Synthesis of mitochondrial protein in the flight muscles of the Colorado beetle

Dit proefschrift met stellingen van Antonius Karel Maria Bartelink, doctorandus in de wiskunde en natuurwetenschappen, geboren te Oldenzaal op 31 januari 1945, is goedgekeurd door de promotoren dr. J. de Wilde, hoogleraar in het dierkundig deel van de planteziektenkunde en dr. A. M. Kroon, hoogleraar in de fysiologische chemie aan de Rijksuniversiteit te Groningen, en de co-promotor dr. C. A. D. de Kort, wetenschappelijk hoofdmedewerker bij de Vakgroep Entomologie.

De rector magnificus van de Landbouwhogeschool,
J. P. H. van der Want

Wageningen, 13 maart 1975

A. K. M. Bartelink

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus, dr. ir. J. P. H. van der Want,
hoogleraar in de virologie,
in het openbaar te verdedigen
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ERRATA

A.K.M.Bartelink

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Paper III

On page 6, the drawings of Figs. 2 and 3 should be interchanged.

On page 7, line 2, "Fig. 4" should read "Fig. 3".

Stellingen

1.
Het juveniel hormoon beïnvloedt de biogenese van vliegspiermitochondriën in de Coloradokever op indirecte wijze.

Dit proefschrift.

2.
De experimenten van Bassi & Feir rechtvaardigen niet hun conclusie, dat het juveniel hormoon bij *Oncopeltus fasciatus* de synthese van het enzym zure fosfatase op transcriptieniveau controleert.

Bassi, S. D. & Feir, D. (1972). Comp. Biochem. Physiol. 41B, 771–776.

3.
De waarnemingen, dat rechtstreekse toediening van schildklierhormonen aan geïsoleerde levermitochondriën de eiwitsynthese in deze organellen stimuleert, hebben geen fysiologische betekenis.

Primack, M. P. & Tapley, D. F. (1974). Endocrinology 94, 871–874.
Primack, M. P., Tapley, D. F. & Buchanan, J. (1971). Biochim. Biophys. Acta 244, 349–352.

4.
De door Kellerman *et al.* bij *Saccharomyces cerevisiae* waargenomen effecten van cycloheximide op de [^{14}C]leucine-incorporatie in mitochondriaal eiwit, duiden niet noodzakelijk op een koppeling tussen de cytoplasmatische en mitochondriale eiwitsynthese.

Kellerman, G. M., Griffiths, D. E., Hansby, J. E., Lamb, A. J. & Linnane, A. W. (1971). In: Autonomy and Biogenesis of Mitochondria and Chloroplasts (Boardman, N. K., Linnane, A. W. & Smillie, R. M., eds.), pp. 346–359. North-Holland Publishing Co., Amsterdam.

5.
De mening van Albin *et al.*, dat tijdens geïnduceerde hartspierhypertrofie bij de rat het gehalte aan cytochroom *c* een index is voor de ontwikkeling van de mitochondriale binnenmembraan, wordt onvoldoende door hun experimenten ondersteund.

Albin, R., Dowell, R. T., Zak, R. & Rabinowitz, M. (1973). Biochem. J. 136, 629–637.

6.
Door Ozawa & Honjo is geenszins aangetoond, dat voor de prognose van het postoperatieve verloop bij patiënten met een maligne levertumor, de concentratie van het cytochroom aa_3 in de niet aangetaste leverkwab een geschikt criterium is.

Ozawa, K. & Honjo, I. (1975). Clin. Sci. Mol. Med. 48, 75–82.

7.
De studie van Von Jagow *et al.* over de samenstelling van de ademhalingsketen bij *Neurospora crassa* kan dienen als paradigma voor soortgelijke onderzoekingen bij animale systemen.

Jagow, G. von, Weiss, H. & Klingenberg, M. (1973). Eur. J. Biochem. 33 140–157.

8.
Bij *Erysiphe graminis* spelen de eigenschappen van de waardplantcuticula hoogstwaarschijnlijk een ondergeschikte rol bij de vorming van appressoria.

