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Studies on Developmental Changes in

Fine Structure and Metabolism in Flight

Muscle of Locusta migratoria L.

by

ALI A.S. AL-ROBAI B.Sc. (Riyadh)

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.... being a thesis presented in candidature

for the degree of Doctor of Philosophy

in the University of Durham

1981

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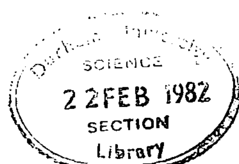
University of Durham



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Finally, I pay tribute to my wife for her endurance and encouragement throughout this work and during the writing of this thesis, which is dedicated to our son Aymen.

ABSTRACT

The fine structure of the median indirect dorsal longitudinal flight muscles has been examined throughout the first week of adult life. During this period, muscle colour changed from white to reddish-brown and the banding pattern characteristic of mature adult flight muscle was established. Associated with these changes there was an increase in myofibril size and the mean number of myosin filaments per myofibril; no significant change was observed in the actin : myosin ratio. There were indications that the number of myofibrils per muscle fibre increase by "longitudinal splitting" of existing myofibrils in the first four days of adult life. A marked increase in mitochondrial size and complexity was noted with increasing age. In addition, total mitochondrial protein increased approximately 10-fold between the 9th day of 5th instar and the 6th day of adult life. However, the increase in mitochondrial size is probably due to both the synthesis of new mitochondrial protein and the fusion of adjacent mitochondria. The mitochondria gradually become arranged in straight columns between the myofibrils by the 5th day of adult life. The relative volume of the sarcoplasmic reticulum (SR) and T-system decreased with age. This was associated with the formation of dyadic junctions and the separation of adjacent myofibrils by sheet(s) of SR. In contrast to the situation, observed in the first few days of adult life, where dyadic junctions are situated near the Z-bands or at an oblique angle to the adjacent myofibrils, in more developed flight muscle they are situated in the region of the A-bands and run parallel to the myofibrils. The distribution of the SR and T-system was affected by the penetration of tracheoles into the muscle fibres. Muscle tracheation was more-or-less fully developed within the 3rd-day of adult life. The relative volume of the tracheoles decreased with age. The physiological implications of these developmental changes in fine structure are discussed.

Mitochondrial phospholipids contained five mean fatty acids at all ages studied; palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). The relative amount of stearic acid (18:0) and the ratio of unsaturated : saturated fatty acids decreased over the period between the 9th-day of the 5th instar and the 15th day of adult life.

Providing a suitable reaction medium was used, oxidative phosphorylation was demonstrable at all ages studied with the following substrates: α-glycerophosphate, pyruvate plus proline and glutamate, but not with succinate.

Allosteric activation of α-glycerophosphate dehydrogenase by Ca^{2+} was demonstrable at all ages studied. No such activation was observed when Mg^{2+} replaced Ca^{2+}. α-Glycerophosphate dehydrogenase activity increased with age.

The properties of SR-ATPase (total ATPase) of *Locusta* flight muscle were similar to those reported for vertebrates skeletal muscle SR-ATPase. Total ATPase activity required Mg^{2+} and was stimulated by Ca^{2+}. Optimal concentrations of Ca^{2+} and Mg^{2+} for total ATPase were 3.19×10^{-6}M (free Ca^{2+}) and 1.5 - 3mM, respectively. The pH optimum was ca. 7.6 for total ATPase activity. The Ca^{2+}-stimulated component of ATPase activity showed similar optima to total ATPase activity. In the presence of Ca^{2+} the apparent Km for ATP was decreased from 0.643mM (Mg^{2+} present) to 0.420mM. There was an approximately 4-fold increase in SR protein per thorax and in the specific activity of total and Ca^{2+}-stimulated ATPase activity. The developmental changes referred to above are discussed in relation to the improved flight performance observed during the first week of adult life in *Locusta migratoria* L.

GLOSSARY

ADP	Adenosine-5'-diphosphate
ADP:O	ADP:O ratio (number of nmoles of ADP esterified per µg AO {microgram atom of oxygen} consumed).
ANS	8-aniline, 1-naphthalene sulphonate
ATP	Adenosine-5'-triphosphate
ATPase	Adenosine-5'-traiphosphatase
BHT	2,6-ditertiary butyl-para-cresol
Bis-Tris propane	1,3-Bis {tris (hydroxymethyl)-methlamino} -propane
BSA	Bovine Serum Albumin
Ca^{2+} ATPase	Calcium-stimulated adenosine 5'-triphosphatase (E.C. 3.6.1.3).
^{o}C	degrees centrigrade
Ci	Curie
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis (β-amino-ethyl (ether) N N'-tetraacetic acid
FCCP	Carbonyl cyanide p-triflurometho xyphenyl hydrazone
GLC	Gas-liquid chromatography
α-GP	α-glycerophosphate
Kapp	Apparent association constant
Km	Michaelis constant
Mg^{2+}-ATPase	Magnesium-dependent adenosine 5'-triphosphatase (EC 3.6.1.3)
M	Molar
NAD^{+}	Nicotinamide-adenine dinucleotide (oxidized form)
NADH	Nicotinamide-adenine dinucleotide (reduced form)
Pi	Inorganic phosphate

RCR	Respiratory control ratio
r.p.m.	revolutions per minute
QO_2	Oxygen consumed expressed in μg AO. mg protein^{-1} hour^{-1}
s.p.	Specific gravity
SR	Sarcoplasmic reticulum
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography
U.V.	Ultra-violet
Vmax	Maximal reaction velocity

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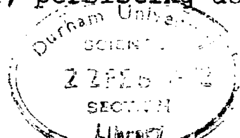
CHAPTER 1

GENERAL INTRODUCTION

The metabolic rate of mature insect flight muscle is amongst the highest known in biology and this is reflected in an approximately 50-100 fold increase in oxygen uptake during flight (SACKTOR, 1965, 1970, 1974; ROWAN and NEWSHOLME, 1979; KAMMER and HEINRICH, 1978; BEENAKKERS *et al.*, 1981). This enormous increase in the expenditure of energy during flight implies that a similar increase occurs in the activity of the respiratory enzymes, substrate flux, oxidative phosphorylation and ATP production (ROWAN and NEWSHOLME, 1979; KAMMER and HEINRICH, 1978). A very efficient system, therefore, is required for the formation of ATP and its conversion to mechanical work. It has been reported that muscle can attain efficiencies in excess of 40% in utilizing metabolic energy for work (WILSON *et al.*, 1981). Indeed, HUDDART (1975) suggests that the muscle fibre can be considered essentially as a machine which converts chemical energy (ATP) into mechanical energy and that the density of the mitochondria may be taken as a measure of the ATP requirement by the muscle.

The major development of *Locusta migratoria* flight muscle takes place during the 5th instar and the early part of adult life (BÜCHER, 1965). However, the precursors of the flight muscles are formed early in development, probably during embryogenesis (VAN MARREWIJIK *et al.*, 1980). Similarly, TIEGS (1955) has shown that, in *Chortoicetes terminifera*, flight muscle is present as undeveloped muscle in the 1st instar larva. In *Schistocerca gregaria*, the future adult pterothoracic musculature is already present at eclosion from the egg (BERNAYS, 1972). However, only the dorsal longitudinal muscles are well developed and these are probably used in hatching. Subsequently, these muscles become non-functional, persisting as rudiments until they develop into the



adult indirect dorsal longitudinal flight muscles (THOMAS, 1954). In *Locusta*, HILL and GOLDSWORTHY (1968) showed that the weight of the flight muscle increased more than 2-fold during the 4th larval instar and about 16-fold in the 5th instar. They also noted that the flight muscles ceased to grow just before and during the period of ecdysis. On the basis of weight, BROSEMER *et al.* (1963) and BÜCHER (1965) have described four phases in the growth of *Locusta* flight muscle in the final larval instar and the 1st week of adult life. These phases are "larval growth" (between the 8th day and the 2nd day before the final ecdysis), "the moulting interval" (from the end of larval growth to the final ecdysis), "the phase of differentiation" (following the final ecdysis up to the 3rd day of adult life) and "the phase of duplication" (between the 3rd and 10th day of adult life). The latter two phases occur in the adult insect during which the dry weight of the flight muscles increased by some 300% (HILL *et al.*, 1968). The locust was then able to sustain flight for several hours (BÜCHER, 1965). BÜCHER (1965) has reported that the above sub-divisions, based on the weight of the muscles, were confirmed when morphological and biochemical properties were compared. As development proceeds the enzyme pattern of young flight muscle develops towards the adult pattern which is quite different (BEENAKKERS *et al.*, 1975). In *Locusta*, BEENAKKERS *et al.* (1975) reported that the ultimate enzyme pattern was established by the 8th day of adult life and that the most profound changes took place over the last few days before and the first 4 days after the final ecdysis.

The wing-beat frequency of tethered *Locusta* (i.e. the rhythm of muscle contraction) increased approximately 2-fold during the first 2 weeks of adult life, whereas the other parameters (wing-stroke angle and stroke-plane angle) remained constant (GEWECKE and KUTSCH, 1979).

Nevertheless, flight speed relative to the surrounding air and lift increased during maturation such that a 2-day old locust could maintain altitude in free flight (KUTSCH and GEWECKE, 1979). However, there was no correlation between flight performance and age in adult animals older than 2 days. KUTSCH and GEWECKE (1979) concluded, therefore, that the basic neuronal flight pattern is determined at the last ecdysis and that the motor output frequency increases with age to match the increase in body weight.

BROSEMER et al. (1963) have investigated the development of subcellular structure together with the enzymatic activities in the flight muscle of Locusta and pointed out that a good correlation exists between ultrastructure and enzyme activities of flight muscle. Similarly, BÜCHER (1965) reported that a parallel development occurred between mitochondrial ultrastructure and mitochondrial enzymes, and concluded that the growth of these organelles is synchronous in all its elements. He stated that "this parallelism between increase in morphological mitochondrial structure and the enzyme content does not leave any doubt about the independent growth of mitochondrial structure". The meaning of "independent growth" has been questioned by DE KORT (1969) who found a good correlation between mitochondrial mass, succinate dehydrogenase and the activity of various other mitochondrial enzymes. However, he could not find proportionality between succinate dehydrogenase activity and that of β-hydroxyacyl-CoA dehydrogenase. He concluded that the growth of mitochondria, in Leptinotarsa decemlineata, is not accompanied by the synchronous growth of all enzymatic components, and stated that "independent growth does not mean the growth of mitochondria can take place independently from other cellular activities". This is in accord with the report that the overall biogenesis of mitochondria takes place by

an interaction between the nuclear - cytoplasmic genetic system and the mitochondrial genetic system (SCHATZ and MASON, 1974).

Associated with the developmental changes referred to above, extra-mitochondrial α-glycerophosphate dehydrogenase was found to increase 20-fold during the phase of differentiation in Locusta (BÜCHER, 1965). This enzyme has been considered as an important aspect of the differentiation of flight muscle (BÜCHER, 1965) and there is a correlation with its activity and the maturity of flight muscle function (BROSEMER, 1965, 1967). However, in contrast to the suggestion that, in Schistocerca vaga, the potentiality for flight may be controlled at the enzymic level by the activity of a single enzyme, extra mitochondrial α-glycerophosphate dehydrogenase (BROSEMER, 1965), DE KORT (1969) failed to find the same substantial increase in extra-mitochondrial α-glycerophosphate dehydrogenase in Leptinotarsa decemlineata flight muscle. He concluded that flight muscle in this insect is limited by the overall enzyme content of the muscle rather than by a single enzyme as suggested by BROSEMER (1965). However, CRABTREE and NEWSHOLME (1972) have found that the maximum activity of mitochondrial α-glycerophosphate dehydrogenase was always much lower than that of the cytosolic enzyme. They suggested, therefore, that the mitochondrial enzyme is the rate limiting factor in the α-glycerophosphate cycle (see Chapter 5).

SACKTOR (1975) has reviewed the mode of control of α-glycerophosphate oxidation in insect flight muscle. He concluded that the release of Ca^{2+} by the sarcoplasmic reticulum (SR), which coincides with the initiation of flight, activates α-glycerophosphate dehydrogenase and therefore α-glycerophosphate oxidation. The ability of the SR of Locusta flight muscle to accumulate and release Ca^{2+} has been demonstrated by TSUKAMOTO et al. (1966) and it may be that the release and

sequestration of Ca^{2+} by the SR represents the mechanism for regulating α-glycerophosphate oxidation by controlling cellular Ca^{2+} concentration (SACKTOR, 1975). The importance of Ca^{2+} in the function of synchronous flight muscle has been emphasised by the study of MARUYAMA *et al.* (1968), who found that the actomyosin ATPase of synchronous flight muscle in *Locusta* was more sensitive to Ca^{2+} than that of asynchronous flight muscle of *Apis mellifera*. They concluded that this difference may be related to the variation in the structural features of these two muscles and that SR development is of great importance in the function of mature *Locusta* flight muscle.

The present study has been carried out to extend our knowledge of the developmental changes in fine structure and mitochondrial function, and to discuss the coordination between structure and function in *Locusta* flight muscle during the first week of adult life. The results of this study are presented in three parts each of which will be introduced separately (Chapters 3, 4 and 5).

CHAPTER 2

GENERAL MATERIALS AND METHODS

Maintenance of the insects

(i) Insectary

The insectary was maintained at a temperature of 30 ± 0.5°C and 60 ± 5% relative humidity. Circulation of air was maintained 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.

(ii) Stock animals

A population of *Locusta migratoria migratorioides* R and F, phase gregaria was reared in cages (43cm x 58cm x 58cm) supplied by Philip Harris Biological Ltd. Each cage was illuminated by a single 40 watt bulb. Food in the form of green grass was supplied daily. There was considerable local variation in temperature and humidity within the cages due to the presence of the bulb. In addition, the relative humidity also increased when fresh food was supplied.

Throughout their development animals were reared at sufficiently high density to ensure their remaining phase gregaria (JOLY and JOLY, 1953).

(iii) Sampling of stock population

A population was prepared for sampling by removing all 5th instar insects at 9 a.m. The population was then examined at 24 hour intervals (9 a.m. each day) and all 5th instar locusts were removed and placed in cylindrical cages (42cm high x 23cm diameter) made of aluminium and acetate sheet. Thus the time of ecdysis must lie between the time the insect was removed and the previous inspection. The mid-point of this period was taken as the time of ecdysis and the insect was aged as 12 hours ± 12 hours

(first day 5th instar) at this time. When adult locusts were required the procedure was the same except that all adult locusts were initially removed from the population.

Treatment of glassware

All routine glassware was soaked overnight in a 2% (w/v) solution of laboratory detergent 'Quadralene', rinsed six times in hot water, six times in cold tap water and finally rinsed four times in distilled water. Glassware was then oven dried. Glass/Teflon homogenizers and polycarbonate centrifuge tubes were allowed to drain at room temperature. All glassware used in lipid experiments was soaked in 50% aqueous nitric acid (AnalaR grade) then rinsed as above. Chromatography tanks and plates were left to drain at room temperature, whilst the rest of the glassware was dried in an oven.

Chemicals

All chemicals were the purest grade commercially available.

Pyruvic acid (sodium salt), proline, DL α-glycerophosphate (α-GP) (disodium salt), succinate, glutamic acid (mono-sodium salt), Tris (hydroxymethyl) amino-methane (Trizma base), bovine serum albumen (BSA) (Fraction V, 69-99%), disodium adenosine triphosphate, disodium adenosine-5'-diphosphate (Sigma grade), ethyleneglycol-bis (β-amino-ethylether), N, N-tetra-acetic acid (sodium salt), imidazole (Free base and grade 1) and carbonyl-cyanide-p-trifluoro-methoxy-phenylhydrazone were purchased from Sigma Chemical Co. Ltd. (Kingston-upon-Thames, Surrey). Cirrasol ALN-WF was a gift from ICI Ltd., Dyestuffs Division. Radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks. Nagarse was obtained from Hughes & Hughes (Enzymes) Ltd. All other chemicals were of analytical reagent grade and were obtained from Boehringer Chem. Co. Ltd. All solutions were prepared in deionised - distilled water or distilled water.

Preparation of mitochondria

The method used employed the proteolytic enzyme, Nagarse, which was first used in the preparation of mitochondria from muscle tissue by CHANCE and HAGIHARA (1961). The essence of this method is the digestion of the myofibrils such that minimal mechanical force is necessary to effect homogenization. Consequently mitochondrial damage is reduced during isolation. The method also obviates the necessity for a low speed centrifugation, which is unsuccessful in preparing mitochondria from insect flight muscle (CHAPPELL and HANSFORD, 1972).

In ice-cold conditions (0-4°C) (unless otherwise stated) 8 locusts (4 males + 4 females) were decapitated by twisting their heads 90° in one direction and then 90° in the other direction. The posterior tip of the abdomen was severed with a pair of scissors and the head removed with the gut attached to it. The thorax and abdomen were then cut open ventrally. The thorax was opened and the fat body removed carefully with tissue paper. Small clean fine forceps were used to cut loose the flight muscles, first laterally and then in the middle. The muscles were immediately transferred to a small beaker containing 10cm^{3} of isolation medium (0-4°C) composed of 0.32M sucrose, 1mM EDTA, 5mM Tris/HCl (pH 7.3). When all the muscles had been removed, they were transferred to a glass homogenizer and 5mg of Nagarse dissolved in 6cm^{3} isolation medium was added. After two passes of the Teflon pestle, by hand, the tissue was allowed to digest for 6 minutes at 0-4°C. During this time 2-3 further passes of the pestle were carried out, again by hand. 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 then washed with another 4cm^{3} of medium and gently squeezed to expel the filtrate. The two filtrates were "pooled" and centrifuged at 4,000g for 8 minutes in an MSE High Speed 18 centrifuge at 0-4°C. The supernatant was discarded and any fatty deposits on the wall of the tubes

removed with a clean paper tissue. The mitochondrial pellet was resuspended in about 10cm^3 isolation medium using a Pasteur pipette and recentrifuged as before. The final pellet was washed very gently by shaking with a small volume of isolation medium to remove the "fluffy" layer on the top of the pellet. The final pellet was resuspended in an appropriate volume of isolation medium to give a mitochondrial protein suspension of approximately 3-20mg/cm^3.

Measurement of oxidation phosphorylation

Oxidative phosphorylation was measured polarographically at 30°C using an oxygen electrode. Two reaction media were normally used.

(i) 154mM KCl
30mM Tris/HCl (pH 7.3) at 30°C
0.04% BSA
30mM K_2HPO_4

(ii) 15mM KCl
2mM EDTA
5mM $MgCl_2$
30mM K_2HPO_4
50mM Tris/HCl (pH 7.3) at 30°C
0.12% BSA

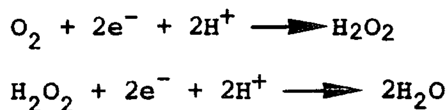
The medium was thoroughly equilibrated to 30°C for several hours to saturate it with air.

Description of the oxygen electrode

Measurements of oxygen concentration were carried out using a Rank oxygen electrode (Rank Bros., Cambridge) which is of similar design to the Clarke oxygen electrode (CLARKE, 1956). It consists of a perspex reaction chamber surrounded by a water jacket which screws onto a perspex base in which a platinum cathode and silver anode is

situated (Fig. 2.1). When in use both were covered with a few drops of 1mM KCl and finally a Teflon membrane. The perspex screw cap, fitted to the top of the reaction chamber, has a small hole through the centre to allow additions to be made to the reaction mixture. The latter is stirred continuously using a magnetic stirrer (Rank Bros., Cambridge).

The principle of the oxygen electrode has been described by DAVIES and BRINK (1943). When a polarising voltage of 0.6 volts is applied between the two electrodes, the platinum being negative to the silver, oxygen undergoes an electrolytic reduction.



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

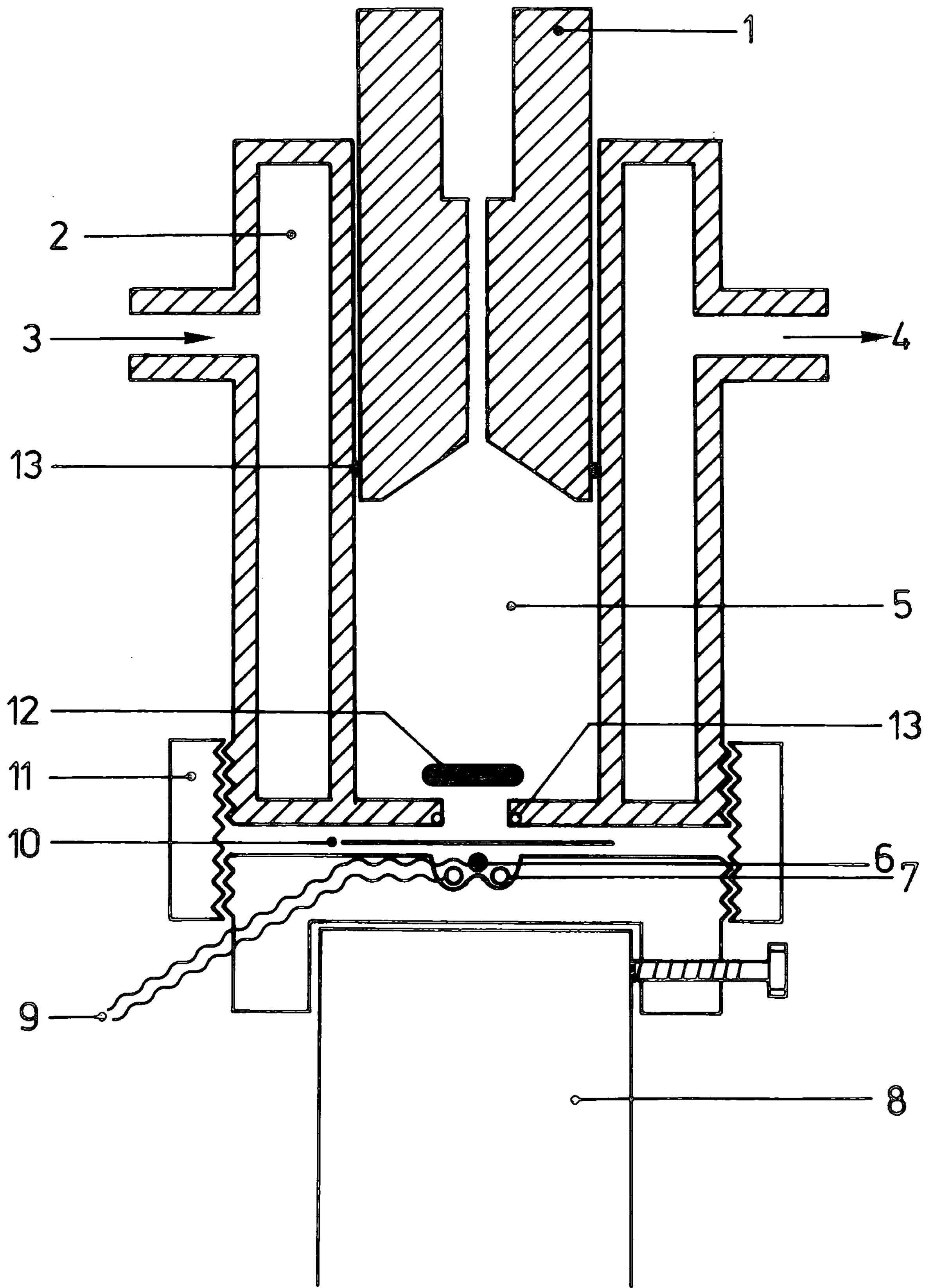
$$H_2O_2 + 2e^- + 2H^+ \longrightarrow 2H_2O$$

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 recorder (Goerz Electron). At 2mV full scale deflection, the recorder baseline was set by reducing the mixture in the reaction chamber with sodium dithionate. This effectively reduced the current across the electrode to zero. The chamber was then washed thoroughly and 2cm^3 reaction medium (saturated with air) pipetted into the reaction chamber. The potential was then adjusted with the helical potentiometer to give full scale deflection on the recorder chart.

Figure 2.1. Diagram of the oxygen electrode used.

1. Screw cap
2. Constant temperature jacket
3. Water in
4. Water out
5. Reaction chamber
6. Platinum electrode (cathode)
7. Annular silver electrode (anode)
8. Magnetic stirrer base
9. Lead in to recorder
10. Teflon membrane
11. Locking ring
12. Magnetic stirrer
13. Rubber ring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Polarographic measurement of oxygen consumption and method of calculating QO_2, RCR and ADP:O ratio

2cm^3 of reaction mixture (unless otherwise stated) were pipetted into the reaction chamber. The perspex screw cap was replaced such that all air bubbles were removed. The reaction medium was allowed to rise about 5mm up to the central hole of the screw cap to minimize contact between the reaction medium and the atmosphere. After about one minute the recorder was adjusted using the helical potentiometer to give full deflection. Oxygen uptake from the medium was followed by determining the rate of change of the reading on the pen recorder after the following additions.

1) 100μl (unless otherwise stated) mitochondrial suspension

2) 10μl of substrate solution (either DL-α-glycerophosphate (α-GP), glutamate, pyruvate plus proline or succinate).

3) 5 or 10μl of 5mM ADP solution made up in 30mM phosphate buffer (pH 6.8 at 0-4°C).

The additions were made by a Hamilton microsyringe. Care was taken to ensure that all air bubbles were expelled from the microsyringe and that the needle did not damage the Teflon membrane over the electrodes. Endogenous rates were neglected throughout this study. The rate which followed the addition of substrate (or after ADP expenditure) is called the substrate rate (i.e. state 4 of CHANCE and WILLIAMS (1955a)). The rate which follows the addition of ADP is called the ADP rate (state 3, CHANCE and WILLIAMS, 1955a).

QO_2 : represents mitochondrial oxygen consumption expressed in terms of μg atom oxygen consumed per mg of mitochondrial protein per hour. It can be calculated from 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 deflection (divisions/minute)

C = initial oxygen content in the reaction mixture saturated at 30°C which has been calculated from results given by DAVISON (1970) to be 0.43μg atom oxygen per cm^3 at 30°C.

R = full scale reading for the oxygen saturated mixture

r = scale reading obtained following addition of sodium dithionate or at anaerobiosis.

RCR : This is defined as the ratio of the respiratory rate in the presence of added ADP (state 3) to the rate observed following its expenditure (state 4) (CHANCE and WILLIAMS, 1956).

$$\text{i.e.} \quad RCR = \frac{\text{State 3 rate}}{\text{State 4 rate}}$$

In some experiments (particularly those involving 5th instar or young adult locusts) the increased rate after ADP addition did not return to a state 4 level. Consequently, it was not possible to measure RCR as indicated above. Under these conditions RCR was estimated by using the ratio of respiratory rate in the presence of ADP to the respiratory rate observed before its addition.

ADP:O ratio : This is defined as the number of μmoles of ADP esterified to ATP for each μg atom of oxygen consumed. ADP:O ratio have been calculated throughout this study using the method described by CHANCE and WILLIAMS (1955b) which relates the amount of oxygen consumed during state 3 rate of respiration to the amount of ADP added.

Determination of protein concentration

The Folin-phenol method of LOWRY *et al.* (1951) was used with slight modification (KASHMEERY, 1977). BSA (Fraction V) was used as standard for calibration curves.

Solution

a) 20% (w/v) sodium carbonate

0.2% (w/v) sodium potassium tartrate

b) 2% (w/v) cupric sulphate

c) This was made up as follows:

5 volumes of A + 0.5 volumes of B + 20 volumes distilled water

d) This was made up by diluting 1 volume of Folin-Ciocalteau's phenol reagent with 10 volumes of distilled water

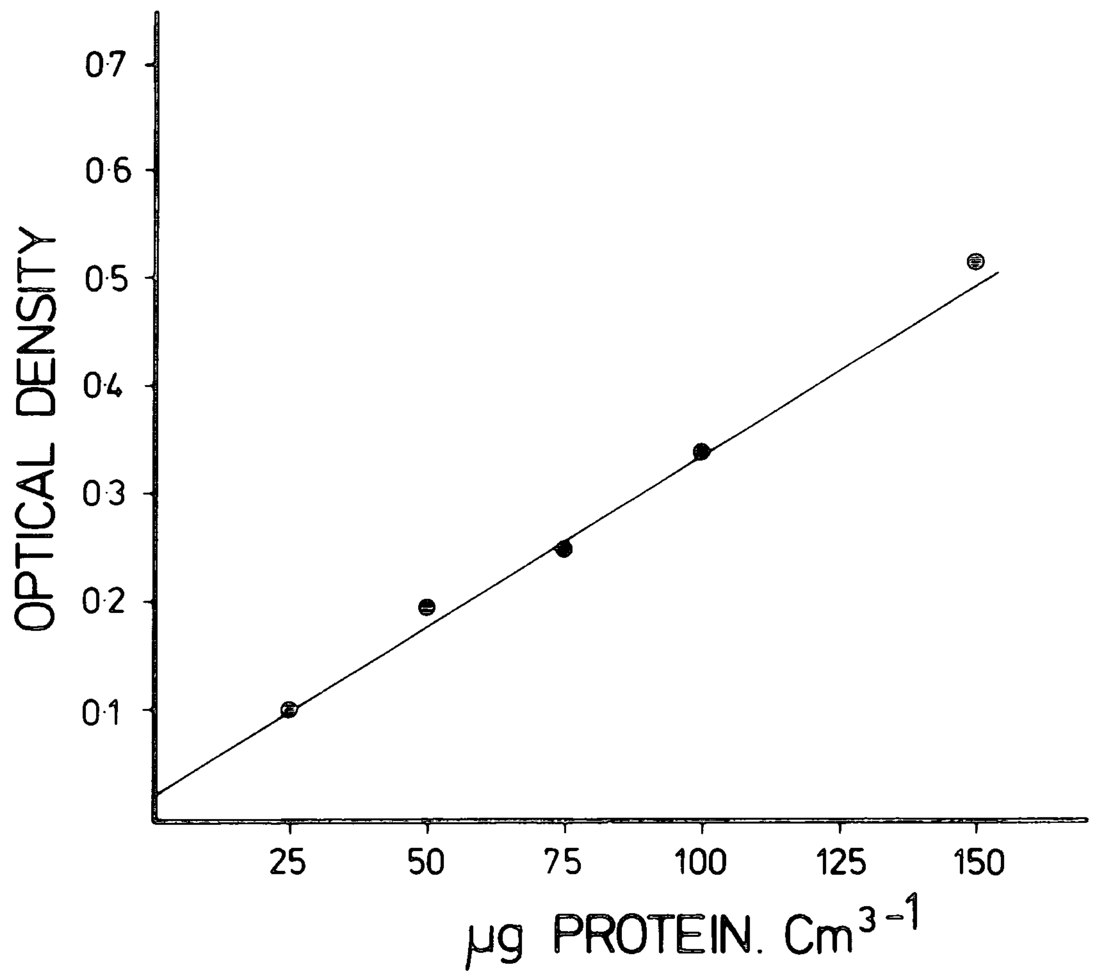
Method

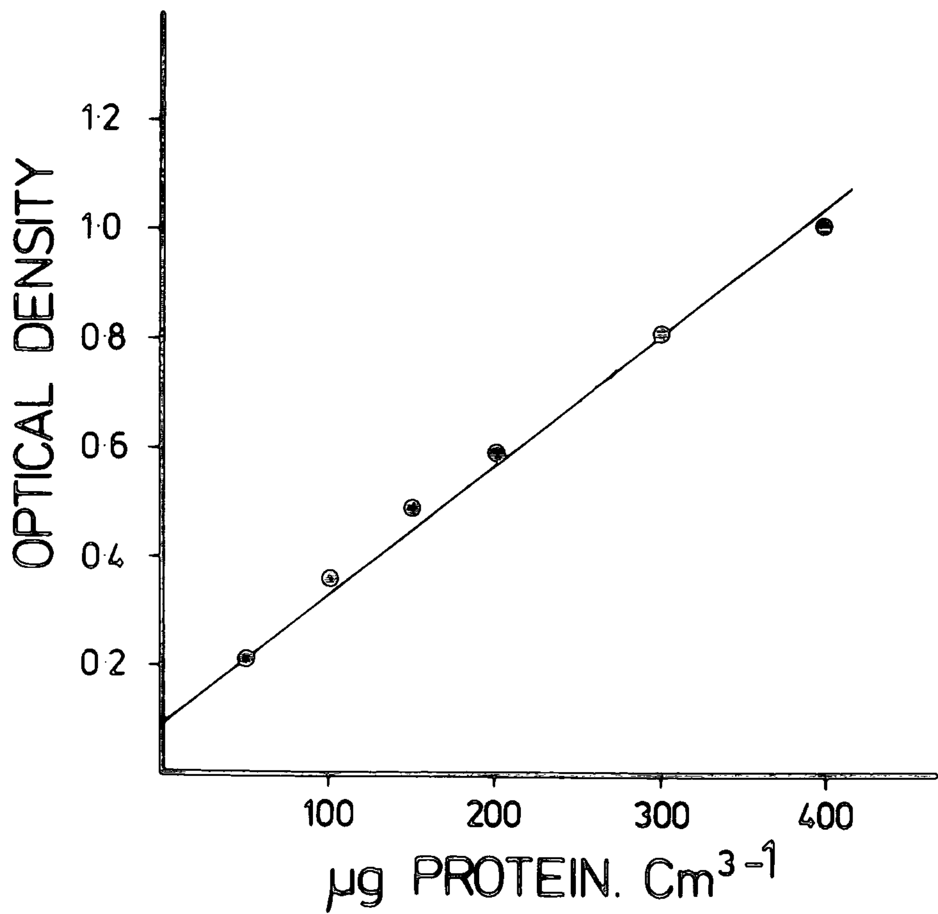
The mitochondrial suspension was diluted with distilled water to give less than $400\mu g/cm^3$, which is the highest concentration of the standards. The latter were made by making a serial dilution from $400\mu g/cm^3$ BSA to give 300, 200, 150, 100 and $0\mu g/cm^3$. In some cases when the protein concentration was likely to be less than 100µg/cm , a further serial dilution was made to give standards of 100, 75, 50, 25 and $0\mu g/cm^3$.

A $1cm^3$ of mixture c) was added to $1cm^3$ of each of the protein solutions and the mitochondrial suspension (or sarcoplasmic reticulum fragments) and allowed to stand at room temperature for 15 minutes. Then $1cm^3$ of reagent d) was added and allowed to stand for a further 30 minutes. The solution was then poured into a glass cuvette (1.0cm length) and the optical density measured against water at 700nm in a Cecil CE 292 spectrophotometer. The relationship between the protein concentration and optical density with the high and low protein concentrations are shown in Figures 2.2 and 2.3, respectively.

Figure 2.2. Representative protein calibration curve for low protein concentration.

Figure 2.3. Representative protein calibration curve for high protein concentration.


0·7
0·6
0·5
0·4
0·3
0·2
0·1
OPTICAL DENSITY
25
50
75
100
125
150
μg PROTEIN. Cm3 -1


1·2
1·0
0·8
0·6
0·4
0·2
OPTICAL DENSITY
100
200
300
400
μg PROTEIN. Cm3 -1

CHAPTER 3

STUDIES ON DEVELOPMENTAL CHANGES IN FLIGHT MUSCLE FINE STRUCTURE, FATTY ACIDS OF MITOCHONDRIAL PHOSPHOLIPIDS AND PROTEIN SYNTHESIS

Introduction

Light microscope studies have made it possible to distinguish three broad categories of flight muscle; tubular, close-packed and fibrillar. Tubular muscle fibres are found in the Odonata, close-packed fibres are typical of the Orthoptera, Trichoptera and Lepidoptera whilst fibrillar muscles are found only in higher insect orders viz. Diptera, Hymenoptera, Hemiptera and Coleoptera. Of the three histological groups of muscles, tubular and close-packed fibres are functionally described as synchronous muscle, implying a 1:1 relationship between the frequency of motor nerve impulses and the mechanical response. In contrast, in fibrillar muscle the contraction frequency greatly exceeds and is relatively independent of the motor nerve impulses (PRINGLE, 1957, 1965; SMITH, 1966a). Such muscles are said to be asynchronous and their structure is well known from the studies of TIEGS (1955), SMITH (1961a, 1966a), SHAFIQ (1963, 1964), ASHHURST (1967), SMITH and SACKTOR (1970) and BAKER (1976).

Detailed ultrastructural studies of the flight muscles of various members of the Odonata (SMITH, 1961b,1966a, 1966b; AUBER, 1967a), Orthoptera (BROSEMER *et al.*, 1963; BÜCHER, 1965; RICHARD *et al.*, 1971; ANSTEE, 1971; ELDER, 1971), Dictyoptera (HAGOPIAN and SPIRO, 1968) and Lepidoptera (SMITH, 1962; AUBER, 1967a,b; REGER, 1967; REGER and COOPER, 1967; BIENZ-ISLER, 1968a,b) have revealed the principal features of these synchronous muscles (see also review by ELDER, 1975). These include the presence of a well-developed sarcoplasmic reticulum which forms a fenestrated curtain around the myofibrils (HAGOPIAN and SPIRO, 1967;

ANSTEE, 1971; ELDER, 1971). The T-system arises from invaginations of the cell membrane and this forms dyadic junctions with the sarcoplasmic reticulum (SR) at the level of the A-bands. Dyadic associations between the T-tubules and the SR membranes thus occur twice per sarcomere length (ELDER, 1971, 1975; HUDDART, 1975).

There is now much evidence to indicate that the SR and the T-system are involved both in the transmission and spread of excitation from the muscle cell membrane to the contractile elements of the myofibrils and in their subsequent relaxation (see reviews by SANDOW, 1965, 1970, 1973; REUBEN et al., 1967; BIANCHI, 1968, 1969; AIDLEY, 1975, HUDDART, 1975; EBASHI, 1976; ENDO, 1977). It seems that the T-system acts by channelling an electrical signal deep into the muscle cell (COSTANTIN, 1970); BASTIAN and NAKAJIMA, 1974). The electrical signal arises as a depolarization of the cell surface membrane and this signal triggers the contractile mechanism. It is suggested that the signal induces the sudden release of Ca^{2+} from the cisternae of the SR. These Ca^{2+} are essential for the splitting of ATP by myosin and the subsequent release of energy which is employed in contraction (SMITH, 1968; ADRIAN et al., 1969a,b). Relaxation occurs when the Ca^{2+} are pumped back into the cisternae of the SR (HASSELBACH, 1964a;TSUKAMOTO et al., 1966). The supply of energy (ATP) is of course maintained by the muscle mitochondria.

In view of the importance of the SR, T-system and mitochondria in muscle function, it is not too surprising to discover that structural variation in these components can be related to the activity of the muscle fibre. Thus in Neoconocephalus robustus it is suggested (ELDER, 1971) that the high incidence of T-tubule invaginations in the mesothoracic flight muscles is an adaptation for achieving a rapid and even spread of excitation throughout the fibre. Similarly, in vertebrate striated muscle, fast acting fibres are characterised by a prominent

T-system and by the regular occurrence of triads and dyads in relation to the band pattern of the myofibrils (FAWCETT and REVEL, 1961; REVEL, 1961; HESS, 1965). The relative volume of the SR is also somewhat greater in fast-acting (with the exception of asynchronous flight muscle) rather than slow-acting muscle (PORTER, 1961; GRINYER and GEORGE, 1969; PAGE, 1969). It is suggested that the greatly increased ratio of SR surface area to myofilament number (SR occupies approx. 19% of total fibre volume; the myofilaments occupy approx. 36% of total fibre volume) is an adaptation to decrease the delay between stimulation and the release of that quantity of Ca^{2+} necessary to initiate the mechanical response (ELDER, 1971). Other differences found by PEACHEY and HUXLEY (1962), and associated with slow fibres in vertebrates, are a paucity of mitochondria, lack of an M-line, weakly defined H-bands and relatively thick Z-bands. In general these features apply to invertebrate muscle also (HAGOPIAN and SPIRO, 1967; JAHAROMI and ATWOOD, 1969; ROSENBLTH, 1969; COHRONE _et al._, 1972; HUDDART and OATES, 1970; ANSTEE, 1971; ELDER, 1971). Fast-acting muscle contains a richer supply of mitochondria, smaller sarcomeres and a more ordered array of thin to thick filaments (3:1) than slow-acting muscle (SMITH, 1968; ELDER, 1975).

Numerous ultrastructual studies on flight muscle development have been carried out on a variety of different species of insects, for example, _Drosophila melanogaster_ (SHAFIQ, 1963, 1964), _Hyalophora cecropia_ (MICHEJDA, 1964), _Apis mellifera_ (HEROLD, 1965), _Lucilia cuprina_ (GREGORY _et al._, 1968), _Antherea pernyi_ (BIENZ-ISLER, 1968), _Leptinotarsa decemlineata_ (DE KORT, 1969), _Homorocoryphus nitidulus_ (ANSTEE, 1971), _Schistocerca gregaria_ (RICHARD _et al._, 1971), _Attagenus megatoma_ (BUTLER and NATH, 1972), _Calliphora erythrocephala_ (ASHHURST, 1967; TRIBE and ASHHURST, 1972), _Musca domestica_ (SOHAL, 1976) and _Manduca sexta_ (RHEUBEN and KAMMER, 1980). The general conclusion is that as development proceeds there is a marked

increase in the size of the myofibrils and mitochondria, and that the density of the mitochondrial cristae increases. Changes are also noted in the SR and the T-system (SMITH, 1961c,1965; BÜCHER, 1965; BIENZ-ISLER, 1968a,DE KORT, 1969; RHEUBEN and KAMMER, 1980; see also review by FINLAYSON, 1975). In general,flight muscle development, in locusts, is completed within 7-8 days after the final ecdysis by which time the various cellular components have developed their typical adult qualities (BROSMER _et al._, 1963; BÜCHER, 1965; KLEINOW _et al._, 1970; RICHARD _et al._, 1971; TANGUAY and CHAUDHARY, 1971; BEENAKKERS _et al._, 1975).

The use of radioactive labelling in _in vivo_ experiments is perhaps the nearest the biologist can come to obtaining information about the cell without disturbing it (ROODYN and WILKIE, 1968). It has been reported that, in _Locusta migratoria_, during the period of maximal biosynthetic activity which takes place within the first few days of adult life, the extent of the incorporation of ^{14}C-isoleucine into flight muscle mitochondrial protein, _in vivo_ (BÜCHER, 1965) and _in vitro_ (BRONSERT and NEUPERT, 1966), is greater than when the mitochondria have become fully developed. Similar results were observed during the incorporation of ^{14}C-serine into mitochondrial phospholipids (BYGRAVE and KAISER, 1969) and various protein fractions of flight muscle during development (KLEINOW _et al._, 1970).

It has been long recognised that lipids play an important role in determining spatial configuration and function of mitochondria; mitochondria depleted of phospholipids artificially, rupture and lose almost all respiratory activity (PETRUSKKA _et al._, 1959; FLEISHER _et al._, 1962). More recently, VAN DEENEN (1972) has pointed out that the fatty acid composition of membrane phospholipids are important in determining the permeability of membranes. It is suggested that membranes containing phospholipids rich in unsaturated fatty acids will be more "fluid" (PASTERRAK, 1977) and

hence more permeable than membranes containing phospholipids rich in saturated fatty acids (CHERQI et al., 1979).

The present study has been carried out to determine the changes in fine structure and protein metabolism which occur in the early days of adult life, in Locusta migratoria. In addition, since the fatty acid composition of membrane phospholipids is important in determining membrane fluidity (and hence permeability), the fatty acid composition of mitochondrial phospholipids has been studied to determine what changes take place in association with mitochondrial growth and development.

Materials and Methods

Electron microscopy

Adult male locusts, Locusta migratoria migratorioides R and F, aged 1-6 days, were killed by twisting their heads 90° in one direction and then 90° in the opposite direction. This broke the "neck" membrane but allowed the gut to remain attached to the head. The posterior tip of the abdomen was then severed and the gut drawn out (still attached to the head). The thorax was then cut open ventrally and pinned out on a cork board, prior to the application of ice-cold (0-4°C) fixative, to reduce muscle contraction during fixation. The musculature was covered with 2.5% glutaraldehyde fixative (see Appendix 3.1) and after 15 minutes the median dorsal longitudinal indirect flight muscles of both the meso- and meta-thoraces were dissected out and placed in a fresh cold fixative at 0-4°C overnight. The tissue was then washed in several changes of cold 0.1M sodium cacodylate buffer (pH 7.3) for total of 6-8 hours followed by post-fixation for 2 hours in 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.3) at room temperature (ca. 20°C). Following dehydration through a graded series of ethanol solutions, the muscles were passed through propylene oxide and embedded in Epon 812 epoxy resin. Sections were cut on a Reichert NK ultratome, mounted

on uncoated copper grids and stained in uranyl acetate followed by lead citrate (REYNOLDS, 1963). They were then examined in an AEI 801 electron microscope.

Preparation of mitochondrial pellets for electron microscopy

Mitochondrial pellets were isolated from adult locusts, aged 1-6 days, as described previously (see General Materials and Methods). Pellets were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.3) as described above. Care was taken to examine sections taken at various levels through the pellets .

Estimation of mitochondrial size

Two different methods were used to measure mitochondrial size:-

(i) Mitochondrial size was determined by measuring their maximum and minimum diameters as seen in electron micrographs of transverse sections through flight muscles. Similar methods have been used in measuring mitochondrial size in *Calliphora erythrocephala* flight muscles (TRIBE and ASHHURST, 1972) and *Calpodes ethlius* fat body (LARSEN, 1970). Only well fixed mitochondria with distinct double membranes and cristae were measured. Mitochondrial size was expressed as the mean profile diameter (i.e. maximum + minimum diameter / 2).

(ii) Mitochondrial size was estimated by tracing mitochondrial profiles from electron micrographs of transverse sections through flight muscle on to standard paper. The tracings were then cut out and weighed; a similar method to that used by FORBES *et al.*, (1972). The results were expressed in arbitrary units. This method was also applied to sections through mitochondrial pellets.

Estimation of myofibril size

Myofibril size was estimated using essentially the same method described in (ii) above.

Tracings from electron micrographs of transverse sections through flight muscles were cut out, weighed and the myofibril size expressed in arbitrary units.

Sarcomere lengths were determined from measurements made on electron micrographs of longitudinal sections through flight muscles.

Relative composition of flight muscle

The relative volumes of the various organelles (excluding nuclei) present in Locusta flight muscle (viz. myofibrils, mitochondria and sarcoplasmic reticulum plus the T-system) and the tracheoles present within the muscle fibres were estimated using essentially the same method as (ii) above. The results were expressed in terms of the percentage total muscle volume.

The determination of ^{14}C-leucine incorporation into flight muscle mitochondrial proteins

Adult animals of known age (see Chapter 2) were injected with 10µl of 2% ethanol containing L-{U-^{14}C}-leucine (radioactive concentration 50µCi/ml; specific activity 354mCi/nmole) using a 50µl Hamilton microsyringe. The needle was inserted into the abdomen, ventro-laterally, between the 3rd-4th abdominal sclerites. Following injection, the locusts were returned to the insectary at 30 ± 0.5°C and 2 animals (1 male + 1 female) were killed as described previously at each of the following times after injection; 30 sec, 30 min, 1 hr, 3 hrs, 5 hrs and 24 hrs. The flight muscles were then dissected out, "pooled" and the mitochondria isolated (see Chapter 2). The washed mitochondrial pellet was then processed using essentially the same methods described by TRIBE and ASHHURST (1972). The pellet was homogenized in 1% trichloroacetic acid (TCA) and centrifuged at 600 r.p.m. for 10 minutes. The precipitate was then washed with 10% TCA at 100°C. After cooling the protein precipitate was centrifuged down again and dissolved in 0.5cm^3

0.4N sodium hydroxide, overnight at room temperature (ca. 20°C). $0.1cm^3$ aliquots were taken and placed in glass liquid scintillation counting vials and $15cm^3$ of NE 262 scintillation cocktail (Nuclear Enterprise Ltd.) were added to each vial. The samples were then counted in a beta/gamma spectometer (NE 8312, Nuclear Enterprise Ltd.). Counts per minute were converted to disintegrations per minute by reference to a quench correction curve (Fig. 3.1).

The determination of ^{14}C-leucine incorporation into total flight muscle protein

The method used was essentially as described in (i) above. The flight muscles were dissected out and homogenized in 10% TCA. After centrifugation, the precipitate was treated as described above except that the final pellet was dissolved in $4cm^3$ of 0.4N sodium hydroxide. $0.2cm^3$ samples in $15cm^3$ of the same scintillation cocktail were counted and corrected for quenching as already described. The results presented in Figures 3.56 a,b,c and d are the mean values of two independent determinations and are expressed as disintegrations per minute per mg protein.

Protein assay

The protein concentration was estimated as described in the General Materials and Methods (Chapter 2).

Analysis of the fatty acid composition of phospholipids extracted from flight muscle mitochondria at different ages

A) Extraction of lipids

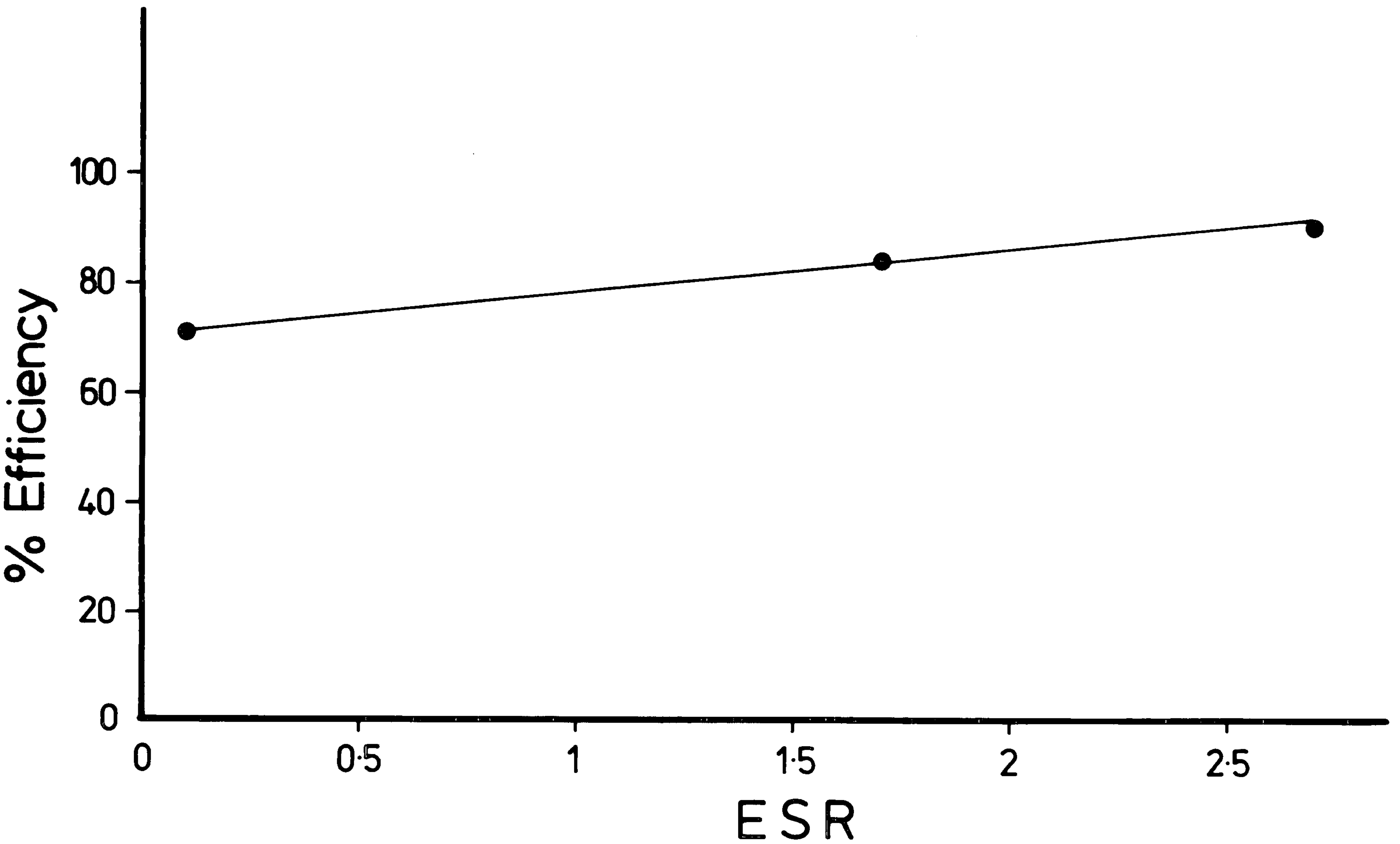
Mitochondrial pellets were isolated from animals of known age as already described in the General Materials and Methods (Chapter 2). These were subjected to two additional washes prior to lipid extraction which was carried out using the method of LEWIS (1978). Mitochondria were

Figure 3.1. Quench-correction graph.

Ordinate: % efficiency

Abscissa: ESR (external standard ratio)

A set of ^{14}C-quenched standards (code number 180060) supplied by Amersham, Bucks, England was used.


% Efficiency
100
80
60
40
20
0
0
0·5
1
1·5
2
2·5
ESR

homogenized in extraction medium I consisting of 33cm^3 chloroform, 17cm^3 methanol and 50mg of the antioxidant, 2,6-ditertiary butyl-para-cresol (BHT). The homogenate was then centrifuged at 5000 r.p.m. for 10 minutes in a "Mistral 2L" centrifuge. The resulting pellet was re-extracted with fresh extraction medium I and following centrifugation the two supernatants were combined in a rotary evaporator flask. The residue was then extracted twice with an extraction medium consisting of 25cm^3 chloroform, 25cm^3 methanol, 1cm^3 conc. HCl and 50mg BHT. Once again, the supernatants of the two steps were "pooled" and mixed with those obtained with extraction medium I. The residue was retained and further extracted twice with a third extraction medium consisting of 25cm^3 chloroform, 25cm^3 methanol, 1cm^3 NH_3 (0.88s.g.) and 50mg BHT. The combined supernatants of these three separate extraction procedures were "pooled" and mixed thoroughly with an equal volume of 0.79% KCl by shaking vigorously for about one minute. This was to extract non-lipid contaminants (FOLCH, LEES and SLOONE-STANLEY, 1957). The resulting emulsion was allowed to stand and complete separation of the organic (bottom) and aqueous (top) phases was facilitated by centrifugation at 2500 r.p.m. for 15 minutes in a "Mistral 2L" centrifuge using a swing-out head (Rotor no. 69166). The aqueous phase was then removed with a Pasteur pipette and discarded. The remaining organic phase was dried overnight over anhydrous sodium sulphate in a ground-glass stoppered flask. The dried mixture was separated from the anhydrous sodium sulphate by centrifugation in glass centrifuge tubes at 2500 r.p.m. as described above. The sodium sulphate deposited was washed twice with chloroform and the dried mixture and the combined washings were then reduced to approximately 1cm^3 in a rotary evaporator 'R' (Buchi, Ltd.) at room temperature.

B) Thin layer chromatographic separation of the phospholipid fractions

The procedure adopted was LEWIS'S (1978) modification of the method described for mammalian phospholipids by VEERKAMP and BROEKUYSE (1976).

(i) Preparation of thin-layer chromatography plates

Five glass plates (20cm x 20cm) thoroughly cleaned and dried as described in the General Materials and Methods section (Chapter 2) were placed in a 'Unoplan' pneumatic holder (Shandon Scientific Ltd.). Plastic gloves were used throughout this procedure. The plates were then washed with chloroform and left to dry. A slurry of silica gel H (Kieselgulr H-Type 60) was made up in 0.1% (w/v) aqueous sodium acetate (approximately 45g silica gel to $100cm^3$ solution was found to be suitable) and vigorously stirred with a clean glass rod until the right consistency was achieved. The slurry was then poured into a 'Unoplan' adjustable spreader with the aperture set at 0.5mm with a Feeler-Gauge. The spreader was then drawn smoothly, and without interruption, across the surface of the glass plates. Air bubbles were excluded by briefly vibrating the plates with a 'Whirlymix' vortex mixer (Fison's Laboratory Apparatus, Loughborough). This also helped to ensure that an absolutely smooth surface was obtained. The plates were then stacked horizontally and dried overnight. Prior to use they were activated in an oven at 110-120^oC for at least 2 hours.

(ii) Application of the lipid extract to the chromatographic plate

The solvent was evaporated from the lipid extract obtained in A above, by means of a stream of dry nitrogen being bubbled through it. Once all the solvent had been removed, aliquots of the lipid sample were applied to the TLC plates as spots, using a microsyringe and the plate dried at 110-120^oC for 5-10 minutes.

Once the plates had cooled, separation of the various lipids was affected by ascending chromatography in large moulded-glass tanks (Shandon Southern Scientific Co. Ltd.) lined with Whatman No. 1 filter paper. The developing solvent consisted of a mixture of 80 volumes petroleum ether (40-60°C boiling point): 20 volumes diethyl ether: 2 volumes of formic acid. This mixture was presented as a shallow layer approximately 1cm from the bottom of the tank. The tank was fully saturated with solvent vapour prior to development of the plates by shaking the tank, and allowing it to stand 1 hour at room temperature. The plate was then placed in the tank and the solvent allowed to rise up the chromatoplate until the solvent front was approximately 1cm from the top of the plate.

(iii) Location of phospholipid fractions

This was done according to the procedure described by GITLER (1972). The plate was dried at 110-120°C for 5-10 minutes and allowed to cool. It was then lightly sprayed with a 0.1% aqueous solution of the sodium salt of 8-aniline, 1-naphthalene sulphonate (ANS) (Eastman Kodak) and irradiated with a U.V. lamp using a 350nm filter. The phospholipid fraction appeared as a bright fluorescent spot against a dark background. These were marked by scraping the silica gel around each spot using a clean needle.

(iv) Preparation of fatty acid methyl-esters

The silica gel, containing the target phospholipid, was scraped off the plate and placed in a bijou bottle. Methanolysis of the phospholipid was effected by adding sufficient boron trifluoride/ methanol (14% w/v) (Sigma), so as to "wet" the entire sample. The bottle was then sealed and the sample heated at 100°C for 15 minutes to ensure that methanolysis was completed. After the sample had cooled a stream of dry nitrogen was blown over it to remove the bulk

of the low boiling point contaminants and to volatilize any residual boron trifluoride/methanol. The fatty acid methyl ester was then eluted from the residue with n-hexane. The solution obtained was concentrated to a small volume in a rotary evaporator or a stream of dry nitrogen, and subjected to gas-liquid chromatographic analysis.

(v) Preparation of the chromatographic column

20g of Gaschrom Q (100-120 mesh) (Applied Science Laboratory) were added to 100cm^3 of 3% (w/v) solution of polyethylene glycol adipate in chloroform. The mixture was then shaken and filtered through a scinterred glass funnel. The residue was first air dried and then heated at 100°C for 1 hour to ensure complete volatilization of any residual chloroform. The dried material, so formed, was poured into a 1m long glass chromatographic column and packed tightly with the aid of a vacuum pump. The column was then conditioned by heating in a stream of dry nitrogen (flow rate 20-25cm^3/minute) at 250°C for 48 hours before use.

(vi) Sample analysis

The mixture of fatty acid methyl esters, prepared as described above, was injected into the GLC by means of a 10μl microsyringe. Prior to use, the microsyringe was washed thoroughly in a sequence of non-polar solvents (acetone, n-hexane, methanol). Periodically, thereafter, between repeated additions of the same sample, the micro-syringe plunger was withdrawn and wiped with a tissue soaked in n-hexane. In addition, cleanliness of the microsyringe was confirmed at frequent intervals by injection of aliquots of n-hexane.

Sample introduction was effected as follows:

(a) n-hexane was taken up and expelled from the microsyringe to remove all air bubbles and to create an effective barrel-plunger seal.

(b) 1µl of n-hexane was taken up into the microsyringe followed by 1µl of air. Then 5µl of the sample to be analysed was taken up followed by another 1µl of air, 1µl of n-hexane and finally a further 1µl of air.

(c) The flexible microsyringe needle was carefully introduced into the column through a rubber septum. The contents were rapidly injected and the needle withdrawn. The microsyringe was then cleaned as indicated above and prepared for another injection.

(vii) Fatty acid indentification and relative composition of mitochondrial phospholipid

Gas liquid chromatographic separation of the samples was carried out using the column described above in a Pye 104 series gas chromatograph at 180°C. Dry white spot nitrogen was used as the carrier gas at a flow rate of 45cm^3 per minute. The resolved sample was detected by flame ionisation (combustion system: hydrogen/air). A signal from the detector was used to trace a chromatograph on the flat bed chart of a Servoscribe recorder (Goerz electron). Typical traces are shown in the Results section. The peaks were tentatively identified by comparing their retention times with those of various standard fatty acid methyl esters (Supelco) under the same conditions.

The relative proportion of the resolved materials was calculated according to the procedure described by CARROLL (1961), which assumes a complete separation of the resolved samples. The product of peak height and retention time gives a value proportional to the area under the peak and consequently the mass of the sample giving that peak (see LEWIS, 1978). In view of the unavoidable variations in the day to day operating conditions, the mass estimate obtained was normalized by expressing the value as a percentage of the total sample applied (i.e. as a proportion of the sum of all the peak areas).

Results

No difference was observed in either the histological or the fine structural appearance of the muscles of the meso- or meta-thoraces. Consequently, the results presented will not distinguish between dorsal longitudinal flight muscles from these two divisions of the thorax.

The flight muscles of newly emerged adult Locusta are relatively small and characteristically "white" in appearance. As growth and development proceed the colour changes gradually to reddish-brown. Associated with this change in colour, there was an increase in the mass of the flight musculature. The possible significance of this change in muscle colour is indicated by the studies of USHERWOOD (1967). He showed that whilst the "red" and "white" fibres of Schistocerca gregaria retractor unguis muscle of the meta-thoracic leg have similar mechanical and electrical properties, the "white" fibres fatigue more rapidly than the "red". In addition, the "red" fibres have higher levels of succinate dehydrogenase and their mitochondrial volume is double that of "white" fibres (ELDER, 1975). A correlation between the increase in the relative amount of cardiolipin (diphosphatidylglycerol), a typical mitochondrial phospholipid, and the change of muscle colour from white to red during the first week of adult life has been suggested, in Locusta (NOVAKOVA et al., 1976) and Periplaneta americana (HELM et al., 1977); the amount of diphosphatidylglycerol doubled and coincided with the appearance of the "red" muscle.

Examination of light micrographs (Fig. 3.2) of transverse sections through the median dorsal longitudinal indirect flight muscles of Locusta shows that the shape and size of each individual fibre profile varies considerably even within the same bundle of fibres. Trachea are clearly seen running between the fibres. In high power

<u>Figure 3.2.</u> A photomicrograph of a transverse section through a part of the median dorsal longitudinal indirect flight muscle of a 6-day old locust.

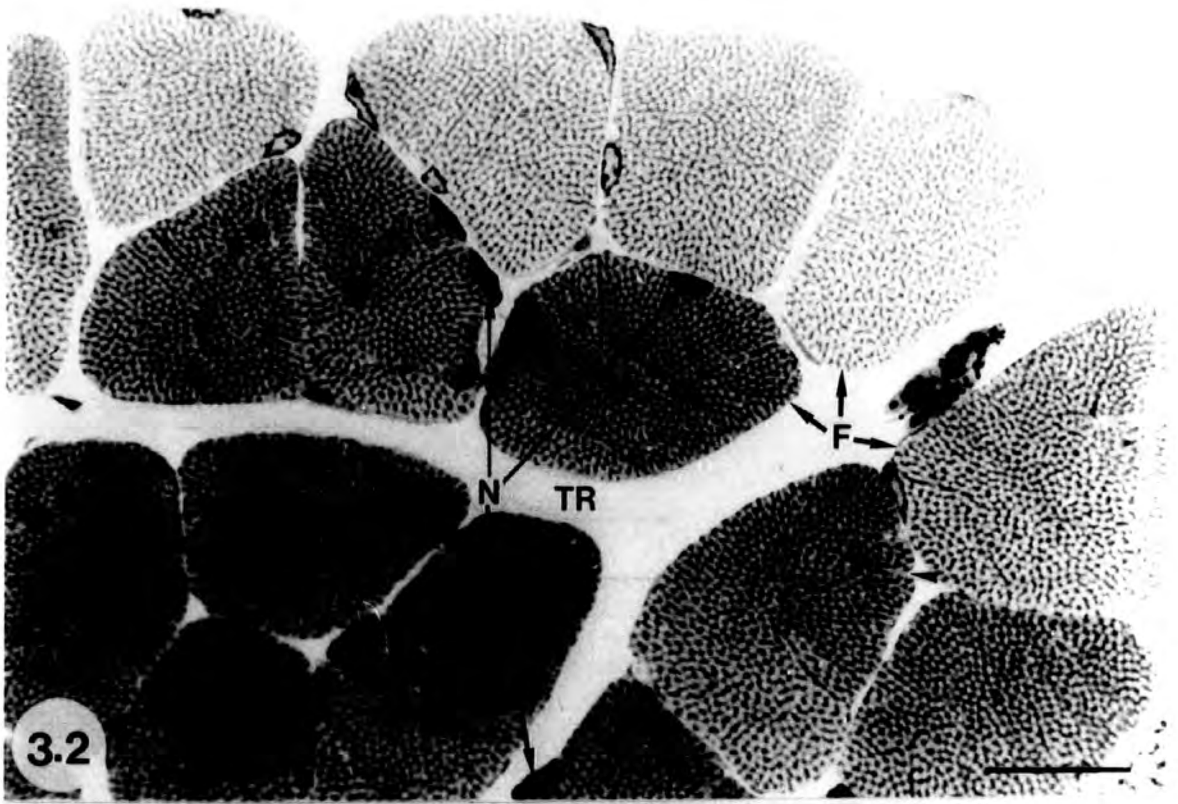
Note the peripheral position of the nuclei (N), the shape and size of the muscle fibres (F). Invagination of the surface cell membrane can be seen (arrow-heads). TR: trachea.

