## *A study of the effects of the juvenile hormone analogue methoprene on the intermediary metabolism of the African migratory locust*

Cotton, Gregory

# A Study of the Effects of the Juvenile Hormone Analogue Methoprene on the Intermediary Metabolism of the African Migratory Locust

by

**Gregory Cotton**

**A Thesis submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy**

**Department of Zoology**

**The University of Durham**

**1989**

## Contents

## List of Figures

## List of Tables

## List of Plates

## Abstract

Insect juvenile hormone (JH) regulates the growth and development of insects. Synthetic analogues of JH (JHAs) have been used as agents of pest control, disrupting the metamorphosis of insects. The purpose of the present study was to determine physiological and biochemical effects of methoprene, a commercial JHA, on certain organs and tissues of the African migratory locust (*Locusta migratoria migratorioides*, phase *gregaria*). Methoprene was topically applied to newly moulted, fifth (final) instar larvae and the subsequent development of the animals was followed.

Cytological development of fat body and dorsal longitudinal flight muscle was studied by light and electron microscopy. Fat body cells of control insects were synthetically active early in the fifth instar, and stored lipid and glycogen in the latter half of the instar. Fat bodies of 8-day old adults were sexually dimorphic, female cells showing high levels of RNA and protein synthesis while male cells were filled with lipid and glycogen stores. Methoprene treatment stimulated the synthetic activity of the cells in fifth instar and adult stadia, especially in female tissue. Cell nuclei were abnormally enlarged, suggesting increased ploidy levels. Levels of lipid and carbohydrate were measured in fat body and haemolymph but methoprene had no obvious effect on them, nor on glycogen phosphorylase activity. However, the JHA affected rates of incorporation of [$^{14}$C]glucose into fat body lipids during the first four days of the fifth instar. Separation of haemolymph proteins by gel electrophoresis revealed an extra protein band in the blood of treated female locusts from the middle of the fifth instar onwards. The same band appeared in the blood of control females only when they reached sexual maturity.

Methoprene treatment disrupted normal development of dorsal longitudinal flight muscles during the fifth instar and early adult life. The JHA reduced

muscle fibre growth but seemed to accelerate myofibril and mitochondrial differentiation in the fifth instar. Treatment also inhibited formation of interfibrillar tracheoles and caused disruption of the myofilaments and sarcoplasmic reticulum in adult muscle. Mitochondria were isolated from flight muscles of mature adults and their respiratory metabolism was measured using an oxygen electrode. Mitochondria from control animals showed high rates of oxygen consumption and good respiratory control. Mitochondria from treated locusts had poor respiratory control and low respiratory rates. Similar results were obtained by *in vitro* applications of methoprene or juvenile hormone to mitochondria.

**Acknowledgements**

I would like to thank my supervisor, Dr. J. H. Anstee, for his constant, almost non-stop, advice and encouragement, without which this work would not have been possible. I would also like to thank Professor K. Bowler for the use of the facilities in his department. Special thanks go to Miss J. Chambers and Mr. T. Gibbons for their invaluable technical assistance. Also, thanks to Mrs. C. Richardson for help with electron microscopy, and to Mr. P. Loftus for his expert maintenance of the insectary.

## Abbreviations

CA – corpus allatum,
ADP – adenosine diphosphate,
AMP – adenosine monophosphate,
ATP – adenosine triphosphate,
BSA – bovine serum albumin,
D.D.S.A. – dodecenylsuccinic anhydride,
DMP 30 – 2,4,6-tri(dimethylaminomethyl)phenol,
DNA – deoxyribonucleic acid,
ER – endoplasmic reticulum,
20HE – 20-hydroxyecdysone,
IGR – insect growth regulator,
JH – juvenile hormone,
JHA – juvenile hormone analogue,
LDH – lactate dehydrogenase,
NAD – nicotinamide-adenine dinucleotide,
NADH – reduced nicotinamide-adenine dinucleotide,
mRNA – messenger RNA,
RCR – respiratory control ratio,
RER – rough endoplasmic reticulum,
RNA – ribonucleic acid,
SER – smooth endoplasmic reticulum,
SR – sarcoplasmic reticulum,
Temed – N,N,N',N'-tetramethylethylenediamine,
Tris – tris(hydroxymethyl)aminomethane,
Vg – vitellogenin.

# Chapter I

## General Introduction

The postembryonic development of insects is characterised by the integration and coordination of growth with regular shedding of the exoskeleton, ultimately leading to metamorphosis into an adult form which then undergoes sexual maturation. The morphogenetic changes involved in such development are primarily regulated by two classes of hormones, the ecdysones and the juvenile hormones (see reviews by RIDDIFORD, 1980, 1985; SRIDHARA, 1981). Ecdysone is produced by the prothoracic glands and, upon release, is converted to the biologically active form of 20-hydroxyecdysone (20HE) by peripheral tissues, most notably the fat body (see review by HOFFMANN and HETRU, 1983). The presence of 20HE in the blood initiates the moulting process in insects, acting upon epidermal cells to stimulate apolysis and the subsequent deposition of fresh cuticle. Juvenile hormone (JH) is secreted by the corpora allata and, acting in conjunction with ecdysone, determines the type of cuticle that is synthesised by the epidermal cells. Consequently, the JH titre in the blood is high at the onset of larval-larval moults to induce the production of larval cuticle. Low levels of JH bring about larval-pupal transformations, while the absence of JH causes metamorphosis to the adult form. The importance of JH in regulating development is demonstrated most clearly in *Bombyx mori* where removal of corpora allata (allatectomy) from young larvae results in pupation at the next moult and, finally, the formation of diminutive adults (BOUNHIOL, 1938). Figure 1.1 shows the levels of JH and ecdysone during the late larval and pupal instars of *Manduca sexta*. JH levels in the blood remain high throughout most of the fourth instar but fall sharply just before the moult to the fifth instar. JH levels rise again in the early part of the instar and then drop to undetectable levels until the larvae become prepupae when JH rises slightly for a brief period. Thereafter, JH is undetectable until the adult stage. Similar changes in the blood titres of these hormones have also been found in hemimetabolous insects such as *Locusta migratoria* (JOHNSON and HILL, 1973; JOLY *et al.*, 1977; BAEHR *et al.*, 1979; HUIBREGTSE-MINDERHOUD *et al.*, 1980) and *Periplaneta americana* (SHAAYA,

1978) although in these animals, which do not have a pupal instar, JH is absent from haemolymph throughout the last larval instar.

In the great majority of insect species studied, the prothoracic glands degenerate and disappear in the pupal or adult stages, although low levels of ecdysone may be still be produced in adult females by the ovaries (see review by SEDLAK, 1985). The corpora allata, however, remain intact after metamorphosis and the role of JH changes to that of a gonadotropin, inducing sexual maturation in the adult insects. The hormone stimulates the synthesis and release of vitellogenin, the yolk protein precursor, by the fat body (see reviews by ENGELMANN, 1979, 1980; WYATT, 1980) and is necessary for oocyte development in the ovary, causing shrinkage of follicle cells which surround the oocytes and so opening up spaces between the cells to give haemolymph-borne vitellogenin access to the oocyte surface (ABU-HAKIMA and DAVEY, 1975; DAVEY, 1981). JH also stimulates the activity of certain accessory glands in both the male and female reproductive systems (see KOEPPE *et al.*, 1985).

The potential of JH as a pest insect control agent was first recognized by WILLIAMS (1956) who found that extracts of JH from adult male Cecropia moths were biologically active when applied to silkworm pupae and prevented normal metamorphosis. Unfortunately, natural juvenile hormones are too unstable to be of commercial use, being rapidly broken down in the environment and by insects. However, since the discovery of SLÁMA and WILLIAMS (1965) that the wood products of certain American conifers contained JH activity, numerous analogues of JH have been described, many of which are more stable and show greater specificity of action than the natural hormone. The more active of these compounds are listed in the reviews of STAAL (1975), HENRICK (1982) and SLÁMA (1985), and the structures of some of these chemicals are shown in Figure 1.2. Five different forms of juvenile hormone have been isolated from various species of Lepidoptera but in all other insects that have been examined only one of these forms, JH III, has been shown to be present (see review by KING, 1983). The epoxide and ester groups of the natural juvenile hormones are the points of greatest structural instability and are particularly susceptible to hydrolysis and hydration, respectively. Such changes, followed by conjugation, provide the major pathways for JH metabolism in insects and result in the loss of most of the molecule's biological activity (see reviews by KING, 1983; HAMMOCK, 1985).

**Figure 1.1 — Titres of Juvenile Hormone and Ecdysone in *Manduca sexta***

Legend: ordinate – hormone titre,
abscissa – days during fourth, fifth and pupal instars,
↓ – time of ecdysis,
(——) – 20-hydroxyecdysone,
(- - - -) – juvenile hormone.

The levels of 20-HE were measured by radioimmunoassay as $\mu$g/ml of haemolymph in the larval instars and $\mu$g/g of fresh weight in the pupal instar. Juvenile hormone was measured by bioassay as ng/ml of haemolymph. (The graph is modified from BOLLENBACHER *et al.*, 1981; RIDDIFORD, 1980)

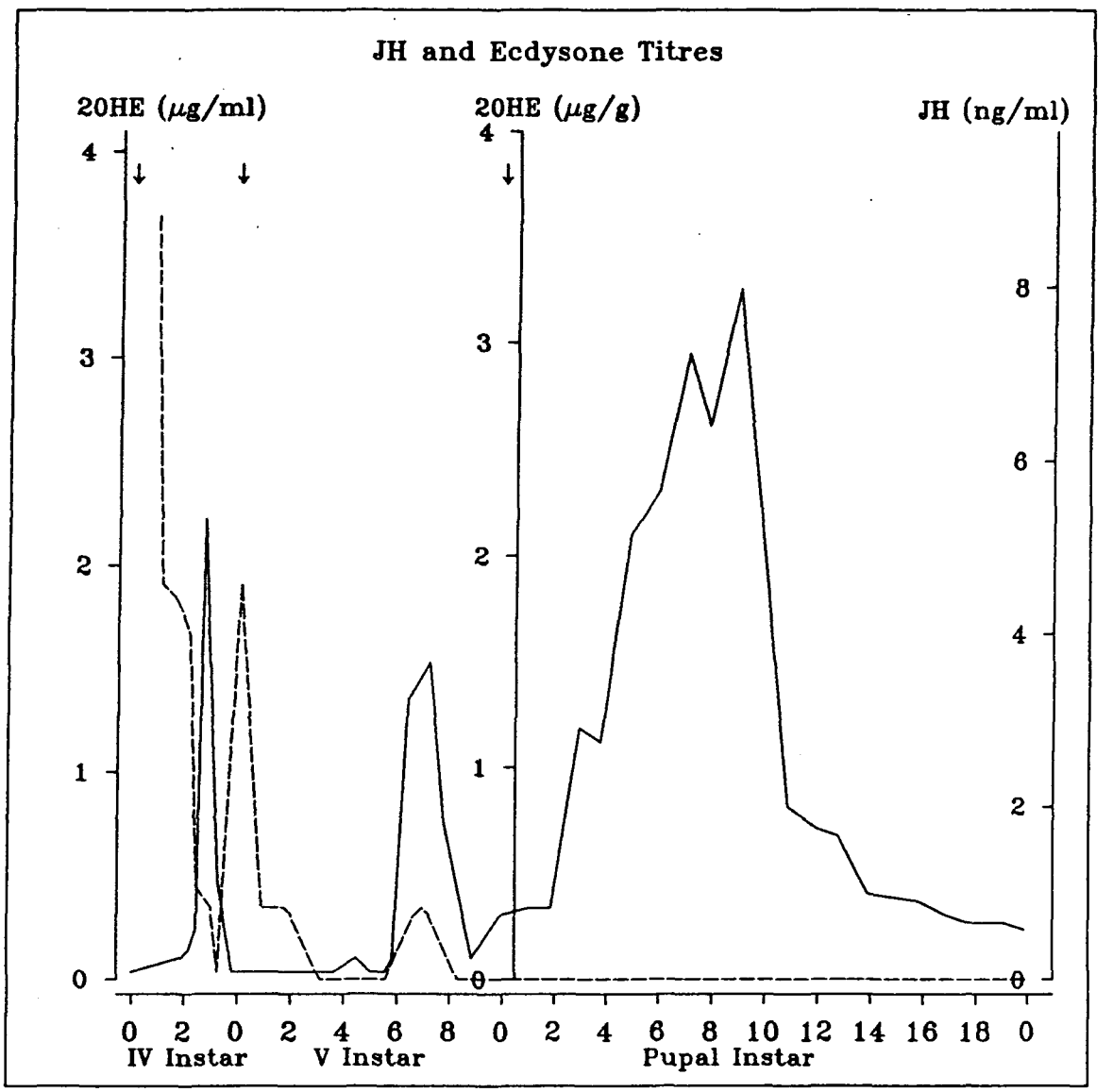
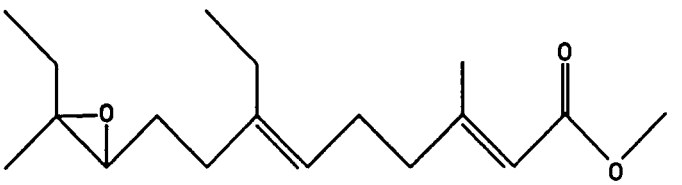

JH and Ecdysone Titres
20HE (μg/ml)
20HE (μg/g)
JH (ng/ml)
4
3
2
1
0
8
6
4
2
0 2 0 2 4 6 8 0 2 4 6 8 10 12 14 16 18 0
IV Instar
V Instar
Pupal Instar

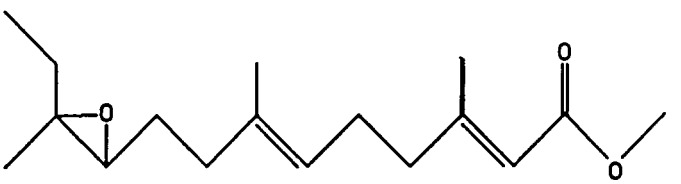
**Figure 1.2 — Chemical Structures of Juvenile Hormones and Juvenile Hormone Analogues**

Key: 1 – juvenile hormone I (RÖLLER *et al.*, 1967),
2 – juvenile hormone II (MEYER *et al.*, 1968),
3 – juvenile hormone III (JUDY *et al.*, 1973),
4 – farnesoic acid (SCHMIALEK, 1963),
5 – methoprene (HENRICK *et al.*, 1973),
6 – hydroprene (HENRICK *et al.*, 1973),
7 – kinoprene (NASSAR *et al.*, 1973).

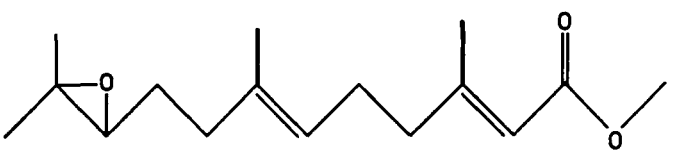
1.



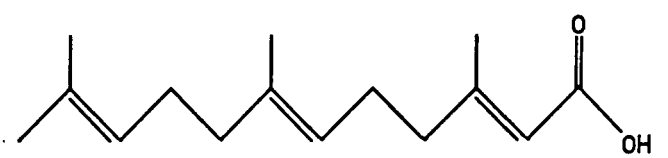
2.



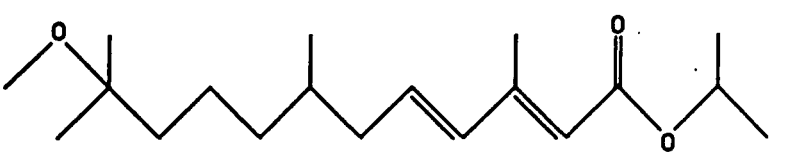
3.



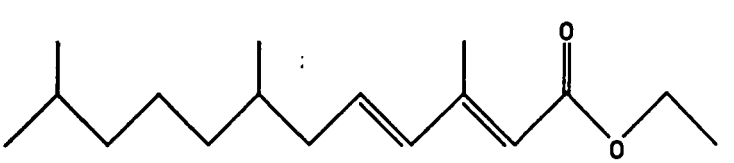
4.



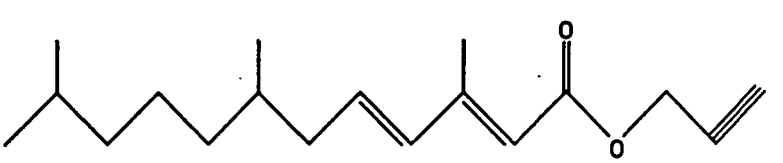
5.



6.



7.



The successful replacement of these groups with more stable structures while retaining JH activity has produced the most commercially important group of known juvenoids (HENRICK, 1982). Not only are such compounds more stable in the environment, but several workers have shown that at least one of these chemicals, methoprene, is not hydrolysed by the insect esterase enzymes which provide the major route for JH degradation in insects (HAMMOCK and QUISTAD, 1981). Methoprene was the first juvenile hormone analogue (JHA) to be registered with the U.S. Environmental Protection Agency for widespread use, against floodwater mosquitoes and as a feed-through horn fly insecticide. It is also used commercially for the control of Pharaoh ants (*Monomorium pharaonis*), the protection of stored tobacco and to increase silk production by larvae of the silkworm *Bombyx mori* (see STAAL, 1975; HENRICK, 1982).

As already mentioned with regard to *Bombyx mori*, elimination of JH from young insect larvae, by either surgical or chemical allatectomy, results in precocious metamorphosis to small and sterile adults (see review by BOWERS, 1985). Equally, raising the titre of JH during the periods when it is normally low or absent disrupts insect development. The reviews of STAAL (1975), VOGEL *et al.* (1979) and SEHNAL (1983) list the various effects that can be caused by applications of JH and JHAs. Treatment of insect eggs with juvenoids can prevent the development of normal larvae by halting embryogenesis at some stage, depending on the species, dose and timing of the application. For example, the eggs of the linden bug, *Pyrrhocoris apterus*, are most sensitive to JHA at the start of embryogenesis, treatment causing embryonic development to stop at blastokinesis (MATOLIN, 1970). JH-active compounds rarely have any effect if applied to early larval instars when the JH titre in the blood is already naturally high. However, the application of JHA to the cockroach *Blattella germanica* during its first larval instar results in high mortalities at the subsequent moult (HANGARTNER and MASNER, 1973). In last-larval and pupal instars, when JH is absent or present only at very low levels, juvenoid treatment can inhibit metamorphosis. Among the Exopterygota, the growth and functioning of insect cells are not usually affected but imaginal differentiation is disrupted, preserving larval characteristics through the final ecdysis (metathetely). For example, POELS and BEENAKKERS (1969) found that implantation of active corpora allata (CA) into *Locusta migratoria* at the start of the fifth (final) larval instar resulted in for-

mation of metathetelic adults with crumpled wings. Larval/adult intermediates have also been produced by treatment of fifth instar *Locusta* with JH (ROUSSEL, 1976) and JH analogues (ROUSSEL, 1973). In similar experiments on locusts, CA implantation and juvenoid treatment induced an extra larval moult with some of the supernumary larvae moulting to near-normal adults (VAN DEN HONDEL-FRANKEN *et al.*, 1980; ROUSSEL and PERRON, 1974). Such supernumary larvae are never perfect replicas of normal larvae because of the different growth rates of various body parts (allometric growth) and these morphometric abnormalities mean that supernumary larvae rarely survive an extra moult. Ovarian development may commence in the juvenoid-induced, extra larval stadia of certain species so that the supernumary larvae contain mature eggs, as in the stick insect *Dixippus morosus* (SOCHA and GELBIČ, 1973). In some species such as *Blattella germanica* (MASNER and HANGARTNER, 1973; MASNER *et al.*, 1975), juvenoids delay or completely block the imaginal moult, resulting in a permanent last larval stage. In the Endopterygota, juvenoids affect both larval-pupal and pupal-adult transformations. Effects range from the formation of perfect supernumary larvae which can moult successfully into fertile adults, such as with *Galleria mellonella* (CIEMIOR *et al.*, 1979), to the production of apparently normal adults which are sterile (see VOGEL *et al.*, 1979; SEHNAL, 1983). Between these two extremes are a myriad of non-viable intermediates combining larval, pupal and adult features (see VOGEL *et al.*, 1979; SEHNAL, 1983). Last instar larvae are usually most sensitive to juvenoid applications when newly moulted. Later treatments result in reduced effects as various tissues gradually become insensitive to JH. For example, the application of methoprene to early last instar larvae of *Choristoneura fumiferana* causes moulting to a supernumary larval instar (RETNAKARAN, 1973). However, treatment of larvae late in the instar usually results in a moult to larval/pupal intermediates, while treatment at the very end of the instar has little effect except for the retention of larval tubercles on the abdomen of the pupa.

The insect used in the present study was the African migratory locust, *Locusta migratoria migratorioides* (R & F), a member of the family of short-horned grasshoppers (Orthoptera: Acrididae). The locust is a suitable subject for physiological studies since it is a large insect, a single animal often providing sufficient tissue for several analyses, and has a relatively short life cycle, allowing develop-

mental studies to be carried out quite quickly. Being a hemimetabolous insect, the five larval instars, or hoppers, resemble the adult except that they are wingless and their gonads are undifferentiated. Locusts can be defined as grasshoppers which are capable of displaying density-dependent polymorphism, the high density type forming large swarms which are migratory. The two phases which may occur are *gregaria*, when in crowded conditions, and *solitaria* when widely dispersed. These two forms differ in coloration, morphology, physiology, behaviour and ecology but the phase differences are continuous and intermediates of all grades can be found between the two extreme types (see review by UVAROV, 1966). In conditions of high humidity, solitary phase *Locusta*, both adult and hopper, are a uniform pale green while they are grey or brown in arid conditions. Gregarious hoppers have a heavy black pattern on a yellow background and the adults are a general pink/brown colour until they reach sexual maturity, when the male becomes yellow. The first indication that JH might affect phase polymorphism in locusts was provided by JOLY and JOLY (1953). They demonstrated that the implantation of extra corpora allata into crowded hoppers of *Locusta migratoria* (phase *gregaria*) caused the haemolymph to change colour from yellow to green within forty-eight hours and the integument to become green after the next moult. Since then, many workers have induced green coloration, a solitary characteristic, in crowded locusts by CA implantation and by the application of JH-active compounds (see reviews by ROWELL, 1971; HARDIE and LEES, 1985), and it has now been shown that the natural JH titre is indeed higher in the haemolymph of solitary *Locusta* (JOLY and JOLY, 1974; JOLY *et al.*, 1977). However, such results do not necessarily mean that JH controls the phase transition of locusts since phase characteristics other than colour are unaffected by CA implantation (see HARDIE and LEES, 1985). Also, several species of grasshopper can exhibit green/brown colour polymorphism without displaying any density-dependent phase polymorphism (ROWELL, 1971), and implantation of CA into hoppers of the grasshopper *Acanthacris ruficornis* was found to produce a green adult form that has never been observed in the field (ROWELL, 1967).

Much work has been done to describe the morphological effects of treatment with juvenoids on many insect species and to assess their potential for pest insect control. However, the physiological and biochemical effects of these compounds are still not well understood. The purpose of this study was to elucidate some of

the effects of the JHA methoprene upon the physiology of the migratory locust during the last larval (fifth) instar and early adult stage.

# Chapter II

# General Materials and Methods

### 2.0.1 Maintenance of the Insects

The insectary was maintained at a temperature of 30°C ± 0.5°C and 60% ± 5% relative humidity. Air circulation was provided by three large electric fans and a slight, continuous air exchange was effected by means of an 'Expelair' ventilator. A constant photoperiod of 12 hours light and 12 hours dark was maintained. Populations of *Locusta migratoria migratorioides* (R & F), phase *gregaria*, were reared in cages (43 cm × 58 cm × 58 cm) supplied by Philip Harris Biological Ltd. Each cage was illuminated by a single 40 Watt bulb, and food in the form of green grass was supplied daily. The insects were reared at sufficiently high densities to ensure their remaining phase *gregaria* (JOLY and JOLY, 1953).

### 2.0.2 Sampling of Stock Population

A cage of stock population was prepared for sampling by removing all fifth instar locusts from the cage at 10 a.m. The population was then examined at 24 hour intervals (10 a.m. each day) and all fifth instar larvae were removed. These insects were 12 hours ± 12 hours old (first day, fifth instar) and were to be used subsequently in experiments. They were immediately treated with test solutions (see below for details) and approximately equal numbers of males and females were placed in 2 litre kilner jars at sufficient densities to ensure their remaining phase *gregaria*. The floors of the kilner jars were lined with tissue paper and wire-mesh perches were placed in the jars to give convenient support for the insects during moulting. The lids of the jars were also made of wire-mesh to permit a free flow of air. Experimental animals were kept in an incubator separated from the stock populations to avoid any contamination with the test solutions. The incubator was maintained at 30°C ± 0.5°C with a 12 hour light/12 hour dark photoperiod but the relative humidity was not controlled. The insects were supplied daily with fresh grass and, at the same time, they were counted and any dead animals were removed.

### 2.0.3 Treatment of Experimental Insects

After their removal from the stock population, newly moulted fifth instar larvae were individually treated with either 475 $\mu$g or 95 $\mu$g of the juvenile hormone analogue methoprene, dissolved in 5 $\mu$l of ethanol. The solution was topically applied to the dorsal surface of the insect's abdomen and thorax. Control locusts were treated in the same manner with 5 $\mu$l of absolute ethanol. After treatment, the animals were kept in kilner jars as described above. In order to study developmental changes in the experimental animals, the insect populations were sampled at intervals from the time of treatment (newly moulted fifth instar) until the animals were sexually mature adults when mating was observed (about eight days after the final moult). Unless stated otherwise, sampling took place on the following days (always at about 10 a.m.): Day 1 of the fifth instar (newly moulted larvae), Day 4 of the fifth instar, Day 7 of the fifth instar, Day 10 of the fifth instar, Day 1 of the adult stadium (newly moulted adults, approximately 11 days after treatment), Day 8 of the adult stadium (sexually mature adults).

### 2.0.4 Estimation of Protein Concentration

In order to estimate the protein concentrations of various tissues and tissue extracts, the Folin's method of LOWRY *et al.* (1951) was employed using the slight modification of KASHMEERY (1977). Folin's solution A was freshly made up before each protein estimation from the following solutions;

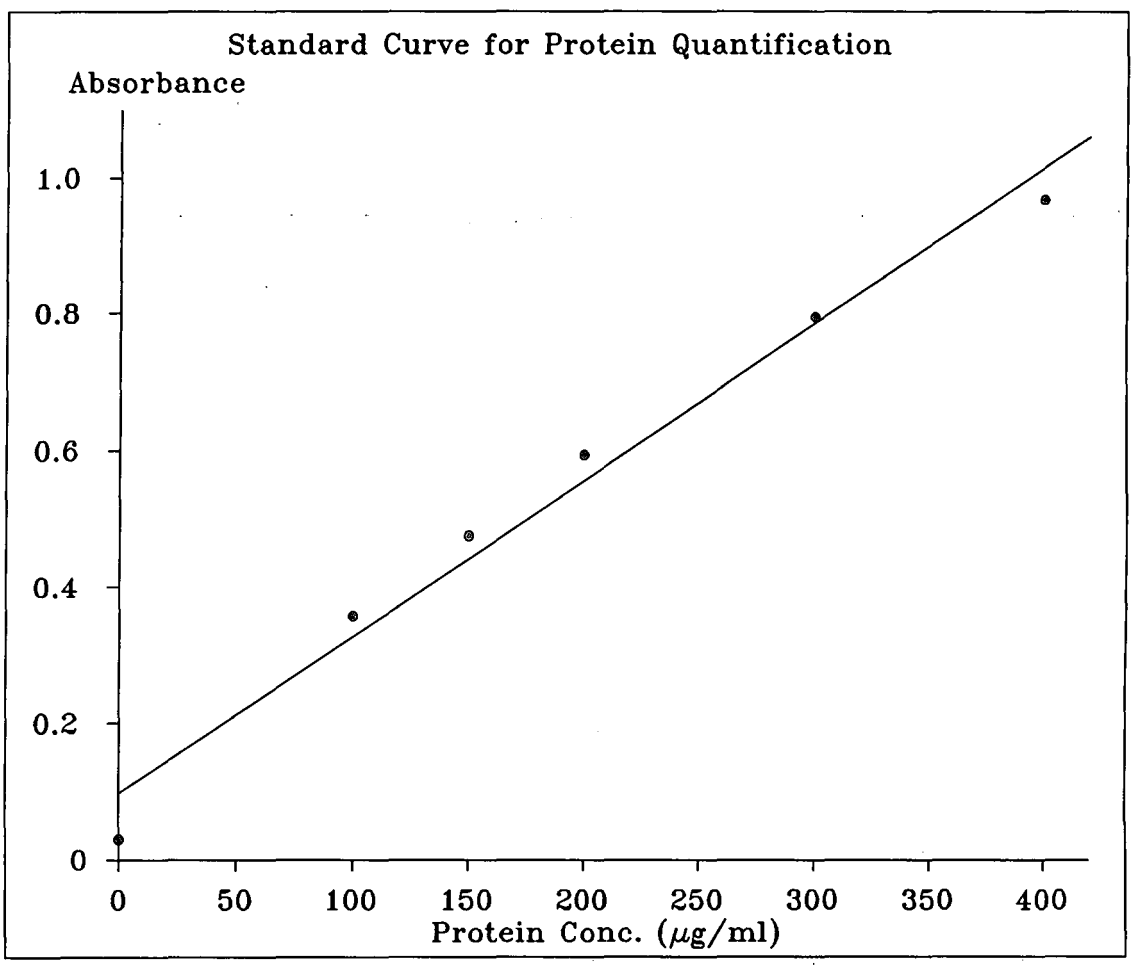
(a) 20% (w/v) sodium carbonate,
0.2% (w/v) sodium potassium tartrate,
(b) 2% (w/v) cupric sulphate.

Equal volumes of (a) and (b) were mixed with 4 volumes of distilled water. Folin's Solution B was prepared by diluting 1 volume of Folin-Ciocalteau's phenol reagent with 10 volumes of distilled water.

Aliquots of tissue extract containing 200–400 $\mu$g of protein were made up to 1 ml with distilled water and equal volumes of Folin's Solution A were added. The solutions were mixed on a Fisons 'Whirlimix' vortex mixer and were then allowed to stand at room temperature for 15 minutes. After the addition of 1 ml of Folin's Solution B, the tube was mixed as before and allowed to stand for a further 30 minutes at room temperature. The optical density of the resulting solution was measured at 700 nm in a LKB Biochrom 'Ultrospec 4050' spectrophotometer.

**Figure 2.1 — Standard Curve of Protein Estimation with Folin's Reagent**

Legend: ordinate – absorbance at 700 nm,
abscissa – protein concentration ($\mu$g BSA/ml).


Standard Curve for Protein Quantification
Absorbance
1.0
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
300
350
400
Protein Conc. (μg/ml)

A calibration curve was constructed with each protein estimation using BSA (Fraction V) as the protein standard. The reaction was linear up to a protein concentration of 400 $\mu$g/ml (see Figure 2.1).

### 2.0.5 Preparation of Material for Electron Microscopy

Tissues removed from experimental animals were prepared for viewing under the electron microscope in the manner described below.

*1. Fixation* for 1 hour at 0–5°C in Karnovsky fixative (KARNOVSKY, 1965) which was made up from the following solutions:

| | | |
|---|---|---|
| Solution A; | 2 g | paraformaldehyde, |
| | 40 ml | dist. $H_2O$, |
| | 2–6 drops | 1 M NaOH, |
| Solution B; | 10 ml | 25% gluteraldehyde, |
| | 40 ml | 0.2 M sodium cacodylate, pH 7.3. |

Solution A was made up by warming the water, adding the paraformaldehyde and then slowly adding the NaOH dropwise until all the precipitate had dissolved. The two solutions were kept at 0–4°C until just before use, when they were mixed.

*2. Post-fixation* in a 1% solution of osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.3, for 1 hour at 0–4°C.

*3. Dehydration* by passage through an alcohol series:

70 % ethanol (3 washes of 5 minutes each),
90 % ethanol (3 washes of 5 minutes each),
100 % ethanol (3 washes of 10 minutes each).

*4. Infiltration* in the following:

| | |
|---|---|
| 100% ethanol/propylene oxide (1:1) | (3 washes of 10 minutes each), |
| propylene oxide | (3 washes of 10 minutes each), |
| propylene oxide/araldite (1:1) | (30 minutes at 45°C), |
| araldite | (30 minutes at 45°C). |

*5. Embedding* in fresh araldite. The araldite mixture was composed of the following:

10 ml araldite,
10 ml D.D.S.A.,
2 ml dibutyl phthalate,
1 ml DMP 30.

After the material was embedded, the araldite was polymerised by incubation at 45°C for 12 hours, and then at 60°C for a further 48 hours.

Ultra-thin sections (silver/silver-gold) of the tissues were cut on a Reichert 'OM U3' ultramicrotome and were mounted on copper grids. The sections were double-stained with uranyl acetate and lead acetate (REYNOLDS, 1963) prior to their examination in a Philips 'EM 400T' electron microscope.

#### 2.0.6 Statistical Analysis of Data

All the methods used for the statistical analysis of data were as described by SOKAL and ROHLF (1969). Student's $t$ tests and paired $t$ tests were used to test the significance of differences between experimental populations. When quotients or ratios were compared, these were first tested for normality of distribution using the Kolmogorov-Smirnov one-sample test and, where necessary, the appropriate transformation was used to produce a normal distribution.

#### 2.0.7 Chemicals

All chemicals used in these experiments were the purest available and were generally supplied by British Drug House Chemicals Ltd. and Sigma Chemical Company Ltd. The methoprene used was a gift from Zoecon Corporation, Palo Alto, California. Glycogen (AMP-free), glucose-6-phosphate and phosphoglucomutase were obtained from Boehringer Corporation (London) Ltd.

#### 2.0.8 Treatment of Glassware

All routine glassware was cleaned prior to use by soaking overnight in a 2% solution of 'Quadralene' laboratory detergent, followed by several rinses in hot tap water and finally in distilled water. Glassware to be used in lipid extractions and estimations were soaked in 50% nitric acid and then rinsed as above. The glassware was oven-dried except for glass/teflon homogenisers and acrylic centrifuge tubes which were allowed to dry at room temperature.

# Chapter III

## Effects of Methoprene upon Morphology and Mortality

### 3.1 Introduction

The effects of juvenile hormone and its analogues upon the general morphology of locusts have already been described in detail in Chapter 1.

### 3.2 Materials and Methods

Newly moulted fifth instar larvae of *Locusta* were topically treated with ethanol or methoprene (either 95 $\mu$g or 475 $\mu$g per insect) as described in General Materials and Methods. The development of the three experimental insect populations was followed for the next twenty days, the numbers of animals dying or undergoing ecdysis being recorded on a daily basis. The gross morphology of the insects was also noted.

In order to make a quantitative determination of any phase dimorphism in the insect populations, the morphometric method of DIRSH (1953) was employed. This required the measurement of the hind femur length (F) and the maximum head width (C) of individual adults using callipers. F/C ratios were constructed and the insect populations were compared statistically according to methods described by SOKAL and ROHLF (1969). More accurate tests of phase polymorphism based on multivariate analysis have been devised by BLACKITH (1972). However, these also require the measurement of wing length which was not possible in the present study since the elytra of JHA-treated animals were badly deformed.

### 3.3 Results

During the fifth instar, there was little difference in appearance between methoprene-treated insects and controls except that animals treated with 475 $\mu$g of the JHA developed a slight green coloration on the sides of the pronotum and thorax. In the adult stage, however, the differences were far more obvious, as shown in Plate 3.1. Control animals were identical in appearance to normal, untreated adults (Plate 3.1, a). Animals which had been treated with

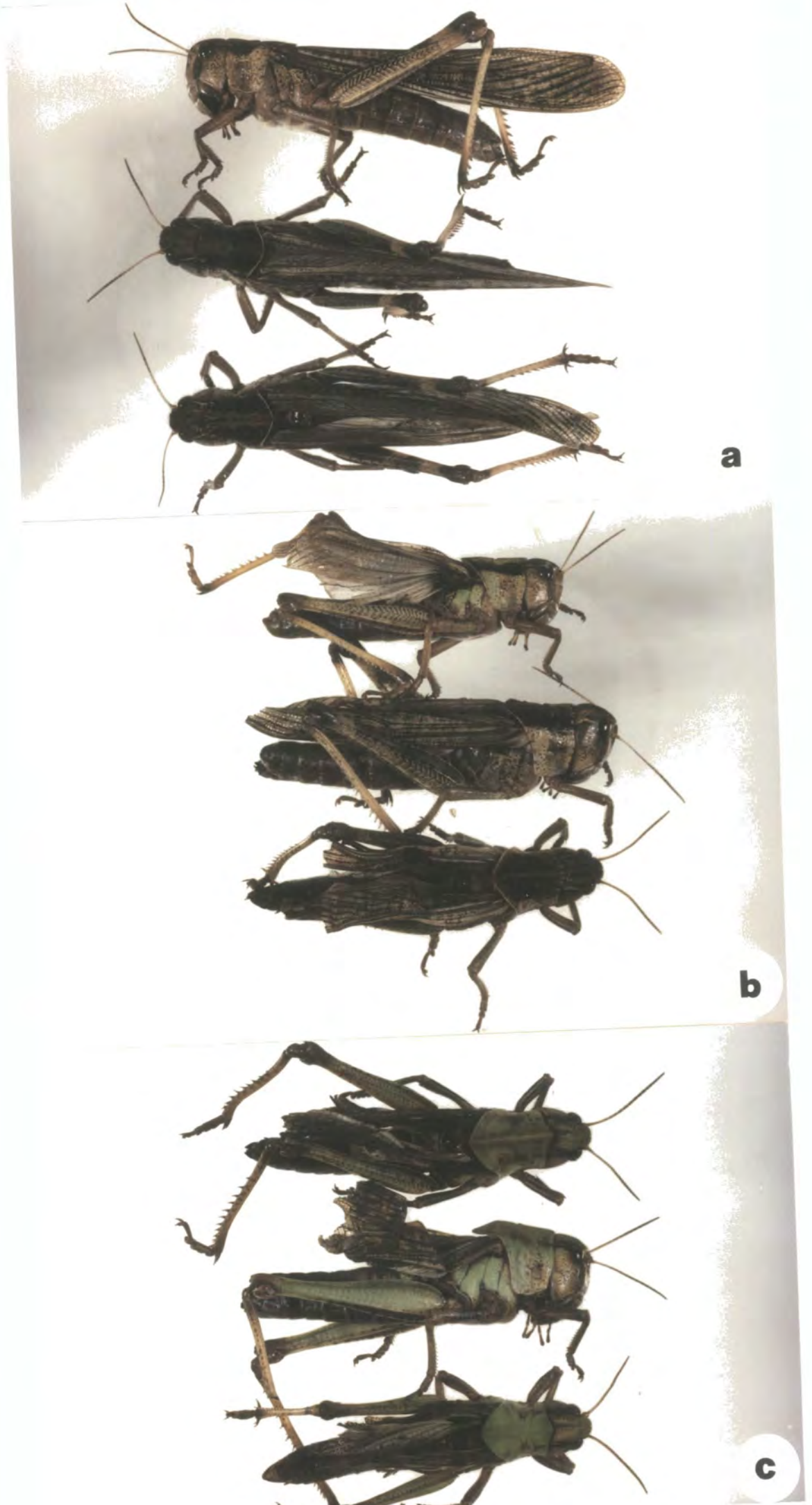
**Plate 3.1 — Photographs of Adult Locusts from Experimental Populations**

**a.** Adults from control population. Note the fully developed wings and the normal body coloration.

**b.** Adults from population which had been treated with 95 $\mu$g of methoprene. Note the deformed wings of the animals. Also note the green pigmentation on the pronotum and thorax, and the deformed hind-legs of some of the animals

**c.** Adults from population which had been treated with 475 $\mu$g of methoprene. Note the deformed wings and hind-legs of the animals. Also note the presence of green pigmentation on head, pronotum, thorax and hind-legs.

The animals are shown at 1.5 $\times$ life-size.


a
b
c

95 $\mu$g of methoprene had crumpled wings and many had deformed hind-legs as well (Plate 3.1, **b**). There were also abnormalities in the insects' colouring, with many having green pigmentation on the pronotum and thorax. These effects were apparent to a far greater degree in the 475 $\mu$g-treated animals, with the head, pronotum, thorax and hind femurs being a near-uniform, bright green (Plate 3.1, **c**). In this population, size differences between the smaller males and larger females were greater than was the case in the controls. Some of the JHA-induced changes in appearance (i.e. cuticle colouring and the disparity in size between sexes) are characteristic of solitary phase *Locusta* (see review by UVAROV, 1966). Therefore, the method of DIRSH (1953) was used to test for phase transformation in the experimental populations. The results of this analysis are given below with the F/C ratios being expressed as quotients. Student's $t$ tests performed on the data showed that the mean F/C quotients of the three populations were not significantly different from each other ($P > 0.05$). Furthermore, the discrepancies between control and treated values were in the opposite direction to that which would be expected if the methoprene-treated animals were in the solitary phase (i.e. solitary phase locusts have higher F/C quotients than gregarious locusts, DIRSH, 1953).

**Table 3.1 — F/C Ratios of Locust Populations**

| Treatment | n | mean F/C quotient |
|---|---|---|
| Control | 34 | 3.036 ± 0.035 |
| 95 $\mu$g JHA | 30 | 3.017 ± 0.025 |
| 475 $\mu$g JHA | 10 | 2.977 ± 0.060 |

(n is the number of insects measured)

Besides the effects of methoprene upon external morphology, on dissecting open experimental animals, it was found that treatment with the JHA also caused changes in the appearances of a number of tissues. By Day 7 of the fifth instar, the haemolymph of treated animals had become bright green and the fat bodies were white or grey. In controls, these tissues were the normal straw-yellow colour. Adults that had previously been treated with 475 $\mu$g of methoprene also

had abnormal dorso-longitudinal and dorso-lateral flight muscles which lacked the normal brown/pink coloration of control flight muscles. These abnormal muscles displayed no contractility when removed from the animals and 475 $\mu$g methoprene-treated adults were never seen to flutter their wings. Such disruption of the flight muscles could not have been due to the deformed elytra of these animals causing atrophy of the muscles since 95 $\mu$g-treated locusts, which had equally deformed wings but whose flight muscles were normal in appearance, frequently flapped their wings.

Figure 3.1 shows the percentage survival of the experimental insect populations against time. The mortality rates of the populations were low and approximately equal until about the ninth day after treatment when the percentage survival of JHA-treated insects began to decline more rapidly than in the control population, the effect being greater with the higher dosage of methoprene. By the twentieth day after treatment (when the experiment was stopped), more than 50% of the control insects had survived whereas there was only 21% and 29% survival among insects treated with 475 $\mu$g and 95 $\mu$g of methoprene respectively (50% mortality was reached in the treated insect populations at about Day 12 with the higher dosage of JHA, and about Day 14 with the lower). The two graphs in Figure 3.2 show the percentages of insects that moulted to adults during the experiment. Figure 3.2 (**a**) represents the percentage of the starting populations which had undergone metamorphosis and reveals that insects dosed with 475 $\mu$g of methoprene moulted earlier than the controls, with 50% of the treated animals having moulted by the ninth day after treatment as against the controls taking 11 days to reach 50% metamorphosis. In contrast, locusts dosed with 95 $\mu$g of methoprene were slightly slower in moulting than the controls and fewer of them had reached adulthood by the end of the experiment (80% as opposed to $\sim$90% of controls and 475 $\mu$g-treated animals). This was not simply due to larger numbers of the 95 $\mu$g-treated insects dying before they had moulted since Figure 3.2 (**b**), which represents the percentage of surviving animals that were adults, shows that a greater proportion of locusts remained as fifth instar larvae in the population treated with 95 $\mu$g of methoprene. By the end of the experiment, only 79% of surviving, 95 $\mu$g-treated locusts were adult whereas 97% of surviving control insects and 100% of 475 $\mu$g-treated animals had moulted. The above results have been given as percentages for ease of comparison but

**Figure 3.1 — % Survival in Experimental Insect Populations**

Legend: ordinate – % survival in insect populations,
abscissa – number of days after treatment,
$\circ$ – control insects,
$\diamond$ – 95 $\mu$g methoprene-treated insects,
$\triangle$ – 475 $\mu$g methoprene-treated insects.

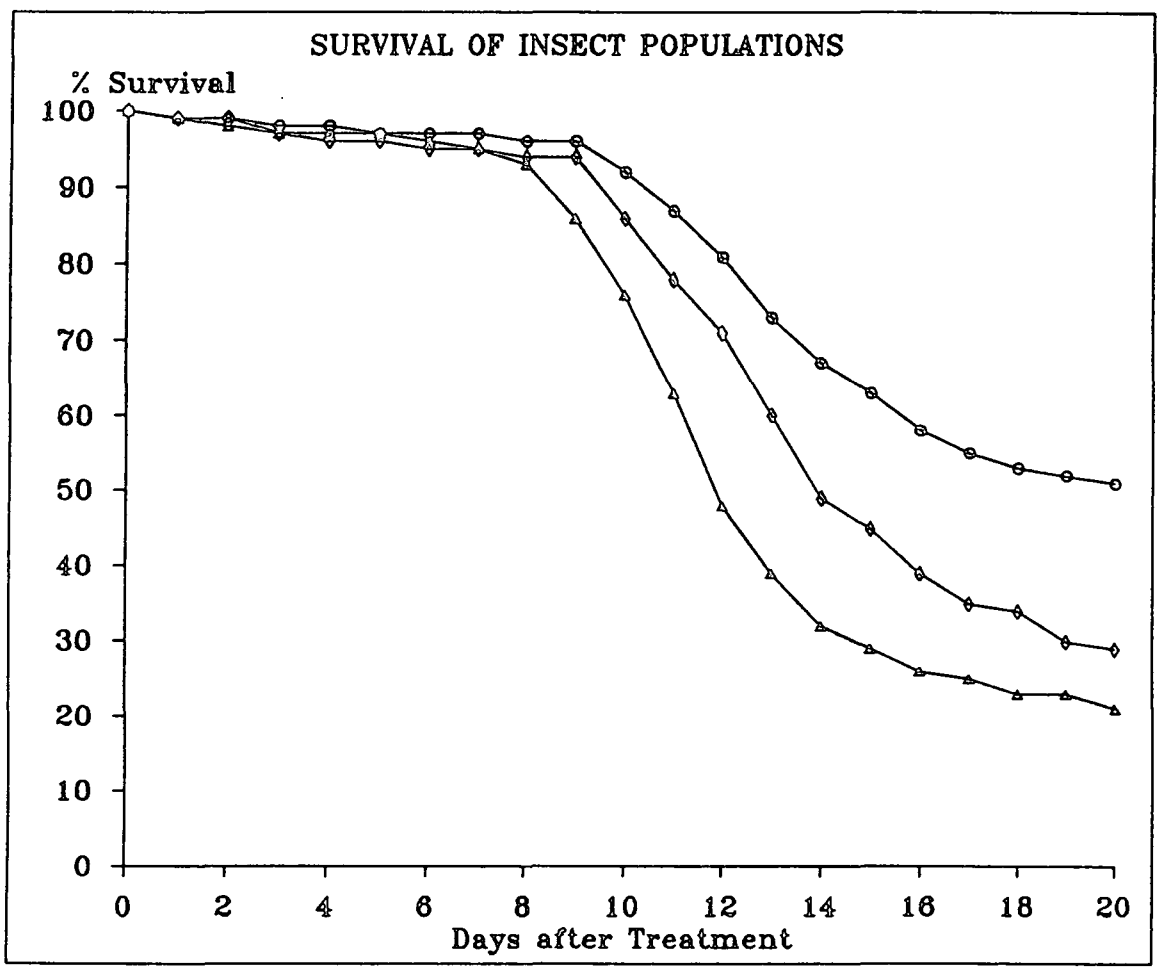

SURVIVAL OF INSECT POPULATIONS
% Survival
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Days after Treatment

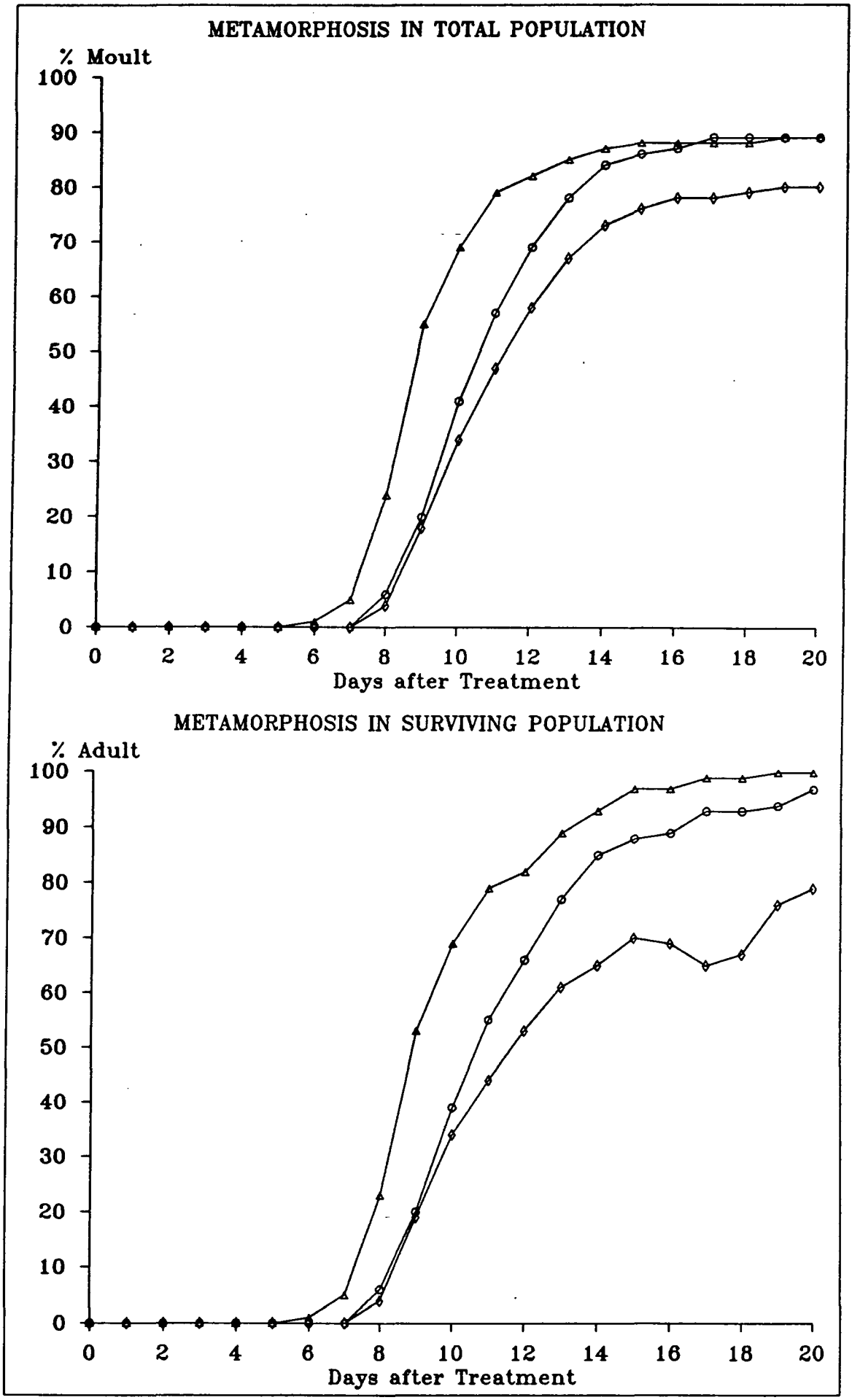
**Figure 3.2 — % Metamorphosis in Experimental Insect Populations**

**a. % Metamorphosis in Total Insect Population**

Legend: ordinate – % of total insect population that has moulted,
abscissa – number of days after treatment,
o – control insects,
◇ – 95 $\mu$g methoprene-treated insects,
△ – 475 $\mu$g methoprene-treated insects.

**b. % Metamorphosis in Surviving Insect Population**

Legend: ordinate – % of surviving insects that have moulted,
abscissa – number of days after treatment,
o – control insects,
◇ – 95 $\mu$g methoprene-treated insects,
△ – 475 $\mu$g methoprene-treated insects.


METAMORPHOSIS IN TOTAL POPULATION
% Moult
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Days after Treatment
METAMORPHOSIS IN SURVIVING POPULATION
% Adult
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Days after Treatment

the actual numbers of locusts that were treated at the start of the experiment were as follows; control insects (755), 475 $\mu$g JHA-treated insects (541), 95 $\mu$g JHA-treated insects (410).

Among the locusts treated with 475 $\mu$g of methoprene, ten adults (5 males and 5 females) underwent an extra moult. Most failed to moult successfully, being unable to shed the old cuticle, and all the supernumary adults died within 24 hours of splitting of the exuviae. The two females that did moult completely were slightly larger than 475 $\mu$g-treated, adult females which had not undergone an extra moult but were otherwise similar in appearance.

### 3.4 Discussion

The colour changes of cuticle and haemolymph, and the deformities of wings and hind-legs seen with methoprene-treated animals after the imaginal moult were typical effects of juvenoid action (see reviews by SEHNAL, 1983; SLÁMA, 1985). The morphometric analysis indicated that there was no phase transformation associated with the colour changes and, in other studies, the F/C ratios of locusts were also found to be unaffected by implantation of active corpora allata (JOLY, 1962; STAAL as cited in ROWELL, 1967). The F/C ratios of locust hoppers increase at each ecdysis during larval growth (UVAROV, 1966) and the mean F/C quotients of the JHA-dosed animals in the present study were lower, albeit not significantly, than the control values. Therefore, this could be an indication that the methoprene-treated insects were in a more juvenile state but measurements of many more animals would have to be made before this could be confirmed. Such a result would not be surprising since the production of metathetelic adults and larval/adult intermediates is a common response to juvenoid treatment (see General Introduction and reviews by VOGEL *et al.*, 1979). The abnormal appearances of fat bodies and flight muscles from methoprene-treated locusts in the present study were similar to those found with CA-implanted locusts in earlier studies (PFEIFFER, 1945; POELS and BEENAKKERS, 1969; VAN DEN HONDEL-FRANKEN *et al.*, 1980). The effects of JH-active compounds upon these tissues are described in greater detail in later chapters.

The application of 475 $\mu$g of methoprene to fifth instar larvae accelerated the onset of metamorphosis and shortened the length of the fifth instar whereas treatment with 95 $\mu$g of the JHA slightly delayed the final moult. Thus, it may

be that the effect of methoprene upon the timing of metamorphosis in locusts is dependent on the concentration of JHA applied. Although a reduction in instar length is the usual response to the implantation of active CA into fifth instar locusts (POELS and BEENAKKERS, 1969; VAN DEN HONDEL-FRANKEN *et al.*, 1980), ROUSSEL and PERRON (1974) found that the injection of juvenoids into fourth instar *Schistocerca gregaria* caused a delay in the subsequent moult. SEHNAL *et al.* (1981) reported that the response of last instar larvae of *Galleria mellonella* to JH application changed with time. Administration of JH to newly moulted larvae accelerated the final moult whereas treatment in the middle of the instar delayed ecdysis. In view of the reduction in sensitivity to JH-active compounds during ageing of the last larval instar (see General Introduction for details), the changes in response seen by SEHNAL *et al.* (1981) may have been due to a reduction in the effective dose of JH at the middle of the last larval instar compared to the start of the instar. The fact that some adults treated with 475 $\mu$g of methoprene in the present study underwent an extra moult suggests that the degeneration of their prothoracic glands, which normally occurs shortly after the imaginal moult, was prevented. GILBERT (1962) demonstrated that the prothoracic glands of *Antheraea polyphemus* remain healthy during supernumary moults caused by JH injection. Such an effect lends credence to the suggestion given above that the methoprene-treated adults might have retained some larval characteristics. In both of the locust populations that were dosed with methoprene, mortality rates were increased during the final moult at the end of the fifth instar. SLÁMA *et al.* (1974) noted that the failure of insects to shed the old cuticle during ecdysis was the most common side effect encountered with the use of chemicals with JH activity. In his review of insect moulting, REYNOLDS (1980) concluded that moulting failure in juvenoid-treated insects was probably due to deformities in the adult integument preventing complete escape from the exuviae.

# Chapter IV

# A Cytological Study of Fat Body Development and its Response to Methoprene

## 4.1 Introduction

The fat body is the principal tissue for intermediary metabolism in the insect and the major storage site for reserve materials, and is therefore analogous to both the liver and the adipose tissue of vertebrates (see reviews by KILBY, 1963; WIGGLESWORTH, 1972; KEELEY, 1985). Fat body cells are capable of a wide range of functions, including the 'metabolism of carbohydrates, lipids and nitrogenous compounds, the synthesis and regulation of blood sugar, storage of glycogen, fat and protein, and synthesis of major blood proteins' (WYATT, 1980). Fat body fine structure has been described in the review articles of LOCKE (1984) and DEAN *et al.* (1985), while developmental changes in fat body structure and function have been well reviewed by LOCKE (1980, 1985). The larval fat body generally acts as a processing organ and, during the early part of each instar, is principally concerned with the synthesis and mobilisation of reserves. Towards the end of the stadium, it switches to growth and cell division in readiness for the coming moult (LOCKE, 1980, 1985). As the larva feeds, the fat body stores excess lipid and glycogen (KILBY, 1963) and produces haemolymph proteins (PRICE, 1973) which may be used at moulting for the construction of the next instar. The tissue undergoes a cycle of these intermoult/moult activities during each larval stadium and this pattern is particularly marked in the final larval instar when the tissue prepares for metamorphosis (LOCKE, 1980). The fat body of the adult insect is primarily a site of storage and production of materials used for reproduction and flight. A major function of the female tissue is the synthesis and release of vitellogenic proteins (ENGELMANN, 1979; HAGEDORN and KUNKEL, 1979; KUNKEL and NORDIN, 1985) but these are not produced by the male fat body (ENGELMANN, 1969). PAN *et al.* (1969) defined vitellogenins as a class of proteins which are precursors of the major yolk proteins of the ovary, the vitellins. In adult female *Locusta*, vitellogenin synthesis first occurs in the fat body about eight days after the final moult and rises to a maximum by

Day 13 when it accounts for 60% of export protein synthesis (CHEN *et al.*, 1979). The rate of vitellogenesis then undergoes a series of fluctuations with successive gonadotrophic cycles (GELLISSEN and EMMERICH, 1978; CHINZEI *et al.*, 1980).

The basic morphology of insect fat bodies has been described by BUYS (1924), and in the reviews of WIGGLESWORTH (1972) and DEAN *et al.* (1985). The tissue is structurally diverse but is typically arranged in lobes or sheets, providing a large surface area exposed to the surrounding haemolymph for rapid metabolite exchange (DEAN *et al.*, 1985). In most insect species studied, the fat body exists as two distinct regions, a peripheral layer which lies adjacent to the integument and is firmly attached to the overlying epidermis, and a central layer loosely surrounding the gut. The predominant cell type is the trophocyte (adipocyte), and in some orders such as Ephemeroptera it is the only cell type present (BUYS, 1924; WIGGLESWORTH, 1972). However, in most insects, the fat body is a composite of two or more cell types, and the fat body of *Locusta migratoria* also contains oenocytes and urocytes (LAUVERJAT, 1977; COUBLE *et al.*, 1979). COUPLAND (1957) described oenocytes from the fat body of the locust *Schistocerca gregaria* as large, 'wine-yellow' cells that were most numerous in the peripheral layer of the fat body and only infrequently found in the central region. Oenocyte fine structure has been described in a number of studies (EVANS, 1967; SMITH, 1968; LOCKE, 1969; GNATZY, 1970; DE LOOF and LAGASSE, 1970; CLARK and DAHM, 1973). In general, the cell consists of a large nucleus and cytoplasm of uniform construction, rich in mitochondria and polysomes, and filled with very extensive smooth endoplasmic reticulum. The plasma membrane is invaginated to form a reticular tubular system. Urocytes or urate cells are found scattered throughout the fat bodies of several insect species, and serve to accumulate uric acid for storage or secretion (WIGGLESWORTH, 1972; DEAN *et al.*, 1985). Urocyte ultrastructure has been detailed in *Periplaneta* (COCHRAN *et al.*, 1979; MULLINS, 1979) and *Locusta* (LAUVERJAT, as cited by MARTOJA and BALLAN-DUFRANÇAIS, 1984). Typically, these cells appear to be degenerate trophocytes with few cytoplasmic structures and most of the cell being taken up by crystalloid spheres of uric acid or urates.

Changes in fat body ultrastructure during larval development have been described for *Philosamia cynthia* (ISHIZAKI, 1965), *Calpodes ethlius* (LOCKE and COLLINS, 1968), *Calliphora erythrocephala* (PRICE, 1969; DE PRIESTER and VAN

DER MOLEN, 1979) and *Sarcophaga bullata* (BENSON, 1965, as cited by PRICE, 1973). In the early stages of the last larval instar, fat body cells from the various species were similar in possessing numerous mitochondria, lipid droplets, Golgi complexes and well-developed endoplasmic reticulum (ER) rich in ribosomes. As the larvae prepared for pupation, there was a great reduction in the ER and in the numbers of mitochondria, while the cells became filled with lipid droplets, glycogen granules and 'proteinaceous spheres'. The latter were usually electron-dense bodies and were thought to be the result of mitochondria and ER being digested by lysosomes. In their detailed study, LOCKE and COLLINS (1968) were able to distinguish three distinct phases of fat body development in the fifth (final) larval instar of *Calpodes ethlius*. Trophocytes of the newly moulted insect were small and contained numerous mitochondria, but little rough endoplasmic reticulum (RER) and no lipid droplets. During the next 66 hours, the cells underwent a phase of preparation for subsequent larval syntheses and were distinguished by high levels of RNA production, as shown by incorporation of [$^3$H]uridine into cytoplasmic RNA (LOCKE, 1970). Many free ribosomes accumulated in the cytoplasm and the cells became intensely basophilic. Small lipid droplets also appeared in the cytoplasm, while Golgi complexes enlarged and began to form secretory vesicles. The second phase of development was characterised by proliferation of the RER and the formation of large secretory vesicles by Golgi complexes as the synthetic activity of the cells increased. Lipid droplets increased in size and substantial deposits of glycogen were formed. In the third phase, synthetic activity was reduced as the trophocytes prepared for the subsequent pupal moult. Protein storage granules appeared in the cells while mitochondria and RER were destroyed by autolysis. Such a pattern of development would also be in agreement with the observations of DE PRIESTER and VAN DER MOLEN (1979) on fat bodies from third instar larvae of *Calliphora*. They noted that, shortly after ecdysis, there was a proliferation of RER, mitochondria and free ribosomes in the trophocytes, while towards the end of the instar, most of the mitochondria and RER were digested by autophagic vacuoles.

Studies of fat body ultrastructure in adult insects are more numerous, including several detailed descriptions of locust fat body in particular, and these have been well reviewed by DEAN *et al.* (1985). There is a marked sexual dimorphism in the appearance of fat body in the adult locust which reflects the sex-specific

role of the tissue in reproduction (MARTOJA and LAUVERJAT, 1964). The fat body of adult male *Schistocerca gregaria* is predominantly a storage organ with large deposits of lipid and glycogen, and sparse cytoplasm concentrated mainly around the cell nuclei (ODHIAMBO, 1967). The tissue contains little RER and few Golgi complexes, indicating low synthetic activity, although it may produce some proteins for the male accessory reproductive glands as has been reported in the migratory grasshopper, *Melanoplus sanguinipes* (FREIDEL and GILLOT, 1976). Also, the role of the male fat body seems to change little with age. IRVINE and BRASCH (1981) reported that during the first two weeks of adult life, the only major changes visible in the fat body cells of male *Locusta migratoria* were increases in cell size and in the volume of the nuclei. In contrast, the fat body of adult female *Locusta* underwent substantial changes in structure during this period (LAUVERJAT, 1977; COUBLE *et al.*, 1979). Fat body cells from newly moulted adults contained extensive lipid and glycogen stores, relatively little cytoplasm, and small nuclei that were indented between lipid droplets (COUBLE *et al.*, 1979; IRVINE and BRASCH, 1981; JENSEN and BRASCH, 1985). RER was limited to short, isolated strands and the few Golgi complexes present showed no synthetic activity (LAUVERJAT, 1977). During the first week of adult life, the Golgi complexes became synthetically active and the strands of RER began to lengthen. Using trophocytes from female *Locusta*, CHEN *et al.* (1979) reported a steady increase in [$^{14}$C]uridine incorporation into RNA from the third to the eighth day of adult life, suggesting an increase in RNA synthesis during this time. LAUVERJAT (1977) noted that, during the same period, trophocyte nucleoli from female locusts became enlarged and multilobed, which may be indicative of heightened ribosome production (see review by DEAN *et al.*, 1985) since cell nucleoli are involved in the synthesis of ribosomal RNA (BUSCH and SMETANA, 1970). When about seven days old, the time when vitellogenin is first produced (CHEN *et al.*, 1979), trophocytes of adult female locusts underwent rapid and conspicuous changes that reached a climax at about Day 15 (LAUVERJAT, 1977; COUBLE *et al.*, 1979). Coincident with a marked reduction in the lipid and glycogen stores, cell nuclei increased in size, their volume doubling or even quadrupling, and Golgi complexes increased in number. Ribosomes entered the cytoplasm and associated with proliferating membranes to form extensive, closely packed RER. The role of RER and Golgi complexes in vitellogenesis were

confirmed when, by immunological methods, vitellogenin was identified and located within cisternae of the RER and in Golgi secretory vesicles of locust fat body cells (CHEN *et al.*, 1976; COUBLE *et al.*, 1979).

In the majority of insects studied, it is now well established that the synthesis of vitellogenin (Vg) by adult female fat body is dependent upon the presence of juvenile hormone in the haemolymph (see reviews by ENGELMANN, 1979, 1980; HAGEDORN and KUNKEL, 1979; KOEPPE *et al.*, 1985). In the case of *Locusta migratoria*, it has been demonstrated that allatectomy of young adult females prevented subsequent Vg synthesis by the fat body, but that this could be restored by the topical application of JH I or methoprene to the insects (CHEN *et al.*, 1976, 1979; CHINZEI and WYATT, 1985). Multiple doses of JH were necessary for maximal stimulation of vitellogenesis, but the more stable methoprene was effective as a single treatment of ~400 $\mu$g per insect (CHEN *et al.*, 1979). DHADIALLA and WYATT (1981, 1983) found that the injection of 200 $\mu$g of methoprene into allatectomised female *Locusta* fully restored vitellogenesis (Vg forming $\geq$60% of total export protein from isolated fat bodies) whereas no Vg synthesis was detected in adult male fat body even after the injection of 500 $\mu$g of methoprene. CHINZEI *et al.* (1982) detected proliferation of both total fat body RNA and Vg mRNA in fat bodies of allatectomised female locusts following the injection of 150 $\mu$g of methoprene. Similarly, the ultrastructural appearance of vitellogenic locust fat body has been shown to be dependent on JH and its analogues (LAUVERJAT, 1977; RINTERKNECHT and ROUSSEL, 1978; COUBLE *et al.*, 1979). Trophocytes of allatectomised animals retained the basic appearance of cells from the previtellogenic phase with small nuclei, little RER and few Golgi complexes. Also, the lipid content of these cells increased to levels even higher than that found in the trophocytes of newly moulted adults. Cell structure was rapidly returned to normal by implantation of active corpora allata (LAUVERJAT, 1977), injection of JH (RINTERKNECHT and ROUSSEL, 1978) or topical application of methoprene (COUBLE *et al.*, 1979). Not only can JH and JHAs restore normal vitellogenic activity to allatectomised females, but they can induce precocious Vg synthesis in immature animals. HILL and IZATT (1973) reported that the implantation of active corpora allata into newly moulted, adult female locusts accelerated the onset of egg development and the incorporation of Vg into oocytes. After injection of mid-fifth instar larvae of *Locusta* with 500 $\mu$g of methoprene, DHADIALLA

and WYATT (1981, 1983) detected high rates of vitellogenesis in fat bodies from female larvae (Vg forming $\geq$60% of total export protein) and even some Vg synthesis in fat bodies from male larvae ($\sim$20% of total export protein).

The purpose of the present study was to establish the normal developmental changes in the fine structure of locust fat body during the last larval and early adult stages, and to elucidate what effect treatment with methoprene has upon this pattern.

## 4.2 Materials and Methods

Newly moulted fifth instar locusts were topically treated with either absolute ethanol or methoprene (475 $\mu$g or 95 $\mu$g per insect). The maintenance, treatment and sampling of these experimental insects were as described in General Materials and Methods. On each day that the animals were sampled, two males and two females were taken from each treatment. The insects were killed by twisting the head to break the neck membrane. The posterior tip of the abdomen was cut off and the head, with the gut attached, was removed. The carcase was cut open ventrally and all readily available fat body was taken from the thorax and abdomen. The peripheral layer of the fat body, which is firmly attached to the epidermis, was not removed.

### 4.2.1 Histological Study of Fat Body Cytology

Fat bodies were removed from the experimental animals and were fixed in Carnoy's fluid (CARNOY, 1887), which is made up of absolute alcohol/chloroform/acetic acid (6:3:1), for 1 hour at room temperature. The tissue was dehydrated by passage through an alcohol series to chloroform in an Elliot 'Automatic Tissue Processor' and was finally embedded in paraffin wax. Serial sections were cut at 1 $\mu$m thickness on a Spencer '820' microtome and these were stained for DNA and RNA with methyl green and pyronin, using the method of BRACHET (1953) as modified by JORDAN and BAKER (1955). This procedure stains DNA a blue/green, RNA is rose red and cell cytoplasm is stained red/purple. In order to determine the level of RNA in the cytoplasm, it is necessary to extract the RNA from one section and compare the level of staining in the cytoplasm with another section that has been stained without prior extraction. BRACHET (1953) used the enzyme ribonuclease for the specific digestion of RNA but it is also possible

to use acid extraction methods (ERICKSON *et al.*, 1949). Heating the section with perchloric or hydrochloric acids will remove RNA but leave the staining of cytoplasm unchanged. However, it may also depolymerise DNA and make this stain red instead of blue/green. The staining protocol is given below.

*1. Dewax* in xylene for 3 minutes.

*2. Hydration* by passage through an alcohol series to water:

100% alcohol (1 minute),
95% alcohol (1 minute),
70% alcohol (1 minute),
dist. water (1 minute).

*3. Differential extraction of RNA.* Use two slides of the same material for test and control. Extract RNA from test slide by incubating in 1 M HCl at 60°C for 10 minutes. Stand control slide in distilled water for an equivalent time.

*4. Stain* for 5 minutes in methyl green-pyronin, made up from the following:

13 ml 0.5% aqueous methyl green (chloroform washed),
37 ml 0.5% aqueous pyronin Y,
50 ml 0.2 M acetate buffer, pH 4.8.

*5. Rinse* in distilled water for 2 seconds and blot lightly until almost dry.

*6. Dehydrate* in tertiary butyl alcohol, two washes of 1 minute each.

*7. Clear* in xylene for 2 minutes.

*8. Mount* in DPX mountant.

#### 4.2.2 Ultrastructural Study of Fat Body

Fat bodies were removed from the experimental animals and were prepared for electron microscopy according to the protocol described in General Materials and Methods.

## 4.3 Results

#### 4.3.1 Histological Study of Fat Body

The fat body of larval and adult *Locusta migratoria* was composed of a series of interconnected, flattened lobes extending throughout the body cavity of the thorax and abdomen, and in the case of control insects, usually deep yellow in colour. Thus, the basic morphology of the tissue was very similar to that of *Schistocerca gregaria* as described by COUPLAND (1957). In this case, the yellow

**Plate 4.1 — Light Micrographs of Control Fat Body**

**a.** Fat body on Day 1 of the fifth instar. Note the small size of cells and numerous, small vacuoles in cytoplasm. Also note small nuclei, containing densely packed DNA. No RNA was detected in nuclei or cytoplasm. Scale = 10 $\mu$m.

**b.** Fat body on Day 4 of the fifth instar. Note increased size of cells compared with Day 1 (**a**), with more plentiful cytoplasm and larger vacuoles. Also note substantial enlargement of nuclei, containing more dispersed DNA. RNA was abundant in both nuclei and cytoplasm. Scale = 10 $\mu$m.

**c.** Fat body on Day 7 of the fifth instar. Note greatly increased vacuolation of cells, and reduced amount of cytoplasm compared with Day 4 (**b**). Also note small size of nuclei with densely packed DNA. Some cytoplasmic RNA remained but none was detected in nuclei. Scale = 10 $\mu$m.

**d.** Fat body on Day 10 of the fifth instar. Note extreme vacuolation of large cells and densely packed DNA of small nuclei. No RNA was detected in either nuclei or cytoplasm. Scale = 10 $\mu$m.

**e.** Fat body on Day 1 of the adult stadium. Note greatly reduced size of cells compared with Day 10 of fifth instar (**d**), with a few, small vacuoles remaining in the cytoplasm. Nuclei were small with densely packed DNA. Both cytoplasm and nuclei stained for RNA. Scale = 10 $\mu$m.

**f.** Fat body of male locust on Day 8 of the adult stadium. Note slightly increased size of cells compared with Day 1 of adult life (**e**), and increased vacuolation of cytoplasm. Nuclei were small with densely packed DNA. RNA was present in the cytoplasm but was not detected in nuclei. Scale = 10 $\mu$m.

**g.** Fat body of female locust on Day 8 of the adult stadium. Note slightly increased cell size compared with Day 1 of adult life (**e**), and increased vacuolation of cytoplasm. Also note substantial enlargement of nuclei and the greater dispersal of DNA. RNA was abundant in cytoplasm and was present in nuclei. Large, discrete bodies of RNA were sometimes present in nuclei (Nu), and were presumably nucleoli. Scale = 10 $\mu$m.

Key: Cy– cytoplasm; N– nucleus; Nu– nucleolus; V– vacuole; large arrow– DNA; small arrow– RNA.

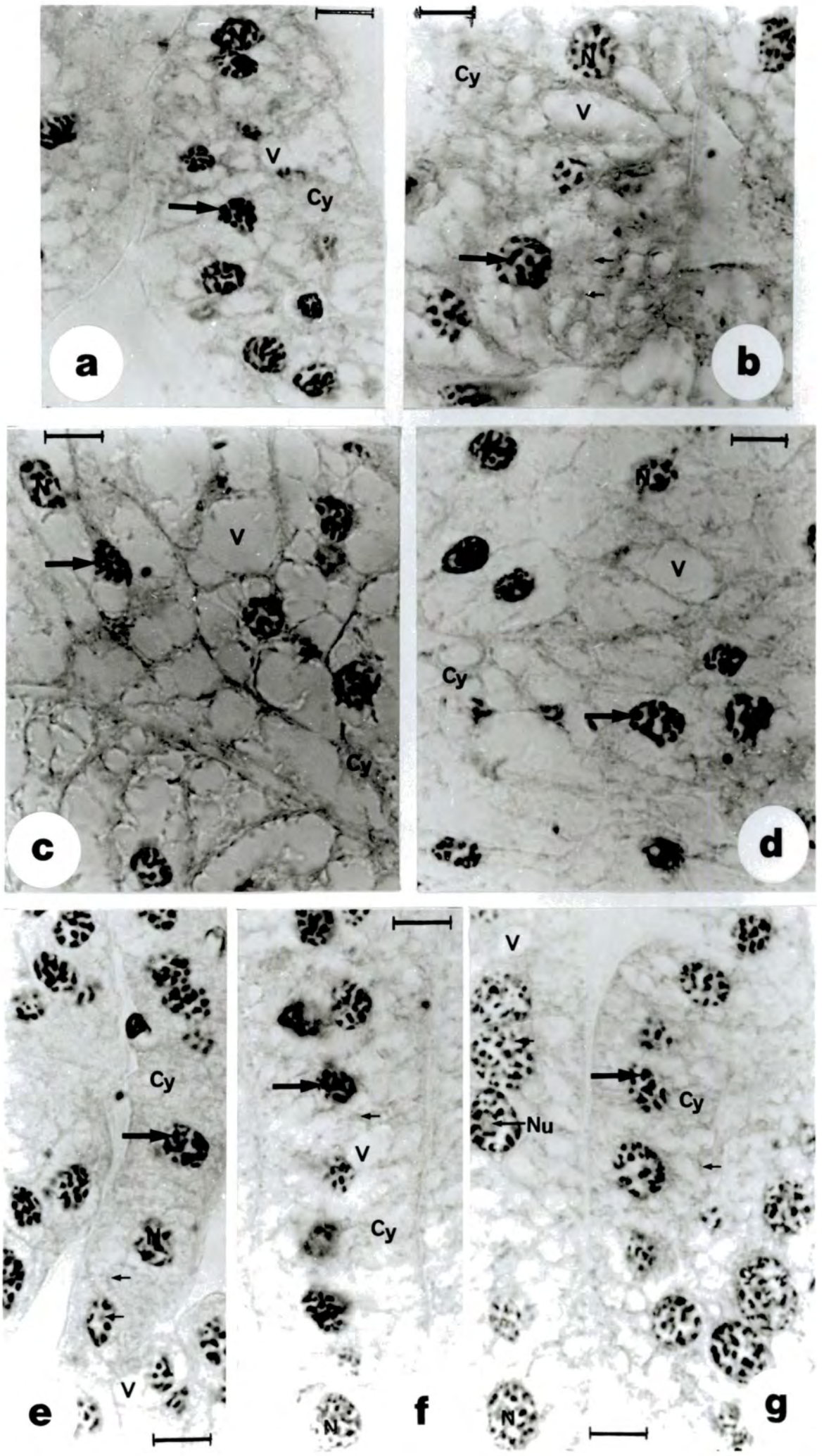

a
V
Cy
b
Cy
V
c
V
Cy
d
V
Cy
e
Cy
N
V
f
V
Cy
N
g
V
Nu
Cy
N

**Plate 4.2 — Light Micrographs of 475 $\mu$g Methoprene-Treated Fat Body, Early Fifth Instar**

**a.** Fat body of male locust on Day 4 of the fifth instar. Note slightly smaller size of cells compared with control tissue (see Plate 4.1, **b**), and smaller vacuoles in cytoplasm. Also note larger size of nuclei, with more dispersed DNA, compared with control. Both cytoplasm and nuclei were heavily stained for RNA. Scale = 10 $\mu$m.

