THE SYNTHESIS OF MACROMOLECULES IN THE LEFT COLLETERIAL GLAND OF *Periplaneta americana* DURING THE REPRODUCTIVE CYCLE.

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ABSTRACT

The synthesis of macromolecules in the left colleterial gland of Periplaneta americana during the reproductive cycle.

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The synthesis of proteins and RNA in the left colleterial gland (LCG) of P. americana during the reproductive cycle are cyclic, highly dynamic processes. The patterns of protein synthesis and accumulation in the gland fluctuate very rapidly throughout the reproductive cycle.

Three major peaks in levels of protein synthesis are detected during the cycle. Two of these, occurring in close succession, take place before the onset of ootheca synthesis, whereas the last peak occurrs shortly before ootheca deposition and extends into the early phases of the new cycle. The patterns of translational activity and protein accumulation in the gland suggest that the LCG could be involved in the production of proteins for export as well as for ootheca synthesis.

The pattern of total RNA synthesis in the LCG closely follows that of protein synthesis during the reproductive cycle. Three major peaks in total RNA synthesis are observed, each of which occurs eight hours before a peak in protein synthesis and corresponds to enhanced production of poly(A)$^-$ RNA. The major increases in poly(A)$^+$ RNA synthesis in the LCG correlate precisely with the peaks in protein synthesis.

The LCG is known to be functionally dependant upon juvenile hormone (JH). However, the transcriptional and translational responses of the gland

to the presence of the hormone *in vitro* are not stereotyped but highly stage-specific. Short term *in vitro* JH III treatment primarily affects RNA synthesis in the LCG, but the transcriptional response of the gland to the presence of the hormone may be positive (RNA synthesis stimulated), negative (inhibition of RNA synthesis) or null, depending on the stage of the cycle.

The stages of high transcriptional activity observed in the LCG during the reproductive cycle correlate precisely with the stages at which *in vitro* JH III treatment elicits a large positive response from the isolated gland.

The immediate translational response of the gland to the presence of JH III *in vitro* is generally negative, except at the stages of high endogenous translational activity.

However, it is not entirely clear whether or not JH III is solely responsible for the initial appearance of the endogenous increases in RNA and protein synthesis observed in the LCG during the reproductive cycle.

It is apparent that JH III is not the only regulatory factor controlling LCG activities during the reproductive cycle.

A major regulatory system controlling the secretory and possibly transcriptional activities of the LCG during the reproductive cycle has been uncovered.

In vitro ecdysterone treatment, of glands isolated at stages far removed from ovulation and the onset of ootheca synthesis, depresses transcriptional activity in the LCG and induces the gland to spontaneously release its luminal content. The inhibitory effects of ecdysterone upon *in vitro* RNA synthesis are only slightly alleviated by JH III when both hormones are present in equal amounts while ecdysone-induced secretory activity is inhibited.

The presence of ovarioles isolated at 64h (onset of *in vivo* LCG secretory activity) in the incubation medium gives rise to a very strong inhibition of RNA synthesis in the LCG while concurrently inducing secretory activity.

CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION.

The striking ability of insects to adapt to and successfully invade all known forms of terrestrial environments has long attracted the attention of biologists with a variety of interests.

Cockroaches (Blattodea) are amongst the oldest and most successful terrestrial insects. Their origin dates back 250 million years, to the Carboniferous period, and fossil records show that they were very abundant at that time (Tillyard, 1937; Sellards, 1904). Modern classification describes about 3500 living species of cockroaches (Rehn, 1951), most of which are of tropical origin, inhabiting a large variety of environments including tropical forests, semi-aquatic habitats, deserts and underground burrows. Some species are wood-boring while a dozen genera are found inhabiting the nests of ants, wasps, termites, the burrows of rodents and caves in association with bats (Chopard, 1938; Roth and Willis, 1961).

The need of man to store food against shortage, particularly food of varied type which would appeal to omnivorous insects, promoted the domiciliary habits of certain species such as Periplaneta americana. Thus, whilst the vast majority of cockroaches in tropical countries continued to live as scavengers outdoors, some species became early cohabitants with man and have remained with him ever since, penetrating almost all parts of the world including polar latitudes such as northern Norway (Marlatt, 1908).

This wide range of terrestrial habitats imposes considerable physiological constraints upon the reproductive capacity of these insects. Cockroaches, amongst other widespread insect subclasses, have evolved mechanisms which successfully alleviate these environmental

pressures, as born out by their ubiquity.

Although a few cockroach families such as the Blaberidae (eg. Diploptera punctata) have evolved a form of true ovoviviparity (Keilin, 1916; Roth and Willis, 1958) as defined by Engelmann (1970), most of the Blattaria families reproduce by a form of oviposition characterized by the formation of an ootheca or egg-case (Cornwell, 1968). The ootheca may be either deposited soon after its formation (eg. Periplaneta americana) or carried externally (eg.Blattela germanica) or internally (eg.Leucophaea maderea) until hatching of the progeny occurs (Fisher, 1928; Pettit, 1946; Engelmann, 1957). The role played by the ootheca is similar to that of the egg shell of all oviparous animals: it isolates the developing embryos from the surroundings and protects them, within limits, against desiccation. To this effect water must be made available to the developing organisms. Water is absorbed by the eggs of all cockroach species during embryonic development (Roth and Willis, 1955) but the amount varies in relation to that present in the ootheca at its formation (Roth, 1967). The initial water content is high in species which deposit the egg-case (P.americana:60-65%) and in those which carry the ootheca externally (B.germanica:62%). It is low in those which incubate the ootheca internally (34-40%) (Roth, 1967; Cornwell, 1968).

The eggs of P. americana are enclosed in a sclerotized egg-case. The raw materials for the production of the ootheca are synthesized and secreted by a pair of highly branched, tubular accessory sex glands known as the colleterial glands (Bordas, 1909).

The right colleterial gland apparently synthesizes and secretes only one product, a β-glucosidase (Brunet and Kent, 1955), whereas the much larger left colleterial gland (LCG; Figs.1a and 1b) produces at

Fig. 1

The left colleterial gland of a mature female of P. americana. The gland was excised at the onset of ootheca synthesis (64 h).

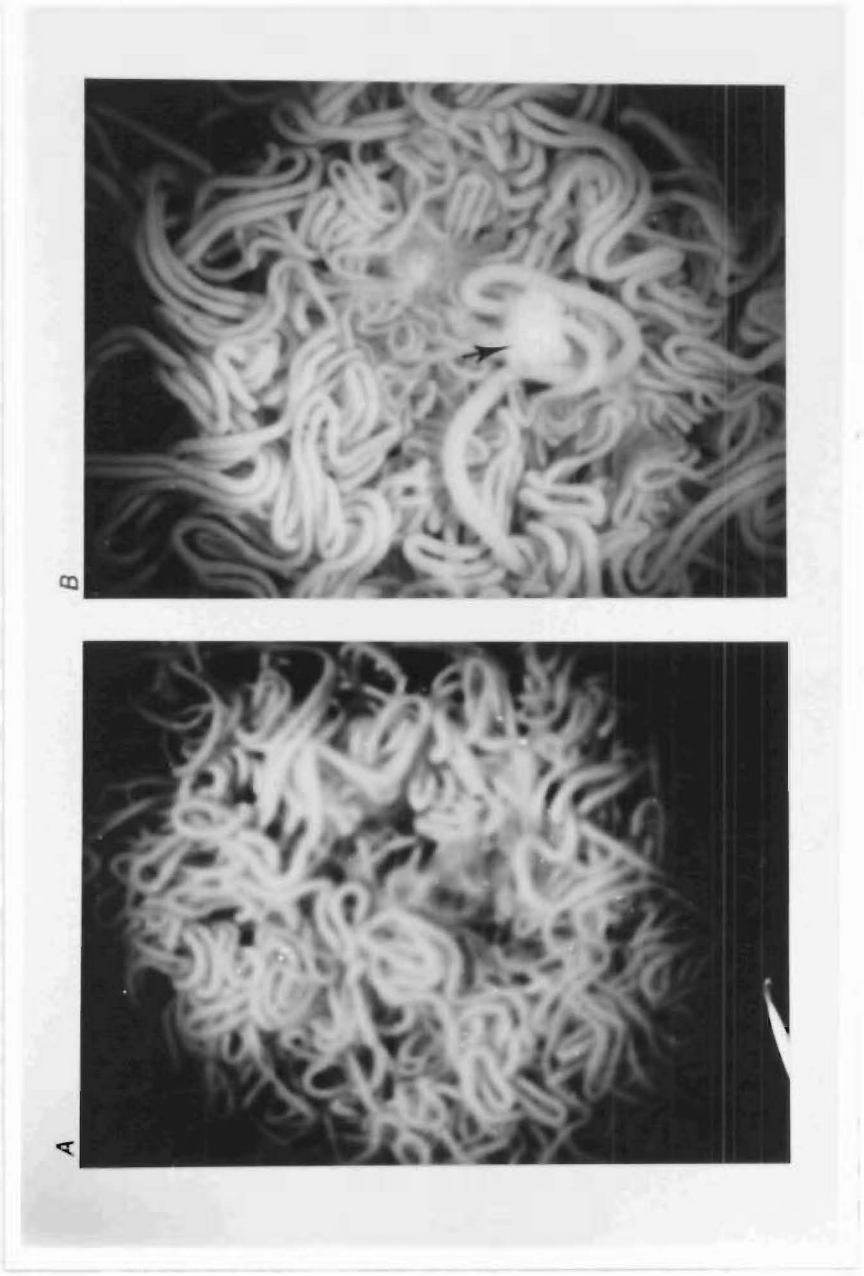
a) Dorsal view.

b) Ventral view. The arrow indicates a globule of secretory material at the cut end of the main secretory duct.

Magnifications:

(a) : x 120

(b) : x 120


B
A

least five structural proteins which can be detected in the oothecal wall (Pau et al., 1971) together with two phenolic glucosides (Pau and Acheson, 1968; Brunet and Kent, 1955), and a polyphenol oxidase (Brunet and Kent, 1955; Pau et al., 1971). When the secretions from the two glands are mixed in the genital vestibulum, β-glucosidase hydrolyzes the glucoside bonds, releasing protocatechuic acid and glucose. The phenolic compound is then oxidized to O-quinone by the phenol oxidase present in the secretion. Tanning of the ootheca now proceeds, the quinone acting as a cross- linking agent on the structural proteins (Brunet and Kent, 1955).

The molecular aspects of ootheca synthesis.

The production of a proteinaceous egg-case vastly increases the complexity of the reproductive process in the Blattaria, more particularly at the level of control of transcriptional activity in the LCG. Experimental evidence has demonstrated that:

i) colleterial gland development is ecdysone dependant (Bodenstein and Sprague, 1959),

ii) accumulation of proteins in nymphal and adult LCG is dependant upon the presence of juvenile hormone (JH) (Scharrer, 1946; Adiyodi, 1968; Shaaya and Bodenstein, 1969),

iii) the synthesis of phenolic glucosides by the LCG is under the control of JH (Bodenstein and Shaaya, 1968; Whitehead, 1969) and is purely female specific. Adult LCG transplanted into males can synthesize the proteins but not the glucosides. This is apparently not through lack of JH in adequate levels, as demonstrated by JH administration and

parabiosis experiments (Shaaya and Bodenstein, 1969), and

iv) exposure of LCG nuclei to JH gives rise to an increase in transcriptional activity (Nair and Menon, 1972). This would then suggest that, in view of the cyclic nature of corpora allata (CA) activity and JH release into the haemolymph during the reproductive cycle (Weaver et al., 1975; Weaver and Pratt, 1977) and the changes in transcriptional activity elicited by JH, protein synthesis and accumulation in the LCG should also present some form of cyclicity. Investigations to date of this aspect of colleterial activity, in P.americana, carried out at the cytological and molecular levels suggest that:

i) the rate of protein accumulation in the LCG is apparently constant (Brunet,1952), and

ii) the rate of [^{3}H]-leucine incorporation into LCG proteins is apparently uniform throughout the duration of the cycle (Weaver, 1981), thereby suggesting that maintenance of polypeptide synthesis in the LCG could require either 1) only low JH levels, or 2) only one exposure to high JH levels and protein synthesis is thereafter continuous and uniform. Yet, a number of reports clearly indicate that protein and RNA synthesis in the LCG not only respond to JH levels but may also require continuous exposure to JH in order to proceed. Bodenstein and Sprague (1959) showed that when JH levels are low, such as just before or after the final molt, protein accumulation in LCG tubules is very slow. When JH levels are high,protein accumulation in the LCG proceeds rapidly (Bodenstein and Sprague, 1959;Shaaya and Bodenstein, 1969). Scharrer (1946) found that in the absence of JH, LCG from females which had been reproductively active prior to allatectomy fail to produce secretory material and Zalokar (1968) showed that activation of the CA gives rise to a rapid increase in RNA synthesis, in the LCG, followed a few hours

later by an increase in glycine incorporation into LCG proteins.

In view of this apparent functional dependence of the LCG upon the presence of JH, what causes the LCG of adult P.americana females allatectomized at emergence to produce low levels of secretion containing structural proteins, calcium oxalate crystals and small amounts of glucosides (Stay et al., 1960); Willis and Brunet, 1966) remains enigmatic.

These conflicting observations give rise to a number of important questions concerning not only the synthetic activities of the LCG and their mode of control, but also the factors controlling the synchronization of oocyte maturation with LCG activity in order to ensure successful oviposition, thereby meeting the reproductive requirements of the species.

AIM OF THE PRESENT STUDY

The work described in this thesis attempts to answer some of the questions arising from the contradictory reports presented above by considering primarily the patterns of gene activity in the left colleterial gland during the reproductive cycle.

1) What are the patterns of protein and RNA synthesis in the LCG during the reproductive cycle? Do they correlate with the known cycles of CA activity?

2) Is there a relationship between the pattern of oocyte development and the synthetic activities of the LCG during the reproductive cycle?

3) What are the specific effects of JH upon the transcriptional and translational activities of the LCG in relation to oocyte development?

4) What is the cue leading to the synchronization of LCG secretory

activity with the onset of ovulation? Is it simply the reported fall in JH levels (Adiyodi, 1967), or the appearance of a specific signal from some components of the reproductive apparatus such as the ovarioles?

The means whereby endocrine factors such as the juvenile hormones can regulate gene activity in a specialized organ are still poorly understood. It is hoped that further knowledge of these aspects may help to provide an insight into the mechanisms whereby a series of interlinked events, involving the contributions of different organs, can be integrated to result in a finite biological function.

CHAPTER TWO

THE PATTERNS OF WEIGHT CHANGES, PROTEIN CONTENT AND PROTEIN SYNTHESIS IN THE LEFT COLLETERIAL GLAND DURING THE REPRODUCTIVE CYCLE.

INTRODUCTION

The eggs of oviparous cockroaches such as P. americana are ovulated into a proteinaceous egg-case, the ootheca, which is then deposited. The mature ootheca is made of sclerotised proteins, resembling in appearance the cuticle of the insect, for which the name of "sclerotin" has been proposed (Pryor, 1940b). However, unlike cuticular scleroprotein, the ootheca is completely lacking in chitin (Campbell, 1929).

The raw materials for the production of the ootheca originate from two tubular, highly branched accessory sex glands known as the colleterial glands (Bordas, 1909). The whitish secretion of the large left gland consists of a mixture of water soluble and insoluble proteins (Pau et al., 1971), two phenolic glucosides (protocatechuic acid glucoside and 4-O-β-D-glucosido-3-hydroxybenzyl alcohol) and a polyphenol oxidase (Brunet and Kent, 1955; Pau and Acheson, 1968). The much smaller right gland apparently releases a β-glucosidase as its only secretory product (Brunet and Kent, 1955). The ootheca is formed from the interaction of these two secretions (Pryor, 1940a), but the number of structural protein species taking part in the formation of the ootheca in P. americana is still unresolved (Pau et al., 1971; Weaver, 1981).

It is generally assumed that the LCG has no function other than the production of the components of the ootheca (Pau et al., 1971; Adiyodi and Adiyodi, 1974). The synthesis of these structural proteins and phenol glucosides have been shown to be under the control of juvenile hormone (JH) (Bodenstein and Sprague, 1959; Willis and Brunet, 1966;

Bodenstein and Shaaya, 1968; Shaaya and Bodenstein, 1969; Weaver, 1981). Yet, little is known of the protein synthetic activities of the LCG during the reproductive cycle. On the basis of cytological studies, Brunet (1952) concluded that protein secretion from the epithelial cells into the lumen proceeds in a constant manner, and he found no evidence which could suggest cyclic changes in the synthetic activities of the gland cells. Weaver (1981) reported that leucine incorporation into LCG proteins is uniform throughout the reproductive cycle of adult females of P. americana. The cyclic release of JH in P. americana has been well documented (Pratt, 1967; Bell, 1969a; Weaver et al.,1975; Weaver and Pratt, 1977), yet Weaver (1981) reported that the cyclic nature of CA activity during the reproductive cycle is not reflected by cyclicity in colleterial protein synthesis. However, Weaver's study was based on one-day and four-day intervals. Thus, rapid changes in the synthetic activities of the LCG may not have been detected. If the colleterial gland of P. americana is to be used as a model system for the investigation of the regulation of protein synthesis by JH (Weaver and Pau, 1982), it is essential that the background information regarding the synthetic activities of the LCG during each oothecal cycle be clarified.

The present study examines, at short time intervals within each reproductive cycle, the relationships between weight changes in the LCG, oocyte development, and in vitro protein synthesis in the LCG. Evidence is presented that reproductively mature females of P. americana do show cyclic changes in terms of LCG protein content and leucine incorporation into colleterial polypeptides. Furthermore, the patterns of extractable proteins of the LCG, as detected by SDS-gel electrophoresis, during each cycle, and their synthesis are not uniform

as implied by previous studies, but are dynamic processes.

MATERIAL AND METHODS

1. Rearing and staging procedures.

Mature females of *P. americana* were taken from large population cages and transferred to clear plastic boxes kept at 25±1°c in constant dim light. Each box contained two females, one of which was marked, and one male. The animals were fed on a mixture of oatmeal and pulverised dog biscuits at a ratio of 7:5 and were supplied with water *ad libitum*. The females took an average of three weeks to adapt to these rearing conditions. During this period, the duration of the oothecal cycle was very erratic, varying from two to six days. After this period of acclimation, the mean duration of the cycle was 112±4h., taking the time of ootheca deposition as 0h of each cycle. Only females which had consistently shown, over four consecutive weeks, a cycle duration of about 112h were used in this study. For preliminary experiments, the reproductive cycle was divided into four periods of 24±2h. However, it was found that the levels of protein synthesis and the electrophoretic patterns of proteins detected at each stage were highly variable between different experiments. The duration of the reproductive cycle was subsequently divided into thirteen periods of eight hours and a final period of ten hours. This mode of timing proved satisfactory and was retained throughout the studies presented here. Under these timing conditions, the onset of ootheca synthesis occurred at 64h, ovulation was initiated between 64 and 72h and ootheca synthesis completed at 88h.

2. Dissection of the LCG and incubation procedures.

The left and right colleterial glands were dissected, as intact as possible, in saline (140mM NaCl, 2.7mM KCl, 1.8 mM $CaCl_2$, 2.4mM $NaHCO_3$) under a binocular microscope, from pre-weighed females taken at each stage of the reproductive cycle. The right gland was subsequently discarded. The isolated LCG was rinsed three times in saline . The wet weight of each LCG, freed of saline as much as possible by gentle blotting on tissue paper, was determined after transfer to a pre-weighed incubation vessel consisting of a siliconized, surface sterilized watch glass and cover glass and 200µl incubation medium. In preliminary tests on the suitability of various incubation media such as those of Yeager (1939); Ludwig et al. (1957); Usherwood, (1963); Wareham et al. (1973), as tabulated by Burton (1975); and that of Robb (1968), it was found that in all these media, the isolated LCG could be maintained for less than 24 hours. The culture medium (Table 1) used throughout the studies presented here was modified from Robb (1968) and from Yamasaki and Narahashi (1959). Isolated LCG s could, in this medium, maintain cycles of spontaneous contraction and relaxation for at least 72 hours.

To determine the rate of protein synthesis, tritiated leucine was used (Weaver, 1981). Although the major colleterial proteins contain different amounts of leucine, it was considered that for a comparative purpose such experimental protocol should produce a reasonable estimate of any variation in incorporation at different time points of the reproductive cycle. For [^{3}H] leucine incorporation in vitro, the washed and pre-weighed LCG was placed in 200µl leucine-free incubation medium supplemented with L[4,5-^{3}H] leucine (The Radiochemical Centre,

Table 1.

Composition of the incubation medium used throughout the present study. In this medium, modified from Robb (1968) and from Yamasaki and Narahashi (1959), isolated LCGs could be maintained in a healthy state for at least 72 h.

Table 1. Incubation medium

Components	Final Concentration
Tris-HCl (pH 7.2)	8.4 mM
NaCl	52.0 mM
KCl	40.0 mM
$MgSO_4 \cdot 7H_2O$	1.2 mM
$MgCl_2 \cdot 6H_2O$	1.2 mM
$CaCl_2$	1.0 mM
Glucose	10.0 mM
Sucrose	100.0 mM
Chloramphenicol	2 µg/ml
Polymixin	5 µg/ml
Amino acids (14-1 and 14-2 of Robb, 1968)	

Amersham, UK. Spec. act. 131Ci/mmole) to a final specific activity of 6.4×10^{-2} µCi/µl. A study of the time course of *in vitro* leucine incorporation by the LCG of females at various stages of the reproductive cycle showed that the rate of incorporation was linear for three to four hours, depending on the stage of the cycle (Table 2). Thus routine labelling was carried out at 25±1°c for three hours. Immediately after labelling, the glands were washed three times in ice cold saline and, unless otherwise indicated, placed in 1.0ml incubation medium at pH 7.8 for 5 min. Under this slightly alkaline condition, LCG from females at all reproductive stages spontaneously released their luminal content at a rate of 150µg protein/5 min. (S_D=48; n=42). The secreted material, released as viscous globules which could be easily picked up, was transfered to 100µl sample buffer (Laemmli, 1970) for electrophoretic analysis. The whole gland was then homogenized in 3.0mM ascorbic acid (Pau *et al.*, 1971) in a tight fitting all-glass manual homogenizer. The tissue homogenate was immediately frozen in liquid air and stored at -20°c until use.

3. Determination of protein content and levels of *in vitro* leucine incorporation into colleterial polypeptides.

To determine the levels of leucine incorporation and the protein content of the LCG, the protein from 70µl aliquots (duplicates) of tissue homogenate was precipitated with 100µl ice cold 10% TCA. The precipitate was pelleted by centrifugation at 18000xg for 10 min. The supernatant was discarded and the pellet was successively washed in 5% TCA, ethanol and ether according to the procedure of Lowry *et al.*

Table 2.

Time-course study of *in vitro* leucine incorporation into colleterial proteins in LCGs isolated at different stages of the reproductive cycle. The isolated glands were incubated at 25 ±1°c, in 200 µl of leucine-free medium supplemented with [4,5-^{3}H] leucine to a final specific activity of 2.5 x 10^{-2} µCi/µl. The levels of radioactivity remaining in the TCA insoluble homogenate were determined by liquid scintillation counting.

Table 2. Time-course study of [^{3}H] leucine *in vitro* incorporation into colleterial polypeptides in LCG s isolated at different time points during the reproductive cycle.

Incubation time (h)	DPM (x 10^3)/ Gland. Stages of the reproductive cycle (h).					
	0	8	24	40	72	96
1	2.94	3.29	1.70	4.10	2.07	1.74
2	5.12	4.87	3.02	7.02	3.61	2.99
3	7.25	6.74	4.11	10.87	6.11	5.01
4	8.31	8.92	4.76	13.92	6.62	6.12

(1951). The final pellet was air dried and dissolved in 500µl 1.0M NaOH. Five µl aliquots (triplicates) of this solution were transferred to glass fibre support (Gupta, 1971) for liquid scintillation counting in a Phillips PW 4540 LSC with a counting efficiency of 59.6%. Neutralized and non-neutralized samples gave identical counts. Protein determination was carried out on 10 and 15µl samples according to the method previously described (Lowry et al., 1951). Bovine serum albumin (Sigma, USA) was used as protein standard. The total protein content and the total level of incorporation were calculated for each gland.

4. Electrophoretic and fluorographic analyses.

The remainder of the tissue homogenate was dissolved in an equal volume of sample buffer (double strength). For electrophoretic analysis of the tissue homogenate, each load was standardized to 40µg protein. Secretory material was also collected in situ from the genital vestibulum of females at 64h of the cycle for electrophoretic analysis. Partially sclerotized oothecae were removed from females at 72h of the cycle. The soft and white part of the egg-case was cut off and all loosely adhering materials were removed. The soft oothecal wall was dissolved in 75µl sample buffer. Due to the low amounts of material available, protein determination was not carried out on the vestibular and oothecal wall samples. The optimum electrophoretic loads were determined by trial.

SDS-gel electrophoresis was initially carried out in 10, 11, 13 and 15% polyacrylamide gels. It was found that the low molecular weight polypeptides, representing the most abundant protein species present in

the LCG, could not be electrophoretically resolved in gels of less than 15% total acrylamide concentration. Electrophoresis was subsequently carried out in 15-22.5% linear gradient polyacrylamide gels. While this procedure gave an excellent level of resolution, the complexities of the electrophoretic patterns were such that the identity of major low molecular weight colleterial polypeptides, differing by only 500 daltons, could not be established with confidence. Attempts were made to further enhance the resolving power of the electrophoretic system. A considerable increase in resolution levels was achieved by using diallyltartardiamide (DATD) as a crosslinker (Späth and Koblet, 1979) in linear gradient acrylamide gels (15-25% acrylamide, 0.8% DATD). In all cases, the acrylamide gradients were stabilized in a 2-5% sucrose gradient. Electrophoresis was performed at room temperature at 180V for eight hours. After electrophoresis, the gels were stained in 0.1% Coomassie Brilliant Blue. Following destaining, the gels were treated according to the method described by Bonner and Laskey (1974) for fluorography. The dried gels were exposed to Agfa-Gevaert X-ray films for 30 days at -20°c.

5. Patterns of oocyte development.

The profiles of terminal (T), penultimate (T-1), and whenever possible, previtellogenic (T-2) oocyte growth were determined by optical measurements. Immediately after dissection of the colleterial glands, the ovarioles were excised from each female and the lengths of T, T-1 and T-2 oocytes measured using an ocular micrometer. For each stage of the reproductive cycle, the lengths of, at least, four oocytes of each type were measured in each of the two ovarioles of the female. These

measurements were repeated in at least three females for each stage of the reproductive cycle.

RESULTS

I)Changes in LCG wet weight during the reproductive cycle.

The LCG presented considerable changes in wet weight over the duration of the reproductive cycle (Fig.1).

Two major phases of increase in gland wet weight were apparent. During the first sixteen hours of the cycle, a 30% increase in gland wet weight, relative to the value recorded at 0h, was observed. This was followed by a weight loss over the next twenty four hours, the gland reaching a minimum weight at 32h. The second phase of increase in gland wet weight occurred between 40 and 64h. The subsequent decrease in gland wet weight corresponded to the period of ootheca synthesis (from 64 to 88h).

II)Changes in protein content of the LCG during the reproductive cycle.

Figure 2a shows the total protein content of the gland isolated at each stage of the reproductive cycle. The protein content of the LCG fluctuated throughout the oothecal cycle. A very substantial fall in total protein was recorded near the beginning of the cycle (from 8 to 24h). During this period 4.4mg of protein, representing 75% of the

Fig. 1

Changes in LCG wet weight. The glands isolated at each stage of the oothecal cycle were weighed immediately after introduction in the incubation medium. Each point is the mean of three individual animals and the SEM is shown for each point.

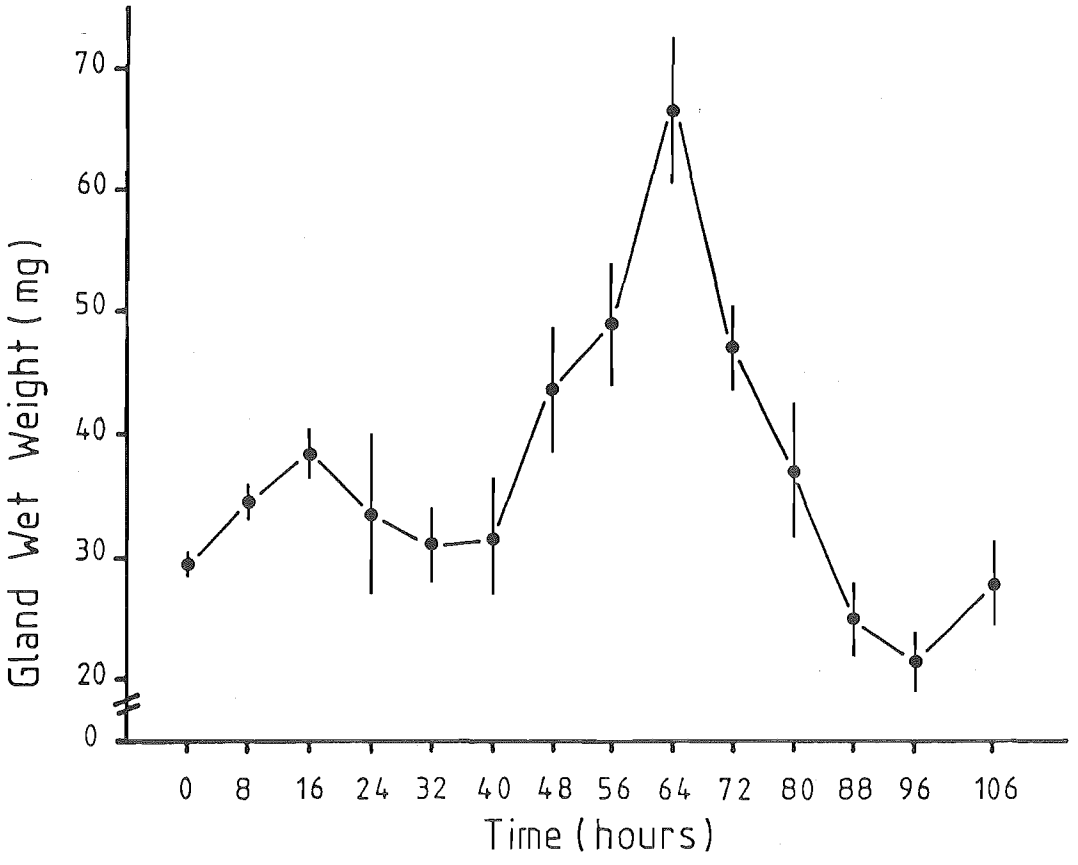

Gland Wet Weight (mg)
70
60
50
40
30
20
0
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

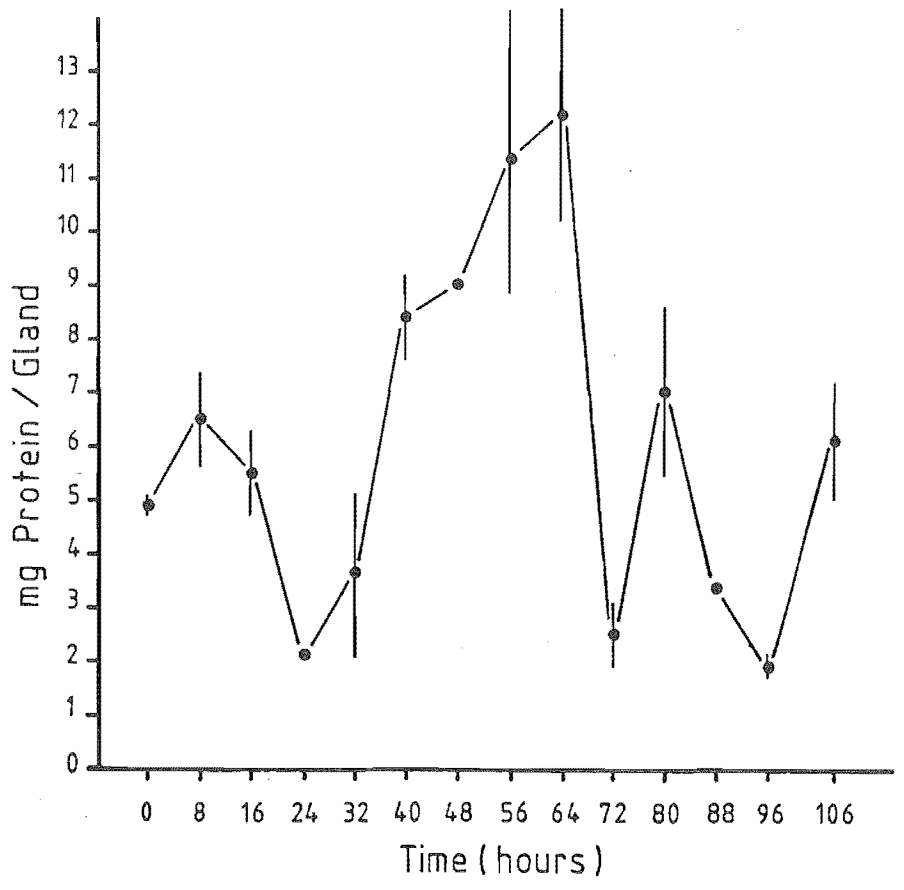
Fig. 2(a)

The total protein content of isolated LCG s was determined from TCA insoluble extracts. Each determination was carried out in triplicate and the mean protein value for each sample was recorded. Each point represents the mean of three animals. Where not shown, the SEM was too small to be included.

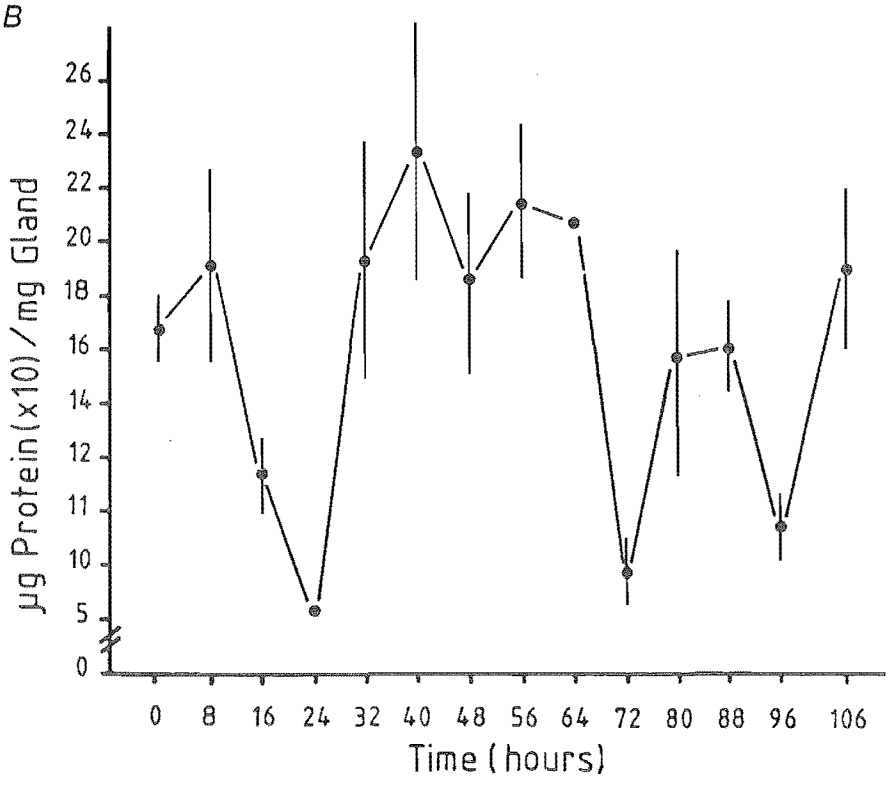
(b)

Ratio of LCG protein to gland weight. Each point is the mean of three individual determinations. Where not shown the SEM was too small to be included.

