STUDIES ON THE JUVENILE HORMONE-CONTROLLED SYNTHESIS

OF VITELLOGENIN IN THE AFRICAN MIGRATORY LOCUST,

LOCUSTA MIGRATORIA MIGRATORIODES R. & F.

By

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ABSTRACT

The biosynthesis of the yolk-precursor protein, vitellogenin
(VG), induced by juvenile hormone (JH) in the fat body of African
migratory locusts, has been studied as a system for analyzing the
mechanism of JH-controlled specific gene activation in insects. In
order to isolate VG mRNA for further characterization, conditions for
the isolation of undegraded VG mRNA-containing polysomes from the fat
body of reproductively active females have been defined. The identity
of these female-specific heavy polysomes as VG polysomes was confirmed
by precipitation of the associated nascent polypeptides with anti-
vitellin serum and by translation of the polysomal RNA in *Xenopus* oocytes.
By EM observation these polysomes were found to contain 40-50 ribosomal
monomers. In normal development, accumulation of ribosome monomers in
the fat body of female locusts began at about day 4 after emergence
(terminal oocyte length = 2 mm), then was followed by the appearance
of VG polysomes beginning at about day 8 (terminal oocyte length = 3 mm).
Vitellogenin polysomal content reached a maximum at about day 14
(terminal oocyte length = 5-6 mm), and then fell to an undetectable
level as the terminal oocytes reached their maximal size (7 mm). In
allatectomized (removal of the source of JH) females VG polysomes can
be induced by ZR-515 (300 μg/animal) treatment. A single treatment
with ZR-515 induced a rapid increase in ribosome monomers and light

polysomes in the first 48 hr, but the maximal production of vitellogenin polysomes was observed at 72 hr. After the decay of this effect (12 days after hormone treatment) a second application of ZR-515 resulted in the rapid formation of vitellogenin polysomes without an initial accumulation of ribosome monomers. These and previous results are consistent with the action of juvenile hormone at the gene level.

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INTRODUCTION

During the development of a zygote into a highly organized-multicellular organism, stable and distinct differences arise between cells. This process, cellular differentiation, involves the preferential synthesis of specific proteins which results in the specialization of a cell with respect to morphology, physiology and/or biosynthetic activity; for example, hemoglobin in erythrocytes, antibodies in lymphocytes, and ovalbumin in the tubular epithelial cells of chick oviduct. At present our understanding of the underlying molecular mechanism(s) of cellular differentiation is incomplete, but it is generally accepted that in molecular terms these differences could have arisen by the selective expression of specific genes. Evidence for two key investigations supports this possibility. First, nuclear transplantation experiments (Gurdon, 1962; Gurdon *et al.*, 1975; Whabl, 1975) showed that nuclei from a variety of adult tissues (skin cells, intestinal cells and lymphocytes) are capable of directing the complete development of normal frogs. This indicates that all the genes required to make other tissues (e.g. nerve, blood, muscle and cartilage) are present in the nucleus of every specialized cell, and are not lost or permanently inactivated. Second, an alternative mechanism for differential gene activity, selective gene amplification, was ruled out by nucleic acid hybridization experiments (reviewed by

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Davidson, 1976). These experiments showed that the genetic information in somatic cells is identical to that of embryonic cells. Since every cell of a higher organism contains a complete set of genetic information, the differences between differentiated cells must be explained in terms of variable rates of gene activity.

Differential gene expression can be regulated at the level of transcription (i.e. RNA synthesis, processing, stabilization or transport) or translation (i.e. protein synthesis or processing). However, in recent years by employing modern biochemical and molecular biological techniques, such as immunochemical analysis, *in vitro* cell-free protein synthesis, complementary DNA preparation, DNA-DNA or DNA-RNA hybridization and recombinant DNA technology, it has emerged that differential gene activation appears to be controlled at the transcriptional level in many systems.

Among many different systems used to investigate the control mechanism of selective gene expression, the induction of the synthesis of specific proteins by developmental hormones has provided evidence which strongly supports the theory that steroid hormones act primarily at the level of transcription (Dawid and Wahli, 1979). In particular, the chick oviduct system has been used as a model system for the study of steroid hormone action and specific gene activity (Palmiter and Schimke, 1973; Palmiter *et al.*, 1976; Cox, 1977; Hynes *et al.*, 1977; Garapin *et al.*, 1978; Mandel *et al.*, 1978; O'Malley *et al.*, 1979). The hormones estrogen and progesterone both induce the oviduct tubular gland cells to synthesize large quantities of the egg white proteins,

of which ovalbumin is one of the major components (Kohler *et al.*, 1969; O'Malley *et al.*, 1969). Unfortunately the tubular gland cells of the chick oviduct, like the target tissues of most other hormone inducible systems, undergo substantial cell-division and cytodifferentiation in response to primary hormone stimulation (Palmiter, 1975). This situation presents major problems in attempting to analyze the early molecular events leading to regulated gene expression. As a result, most of the analysis of the initial events in ovalbumin induction has been conducted during secondary stimulation, which is not complicated by cell proliferation. Clearly alternative systems must be developed to look at the early events of hormone action, since differences may exist between primary and secondary responses of the target tissue to hormones.

Vitellogenesis, the formation of egg yolk, is a major developmental process during oogenesis in egg-laying vertebrates and invertebrates (Clemens, 1974; Wallace and Bergink, 1974; Engelmann, 1979; Hagedorn and Kunkel, 1979). This process, which involves the expression of vitellogenin (the female-specific egg yolk precursor proteins, VG) genes, is under the obligatory control of estrogen in egg-laying vertebrates (Wangh and Knowland, 1975; Green and Tata, 1976; Gruber *et al.*, 1979; Jost and Pehling, 1976a,b; Tata, 1976) and of juvenile hormone (JH) in most insects (Chen *et al.*, 1976, 1979; Koeppe and Ofengand, 1976; Kunkel and Pan, 1976). Vitellogenin in all species studied is a multicomponent glycoprotein that is synthesized in the liver of vertebrates or in the fat body of insects, secreted into the circulation, and is then taken up by the developing oocytes (Bergink

et al., 1974; Deeley *et al.*, 1975, 1977a,b; Christmann *et al.*, 1977; Ohlendorf *et al.*, 1977; Wyatt and Pan, 1978; Engelmann, 1979; Hagedorn and Kunkel, 1979). The fact that vitellogenin production can be reversibly induced in the liver of vertebrates by estrogen or in the fat body of adult female insects by JH makes it an ideal system for studying the selective expression of the genes controlled by these hormones. The recent development of primary hepatocyte cultures (in vertebrates) or organ cultures (in invertebrates) capable of undergoing a complete vitellogenic response has further provided the opportunity for radiolabelling of newly-synthesized RNA to a high specific activity, and has therefore permitted the study of the initial transcriptional and post-transcriptional events after hormone treatment (Wahli *et al.*, 1978; Chen and Wyatt, 1980).

Vitellogenin synthesis can be induced by estradiol in the liver of male *Xenopus* (Wallace and Jared, 1968b), rooster (Bergink *et al.*, 1973; Jost and Pehling, 1976) and rainbow trout (Chen, unpublished results). This phenomenon has made possible the detailed analysis of the estrogen induction process under experimental conditions with an undetectable background level of vitellogenin synthesis. In contrast, the titer of juvenile hormone in almost all insect species studied (Wyatt, 1972) is usually slightly higher in the male, but does not induce vitellogenin synthesis. By giving exogenous applications of juvenile hormone to male locusts the hormone titer has been raised to even higher levels, yet synthesis of vitellogenin remained undetectable (Chen and Wyatt, 1980). These results suggest that the vitellogenin

genes in male locusts are permanently unresponsive to activation by
juvenile hormone; therefore further investigation of this unique
property of juvenile hormone-controlled gene expression in insects is
indicated.

Vitellogenin synthesis has been studied extensively at the
subcellular level in the South American cockroach, *Leucophaea maderae* (F.), in
which juvenile hormone has been shown to induce vitellogenin synthesis (Brookes,
1969; Engelmann, 1974, 1976, 1979; Koeppe and Ofengand, 1976). However
molecular studies in this system are complicated since the cockroach
contains an intracellular symbiotic bacterium which makes the interpre-
tation of biochemical data tenuous (Chen *et al.*, 1976; Sridhara *et al.*,
1978; Houk and Griffiths, 1980). In order to pursue the molecular and
biochemical studies of juvenile hormone action, the African migratory
locust, *Locusta migratoria migratorioides* R.& F. (Fig. 1), has been selected
as an alternate experimental animal. The locust was chosen because
the fat body is free of any intracellular symbiotes, and egg maturation
has been shown to be under the obligatory control of juvenile hormone
(Chen *et al.*, 1976, 1979). Also, the induction of vitellogenin syn-
thesis in the locust fat body has recently been accomplished *in vitro*
Abu-Hakima and Wyatt, 1980), and finally, locusts are large animals,
inexpensive, easily reared with a short generation time. Therefore
the locust fat body system has a number of advantages which make it
a very feasible system for the investigation of the molecular mechanisms
of juvenile hormone-controlled vitellogenin gene expression during
oogenesis.

Figure 1. Mature male and female locusts, *Locusta migratoria migratorioides*. A side view of the locusts against a background of wheat seedlings; the male is above the female.
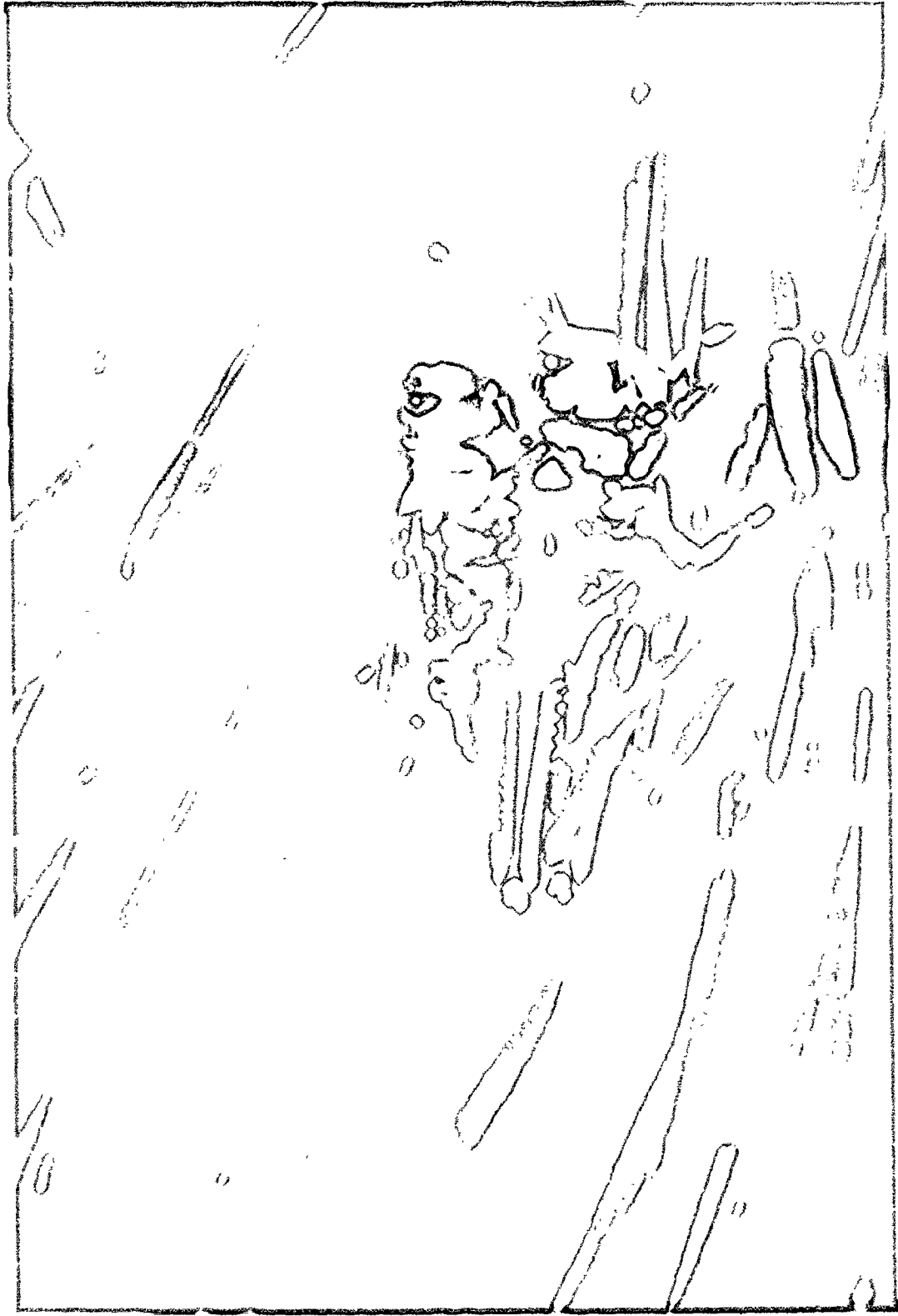
In order to present the rationale and objectives of this work,
a general description of the biosynthesis of the vitellogenins (yolk
protein precursors) induced by juvenile hormone in insects, in particular
the African migratory locust, will be discussed in the following sections.

Vitellogenins and Egg Yolk Proteins

The egg yolk proteins of a wide variety of oviparous vertebrates
and invertebrates form microcrystals (yolk granules, or platelets) in
the cell body of the oocyte (Wallace, 1963, 1965; Clemens, 1974;
Christmann *et al.*, 1977; Ohlendorf *et al.*, 1977, 1978; Engelmann, 1979;
Hagedorn and Kunkel, 1979). The physical properties and large
available quantities of these complexes in the egg yolk have made their
purification and characterization relatively straightforward. In all
the vertebrates studied there are two major classes of yolk proteins,
phosphoprotein (phosvitin) and a lipoprotein (lipovitellin); in contrast,
insects have one multicomponent glycoprotein, vitellin. Some properties
of avian and amphibian phosvitin, lipovitellin and their precursor
protein, vitellogenin, are summarized in Table I. Phosvitin contains
a large amount of serine (56% of the total amino acids), and the
majority of these residues have been phosphorylated in their hydroxyl
side chains. It also has a moderate number of carbohydrate moieties
(10% by weight) consisting of three mannose, three galactose, five
N-acetylglucosamine and two sialic acid residues arranged in a branched
structure attached to the polypeptide via asparagine residues (Shainkin
and Perlmann, 1971a,b). The lipovitellin of *Xenopus laevis*, in contrast

TABLE I

Some Chemical Properties of the Principal Egg Yolk Proteins and their Precursors in Xenopus and Chicken

Proteins	Animal	Molecular Weight X10^{-3}		Amino Acid Residues (mol%)		P* (%)	Lipid (%)	(CH$_2$O)$_n$** (%)
		Native	Subunits	Serine	Methionine			
Phosvitin	Xenopus		32	56	<0.4	10.0	0.5	10.0 (1)
	Xenopus		17	56	-	10.0	-	- (2)
	Chicken		32	56	<0.4	10.1	0.3	11.3 (1)
	Chicken		34, 28	57, 50	-	9.5, 9.2	-	- (3)
Lipovitellin	Xenopus	115		6	2.2	0.5	20.0	0.3 (1)
	Xenopus		105, 35.5, 32	-	-	-	-	- (2)
	Chicken	130		5.	2.1	0.4	21.1	0.3 (1)
	Chicken	170	140, 30	-	-	-	17.5	- (3)
Vitellogenin	Xenopus	490-600	215	12	2	1.6	12	2 (1)
	Chicken	500	240	14	2	2.4	12	2 (1)

(1) Tata and Smith, (1979).
(2) Ohlendorf et al., (1977).
(3) Christmann et al., (1977).

*Phosphorus; **Carbohydrate; - not determined

has a low serine and carbohydrate content and a moderately high lipid
content (about 20% by weight), 75% of which is phospholipids (Ohlendorf
et al., 1977). While multiple phosvitin and lipovitellin components
which differ with respect to molecular weight and composition have been
detected, this variation has been attributed to different degrees of
phosphorylation, glycosylation and lipidation of the same polypeptide
(Tata and Smith, 1979).

The main properties of amphibian and avian vitellogenins are
also summarized in Table I. In frogs the native vitellogenin (MW 4.9-
6.0 x 10^5) is composed of two identical subunits of 2.1-2.2 x 10^5
daltons as judged by polyacrylamide gel electrophoresis under denaturing
conditions (Bergink and Wallace, 1974; Clemens, 1974; Berridge et al., 1976;
Tata, 1976; Penning et al., 1977). Similarly, chicken vitellogenin
has been reported to be a protein dimer with a subunit size of 2.4-
2.5 x 10^5 daltons (Deeley et al., 1975; Wetekam et al., 1975; Gruber
et al., 1976; Jost and Pehling, 1976b; Christmann et al., 1977; Gordon
et al., 1977). Recent studies on the nature and number of subunits
in Xenopus and chicken vitellogenins, phosvitins and lipovitellins
(Bergink and Wallace, 1974; Christmann et al., 1977; Ohlendorf et al.,
1977, 1978) have revealed a precursor-product relationship for these
molecules. Each subunit from chicken vitellogenin appears to have one
lipovitellin component (MW 170,000) and two distinct phosvitin poly-
peptide residues (MW 28,000 and 34,000) while in amphibians each subunit
is composed of three distinct lipovitellin subunits (MW 105,000, 35,000,
and 32,000) and one phosvitin moiety (MW 17,000).

In all the insect species that have been studied, vitellogenin and vitellin are found to be immunologically and electrophoretically identical (Wyatt and Pan, 1978; Engelmann, 1979; Hagedorn and Kunkel, 1979). Therefore antiserum prepared against total egg extract and absorbed with male hemolymph can be used to identify insect vitellogenins in the hemolymph and vitellins in the eggs. Like vertebrate vitellogenins and lipovitellins, insect vitellogenins and vitellins are generally insoluble in solutions of low ionic strength, and can thus be purified by precipitation with dilution or dialysis from high salt extraction solutions (Dejmal and Brookes, 1972). Some chemical properties of vitellogenins and vitellins of several representative insect species are summarized in Table II. Both vitellogenin and vitellin are lipoglycoproteins with lipid contents of 6.9-15.7%, and carbohydrate contents of 1-14%. The native molecular weights of vitellogenin and vitellin range from 500,000 to 600,000 daltons in most of the insect species except *Diptera*, which range between 220,000 and 380,000 daltons (Engelmann, 1979; Hagedorn and Kunkel, 1979; Harnish, 1979). Upon denaturation of vitellogenin and vitellin with SDS, several subunit polypeptides are released. For instance, denaturation of locust vitellogenin and vitellin with sodium dodecyl sulfate releases 8 polypeptides of 125,000 to 52,000 daltons, which do not occur in simple molar proportions (Chen *et al.*, 1976, 1978; Chen, 1980). It is possible that these subunits of vitellogenin represent products of intracellular processing in the fat body before secretion into the hemolymph. By pulse-labelling of locust fat body *in vitro* with

TABLE II

Characteristics of Some Insect Vitellogenin (VG) and Vitellin (VN)

Order and Species	VG or VN	Molecular Weight (X10^{-3})			Lipids (%)	Carbohydrate (%)	References
		Native Protein	Denatured Protein	Primary Polypeptides			
Dictyoptera							
Leucophaea maderae (Fab.)	VG, VN	560	118, 96, 87, 57	260, 179	6.9	8.6	1, 2, 3
Blattella germanica (L.)	VG	659	100, 52		15.7	4.5	4
Orthoptera							
Locusta migratoria R.+F.	VG, VN	550	126, 117, 112, 104, 96, 64, 57, 54	235, 225	9.6	14.0	5, 6
Lepidoptera							
Hyalophora cecropia (L.)	VG, VN	510	180, 47	220	9.4	1.0	3, 7, 8
Manduca sexta (L.)	VG, VN	260	180, 50		12.0	3.0	9
Philosamia cynthia (D.)	VG, VN	500	230, 55		10.0	2.5	10, 11, 12
Coleoptera							
Tenebrio molitor L.	VG	460	160, 150, 145, 104, 56, 41	204			8
Diptera							
Aedes aegypti (L.)	VN	350	175, 165	175, 165		10.5, 5.8	8, 13

Species						
Drosophila melanogaster Meig.	VG, VN	220	45, 45, 44	46, 45, 44	2.7	8, 14, 15, 16
Drosophila virilis Sturt.	VN		49		1.2	16
Culex pipiens L.	VN	380	160, 82			17
Calliphora erythrocephala (Meig.)	VG	225	50, 47	ca. 50		18
Hemiptera						
Triatoma protracta (U.)	VN	437				19
Rhodnius prolixus St.	VN	460				20

1) Dejmal and Brookes, (1972)
2) Koeppe and Ofengand, (1976)
3) Engelmann, (1976)
4) Kunkel and Pan, (1976)
5) Chen et al., (1978)
6) Chen, (1980)
7) Pan and Wallace, (1974)
8) Harnish, (1979)
9) Mundall and Law, (1977)
10) Chino et al., (1969)

11) Chino et al., (1976)
12) Chino et al., (1977)
13) Hagedorn and Judson, (1972)
14) Bownes and Hames, (1977)
15) Warren and Mahowald, (1979)
16) Kambysellis, (1979)
17) Atlas et al., (1978)
18) Fourney, (1979)
19) Mundall and Engelmann, (1977)
20) Chalaye, (1979)

labelled amino acids (Chen *et al.*, 1978) and also by translation of poly(A)-containing RNA of locust fat body in *Xenopus* oocytes (Chen, 1980) it has been concluded that mature vitellogenin is the cleavage product of two primary translation subunits of 235,000 (VG_1) and 225,000 (VG_2) daltons, presumably coded for by two different structural genes. A similarity between the molecular weights of the primary translation units of four insect orders and the native molecular weights of the *Diptera* suggests that insect vitellogenin genes may be similar in structure and organization. Recently Harnish (1979) has reported that the vitellogenins of representatives of six insect orders appear to be derived from primary polypeptides of molecular weight about 225,000, and that in some species at least, more than one gene is involved. This situation with respect to the size of the initial polypeptides and the existence of multiple vitellogenin genes is analogous to that in amphibians (Wahli *et al.*, 1979; Felber *et al.*, 1980).