**b.** Fat body of female locust on Day 4 of the fifth instar. Note reduced level of vacuolation in cells compared with male tissue (**a**) and, especially, control fat body (see Plate 4.1, **b**). Also note greatly enlarged nuclei with widely dispersed DNA. Both cytoplasm and nuclei heavily stained for RNA. Scale = 10 $\mu$m.

**c.** Fat body of male locust on Day 7 of the fifth instar. Note greatly increased vacuolation and increased size of cells compared with Day 4 (**a**). Also note reduced size of nuclei and densely packed DNA. The cells were similar to control tissue (see Plate 4.1, **c**) except that nuclei were still abnormally large. RNA was still present in cytoplasm but was not detected in nuclei. Scale = 10 $\mu$m.

**d.** Fat body of female locust on Day 7 of the fifth instar. Note similarity with 4 day old tissue (**b**), apart from slightly increased size of the cytoplasmic vacuoles. Cytoplasm was still abundant within cells and nuclei were very large with widely dispersed DNA. RNA was abundant in both cytoplasm and nuclei. Scale = 10 $\mu$m.

**e.** Fat body of female locust on Day 7 of the fifth instar. Note very small size of cells, without any visible vacuolation. Also note presence of several mitotic bodies (open arrows). The remaining nuclei were smaller than in other treated, female fat body (**d**) but were larger than those of control tissue (see Plate 4.1, **c**). RNA was present in both cytoplasm and nuclei. Scale = 10 $\mu$m.

Key: Cy– cytoplasm; N– nucleus; V– vacuole; large arrow– DNA; small arrow– RNA; open arrow– mitotic body.

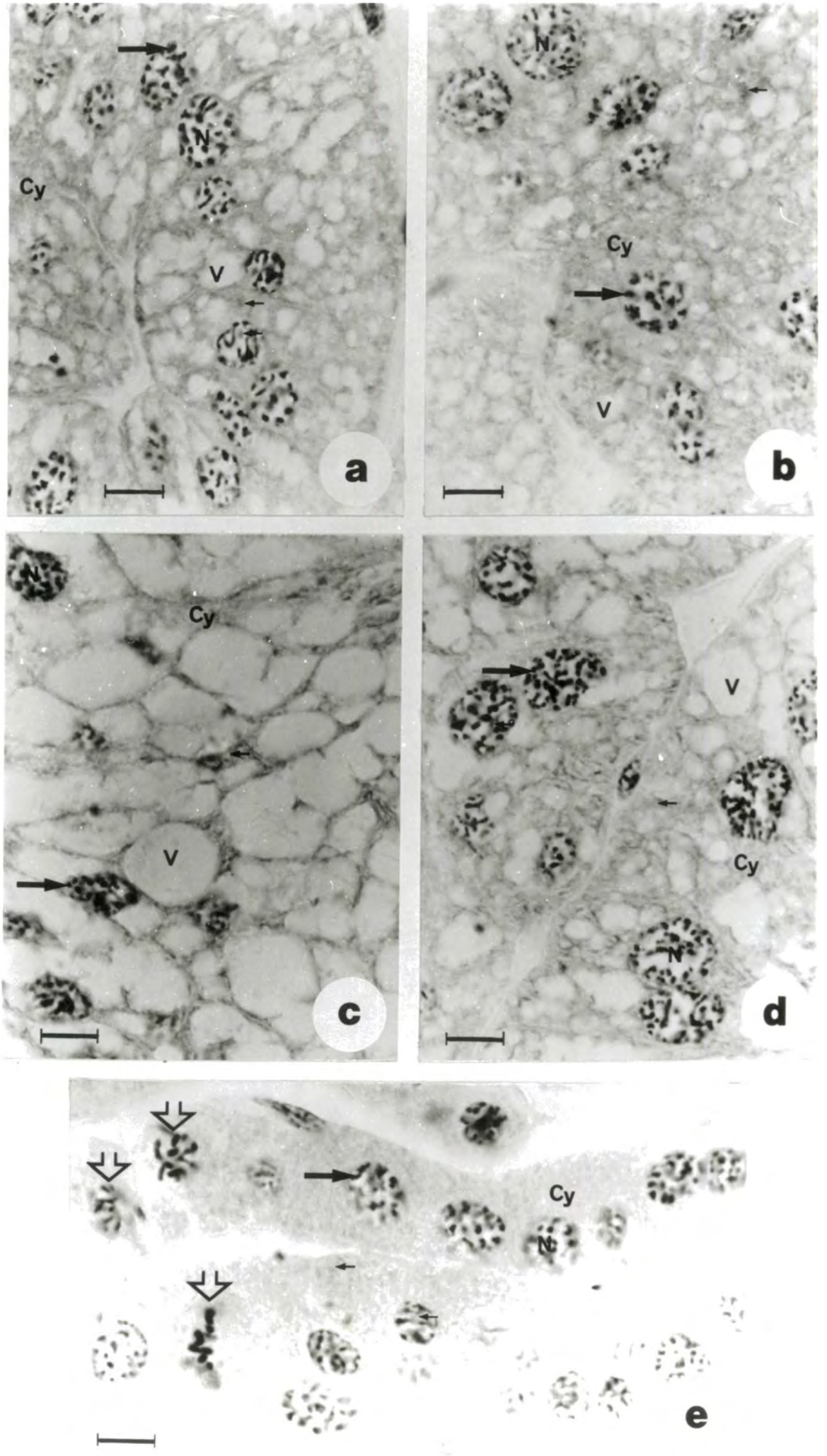

Cy
N
V
a
N
Cy
V
b
N
Cy
V
c
V
Cy
N
d
Cy
N
e

**Plate 4.3 — Light Micrographs of 475 $\mu$g Methoprene-Treated Fat Body, Late Fifth Instar and Adult**

**a.** Fat body of male locust on Day 10 of the fifth instar. Note extreme vacuolation of large cells and densely packed DNA of small nuclei, similar to control tissue (see Plate 4.1, **d**). No RNA was detected. Scale = 10 $\mu$m.

**b.** Fat body of female locust on Day 10 of the fifth instar. Note extreme vacuolation of large cells and densely packed DNA of nuclei. Nuclear size was greatly reduced compared with Day 7 of the fifth instar (Plate 4.2, **d** & **e**) but was larger than in male treated tissue (**a**) and control fat body (see Plate 4.1, **d**). Scale = 10 $\mu$m.

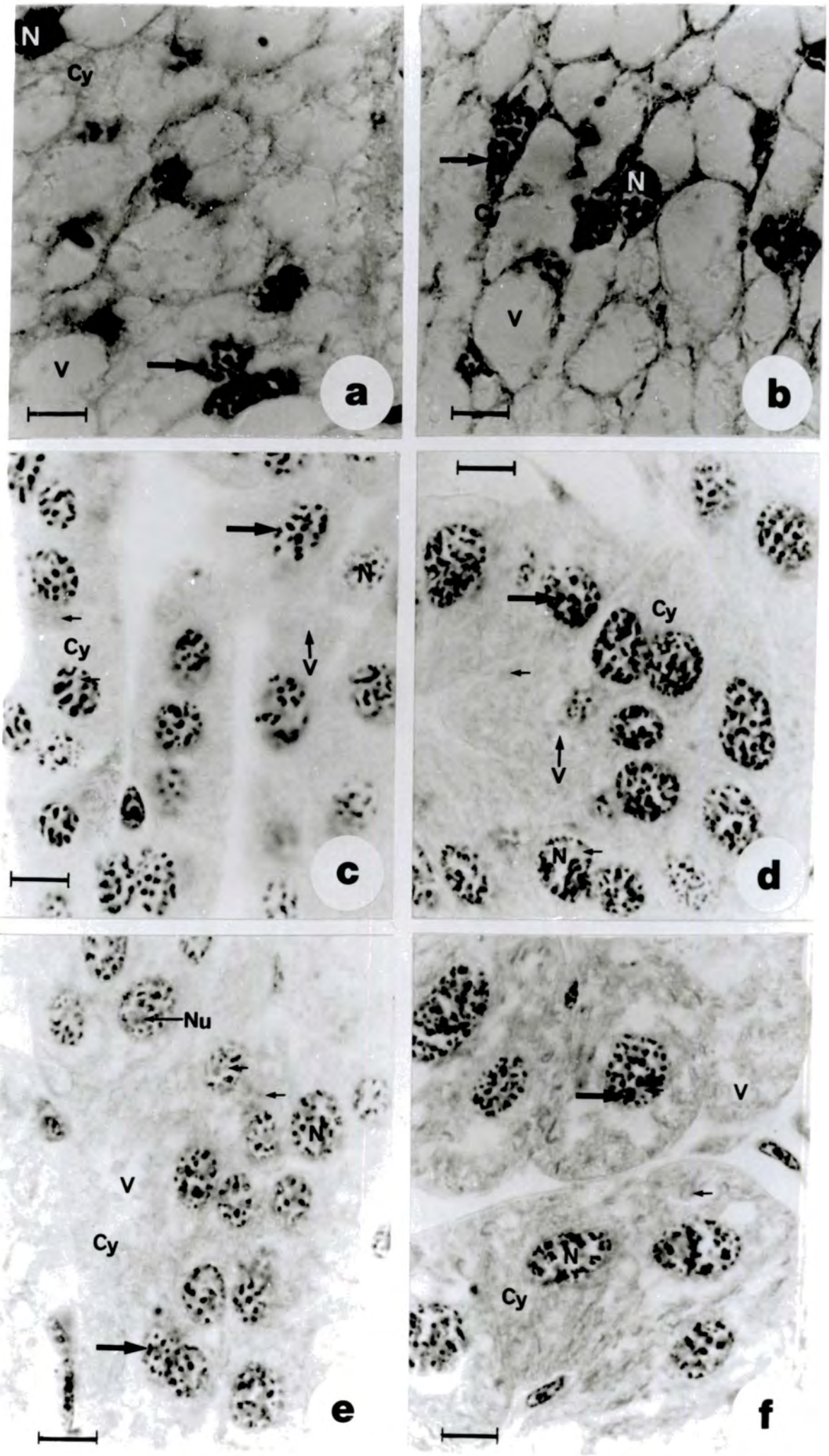
**c.** Fat body of male locust on Day 1 of the adult stadium. Note greatly reduced size of cells compared with Day 10 of the fifth instar (**a**), with only a few, small vacuoles remaining in cytoplasm. Nuclei were increased in size and contained widely spaced DNA. Note reduced cytoplasmic vacuolation and larger nuclear size compared with control tissue (see Plate 4.1, **e**). RNA was plentiful in cytoplasm and nuclei. Scale = 10 $\mu$m.

**d.** Fat body of female locust on Day 1 of the adult stadium. Note reduced size and greatly reduced vacuolation of cells compared with Day 10 of the fifth instar (**b**). Also note increased size of nuclei with widely spaced DNA. Cells and especially nuclei were larger than in male treated tissue (**c**) and control fat body (see Plate 4.1, **e**). RNA was abundant in cytoplasm and nuclei. Scale = 10 $\mu$m.

**e.** Fat body of male locust on Day 8 of the adult stadium. Note increased size and vacuolation of cells compared with Day 1 (**c**). Also note slight increase in nuclear size. Cytoplasmic vacuolation was less than in control tissue (see Plate 4.1, **f**), while nuclei were larger with more widely spaced DNA. RNA was abundant in cytoplasm and nuclei. Scale = 10 $\mu$m.

**f.** Fat body of female locust on Day 8 of the adult stadium. Note increased size and vacuolation of cells compared with Day 1 (**d**). Also note slight increase in nuclear size. Level of vacuolation of cytoplasm was less than in control tissue (see Plate 4.1, **g**), but cells and nuclei were larger. RNA was abundant in cytoplasm and nuclei. Scale = 10 $\mu$m.

Key: Cy– cytoplasm; N– nucleus; Nu– nucleolus; V– vacuole; large arrow– DNA; small arrow– RNA.


N
Cy
V
a
N
V
b
Cy
V
c
Cy
N
V
d
Nu
N
V
Cy
e
V
N
Cy
f

colour of the fat bodies was ascribed to the presence of large numbers of yellow-pigmented lipid droplets within the cells. In the present study, methoprene-treated fat bodies were similar to control tissues in their basic structure but were often white or pale grey in colour, apparently lacking the normal yellow pigmentation.

Plate 4.1 provides views of fat body from control locusts at regular intervals during the fifth instar and into the adult stage. The lobes of fat body tissue were typically one or two cells thick and, until Day 8 of adult life, there was no apparent difference between fat bodies from male and female animals. The levels of staining for RNA in cytoplasm and nuclei are not clearly shown in the micrographs. Therefore, the amounts of RNA found in the various fat bodies are given, in arbitrary values, in Table 4.1 below. Fat bodies of newly moulted, fifth instar locusts (12 hours $\pm$ 12 hours old) consisted of small cells with vacuolated cytoplasm, the vacuoles presumably being reserves of lipid and/or glycogen (Plate 4.1, **a**). The nuclei of the cells were also small with densely packed chromatin (DNA), but neither nuclei nor cytoplasm showed any staining for RNA. By Day 4 of the fifth instar, the fat body cells had greatly enlarged, as had the vacuoles in the cytoplasm and the cell nuclei (Plate 4.1, **b**). Particles of RNA were present in the cytoplasm, suggesting that the cells were involved in protein synthesis, and within the nuclei, indicating active transcription. On the seventh day of the instar (Plate 4.1, **c**), the cells were heavily vacuolated, with large spaces filling most of the cells so that only peripheral strands of cytoplasm remained. The cell nuclei were smaller than on Day 4 and, since RNA was no longer in evidence, apparently transcriptionally quiescent. However, RNA was still present in the remaining cytoplasm, suggesting that the cells were still actively synthetic. In contrast, RNA was no longer detectable in fat bodies on Day 10 of the fifth instar (Plate 4.1, **d**), suggesting that the synthetic activity of the tissue was relatively low. At this stage, fat body cells probably served mainly for the storage of reserve materials since the cells were filled with large vacuoles and contained very little cytoplasm. The nuclei were small with densely packed chromatin. After the final moult, the fat body cells were greatly reduced in size and only small vacuoles remained in the cells (Plate 4.1, **e**). Presumably, the rest of the stored material was used during metamorphosis. The tissue was similar in appearance to the fat body of the 1-day old fifth instar insect (Plate 4.1, **a**) ex-

cept that the nuclei were slightly larger. The presence of RNA in both cytoplasm and nuclei suggests that the cells were actively transcribing RNA in preparation for adult syntheses. The fat bodies of 8-day old adults showed, for the first time, sexual dimorphism. The cells of male fat body (Plate 4.1, **f**) had increased in size since the moult and were more extensively vacuolated. The cytoplasm stained for RNA so the cells were possibly synthetically active, but there was no sign of RNA within the small nuclei, suggesting that little or no transcription was taking place. In contrast, the nuclei of female trophocytes were large and, to judge from the intranuclear RNA, transcriptionally active (Plate 4.1, **g**). The cells contained numerous, small vacuoles but the abundance of RNA in the cytoplasm and nuclei suggested that the cells may have been preparing for the intense synthetic activity of vitellogenesis.

**Table 4.1 — Levels of RNA Detected in Fat Body**

| Day | Control | | | | 475 $\mu$g Metho. | | | |
|---|---|---|---|---|---|---|---|---|
| | Male | | Female | | Male | | Female | |
| | Nu | Cy | Nu | Cy | Nu | Cy | Nu | Cy |
| 1f | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4f | 2 | 2 | 2 | 2 | 3 | 3 | 4 | 4 |
| 7f | 0 | 1 | 0 | 1 | 0 | 1 | 3 | 3 |
| 10f | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1a | 1 | 1 | 1 | 1 | 2 | 2 | 3 | 3 |
| 8a | 0 | 1 | 2 | 2 | 2 | 2 | 4 | 4 |

The levels of staining for RNA are given as values in the range 0–4, '0' meaning that no RNA was detected whereas '4' meant that it was extremely abundant.

Legend: Day *x*f – Day *x* of the fifth instar,
Day *x*a – Day *x* of the adult stadium,
Nu – Nucleus,
Cy – Cytoplasm.

The *in vivo* application of 475 $\mu$g of methoprene to newly moulted fifth instar locusts caused substantial changes in fat body morphology. These effects were more extreme in female animals so that a marked sexual dimorphism in fat body structure was seen throughout the developmental period which was studied. On Day 4 of the fifth instar, fat body cells from treated males (Plate 4.2, **a**) were slightly smaller than those of 4-day old control animals (see Plate 4.1, **b**), and contained smaller cytoplasmic vacuoles. Cell nuclei, however, were larger in the treated tissues with more widely spaced chromatin, and RNA was more abundant in both cytoplasm and nuclei (see Table 4.1). Treated female tissue of the same age (Plate 4.2, **b**) also showed reduced cytoplasmic vacuolation, increased nuclear size and increased staining for RNA, but to a greater degree than was found in male fat body, especially with regard to nuclear size. On Day 7 of the fifth instar, treated male fat body (Plate 4.2, **c**) was similar to control tissue of the same age (see Plate 4.1, **c**), the cells being extensively vacuolated and containing little cytoplasm, with reduced levels of RNA. The nuclei, although slightly larger than those of control fat body, were reduced in size compared with 4-day old treated tissue (Plate 4.2, **a**) and contained closely packed chromatin. The morphology of the female fat bodies was very different. In one case (Plate 4.2, **d**), the cells still contained extensive cytoplasm, though the level of vacuolation was greater than that of 4-day old tissue (Plate 4.2, **b**). RNA was abundant, and the cell nuclei were extremely large with widely spaced chromatin. The fat body cells from the other methoprene-treated, 7-day old female were much smaller and without any cytoplasmic vacuoles (Plate 4.2 **e**). Most of the nuclei were large with dispersed chromatin, and RNA was present in both cytoplasm and nuclei. However, a sizeable proportion of the cells appeared to be undergoing mitosis. By the tenth day of the fifth instar, treated fat body cells, both male (Plate 4.3, **a**) and female (Plate 4.3, **b**), showed extreme vacuolation with little cytoplasm remaining, and an absence of RNA, similar to control tissue of the same age (see Plate 4.1, **d**). The nuclei of treated fat bodies had reduced in size since Day 7 of the fifth instar (Plate 4.2, **c**, **d** & **e**) and their DNA was closely packed, but they were still slightly larger than those of 10-day old controls, with treated female tissue having the greatest nuclear size. Immediately after the final moult, the fat body cells of treated male locusts were very small, about the same size as those of newly moulted, adult controls (see Plate 4.1, **e**). However, in the treated

tissues, cell cytoplasm was less vacuolated, nuclei were substantially larger, and RNA was more abundant in both cytoplasm and nuclei. In treated female tissues of the same age (Plate 4.3, **d**), these differences were more extreme, especially with regard to nuclear size, and the cells were also enlarged. By Day 8 of the adult stadium, trophocytes from treated male insects (Plate 4.3, **e**) had enlarged and were more or less equivalent in size to the fat body cells of 8-day old control adults (see Plate 4.1, **f**). The differences between control and treated fat bodies remained essentially the same as on Day 1 of adult life, with treated tissues having enlarged nuclei, fewer and smaller cytoplasmic vacuoles, and greater amounts of RNA in both nucleus and cytoplasm. The fat body cells from treated females of the same age (Plate 4.3, **f**) were increased in size and showed greater cytoplasmic vacuolation compared with tissue from newly moulted adults (Plate 4.3, **d**), and the cell nuclei were also slightly increased in size. Compared with fat body from control females on Day 8 of the adult instar (see Plate 4.1, **g**), the treated cells were larger and less heavily vacuolated, while the cell nuclei were much larger. RNA was also more abundant in both cytoplasm and nuclei of treated fat bodies (see Table 4.1).

### 4.3.2 Fat Body Ultrastructure

Apart from the basic trophocyte, locust fat bodies also contained oenocytes and urocytes, and these two cell types, from control fat body on Day 10 of the fifth instar, are shown in Plate 4.4. The oenocyte (Plate 4.4, **a** & **b**) had a characteristic appearance which was very different from that of surrounding trophocytes. The nucleus was large with very little heterochromatin, while the cytoplasm was rich in mitochondria, polysomes and granular bodies. The cell also contained an extensive reticular tubular system, and some elements of this system had surrounded and apparently isolated parts of the cell cytoplasm (Plate 4.4, **b**). There was no discernible smooth endoplasmic reticulum (SER) in the cell whereas this is normally extremely abundant in oenocytes (see Introduction). However, in oenocytes from *Calpodes ethlius* (LOCKE, 1969) and *Culex pipiens* (GNATZY, 1970), it was seen that, at the approach of larval and pupal moults, SER diminished and the cells underwent a phase of autophagy, with the reticular tubular system enclosing and isolating organelles and small volumes of cytoplasm for subsequent digestion. In the present study, the lack of SER and the presence

**Plate 4.4 — Electron Micrographs of Oenocyte and Urocyte from Control Fat Body**

**a.** Oenocyte on Day 10 of the fifth instar. Note the large size of the nucleus and the presence of several lipid droplets in the cytoplasm. Also note the very different appearance of the cytoplasm compared with that of surrounding trophocytes. Scale = 2 $\mu$m.

**b.** Detail of oenocyte. Note the presence of an extensive reticular tubular system running throughout the cytoplasm. Also note the presence of numerous mitochondria, polysomes and granular bodies within the cytoplasm. Several isolation bodies were also present, small areas of cytoplasm which appeared to be completely enclosed by elements of the tubular system. Scale = 1 $\mu$m.

**c.** Urocyte on Day 10 of the fifth instar. Note the typically slender and elongate profile of the cell. Apart from the nucleus, the only other easily recognisable contents of the cell were urate spherules. Scale = 2 $\mu$m.

**d** & **e.** Detail of urocyte. Note the large variety of forms of urate spherules. Usually, these were composed of an electron-dense core surrounded by one or two rings of granular or fibrous material. Occasionally, organelles such as mitochondria were also seen in the cells. Scale = 0.5 $\mu$m.

Key: G– granular body; Hc– heterochromatin; IB– isolation body; L– lipid droplet; M– mitochondrion; N– nucleus; Oc– oenocyte; P– polysome; Tc– trophocyte; TE– tubular element; Uc– urocyte; US– urate spherule.

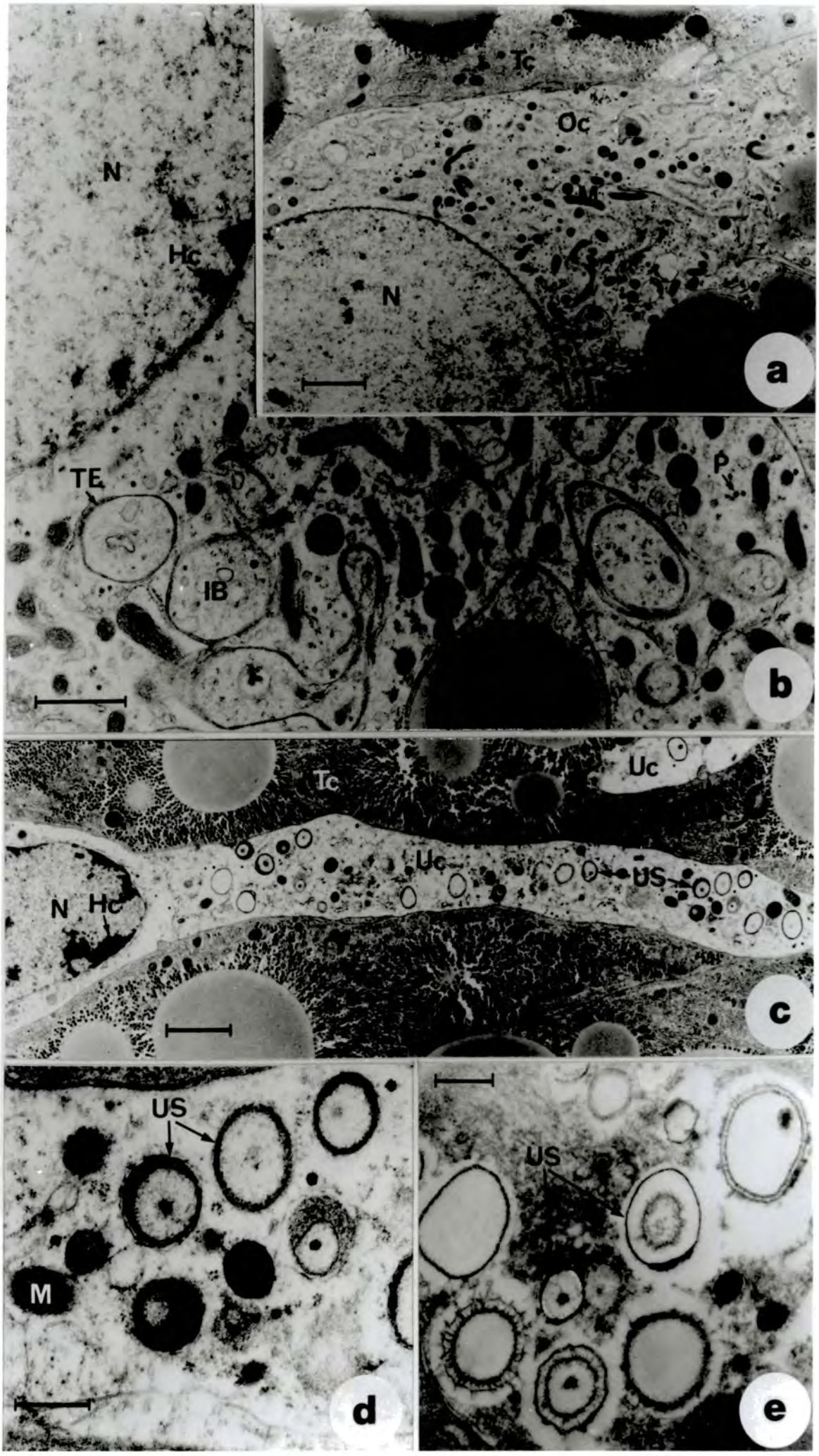

N
Hc
Tc
Oc
N
a
TE
IB
P
b
Tc
Uc
Uc
US
N
Hc
c
US
M
d
US
e

**Plate 4.5 — Electron Micrographs of Control Fat Body, Day 1 of the Fifth Instar**

**a** & **b**. Trophocytes from newly moulted fifth instar locusts. The majority of trophocytes were similar to those shown in micrograph **a**. Note the small size of the cells, mainly taken up by lipid droplets. Also note the large amounts of heterochromatin packed in the cell nuclei, and numerous granular bodies in the cytoplasm. A sizeable minority of fat body cells were similar to that shown in micrograph **b**. Note the presence of extremely abundant RER in the cytoplasm, and the scarcity of lipid droplets compared with micrograph **a**. Also note the presence of numerous, large mitochondria, some of which appeared to be dividing (arrow). Scale = 2 $\mu$m.

**c**. Detail of cytoplasm from micrograph **a**. Note the presence of numerous, large mitochondria, and of short scattered strands of RER. Scale = 0.5 $\mu$m.

**d**. Detail of cytoplasm from micrograph **b**. Note the presence of large amounts of RER, and the swollen nature of both RER and the nuclear membranes. Also note the large size of the mitochondria, some of which appeared to be dividing (arrow). Granular bodies and Golgi complexes were common. Scale = 0.5 $\mu$m.

**e**. Detail of cytoplasm showing the irregular, speckled appearance of granular bodies. Scale = 0.5 $\mu$m.

Key: G– granular body; Go– Golgi complex; Hc– heterochromatin; L– lipid droplet; M– mitochondrion; N– nucleus; NM– nuclear membrane; RER– rough endoplasmic reticulum.

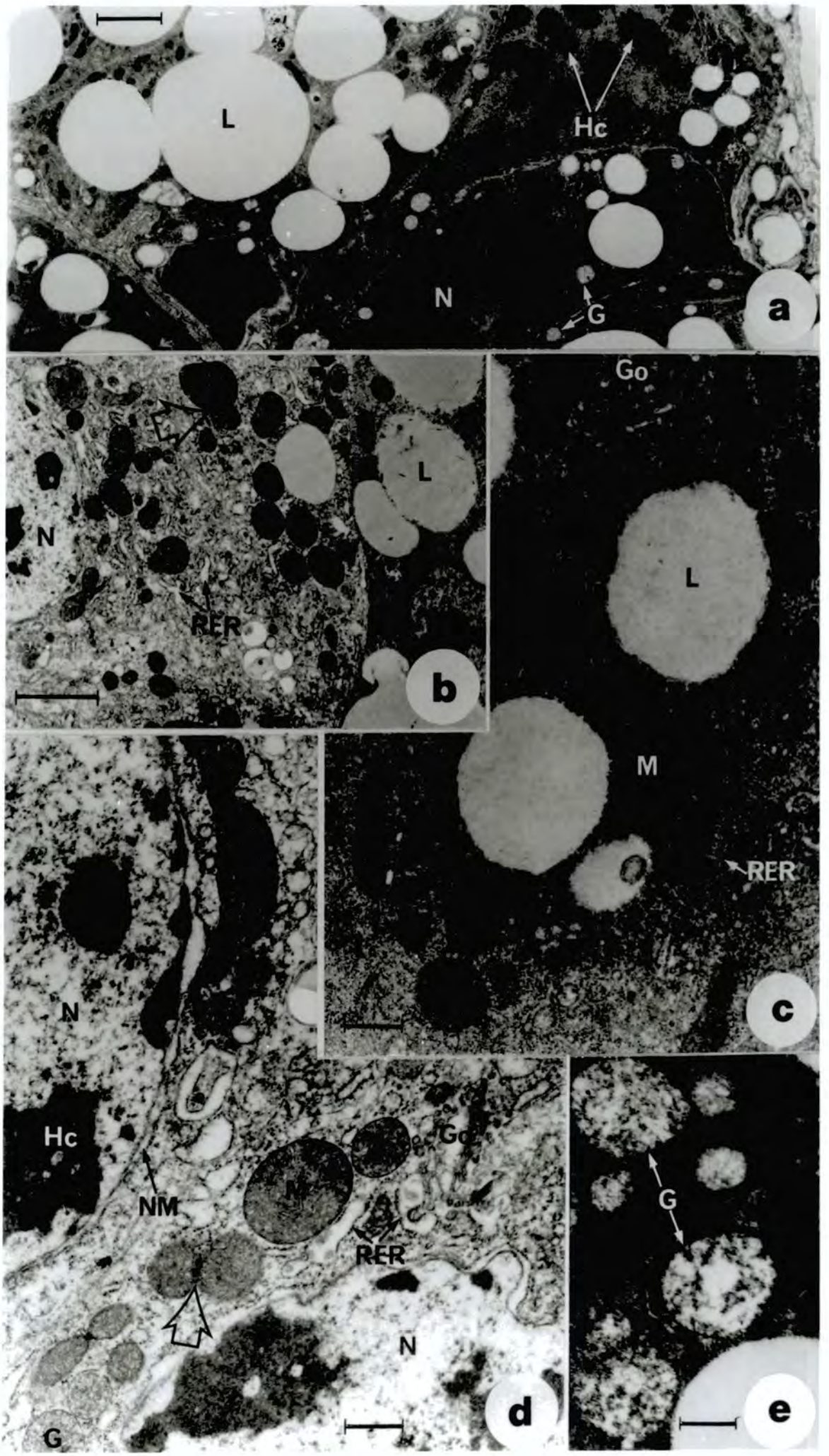

L
Hc
N
G
a
L
N
RER
b
Go
L
M
RER
c
N
Hc
NM
Go
M
RER
N
G
d
G
e

**Plate 4.6 — Electron Micrographs of Control Fat Body, Day 4 of the Fifth Instar**

**a**, **b** & **c**. Trophocytes from 4-day old fifth instar locusts. Note the small size and numbers of lipid droplets compared with Day 1 of the fifth instar (see Plate 4.5, **a** & **b**). Also note the presence of numerous mitochondria and Golgi complexes, and the well-developed RER. Inclusion bodies were occasionally seen (**b**), and the cell nuclei were large with scattered bodies of heterochromatin (**c**). Scale = 2 $\mu$m.

**d** & **e**. Detail of cytoplasm from micrograph **b**. Note the slightly swollen nature of the extensive RER. Also note the large numbers of secretory vesicles around the Golgi complexes, indicating high levels of synthetic activity. Note the appearance of the inclusion body in micrograph **d**, which seemed to contain layered, electron-dense lamellae. Also note the very close association between an inclusion body and a Golgi complex in micrograph **e** (arrow). Scale = 1 $\mu$m.

**f**. Detail of cytoplasm showing various forms of inclusion body that were seen in 4-day old fifth instar fat body. Scale = 1 $\mu$m.

Key: Go– Golgi bodies; Hc– heterochromatin; In– inclusion body; L– lipid droplet; M– mitochondrion; N– nucleus; RER– rough endoplasmic reticulum; SV– secretory vesicle; Uc– urocyte.

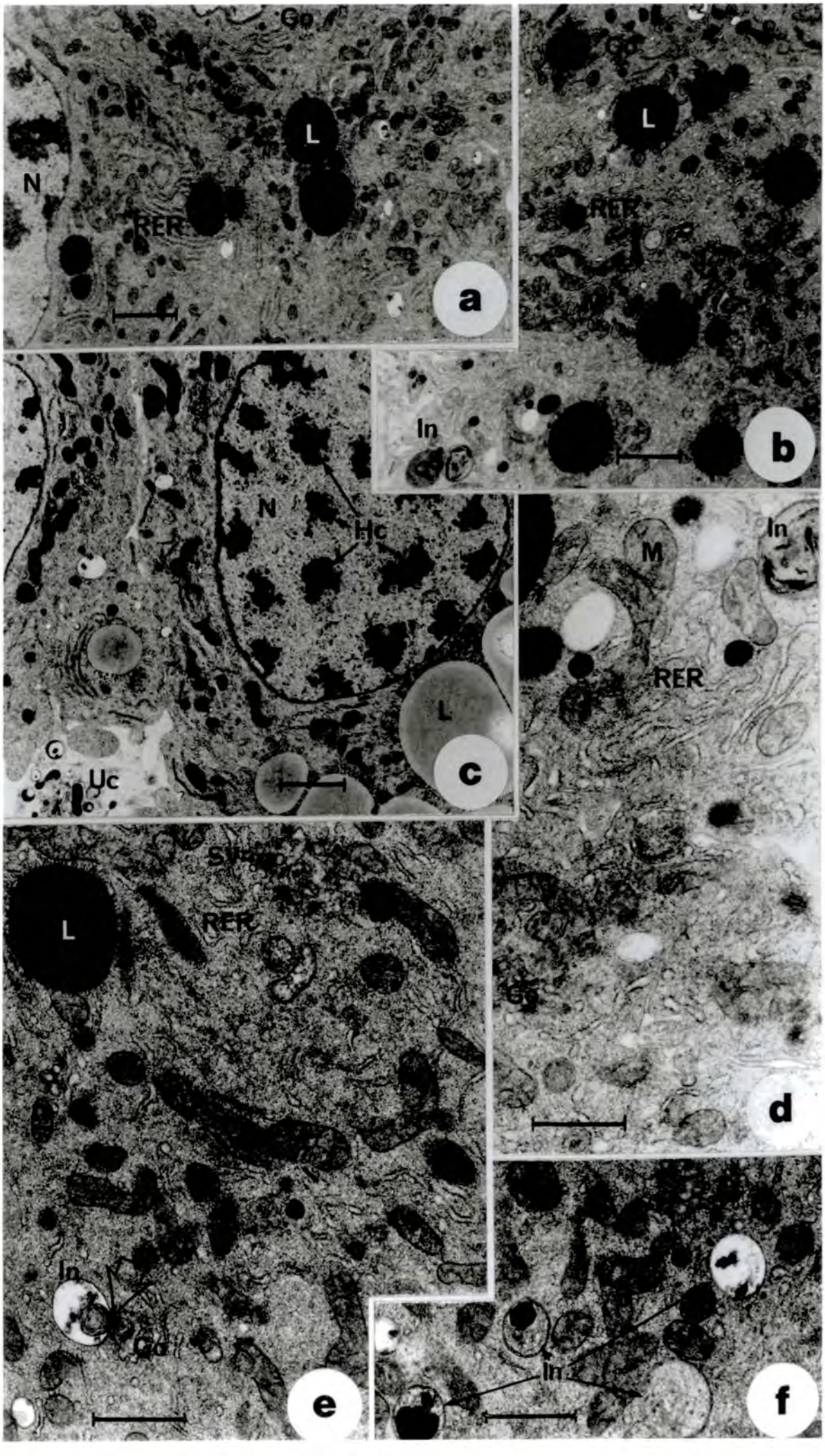

Go
L
N
RER
a
L
RER
In
b
N
Hc
L
Uc
c
In
M
RER
d
L
RER
In
Go
e
In
f

**Plate 4.7 — Electron Micrographs of Control Fat Body, Day 7 of the Fifth Instar**

**a, b** & **c**. Trophocytes from 7-day old fifth instar locusts. Note the large variation in the amount of stored lipid and glycogen within different cells, with some cells having relatively little reserve material (**a**). Other cells contained more lipid and glycogen so that the stored material occupied most of the cell volume and the cell cytoplasm became restricted to areas around the nucleus and along the plasma membrane (**b** & **c**). Scale = 2 $\mu$m.

**d**. Detail of cytoplasm from micrograph **a**. Note the presence of extensive, swollen RER and active Golgi complexes. Scale = 0.5 $\mu$m.

**e**. Detail of cytoplasm from micrograph **b**. Note the presence of numerous mitochondria and RER in the remaining cytoplasm, and of electron-dense inclusion bodies. Scale = 1 $\mu$m.

**f**. Detail of cytoplasm from micrograph **c**. Note the presence of numerous mitochondria and RER in the remaining cytoplasm, and of electron-dense inclusion bodies. Scale = 0.5 $\mu$m.

Key: Gl– glycogen; Go– Golgi complex; In– inclusion body; L– lipid droplet; M– mitochondrion; N– nucleus; Nu– nucleolus; PM– plasma membrane; RER– rough endoplasmic reticulum.

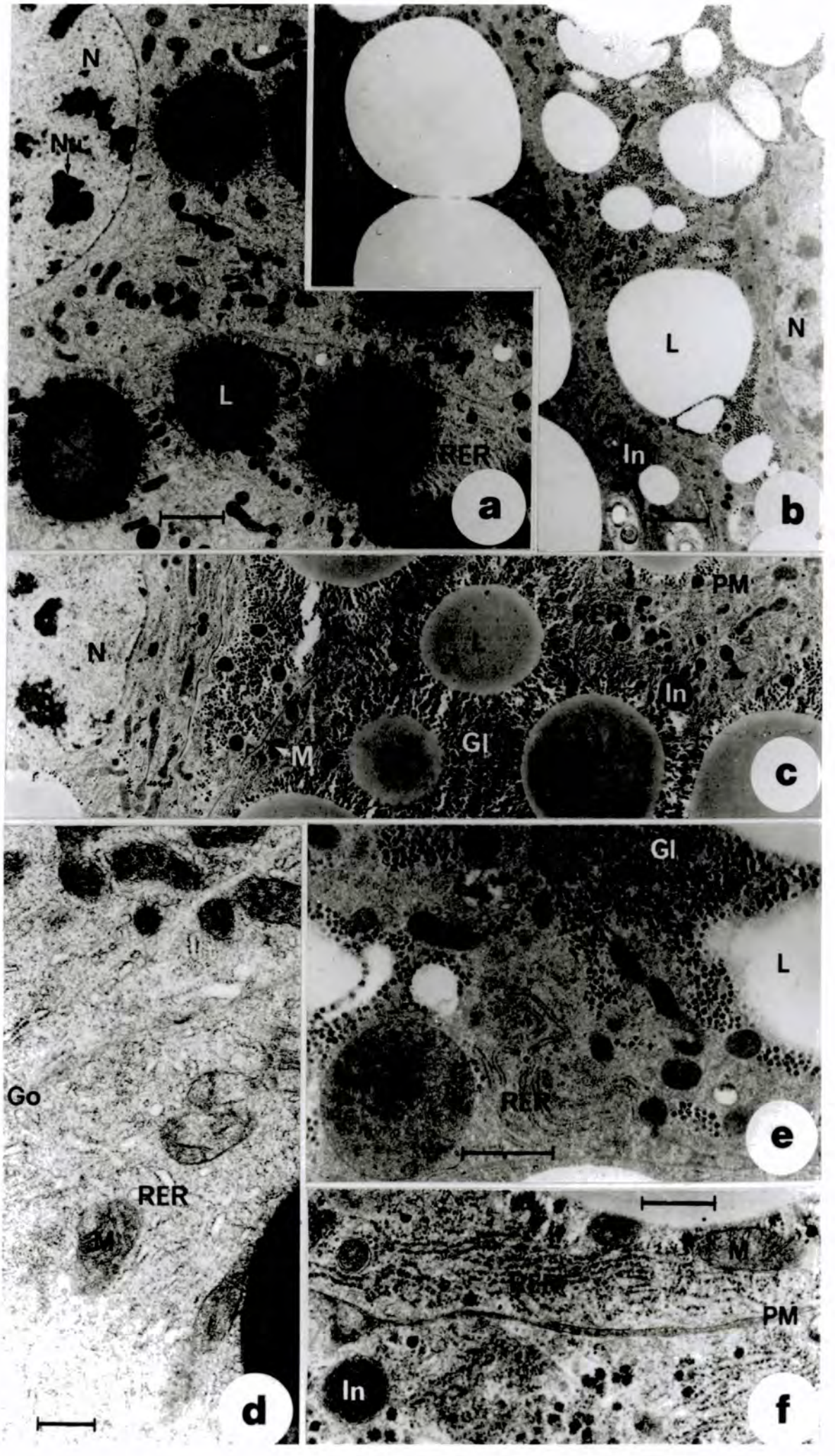

N
Nu
L
RER
a
L
N
In
b
N
M
GI
L
In
PM
c
Go
RER
d
GI
L
RER
e
M
PM
In
f

**Plate 4.8 — Electron Micrographs of Control Fat Body, Day 10 of the Fifth Instar**

**a** & **b**. Trophocytes from 10-day old fifth instar locusts. Note the large amounts of lipid and, especially, glycogen stored within the cells and occupying nearly all of the cell volume. Also note the presence of large vacuoles within the glycogen stores of many cells (**b**). Note the presence of numerous large, electron-dense inclusion bodies. The remaining cytoplasm was limited to thin strands along the plasma membrane and in the perinuclear region. Scale = 2 $\mu$m.

**c**. Detail of cytoplasm from micrograph **a**. Note the large and active Golgi complexes, their secretory vesicles being filled with electron-dense material. Also note the numerous mitochondria and RER in the residual cytoplasm. Scale = 0.5 $\mu$m.

**d**. Details of cytoplasmic inclusion bodies seen in 10-day old fifth instar fat body. Note the presence of multiple, layered lamellae in many of the inclusion bodies (solid arrow). Also note the presence of what appeared to be small bodies of cytoplasm within certain inclusion bodies (open arrow). Scale = 0.5 $\mu$m.

Key: Gl– glycogen; Go– Golgi complex; Hc– heterochromatin; In– inclusion body; L– lipid droplet; M– mitochondrion; N– nucleus; Nu– nucleolus; PM– plasma membrane; RER– rough endoplasmic reticulum; SV– secretory vesicle; V– vacuole.

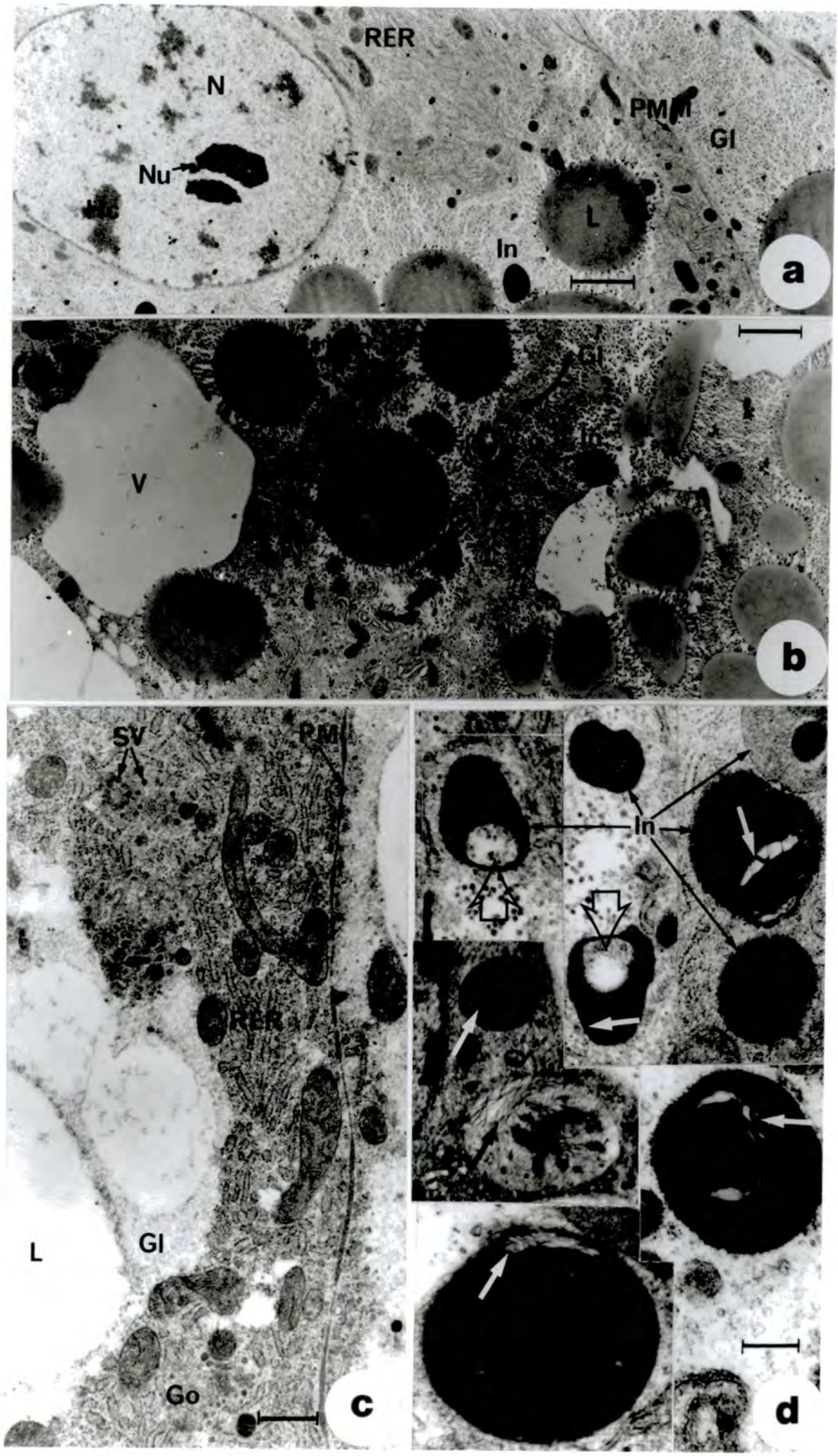

RER
N
PM
Gl
Nu
L
In
a
Gl
In
V
b
SV
PM
RER
L
Gl
Go
c
In
d

**Plate 4.9 — Electron Micrographs of Control Fat Body, Day 1 of the Adult Stadium**

**a** & **b**. Trophocytes from 1-day old adult locusts. Note the presence of numerous, small lipid droplets in the cytoplasm. In some cells (**b**), deposits of glycogen were also in evidence. Also note the presence of numerous granular bodies (**a**) and inclusion bodies (**b**). Scale = 2 $\mu$m.

**c** & **d**. Details of cytoplasm from micrographs **a** and **b**, respectively. Note that the RER was generally present as short, isolated strands (**c**), but was occasionally more extensive (**d**). Note the presence of numerous mitochondria and of large, active Golgi complexes. Also note the appearance of the granular bodies, similar to those seen in trophocytes from 1-day old fifth instar locusts (see Plate 4.5). Scale = 0.5 $\mu$m.

**e**. Typical nucleus of trophocytes from 1-day old adult locusts. Note the presence of numerous bodies of heterochromatin and of multiple nucleoli. Scale = 2 $\mu$m.

Key: G– granular body; Gl– glycogen; Go– Golgi complex; Hc– heterochromatin; In– inclusion body; L– lipid droplet; M– mitochondrion; N– nucleus; Nu– nucleolus; RER– rough endoplasmic reticulum; Uc– urocyte, US– urate spherule.

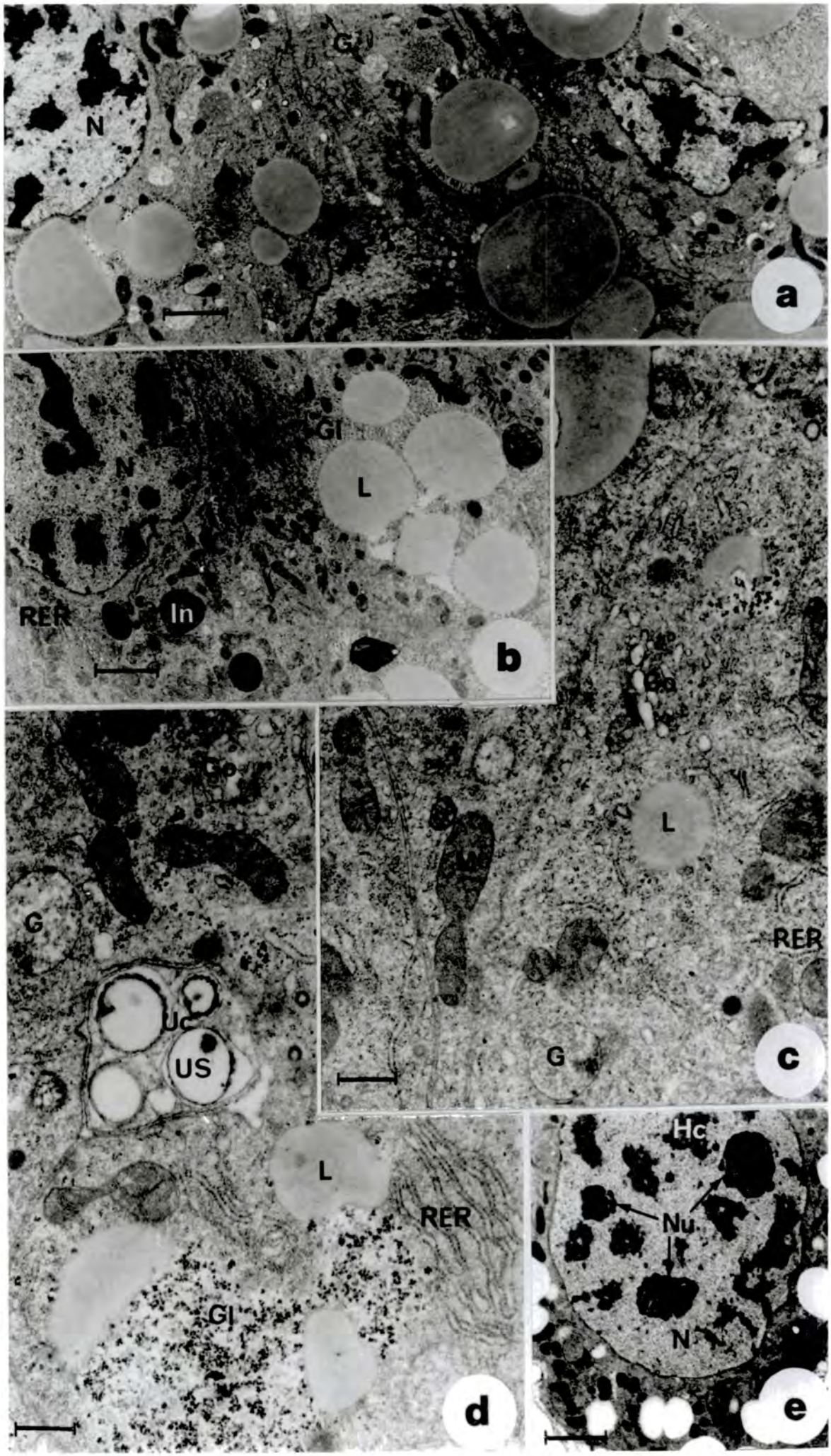

N
Gl
a
Gl
N
L
RER
In
b
L
RER
G
c
G
Uc
US
L
RER
Gl
d
Hc
Nu
N
e

**Plate 4.10 — Electron Micrographs of Control Fat Body, Day 8 of the Adult Stadium**

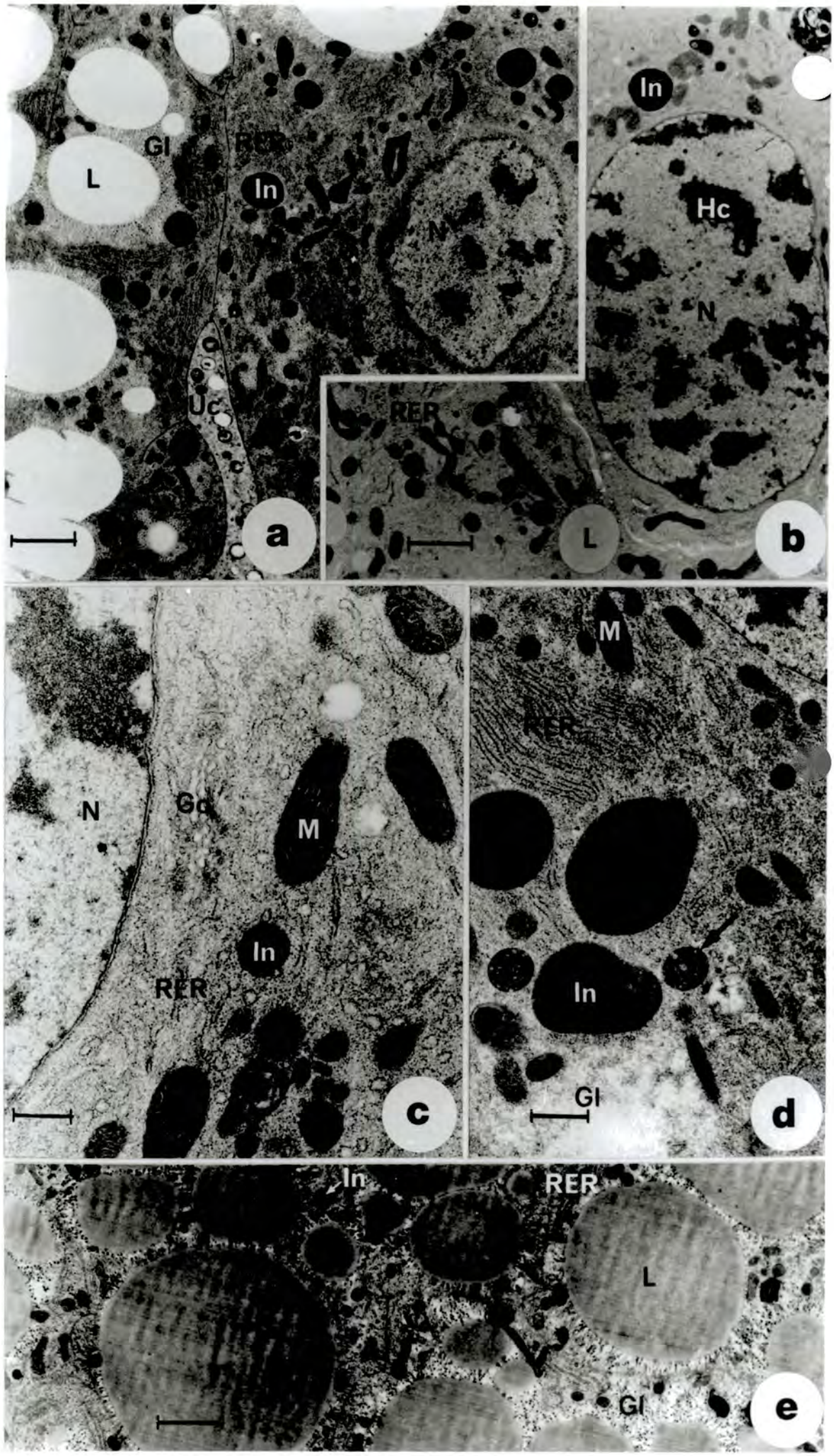
**a** & **b**. Trophocytes from 8-day old adult female locusts. Note the extensive RER and numerous mitochondria in the abundant cytoplasm of the cells. Many cells contained moderate amounts of stored lipid and glycogen (**a**) but in others there was almost no reserve material (**b**). Also note the presence of many large, electron-dense inclusion bodies. Scale = 2 $\mu$m.

**c**. Detail of cytoplasm from micrograph **a**. Note the extensive, slightly swollen RER. Scale = 0.5 $\mu$m.

**d**. Detail of cytoplasm from micrograph **a**. Note the large size and electron-dense nature of the inclusion bodies. Also note the multivesicular appearance of many of the inclusion bodies, especially the smaller ones (arrow). Scale = 0.5 $\mu$m.

**e**. Trophocytes from 8-day old adult male locusts. Note the large amounts of stored lipid and glycogen, occupying the greater part of the cells. The cytoplasm contained numerous mitochondria and extensive RER but was limited to thin strands at the perimeter of the cells. Scale = 2 $\mu$m.

Key: Gl– glycogen; Go– Golgi complex; Hc– heterochromatin; In– inclusion body; L– lipid droplet; M– mitochondrion; N– nucleus; RER– rough endoplasmic reticulum; Uc– urocyte.


Gl
L
In
N
Uc
a
In
Hc
N
RER
L
b
N
Gc
M
In
RER
c
M
RER
In
Gl
d
In
RER
L
Gl
e

of isolation bodies formed from the tubular reticular system suggest that a similar autophagic phase may take place in locust oenocytes at the end of the fifth instar. However, oenocytes were only very rarely encountered in locust fat bodies and no attempt was made to follow their structural changes during development nor to determine their responses, if any, to the application of methoprene. Urocytes were found more frequently in locust fat bodies and a typical cell is shown in Plate 4.4 (**c**). The cells were usually thin and elongate, and so were most often encountered as small transverse sections. The nucleus contained large patches of heterochromatin and was similar in appearance to trophocyte nuclei (e.g. see Plate 4.6, **c**). The cytoplasm was mainly taken up by urate spherules which were very heterogeneous in appearance but generally consisted of an electron-dense core surrounded by one or two rings of fibrous or granular structure (Plate 4.4, **d** & **e**). Preliminary observations revealed no obvious changes in their structure during development nor after the application of methoprene, and a more detailed examination was not attempted.

The trophocytes from newly moulted fifth instar *Locusta* were small and the majority appeared to be metabolically quiescent, most of the cells' volume being taken up by lipid stores (Plate 4.5, **a**) while only short, scattered strands of RER were present in the cytoplasm and Golgi complexes were infrequent (Plate 4.5, **c**). However, a number of trophocytes seemed to show greater synthetic activity (Plate 4.5, **b** & **d**), having much less stored lipid and extremely abundant RER which was also very swollen, indicative of intense synthetic activity (COUBLE *et al.*, 1979). Golgi complexes were also abundant, as were the mitochondria, several of which appeared to be dividing. The newly moulted fat body cells also contained numerous bodies with a granular structure (Plate 4.5, **e**) whose possible function is considered in the Discussion. By Day 4 of the fifth instar, fat body cell cytoplasm was much more abundant and stored lipid was limited to relatively few, small droplets (Plate 4.6, **a** & **b**). The RER was extensive and slightly swollen while Golgi complexes were large with many secretory vesicles, suggesting a high level of synthetic activity in the cells (Plate 4.6, **d** & **e**). Granular bodies were no longer in evidence but the cells now contained a variety of inclusion bodies, many of which contained layered or whorled lamellae (Plate 4.6, **d**, **e** & **f**). By the seventh day of the fifth instar, fat body cells had accumulated larger amounts of reserve material (Plate 4.7, **a**, **b** & **c**). Lipid droplets had increased

in both size and number, and for the first time substantial stores of glycogen granules were detectable in most cells. Inclusion bodies were also more common within the trophocytes. As the levels of stored material increased, the amount of cytoplasm within the cells was reduced and, in some cases, was restricted to thin strands around the nucleus and along the plasma membranes (Plate 4.7, **c**). However, many cells still contained extensive, swollen RER and large Golgi complexes, indicating that the fat body was still synthetically active (Plate 4.7, **d**). On Day 10 of the fifth instar, fat body cells were filled with lipid droplets and glycogen granules (Plate 4.8, **a** & **b**). Within the deposits of glycogen, large vacuoles were often present which did not seem to be merely lipid vacuoles that had been emptied of their contents during the fixation process (Plate 4.8, **b**). LOCKE (1985) noted that similar vacuoles appeared within the glycogen stores of both fat body and epidermal tissues of *Calpodes ethlius* as carbohydrate reserves reached their maximal extent towards the end of each larval instar. He suggested that the glycogen might be converted to soluble precursors in order to allow rapid mobilisation during its redeployment, but this has yet to be confirmed. The residual cytoplasm of the cells was packed with RER, and numerous Golgi bodies were producing secretory vesicles, indicating that active syntheses were still taking place (Plate 4.8, **c**). Many large inclusion bodies were present in the cells. A number of different forms were seen (Plate 4.8, **d**) but the fact that many contained layered or whorled lamellae suggested that they could be of similar origin. The trophocytes of newly moulted adults were much depleted of lipid and especially glycogen (Plate 4.9, **a** & **b**), suggesting that most of the fat bodies' reserve materials stores were mobilised during the final moult. The tissue was similar in many ways to that of newly moulted fifth instar locusts (see Plate 4.5), containing numerous granular bodies (Plate 4.9, **a**) and the cytoplasm seeming to be metabolically quiescent, with short, scattered strands of RER and few Golgi complexes (Plate 4.9, **c** & **d**). Occasionally, trophocyte nuclei were seen to contain multiple nucleoli (Plate 4.9, **e**), suggesting that the cells were polyploid (LOCKE, 1980, 1985). Fat bodies from 8-day old adults showed, for the first time, sexual dimorphism in their ultrastructure. Trophocytes from female insects contained little reserve material, and cell cytoplasm was very abundant (Plate 4.10, **a** & **b**). The cells appeared synthetically active, having extensive and often swollen RER, and numerous Golgi complexes (Plate 4.10, **c** & **d**). Large,

electron-dense inclusion bodies were also abundant in the tissue (Plate 4.10, **a**), many of which had a multivesicular appearance (Plate 4.10, **d**). In contrast, male fat body had a metabolically quiescent appearance, with ample stores of lipid and glycogen and little sign of synthetic activity in the residual cytoplasm (Plate 4.10, **e**).

The *in vivo* application of methoprene to newly moulted fifth instar locusts caused substantial changes in the ultrastructure of the fat body during the insects' subsequent development. However, the effects were generally less extreme than those seen under the light microscope and, although changes were slightly more marked in female fat bodies, there was little sexual dimorphism in the response of the tissue to methoprene treatment. Also, both concentrations of the JHA that were used, 475 $\mu$g and 95 $\mu$g per insect, usually had similar effects on the tissue. On Day 4 of the fifth instar, trophocytes from methoprene-treated insects contained abundant cell cytoplasm whereas stored material was limited to relatively few, small lipid droplets (Plate 4.11, **a** & **b**). RER was swollen and very extensive, and Golgi complexes were numerous and prominent, with many secretory vesicles, (Plate 4.11, **c** & **d**). In the nuclei of 475 $\mu$g-treated fat body, nucleoli were especially large and multilobed (Plate 4.11, **a**), while in the cytoplasm, the RER was particularly abundant and swollen (Plate 4.11, **c**). Inclusion bodies were also seen in the trophocytes (Plate 4.11, **c** & **d**), and occasionally isolation bodies were seen as well, some of which seemed to contain fragments of RER (Plate 4.11, **e**). The tissue was similar in appearance to control fat body of the same age (see Plate 4.6) except that RER was slightly more extensive and swollen in methoprene-treated cells. By the seventh day of the fifth instar, the levels of stored lipid in treated fat body cells had slightly increased (Plate 4.12, **a** & **d**), but were lower than those found in control tissue of the same age (see Plate 4.7), and there were no visible deposits of glycogen. The cytoplasm still contained extensive RER and numerous, large Golgi complexes, especially the 475 $\mu$g-treated fat bodies (compare **b** & **c** with **e**, Plate 4.12). Occasionally, discrete bodies were seen within trophocyte nuclei (Plate 4.12, **f**) which were similar in appearance to the nuclear bodies described by JENSEN and BRASCH (1985) in the fat body of adult *Locusta*. On Day 10 of the fifth instar, treated fat body cells were filled with deposits of lipid and glycogen while cell cytoplasm was reduced to peripheral strands (Plate 4.13, **a** & **b**), similar to control fat body of equivalent

## Plate 4.11 — Electron Micrographs of Methoprene-Treated Fat Body, Day 4 of the Fifth Instar

**a.** Trophocyte from 4-day old fifth instar locust previously treated with 475 $\mu$g of methoprene. Note the presence of scattered bodies of heterochromatin and multiple, multilobed nucleoli within the nucleus. Note the extreme abundance of RER in the cytoplasm, more extensive than that of control tissue of the same age (see Plate 4.6). Numerous mitochondria and small lipid droplets were also present in the cytoplasm. Scale = 2 $\mu$m.

**b.** Trophocyte from 4-day old fifth instar locust previously treated with 95 $\mu$g of methoprene. Note the presence of very extensive RER, numerous mitochondria and small lipid droplets, similar in appearance to micrograph **a**. Scale = 2 $\mu$m.

**c.** Detail of cytoplasm from micrograph **a**. Note the slightly swollen nature of the extensive RER and the large numbers of secretory vesicles around the large Golgi complexes. Also note the presence of occasional inclusion bodies. Scale = 0.5 $\mu$m.

**d.** Detail of cytoplasm from micrograph **b**. Note the similarity in general appearance with micrograph **c** except that the RER seemed to be less swollen. Scale = 0.5 $\mu$m.

**e.** Detail of cytoplasm from 95 $\mu$g methoprene-treated tissue. Occasional isolation bodies were seen in the cytoplasm. Where the contents were discernible, these structures seemed to contain fragments of RER (open arrow). Scale = 0.5 $\mu$m.

Key: Go– Golgi complex; Hc– heterochromatin; IB– isolation body; In– inclusion body; L– lipid droplet; M– mitochondrion; N– nucleus; Nu– nucleolus; RER– rough endoplasmic reticulum; SV– secretory vesicle.

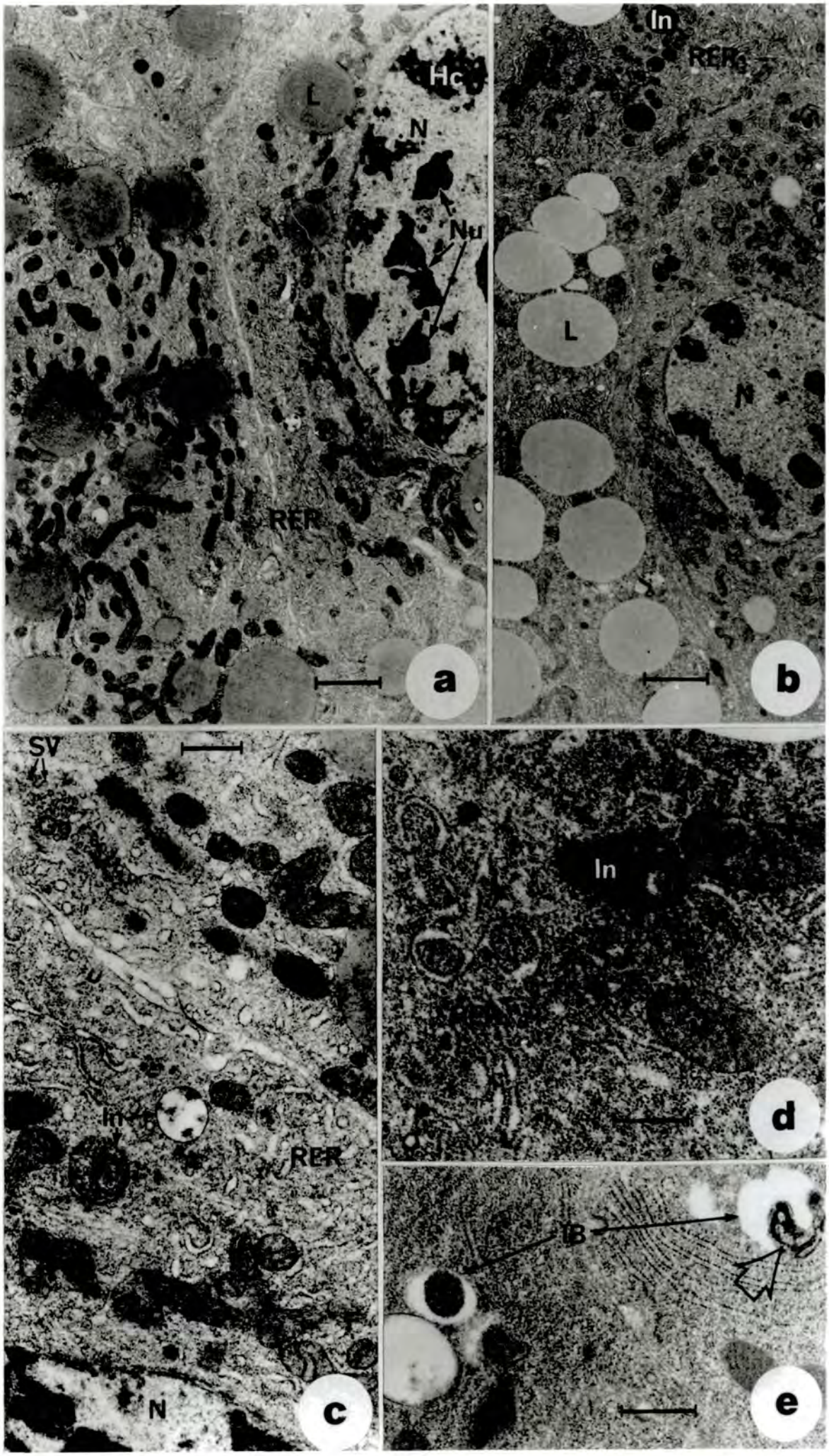

Hc
L
N
Nu
RER
a
In
RER
L
N
b
SV
In
RER
N
c
In
d
IB
e

**Plate 4.12 — Electron Micrographs of Methoprene-Treated Fat Body, Day 7 of the Fifth Instar**

**a.** Trophocytes of 7-day old fifth instar locust previously treated with 475 $\mu$g of methoprene. Note the presence of moderate amounts of stored lipid, less than was normally found in control tissue of the same age (see Plate 4.7). Also note the presence of numerous, large inclusion bodies in the cytoplasm. Scale = 2 $\mu$m.

**b** & **c.** Details of cytoplasm from 7-day old, 475 $\mu$g-treated trophocytes. Note the presence of extensive RER which was often found in closely packed layers (**b**) although it was usually more scattered (**c**). Also note the presence of active Golgi complexes and numerous mitochondria. Scale = 0.5 $\mu$m.

**d.** Trophocytes of 7-day old fifth instar locusts previously treated with 95 $\mu$g of methoprene. Note the presence of moderate amounts of stored lipid, extensive RER and large, electron-dense inclusion bodies. The cells were similar in appearance to those from 475 $\mu$g-treated insects (**a**). Scale = 2 $\mu$m.

**e.** Detail of cytoplasm from micrograph **d**. Note the presence of active Golgi complexes, numerous mitochondria and extensive, scattered RER, similar to the appearance of the cytoplasm in micrograph **c**. Scale = 0.5 $\mu$m.

**f.** Detail of nucleus from 7-day old, 475 $\mu$g-treated trophocyte. Note the characteristic appearance of a nuclear body. Such structures were occasionally seen in the trophocyte nuclei of both 475 $\mu$g- and 95 $\mu$g-treated insects. Scale = 0.5 $\mu$m.

Key: Go– Golgi complex; Hc– heterochromatin; In– inclusion body; L– lipid droplet; M– mitochondrion; N– nucleus; NB– nuclear body; RER– rough endoplasmic reticulum.

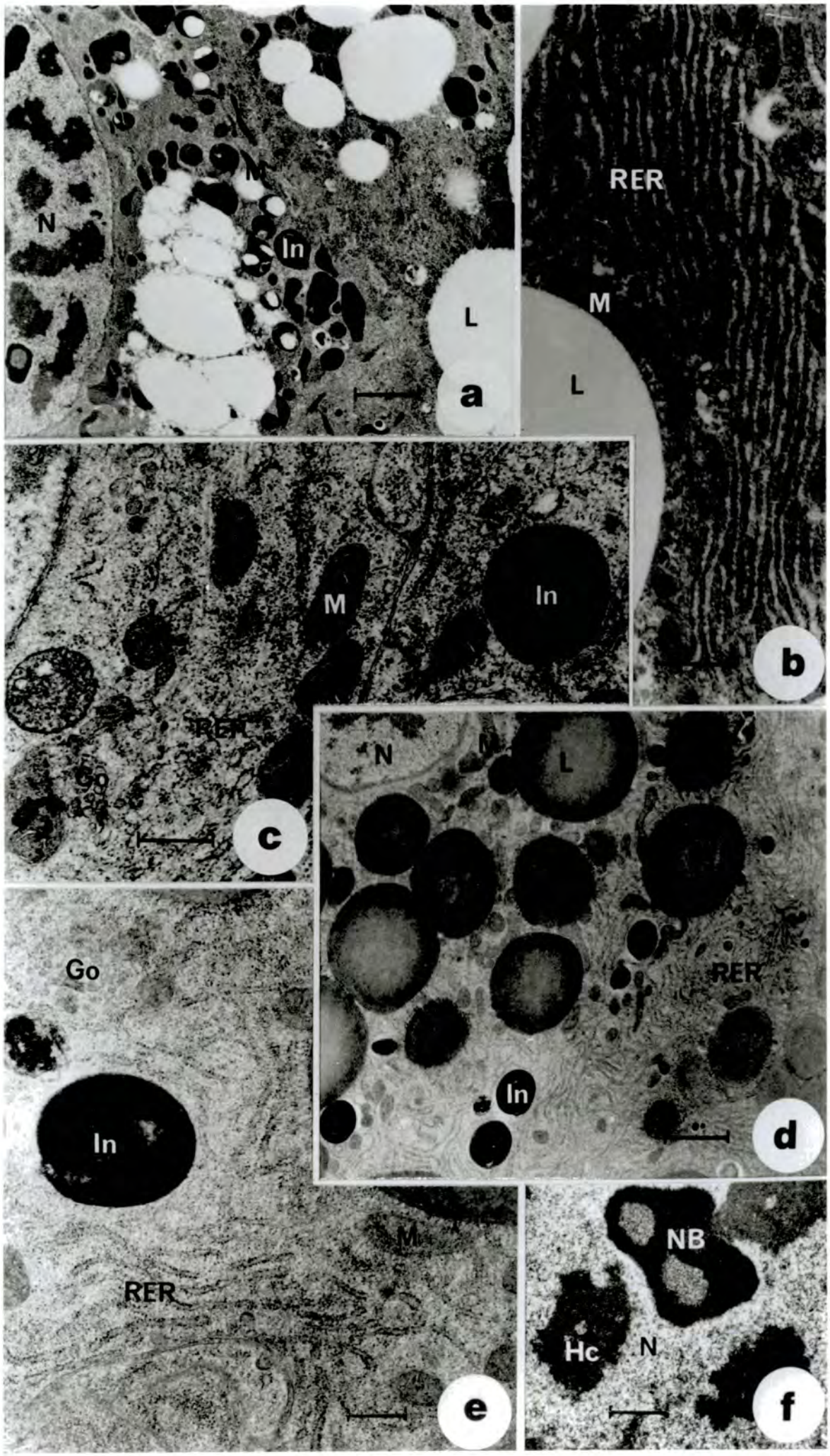

N
M
In
L
a
RER
M
L
b
M
In
RER
Go
c
N
M
L
RER
In
d
Go
In
M
RER
e
NB
Hc
N
f

**Plate 4.13 — Electron Micrographs of Methoprene-Treated Fat Body, Day 10 of the Fifth Instar and Day 1 of the Adult Stadium**

**a.** Trophocytes of 10-day old fifth instar locust previously treated with 475 $\mu$g of methoprene. Note the extensive lipid and glycogen deposits nearly filling the cells. Also note the presence of several large inclusion bodies. Cytoplasm was reduced to a thin layer lying against the plasma membrane, mainly filled with closely packed RER. Scale = 2 $\mu$m.

**b.** Trophocytes of 10-day old fifth instar locust previously treated with 95 $\mu$g of methoprene. Note their similarity with the cells in micrograph **a**. Cells were mainly taken up by lipid and glycogen deposits. Note the presence of vacuoles within the stored glycogen, similar to control tissue of the same age (see Plate 4.8). Also note the presence of numerous inclusion bodies, smaller than those in micrograph **a**. Residual cytoplasm lay along the periphery of the cells mainly taken up by closely packed RER. Scale = 2 $\mu$m.

**c.** Details of cytoplasm from 10-day old, 475 $\mu$g-treated trophocytes. Note the presence of multilamellar structures, composed of multiple, whorled membranes. Also note the presence of numerous mitochondria, active Golgi complexes and RER in the residual cytoplasm. Scale = 0.5 $\mu$m.

**d.** Trophocytes of 1-day old adult locust previously treated with 475 $\mu$g of methoprene. Note the presence of small lipid droplets, numerous mitochondria and extensive RER in the cytoplasm. Also note the absence of glycogen deposits and granular bodies, unlike control tissue of the same age (see Plate 4.9). Scale = 2 $\mu$m.

**e.** Trophocytes of 1-day old adult locust previously treated with 95 $\mu$g of methoprene. Note the very small amount of lipid stored in the cells. The cytoplasm contained numerous mitochondria and extensive RER . Also note the presence of occasional inclusion bodies and granular bodies. Scale = 2 $\mu$m.

**f.** Detail of cytoplasm from micrograph **e**. Note the slightly swollen nature of the RER and the active appearance of Golgi complexes. Scale = 0.5 $\mu$m.