Yang, S. L. & Ellingboe, A. H. (1972). Phytopathology 62, 708–714.

A. K. M. Bartelink
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Voorwoord

Verscheidene mensen zijn mij de afgelopen jaren tijdens mijn promotie-onderzoek behulpzaam geweest. Een aantal van hen wil ik graag aan het begin van dit proefschrift noemen.

Professor De Wilde, U ben ik zeer dankbaar voor de mogelijkheid, die U mij geboden heeft dit onderzoek op het Laboratorium voor Entomologie te verrichten. Uw brede wetenschappelijke belangstelling en aanstekelijk enthousiasme hebben inspirerend gewerkt. Veel dank ben ik ook verschuldigd aan prof. dr. A. M. Kroon, die bereid was mede leiding te geven aan het onderzoek. Beste Ab, ik beschouw dit als een voorrecht. Jouw gedegen kennis en oorspronkelijke ideeën zijn voor mij van grote waarde geweest. De probleemstelling van dit proefschrift is gesuggereerd door dr. C. A. D. de Kort, die ook tijdens de eerste twee jaar van het onderzoek de directe supervisie had. Jouw bijdrage, Stan, heb ik erg gewaardeerd. De vele discussies, die je met mij hebt gevoerd, waren zeer stimulerend.

Veel profijt heb ik gehad van het werk van Manu Brouwers, Hanneke Drosopoulos-van Albada, Wim van der Klauw, René Schuurmans en Betty Valk, die in het kader van hun doctoraalstudie een aantal deelproblemen van het onderzoek hebben opgelost. De prettige assistentie van Marian Broeders-van Roestel tijdens de eindfase van het onderzoek wil ik hier eveneens vermelden. Verder gaat mijn dank uit naar Wim Piet Gijzel (spectrofotometrische adviezen), dr. G. J. J. Kortstee (microbiologische hulp), Wil van Marrewijk (aminozuuranalyses) en dr. M. A. J. van Montfort (Mitscherlichberekeningen). Ook mijn collega's Frans Dieleman, Rudi Rabbinge en Hugo Schooneveld ben ik erkentelijk voor hun hulp en deskundige adviezen.

De heer W. C. Th. Middelplaats en Frits von Planta verzorgden het tekenwerk en Ria Bodt typte het manuscript voor het vierde artikel. De Engelse tekst is, tenzij anders vermeld, gecorrigeerd door dr. W. Mordue. De heer R. J. P. Aalpol (Pudoc) was behulpzaam bij het uitgeven van dit proefschrift.

Tenslotte dank ik alle medewerkers van het Laboratorium voor Entomologie voor hun interesse en vriendschap.

Nijmegen,
januari 1975

Ton Bartelink

Contents

List of abbreviations and symbols

ADP	adenosine 5′-diphosphate
AMP	adenosine 5′-monophosphate
ATP	adenosine 5′-triphosphate
Ci	curie ($3.7 \times 10^{10} s^{-1}$)
DNA	deoxyribonucleic acid
d.p.m.	disintegrations/min
EDTA	ethylenediaminetetra-acetate
EGTA	ethanedioxybis(ethylamine)tetra-acetate
g	acceleration due to gravity ($981\ cm \cdot s^{-2}$)
M	molar
mol	mole
NAD	nicotinamide-adenine dinucleotide
NAD^+	oxidized nicotinamide-adenine dinucleotide
p	probability
Pi	orthophosphate (inorganic)
rev.	revolutions
RNA	ribonucleic acid
S.D.	standard deviation
S.E.M.	standard error of estimate of mean value
spec.	species
Tricine	*N*-tris(hydroxymethyl)methylglycine
Tris	tris(hydroxymethyl)methylamine
tRNA	transfer ribonucleic acid
U	uniformly labelled
v/v	volume/volume
w/v	weight/volume