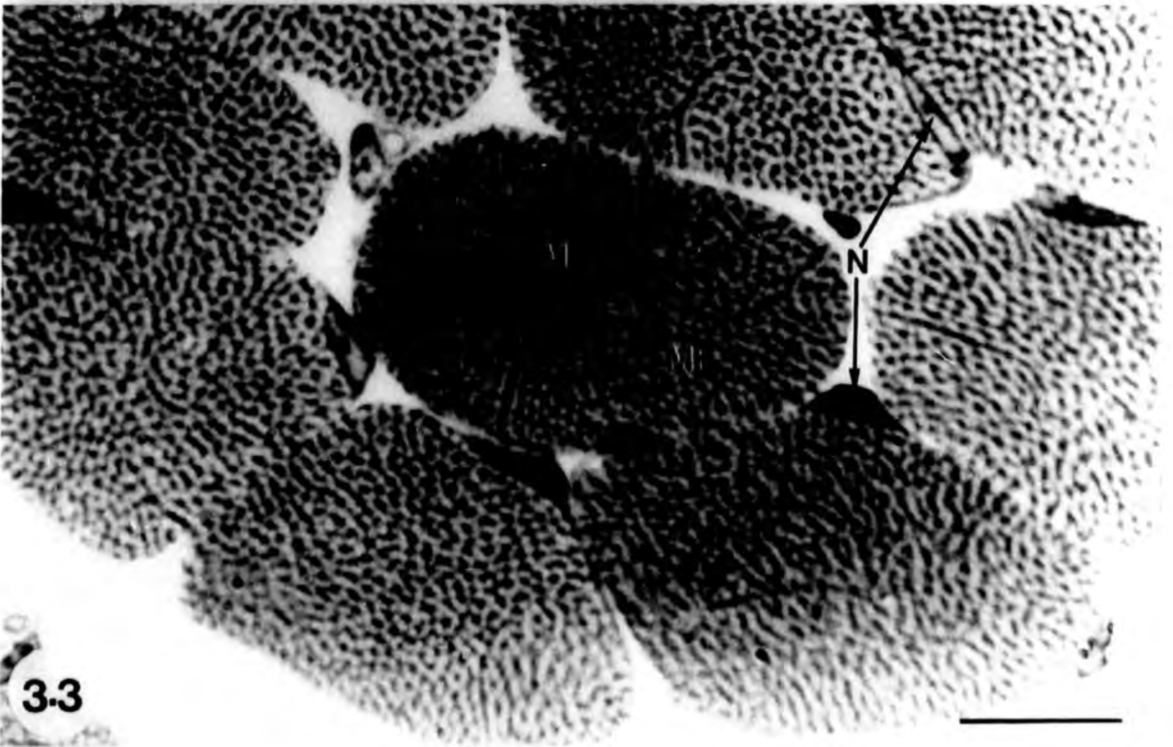
Scale 25μm

<u>Figure 3.3.</u> A high power photomicrograph of a transverse section through a part of the median dorsal through longitudinal flight muscle from an animal aged 6 days.

Note that myofibrils (MF) are arranged in a more-or-less radial fashion. M: mitochondria; N: nucleus

Scale 20μm


N
TR
F
3.2


N
3.3

light micrographs (Fig. 3.3) whilst it appears that the majority of myofibrils are less regular, some seem to be arranged in a more-or-less radial fashion. The peripheral position of the nuclei and their presence under the cell membrane is clearly seen in Figs 3.2 and 3.3. This represents the normal position of these organelles in the vast majority of muscle cells examined. However, occasionally one nucleus was encountered which was positioned within a muscle fibre (Fig. 3.4). In the early stages of muscle development, nuclei are situated in the middle of the myoblast, and as growth and development proceed the increase in muscle cell components tends to push the nuclei to a peripheral position (GOLDSPINK, 1974). The above observation would seem to suggest, therefore, that all muscle differentiation is not complete at this stage (3-day old adult).

Mitochondria, sarcoplasmic reticulum and T-system, and their connections (dyad or triad) are visible between the myofibrils in all ages studied (see Figs 3.5, 3.6, 3.7 and 3.8). The sarcolemma which surrounds each muscle fibre consists of a typical cell membrane and layer of amorphous material or basement membrane (Fig. 3.9). It is within this amorphous material that the tracheoles supplying the fibre are found.

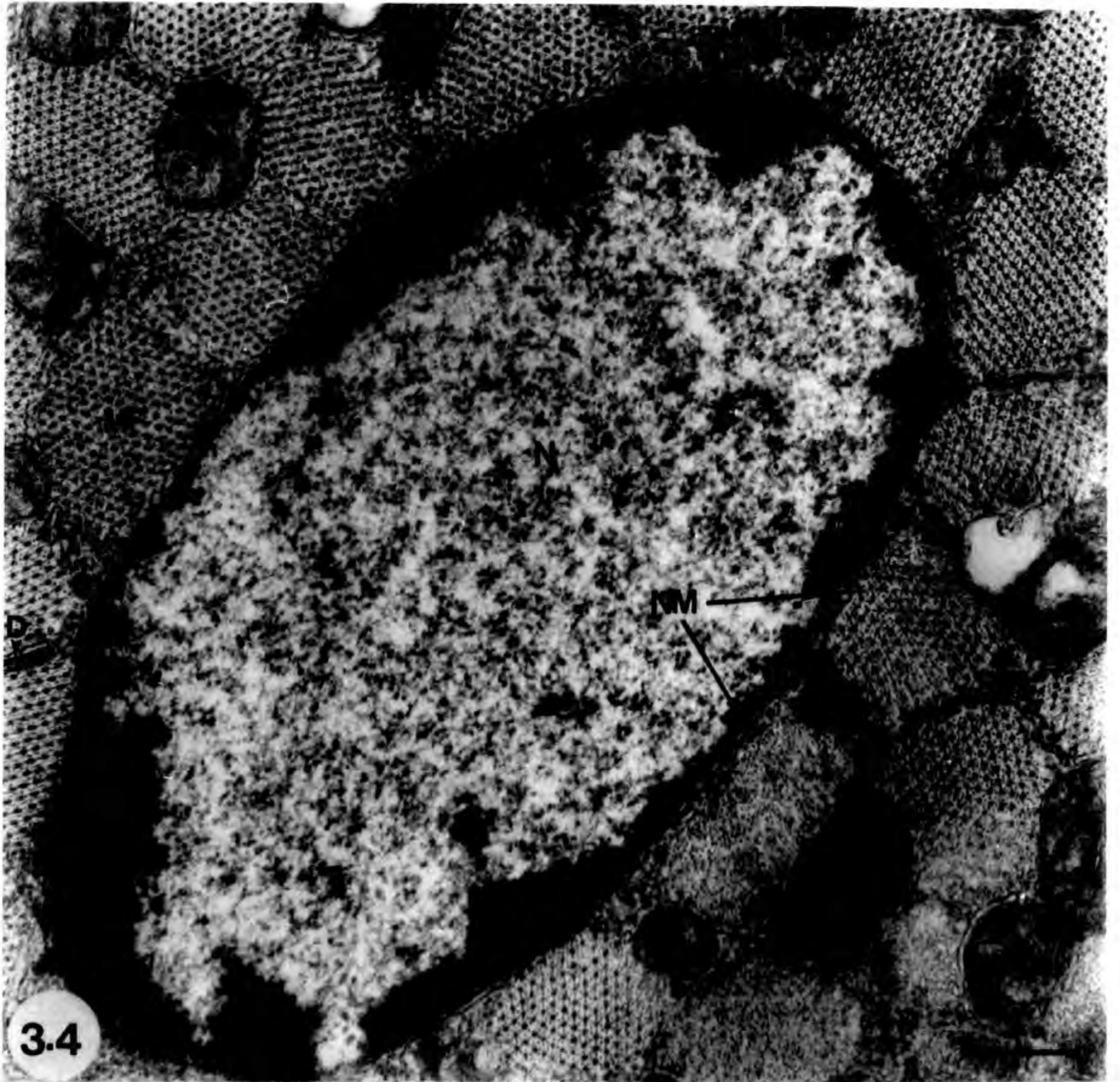
Myofibrils

As mentioned earlier, the myofibrils and mitochondria represent the bulk of the muscle fibre with the nuclei situated in a peripheral position. The arrangement of the myofibrils is irregular (Fig. 3.9) and described as close-packed (BÜCHER, 1965). In longitudinal sections from 6-day old adult animals (Fig. 3.10), the myofibrils are usually in almost perfect parallel register, as witnessed by the relatively straight rows of Z-bands. The latter divide the myofibrils into sarcomeres. These sarcomeres exhibit the band pattern typical of striated muscle. Distinctive I-bands can be seen on either side of the Z-band; the majority of the sarcomere length being represented

Figure 3.4. An electron micrograph of a transverse section through a portion of a muscle fibre from an animal aged 3 days.

Note the position of the nucleus (N) in the middle of the muscle fibre. M: mitochondrion; MF: myofibril; D: dyad; NM: nuclear membrane.

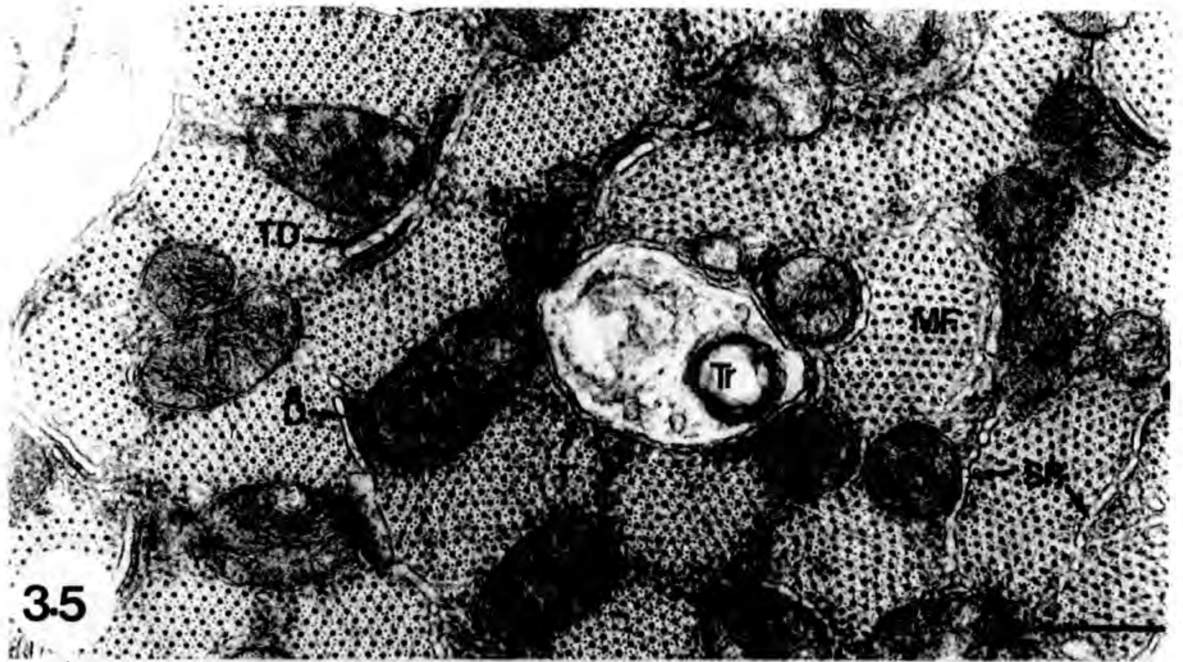
Scale 0.5μm



N
NM
3.4

Figure 3.5. A transverse section through a muscle fibre from a 1-day old locust showing the typical appearance. Mitochondria (M), myofibril (MF), dyad (D), triad (TD) and sarcoplasmic reticulum (SR). Note the size and shape of the mitochondria and the presence of relatively few cristae. Tracheolar cell (Tr) situated in a dilation of the T-system (T).
Scale 0.5μm

Figure 3.6. A transverse section through a muscle fibre from an animal aged 2 days illustrating the typical appearance, size and shape of the muscle components. It can be seen that the mitochondria (M) still contain relatively few cristae. Tr: tracheole; TD: triad; SR: sarcoplasmic reticulum; S: non-fenestrated sarcoplasmic reticulum; T: T-system; MF: myofibril; M: mitochondria.
Scale 0.5μm


TD
MF
Tr
SR
3.5

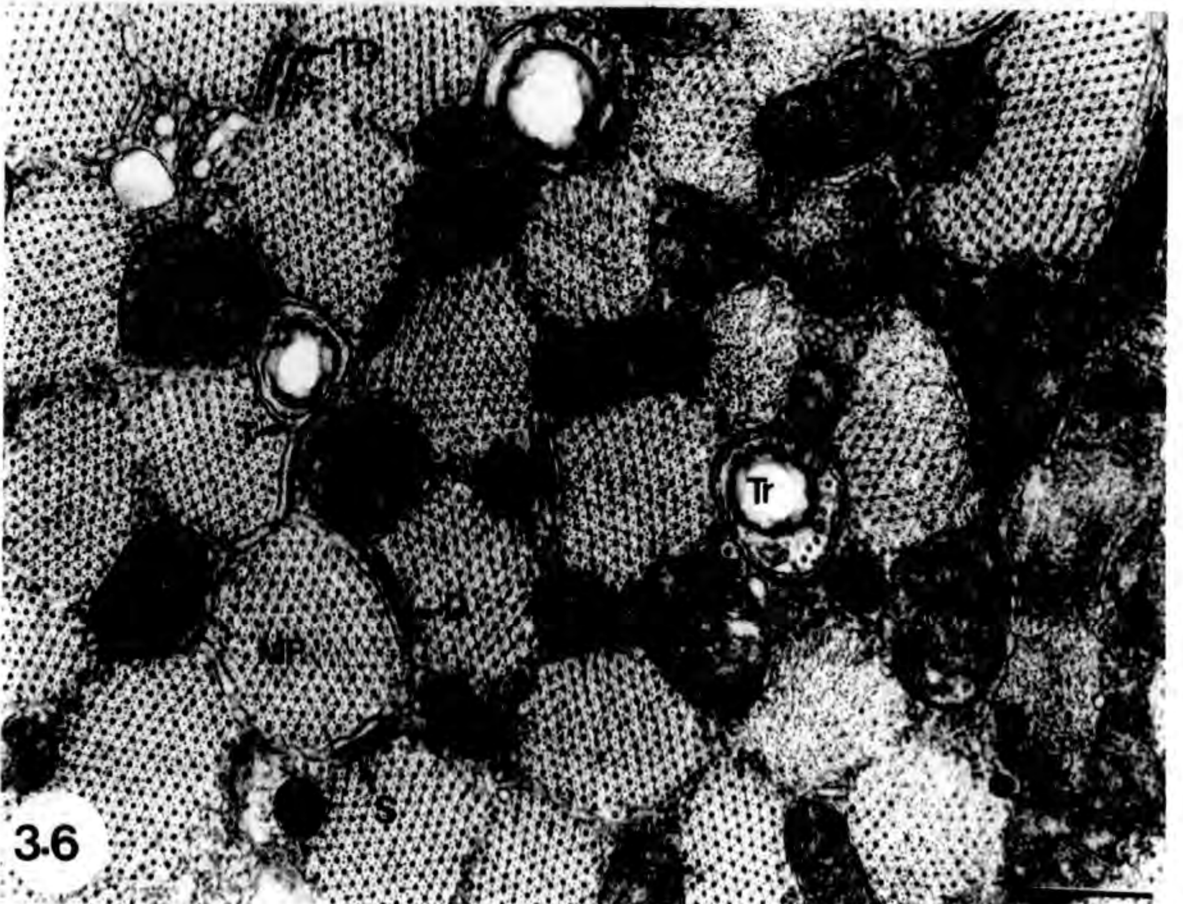

TD
Tr
MF
S
3.6

Figure 3.7. A transverse section through a muscle fibre from an animal aged 3 days showing the typical appearance, size and shape of the muscle components at this age. Note the increase in myofibril (MF) size, myosin number and the variation in mitochondrial (M) size compared with Figures 3.5 and 3.6. D: dyad, SR: sarcoplasmic reticulum; T: T-system.

Scale 0.5μm

Figure 3.8. A transverse section through a muscle fibre from a 5-day old adult locust. The myofibrils (MF) and mitochondria (M) show a further increase in size compared with Figures 3.5, 3.6 and 3.7. Note the increase in mitochondrial size and the number of cristae (Cr). A number of electron dense granules (arrow-heads) are visible in the mitochondria. The sarcoplasmic reticulum (SR) is abundant and completely encircles each myofibril; in some cases two sheets of SR separate the myofibrils.

D: dyad; T: T-system.

Scale 0.5μm

3.7

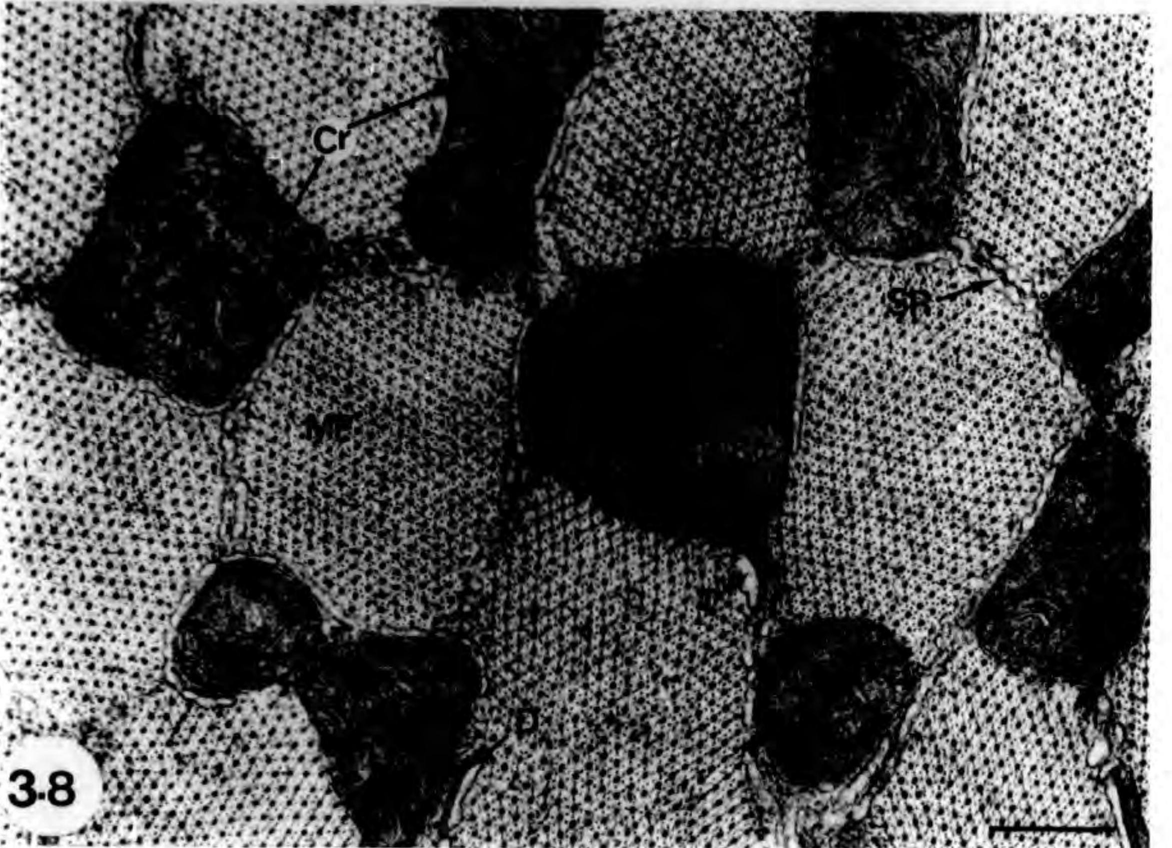
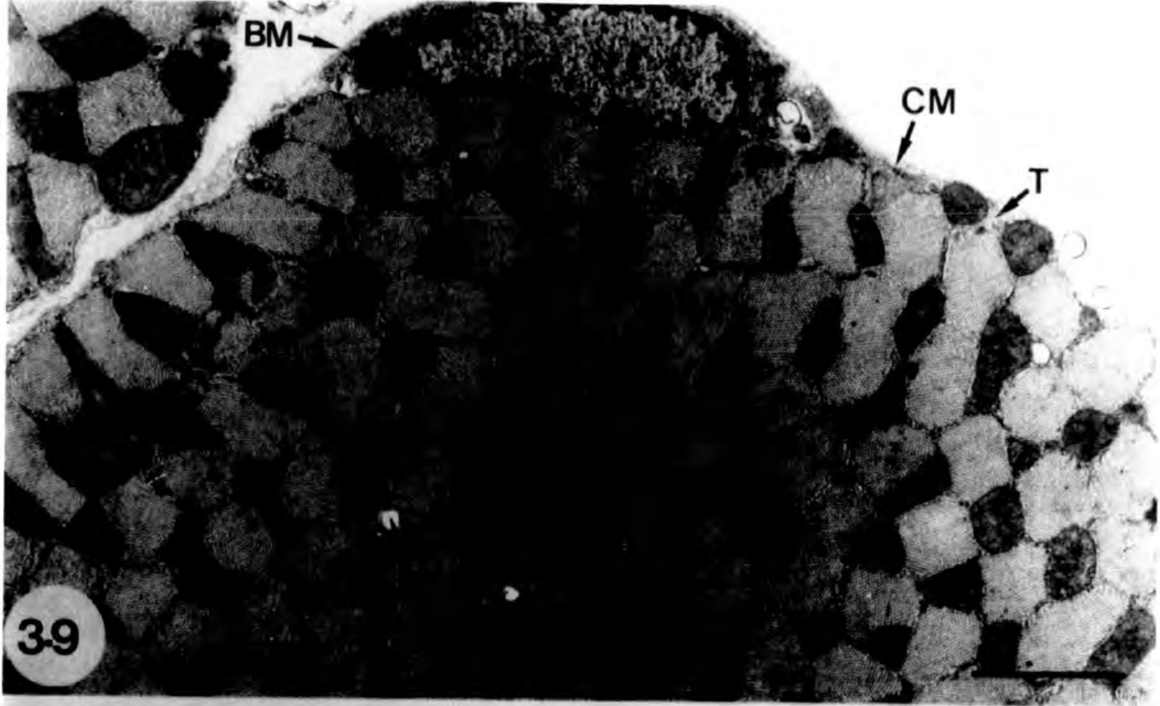


Figure 3.9. A transverse section through a muscle fibre from a 6-day old locust.
Note the variation in size and shape of the myofibrils (MF) and mitochondria (M). A layer of amorphous material forms the basement membrane (BM) of the muscle fibre and one nucleus (N) is seen lying immediately beneath the cell membrane (CM) (see also Figure 3.12). Invagination of the cell membrane can be seen to give rise to the T-system (T) (see also Figures 3.24, 3.28 and 3.51). Tr: tracheole
Scale 2.5μm

Figure 3.10. A longitudinal section through the muscle fibre from a 6-day old locust. The sarcomere is delimited by Z-bands. The I- and A-bands are clearly defined at this age. Dyads (arrow-heads) can be seen normally in indentation of adjacent mitochondria midway between the H-band, which is characterized by a localized scattering of small granules at the centre of the A-band, and the I-band. Note also that individual mitochondria extend more than 4 sarcomere lengths.
M: mitochondrion; MF: myofibril; SR: sarcoplasmic reticulum; G: glycogen.
Scale 1.5μm

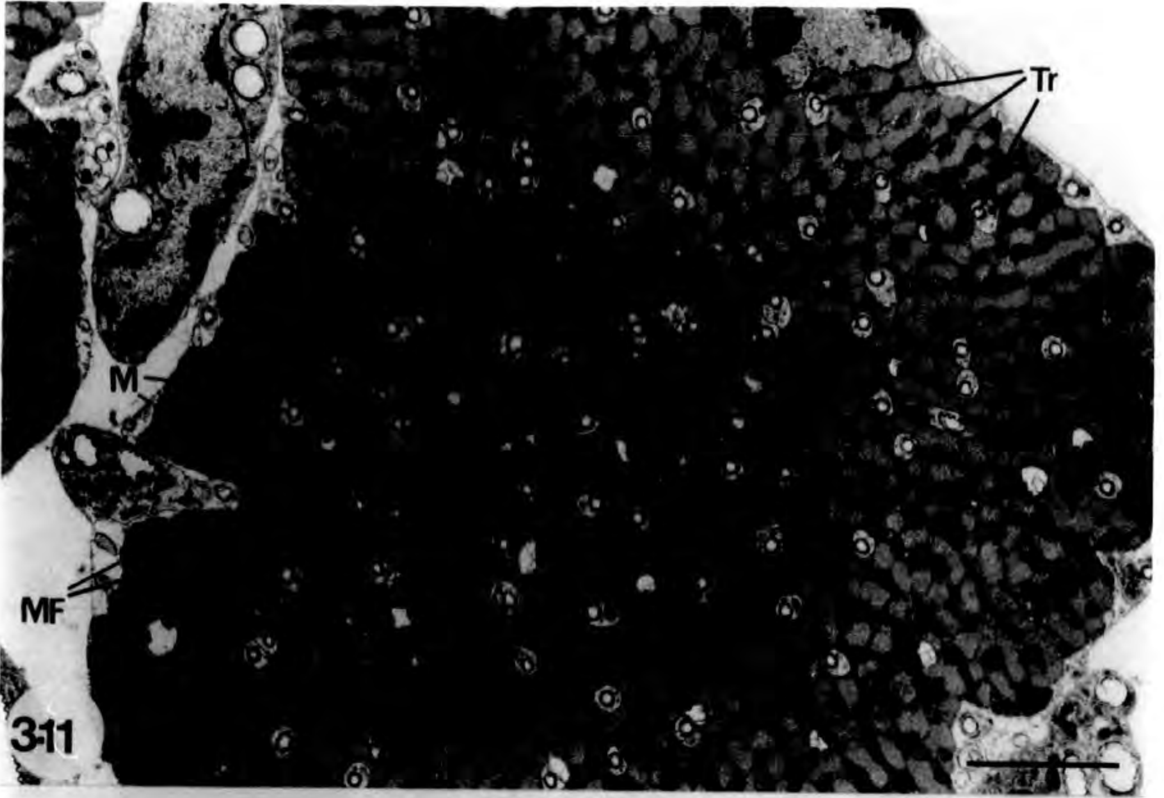

BM
CM
T
3.9

by the A-band (Fig. 3.10). There is a light H-band in the middle of the A-band, within which one can see a localized scattering of small granules. Similar granules have been observed in flight muscle of Tenebrio molitor (SMITH, 1960), Aeshna sp. (SMITH, 1961b) and Homorocoryphus nitidulus (ANSTEE, 1971). These granules are thought to represent the M-line, which is normally absent from insect synchronous flight muscle.

There was considerable variation in the shape of the myofibrillar profiles as seen in transverse sections. This was so for all ages studied (cf. Figs 3.11, 3.12 and 3.13). The shapes ranged from more-or-less circular to oblong in the first 4 days following the final ecdysis, whilst in 6-day old adults most myofibrils were polygonal (Fig. 3.9). This finding differs from that of BÜCHER (1965), who reported that the variation in myofibrillar profile shape disappears after the final ecdysis. By the 3rd day of adult life, there was a tendency for some of the peripheral myofibrils to be somewhat larger in size than the more centrally placed ones (Fig. 3.12). Careful examination of the arrangement of myofilaments in sections taken at various levels through a sarcomere shows that whereas thin and thick filaments are present in the A-band (Fig. 3.14), only thin filaments are present in the region corresponding to the I-band (Fig. 3.15). In addition, it is noticeable that the thick filaments appear elliptical in cross-sections in the H-band (Fig. 3.16), compared with more circular profiles presented in the region of the A-band. The thick filaments appear to have a less dense core especially those situated in the peripheral region of the myofibril (Fig. 3.14). The typical hexagonal arrangement with 6 actin filaments around each myosin is clearly seen in transverse sections of 5 and 6-day old locusts (Fig. 3.14).

Figure 3.11. Low power electron micrograph of a transverse section through a muscle fibre of a 1-day old adult locust showing the typical appearance of flight muscle at this age.
Note the presence of numerous tracheoles (Tr) and that cytoplasm of tracheoblast occupies a large area of the total muscle volume. Numerous small mitochondria (M) are seen in close association. MF: myofibril; N: nucleus.
Scale 4μm

Figure 3.12. An electron micrograph of a transverse section through a muscle fibre from a 3-day old adult locust. The peripheral region of four fibres is shown. Note the variation in size and shape between the peripheral (arrow-heads) and more-centrally placed myofibrils (MF). The latter tend to be smaller in size and more circular in shape. Mitochondrial shape ranges from oval to circular. N: nucleus; M: mitochondrion; Tr: tracheole; CM: cell membrane; BM: basement membrane; TR: trachea.
Scale 2.5μm


Tr
M
MF
3·11

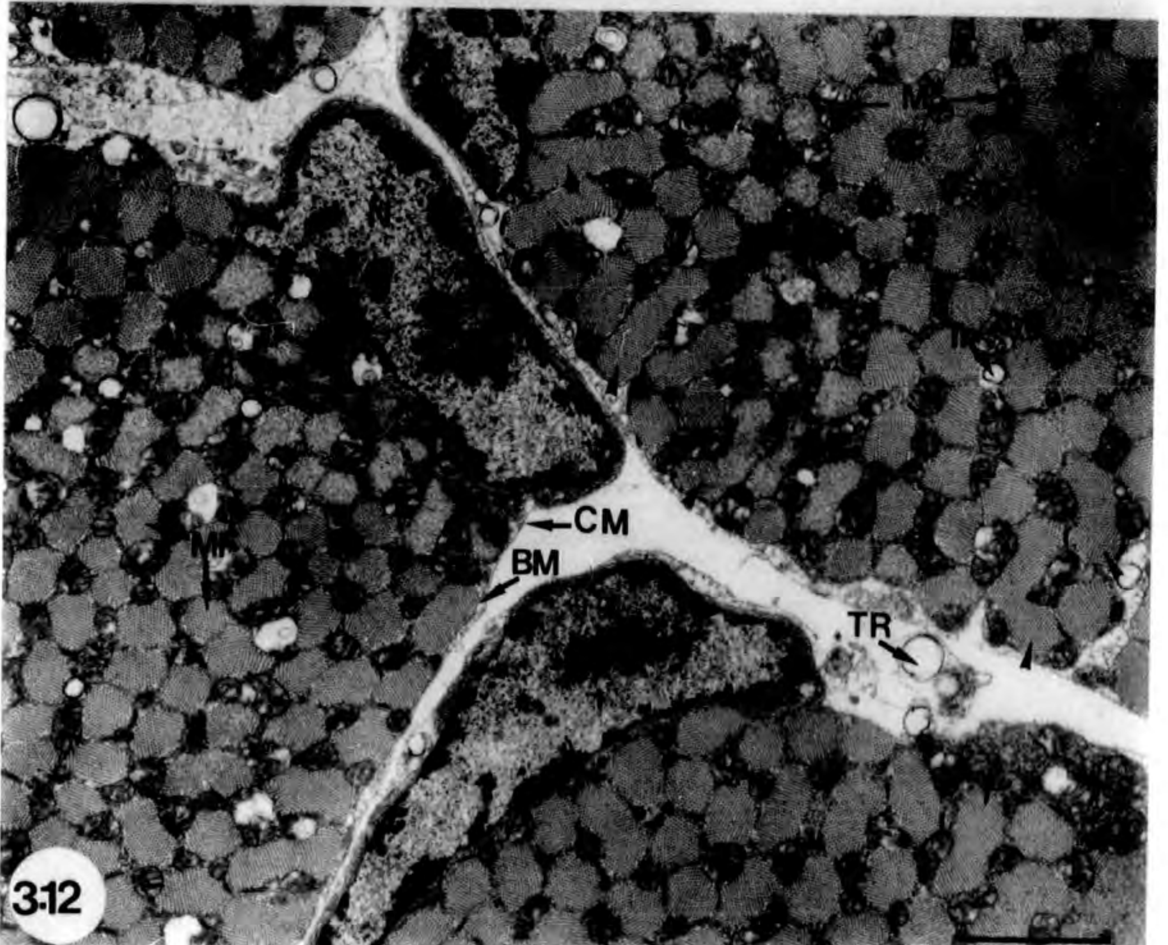

M
CM
BM
TR
3·12

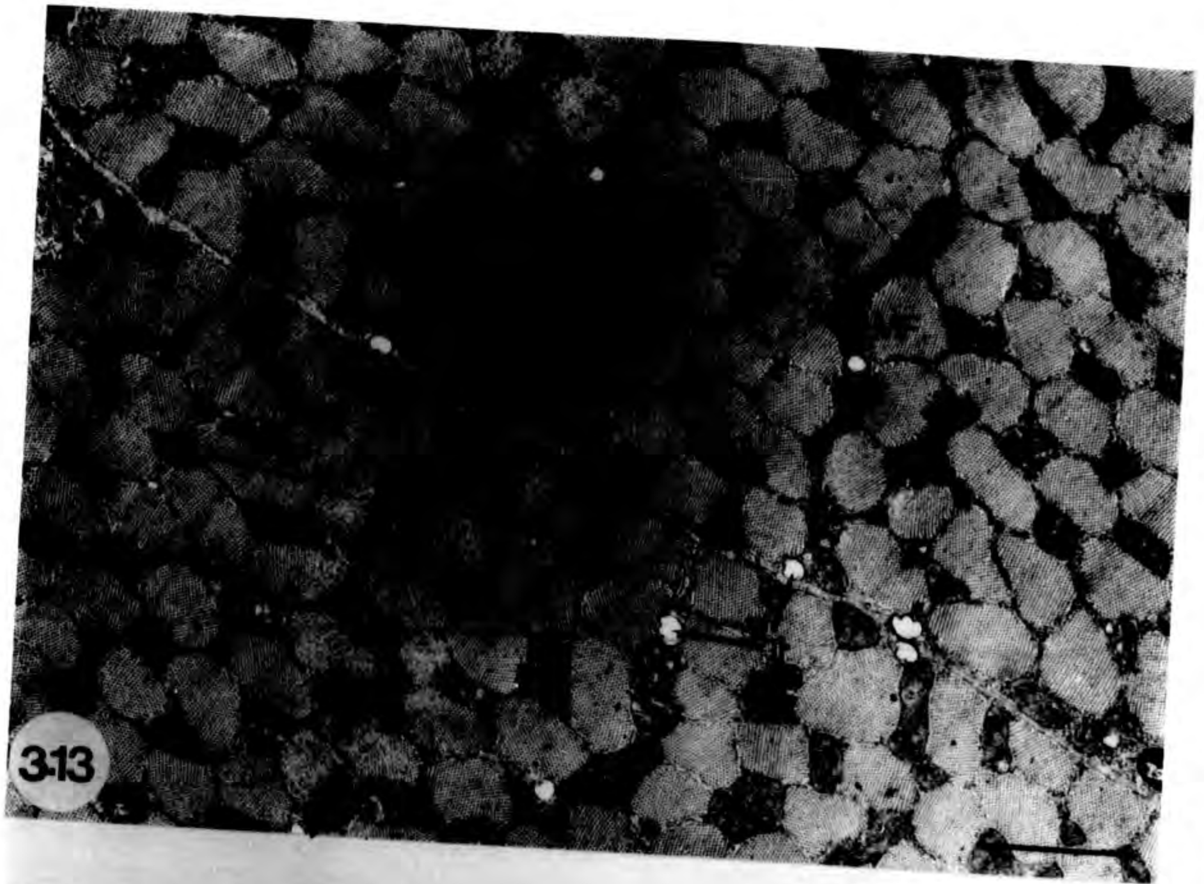
Figure 3.13. An electron micrograph of the peripheral region of two muscle fibres from a 5-day old adult locust. Note the enormous increase in size and change in shape of the mitochondria (M) and myofibrils (MF) compared with those seen in Figure 3.12. The tracheoles (Tr) occupy a small area of the total muscle volume (cf. Figures 3.11 and 3.12).
Scale 2.5μm

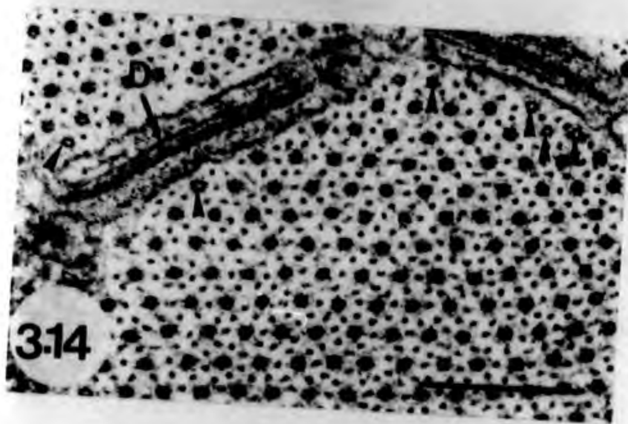
Figure 3.14. A transverse section through a myofibril of a 5-day old adult locust at the level of the A-band. Note the presence of actin (thin) and myosin (thick) filaments. The peripheral myosin filaments appear to have a less dense core (arrow-heads). D: dyad.
Scale 0.25μm

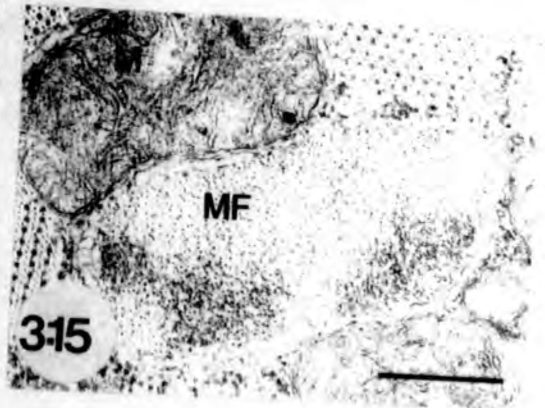
Figure 3.15. A transverse section through a myofibril (MF) of a 5-day old adult locust at the level of the I-band, showing only actin filaments at this level.
M: mitochondria.
Scale 0.5μm

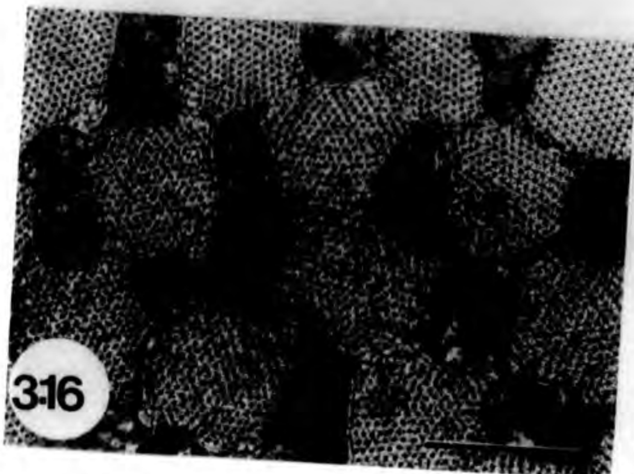
Figure 3.16. A transverse section through the muscle fibre of a 3-day old adult locust at the level of the H-band (H). Note that only thick filaments are present.
M: mitochondrion; MF: myofibril ; SR: sarcoplasmic reticulum.
Scale 1μm

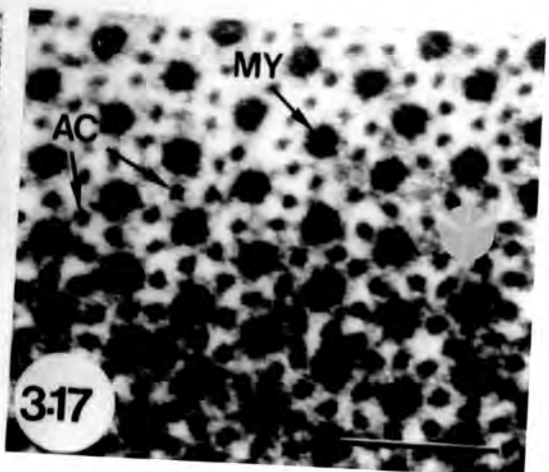
Figure 3.17. A high power electron micrograph of a transverse section through the A-band of a 1-day old adult locust. Note that occasionally more than 6 actin filaments (AC) surround each myosin filament (My) at this age.
Scale 0.1μm


3·13


D
3·14


MF
3·15


3·16


MY
AC
3·17

Each thin filament is equidistant between adjacent pairs of thick filaments. A similar arrangement has been described elsewhere in insect flight muscle, for example, in Periplaneta americana (SPIRO and HAGOPIAN, 1967) and Neoconocephalus (ELDER, 1971). However, occasionally 6-9 actin filaments were observed around each myosin filament. This latter condition was more common in muscles from adult Locusta aged between 1-4 days (Fig. 3.17) suggesting that the actin to myosin ratio may change with age (see Table 3.1). Nevertheless, the actin : myosin ratio of 3.33 ± 0.12 ($n = 7$) on day one was not significantly different from the value of 2.91 ± 0.14 ($n = 7$) in 6 day old adults. These ratios of approximately 3:1 are in good agreement with those encountered in other insect flight muscles which have been examined (SMITH, 1966a; ANSTEE, 1971; HUDDART, 1975). Whilst the deviation from a perfect 3:1 actin/myosin ratio may reflect developmental differences, an alternative explanation is possible. The state of the fixed muscle is known to affect the orbital number of thin filaments around each thick filament (AUBER, 1967a; HOYLE, 1967). Thus muscle fixed in the contracted state frequently exhibits a double overlap of thin filament in the centre of the A-band. Such overlappings have been described by HAGOPIAN (1966) and ELDER (1971).

In longitudinal sections of muscle from one day adult Locusta (Fig. 3.18) the cross-striations are poorly defined and the Z-bands are irregularly arranged. Similar observations have been noted in larval flight muscle of Manduca sexta (RHEUBEN and KAMMER, 1980). In Locusta, adjacent to the Z-band is a lighter region which represents the early appearance of the I-band (Fig. 3.18). During this stage the H-band is difficult to recognize. Similar observations have been reported in Leptinotarsa decemlineata (DE KORT, 1969). Gradually these features undergo developmental changes and the typical structural pattern of Locusta flight muscle is established by day 6 of adult life

Table 3.1. Effect of age on myofibril size, sarcomere length, myosin number and actin/myosin ratio of flight muscle of adult *Locusta migratoria*.

Age	myofibril size	sarcomere length (μm)	myosin number per myofibril	actin/myosin ratio
1	5.69 ± 0.20 (72)	2.84 ± 0.04 (82)	95 ± 3 (38)	3.28 ± 0.12 * (7)
2	8.49 ± 0.23 (92)	2.99 ± 0.01 (51)	141 ± 4 (36)	-
3	11.20 ± 0.39 (54)	3.06 ± 0.04 (30)	200 ± 6 (41)	2.97 ± 0.13 * (7)
4	18.29 ± 0.42 (42)	2.91 ± 0.22 (44)	248 ± 10 (12)	-
5	21.65 ± 0.65 (55)	3.18 ± 0.04 (69)	346 ± 9 (37)	-
6	21.54 ± 1.16 (35)	3.19 ± 0.02 (82)	391 ± 12 (19)	2.91 ± 0.14 * (7)

Determined by tracing myofibrillar profiles from electron micrographs on to standard paper. These were then cut-out, weighed and expressed in arbitrary units.

* Not significantly different

The figures in parentheses indicate the number of determinations.

<u>Figure 3.18.</u> A longitudinal section through the muscle fibre of a 1-day old adult locust.

Note the poorly developed banding pattern of the sarcomere and the irregular Z-bands (cf. Figure 3.10). Also noticeable is the lighter area adjacent to Z-bands which indicate the developing I-bands. The splitting (SP) of a single myofibril is also seen at this age.

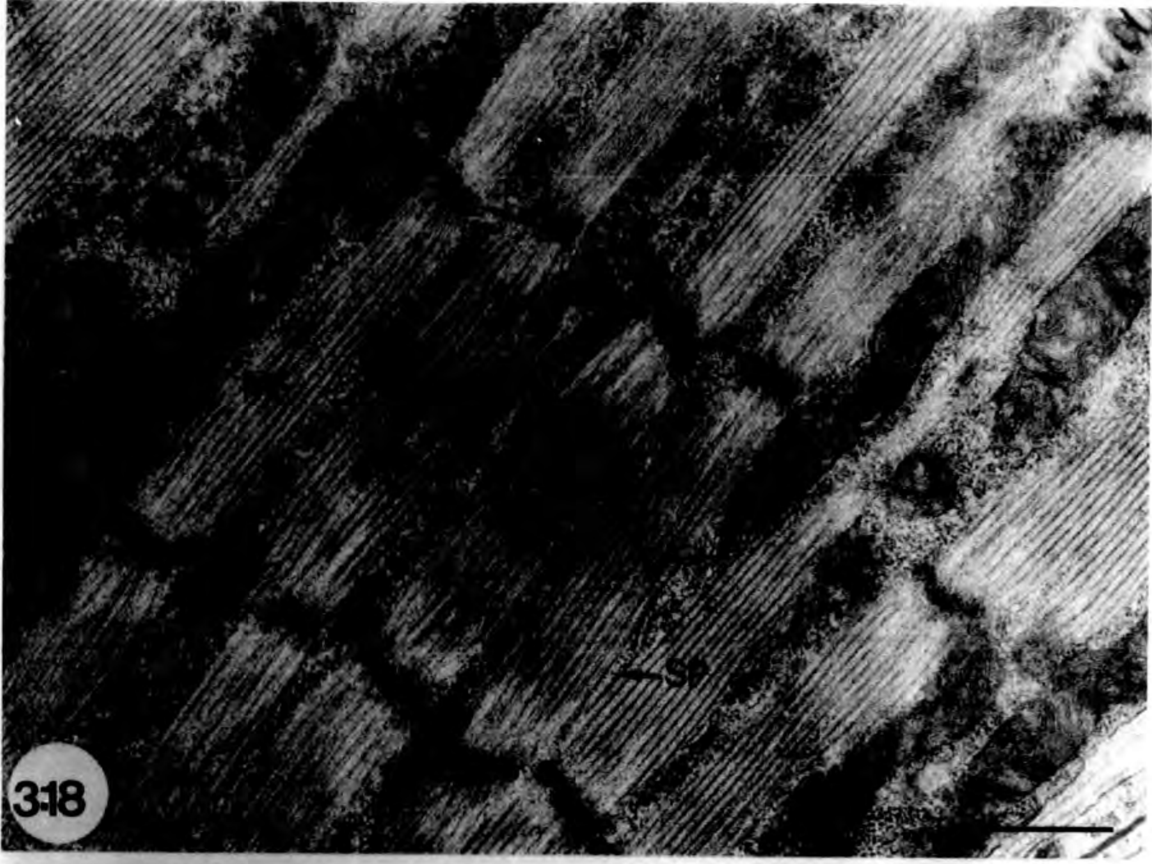
M: mitochondrion; MF myofibril; D: dyad; G: glycogen.

Scale 1μm

<u>Figure 3.19.</u> A longitudinal section through the muscle fibre of a 2-day old adult locust.

Note that the mitochondria (M) are more-or-less arranged in columns between the myofibrils (MF), the Z-bands are more regular and so are the I-bands. D: dyad; Cr: cristae.

Scale 0.5μm


3:18
3:19
D

(cf. Figs 3.18, 3.19, 3.20 and 3.10). Over the period studied, there was a significant increase in the average sarcomere length (see Table 3.1).

GOLDSPINK (1970) has shown that the number of myofibrils increases during muscle growth in the mouse. He obtained strong evidence to suggest that this proliferation of myofibrils is the result of longitudinal splitting of existing myofibrils once they attain a certain size. Examination of Figs 3.20, 3.21, 3.22 and 3.23, clearly indicates that this process may also be taking place in flight muscle of 1-4 day old adult Locusta. Furthermore, this process appears to be more-or-less complete by the 4th day of adult life. However, "myofibrillar splitting" was occasionally observed in 4-day old locusts where it appeared to be restricted to the more peripheral myofibrils. This is shown in Figure 3.24 where a dyadic junction is present in the middle of a myofibril. On the basis of this observation one might suggest that most "myofibrillar splitting" takes place in early adult life and affects myofibrils throughout the muscle, but that this process seems to continue in the larger peripheral myofibrils.

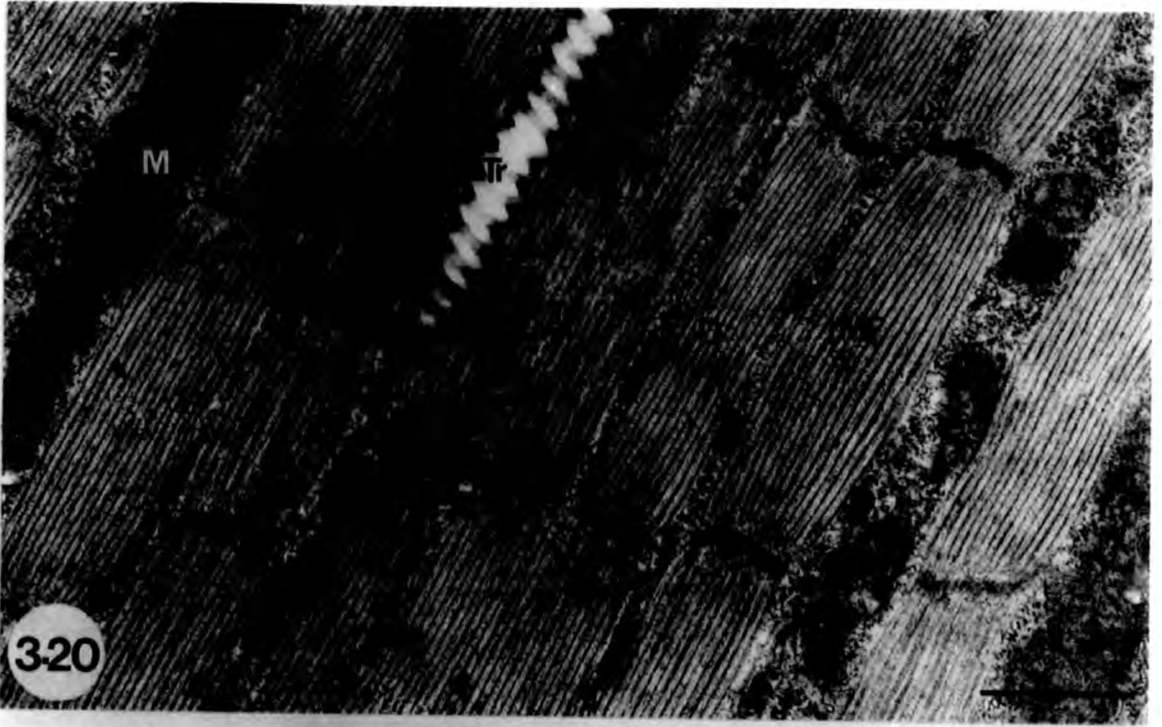
Examination of Figs 3.5, 3.6, 3.7 and 3.8 indicates that following the increase in the numbers of myofibrils, there is an increase in the number of actin and myosin filaments per myofibril as the latter increase in size. This observation is confirmed by counting the number of myosin filaments per myofibril, the actin to myosin ratio and by measuring myofibrillar profile size at different ages (Fig. 3.25). There was a 4-fold increase in myofibril size and the number of myosin filaments per myofibril over the first 6 days of adult life. This indicates a close relationship between the number of myosin filaments per myofibril and myofibril size. The proportion of total muscle volume occupied by the myofibrils increased with age (Table 3.2);

Figure 3.20. A longitudinal section through a muscle fibre of a 3-day old adult locust.
Note the indication of splitting (SP) of a single myofibril (MF). The normal banding pattern is clearly seen. SR: sarcoplasmic reticulum; M: mitochondria; D: dyad; Tr: tracheole; G: glycogen; Z: Z-band.
Scale 1μm

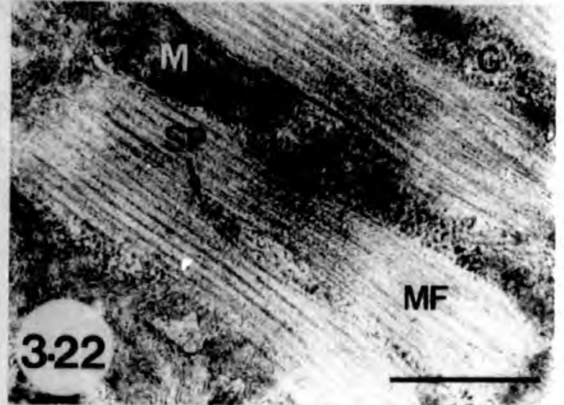
Figure 3.21. A longitudinal section through a muscle fibre of a 1-day old adult locust.
Similar myofibrillar splitting (SP) as indicated in Figure 3.20 is apparent. Note the position occupied by the dyad (D) and its oblique arrangement in relation to the myofibril (MF) (see inset). SR: sarcoplasmic reticulum.
Scale 0.5μm

Figure 3.22. A longitudinal section through a muscle fibre of a 1-day old adult locust.
Note once again that myofibrillar splitting (SP) is taking place in single myofibril. M: mitochondrion; MF: myofibril; G:glycogen.
Scale 0.8μm

Figure 3.23. A longitudinal section, through a muscle fibre of a 4-day old locust, showing that the myofibril splitting is taking place at this age.
MF: myofibril; M: mitochondria; SP: splitting; Z: Z-bands; Tr: tracheole.
Scale 1.5μm


M
Tr
3·20

D
MF
3·21


M
C
MF
3·22

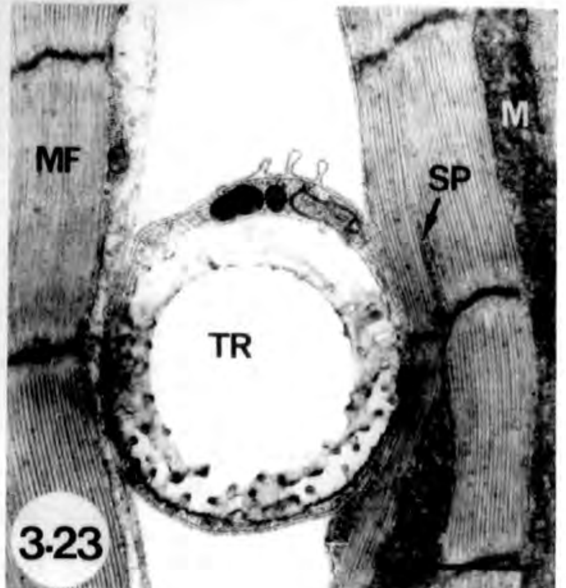

MF
M
SP
TR
3·23

Figure 3.24. A transverse section through a muscle fibre of a 4-day old adult locust.

Note the presence of dyadic junctions in the middle of two peripheral myofibrils.

MF: myofibril; M: mitochondrion; D: dyad; CM: cell membrane; T: T-system.

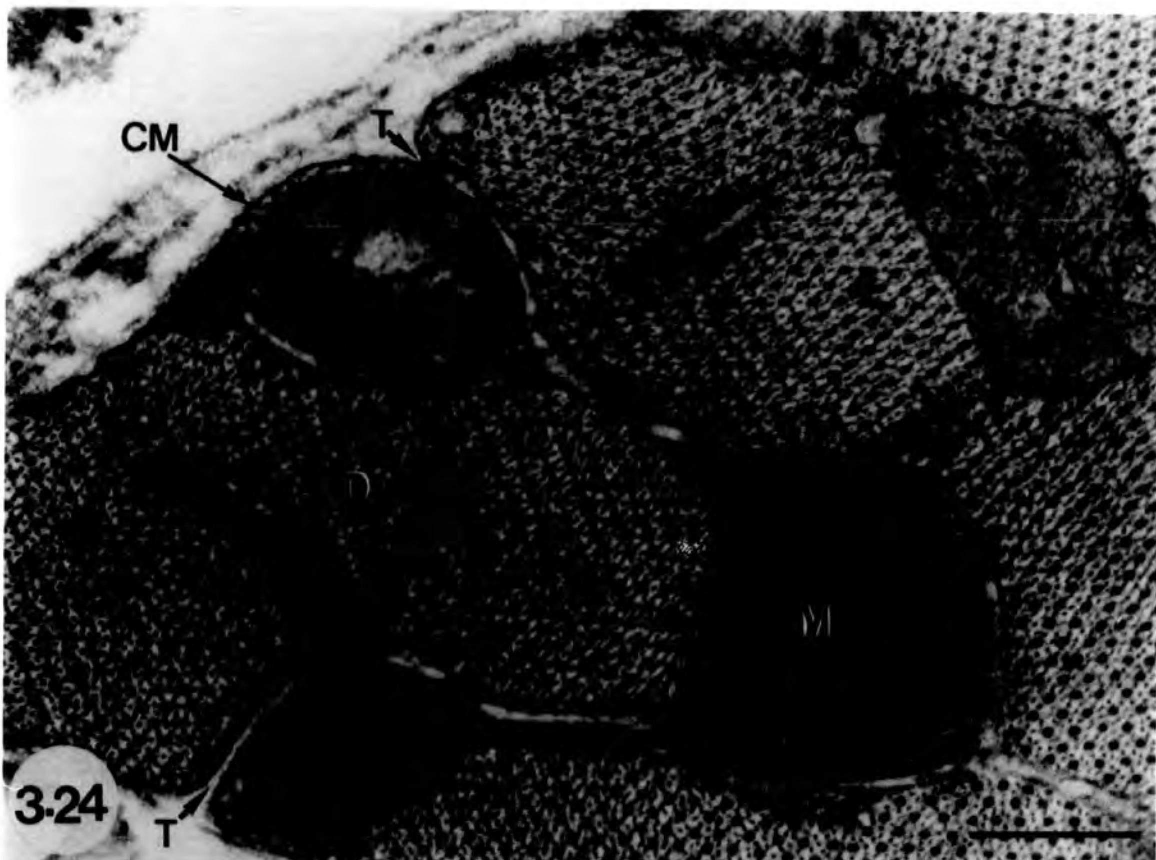
Scale 0.4μm

Figure 3.26. A longitudinal section through a muscle fibre of a 2-day old adult locust.

Note the columns of mitochondria of different sizes between the myofibrils.

M: mitochondrion; MF: myofibril; Z: Z-band; D: dyad; Tr: tracheole.

Scale 2.5μm


CM
T
3·24
T

D
M
3·26

Figure 3.25. Effect of age on myofibril size and the number of myosin filaments per myofibril (data taken from Table 3.1.).

O myofibril size

Δ myosin number

The figures in parentheses indicate the number of myofibrils measured or the number of myofibrils from which the myosin filaments were counted.

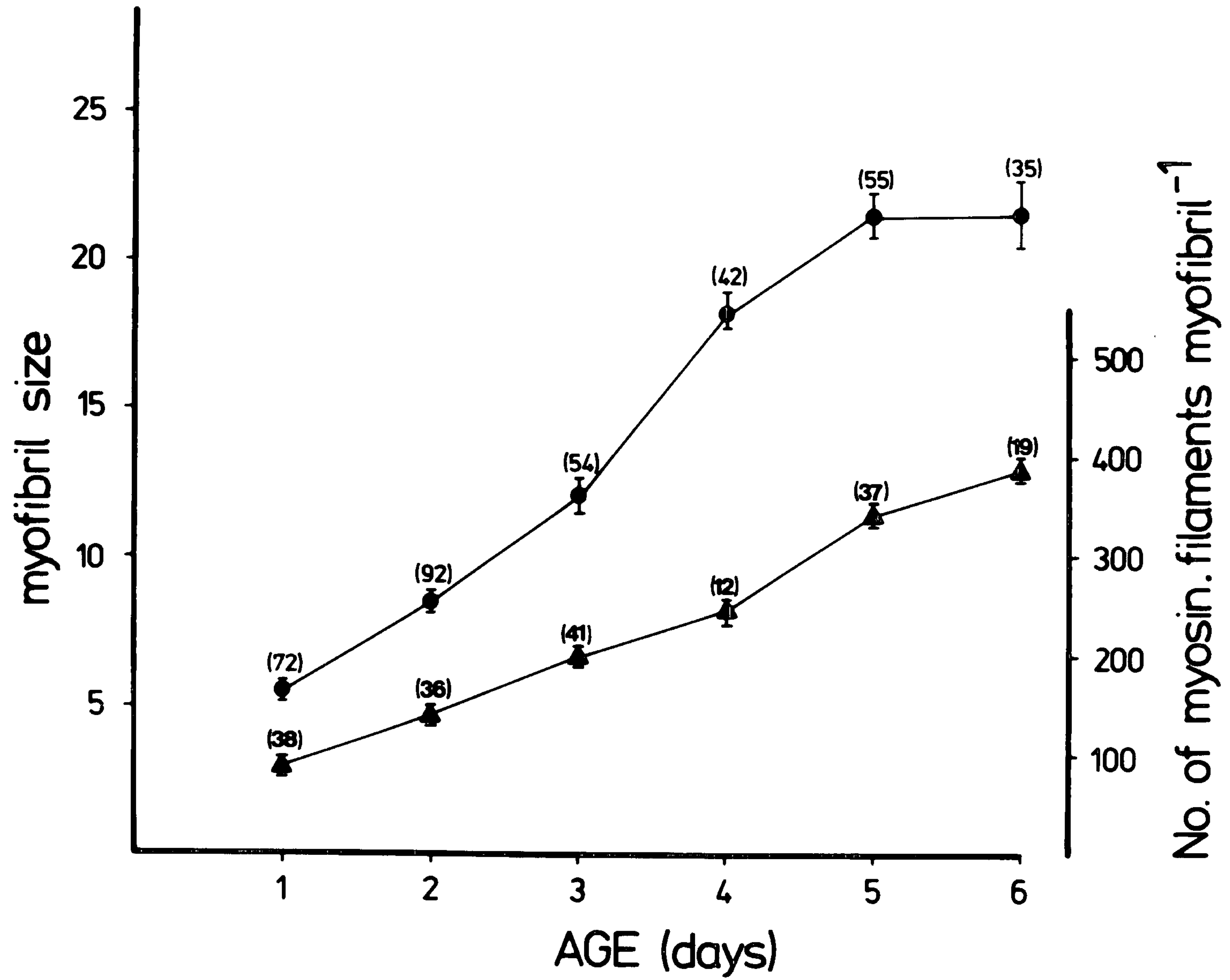

myofibril size
25
20
15
10
5
No. of myosin. filaments myofibril^{-1}
500
400
300
200
100
AGE (days)
1
2
3
4
5
6
(72)
(92)
(54)
(42)
(55)
(35)
(38)
(36)
(41)
(12)
(37)
(19)

Table 3.2. The effect of age on the relative proportion of the flight muscle components of *Locusta migratoria*

Age in days	myofibril	mitochondria	sarcoplasmic reticulum	tracheoles
1	52.26 ± 1.51* (5)	24.66 ± 1.38 (5)	15.98 ± 0.93 (5)	7.15 ± 1.43 (5)
2	59.54 ± 2.13 (6)	22.87 ± 2.25 (6)	13.71 ± 1.22 (6)	3.50 ± 1.01 (6)
3	64.76 ± 1.66 (6)	22.04 ± 1.76 (6)	12.36 ± 0.78 (6)	1.53 ± 0.04 (6)
5	61.55 ± 1.43 (8)	26.40 ± 1.46 (8)	11.14 ± 0.70 (8)	1.46 ± 0.92 (8)
6	59.99 ± 2.31* (7)	28.52 ± 1.70 (7)	10.83 ± 0.94 (7)	0.96 ± 0.61 (7)

The data represent the mean percentage ± S.E.M. of total muscle volume occupied by each component and were obtained by weighing component profiles traced from E.M. graphs (see Materials and Methods).

The figures in parentheses indicate the number of determinations.

*$p > 0.001$

being 52.26 ± 1.51% and 59.66 ± 2.31% ($p > 0.001$) in 1-day old and 6-day old adults respectively.