A


mg Protein / Gland
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

B


µg Protein(x10) / mg Gland
0
5
10
11
12
14
16
18
20
22
24
26
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

protein content at 0h, was lost. This was followed by a rapid increase in total protein content over the next forty hours, leading to a final gain of 10.15mg protein, representing an increase of 172% over the 0h value. The second massive fall in protein content occurred at the onset of ootheca synthesis, between 64 and 72h, representing a loss of 9.8mg protein. During ootheca synthesis, between 72 and 80h, an increase in protein content was observed, followed by a decrease, the protein content of the gland reaching a minimum at 96h, eight hours after completion of the ootheca.

The changes in gland wet weight over the duration of the oothecal cycle generally followed the pattern of protein accumulation. However, between 8 and 16h, the total protein content of the gland fell by 1.0mg while the gland wet weight increased by 3.9mg. Between 32 and 40h, the protein content of the gland increased by 4.8mg, whereas the wet weight of the gland remained practically unchanged (compare Figs. 1 and 2a). The contribution of total protein to LCG wet weight during the reproductive cycle is shown in Fig.2b. There were three stages during the reproductive cycle (24, 72 and 96h) where total protein contributed very little to the gland wet weight.

III) Profile of [^{3}H] leucine incorporation into colleterial protein.

Since the total protein content of the LCG fluctuated throughout the reproductive cycle, it became necessary to determine if these changes were due to de novo protein synthesis in the gland or to the uptake of haemolymph proteins. The results of this investigation are presented in Fig.3a.

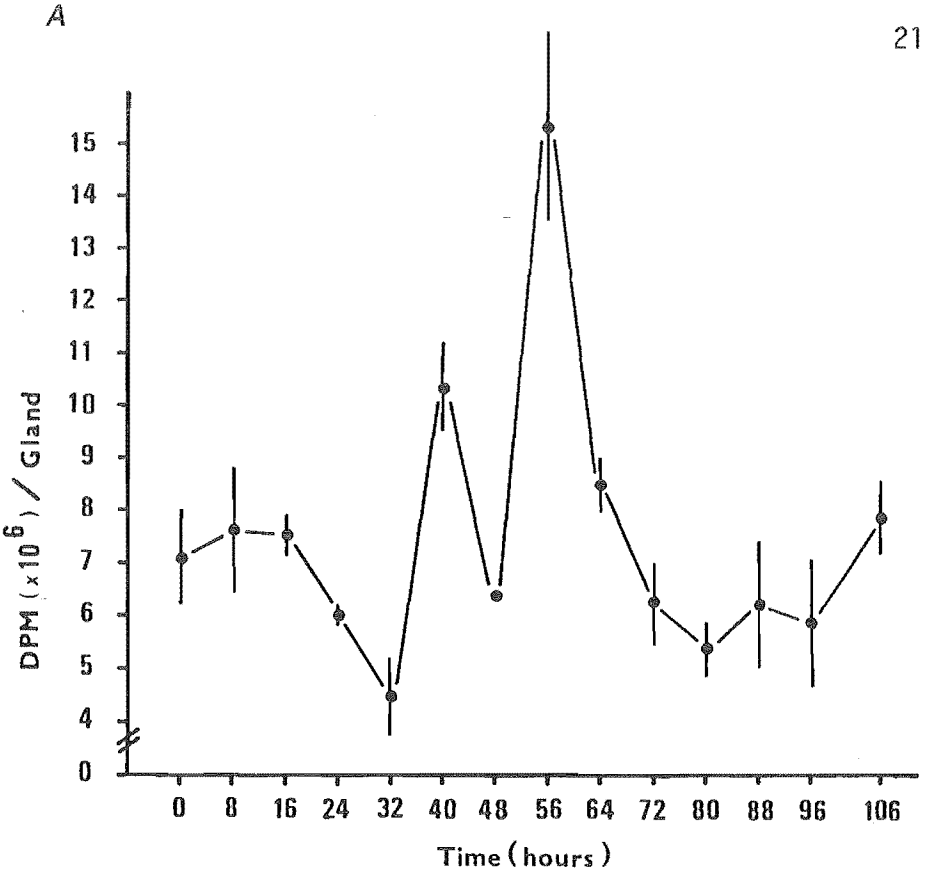
Fig. 3(a).

Levels of leucine incorporation into colleterial proteins determined at each stage of the reproductive cycle after *in vitro* incubation. The isolated LCG s were incubated for 3 h in the presence of [4,5-^{3}H] leucine. The TCA insoluble radioactivity remaining in the gland homogenate was determined in triplicate for each sample. Each point represents the mean of three separate experiments. Where not shown the SEM was too small to be included.

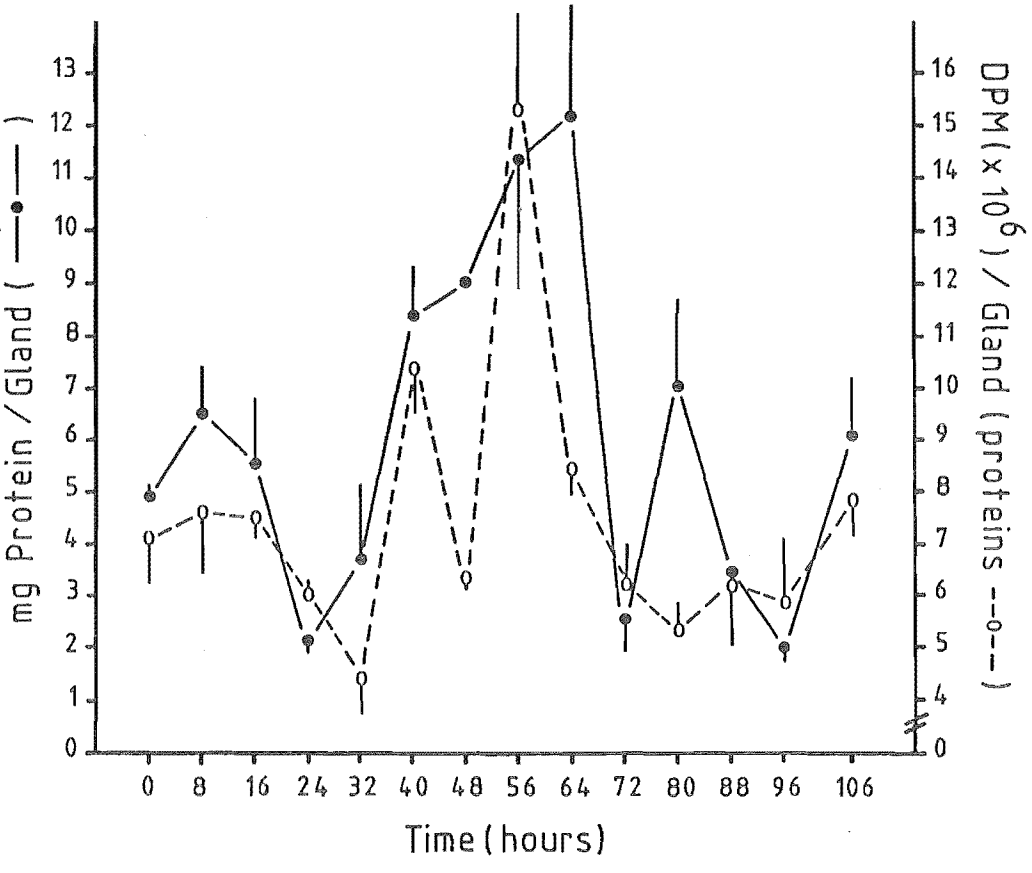
(b)

Comparison between protein content (solid line) and leucine incorporation into colleterial polypeptides (dotted line) in individual LCG s at each stage of the reproductive cycle.

A


DPM (x10^6) / Gland
0 4 5 6 7 8 9 10 11 12 13 14 15
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

B


mg Protein / Gland (—●—)
DPM (x 10^6) / Gland (proteins --o--)
0 1 2 3 4 5 6 7 8 9 10 11 12 13
0 4 5 6 7 8 9 10 11 12 13 14 15 16
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

The pattern of leucine incorporation into colleterial polypeptides closely followed that of the changes in total protein content of the gland during the reproductive cycle from 0 to 72h. Two very large peaks in levels of incorporation were detected between 32 and 40h and between 48 and 56h. The first increase in leucine incorporation was initiated soon after the onset of the major phase of protein accumulation in the gland (Fig.3b). The second increase, initiated at 48h, corresponded to the final phase of protein accumulation in the gland, shortly before the onset of ootheca synthesis (64h). From this time point onward, and until the end of ootheca synthesis, the levels of leucine incorporation into colleterial protein decreased very rapidly. A third, albeit slower, increase in incorporation levels occurred from about 106h, shortly before ootheca deposition, to 8h after oviposition (new cycle) and was concurrent with a 4.55mg increase in protein content of the gland, a value comparable to that recorded during the first burst in incorporation levels (4.75mg between 32 and 40h; Fig.3b). However, one increase in LCG protein content (between 72 and 80h) did not correspond to a rise in incorporation levels.

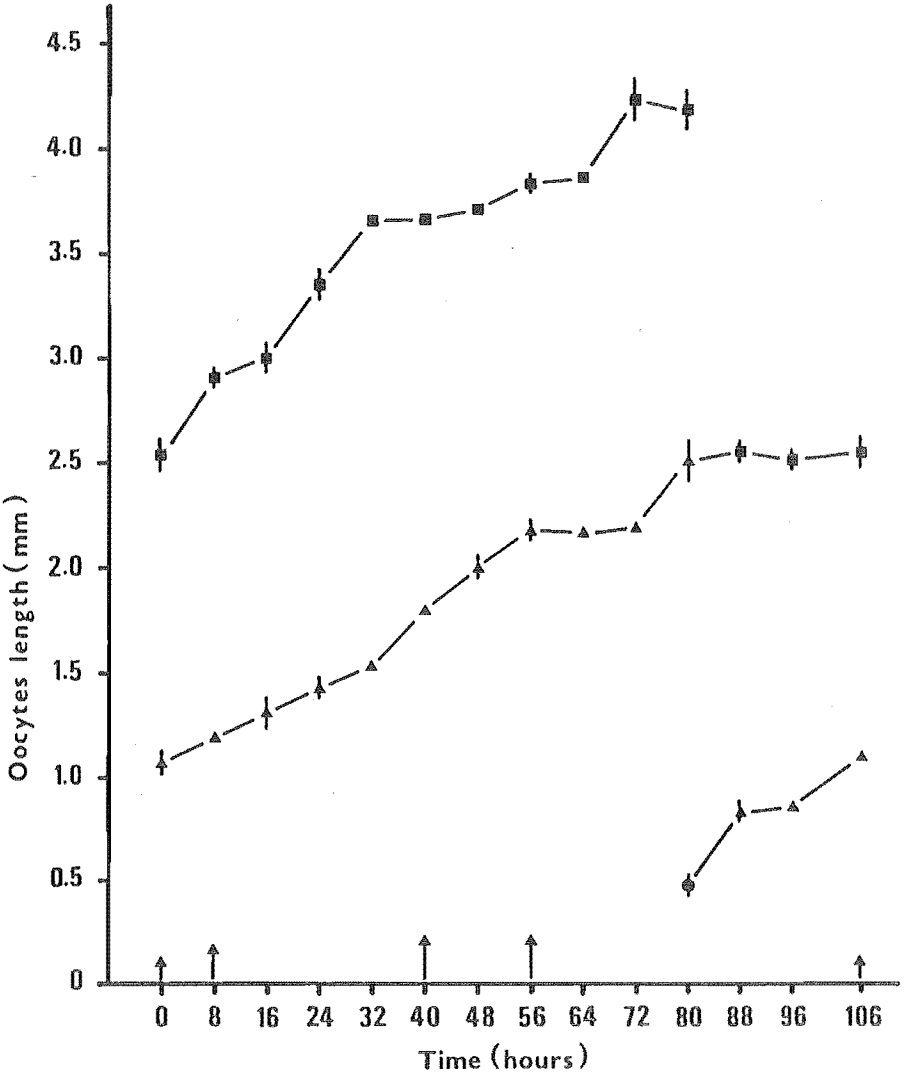
IV) Patterns of oocyte growth during the reproductive cycle.

The patterns of T and T-1 oocyte elongation over the duration of the reproductive cycle are presented in Fig.4. The stages at which increases in the levels of leucine incorporation into colleterial polypeptides occurred are indicated on the abscissa.

The major elongation phase of T oocytes occurred between 0 and 32h. The rate of growth of T-1 oocytes over this period was markedly lower

Fig. 4

The lengths of T (■), T-1 (▲) and T-2 (●) oocytes at each stage of the reproductive cycle measured immediately after dissection of the ovarioles. For each set of ovarioles (two per animal) the length of four to six oocytes of each type (T, T-1 and when possible T-2) were measured and the mean size of each type of oocyte for each experimental animal was recorded. Each point represents the mean oocyte lengths in three animals. The arrows indicate the timing of high leucine incorporation into colleterial polypeptides.


4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Oocytes length (mm)
0
8
16
24
32
40
48
56
64
72
80
88
96
106
Time (hours)

than that of T oocytes. From 32 to 56h, the rate of T oocytes elongation fell from 3.44%/h to 0.94%/h, while that of T-1 oocytes increased rapidly from 1.41%/h to 3.13%/h. The last major elongation phase of T oocytes occurred between 64 and 72h, shortly before ovulation, whereas that of T-1 oocytes took place 8h later.

V) Electrophoretic analyses of colleterial proteins.

a) LCG tissue homogenate.

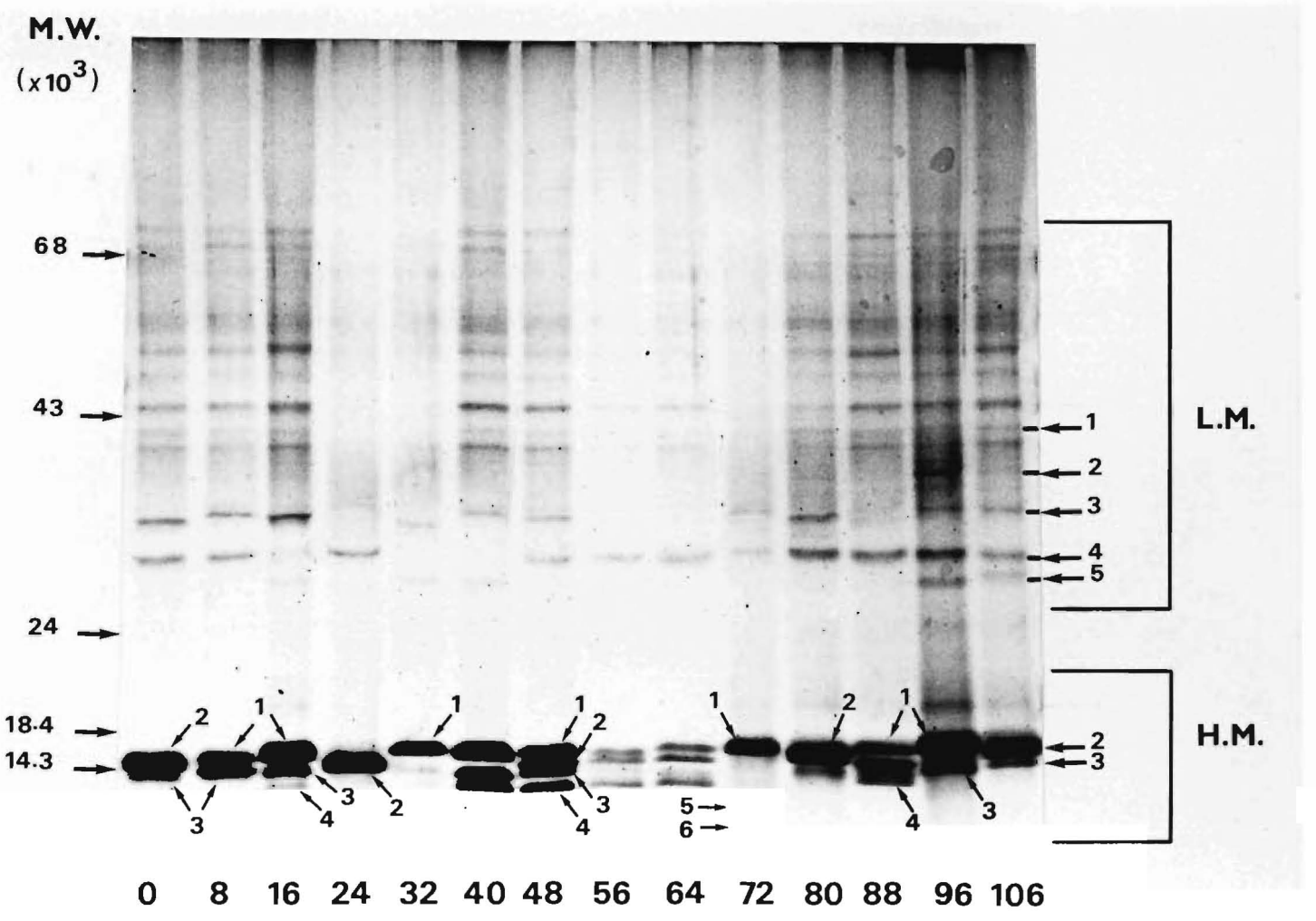
Figure 5 presents a typical electrophoreogram obtained from the homogenate of LCG. The pattern of proteins present in the LCG showed considerable qualitative and quantitative changes from one stage to another over the duration of the reproductive cycle. The proteins detected can be horizontally divided into two groups.

i) The low mobility group (LM), representing proteins larger than 25000 daltons, consisted of at least thirteen polypeptides with apparent molecular weights ranging from 27000 to 75000 daltons. In order to enhance comparison with protein species detected from other sources (see below) only four of these electrophoretic bands were numbered in order of decreasing molecular weights.

ii) The high mobility group (HM), representing proteins with a molecular weight smaller than 25000 daltons, contained the most abundant species of polypeptides present in the LCG during the reproductive cycle. This group consisted of at least four major bands (labelled 1 to 4) and two minor bands (labelled 5 and 6). This group of proteins showed rapid and striking qualitative changes over most of the reproductive cycle.

Fig. 5

SDS-polyacrylamide gel electrophoresis of polypeptides from tissue homogenate of LCG s isolated at each stage of the reproductive cycle. Electrophoresis was carried out in 15-22.5% polyacylamide gradient gels at 160 V for 8 h. The proteins used as molecular weight standards were bovin serum albumin (68.0 Kd), albumin (43.0 Kd), pepsin (34.7 Kd), trypsinogen (24.0 Kd), β-lactoglobulin (18.4 Kd) and lysozyme (14.3 Kd). Electrophoregram stained with 0.1% Coomassie Brilliant Blue showing the major polypeptides present in the LCG homogenate at each stage of the reproductive cycle.


M.W.
(x10³)
68
43
24
18·4
14·3
L.M.
H.M.
1
2
3
4
5
6
0
8
16
24
32
40
48
56
64
72
80
88
96
106
Time (hours)

Some of the electrophoretic patterns presented at specific time points in the early phases of the cycle seemed to reappear at later stages. The electrophoretic pattern of major low molecular weight polypeptides presented by LCG s at 24h of the cycle was practically identical to those resolved at 80 and 106h. However, the very small molecular weight differences between the various members of the HM group (about 500 daltons) made their individual identification at different stages of the oothecal cycle difficult. The LCG s used here were artificially induced to release their luminal content. As a result, at most of the time points analysed, polypeptides 3 and 4 appeared to be present at low levels (16, 32, 72, 96 and 106h) or to be totally absent from the tissue homogenate (8 and 24h).

The electrophoretic patterns obtained with the SDS-DATD-acrylamide system from homogenate of LCG s at the different stages of the reproductive cycle are presented in Fig.6. The glands used in these analyses were not induced to secrete in vitro, and polypeptides 3 and 4 were present at all stages in the tissue homogenate. The electrophoretic patterns characterizing each stage of the reproductive cycle, essentially identical to those obtained with the SDS-polyacrylamide system, were resolved with a clarity not afforded by the previous analytical procedure. This considerable increase in levels of resolution enabled accurate comparison of the stage-specific electrophoretic patterns to be carried out.

The electrophoretic patterns characterizing each stage of the reproductive cycle were very consistent between different experiments (Table 3). The overall distribution of low molecular weight polypeptide species found in the LCG homogenate over the duration of the reproductive cycle appeared to consist of three recurrent

Fig. 6

Electrophoretic patterns obtained from LCG tissue homogenate with the SDS-DATD-acrylamide electrophoretic system. The samples were different from those used in Fig. 5 and the loading sequence was chosen in order to enhance comparison of electrophoretic patterns between different stages of the reproductive cycle. Each sample load was standarized to 40 µg protein.

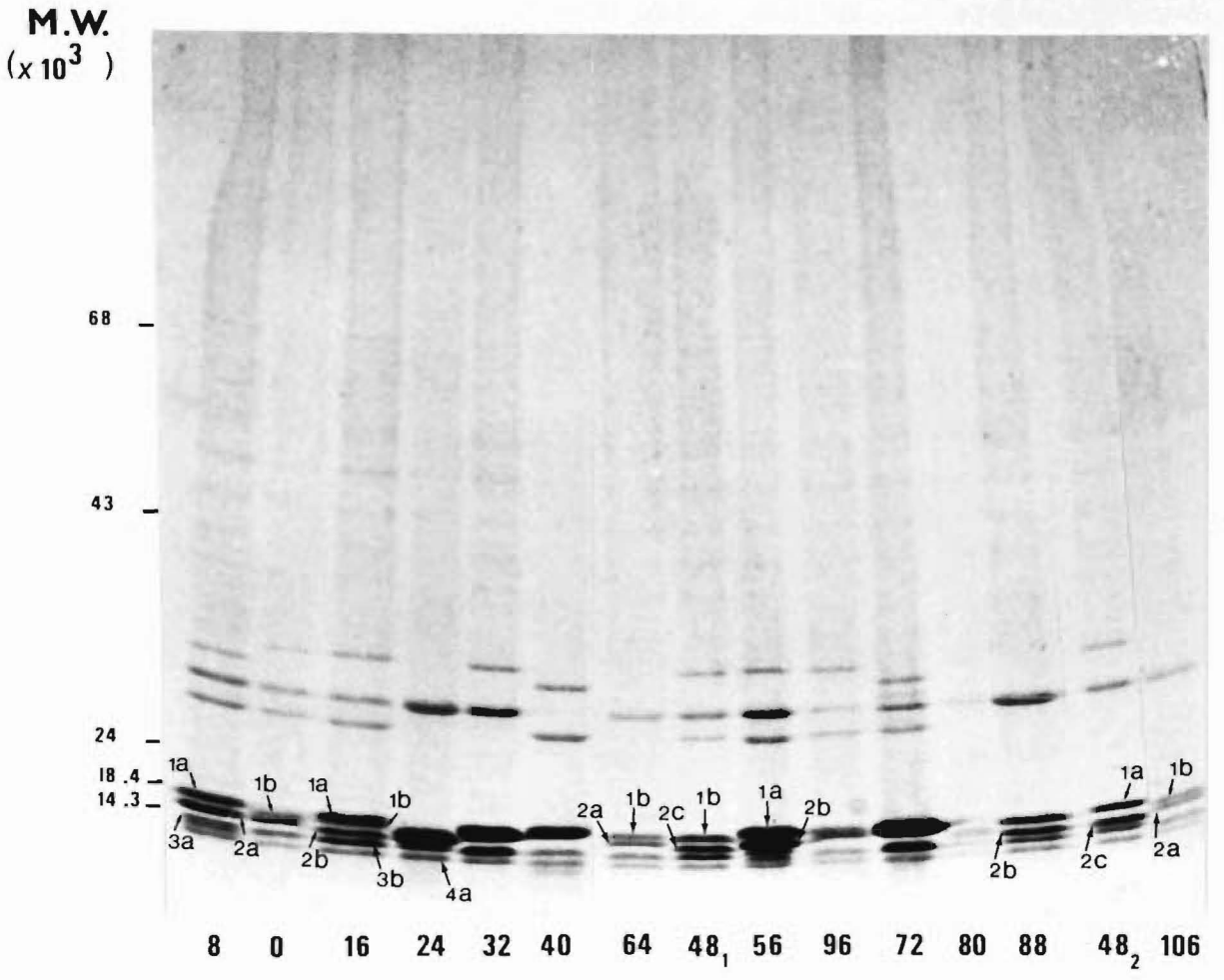

M.W.
(x 10³)
68
43
24
18.4
14.3
1a
1b
1a
1b
3a
2a
2b
3b
4a
2a
1b
2c
1b
1a
2b
1a
1b
2b
2c
2a
8
0
16
24
32
40
64
48₁
56
96
72
80
88
48₂
106
Time (hours)

Table 3.

Consensus patterns of distribution of low molecular weight major colleterial proteins characterizing the stage-specific electrophoretic patterns presented by LCG homogenate at all stages of the reproductive cycle.

*[1] : Polypeptide 2 or variants thereof were sometimes (F= 6.7%) absent from the electrophoretic patterns obtained with this stage.

*[2] : At this time point, a variant of polypeptide 2 occasionally (F= 4.1%) appeared in the electrophoretic patterns.

*[3] : A variant of polypeptide 1 was infrequently ($\bar{x}$ F< 5%) detected in the electrophoretic patterns obtained with these stages.

F : frequency of detection.

Table 3. The distribution of low molecular weight major colleterial polypeptides characterizing the stage-specific electrophoretic patterns presented by the LCG during the reproductive cycle.

Pattern	Time of occurrence (h)	Polypeptides present
1	8, 16*, 48, 56, 64, 88.	1, 2, 3 and 4
2	32, 40, 72 96*.	1, 3 and 4
3	0*, 24*, 80, 106*.	2, 3 and 4

electrophoretic patterns (Table 3) dispersed amongst the different stages of the reproductive cycle. However, the four major polypeptide species constituting this group showed some variability in terms of their specific apparent molecular weights (Table 4). The appearance of any one variant in a given pattern was very unpredictable (Figs.7a and 7b), and seemed to be due purely to individual genetic variation. Some variant forms such as 1a-b and 2a-b, were very common while others were found very rarely (2c,3c and 4b) in the stage specific electrophoretic patterns. The electrophoreogram of a given LCG never presented simultaneously more than two variant forms for a given polypeptide species (Figs 5 to 7) although, at a population level, proteins 2 and 3 seemed to be present in three different forms (Table 4). Similar effects were observed at the level of the major polypeptides composing the LM group (Fig. 7b; Table 4).

b) LCG luminal material.

The electrophoretic patterns obtained with material spontaneously secreted by isolated LCG s (Fig. 8) indicated that all the major polypeptide species present in the tissue homogenate at each stage of the reproductive cycle were also present in the luminal content of the gland.

c) Proteins from the genital vestibulum and the oothecal wall.

The electrophoregrams of secretory material collected in situ from the genital vestibulum (labelled G.V.) at the onset of ootheca synthesis (64h) revealed the presence of eleven polypeptides with electrophoretic mobilities similar to proteins found in LCG homogenate and luminal material (Figs. 9a and 9b). Samples of non-sclerotized

Table 4.

The different apparent variant forms of the major low molecular weight colleterial proteins detected by unidimensional electrophoresis during the course of the present studies. The samples analysed included LCG homogenate and secretory material at all stages of the reproductive cycle, material collected from the genital vestibulum as well as extracts from oothecal walls. The frequency of detection of each variant form and combinations thereof is indicated except for the LM group were only a few samples (27) could be accurately analysed.

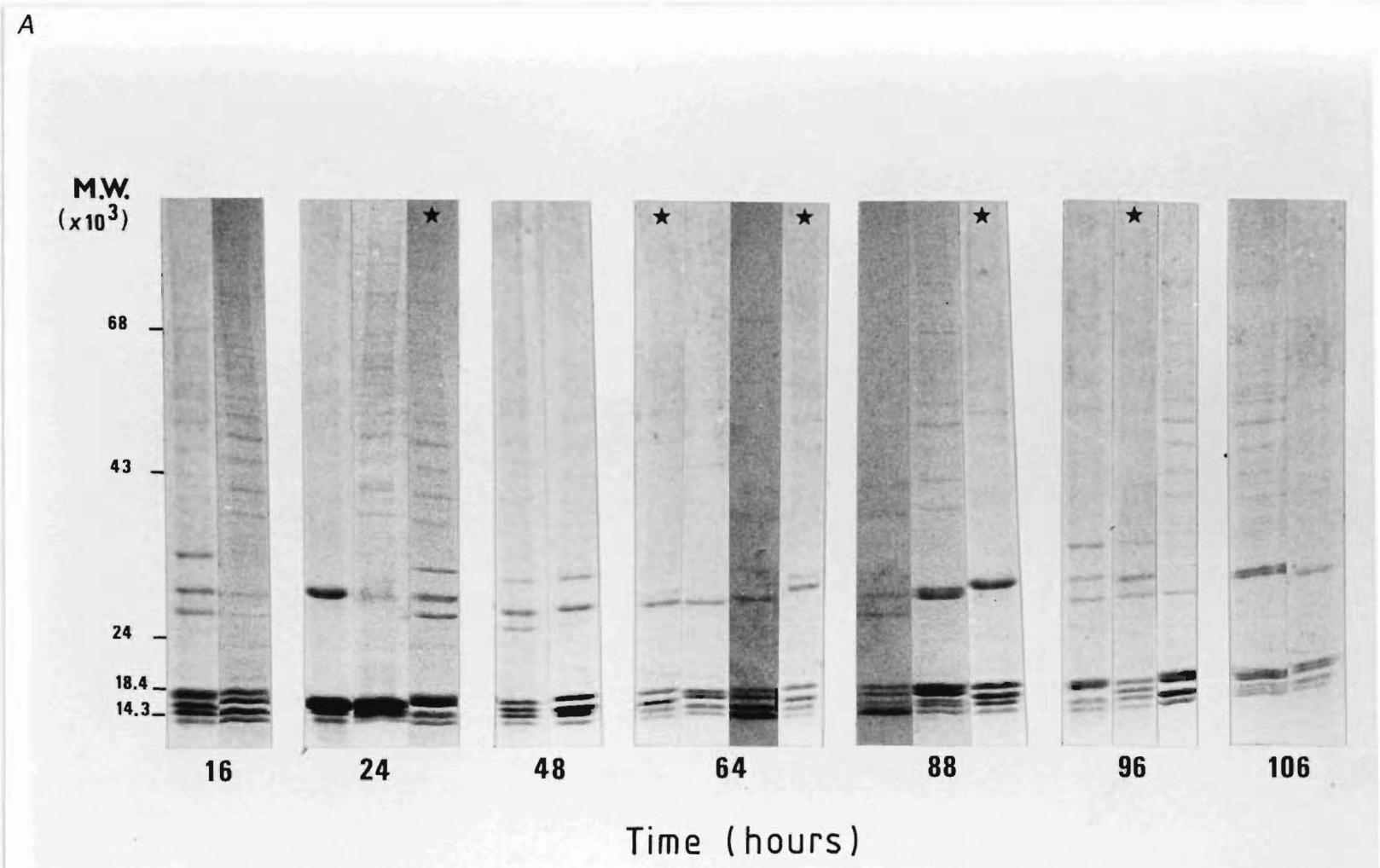
Table 4. The different variant forms of the major low molecular weight colleterial proteins and their frequency of detection in the stage-specific electrophoretic patterns.

