CYTOCHEMISTRY OF THE DIFFERENTIATING FLIGHT MUSCLES OF THE DESERT LOCUST *SCHISTOCERCA GREGARIA* FORSKAL

by

LYNN CAROL PANAR

B.Sc., Simon Fraser University, 1972

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Biological Sciences

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SIMON FRASER UNIVERSITY

August, 1974

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APPROVAL

Name: Lynn Carol Panar

Degree: Master of Science

Title of Thesis: Cytochemistry of the Differentiating Flight Muscles of the Desert Locust *Schistocerca gregaria* Forskal

Examining Committee:

Chairman:

K. K. Nair
Senior Supervisor

P. Belton

J. M. Webster

C. L. Kemp

Date Approved: July 29, 1974

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Author: [signature]
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Lynn Carol Panar
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ABSTRACT

The purpose of this investigation was to study the cytochemical changes in the metathoracic median dorsal longitudinal flight muscle of the female desert locust, *Schistocerca gregaria*, during its differentiation from the last day of the fourth nymphal instar, through the fifth nymphal instar up to the eighth day after the imaginal moult. The parameters examined were: I. changes accompanying nuclear differentiation, with respect to: (1) DNA content, (2) transcriptional activity, (3) degree of chromatin condensation and (4) nuclear area. II. changes accompanying muscle fibre differentiation, considering: (1) RNA content, (2) protein content and (3) cross-sectional fibre area.

The results of the incorporation of thymidine (methyl ^{3}H) into muscle nuclei show that DNA synthesis occurs just prior to the final nymphal moult, three days thereafter and, to a lesser degree, five days after the imaginal moult.

Although DNA synthesis occurs during differentiation microspectrophotometric analysis of Feulgen-stained nuclei shows that polyploidization is not a phenomenon associated with the differentiation of these muscles. The nuclei remain diploid throughout the developmental period.

By "staining" with ^{3}H-Actinomycin D, followed by autoradiography, the changes in transcriptional activity coincident with flight muscle development were examined. At the beginning of the developmental period the transcriptional activity is relatively

high and remains so up until about the middle of the fifth instar after which time it declines fairly steadily. These changes in transcriptional activity are accompanied by changes in the degree of chromatin condensation. Generally, the periods of high transcriptional activity exhibit less condensation of the chromatin.

The originally high transcriptional activity is accompanied by a steady over-all increase in total RNA content during the developmental period; and in turn a large increase in total protein content. The cross-sectional area of the muscle fibres increases significantly during the growth and differentiation of the flight muscles.

ACKNOWLEDGEMENTS

My sincere thanks are due to Dr. K. K. Nair for his constant guidance and encouragement during the research for and the writing of this thesis; to Dr. P. Belton and Dr. J. M. Webster for their helpful comments and suggestions; to Mr. M. Horta for rearing the insects; to Dr. Vic Bourne for his technical assistance; and to the National Research Council for their award of a scholarship.

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INTRODUCTION

The indirect flight muscles of most insects are made up of large sized fibrils arranged into giant fibres several hundred μm in diameter (Sacktor, 1970). The fibrils (myofibrils) are discrete groups of interdigitating arrays of two sets of filaments, thick myosin filaments, 150 Å in diameter, and thin actin filaments, 50 Å in diameter, and transverse Z-membranes. The fibrils are surrounded by mitochondria, sarcoplasmic reticulum, nuclei and sarcoplasmic sap (Maruyama, 1965). The fibres are characteristically invaded by a rich tracheolar system (Sacktor, 1970). The fibres are arranged around a central trachea which has radiating branches (Ashhurst, 1967), penetrating, in large numbers, into the interior of the fibres (Tiegs, 1955), thus facilitating oxygen transport by diffusion.

The muscle nuclei are usually few in number and are generally located peripherally immediately under the sarcolemma (Tiegs, 1955). The numerous mitochondria are slablike and very large (Smith, 1966) and are arranged in closely-packed elongate columns, between the fibrils (Sacktor, 1970).

The development of insect flight muscles is a complex process differing from order to order and indeed from species to species. This is evident from the monumental work of Tiegs (1955) who examined in detail the developing flight muscles of various orders of insects. Among the Acridiidae he has described the postembryonic development of these muscles in the plague locust *Chortoicetes terminifera*. In the minute first instar

nymph, all the main muscles of the adult can be distinguished, in a very rudimentary condition. During development the fibres both increase in diameter and in number. The fibre enlargement usually takes place during the early growth period of the nymph. In the third, penultimate instar, the fibres begin to cleave. In the final instar, the muscle, whose growth has, till now, merely kept pace with that of the nymph, accelerates its development in preparation for the new function of flight. The muscles enlarge up to three or four times their diameter at the inception of the instar, while the nymph enlarges by about twice.

Bocharova-Messner and Yanchuk (1966) observed a similar pattern of flight muscle development in the domestic cricket, _Acheta domestica_. In addition they noted that the intensive preparation for new duties associated with intensification of energy balance, begins during the last third of the final nymphal instar.

Scudder (1971) and Scudder and Hewson (1971) studied the flight muscle development in the Hemipterans _Cenocorixa bifida_ and _Oncopeltus fasciatus_ respectively. They found that the dorsolongitudinal flight muscles of the first instar disappear and are replaced in later instars by new muscles that function in the adult. During the first instar there are four, fully formed, striated dorsolongitudinal muscle fibres. During the second instar, they become surrounded by basiphilic cells with large prominent nuclei. In the third instar these basiphilic

cells appear to have invaded and fragmented the original muscle fibres. The fourth instar is characterized by the predominance and organization of the basiphilic cells into four discrete groups, closely packed together. In the final nymphal instar and for some time after the imaginal moult these groups of cells grow and differentiate into functional flight muscles.

In recent years considerable research has been carried out on the ultrastructure of differentiating muscles of insects particularly in holometabolous species. The development of the flight muscles has been described in *Simulium ornatum* (Grant, 1961), *Drosophila melanogaster* (Shafiq, 1963), *Antherea pernyi* (Błotna and Michejda, 1966), *Galleria melonella* (Sahota and Beckel, 1967), *Lucilia cuprina* (Gregory et al., 1968; Peristianis and Gregory, 1971) and *Calliphora erythrocephala* (Crossley, 1972). Most of these studies have shown that in holometabolous insects the larval muscles degenerate at pupation and the imaginal muscles are formed by the multiplication and fusion of myoblasts into syncytia. The steps involved in the formation of muscle syncytia have been studied in depth using cultured voluntary striated muscles of vertebrates examined with the aid of time-lapse cinematography (Capers, 1960; Cooper and Konigsberg, 1961).

According to the reviews by Goodman (1957) and Murray (1960) amitosis has been implicated in the formation of the syncytium, whereas Cooper and Konigsberg (1961) cast considerable doubt on the evidence which had been presented to support

"amitotic" nuclear division as a mechanism of the origin of multinuclearity of muscle cells. Goodman (1957) and Murray (1960) had drawn attention to deformities imparting to such nuclei a "dumbbell" shape. Cooper and Konigsberg (1961) found that this type of deformity does not lead to direct division of the nucleus. The dumbbell shape is assumed transitionally, the nucleus later resuming its elliptical shape.

Capers (1960) noted from his observations that in the course of rotation and migratory activity of the nuclei, a great deal of distortion commonly occurs. During migration, oval nuclei often turn so that their long axes are perpendicular to those of the muscle "straps". This frequently results in the formation of folds in the nuclear membrane some of which occur between two nucleoli of the same nucleus. Capers (1960) suggests that this may explain numerous reports that nuclei divide into sectors each with its own nucleolus. However, he found that such divisions are not permanent but disappear as soon as the nucleus has completed its rotation.

Capers (1960) and Cooper and Konigsberg (1961) found that no mitosis, amitosis, or nuclear budding could be observed during the course of muscle development. Cooper and Konigsberg (1961) noted that despite the complete absence of mitotic phenomena in the multinucleate cells, mitoses are numerous among adjacent mononucleated elements in culture. It seems unlikely, therefore, that culture conditions *per se* prevented mitosis. Cooper and Konigsberg (1961) as well as Capers (1960)

reported that myoblast fusion was the only demonstrable way of giving rise to multinucleation.

This question seems to have been resolved as reported in Fischman's (1972) most recent review. Therein he states clearly that "multinucleation in muscle cells results from the cytoplasmic fusion of myogenic cells and not from amitosis or some other process involving the multiplication of myotube nuclei without cytokinesis." Evidence for this is obtained from five principal types of experiments. These include: direct morphological analysis of light and electron microscopy of both *in vitro* and *in vivo* systems; quantitative DNA measurements by Feulgen microspectrophotometry showing all nuclei within myotubes to be diploid; autoradiographic analysis of nuclear synthesis using tritiated thymidine as a labelled precursor of DNA which is found not to be incorporated into the nuclear DNA of myotubes; inhibitor studies in which DNA synthesis is blocked by X-rays, nitrogen mustards, folic acid antagonists, colchicine etc., resulting in the prevention of the replication of mononucleated cells but not myotube formation; and finally fusion studies using genetic markers.

In the regenerating limb muscles of *Periplaneta americana*, Cowden and Bodenstein (1961) found that when the mononucleated myoblasts reach a sufficient population density, lying as they do in a space between myotubes, they form interdigitating chains and cytoplasmic outlines become indistinct. They found no mitotic figures even in the multinucleate myotubes. Bhakthan *et al.*

(1971) while examining the regeneration of flight muscles in the bark beetle, _Ips paraconfusus_ (_I. confusus_) found that mononucleated myoblasts appear and apparently fuse with other myoblasts to form multinucleated cells. From the above it may be concluded that the multinucleate condition of the myofibres of insect muscles is achieved by the fusion of myoblasts rather than by mitosis.

Further development of the flight muscles includes a considerable change in the weight of the muscle. This is of course especially noticeable in the hemimetabolous insects where the growth and differentiation takes place over a longer period of time. In the desert locust _Schistocerca gregaria_, the flight muscles grow throughout the fourth and fifth instar, ceasing just prior to and during the ecdyses. Hill and Goldsworthy (1968) calculated that the flight muscle increases in weight by 238% during the fourth instar and by 1624% during the fifth instar. This somatic growth continues after the imaginal moult for about eight to ten days during which time the male experiences an overall 90% increase in dry weight (Walker _et al._, 1970), and the female a 110% increase (Hill _et al._, 1968). Although these increases in body weight are not entirely due to growth of the flight muscles, this probably constitutes a large percentage of it.

These structural changes are accompanied by biochemical changes as well. Much of the biochemical analysis of flight muscle development has been carried out on various Orthopteran

insects. Bücher (1965) has done substantial work using Locusta migratoria. He examined the following biochemical parameters; non-extractable protein made up partly by mitochondrial fractions but chiefly by the contractile proteins; the glycerol-1-phosphate oxidase representing the constant proportion complement of mitochondrial enzymes; and finally four extra-mitochondrial enzymes involved in the breakdown of carbohydrate: glyceraldehyde-3-phosphate dehydrogenase, glycerol-1-phosphate dehydrogenase (GDH), lactose dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PDH). During the first phase of the fifth instar the exponential increase of all parameters is almost equal. During the moulting interval the increase in LDH shows a peak, which can possibly be attributed to the growth of the tracheoblasts. Next, the activity of GDH increases by a factor of 20 (Brosemer et al., 1963), whereas that of LDH decreases by a factor of three. Activities of G6PDH and some other enzymes almost disappear. From about the fifth to the eighth day of imaginal life there is an almost exact duplication of the extramitochondrial enzyme activities.

The tremendous increase in GDH after the last moult is due to net enzyme synthesis de novo, and not the activation of a preexisting apoenzyme (Brosemer, 1965). Two other enzymes, not mitochondrial, also show a significant increase in activity during development. These are β-hydroxyacyl-CoA-dehydrogenase and β-ketoacylthiolase (Beenakkers, 1963). This may be correlated with flight metabolism.

Growth and differentiation of insect flight muscles is an orderly but complex process. It is undoubtedly carefully controlled and guided as are most developmental phenomena in insects. Numerous experiments have been done in an attempt to elucidate those control mechanisms that are related to somatic growth in general (see reviews by: Doane, 1973; Gilbert, 1964; Wigglesworth, 1964). Poels and Beenakkers (1969) and Beenakkers (1973) observed that implantation of active corpus allatum inhibited differentiation of flight muscles in *L. migratoria*. From this they proposed that the trigger for differentiation of the muscles is the low titre of corpus allatum hormone. The specific mechanisms involved in this process remain to be elucidated.

