ± 4,856	20,165 ± 2,896	61.7 ± 18.1

Perivisceral and paragonadal fat bodies were dissected from mature adult females, with terminal oocyte length between 2.5 to 4.5 mm, and 6 day old fifth instar females injected with 300 µg methoprene on day 3 after the fourth molt, were assayed for [^{3}H]leucine incorporation in vitro.

* assayed after dissecting free from ovarian tissue.

† assayed along with gonadal tissue.

of the synthetic activities of the perivisceral and paragonadal fat body from mature adult females by incubation in the presence of [^{3}H]leucine, revealed that the latter was slightly more active in secreting Vg (expressed as a percentage of total secreted proteins) than the former (Table 6). Of the total Vg secreted by the two fat bodies, paragonadal fat bodies contributed about 20%. The relative contribution of Vg by the paragonadal fat body is probably an underestimate, since it was difficult to dissect all of it intact. In one experiment, when ovaries, dissected free from paragonadal fat body, were cultured for 3 h in the presence of [^{3}H]leucine, no immunoprecipitable Vg could be detected in the medium.

To find whether a similar differentiation existed in the fat body of fifth larval instars, individual fifth instar females were injected with 300 µg methoprene and their fat bodies assayed 3 days later for synthesis of Vg *in vitro* (Table 6). The paragonadal fat body was assayed together with the undeveloped gonadal tissue, from which it could not be dissected. Hence, the mean value of Vg (as percent of total proteins) was lowered due to the synthesis of non-Vg proteins by the gonadal tissue. However, the relative contribution of secreted Vg by the fat bodies was in the same proportions as observed in the adult females.

Discussion

In *L. migratoria*, JH-dependent Vg synthesis is normally limited to the fat body of adult females. After allatectomy, either by surgery

or treatment with precocene, Vg synthesis can be induced by treatment with JH or an analog such as methoprene. Methoprene has been used in this study because of its greater stability *in vivo* than natural JH (Henrick *et al.*, 1973). In *Locusta*, injected JH is rapidly degraded and excreted (Erley *et al.*, 1975) and repeated doses are required for induction of vitellogenesis (Chen *et al.*, 1976), so that developmental changes in response might largely reflect changing rates of degradation. In the present work, methoprene was injected in mineral oil, which is a more effective route of administration (ED_{50} = 30 - 40 µg/locust) than topical application (ED_{50} = 150 µg; Chen *et al.* 1979). This corroborates a report that JH is more effective in stimulation of egg development when injected in oil than when applied topically to locusts (Lazarovici and Pener, 1977). In addition, the duration of response was increased. After injection of 150 µg of methoprene in oil into 8-14 day old precocene-treated adults, Vg synthesis was maximal on days 4-5 and still active on day 7, whereas after topical application of 250 µg synthesis was maximal on day 3 and had declined almost to zero by day 7 (Chen *et al.*, 1979). The longer sustained effect after injection may be due to gradual release of the hormone analog from oil droplets where it is protected from degradation.

Another JH analog, hydroprene, was about four times less effective than methoprene in producing a half-maximal stimulation of Vg synthesis in adult females treated with precocene. The relative potencies of methoprene and hydroprene in stimulating Vg synthesis are in agreement with results obtained on the binding specificity of the

putative JH receptor, from the fat body of the adult L. migratoria (Roberts and Wyatt, 1983).

Precocenes have been shown to selectively destroy the corpora allata in several insect species (Unnithan et al., 1977; Pener et al., 1978; Feyereisen et al., 1981; Bowers and Feldlaufer, 1982) and can, therefore, be used as an alternative to surgical allatectomy (Chinzei et al., 1982). However, different effects on Vg synthesis and oocyte growth in precocene II injected or surgically allatectomized vitellogenic females of L. migratoria have been reported (Pines et al., 1980; Lubzens et al., 1981; but see Chapter V). Although the assay used was not directly comparable to the above studies, I did not find any difference in the level of methoprene-stimulated Vg synthesis in adult females which had been treated with precocene II or surgically allatectomized within 1 day after ecdysis. Therefore, treatment with precocene has been used as an acceptable alternative to surgical allatectomy.

In the adult female locust, the fat body becomes competent to synthesize Vg in response to injected JH analog on days 4-5, about 3 days before synthesis is normally turned on by JH released from the corpora allata (Johnson and Hill, 1975; Rembold, 1981). Competence increases quantitatively to about day 8 and then remains at a maximal level at least until day 14. In O. fasciatus, the fat body has been shown to acquire the ability to respond to JH by making the mature form of Vg about 2 days before it is normally made in the adult female (Kelly and Hunt, 1982).

In the adult male, only a very small but statistically significant

response, assayed as fat body production of immunochemically precipitable protein, was observed with the highest does of methoprene used. Since no accumulation of Vg in the hemolymph could be detected by immunodiffusion techniques, the nature of the immunoprecipitated radioactivity from adult male fat body is questionable. Moreover, by using a cloned Vg hybridization probe, $mRNA_{Vg}$ could not be detected in fat bodies from methoprene treated adult males (Chapter V).

In fourth and fifth instar larvae of both sexes, which do not normally produce Vg, synthesis could be stimulated by high doses of JH analog. Induced synthesis was maximal in mid-instar, and declined before the larval-adult ecdysis, to be restored several days after the ecdysis, in females only, with increased sensitivity, as described above. Thus, the sex-limitation on expression of the Vg genes is not fully established until after the metamorphic molt. Quantitatively, the fifth instar female and male had an ED_{50} about 3 times that for the adult female, although the male had a much lower level of maximal response. These doses are expressed per individual and the differences become even greater when adjusted for body weights (about 1 g for a mid-fifth stage larva and 2 g for an adult). Fifth-stage larvae, therefore, exhibit low sensitivity to JH induction, but higher in the female than the male, and in the adult the sensitivity of the female tissue is much increased and that of the male further diminished. The sexual differentiation of gene inducibility is therefore a graded and not an all-or-none developmental process.

In the fifth stage larvae of both sexes, the stimulation of Vg synthesis with methoprene was accompanied by concurrent increases in

the DNA content of the fat body. When the fat bodies of female fifth instars were assayed for the incorporation of [^{3}H]thymidine at various times after injecton of methoprene on day 3, thymidine incorporation was maximal after 12 h and declined thereafter. Thymidine incorporation was also stimulated in control oil-injected female fifth instars, but to a lesser extent. The stimulation of thymidine incorporation after injection may therefore be partially a result of integumentary injury, as observed for RNA and protein synthesis in certain Lepidopteran pupae (Stevenson and Wyatt, 1962; Wyatt and Linzen, 1964; Berry et al., 1967). However, the significantly higher incorporation of thymidine into fat body after hormone treatment argues against an effect totally due to injury.

DNA synthesis and increase in nuclear ploidy levels have been shown to accompany Vg synthesis in fat body of adult L. migratoria (Nair et al., 1981; Irvine and Brasch, 1981). The significance of this relationship is not known but it may serve to provide additional copies of the genome and increased capability for transcription and protein synthesis. The stimulation of DNA synthesis and Vg synthesis in fat body of fifth instar larvae, which are already active in protein synthesis (see below), provides additional support for the above hypothesis. Any causal relationship, however, has to be proven by the use of inhibitors of DNA synthesis.

JH stimulation of adult female fat body is highly selective for Vg synthesis. Fat body of adults of both sexes also shows a relatively low level stimulation of non-Vg protein synthesis (Gellissen and Wyatt, 1981). This presumably includes proliferation of ribosomes,

endoplasmic reticulum and other organelles and enzymes required for the JH-dependent transition of the cells from a predominantly storage to an actively biosynthetic role (Couble et al., 1979). Larval fat body, in contrast, showed a decrease in production of proteins other than Vg when stimulated by methoprene. The mid-instar larval tissue is already active in protein synthesis (Turner and Loughton, 1975) and the stimulated expression of Vg genes may decrease synthesis of other proteins through competition for components needed for translation. Larval stage Vg synthesis is, in any case, a pharmacological process which probably does not occur in nature.

The available information on other insects indicates that Vg, while not occurring generally in males, is sometimes produced in males of some species. In the bug, Rhodnius prolixus, Vg occurs normally in hemolymph of adult males at levels similar to those in females (Chalaye, 1979). In the cockroach, Diploptera punctata, Vg synthesis can be induced in adult males by implanting active female corpora allata or injecting a JH analog (Mundall et al., 1979). In the roach, B. germanica, trace levels of Vg can be induced in adult males with JH analog, but the response is much stronger in larval males and still stronger in larval females (Kunkel, 1981; J.G. Kunkel, personal communication). L. migratoria appears to be similar to B. germanica except that Vg is not clearly inducible in adult males.

In Drosophila, the situation is somewhat different. Yolk polypeptides (YPs) are made both in the fat body and in ovarian follicle cells (Brennan et al., 1982), and synthesis can be induced in isolated female abdomens both by methoprene and by ecdysterone

(Postlethwait et al., 1980). In adult males, in which YPs are not normally present, synthesis could be induced by ecdysterone but not by methoprene. It is interesting that the response to the steroidal inducer (ecdysterone), in contrast to JH, is not sex-limited, since the same is true of the steroidal inducer (estradiol) in oviparous vertebrates.

The possibility that the sex-related differentiation of female and male locust fat body during the metamorphic molt might be due to differences in hormone levels or other aspects of the internal milieu, has been ruled out by the demonstration that female fat body, after metamorphosis within a male locust, is vitellogenically active. At the cellular level, one could postulate that the sex difference might depend on the presence of JH receptors; however, adult male fat body replicates DNA (Nair et al., 1981; Irvine and Brasch, 1981) and increases its synthesis of non-Vg proteins (Gellissen and Wyatt, 1981) in response to JH, so that adult male fat body is a JH target tissue. The sex-related differences in competence for Vg synthesis must reflect programming at the nuclear level. The gene programming differences are determined at least by the fifth larval instar (and probably much earlier) but are fully manifested only after the metamorphic molt. The key role of a metamorphic molt is confirmed by results obtained with precocious fifth instar adults of both sexes, which showed that molting in the absence of JH, rather than a critical number of molts, established the adult sex differentiation of the fat body. A molt in the presence of ecdysone and the absence of JH has similarly been shown to be required for development of ovarian

competence to take up yolk in females of P. americana (Bell and Sams, 1975). More recently, it has been shown for O. fasciatus that competence in the fat body to respond to JH by making the mature form of Vg and in the ovary to deposit yolk developed only after the metamorphic molt (Kelly and Hunt, 1982).

A L. migratoria Vg gene is located on the X chromosome (Bradfield and Wyatt, 1983), which results in a male-female dosage difference that may contribute to levels of expression, but other genes concerned with development of sex phenotype must influence Vg gene inducibility. In Drosophila, study of sex mutants and intersexes has led to the conclusion that capacity for yolk protein synthesis is associated with female phenotype, regardless of sex chromosome constitution (Bownes and Nothiger, 1981).

For the developmental changes in fat body responsiveness to JH which are, in part, common to both sexes, effects of other hormones may be important. In particular, the decline in competence for Vg synthesis and the JH-stimulated DNA synthesis towards the end of the fifth instar may be related to a steep rise in ecdysteroid titer at this time, preceding the molt (Baehr et al., 1979). This has been suggested and supported by demonstration of ecdysterone inhibition of JH-stimulated Vg synthesis in larval B. germanica (Kunkel, 1981). An antagonistic effect of ecdysterone on JH-stimulated hemoglobin synthesis in fat body of Chironomus larvae has also been reported (Mandalos and Laufer, 1980). The results of preliminary experiments with fifth-instar female locusts indicate that injection of ecdysterone can cause decreased responsiveness of fat bodies to

methoprene in producing Vg. However, more experiments need to be done before drawing any conclusions on these interactions. At the cellular and molecular levels, tissue competence to respond to JH may be influenced quantitatively by the number and distribution of JH receptors or the ability of JH to enhance DNA synthesis. The localization of at least one Vg gene on the X-chromosome (Bradfield and Wyatt, 1983), and the higher fat body ploidy levels reported for the adult female than the male (Nair et al., 1981; Irvine and Brasch, 1981) would amplify the female-male differences in Vg gene copy number which, in part, could be responsible for the different response to JH. It would be interesting to investigate whether the decrease or lack of Vg response to methoprene towards the end of the fifth instar is due to a block in the transcription or translation of $mRNA_{Vg}$.

Developmental acquisition of gene inducibility by estrogen has been studied in amphibia and birds. In Xenopus, Vg synthesis becomes inducible at stage 62 of metamorphosis; although closely correlated with thyroid hormone-dependent morphological development, experiments with antagonists indicate that thyroid hormone does not directly influence Vg gene programming (May and Knowland, 1980; Wangh, 1982). Estrogen receptors are present before the genes become inducible, and the ability of the hormone to cause an increase of these in the nucleus may be important (May and Knowland, 1981). In the chick, Vg becomes estrogen-inducible about day 12 of embryonic development (Lazier, 1978), whereas VLDL apoproteins B and II become inducible about 2 days earlier (Elbrecht et al., 1981). The acquisition of inducibility of VLDL apo II is accompanied by loss of methylation from

the DNA at two specific sites flanking the gene, which may be important in programming for expression (Colgan et al., 1982).

The sex- and stage-related variation exhibited by L. migratoria fat body make the system favorable for analysis of the molecular basis of gene inducibility. One aspect of this using a cloned Vg gene sequence is presented in Chapter V.

IV. STIMULATION OF VITELLOGENIN SYNTHESIS IN FAT BODIES BY METHOPRENE ADDED IN VITRO

Introduction

The roles of JH and ecdysone in stimulating Vg synthesis in insects have been demonstrated mainly by allatectomy, decapitation or isolation of abdomens followed by administration of either natural hormones or active analogs (Engelmann, 1969; Chen et al., 1976; Koeppe and Offengand, 1976; see also reviews by Fuchs and Kang, 1981; Postlethwait and Jowett, 1981; Bownes, 1982a). The results of these experiments in vivo, although often convincing as to the requirement for the hormone in question, leave open the possibility that it may act either indirectly via stimulation of other endocrine glands or tissues, or only in the presence of some additional factor, such as a peptide hormone. Therefore, the direct action of a hormone on a tissue must be demonstrated in vitro.

Although considerable progress has been made in obtaining direct stimulation of Vg synthesis by estradiol in amphibian liver tissue or primary liver cell cultures (Wangh and Knowland, 1975; Green and Tata, 1976; Wangh et al., 1979; Stanchfield and Yager, 1978; 1980; Searle and Tata, 1981; Wangh and Schneider, 1982), the success of comparable experiments with insect fat body has been limited. Fallon et al. (1974) reported a strong in vitro stimulation of Vg synthesis by ecdysterone in A. aegypti fat body. However, in subsequent

investigations only a weak response was reproducible (Hagedorn et al., 1978; Borovsky, 1981). Attempts to induce Vg synthesis in female and male fat bodies of Drosophila cultured in the presence of ecdysterone have been unsuccessful (Bownes, 1982b).

Both in the mosquito and Drosophila the regulation of Vg synthesis involves both ecdysterone and JH. In L. migratoria, so far as is known, synthesis of Vg in the fat body is under the strict control of JH, and during peak hormonal response Vg is the predominant secreted protein, hence providing an ideal system for attempting the induction of Vg synthesis by JH in vitro. The only two reports that have appeared on the stimulation of Vg synthesis in L. migratoria fat body by JH or methoprene in vitro (Wyatt et al., 1976; Abu-Hakima, 1981) have originated from the same laboratory as this investigation. The work reported in this chapter is a reevaluation of the procedures and the data presented in these reports.