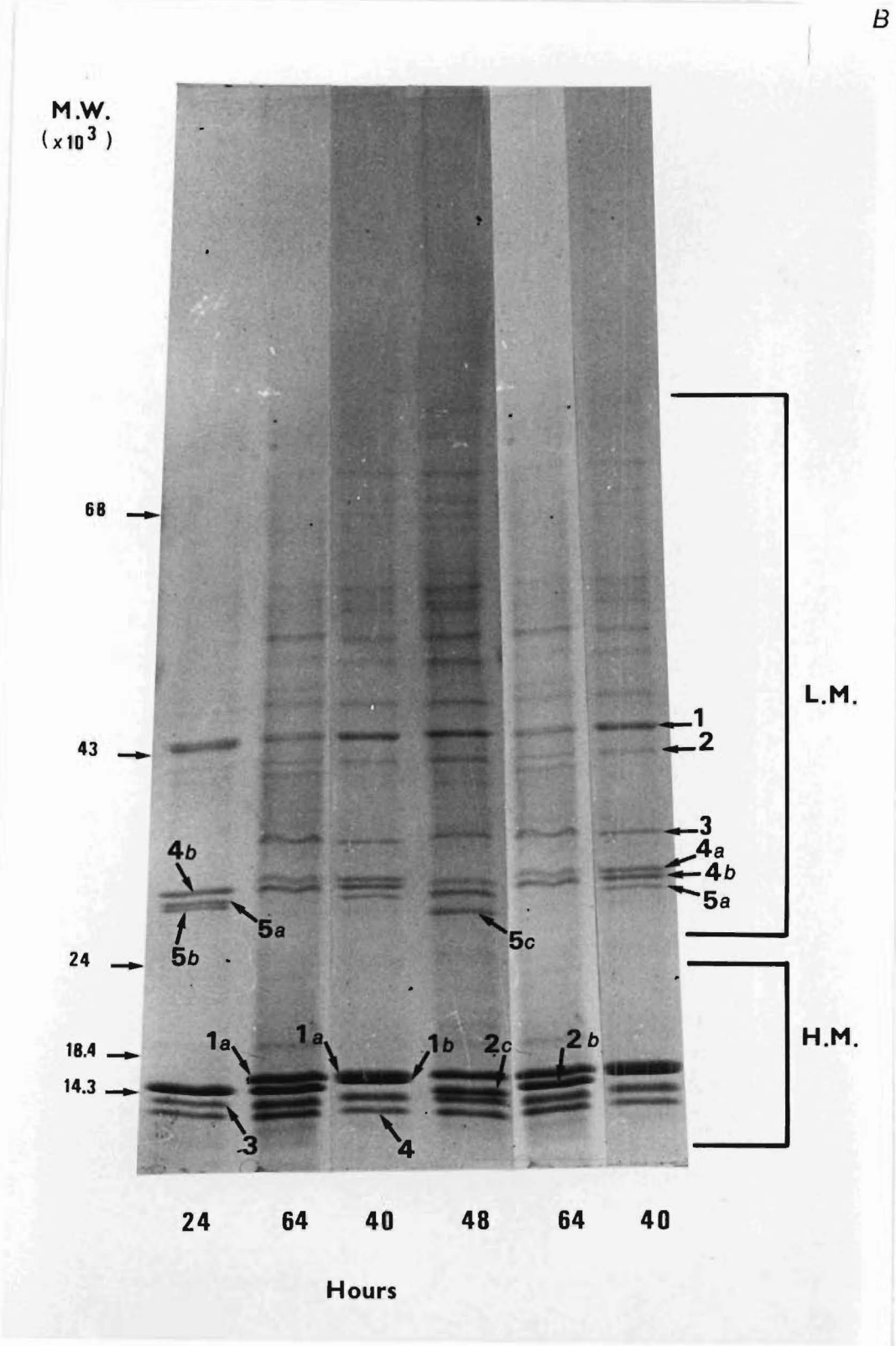
Group	Polypeptide	Mean M.W. (Kd)	Variant	Specific M.W.(Kd)	Pattern	F	n
L.M.	4	30.5	4a	30.8			
			4b	30.3			
	5	29.5	5a	29.7			
			5b	29.2			
H.M.	1	15.0	1a	15.2	1a	36.4	150
			1b	14.9	1b	42.3	
					1a + 1b	21.3	
	2	14.6	2a	14.7	2a	37.3	150
			2b	14.5	2b	23.1	
			2c	14.4	2c	7.3	
					2a + 2b	24.1	
					2b + 2c	8.2	
					2a + 2c	0	
	3	13.9	3a	14.2	3a	4.2	200
			3b	14.0	3b	65.8	
			3c	13.8	3c	24.2	
					3a + 3b	2.5	
					3b + 3c	4.0	
					3a + 3c	0	
	4	13.0	4a	13.1	4a	93.7	200
			4b	12.8	4b	4.2	
					4a + 4b	2.1	

Fig. 7

Electrophoreograms of LCG tissue homogenate obtained with SDS-DATD-acrylamide linear gradient gels (15-25% acrylamide) highlighting the occurrence of variant forms amongst the polypeptides composing the HM (a) and LM (b) groups. The patterns shown in Fig(a) were obtained during the course of five different experiments, while those shown in Fig(b) were taken from a single gel.

* : Patterns seldom observed at these stages of the reproductive cycle.


A
M.W.
(x10³)
68
43
24
18.4
14.3
16
24
48
64
88
96
106
Time (hours)


B
M.W.
(x10³)
68
43
24
18.4
14.3
4b
5a
5b
5c
1a
1a
1b
2c
2b
3
4
1
2
3
4a
4b
5a
L.M.
H.M.
24
64
40
48
64
40
Hours

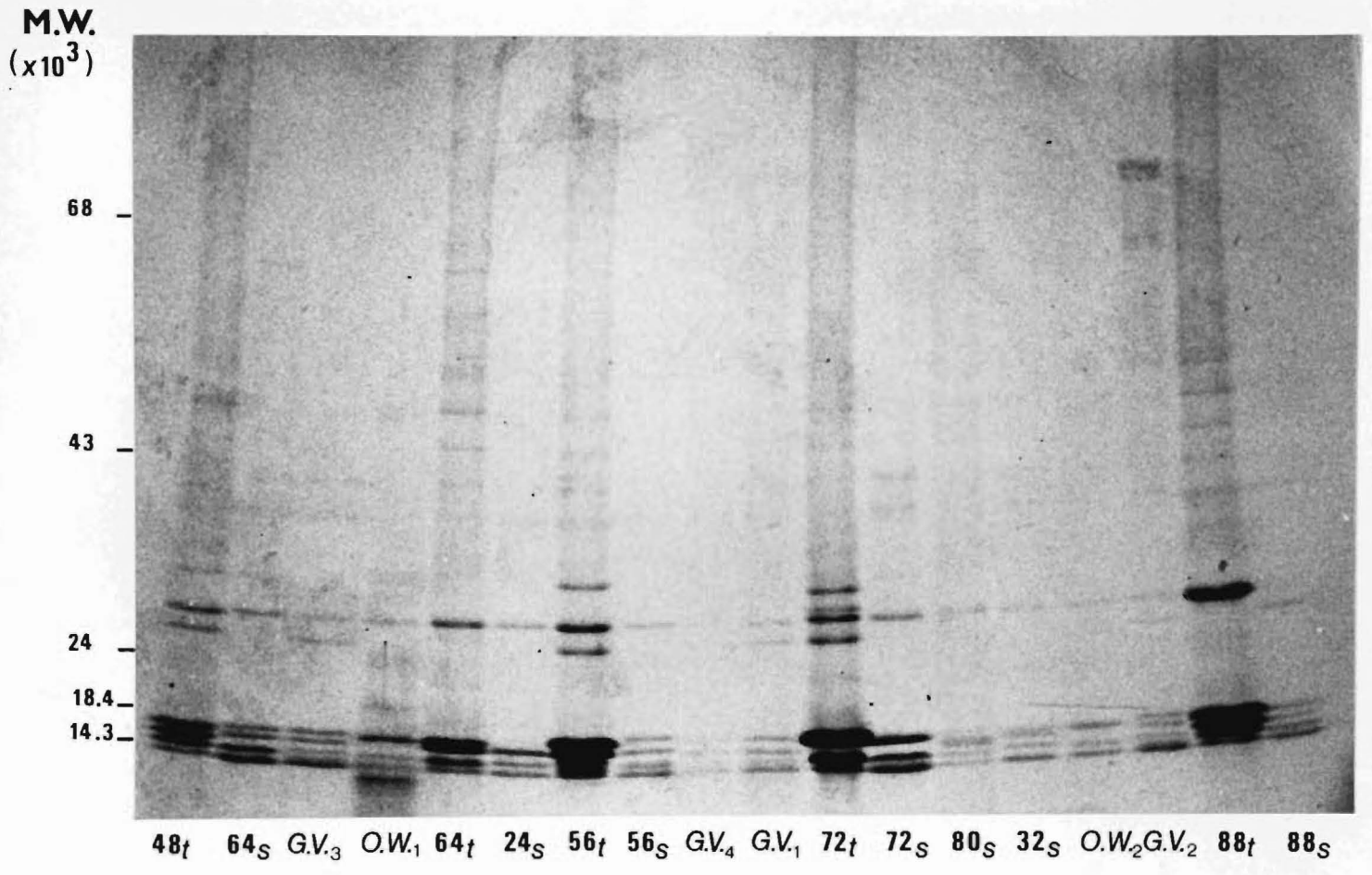

M.W.
(x10³)
68
43
24
18.4
14.3
48t
64s
G.V.3
O.W.1
64t
24s
56t
56s
G.V.4
G.V.1
72t
72s
80s
32s
O.W.2
G.V.2
88t
88s
Time (hours)

Fig. 8

Comparison of electrophoretic patterns presented by LCG tissue homogenate (t) and material resulting from induced secretory activity (s) in LCG s isolated at different stages of the reproductive cycle. Material collected *in situ* from the genital vestibulum (G.V.) of different animals and extracts from newly formed oothecal walls (O.W.) were also included.

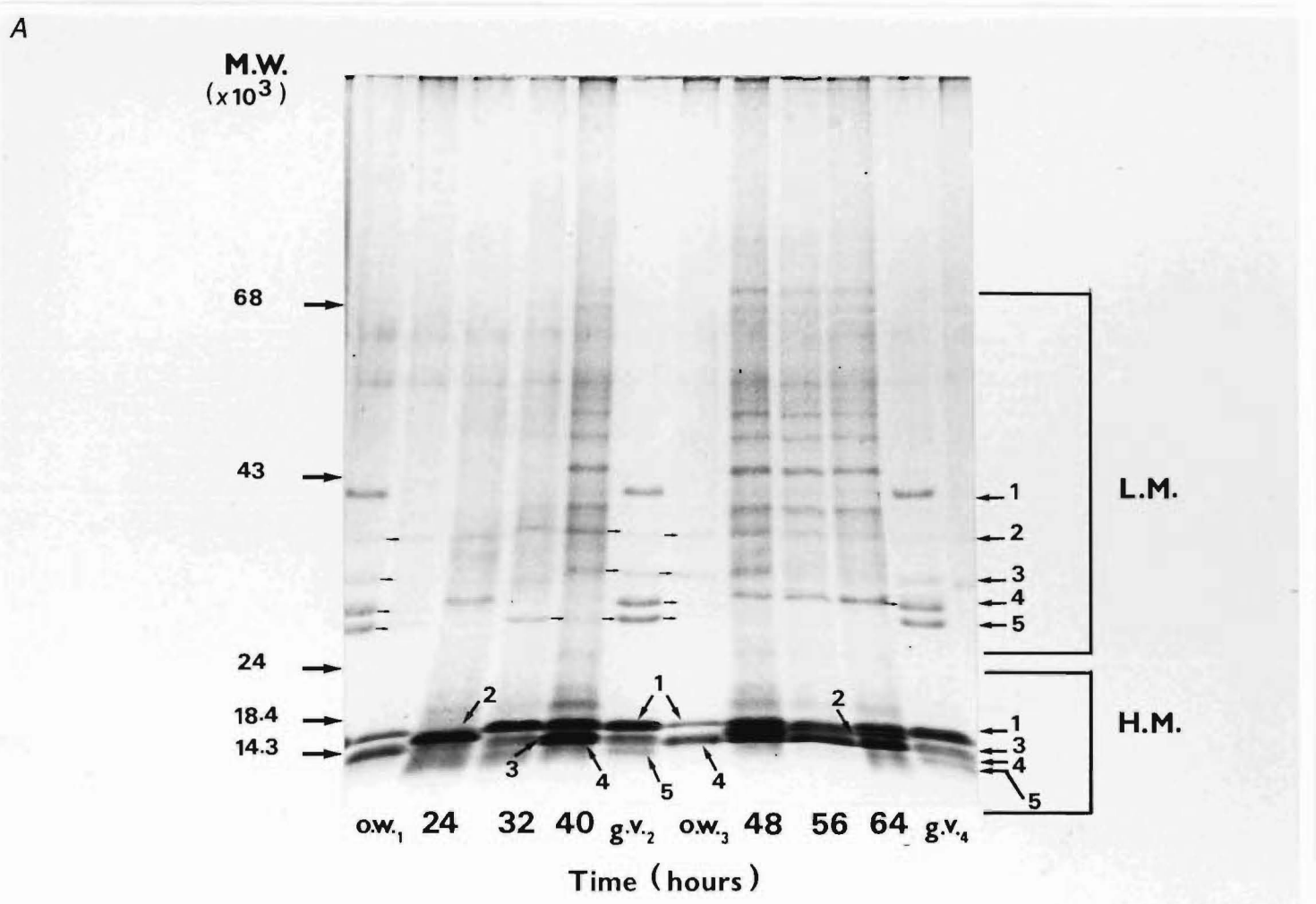
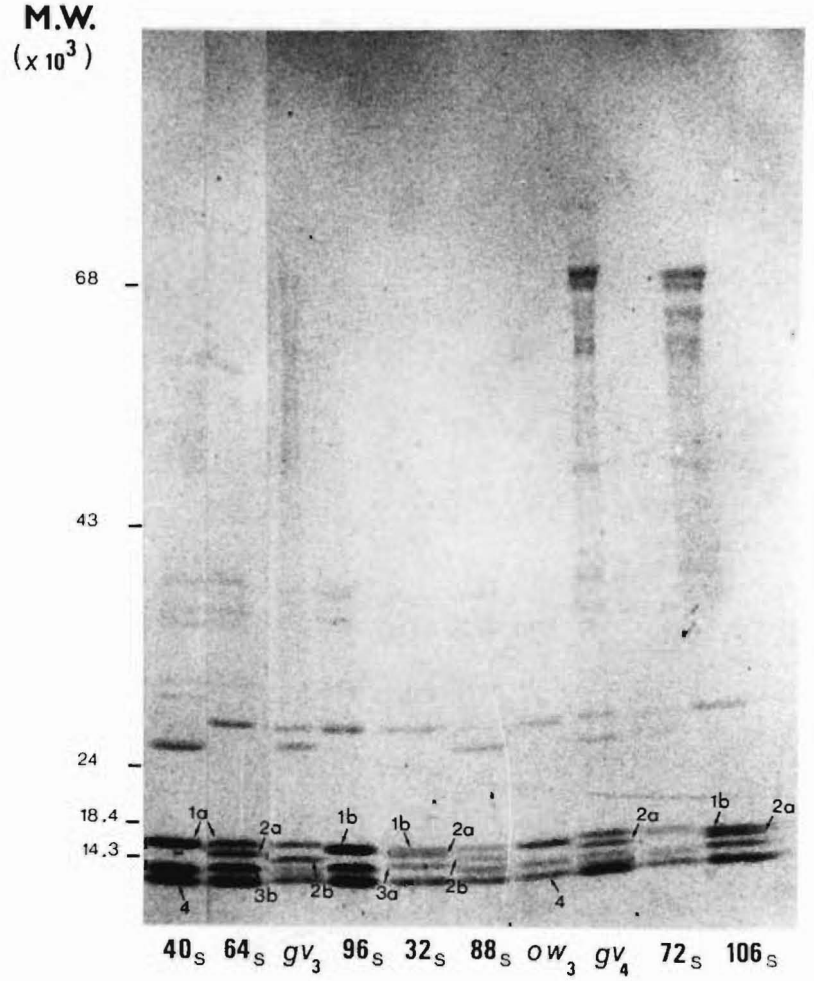

A
M.W.
(x10³)
68
43
24
18.4
14.3
L.M.
H.M.
1
2
3
4
5
o.w.₁ 24 32 40 g.v.₂ o.w.₃ 48 56 64 g.v.₄
Time (hours)

Fig. 9

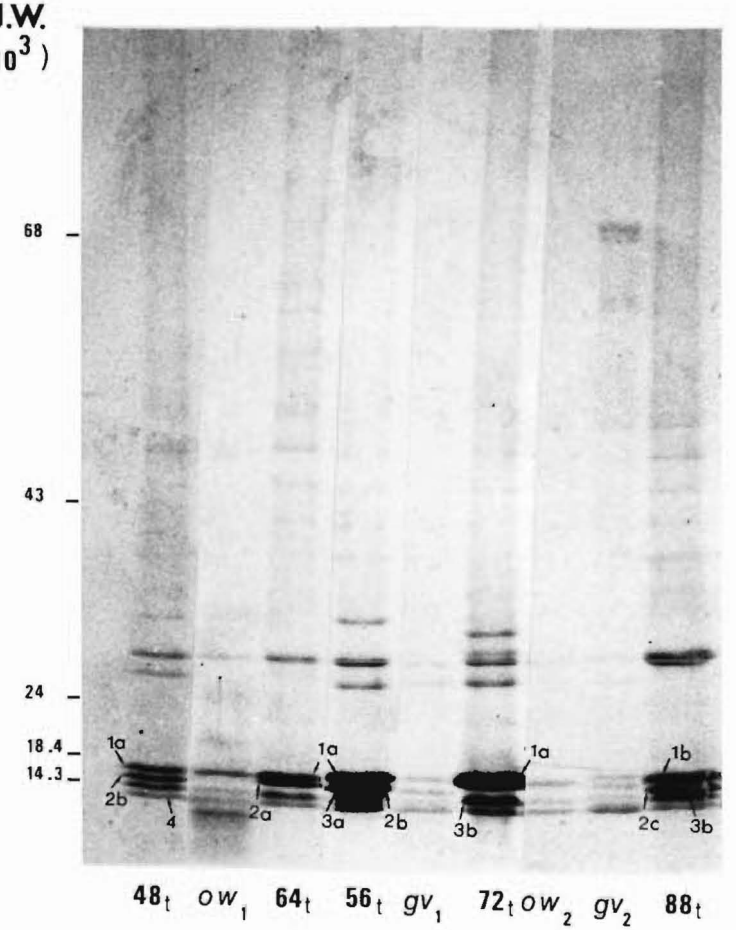
Electrophoretic comparison of polypeptides from LCG homogenate, material secreted *in vitro* by isolated glands, samples collected *in situ* from the genital vestibulum of several different animals at 64 h of the reproductive cycle and oothecal wall extracts of newly formed oothecae removed from different animals at 72 h (the samples were different from those in Fig. 8). The homogenate samples in Fig.(a) were identical to those of Fig. 5, whereas those of Figs. (b) and (c) were obtained during three different experiments. Electrophoresis was carried out in SDS-polyacrylamide (a) and SDS-DATD-acrylamide (b and c) linear gradient gels. The sample preparation, sample size and conditions of electrophoresis were as indicated in the text. However, the SDS-polyacrylamide gradient was 10-20%.

C



Time (hours)

B



Time (hours)

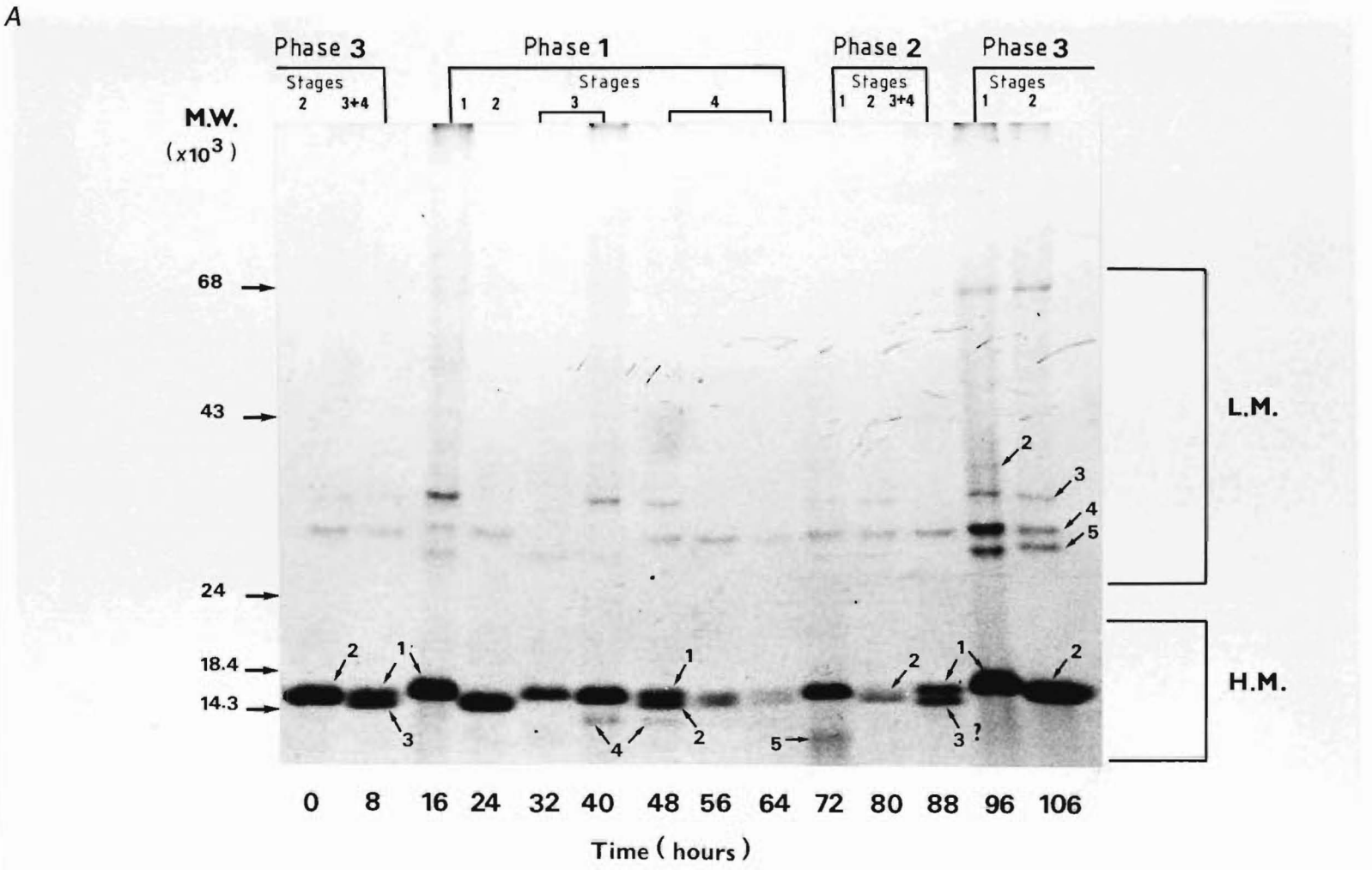
oothecal walls (labelled O.W.) contained eight polypeptides which were also detected in material from the genital vestibulum (Figs. 9a and 9b). Protein LM 2 was abundant in the genital vestibulum and in traces in the gland extracts (Figs. 5 to 7) but it was not detected in the oothecal wall. Taking into consideration the effects due to individual variation, the electrophoretic patterns presented by vestibular and oothecal materials obtained from randomly selected females were very consistent.

VI) Patterns of protein synthesis in the LCG during the oothecal cycle

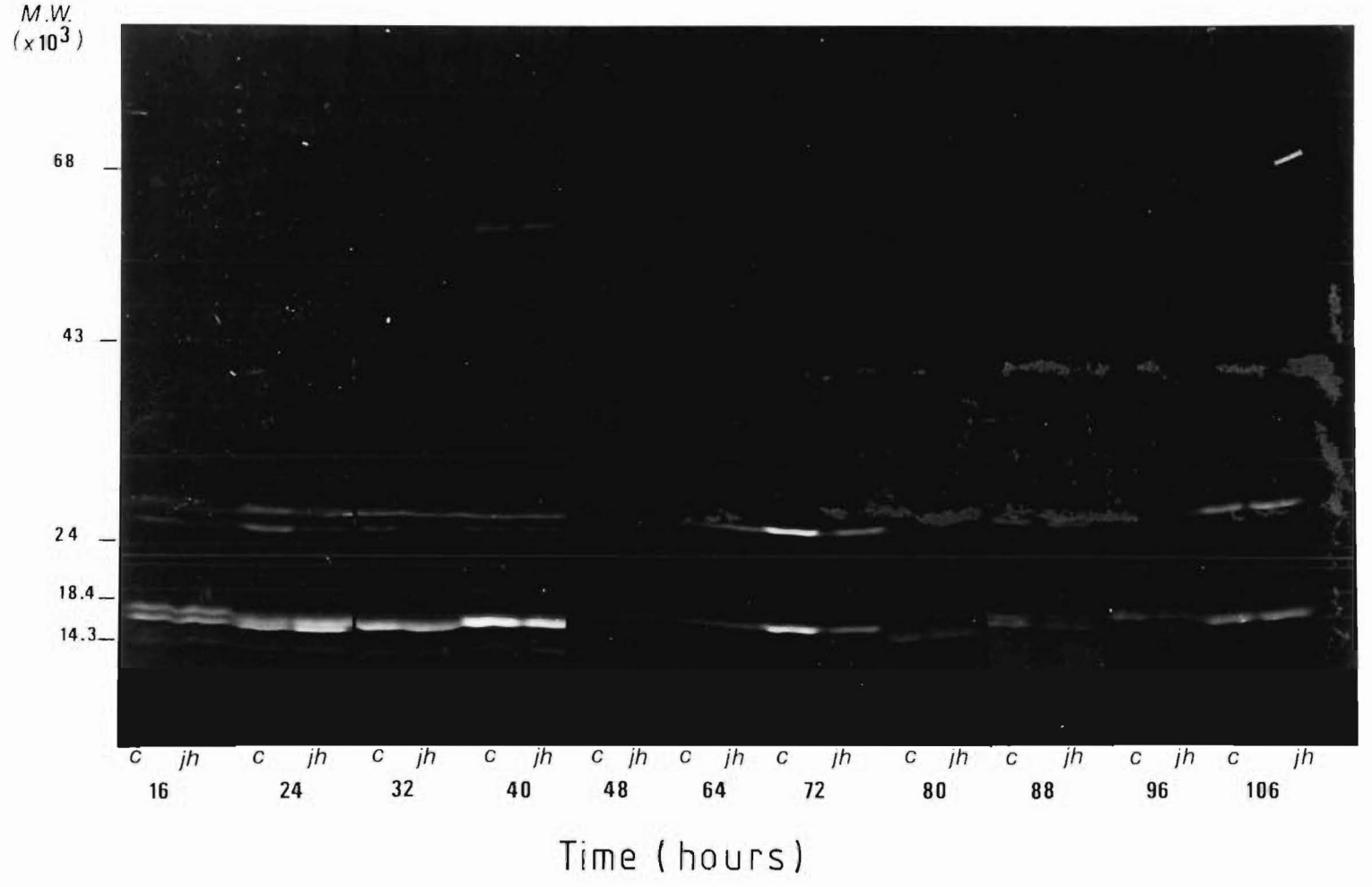
The fluorographic patterns (Figs. 10a and 10b) indicate that polypeptides LM 3 to 6 and HM 1 to 5 were synthesized by the LCG during the reproductive cycle, albeit at different times. Taking into consideration the effects of individual genetic variation, the general pattern of protein synthesis in the LCG over the duration of the reproductive cycle appeared to consist of three very similar phases, each containing four different patterns of synthetic activities (stages 1 to 4) especially evident in the HM group. The first (an arbitrary designation) of these apparently repetitive phases was initiated at 16h (stage 1). This was followed by stage 2 at 24h, then stage 3 from 32 to 40h and stage 4 from 48 to 64h. The second repetitive phase appeared to start soon after the onset of ovulation, with stage 1 occurring at 72h, stage 2 occurring at 80h while stages 3 and 4 appeared to be contracted into one single stage at 88h. Eight hours after completion of the ootheca, at 96h, the third repetition of this cycle of synthetic activity started with a pattern of synthesis nearly identical to those observed at 16 and 72h (stage 1) followed by stage 2 at 106 and 0h.

Fig. 10

Fluorographs showing the polypeptide species synthesized in the LCG at each stage of the reproductive cycle. Fig.(a) was developed from the gel presented in Fig. 5. The apparent repetitive phases of translational activities and their different stages are indicated. The patterns presented in Fig.(b) were obtained from two different fluorographs developed from SDS-DATD-acrylamide gradient gels representing different experiments.


A
Phase 3
Phase 1
Phase 2
Phase 3
Stages
2 3+4
Stages
1 2 3 4
Stages
1 2 3+4
Stages
1 2
M.W.
(x10³)
68
43
24
18.4
14.3
L.M.
H.M.
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

B


M.W.
(x10³)
68
43
24
18.4
14.3
c jh c jh c jh c jh c jh c jh c jh c jh c jh c jh c jh
16 24 32 40 48 64 72 80 88 96 106
Time (hours)

Stages 3 and 4 contracted at 8h. Several differences between each of these apparently repetitive phases were apparent in the patterns of synthesis of the HM group (bands 1 and 2). Apart from the high level of heterogeneity found in the electrophoretic mobilities of these two polypeptide species, some individuals presented deviations from the stage-specific patterns of synthesis. At 16h, the presence of a variant of polypeptide HM 2 was often seen in the pattern of synthesis, although its identity was very unpredictable. A similar observation applied to the patterns presented by various LCG s at 24, 96 and 106h (Table 4). However, the appearance in the patterns of variant forms of a polypeptide normally absent from the consensus electrophoretic pattern specific to any given stage of the reproductive cycle, was rare. Table 5 summarizes the protein species detected from various sources and their timing of synthesis.

DISCUSSION

The present study revealed that the total protein content of the LCG fluctuates throughout the reproductive cycle with a clear cyclic pattern. In the cycle duration used here, ootheca synthesis is visually detectable at 72h of each reproductive cycle and the egg-case is completely formed at 88h, its anterior end sealed but not yet sclerotized. Thus, while the massive fall in protein content detected between 64 and 72h and possibly that observed between 80 and 88h, can be explained in terms of ootheca synthesis, the substantial changes in total protein recorded between 8 and 24h and again between 88 and 96h cannot

Table 5.

Distribution and timing of synthesis of the major colleterial polypeptides during the reproductive cycle. Distinction between variant forms of any given polypeptide were not considered and the molecular weights indicated represent the mean apparent value for each major colleterial protein. The numbers in parentheses indicate proteins in oothecal wall and LCG secretion detected by Pau *et al.* (1971).

Table 5. Molecular weights, distribution and timing of synthesis of the major colleterial polypeptides during the reproductive cycle.

	Band number	M.W. (x 10^3)	LCG Homogenate	LCG Secretion	Vestibular Material	Oothecal Wall	Time of synthesis (h)
L.M.	1 (4)	45.0	+	?	-	-	N.D.
	2	37.5	+	+	+	+	96; low levels at 0,8 and 106 h
	3	33.0	+	+	+	+	0,8,16°,40,48,72,80,96, 106 h
	4	30.5	+	+	+	+	All except 32 and 40 h
	5	29.5	+	+	+	+	All except 32,40,64 and 88 h
H.M.	1 (3)	15.0	+	+	+	+	All except 24*,80 and 106*h
	2	14.5	+	+	-	-	All except 32,40,72 and 96*h
	3 (2)	14.0	+	+	+	?	All stages
	4 (1)	13.0	+	+	+	+	All stages

be accounted for by the utilization of proteins for the synthesis of the egg-case. At these stages, the genital vestibulum of the experimental animals was invariably found to be empty of secretory material. Although turnover of colleterial proteins at these stages cannot be ruled out, it is more probable that the decreases could be due to loss through export of colleterial proteins into the haemolymph.

Proteins are not the only materials synthesized and secreted by the LCG. Phenol glucosides and calcium oxalate crystals are also major components of the LCG secretory products (Brunet and Kent, 1955; Stay et al., 1960; Bodenstein and Shaaya, 1968). The lack of correspondence between total protein content and wet weight of the gland is probably due to the non-concordant increase in the levels of non-proteinaceous components and proteins in the gland. The non-proteinaceous components involved here have not been investigated. They could include phenolic glucosides, water, as well as unidentified materials.

A functional relationship might exist between the patterns of protein accumulation and synthesis in the LCG and the concurrent profiles of oocyte maturation during the reproductive cycle. The present study indicates that vitellogenesis in T oocytes terminates at about 56h. The second peak in levels of protein synthesis in the LCG occurs at 56h while the maximum in protein content of the gland is reached eight hours later, at 64h. Bell (1969b) reported that, during the reproductive cycle, females of P. americana increase their food intake toward the end of vitellogenesis in T oocytes, when the rise in haemolymph proteins is almost at peak level.

There are two studies on the rate of protein synthesis in the LCG of the cockroaches Blatella germanica (Zalokar, 1968) and P. americana (Weaver, 1981). It should be pointed out that B. germanica

carries the ootheca until hatching of the progeny occurs, in contrast to P. americana which deposits the ootheca before maturation of the embryos. Zalokar reported a gradual and linear increase in the level of glycine incorporation into colleterial proteins over the period preceding synthesis of the egg-case; and Weaver reported a uniform rate of leucine incorporation into oothecin (polypeptides HM 1 to 4 in the present study) during the reproductive cycle. The present investigation, in contrast to the above studies, indicates that leucine incorporation in the LCG of P. americana fluctuates throughout the reproductive cycle. These discrepancies may be due to different experimental protocols being followed. In the present study, protein synthetic activity was assayed every eight hours throughout the cycle, while previous studies measured the synthetic activities of the LCG on a twenty four hour basis. Thus, rapid changes detected in this study may not have been detected by a twenty four hour protocol. During the course of this study, it was noted that practically all females were prone to show random and short lived lengthening or shortening of their reproductive cycle, irrespective of the time spent under controlled rearing conditions. In order to obtain reproducibility it was found absolutely essential to use females showing consistent reproductive synchrony.

Although high rates of leucine incorporation can often be correlated with increases in total protein content of the gland, this is not the rule. For instance, between 24 and 32h, and between 72 and 80h the rate of leucine incorporation decreases although the protein content of the gland increases. Uptake of haemolyph proteins may explain the increases in the protein content of the LCG during these periods when the level of leucine incorporation is low. It has been suggested that

the epithelial cells of the LCG sequester and hydrolyze blood proteins to obtain amino acids for the synthesis of oothecal structural proteins (Adiyodi, 1968; Bell, 1969).

The major electrophoretic analysis on oothecal structural proteins of P. americana has been that of Pau et al. (1971). They found five major proteins which were common to both the secretion of the LCG and oothecal wall extracts. In the present investigation at least twenty protein species were detected in the artificially induced secretion, eleven in the material from the genital vestibulum and eight in the extracts of the white oothecal wall. The discrepancy in the number of proteins found in the three sources may be due to a number of reasons.

i) Although sclerotization had not had any visible effects on the oothecal wall at the time of collection, the cross-linking reaction between the o-quinones and proteins could have started and therefore may have effectively made some proteins unextractable.

ii) The poor solubilities of the vestibular material and the LCG secretion may render electrophoretic comparison difficult.

iii) The LCG secretion was induced artificially and therefore may not represent the true secretion in vivo.

It was suggested earlier in the discussion that the LCG may be involved in the synthesis of specific proteins for export. This contention is further reinforced by the detection of a major colleterial protein, HM 2. Its synthesis was detected at ten points of the reproductive cycle, but this polypeptide was detected in neither the vestibular material nor in the oothecal wall. It was detected in the secretion. However, this may be the result of artificial release from the epithelial cells. Furthermore, this protein invariably disappears from the tissue extract shortly after its synthesis. It is interesting

to note that this protein has not been detected by previous workers (Pau et al., 1971; Weaver, 1981). This may be due to the improved resolution in the gradient gels used in the present study. If the LCG has, as is generally assumed, the production of oothecal structural proteins for unique function (Pau et al., 1971; Weaver and Pau, 1982), then what would be the purpose of the HM 2 protein? It could be argued that the apparent changes in the electrophoretic patterns at different stages of the reproductive cycle do not represent actual changes in the pattern of gene activity but merely reflect individual genetic variation with respect to the presence or absence of different members of the same multigene family. If this was correct, it would then be expected that the electrophoretic patterns of material from the genital vestibulum or ootheca wall extracts obtained from randomly selected adult females would present at least some degree of variability as to the specific protein species to be found in these materials. Yet, despite numerous electrophoretic analyses of such samples, this has never been the case.