Juvenile Hormone

Structure, bioassay and occurrence of juvenile hormone

By a variety of chemical and biological techniques three juvenile hormones have been identified in insects (see reviews: Staal, 1972; Schooley, 1977). They are homologous sesquiterpenes containing an epoxide function. A rich source of juvenile hormone activity was first discovered in an ether extract of the abdomens of adult male *Cecropia* moths by Williams (1956). The chemical structure of the purified hormone was determined by Röller *et al.* (1967) as a

methyl-10,11-epoxy-7-ethyl-3,11-dimethyl-2-trans-6-trans tridecadienoate
(JH I, Fig. 2). A second minor component of this "*Cecropia* oil" was
later isolated by Meyer *et al.* (1968) and shown to be a C_{17} homologue
(JH II, Fig. 2). The third JH was isolated *in vitro* from media used
to culture the adult female corpora allata of *Manduca sexta* (Judy *et
al.*, 1973) and was shown to be a C_{16} homologue of the *Cecropia* hormone —
(JH III, Fig. 2). A prerequisite for the isolation of any of the three
known juvenile hormones has been the availability of reliable, sensi-
tive and at least semi-quantitative procedures to monitor hormonal
activity during the purification process. At present the most common
technique used is the simple and sensitive *Galleria mellonella* (L.) pupal
bioassay. This bioassay involves the wounding of wax moth pupae on
the mesothorax (approximately $1mm^2$) and the application of the test
material dissolved in a mineral oil-molten paraffin wax mixture.
A positive response is noted by the appearance of patches of pupal
cuticle over the wound after the adult molt. With a single exception
(Lanzrein *et al.*, 1975) JH III appears to be the major form of the
hormone found in insects other than the Lepidoptera (Table III).

Biosynthesis of juvenile hormone

The corpus allatum (CA) is the site of synthesis and release
of juvenile hormone in all insects studied (Engelmann, 1970;
Wigglesworth, 1970). Juvenile hormone produced by the CA has now
been isolated and identified in a wide number of insects, primarily
in vitro (Schooley, 1977). Recently Tobe and Saleuddin (1977) have

Figure 2.   Chemical structures of the three known insect
juvenile hormones and an active juvenile
hormone analog, ZR-515.

15A

JH—I

JH—II

JH—III

ZR—515

COOCH₃

COOCH₃

COOCH₃

COOCH(CH₃)₂

C₂H₅

CH₃

CH₃

C₂H₅ O H

C₂H₅ O H

CH₃ O H

O
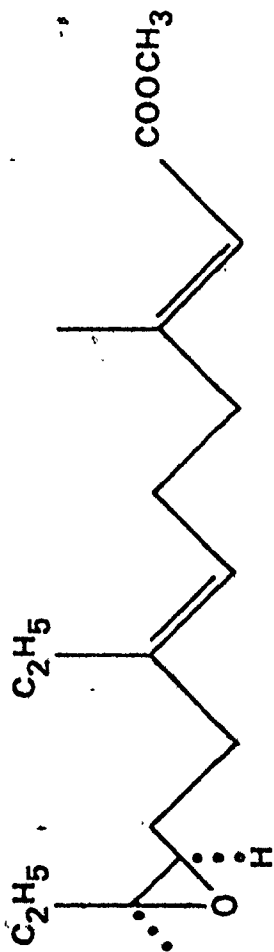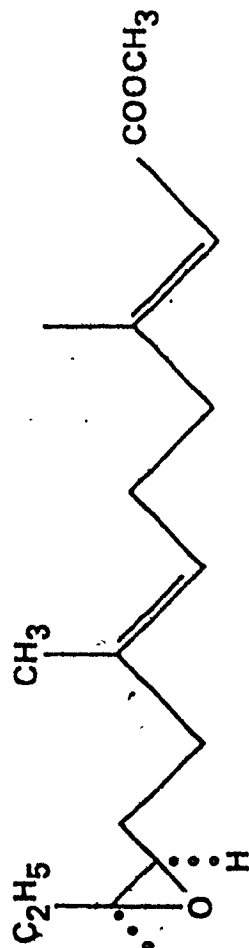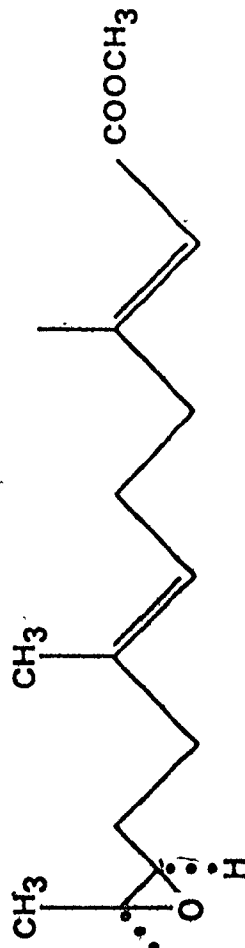
TABLE III

IDENTIFICATION OF THE JUVENILE HORMONES OF VARIOUS INSECTS [SCHOOLEY. 1977]

Genus/Species	(Order)	Stage	Juvenile Hormone		
			JH I	JH II	JH III
Hyalophora cecropia	(Lepidoptera)	ad. m	++	+	+
"	"	"			
Hyalophora gloveri	"	"	+	+	
Samia cynthia	"	"	++	+	
Manduca sexta	"	"	++	+	
Heliothis virescens	"	ad. f	+	+	+
Melolontha melolontha	(Coleoptera)	ad. f		++	+
Tenebrio molitor	"	ad. m,f			+
"	"	ad. m,f			+
"	"	ad. f			+
Leptinotarsa decemlineata	"	ad. m,f			+
Apis mellifera	(Hymenoptera)	ad. f workers			+
Schistocerca vaga	(Orthoptera)	ad. f			+
Gastrimargus africanus	"	ad. f			+
Schistocerca gregaria	"	ad. m,f			+
"	"	larvae & ad. m,f			+
Periplaneta americana	(Dictyoptera)	ad. f			+
Blatta orientalis	"	ad. m,f			+
Leucophaea maderae	"	ad. m,f			+
Nauphoeta cinerea	"	ad. m,f			+
"	"	ad. f	trace	trace	+
"	"	larvae	+	+	+

Abbreviations are as follows: adult (ad.), present (+), present as major component (++), male (m), female (f).

determined the subcellular localization of newly-synthesized juvenile

hormone by electron microscopic autoradiography and found it is pro-

duced in all cells of the CA of the desert locust *Schistocerca gregaria* (S.).

At present the complete biosynthetic pathway of the three juvenile

hormones has not been worked out due to the impermeability of the CA

in vitro to some of the labelled putative precursors (Reibstein *et al.*,

1976; Schooley *et al.*, 1976). However, cell-free CA enzyme systems

have been used to isolate specific intermediates in this pathway. On

the basis of these results it is suggested that JH I is synthesized

from one mevalonate and two homomevalonate molecules, JH II from one

homomevalonate and two mevalonate molecules and JH III from three

mevalonate molecules. The final steps in juvenile hormone biosynthesis

involve methylation at C-1 and and epoxidation at the C-10, C-11

positions in an as yet undetermined order (Fig. 3).

The nature of the mechanism(s), nervous and/or hormonal, regulating the

production of juvenile hormone in the corpora allata has been established

for a number of insects (Gilbert *et al.*, 1980). The corpora allata

are innervated by neurosecretory fibers from the brain and ordinary

neurons. Juvenile hormone production can then be regulated by either

allatropins and allatahibins, and by nervous stimuli. Studies with

implanted glands and with CA *in vitro* have suggested the existence of

neurosecretory stimulation and neural inhibition of hormone production

in adult female insects where juvenile hormone plays a gonadotropic

role.

Figure 3. Proposed scheme for the biosynthesis of the three
juvenile hormones. (1) acetyl-CoA; (2) mevalonic
acid; (3) isopentenyl pyrophosphate; (4) propionyl-
CoA; (5) homomevalonic acid; (6) 3-methylene pentyl
pyrophosphate; (8) JH I-acid; (9) JH I; (11) JH II-
acid; (12) JH II; (13) farnesyl pyrophosphate;
(14) JH III-acid; (15) JH III. SAM, S-adenosyl-
methionine. (After Schooley *et al.*, 1976, and
Reibstein *et al.*, 1976.)

3 [structure (1)] → [structure (2)] → ATP, CO_2, H_2O → [structure (3)]

(1)   (2)   (3)

[structure (4)] + 2 [structure (1)] → [structure (5)] → ATP, CO_2, H_2O → [structure (6)]

(4)   (1)   (5)   (6)

JH I

2 [structure (6)] + [structure (3)] → → [structure (7)]

(6)   (3)   (7)

↓ NADPH, O_2

[structure (9)] ← SAM ← [structure (8)]

(9)   (8)

JH II

[structure (6)] + 2 [structure (3)] → → [structure (10)]

(6)   (3)   (10)

↓ NADPH, O_2

[structure (12)] ← SAM ← [structure (11)]

(12)   (11)

JH III

3 [structure (3)] → → [structure (13)]

(3)   (13)

↓ NADPH, O_2

[structure (15)] ← SAM ← [structure (14)]

(15)   (14)
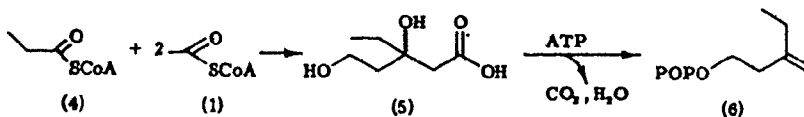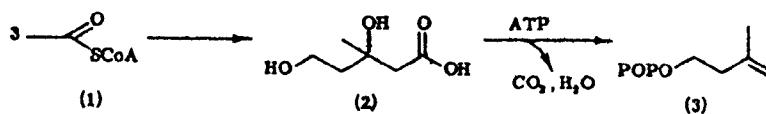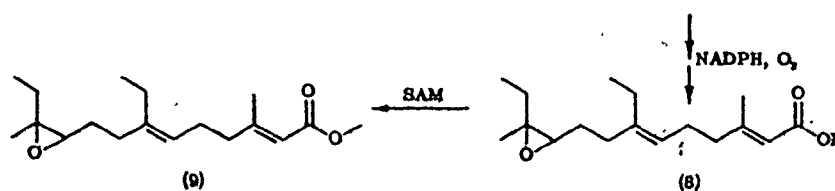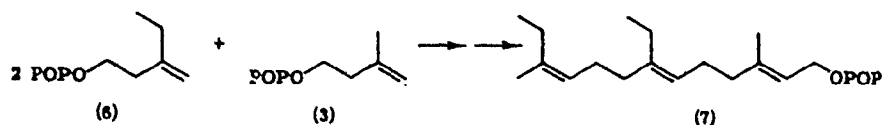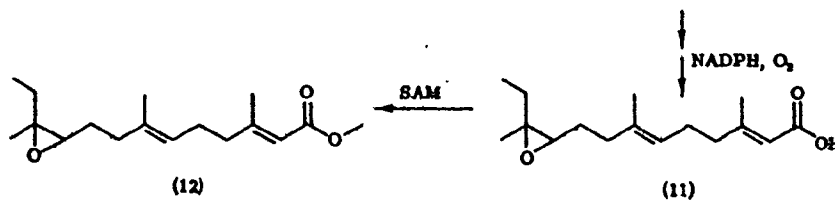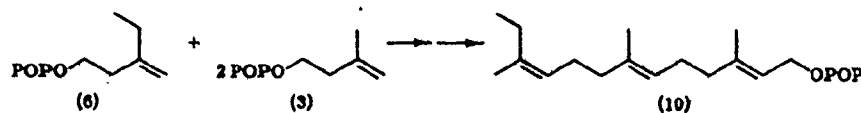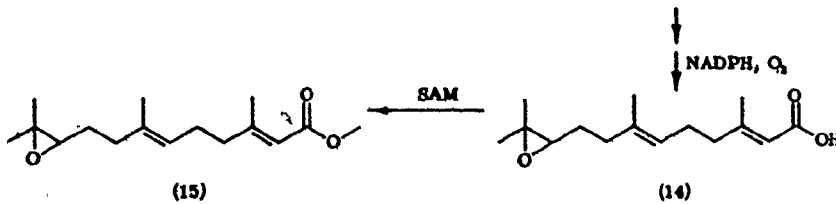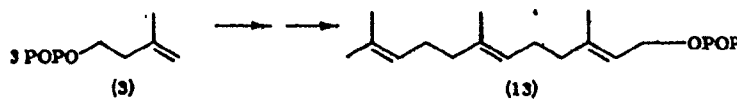
After synthesis the juvenile hormones are immediately secreted into the hemolymph where they are bound by specific hydrophilic carrier proteins. · These proteins serve to protect the hormone from degradative enzymes during transport to the target tissue (Sandburg *et al.*, 1975b). Catabolism of the juvenile hormone has been shown to occur through two major mechanisms: the hydrolysis of the ester to the acid, and the hydration of the epoxide group to the diol (Erley *et al.*,1975; Fig. 4). Both forms are biologically inactive. In either case the next step is the formation of the acid-diol which subsequently conjugates with sulfates for excretion. The most common metabolite is the juvenile hormone acid since general carboxyesterases are released at intervals into the circulation (Sanburg *et al.*, 1975a).

Therefore the titer of JH is regulated by the biosynthesis and degradation of the hormone, and also by the presence or absence of specific juvenile hormone carrier proteins.

Biological activity of juvenile hormone

(1) The role of juvenile hormone in metamorphosis:

Since the rigid exoskeleton of an insect restricts the growth of the larva, a periodic shedding (ecdysis) of this cuticle and the production of a new, larger version is required. The process of insect development proceeds through a series of distinct stages (instars) which are divided by molts. The sequence of larval-larval molts occurs with only minor alterations in morphological features before they are transformed into adults. The adult metamorphosis occurs either

Figure 4. Catabolism of juvenile hormone in the insect.
(I) JH I; (II) JH I-acid; (III) JH I-diol;
(IV) JH-acid-diol. The breakdown of JH II and
III occurs similarly. Dotted line represents
minor pathway. (After Erley *et al.*, 1975.)

(I)

(II)

(III)

(IV)

conjugates
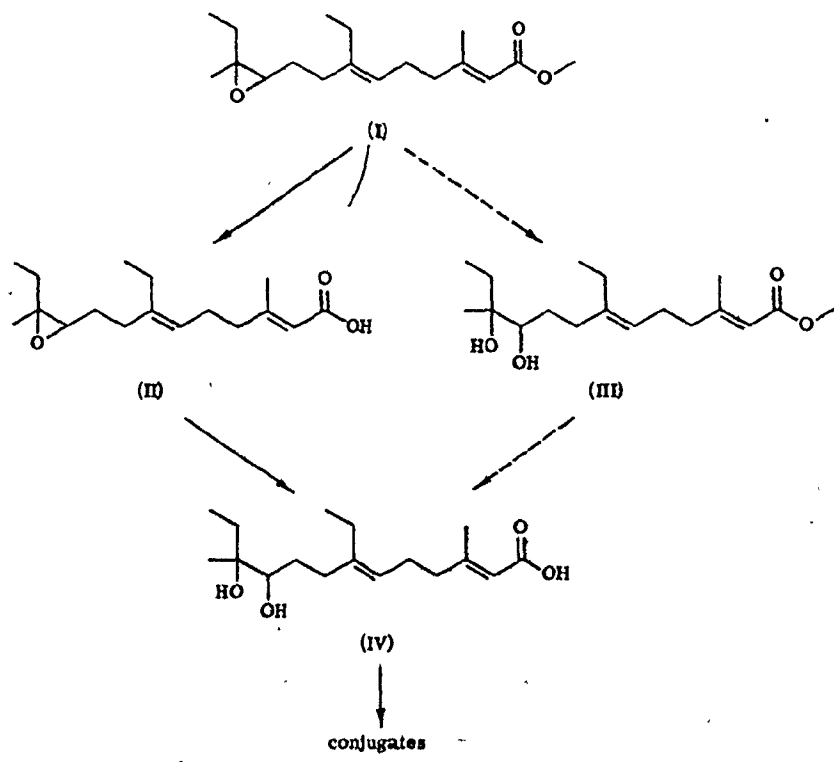
directly with a larval-adult molt (Hemimetabola) or indirectly with an intermediate larval-pupal molt before the final molt to the adult (Holometabola).

The control of the molting process involves four hormones: ecdysiotropin, ecdysterone, allatropin and juvenile hormone. Environmental and physical stimuli trigger the production of ecdysiotropin by neurosecretory cells in the brain; it is released from the paired neurohemal organs for these cells, the corpora cardiaca. This neural hormone travels in the hemolymph to the ecdysial gland where the prohormone, ecdysone, is produced and secreted. Ecdysone is then converted by other tissues, primarily the fat body, to the molting hormone ecdysterone (20-hydroxyecdysone). This active hormone is released into the hemolymph, travels to the target tissue (epidermis) and initiates the next molt. The process of molting consists of the retraction of the epidermis from the old cuticle (apolysis), secretion of new cuticle and the ultimate shedding of the partially digested old cuticle (ecdysis). The type of new cuticle formed depends on the juvenile hormone titer which is, in part, influenced by the allatropin-stimulated biosynthesis of juvenile hormone in the corpora allata. High concentrations of juvenile hormone result in a larval-larval molt, a lower titer produces a larval-pupal transformation and pupal-adult metamorphosis occurs in the absence of juvenile hormone (Fig. 5).