Results

Fat Body Culture Conditions

Initially, I attempted to demonstrate fat body viability for at least 3 days in culture using the procedure and culture conditions described by Abu-Hakima (1981). However, a modification in the procedure was made necessary due to the chronic appearance of unidentified mould fungus in fat bodies that had been in culture for about 30 h. The loss of fat body cultures due to fungus contamination

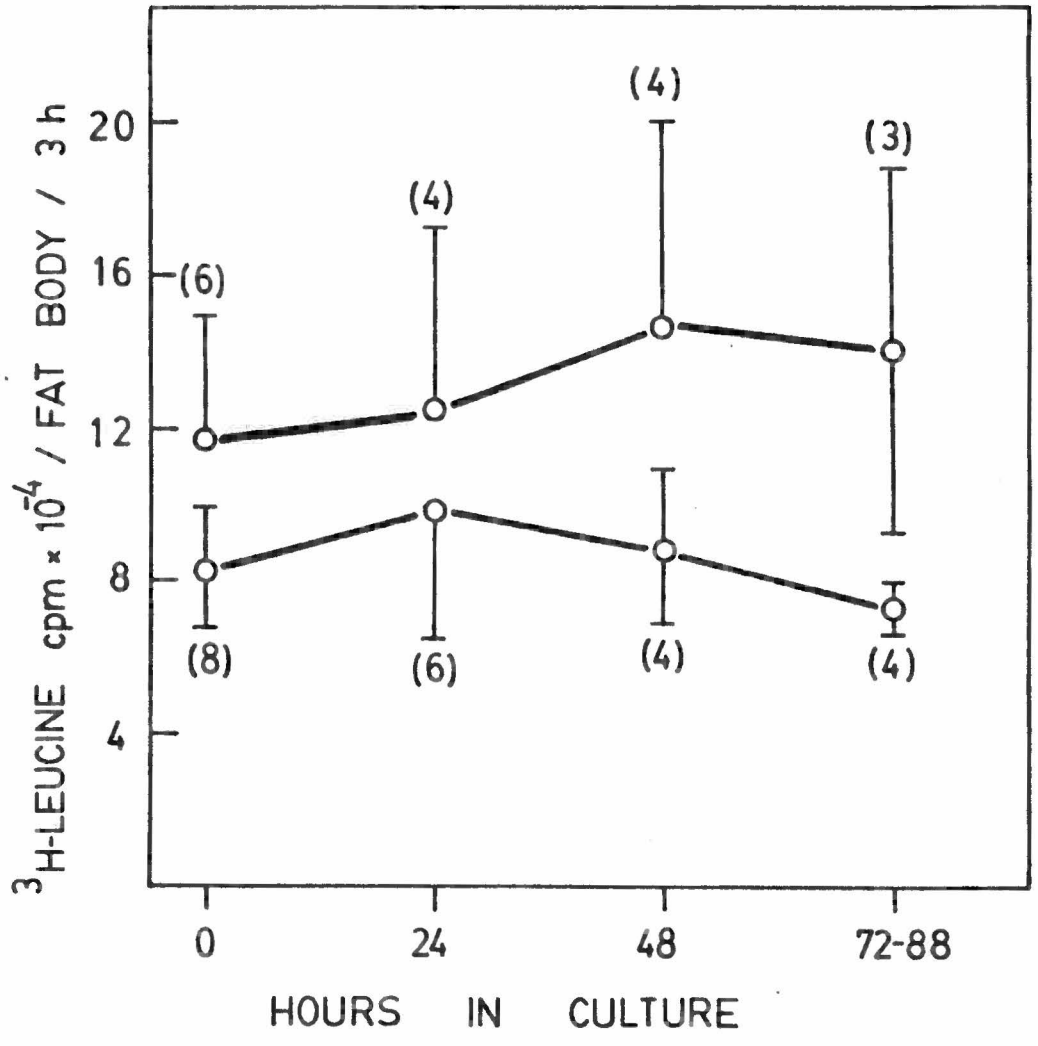
was almost 100% during the summers and about 30-40% during January to April. Therefore, all the tissue culture work had to be restricted to the first quarter of each year. The following two approaches were made to reduce the fungus contamination: (1) After it had been reported that the methyl ester of amphotericin B was a useful anti-fungal agent for tissue culture work (Schaffner, 1979), this antibiotic was substituted for rimocidin sulfate in the S-20 medium. However, it was no more effective than rimocidin sulfate in controlling fungus contamination in fat body cultures, so that rimocidin sulfate was retained in the medium. (2) Due to the nature of the insect respiratory system, it seemed probable that fungal spores might reside in the tracheae and tracheal sacs that would normally accompany the fat body. To reduce this probable source of fungus, large air sacs and easily dissectable tracheae were carefully removed from the fat bodies before culturing them. With this procedure, only about 17% of the fat body cultures were lost to fungus in experiments performed between January and April.

In spite of this major problem, some encouraging results were obtained for the long term maintainance of fat bodies in vitro. As a test of satisfactory tissue viability, the protein synthesizing activity of the fat body was measured during 3 h incorporation after different periods of culturing (figure 12). Fat bodies taken from adult females in peak vitellogenesis (oocyte length 3-5 mm; Gellissen and Wyatt, 1981) incorporated leucine into total secreted proteins at an almost constant rate during a 72 h culture period, which suggested very little, if any, loss of cell viability. A similar experiment

Figure 12

Protein secretion by fat bodies of adult female L. migratoria maintained in S-20 medium.

Fat bodies from 14-16 day old adult females (oocyte length 3-5 mm) were dissected out and freed of tracheal sacs and major tracheae. Individual fat bodies were cultured in 35 mm diameter plastic tissue culture dishes with 2 ml S-20 medium as described under Methods. At the indicated times, fat bodies were rinsed in incorporation medium and assayed for incorporation of [^{3}H]leucine over 3 h into secreted total proteins. The two curves represent data from two series of experiments done a year apart. The points represent the means $\pm$ S.E.M. of the number of fat bodies indicated in parenthesis.


^{3}H-LEUCINE cpm × 10^{-4} / FAT BODY / 3 h
20
16
12
8
4
(6)
(4)
(4)
(3)
(8)
(6)
(4)
(4)
0
24
48
72-88
HOURS IN CULTURE

repeated after a year gave the same results which suggested that the fat body culture conditions were reproducible (figure 12). The large variance in the data is mainly due to the different physiological states of females and differences in the sizes of fat bodies after removal of tracheae.

Stimulation of Vitellogenin Synthesis in Fat Body by Methoprene Added in vitro

In this and the next chapter the terms primary and secondary stimulation represent, respectively, a first time exposure of the fat body to methoprene and a second exposure to methoprene after complete withdrawal from primary stimulation either by endogenous JH or by experimentally administered JH analog.

Fat bodies for testing the primary response in vitro were obtained from adult female locusts 14 days after they had been treated with precocene II on the day of adult ecdysis. Based on the kinetic data on primary stimulation of Vg synthesis in fat bodies after injection of methoprene into adult locusts (see Chapter V), it was thought necessary to expose the fat bodies to methoprene for 72 h for a maximal response of Vg synthesis. After 72 h of incubation in the presence or absence of methoprene, fat bodies were assayed for the incorporation of [^{3}H]leucine into secreted Vg and total proteins. When methoprene was added to the culture medium at a dose of 200 µg/ml, incorporation of leucine into Vg was not stimulated, although

protein synthesis was active. However, at a level of 400 µg/ml S-20 medium, some fat bodies responded with stimulated incorporation of leucine into Vg (Table 7). The response, however, was generally weak and very variable.

Due to the difficulty in obtaining consistent primary stimulation of Vg synthesis in fat bodies cultured in the presence of methoprene and the shorter time required for maximal secondary stimulation in vivo, it was decided to attempt the latter response in vitro. Fat bodies for secondary stimulation of Vg synthesis were taken from adult females twelve days after they had been surgically allatectomized during peak vitellogenesis (14-16 day old). After 48 h of culturing in the presence (400 µg/ml) or absence of methoprene, fat bodies were assayed for incorporation of [^{3}H]leucine into Vg and total proteins (Table 8). In the control cultures, although protein synthesis was active, there was no incorporation of leucine into Vg. Fat bodies maintained in the presence of the hormone analog, on the other hand, showed a strong leucine incorporation into Vg (averaging 19 percent of the incorporation into total proteins). This specific response obtained in vitro was about 25% of that observed for the incorporation of leucine into Vg by fat bodies stimulated in vivo with 150 µg methoprene (secondary response; Chapter V; 5.2×10^{-5} cpm in Vg = 77% of cpm in total protein).

Discussion

A direct, although not always easy method to demonstrate the

Table 7

Primary stimulation of protein synthesis in the fat body of L. migratoria by methoprene added in vitro

Level of Methoprene	N	N*	[^{3}H]leucine cpm/fat body/3 h (mean ± S.E.M.) Vg	TCA	% Vg
0 µg/ml					
Expt. 1	3		0	57,261 ± 17,763	0
2	1		0	23,165	0
3	1		0	54,250	0
400 µg/ml					
Expt. 1	4	(2)	464 ± 490	45,292 ± 9,315	0.7
2	1	(1)	7,125	130,740	5.4
3	5	(3)	20,451 ± 21,863	54,867 ± 32,912	13.3

Fat bodies were dissected out from adult female locusts 14 days after precocene treatment on the day of adult ecdysis and first cultured for 8 h in 35 mm diameter plastic petri dishes containing 2 ml S-20 medium. Methoprene in hexane or hexane alone was evaporated on PEG coated glass petri dishes and 2 ml S-20 medium added. Fat bodies adapted to S-20 medium were then transferred into the glass petri dishes. After 72 h in culture with and without methoprene, the fat

Table 7. (cont'd)

bodies were assayed for incorporation of [^{3}H]leucine into secreted Vg and total proteins as described under Methods.

N* represent the number of fat bodies in each experiment that responded to methoprene in producing Vg.

Table 8

Secondary stimulation of protein synthesis in the fat body of *L. migratoria* by methoprene added *in vitro*

Level of Methoprene (µg/ml)	N	[^{3}H]leucine cpm/fat body (mean ± S.E.M.)		% Vg
		Vg	Total Protein	
0	7	0	71,414 ± 16,342	0
400	11	21,440 ± 9,802	88,455 ± 17,399	19.2

14-16 day old adult female locusts in peak vitellogenesis were surgically allatectomized and kept for 12 days. Fat bodies were then dissected out from individuals which did not show Vg in their hemolymph, and first cultured for 8 h in 35 mm diameter plastic petri dishes containing 2 ml S-20 medium. Methoprene (400 µg/ml) in hexane or hexane alone was evaporated on PEG coated glass petri-dishes and 2 ml S-20 medium added. Fat bodies conditioned in S-20 medium were then transferred into the glass petri dishes. After 48 h in culture with or without methoprene, the fat bodies were assayed for incorporation of [^{3}H]leucine into secreted Vg and total proteins as described under Methods.

action of a hormone on a tissue is by culture *in vitro*. Two basic requirements for being able to do so are to establish satisfactory culture conditions for the tissue and to optimize conditions for a hormonal response that is quantitatively comparable to the response obtained *in vivo*.

In the present investigation the functional integrity of the fat body, reflected by the rate of protein synthesis, was maintained for at least 72 h of culture *in vitro* in S-20 medium. This should allow sufficient time for secondary or primary hormonal stimulation. The assay conditions to monitor the protein synthetic activity of the cultured fat bodies were identical to those utilized by Abu-Hakima (1981). However, the level of leucine incorporation into total proteins from adult female fat body (expressed as cpm/fat body/3 h) was about 15 times that reported by Abu-Hakima (1981), a difference for which no explanation is apparent.

In order to obtain a secondary hormonal stimulation of Vg synthesis in *L. migratoria* fat bodies *in vitro*, Abu-Hakima (1981) used fat bodies from chemically allatectomized adult females two weeks after injecting them with 30-100 µg methoprene in mineral oil. With this procedure she reported a strong secondary stimulation of Vg synthesis in fat bodies cultured in the presence of only 10 and 50 µg/ml methoprene. However, a basic problem in the above procedure is the slow decay of the primary stimulation by the very stable synthetic analog. When locusts allatectomized soon after adult ecdysis were injected with 150 µg methoprene in mineral oil, circulating levels of Vg could be detected in their hemolymph even after 4 weeks, reflecting

the long lasting effects of injected methoprene (Chinzei et al., 1982; Chinzei and Wyatt, in preparation). Moreover, as shown in figure 4, fat bodies from methoprene injected females were active in Vg synthesis even 7 days after hormone treatment. In order to study changes in RNA during the secondary response, Chinzei et al. (1982) used locusts about 5 weeks after primary stimulation with methoprene. These observations place in question the nature of secondary stimulation of Vg synthesis in locust fat bodies taken only two weeks after injection of methoprene, as reported by Abu-Hakima (1981).

With the above results in mind, I adopted a different approach to prepare fat bodies for secondary stimulation. Adult females (14-16 day old) were surgically allatectomized during peak vitellogenesis. In this way, fat bodies were primed with endogenous JH and surgical removal of the corpus allatum resulted in an acute withdrawal of the hormone. This procedure also avoided the need to work with fat bodies from aged locusts. Twelve days after allatectomy, fat bodies of operated females which did not show detectable levels of Vg in the hemolymph were used for secondary stimulation in vitro. In contrast to the report of Abu-Hakima (1981), I could not obtain a secondary stimulation of Vg synthesis in fat bodies cultured in the presence of methoprene levels less than 400 µg/ml. Levels of 50-150 µg/ml which are sufficient to induce Vg synthesis in vivo were ineffective. The differences in response with lower levels of methoprene cannot be explained entirely as due to fat body culture conditions since the present study used conditions similar to those used by Abu-Hakima (1981), and the rates of leucine incorporation into Vg as well as

total proteins were substantially higher (discussed above).

Primary stimulation of Vg synthesis in fat bodies of L. migratoria with JH or methoprene added in vitro has been attempted twice before this investigation with irreproducible results (Wyatt et al., 1976; Abu-Hakima, 1981). In a first attempt to demonstrate the stimulation of Vg synthesis in fat bodies with hormone added in vitro, Wyatt et al. (1976) used their own formulation of culture medium. Without presenting data on the functional integrity of fat bodies maintained in their medium, they reported that by exposing fat bodies of allatectomized locusts for only 36 h to 90 µg JH-I in vitro (30 µg JH-I every 12 h) incorporation of leucine into Vg was stimulated. Abu-Hakima (1981) used Landureau's S-20 medium and reported satisfactory maintenance of fat bodies cultured for up to 96 h. She found it necessary to culture the fat bodies for 72 h in the presence of methoprene (100 and 200 µg/ml) to stimulate Vg synthesis in vitro. In the present investigation, I was unsuccessful in obtaining a primary stimulation of Vg synthesis in fat bodies cultured in the presence of 200 µg/ml methoprene, but by using twice this level of methoprene, variable and weak stimulation of leucine incorporation into Vg was obtained. In comparison to the results reported earlier, however, the stimulation of leucine incorporation into Vg from fat bodies that did respond was several times higher. In view of the problems discussed above in the procedure used by Wyatt et al. (1976), and the lack of success in reproducing the results of Abu-Hakima (1981) the data in both reports have to be viewed critically.

The results presented in this chapter demonstrate a definite

improvement in the maintenance of locust fat bodies *in vitro*. In terms of the hormonal induction of Vg synthesis *in vitro*, it is not yet clear whether the limited success and low response reported are due to lack of optimal conditions for fat body response, or whether another factor, in addition to JH or an analog, may be required. Similar difficulties have been reported for the stimulation of Vg synthesis in fat bodies of unfed mosquitoes cultured in the presence of ecdysterone (Borovsky, 1981). A requirement for thyroxine has been shown for estrogen-stimulated Vg synthesis in *Xenopus* liver cultures (Wang and Schneider, 1982). Therefore, it is worthwhile to try culturing locust fat bodies in the presence of brain homogenates, whole brains, or corpora cardiaca in addition to JH or an analog. Perhaps, initially, instead of adding JH or methoprene, corpora allata should be co-cultured with fat bodies to provide a continuous supply of natural JH.

In this investigation, very high levels of methoprene (400 µg/ml) were required to stimulate Vg synthesis in fat bodies *in vitro*. This amount far exceeds the reported solubility of this analog (1.4 µg/ml; Kramer *et al.*, 1976) which might in fact have worked to an advantage. The excess level of methoprene could have served as a reserve which dissolved gradually as the portion already in solution was slowly degraded if it existed as an emulsion in the medium, the fine droplets could have become bound to lipoidal cell membranes, and hence be able to act without actually dissolving. An important factor determining the effectiveness of a hormone on a tissue *in vitro* is its relative stability; this can necessitate its use at unphysiological levels

(Searle and Tata, 1981). Very little is known about the stability of methoprene relative of JH. However, as an indicator of relative biological stability, Chen et al. (1976) reported that in contrast to a single dose of methoprene, repeated doses of JH-I were required to stimulate Vg synthesis in locusts. One of the reasons for the instability of JH is due to an attack by specific esterases, which in nature is circumvented by the protection afforded by JH binding proteins (Sandburg et al., 1975). Therefore, if JH is to be used for stimulation of Vg synthesis in fat body in vitro, it may be worthwhile to include JH binding protein in the culture medium. However, to convincingly demonstrate the specificity of action of a synthetic analog for stimulation of Vg synthesis in fat bodies in vitro a control with a hormonally inactive compound would be necessary.

In spite of the failure, so far, to reproduce fully in vitro the degree of specific stimulation of fat body of L. migratoria, that is obtained with JH analog in vivo, the results obtained in this study do demonstrate the direct action of methoprene on the fat body. The success obtained is sufficient to indicate use of the system for experiments on the effects of inhibitors and on the stimulation of $mRNA_{Vg}$ synthesis in vitro.