From the foregoing it is evident that a number of biochemical events associated with muscle differentiation in insects are being brought to light. However much also still remains to be studied. In an attempt to further clarify some of the processes involved in differentiation of flight muscles the following study was undertaken. The objectives of this study were: I. to determine changes accompanying nuclear differentiation, with respect to: (1) DNA content, (2) transcriptional activity, (3) degree of chromatin condensation and (4) nuclear area. II. to determine the changes accompanying muscle fibre differentiation, considering: (1) RNA content, (2) protein content and (3) cross-sectional fibre area.

METHODS AND MATERIALS

The desert locusts *Schistocerca gregaria* used in this study were raised on a diet of whole wheat bran and fresh grass (Hunter-Jones, 1956), and reared under a constant light regime at 35 - 38° C and 60 - 70% R. H. Under these conditions the fifth instar nymph moulted to an adult in about six days.

Histological Preparations:

This study was confined to the metathoracic median dorsal longitudinal muscles. Figure 1 is a pictorial representation of the over-all change in size that this particular muscle undergoes during its differentiation. The changes characterizing muscle growth and differentiation are much exaggerated in females as compared with those in the males (Hill *et al.*, 1968; Walker *et al.*, 1970) and so only females were used in this study.

The flight muscles begin differentiating towards the end of the fourth nymphal instar, and continue through the fifth nymphal instar and into about eight days of imaginal life (Hill *et al.*, 1968). Therefore, the following age groups were chosen for study: last day of the fourth nymphal instar; first, third, fifth and last day of the fifth nymphal instar; first, third, fifth and eighth day after the imaginal moult. Three to five insects were used for each group.

Nucleus:

For studies on deoxyribonucleic acid (DNA) synthesis, locusts were injected under CO_2 anaesthesia with 10 µl of thymidine (methyl H^3) (2 Ci/mM, Amersham/Searle, Chicago) through

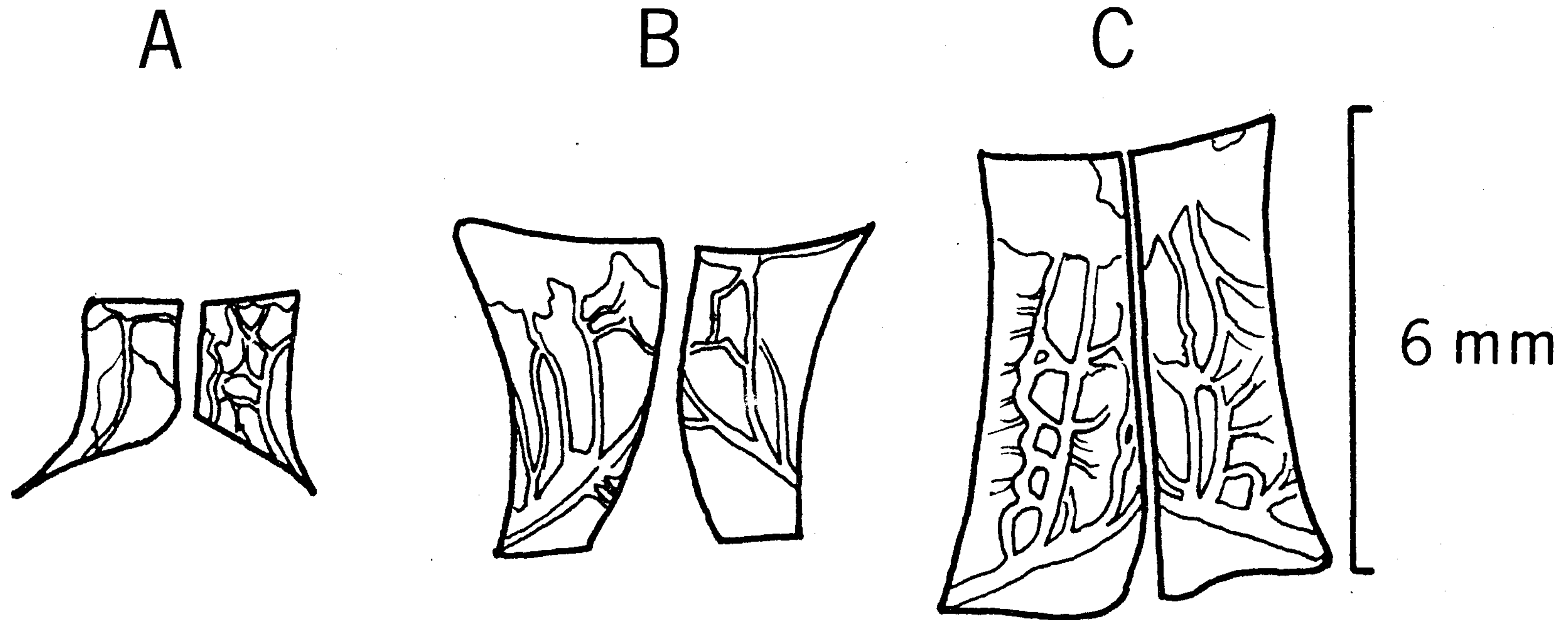
Figure 1. The metathoracic dorsal longitudinal flight muscle of female *S. gregaria* at different stages of development. These diagrams are pictorial representations of a ventral view of the pair of muscles.

A. end of fourth nymphal instar

B. middle of fifth nymphal instar

C. five-day-old adult

METATHORACIC DORSAL LONGITUDINAL FLIGHT MUSCLE (*S. gregaria*)



the arthrodial membrane at the base of the second pair of legs. After injection, the insects were returned to 36±1° C for four hours. The flight muscles were then dissected out and squashes were made on gelatinized (0.5%) slides using the dry ice technique (Conger and Fairchild, 1953). After removing the cover slips, the slides were air-dried for 10 minutes, and fixed in ethanol:acetic acid (3:1) for two hours. After hydration the slides were treated with cold trichloracetic acid (TCA) for 15 minutes and washed in nonradioactive 0.1% thymidine solution for 20 minutes to remove unbound radioactive thymidine. The slides were air-dried and then coated with NTB-2 nuclear emulsion (Eastman Kodak) according to Baserga and Malamud (1969). Some slides were treated with ribonuclease (RNase) (Sigma Chemical Co., St. Louis, Mo.), 1 mg/ml for 1 hour (Deitch, 1966) and then coated with nuclear emulsion. Preparations were exposed for four weeks at 4° C and then processed and stained in methyl green/pyronin (Pearse, 1968). At least 500 nuclei per age group were examined for labelling and from these data the percentage of labelled nuclei was calculated.

To examine changes, if any, in the total DNA content and its concentration during the developmental period, ethanol:acetic acid (3:1) fixed squashes of muscles were hydrolyzed in 3.5N hydrochloric acid (HCl) at 37° C (Fand, 1972).

To determine the optimun hydrolysis time squashes of flight muscle of two different age groups, one-day-old and final day fifth instar nymphs, were hydrolyzed in 3.5N HCl for 10, 15,

20 and 25 minutes and stained in Schiff's reagent (Basic Fuchsin, Harleco, Philadelphia) prepared according to Deitch (1966). The optimum hydrolysis time was 20 minutes in both cases (Table I). To standardize the technique, slides were stabilized in 37° C distilled water before hydrolysis, and were chilled after hydrolysis in cold (4° C) 1N HCl to halt this process. The slides were then stained in Schiff's reagent for one hour and, after processing, were mounted in oil of matching refractive index (^nD = 1.56, Cargille, Cedar Grove, N. J.).

Extinction measurements on 100 nuclei per age group were taken at 570 nm using a Scanning Microscope Photometer (SMP; Carl Zeiss, Oberkochen, W. Germany). These readings were compared with those obtained from similarly fixed and stained spermatids (1C) to determine the DNA class of flight muscle nuclei.

The technique of "staining" by ^{3}H-Actinomycin D (AMD) followed by autoradiography (Brachet and Ficq, 1965) was used to determine whether the transcriptional activity of the nuclei changes during differentiation. For this, flight muscles were dissected out and fixed in cold (4° C) Carnoy (Brachet and Hulin, 1969) for four hours. The muscles were then dehydrated, embedded in paraffin and cut at 5 µm thickness. The sections were deparaffinized, hydrated and transferred to a ^{3}H-AMD solution (5 µCi/ml, Sp. act. 3.8 Ci/mM, Schwartz/Mann, Orangeburg, N. Y.) in the dark. After one hour the slides were rinsed in distilled water and transferred to a

TABLE I

Mean extinction of Feulgen-stained flight muscle nuclei of *S. gregaria* as a function of hydrolysis in 3.5N HCl at 37° C

Hydrolysis Time	M.E.[1] ± S.E.*	M.E.[2] ± S.E.
10	0.34 ± 0.007	0.31 ± 0.009
15	0.35 ± 0.009	0.44 ± 0.011
20	0.39 ± 0.014	0.46 ± 0.013
25	0.28 ± 0.012	0.36 ± 0.009

Total number of nuclei measured per group was 25.

[1] One-day-old fifth instar nymph.

[2] Last day fifth instar nymph

* S.E. = standard error

Analysis of variance showed that the change in each age group was significant ($P < 0.05$).

nonradioactive AMD (Calbiochem, San Diego, Calif.) solution having a concentration of 10 - 20 mg/ml for one hour. This was followed by washing in tap water for 20 hours, after which the slides were rinsed in two changes of distilled water and four changes of 70% ethanol (Ebstein, 1969). They were then air-dried and coated with NTB-2 nuclear emulsion (Eastman Kodak). After three days of exposure at 4° C, the slides were processed and stained with methyl green/pyronin. Using a square graticule the number of silver grains per grid (6.25 μm^2) of nuclear area was determined for 100 nuclei per age group.

Cytoplasm:

To examine changes in the ribonucleic acid (RNA) and protein levels during development, flight muscles were dissected out and fixed overnight in 3% glutaraldehyde in phosphate buffer (pH 7.4). The tissue was then washed in two changes of buffer, slowly dehydrated, cleared and embedded in paraffin. Five micron sections were cut, deparaffinized, hydrated and washed in three 20-minute changes of tap water before staining.

Sections for RNA analysis were stained with the basic thiazine dye Azure B (Allied Chemical, Morristown, N. J.), according to Flax and Himes (1952). The optimum staining period at 40° C was two hours. The pH of the staining solution was adjusted to 4.0 by the addition of potassium biphthalate crystals (Deitch, 1966).

Sections for protein analysis were stained with the triphenylmethane dye Coomassie Blue (Edward Gurr Ltd., London,

England). The staining procedure was modified from that described for gel electrophoresis. (See Appendix I.) A 1% stock solution was dissolved in 12% TCA to make a 0.1% staining solution. Staining was done at 37° C for 15 minutes followed by destaining in 7% glacial acetic acid for 15 minutes at room temperature.

Extinction measurements for both the Azure B and Coomassie Blue stained sections mounted in oil ($^{n}D = 1.56$) were made at 590 and 595 nm respectively. The extinction values were determined for the cross-sections of 100 muscle fibres per age group in both these studies.

Microspectrophotometry:

The Feulgen, Azure B and Coomassie Blue stained slides were examined with the SMP. The SMP has a mechanical stage that exhibits the comb-type movement. According to Zimmer (1970), the fine scanning stage can be shifted at a rate of 60 measuring steps per second, being driven by two stepping motors which receive the necessary pulses from the control unit. Measurements are performed in the interval between the steps, whose length and size of area can be varied (Zimmer, 1970).

The connection of this machine to a digital computer creates practically unlimited possibilities for the digital analysis of microscope images. The SMP used in this study was on-line with a PDP-12 computer (Digital Equipment Corporation, Maynard, Mass.). The program used is called APAMOS (Automatic Photometric Analysis of Microscopic Objects by Scanning), a

variation of the original program, TICAS (Taxonomic Intracellular Analytical System), described in detail by Weid et al. (1968).

The CRT unit allows the display of the measured points of various extinctions. In addition, an editing program allows the operator to exclude measurements from adjacent cells.

All measurements were carried out with ultrafluar objective (x100, N. A. 1.25) and condenser (N. A. 0.8) lenses at 1.0 µm step size along the X and Y axes. The diameter of the measuring aperture was 1.0 µm for nuclei and 1.6 µm for fibres.

Figure 2 represents a print-out of the extinction values of each of the measured points of a Feulgen-stained nucleus. The machine will also print out the data as follows:

X= steps   Y= lines   A=   TE=   ME=

X represents the number of steps on the x axis and Y the number of lines on the y axis. A represents the area as determined by the number of measured points having a transmission between 5 and 95%. TE is the total extinction. It represents the total of all the values presented in figure 2. ME is the mean extinction or the average of all these values.