List of enzymes

Trivial name	Systematic name*	Number*
Acid phosphatase	Orthophosphoric-monoester phosphohydrolase (acid optimum)	3.1.3.2
ATPase	ATP phosphohydrolase	3.6.1.3 (formerly 3.6.1.4)
Cytochrome *c* oxidase	Ferrocytochrome *c*: oxygen oxidoreductase	1.9.3.1
α-Glycerophosphate dehydrogenase	*sn*-Glycerol-3-phosphate: NAD^+ 2-oxidoreductase	1.1.1.8
Lactate dehydrogenase	L-Lactate: NAD^+ oxidoreductase	1.1.1.27
Nagarse proteinase	—	3.4.21.14
Pyruvate kinase	ATP: pyruvate 2-*O*-phosphotransferase	2.7.1.40
Ribonuclease	Ribonucleate 3′-pyrimidino-oligonucleotidohydrolase (formerly	3.1.4.22 2.7.7.16)
Succinate dehydrogenase	Succinate: ferricytochrome *c* oxidoreductase	1.3.99.1

* According to the recommendations of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry.

General introduction

In the Colorado beetle (*Leptinotarsa decemlineata* Say) the structural state of the flight muscles and their mitochondria is under photoperiodic control. Beetles reared at long photoperiods show marked development of the flight muscles following adult emergence. This development, in which there is the formation of an extensive sarcosomal system, is completed within two weeks (de Kort, 1969; de Kort & Bartelink, 1972; de Kort *et al.*, 1973). In animals reared under short-day conditions flight-muscle development is initiated, but stops precociously and the muscles soon undergo pronounced atrophy. The onset of the degeneration process is marked by a decrease in the activities of several mitochondrial enzymes (Stegwee *et al.*, 1963; Stegwee, 1964; de Kort, 1969; de Kort & Bartelink, 1972).

Long-day beetles exhibit feeding and reproductive behaviour, but short photoperiods induce diapause: the critical photoperiod being about 15 hours (de Wilde, 1969). This response is mediated by the endocrine system (de Wilde, 1968; de Wilde & de Loof, 1973), and enables the insect to survive the unfavourable season. The degeneration of the flight-muscle mitochondria largely accounts for the low rate of oxygen consumption observed during dormancy (de Wilde & Stegwee, 1958; Stegwee, 1964).

The above studies left unanswered which mechanism was responsible for the cessation in mitochondrial formation at short photoperiods. This question is even more intriguing since biogenesis of mitochondria involves the activity of both the nucleo-ergastoplasmic genetic system and the intrinsic mitochondrial machinery of transcription and translation. Part of the mitochondrial proteins are coded for by the nuclear genome and synthesized on cytoplasmic ribosomes, the other proteins are formed within the confines of the organelle itself (Borst, 1972; Kroon *et al.*, 1972; de Vries, 1973; Schatz & Mason, 1974). Thus, it is interesting to find out whether in short-day beetles the extrinsic synthesis of mitochondrial proteins becomes deficient or whether it is the intramitochondrial protein synthesis which is affected. Information on this point may lead to an understanding of the factors regulating the activity of both translation systems.

The present thesis is based on four papers. In Paper I conditions are defined for the assessment *in vivo* of extra- and intramitochondrial contributions to the amino acid incorporation into mitochondrial protein. Paper II deals with the elaboration of an incubation medium to study the incorporation activity of isolated flight-muscle mitochondria *in vitro*. The procedures devised are applied in an investigation (Paper III) of mitochondrial biogenesis in Colorado beetles reared under long-day conditions. Paper III further describes the accumulation in these beetles of mitochondrial cytochromes and some respiratory enzymes. Similar studies conducted with short-day animals are presented in Paper IV. As the juvenile hormone is of particular importance in the photoperiodic response, this paper also includes experiments on the effects of administration of the hormone.