(2) The role of juvenile hormone in reproduction:

After adult emergence the corpora allata again begin to

synthesize and release juvenile hormone. In the adult female of most

species juvenile hormone has been shown to affect one or more of the

following processes: (a) maturation of previtellogenic oocytes, (b)

initiation of vitellogenin synthesis in the fat body, and (c) induction

of vitellogenin uptake into the developing oocyte (Sridhara *et al.*,

1978; Engelmann, 1979; Hagedorn and Kunkel, 1979). Juvenile hormone

has also been shown in *Rhodnius prolixus* S. to stimulate the growth of

ovarian follicle cells, enabling the oocytes to take up vitellogenin

(Abu-Hakima and Davey, 1977). Hormonal regulation of vitellogenin synthesis

in the fat body of *Locusta migratoria* has been demonstrated *in vitro*

(Wyatt *et al.*, 1976). Bell (1969) has shown that in *Periplaneta*

americana L. the injected vitellogenin is not incorporated into oocytes

unless juvenile hormone is present. In the male, juvenile hormone is

important in some species for the maturation of the accessory glands

(DeWilde and DeLoof, 1973; Leopold, 1976) or for the regulation of

reproductive behaviour (Truman and Riddiford, 1977).

Hormonal Control of Vitellogenin Synthesis

Regulation of vitellogenin synthesis in vertebrates

The estrogen induction of vitellogenin synthesis in the liver

of egg-laying vertebrates has been intensively investigated as a model

system for the analysis of different molecular events leading to

regulated gene expression (Clemens, 1974; Wallace and Bergink, 1974;

Tata, 1976; Tata and Smith, 1979). The specificity and kinetics of
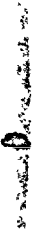
Figure 5.   Scheme of the hormonal control of insect molting
and metamorphosis in homoletabolous insects.
(Summarized by T.T. Chen.)

Light Temperature Food Intake Body Size

etc.

Brain

Ecdysiotropin
(PTTH)

Corpora Cardiaca
→ Other
Neurohormones

Corpora Allata

JH

Fat Body,
Ovary, etc
(in adult)

Prothoracic
Glands
(Ecdysial)

JH

Ecdysone

Ecdysone

Ecdysone

Ecdysone

Larval
Cuticle

Pupal
Cuticle

Adult Cuticle
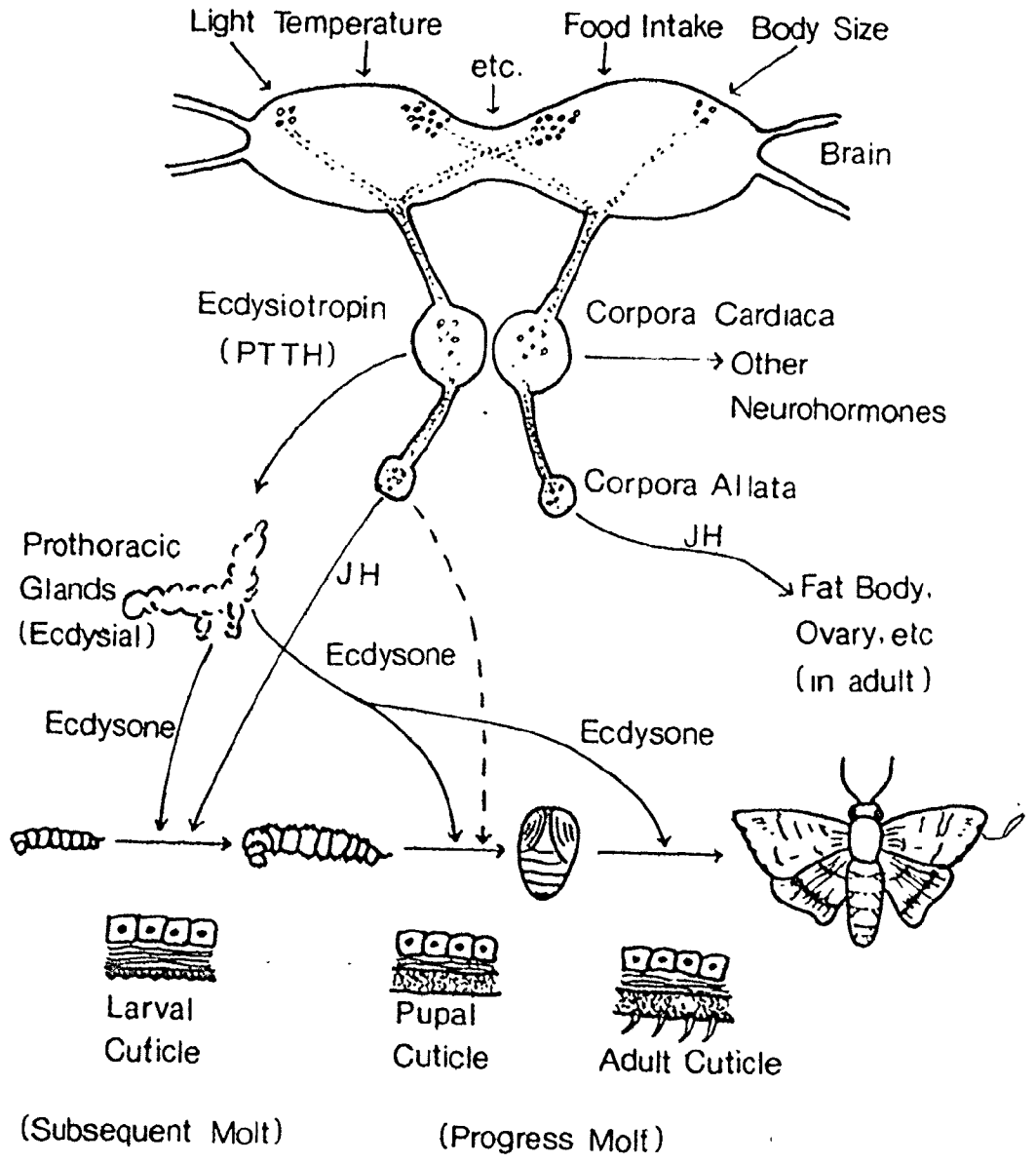
(Subsequent Molt)

(Progress Molt)

vitellogenin production in response to estrogen in the liver of male and female *Xenopus* and chicken are particularly well characterized (Follet and Redshaw, 1968; Wallace and Dumont, 1968; Wallace and Jared, 1968a,b; Clemens, 1974; Bergink *et al.*, 1974; Wallace and Bergink, 1974; Gruber *et al.*, 1976). Since males normally do not produce this yolk protein the estrogen-induced vitellogenin synthesis and secretion in males offer an ideal system in which the primary response of a tissue to hormone treatment can be observed. After an injection of estrogen, vitellogenin production in males can be detected within 12 hr (*Xenopus*), becomes maximal after 8 to 10 days and is undetectable after 30 days. In the rooster a similar but more rapid response is observed. Vitellogenin is first detected between 4 to 6 hr after hormone stimulation, is maximal by 2 to 4 days and stops after 12 to 14 days. Estrogen has also recently been shown to induce vitellogenin synthesis in liver organ culture and hepatocyte culture (Wangh and Knowland, 1975; Green and Tata, 1976; Felber *et al.*, 1978). These results have firmly established that estrogen is the only controlling factor necessary for vitellogenin synthesis.

Regulation of vitellogenin synthesis in insects

The hormone induction of vitellogenisis in the fat body of a wide variety of insects is another system being used for the investigation of the mode of hormone action, analogous to the estrogen stimulation of vitellogenin synthesis in the liver of amphibians and birds (Engelmann, 1979; Hagedorn and Kunkel, 1979). Unlike vertebrates,

insects have retained two developmental hormones, ecdysone (a steroid) and juvenile hormone (a sesquiterpenoid) to control adult reproductive maturation. Juvenile hormone has been shown to regulate oogenesis directly in the female of insects, such as the cockroach (Brookes, 1969; Engelmann, 1969), the Monarch butterfly (Pan and Wyatt, 1976) and the African migratory locust (Chen et al., 1976, 1979). In the mosquito Aedes aegypti and Culex pipiens, however, both ecdysone and juvenile hormone are required for egg maturation (Hagedorn, 1974; Flanagan and Hagedorn, 1977; Srdic et al., 1979) and in the saturnid moths (silk moth) egg maturation does not appear to depend directly upon either of these hormones (Pan, 1977). Furthermore, by the use of organ culture techniques it has recently been demonstrated that the cultured fat body of the female African migratory locust can respond to JH to induce vitellogenin synthesis (Wyatt et al., 1976; Chen and Wyatt, 1980). This firmly establishes that juvenile hormone can act directly on the fat body in the absence of any other hormonal factors. With only a few exceptions (Chalaye, 1979; Mundall and Tobe, 1979) most male insects, including the African migratory locust, do not produce vitello-genin (Wyatt, 1972). Therefore investigation of the molecular nature of the sexual dimorphism in insects may be important for the under-standing of the mechanism of regulated gene expression.

Hormonal Control of Vitellogenin Synthesis at the Molecular Level

Amphibian and avian

In Xenopus and chicken the production of vitellogenin in the

liver following estrogen stimulation may be a result of the synthesis of the corresponding mRNA or the translation of stored vitellogenin mRNA. The presence of vitellogenin mRNA in *Xenopus* (Shapiro *et al.*, 1976; Skipper and Hamilton, 1977; Farmer *et al.*, 1978) and in chicken liver (Mullinex *et al.*, 1976) was initially quantified by its translational activity *in vitro*. The appearance of vitellogenin mRNA in these systems was identified using the ability of vitellogenin mRNA-containing polysomes or the polysomal RNA to direct the synthesis of immuno-precipitable vitellogenin polypeptides in a cell-free protein synthesizing system (Berridge *et al.*, 1976; Jost and Pehling, 1976a; Mullinix *et al.*, 1976; Roskam *et al.*, 1976a,b; Bast *et al.*, 1977; Skipper and Hamilton, 1977). These studies showed that translationally active mRNA accumulated in the cytoplasm only after hormone stimulation. However they do not rule out the possible presence of a supply of vitellogenin mRNA which is inactive as a template for *in vitro* translation until estrogen treatment. Therefore complementary DNA (cDNA) prepared from purified vitellogenin mRNA was used as a hybridization probe to determine the number of vitellogenin mRNA sequences present (Baker and Shapiro, 1977; Deeley *et al.*, 1977; Felber *et al.*, 1978; Jost *et al.*, 1978; Ryffel *et al.*, 1977; Baker and Shapiro, 1978). In most of these studies the rapid accumulation of mRNA has been directly correlated with the rate of synthesis of vitellogenin as measured by its appearance in the circulation, in the tissue, or in the medium of organ cultures of liver from animals treated with estrogen *in vivo*. This evidence has led to the conclusion that the synthesis

of vitellogenin is a function of the appearance of newly-transcribed vitellogenin messenger RNA.

Another feature of the action of most growth and developmental hormones is a lag period preceding the onset of the synthesis of specific proteins in the target tissue (Tata, 1970). In the liver of birds and amphibians exposed for the first time to estrogen there is a lag period before vitellogenin synthesis begins. The length of this lag period in absolute terms depends on the animal system (birds having a shorter lag period than do amphibians), whether the induction is in tissue culture or *in vivo*, whether the protein is measured in the blood or inside the cell, and upon the sensitivity of the assay method used to measure the level of mRNA or protein (Clemens *et al.*, 1975; Berridge *et al.*, 1976; Green and Tata, 1976; Gruber *et al.*, 1976; Baker and Shapiro, 1978; Farmer *et al.*, 1978). After the full course of primary stimulation the production of vitellogenin returns to a zero level. If a second dose of estrogen is given several significant differences are noted: the lag period is dramatically shortened, protein synthetic capacity at peak production is greater than that in primary stimulation, and synthesis reaches its maximum more quickly. These results suggest the possible existence of some kind of rate-limiting steps or factors essential for the specific expression of vitellogenin genes at the transcriptional and/or the translational level. Studies on the kinetics of vitellogenin mRNA accumulation during primary and secondary induction by estrogen have revealed that the rate of mRNA accumulation in the secondary stimulation is higher than that of the primary stimulation

(Baker and Shapiro, 1977, 1978; Deeley *et al.*, 1977a,b). However, this information alone cannot completely account for the observed change in the length of the lag period reported previously. Data from several investigations have shown that the induction of vitellogenin synthesis is accompanied by a massive accumulation of total RNA and a proliferation of the endoplasmic reticulum (Clemens, 1974; Tata and Baker, 1975; Lewis *et al.*, 1976). These varied responses of the hepatocytes to estrogen stimulation may suggest an involvement in the production of vitellogenin at the translational or post-translational level.

Insect

The biosynthetic processes related to the production of vitellogenin in the locust fat body have been examined by Chen *et al.* (1976, 1979). Vitellogenin synthesis becomes detectable about 7 days after eclosion and rises to a maximum at day 13 where vitellogenin accounts for approximately 60% of the total protein output of the cells. A steady increase in the incorporation of uridine, into total RNA during the period of maturation is accompanied by a sudden increase in tissue RNA content at about the onset of vitellogenin production and is followed by a decline. An 8-fold increase in total RNA content and a 2-fold increase in DNA content occur prior to vitellogenin synthesis. Associated with these metabolic changes, cytological examination of the fat body cells has revealed a marked increase in the abundance of ribosomes and endoplasmic reticulum (Lauverjat, 1977; Couble *et al.*, 1979). Vitellogenin synthesis and the increase in RNA and DNA content

depend upon the appearance of juvenile hormone, as removal of the corpora allata prevents these cellular and molecular changes (Chen *et al.*, 1976, 1979). Treatment of allatectomized locusts with JH I or an active analog induces vitellogenin synthesis. Recently Chinzei *et al.* (1980) have developed a photographic scanning technique to quantify the accumulation of vitellogenin mRNA in denaturing-agarose gels. Using this technique vitellogenin mRNA accumulation is shown to parallel the initial increase in vitellogenin synthesis up to the stage of maximal production, after which vitellogenin synthesis drops to zero while vitellogenin mRNA accumulation remains high.

While these data suggest that juvenile hormone acts at the level of transcription initially, it also implies that some mechanism of translational control is present. Confirmation of these results awaits the development of a more sensitive probe for the quantification of vitellogenin mRNA accumulation.

Rationale and Objectives

As a consequence of the above literature survey it is apparent that our understanding of the molecular mechanisms of juvenile hormone-controlled specific gene activation in insects is incomplete.

The main objectives of this work are: (a) to prepare and characterize undegraded vitellogenin mRNA-containing polysomes from the fat body of the locust, *Locusta migratoria*. These polysomes may serve as a rich source for the isolation of vitellogenin mRNA. Pure

vitellogenin mRNA can serve as template for the development of a
specific complementary DNA probe for the analysis of molecular events
leading to juvenile hormone-regulated expression of the vitellogenin
genes in *Locusta;* (b) to characterize the changes in the content of
vitellogenin mRNA-containing polysomes in the fat body of adult females
during their gonadotropic cycle; (c) to investigate the kinetics of
the appearance of polysomes during primary and secondary induction of
vitellogenin synthesis by an active juvenile hormone analog.

MATERIALS AND METHODS

Insects

Stocks of the African migratory locust (*Locusta migratoria*)
obtained from Dr. G.R. Wyatt (Queen's University, Kingston) were
reared in the laboratory under crowded conditions as described by
Chen *et al.* (1978). Groups of up to 50 adult females were reared with
an equal number of males from the second or third day after eclosion
in a cage of 0.3 m^3. The temperature was maintained at 37°C for 12
hr each day by a 60 watt light bulb, and fell to room temperature at
night (about 24°C). Barley and wheat seedlings were provided daily,
with a supply of bran continuously available. Oviposition began about
2 weeks after emergence and was repeated at about 1 wk intervals.
Eggs were deposited in cups filled with moist sand. The cups were then
removed, covered with plastic wrap and incubated at 37°C. They hatched
in about 10 days.

Chemicals

A juvenile hormone analog, ZR-515 (methoprene isopropyl-11-
methoxyl-3,7,11 trimethyldodeca-2,4-dienoate; Henrick *et al.*, 1973)
was acquired from Zoecon Corporation (Palo Alto, California; Fig. 2).

[^{3}H]-leucine (20 Ci/mmole) and [^{35}S]-methionine (500 Ci/mmole) were purchased from New England Nuclear (Boston, Mass). Trisma base (tris (hydroxymethyl) aminomethane), TES (N-tris (hydroxymethyl) methyl-2-4 aminomethane sulfonic acid), EGTA (ethyleneglycol-bis-(β-amino-ethyl-ether) N, N'-tetra-acetic acid) and PMSF (phenylmethyl-sulfonyl fluoride were purchased from Sigma Chemical Company (St. Louis, Missouri). SDS (sodium dodecyl sulphate) was purchased from BDH Chemicals (Toronto, Montreal, Vancouver). Magnesium chloride (6-hydrate) and potassium chloride were purchased from the J.T. Baker Chemical Company (Phillipsburg, New Jersey) and Eriochrome Black-T was a gift from Dr. O. Hermann of the Department of Chemistry, McMaster University. Purified vitellin, prepared by the method of Chen *et al.* (1978), was a gift from Dr. T.T. Chen, Department of Biology, McMaster University.

Antiserum against Purified Vitellin

Antiserum against purified vitellin (which is immunologically identical to vitellogenin) was prepared by the method of Chen *et al.* (1978). Solutions of purified vitellin (4 mg of protein in 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.1) containing 1 mM PMSF and 0.4 M NaCl) were mixed with equal volumes of Freund's complete adjuvant (Difco). This mixture was injected intramuscularly into rabbits three times at 2 wk intervals, and two wks after the last injection the rabbits were bled and sera was prepared. The blood was allowed to clot for 20 min at room temperature, for 1 hr at 4°C and was then centrifuged

at 5,000 x g for 20 min to remove cells and debris. This serum was rendered specific for locust vitellin by absorption with adult male hemolymph (Chen *et al.*, 1978), and was stored in small portions at -20°C.

Hormone Treatment

Allatectomy was performed with a procedure adapated by Chen *et al.* (1978) from that of Strong (1963) on 1-2 day old females. Groups of allatectomized females were then kept in separate cages for 2 wks before they were used in any experiment. The absence of vitellogenin in the hemolymph of each individual was tested by Ouchterlony immunodiffusion (Ouchterlony and Nilssen, 1973) with anti-vitellin serum (Fig. 6). In petri plates (30 mm) covered with a 2 mm thick layer of 1% agarose in 0.9% NaCl seven wells were made, six peripheral and one central. The central well was filled with 25 μl of antiserum. Each peripheral well was filled with 10 μl of hemolymph from allatectomized females or with 10 μl of purified vitellin solution (5 μg protein). The plates were then incubated at room temperature overnight in a moist chamber. The presence and absence of antigen-antibody precipitin arcs were recorded. After confirming the absence of vitellogenin, each individual was injected with 300 μg of ZR-515 in 10 μl of olive oil at the base of the first abdominal segment, near the leg. A second dose of hormone was administered two weeks after the first injection, in a similar manner.

Figure 6. Representative Ouchterlony immunodiffusion plate used
to detect the presence or absence of vitellogenin in
the hemolymph. The centre well contains anti-vitellin
serum and the peripheral wells contain vitellin (VN),
or hemolymph from a reproductively active female (MF),
immature female (IF), allatectomized female (CA$^-$),
or allatectomized female treated with ZR-515 (Ca$^-$ +
ZR-515).