Data Analysis:

The data were analysed using one way analysis of variance (Burkhardt, 1964).

DNA content was expressed as $\log_2$ of total extinction. This was for the purpose of determining if polyploidy is occurring in the developing flight muscles since an increase by one unit in the $\log_2$ value represents a doubling of the real value

Figure 2. A matrix of extinction values for each of the points measured in a Feulgen-stained flight muscle nucleus.

SFU CYTOLAB PANAR OCT29 73

16 21 23 11
7 20 20 28 17 16 6
9 22 22 29 27 34 29 12
20 26 36 38 32 26 27 16
17 24 33 43 37 40 37 32 10
18 26 33 34 40 45 44 32 30
7 27 25 23 29 38 50 55 35 21
19 21 30 21 23 31 50 49 40 9
9 21 25 33 41 36 40 55 43 28
15 24 38 48 49 41 54 57 38 11
20 30 39 43 24 45 54 37 26
7 23 37 39 21 39 41 39 33
15 31 31 30 46 33 24 32
21 33 31 39 20 9 28
23 27 37 24 29 23
14 24 25 28

(Mittwoch, *et al*., 1966; Fox, 1969).

For determination of the relative frequency of occurrence of extinction values in Feulgen-stained nuclei a computer program modified from the original version (Bartels *et al*., 1974) was used. This program counts the frequency of occurrence of extinction values within the range of 0.0 to 1.8 at intervals of 0.1 for each nucleus. From these a composite histogram for the entire population is compiled.

RESULTS

DNA Synthesis:

The results from the thymidine (methyl H^3) incorporation are summarized in figure 3. Peaks of DNA synthesis occur during the fourth instar, just prior to the final nymphal moult, and on the third day of the fifth nymphal instar. Synthesis declines rapidly and ceases completely prior to the imaginal moult. There is one other, smaller peak, five days after the imaginal moult. It is pertinent to mention here that RNase treatment had no effect on the extent of labelling in these nuclei.

DNA Content:

The data from Feulgen microspectrophotometry indicate that no clear-cut polyploidy is occurring during differentiation (Fig. 4). It seems that the flight muscle nuclei are diploid and remain thus throughout development.

DNA Transcriptional Activity:

The transcriptional activity is relatively high to begin with and remains so, with no significant change, up to the fifth day of the fifth instar. It then declines steadily until about the third day of adult life after which time it rises slightly (Fig. 5). The overall change is statistically significant ($P < 0.05$).

The changes in transcriptional activity may be expected to be coincident with changes in the degree of condensation of the chromatin. Transcriptionally active chromatin would assume a less condensed state than transcriptionally inactive chro-

Figure 3. Thymidine (methyl H^3) incorporation into S. gregaria flight muscle nuclei. Total number of nuclei examined per age group was at least 500.

* No labelled nuclei could be detected in these groups.

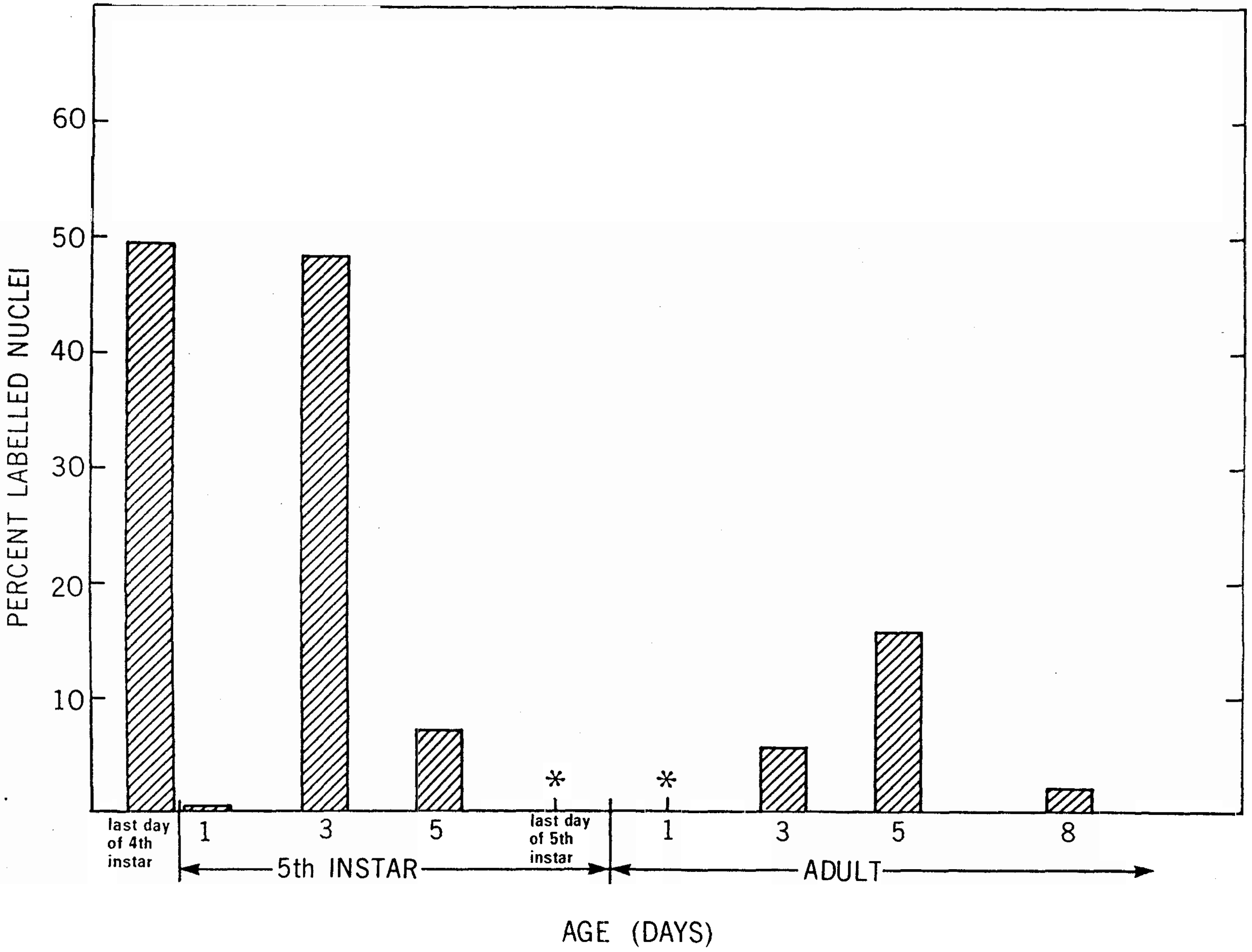

PERCENT LABELLED NUCLEI
60
50
40
30
20
10
*
*
last day of 4th instar
1
3
5
last day of 5th instar
1
3
5
8
5th INSTAR
ADULT
AGE (DAYS)

Figure 4. DNA content of _S. gregaria_ flight muscle nuclei expressed as $\log_2$ of total extinction.

A: last day of fourth nymphal instar; B, C, D, E: first, third, fifth and last day of fifth nymphal instar, respectively; F, G, H, I: first, third, fifth and eighth day of adult, respectively; J: spermatids. Total number of nuclei measured per age group was 100. 20 spermatid nuclei were measured to obtain the haploid value.

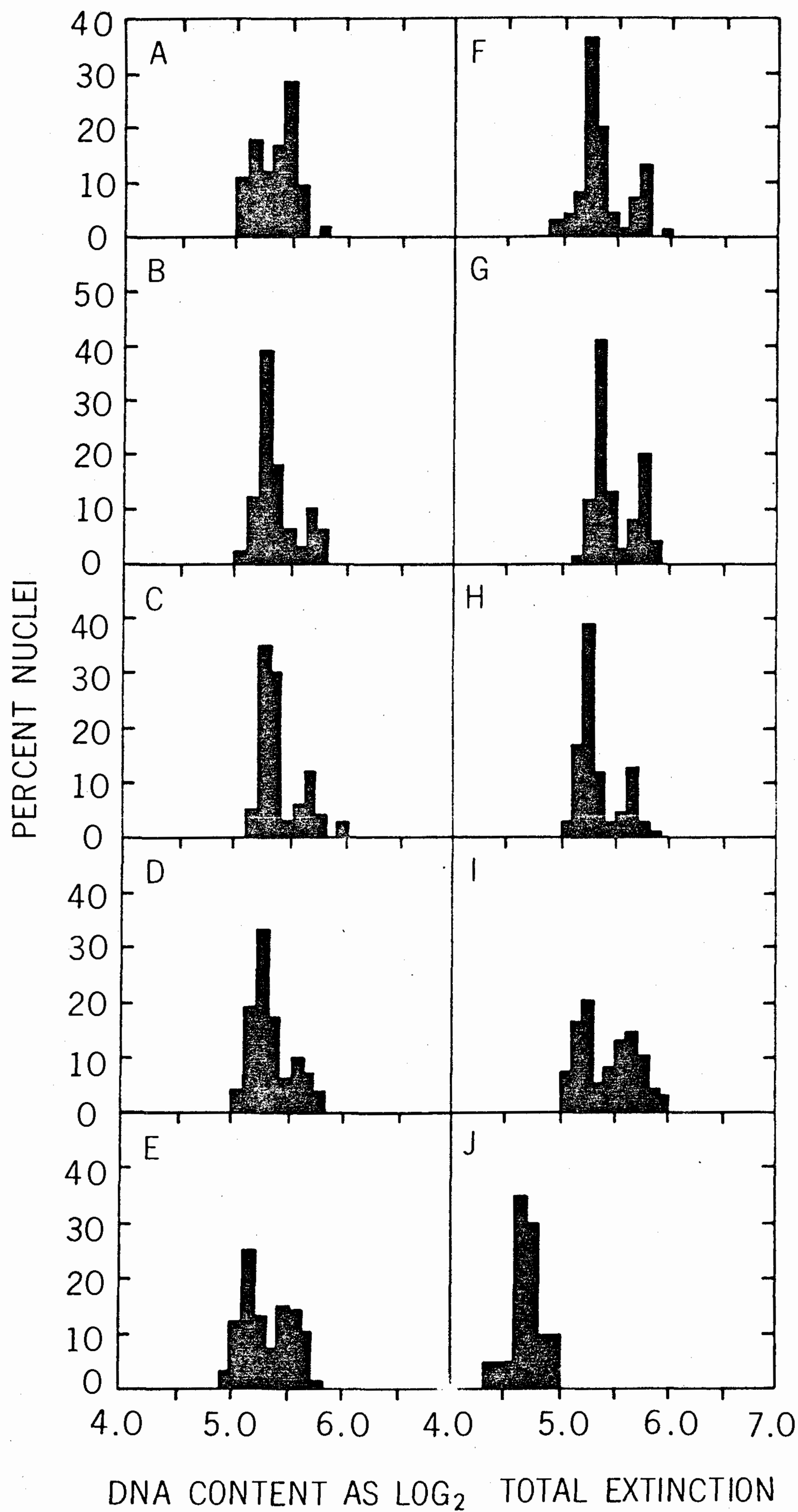
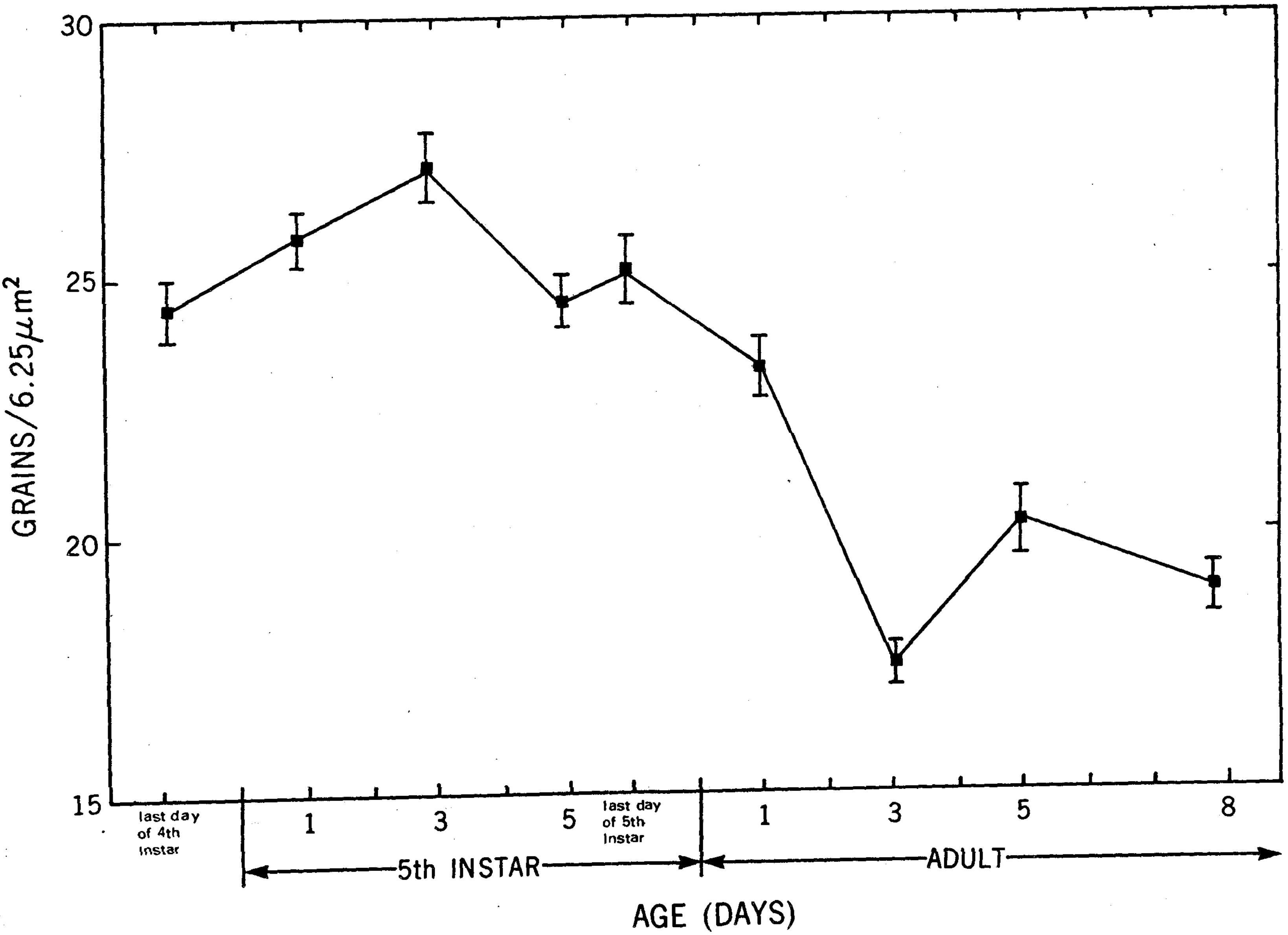