References

Borst, P. (1972). Mitochondrial nucleic acids. Annu. Rev. Biochem. 41, 333–376.

de Kort, C. A. D. (1969). Hormones and the structural and biochemical properties of the flight muscles in the Colorado beetle. Ph. D. Thesis, University of Nijmegen.

de Kort, C. A. D. & Bartelink, A. K. M. (1972). Biochemical maturation of flight muscle mitochondria in the Colorado beetle, *Leptinotarsa decemlineata* Say. Comp. Biochem. Physiol. 43B, 1009–1017.

de Kort, C. A. D., Bartelink, A. K. M. & Schuurmans, R. R. (1973). The significance of L-proline for oxidative metabolism in the flight muscles of the Colorado beetle, *Leptinotarsa decemlineata*. Insect Biochem. 3, 11–17.

de Vries, H. (1973). The protein-synthetic system of rat-liver mitochondria. Its characterization and its response to inhibitors. Ph. D. Thesis, State University of Groningen.

de Wilde, J. (1968). Hormones and diapause. Proc. Third Intern. Congr. Endocrinol. Mexico, D.F. 356–364.

de Wilde, J. (1969). Diapause and seasonal synchronization in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). Symp. Soc. Exp. Biol. 23, 263–284.

de Wilde, J. & de Loof, A. (1973). Reproduction-Endocrine control. In: The Physiology of Insecta (Rockstein, M., ed.), vol. 1, 2nd edn., pp. 97–157. Academic Press, Inc., New York.

de Wilde, J. & Stegwee, D. (1958). Two major effects of the corpus allatum in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). Arch. Néerl. Zool. (Suppl.) 13, 277–289.

Kroon, A. M., Agsteribbe, E. & de Vries, H. (1972). Protein synthesis in mitochondria and chloroplasts. In: The Mechanism of Protein Synthesis and its Regulation (Bosch, L., ed.), pp. 539–582. North-Holland Publishing Co., Amsterdam.

Schatz, G. & Mason, T. L. (1974). The biosynthesis of mitochondrial proteins. Annu. Rev. Biochem. 43, 51–87.

Stegwee, D. (1964). Respiratory chain metabolism in the Colorado potato beetle. II. Respiration and oxidative phosphorylation in 'sarcosomes' from diapausing beetles. J. Insect Physiol. 10, 97–102.

Stegwee, D., Kimmel, E. C., de Boer, J. A. & Henstra, S. (1963). Hormonal control of reversible degeneration of flight muscle in the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera). J. Cell Biol. 19, 519–527.

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Synthesis of Mitochondrial Protein in the Flight Muscles of the Colorado Beetle

DIFFERENTIATION *IN VIVO* BETWEEN EXTRA- AND INTRA-MITOCHONDRIAL CONTRIBUTIONS TO THE AMINO ACID INCORPORATION INTO MITOCHONDRIAL PROTEIN

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By using cycloheximide, an inhibitor of cytoplasmic protein synthesis, conditions were investigated to estimate *in vivo* the extra- and intra-mitochondrial contributions to the synthesis of organelle protein in the flight muscles of Colorado beetles. With 4-day-old beetles about 15% of the [^{14}C]leucine incorporation into mitochondrial protein is resistant to the action of cycloheximide. The incorporation into cytosol protein is inhibited by more than 99.5% with cycloheximide. During the first hour after precursor administration the incorporation into mitochondrial protein proceeds, in both the presence and the absence of cycloheximide, at a more-or-less linear rate with time. The cycloheximide-resistant amino acid incorporation is sensitive to the inhibitor of mitochondrial protein synthesis, chloramphenicol. The uncertainties inherent in the use of cycloheximide were discussed in arriving at the conclusion that about 15% of the mitochondrial protein is formed inside the organelle.

In the past decade our knowledge of the mechanism of mitochondriogenesis has increased considerably. Mitochondria have been shown to contain genetic information and an intrinsic system for protein synthesis (for reviews see Borst, 1972; Kroon *et al.*, 1972). The properties of the mitochondrial DNA, RNA, polymerases, aminoacyl-tRNA synthetases, ribosomes and protein factors differ from those of the nuclear and cytoplasmic counterparts. The response of mitochondria to antibiotics exemplifies this. Mitochondrial protein synthesis is impaired by chloramphenicol and carbomycin, agents that also interfere with bacterial ribosome functioning but that do not have any effect on the cytoplasmic ribosomes of eukaryotes. Cycloheximide, on the contrary, inhibits protein synthesis by cytoplasmic ribosomes without affecting mitochondrial and bacterial ribosomes.