V. KINETICS OF VITELLOGENIN mRNA ACCUMULATION AND RELATIVE RATES OF VITELLOGENIN SYNTHESIS IN FAT BODIES STIMULATED BY METHOPRENE IN VIVO

Introduction

The study of hormonally regulated systems in which large amounts of differentiated cell products are synthesized has contributed much to understanding of the molecular basis of hormone action and gene regulation. A large part of this knowledge has come from the extensive research on steroid regulated protein synthesis in transformed cell lines (reviewed in Thompson et al., 1980; Johnson et al., 1980), chick oviduct (Schimke et al., 1975; O'Malley et al., 1979) and the liver of oviparous vertebrates (Tata and Smith, 1979; Shapiro, 1982). The results of these studies have indicated that steroid hormones act at the transcriptional level.

In insects, our knowledge on the molecular basis of hormonally regulated gene expression is still in its infancy. Of the two lipoidal insect growth and developmental hormones, ecdysone and juvenile hormone, the molecular action of the former is better studied. The suggestion by Karlson (1963) that steroid hormones act upon genes had a strong influence in promoting research on the actions of these hormones at the molecular level. It was, however, only recently that direct evidence of the localization of a steroid (ecdysone) on those chromosomal loci that it activates was provided by Groneymeyer and Pongs (1980). This major contribution was possible

mainly due to the presence of polytene chromosomes in the nuclei of many larval tissues in the Diptera where the hormone induced pattern of transcription is reflected by puffing activity. While these significant advances have been made with ecdysone, much less work has been done with juvenile hormone.

A favourable system for the investigation of juvenile hormone action and hormonal control of specific gene expression in insects is vitellogenin synthesis. In Drosophila, despite its unique advantage for genetic analysis, Vg synthesis is under the control of both ecdysterone and JH. The specific roles of these hormones are not clearly defined, hence complicating the analysis of the molecular processes.

In L. migratoria, as described in the preceding chapters, Vg synthesis in the fat body of the adult female is under the control of JH. the results so far obtained suggest that JH regulates the synthesis of vitellogenin at the transcriptional level. However, the times at which $mRNA_{Vg}$ and Vg synthesis commence after the administration of JH or methoprene have not been clearly established (Chen et al., 1979; Chinzei et al., 1982). Chen et al. (1976) found that after topical application of 250 µg methoprene to allatectomized females, Vg synthesis was first detectable by 24 h, and increased sharply after 48 h to a maximum rate at 72 h, and declined thereafter. A second application of methoprene, 13 days after the first, evoked a rapid increase in Vg synthesis, which reached a maximal rate by 48 h. Chinzei et al. (1982) investigated the kinetics of $mRNA_{Vg}$ accumulation during primary and secondary stimulation of locusts injected with

methoprene. In the absence of molecular hybridization probes, these authors quantified $mRNA_{Vg}$ by photometric scanning of negatives of ethidium bromide stained gels on which fat body RNA had been resolved by electrophoresis. This method however, was relatively insensitive, and could not detect low levels of $mRNA_{Vg}$ which might have been present at early times of hormonal induction.

Therefore, in order more clearly to establish the time course of mRNA accumulation as a consequence of Vg gene activation and correlate it to the rate of Vg synthesis, I have made use of a cloned Vg gene fragment and purified $mRNA_{Vg}$ as a standard in a dot-hybridization technique. This assay is highly sensitive and capable of detecting complementary $mRNA_{Vg}$ sequences which exist at a level of two $mRNA_{Vg}$ molecules/fat body cell. I have used this technique to investigate the accumulation of $mRNA_{Vg}$ and rate of Vg synthesis in primary and secondary induction in adult female locusts, and after administration of methoprene to fifth instar larvae. Data have also been obtained on the decay kinetics of $mRNA_{Vg}$ and rate of Vg synthesis after withdrawal of JH from vitellogenic females.

The results presented in this chapter represent, to date, the only information on the kinetics of accumulation and decay of JH inducible gene transcripts quantified by a cloned hybridization probe.

Results

Isolation of Vitellogenin mRNA and Hybridization Specificity of DNA Sequences in p144-4.6 to $mRNA_{Vg}$

As a prerequisite for the assay of $mRNA_{Vg}$ by the dot-hybridization technique, it was necessary to isolate pure $mRNA_{Vg}$ for use as a reference standard and to demonstrate that the cloned locust DNA sequences in p144-4.6 were complementary only to $mRNA_{Vg}$.

Vitellogenin mRNA was purified by the procedure outlined by Chinzei *et al.* (1982). The poly(A)-containing fraction was first enriched from mature female fat body RNA by binding to oligo(dT)-cellulose. Upon sucrose density gradient centrifugation of the $poly(A)^+$ RNA, three major peaks, detected by $A_{260\ nm}$, were separated, and the RNA from each was precipitated. Fractions at the bottom of the gradient were also pooled and the nucleic acids precipitated. RNA from these steps was analyzed by electrophoresis in agarose-methyl mercury hydroxide gels and stained with ethidium bromide (figure 13). After sucrose density gradient centrifugation of $poly(A)^+$ RNA, RNA from one peak fraction (figure 13, lane C) was almost entirely (> 95%) $mRNA_{Vg}$. Additional $mRNA_{Vg}$ (about half of the amount in the above fraction) had migrated to the bottom of the sucrose gradient (figure 13, lane f). However, only $mRNA_{Vg}$ from the peak fraction was used as a standard for the dot-hybridization technique.

Figure 13

Electrophoretic analysis of RNA samples from steps in purification of $mRNA_{Vg}$ from fat body of mature adult female L. migratoria.

RNA samples were electrophoresed in a 1.2% agarose gel containing methyl mercuric hydroxide. The gel was stained in ethidium bromide and photographed under ultraviolet light. (a) 14 µg female fat body total RNA; (b) 2 µg poly(A)$^+$ RNA after oligo(dT)-cellulose chromatography of (a); (c-e) peak fractions after sucrose gradient centrifugation of poly(A)$^+$ RNA; (c) 0.8 µg of peak fractions containing $mRNA_{Vg}$; (d) 1.9 µg of peak fractions from the top of the gradient; (f) 1.6 µg of the pooled fractions at the bottom of the gradient.

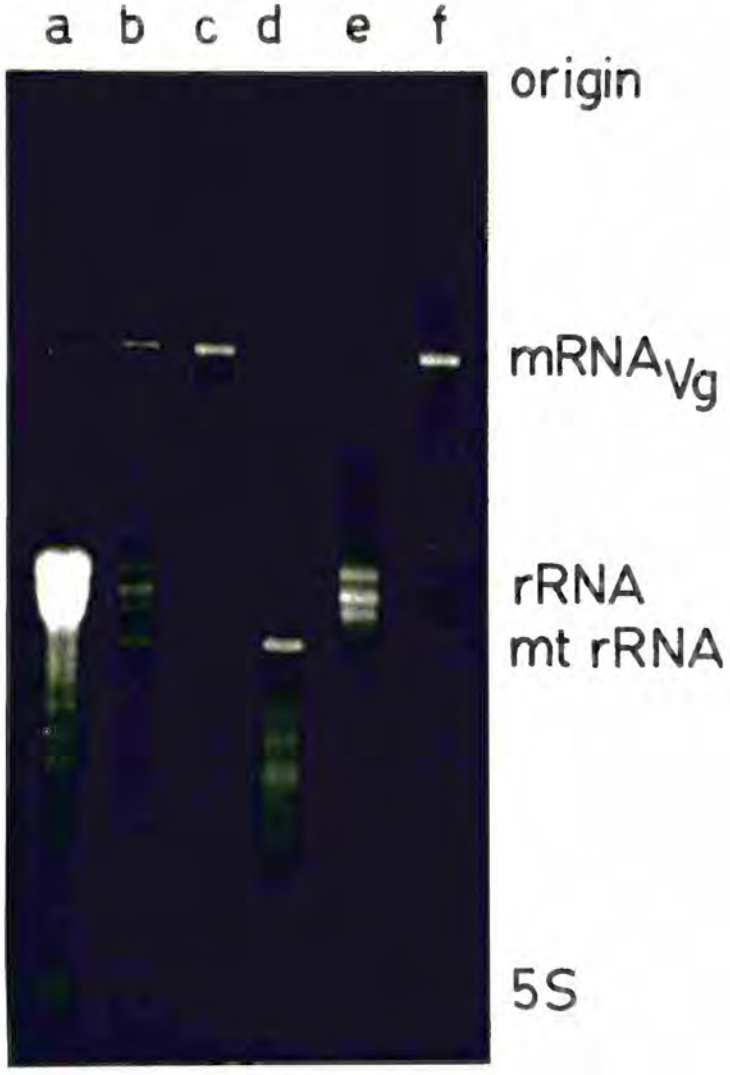
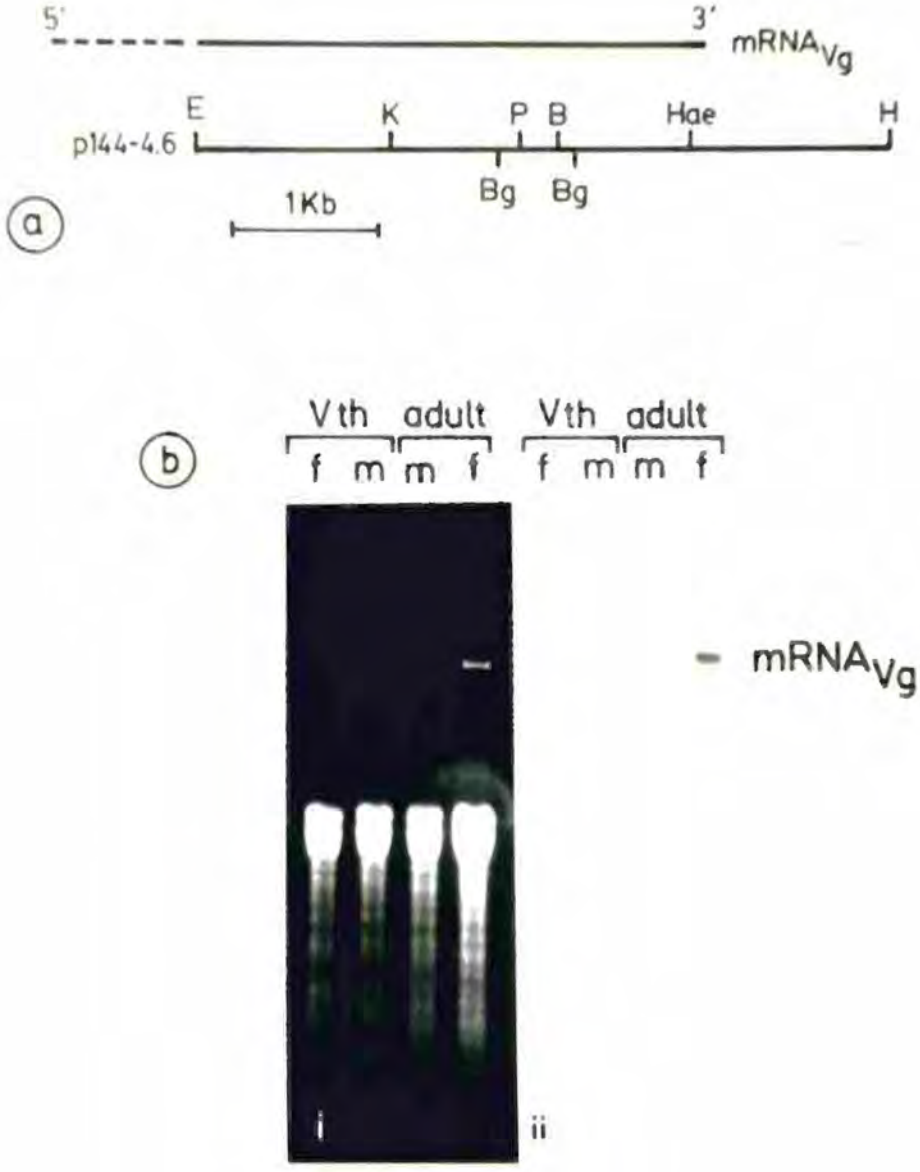

a b c d e f
origin
mRNA$_{Vg}$
rRNA
mt rRNA
5S

Figure 14

Restriction map (a) and hybridization specificity (b) of p144-4.6 for $mRNA_{Vg}$

a, restriction map of the 4.6 kb Eco RI-Hind III fragment of the sub-cloned p144-4.6 (with permission from J. Locke). The location and orientation of $mRNA_{Vg}$ with respect to the restriction sites is shown above the map. E, Eco RI; Hae, Hae III; B, Bam HI; Bg, Bgl II; P, Pst 1; K, Kpn 1. The scale in kb is shown below the map. b, (i) Total RNA samples from fat bodies of female (f) and male (m) fifth instar larvae and mature adult locusts were electrophoresed in a 1.2% agarose gel containing methyl mercuric hydroxide. The gel was stained with ethidium bromide; (ii) the electrophoresed RNA samples on the gel were transferred onto Gene Screen and hybridized to nick translated p144-4.6 DNA. 10 µg RNA samples, except for 15 µg of adult female fat body RNA, were loaded per lane. The northern blot was exposed at $-70^{o}C$ for 4 h.


5'
3'
mRNA Vg
E
K
P
B
Hae
H
p144-4.6
Bg
Bg
1Kb
a
b
Vth
adult
Vth
adult
f
m
m
f
f
m
m
f
mRNA Vg
i
ii

The sub-clone p144-4.6, containing L. migratoria DNA sequences complementary to $mRNA_{Vg}$ inserted in the plasmid pAT 153, was derived from a clone of locust DNA in bacteriophage λ Charon 4 by John Locke (Wyatt et al., 1981). A detailed restriction map for p144-4.6 and the location and orientation of coding sequences have been worked out (Locke, personal communication) and are shown in figure 14a. That p144-4.6 contained sequences complementary to $mRNA_{Vg}$, as already demonstrated by John Locke, was confirmed by northern blot analysis. RNA from fat body of fifth larval instar and adult female and male locusts was electrophoretically separated on agarose gels under denaturing conditions (figure 14b). A northern blot of this gel probed with [^{32}P] labelled p144-4.6 showed strong hybridization only to a female-specific RNA with same mobility as the visible $mRNA_{Vg}$ on the stained gel.

Assay of $mRNA_{Vg}$ Sequences by the Dot-Hybridization Technique

In order to quantify $mRNA_{Vg}$ in locust fat body, the diazotized paper disc hybridization procedure described by Searle and Tata (1981) was tried initially. To compare the binding capacity of RNA to diazotized paper or nitrocellulose filter membrane, known quantities of $mRNA_{Vg}$ were bound to cut paper squares of both types and hybridized to nick-translated p144-4.6 in a single hybridization bag. Samples with a single concentration of $mRNA_{Vg}$ bound in the presence of varied amounts of male fat body RNA (2 to 11 μg) were included. The squares were washed to remove non-specifically bound probe and the amount of

specific hybridization was determined by counting in a liquid scintillation counter. Two points were evident: (i) The diazotized paper had a greater binding capacity than the nitrocellulose, although for both there was a good correlation of the amount of $mRNA_{Vg}$ applied and the cpm hybridized; (ii) The cpm hybridized for a given concentration of $mRNA_{Vg}$ remained constant irrespective of the amount of carrier RNA in a sample. However, technical difficulties were encountered in the application of RNA samples and during the hybridization step with this procedure. The use of the diazotized paper was generally more tedious than the use of nitrocellulse paper.