A
B
C
D
E
F
G
H
I
J
PERCENT NUCLEI
4.0
5.0
6.0
4.0
5.0
6.0
7.0
DNA CONTENT AS LOG2 TOTAL EXTINCTION

Figure 5. Transcriptional activity of *S. gregaria* flight muscle nuclei based on the binding of ^{3}H-Actinomycin D to chromatin. Each point represents the mean ± standard error of 100 measurements.



30
25
20
15
GRAINS/6.25μm²
last day of 4th instar
1
3
5
last day of 5th instar
1
3
5
8
5th INSTAR
ADULT
AGE (DAYS)

matin. This would be reflected by the lower percentage of high density points for less condensed chromatin (Fig. 6).

There are few high density points in the last day of the fourth nymphal instar but their frequency increases gradually until the first day after the imaginal moult. The third day of adult life is characterized by a decrease in the frequency distribution of high density points. A further increase is evident on the fifth day.

Nuclear Area:

The changes in the size of the nuclei over the developmental period are summarized in figure 7. The area is seen to decrease at both moults and also as maturation is achieved. These changes are statistically significant ($P < 0.05$).

RNA:

Azure B microspectrophotometry carried out on cross-sections of flight muscle fibres indicates an overall increase in the total level of RNA during development (Fig. 8). This trend is interrupted only at the time of the two moults after which the total RNA level continues to increase. In both cases, the rise resumes immediately after the ecdysis. The apparent decline in the amount of RNA between the first and third days after the ultimate moult is not statistically significant ($P > 0.05$).

Other than a slight increase at the very onset of development, the concentration of RNA declines slowly, but steadily throughout this period. Although the overall change

Figure 6. Relative frequency of occurrence of partial extinction values in Feulgen-stained _S. gregaria_ flight muscle nuclei.

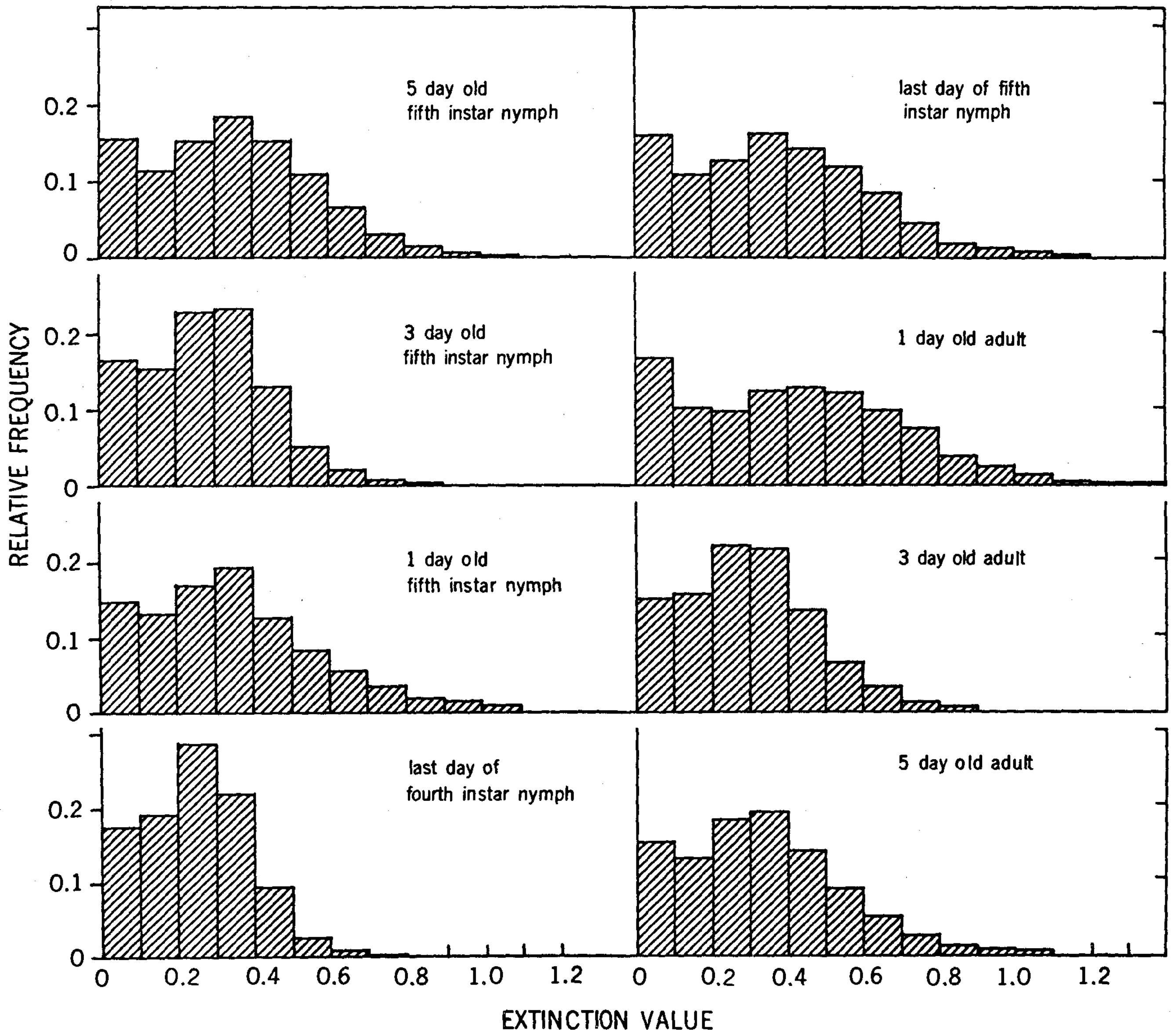

5 day old
fifth instar nymph
last day of fifth
instar nymph
3 day old
fifth instar nymph
1 day old adult
1 day old
fifth instar nymph
3 day old adult
last day of
fourth instar nymph
5 day old adult
RELATIVE FREQUENCY
0
0.1
0.2
EXTINCTION VALUE
0
0.2
0.4
0.6
0.8
1.0
1.2

Figure 7. Area of *S. gregaria* flight muscle nuclei. Each point represents the mean ± standard error of 100 measurements. A nucleus with a 100 μm step size is equivalent to 76 μ^2 in area.

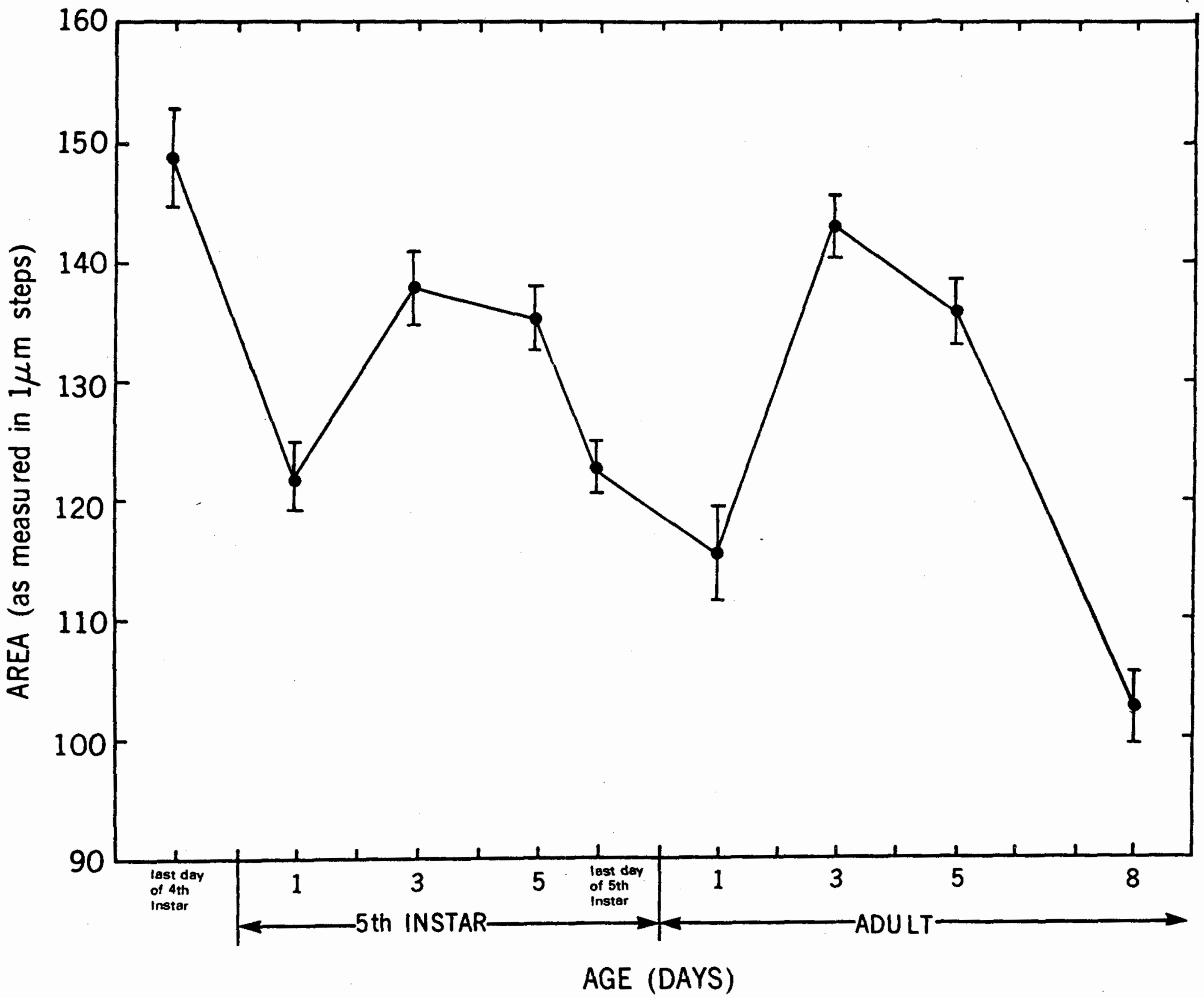
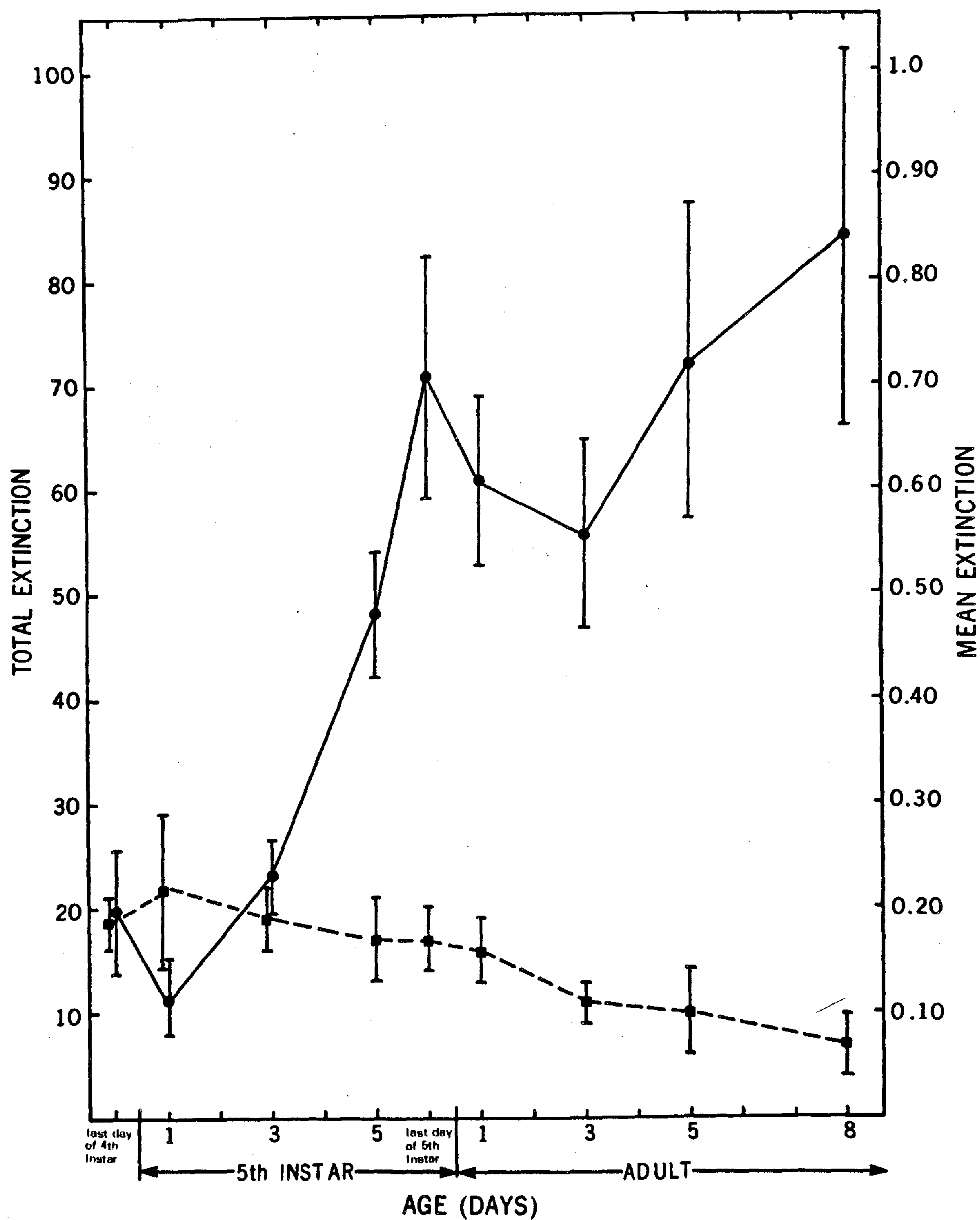