Besides the intrinsic genetic system there is also an extramitochondrial contribution to the biogenesis of mitochondria. The information content of mitochondrial DNA is far from sufficient to code for all mitochondrial proteins. Most are coded by nuclear genes, synthesized on cytoplasmic ribosomes and subsequently transferred to the mitochondria. With regard to cytochrome *c* this chain of events is now well documented (Sherman & Stewart, 1971).

Mitochondriogenesis thus comprises the assembly of proteins synthesized at different locations in the cell. Particularly with respect to the regulation of the two translation systems our knowledge is very incomplete. The series of studies we started on mitochondria in developing flight muscles of the Colorado beetle is associated with this.

The development of flight muscles in the Colorado beetle occurs mainly during the adult stage. This development involves a 50-fold increase in mitochondrial mass. As a result 14 days after the imaginal moult the mitochondria occupy about one-third of the muscle volume (de Kort, 1969). However, complete development only takes place if the beetles are reared at long photoperiods. When the animals are bred under short-day conditions, a treatment which induces diapause (de Wilde *et al.*, 1959), the development of the flight muscles and their mitochondria ceases soon after the moult. This arrest is followed by a complete degeneration (Stegwee, 1964; de Kort, 1969; de Kort & Bartelink, 1972). From experiments of de Kort (1969) it is evident that the photoperiodic effects on flight-muscle development are mediated by the endocrine system. These observations suggest that flight-muscle mitochondria of the Colorado beetle are an interesting object for studying mitochondriogenesis. The usefulness of insect flight-muscle mitochondria for such studies has been demonstrated (Kleinow *et al.*, 1971).

The present paper deals with experiments to work out conditions for determinations of extra- and intramitochondrial contributions *in vivo* to the synthesis of mitochondrial protein in the flight muscles. This was

accomplished by making use of the above mentioned differential sensitivity of cytoplasmic and mitochondrial ribosomes to cycloheximide.

Materials and Methods

Materials

Animals. Adults of the Colorado beetle (*Leptinotarsa decemlineata* Say) were obtained from the internal laboratory breeding stock. The beetles were reared at 25°C on fresh potato foliage as described by de Wilde (1957) and de Kort (1969). The daily length of the photophase amounted to 18h (long photoperiod). Unless otherwise stated the experiments were performed with 16h-starved beetles 4 days after adult emergence (the young adults emerge from the soil at the second day of the imaginal stage).

Chemicals. From Sigma Chemical Co., St. Louis, Mo., U.S.A., the following were purchased: cycloheximide, D(−)-*threo*-chloramphenicol, bovine serum albumin (fraction V powder), yeast RNA (type XI) and fish sperm DNA (type VI). L-[U-^{14}C]Leucine (331mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Nagarse was obtained from Teikoku Chemical Industry Co. Ltd., Higashi-Ku, Osaka, Japan, and succinate, cytochrome *c*, ATP and 4-nitrophenyl phosphate were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Oligomycin was a gift of Professor J. Dekker, Laboratory of Phytopathology, Agricultural University, Wageningen, The Netherlands. All other chemicals were of analytical-reagent grade. The solutions were prepared in deionized distilled water.

Methods

Injection of antibiotics and radioisotope. Antibiotics and L-[^{14}C]leucine, dissolved in Ringer solution (Ephrussi & Beadle, 1936), were injected abdominally as described by Schooneveld (1970), each beetle receiving 1μl of freshly prepared antibiotic solution and 0.1μCi of [^{14}C]leucine (331mCi/mmol) in a volume of 2μl. The 50μl Hamilton 705 SN micro-syringes (Micromesure N.V., The Hague, The Netherlands) used were fitted with a 31-gauge needle and operated by means of a Hamilton PB600-1 repeating dispenser. This device allows the delivery of microlitre quantities with a reproducibility of ±1%.