Key: G– granular body; Gl– glycogen; Go– Golgi complex; In– inclusion body; L– lipid droplet; M– mitochondrion; ML– multilamellar body; N– nucleus; PM– plasma membrane; RER– rough endoplasmic reticulum; V– vacuole.

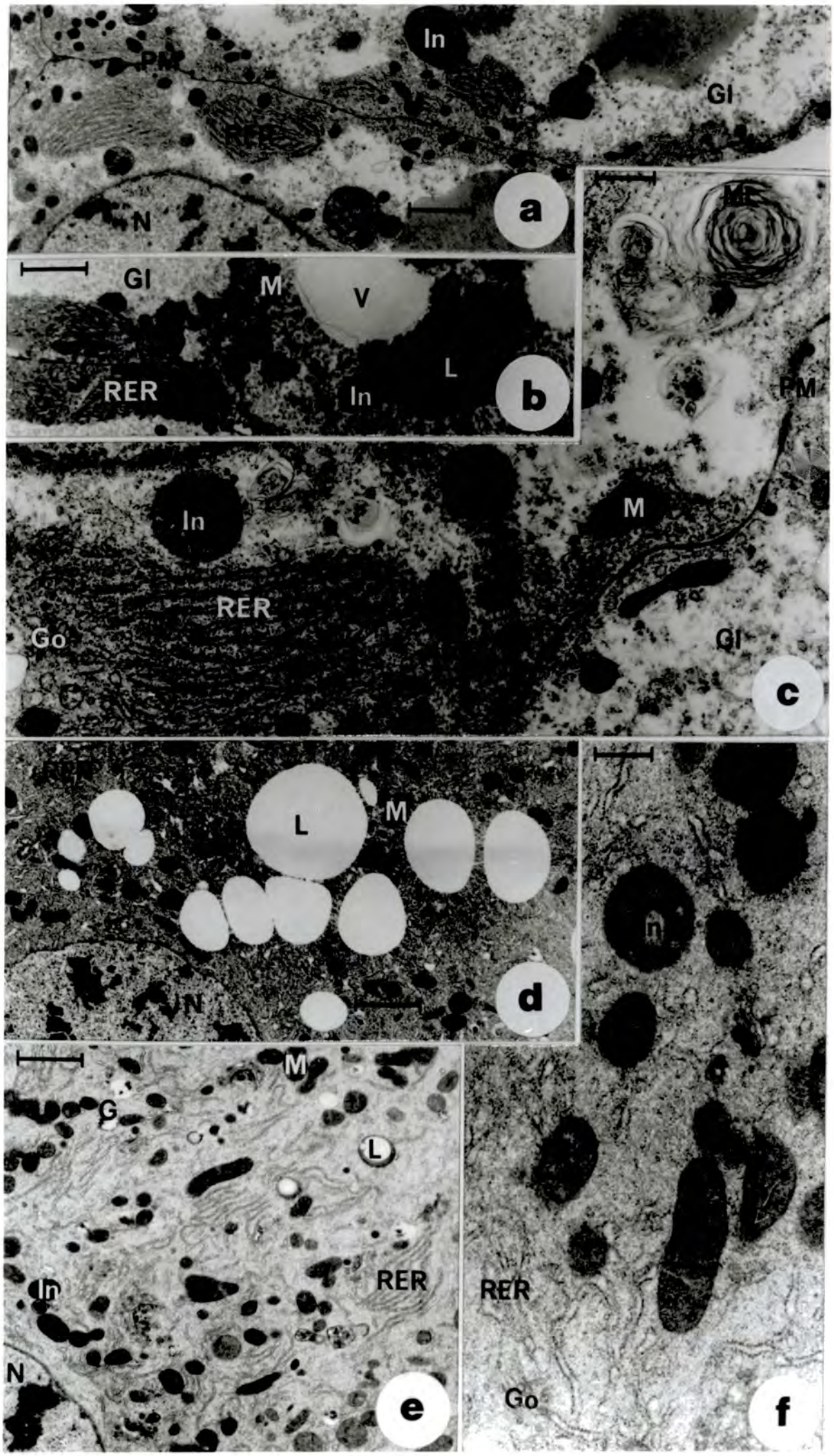

In
PM
Gl
RER
N
a
Gl
M
V
L
RER
In
b
ME
PM
M
In
RER
Go
Gl
c
L
M
N
d
n
M
G
L
RER
In
N
e
RER
Go
f

**Plate 4.14 — Electron Micrographs of Methoprene-Treated Fat Body, Day 8 of the Adult Stadium**

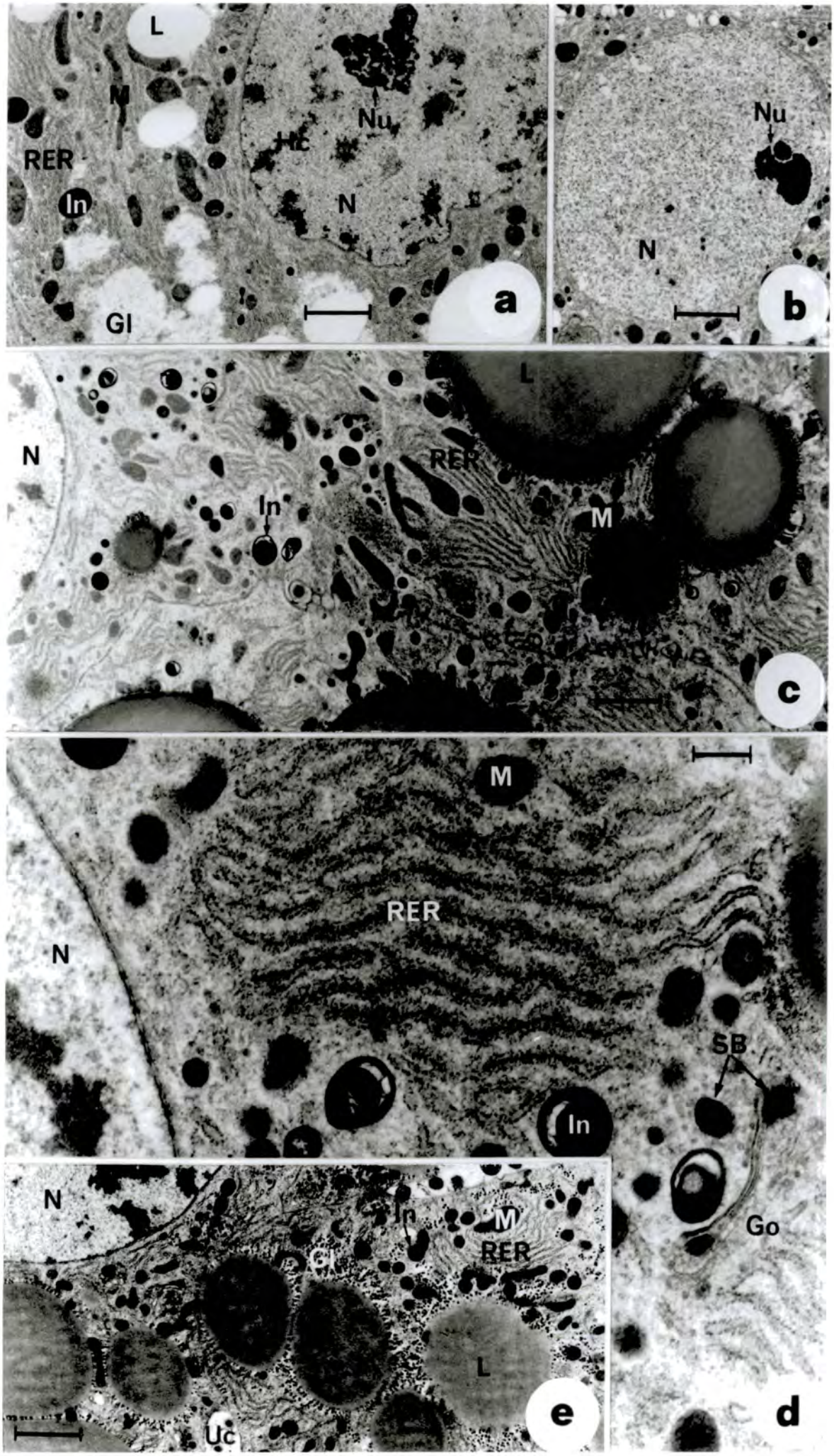
**a** & **b**. Trophocytes from 8-day old, adult female locusts previously treated with 475 $\mu$g of methoprene. Note the presence of moderate deposits of lipid and glycogen. Also note the presence of numerous mitochondria and inclusion bodies in the cytoplasm, and the abundant nature of the RER. In most cells, the nuclei contained large, multilobed nucleoli and small, scattered bodies of heterochromatin (**a**). However, in a number of nuclei, heterochromatin seemed to be completely lacking (**b**). Scale = 2 $\mu$m.

**c**. Trophocytes from 8-day old, adult female locusts previously treated with 95 $\mu$g of methoprene. Note the similarity in the appearance of the cell cytoplasm with that of 475 $\mu$g-treated tissue (**a**), there being numerous mitochondria and inclusion bodies, and extensive RER. Also note the slightly more extensive lipid deposits in the 95 $\mu$g-treated fat body. Scale = 2 $\mu$m.

**d**. Detail of cytoplasm from micrograph c. Note the slightly swollen nature of the RER. Also note the very large size of the Golgi complexes and their electron-dense secretory bodies. Scale = 0.5 $\mu$m.

**e**. Trophocytes from 8-day old, adult male locusts previously treated with 475 $\mu$g of methoprene. Note the presence of abundant lipid and glycogen stores occupying most of the cells' volume. The remaining cytoplasm contained numerous mitochondria and extensive RER, and seemed to be slightly more substantial than that in male control fat body of the same age (see Plate 4.10, **e**). Scale = 2 $\mu$m.

Key: Gl– glycogen; Go– Golgi complex; Hc– heterochromatin; In– inclusion body; L– lipid droplet; M– mitochondria; N– nucleus; Nu– nucleolus; RER– rough endoplasmic reticulum; SB– secretory body; Uc– urocyte.


L
M
RER
In
Nu
Hc
N
Gl
a
Nu
N
b
L
N
RER
In
M
c
M
RER
N
SB
In
Go
d
N
In
M
RER
Gl
L
e
Uc

age (see Plate 4.8). Fat body treated with 475 $\mu$g of methoprene was distinguished by the large size of its inclusion bodies and the presence of occasional multilamellar bodies, composed of numerous whorled membranes (Plate 4.13, **a** & **c**). Fat bodies from newly moulted adult locusts contained very little stored lipid and no deposits of glycogen (Plate 4.13, **d** & **e**), less reserve material than was seen in control tissue of the same age (see Plate 4.9). Methoprene-treated fat bodies were also distinguished by the lack of granular bodies in the cytoplasm, very few in 95 $\mu$g-treated tissue (Plate 4.13, **e**) and none at all in 475 $\mu$g-treated cells (Plate 4.13, **d**). The presence of abundant, slightly swollen RER and numerous Golgi complexes in the cytoplasm (Plate 4.13, **f**) suggested that fat bodies from 1-day old, methoprene-treated adults were synthetically active. The fat bodies from 8-day old, treated adults displayed a sexual dimorphism in their ultrastructure (Plate 4.14) which was similar to that found in control animals of the same age (see Plate 4.10). Trophocytes from 475 $\mu$g-treated female insects contained little reserve material (Plate 4.14, **a**), while lipid stores were slightly more abundant in tissue from 95 $\mu$g-treated females (Plate 4.14, **c**). However, in both cases, cell cytoplasm was very extensive. Trophocyte nuclei from animals dosed with 475 $\mu$g of methoprene usually contained large, multilobed nucleoli and small, scattered bodies of heterochromatin (Plate 4.14, **a**), but several nuclei appeared to be completely devoid of heterochromatic material (Plate 4.14, **b**). The methoprene-treated fat bodies showed signs of greater synthetic activity than that of tissue from control females of the same age (see Plate 4.10) since RER was slightly more extensive and swollen, and Golgi complexes were extremely large and active, producing large, electron-dense secretory bodies (Plate 4.14, **d**). Fat body cells from male animals were mainly taken up by lipid and glycogen stores (Plate 4.14, **e**). However, the residual cytoplasm, containing numerous mitochondria and extensive RER, seemed more substantial than that in male control fat body of the same age (see Plate 4.10, **e**).

## 4.4 Discussion

During the present study, fat bodies of control insects seemed to follow the same pattern of development during the last larval instar that has been described in other insect species (see Introduction for details). In newly moulted larval locusts, trophocytes were small and mainly appeared to be metabolically

quiescent. The cells rapidly increased in size, and by Day 4 were adapted for intense synthetic activity, with extensive RER and numerous mitochondria and golgi complexes. The cells progressively accumulated reserve material during the instar so that, by Day 10, they were filled with stored lipid and carbohydrate. At the same time, RER and mitochondria were greatly diminished in extent, while numerous inclusion bodies arose. In the adult stadium, the 8-day old locusts of the present study showed a marked sexual dimorphism in the fine structure of their fat bodies, similar to that reported by other workers in adult locusts (see Introduction). Male fat body cells were predominantly concerned with the storage of reserve material whereas female trophocytes seemed to be adapted for the high levels of synthetic activity required for vitellogenesis, having extensive RER, active golgi complexes and many mitochondria. However, the fat bodies from newly emerged adults in the present study seemed to be unusual in containing relatively little stored material. In the literature, fat body from newly moulted adult locust is usually described as being filled with lipid and glycogen deposits so that the cell nuclei are indented by lipid droplets (COUBLE *et al.*, 1979; IRVINE and BRASCH, 1981; JENSEN and BRASCH, 1985). These differences might be due to discrepancies in the insects' diets since, unlike those kept in the present study, the locusts maintained by the above authors were given a mixture of bran, skimmed milk and brewer's yeast in addition to daily supplies of grass or wheat seedlings (CHEN *et al.*, 1978). Such dietary supplements might allow larger amounts of reserve material to be stored during the final moult than would be possible if fed on grass alone as was done in the present study (see General Materials and Methods).

Numerous granular bodies were found in trophocytes from newly moulted control locusts of the present study, both fifth instar and adult insects. Whether these bodies were present during the rest of the stadia is unknown, but they were certainly far more scarce. In view of their structure, these bodies were probably peroxisomes. These are vacuoles containing various oxidases, usually with catalase, which are bounded by a single unit membrane (see reviews by LOCKE, 1984, 1985). CASSIER and FAIN-MAUREL (1972) identified peroxisomes in oenocytes of *Locusta migratoria* and these were very similar in appearance to the granular bodies of the present study. In the fat body of *Calpodes ethlius*, peroxisomes were formed at the beginning of the fourth instar and were destroyed after ecdysis to

the fifth (final) larval instar when a new population arose which was lost prior to pupation (LOCKE and MCMAHON, 1971). Thus, the peroxisomes seemed to 'develop only for use in a particular stadium and undergo repeating cycles of formation and destruction' (LOCKE, 1984). The organelles also became progressively smaller during the phase of intermoult synthetic activity which, together with the growing size of the trophocytes, caused them to become increasingly scarce during each stadium (LOCKE and MCMAHON, 1971). If a similar pattern of development occurs in locust fat body, this would explain the present observation of numerous granular bodies only in tissue from newly moulted insects. In methoprene-treated insects, the numbers of granular bodies in trophocytes from 1-day old adults were greatly reduced, there being very few in tissue from insects dosed with 95 $\mu$g of methoprene while none were seen in the fat bodies of 475 $\mu$g-treated insects. Unfortunately, very little work has been done on the function of peroxisomes in insect cells (see review by LOCKE, 1985), so the significance of their non-appearance cannot be assessed.

In the present study, locust fat body cells contained numbers of inclusion bodies which, in their structure and in the timing of their greatest abundance (the end of the fifth instar), were similar to the 'proteinaceous spheres' described in earlier studies of insect fat body (see Introduction for details). These were thought to be autophagic vacuoles formed by the digestion of RER and mitochondria towards the end of a larval instar (see reviews by PRICE, 1973; LOCKE, 1985). The multilamellar nature of many of the inclusion bodies found in the present study was akin to that of autophagic vacuoles seen in the fat body of *Calpodes ethlius* (LOCKE and SYKES, 1975), and in the reviews of lysosome structure by DE DUVE and WATTIAUX (1966) and PITT (1975). Similar structures were also found in fat bodies of adult female *Locusta* during the post-vitellogenic, lysosomal phase (LAUVERJAT, 1977; COUBLE *et al.*, 1979). However, inclusion bodies were seen in locust fat bodies at every age that was examined in the present study, which suggests either that many of the structures labelled as inclusion bodies had different functions to that proposed above, or that the timing of autophagic activity in locust fat body is less closely regulated than that which seems to be the case in holometabolous insects (see DEAN *et al.*, 1985; LOCKE, 1985). The inclusion bodies seen in methoprene-treated fat bodies in the present study were similar to those seen in control insects. However, on Day 10 of the fifth instar, tropho-

cytes from locusts treated with 475 $\mu$g of methoprene also contained a number of multilamellar bodies (Plate 4.13). Their arrangement of whorled membranes was very similar to that of the residual bodies described by PITT (1975). These are post-lytic structures which have little or no hydrolase activity and contain the indigestible remains of autophagy. The multilamellar bodies of the present study lacked the electron-dense matrix that was typical of many of the inclusion bodies (presumably autophagic vacuoles). This may be indicative of a lack of hydrolytic enzymes since LOCKE and SYKES (1975) found that in sections of fat body from *Calpodes ethlius* prepared for electron microscopy, the phosphatase enzymes of autolytic bodies would react with lead stains (such as lead citrate as used in the present study) to form electron-dense precipitates. Large numbers of residual bodies may accumulate in tissues where there is an abnormality in lysosome metabolism preventing the normal degradation of material (see reviews by DE DUVE and WATTIAUX, 1966; PITT, 1975), and there is some evidence that juvenoids can disrupt hydrolytic activity in insect fat bodies. SASS and KOVÁCS (1977, 1980) found that treatment of larval *Mamestra brassicae* with ecdysone accelerated the appearance of autophagic bodies within the fat body, but that the application of a JHA to young, last instar larvae prevented later induction of autophagy by ecdysone. Also, in the penultimate larval instar of *Mamestra*, fat body showed no autophagic response to exogenous ecdysone until 24 hours before ecdysis, the time at which the previously high haemolymph titre of JH declined (SASS and KOVÁCS, 1977). In pupal *Spodoptera mauritia*, MATHAI and NAIR (1984) measured the levels of acid and alkaline phosphatases, which can be used as cytochemical markers for hydrolytic activity (LOCKE and SYKES, 1975; LARSEN, 1976; DINGLE, 1977). They reported a significant reduction in the enzymes' activities following topical application of the JHA hydroprene to newly moulted pupae. Thus, the present detection of multilamellar bodies in fat bodies from locusts treated with 475 $\mu$g of methoprene at the time of greatest hydrolytic activity may be an indication of disrupted lysosome metabolism in the tissue. However, WALKER (1965) found 'myelin bodies' in the fat body of *Philosamia cynthia* towards the end of the last larval instar which were similar in form to the multilamellar bodies of the present study. Therefore, the appearance of such structures in locust trophocytes could be a normal occurrence at the end of the last larval instar. Their presence only in tissue from methoprene-treated

animals on Day 10 of the fifth instar might simply be due to these insects being at a slightly more advanced stage of development since it was seen in Chapter 3 that treatment with 475 $\mu$g of methoprene shortened the length of the fifth instar and induced early moulting.

The present study of fat body histology showed that the application of 475 $\mu$g of methoprene leads to a reduction in the vacuolation of trophocytes at most of the ages that were examined, suggesting a decrease in the amount of reserve material accumulated by the fat body. This was corroborated by ultrastructural analysis of the tissue which generally revealed lower levels of stored lipid and glycogen in the fat body cells of methoprene-treated animals. Such a result would be compatible with the reports of LAUVERJAT (1977) and COUBLE *et al.* (1979) who found that allatectomy of adult *Locusta* resulted in abnormal accumulations of lipid and glycogen in fat body cells. Numerous other studies have also demonstrated excessive levels of fat body lipid following allatectomy (see reviews by STEELE, 1976, 1985; BEENAKKERS *et al.*, 1981), while HILL and IZATT (1974) reported that implantation of extra corpora allata into intact female *Schistocerca gregaria* reduced fat body lipid to less than one third of that in normal adults by ten days after the operation. In addition, RÖSELER and RÖSELER (1988) found that topical applications of JH to newly emerged queens of *Bombus terrestris* reduced the lipid and glycogen content of their fat bodies. The present study of locust fat body showed that methoprene treatment also increased the amount of material staining for RNA and the proliferation of RER in both fifth instar and adult locusts, indicating a stimulation of RNA and protein synthesis. Several investigations have shown that protein synthesis in insects is inhibited by allatectomy and stimulated by application of JH or implantation of CA (see review by STEELE, 1976). THOMAS and NATION (1966a, 1966b) found that allatectomy of adult female *Periplaneta americana* lead to a marked reduction in the incorporation of radiolabelled amino acids into haemolymph proteins and an abnormal decrease in whole body protein levels. Similarly, the rate of incorporation of $^{14}$C-labelled protein hydrolysate into proteins of the fat body haemolymph and ovary was reduced in female *Locusta* after removal of the CA (MINKS, 1967). In allatectomised females of *Leucophaea maderae*, both implantation of CA and application of synthetic JH increased the incorporation of [$^{14}$C]leucine into serum proteins by 100% (ENGELMANN, 1971).

Implantation of CA into decapitated females of *Nauphoeta cinerea* tripled the incorporation of [$^{14}$C]alanine into fat body proteins (LÜSCHER, 1968) and the injection of JH into fifth instar *Oncopeltus fasciatus* doubled [$^{3}$C]leucine incorporation (BASSI and FEIR, 1971). Under the light microscope, it was seen that the response to methoprene treatment in the present study was more extreme in fat bodies from female insects than in male tissue. This would suggest that the application of methoprene stimulated the production of female-specific material. In view of the role of JH in the manufacture of yolk protein precursors by female fat body (see Introduction for details), the most obvious candidate for such a methoprene-inducible product would be vitellogenin. This suggestion is consistent with the results of DHADIALLA and WYATT (1981, 1983) who caused precocious vitellogenin production in fat bodies from fifth instar *Locusta* by the prior injection of methoprene, and found that the response was greater in tissue from female animals than in fat bodies from males (see Introduction for further details). However, some of the apparent increase in fat body protein synthesis seen in the methoprene-treated locusts of the present study could have been due to enhanced production of larval proteins. GOLTZENÉ-BENTZ *et al.* (1972) reported a reduction in the rate of increase in total body protein during the fifth instar of *Locusta migratoria* following allatectomy of the insects, while PRICE (1968) found that ligaturing of third instar *Calliphora erythrocephala* to isolate the CA from the fat body accelerated the normal decline in fat body protein synthesis prior to pupation. These results suggest that JH, presumably at very low concentrations, may regulate the manufacture of larval proteins by the fat body as well as vitellogenin in adult females. Furthermore, GELLISSEN and WYATT (1981) found that inactivation of the CA (by the application of precocene) in adult *Locusta* not only prevented vitellogenin synthesis in females but also diminished the production of other fat body proteins in both sexes.

The present study showed changes in the structure of trophocyte nuclei following the application of methoprene. Under the light microscope, it was seen that methoprene treatment caused an enlargement of fat body cell nuclei, especially in tissue from female insects. In control insects, trophocyte nuclei were at their largest on Day 4 of the fifth instar and Day 8 of adult life, especially in female tissue. Since these were the times of maximal staining for RNA in the cells, it would appear that nuclear size is related to transcription rates. Thus,

the increased nuclear size of methoprene-treated fat bodies may be another indication of heightened RNA synthesis. However, nuclear enlargement may also be indicative of increased ploidy levels in the fat body cells. In common with several other insect species (see reviews by NAGL, 1978; DEAN *et al.*, 1985), locust fat body cells become increasingly polyploid during the fifth instar and adult stadium. KOOMAN and NAIR (1982) found that fat body cells from female *Schistocerca gregaria* were predominantly tetraploid in the fifth (final) larval instar, but that there was a gradual increase in octaploid nuclei during the instar (from 8% to 20% of total nuclei). In the adult locust, there were further increases in fat body cell ploidy levels, from mostly tetraploid (4N) in 1-day old and 3-day old adults, to mainly 8N nuclei on the fifth day, and a marked rise in the number of 16N nuclei by Day 8 (KOOMAN and NAIR, 1982). Fat body cells of *Locusta migratoria* also became increasingly polyploid during adult life (IRVINE and BRASCH, 1981; NAIR *et al.*, 1981). In female *Locusta*, trophocytes were chiefly 4N, 8N and 16N on the first, seventh and fifteenth days of adult life, respectively. Male fat body was also predominantly 4N on Day 1 but underwent less replication to become mainly octaploid by Day 15. As the trophocytes increased in ploidy levels, IRVINE and BRASCH (1981) noted a corresponding increase in nuclear size, while KOOMAN and NAIR (1982) found that the total area of nuclei in locust fat body squashes increased linearly with ploidy level. Also, the normal polyploidisation of adult fat body cells was shown to be JH dependent since it was prevented by allatectomy but could be restored by injection or topical application of methoprene (IRVINE and BRASCH, 1981; NAIR *et al.*, 1981). The present observation of enlarged trophocyte nuclei in methoprene-treated locusts may therefore be an indication of increased polyploidisation. Since growth by DNA replication and cell enlargement to form polyploid cells is especially common in tissues that are adapted for the rapid synthesis of large amounts of particular proteins (BRODSKY and URYVAEVA, 1977; NAGL, 1978), increased ploidy levels in methoprene-treated trophocytes may be another indication of stimulated protein syntheses. Also, the greater size, and possibly ploidy level, of fat body cell nuclei in female methoprene-treated locusts compared with males is similar to the situation normally found in adult locusts (see above), suggesting that methoprene treatment may be causing precocious nuclear development. Further support for this hypothesis may come from the observation of nuclear bodies

within trophocytes from methoprene-treated, fifth instar locusts of the present study (see Plate 4.12, **f**). These structures were very similar in appearance to the nuclear bodies described by JENSEN and BRASCH (1985) in fat body nuclei of adult *Locusta*, which were seen to increase in number during sexual maturation. Their normal proliferation was prevented by allatectomy and restored by the application of methoprene, indicating a dependence on the presence of JH or its analogues (JENSEN and BRASCH, 1985). Such premature nuclear development would lend credence to the suggestion made above that methoprene treatment stimulated vitellogenesis in fifth instar, female locusts.

The present investigation also occasionally revealed further abnormalities in fat body structure as a result of methoprene treatment. In the histological study, one of the fat bodies taken from treated females on Day 7 of the fifth instar was seen to contain mitotic bodies (see Plate 4.2, **e**). Since fat body cells are thought to undergo mitotic division only at the time of moulting (see reviews by LOCKE, 1980; DEAN *et al.*, 1985), it would appear that this particular insect was about to moult to the adult. This would agree with the lack of cytoplasmic vacuolation in the trophocytes since the present study showed that stored material was lost from the fat body at the time of metamorphosis. However, in Chapter 3 it was seen that, although the application of 475 $\mu$g of methoprene to 1-day old fifth instar locusts shortened the length of the instar and induced early moulting, less than 5% of such treated insects had moulted to adults by six days after treatment. An alternative explanation for the presence of the mitotic bodies could be that the cells were undergoing endomitosis to attain a higher ploidy level, as was discussed earlier. According to BRODSKY and URYVAEVA (1977), however, endomitosis in insect cells is characterised by the retention of an intact nuclear membrane throughout the process of DNA replication. In the present study, no nuclear membranes were visible around the mitotic bodies. Under the electron microscope, it was seen that the application of 475 $\mu$g of methoprene leads to a noticeable, and occasionally extremely marked, reduction in the amount of heterochromatin present in trophocyte nuclei of 8-day old adults. Heterochromatin is considered to be transcriptionally-inactive DNA condensed with protein (KRSTIĆ, 1979; DEAN *et al.*, 1985) so that the apparent lack of heterochromatin could be the result of extremely high rates of RNA synthesis. However, the histochemical analysis of comparable fat body tissue did not reveal the exceptional

levels of RNA which would be expected. There have been so few attempts to correlate nuclear ultrastructure with function that no firm conclusion can be drawn from the appearance of these nuclei other than to state their abnormality.

# Chapter V

# Effects of Methoprene upon Lipid, Carbohydrate and Protein Metabolism

## 5.1 Introduction

The fat body is the principal storage site of lipid and carbohydrate (see previous chapter for details), which are mainly deposited as triglyceride (triacylglycerol) and glycogen, respectively (see review by KEELEY, 1985). In the haemolymph, carbohydrate is chiefly transported as the non-reducing disaccharide trehalose (STEELE, 1985; FRIEDMAN, 1985), while diglyceride (diacylglycerol) is the major lipid component, most of this being bound to the carrier protein lipophorin, although a large proportion may be taken up by vitellogenin in reproductive females (STEELE, 1985; DOWNER, 1985). The exact pathway by which triglyceride is broken down to diglyceride has not yet been established (see review by FRIEDMAN, 1985). Glycogen is broken down to free or phosphorylated glucose before being either further metabolised or converted to trehalose for transport. The rate-limiting enzyme controlling glycogen breakdown is glycogen phosphorylase which cleaves the terminal glucosyl residue from the parent glycogen according to the following scheme:

$$\text{Glycogen(Glucose}_n\text{)} + \text{Pi} \rightleftharpoons \text{Glycogen}_{(n-1)} + \text{Glucose-1-P.}$$

Although the reaction is reversible, in practice it is unidirectional towards glucose-1-P synthesis because of the high concentration of inorganic phosphate in the cell (see review of insect phosphorylase by STEELE, 1982). Glycogen phosphorylase occurs in two interconvertible forms within the same cell, one active ($a$) and the other inactive ($b$). In fact, phosphorylase $b$ can be made to show full activity *in vitro* by the addition of AMP to the assay medium. However, the activity of the $b$ form *in vivo* is negligible and the physiological activity of phosphorylase is dependent on the absolute amount of the $a$ form.

As mentioned in the previous chapter, HILL and IZATT (1974) found that the implantation of extra corpora allata into adult female *Schistocerca gregaria*

reduced the levels of fat body lipid, while topical applications of JH to newly moulted female *Bombus terrestris* reduced both the lipid and glycogen content of their fat bodies (RÖSELER and RÖSELER, 1988). Also, numerous studies have shown excessive accumulations of lipid in the fat bodies of allatectomised insects (see reviews by STEELE, 1976, 1985; BEENAKKERS *et al.*, 1981). For example, BAILEY *et al.* (1975) found that the average lipid content of fat body from male *Locusta migratoria* rose from 30.2 mg to 68.9 mg by 25 days after allatectomy, while lipid levels rose from 46.1 mg to 69.5 mg in female locusts. Similar elevations in fat body lipid stores have been reported for allatectomised *Schistocerca*, both male (WALKER and BAILEY, 1971b) and female (HILL and IZATT, 1974), and the operation increased total body lipid in *Melanoplus differentialis* (PFEIFFER, 1945), *Locusta* (STRONG, 1968a, 1968b) and *Schistocerca* (ODHIAMBO, 1966). MINKS (1967) also found increased levels of fat body lipid by 20 days after allatectomy of adult female *Locusta* but found no extra accumulation of fat deposits in operated males. The effect is not due to injury from the operation since reimplantation of corpora allata can restore elevated fat body lipid deposits to normal levels (ODHIAMBO, 1966; BEENAKKERS, 1969; HILL and IZATT, 1974). In addition, allatectomy has been shown to affect carbohydrate storage in the fat body, causing increased glycogen accumulation in fat bodies of both male and female *Locusta* (MINKS, 1967), and in females of various dipterans (see review by STEELE, 1976). Similar results were found with whole body extracts from larval and adult *Carausius morosus* (L'HELIAS, 1953) and adult female *Pyrrhocoris apterus* (JANDA and SLÁMA, 1965). The only exceptions to this pattern that have been reported are adult female *Aedes taeniorhyncus* (VAN HANDEL and LEA, 1970) and adult male *Blaberus discoidalis* (MANNIX and KEELEY, 1980). In both cases, allatectomy was reported to have no effect on the levels of fat body lipid and glycogen. However, VAN HANDEL and LEA (1970) only examined the mosquitoes for three days following the operation, which may have been insufficient time to see any effects. Furthermore, in pupal *Aedes aegypti*, DOWNER *et al.* (1975) reported a substantial decrease in lipid and glycogen levels following the application of methoprene. BUTTERWORTH and BODENSTEIN (1969) found that implantation of CA or injection of synthetic JH into adult male *Drosophila melanogaster* increased the lipid and glycogen content of the animals' fat bodies, opposite to the expected result. Nevertheless,

allatectomy of adult female *Drosophila* resulted in the accumulation of excess fat body lipid (VOGT, 1949), and in sterile mutants of *Drosophila* CA inactivity was found to be accompanied by fat body hypertrophy (DOANE, 1961). Thus, it would appear that accumulation of lipid and carbohydrate in the fat body is increased by low JH levels and reduced by high titres of juvenile hormone, and the ultrastructural examination described in Chapter 4 suggests that methoprene treatment may have had a comparable effect upon locust fat body in the present study.

The JH-dependent changes in fat body lipid and carbohydrate levels reported above must be due to changes in either the synthetic and/or metabolic rates of the reserve materials. The effect does not seem to be due to changes in food consumption since allatectomy has no effect on the feeding activity of *Locusta* (STRONG, 1968a; BEENAKKERS and VAN DEN BROEK, 1974) and *Schistocerca* (ODHIAMBO, 1966; HILL and IZATT, 1974). Observations that allatectomised locusts still showed normal hyperlipaemia following flight activity (LEE and GOLDSWORTHY, 1975, 1976) or the injection of adipokinetic hormone (GOLDSWORTHY *et al.*, 1972; MWANGI and GOLDSWORTHY, 1980) indicate that lipid mobilisation is not impaired. This conclusion is further supported by the reports of haemolymph lipid concentrations remaining unchanged after removal of CA from *Locusta* (BEENAKKERS, 1969; BAILEY *et al.*, 1975) and *Schistocerca* (WALKER and BAILEY, 1971b; HILL and IZATT, 1974). There is evidence that JH levels may affect the *de novo* synthesis of fat body lipid. VROMAN *et al.* (1965) measured the incorporation of radiolabelled acetate into total body lipid of adult female *Periplaneta americana* and demonstrated that allatectomy significantly increased the incorporation into both fatty acids and triglyceride. Allatectomy also increased incorporation of [$^{14}$C]acetate into total fat body lipid of adult *Schistocerca gregaria* (WALKER and BAILEY, 1971a; HILL and IZATT, 1974), and increased the activities of a number of fat body enzymes which are involved in lipogenesis (WALKER and BAILEY, 1971c). Furthermore, GILBERT (1967) showed that *in vitro* additions of CA extract to fat body from *Leucophaea maderae* inhibited the incorporation of [$^{14}$C]palmitate into lipids. Less work has been done with regard to the effects of JH upon carbohydrate metabolism. RÖSELER and RÖSELER (1988) found that application of JH to newly moulted queens of *Bombus terrestris* resulted in a lower fat body glycogen content 4 days later and a reduction

in the incorporation of [$^{14}$C]UDP-glucose into glycogen, indicating a lower specific activity of glycogen synthase. WRIGHT and RUSHING (1973) reported that the utilisation of fat body glycogen in pupal *Stomoxys calcitrans* was inhibited by the application of a JHA, but glycogen phosphorylase activity was found to be unaffected by the treatment (WRIGHT *et al.*, 1975). In contrast, exposure of newly moulted, last-instar larvae of *Aedes aegypti* to methoprene reduced the level of fat body glycogen phosphorylase, both active and inactive, in 3-day old larvae, while in pupae the ratio of active to inactive phosphorylase was lowered (GORDON and BURFORD, 1984).

STEELE (1976) proposed that the excess accumulation of lipid and carbohydrate in the fat bodies of allatectomised insects was possibly a secondary effect resulting from reduced metabolic demand due to inhibition of protein synthesis in the fat body. Several investigations have shown that protein synthesis in insects is decreased by allatectomy and increased by injection of JH or implantation of active corpora allata (for details, see Chapter 4 and STEELE, 1976). The majority of these experiments were performed on adult female insects and involved the production of a female-specific protein which was considered to be the yolk protein precursor, vitellogenin (STEELE, 1976). The cytological study of fat body structure described in Chapter 4 indicated that treatment with methoprene also stimulated the synthesis of fat body proteins, with tissue from female animals showing greater stimulation. Vitellogenin (Vg) is not normally stored in fat body but is immediately released into haemolymph and carried to the ovaries where it is taken up by developing oocytes (see review by KEELEY, 1985). In view of this, it would seem most likely to find further evidence for stimulation of Vg synthesis in the haemolymph of methoprene-treated locusts rather than in the fat body. A large body of information on insect haemolymph proteins has built up (see reviews by WYATT and PAN, 1978; TOMINO, 1985) and certain generalities can be made which are also applicable to *Locusta* (e.g. MINKS, 1967; TOBE and LOUGHTON, 1967). In immature insects, the concentration of total haemolymph protein undergoes cyclical fluctuations, increasing steadily during each larval instar, falling dramatically at the time of the moult and then rising again in the next instar. In adults, blood protein concentrations increase until the animals reach sexual maturity, at which point protein concentration tends to remain constant in males, while in females it exhibits periodic changes which

coincide with the reproductive cycles. Usually only a few major proteins are present in the haemolymph, although the number of proteins that can be separated varies with the species and the sensitivity of the detection method. The protein pattern may alter both qualitatively and quantitatively depending on the animal's stage of development and, in some cases, its sex. Relatively little work has been done to determine developmental changes in the blood protein patterns of *Locusta* and the various attempts to separate haemolymph proteins by gel electrophoresis have yielded very different results, with between 7 and 19 protein bands being found (DUKE, 1966; MCCORMICK and SCOTT, 1966; MINKS, 1967; TOBE and LOUGHTON, 1967; BENTZ, 1969; BENTZ *et al.*, 1970; EMMERICH and HARTMANN, 1973; PELED and TIETZ, 1973; TURNER and LOUGHTON, 1975; WHEELER and GOLDSWORTHY, 1983). Some of the reports on locust blood proteins suggested that there was little qualitative difference between the protein patterns of fifth instar animals and adults, and between males and females (DUKE, 1966; TOBE and LOUGHTON, 1967; BENTZ *et al.*, 1970). In contrast, MINKS (1967) found an extra protein band in the haemolymph of mature adult females. A similar sex-specific protein band was seen to arise in the blood of adult female locusts during sexual maturation by EMMERICH and HARTMANN (1973). Such female-specific proteins have been detected in many other insect species and have been identified as vitellogenins (see reviews by WYATT and PAN, 1978; ENGELMANN, 1979). In *Locusta*, vitellogenin normally first appears in the haemolymph of adult females seven or eight days after the final moult (GELLISSEN and EMMERICH, 1978) but, except for Vg, the blood protein patterns of male and female locusts appear to be similar (MINKS, 1967; EMMERICH and HARTMANN, 1973).

Apart from vitellogenin, there have been very few reports on the effects of JH upon individual haemolymph proteins. However, there is some evidence that JH and its analogues may affect esterase activity in insect haemolymph. Two classes of esterase can be detected in the haemolymph and they are thought to play a major role in JH metabolism since the main pathway for JH degradation seems to be via ester hydrolysis (see reviews by DE KORT and GRANGER, 1981; HAMMOCK, 1985). The major group of non-specific carboxylesterases readily hydrolyses free juvenile hormone but cannot hydrolyse JH which is bound to its carrier protein, whereas the smaller class of JH-specific esterases efficiently

metabolises both free and protein-bound JH. In *Trichoplusia ni*, the application of JH-active compounds late in the final larval instar lead to an increase in the normal prepupal esterase peak, whereas allatectomy caused a decrease in esterase activity (SPARKS and HAMMOCK, 1979; JONES and HAMMOCK, 1983; SPARKS, 1984). Similar results have been reported with late last instar larvae and pupae of other Lepidopteran species such as *Manduca sexta* (SPARKS *et al.*, 1983), *Galleria mellonella* (REDDY *et al.*, 1979; MCCALEB and KUMARAN, 1980) and *Hyalophora cecropia* (WHITMORE *et al.*, 1972). Among other insect orders, ROTIN *et al.* (1982) found that allatectomy of adult females of the viviparous cockroach *Diploptera punctata* caused a decline in JH esterase activity and that this was restored in a dose-dependent manner by the application of the JH analogue hydroprene. Similarly, the application of JH I and three JH analogues to Colorado beetle increased JH-specific esterase activity in diapausing adults (KRAMER, 1978). A number of exceptions to this pattern have been found, however, and treatment with methoprene had no effect on esterase levels in adult *Trichoplusia ni* (VENKATESH *et al.*, 1988). The application of JH to young last instar larvae of *Galleria mellonella* actually reduced esterase activity, opposite to the result found with prepupal larvae (REDDY *et al.*, 1979). Both allatectomy and treatment with JH-active compounds decreased the naturally high esterase levels of adult Colorado beetles reared in a short daylength regime (KRAMER, 1978), and DOWNER *et al.* (1975) reported that the levels of non-specific esterases in pupal *Aedes aegypti* were reduced by prior treatment of larvae with methoprene.

The purpose of the experiments described in the present chapter was to clarify the effects of methoprene treatment upon lipid, carbohydrate and protein metabolism which were suggested by the results of Chapter 4.

## 5.2 Materials and Methods

The experimental animals used in this study were topically treated with 475 $\mu$g of methoprene in the manner described in General Materials and Methods. Unless otherwise stated, they were also subsequently maintained and sampled according to the procedures in General Materials and Methods. All reagents used were Anala R grade or purest available.

### 5.2.1 Extraction of Lipid and Carbohydrate

Haemolymph was collected from animals by the method described by STERNBURG and CORRIGAN (1959). A small hole was cut in the vertex of the insect's head and the exposed air sacs and brain were removed. The tarsi were cut from each hind leg to create an 'open system' through the animal. The insect was fixed over a centrifuge tube with plasticine so that the hole in the vertex was directly over the top of the tube. The insect's mouth was shielded from the tube with cotton wool to prevent contamination of the haemolymph by regurgitated gut contents. Animals so prepared were placed in a MSE 'Centaur 2' bench centrifuge and spun at 500 r.p.m. for 5 minutes. The haemolymph was thrown down as a clear serum with most of the haemocytes massed at the bottom of the tube. A Gilson micro-pipette was used to transfer the haemolymph to a glass homogenisation tube. To remove the animal's fat body, the posterior tip of the abdomen was cut off and the head, with the gut attached, was pulled away. The carcase was cut open ventrally and all readily visible fat body was taken from the thorax and abdomen. This was gently blotted on tissue paper before being weighed.

Lipid and carbohydrate were extracted using the method described by VAN HANDEL (1965). Tissue from equal numbers of male and female animals was pooled to give 250 $\mu$l of haemolymph and 30 mg of fat body. Immediately after removal from the insect, each tissue was placed in a glass homogenisation tube containing 4 ml of ice-cold chloroform/methanol (1:1 v/v) with two drops of saturated sodium sulphate. This was homogenised for 2 minutes with a Teflon pestle rotating at 3,000 r.p.m. on a Tri-R 'Stir-R' homogeniser. The homogenate was centrifuged for 10 minutes at 1,000 r.p.m. in a MSE 'Coolspin' centrifuge at 0–4°C and the supernatant was decanted into a boiling tube. The residual pellet was resuspended in 4 ml of ice-cold chloroform/methanol (1:1) and then homogenised and centrifuged as before. The supernatants were pooled and, after the addition of 4 ml of chloroform and 2.75 ml of distilled water, were mixed on a Fisons 'Whirlimix' vortex mixer. The aqueous and lipid phases were separated by centrifugation at 1,500 r.p.m. for 1 hour in a 'Coolspin' centrifuge at 0–4°C. The aqueous phase was removed to a separate tube with a Pasteur pipette and then 4 ml of chloroform and 2.75 ml of distilled water were added to the organic phase which was mixed and centrifuged as before. The aqueous phase was again

removed and pooled with the previous one. The washed lipid extract was then placed in a Techne 'DG-1 Block Digestor' set at 40°C and evaporated to dryness by passing pure, dry nitrogen over it. The lipid extract was redissolved in 2 ml of chloroform and was sealed in a glass vial under a nitrogen atmosphere. The vials were stored in the dark at −20°C until required.

The pellet which had been formed earlier was resuspended in 4 ml of 66% ethanol saturated with sodium sulphate. This suspension was homogenised in a glass homogenisation tube for 2 minutes with a Teflon pestle rotating at 2,000 r.p.m., and was then centrifuged for 10 minutes at 1,000 r.p.m. in a MSE 'Centaur 2' bench centrifuge. The supernatant, containing the non-glycogen carbohydrate, was added to the aqueous washings from the lipid extraction. The pellet was resuspended in 2 ml of 66% ethanol by mixing on a vortex mixer. The suspension was centrifuged as before and the supernatant was pooled with the washings from the lipid extraction. The pellet was heated in a water bath at 100°C to drive off residual ethanol and so prevent foaming at the next step. The pellet was dissolved in 1 ml of 30% KOH and heated to 100°C for 15 minutes in a water bath. The digest was allowed to cool and 4 ml of absolute ethanol was added in order to precipitate glycogen adsorbed on the sodium sulphate. This was spun down by centrifugation at 1,000 r.p.m. for 10 minutes in a bench centrifuge. The supernatant was carefully removed with a Pasteur pipette and the glycogen was dissolved in an appropriate volume of distilled water. The carbohydrate extracts prepared above were stored at 4°C in Parafilm-sealed tubes for up to 4 days before being quantified.

### 5.2.2 Quantification of Total Lipid

The method used to quantify lipid in the tissue extracts was essentially that described by ZÖLLNER and KIRSCH (1962). It was performed on lipid extracts from fat body, as prepared above, and on haemolymph without prior extraction. Aliquots containing 30–120 $\mu$g of lipid were digested with 1 ml of concentrated sulphuric acid at 100°C for 10 minutes in a Techne 'DG-1 Block Digestor'. The tubes were cooled and 5 ml of sulphosphovanillin reagent (4 parts orthophosphoric acid to 1 part 0.6% aqueous vanillin solution) was added to the digest. After standing at room temperature for 40 minutes, the absorbance was measured at 530 nm in a LKB Biochrom 'Ultrospec 4050' spectrophotometer. The

**Figure 5.1 — Quantification and Separation of Lipid Standards**

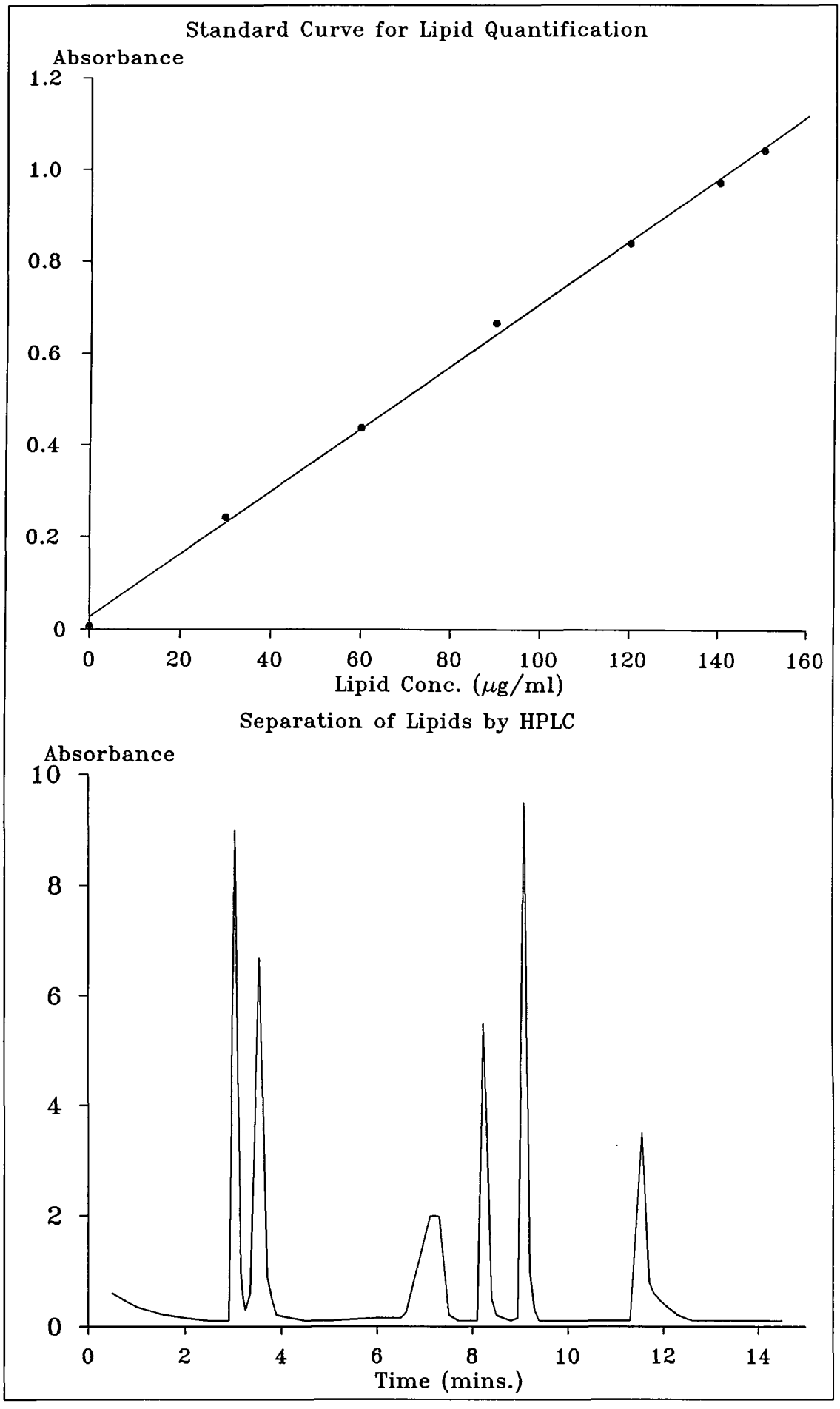
**a. Quantification of Lipid with Sulphosphovanillin Reagent**

Legend: ordinate – absorbance at 530 nm,
abscissa – lipid concentration ($\mu$g/ml).

**b. A Typical Separation of Neutral Lipids by HPLC**

Legend: ordinate – absorbance at 206 nm,
abscissa – time after injection of sample (minutes).

The retention times of the various lipid classes were as follows: cholesteryl esters, 3.03 min.; triglycerides, 3.53 min.; free fatty acids, 7.20 min.; diglycerides, 8.73 min.; cholesterol, 9.02 min.; monoglycerides, 11.55 min.


Standard Curve for Lipid Quantification
Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
Lipid Conc. (μg/ml)
Separation of Lipids by HPLC
Absorbance
10
8
6
4
2
0
0
2
4
6
8
10
12
14
Time (mins.)

reaction was linear up to 150 $\mu$g of lipid (see Figure 5.1, **a**) and a standard curve was estimated at each determination using cholesterol (Sigma Co.) as the lipid standard.

### 5.2.3 Separation and Quantification of Neutral Lipids

Initially, the lipid extracts were separated by thin layer chromatography (TLC) using the method of SKIPSKI *et al.* (1965). The solvent system consisted of petroleum ether/diethyl ether/acetic acid (80:20:1) and the separated lipid fractions were detected by means of iodine vapour (MANGOLD and MALINS, 1960). This method is fully described below. The lipid fractions were scraped into centrifuge tubes and quantified by 'charring' with concentrated sulphuric acid as described by MARSH and WEINSTEIN (1966) and KRITCHEVSKY *et al.* (1973). However, when lipid standards were processed in the above manner, highly variable results were obtained. Therefore, when high performance liquid chromatography (HPLC) became available, it was decided to use this method for all future lipid separation and quantification.

HPLC analyses were made with a Gilson apparatus (two Model 303 pumps, a Model 802c manometric module, Model 811 dynamic mixer, 'HM Holochrome' variable wavelength UV detector and a Model 702 solvent programmer software) linked to a Rheodyne sample injection valve (Model 7125) and a Trivector 'Trio' chromatographic data processor. The method used was a modification of that described by HAMILTON and COMAI (1984) and was carried out on 20 $\mu$l aliquots of the lipid extracts of fat body and haemolymph prepared above. Before analysis by HPLC, the lipid extracts were evaporated to dryness and redissolved in an appropriate volume of Solvent A (see below). The lipids were separated on a 250 $\times$ 4.6 mm stainless-steel column prepacked with 5 $\mu$m particles of 'Spherisorb ODS2' (Anachem Ltd.) and eluted with a linear gradient of hexane–propanol at a flow rate of 1.0 ml/min. Solvent A consisted of hexane/conc. $H_2SO_4$ (100:0.01 v/v) and Solvent B contained isopropanol/conc. $H_2SO_4$ (100:0.01 v/v). The solvent gradient programme used was according to the following scheme:

| Time (minutes) | 0 | 3 | 7 | 9 | 15 |
|---|---|---|---|---|---|
| Solvent A | 99.5% | 99.5% | 75% | 70% | 70% |
| Solvent B | 0.5% | 0.5% | 25% | 30% | 30% . |

To equilibrate the column, the system was run at 99.5% Solvent A for 5 min-

utes before each separation, the sample being injected at time 0. The UV detector was set at 206 nm and the lipid peaks were quantified using the integrator. This was calibrated at the start of each day with samples of standard lipids dissolved in suitable volumes of Solvent A. The absorbance of low wavelength UV by lipids arises primarily from double bonds in the molecule (JUNGALWALA *et al.*, 1976). Therefore, the standards used for calibration contained only mono-unsaturated fatty acid moieties. Initially, the standards used were cholesterol and the mono-glycerides, diglycerides, triglycerides, free fatty acids and cholesteryl esters based upon either oleic acid (18-carbon chain) or eicosenoic acid (20-carbon chain), all obtained from Sigma Co. When it was established that lipids of the same class eluted as a single peak, later calibrations were carried out with just the standards derived from oleic acid. A chromatogram of a typical separation of the lipid standards is shown in Figure 5.1 (**b**).

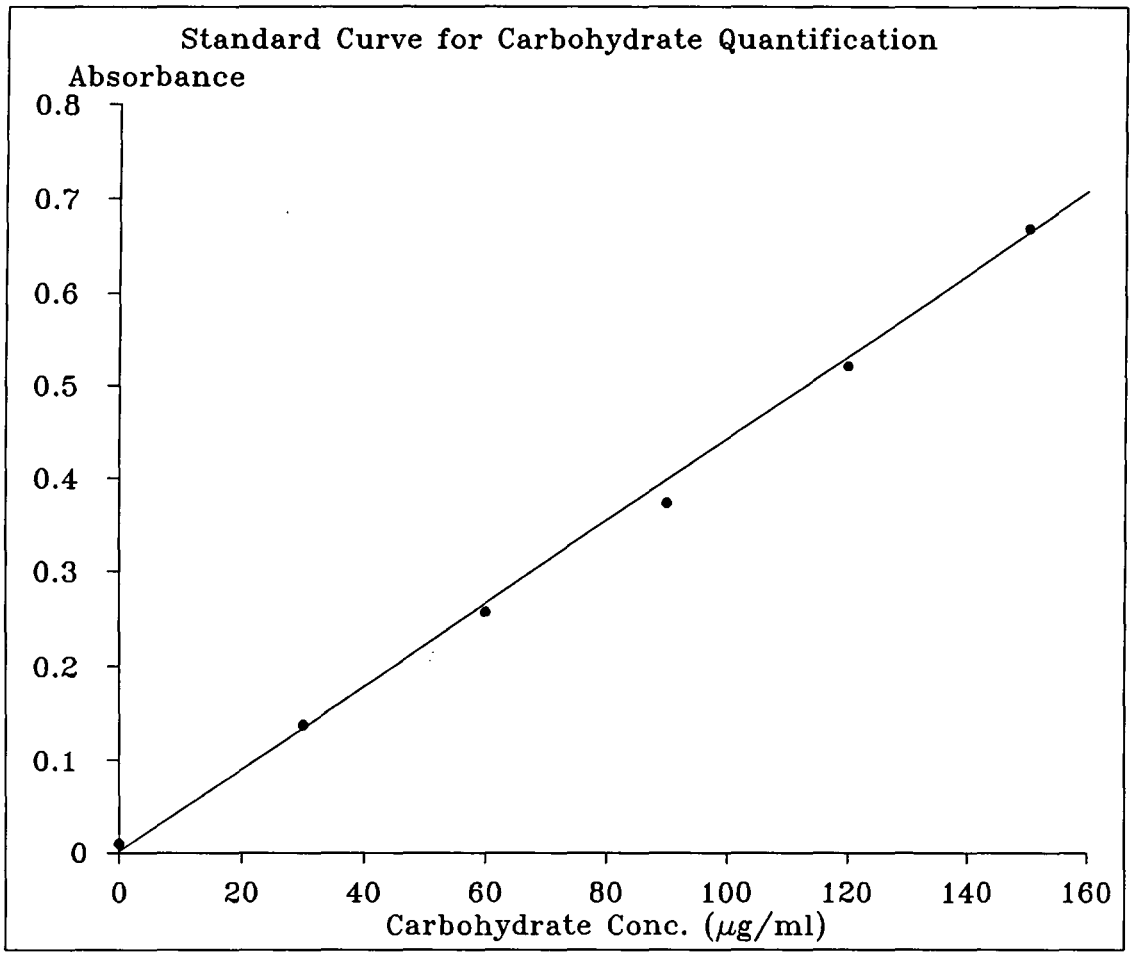
### 5.2.4 Quantification of the Carbohydrate Extracts

Carbohydrate extracts were quantified according to the method described by ROE (1955) using anthrone reagent. This was composed of 0.05% (w/v) anthrone and 1% (w/v) thiourea dissolved in 66% (v/v) sulphuric acid. Thiourea acted as an anti-oxidant to inhibit the oxidation of anthrone in $H_2SO_4$ and the solution was made up fresh each week. Aliquots of extract containing less than 150 $\mu$g of carbohydrate were made up to 0.5 ml with distilled water and 5 ml of anthrone reagent was then added. After mixing on a vortex mixer, the tubes were heated to 90°C for 15 minutes in a Techne 'DG-1 Block Digestor'. They were rapidly cooled in an ice-water bath and then stored in the dark at room temperature for 30 minutes. The absorbance was read at 620 nm in a 'Ultrospec 4050' spectrophotometer. The reaction was linear up to 150 $\mu$g of carbohydrate (see Figure 5.2) and a standard curve was estimated at each determination using Anala R grade glucose as the standard.

Isolation of trehalose from other non-glycogen carbohydrates was made possible by its extreme stability to acid and alkali (WYATT and KALF, 1957). Appropriate aliquots of non-glycogen carbohydrate extract were evaporated to dryness and redissolved in 0.2 ml of 0.1 M sulphuric acid. The tubes were capped with foil and placed in a water bath at 100°C for 10 minutes to hydrolyse sucrose. In order to destroy reducing sugars, the extracts were then made alkaline by the

**Figure 5.2 — Quantification of Carbohydrate with Anthrone Reagent**

Legend: ordinate – absorbance at 620 nm,
abscissa – concentration of glucose ($\mu$g/ml).


Standard Curve for Carbohydrate Quantification
Absorbance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
Carbohydrate Conc. (μg/ml)

addition of 0.15 ml of 6 N NaOH and again heated to 100°C for 10 minutes. The samples were then quantified with anthrone as described above.

### 5.2.5 Determination of *De Novo* Lipid Synthesis in the Fat Body

The experimental insect populations used in this investigation were treated and maintained as described in General Materials and Methods, but the animals were sampled as two, three, four and five day old fifth instar larvae. On each of these days, an estimation of *de novo* lipid synthesis was made on the pooled fat bodies of one male and one female insect. Lipid neogenesis was determined by injecting animals with radiolabelled glucose and measuring its subsequent conversion into lipid. The insects to be sampled were each injected with 5 $\mu$l of D-(U-$^{14}$C)-glucose (specific activity 270 mCi/mmol, 200 $\mu$Ci/ml; Amersham International) which contained 1 $\mu$Ci of radioactivity. These animals were maintained at 30°C for 1 hour and were then killed and their fat bodies removed in the manner described previously in this chapter. The fat bodies for each determination were pooled in a glass homogenisation tube. To this was added 2 ml of ice-cold chloroform/methanol (1:1 v/v) and the tissue was homogenised for 2 minutes with a Teflon pestle rotating at 3,000 r.p.m. The homogenate was centrifuged for 5 minutes at 1,500 r.p.m. in a 'Centaur 2' centrifuge and the supernatant decanted into a clean tube. The pellet was resuspended in 2 ml of chloroform/methanol (1:1) and the tissue was homogenised and centrifuged as before. The supernatant fractions were pooled and, after the addition of 1.25 ml of chloroform and 1.25 ml of distilled water, mixed on a 'Whirlimix' vortex mixer. The tissue was centrifuged as before to separate the aqueous and lipid fractions and the aqueous phase was removed with a Pasteur pipette and discarded. To wash the organic phase, 4.2 ml of synthetic top phase was added and then mixed and centrifuged as before. The upper layer was removed with a Pasteur pipette and discarded. This washing procedure was repeated two further times and the washed lower phase was transferred to a clean conical tube. The lipid extract was evaporated to dryness by passing pure, dry nitrogen over it and then redissolved in 100 $\mu$l of chloroform. In order to determine the total level of radioactivity in the lipid extract, a 20 $\mu$l aliquot of the extract was transferred to a scintillation vial and evaporated to dryness to prevent quenching by chloroform. To this was added 10 ml of 'Liquiscint' scintillator fluid (National Diagnostics) and the vial

was shaken and allowed to stand for 30 minutes. The vial was then counted in a Packard 'Tri-Carb 300C' liquid scintillation system which had previously been calibrated with $^{14}C$ standards of known activity.

The remaining 80 $\mu$l of lipid extract was used to determine the levels of radioactivity in various lipid classes. Neutral lipids were separated by TLC, using the method of SKIPSKI *et al.* (1965). The chromatoplates used were glass plates (200 mm × 200 mm × 3 mm) covered with a 250 $\mu$m thick layer of silica gel, type H (Sigma Co.). Before use, the plates were 'activated' at 110°C for 1 hour. After the plates had cooled, the remaining lipid extracts were carefully applied to the gel surface with a microsyringe (Terumo), about 2 cm from the bottom of the plate. The chromatoplates were developed by ascending chromatography using the solvent system of MANGOLD and MALINS (1960) which consisted of petroleum ether/diethyl ether/acetic acid (80:20:1 v/v). This solvent was added, to a depth of about 1 cm, to large, glass developing tanks (Shandon Scientific) lined with Whatmann No.1 filter paper. When the tanks were fully saturated with solvent vapour, the chromatoplates were inserted and the solvent was allowed to rise up them until the solvent front was about 2 cm from the top. The plates were then removed from the tanks and residual solvent was driven off by air-drying. In order to locate separated lipid classes, the chromatoplates were placed in a tank of iodine vapour. The lipids took up the iodine vapour to become brown spots and the different lipid classes could be identified by comparison with standards which were run at the same time (MANGOLD and MALINS, 1960; SIMS and LAROSE, 1962). The plates were removed from the tank and the positions of the lipid spots were marked before the iodine was sublimed off by warming the plates. Each lipid spot was scraped off into a separate scintillation vial and 10 ml of 'Liquiscint' scintillator fluid was added. The vials were shaken and allowed to stand for 30 minutes before being counted in a 'Tri-Carb 300C' liquid scintillation system.

#### 5.2.6 Estimation of Fat Body Glycogen Phosphorylase Activity

Newly moulted fifth instar locusts were used to investigate the *in vivo* effects of 475 $\mu$g of methoprene upon glycogen phosphorylase activity, and were treated and maintained as described in the General Materials and Methods. The insects were killed at known times after treatment and their fat bodies were

removed in the manner described below. Animals used to study the *in vitro* effects of methoprene and JH III were untreated, newly moulted fifth instar larvae (12 hours ± 12 hours old). Enzyme activity was assayed in the direction of glycogen breakdown according to the method of CHILDRESS and SACKTOR (1970). The principle of the enzyme assay is as follows:

1) $(\text{Glucose})_n + \text{Pi} \xrightarrow{a} (\text{Glucose})_{n-1} + \text{Glucose-1-P}$

2) $\text{Glucose-1-P} \xrightarrow{b} \text{Glucose-6-P}$

3) $\text{Glucose-6-P} + \text{NADP}^+ \xrightarrow{c} \text{Gluconate-6-P} + \text{NADPH} + \text{H}^+$ , where

$a$ = phosphorylase a or phosphorylase b + AMP,

$b$ = phosphoglucomutase,

$c$ = Glucose-6-P dehydrogenase.

The rate of change in the absorbance at 340 nm ($\Delta$E/min) is a measure of the reduction of NADP and, therefore, of phosphorylase activity. The specific activity of the enzyme was calculated in the following way;

$$\text{Volume activity} = \frac{V}{\in \times d \times v} \times \Delta\text{E/min} \quad [\text{U/ml sample}]$$

$$\text{Specific activity} = \frac{\text{Volume activity}}{P} \quad [\text{U/mg protein}] \quad \text{, where}$$

$V$ = final volume of assay mixture (1.05 ml),

$\in$ = extinction coefficient (6.22 $\text{cm}^2/\mu$mole at 340 nm),

$d$ = distance of light path (1.0 cm),

$v$ = volume of enzyme sample (0.05 ml),

$P$ = protein concentration of enzyme sample.

All reagents used were Anala R grade or purest available and supplied by Boehringer Corporation, BDH or Sigma Company. The method used to measure fat body glycogen phosphorylase activity was that of VAN MARREWIJK *et al.* (1980), using the following media:

| | | |
|---|---|---|
| isolation medium; | 5 mM | EDTA, |
| | 20 mM | NaF, |
| | 50 mM | phosphate buffer, pH 7.0, |
| reaction medium; | 2 mM | EDTA, |
| | 5 mM | imidazol, |
| | 1.4 mM | dithio-erythritol, |
| | 5 mM | magnesium acetate, |
| | 0.6 mM | NADP, |
| | 4 $\mu$M | glucose-1,6-diphosphate, |
| | 2 mg/ml | glycogen (AMP-free), |
| | 4 units/ml | phosphoglucomutase, |
| | 0.8 units/ml | glucose-6-phosphate dehydrogenase, |
| | 40 mM | phosphate buffer, pH 7.0. |

In order to measure total phosphorylase activity, 2 mM AMP (final concentration) was included in the reaction medium.

Fat bodies from equal numbers of male and female animals (usually three of each sex) were extracted. The animals were killed by twisting the head to break the neck membrane. After cutting off the last abdominal segment, the head with the attached gut was pulled away. The carcase was cut open along the ventral surface and the fat body removed. The fat bodies were pooled in 2 ml of ice-cold isolation medium and homogenised for 2 minutes with a Teflon pestle rotating at 3,000 r.p.m. on a Tri-R 'Stir-R' homogeniser. The homogenate was centrifuged at 35,000 g for 15 minutes in a MSE 'Prepspin 50' ultracentrifuge at 0–4°C. The infranatant, between the pelleted material and the upper layer of fatty material, was used immediately for enzyme assay. Cuvettes containing 1 ml aliquots of reaction medium were placed in the cell of a Pye Unicam 'SP8-100' ultraviolet spectrophotometer. The cell temperature was maintained at 25°C and the cuvettes were preincubated for 10 minutes. Reactions were started by the addition of 50 $\mu$l of fat body extract. The solutions were mixed with a small stirring rod and the change in absorbance was read at 340 nm, at 1 minute intervals, for 5 minutes. Active phosphorylase was assayed in the absence of AMP, while total phosphorylase activity was measured in the presence of 2 mM AMP.

In order to investigate the *in vitro* effects of methoprene and JH III upon

phosphorylase activity, the enzyme was assayed by the method described above but extra additions were made to the cuvettes. In control incubations, 5 $\mu$l of absolute ethanol was added to the reaction medium prior to the addition of fat body extract. In treated cuvettes, 5 $\mu$l of ethanol containing either 475 $\mu$g of methoprene or 408 $\mu$g of JH III was added to the medium prior to the addition of extract.

### 5.2.7 Estimation of Protein

The protein content of haemolymph samples and of fat body extracts used to determine glycogen phosphorylase activity was measured using the Folin's method of LOWRY *et al.* (1951) as described in General Materials and Methods.

### 5.2.8 Separation and Quantification of Haemolymph Proteins

The haemolymph used in the present study was initially collected by the method described by STERNBURG and CORRIGAN (1959), and consisted of blood pooled from equal numbers of males and females (see above for details). In a later experiment, haemolymph was taken from individual insects by puncturing the soft membrane behind the animal's hind leg and removing blood with a 50 $\mu$l micro-cap. Blood was taken from four males and four females on each sampling day and used immediately for gel electrophoresis. The remainder was then stored frozen at $-20$°C under a nitrogen atmosphere until it was employed in the estimation of total haemolymph protein (see above).

Haemolymph proteins were separated by vertical polyacrylamide slab gel electrophoresis, based on the method of RAYMOND and WEINTRAUB (1959) and using the following solutions:

| | | |
|---|---|---|
| acrylamide solution; | 28.5% (w/v) | acrylamide, |
| | 1.5% (w/v) | N'N-methylene-bis-acrylamide; |
| protein carrier medium; | 20% (v/v) | glycerol, |
| | 0.1% (w/v) | Bromophenol Blue, |
| | 5 mM | EDTA, |
| | 54 mM | glycine, |
| | 52 mM | Tris/HCl buffer, pH 9.7; |
| resolving gel buffer; | 1.5 M | Tris/HCl buffer, pH 8.9; |

upper tank buffer; 54 mM glycine,
52 mM Tris/HCl buffer, pH 9.7;
lower tank buffer; 100 mM Tris/HCl buffer, pH 8.1.

The proteins were separated on 4% polyacrylamide gels which were made up in the manner described by DAVIS (1964) from the following:

20 ml dist. $H_2O$,
5.5 ml acrylamide solution,
16.0 ml resolving gel buffer,
60 $\mu$l Temed,
2 ml 90 mM ammonium persulphate (this was added last to cause polymerisation of the gel).

Each gel was formed in a mould comprised of two 18.5 cm × 20 cm glass plates (one a plain plate, the other having a notch 2 cm deep and 16 cm wide in the top) separated by 2 mm-thick spacers and held together with bulldog clips. The bottom and sides of the mould were temporarily sealed with silicone rubber tubing. The mould was held upright and the gel mixture was poured into it. When the mould was full, a plastic toothed comb was inserted in the top to form sample wells in the gel. After the gel had set, the comb and rubber tubing were removed and the mould was placed in an electrophoresis chamber. The bottom of the mould was positioned within the lower tank of the chamber while the top was clamped against the seal of the upper tank. The upper and lower tank buffers were poured into their respective tanks so that both the top and bottom of the gel were submerged. Platinum wires were strung along the floors of both upper and lower tanks and were electrically connected to a 'Vokam SAE 2761' constant current D.C. power supply (Shandon Scientific), the upper tank being connected to the cathode and the lower tank to the anode. A 20 mA current was passed through the apparatus for 1 hour to equilibrate the gel, after which protein samples could be separated on it.

Haemolymph samples were mixed with protein carrier medium (1:1 v/v) and 20 $\mu$l aliquots were loaded onto the top of the gel in separate sample wells with a Hamilton syringe. The apparatus was then run at 36 mA current for about 12 hours, until the Bromophenol Blue tracer dye from the protein carrier medium was ~2 cm from the bottom of the gel. The mould was removed from the electrophoresis chamber and the glass plates were detached to isolate the gel.

Protein bands were located by staining the gel with Coomassie Blue dye in the manner described by MEYER and LAMBERT (1965). 2.5 g of Coomassie Blue was dissolved in 500 ml of methanol and to this was added 70 ml of acetic acid and 500 ml of distilled water. Before use, the solution was filtered to remove any particles of dye that had not dissolved. Gels were stained by immersion in a bath of the dye solution for ~45 minutes during which the bath was gently shaken. Excess dye was then removed from the gel by washing several times in a mixture of methanol/acetic acid/distilled water (5:7:88 v/v). The gels were analysed with a Helena Lab. 'auto scanner' which determined the relative concentrations of the protein bands in each sample. By combining this information with the estimation of the haemolymph's total protein concentration, the actual concentration of each of the protein bands could be deduced.

On each sampling day, duplicate gels were run in the manner described above but were specifically stained to detect esterase activity using the method described by MARKERT and HUNTER (1959). The isolated gels were first incubated for 5 minutes in a bath of substrate solution. This consisted of 1.6 ml of 1% $\alpha$-naphthyl acetate solution in ethanol made up to 100 ml in 0.2 M acetate buffer (acetic acid/sodium acetate) at pH 5.0. The gel was then stained with a solution of Fast Blue B dye (200 mg in 100 ml acetate buffer) for 15 minutes and was finally rinsed in acetate buffer to remove excess stain. The solutions were freshly prepared for each determination.

## 5.3 Results

### 5.3.1 Effects of Methoprene on Lipid and Carbohydrate Levels

Known amounts of cholesterol, glycogen and glucose were processed through the extraction and quantification protocols described above in order to determine the efficiency of the procedures. It was found that 90–95% of the cholesterol, 80–90% of the glucose and 60–70% of the glycogen were detected.

Five experiments, using independent insect populations, were undertaken in order to investigate the effects of treatment with 475 $\mu$g of methoprene upon lipid and carbohydrate levels in locust fat body and haemolymph. Figure 5.3 shows the mean wet weights of locust fat bodies and, despite large variations between individuals of the same age, reveals a pattern of fluctuating fat body

**Figure 5.3 — Fat Body Wet Weight**

Legend:
ordinate – mean wet weight of fat body (mg),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

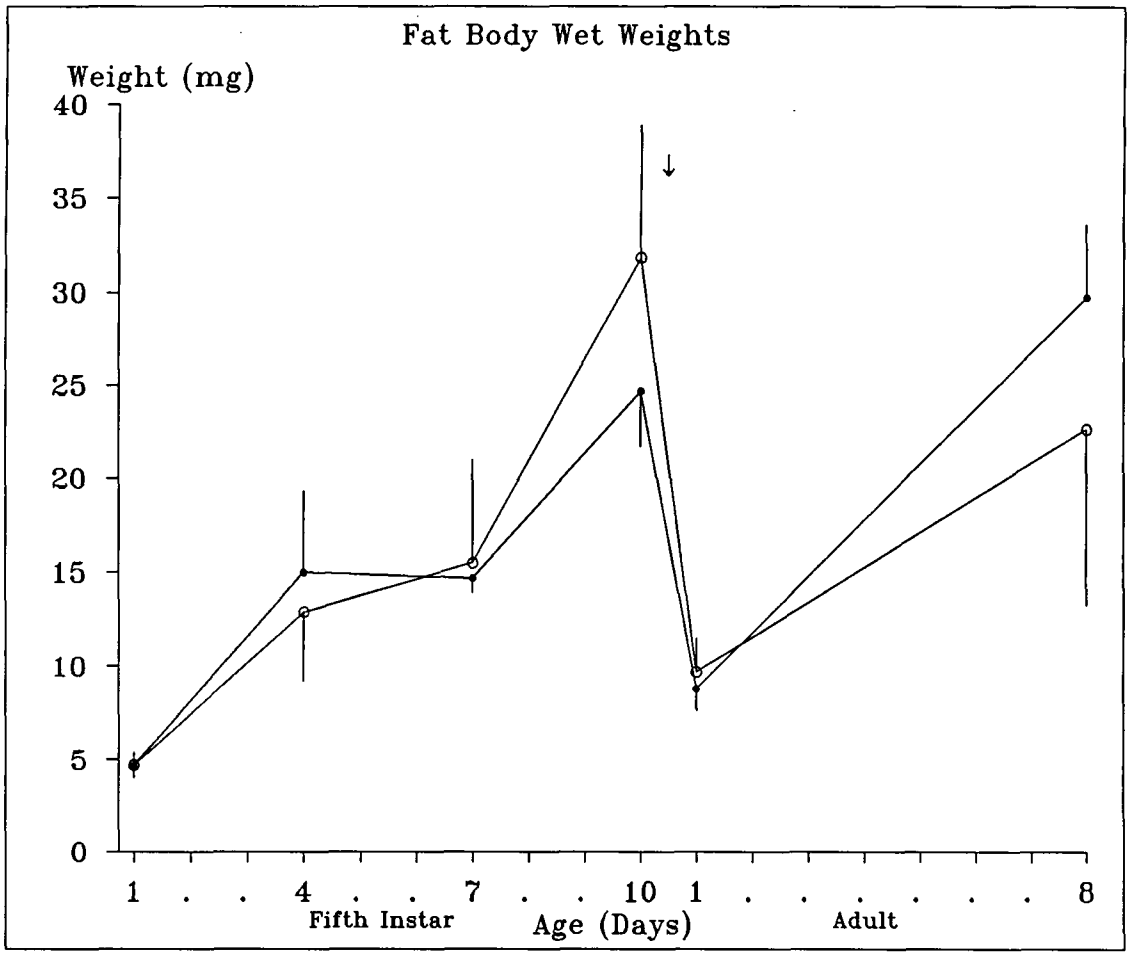

Fat Body Wet Weights
Weight (mg)
40
35
30
25
20
15
10
5
0
1
4
7
10
1
8
Fifth Instar
Age (Days)
Adult

**Figure 5.4 — Levels of Total Lipid in Fat Body**

**a. Amount of Lipid per Single Fat Body**

Legend: ordinate – amount of lipid in a single fat body ($\mu$g),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

**b. Concentration of Fat Body Lipid**

Legend: ordinate – concentration of lipid ($\mu$g/mg fat body),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

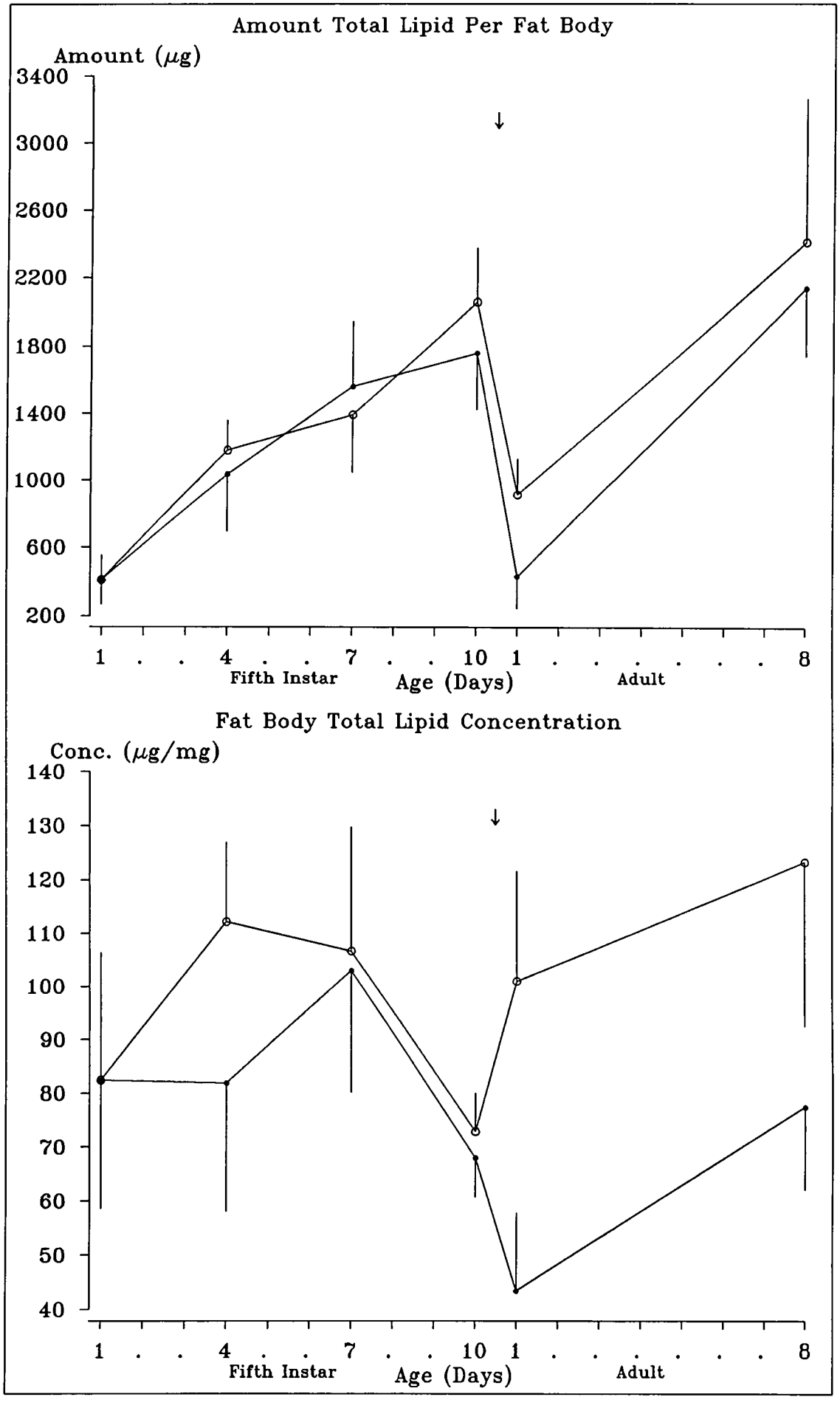

Amount Total Lipid Per Fat Body
Amount (μg)
3400
3000
2600
2200
1800
1400
1000
600
200
1
4
7
10
1
8
Fifth Instar
Age (Days)
Adult
Fat Body Total Lipid Concentration
Conc. (μg/mg)
140
130
120
110
100
90
80
70
60
50
40
1
4
7
10
1
8
Fifth Instar
Age (Days)
Adult

**Figure 5.5 — Levels of Triglyceride in Fat Body**

The levels of triglyceride detected are given in arbitrary units due to the lipid classes being quantified with a UV absorbance detector. UV absorbance is primarily due to carbon-carbon double bonds (see Materials and Methods for details) and, since the lipids' level of unsaturation was not known, the actual concentration of the lipid classes could not be calculated.