AREA (as measured in 1μm steps)
160
150
140
130
120
110
100
90
last day of 4th instar
1
3
5
last day of 5th instar
1
3
5
8
5th INSTAR
ADULT
AGE (DAYS)

Figure 8. Cytoplasmic RNA content (total extinction) and its concentration (mean extinction) of S. gregaria flight muscle fibres. Each point represents the mean ± standard deviation of 100 measurements.

●———● total extinction

■- - - - -■ mean extinction



100
90
80
70
60
50
40
30
20
10
TOTAL EXTINCTION
1.0
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
MEAN EXTINCTION
last day of 4th instar
1
3
5
last day of 5th instar
1
3
5
8
5th INSTAR
ADULT
AGE (DAYS)

appears small, analysis of variance indicates that it is significant ($P < 0.05$).

Protein:

The changes in the amount of total protein in the muscle fibres follows very closely those changes in the total RNA levels (Figs. 8, 9). As with the RNA, the total protein level decreases significantly ($P < 0.05$) at the time of both the final nymphal moult and the imaginal moult.

The concentration of protein rises at the beginning of development and then decreases slowly and steadily throughout the remainder of the growth and differentiation period (Fig. 9).

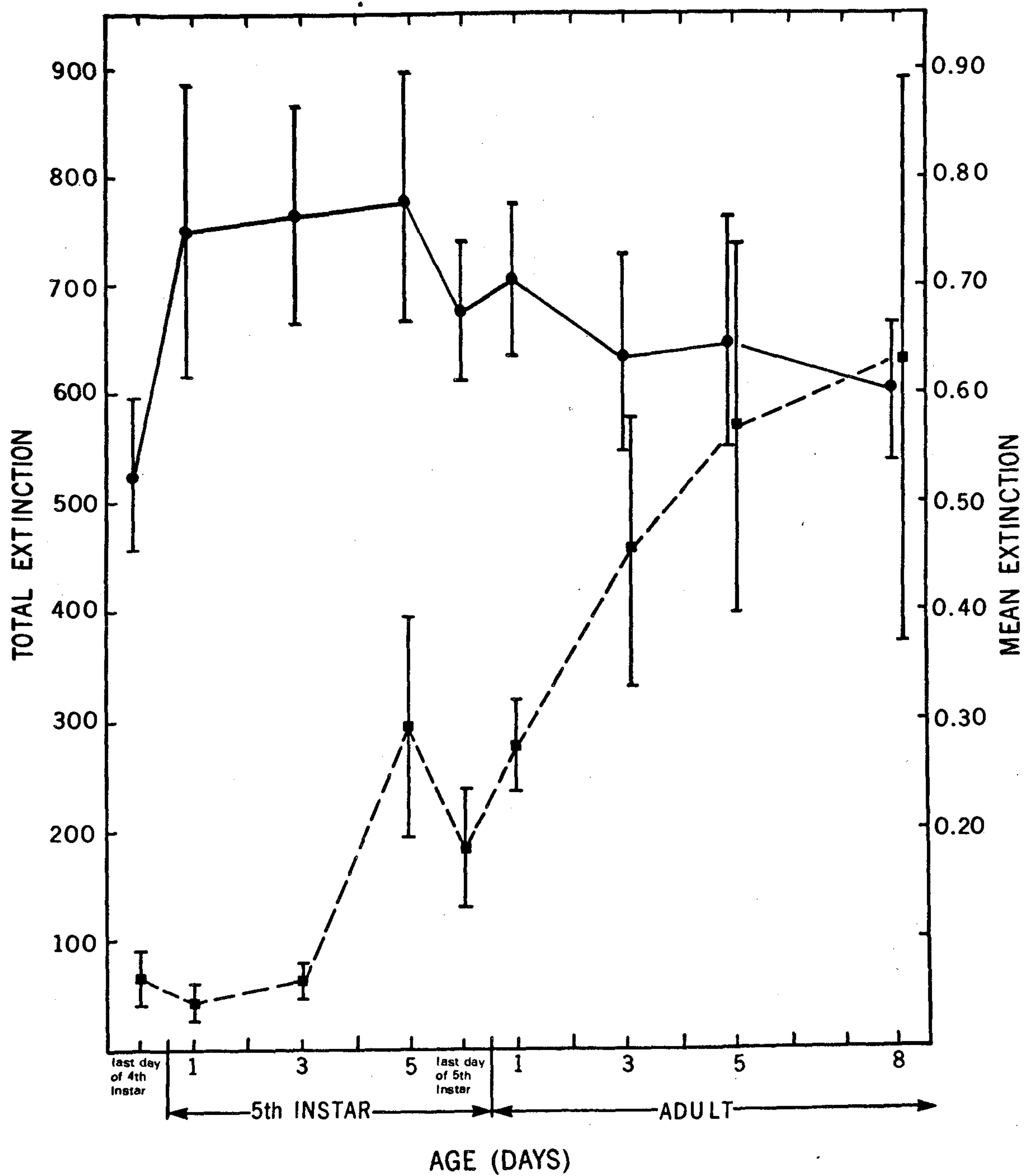
Cross-sectional Fibre Area:

The growth and differentiation of the insect flight muscles is reflected in changes occurring in the cross-sectional area of the muscle fibres. Except for interruptions at the time of both the moults, the cross-sectional area of the fibres increases rapidly throughout the developmental period (Fig. 10). During the fifth nymphal instar the area increases by 800% with a subsequent 350% increase in area during the first eight days of imaginal life. This statistically significant ($P < 0.05$) increase of about 12-fold is not surprising in view of the overall increase in weight that occurs.

Figure 9. Cytoplasmic protein content (total extinction) and its concentration (mean extinction) of S. gregaria flight muscle fibres. Each point represents the mean ± standard deviation of 100 measurements.

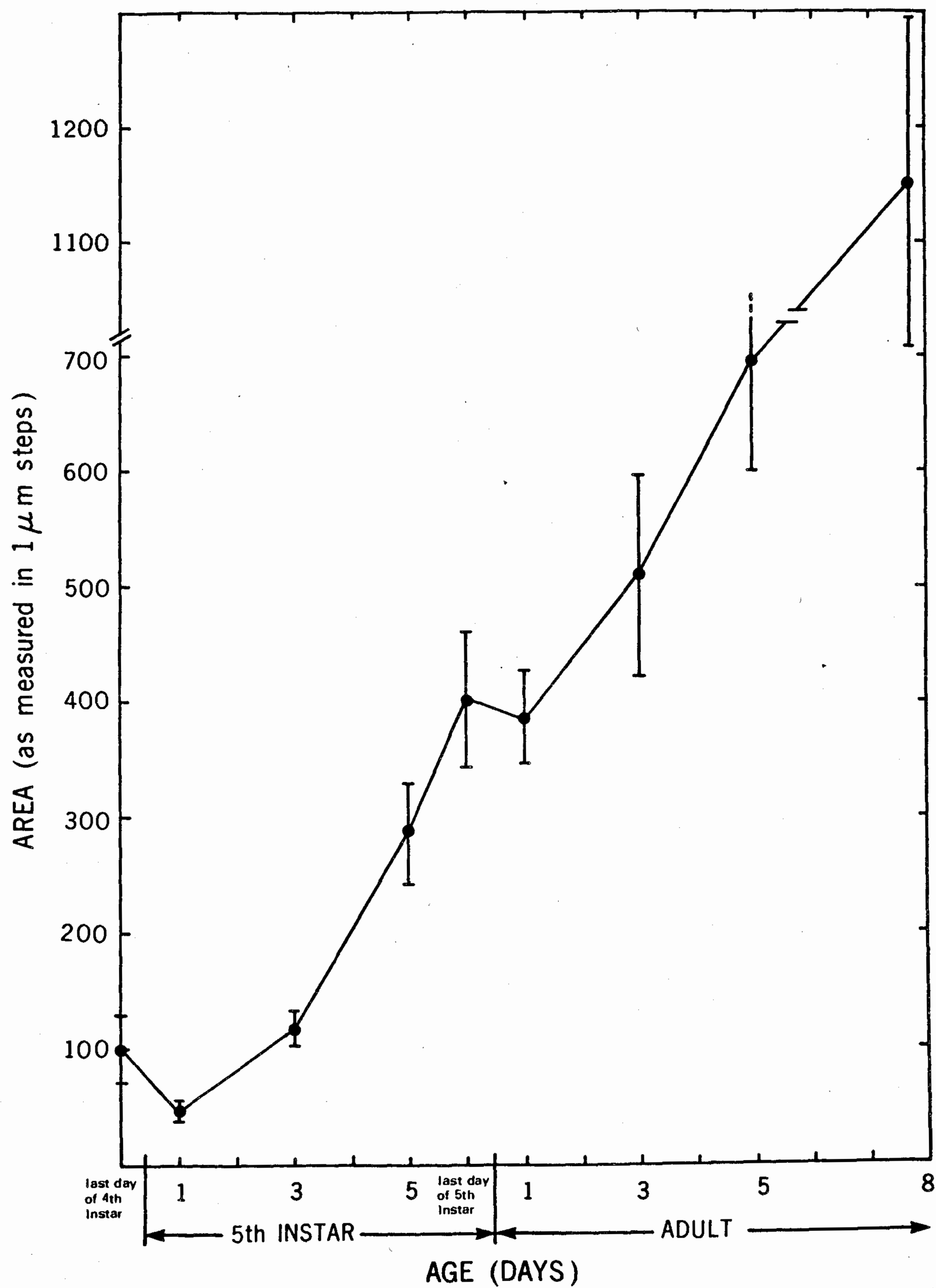
■------● total extinction

●———● mean extinction



900
800
700
600
500
400
300
200
100
TOTAL EXTINCTION
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
MEAN EXTINCTION
last day of 4th instar
1
3
5
last day of 5th instar
1
3
5
8
5th INSTAR
ADULT
AGE (DAYS)

Figure 10. Cross-sectional area of S. gregaria flight muscle fibres expressed in terms of a 1 μm step size. Each point represents the mean ± standard error of 100 measurements.