CA⁻+ZR515

CA⁻

VN

VN

MF

IF
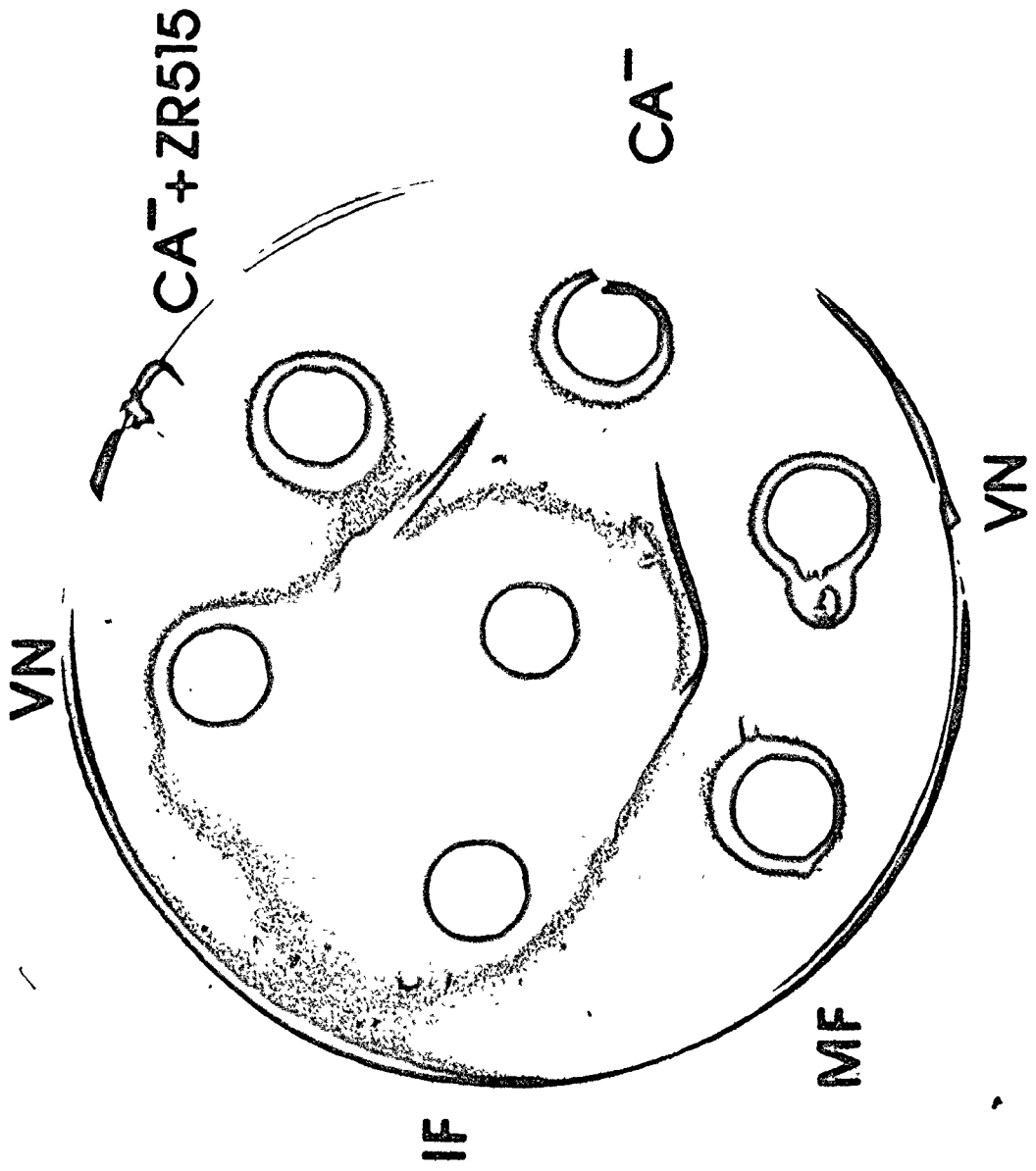
Preparation of Polysomes

Locusts were opened ventrally and the gut and gonads removed (Fig. 7). After flooding with locust Ringer solution (Chen *et al.*, 1978; 20 mM TES, pH 7.2, containing 8 mM $MgCl_2$, 150 mM KCl, 120 mM NaCl, 60 mM sucrose, 5 mM D-glucose) the fat body tissue was carefully removed from the abdominal body wall, rinsed once in the ice-cold modified locust Ringer solution and once in the homogenization buffer (200 mM Tris, pH 9.0, containing 200 mM sucrose, 0-600 mM KCl, 25-75 mM $MgCl_2$, 0-45 EGTA, 1% Triton X-100, 5 μg/ml cycloheximide and 7 mM β-mercaptoethanol). Fat body from 4-6 locusts (about 0.6 g of tissue wet weight) was homogenized in 1.5 ml of the ice-cold homogenization buffer in a Dounce homogenizer with pestle "B" (15 strokes). The homogenate was centrifuged twice for 10 min at 10,000 x g. A 0.5 ml sample of the post-mitochondrial supernatant was layered onto a 16 ml ·linear sucrose gradient (15-60% (W/V) sucrose in 40 mM Tris-HCl buffer, pH 9.0, containing 200 mM KCl, 5 mM EGTA and 35 mM $MgCl_2$). The gradients were centrifuged for 4 hr (5 hr for Figs. 13c, 14c, 15, 17, 20 and 21) at 23,000 rpm in a SW 27.1 rotor (Beckman) at 4°C. Fractions of 0.5 ml were collected and the absorbance profile monitored by an Isco UV analyzer (UA 5) at 254 nm.

To label the nascent vitellogenin peptides associated with polysomes, the fat body tissue was incubated in 2 ml of the incorporation medium (Wyatt *et al.*, 1976) containing 40 μCi of [³H]-leucine for ten min at 30°C. After incubation the polysomes were prepared

Figure 7. Internal body structure of reproductively active

female locust, opened ventrally with the digestive

tract and gonads removed. The loose light-coloured

net-like structure is fat body tissue.

by the method described above. The $[^3H]$-leucine labelled vitello-
genin nascent peptides in each fraction were precipitated with anti-
vitellin serum and carrier vitellin according to the method described
by Chen et al. (1979). Each immunoprecipitate was collected on a
Millipore filter and washed with about 40 ml of 0.1 M sodium phosphate
buffer (pH 7.5, containing 0.9% NaCl, 1% Triton X-100 and 1% sodium
deoxycholate). The filter was dried and counted in 5 ml of a Triton
X-100 toluene based scintillator (2 l of toluene, 1 l of Triton
X-100 and 17 g of omnifluor).

Extraction of Polysomal RNA

Polysomes were prepared and collected from ten 16 ml sucrose
gradients. Each gradient was fractionated into six regions and then
identical regions were combined. Total RNA in each region was
extracted by the phenol-chloroform method of Palmiter (1974), modified
by Chen et al. (1980). To each pooled sample region, equal volumes
of phenol saturated with buffer (pH 5.0, containing 100 mM NaAc, 25 mM
NaCl, 35 mM $MgCl_2$, 25 mM EGTA) and chloroform were added and the
mixture was vigorously shaken for 20 minutes. RNA in the aqueous
phase was precipitated with 2.0-2.5 volumes of absolute ethanol at
-20°C overnight. The crude precipitate was then washed several times
in 3 M sodium acetate and once with 70% ethanol containing 0.3 M
sodium acetate. The RNA was dissolved in sterile double distilled
water, lyophilized, dissolved in the injection medium formulated by
Lane et al. (1971) and kept at -20°C.

Translation of Vitellogenin mRNA in *Xenopus* Oocytes

The vitellogenin mRNA activity in the polysomal RNA was assayed in a translational system derived from *Xenopus* oocytes (Lane *et al.*, 1971). For each RNA preparation thirty oocytes were each micro-injected with 40-80 nl of the RNA solution (Lane *et al.*, 1971). Control oocytes were injected with the same volume of the injection medium alone. The oocytes were incubated in 0.3 ml of incorporation medium (Lane *et al.*, 1971) containing 80 μCi of [^{35}S]-methionine at 20°C for 18 hr, and then homogenized in 1 ml of a 0.05 M Tris buffer (pH 8.1) containing 0.4 M NaCl, 1 mM PMSF, 1% deoxycholate, 1% Triton X-100 and 0.2% SDS (Chen, 1980). After a 1 hr centrifugation at 30,000 x g the supernatant was collected and the [^{35}S]-methionine labelled oocyte proteins were precipitated with specific anti-vitellin serum. Each immunoprecipitate was washed several times with a 0.1 M sodium phosphate buffer (pH 7.5, containing 0.9% NaCl, 1% Triton X-100 and 1% sodium deoxycholate), analyzed on 5% SDS-polyacrylamide slab gels and visualized by fluorography (Chen *et al.*, 1978).

SDS-Polyacrylamide Gel Electrophoresis

SDS (0.1%) - polyacrylamide (5%) gels were prepared according to Chen *et al.* (1978). Protein samples (100 μg) in 0.1 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 10% glycerol, 3% SDS, 1% β-mercaptoethanol and 0.002% bromophenol blue were denatured at 100°C for 3 min and 50 μl of each denatured sample was applied to each well.

Electrophoresis was carried out in a Tris-glycine buffer (25 mM Tris, 0.19 M glycine containing 0.1% SDS) at a constant voltage of 30 V.

Fluorography

After electrophoresis polyacrylamide gels were prepared for fluorography by the method of Laskey and Mills (1975). The gels were fixed in 7% acetic acid, equilibrated with dimethylsufoxide (DMSO) and impregnated in a solution of 22.2% (W/V) omnifluor in DMSO. Gels were then washed in water for at least 2 hrs, dried under vacuum at about 90-100°C and exposed to Kodak RPX-Omat film (RP/R) at -70°C. Films were developed in a Kodak automatic film processor.

Electron Microscopy

A drop of the polysome suspension from the desired region of the sucrose gradient was placed on a formvar-coated carbonized copper grid of 300 mesh for 1 min, to allow the polysomes to adhere to the surface, and the excess was drawn off by blotting with filter paper. Each grid was pretreated with glycerol (0.01%) to remove any electro-static charges. The preparation was stained with an ice-cold aqueous solution containing 2% uranyl acetate in water for 1 min (Roskam *et al.*, 1976a), rinsed with several drops of distilled water and allowed to dry. Grids were examined and photographed on a Phillips EM 300 electron microscope at a magnification of 60,420 X which was calibrated

using a grating replica. Measurements of particle size were made on
a photographic print of magnification approximately 196,000 X.

RESULTS

Conditions for the Preparation of Polysomes

Since at least 60% of the total export proteins synthesized
by the fat body of reproductively active female locusts are vitello-
genin (Chen *et al.*, 1979) a significant portion of the total fat body
polysomes is expected to contain vitellogenin mRNA. As reported by
Chen (1980) the molecular weights of locust vitellogenin subunit
polypeptides are 235,000 and 225,000. These primary translation
products are similar in size to those of avian and amphibian vitello-
genins (170,000 to 240,000 daltons; Jost and Pehling, 1976a,b; Tata,
1976). Therefore locust vitellogenin polysomes are expected to be
about the same size as those of frogs and birds and to have between 40
and 60 ribosomes (Palacios *et al.*, 1972; Roskam *et al.*, 1976a).
However, attempts to prepare locust vitellogenin polysomes employing
conditions used successfully to isolate polysomes from hormone-induced
male *Xenopus* liver (Berridge *et al.*, 1976) and rooster liver (Jost
and Pehling, 1976b; Roskam *et al.*, 1976a,b) were unsuccessful due to
endogenous ribonuclease activity. Consequently a buffer which contains
200 mM Tris-HCl (pH 9.0), 400 mM KCl, 35 mM $MgCl_2$, 25 mM EGTA, 200 mM
sucrose, 7 mM β-mercaptoethanol and 5 μg/ml cycloheximide was developed
for the homogenization of fat body tissue. Subsequent purification and

analysis of polysomes were performed in the same buffer, except that the concentrations of EGTA and Mg^{2+} were 5 mM and 35 mM respectively while cycloheximide and β-mercaptoethanol were omitted. Fat body from four females (approximately 0.6 g of tissue) was efficiently homogenized in 1.5 ml of the homogenization buffer. With these conditions a large broad peak of fast sedimenting polysome was prepared from the fat body of reproductively active female locusts (Fig. 8).

In order to obtain reproducibly higher yields of large poly-somes, the optimum concentrations of EGTA, Mg^{2+} and K^+ in the homogeniza-tion buffer were determined. The effects of different concentrations of EGTA on the yield of heavy polysomes are presented in Figs. 9a-d. Without EGTA (Fig. 9a), a high monosome peak, as indicated by the arrow labelled 80S, was apparent and the yield of large polysomes was low. Use of even low concentrations of EGTA resulted in the appearance of large polysomes and a reduction in the amount of monosomes (Figs. 9b and c). The height of the heavy polysome peak relative to the height of the monosome peak indicates that the maximum yield of large polysomes is obtained with a concentration of 35 mM EGTA (Fig. 9d). A higher concentration of EGTA (45 mM) resulted in a reduction of the number of large polysomes (data not shown).

The influence of different concentrations of Mg^{2+} in the homogenization buffer on the yield of fast sedimenting polysomes is shown in Figs. 10a-f. A low concentration of Mg^{2+} (Fig. 10a) during homogenization produced polysome profiles with a low yield of small molecular weight polysomes and a high peak in the monosome region.

Figure 8. Polysome profile from the fat body of reproductively
active females. Fat body from 4 females was homogenized
at 4°C in 1.5 ml of 0.2 M Tris-HCl, pH 9.0, containing
25 mM EGTA, 35 mM $MgCl_2$, 0.4 M KCl, 0.2 M sucrose, 1%
Triton X-100, 5 µg/ml cycloheximide and 7 mM β-mercapto-
ethanol (0.5 ml) was layered on a 15-60% (W/V) linear
sucrose gradient (see Materials and Methods). T, top;
B, bottom of the gradient.

Figure 9. Effect of EGTA on the polysome profile of the reproduct-

ively active female fat body. Fat body from 4 females

was homogenized at 4°C in 1.5 ml of 0.2 M Tris-HCl,

pH 9.0, containing 0.4 M KCl, 0.2 M sucrose, 1% Triton

X-100, 7 mM β-mercaptoethanol, 5 μg/ml cycloheximide

and various concentrations of EGTA and $MgCl_2$ as indicated:

(a) 0 mM EGTA, 20 mM $MgCl_2$; (b) 15 mM EGTA, 35 mM

$MgCl_2$; (c) 25 mM EGTA, 45 mM $MgCl_2$; (d) 35 mM EGTA,

55 mM $MgCl_2$. The absorbance at 80S (labelled arrow about

fraction 7) indicates the presence of ribosome monomers,

and the unlabelled arrow in (d) indicates the position

of the heaviest polysomes (about fraction 22). The

optimum concentration of EGTA (35 mM) was selected by

considering the polysome to monosome ratio, size of

polysomes and the reproducibility of the profile.

FRACTIONS

A_{254nm}

44A

Figure 10. Effect of Mg^{2+} on the polysome profile of the

reproductively active female fat body. Fat body from

4 females was homogenized at 4°C in 1.5 ml of 0.2 M

Tris-HCl, pH 9.0, containing 35 mM EGTA, 0.4 M KCl,

0.2 M sucrose, 1% Triton X-100, 7 mM β-mercaptoethanol,

5 μg/ml cycloheximide and concentrations of $MgCl_2$ as

indicated: (a) 35 mM; (b) 40 mM; (c) 45 mM; (d) 55 mM;

(e) 65 mM; (f) 85 mM. The profile was obtained by

measuring the absorbance of individual fractions.

The optimum concentration of Mg^{2+} (55 mM) was selected

by considering the polysome to monosome ratio, size

of polysomes, and reproducibility of the profile.
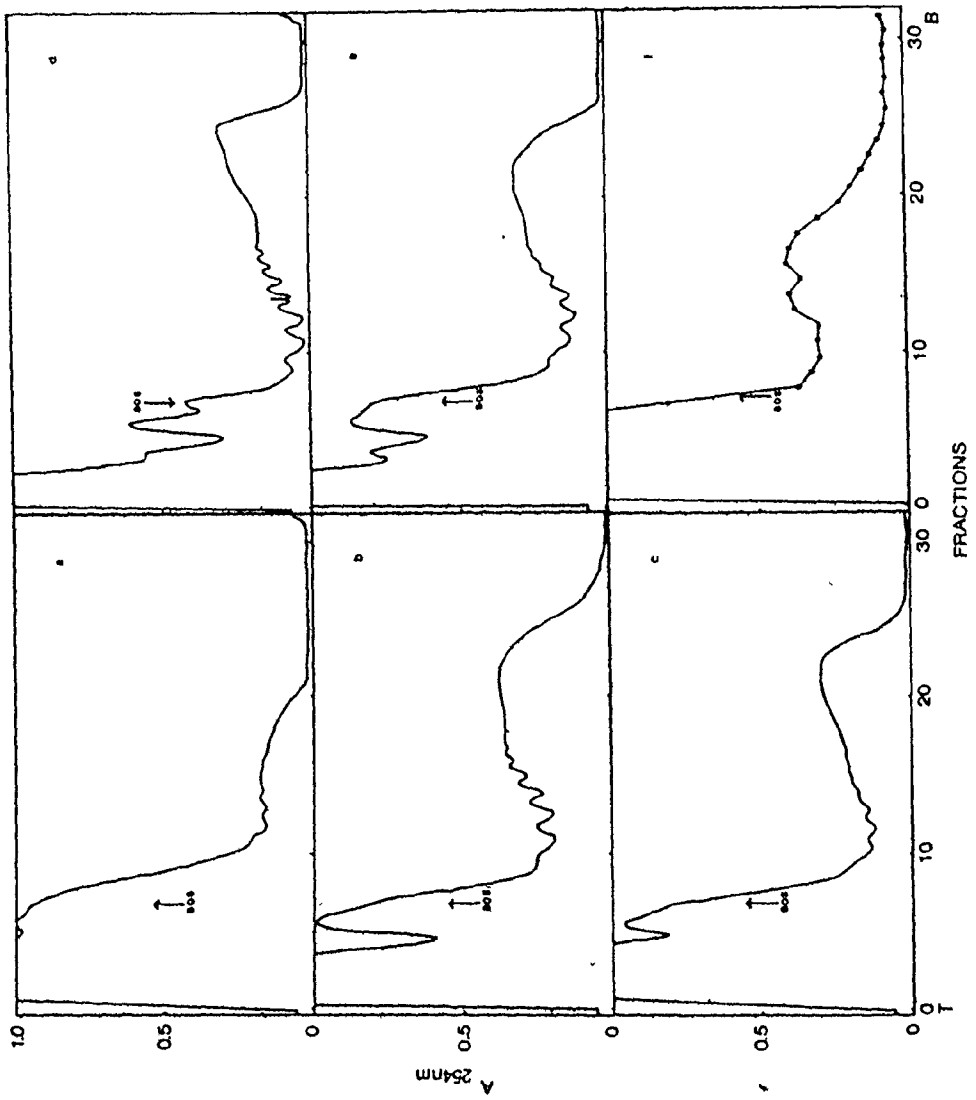
A small increase in the concentration of Mg^{2+} (Fig. 10b) resulted in a significant increase in the yield of total polysomes, especially high molecular weight polysomes. A concentration of 55 mM Mg^{2+} (Fig. 10d) gave the highest yield of heavy polysomes with a prominent peak appearing at the fast-sedimenting edge of the polysome profile and a low 80S peak. The concentration of 55 mM Mg^{2+} is critical since polysome profiles prepared in homogenization buffers containing slightly lower (45 mM, Fig. 10e) concentrations of Mg^{2+} showed a significant loss of large polysomes. At a concentration of 85 mM Mg^{2+} (Fig. 10f) two distinct peaks appeared in the small polysome region, which may be due to breakage of large polysomes.

Figs. 11a-f show the effects of K^+ in the homogenization buffer on the yield of heavy polysomes. A wide range of concentrations of K^+ (100-400 mM) gave a high yield of large polysomes, as was evident from the appearance of a large, slightly skewed peak near the bottom of the gradient (Fig. 11c-e). Above and below this concentration range there is a specific loss of large polysomes (Fig. 11b and f). At zero or low (50 mm) concentrations of K^+ in the homogenization buffer the small polysome region became prominent, which may have resulted from the non-specific aggregation of ribosomes or selective breakdown of large polysomes (Figs. 11a and b).