**a. Amount of Triglyceride per Single Fat Body**

Legend: ordinate – amount of triglyceride in a single fat body (units),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

**b. Concentration of Fat Body Triglyceride**

Legend: ordinate – concentration of triglyceride (units/mg fat body),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

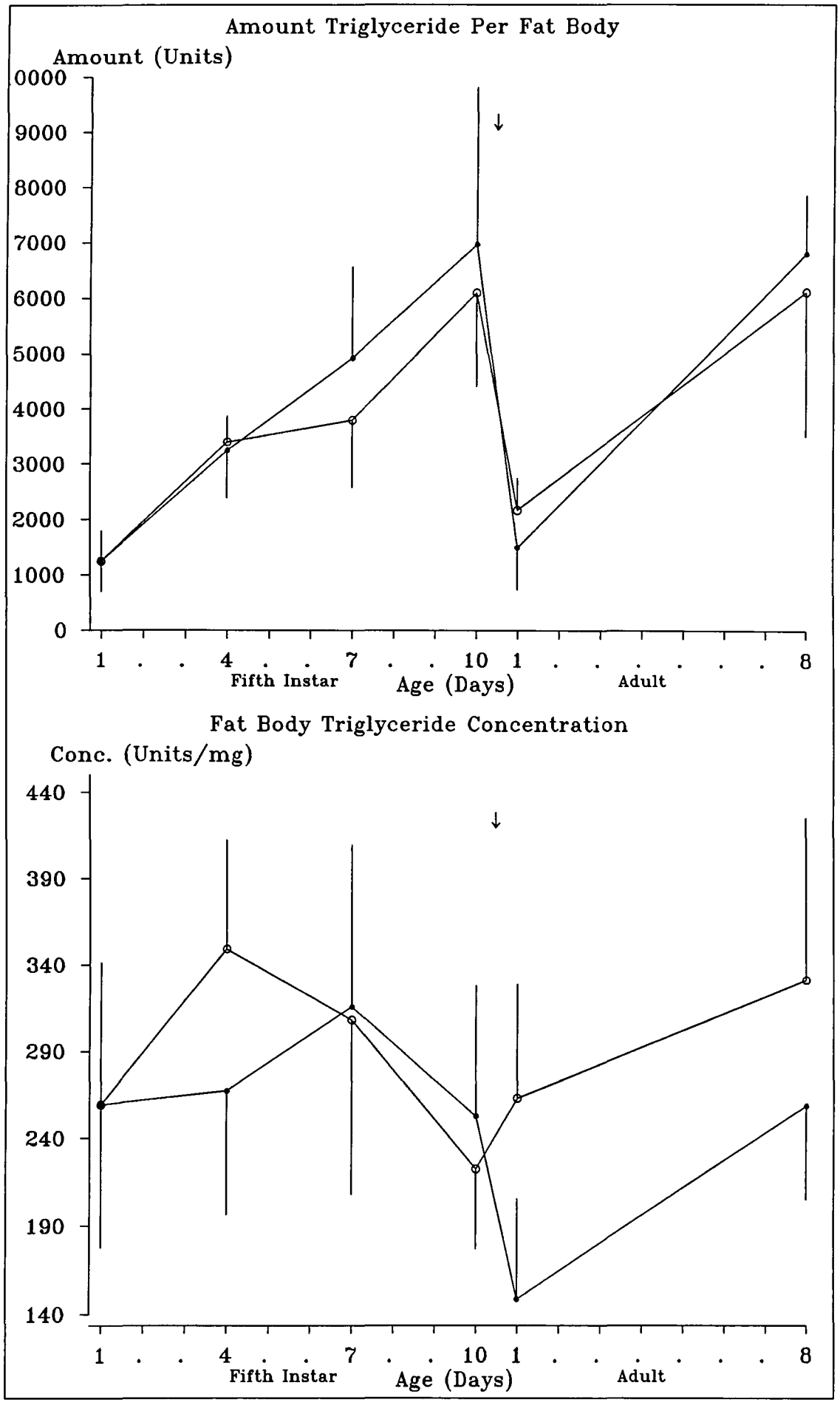

Amount Triglyceride Per Fat Body
Amount (Units)
0000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult
Fat Body Triglyceride Concentration
Conc. (Units/mg)
440
390
340
290
240
190
140
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult

**Figure 5.6 — Levels of Glycogen in Fat Body**

**a. Amount of Glycogen per Single Fat Body**

Legend: ordinate – amount of glycogen in a single fat body ($\mu$g),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

**b. Concentration of Fat Body Glycogen**

Legend: ordinate – concentration of glycogen ($\mu$g/mg fat body),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

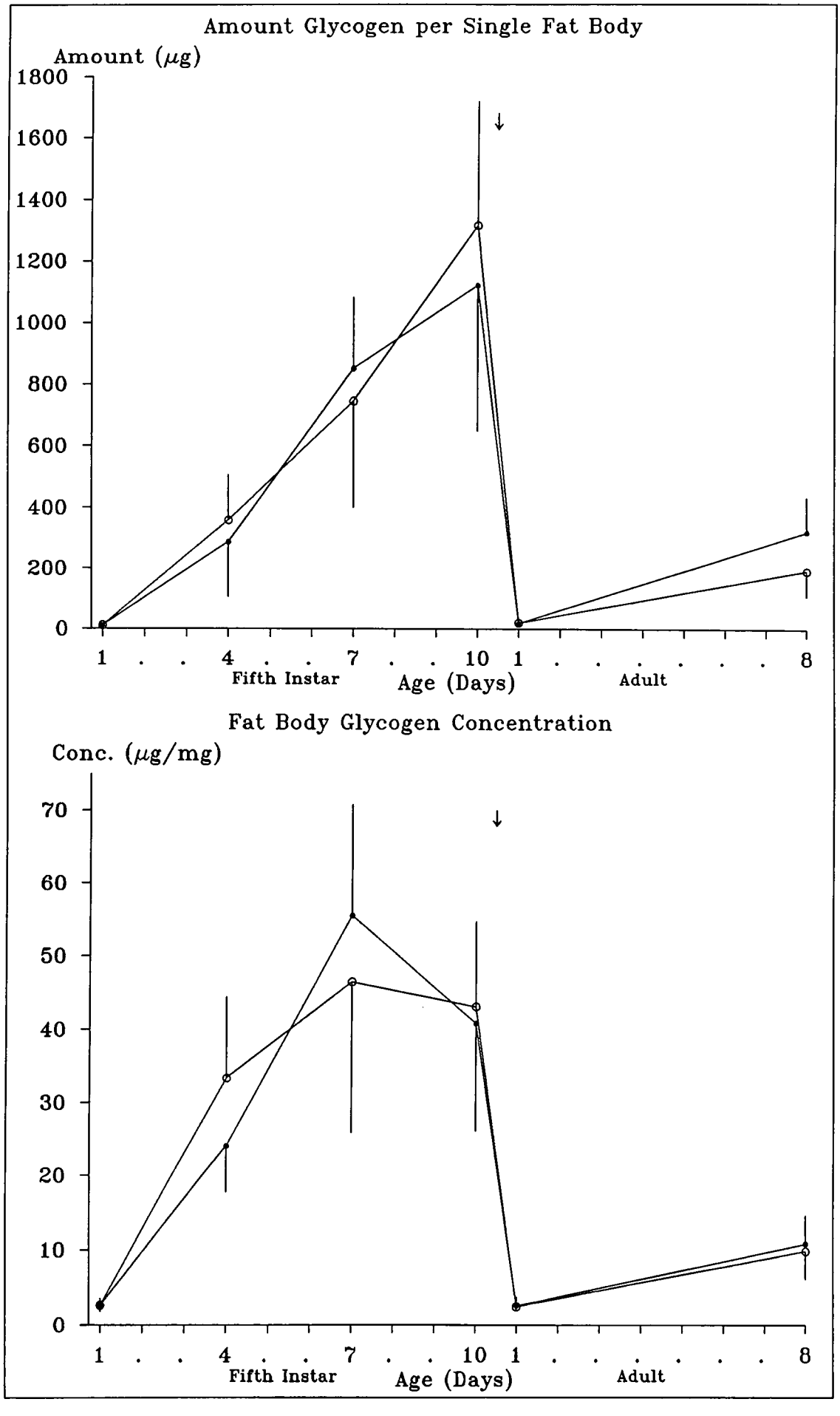

Amount Glycogen per Single Fat Body
Amount (μg)
1800
1600
1400
1200
1000
800
600
400
200
0
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult
Fat Body Glycogen Concentration
Conc. (μg/mg)
70
60
50
40
30
20
10
0
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult

**Figure 5.7 — Levels of Non-Glycogen Carbohydrate in Fat Body**

**a. Amount of Non-Glycogen Carbohydrate per Single Fat Body**

Legend: ordinate – amount of carbohydrate in a single fat body ($\mu$g),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

**b. Concentration of Fat Body Non-Glycogen Carbohydrate**

Legend: ordinate – concentration of carbohydrate ($\mu$g/mg fat body),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

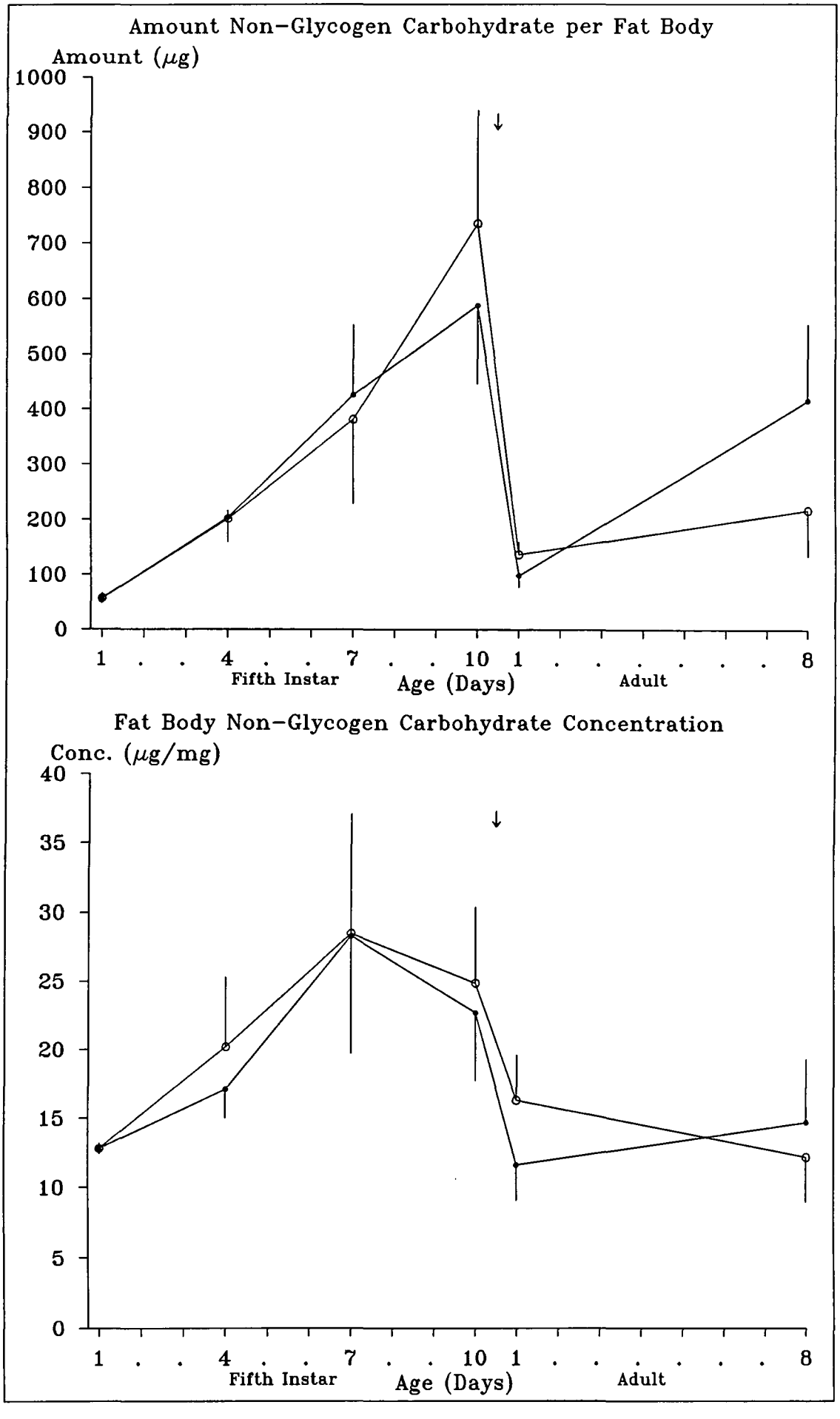

Amount Non-Glycogen Carbohydrate per Fat Body
Amount (μg)
1000
900
800
700
600
500
400
300
200
100
0
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult
Fat Body Non-Glycogen Carbohydrate Concentration
Conc. (μg/mg)
40
35
30
25
20
15
10
5
0
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult

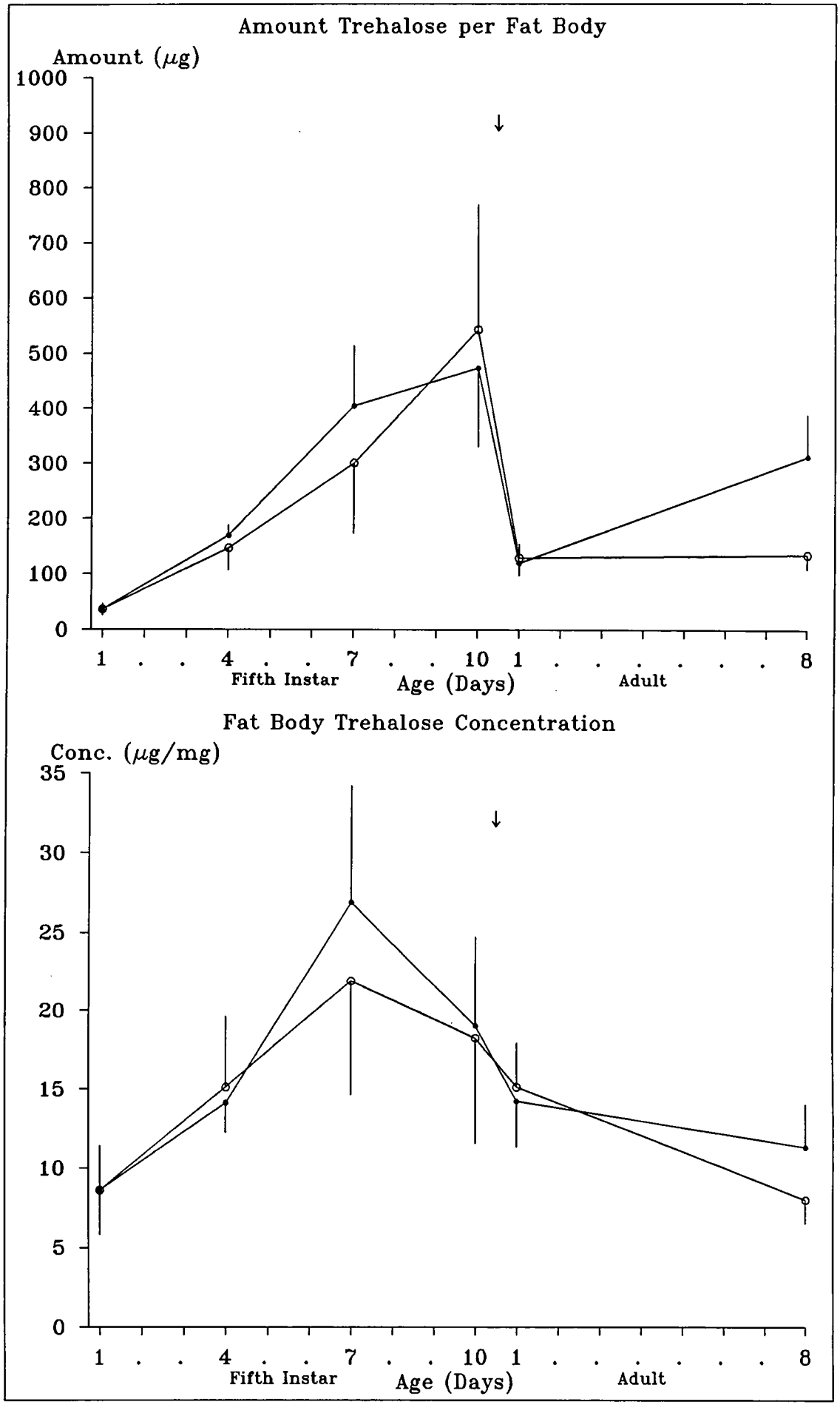
**Figure 5.8 — Levels of Trehalose in Fat Body**

**a. Amount of Trehalose per Single Fat Body**

Legend: ordinate – amount of trehalose in a single fat body ($\mu$g),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

**b. Concentration of Fat Body Trehalose**

Legend: ordinate – concentration of trehalose ($\mu$g/mg fat body),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.


Amount Trehalose per Fat Body
Amount (μg)
1000
900
800
700
600
500
400
300
200
100
0
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult
Fat Body Trehalose Concentration
Conc. (μg/mg)
35
30
25
20
15
10
5
0
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult

weight during the fifth instar and early adult life. The wet weight of control fat bodies increased throughout the fifth instar, particularly in the latter part of the stadium, and then decreased sharply at the time of the final moult. By Day 8 of adult life, fat body weights varied considerably between individual animals, having risen substantially in some insects while in others the tissue was little heavier than those of 1-day old adults. Fat bodies from locusts treated with 475 $\mu$g of methoprene were generally similar in weight to tissue from control animals of the same age. Although methoprene-treated fat bodies appeared to be substantially lighter than equivalent controls on Day 10 of the fifth instar and heavier on Day 8 of adult life, Student's $t$ tests showed that there were no significant differences between control and treated fat body weights on any of the days sampled ($P > 0.05$). Figures 5.4–5.8 show the mean levels of lipid and carbohydrate extracted from locust fat bodies, both as amounts (in $\mu$g) per single fat body and as concentrations ($\mu$g/mg of fat body). The mean amounts of lipid per single fat body are displayed in Figure 5.4 (**a**) and show that in both control and methoprene-treated tissues the amount of fat body lipid increased steadily throughout the fifth instar, decreased sharply during the final moult and had increased dramatically by the eighth day of adult life. A different pattern was revealed when lipid levels were considered as concentrations in $\mu$g/mg wet weight (Figure 5.4, **b**). Lipid concentration in control fat bodies increased from Day 1 of the fifth instar to reach a maximum in on Day 4, and then declined during the rest of the instar. The concentration rose dramatically in 1-day old adults and showed a further increase by Day 8 of adult life. In methoprene-treated fat bodies, lipid concentration changed very little during the first four days of the fifth instar but then rose to a maximum on Day 7. The lipid concentration declined during the rest of the instar and during the final moult, but had risen slightly by Day 8 of the adult stadium. Although the fat body lipid concentration of 1-day old adults was considerably lower in methoprene-treated animals compared with controls, Student's $t$ tests performed on the data showed no significant difference between control and treated results on any of the days sampled ($P > 0.05$). Separation of lipid extracts by HPLC revealed that, at all times when samples were taken, >95% of fat body lipids were in the form of triglycerides. The levels of triglyceride detected are presented in Figure 5.5 and, not surprisingly, the graphs are very similar in appearance to those of Figure 5.4. The levels of fat body

glycogen, non-glycogen carbohydrate and trehalose are shown in Figures 5.6, 5.7 and 5.8, respectively. Glycogen was the most plentiful of the three classes of carbohydrate but was still much less abundant than fat body lipid at all ages that were examined (compare Figures 5.4 and 5.6). The carbohydrates from control fat bodies showed similar developmental changes in their levels. The amount of each carbohydrate class per single fat body increased throughout the fifth instar, dropped suddenly at the time of the final moult and increased slightly again by the eighth day of adult life. Carbohydrate concentration increased to a maximum on Day 7 of the fifth instar and declined slightly by Day 10. The concentration decreased more sharply during the final moult and there was little difference between values on Day 1 and Day 8 of the adult stadium. Methoprene-treated fat bodies had similar levels of carbohydrates and Student's $t$ tests, performed on the data, showed that there was no significant difference between control and treated results on any of the days sampled ($P > 0.05$). Comparison of Figures 5.7 and 5.8 reveals that most of the non-glycogen carbohydrate present in the fat body was in the form of trehalose.

Figures 5.9–5.11 show the mean levels of lipid and carbohydrate in haemolymph from control and methoprene-treated locusts, calculated from five experiments upon independent insect populations. Lipid concentrations in the haemolymph of control locusts increased steadily throughout the fifth instar, dropped sharply at the time of the final moult, and then rose substantially during the first eight days of adult life (Figure 5.9, **a**). Haemolymph from methoprene-treated animals contained similar levels of lipid and there was no significant difference between control and treated results on any of the days that were sampled ($P > 0.05$, Student's $t$ tests). When the lipid extracts were separated by HPLC, the major lipid classes detected were triglycerides, diglycerides and cholesteryl esters which respectively accounted for ~70% , ~20% and ~10% of the total lipid. However, the total levels of lipid detected by HPLC bore no relation to the levels quantified with sulphosphovanillin reagent (compare **a** and **b** of Figure 5.9). This suggests that a significant proportion of lipid present in the haemolymph was completely saturated and so not detected by the UV monitor of the HPLC (see Materials and Methods for explanation). Haemolymph contained high concentrations of non-glycogen carbohydrate, mainly in the form of trehalose (Figure 5.10, **a&b**). In control haemolymph, carbohydrate concentration increased during the fifth instar

**Figure 5.9 — Levels of Lipid in Haemolymph**

**a. Concentration of Haemolymph Lipid**

Legend: ordinate – concentration of lipid ($\mu$g/$\mu$l haemolymph),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

**b. Estimation of Haemolymph Lipid by HPLC**

Legend: ordinate – concentration of lipid (units/$\mu$l haemolymph),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

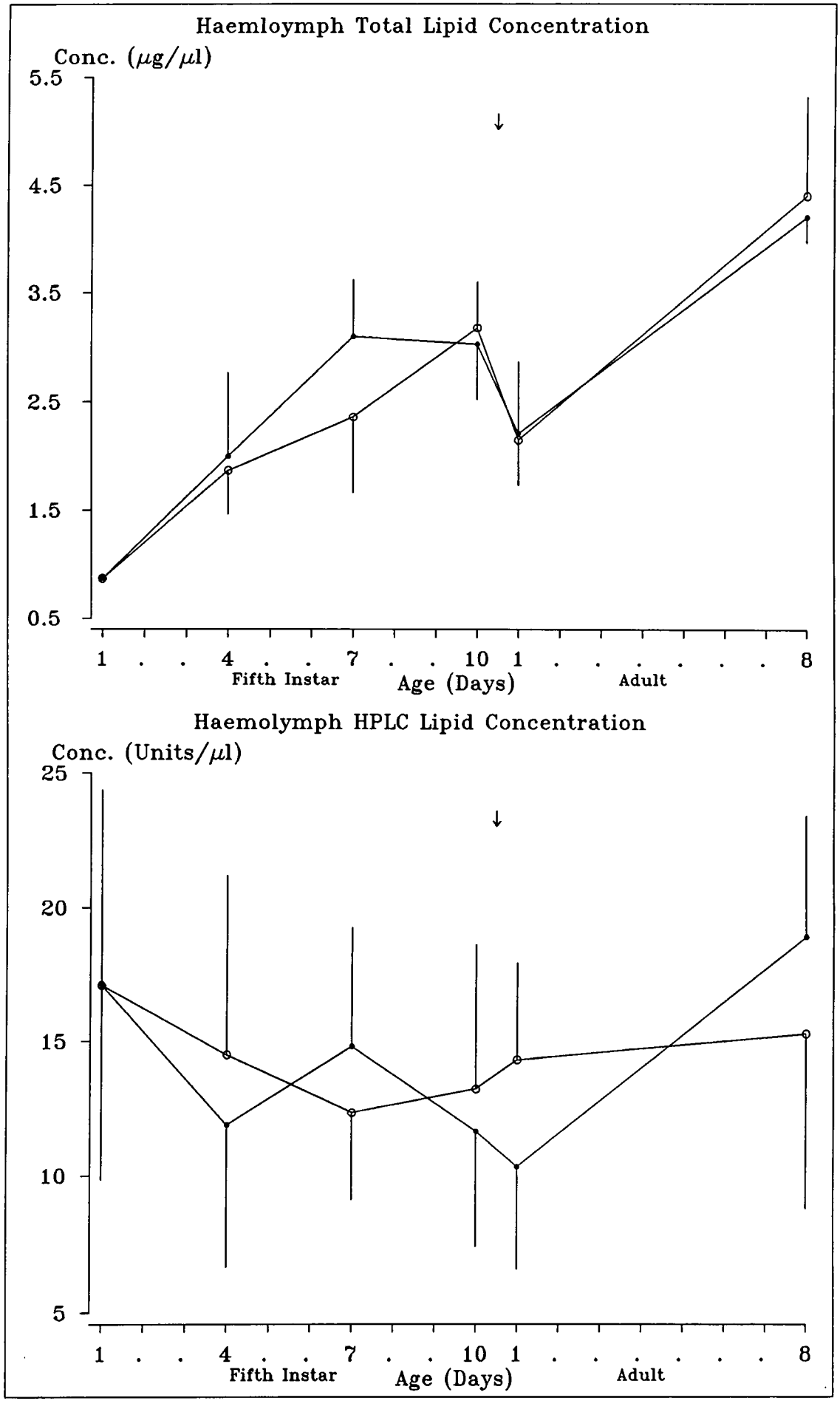

Haemloymph Total Lipid Concentration
Conc. (μg/μl)
5.5
4.5
3.5
2.5
1.5
0.5
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult
Haemolymph HPLC Lipid Concentration
Conc. (Units/μl)
25
20
15
10
5
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult

**Figure 5.10 — Levels of Non-Glycogen Carbohydrate in Haemolymph**

**a. Concentration of Haemolymph Non-Glycogen Carbohydrate**

Legend: ordinate – concentration of carbohydrate ($\mu$g/$\mu$l haemolymph),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

**b. Concentration of Haemolymph Trehalose**

Legend: ordinate – concentration of trehalose (units/$\mu$l haemolymph),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

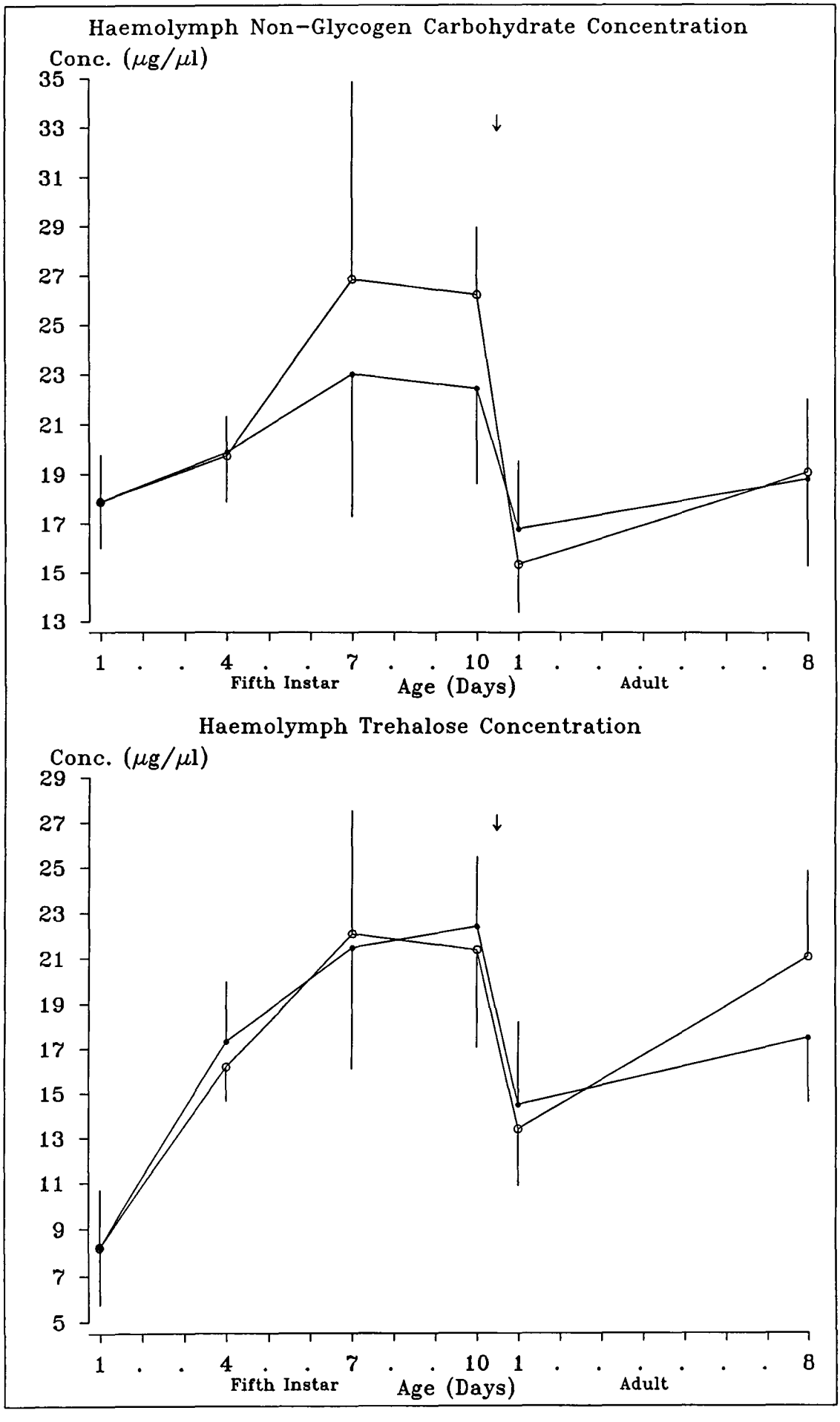

Haemolymph Non-Glycogen Carbohydrate Concentration
Conc. (μg/μl)
35
33
31
29
27
25
23
21
19
17
15
13
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult
Haemolymph Trehalose Concentration
Conc. (μg/μl)
29
27
25
23
21
19
17
15
13
11
9
7
5
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult

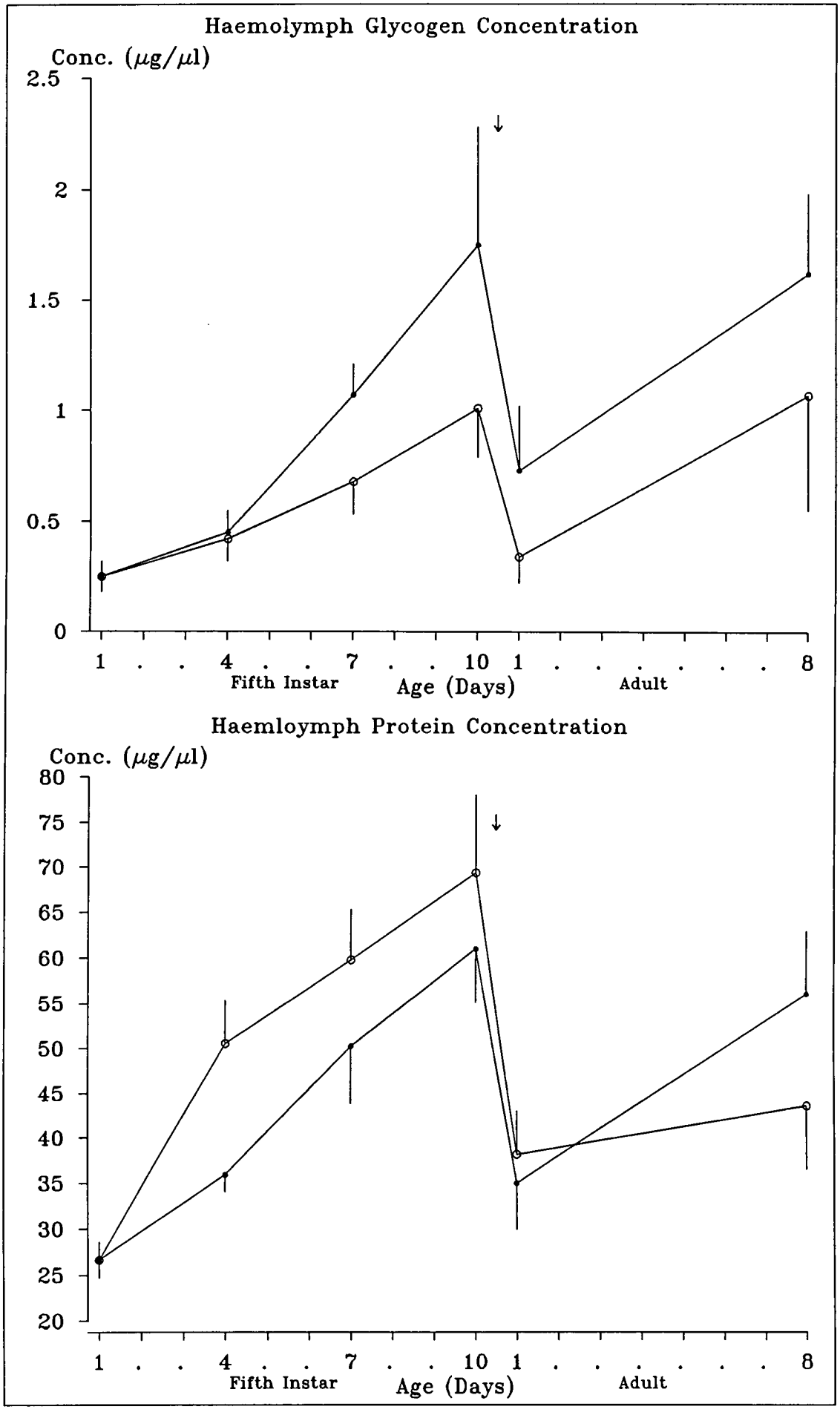
**Figure 5.11 — Levels of Glycogen and Protein in Haemolymph**

**a. Concentration of Haemolymph Glycogen**

Legend: ordinate – concentration of glycogen ($\mu$g/$\mu$l haemolymph),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.

**b. Concentration of Haemolymph Protein**

Legend: ordinate – concentration of protein ($\mu$g/$\mu$l haemolymph),
abscissa – age of insects (days),
↓ – time of final moult,
o – control locusts,
• – methoprene-treated locusts,
| – one standard error of mean.


Haemolymph Glycogen Concentration
Conc. (μg/μl)
2.5
2
1.5
1
0.5
0
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult
Haemloymph Protein Concentration
Conc. (μg/μl)
80
75
70
65
60
55
50
45
40
35
30
25
20
1 . . 4 . . 7 . . 10 1 . . . . . 8
Fifth Instar
Age (Days)
Adult

to reach a maximum on Day 7. The concentration declined slightly in the latter part of the instar, fell abruptly during the moult to the adult, and rose slightly in 8-day old adults. Carbohydrate concentrations underwent similar changes in methoprene-treated animals, although there was noticeably less non-glycogen carbohydrate in methoprene-treated haemolymph on Days 7 and 10 of the fifth instar compared with the equivalent controls. However, the differences between control and treated values were not significant at any age examined ($P > 0.05$, Student's $t$ tests). Glycogen was present in the haemolymph only at low levels though it followed the familiar pattern of developmental changes, increasing in concentration during the fifth instar, dropping sharply at the moult from larva to adult, and then rising again during the next eight days (Figure 5.11, a). After Day 4 of the fifth instar, glycogen concentrations were consistently higher in control haemolymph compared with blood from methoprene-treated animals but the differences were not significant on any of the days examined ($P > 0.05$, Student's $t$ tests).

### 5.3.2 Effects of Methoprene on *De Novo* Lipid Synthesis

The incorporation of radiolabelled glucose into fat body lipids was measured for the first four days of the fifth instar in control and 475 $\mu$g methoprene-treated locusts. Fat body lipid extracts were separated by TLC into triglycerides, free fatty acids, diglycerides and monoglycerides. The latter also contained more polar substances. The results are shown in Table 5.1. Triglycerides always contained by far the greatest levels of radioactivity while only very small amounts of radiolabelled diglycerides and free fatty acids were detected on any of the days sampled. In control fat bodies, there was little incorporation of [$^{14}$C]glucose into any lipid class on the first two days of the fifth instar. On Day 3, however, there was a more than tenfold increase in the amount of radiolabelled triglyceride in fat body tissue and a smaller but still significant increase in $^{14}$C incorporation into the 'monoglyceride' lipids. This increased level of radiolabelling was maintained on the fourth day of the fifth instar. In methoprene-treated fat bodies, [$^{14}$C]glucose incorporation into triglycerides and 'monoglycerides' was significantly higher on Days 1 and 2 of the fifth instar than in control tissue. On Day 3, a marked increase in the levels of radiolabelled triglyceride and 'monoglyceride' was observed, similar to that seen in control fat bodies. However, the

**Table 5.1 — Fat Body *De Novo* Lipid Synthesis**

Legend: n – number of determinations,
Time – day of fifth instar when animals killed,
475 $\mu$g Metho. – 475 $\mu$g of methoprene.
The levels of radiolabel incorporation are given as d.p.m. $\times 10^3$.

**Radiolabel Incorporation into Fat Body Lipids**

| Time | Treatment | n | Total Lipid | Lipid Class | | | |
|---|---|---|---|---|---|---|---|
| | | | | Triglyceride | Diglyceride | 'Monoglyceride' | Free Fatty Acid |
| Day 1 | Control | 4 | 1.83 ± 0.36 | 0.97 ± 0.27 | 0.20 ± 0.01 | 0.36 ± 0.07 | 0.45 ± 0.07 |
| | 475 $\mu$g Metho. | 4 | 12.46 ± 1.00 | 7.78 ± 1.37 | 0.19 ± 0.01 | 0.56 ± 0.09 | 1.64 ± 0.21 |
| Student's $t$ tests | | | P < 0.01 | P < 0.05 | P > 0.05 | P > 0.05 | P < 0.05 |
| Day 2 | Control | 4 | 2.77 ± 0.13 | 1.20 ± 0.09 | 0.15 ± 0.01 | 0.29 ± 0.02 | 0.73 ± 0.03 |
| | 475 $\mu$g Metho. | 4 | 11.45 ± 1.35 | 7.90 ± 0.90 | 0.18 ± 0.01 | 0.65 ± 0.09 | 1.73 ± 0.14 |
| Student's $t$ tests | | | P < 0.05 | P < 0.02 | P > 0.05 | P > 0.05 | P < 0.05 |
| Day 3 | Control | 4 | 50.66 ± 7.45 | 37.41 ± 6.46 | 0.32 ± 0.02 | 0.78 ± 0.08 | 3.12 ± 0.20 |
| | 475 $\mu$g Metho. | 4 | 38.14 ± 7.60 | 27.13 ± 6.32 | 0.31 ± 0.03 | 1.09 ± 0.05 | 3.71 ± 0.47 |
| Student's $t$ tests | | | P > 0.05 | P > 0.05 | P > 0.05 | P < 0.05 | P > 0.05 |
| Day 4 | Control | 4 | 45.33 ± 3.42 | 37.25 ± 1.19 | 0.46 ± 0.03 | 1.52 ± 0.17 | 3.83 ± 0.04 |
| | 475 $\mu$g Metho. | 4 | 16.33 ± 4.99 | 10.60 ± 3.59 | 0.21 ± 0.01 | 1.08 ± 0.25 | 2.75 ± 0.64 |
| Student's $t$ tests | | | P < 0.02 | P < 0.05 | P < 0.02 | P > 0.05 | P > 0.05 |

rate of $^{14}C$ incorporation into triglycerides had decreased substantially in 4-day old, methoprene-treated fat bodies and was significantly lower than that found in control tissue.

### 5.3.3 Effects of Methoprene on Glycogen Phosphorylase Activity

The rate-limiting enzyme in glycogen metabolism is glycogen phosphorylase (see Introduction for further details). The enzyme's activity was measured in fat body homogenates in order to determine whether methoprene treatment affected carbohydrate mobilisation. *In vitro* studies were performed on tissue extracts from newly moulted fifth instar locusts, to which various additions were made in the incubation vial (see Materials and Methods). The results of this analysis are shown in Table 5.2 (**a**), and Student's *t* tests performed on the data demonstrated that none of the treatments had any significant effect on activity ($P > 0.05$). The *in vivo* effects of methoprene were investigated by measuring fat body phosphorylase activity in fifth instar locusts at various times after they had been topically treated with the JHA (see Materials and Methods). The results are shown in Table 5.2 (**b**) and reveal that the % of phosphorylase in the active *a* form declines significantly within 24 hours of the topical applications being made ($P < 0.05$, Student's *t* tests). However, enzyme activity in methoprene-treated fat body was not significantly different from that in control tissue at any of the times examined ($P > 0.05$).

### 5.3.4 Effects of Methoprene on Total Haemolymph Protein

The total protein concentrations of haemolymph samples were determined using Folin's reagent (see General Materials and Methods for details). Haemolymph obtained primarily for the quantification of lipid and carbohydrate consisted of blood pooled from equal numbers of male and female insects (see Materials and Methods for details). In a later experiment (Expt. VI), blood samples from male and female insects were stored separately. However, it was found that blood protein concentrations were not significantly different between male and female insects at any of the ages examined. Therefore, the results from all experiments were pooled to give the levels shown in Figure 5.11 (**b**). In both control and methoprene-treated animals, haemolymph protein concentration increased throughout the fifth instar and then fell markedly after the final moult, almost

**Table 5.2 — Glycogen Phosphorylase Activity**

**a. *In Vitro* Effects of Methoprene on Glycogen Phosphorylase**

Legend:
% Activity – % of phosphorylase in active *a* form,
n – number of determinations,
475 $\mu$g Metho. – 475 $\mu$g of methoprene applied in 5 $\mu$l of ethanol.

Glycogen phosphorylase specific activity is given in units of U/mg protein (see Materials and Methods for further details).

**b. *In Vivo* Effects of Methoprene on Glycogen Phosphorylase**

Legend:
Time – time after treatment (hours),
% Activity – % of phosphorylase in active *a* form,
n – number of determinations,
475 $\mu$g Metho. – 475 $\mu$g of methoprene.

Glycogen phosphorylase specific activity is given in units of U/mg protein.

**_In Vitro_ Effects of Methoprene on Glycogen Phosphorylase**

| Treatment | n | Specific Activity | | % Activity |
|---|---|---|---|---|
| | | Phosphorylase *a* | Total Phosphorylase | |
| Untreated | 5 | 0.651 ± 0.069 | 1.296 ± 0.060 | 50% |
| 5 $\mu$l Ethanol | 6 | 0.571 ± 0.059 | 1.153 ± 0.090 | 50% |
| 475 $\mu$g Metho. | 6 | 0.542 ± 0.055 | 1.158 ± 0.064 | 47% |

**_In Vivo_ Effects of Methoprene on Glycogen Phosphorylase**

| Time | Treatment | n | Specific Activity | | % Activity |
|---|---|---|---|---|---|
| | | | Phosphorylase *a* | Total Phosphorylase | |
| 0 | Control | 4 | 0.288 ± 0.099 | 0.886 ± 0.160 | 33% |
| | 475 $\mu$g Metho. | 4 | 0.288 ± 0.099 | 0.886 ± 0.160 | 33% |
| 1 | Control | 3 | 0.346 ± 0.027 | 0.884 ± 0.020 | 39% |
| | 475 $\mu$g Metho. | 3 | 0.458 ± 0.017 | 0.987 ± 0.055 | 46% |
| 3 | Control | 4 | 0.131 ± 0.043 | 0.993 ± 0.180 | 13% |
| | 475 $\mu$g Metho. | 4 | 0.122 ± 0.049 | 0.993 ± 0.220 | 12% |
| 6 | Control | 4 | 0.086 ± 0.025 | 0.729 ± 0.161 | 12% |
| | 475 $\mu$g Metho. | 4 | 0.078 ± 0.010 | 0.858 ± 0.209 | 9% |
| 24 | Control | 4 | 0.086 ± 0.023 | 0.576 ± 0.190 | 15% |
| | 475 $\mu$g Metho. | 4 | 0.130 ± 0.028 | 0.657 ± 0.241 | 20% |
| 48 | Control | 4 | 0.073 ± 0.011 | 0.322 ± 0.052 | 23% |
| | 475 $\mu$g Metho. | 4 | 0.089 ± 0.008 | 0.395 ± 0.065 | 23% |

to the levels at the beginning of the fifth instar. By the eighth day of adult life, protein levels had increased again slightly, especially in methoprene-treated animals. Student's $t$ tests performed on the data demonstrated that blood protein concentrations from control and treated insects were not significantly different from each other at any of the ages examined ($P > 0.05$).

### 5.3.5 Effects of Methoprene on Haemolymph Protein Patterns

Haemolymph proteins were separated by polyacrylamide gel electrophoresis and located by staining with Coomassie Blue. Up to eight protein bands could be distinguished in a single sample (see Plate 5.1, **a**), but in most cases two of the faster-moving bands (bands 2 and 3) were so closely associated that they could not be separated either by sight or by the gel scanner. Therefore, these have been recorded as a single peak in the results. Initially, the blood samples used were pooled from equal numbers of males and females, and two experiments, with independent insect populations, were undertaken (Expts. IV and V). The results of these experiments are shown in Figure 5.12. The results of Expt. VI, in which blood from individual males and females were separated, are given in Figures 5.13 and 5.14. Protein bands 1 and '2&3' were always present in both sexes and at all ages examined, and were generally the major protein bands of the haemolymph protein. Bands 6 and 8 were usually only present at very low concentrations and were often not detectable. Protein bands 5 and 7 were present at higher concentrations but, like bands 6 and 8, there was no obvious pattern to the changes in their levels during the insects' development. At all the ages examined, there was no significant difference in the concentrations of all the aforementioned protein bands between haemolymph from control and methoprene-treated animals nor between males and females ($P > 0.05$, Student's $t$ tests). Protein band 4, however, was found only in the blood of female locusts and, in control animals, only on the eighth day of adult life (see Figures 5.13 and 5.14). In contrast, band 4 was present in the haemolymph of methoprene-treated, female locusts from Day 7 of the fifth instar onwards.

Examination of acrylamide gels that had been stained with $\alpha$-naphthyl acetate and Fast Blue B revealed the main band of protein(s) with esterase activity was associated with protein band 7 (see Plate 5.1). From Day 4 of the fifth instar onwards, there were also a number of much fainter esterase bands. It was

**Plate 5.1 — Electrophoresis Gels of Haemolymph Proteins**

**a.** Gel stained with Coomassie Blue showing the total protein bands of blood samples from 10-day old fifth instar locusts. Note the presence of up to eight separate protein bands in each sample, and that protein band 4 is present only in haemolymph from methoprene-treated females (t3 & t4).

**b.** Duplicate gel to that shown in **a** except that it was stained with $\alpha$-naphthyl acetate and Fast Blue B to show proteins with esterase activity. Note the presence of one main esterase band which corresponded with protein band 7 of **a**. Also note the presence of other, much weaker-staining esterase bands (arrows).

Key: c1 & c2– blood samples from control males; c3 & c4– blood samples from control females; t1 & t2– blood samples from treated males; t3 & t4– blood samples from treated females; TD– tracer dye.

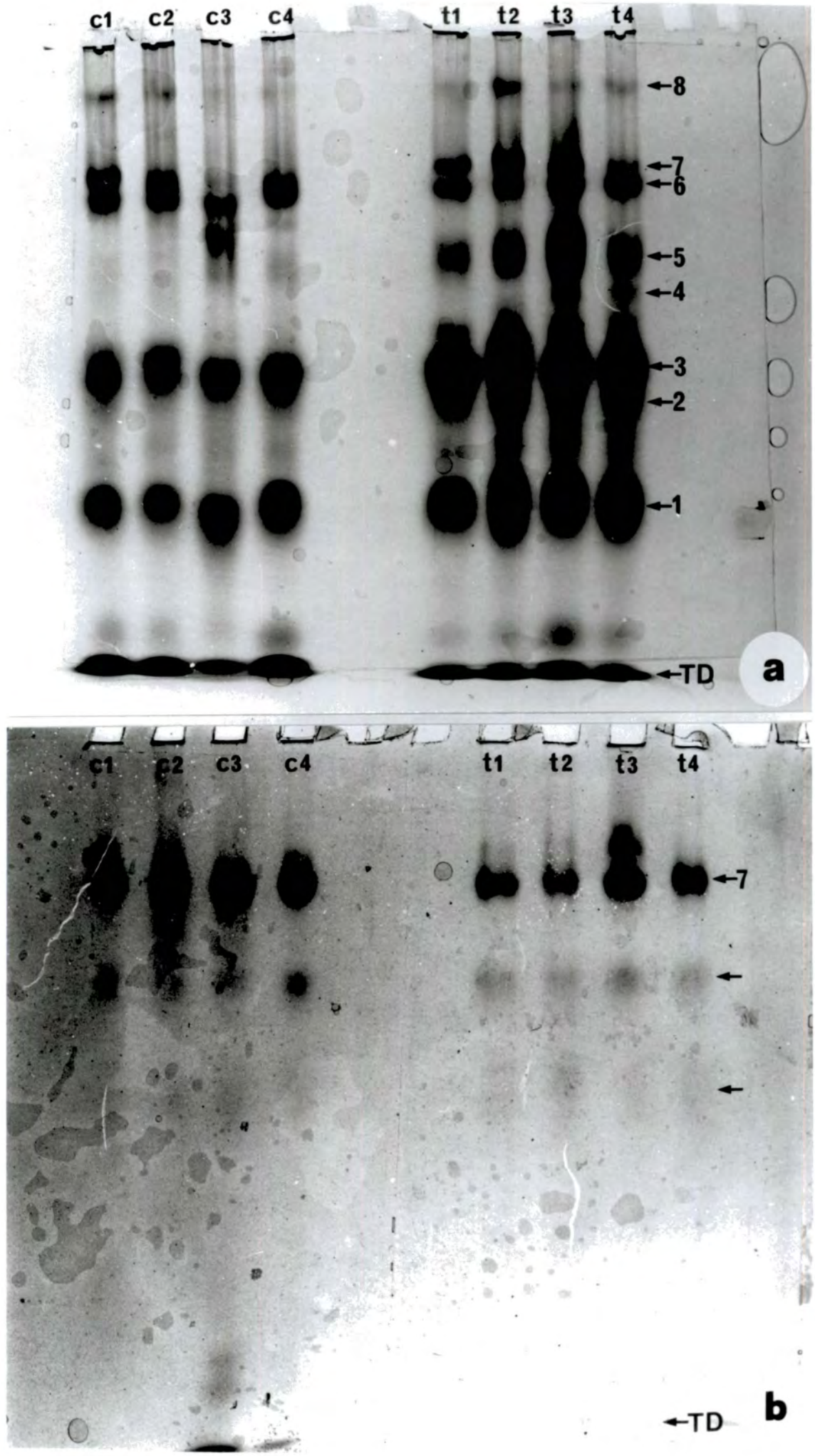

c1
c2
c3
c4
t1
t2
t3
t4
8
7
6
5
4
3
2
1
TD
a
c1
c2
c3
c4
t1
t2
t3
t4
7
TD
b

**Figure 5.12 — Haemolymph Protein Bands from Pooled Male and Female Blood**

Legend:
ordinate – protein concentration ($\mu$g/$\mu$l),
abscissa – protein band number (see Plate 5.1),
Day *n*f – day *n* of the fifth instar,
Day *n*a – day *n* of the adult stadium,
light shading – results for control insects,
dark shading – results for methoprene-treated insects.

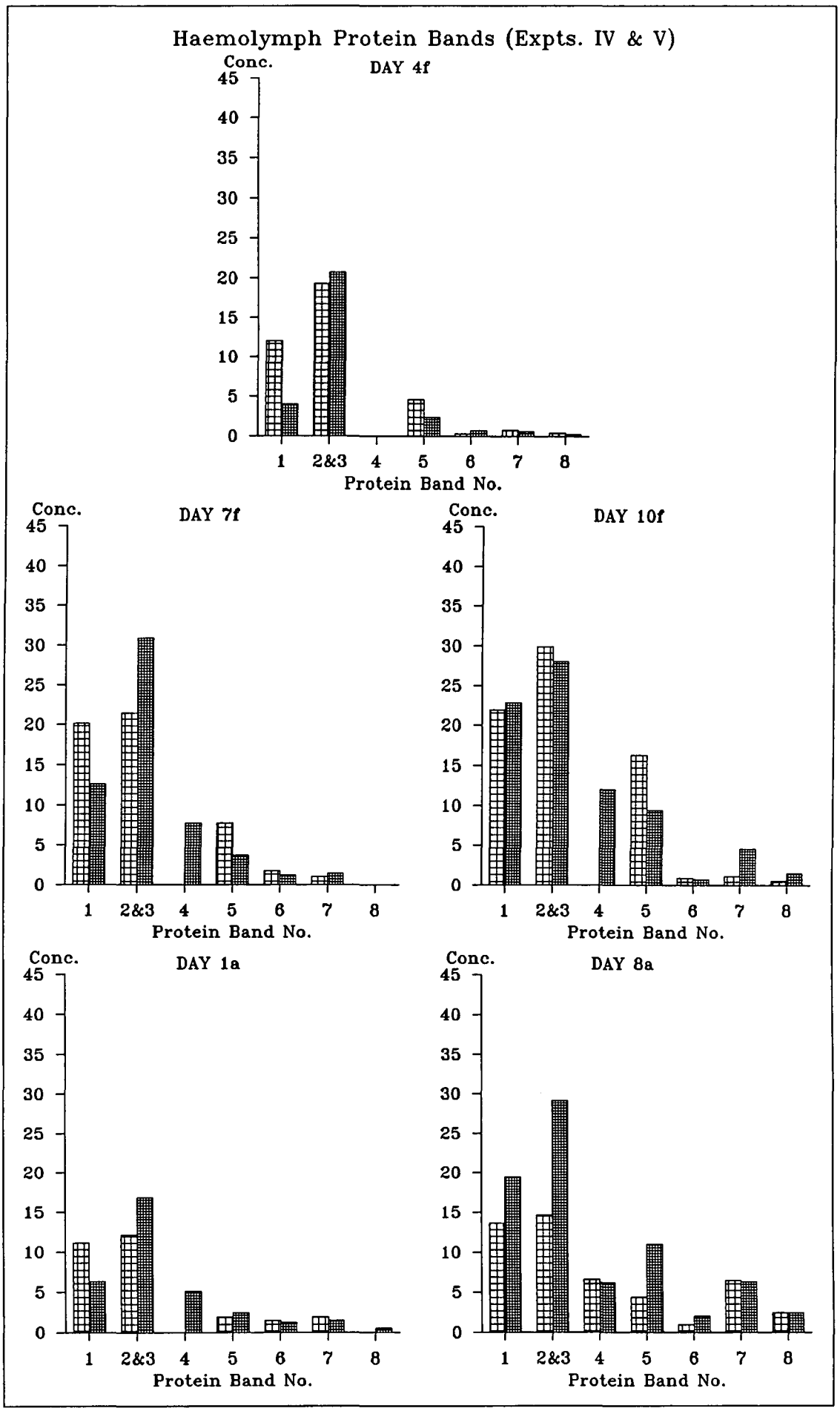

Haemolymph Protein Bands (Expts. IV & V)
Conc.
DAY 4f
45
40
35
30
25
20
15
10
5
0
1
2&3
4
5
6
7
8
Protein Band No.
Conc.
DAY 7f
Protein Band No.
Conc.
DAY 10f
Protein Band No.
Conc.
DAY 1a
Protein Band No.
Conc.
DAY 8a
Protein Band No.

**Figure 5.13 — Haemolymph Protein Bands from Male Insects**

Legend:

ordinate – protein concentration ($\mu$g/$\mu$l),
abscissa – protein band number (see Plate 5.1),
Day *n*f – day *n* of the fifth instar,
Day *n*a – day *n* of the adult stadium,
light shading – results for control insects,
dark shading – results for methoprene-treated insects,
vertical line – one standard error of mean value.



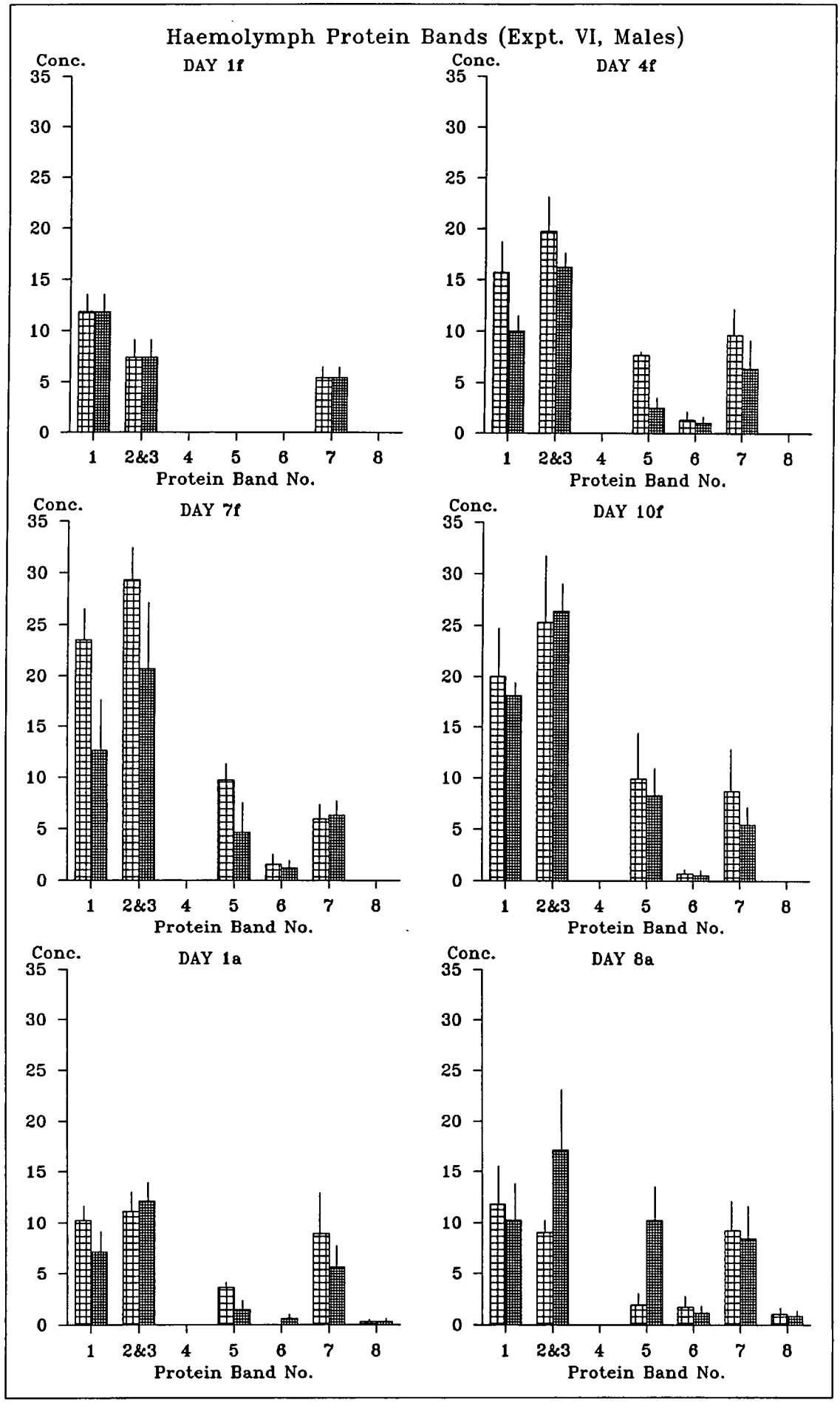
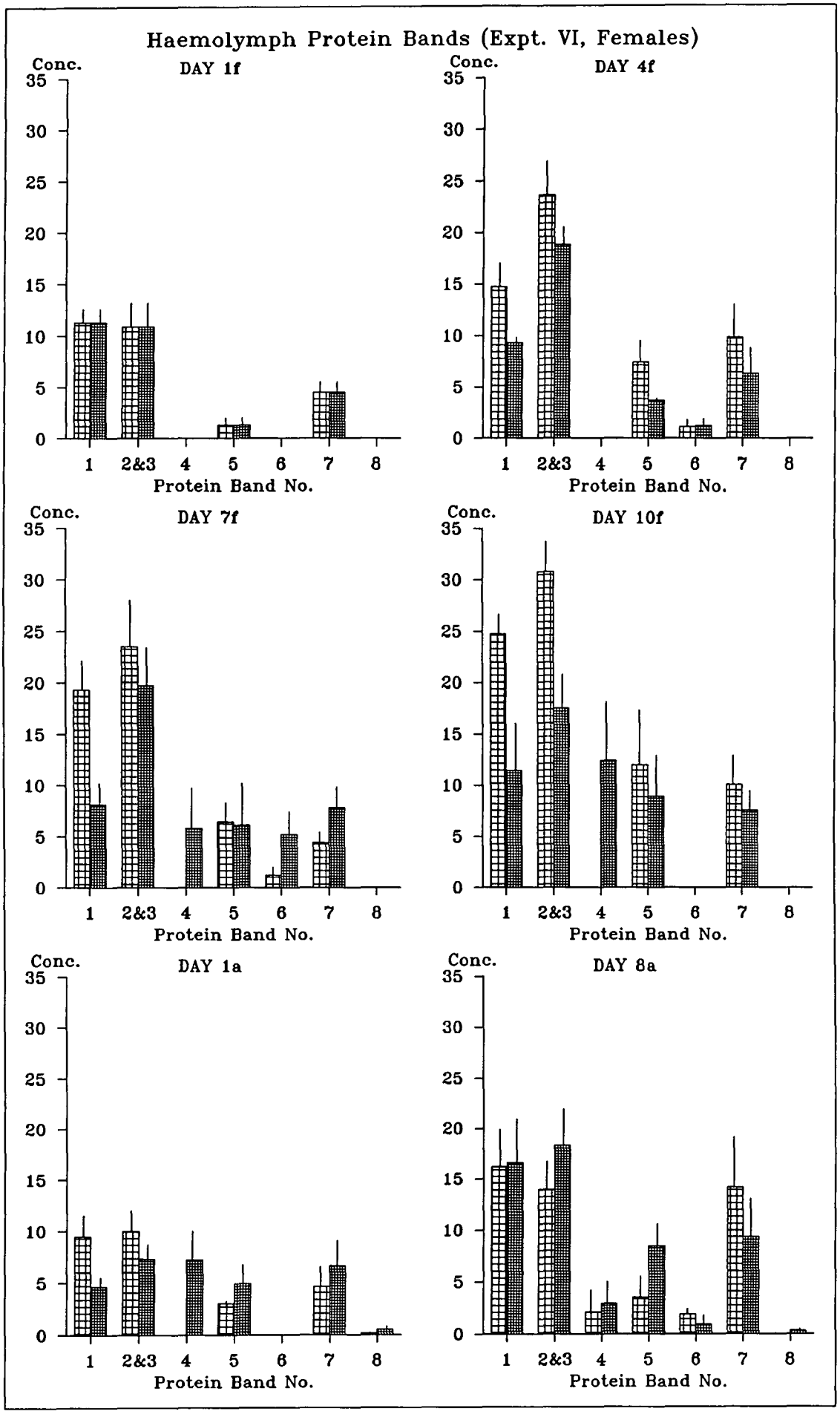

Haemolymph Protein Bands (Expt. VI, Males)
Conc.
DAY 1f
35
30
25
20
15
10
5
0
1
2&3
4
5
6
7
8
Protein Band No.
Conc.
DAY 4f
Protein Band No.
Conc.
DAY 7f
Protein Band No.
Conc.
DAY 10f
Protein Band No.
Conc.
DAY 1a
Protein Band No.
Conc.
DAY 8a
Protein Band No.

**Figure 5.14 — Haemolymph Protein Bands from Female Insects**

| | | |
|---|---|---|
| Legend: | ordinate – | protein concentration ($\mu$g/$\mu$l), |
| | abscissa – | protein band number (see Plate 5.1), |
| | Day *n*f – | day *n* of the fifth instar, |
| | Day *n*a – | day *n* of the adult stadium, |
| | light shading – | results for control insects, |
| | dark shading – | results for methoprene-treated insects, |
| | vertical line – | one standard error of mean value. |


Haemolymph Protein Bands (Expt. VI, Females)
DAY 1f
DAY 4f
DAY 7f
DAY 10f
DAY 1a
DAY 8a
Conc.
35
30
25
20
15
10
5
0
1
2&3
4
5
6
7
8
Protein Band No.

not possible to determine the amount of esterase protein on the gels with the 'auto scanner' because this only measured the relative amounts of material in the various protein bands of a sample. However, there was no apparent difference between control and treated samples in the staining intensity of the main esterase band. Nor were there any consistent differences in the numbers or staining intensities of the secondary esterase bands.

## 5.4 Discussion

The levels of lipid and carbohydrate in locust fat bodies and haemolymph which were found in the present study are comparable with results from previous studies (e.g. MINKS, 1967; STRONG, 1968a; HILL and IZATT, 1974; BAILEY *et al.*, 1975). Although the amounts of lipid and carbohydrate per single fat body increased throughout the last larval stadium, their concentrations per mg wet weight of fat body decreased from Day 7 to Day 10 of the fifth instar. This is in apparent contradiction with the observation made in Chapter 4 that fat body cells from 10-day old fifth instar locusts were almost entirely given over to the storage of lipid and carbohydrate deposits. However, this could be explained by the presence of many large vacuoles within such cells' glycogen stores (see Plate 4.8, **b**, of Chapter 4). LOCKE (1985) suggested that similar vacuoles seen in the fat body cells of *Calpodes ethlius* might be due to the conversion of stored glycogen to soluble sugars in order to allow its rapid mobilisation. In the present study, the fact that non-glycogen carbohydrate in fat bodies also decreased in concentration from Day 7 to Day 10 of the fifth instar argues against a similar process occurring in locust fat body. Comparison of Figures 5.4 and 5.5 reveals a high degree of similarity between graphs of fat body lipid detected by sulphosphovanillin reagent and of fat body lipid detected by HPLC (almost entirely triglyceride). This indicates that neither the proportions of the various lipid classes nor their degree of unsaturation changed significantly during the fifth instar and early adult life of *Locusta*. The similarity between control and treated results in the two figures also suggests that the application of methoprene had no significant effect on the level of lipid saturation in the fat body. Thus, there is no support in the present study for the proposal of STEPHEN and GILBERT (1969, 1970) that JH acts to suppress desaturase activity in the fat body and so raise the relative concentration of saturated fatty acids. This suggestion was due to their

observation that, in the final larval instar and early adult life of *Hyalophora cecropia*, changes in the amounts of total body lipids and in their degree of saturation corresponded with fluctuations in the JH titre. In Chapter 4, it was observed that fat body cells of methoprene-treated animals seemed to contain less stored lipid than did control tissue of the same age. The results from the current chapter show that, although lipid concentrations were consistently lower in methoprene-treated fat bodies compared with the controls (Figure 5.4, **b**), the differences were not statistically significant at any of the times that measurements were taken. Also, treatment with methoprene had no significant effect on the levels of carbohydrate in the fat body. Thus, any effect that the JHA might have had upon lipid and carbohydrate storage in the fat body was relatively minor compared to the normal variation in the levels of these materials. This result tends to support the proposal of STEELE (1976) that the effects of JH on the levels of fat body lipid and carbohydrate were simply secondary effects resulting from its stimulation of fat body protein synthesis. Also, it would appear that treatment of locusts with methoprene had much less effect on the accumulation of fat body lipid and carbohydrate than removal and implantation of corpora allata has been seen to do in other studies (see Introduction for details).

In several insect species, it has been demonstrated that *de novo* lipid synthesis, determined by incorporation of radiolabelled acetate or palmitate into fat body lipids, is increased by allatectomy (see Introduction for details). The application of methoprene to fifth instar locusts of the present study also caused a marked change in lipid neogenesis in their fat bodies. The very low rate of [$^{14}$C]glucose incorporation in the first two days of the instar compared with Days 3 and 4 suggested that *de novo* lipid synthesis was initially at a basal, resting level. The significantly higher levels of lipid neogenesis seen in methoprene-treated fat bodies on these two days might then simply be due to an increased metabolic rate in these insects (such an increase is suggested by their accelerated rate of development as shown in Chapter 3 and the greater capacity of their fat bodies for synthetic activity as shown in Chapter 4). In contrast, when lipid neogenesis was greatly increased on the third and fourth days of the fifth instar, the levels of radiolabel incorporated by treated fat bodies were lower, especially on Day 4. GOKULDAS *et al.* (1988) found that *in vitro* incorporation of [$^{14}$C]acetate into lipids by fat bodies from *Schistocerca gregaria* suddenly peaked on Day 4 of

the fifth (final) larval instar and declined rapidly thereafter. Assuming that a similar pattern was followed in *Locusta*, it would seem that methoprene treatment in the present study reduced the normal peak of fat body lipid synthesis in the early part of the fifth instar. However, an alternative explanation could be that a greater proportion of lipid newly synthesised by the fat bodies in methoprene-treated locusts was immediately exported to other parts of the body.

Neither *in vitro* nor *in vivo* applications of methoprene had any significant effect on fat body glycogen phosphorylase activity whether considering the total level of enzyme present or the proportion of the enzyme in the active *a* form. This is in contrast to the results of GORDON and BURFORD (1984) who detected a reduction in fat body glycogen phosphorylase activity in both larval and pupal *Aedes aegypti* following the treatment of newly moulted, last-instar larvae with methoprene. However, this response has not been reported in any other species and WRIGHT *et al.* (1975) found that the application of a JHA to pupal *Stomoxys calcitrans* had no effect on the specific activity of fat body glycogen phosphorylase even though utilisation of fat body glycogen was inhibited by the treatment (WRIGHT and RUSHING, 1973). It is of interest that in the present study methoprene treatment had no apparent effect on glycogen phosphorylase activity nor on the levels of haemolymph and fat carbohydrate, but did have a significant effect on the rate of conversion of glucose into fat body lipid. The results are too fragmentary for any conclusions to be drawn but this is obviously an area that merits further study.

The present analysis of total protein concentration in haemolymph of control insects revealed a pattern of change during the fifth instar, a steady increase in protein concentration throughout the stadium followed by a sudden fall at the time of the moult, which is typical of most insect species that have been studied (see review by WYATT and PAN, 1978). Unlike the majority of cases listed by WYATT and PAN (1978), the adult insects of the present study showed only a slightly increased haemolymph protein concentration in 8-day old insects compared with newly moulted adults. However, other workers have also found little change in protein concentration during the maturation of adult locusts (TOBE and LOUGHTON, 1967; KULKARNI and MEHROTRA, 1970), and in those studies where increased blood protein levels were reported, there are wide variations in the protein concentrations detected (HILL, 1962; MINKS, 1967; HILL *et al.*, 1968;

ELLIOT and GILLOT, 1977; GELLISSEN and EMMERICH, 1978). As mentioned in the Introduction, the various attempts to separate locust haemolymph proteins by gel electrophoresis have yielded very different numbers of protein bands. However, the pattern of eight bands found in the present study is similar to the results of MINKS (1967) and EMMERICH and HARTMANN (1973), allowing for the slightly different techniques that were used. In control animals of the present study, protein band 4 was restricted to the haemolymph of 8-day old adult females. Thus, its identity is most probably the yolk protein precursor, vitellogenin, since this protein is similarly limited in its appearance to blood of adult female locusts (see reviews by WYATT and PAN, 1978; ENGELMANN, 1979). The identities of the other protein bands are more problematical except for band 7 which, in part at least, was made up of esterase protein(s). This main band of material staining for esterase was presumably due to the non-specific carboxylesterases which form the bulk of blood esterases and which readily hydrolyse $\alpha$-naphthyl acetate (see reviews by DE KORT and GRANGER, 1981; HAMMOCK, 1985). It was impossible to tell which, if any, of the other esterase bands detected were due to JH-specific esterase. Such specific esterases are known to have low activity on $\alpha$-naphthyl acetate and are normally present in the haemolymph only at low concentrations (see HAMMOCK, 1985) so that the extreme faintness of the secondary esterase bands is not surprising.

Blood protein concentrations in methoprene-treated locusts followed roughly the same developmental changes as were found in control animals, there being no significant difference between the protein levels in the two experimental populations at any of the ages that were studied. A similar result was found with the haemolymph protein bands separated by electrophoresis, with one exception. Protein band 4 was sex-specific, as was the case in control animals, but in methoprene-treated insects this band was present in blood of female locusts from Day 7 of the fifth instar onwards. As mentioned above, protein band 4 probably represents vitellogenin (Vg). These results would, therefore, tend to support the proposal made in Chapter 4 that treatment with methoprene induced precocious vitellogenesis. DHADIALLA and WYATT (1981, 1983) reported that treatment of fifth instar *Locusta* with methoprene stimulated Vg production in fat bodies from both female and, to a lesser extent, male animals. In the present study, there was no evidence for Vg production in methoprene-treated males. However,

DHADIALLA and WYATT (1981, 1983) not only used a more sensitive detection method (immuno-precipitation of Vg with specific antibodies) but they treated insects by injection of 500 $\mu$g of methoprene rather than by topical application. Thus, the actual concentration of JHA experienced by the animals' tissues was probably substantially higher than that received by the insects of the present study.

Several studies have indicated that JH-active compounds can alter the levels of haemolymph esterases (see Introduction for details). In the present study, there was no apparent difference between blood samples from control and methoprene-treated locusts in the levels of esterase activity. However, as mentioned above, the method used to detect esterase activity was not very sensitive to JH-specific esterases. The ideal assay method for JH esterase requires the use of radiolabelled JH (HAMMOCK and QUISTAD, 1981; HAMMOCK and ROE, 1985) but the very high cost of this compound precluded its use in the present study. However, several workers have successfully demonstrated JH-specific esterase activity in *Locusta* with $\alpha$-naphthyl acetate (EMMERICH and HARTMANN, 1973; ERLEY *et al.*, 1975; PETER *et al.*, 1979) so that any substantial change in JH-specific esterase activity in methoprene-treated animals of the present study should have been detected. Therefore, it must be concluded that the application of methoprene had no major effect on the levels of JH-specific esterases in locust haemolymph, nor on the levels of carboxylesterases.