AREA (as measured in 1 μm steps)
1200
1100
700
600
500
400
300
200
100
last day of 4th instar
1
3
5
last day of 5th instar
1
3
5
8
5th INSTAR
ADULT
AGE (DAYS)

DISCUSSION

Although there has been a considerable amount of research on differentiation of striated muscles of vertebrates, little is known on nuclear differentiation of muscles (see reviews by Fischman, 1972; Holtzer *et al.*, 1972, Goldspink, 1972). The available data indicate that DNA synthesis is seen only in the mononucleated myoblasts and once a syncytium is formed by the fusion of these cells, DNA synthesis ceases. The muscle nuclei remain diploid throughout the life of the organism.

Information on the cytochemistry of insect muscle nuclei during development and metamorphosis is also scanty. The results obtained in my study show that DNA synthesis occurs during the period of flight muscle differentiation in *S. gregaria*. During this time there are three distinct peaks of high synthetic activity (Fig. 3).

Krishnakumaran *et al.* (1967) studied the pattern of DNA synthesis in different tissues of Lepidopterous insects during growth and metamorphosis. They observed DNA synthesis in muscle cells not only during the larval instars, but also during pupal-adult ecdysis. They also noticed labelling of muscle nuclei in adult cockroaches and mealworms. These observations led Krishnakumaran *et al.* (1967) to hypothesize that DNA synthesis at certain times during development may be for elimination of various "biases" thus enabling the cells to be reprogrammed. Although this hypothesis is an interesting one its validity has yet to be established.

The fact that DNA synthesis occurs in the flight muscles during differentiation raises possibilities concerning its purpose. Does this indicate polyploidization of nuclei or their duplication?

Throughout the developmental period the $\log_2$ of the total extinction values of the flight muscle nuclei remain between 5.0 and 6.0 (Fig. 4). This represents a diploid condition. These observations conform with those obtained in vertebrate skeletal muscles by others (Lash *et al.*, 1957; Firket, 1958; Basleer, 1962; Cox and Simpson, 1970).

This rules out polyploidization as the reason for DNA synthesis. Another possibility is nuclear fission. Examination of nuclear area shows that it undergoes statistically significant changes over the developmental period. These changes ($\pm$ 20%) do not appear substantial enough to support the possibility of fission (Fig. 7).

The absence of any mitotic or amitotic figures as well as a lack of polyploidization in these fibres would suggest a different function for the newly synthesized DNA. From the data I have it is difficult to establish that this new DNA is metabolic DNA (Pelc, 1972). In the locust, the "precursor" muscle begins synthesizing its components immediately after the moult into the fifth instar. This involves the synthesis of certain molecules like myosin. In systems which synthesize large quantities of proteins it will be advantageous to have additional gene copies for transcription. Pelc (1972) suggests

that "owing to wear and tear some copies become unusable and are periodically renewed." In light of this it is tempting to suggest that the cycles of DNA synthesis in these nuclei at certain periods of development represent the synthesis of the necessary copies of the genome that transcribe for special proteins like myosin and certain enzyme systems.

DNA transcriptional activity is an essential, on-going process in both developing and mature tissues. Bonner and Huang (1963) suggest that histones, basic nuclear proteins, are involved in the regulation of gene expression at the transcriptional level. They surmise that changes in the binding of histones to the template would make different segments of DNA available for transcription. Actinomycin D is an antibiotic which binds specifically to the histone-free guanosine-containing sites of the DNA molecule (Müller and Crothers, 1968; Zajicek et al., 1970).

It has been shown conclusively, that the binding of AMD is dependent upon the state of repression of the chromatin, that is, an AMD binding profile reflects changes in the degree of association between DNA and chromosomal proteins at different stages of the cell cycle (Brachet and Ficq, 1965; Berlowitz et al., 1969; Badr, 1972; Pederson and Robbins, 1972).

Brachet and Malpoix (1971) state that AMD binding decreases when the genome is repressed. This binding decreases towards the final phase of differentiation especially in those cells that synthesize one major protein. It has also been shown that

AMD binding increases in lymphocytes stimulated by phytohemagglutinine (Darzcynkiewicz et al., 1969; Ringertz et al., 1969; Ringertz and Bolund, 1969 a, b; Rigler et al., 1969), and in the colleterial gland nuclei of Periplaneta americana stimulated by juvenile hormone (Nair and Menon, 1972).

From the ^{3}H-AMD study (Fig. 5) we see that in general as differentiation nears completion the originally high transcriptional activity declines. The high transcriptional activity in the early stages is probably indicative of increased mRNA production for the synthesis of special proteins such as myosin and actin. These results support the findings of Vogell (1962) who observed large amounts of polysomes in electronmicrographs of L. migratoria "precursor muscle".

The chromatin in nuclei exists either in diffused form (euchromatin) or in a condensed form (heterochromatin). It is believed that euchromatin is transcriptionally active, whereas the heterochromatin is transcriptionally inactive (Keifer et al., 1973). If this is so analysis of the chromatin pattern would give some information on the genetic activity of the nucleus. Frequency distribution of points in a Feulgen-stained nucleus would show whether there are points with high extinction values. These would represent the regions of condensation in the chromatin.

The results of the ^{3}H-AMD study suggest that a frequency distribution of the extinction values of Feulgen-stained flight muscle nuclei should exhibit more high density points as the age increases. Generally, this is true. However, one other

factor must also be considered. A change in the physical state of the chromatin may be indicative not only of the level of transcriptional activity but also of the occurrence of DNA synthesis. Indeed, those two age groups wherein no DNA synthesis was detected (last day of the fifth nymphal instar and one-day-old adult) also have the highest percentage of high density points (Fig. 6). Furthermore, those two age groups showing the highest percentage of thymidine (methyl ^{3}H) incorporation (last day of the fourth nymphal instar and three-day-old fifth instar nymph) have the lowest percentage of high density points. Therefore, as expected, where the chromatin exhibits low condensation and presumably, therefore, high synthetic activity, the frequency distributions show fewer high density points.

Extensive work has been done on flight muscle development in the *L. migratoria*, an insect closely related to *S. gregaria*. On the basis of fresh weight of the muscle the developmental process falls into four distinct phases (Brosemer *et al.*, 1963; Bücher, 1965). This has been confirmed by ultrastructural and biochemical studies. The first phase, "larval growth", comprises most of the fifth instar. It is characterized by an increase, on a logarithmic scale, of all cellular components. Although the nuclei are excluded from this extensive growth, they do remain highly active at this time. The "moulting interval" begins just prior to the imaginal moult and ends a day or two after ecdysis. At this time the increase in weight ceases. Furthermore, the muscles undergo tracheole invasion. The "dif-

ferentiation phase" proceeds until about the third day after the imaginal moult. It is during this period that growth resumes coupled with differentiation of the morphological and enzymological patterns characteristic of the functional flight muscle. After differentiation, the "duplication phase" proceeds until about the eighth day of imaginal life. At this time, many major components are duplicated.

An examination of the changes in total RNA and protein as well as in cross-sectional area of the fibres show that the development in S. gregaria is very similar to that in L. migratoria (Figs. 8, 9, 10).

In all three of the parameters examined the "larval growth phase" is characterized by considerable increases. The apparent decrease in fibre area during the "moulting interval" is not statistically significant. However, there has obviously been a cessation of growth. Indeed total protein and total RNA levels decline slightly at this time. Although they were expected to increase during the "differentiation phase", the reason they did not may be linked to the transcriptional activity. The transcriptional activity is relatively high at the beginning of the "larval growth phase", but it declines about midway through this phase and does not increase again until the "differentiation phase" has begun. Therefore the increases in total RNA and protein content expected after the imaginal moult would not occur until after this significant increase in transcriptional activity. And so the "duplication phase" is not surprisingly associated with substantial increases in all three components.

The overall increase in total RNA content was predictable considering the initially high transcriptional activity in the developing flight muscle nuclei. Furthermore, in view of the tremendous increase in size that the flight muscle undergoes (Hill and Goldsworthy, 1968; Hill *et al.*, 1968), the changes in total protein content are not surprising. And finally, with such a substantial increase in protein, the major constituent of muscle (Maruyama, 1965), the changes in cross-sectional muscle fibre area are to be expected. Indeed both the total protein content and the cross-sectional area increase by a factor of 12. Bücher (1965) found a similar increase in the fresh weight of *L. migratoria* flight muscles during their development.

The concentration of RNA in the flight muscles decreases throughout development while the total RNA content increases. This is because the cross-sectional area of the muscle fibres is increasing at a higher rate than the total RNA. And so, the changes in the concentration of protein or RNA in the developing flight muscles is a function of the changes in total protein and RNA and the changes in fibre area.

There is one final point to be discussed and that is the significance of the moulting interval. In *L. migratoria* the developing flight muscles do not have a tracheole system until after the final ecdysis (Bücher, 1965). Indeed the moulting interval is characterized by the invagination of tracheoblasts. Bücher (1965) suggested that development could proceed only so far under the anaerobic conditions that would occur in the

absence of interfibrillar tracheae. Therefore, before development can continue, the tracheole system would have to be reconstructed. There is some evidence from this study that the same situation exists in S. gregaria. Firstly, there is this characteristic interruption in development during the moulting interval indicated by the cessation in the increase in total RNA and protein content and cross-sectional fibre area. Secondly, at this time, the distance between the muscle fibres is seen to increase greatly which could indicate tracheolar invagination.

CONCLUSIONS

Cytochemical analysis of flight muscle differentiation in *S. gregaria* showed the following: --

(1) The flight muscle nuclei remained diploid throughout the period of differentiation.

(2) Although the nuclei are diploid, cyclical synthesis of DNA is seen during differentiation. This may represent amplification of certain segments of the genome.

(3) The transcriptional activity of these nuclei is high to begin with but gradually declines towards the end of differentiation.

(4) RNA and protein content increase during the differentiation period by a factor of four and twelve respectively.

(5) The cross-sectional area of the fibres increase by a factor of 12.

REFERENCES [1]

Alfert, M. and I. I. Geschwind. 1953. A selective staining method for the basic proteins of cell nuclei. Proc. Nat. Acad. Sci., 39:991-999.

Ashhurst, D. E. 1967. The fibrillar flight muscles of giant water-bugs: an electron-microscope study. J. Cell Sci., 2:435-444.

Badr, E. A. 1972. Cytophotometric studies on histones in different types of cells as compared to the degree of binding of ^{3}H-actinomycin D to their nuclei. Arch. Biol., 83:21-27.

Baserga, R. and D. Malamud. 1969. Modern methods in experimental pathology autoradiography: techniques and applications. Harper and Row, N. Y., Evanston and London.

Basleer, R. 1962. Etude l'augmentation du nombre de noyaux dans des bourgeions musculaired cultives _in vitro_. Z. anat. Entwicklung., 123:184-205.

Bartels, P. H., G. Bahr, W. Jeter, G. Olson, J. Taylor and G. Weid. 1974. Evaluation of correlational information in digitized cell images. J. Histochem. Cytochem., 22:69-79.

Beenakkers, A. M. 1963. Enzyme der dettsaure-oxydation in den flugmuskeln von _Locusta migratoria_ wahrend ihrer entwicklung. Biochem. Z., 337:436-439.

Beenakkers, A. M. 1973. The influence of corpora allata on the flight muscle development in locusts. J. Endocrinol., 57:52.