Mitochondria

Mitochondria are abundant in muscle fibres at all ages studied and are packed between the myofibrils (Figs 3.5, 3.6, 3.7 and 3.8). The mitochondrial arrangement is clearly shown in longitudinal sections where they appear as columns of different sizes (Figs 3.26 and 3.27). As reported for Homorocoryphus (ANSTEE, 1971) there is no obvious alignment with the striation pattern of the myofibrils. This is in contrast to the finding of SMITH (1962) who reported that in the fibrillar flight muscle of the wasp, Polistes sp., the mitochondria are aligned with each half sarcomere, i.e. between the Z- and M-bands. MICHEJDA (1964) reported that, in Hyalophora cecropia flight muscle, there is a single row of mitochondria situated between adjacent myofibrils in the proportion of three mitochondria per sarcomere.

In the present study, the main changes observed in these organelles, associated with age, involved increased size, number and density of cristae. Transverse sections through flight muscles of 1-day old adult locusts are characterized by the presence of numerous small elliptical or circular shaped mitochondria packed between the myofibrils (Figs 3.5 and 3.11). They contain fewer cristae and their matrices are relatively large (Fig. 3.28). In longitudinal sections (Fig. 3.29) the mitochondria are seen between the myofibrils and their non-overlapping arrangement in columns is incomplete. It can be seen also that, at this stage, there is more than one mitochondrion between adjacent myofibrils. By the 3rd day of adult life, the mitochondria are arranged in columns with little or no indications of overlapping (Fig. 3.27). However, examination of transverse sections from this stage indicate that aggregated mitochondria occur in the peripheral region of the fibres suggesting that the development of single

Figure 3.27. A longitudinal section through a muscle fibre of a 5-day old adult locust.
More than two mitochondria (M) are present per sarcomere. Note also that the sarcoplasmic reticulum (SR) at this age separates each myofibril (MF) completely (two sheets of it in some cases). Cr: cristae; D: dyad; G: glycogen; Tr: tracheole.
Scale 1μm

Figure 3.28. A transverse section through a muscle fibre of a 1-day old adult locust.
Note that the mitochondria (M) contain few cristae and some of the mitochondria appear to be in close contact with the adjacent ones. Note also the close association between a dyad (D) and a triad (TD) and a mitochondria. MF: myofibril; T: T-system; Tr: tracheole.
Scale 0.4μm

D
SR
G
MF
Tr
3.27

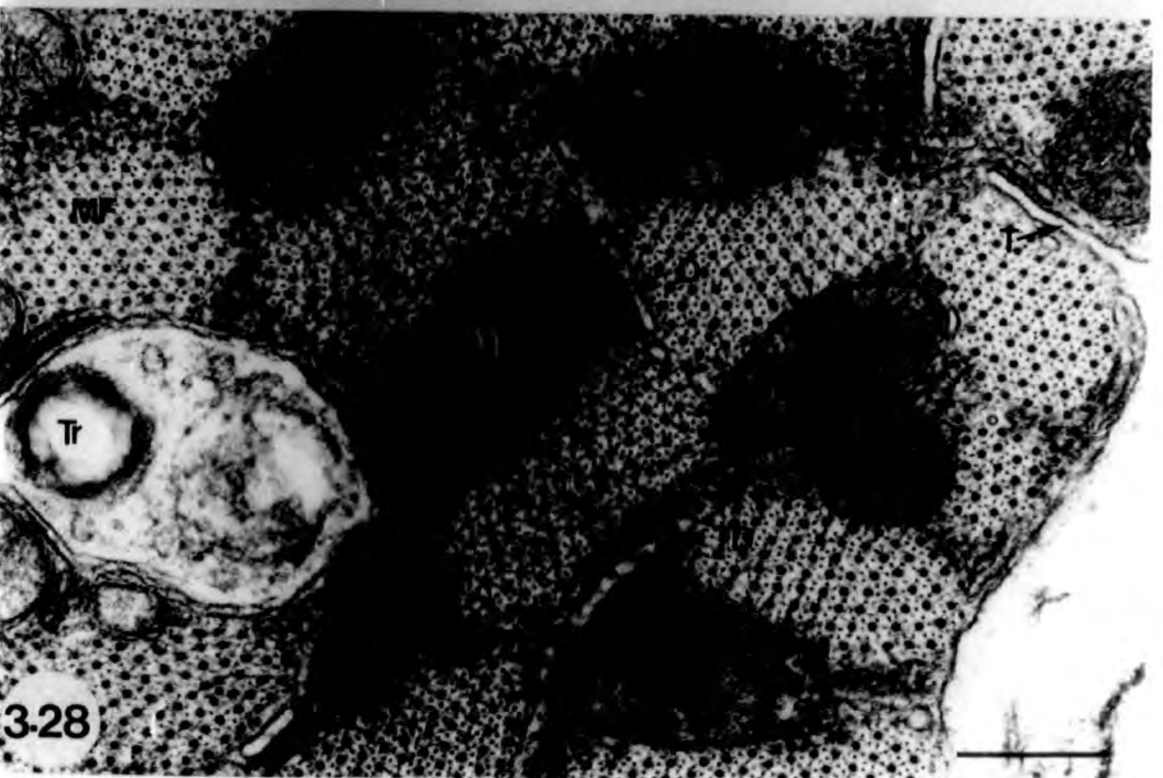
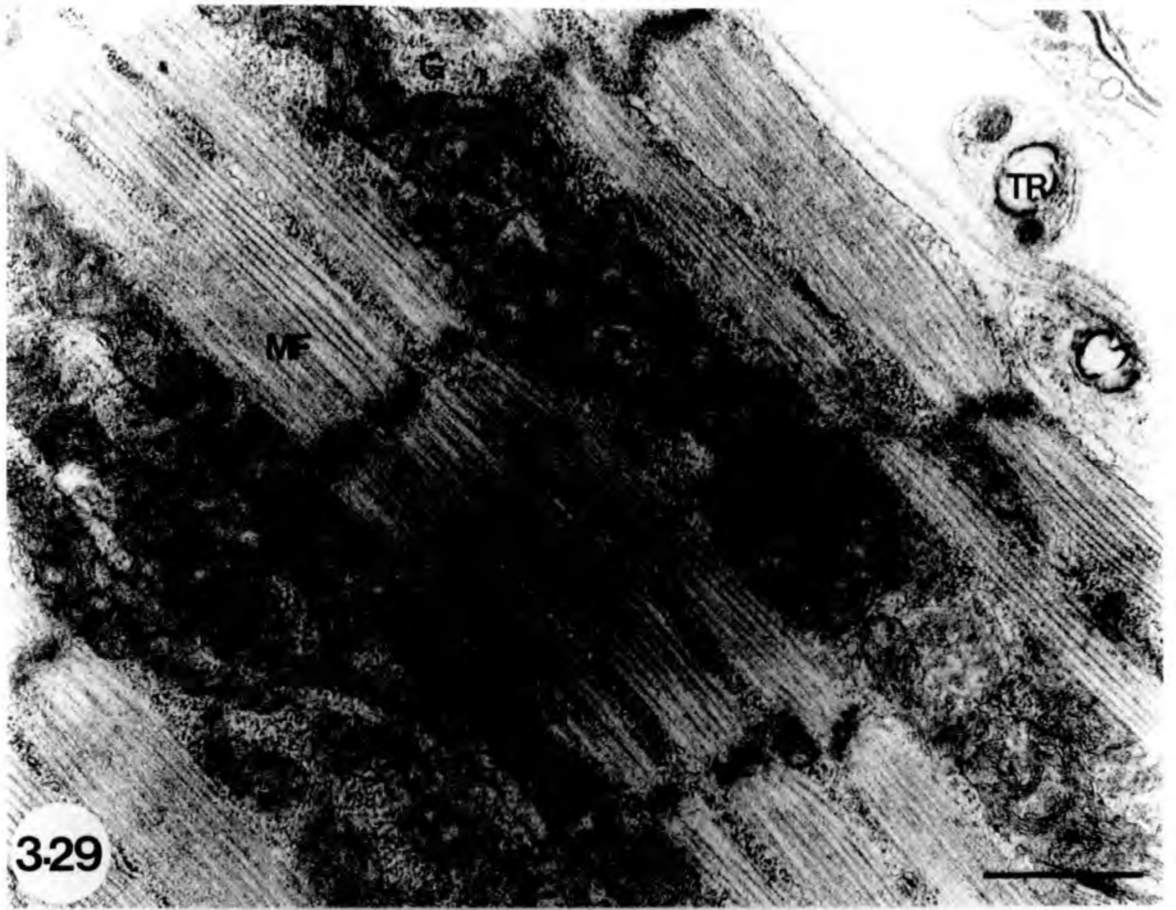
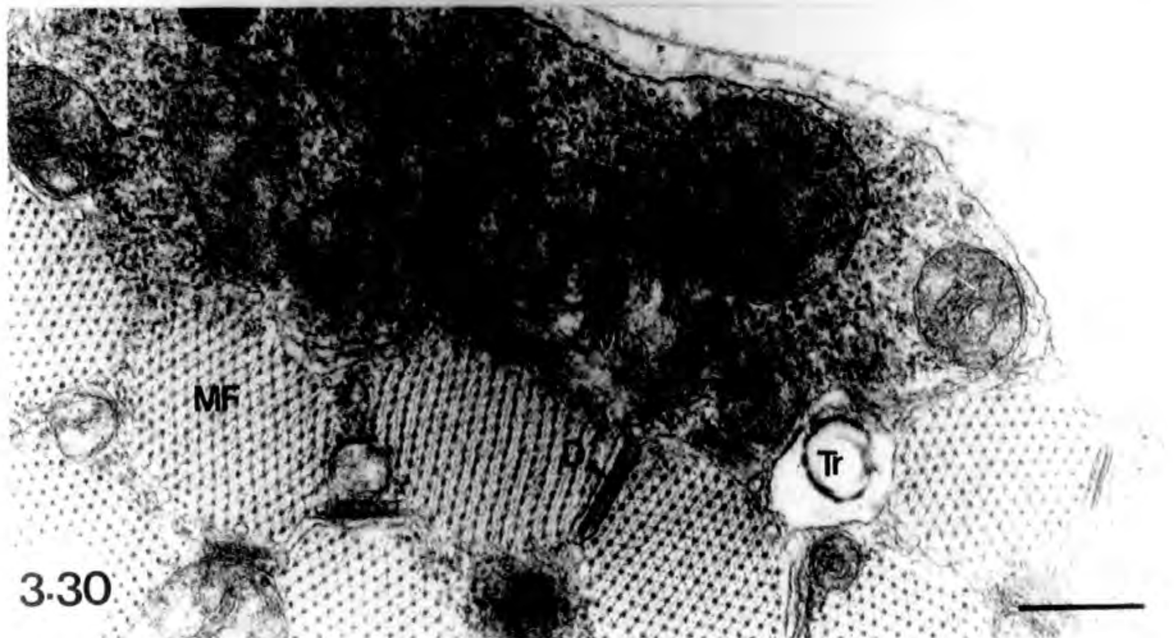


Figure 3.29. A longitudinal section through a muscle fibre of a 1-day old adult locust.
At this early stage of development incomplete columns of mitochondria (M) are clearly seen between the myofibrils (MF). Note the presence of dyads (D) between two adjacent myofibrils. SR: sarcoplasmic reticulum; SP: splitting; T: tracheole; G: glycogen.
Scale 1μm

Figure 3.30. A transverse section through a muscle fibre of a 3-day old adult locust.
Note the aggregation of mitochondria (M) in the peripheral region of the fibre. D: dyad; MF: myofibril; Tr: tracheole.
Scale 0.5μm


G
TR
MF
3.29


MF
D
Tr
3.30

mitochondrial column between myofibrils is not complete at this stage (Fig. 3.30). This phenomenon disappears as growth proceeds and by the 5th and 6th day of adult life the mitochondria exhibit marked changes in their internal structure (Fig. 3.10). At this stage single mitochondrial columns can be seen clearly (Fig. 3.10) and the mitochondria become so compressed between adjacent myofibrils that they appear polygonal in transverse sections (Fig. 3.9). In addition the number of mitochondria per sarcomere length changed with age; up to three mitochondria per sarcomere length can be seen in 1-3 day old adults (Fig. 3.27 and 3.31), but by the 5th day of adult life single mitochondria are observed extending over the length of two or more sarcomeres (Fig. 3.32). This elongation is continued, such that in 6-day old locusts a single mitochondrion may extend the length of up to 5 sarcomeres (Fig. 3.10).

The results shown in Table 3.3 summarize the changes in mitochondrial size with age. The mean mitochondrial diameter in situ showed little change during the first 4 days of adult life. However, by day 6 the mean diameter was approximately double that of newly ecdysed adults. A similar result was obtained when mitochondrial mean size was estimated by tracing their profiles on to standard paper, followed by their being cut out and weighed. The overall increase in size was even more apparent by this method. Again little change was noted over 1-3 days of adult life, but by the 4th day an increase in size was apparent whilst by day 6 there had been a 4.4 fold increase in mitochondrial size compared with newly ecdysed insects. Figure 3.33 shows the variation in mitochondrial size at various ages. The trend towards increasing mitochondrial size with age is again clearly visible.

The proportion of total muscle volume occupied by mitochondria changed little over the period studied, being ca. 24.7% on the first day and 28.5% on the 6th day of adult life (see Table 3.3). Whilst a

Figure 3.31. A longitudinal section through a muscle fibre of a 3-day old adult locust.

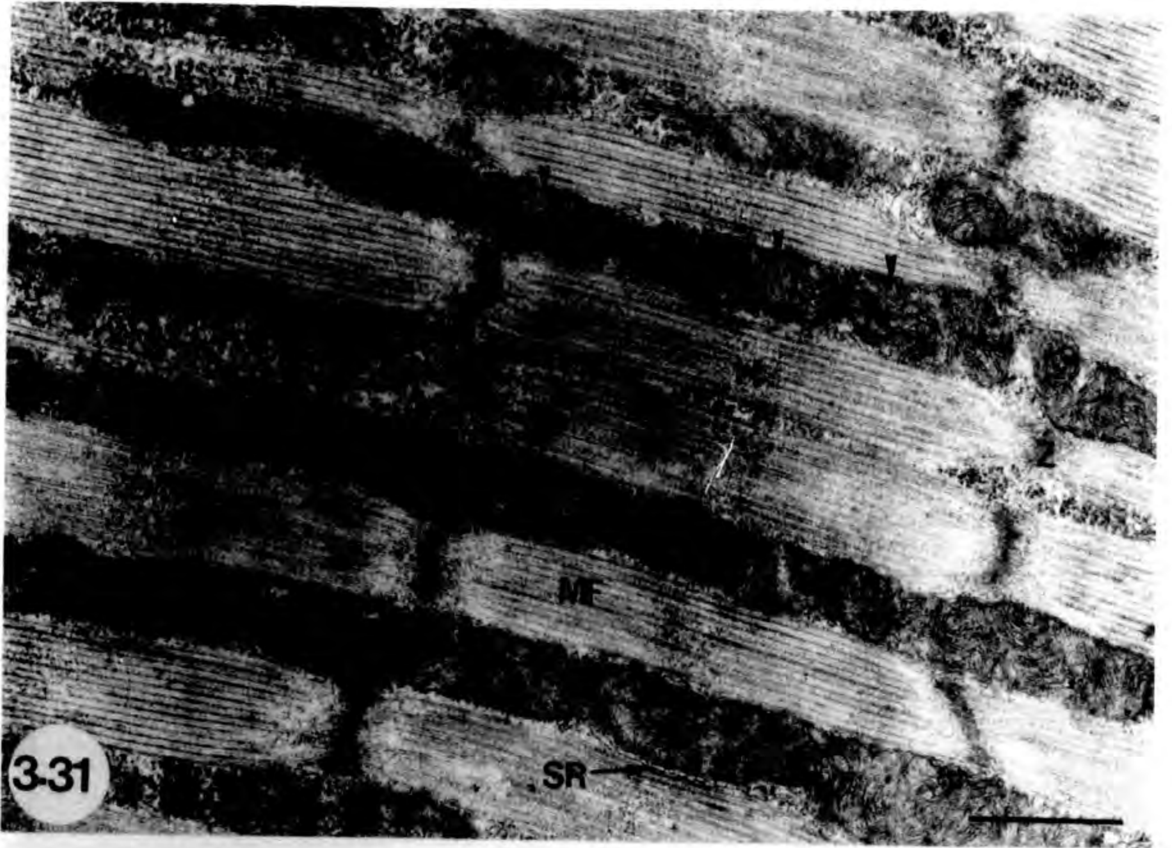
The number of mitochondria (M) per sarcomere varies and it can be seen that there are up to three mitochondria per sarcomere length (arrow-heads) at this age. Z: Z-band; H: H-band; MF: myofibrils; SR: sarcoplasmic reticulum; G: glycogen.

Scale 1μm

Figure 3.32. A longitudinal section through a muscle fibre of a 5-day old adult locust.

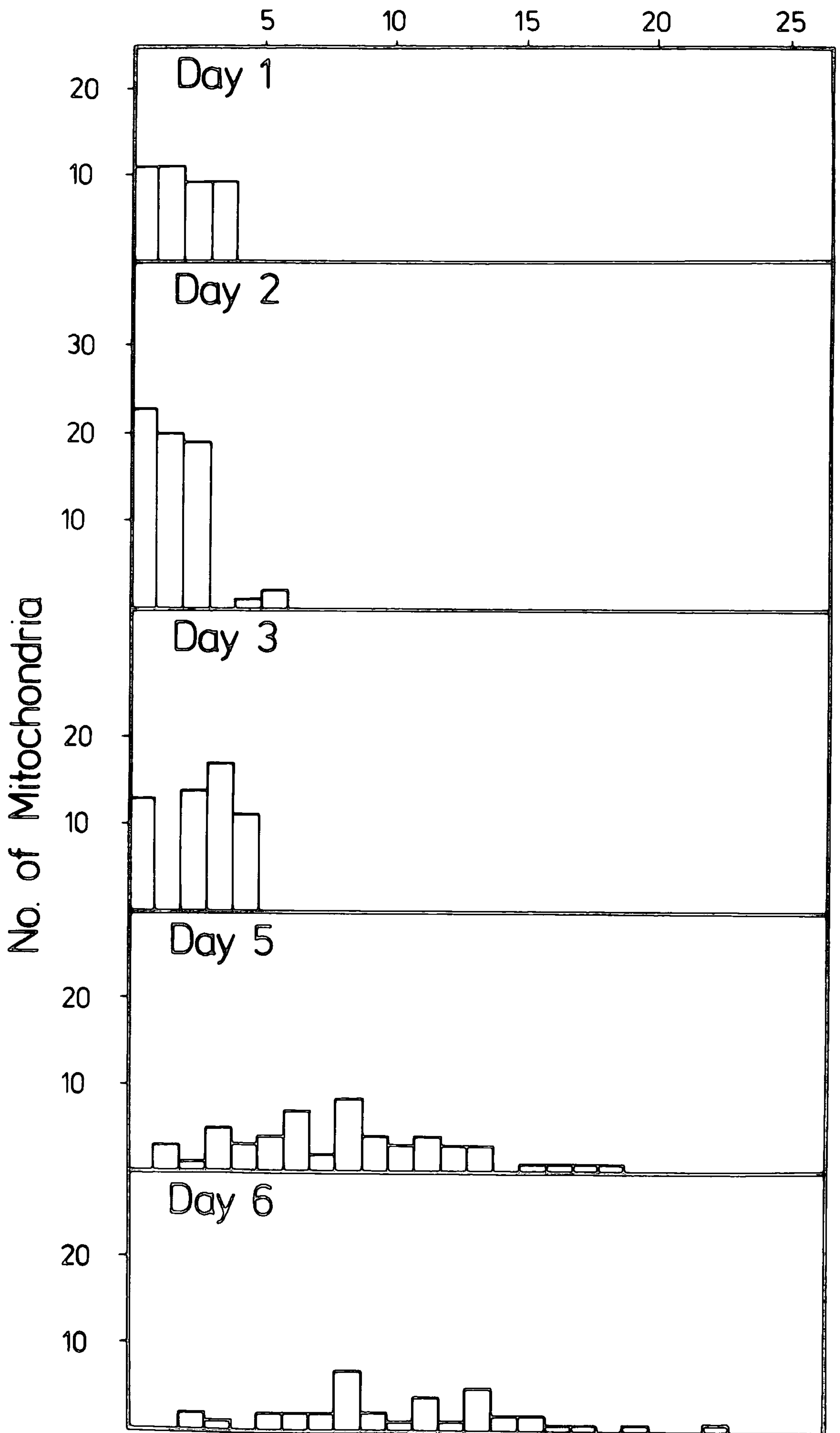
Note that a single mitochondrion (M) extends for more than two sarcomere lengths whilst others extend less than one sarcomere length. Note also the dyad (D) is midway between the I- and H-bands. Sarcoplasmic reticulum (SR) is also seen between the mitochondria (M) and the myofibril (MF). G: glycogen; H: H-band; Z: Z-band.

Scale 1μm


MF
SR
3-31

MF
SR
D
H
D
3-32

Figure 3.33. Histograms showing size distribution of flight muscle mitochondria from *Locusta migratoria* at different ages *in situ*. The data were obtained by tracing the mitochondria from electron micrographs. These were then cut out and weighed. The results were expressed in arbitrary units.



Mitochondrial Size
5
10
15
20
25
Day 1
20
10
Day 2
30
20
10
Day 3
20
10
Day 5
20
10
Day 6
20
10
No. of Mitochondria

Table 3.3. Effect of age on mitochondrial size *in situ* and *in vitro*

Age in days	(1) Mitochondrial size (in arbitrary units)		(2) Mitochondrial diameter *in situ*		
	in situ	*in vitro*	maximum (μm)	minimum (μm)	mean
1	2.62 ± 0.17 (42)	13.21 ± 0.45* (223)	0.50 ± 0.02 (80)	0.36 ± 0.01 (80)	0.43 ± 0.01 (80)
2	2.28 ± 0.15 (71)	20.03 ± 0.51* (186)	0.49 ± 0.02 (86)	0.34 ± 0.01 (86)	0.42 ± 0.01 (86)
3	2.96 ± 0.18 (80)		0.57 ± 0.01 (87)	0.43 ± 0.01 (87)	0.51 ± 0.01 (87)
4	6.23 ± 0.42 (50)	21.32 ± 1.08 (103)	0.55 ± 0.02 (38)	0.36 ± 0.01 (38)	0.46 ± 0.02 (38)
5	8.96 ± 0.55 (55)	18.15 ± 0.44 (255)	1.05 ± 0.01 (59)	0.68 ± 0.01 (59)	0.86 ± 0.02 (59)
6	11.50 ± 0.83 (36)	21.44 ± 1.25 (136)	1.22 ± 0.03 (95)	0.75 ± 0.05 (95)	0.99 ± 0.02 (95)

The results are expressed as mean ± S.E.M. The figures in parentheses indicate the number of determinations.

*$p < 0.001$

(1) Determined by tracing myofibrillar profiles from E.M. graphs on to standard paper etc. (see Materials and Methods)

(2) Determined by direct measurement from E.M. graphs.

slight decrease in muscle mitochondrial volume was noted in 2 and 3-day old adults, this change was not statistically significant.

Isolated Mitochondria

Mitochondria extracted from flight muscle of 1-6 day old adult *Locusta* were examined by electron microscopy. Sections through the various mitochondrial pellets revealed that the preparations contained few contaminating myofibrils (Fig. 3.34, 3.35, 3.36 and 3.37). The general appearance of extracted mitochondria was somewhat different from those fixed *in situ*. Their shape *in vitro* was less irregular and they appeared approximately spherical. Similar observations have been reported for mitochondria isolated from *Calliphora erythrocephala* flight muscle (TRIBE and ASHHURST, 1972). However, the presence of intact outer and inner membranes (Fig. 3.38, 3.39, 3.40 and 3.41) indicate that the method of extraction used, in this study, gives a relatively good preparation with little indication of mechanical damage. Two distinct mitochondrial configurations were recognizable; one exhibiting a condensed structure with an electron dense matrix and relatively undilated cristae (reduced intra-cristal space). This mitochondrial type (I) represents only a minute proportion of the total (Figs 3.36, 3.37, 3.42, 3.43, 3.44 and 3.45) and such mitochondria are much smaller than the type II mitochondria which exhibit dilated cristae and represent the majority of the mitochondrial population.

As with the *in situ* mitochondria, isolated mitochondria showed a marked age-dependent change in fine structure, particularly in the increased number of cristae present in mitochondria from older insects (cf. Figs 3.34, 3.35, 3.36 and 3.37). Table 3.3 shows the mean size of isolated mitochondria from 1-6 day of adult life. With the exception of 1-day old adults ($p < 0.001$), no significant differences were observed when compared with that of 6-day old adults. This is further supported when the distribution of mitochondrial size with age is examined (Fig. 3.46).

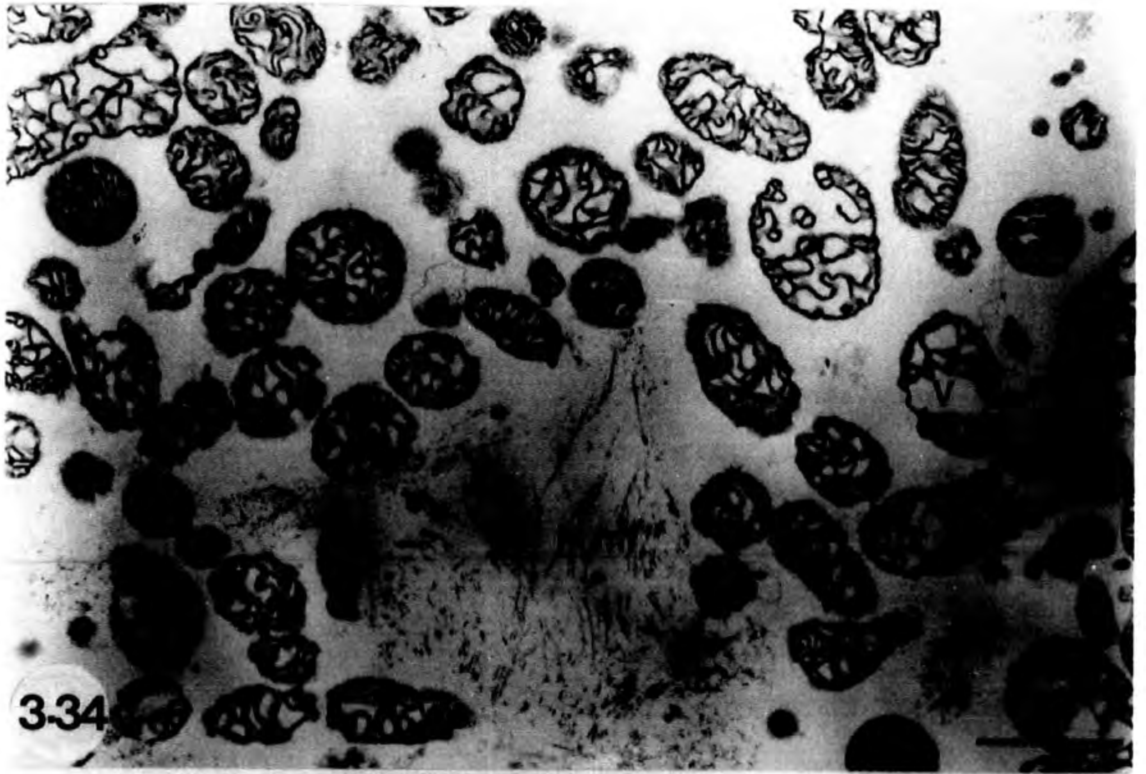
Figure 3.34. Electron micrographs showing mitochondria isolated from the flight muscle of 1-day old adult locusts.

Note the fragments of myofibril (Fr.mf) in the background, some mitochondria are vacuolated (V). The majority of the mitochondria are intact and contain few cristae.

Scale 1.5µm

Figure 3.35. Electron micrograph showing mitochondria isolated from the flight muscle of 2-day old adult locusts. Vacuolated (V) and fractured (Fr) mitochondria can be seen. The majority of the mitochondria are intact.

Scale 1.5µm


V
3·34

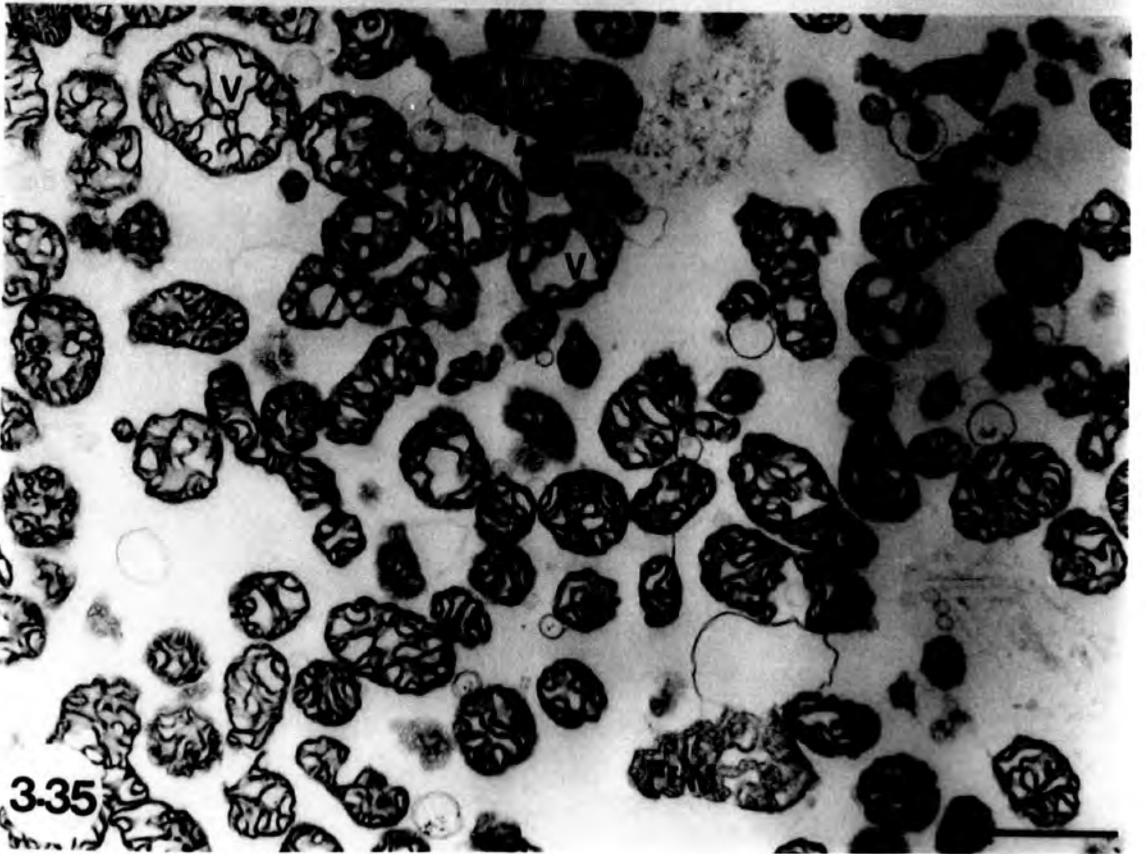

V
V
3·35

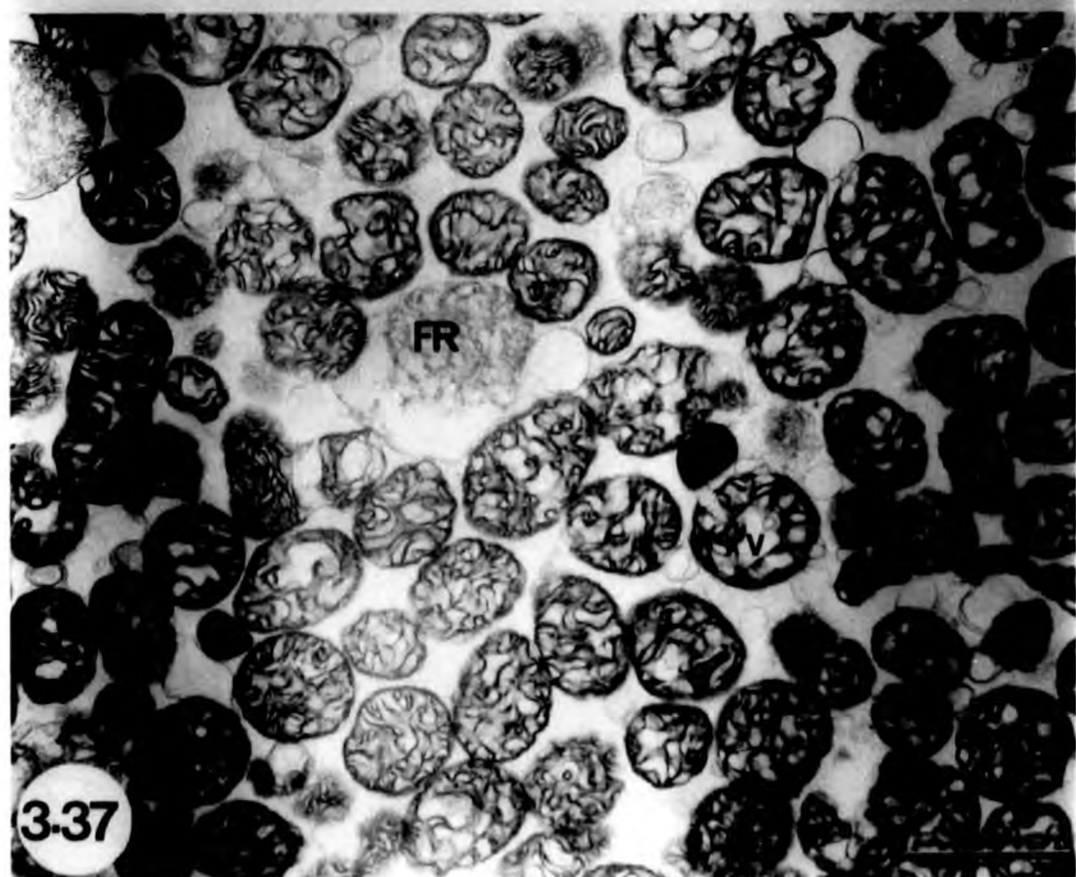
Figure 3.36. Electron micrograph showing mitochondria isolated from the flight muscle of 5-day old adult locusts. The majority of mitochondria are intact and contain more cristal membrane (cf. Figures 3.34 and 3.35). V: vacuolated mitochondria; CF.r: cristal fragments.
Scale 1.5μm

Figure 3.37. Electron micrograph showing mitochondria isolated from the flight muscle of 6-day old adult locusts. The appearance of the mitochondria of this age is similar to that shown in Figure 3.36. FR: fragmented mitochondria.
Scale 1.5μm

3-36

V

C-Fr

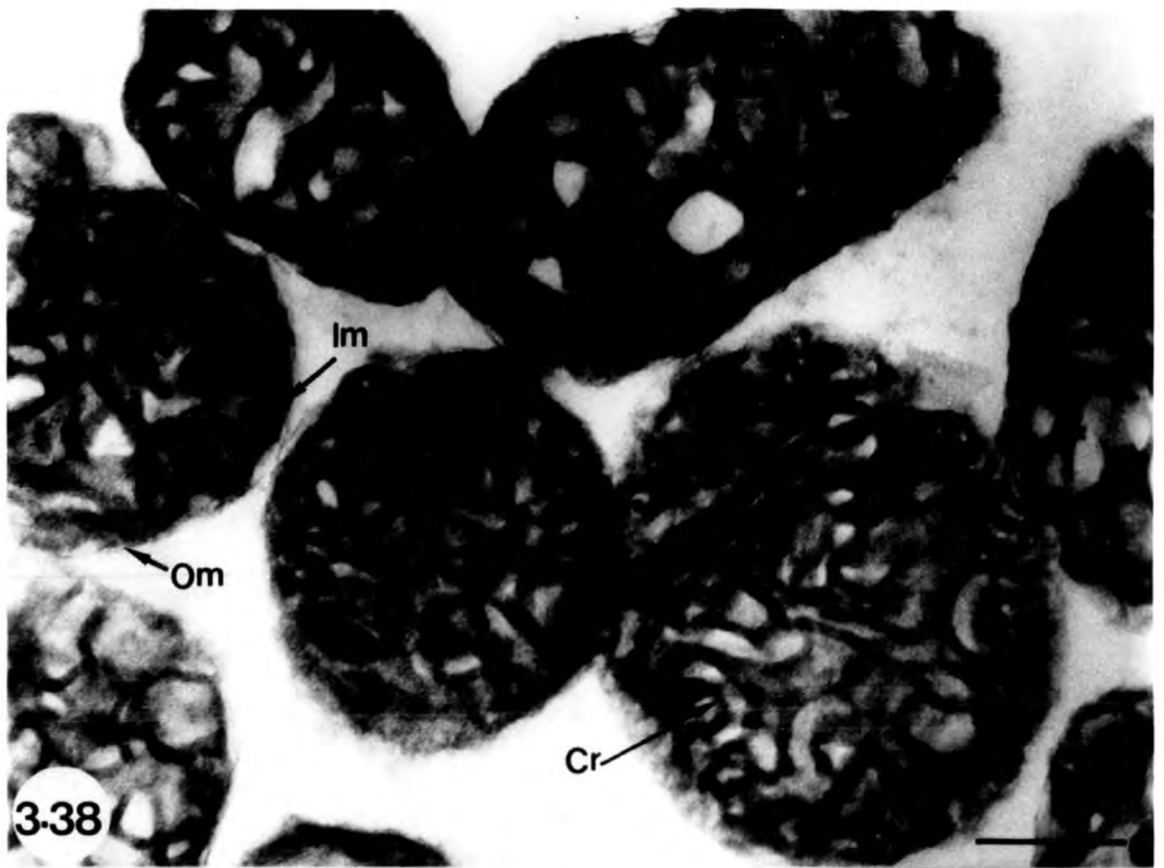


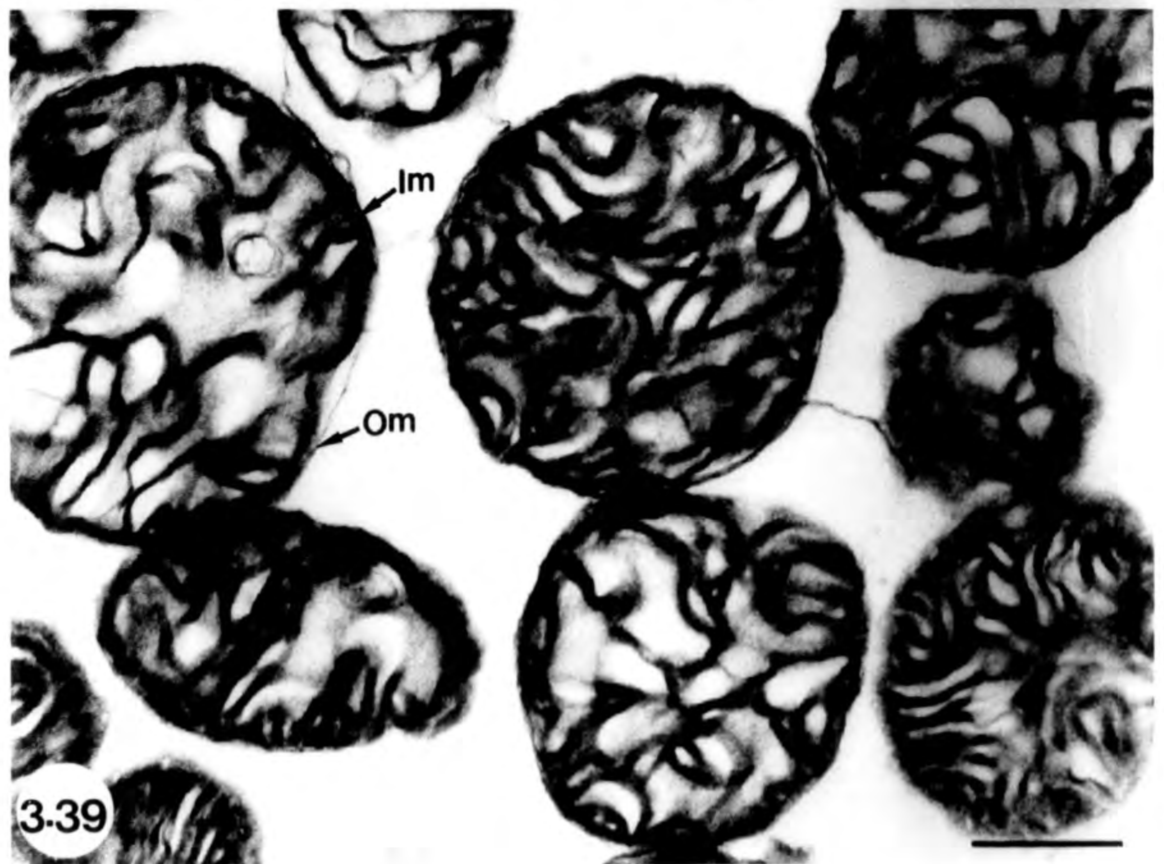
Figures 3.38, 3.39, 3.40 and 3.41.

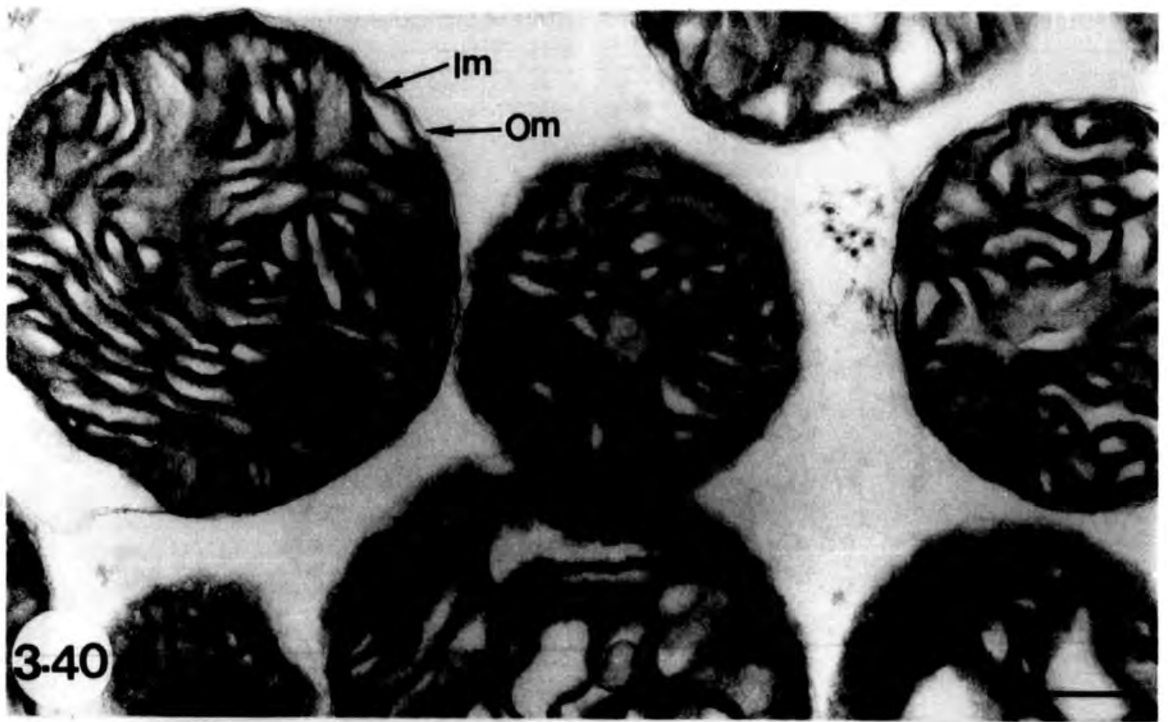
High power electron micrographs showing the ultrastructure of mitochondria isolated from flight muscle of 1, 2, 5 and 6-day old adult locusts, respectively. Note the presence of outer (Om) and inner (Im) mitochondrial membrane indicating that mitochondria are intact.

ICS: intra-cristal space; Cr: cristae.

Scale 0.4μm for Figures 3.38, 3.39, 3.40 and 0.25μm for Figure 3.41.


Im
Om
Cr
3.38


Im
Om
3.39

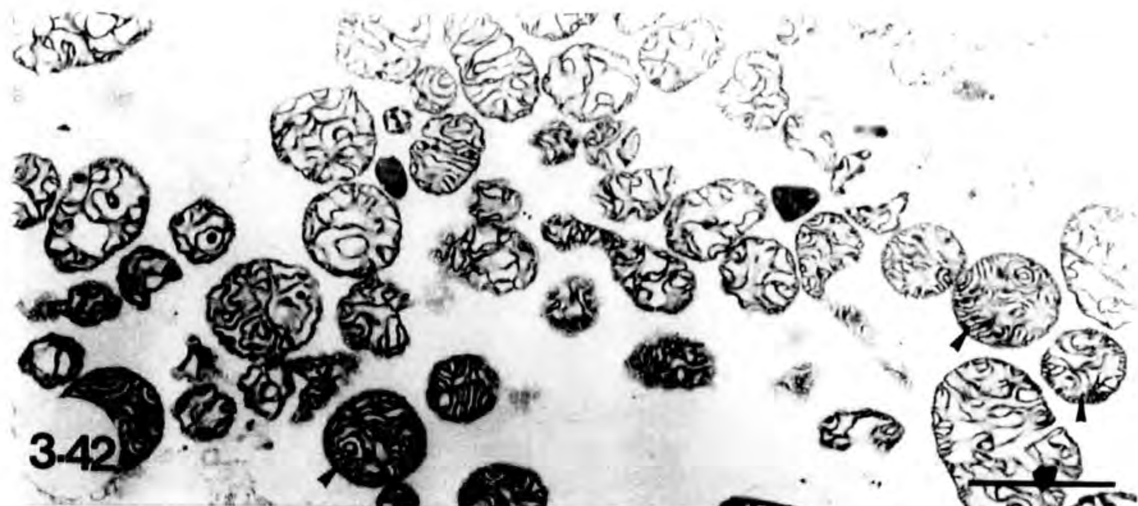


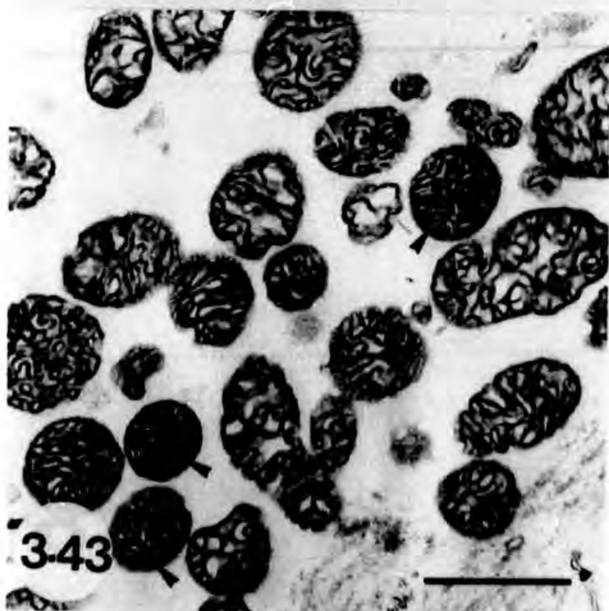
Cr
ICS
Om
Im
3-41

Figures 3.42, 3.43, 3.44 and 3.45.

Electron micrographs showing mitochondria isolated from the flight muscle of 1, 2, 5 and 6-day old adult locusts, respectively. Note the presence of type I (arrow-heads) and type II mitochondria at all ages.

Scale 1.5μm


3-42


3-43

V
3-44

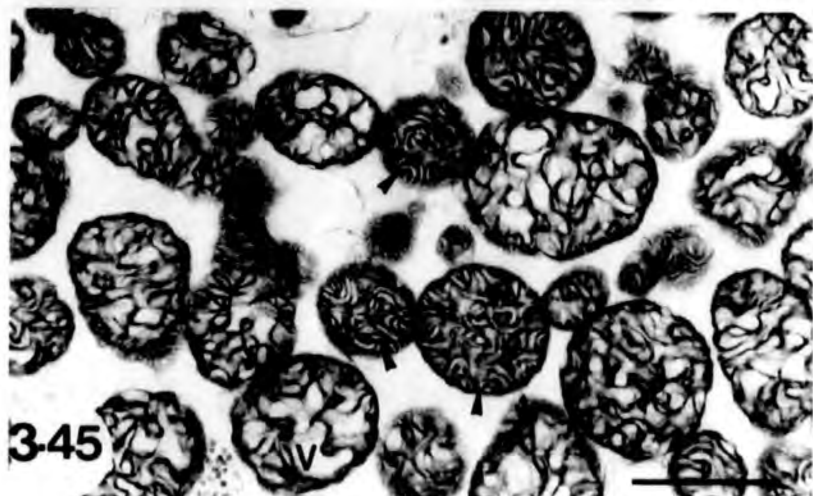
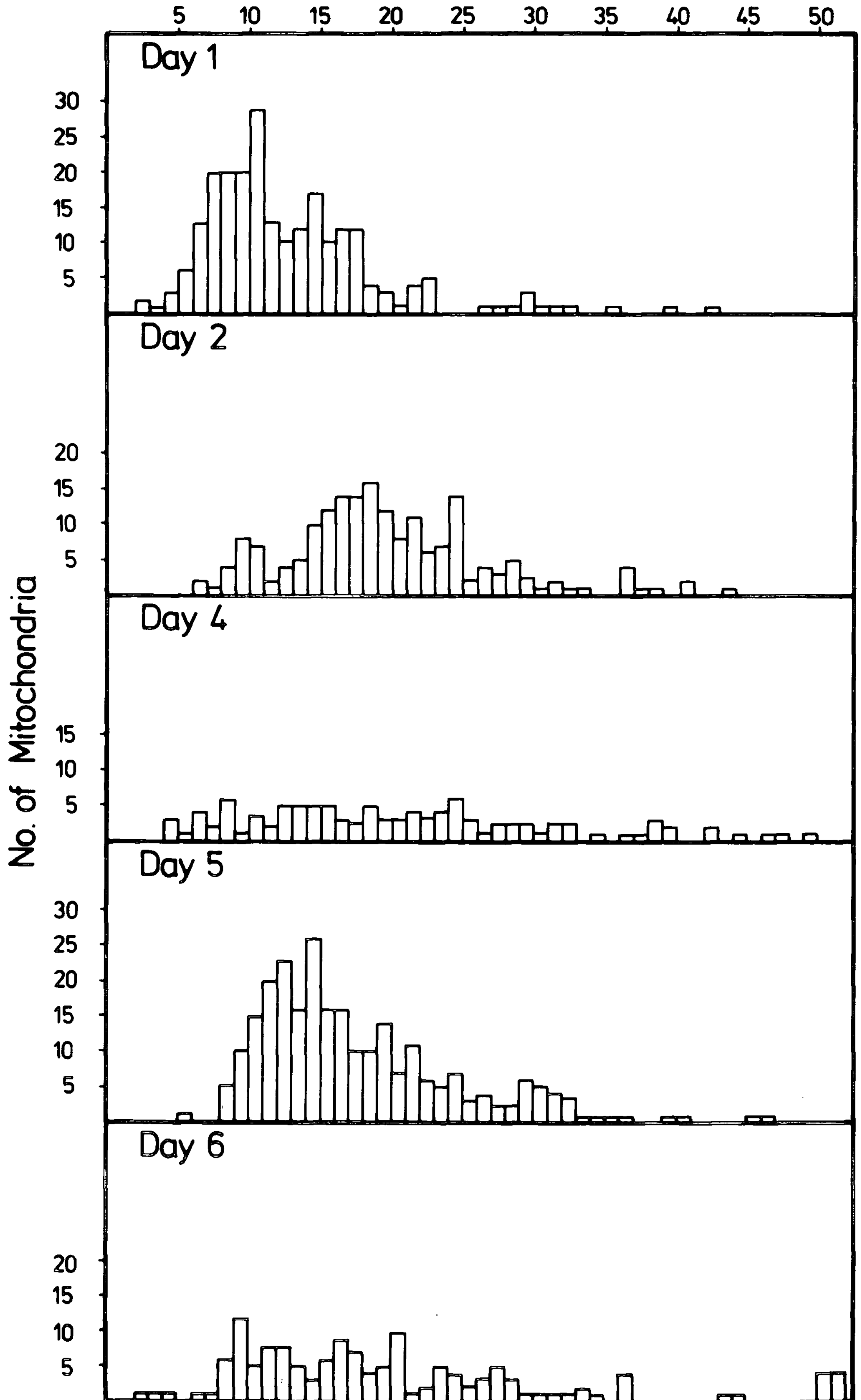

3-45
V

Figure 3.46. Histograms showing the size distribution of isolated flight muscle mitochondria from *Locusta migratoria*.

The data were obtained as described in the legend to Figure 3.33.



Size of isolated Mitochondria
5
10
15
20
25
30
35
40
45
50
Day 1
30
25
20
15
10
5
Day 2
20
15
10
5
Day 4
15
10
5
Day 5
30
25
20
15
10
5
Day 6
20
15
10
5
No. of Mitochondria

Sarcoplasmic reticulum and T-system

Examination of sections (Fig. 3.47) through flight muscle of Locusta revealed that the fenestrations or pores in the sarcoplasmic reticulum are formed by fusion of its membranes such that cisternae do not communicate with the sarcoplasm. The distribution of the fenestrated SR over most of the myofibrillar surface can be clearly seen. This is further substantiated by examination of longitudinal sections (Figs 3.20 and 3.32), which indicate that the SR surrounds each myofibril and separates adjacent myofibrils completely.

The association between a single T-system tubule and the non-fenestrated type of SR to form dyadic junctions is shown in Figs 3.20 and 3.48. Such junctions are recognizable by virtue of the fact that the membranes of the junction exhibit an increase in electron density (HAGOPIAN and SPIRO, 1967). No definite communication can be seen between the components of the dyadic junction, but the space between the unit membrane of the T-system and that of SR is frequently filled with an electron dense material. This density is due to numerous evenly spaced thickenings of the membrane of the reticular component of each dyadic junction, which project towards the T-system (Fig. 3.49). Similar structures have been reported in other insect muscles, for example, in Megoura viciae (SMITH, 1965), Phormia regina (SMITH and SACKTOR, 1970), Schistocerca gregaria (PIEK and NJIO, 1979), Homorocoryphus nitidulus (ANSTEE, 1971) and Neoconocephalus (ELDER, 1971). At the region of the A-band, the mitochondria are indented on each side almost midway between the Z-band and the region corresponding to H-band (Figs 3.10 and 3.32). Within each of these indentations one can see an association between the SR and T-system to form a dyad. There are four such structures per sarcomere, two on each side (Fig. 3.32). Similar arrangements of dyadic junctions have been reported in other species (ANSTEE, 1971; ELDER, 1971). In transverse sections which pass

Figure 3.47. Transverse section, through the muscle fibre of a 5-day old adult locust, showing the distribution of fenestrated sarcoplasmic reticulum (SR) around the myofibrils (MF).

Note the separation of adjacent myofibrils by one or two sheets of sarcoplasmic reticulum (see also Figure 3.27). Note also the pores (arrow-heads) in the fenestrated sarcoplasmic reticulum envelope.

M: mitochondria

Scale 0.5μm

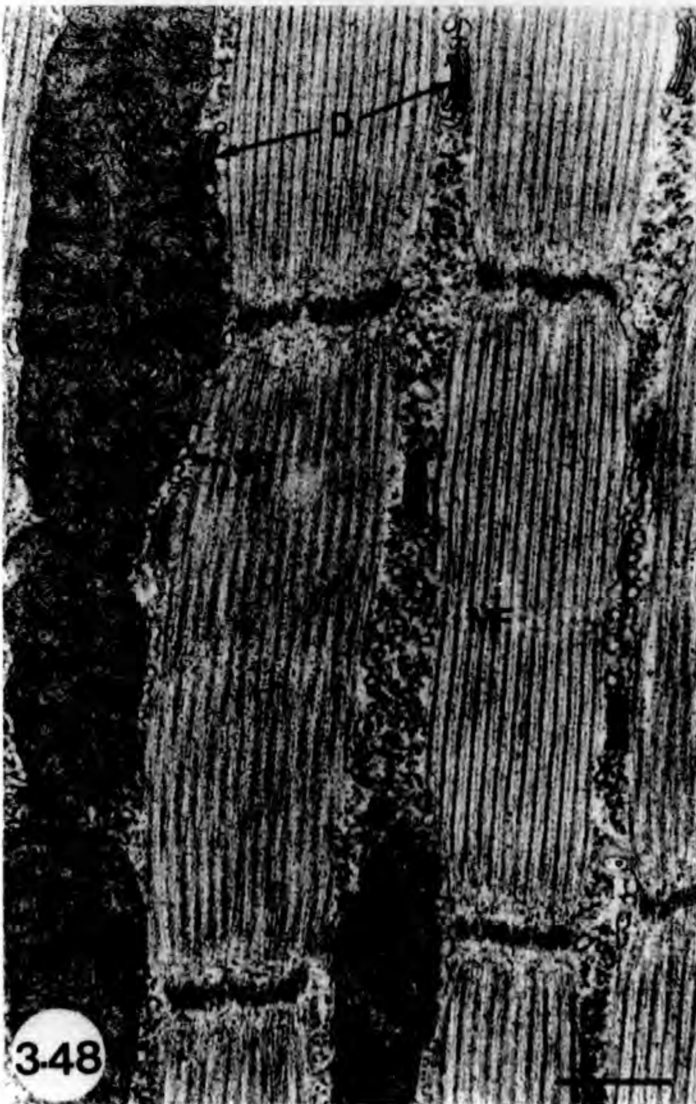
Figure 3.48. A longitudinal section through the muscle fibre of a 5-day old adult locust. Dyads (D) can be seen in slight mitochondrial indentations midway between the I- and the H-bands or in a similar position between adjacent myofibrils (MF) (cf. Figures 3.19, 3.21 and 3.29). SR: sarcoplasmic reticulum; G: glycogen; M: mitochondria.

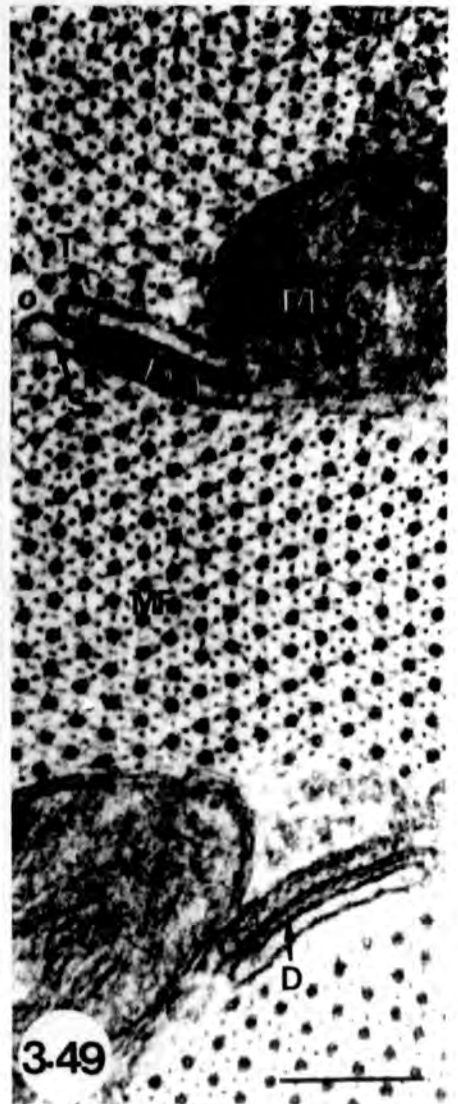
Scale 0.5μm

Figure 3.49. A high power electron micrograph of a transverse section of a muscle fibre showing a dyadic association (D) between a T-system tubule (T) and non-fenestrated sarcoplasmic reticulum (S). Note the close contact between dyads and mitochondria. Note also the evenly spaced thickenings along the membrane of the reticular component of the dyad (arrow-heads). M: mitochondria; MF: myofibril.

Scale 0.25μm

3.47


D
MF
3.48


MF
D
3.49

through a sarcomere at the level of the dyads, one can see that such structures form nearly a complete ring around the myofibril (Fig. 3.50).

The main changes in the SR and T-system effected by growth and development are the formation of dyads and the separation of the adjacent myofibrils by a sheet of SR. The T-system originates from the muscle plasma membrane at irregular intervals and penetrates the muscle fibre to divide it into myofibrils (see Fig. 3.51). In longitudinal sections taken from 1-day old adults (Fig. 3.29), dyads can be seen between the myofibrils. Careful examination during this early stage reveals that dyads tend to be situated nearer the Z-bands than previously described and the dyadic elements are at an oblique angle to the adjacent myofibrils rather than being parallel to them (Fig. 3.21). A similar positioning of the dyads near the Z-band was also seen in 2-day old (Fig. 3.19) and 3-day old locusts (Fig. 3.20). In contrast, by the 6th day of adult life the dyads come to occupy the position described earlier. The proportion of the total muscle fibre volume occupied by the SR and T-system from locusts of different ages are summarized in Table 3.2. The volume drops from approximately 16% in 1-day old adults to approximately 11% in 6-day old adult locusts ($p < 0.01$). This might suggest that the SR and T-system become established within the first 3-4 days of adult life, prior to the growth of the mitochondria and the myofibrils. Alternatively, it may be that the growth rate of SR and T-system is slower than that of the other muscle components.

Tracheole systems

Figure 3.52 shows a tracheal branch in close proximity to a muscle fibre. Such trachea give rise to tracheoles which penetrate individual muscle fibres. In transverse sections taken from flight muscle of 1-day old adults (Fig. 3.11), one can see large numbers of tracheoles within

Figure 3.50. An electron micrograph of a transverse section, through the A-band of a 3-day old adult locust, showing the abundance of dyads (D) in this region. The dyads encircle the myofibril (MF) forming an almost complete ring around it. T: T-system; M: mitochondria

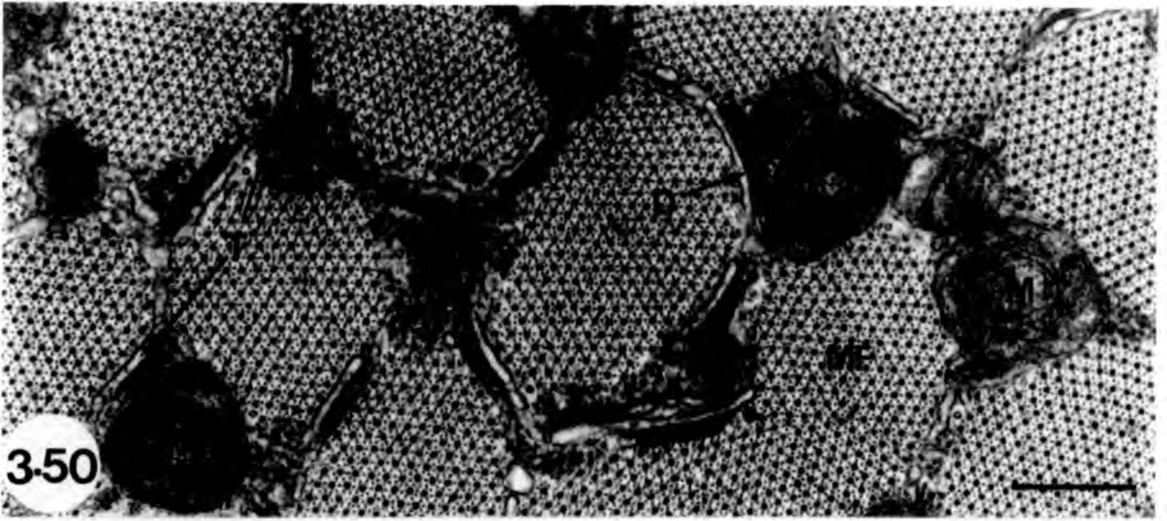
Scale 0.5μm

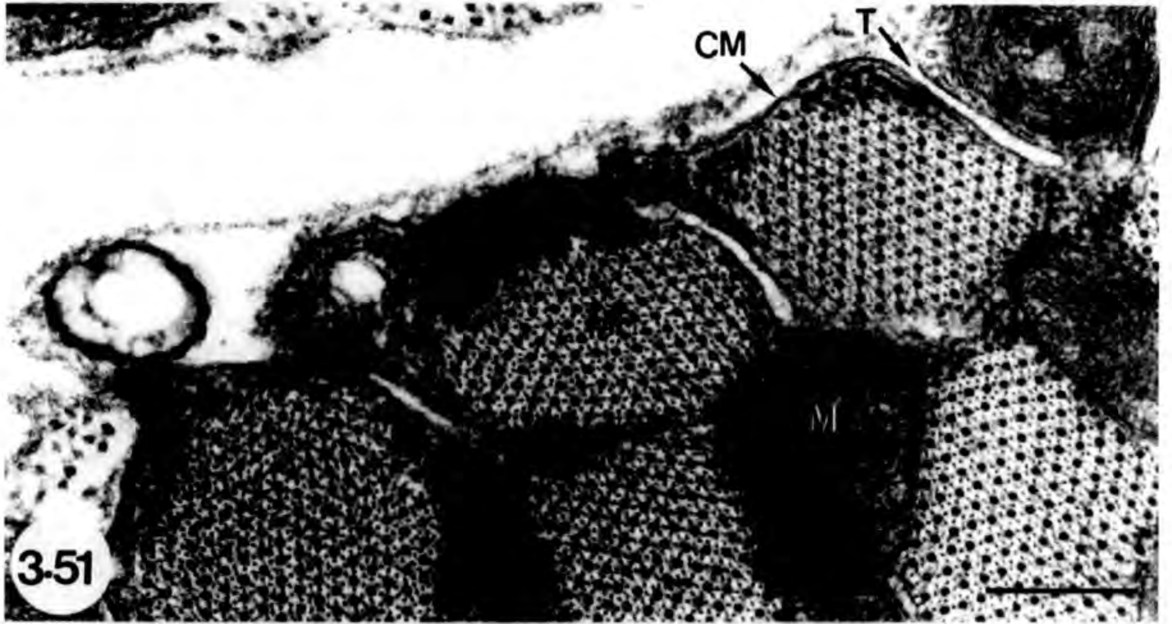
Figure 3.51. A transverse section through the peripheral region of a muscle fibre, showing the invagination of the cell membrane (CM) which gives rise to the T-system (T) (see also Figures 3.9, 3.24 and 3.28). M: mitochondria; MF: myofibril.