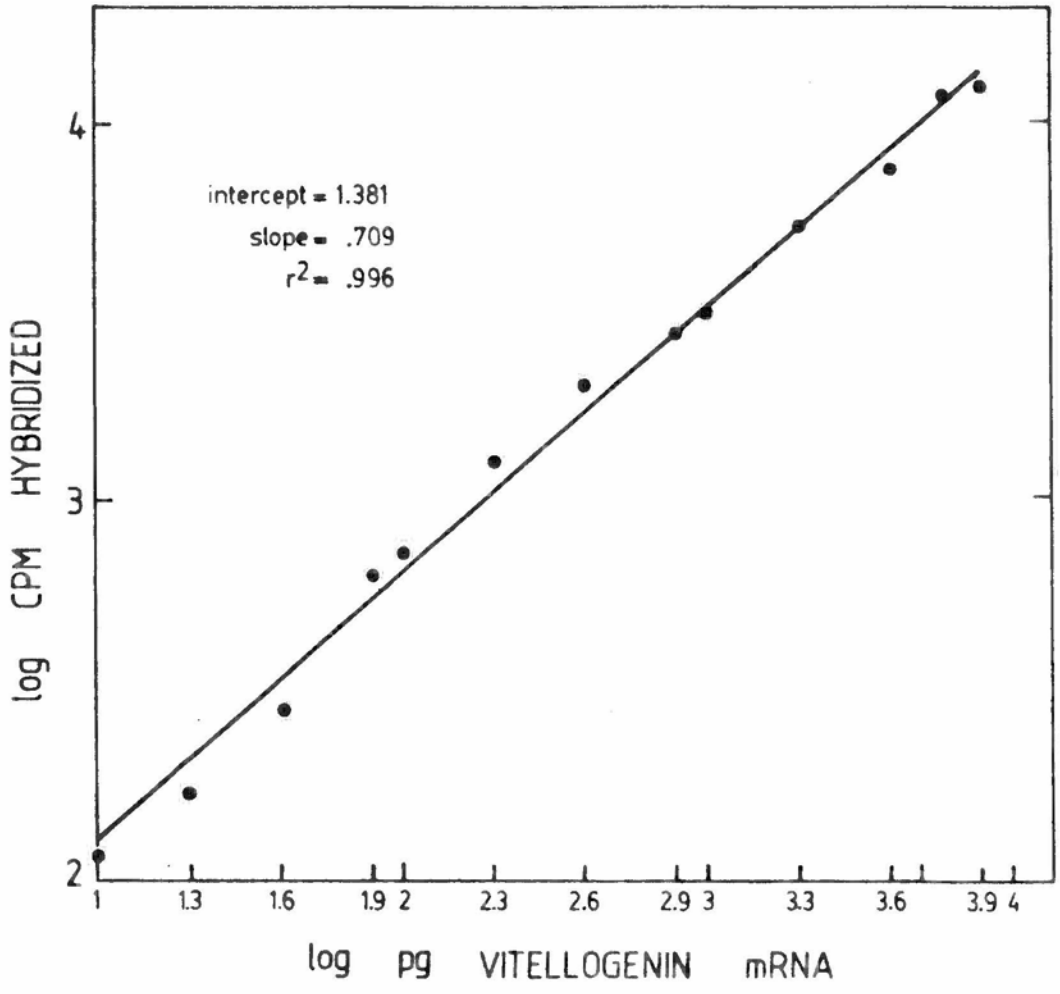
With the availability of the commercial Hybri-dot apparatus, the procedure was modified. Instead of using squares of paper, RNA samples were applied to spots 3 mm in diameter on a nitrocellulose sheet. Many more RNA samples could be applied in much less time, and problems such as the stacking of squares of paper in the hybridization bag were avoided.

When the modified procedure was followed with known concentrations of a standard $mRNA_{Vg}$ preparation and the cpm hybridized plotted against the amount of $mRNA_{Vg}$ applied, a linear relationship was obtained. A log-log plot was used in order to portray the data from a wide range of mRNA levels, and the linear relationship was maintained over three orders of magnitude (figure 15). The lower limit of detection of $mRNA_{Vg}$ amount was 10 pg, which was equivalent to 2 molecules $mRNA_{Vg}$/fat body cell in the preparations used. RNA samples expected to have a high content of $mRNA_{Vg}$ were diluted appropriately so that the cpm hybridized fell in the middle range of the curve.

Figure 15

A standard curve for the quantification of $mRNA_{Vg}$ by the dot-hybridization assay.

Purified $mRNA_{Vg}$ (0-8,000 pg) samples in 20 x SSC in the presence of 2 µg total RNA from adult male fat body were applied in duplicate onto a nitrocellulose sheet using the Hybridot^{Tm} apparatus. The nitrocellulose sheet was baked and soaked in the prehybridization buffer. Hybridization was carried out with [^{32}P] labelled p144-4.6 DNA. After hybridization the sheet was washed and autoradiographed. With the autoradiogram as a template, spots containing RNA and hybridized probe were cut out and counted in a Beckman liquid scintillation counter. After subtracting the background value (80 cpm), a logarithmic plot of cpm hybridized and amount of $mRNA_{Vg}$ (pg) applied gave a linear relationship with a high correlation coefficient.


intercept = 1.381
slope = .709
r^2 = .996
log CPM HYBRIDIZED
4
3
2
1
1.3
1.6
1.9
2
2.3
2.6
2.9
3
3.3
3.6
3.9
4
log pg VITELLOGENIN mRNA

Changes in the Fat Body of Adult Females After Primary and Secondary Stimulation by Methoprene in vivo

With locust $mRNA_{Vg}$ purified, a specific hybridization probe for $mRNA_{Vg}$ available and the technique to quantify $mRNA_{Vg}$ standardized, it was possible to carry out experiments to correlate the rate of Vg synthesis and accumulation of $mRNA_{Vg}$ in fat bodies after primary and secondary stimulation by methporene.

In the experimental protocol, fat bodies from individual adult female locusts after injection of methoprene were used to determine the rates of synthesis of Vg and total secreted protein, and the levels of $mRNA_{Vg}$ and total cellular RNA.

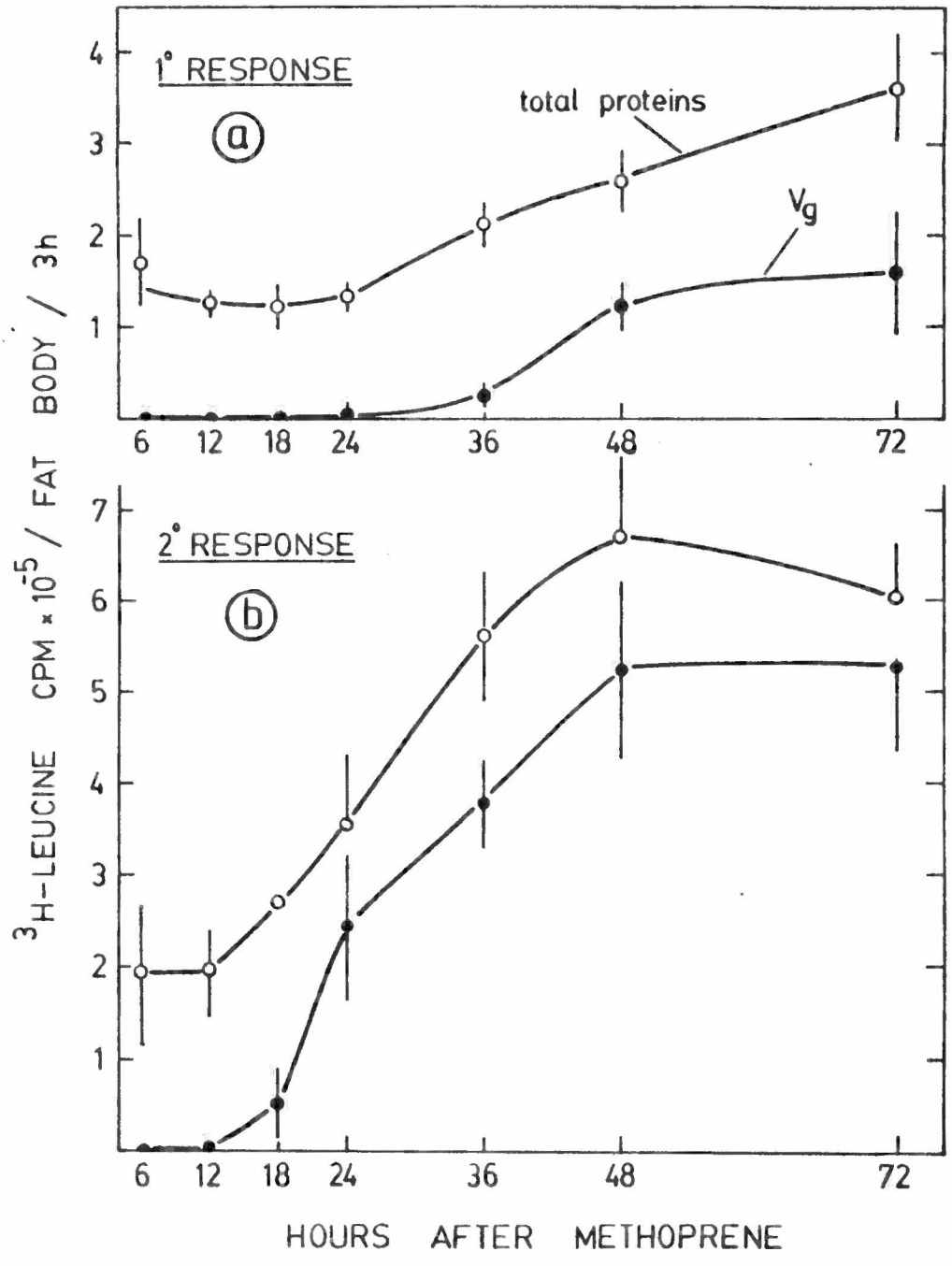
Relative rate of protein synthesis. At various times after primary and secondary stimulation of adult female locusts, protein synthesis by fat bodies was assayed by incorporation of [^{3}H]leucine into Vg and total secreted proteins as described under Methods. Labelled vitellogenin was first detected in the culture medium of some fat bodies 24 h after primary stimulation, after which time it increased gradually to maximal levels of secretion by 48 - 72 h (averaging 41-47% of total secreted proteins; figure 16a). During this time after methoprene treatment, total secreted proteins more than doubled. Fat bodies of locusts injected with mineral oil alone were not stimulated to synthesize Vg or increase their total protein output (data not shown).

Figure 16

Relative rates of vitellogenin and total secreted protein synthesis after primary and secondary stimulation of fat bodies from adult female L. migratoria by methoprene in vivo.

For primary stimulation (a) 14 day adult female locusts, in which the corpora allata had been inactivated by treatment with precocene, were injected with 150 µg methoprene in mineral oil. For secondary stimulation (b) 14-16 day adult females in peak vitellogenesis were allatectomized surgically, and two weeks later, when Vg could not be detected in the hemolymph, injected with 150 µg methoprene in mineral oil.

At the indicated times after primary and secondary stimulation, fat bodies were dissected out and assayed for incorporation of [^{3}H] leucine into secreted Vg (●) and total proteins (o) during 3 h as described under Methods. The points are means ± S.E.M. from three individual fat bodies.


1° RESPONSE
a
total proteins
Vg
2° RESPONSE
b
^{3}H-LEUCINE CPM × 10^{-5} / FAT BODY / 3h
HOURS AFTER METHOPRENE
1
2
3
4
5
6
7
12
18
24
36
48
72

Fourteen days after surgical removal of corpora allata of adult females in peak vitellogenesis, Vg could no longer be detected in the hemolymph. When assayed in vitro, the fat bodies of such locusts did not incorporate leucine into Vg, and the incorporation into total proteins had declined to a level that existed before primary stimulation. After a secondary stimulation with 150 µg methoprene, the fat bodies began to produce Vg with a much shorter lag period (about 12 h) and the rate increased more steeply than after primary stimulation (figure 16b). Seventy two hours after methoprene administration, fat bodies during secondary stimulation incorporated leucine into Vg at a rate about 4 times that observed during primary stimulation. At this time in the secondary response about 86% of the total secreted proteins was Vg.

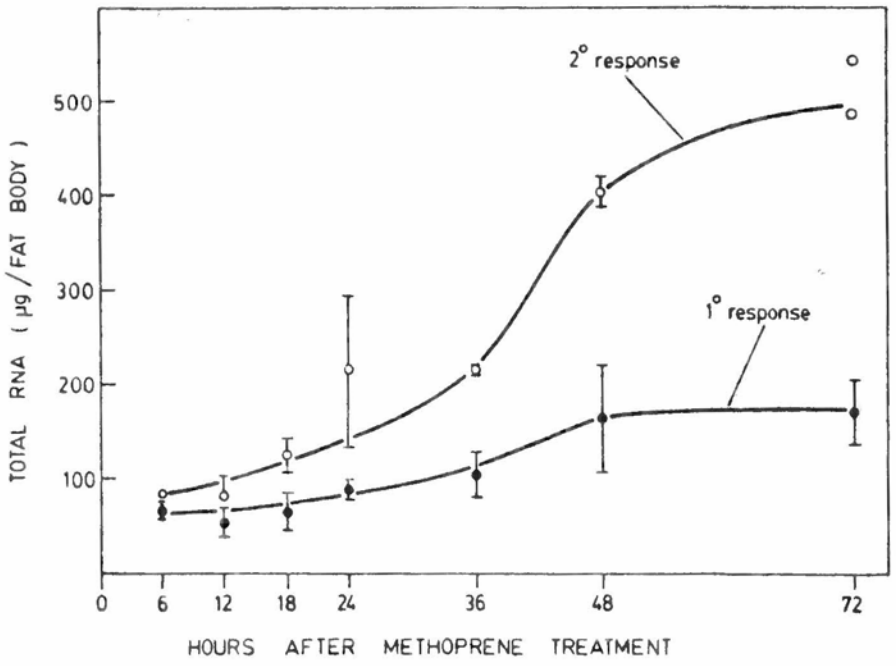
Changes in total RNA. Fat bodies from the above experiment were used to follow changes in total RNA and $mRNA_{Vg}$ during methoprene-induced primary and secondary responses. Total RNA was extracted from individual fat bodies and the yield determined. These values have been used to indicate changes during the hormonal response, but, because of losses during extraction and precipitation, they are low estimates of true tissue RNA content. In precocene-treated locusts injected with mineral oil, the RNA content remained at about 60 µg/fat body. After primary stimulation of precocene-treated locusts with methoprene, total fat body RNA increased gradually from the basal level to about 165 µg/fat body by 48-72 h (figure 17).

After decay of the primary stimulation of fat bodies by endogenous

Figure 17

Total RNA content of fat body after primary and secondary stimulation with methoprene.

Adult female locusts were injected with 150 µg methoprene for primary (•) and secondary (o) stimulation as described in figure 16. Fat bodies were dissected out at the times shown after hormone treatment and used individually for extraction of RNA by the SDS-phenol-chloroform method. RNA yield was estimated by $A_{260\ nm}$. Each point is a mean ± S.E.M. from 3 fat bodies except two for the 72 h point after secondary stimulation.


2° response
1° response
500
400
300
200
100
TOTAL RNA (µg / FAT BODY)
0
6
12
18
24
36
48
72
HOURS AFTER METHOPRENE TREATMENT

levels of JH, induced by surgical allatectomy of vitellogenic females, total RNA had dropped from about 360 µg/fat body (value obtained from the experiment on decay kinetics of $mRNA_{Vg}$ and Vg; data not shown) to about 85 µg/fat body (figure 17). Secondary stimulation of allatectomized locusts with 150 µg methoprene evoked an increase in fat body RNA which was more rapid than observed after primary stimulation, and after 72 h total RNA had reached a level of 500 µg/fat body.

Electrophoretic and northern blot analysis of female fat body RNA. Before $mRNA_{Vg}$ content in female fat body RNA samples was quantified, samples of RNA representing various times after hormonal stimulation were analysed by electrophoresis followed by northern blot analysis of the separated RNA. When samples of RNA from fat bodies after primary stimulation were electrophoretically separated on an agarose gel and stained with ethidium bromide, an $mRNA_{Vg}$ band was visible in RNA samples obtained after 48 and 72 h (figure 18A), a result in agreement with that obtained by Chinzei et al. (1982). However, on a northern blot of the gel probed with radiolabelled p144-4.6, hybridization to $mRNA_{Vg}$ could be detected as early as 36 h after primary stimulation (figure 18B). On a longer exposure of the probed blot (not shown), a faint signal corresponding to $mRNA_{Vg}$ was evident in RNA samples from fat bodies 24 h after methoprene treatment. In RNA from control treated fat bodies neither a stained $mRNA_{Vg}$ band nor any hybridization signal on the northern blot was present.

When samples of RNA extracted from fat bodies at various times

Figure 18

Electrophoretic and northern blot analysis of RNA from fat body of adult female L. migratoria after primary stimulation with methoprene in vivo.

Fat bodies were taken from locusts 6-72 h after primary stimulation, assayed for their rates of Vg and total protein synthesis (figure 16), and used for total RNA extraction. (A), Representative total RNA samples (10 µg/lane) from the fat bodies at the indicated times after hormone or control (72 c) treatment were separated by electrophoresis on 1.2% agarose gels in the presence of methyl mercuric hydroxide and the gel stained in ethidium bromide. (B), The separated RNA samples were blotted onto Gene Screen and hybridized to [^{32}P]labelled p144-4.6. The blot was washed and autoradiographed as described under Methods.