Another anomaly is observed in the protein HM 1. This protein is synthesized in three different periods of the reproductive cycle and only one can be related to ootheca synthesis (that is between 32 and 72h). More importantly, this protein synthesized between 8 and 16h and between 88 and 96h is never accumulated in the tissue after its synthesis. Yet a protein with similar electrophoretic mobility was detected in the vestibular material and in the oothecal wall. HM 1 is similar in molecular weight to oothecin III of Pau (1981) and Weaver (1981). Thus the timing of its synthesis and the pattern of accumulation are apparently inconsistent with its assumed structural role. A possible explanation is that there are two proteins with very similar molecular weights, which cannot be separated in SDS-gel electrophoresis; one is an

oothecal structural protein synthesized prior to ootheca formation (from 32 to 72h); the other one is synthesized at times apparently unrelated to ootheca formation but is exported, and is thus never accumulated for a long enough period in the LCG to allow for its detection after synthesis. However, it must be stressed that post-translational modifications may also explain the lack of accumulation as well as the detection of several variant forms of the major colleterial polypeptides.

The significance of the apparently repetitive phases in the pattern of protein synthesis is not clear. Whether or not this cyclic pattern is related to the cyclic release of JH during the reproductive cycle in P. americana (Weaver et al., 1975; Weaver and Pratt, 1977) is not yet clear. Oocyte maturation (Scharrer, 1946; Thomas and Nation, 1966; Lüsher and Lanzrein, 1976; Lanzrein et al., 1978; Stay and Tobe, 1977; Tobe and Stay, 1977) is known to be under the control of JH. In the present study, a good correlation was obtained between the timing of maximum leucine incorporation into colleterial polypeptides and the periods of maximum rate of growth of T and T-1 oocytes. The effects of JH on the synthesis of specific colleterial proteins and the changes in the endogenous titre of JH during the reproductive cycle must be known before any conclusion can be made.

SUMMARY

This study compared the wet weight, protein content and protein synthesis in the LCG at different time points during the reproductive cycle of the cockroach Periplaneta americana. It was found that:

1) The wet weight of the gland fluctuates throughout the cycle. Only one of these changes can be related to egg-case synthesis.

2) The protein content of the gland also fluctuates with three cyclic changes. Again, only one of these changes can be correlated with ootheca synthesis.

3) Leucine incorporation *in vitro* into colleterial protein is not uniform throughout the cycle. The highest incorporation levels coincide with the greatest increases in the protein content of the gland and with the greatest rate of oocyte growth.

4) Electrophoretic analyses of the proteins extracted from the LCG reveals qualitative and quantitative changes. Of all the protein species detected in the gland extracts, at least eight were also detected in the artificially induced secretion, material collected from the genital vestibulum and the newly formed egg-case. All these proteins are synthesized in the gland.

5) The timing of synthesis of some major colleterial proteins, in relation to ootheca synthesis, and the lack of long term accumulation after synthesis suggests that the LCG could be involved in the production of proteins for export as well as for ootheca synthesis.

CHAPTER THREE

PATTERNS OF TRANSCRIPTIONAL ACTIVITIES IN RELATION TO TRANSLATIONAL ACTIVITIES IN THE LEFT COLLETERIAL GLAND DURING THE REPRODUCTIVE CYCLE

INTRODUCTION

The left colleterial gland (LCG) of Periplaneta americana has recently been recognized as a promising organ for the study of gene regulation by juvenile hormones (JH) (Weaver 1981; Weaver and Pau 1982). Indeed, this organ presents substantial advantages over the fat body system as an experimental tool. The LCG is large, easy to dissect in relatively intact condition and can be maintained in culture for long periods (Chapter II, p 11). Moreover, the LCG produces large quantities of proteins at a rapid rate (Bodenstein and Shaaya 1968). The predominant protein species produced by this accessory sex gland have been electrophoretically characterised (Pau et al. 1971; Pau 1981; Iris and Sin 1984). However, in order to utilize the LCG as an investigative tool in the study of the regulation of protein synthesis by JH, the patterns of macromolecule synthesis in this organ during the reproductive cycle must be known. The synthetic activities of the LCG in relation to the oothecal cycle and the effects of JH upon them are not yet fully understood. Studies investigating these aspects are few and present a confusing picture with conflicting evidence (see Zalokar 1968; Shaaya and Bodenstein 1969; Weaver et al. 1975 ; Weaver 1981). In the previous chapter, it was shown that, contrary to previous reports (Weaver 1981), protein synthesis in the LCG fluctuates during each reproductive cycle. The cyclic changes coincide with the known periodic release of JH during the reproductive cycle. It is still, however, very unclear whether or not the changes in patterns of protein synthesis are the results of activated translation of pre-existing m-RNA or specific gene activation. No detailed study has so far been carried out on the patterns of RNA synthesis in the LCG during the reproductive cycle. In view of the

stimulatory effects of JH upon transcriptional activity (Zalokar 1968; Nair and Menon 1972; Wyatt et al. 1980) and the very rapid changes in patterns of protein synthesis occuring in the LCG during the reproductive cycle (Chapter II, section VI, pp 36-39) ,it is essential that the patterns of RNA synthesis be known in order to further our understanding of gene regulation by the juvenile hormones.

MATERIAL AND METHODS

1. Rearing and tissue culture conditions.

Mature females of P. americana were reared, staged and the LCG excised and cultured according to the protocol described in the previous chapter (Sections 1 and 2, pp 10-11).

2. Incorporation of nucleosides into colleterial RNA.

Incubations of isolated LCG s were carried out in 100 µl of complete incubation medium (Chapter II, section 2, p 11 and Table 1,p 12) supplemented with equal amounts of [5,6-^{3}H] uridine, [2-^{3}H] adenosine, [5-^{3}H] cytidine and [8-^{3}H] guanosine (The Radiochemical Centre, Amersham, U.K.; spec. act. 50 Ci, 20 Ci, 29 Ci and 11 Ci/mMol respectively) to a final total specific activity of 0.25 µCi/µl. The rate of tritium incorporation into nucleic acids was found to be linear over a period of 4 to 5 hours depending on the stage of the cycle. Routine nucleic acid labelling was thus carried out at 25°C ± 1°C for 3.5 h. In

order to insure the validity of the correlation between RNA synthesis and leucine incorporation into LCG polypeptides, RNA and protein synthesis were assayed simultaneously by dual labelling procedures using leucine-free medium supplemented with L-[U-^{14}C] leucine (The Radiochemical Centre, Amersham, U.K.; spec. act. 340 mCi/m Mol) to a final specific activity of 1.25 x 10^{-2} µCi/µl, and containing the nucleosides as above.

3. RNA extraction and purification.

All glassware, Eppendorf tubes and distilled water used were autoclaved. The buffers were sterilized by membrane filtration (0.45 µm). Extraction of nucleic acids from individual LCG s were carried out according to the method described for Drosophila melanogaster by Barnett et al. (1980) with the following modifications. Each gland was frozen and broken down with five strokes in an all-glass manual homogenizer. Then 100 µl of 150 mM spermine was added and the tissue was further homogenised with two to three stokes. A 100 µl aliquot of extraction buffer (x2, pH 9.0) was added and homogenization was continued until total disruption of the gland was achieved. The tissue homogenate was transferred to diethyl-pyrocarbonate (0.2% DEPC) treated, sterile Eppendorf tubes kept on ice. A 20 µl aliquot was taken for liquid scintillation counting, protein determination and electrophoretic analysis on SDS polyacrylamide gels.The remaining tissue homogenate was used for RNA extraction. The all glass homogenizer was rinsed twice with 200 µl 88% phenol:$CHCl_3$:iso-amyl alcohol (v/v/v, 25:25:1). The solvent washes were added to the tissue homogenate. After addition of an equal volume of buffer saturated phenol, the Eppendorf tubes containing the

tissue homogenate and organic solvents were shaken for 10 min and centrifuged at 18000xg for 10 min at 4°c. The aqueous phase was collected by suction, 100 µl of extraction buffer and 300 µl of phenol:$CHCl_3$: isoamyl alcohol were added to the interphase and the organic phase and the above operation repeated. The aqueous phase was collected and the organic phase was discarded. Then 100 µl of extraction buffer and 250 µl of $CHCl_3$ were added to the flocculent material for further extraction. After shaking and centrifugation, the aqueous phase was collected and the organic phase was discarded. Fifty µl of extraction buffer was added to the thin, white,tightly packed interphase. The flocculent material was thoroughly resuspended by shaking for 10 min and 250 µl of $CHCl_3$ was then added. The tubes were shaken for 5 min, centrifuged for 5 min, the aqueous phase collected and the flocculent interphase extracted once more. All the aqueous fractions were pooled and re-extracted with 300 µl of $CHCl_3$ until complete disappearance of an interphase (usually twice). The final aqueous phase was adjusted to 400 mM NaOAc and the nucleic acid was precipitated by addition of 2.5 volumes of cold absolute ethanol:ether (v/v,2:1) and storage at -20°c overnight or at -70°c for 3.5-4 h. The nucleic acids were pelleted by centrifugation at 18000xg for 15 min at 4°c. The pellet was resuspended in 3.0 M NaOAc and left standing at 0°c for 1.5 to 2 h. The extracted nucleic acids were subsequently washed twice in cold 70% ethanol, dried under vacuum and dissolved in 300 µl of 10 mM NaOAc (total volume) for spectrophotometric analysis, quantification and liquid scintillation counting. The nucleic acid solution was then treated with DNase I (RNase-free; Sigma) for 30 min at 0°c according to the method of Brown and Suzuki (1974). The remaining RNA was re-extracted once with phenol:$CHCl_3$ (v/v, 1:1), and twice with $CHCl_3$, precipitated with absolute ethanol:ether (v/v, 2:1),

washed three times and reanalysed as above. This procedure gave a final product in high yield, free of any detectable protein and DNA contamination. Control DNase treatments carried out on a) calf liver RNA, and b) adult D. melanogaster total RNA, obtained by the above method, (range 30 µg to 210 µg RNA/ml), for as long as 12 hours showed no substantial RNA loss due to residual RNase activity. The final RNA pellet was kept under absolute ethanol at -20°c until used.

4. Nucleic acid quantification and determination of tritium incorporation levels.

The purity of the nucleic acids extracted was determined by U.V. absorbtion spectra (190nm to 350nm), and RNA was quantified by 1) U.V. spectrophotometric measurement (A_{260}) and 2) RNA quantitative estimation according to the orcinol method of Huberman and Attardi (1966) on 50 µl aliquots. In both cases, calf liver RNA (Sigma) was used as standard. In view of the good agreement obtained between the orcinol and spectrophotometric methods for RNA quantification, routine determinations were carried out on the basis of A_{260} and U.V. absorbtion spectra.

The levels of tritium incorporation into total extractable nucleic acids and total extractable RNA were estimated by liquid scintillation counting of 10 µl aliquots (duplicates) in a Beckmann LS 2800 LSC with a counting efficiency of 59.3%. The levels of radiolabel incorporation into total nucleic acids and proteins were estimated by dual label scintillation counting of TCA precipitated homogenate resolubilised in 1.0 M NaOH (5 µl aliquots, duplicates), with counting efficiencies of

59.3% (^{3}H) and 79.4% (^{14}C).

5. Purification of poly(A)$^{+}$ RNA.

Poly(A)$^{+}$ RNA was isolated from total extractable RNA by chromatography on oligo(dT) cellulose (Sigma) according to the method of Aviv and Leder (1972) modified as follows. The binding buffer was 10.0 mM Tris-HCl, pH 7.6; 1.0 mM ethylene-diamine-tetra-acetic acid (EDTA); 400 mM NaOAc; 0.05% SDS (sterile). The elution buffer was 10.0 mM Tris-HCl, pH 7.6; 1.0 mM EDTA (sterile). Each sample was passed through two oligo(dT) columns. The unbound U.V. absorbing fractions were pooled, freeze-dried and redissolved in 500 µl sterile distilled water and the RNA precipitated. The poly(A)$^{+}$ fractions were pooled and treated in the same manner but adjusted to 400 mM NaOAc before precipitation. After two successive washings in 70% ethanol, the RNA pellet was redissolved in 300 µl of sterile distilled water for analyses. The purified poly(A)$^{-}$ RNA and poly(A)$^{+}$ RNA were stored under absolute ethanol at -20°C until used. The efficiency of the oligo(dT) cellulose columns was tested using total RNA extracted from D. melanogaster. The amount of total RNA loaded, ranging from 5.0 to 100 µg RNA/250 µl, had no effect on the efficiency of the columns. Freshly packed columns gave a poly(A)+ RNA yield corresponding to 4.51% ±0.13% of the total RNA loaded. This compared very well with the reported percentage of adult D. melanogaster total RNA represented by poly(A)$^{+}$ RNA (about 5.0%; Barnett et al. 1980; Schlief and Wensink,1981). However, columns that had been used more than five times produced eluates giving high absorbances at 260nm (Fig. 1b) even when no RNA was passed through (λ max:260 to 272 nm). Thus each oligo(dT) cellulose column was

subsequently regenerated for not more than four times.

6. Protein determination and electrophoretic analyses.

These analyses were carried out to verify the accuracy of the staging procedure. The protein content of each LCG was determined following the procedure previously described (Lowry et al. 1951).

Electrophoresis was carried out on linear gradient gels of acrylamide crosslinked with diallyltartardiamide (15 to 25% acrylamide;0.8% DATD). The buffer system was that of Laemmli (1971). Sample preparation and conditions of electrophoresis were as previously described (Chapter II, section 4, pp 15-16).

RESULTS

I) Efficiency of various RNA extraction procedures.

For the purpose of the present study, it was important to examine the efficiency of various procedures for RNA extraction. Five published methods were compared and one of these was eventually modified to obtain reproducible yields of relatively pure RNA. Table 1 shows the yields of RNA extracted from 88 h colleterial glands by the four protocols.

The method of Arthur et al. (1979) gave poor yields of nucleic acids from the LCG. Following DNase treatment, the UV spectra presented by the final product (Fig. 2a) indicated phenol contamination (large peak at 270 nm) as well as unsatisfactory deproteinization (shoulder at 280 nm). Attempts to recover poly $(A)^+$ RNA from this material were

Fig. 1

U.V. absorbtion spectra obtained with calf liver RNA (a; 45 µg RNA) and the eluate from an oligo (dT) cellulose column (b) regenerated for the fifth time. Buffers only had been passed through the column. The A_{260} value obtained from the RNA-free eluate corresponded to 50 µg RNA.

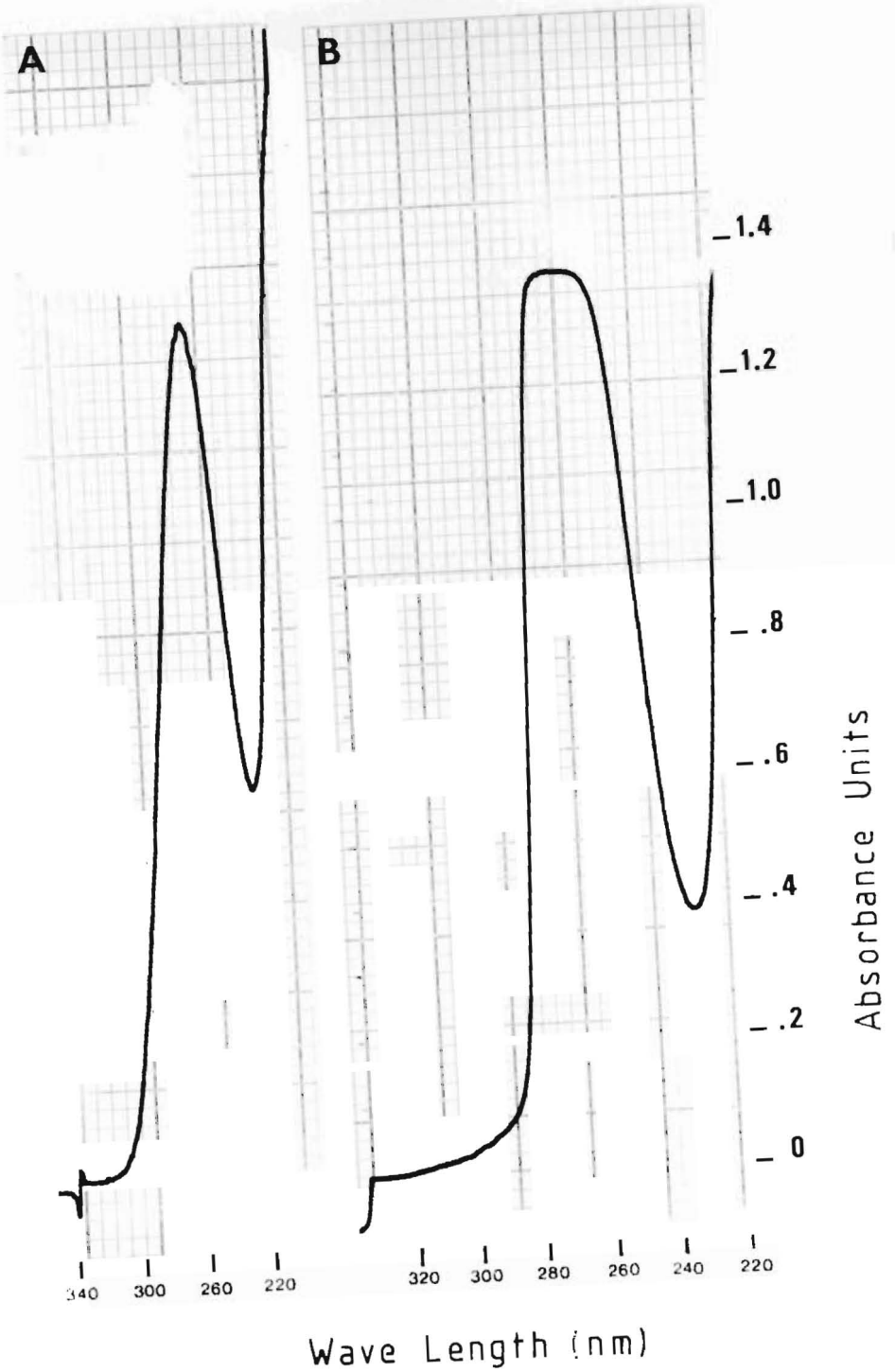

A
B
1.4
1.2
1.0
.8
.6
.4
.2
0
Absorbance Units
340
300
260
220
320
300
280
260
240
220
Wave Length (nm)

Fig. 2

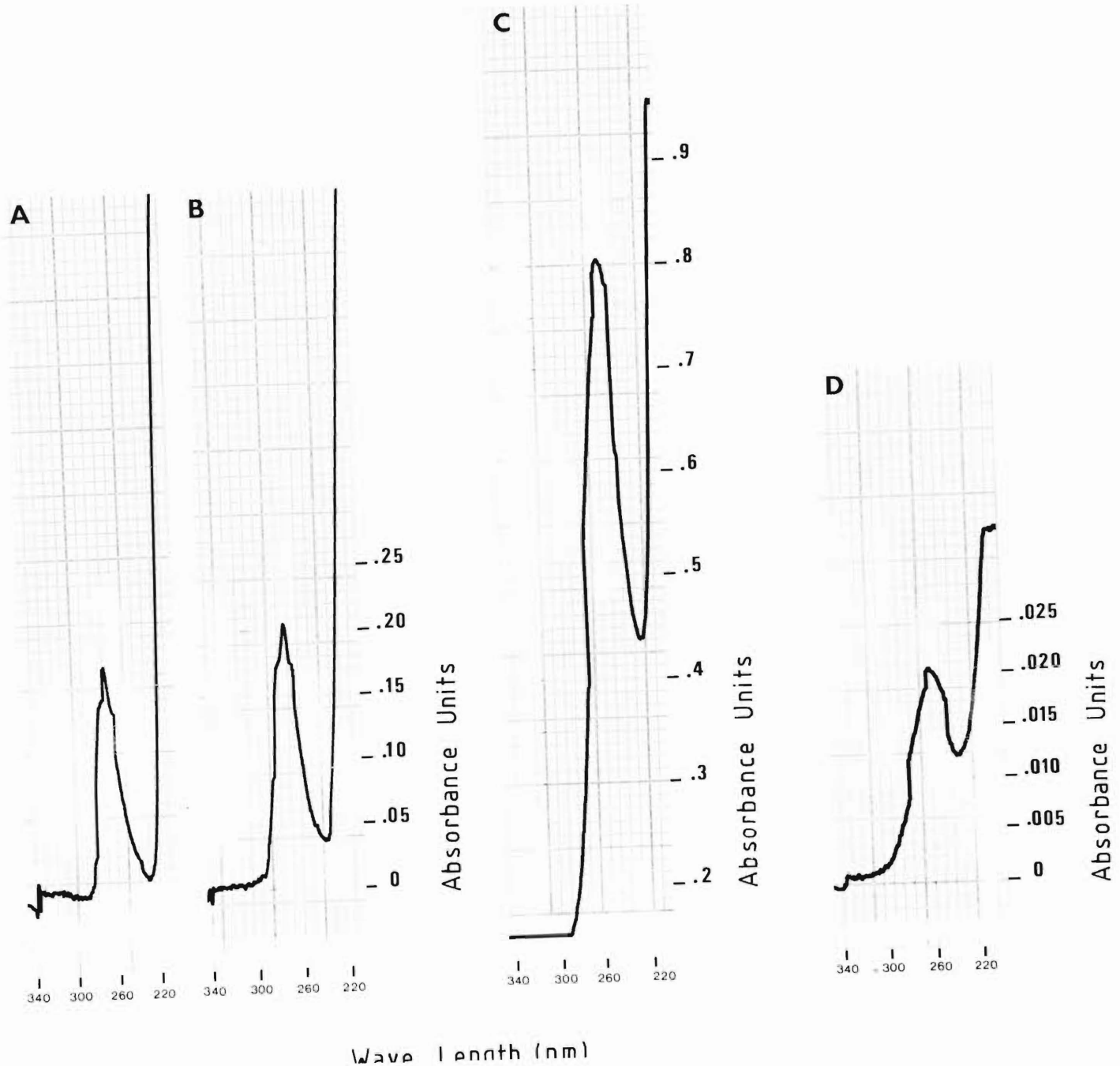
U.V. absorbtion spectra obtained with DNase treated colleterial RNA extracted from single LCGs according to the methods of:

(a) Arthur et al. (1979),

(b) White and De Lucca (1977; pH 9.0 procedure),

(c) White and De Lucca (1977; SDS-phenol-$CHCl_3$ procedure).

Fig. (d) shows the absorbtion spectrum of poly(A)$^+$ RNA recovered from colleterial total RNA extracted by the SDS-phenol-$CHCl_3$ method.


A
B
C
D
.25
.20
.15
.10
.05
0
.9
.8
.7
.6
.5
.4
.3
.2
.025
.020
.015
.010
.005
0
Absorbance Units
340
300
260
220
Wave Length (nm)

unsuccessful. The procedures described by White and De Lucca (1977) for the extraction of RNA from locust fat body (pH 9.0) and whole Drosophila (SDS-phenol-$CHCl_3$) were also tested. The "pH 9.0" protocol gave, with the LCG, results equivalent to those obtained with the method of Arthur et al. (Fig. 2b), whereas the phenol-$CHCl_3$ procedure produced considerably higher RNA yields (Fig. 2c). UV spectral analyses of the final product indicated that while phenol contamination had been removed, deproteinization was unsatisfactory and the RNA obtained was heavily contaminated by a variety of organic materials (shoulders at 248, 255 and 265 nm). Low levels of poly $(A)^+$ RNA could be recovered from these samples by affinity chromatography (Fig. 2d). Attempts were made to modify this method so as to improve yield and purity of the final product. By using buffers at pH 9.0, repeatedly re-extracting the flocculent interphase in fresh buffer and organic solvents, and thoroughly purifying the pooled aqueous phases before precipitating the nucleic acids, the yield and purity of the final product were considerably increased (Fig. 3a). However, phenol contamination remained a problem and the amounts of poly $(A)^+$ RNA recovered were low (Fig. 3b). The procedure described by Barnett et al. (1980) for the extraction of RNA from D. melanogaster, used unmodified on the LCG, gave results equivalent to those obtained with the second protocol of White and De Lucca (Fig. 4a). However, the poly $(A)^+$ RNA yield was considerably greater (Fig. 4b). Fully modified, this method was found to produce consistently good yields of relatively pure RNA (compare Figs 5a, 1a and 6b) from which high levels of poly $(A)^+$ RNA could be recovered (Fig. 5b). The quantitative efficiency of this protocol was tested on known amounts of calf liver RNA (Sigma, USA) mixed with colleterial secretion. The level of calf liver RNA recovery was 91.8± 2.1% (n=4)

Fig. 3

U.V. absorbtion spectra of total colleterial RNA (a; post-DNase) and the corresponding poly(A)$^+$ RNA fraction (b) isolated by affinity chromatography on oligo (dT) cellulose, following extraction from a single gland according to the modified version of the SDS-phenol-$CHCl_3$ method of White and De Lucca (1977).

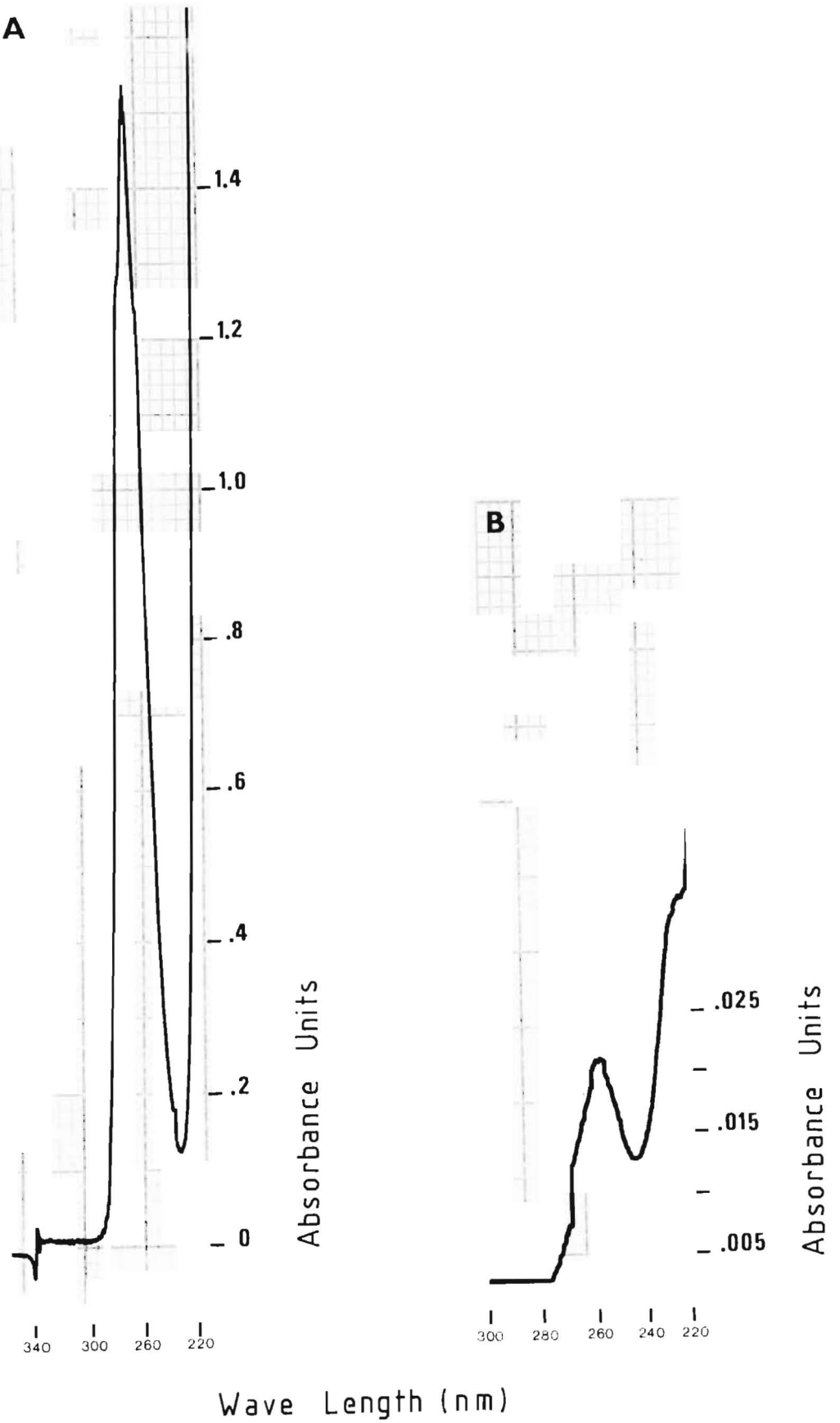

A
1.4
1.2
1.0
.8
.6
.4
.2
0
Absorbance Units
340
300
260
220
B
.025
.015
.005
Absorbance Units
300
280
260
240
220
Wave Length (nm)

Fig. 4

U.V. absorbtion spectra of colleterial RNA extracted from a single LCG according to the method of Barnett *et al.* (1980).

(a) Total colleterial RNA after DNase digest,

(b) Poly(A)$^+$ RNA fraction isolated from total RNA.

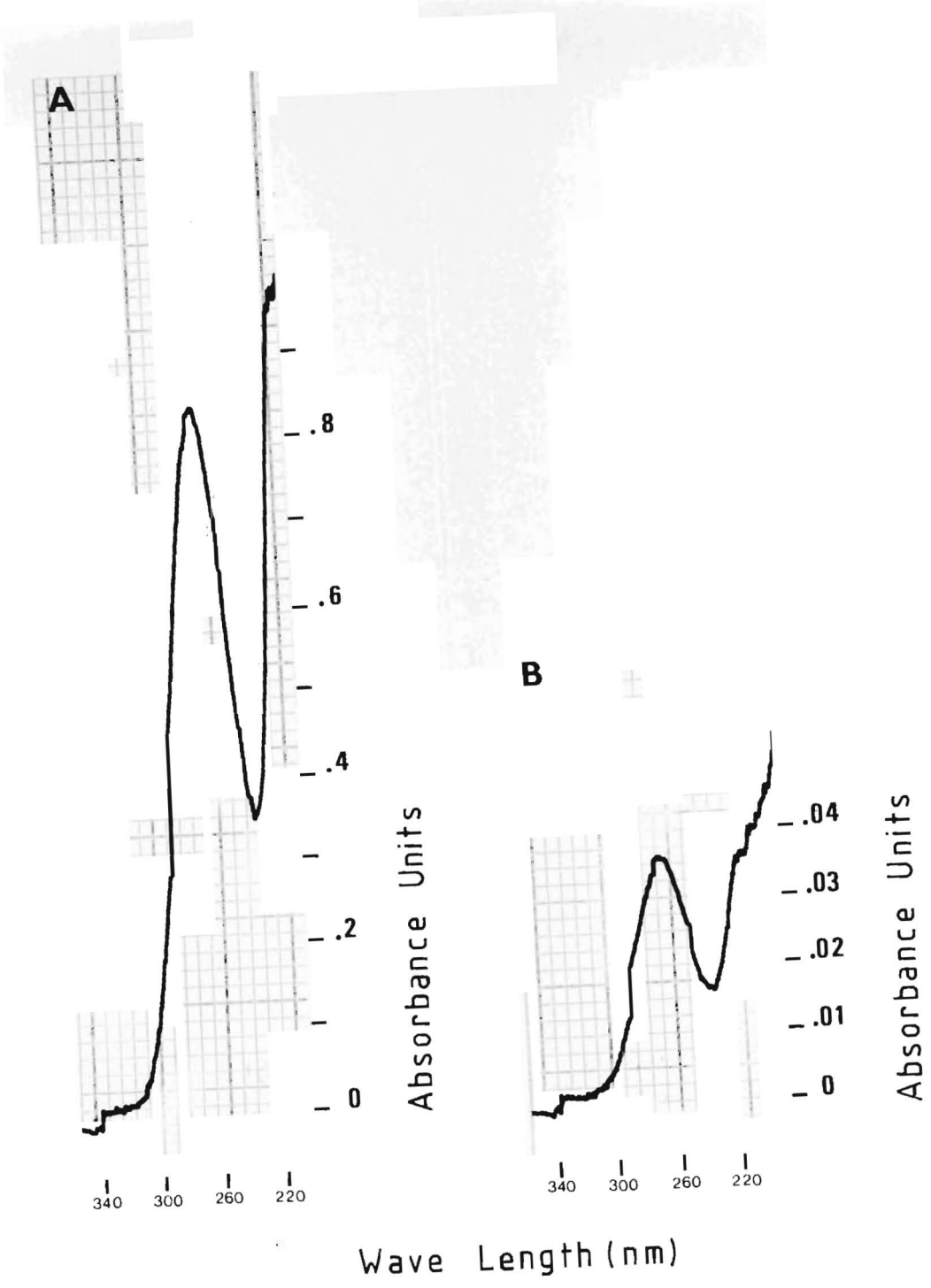

A
.8
.6
.4
.2
0
Absorbance Units
340
300
260
220
B
.04
.03
.02
.01
0
Absorbance Units
340
300
260
220
Wave Length (nm)

Fig. 5

U.V. absorbtion spectra presented by colleterial RNA extracted from a single LCG, isolated at 88 h of the cycle, according to the modified version of the method of Barnett et al. (1980).

(a) Total RNA (DNase treated),

(b) Poly(A)$^+$ RNA fraction recovered from total RNA.