Preparation of subcellular fractions. At specified times after the injections the beetles, kept at 25°C, were decapitated and freed from elytra, wings, legs and abdomens. The thoraces of six beetles (three females and three males) treated identically were bisected and placed in 6ml of a preparation medium containing 0.25M-sucrose, 5mM-Tris–HCl buffer, pH7.4 at 20°C, 1mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate] and 10mM-L-[^{12}C]leucine. After the addition of 1.4mg of bacterial proteinase (Nagarse), dissolved in 1ml of preparation medium, the thoraces were briefly homogenized in a Dounce homogenizer (two strokes of the Teflon pestle). The suspension was incubated for 15min at 0°C, after which the homogenization was repeated. The homogenate was filtered through four layers of muslin and diluted with 10ml of preparation medium. The mitochondria were sedimented by centrifugation at 6000*g* for 5min in a Sorvall SS-1 centrifuge. After decantation the centrifuge tubes were cleaned with paper tissue and the 'fluffy' layer was removed by gentle agitation with a small volume of medium. The pellet was suspended in 8ml of preparation medium and re-centrifuged. The sedimented mitochondria were finally suspended in 1.2ml of preparation medium. The whole procedure, which is an adaptation of the method of Chappell & Hansford (1969), was performed at 0–4°C.

For the preparation of the 100000*g* supernatant the six thoraces were homogenized, with omission of Nagarse, in 7ml of preparation medium by four strokes of the Dounce homogenizer. The homogenate was passed through four layers of muslin and centrifuged for 5min at 6000*g*. The supernatant fluid was then centrifuged in a Christ 9530 rotor at 100000*g* during 60min. The resulting supernatant was carefully collected with the aid of a pipette. This preparation procedure was also carried out at 0–4°C.

Determination of radioactivity. Portions of the mitochondrial suspension or 100000*g* supernatant were added to equal volumes of cold 10% (w/v) trichloroacetic acid, containing 10mM-L-[^{12}C]leucine. After 10min the precipitates were recovered by centrifugation and then washed as described by Hawley & Greenawalt (1970). The final sediments were dissolved in 1M-NaOH. To 1.0ml of the solutions, 0.1ml of 11M-HCl and 10ml of toluene–Triton X-100 (2:1, v/v) liquid scintillant was added, the toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene (cf. Madsen, 1969). The samples were counted for radioactivity with a Nuclear–Chicago Mark I liquid-scintillation counter. The counting efficiency was 79–81%.

Determinations of enzyme activity. The activity of succinate dehydrogenase (EC 1.3.99.1) was determined spectrophotometrically as described previously (de Kort & Bartelink, 1972). ATPase* activity (EC 3.6.1.4) was measured in a medium containing 75mM-KCl, 50mM-sucrose, 50mM-Tris–acetate buffer, pH7.4, 1mM-EDTA, 6mM-$MgCl_2$ and 5mM-ATP. After a 15min incubation period the reaction was terminated by the addition of 10% (w/v) trichloroacetic acid. The amount of liberated phosphate was

* Abbreviation: ATPase, adenosine triphosphatase.

determined by the method of Fiske & SubbaRow (1925). The activity of acid phosphatase (EC 3.1.3.2) was measured with 4-nitrophenyl phosphate as a substrate. The reaction medium contained 50mM-sodium citrate buffer, pH4.8, 0.1% (v/v) Triton X-100 and 5.5mM-4-nitrophenyl phosphate. After incubation for 30min a double volume of 0.2M-NaOH was added and the extinction read at 405nm. The amount of 4-nitrophenol released was determined from a standard curve. All enzyme assays were performed at 25°C.

Determinations of protein, RNA and DNA. Protein content of the solutions in 1M-NaOH was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Samples for determinations of RNA and DNA were prepared by adding equal volumes of cold 0.4M-$HClO_4$ and washing the resulting precipitates as described by Tanguay & Chaudhary (1971). The final residues were extracted with 0.5M-$HClO_4$ at 90°C for 60 min. RNA content was measured in the extracts by the orcinol method as described by Schneider (1957), with yeast RNA as the standard. DNA was determined by the diphenylamine test (Burton, 1956). Fish sperm DNA served as the standard. Corrections for deoxyribose destruction during the extraction were made as indicated by Løvtrup (1962).