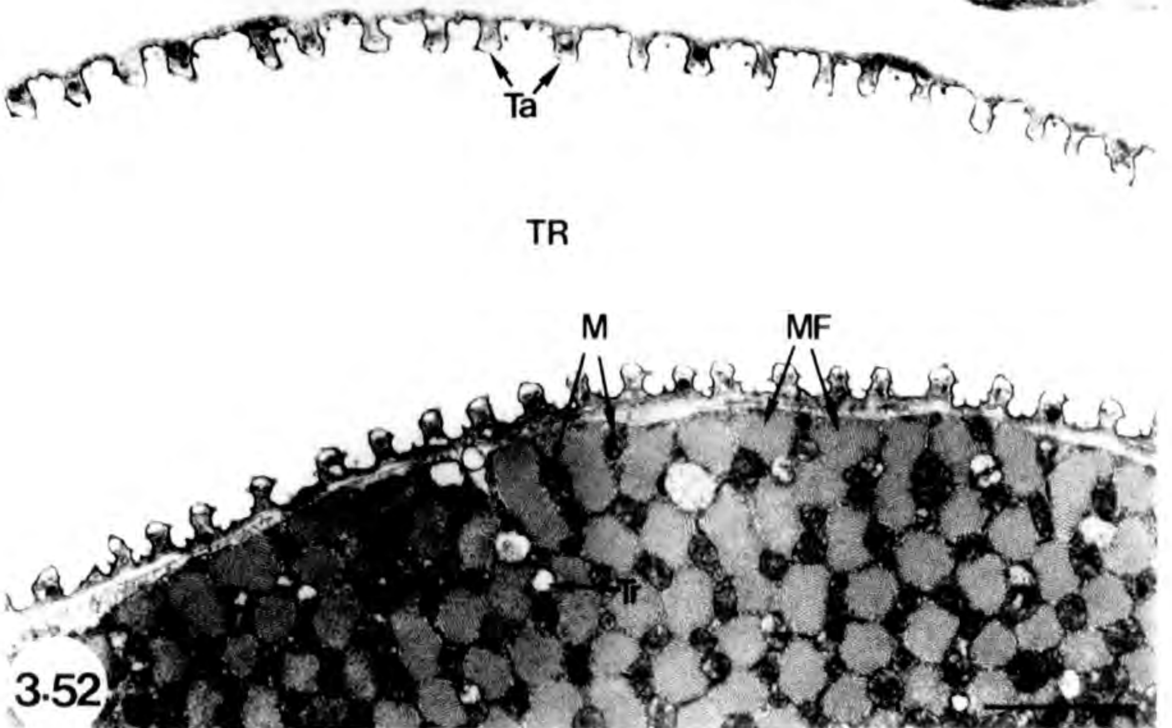
Scale 0.4μm

Figure 3.52. A transverse section, through the peripheral region of a muscle fibre of a 3-day old adult locust, showing the close association between a trachea (TR) and a muscle fibre. M: mitochondria; MF: myofibril; Tr: tracheole; Ta: taenidia.

Scale 2.25μm


3.50


CM
T
3.51


Ta
TR
M
MF
Tr
3.52

the muscle fibres, indicating a very efficient oxygen supply. In higher magnification (Figs 3.5 and 3.6), individual tracheoles are seen to be surrounded by their own plasma membrane and that of the muscle cell, which they draw with them during invagination. The luminal walls of these tracheoles possess small taenidial foldings, which prevent them from collapsing during muscle contraction. The proportion of total muscle fibre volume occupied by tracheoles falls with increasing age (Table 3.2) (cf. Figs 3.11, 3.12, 3.13 and 3.9). Thus tracheoles occupy approximately 7.0% of the total muscle volume in 1-day old adult insects, but only ca. 1% in 6-day old adult animals ($p < 0.01$). These observations suggest that the growth and final differentiation of this system take place during the first 2-4 days after the final ecdysis, possibly prior to the "duplication phase" (BROSEMER _et al._, 1963; BÜCHER, 1965), during which all the components of the flight muscle (excluding nuclei) are reported to duplicate (BÜCHER, 1965). Failure of the tracheoles to keep pace with this duplication would explain the decrease in their proportion of the total muscle volume with increasing age.

Age-dependent changes in mitochondrial protein content of locust flight muscle

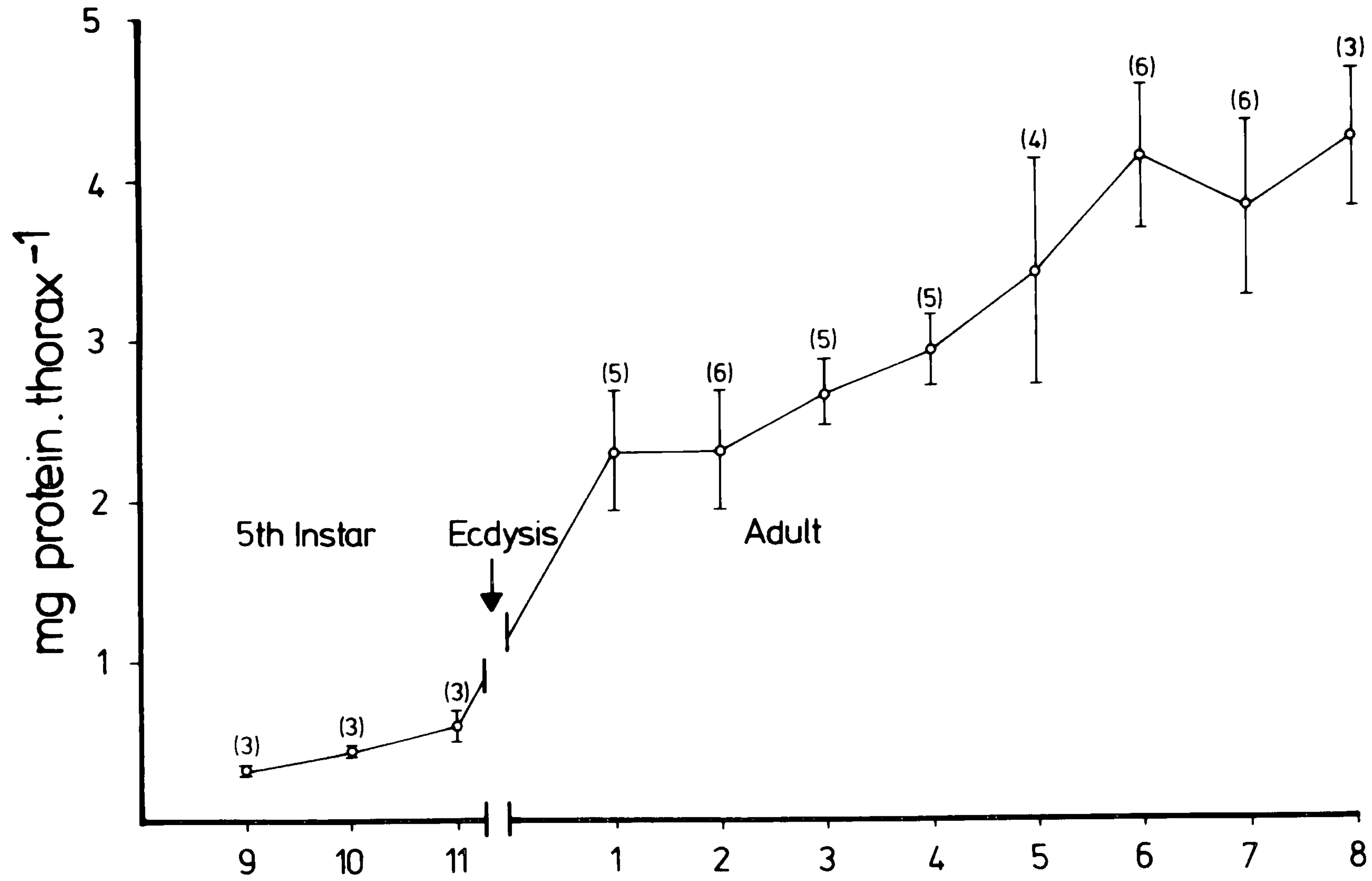
Relative amounts of locust flight muscle mitochondrial protein were estimated throughout the late 5th instar and early adult life. Equal numbers of male and female insects were employed at each age to ensure that any differences were unrelated to sex. The results obtained are shown in Fig. 3.53. Flight muscle mitochondrial protein per thorax changed little in amount over days 9-11 of the 5th instar. There was approximately 0.4mg of mitochondrial protein extracted per thorax. However, in newly ecdysed adults a marked increase in mitochondrial protein (2.3 ± 0.43mg, 5 determinations) was noted compared with the late 5th instar levels. After the final ecdysis, the amount of

Figure 3.53. Effect of age on the content of mitochondrial protein per thorax.

The figures in parentheses indicate the number of separate determinations.

Ordinate: mg protein. $thorax^{-1}$

Abscissa: Age in days


mg protein . thorax⁻¹
5
4
3
2
1
5th Instar
Ecdysis
Adult
(3)
(3)
(3)
(5)
(6)
(5)
(5)
(4)
(6)
(6)
(3)
9
10
11
1
2
3
4
5
6
7
8

mitochondrial protein continued to increase up to ca. 6th day of adult life, when it tended to level off at approximately 4mg per thorax; little change being observed over the next 2 days.

The incorporation of ^{14}C-leucine into mitochondrial protein during development

The incorporation of ^{14}C-leucine into flight muscle mitochondria *in vivo*, was measured at various times after injection into adult animals aged 1,3,5 and 7 days. The specific activity of the incorporation into mitochondrial protein, as a function of time after injection, at all ages studied, is shown in Figs 3.54 a,b,c and d). It can be seen that the initial rates of incorporation are high and that after ca. 1 hour the rate of incorporation decreases. In general, maximum levels of incorporation of ^{14}C-leucine into mitochondrial protein were recorded in 3-day old locusts (see Fig. 3.55). At all ages studied the specific activity was considerably reduced at 24 hours after injection.

Incorporation of ^{14}C-leucine into total flight muscle protein during development

The incorporation of ^{14}C-leucine into *Locusta* flight muscle was determined in adult animals aged 1,3,5 and 7 days after the final ecdysis. Figures 3.56 a,b,c and d show the specific activities obtained at different times after injection for the different ages studied. The rate of incorporation tended to be more rapid initially and then to level off at the maximal level of incorporation which was observed approximately 3 hours after the injection, at all ages studied. However, in the case of 1-day old locusts, the rate and level of incorporation was very low throughout.

Figure 3.54. The effect of age on ^{14}C-leucine incorporation into mitochondria protein.

a: Day 1 of adult life

b: Day 3 of adult life

c: Day 5 of adult life

d: Day 7 of adult life

The figures in parentheses indicate the number of separate experiments.

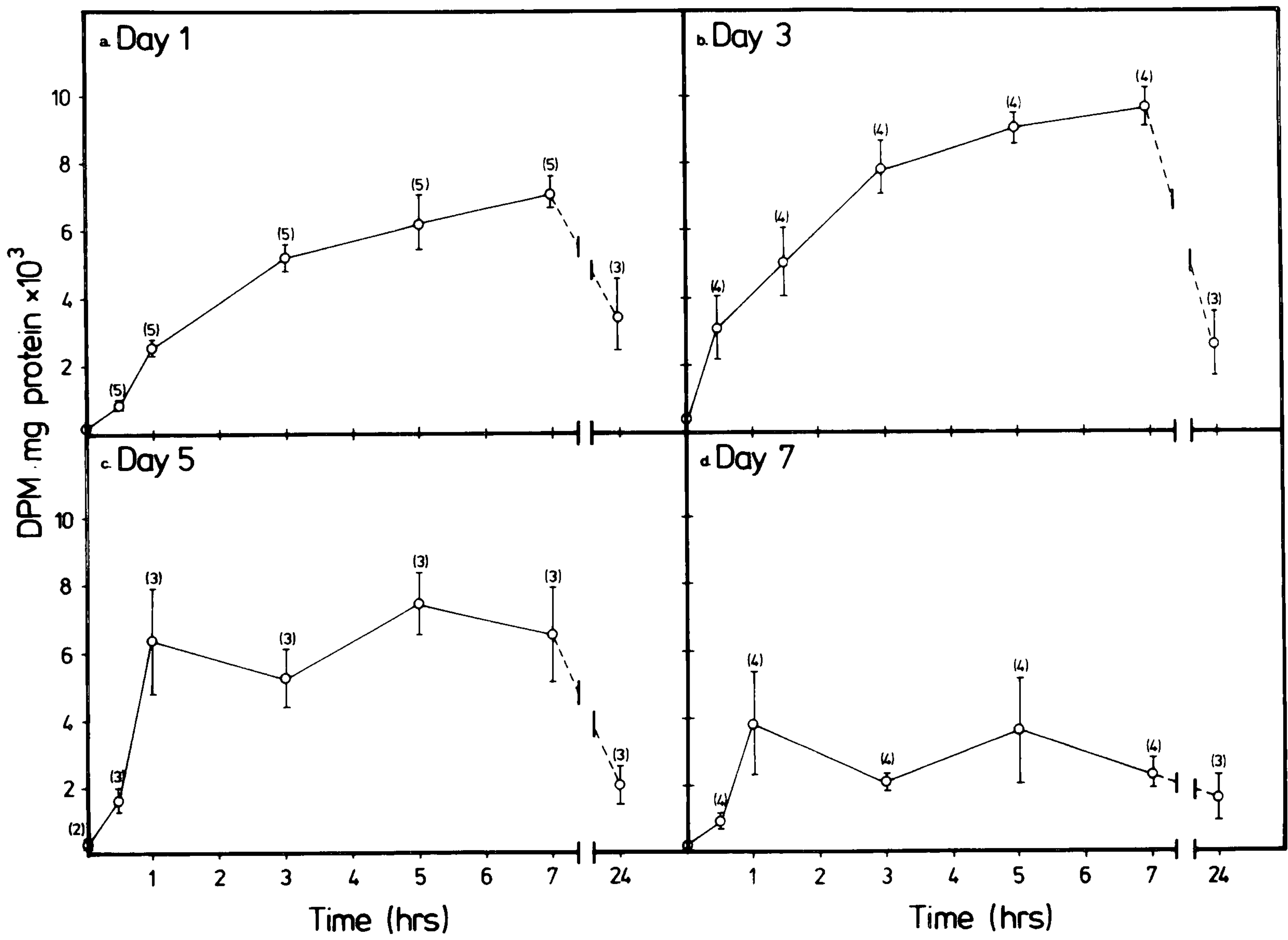

a. Day 1
b. Day 3
c. Day 5
d. Day 7
DPM . mg protein ×10³
Time (hrs)
Time (hrs)

Figure 3.55. Histograms of ^{14}C-leucine incorporation into mitochondria from *Locusta migratoria* flight muscle showing the maximum protein incorporation at different times of incubation and age.

The time of incubation was as follows:

A. 0-1 minute

B. 30 minutes

C. 1 hour

D. 5 hours

E. 7 hours

F. 24 hours

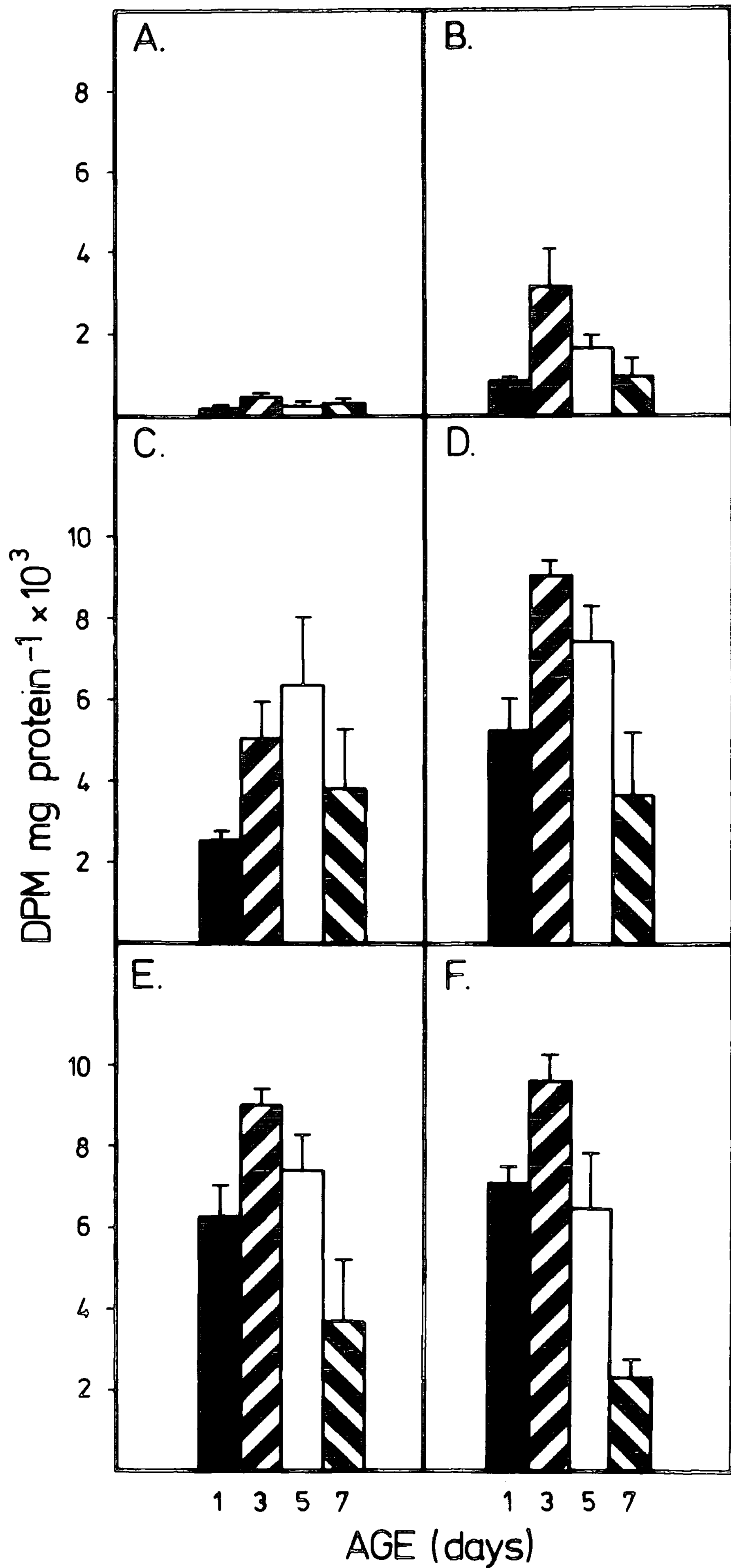

A.
B.
C.
D.
E.
F.
8
6
4
2
10
8
6
4
2
10
8
6
4
2
DPM mg protein $^{-1}$ x 10^3
1 3 5 7
1 3 5 7
AGE (days)

Figure 3.56. Effect of age on the ^{14}C-leucine incorporation into *Locusta migratoria* flight muscle at different ages.

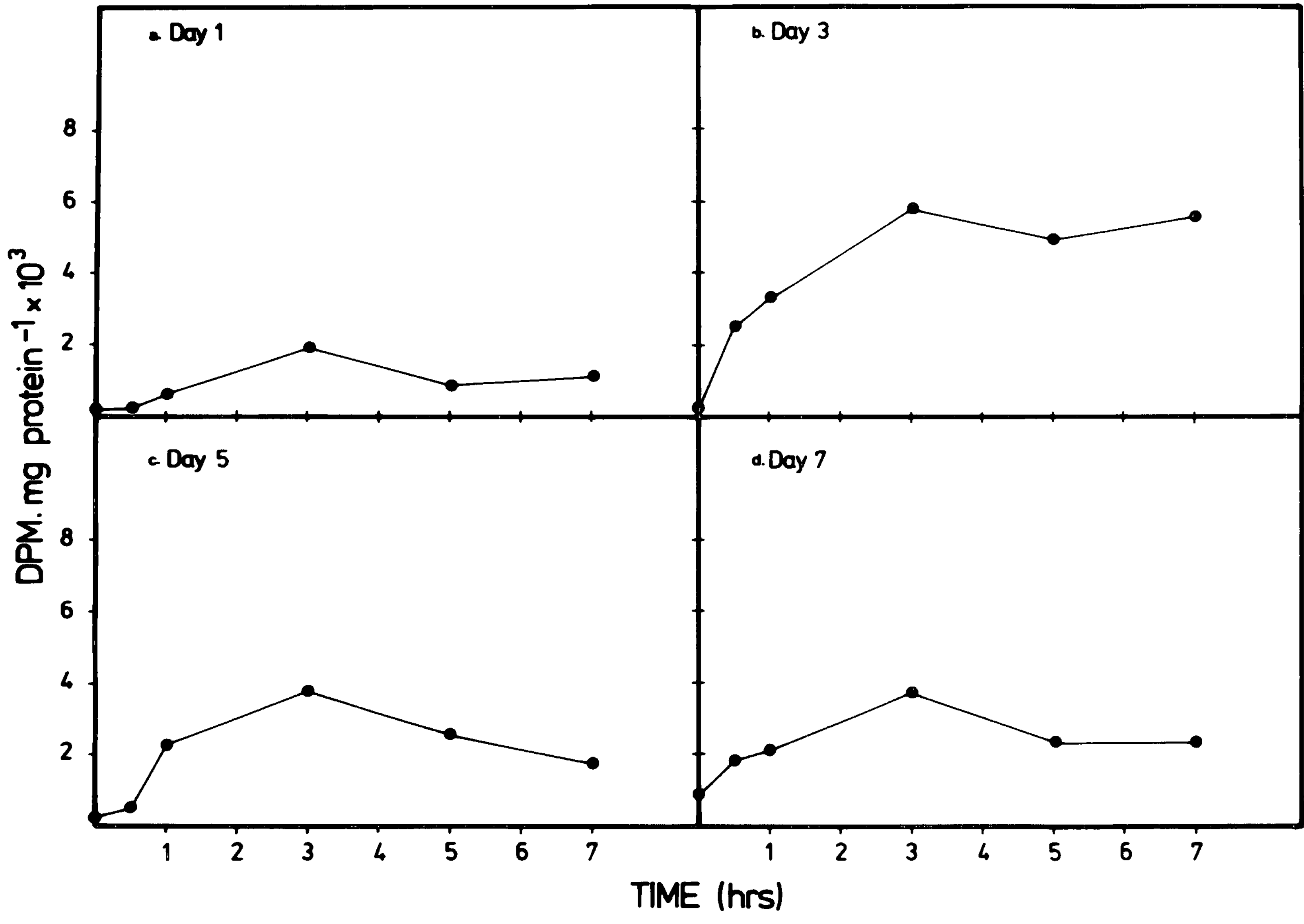
a. 1-day old adult

b. 3-day old adult

c. 5-day old adult

d. 7-day old adult

The data represent the mean of two separate experiments.



a. Day 1
b. Day 3
c. Day 5
d. Day 7
DPM. mg protein^{-1} x 10^3
8
6
4
2
1
2
3
4
5
6
7
TIME (hrs)

Fatty acid composition of flight muscle mitochondrial phospholipids during development

Typical chromatographic traces, obtained with a 1m glass spiral column packed with 3% polyethylene glycol adipate on a Gaschrome Q inert support, are illustrated in Figs 3.57 and 3.58 a,b,c and d. Figure 3.57 illustrates the fatty acid composition of the authentic standards and their retention times. These were used to identify the fatty acid composition of the mitochondrial phospholipid samples. Figure 3.58 illustrates the fatty acid composition of phospholipids, extracted from 9-day old 5th instar (a), 2-day (b), 5-day (c) and 15-day (d) old adults. These traces show that palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) are the major fatty acid constituents of mitochondrial phospholipids. There are in addition a number of small peaks of unknown origin. However, these represented less than 1% of the total phospholipid fatty acids present and were therefore omitted from the calculations.

Table 3.4 shows the fatty acid composition of flight muscle mitochondrial phospholipids of Locusta. It can be seen that the major fatty acid component is linolenic acid (18:3) which represented more than 40% of the total fatty acids present, at all ages studied, and more than 60% of the total unsaturated fatty acid composition. Apart from stearic acid (18:0) little change was noted in the relative amounts of the various fatty acids present at the different ages (see Table 3.4). Stearic acid (18:0) showed a significant decrease from 18.90 ± 1.00% of the total fatty acids present in 9-day old 5th instar locusts to 12.13 ± 0.52% in 15-day old adults ($p < 0.001$).

Table 3.5 shows the relative proportion of saturated and unsaturated fatty acids at the various ages. It can be seen that there is a significant change in the ratio of these two groups. Thus the unsaturated/saturated ratio was 2.30 ± 0.30 in 9-day old 5th instar locusts whereas this had increased to 3.30 ± 0.30 by the 15th day of adult life ($p < 0.02$).

Figure 3.57. Typical chromatograph illustrating the fatty acid composition of the authentic standards and their retention time (mins). The method of operating and conditions are described in the text.

BHT, 2,6-ditertiary-para-cresol (front)

16:0, Palmitic acid

16:1, Palmitoleic acid

18:0, Stearic acid

18:1, Oleic acid

18:2, Linoleic acid

18:3, Linolenic acid

20:0, Arachnidic acid

20:1, Eicosenic acid

20:2, Eicosadienic acid

20:3, Eicosatrieoic

22:0, Behenic acid

22:1, Erucic acid

24:1, Nervonic acid

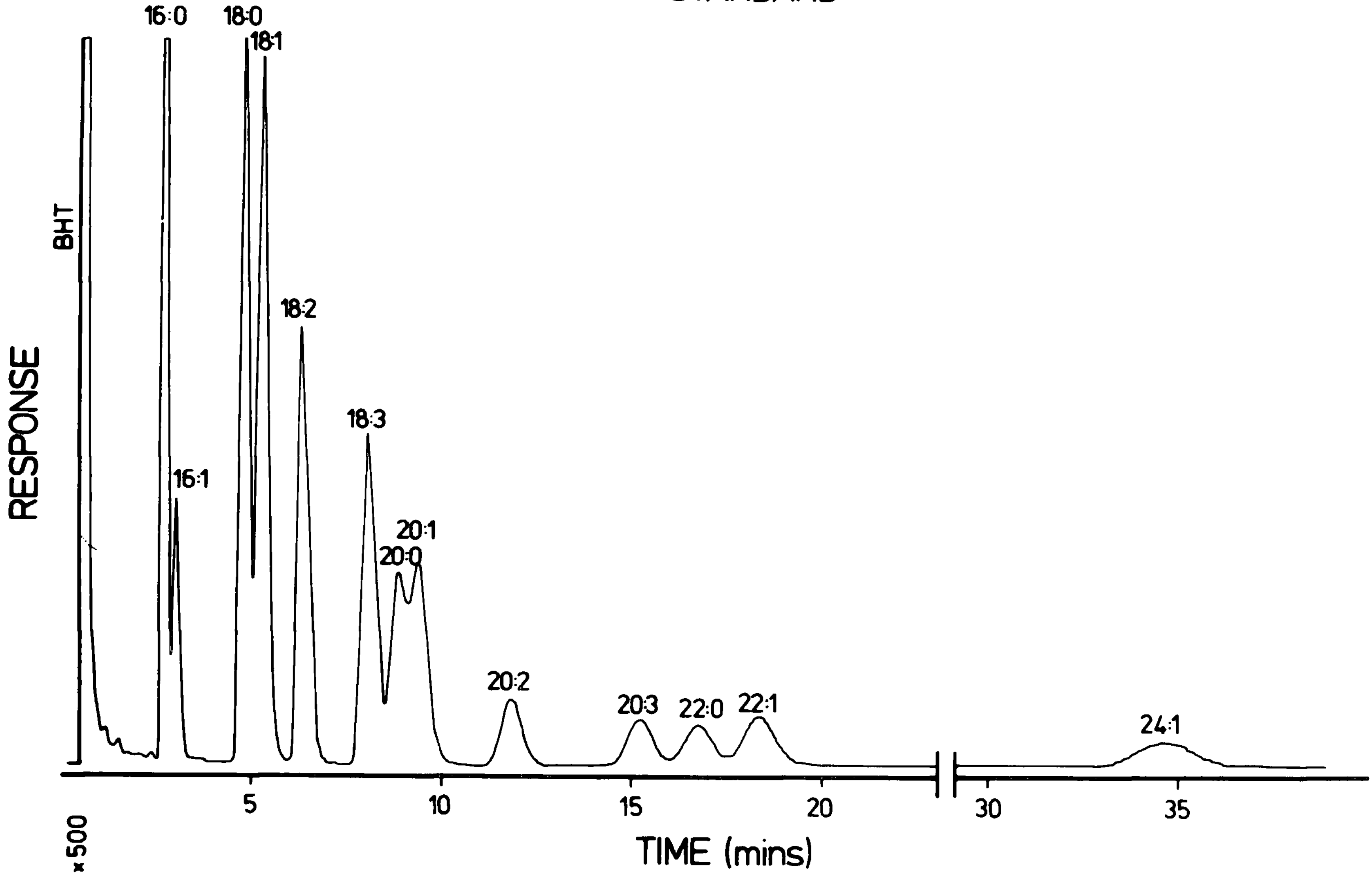

STANDARD
16:0
18:0
18:1
BHT
16:1
18:2
18:3
20:1
20:0
20:2
20:3
22:0
22:1
24:1
RESPONSE
x500
5
10
15
20
30
35
TIME (mins)

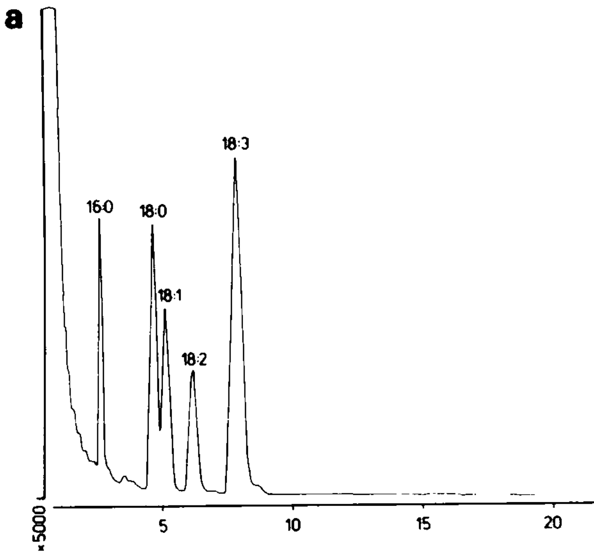
Figure 3.58. Representative GLC traces showing the fatty acid composition of flight muscle mitochondrial phospholipid in locusts at different ages.

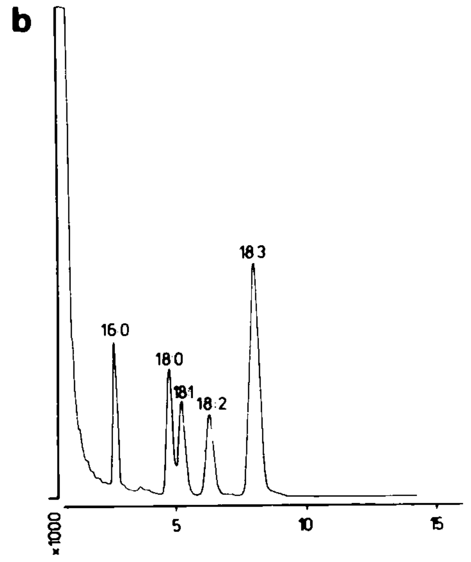
a. 9-day old of 5th instar larvae

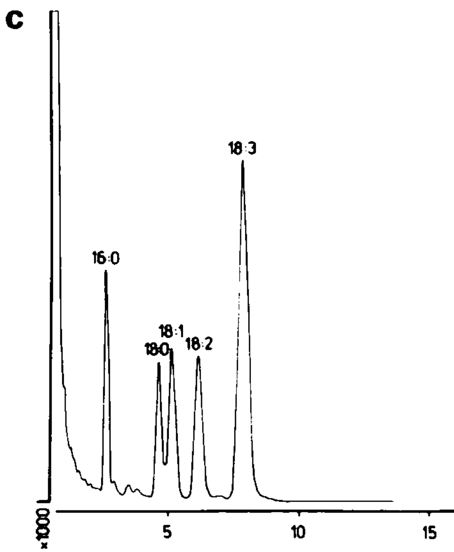
b. 2-day old adult

c. 5-day old adult

d. 15-day old adult


a
16:0
18:0
18:1
18:2
18:3
×5000
5
10
15
20


b
16:0
18:0
18:1
18:2
18:3
×1000
5
10
15


c
16:0
18:0
18:1
18:2
18:3
×1000
5
10
15

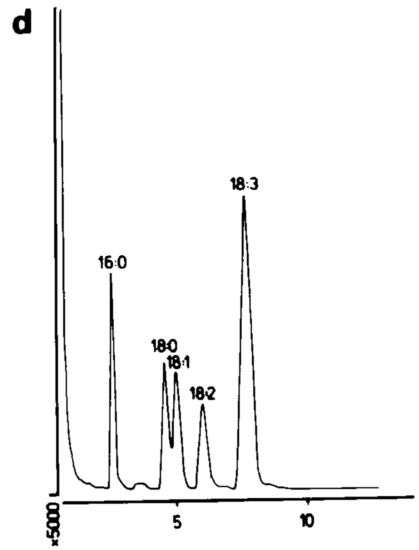

d
16:0
18:0
18:1
18:2
18:3
×5000
5
10

Table 3.4. Effect of age on the total fatty acid composition of mitochondrial phospholipids extracted from flight muscle of *Locusta migratoria* at different ages.

	Fatty acids (% of mitochondrial phospholipid ± S.E.M.)					
Age in days	16 : 0	18 : 0	18 : 1	18 : 2	18 : 3	n
9-day old of 5th instar	12.22 ± 1.84	18.91 ± 1.00*	13.42 ± 1.71	12.84 ± 0.31	42.51 ± 4.12	5
1-day old adult	11.87 ± 2.10	18.92 ± 2.50	14.83 ± 3.00	12.93 ± 1.12	41.45 ± 6.41	3
3-day old adult	10.63 ± 0.81	15.13 ± 0.21	14.41 ± 1.22	14.22 ± 1.40	45.61 ± 3.10	4
5-day old adult	10.63 ± 1.10	13.13 ± 0.98	13.40 ± 1.21	13.02 ± 1.63	49.82 ± 1.62	3
7-day old adult	11.22	15.35	14.42	16.77	42.24	2
9-day old adult	10.04 ± 0.20	12.24 ± 0.90	13.49 ± 1.24	13.20 ± 1.50	51.03 ± 1.89	3
11-day old adult	9.93 ± 0.64	12.81 ± 0.83	13.32 ± 0.22	12.42 ± 0.31	51.52 ± 0.30	3
15-day old adult	11.30 ± 0.92	12.13 ± 0.52*	15.43 ± 0.62	12.71 ± 1.70	48.43 ± 1.29	3

Fatty acid shorthand notion: Palmitic acid 16 : 0, Stearic acid 18 : 0, Oleic acid 18 : 1, Linoleic acid 18 : 2, Linolenic acid 18 : 3.

n: the number of experiments

*$p = 0.001$

Table 3.5. Effect of age on the percentage distribution of unsaturated and saturated fatty acids of mitochondrial phospholipids extracted from developing flight muscle of *Locusta migratoria* and their ratio.

	% of total fatty acid content			
Age in days	saturated (1) fatty acid	unsaturated (2) fatty acid	Ratio (2 : 1)	n
9-day old of 5th instar	31.35 ± 2.73*	68.65 ± 2.73	2.19 ± 0.32**	5
1-day old adult	30.75 ± 4.52	69.25 ± 4.52	2.25 ± 0.5	3
3-day old adult	25.56 ± 0.90	74.44 ± 0.90	2.91 ± 0.15	3
5-day old adult	23.85 ± 2.10	76.15 ± 2.10	3.19 ± 0.40	4
7-day old adult	2.65	73.60	3.49	2
9-day old adult	22.31 ± 0.90	77.80 ± 0.90	3.40 ± 0.18	3
11-day old adult	22.74 ± 0.61	77.30 ± 0.61	3.41 ± 0.11	3
15-day old adult	23.40 ± 1.44*	76.60 ± 1.44	3.31 ± 0.32**	3

*$p > 0.02$
**$p > 0.02$

Discussion

The present study has shown that marked changes in flight muscle mitochondrial fine structure take place during the first 6 days of adult life. These changes include an increase in mitochondrial size and the number and density of their cristae. Similar changes have been reported by BROSEMER et al. (1963) and BÜCHER (1965). Furthermore, it has been shown that associated with these structural changes there is an increase in mitochondrial enzymes (BROSEMER et al., 1963; BEENAKKERS, 1963) and those enzymes important in catabolic pathways (BEENAKKERS et al., 1975). In Locusta migratoria, the mitochondria (in situ) changed in size during the first 2-3 days of adult life. However, by day 6 the mitochondria were approximately 4-fold larger than in 1-day old insects, and their diameter had more-or-less doubled. Mitochondria constituted ca. 28.5% of the total muscle fibre volume in 6-day old locusts compared with ca. 24.7% in 1-day old adults. These values compare well with those of BROSEMER et al. (1963) and BÜCHER (1965) who reported that, in Locusta, the relative volume occupied by the mitochondria increased from 6% to 23% between the 8th day before and the 1st day after the final ecdysis. By the 8th day after the final ecdysis the mitochondria attained their maximum relative volume of 30%. BÜCHER (1965) demonstrated that in Locusta the total flight muscle mass increased by a factor of 10 during the entire developmental period. Thus the total increase in the muscle chondriome is about 50-fold. Similarly RICHARD et al. (1971) reported that, in Schistocerca gregaria, the mass of the flight muscle increased 13-fold between the beginning of the 5th instar and the onset of sexual maturity, and that the increase in mitochondrial volume was comparable with that of Locusta. In Leptinotarsa decemlineata, DE KORT (1969) has shown that the flight muscle mitochondria occupied 4% of the total muscle volume at the time of adult emergence, and that by the 12th day

of adult life this volume had increased to 30%. It is interesting to note that, in mature flight muscle of different species, there are variations in the proportion of the total muscle volume occupied by mitochondria. For example, mitochondria occupy 44% of the total flight muscle in Neoconocephalus robustus (JOSEPHSON and ELDER, 1968; ELDER, 1971), 28.5% (present study) and 30% (BÜCHER, 1965) in Locusta and 40% in Aeshna (SMITH, 1961b). In general, it seems that, in synchronous flight muscle, the more active the flight muscle is the more mitochondria it contains (HOYLE, 1969).

Similar changes to those described above have been reported elsewhere, for example, in Phormia regina (WATNABE and WILLIAMS, 1953), in Musca domestica (ROCKSTEIN and BHATNAGER, 1965; SOHAL et al., 1972) and Calliphora erythrocephala (TRIBE and ASHHURST, 1972). LEVENBROOK and WILLIAMS (1956) reported that, in Phormia regina, the wing-beat frequency is maximum by about the 7th day of adult life, and that the number of mitochondria is independent of age (6.7×10^8/thoracic flight muscle). However, this improved flight performance was associated with a 3-fold increase in mitochondrial dry weight in the first week of adult life. In Hyalophora cecropia (MICHEJDA, 1964), the average mitochondrial diameter increased from 0.35μm on the 5th day to 0.8μm on the 20th day of pupal life by which time the mitochondria : myofibril ratio, characteristic of the adult moth, had been established. This is in sharp contrast to the situation observed in Locusta in the present study, where marked changes in the mitochondria : myofibril ratio were observed after the final ecdysis.

Several researchers have reported the presence of mitochondria of differing fine structure in the flight muscles of insects. For example, GREGORY et al. (1968) have described two types of mitochondria in flight muscle of Lucilia cuprina; one type present in the pupa, in which both tubular and lamellar cristae were observed and a second type, found in the adult, in which only lamellar cristae occur.

ELDER (1971) has reported two distinct mitochondrial configurations (normal and vesiculated) in the very fast contracting synchronous flight muscle of mature *Neoconocephalus robustus*. Similarly, SIMON *et al*. (1969) described two types of mitochondria (Type A and B) in *Musca domestica* flight muscle. In contrast, only one mitochondrial type was observed in 6-day old adults in the present study. These possessed characteristically dense matrices and numerous densely packed cristae and are not too different from Type B mitochondria described by SIMON *et al*. (1969). Type A mitochondria which are characterised by less dense matrices and few cristae were only observed in 1-day old adult *Locusta*. Similar observations have been reported in *Homorocoryphus nitidulus* (ANSTEE, 1971) and in *Attagenus megatoma* (BUTLER and NATH, 1972).

In contrast to the present studies reported above on *Locusta*, two distinct types of mitochondria were frequently found in sections through pellets of isolated mitochondria, at all ages studied. The exact significance of these two structural types is uncertain. However, there is evidence to suggest that associated with the functional state of the mitochondria there are changes in fine structure. TRIBE and ASHHURST (1972) have reported a variety of conformational changes in mitochondria isolated from *Calliphora erythrocephala* flight muscle, which can be correlated with their different respiratory states (i.e. State III and IV, as defined by CHANCE and WILLIAMS, 1955a). They concluded that these changes are comparable to those seen in mammalian mitochondria (GOYER and KROLL, 1969; HACKENBROCK, 1966; PENNISTOR *et al*. (1968), though it has been suggested by STONER and SIRAK (1969) and BUTLER and JUDAH (1970) that some of the conformations might be artefacts. However, SMITH *et al*. (1970) have reported that conformational changes can be induced *in situ* in the flight muscle mitochondria of *Phormia regina* and *Musca domestica* by the injection of Chloramphenical, an inhibitor of mitochondrial protein synthesis.

As mentioned in the Results section, the main size change in isolated mitochondria was observed between the 1st and 2nd days of adult life. Thereafter, little change in mean mitochondrial size was observed during the period of this study. These findings contrast markedly with those reported by TRIBE and ASHHURST (1972) for isolated mitochondria from the flight muscle of Calliphora erythrocephala. They showed that there was good agreement between the mean size of isolated mitochondria from a given stage and their size in situ. Both in situ and in vitro mitochondria increased in size with age. However, in a similar study on mitochondrial size in situ and in vitro in Hyalophora cecropia, MICHEJDA (1964) showed that isolated mitochondria were larger than those observed in situ at the same age. He suggested that such an increase in the size of isolated mitochondria might be due either to swelling or to their removal from the influence of intra-tissue forces.

The fatty acid composition of membrane phospholipids is known to affect the fluidity of the membrane, that is, the ability of the component molecules to move in a lateral direction independently of each other (VAN DEENEN, 1972; PASTERRAK, 1977; CHERQI et al., 1979). Thus membranes containing phospholipids rich in unsaturated fatty acids form less compact bilayers (more fluid) than those containing phospholipids rich in saturated fatty acids. In the present study, it was shown that the fatty acids present in mitochondrial phospholipids of Locusta migratoria did not change over the period studied (9-day 5th instar - 15-day old adults). There was, however, a significant decrease in the relative amount of stearic acid (18:0) present in older insects. Furthermore, the unsaturated : saturated fatty acids ratio increased significantly with age, and this suggests that the mitochondrial membrane in early adult life may be less fluid than in more mature insects. Recently, CHERQI et al., (1979) has suggested that in rats, increased fluidity

of adipocyte plasma membrane is associated with an increase in permeability. A similar relationship in Locusta mitochondria would imply increased permeability with the onset of flight muscle maturity and this in turn would permit greater metabolic activity.

At no time in the present study, on adult flight muscle, were any structures observed which might be taken to represent incomplete mitochondria, or mitochondrial precursors. The observations are consistent with those reported by BÜCHER (1965) that the increase in the chondriome, in Locusta flight muscle, is mainly due to the growth of existing mitochondria. However, BROSEMER et al. (1963) and RICHARD et al (1971) have reported mitochondrial division in developing flight muscle of Locusta migratoria and 5th instar Schistocerca gregaria. Similarly, DE KORT (1969) reported a significant increase in mitochondrial numbers in flight muscle of Leptinotarsa decemlineata during the first few days after the final ecdysis. He concluded that this was due to mitochondrial division. The latter has also been reported in the fat body of Calpodes ethlius (LARSEN, 1970). No signs of mitochondrial division were observed in the present study. It must be concluded, therefore, that if mitochondrial division takes place in the flight muscle of Locusta, it does so prior to the final ecdysis, as in Schistocerca gregaria (RICHARD et al., 1971).

In Locusta, it was noted that as development proceeded there was a decrease in the number of mitochondria per sarcomere indicating mitochondrial elongation. A similar phenomenon has been reported by HEROLD (1965), in Apis mellifera, who suggested this was achieved by the fusion of adjacent mitochondria. Such mitochondrial fusion at various stages of development has been suggested elsewhere, e.g. in Hyalophora cecropia (MICHEJDA, 1964), in Lucilia cuprina (GREGORY et al., 1968), in Musca domestica (SOHAL and ALLISON, 1971; SOHAL et al., 1972; SOHAL, 1976; WEEB and TRIBE, 1974), in Calliphora erythrocephala (TRIBE

and ASHHURST, 1972; WEEB and TRIBE, 1974) and in Attagenus megatma (BUTLER and NATH, 1972). TRIBE and ASHHURST (1972) showed that mitochondrial size in Calliphora flight muscle increased from 1.66μm in 2-day old adults to 2.28μm by the 10th day of adult life. However, during this period they found no increase in the relative volume of the muscle mitochondria. Furthermore, autoradiographic studies revealed that newly synthesized protein could account for only a 4-8% increase in mitochondrial size, whilst a 25% size increase was noted in electron microscopical and Coulter counter studies (see also WEEB and TRIBE, 1974). They concluded that these observations were best explained by mitochondrial fusion. A similar conclusion was recorded by SOHAL (1976) with Musca domestica. He reported that whilst the number of mitochondria was reduced by more than 44% between the 1st and 9th day of adult life, the mean area of individual mitochondrial profiles increased by 143% and the relative area of the sarcoplasm occupied by mitochondria increased by 43%. Moreover, SOHAL (1976) described the formation of highly elongated and irregularly shaped mitochondria, which are often referred to as "giant" mitochondria by side-to-side and end-to-end, as well as oblique fusion. On the basis of the present study, it appears that in Locusta, end-to-end fusion is the major mechanism by which mitochondrial elongation is effected. The structural mechanism of fusion has been described by SOHAL (1976). Initially joining of the outer mitochondrial membranes into a single thick membrane occurs followed by the development of regularly patterned cristae at the site of fusion. The nature of the factor(s) causing mitochondrial fusion is unknown. However, TANDLER et al. (1968) have suggested that mitochondrial fusion may be related to the intracellular distribution of membrane components such as phospholipids. It has been demonstrated that in Neurospora, low concentrations of choline, a precursor of lecithin (choline phosphoglyceride), in the medium

resulted in an increase in mitochondrial size (LUCK, 1965). It is not known whether mitochondrial fusion in insect flight muscle is related to such changes in the intracellular concentration of membrane macromolecules or not.

The specific activity of ^{14}C-leucine incorporation into flight muscle mitochondrial protein of adult *Locusta*, was maximal in 3-day old animals. Similarly, in *Schistocerca gregaria*, RICHARD *et al.* (1971) showed that the incorporation of ^{14}C-phenylalanine into mitochondrial protein was maximal in 2-day old adults and that mitochondrial protein synthesis decreased in older adults. KLEINOW *et al.* (1970) showed that ^{14}C-iso-leucine incorporation into adult flight muscle mitochondrial protein of *Locusta* decreased with age. Recently, VAN MARREWIJIK *et al.* (1980) showed that the relative amount of the free leucine is not constant in developing flight muscle of *Locusta migratoria*; the leucine concentration was greatest in 1-day old adult locusts, being 1.82μmoles/gm fresh weight and then decreased dramatically to 0.63μmoles/gm fresh weight by the 4th day and then increased slightly to 0.85μmoles/gm fresh weight by day 8. Such changes in the size of the "leucine pool" clearly have implications for the present studies on ^{14}C-leucine incorporation into mitochondrial protein. It may, therefore, be significant that the changing size of the "leucine pool" is the inverse of ^{14}C-leucine incorporation into mitochondrial protein. The peak incorporation of ^{14}C-leucine, observed in the 3-day old adult locusts, may be partly due to the reduced size of the "leucine pool" at this time.

The present study showed that the amount of mitochondrial protein per thorax increased approximately 7-fold from the 9th day of 5th instar to the 6th day of adult life. More than half of this increase took place during the final ecdysis. This substantial increase in mitochondrial protein per thorax over the period of the final ecdysis may be explained in two ways: (1) The increase may be due to an actual

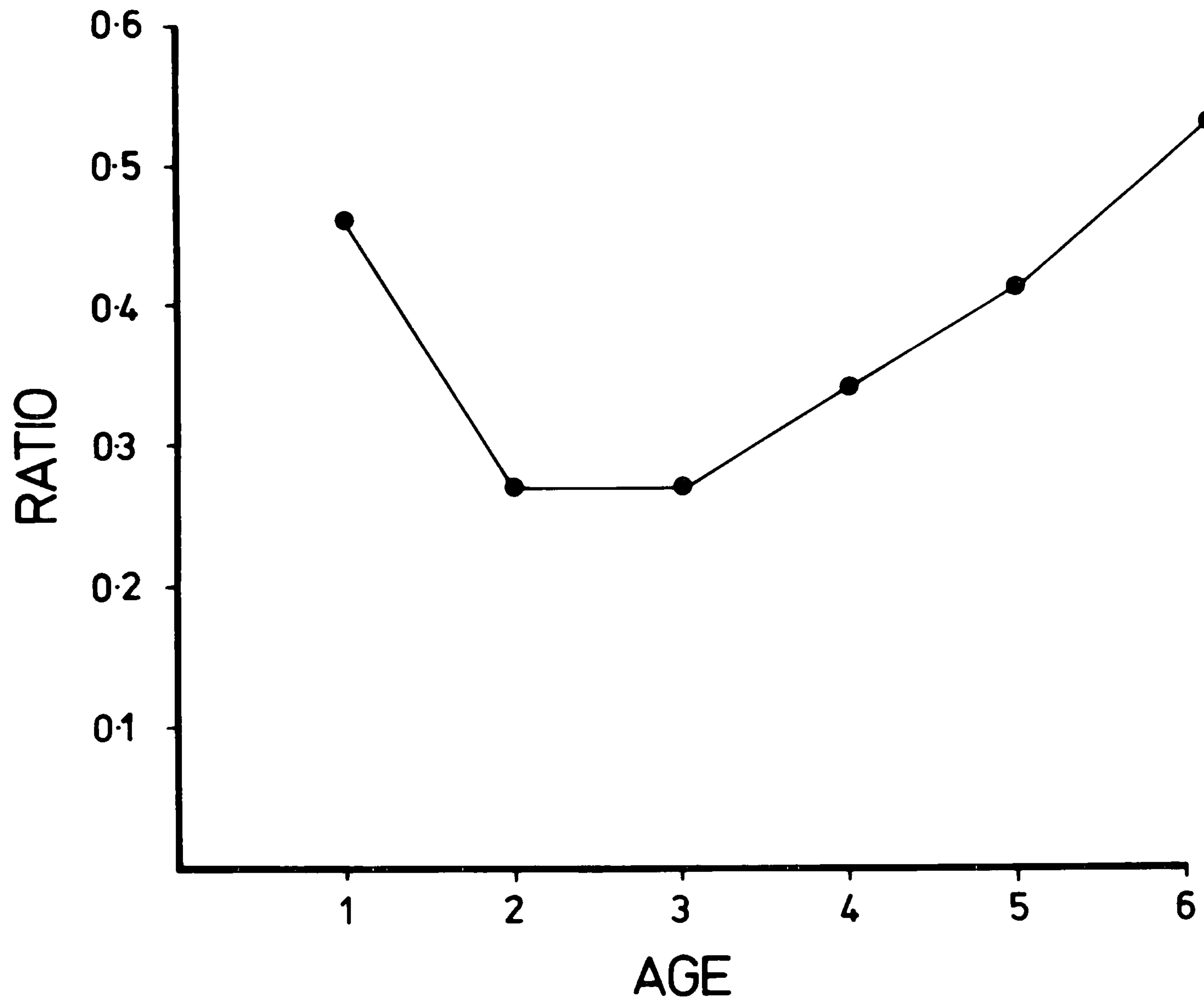
increase in protein synthesis during the period of the final ecdysis. (2) The increase may be due to improved extraction of mitochondria in the adult. It is possible that with pre-adult muscle the mitochondria which are smaller may be more fragile. This would lead to a decrease in the yield of the mitochondria extracted from 5th instar flight muscle. Whether the large differences in the level of mitochondrial protein observed between the 9th day of the 5th instar and the 1st day of adult life in the present study are real differences or exaggerated by variation in the efficiency of mitochondrial extraction is uncertain.. Nevertheless, the increase in size and cristal density of the mitochondria in 1-6 day old adult locusts suggest that an increase in mitochondrial protein is certainly occurring at this time. Other workers have reported large changes in mitochondrial protein at the time of final ecdysis. HOLMES and KEELEY (1975) reported an increase in mitochondrial protein from 4mg to 12.8mg per thorax over the period of final ecdysis in _Heliothis virescens_. In _Lucilia cuprina_, LENNIE and BIRT (1967) observed two periods of incorporation of protein into flight muscle mitochondria at the expense of soluble protein. The first period of protein incorporation was recorded immediately following pupation and the second period was during the pupal-adult ecdysis. WALKER and BIRT (1969), also working with _Lucilia cuprina_, reported that there was an increase in the specific gravity of the mitochondrial population over the period of the final ecdysis. In the present study a 3.2-fold increase was observed in mitochondrial protein during the first 6-days of adult life. Similar increases in thoracic mitochondrial protein (2.6-fold) have been reported for _Musca domestica_ (VAN DEN BERGH, 1962) and _Glossina morsitans_ (BURSELL, 1973) during the first 3 weeks of adult life, whilst RICHARD _et al_. (1971) reported a 7-fold increase in total mitochondrial protein from 4th day of 5th instar to 20th day of adult

life. It seems, therefore, that whilst mitochondrial growth is in part due to mitochondrial fusion (see earlier) nevertheless, considerable incorporation of new protein into mitochondria is also involved.

The change in the ratio of mitochondrial size to myofibrillar size suggest that the growth rates of these organelles are not constant (see Fig. 3.59). The ratio decreased from 0.45 on day 1 to 0.27 on day 3 of adult life and then increased to a value of 0.53 by day 6 of adult life. This latter value is similar to that reported by MICHEJDA (1964) in *Hyalophora cecropia*. When the relative proportions of total muscle volume occupied by mitochondria and myofibrils (see Table 3.2) were compared, a similar pattern emerged, i.e. an initial decrease in the ratio followed by an increase. On this basis, one can deduce that myofibrillar growth proceeds mitochondrial enlargement, but the latter subsequently restores the original relationship between the contractile machinery and the organelles supplying the energy for contraction.

RICHARD *et al.* (1971) have shown that the actin and myosin fractions constitute the major protein present in the flight muscle of *Schistocerca gregaria*. Furthermore, the relative proportion of these proteins increased from 50.9% of total muscle protein in 1-day old adults to 59.9% by the 4th day of adult life before stabilizing at this level. BÜCHER (1965) has reported that there is a substantial increase in the number of myofibrils in individual muscle fibres (from ca. 30 to 1000) during the "phase of duplication", from the 3rd-8th day following the final ecdysis. This observation is consistent with the fact that "longitudinal splitting" of myofibrils was observed in the present study during the first 4 days of adult life. As mentioned earlier, GOLDSPINK (1970) was the first to provide electron micrographs as evidence for myofibrillar splitting. He suggested that this mechanism explained the substantial increase in the number of

Figure 3.59. Effect of age on the ratio between mitochondrial size and myofibrillar size in flight muscle of *Locusta migratoria*.
The ratios were calculated from the mean values shown in Tables 3.3 and 3.1 for mitochondrial and myofibrillar size, respectively.


RATIO
0·6
0·5
0·4
0·3
0·2
0·1
1
2
3
4
5
6
AGE

myofibrils per muscle fibre in post-natal mouse development. Since this time myofibrillar splitting has been reported in insect and crustacean muscles (HUDDART, 1975). GOLDSPINK (1971) proposed a mechanism to explain "longitudinal splitting". He observed that the peripheral actin filaments run slightly obliquely from the Z-band, and suggested that when tension is developed by two adjacent sarcomeres the oblique pull on the actin filaments will produce stress in the centre of the Z-band. When the myofibril attains a certain thickness the tension developed will be sufficient to tear a hole in the centre of the Z-band and the rip would then extend to the end of the Z-band. The advantage of such a splitting process is that it allows the SR and T-system to develop at the same rate as the contractile apparatus and permits the mitochondria to become interspersed between the myofibrils (GOLDSPINK, 1970). The latter is important in that it ensures the availability of energy for mechanical activity. GOLDSPINK (1970) found that splitting myofibrils were about twice the size of non-splitting myofibrils. He concluded that, in mouse muscle development, this was the mechanism whereby myofibrils increase in number within a muscle rather than "new" myofibrils arising by *de novo* synthesis as has been reported in embryonic chick (FISCHMAN, 1967) and in the early stages of development in *Drosophila melanogaster* (SAFIQE, 1963) and *Calliphora erythrocephala* (AUBER, 1969). Thus "longitudinal splitting" of myofibrils would result in the production of smaller myofibrils. From the physiological stand-point, the latter is very important in fast-acting synchronous flight muscle, because the larger the myofibrils size, the greater is the distance between the central myofilaments and the surrounding SR (FARENBACH 1963; AUBER, 1967b;EBASHI and ENDO, 1968; ELDER, 1971). In other words, the Ca^{2+} would need a longer time to diffuse to the central myofilaments to trigger the contraction. This agrees with the fact that small

myofibrils are reported to be mechanically more active (ELDER, 1971, 1975). In contrast, in asynchronous flight muscle, myofibrillar splitting is not found beyond the early stages of formation in spite of a large addition of myosin filaments. Consequently the myofibrils grow to a very large size (AUBER, 1969).

The present study showed that in the first 4 days after the final ecdysis there was a 3.2-fold increase in myofibril size. A clear levelling off was seen around the 4th-5th day. This increase in myofibril size is also reflected in an increase in the number of myosin filaments per myofibril. Similarly, BURSELL et al. (1971) reported a marked correspondence between the increase in myofibril volume and the estimated contractile protein during the growth of Glossina morsitans. They found that the bulk of the contractile protein was synthesised by the 8th day of adult life. Working with the same species, ANDERSON and FINLAYSON (1973) confirmed this result by showing that the number of myosin filaments per myofibril increased during the same period. AUBER (1969) found that in Calliphora erythrocephala the number of thick filaments visible in a cross-section of a single myofibril of a dorsal longitudinal muscle fibre increased from 669-1186 at the time of ecdysis to a maximum of around 2000 after 10 days of adult life. In the present study, whilst the number of myosin filaments increased with age, no statistically significant changes in actin : myosin ratio (ca. 3:1) could be demonstrated over the 6-day period studied. It would appear, therefore, that both actin and myosin filaments increase at a more-or-less equivalent rate. This is in contrast to the finding of VALVASSORI et al. (1978), who reported that in the dragonfly Aeschna mixta the actin : myosin filaments ratio is quite high initially (ca. 4-4.5 : 1) but rapidly drops to the final 3:1 ratio when the myofibrils are well developed. Similar changes in the number of actins around each myosin have been reported

between the "slow larval" and "fast adult" of Manduca sexta dorsal longitudinal muscle (RHEUBEN and KAMMER, 1980). The ratio of 3:1 between actin and myosin observed in Locusta flight muscle is of widespread occurrence in flight muscle of both synchronous and asynchronous type, although several exceptions have been noted in synchronous flight muscle of certain species (SMITH, 1966a;PRINGLE, 1972; ELDER, 1975; HUDDART, 1975).

From the functional point of view, AUBER (1966) noted the correspondence of a slow work rhythm to a high ratio (greater than 3:1) of thin to thick filaments. In flight muscle this is illustrated by the 4:1 ratio found in butterflies Vanessa pieris in which the wing frequency is less than 10Hz (AUBER, 1967a,b) and 3:1 ratio in the flight muscle of Neoconocephalus robustus, which employs a maximum frequency of 212Hz (JOSEPHSON and HALVERSON, 1971). It would appear, therefore, that in flight muscle the hexagonal arrangement of actin and myosin is the most efficient for maximum force and speed of myofibrillar contraction (AUBER, 1967a). However, JAHROMI and ATWOOD (1969) reported that in Periplaneta americana leg, despite differences in the ratio of actin to myosin filaments in the A-band region of fibres of muscle 136 and 137 (6 actin : 1 myosin) and muscle 135a and 135c (3 actin : 1 myosin), no differences could be detected in the speed of contraction nor in the ability of these muscles to develop significantly different tensions.

The process whereby more actin and myosin are added to the myofibril is not known with certainty (GOLDSPINK, 1974). However, the study of AUBER (1965, 1969) on the addition of myofilaments in developing Calliphora erythrocephala has shown that myosin is added at the periphery of the myofibrils. The "new" myosin filaments often show a smaller diameter compared with that found in the middle of the myofibril, indicating that the construction of these peripheral

filaments may be incomplete. MORKIN (1970) has studied this phenomenon by using radioautography in conjunction with electron microscopy during the assembly of post-natal muscle fibre in chicken. He reported that the newly formed contractile proteins (both actin and myosin) are added to the outside of the peripheral region of the myofibril which does seem reasonable as it is difficult to imagine new myosin filaments being produced at, or being attached, to the centre of the myofibril (GOLDSPINK, 1974).

The average sarcomere length increased from 2.34μm in 1-day old adult locusts to 3.19μm in 6-day old adult locusts ($p > 0.001$). This was accompanied by the appearance of a well defined I-band. Similar increases have been reported in vertebrate muscle (GOLDSPINK, 1968) and in invertebrate muscles (ARONSON, 1961; SHAFIQ, 1963; AUBER, 1965, 1969). Two explanations of increasing sarcomere length have been suggested: (i) the deposition of new protein sub-units to the end of the myofilaments; (ii) the result of the sliding filament mechanism. AUBER (1969) has measured the sarcomere length in Calliphora erythrocephala during development, and reported that, whilst the sarcomere length increased from 2μm on the 6th day before to 3.2μm at ecdysis, no significant change in I-band size was noted at any stage. He concluded, therefore, that some increase in myofilaments length was taking place by deposition of new protein. However, GOLDSPINK (1968) has shown that in mice the sarcomeres which increase from 2.3μm in new born animals to 2.8μm in the adult do so by the sliding-myofilament mechanism rather than by a change in myofilament length. Whilst it is difficult to reach a final conclusion about the method of sarcomere elongation in Locusta, on the basis of the present study, the fact that the I-bands increase in size with increasing sarcomere length suggests that the sliding-myofilament explanation is the more appropriate here.

As indicated above, concomitant changes occur in SR and T-system during the growth and development of the other components. The SR begins as a network of tubules between the myofibrils and growth, and development takes place by the formation of dyadic junctions between SR and T-system at the level of the A-band. Similar results have been reported in synchronous slight muscle of *Antherea pernyi* (BIENZ-ISLER, 1968b). Throughout the present study both the SR and T-system were affected by the penetration of the tracheoles. These arise from tracheoblasts which invade the muscle fibres of locust flight muscle during the first few days before and after the final ecdysis (BÜCHER, 1965). As the tracheoblasts penetrate the muscle they draw the muscle cell membrane with them and consequently participate in distributing the T-system between the myofibril (SMITH, 1961, 1965; BÜCHER, 1965; DE KORT, 1969).