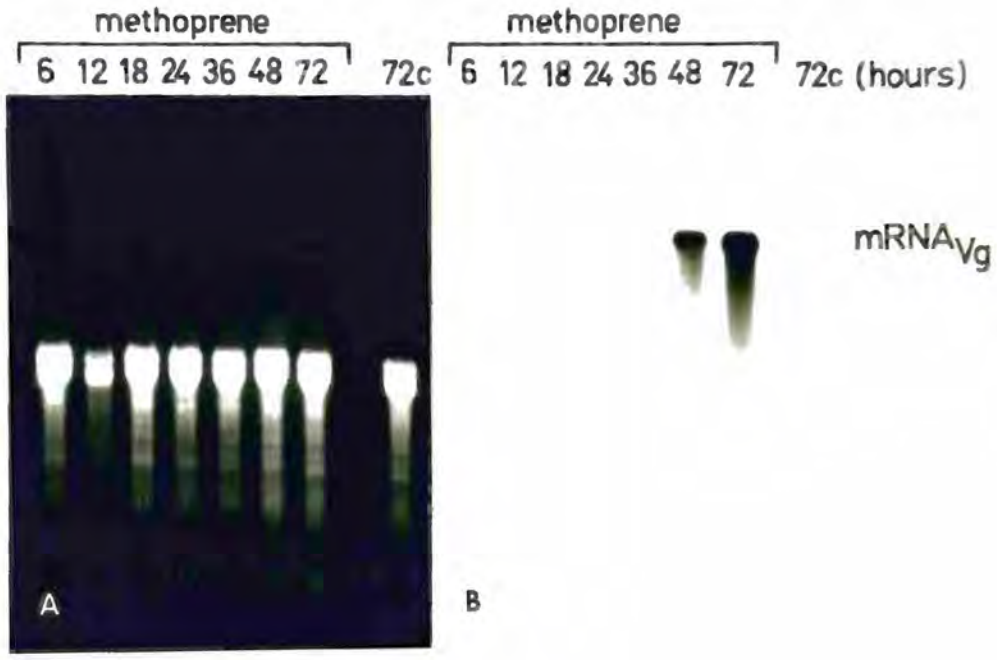
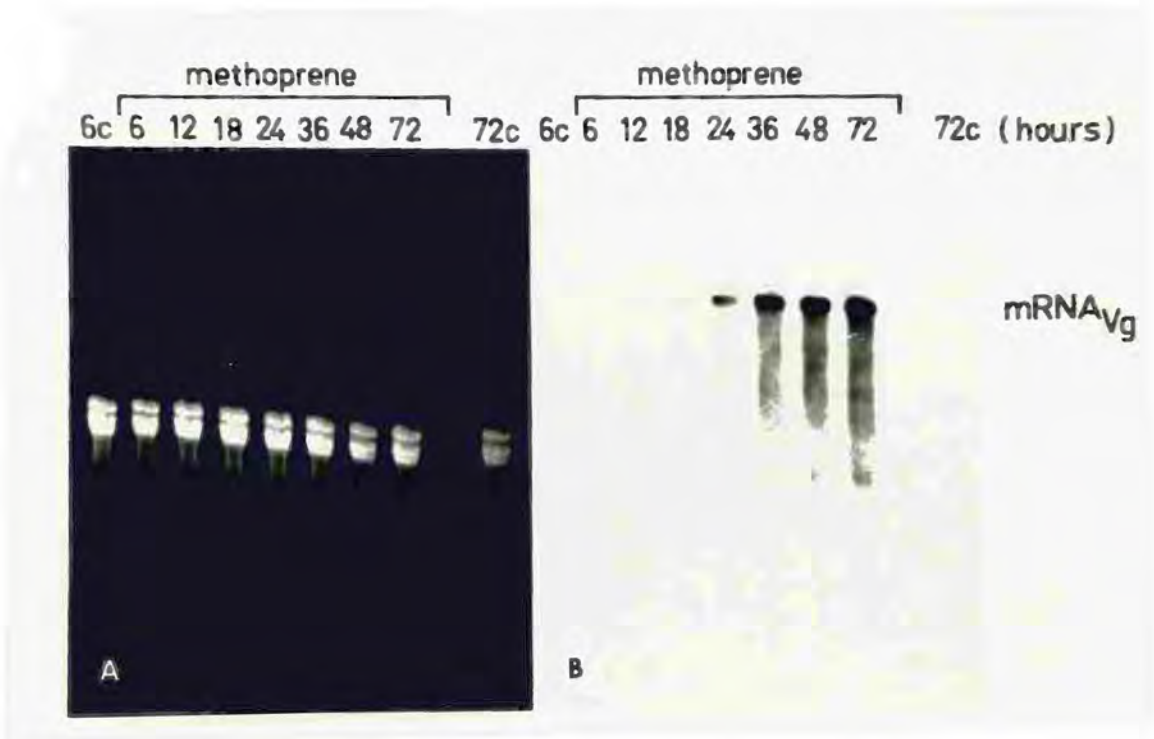

methoprene
6 12 18 24 36 48 72
72c
methoprene
6 12 18 24 36 48 72
72c (hours)
mRNA$_{Vg}$
A
B

Figure 19

Electrophoretic and northern blot analysis of total RNA from fat body of adult female L. migratoria after secondary stimulation with methoprene in vivo.

Fat bodies taken from locusts 6-72 h after secondary stimulation were assayed for incorporation of [^{3}H]leucine into Vg and total proteins (figure 16), and then used for total RNA extraction. (A) Representative total RNA samples (10 µg/lane) from the fat bodies at the indicated times after hormone or control (6 c and 72 c) treatment were electrophoresed on 1.2% agarose gels in the presence of methyl mercuric hydroxide and the stained gel photographed under ultraviolet light. (B) The separated RNA samples were transferred onto Gene Screen and hybridized to nick translated p144-4.6. The blot was washed and autoradiographed as described under Methods.


methoprene
6c 6 12 18 24 36 48 72 72c
methoprene
6c 6 12 18 24 36 48 72 72c (hours)
mRNA$_{Vg}$
A
B

after secondary hormonal stimulation were electrophoresed and the gel stained with ethidium bromide, $mRNA_{Vg}$ was visible as a faint band in the 24 h sample (figure 19A) and the intensity of the message band increased between 36 and 72 h. By northern blot analysis, there was a clear radioactive band corresponding to $mRNA_{Vg}$ in samples taken as early as 12 h after methoprene treatment. With sufficient exposure of the blot, a faint band of $mRNA_{Vg}$ hybridization was detectable at 6 h; however, this may not reflect secondary hormonal stimulation since similar weak specific hybridization was detected in 6 h and 72 h control (mineral oil injected) samples (figure 19B).

Kinetics of $mRNA_{Vg}$ accumulation. The accumulation of $mRNA_{Vg}$ in fat bodies stimulated by methoprene (primary and secondary response) was followed quantitatively by a sensitive dot-hybridization technique. With this technique as little as 2 $mRNA_{Vg}$ molecules/fat body cell could be detected reliably. Fat bodies of precocene-treated females injected with mineral oil did not contain detectable levels of $mRNA_{Vg}$.

After primary stimulation, $mRNA_{Vg}$ sequences in total RNA of fat body cells remained undetectable up to 18 h. Twenty four hours after methoprene administration, $mRNA_{Vg}$ sequences were clearly detectable (figure 20) and accumulated at a slow rate (370 molecules/h/fat body cell) for the next 12 h. A striking feature of the primary response was the 25-fold increase in $mRNA_{Vg}$ accumulation rate (9,000 molecules/h/fat body cell) between 36 and 48 h after hormonal stimulation.

Two weeks after the surgical removal of corpora allata of

vitellogenic females, when Vg in the hemolymph was no longer detectable, a second injection of methoprene stimulated accumulation of $mRNA_{Vg}$ sequences after a much shorter lag period than for primary stimulation. However, unlike the situation in the control fat bodies for the primary response, $mRNA_{Vg}$ at a level of about 270 molecules/fat body cell were detectable 6 h after mineral oil injection into females for the secondary response, and a similar level of $mRNA_{Vg}$ was present in hormone-treated fat bodies taken after 6 h. The accumulation of these sequences increased at a slow rate (438 molecules/h/fat body cell) only during 12-18 h, and at a somewhat faster rate thereafter.

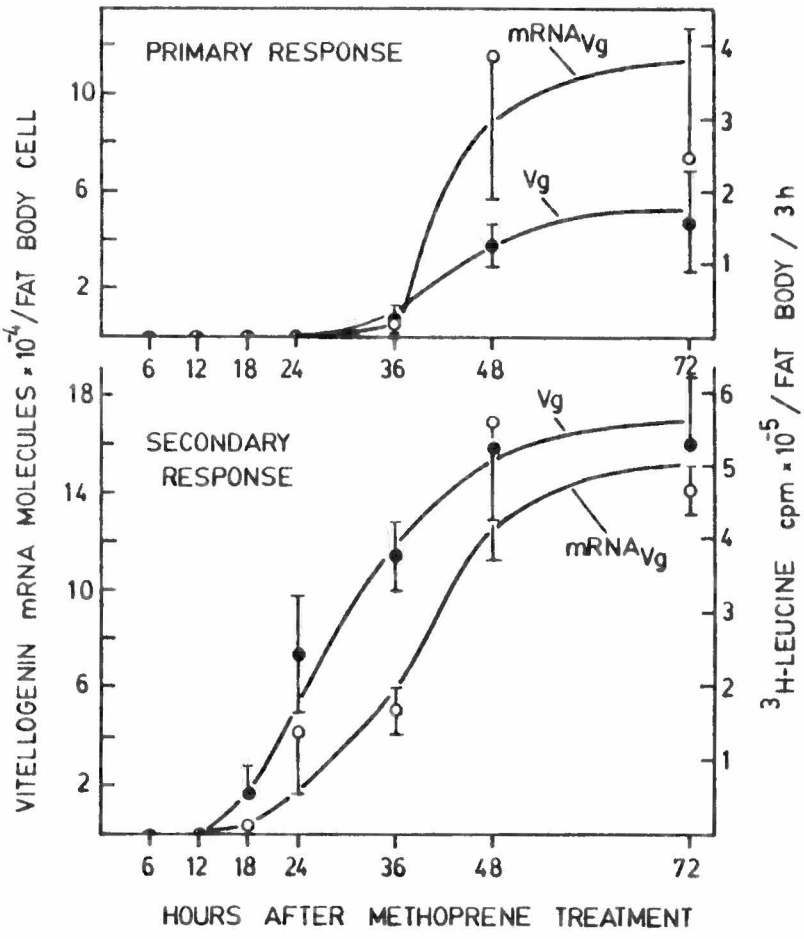
The pattern of increasing rate of $mRNA_{Vg}$ synthesis was less distinctively biphasic in the secondary than in the primary response. Too detailed an analysis of the curves is not possible because of the large variances of the points. However, between 36-48 h after hormonal stimulation, the slopes for $mRNA_{Vg}$ accumulation were similar for the primary and secondary responses (figure 20), and in both types of responses, maximal levels of $mRNA_{Vg}$ had accumulated 48-72 h after hormone treatment.

Experimental data for the relative rate of Vg synthesis, taken from figure 16, are plotted along with data on accumulation of $mRNA_{Vg}$ to facilitate comparison (figure 20). The quantitative relations of these curves will be discussed below.

Figure 20

Kinetics of $mRNA_{Vg}$ accumulation and relative rate of Vg synthesis after primary and secondary stimulation of fat bodies from adult female L. migratoria by methoprene in vivo.

Adult female locusts were injected with 150 µg methoprene in mineral oil for primary and secondary stimulation and at the indicated times their fat bodies were assayed for incorporation of [^{3}H]leucine into Vg (•) during a 3 h incubation in vitro as described in figure 16. Total RNA, extracted from the same individual fat bodies, was assayed for $mRNA_{Vg}$ by the dot-hybridization assay as described under Methods. The numbers of $mRNA_{Vg}$ molecules per fat body cell (o) were calculated as also detailed under Methods. Each point is a mean ± S.E.M. of three individual fat bodies.


PRIMARY RESPONSE
mRNA$_{Vg}$
Vg
SECONDARY RESPONSE
Vg
mRNA$_{Vg}$
VITELLOGENIN mRNA MOLECULES • 10^{-4}/FAT BODY CELL
^{3}H-LEUCINE cpm • 10^{-5}/FAT BODY / 3 h
HOURS AFTER METHOPRENE TREATMENT
6 12 18 24 36 48 72
2 6 10
2 6 10 14 18
1 2 3 4
1 2 3 4 5 6

Decay Kinetics of $mRNA_{Vg}$ and Rate of Vitellogenin Synthesis During Withdrawal After Primary Stimulation of Fat Body By Endogenous JH.

The decay of $mRNA_{Vg}$ and of the rate of Vg synthesis was investigated by surgically allatectomizing 14-16 d adult females which were in peak vitellogenesis (determined by measuring oocyte length of a few from a group of females). At different times after allatectomy, fat bodies were dissected out and first assayed for the incorporation of [^{3}H]leucine into Vg and total proteins. The same fat bodies were then used for RNA extraction and $mRNA_{Vg}$ was assayed as described earlier.

In the fat bodies of unoperated females $mRNA_{Vg}$ had accumulated to about 1.6×10^5 molecules/fat body cell (day 0, figure 21). After surgical allatectomy of vitellogenic females, which resulted in an acute withdrawal of JH, $mRNA_{Vg}$ decayed with first order kinetics showing a half-life of about 24 h. Fifteen days after allatectomy, when $mRNA_{Vg}$ could not be detected as a stained band on agarose-methyl mercury hydroxide gels (inset, figure 21), there were about 5 $mRNA_{Vg}$ molecules/fat body cell detectable by the dot hybridization technique.

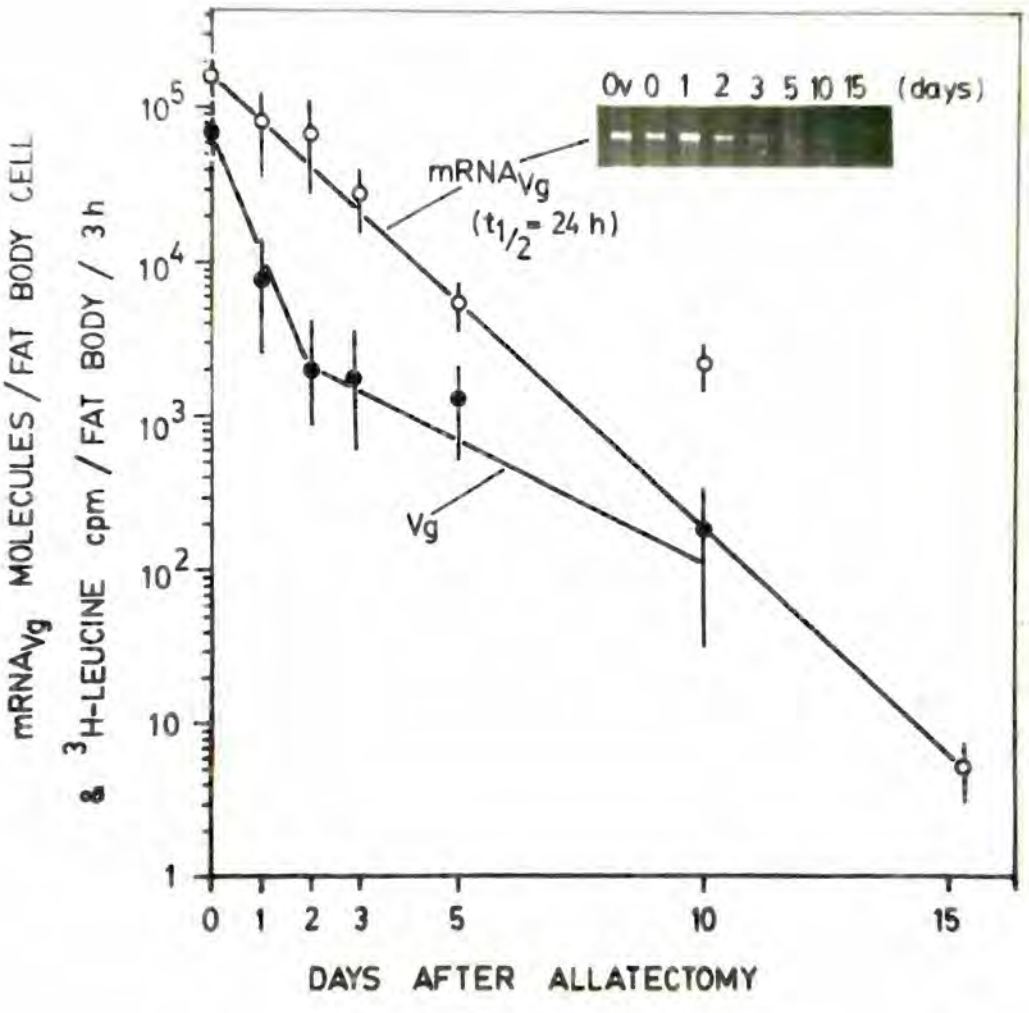
During peak vitellogenesis, Vg constituted about 67% of the total secreted proteins synthesized. After allatectomy the rate of leucine incorporation into Vg declined with biphasic characteristics (figure 21). There was a dramatic decrease in Vg synthesis for two days after allatectomy (to 3% of the total secreted proteins) and thereafter it decreased gradually to extremely low levels. Fifteen days after the

Figure 21

Decay kinetics of $mRNA_{Vg}$ and rate of vitellogenin synthesis after acute withdrawal of JH in mature adult female L. migratoria.