Determination of leucine specific radioactivity. Six thoraces were homogenized in 6ml of cold water with a Potter–Elvehjem (glass–Teflon) homogenizer (A. H. Thomas Co., Philadelphia, Pa., U.S.A.). The free amino acids were extracted from the homogenate and analysed as described by de Zwaan & van Marrewijk (1973). The volumes of the solvents used during the extraction procedure were adjusted to the small amount of material present. The ethanol residue was discarded and the second chloroform treatment as well as the fractionation on Amberlite were omitted from the procedure.

Results

Characteristics of the mitochondrial fraction from flight muscles

Some biochemical characteristics of mitochondrial preparations from flight muscles of the Colorado beetle are listed in Table 1. Since in the preparation procedure a proteinase was used, it was impossible to estimate in the usual way the recoveries of the enzymes from the homogenate. We therefore chose the following procedure. The thoraces of 60 beetles were longitudinally bisected and the halves divided between two glass mortars. The thorax parts in the first mortar were homogenized by a Potter–Elvehjem-type Teflon pestle (A. H. Thomas Co.) in 35ml of preparation medium without added leucine. The motor-driven pestle rotated at 170rev./min (three periods of 30s). The resulting homogenate was filtered through one layer of nylon gauze. The preparation was performed at 0–4°C. The mitochondria were isolated from the thorax halves in the second mortar as described in the Materials and Methods section, except that the medium without added leucine was used and the volumes were quintupled. For the calculation of the recovery values, the total enzyme activities and DNA and RNA contents of the mitochondrial preparation were compared with those of the filtered homogenate.

The recovery of the mitochondrial inner-membrane enzyme succinate dehydrogenase indicates that the preparation procedure yielded about 50% of the mitochondria present in the thoraces. The ATPase activity in the mitochondrial preparations showed a high sensitivity for the inhibitor oligomycin. It can

Table 1. *Characteristics of mitochondrial preparations from flight muscles*

The reported experiments were carried out on 12-day-old beetles, which contain full-grown flight muscles with an extensive sarcosomal system. For further details see the text. Specific activity is expressed as μmol of substrate/h per mg of protein and content as μg/mg of protein.

	Recovery (% of homogenate)		Specific activity or content	
	Expt. a	Expt. b	Expt. a	Expt. b
Succinate dehydrogenase	43	54	14.8	15.8
ATPase	17	10	9.4	7.2
+oligomycin*			0.6	0.7
Acid phosphatase	7.5	6.9	0.40	0.36
DNA	8.5	9.2	5.4	5.2
RNA	4.1	5.0	20.1	18.0
Protein	12	13		

* 10–20μg of oligomycin/mg of protein.

be deduced from the recovery of acid phosphatase activity that the preparations were contaminated with lysosomes. The observed values of the DNA and RNA contents of the mitochondrial fraction greatly exceed those reported for purified preparations of mature flight-muscle mitochondria from the blowfly *Lucilia cuprina*, which contained 1.4μg of DNA and 5μg of RNA per mg of protein (Lennie *et al.*, 1967). Tanguay & Chaudhary (1971) found 1.2μg of DNA per mg of protein in mitochondria isolated from full-grown flight muscles of the locust *Schistocerca gregaria*, and Kleinow & Neupert (1970) estimated with *Locusta migratoria* 4.6μg of RNA per mg of mitochondrial protein. Therefore the possibility of contamination of our mitochondrial fraction by nuclei and microsomal fractions is not unlikely. In view of the relatively high nucleic acid to protein ratio usually encountered in such components a contamination in terms of protein is not necessarily disquieting. In the fully developed flight muscles of the Colorado beetle about 90% of the volume is occupied by myofibrils and mitochondria (de Kort, 1969). Nuclei, tracheoles, sarcoplasmic reticulum, ribosomes, lysosomes etc. make up the remaining volume. Thus despite the high nucleic acid content of the mitochondrial fraction and the retention of acid phosphatase activity, we think that the contamination by non-mitochondrial protein cannot be of a severe character.