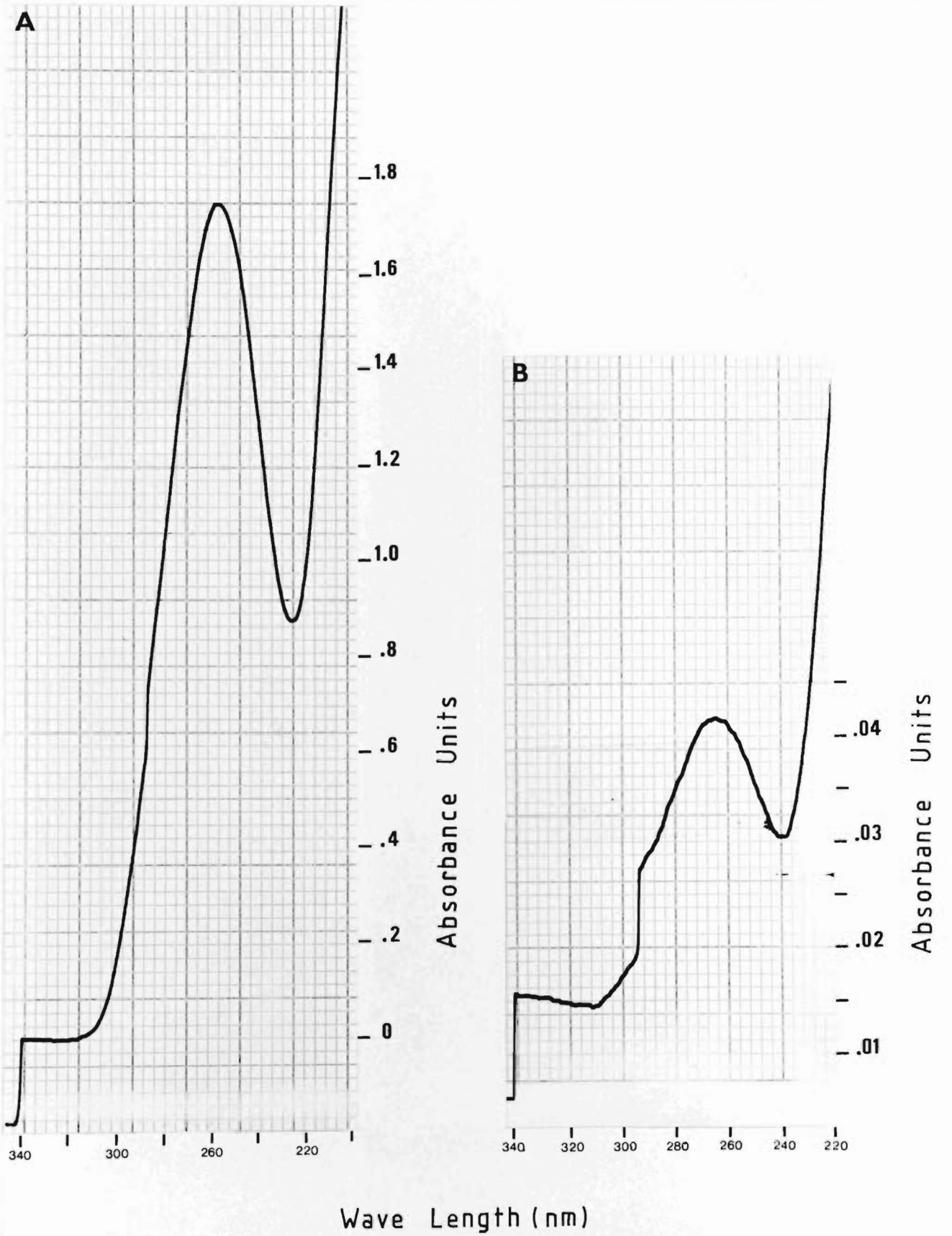

A
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
Absorbance Units
340
300
260
220
B
.04
.03
.02
.01
Absorbance Units
340
320
300
280
260
240
220
Wave Length (nm)

Fig. 6

Assessment of the quantitative efficiency of the modified version of the RNA extraction procedure of Barnett *et al.* (1980).

Known amounts of calf liver RNA (range: 5 to 85 µg) were mixed with material released *in vitro* by isolated LCGs. The RNA was then extracted according to the protocol described, including DNase treatment, and analysed and quantified by U.V. spectrophotometry.

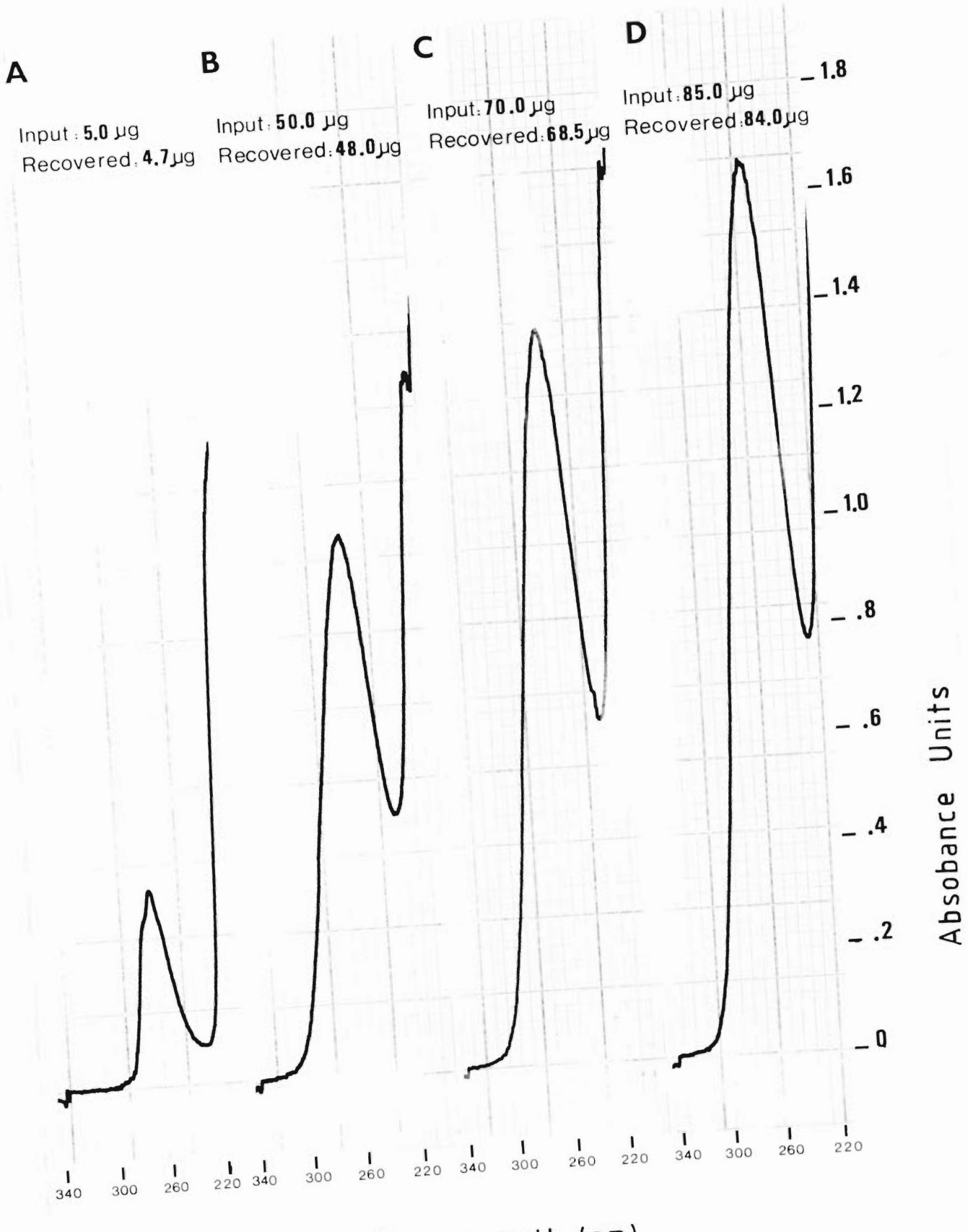

A
Input : 5.0 µg
Recovered : 4.7µg
B
Input : 50.0 µg
Recovered : 48.0µg
C
Input : 70.0 µg
Recovered : 68.5µg
D
Input : 85.0 µg
Recovered : 84.0µg
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
Absobance Units
340 300 260 220 340 300 260 220 340 300 260 220 340 300 260 220
Wave Length (nm)

Table 1.

Assessment of various RNA extraction procedures on single LCGs isolated at 88 h of the reproductive cycle.

1: Method of Arthur *et al.* (1979).

2: Method of White and De Lucca (1977; pH 9.0).

3: Method of White and De Lucca (1977; SDS-phenol-$CHCl_3$).

4: Modified version of method 3.

5: Method of Pau (1981).

6: Method of Barnett *et al.* (1980).

7: Modified version of method 6.

Table 1. Assessment or RNA extraction procedures on LCG's isolated from females at 88 h of the reproductive cycle.

METHODS	TOTAL N.A. YIELD per LCG (µg DNA+RNA)	TOTAL RNA YIELD Per LCG (µg)	POLY(A)$^+$ RNA (% of purified RNA)
1 and 2	18.8 ± 2.2 (n = 4)	18.8 ± 2.2	Not measurable
3	32.3 ± 8.8 (n = 4)	24.6 ± 8.0	1.95 ± 0.75
4	104.0 ± 10.7 (n = 4)	46.5 ± 9.6	3.10 ± 1.85
5	68.6 ± 9.0 (n = 4)	N.M.	N.M.
6	149.5 ± 17.0 (n = 4)	53.2 ± 6.4	3.60 ± 1.30
7	75.7 ± 3.3 (n = 4)	49.0 ± 1.6	4.40 ± 0.90

over the range 50 to 85 µg initial RNA input (Fig. 6).

II) Changes in LCG RNA content and synthesis in relation to protein synthetic activity during the oothecal cycle.

a) Total RNA content.

The amount of purified total RNA extracted from single glands at different stages of the oothecal cycle are shown in Fig.7a. A single phase of rapid increase in total extractable RNA was apparent over the period extending from 0 to 40 h. This was followed by a substantial fall over the next twenty four hours (from 40 to 72 h), after which the amount of total RNA extracted from each gland remained at a relatively stable level until the end of the cycle. The major phase of increase in the levels of total extractable RNA (from 16 to 32 h) occurred during a period of decrease in leucine incorporation into colleterial proteins (Fig. 7b). The largest amount of purified total RNA was obtained from LCG s at 40 h of the reproductive cycle.

b) Profile of [^{3}H] nucleosides incorporation into colleterial total RNA during the reproductive cycle.

The patterns of total RNA synthesis in the LCG fluctuated throughout the duration of the reproductive cycle (Fig.8a). Three large increases in levels of radiolabel incorporation were detected between 24

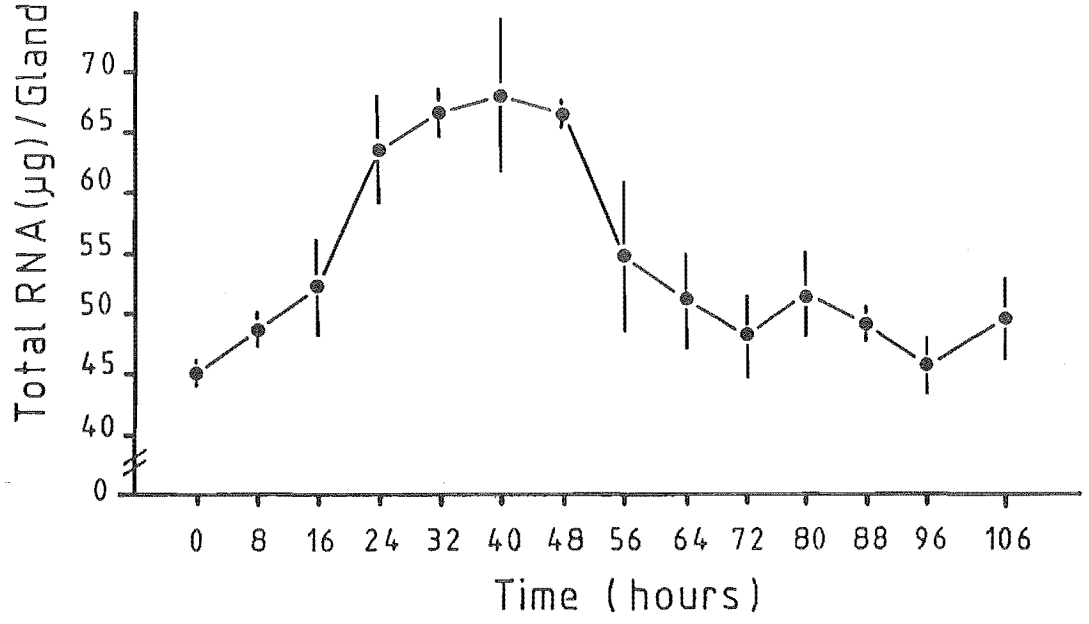
Fig. 7(a)

Changes in total RNA content of single LCGs during the reproductive cycle in mature females of P. americana. Each point represents the mean of, at least, three individual determinations. The SEM is shown for each point.

(b)

Comparison of the patterns of leucine incorporation into colleterial polypeptides (solid line) and the concurrent changes in total RNA content of single LCGs (dotted line) isolated at each stage of the reproductive cycle. Each point is based on, at least, three different animals. The vertical bars represent the SEM of each point.

A


Total RNA (µg) / Gland
70
65
60
55
50
45
40
0
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

B

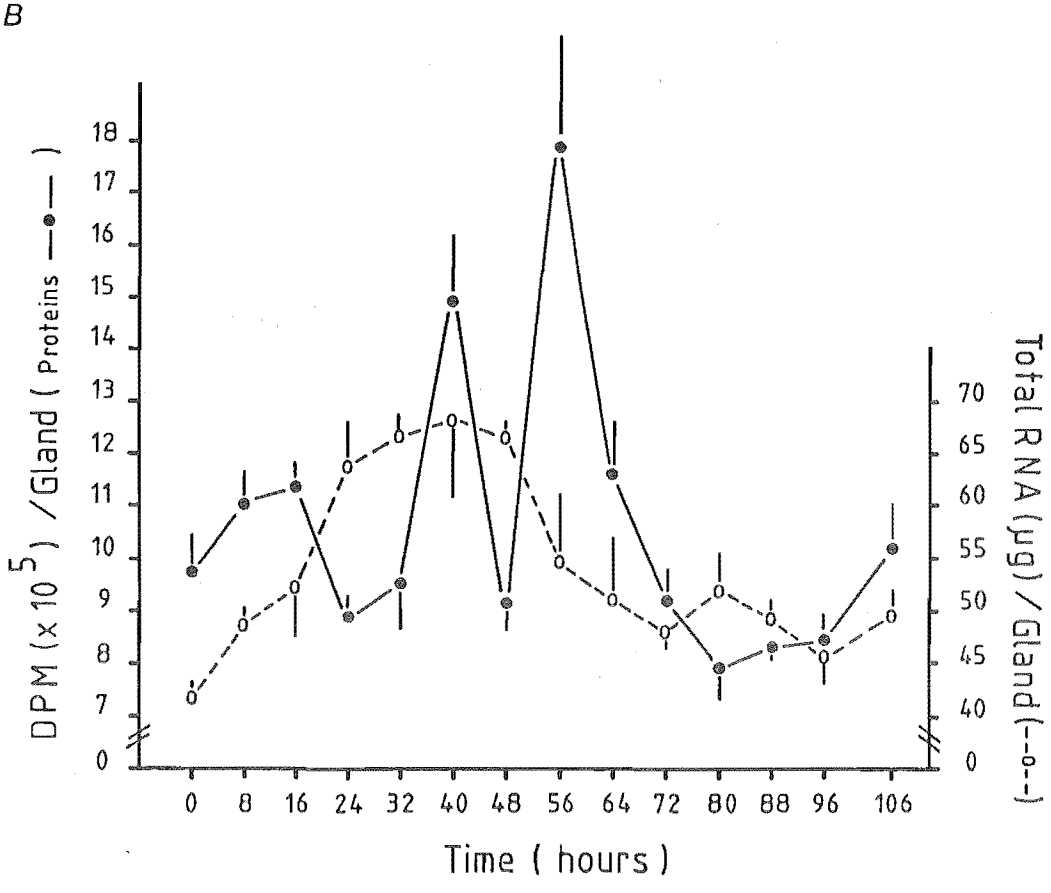

DPM (x 10^5) / Gland (Proteins —•—)
18
17
16
15
14
13
12
11
10
9
8
7
0
Total RNA (µg) / Gland (--o--)
70
65
60
55
50
45
40
0
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

Fig. 8(a)

Total RNA synthesis in individual LCGs determined at each stage of the reproductive cycle after *in vitro* incubation in the presence of tritiated nucleosides.

(b)

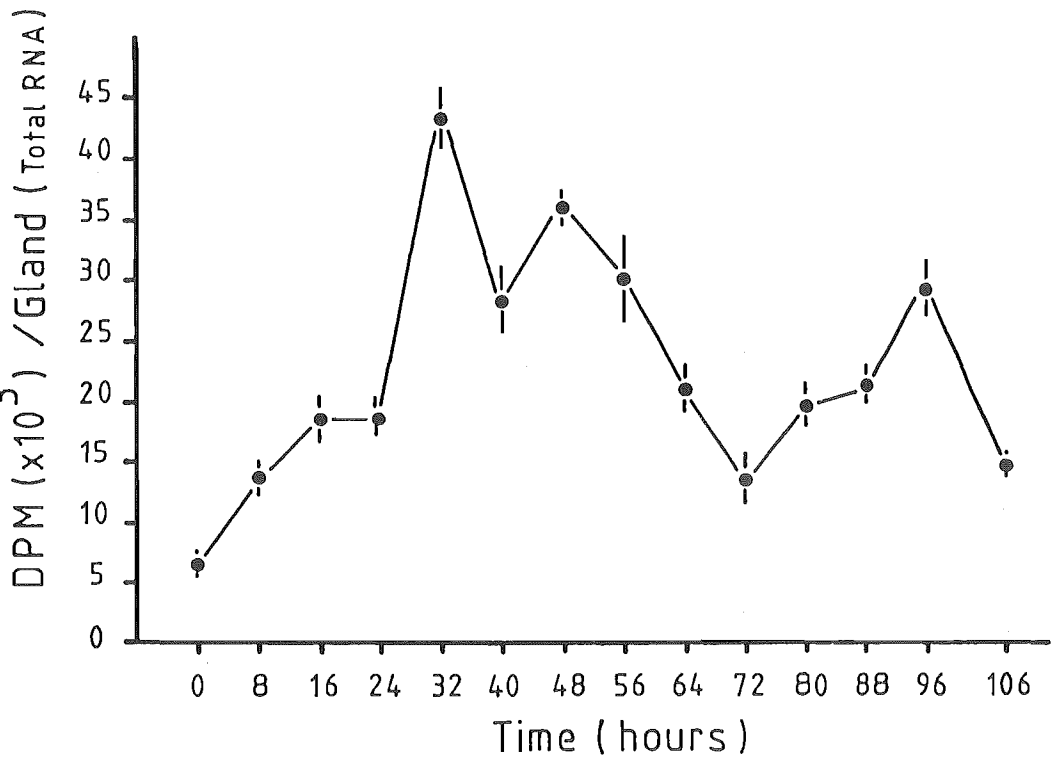
The translational activity (solid line) compared to the concurrent patterns of transcriptional activity (dotted line) in individual LCGs at each stage of the reproductive cycle.

(c)

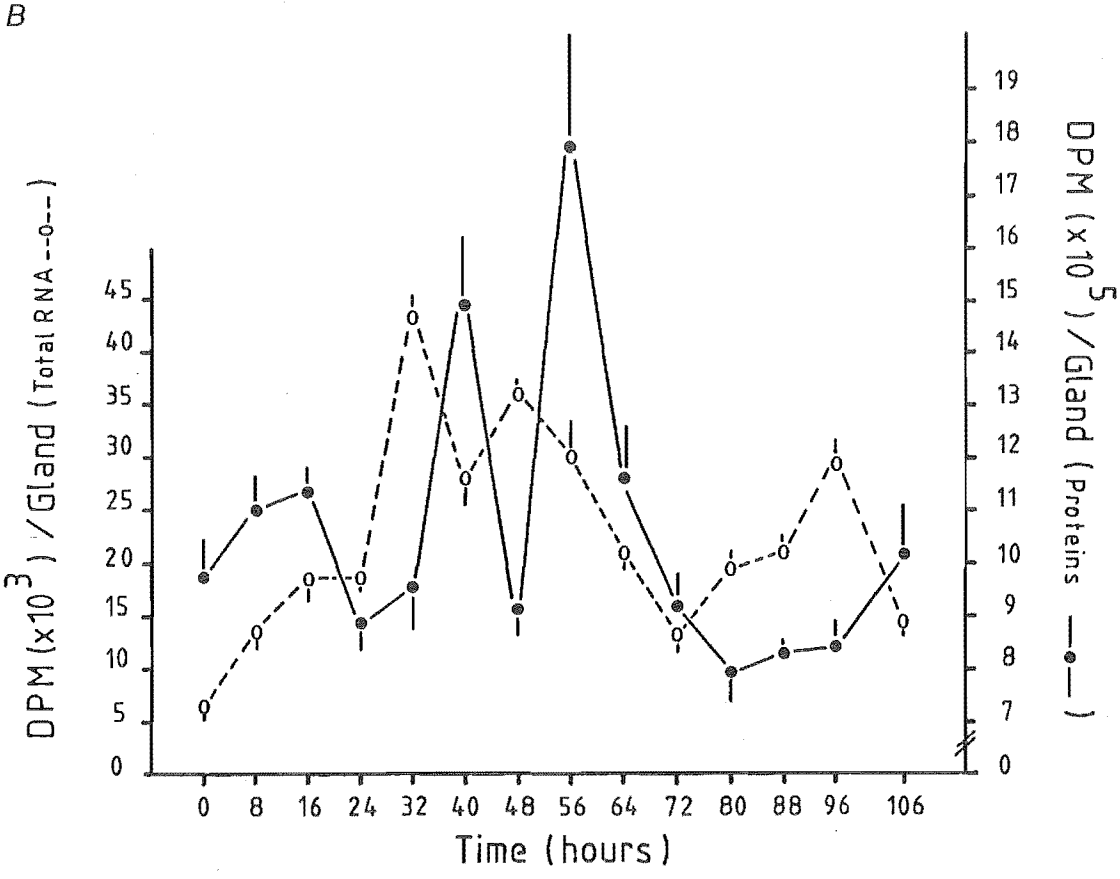
Comparison between total RNA synthesis (solid line) and total RNA content (dotted line) in single LCGs during the oothecal cycle.

Each point is the mean of, at least, three different animals and the SEM is shown for each point.

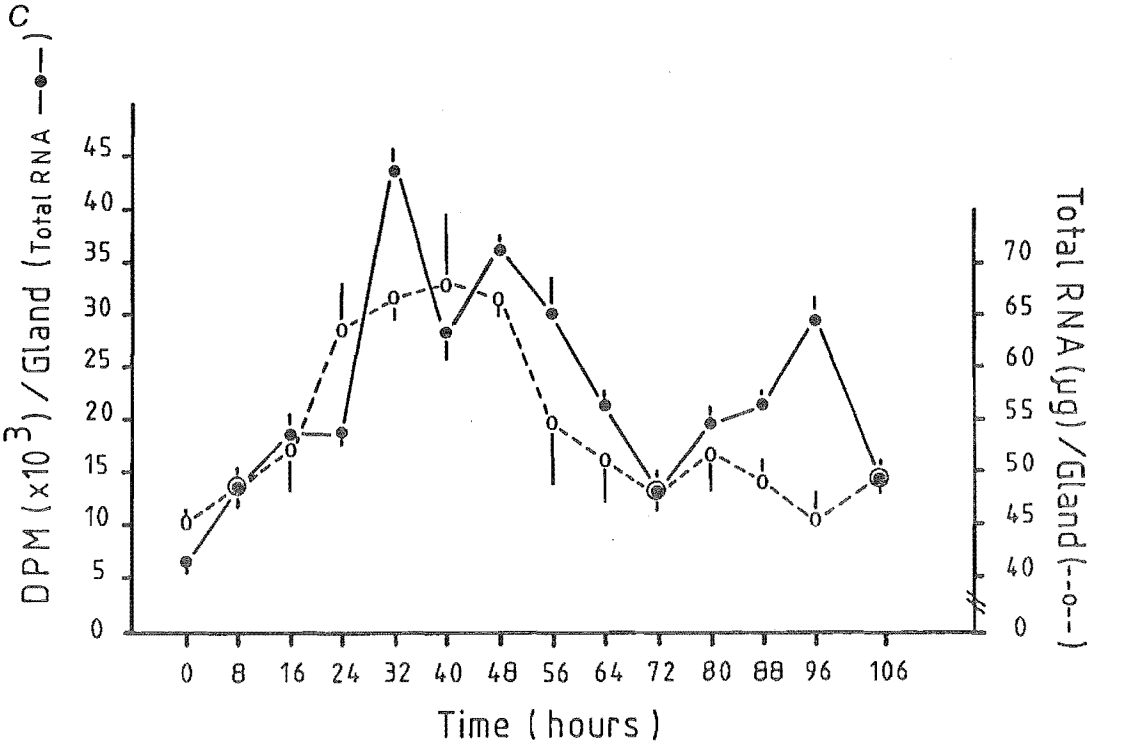
A


DPM (x10^3) /Gland (Total RNA)
0
5
10
15
20
25
30
35
40
45
0
8
16
24
32
40
48
56
64
72
80
88
96
106
Time (hours)

B


DPM (x10^3) / Gland (Total RNA --o--)
DPM (x10^5) / Gland (Proteins —●—)
Time (hours)
0 8 16 24 32 40 48 56 64 72 80 88 96 106
0 5 10 15 20 25 30 35 40 45
0 7 8 9 10 11 12 13 14 15 16 17 18 19

C


DPM (x10^3) / Gland (Total RNA —●—)
Total RNA (µg) / Gland (--o--)
Time (hours)
0 8 16 24 32 40 48 56 64 72 80 88 96 106
0 5 10 15 20 25 30 35 40 45
0 40 45 50 55 60 65 70

and 32 h, 40 and 48 h, and 72 and 96 h. The levels of total RNA synthesis decreased very rapidly during the sixteen hours preceding ootheca synthesis, between 56 and 72h, reaching a minimum shortly after the onset of ovulation, at 72 h. A second very sharp decrease in total RNA synthesis occurred at the very end of the reproductive cycle, between 96 and 106 h and continued until ootheca deposition (0 h, new cycle). Two major features were apparent in these patterns. 1) Except for the first increase in incorporation levels (0 to 16 h), all the major peaks in total RNA synthesis invariably preceded the peak levels of leucine incorporation into colleterial polypeptides by eight hours (Fig. 8b), and, 2) From 0 to 72 h, the profile of total RNA synthesis closely followed the changes in patterns of total extractable RNA (Fig. 8c). However, from 72 h until the end of the cycle, the large changes in total RNA synthesis were not reflected by similar changes in the amounts of RNA extracted from the gland.

c) Poly(A)$^-$ RNA.

The levels of poly(A)$^-$ RNA obtained at each stage of the reproductive cycle are shown in Fig. 9a. Although the overall pattern follows closely that of extractable total RNA (compare Figs. 7a and 9a), a substantial change in RNA distribution, in terms of poly(A)$^-$ and poly(A)$^+$ RNA, was recorded from 32 to 72 h. The levels of total extractable RNA reached a maximum at 40 h and subsequently declined rapidly to a minimum at 72 h. The peak levels of poly(A)$^-$ RNA were obtained with total RNA extracted from LCG s at 24 and 32 h of the cycle, a period during which leucine incorporation was at its lowest level (see Fig 8b). From 32 h to 56 h ,the poly(A)$^-$ RNA content of the

glands decreased rapidly. This was followed by a period of minor fluctuations, from 56 to 96 h, which followed precisely the pattern of total extractable RNA. The smallest amount of poly(A)$^-$ RNA was obtained from glands at 106 h in the cycle. This corresponded to a stage of high levels of leucine incorporation. The profile of nucleosides incorporation into poly(A)$^-$ RNA was similar to that of total extractable RNA (Fig. 9b).

d) Poly(A)$^+$ RNA.

The amount of poly(A)$^+$ RNA in the LCG fluctuated in a cyclic manner throughout the reproductive cycle. The changes in the levels of poly(A)$^+$ RNA recovered at each stage of the oothecal cycle are shown in Fig. 10a. Three large increases in levels of poly(A)$^+$ RNA were recorded at 40, 56 and 106 h. A fourth, albeit considerably smaller, increase was detected at 8 h. The amounts of poly(A)$^+$ RNA recovered at peak levels (40, 56 and 106 h) reached exceptionally high values (10.4, 10.1 and 10.0 μg/gland respectively), whereas at most other stages the amounts recovered oscillated around 2 μg/gland. These substantial fluctuations in poly(A)+ RNA levels were reflected in the pattern of nucleoside incorporation into this RNA fraction (Fig. 10b). Four peaks in poly(A)$^+$ RNA synthesis were recorded at 8, 40, 56 and 106 h. These peaks corresponded exactly with the peaks in poly(A)$^+$ RNA content. Furthermore, the timing of high leucine incorporation into LCG proteins corresponded with the peak synthesis in poly(A)$^+$ RNA (Fig. 10b). However, a comparison of Figs. 10a and 10b shows that whilst a considerable increase in poly(A)$^+$ RNA synthesis was recorded at 8 h, the concurrent increase in poly(A)$^+$ RNA level was much smaller. The

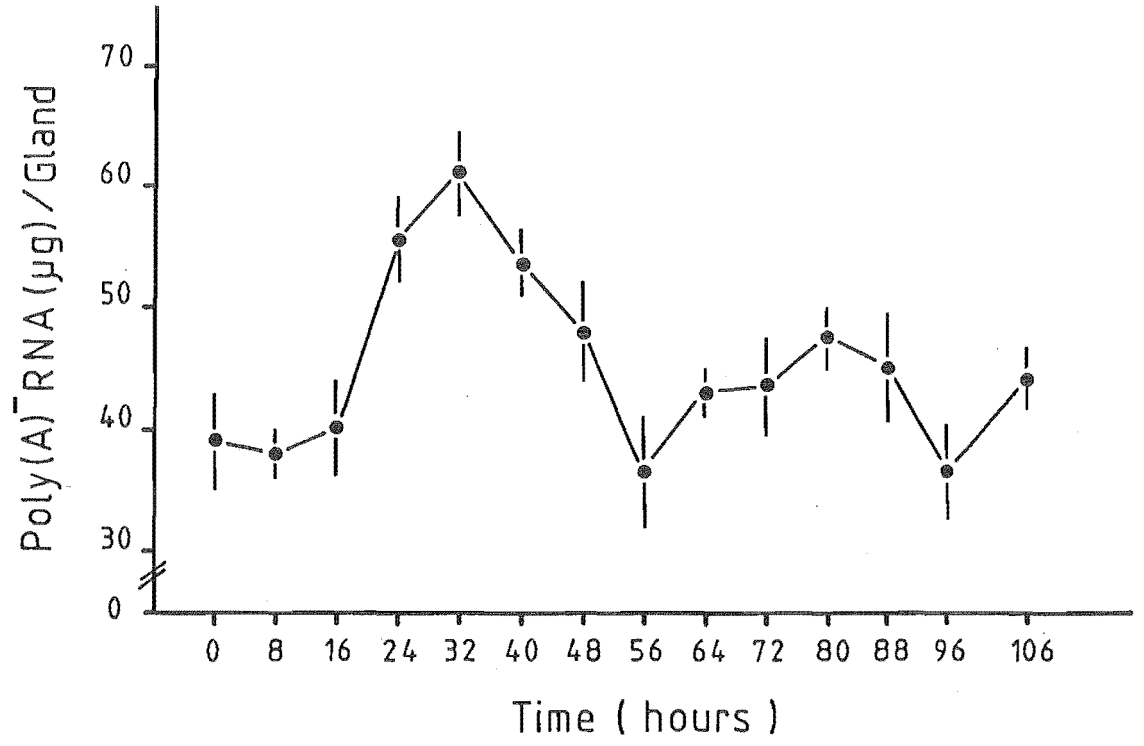
Fig. 9(a)

Poly(A)$^-$ RNA content of LCGs isolated at each stage of the reproductive cycle. Each point represents the mean of, at least, three animals and the SEM is shown for each point.

(b)

Levels of poly(A)$^-$ RNA synthesis in individual LCGs at each stage of the reproductive cycle. The SEM is shown for each point (n= 3-5).

A


Poly(A)⁻RNA (µg) / Gland
70
60
50
40
30
0
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

B

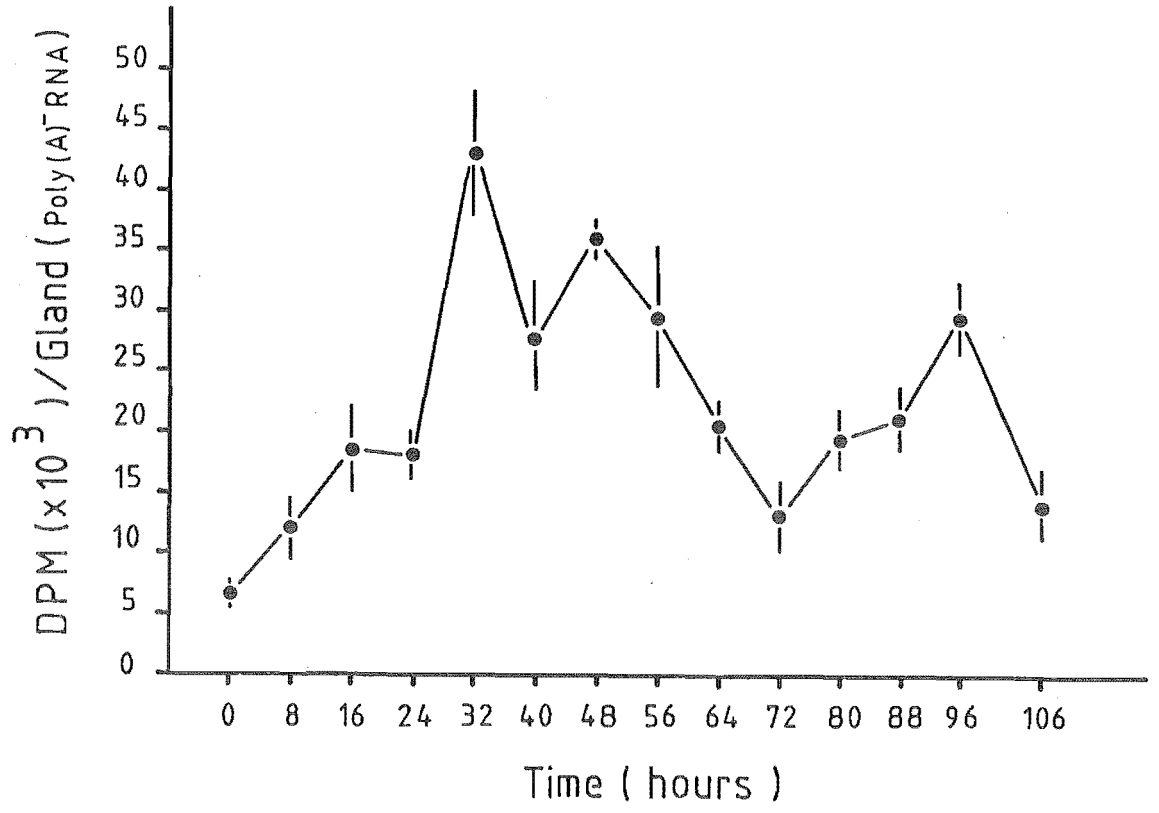

DPM (x10³) / Gland (Poly(A)⁻RNA)
50
45
40
35
30
25
20
15
10
5
0
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

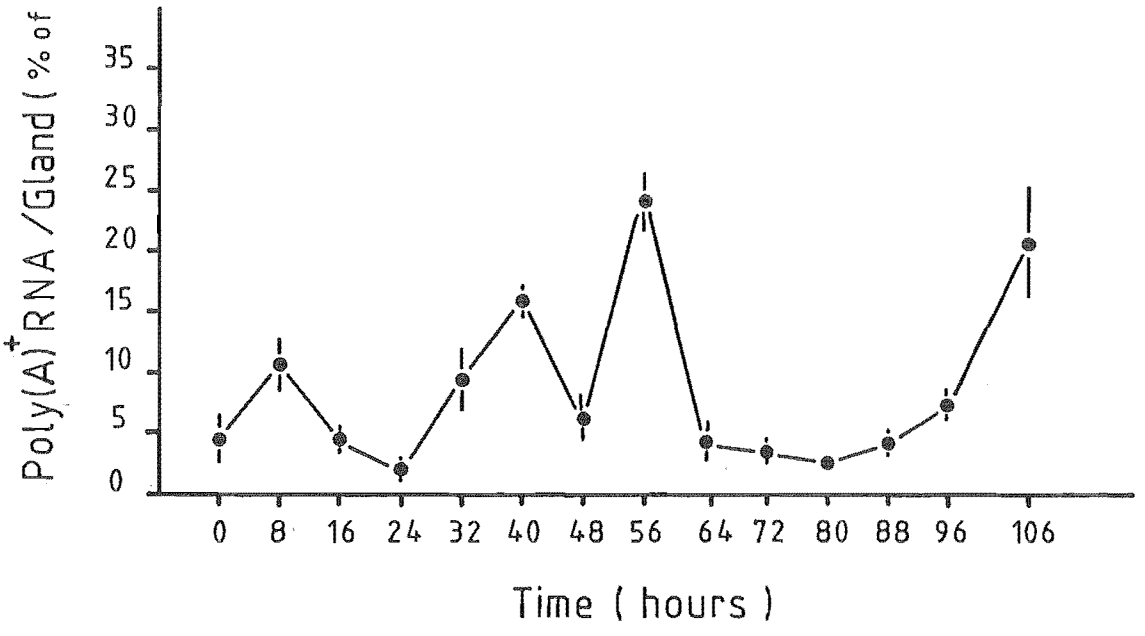
Fig. 10(a)

Changes in poly(A)$^+$ RNA content of single LCGs during the reproductive cycle. Each point is based on 3 to 5 individual determinations. Where not shown, the SEM was too small to be included.