A correlation between the degree of SR development and mechanical function has been suggested (EDWARDS *et al.*, 1956). Similarly, SMITH (1962a) has suggested that a good correlation exists between the amount of SR (excluding asynchronous flight muscle) and the rate of relaxation found in insect flight muscle. TYRER (1973) has reported that in *Locusta* inter-segmental muscle, the maximal contraction rate is attained before the maximal relaxation rate and the former coincides with the development of the T-system and dyads. Subsequently, the full relaxation is attained when the SR is fully developed. Whether this is also true in *Locusta* flight muscle remains to be established.

CHAPTER 4

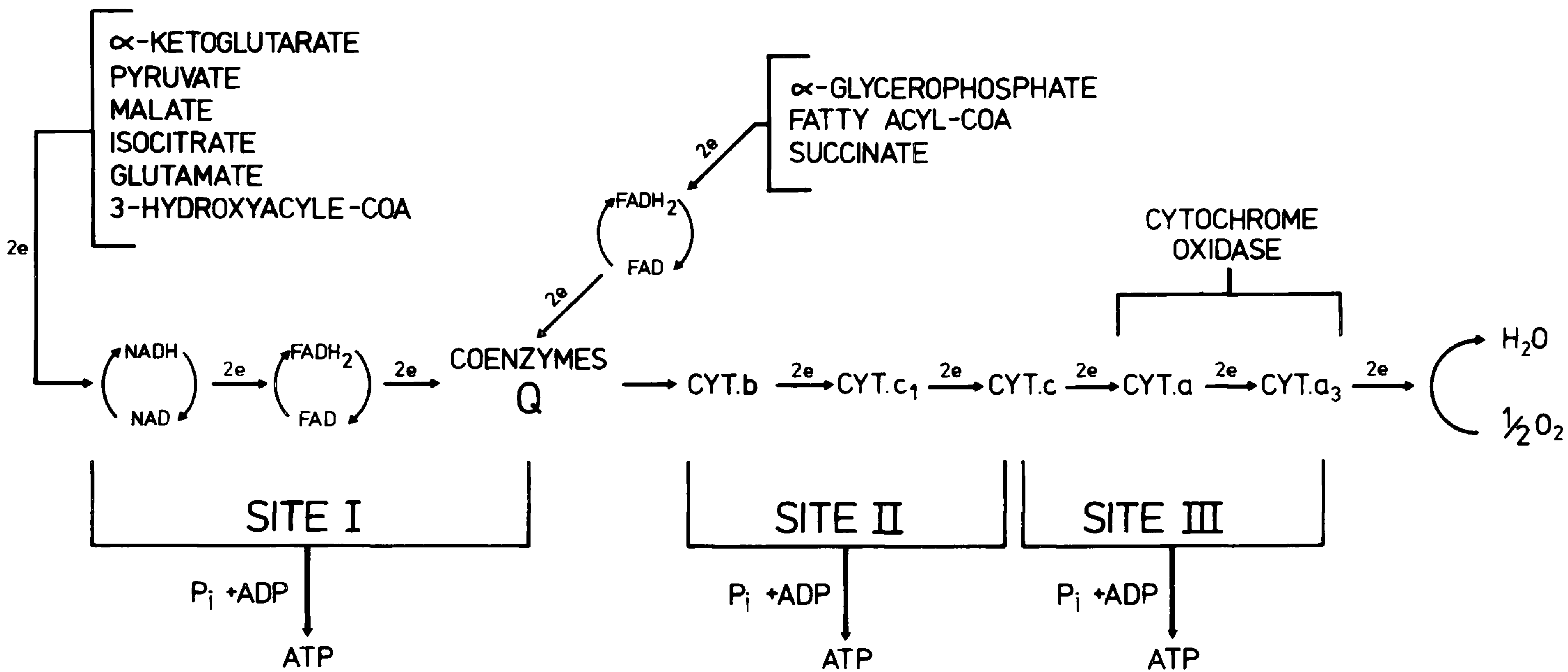
AGE RELATED CHANGES IN THE RESPIRATORY PHYSIOLOGY AND ENERGY PRODUCTION IN THE FLIGHT MUSCLE OF *LOCUSTA MIGRATORIA*

Introduction

The flight muscle mitochondria of adult *Locusta migratoria* occupies approximately 30% of the total flight muscle volume (see Chapter 3). These organelles contain the enzymes of the tricarboxylic acid cycle, the β-oxidation pathway and the respiratory chain (review by DE HAAN *et al*., 1973). The oxidation of the respiratory fuels by mitochondria leads to the production of hydrogen atoms (or the electrons derived from them) which then pass through the respiratory chain. Associated with the electron transport to oxygen via the respiratory chain, a coupling process links the reoxidation of the electron carrier to the phosphorylation of ADP to ATP at different sites along the chain (see Fig. 4.1). The oxidation of the NAD-linked substrates leads to the phosphorylation of ADP at three sites along the respiratory chain and to the formation of three molecules of ATP per atom of oxygen reduced (ADP:O ratio = 3). Other metabolites such as succinate and α-glycerophosphate are dehydrogenated by flavoproteins and consequently bye-pass the first phosphorylation site in the chain; the electrons therefore feed directly into ubiquinone with the result that only two molecules of ATP are formed (at sites II and III, Fig. 4.1) per atom of oxygen reduced (ADP:O ratio = 2).

The energy for muscular contraction during flight in *Locusta* is derived largely from the aerobic degradation of the final products of carbohydrates and lipids (BEENAKKERS, 1969; ANDY, 1970; see also reviews on insect flight muscle metabolism by BAILEY, 1975; SACKTOR, 1975, KAMMER and HEINRICH, 1978). Carbohydrate serves as the initial substrate, whereas

Figure 4.1. A schematic diagram of the respiratory chain and the points of entry of electrons from various substrates (after LEHNINGER, 1975).


∝-KETOGLUTARATE
PYRUVATE
MALATE
ISOCITRATE
GLUTAMATE
3-HYDROXYACYLE-COA
2e
NADH
NAD
2e
FADH$_2$
FAD
2e
COENZYMES
Q
∝-GLYCEROPHOSPHATE
FATTY ACYL-COA
SUCCINATE
2e
FADH$_2$
FAD
2e
CYT.b
2e
CYT.c$_1$
2e
CYT.c
2e
CYT.a
2e
CYT.a$_3$
2e
CYTOCHROME
OXIDASE
H_2O
$\frac{1}{2}O_2$
SITE I
SITE II
SITE III
P_i +ADP
ATP
P_i +ADP
ATP
P_i +ADP
ATP

during prolonged flight the utilization of stored lipid comes to prominence (WEIS-FOGH, 1952; MAYER and CANDY, 1969; JUTSUM and GOLDSWORTHY, 1976; VAN DER HORST et al., 1980; BEENAKKERS et al., 1981).

The respiratory metabolism of insect flight muscle has been extensively studied in various orders (reviews by SACKTOR, 1965, 1970, 1974, 1975). It is generally accepted that the respiratory organelles (sarcosomes) isolated from insect flight muscles are homologous with mitochondria from other animal tissues, such as liver, heart and skeletal muscle. WATANABE and WILLIAMS (1951, 1953) were the first to isolate such organelles from the flight muscle of the blowfly Phormia regina. They stated that the maintenance of the normal architecture of isolated mitochondria and the absence of swelling or shrinkage, must be regarded as important morphological criteria for establishing mitochondrial integrity. The latter is very important in preserving normal mitochondrial function. The isolation of mitochondria from insect flight muscle involves homogenization, centrifugation and resuspension. All these mechanical manipulations may cause damage to mitochondrial structure and function. CHEFURKA (1965) has suggested that discrepancies between the results of various early workers, using mitochondria from different tissue sources, was due largely to differences in methodology. Such differences involved both the methods used to assay respiratory activity and also the methods used in mitochondrial preparation. Various factors that may alter the stability of isolated mitochondria and cause a deterioration in their function have been enumerated by CHEFURKA (1965) and HARVEY and HASKELL (1966). These include the composition of the isolation and reaction media, the extent of homogenization, ageing of mitochondria in vitro, concentration of the reactants (substrate, ADP and phosphate) and the age of the insect.

The various methods of isolation of insect mitochondria are based largely on the techniques originally reported by WATANABE and WILLIAMS (1951). Since this time, numerous researchers have used this basic method with slight modifications to study mitochondria from a variety of different insect species, for example, from *Calliphora erythrocephala* (LEWIS and SLATER, 1954; TRIBE, 1967; DAVISON and BOWLER, 1971; BOWLER and KASHMEERY, 1981), *Apis mellifera* (BALBONI, 1965), *Periplaneta americana* (COCHRAN, 1963), *Prodenia eridania* (STEVENSON, 1966), *Musca domestica* (SACKTOR and COCHRAN, 1958; GREGG *et al.*, 1959, 1960. VAN DEN BERGH, 1962), *Leptinotarsa decemlineata* (STEGWEE and VAN KAMMEN-WERTH, 1962) and *Locusta migratoria* (KLINGENBERG and BÜCHER, 1959; MINKS, 1967).

More recently, a number of workers have used the proteolytic enzyme, Nagarse, in the isolation of insect mitochondria, for example, from flight muscle of *Lucilia cuprina* (BYGRAVE *et al.*, 1975), *Sarcophaga bullata* (WOHLRAB, 1976), *Manduca sexta* (HANSFORD and JOHNSON, 1976), *Leptinotarsa decemlineata* (WEEDA *et al.*, 1980), *Calliphora erythrocephala* (BOWLER and KASHMEERY, 1981) and *Leptinotarsa decemlineata* and *Locusta migratoria* (KHAN and DE KORT, 1978). Nagarse was first used by CHANCE and HAGIHARA (1961) to isolate mitochondria from heart muscle, and later by CHAPPELL and HANSFORD (1972). The latter authors stated that the essence of this method is the total digestion of the myofibrils and the elimination of the necessity for low-speed centrifugation. Furthermore, the myofibril-digestion facilitates mitochondrial extraction by mild homogenization.

It has been known that the composition of the reaction medium is an important factor in determining the respiratory activity of isolated mitochondria (CHEFURKA, 1965; HARVEY and HASKELL, 1966). A wide variety of reaction media have been used in studies on flight muscle mitochondrial function from various species, for example, in studies on

mitochondria from Musca domestica (BIRT, 1961; VAN DEN BERGH and SLATER, 1962), Hyalophora cecropia (MICHEJDA, 1964), Phormia regina (SACKTOR and CHILDRESS, 1967; BULOS et al., 1972), Apis mellifera (BALBONI, 1968), Calliphora erythrocephala (HANSFORD, 1972; TRIBE and ASHHURST, 1972; BOWLER and KASHMEERY, 1981) and Sarcophaga nodosa and Glossina morsitans (SLACK and BURSELL, 1976a).

The age of the experimental animal has been regarded as important with an undeniable influence on the properties and respiratory efficiency of the isolated mitochondria. BAKER (1976) has reported that the attainment of optimal flight performance in dipteran insects is intimately linked with the maturation of the metabolic processes. This implies an increase in the activities of enzymes associated with the energy yielding pathways and the accumulation of fuels necessary for flight. The development of the main metabolic pathways of Calliphora erythrocephala, Locusta migratoria and Philosamia cynthia have been reported by BEENAKKERS et al. (1975). Associated with the developmental changes in the biochemical properties of the flight muscles in these insects, the complexity of mitochondrial ultrastructure increased with age (see also Chapter 3). WOHLRAB (1976) has shown that the ability of mitochondria to oxidize various substrates increased with age in Sarcophaga bullata.

It has been reported that the wing-beat frequency of Drosophila funebris and Phormia regina increase with age to a maximum after the 6th day of adult life (LEVENBROOK and WILLIAMS, 1956). This has been correlated to respiratory performance and the mitochondrial content of cytochrome c. LEWIS and SLATER (1954) showed that isolated mitochondria of the blowfly, Calliphora erythrocephala, exhibited submaximal oxidative phosphorylation during the first week of adult life, whereas VAN DEN BERGH (1962) showed that maximal respiratory activity could be demonstrated at all times during the first 3 weeks of adult life in

Musca domestica. BALBONI (1967) reported little or no respiratory control by ADP on oxidative phosphorylation in mitochondrial preparations from young honeybee, *Apis mellifera*. However, he suggested that this may not necessarily mean that the mitochondria lacked these functions, but may be due to an age-related mitochondrial fragility. A systematic study of mitochondrial performance during the development of the *Hyalophora cecropia*, over the period from 15th day old pupal development up to the 8th day of adult life, showed that oxidative phosphorylation remained approximately at the same level in all stages studied (MICHEJDA, 1964). Moreover, in the early stages the oxidation of α-glycerophosphate, pyruvate plus fumarate or malate was twice as high as that of succinate. Increasing age resulted in the reverse, that is, the oxidation of succinate increased whereas that of NAD-linked substrates decreased.

Several studies have shown that the activity of the intra-mitochondrial enzymes increase with age to reach maximal levels at the time of maturity, for example, in flight muscle mitochondria of *Leptinotarsa decemlineata* (DE KORT, 1969), *Musca domestica* (ROCKSTEIN, 1972; BEEZELEY *et al.*, 1974) and *Locusta migratoria* (BROSEMER *et al.*, 1963; BÜCHER, 1965). In addition, the study of BEENAKKERS *et al.* (1975) showed that the key enzymes in the metabolic pathways reach maturity within the first week of adult life in *Locusta*. These observations are consistent with the changes in flight muscle ultrastructure observed during the first 6 days of adult life (Chapter 3). However, KLINGENBERG and BÜCHER (1959) reported that respiratory control could only be demonstrated when locusts become 10-15 day old adults. This implies that mitochondrial respiration is uncoupled during the early stages of locust flight muscle development.

In view of the role played by flight muscle mitochondria in the synthesis of ATP, which is frequently referred to as the energy currency of the living cell (SLATER, 1972), the respiratory activity of developing mitochondria has been studied in Locusta to detect changes that may occur in mitochondrial function with age.

Materials and Methods

Unless otherwise stated, locusts of known age were used and they were reared and collected as described previously (Chapter 2). Similarly, the methods of flight muscle mitochondrial extraction and the method of determination of QO_2 (respiratory rate of state 3 and state 4), RCR and ADP:O ratio have already been mentioned in Chapter 2.

Results

Effect of reaction media on substrate oxidation and ADP phosphorylation by isolated flight muscle mitochondria

It was impossible to test all the reaction media that have been reported by various workers in the field of mitochondrial research. However, four reaction media were tested to establish the most suitable for the measurement of the respiratory activity of isolated mitochondria from developing flight muscle of Locusta. Table 4.1 shows the composition of these media. Those media which enabled good RCR and ADP:O ratios to be demonstrated were taken to be the most suitable and these were used in all subsequent studies unless otherwise stated.

Table 4.2 shows a representative set of data obtained in a single series of experiments using mitochondria isolated from 8-day old adults. It can be clearly seen that in all cases, apart from reaction medium 2, α-glycerophosphate was rapidly metabolized. However, the inclusion of BSA in the reaction media was necessary for oxidative phosphorylation (i.e. ADP:O ratio) to be measured with this substrate. Similarly, RCR

Table 4.1. The composition of the reaction media used.

Composition (mM)	Reaction medium			
	1	2	3	4
KCl	154	154	15	15
Tris	10	10	50	50
K_2HPO_4	30	30	30	30
EDTA	-	-	2	2
$MgCl_2$	-	5	5	5
BSA (%)	0.04	-	-	0.12

All media were adjusted to pH 7.3 at 30°C with HCl.

Table 4.2. The effect of different reaction media and substrates on the respiratory activity of isolated mitochondria from 8-day old adult Locusts.

		Reaction media											
		1			2			3			4		
Substrate		α-GP	pyruvate + proline	glutamate	α-GP	pyruvate + proline	glutamate	α-GP	pyruvate + proline	glutamate	α-GP	pyruvate + proline	glutamate
QO_2	state 4	5.19	2.40	2.40	2.62	2.50	0.82	7.21	3.91	3.82	6.55	5.30	4.25
	state 3	12.55	13.57	6.16	4.11	9.21	1.92	16.19	7.31	5.63	14.67	17.94	10.66
RCR		2.42	5.65	2.73	1.57*	3.68	2.81*	2.25*	1.78	1.53	2.24	3.39	2.68
ADP:O		1.32	2.82	2.82	n.m.	2.56	n.m.	n.m.	2.72	2.59	1.70	3.20	3.10

State 3 and 4 respiration expressed as μg AO. mg protein^{-1}. hour^{-1}.

Substrate concentration was 5mM throughout.

See Table 4.1. for the composition of the reaction media.

n.m. = not measurable.

(*) indicates RCR was calculated as the ratio of state 3 activity to state 2 (i.e. before ADP added).

Typical set of data representative for three experiments (see Appendix 4.1.).

was lower in the absence of BSA. The apparently high RCR value obtained with medium 3 is due to the different method of calculation (see footnote to Table 4.2).

Similarly, with pyruvate plus proline as the substrate, the best results, in terms of good RCR values, were observed when BSA was included in the reaction media (i.e. with media 1 and 4, Table 4.2). Nevertheless, normal ADP:O ratios were observed with all four reaction media. Once again, the best results were observed with reaction media 1 and 4.

Glutamate was oxidized by isolated mitochondria in all four reaction media tested, with good RCR and near theoretical values for ADP:O ratios being recorded; except with medium 2.

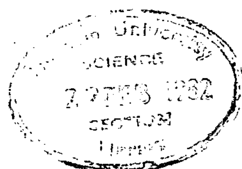
On the basis of these findings, it was concluded that reaction media 1 and 4 were the most suitable and consequently these two media were used in subsequent studies carried out on developing flight muscle mitochondria.

The utilization of various substrates by isolated mitochondria

Pyruvate plus proline

Presented in Figure 4.2 is a representative example of the pattern of response to successive additions of ADP, when the substrate used was pyruvate plus proline. Table 4.3 shows the mean data extracted from such oxygen electrode traces. It can be clearly seen that, in the presence of medium 4 and substrate (state 2, CHANCE and WILLIAMS, 1955a), the mitochondrial respiratory rate was negligible (see Fig. 4.2). Addition of 0.5μmoles of ADP evoked a sharp increase in the rate of respiration (state 3, CHANCE and WILLIAMS, 1955a). Eventually, the respiratory rate returned to a level not too different from the prestimulated one (state 4). It is suggested that this occurs when the added ADP has been phosphorylated to ATP (CHANCE and WILLIAMS, 1955b). A similar response was observed with subsequent additions of ADP to the reaction chamber.

Figure 4.2. A representative polarographic trace showing the respiratory response of mitochondria to successive ADP additions.
The mitochondria were isolated from 8-day old adults and the substrate was pyruvate + proline in the presence of reaction medium 4 (see Table 4.1.).



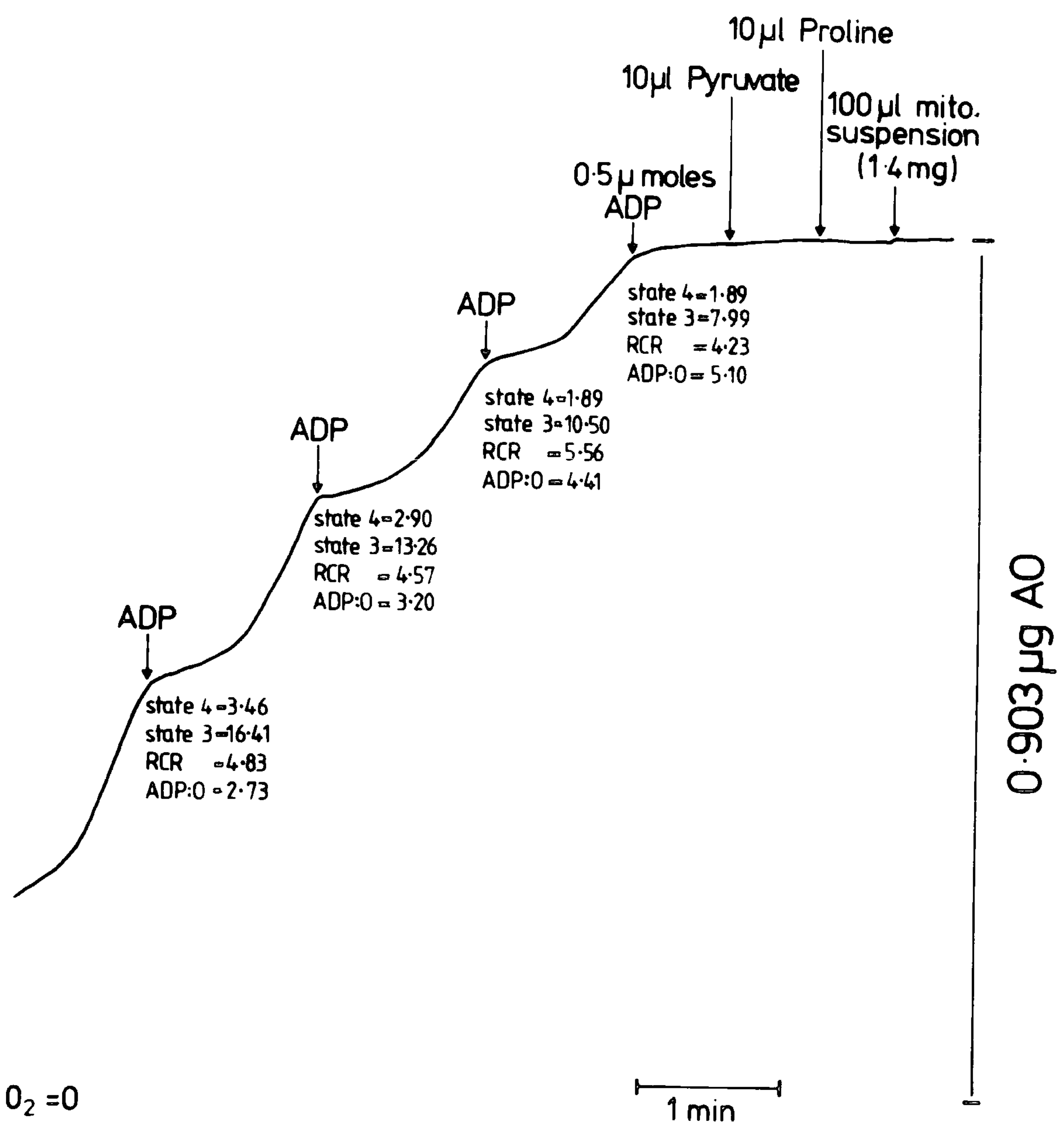

10µl Proline
10µl Pyruvate
100µl mito. suspension (1·4mg)
0·5µ moles ADP
ADP
ADP
ADP
state 4=1·89
state 3=7·99
RCR =4·23
ADP:0= 5·10
state 4=1·89
state 3=10·50
RCR =5·56
ADP:0=4·41
state 4=2·90
state 3=13·26
RCR =4·57
ADP:0 = 3·20
state 4=3·46
state 3=16·41
RCR =4·83
ADP:0 =2·73
0·903µg AO
0₂ =0
1 min

Table 4.3. The effect of successive ADP additions on the mitochondrial respiration with pyruvate (5mM) plus proline (mM) as substrate.

ADP addition No.	QO_2		RCR	ADP:O ratio
	state 4	state 3		
1	2.05 ± 0.54	12.57 ± 2.81	6.99 ± 1.52	3.89 ± 0.67
2	2.12 ± 0.19	13.81 ± 2.47	6.42 ± 0.80	3.54 ± 0.52
3	2.73 ± 0.69	14.62 ± 2.25	5.83 ± 0.80	2.94 ± 0.32
4	2.64 ± 0.68	16.34 ± 3.83	6.48 ± 0.58	2.63 ± 0.16
p (1 vs 4)	n.s.	n.s.	n.s.	n.s.

The data represent the mean obtained in four separate experiments ± S.E.M.
Reaction medium 4 was used (see Table 4.1. for composition).
n.s. : not significant.

Reference to Table 4.3 shows that there was no significant change in respiratory parameters with successive additions of ADP. However, the oxygen electrode trace shown in Figure 4.2 indicates that there was an increase in the respiratory rate with each successive ADP addition. This discrepancy may be due to the high standard error encountered (see Table 4.3).

A significant change in respiratory rate of state 4 ($p > 0.02$) and state 3 ($p > 0.001$) respiration was observed between the first and fourth addition of ADP, when reaction medium 1 was used (see Table 4.4). In addition, the ADP:O ratio changed significantly ($p < 0.001$) between the first and fourth addition of ADP. However, no significant change was observed in RCR.

Glutamate

The pattern of response to successive ADP additions, when glutamate is metabolized by isolated mitochondria (Fig. 4.3), was qualitatively similar to that of pyruvate plus proline. The data presented in Table 4.5 clearly shows the effect of successive additions of ADP on the state 3 and 4 respiratory rates. When reaction medium 4 was used, the effect was similar to that observed with pyruvate plus proline (see Table 4.4); the state 3 and state 4 respiratory rates increased with each ADP addition, once again little change was observed in RCR but a significant decrease ($p < 0.02$) was recorded in ADP:O ratio between the initial and fourth addition of ADP (Table 4.5).

Table 4.6 represents the data derived from experiments in which assays were carried out in reaction medium 1. There was a significant increase in the state 4 respiratory rate ($p > 0.02$) and that of state 3 ($p < 0.02$) between the initial and fourth addition of ADP. However, little change was observed in either RCR or the ADP:O ratio.

Table 4.4. The effect of successive ADP additions on the mitochondrial respiration with pyruvate (5mM) plus proline (5mM) as substrate.

ADP addition No.	QO_2		RCR	ADP:O ratio
	state 4	state 3		
1	1.70 ± 0.18	8.64 ± 1.17	5.07 ± 0.33	5.54 ± 0.27
2	1.98 ± 0.26	16.92 ± 2.20	5.60 ± 0.11	4.71 ± 0.27
3	2.74 ± 0.32	13.9 ± 1.96	5.11 ± 0.21	3.45 ± 0.15
4	3.29 ± 0.66	17.01 ± 2.26	5.27 ± 0.42	2.71 ± 0.06
p (1 vs 4)	> 0.02	> 0.01	n.s.	< 0.001

The data represent the mean obtained in four separate experiments ± S.E.M.
Reaction medium 1 was used (see Table 4.1. for composition).
n.s. : not significant

Figure 4.3. A representative polarographic trace showing the respiratory response of mitochondria to successive ADP additions.

The mitochondria were isolated from 8-day old adults and the substrate was glutamate in the presence of reaction medium 4 (see Table 4.1).

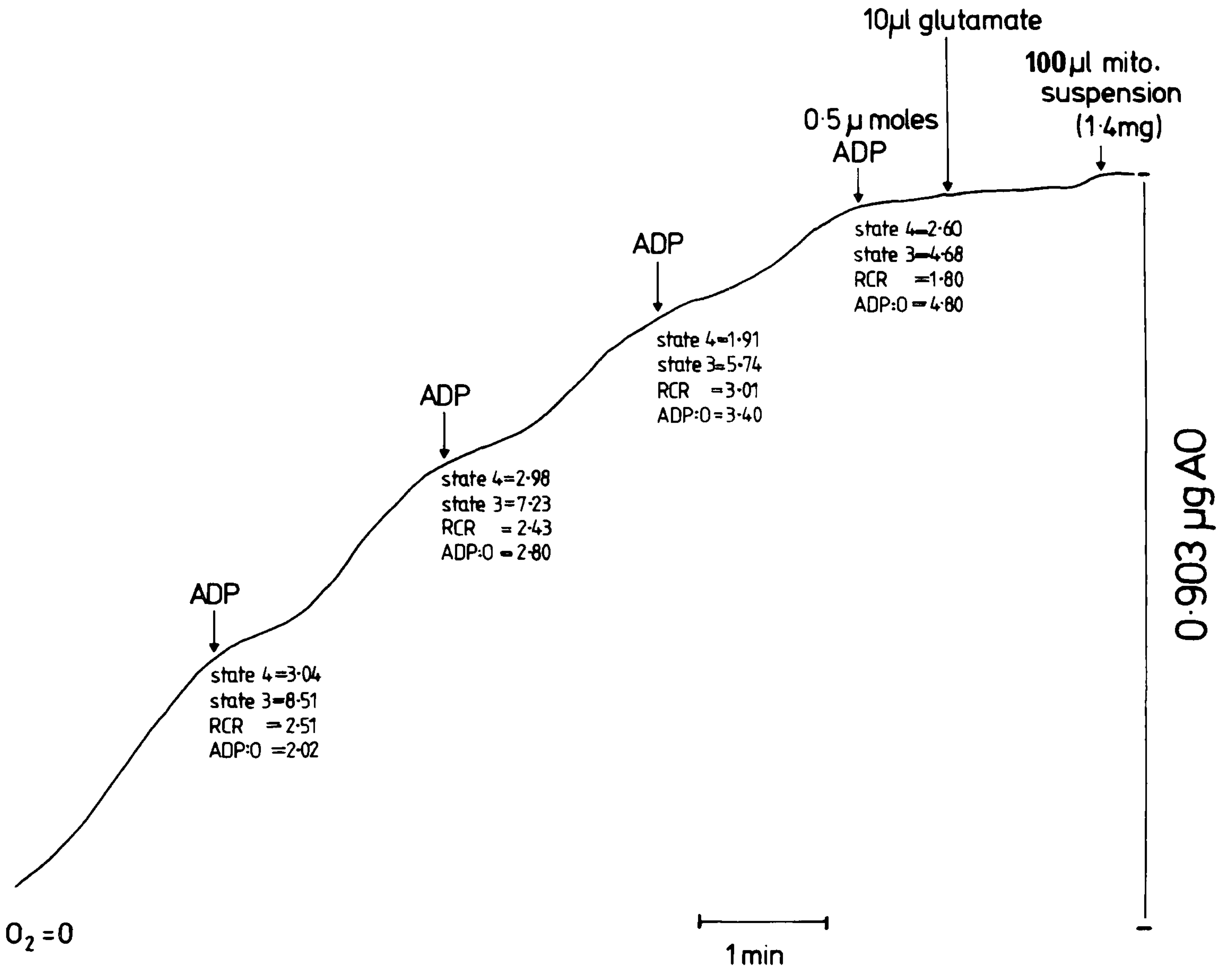

10µl glutamate
100µl mito. suspension (1·4mg)
0·5 µ moles ADP
ADP
ADP
ADP
state 4=2·60
state 3=4·68
RCR =1·80
ADP:0 =4·80
state 4=1·91
state 3=5·74
RCR =3·01
ADP:0=3·40
state 4=2·98
state 3=7·23
RCR =2·43
ADP:0 =2·80
state 4=3·04
state 3=8·51
RCR =2·51
ADP:0 =2·02
0·903 µg AO
O_2 =0
1 min

Table 4.5. The effect of successive additions of ADP on the mitochondrial respiration with glutamate as substrate (5mM).

ADP addition No.	QO_2		RCR	ADP:O ratio
	state 4	state 3		
1	1.39 ± 0.41	5.49 ± 0.37	4.9 ± 1.13	3.61 ± 0.41
2	1.70 ± 0.28	6.38 ± 0.36	4.05 ± 0.64	2.91 ± 0.19
3	2.49 ± 0.18	7.73 ± 0.59	3.14 ± 0.29	2.59 ± 0.19
4	2.71 ± 0.31	8.66 ± 0.55	3.22 ± 0.32	2.28 ± 0.12
p (1 vs 4)	$p > 0.02$	$p < 0.01$	n.s.	$p < 0.02$

The data represent the mean obtained in four separate experiments ± S.E.M.
Reaction medium 4 was used (see Table 4.1. for composition).
n.s. : not significant.

Table 4.6. The effect of successive ADP additions on the mitochondrial respiration with glutamate as substrate (5mM).

ADP addition No.	QO_2		RCR	ADP:O ratio
	state 4	state 3		
1	2.59 ± 0.24	6.13 ± 0.72	2.48 ± 0.52	3.22 ± 0.60
2	2.52 ± 0.49	8.68 ± 1.60	3.75 ± 0.79	2.87 ± 0.24
3	3.09 ± 0.26	10.01 ± 1.66	3.31 ± 0.58	2.54 ± 0.12
4	3.74 ± 0.29	11.65 ± 1.51	2.96 ± 0.33	2.24 ± 0.10
p (1 vs 4)	> 0.02	< 0.02	n.s.	n.s.

The data represent the mean obtained in four separate experiments ± S.E.M.
Reaction medium 1 was used (see Table 4.1 for composition).
n.s. : not significant.

α-Glycerophosphate

In contrast to the traces of the two substrates referred to above, when α-glycerophosphate was used as substrate, the state 2 respiratory rate was significant (see Fig. 4.4). However, the response to successive additions of ADP to the reaction chamber was similar to that observed with both pyruvate plus proline and glutamate. The oxygen electrode trace referred to above was obtained from respiratory activity of isolated mitochondria assayed in reaction medium 4. The data presented in Table 4.7 shows that, apart from a significant decrease ($p < 0.001$) in ADP:O ratio between the initial addition of ADP and the fourth such addition, little change was observed in the state 3 and state 4 respiratory rates and the RCR.

When reaction medium 1 was used, no significant change in ADP:O ratio, RCR or state 3 and state 4 respiration was observed between the initial and third ADP addition (Table 4.8).

Succinate

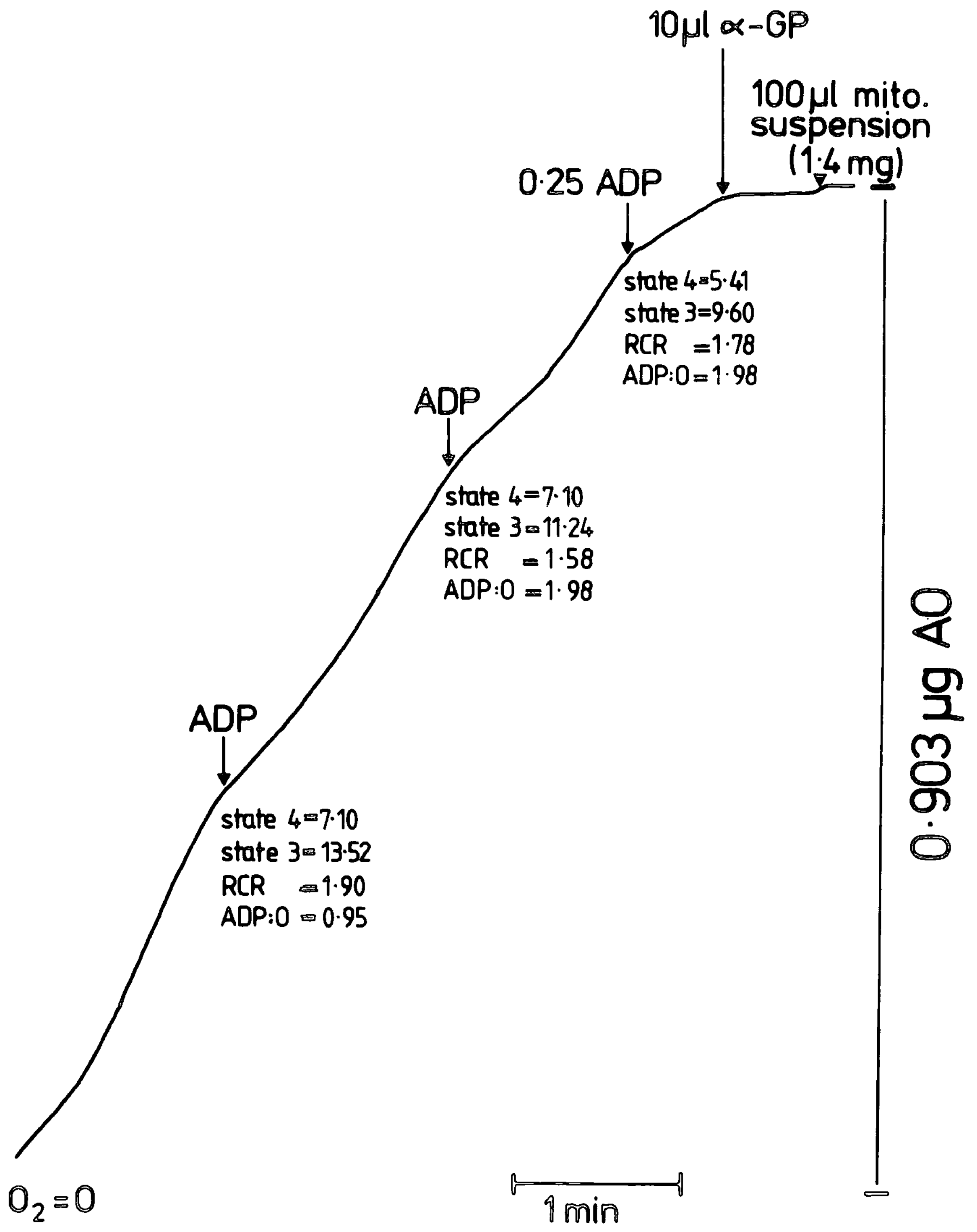
When succinate was used, the pattern of response to the addition of ADP was completely different from that of the other substrates referred to above. As with the other substrates ADP-stimulated respiration, however, the ADP-stimulated rate remained unchanged over the period of measurement (Fig. 4.5). Consequently, an ADP:O ratio could not be calculated by using the CHANCE and WILLIAMS methods (1955b). The RCR was calculated as the ratio between the respiratory rate after ADP addition and that before (state 2).

Effect of proline on pyruvate utilization

SACKTOR and WORMSER-SHAVIT (1966) have suggested that, when pyruvate is used as substrate, it is necessary to include proline so that the tricarboxylic acid cycle intermediates, which may leak out during isolation, are replaced. To determine the extent to which this

Figure 4.4. A representative polarographic trace showing the respiratory response of mitochondria to successive ADP additions.

The mitochondria were isolated from 8-day old adults and the substrate was α-glycerophosphate in the presence of reaction medium 4 (see Table 4.1).



10µl ∝-GP
100µl mito. suspension (1.4mg)
0·25 ADP
state 4=5·41
state 3=9·60
RCR =1·78
ADP:O=1·98
ADP
state 4=7·10
state 3=11·24
RCR =1·58
ADP:O =1·98
ADP
state 4=7·10
state 3=13·52
RCR =1·90
ADP:O =0·95
O2=0
1 min
0·903 µg AO

Table 4.7. The effect of successive ADP additions on the mitochondrial respiration with α-glycerophosphate as substrate (5mM).

ADP addition No.	QO_2		RCR	ADP:O ratio
	state 4	state 3		
1	1.93 ± 0.25	3.92 ± 0.38	2.08 ± 0.12	1.55 ± 0.01
2	2.24 ± 0.32	4.10 ± 0.50	1.91 ± 0.13	1.24 ± 0.04
3	2.2 ± 0.27	4.18 ± 0.45	1.92 ± 0.08	1.14 ± 0.034
p (1 vs 3)	n.s.	n.s.	n.s.	$p < 0.001$

The data represent the mean obtained in five separate experiments ± S.E.M. Mitochondria were isolated from 1-day old adult locusts and reaction medium 4 was used (see Table 4.1 for composition).
n.s. : not significant.

Table 4.8. The effect of successive ADP additions on mitochondrial respiration with α-glycerophosphate as substrate (5mM).

No. of ADP additions	QO_2		RCR	ADP:O
	State 4	State 3		
1	6.98 ± 1.23	14.51 ± 2.73	2.08 ± 0.11	2.28 ± 0.15
2	7.43 ± 1.36	15.26 ± 3.04	2.15 ± 0.22	2.07 ± 0.07
3	7.29 ± 1.17	17.51 ± 2.98	2.45 ± 0.26	2.15 ± 0.05
1:3 (P)	n.s.	n.s.	n.s.	n.s.

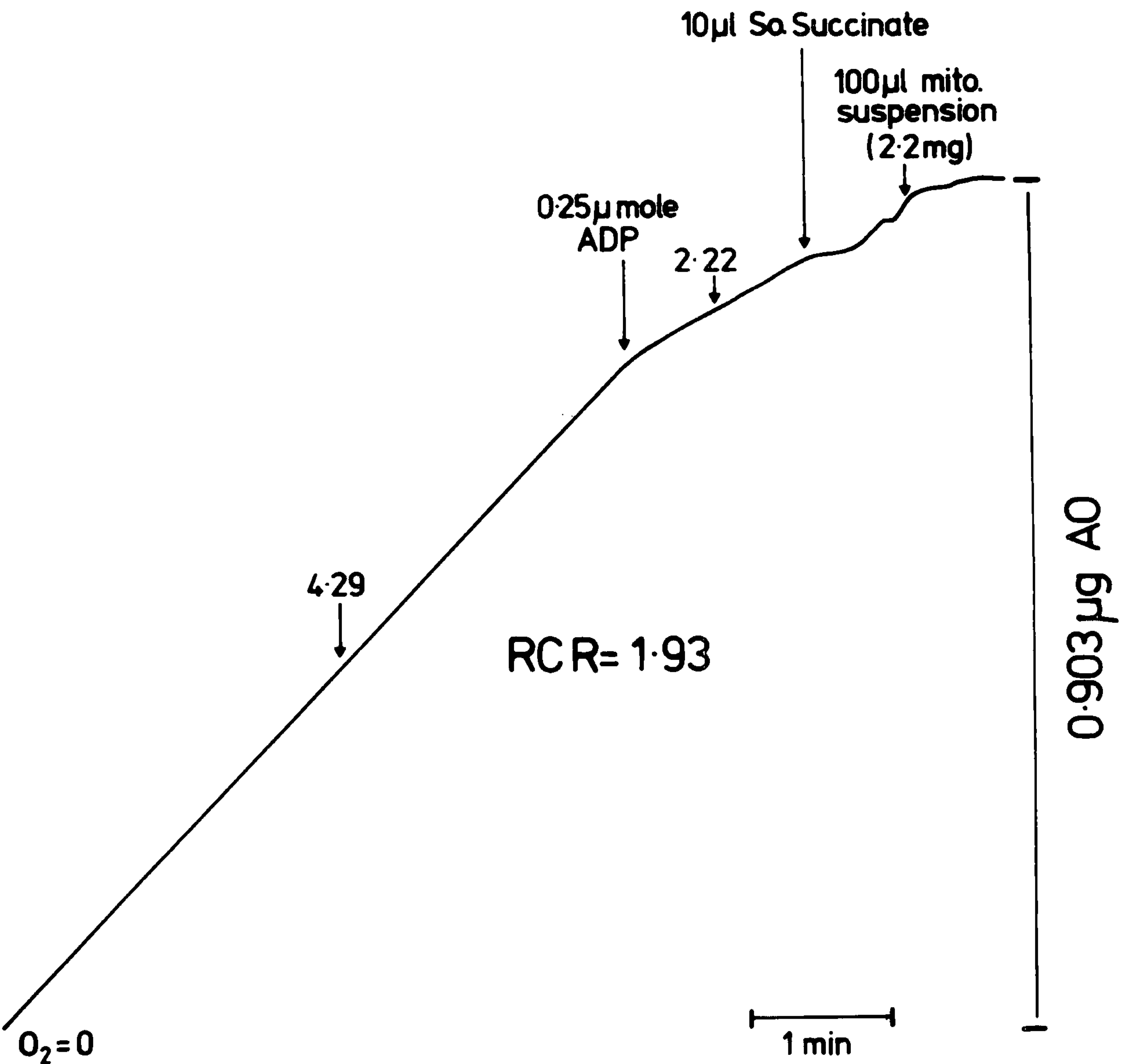
Each value represents the mean ± S.E.M. of four separate experiments.

Reaction medium 1 was used (see Table 4.1.).

n.s. : not significant.

Figure 4. 5. A representative polarographic trace showing the respiratory response of mitochondria to successive ADP additions.

The mitochondria were isolated from 8-day old adults and the substrate was succinate in the presence of reaction medium 4 (see Table 4.1).


10µl Sa Succinate
100µl mito.
suspension
(2·2mg)
0·25µ mole
ADP
2·22
4·29
RCR= 1·93
0·903µg AO
O2=0
1 min

applied to the flight muscle mitochondria of Locusta migratoria a series of experiments were conducted using reaction media 1 and 4. The data obtained from such studies are presented in Table 4.9. It can be clearly seen that, with both reaction media, there was no significant change in all respiratory parameters. This suggests that isolated mitochondria from Locusta flight muscle do not require proline for pyruvate utilization (i.e. the tricarboxylic acid cycle is available). However, to ensure that this was so in all stages of Locusta mitochondria during development, especially in the younger stages when mitochondria are more fragile, proline was included when pyruvate was used as substrate.

Developmental changes in the energetic capabilities of isolated mitochondria from Locusta flight muscle

The essence of this study is to establish whether developing flight muscle mitochondria of Locusta contain the systems which can couple oxidation of various substrates to ADP phosphorylation. The results referred to above suggest that the two reaction media (1 and 4) gave good respiratory activity for all parameters studied and, therefore, these were used to assess the energetic capabilities of isolated mitochondria. As was indicated above, the respiratory parameters occasionally show changes with successive ADP additions. This makes it difficult to evaluate the developmental changes in mitochondrial function. To overcome this problem a similar number of ADP additions were employed for each preparation at each age and the mean value calculated.

Pyruvate plus proline - supporting respiration

Figures 4.6 and 4.7 show the rate of oxygen consumption observed with mitochondria isolated from flight muscles at different ages, using medium 1 and 4, respectively. No significant change in state 4

Table 4.9. The effect of proline on pyruvate oxidation by mitochondria isolated from 10-day old adult locusts.

Substrate	Reaction medium	QO_2		RCR	ADP:O	n
		State 4	State 3			
Pyruvate (10mM)	1	0.69 ± 0.21	8.23 ± 0.41	14.65 ± 3.44	4.18 ± 0.37	4
Pyruvate (10mM) Proline (5mM)	1	1.23 ± 0.23	8.69 ± 0.56	7.72 ± 1.67	3.94 ± 0.39	4
Pyruvate (10mM)	4	0.59 ± 0.14	6.88 ± 0.63	13.05 ± 2.88	4.13 ± 0.40	3
Pyruvate (10mM) Proline (5mM)	4	0.99 ± 0.20	5.66 ± 0.50	6.26 ± 1.50	3.54 ± 0.29	3

The composition of the different reaction media are given in Table 4.1.

n: the number of separate experiments.

The data are expressed as the mean ± S.E.M.

QO_2: μg AO mg protein^{-1} hour^{-1}.

respiration was observed over the adult period studied irrespective of which reaction medium was used. However, when reaction medium 4 was used, the state 4 rate of respiration increased approximately 3-fold between the 9th day of the 5th instar and the 4th day of adult life (see Fig. 4.7). In contrast, state 3 rate of respiration increased significantly ($p < 0.001$) between the 3rd and 7th day of adult life when medium 1 was used (Fig. 4.6). No significant differences were observed in this period when reaction medium 4 was used. Nevertheless, there was a significant difference between the state 3 rate of respiration of 4-day old adult mitochondria and those of late 5th instar animals ($p = 0.001$) and between the 1st and 4th day of adult life when reaction medium 4 was used ($p < 0.02$) (see Fig. 4.7). Apart from this, the main differences observed when the two reaction media were used concerns the levels of activity measured; the maximal level of state 3 rate of respiration with medium 1 being approximately 20.0μg AO mg $protein^{-1}$ $hour^{-1}$ which is almost twice that observed with reaction medium 4.

No marked variation in ADP:O ratio was observed between the 9th day of the 5th instar and the 7th day of adult life when reaction medium 4 was used (Fig. 4.8). In contrast, the ADP:O ratio could not be measured prior to the 2nd day of adult life when medium 1 was used (Fig. 4.9). Yet another difference was the effect of the two media on RCR. The value for RCR was largely unchanged throughout the period studied (Fig. 4.8) when medium 4 was used. In contrast, when medium 1 was used RCR increased from 1.53 ± 0.29 ($n = 4$) on the 1st day of adult life to 8.27 ± 0.2 ($n = 3$) by day 8. It is clear, therefore, that the choice of reaction medium is important in assessing the respiratory competence of isolated mitochondria at different ages (based on RCR and ADP:O ratio); medium 4 would have been considered superior to medium 1 up to the 1st day of adult life whereas medium 1 would have been preferred thereafter.

Figure 4.6. Effect of age on the respiratory rate of mitochondria isolated from flight muscle of *Locusta*.

The substrate was pyruvate (5mM) plus proline (5mM) in the presence of reaction medium 1.

Ordinate: QO_2 is expressed in µg AO. mg protein^{-1}. hour^{-1}

Abscissa: Age in days

The figures in parentheses indicate the number of separate determinations for state 4 and state 3 respiration.

⊙ state 4 respiration

O state 3 respiration

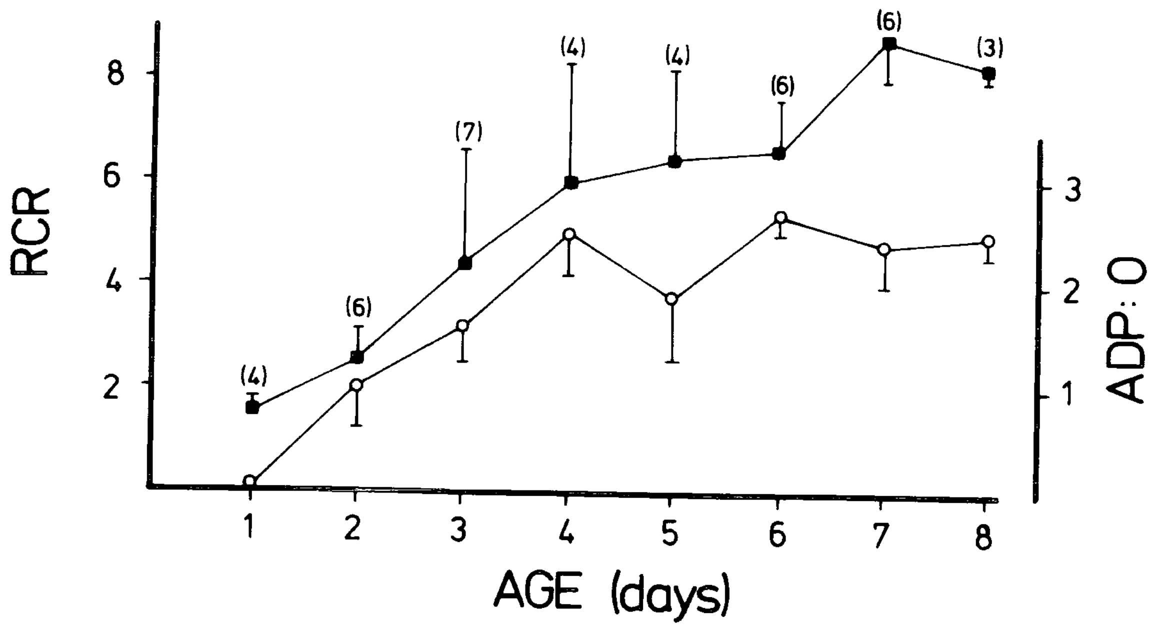
Figure 4.9. Effect of age on RCR and ADP:O ratio of mitochondria isolated from flight muscle of *Locusta*.

The substrate was pyruvate (5mM) plus proline (5mM) in the presence of reaction medium 1.

The figures in parentheses indicate the number of determinations for RCR and ADP:O ratio.

⊡ RCR

O ADP:O ratio


RCR
ADP: O
(4)
(6)
(7)
(4)
(4)
(6)
(6)
(3)
8
6
4
2
3
2
1
1 2 3 4 5 6 7 8
AGE (days)

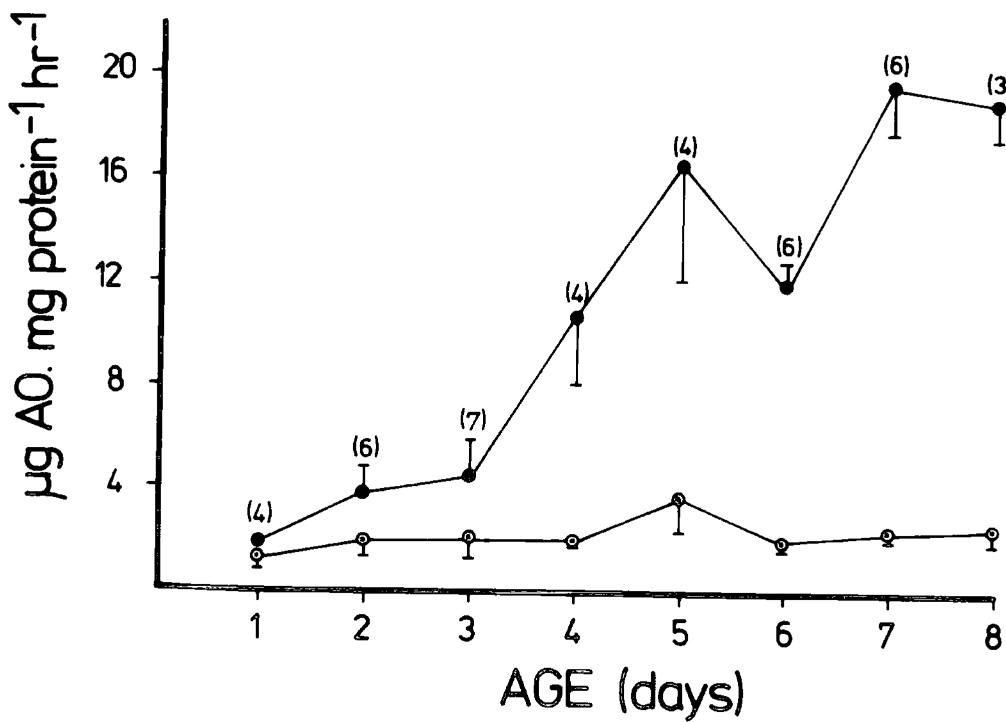

µg AO. mg protein^{-1} hr^{-1}
20
16
12
8
4
(4)
(6)
(7)
(4)
(4)
(6)
(6)
(3)
1 2 3 4 5 6 7 8
AGE (days)

Figure 4.7. Effect of age on the respiratory rate of mitochondria isolated from flight muscle of *Locusta*.

The substrate was pyruvate (5mM) plus proline (5mM) in the presence of reaction medium 4.

Ordinate: QO_2 is expressed in µg AO. mg protein^{-1}. hour^{-1}

Abscissa: Age in days

The figures in parentheses indicate the number of separate determinations for state 4 and state 3 respiration.

⊙ state 4 respiration

○ state 3 respiration

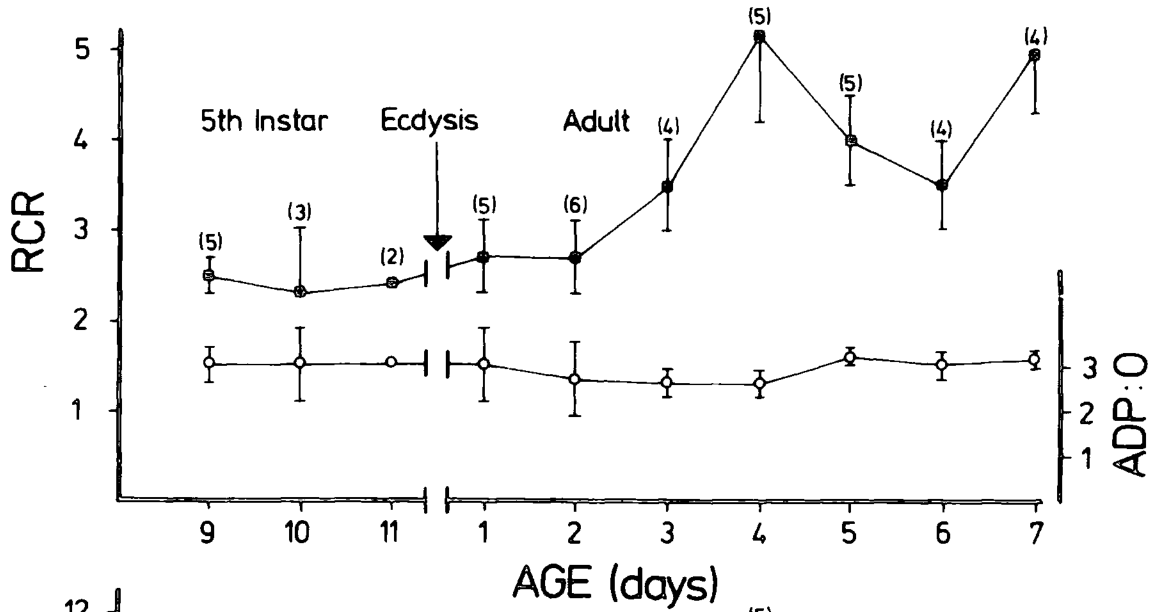
Figure 4.8. Effect of age on RCR and ADP:O ratio of mitochondria isolated from flight muscle of *Locusta*.

The substrate was pyruvate (5mM) plus proline (5mM) in the presence of reaction medium 4.

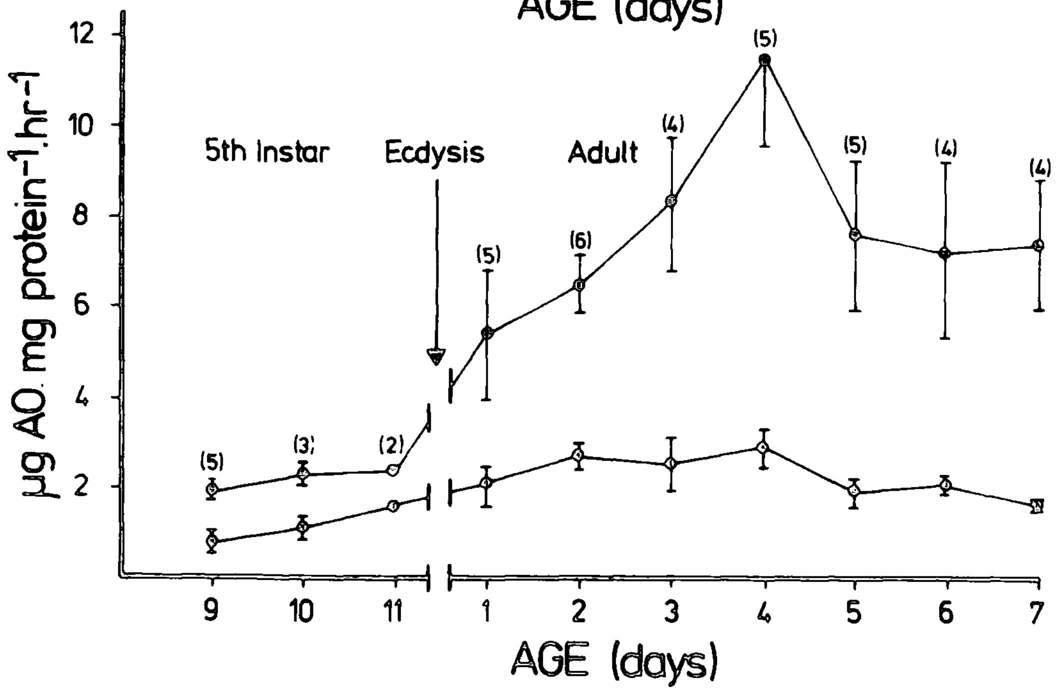
The figures in parentheses indicate the number of determinations for RCR and ADP:O ratio.

□ RCR

O ADP:O ratio



RCR
5
4
3
2
1
5th Instar
Ecdysis
Adult
(5)
(3)
(2)
(5)
(6)
(4)
(5)
(5)
(4)
(4)
ADP:O
3
2
1
9
10
11
1
2
3
4
5
6
7
AGE (days)



µg AO. mg protein-1.hr-1
12
10
8
6
4
2
5th Instar
Ecdysis
Adult
(5)
(3)
(2)
(5)
(6)
(4)
(5)
(5)
(4)
(4)
9
10
11
1
2
3
4
5
6
7
AGE (days)

α-Glycerophosphate - supporting respiration

Figures 4.10and 4.11 show the state 3 and state 4 respiratory rates obtained, when α-glycerophosphate was metabolized by developing flight muscle mitochondria in reaction media 1 and 4, respectively. The age-dependent pattern of activity was different depending on the reaction medium used. Thus the rate of respiration increased significantly for state 3 ($p < 0.05$) and state 4 ($p = 0.001$) between the 9th day of 5th instar and the 1st day of adult life, when reaction medium 4 was used. No significant change was observed thereafter in the two states. In contrast, mitochondria assayed in reaction medium 1 exhibited little change in the state 3 rate of respiration during the first 6 days of adult life but by the 7th day of adult life the mean value increased approximately 3-fold (Fig. 4.10). It is perhaps significant that ADP:O ratios were only measurable after the 5th day of adult life, when reaction medium 1 was used (Fig. 4.12) and that both state 3 and state 4 rates of respiration increased substantially at this time. However, no significant change was observed in ADP:O ratio values when reaction medium 4 was used (Fig. 4.13). Furthermore, there was no major changes in RCR in all ages studied when reaction medium 4 was used (Fig. 4.13). Similar RCR values were obtained when reaction medium 1 was used (Fig. 4.12), but prior to the 5th day it was necessary for the RCR to be estimated as the ratio of the respiratory rate in the presence of ADP to that before ADP addition (state 2).

Glutamate - supporting respiration

Figure 4.14 shows the rates of mitochondrial oxygen consumption as a function of age when reaction medium 1 was used. There was no significant change in the state 3 rate of respiration during the first 6 days of adult life; the rate being approximately 4 to 6μg AO mg $protein^{-1}$ $hour^{-1}$. However by day 7 of adult life the rate of respiration had

Figure 4.10. Effect of age on the respiratory rate of mitochondria isolated from flight muscle of *Locusta*.

The substrate was α-glycerophosphate (5mM) in the presence of reaction medium 1.

Ordinate: QO_2 is expressed in μg AO. mg protein^{-1}. hour^{-1}

Abscissa: Age in days

The figures in parentheses indicate the number of separate determinations for state 4 and state 3 respiration.

◎ state 4 respiration

○ state 3 respiration

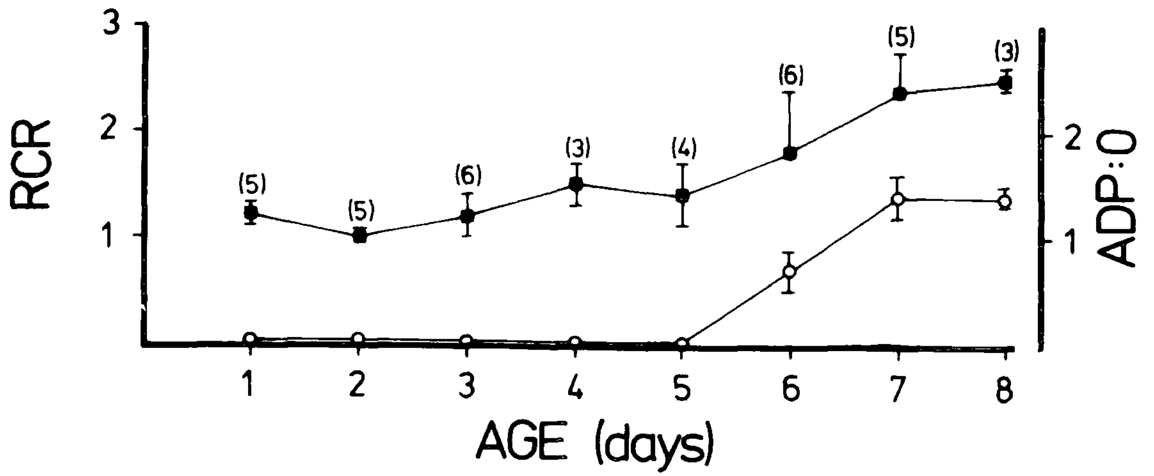
Figure 4.12. Effect of age on RCR and ADP:O ratio of mitochondria isolated from flight muscle of *Locusta*.

The substrate was α-glycerophosphate (5mM) in the presence of reaction medium 1.