# Chapter VI

# A Cytological Study of Flight Muscle Development and its Response to Methoprene

## 6.1 Introduction

The indirect flight muscles of adult insects consist of two paired groups, the dorso-ventral muscles and the dorsal longitudinal muscles. Contraction of the dorso-ventral muscles lowers the tergal region of the thorax and causes elevation of the wings due to their articulation with the thoracic walls. The dorsal longitudinal muscles force the tergum upwards by their contraction and so depress the wings (for a more detailed description, see ELDER, 1975). This arrangement means that the muscles only need to contract a very short distance in order to effect a full movement of the wings and WEIS-FOGH (1956) has estimated that the dorsal longitudinal muscles of *Locusta migratoria* shorten by as little as 5% of the resting length, both *in vivo* and *in vitro*. The near isometric operating conditions of these muscles are reflected in their structure (see below). Insect muscles are made up of a number of muscle fibres, each of which consists of many myofibrils lying in a multinucleate matrix (the sarcoplasm) and surrounded by a plasma membrane (the sarcolemma). The sarcoplasm also contains mitochondria and the membrane systems of the sarcoplasmic reticulum and transverse tubular system (see reviews of insect muscle structure by PRINGLE, 1974; ELDER, 1975; SMITH, 1984). With the light microscope, it is usually possible to distinguish three basic categories of insect flight muscle, based on the distribution of myofibrils within the muscle fibres (PRINGLE, 1957). In tubular muscle, characteristic of the Odonata and Dictyoptera, a central column of nuclei is surrounded by radially arranged rows of myofibrils, with mitochondria lying between the fibril rows. Close-packed muscle is found in the higher Orthoptera (including *Locusta*, BÜCHER, 1965), Trichoptera and Lepidoptera. The myofibrils are packed tightly throughout the whole muscle fibre and separated from each other by large mitochondria, while the nuclei are flattened and peripheral. Fibrillar flight muscles are commonly of large diameter and are found only in the Coleoptera, Hymenoptera, Diptera and Hemiptera. Large, cylindrical fibrils fill the fibre,

peripheral nuclei are arranged in longitudinal rows, and longitudinal columns of large mitochondria fill the spaces between the myofibrils. PRINGLE (1957) also distinguished two functionally discrete groups of insect flight muscle, synchronous (tubular and close-packed) and asynchronous (fibrillar). In synchronous muscles, there is a 1:1 ratio between the frequency of motor nerve impulses and the frequency of muscular contractions whereas, in asynchronous muscles, the contraction rate greatly exceeds, and is relatively independent of, the impulse frequency of the motor nerves. Insect muscles are cross-striated, their myofibrils showing characteristic banding patterns when suitably prepared (e.g. SCHÄFER, 1891) and the bands of adjacent myofibrils being transversely aligned so that the whole muscle fibre appears striated (see reviews by ELDER, 1975; SMITH, 1984). The banding pattern is similar to that found in vertebrate striated muscle with the two main bands, the darker A band and the lighter I band, alternating along the length of the myofibril (SMITH, 1972). In the middle of each I band is a dark line, the Z disc, and the unit of length between two Z discs is called the sarcomere. In the middle of the A band is a lighter region, the H band, which is bisected by a darker line, the M line. The striation pattern is due to the arrangement of thick filaments (myosin) and thin filaments (actin) (HANSON and HUXLEY, 1953, 1955; HANSON, 1956). The thick filaments run the length of the A band, and the M line is caused by cross-links between these filaments. The thin filaments are attached to the Z disc and run through the I bands and into the A band up to the H band. However, this pattern is slightly different in close-packed muscle, as shown in ultrastructural studies of flight muscle from Orthopterans *Locusta migratoria* (VOGELL *et al.*, 1959; BROSEMER *et al.*, 1963; BÜCHER, 1965; AL-ROBAI, 1981), *Schistocerca gregaria* (RICHARD *et al.*, 1971), *Neoconocephalus robustus* (ELDER, 1971) and *Homorocoryphus nitidulus* (ANSTEE, 1971), and from various Lepidoptera (AUBER, 1967a, 1967b; REGER, 1967; REGER and COOPER, 1967; BIENZ-ISLER, 1968a, 1968b). In general, the H band is indistinct with no visible M line, although this zone is sometimes marked by the presence of small granules. The sarcomeres are short and the I bands are especially narrow which may be a reflection of the short contraction distance of indirect flight muscle (ELDER, 1975). In transverse sections, the myofilaments display a regular arrangement of 6 thin filaments in orbit round each thick filament with an actin/myosin ratio of 3:1. This arrangement, first

described by HUXLEY and HANSON (1957), seems to be common to all insect flight muscles (PRINGLE, 1974; SMITH, 1984).

Insect flight muscle is reported to be the most metabolically active tissue known (WEIS-FOGH, 1964; SACKTOR, 1970) and, in flight, the insect's respiratory rate may increase by as much as 50–100 times the resting rate. The high energy requirements of the tissue are met by the large and numerous flight muscle mitochondria which can occupy up to 30–40% of total muscle fibre volume (ELDER, 1975). In order to supply the mitochondria with sufficient oxygen, the flight muscles are richly supplied with tracheae and, with the exception of certain Odonata (SMITH, 1966b), the tracheoles invaginate into the muscle cells to terminate on the mitochondria, thus reducing the intracellular diffusion pathway for oxygen (WIGGLESWORTH and LEE, 1982; WIGGLESWORTH, 1983). This system is so efficient that virtually no oxygen debt is built up during flight (SACKTOR, 1970; WIGGLESWORTH, 1983) and studies of the catabolic enzyme systems of insect flight muscles, including those of *Locusta* (BEENAKKERS *et al.*, 1975), show that they have very little capacity for anaerobic metabolism (KAHN and DE KORT, 1978; BEENAKKERS *et al.*, 1985). The sarcoplasmic reticulum (SR) of the muscle fibre is considered to be involved in the coupling of excitatory nerve impulses with muscular contraction (see review by EBASHI, 1980). Nervous stimulation of muscle fibres induces the release of calcium ions from the SR into the sarcoplasm which causes contraction of the myofibrils. The SR is particularly well developed in synchronous flight muscles where the rate of muscular contraction is high (see reviews by SMITH, 1965b, 1966a). Sarcoplasmic reticulum was estimated to occupy 5% of the volume of dragonfly flight muscle (SMITH, 1966b), while it took up 19% of fibre volume in the very fast-contracting, stridulatory muscles of the male tettigoniid *Neoconocephalus* (ELDER, 1971). In contrast, the SR is sparse and poorly developed in asynchronous flight muscles where muscular contraction is relatively independent of nervous stimulation (see ELDER, 1975), occupying only 0.2% of fibre volume in the fibrillar flight muscles of *Phormia regina* (SMITH and SACKTOR, 1970). Generally, the SR forms a fenestrated curtain of cisternae surrounding the myofibrils and running the entire length of the muscle fibre without interruption (see reviews b y SMITH, 1965b, 1966a). In vertebrate muscle the SR is segmentally arranged, being divided at intervals by transversely oriented tubules of the T system to form 'triads' (ANDERSSON-

CEDERGREN, 1959). In insect muscle, the T-tubules run alongside, and in close apposition to the cisternae of the SR to form 'dyads', comparable to the triads of vertebrate muscle (SMITH, 1966a). Triads have also been reported in insect muscle, being found where a T-tubule runs between two adjacent sheets of SR (SMITH, 1966a). The T-tubule system of synchronous muscle is very regular in its arrangement, the tubules pass transversely into the muscle fibre at the A band region of the sarcomere, forming two dyads per sarcomere unit, midway between the Z and H bands. As was found in vertebrate muscle (HUXLEY, 1964), the T-tubules of insect muscle fibres are continuous with the plasma membrane (SMITH, 1966b; SMITH and ALDRICH, 1971) and can be seen as invaginations of the sarcolemma (HAGOPIAN and SPIRO, 1967). According to the present concept of excitation/contraction coupling, when a nerve impulse arrives at the sarcolemma, the resulting membrane depolarisation is propagated deep into the muscle fibres by the T-tubules and transmitted to the SR via dyadic connections, thus minimising the delay between stimulation and maximal activation (see reviews by SMITH, 1966a; AIDLEY, 1985).

Ultrastructural studies of flight muscle development have been carried out on a number of insect species, including *Locusta migratoria* (BROSEMER *et al.*, 1963; BÜCHER, 1965; AL-ROBAI, 1981; VAN DEN HONDEL-FRANKEN and FLIGHT, 1981), *Schistocerca gregaria* (RICHARD *et al.*, 1971), *Periplaneta americana* (TEUTSCH-FELBER, 1970), *Homorocoryphus nitidulus* (ANSTEE, 1971), *Antheraea pernyi* (BIENZ-ISLER, 1968b; NÜESCH, 1985), *Manduca sexta* (RHEUBEN and KAMMER, 1980), *Hyalophora cecropia* (MICHEJDA, 1964), *Apis mellifera* (HEROLD, 1965) and *Calliphora erythrocephala* (AUBER, 1969; TRIBE and ASHURST, 1972). The flight muscles of several hemimetabolous insects are already present at, or shortly after, hatching but remain small and undifferentiated until the last larval stage when they start to develop (TIEGS, 1955; PRINGLE, 1957; HINTON, 1959). This pattern may be true for *Locusta*, where flight muscle rudiments are present by the second larval instar but their development does not begin until the fourth moult (BROSEMER *et al.*, 1963; BÜCHER, 1965). During the maturation of the flight muscle, BROSEMER *et al.* (1963) identified four distinct phases of development, called larval growth, moulting interval, phase of differentiation and phase of duplication. At the start of the fifth instar, the fibres of the precursor muscle are small and only slightly differentiated, some

small myofibrils being present with irregular cross-striations. The mitochondria are very small and sparse, occupying only about 6% of the volume of the muscle, and large amounts of glycogen are present in the sarcoplasm (BÜCHER, 1965). Larval growth lasts for about the first seven days of the fifth instar and is characterised by a rapid increase in the numbers of myofibrils, by longitudinal splitting of existing fibrils and proliferation of the myofilaments, and of the mitochondria, but with little differentiation. There is a temporary pause in muscle growth during the moulting interval, from just before to just after the imaginal moult, at which time the process of muscle fibre tracheolation is initiated. Two or three days before the final moult, the muscle fibres are penetrated by cytoplasmic extensions from surrounding tracheoblasts and small tracheoles form within these processes at the time of moulting (BÜCHER, 1965; VAN DEN HONDEL-FRANKEN and FLIGHT, 1981). The differentiation phase lasts for the first few days of adult life, during which the formation of interfibrillar tracheoles is completed and muscle growth is resumed with a full differentiation of flight muscle structure and metabolic enzyme pattern. At this point, the locusts are able to flutter their wings but cannot yet fly. Finally, there is a duplication phase, from about Day 5 to Day 8 of the adult, in which the structural and enzymatic components of the flight muscle are duplicated, the size and numbers of myofibrils and mitochondria increasing substantially. The growth in mitochondrial size is particularly marked so that, in the mature flight muscle, mitochondria take up 30% of flight muscle volume (BROSEMER *et al.*, 1963; BÜCHER, 1965). In the close-packed flight muscles of *Schistocerca* (RICHARD *et al.*, 1971) and *Homorocoryphus* (ANSTEE, 1971), adult growth is also marked by decreasing levels of sarcoplasmic glycogen so that little remains in the mature muscle.

Several studies have shown that elevated levels of JH during an insect's last larval instar can prevent the normal development of flight muscle. Thus, POELS and BEENAKKERS (1969) found that implantation of active corpora allata into newly moulted fifth instar *Locusta migratoria* substantially reduced the subsequent, normal increase in dry weight and protein content of the dorsal longitudinal flight muscles. On the other hand, allatectomy of fourth instar *Locusta* stimulated precocious flight muscle development such that the tissue acquired the adult pattern of metabolic enzymes after the insects had moulted to small adultoid forms (BEENAKKERS and VAN DEN BROEK, 1976). The same work-

ers also reported that the flight muscles of CA-implanted, fifth instar locusts failed to develop properly and remained metabolically undifferentiated up to two weeks after their moult to imperfect adults. These results were confirmed by VAN DEN HONDEL-FRANKEN *et al.* (1980) who found that implantation of active CA into 1-day old, fifth instar *Locusta* gave rise to an extra larval stage. Throughout this supernumary larval instar, development of the dorsal longitudinal flight muscles was retarded with regard to metabolic enzyme activity. In a related study, VAN DEN HONDEL-FRANKEN and FLIGHT (1981) investigated the process of tracheolation of flight muscle fibres in CA-implanted locusts. Invagination of tracheoblasts into the muscle fibres was delayed until the end of the extra larval instar. Further experiments with *Locusta* demonstrated that implantation of active CA on Days 5 and 6 of the fourth instar completely prevented the normal invagination of tracheoblasts into flight muscle fibres at the end of the fifth instar, whereas implantation into 2-, 3- and 5-day old fifth instar larvae caused only partial and decreasing inhibition of flight muscle tracheolation (VAN DEN HONDEL-FRANKEN, 1982). It was concluded that the initiation of flight muscle development was correlated with the virtual absence of JH from the haemolymph and was disrupted by high JH titres, but that JH rapidly became ineffective once development was in progress. VAN DEN HONDEL-FRANKEN (1982) suggested that the loss of sensitivity to JH might be associated with the small, intermoult peak in haemolymph ecdysteroid which occurs in *Locusta* on about the third day of the last larval instar (BAEHR *et al.*, 1979).

In view of the above reports and the abnormal appearance of flight muscle seen in methoprene-treated locusts of the present study (see Chapter 3), the application of methoprene to fifth instar *Locusta* might be expected to have a profound effect on flight muscle development. The purpose of the present study was to make a detailed cytological investigation of flight muscle development in the hopes of clarifying any disruptive effects that methoprene might have.

## 6.2 Materials and Methods

Newly moulted fifth instar locusts were topically treated with either absolute ethanol or methoprene (475 $\mu$g or 95 $\mu$g per insect). The maintenance, treatment and sampling of these experimental insects were as described in General Materials and Methods. On each day that the animals were sampled, two males and two

females were taken from each treatment. The insects were killed by twisting the head to break the 'neck' membrane. The posterior tip of the abdomen was cut off and the head, with the gut attached, was removed. The carcase was cut open ventrally and the fat body overlying the flight muscles was removed with tissue paper. The dorsal longitudinal flight muscles were then dissected out and prepared for electron microscopy according to the protocol described in General Materials and Methods.

In order to estimate the relative volume occupied by mitochondria within muscle fibres, a square lattice of dots was superimposed on electron micrographs of the tissue and the numbers of dots lying over mitochondria or over other components of the muscle fibre were counted. From these values it was possible to calculate the relative area of mitochondria on each micrograph and, by combining the results, to determine the relative volume of mitochondria within the muscle fibres. The principles of this type of analysis are described by STEER (1981).

## 6.3 Results

In the present study, there was no observable difference between the sexes with regard to flight muscle development, neither with control animals nor experimentals. Therefore, the following descriptions of developing flight muscles make no distinction between tissues from male and female animals.

Representative cross-sections of fibres from the dorsal longitudinal flight muscles of control insects are shown in Plate 6.1. During the fifth instar, the muscle fibres steadily grew in diameter while their peripheral nuclei remained constant in size (Plate 6.1, **a**, **b** & **c**). There was little further growth immediately after the final moult (Plate 6.1, **d**) but this was later resumed so that, by Day 8 of adult life, the fibres were greatly enlarged (Plate 6.1, **e** & **f**). The muscle fibres from 8-day old adults showed large variations in size (compare **e** and **f** of Plate 6.1), indicating that flight muscle development was not yet finished in some individuals. Plates 6.2 (**a** & **b**) and 6.3 (**a** & **b**) show the fine structure of control flight muscle fibres on Day 4 of the fifth instar. The fibres contained small myofibrils and very small and sparse mitochondria (Plate 6.2, **a**, and Plate 6.3, **a**). T-tubules were present and dyads had already formed between these and the rudimentary sarcoplasmic reticulum (Plate 6.2, **a**). Within the myofibrils, the myofilaments were irregularly arranged (Plate 6.2, **b**) and showed relatively poor

**Plate 6.1 — Low Power Electron Micrographs of Control Flight Muscle Fibres, Transverse Sections**

**a.** Day 4 of the fifth instar, scale = 10 $\mu$m.
**b.** Day 7 of the fifth instar, scale = 10 $\mu$m.
**c.** Day 10 of the fifth instar, scale = 10 $\mu$m.
**d.** Day 1 of the adult stadium, scale = 10 $\mu$m.
**e** & **f.** Day 8 of the adult stadium, scale = 10 $\mu$m.

Dorsal longitudinal flight muscle consisted of muscle fibres with peripheral nuclei. There was large variation in fibre size within each muscle but the fibres shown represented the average size. The muscle fibres increased in size during the fifth instar (**a**, **b** & **c**) but there was little further growth in the newly moulted adults (**d**). By Day 8 of adult life, the fibres were substantially enlarged although muscle fibres varied greatly in size between individual adults (compare **e** and **f**). Key: MF– muscle fibre; N– nucleus.

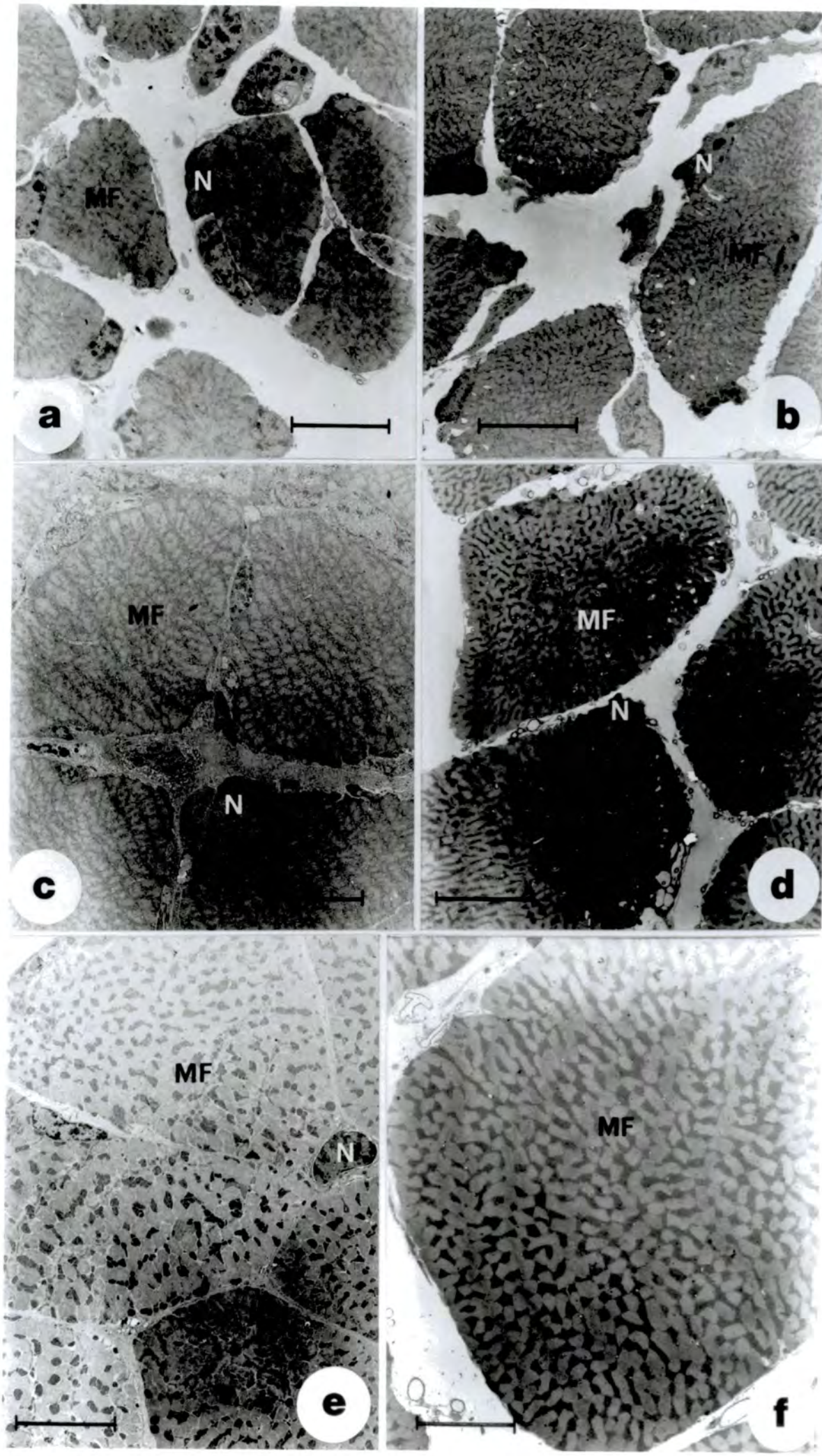

MF
N
a
N
MF
b
MF
N
c
MF
N
d
MF
N
e
MF
f

**Plate 6.2 — Electron Micrographs of Control Flight Muscle, Fifth Instar, Transverse Sections**

**a.** Muscle fibre on Day 4 of the fifth instar. Note the small size of the myofibrils and the rudimentary SR running between them. T-tubules can be seen, penetrating from the surface of the muscle fibre and forming dyads with SR. Note the small size and scarcity of mitochondria. Scale = 0.5 $\mu$m.

**b.** Detail of 4-day old fifth instar fibre. Note the disordered and widely spaced arrangement of myofilaments in the fibrils. Note also the very few cristae and electron-lucid matrix within mitochondria. Scale = 0.5 $\mu$m.

**c.** Muscle fibre on Day 7 of the fifth instar. Note the increased size of both myofibrils and mitochondria compared with Day 4 (**a**), and the increased mitochondrial population. Scale = 0.5 $\mu$m.

**d.** Detail of 7-day old fifth instar fibre. Note the disordered arrangement myofilaments within myofibrils, and the presence of glycogen granules between the fibrils. Mitochondria had few cristae and electron-lucid matrices. Occasional cytoplasmic processes from tracheoblasts were seen, enclosed within dilated T-tubules. Scale = 0.5 $\mu$m.

**e.** Muscle fibre on Day 10 of the fifth instar. Note the reduced size of the myofibrils and the increased numbers of mitochondria compared to Day 7 (**c**). The mitochondria were also increased in size, the SR was more extensive and dyadic connections with T-tubules were numerous. Scale = 0.5 $\mu$m.

**f.** Detail of 10-day old fifth instar fibre. Note the closely packed but disordered arrangement of myofilaments in the myofibrils. Mitochondria contained few, widely spaced cristae and the mitochondrial matrix was electron-lucid. Scale = 0.5 $\mu$m.

Key: c– mitochondrial crista; D– dyad; F– myofibril; Gl– glycogen; M– mitochondrion; m– myosin filament; SR– sarcoplasmic reticulum; T– T-tubule; Tb– tracheoblast.

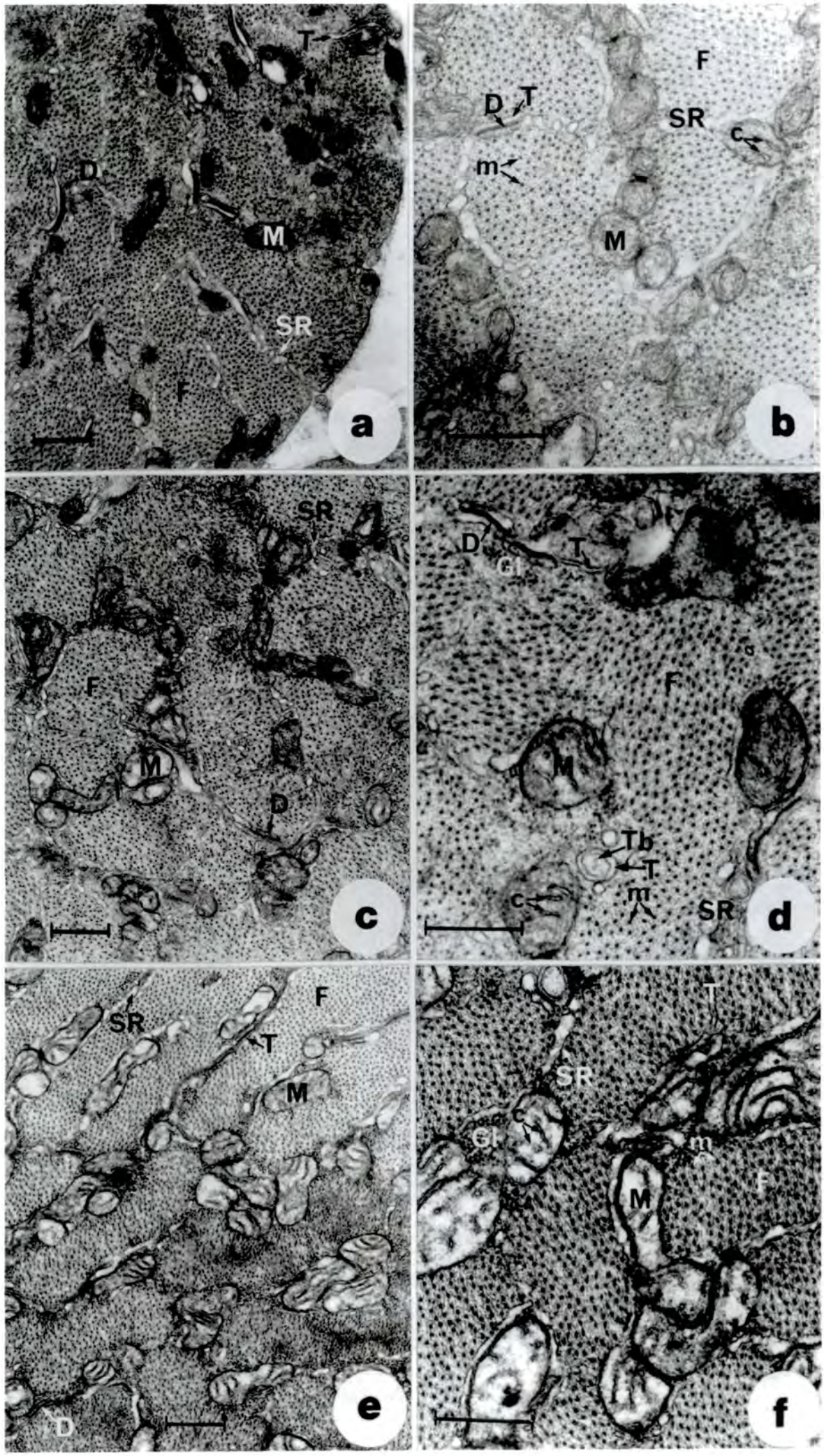

T
D
M
SR
F
a
F
D
T
SR
c
m
M
b
SR
F
M
D
c
D
Cl
T
F
M
Tb
T
m
c
SR
d
SR
F
T
M
D
e
T
SR
c
Cl
m
M
F
f

**Plate 6.3 — Electron Micrographs of Control Flight Muscle, Fifth Instar, Longitudinal Sections**

**a.** Muscle fibre on Day 4 of the fifth instar. Note the small size of the myofibrils and the lack of strict parallel register between the adjacent, indistinct Z discs. Note also the small size and scarcity of the mitochondria. Scale = 2 $\mu$m.

**b.** Detail of 4-day old fifth instar fibre. Note the diffuse, ill-defined appearance of the Z discs and the poor longitudinal alignment of the myofilaments. Occasional dyads were seen , formed between the sparse SR and T-tubules. Note the large amounts of glycogen between myofibrils. Average sarcomere length was $\sim$1.6 $\mu$m. Scale = 0.5 $\mu$m.

**c.** Muscle fibre on Day 7 of the fifth instar. Note the increased width of the myofibrils compared with Day 4 (**a**), and the greatly increased numbers of mitochondria. Also note the lack of strict parallel register between Z discs. Scale = 2 $\mu$m.

**d.** Detail of 7-day old fifth instar fibre. Note the increased definition of the Z discs compared with Day 4 (**b**), and the increased size of the mitochondria. Myofilaments showed poor longitudinal alignment. Note the presence of dilated T-tubules (arrows). Average sarcomere length was $\sim$1.2 $\mu$m. Scale = 0.5 $\mu$m.

**e.** Muscle fibre on Day 10 of the fifth instar. Note the increased parallel register of the Z discs compared with Day 7 (**c**). Also note the longitudinal splitting of myofibrils (arrow). Scale = 2 $\mu$m.

**f.** Detail of 10-day old fifth instar fibre. Note the increased longitudinal alignment of myofilaments compared with Day 7 (**d**). Average sarcomere length was $\sim$1.9 $\mu$m. Scale = 0.5 $\mu$m.

Key: D– dyad; F– myofibril; Gl– glycogen; M– mitochondrion; m– myosin filament; N– nucleus; S– sarcomere; SR– sarcoplasmic reticulum; T– T-tubule; Z– Z disc.

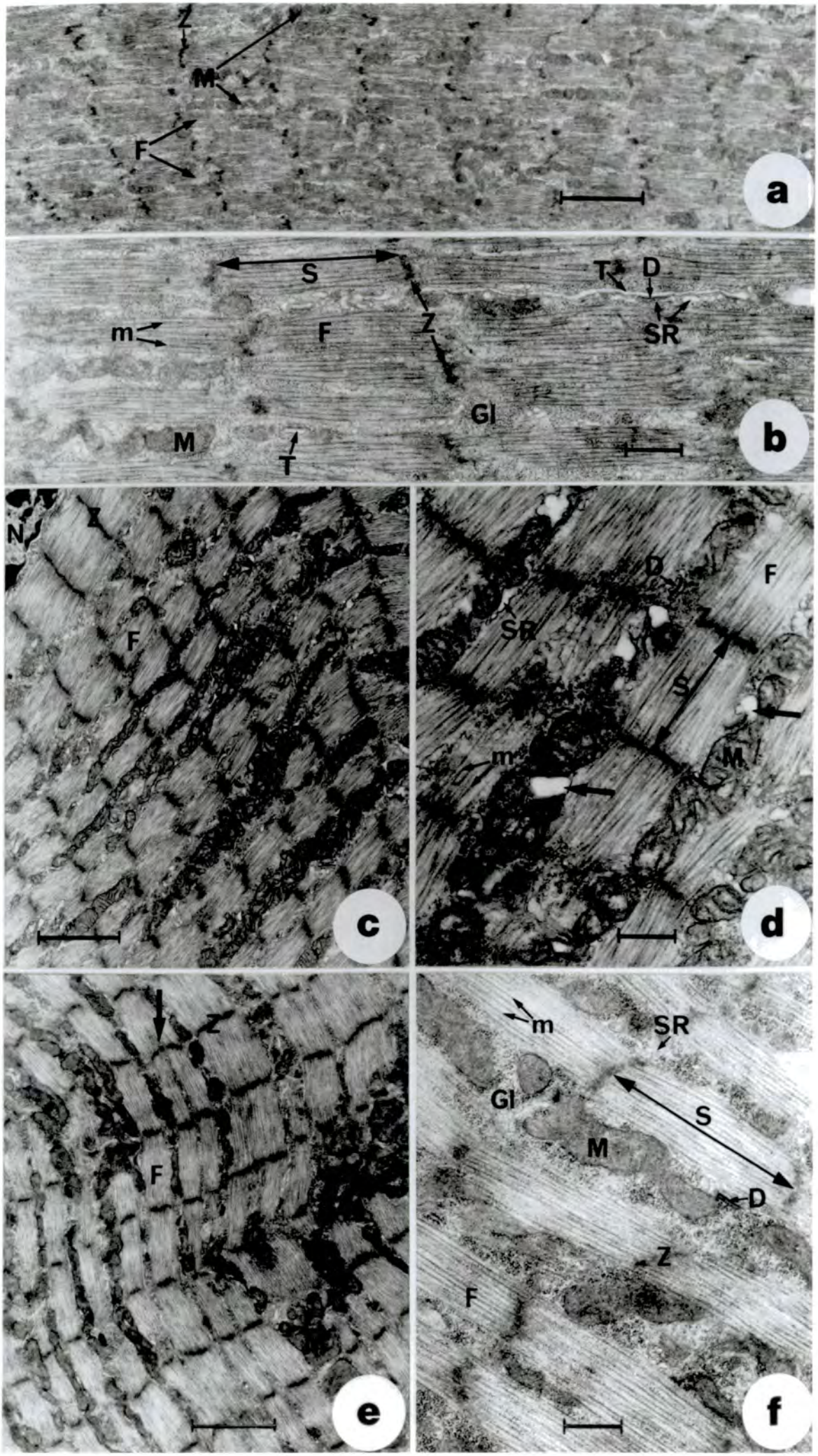

Z
M
F
a
S
T
D
m
F
Z
SR
Gl
M
T
b
N
Z
F
c
D
F
SR
S
m
M
d
Z
F
e
m
SR
Gl
S
M
D
Z
F
f

**Plate 6.4 — Electron Micrographs of Control Flight Muscle, Adult, Transverse Sections**

**a.** Muscle fibre on Day 1 of adult stadium. Note the increased size and numbers of mitochondria, and the more extensive SR, compared with Day 10 of the fifth instar (Plate 6.2, **e**). Also note the presence of interfibrillar tracheoles. Scale = 0.5 $\mu$m.

**b.** Detail of 1-day old adult fibre. Note the thick layer of tracheoblast cytoplasm enclosing interfibrillar tracheoles. Also note the much more ordered arrangement of myofilaments in the fibrils compared with Day 10 of the fifth instar (Plate 6.2, **f**). The mitochondria contained numerous, closely packed cristae in an electron-dense matrix. Scale = 0.5 $\mu$m.

**c.** Muscle fibre on Day 8 of the adult stadium. Note the greatly enlarged mitochondria, myofibrils and SR compared with Day 1 (**a**). Occasionally, the I bands and Z discs of myofibrils could be seen as well as A bands. Scale = 0.5 $\mu$m.

**d.** Detail of 8-day old adult fibre. Note the highly ordered arrangement of myosin filaments in the myofibrils, forming a regular hexagonal pattern. Also note irregularities in the numbers of actin filaments surrounding each myosin filament. Both actin and myosin filaments were present in the A bands of the myofibrils whereas only actin filaments were seen in the I bands. Note the lack of tracheoblast cytoplasm around interfibrillar tracheoles. Also note the densely packed cristae and electron-opaque matrix of mitochondria, and the large cisternae of the SR. Scale = 0.5 $\mu$m.

Key: A– A band of sarcomere; a– actin filament; c– mitochondrial crista; D– dyad; F– myofibril; Gl– glycogen; I– I band of sarcomere; M– mitochondrion; m– myosin filament; SR– sarcoplasmic reticulum; T– T-tubule; Tb– tracheoblast; Tr– tracheole; Z– Z disc.

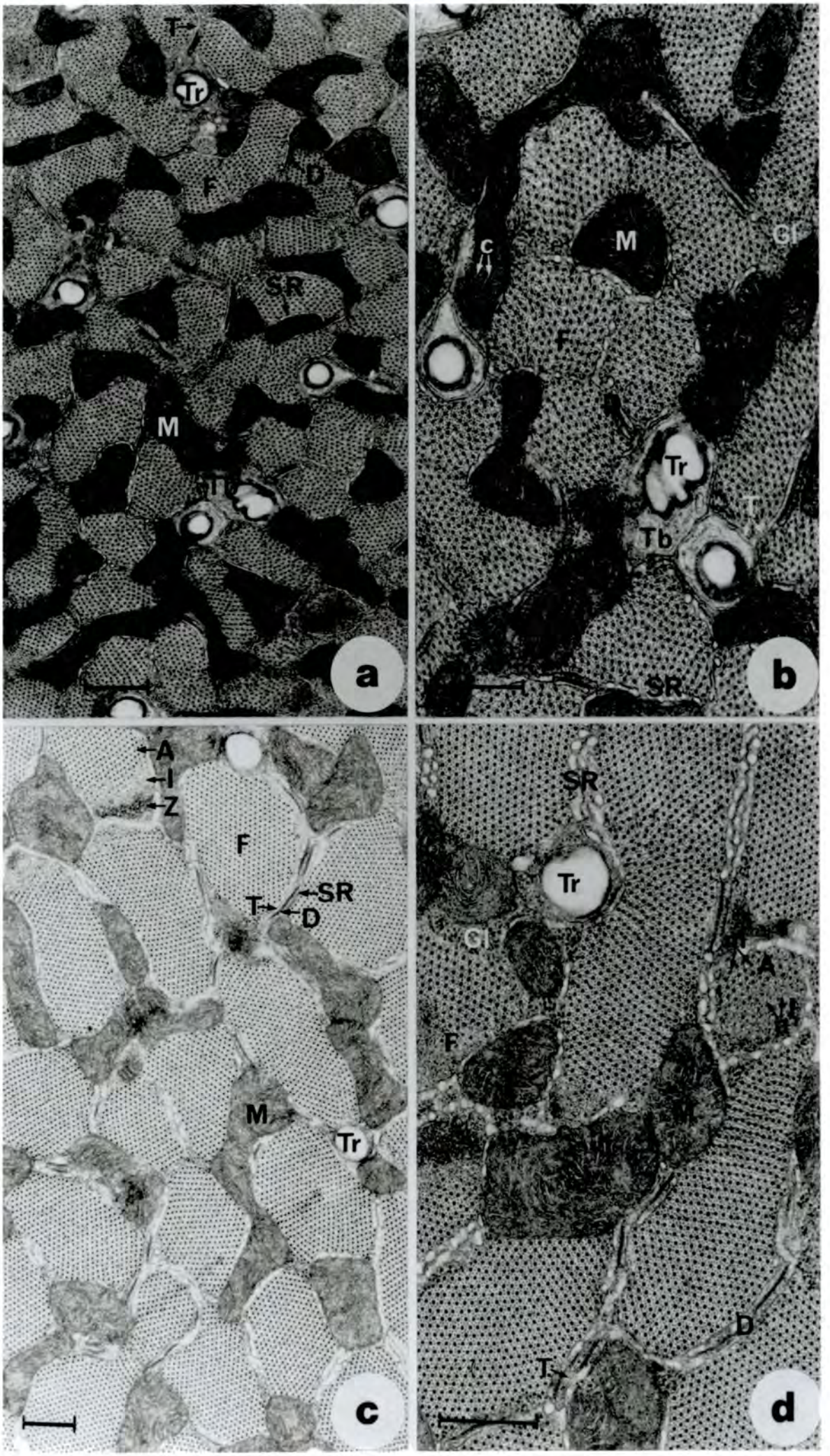

T
Tr
F
D
SR
M
Tr
a
T
c
M
Gl
F
Tr
T
Tb
SR
b
A
I
Z
F
T
SR
D
M
Tr
c
SR
Tr
Gl
A
F
D
T
d

**Plate 6.5 — Electron Micrographs of Control Flight Muscle, Adult, Longitudinal Sections**

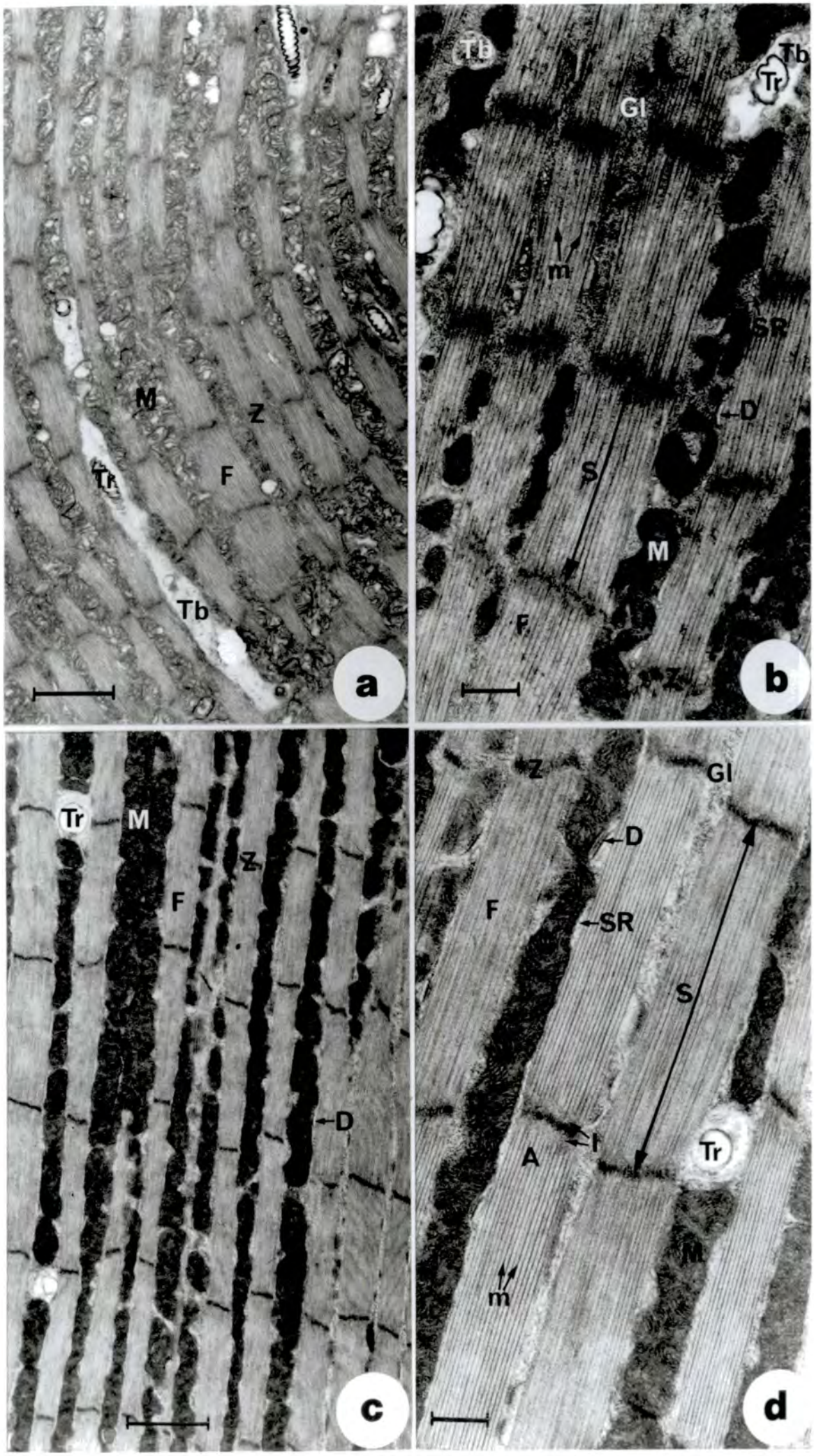
**a.** Muscle fibre on Day 1 of the adult stadium. Note the presence of interfibrillar tracheoles within cytoplasmic processes of tracheoblasts. Also note the electron-lucid matrices of the mitochondria. Scale = 2 $\mu$m.

**b.** Detail of 1-day old adult fibre. Note the reduced definition of the Z discs compared with Day 10 of the fifth instar (Plate 6.3, **f**). Also note the densely packed cristae and electron-opaque nature of the mitochondria, in contrast to those in micrograph **a**. The myofilaments were in good longitudinal alignment and the average sarcomere length was $\sim$1.9 $\mu$m. Scale = 0.5 $\mu$m.

**c.** Muscle fibre on Day 8 of the adult stadium. Note the elongate nature of the mitochondria and their arrangement into columns running alongside the myofibrils. Also note the lack of strict parallel register between adjacent Z discs. Scale = 2 $\mu$m.

**d.** Detail of 8-day old adult fibre. Note the precise longitudinal alignment of the myofilaments, and the appearance of the mitochondria, with closely packed cristae in an electron-dense matrix. Also note the presence of plentiful amounts of glycogen. Separate A bands and I bands were visible in the myofibrils. Average sarcomere length was $\sim$3.2 $\mu$m. Scale = 0.5 $\mu$m.

Key: A– A band of sarcomere; D– dyad; F– myofibril; Gl– glycogen; I– I band of sarcomere; M– mitochondrion; m– myosin filament; S– sarcomere; SR– sarcoplasmic reticulum; T– T-tubule; Tb– tracheoblast; Tr– tracheole; Z– Z disc.


M
Z
F
Tr
Tb
a
Tb
Tr
Tb
Gl
m
SR
D
S
M
F
Z
b
Tr
M
Z
F
D
c
Z
Gl
D
F
SR
S
I
A
Tr
M
m
d

longitudinal alignment (Plate 6.3, **b**). The Z discs of the sarcomeres were not in good parallel register (Plate 6.3, **a**) and lacked definition in their structure (Plate 6.3, **b**). The appearance of the muscle fibres in longitudinal section was similar to that of locust precursor flight muscle as described by BROSEMER *et al.* (1963), with poorly differentiated myofibrils, few mitochondria and plentiful amounts of interfibrillar glycogen (Plate 6.3, **b**). By the seventh day of the fifth instar, mitochondria had greatly increased in both size and numbers (Plate 6.2, **c**) but were still immature in structure with few cristae and electron-lucid matrices (Plate 6.2, **d**). Many of the myofibrils were considerably increased in width (Plate 6.3, **c**) but the average sarcomere length had decreased from $\sim$1.6 $\mu$m to $\sim$1.2 $\mu$m (compare **b** and **d** of Plate 6.3). The Z discs were now more clearly defined but the myofilaments still showed poor longitudinal alignment (Plate 6.3, **d**) and there was no apparent order to their arrangement within the myofibrils (Plate 6.2, **d**). At this time, occasional cytoplasmic protrusions from surrounding tracheoblasts were seen in the muscle fibres (Plate 6.2, **d**). These processes were always present within dilations of the T-tubule system and enlarged T-tubules were frequently found (Plate 6.3, **d**), possibly in order to accommodate further invaginations by tracheoblasts. By Day 10 of the fifth instar, the sarcoplasmic reticulum (SR) was more extensive and there were further increases in mitochondrial size and number (Plate 6.2, **e**). The myofibrils were reduced in width, apparently due to longitudinal splitting of existing fibrils (Plate 6.3, **e**). The myofilaments were more closely packed in the myofibrils, though still not ordered in their arrangement (Plate 6.2, **f**), and now showed much better longitudinal alignment in lengthened sarcomeres (Plate 6.3, **f**). In newly moulted adults, interfibrillar tracheoles were present in the muscle fibres, enclosed within the cytoplasmic processes of tracheoblasts (Plate 6.4, **a**). Mitochondria had undergone substantial increases in size and numbers (Plate 6.4, **a**), and had acquired closely packed cristae and electron-dense matrices (Plate 6.4, **b**), reminiscent of mitochondria from mature flight muscle (BÜCHER, 1965). However, in some muscle fibres, mitochondria retained the electron-lucid matrix of organelles from fifth instar tissue (Plate 6.5, **a**). The SR was more extensive with greater numbers of dyads (Plate 6.4, **a**). The myofilaments showed a more ordered arrangement within the myofibrils (Plate 6.4, **b**) and their longitudinal alignment was also better (Plate 6.5, **b**). There was no further increase in sarcomere length while the

Z discs had thickened and lost some definition of structure (Plate 6.5, **b**). By the eighth day of adult life, there was a substantial enlargement of myofibrils and mitochondria (Plate 6.4, **c**). This was especially noticeable in longitudinal sections (Plate 6.5, **c** & **d**), with single mitochondria frequently extending over more than one sarcomere unit and the sarcomeres themselves having lengthened to $\sim$3.2 $\mu$m (AL-ROBAI, 1981, estimated the average sarcomere length to be 3.2 $\mu$m in the dorsal longitudinal flight muscle of mature *Locusta*). The SR was very extensive, forming numerous dyadic connections with T-tubules (Plate 6.4, **c**), and the SR cisternae were greatly enlarged (Plate 6.4, **d**). Interfibrillar tracheoles were no longer surrounded by a layer of tracheoblast cytoplasm (Plate 6.4, **c**), suggesting that their development was completed. The myofilaments were now in strict longitudinal alignment and, for the first time, A bands and I bands could be discerned in the sarcomeres (Plate 6.5, **d**). The narrowness of the I bands probably reflected the very short contraction distance of the muscle (WEIS-FOGH, 1956). In transverse sections, it could be seen that the myosin filaments were arranged in a highly ordered pattern, but there was still some variation in the numbers of actin filaments surrounding each rod of myosin (Plate 6.4, **d**). This irregularity, together with the imperfect parallel register of the Z discs (Plate 6.5, **c**) and the continued presence of interfibrillar glycogen (Plate 6.5, **d**), suggests that the development of the muscle fibres was not yet complete. To summarise, the fifth instar was characterised by growth of the dorsal longitudinal flight muscle fibres but with relatively little differentiation of myofibrils and mitochondria. There was little increase in the cross-sectional area of the fibres during the final moult but there was a marked increase in differentiation, and the formation of interfibrillar tracheoles commenced. By Day 8 of the adult stadium, both mitochondria and myofibrils had grown substantially in size, and structural differentiation was more-or-less complete. Thus, the development of control flight muscle fibres was similar to the descriptions of locust flight muscle development by BROSEMER *et al.* (1963) and BÜCHER (1965).

The application of 475 $\mu$g of methoprene to newly moulted fifth instar locusts greatly disrupted the growth and differentiation of flight muscle fibres. Plate 6.6 shows representative cross-sections of such muscle fibres. On Day 4 of the fifth instar (Plate 6.6, **a**), the muscle fibres were about the same size as those from control animals of the same age (see Plate 6.1, **a**), but their nuclei were substan-

**Plate 6.6 — Low Power Electron Micrographs of 475 $\mu$g Methoprene-Treated Flight Muscle Fibres, Transverse Sections**

**a**. Day 4 of the fifth instar, scale = 10 $\mu$m.
**b**. Day 7 of the fifth instar, scale = 10 $\mu$m.
**c**. Day 10 of the fifth instar, scale = 10 $\mu$m.
**d**. Day 1 of the adult stadium, scale = 10 $\mu$m.
**e**. Day 8 of the adult stage, scale = 10 $\mu$m.

The dorsal longitudinal flight muscle consisted of muscle fibres with peripheral nuclei. There was large variation in fibre size within each muscle but the fibres shown represented the average size. The muscle fibres slightly increased in size during the fifth instar (**a**, **b** & **c**) but much less than the growth rate of control fibres (see Plate 6.1, **a**, **b** & **c**). During early adult life (**d** & **e**), there was more substantial enlargement of the fibres, but they remained much smaller than those of control adults (see Plate 6.1, **d**, **e** & **f**). In 4-day old fifth instar tissue (**a**) and fibres of newly moulted adults (**d**), the peripheral nuclei were abnormally large.

Key: MF– muscle fibre; N– nucleus.

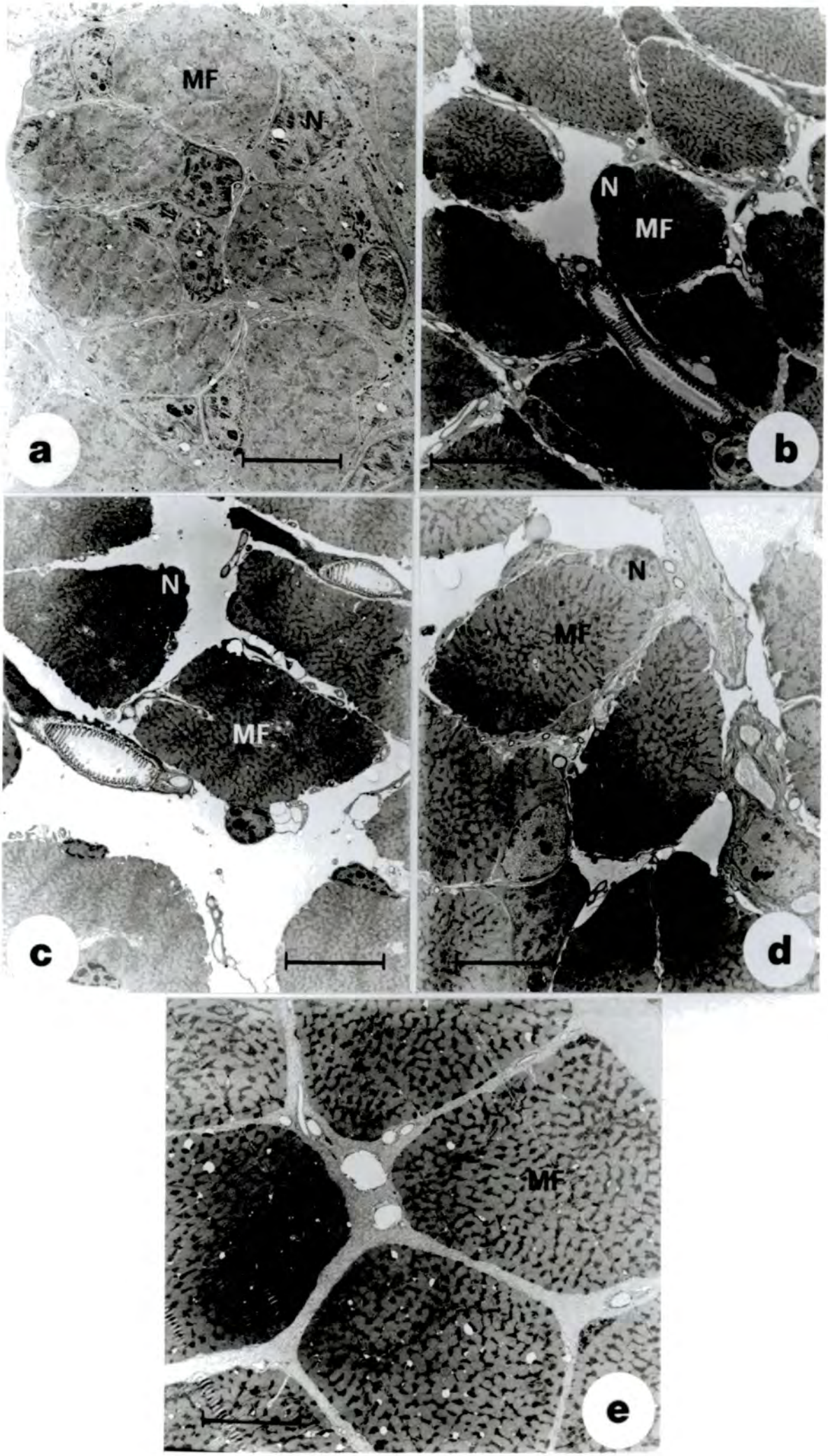

MF
N
a
N
MF
b
N
MF
c
N
MF
d
MF
e

**Plate 6.7 — Electron Micrographs of 475 $\mu$g Methoprene-Treated Flight Muscle, Fifth Instar, Transverse Sections**

**a.** Muscle fibre on Day 4 of the fifth instar. Note the very large size of the myofibrils and the scarcity of the small mitochondria lying between them. T-tubules were present and had formed dyadic connections with the rudimentary SR. Scale = 0.5 $\mu$m.

**b.** Detail of 4-day old fifth instar fibre. Note the widely spaced and disordered arrangement of the myofilaments in the fibrils. The cisternae of the SR were abnormally swollen but mitochondria were normal in appearance (compare with Plate 6.2, **b**). Scale = 0.5 $\mu$m.

**c.** Muscle fibre on Day 7 of the fifth instar. Note the greatly reduced size and increased numbers of myofibrils compared with Day 4 (**a**). Mitochondria were also much more numerous but their size remained unchanged. The SR was very extensive, forming numerous dyadic connections with T-tubules. Scale = 0.5 $\mu$m.

**d.** Detail of 7-day old fifth instar fibre. Note the ordered, regular arrangement of myosin filaments in the myofibrils. There were large variations in the numbers of actin filaments orbiting each myosin rod. Scale = 0.5 $\mu$m.

**e.** Muscle fibre on Day 10 of the fifth instar. Note the presence of cytoplasmic processes from tracheoblasts, containing tracheoles and lying within dilated T-tubules. Myofibrils and mitochondria were slightly increased in size compared with Day 7 (**c**). Scale = 0.5 $\mu$m.

**f.** Detail of 10-day old fifth instar fibre. Note the decreased order in the arrangement of myofilaments compared with Day 7 (**d**). Scale = 0.5 $\mu$m.

Key: c– mitochondrial crista; D– dyad; F– myofibril; Gl– glycogen; M– mitochondrion; m– myosin filament; SR– sarcoplasmic reticulum; T– T-tubule; Tb– tracheoblast; Tr– tracheole.

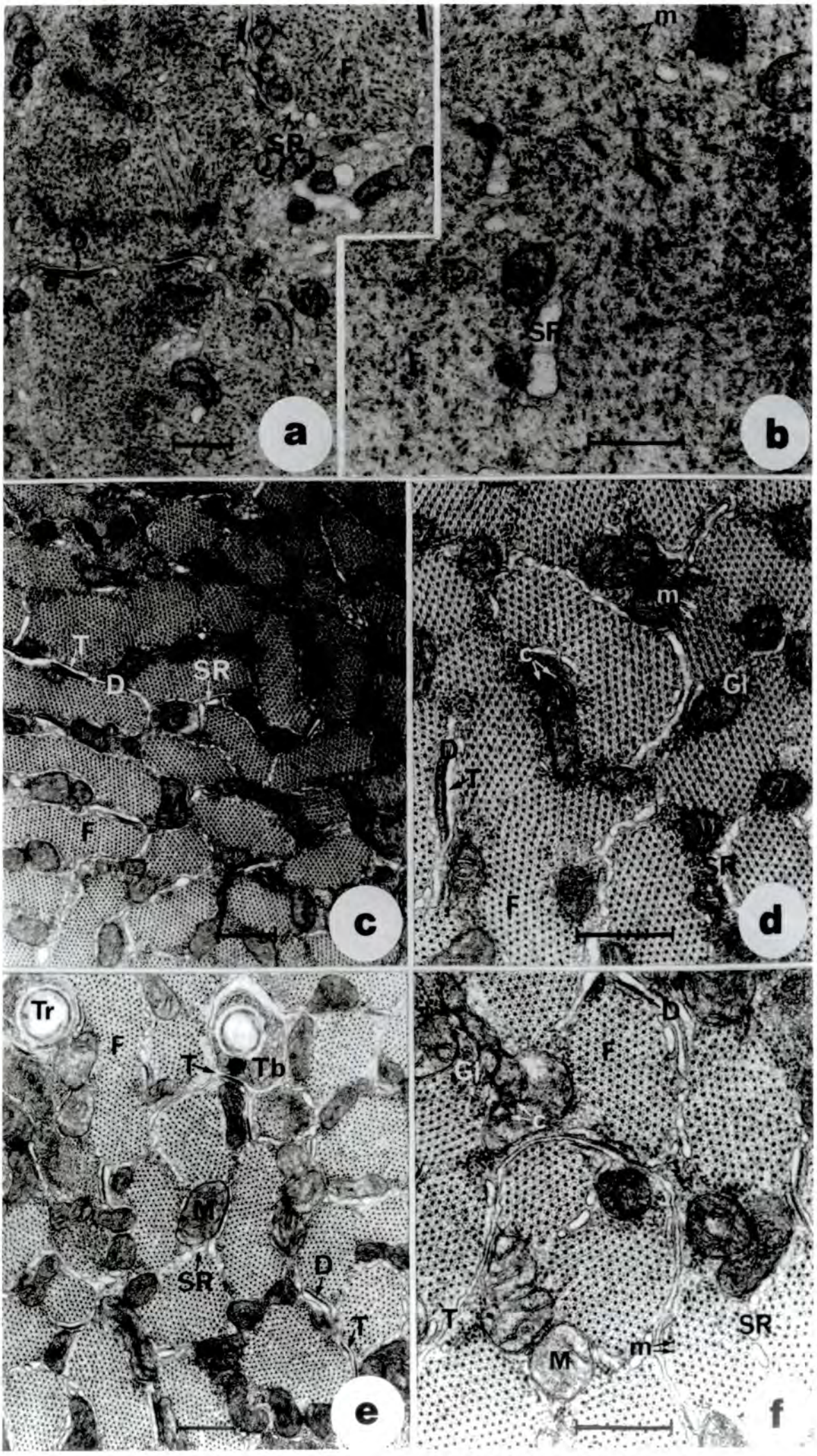

T
F
SR
a
m
SR
b
T
D
SR
F
c
m
C
GI
D
T
SR
F
d
Tr
F
T
Tb
M
SR
D
T
e
D
F
C
T
SR
M
m
f

**Plate 6.8 — Electron Micrographs of 475 $\mu$g Methoprene-Treated Flight Muscle, Fifth Instar, Longitudinal Sections**

**a.** Muscle fibre on Day 4 of the fifth instar. Note the extreme width of the myofibrils and the scarcity of mitochondria lying between them. The Z discs showed relatively good parallel register compared with control tissue on Day 4 of the fifth instar (see Plate 6.3, **a**). Scale = 2 $\mu$m.

**b.** Detail of 4-day old fifth instar fibre. Note the poor longitudinal alignment and wide spacing of the myofilaments. Z discs were thick but clearly defined. Mitochondria were similar in size and appearance to those of 4-day old fifth instar control tissue (see Plate 6.3, **b**). Dyads, glycogen granules and SR were visible between the myofibrils. Average sarcomere length was ~1.5 $\mu$m. Scale = 0.5 $\mu$m.

**c.** Muscle fibre on Day 7 of the fifth instar. Note the much reduced width of myofibrils compared with Day 4 (**a**), and the far more numerous mitochondria arranged in columns running alongside the myofibrils. Also note the near-perfect parallel register of the Z discs. Scale = 2 $\mu$m.

**d.** Detail of 7-day old fifth instar fibre. Note the strict longitudinal alignment of myofilaments, and the clearly defined Z discs in the myofibrils. Also note the elongate profiles of the mitochondria, and their numerous cristae in an electron-dense matrix. Average sarcomere length was ~1.9 $\mu$m. Scale = 0.5 $\mu$m.

**e.** Muscle fibre on Day 10 of the fifth instar. Note the good parallel register, but indistinct structure of the Z discs. Scale = 2 $\mu$m.

**f.** Detail of 10-day old fifth instar fibre. Note the extreme width and diffuse appearance of the Z discs. Also note the precise longitudinal alignment of myofilaments. Average sarcomere length was ~1.6 $\mu$m. Scale = 0.5 $\mu$m.

Key: D– dyad; F– myofibril; Gl– glycogen; M– mitochondrion; m– myosin filament; N– nucleus; S– sarcomere; SR– sarcoplasmic reticulum; Z– Z disc.

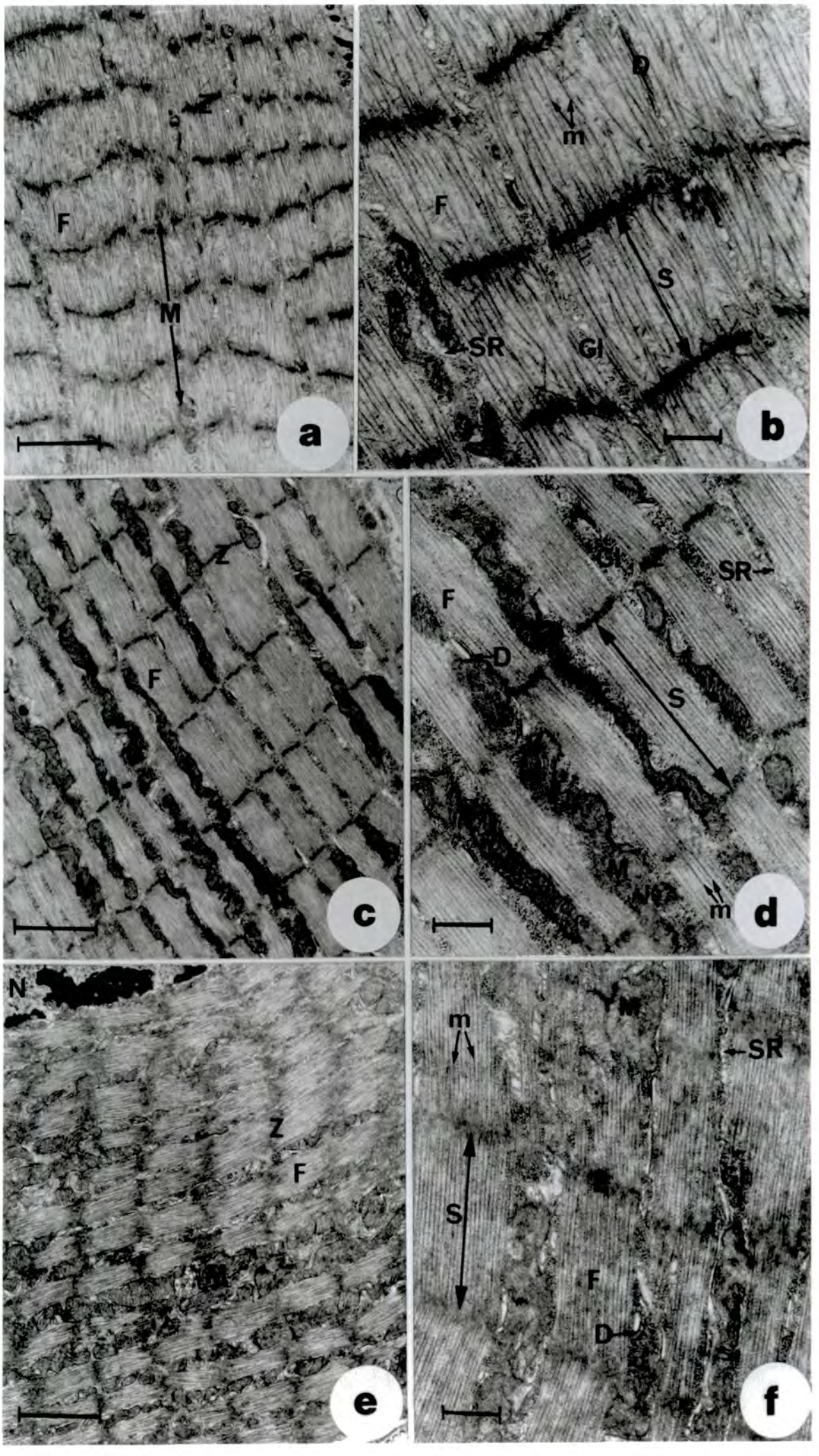

Z
F
M
a
D
m
F
S
SR
GI
b
Z
F
c
SR
F
D
S
M
m
d
N
Z
F
e
m
M
SR
S
F
D
f

**Plate 6.9 — Electron Micrographs of 475 $\mu$g Methoprene-Treated Flight Muscle, Adult, Transverse Sections**

**a.** Muscle fibre on Day 1 of the adult stadium. Note the increased size of myofibrils and mitochondria compared with Day 10 of the fifth instar (Plate 6.7, **e**). The SR was extensive and formed numerous dyadic connections with T-tubules. Scale = 0.5 $\mu$m.

**b.** Detail of 1-day old adult fibre. Note the increased disorder in the arrangement of myofilaments compared with Day 10 of the fifth instar (Plate 6.7, **f**). Mitochondria contained numerous cristae in an electron-dense matrix. Scale = 0.5 $\mu$m.

**c.** Muscle fibre on Day 8 of the adult stadium. Note the increased size of myofibrils and, especially, mitochondria compared with Day 1 (**a**). Also note the greatly enlarged SR. Scale = 0.5 $\mu$m.

**d.** Detail of 8-day old adult fibre. Note the completely disordered arrangement of myofilaments in the myofibrils. Compare the extremely swollen cisternae of the SR with those of control tissue (see Plate 6.4, **d**), but also note the apparently normal dyadic connections with T-tubules. The large mitochondria were also normal in appearance. Scale = 0.5 $\mu$m.

Key: c– mitochondrial crista; D– dyad; F– myofibril; Gl– glycogen; M– mitochondrion; m– myosin filament; SR– sarcoplasmic reticulum; T– T-tubule.

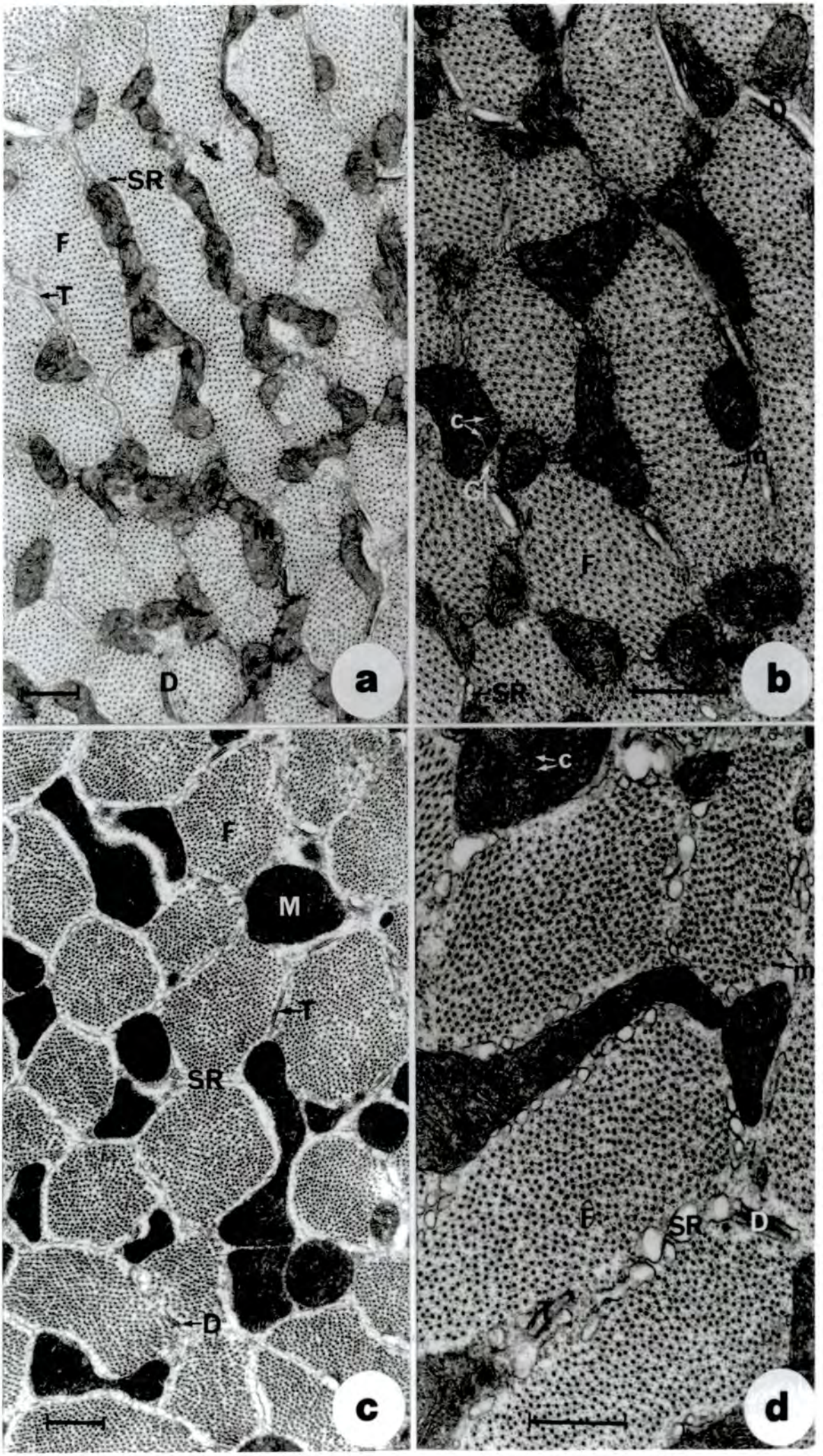

SR
F
T
D
a
C
M
b
F
M
T
SR
D
c
C
M
F
SR
D
d

**Plate 6.10 — Electron Micrographs of 475 $\mu$g Methoprene-Treated Flight Muscle, Adult, Longitudinal Sections**

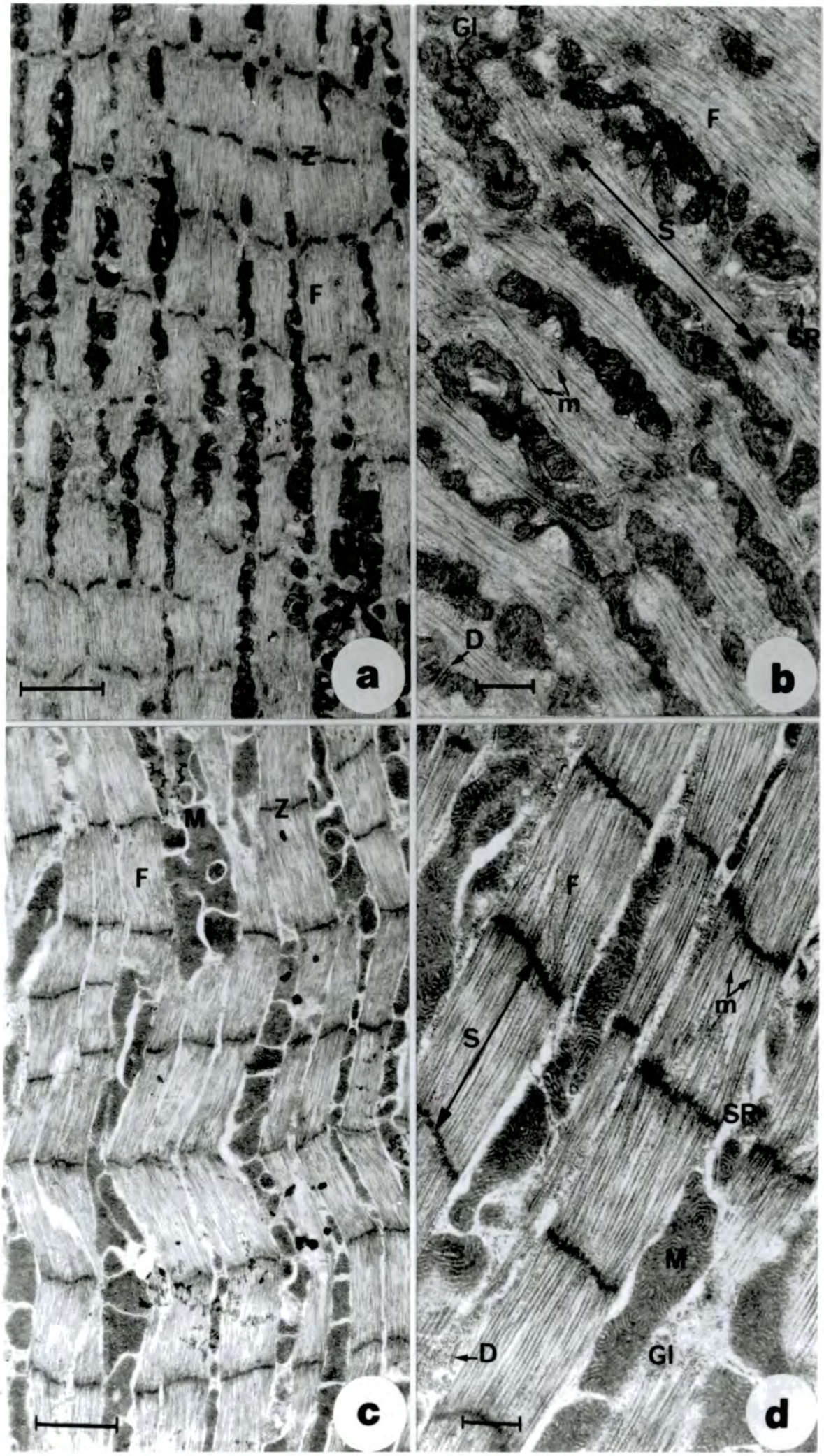
**a.** Muscle fibre on Day 1 of the adult stadium. Note the decreased parallel register of Z discs compared with Day 10 of the fifth instar (Plate 6.8, **e**). Also note the scarcity of mitochondria compared with control tissue (see Plate 6.5, **a**). Scale = 2 $\mu$m.

**b.** Detail of 1-day old adult fibre. Note the indistinct appearance of the Z discs, though less diffuse compared with Day 10 of the fifth instar (Plate 6.8, **e**). Also note the poor longitudinal alignment of the myofilaments. Average sarcomere length was ~2.3 $\mu$m. Scale = 0.5 $\mu$m.

**c.** Muscle fibre on Day 8 of the adult stadium. Note the increased size of mitochondria compared with Day 1 (**a**), but their smaller size than those of control tissue (see Plate 6.5, **c**). Also note the much shorter length of the sarcomeres compared with the controls. The Z discs were in poor parallel register. Scale = 2 $\mu$m.

**d.** Detail of 8-day old adult fibre. Note the poor longitudinal alignment of the myofilaments, and the enlarged SR compared with control tissue (see Plate 6.5, **d**). The Z discs were more clearly defined compared with Day 1 (**b**). Mitochondria contained numerous, closely packed cisternae in an electron-dense matrix. Average sarcomere length was ~1.9 $\mu$m. Scale = 0.5 $\mu$m.

Key: D– dyad; F– myofibril; Gl– glycogen; M– mitochondrion; m– myosin filament; S– sarcomere; SR– sarcoplasmic reticulum; Z– Z disc.