Bhakthan, N. M., K. K. Nair and J. H. Borden. 1971. Fine structure of degenerating and regenerating flight muslces in a bark beetle, _Ips confusus_. II. Regeneration. Can. J. Zool., 49:85-89.

Bennett, J. and K. J. Scott. 1971. Quantitative staining of fraction 1 protein in polyacrylamide gels using Coomassie Brilliant Blue. Anal. Biochem., 43:173-182.

Berlowitz, L., D. Pallotta and C. H. Sibley. 1969. Chromatin and histones: binding of tritiated actinomycin D to heterochromatin in mealy bugs. Sci., 164:1527-1529.

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[1] Includes references cited in body of text as well as in the appendix.

Bocharova-Messner, O. M. and K. A. Yanchuk. 1966. Changes in the ultrastructure of the wing muscles of the domestic cricket (Acheta domestica L.) in ontogenesis. Akademiia Nauk U.S.S.R. Doklady, 170:657-660.

Bonner, J. and R. C. Huang. 1963. Properties of chromosomal nucleohistone. J. Mol. Biol., 6:169-174.

Błotna, M. and J. Michejda. 1966. The ultrastructure of flight muscles in development of Antherea pernyi Guer. Poznaskie towarzystow pyzyjaciol Nauk Bull. Serie D. Sciences Biol. Poznan., 7:85-94.

Brachet, J. 1957. Biochemical Cytology. Academic Press, New York.

Brachet, J. and A. Ficq. 1965. Binding sites of ^{14}C-actinomycin D in amphibian ovocytes and an autoradiography technique for the detection of cytoplasmic DNA. Exptl Cell Res., 38:153-159.

Brachet, J. and N. Hulin. 1969. Binding of tritiated actinomycin and cell differentiation. Nature, 222:481-482.

Brachet, J. and P. Malpoix. 1971. Macromolecular synthesis and nucleocytoplasmic interactions in early development, p. 263-316. In: M. Abercombie and J. Brachet (eds.). Adv. Morphogenesis Vol. 9. Academic Press, N. Y. and London.

Brosemer, R. W., W. Vogell and Th. Bücher. 1963. Morphologische und enzymatische muster bei der entwicklung indirekter flugmuskeln von Locusta migratoria. Biochem. Z., 338: 854-910.

Brosemer, R. W. 1965. The effect of puromycin and actinomycin D on the development of grasshopper flight muscle glycerolphosphate dehydrogenase. Biochim. Biophys. Acta, 99:388-399..

Bücher, Th. 1965. Formation of the specific structural and enzymic pattern of the insect flight muscle. Biochem. Soc., Lond., 25:15-28.

Burkhardt, H. III. 1964. Analysis of variance-PDP-5/8. Decus Program Library, Decus No. 5/8-9.

Capers, C. R. 1960. Multinucleation of skeletal muscle in vitro. J. Biophys. Biochem. Cytol., 7:559-566.

Conger, A. and A. M. Fairchild. 1953. Quick-freeze method for making smear slides permanent. Stain Tech., 28:281-289.

Cooper, W. G. and I. R. Konigsberg. 1961. Dynamics of myogenesis _in vitro_. Anat. Rec., 140:195-206.

Cowden, R. R. and D. Bodenstein. 1961. A cytochemical investigation of striated muscle differentiation in regenerating limbs of the roach, _Periplaneta americana_. Embryol., 6:36-50.

Cox, P. C. and S. B. Simpson. 1970. A microphotometric study of myogenic lizard cells grown _in vitro_. Devel. Biol., 23:433-443.

Chrambach, A., R. A. Reisfeld, M. Wychoff and J. Zaccari. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal. Biochem., 20:150-154.

Crossley, A. C. 1972. Ultrastructural changes during transition of larval to adult intersegmental muscle at metamorphosis in the blowfly _Calliphora erythrocephala_. I. Dedifferentiation and myoblast fusion. J. Embryol. exp. Morphol., 27: 43-74.

Darzcynkiewicz, Z., B. D. Gledhill and N. R. Ringertz. 1969. Changes in deoxyribonucleoprotein during spermiogenesis in the bull. Exptl Cell Res., 58:435-438.

Deitch, A. D. 1955. Microspectrophotometric study of the binding of the anionic dye, Naphthol Yellow S, by tissue sections and by purified protein. Lab. Invest., 4:324-351.

Deitch, A. D. 1961. An improved Sakaguchi reaction for microspectrophotometric use. J. Histochem. Cytochem., 9:477-483.

Deitch, A. D. 1966. Cytophotometry of nucleic acids, p. 327-354. In: G. L. Weid (ed.) Introduction to Quantitative Cytochemistry. Academic Press, New York and London.

Doane, W. 1973. Role of hormones in insect development, p. 291-497. In: S. J. Counce and C. H. Waddington (eds.) Developmental Systems: Insecta Vol. 2. Academic Press, London and New York.

Ebstein, B. S. 1969. The distribution of DNA within the nucleoli of the amphibian oocyte as demonstrated by tritiated actinomycin D radioautography. J. Cell Sci., 5: 27-44.

Edwards, F. J. 1969. Development and histolysis of the indirect flight muscles in Dysdercus intermedius. J. Insect Physiol., 15:1591-1599.

Fand, S. 1972. Environment conditions for optimal Feulgen hydrolysis, p. 211-222. In: G. L. Weid and G. F. Bahr (eds.). Introduction to Quantitative Cytochemistry-II. Academic Press, N. Y. and London.

Fazekas de St. Groth, S., R. G. Webster and A. Datyner. 1963. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. Biochim. Biophys. Acta, 71:377-391.

Ficq, A. 1955. Etude autoradiographique du metabolisme des proteines et des acides nucleiques au cours de l'oogenese chez les bactraciens. Exptl Cell Res., 9:286-293.

Firket, H. 1958. Researches sur la synthese des acides desocyribonucleiques et la preparation a la mitose dans des cellulles cultivees in vitro. Arch. Biol., 69:1-166.

Fischman, D. E. 1972. Development of striated muscle, p. 75-148. In: G. H. Bourne (ed.) The Structure and Function of Muscle Vol. 1. Academic Press, N. Y. and London.

Fishbein, W. N. 1972. Quantitative densitometry of 1-50 g protein in acrylamide gel slabs with Coomassie blue. Anal. Biochem., 46:388-401.

Flax, M. H. and M. H. Himes. 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. Physiol. Zool., 25:297-311.

Fox, D. P. 1969. DNA values in somatic tissues of Dermestes (Dermistidae: Coleoptera) I. Abdominal fat body and testis wall of the adult. Chromosoma (Berl.), 28:445-456.

Gilbert, L. I. 1964. Physiology of growth and development: endocrine aspects, p. 248-336. In: M. Rockstein (ed.) The Physiology of Insecta Vol. 1. Academic Press, N. Y. and London.

Goldspink, G. 1972. Postembryonic growth and differentiation of striated muscle, p. 179-236. In: G. H. Bourne (ed.) The Structure and Function of Muscle Vol. 1. Academic Press, N. Y. and London.

Gomori, G. 1956. Histochemical methods for protein-bound sulphydryl and disulphide groups. Quart. J. Microscop. Sci., 97:1-9.

Goodman, G. C. 1957. On the regeneration and redifferentiation of mammalian striated muscle. J. Morphol., 100:27-82.

Grant, V. J. 1961. Origin and differentiation of the imaginal indirect flight muscles of Simulium ornatum Meigen (Diptera, Nematocera). J. Zool. Soc. India, 13:174-179.

Gregory, D. W., R. W. Lennie and L. M. Birt. 1968. An electron-microscopic study of flight muscle development in the blow-fly Lucilia cuprina. J. Roy. Microscop. Soc., 88:151-175.

Herold, R. C. 1965. Development and ultrastructural changes of sarcosomes during honey bee flight muscle development. Develop. Biol., 12:269-286.

Hill, L. and G. J. Goldsworthy. 1968. Growth, feeding activity and the utilization of reserves in larvae of Locusta. J. Insect Physiol., 14:1085-1098.

Hill, L., A. J. Luntz, and P. A. Steele. 1968. The relationships between somatic growth, ovarian growth, and feeding activity in the adult desert locust. J. Insect Physiol., 14:1-20.

Holtzer, H., H. Weintraub, R. Mayne and B. Mochan. 1972. The cell cycle, cell lineages and cell differentiation, p. 229-259. In: A. A. Moscona and A. Monroy (eds.) Current Topics in Developmental Biology, Vol. 7. Academic Press, N. Y. and London.

Hunter-Jones, P. 1956. Instructions for Rearing and Breeding Locusts in the Laboratory. Anti-Locust Research Centre, London.

Keifer, R., G. Keifer, R. Salm, R. Rossner and W. Sandritter. 1973. A method for the quantitative evaluation of eu- and heterochromatin in interphase nuclei using cytophotometry and pattern analysis. Beitr. Path. Bd., 150: 163-173.

Krishnakumaran, A., S. J. Berry, H. Oberlander and H. A. Schneiderman. 1967. Nucleic acid synthesis during insect development--H. Control of DNA synthesis in the cecropia silkworm and other saturniid moths. J. Insect Physiol., 13:1-57.

Lash, J. W., H. Holtzer and H. Swift. 1957. Regeneration of mature skeletal muscle. Anat. Rec., 128:679-693.

Maizel, J. V. Jr. 1966. Acrylamide-gel electrophorograms by mechanical fractionation: Radioactive adenovirus proteins. Sci., 151:988-990.

Maruyama, K. 1965. The biochemistry of the contractile elements of insect muscle, p. 451-481. In: M. Rockstein (ed.) The Physiology of Insecta. Academic Press, New York.

Mazia, D., P. A. Brewer and M. Alfert. 1953. The cytochemical staining and measurement of protein with mercuric bromphenol blue. Biol. Bull., 104:57-67.

Meyer, T. S. and B. L. Lamberts. 1965. Use of Coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide-gel strips. Biochim. Biophys. Acta, 107:144-145.

Mittwoch, U., H. Kalmus and W. Webster. 1966. Deoxyribonucleic acid values in dividing and nondividing cells of male and female larvae of the honey bee. Nature, 210:264-266.

Müller, W. and D. M. Crothers. 1968. Studies of the binding of actinomycin and related compounds to DNA. J. Mol. Biol., 35:251-290.

Murray, M. R. 1960. Skeletal muscle tissue in culture, p. 111-154. In: G. H. Bourne (ed.) The Structure and Function of Muscle. Academic Press, New York.

Nair, K. K. and M. Menon. 1972. Detection of juvenile-hormone-induced gene activity in the colleterial gland nuclei of *Periplaneta* by actinomycin D 'staining' technique. *Experentia*, 28:577.

Pearse, A. G. E. 1968. Histochemistry: Theoretical and Applied, Vol. 1. J. A. Churchill Ltd., London.

Pederson, T. and E. Robbins. 1972. Chromatin structure and the cell division cycle. J. Cell Biol., 55:322-327.

Pelc, S. R. 1972. Metabolic DNA in ciliated protozoa, salivary gland chromosomes and mammalian cells, p. 327-355. In: G. H. Bourne and J. F. Danielli (eds.) International Review of Cytology Vol. 32. Academic Press, N. Y. and London.

Peristianis, G. C. and D. W. Gregory. 1971. Early stages of flight muscle development in the blowfly *Lucilia cuprina*: A light and electron microscopic study. *J. Insect Physiol.*, 17:1005-1022.

Poels, C. L. M. and A. M. Beenakkers. 1969. The effects of corpus allatum implantation on the development of flight muscles and fat body in *Locusta migratoria*. Ent. exp. appl., 12:312-324.

Rasch, E. and H. Swift. 1960. Microphotometric analysis of the cytochemical Millon action. J. Histochem. Cytochem., 8:4-17.

Rigler, R., D. Killander, L. Bolund and N. R. Ringertz. 1969. Cytochemical characterization of deoxyribonucleoprotein in individual cell nuclei. Exptl Cell Res., 55:215-224.

Ringertz, N. R. and L. Bolund. 1969a. Actinomycin binding capacity of deoxyribonucleoprotein. Biochim. Biophys. Acta, 174:147-154.

Ringertz, N. R. and L. Bolund. 1969b. "Activation" of hen erythrocyte deoxyribonucleoprotein. Exptl Cell Res., 55:205-214.

Ringertz, N. R., Z. Darzcynkiewicz and L. Bolund. 1969. Actinomycin binding properties of stimulated human lymphocytes. Exptl Cell Res., 56:411-417.