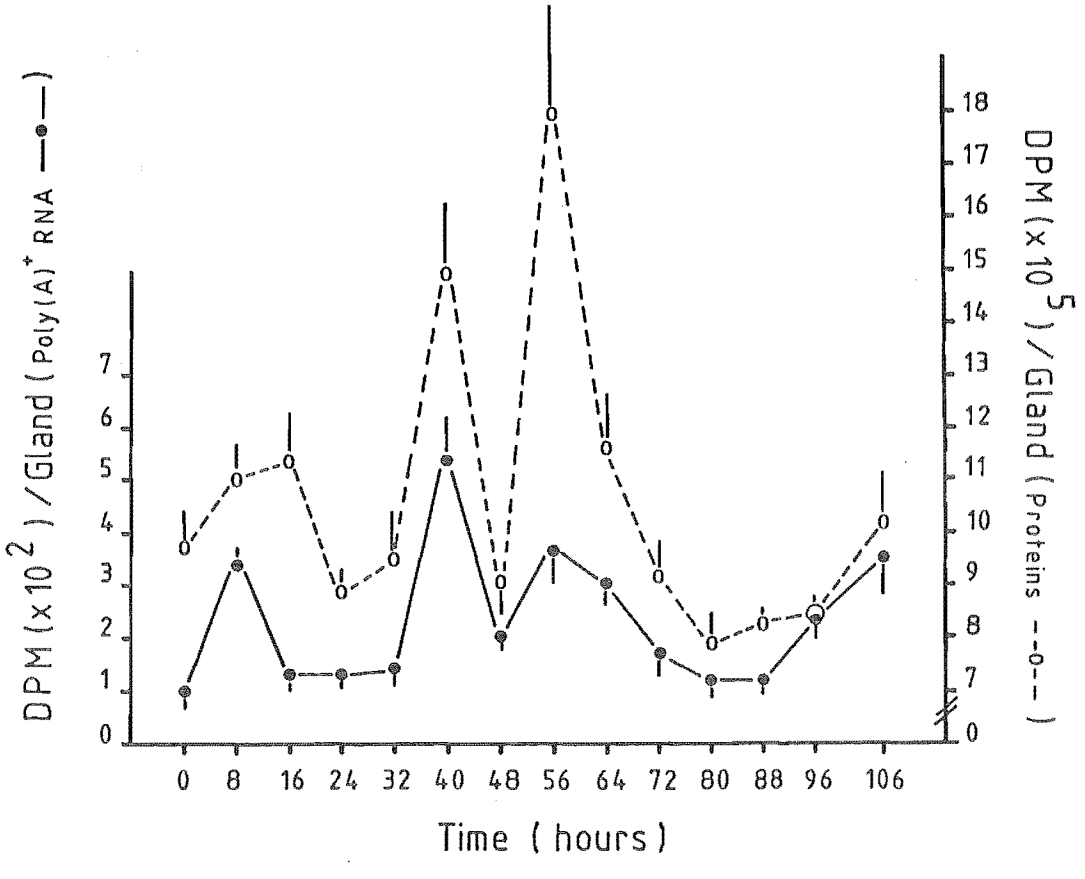
(b)

Comparison between the profiles of poly(A)$^+$ RNA synthesis and translational activity in individual LCGs at each stage of the reproductive cycle. The level of radiolabel incorporated into the poly(A)$^+$ RNA fraction was determined in duplicate for each sample. Each point is the mean of, at least, three sets of duplicates and the SEM is shown for each point.

A


Poly(A)+RNA /Gland (% of total RNA)
35
30
25
20
15
10
5
0
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

B


DPM (x10^2) /Gland (Poly(A)+ RNA —•—)
DPM (x10^5) / Gland (Proteins --o--)
0 1 2 3 4 5 6 7
0 7 8 9 10 11 12 13 14 15 16 17 18
0 8 16 24 32 40 48 56 64 72 80 88 96 106
Time (hours)

reverse effect was observed at 56 h. Between 16 and 32 h, the levels of poly(A)$^+$ RNA isolated from each gland showed an initial decrease followed by a sharp increase whereas, during the same period, poly(A)$^+$ RNA synthesis remained at a practically constant level.

DISCUSSION

The present study investigated transcriptional activities in relation to translational activities in the LCG during the reproductive cycle. The pattern of leucine incorporation into colleterial polypeptides obtained in the present study was essentially identical to that previously determined (chapter II, section III, pp 20-22 and Figs 3a-b, p 21), thereby independantly confirming the timing of the cyclic phases of translational activity in the LCG during the reproductive cycle. The amount of total extractable RNA obtained from single glands, while consistent for any given stage of the reproductive cycle, were lower than the levels reported by Pau (1981). The highest yield of purified RNA obtained in the present study was of the order of 70 µg/gland, as compared with 100 µg/gland reported by Pau. In the assessment of RNA extraction procedures, the urea method, as used by this author, was tested on the LCG at 88 h of the cycle. This protocol gave slightly lower and less reproducible nucleic acids yields (pre-DNase extracts) than the procedure followed in the present study. This could be due to differences in handling procedures. The discrepancy between the final amounts of RNA obtained here and those reported by Pau may result from the inclusion of DNase digestion in the purification procedure followed in the present investigation. It was found that the RNA content of the LCG as well as

RNA synthesis in this organ fluctuate throughout the reproductive cycle in a cyclic manner. There appears to be a strong temporal relationship between peak activities in RNA and protein synthesis. The RNA content of the gland presents a single phase of increase and decrease over the duration of the cycle. The phase of increase occurs over a forty eight hour period starting immediately after ootheca deposition (0 h). The peak in RNA content is reached at 24 h and maintained for a further twenty four hours (until 48 h). The pre-ovulatory period, from 48 to 72h, is characterized by a sharp fall in RNA content. The time of ovulation at about 72 h (Chapter II, section 1, p 10) corresponds to the beginning of a period of low RNA content which is maintained until ootheca deposition (0 h, new cycle). Total RNA synthesis, however, occurs in bursts. Two of the three major peaks in transcriptional activity detected during the reproductive cycle take place during the phase of increase in total extractable RNA content of the gland (32 and 48 h respectively). The third peak occurs during the post-ovulatory period, at 96 h, shortly before ootheca deposition. The patterns presented by the total RNA content and synthesis in the gland strongly suggest that considerable RNA turnover is taking place in the LCG during the reproductive cycle, particularly during the forty hours preceding ovulation (from 32 to 72 h).

The profiles of poly(A)$^+$ RNA content and synthesis correlate precisely with the pattern of translational activity in the LCG during the reproductive cycle, thereby indicating that the increases in translational activities are probably not dependant upon activated translation of pre-existing m-RNA but concurrent with de novo poly(A)$^+$ RNA synthesis. The patterns presented by the levels of poly(A)$^-$ RNA in the LCG during the reproductive cycle demonstrate not

only that this RNA fraction does not remain at a relatively stable level throughout the cycle but also that there is no apparent quantitative relationship between this fraction and the levels of leucine incorporation into colleterial proteins. The major peaks in protein synthesis are occurring in the pre-ovulatory period, at 16, 40 and 56h. During this period, the poly(A)$^-$ RNA content of the gland shows a single phase of increase peaking at 24 and 32 h, two stages at which translational activity is low. At the time of occurrence of the third major peak in protein synthesis (56 h), the poly(A)$^-$ RNA content of the LCG has fallen to one of the lowest levels recorded during the cycle. Moreover, leucine incorporation shows a rapid increase toward the end of the oothecal cycle (from 88 to 106 h). During this period, the poly(A)$^-$ RNA content of the gland decreases, reaching a minimum at 106 h. These observations strongly support the occurrence of high RNA turnover in the LCG indicated by the profile of total RNA extracted from the LCG at each stage of the oothecal cycle. The patterns of total extractable RNA and poly(A)$^-$ RNA synthesis do, however, bear a clear temporal relationship with that of leucine incorporation. The eight hours lag between these two processes strongly suggests that, while bursts of poly(A)$^+$ RNA synthesis occur concurrently with phases of high translational activity in the LCG, these transcriptional and translational events are almost invariably preceded by bursts of poly(A)$^-$ RNA synthesis. A similar lag period between RNA and protein synthesis has been reported to occur *in vivo* in the LCG of *Blattela germanica*. Zalokar (1968) showed that spontaneous deposition and artificial removal of the ootheca result in a rapid increase in RNA synthesis followed by a decline. Protein synthesis increased only after a lag of six to eight hours. In allatectomized mature *P. americana*

females, topical application of JH analogues result in increased transcriptional activity in LCG nuclei (Nair and Menon 1972). However, in view of the rapid changes in patterns of protein synthesis and the apparently high level of RNA turnover occurring in the LCG during the reproductive cycle, it seems likely that the high levels of transcriptional activity preceding the major periods of leucine incorporation into colleterial proteins, could also involve the concurrent production and degradation of specific colleterial m-RNA species. This suggestion is reinforced by the detection of highly repetitive phases of translational activities involving the production of, at least, two major colleterial proteins. Three repetitive phases of protein synthesis had been detected during the reproductive cycle, at 16, 72 and 96 h (Chapter II, section VI, pp 36-39 and Figs 10a and 10b), The electrophoretic patterns obtained in the present study were essentially identical to those presented in the previous chapter and confirmed these phenomena. These recurrent phases of translational activity, which could not be suitably explained, are constituted of four different stages. Each phase appears to be consistently initiated by high levels of synthesis of the 15.0 Kd protein (HM 1; at 16, 72 and 96h). The subsequent stages are characterized by the predominant synthesis of the 14.55 Kd polypeptide (HM 2; at 24, 80, 106 and 0 h), or high synthetic levels of both HM 1 and HM 2 (at 48 and 56 h). Each phase appears to terminate with the synthesis of either HM 2 only (32 h) or HM 2 and HM 1 (at 8, 64 and 88 h). Coincidentally, the initiation stage of two of these repetitive phases corresponds to a shoulder preceding large increases in total extractable RNA and poly(A)$^-$ RNA synthesis (16 and 72 h). The timing of initiation of each phase correlates with low poly(A)$^+$ RNA levels in the LCG preceding two of the major phases of

increases in this RNA fraction (16, 72 and 96 h). This may indicate that the sequence of translational events within each recurrent phase, as well as the periodicity of these phases during the reproductive cycle, could be directly linked with the pattern of poly(A)$^-$ RNA synthesis. Whether or not the production of specific t-RNA species could be involved in these phenomena is not known.

The juvenile hormones have been demonstrated to affect translational events in insect systems by a number of strategies. In the imaginal discs of D. melanogaster larvae, Nishirua and Fristrom (1975) showed that JH stimulates RNA polymerase I activity (r-RNA synthesis) by about two fold. In Locusta migratoria fat body, JH treatment results in the selective synthesis of vitellogenine m-RNA not only in reproductively mature females but also in male and female larval stages (Wyatt et al. 1981; Chienzi et al. 1981). In the cockroach Leucophaea madera, JH I treatment gives rise to increases in the level of thymidine kinase synthesis while promoting the up-take of this enzyme into the ovarian follicle cells and concurrently stimulating DNA synthesis in these cells [gene amplification? (Koeppe and Wellman 1979; Koeppe et al. 1980a; Koeppe et al. 1980b)]. Whether or not the transcriptional events, preceding by eight hours the major phases of poly(A)$^+$ RNA synthesis and protein synthesis that were detected in the present study, could be imputed to the effects of JH III remains to be elucidated.

SUMMARY

The present study investigated the patterns of in vitro RNA

synthesis in relation to protein synthesis in the LCG at short time intervals during each reproductive cycle. Evidence was presented showing that:

1) In mature P. americana females, total RNA synthesis in the LCG is a cyclic, highly dynamic process.

2) The major increases in total RNA synthesis primarily correspond to increases in poly(A)$^-$ RNA production.

3) These major increases in poly(A)$^-$ RNA synthesis precede by eight hours the stages of high leucine incorporation into colleterial polypeptides whereas,

4) the pattern of poly (A)$^+$ RNA synthesis follows that of leucine incorporation.

5) The increases in levels of colleterial protein synthesis appear to be dependent upon de novo m-RNA synthesis.

CHAPTER FOUR

THE HORMONAL CONTROL OF THE SYNTHETIC AND SECRETORY ACTIVITIES OF THE LEFT COLLETERIAL GLAND DURING THE REPRODUCTIVE CYCLE.

INTRODUCTION

The left colleterial gland (LCG) of P.americana synthesizes, accumulates and secretes the structural components of the ootheca (Bodenstein and Sprague, 1959; Bodenstein and Shaaya,1968, Pau et al., 1971). To fullfil its biological function, the LCG must release its accumulated secretion shortly before ovulation (Adiyodi and Adiyodi, 1975). The LCG is functionally dependant upon juvenile hormone (JH) (Scharrer, 1946; Bodenstein and Sprague, 1959, Bodenstein and Shaaya, 1968, Adiyodi, 1968; Emmerich and Barth, 1968; Shaaya and Bodenstein, 1969; Whitehead, 1969) although some aspects of LCG synthetic activities, such as the production of phenolic glucosides, seem to be purely female specific [(Shaaya and Bodenstein, 1969); see chapter I, p 4].

The synthesis and release of JH in mature females of P. americana during the reproductive cycle occurs cyclically, with at least two peaks of JH release corresponding to waves of oocyte maturation (Weaver et al., 1975; Weaver and Pratt, 1977). It has been shown that protein and RNA synthesis in the LCG during the reproductive cycle are cyclic events correlating with the known phases of JH release (Chapter III, section II b, pp 62-66).

Experimental evidence indicates that, in a number of insect species, including some Blattaridae, the stimulation of protein synthesis is mediated via enhancement of transcriptional activity in the tissues under study (Zalokar, 1968, Nair and Menon, 1977; Wyatt et al., 1982; Chinzei et al.,1982; Kovalic and Koeppe, 1982). In the present studies, it has been shown that the major peaks in RNA synthesis detected in the LCG during the reproductive cycle reflect increases in the production of poly

$(A)^-$ RNA and occur eight hours before the stages of increase in translational activity, the peaks in poly $(A)^+$ RNA synthesis corresponding precisely with the major increases in protein synthesis (Chapter III, section II d, pp 67-70).

It has long been known that ovulation in P. americana is accompanied by a sharp fall in corpora allata (CA) activity, and hence JH synthesis and release (Adiyodi, 1967; Hentschel, 1972; 1975). This endogenous decline in CA activity can be mimicked, in a dose-dependant fashion, by ecdysterone treatment in vivo (Tobe and Stay, 1980; Stay et al., 1980) but not in vitro (Friedel et al., 1980). Ecdysteroids have been detected in the h molymph and ovaries of mature, reproductively active females of several cockroach species (Imboden et al., 1978; Bulliere et al., 1979; Tobe and Stay, 1980; Stay et al., 1980; Friedel et al., 1980), including P. americana (Weaver et al., 1985), shortly before the onset of ovulation.

Very little is known concerning the mechanisms governing the synthetic and secretory activities of the LCG in relation to the reproductive cycle. The process of reproduction in P. americana can be considered as completed once the ootheca is fully formed and ready for deposition. However, in order to achieve successful reproduction, the release of accumulated secretory materials from the LCG and the process of ovulation must be stringently and accurately coordinated. What factors control this synchronization of events, let alone their possible mode of action upon the LCG, is totally unknown.

The patterns of macromolecule synthesis in the LCG throughout the reproductive cycle have been established (Chapters II and III). However, the detailed effects of endocrine factors such as JH and ecdysterone upon the patterns of transcriptional, translational and secretory activities

of the LCG at specific time points during the reproductive cycle and in relation to ovulation are still unknown. The detection of relatively high levels of ecdysterone in the heamolymph of mature P. americana females at the time of chorion formation (Weaver et al., 1985) may yet indicate a functional link between oocyte development and certain aspects of LCG activities in relation to the patterns of JH release during the reproductive cycle.

MATERIAL AND METHODS

1. Rearing and tissue culture conditions.

Mature females of P. americana were reared, staged and the LCG isolated and cultured according to the protocol previously described (Chapter II, section 1, pp 10-11).

2. Incorporation of radiolabel into colleterial RNA and polypeptides.

For the purpose of the present study, each isolated LCG was divided into two parts. This was achieved by sectioning one of the two major collecting ducts at the junction with the main excretory duct, producing two half LCG of approximately equal size. Incubations of isolated half LCG s were carried out in 60 µl of leucine-free medium (Chapter II,

section 2 , p 11) containing 5.0% (v/v) glycerol, supplemented with [5,6-^{3}H] uridine and L-[u-^{14}C] leucine (The Radiochemical Centre, Amersham, U.K. Spec. act. 50 Ci and 340 mCi/mMol respectively) to final specific activities of 1.33×10^{-1} and 4.20×10^{-3} µCi/µl respectively.

3. Hormone assays and conditions of incubation.

The stock solutions of hormones used in the present study were prepared from purified juvenile hormone III and 20-hydroxyecdysone batches (Sigma, USA, lots 12F-03591 and 80F-0247 respectively) by dilution in pure glycerol: absolute ethanol (v/v 1:1, JH III) and in absolute ethanol (ecdysone) to a final stock concentration of 1.0 µg/µl.

A study of the in vitro dose-response to JH III by LCG's isolated at different stages of the reproductive cycle, over the range 0.20 to 3.6 µg JH III/incubation (60 µl), showed the least JH III dose producing reproducible effects to vary between 600 ng and 1.8 µg per incubation, depending on the stage of the cycle (Table 1). Routine incubations were thus carried out at 25 ±1°c for 3.5h in the presence of 1.2 µg JH III per incubation.

a) JH III assay.

The effects of JH III upon transcriptional and translational activities were assessed on sister complementary glands. One half of any given LCG was used as control and incubated in hormone-free medium, containing the radiolabelled tracers, supplemented with the appropriate volume of hormone solvent. The other half of the gland was used for

Table 1.

Dose-response study of the effects of JH III upon the transcriptional and translational activities of LCGs isolated at 32 h of the reproductive cycle. The isolated glands were treated according to the protocol described. The sample size for each JH III dose was 3 half glands.

Table 1. Effects of different JH III doses upon *in vitro* RNA and protein synthesis in LCG s isolated at 32 h of the reproductive cycle.

JH III dose (µg/60 µl)	DPM (x 10)/µg RNA		DPM (x 10^2)/µg protein	
	Control	Hormone	Control	Hormone
.2 (n = 3)	19.1 ± 4.2	19.2 ± 5.6	31.6 ± 6.0	29.1 ± 4.7
.6 (n = 3)	12.6 ± 6.6	8.9 ± 9.3	37.6 ± 7.4	20.8 ± 6.2
1.0 (n = 3)	25.2 ± 2.1	17.5 ± 2.6	27.3 ± 5.1	17.8 ± 5.9
1.8 (n = 3)	25.1 ± 3.0	18.1 ± 4.1	32.4 ± 6.8	21.1 ± 7.4
3.6 (n = 3)	24.9 ± 4.7	30.0 ± 6.8	24.3 ± 5.9	36.1 ± 10.4

hormone assay. Following incubation, each half gland was rinsed three times in ice-cold saline and individually frozen for RNA extraction.

b) Ecdysterone assay.

The effects of hyroxyecdysterone (1.2 µg/ incubation) in the absence and in the presence of JH III as well as the effects of ovarioles isolated at 64h of the reproductive cycle were assessed in terms of LCG transcriptional and secretory activities only. Routine hormone and ovarioles assays were carried out on LCG s isolated from females at 32h of the reproductive cycle. Each half gland was incubated in complete medium (Chapter II, section 2, p 11) containing 5.0% glycerol and supplemented with [5,6-^{3}H) uridine as above. The conditions of incubation were identical to those followed for JH III. Incorporation of tritium into colleterial RNA was stopped by rinsing the half glands in ice-cold saline. Each half gland was subsequently frozen individually and rapidly homogenized to a paste in a manual all-glass homogenizer. Then 75 µl of 150 mM spermine were added and the tissue further homogenized until total disruption of the gland was achieved. The tissue homogenate was transfered to a sterile Eppendorf tube kept on ice. The homogenizer was rinsed twice with 75 µl of 150 mM spermine and the washings added to the homogenate. A 50 µl aliquot, taken for scintillation counting and protein determination, was immediately TCA treated. The remainder of the homogenate was frozen and stored at -20°c. Total RNA was not extracted from these samples.

4. RNA extraction and purification

Total RNA was extracted from each half gland according to the method of Barnett et al. (1980), modified as indicated in the previous chapter (section 3, pp 49-51), following the protocol described for single glands.

5. RNA quantification and determination of tritium incorporation levels.

The purity of the total RNA extracted from each half gland was determined by U.V. absorbtion spectrophotometry (190 to 350 nm). Quantification was based on A_{260}, using calf liver RNA (Sigma) as standard.

The levels of tritium incorporated into total extractable RNA and total cellular RNA (not extracted) were measured by liquid scintillation counting of 10 µl aliquots (duplicates) in a Beckman LS 2800 LSC with a counting efficiency of 59.3%.

6. Protein determination and measurments of leucine incorporation levels into colleterial polypeptides.

The total protein content of each half gland was determined from TCA precipitated homogenate according to the procedure of Lowry et al. (1951). Tissue homogenate treatment for protein determination and scintillation counting was as previously described (Chapter II, section 3 pp 13-15). Liquid scintillation counting was carried out using dual label procedures with counting efficiencies of 59.3% (^{3}H) and 79.4% (^{14}C).

7. Electrophoretic and fluorographic analyses.

Electrophoresis was carried out in diallytartardiamide (DATD) crosslinked linear gradient acrylamide gels (15 to 25% acrylamide, 0.8% DATD) stabilized with a 2 to 5% sucrose gradient. The buffer system was that of Laemmli (1970). Sample preparation, electrophoretic loads and running conditions were as previously described (Chapter II, section 4, pp 15-16).

For fluorography, the gels were treated according to the method of Bonner and Laskey (1974). The dried gels were exposed to Agfa-Gevaert X-ray films for thirty days at room temperature.

The levels of specific translational activities were determined by slicing out the electrophoretic bands of interest from freshly destained gels. The bands thus excised and the intervaling gel strips were individually dissolved in 88mM sodium periodate according to the method of Späth and Koblet (1979). The levels of leucine incorporated into each electrophoretic strip were determined by liquid scintillation counting (50 µl aliquots, duplicates) with a counting efficiency of 97.4%.

RESULTS

I) Effects of in vitro JH III treatment upon LCG synthetic activities at specific time points during the reproductive cycle.

a) Transcriptional activity.

The transcriptional response to *in vitro* JH III treatment from the LCG at different stages of the reproductive cycle are shown in Fig. 1. The gland did not respond in a uniform manner to the presence of JH III at different stages of the oothecal cycle. The level of transcriptional response obtained appeared to be highly stage specific.

The overall pattern of *in vitro* RNA synthesis in the LCG, in response to the presence of JH III, appeared to consist of alternating phases of stimulated and inhibited transcriptional activity with a periodicity of about sixteen hours. These effects were most noticable over a sixty four hour period starting at 32h and terminating at 96h of the reproductive cycle. Large positive responses (increased translational activity) were obtained with glands isolated at 32, 40 and 48h of the cycle. Two of these stages (32 and 48h) corresponded to phases of already high endogenous transcriptional activity.

During the periods immediately preceding and following the onset of ovulation (64 and 72h respectively) the LCG responded negatively, if at all, to the presence of the hormone, RNA synthesis falling significantly (5% level) as compared to the control levels. Over the next sixteen hours, from 80 to 96h, RNA synthesis was slightly (not significant at 5% level) stimulated by the presence of JH III.

Glands isolated at 24h, eight hours before the first endogenous peak in RNA synthesis, and at 106h, eight hours after the last endogenous increase in transcriptional activity, did not respond, at the transcriptional level, to the presence of JH III.

b) Translational activity.

The effects of short term *in vitro* JH III treatment upon protein

Fig. 1

Effects of JH III *in vitro* treatment upon the transcriptional activities of the LCG at specific time points during the reproductive cycle. Each isolated gland was divided into two halves. One half was used as control (C) and the complementary half subjected to JH III treatment (JH). Determination of transcriptional activity levels were carried out in duplicates on total RNA extracted from each half gland. Each point represents the mean of four animals and the SEM is shown for each stage.

The arrows indicate

1) the onset of *in vivo* LCG secretory activity,
2) the onset of ovulation, and
3) the end of ootheca synthesis.

* : Transcriptional activity levels in hormone treated half glands significantly different (5% level, paired t-test) from that of their control counterparts.

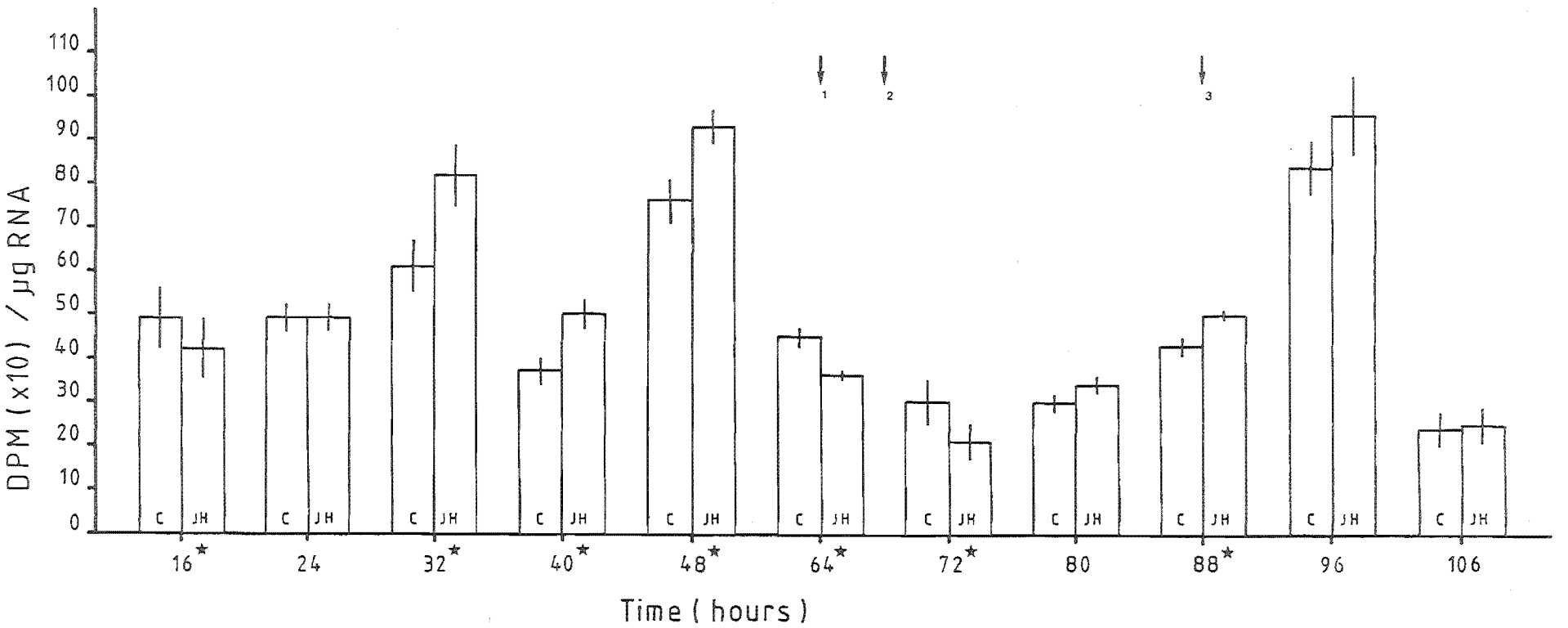

DPM (x10) / µg RNA
110
100
90
80
70
60
50
40
30
20
10
0
C
JH
1
2
3
16*
24
32*
40*
48*
64*
72*
80
88*
96
106
Time (hours)

Fig. 2

Effects of JH III *in vitro* treatment upon the translational activities of LCG isolated at specific time points during the reproductive cycle. The TCA insoluble radioactivity remaining in aliquots of each half gland homogenate was determined, in duplicate, by liquid scintillation counting using a dual label counting procedure. The counting efficiencies were 59.3% (^{3}H) and 79.4% (^{14}C)

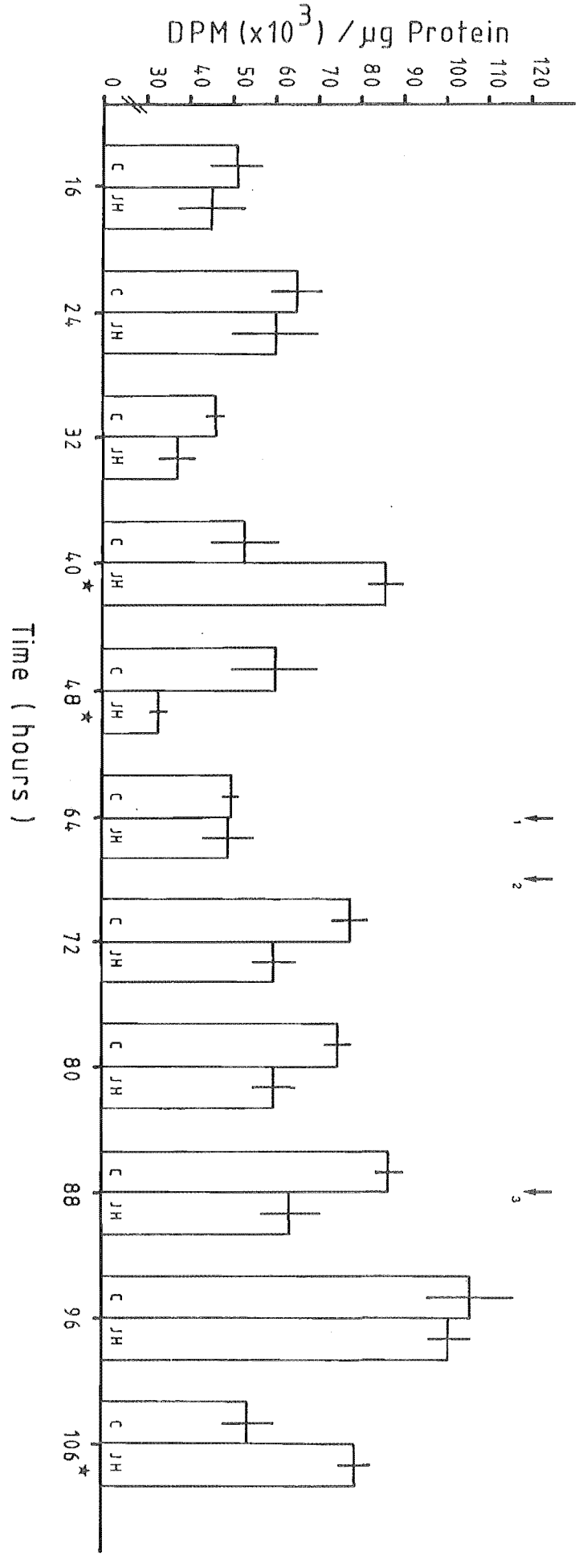
The arrows indicate

1) the onset of *in vivo* LCG secretory activity,

2) the onset of ovulation, and

3) the end of ootheca synthesis.

* : Translational activity levels in hormone treated half glands significantly different (5% level, paired t-test; n=4) from that of their control counterparts.


DPM (x10³) / µg Protein
0
30
40
50
60
70
80
90
100
110
120
C
JH
16
24
32
40
48
64
72
80
88
96
106
Time (hours)
1
2
3

synthesis in the LCG at different time points during the reproductive cycle are shown in Fig. 2.

With the exception of glands isolated at 24, 40, 64 and 106h of the cycle, JH III inhibited in vitro protein synthesis in the LCG. The magnitude of these short term inhibitory effects fluctuated in a stage dependant manner. Marked inhibitory responses were observed in glands isolated at 32 and 48h, two stages of high endogenous levels of RNA synthesis, and during the period of ootheca formation, from 72 to 88h.

The two stages at which JH III treatment was found to significantly enhance in vitro translational activity (40 and 106h) corresponded to time points at which endogenous levels of protein and poly $(A)^+$ RNA synthesis in the LCG were already high.

At most of the time points where short term JH III in vitro treatment significantly stimulated transcriptional activity, it also strongly inhibited protein synthesis in the LCG, with the exception of glands isolated at 40h where both processes were stimulated concurrently (compare Figs. 1 and 2).

Glands isolated at 24, 64 and 96h did not show any changes in the overall levels of translational activity in response to JH III treatment.