Z
F
a
Gl
F
S
SR
m
D
b
M
Z
F
c
F
m
S
SR
M
D
Gl
d

tially larger. During the rest of the fifth instar (Plate 6.6, **b** & **c**), there was little increase in muscle fibre size and the fibres were much smaller than those from control animals (see Plate 6.1, **b** & **d**), indicating that methoprene treatment greatly reduced flight muscle growth. There was some enlargement of the muscle fibres during early adult life (compare **d** and **e** of Plate 6.6), but much less than that found in the controls (see Plate 6.1, **d**, **e** & **f**). Treatment with 475 $\mu$g of methoprene also had a profound effect on the fine structure of the muscle fibres. In the flight muscles of 4-day old fifth instar animals, although the treated fibres were the same size in cross-section as the control fibres (see above), they contained fewer myofibrils which were extremely large (Plate 6.7, **a**). Within the fibrils, the myofilaments were widely spaced and very disordered (Plate 6.7, **b**). The SR seemed unusually sparse (Plate 6.7, **a**) with swollen cisternae (Plate 6.7, **b**), but dyadic connections with T-tubules were normal in appearance. The mitochondria were also scarce (Plate 6.7, **a**) but were similar in size and structure to those in control tissue (Plate 6.7, **b**). The scarcity of mitochondria and width of the myofibrils were also apparent in longitudinal section (Plate 6.8, **a**) where it could be seen that the Z discs were clearly defined and in better parallel register than those of control tissue of the same age (see Plate 6.3, **a**). The sarcomeres were relatively short, $\sim$1.5 $\mu$m, and the myofilaments showed poor longitudinal alignment (Plate 6.8, **b**). Thus, the myofibrils resembled those of 7-day old fifth instar control fibres (see Plate 6.3, **c** & **d**) more closely than those from 4-day old controls (see Plate 6.3, **a** & **b**). By Day 7 of the fifth instar, both mitochondria and myofibrils were greatly increased in number in treated muscle fibres, the latter also being much reduced in size (Plate 6.7, **c**). Around the fibrils, the SR was very extensive and well organised, forming numerous dyads with T-tubules. The myofilaments showed precise longitudinal alignment along the sarcomeres (Plate 6.8, **d**), and the myosin rods were arranged in a highly ordered pattern within the myofibrils (Plate 6.7, **d**). The Z discs were in near-perfect parallel register (Plate 6.8 **c**) and the general level of myofibril differentiation was better than that found in 10-day old fifth instar control muscle, let alone 7-day old tissue (see Plates 6.2 and 6.3). In cross-sections of treated muscle fibres, mitochondrial profiles were small (Plate 6.7, **c**), being no larger than those from 4-day old muscle fibres, but they were longitudinally elongated (Plate 6.8, **c**) in a similar manner to that found in adult flight muscle (see Plate 6.5, **c**). Also, mito-

chondrial cristae were closely packed within an electron-dense matrix (Plate 6.8, **d**). Thus, the level of organisation of treated, 7-day old fifth instar muscle fibre was such that it could almost have been a miniature simulacrum of mature flight muscle fibre. On the tenth day of the fifth instar, some interfibrillar tracheoles were already present in treated muscle fibres, enclosed in cytoplasmic processes from tracheoblasts (Plate 6.7, **e**). Myofibrils and mitochondria had slightly increased in size since Day 7 (compare **c** and **e** of Plate 6.7), but the latter were abnormally small compared with those from control tissue of the same age (see Plate 6.2, **e**). The arrangement of myofilaments in the myofibrils had lost some of the order seen in the 7-day old fibres (compare **d** and **f** of Plate 6.7). In longitudinal section, the Z discs were still in good parallel register (Plate 6.8, **e**) but had thickened and become very diffuse and indistinct (Plate 6.8, **f**). The precise longitudinal alignment of the myofilaments remained but the sarcomeres had shortened slightly, from $\sim$1.9 $\mu$m to $\sim$1.6 $\mu$m (Plate 6.8, **f**). By Day 1 of adult life, myofibrils and mitochondria had substantially increased in size (Plate 6.9, **a**) but the arrangement of myofilaments had become more disordered (Plate 6.9, **b**) and their longitudinal alignment was relatively poor (Plate 6.10, **b**). Sarcomere length had increased to $\sim$2.3 $\mu$m, the Z discs were still thickened and poorly defined (Plate 6.10, **b**), and now showed relatively poor parallel register (Plate 6.10, **a**). There were further increases in the cross-sectional profiles of myofibrils and mitochondria by Day 8 of the adult stadium (Plate 6.9, **c**), and these were approximately normal in size (compare with Plate 6.4, **c**). However, in longitudinal section (Plate 6.10, **c**), it was seen that both mitochondria and sarcomeres were much shorter than those of adult control tissue (see Plate 6.5, **c**). The arrangement of myofilaments in the myofibrils was disordered (Plate 6.9, **d**) and their longitudinal alignment was poor (Plate 6.10, **d**). The parallel register of the Z discs was also poor (Plate 6.10, **c**), though these now had a more definite structure than in the newly moulted adult (Plate 6.10, **d**). However, the most obvious abnormality of the treated muscle fibres was the grossly enlarged SR (Plate 6.9, **c**) made up of extremely swollen cisternae (Plate 6.9, **d**), although the dyads formed with T-tubules were still apparently normal (compare with Plate 6.4, **c** & **d**).

In animals dosed with 95 $\mu$g of methoprene, the rate of growth of flight muscle fibres was the same as in control animals (results not shown) but there

**Plate 6.11 — Electron Micrographs of 95 $\mu$g Methoprene-Treated Flight Muscle, Fifth Instar, Transverse and Longitudinal Sections**

**a.** Transverse section of muscle fibre on Day 4 of the fifth instar. Note the abnormally large size of the myofibrils compared with control tissue (see Plate 6.2, **a**). Also note the small size and scarcity of mitochondria. T-tubules were present, forming dyadic connections with the rudimentary SR. Scale = 0.5 $\mu$m.

**b.** Detail of 4-day old fifth instar fibre. Note the disordered and widely spaced arrangement of myofilaments in the fibrils. Note also the very few cristae and electron-lucid matrix within mitochondria. Scale = 0.5 $\mu$m.

**c.** Longitudinal section of muscle fibre on Day 4 of the fifth instar. Note the lack of strict parallel register between the ill-defined Z discs and the scarcity of the small mitochondria, similar to the appearance of control tissue (see Plate 6.3, **a**). Scale = 2 $\mu$m.

**d.** Longitudinal section of muscle fibre on Day 7 of the fifth instar. Note the increased width of the myofibrils compared with Day 4 (**c**), similar to the appearance of control tissue (see Plate 6.3, **c**). Also note the better parallel register and more clearly defined nature of the Z discs compared with those of the controls. The numbers of mitochondria were increased compared with Day 4 (**c**), but less so than was seen in the control fibres (Plate 6.3, **c**). Scale = 2 $\mu$m.

**e.** Transverse section of muscle fibre on Day 7 of the fifth instar. Note the increased numbers of mitochondria compared with Day 4 (**a**). Also note the presence of several very dilated T-tubules (arrows). Myofibrils and mitochondria were similar in size to those of Day 4. Scale = 0.5 $\mu$m.

**f.** Detail of 7-day old fifth instar fibre. Note the disordered arrangement of the myofilaments, more closely packed than those in control fibrils (see Plate 6.2, **d**). The mitochondria contained few cristae in an electron-lucid matrix. Scale = 0.5 $\mu$m.

Key: D– dyad; F– myofibril; Gl– glycogen; M– mitochondrion; m– myosin filament; N– nucleus; SR– sarcoplasmic reticulum; T– T-tubule; Z– Z disc.

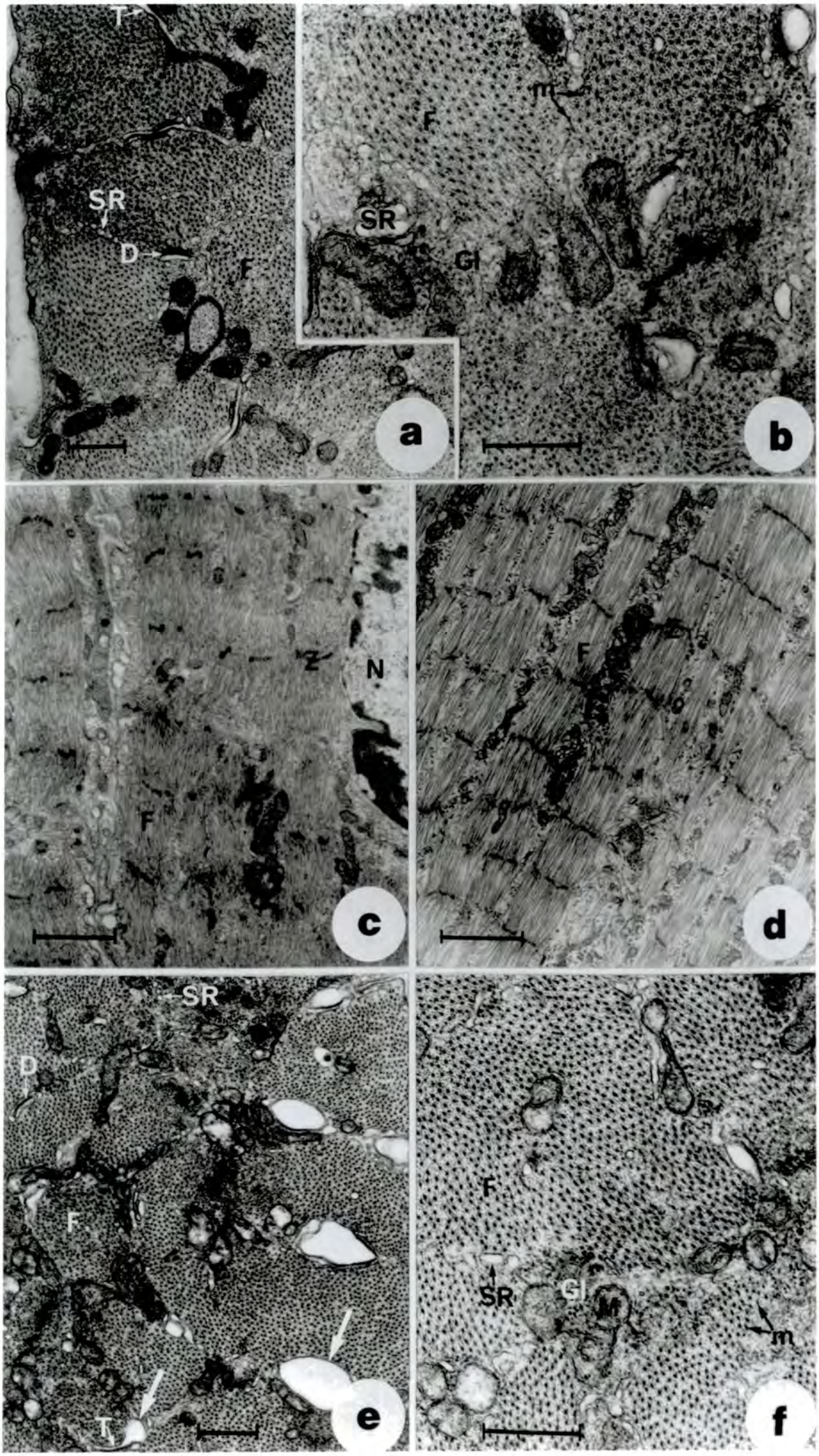

T
SR
D
F
a
m
F
SR
Gl
b
Z
N
F
c
F
d
SR
D
F
T
e
F
SR
Gl
M
m
f

**Plate 6.12 — Electron Micrographs of 95 $\mu$g Methoprene-Treated Flight Muscle, Fifth Instar and Adult, Transverse and Longitudinal Sections**

**a.** Transverse section of muscle fibre on Day 10 of the fifth instar. Note the presence of cytoplasmic processes from tracheoblasts containing tracheoles. Also note the reduced size and increased numbers of myofibrils compared with Day 7 of the fifth instar (Plate 6.11, **e**). Mitochondria were also more numerous and were increased in size. However, both myofibrils and mitochondria were unusually small compared with control tissue (see Plate 6.2, **e**). The SR was extensive, forming numerous dyads with T-tubules. Scale = 0.5 $\mu$m.

**b.** Detail of 10-day old fifth instar fibre. Note the arrangement of myofilaments in the myofibrils, slightly more ordered than those of control muscle fibres (see Plate 6.2, **f**). Scale = 0.5 $\mu$m.

**c.** Longitudinal section of muscle fibre on Day 10 of the fifth instar. Note the presence of interfibrillar tracheoles and cytoplasmic processes of tracheoblasts. Also note the similarity of the tissue structure with control muscle fibre (see Plate 6.3, **e**). Scale = 2 $\mu$m.

**d.** Detail of 10-day old fifth instar fibre. Note the strict longitudinal alignment of the myofilaments and the clearly defined Z discs. Also note the elongate profiles of the mitochondria, which contained numerous cristae in an electron-dense matrix. Scale = 0.5 $\mu$m.

**e.** Transverse section of muscle fibre on Day 1 of the adult stadium. Note the increased size of myofibrils and mitochondria compared with Day 10 of the fifth instar (**a**). Also note the extremely disordered arrangement of myofilaments in the myofibrils. Scale = 0.5 $\mu$m.

**f.** Longitudinal section of muscle fibre on Day 1 of the adult stadium. Note the very diffuse and ill-defined appearance of the Z discs within the myofibrils. Scale = 2 $\mu$m.

Key: D– dyad; F– myofibril; Gl– glycogen; M– mitochondrion; m– myosin filament; S– sarcomere; SR– sarcoplasmic reticulum; T– T-tubule; Tb– tracheoblast; Tr– tracheole; Z– Z disc.

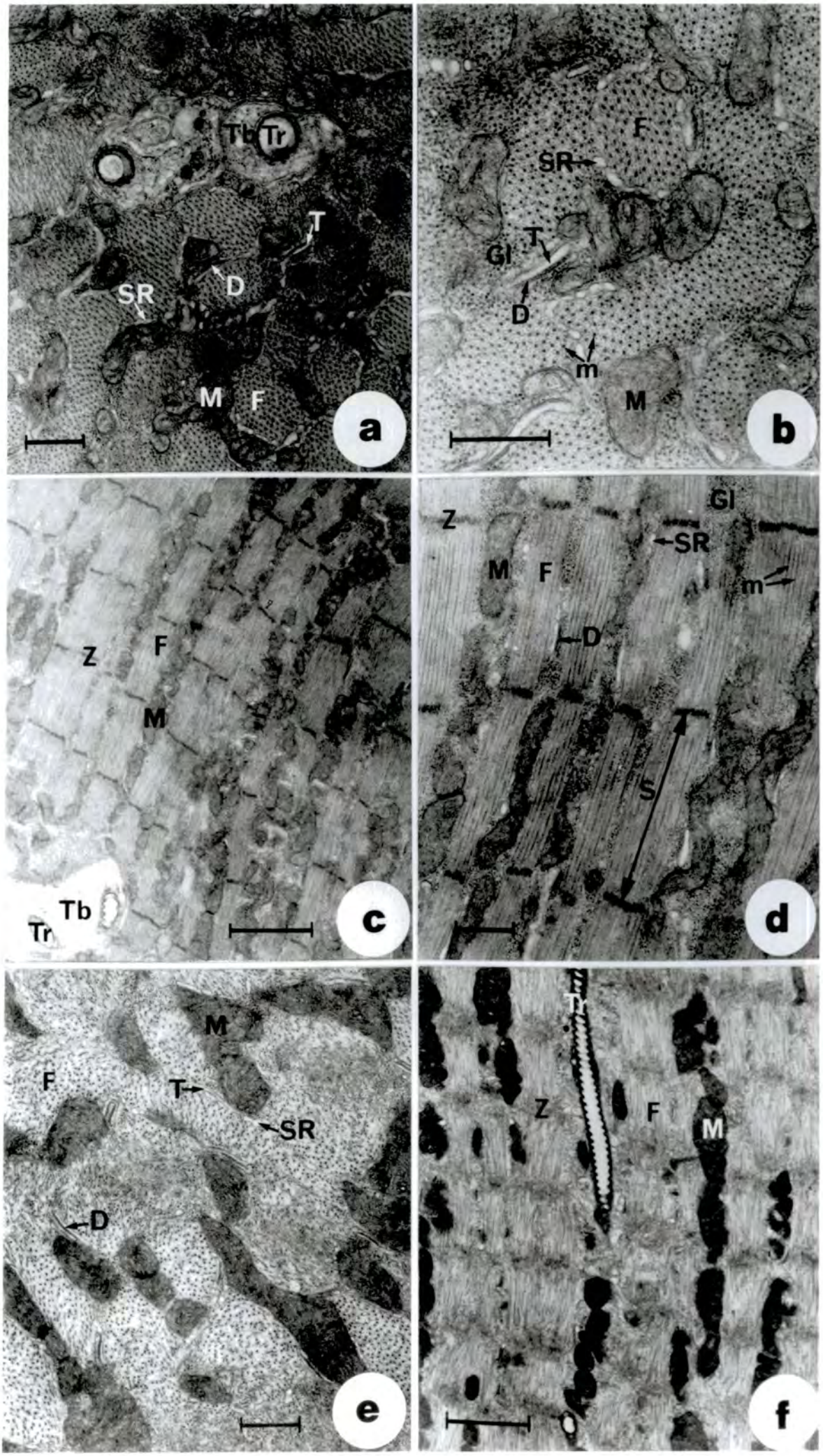

Tb
Tr
T
D
SR
M
F
a
F
SR
T
Gl
D
m
M
b
Z
F
M
c
Tb
Tr
Z
Gl
SR
M
F
m
D
S
d
M
F
T
SR
D
e
Tr
Z
F
M
f

**Plate 6.13 — Electron Micrographs of 95 $\mu$g Methoprene-Treated Flight Muscle, Adult, Transverse and Longitudinal Sections**

**a.** Transverse section of muscle fibre on Day 8 of the adult stadium. Note the large size of mitochondria and myofibrils, and the well-developed SR which formed numerous dyads with T-tubules. Also note the lack of tracheoblast cytoplasm surrounding interfibrillar tracheoles. Scale = 0.5 $\mu$m.

**b.** Detail of 8-day old adult fibre. Note the more irregular arrangement of myofilaments within the myofibrils compared with that found in control tissue (see Plate 6.4, **d**). Also note the numerous, densely packed cristae of the mitochondria, within an electron-dense matrix. Scale = 0.5 $\mu$m.

**c.** Longitudinal section of muscle fibre on Day 8 of the adult stadium. Note the strict parallel register of adjacent Z discs and the regular array of dyads along the myofibrils, two per sarcomere length (arrows). Also note separate A bands and I bands clearly visible in the sarcomeres. The longitudinally elongated mitochondria were arranged in columns running alongside the myofibrils. Scale = 2 $\mu$m.

**d.** Detail of 8-day old adult fibre. Note the clearly defined structure of the Z discs and the strict longitudinal alignment of the myofilaments. Also note the small amount of interfibrillar glycogen present compared with control tissue (see Plate 6.5, **d**). Average sarcomere length was ~2.5 $\mu$m. Scale = 0.5 $\mu$m.

Key: A– A band of sarcomere; c– mitochondrial crista; D– dyad; F– myofibril; Gl– glycogen; I– I band of sarcomere; M– mitochondrion; m– myosin filament; S– sarcomere; SR– sarcoplasmic reticulum; T– T-tubule; Tr– tracheole; Z– Z disc.

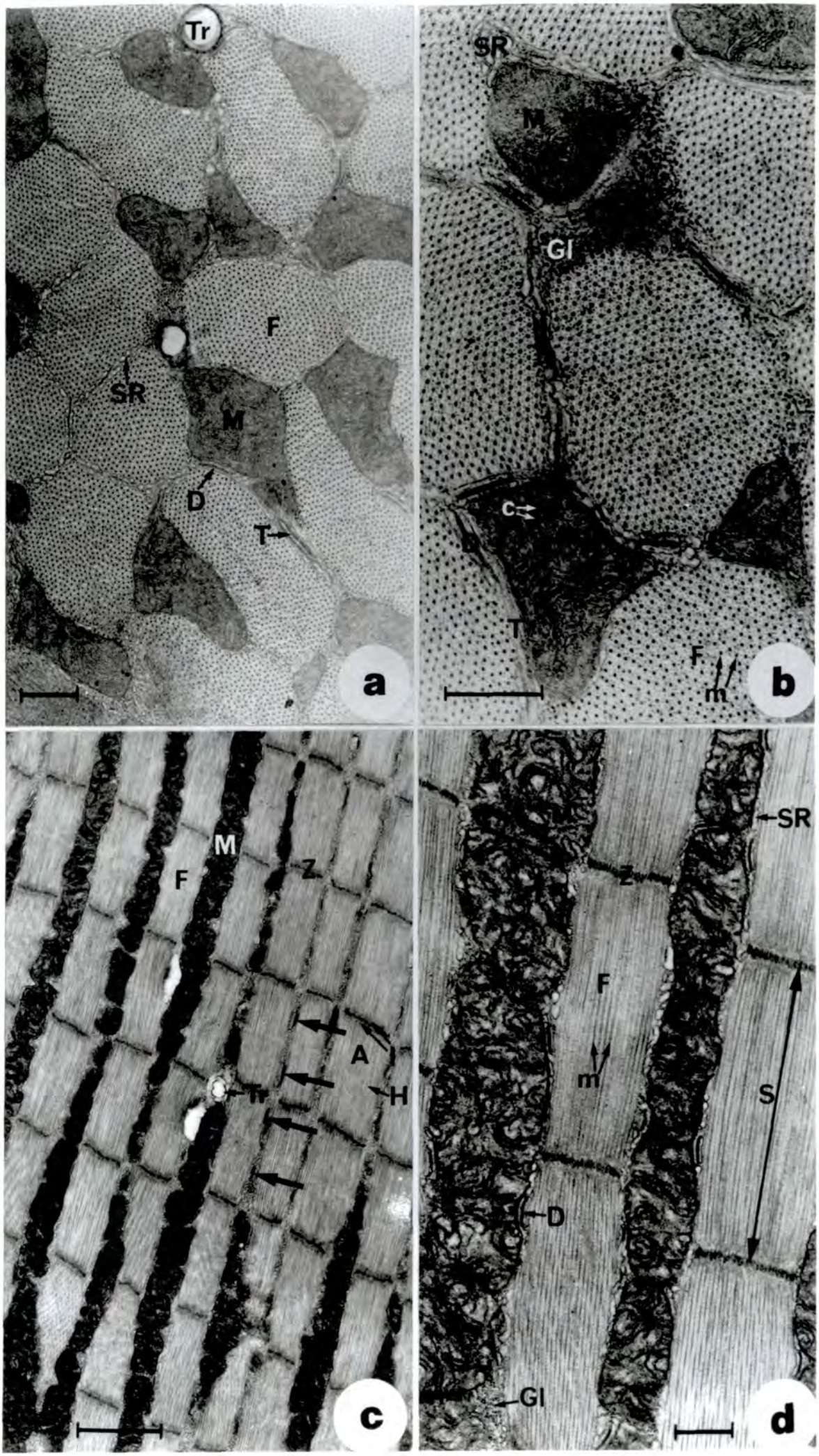

Tr
SR
M
F
D
T
a
SR
M
Gl
c
T
F
m
b
F
M
Z
A
H
Tr
c
SR
Z
F
m
S
D
Gl
d

**Plate 6.14 — Electron Micrographs of Control and Methoprene-Treated Flight Muscle, 8-Day Old Adult, Transverse Sections**

**a.** Muscle fibres of control insects on Day 8 of the adult stadium. Scale = 2 $\mu$m.

**b.** Muscle fibres of 475 $\mu$g methoprene-treated insects on Day 8 of the adult stadium. Scale = 2 $\mu$m.

**c.** Muscle fibres of 95 $\mu$g methoprene-treated insects on Day 8 of the adult stadium. Scale = 2 $\mu$m.

Note the presence of numerous interfibrillar tracheoles within the muscle fibres of control insects (**a**), whereas there were far fewer in the fibres from locusts treated with methoprene, both 475 $\mu$g (**b**) and 95 $\mu$g (**c**).

Key: F– myofibril; M– mitochondrion; N– nucleus; Tr– tracheole.

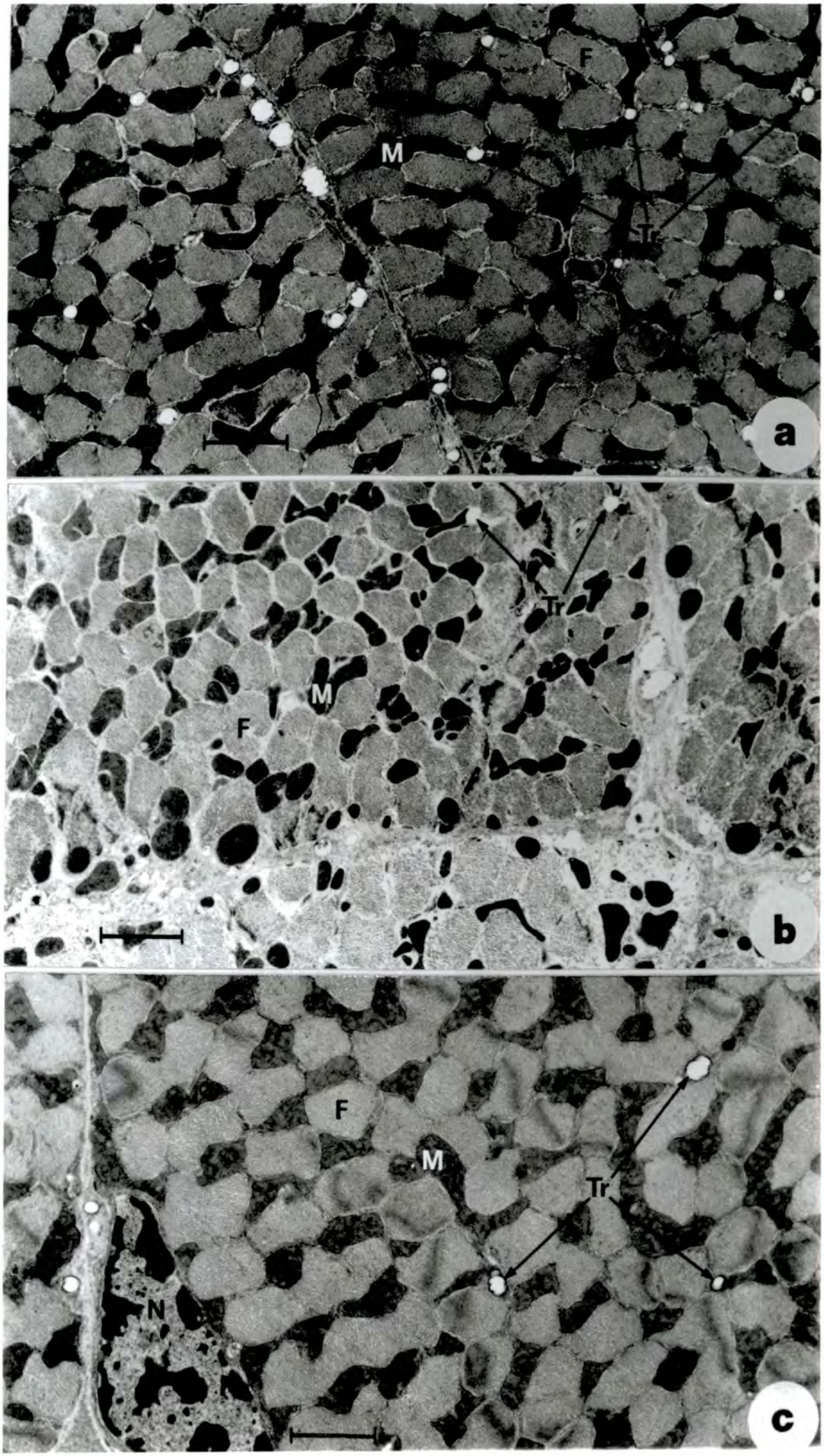

F
M
Tr
a
Tr
M
F
b
F
M
Tr
N
c

**Table 6.1 — Percentage of Flight Muscle Occupied by Mitochondria**

The percentages of flight muscle fibres made up of mitochondria were estimated from a series of micrographs showing transverse sections of muscle fibres, using a dot matrix. The mean values for each age and treatment are given in the table with standard errors (the values were first tested for normality of distribution).

Legend: Day *x*f – Day *x* of the fifth instar,
Day *x*a – Day *x* of the adult stadium,
n – number of micrographs examined.

**Percentage of Flight Muscle Occupied by Mitochondria**

| Treatment | Age of Insect | | | | |
|---|---|---|---|---|---|
| | Day 4f (n) | Day 7f (n) | Day 10f (n) | Day 1a (n) | Day 8a (n) |
| Control | 13.6 ± 0.9 (13) | 22.1 ± 1.7 (15) | 19.9 ± 1.1 (14) | 29.4 ± 1.2 (21) | 30.7 ± 1.6 (20) |
| 475 $\mu$g Metho. | 8.0 ± 1.4 (6) | 21.6 ± 1.5 (9) | 25.5 ± 1.9 (9) | 23.7 ± 1.3 (9) | 17.9 ± 1.2 (9) |
| 95 $\mu$g Metho. | 8.0 ± 0.5 (9) | 14.3 ± 0.9 (8) | 21.2 ± 0.9 (12) | 22.3 ± 0.7 (12) | 25.9 ± 1.0 (13) |
| Student's $t$ tests | | | | | |
| Control/ 475 $\mu$g Metho. | P< 0.01 | P> 0.05 | P< 0.05 | P< 0.005 | P< 0.001 |
| Control/ 95 $\mu$g Metho. | P< 0.001 | P< 0.01 | P> 0.05 | P< 0.001 | P< 0.05 |

were some differences in fine structural details. On Day 4 of the fifth instar, myofibrils from the flight muscles of treated locusts were noticeably larger than those of controls, and the interfibrillar mitochondria were scarcer (Plate 6.11, **a**). However, fine structural details were otherwise similar to control tissue, in both transverse sections (Plate 6.11, **b**) and longitudinal sections (Plate 6.11, **c**). On Day 7 of the fifth instar, mitochondria were still abnormally scarce in the treated flight muscle and were now smaller than the comparable control organelles as well (Plate 6.11, **e**). The myofibrils were of normal size but their level of differentiation may have been higher than those of control tissue since myofilaments within the myofibrils were more closely packed (Plate 6.11, **f**), while in longitudinal section, Z discs were more clearly defined and in better parallel register (Plate 6.11, **d**). There were also dilations in the T-tubule system (Plate 6.11, **e**) which were much larger than those seen in 7-day old control muscle fibres (see Plate 6.3, **d**). By Day 10 of the fifth instar, interfibrillar tracheoles had appeared in the muscle fibres of 95 $\mu$g-treated animals, enclosed within cytoplasmic processes from tracheoblasts (Plate 6.12, **a**). Both myofibrils and mitochondria had smaller cross-sectional profiles than those of control tissue (compare Plate 6.12, **a**, with Plate 6.2, **e**), but their level of differentiation seemed to be slightly higher since myofilaments within the myofibrils were more ordered (compare Plate 6.12, **b**, with Plate 6.2, **f**). In longitudinal section (Plate 6.12, **c**), the general appearance of the tissue was similar to that of control muscle fibre (see Plate 6.3, **e**). In fine detail, however, the myofibrils of the treated tissue had clearly defined Z discs and myofilaments in good longitudinal alignment, while the mitochondria were elongated with electron-dense matrices (Plate 6.12, **d**). Thus, the tissue's level of differentiation was more similar to that of 475 $\mu$g-treated muscle fibre on Day 7 of the fifth instar (see Plate 6.8, **d**) rather than 10-day old fifth instar control tissue (see Plate 6.3, **f**). After the final moult, the arrangement of myofilaments within 95 $\mu$g methoprene-treated myofibrils was very disordered (Plate 6.12, **e**), and Z discs had become thickened and ill-defined (Plate 6.12, **f**). By Day 8 of adult life, flight muscle myofibrils and mitochondria had greatly increased in size (Plate 6.13, **a** & **c**), and were now slightly larger than those of adult control animals (see Plate 6.4, **c**, and Plate 6.5, **c**). The level of differentiation of the treated muscle fibres also seemed to be higher than the controls' in some ways. Myofibrillar Z discs were arranged in precise parallel register and the

regular arrangement of dyads along the myofibrils, two per sarcomere length, was obvious (Plate 6.13, **c**). Also, the amount of interfibrillar glycogen was less than that found in control tissue and Z disc structure was more clearly defined (Plate 6.13, **d**). However, the myofibrils lacked the highly ordered arrangement of myosin filaments that was seen in control fibrils (compare Plate 6.13, **b**, with Plate 6.4, **d**), and the sarcomeres were substantially shorter than those of control tissue, being only ~2.5 $\mu$m in length (compare Plate 6.13, **d**, with Plate 6.5, **d**).

Both the concentrations of methoprene that were used seemed to accelerate the onset of tracheolation of the flight muscle fibres, interfibrillar tracheoles being present in treated muscle fibres by Day 10 of the fifth instar whereas they were first seen in control tissue after the final moult (see above). However, Plate 6.14 clearly shows that, on Day 8 of adult life, there were far fewer interfibrillar tracheoles in methoprene-treated flight muscles (Plate 6.14, **b** & **c**) than in control fibres (Plate 6.14, **a**). Thus, both concentrations of methoprene strongly inhibited the tracheolation of locust flight muscle fibres. The application of methoprene also seemed to affect mitochondrial growth in the developing muscle fibres. In order to investigate this further, an estimation was made of the relative volume of flight muscle fibres occupied by mitochondria, using low power, cross-sectional micrographs of the tissues (for details, see Materials and Methods). The results, given in Table 6.1, suggest that mitochondria in control tissue underwent an initial period of expansion in the early part of the fifth instar and then remained more or less unchanged until the final moult. At this point, there was a very marked increase in the proportion of muscle fibre taken up by mitochondria, reaching the level found in mature flight muscle (BÜCHER, 1965), and this was maintained during the subsequent increase in fibre size. Treatment with both concentrations of methoprene significantly reduced the density of mitochondria in muscle fibre from 4-day old fifth instar insects. In fact the proportion of fibre volume taken up by mitochondria was only slightly higher than that found by BÜCHER (1965) in the precursor muscle of newly moulted fifth instar locust (6%). However, in animals treated with 475 $\mu$g of the JHA, mitochondrial density increased rapidly in the muscle fibres until, by Day 10 of the fifth instar, it was significantly higher than that of control tissue. Immediately after the final moult, the relative volume of mitochondria in the treated muscle fibres was slightly reduced and, by Day 8 of adult life, this had declined further so that it

was barely more than half that of control tissue. In locusts treated with 95 $\mu$g of methoprene, the proportion of muscle fibre taken up by mitochondria increased only slightly during the early part of the fifth instar, but then more rapidly to reach levels comparable with the control tissue by the end of the instar. There was little change in mitochondrial density immediately after the final moult, but this increased slightly during subsequent muscle fibre growth growth so that the relative volume of mitochondria was about 80% of that in control flight muscle by Day 8 of the adult stadium. Treatment with 475 $\mu$g of methoprene greatly reduced muscle fibre growth during the fifth instar so that the abnormally high mitochondrial density in late fifth instar flight muscle could be due to a lesser inhibition of mitochondrial growth compared with other components of the muscle fibres. In view of the reduced size of mitochondria in the treated flight muscle, this explanation seems more likely than methoprene actually stimulating mitochondrial growth. Further support is given by the effect of 95 $\mu$g of methoprene on flight muscle. Here, there was little or no reduction in muscle fibre growth but the volume taken up by mitochondria was significantly reduced at most stages of development compared with control flight muscle.

## 6.4 Discussion

In control flight muscle fibres, there was a decrease in sarcomere length between Day 4 and Day 7 of the fifth instar. It is most unlikely that the myofibrils were functional at that age so the shortening of 7-day old sarcomeres could not be due to myofibril contraction, besides which, the the narrowness of the I bands in locust flight muscle means that the sarcomeres can only contract for a very small percentage of their total length (WEIS-FOGH, 1956). Of course, the reduction in sarcomere length might simply be due to realignment of myofilaments within the myofibrils, but BÜCHER (1965) reported that there was an increase in the number of Z discs (i.e. sarcomeres) along the length of locust flight muscle fibres during the early part of the fifth instar. In *Calliphora*, extra sarcomeres are produced in growing flight muscle fibres by division of Z discs within myofibrils (AUBER, 1969; HOULIHAN and NEWTON, 1979) rather than by the serial addition of new sarcomeres at the ends of muscle fibres as has been found with vertebrate muscles (see GOLDSPINK, 1980). Thus, it is possible that the decrease in sarcomere length noted in the present study could have been due to the interstitial splitting

of Z discs to produce extra sarcomeres. The Z discs from control flight muscle fibres were slightly disrupted in newly moulted adults, seeming to have thickened and lost some definition of structure. One would expect a sudden increase in flight muscle length at the final moult due to the increase in size of the thorax. It is, therefore, possible that the apparent widening and loosening of the Z discs served to accommodate stretching of the muscle fibres. Such a phenomenon has not been described in other ultrastructural studies of locust flight muscle development, although this might not be surprising if the condition is very transient. However, there may be some support from BÜCHER (1965) who noted that, in polarised light, the flight muscles of newly moulted locusts, both fifth instar and adult, appeared to be empty under the light microscope. In the precursor muscle of young fifth instar animals, it was suggested that this was due to the irregular cross-striations of neighbouring myofibrils. This could not be the explanation for adult flight muscle where myofibril striations were in parallel register, and BÜCHER (1965) suggested that the optical effect might due to deterioration of A and I band protein structure, caused by stretching. The stretching of Z discs has been reported in insect muscles other than flight muscles, which may lend some credence to the present proposal. During the oviposition of female *Locusta*, some abdominal intersegmental muscles are extended by up to ten times their resting length. This is permitted by fragmentation of the the Z discs to form small Z bodies which, themselves, then stretch (JORGENSEN and RICE, 1983). Z disc disruption in the flight muscles of newly moulted adult locusts was more apparent in methoprene-treated animals than in the controls, which might indicate a reduced ability to recover from the effects of muscle stretching. However, the fact that the most extreme example of Z disc derangement was from an animal dosed with 95 $\mu$g of JHA suggests that this effect was not dose dependent, and that the differences in the degree of disruption might simply be due to the time elapsed after adult ecdysis.

In both control and methoprene-treated flight muscles fibres, the interfibrillar tracheoles and tracheoblasts were seen to lie inside dilations of the T-tubule system. This is in agreement with other studies of insect flight muscle tracheolation which have shown that the tracheoblast processes invaginate within sheaths of muscle fibre plasma membrane and so are never actually intracellular (see reviews by WIGGLESWORTH, 1983; SMITH, 1984). In the insect species *Tenebrio*

*molitor* (SMITH, 1961) and *Megoura viciae* (SMITH, 1965a), the T-tubules of the flight muscles originate from these circumtracheolar invaginations of the plasma membrane. Studies of flight muscle surface membranes from *Pieris brassicae* (WIGGLESWORTH and LEE, 1982) and *Apis mellifera* (SMITH, 1984) have revealed somewhat irregular and sparse T-tubule invaginations. SMITH (1984) has suggested that, in these cases, much of the flight muscle T-tubule system is derived from circumtracheolar indentations of the plasma membrane. However, the present investigation of locust flight muscle development has demonstrated that the T-tubule system was highly developed in the muscle fibres before tracheolation took place, and that it seemed to be normal in extent in methoprene-treated tissue where the numbers of interfibrillar tracheoles were much reduced. Thus, in the flight muscles of *Locusta*, it appears that the tracheoles simply pass down existing T-tubules and contribute very little to their distribution, as has been suggested for the flight muscles of *Antheraea pernyi* (BIENZ-ISLER, 1968b) and *Phormia regina* (BEINBRECH, 1969). Both the concentrations of methoprene used in the present study had similar effects on the tracheolation of the flight muscle fibres, hastening the first appearance of interfibrillar tracheoles and, as mentioned above, greatly reducing the total level of tracheolation. BROSEMER *et al.* (1963) and BÜCHER (1965) reported that interfibrillar tracheoles appeared within the dorsal longitudinal flight muscles of *Locusta* immediately after the final moult, as was found in this investigation with regard to control animals. However, VAN DEN HONDEL-FRANKEN and FLIGHT (1981) observed interfibrillar tracheoles in the flight muscles of *Locusta* on the last day of the fifth instar. Thus, it may be that tracheoles first arise within the flight muscle fibres just before, rather than just after, the final moult (if the first appearance of interfibrillar tracheoles is only just before the moult, it could easily have been overlooked in the earlier studies). If this is the case, then the early occurence of interfibrillar tracheoles in the methoprene-treated animals of the present study might simply have been due to the accelerated development of these animals (i.e. the shortened length of the fifth instar as reported in Chapter 3) rather than a fundamental disruption of flight muscle development. The reduced level of flight muscle tracheolation in these animals is in agreement with the findings of VAN DEN HONDEL-FRANKEN and FLIGHT (1981) and VAN DEN HONDEL-FRANKEN (1982) who investigated the effects of CA implantation into fourth and fifth instar *Locusta* (see Introduction

for details). BROSEMER *et al.* (1963) noted that the invagination of tracheoblasts into flight muscle fibres of *Locusta* was coincident with the maximal activity of lactate dehydrogenase (LDH) in the tissue. LDH activity increased during the fifth instar until the day before the final moult, and then declined again during early adult life. It was suggested that the two events might be correlated so that lactic acid or other metabolites produced during anaerobic metabolism were attractive to the growing tracheoblasts. This is supported by the work of PIHAN (1972) on the morphogenesis of the tracheal system in pupal Diptera (*Calliphora erythrocephala* and *Tipula flavolineata*), in which the injection of small amounts of lactate into the haemolymph caused diffuse hypertracheation. Implantation of active CA into fifth instar *Locusta* prevented the normal rise in flight muscle LDH activity at the time of the fifth moult (VAN DEN HONDEL-FRANKEN *et al.*, 1980) and the related study by VAN DEN HONDEL-FRANKEN and FLIGHT (1981) showed that flight muscle tracheolation was also prevented. Another possible factor in the control of tracheoblast growth is the presence of ecdysone. In fifth instar *Locusta*, the titre of haemolymph ecdysteroid hormones increases during the latter half of the instar to peak about 48 hours before the final moult (BAEHR *et al.*, 1979), and is therefore coincident with tracheoblast invagination of the flight muscles. There is evidence that ecdysone can stimulate the growth of tracheoblasts in *Bombyx mori* (BEAULATON, 1968), so the premoult ecdysone peak in *Locusta* could well control flight muscle tracheolation. VAN DEN HONDEL-FRANKEN and FLIGHT (1981) suggested that CA implantation might prevent flight muscle tracheolation either by reducing ecdysone production, as was found in JH-treated, last instar larvae of *Blattella germanica* (MASNER *et al.*, 1975), or by preventing the development of the tracheoblasts to an ecdysone-sensitive condition.

The application of 475 $\mu$g of methoprene to newly moulted fifth instar locusts greatly reduced the growth of flight muscle fibres in both the fifth instar and the adult. In such animals, there was also substantial proliferation of synthetic apparatus (rough endoplasmic reticulum and Golgi complexes) in fat body cells (see Chapter 4). This raises the possibility that the inhibition of flight muscle growth was an indirect result of fat body stimulation, due to the re-channelling of resources from the growing flight muscles to the fat body. However, this is unlikely since treatment with 95 $\mu$g of methoprene led to an almost identical

increase in the apparent synthetic activity of fat body cells and yet the growth of flight muscle fibres was undiminished. Treatment with 475 $\mu$g of methoprene also had a marked effect on the fine structure of flight muscle fibres such that tissue from 4-day old fifth instar locusts had some resemblance to that from 7-day old fifth instar controls, while the 7-day old tissue was almost a miniature version of mature flight muscle. These observations suggest that, although muscle growth was reduced by methoprene treatment, the actual differentiation of the muscle fibres may have been greatly accelerated so that this process was more or less complete by Day 7 of the fifth instar. Certainly, there was no further increase in the level of differentiation at later stages in development, rather, there was deterioration in structural organisation. LEE and GOLDSWORTHY (1975) reported that JH could accelerate the development of flight performance in adult locusts. In adult male *Locusta*, flight activity normally peaked about 18 days after moulting and then rapidly declined. Allatectomy of young adults resulted in reduced flight performance for about the first 18 days of adult life but older allatectomised animals flew better than controls of the same age, and when 18-day old adults were allatectomised, they subsequently flew better than the controls (LEE and GOLDSWORTHY, 1975). The application of juvenile hormone to allatectomised animals counteracted the effects of the operation to some extent so that the age-dependent changes in flight activity more closely resembled those of the controls. It was suggested that these results were due to differential ageing of allatectomised and control insects (LEE and GOLDSWORTHY, 1975), and it has been reported that the life-span of allatectomised adult locusts is much longer than that of controls (PENER, 1972). However, such a generalised acceleration of development cannot explain the results of 475 $\mu$g methoprene treatment in which the differentiation of muscle fibre structure was completely out of synchrony with other aspects of muscle development such as tracheolation. If such muscle fibres were indeed fully differentiated by Day 7 of the fifth instar, could the subsequent deterioration of muscle fibre structure be due to premature senescence in the tissue? Unfortunately, structural studies on ageing in flight muscles have been limited to insects with fibrillar flight muscles, which may not be relevant to the present case. The results of these studies have been reviewed by FINLAYSON (1975) and SOHAL (1985) and, although there is considerable disagreement between different authors and different species, the degenerative changes tend to be

of limited severity. These are most commonly concerned with the loss of interfibrillar glycogen and the loss and disorganisation of mitochondrial cristae (SOHAL, 1985). In the present study, however, the SR and myofibrils were most severely disrupted while the mitochondria remained relatively unscathed.

In 475 $\mu$g methoprene-treated animals, degenerative changes in the myofibril organisation of flight muscles were first seen on Day 10 of the fifth instar. If this was, in fact, the time at which muscle degradation began, then it might be associated with the premoult ecdysteroid peak which occurs at about this time (BAEHR *et al.*, 1979). A similar situation is found in the programmed cell death of larval muscles during metamorphosis (see reviews by LOCKSHIN *et al.*, 1980; LOCKSHIN, 1985). In most instances, the degenerative process involves invasion of the muscles by phagocytes (e.g. *Calliphora erythrocephala*, CROSSLEY, 1968) or the appearance of autophagic vacuoles in the tissue (e.g. *Dendroctonus pseudotsugae*, SAHOTA, 1975). However, in certain cases such as intersegmental abdominal muscles in adult *Rhodnius prolixus* (AUBER-THOMAY, 1979) and flight muscles in adult *Acheta domestica* (SRIHARI *et al.*, 1975), the tissues deteriorate without an obvious mechanism for degradation. In the latter case especially, the disruption of myofilaments resembled that found in the present study with flight muscles from 8-day old adults that had been treated with 475 $\mu$g of methoprene. Thus, the disruption of the treated flight muscles might be due to re-programming of the muscle cells by methoprene, causing the fibres to degenerate. In the cricket *Acheta domestica*, flight muscle degeneration in the adult is stimulated by an increase in the haemolymph titre of JH (CHUDAKOVA and BOCHAROVA-MESSNER, 1968; CHUDAKOVA, 1978). Juvenile hormone has similar effects in several other species where reproductive and flight activities seem to be mutually exclusive, such as *Dysdercus intermedius* (EDWARDS, 1970), *Ips paraconfusus* (UNNITHAN and NAIR, 1977) and *Dendroctonus pseudotsugae* (SAHOTA, 1975). However, such effects are an adaptation to the adult insects' life strategies rather than a fundamental response of the flight muscle (PENER, 1985) and, in *Leptinotarsa decemlineata*, JH has exactly the opposite effect, stimulating flight muscle regeneration (DE WILDE and STEGWEE, 1958; DE KORT, 1969).

In 8-day old adults, one of the most obvious effects of treatment with 475 $\mu$g of methoprene was the extreme enlargement of the sarcoplasmic reticulum. According to the present concept of the control of muscular contraction (see reviews

by EBASHI, 1980; AIDLEY, 1985), when a nerve impulse arrives at the muscle fibre plasma membrane, the resulting depolarisation is transmitted to the SR and stimulates the release of calcium ions into the sarcoplasm. It is the high intracellular level of $Ca^{2+}$ which causes the myofibrils to contract. Muscular relaxation follows the re-uptake of $Ca^{2+}$ by the SR, at the expense of ATP, restoring the low resting level of $Ca^{2+}$ in the sarcoplasm. TSUKAMOTO *et al.* (1966) has shown that vesicles of SR isolated from locust flight muscle will bind $Ca^{2+}$ in the presence of ATP and will inhibit the calcium-dependent ATPase of locust and rabbit myofibrils. The enlarged SR cisternae in methoprene-treated flight muscle fibres of the present study raises the possibility that the properties of the SR membranes were disrupted. Alternatively, if the muscle fibre plasma membrane had been made more permeable to calcium, allowing greater influx of the ions, one would also expect to see hypertrophy of the SR. In either case, there would be an abnormally high intracellular $Ca^{2+}$ concentration. Chronic elevation of intracellular $Ca^{2+}$ can have toxic effects on cells and this condition has been linked with certain forms of muscular dystrophy (see MCCOMAS, 1977). Indeed, the characteristic features of dystrophic muscle include degenerating myofibrils and dilated sarcoplasmic reticulum. Thus, some of the structural disruption seen in methoprene-treated flight muscle fibres may be secondary effects due to calcium poisoning.

# Chapter VII

## Effects of Methoprene on the Respiratory Metabolism of Flight Muscle Mitochondria

### 7.1 Introduction

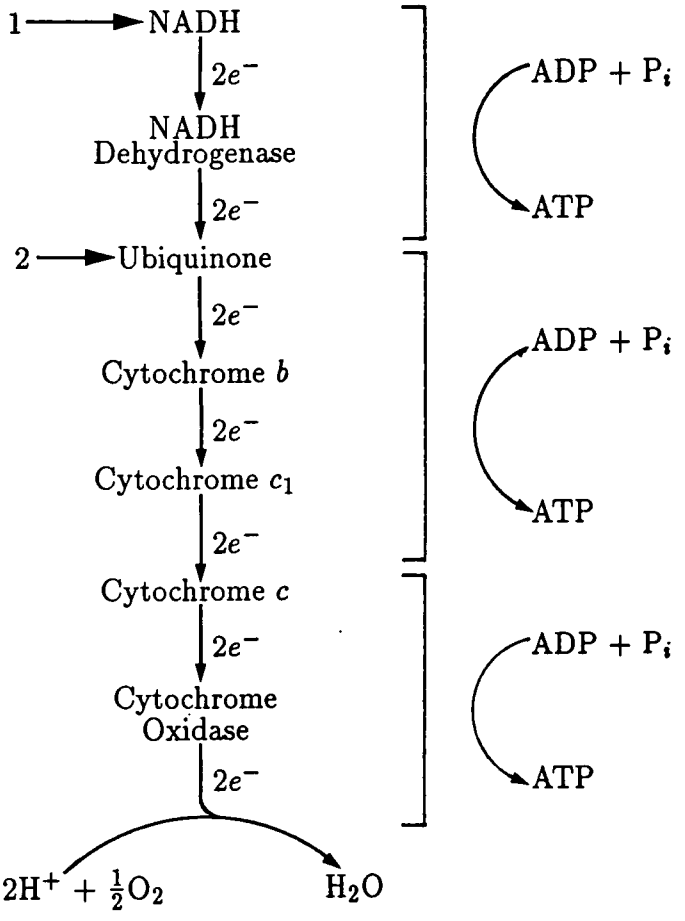
The mitochondrion contains the enzymes of the citric acid cycle, $\beta$-oxidation pathway and the electron-transport chain. These serve to 'to catalyze the oxidation of organic cell nutrients by molecular oxygen to yield carbon dioxide and water' (LEHNINGER, 1982). The energy released by such reactions is used to generate adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate. Specifically, the metabolism of respiratory substrates leads to the production of hydrogen ions and their associated electrons. The latter pass down the electron-transport chain to oxygen and this energy-yielding process is coupled to the phosphorylation of ADP (see Figure 7.1). ATP, the major energy source for the cell, is then passed out of the mitochondria into the cytosol where it is used to carry out cellular work. In intact mitochondria, electron transport only proceeds at a maximal rate when the incubation medium contains both phosphate and ADP, but when ADP is lacking, the respiration rate is very low (LEHNINGER, 1982). Hence, the rate of electron transport is controlled by the concentration of ADP, being very high when ADP is present and then falling to a 'resting' level when the ADP has been phosphorylated. This phenomenon is called respiratory control or acceptor control, and the respiratory control ratio (RCR) is an index of the rate of mitochondrial respiration in the presence of ample ADP against the respiration rate in the absence of ADP. Normally, the RCR is quite high and may be 10 or more in intact mitochondria (LEHNINGER, 1982) but aged or damaged mitochondria lose the ability to synthesise ATP (i.e. oxygen consumption and ADP phosphorylation are 'uncoupled') and the ratio falls to 1.0. Thus, the RCR is a convenient measure of the structural integrity of isolated mitochondria.

In most cells, over 90% of the oxygen that is consumed is used by the mitochondria (LEHNINGER, 1982) so that the rate of oxygen uptake by a cell (or an organism) is a reflection of mitochondrial activity. ROUSSEL (1963) found

**Figure 7.1 — Electron-Transport Chain**

The figure shows, in diagrammatic form, the respiratory chain in mammalian mitochondria. Electrons from substrates which are dehydrogenated by NAD-linked dehydrogenases (see Figure 7.2) enter the respiratory chain at point '1'. Such substrates include $\alpha$-ketoglutarate, pyruvate, malate, isocitrate, glutamate and 3-hydroxyacyl-CoA. Electrons from substrates which are dehydrogenated by flavin-linked dehydrogenases enter the respiratory chain at point '2'. Such substrates include succinate, fatty acyl-CoA and glycerol phosphate. (Adapted from LEHNINGER, 1975).

**Electron Transport Chain**



that allatectomy of adult female *Locusta* caused a significant decrease in the insects' oxygen consumption. Similar reductions in oxygen uptake following CA removal have been reported in most insect species that have been studied, while the implantation of CA, particularly into allatectomized animals, has tended to increase oxygen uptake (see review by STEELE, 1976). Some exceptions have been found, however, and neither allatectomy nor CA implantation had any effect on the respiratory metabolism of the larvae and adults of *Carausius morosus* (NEUGEBAUER, 1961; COURGEON, 1966). In general, the addition of CA extracts to isolated tissues and tissue homogenates also stimulated oxygen consumption, most notably in fat body (STEELE, 1976). For example, RALPH and MATTA (1965) prepared homogenates of fat body and flight muscle from three species of cockroach and found that the respiratory rates of the two tissues were increased by ~40% and ~20% respectively, following the addition of CA extracts. LÜSCHER (1968) reported a 78% increase in the oxygen consumption of fat body from *Nauphoeta cinerea* after CA implantation into decapitated adult females. STEELE (1976) suggested that these heightened respiratory rates might be linked with the reported JH-induced increase in protein synthesis by the fat body (for details of this effect, see Chapter 4). The treatment of insects with JH and JHAs can also affect oxygen uptake, and STEEN (1961) noted that, in pupae of *Antheraea polyphemus*, respiration was stimulated by the injection of cecropia oil, a JH extract from adult male *Hyalophora cecropia* (WILLIAMS, 1956). By injecting the juvenoid farnesyl methyl ether into pupal *Tenebrio molitor*, SCHMIALEK and DREWS (1965) reduced the depression of oxygen consumption that normally occured during this stage of development. SLÁMA and KRYPSIN-SORENSEN (1979) found that some JHAs could also greatly increase the respiratory metabolism of last instar larvae of *Dermestes maculatus* when included in the insects' diet. They observed that the excess amount of energy released by hypermetabolism was partly converted to heat and suggested that this could be due to the uncoupling of oxygen consumption from the phosphorylation of ADP.

The above experiments show that respiratory metabolism is increased by JH and its analogues but not whether this is due to a direct effect on mitochondrial activity. CLARKE and BALDWIN (1960) made preliminary studies on the oxygen consumption of mitochondria isolated from the flight muscles and fat bodies of adult *Locusta migratoria*. Using succinate as a substrate, they found that the

respiratory rates of mitochondria from both tissues were increased by the addition of CA extract to the incubation mixture, indicating a direct stimulation by JH. MINKS (1967) also investigated the respiratory metabolism of isolated mitochondria from flight muscle and fat body of *Locusta*, but he employed the substrate systems pyruvate/malate and $\alpha$-glycerophosphate. The addition of CA homogenates made no difference to the recorded rates of oxygen consumption but did increase the values of the P/O ratios ($\mu$moles of inorganic phosphate taken up per $\mu$g atom oxygen consumed) which suggested an improvement in the coupling of oxygen consumption to ADP phosphorylation. However, when certain JH-active substances (cecropia oil, farnesol and farnesyl methyl ether) were added to mitochondrial incubations at concentrations of $10^{-2}$ and $10^{-3}$ M, both oxygen uptake and the P/O ratios were lowered by $\sim$20% . At more dilute concentrations ($10^{-6}$ to $10^{-8}$ M), cecropia oil caused a marked increase in the P/O ratio but farnesol and its derivative had no effect. The importance of substrate in determining the effect of juvenile hormone on mitochondrial activity was indicated by FIRSTENBERG and SILHACEK (1973). Using mitochondria extracted from whole body homogenates of larval *Plodia interpunctella*, they found that the *in vitro* addition of JH strongly inhibited respiratory metabolism when using the substrate systems $\alpha$-ketoglutarate, glutamate, malate, pyruvate/malate or pyruvate/glutamate. However, the oxidation of $\alpha$-glycerophosphate and ascorbate was unaffected by JH, while the hormone stimulated succinate oxidation. If one considers the points of entry of the various substrate oxidations into the electron transport chain (see Figure 7.1), it can be seen that JH only inhibited the oxidation of those substrate systems which are NAD-linked (i.e. which enter the respiratory chain at NADH). Substrate oxidations which enter the chain at the later points of ubiquinone (e.g. succinate and $\alpha$-glycerophosphate) and cytochrome *c* (e.g. ascorbate) were not inhibited by JH. FIRSTENBERG and SILHACEK (1973) performed a further series of experiments in which they preincubated mitochondrial suspensions at 30°C for 45 minutes. Such 'aged' organelles were incapable of pyruvate/malate oxidation but oxidised NADH at a much greater rate than did fresh mitochondria. It was suggested that the preincubation served to increase mitochondrial permeability, thus facilitating the entry of NADH, but that it also permitted the loss of certain cofactors necessary for pyruvate/malate oxidation. The addition of JH to aged mitochondria strongly inhibited NADH oxidation

just as it did with pyruvate/malate oxidation by fresh mitochondria. It was proposed that JH inhibited the transfer of electrons between NADH and ubiquinone, preventing the oxidation of NAD-linked substrates.

While these results may help to clarify the inhibitory effects of JH upon mitochondrial activity, they do not explain the hormone's stimulation of succinate oxidation. Several workers have reported that intermediates of the citric acid cycle such as succinate (see Figure 7.2), and NADH are not effective respiratory substrates for isolated insect mitochondria (CHANCE and SACKTOR, 1958; VAN DEN BERGH and SLATER, 1962; CHILDRESS and SACKTOR, 1966; CHILDRESS *et al.*, 1967; SACKTOR and CHILDRESS, 1967). A possible explanation was provided by VAN DEN BERGH and SLATER (1962), who found that mitochondria from blowfly flight muscle were not readily permeable to citric acid cycle intermediates. In a later study, VAN DEN BERGH (1964) demonstrated that succinate, malate, citrate and isocitrate cannot penetrate mitochondria isolated from housefly or locust. However, respiratory rates with these substrates could be greatly increased by subjecting mitochondria to sonic disintegration or alternate freezing and thawing, treatments which caused disruption of the mitochondrial membranes (VAN DEN BERGH and SLATER, 1962; SACKTOR and CHILDRESS, 1967). In contrast, these procedures did not stimulate oxygen consumption when using pyruvate or $\alpha$-glycerophosphate (to which mitochondria are readily permeable) as the substrate. In view of these results, STEELE (1976) suggested that the JH-mediated increase in succinate oxidation (see above) could be due to the hormone having a similar disruptive effect on the plasma membranes of mitochondria, increasing their permeability to citric acid cycle intermediates. This would be compatible with the proposal of FIRSTENBERG and SILHACEK (1973) that JH inhibited the transfer of electrons between NADH and ubiquinone, since all the components of the electron transport chain are embedded in the inner membrane of the mitochondrion (LEHNINGER, 1982). Therefore, any damage to the mitochondrial membranes could affect the passage of electrons between the carrier molecules. CHEFURKA (1978) applied juvenile hormones, JH I, JH II and JH III, and JH analogues, methoprene and epofenonane, to isolated mouse liver mitochondria. Since succinate was used as the respiratory substrate, any JH-mediated inhibition of electron transfer between NADH and ubiquinone would not have been apparent. However, IGR treatment did cause uncoupling of oxida-

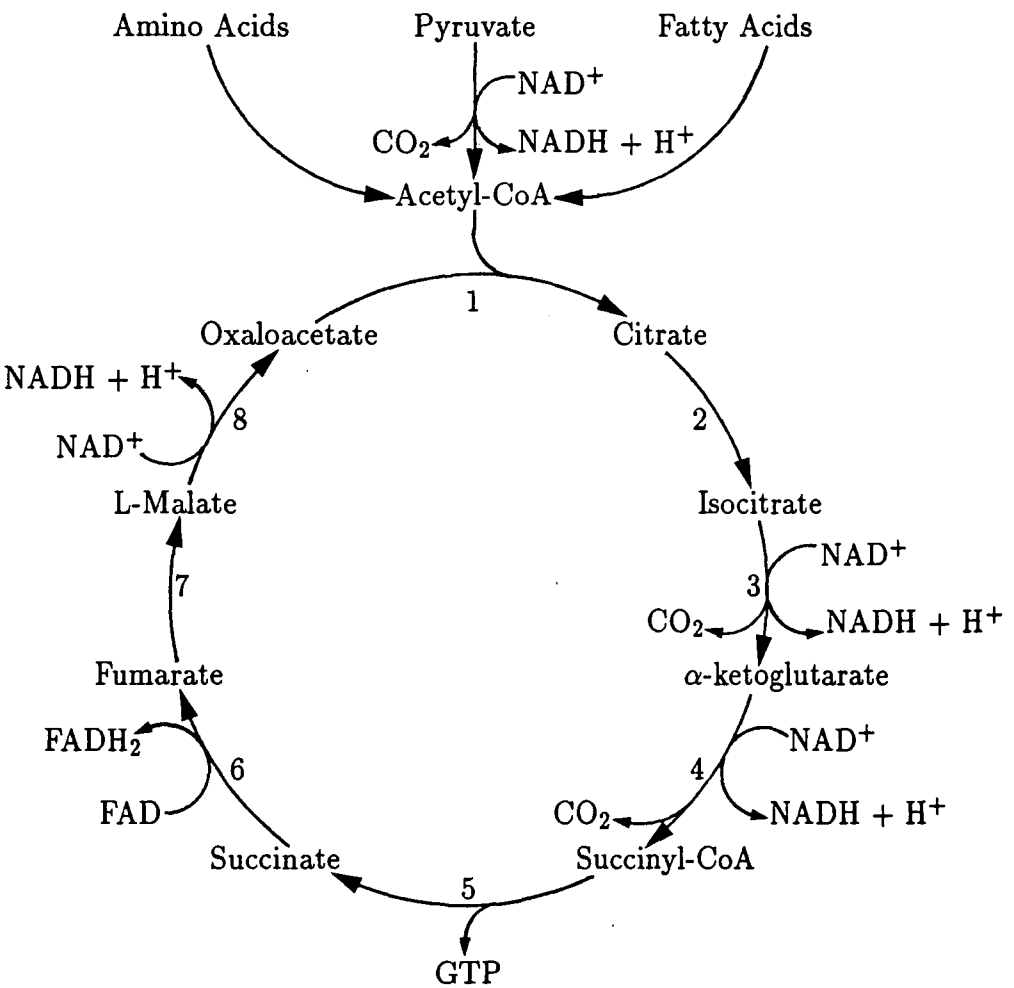
**Figure 7.2 — Citric Acid Cycle**

The enzymatic reactions of the citric acid cycle take place within the inner compartment of the mitochondrion. The production of acetyl-CoA from pyruvate is performed by the pyruvate dehydrogenase complex which is also situated within the mitochondrion.

Legend: 1 – citrate synthase,
2 – aconitase,
3 – isocitrate dehydrogenase (NAD-linked),
4 – $\alpha$-ketoglutarate dehydrogenase (NAD-linked),
5 – succinyl-CoA synthetase,
6 – succinate dehydrogenase (flavin-linked),
7 – fumarase,
8 – malate dehydrogenase (NAD-linked).

(Adapted from LEHNINGER, 1975).

**Citric Acid Cycle**



tive phosphorylation so that all respiratory control was lost and oxygen consumption continued at a maximal rate indefinitely. The coupling of electron transport with the phosphorylation of ADP is dependent on the impermeability of the inner mitochondrial membrane to various ions (e.g. $H^+$, $OH^-$, $K^+$ and $Cl^-$), and uncoupling agents generally act as protonophores, allowing the passage of $H^+$ ions across the membrane (LEHNINGER, 1982). CHEFURKA (1978) showed that the uncoupled mitochondria were indeed more permeable to a number of ions, including $H^+$. Further support for the proposal that JH has a disruptive effect on membranes was provided by COHEN and GILBERT (1972) who noted that the application of JH to two insect cell cultures, derived from ovarian cells of *Antheraea eucalypti* (GRACE, 1962) and *Trichoplusia ni* (HINK, 1970), caused swelling of the cells and the appearance of lesions in the plasma membranes. BAUMANN (1968) investigated the *in vitro* effects of three compounds with JH activity (dodecyl methyl ether, farnesyl methyl ether and farnesyl methyl amine) upon cell membranes from the salivary glands of *Galleria mellonella.* He found that the JHAs caused depolarisation of the membranes by increasing their conductivity. In a later study (BAUMANN, 1969), he tested the same JHAs on synthetic membranes composed of lipid bilayers and again recorded an increase in membrane conductance, indicating that the compounds' effects on plasma membranes were due to a direct interaction with membrane lipids. Several workers have described the lipophilic and surfactant properties of juvenile hormone and its readiness to absorb to non-specific sites including blood cells and basement and plasma membranes (AKAMATSU *et al.*, 1975; LAW, 1980; GOODMAN and CHANG, 1985; HAMMOCK, 1985) and the above observations suggest that the disruption of mitochondrial membranes by JH and some JHAs is due to the hydrophobicity of such sesquiterpenoid compounds.

In Chapter 6, it was seen that the treatment of newly moulted fifth instar *Locusta* with methoprene affected flight muscle structure. Similarly, VAN DEN HONDEL-FRANKEN and co-workers found that the implantation of active corpora allata (CA) into young fifth instar *Locusta* prevented the normal growth of the animals' flight muscles, both in terms of ultrastructural development (see Chapter 6 for details) and the differentiation of the adult pattern of metabolic enzymes. Following CA implantation into newly-moulted fifth instar larvae, VAN DEN HONDEL-FRANKEN *et al.* (1980) measured the specific activities of certain key

metabolic enzymes from the flight muscles; glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase (glycolysis), $\alpha$-glycerophosphate dehydrogenase (glycerophosphate cycle), 3-hydroxyacyl-CoA dehydrogenase ($\beta$-oxidation) and citrate synthase (citric acid cycle). Operated animals underwent an extra larval stage and, consequently, an extra moult. Throughout this supernumary larval instar, the enzymes' activities were abnormally low except in the case of lactate dehydrogenase. By examining the ratios of the enzyme activities, it was seen that the relative importance of $\beta$-oxidation against glycolysis increased during development, but at a slower rate in the CA-implanted animals. Locust flight muscle development is characterised by a large change in enzyme patterns, with aerobic metabolism (e.g. glycerophosphate cycle, $\beta$-oxidation and citric acid cycle) becoming relatively more important than anaerobic glycolysis, especially with regard to lactate dehydrogenase activity (BROSEMER *et al.*, 1963; BEENAKKERS *et al.*, 1975). Therefore, CA implantation would appear to retard flight muscle development during the extra larval instar (VAN DEN HONDEL-FRANKEN *et al.*, 1980). A few of the supernumary larvae successfully moulted to adults, almost normal in appearance. In these animals, the cytoplasmic enzymes of the glycerophosphate cycle and glycolytic pathway reached levels approximately equal to those of the controls, indicating recovery of the flight muscles from the effects of the operation. In contrast, the activities of the mitochondrial enzymes, 3-hydroxyacyl-CoA dehydrogenase and citrate synthase, were still much lower than the relevant control values and it was suggested that mitochondriogenesis was irreversibly inhibited by CA implantation. However, such a result could either be a consequence of inhibition of flight muscle development by implanted CA, or due to JH having a direct action on mitochondria. STEPIEN *et al.* (1988) studied both the direct and indirect effects of JH-active compounds (including the JHAs methoprene and hydroprene) upon mitochondrial enzyme activities in isolated insect cells. They used the Kc 0% cell line established from cells of embryonic *Drosophila melanogaster* (ECHALIER and OHANESSIAN, 1969; ECHALIER, 1976). Addition of JH I, JH II or JH III to the culture medium had no effect on the activities of enzymes from the citric acid cycle (malate dehydrogenase and citrate synthase), but caused an increase in cytochrome oxidase activity, and in the incorporation of radiolabelled methionine into mitochondrial proteins (a measurement of mitochondrial protein synthesis). Methoprene and, to a lesser

degree, hydroprene had similar effects but only at a concentration of $10^{-6}$ M in the culture medium, which was 1000-fold higher than that of the juvenile hormones ($10^{-9}$ M). Further studies were carried out with JH II, the most potent of the compounds used, which showed that, while cytochrome oxidase activity was stimulated by $10^{-9}$ M JH, a concentration of $10^{-4}$ M inhibited enzyme activity. However, when mitochondria were isolated from Kc cells and subsequently incubated with optimal concentrations of JH II ($10^{-9}$ to $10^{-6}$ M), there was no increase in cytochrome oxidase activity nor in [$^{35}$S]methionine incorporation. It was proposed that the stimulation of mitochondrial activity by JH was an indirect effect, requiring the presence of a cytoplasmic 'trigger', while the inhibition of cytochrome oxidase by higher concentrations of the hormone was probably due to disorganisation of the enzyme's subunits in the mitochondrial membrane.

In view of the work reported above and the structural abnormalities of flight muscles from methoprene-treated locusts seen in Chapter 6, it was decided to investigate the effects of methoprene upon the respiratory metabolism of flight muscle mitochondria. It has been reported that mitochondria isolated from flight muscles of *Phormia regina* quickly lose the ability to oxidise pyruvate, but that respiration could be restored by the addition of proline, though not by other amino acids nor intermediates of the citric acid cycle (CHILDRESS and SACKTOR, 1966; SACKTOR and CHILDRESS, 1967). By using [$^{14}$C]pyruvate and [$^{14}$C]proline, SACKTOR and CHILDRESS (1967) showed that the augmented respiration was due to increased pyruvate oxidation rather than the oxidation of proline. Blowfly mitochondria are readily permeable to proline and it was suggested that the proline underwent intramitochondrial metabolism to provide citric acid cycle intermediates, necessary for the oxidation of acetyl-CoA which can build up during periods of intense mitochondrial activity (SACKTOR, 1970). Using an improved method of mitochondrial extraction, SLACK and BURSELL (1976a, 1976b) showed that mitochondria from *Sarcophaga nodosa* and *Phormia regina* were capable of rapid pyruvate utilisation in the absence of proline. They suggested that the reported requirement for a proline 'primer' was due to the use of inappropriate extraction methods which damaged the mitochondria and allowed the citric acid cycle intermediates to leak out. AL-ROBAI (1981) also found that proline was not necessary for the oxidation of pyruvate by mitochondria isolated from mature adult *Locusta migratoria*, and that its presence did

not cause a significant change in respiration. Nevertheless, in their review of flight muscle metabolism, BEENAKKERS *et al.* (1985) stated that proline provided citric acid cycle intermediates during flight-induced increases in oxidation 'in orthopteran, lepidopteran, hymenopteran and a number of dipteran species'. Therefore, in the present study, it was decided to include proline with pyruvate substrate in order to ensure the latter's rapid oxidation by isolated flight muscle mitochondria. The purpose of this study was to determine the effects, both *in vivo* and *in vitro*, of the JHA methoprene and of JH III upon the respiratory metabolism of flight muscle mitochondria from adult *Locusta migratoria*.

## 7.2 Materials and Methods

The animals used to investigate the *in vivo* effects of methoprene were individually treated, as newly moulted fifth instar larvae, with either 475 $\mu$g or 95 $\mu$g of methoprene in 5 $\mu$l of ethanol by topical application. Also some animals were topically treated with 408 $\mu$g of JH III (the molar equivalent to 475 $\mu$g of methoprene) in 5 $\mu$l of ethanol. Control animals were treated with 5 $\mu$l of absolute ethanol. All animals were subsequently maintained as described in General Materials and Methods and were killed as 10–15 day old adults.

Studies on the *in vitro* effects of the JH and JHA were carried out on mitochondria extracted from untreated adult locusts that were sexually mature but not accurately aged.

### 7.2.1 Extraction of Mitochondria from Flight Muscle

The method of mitochondrial extraction was similar to that described by CHANCE and HAGIHARA (1961), and CHAPPELL and HANSFORD (1972). The isolation medium consisted of;

0.32 M sucrose,
1.0 mM EDTA,
5.0 mM Tris/HCl buffer at pH 7.3.

Equal numbers of male and female insects were used for each mitochondrial extraction (normally 4 of each). Animals were killed by twisting the head to break the neck membrane. The posterior tip of the abdomen was cut off and the head, with the gut attached, was removed. The carcase was cut open ventrally and the fat body overlying the flight muscles carefully removed with tissue paper.

The flight muscles were dissected out and pooled in 10 ml of ice-cold isolation medium in a glass homogenisation tube to which was added 5 mg of proteinase (Nagarse or Pronase E were found to be suitable) dissolved in 5 ml of isolation medium. The proteinase digested the myofibrils so that minimal mechanical force was needed to effect homogenisation. Consequently, mitochondrial damage was reduced during isolation (CHAPPELL and HANSFORD, 1972). Two gentle passes of the teflon pestle were made by hand and the tissue was then allowed to digest for 6 minutes at 0–4°C, with three further passes of the pestle being made during this time. The resulting suspension was filtered through four layers of muslin (which had previously been boiled in distilled water and soaked in ice-cold isolation medium). The residue was washed through with a further 5 ml of isolation medium and the muslin was gently squeezed to expel the filtrate. The filtrate was centrifuged at 4,000 g for 8 minutes in a MSE 'Coolspin' centrifuge at 0–4°C. The supernatant was discarded and the mitochondrial pellet resuspended in 10 ml of ice-cold isolation medium using a Pasteur pipette. The suspension was re-centrifuged as before and the supernatant again discarded. The 'fluffy' layer on top of the final pellet was removed by washing it gently with a small volume of fresh isolation medium. The latter was then decanted off and the remaining pellet resuspended in an appropriate volume of isolation medium to give a mitochondrial protein suspension of approximately 10–30 mg/ml.

### 7.2.2 Description of the Oxygen Electrode

Measurements of mitochondrial respiration were carried out using a Rank oxygen electrode (Rank Bros., Cambridge) which is of similar design to the Clark oxygen electrode (CLARK, 1956). It consists of a perspex reaction chamber surrounded by a water jacket and this unit screws onto a perspex base where a platinum cathode and silver anode are situated. When in use, these electrodes are covered with a few drops of 1 M KCl and this electrolyte is separated from the reaction vessel by a teflon membrane. A perspex screw cap fits into the top of the reaction chamber and has an injection port bored through the centre to allow additions to be made to the reaction mixture. The mixture in the reaction chamber is continuously stirred by a magnetic stirrer (Rank Bros., Cambridge).

The principle of the oxygen electrode has been described by DAVIES and BRINK (1942). When a polarising voltage of 0.6 volts is applied between the two

electrodes, oxygen undergoes an electrolytic reduction:

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$$

$$H_2O_2 + 2e^- + 2H^+ \longrightarrow 2H_2O.$$

The current produced is directly proportional to the oxygen concentration of the reaction medium.

The current flowing through the electrode system was passed through a helical potentiometer in series with the platinum electrode, and the voltage developed across this resistance was fed into a servoscribe pen recorder (Goerz Electronics) set at 2 mV sensitivity. The recorder baseline was set by reducing the medium in the reaction chamber with sodium dithionite. This effectively reduced the current across the electrode to zero. To give a full scale deflection on the pen recorder, reaction medium saturated with air was pipetted into the reaction chamber and the potential adjusted with the helical potentiometer.

### 7.2.3 Measurement of Mitochondrial Respiration

Mitochondrial oxygen consumption was measured at 30°C ± 0.1°C using the oxygen electrode described above. The reaction medium employed was that described by KASHMEERY (1977) and was successfully used by AL-ROBAI (1981) with mitochondria isolated from locust flight muscle. It consisted of;

154 mM KCl,
0.4 mg/ml BSA,
10 mM Tris,
30 mM Phosphate buffer, pH 7.3.

After thorough equilibration at 30°C to saturate it with air, 2 ml of the reaction medium was pipetted into the reaction chamber. The perspex screw cap was replaced so that all air bubbles were removed and the reaction medium was allowed to rise about 5 mm up the injection port to minimize the medium's contact with the air. Oxygen uptake from the medium was monitored with the pen recorder during the following additions:

(1) 100 $\mu$l (unless otherwise stated) of mitochondrial suspension,
(2) 10 $\mu$l of substrate solution (1 M pyruvate + 1 M proline),
(3) 10 $\mu$l of 50 mM ADP solution made up in 30 mM phosphate buffer, pH 6.8.

These additions were made with microsyringes (Hamilton or Terumo) and care was taken to ensure that the needle did not damage the teflon membrane over the electrodes.

In the experiments investigating the *in vitro* effects of the IGRs on mitochondrial function, various concentrations of methoprene or JH III (in 5 $\mu$l of ethanol) were added to the chamber before the introduction of ADP. In control determinations, 5 $\mu$l of absolute ethanol replaced the IGRs. The same mitochondrial extraction was used for all control and treated runs in each independent series of determinations. Also, the control determinations were performed at regular intervals between the treated runs to ensure that any differences between control and treated results could not be due to ageing and deterioration of the mitochondrial suspension.

The rate of mitochondrial oxygen consumption ($QO_2$) is expressed in terms of $\mu$g atoms of oxygen per mg of mitochondrial protein per hour. It can be calculated using the following equation:

$$QO_2 = \frac{P \times C \times 60}{(R - r) \times \text{protein concentration}} \quad \text{, where}$$

P = rate of change of chart reading (divisions/minute),

C = initial oxygen content in reaction mixture (0.43 $\mu$g atoms oxygen/ml at 30°C; DAVISON, 1970),

R = chart reading in presence of air-saturated medium,

r = chart reading following addition of sodium dithionite.

The $QO_2$ which followed the injection of ADP is called the state 3 rate and the $QO_2$ after ADP expenditure is called the state 4 rate (CHANCE and WILLIAMS, 1955a). Figure 7.3 represents a typical oxygen electrode trace, showing how rates of oxygen consumption were determined.