From these studies it is concluded that the optimum concentrations of K^+, Mg^{2+} and EGTA in the homogenization buffer are 200 mM, 55 mM and 35 mM respectively.
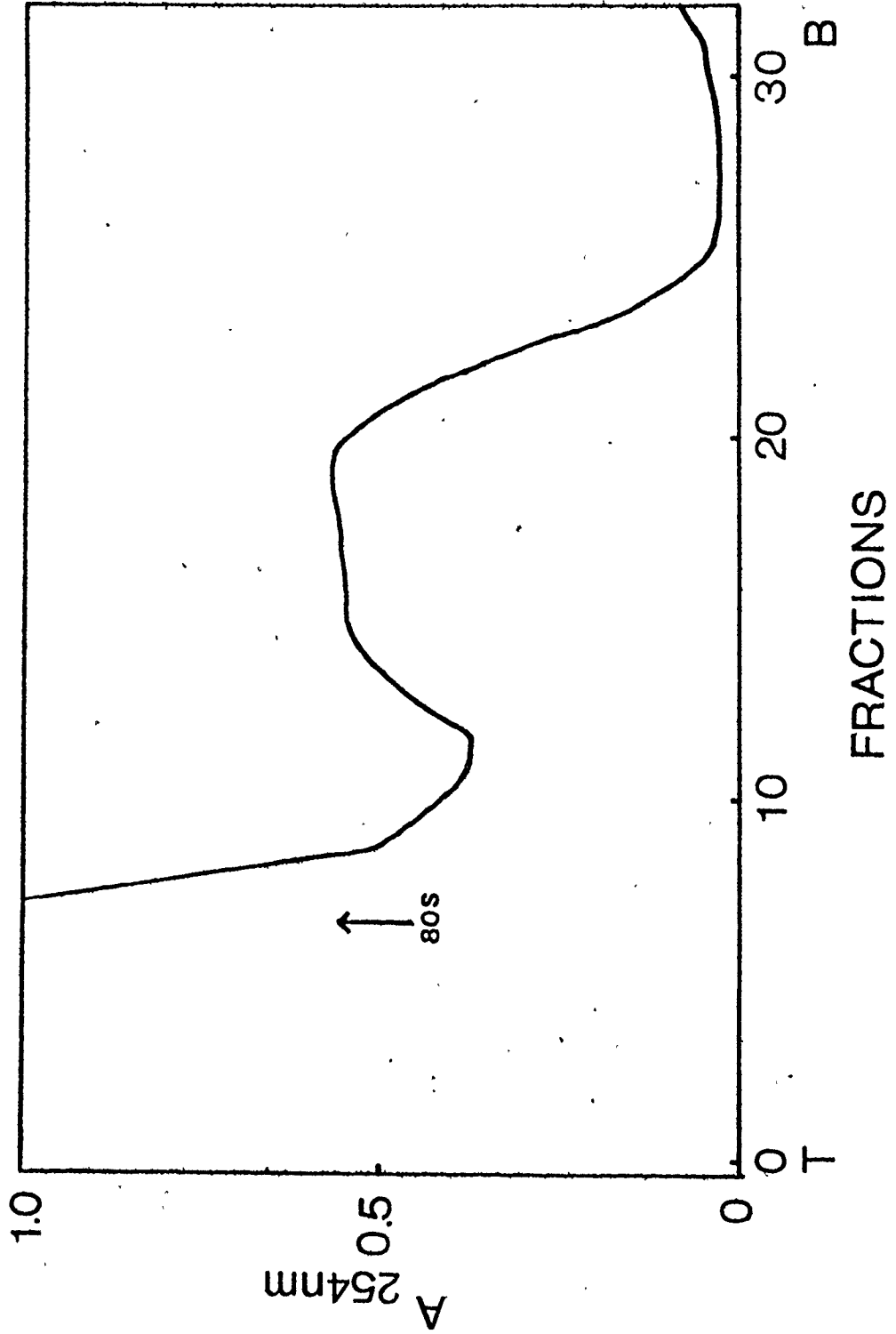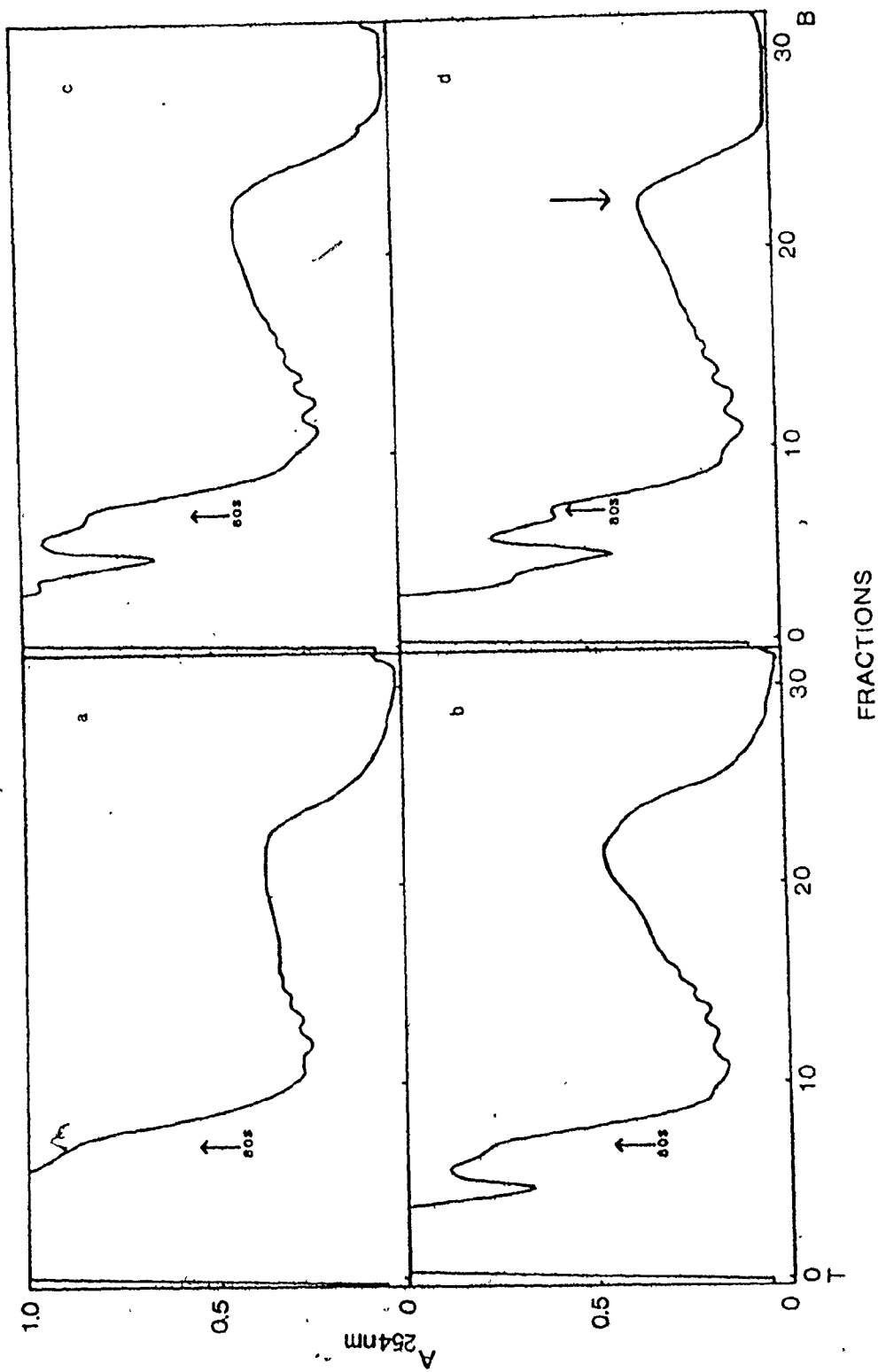
Figure 11. Effect of K^+ on the polysome profile of the female fat body. Fat body from 4 females was homogenized at 4°C in 1.5 ml of 0.2 M Tris-HCl, pH 9.0, containing 35 mM EGTA, 55 mM $MgCl_2$, 0.2 M sucrose, 1% Triton X-100, 7 mM β-mercaptoethanol, 5 μg/ml cycloheximide and concentrations of KCl as indicated: (a) 0 mM; (b) 50 mM; (c) 100 mM; (d) 200 mM; (e) 400 mM; (f) 600 mM. The optimum concentration of K^+ (200 mM) was selected by considering the polysome to monosome ratio, size of polysomes, and the reproducibility of the profile.
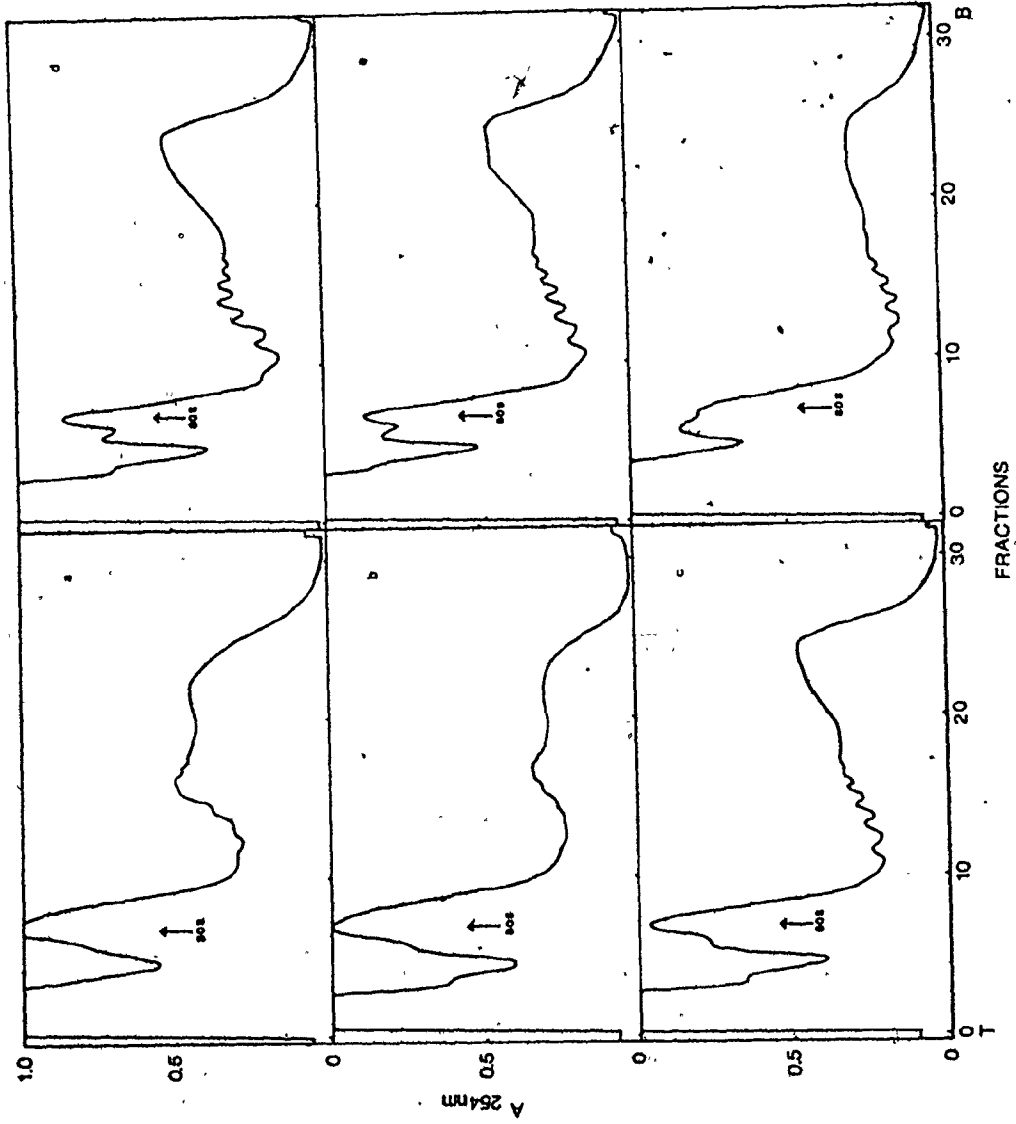
Characterization of Polysomes

Sensitivity to ribonuclease and EDTA treatment

Incubation of the post-mitochondrial fraction with 0.6 µg of
RNase-A from bovine pancreas at 4°C for 20 min resulted in the appear-
ance of a large monosome peak, and the complete elimination of all
polysome peaks present in the control profile (Fig. 12a and b).
Inclusion of EDTA in the post-mitochondrial fraction at a concentration
of 40 mM produced a profile (Fig. 12c) similar to the RNase-treated
profile (Fig. 12b) in which all the polysomes were dissociated into
ribosomal subunits. These results suggest that the fast-sedimenting
material in the polysome profiles did not result from non-specific aggre-
gation of ribosome monomers and small polysomes.

Identification of vitellogenin-synthesizing polysomes by direct immunoprecipitation

Analysis of the polysome profile from fat body of reproductively-
active females revealed the presence of a predominant population of
heavy polysomes at the fast-sedimenting edge of the profile (Fig. 13a)
which was not present in the polysome profiles prepared from the fat
body of immature females (Fig. 13c) or males of any age (Fig. 13b).
The presence of nascent vitellogenin peptides associated with these
female-specific polysomes was analyzed by pulse-labelling of the fat
body with [^{3}H]-leucine, followed by direct immunoprecipitation from
sucrose gradients with vitellin antiserum. A peak of [^{3}H]-leucine

Figure 12. Sensitivity of polysomes to RNase-A and EDTA treatment. Fat body from 4 mature females was homogenized in 1.5 ml of the optimized homogenation buffer (HB: 0.2 M Tris-HCl, pH 9.0, containing 35 mM EGTA, 55 mM $MgCl_2$, 0.4 M KCl, 0.2 M sucrose, 1% Triton X-100, 5 µg/ml cyclo-heximide and 7 mM β-mercaptoethanol). After treatment with RNase-A or EDTA, 0.5 ml of the post-mitochondrial supernatant was analyzed on 15-60% (W/V) linear sucrose gradients (see Materials and Methods). (a) control; (b) RNase-A; (c) EDTA (40 mM).
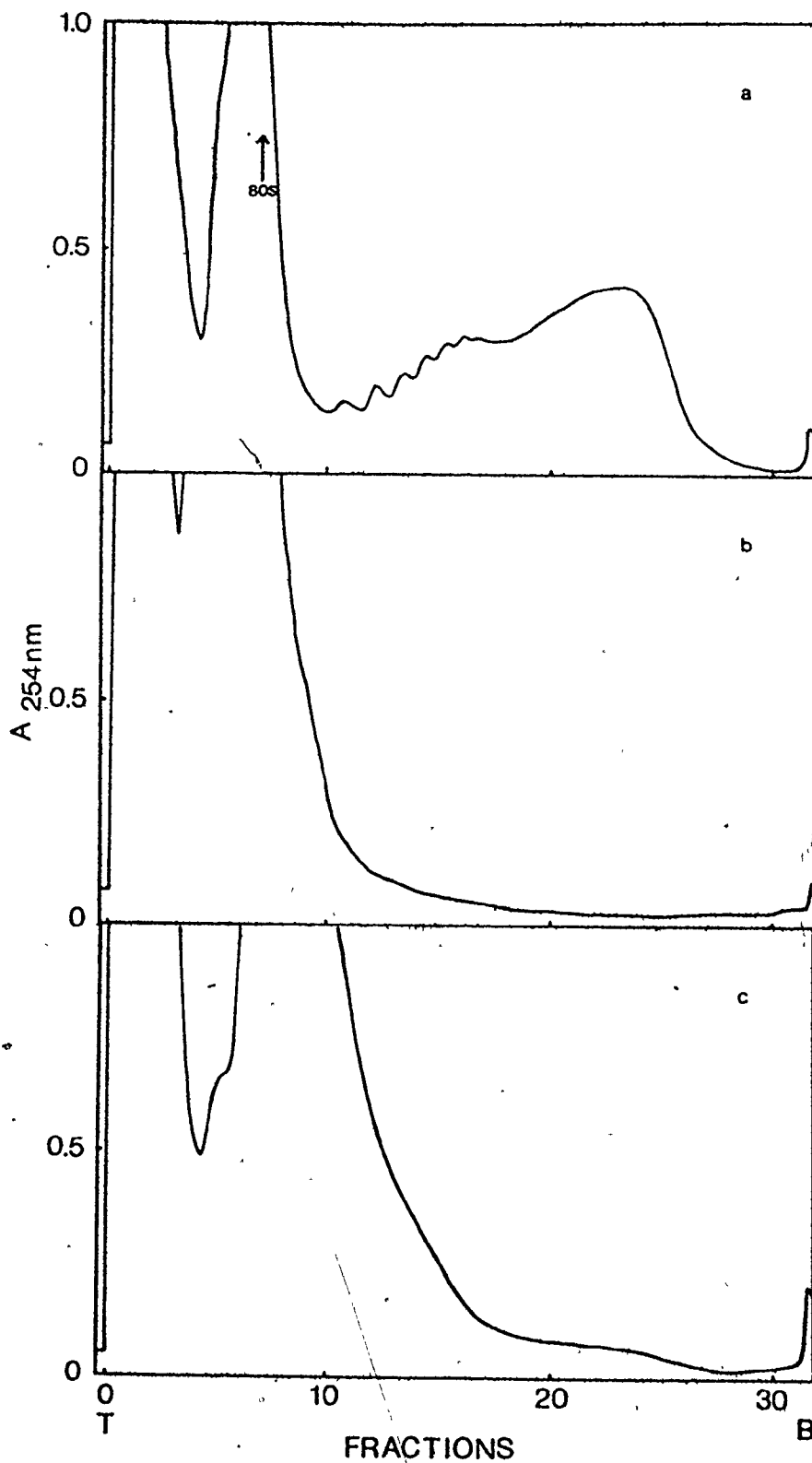
Figure 13. Polysome profiles from fat body of male and female
locusts. Fat body from 4-6 locusts was homogenized
in the optimized HB at 4°C, and 0.5 ml of the post-
mitochondrial supernatant was analyzed on 15-60%
(W/V) linear sucrose gradients. (a) reproductively
active females; (b) reproductively active males;
(c) immature females (2 days after adult ecdysis).
This preparation was centrifuged for 5 hr.