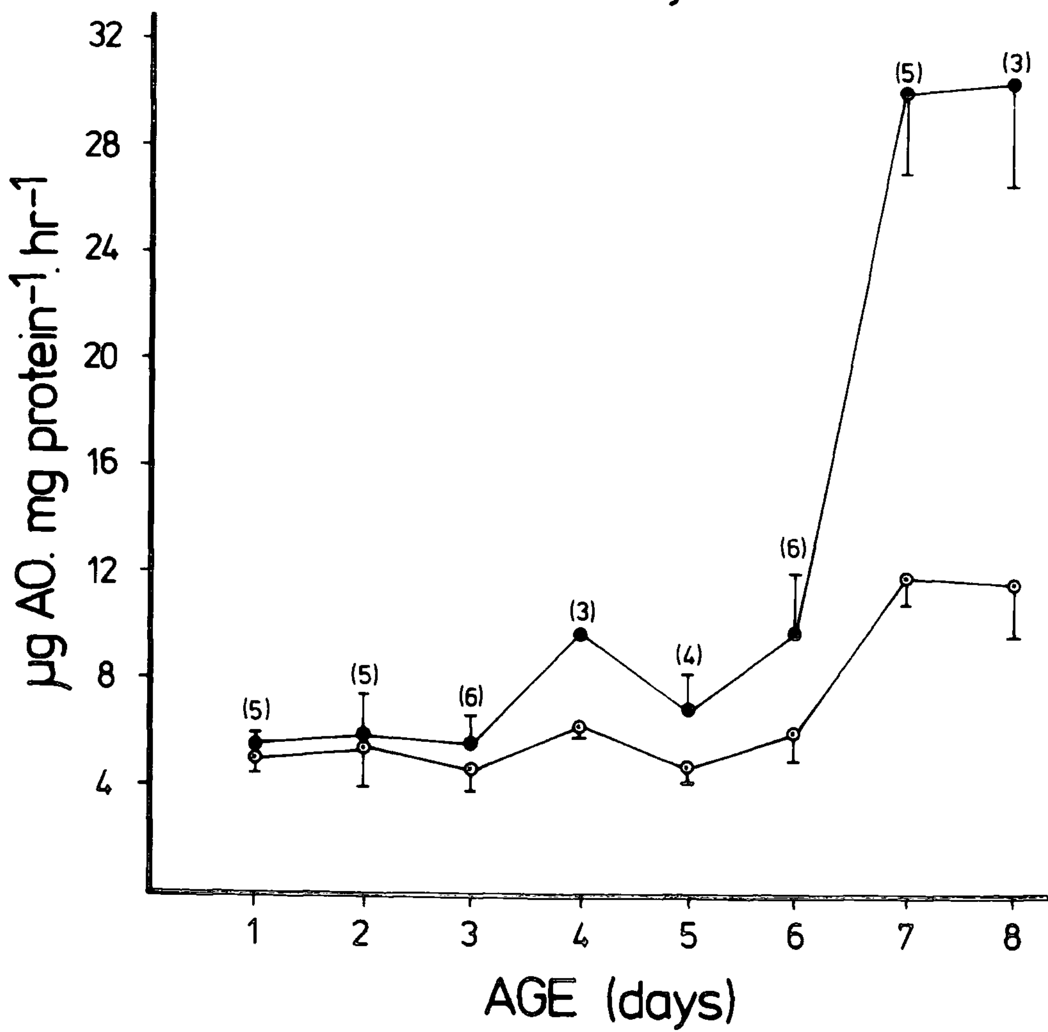
The figures in parentheses indicate the number of determinations for RCR and ADP:O ratio.

□ RCR

○ ADP:O ratio



RCR
ADP:O
3
2
1
(5)
(5)
(6)
(3)
(4)
(6)
(5)
(3)
1
2
3
4
5
6
7
8
AGE (days)



µg AO. mg protein⁻¹. hr⁻¹
32
28
24
20
16
12
8
4
(5)
(5)
(6)
(3)
(4)
(6)
(5)
(3)
1
2
3
4
5
6
7
8
AGE (days)

Figure 4.11. Effect of age on the respiratory rate of mitochondria isolated from flight muscle of *Locusta*.

The substrate was α-glycerophosphate in the presence of reaction medium 4.

Ordinate: QO_2 is expressed in μg AO. mg protein^{-1}. hour^{-1}

Abscissa: Age in days

The figures in parentheses indicate the number of separate determinations for state 4 and state 3 respiration.

⊙ state 4 respiration

O state 3 respiration

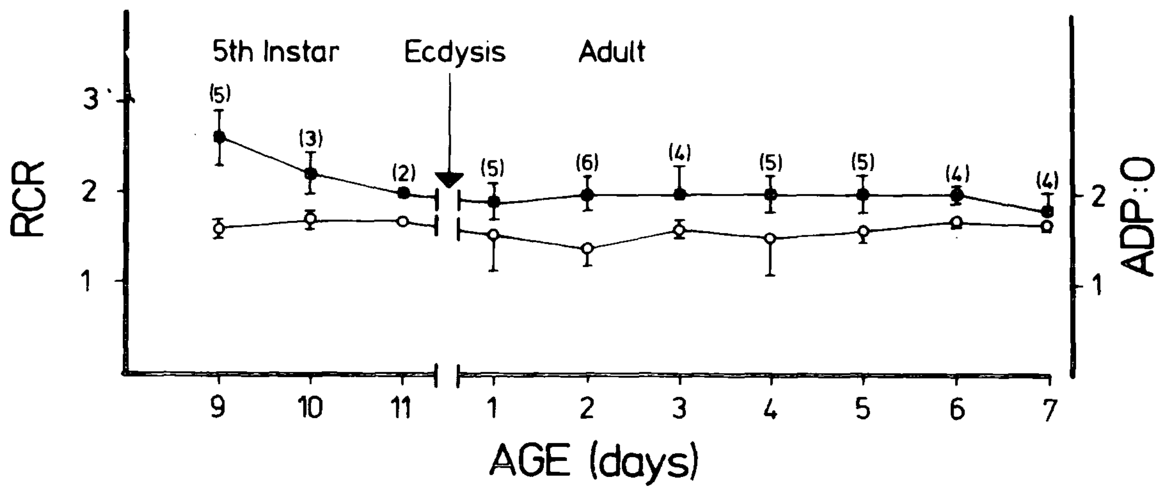
Figure 4.12. Effect of age on RCR and ADP:O ratio of mitochondria isolated from flight muscle of *Locusta*.

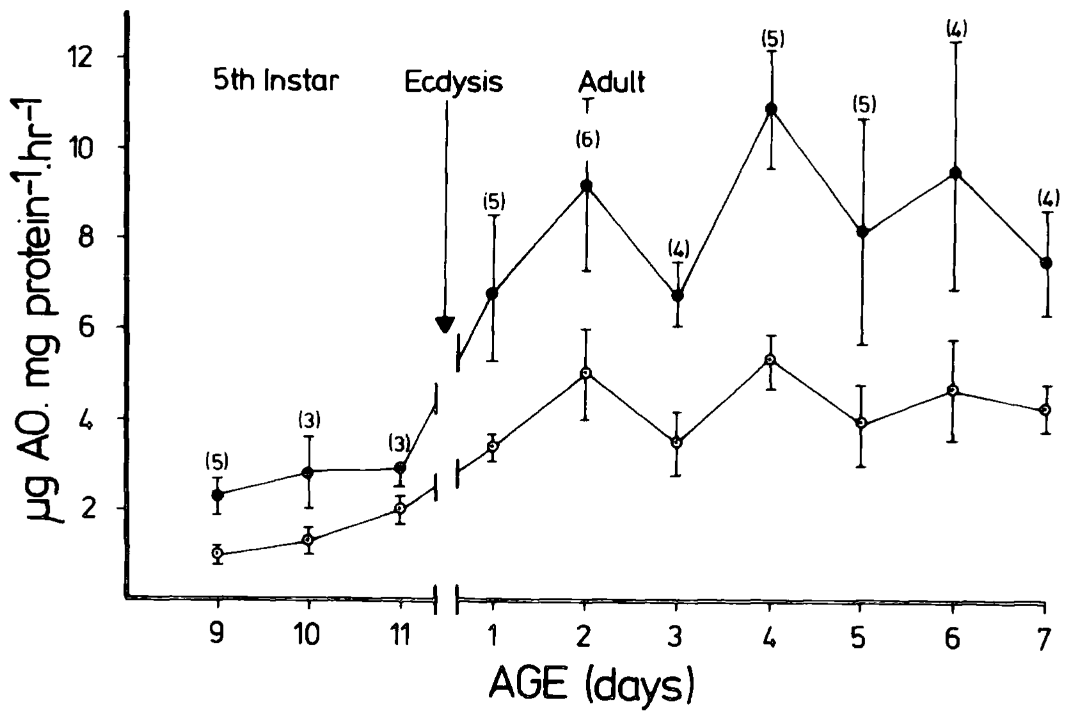
The substrate was α-glycerophosphate (5mM) in the presence of reaction medium 4.

The figures in parentheses indicate the number of determinations for RCR and ADP:O ratio.

⊠ RCR

O ADP:O ratio


5th Instar
Ecdysis
Adult
RCR
ADP:O
(5)
(3)
(2)
(5)
(6)
(4)
(5)
(5)
(4)
(4)
3
2
1
2
1
9
10
11
1
2
3
4
5
6
7
AGE (days)


5th Instar
Ecdysis
Adult
μg AO. mg protein-1.hr-1
(5)
(3)
(3)
(5)
(6)
(4)
(5)
(5)
(4)
(4)
12
10
8
6
4
2
9
10
11
1
2
3
4
5
6
7
AGE (days)

increased approximately 3-fold. There was no significant change in the state 4 rate of respiration during the period studied; the state 4 rate of respiration being approximately 2 - 4μg AO mg protein^{-1} hour^{-1} (Fig. 4.14).

In contrast, when reaction medium 4 is used (Fig. 4.15), the state 3 rate of respiration increased approximately 2-fold between the 11th day of the 5th instar and the 1st day of adult life. No significant change in state 3 rate of respiration was observed in adult animals over the period studied. State 4 rate of respiration showed a similar pattern of response to that of state 3 (see Fig. 4.15).

Whilst the RCR increased significantly ($p < 0.02$) between the 1st and 7th day of adult life when reaction medium 1 was used (Fig. 4.16), no significant change was observed when reaction medium 4 was used (Fig. 4.17). Furthermore, RCR values obtained with adult mitochondria were similar to those observed with mitochondria from late 5th instar animals (Fig. 4.17). The oxidation of glutamate was coupled to ADP phosphorylation at all ages studied, irrespective of the reaction medium used (see Figs 4.16 and 4.17).

<u>Succinate - supporting respiration</u>

Figures 4.18 and 4.19 show the state 3 and state 4 respiratory rates obtained when succinate is oxidised by mitochondria isolated from locusts of various ages, in the presence of reaction media 1 and 4, respectively. Little significant change in state 3 and state 4 rates of respiration was noted with adult mitochondria over the period studied irrespective of the reaction medium used, except that, in the presence of reaction medium 1 (Fig. 4.18) the state 3 rate of respiration observed on the 8th day of adult life was significantly higher ($p < 0.01$) than that observed on day 1.

<u>Figure 4.14.</u> Effect of age on the respiratory rate of mitochondria isolated from flight muscle of <u>Locusta</u>.

The substrate was glutamate (5mM) in the presence of reaction medium 1.

Ordinate: QO_2 is expressed in μg AO. mg protein^{-1}. hour^{-1}

Abscissa: Age in days

The figures in parentheses indicate the number of separate determinations for state 4 and state 3 respiration.

⊙ state 4 respiration

O state 3 respiration

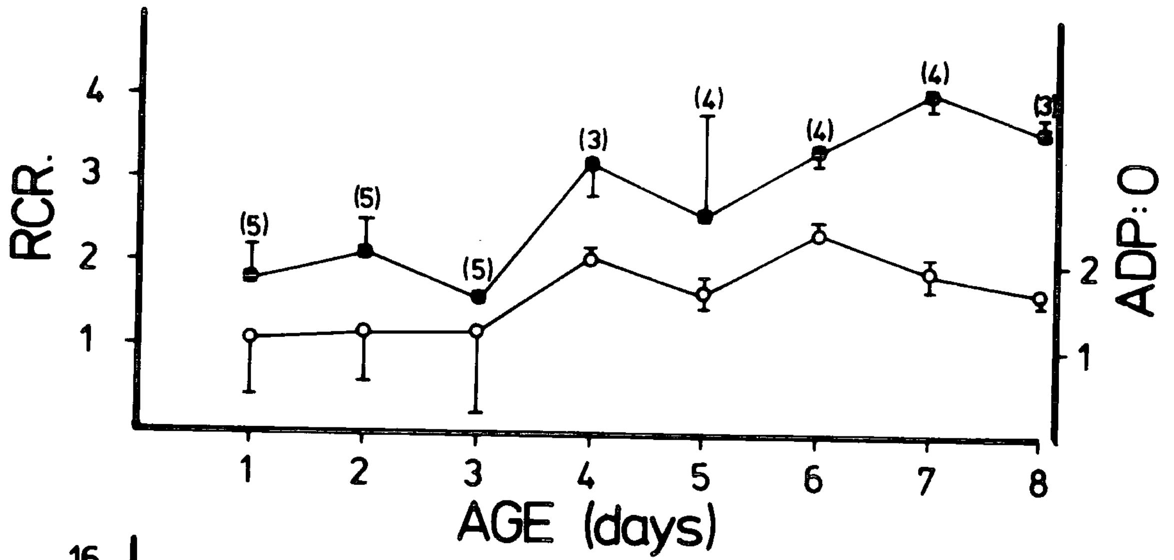
<u>Figure 4.16.</u> Effect of age on RCR and ADP:O ratio of mitochondria isolated from flight muscle of <u>Locusta</u>.

The substrate was glutamate (5mM) in the presence of reaction medium 1.

The figures in parentheses indicate the number of determinations for RCR and ADP:O ratio.

□ RCR

O ADP:O ratio


RCR.
4
3
2
1
ADP:O
2
1
(5)
(5)
(5)
(3)
(4)
(4)
(4)
(3)
1 2 3 4 5 6 7 8
AGE (days)

μgAO. mg protein-1.hr-1
16
14
12
10
8
6
4
2
(5)
(5)
(5)
(3)
(4)
(4)
(4)
(3)
1 2 3 4 5 6 7 8
AGE (days)

Figure 4.15. Effect of age on the respiratory rate of mitochondria isolated from flight muscle of *Locusta*.

The substrate was glutamate (5mM) in the presence of reaction medium 4.

Ordinate: QO_2 is expressed in μg AO. mg protein^{-1}. hour^{-1}

Abscissa: Age in days

The figures in parentheses indicate the number of separate determinations for state 4 and state 3 respiration.

⊙ state 4 respiration

O state 3 respiration

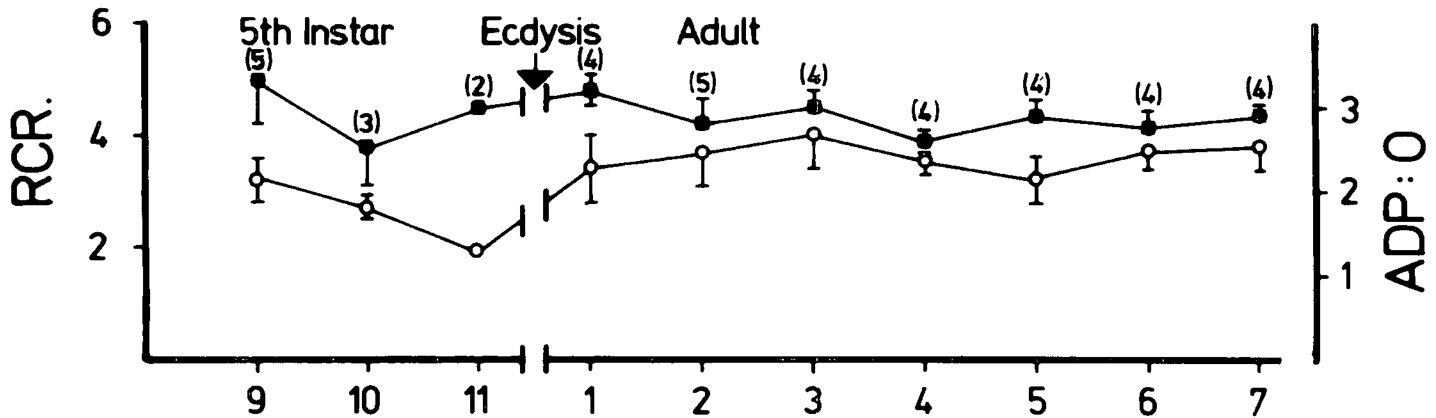
Figure 4.17. Effect of age on RCR and ADP:O ratio of mitochondria isolated from flight muscle of *Locusta*.

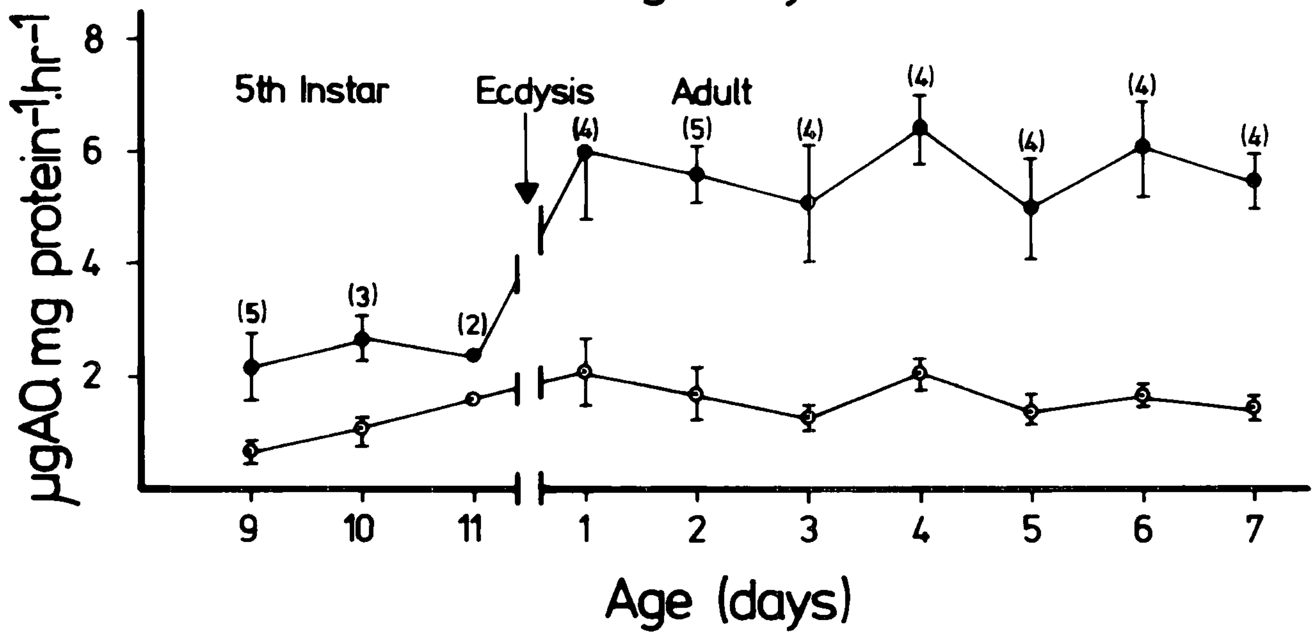
The substrate was glutamate (5mM) in the presence of reaction medium 4.

The figures in parentheses indicate the number of determinations for RCR and ADP:O ratio.

▣ RCR

O ADP:O ratio


RCR.
ADP : O
5th Instar
Ecdysis
Adult
Age (days)


μgAO mg protein⁻¹.hr⁻¹
5th Instar
Ecdysis
Adult
Age (days)

When reaction medium 4 was used, the state 3 rate of respiration increased significantly ($p < 0.05$) between the 9th day of 5th instar and the 2nd day of adult life; the rate of oxygen consumed increased approximately 4.5-fold over this period. No significant change was observed in state 4 rate of respiration over the same period.

Throughout this study, no coupling between succinate oxidation and ADP phosphorylation was observed as defined by CHANCE and WILLIAMS (1955b). However, the addition of ADP did effect a stimulation of respiration (see Fig. 4.5). No significant change was demonstrated in RCR at all ages studied irrespective of the reaction medium used (Figs 4.20 and 4.19).

Effect of $MgCl_2$ on the respiratory activity of isolated mitochondria

Reference to Figures 4.8, 4.9, 4.12 and 4.13 indicate that the ability of isolated mitochondria to effect oxidative phosphorylation of ADP is dependent on the reaction medium. Thus near theoretical values for ADP:O ratio were obtained at all ages from the 9th day of 5th instar to the 7th day of adult life when reaction medium 4 was used. In contrast, ADP:O ratios could only be measured in post 5-day old adults and post 1-day old adults with α-glycerophosphate (Fig. 4.12) and pyruvate plus proline (Fig. 4.9), respectively, when reaction medium 1 was used. This might suggest that reaction medium 4 is the more appropriate. However, it also indicates that the mitochondrial requirements, for the demonstration of oxidative phosphorylation, have changed with age. One difference between the 2 reaction media is the absence of $MgCl_2$ from reaction medium 1. Mg^{2+} are known to be important in stabilizing mitochondrial activity (see Discussion). It was decided, therefore, to determine the extent to which the presence of Mg^{2+} might affect mitochondrial function in 4-day old adult locusts.

Figure 4.18. Effect of age on the respiratory rate and RCR of mitochondria isolated from flight muscle of *Locusta*.

The substrate was succinate (5mM) in the presence of reaction medium 1.

The figures in parentheses indicate the number of separate determinations for state 4 and state 3 respiration.

⊙ state 4 respiration

O state 3 respiration

□ RCR

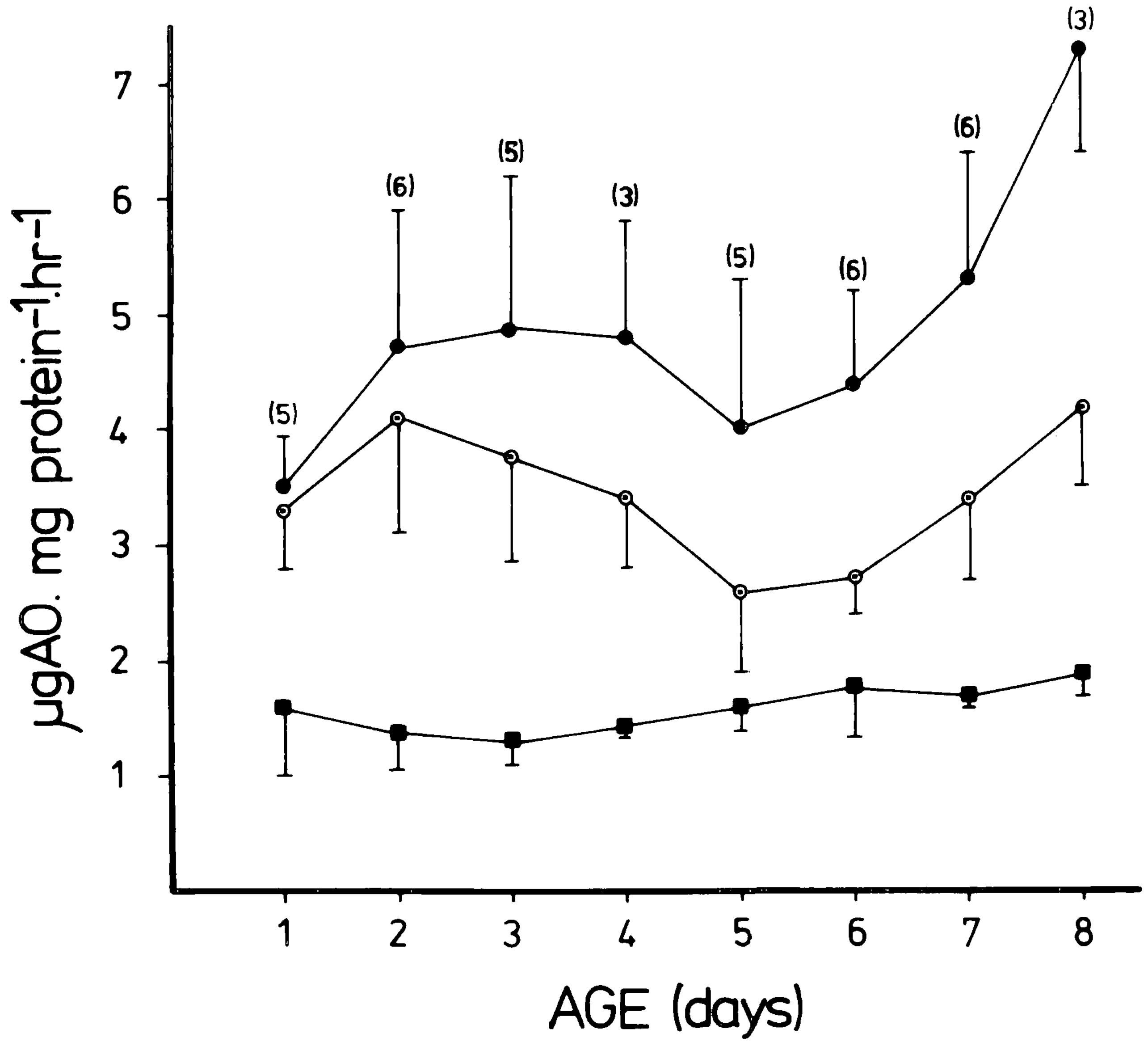

μgAO. mg protein-1.hr-1
7
6
5
4
3
2
1
(5)
(6)
(5)
(3)
(5)
(6)
(6)
(3)
1
2
3
4
5
6
7
8
AGE (days)

Figure 4.19. Effect of age on the respiratory rate of mitochondria isolated from flight muscle of *Locusta*.

The substrate was succinate (5mM) in the presence of reaction medium 4.

Ordinate: QO_2 is expressed in μg AO. mg protein^{-1}. hour^{-1}

Abscissa: Age in days

The figures in parentheses indicate the number of separate determinations for state 4 and state 3 respiration.

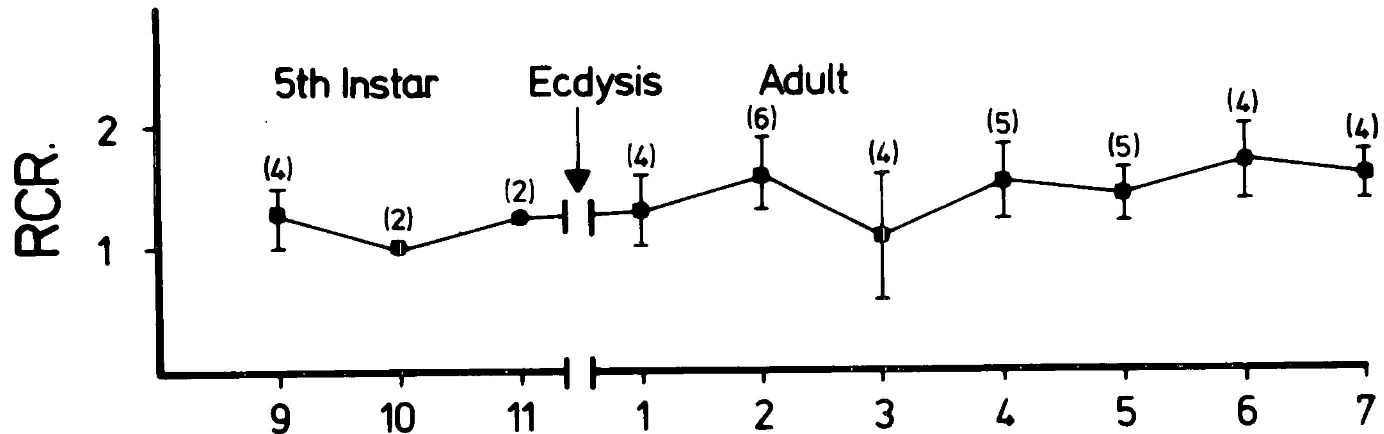
⊙ state 4 respiration

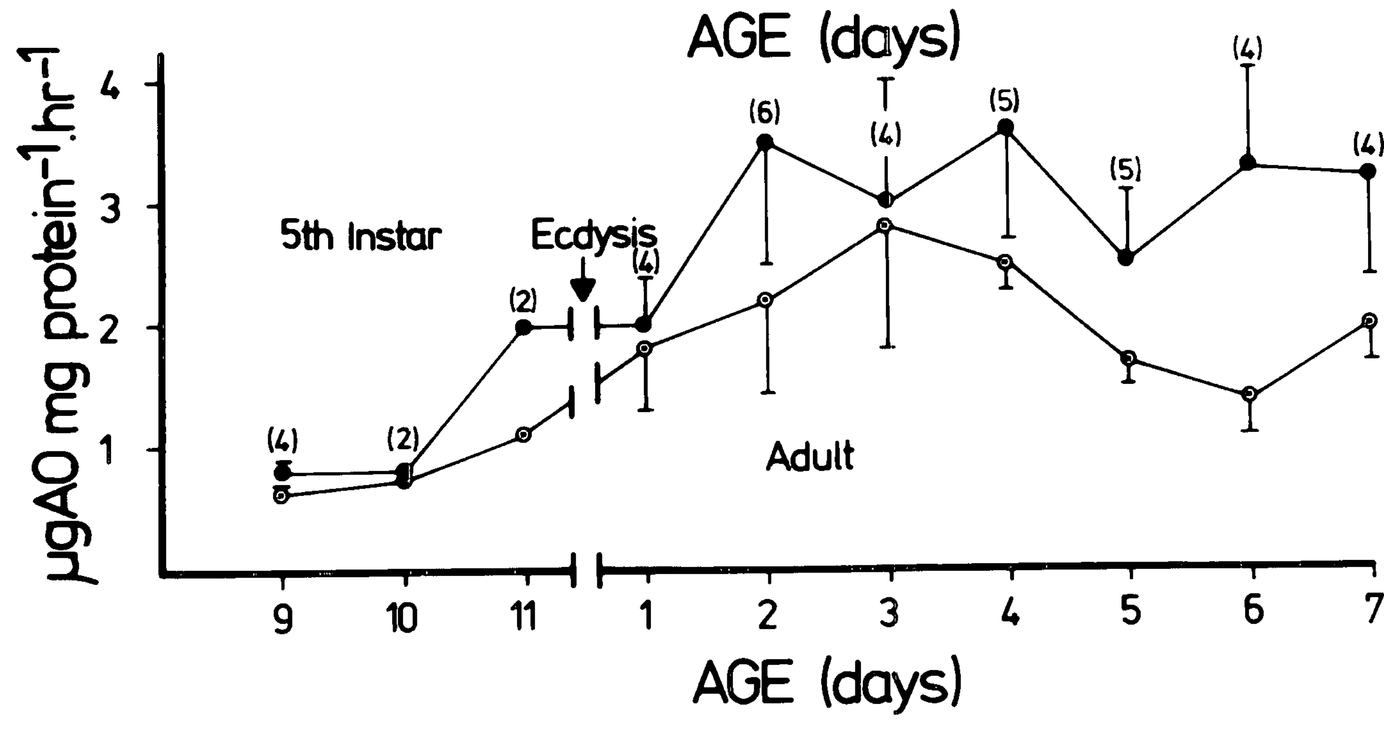
○ state 3 respiration

Figure 4.20. Effect of age on the RCR of mitochondria isolated from flight muscle of *Locusta*.

The substrate was succinate (5mM) in the presence of reaction medium 4.

The figures in parentheses indicate the number of determinations.


RCR.
5th Instar
Ecdysis
Adult
AGE (days)


µgAO mg protein^{-1}.hr^{-1}
5th Instar
Ecdysis
Adult
AGE (days)

<u>Figure 4.21.</u> The effect of Mg^{2+} on the oxidation of α-glycerophosphate by <u>Locusta</u> flight muscle mitochondria.

Reaction medium 1 was used and Mg^{2+} was added to give the final concentration indicated.

α-glycerophosphate was 6.7mM.

Ordinate: QO_2 is expressed in μg AO. mg protein^{-1}. hour^{-1}

Abscissa: Mg^{2+} concentration (mM)

○ state 3 respiration

◎ state 4 respiration

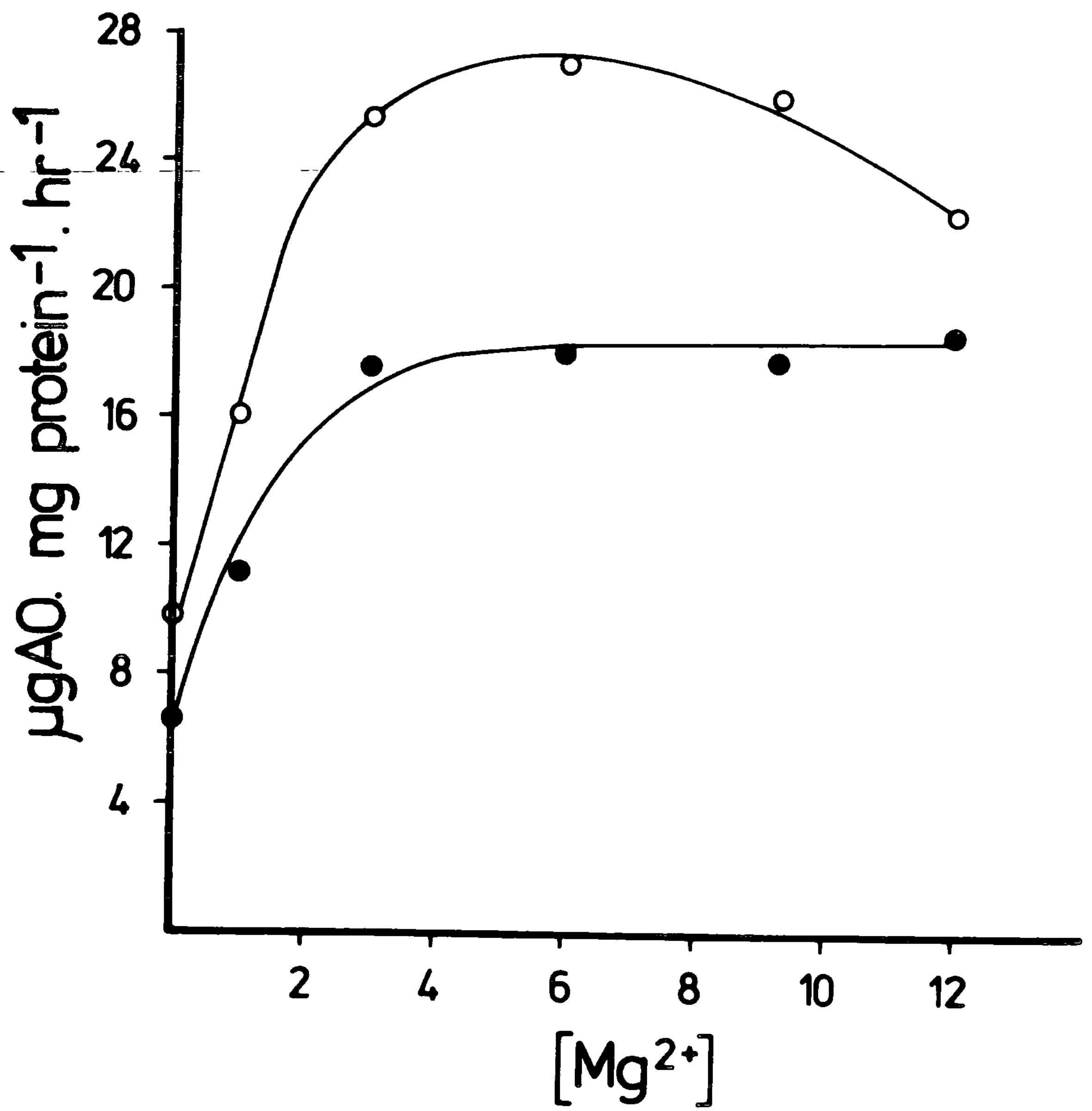

µgAO. mg protein⁻¹. hr⁻¹
28
24
20
16
12
8
4
2
4
6
8
10
12
[Mg²⁺]

Table 4.10. Effect of Mg^{2+} on the oxidation of α-glycerophosphate or pyruvate by *Locusta* flight muscle mitochondria isolated from 4-day old adults.

Substrate (mM)	Mg^{2+} (mM)	QO_2		RCR	ADP:O
		state 4	state 3		
α-glycerophosphate					
(6.7)	0.0	6.61	9.90	1.50	n.m.
(6.7)	6.7	16.30	27.00	1.66	1.30
(6.7)	13.4	18.60	22.23	1.20	1.34
13.4	0.0	10.77	14.34	1.34	n.m.
pyruvate					
(6.7)	0.0	3.67	28.10	8.03	3.98
(6.7)	3.3	6.34	11.32	1.79	3.90

Typical sets of data representative of four separate experiments (see Appendix 4.2 for mean ± S.E.M.). Reaction medium 1 was used.
Mg^{2+} was added to the reaction chamber to give the concentration indicated.
n.m. : not measurable and RCR in these cases was calculated as indicated previously (see footnote to Table 4.2).

Figure 4.21 shows the effect of different concentrations of $MgCl_2$ on the state 3 and state 4 respiratory rates in the presence of a constant concentration of α-glycerophosphate (6.7mM). This experiment was performed by utilizing reaction medium 1 to which the Mg^{2+} was added to the reaction chamber. Both respiratory rates increased with increasing $MgCl_2$ concentration up to 3mM. Further increases in $MgCl_2$ concentration had little effect on state 4 respiratory rate but increasing $MgCl_2$ concentration above 9mM resulted in a decreased stimulation of the state 3 rate of respiration.

Table 4.10 shows the mitochondrial response to the presence or absence of $MgCl_2$ in reaction medium 1. The presence of Mg^{2+} was essential for demonstrating ADP:O ratio and high state 3 and state 4 rates of respiration, when α-glycerophosphate was the substrate. The effect of this cation on the metabolism of pyruvate plus proline was somewhat different from that observed with α-glycerophosphate. Whilst state 4 rate of respiration was increased by approximately 42% by the inclusion of 3.3mM $MgCl_2$ in reaction medium 1, the state 3 rate of respiration decreased by approximately 60% (see Table 4.10). Consequently, the RCR decreases from 8.03 in the absence of $MgCl_2$ to 1.79 in its presence. No significant change was noted in the ADP:O ratio obtained irrespective of whether Mg^{2+} were included in the medium or not (Table 4.10).

Discussion

The present study indicates that the composition of the reaction medium is of great importance in assessing the functional competence of isolated mitochondria. Throughout this study mitochondria isolated from *Locusta migratoria* flight muscle required BSA in order for good respiratory parameters to be maintained. Similar results have been reported elsewhere. SACKTOR (1954) showed that BSA was required to

demonstrate oxidative phosphorylation in Musca domestica flight muscle mitochondria. In contrast, GREGG et al. (1960), VAN DEN BERGH and SLATER (1962) and COCHRAN (1963) have reported rapid oxidation and high P:O ratios in the absence of BSA. However, BALBONI (1968) found that BSA stabilized mitochondrial respiratory activity and inhibited spontaneous swelling of isolated mitochondria. The "stabilizing effect" (BALBONI, 1968) or "protective action" (HARVEY and HASKEL, 1966; CHILDRESS and SACKTOR, 1966; MATSUOK and NAKAMURA, 1979) of BSA was due to its capacity to bind free fatty acids (BJORNTORP, 1964; MATSUOK and NAKAMURA, 1979), which have been reported to stimulate mitochondrial swelling (LEHNINGER and REMMERT, 1959). In this way BSA is claimed to restore full coupling efficiency to isolated mitochondria (WARSHAW, 1969; DOW, 1967).

In addition to the presence of BSA in the reaction medium, certain other requirements were also necessary in association with the different substrates. Thus Mg^{2+} was necessary in the reaction medium for maintaining near theoretical values for the ADP:O ratio when α-glycerophosphate was used as the substrate, whereas the role of this cation is complicated when the substrate is pyruvate plus proline. In the present study, although mitochondria isolated from flight muscle of 2-8 day old adult insects required no Mg^{2+} when pyruvate plus proline was the substrate, this cation was essential for demonstration of oxidative phosphorylation by mitochondria isolated from late 5th instar and 1-day old adult insects. Similarly, Mg^{2+} was necessary for the demonstration of oxidative phosphorylation by mitochondria isolated from mature flight muscle of the blowfly, Calliphora erythrocephala (KASHMEERY, 1977) when α-glycerophosphate was the substrate but was best excluded from the reaction medium when the substrate was pyruvate plus proline. SLACK and BURSELL (1976b) showed that Mg^{2+} concentrations below 10^{-5}M had no significant effect on the respiratory parameters of

mitochondria isolated from the flight muscle of Sarcophaga nodosa when pyruvate was the substrate. However, Mg^{2+} concentrations greater than $10^{-5}M$ increased state 4 rates of respiration to a peak at about $10^{-3}M$ Mg^{2+}, followed by a decline (at > $10^{-3}M$ Mg^{2+}) and a secondary increase (at > $10^{-2}M$ Mg^{2+}). These changes were associated with inverse changes in the levels of state 3 respiration. These researchers concluded that the changes caused by Mg^{2+} (above $10^{-5}M$) were associated, in part, with a low level of ion translocation and, in part, with the activity of a Mg^{2+}-sensitive ATPase.

It has long been known that free Ca^{2+} has a damaging effect on the respiratory function of isolated mitochondria (LEHNINGER, 1962; CHANCE, 1965) and, therefore, calcium chelaters (e.g. EDTA) are widely used in isolation media to decrease the concentration of free Ca^{2+} and hence eliminate its damaging effect. BÜCHER and KLINGENBERG (1959) found that isolated mitochondria from Locusta migratoria exhibited, respiratory control only when the isolation medium contained at least 1mM EDTA. However, a low concentration of free Ca^{2+} was reported to be required when α-glycerophosphate was used as substrate (SLACK and BURSELL, 1977). The latter authors reported that the difference between the oxidation of pyruvate and α-glycerophosphate is that the relatively labile phosphorylation system, which is involved in the state 3 respiration of both, can be replaced as a releaser of the respiratory chain by the Ca^{2+}-uptake system when the oxidation is supported by α-glycerophosphate, but not when it is supported by pyruvate. However, utilization of both substrates was affected by higher Ca^{2+} concentrations and the state 3 respiration decreased steeply to zero as Ca^{2+} concentration was raised from 10^{-4} to $10^{-3}M$. It is possible, therefore, that the stabilizing effect of Ca^{2+} on the metabolism of α-glycerophosphate may be the result of competition between endogenous Ca^{2+} and added Mg^{2+} for the less specific chelating agent, EDTA (SACKTOR, 1974). The latter author suggested that the addition of Mg^{2+}

to the reaction medium leads to a partial release of Ca^{2+} from EDTA, which was originally derived from the muscle and chemical agents. It may well be that this was also the case in the present study on Locusta and that sufficient free Ca^{2+} is present in adult mitochondria to make Mg^{2+} addition unnecessary when pyruvate plus proline is the substrate. However, further studies are required to establish whether this is indeed so.

The addition of proline, when pyruvate was the substrate, did not alter the state 3 rate of oxygen consumption but slightly increased that of state 4 respiration. Similar results have been reported by SLACK and BURSELL (1976a) for mitochondria isolated from the flight muscles of Sarcophaga nodoso and Phormia regina. The latter authors have suggested that the difference between their results and those reported by many other workers concerning the need for a "primer" or "sparker", such as bicarbonate (HANSFORD, 1972), malate (VAN DEN BERGH, 1964; STEVENSON, 1968; TRIBE and ASHHURST, 1972) and proline (SACKTOR and CHILDRESS, 1967; SACKTOR, 1970, 1974), are due to the use by such workers of inappropriate extraction methods. It has been suggested that the less functionally intact the mitochondria are, the greater the sensitivity towards the external conditions (SLACK, 1975). SLACK and BURSELL (1976b) have shown that mitochondria isolated from dipteran flight muscle utilize pyruvate at very high rates without the requirement for a primer when the isolation medium contained potassium-D-aspartate and resuspension was effected by means of gentle magnetic stirring. However, KHAN and DE KORT (1978) found that potassium-D aspartate strongly inhibited respiration rates of beetles and Locusta flight muscle mitochondria. SLACK and BURSELL (1976b) concluded that many of the results reported with insect mitochondria may be regarded as artefacts of the isolation methods used. Since proline was unnecessary for pyruvate oxidation in their studies, SLACK and BURSELL

(1976b) rejected the suggestion that the decrease in proline concentration, during the early phases of flight in Phormia regina (SACKTOR and WORMSER-SHAVIT, 1966), plays an important role in re-establishing the full function of the tricarboxylic acid cycle by the formation of oxaloacetate. Thus they regard proline as a supplementary substrate rather than a "primer", and suggest that its availability in haemolymph and muscle make it a useful source of additional energy during the initial phase of flight. This was supported by the more recent studies on the utilization of proline and pyruvate by Leptinotarsa decemlineata flight muscle mitochondria (WEEDA et al., 1980) which suggest that in the presence of low proline concentration, pyruvate and proline oxidation are fully additive, whereas at higher concentrations of proline, pyruvate oxidation is completely turned off by proline oxidation.

Throughout this study, the respiratory rates and ADP:O ratios varied with successive additions of ADP. However, the mean values showed no significant difference in the RCR and tend to mask this effect in some cases (see Results section). Similar changes have been reported elsewhere. SACKTOR (1974) showed that following the second addition of ADP there was an 11% decrease (from 2.98 to 2.64) in ADP:O ratio and a 48% increase in RCR (from 26 to 50) (calculated from data given by SACKTOR, 1974). BURSELL and SLACK (1976) showed that successive additions of ADP resulted in a progressive increase in the state 4 rates of respiration. RCR values, calculated from this data, decreased from 131.5 following the first ADP addition to 27.3 following the third addition. They suggested that these changes were due to the activity of mitochondrial ATPase. The latter produces progressively higher ADP concentrations which require extra oxygen for phosphorylation to ATP and therefore leads to a delay in the transition from state 3 to state 4 (SLACK and BURSELL, 1976b). The

present decline in ADP:O ratio following successive ADP addition may, therefore, be explained in these terms. In contrast, CHILDRESS and SACKTOR (1966) have shown that in Phormia regina, flight muscle mitochondrial respiration was little affected by successive ADP additions.

An alternative explanation to the one given above is that the high values for the ADP:O ratio, observed following the first 2 additions of ADP, when pyruvate plus proline or glutamate were used as substrate, may be due to changes in the permeability of the mitochondrial membranes. CHAPPELL and HANSFORD (1972) have suggested that the isolation of mitochondria by the Nagarse enzyme method may alter some of the membrane properties, lower the content of the endogenous tricarboxylic acid cycle intermediates and inactivate the inner membrane carriers. However, the present study has shown that isolated mitochondria exhibit good respiratory control and oxidative phosphorylation, indicating that at least in adult locusts, the isolated mitochondria were intact and not adversely affected by the Nagarse extraction method.

The results presented above show quite conclusively that, providing a suitable reaction medium was used, the mitochondria isolated from developing flight muscle of Locusta migratoria contained the metabolic systems necessary for the coupling between phosphorylation and the oxidation of the various substrates used, with the exception of succinate (see Results section) at all stages studied. This result is in contrast to the early report by KLINGENBERG and BÜCHER (1959) that respiratory control was not demonstrable before the 10th - 15th day of adult life. However, BÜCHER (1965) suggested that the "anlage" of the last instar of Locusta contains fully functional but very small mitochondria. It has also been reported (POLLAK and SUTTON, 1980) that the development of mitochondrial function is often an early and essential part of differentiation since many aspects of cellular

differentiation depend on the energy provided by these organelles. Similarly, MICHEJDA (1964) has reported that the flight muscle mitochondria of Hyalophora cecropia demonstrated equally efficient phosphorylation (P:O) at all developmental stages (15th day before to 8th day after final ecdysis). However, he showed that respiratory control by ADP, when glutamate was the substrate, was low before the final ecdysis, reached its maximum at the time of ecdysis to adult and was then lost in mitochondria from older adults. VAN DEN BERGH (1962) found that respiratory function was well developed and comparable at all stages (from 24 hours after the final ecdysis up to the end of the 3rd week of adult life) in Musca domestica, whereas LEWIS and SLATER (1954) found low P:O ratios with mitochondria isolated from the blowfly, Calliphora erythrocephala, younger than 10-day old adult. Similar observations, to the latter, have been reported in mitochondria isolated from the flight muscle of Apis mellifera (BALBONI, 1967) and Calliphora erythrocephala (TRIBE, 1967). The present study revealed that the two reaction media used to assess the developmental competence of isolated mitochondria gave different results. Thus, the reaction medium most suitable for mitochondria from young adult locusts was less suitable for those from older adult animals. This suggests that the study of the mitochondrial competence is reaction medium dependent, and that this dependence changes with development. This is in agreement with the statement by CRABTREE and NEWSHOLME (1975) that since the metabolic pathways consist of a large number of different and sometimes complex reactions, it was not possible to establish optimal conditions for all reactions in one reaction medium.

The flight muscle of Locusta showed substantial developmental changes during the first week of adult life (see Chapter 3). It will be recalled that these changes affected both the contractile machinery and the mitochondria. The results presented in this Chapter have shown

that associated with the fine structural changes in the mitochondria there is a marked increase in their oxidative capacity. BURSELL (1973) has suggested that in *Glossina morsitans* the wing-beat frequency may be limited by the rate at which energy can be supplied to the contractile machinery. More recently, HERSCH *et al*. (1978) have suggested that it is the energy producing systems (i.e. Krebs cycle enzymes, cytochrome c content, increased size and structural complexity of mitochondria and glycolytic enzymes) which limit muscle activity and develop synchronously at the appropriate age. In *Locusta*, a marked increase in mitochondria respiration per mg protein was noted in the first week of adult life indicating an increase in mitochondrial oxidative activity at the time flight performance is known to improve. Similarly, STREUMER-SVOBODOVA and DRAHOTA (1977) have suggested that the increase in oxidative capacity of mitochondria of various tissues during early post-natal development of vertebrates is in close correlation with the increase in activity of these muscles.

CHAPTER 5

CHARACTERIZATION OF SR-ATPase with PARTICULAR EMPHASIS ON THE DEVELOPMENTAL CHANGES IN SR FUNCTION AND α-GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY

Introduction

It is now recognised that Ca^{2+} act as activators and regulators of many biological processes (CHEUNG, 1980). The most thoroughly documented are the excitation - contraction coupling in skeletal, cardiac and smooth muscle (SANDOW, 1965, 1970; EBASHI and ENDO, 1968; HUDDART, 1975; EBASHI, 1976) and the activation of certain important metabolic enzymes (KRESINGER, 1976; CARFOLI and CROMPTON, 1978; DENTON and McCORMACK, 1980a). As mentioned earlier, the stimulus for the contraction of muscle is the arrival of an electrical impulse, via a motor nerve, at the end-plate or neuromuscular junction. When the excitatory impulse spreads over the cell membrane, the surface membrane becomes depolarized. This depolarization is channelled into the fibre via the T-system and usually takes the form of an action potential (COSTANTIN, 1970; BASTIAN and NAKAJIMA, 1974) which exerts an influence (TAYLOR and GODT, 1976) via the triadic and dyadic junctions (FRANZINI-ARMSTRONG, 1975, 1980) on the SR causing increased permeability (TAYLOR and GODT, 1976; MEECH, 1976). As a result, Ca^{2+} are released from the terminal cisternae of the SR (TAYLOR and GODT, 1976; ENDO, 1977) in which it is normally segregated in relaxing muscle (JORGENSEN et al., 1979). The extremely rapid discharge of Ca^{2+} from the SR leads to an increased intracellular concentration of Ca^{2+}; probably in the range of 5×10^{-5}M (DAVIES, 1963). This, in turn, triggers contraction by removing the inhibitory effect of the regulatory proteins, troponin and tropomyosin, on actin (reviews by EBASHI and ENDO, 1968; EBASHI et al., 1969, 1976; EBASHI, 1980; WEBER and MURRY, 1973; PERRY, 1979). Thus in

the presence of Ca^{2+} actin and myosin interaction can occur and contraction takes place. As long as motor-nerve impulses continue to arrive at the cell membrane, Ca^{2+} remain in the sarcoplasm and keep the muscle in a contracted state. When the motor-nerve impulses cease, the cell membrane and the SR regain their original permeability patterns. The Ca^{2+} present in the sarcoplasm are then transported, in an energy - requiring process (EBASHI and LIPMANN, 1962), across the SR membranes into the cisternae of the SR. Thus the concentration of Ca^{2+} in the sarcoplasm is brought to low resting concentration (PORTZEHL et al., 1964). The inhibitory effect of Ca^{2+} on the regulatory system which prevented the interaction between actin and myosin is removed and consequently relaxation occurs (reviews by EBASHI and ENDO, 1968; EBASHI et al., 1969, 1976; EBASHI, 1980; WEBER and MURRY, 1979; PERRY, 1979).

The energy-requiring active transport of Ca^{2+} from the sarcoplasm into the cisternae of the SR has been studied by biochemical methods (MARTONOSI and FERETOS, 1964a,b; WEBER et al., 1966; see also reviews by HASSELBACH, 1964a; INESI, 1972). Approximately 30 years ago MARSH (1951, 1952) showed that aqueous extracts prepared from rabbit skeletal muscle contained a factor (the so called relaxing factor) capable of inhibiting ATPase activity of myofibrils. Subsequently, KUMAGAI et al. (1955) reported the presence of a membrane ATPase, distinct from the myofibrillar ATPase, which induced muscle relaxation. Furthermore, it was shown that this ATPase was the same as that isolated by KIELLEY and MEYERHOF (1948a,b) and known as "KIELLEY-MEYERHOF granular ATPase". In 1958, EBASHI showed that this ATPase and the relaxing factor purified together and that the relaxing factor was dependent on ATPase activity. EBASHI and LIPMANN (1962) showed that the membrane fraction showing ATPase activity was largely made up of small resealed vesicles of SR, which can be isolated in the microsomal fraction. They suggested that

the massive accumulation of Ca^{2+} by such preparations could be explained by the existence of an energy-requiring system for the transport of Ca^{2+} into the membrane vesicles (see also NAGAI *et al.*, 1960; MUSCATELLO *et al.*, 1962).

To date the majority of studies on this system have been carried out on vertebrate muscle (see reviews by HASSELBACH, 1964a; INESI, 1972; MacLENNAN and HOLLAND, 1975). However, TSUKOMOTO *et al.* (1966) reported that the SR from flight muscle of *Locusta migratoria* exhibited properties similar to those of the corresponding fraction from vertebrate skeletal muscle (HASSELBACH, 1964a). Indeed, the granular SR fraction from *Locusta* flight muscle was very effective in inhibiting myofibrillar ATPase, whether the latter was prepared from the same muscle or from rabbit skeletal muscle. Evidence was put forward to suggest that the granules were acting by removing Ca^{2+} from the myofibrils because of their strong Ca^{2+}-binding capacity. It was concluded that insect sarcoplasmic reticula granules, like those of vertebrates, are capable of accumulating Ca^{2+} (HUDDART *et al.*, 1974) and acting as relaxing factors (MARUYAMA, 1974). Therefore, all muscles, both vertebrate and invertebrate, can be considered identical systems in so far as the chemical mechanism of contraction and relaxation is concerned (TSUKOMOTO *et al.*, 1966; MARUYAMA, 1974).

In their early studies KIELLEY and MEYERHOF (1948) showed that the ATPase from SR was activated by Mg^{2+} and inhibited by Ca^{2+} at mmolar concentrations. This has subsequently been confirmed by HASSELBACH and MAKINOSE (1961) (see also review by HASSELBACH, 1964a) who showed that the effect of Ca^{2+} is dependent on its concentration. Thus in contrast to the inhibitory effect of Ca^{2+} just reported, µmolar concentration of Ca^{2+} stimulated SR-ATPase activity.

In biochemical studies on muscle SR fractions, two distinct ATPases are reported (see HASSELBACH, 1964a,b; INESI, 1972). (i) Basal ATPase (HASSELBACH and MAKINOSE, 1961, 1962; YAMAMOTO and TONOMURA, 1967; HOLLAND and PERRY, 1969), which has been variously described, Mg^{2+}-activated ATPase, Mg^{2+} + EGTA-activated Ca^{2+}-insensitive ATPase (MARTONOSI et al., 1972; BOLLAND et al., 1974) and Mg^{2+}-dependent ATPase (COSSINS, 1974). This enzyme exhibits low activity in the presence of oxalate (HASSELBACH and MAKINOSE, 1961, 1962; HOLLAND and PERRY, 1969) or EGTA (MARTONOSI et al., 1972; BOLLAND et al., 1974; COSSINS, 1974). (ii) Total (Basal + extra) ATPase (HOLLAND and PERRY, 1969) or Mg^{2+} + Ca^{2+}-activated ATPase (MARTONOSI et al., 1972; BOLLAND et al., 1974; COSSINS, 1974), which is observed in the presence of Ca^{2+} and Mg^{2+} together. The difference in activity between (i) and (ii) is referred to as either "extra" ATPase, Ca^{2+}-activated ATPase (HASSELBACH, 1964a; HOLLAND and PERRY, 1969; MARTONOSI et al., 1972; BOLLAND et al., 1974), Ca^{2+}-dependent ATPase (YAMAMOTO and TONOMURA, 1967) or Ca^{2+}-stimulated ATPase (COSSINS, 1974) and it is this activity which has been correlated with Ca^{2+} -uptake by the SR (HASSELBACH and MAKINOSE, 1961; HASSELBACH, 1964b; WEBER et al., 1966; YAMADA et al., 1970; HASSELBACH et al., 1970). Whilst numerous workers have studied the properties of this ATPase activity from SR of vertebrate skeletal muscle (MARTONOSI and FERETOS, 1964b; WEBER et al., 1966; YAMAMOTO and TONOMURA, 1967; MARTONOSI, 1968; MacLENNAN, 1970) (see also review by INESI, 1972; MacLENNAN and HOLLAND, 1975; TADA et al., 1978), information on insect material is extremely limited; a notable exception being the recent study by VOLMER (1978). It is now well established that the Ca^{2+}-transporting ATPase from sarcoplasmic reticulum is Mg^{2+}-dependent (mmolar conc.) and stimulated by Ca^{2+} (µmolar free Ca^{2+}). The Ca^{2+}-stimulated ATPase from freshwater crayfish muscle required, in addition to Mg^{2+} and Ca^{2+}, K^{+} for activation

(COSSINS and BOWLER, 1976) while GREEN *et al.* (1976) and RUBIN and KATZ (1967) have shown that ATPase from SR of rat muscle and rabbit muscle, respectively, was stimulated above Mg^{2+}-dependent ATPase by K^{+}. VOLMER (1978) reported that SR-ATPase from *Locusta* SR reaches a maximum level at 100mM KCl.

The accumulation of Ca^{2+} from the sarcoplasm by SR ATPase is thought to be the rate-limiting step in relaxation (HASSELBACH, 1964b; EBASHI and ENDO, 1968; SANDOW, 1970; BRIGGS *et al.*, 1977; ENDO, 1977) and it is possible to relate the rate of relaxation to the ability of the SR to accumulate Ca^{2+} (BRIGGS *et al.*, 1977). For example, FIEHN and PETER (1971) and BRIGGS *et al.* (1977) have shown that the relative Ca^{2+}—uptake by the SR from slow and fast muscle corresponds closely with their relative rates of relaxation. Furthermore, it would appear that the differences in relaxation time between various muscles may be accounted for by the amount and properties of their SR (BRIGGS *et al.*, 1977). The Ca^{2+}-uptake activity of SR microsomes is thought to reflect the activity in the intact skeletal muscle (FANBURG *et al.*, 1968) and can be correlated with the type of muscle from which it is isolated (PEITTE and HEILMANN, 1980). Recently, chronic electro-stimulation (HEILMANN and PEITTE, 1980) has shown that, after two days of stimulation, a progressive transformation of SR of fast-twitch rabbit muscle can be induced by the frequency pattern received from a slow-twitch muscle. This transformation includes a decrease in Ca^{2+}-dependent ATPase, Ca^{2+}-transport, Ca^{2+}, Mg^{2+}-dependent phosphoprotein and a rearrangement of the electrophoretic polypeptide and phosphoprotein pattern. In addition, the transformation is accompanied by an increase in time-to-peak of twitch contraction and half relaxation time which occurs before conversion of the myosin light chain pattern. They concluded that this observation emphasized

the role played by SR in determining the twitch characteristics of fast- and slow twitch muscle.

During the growth of an animal the speed of contraction and relaxation increase in vertebrate skeletal muscle (CLOSE, 1964; BUTLER et al., 1966) and in intrasegmental muscle of Locusta (TYRER, 1969, 1973). These changes have been correlated with the development of the T-system and the SR (TYRER, 1973). Similarly, developmental changes have been reported in the functional properties of SR from rabbit (SZABLES et al., 1967; HOLLAND and PERRY, 1969) and chicken (FANBURG et al., 1968; MARTONOSI et al., 1972, 1980; BOLLAND et al., 1974) and in ultrastructure (TILLACK et al., 1974).

In Locusta migratoria flight muscle, there appears to be a good correlation between the changes in ultrastructure (see Chapter 3) associated with development and the development of the enzyme pattern important in catabolic pathways of flight metabolism (BEENAKKERS et al., 1975). Associated with these developmental changes is the fact that Ca^{2+} must move between SR and sarcoplasm and back to control the contraction - relaxation cycle (ELDER, 1975).

It is thought that the maximal activity of Ca^{2+}-transporting ATPase and Ca^{2+}-uptake by the SR of vertebrate skeletal muscle, is preceded by the appearance of Mg^{2+}-dependent ATPase and is associated with the development of the SR (FANBURG et al., 1968; HOLLAND and PERRY, 1969; MARTONOSI et al., 1972; BOLLAND et al., 1974). This is consistent with the suggestion that the speed of contraction and relaxation of mature skeletal muscle is determined by its Ca^{2+}-transporting activity (SANDOW, 1965).

In view of the information presented above, the development of the SR ATPase system is clearly an important factor in regulating mechanical activity by controlling sarcoplasmic levels of Ca^{2+} concentration.

The latter, in turn, plays a significant role in mitochondrial function. A number of mitochondrial enzymes are known to be influenced by Ca^{2+} (HANSFORD and CHAPPELL, 1967; VAUGHAN and NEWSHOLME, 1969; ZAMMIT and NEWSHOLME, 1976; ROCHE et al., 1980; DENTON and McCORMACK, 1980a), for example, α-glycerophosphate dehydrogenase (HANSFORD and CHAPPELL, 1967; DONNELLAN and BEECHEY, 1969). Associated with the complete development of flight muscle of Locusta migratoria, there is an approximately 5-fold increase in the total activity of α-glycerophosphate dehydrogenase (EC 1.1.99.5) and a 50-fold increase in the chondriome (BROSEMER et al., 1963; BÜCHER, 1965). α-Glycerophosphate dehydrogenase is bound to the inner membrane of Locusta flight muscle mitochondria (ZEBE and McSHAN, 1957). More recently, studies by VAUGHAN and NEWSHOLME (1970), KLINGENBERG and BUCHHOLZ (1970), DONNELLAN et al. (1970), BALBONI (1972) and LLOYD and HARRISON (1974) suggest that this enzyme is, in fact, located on the outer surface of the inner mitochondrial membrane. However, SLACK and BURSELL (1977) suggest that α-glycerophosphate dehydrogenase is located on the inner rather than outer surface of the inner mitochondrial membrane. The coupling between mitochondrial α-glycerophosphate dehydrogenase and cytoplasmic α-glycerophosphate dehydrogenase (EC 1.1.1.8), provides insect flight muscle with a highly efficient system (the α-glycerophosphate shuttle) for the oxidation of extra-mitochondrial NADH generated during glycolysis (ZEBE and McSHAN, 1957; ZEBE et al., 1959; SACKTOR, 1965) (see Fig. 5.1.). The α-glycerophosphate shuttle or cycle assumes a highly significant role in the overall metabolism of flight muscle compensating for the very low activity of lactate dehydrogenase present in this tissue (SACKTOR, 1955; ZEBE and McSHAN, 1957).

It has been shown that the inclusion of EDTA (ESTABROOK and SACKTOR, 1958) or EGTA (HANSFORD and CHAPPELL, 1967) in the incubation media resulted in the inhibition of α-glycerophosphate oxidation by

Figure 5.1. A schematic diagram of the α-glycerophosphate shuttle showing its connection with glycolysis in insect flight muscle (after SACKTOR, 1975). The enzymes involved at the various points are as follows:

(1) Phosphofructokinase

(2) Aldolase

(3) Glyceraldehyde-3-phosphate dehydrogenase

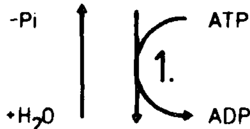
(4) Triose phosphate isomerase

(5) Cytoplasmic α-glycerophosphate dehydrogenase

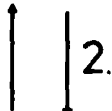
(6) Mitochondrial α-glycerophosphate dehydrogenase

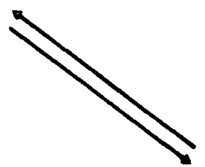
GLYCOLYSIS

FRUCTOSE-6-PHOSPHATE

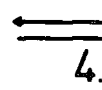


FRUCTOSE-1,6-DIPHOSPHATE

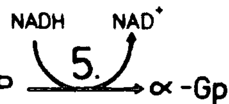




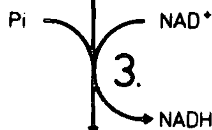
GLYCERALDEHYDE-3-PHOSPHATE



DHAP



α-Gp



1,3-DIPHOSPHATE GLYCERATE

CYTOPLASM

α-Gp CYCLE

PYRUVATE

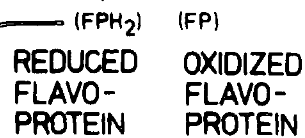
MITOCHONDRIAL

MEMBRANE

CITRIC ACID CYCLE

DHAP

α-Gp



REDUCED FLAVO-PROTEIN

OXIDIZED FLAVO-PROTEIN

MITOCHONDRIA

H_2O ← $\frac{1}{2}O_2$

RESPIRATORY CHAIN

flight muscle mitochondria from Musca domestica and Calliphora vomitora, respectively. This inhibition was relieved by the divalent metal ions, Ca^{2+} and Mg^{2+} or by increasing the substrate concentration. Indeed, HANSFORD and CHAPPELL (1967) showed that μmolar concentration of Ca^{2+} were sufficient to induce a rapid rate of α-glycerophosphate oxidation by flight muscle mitochondria from Calliphora vomitora. Furthermore, a plot of enzyme activity verus concentration of α-glycerophosphate revealed a sigmoidal relationship in the absence of Ca^{2+}. At 2mM α-glycerophosphate, which is the concentration found in the flight muscle of the blowfly, Phormia regina (SACKTOR and WORMSER-SHAVIT, 1966), the inclusion of Ca^{2+} in the incubation medium effected a 10-fold increase in the rate of oxidation. It was, therefore, suggested that the action of Ca^{2+} is to lower the apparent Km for α-glycerophosphate (HANSFORD and CHAPPELL, 1967) by acting as a co-operative heterotropic allosteric effector on the allosteric site of the enzyme (DONNELLAN and BEECHEY, 1969). This activation of α-glycerophosphate dehydrogenase by μmolar Ca^{2+}, has subsequently been demonstrated with flight muscle mitochondria and submitochondrial particles from various insects, including Sarcophaga barbata, Pieris brassicae, Apis mellifera, Schistocerca gregaria and Musca domestica (DONNELLAN and BEECHEY, 1969), Phormia regina (CARFOLI and SACKTOR, 1972). LLOYD and HARRISON (1974) have shown that the oxidation of 0.5mM α-glycerophosphate by Locusta flight muscle mitochondria increased 5-fold by addition of very low concentrations of Ca^{2+}.