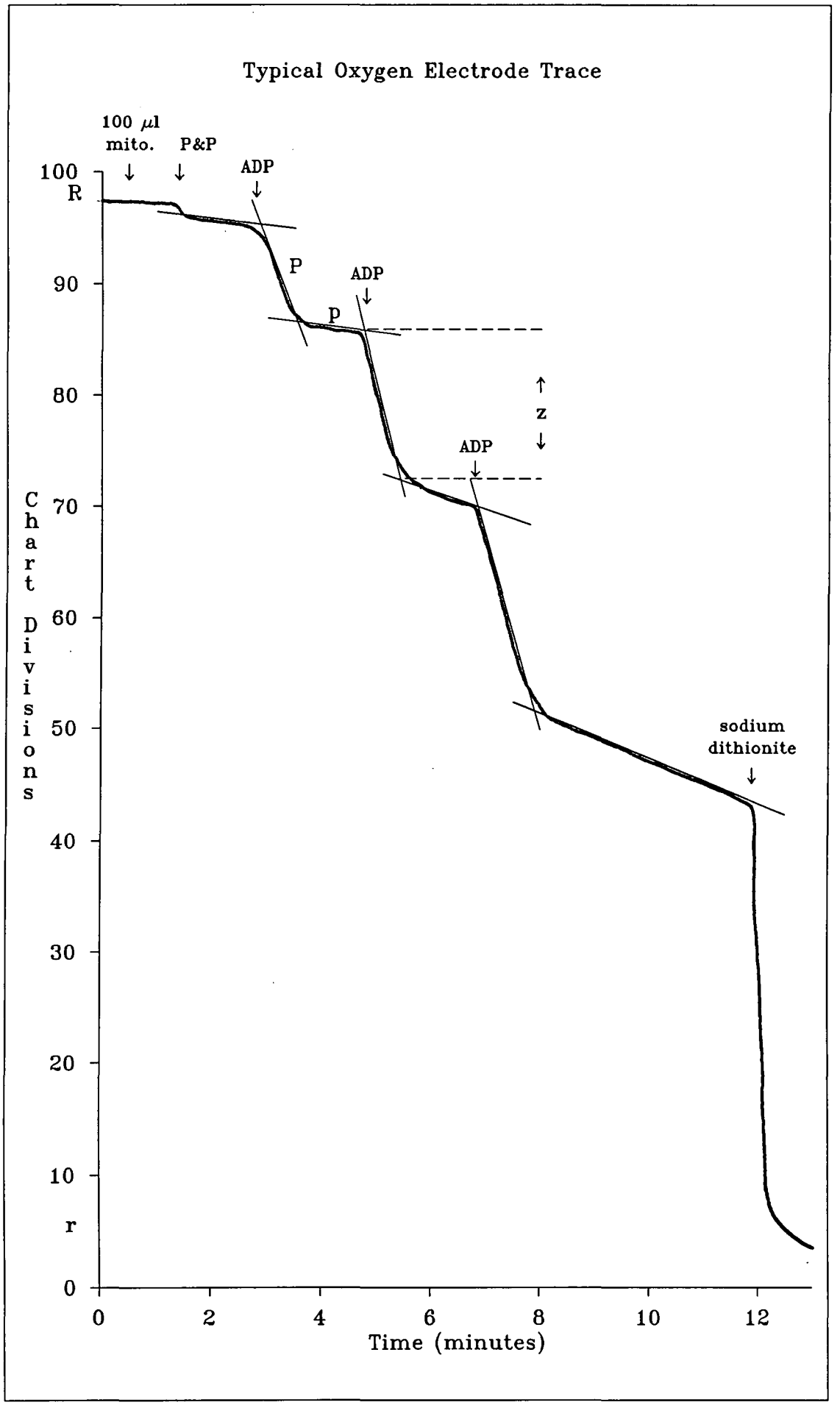
The respiratory control ratio (RCR) was determined as the ratio of the respiratory rate in the presence of ADP (state 3) to the rate following its expenditure (state 4),

$$\text{i.e.} \quad RCR = \frac{\text{state 3 rate}}{\text{state 4 rate}} \quad \text{(CHANCE and WILLIAMS, 1956).}$$

The ADP/O ratio was determined from the number of $\mu$moles of ADP esterified to ATP for each $\mu$g atom of oxygen consumed. The ratios have been

**Figure 7.3 — A Typical Oxygen Electrode Trace**

Legend: ordinate – chart divisions (= oxygen),
abscissa – time (minutes),
R – chart reading for air-saturated medium,
r – chart reading for oxygen-depleted medium,
P – rate of change in presence of ADP,
(divisions/minute),
p – rate of change after ADP expenditure,
z – divisions (= oxygen) used during state 3 respiration,
P& P – pyruvate and proline.


Typical Oxygen Electrode Trace
100 µl mito.
P&P
ADP
ADP
ADP
P
P
z
sodium dithionite
100
R
90
80
70
60
50
40
30
20
10
r
0
Chart Divisions
0
2
4
6
8
10
12
Time (minutes)

calculated using the method described by CHANCE and WILLIAMS (1955b) which relates the amount of oxygen consumed during state 3 respiration to the amount of ADP added (see Figure 7.3).

### 7.2.4 Determination of Mitochondrial Protein Concentration

The protein content of the mitochondrial extracts was determined using the Folin's method of LOWRY *et al.* (1951). See General Materials and Methods for details.

## 7.3 Results

### 7.3.1 Effects of *In Vivo* Treatments with IGRs

Table 7.1 shows the protein concentrations of mitochondrial suspensions isolated from the flight muscles of adult locusts which had undergone *in vivo* treatment with methoprene or ethanol when newly moulted fifth instar animals. From these results, it was possible to calculate the average amount of mitochondrial protein that was extracted from each animal. Significantly less protein was extracted from those insects that were treated with 475 $\mu$g of methoprene than from the corresponding controls. In contrast, there was no significant difference between the amounts of protein isolated from animals treated with 95 $\mu$g of the JHA and the controls.

Five experiments, using independent insect populations, were undertaken in order to study the effects of *in vivo* treatment with 475 $\mu$g of methoprene upon mitochondrial respiration. Figure 7.4 shows some typical oxygen electrode traces from one of these experiments and illustrates the impaired respiratory control and reduced respiratory rates that were found with 'treated' mitochondria (Figure 7.4, **b**). In some determinations the results were more spectacular with a complete loss of respiratory control and a progressive inhibition of oxygen consumption (Figure 7.4, **c**). In such cases, the mitochondria were no longer capable of displaying a steady respiration rate and oxygen consumption was deemed to be not measureable. The results of the five investigations into the *in vivo* effects of 475 $\mu$g of methoprene are listed in Table 7.2 at the end of this chapter. Whilst substantial variation was encountered between corresponding values from the independent populations, student's $t$ tests of the mean results showed that methoprene treatment caused significant reductions in the state 3

**Table 7.1 — Mitochondrial Pellet Protein Concentration**

Legend: Protein conc. – protein concentration of mitochondrial suspensions ($\mu$g/ml),

Amnt. Protein/ Insect – average amount of mitochondrial protein extracted from each insect ($\mu$g),

$\bar{x}$ – mean values.

**Mitochondrial Pellet Protein Concentration**

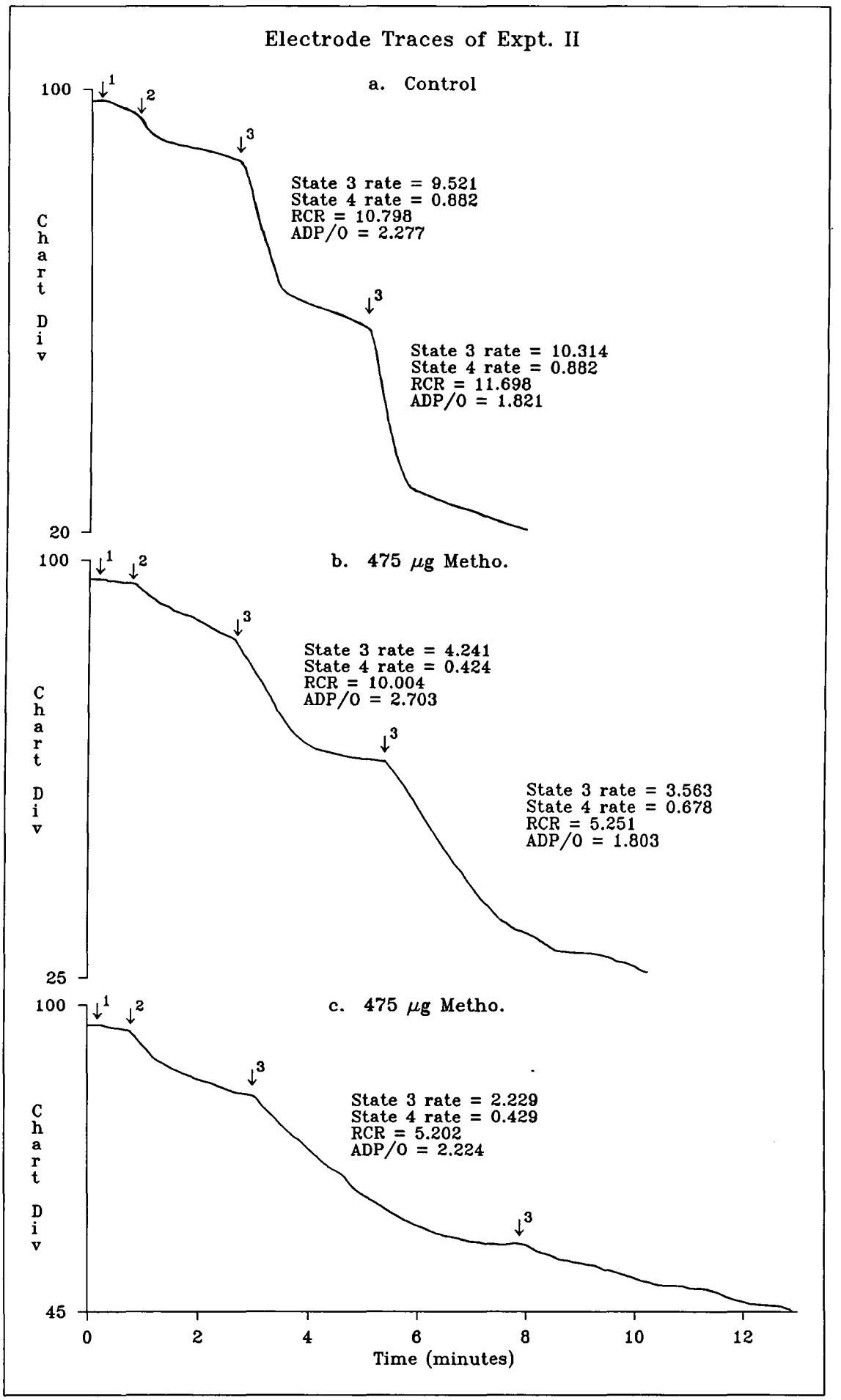
| Expt. | treatment | Protein conc. | Amnt. Protein/ Insect | Expt. | treatment | Protein conc. | Amnt. Protein/ Insect |
|---|---|---|---|---|---|---|---|
| I | Control | 12.45 | 1.04 | VI | Control | 12.58 | 1.05 |
| | 475 $\mu$g Metho. | 6.31 | 0.53 | | 95 $\mu$g Metho. | 10.29 | 0.86 |
| II | Control | 20.76 | 1.30 | VII | Control | 15.78 | 1.32 |
| | 475 $\mu$g Metho. | 10.90 | 0.61 | | 95 $\mu$g Metho. | 15.15 | 1.26 |
| III | Control | 19.36 | 1.21 | VIII | Control | 30.77 | 1.28 |
| | 475 $\mu$g Metho. | 25.31 | 0.53 | | 95 $\mu$g Metho. | 29.97 | 1.25 |
| IV | Control | 17.62 | 1.10 | IX | Control | 28.63 | 1.19 |
| | 475 $\mu$g Metho. | 7.88 | 0.33 | | 95 $\mu$g Metho. | 20.51 | 0.86 |
| V | Control | 15.81 | 0.99 | X | Control | 22.36 | 0.93 |
| | 475 $\mu$g Metho. | 9.77 | 0.24 | | 95 $\mu$g Metho. | 21.56 | 0.90 |
| | | | | | | | |
| $\bar{x}$ | Control | — | 1.13 $\pm$ 0.06 | $\bar{x}$ | Control | — | 1.15 $\pm$ 0.07 |
| $\bar{x}$ | 475 $\mu$g Metho. | — | 0.45 $\pm$ 0.07 | $\bar{x}$ | 95 $\mu$g Metho. | — | 1.03 $\pm$ 0.10 |
| Student's $t$ test | | — | P< 0.001 | Student's $t$ test | | — | P> 0.05 |
| Paired $t$ test | | — | P< 0.001 | Paired $t$ test | | — | P> 0.05 |

**Figure 7.4 — Oxygen Electrode Traces; *In Vivo* Treatments with 475 $\mu$g Methoprene**

The figure displays representative oxygen electrode traces from Expt. II. Trace **a** shows a typical determination of oxygen consumption by mitochondria from the control animals. Traces **b** and **c** show determinations obtained with mitochondria from the methoprene-treated animals. Trace **b** exhibited the least deviation from the control pattern while **c** showed the greatest disruption of respiratory metabolism. The other three determinations that were made with 'treated' mitochondria lay between these two extremes.

Legend: ordinate – chart divisions (= oxygen),
abscissa – time (minutes),
$\downarrow^1$ – addition of mitochondria,
$\downarrow^2$ – addition of pyruvate and proline,
$\downarrow^3$ – addition of ADP.

All the traces are drawn to the same scale. The rate of oxygen consumption was deemed to be not measureable when it was no longer possible to determine a steady rate of oxygen depletion.


Electrode Traces of Expt. II
a. Control
100
20
Chart Div
State 3 rate = 9.521
State 4 rate = 0.882
RCR = 10.798
ADP/O = 2.277
State 3 rate = 10.314
State 4 rate = 0.882
RCR = 11.698
ADP/O = 1.821
b. 475 μg Metho.
100
25
Chart Div
State 3 rate = 4.241
State 4 rate = 0.424
RCR = 10.004
ADP/O = 2.703
State 3 rate = 3.563
State 4 rate = 0.678
RCR = 5.251
ADP/O = 1.803
c. 475 μg Metho.
100
45
Chart Div
State 3 rate = 2.229
State 4 rate = 0.429
RCR = 5.202
ADP/O = 2.224
0
2
4
6
8
10
12
Time (minutes)

rates and RCRs (see Table 7.2). The mean state 3 rate of treated mitochondria was 36% of the mean control rate after the first ADP addition and 24% of the control rate after the second ADP addition, while the mean treated RCR was 61% and 39% of the mean control value following the first and second ADP additions respectively. Examination of the individual experiments revealed that, where they were measureable, the results obtained with 'methoprene-treated' mitochondria were consistently lower than the associated control values with regard to all four respiratory parameters that were recorded (state 3 rate, state 4 rate, RCR, and ADP/O ratio). Therefore, paired $t$ tests were performed on the data (see Table 7.2) and these indicated that the differences between control and treated results were statistically significant for all four parameters. The discrepancies were always greater following the second addition of ADP, as shown above by the mean state 3 rates and RCRs. Similarly, the mean state 4 rate had decreased to 62% of the mean control rate after the first ADP application and to 59% of the control following the second. The ADP/O ratio was least affected, being 97% and 80% of the control values after the first and second ADP additions respectively. However, it must be noted that, in calculating the average values of the parameters for each experiment, only those determinations which gave measureable results could be used. Therefore, since some of the determinations with methoprene-treated mitochondria did not give measureable respiratory rates, the differences between control and treated values which are quoted above underestimate the true effects of the JHA.

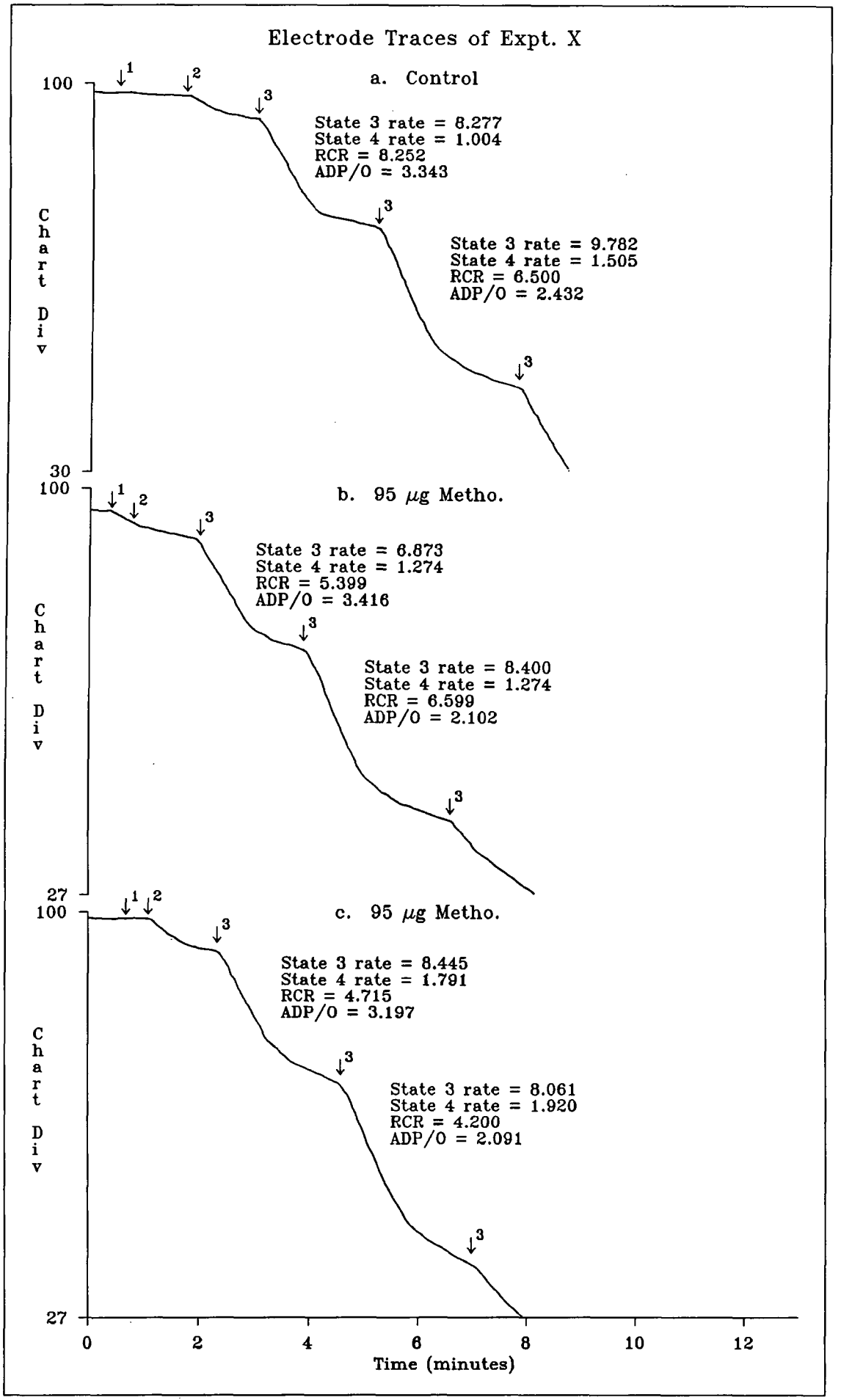
The results of investigations into the *in vivo* effects of 95 $\mu$g of methoprene upon mitochondrial function are given in Table 7.3 at the end of the chapter. Figure 7.5 shows some typical oxygen electrode traces from one of these experiments and it can be seen that the JHA treatment caused a slight reduction in respiratory control. Again, five independent insect populations were studied and there were large variations between comparable results from these experiments. Student's $t$ tests indicated that there was no significant difference between the mean control results and the mean treated values (see Table 7.3). However, the differences between the treated values of the separate experiments and their corresponding controls were consistent, the differences again being greater after the second injection of ADP, and paired $t$ tests suggested that some of these discrepancies were significant (see Table 7.3). The RCRs were significantly lower in the

**Figure 7.5 — Oxygen Electrode Traces; *In Vivo* Treatments with 95 $\mu$g Methoprene**

The figure displays representative oxygen electrode traces from Expt. X. Trace **a** shows a typical determination of oxygen consumption by mitochondria from the control animals. Traces **b** and **c** show two determinations obtained with mitochondria from the methoprene-treated animals. Trace **b** represents the least impairment of respiratory capacity that was recorded and Trace **c**, the greatest.

Legend: ordinate – chart divisions (= oxygen),
abscissa – time (minutes),
$\downarrow^1$ – addition of mitochondria,
$\downarrow^2$ – addition of pyruvate and proline,
$\downarrow^3$ – addition of ADP.

All the traces are drawn to the same scale.


Electrode Traces of Expt. X
a. Control
State 3 rate = 8.277
State 4 rate = 1.004
RCR = 8.252
ADP/O = 3.343
State 3 rate = 9.782
State 4 rate = 1.505
RCR = 6.500
ADP/O = 2.432
b. 95 μg Metho.
State 3 rate = 6.873
State 4 rate = 1.274
RCR = 5.399
ADP/O = 3.416
State 3 rate = 8.400
State 4 rate = 1.274
RCR = 6.599
ADP/O = 2.102
c. 95 μg Metho.
State 3 rate = 8.445
State 4 rate = 1.791
RCR = 4.715
ADP/O = 3.197
State 3 rate = 8.061
State 4 rate = 1.920
RCR = 4.200
ADP/O = 2.091
Chart Div
100
30
27
0
2
4
6
8
10
12
Time (minutes)

treated populations after both applications of ADP, the mean treated RCRs being 86% and 82% of the control values after the first and second ADP additions respectively. With respect to the state 3 and state 4 rates, methoprene-treated values were significantly different from the controls only after the second application of ADP, but not after the first addition. Following the second ADP injection, the mean treated state 3 rate was 90% of the mean control rate while the mean treated state 4 rate was some 5% higher than that of the control. No significant difference was found between the control and treated values of the ADP/O ratios after both ADP additions.

Table 7.4 at the end of the chapter shows the results from Expt. XI, which tested the effects of *in vivo* treatment with 408 $\mu$g of JH III upon mitochondrial respiration. Only one insect population (i.e. one control and one treated mitochondrial preparation) was studied and student's $t$ tests indicated that there was no significant difference between the controls and the treated values (see Table 7.4).

### 7.3.2 Effects of *In Vitro* Treatments with IGRs

Investigations into the *in vitro* effects of methoprene and JH III upon the respiratory metabolism of flight muscle mitochondria were made with extractions from untreated adult locusts, as described in Materials and Methods. Preliminary studies established that the addition of absolute ethanol (up to 20 $\mu$l) to the reaction mixture had no effect on mitochondrial oxygen consumption (e.g. see Table 7.5 at the end of the chapter). On this basis, it was considered unnecessary to have untreated determinations as well as controls in subsequent studies. This was of some importance, since only a limited number of determinations could be made with a mitochondrial extract before it began to deteriorate (extracts began to lose respiratory capacity 2–3 hours after their preparation).

Figure 7.6 shows some typical oxygen electrode traces from Expt. XII, in which the time between the addition of methoprene (or ethanol) and the addition of ADP was alterred. The complete results of this experiment are given in Table 7.6 at the end of the chapter. Each determination was performed with 100 $\mu$l of mitochondrial extract and, in all treated runs, 95 $\mu$g of methoprene was injected. Therefore, the concentration of methoprene within the reaction chamber was constant. Lengthening the time between the injection of ethanol and

**Figure 7.6 — Oxygen Electrode Traces; *In Vitro* Treatments with Methoprene, Experiment XII**

The figure displays representative oxygen electrode traces from Expt. XII in which the timing of *in vitro* additions was changed. The uppermost trace displays a typical control determination with a time of 120 seconds having elapsed between the injection of ethanol and the first addition of ADP. The lower three traces show the effect of increasing the time between the injection of 95 $\mu$g of methoprene and the first ADP addition (30, 60 and 100 seconds respectively). The longer the elapsed time, the greater the inhibition of oxygen consumption and respiratory control.

Legend: ordinate – chart divisions (= oxygen),
abscissa – time (minutes),
$\downarrow^1$ – addition of 100 $\mu$l mitochondria, plus pyruvate and proline,
$\downarrow^2$ – addition of ethanol/methoprene,
$\downarrow^3$ – addition of ADP.

All the traces are drawn to the same scale. The rate of oxygen consumption was deemed to be not measureable when it was no longer possible to determine a steady rate of oxygen depletion.

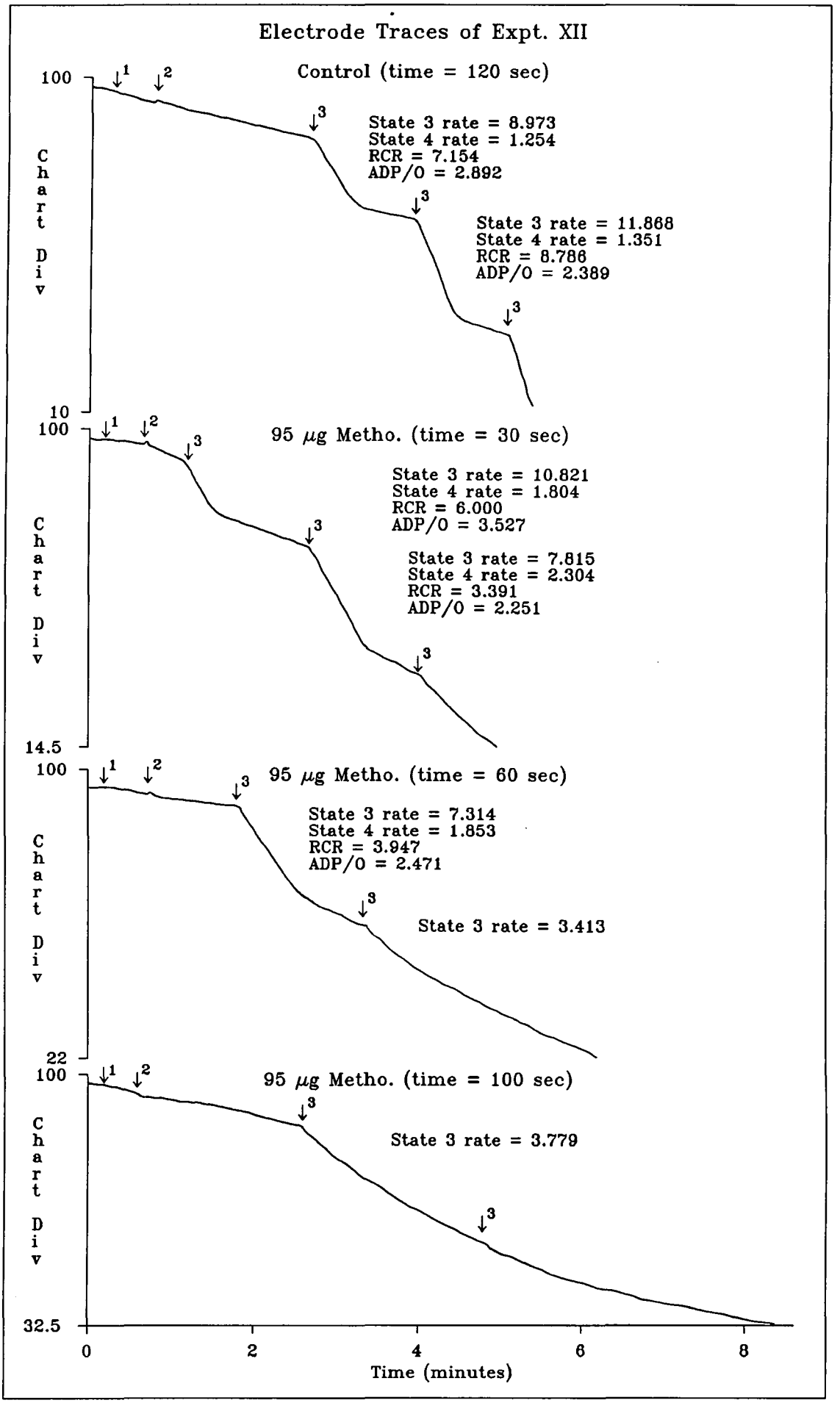

Electrode Traces of Expt. XII
Control (time = 120 sec)
State 3 rate = 8.973
State 4 rate = 1.254
RCR = 7.154
ADP/O = 2.892
State 3 rate = 11.868
State 4 rate = 1.351
RCR = 8.786
ADP/O = 2.389
95 μg Metho. (time = 30 sec)
State 3 rate = 10.821
State 4 rate = 1.804
RCR = 6.000
ADP/O = 3.527
State 3 rate = 7.815
State 4 rate = 2.304
RCR = 3.391
ADP/O = 2.251
95 μg Metho. (time = 60 sec)
State 3 rate = 7.314
State 4 rate = 1.853
RCR = 3.947
ADP/O = 2.471
State 3 rate = 3.413
95 μg Metho. (time = 100 sec)
State 3 rate = 3.779
Chart Div
100
10
14.5
22
32.5
0
2
4
6
8
Time (minutes)

**Figure 7.7 — Oxygen Electrode Traces; *In Vitro* Treatments with Methoprene, Experiment XIII**

The figure displays representative oxygen electrode traces from Expt. XIII in which different amounts of mitochondrial suspension were added to the reaction medium. The uppermost trace represents a typical control determination, with 5 $\mu$l of ethanol and 120 $\mu$l of mitochondrial suspension being injected. The other three traces show the effect of 95 $\mu$g of methoprene upon mitochondria. As smaller volumes of mitochondrial suspension were used in the determinations, the disruption of mitochondrial activity by methoprene became greater.

Legend: ordinate – chart divisions (= oxygen),
abscissa – time (minutes),
$\downarrow^1$ – addition of mitochondria,
plus pyruvate and proline,
$\downarrow^2$ – addition of ethanol/methoprene,
$\downarrow^3$ – addition of ADP.

All the traces are drawn to the same scale. The rate of oxygen consumption was deemed to be not measureable when it was no longer possible to determine a steady rate of oxygen depletion.

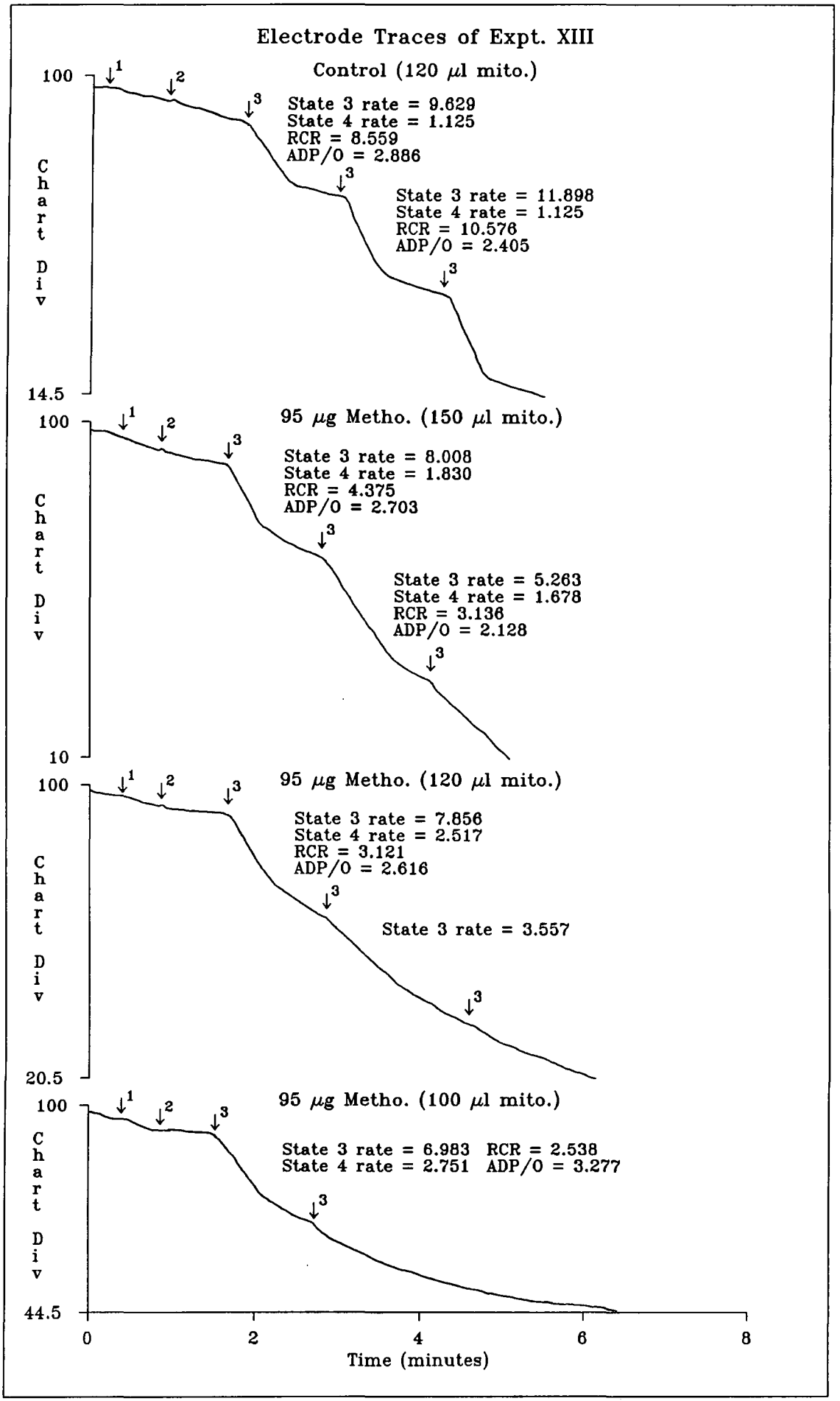

Electrode Traces of Expt. XIII
Control (120 μl mito.)
State 3 rate = 9.629
State 4 rate = 1.125
RCR = 8.559
ADP/O = 2.886
State 3 rate = 11.898
State 4 rate = 1.125
RCR = 10.576
ADP/O = 2.405
95 μg Metho. (150 μl mito.)
State 3 rate = 8.008
State 4 rate = 1.830
RCR = 4.375
ADP/O = 2.703
State 3 rate = 5.263
State 4 rate = 1.678
RCR = 3.136
ADP/O = 2.128
95 μg Metho. (120 μl mito.)
State 3 rate = 7.856
State 4 rate = 2.517
RCR = 3.121
ADP/O = 2.616
State 3 rate = 3.557
95 μg Metho. (100 μl mito.)
State 3 rate = 6.983 RCR = 2.538
State 4 rate = 2.751 ADP/O = 3.277
Chart Div
100
14.5
10
20.5
44.5
0
2
4
6
8
Time (minutes)

**Figure 7.8 — Oxygen Electrode Traces; *In Vitro* Treatments with Methoprene, Experiment XIII**

The figure displays representative oxygen electrode traces from Expt. XIII, in which the volume of mitochondrial suspension added to the reaction chamber remained constant (120 $\mu$l) while the amount of methoprene injected was changed. The uppermost trace shows a typical control determination, with 5 $\mu$l of ethanol and 120 $\mu$l of mitochondrial suspension being injected. The lower three traces show the effects of methoprene upon mitochondria. Mitochondrial activity was increasingly inhibited by the addition of progressively larger amounts of methoprene (57, 76 and 95 $\mu$g).

Legend: ordinate – chart divisions (= oxygen),
abscissa – time (minutes),
$\downarrow^1$ – addition of mitochondria,
plus pyruvate and proline,
$\downarrow^2$ – addition of ethanol/methoprene,
$\downarrow^3$ – addition of ADP.

All the traces are drawn to the same scale. The rate of oxygen consumption was deemed to be not measureable when it was no longer possible to determine a steady rate of oxygen depletion.

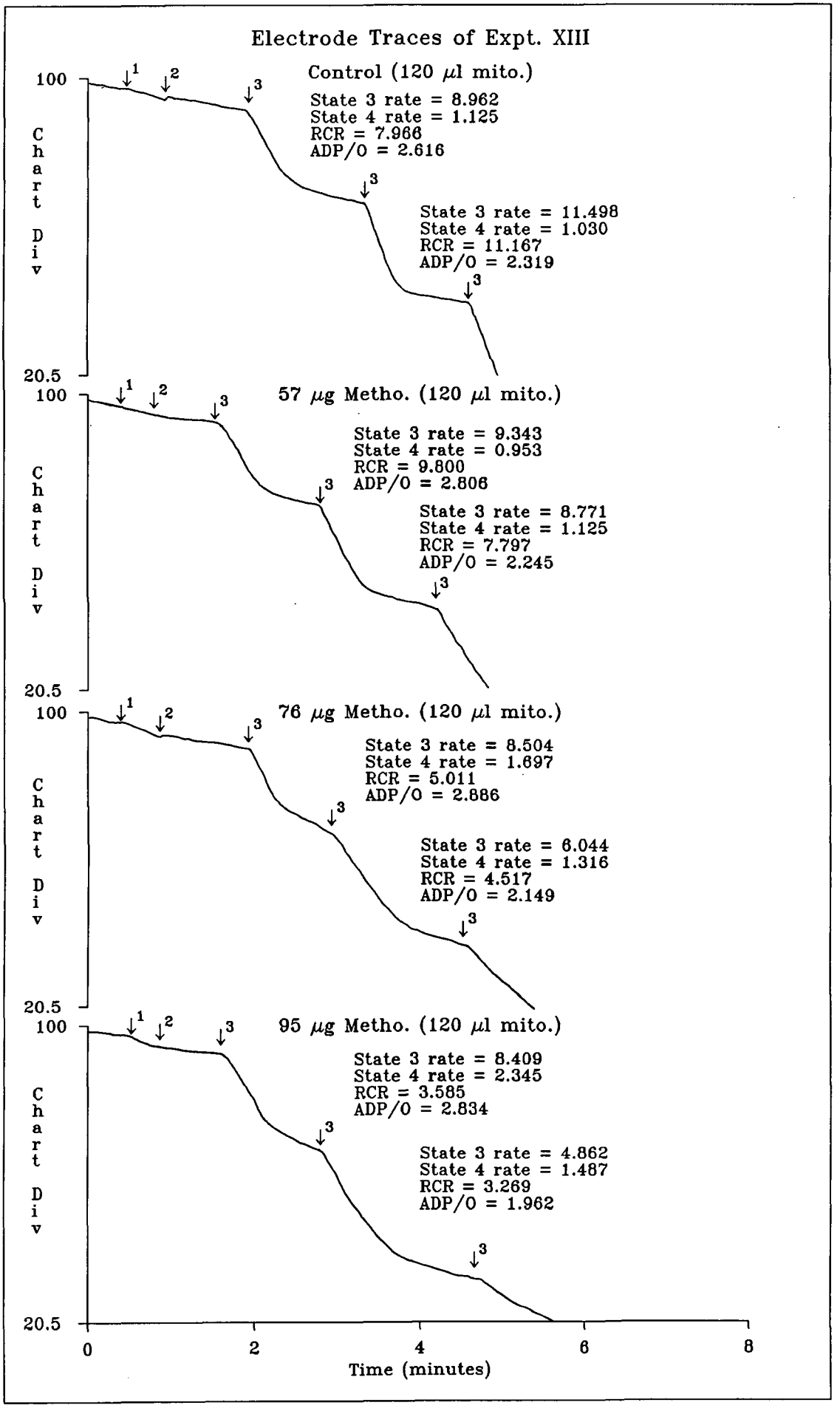

Electrode Traces of Expt. XIII
Control (120 μl mito.)
State 3 rate = 8.962
State 4 rate = 1.125
RCR = 7.966
ADP/O = 2.616
State 3 rate = 11.498
State 4 rate = 1.030
RCR = 11.167
ADP/O = 2.319
57 μg Metho. (120 μl mito.)
State 3 rate = 9.343
State 4 rate = 0.953
RCR = 9.800
ADP/O = 2.806
State 3 rate = 8.771
State 4 rate = 1.125
RCR = 7.797
ADP/O = 2.245
76 μg Metho. (120 μl mito.)
State 3 rate = 8.504
State 4 rate = 1.697
RCR = 5.011
ADP/O = 2.886
State 3 rate = 6.044
State 4 rate = 1.316
RCR = 4.517
ADP/O = 2.149
95 μg Metho. (120 μl mito.)
State 3 rate = 8.409
State 4 rate = 2.345
RCR = 3.585
ADP/O = 2.834
State 3 rate = 4.862
State 4 rate = 1.487
RCR = 3.269
ADP/O = 1.962
Chart Div
100
20.5
0
2
4
6
8
Time (minutes)

**Figure 7.9 — Oxygen Electrode Traces; *In Vitro* Treatments with Methoprene and JH III, Experiment XIV**

The figure shows some typical traces from Expt. XIV in which *in vitro* additions of both methoprene and JH III were made. The control (topmost) trace indicates that the mitochondria were fully functional, but the addition of 95 $\mu$g of methoprene (middle two traces) and 82 $\mu$g of JH III (bottom trace) markedly inhibited oxygen consumption. In only one determination of the experiment (second trace down) was it possible to measure a stable respiratory rate after the addition of JHA or JH, which were at a concentration of 182 nmoles/mg mitochondrial protein.

Legend: ordinate – chart divisions (= oxygen),
abscissa – time (minutes),
$\downarrow^1$ – addition of mitochondria,
– plus pyruvate and proline,
$\downarrow^2$ – addition of ethanol/IGR,
$\downarrow^3$ – addition of ADP.

All the traces are drawn to the same scale. The rate of oxygen consumption was deemed to be not measureable when it was no longer possible to determine a steady rate of oxygen depletion.

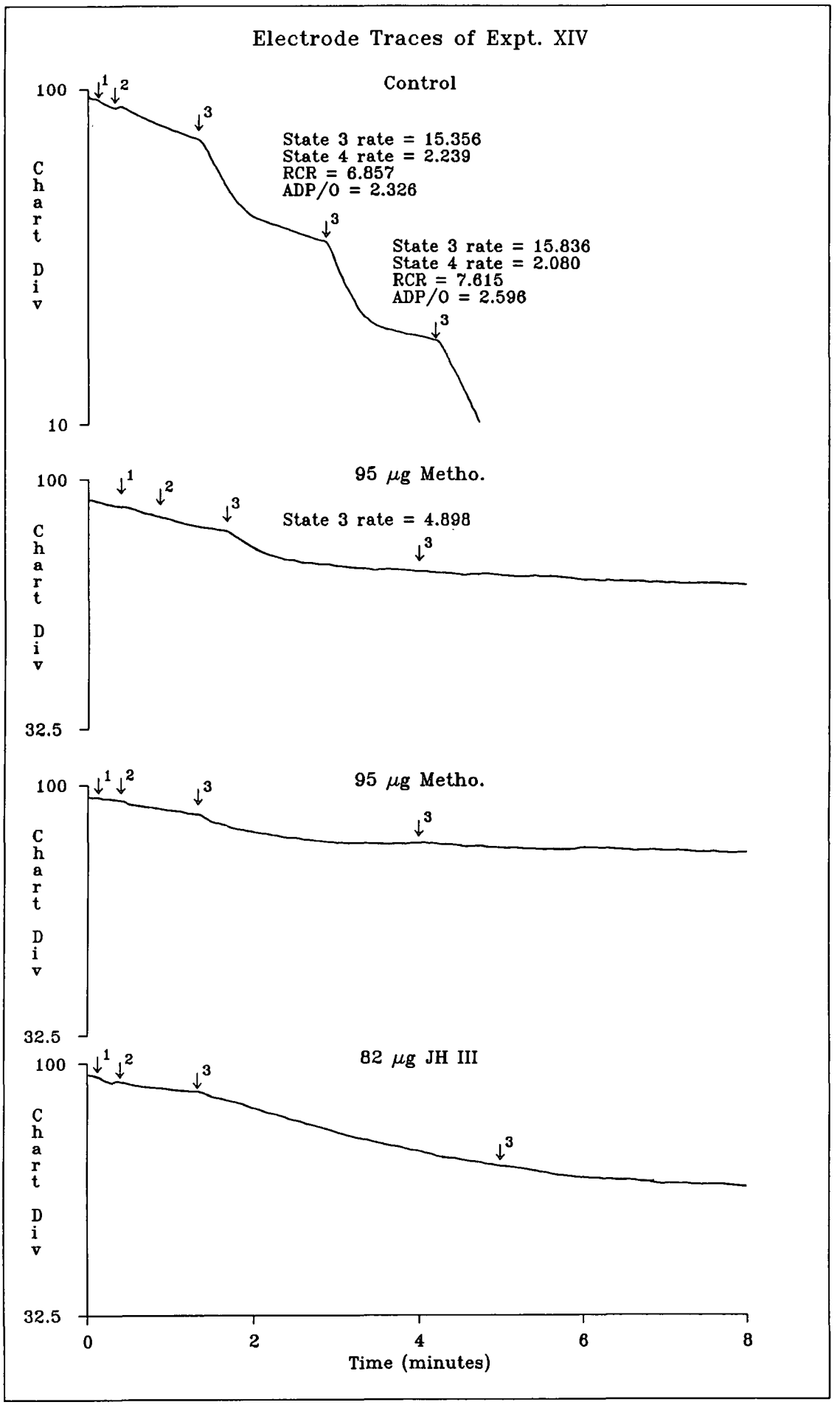
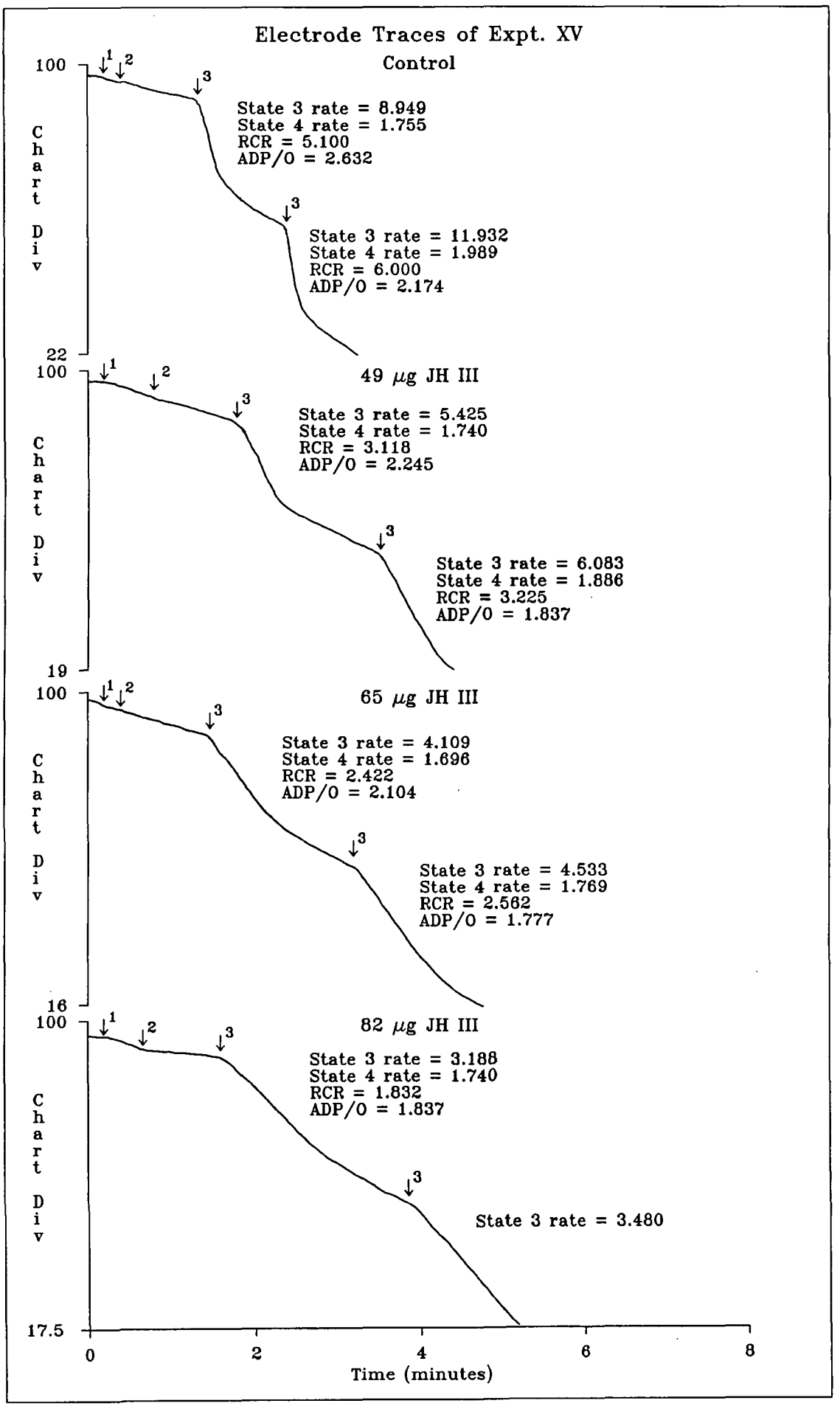

Electrode Traces of Expt. XIV
Control
100
10
Chart Div
1
2
3
State 3 rate = 15.356
State 4 rate = 2.239
RCR = 6.857
ADP/O = 2.326
State 3 rate = 15.836
State 4 rate = 2.080
RCR = 7.615
ADP/O = 2.596
95 μg Metho.
100
32.5
State 3 rate = 4.898
95 μg Metho.
82 μg JH III
0
2
4
6
8
Time (minutes)

**Figure 7.10 — Oxygen Electrode Traces; *In Vitro* Treatments with JH III, Experiment XV**

The figure shows some typical traces from Expt. XV in which different amounts of JH III were added. The control (topmost) trace indicates that the mitochondria were fully functional, but the addition of increasing amounts of JH III (lower three traces) caused progressively greater inhibition of respiratory metabolism.

Legend: ordinate – chart divisions (= oxygen),
abscissa – time (minutes),
$\downarrow^1$ – addition of mitochondria,
– plus pyruvate and proline,
$\downarrow^2$ – addition of ethanol/IGR,
$\downarrow^3$ – addition of ADP.

All the traces are drawn to the same scale. The rate of oxygen consumption was deemed to be not measureable when it was no longer possible to determine a steady rate of oxygen depletion.


Electrode Traces of Expt. XV
Control
State 3 rate = 8.949
State 4 rate = 1.755
RCR = 5.100
ADP/O = 2.632
State 3 rate = 11.932
State 4 rate = 1.989
RCR = 6.000
ADP/O = 2.174
49 μg JH III
State 3 rate = 5.425
State 4 rate = 1.740
RCR = 3.118
ADP/O = 2.245
State 3 rate = 6.083
State 4 rate = 1.886
RCR = 3.225
ADP/O = 1.837
65 μg JH III
State 3 rate = 4.109
State 4 rate = 1.696
RCR = 2.422
ADP/O = 2.104
State 3 rate = 4.533
State 4 rate = 1.769
RCR = 2.562
ADP/O = 1.777
82 μg JH III
State 3 rate = 3.188
State 4 rate = 1.740
RCR = 1.832
ADP/O = 1.837
State 3 rate = 3.480
Chart Div
100
22
19
16
17.5
0
2
4
6
8
Time (minutes)

the first addition of ADP had no effect on the control results. However, in the methoprene-treated runs, there was a progressive deterioration in mitochondrial function as the time between JHA injection and ADP addition was increased (20–100 seconds) until all respiratory control was lost after the first injection of ADP. Since the effect of treatment changed with the time elapsed, in later experiments the first ADP injection was always made precisely one minute after the addition of methoprene or JH III. In Expt. XIII, a series of determinations were made in which different volumes of mitochondrial suspension (100–150 $\mu$l) and different amounts of methoprene (57–95 $\mu$g) were added to the reaction chamber. The results of this experiment are given in Table 7.7 at the end of the chapter and some representative traces are shown in Figures 7.7 and 7.8. It can be seen that methoprene's disruptive effect upon mitochondrial respiration increased as larger amounts of methoprene or smaller volumes of mitochondrial suspension were injected into the chamber. Therefore, in the tables of results, the amounts of methoprene and JH III injected during *in vitro* experiments are converted to concentrations ($\mu$g of IGR per mg of mitochondrial protein) to take account of the fluctuating amounts of protein in different mitochondrial extractions. Three further *in vitro* experiments were carried out in which varying amounts of JH III and methoprene were injected into the reaction chamber of the apparatus. Representative traces from Expt. XIV are displayed in Figure 7.9, showing that high concentrations of methoprene (56.5 $\mu$g/mg protein) and JH III (48.5 $\mu$g/mg protein) completely inhibited mitochondrial oxygen consumption. Figure 7.10 shows the results of injecting different amounts of JH III. The hormone had an effect similar to that of methoprene, causing a disruption of mitochondrial respiration which increased with increasing amounts of JH III.

The mean results from all the *in vitro* experiments are listed in Table 7.8 at the end of the chapter. Large variations were encountered among the control results from the different experiments (different mitochondrial extractions). Therefore, to allow easier comparison of experiments, the results were normalised (i.e. the treated results were expressed as proportions of the corresponding control values), and the concentrations of methoprene and JH III were converted from $\mu$g/mg protein to nmoles per mg protein in order to permit comparison of the two treatments (see Table 7.9, at the end of the chapter). At the highest concentrations of JH and JHA (182 nmoles/mg protein), respiration was rapidly

inhibited, and in only one of the determinations, with methoprene, was there any response to the addition of ADP. At lower concentrations (~97–110 nmoles methoprene and 75 nmoles JH III), respiratory control was lost after the second ADP addition and no subsequent state 4 rate or ADP/O ratio could be determined. With both compounds, the effects of treatment were greater after the second addition of ADP than after the first, though this was far more marked with methoprene. Except at concentrations where mitochondrial function was completely inhibited, methoprene tended to increase the state 4 rate, whereas it was either unaffected or slightly inhibited by JH III. In other respects, the two treatments had similar effects, reducing state 3 rates, RCRs and, to a lesser extent, ADP/O ratios. In terms of molar activity, JH III was more potent than methoprene. Again, it should be noted that the average values quoted in Tables 7.8 and 7.9 are derived only from the determinations that gave measureable results and therefore underestimate the effects of the higher concentrations of the IGRs, which caused a complete inhibition of respiration in some determinations.

## 7.4 Discussion

In the experiments described above, the majority of mitochondrial extractions from control or untreated animals exhibited good respiratory control and coupling of oxygen consumption to ADP phosphorylation, the results comparing favourably with those from other studies (e.g. STEGWEE and VAN KAMMEN-WERTHEIM, 1962; VAN DEN BERGH and SLATER, 1962; CHILDRESS and SACKTOR, 1966; MINKS, 1967; AL-ROBAI, 1981). This indicates that the isolated mitochondria were intact and that they contained all the metabolic systems necessary for oxidative phosphorylation. When mitochondria metabolise substrates which have NAD-linked oxidations, such as pyruvate, three molecules of ADP are phosphorylated to ATP for every pair of electrons that passes down the electron-transport chain (see Figure 7.1). Therefore, the respiration of pyruvate should, theoretically, give an ADP/O quotient of 3.0, while lower values would suggest a certain degree of uncoupling of ATP synthesis from the transfer of electrons (i.e. electron transport continues but it is no longer linked to the phosphorylation of ADP). However, in the present study, some of the ADP/O values recorded after the first ADP addition were greater than the theoretical maximum of three. Similar results have been recorded elsewhere (VAN DEN BERGH and SLATER,

1962; TRIBE and ASHURST, 1972; AL-ROBAI, 1981) but no explanation for this phenomenon has been provided.

Direct additions of methoprene and JH III to isolated mitochondria had very damaging effects on respiratory metabolism, in agreement with the proposal of STEELE (1976) that the *in vitro* action of JH is to disrupt mitochondrial membranes. The higher concentrations of JH and JHA caused a rapid loss of mitochondrial respiration, which would be expected if NADH–ubiquinone electron transport was inhibited, as suggested by FIRSTENBERG and SILHACEK (1973). JH III was the more potent of the two compounds in terms of molar activity and this may be due to the hormone's greater hydrophobicity (GOODMAN and CHANG, 1985) since the more lipophilic compound would more readily interact with membrane lipids, the probable site of JH action on membranes (BAUMANN, 1968, 1969). Where respiratory rates were measureable, methoprene lowered the state 3 rate but stimulated the state 4 rate and this reduction of respiratory control suggests that methoprene may also have an uncoupling effect, as was found by CHEFURKA (1978) on mouse liver mitochondria. Methoprene-induced disruption of mitochondrial membranes might be expected to cause a loss of respiratory control since the uncoupling of oxidative phosphorylation can be achieved by increasing mitochondrial permeability to hydrogen ions (LEHNINGER, 1982). In the present study, methoprene increased state 4 rate respiration at concentrations of 75–117 nmoles per mg of mitochondrial protein, whereas JH III seemed to have no stimulatory effect. In contrast, CHEFURKA (1978) reported that juvenile hormones (JH I, II and III) were far more effective than methoprene in uncoupling oxidative phosphorylation in mouse liver mitochondria. However, the concentrations that he required for maximal stimulation of state 4 respiration (~400–500 nmoles/mg protein for the JHs and ~2,500 nmoles/mg protein for methoprene), were much higher than those used in the present study, suggesting that insect mitochondria may be considerably more sensitive to IGR-mediated damage than mammalian mitochondria.

The *in vivo* application of 475 $\mu$g of methoprene to fifth instar locusts significantly reduced the amount of mitochondrial protein which could later be extracted from the adults. The average amount of protein extracted from each methoprene-treated animal was ~40% of that isolated from control locusts. This agrees with the results of Chapter 6 which described flight muscle structure.

Flight muscle fibres from adults which had been treated earlier with 475 $\mu$g had smaller cross-sectional areas than those from control animals. Also, the ultrastructural study showed that, in 8-day old adults, mitochondria occupied 31% of the volume of control flight muscle fibres whereas they only accounted for 18% of the volume of 475 $\mu$g-treated flight muscle fibres, and 26% of 95 $\mu$g-treated muscle fibres (i.e. 58% and 84% of control values for 475 $\mu$g and 95 $\mu$g treatments respectively). Although there were no apparent signs of mitochondrial damage in the ultrastructural study, another reason for the abnormally low yields of mitochondria from methoprene-treated animals could be that they were unusually fragile, possibly due to changes in membrane properties. If so, the mitochondria would be more susceptible to injury and destruction during the extraction procedure. This could account for the reduced respiratory control of the isolated mitochondria, indicative of structural damage (LEHNINGER, 1982). Increased mitochondrial fragility could also explain the observation that treated mitochondria showed greater impairment of respiratory metabolism after the second addition of ADP. In the reaction chamber, the mitochondria were subjected to both the mechanical forces of stirring and a higher temperature, and the more frail organelles would be expected to degrade faster.

The similarity between the results from *in vitro* and *in vivo* treatments with methoprene raises the possibility that the mode of action of the JHA was similar in both cases, presumably causing a loss of structural integrity of the inner mitochondrial membrane by penetrating the lipid bilayer. This requires that methoprene penetrated the flight muscle cells after its *in vivo* application and reacted directly with the cell mitochondria. It now seems probable that the physiological mode of action of JH is similar to that proposed for steroid hormones (YAMAMOTO and ALBERTS, 1976) so that, when the hormone reaches a target cell, it is bound to a cytoplasmic receptor which transports it to the nucleus where it binds to sites on the chromatin in order to affect transcription (ROBERTS and WYATT, 1983). High-affinity binding of JH to cytosolic proteins has been detected in various insect tissues such as epidermis of *Drosophila melanogaster* (KLAGES *et al.*, 1980), fat body and ovary of *Leucophaea maderae* ENGELMANN, 1981a, 1981b; KOEPPE *et al.*, 1981) and fat body of *Locusta migratoria* (ROBERTS and WYATT, 1983). Because of the *in vitro* effects of JH III upon mitochondria and its general surfactant properties, such a JH-receptor would be necessary to

prevent intracellular JH from reacting with a number of membrane-bound organelles (GOODMAN and CHANG, 1985). Furthermore, it has been demonstrated that cytosolic JH-receptors will bind methoprene, albeit less strongly than JH (KLAGES *et al*, 1980; KOEPPE *et al.*, 1981; ROBERTS and WYATT, 1983). Thus, it would appear that there are mechanisms available which would permit the entry of methoprene into insect cells. Also, the relatively weak binding of methoprene to intracellular JH-receptors suggests that bound methoprene might disassociate from the binding protein and so be free to react with mitochondria, as well as many other cellular components. However, such a mode of action would require either that there be methoprene still freely available in the locust 20–25 days after it had been applied, or that mitochondria affected by methoprene early in the fifth instar survived with disrupted membranes up to the fifteenth day of adult life. Neither explanation seems likely. Also, such non-specific binding of cellular components would affect many other insect tissues as well and one would expect a much greater incidence of immediate toxicity than was recorded in the present study (see Chapter 3).

An alternative explanation for the *in vivo* response of flight muscle mitochondria to methoprene is that the JHA penetrated the nucleus and affected RNA transcription, preventing normal tissue development. AL-ROBAI (1981) suggested that mitochondria from the flight muscles of young adult locusts may be more fragile than those of mature adults. Thus, it is possible that mitochondria extracted from JHA-dosed adults were still in an immature state and, hence, were abnormally fragile. If such mitochondria were, indeed, not fully developed, their impaired respiratory function could also be explained by their being metabolically juvenile. The normal development of locust flight muscle begins after the fourth moult and is completed by the eighth day of the adult instar (BÜCHER, 1965). During this period, there is a marked change in the pattern of metabolic enzymes, both cytosolic and mitochondrial (BROSEMER *et al.*, 1963; BEENAKKERS *et al.*, 1975). VAN DEN HONDEL-FRANKEN *et al.* (1980) found that the implantation of CA into young fifth instar *Locusta* delayed the development of the adult enzyme pattern in flight muscle (see the Introduction for details). The activities of those mitochondrial enzymes which were studied appeared to be permanently reduced and it was suggested that mitochondriogenesis was irreversibly inhibited by the operation. The respiratory metabolism

of immature mitochondria was measured by AL-ROBAI (1981), who investigated developmental changes in the flight muscle of *Locusta*. During the period from Day 1 to Day 8 of the adult instar, there were pronounced changes in the ability of the mitochondria to oxidise pyruvate/proline. The state 3 rate increased from ~2–19 $\mu$g atoms oxygen/mg protein/hour (average values from a number of subsequent ADP additions), state 4 rate from ~1.5–2.5 (not significantly different), and the RCR from ~1.5–8.3. The ADP/O ratio was not measureable until the second day of the adult instar (~1.0) and rose to ~2.5 $\mu$moles ADP/$\mu$g atom oxygen by Day 8. In the present study, the equivalent values for mitochondria of 475 $\mu$g methoprene-treated animals and controls respectively, were as follows; 3.4 and 11.1 for the state 3 rate, 0.9 and 1.4 for state 4 rates, 4.4 and 8.4 for the RCR, and 2.0 and 2.2 for the ADP/O ratio. Although the pattern of changes is similar, the magnitude of the differences between mitochondria from immature and mature adults do not exactly match those between methoprene-treated and control animals. This suggests that the *in vivo* effects of methoprene on mitochondrial activity may not be solely due to the maintenance of the organelles' enzymatic capabilities in a juvenile state. Such a conclusion is to be expected if one considers the results of the ultrastructural study in Chapter 6. The topical application of methoprene to fifth instar locusts did not simply inhibit or delay the maturation of the flight muscles but, rather, caused abnormal development of the tissue and actually seemed to accelerate muscle fibre differentiation during the fifth instar.

In the one experiment that tested the *in vivo* effects of JH III, the respiration of mitochondria from treated locusts was no different from that of control mitochondria. Not only was there no effect on mitochondrial activity, but the JH III-treated animals showed none of the subsequent morphological changes (e.g. green coloration of cuticle and haemolymph, deformed wings etc.) that were associated with the application of methoprene in the present study, or with the implantation of corpora allata by other workers (e.g. POELS and BEENAKKERS, 1969). Therefore, it would appear that the topically applied JH III either failed to penetrate the insects or that it was metabolised before it could affect any susceptible tissues. Insects produce JH-specific esterases during the last larval instar, presumably to ensure a low JH titre for metamorphosis (see review by DE KORT and GRANGER, 1981), and it has been found that the morphological ac-

tivity of exogenously applied JH is very dependent on the method of application. JH extracts from adult male cecropia moths had no effect on fifth instar larvae of *Rhodnius* when topically applied or if injected into the haemocoel as an emulsion whereas the hormone was biologically active when injected as a dilute solution in a drop of oil or in a mixture with paraffin wax (GILBERT and SCHNEIDERMAN, 1960; WIGGLESWORTH, 1969).

**Table 7.2 — Mitochondrial Respiration; *In Vivo* Effects of 475 $\mu$g Methoprene**

Legend:
- n – number of determinations (the results shown are the average values),
- $*n$ – only $n$ number of determinations were measureable,
- ** – none of the determinations gave a measureable result,
- $\bar{x}$ – mean values.

State 3 rates and state 4 rates are in units of $\mu$g atoms of oxygen per mg of mitochondrial protein per hour. Student's $t$ tests were performed on the mean values while paired $t$ tests were performed on the paired values of the individual experiments.

**Effects of 475 $\mu$g Methoprene, *in vivo* Treatment**

| Expt. | treatment | n | First ADP addition | | | | Second ADP addition | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | state 3 rate | state 4 rate | RCR | ADP/O | state 3 rate | state 4 rate | RCR | ADP/O |
| I | Control | 2 | 5.64 | 0.71 | 8.17 | 2.51 | 5.50 | 1.13 | 5.80 | 2.70 |
| | 475 $\mu$g Metho. | 2 | 1.69 | 0.46*1 | 3.83*1 | 2.39*1 | ** | ** | ** | ** |
| II | Control | 3 | 9.83 | 0.95 | 10.17 | 2.43 | 13.42 | 1.01 | 13.50 | 1.93 |
| | 475 $\mu$g Metho. | 5 | 2.70 | 0.46 | 6.39 | 2.38 | 2.73*2 | 0.62*2 | 4.33*2 | 1.68*2 |
| III | Control | 4 | 14.12 | 1.87 | 7.61 | 2.06 | 20.08 | 1.85 | 11.36 | 1.69 |
| | 475 $\mu$g Metho. | 4 | 4.72 | 0.91 | 5.24 | 1.97 | 4.71 | 0.85 | 5.59 | 1.62 |
| IV | Control | 7 | 10.09 | 1.52 | 6.76 | 2.50 | 12.05 | 2.14 | 5.65 | 1.78 |
| | 475 $\mu$g Metho. | 6 | 4.34 | 1.16 | 4.61 | 2.50 | 3.49 | 1.36 | 2.67 | 1.56 |
| V | Control | 5 | 10.17 | 1.25 | 8.37 | 2.63 | 10.00 | 1.59 | 6.31 | 1.79 |
| | 475 $\mu$g Metho. | 5 | 4.29 | 0.91 | 4.85 | 2.56 | 2.11 | 0.92*3 | 2.83*3 | 1.49*3 |
| | | | | | | | | | | |
| $\bar{x}$ | Control | | 9.97 ± 1.34 | 1.26 ± 0.20 | 8.21 ± 0.56 | 2.43 ± 0.10 | 12.21 ± 2.38 | 1.55 ± 0.21 | 8.52 ± 1.63 | 1.98 ± 0.19 |
| $\bar{x}$ | 475 $\mu$g Metho. | | 3.55 ± 0.58 | 0.78 ± 0.14 | 4.99 ± 0.42 | 2.36 ± 0.10 | 3.26 ± 0.56 | 0.94 ± 0.15 | 3.85 ± 0.69 | 1.59 ± 0.04 |
| Student's $t$ test | | | P< 0.01 | P> 0.05 | P< 0.02 | P> 0.05 | P< 0.02 | P> 0.05 | P< 0.05 | P> 0.05 |
| Paired $t$ test | | | P< 0.01 | P< 0.02 | P< 0.002 | P< 0.05 | P< 0.01 | P< 0.02 | P< 0.05 | P< 0.05 |

**Table 7.3 — Mitochondrial Respiration; *In Vivo* Effects of 95 $\mu$g Methoprene**

Legend: n – number of determinations
(the results shown are the average values),
$\bar{x}$ – mean values.

State 3 rates and state 4 rates are in units of $\mu$g atoms of oxygen per mg of mitochondrial protein per hour. Student's $t$ tests were performed on the mean values while paired $t$ tests were performed on the paired values of the individual experiments.

**Effects of 95 $\mu$g Methoprene, *in vivo* Treatment**

| Expt. | treatment | n | First ADP addition | | | | Second ADP addition | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | state 3 rate | state 4 rate | RCR | ADP/O | state 3 rate | state 4 rate | RCR | ADP/O |
| VI | Control | 3 | 3.02 | 0.77 | 3.90 | 3.18 | 2.84 | 0.61 | 4.61 | 2.73 |
| | 95 $\mu$g Metho. | 3 | 2.64 | 0.79 | 3.40 | 3.08 | 2.36 | 0.79 | 3.10 | 2.93 |
| VII | Control | 5 | 6.86 | 1.22 | 5.46 | 3.64 | 7.67 | 1.15 | 6.42 | 1.98 |
| | 95 $\mu$g Metho. | 5 | 6.40 | 1.37 | 4.65 | 3.75 | 6.04 | 1.17 | 5.11 | 2.61 |
| VIII | Control | 4 | 11.96 | 2.33 | 5.15 | 3.78 | 17.05 | 2.73 | 6.37 | 2.77 |
| | 95 $\mu$g Metho. | 4 | 10.55 | 2.36 | 4.54 | 3.50 | 15.79 | 2.80 | 5.65 | 2.79 |
| IX | Control | 4 | 14.11 | 2.90 | 4.78 | 3.22 | 11.93 | 2.38 | 5.75 | 2.69 |
| | 95 $\mu$g Metho. | 3 | 15.47 | 3.57 | 4.33 | 3.00 | 11.15 | 2.51 | 4.56 | 3.00 |
| X | Control | 4 | 7.84 | 1.15 | 7.22 | 3.31 | 8.04 | 1.46 | 5.47 | 2.09 |
| | 95 $\mu$g Metho. | 4 | 7.63 | 1.34 | 5.90 | 2.93 | 7.54 | 1.50 | 5.18 | 1.98 |
| | | | | | | | | | | |
| $\bar{x}$ | Control | | 8.76 $\pm$ 1.95 | 1.68 $\pm$ 0.40 | 5.30 $\pm$ 0.55 | 3.43 $\pm$ 0.12 | 9.51 $\pm$ 2.37 | 1.67 $\pm$ 0.39 | 5.72 $\pm$ 0.33 | 2.45 $\pm$ 0.17 |
| $\bar{x}$ | 95 $\mu$g Metho. | | 8.54 $\pm$ 2.15 | 1.89 $\pm$ 0.49 | 4.56 $\pm$ 0.40 | 3.25 $\pm$ 0.16 | 8.58 $\pm$ 2.29 | 1.75 $\pm$ 0.39 | 4.72 $\pm$ 0.44 | 2.66 $\pm$ 0.18 |
| Student's $t$ test | | | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 |
| Paired $t$ test | | | P> 0.05 | P> 0.05 | P< 0.01 | P> 0.05 | P< 0.02 | P< 0.05 | P< 0.02 | P> 0.05 |

**Table 7.4 — Mitochondrial Respiration; *In Vivo* Effects of 408 $\mu$g of JH III**

State 3 rates and state 4 rates are in units of $\mu$g atoms of oxygen per mg of mitochondrial protein per hour. Student's $t$ tests were performed on the average values.

**Table 7.5 — Mitochondrial Respiration; *In Vitro* Effects of Absolute Ethanol**

State 3 rates and state 4 rates are in units of $\mu$g atoms of oxygen per mg of mitochondrial protein per hour.

**Expt. XI; Effects of 408 $\mu$g JH III, *in vivo* Treatment**

| Treatment | n | First ADP addition | | | | Second ADP addition | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | state 3 rate | state 4 rate | RCR | ADP/O | state 3 rate | state 4 rate | RCR | ADP/O |
| Control | 4 | 7.79 (± 0.45) | 0.58 (± 0.06) | 14.10 (± 2.10) | 2.70 (± 0.12) | 11.65 (± 1.05) | 0.77 (± 0.08) | 15.68 (± 1.09) | 1.96 (± 0.06) |
| 408 $\mu$g JH III | 4 | 8.17 (± 0.79) | 0.50 (± 0.10) | 18.35 (± 4.15) | 2.91 (± 0.04) | 12.16 (± 0.69) | 0.74 (± 0.12) | 18.14 (± 3.96) | 2.00 (± 0.09) |
| Student's *t* test | | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 | P> 0.05 |

**Effects of Ethanol, *in vitro* Additions**

| Treatment | First ADP Addition | | | | Second ADP Addition | | | |
|---|---|---|---|---|---|---|---|---|
| | state 3 rate | state 4 rate | RCR | ADP/O | state 3 rate | state 4 rate | RCR | ADP/O |
| Untreated | 6.08 | 0.90 | 6.77 | 3.44 | 9.11 | 1.01 | 9.01 | 3.00 |
| Untreated | 4.68 | 0.73 | 6.44 | 2.99 | 9.02 | 0.89 | 10.18 | 2.61 |
| 5 $\mu$l Ethanol | 6.41 | 0.84 | 7.62 | 3.00 | 9.78 | 1.13 | 8.70 | 2.81 |
| 10 $\mu$l Ethanol | 3.89 | 0.57 | 6.84 | 3.06 | 6.41 | 0.73 | 8.81 | 2.59 |
| 15 $\mu$l Ethanol | 5.51 | 0.61 | 8.98 | 3.12 | 7.35 | 0.73 | 10.11 | 2.81 |
| 15 $\mu$l Ethanol | 4.56 | 0.61 | 7.43 | 3.18 | 6.58 | 0.73 | 9.05 | 2.73 |
| 20 $\mu$l Ethanol | 7.84 | 1.31 | 6.00 | 3.36 | 12.44 | 1.48 | 8.42 | 2.92 |
| 20 $\mu$l Ethanol | 4.86 | 0.71 | 6.90 | 2.95 | 6.05 | 0.77 | 7.82 | 2.56 |

**Table 7.6 — Mitochondrial Respiration; *In Vitro* Effects of Methoprene, Experiment XII**

Legend: Time – time (in seconds) between the treatment and the first addition of ADP,
** – the result was not measureable.

State 3 rates and state 4 rates are in units of $\mu$g atoms of oxygen per mg of mitochondrial protein per hour.

**Expt. XII; Effects of Timing of *in vitro* Additions**

| Treatment | Time | First ADP addition | | | | Second ADP addition | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | state 3 rate | state 4 rate | RCR | ADP/O | state 3 rate | state 4 rate | RCR | ADP/O |
| 5 $\mu$l Ethanol | 20 | 11.02 | 1.23 | 8.98 | 3.37 | 14.70 | 1.45 | 10.11 | 2.73 |
| 5 $\mu$l Ethanol | 60 | 9.31 | 1.36 | 6.86 | 3.31 | 9.89 | 1.16 | 8.50 | 3.12 |
| 5 $\mu$l Ethanol | 120 | 8.97 | 1.25 | 7.15 | 2.89 | 11.87 | 1.35 | 8.79 | 2.39 |
| 5 $\mu$l Ethanol | 120 | 10.26 | 1.24 | 8.31 | 3.10 | 12.54 | 1.14 | 11.00 | 2.54 |
| 95 $\mu$g Metho. | 20 | 11.77 | 1.77 | 6.67 | 2.92 | 11.77 | 2.45 | 4.80 | 2.40 |
| 95 $\mu$g Metho. | 30 | 10.82 | 1.80 | 6.00 | 3.53 | 7.82 | 2.30 | 3.39 | 2.25 |
| 95 $\mu$g Metho. | 30 | 11.35 | 2.81 | 4.03 | 2.67 | 8.73 | 3.49 | 2.50 | 2.19 |
| 95 $\mu$g Metho. | 60 | 7.31 | 1.85 | 3.95 | 2.47 | 3.41 | ** | ** | ** |
| 95 $\mu$g Metho. | 60 | 9.31 | 2.91 | 3.20 | 2.23 | 5.92 | ** | ** | ** |
| 95 $\mu$g Metho. | 60 | 5.92 | ** | ** | ** | ** | ** | ** | ** |
| 95 $\mu$g Metho. | 60 | 3.57 | ** | ** | ** | ** | ** | ** | ** |
| 95 $\mu$g Metho. | 80 | 2.62 | ** | ** | ** | ** | ** | ** | ** |
| 95 $\mu$g Metho. | 100 | 3.78 | ** | ** | ** | ** | ** | ** | ** |

**Table 7.7 — Mitochondrial Respiration; *In Vitro* Effects of Methoprene, Experiment XIII**

Legend: Conc. – concentration of methoprene as $\mu$g per mg mitochondrial protein,
** – the result was not measureable.

State 3 rates and state 4 rates are in units of $\mu$g atoms of oxygen per mg of mitochondrial protein per hour.