Adult females (14-16 day old) in peak vitellogenesis were allatectomized surgically. At the indicated times after surgery, the fat bodies were dissected out and assayed for incorporation of [^{3}H]leucine into Vg (o) during a 3 h incubation in vitro as described under Methods. The above fat bodies were then individually used for the extraction of total RNA. The amount of $mRNA_{Vg}$ in each total RNA preparation was quantified by the dot-hybridization assay and molecules of $mRNA_{Vg}$ per fat body cell calculated as described under Methods. Each point is a mean $\pm$ S.E.M. from 3 individual fat bodies.

The inset is a photograph of the portion of a stained agarose-methyl mercuric hydroxide gel showing $mRNA_{Vg}$ after electrophoresis of representative fat body total RNA samples (above) and total RNA extracted from fat body of an ovipositing female (Ov).


mRNA$_{Vg}$ MOLECULES / FAT BODY CELL
& ^{3}H-LEUCINE cpm / FAT BODY / 3 h
10^5
10^4
10^3
10^2
10
1
Ov 0 1 2 3 5 10 15 (days)
mRNA$_{Vg}$
($t_{1/2}$ = 24 h)
Vg
0 1 2 3 5 10 15
DAYS AFTER ALLATECTOMY

surgery when leucine was no longer perceptibly incorporated into Vg, $mRNA_{Vg}$ was still detectable by the dot-hybridization technique. By this time both the total RNA and total protein synthesis had dropped to the levels observed earlier before methoprene stimulation (figure 16 and 17).

Changes in the Rate of Protein Synthesis and $mRNA_{Vg}$ Level in Fat Bodies of Fifth Instar Females Stimulated by Methoprene.

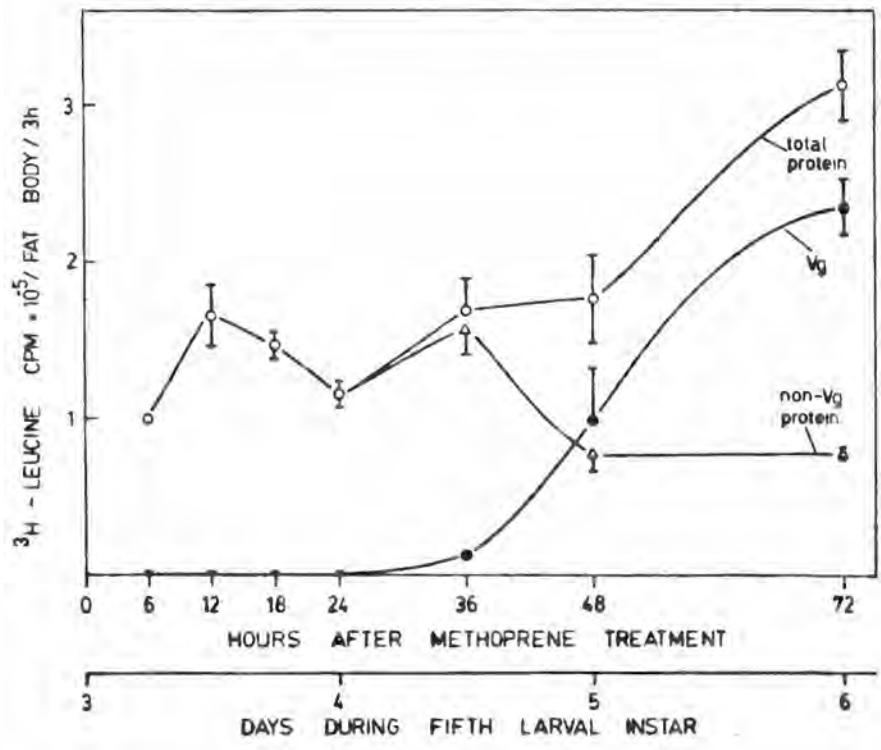
Since during the fifth stadium, the locust fat body can respond to high doses of methoprene by producing Vg, it was of interest to study the kinetics of the response at the mRNA and protein level. In this stage, during the normal exposure to endogenous levels of JH, Vg is not produced. Fifth instar females were injected on day 3 with 300 µg methoprene in mineral oil or with mineral oil alone and at various times thereafter, the fat bodies were assayed for protein synthesis and levels of total RNA and $mRNA_{Vg}$ as described above.

After methoprene injection, leucine incorporation into Vg was detectable with a lag of about 24 h (figure 22). After a slow increase over the next 12 h, the fat bodies produced Vg at an accelerated rate. Seventy two hours after methoprene treatment, 75 percent of the total secreted proteins was Vg. Fat bodies from mineral oil-injected larvae did not produce Vg. The amount of leucine incorporated into TCA-precipitable protein was variable for the first 36 h after methoprene treatment, after which time there was an increase. Between 48 and 72 h, incorporation into total proteins and

Figure 22

Relative rates of vitellogenin and total protein synthesis after stimulation of fat bodies from female fifth larval instar L. migratoria by methoprene in vivo.

Female fifth instar larvae, 3 days after the fourth molt, were injected with 300 µg methoprene in 5 µl mineral oil or 5 µl mineral oil alone. At the indicated times after treatment, their fat bodies were dissected out and assayed for the incorporation of [^{3}H]leucine into Vg (•), total proteins (o) and non-Vg proteins (Δ; calculated by difference) during 3 h as described under Methods. Along the X-axis, the corresponding days of development during the fifth instar are also shown. The points are means ± S.E.M. from three individual fat bodies.


^3H - LEUCINE CPM • 10^{-5}/ FAT BODY / 3h
3
2
1
total protein
Vg
non-Vg protein
0
6
12
18
24
36
48
72
HOURS AFTER METHOPRENE TREATMENT
3
4
5
6
DAYS DURING FIFTH LARVAL INSTAR

Vg increased in parallel, which suggested that the increase in total proteins was due to the increase in Vg synthesis. In fact, between 36 and 72 h, the non-Vg proteins (calculated by difference) decreased, in agreement with the results obtained earlier (figure 10).

RNA was extracted from fat bodies of the hormone-treated fifth instar females and analyzed by electrophoresis. On the stained gel, $mRNA_{Vg}$ was visible as a band in samples from fat bodies exposed to methoprene for 48 and 72 h (figure 23A). A northern blot of the same gel probed with ^{32}P- nick-translated p144-4.6 revealed weak hybridization to $mRNA_{Vg}$ in fat body RNA from females injected with methoprene 36 h earlier (figure 23B). $mRNA_{Vg}$ was not detectable in RNA from control fat bodies. This developmental profile of $mRNA_{Vg}$ after hormone treatment is remarkably similar to that obtained during primary response in the adult female (compare with figure 18A and B).

The accumulation of $mRNA_{Vg}$ in the fat body after methoprene treatment of fifth instars was quantified by the dot-hybridization technique and compared to the relative rates of leucine incorporation into Vg (figure 24). The number of $mRNA_{Vg}$ molecules per fat body cell could not be calculated since the DNA ploidy level in the larval fat body is not known. In methoprene-stimulated fat bodies, $mRNA_{Vg}$ accumulated with biphasic kinetics, characteristic of the response during primary stimulation of adult female fat bodies (compare with figure 20). After hormone treatment, $mRNA_{Vg}$ accumulated at a slow rate of 430 pg/h/fat body between 24-36 h, and then increased dramatically over the next 12 h (about 16 ng/h/fat body). While $mRNA_{Vg}$ had probably reached a maximal level between 48-72 h after

Figure 23

Electrophoretic and northern blot analysis of total RNA samples from fat bodies of female fifth instar larval L. migratoria after stimulation with methoprene in vivo.

Female fifth instar larvae were injected with 300 µg methoprene in 5 µl mineral oil or mineral oil alone on day 3 after the fourth molt. At 6-72 h after hormone or control treatment, the fat bodies were first assayed for Vg and total protein synthesis (figure 22) and then used for extraction of total RNA. (A) Representative RNA samples (10 µg/lane) from indicated times after hormone or control (6 c and 72 c) treatment were electrophoretically separated in a 1.2% agarose gel containing methyl mercuric hydroxide and stained with ethidium bromide. (B) The RNA samples separated on the gel were blotted onto Gene Screen and hybridized to nick-translated p144-4.6. The blot was washed and autoradiographed as described under Methods.

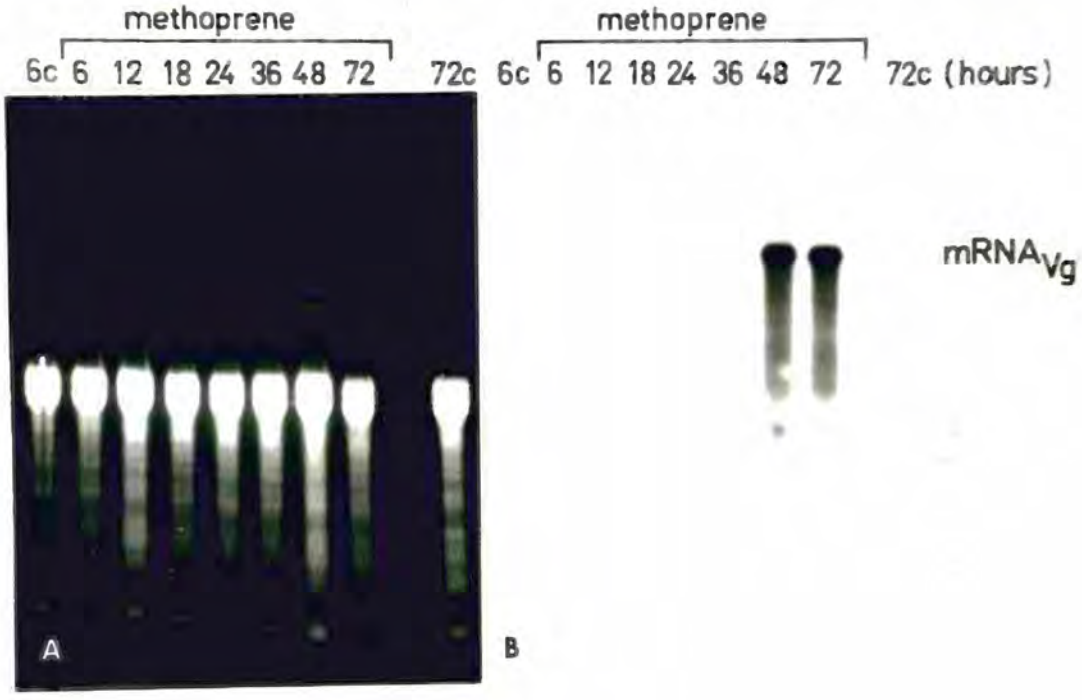
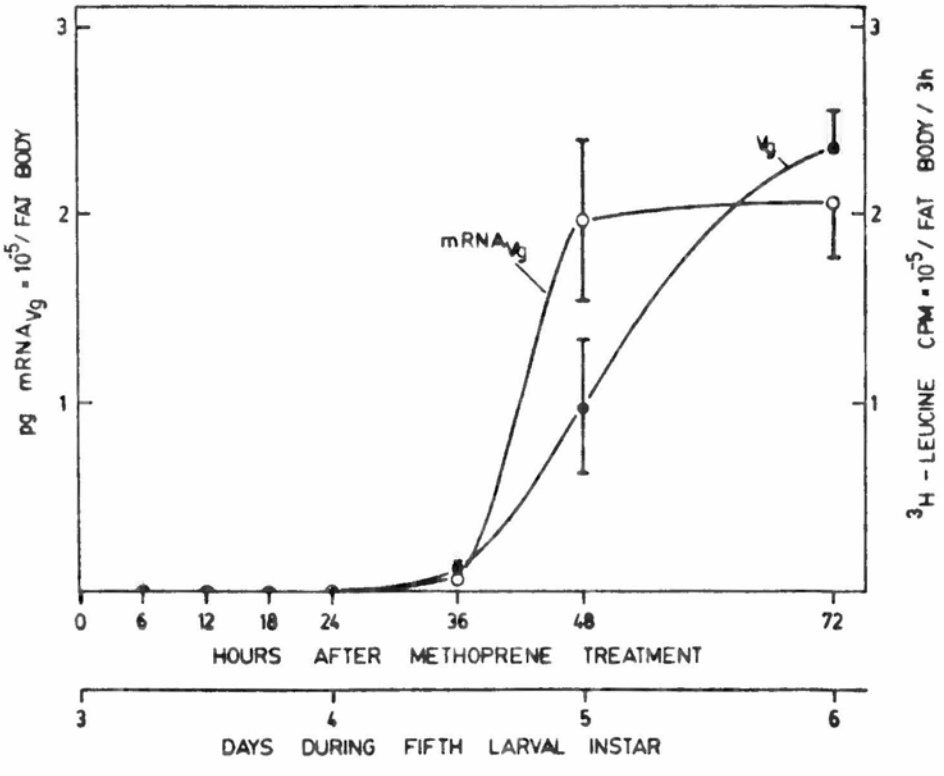

methoprene
methoprene
6c 6 12 18 24 36 48 72 72c 6c 6 12 18 24 36 48 72 72c (hours)
mRNA Vg
A
B

Figure 24

Kinetics of $mRNA_{Vg}$ accumulation and the relative rate of vitellogenin synthesis after stimulation of fat bodies from fifth instar female larvae of L. migratoria injected with methoprene.

Female fifth instar larvae were injected with 300 µg methoprene in mineral oil on day 3 after the fourth larval molt and at the indicated times their fat bodies were assayed for incorporation of [^{3}H]leucine into Vg (•) during a 3 h incubation in vitro as described in figure 22. Total RNA was extracted from the same individual fat bodies and each RNA sample was assayed for the amount of $mRNA_{Vg}$ by the dot-hybridization technique as described under Methods. The data points are the mean ± S.E.M. from three individual fat bodies.


pg mRNA$_{vg}$ • 10^{-5}/ FAT BODY
^{3}H - LEUCINE CPM • 10^{-5}/ FAT BODY / 3h
mRNA$_{vg}$
Vg
0
6
12
18
24
36
48
72
HOURS AFTER METHOPRENE TREATMENT
3
4
5
6
DAYS DURING FIFTH LARVAL INSTAR

methoprene treatment, the rate of Vg synthesis continued to increase until at least 72 h.

Relative Levels of Vitellogenin Gene Expression in Fat Bodies from Fifth Instar Larval and Adult Female and Male L. migratoria.

The relative levels of $mRNA_{Vg}$ in fat body RNA from female and male locusts 72 h after methoprene treatment (300 µg/fifth instar larva and 150 µg/adult) were quantified by the dot-hybridization technique. Since the size differences in fat bodies between the two sexes and the stages would generate differences in the amount of $mRNA_{Vg}$ per fat body, the results were expressed as $mRNA_{Vg}$ molecules per DNA equivalent of a haploid genome (Table 9).

Within the sensitivity limits of the dot-hybridization technique, $mRNA_{Vg}$ was not detectable in RNA from fat bodies of adult males exposed to methoprene. However, as would be expected, based on results in Chapter III, a significant amount of $mRNA_{Vg}$ was present in fat bodies of hormone-treated fifth larval instar males. This level of $mRNA_{Vg}$ was about 2 orders of magnitude less than that found in fat body from fifth instar females treated with methoprene. The difference between the amounts of $mRNA_{Vg}$ per haploid genome in fifth instar and adult females was, however, only about two-fold.

Inhibition of $mRNA_{Vg}$ Transcription in Late, Female Fifth Instar Larvae

The observation made in Chapter III that late fifth instar larvae

Table 9

Vitellogenin mRNA levels in fat bodies from larval and adult male and female L. migratoria†

Stage	Haploid genomes per fat body*	Vitellogenin mRNA molecules per fat body	Vitellogenin mRNA molecules per haploid genome
Male			
Vth Instar	7.8×10^6 (a)	3.3×10^9	4.2×10^2
Adult	2.8×10^6 (b)	- (c)	-
Female			
Vth Instar	1.2×10^7 (a)	6.4×10^{10}	5.3×10^3
Adult-Primary	1.2×10^7 (b)	1.1×10^{11}	9.2×10^3
Secondary	2.6×10^7 (d)	1.9×10^{11}	7.3×10^3

† Fat bodies were analysed after 3 days of exposure to methoprene

* 1 haploid genome = 6.3 pg DNA (Rees et al., 1978)

(a) Values derived from the estimated DNA content of fifth instar male and female fat bodies after stimulation by 300 µg methoprene in vivo.

(b) Value derived from data on DNA content given by Irvine and Brasch (1981). Adult locusts were injected with 150 µg methoprene.