The present study was undertaken to determine some of the properties of the SR-ATPase of Locusta flight muscle and to determine whether the activity of this enzyme changes with development. In addition, the ability of isolated mitochondria to oxidize α-glycerophosphate and the effect of Ca^{2+}, Mg^{2+} and substrate concentrations on this process have been studied, at various ages, to determine whether changes in the allosteric behaviour are associated with development.

Materials and Methods

The maintenance of insects and the methods of sampling were the same as those described in the General Materials and Methods (Chapter 2).

A) Preparation of SR microsomal fraction from *Locusta migratoria* flight muscle

The method employed was essentially the same as that described by COSSINS (1974).

Unless otherwise stated, 10 adult animals (5 males + 5 females) were killed as described in the preparation of mitochondria (see General Materials and Methods (Chapter 2)). The flight muscles were quickly dissected out and collected in a small beaker standing on crushed ice, and containing 10cm^3 of an ice-cold homogenization medium (1) consisting of 100mM KCl, 10mM imidazole/HCl, pH 7.1. The "pooled" muscles were transferred to an ice-cold homogenization tube and homogenization was carried out with a Teflon pestle (clearance 0.1-0.5mm). Ten passes of the pestle, by hand, were sufficient to break up the flight muscle to small fragments. The resulting homogenate was then distributed into two polycarbonate centrifuge tubes (M.S.E. Ltd.) and centrifugation was carried out at 1,000g (Mistral 2L, M.S.E. Ltd.) for 30 minutes at 0-4°C to remove nuclei, connective tissue and cell debris. The supernatant (a) was stored on ice, and the pellet obtained from this spin was re-extracted as described above using isolation medium (1). The second pellet was discarded and the supernatant (b) retained and combined with supernatant (a). The combined supernatants (a + b) were centrifuged at 13,000g at 0-4°C for 30 minutes in a High Speed 18 refrigerated centrifuge (M.S.E. Ltd.) to remove remaining nuclei, myofibrils and mitochondria. Once again the pellet was discarded and the supernatant

which contained microsomes (SR fragments), soluble protein, myofibrils and mitochondrial contaminants retained. This was then centrifuged at 35,000g in an M.S.L. Automatic Superspeed 40 (head number 2409) for 60 minutes at 0-4°C. The supernatant was discarded and the pellet retained and resuspended in a concentrated KCl medium (2), consisting of 0.6M KCl, 10mM imidazole/HCl, pH 7.1, by homogenization, in order to solubilise contaminant fragments of actomyosin (MARTONOSI and FERETOS, 1964b).

The pellet was finally sedimented at 35,000g for 60 minutes at 0-4°C, and the final pellet was suspended by homogenization in an appropriate volume of sucrose medium (3), consisting of 0.3M sucrose, 10mM imidazole/HCl, pH 7.1. The latter medium has been found to protect the microsomal preparation from deterioration during storage (COSSINS, 1974). The protein concentration was between 60-100μg/cm^3.

B) <u>Assay of ATPase activity</u>

(i) <u>Ionic media</u>

(a) Basal ATPase (Mg^{2+}-dependent ATPase)

The Mg^{2+}-dependent ATPase activity was determined in a reaction medium with the following final ionic composition.

3mM $MgCl_2$

0.5mM EGTA

100mM KCl

75mM Sucrose

25mM Bis-Tris Propane/HCl (pH 7.6) at 30°C

(b) Total ATPase (Ca^{2+}-Mg^{2+}-ATPase)

The total ATPase activity was determined in reaction medium with the following ionic composition.

3mM $MgCl_2$

0.5mM EGTA

0.5mM $CaCl_2$

100mM KCl

75mM Sucrose

25mM Bis-Tris Propane/HCl (pH 7.6) at 30°C

In cases where the composition of the reaction media and pH were different, they will be described in the text.

(ii) Stopping mixture

This consisted of a 1:1 mixture of 1% cirrasol ALN-WF and 1% ammonium molybdate in 0.9M sulphuric acid (ATKINSON et al., 1973).

(iii) Experimental procedure

Incubations were normally carried out at 30 ± 0.1°C for 20 minutes (unless otherwise stated).

Enzyme activity was assayed either in 150 x 25mm "Pyrex" boiling tubes, or "Pyrex" glass centrifuge tubes. Assay tubes containing the appropriate ionic media were thermoequilibrated for 15 minutes at bath temperatures before starting the reaction. The reaction media consisted of $1cm^3$ of the appropriate ionic medium and $0.5cm^3$ of 12mM Tris-ATP (see Appendix 5.1). The reaction was started by the addition of $0.5cm^3$ of the microsomal suspension bringing the final volume up to $2cm^3$. After the period of incubation, the reaction was stopped by adding $4cm^3$ of the stopping mixture and the tubes were quickly removed from the water bath and left to stand at room temperature for 10 minutes to allow the yellow colour to develop. They were then transferred to crushed ice for storing. Control tubes were set up in each experiment. These tubes contained the normal reaction medium and $0.5cm^3$ Tris-ATP. They were incubated for the same period as the experimental tubes. However, the microsomal suspension was not added until immediately

after the addition of the stopping mixture.

(iv) Analysis of inorganic phosphate

Following centrifugation at 1,000g in M.S.E. Mistral 2L centrifuge for 10 minutes at 0-4°C, to remove any protein which precipitated, the supernatant was poured into clean, precooled "Pyrex" tubes and stored on ice. The intensity of the yellow colour which developed was read at 390nm on a Cecil CE 292 spectrophotometer. The resulting absorbancy values were converted to concentration of inorganic phosphate by reference to a standard calibration curves (see Appendix 5.2) (ATKINSON *et al.*, 1973). All results were expressed as nmoles Pi liberated mg.protein^{-1}.minute^{-1}.

(v) Estimation of Ca^{2+}-stimulated ATPase

The Mg^{2+}-dependent ATPase and Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activities were calculated by subtracting the values for the control tubes from those obtained with the experimental tubes. The Ca^{2+}-stimulated ATPase (extra ATPase) activity was calculated as the difference between Mg^{2+}-dependent ATPase activity and Ca^{2+} + Mg^{2+}-ATPase activity.

(vi) Protein determination of SR

These were made by the method of LOWRY *et al.* (1951) using BSA fraction V (Sigma Chemical Co.) as standard (see Chapter 2). From knowing the number of animals used in the preparation of SR fragments and the volume of the final microsomal suspension, the amount of SR protein per thorax was calculated.

(vii) Treatment of saturation kinetics data

The data were treated graphically using the LINEWEAVER and BURK (1934) plot which modifies the Michaeles - Menten equation. $\frac{1}{\{s\}}$ was plotted against $\frac{1}{v}$ (s = substrate concentration and v = reaction velocity).

Vmax was calculated from $\frac{1}{\text{intercept}}$ on the y axis and Km from $\frac{1}{\text{intercept}}$ on the x axis. The slopes were calculated by using regression analysis (SNEDECOR and COCHRAN, 1967).

C) The effect of Ca^{2+}, Mg^{2+} and α-glycerophosphate concentration on the activity of α-glycerophosphate dehydrogenase from developing flight muscle mitochondria

The activity of this enzyme was determined by measuring the uptake of oxygen; oxygen is the terminal electron acceptor in mitochondria. The method employed was essentially as described by KASHMEERY (1977). This involved the inclusion of the uncoupling agent, carbonyl-cyanide-p-trifluoro-methoxy-hydrazone (FCCP), in the incubation medium to ensure the elimination of the role played by the respiratory chain during α-glycerophosphate oxidation.

α-Glycerophosphate dehydrogenase activity of isolated mitochondria was monitored in the presence of varying concentrations of α-glycerophosphate. All reaction media were fully equilibrated with air at 30°C for at least 2 hours prior to beginning the experiment. $2cm^3$ of the appropriate reaction medium was pipetted into the reaction chamber (see Fig. 2.1 in Chapter 2). Following the exclusion of all air bubbles from the reaction chamber, 3μl of an ethanolic solution of FCCP was added to give a final concentration of 0.5μM. This was followed by 50μl of the appropriate mitochondrial suspension. The reaction was started by the addition of 40μl α-glycerophosphate substrate. The final substrate concentrations used were as follows: 0.2, 0.4, 0.8, 1.2, 1.6, 3.3, 8.2, 13, 20, 32, 43, 54mM. All additions were carried out using a microsyringe. The complete range of α-glycerophosphate concentrations was studied with each mitochondrial preparation.

The following three reaction media were used.

(i) Calcium free medium

100mM KCl

10mM Tris/HCl, pH 7.1 at 30°C

* 1mM EGTA

(ii) Calcium containing medium

100mM KCl

10mM Tris/HCl, pH 7.1 at 30°C

1mM EGTA

1mM $CaCl_2$ (free Ca=1.43×10^{-5}M)

(iii) Magnesium containing medium

100μM KCl

5mM $MgCl_2$

10mM Tris/HCl, pH 7.1 at 30°C

* 1mM EGTA

* The inclusion of EGTA ensured the elimination of Ca^{2+}.

D) <u>Calculation of the concentration of free Ca^{2+} in a Ca-EGTA buffer system</u>

A low level of free Ca^{2+} concentration is known to activate and control a number of biological processes, such as the excitation-contraction coupling and relaxation (see Introduction). The presence of significant concentrations of Ca^{2+} from extraneous sources and of an unknown number of Ca^{2+} binding sites in mitochondrial and SR preparations, makes it difficult to know the free Ca^{2+} concentration accurately. This problem can be overcome by the use of a Ca^{2+}-buffer system, using substances which selectively bind to divalent ions. One of the best known substance of this type is EDTA which binds Ca^{2+} about 10^2 more

effectively than Mg^{2+}. Recently, however, EDTA has been replaced by EGTA which can bind Ca^{2+} over 10^5 times more effectively.

Normally only the binding of Ca^{2+} by the forms of EGTA with three negative charges (HL^{3-}) and four negative charges (L^{4-}) are considered (CALDWELL, 1970).

At given conditions of pH, ionic strength and temperature, the apparent association constant k_1 and k_2 for the two forms of Ca-EGTA can be defined as

$$k_1 = [MeHL^-] / [Me^{2+}][HL^{3-}] \text{ and}$$

$$k_2 = [MeL^{2-}] / [Me^{2+}][L^{4-}] \text{ where}$$

$[MeHL^-]$ and $[MeL^{2-}]$ represent the concentration of Ca-EGTA for the two forms of binding. $[HL^{3-}]$ and $[L^{4-}]$ represent the total ligand that is not complexed, and $[Me^{2+}]$ represent the uncomplexed Ca^{2+} concentration. A combined apparent association constants K (Kapp) can be defined as

$$K = k_1 + k_2 = \frac{[MeL]}{[Me^{2+}][L]} \text{ where}$$

$[MeL]$, $[L]$ and $[Me^{2+}]$ represent total concentration of Ca-EGTA complex, uncomplexed EGTA and the remaining free Ca^{2+} concentration, respectively.

The latter equation was used to calculate the free Ca^{2+} concentration, using the corrected values of the apparent association constant (see Appendix 5.3) by a process of successive approximations (CALDWELL, 1970; COSSINS, 1974). Initially it was assumed that all available Ca^{2+} was bound by EGTA, so that $[MeL]$ was equal to the total EGTA remaining uncomplexed. The value of K, $[MeL]$ and $[L]$ was used to calculate a value of Ca^{2+} concentration. Subsequently, the values of $[MeL]$ and $[L]$ were adjusted to account for free Ca^{2+} concentrations calculated previously. This process was repeated until reasonably steady values of Ca^{2+} concentration were reached. The effect of Mg^{2+} on the equilibrium was not estimated.

The relationship between the total Ca^{2+} concentration in the system and the free Ca^{2+} concentration in the presence of 0.5mM and 1mM EGTA is shown in Figures 5.2a and b.

Results

In the present study, it was assumed that microsomal fractions prepared from Locusta migratoria flight muscle, as described in the Materials and Methods, consist mainly of fragments of SR (COSSINS, 1974). The extraction step with 0.6M KCl yielded a microsomal preparation believed to be free of actomyosin for this high concentration of KCl dissolves actomyosin but does not effect SR protein (MARTONOSI, 1968; INESI, 1970). However, the possibility that there may be sarcolemmal and mitochondrial contamination cannot be ruled out.

Time course of ATP hydrolysis

Mg^{2+}-dependent ATPase was determined in an incubation medium containing 3mM $MgCl_2$ and 0.5mM EGTA, and Ca^{2+} + Mg^{2+}-ATPase (total ATPase in an incubation medium containing 0.5mM Ca-EGTA (free Ca^{2+} = 3.19×10^{-6}) in addition to 3mM $MgCl_2$. Pairs of tubes, experimental and blank, were incubated for different periods of time (0-30 min.) at 30°C. The buffer system used was 25mM Bis-Tris propane (pH 7.6) and 3mM Tris-ATP used as substrate. The results are shown in Figure 5.3. It can be seen that the activity of Ca^{2+} - Mg^{2+}-ATPase, Ca^{2+}-stimulated ATPase and Mg^{2+}-dependent ATPase are linear with time over the 30 min period studied. In all subsequent studies incubations were run routinely for 20 minutes only.

Effect of pH on ATPase activity

The basic incubation medium consisted of 100mM KCl, 75mM sucrose, 0.5mM EGTA and 3mM Tris-ATP. Two distinct buffer systems were used to test the dependence of the enzyme on pH and to establish the optimum

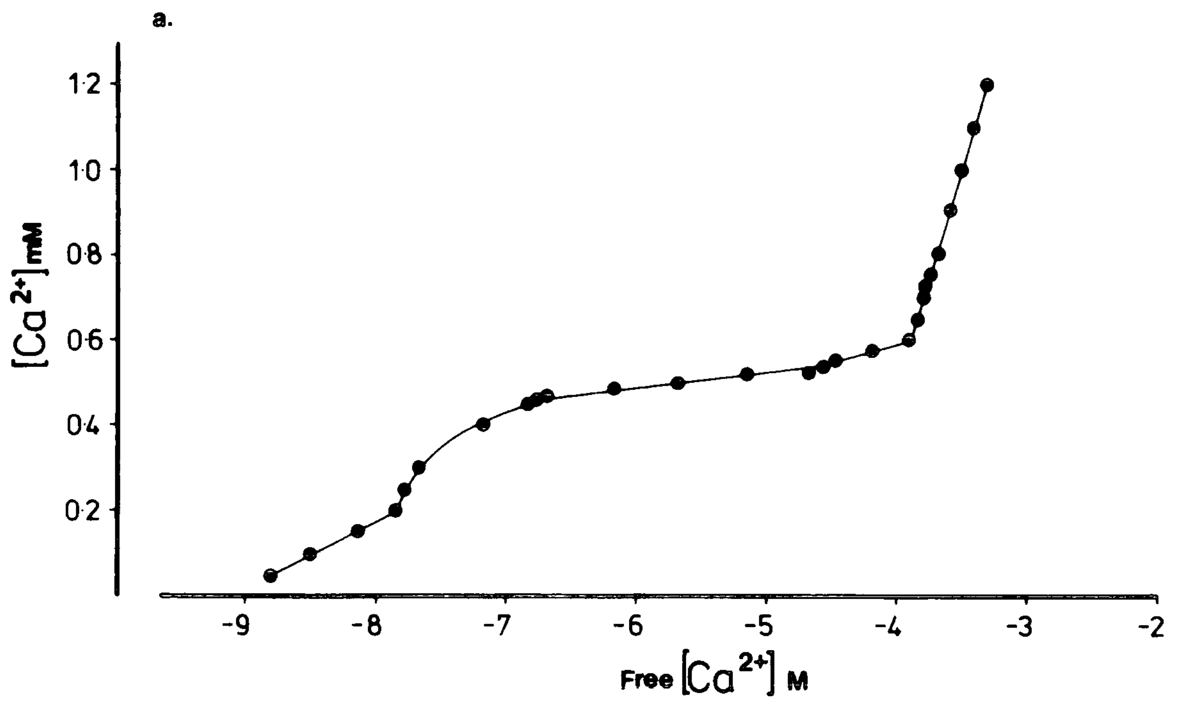
Figure 5.2. The relationship between Ca^{2+} concentration and free Ca^{2+} concentration at pH 7.6 and pH 7.1 at 30°C.

a. at pH 7.6

The EGTA concentration was 0.5mM and K_{app} was taken to be $10^{7.69} \equiv 4.87 \times 10^{7}$ (see Appendix 5.3).

b. at pH 7.1

The EGTA concentration was 1mM and K_{app} was taken to be $10^{6.69} \equiv 4.89 \times 10^{6}$ (see Appendix 5.3).


a.
1·2
1·0
0·8
0·6
0·4
0·2
$[Ca^{2+}]_{mM}$
-9
-8
-7
-6
-5
-4
-3
-2
Free $[Ca^{2+}]$ M

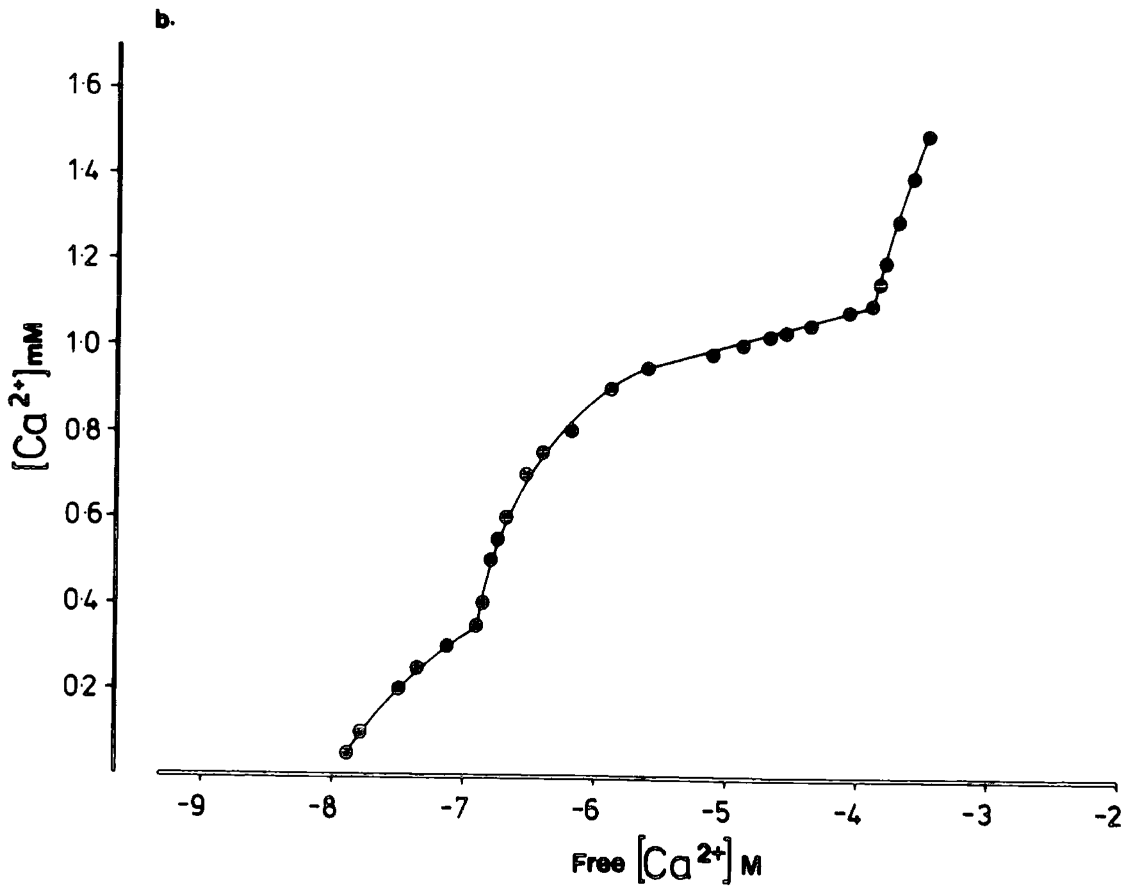

b.
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
$[Ca^{2+}]_{mM}$
-9
-8
-7
-6
-5
-4
-3
-2
Free $[Ca^{2+}]$ M

Figure 5.3. Effect of time on the hydrolysis of ATP by SR-ATPase from *Locusta migratoria* flight muscle.

The condition of incubation and the composition of the reaction media are fully described in the text.

Ordinate: ATPase activity (nmole Pi liberated. mg $protein^{-1}$. $minute^{-1}$)

Abscissa: Time (mins)

● Total (Ca^{2+} - Mg^{2+})-ATPase

▲ Ca^{2+}-stimulated-(Mg^{2+}-dependent)-ATPase

■ Mg^{2+}-dependent ATPase

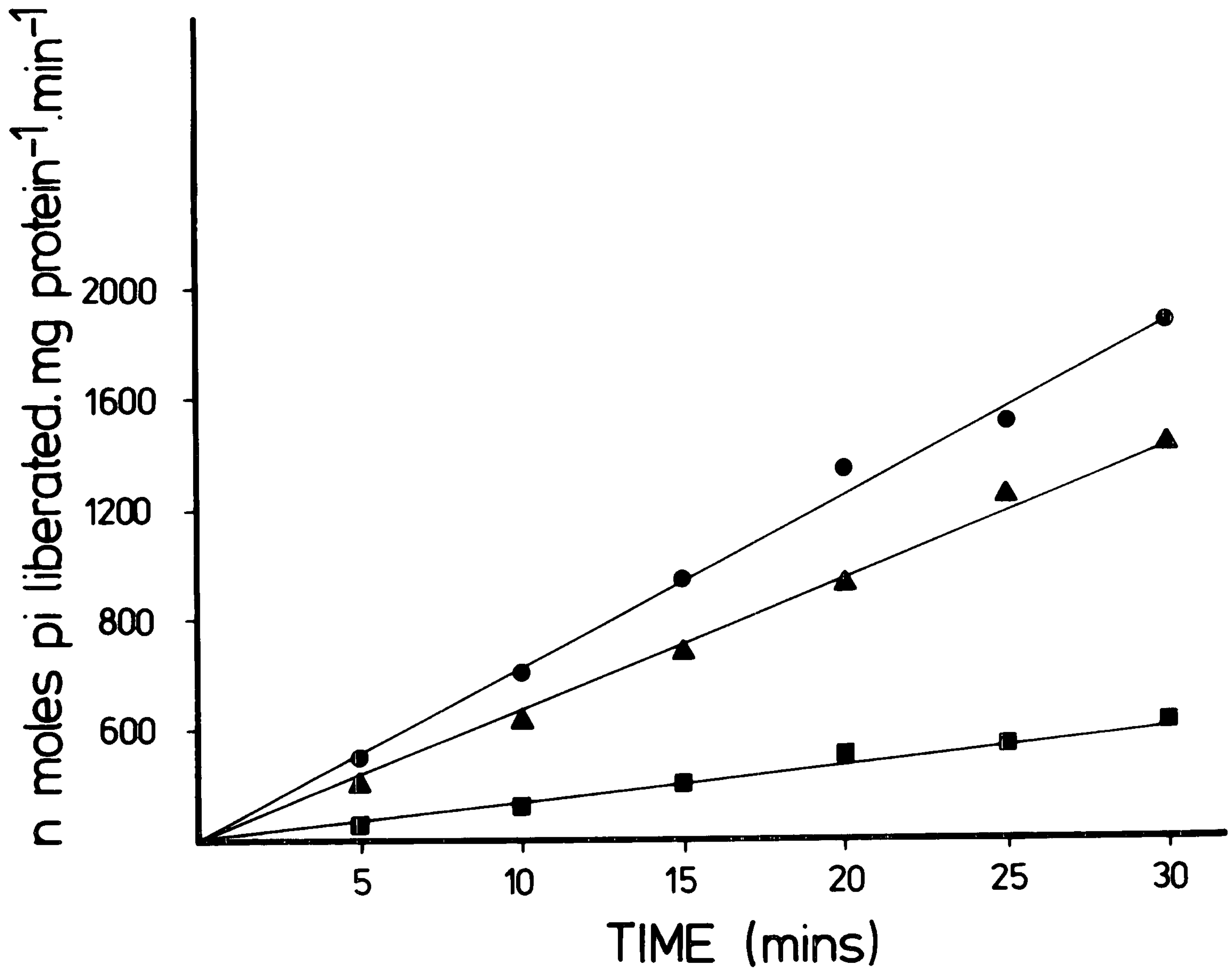

n moles pi liberated.mg protein⁻¹.min⁻¹
2000
1600
1200
800
600
5
10
15
20
25
30
TIME (mins)

Figure 5.4a. Effect of pH on the activity of SR-ATPase extracted from Locusta migratoria flight muscle. The data were obtained in the presence (●) and absence (■) of 3.19×10^{-6}M free Ca^{2+}.

The stimulation due to the presence of Ca^{2+} (▲) represents the difference between (●) and (■).

Typical experiment representative of four separate experiments (see Appendix 5.4a for mean ± S.E.M.).

Ordinate: ATPase activity (nmole Pi liberated. mg $protein^{-1}$. $minute^{-1}$

Abscissa: pH

A Bis-Tris propane/HCl buffer system was used.

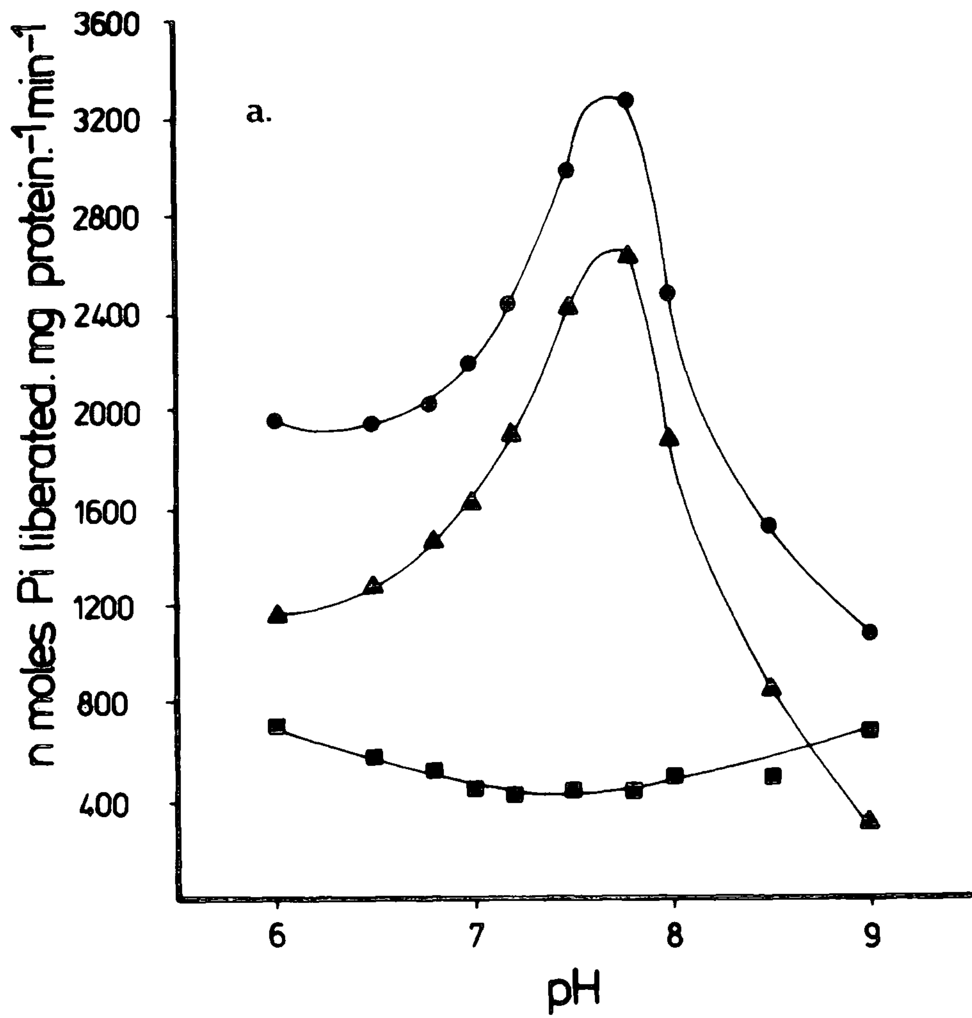
■ Mg^{2+}-dependent ATPase

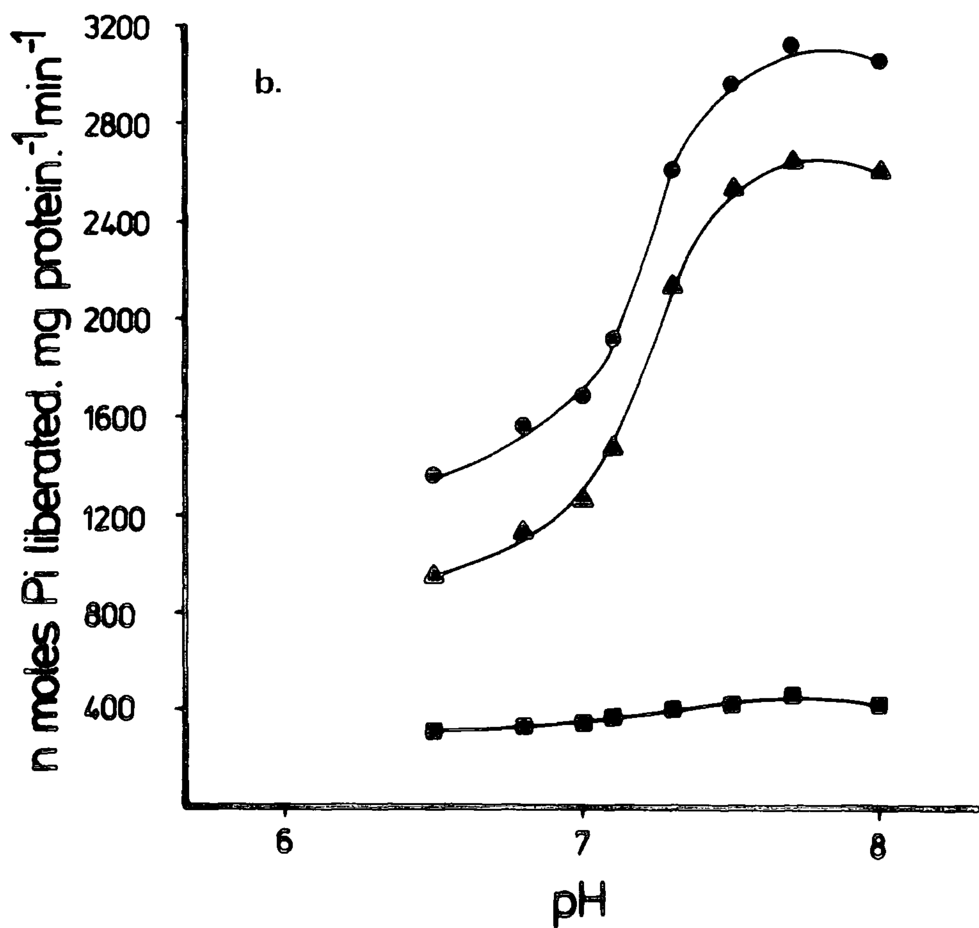
▲ Ca^{2+}-stimulated-(Mg^{2+}-dependent)-ATPase

● Total-(Ca^{2+} + Mg^{2+})-ATPase

Figure 5.4b. Effect of pH on the activity of SR-ATPase extracted from Locusta migratoria flight muscle. Details are as given in Figure 5.4a except that imidazole/HCl was the buffer system used.

Typical experiment representative of four separate experiments (see Appendix 5.4b for mean ± S.E.M.).


a.
n moles Pi liberated. mg protein.-1min-1
3600
3200
2800
2400
2000
1600
1200
800
400
6
7
8
9
pH


b.
n moles Pi liberated. mg protein.-1min-1
3200
2800
2400
2000
1600
1200
800
400
6
7
8
pH

conditions for future study. These were either 25mM imidazole/HCl (to provide a pH range from 6.5 to 8) or 25mM Bis-Tris propane/HCl (to provide a pH range from 6 to 9). Mg^{2+}-dependent ATPase was determined in suitably buffered incubation media (basic) containing 3mM $MgCl_2$. Total ATPase activity was determined in suitably buffered (basic) incubation media containing 3mM $MgCl_2$ and 0.5mM $CaCl_2$. Although changes in pH induce changes in the free concentration of Ca^{2+} and Mg^{2+} (AMOS _et al_., 1976), no attempt was made to compensate for this fact. Figures 5.4a and b (see also Appendix 5.4) show the relationship between Mg^{2+}-dependent ATPase, total ATPase and Ca^{2+}-stimulated ATPase activity and pH. The pH optimum was approximately 7.6 for total ATPase and Ca^{2+}-stimulated ATPase irrespective of the buffer system used. Similarly, maximal activity of total ATPase and Ca^{2+}-stimulated ATPase was little affected by the buffer used. Mg^{2+}-dependent activity, on the other hand, showed little change over the pH range studied.

Effect of $MgCl_2$ concentration on ATPase activity

Mg^{2+}-dependent ATPase and total ATPase activities were assayed as described previously except that the $MgCl_2$ concentration was varied between 0-12mM. The relationships between Mg^{2+} concentration and ATPase activities are shown in Figure 5.5. (see also Appendix 5.5). Little or no ATPase activity was demonstrated in the absence of Mg^{2+}. A marked increase in specific activity of Mg^{2+}-dependent ATPase, total ATPase and Ca^{2+}-stimulated ATPase was observed by the inclusion of Mg^{2+}; maximal ATPase activities being obtained between 1.5-3mM $MgCl_2$ (see Fig. 5.5 and also Appendix 5.5). Reference to Figure 5.5 and Appendix 5.5 shows that increasing Mg^{2+} concentration further resulted in a decrease in the stimulated level of ATPase activities. Thus maximal Ca^{2+}-stimulated ATPase activity was reduced by ca. 72% when $MgCl_2$

Figure 5.5. Effect of Mg^{2+} concentration on the activity of SR-ATPase extracted from Locusta migratoria flight muscle.

The data were obtained in the presence (●) and absence (■) of 3.19 x 10^{-6}M free Ca^{2+} concentration.

The stimulation due to Ca^{2+} (▲) represents the difference between (●) and (■).

Typical set of data representative of five separate experiments (see Appendix 5.5 for mean ± S.E.M.).

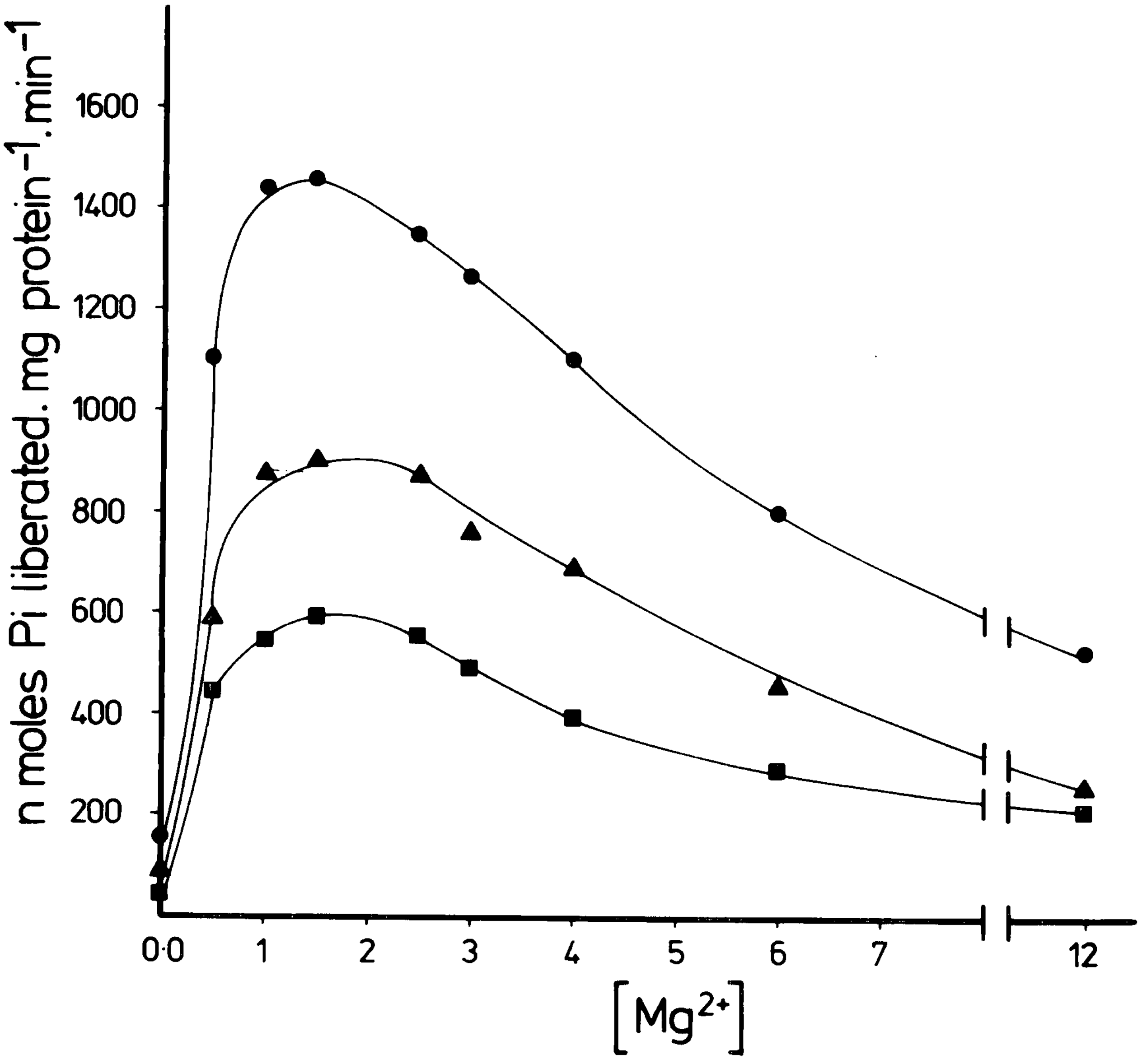
Ordinate: ATPase activity (nmole Pi liberated. mg $protein^{-1}$. $minute^{-1}$

Abscissa: Mg^{2+} concentration (mM)

■ Mg^{2+}-dependent ATPase

▲ Ca^{2+}-stimulated-(Mg^{2+}-dependent)-ATPase

● Total-(Mg^{2+} + Ca^{2+})-ATPase



n moles Pi liberated. mg protein^{-1}. min^{-1}
1600
1400
1200
1000
800
600
400
200
0·0
1
2
3
4
5
6
7
12
$[Mg^{2+}]$

concentration was increased to 12mM. Similar reductions in the specific activity of total ATPase and Mg^{2+}-dependent ATPase were observed (ca. 64%). The maximal level of Ca^{2+}-stimulated ATPase activity represented ca. 61% of total ATPase activity.

Effect of free Ca^{2+} concentration on ATPase activity

Mg^{2+}-dependent ATPase activity was assayed in incubation media in which the free Ca^{2+} concentration was varied between $0 - 1.50 \times 10^{-3}$M by means of a Ca-EGTA buffer system. The results obtained in a typical experiment are shown in Figure 5.6 (see also Appendix 5.6). The ATPase activity exhibited a high sensitivity to free Ca^{2+} concentration. Maximal activity (i.e. total ATPase activity) was observed at 3.19×10^{-6}M. Increasing the free Ca^{2+} concentration above this level resulted in a reduction in the stimulated level of activity (see Fig. 5.6). At maximal activity the Ca^{2+}-stimulated ATPase represented more than 70% of the total activity present.

Effect of ATP concentration on ATPase activity

The effect of ATP concentration on enzyme activity was measured by incubating the membrane preparation with 3.19×10^{-6}M free Ca^{2+}, 25mM Bis-Tris propane/HCl (pH 7.6), 100mM KCl, 75mM sucrose and various concentrations of ATP (Tris salt) over the range of 0.05 - 3mM. Figure 5.7 shows the relationship between total ATPase, Ca^{2+}-stimulated ATPase and Mg^{2+}-dependent ATPase activities and ATP concentrations for a typical set of data. A Lineweaver-Burk plot of such data is shown in Figure 5.8. Such plots were used in calculating the apparent Michaelis Constant (Km) and Vmax for total ATPase, Ca^{2+}-stimulated ATPase and Mg^{2+}-dependent ATPase (Table 5.1). The results show that in the presence of Ca^{2+} Vmax increased substantially. Furthermore, the values for Km indicate that in the presence of Ca^{2+} there is an increased enzyme affinity for ATP.

Figure 5.6. Effect of free Ca^{2+} concentration on the activity of SR-ATPase extracted from *Locusta migratoria* flight muscle.

The data were obtained in the presence of Mg^{2+} (●) and Ca^{2+} + Mg^{2+} (⊖). The Mg^{2+} concentration was 3mM throughout.

Typical experiment representative of five separate experiments (see Appendix 5.6. ± S.E.M.).

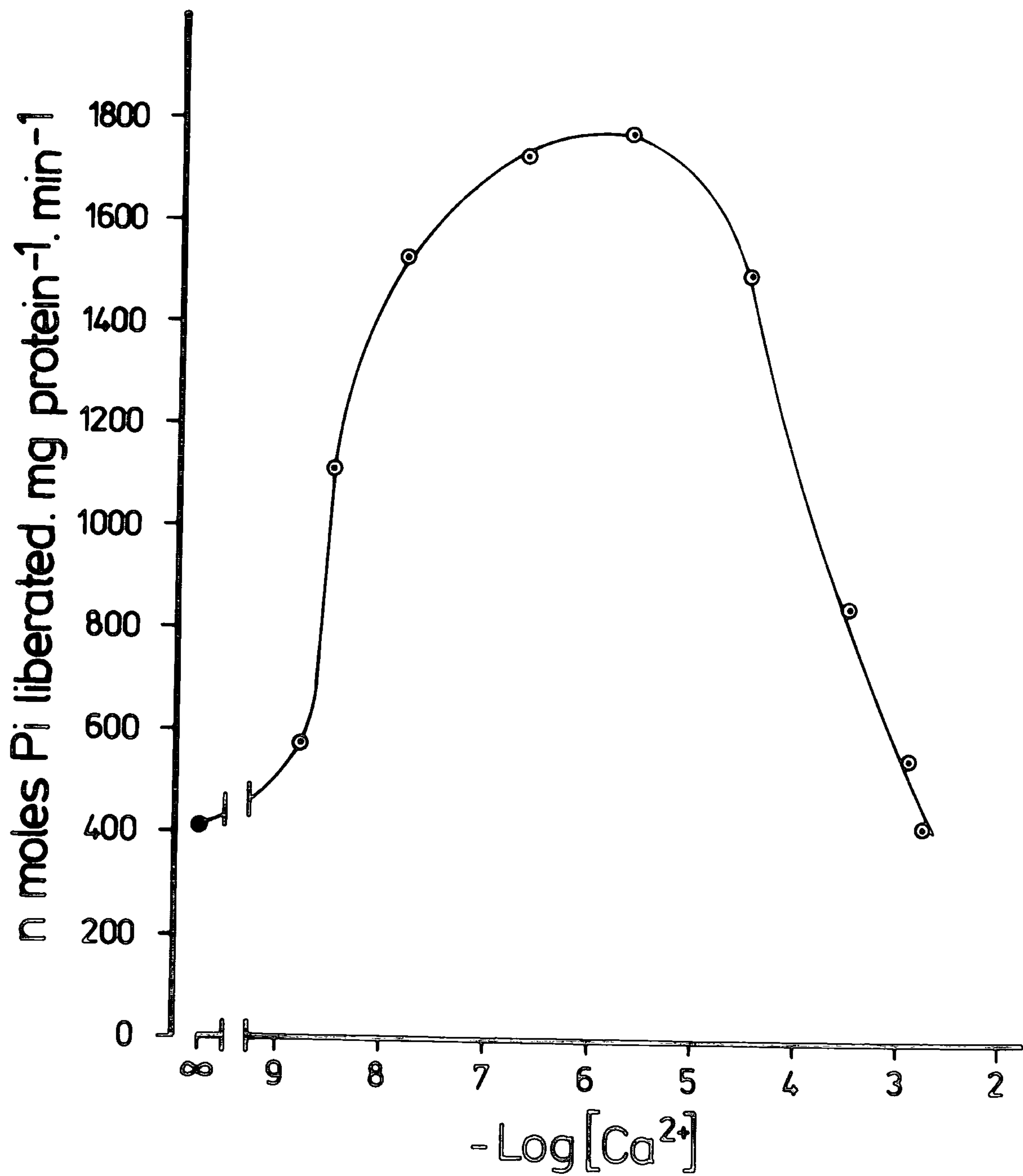
Ordinate: ATPase activity (nmole Pi liberated. mg protein^{-1}. minute^{-1}

Abscissa: negative logarithm of free Ca^{2+} concentration (M).

The free Ca^{2+} concentration was calculated from Figure 5.2a.

● Mg^{2+}-dependent ATPase

⊖ Total (Mg^{2+} + Ca^{2+})-ATPase



n moles Pi liberated. mg protein-1. min-1
1800
1600
1400
1200
1000
800
600
400
200
0
∞
9
8
7
6
5
4
3
2
-Log[Ca2+]

Figure 5.7. Effect of ATP concentration on the activity of SR-ATPase extracted from *Locusta migratoria* flight muscle.

The data were obtained in the presence (●) and absence (■) of 3.19×10^{-6}M free Ca^{2+}. The stimulation due to Ca^{2+} (△) represents the difference between (●) and (■).

Typical experiments representative of six separate experiments.

Ordinate: ATPase activity (nmole Pi mg protein^{-1} minute^{-1})

Abscissa: ATP concentration (mM)

■ Mg^{2+}-dependent ATPase

△ Ca^{2+}-stimulated-(Mg^{2+}-dependent)-ATPase

● Total (Ca^{2+} + Mg^{2+})-ATPase

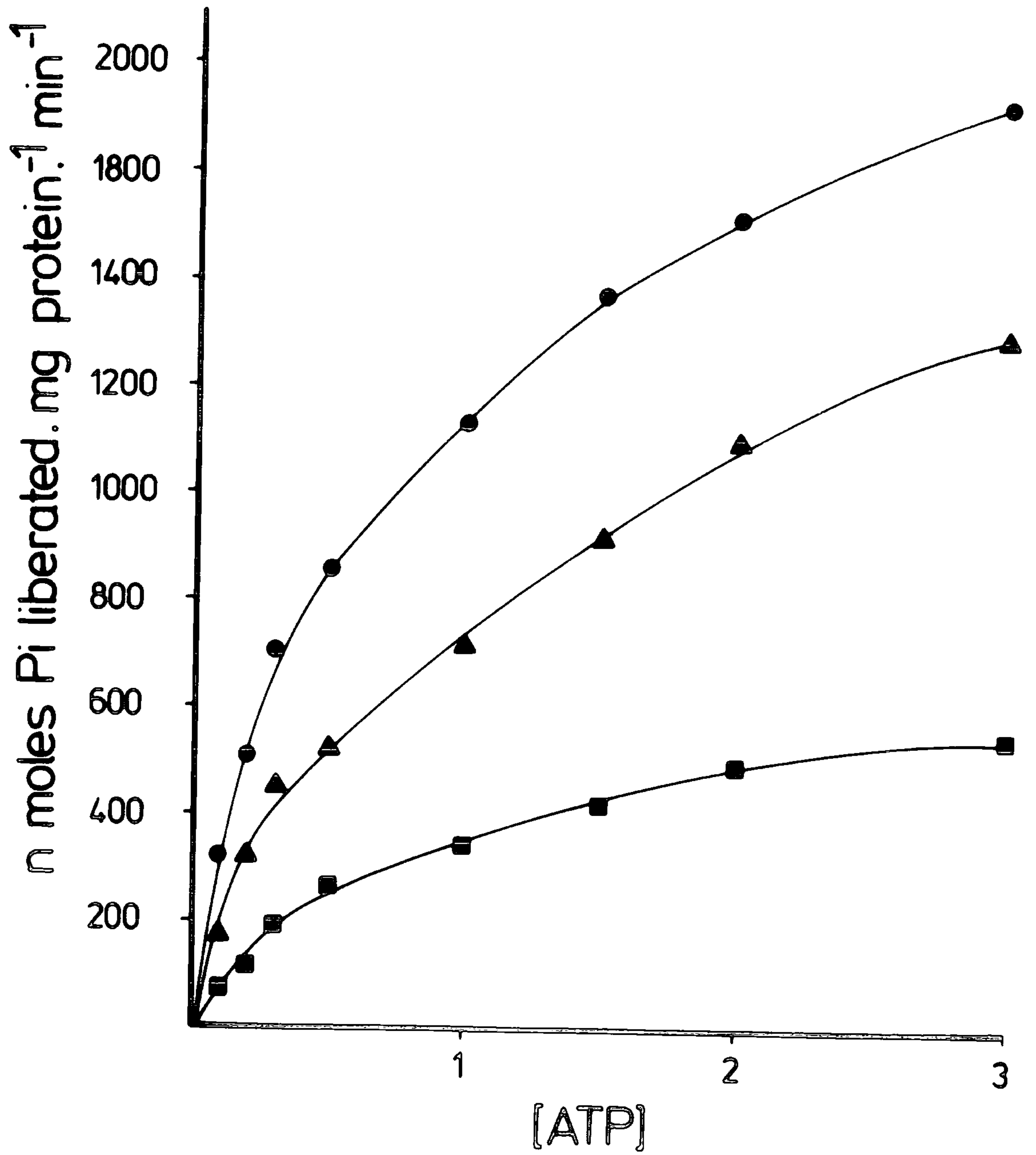

n moles Pi liberated. mg protein.-1 min-1
2000
1800
1400
1200
1000
800
600
400
200
1
2
3
[ATP]

Figure 5.8. Lineweaver-Burk plot of SR-ATPase activity as function of ATP concentration.

The data were obtained in the presence (■) and absence (●) of 3.19 x 10^{-6}M free Ca^{2+}. The stimulation due to Ca^{2+} (▲) represents the difference between (■) and (●).

Ordinate: reciprocal of ATPase activity (nmole Pi liberated. mg $protein^{-1}$ $minute^{-1}$) x 10^{-3}

Abscissa: reciprocal of ATP concentration (mM)

● Mg^{2+}-dependent ATPase

▲ Ca^{2+}-stimulated-(Mg^{2+}-dependent)-ATPase

■ Total-(Mg^{2+} + Ca^{2+})-ATPase

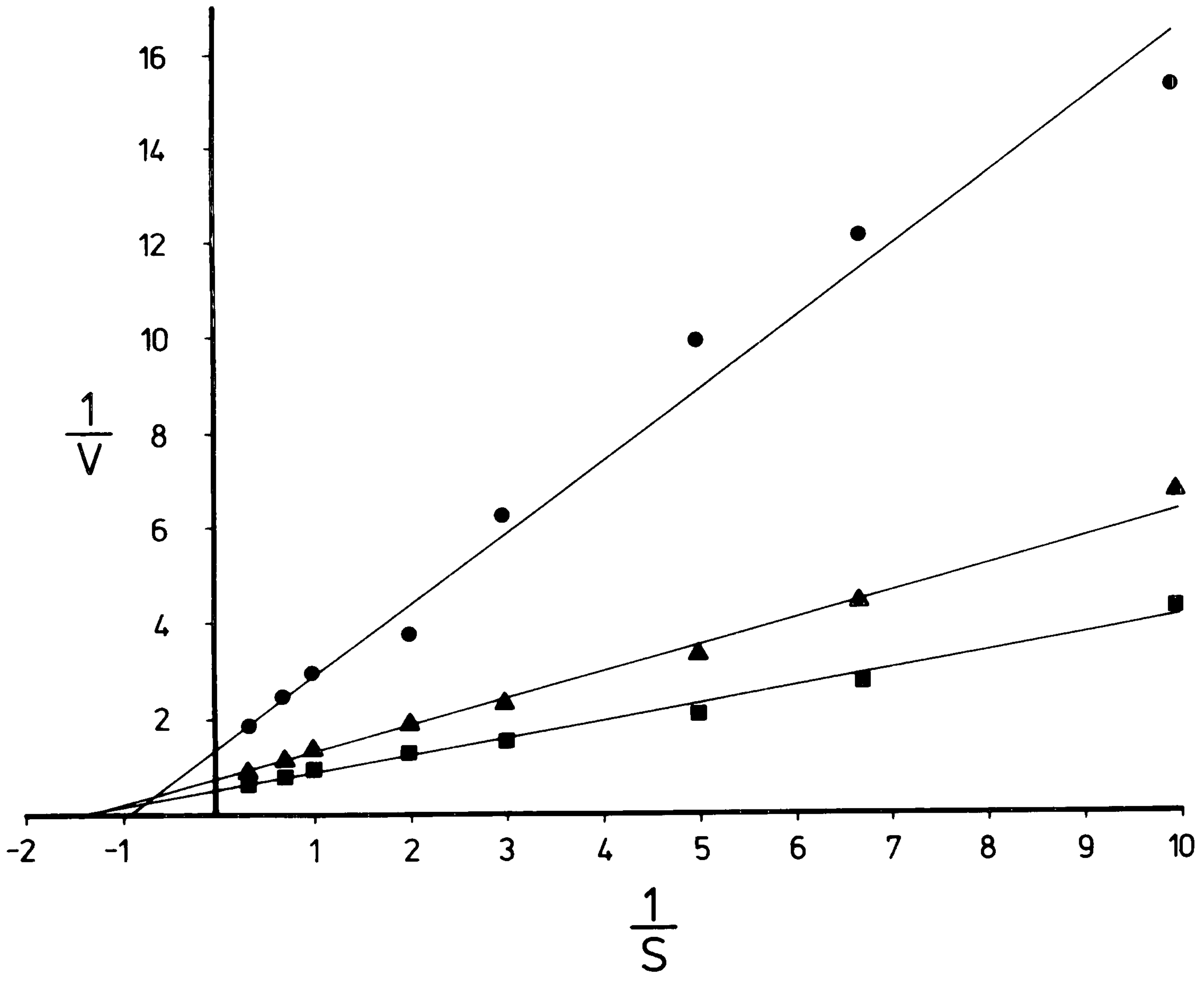

16
14
12
10
8
6
4
2
1/V
-2
-1
1
2
3
4
5
6
7
8
9
10
1/S

Table 5.1. Km and Vmax values for sarcoplasmic reticulum ATPase extracted from *Locusta migratoria* flight muscle.

	n	Km (mM ± S.E.M.)	Vmax (nmole) Pi mg protein min^{-1}
Mg^{2+}-dependent ATPase	6	0.643 ± 0.049	442.0 ± 55.0
Total (Mg^{2+} + Ca^{2+}) ATPase	6	0.420 ± 0.028	2453.0 ± 278.0
Ca^{2+}-stimulated ATPase	6	0.377 ± 0.051	1532.0 ± 370.0

Changes in sarcoplasmic reticulum microsomal protein content of flight muscle during development

The amount of sarcoplasmic reticulum microsomal protein per animal (i.e. μg protein. $thorax^{-1}$) was calculated from protein determinations carried out on the microsomal preparation used to measure ATPase activity during development (see Materials and Methods). The assumption has been made that the amounts of sarcoplasmic reticulum microsomal protein extracted from the flight muscles is a guide to the actual amount of sarcoplasmic reticulum protein present in the intact muscle. The results obtained are shown in Figure 5.9. It can be seen that the amount of sarcoplasmic reticulum protein increased with age. The overall increase being approximately 4-fold from the 9th day of 5th instar (20 animals being used, 10 males + 10 females) to the 8th day of adult life.

Developmental changes in ATPase activity of SR extracted from *Locusta* flight muscle

Mg^{2+}-dependent ATPase, total ATPase and Ca^{2+}-stimulated ATPase activities were determined during flight muscle development. The study was carried out over the period from 9th day of 5th instar to 8th day of adult life. Tris-ATP and Mg^{2+} concentration (3mM) were kept in proportion of 1:1 in this series of experiments. The relationships between the specific activities of Mg^{2+}-dependent ATPase, total ATPase and Ca^{2+}-stimulated ATPase and age are shown in Figure 5.10. It can be seen that total ATPase activity increased gradually from the 9th day of 5th instar (764.30 ± 84.99 nmoles Pi liberated mg $protein^{-1}$ $minute^{-1}$) to 6-day old adults (2303.24 ± 299.89 nmoles Pi liberated mg $protein^{-1}$ $minute^{-1}$) and then levelled off. The overall increase was approximately 3-fold.

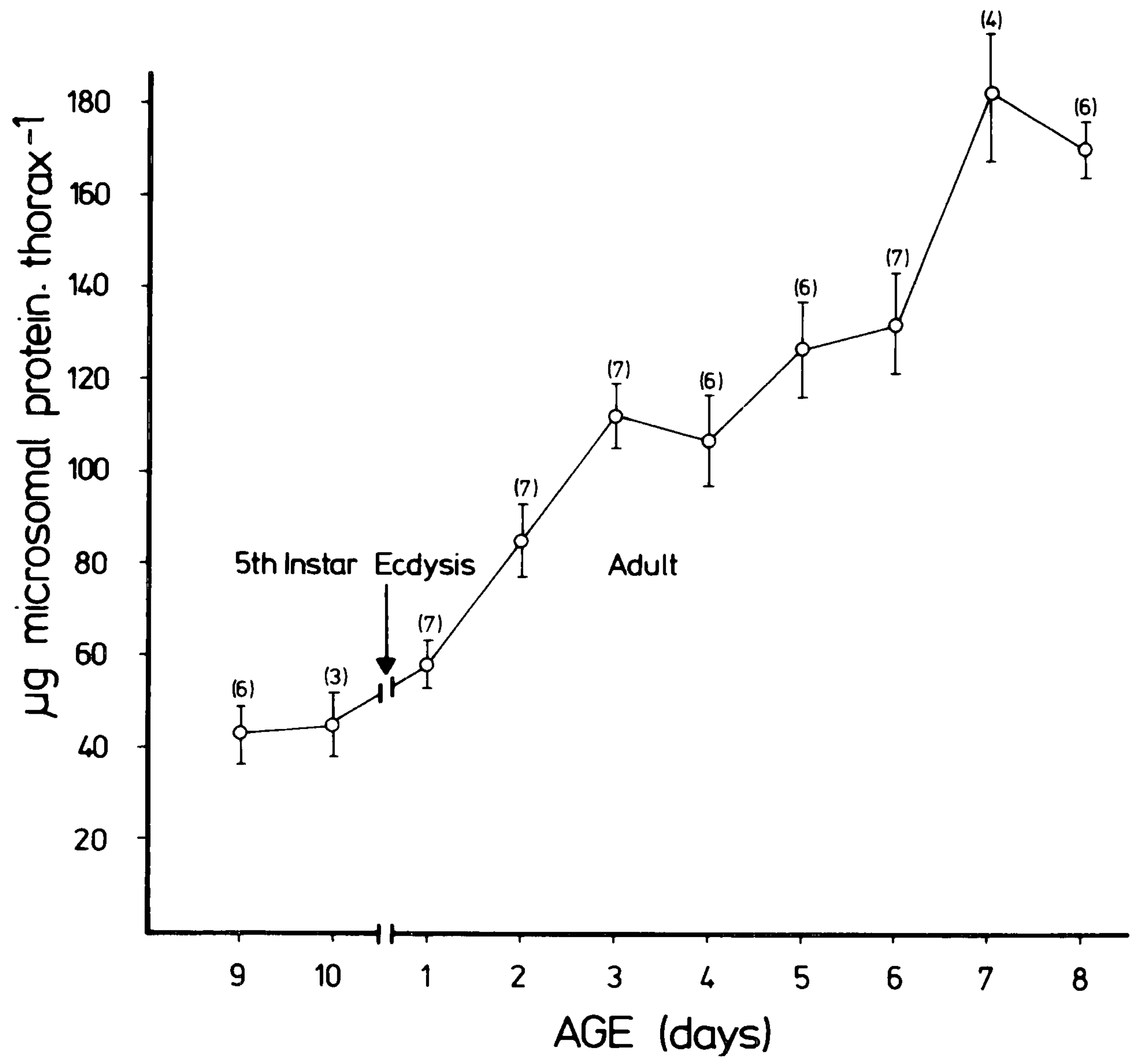
Figure 5.9. Effect of age on the amount of SR microsomal protein per thorax.

The data were calculated from the protein determination made on the microsomal preparations used to study developmental changes in ATPase activity.

Ordinate: protein concentration (μg microsomal protein. thorax^{-1})

Abscissa: Age in days

The figures in parentheses indicate the number of determinations.



μg microsomal protein. thorax-1
180
160
140
120
100
80
60
40
20
5th Instar Ecdysis
Adult
(6)
(3)
(7)
(7)
(7)
(6)
(6)
(7)
(4)
(6)
9
10
1
2
3
4
5
6
7
8
AGE (days)

Figure 5.10. The developmental changes in the activity of SR-ATPase from Locusta migratoria flight muscle.

The incubation conditions were similar to those described in Figure 5.5. except that Tris-ATP and Mg^{2+} concentration (3mM) were kept in proportion of 1:1.

Ordinate: ATPase activity (nmole Pi liberated $protein^{-1}.minute^{-1}$)

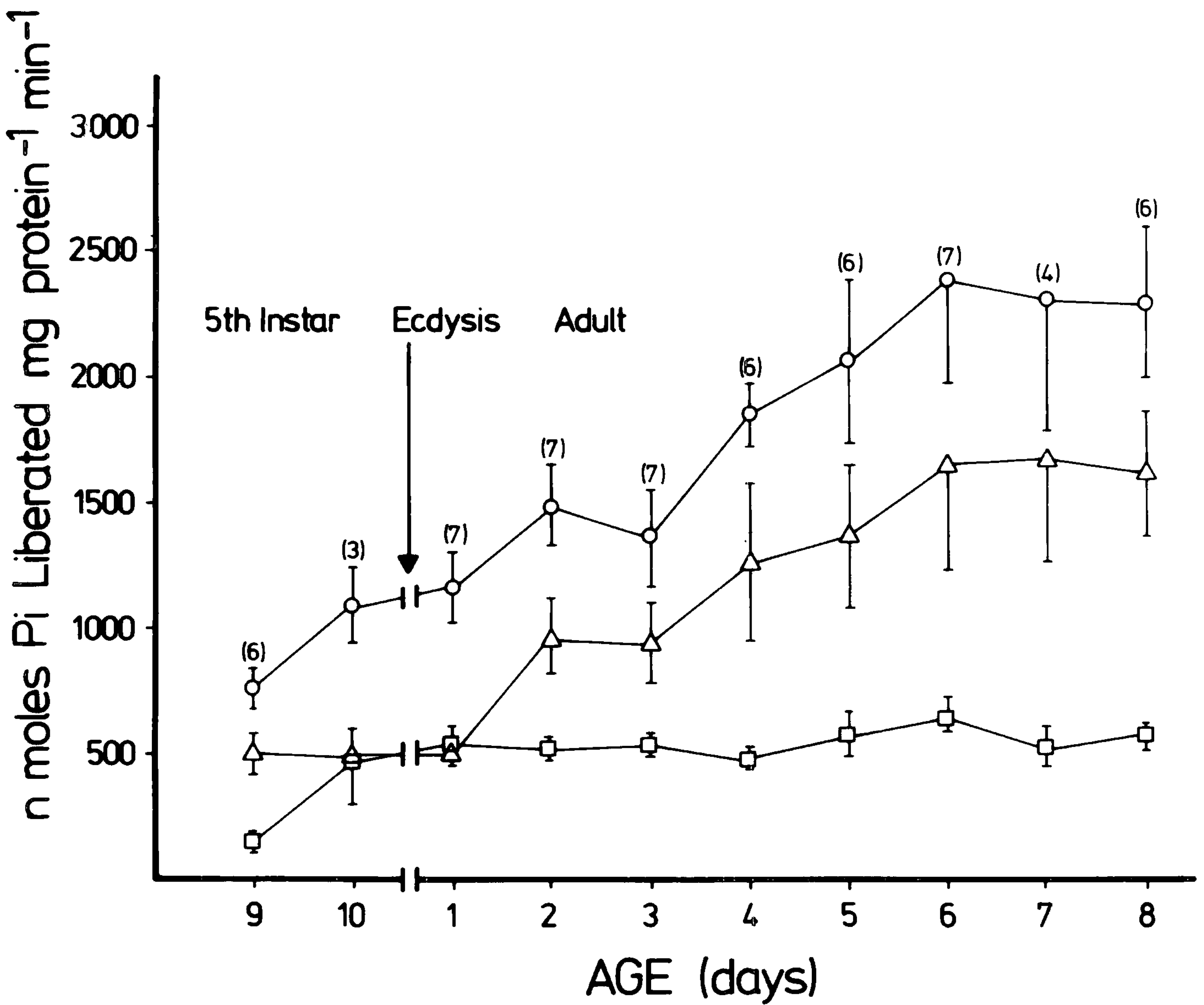
Abscissa: Age (days)

The numbers in brackets on the Figure indicate the number of experiments.