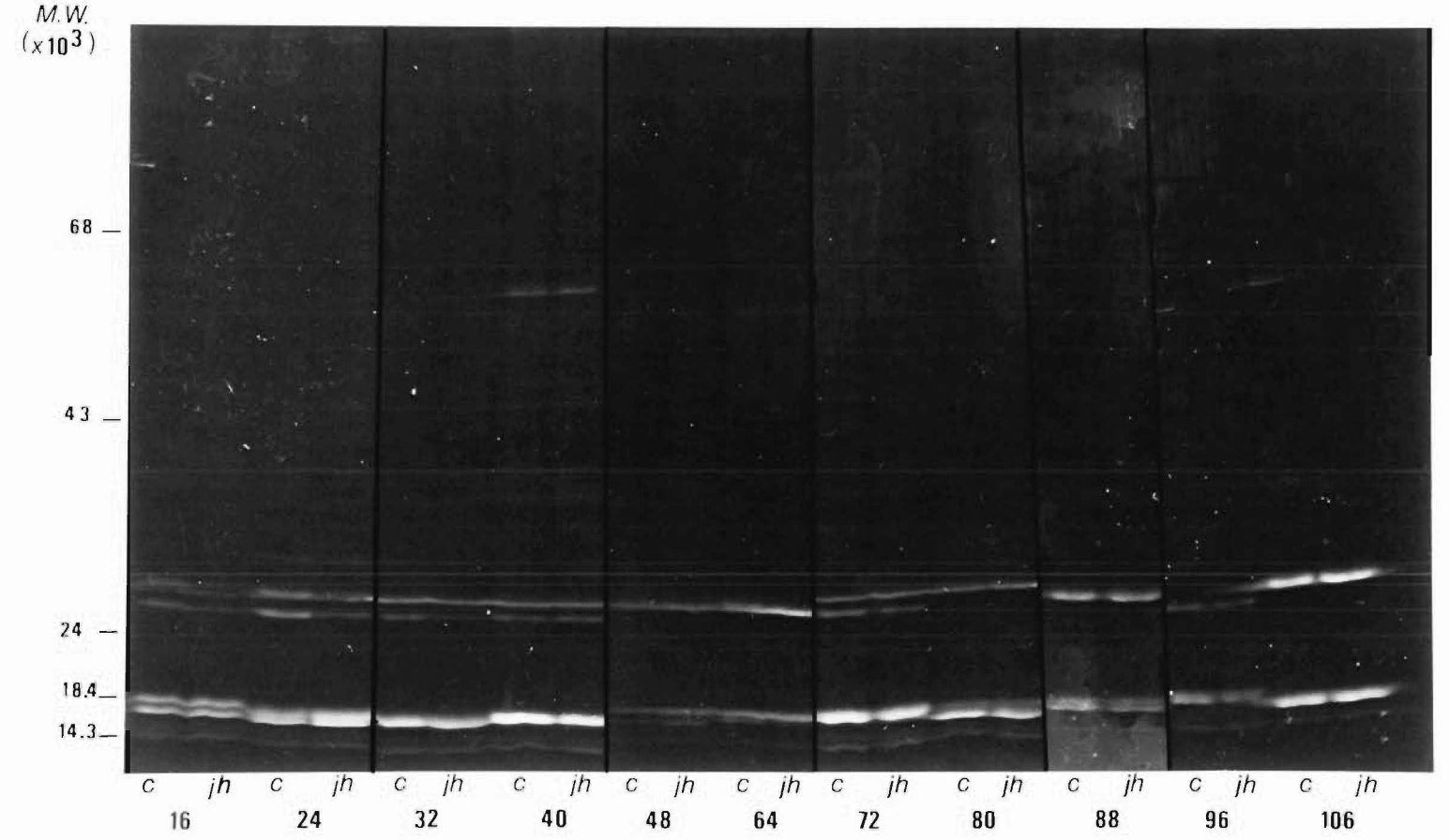
II) Electrophoretic analyses of in vitro translational activities in LCG s exposed to JH III treatment at different stages of the reproductive cycle.

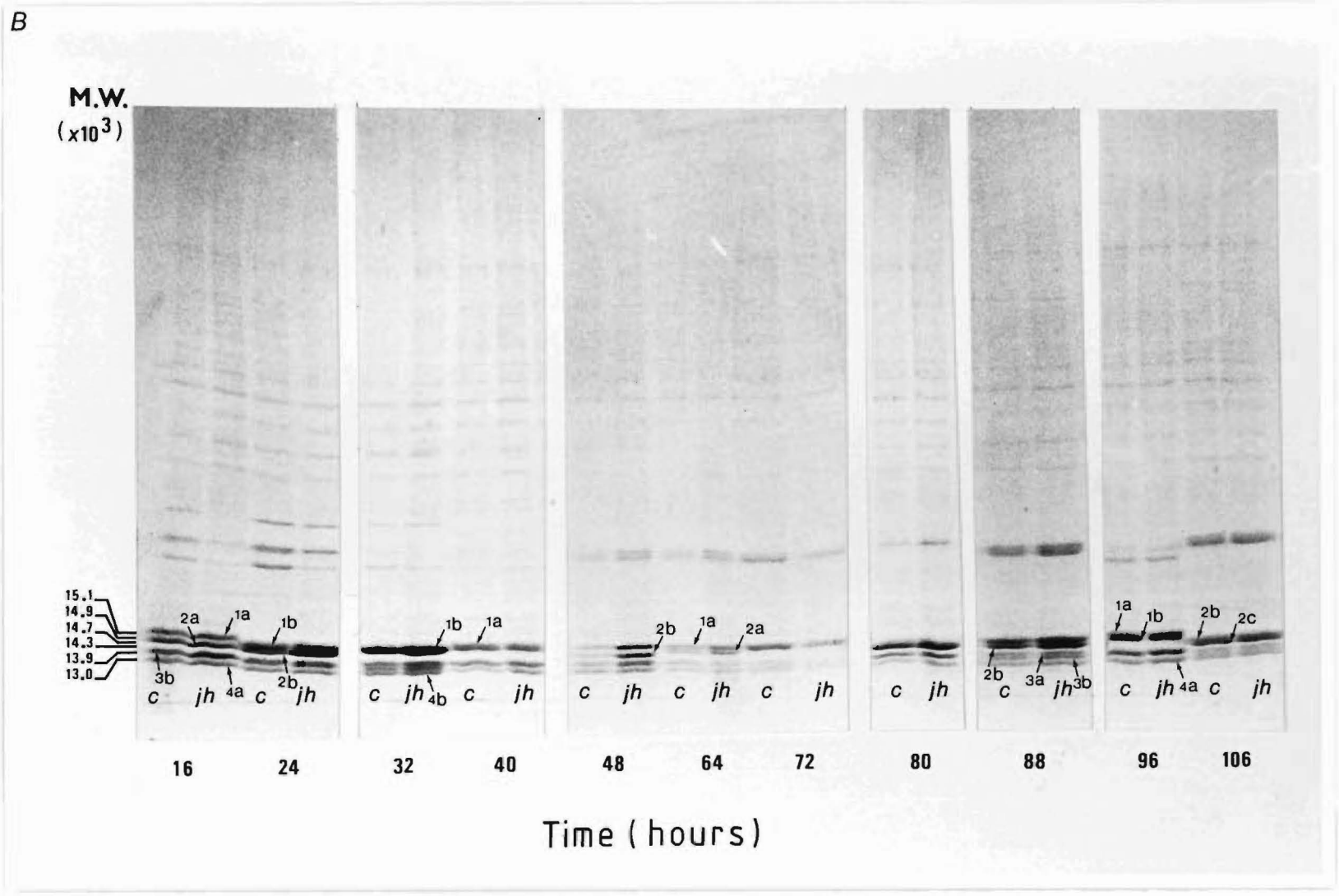
a) Fluorographic analyses.

Fig. 3

Fluorographic analyses (a) of the patterns of protein synthesis in control (C) and JH III treated (JH) half glands. The patterns shown were developed from six SDS-DATD-acrylamide linear gradient gels (b), corresponding to four different experiments.

A


M.W.
(x10³)
68
43
24
18.4
14.3
c jh c jh c jh c jh c jh c jh c jh c jh c jh c jh c jh
16 24 32 40 48 64 72 80 88 96 106
Time (hours)


B
M.W.
(x10³)
15.1
14.9
14.7
14.3
13.9
13.0
2a
1a
1b
3b
2b
4a
1b
1a
4b
2b
1a
2a
2b
1a
3a
3b
1a
1b
2b
2c
4a
c
jh
16
24
32
40
48
64
72
80
88
96
106
Time (hours)

Short term *in vitro* JH III treatment did not give rise to qualitative changes in the electrophoretic patterns of translational activities presented by the LCG at different stages of the oothecal cycle. The electrophoretic patterns of protein synthesis presented by JH III treated half glands were qualitatively identical to those of their control counterparts at all stages investigated (Fig. 3).

b) Patterns of specific translational activities.

Although the protein species synthesised *in vitro* by control and JH III treated half glands were electrophoretically identical at any given stage of the oothecal cycle, the levels of synthesis of at least four major colleterial proteins, with apparent molecular weights (MW) smaller than 25000 daltons, were quantitatively affected by the presence of JH III in a stage specific manner (Fig. 4 and Table 2).

The increases in translational activities recorded at 40 and 106h in response to short term JH III treatment were found to be of a general character. The synthesis of all the major low MW polypeptide species produced by the LCG at these stages was increased in a uniform manner (Fig. 4 D and K).

The inhibition of LCG translational activity in the presence of JH III observed to occur in glands isolated during the early phases of the reproductive cycle (16, 32 and 48h) as well as during the post-ovulatory period (72, 80 and 88h) presented a high degree of specificity. While short term JH III treatment of glands isolated at 16 and 48h of the cycle resulted in uniformly inhibited synthesis of all the major low MW polypeptides produced in the gland at these stages (Fig. 4 A and E), the

Fig. 4

The patterns of specific translational activities in control (C) and JH III treated (JH) half glands were assessed on the major low molecular weight colleterial polypeptides only. The electrophoretic bands of interest were sliced out of freshly destained SDS-DATD-acrylamide gradient gels. The bands thus excised and the intervening gel strips were individually dissolved in sodium periodate. The levels of leucine incorporated into each electrophoretic stip were determined in duplicate by liquid scintillation counting. Each electrophoretic sample load was standardized to 40 µg protein.

The electrophoretic patterns shown were obtained from eight gels representing four different experiments. The figures beside each individual pattern indicate the mean DPM recorded in the corresponding individual band or group of bands.

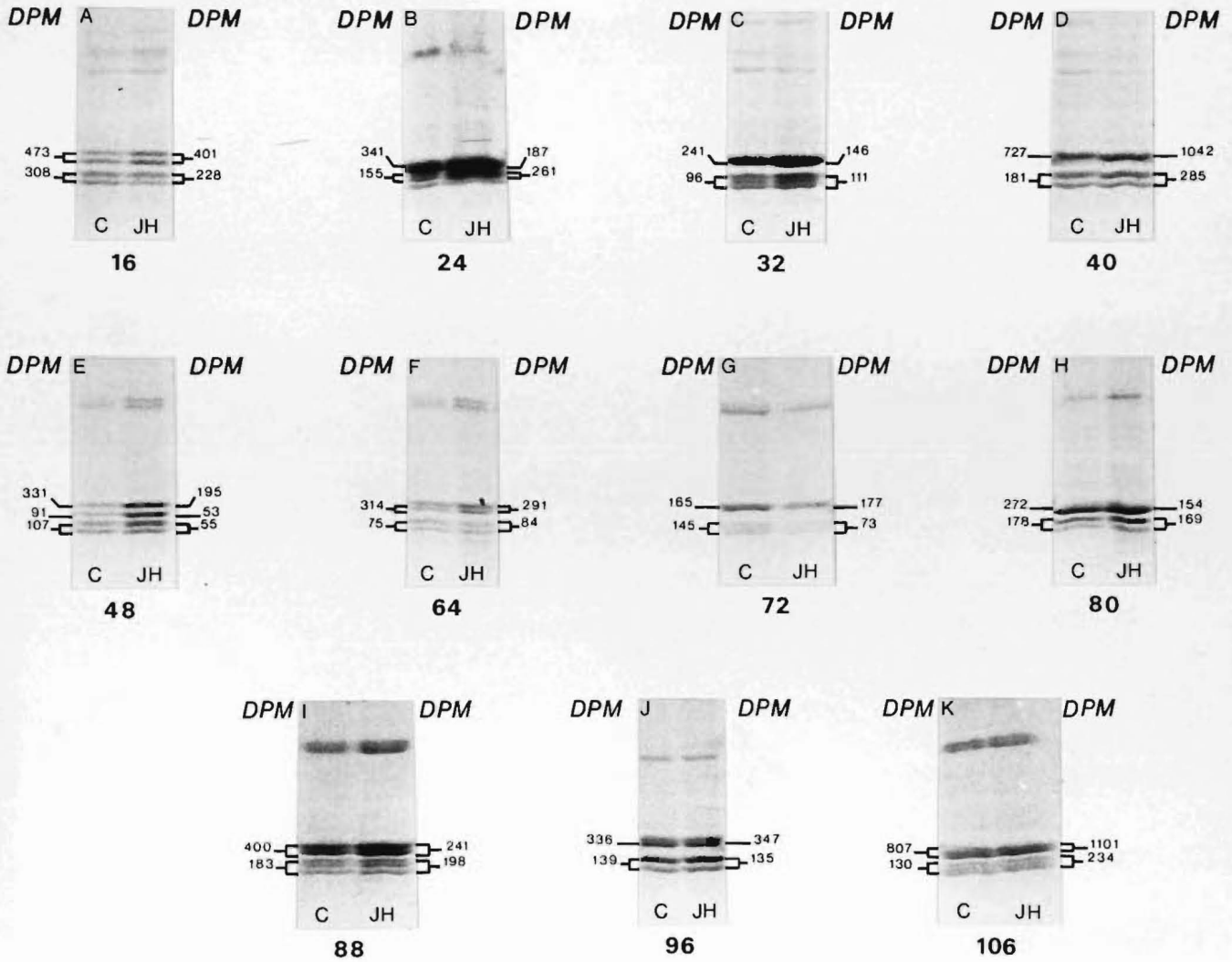

DPM A DPM
473
308
401
228
C JH
16
DPM B DPM
341
155
187
261
C JH
24
DPM C DPM
241
96
146
111
C JH
32
DPM D DPM
727
181
1042
285
C JH
40
DPM E DPM
331
91
107
195
53
55
C JH
48
DPM F DPM
314
75
291
84
C JH
64
DPM G DPM
165
145
177
73
C JH
72
DPM H DPM
272
178
154
169
C JH
80
DPM I DPM
400
183
241
198
C JH
88
DPM J DPM
336
139
347
135
C JH
96
DPM K DPM
807
130
1101
234
C JH
106

Table 2.

The levels of leucine incorporated into specific polypeptides in JH III treated half glands at different stages of the reproductive cycle are expressed as a percentage of the corresponding control levels. Brackets indicate electrophoretic bands which could not be excised individually and were dissolved and counted together. For all stages analysed, each band or group of bands, obtained from three different LCGs, were excised from four gels run in pairs.

Table 2. Effects of JH III treatment upon specific translational activity *in vitro* in the LCG during the reproductive cycle.

Polypeptide	M.W.	Time (hours)										
	(x 10^3)	16	24	32	40	48	64	72	80	88	96	106
1a	15.1	84.8 ±9.3			143.4 ±5.2	59.0 ±22.1	92.9 ±18.2	107.3 ±18.8			103.1 ±11.5	
1b	14.9			60.6 ±12.0						60.3 ±10.6		
2a	14.7		54.9 ±8.6			59.0 ±16.4			56.8 ±16.1			136.5 ±11.1
2b	14.3									108.2 ±18.5		
3 & 4	13.9/13.0	74.0 ±11.1	170.9 ±10.4	115.0 ±16.8	157.0 ±7.5	51.0 ±16.7	112.9 ±15.5	50.6 ±10.5	95.1 ±20.0		98.2 ±24.3	180.0 ±23.2

synthesis of very specific polypeptides only was found to be inhibited in glands isolated at 32, 72, 80 and 88h. At three of these stages (32, 80 and 88h) the synthesis of a major polypeptide with an apparent MW of 15100 daltons was repressed to about 60% of the control levels (Fig. 4 C, H and I), whereas that of a 14000 daltons major colleterial polypeptide was strongly inhibited at 72h (Fig. 4 G). The synthesis of the other major low MW proteins produced by the LCG at these stages remained at levels equivalent to those observed in the control half glands. Both of these major colleterial proteins are structural components of the ootheca.

The overall translational activity of glands isolated at 24, 64 and 96h of the reproductive cycle was apparently unaffected by the presence of JH III (Fig. 2). However, this apparent null response to short term in vitro hormone treatment was found to be true only in glands isolated at 64 and 96h, where the levels of synthesis of all the major low MW polypeptides produced at these stages were practically identical in JH III treated and control sister half glands (Fig. 4 J and F). The translational response to the presence of JH III obtained with glands isolated at 24h was found to be a simultaneous decrease in the level of synthesis of a 14500 daltons protein (down to 55% of the control level) while that of at least one of two major proteins, with respective MW s of 14000 and 13000 daltons was stimulated by about twofold with respect to the control (Fig. 4 B). The differences in relative abundance of these proteins as well as their inequal leucine content were probably responsible for the apparent null effect in terms of overall leucine incorporation levels in response to JH III treatment.

III) Effects of ecdysterone and mature whole ovarioles upon the transcriptional and secretory activities of the LCG at 32h of the reproductive cycle.

In an attempt to clarify the nature of the relationship between LCG synthetic and secretory activities in relation to ovulation and the onset of ootheca synthesis, glands isolated at 32 and 64h of the reproductive cycle were incubated in the presence of ecdysterone as well as in the presence of whole ovarioles (the presumed source of ecdysteroids in the adult female) isolated at two specific stages of terminal oocyte development. These two stages of the reproductive cycle (32 and 64h) were chosen in relation to their relative temporal position with respect to a) ovulation and the onset of ootheca synthesis, b) the major peaks in the patterns of macromolecules synthesis presented by the LCG over the duration of the reproductive cycle and the in vitro effects of JH III thereupon and c) the peak in haemolymph ecdysteroid levels.

a) Transcriptional activity.

Half glands isolated at 32h of the reproductive cycle and incubated in the presence of ecdysterone showed a strong reduction in transcriptional activity as compared to their control counterparts (Table 3).

The presence of two complete sets of ovarioles (the full complement in an adult female), isolated from the females which supplied the LCG s (32h), did not produce any distinguishable effects upon the overall transcriptional activities presented by control as well as ecdysone

Table 3.

The effects of ecdysterone and mature ovarioles, isolated shortly before ovulation (64 h), upon *in vitro* transcriptional and secretory activities in half LCGs were assessed on glands isolated at 32 h of the cycle. Each gland was divided into two parts of approximately equal size and the resultant halves were subjected to different treatments. The levels of tritium incorporated into total cellular RNA (not extracted) are expressed as a function of the protein content of each half gland. The numbers in parentheses indicate complementary halves of the same LCG.

Table 3. Effects of ecdysterone and mature ovarioles at different stages of T oocyte development upon *in vitro* transcriptional activity in the LCG at 32 h of the reproductive cycle.

Treatment	n	DPM/µg protein ($\bar{x}$)	S_D	% incorporation over control	Secretion spontaneously released (µg/half gland/3.5 h)
Control (1, 2)	2	26.00	2.28	100 ± 8.8	Not measurable
32 h ovarioles (3, 4)	2	26.20	1.37	100.8 ± 5.3	Not measurable
JH III (5, 6)	2	33.30	2.10	127.6 ± 8.1	170 ± 60
Ecdysterone (1, 2, 5, 6)	4	10.17	2.86	38.97 ± 8.8	1480 ± 380
Ecdysterone + 32 h ovarioles (3, 4)	2	11.70	1.00	44.82 ± 3.9	1540 ± 410
Ecdysterone + JH III (w/w1:1) (7, 8, 9)	3	16.60	2.86	63.60 ± 11.0	103 ± 70
64 h ovarioles (7, 8, 9)	3	3.59	0.57	13.74 ± 2.20	670 ± 130

treated half glands. The levels of RNA synthesis observed in these glands were practically identical to those recorded for LCG s incubated in the absence of ovarioles at this early stage of T-oocyte development.

Treatment with JH III produced, as expected at this particular stage of the reproductive cycle (32h), an increase in the transcriptional activities of the control half glands, whereas ecdysterone treatment on their complementary halves resulted in the usual marked inhibition of RNA synthesis. The strong in vitro inhibitory effects of ecdysterone upon LCG transcriptional activity were only slightly alleviated by the addition of an equal amount of JH III.

The presence in the incubation medium of two complete sets of ovarioles isolated from females at 64h of the reproductive cycle, shortly before the onset of ovulation, depressed in vitro RNA synthesis in LCG s isolated at 32h of the cycle to very low levels (a decrease of at least sevenfold over the control value). The inhibitory potency of the 64h ovarioles upon in vitro RNA synthesis in the LCG, as measured at 32h of the reproductive cycle, was greater than three times that of ecdysterone, as used in the present study.

The LCG s of the females which supplied the 64h ovarioles were assessed for their transcriptional responses to the presence of ovarioles isolated at 32h and to that of JH III.The results obtained (Table 4) were in agreement with the levels of in vitro transcriptional activities expected at this stage of the cycle in response to the presence and absence JH III, thus indicating that ovarioles isolated during the active phase of yolk deposition in T and T-1 oocytes did not apparently affect the levels of in vitro RNA synthesis in LCG's isolated at 32 as well as at 64h of the reproductive cycle.

Table 4.

The effects of JH III and mature ovarioles, isolated during active vitellogenesis in T-oocytes (32 h), upon transcriptional activity in the LCG were assessed on glands isolated at 64 h of the reproductive cycle. The glands were obtained from the females which supplied the ovarioles containing T-oocytes in the process of chorion deposition. The glands were treated as previously described (see Table 2.). The levels of transcriptional activity observed in the isolated LCGs under these experimental conditions are expressed as a percentage of the control levels recorded at 32 and 64 h.

(a) The mean level of tritium incorporated into total extractable RNA in control half glands at 64 h was 72.0% of that recorded for controls at 32 h of the cycle (see Fig. 1).

(b) The mean level of tritium incorporated into total extractable RNA in JH III treated half glands at 64 h was 77.8% of that recorded for their control counterparts (see Fig. 1).

Table 4. Effects of JH III and mature ovarioles isolated at 32h upon *in vitro* transcriptional activity in the LCG at 64h Of the reproductive cycle (onset of ootheca synthesis *in vivo*).

Treatment	n	DPM/ µg protein ($\bar{X}$)	S_D	% incorporation over a)32h and b) 64h controls
32h ovarioles (10,11,12)	3	17.90	1.20	68.80 ± 6.7 (a)
JH III + 32h ovarioles (10,11,12)	3	13.60	0.90	76.00 ± 6.6 (b)

b) Secretory activity.

Appart from its inhibitory effects upon *in vitro* RNA synthesis in LCG s isolated at 32h, a stage far removed from ovulation, the presence of ecdysterone induced copious release of secretory material from the isolated half glands (1.51±0.4mg/half gland; Fig. 5a). The control half glands, incubated in the absence of ecdysone, with or without ovarioles isolated at 32h of the cycle, did not spontaneously release any secretory material over the period of incubation (Fig. 5b).

The presence of ovarioles isolated at 64h of the cycle induced release of secretory material from 32h half glands (Fig. 5c), although not as effectively as ecdysterone (0.67±0.32mg/half gland).

JH III was found to be very ineffective as an inducer of *in vitro* secretory activity (Fig. 5d). Glands isolated at 32h and incubated in the presence of JH III did release very small amounts of secretory material (0.17±0.6mg/half gland) but this effect could not be meaningfully compared to those of ecdysterone or of ovarioles isolated at 64h of the cycle. However, the stimulatory effects of hydroxyecdysterone upon LCG secretory activities *in vitro* were abolished in the presence of JH III (not shown). Glands isolated at 32h and incubated in the presence of both hormones released small amounts of secretory material comparable to that obtained in the presence of JH III alone (about 1.0mg/half gland).

DISCUSSION

The present study demonstrated, for the first time, the *in vitro*

Fig. 5

The inductive potency of ecdysterone (a), ovarioles excised at 32h (b) and 64h (c) of the reproductive cycle, and that of JH III (d) upon *in vitro* LCG secretory activity was tested on glands isolated at 32h, a stage far removed from the onset of *in vivo* ootheca synthesis (64h).

The arrows in Figs. a, c, and d indicate secretory material released during experiments by the isolated half glands (the secretory material is diffuse in Fig. d).

The arrow in Fig. b indicates the cut end of the main secretory duct.

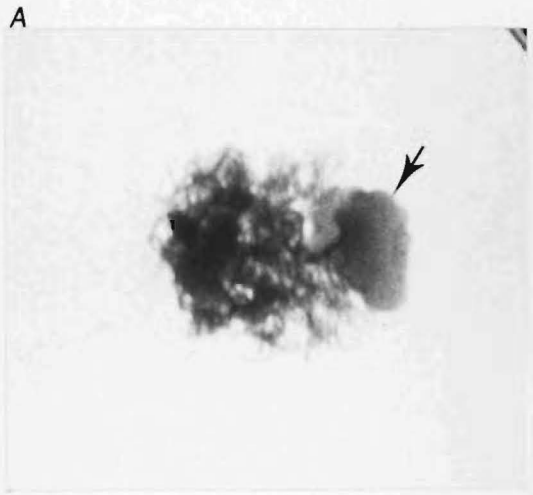
Magnifications:

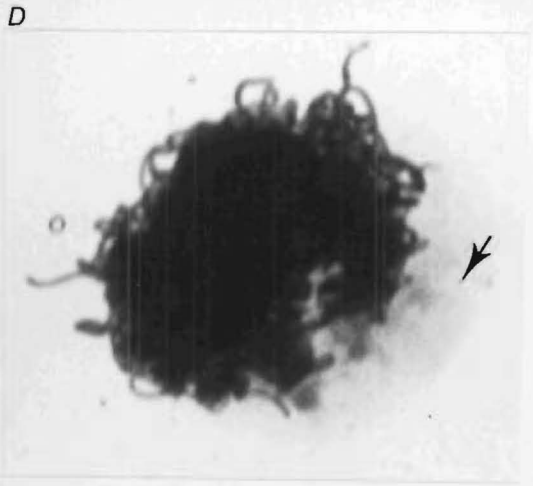
a) x 50

b) x 120

c) x 120

d) x 70





effects of JH III treatment upon the synthetic activities of the LCG at various time points during the reproductive cycle and outlined the means whereby LCG synthetic and secretory activities might be coordinated with oocyte development.

The short term effects of JH III primarily center upon RNA metabolism in the LCG during the reproductive cycle. However, the mode of action of the hormone at this level is not uniform. The transcriptional response of the LCG to the presence of JH III is highly stage-specific. The endogenous patterns of transcriptional activities in the LCG present three large peaks in RNA synthesis occuring at 32, 48 and 96h (Chapter III, section IIb, pp 62-66). In vitro JH III treatment of the LCG at these particular stages leads to further increases in transcriptional activities, thus indicating that JH III potentiates the existing increases in RNA synthesis. However, it is not entirely clear whether or not JH III release from the CA is solely responsible for the initial appearance of these endogenous increases. Hormonal in vitro treatment of glands isolated at 24 and 106h, respectively eight hours before and after the first and third peaks in endogenous transcriptional activities, failed to produce a change in levels of RNA synthesis, whereas similar treatment of glands isolated at 40 and 88h, two stages preceding a peak in RNA synthesis, gave an increase in transcriptional activities.

These observations demonstrate that the mere presence of JH III in vitro does not elicit a stereotyped transcriptional response from the LCG at any given time during the reproductive cycle, but also, and more importantly, suggest that JH III constitutes only one link, albeit an indispensable one, in a chain of control elements regulating the synthetic activities of the LCG during the reproductive cycle. The stage specific form of in vitro response to the presence of JH III elicited

from the gland during the reproductive cycle seems to be governed by physiological factors which may not originate directly from the gland itself. In order to respond transcriptionally to the hormone, the LCG must apparently achieve a state of "competence". This state of physiological receptiveness to the presence of the hormone can be either positive or negative since it may lead to either stimulation or inhibition of RNA synthesis in a stage specific manner. The failure of the gland to respond transcriptionally to the presence of the hormone at certain specific stages (24 and 106h) may be taken to indicate that the competence to respond transcriptionally to JH III is a dynamic process in the LCG which may be independant of the direct effects of the hormone upon the gland.

The precise nature of the short term effects of JH III treatment upon in vitro translational activities in the LCG during the reproductive cycle is very uncertain.

The stages at which JH III treatment produced significant increases in the levels of in vitro protein synthesis in the LCG corresponded precisely with the points at which endogenous peaks in translational activities are known to occur during the reproductive cycle (Chapters II and III, sections III and IIb, pp 20-22; 62-66). Thus, in terms of its stimulatory effects upon RNA and protein synthesis, JH III treatment clearly potentiates a pre-existing synthetic condition in the LCG. The potentiation of translational activities is apparently non-specific since the levels of synthesis of each major low molecular weight polypeptide produced by the gland at these stages is uniformly increased.

However, most of the inhibitory effects of JH III treatment upon LCG translational activities in vitro cannot be readily explained.

JH III treatment results in a marked inhibition of in vitro

protein synthesis at five stages of the reproductive cycle (32, 48, 72, 80 and 88h). At three of these stages (32, 80 and 88h) this inhibition, of a very specific character, takes place in the face of stimulated transcriptional activity. The LCG during the ovulatory and post-ovulatory periods (from 64 to about 88h) is normally not exposed to high endogenous levels of JH III (Weaver et al., 1975; Weaver and Pratt, 1977). The specificity of the JH III induced inhibition of in vitro protein synthesis of particular polypeptides at these stages may be indicative of an RNA catabolic mechanism controlled by JH III in the LCG. Whether or not the observed effects are due to the degradation of very specific m-RNA molecules is not known. From these observations it is, however, apparent that short term JH III treatment can affect in vitro translational activity in the LCG quite independently from RNA synthesis. It has been shown that, in the LCG during the reproductive cycle, the major peaks in translational activities occur concurrently with a marked stimulation of poly(A)$^+$ RNA synthesis and follow by eight hours the major increases in transcriptional activities which primarily represent a stimulation of poly(A)$^-$ RNA synthesis (Zalokar, 1968; Chapter III, sections IIb-d, pp 62-70). It is now apparent that short term JH III treatment of the LCG at any given stage of the reproductive cycle does not elicit the production of new polypeptide species in the gland, nor does an in vitro, JH III induced, significant increase in RNA synthesis necessarily correspond to a concurrent increase in translational activities. Moreover, significant increases in the levels of protein synthesis in the LCG, be they endogenous or JH III induced in vitro, almost always correlate with low levels of transcriptional activity (Chapter III, section IIb, pp 62-66). Although these observations cannot be taken as an indication that JH III is without direct effects upon

m-RNA synthesis in the LCG, they certainly constitute a strong suggestion that, in the LCG, the patterns of translational activities during the reproductive cycle could be strongly and directly influenced by those of poly(A)$^-$ RNA synthesis which seem to be under the direct influence of JH III.

A major regulatory system controlling the secretory and possibly transcriptional activities of the LCG during the reproductive cycle has now been uncovered for the first time.

It has long been known that the secretory activity of the LCG is synchronized with ovulation (Bordas, 1909). However, very little is known of the mechanisms controlling ovulation and less so of those coordinating LCG secretory activity and oocyte development in P. americana (reviewed by Adiyodi and Adiyodi, 1975, Raabe, 1982; Weaver et al., 1985). The results obtained in the present study clearly indicate that factors of ovarian origin, present in the ovarioles shortly before and during ovulation only, are capable of inducing secretory activity in vitro and strongly inhibiting RNA synthesis in LCG s isolated at a stage far removed from ovulation and the onset of ootheca synthesis.

While ecdysterone acts as a strong inhibitor of in vitro RNA synthesis in the LCG, ovarioles containing chorionated oocytes are extremely potent repressors of in vitro transcriptional activity in the gland, much more so than ecdysterone. On the other hand the steroid hormone is a stronger inducer of in vitro LCG secretory activity than are ovarioles isolated shortly before ovulation. In the presence of JH III the induced secretory activities are inhibited.

These observations suggest that the strong inhibiton of LCG transcriptional activity in vitro, although originating from the ovarioles, is probably not ecdysterone but some other unknown ovarian

factor.

The results presented here may be of some importance in clarifying the mechanisms regulating some of the reproductive processes in P. americana when considered in the light of other reports.

In view of the patterns of macromolecule synthesis presented by the LCG, it is probable that there are three bursts of JH release during the reproductive cycle, two of which occur in close succession. P. americana presents a continuous and rhythmic reproductive cycle (Bell, 1969b) and at any given time two oocytes in any given ovariole are at different stages of vitellogenesis. The first increase in JH levels (an arbitrary designation) seems to take place around the time of vitellogenesis initiation in penultimate oocytes (T-1), and corresponds to the terminal phase of yolk deposition in the terminal (T) oocytes (Weaver et al., 1975; 1985, Chapter II, section IV, pp 22-24). During this period, the rate of T-1 oocyte growth is seen to increase rapidly while T oocyte elongation is abruptly stopped (Chapter II). At the same time , the levels of poly(A)$^-$ RNA synthesis in the LCG, presumably under the influence of JH, increases considerably followed, eight hours later, by large increases in translational activity and poly(A)$^+$ RNA synthesis (Chapter II, section III, pp 20-22 and Chapter III, section IIb, pp 62-66). These phases of high synthetic activities in the LCG end soon after the second burst of JH release, shortly before the onset of chorion formation by the T oocytes (Weaver et al., 1985; Chapters II and III).

It now seems likely that chorion deposition sets in motion a far reaching cascade of events. At this particular stage of oocyte development, the ovarioles start releasing ecdysterone (Weaver et al., 1985). During this period and until the onset of ovulation, the growth

rate of T and T-1 oocytes increases (Chapter II) while CA activity is inhibited (Adiyodi, 1966). At the same time, transcriptional and translational activities in the LCG decrease sharply while the gland starts releasing its accumulated secretions into the genital vestibulum (Chapters II and III).

The results presented here suggest that the initial fall in the synthetic activities of the gland at the onset of *in vivo* ootheca synthesis, shortly before ovulation, is not due to a fall in JH levels (CA inactivation) but could be caused by the release of ecdysterone and some as yet unknown factor from the ovarioles. At this particular point in the reproductive cycle, *in vitro* JH III treatment has no effects upon protein synthesis while producing a further decrease in RNA synthesis.