(c) Precocene treated males were injected with 500 µg methoprene in 5 µl mineral and after 3 days their fat body RNA assayed for $mRNA_{Vg}$ as described under Methods.

(d) Value from an experiment whose data is not shown.

of either sex cannot be induced to produce Vg after injection of high doses of methoprene (figure 7), posed the question about the molecular level of this inhibition. To answer this, total fat body RNA extracted from 7 day old female fifth instar larvae injected with 300 µg methoprene in mineral oil or mineral oil alone 48 h earlier was separated by electrophoresis under denaturing conditions. As shown in figure 25, a stained $mRNA_{Vg}$ band was not visible in total fat body RNA samples from either hormone or control treated larvae. A northern blot of this gel probed with nick-translated p144-4.6 and autoradiographed, also did not reveal any hybridization to lanes containing separated larval fat body RNA, thus demonstrating that the absence of Vg synthesis in late fifth instar female fat body is due to the lack of $mRNA_{Vg}$. A block in transcription of $mRNA_{Vg}$ might be imposed by the rising titers of ecdysone at this time in preparation for the metamorphic molt.

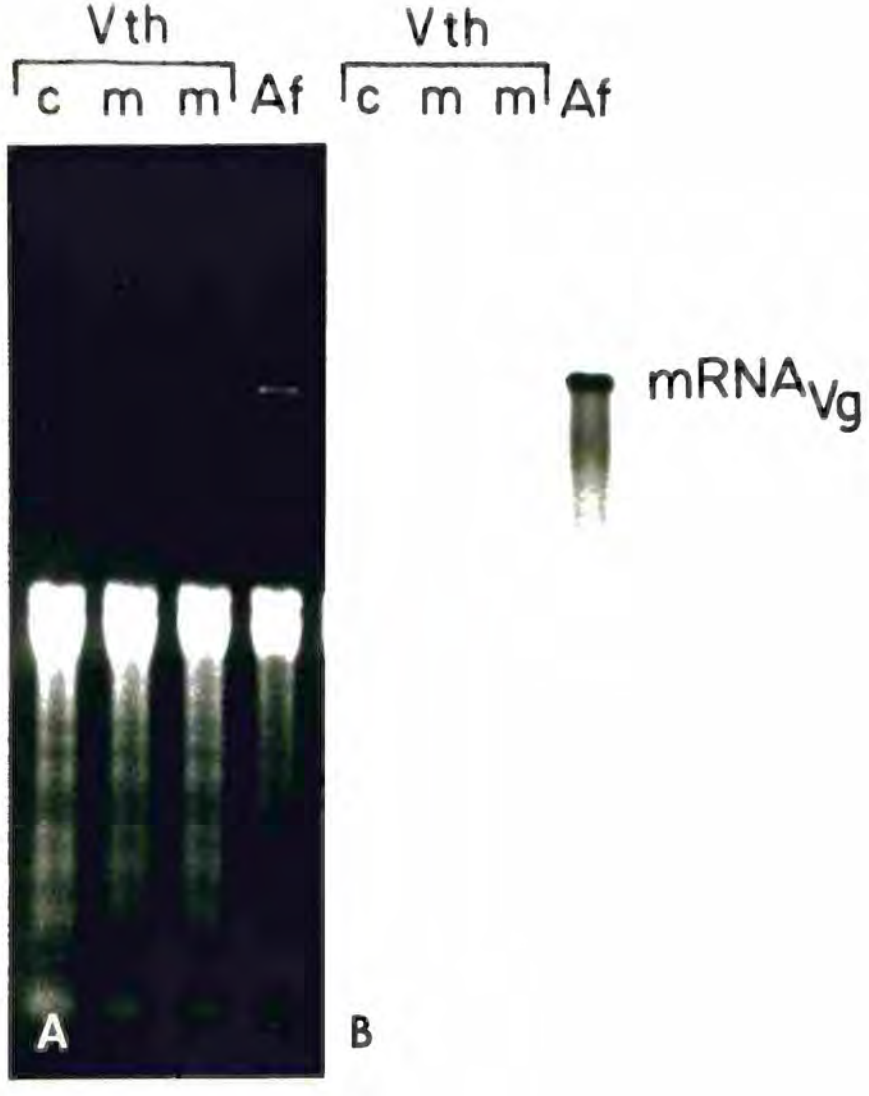
Discussion

In order to contribute to understanding of the molecular basis of JH action in stimulating Vg synthesis in the fat body of L. migratoria, I have focussed my attention at the transcriptional and translational level. To correlate the rate of accumulation of $mRNA_{Vg}$ and total RNA to the relative rates of Vg and total protein synthesis the amounts of these molecules were measured in individual fat bodies. This avoided the need to draw conclusions from experiments performed by different researchers using different methods, especially in the

Figure 25

Transcriptional control of $mRNA_{Vg}$ synthesis in late fifth larval instar female L. migratoria.

Female fifth instar larvae were injected with 300 µg methoprene in 5 µl mineral oil or mineral oil alone on day 5 after the fourth larval molt. 48 h after hormone or control treatment, fat bodies were dissected out and their total RNA was extracted. (A), Total RNA (10 µg/lane) from fat bodies of methoprene (m) or control (c) treated fifth instar females and of sexually mature adult female (Af) were separated by electrophoresis in a 1.2% agarose gel containing methyl mercuric hydroxide. The gel was stained with ethidium bromide. (B), After electrophoresis, RNA from the gel was transferred onto Gene Screen and hybridized to [^{32}P]labelled p144-4.6 DNA. The blot was washed and autoradiographed as described under Methods.


Vth
c m m Af
Vth
c m m Af
mRNA Vg
A
B

mode of hormone application, as discussed earlier.

Assay for the Measurement of $mRNA_{Vg}$

The rate of $mRNA_{Vg}$ accumulation in hormone or control treated fat bodies was determined, against a purified $mRNA_{Vg}$ standard, by a highly sensitive dot-hybridization technique. The hybridization probe contained DNA sequences from a Vg gene complementary to the 3′ end of $mRNA_{Vg}$. Quantities of $mRNA_{Vg}$ as low as 10 pg could reliably be detected and a linear response was obtained upto 8 ng. As opposed to quantitative liquid hybridization which has been used to measure $mRNA_{Vg}$ in chicken (Deeley *et al.*, 1977 a, b; Jost *et al.*, 1978) and *Xenopus* liver (Baker and Shapiro, 1977, 1978), the dot-hybridization technique is much simpler in design, easy to perform and just as sensitive. Recently, Chinzei *et al.* (1982) measured the amount of *Locusta* $mRNA_{Vg}$ by photometric scanning of negatives of ethidium bromide stained agarose methyl mercury hydroxide gels on which total RNA had been separated along with tRNA standards. The lower limit of detection by this method was 50 ng $mRNA_{Vg}$, which is about 5,000 times more than the minimum amount detectable by the dot-hybridization assay.

The cloned probe used for assay of $mRNA_{Vg}$ probably hybridized with only a portion of the total $mRNA_{Vg}$ sequences. Evidence based on pulse labelling in fat body and translation of $mRNA_{Vg}$ in *Xenopus* oocytes (Chen, 1980), and on translation in a reticulocyte cell-free system (G.R. Wyatt and E.R. Belland, unpublished data), suggested that

locust $mRNA_{Vg}$ consists of two populations of messages transcribed from separate genes. Recently, cloned DNA sequences representing two distinct Vg genes (A and B) have been isolated from recombinant DNA genomic libraries. The cloned DNA sequences representing the two Vg genes do not share sequence homology (by cross-hybridization experiments) but appear to be coordinately expressed (J. Locke, personal communication). Under the stringent washing conditions employed in the dot-hybridization assay of $mRNA_{Vg}$, only transcripts from the A gene should have been detected. However, since the purified $mRNA_{Vg}$ used as a standard represents transcripts encoded by both the genes the estimated levels of $mRNA_{Vg}$ should represent the absolute amounts in fat body RNA samples. Since genes A and B may not be under identical regulation, it will be of interest to perform assays with a probe for gene B and compare the results.

Related to the absolute levels of $mRNA_{Vg}$ is the fact that losses of RNA undoubtedly occurred during the RNA extraction procedure. The value of total RNA from fat body of vitellogenic females obtained in this investigation (an average of 360 µg/fat body; data not shown) is about two-thirds of the value reported by Chinzei *et al.* (1982). James *et al.* (1982) extracted RNA from fat bodies of vitellogenic females using a different extraction procedure from Chinzei *et al.* (1982) and this investigation and reported a 25% loss of RNA. However, after compensating for the loss, their value of 180 µg total RNA/fat body is still half the amount I recovered from fat body of vitellogenic female. The percent loss incurred during extraction of fat body RNA in this investigation should be investigated by

co-extracting a known quantity of radio-labelled carrier RNA.

Quantitative Changes in $mRNA_{Vg}$, Rates of Vg Synthesis and Total RNA after Hormonal Stimulation of Fat Body

The developmental profile of $mRNA_{Vg}$ in fat bodies exposed to a primary dose of methoprene was analysed both qualitatively and quantitatively. In fat body RNA samples taken during primary response and separated by electrophoresis, $mRNA_{Vg}$ was visible as a stained band 48 h after hormone treatment, a result in agreement with that reported by Chinzei et al. (1982). However, by northern blot or quantitative dot-hybridization analysis, $mRNA_{Vg}$ was detectable in some fat bodies after only 18 h and in all after 24 h. After this time $mRNA_{Vg}$ accumulated with biphasic kinetics; there was a slow increase between 24 - 36 h, and a rapid one between 36 - 48 h. The biphasic response during primary stimulation has also been observed for $mRNA_{Vg}$ accumulation in the liver of oviparous vertebrates (Deeley et al., 1977 b; Baker and Shapiro, 1978). During secondary stimulation, the persisting low levels of $mRNA_{Vg}$ (probably conserved from primary stimulation) began to increase with a diminished lag of 12 - 18 h. By 48 h, in both primary and secondary stimulation, there were, on the average, $1 - 1.6 \times 10^5$ molecules of $mRNA_{Vg}$/fat body cell. The pattern of $mRNA_{Vg}$ accumulation during early secondary response is in agreement with that reported by Chinzei et al. (1982). However, the quantitative data reported above differ substantially from that reported by the latter authors. They reported values of about 0.3 and

1.8 µg $mRNA_{Vg}$/fat body (equivalent to 7×10^4 and 4×10^6 molecules $mRNA_{Vg}$/fat body cell) 48 h after primary and secondary stimulation, respectively. While the above values for the primary response are in general agreement, the calculated value for secondary response reported by Chinzei et al. (1982) is about 25 times higher than that obtained in this investigation. This discrepancy in results could be due to the different approaches used for secondary stimulation of locusts; an acute withdrawal of JH by surgical allatectomy of normal vitellogenic females (present investigation) compared to a slow decay of the primary effects of methoprene (Chinzei et al., 1982). With the latter approach, due to stability of the injected methoprene (discussed in Chapter III), it is possible that, although Vg was not detectable in the hemolymph of locusts five weeks after primary stimulation, the effect of methoprene continued to be exerted on $mRNA_{Vg}$ as well as total RNA (discussed below).

The relative rates of $mRNA_{Vg}$ accumulation in the fat body of fifth instar females after injection of methoprene were also investigated. Vitellogenin mRNA accumulated with biphasic kinetics which were remarkably similar to those observed for primary stimulation discussed above. It is interesting to note that, although fat bodies in fifth instar females are exposed to a low level of endogenous JH (Johnson and Hill, 1973; Baehr et al., 1979) $mRNA_{Vg}$ was first detectable 24 h after methoprene treatment, and not at the earlier times observed in secondary stimulation.

The results on early $mRNA_{Vg}$ accumulation and relative rates of Vg synthesis during primary and secondary stimulation in the adult female

were compared, in order to establish any temporal relationship. In primary stimulation, Vg synthesis was first detectable after a lag of 24 h and increased slowly to 36 h, corresponding to the first appearance and early slow increase of $mRNA_{Vg}$. Between 36 and 48 h, however, the rate of Vg synthesis increased less steeply than the accumulation of $mRNA_{Vg}$, and apparently reached saturating levels by 48-72 h. These results suggest that the initiation of Vg synthesis depended on the appearance of newly transcribed message, but with continued accumulation of mRNA, the rate of translation was limited by other components of the protein synthesizing system. During secondary stimulation, Vg synthesis commences with a much shorter lag and before a significant increase in the level of $mRNA_{Vg}$. Between 18 and 48 h the curves for $mRNA_{Vg}$ and Vg synthesis rose roughly in parallel. It is interesting to note that despite the similar levels of $mRNA_{Vg}$ accumulated 48 h after primary and secondary stimulation, the rate of Vg synthesis was much higher in the secondary response, which suggests that mRNA is being translated with greater efficiency.

Elevation of rRNA and tRNA is common during the adaptation of cells for increased protein synthesis under hormonal stimulation (Tata, 1980). Differences in the relative amounts of total RNA (chiefly rRNA and tRNA) observed after primary and secondary stimulation might explain the apparent differences in the efficiency of translation, discussed above. After primary stimulation a small, gradual increase in total RNA was seen. Two weeks after the removal of corpora allata from vitellogenic females, the level of RNA had dropped to the level observed before the primary response.

Administration of methoprene at this time evoked a rapid increase in total RNA to much higher levels. Seventy two hours after hormonal stimulation there was 3 times as much total RNA in the secondary as in the primary response. This pattern of total RNA accumulation contrasted from that observed by Chinzei et al. (1982), who reported a steep rise in total RNA during primary stimulation but only a small subsequent decline and relatively small increase during secondary stimulation. This discrepancy between results is probably due to the different methods of obtaining locusts for secondary stimulation, as discussed earlier.

Regulatory Mechanisms for the Molecular Basis of Juvenile Hormone Action

The current model of steroid hormone regulation of protein synthesis is that the rate of induced protein synthesis is a function of the cytoplasmic concentration of mRNA (Deeley et al., 1977a, b; Baker and Shapiro, 1977, 1978; Palmiter 1977; Moen and Palmiter, 1981). The results reported in this chapter are partially consistent with this model. However, the lower level of Vg synthesis, relative to mRNA, during primary stimulation and enhanced synthesis during secondary stimulation further suggest that a change in the level or nature of some translational component(s) that occurs during primary induction may persist so that subsequent secondary stimulation involves replenishment of $mRNA_{Vg}$. These results indicate that translational as well as transcriptional mechanisms determine the

kinetics of the JH regulated Vg gene expression.

The balance of factors involved in controlling the rate of Vg synthesis after methoprene treatment of fifth instar females may be different. Fat body in mid-fifth instar is already active in protein synthesis (Turner and Loughton, 1975). Vg synthesis (from 24-36 h) is initially dependent on the availability of newly transcribed $mRNA_{Vg}$ and may become limited by the availability of components needed for translation, for which other messages are competing. As the synthesis of non-Vg proteins decreases at about 36 h after methoprene treatment, perhaps as part of a programmed response in preparation for the metamorphic molt, the existing translational apparatus is made available for Vg synthesis, which then rises to a much more rapid rate than observed during primary stimulation in the adult.

A characteristic feature of the primary response in adult females was the long delay before $mRNA_{Vg}$ was detectable. This lag was shorter in the secondary response but still long compared to the lag period of 30 min to 3 h reported for steroid-induced specific protein synthesis in the liver and oviduct of some vertebrates (McKnight *et al.*, 1975; Palmiter *et al.*, 1976; Deeley *et al.*, 1977 b; Baker and Shapiro, 1978; Seaver *et al.*, 1980). The lag has important implications in that there are as yet undefined, rate-limiting events between JH receptor binding and the dramatic acceleration of $mRNA_{Vg}$ production that occurs many hours later. In the absence of available data, any suggestions on the nature of these rate-limiting events are only speculative. The initial transcription of $mRNA_{Vg}$ may depend on processes like the build-up of RNA polymerases or some other

intermediary proteins. The latter may in turn be responsible for inducing $mRNA_{Vg}$ transcription. A role of intermediary proteins has been suggested in the estrogen induced $mRNA_{Ov}$ synthesis in the chick oviduct *in vitro* (McKnight, 1978). It has been shown that in the ecdysone induced puffs in salivary gland chromosomes of *Drosophila*, the appearance of the late puffs was dependent on the products of early puff genes (Ashburner *et al.*, 1974; Walker and Ashburner, 1982). If a similar situation prevails in the JH dependent $mRNA_{Vg}$ transcription in fat body of *L. migratoria* then the action of JH on Vg gene expression must be viewed as an indirect one. This hypothesis can be tested with inhibitors of protein synthesis if a consistent primary induction of Vg synthesis can be obtained *in vitro*. The lag period could also be required for the build-up of a critical level of JH receptors or for all or a combination of the above steps.