1.0

0.5

0

↑
80S

a

A_{254nm}

0.5

↑
80S

0

b

0.5

80S
↓

c

0

0   T   10   20   30   B
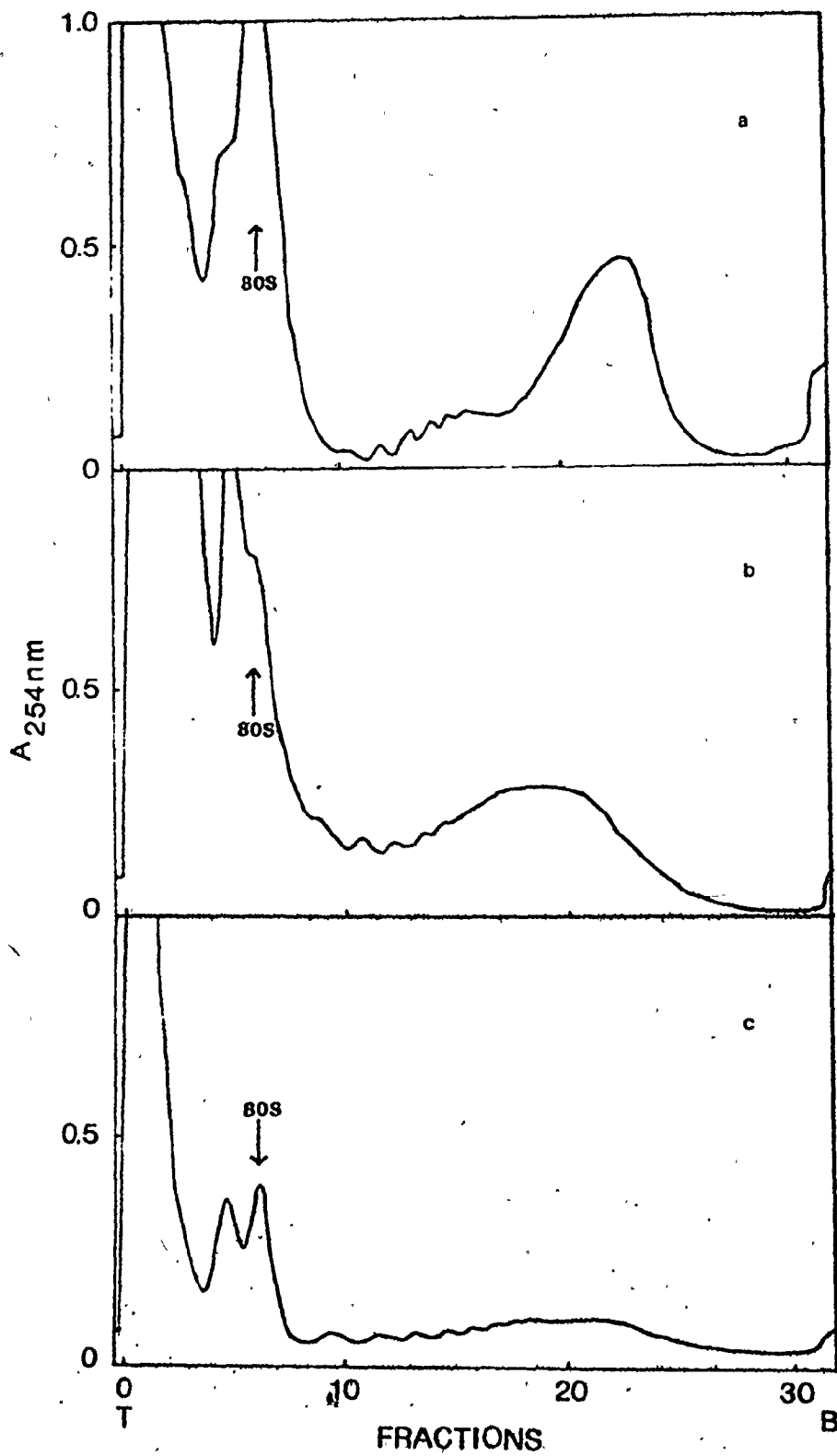
FRACTIONS

labelled vitellogenin nascent peptides was observed, corresponding to the rapidly-sedimenting peak of the polysome profile prepared from the fat body of reproductively active female locusts (Fig. 14a). When sucrose gradient fractions from reproductively active males and/or young female locusts were precipitated with anti-vitellin serum and vitellin, no incorporation of radioactivity into the heavy polysome region was observed (Fig. 14b and c). These results indicate that this female-specific heavy polysome peak contains vitellogenin mRNA. It was also seen in Figure 14a that substantial amounts of the immuno-logically reacting material spread into the region of small polysomes. This could be the result of partial degradation of a portion of the vitellogenin polysomes during preparation.

Identification of vitellogenin-synthesizing polysomes by translation of polysomal RNA in *Xenopus* oocytes

The integrity of the mRNA in vitellogenin polysomes was determined by translation in *Xenopus* oocytes (Fig. 15). Polysomal RNA isolated by the method of chloroform-phenol extraction from six regions of the polysome profile of reproductively active female locusts was micro-injected into *Xenopus* oocytes for translation. Newly-synthesized [^{35}S]-methionine labelled vitellogenin polypeptides were immunoprecipitated with anti-vitellin serum and vitellin, and then analyzed by SDS-poly-acrylamide gel electrophoresis and fluorography. These results showed that RNA isolated from the rapidly sedimenting polysome peak was able to direct the synthesis of two polypeptides which were immunologically

Figure 14. Immuno-characterization of vitellogenin-synthesizing
polysomes. Fat body from 4-6 male or female locusts
was pulse-labelled with [^{3}H]-leucine (40 µCi) for 10
min at 30°C. The labelled vitellogenin nascent poly-
peptides in each fraction were precipitated with anti-
vitellin serum (————), A$_{254}$ nm; (—●—●—) [^{3}H]-
leucine labelled vitellogenin nascent peptides.
(a) reproductively mature females (12 days after adult
ecdysis); (b) reproductively mature males (12 days
after adult ecdysis); (c) immature females (2 days
after adult ecdysis). This preparation was centri-
fuged for 5 hr.

FRACTIONS
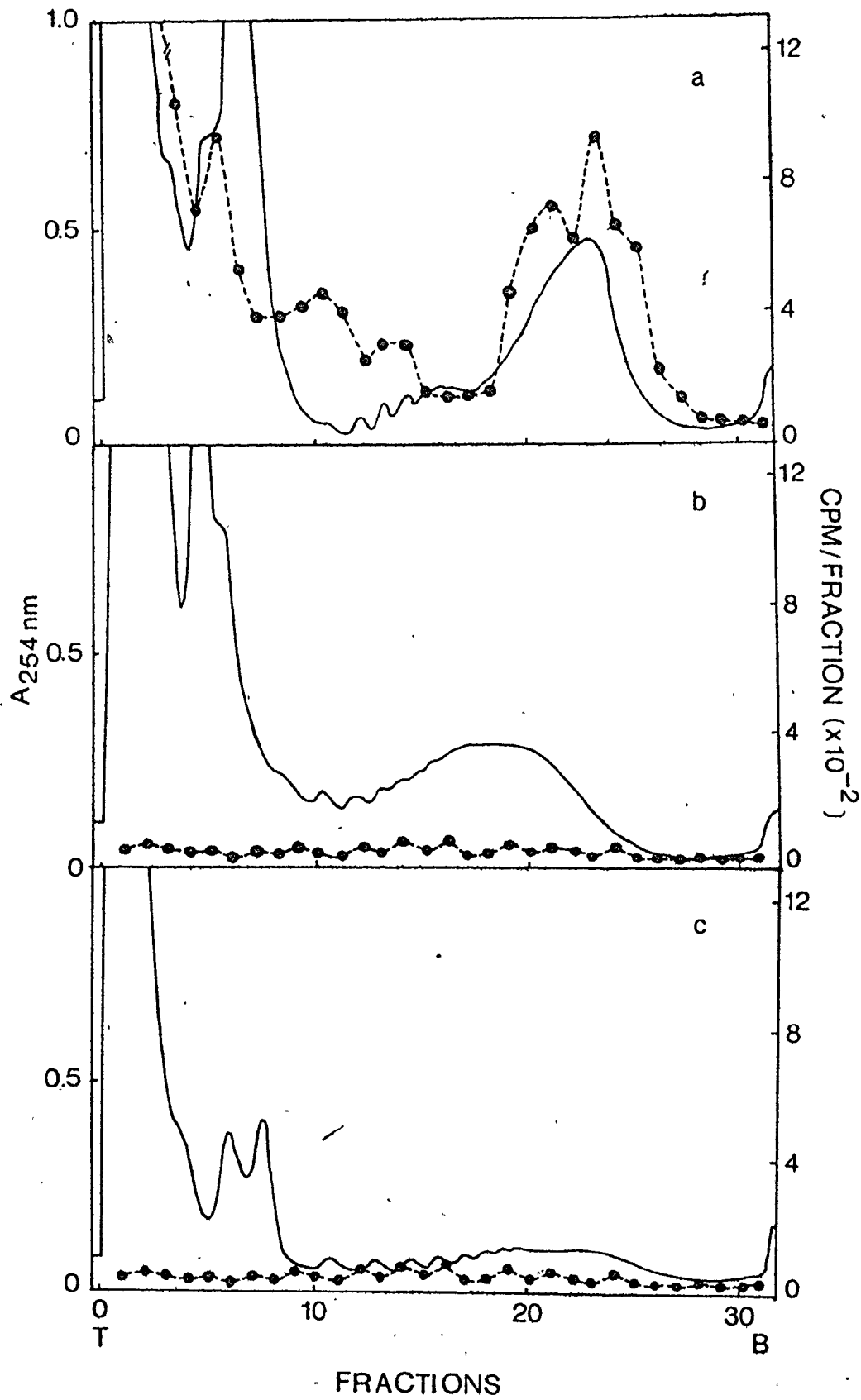
Figure 15. Translation of locust polysomal RNA in *Xenopus* oocytes.

Batches of 30 oocytes each injected with 40-60 nl of

RNA isolated from polysomes of reproductively active

females were labelled with 80 μCi of [^{35}S]-methionine.

The labelled soluble oocyte proteins were precipitated

with anti-vitellin serum, analyzed on 5% SDS-poly-

acrylamide gels and fluorographed according to the

method described by Chen *et al.* (1978). The R_f values

for VG_1 and VG_2 are 0.23 and 0.27, respectively.

Vg₁
Vg₂
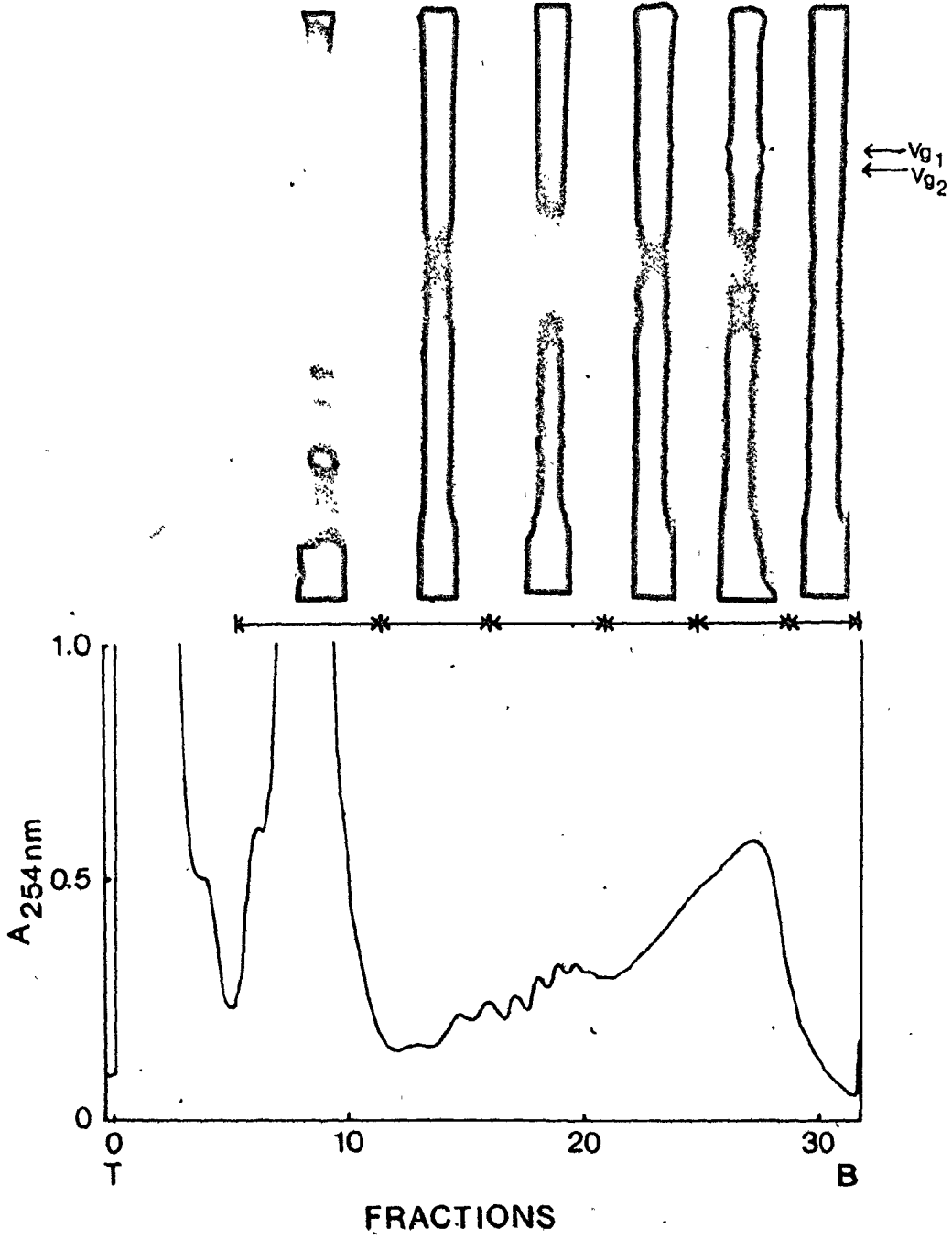
and electrophoretically identical to the authentic unprocessed vitello-
genin polypeptides VG_1 and VG_2 (Chen, 1980). Several lower molecular
weight polypeptides were also observed. These small peptides, which
appear to be similar to some of the intracellularly processed products
of VG_1 and VG_2 in fat body cells (Chen et al., 1976, 1978), indicate
that some processing of these large vitellogenin subunits may also be
occurring in the Xenopus oocytes (Chen, 1980). These results confirm
that intact vitellogenin mRNA can be prepared using this technique from
the fat body of reproductively active female locusts.

Size

Polysomes from the heavy peak were examined by electron micro-
scopy (Fig. 16). The polysomes appear to be large and unaggregated.
Depending on the batch examined, the average number of ribosomes per
molecule of mRNA in the heavy polysome peak is between forty and fifty
(Fig. 16).

Sequential Changes in the Vitellogenin Polysome Content of the Normal Female Fat Body

The sequential changes in the polysome profile of the fat body
tissue from female locusts in their first gonadotropic cycle were
investigated using sucrose gradient centrifugation, since vitellogenin
polysomes can easily be distinguished from other polysomes due to
their high rate of sedimentation. The rate of development of individual

Figure 16. Electron micrograph of polysomes. The horizontal

bar is 0.12 μm.
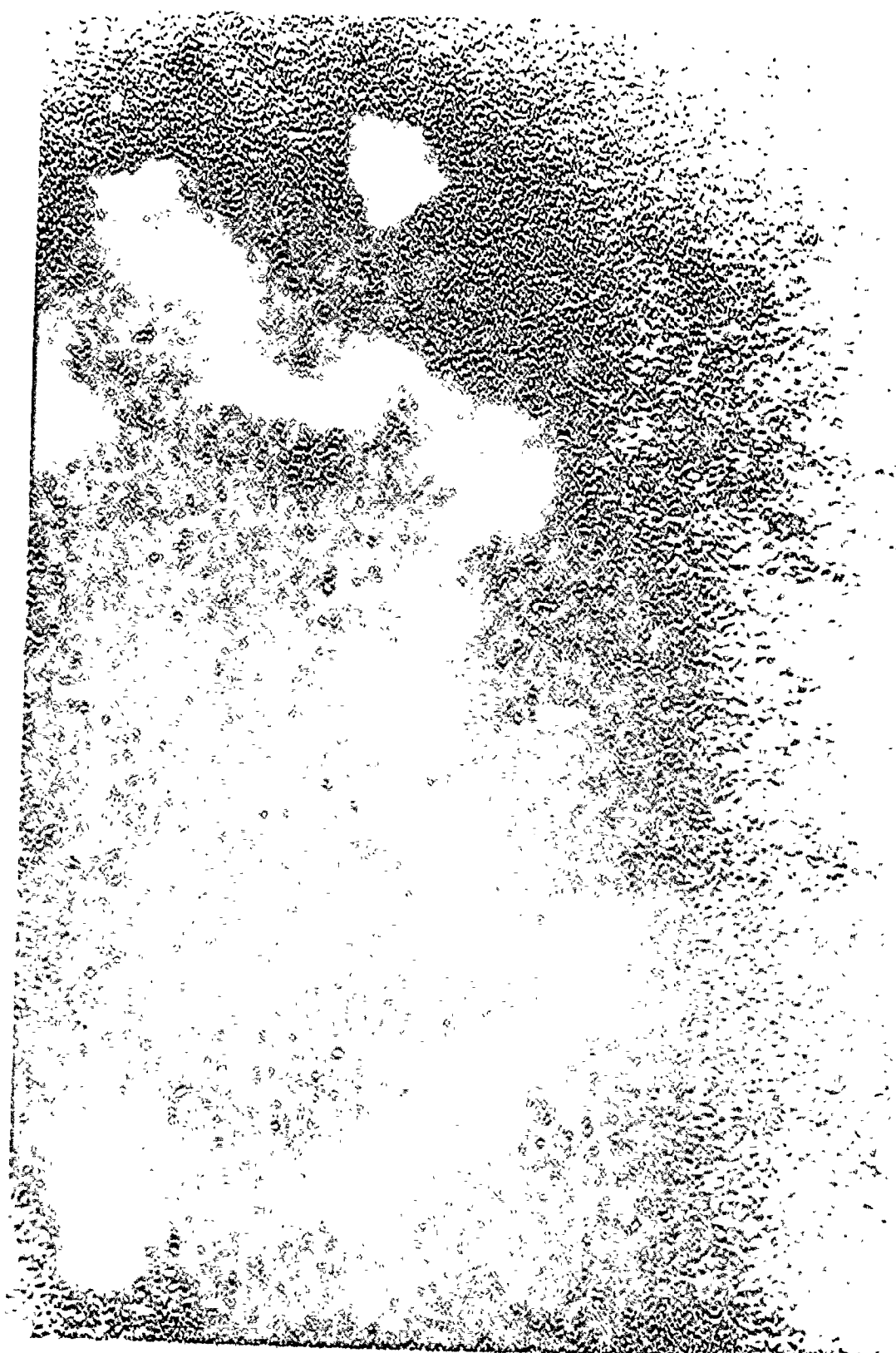
female locusts reared under identical conditions varied to the extent
that age after emergence could not be used as a reliable measure of
maturity. Gellissen and Emmerich (1978), however, showed that oocyte
length is correlated with developmental state. Therefore in the
following experiments terminal oocyte length was used as an index of
maturity. At each developmental stage fat bodies from four females
were used in the analysis of the vitellogenin polysome content, with
representative profiles appearing in Figs. 17a-f.

Newly-emerged females characteristically contained terminal
oocytes measuring less than 1 mm in length. The oocytes then increased
to their maximum length of 7 mm just before oviposition. From day 0
to 6 after adult ecdysis (terminal oocytes less than 2 mm) vitellogenin-
synthesizing polysomes were absent from the polysome pattern (Fig. 17a).
The amount of absorbance in the ribosomal subunit peaks (40S and 60S),
the monosome peak (80S) and the entire polysome region was low, indi-
cating that little protein synthetic activity was occurring in the fat
body. At an oocyte length of 2 mm the appearance of a small absorbance
peak in the large polysome region marked the first detectable indi-
cation of the presence of vitellogenin-containing polysomes (Fig. 17b).
Accompanying this event there was a large increase in the amount of
absorbance in the monosome peak and a general increase in the peaks
in the small polysome region. This represents a massive accumulation
of ribosomes and a significant increase in the level of protein
synthesis in the fat body. Further growth of the oocytes (length 3 mm)
was accompanied by a significant decrease in the amount of absorbance

Figure 17. Changes in vitellogenin polysome contents of adult
female *L. migratoria* at different stages of develop-
ment. Fat body from 4 locusts at different stages
of development was homogenized in 1.5 ml of the
optimized HB_d and 0.5 ml of the post-mitochondrial
fraction was analyzed on 15-60% (W/V) linear sucrose
gradients. The centre of the vitellogenin polysome
peak in each gradient is indicated by an arrow.
The length of the terminal oocyte was used as an
indicator to judge the stages of development. (a)
oocyte length smaller than 1 mm; (b) oocyte length
2 mm; (c) oocyte length 3 mm; (d) oocyte length 4 mm;
(e) oocyte length 5-6 mm; (f) oocyte length 7 mm.