Sacktor, B. 1970. Regulation of intermediary metabolism with special reference to the control mechanism in insect flight muscle, p. 267-347. In: J. W. Beament, J. E. Treherne and V. B. Wigglesworth (eds.) Advances in Insect Physiology Vol. 7. Academic Press, London and New York.

Sahota, T. S. and W. E. Beckel. 1967. The influence of epidermis on the developing flight muscles in Galleria mellonella. Can. J. Zool., 45:421-434.

Scudder, G. G. E. 1971. The postembryonic development of the indirect flight muscles in Cenocorixa bifida (Hung.) (Hemiptera:Corixidae). Can. J. Zool., 49:1387-1398.

Scudder, G. G. E. and R. J. Hewson. 1971. The postembryonic development of the indirect flight muscles in Oncopeltus fasciatus (Dallas) (Hemiptera:Lygaedia). Can. J. Zool., 49:1377-1386.

Shafiq, S. 1963. Electron microscopic studies on the indirect flight muscles of Drosophila melanogaster II. Differentiation of myofibrils. J. Cell Biol., 17:363-373.

Shirer, D. L. 1969. Curfit. Decus Program Library, Decus No. Focal 8-63.

Smith, D. S. 1966. The organization of flight muscle fibres in the Odonata. J. Cell Biol., 1:109-126.

Smith, D. S. 1968. Insect Cells. Oliver and Boyd, Edinburgh.

Tiegs, O. W. 1955. The flight muscles of insects -- their anatomy and histology; with some observations on the structure of striated muscle in general. Phil. Trans. Roy. Soc. London, 238:221-359.

Vesterberg, O. 1971. Staining of protein zones after iso-electric focusing polyacrylamide gels. Biochim. Biophys. Acta, 243:345-348.

Vogell, W. 1962. S. B. Ges. Beford. ges. Naturwiss. Marburg 83/84, 297, as cited in Bücher, Th. 1965.

Walker, P. R., L. Hill and E. Bailey. 1970. Feeding activity, respiration and lipid and carbohydrate content of the male desert locust during adult development. J. Insect Physiol., 16:1001-1015.

Weid, G. L., P. H. Bartels, G. F. Bahr, and D. G. Oldfield. 1968. Taxonomic intra-cellular analytic system (TICAS) for cell identification. Acta Cytol., 12:177-201.

Wigglesworth, V. B. 1964. The hormonal regulation of growth and reproduction in insects, p. 215-226. In: J. W. Beament, J. E. Treherne and V. B. Wigglesworth (eds.) Advances in Insect Physiology Vol. 2. Academic Press, New York and London.

Zajicek, D., F. J. Swartz and A. D. Floyd. 1970. *Ascaris suum* and *Toxocara canis*: Radioautographic detection of DNA in Feulgen-negative muscle nuclei. Exptl Parasitol., 27:516-523.

Zimmer, H. 1970. Automatic analysis of microscopic images. Zeiss Information, 74:126-132.

Appendix I

Introduction:

There is an unfortunate gap among histochemical techniques for quantitative protein stains. Gomori (1956)[1] has described the problems associated with those protein stains which react with the sulphydryl and disulphide groups. There are a few stains which have been used successfully in the past for protein microspectrophotometry. However, the majority of these are for histones. These include Fast Green (Alfert and Geschwind, 1953), the Sakaguchi reaction (Deitch, 1961) and α Naphthol Yellow S (Deitch, 1955). However, there are problems associated with use of these techniques. The Sakaguchi reaction product is of low intensity (Deitch, 1966) and furthermore its development and preservation are technically difficult (Pearse, 1968). α Naphthol Yellow does not stain intensely and is rather diffuse (Brachet, 1957).

The commonly used stain for general protein determination is Millon's. However, due to numerous problems Rasch and Swift (1960) found that this technique is really only valuable if an accuracy of no greater than 20% is desired.

Mercuric Bromophenol blue (Mazia *et al.*, 1953) is another commonly used protein stain. Ficq (1955) found that the stain intensity of Bromophenol blue is strongly influenced by the presence or absence of RNA.

The purpose of this investigation was to determine whether

[1]References cited in the appendix are listed in the general reference list from p. 39.

Coomassie Brilliant blue could be used for the estimation of proteins in tissue sections by cytophotometric methods. It has been used extensively in electrophoresis (Fazekas de St. Groth et al., 1963; Meyer and Lamberts, 1965; Maizel, 1966; Chrambach et al., 1967; Bennett and Scott, 1971; and Fishbein, 1972).

Fazekas de St. Groth et al. (1963), Chrambach et al., (1967) and Fishbein (1972), have shown that the intensity of the stain bound to proteins was proportional to the concentration of proteins.

Methods and Materials:

The dorsal longitudinal flight muscle of a five-day-old fifth instar nymph of *S. gregaria* was fixed by freeze-substitution (Pearse, 1968) and embedded in paraffin. Five micron sections were cut and placed on quartz slides. Some sections were treated with ribonuclease (Sigma Chemical Co., St. Louis, Mo.) (1.0 mg/ml) for 1 hour at 37° C (Deitch, 1966). These and the control sections were then dehydrated through grades of ethanol and mounted in glycerine ($^{n}D = 1.459$). The extinction measurements were made at 280 nm in the SMP as described on p. 15 . The diameter of the scanning aperture was 1.16 µm and the step size was 2 µm.

These same sections were then stained with Coomassie Blue (Edward Gurr Ltd., London, England) after refixation in 12.5% trichloroacetic acid (TCA) for 30 minutes.

Since the concentration of the dye solution does not appear to be a crucial factor when used between 0.05 and 1.0%

(Maizel, 1966; Chrambach et al., 1967; Vesterberg, 1971; and Fishbein, 1972), in my study the stain was made up from a 1% stock solution, by a 1:20 dilution in 12.5% TCA (Chrambach et al., 1967). These and all other solutions preceding destaining were made up with glass distilled water as suggested by Fazekas de St. Groth et al. (1963). The sections were stained for one hour at 37° C and destained in 7% acetic acid (Maizel, 1966) for one hour at room temperature. They were then mounted in oil (^{n}D = 1.56, Cargille, U. S. A.). Extinction measurements of these sections were taken at 590 nm using the same aperture and step size as before.

The data were submitted to "Curfit" analysis, a Decus program (Shirer, 1969). This program fits weighted or unweighted data to a straight line on a Cartesian, log-log, or semi-log graph. It calculates the slope and the intercept of the line, the standard error in these values, the regression coefficient and other measures of the "goodness" of fit. This was used therefore to enable comparison of the slopes using the student t-test.

Results and Discussion:

Coomassie Blue is a triphenylmethane dye which gives an exceptionally high colour intensity (Fazekas de St. Groth et al., 1963).

The total extinction for increasing amounts of proteins in unstained locust flight muscle sections determined by ultraviolet microspectrophotometry, is presented in figure 11A and B.

Figure 11. The relationship between total extinction and the amount of proteins in sections of fifth instar _S. gregaria_ flight muscle.

A: measured at 280 nm

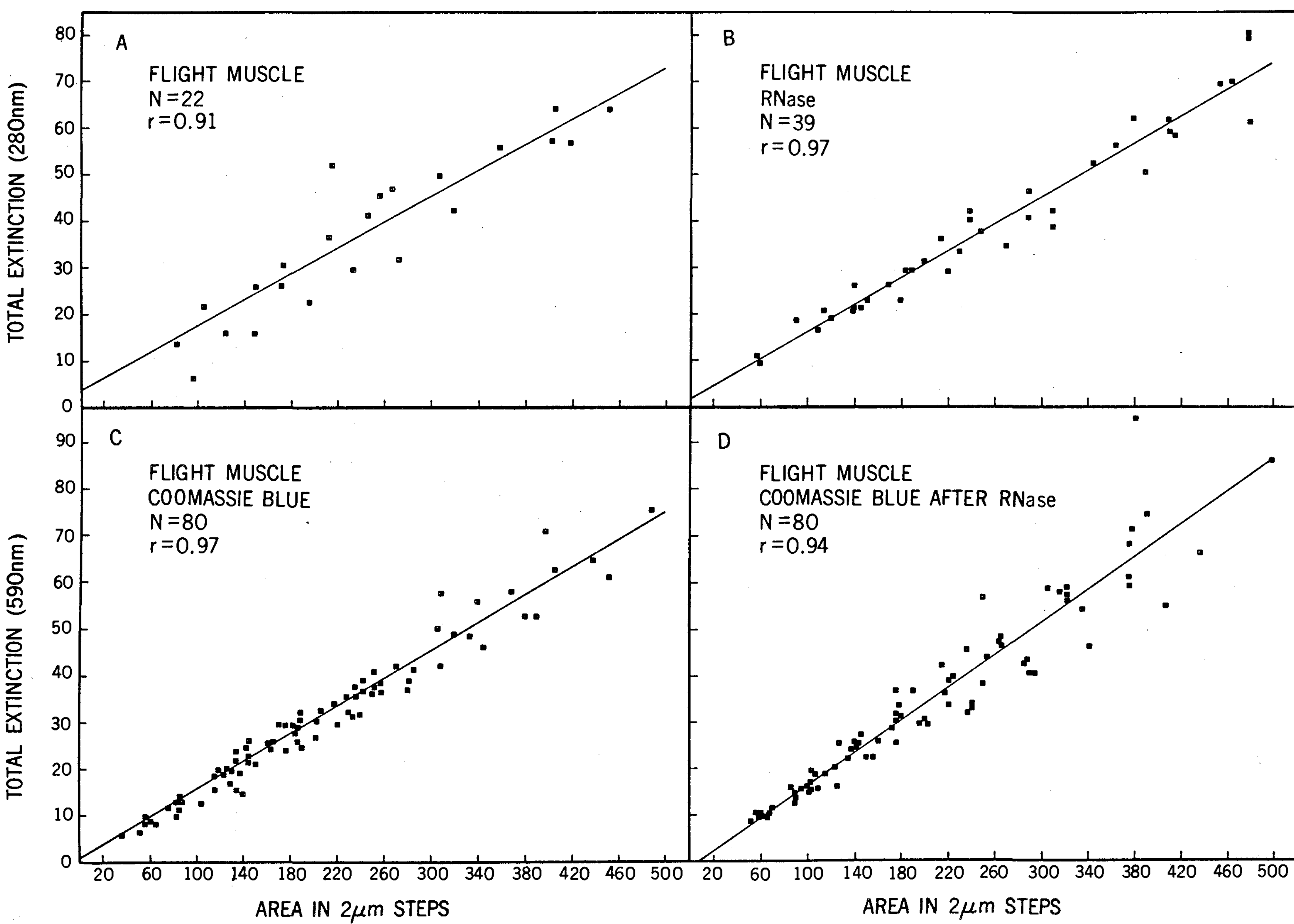
B: same as above after RNase treatment for one hour at 37° C

C: measured at 590 nm after staining with Coomassie blue

D: same as C preceded by RNase treatment

N: sample size

r: regression coefficient



TOTAL EXTINCTION (280nm)
A
FLIGHT MUSCLE
N=22
r=0.91
B
FLIGHT MUSCLE
RNase
N=39
r=0.97
TOTAL EXTINCTION (590nm)
C
FLIGHT MUSCLE
COOMASSIE BLUE
N=80
r=0.97
D
FLIGHT MUSCLE
COOMASSIE BLUE AFTER RNase
N=80
r=0.94
AREA IN 2μm STEPS
AREA IN 2μm STEPS

Figure 11B is for flight muscle sections previously treated with ribonuclease. Figures 11C and D represent the protein content of the same sections after staining with Coomassie blue. The statistical data from these four graphs is also presented in Table II.

Although the two ways of measuring protein content, i.e., u.v. and Coomassie blue microspectrophotometry, would be expected to yield different absolute results, both the trends and slope of the resulting lines should be very similar. Table II and figure 11 show this to be the case.

Statistical analysis showed no significant differences between any two of these four populations when resulting slopes were compared.

Conclusions:

It appears that Coomassie blue can be used as a quantitative stain for cytochemical analysis of proteins. Furthermore, the presence of RNA does not interfere with the Coomassie Blue staining whereas it does with mercuric Bromophenol blue staining (Ficq, 1955).

It would be desirable to test the influence of other factors like the temperature of the staining bath and the minimum staining time for each type of tissue to be examined.

TABLE II

Regression coefficient analysis of data in Fig. 11

Sample	N	y-intercept	slope	r
A	22	3.77	0.138	.91
B	39	0.97	0.144	.97
C	80	0.09	0.149	.97
D	80	-1.58	0.176	.94

A-D: as in Fig. 11

N: sample size

r: regression coefficient