One of the effects of steroid hormones is to confer metabolic stability on mRNAs by an unknown mechanism (Palmiter and Carrey, 1974; Shapiro, 1982). The biphasic nature of $mRNA_{Vg}$ accumulation during the primary response could be explained by increased stabilization of $mRNA_{Vg}$ after 12 h, and the earlier, more rapid response during secondary stimulation could be due to the immediate recall of this stabilization.

Decay Kinetics of $mRNA_{Vg}$ and Rate of Vitellogenin Synthesis After Withdrawal of Juvenile Hormone

The decay kinetics of $mRNA_{Vg}$ and rate of Vg synthesis were

investigated by surgically removing the source of JH from vitellogenic females, thus providing an acute withdrawal of the hormone. In unoperated females the accumulated level of $mRNA_{Vg}$ was about 1.6×10^5 molecules/fat body cell. James et al. (1982), using a cloned cDNA fragment complementary to $mRNA_{Vg}$ as a hybridization probe in a quantitative disc-hybridization assay (Searle and Tata, 1981) reported 1.5×10^5 $mRNA_{Vg}$ copies/cell in the fat body of a fully vitellogenic female, which is in general agreement with values reported in this investigation. After the surgical allatectomy of vitellogenic females, $mRNA_{Vg}$ decayed with a half-life of 24 h. The reported half-life values of $mRNA_{Vg}$ in chicken and Xenopus during withdrawal of estrogen are < 3 h and 45 h, respectively (Wiskocil et al., 1980; Shapiro, 1982). Palmiter and Carrey (1974) reported that $mRNA_{Ov}$ decayed with a half-life of 2 - 3 h during withdrawal of estrogen. However, in the presence of estrogen, the half-lives of $mRNA_{Vg}$ in chicken and Xenopus and $mRNA_{Ov}$ in chicken were much longer; 22, 800(!) and 24 h, respectively (Palmiter and Carrey, 1974; Wiskocil et al., 1980; Shapiro, 1982). In light of these results, it is possible that in the presence of JH, the half-life of $mRNA_{Vg}$ is much longer. This might be experimentally determined by culturing vitellogenic fat bodies in vitro in the presence of JH or methoprene and levels of α-amanitin that would inhibit RNA polymerase II activity. Measurements of $mRNA_{Vg}$ levels over a period of time should then give an estimate of the half-life of this transcript.

Unlike the decay of $mRNA_{Vg}$ during withdrawal of JH, the rate of Vg synthesis decayed with apparently biphasic kinetics. During two days

after allatectomy, Vg synthesis dropped from an initial 67% to 3% of total secreted protein synthesis, and thereafter the rate of Vg synthesis decayed slowly to zero level by day 15 - 18. At this time there were still low levels of $mRNA_{Vg}$. These results and those of early secondary response suggest that $mRNA_{Vg}$ is conserved during hormone withdrawal. In contrast to the above findings on the decay rate of Vg synthesis after allatectomy, Pines et al. (1980) reported that 3 days after surgical allatectomy of vitellogenic females, fat bodies, when incubated in vitro, actively synthesized Vg, although synthesis of Vg in vivo had ceased shortly after allatectomy. I have no explanation for this disparity in results, suffice it to say that fat bodies of allatectomized vitellogenic females even when cultured in the presence of low doses of methoprene did not respond by synthesizing Vg (Chapter IV).

Relative Level of Vitellogenin Gene Expression in Fifth Instar and Adults of Both Sexes

In order to explore the sex- and stage- related differences in the inducibility of Vg synthesis (Chapter III), the accumulation of $mRNA_{Vg}$ in fat bodies of fifth instar larvae and adults of both sexes was measured 3 days after hormone treatment. The results were expressed as $mRNA_{Vg}$ molecules per haploid genome for comparative purposes. Vitellogenin mRNA was not detectable in the fat body of adult males injected with high doses of methoprene, confirming the conclusion in Chapter III that Vg genes in the adult male are repressed. On the

other hand, $mRNA_{Vg}$ was detectable in the fat body of fifth instar males. The level of $mRNA_{Vg}$/haploid genome, however, was an order of magnitude less than that in fifth instar female fat body, which in turn was about 1.7 times less than that in adult female fat body. These results reflect the relative response of fat bodies from the two stages of both sexes to methoprene (figure 11) and once again demonstrate the dependence of Vg synthesis on the transcription of $mRNA_{Vg}$.

A L. migratoria Vg gene (represented by the same DNA sequence used as a hybridization probe in this investigation) has been localized to the X-chromosome (Bradfield and Wyatt, 1983). This gene is present at one copy per haploid genome in females but at only one-half the number in males. It has been suggested that somatic polyploidy may serve to provide multiple copies of the genome from which specific genes can be selectively expressed to supply the required levels of their transcripts. In Locusta, the adult fat body, under the influence of JH, undergoes polyploidization, but the ploidy level in males is only half as much as in females, hence creating a further difference in the Vg gene number. In the present investigation, DNA synthesis was also enhanced in the fat bodies of female and male fifth instar larvae, although to a lesser degree in the male (Chapter III), presumably producing ploidy differences between the sexes. The combined effect of twice the number of sex-chromosomes and higher ploidy levels in the female locust fat body may contribute to differences in expression. However, by itself this is insufficient to explain the ten-fold difference in $mRNA_{Vg}$ levels seen in methoprene

treated fifth instar larvae. Moreover, it certainly cannot explain the total difference in gene expression seen in adults. Therefore, this must be due to programming for expressibility imposed on genes through the actions of other genes concerned with sex phenotype.

It is apparent from the foregoing discussion that there is still a large gap in our understanding of the molecular basis of JH action. However, the results presented in this investigation and others (Engelmann, 1981; Koeppe et al., 1981; Roberts and Wyatt, 1983) are a beginning towards an understanding of this important and fascinating problem.

VI. CONCLUSIONS

Although the precise developmental and molecular mechanisms regulating Vg genes remain obscure, several features of these processes have been revealed in recent studies on oviparous vertebrates. Relatively much less is known of the corresponding mechanisms in insects. In this report, three different lines of research were pursued to enhance understanding of molecular aspects of the regulation of Vg genes by JH. In this final section, the main conclusions from this work will be summarized, some further questions will be raised, and some approaches to answering them suggested.

Throughout the course of this study, methoprene has been used as a synthetic substitute for JH, because of its greater chemical and biological stability. However, it should not be assumed without question that methoprene provides an adequate model for the subcellular actions of JH. Therefore, at some stage it will be necessary to compare the molecular aspects of Vg gene activation by methoprene with those produced by natural JH. Nevertheless, methoprene and other synthetic analogs and "JH mimics" exert biological effects very similar to those of JH (Henrick et al., 1976) and efficiently reverse the effects of allatectomy, which strongly suggests a similar basic mode of action.

The study of the development of fat body competence to respond to methoprene in producing Vg yielded some interesting results: (i) Vg gene expression is JH-inducible in both female and male fifth instar

larvae, but only in female adults; (ii) the transition from the inducible to an uninducible stage in males occurs during or after the metamorphic molt; and (iii) there is also a change in responsiveness of fat body to methoprene during the fifth instar of both sexes. These results establish a biological framework for investigation of the mechanisms underlying the transition between expressible and non-expressible states of the Vg genes.

The structural organization of interphase chromatin is thought to play an important role in regulating eukaryotic gene expression. The observation that the puffing patterns of dipteran polytene chromosomes are correlated with the appearance of specific transcriptional products during normal development or after hormone treatment provides support for the proposal that transcription may be controlled, at least in part, by changes in the architecture of the chromatin complex (Spradling et al., 1975). At the biochemical level, the transition from an active to inactive (or vice versa) chromatin can be investigated by using pancreatic DNase, to which transcriptionally active genes are exceedingly sensitive (Weintraub and Groudine, 1976; Garel and Axel, 1976). DNase I sensitivity of active genes is not restricted to the coding regions of these genes but extends far upstream and downstream, and limited sequences in the 5'-flanking region exhibit hypersensitivity to the enzyme. Although the structural basis and the functional significance of DNase I sensitivity are not clearly understood, the hypersensitivity may be related to the regulatory sequences that are currently being defined in association with eukaryotic genes. It would be worthwhile to

investigate conformational changes in the locust Vg genes, using DNase I as a probe, during the fifth instar and after the metamorphic molt in both male and female fat body. Nuclear acidic proteins may also be important in regulating gene expression (Spelsberg, 1982). In relation to the question of repression of Vg genes in the adult male locust, a possible regulatory role of these proteins should be investigated.

At the hormonal level, the action of JH in regulating Vg synthesis might be influenced by ecdysone. In late fifth instars, two days before the metamorphic molt, Vg synthesis could not be induced by high doses of methoprene, perhaps due to the rising titer of ecdysone at this time, and $mRNA_{Vg}$ could not be detected in RNA extracted from fat bodies exposed to methoprene in late fifth instar females. In the adult female, ecdysone has been found to accumulate in the ovaries as the oocytes mature (Lagueux et al., 1979), when synthesis of Vg declines (Gellissen and Wyatt, 1981). On the other hand, the sex-specific competence of the adult fat body to synthesize Vg was established after a molt induced by ecdysone (in the absence of JH). These observations suggest possible participation of ecdysone in regulating Vg gene expression, that needs to be investigated.

In the kinetics of $mRNA_{Vg}$ induction in adult and fifth instar females, a characteristic feature was a long lag before $mRNA_{Vg}$ could be detected. Possible explanations of this lag were discussed in the last Chapter. Of these, the possibility of intermediate proteins induced by JH, which may be required for Vg gene expression, should be investigated. This can be done by looking at effects of protein

synthesis inhibitors on $mRNA_{Vg}$ synthesis in fat bodies stimulated by JH *in vitro*. The cytoplasmic levels of $mRNA_{Vg}$ in the presence or absence of protein synthesis inhibitors can be measured by the simple dot-hybridization assay described by White and Brancroft (1982), which eliminates the need to purify RNA from each tissue sample.

The stimulation of Vg synthesis in adult female locusts by JH is correlated with DNA synthesis and elevated polyploidization (Nair *et al.*, 1981; Irvine and Brasch, 1981). In fifth stage larvae, at least, as reported in Chapter III, methoprene-induced $mRNA_{Vg}$ and Vg synthesis were preceded by enhanced DNA synthesis. This raises questions about the role of DNA synthesis during the lag period and its possible relationship to active transcription of the Vg genes. The use of specific inhibitors of DNA synthesis, such as hydroxyurea, applied to fat body incubated *in vitro*, would help in answering these questions.

The current model of steroid hormone action involves the binding of the steroid to cytoplasmic receptors which are then translocated to the nucleus where they act at specific chromatin sites (Yamamoto and Alberts, 1976). Putative receptor proteins for JH have only recently been identified and partially characterized (Engelmann, 1981; Koeppe *et al.*, 1981; Roberts and Wyatt, 1983), and their role in mediating JH action is not yet established. As a first step towards understanding the presumably receptor-mediated JH action, the cytoplasmic and nuclear distribution of these receptor proteins, before and after exposure of the fat bodies to the hormone, needs to be investigated. The relative levels of these receptors in fat bodies

of adult and fifth instar locusts of both sexes might contribute to explaining differences in responsiveness to JH.

In the present investigation, the accumulation of $mRNA_{Vg}$ in the fat body of locusts after methoprene administration was measured. However, the accumulated levels of $mRNA_{Vg}$ are a resultant of the rates of synthesis and degradation. This poses the question, whether JH regulates only the transcription or also the stability of $mRNA_{Vg}$. To answer this, it will be important to measure transcription rates in isolated nuclei, and to obtain estimates of the half-life of $mRNA_{Vg}$ when JH is present.

Finally, the result on the accumulation of $mRNA_{Vg}$ and relative rates of Vg synthesis in fat bodies after primary and secondary stimulation with methoprene *in vivo*, suggests the existence of translational as well as transcriptional controls. The translation of $mRNA_{Vg}$ is perhaps initially limited by the availability of ribosomes, as proposed for the steroid regulated Vg synthesis in male *Xenopus* liver (Farmer *et al.*, 1978). After withdrawal of JH during the vitellogenic phase, the much more rapid decline in Vg synthesis than in $mRNA_{Vg}$ level suggests the sequestration of mRNA in some non-translatable form, possibly as ribonucleoprotein particles. Such a suggestion has already been made on the basis of the apparent conservation of untranslated $mRNA_{Vg}$ towards the end of a natural gonadotrophic cycle (Chinzei *et al.*, 1982). These possibilities could be tested by examining the profiles of Vg-synthesizing polyribosomes, free ribosomes, and other ribonucleoprotein particles, along with assays of $mRNA_{Vg}$, in fat body at different states of hormonal

stimulation.

For analysis of the role of JH in regulating insect metamorphosis, much detailed information has been accumulated on the endocrinology of the tobacco hornworm, Manduca sexta (Riddiford and Kiely, 1981). These elegant studies place M. sexta in the forefront for understanding of the morphogenetic actions of JH on cellular and molecular events occurring in the epidermis during the initiation of metamorphosis. The results presented here on L. migratoria concerning the sex- and stage-related development of fat body competency to synthesize Vg, induction in vitro of Vg synthesis in fat bodies cultured in the presence of methoprene and the relative changes of molecular events in the fat body after hormonal stimulation, make this species the "white rat" of insects for the study of the molecular mechanisms underlying the action of the multi-functional terpenoid, JH. Using Vg synthesis in L. migratoria as a model system, we may anticipate significant advances in our understanding of JH action in the coming years.

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APPENDIX

LOCUST RINGER AND INCORPORATION MEDIUM FOR CULTURE OF *L. migratoria* FAT BODY

The chemicals and amounts used to prepare locust Ringers and the (^{3}H)leucine incorporation medium were exactly as described by Wyatt *et al.* (1976), except that the final pH was adjusted to 7.0 instead of 7.2.

Locust Ringer:

Components	mg/100 ml
Inorganic salts	
NaCl	702
KCl	112
$CaCl_2$	44
$MgCl_2 \cdot 6H_2O$	160.2
Na_2HPO_4	35.5
TES	458
Sucrose	2500

Incorporation medium:

In addition to the components in the locust Ringers, excluding Sucrose, the incorporation medium contained the following:

Components	mg/100 ml	Components	mg/100 ml
Amino acids		Amino acids (Cont'd)	
Alanine	44.6	Threonine	23.8
Arginine-HCl	105.4	Tryptophan	40.8
Asparagine-H_2O	75	Tyrosine	18.1
Aspartic acid	66.5	Valine	23.4
Cysteine-HCl.H_2O	35.1	Lysine-HCl	91.5
Glutamic acid	73.5	Leucine	0.39
Glutamine	73		
Glycine	75	Sugars	
Histidine-HCl.H_2O	314	D-Glucose	90.5
Isoleucine	26.2	Trehalose	756.5
Methionine	29.8		
Phenylalanine	33	Antibiotics	
Proline	57.5	Streptomycin-SO_4^{--}	2.5
Serine	105	Penicilin G (Na^+)	1.5

L-Amino acids were used. The medium was adjusted to pH 7.0 with 1N NaOH and sterilized by filteration.

S-20 MEDIUM FOR CULTURE OF L. migratoria FAT BODY

The S-20 medium for long term culture of locust fat body was prepared as described by Landureau (1976), with slight modifications. The medium consisted of the following components:

Components	mg/100 ml	Components	mg/100 ml
Amino acids		Amino acids (Cont'd)	
Arginine-HCl	75	Tryptophan	10
Aspartic acid	50	Tyrosine	10
Cysteine-HCl	37.5	Valine	10
Glutamic acid	150		
Glutamine	7.5	Vitamins	
Glycine	75	d-biotin	.005
Histidine	20	Ca-pentothenate	.1
Isoleucine	10	Choline-HCl	25.
Leucine	20	Cyanocobalamine	.02
Lycine-HCl	20	Folic acid	.005
Methionine	25	Inositol	.05
Phenylalanine	10	Nicotinamide	.025
Proline	65	Pyridoxine-HCl	.025
Serine	3.5	Riboflavin	.05
Threonine	10	Thiamine-HCl	.2

S-20 Medium (Cont'd)

Components	mg/100 ml	Components	mg/100 ml
Inorganic salts		Sugar	
KCl	105	Glucose	400
NaCl	850		
$MgSO_4 \cdot 7H_2O$	125	Antibiotics	
$CaCl_2 \cdot 2H_2O$	50	Penicilin-G(Na^+)	12.5
$MnSO_4 \cdot H_2O$	3.5	Streptomycin-SO_4^{--}	10
Na_2HPO_4	35.5	Rimocidin Sulphate	2
HEPES	238		

L-Amino acids were used. The medium was diluted with distilled water by 10% and the pH adjusted to 7.0 with 1N NaOH. The medium was sterilized by filtration.

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