Taking into consideration the information currently available concerning the patterns of CA activities in relation to oocyte development, it is apparent that the CNS-CA complex is kept continuously informed about the state of the oocytes (reviewed by Adiyodi and Adiyodi, 1974; Tobe and Stay, 1982; Bell and Adiyodi, 1982), although the means whereby the information is transmitted to these centres remain unknown. In view of the results of the present study and the reports that ecdysterone can inhibit CA activity *in vivo* but not *in vitro* (Stay *et al.*, 1980; Friedel *et al.*, 1980), it could be speculated that, although the levels of ecdysterone produced by the ovarioles shortly before ovulation are relatively low (Weaver *et al.*, 1985), ovarian ecdysterone could be instrumental in triggering the inhibition of CA activity by indirect action. This putative involvement of ecdysterone in the initiation of CA repression, is in agreement with a large body of literature (reviewed by Tobe and Stay, 1982; Weaver *et al.*, 1985),

including the findings of Bulliere et al. (1979); Friedel et al., 1980 and Stay et al., 1980. Considering the necessity of CA inactivation for ovulation to occur (Hentschel, 1972; 1975), and the inhibition by JH III of ecdysterone induced LCG in vitro secretory activity, ecdysterone appears as a likely candidate for the roles of messenger to the CNS-CA complex concerning the status of T oocytes development at the time of chorion formation, as well as that of coordinator in the synchronization of LCG secretory activity with the onset of ovulation. These two processes require that JH levels be low, but, to be induced, LCG secretory activity also requires the presence of an ovarian factor which appears to be ecdysterone.

The period of ootheca synthesis corresponds to a very slow rate of oocyte growth as well as low levels of synthetic activities in the LCG (Chapters II and III).

The third burst in JH release appears to occur shortly after completion of the ootheca and probably extends until soon after ootheca deposition (new cycle). Over this period, the rate of T and T-1 oocyte growth increases markedly and transcriptional as well as translational activities in the LCG are stimulated (Chapters II and III). As was the case during the first two bursts of JH release, the synthesis of poly(A)$^-$ RNA represents the bulk of the increase in LCG transcriptional activity, presumably under the influence of JH III, with increases in protein and poly(A)$^+$ RNA synthesis following after an eight hours lag. The period between the end of this last increase in JH levels and the appearance of the first burst in the new cycle, sees no change in the growth rates of T and T-1 oocytes. During this period, translational activity in the LCG is falling rapidly while transcriptional activity increases steadily (Chapters II and III).

What causes the LCG to synthetically respond in a highly stage specific manner to the presence of JH III is not known. The form of *in vitro* synthetic responses which will be elicited from the gland by JH III at any given stage of the reproductive cycle is apparently not due to the direct effects of the hormone upon the gland.

Whether or not these stage specific effects are associated with the factors governing the patterns of CA activity in relation to oocyte development and their effects upon the reproductive apparatus as a whole remains to be elucidated.

SUMMARY

The present study investigated the *in vitro* effects of JH III and ecdysterone upon the synthetic and secretory activities of the LCG of mature females of *P. americana* at several specific time points during the reproductive cycle. Evidence was presented demonstrating that:

1) Short term *in vitro* JH III treatment primarily affects RNA synthesis.

2) The transcriptional response elicited from the LCG by the presence of JH III is not uniform throughout the reproductive cycle. The response may be positive (stimulated transcriptional activity), negative (inhibition of RNA synthesis) or null, depending on the stage of the cycle.

3) The stages of high transcriptional activity levels observed in the LCG during the reproductive cycle correlate precisely with the stages at which *in vitro* JH III treatment elicites a large positive response

from the isolated gland.

4) The immediate translational response to short term in vitro JH III treatment is generally negative (decreased translational activity), except at the stages of high translational activity in the LCG.

5) In vitro ecdysterone treatment depresses transcriptional activity in the LCG and induces spontaneous release of secretory material at stages far removed from ovulation.

6) The short term inhibitory effects of ecdysterone upon RNA synthesis are only slightly alleviated by JH III when both hormones are present in equal amounts while ecdysone induced spontaneous secretory activity is inhibited.

7) The presence of ovarioles isolated at 64h of the reproductive cycle (onset of in vivo LCG secretory activity) in the incubation medium gives rise to a very strong inhibition of RNA synthesis in the LCG and induces spontaneous secretory activity.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION

In most insects, oviposition follows ovulation very closely (Adiyodi and Adiyodi, 1974). The two events are almost synchronous and could be regulated, to some extent, by the same control mechanisms. The Blattaria however, group their eggs into distinct batches arranged in two rows (Fisher, 1928; Gould and Deay, 1938, Rau, 1940), P.americana placing 18 to 28 eggs (most commonly 22-24) within each ootheca (Griffiths and Tauber, 1942), thus temporally separating ovulation and oviposition. Reproduction in the Blattaria is a cyclic event (Engelmann, 1960; Engelmann and Rau 1965; Barth, 1968; Bell, 1969; Barth and Bell, 1970).

I) MECHANISMS OF REPRODUCTION IN P.americana.

The physiological mechanisms pertaining to insect reproduction, most particularly in cockroaches, have been the object of intensive studies for nearly four decades. Reproduction takes place as the result of many processes that occur sequentially in time and are different in nature.

Reproduction in P.americana is composed of four distinct phases:

a) Previtellogenesis (vitellarium differentiation).

b) Vitellogenesis.

c) Ovulation.

d) Ootheca synthesis.

The reproductive process as a whole is governed by extrinsic factors such as food (Willis and Lewis, 1957; Engelmann and Rau, 1965; Mills et al., 1966; Bell, 1969b; Bell, 1971); temperature (Gunn, 1935; Roth and

Willis, 1956; Mills et al., 1966; Adiyodi and Nayar, 1965; Adiyodi and Adiyodi, 1974); mating (Griffiths and Tauber, 1942; Roth and Willis, 1956; Adiyodi and Adiyodi, 1975); predominant light intensities and the density and behaviour of conspecific individuals (Cornwell, 1968; Roth, 1981); and by intinsic factors primarily originating from the endocrine system including the pars intercerebralis-corpora cardiaca (PI-CC) complex and the corpora allata (CA).

These two regions of the brain are functionally closely associated and are respectively located in the anterior part and median posterior parts (retrocerebral complex) of the supraoesophageal ganglion (Arnold, 1960; Willey, 1961) and constitute the major sources of neurosecretions and hormones demonstrated to affect reproduction (reviewed by Tobe and Stay, 1982). Certain cells in these regions have, in addition to their ability to transmit nerve impulses, the ability to release hormones and neurosecretions into the circulatory system and effect a response from target organs.

The duration of the reproductive cycle is determined by the interplay between these introceptive and extroceptive cues, some acting at very specific stages of the reproductive cycle while others are very unspecific in this respect.

A) Previtellogenesis and oocyte development.

Vitellarium development and cytoplasmic growth of the previtellogenic oocytes in insects have been shown by in vivo and in vitro studies to require the presence of ecdysteroids as well as an unidentified brain neurohormone (Mordue, 1965; Laverdure, 1972). These findings can be correlated with those of Bell and Sams (1975) who

reported that the ovaries of last instar nymphs of P.americana must be exposed to ecdysterone in the absence of JH in order to become vitellogenic when exposed to JH at a later time. Moreover, the detection of ecdysteroids in the heamolymph and the ovaries of several cockroach species (Imboden et al., 1978; Bulliere et al., 1979;Tobe and Stay, 1980; Stay et al., 1980; Friedel et al., 1980), including P.americana (Weaver et al., 1985) together with the observation that ecdysterone inhibits JH synthesis in a dose dependent fashion in vivo (Stay et al., 1980;Friedel et al., 1980) but not in in vitro (Friedel et al., 1980) suggest that the allatostatic effect is mediated by the CNS.

These findings correlate very precisely with the results obtained in the present study. It was found that, as expected in view of the report of Weaver et al. (1985), ovarioles isolated shortly before ovulation appeared to release ecdysterone. It was also shown that pre-vitellogenic oocytes initiate their active growth period during ootheca synthesis, between 80 and 88h. At these stages, the ovarioles appeared to have ceased to release ecdysterone. This period was immediately followed by an apparent long burst of JH release during which the formerly pre-vitellogenic oocytes (now T-1) enter an active vitellogenic phase (Chapter II, section IV, pp 22-24 and figure 4, p 23). Moreover, during the period immediately preceding ovulation, The LCG not only presented a fall in synthetic activities but also responded negatively to the presence of JH III. This decrease in RNA and protein synthesis appeared to be associated with the release of an inhibitor produced by the ovarioles concurrently with ecdysterone. Whether or not the sharp fall in LCG synthetic activities during this phase of the reproductive cycle is solely due to the effects of this unidentified ovarian factor is not

known. However, it is very clear that JH III levels do not, at this stage, regulate the synthetic functions of the LCG. The observations relating to the patterns of oocyte development and the synthetic response of the LCG during exposure to ecdysterone, followed by the appearance of a peak in JH levels, correlate well with the suggestion that CA inhibition appears to be associated with the appearence of ecdysterone and is probably mediated by the CNS through the release of an allatostatic neurohormone (see Fig. 1, p 122).

B) Vitellogenesis and oocyte maturation.

This aspect of oocyte development is directly under the influence of the juvenile hormones. It has been demonstrated in several insect species, including P.americana, that JH is released from the CA as soon as it is synthesized (Pratt et al., 1975; Weaver and Pratt, 1977). It is thus the biosynthesis of JH rather than release from the CA which ultimately regulates the amount of JH present in the haemolymph. Using radiochemical assays, the changes in rate of JH synthesis have been followed in P.americana adult females in relation to the reproductive cycle (Weaver et al., 1975; Weaver and Pratt, 1977). From these studies, it became apparent that the CA present at least two cyclic peaks of JH synthesis and release, associated with waves of oocyte development, indicating that CA activity is synchronized with oocyte maturation. In view of the continuous and rhythmic nature of the reproductive cycle in P.americana, and the lack of detailed information regarding :

a) The probable timing of the bursts of JH release, and

b) the concurrent patterns of oocyte development,

it had not been possible to correlate a given peak in JH synthesis with a given wave of oocyte development.

The present studies have provided some of this information. In view of the good correlations reported between the cycles of JH release from the CA and the profile of oocyte development (see above) as well as the patterns of macromolecule synthesis in the LCG (Zalokar, 1968; Chapters II and III), it is probable that there are three bursts of JH release during the reproductive cycle. Each of these peaks seems to be associated with a particular phase of vitellogenesis in T and T-1 oocytes in alternation. The first burst of JH release (at about 32h) appears to correspond to the onset of the active phase of vitellogenesis in T-1 oocytes. The second peak in JH release follows very closely (at about 48h) and correlates with the late phase of yolk deposition in T oocytes, shortly before the onset of chorion formation. The third peak in JH levels seems to occur soon after completion of the ootheca (at about 96h) and probably extends over a twenty four-hour period, until eight to sixteen hours after ootheca deposition. This burst correlates with the initiation of vitellogenesis in T-1 oocytes and with a very active phase of yolk deposition in T oocytes.

The juvenile hormones have an extensive regulatory function upon the entire reproductive process in P. americana as well as other cockroach species. Vitellogenin synthesis in the fat body; vitellogenin uptake into the oocytes and yolk formation, that is the whole process of oocyte development, requires the presence of JH (Thomas and Nation, 1966; Engelmann, 1968; 1970; Koeppe et al., 1980; de Kort et al., 1981). During vitellogenesis, JH not only regulates vitellogenin uptake but also DNA synthesis in the follicle cells (Koeppe and Wellman, 1980; Koeppe et al., 1980). However, a number of reports also indicate that other

neuroendocrine centres could be actively involved in the control of oocyte development.

Variations in the activity of the PI-CC neurosecretory system have been correlated with vitellogenesis in several insect genera including the Blattaria (reviewed by Raabe, 1982). In P.americana, material from the PI-CC complex is released in large amount twice during each reproductive cycle:

i) one release prior to ovulation, and

ii) another immediately after oviposition (Adiyodi, 1967),

thus indicating a functional periodicity in the activity of the PI-CC neurosecretory cells in apparent synchrony with the reproductive cycle.

Accumulation of neurosecretory material in the PI-CC complex of cockroaches has been reported to occur under experimental conditions resulting in ovarian suppression such as starvation, ovariectomy (Adiyodi and Bern, 1968), and administration of an ovary inhibiting extract of crab eyestalks (Adiyodi and Adiyodi, 1974).

A direct role of the PI-CC neurosecretions upon oocyte maturation has not been demonstrated in P.americana where integrity of the PI-CC complex does not appear to be indispensable for ootheca production once the reproductive cycle has been initiated (Engelmann and Penney, 1966; Adiyodi, 1974). However, the possibility of an indirect action upon the rate of oocyte development cannot be ruled out.

Several exogenous factors play a major controlling role at this level.

-Deprivation of food over an extended period causes:

i) an abrupt curtailing of vitellogenesis (Bell, 1971; Brousse- Gaury, 1977).

ii) inactivity of the CA (von Harnack, 1961).

iii) oocyte resorption (Bell and Adiyodi ,1982).

Subsequent feeding results in resumption of CA activity and oocyte development.

-The sensory stimulation elicited by mating is important in maintaining JH synthesis, and thus CA activity and vitellogenesis, at peak level. Although enforced virginity in adult P.americana females results in a marked decrease in JH synthesis and yolk deposition as well as a virtual disappearance of cyclicity in CA activity (Weaver and Pratt, 1977), oocyte maturation can still proceed without mating, albeit at a much slower rate (Roth and Willis, 1956), once the reproductive cycle has been initiated (ie. in parthenogenesis). The stimulus elicited by mating may be purely mechanical in nature as inferred by a number of studies:

i) castrated males are capable of stimulating oocyte development in Diploptera, Nauphoeta and Leucophaea (Roth and Stay, 1961; Roth, 1964),

ii) purely mechanical stimulation in the total absence of males causes egg maturation in Diploptera and Leucophaea (Roth and Stay, 1961),

iii) transection of the ventral nerve cord prior to or shortly after mating prevents CA activation (Engelmann, 1959; Roth and Stay, 1961), and

iv) copulation may induce the release of secretion from the PI-CC complex in some Blattaria species (Barth, 1968).

However,mechanical stimulation of the genitalia during periods of sexual non-receptivity has no influence on CA activity in the adult female (Engelmann, 1970).

C) Ovulation.

The causative mechanisms of ovulation are imperfectly understood,

but there are reasons to believe that both neural and neuroendocrine controls may be implicated. In P. americana, ovulation is attended by a steep, if transient, decline in CA activity and a burst of neurosecretory release from the PI-CC complex, which could include biogenic amines. Inhibitor studies suggest that dopamine is involved in the suppression of CA activity at the onset of ovulation; a suppression which cannot be elicited in biogenic amines depleted females and results in a failure to ovulate (Hentschel, 1972; 1975).

Extrinsic factors do also show some effects on the incidence and progress of ovulation. Exposure to low temperature apparently inhibits the onset of ovulation (Adiyodi and Adiyodi, 1974), and mating could also play an important role since ovulation in virgin females is often only partial whereas mated females normally ovulate all their mature eggs (Roth and Stay, 1962).

D) Ootheca formation.

As indicated earlier, the structural and enzymatic components required for ootheca synthesis are produced and secreted by the colleterial glands. The synthesis and accumulation of the structural components of the ootheca was widely assumed to be the only function of the LCG during the reproductive cycle. While this remains a major aspect of LCG function during the oothecal cycle, it appears that this organ may also be involved in the very active synthesis of polypeptides which do not take part in the formation of the ootheca but are probably produced for export (Chapter II, sections V and IV, pp 24-39).

The accumulation of secretory material in the glands is stimulated by JH (Bodenstein and Sprague, 1959; Bodenstein and Shaaya, 1968;

Adiyodi, 1968; Emmerich and Barth, 1968).

In this respect, it is thought that JH most probably stimulates translational activity in the LCG. The present studies demonstrated that this is not necessarily a direct response and that JH III is not the only regulatory element controlling the synthetic activities of the LCG during the reproductive cycle (see below).

In order to produce a progeny, the fertilized oocytes must be protected from desiccation and the secretory activity of the colleterial glands must be synchronised with the ovulation process. However, little was known of the mechanisms whereby the release of accumulated secretory material is elicited from the glands at the onset of ovulation. At this point of the reproductive cycle, CA activity is at a minimum. Reserpin treatment prevents this fall in JH synthesis and leads to a failure to ovulate and an inhibition of secretion release from the colleterial glands (section C). Moreover, treatment with antigonadotropic and antioestrogenic drugs such as clomiphen (1[-p-(diethylamino-ethoxy)phenyl-1]-1-2,diphenyl-2-chloroethylene) (Adiyodi and Nayar, 1965) gives rise to:

i) precocious and gross discharge of neurosecretory material from the PI-CC complex,

ii) strong reduction in CA activity levels, and

iii) desynchronisation of ovulation and colleterial secretory activity, leading to eggs being expelled individually with, and in absence of, colleterial secretion, depending on the reproductive stage at which treatment was administered.

This latter effect has been reported to occur spontaneously in laboratory colonies (Roth and Willis, 1954).

It is, at the moment, difficult to interpret the latter experimental

data. It is highly probable that administration of clomiphen results in a wide range of damaging effects on the neuroendocrine system as a whole and this would, naturally, affect the reproductive mechanism. Hence, whether or not the deregulation of ovulation and oviposition are primarily due to the effects of this drug on CA activity alone remains highly debatable.

The present study demonstrated clearly that ecdysterone acts as a potent inducer of secretory activity in glands isolated at a stage where secretory activity is not normally seen in vivo. The isolated LCGs did not release appreciable quantities of secretory material when incubated in the absence of ecdysterone or in the presence of JH III. The presence of ovarioles isolated during chorion deposition in T oocytes caused the isolated glands to release their luminal content. Moreover, the presence of JH III concurrently with ecdysterone abolished the induction of secretory activity. Thus the in vitro induction of LCG secretory activity apparently requires low JH levels concurrently with the presence of ecdysterone. These finding are in good agreement with the reports outlined above.

II) THE INTEGRATED CONTROL OF REPRODUCTION.

From the above considerations, it is apparent that the control of reproduction ultimately rests with the ability of the CNS to integrate incoming extrinsic information with the internal physiological status which, in turn, depends upon 1) endogenous rhythms and, 2) environmental factors. Certain phases of the reproductive cycle require high CA activity levels (vitellogenesis and post-ovulatory periods), while

others, to be elicited, require low levels of CA activity (onset of ovulation and ootheca synthesis and development of previtellogenic oocytes). It is therefore the profile of CA activity which directs the flow and timing of the different phases of reproduction. However, the CNS plays a powerful overiding role in the control of CA activity. If two stimuli of opposing nature are simultaneously present, the CNS integrates appropriately. Nutritional factors, sensory stimuli and endogenous humoral/neurohormonal factors can also affect CA activity directly and indirectly. A degree of interaction between these divers modes of CA regulation is apparently present at all times during the reproductive cycle. The end result is the integrated control of the reproductive cycle in a manner which benefits the species best. The reproductive process, which can be initiated and completed if enough reserves are available and/or if the CA are sufficiently stimulated (artificially and otherwise), does not occur spontaneously under unfavourable conditions, and can be abruptly curtailed. Nevertheless, the mechanism whereby JH can effect a cellular response from its numerous target tissues is still very poorly understood. Numerous reports indicate that JH has a marked stimulatory effect upon the rate of synthesis of some very specific proteins such as vitellogenin (Thomas and Nation, 1966; Adiyodi and Nayar, 1966; 1967; Bell, 1969; 1970), ovarian thymidine kinase and a JH-specific carrier protein (Koeppe et al., 1981; Kovalic and Koeppe, 1982). In some instances, it has been shown that JH acts at the genomic level by stimulating transcriptional activity (RNA synthesis) which then leads to increased production of specific polypeptides. This is the case for vitellogenin (Chienzi et al., 1982) and thymidine kinase (Koeppe et al., 1981). Whether or not this stimulation of highly specific transcriptional activity is mediated by JH directly is still

unresolved. Due to the wide variety of effects reported to be associated with JH, the ultimate mode of action of this hormone upon gene activity in its target cells remains largely mysterious.

The present study indicates that JH III appears to primarily affect RNA metabolism in the LCG during the reproductive cycle. However, in this specialized insect tissue, the effects associated with the presence of JH III *in vitro* are complex. The terpenoid hormone certainly potentiates RNA and protein synthesis in the LCG, but the tissue does not respond to the hormone in a stereotyped manner. The synthesis of very specific colleterial proteins will be affected at some stages of the cycle, whereas at other time points, the translational response will be generalized or null.

The synthesis of poly(A)$^-$ RNA appears to be directly affected, in a stage specific manner, by JH III. The patterns of poly(A)$^-$ RNA synthesis appear, in turn, to affect those of protein and possibly poly(A)$^+$ RNA synthesis in the LCG. Nevertheless, the results presented in this thesis clearly indicate that despite the apparently strong link between the patterns of transcriptional and translational activities, both processes can still be affected independently by JH III.

It is now evident that the ultimate effects of JH III upon the synthetic activities of the LCG are subject to modulation by other factors. The probable origin of some of these co-regulatory elements, their effects as well as their time of appearance in the LCG system, have been resolved. However, the probable identity of only one of these modulating elements is known.

CONCLUSION

The control of reproduction in P. americana, although still poorly understood, is quite obviously a very complex process. Several different mechanisms are continuously interacting to produce an integrated form of control.

The left colleterial gland is an integral part of the reproductive apparatus of P. americana, and successful reproduction is largely dependent upon its synthetic and secretory activities. Animals possessing perfectly functional ovarioles but a defective LCG simply cannot reproduce: the oocytes are expelled individually and desiccate very rapidly, thereby defeating the whole process of oocyte maturation, ovulation and fertilization. The synthetic and secretory activities of this organ are therefore stringently and very accurately controlled during the reproductive cycle. It has been thought, until very recently, that JH was primarily responsible for the regulation of the synthetic and possibly the secretory activities of the LCG during the reproductive cycle. The mechanism was assumed to be based upon a simple positive mode of control: high JH levels meant enhanced synthetic activities and low JH levels corresponded to a slow rate of protein synthesis and accumulation in the gland. It is now evident that the system regulating LCG activities during the reproductive cycle is considerably more complex.

Figure 1 summarizes the present state of knowledge regarding the mechanisms whereby the synthetic and secretory activities of the LCG are regulated during the oothecal cycle.

The CA are by far the most important endocrine centers in terms of insect reproductive physiology. The secretory products of the CA are the terpenoid hormones known as the juvenile hormones (JH) (Wigglesworth,

Fig. 1

Schematic representation of the present state of knowledge regarding the mechanisms controlling the synthetic and secretory activities of the LCG during the reproductive cycle.

CNS = Central nervous system (supraoesophageal ganglion).

PI-CC = Pars intercerebralis-corpora cardiaca complex.

CA = Corpora allata.

1 : Hentschel, 1972; 1979; Adiyodi and Nayar, 1965.

2 : Tobe and Stay, 1977; Barth and Sroka, 1975; Fraser and Pipa, 1977.

3 : Friedel et al., 1980; Stay et al., 1980.

4 : Weaver et al., 1984.

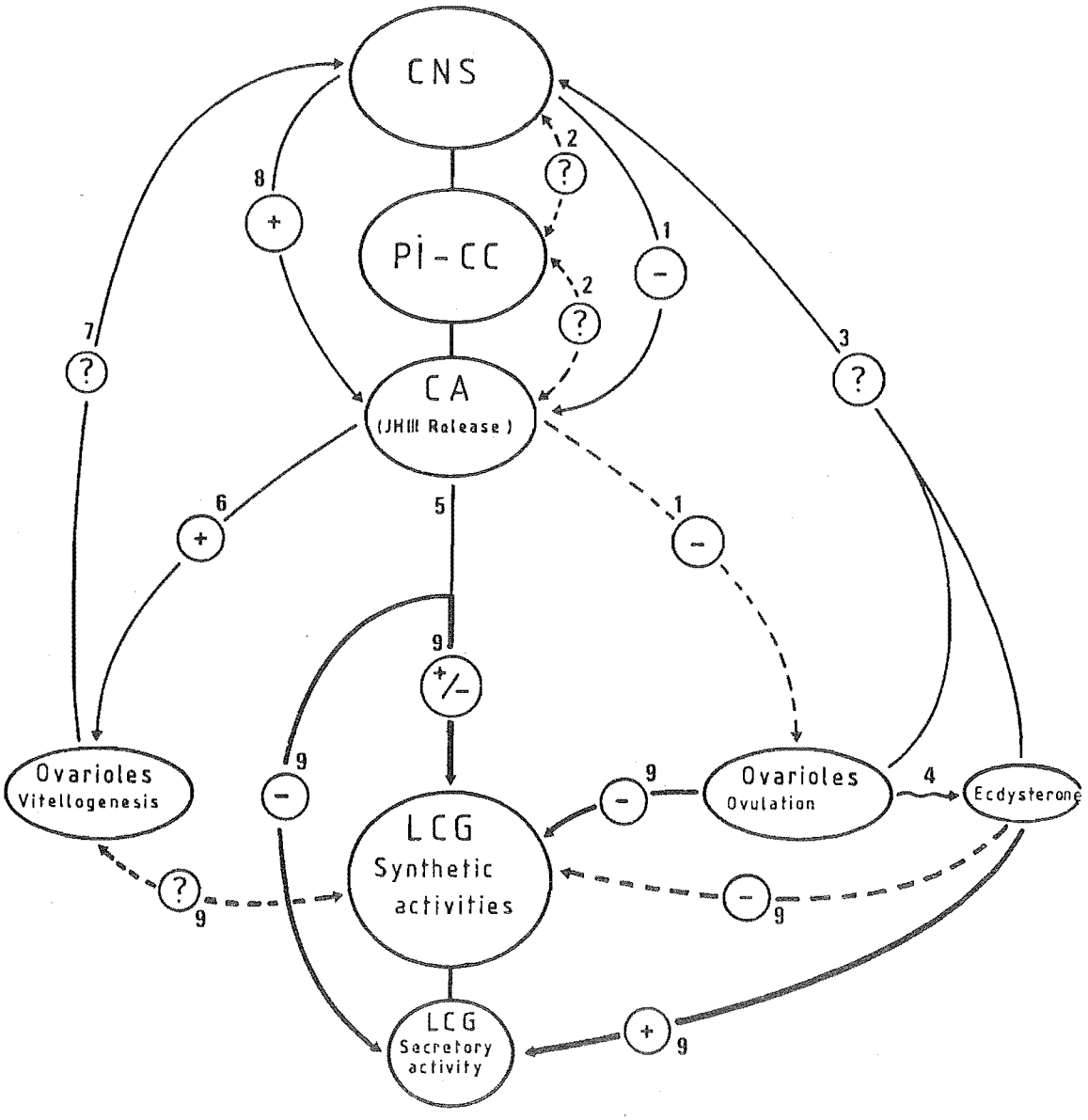
5 : Bodenstein and Sprague, 1959; Bodenstein and Shaaya, 1968.

6 : Thomas and Nation, 1966; De Kort et al., 1981.

7 : Adiyodi and Adiyodi, 1974; Laverdure, 1972.

8 : Tobe and Stay, 1979; 1980.

9 : Present study.


CNS
PI-CC
CA
(JHIII Release)
Ovarioles
Vitellogenesis
Ovarioles
Ovulation
Ecdysterone
LCG
Synthetic
activities
LCG
Secretory
activity
?
+
−
+/−

1940). In most insect species investigated the CA is capable of producing three types of JH [C_{18}, C_{17} and C_{16}, commonly known as JH I, JH II and JH III respectively (Kunkel, 1973; Lanzrein et al., 1975; Lüscher and Lanzrein, 1976; Lanzrein et al., 1978; Lanzrein, 1979)]. The CA of adult P.americana appear to synthesize and release the C_{16} form only (JH III) (Pratt et al., 1978; Hamnett and Pratt, 1978). Recent studies indicate that JH III is the predominant form in most adult insects (Lanzrein et al., 1978; Schooley et al., 1978; Trautmann et al., 1976).

The juvenile hormones play a central role and are involved in all the mechanisms related to reproduction. The direct stimulatory effects of JH upon the production of sex pheromones (Barth, 1962; 1968; Emmerlich and Barth, 1968) and the sexual receptivity of adult females (Engelmann and Barth, 1968) have been demonstrated in at least two cockroach species (Byrsotria and Leucophaea). The development and functioning of both adult male and female accessory sex glands are under the influence of these hormones (Scharrer, 1946; Bodenstein and Sprague, 1959; Willis and Brunet, 1966; Bodenstein and Shaaya, 1968; Shaaya and Bodenstein, 1969; Chen, 1984).

The cyclic nature of CA activity during the reproductive cycle has been well documented in P. americana (Pratt, 1967; Bell, 1969; Weaver et al., 1975; Weaver and Pratt, 1977). Although the CA are known to possess a self regulating component in the form of a negative/positive feedback based on JH haemolymph levels (Tobe and Stay, 1979; 1980), the means whereby the cycles of CA activity are regulated in relation to the patterns of oocyte development are still poorly understood. A considerable body of literature indicates that components of the CNS, particularly the PI-CC complex, could play an important regulatory role.

In insects, the PI-CC complex has been suggested to play a dual role in reproduction:

i) regulation of the corpora allata by allatostatic (inhibitory) and allatotropic (stimulatory) factors transmitted through the neurosecretory fiber terminals which are in contact with the CA (Fraser and Pipa, 1977; Barth and Sroka, 1975; Tobe and Stay, 1977), and

ii) stimulation of vitellogenin synthesis in the fat body during vitellogenesis (Wyss-Hüber and Lüscher, 1966; 1972).

However, a direct relationship between the release of neurosecretory material from the PI-CC complex and the cycles of CA activity has not been demonstrated in P. americana.

JH III plays an important role in the control of LCG synthetic activities during the reproductive cycle. The major peaks in RNA and protein synthesis in the LCG during the oothecal cycle correlate very precisely with the known cycles of JH III release from the CA (Chapters II and III). However, the ultimate effects of the hormone upon the synthetic activities of the gland are not uniform. JH III can stimulate or inhibit RNA as well as protein synthesis in the LCG, depending on the stage of the cycle. Although the effects of short term JH III treatment upon the synthetic activities of the LCG in vitro were investigated in some depth, the mechanism whereby the hormone can affect, positively or negatively, RNA synthesis and protein synthesis concurrently as well as independently are not understood. There is little doubt that JH III probably directly affects poly(A)$^+$ RNA metabolism in the LCG. However, whether or not these effects are

i) highly specific,

ii) mainly catabolic, and

iii) concurrent with and dependent upon specific patterns of poly(A)$^-$

RNA synthesis remains to be elucidated.

These observations strongly suggests that other regulatory elements are effectively priming the synthetic response of the gland in anticipation of the presence or absence of the hormone. What these regulatory factors could be is totally unknown. An indication as to their possible origin might be found in the observation that the ovarioles, shortly before ovulation, appear to release a very potent inhibitor of RNA synthesis in the LCG. Whether or not the ovarioles release such factors at several time points during the reproductive cycle is not known, nor is the nature of this inhibitor.

The ovarioles clearly play an important regulatory role during the reproductive cycle. Relatively high levels of ecdysterone, released by the ovaries, have been detected in the haemolymph of P. americana during the period of chorion formation and at the onset of ovulation (Weaver et al., 1985). The endocrine activity of the ovarioles appears to be short lived and does not extend past the end of the ovulatory period. The possible function of ecdysteroids in the Blattaria is disputed. There are indications that ecdysterone may play an important role in the development of pre-vitellogenic oocytes (Mordue, 1965; Laverdure, 1972) and that it may also be indirectly involved in the inhibition of CA activity shortly before the onset of ovulation (Bulliere et al., 1979;Tobe and Stay, 1980; Stay et al., 1980; Friedel et al., 1980). Weaver et al. (1985) believe that it is only an evolutionary left-over with no significance in terms of reproductive biology in the Blattaria.

The present study presents evidence that ecdysterone is directly involved in the control of LCG secretory activity during the reproductive cycle of P. americana. The observation that the presence of JH III

concurrently with ecdysterone abolishes the induced secretory activity supports the suggestion that the ovarioles, through the release of ecdysterone, could be instrumental in triggering the inhibition of CA activity required for ovulation to occur.

It is, however, apparent that the ovarioles are not directly associated with the increases in synthetic activities of the LCG, at least during the early stages of the reproductive cycle (Chapter IV, section IIIa, pp 94-96). Nevertheless, an indirect role of ovarian factors in the regulation of CA activity during the early phases of the reproductive cycle cannot be ruled out.

FINAL COMMENTS

Although the work presented in this thesis provides answers to several poorly understood aspects of LCG biology, it also gives rise to many more questions.

The precise functions of the LCG during the reproductive cycle are not entirely clear. The LCG appears to produce large quantities of polypeptides for export. But what the possible targets for this considerable outflow of proteins from the gland might be is not known. It could be speculated that oocytes in their active phase of development might constitute a possible end point. Indeed, there is a close relationship between the relative positions of the LCG and the ovarioles. Moreover, the tubules of the LCG are often seen in intimate physical contact with the ovarioles, particularly during the early phases of the reproductive cycle. Whether or not these observations could be indicative of a relationship other than a purely incidental one remains highly

debatable.

The considerable quantities of major low molecular weight polypeptides produced by the LCG during the reproductive cycle strongly suggests that the genes coding for these proteins are probably greatly amplified. The very high levels of poly(A)$^+$ RNA recovered from the gland at specific stages of the reproductive cycle, together with the observation that high levels of DNA synthesis seem to occur concurrently with the increases in poly(A)$^-$ RNA synthesis (data not included in this thesis) suggest that gene amplification could be a dynamic process in the LCG during the reproductive cycle. Practically nothing is known of the patterns of DNA synthesis in the LCG during the reproductive cycle. Yet several reports indicate that JH treatment leads to stimulation of DNA synthesis in ovarian follicle cells. Could the eight hours lag between the peaks in transcriptional activity and the increases in protein synthesis observed to occur in the LCG be representative of a period of active gene amplification preceding the major increases in translational activities? If so, what is the relationship between the patterns of poly(A)$^-$ RNA synthesis and the mechanisms of gene amplification (if any)?

Finally, the electrophoretic patterns of protein synthesis in the LCG change very rapidly during the reproductive cycle, yet JH III *in vitro* treatment failed to produce qualitative changes in these patterns. Is it because JH III cannot directly induce changes in the patterns of structural gene expression? If so, what exactly causes the apparent changes? How are the genes coding for the major colleterial proteins controlled? Are they grouped on the same chromosome? Are they dispersed throughout the genome?

Most of these questions arise directly from the work presented here.

Although a great deal remains to be done, an understanding of the mechanism controlling the synthetic activities of the LCG in relation to oocyte development has been achieved. The means whereby the secretory activities of the gland are synchronized with ovulation have been uncovered. During the course of this study, several highly sensitive analytical techniques have been developed, including a reliable method of nucleic acid extraction and purification from small tissue samples and a powerful unidimensional electrophoretic technique.

It is hoped that the technical and factual information contained in this thesis will not only assist but also motivate further investigation of this difficult but fascinating system.

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