○ Total (Ca^{2+} + Mg^{2+})-ATPase

△ Ca^{2+}-stimulated (Mg^{2+}-dependent)-ATPase

□ Mg^{2+}-dependent ATPase



n moles Pi Liberated mg protein⁻¹ min⁻¹
3000
2500
2000
1500
1000
500
5th Instar
Ecdysis
Adult
(6)
(3)
(7)
(7)
(7)
(6)
(6)
(7)
(4)
(6)
9
10
1
2
3
4
5
6
7
8
AGE (days)

No change in specific activity of Ca^{2+}-stimulated ATPase was observed between the 9th day of 5th instar and the 1st day of adult life. However, Ca^{2+}-stimulated ATPase activity increased gradually from 1st day of adult life to 6-day old adults and then levelled off. The overall increase was similar to that of total ATPase. The specific activity of the Ca^{2+}-stimulated ATPase represented ca. 70% of the total ATPase activity of the 6-day old adult locusts, compared with a value of approximately 44% in 1-day old adult animals.

In contrast to the developmental changes described above, the mean Mg^{2+}-dependent ATPase (basal ATPase) activity showed no significant change in activity over the period studied. However, this may be due to the large standard errors encountered.

Intramitochondrial α-glycerophosphate dehydrogenase activity during development

The ability of *Locusta migratoria* flight muscle mitochondria to oxidize α-glycerophosphate was determined over a range of substrate concentrations (0-54mM) in the presence and absence of $MgCl_2$ and $CaCl_2$. The mitochondria used were isolated from either 9-day old 5th instar larvae, 2-day old or 9-day old adults. The results obtained are shown in Figures 5.11a,b and c and Table 5.2. Reference to Table 5.2 shows that the effect of the two cations is dependent on substrate concentration. Thus in the presence of 20mM α-glycerophosphate, the dehydrogenase activity was comparable irrespective of whether Ca^{2+} or Mg^{2+} were included in the reaction media or not. At this substrate concentration α-glycerophosphate dehydrogenase activity increased approximately 5.7-fold between the 9th day of 5th instar (ca. 2.4μg Ao mg protein^{-1} hour^{-1}) and the 9th day of adult life (ca. 14μg Ao mg protein^{-1} hour^{-1}). In contrast, at low substrate concentration (3.3mM) allosteric stimulation by Ca^{2+} is clearly demonstrated (see

Figures 5.11a,b and c. Effect of Ca^{2+}, Mg^{2+} and substrate concentration on the activity of α-glycerophosphate dehydrogenase from *Locusta migratoria* flight muscle mitochondria.

α-glycerophosphate was varied between 0 and 54mM. Free Ca^{2+} concentration (1.43×10^{-5}M) was calculated from Figure 5.2b. Incubation condition is described in the text. a,b and c are typical results, representative for three experiments (see Appendix 5.8a,b and c).

a. 9-day old of 5th instar larvae

b. 2-day old adult

c. 9-day old adult

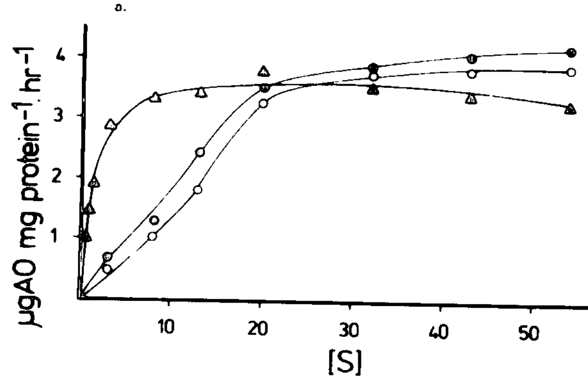
Ordinate: α-glycerophosphate dehydrogenase activity (μg AO. mg $protein^{-1}$. $hour^{-1}$)

Abscissa: α-glycerophosphate concentration {S}

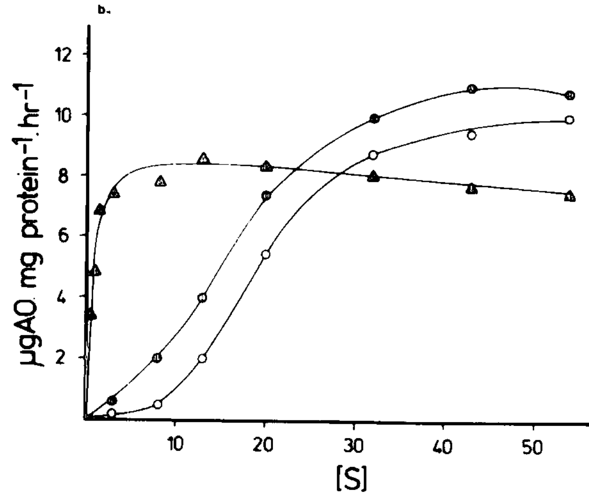
▲ medium containing Ca^{2+}

● medium containing Mg^{2+}

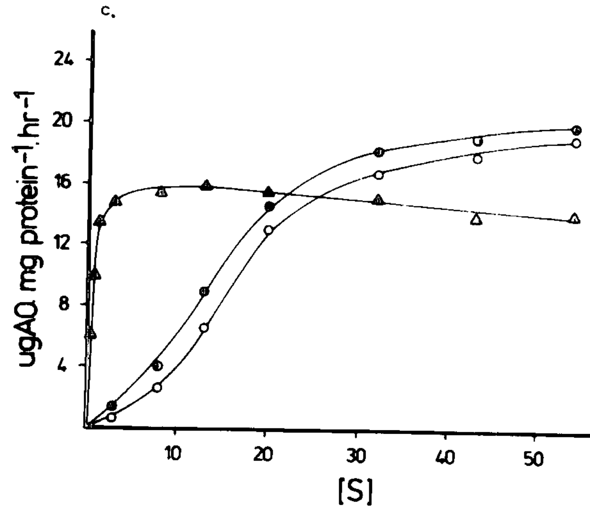
○ medium lacking Ca^{2+} + Mg^{2+}



a.
μgAO mg protein-1.hr-1
[S]



b.
μgAO mg protein-1.hr-1
[S]



c.
ugAO mg protein-1.hr-1
[S]

Table 5.2. Effect of Ca^{2+} and Mg^{2+} on the oxidation of α-glycerophosphate by *Locusta migratoria* flight muscle mitochondria.

α-glycerophosphate (mM)	Age (in days)	QO_2 (μg AO. mg $protein^{-1}$. $hour^{-1}$)		
		medium containing Ca^{2+}	medium containing Mg^{2+}	medium lacking Ca^{2+} and Mg^{2+}
20	9 day old of 5th instar	2.30	2.13	2.81
	2 day old adult	8.40	7.40	8.80
	9 day old adult	13.41	14.81	13.50
3.3	9 day old of 5th instar	2.90	0.23	0.18
	2 day old adult	7.40	0.62	0.20
	9 day old adult	14.34	0.94	0.50

Typical set of data representative of three separate experiments (see Appendix 5.7).
The composition of the reaction media are given in the text.
Free Ca^{2+} concentration (1.43×10^{-5}M) was calculated from Figure 5.2b.

Table 5.2 and Figs 5.11a,b and c). In addition, it can be seen that in the presence of Ca^{2+} higher concentrations of α-glycerophosphate result in a very slight decline in the maximal level of dehydrogenase activity. When Mg^{2+} was substituted for Ca^{2+} no allosteric stimulation of α-glycerophosphate dehydrogenase activity was observed (Fig. 11a,b and c).

No differences in the allosteric effect of Ca^{2+} on α-glycerophosphate oxidation were demonstrated at the various ages studied; Ca^{2+} stimulated α-glycerophosphate dehydrogenase in a similar manner (Figs 5.11a,b and c).

Discussion

The presence of a Mg^{2+}-dependent ATPase (basal ATPase), Ca^{2+} + Mg^{2+}-ATPase (total ATPase) and Ca^{2+}-stimulated ATPase (extra ATPase) have been demonstrated in microsomal preparations from SR of *Locusta migratoria* flight muscle. Similar enzymes have been reported in SR from skeletal muscle of various animals (see Introduction). The present results are consistent with those observed by VOLMER (1978), who reported that the total ATPase activity from *Locusta* flight muscle showed no marked differences to the enzyme prepared from rabbit skeletal muscle (MacLENNAN, 1970) with regard to the effect of various cations and pH on the activity.

In the present study, the concentration of Mg^{2+} effecting maximal stimulation, in the presence of 0.5mM Ca-EGTA (free Ca^{2+} = 3.19×10^{-6}M) and 3mM Tris-ATP, was 2.25 ± 0.34 (n = 6). This Mg^{2+} concentration is similar to that reported by YAMAMOTO and TONOMURA (1967), working with SR from rabbit dorsal muscle. They showed that ATPase activity was not affected by increasing the Mg^{2+} concentration from 0.5mM to 5mM and routinely used 3mM Mg^{2+} in their studies. Somewhat higher concentrations have been reported elsewhere; MacLENNAN (1970) observed maximal activity of purified chicken skeletal muscle ATPase with 5mM Mg^{2+}. VOLMER (1978) and WIERICHS *et al.* (1980) reported maximal

activity in the presence of 10mM Mg^{2+} with ATPase from Locusta flight muscle and pig heart muscle, respectively. The differences in the Mg^{2+} concentration, which are required to achieve maximal activity, may possibly be accounted for by differences in methods of preparation. The latter has been shown to greatly influence the cation concentration within a given membrane fraction. For example, CAMPBELL *et al.* (1980) have shown that "light" and "heavy" vesicles isolated from SR of rabbit contain very different concentrations of Ca^{2+} per unit weight of protein. However, KCl washing reduced the Ca^{2+} in both preparations to the same (very much lower) level. Mg^{2+} concentration was similarly reduced by KCl washing. MacLENNAN and HOLLAND (1976) have reported that the response of Ca^{2+}-sensitive ATPase (extra ATPase) to Ca^{2+}, Mg^{2+} and ATP concentration in the medium is complex; the concentration of each of these three components influences the effect of the other two components on the overall reaction. It is clear, therefore, that the more purified preparations contain lower Mg^{2+} and Ca^{2+} concentrations and that this may, in turn, effect the levels of Mg^{2+} and Ca^{2+} required, *in vitro*, to affect maximal stimulation. This could well explain the differences in the optimal level of Mg^{2+} reported by VOLMER (1978) using purified SR preparation and those obtained in the present study.

Unlike Mg^{2+}-dependent ATPase, the Ca^{2+} + Mg^{2+}-ATPase (total ATPase activity) require Ca $^{+}$ for activation. The Ca^{2+} concentration giving maximal activity, in the presence of 3mM Mg^{2+} and ATP, was 0.5mM Ca-EGTA (free Ca^{2+} = 3.19 x 10^{-6}M). This is in good agreement with values of 5 x 10^{-6}M free Ca^{2+} reported by VOLMER (1978) in Locusta flight muscle. Somewhat, lower free Ca^{2+} concentrations have been reported elsewhere; HASSELBACH *et al.* (1970) obtained maximal activity of Ca^{2+}-stimulated ATPase of rabbit skeletal muscle in the presence of 4 x 10^{-7}M Ca^{2+} whilst WIERICHS *et al.* (1980) reported maximal total

ATPase activity with pig heart SR preparation at 4.8 x 10^{-7}M Ca^{2+}. MARTONOSI and FERETOS (1964b) observed that the Ca^{2+} concentration required for optimal activation of total ATPase from rat skeletal muscle, in the presence of 0.1M KCl, 4-5mM ATP and 4-5mM Mg^{2+} was about 5 x 10^{-6} to 5 x 10^{-5}M. Similar free Ca^{2+} concentrations have been reported for the purified ATPase from rabbit skeletal muscle (MacLENNAN, 1970; MØLLER _et al._, 1980) and microsomes from the SR of freshwater crayfish muscle (COSSINS, 1974). INESI _et al._ (1970) have shown that the free Ca^{2+} concentration required to give maximal incorporation of ATP terminal phosphate into SR of rabbit hind-leg white muscle and Ca^{2+}-stimulated ATPase activity was identical (Km = 1 x 10^{-7}M). They suggest, therefore, that this similarity indicates that a phosphate-membrane complex is an intermediate in the mechanism of Ca^{2+}-dependent ATP hydrolysis (YAMAMOTO and TONOMURA, 1967; MARTONOSI, 1967).

The pH optimum of total ATPase and Ca^{2+}-stimulated ATPase was observed at approximately pH 7.6 irrespective of whether imidazole/HCl or Bis-Tris propane/HCl buffer systems were used. Similar pH optima in the ranges of pH 7.4-7.6 have been reported elsewhere; _Locusta_ flight muscle (VOLMER, 1978) and rabbit skeletal muscle (YAMAMOTO and TONOMURA, 1967; MacLENNAN, 1970). In contrast to total ATPase and Ca^{2+}-stimulated ATPase, Mg^{2+}-dependent ATPase activity was little affected by pH over the range studied. YAMAMOTO and TONOMURA (1967) showed that rabbit skeletal muscle Mg^{2+}-ATPase exhibited optimum pH at 5.9, below the lowest pH examined in the present study. The role played by Mg^{2+}-dependent ATPase in Ca^{2+} transport activity is uncertain as indeed is the nature and source of this enzyme. YAMAMOTO and TONOMURA (1967) showed that Mg^{2+}-dependent ATPase was not affected by specific inhibitors of Ca^{2+}-stimulated ATPase such as

n-ethylmaleimide, but that it was inhibited by Mg^{2+}-dependent ATPase inhibitors, such as azide, oligomyosin and high concentration of 2,4-dinitrophenol (FERNANDEZ _et al._, 1980). In addition, the Mg^{2+}-dependent ATPase was completed abolished when SR membranes were solubilized by cholate, deoxycholate and Triton X-100 (YAMAMOTO and TONOMURA, 1967; WALTER and HASSELBACH, 1973; McFARLAND and INESI, 1970: FERNANDEZ _et al._, 1980). Such studies indicate that the Mg^{2+}-dependent ATPase remains insoluble in detergents, whereas most of the SR protein and phospholipid is solubilized in the non-ionic detergent Triton X-100 (FERNANDEZ _et al._, 1980). These researchers also reported that all Ca^{2+}-stimulated ATPase activity was associated with the soluble fraction of the heavy and intermediate fractions which sedimented in discontinuous sucrose density gradient and displayed high Ca^{2+}-uptake activity. Furthermore, the insoluble fractions were devoid of the 100,000 dalton polypeptide, which is known to be associated with the Ca^{2+}-transporting ATPase, and contains high levels of the mitochondrial enzyme, cytochrome oxidase. The activity of the latter enzyme, which suggests mitochondrial contamination, has been demonstrated in microsomal fractions from SR of _Locusta_ flight muscle (VOLMER, 1978). BOLLAND _et al._ (1974) have reported that the Mg^{2+}-dependent ATPase, which is associated with the membrane fraction of chicken skeletal muscle, can be separated from the Ca^{2+}-transporting elements of SR. They concluded that this ATPase (Mg^{2+}-dependent ATPase) may represent an entirely independent function from that of the Ca^{2+} pump. The recent study of FERNANDEZ _et al._ (1980) referred to above suggested that the Mg^{2+}-dependent ATPase is not an intrinsic enzymatic activity of SR, since the highly purified and active SR obtained by Ca^{2+} phosphate loading is partially devoid of Ca^{2+}-independent ATPase activity. They also suggest that the Mg^{2+}-dependent ATPase originates from mitochondrial contamination (F_1 ATPase) and other membranes present in SR preparations.

Saturation kinetics of total ATPase and Ca^{2+}-stimulated ATPase were carried out at constant Mg^{2+} concentrations. The Lineweaver-Burk plot showed a single straight line over the range of Tris-ATP used (0.05-3mM). The substrate concentration yielding half maximal activity (Km) was 0.420 ± 0.028mM and 0.377 ± 0.051mM ATP (n = 6) for total ATPase and Ca^{2+}-stimulated ATPase, respectively. Similar results have been reported elsewhere for other tissues (PANET et al., 1971; SULAKHE et al., 1973; WALTER and HASSELBACH, 1973). However, HORGON (1974) has shown that at constant concentrations of Mg^{2+} the Lineweaver-Burk plot of Ca^{2+}-stimulated ATPase from rabbit muscle displayed non-linear relationships, although a linear relationship was observed when Mg^{2+}/ATP ratio was kept at unity. He also noted that the relationships approach linearity either in aged SR or by the addition of small quantities of Triton X-100. A biphasic relationship, which corresponds to high affinity (low Km) and low affinity (high Km) of enzyme activity has been reported by several workers for Ca^{2+}-stimulated ATPase (YAMAMOTO and TONOMURA, 1967; INESI et al., 1967; COSSINS, 1974; NEET and GREEN, 1977; MØLLER et al., 1980) and other processes associated with its activity, such as phosphorylation of SR with the terminal phosphate of ATP (INESI et al., 1970), and Ca^{2+}-uptake by rabbit skeletal muscle SR (INESI et al., 1967; WEBER et al., 1966). MEISSNER (1973) has shown that solubilized ATPase gives a single straight line, giving a Km value at 16μm, whereas "vesicular" ATPase shows a biphasic relationship. It was thought that the high affinity and low affinity properties may represent two enzyme systems, but the studies of YAMAMOTO and TONOMURA (1967) have shown a remarkable similarity in the biochemical properties of the two systems. These properties include the dependency of ATPase on pH, the Ca^{2+}-uptake/ATP hydrolysis ratios, n-ethylmaleimide inhibitor and temperature. In addition COSSINS (1974) has shown that the mean Arrhenius activation energy of the high and low affinity

activities were identical. These researchers concluded that the high and low affinity of this enzyme are attributed to a single enzyme and that high ATP concentration acts upon the enzyme, not only as a substrate (Mg-ATP) but also as a regulator controlling the substrate-binding and rate of decomposition of the phosphorylated enzyme. This conclusion has been confirmed by several workers with preparations capable of accumulating Ca^{2+} (see review by BOYER and ARIKI, 1980). There are conflicting reports in the literature concerning the concentrations of substrate required to effect activation of the rate of Pi formation. Several workers have shown activation above 1×10^{-4}M ATP (HORGAN, 1974; NEET and GREEN, 1977; DUPONT, 1977; MØLLER *et al.*, 1980), whereas others have shown a much lower concentration for activation, of the order of 3×10^{-5}M (YAMAMOTO and TONOMURA, 1967; KANAZAWA *et al.*, 1971; PANET *et al.*, 1971; VIANNA, 1975; YATES and DUANCE, 1976). COSSINS (1974) study showed activation above 0.5mM ATP, and suggested that this property may have a physiological advantage *in vivo* changing the activity of the enzyme according to the availability of substrate. However, the function of this phenomena in *Locusta* SR-ATPase is uncertain for the level of ATP, although its distribution is totally unknown in muscle cells (BYGRAVE, 1967), does not change significantly between rest and flight (up to 2 hours) being approximately 5.05 ± 0.44 µmole g fresh weight^{-1} (WORM and BEENAKKERS, 1980).

Developmental changes in the amount of SR protein and the specific activity of total ATPase, Mg^{2+}-dependent ATPase and Ca^{2+}-stimulated ATPase of SR microsomes, extracted from *Locusta* flight muscle, were studied in the late days of the 5th instar and the first week after the final ecdysis. The amount of SR microsomal protein increased approximately 4-fold over this period. This result is consistent with the ultrastructural changes reported earlier (Chapter 3). It has been

shown that the development of flight muscle was accompanied by changes in the amount of SR between myofibrils. Similar observations have been reported in chicken skeletal muscle (MARTONOSI et al., 1972; HOLLAND et al., 1974; MARTONOSI et al., 1980), where it was shown that the increase in SR protein is reflected by an increase in density and a decline in the phospholipid : protein ratio. However, RICHARD et al. (1971) reported that the protein concentration of SR from Schistocerca gregaria flight muscle decreased during the last day of 5th instar and remained more or less constant in the adult animals. Similarly, HOLLAND and PERRY (1969) have shown that the protein concentration of crude SR from rabbit Longissimus dorsi muscle decreased during development to the adult value.

The Ca^{2+}-transporting enzyme of various skeletal muscles is reported to have a molecular weight of ca. 100,000 on the basis of polyacrylamide gel electrophoresis (MARTONOSI and HALPIN, 1971; MacLENNAN, 1970; MacLENNAN et al., 1971; BOLLAND et al., 1974; MARTONOSI et al., 1980) and constitutes 60-80% of the protein content of SR isolated from adult animals (MARTONOSI and HALPIN, 1971; MacLENNAN and HOLLAND, 1976; TADA et al., 1978). The protein composition of extracted SR microsomes from different ages of chicken skeletal muscle during development shows a number of protein bands (MARTONOSI et al., 1972, 1980; BOLLAND et al., 1974). The M protein, which has been identified in rabbit sarcoplasmic reticulum with Ca^{2+}-transporting ATPase (MARTONOSI, 1969; MacLENNAN et al., 1972), represents a minor component during embryonic development of chicken but increases in concentration to ca. 60-70% of the microsomal protein in the adult. MARTONOSI et al. (1980) reported that the Ca^{2+} ATPase content increased more than 20-fold during chicken development. In addition, TILLACK et al. (1974) and MARTONOSI et al. (1980) reported that the sharp increases in Ca^{2+}-transport and Ca^{2+}-sensitive

ATPase activity (MARTONOSI *et al.*, 1972; BOLLAND *et al.*, 1974; MARTONOSI *et al.*, 1980) in chicken skeletal muscle development were accompanied by increases in the density of the 75-85A° Freeze-etch particles. These particles represent a cluster of about four ATPase molecules (MARTONOSI *et al.*, 1980) and represent a dominant feature of SR membrane isolated from animals, rabbit skeletal and heart muscle (DEAMER and BASKIN, 1969; BASKIN and DEAMER, 1969), lobster abdominal muscle (BASKIN, 1971) and chicken skeletal muscle (TILLACK *et al.*, 1974).

The specific activity of Ca^{2+}-stimulated ATPase and total ATPase increased approximately 4-fold over the period studied. Similar developmental changes in SR function have been reported in various skeletal muscle of chicken *in vivo* (FANBURG *et al.*, 1968; HOLLAND and PERRY, 1969; MARTONOSI *et al.*, 1972; BOLLAND *et al.*, 1974; MARTONOSI *et al.*, 1980) and *in vitro* (LOUGH *et al.*, 1972), and rabbit (HOLLAND and PERRY, 1969). This suggests that most of the protein increase referred to above is probably due to Ca^{2+}-ATPase increase during SR development.

The present study showed that whilst there was a parallel increase in the activity of total ATPase and Ca^{2+}-stimulated ATPase over the period studied (maximum being at 6-day old adults), Mg^{2+}-dependent ATPase reached maximal activity at approximately final ecdysis. Other researchers have reported parallel development of the Ca^{2+}-activated ATPase, Mg^{2+}-activated ATPase and total ATPase in chicken skeletal muscle (MARTONOSI *et al.*, 1972; BOLLAND *et al.*, 1974; HOLLAND and PERRY, 1969). In contrast, FANBURG *et al.* (1968) and LOUGH (1972) reported that whilst total ATPase activity rose significantly during development, Ca^{2+}-stimulated ATPase changed very little over the same period of chicken development. Maximal Ca^{2+}-uptake activity is reported to be correlated with total ATPase (FANBURG *et al.*, 1968; LOUGH *et al.*, 1972; VOLMER, 1978) and Ca^{2+}-stimulated ATPase activity (HOLLAND and PERRY, 1969;

MARTONOSI et al., 1972; BOLLAND et al., 1974). The rise in Mg^{2+}-dependent ATPase activity of SR of Locusta, which occurred much earlier than the increase in the Ca^{2+}-stimulated ATPase and total ATPase activities, is in agreement with the finding of HOLLAND and PERRY (1969), MARTONOSI et al. (1972) and BOLLAND et al. (1974) with skeletal muscle of rabbit and chicken. However, as indicated earlier in this discussion Mg^{2+}-dependent ATPase activity may be mitochondrial in origin and not related to the Ca^{2+}-transporting system of the SR.

There is much discussion concerning the mechanism whereby ATPase functioning is developed and regulated. The observation that Mg^{2+}-dependent ATPase activity reached maximum values before Ca^{2+}-uptake leads to the suggestion that the Ca^{2+}-transport system consisted of two components, the ATPase and a Ca^{2+}-transport coupling factor (HOLLAND and PERRY, 1969). According to this suggestion the so called "coupling factor", may link ATPase activity to Ca^{2+}-transport. HOLLAND and PERRY (1969) further suggest that "coupling factor" is present only in membranes of fully developed SR. The correlation between the decline in Mg^{2+}-dependent ATPase activity during development (HOLLAND and PERRY, 1969; BOLLAND et al., 1974), the enhancement of Ca^{2+}-transport and the increase in microsomal density lead to the suggestion (BOLLAND et al., 1974) that the Mg^{2+}-dependent ATPase may represent a precursor of the Ca^{2+}-transport ATPase which aquires Ca^{2+}-sensitivity at relatively late stages in development. Furthermore, they suggest that the hypothetical coupling factor may be a protein subunit which links the various elements of the Ca^{2+}-pump into a functioning complex. An alternative explanation of ATPase functional development suggests that the assembly of the transport ATPase into a functional enzyme complex may proceed only when the phospholipid and fatty acid composition of the membranes becomes optimal (MARTONOSI et al., 1972; BOLLAND et al., 1974). More recently, MARTONOSI et al. (1980) have suggested two alternative theories to explain the

increase in Ca^{2+}-transport activity in developing SR of chicken skeletal muscle. This increase may arise: (i) from the appearance of more and more SR in the cell with the characteristic properties observed in fully developed muscle, or (ii) that the SR evolves from endoplasmic reticulum of myoblasts by the insertion of Ca^{2+}-transport ATPase molecules, synthesised on membrane bound polysomes (BOLLAND et al., 1974), into the phospholipid-rich endoplasmic reticulum membrane. The process of insertion continues until the Ca^{2+}-transport ATPase of the membrane approaches physical saturation. Furthermore, they indicated that the latter theory is consistent with observations that the density of the 75-85A° freeze-etch particles, which is a dominant feature of SR membrane isolated from adult animals (DEAMER and BASKIN, 1961; BASKIN and DEAMER, 1969; BASKIN, 1971; TILLACK et al., 1974), increase with development of chicken skeletal muscle (TILLACK et al., 1974; MARTONOSI et al., 1980).

In the present study, α-glycerophosphate dehydrogenase activity increased approximately 5-fold between the 9th day of 5th instar and 9th day of adult life. This change in activity might be explained in two ways: (i) an actual increase in the amount of enzyme protein. This would be consistent with the increase in the amount of mitochondrial protein reported earlier (see Chapter 3), (ii) a change in the activity of fixed or nearly fixed amounts of enzyme protein. As was mentioned earlier α-glycerophosphate dehydrogenase is allosterically stimulated by Ca^{2+}. It might be, therefore, that the increase in activity with age is related to the development of or changes in this property. However, this possibility is excluded in the case of mitochondrial α-glycerophosphate dehydrogenase of *Locusta* flight muscle because the allosteric behaviour in relation to Ca^{2+} was demonstrated at all ages studied. It would seem, therefore, that the increased activity is due to an increase in enzyme protein. This is consistent with the finding of BÜCHER (1965), who

reported that the quotient of increase in the membrane profile of the mitochondrial and enzymatic activity of α-glycerophosphate dehydrogenase is constant within experimental error of the analysis. Other workers have reported increases in α-glycerophosphate dehydrogenase activity associated with flight muscle mitochondrial development. LENNIE and BIRT (1967) reported that, in Lucilia cuprina myoblasts, small mitochondria (i.e. less than 1μm diameter) exhibit α-glycerophosphate dehydrogenase and that its specific activity increased 1.4-fold between the 1½ days before to 7 days after the pupal - adult ecdysis. In contrast, with large mitochondria (1-10μm in diameter) the specific activity increased approximately 7-fold during the same period. Other mitochondrial enzymes (e.g. malic dehydrogenase, NAD^+-linked isocitric dehydrogenase and cytochrome oxidase) increase 5-10-fold more or less synchronously. The present study as well as that of BROSEMER et al. (1963) and BÜCHER (1965) showed that in Locusta, mitochondrial α-glycerophosphate dehydrogenase activity continued to increase during early adult life. Similar results were observed by BALBONI (1967) for Apis mellifera. BEEZELEY et al. (1974) showed fluctuations in the activity of this enzyme in Musca domestica; activity being higher in 4, 14 and 21-day old flies as compared to 7 and 8-day old flies. In contrast, HOLMES and KEELEY (1975) found that, in Heliothis viresens, α-glycerophosphate dehydrogenase activity peaked 2 days before the final ecdysis. This may indicate that mitochondria of Heliothis flight muscle are fully developed prior to the adult ecdysis. Support for this comes from the fact that Heliothis is capable of flight shortly after the final ecdysis whilst in Locusta sustained flight is not possible until approximately the 8th day of adult life (BÜCHER, 1965).

The effect of Ca^{2+} on the activity of α-glycerophosphate dehydrogenase was essentially the same as that reported by many other workers (see Introduction). The present study showed that the Mg^{2+} had no stimulatory effect on α-glycerophosphate dehydrogenase. This suggests that Mg^{2+} could not substitute the role played by Ca^{2+}. Indeed, DONNELLAN and BEECHEY (1969) reported that Mg^{2+} is ten thousand times less effective than Ca^{2+} in activating α-glycerophosphate dehydrogenase _in vitro_.

CHAPTER 6

CONCLUSIONS

The present study has concerned the growth and development of the flight muscles in *Locusta* with particular reference to those systems involved in energy supply and utilization. The data obtained from morphometric studies (Chapter 3) revealed that both mitochondria and myofibrils increased in size, and that the relative proportion of the muscle constituents changed with increasing age. These changes are consistent with earlier reports (BROSEMER *et al.*, 1963; BÜCHER, 1965; RICHARD *et al.*, 1971), which suggested that poor flight performance during the early stages of adult development in locusts may be due to a limited capacity to elaborate and utilise energy for flight (i.e. incomplete structural and functional development). The mechanisms whereby mitochondria and myofibrils increase in size have been discussed in detail previously (see Chapter 3). It was concluded that, although mitochondrial protein content increased during the first week of adult life, the increase in mitochondrial size was probably due largely to fusion of adjacent mitochondria. These developmental changes were accompanied by an increase in the number and density of the cristae per mitochondrion. The fatty acid composition of the mitochondrial phospholipids (Chapter 3) and the allosteric properties of mitochondrial α-glycerophosphate dehydrogenase (Chapter 5) were similar at all ages studied. Furthermore, mitochondrial phosphorylation was fully developed at all ages studied (see Chapter 4). This is in agreement with the reports that, during the formation of *Locusta migratoria* flight muscle mitochondria, the morphologically visible mitochondrial elements are enzymatically fully equipped at an early stage (BROSEMER *et al.*, 1963; BÜCHER, 1965). Whilst the above results seem to be in agreement with the suggestion that mitochondrial development is synchronous in all

elements (BÜCHER, 1965), the significant decrease ($p < 0.02$) in the ratio of unsaturated : saturated fatty acids in mitochondrial phospholipid, the substantial decrease ($p < 0.001$) in stearic acid (18:0) content and the increase in the specific activity of mitochondrial α-glycerophosphate dehydrogenase suggests asynchronous development. This is consistent with the studies of DE KORT (1969) on *Leptinotarsa decemlineata* flight muscle. Associated with the increased mitochondrial development, myofibril size and the number of myosin filaments within each individual myofibril, increased approximately 4-fold, in *Locusta*, over the period studied. Similarly, BÜCHER (1965) showed that, during the "phase of duplication", there was a 2-fold increase in the number of myosin filaments and that the number of myofibrils increased from about 30 to approximately 1000 in each muscle fibre over the whole period of development. More recently, VAN DEN HONDEL-FRANKEN and FLIGHT (1981) reported that the muscle fibre diameter of the metathoracic dorsal longitudinal flight muscle of *Locusta* increased approximately 3-fold between the 5th day before and 10th day after the final ecdysis. On the basis of the present study, it was concluded that the increase in the number and size of the myofibrils was probably the result of "longitudinal splitting" (GOLDSPINK, 1970, 1974; see also HUDDART, 1974) and the addition of contractile protein (myosin and actin) into the peripheral region of the new myofibrils (see GOLDSPINK, 1974). As the above changes were taking place the characteristic structural features of the mature adult flight muscle were being assumed. The relative proportion (by volume) of myofibrils to mitochondria was more than 2:1 at all ages studied; being maximal on the 3rd day of adult life. In the present study, the more-or-less parallel changes in the relative proportion of myofibrils and mitochondria referred to above support the general view that mitochondrial formation is strictly coordinated with the formation of other cellular constituents in *Locusta migratoria*.

This is in agreement with the studies of KLEINOW *et al.* (1970). The physiological importance of an increase in the mass of contractile protein and mitochondria has been indicated by BURSELL (1973), who reported that the former promoted an increase in the frequency of muscle contraction in *Glossina morsitans*. The latter, in turn, results in an increase in the demand for energy, which is fulfilled by an increase in the chondriome. The relationship between myofibrillar and mitochondrial development observed in the present study would seem to be consistent with this interpretation in *Locusta*. It was mentioned earlier (Chapter 3) that, as the maturation of locust flight muscle proceeds, there was a distinct change in colour from white in the newly ecdysed adult to reddish-brown in more developed flight muscle. This was associated with an increase in the mitochondrial mass, α-glycerophosphate dehydrogenase activity and the rate of respiration (Chapter 4). However, isolated mitochondria were capable of effecting coupled oxidation with the various substrates used (except succinate) at all ages studied, when supported by a suitable reaction medium. On this basis it is clear that the reports of poor coupling between oxidation and phosphorylation in mitochondria from younger adult locusts (KLINGENBERG and BÜCHER, 1959) (see Chapter 4) must be refuted and it must be concluded that failure to demonstrate oxidative phosphorylation and respiratory control at this time is due, at least partly, to the use of an inappropriate reaction medium. Alternatively, the maintenance of the integrity of isolated mitochondria, which is essential in considering their energetic capabilities, may have been differentially affected by age with consequent functional changes. Ultrastructural studies suggest that mitochondria may be more fragile in the young adult compared with the more mature insect. This is consistent with the study of BALBONI (1967) on honeybee flight muscle, who reported that the little or no capacity for respiratory control or

oxidative phosphorylation does not mean the mitochondria from newly ecdysed bees actually lack these attributes. It may be more a reflection of an age-related mitochondrial fragility. The present study employed the Nagarse extraction method (see Chapter 2) which reduces the need for vigorous mechanical manipulation to separate the mitochondria from the myofibrils and this may explain why good respiratory control and oxidative phosphorylation were observed in the young insect preparation.

In the present study, tracheolization of the flight muscle was already well established in the newly ecdysed adult (see Chapter 3). As development proceeded there was a reduction in the tracheoblast cytoplasm, a decrease in the relative proportion of the total muscle fibre volume occupied by tracheoles, and the tracheoblast cellular sheath enclosing the intima of the interfibrillar tracheoles became very attenuated (see also VAN DEN HONDEL-FRANKEN and FLIGHT, 1981). Such developmental changes resulted in a reduction in the diffusion path between the tracheolar lumen and the mitochondria thus facilitating the supply of oxygen, which is conveyed via trachae, to these organelles (EDWARDS and RUSKA, 1955).

The present study and that of VOLMER (1978) have demonstrated that the properties of the Ca^{2+}-transporting ATPase of SR are similar to those of vertebrate skeletal muscle described elsewhere (see Chapter 5). In view of the fact that there is 1:1 correspondence between muscle fibre depolarization and contraction, which characterizes the synchronous type of insect flight muscle, SR function is clearly of great importance in the coordination of these processes by controlling the sarcoplasmic Ca^{2+} concentration. It is perhaps significant therefore that SR protein increased approximately 4-fold between the 9th day of 5th instar and 7th day of adult life as more-or-less did the specific

activity of the Ca^{2+}-transporting ATPase (Chapter 5), and that considerable development of the SR and its association with the T-system was observed in the early days of adult life (Chapter 3). The importance of free Ca^{2+} concentration in the metabolic activity of insect flight muscle has been highlighted by SACKTOR (1975). He reported that, in Phormia regina, at least five enzymes, which participate in the regulation of the rest/flight transition, are known to be sensitive to low concentrations of Ca^{2+}. These included two extra-mitochondrial enzymes (actomyosin ATPase and phosphorylase b kinase) and three mitochondrial enzymes (α-glycerophosphate dehydrogenase, pyruvate dehydrogenase and NAD-linked isocitrate dehydrogenase). SACKTOR (1975) suggested that the initiation of contraction by increased sarcoplasmic free Ca^{2+} concentration, which activates the actomyosin ATPase, also stimulated metabolic activity. It is known that the intense rate of glycogenolysis observed at the initiation of flight, is the result of the conversion of the inhibited phosphorylase b to the active phosphorylase a (CHILDRESS and SACKTOR, 1970) by phosphorylase a kinase. The latter is, in turn, activated by Ca^{2+} (HANSFORD and SACKTOR, 1970). α-Glycerophosphate dehydrogenase is probably activated by the same Ca^{2+} "pool" which activates the phosphorylase and actomyosin ATPase enzymes since it is located on the outer surface of the inner mitochondrial membrane (SACKTOR, 1975). The fact that Ca^{2+} was an allosteric activator of α-glycerophosphate dehydrogenase at all ages studied indicates the importance of this cation in metabolic control in Locusta. It is clear, therefore, that the development of SR is an important factor in the function of flight muscle. Since it is generally accepted that Ca^{2+}-transporting ATPase is a pre-requisite for the appearance of Ca^{2+}-transporting function of SR (FANBURG et al., 1968; HOLLAND and PERRY, 1969; MARTONOSI et al., 1972; BOLLAND et al., 1974), it is tempting to suggest that the

developmental changes observed in SR and its associated ATPase activity are important factors in explaining the improvement in flight performance noted in the developing adult *Locusta* (BÜCHER, 1965; GEWECKE and KUTSCH, 1979). Furthermore, the development of the T-system and its dyadic association with SR might be expected to facilitate the transmission of nervous stimulation to the muscle fibres and thereby influence the efficiency of the flight mechanism.

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Appendix 3.1.

Preparation of the median longitudinal indirect flight muscle for electron microscopy.

(a) Fixative

2.5% glutaraldehyde

0.1M sodium cacodylate buffer (pH 7.3)

0.32M sucrose

(b) Post Fixative

1% osmium tetroxide

0.1M cacodylate buffer (pH 7.3)

(c) Embedding medium (epoxy resin)

Epon 812	47g
DDSA	21g
MNA	32g
DMP-30	$1.4cm^3$

Procedure

After the thorax was pinned out on a cork board and covered with fixative for 15 minutes, the following procedure was used.

(i) the median dorsal longitudinal indirect flight muscles from the meso- and meta-thoraces were dissected out and placed in fresh cold fixative (a) at 0-4°C for overnight.

(ii) the tissues were washed in several changes of 0.1M cacodylate (pH 7.3) and left overnight.

(iii) post-fixation was carried out in 1% aqueous osmium tetroxide in 0.1M cacodylate buffer (pH 7.3) (b) at room temperature for two hours.

(iv) after post-fixation, the tissues were washed in 0.1M sodium cacodylate buffer for 30 minutes and dehydrated in a graded

series of ethanol solutions (50%, 70%, 95% and absolute ethanol) for 10 minutes each.

(v) the tissues were then passed through propylene oxide (2 x 15 minutes) and left overnight in a 50/50 mixture of propylene oxide and embedding medium (c).

(vi) the samples were infiltrated for 6 hours in absolute epon resin (c). They were then transferred to foil dishes containing absolute epon resin and were polymerized at 60°C for 48 hours.

Appendix 4.1. **The effect of different reaction media and substrates on the respiratory activity of isolated mitochondria from 8-day old adult locusts.** Detailed description found in the representative experiment (see Table 4.2.)

		Reaction media											
		1			2			3			4		
Substrate		α-GP	pyruvate + proline	glutamate	α-GP	pyruvate + proline	glutamate	α-GP	pyruvate + proline	glutamate	α-GP	pyruvate + proline	glutamate
QO_2 μg AO mg protein^{-1} hour^{-1}	State 4	6.95 ± 1.02	3.02 ± 0.36	2.37 ± 0.49	3.38 ± 0.43	1.69 ± 0.47	1.44 ± 0.45	7.96 ± 0.43	2.95 ± 0.56	2.72 ± 0.60	8.59 ± 1.18	4.24 ± 0.62	4.03 ± 0.35
	State 3	13.87 ± 0.76	16.22 ± 1.53	7.46 ± 0.80	4.33 ± 0.13	6.61 ± 1.50	2.96 ± 0.75	18.39 ± 1.21	6.21 ± 0.64	6.43 ± 0.46	17.62 ± 1.70	15.39 ± 1.48	11.12 ± 0.94
RCR		1.55 ± 0.44	5.50 ± 0.12	3.46 ± 0.71	1.56 ± 0.07	4.31 ± 0.34	2.28 ± 0.27	2.34 ± 0.05	2.25 ± 0.20	2.66 ± 0.62	2.21 ± 0.05	2.87 ± 0.21	2.83 ± 0.08
ADP:O		1.32 ± 0.10	2.85 ± 0.05	2.97 ± 0.08	n.m.	2.56 ± 0.50	n.m.	n.m.	2.70 ± 0.21	2.86 ± 0.18	1.68 ± 0.10	3.17 ± 0.20	2.98 ± 0.07

Appendix 4.2. Effect of Mg^{2+} on the oxidation of α-glycerophosphate or pyruvate by *Locusta* flight muscle mitochondria isolated from 4-day old adults.

Substrate (mM)	Mg^{2+} (mM)	QO_2		RCR	ADP:O
		state 4	state 3		
α-glycerophosphate (6.7)	0.0	7.08 ± 0.51	10.50 ± 0.73	1.50 ± 0.03	n.m.
(6.7)	6.7	16.77 ± 0.68	27.30 ± 0.33	1.65 ± 0.05	1.33 ± 0.06
(6.7)	13.4	18.28 ± 0.58	22.98 ± 0.48	1.26 ± 0.05	1.42 ± 0.05
(13.4)	0.0	11.16 ± 0.42	14.59 ± 0.27	1.32 ± 0.04	n.m.
pyruvate (6.7)	0.0	3.77 ± 0.58	28.11 ± 1.25	7.56 ± 1.16	3.77 ± 0.23
(6.7)	3.3	5.86 ± 0.60	11.87 ± 0.93	2.05 ± 0.13	3.66 ± 0.24

Appendix 5.1.

Convertion of ATP from the disodium salt to ATP-Tris

(i) Re-charge the Dowex resin

Adenosine triphosphate (ATP) was purchased as the disodium salt, which was converted into Tris-ATP by using ion exchange Dowex resin. The Dowex was converted into its H^+ form (re-charged) before use. This was achieved as follows: The Dowex was well rinsed with distilled water in a Buchner funnel and the wet weight was noted. It was then washed in 3N HCl (AnalaR) using $30cm^3$ of acid per 5gm weight of resin. This was followed by washing in distilled water until the effluent had a pH between 3-4. At this stage all the residual acids were removed from the resin and it was in its charged form. It was then resuspended in an equal volume of distilled water and stored at 0-4°C until required.

(ii) Preparation of the Tris salt of ATP

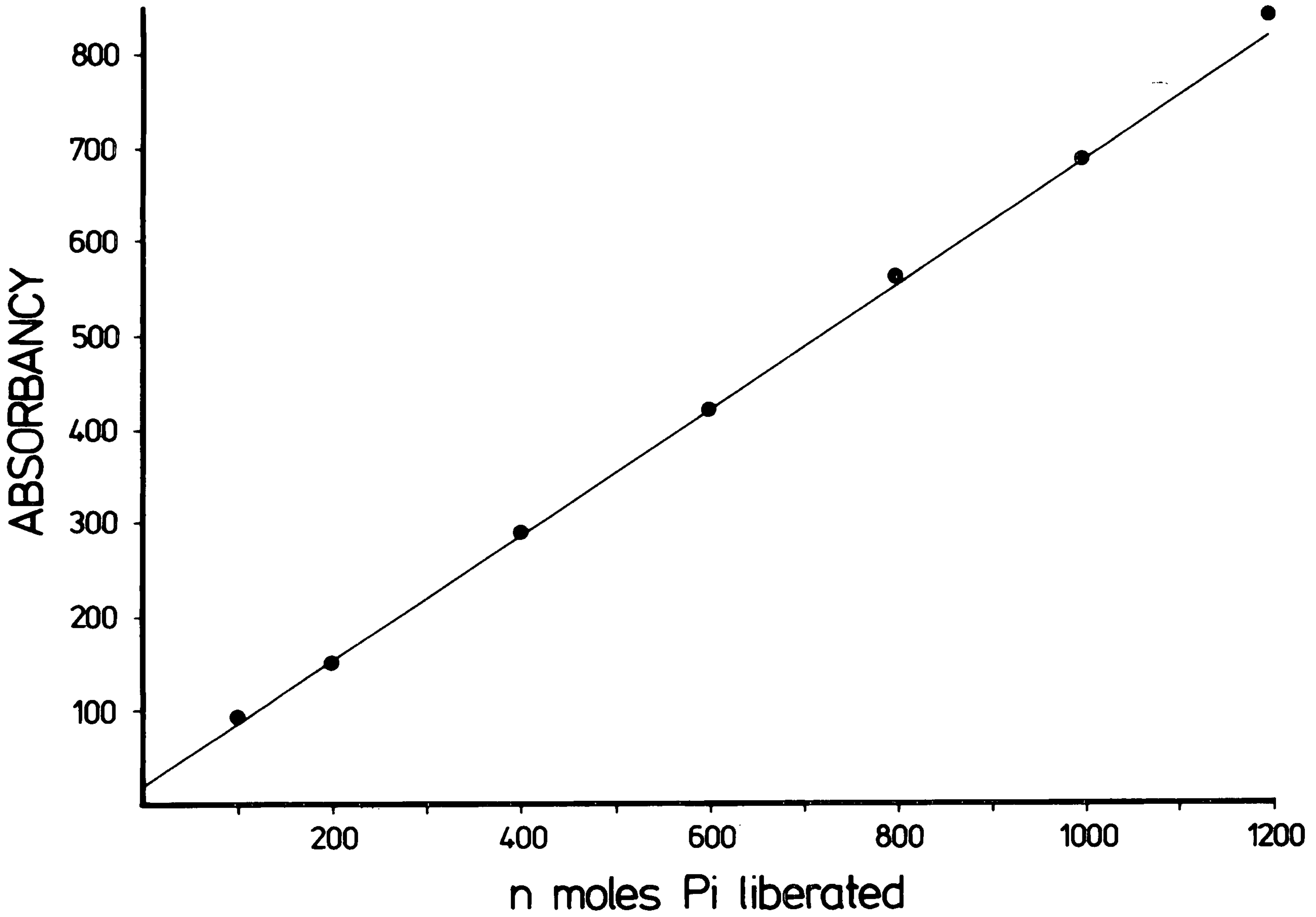
A known quantity of adenosine triphosphate (ATP) was dissolved in a known small quantity of the H^+ Dowex resin and thoroughly mixed using a "Whirlimixer" (Fison's Scientific Apparatus) for a few minutes. The resin was allowed to sediment and the supernatant removed and retained. The resin was washed three times with a small quantity of distilled water, and mixed thoroughly each time. This ensured the removal of all the H-ATP from the Dowex. The supernatants from each washing were "pooled". At this state the ATP was in its H^+ form and it was converted to the Tris salt by the addition of drops of 2M Tris until the appropriate pH was reached. It was then made up to the required volume with deionized water and stored at 20°C.

Appendix 5.2.

Construction of standard calibration curve for inorganic phosphate

A stock solution containing 0.6μmoles of phosphate (as KH_2PO_4) was serially diluted to give 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05 and 0.0 μmoles Pi per cm^2. $2cm^2$ of each standard solution were pipetted into M.S.E. centrifuge tubes followed by $4cm^2$ of the stopping mixture (see Chapter 5). The tubes were then left to stand for 10 minutes at room temperature. The intensity of the yellow colour, which developed during this time was determined by measuring the absorbancy on a Cecil Ce 292 spectrophotometer at 390nm. Figure 5.2.1. shows the relationship between absorbancy and inorganic phosphate concentration.

Figure 5.2.1. Typical example of a standard inorganic phosphate calibration curve.


ABSORBANCY
800
700
600
500
400
300
200
100
200
400
600
800
1000
1200
n moles Pi liberated

Appendix 5.3.

Calculation of the "apparent association constant" (Kapp) for Ca-EGTA under different pH and temperature

Different investigators have used different values for the "apparent association constant" in the calculation of free Ca^{2+} concentration (WEBER and MURRAY, 1973). In the present study, calculations are based on values for the association constant given by SCHATZMANN (1973) and AMOS *et al.* (1976). These values were then corrected for the changes in pH and temperature.

The relationship between the equilibrium constant (K) and temperature (T) is given by the equation:

$$\text{Log } K = \frac{-\Delta H}{2.303R} \cdot \frac{1}{T} \quad \text{where}$$

H = enthalpy, R = gas constant, T = absolute temperature

On the assumption $-\Delta H$ does not vary with temperature, the equation becomes

$$\text{Log } K \propto \frac{1}{T}$$

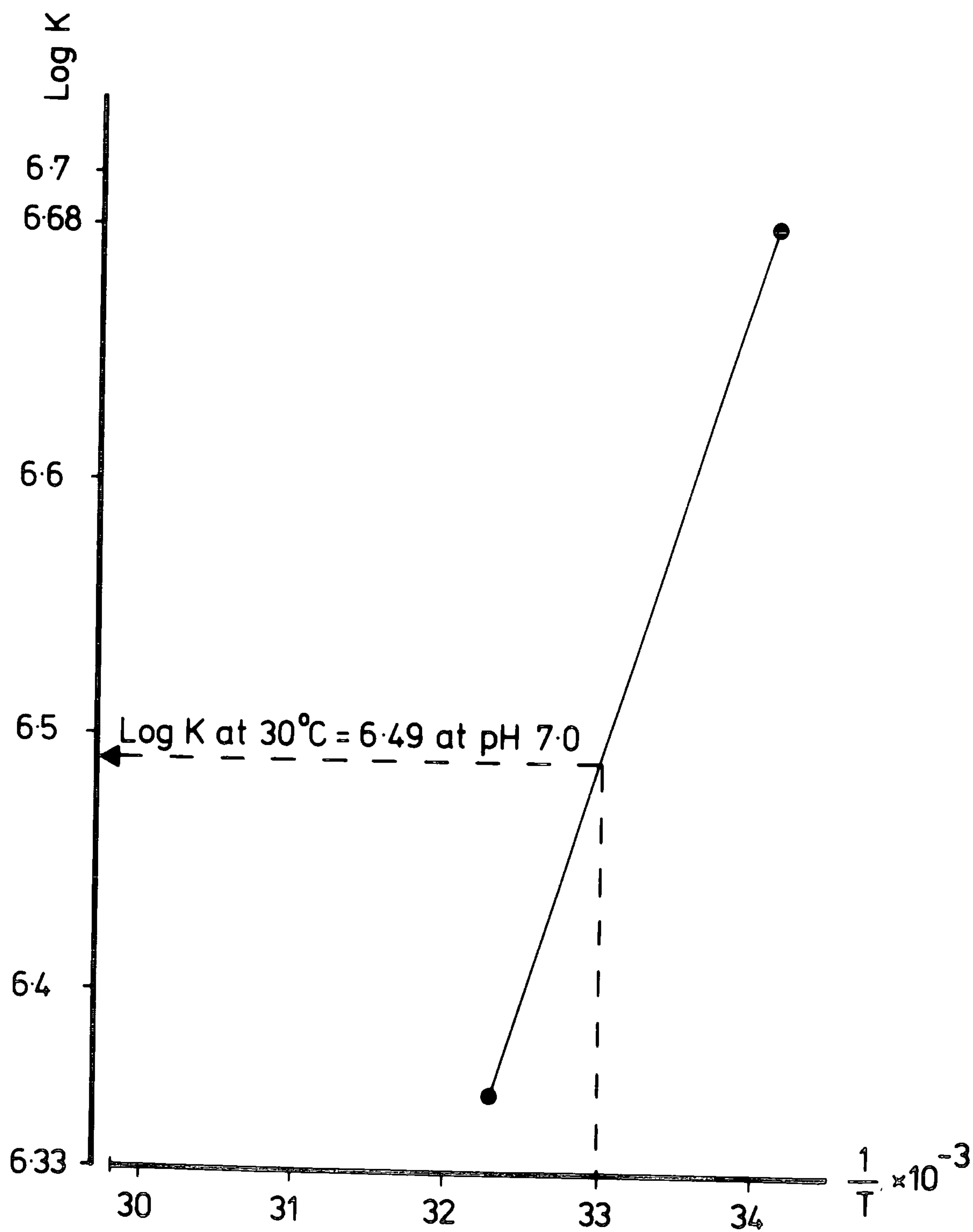
From a plot of K versus $\frac{1}{T}$ at pH 7.0 (Figures 5.3.1), Kapp at 30°C was calculated to be $10^{6.49}$ at pH 7.0. However, AMOS *et al.* (1976) have shown that Kapp is effected by pH, such that the Log_{10} Kapp is increased by a factor of 1.2 when the pH is changed from pH 7.0 to pH 7.6 and by 0.2 when pH is increased from pH 7.0 to pH 7.1 at 30°C. In this study Kapp at pH 7.6 and 7.1 have been calculated at 30°C on the assumption that Kapp will be similarly affected by pH at 30°C and 20°C. On this basis Kapp at 30°C was taken to equal $10^{6.69}$ at pH 7.1 and $10^{7.69}$ at pH 7.6.

Figure 5.3.1.- shows the relationship between Log K and $\frac{1}{T}$.

K: apparent association constant

T : absolute temperature

The two points indicated on the Figure represent Kapp of $10^{6.36}$ at 35°C and pH 7.0 (SCHATZMANN, 1973) and $10^{6.38}$ at 20°C and pH 7.0 (AMOS *et al.*, 1976).



Log K
6·7
6·68
6·6
6·5
6·4
6·33
Log K at 30°C = 6·49 at pH 7·0
30
31
32
33
34
1/T ×10⁻³

Appendix 5.4. Effect of pH on the activity of SR-ATPase extracted from flight muscle of *Locusta migratoria*.

(a) Bis-Tris propane/HCl was used as buffer system

pH	ATPase activity (nmole Pi liberated mg protein^{-1}. hour^{-1})		
	Mg^{2+}-dependent ATPase	Total (Ca^{2+} + Mg^{2+}) ATPase	Ca^{2+}-stimulated ATPase
6.0	813.00 ± 101.08	1774.33 ± 136.34	961.33 ± 30.07
6.5	740.67 ± 133.31	1805.00 ± 151.00	1064.45 ± 26.36
6.8	587.50 ± 56.75	1886.56 ± 156.43	1182.25 ± 50.60
7.0	642.00 ± 103.53	2061.45 ± 106.71	1419.46 ± 59.33
7.2	648.67 ± 118.66	2310.33 ± 119.93	1661.67 ± 69.09
7.5	676.45 ± 115.28	2918.56 ± 198.08	2248.00 ± 139.07
7.8	628.54 ± 98.57	2736.00 ± 156.48	2107.35 ± 71.88
8.0	717.33 ± 113.54	2369.33 ± 75.10	1652.00 ± 38.79
8.5	762.00 ± 113.78	1615.67 ± 63.05	853.64 ± 53.75
9.0	856.33 ± 84.22	1257.43 ± 155.99	400.67 ± 186.28

(b) Imidazole/HCl was used as buffer system

pH	ATPase activity (nmole Pi liberated mg $protein^{-1}$. $hour^{-1}$)		
	Mg^{2+}-dependent ATPase	Total (Ca^{2+} + Mg^{2+}) ATPase	Ca^{2+}-stimulated ATPase
6.5	339.67 ± 37.60	1332.30 ± 35.18	986.00 ± 72.98
6.8	356.66 ± 43.38	1543.00 ± 105.86	1187.35 ± 149.25
7.0	360.30 ± 34.92	1643.45 ± 141.83	1282.46 ± 169.68
7.1	370.00 ± 35.31	1863.30 ± 220.85	1493.00 ± 239.02
7.3	395.60 ± 37.83	2584.60 ± 398.75	2189.50 ± 390.29
7.5	418.30 ± 11.78	3141.00 ± 424.53	2723.00 ± 413.38
7.7	460.00 ± 54.98	3082.00 ± 444.56	2622.75 ± 426.08
8.0	427.60 ± 30.94	2907.00 ± 485.55	2479.30 ± 461.30

Appendix 5.5. Effect of Mg^{2+} concentration on the activity of SR-ATPase extracted from flight muscle of *Locusta migratoria*.

Mg^{2+} (mM)	ATPase activity (nmole Pi liberated mg $protein^{-1}$. $minute^{-1}$)		
	Mg^{2+}-dependent ATPase	Total (Ca^{2+} + Mg^{2+}) ATPase	Ca^{2+}-stimulated ATPase
0.0	23.00 ± 7.77	107.25 ± 27.26	76.50 ± 24.72
0.5	285.75 ± 69.47	950.33 ± 168.86	543.00 ± 132.94
1.0	377.25 ± 79.48	1105.00 ± 236.00	735.00 ± 162.55
1.5	456.35 ± 73.42	1241.25 ± 235.48	790.75 ± 164.05
2.5	482.75 ± 60.45	1384.00 ± 152.47	901.25 ± 104.63
3	496.00 ± 23.01	1344.26 ± 121.71	835.75 ± 39.28
4	413.00 ± 49.00	1145.00 ± 137.74	732.42 ± 62.95
6	359.25 ± 49.87	885.50 ± 76.56	528.34 ± 80.56
12	305.45 ± 52.42	693.75 ± 56.49	388.00 ± 63.18

Appendix 5.6. Effect of free Ca^{2+} concentration on the activity of SR-ATPase extracted from flight muscle of *Locusta migratoria*.

Free Ca^{2+} concentration (M)	ATPase activity (nmole Pi liberated mg $protein^{-1}$. $hour^{-1}$)	
	Mg^{2+}-dependent ATPase	Total (Ca^{2+} + Mg^{2+}) ATPase
0.0	558.25 ± 72.66	
2.27×10^{-9}		750.75 ± 50.97
5.12×10^{-9}		1261.27 ± 77.29
2.04×10^{-8}		1658.49 ± 94.30
3.00×10^{-7}		1833.00 ± 104.07
3.19×10^{-6}		1929.54 ± 84.21
5.00×10^{-5}		1640.67 ± 79.43
5.63×10^{-4}		1031.50 ± 72.04
1.00×10^{-3}		751.58 ± 70.45
1.50×10^{-3}		625.00 ± 80.31

Appendix 5.7. Effect of Ca^{2+} and Mg^{2+} on the oxidation of α-glycerophosphate by *Locusta migratoria* flight muscle mitochondria at different ages.

α-glycerophosphate (mM)	Age (in days)	QO_2 (μg AO. mg protein^{-1}. hour^{-1})					
		Medium containing Ca^{2+}		Medium containing Mg^{2+}		Medium lacking Ca^{2+} and Mg^{2+}	
		Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
20	9 day old of 5th instar	2.75	2.19	2.09	2.93	1.96	3.12
	2 day old adult	9.53	10.21	8.52	7.13	9.25	8.67
	9 day old adult	14.57	15.71	16.39	14.27	13.93	15.32
3.3	9 day old of 5th instar	3.42	2.87	0.19	0.28	0.21	0.28
	2 day old adult	8.32	9.12	0.57	0.74	0.31	0.39
	9 day old adult	16.34	14.92	0.96	1.02	0.58	0.47

Appendix 5.8. Effect of Ca^{2+} and Mg^{2+} on the oxidation of α-glycerophosphate by *Locusta* flight muscle mitochondria.

(a) mitochondria isolated from flight muscle of 9-day old 5th instar locusts.

	QO_2 (μg AO. mg $protein^{-1}$. $hour^{-1}$)		
α-glycerophosphate (mM)	medium containing Ca^{2+}	medium containing Mg^{2+}	medium lacking Ca^{2+} and Mg^{2+}
0.5	1.33 ± 0.17	-	-
1.0	1.75 ± 0.15	-	-
1.5	2.30 ± 0.20	-	-
3.0	2.95 ± 0.05	0.95 ± 0.28	0.57 ± 0.09
8.0	3.87 ± 0.47	2.07 ± 0.70	1.37 ± 0.47
13.0	4.33 ± 0.79	3.40 ± 0.80	2.17 ± 0.42
20.0	4.83 ± 0.71	4.50 ± 1.00	3.75 ± 0.63
32.0	4.30 ± 0.60	4.78 ± 0.86	4.68 ± 0.91
43.0	4.37 ± 0.69	5.17 ± 0.93	4.93 ± 1.04
54.0	3.80 ± 0.56	5.07 ± 0.52	4.62 ± 0.48

The data represent the mean of three individual experiments ± S.E.M.

(b) mitochondria isolated from flight muscle of 2-day old adult locusts.

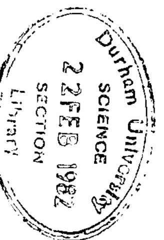
	QO_2 (μg AO. mg protein^{-1}. hour^{-1})		
α-glycerophosphate (mM)	medium containing Ca^{2+}	medium containing Mg^{2+}	medium lacking Ca^{2+} and Mg^{2+}
0.5	3.83 ± 0.23	-	-
1.0	5.43 ± 0.39	-	-
1.5	7.47 ± 0.32	-	-
3.0	7.93 ± 0.32	0.75 ± 0.38	0.93 ± 0.42
8.0	8.37 ± 0.35	2.20 ± 0.47	2.13 ± 0.83
13.0	8.93 ± 0.33	3.87 ± 0.87	4.40 ± 1.25
20.0	9.30 ± 0.63	8.30 ± 0.85	7.83 ± 1.20
32.0	8.63 ± 0.58	12.33 ± 1.30	10.77 ± 1.08
43.0	8.60 ± 0.90	12.50 ± 0.76	11.67 ± 1.17
54.0	8.53 ± 0.67	12.26 ± 0.93	11.60 ± 0.95

The data represent the mean of three individual experiments ± S.E.M.

(c) mitochondria isolated from flight muscle of 9-day old adult locusts.

	QO_2 (μg AO. mg $protein^{-1}$. $hour^{-1}$)		
α-glycerophosphate (mM)	medium containing Ca^{2+}	medium containing Mg^{2+}	medium lacking Ca^{2+} and Mg^{2+}
0.5	4.83 ± 0.73	-	-
1.0	7.80 ± 1.47	-	-
1.5	12.00 ± 1.40	-	-
3.0	13.43 ± 1.0	1.40 ± 0.35	0.93 ± 0.24
8.0	14.07 ± 1.05	3.87 ± 0.10	2.70 ± 0.72
13.0	13.8 ± 1.36	7.87 ± 0.13	5.8 ± 1.47
20.0	13.26 ± 1.29	12.93 ± 1.87	11.70 ± 2.15
32.0	13.13 ± 1.07	16.87 ± 1.84	15.70 ± 1.90
43.0	12.90 ± 0.73	17.20 ± 1.90	16.47 ± 1.74
54.0	12.30 ± 1.0	17.70 ± 1.78	16.33 ± 2.03

The data represent the mean of three individual experiments ± S.E.M.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