**Expt. XIII; Effects of Relative Amounts of *in vitro* Additions**

| Amount Mito. | Amount Treatment | Conc. Metho. | First ADP addition | | | | Second ADP addition | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | state 3 rate | state 4 rate | RCR | ADP/O | state 3 rate | state 4 rate | RCR | ADP/O |
| 150 $\mu$l | 5 $\mu$l Ethanol | — | 10.07 | 1.07 | 9.43 | 2.63 | 14.19 | 1.07 | 13.29 | 2.33 |
| 120 $\mu$l | 5 $\mu$l Ethanol | — | 9.63 | 1.13 | 8.56 | 2.89 | 11.90 | 1.13 | 10.58 | 2.41 |
| 100 $\mu$l | 5 $\mu$l Ethanol | — | 8.96 | 1.13 | 7.97 | 2.62 | 11.50 | 1.03 | 11.17 | 2.32 |
| 150 $\mu$l | 57 $\mu$g Metho. | 14.5 $\mu$g Metho. | 9.38 | 0.92 | 10.25 | 2.63 | 9.61 | 0.92 | 10.50 | 2.22 |
| 150 $\mu$l | 76 $\mu$g Metho. | 18.7 $\mu$g Metho. | 9.06 | 1.13 | 8.03 | 2.73 | 8.16 | 1.13 | 7.23 | 2.20 |
| 120 $\mu$l | 57 $\mu$g Metho. | 18.7 $\mu$g Metho. | 9.34 | 0.95 | 9.80 | 2.81 | 8.77 | 1.13 | 7.80 | 2.25 |
| 150 $\mu$l | 95 $\mu$g Metho. | 24.2 $\mu$g Metho. | 8.01 | 1.83 | 4.38 | 2.70 | 5.26 | 1.68 | 3.14 | 2.13 |
| 150 $\mu$l | 95 $\mu$g Metho. | 24.2 $\mu$g Metho. | 8.30 | 1.50 | 5.55 | 2.55 | 5.83 | 1.42 | 4.11 | 2.13 |
| 120 $\mu$l | 76 $\mu$g Metho. | 24.2 $\mu$g Metho. | 8.50 | 1.70 | 5.01 | 2.89 | 6.04 | 1.32 | 4.52 | 2.15 |
| 120 $\mu$l | 76 $\mu$g Metho. | 24.2 $\mu$g Metho. | 7.86 | 2.06 | 3.82 | 2.83 | 5.32 | 1.41 | 3.77 | 2.08 |
| 120 $\mu$l | 95 $\mu$g Metho. | 30.2 $\mu$g Metho. | 9.92 | 1.70 | 5.84 | 2.89 | 8.77 | 1.41 | 6.22 | 2.20 |
| 120 $\mu$l | 95 $\mu$g Metho. | 30.2 $\mu$g Metho. | 8.41 | 2.35 | 3.59 | 2.83 | 4.86 | 1.49 | 3.27 | 1.96 |
| 120 $\mu$l | 95 $\mu$g Metho. | 30.2 $\mu$g Metho. | 7.91 | 1.64 | 4.83 | 2.38 | 3.92 | ** | ** | ** |
| 120 $\mu$l | 95 $\mu$g Metho. | 30.2 $\mu$g Metho. | 7.86 | 2.52 | 3.12 | 2.62 | 3.56 | ** | ** | ** |
| 100 $\mu$l | 95 $\mu$g Metho. | 36.2 $\mu$g Metho. | 6.98 | 2.75 | 2.54 | 3.277 | ** | ** | ** | ** |

**Table 7.8 — Mitochondrial Respiration; *In Vitro* Effects of Methoprene and JH III**

Legend: n – number of determinations
(the results shown are the mean values),
$*n$ – only $n$ number of determinations were measureable,
** – none of the determinations gave a measureable result,

State 3 rates and state 4 rates are in units of $\mu$g atoms of oxygen per mg of mitochondrial protein per hour.

**Effects of Methoprene and JH III, *in vitro* Additions**

| Expt. | treatment | n | First ADP addition | | | | Second ADP addition | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | state 3 rate | state 4 rate | RCR | ADP/O | state 3 rate | state 4 rate | RCR | ADP/O |
| XII | Control | 4 | 9.51 ± 0.38 | 1.28 ± 0.04 | 7.44 ± 0.44 | 3.10 ± 0.12 | 11.43 ± 0.79 | 1.22 ± 0.07 | 9.43 ± 0.79 | 2.68 ± 0.22 |
| | 33.6 μg Metho. | 4 | 6.53 ± 1.21 | 2.38*2 | 3.57*2 | 2.35*2 | 4.67*2 | ** | ** | ** |
| XIII | Control | 3 | 9.55 ± 0.32 | 1.11 ± 0.02 | 8.65 ± 0.43 | 2.71 ± 0.09 | 12.53 ± 0.84 | 1.07 ± 0.03 | 11.68 ± 0.82 | 2.35 ± 0.03 |
| | 14.5 μg Metho. | 1 | 9.38 | 0.92 | 10.25 | 2.63 | 9.61 | 0.92 | 10.50 | 2.22 |
| | 18.7 μg Metho. | 2 | 9.20 | 1.04 | 8.91 | 2.77 | 8.47 | 1.13 | 7.51 | 2.22 |
| | 24.2 μg Metho. | 4 | 8.17 ± 0.15 | 1.77 ± 0.12 | 4.69 ± 0.38 | 2.74 ± 0.08 | 5.61 ± 0.19 | 1.46 ± 0.08 | 3.88 ± 0.29 | 2.12 ± 0.01 |
| | 30.2 μg Metho. | 4 | 8.52 ± 0.48 | 2.05 ± 0.22 | 4.34 ± 0.62 | 2.68 ± 0.12 | 5.28 ± 1.20 | 1.45*2 | 4.74*2 | 2.08*2 |
| | 36.2 μg Metho. | 1 | 6.98 | 2.75 | 2.54 | 3.28 | ** | ** | ** | ** |
| XIV | Control | 6 | 14.30 ± 0.75 | 2.32 ± 0.06 | 6.16 ± 0.31 | 2.34 ± 0.34 | 15.95 ± 1.15 | 2.13 ± 0.11 | 7.49 ± 0.37 | 2.20 ± 0.23 |
| | 56.5 μg Metho. | 3 | 4.90*1 | ** | ** | ** | ** | ** | ** | ** |
| | 48.5 μg JH III | 3 | ** | ** | ** | ** | ** | ** | ** | ** |
| XV | Control | 3 | 8.12 ± 0.92 | 1.71 ± 0.16 | 4.74 ± 0.19 | 2.86 ± 0.25 | 10.97 ± 0.86 | 2.05 ± 0.07 | 5.36 ± 0.38 | 2.22 ± 0.08 |
| | 23.2 μg Metho. | 2 | 9.10 | 3.03 | 3.18 | 2.67 | 6.56 | 2.84 | 2.26 | 1.94 |
| | 11.9 μg JH III | 2 | 5.51 | 1.73 | 3.18 | 2.26 | 6.09 | 1.84 | 3.31 | 1.85 |
| | 15.9 μg JH III | 2 | 4.04 | 1.73 | 2.34 | 2.04 | 4.47 | 1.77 | 2.53 | 1.72 |
| | 19.9 μg JH III | 2 | 3.06 | 1.66 | 1.85 | 1.85 | 3.36 | ** | ** | ** |
| XVI | Control | 2 | 8.55 | 1.19 | 7.50 | 3.58 | 10.29 | 1.27 | 8.29 | 2.99 |
| | 6.1 μg Metho. | 3 | 8.52 ± 0.48 | 1.15 ± 0.11 | 7.50 ± 0.58 | 3.69 ± 0.28 | 8.47 ± 0.06 | 1.18 ± 0.04 | 7.22 ± 0.28 | 3.11 ± 0.28 |

**Table 7.9 — Mitochondrial Respiration; *In Vitro* Effects of Methoprene and JH III, Normalised Results**

Legend: n – number of determinations
(the results shown are the mean values),
$*n$ – only $n$ number of determinations were measureable,
** – none of the determinations gave a measureable result,

**Normalised Effects of Methoprene and JH III, *in vitro* Additions**

| Treatment | n | First ADP addition | | | | Second ADP addition | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | state 3 rate | state 4 rate | RCR | ADP/O | state 3 rate | state 4 rate | RCR | ADP/O |
| Control | – | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 6.1 $\mu$g Metho. = 19.6 nmoles | 3 | 100 ± 5 | 97 ± 9 | 100 ± 8 | 103 ± 8 | 82 ± 0.5 | 93 ± 3 | 87 ± 3 | 104 ± 10 |
| 14.5 $\mu$g Metho. = 46.7 nmoles | 1 | 98 | 83 | 118 | 97 | 77 | 85 | 90 | 95 |
| 18.7 $\mu$g Metho. = 60.2 nmoles | 2 | 96 | 94 | 103 | 102 | 68 | 105 | 64 | 95 |
| 23.2 $\mu$g Metho. = 74.7 nmoles | 2 | 112 | 177 | 67 | 93 | 60 | 139 | 42 | 88 |
| 24.2 $\mu$g Metho. = 77.9 nmoles | 4 | 86 ± 2 | 160 ± 11 | 54 ± 4 | 101 ± 3 | 45 ± 2 | 136 ± 7 | 33 ± 3 | 90 ± 0.5 |
| 30.2 $\mu$g Metho. = 97.3 nmoles | 4 | 89 ± 5 | 185 ± 20 | 50 ± 7 | 99 ± 4 | 42 ± 10 | 135*2 | 41*2 | 88*2 |
| 33.6 $\mu$g Metho. = 108.2 nmoles | 4 | 69 ± 13 | 186*2 | 48*2 | 76*2 | 41*2 | ** | ** | ** |
| 36.2 $\mu$g Metho. = 116.6 nmoles | 1 | 73 | 249 | 29 | 121 | ** | ** | ** | ** |
| 56.5 $\mu$g Metho. = 182.0 nmoles | 3 | 34*1 | ** | ** | ** | ** | ** | ** | ** |
| 11.9 $\mu$g JH III = 44.6 nmoles | 2 | 68 | 102 | 67 | 79 | 56 | 90 | 62 | 83 |
| 15.9 $\mu$g JH III = 59.7 nmoles | 2 | 50 | 102 | 49 | 71 | 41 | 86 | 47 | 78 |
| 19.9 $\mu$g JH III = 74.7 nmoles | 2 | 38 | 97 | 39 | 65 | 31 | ** | ** | ** |
| 48.5 $\mu$g JH III = 182.1 nmoles | 3 | ** | ** | ** | ** | ** | ** | ** | ** |

## Bibliography


ABU-HAKIMA, R. and DAVEY, K. G. (1975). Two actions of juvenile hormone on the follicle cells of *Rhodnius prolixus*. *Can. J. Zool.* **53**, 1187–1188.

AIDLEY, D. J. (1985). Muscular contraction. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 5, pp. 407–437. Pergamon Press, Oxford.

AKAMATSU, Y., DUNN, P. E., KEZDY, F. G., KRAMER, F. J., LAW, J. H., REIBSTEIN, D. and SANBURG, L. L. (1975). Biochemical aspects of juvenile hormone action in insects. In *Control Mechanisms in Development* (R. H. Meintz and E. Davis, eds.), pp. 123–149. Plenum Press, New York.

AL-ROBAI, A. A. S. (1981). Studies on developmental changes in fine structure and metabolism in flight muscle of *Locusta migratoria* L. Ph.D. Thesis, University of Durham.

ANDERSSON-CEDERGREN, E. (1959). Ultrastructure of motor end plate and sarcoplasmic components of mouse skeletal muscle fiber as revealed by threee-dimensional reconstructions from serial sections. *J. ultrastruc. Res. Suppl.* **1**, 5–191.

ANSTEE, J. H. (1971). An ultrastructural study of tettigoniid muscle in relation to function. *J. Insect Physiol.* **17**, 1983–1994.

AUBER, M. J. (1967a). Distribution of the two kinds of myofilaments in insect muscles. *Am. Zool.* **7**, 451–456.

AUBER, M. J. (1967b). Paticularités ultrastructurales des myofibrilles des muscles du vol chez les Lépidoptères. *C. R. Acad. Sci. Paris, Ser. D* **264**, 621–624.

AUBER, M. J. (1969). La myofibrillogenese du muscle strié: I. Insectes. *J. Microsc., Paris* **8**, 197–232.

AUBER-THOMAY, M. (1979). Changes in the fine structure of the ventral intersegmental abdominal muscles in the adult of *Rhodnius prolixus*. *Tissue Cell* **11**, 655–662.

BAEHR, J.-C., PORCHERON, P., PAPILLON, M. and DRAY, F. (1979). Haemolymph levels of juvenile hormone, ecdysteroids and protein during the last two larval instars of *Locusta migratoria*. *J. Insect Physiol.* **25**, 415–421.

BAILEY, E., HORNE, J. A., IZATT, M. E. G. and HILL, L. (1975). The effects of allatectomy on the lipid composition of the fat body and haemolymph of adult *Locusta*. *Comp. Biochem. Physiol.* **52B**, 525–528.

BASSI, S. D. and FEIR, D. (1971). Effects of juvenile hormone on the rates of protein synthesis in *Oncopeltus fasciatus*. *Insect Biochem.* **1**, 428–432.

BAUMANN, G. (1968). Zur wirking des juvenilhormons: elektrophysiologische messungen an der zellmembran der speicheldrüse von *Galleria mellonella*. *J. Insect Physiol.* **14**, 1459–1476.

BAUMANN, G. (1969). Juvenile hormone: effect on bimolecular lipid membranes. *Nature, Lond.* **223**, 316–317.

BEAULATON, J. (1968). Modifications ultrastructurales de trachéoles et genèse de petites trachées et trachéoles chez les vers à soie en période de mue. *J. Microsc., Paris* **7** 621–646.

BEENAKKERS, A. M. TH. (1969). The influence of corpus allatum and corpus cardiacum on lipid metabolism in *Locusta migratoria*. *Gen. comp. Endocrinol.* **13**, 492.

BEENAKKERS, A. M. TH. and VAN DEN BROEK, A. TH. M. (1974). Influence of juvenile hormone on growth and digestion in fifth instar larvae and adults of *Locusta migratoria*. *J. Insect Physiol.* **20**, 1131–1142.

BEENAKKERS, A. M. TH. and VAN DEN BROEK, A. TH. M. (1976). Juvenile hormone and the development of locust muscles. *Gen. comp. Endocrinol.* **29**, 295.

BEENAKKERS, A. M. TH., VAN DEN BROEK, A. TH. M. and DE RONDE, TH. J. A. (1975). Development of catabolic pathways in insect flight muscles, a comparative study. *J. Insect Physiol.* **21**, 849–859.

BEENAKKERS, A. M. TH., VAN DER HORST, D. J. and VAN MARREWIJK, W. J. (1981). Role of lipids in energy metabolism. In *Energy Metabolism in Insects* (R. G. H. Downer, ed.), pp. 53–100. Plenum Press, New York.

BEENAKKERS, A. M. TH., VAN DER HORST, D. J. and VAN MARREWIJK, W. J. (1985). Biochemical processes directed to flight muscle metabolism. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 10, pp. 451–486. Pergamon Press, Oxford.

BEINBRECH, G. (1969). Zur Flugmuskelentwicklung von *Phormia regina*: Beziehungen zwischen dem sarcotubulären und dem Trachealsystem. *Zool. Anz. Suppl.* **33**, 401–407.

BENTZ, F. (1969). Variations protéiniques de l'ovaire de *Locusta migratoria* (L.) (Orthoptère) en première phase de maturation. *C. R. Acad. Sci. Paris, Ser. D* **269**, 494–496.

BENTZ, F., GIRARDIE, A. and CAZAL, M. (1970). Étude électrophorétique des variations de la protéinémie chez *Locusta migratoria* pendant la maturation sexuelle. *J. Insect Physiol.* **16**, 2257–2270.

BIENZ-ISLER, G. (1968a). Elektronenmikroskopische Untersuchungen über die imaginale Struktur der dorsolongitudinalen Flugmuskeln von *Antheraea pernyi* Guer. (Lepidoptera). *Acta Anat.* **70**, 416–433.

BIENZ-ISLER, G. (1968b). Elektronenmikroskopische Untersuchungen über die Entwicklung der dorsolongitudinalen Flugmuskeln von *Antheraea pernyi* Guer. (Lepidoptera). *Acta Anat.* **70**, 524–553.

BLACKITH, R. E. (1972). Morphometrics in acridolgy: A brief survey. *Acrida* **1**, 7–15.

BOLLENBACHER, W. E., SMITH, S. L., GOODMAN, W. and GILBERT, L. I. (1981). Ecdysteroid titer during the larval to pupal to adult development of *Manduca sexta*. *Gen. comp. Endocrinol.* **44**, 302–306.

BOUNHIOL, J. J. (1938). Recherches expérimentales sur le determinisme de la métamorphose chez les Lépidoptères. *Bull. biol. Suppl.* **24**, 1–199.

BOWERS, W. S. (1985). Antihormones. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 8, pp. 551–564. Pergamon Press, Oxford.

BRACHET, J. (1953). The use of basic dyes and ribonuclease for the cytochemical detection of ribonucleic acid. *Quart. J. micr. Sci.* **94**, 1–10.

BRODSKY, W. Y. and URYVAEVA, I. V. (1977). Cell polyploidy: its relation to tissue growth and function. *Int. Rev. Cytol.* **50**, 275–328.

BROSEMER, R. W., VOGELL, W. and BÜCHER, TH. (1963). Morphologische und enzymatische Muster bei de Entwicklung indirekter Flugmuskeln von *Locusta migratoria*. *Biochem. Z.* **338**, 854–910.

BÜCHER, TH. (1965). Formation of the specific structural and enzymic pattern of the insect flight muscle. *Biochem. Soc. Symp.* **25**, 15–28.

BUSCH, H. and SMETANA, K. (1970). *The Nucleolus.* Academic Press, London.

BUTTERWORTH, F. M. and BODENSTEIN, D. (1969). Adipose tissue of *Drosophila melanogaster* IV. The effect of the corpus allatum and synthetic juvenile hormone on the tissue of the adult male. *Gen. comp. Endocrinol.* **13**, 68–74.

BUYS, K. S. (1924). Adipose tissue in insects. *J. Morph.* **38**, 485–527.

CARNOY, J. B. (1887). *Cellule* **3**, 276.

CASSIER, P. and FAIN-MAUREL, M.-A. (1972). Caractères infrastructureaux et cytochimiques des oenocytes de *Locusta migratoria migratorioides* en rapport avec les mues et les cycles ovariens. *Arch. Anat. microsc. Morphol. exp.* **61**, 357–380.

CHANCE, B. and HAGIHARA, B. (1961). Symposium of intracellular respiration: phosphorylating and non-phosphorylating oxidation reactions. In *Proceedings of the Fifth International Congress of Biochemistry, Moscow, 1961* (E. C. Slater, ed.), vol. 5, p. 3. Pergamon Press, Oxford, 1963.

CHANCE, B. and SACKTOR, B. (1958). Respiratory metabolism of insect flight muscle II. Kinetics of respiratory enzymes in flight muscle sarcosomes. *Arch. Biochem. Biophys.* **76**, 509–531.

CHANCE, B. and WILLIAMS, G. R. (1955a). A simple and rapid assay of oxidative phosphorylation. *Nature, Lond.* **175**, 1120–1124.

CHANCE, B. and WILLIAMS, G. R. (1955b). Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J. biol. Chem.* **217**, 383–393.

CHANCE, B. and WILLIAMS, G. R. (1956). The respiratory chain and oxidative phosphorylation. *Adv. Enzymol.* **17**, 65–134.

CHAPPELL, J. B. and HANSFORD, R. G. (1972). Preparation of mitochondria from animal tissues and yeasts. In *Subcellular Components, Preparation and Fractionation* (G. D. Birnie, ed.), pp. 77–91. Butterworth & Co, London.

CHEFURKA, W. (1978). Sesquiterpene juvenile hormones: novel uncouplers of oxidative phosphorylation. *Biochem. biophys. Res. Commun.* **83**, 571–578.

CHEN, T. T., COUBLE, P., ABU-HAKIMA, R. and WYATT, G. R. (1979). Juvenile hormone-controlled vitelogenin synthesis in *Locusta migratoria* fat body. Hormonal induction *in vivo*. *Dev. Biol.* **69**, 59–72.

CHEN, T. T., COUBLE, P., DE LUCCA, F. L. and WYATT, G. R. (1976). Juvenile hormone control of vitellogenin synthesis in *Locusta migratoria*. In *The Juvenile Hormones* (L. I. Gilbert, ed.), pp. 505–529. Plenum Press, New York.

CHEN, T. T., STRAHLENDORF, P. and WYATT, G. R. (1978). Vitellin and vitellogenin from locusts (*Locusta migratoria*): properties and post-translational modifications in the fat body. *J. biol. Chem.* **253**, 5324–5331.

CHILDRESS, C. C. and SACKTOR, B. (1966). Pyruvate oxidation and the permeability of mitochondria from blowfly flight muscle. *Science, N.Y.* **154**, 268–270.

CHILDRESS, C. C. and SACKTOR, B. (1970). Regulation of glycogen metabolism in insect flight muscle. Purification and properties of phosphorylase *in vitro* and *in vivo*. *J. biol. Chem.* **245**, 2927–2936.

CHILDRESS, C. C., SACKTOR, B. and TRAYNOR, D. R. (1967). Function of carnitine in the fatty acid oxidase-deficient insect flight muscle. *J. biol. Chem.* **242**, 754–760.

CHINZEI, Y., CHINO, H. and WYATT, G. R. (1980). Purification and properties of of vitellogenin and vitellin from *Locusta migratoria*. *Insect Biochem* **11**, 1–8.

CHINZEI, Y., WHITE, B. N. and WYATT, G. R. (1982). Vitellogenin mRNA in locust fat body: isolation and quantitative changes induced by juvenile hormone. *Can. J. Biochem.* **60**, 243–251.

CHINZEI, Y. and WYATT, G. R. (1985). Vitellogenin titre of haemolymph of *Locusta migratoria* in normal adults, after ovariectomy, and in response to methoprene. *J. Insect Physiol.* **31**, 441–445.

CHUDAKOVA, I. (1978). Analysis of varying effects of juvenile hormone on muscles of the house cricket (*Acheta domestica*). *Gen. comp. Endocrinol.* **34**, 114.

CHUDAKOVA, I. and BOCHAROVA-MESSNER, O. M. (1968). Endocrine regulation of the state of the wing musculature in the imago of the house cricket (*Acheta domestica* L.). *Dokl. Akad. Nauk. SSSR* **179**, 489–492.

CIEMIOR, K. E., SEHNAL, F. and SCHNEIDERMAN, H. A. (1979). Moulting, growth and survival of *Galleria mellonella* L. (Lep., Pyralidae) treated with juvenoids. *Z. Ang. Ent.* **88**, 414–425.

CLARK JR., L. C. (1956). Monitor and control of of blood and tissue oxygen tension. *Trans. Am. Soc. artif. intern. Organs* **2**, 41–65.

CLARK, M. C. and DAHM, P. A. (1973). Phenobarbital-induced, membrane-like scrolls in the oenocytes of *Musca domestica* Linnaeus. *J. Cell Biol.* **56**, 870–875.

CLARKE, K. U. and BALDWIN, R. W. (1960). The effect of insect hormones and 2,4-dinitrophenol on the mitochondrion of *Locusta migratoria* L. *J. Insect Physiol.* **5**, 37–46.

COCHRAN, D. G., MULLINS, D. E. and MULLINS, K. J. (1979). Cytological changes in the fat body of the American cockroach, *Periplaneta americana*, in relation to dietary nitrogen levels. *Ann. entomol. Soc. Am.* **72**, 197–205.

COHEN, E. and GILBERT, L. I. (1972). Metabolic and hormonal studies on two insect cell lines. *J. Insect Physiol.* **18**, 1061–1076.

COUBLE, P., CHEN, T. T. and WYATT, G. R. (1979). Juvenile hormone-controlled vitellogenin synthesis in *Locusta migratoria* fat body: cytological development. *J. Insect Physiol.* **25**, 327–337.

COUPLAND, R. E. (1957). Observations on the normal histology and histochemistry of the fat body of the locust (*Schistocerca gregaria*). *J. exp. Biol.* **34**, 290–296.

COURGEON, A.-M. (1966). Étude de l'intensité respiratoire de *Carausius morosus* Br., au cours des deux derniers stades larvaires, après ablation des *corpora allata*. *C. R. Acad. Sci. Paris, Ser. D* **262**, 1272–1275.

CROSSLEY, A. C. (1968). The fine-structure and mechanism of breakdown of the intersegmental muscles of the blowfly *Calliphora erythrocephala*. *J. Insect Physiol.* **14**, 1389–1407.

DAVEY, K. G. (1981). Hormonal control of vitellogenin uptake in *Rhodnius prolixus* Stal. *Am. Zool.* **21**, 701–705.

DAVIES, P. W. and BRINK JR., F. (1942). Microelectrodes for measuring local oxygen tension in animal tissues. *Rev. sci. Instrum.* **13**, 524–533.

DAVIS, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 404.

DAVISON, T. F. (1970). An investigation into the factors involved in acclimatization to temperature and death at high temperature in *Calliphora erythrocephala* (Meig.). Ph.D. Thesis, University of Durham.

DEAN, R. L., LOCKE, M. and COLLINS, J. V. (1985). Structure of the fat body. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 3, pp. 155–210. Pergamon Press, Oxford.

DE KORT, C. A. D. (1969). Hormones and the structural and biochemical properties of the flight muscles in the Colorado beetle. *Meded. Landbhogesch. Wageningen* **69**, 1–63.

DE KORT, C. A. D. and GRANGER, N. A. (1981). Regulation of the juvenile hormone titer. *Ann. Rev. Entomol.* **26**, 1–28.

DE WILDE, J. and STEGWEE, D. (1958). Two major effects of the corpus allatum in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). *Arch. Néerl. Zool.* **13**, 277–289.

DHADIALLA, T. S. and WYATT, G. R. (1981). Competence for juvenile hormone-stimulated vitellogenin synthesis in the fat body of female and male *Locusta migratoria*. In *Juvenile Hormone Biochemistry* (G. E. Pratt and G. T. Brooks, eds.), pp. 257–262. Elsevier/North-Holland Biomedical Press, Amsterdam.

DHADIALLA, T. S. and WYATT, G. R. (1983). Juvenile hormone-dependent vitellogenin synthesis in *Locusta migratoria* fat body: inducibility related to sex and stage. *Dev. Biol.* **96**, 436–444.

DINGLE, J. T. (1977). *Lysosomes. A Laboratory Handbook*, second edition. Elsevier/North-Holland Biomedical Press, Amsterdam.

DIRSH, V. M. (1953). Morphometrical studies on phases of the desert locust (*Schistocerca gregaria* Forskål). *Anti-Locust Bull.* **16**, 1–34.

DOANE, W. W. (1961). Developmental physiology of the mutant female sterile (2) adipose of *Drosophila melanogaster*. III. Corpus allatum complex and ovarian transplantations. *J. exp. Zool.* **146**, 275–298.

DOWNER, R. G. H. (1985). Lipid metabolism. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 10, pp. 77–113. Pergamon Press, Oxford.

DOWNER, R. G. H., WIEGAND, M. and SMITH, S. M. (1975). Suppression of pupal esterase activity in *Aedes aegypti* (Diptera: Culicidae) by an insect growth regulator. *Experientia* **31**, 1239–1240.

DUKE, E. J. (1966). Lymph protein patterns in the development of *Locusta migratoria migratorioides*. *Comp. Biochem. Physiol.* **17**, 661–664.

DE DUVE, C. and WATTIAUX, R. (1966). Functions of lysosomes. *Ann. Rev. Physiol.* **28**, 435–492.

EBASHI, S. (1980). The regulation of muscle contraction. *Proc. R. Soc.* **207B**, 259-286.

ECHALIER, G (1976). *In vitro* established line of *Drosophila* cells and application in physiological genetics. In *Invertebrate Tissue Culture* (E. Kurstak and and K. Maramorosch, eds.), pp. 131–150. Academic Press, London.

ECHALIER, G. and OHANESSIAN, A. (1969). Isolement, en cultures *in vitro*, de lignées cellulaires diploïdes de *Drosophila melanogaster*. *C. R. Acad. Sci. Paris, Ser D* **268**, 1771–1774.

EDWARDS, F. J. (1970). Endocrine control of flight muscle histolysis in *Dysdercus intermedius*. *J. Insect Physiol.* **16**, 2027–2031.

ELDER, H. Y. (1971). High frequency muscles used in sound production by a katydid. II. Ultrastructure of the singing muscles. *Biol. Bull.* **141**, 434–448.

ELDER, H. Y. (1975). Muscle structure. In *Insect Muscle* (P. N. R. Usherwood, ed.), pp. 1–74. Academic Press, London.

ELLIOT, R. H. and GILLOTT, C. (1977). Changes in the protein concentration and volume of the haemolymph in relation to yolk deposition, ovariectomy, allatectomy and cautery of the median neurosecretory cells in *Melanoplus sanguinipes*. *Can. J. Zool.* **55**, 97–103.

EMMERICH, H. and HARTMANN, R. (1973). A carrier lipoprotein for juvenile hormone in the haemolymph of *Locusta migratoria*. *J. Insect Physiol.* **19**, 1663–1675.

ENGELMANN, F. (1969). Endocrine-stimulated synthesis of sex-specific and non-specific proteins in *Leucophaea maderae*. *Gen. comp. Endocrinol.* **13**, 503–504.

ENGELMANN, F. (1971). Juvenile hormone-controlled synthesis of female-specific protein in the cockroach *Leucophaea maderae*. *Arch. Biochem. Biophys.* **145**, 439–447.

ENGELMANN, F. (1979). Insect vitellogenin: identification, biosynthesis and role in vitellogenesis. *Adv. Insect Physiol.* **14**, 49–108.

ENGELMANN, F. (1980). Endocrine control of vitellogenin synthesis. In *Insect Biology in the Future* (M. Locke and D. S. Smith, eds.), pp. 311–324. Academic Press, London.

ENGELMANN, F. (1981a). The identification of juvenile hormone binding proteins in the fat bodies of *Leucophaea maderae*. In *Juvenile Hormone Biochemistry* (G. E. Pratt and G. T. Brooks, eds.), pp. 263–270. Elsevier/-North-Holland Biomedical Press, Amsterdam.

ENGELMANN, F. (1981b). Heterogeneity of juvenile hormone binding compounds in fat bodies of a cockroach. *Mol. cell. Endocrinol.* **24**, 103–112.

ERICKSON, R. O., SAX, K. O. and OGAR, M. (1949). Perchloric acid in the cytochemistry of pentose nucleic acid. *Science* **110**, 472–473.

ERLEY, D., SOUTHARD, S. and EMMERICH, H. (1975). Excretion of juvenile hormone and its metabolites in the locust, *Locusta migratoria*. *J. Insect Physiol.* **21**, 61–70.

EVANS, J. J. T. (1967). Development and ultrastructure of the fat body cells and oenocytes of the Queensland fruit fly, *Dacus tryoni* (Frogg.). *Z. Zellforsch Mikrosk. Anat.* **81**, 49–61.

FINLAYSON, L. H. (1975). Development and degeneration. In *Insect Muscle* (P. N. R. Usherwood, ed.), pp. 75–149. Academic Press, London.

FIRSTENBERG, D. E. and SILHACEK, D. C. (1973). Juvenile hormone regulation of oxidative metabolism in isolated insect mitochondria. *Experientia* **29**, 1420–1422.

FREIDEL, T. and GILLOT, C. (1976). Extraglandular synthesis of accessory reproductive gland components in male *Melanoplus sanguinipes*. *J. Insect Physiol.* **22**, 1309–1314.

FRIEDMAN, S. (1985). Carbohydrate metabolism. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 10, pp. 43–76. Pergamon Press, Oxford.

GELLISSEN, G. and EMMERICH, H. (1978). Changes in the titer of vitellogenin and of diglyceride carrier lipoprotein in the blood of adult *Locusta migratoria*. *Insect Biochem.* **8**, 403–412.

GELLISSEN, G. and WYATT, G. R. (1981). Production of lipophorin in the fat body of adult *Locusta migratoria*: comparison with vitellogenin. *Can. J. Biochem.* **59**, 648–654.

GILBERT, L. I. (1962). Maintenance of the prothoracic gland by the juvenile hormone in insects. *Nature, Lond.* **193**, 1205–1207.

GILBERT, L. I. (1967). Changes in lipid content during the reproductive cycle of *Leucophaea maderae* and effects of the juvenile hormone on lipid metabolism *in vitro*. *Comp. Biochem. Physiol.* **21B**, 237–257.

GILBERT, L. I. and SCHNEIDERMAN, H. A. (1960). The development of a bioassay for the juvenile hormone of insects. *Trans. Am. microsc. Soc.* **79**, 38–67.

GNATZY, W. (1970). Struktur und Entwicklung des Integuments und der Oenocyten von *Culex pipiens* L. (Dipt.). *Z. Zellforsch Mikrosk. Anat.* **110**, 401–433.

GOKULDAS, M., HUNT, P. A. and CANDY, D. J. (1988). The inhibition of lipid synthesis *in vitro* in the locust, *Schistocerca gregaria*, by factors from the corpora cardiaca. *Physiol. Entomol.* **13**, 43–48.

GOLDSPINK, D. F. (1980). Physiological factors influencing protein turnover and muscle growth in mammals. In *Development and Specialization of Skeletal Muscle* (D. F. Goldspink, ed.), pp. 65–89. Cambridge University Press, Cambridge.

GOLDSWORTHY, G. J., JOHNSON, R. A. and MORDUE, W. (1972). *In vivo* studies on the release of hormones from the corpora cardiaca of locusts. *J. comp. Physiol.* **79**, 85–96.

GOLTZENÉ-BENZ, F., JOLY, L., BREHÉLIN, M. and HOFFMAN, J. A. (1972). Influence des corpora allata sur la proteinemie de larves de stade V et d'imago femelles de *Locusta migratoria* L. (Insecte orthoptère). *C. R. Acad. Sci. Paris, Ser. D* **274**, 1059–1062.

GOODMAN, W. G. and CHANG, E. S. (1985). Juvenile hormone cellular and haemolymph binding proteins. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.) vol. 7, pp. 491–510. Pergamon Press, Oxford.

GORDON, R. and BURFORD, I. R. (1984). Effects of methoprene, a juvenile hormone analogue, on the larval and pupal stages of the yellow fever mosquito, *Aedes aegypti*. *J. Insect Physiol.* **30**, 279–286.

GRACE, T. D. C. (1962). Establishment of four strains of cells from insect tissues grown *in vitro*. *Nature, Lond.* **195**, 788–789.

HAGEDORN, H. H. and KUNKEL, J. G. (1979). Vitellogenin and vitellin in insects. *Ann. Rev. Entomol.* **24**, 475–505.

HAGOPIAN, M. and SPIRO, D. (1967). The sarcoplasmic reticulum and its association with the T system in an insect. *J. Cell Biol.* **32**, 535–545.

HAMILTON, J. G. and COMAI, K. (1984). Separation of neutral lipids and free fatty acids by high-performance liquid chromatography using low wavelength ultraviolet detection. *J. Lipid Res.* **25**, 1142–1148.

HAMMOCK, B. D. (1985). Regulation of juvenile hormone titer: degradation. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.) vol. 7, pp. 431–472. Pergamon Press, Oxford.

HAMMOCK, B. D. and QUISTAD, G. B. (1981). Metabolism and mode of action of juvenile hormone, juvenoids and other insect growth regulators. In *Progress in Pesticide Biochemistry* (D. H. Hutson and T. R. Roberts, eds.), vol. 1, pp. 1–83. John Wiley & Sons, New York.

HAMMOCK, B. D. and ROE, R. M. (1985). Analysis of juvenile esterase activity. In *Methods in Enzymology* (S. P. Colowick and N. O. Kaplan, eds.) vol. 111, pp. 487–494. Academic Press, London.

HANGARTNER, W. and MASNER, P. (1973). Juvenile hormone: inhibition of ecdysis in larvae of the German cockroach, *Blattella germanica*. *Experientia* **29**, 1358–1359.

HANSON, J. (1956). Studies on the cross-striation of the indirect flight myofibrils of the blowfly *Calliphora*. *J. biophys. biochem. Cytol.* **2**, 691–709.

HANSON, J. and HUXLEY, H. E. (1953). The structural basis of the cross-striations in muscle. *Nature, Lond.* **172**, 530–532.

HANSON, J. and HUXLEY, H. E. (1955). The structural basis of contraction in striated muscle. *Symp. Soc. exp. Biol.* **9**, 228–264.

HARDIE, J. and LEES, A. D. (1985). Endocrine control of polymorphism and polyphenism . In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.) vol. 8, pp. 441–490. Pergamon Press, Oxford.

HENRICK, C. A. (1982). Juvenile hormone analogs: structure-activity relationships. In *Insecticide Mode of Action* (J. R. Coats, ed.), pp. 315–402. Academic Press, London.

HENRICK, C. A., STAAL, G. B. and SIDDALL, J. B. (1973). Alkyl-3,7,1-trimethyl-2,4-dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. *Agr. Food Chem.* **21**, 354–359.

HEROLD, R. C. (1965). Development and ultrastructural changes of sarcosomes during honey bee flight muscle development. *Dev. Biol.* **12**, 269–286.

HILL, L. (1962). Neurosecretory control of haemolymph protein concentration during ovarian development in the desert locust. *J. Insect Physiol.* **8**, 609–619.

HILL, L. and IZATT, M. E. G. (1973). Hormonal control of lipid and protein metabolism in the adult desert locust. *J. Endocrinol.* **57**, l–li.

HILL, L. and IZATT, M. E. G. (1974). The relationships between corpora allata and fat body and haemolymph lipids in the adult female desert locust. *J. Insect Physiol.* **20**, 2143–2156.

HILL, L., LUNTZ, A. J. and STEELE, P. A. (1968). The relationships between somatic growth, ovarian growth and feeding activity in the adult desert locust. *J. Insect Physiol.* **14**, 1–20.

HINK, W. F. (1970). Established insect cell line from the cabbage looper *Trichoplusia ni. Nature, Lond.* **226**, 466–467.

HINTON, H. E. (1959). How the indirect flight muscles of insects grow. *Sci. Prog., Lond.* **47**, 321–333.

HOFFMANN, J. A. and HETRU, C. (1983). Ecdysone. In *Endocrinology of Insects* (R. G. H. Downer and H. Laufer, eds.), pp. 65–88. Alan R. Liss, New York.

HOULIHAN, D. F. and NEWTON, J. R. L. (1979). Sarcomere formation and longitudinal growth in the developing flight muscles of *Calliphora*. *J. Insect Physiol.* **25**, 879–893.

HUIBREGTSE-MINDERHOUD, L., VAN DEN HONDEL-FRANKEN, M. A. M., VAN DER KERK-VAN HOOF, A. C., BIESSELS, H. W. A., SALEMINK, C. A., VAN DER HORST, D. J. and BEENAKKERS, A. M. TH. (1980). Quantitative determination of the juvenile hormones in the haemolymph of *Locusta migratoria* during normal development and after implantation of corpora allata. *J. Insect Physiol.* **26** 627–631.

HUXLEY, H. E. (1964). Evidence for continuity between the central elements of the triads and extracellular space in frog sartorius muscle. *Nature, Lond.* **202**, 1067–1071.

HUXLEY, H. E. and HANSON, J. (1957). Preliminary observations on the structure of insect flight muscle. In *Electron Microscopy. Proceedings of the Stockholm Conference, 1956*, pp. 202–203. Almqvist & Wiksells, Stockholm.

IRVINE, D. J. and BRASCH, K. (1981). The influence of juvenile hormone on polyploidy and vitellogenesis in the fat body of *Locusta migratoria*. *Gen. comp. Endocrinol.* **45**, 91–99.

ISHIZAKI, H. (1965). Electron microscopic study of changes in the subcellular organization during metamorphosis of the fat body cell of *Philosamia cynthia ricini* (Lepidoptera). *J. Insect Physiol.* **11**, 845–855.

JANDA, V. and SLÁMA, K. (1965). Über den Einfluss von Hormonen auf den Glykogen-, Fett- und Stickstoffmetabolismus bei den Imagines von *Pyrrhocoris apterus* L. (Hemiptera). *Zool. Jb.* **71**, 345–358.

JENSEN, A. L. and BRASCH, K. (1985). Nuclear development in locust fat body: the influence of juvenile hormone on inclusion bodies and the nuclear matrix. *Tissue Cell* **17**, 117–130.

JOHNSON, R. A. and HILL, L. (1973). The activity of the corpora allata in the fourth and fifth instars of the migratory locust. *J. Insect Physiol.* **19**, 1921–1932.

JOLY, L. and JOLY, P. (1974). Comparaison de la phase grégaire et de la phase solitaire de *Locusta migratoria migratorioides* (Orthoptère) du point de vue de la Teneur de leur hémolymphe en hormone juvénile. *C. R. Acad. Sci. Paris, Ser. D* **279**, 1007–1009.

JOLY, L., HOFFMAN, J. and JOLY, P. (1977). Controle humoral de la différenciation phasaire chez *Locusta migratoria migratorioides* (R. & F.) (Orthoptères). *Acrida* **6**, 33–42.

JOLY, P. (1962). Rôle joué par les corpora allata dans la réalisation du polymorphisme de phase chez *Locusta migratoria* L. *Colloq. int. Cent. natl. Rech. sci.* **114**, 77–88.

JOLY, P. and JOLY, L. (1953). Résultats de greffes de corpora allata chez *Locusta migratoria migratorioides* L. *Ann. Sci. nat. Zool.* **15**, 331–345.

JONES, G. and HAMMOCK, B. D. (1983). Prepupal regulation of juvenile hormone esterase through direct induction by juvenile hormone. *J. Insect Physiol.* **29**, 471–475.

JORDAN, B. M. and BAKER, J. R. (1955). A simple pyronine/methyl green technique. *Quart. J. micr. Sci.* **96**, 177–179.

JORGENSEN, W. K. and RICE, M. J. (1983). Superextension and supercontraction in locust ovipositor muscles. *J. Insect Physiol.* **29**, 437–448.

JUDY, K. J., SCHOOLEY, D. A., DUNHAM, L. L., HALL, M. S., BERGOT, B. J. and SIDDALL, J. B. (1973). Isolation, structure and absolute configuration of a new natural insect juvenile hormone from *Manduca sexta*. *Proc. natl. Acad. Sci. U.S.A.* **70**, 1509–1513.

JUNGALWALA, F. B., EVANS, J. E. and MCCLUER, R. H. (1976). High-performance liquid chromatography of phosphatidylcholine and sphingomyelin with detection in the region of 200nm. *Biochem. J.* **155**, 55–60.

KAHN, M. A. and DE KORT, C. A. D. (1978). Further evidence for the significance of proline as a substrate for flight in the Colorado potato beetle, *Leptinotarsa decemlineata*. *Comp. Biochem. Physiol.* **60B**, 407–411.

KARNOVSKY, M. J. (1965). The localization of cholinesterase activity in rat cardiac muscle by electron microscopy. *J. Cell Biol.* **23**, 217–232.

KASHMEERY, A. M. S. (1977). An investigation into the factors involved in heat death and thermal sensitivity in *Calliphora vicina* R-D. (A biochemical study). Ph.D. thesis, University of Durham.

KEELEY, L. L. (1985). Physiology and biochemistry of the fat body. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.) vol. 3, pp. 211–248. Pergamon Press, Oxford.

KILBY, B. A. (1963). The biochemistry of the insect fat body. *Adv. Insect Physiol.* **1**, 112–174.

KING, D. S. (1983). Chemistry and metabolism of the juvenile hormones. In *Endocrinology of Insects* (R. G. H. Downer and H. Laufer, eds.), pp. 57–64. Alan R. Liss, New York.

KLAGES, G., EMMERICH, H. and PETER, M. G. (1980). High-affinity binding sites for juvenile hormone I in the larval integument of *Drosophila hydei*. *Nature, Lond.* **286**, 282–285.

KOEPPE, J. K., FUCHS, M., CHEN, T. T., HUNT, L.-M., KOVALICK, G. E. and BRIERS, T. (1985). The role of juvenile hormone in reproduction. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.) vol. 8, pp. 165–203. Pergamon Press, Oxford.

KOEPPE, J. K., KOVALICK, G. E. and LAPOINTE, M. C. (1981). Juvenile hormone interactions with ovarian tissue in *Leucophaea maderae*. In *Juvenile Hormone Biochemistry* (G. E. Pratt and G. T. Brooks, eds.), 215–231. Elsevier/North-Holland Biomedical Press, Amsterdam.

KOOMAN, C. and NAIR, K. K. (1982). Polyploidization in the fat body of the desert locust *Schistocerca gregaria* during growth and metamorphosis. *Cytobios* **34**, 25–34.

KRAMER, S. J. (1978). Regulation of the activity of JH-specific esterases in the Colorado potato beetle *Leptinotarsa decemlineata*. *J. Insect Physiol.* **24**, 743–747.

KRITCHEVSKY, D., DAVIDSON, L. M., KIM, H. K. and MALHOTRA, S. (1973). Quantification of serum lipids by a simple TLC-charring method. *Clinica chim. Acta* **46**, 63–68.

KRSTIĆ, R. V. (1979). *Ultrastructure of the Mammalian Cell. An Atlas.* Springer Verlag, Berlin.

KULKARNI, A.P. and MEHROTRA, K. N. (1970). Amino acid nitrogen and proteins in the haemolymph of adult desert locust, *Schistocerca gregaria*. *J. Insect Physiol.* **16**, 2181–2199.

KUNKEL, J. G. and NORDIN J. H. (1985). Yolk proteins. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.) vol. 1, pp. 83–111. Pergamon Press, Oxford.

LARSEN, W. J. (1976). Cell remodelling in the fat body of an insect. *Tissue Cell* **8**, 73–92.

LAUVERJAT, S. (1977). L'evolution post-imaginale du tissu adipeux femelle de *Locusta migratoria* et son controle endocrine. *Gen. comp. Endocrinol.* **33**, 13–34.

LAW, J. H. (1980). Lipid-protein interactions in insects. In *Insect Biology in the Future* (M. M. Locke and D. S. Smith, eds.), pp. 295–309. Academic Press, London.

LEE, S. S. and GOLDSWORTHY, G. J. (1975). Allatectomy and flight performance in male *Locusta migratoria. J. comp. Physiol.* **100**, 351–359.

LEE, S. S. and GOLDSWORTHY, G. J. (1976). The effect of allatectomy and ovariectomy on flight performance in female *Locusta migratoria migratorioides* (R. and F.). *Acrida* **5**, 169–180.

LEHNINGER, A. L. (1975). *Biochemistry*, second edition. Worth Publishers, New York.

LEHNINGER, A. L. (1982). *Principles of Biochemistry.* Worth Publishers, New York.

L'HELIAS, C. (1953). Role des corpora allata dans le metabolisme des flucides, de l'azote et des lipides chez le phasme *Dixippus morosus. C. R. Acad. Sci. Paris, Ser. D* **236**, 2164–2166.

LOCKE, M. (1969). The ultrastructure of the oenocytes in the molt/intermolt cycle of an insect. *Tissue Cell* **1**, 103–154.

LOCKE, M. (1970). The molt/intermolt cycle in the epidermis and other tissues of an insect, *Calpodes ethlius. Tissue Cell* **2**, 197–223.

LOCKE, M. (1980). The cell biology of fat body development. In *Insect Biology in the Future* (M. M. Locke and D. S. Smith, eds.), pp. 227–252. Academic Press, London.

LOCKE, M. (1984). The structure and development of the vacuolar system in the fat body of insects. In *Insect Ultrastructure* (R. C. King and H. Akai, eds.), vol. 2, pp. 151–197. Plenum Press, New York.

LOCKE, M. (1985). A structural analysis of post-embryonic development. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.) vol. 2, pp. 87–149. Pergamon Press, Oxford.

LOCKE, M. and COLLINS, J. V. (1965). Protein uptake into multivesicular bodies and storage granules in the fat body of an insect. *J. Cell Biol.* **36**, 453–483.

LOCKE, M. and MCMAHON, J. T. (1971). The origin and fate of microbodies in the fat body of an insect. *J. Cell Biol.* **48**, 61–78.

LOCKE, M. and SYKES, A. K. (1975). The role of the golgi complex in the isolation and digestion of organelles. *Tissue Cell* **7**, 143–158.

LOCKSHIN, R. A. (1985). Programmed cell death. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 2, pp. 301–317. Pergamon Press, Oxford.

LOCKSHIN, R. A., COLON, A. D. and DORSEY, A. M. (1980). Control of muscle proteolysis in insects. *Fed. Proc.* **39**, 48–52.

DE LOOF, A. and LAGASSE, A. (1970). Juvenile hormone and the ultrastructural properties of the fat body of the adult Colorado beetle, *Leptinotarsa decemlineata* Say. *Z. Zellforsch Mikrosk. Anat.* **106**, 439–450.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951). Protein measurement with the Folin-phenol reagent. *J. biol. Chem.* **193**, 265–275.

LÜSCHER, M. (1968). Hormonal control of respiration and protein synthesis in the fat body of the cockroach *Nauphoeta cinerea* during oöcyte growth. *J. Insect Physiol.* **14**, 499–511.

MANGOLD, H. K. and MALINS, D. C. (1960). Fractionation of fats, oils and waxes on thin layers of silicic acid. *J. Am. Oil Chem. Soc.* **37**, 383–385.

MANNIX, J. J. and KEELEY, L. L. (1980). Age and endocrine effects on fat body metabolite composition in adult male *Blaberus discoidalis* cockroaches. *J. exp. Zool.* **212**, 113–117.

MARKERT, C. L. and HUNTER, R. L. (1959). The distribution of esterases in mouse tissues. *J. Histochem. Cytochem.* **7**, 42–49.

MARSH, J. B. and WEINSTEIN, D. B. (1966). Simple charring method for the determination of lipids. *J. Lipid Res.* **7**, 574–576.

MARTOJA, R. and BALLAN-DUFRANÇAIS, C. (1984). The ultrastructure of the digestive and excretory organs. In *Insect Ultrastructure* (R. C. King and H. Akai, eds.), vol. 2, pp. 199–268. Plenum Press, New York.

MARTOJA, R. and LAUVERJAT, S. (1964). Charactéres histochimiques et dimorphisme sexuel du corps gras chez les insectes Orthoptères. *Bull. Soc. Zool. Fr.* **89**, 339–383.

MASNER, P. and HANGARTNER, W. (1973). Ecdysone: an antagonist of juvenile hormone in the control of cuticle synthesis in the German cockroach (*Blattella germanica*). *Experientia* **29**, 1550–1551.

MASNER, P., HANGARTNER, W. and SUCHY, M. (1975). Reduced titres of ecdysone following juvenile hormone treatment in the German cockroach *Blattella germanica*. *J. Insect Physiol.* **21**, 1755–1762.

MATHAI, S. and NAIR, S. K. (1984). Effects of a juvenile hormone analogue on acid and alkaline phosphatase activities in the pupae of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). *Comp. Physiol. Ecol.* **9**, 253–256.

MATOLIN, S. (1970). Effects of a juvenile hormone analogue on embryogenesis in *Pyrrhocoris apterus* L. *Acta Ent. Bohemoslov.* **67**, 9–12.

MCCALEB, D. C. and KUMARAN, A. K. (1980). Control of juvenile hormone esterase activity in *Galleria mellonella* larvae. *J. Insect Physiol.* **26**, 171–177.

MCCOMAS, A. J. (1977). *Neuromuscular Function and Disorders.* Butterworths, London.

MCCORMICK, F. W. and SCOTT, A. (1966). A protein fraction in locust haemolymph associated with the moulting cycle. *Experientia* **22**, 228–229.

MEYER, A. S., SCHNEIDERMAN, H. A., HANZMAN, E. and KO, J. H. (1968). The two juvenile hormones from the cecropia silk moth. *Proc. natl. Acad. Sci. U.S.A.* **60**, 853–860.

MEYER, T. S. and LAMBERT, B. L. (1965). Use of coomassie brilliant blue R250 for the electrophoresis of microgram quantities of partid saliva proteins on acrylamide-gel strips. *Biochim. Biophys. Acta* **107**, 144–145.

MICHEJDA, J. (1964). Physiology and structure of flight-muscle sarcosomes in silkworm *Hyalophora cecropia*. *Bull. Soc. Amis Sci. Lett. Poznan, Ser. D* 4, 61–102.

MINKS, A. K. (1967). Biochemical aspects of juvenile hormone action in the adult *Locusta migratoria*. *Archs. Néerl. Zool.* **17**, 175–258.

MULLINS, D. E. (1979). Isolation and partial characterization of uric acid spherules obtained from cockroach tissues (Dictyoptera). *Comp. Biochem. Physiol.* **62A**, 699–705.

MWANGI, R. W. and GOLDSWORTHY, G. J. (1980). The effects of allatectomy and ovariectomy on haemolymph proteins and lipid mobilization in *Locusta*. *J. Insect Physiol.* **26**, 741–747.

NAGL, W. (1978). *Endopolyploidy and Polyteny in Differentiation and Evolution.* North-Holland, Amsterdam.

NAIR, K. K., CHEN, T. T. and WYATT, G. R. (1981). Juvenile hormone stimulated polyploidy in adult locust fat body. *Dev. Biol.* **81**, 356–360.

NASSAR, S. G., STAAL, G. B. and ARMANIOUS, N. I. (1973). Effects and control potential of insect growth regulators with juvenile hormone activity on the greenbug. *J. econ. Entomol.* **66**, 847–850.

NEUGEBAUER, W. (1961). Wirkung der Extirpation und Transplantation der Corpora allata auf den Sauerstoffverbrauch, die Eibildung und den Fettkörper von *Carausius* (*Dixippus*) *morosus*. *Arch. Entwicklungsmech. Org.* **153**, 314–352.

NÜESCH, H. (1985). Control of muscle development. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.) vol. 2, pp. 425–452. Pergamon Press, Oxford.

ODHIAMBO, T. R. (1966). The metabolic effects of the corpus allatum hormone in the male desert locust. I. Lipid metabolism. *J. exp. Biol.* **45**, 45–50.

ODHIAMBO, T. R. (1967). The fine structure and histochemistry of the fat body in the locust, *Schistocerca gregaria*. *J. Cell Sci.* **2**, 235–242.

PAN, M. L., BELL, W. J. and TELFER, W. H. (1969). Vitellogenic blood protein synthesis by insect fat body. *Science* **165**, 393–394.

PELED, Y. and TIETZ, A. (1973). Fat transport in the locust, *Locusta migratoria*: the role of protein synthesis. *Biochim. Biophys. Acta* **296** 499–509.

PENER, M. P. (1972). The corpus allatum in adult acridids: the interrelation of its functions and possible correlations with the life-cycle. In *Proceedings of the International Study Conference on the Current and Future Problems of Acridology, London 1970* (C. F. Hemming and T. H. C. Taylor, eds.), pp. 135–147. Centre for Overseas Pest Research, London.

PENER, M. P. (1985). Hormonal effects on flight and migration. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 8, pp. 491–550. Pergamon Press, Oxford.

PETER, M. G., GUNAWAN, S., GELLISSEN, G. and EMMERICH, H. (1979). Differences in hydrolysis and binding of homologous juvenile hormones in *Locusta migratoria* haemolymph. *Z. Naturforsch., Ser. C* **34**, 588–598.

PFEIFFER, I. W. (1945). Effect of the corpora allata on the metabolism of the adult female grasshopper. *J. exp. Zool.* **99** 183–233.

PIHAN, J. C. (1972). Facteurs intervenant au cours de la morphogenèse du système tracheen chez les insectes Diptères. *Bull. Soc. zool. France* **97**, 351–361.

PITT, D. (1975). *Lysosomes and Cell Function.* Longman Group, Harlow.

POELS, C. L. M. and BEENAKKERS, A. M. TH. (1969). The effects of corpus allatum implantation on the development of flight muscles and fat body in *Locusta migratoria*. *Entomol. exp. Appl.* **12**, 312–324.

PRICE, G. M. (1969). Factors affecting protein synthesis by the fat body of the larva of the blowfly *Calliphora erythrocephala*. *Biochem. J.* **108**, 19p–20p.

PRICE, G. M. (1973). Protein and nucleic acid metabolism in insect fat body. *Biol. Rev.* **48**, 333–375.

DE PRIESTER, W. and VAN DER MOLEN, L. G. (1979). Premetamorphic changes in the ultrastructure of *Calliphora* fat cells. *Cell Tis. Res.* **198**, 79–93.

PRINGLE, J. W. S. (1957). *Insect Flight.* Cambridge University Press, Cambridge.

PRINGLE, J. W. S. (1974). Locomotion: flight. In *The Physiology of Insecta* (M. Rockstein, ed.), second edition, vol. 3 pp. 453–476. Academic Press, London.

RALPH, C. L. and MATTA, R. J. (1965). Evidence for hormonal effects on metabolism of cockroaches from studies of tissue homogenates. *J. Insect Physiol.* **11**, 983–991.

RAYMOND, S. and WEINTRAUB, L. (1959). Acrylamide as a supporting medium for zone electrophoresis. *Science* **130**, 711.

REDDY, G., HWANG-HSU, K. and KUMARAN, A. K. (1979). Factors influencing juvenile hormone esterase activity in the wax moth, *Galleria mellonella*. *J. Insect Physiol.* **25**, 65–71.

REGER, J. F. (1967). The organization of sarcoplasmic reticulum in direct flight muscle of the Lepidopteran *Achalarus lyciades*. *J. ultrastruct. Res.* **18**, 595–599.

REGER, J. F. and COOPER, D. P. (1967). A comparative study on the fine structure of the basalar muscle of the wing and the tibial extensor muscle of the leg of the Lepidopteran *Achalarus lyciades*. *J. Cell Biol.* **33**, 531–542.

RETNAKARAN, A. (1973). Hormonal induction of supernumary instars in the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *Can. Entomol.* **105**, 459–461.

REYNOLDS, E. S. (1963). The use of lead citrate at a high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208–212.

REYNOLDS, S. E. (1980). Integration of behaviour and physiology in ecdysis. *Adv. Insect Physiol.* **15**, 475–595.

RHEUBEN, M. B. and KAMMER, A. E. (1980). Comparison of slow larval and fast adult muscle inervated by the same motor neurone. *J. exp. Biol.* **84**, 103–118.

RICHARD, C., TANGUAY, R. and CHAUDHARY, K. D. (1971). Nucleic acid and protein variations in relation to ultrastructural changes in the developing thoracic muscles of the African desert locust *Schistocerca gregaria* Forsk. *Cytobios* **3**, 145–160.

RIDDIFORD, L. M. (1980). Insect endocrinology: action of hormones at the cellular level. *Ann. Rev. Physiol.* **42**, 511–528.

RIDDIFORD, L. M. (1985). Hormone action at the cellular level. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 8, pp. 37–84. Pergamon Press, Oxford.

RINTERKNECHT, E. and ROUSSEL, J.-P. (1978). Modifications ultrastructurales induites par les hormones juveniles dans les cellules du corp gras de *Locusta migratoria* L. *Bull. Soc. Zool. Fr.* **103**, 359–366.

ROBERTS, P. E. and WYATT, G. R. (1983). Juvenile hormone binding by components of fat body cytosol from vitellogenic locusts. *Mol. cell. Endocrinol.* **31**, 53–69.

ROE, J. H. (1955). Determination of sugar in blood and spinal fluid with anthrone reagent. *J. biol. Chem.* **212**, 335–338.

ROHDENDORF, E. B. and SEHNAL, F. (1973). Inhibition of reproduction and embryogenesis in the firebrat, *Thermobia domestica*, by juvenile hormone analogues. *J. Insect Physiol.* **19**, 37–56.

RÖLLER, H., DAHM, K. H., SWEENEY, C. C. and TROST, B. M. (1967). Die struktur des juvenilhormon. *Angew. Chem.* **79**, 190–191.

RÖSELER, P.-F. and RÖSELER, I. (1988). Influence of juvenile hormone on fat body metabolism in ovariolectomized queens of the bumblebee, *Bombus terrestris*. *Insect Biochem.* **18**, 557–563.

ROTIN, D., FEYEREISEN, R., KOENER, J. and TOBE, S. S. (1982). Haemolymph juvenile hormone esterase activity during the reproductive cycle of the viviparous cockroach *Diploptera punctata*. *Insect Biochem.* **12**, 263–268.

ROUSSEL, J.-P. (1963). Consommation d'oxygene après ablation des corpora allata chez des femelles adultes de *Locusta migratoria* L. *J. Insect Physiol.* **9**, 721–725.

ROUSSEL, J.-P. (1973). Action de substances mimétiques de l'hormone juvénile sur le rhythme cardiaque de *Locusta migratoria* L. et de *Schistocerca gregaria* Forsk. *C. R. Acad. Sci. Paris, Ser. D* **277**, 1053–1056.

ROUSSEL, J.-P. (1976). Activité comparée des hormones juvéniles en C-18 (JH-I) et en C-16 (JH-III) chez *Locusta migratoria*. *J. Insect Physiol.* **22**, 83–88.

ROUSSEL, J.-P. and PERRON, J. M. (1974). Action de substances mimétiques de l'hormone juvénile sur *Schistocerca gregaria* Forsk. *Arch. Zool. exp. gén.* **115**, 251–262.

ROWELL, C. H. F. (1967). Corpus allatum implantation and green/brown polymorphism in three African grasshoppers. *J. Insect Physiol.* **13**, 1401–1412.

ROWELL, C. H. F. (1971). The variable coloration of the acridoid grasshoppers. *Adv. Insect Physiol.* **8**, 145–198.

SACKTOR, B. (1970). Regulation of intermediary metabolism, with special reference to the control mechanisms in insect flight muscle. *Adv. Insect Physiol.* **7**, 267–347.

SACKTOR, B. and CHILDRESS, C. C. (1967). Metabolism of proline in insect flight muscle and its significance in stimulating the oxidation of pyruvate. *Arch. Biochem. Biophys.* **120**, 583–588.

SAHOTA, T. S. (1975). Effect of juvenile hormone on acid phosphatases in the degenerating flight muscles of the douglas-fir beetle, *Dendroctonus pseudotsugae*. *J. Insect Physiol.* **21**, 471–478.

SASS, M. and KOVÁCS, J. (1977). The effect of ecdysterone on the fat body cells of the penultimate larvae of *Mamestra brassicae*. *Cell Tiss. Res.* **180**, 403–409.

SASS, M. and KOVÁCS, J. (1980). The effects of actinomycin D, cycloheximide and puromycin on the 20-hydroxyecdysone induced autophagocytosis in larval fat body cells of *Pieris brassicae*. *J. Insect Physiol.* **26**, 569–577.

SCHÄFER, E. A. (1891). On the minute structure of the muscle-columns or sarcostyles which form the wing-muscles of insects. *Proc. R. Soc.* **49**, 280–286.

SCHMIALEK, P. (1963). Metamorphoschemmung von *Tenebrio molitor* durch Farnesylmethyläther. *Z. Naturforsch.* **186**, 513–515.

SCHMIALEK, P. and DREWS, G. (1965). Wirkung von Farnesylmethyläther auf die Atmung von *Tenebrio molitor* puppen. *Z. Naturforsch.* **206**, 214–215.

SEDLAK, B. J. (1985). Structure of endocrine glands. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 7, pp. 25–60. Pergamon Press, Oxford.

SEHNAL, F. (1983). Juvenile hormone analogues. In *Endocrinology of Insects* (R. G. H. Downer and H. Laufer, eds.), pp. 657–672. Alan R. Liss, New York.

SEHNAL, F., MARÓY, P. and MALÁ, J. (1981). Regulation and significance of ecdysteroid titre fluctuation in lepidopterous larvae and pupae. *J. Insect Physiol.* **27**, 535–544.

SHAAYA, E. (1978). Ecdysone and juvenile hormone activity in the larvae of the cockroach *Periplaneta americana*. *Insect Biochem.* **8**, 193–195.

SIMS, R. A. and LAROSE, J. A. G. (1962). The use of iodine vapour as a general detecting agent in the thin layer chromatography of lipids. *J. Am. Oil Chem. Soc.* **39**, 232.

SKIPSKI, V. P., SMOLOWE, A. F., SULLIVAN, R. C. and BARCLAY, M. (1965). Separation of lipid classes by thin-layer chromatography. *Biochim. Biophys. Acta* **106**, 386–396.

SLACK, E. N. and BURSELL, E. (1976a). The isolation of mitochondria from dipteran flight muscle. *Biochim. Biophys. Acta* **449**, 491–499.

SLACK, E. and BURSELL, E. (1976b). Oxidation of pyruvate by mitochondria isolated from the flight muscles of blowflies. *Insect Biochem.* **6**, 637–647.

SLÁMA, K. (1985). Pharmacology of insect juvenile hormones. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology.* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 11, pp. 357–394. Pergamon Press, Oxford.

SLÁMA, K. and KRYPSIN-SORENSEN, I. (1979). Hypermetabolic response induced by juvenile hormone analogues in an insect. *Z. Naturforsch., Ser. C* **34**, 599–607.

SLÁMA, K., ROMAŇUK, M. and ŠORM, F. (1974). *Insect Hormones and Bioanalogues.* Springer-Verlag, Vienna.

SLÁMA, K. and WILLIAMS, C. M. (1965). Juvenile hormone activity for the bug *Pyrrhocoris apterus*. *Proc. natl. Acad. Sci., U.S.A.* **54**, 411–414.

SMITH, D. S. (1961). The structure of fibrillar flight muscle: a study made with special reference to the membrane systems of the fibre. *J. biophys. biochem. Cytol.* **10** (No. 4 Supplement), 123–158.

SMITH, D. S. (1965a). The organization of flight muscle in an aphid, *Megoura viciae* (Homoptera): with a discussion on the fine structure of synchronous and asynchronous striated muscle fibres. *J. Cell Biol.* **27**, 379–393.

SMITH, D. S. (1965b). The flight muscles of insects. *Sci. Am.* **212**(6), 77–88.

SMITH, D. S. (1966a). The organization and function of the sarcoplasmic reticulum and T-system of muscle cells. *Prog. Biophys. mol. Biol.* **16**, 109–142.

SMITH, D. S. (1966b). The organization of flight muscle fibers in the Odonata. *J. Cell Biol.* **28**, 109–126.

SMITH, D. S. (1968). *Insect Cells: Their Structure and Function.* Oliver and Boyd, Edinburgh.

SMITH, D. S. (1972). *Muscle: A Monograph.* Academic Press, New York.

SMITH, D. S. (1984). The structure of insect muscles. In *Insect Ultrastructure* (R. C. King and H. Akai, eds.), vol. 2, pp. 111–150. Plenum Press, New York.

SMITH, D. S. and ALDRICH, H. C. (1971). Membrane system of freeze-etched striated muscle. *Tissue Cell* **3**, 261–281.

SMITH, D. S. and SACKTOR, B. (1970). Disposition of membranes and the entry of haemolymph-borne ferritin in flight muscle fibers of the fly *Phormia regina*. *Tissue Cell* **2**, 355–374.

SOCHA, R. and GELBIČ, I. (1973). Stages in the differentiation of ovarian development in *Dixippus morosus* as revealed by the effects of juvenoids. *Acta Ent. Bohemoslov.* **70**, 303–312.

SOHAL, R. S. (1985). Aging in insects. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology.* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 10, pp. 595–631. Pergamon Press, Oxford.

SOKAL, R. R. and ROHLF, F. J. (1969). *Biometry: the principles and practice of statistics in biological research.* W. H. Freeman & Co., San Francisco.

SPARKS, T. C. (1984). Effects of juvenile hormone I and the anti-juvenile hormone fluoromevalonolactone on development and juvenile hormone esterase activity in post-feeding last-stadium larvae of *Trichoplusia ni* (Hübner). *J. Insect Physiol.* **30**, 225–234.

SPARKS, T. C. and HAMMOCK, B. D. (1979). Induction and regulation of juvenile hormone esterases during last larval instar of the cabbage looper, *Trichoplusia ni*. *J. Insect Physiol.* **25**, 551–560.

SPARKS, T. C., HAMMOCK, B. D. and RIDDIFORD, L. M. (1983). The haemolymph juvenile hormone esterase of *Manduca sexta* (L.) – inhibition and regulation. *Insect Biochem.* **13**, 529–541.

SRIDHARA, S. (1981). Macromolecular changes during insect metamorphosis. In *Metamorphosis: A Problem in Developmental Biology* (L. I. Gilbert and E. Freiden, eds.), second edition, pp. 177–216. Plenum Press, New York.

SRIHARI, T., GUTMANN, E. and NOVAK, V. J. A. (1975). Effect of ecdysterone and juvenoid on the developmental involution of flight muscles in *Acheta domestica*. *J. Insect Physiol.* **21**, 1–8.

STAAL, G. B. (1975). Insect growth regulators with juvenile hormone activity. *Ann. Rev. Entomol.* **20**, 417–460.

STEELE, J. E. (1976). Hormonal control of metabolism in insects. *Adv. Insect Physiol.* **12**, 239–323.

STEELE, J. E. (1982). Glycogen phosphorylase in insects. *Insect Biochem.* **12**, 131–147.

STEELE, J. E. (1985). Control of metabolic processes. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. Kerkut and L. I. Gilbert, eds.), vol. 8, pp. 99–145. Pergamon Press, Oxford.

STEEN, J. B. (1961). The effect of juvenile hormone on the respiratory metabolism of silkworm pupae, as recorded with a new semi-micro device. *Acta Physiol. Scand.* **51**, 275–282.

STEER, M. W. (1981). *Understanding Cell Structure.* Cambridge University Press, Cambridge.

STEGWEE, D. and VAN KAMMEN-WERTHEIM, A. R. (1962). Respiratory chain metabolism in the Colorado potato beetle – I. Respiration and oxidative phosphorylation in sarcosomes from active beetles. *J. Insect Physiol.* **8**, 117–126.

STEPHEN, W. F. and GILBERT, L. I. (1969). Fatty-acid biosynthesis in the silk moth, *Hyalophora cecropia*. *J. Insect Physiol.* **15**, 1833–1854.

STEPHEN, W. F. and GILBERT, L. I. (1970). Alterations in fatty-acid composition during the metamorphosis of *Hyalophora cecropia*: correlations with the juvenile hormone titre. *J. Insect Physiol.* **16**, 851–864.

STEPIEN, G., RENAUD, M., SAVRE, I. and DURAND, R. (1988). Juvenile hormone increases mitochondrial activities in *Drosophila* cells. *Insect Biochem.* **18**, 313–321.

STERNBURG, J. and CORRIGAN, J. (1959). Rapid collection of insect blood. *J. econ. Entomol.* **52**, 538–539.

STRONG, L. (1968a). The effect of enforced locomotor activity on lipid content in allatectomized males of *Locusta migratoria migratorioides*. *J. exp. Biol.* **48**, 625–630.

STRONG, L. (1968b). Locomotor activity, sexual behaviour, and the corpus allatum hormone in males of *Locusta*. *J. Insect Physiol.* **14**, 1685–1692.

TEUTSCH-FELBER, D. (1970). Experimentelle und histologische Untersuchungen an der Thoraxmusculatur von *Periplaneta americana* L. *Rev. Suisse Zool.* **77**, 481–523.

THOMAS, K. K. and NATION, J. L. (1966a). Control of a sex-limited haemolymph protein by corpora allata during ovarian development in *Periplaneta americana*. *Biol. Bull.* **130**, 254–264.

THOMAS, K. K. and NATION, J. L. (1966b). RNA, protein and uric acid content of body tissues of *Periplaneta americana* (L) as influenced by corpora allata during ovarian development. *Biol. Bull.* **130**, 442–449.

TIEGS, O. W. (1955). The flight muscles of insects – their anatomy and histology; with observations on the structure of striated muscle in general. *Phil. Trans. R. Soc.* **238B**, 221–348.

TOBE, S. S. and LOUGHTON, B. G. (1967). The development of blood protein in the African migratory locust. *Can. J. Zool.* **45**, 975–984.

TOMINO, S. (1985). Major plasma proteins of *Bombyx mori*. *Zool. Sci.* **2**, 293–303.

TRIBE, M. A. and ASHURST, D. E. (1972). Biochemical and structural variations in the flight muscle mitochondria of ageing blowflies, *Calliphora erythrocephala*. *J. Cell Sci.* **10**, 443–469.

TSUKAMOTO, M., NAGAL, Y., MARUYAMA, K. and AKITA, Y. (1966). The occurance of relaxing granules in the muscles of the locust, *Locusta migratoria*. *Comp. Biochem. Physiol.* **17**, 569–581.

TURNER, A. E. and LOUGHTON, B. G. (1975). *In vitro* protein synthesis by tissues of the fifth instar locust. *Insect Biochem.* **5**, 791–804.

UNNITHAN, G. C. and NAIR, K. K. (1977). Utrastructure of juvenile hormone-induced degenerating flight muscles in a bark beetle, *Ips paraconfusus*. *Cell Tiss. Res.* **185**, 481–490.

UVAROV, B. (1966). *Grasshoppers and Locusts*, vol. 1. Cambridge University Press, Cambridge.

VAN DEN BERGH,, S. G. (1964). Pyruvate oxidation and the permeability of housefly sarcosomes. *Biochem. J.* **93**, 128–136.

VAN DEN BERGH, S. G. and SLATER, E. C. (1962). The respiratory activity and permeability of housefly sarcosomes. *Biochem. J.* **82**, 362–371.

VAN HANDEL, E. (1965). Micro-separation of glycogen, sugars and lipids. *Analyt. Biochem.* **11**, 266–271.

VAN HANDEL, E. and LEA, A. O. (1970). Control of glycogen and fat metabolism in the mosquito. *Gen. comp. Endocrinol.* **14**, 381–384.

VAN DEN HONDEL-FRANKEN, M. A. M. (1982). Critical period of sensitivity to juvenile hormone for the invagination of tracheoblasts into the developing flight muscle fibres of *Locusta migratoria*. *Gen. comp. Endocrinol.* **47**, 131–138.

VAN DEN HONDEL-FRANKEN, M. A. M. and FLIGHT, W. F. G. (1981). Tracheolization and the effects of implantation of corpora allata on the invagination of tracheoblasts into the developing flight muscle fibres of *Locusta migratoria*. *Gen. comp. Endocrinol.* **43**, 503–518.

VAN DEN HONDEL-FRANKEN, M. A. M., VAN DEN BROEK, A. TH. M. and BEENAKKERS, A. M. TH. (1980). Flight muscle development in *Locusta migratoria*: effects of implantation of corpora allata on the attainment of metabolic enzyme activities. *Gen. comp. Endocrinol.* **41**, 477–486.

VAN MARREWIJK, W. J. A., VAN DEN BROEK, A. TH. M. and BEENAKKERS, A. M. TH. (1980). Regulation of glycogenolysis in the locust fat body during flight. *Insect Biochem.* **10**, 675–679.

VENKATESH, K., CRAWFORD, C. L. and ROE, R. M. (1988). Characterization and the developmental role of plasma juvenile hormone esterase in the adult cabbage looper, *Trichoplusia ni*. *Insect Biochem.* **18**, 53–61.

VOGEL, W., MASNER, P., GRAF, O. and DORN, S. (1979). Types of response of insects on treatment with juvenile hormone active insect growth regulators. *Experientia* **35**, 1254–1256.

VOGELL, W., BISHAI, F. R., BÜCHER, M., KLINGENBERG, M., PETTE, D. and ZEBE, E. (1959). Über strukturelle und enzymatische Muster in Muskeln von *Locusta migratoria*. *Biochem. Z.* **332**, 81–17.

VOGT, M. (1949). Fettkörper und Öenocyten der *Drosophila* nach Extirpation der adulten Ringdrüse. *Z. Zellforsch. mikrosk. Anat.* **34**, 160–164.

VROMAN, H. E., KAPLANIS, J. N. and ROBBINS, W. E. (1965). Effect of allatectomy on lipid biosynthesis and turnover in the female American cockroach, *Periplaneta americana* (L). *J. Insect Physiol.* **11**, 897–904.

WALKER, P. R. (1965). The structure of the fat body in normal and starved cockroaches as seen with the electron microscope. *J. Insect Physiol.* **11**, 1625–1631.

WALKER, P. R. and BAILEY, E. (1971a). Effect of allatectomy on fat body lipid metabolism of the male desert locust during adult development. *J. Insect Physiol.* **17**, 813–821.

WALKER, P. R. and BAILEY, E. (1971b). Effect of allatectomy on the growth of the male desert locust during adult development. *J. Insect Physiol.* **17**, 1125–1137.

WALKER, P. R. and BAILEY, E. (1971c). Effect of allatectomy on fat body lipogenic enzymes of the male desert locust during adult development. *J. Insect Physiol.* **17**, 1359–1369.

WEIS-FOGH, T. (1956). Tetanic force and shortening in locust flight muscle. *J. exp. Biol.* **33**, 668–684.

WEIS-FOGH, T. (1964). Diffusion in insect wing muscle, the most active tissue known. *J. exp. Biol.* **41**, 229–256.

WHEELER, C. H. and GOLDSWORTHY, G. J. (1983). Qualitative and quantitative changes in *Locusta* haemolymph proteins and lipoproteins during ageing and adipokinetic hormone. *J. Insect Physiol.* **29**, 339–347.

WHITMORE JR., D., WHITMORE, E. and GILBERT, L. I. (1972). Juvenile hormone induction of esterases: a mechanism for the regulation of juvenile hormone titer. *Proc. natl. Acad. Sci., U.S.A.* **69**, 1592–1595.

WIGGLESWORTH, V. B. (1969). Chemical structure and juvenile hormone activity: comparative tests on *Rhodnius prolixus*. *J. Insect Physiol.* **15**, 73–94.

WIGGLESWORTH, V. B. (1972). *The Principles of Insect Physiology*, seventh edition. Chapman & Hall, London.

WIGGLESWORTH, V. B. (1983). The physiology of insect tracheoles. *Adv. Insect Physiol.* **17**, 85–148.

WIGGLESWORTH, V. B. and LEE, W. M. (1982). The supply of oxygen to the flight muscles of insects: a theory of tracheole physiology. *Tissue Cell* **14**, 501–518.

WILLIAMS, C. M. (1956). The juvenile hormone of insects. *Nature, Lond.* **178**, 212–213.

WRIGHT, J. E. and RUSHING, D. D. (1973). Glycogen in pupal and adult stable flies as affected by a juvenile hormone analogue. *Ann. ent. Soc. Am.* **66**, 274–276.

WRIGHT, J. E., CROOKSHANK, H. R. and RUSHING, D. D. (1975). Glycogen phosphorylase activity in pharate adults of the stable fly and the effects of a juvenile hormone analogue. *J. Insect Physiol.* **19**, 1575–1578.

WYATT, G. R. (1980). The fat body as a protein factory. In *Insect Biology in the Future* (M. Locke and D. S. Smith, eds.), pp. 201–225. Academic Press, New York.

WYATT, G. R. and KALF, G. F. (1957). Chemistry of insect physiology II. Trehalose and other carbohydrates. *J. gen. Physiol.* **40**, 833.

WYATT, G. R. and PAN, M. L. (1978). Insect plasma proteins. *Ann. Rev. Biochem.* **47**, 779–817.

YAMAMOTO, K. R. and ALBERTS, B. M. (1976). Steroid receptors: elements for modulation of eukaryotic transcription. *Ann. Rev. Biochem.* **45**, 721–746.

ZÖLLNER, N. and KIRSCH, K. (1962). Zeitschrift fur die gesont. *Epl. Medigin* **135**, 545–561.