FRACTIONS
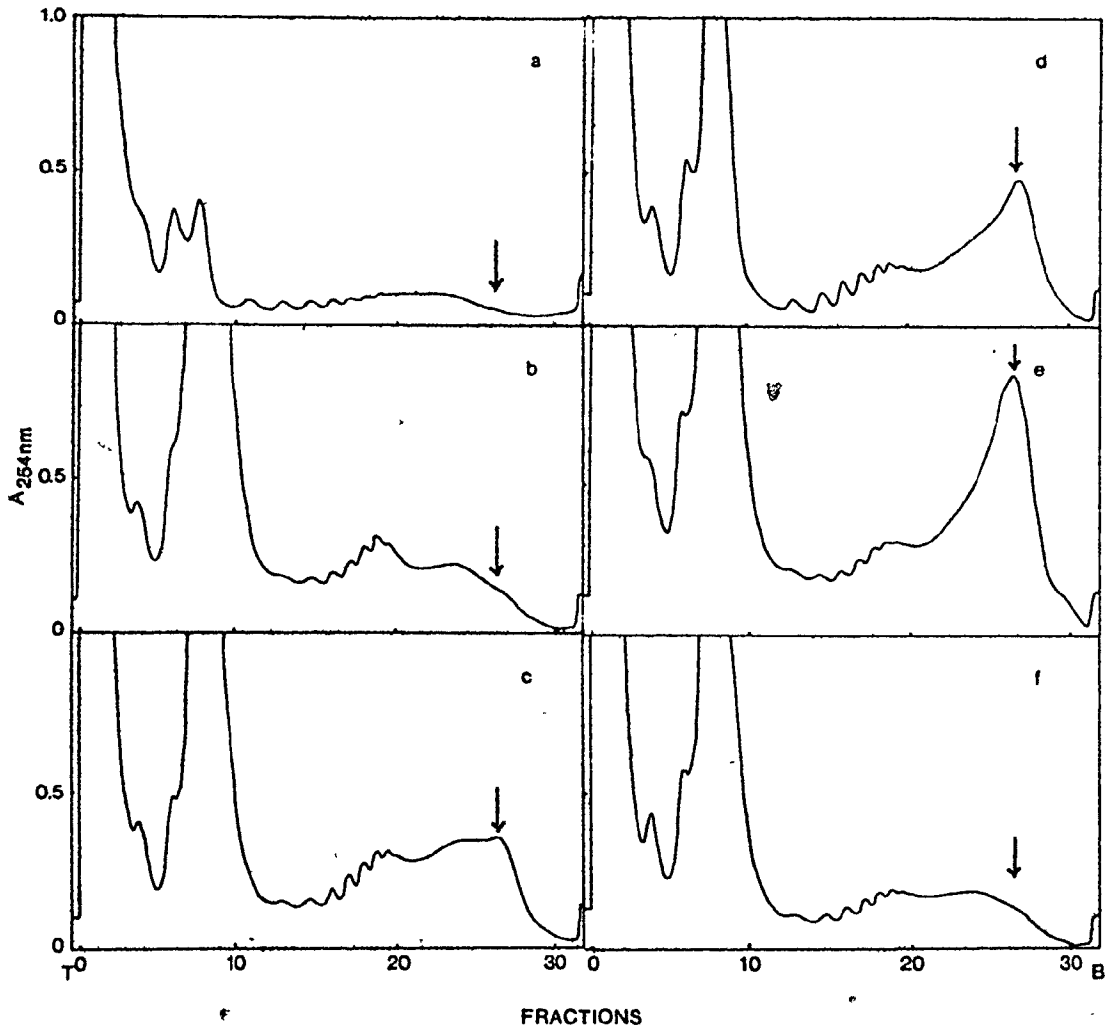
in the monosome peak, and a corresponding increase in the polysome peak containing vitellogenin-synthesizing polysomes (Fig. 17c). This was followed by the continued accumulation of vitellogenin mRNA-containing polysomes, without a significant decrease in the monosome peak (Fig. 17d). The maximum accumulation of vigellogenin polysomes occurred at an oocyte length of 5 to 6 mm (Fig. 17e). At maturity (oocyte length 7 mm) the vitellogenin-synthesizing polysome peak decreased to an undetectable level, while a large monosome peak remained (Fig. 17f). The profile of fully mature female locust fat body was similar to that of the immature female just prior to the onset of the rapid formation of vitellogenin-containing polysomes. From many repeated experiments it was concluded that the observed change in the content of vitellogenin polysomes in the fat body of female locusts was characteristic of the developmental stage and did not result from variation among individual animals.

Induction of Vitellogenin mRNA-containing Polysomes by an Active Juvenile Hormone Analog, ZR-515

It was reported by Chen *et al.* (1976, 1979) that the synthesis of vitellogenin in the locust can be prevented by allatectomy, and restored by treatment with juvenile hormone or an active synthetic analog, ZR-515. To study the effect of JH on the formation of heavy polysomes, allatectomized female locusts were injected with 300 µg of SR-515 dissolved in olive oil. Three days after hormone treatment polysomes from the fat body of females were prepared using sucrose

Figure 18. Polysomes induced by ZR-515 in allatectomized female

locusts. Fat body from 4-6 allatectomized and ZR-515

treated females (300 µg/animal; duration of treatment

72 hr) was homogenized in the optimized HB, and 0.5 ml

of the post-mitochondrial supernatant was analyzed

on 15-60% (W/V) linear sucrose gradients. (a)

reproductively active female; (b) allatectomized female

treated with ZR-515; (c) allatectomized female treated

with olive oil.
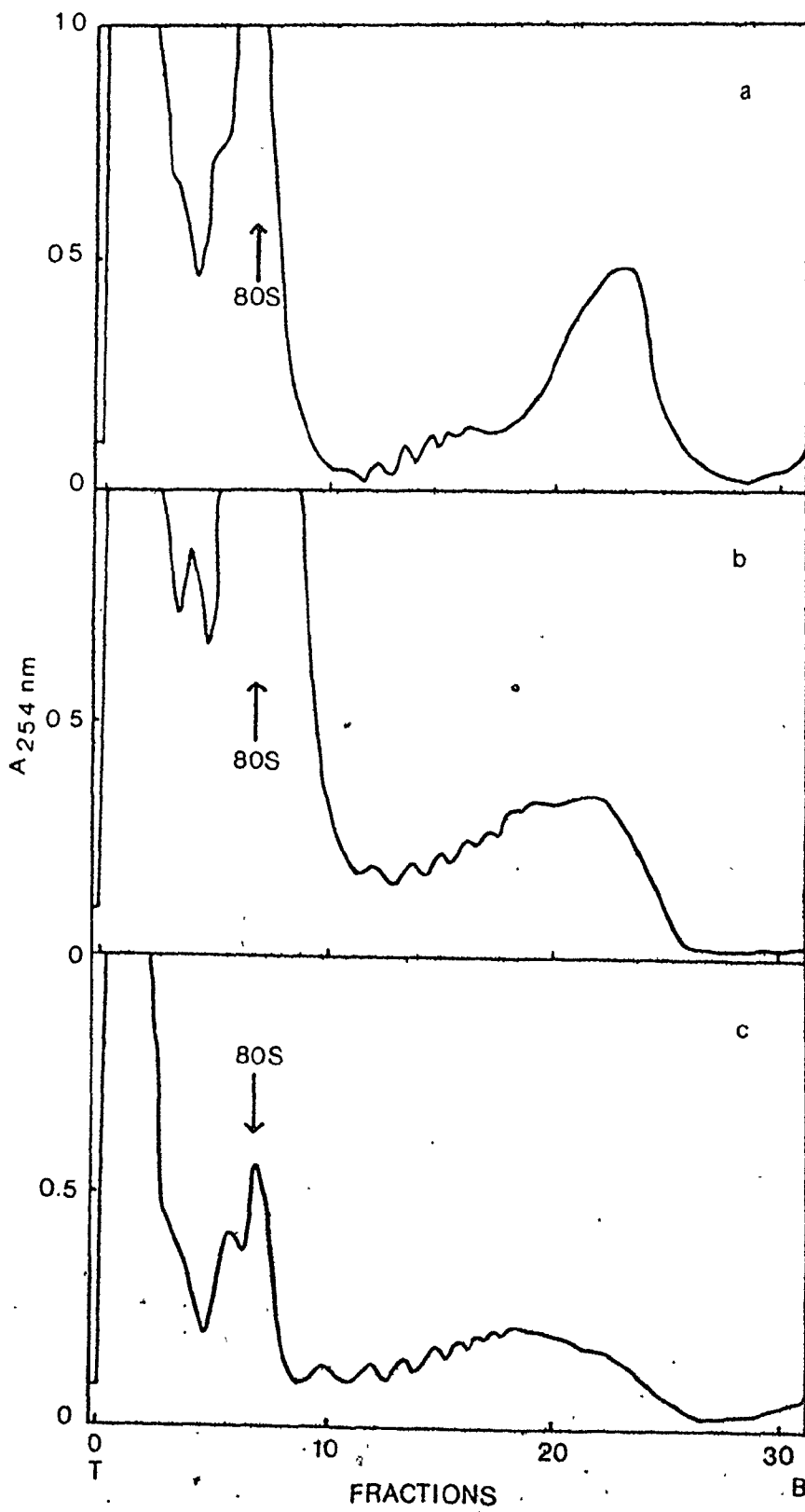
gradients and the results are presented in Figures 18a-c. Allatectomy prevented the formation of female-specific polysomes which are normally found in the fat body of reproductively active females (Figs. 18a and c), whereas application of 300 µg/animal of ZR-515 restored the formation of the female-specific polysomes (Fig. 18b). These hormone-induced female-specific polysomes were found to contain vitellogenin mRNA since a peak of [^{3}H]-leucine labelled vitellogenin-nascent peptides was found associated with the heavy polysome fractions (Figs. 19a-c).

In their studies on the *in vivo* induction of vitellogenin synthesis in allatectomized female locusts Chen *et al.* (1979) showed that a single treatment of these females with 250 µg/animal of ZR-515 resulted in a slow rise in the rate of vitellogenin synthesis for the first 48 hr, followed by a rapid increase in the vitellogenin production to a maximum at 72 hr. Application of a second dose of ZR-515 produced a rapid rise in rate of vitellogenin production after a shorter lag, and reached a maximum within 48 hr. The nature of these differences was investigated by studying the sequential changes in the vitellogenin polysome content of the fat body following primary and secondary stimulation with ZR-515 (300 µg/animal each dose). Within 48 hr after ZR-515 treatment, the content of ribosome monomers and light weight polysomes increased substantially, mainly due to increased ribosome synthesis (Figs. 20a-c). By 72 hr the character-istic peak of vitellogenin-synthesizing polysomes had reached its maximum, and then fell to near zero levels within the next 24 hr (Figs. 20d-f). When a second dose of ZR-515 (300 µg/animal) was applied to

Figure 19. Vitellogenin polysomes induced by ZR-515 in alla-
            tectomized female locusts. Fat body from 4-6 alla-
            tectomized and ZR-515 treated females (300 µg/animal;
            duration of treatment 72 hr) was pulse-labelled with
            40 µCi of [^{3}H]-leucine in 2 ml of the incubation
            medium for 10 min at 30°C. Labelled vitellogenin
            nascent peptides associated with polysomes were pre-
            cipitated with specific anti-vitellin serum. (———),
            A_{254} nm (—•—•—), vitellogenin nascent peptides.
            (a) reproductively active female; (b) allatectomized
            female treated with ZR-515; (c) allatectomized female
            treated with olive oil.

FRACTIONS
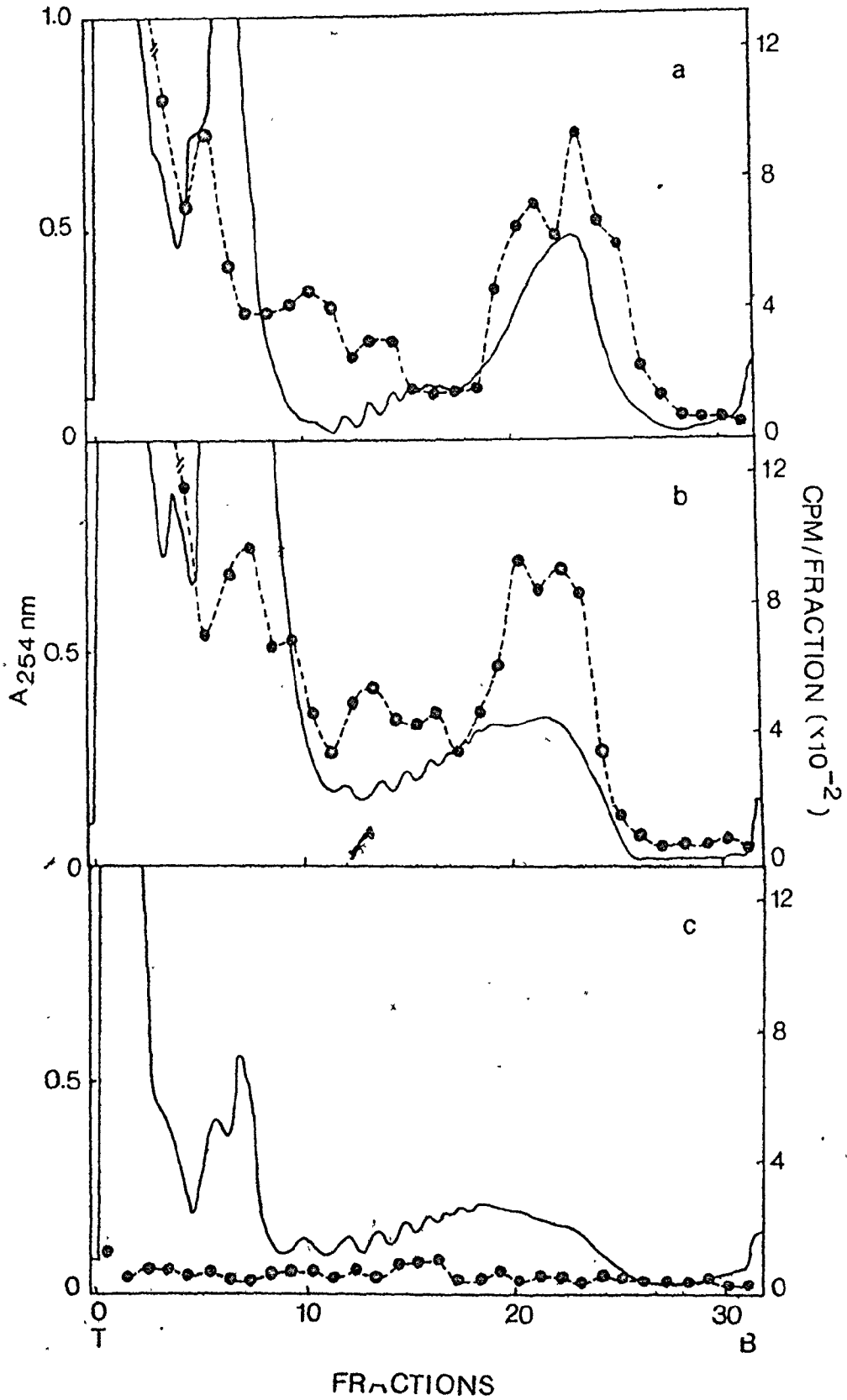
Figure 20: Time course of the appearance of vitellogenin-
synthesizing polysomes during primary inducation.
ZR-515 (300 μg/animal in olive oil) was injected
into allatectomized females, and the polysome
profiles were prepared from 4 fat bodies at the
times indicated. (a) 0 hr; (b) 24 hr; (c) 48 hr;
(d) 72 hr; (e) 96 hr; (f) 144 hr. The centre of
the vitellogenin polysome peak is indicated with
an arrow.

FRACTIONS
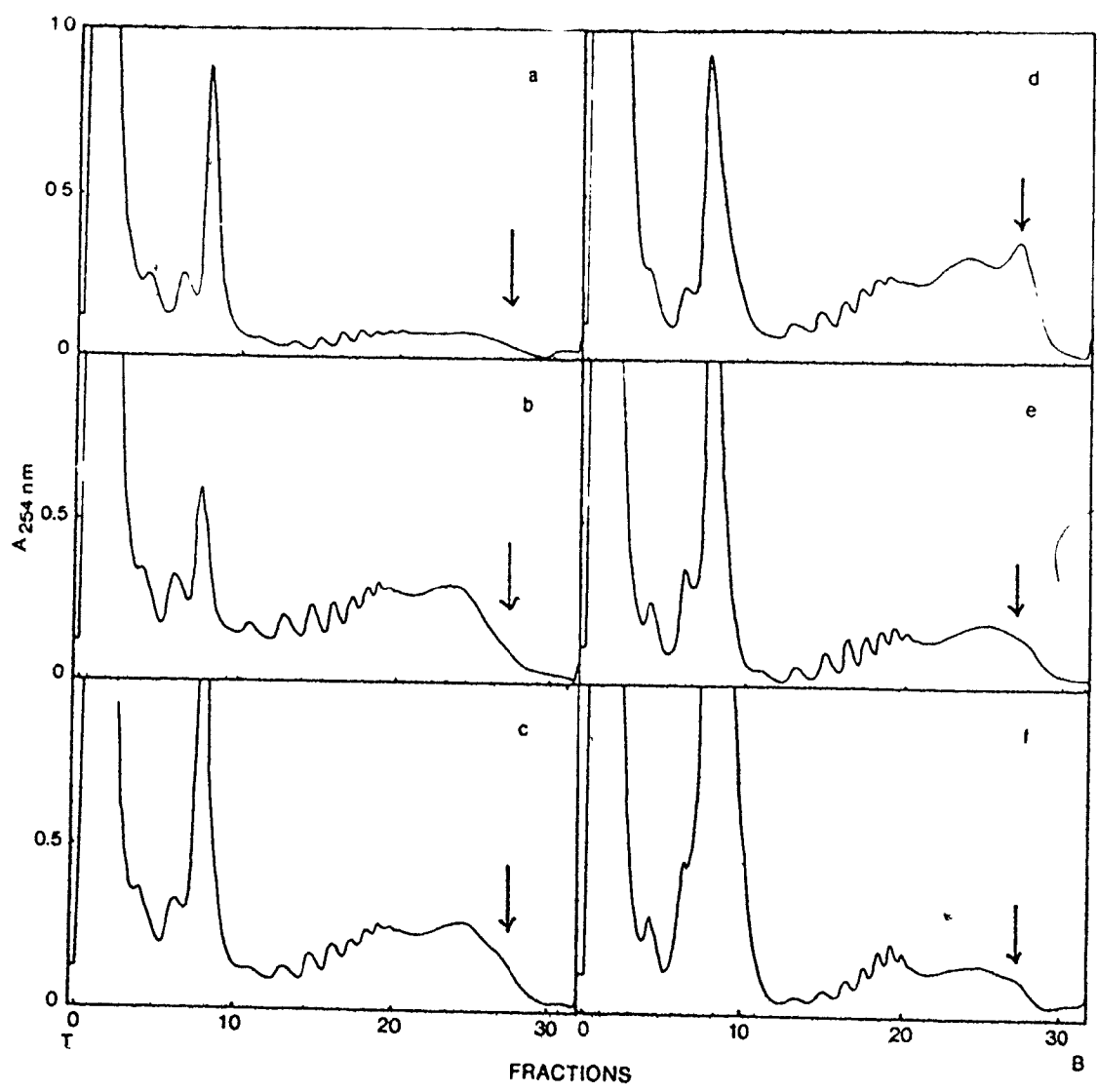
animals 14 days after the primary stimulation, it resulted in an earlier appearance as well as a greater increase in the vitellogenin polysome content of the fat body (Figs. 21a-d).

Figure 21. Time course of the appearance of vitellogenin-

synthesizing polysomes during secondary induction.

Fourteen days after the primary dose of ZR-515

(300 μg/animal) each animal was treated with the

same amount of ZR-515. At different times after

treatment 4 fat bodies were used for polysome

preparation. (a) 0 hr; (b) 24 hr; (c) 48 hr;

(d) 72 hr. The centre of the vitellogenin poly-

some peak is indicated with an arrow.
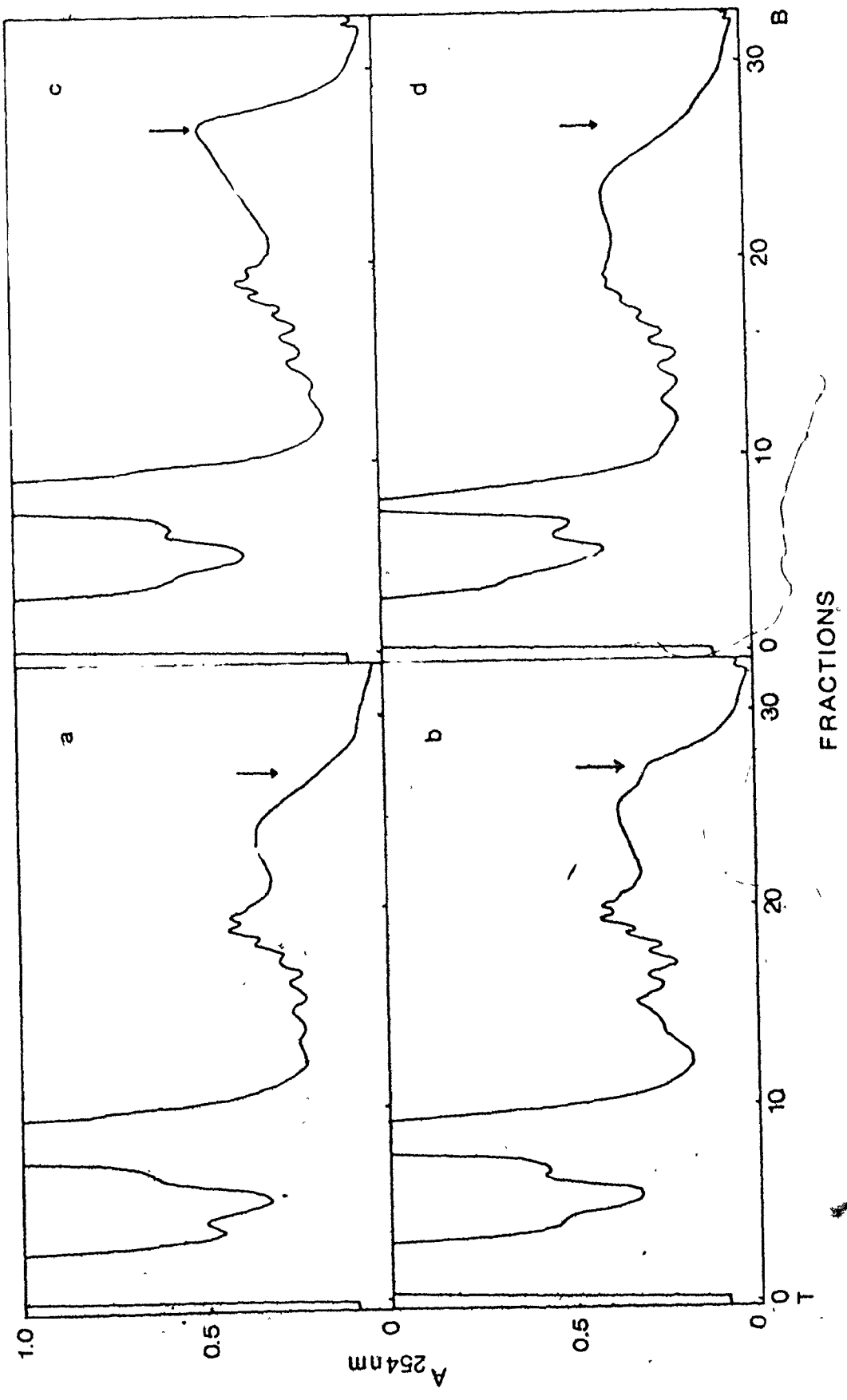
DISCUSSION

The juvenile hormone-induced synthesis of vitellogenin in locusts provides an opportunity for analyzing the primary response of a tissue to a hormone in a system where cellular proliferation is not involved. Also, a sexual dimorphism exists in locusts which serves to prevent vitellogenin induction in males. This phenomenon may be important for elucidating the control mechanism of differential gene expression during development and differentiation. Finally, vitellogenin is synthesized in large amounts by a well-characterized and homogeneous population of cells in response to a clearly-defined hormonal stimulus *in vitro*. Therefore the locust fat body system is a readily analyzable system well suited for the study of regulated gene expression.

Definition of conditions for the isolation of intact polysomes may facilitate the study of vitellogenin production with respect to the importance of post-translational modifications for secretion and may also serve as a rich source for the isolation of vitellogenin mRNA. Vitellogenin is a multicomponent precursor protein which is chemically modified before it is secreted into the hemolymph. Immunological and histochemical analysis of the vitellogenin polysomes may provide some insight into the actual processing steps which occur during the production of this precursor protein (Tata, 1973; Shore and Tata, 1977; Tata and Smith, 1979).

Isolation of vitellogenin mRNA is a prerequisite for the quantitative analysis of the early molecular events involved in the induction of vitellogenin synthesis in the fat body of the locust by juvenile hormone. It is necessary to determine the accumulation of specific vitellogenin mRNA sequences during hormone stimulation in order to distinguish between possible transcriptional or translational control mechanisms. This information has been obtained for other hormone-induced proteins through the use of cell-free systems for translation and by DNA-RNA hybridization studies with the corresponding complementary DNA probe (see reviews: Palmiter, 1975; Ryffel, 1978; O'Malley et al., 1979; Tata and Smith, 1979; Westley, 1979). However, the sensitivity of the immunological assay for vitellogenin synthesis has been shown to be somewhat unreliable unless the immunoprecipitable translation products are critically evaluated (Mullinix et al., 1976; Burns et al., 1978). More recently the use of translational analysis as a quantitative tool in the estimation of the number of mRNA sequences has been superseded by cDNA hybridization analysis (see reviews: Dawid and Wahli, 1979; O'Malley et al., 1979; Tata and Smith, 1979). The conventional method for the preparation of a specific cDNA probe involves the use of a purified mRNA as a template for transcription using reverse transcriptase from avian myeloblastosis virus (Monahan et al., 1976). A specific mRNA can easily be prepared from the corresponding mRNA-containing polysomes isolated by immunoprecipitation (Ab et al., 1976; Jost and Pehling, 1976a; Jost et al., 1978).

In this study, a technique for the preparation of vitellogenin
polysomes from which vitellogenin mRNA can be isolated was developed.
The large sizes of the two locust vitellogenin primary subunit poly-
peptides (235,000 and 225,000 daltons; Chen *et al.*, 1980) suggest that
vitellogenin polysomes will be part of, if not the heaviest polysomes
present in the fat body cells. This has been found to be true for
avian and amphibian vitellogenin polysomes which sediment with an
S-value of 220 (Tata and Smith, 1979). At the peak of vitellogenin
synthesis in locusts these proteins account for at least 60% of the
total fat body protein output (Chen *et al.*, 1976, 1978). Vitellogenin
polysomes then should be the predominant species of heavy polysomes
present in the fat body of reproductively active females. In avian
and amphibian liver, after tissue homogenization, high levels of
ribonucleases become exposed to polysomes and cause their degradation.
In these systems, however, control of ribonuclease activity has been
accomplished by homogenization of tissue at low temperature (4°C) in
a buffer of high pH and high ionic strength which includes various
ribonuclease inhibitors; heparin, diethylpyrocarbonate, β-mercapto-
ethanol or cycloheximide (Berridge *et al.*, 1976). Using conditions
established by Berridge *et al.* (1976) I could not prepare heavy poly-
somes from the fat body of locusts. However, Engelmann (1977) by
including the Ca^{2+} chelator (EGTA) in the homogenization buffer
succeeded in preparing heavy polysomes from the fat body of vitellogenic
female cockroaches, *Leucophaea maderae*. By supplementing the homogeni-
zation and the sucrose gradient buffers in this study with EGTA, I

was also able to prepare large polysomes from the fat body of reproductively active female locusts. These polysomes were shown to be sensitive to EDTA and RNase-A treatment. The above results indicate that insect fat body may contain Ca^{2+}-dependent ribonucleases.

In order to optimize the recovery of vitellogenin polysomes, conditions that favour the yield of large polysomes were determined. The concentrations of EGTA, Mg^{2+} and K^+ in the homogenization buffer are the factors most likely to influence the integrity of large polysomes (Palmiter, 1974). A systematic evaluation of the effects of different concentrations of these components was then undertaken.

The presence of EGTA in the homogenization buffer was found to be essential for the isolation of heavy polysomes; without EGTA variable degradation of the polysomes occurred. Inclusion of even a low concentration (15 mM) of EGTA was found to improve both the reliability and the yield of heavy polysomes, with the maximum yield of large polysomes being obtained at an EGTA concentration of 35 mM. Presumably this represents the necessary concentration of EGTA needed to minimize the Ca^{2+}-dependent RNase activity present in the fat body.

Mg^{2+} is known to be essential for the maintenance of the association of ribosomal subunits (Vignais *et al.*, 1972; Sperrazza *et al.*, 1980) and small changes in Mg^{2+} concentration were found to have significant effects on polysome integrity. A concentration of 55 mM Mg^{2+} was shown to give profiles with a large peak of fast-sedimenting polysomes. While EGTA chelates both Ca^{2+} and Mg^{2+}, the Ca^{2+}-EGTA complex (log K = 10.7) is significantly more stable than

the Mg^{2+}-EGTA complex (log K = 5.4) (Laitinen, 1960). This difference in the formation constants indicates that Ca^{2+} will be selectively and irreversibly complexed in the homogenate. However since some Mg^{2+} will also be bound, the actual concentration of Mg^{2+} in the optimized homogenization buffer (55 mM) will be reduced by the uncomplexed EGTA. Considering this, the optimized concentration of Mg^{2+} in the homogenization buffer must be between 20-55 mM, which is similar to those values (5-50 mM) reported by other investigators in a variety of plant and animal systems (Verger, 1972; Davies et al., 1972; Gelinas and Kafatos, 1973; Berridge et al., 1976; Jost and Pehling, 1976b; Jost et al., 1978). At these concentrations, Mg^{2+} might also be inhibiting RNase activity in this system (Gribnau et al., 1970). A decreased yield of large polysomes at higher concentrations of Mg^{2+} in this study may be caused by Mg^{2+} precipitation of polysomes. Palmiter (1974) has reported a procedure for the effective precipitation of all ribosomes and polysomes from a crude homogenate with the addition of high concentrations of Mg^{2+} (60-100 mM).

Over a wide range of concentrations of K^+ (100-400 mM) a high recovery of heavy polysomes was possible. These concentrations of K^+ correspond to the monovalent cation range reported by Palmiter (1974) which prevents the precipitation of ribosomes and polysomes by Mg^{2+}, since high concentrations of K^+ compete effectively with Mg^{2+} for ribosome binding sites (Petermann and Pavelovec, 1967). At these optimized concentrations basic proteins such as ribonucleases may be removed from the ribosomes; therefore degradation of polysomes may

be reduced (Davies *et al.*, 1972). An accumulation of small polysomes· and a loss of heavy polysomes were observed at low concentrations of K^+ (0-50 mM). This may be due to nonspecific aggregation of ribosome monomers, or to partial degradation of the large polysomes which have been reported to be more sensitive to ribonuclease activity than small polysomes (Palmiter, 1974) or to the selective precipitation of large polysomes which would be lost in the preparation of the post-mitochondrial supernatant. At a very high concentration of K^+ (600 mM), the yield of heavy polysomes is again reduced. This concentration of K^+ could cause the ribosomes to dissociate for it is a strong contributor to the ionic strength of the homogenization buffer (Sperrazza *et al.*, 1980).

Since the heavy polysomes of reproductive females were prepared in a buffer containing 55 mM Mg^{2+}, it is possible that they could represent an aggregation of small polysomes. However, this is very unlikely, since, on the one hand, mature male fat body or that of immature or allatectomized females treated under identical conditions did not give this size class of polysomes; on the other hand, the use of lower concentrations of Mg^{2+} (40-45 mM) did not affect the amount of absorbance of the small polysomes substantially. Therefore the concentrations of EGTA (35 mM), Mg^{2+} (55 mM) and K^+ (200 mM) in the homogenization buffer were found to give the maximum yield of polysomes in this system. Under these conditions a unique class of large polysomes appears to be characteristic of the fat body from reproductively active female locusts.

Large vitellogenin polysomes containing 40-60 ribosomes have been isolated and identified in the liver of rooster (Roskam *et al.*, 1976a,b) and *Xenopus* (Berridge *et al.*, 1976) by immunochemical methods. In these two vertebrates the molecular weights of the primary translation products from these polysomes appear to be in the order of 200,000. In locusts, the female-specific heavy polysomes were shown to contain vitellogenin mRNA by immunoprecipitation of associated nascent polypeptides with anti-vitellin antiserum, and by electron microscopy to be large polysomes with 40-50 ribosomes. Since the translation products directed by locust polysomal RNA in *Xenopus* oocytes were indistinguishable from those of poly(A)-containing RNA or authentic VG_1 and VG_2 produced in intact fat body cells (Chen, 1980), the vitellogenin polysomes that were prepared in this study must be intact. Furthermore, the unique polysome profile of reproductively active females indicates that this tissue is primarily making vitellogenin during reproduction. Therefore, the fat body of reproductively mature females can serve as a ready source for the isolation of vitellogenin mRNA.

A characteristic feature of all developmental systems under hormonal control is the marked enhancement of overall protein synthetic capacity (Tata, 1970). In their studies on the major biochemical events occurring in the fat body of adult female locusts during the first gonadotropic cycle, Chen *et al.* (1976, 1979) reported that the rate of total RNA synthesis increased steadily during the period of maturation to a maximum at about the time of initiation of vitellogenin

synthesis (day 7-8 after emergence) and then declined thereafter.
These biosynthetic processes are accompanied by cytological changes
including the massive accumulation of ribosomes, followed by prolifera-
tion of the rough endoplasmic reticulum and the Golgi complex (Couble
et al., 1979). By quantitative measurement of a presumptive vitello-
genin mRNA band on a denaturing agarose gel, Chinzei et al. (1980)
found that the total RNA in the fat body increased in the early part
of the gonadotropic cycle, followed by vitellogenin mRNA accumulation,
and that both reached maxima at 4-5 mm oocyte length when vitello-
genin synthesis was at its peak. During the rising phase, there is
generally a good correlation between vitellogenin mRNA level and the
rate of vitellogenin synthesis. In the second part of the cycle,
while vitellogenin production is declining, total RNA decreases but
not to its previtellogenic level. Furthermore the amount of vitello-
genin mRNA decreases only slightly, remaining quite high at 7 mm
oocyte length. The correlation between mRNA and vitellogenin synthesis
thus appears to break down at this stage.

In this study the participation of vitellogenin mRNA and
ribosomes in protein synthesis during the first vitellogenin cycle
was investigated by following the occurrence of vitellogenin-specific
polysomes. At 2 mm oocyte length, there was an obvious accumulation
of ribosome monomers and a trace of vitellogenin polysomes. The
vitellogenin polysomes reached maximal amounts at about 5 mm oocyte
length, and by 7 mm had disappeared. The disappearance of vitellogenin
polysomes correlates with the cessation of vitellogenin production

reported by Chen *et al.* (1979). The persistance of vitellogenin mRNA at this stage, however, suggests that this message may be preserved under some form of translational inhibition. Further investigation of these events must be undertaken in order to verify the results obtained.

The temporal sequence of molecular events in vitellogenin gene expression can be followed with more precision in fat bodies from locusts that have been treated with juvenile hormone or JH active analog (ZR-515) after allatectomy (Chen *et al.*, 1979). As reported by Chen *et al.* (1979) after a single treatment with ZR-515 the rate of vitellogenin synthesis rose slowly for the first 48 hr, then steeply to a maximum at 72 hr and was followed by a sharp drop. After the decay of the primary response a second application of ZR-515 produced a rapid rise in vitellogenin synthesis with a very short lag period. The cytological picture, studied with the electron microscope, showed a rapid build-up of rough endoplasmic reticulum during the first two days after hormone treatment, followed by the appearance of arrays of packed ribosome-studded membranes that are believed to be inactive in protein synthesis (Couble *et al.*, 1979).

Similar primary and secondary induction events have been reported for the estrogen-induced synthesis of vitellogenin in the liver of rooster (Bergink *et al.*, 1973; Deeley *et al.*, 1977a,b) and *X. laevis* (Green and Tata, 1976; Baker and Shapiro, 1977, 1978; Ryffel *et al.*, 1977; Ryffel, 1978), and for ovalbumin in chick oviduct (Palmiter *et al.*, 1976). In avian and amphibian liver the induction of vitellogenin

synthesis is also characterized by a massive initial accumulation
of total RNA (Jost *et al.*, 1973; Tata and Baker, 1975; Lewis. *et al.*,
1976), which presumably is ribosomal RNA (Wittliff and Kenney, 1972;
Lewis *et al.*, 1976; Bast *et al.*, 1978). Cytologically a massive
proliferation of the membranes of the endoplasmic reticulum and Golgi
complex also occurs (Lewis *et al.*,1976). By the use of vitellogenin
cDNA as a probe for the quantitative measurement of the level of
vitellogenin mRNA in rooster or *Xenopus* during primary and secondary
stimulation by estradiol, it was observed that the lag phase of
vitellogenin mRNA accumulation in the secondary induction was shorter
than that of the primary induction (Deeley *et al.*, 1977a,b; Baker and
Shapiro, 1978). These results are in agreement with those observed
in locusts. However using a cell-free translation system derived from
a reticulocyte lysate to quantify the level of vitellogenin mRNA,
Farmer *et al.* (1978) recently reported conflicting data showing very
similar increases in vitellogenin mRNA appearance during primary and
secondary induction in *Xenopus*. These authors suggested that the
long lag phase in the induction of vitellogenin synthesis after primary
estrogen stimulation was due to delayed formation of polysomes.
However, the sensitivity and reproducibility of quantitative *in vitro*
translational analysis must be reviewed critically because of the
inherited variability due to factors affecting initiation and elongation.

In considering possible mechanisms for the cellular memory
effect during secondary induction in locusts, it is important to
know whether the rate of vitellogenin mRNA synthesis is accelerated

or whether the translation of vitellogenin mRNA has become more
efficient due to some factor(s), such as the retention of ribosomes.
The levels of total RNA and vitellogenin mRNA in fat body during
primary and secondary stimulation with ZR-515 have also been measured
by Chinzei *et al.* (1980). These results showed that the rate of
vitellogenin mRNA accumulation in the fat body during secondary stimula-
tion by ZR-515 was faster than that of the primary induction. In order
to investigate the possible involvement of translational control by
juvenile hormone in this system the kinetics of the appearance of
vitellogenin mRNA-containing polysomes during primary and secondary
stimulation were followed.

The time course of vitellogenin polysome formation after
primary stimulation with ZR-515 in allatectomized locusts exhibits a
lag phase during which large amounts of free-ribosomes are accumulated,
followed by a rapid increase in the content of vitellogenin polysomes
to a maximum at 72 hr. Vitellogenin polysome content then decreases,
and by 120 hr is barely detectable. From the polysome profiles through-
out primary induction, the number of free ribosomes appears to
accumulate continuously, but since the peak extends off-scale,
a truly quantitative interpretation is not possible. After the
decline of the number of vitellogenin polysomes to a near-zero level,
a second dose of the hormone produces the rapid reappearance of
these polysomes within 48 hr without prior accumulation of ribosomes.
Furthermore, the magnitude of the induction in the secondary stimulation

is higher than that in the primary stimulation. These observed
differences in the rates of vitellogenin polysome formation during
primary and secondary stimulation by juvenile hormone could be in part
explained as the result of differential rates of vitellogenin mRNA
production. Therefore it appears that the primary action of juvenile
hormone on the locust fat body involves selective stimulation of trans-
cription of vitellogenin genes, accompanied by synthesis of rRNA
and build-up of the protein-synthesizing apparatus. However, these
results do not exclude the possibility of the translational control of
vitellogenin synthesis in this system. Chinzei *et al.* (1980) have
further reported the continued accumulation of vitellogenin mRNA for
several days past the time of maximal vitellogenin polysome formation
and cellular vitellogenin production in both primary and secondary
responses. This may indicate the involvement of some rate-limiting
factors or mechanisms essential for maintaining active translation of
vitellogenin mRNA during hormone action. The presence of these factors
may be dependent upon the maintenance of a sensitive threshold level
of JH in the target tissue. Therefore it is possible that the
expression of vitellogenin genes in locusts could be controlled at
both transcriptional and translational levels by juvenile hormone.
Further studies should be directed to investigate this problem.

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