Electron-microscopic investigation has shown that the mitochondrial fraction was free from fibrillar material (de Kort & Bartelink, 1972). Data on respiratory and phosphorylating activities of the mitochondria were recorded in previous papers (de Kort & Bartelink, 1972; de Kort *et al.*, 1973).

Incorporation of [^{14}C]leucine into mitochondrial protein

The specific radioactivities of the protein from mitochondrial fractions isolated at different times after the injection of [^{14}C]leucine are shown in Fig. 1. During the first hour the [^{14}C]leucine incorporation follows a more-or-less linear course. Later on the incorporation rate declines. The incorporation of [^{14}C]leucine into the protein of the other fractions that are obtained during the preparation of mitochondria is shown in Table 2. When considering the recovery values for radioactivity and protein one should bear in mind the use of Nagarse.

Effects of antibiotics on [^{14}C]leucine incorporation

In Fig. 2 the effects of different amounts of cycloheximide on the [^{14}C]leucine incorporation into mitochondrial and 100000*g* supernatant protein are shown. After the administration of 1μg of this cytoplasmic ribosomal inhibitor per animal the radioactivity incorporated into mitochondrial protein is decreased to about 13% of the control value. An increase in the amount of administered cycloheximide up to 5–10μg per animal does not lead to a further inhibition. These quantities of cycloheximide inhi-

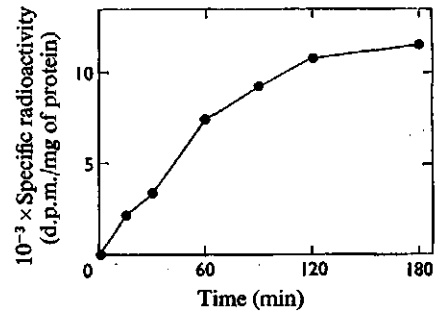

Fig. 1. *Incorporation of [^{14}C]leucine into mitochondrial protein as a function of time*

4-day-old beetles which contain rapidly developing flight muscles were injected with 0.1μCi of [^{14}C]-leucine (331mCi/mmol). At the indicated times the flight-muscle mitochondria were isolated and processed as described in the Materials and Methods section. The animals received no antibiotic injection.

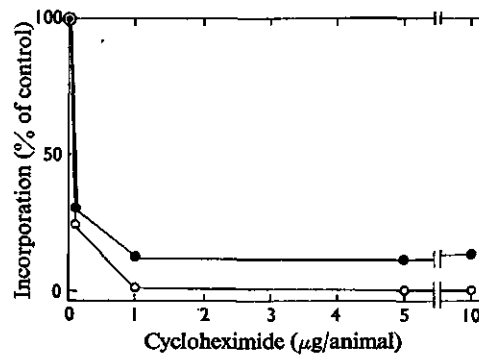

Fig. 2. *Effect of cycloheximide on [^{14}C]leucine incorporation into mitochondrial protein and the protein of the 100000g supernatant*

The antibiotic, dissolved in Ringer solution, was administered 10min before the injection of 0.1μCi of [^{14}C]leucine per animal. After a 30min labelling period the thoraces were fractionated as indicated in the Materials and Methods section. The 100% value for the mitochondrial fraction amounted to 3013d.p.m./mg of protein; the control of the 100000*g* supernatant was 3402d.p.m./mg of protein. ●, Mitochondrial protein; ○, 100000*g* supernatant protein.

Table 2. *Distribution of radioactivity in fractions from flight muscles*

Six beetles each received 0.1 μCi of [^{14}C]leucine. After 30min the thoraces were isolated and fractionated as described in the Materials and Methods section. There was no injection of antibiotics.

Fraction	Specific radioactivity (d.p.m./mg of protein)	Recovery (% of homogenate)	
		Radioactivity	Protein
Homogenate	3191	100	100
Filtrate	3110	93	96
Supernatant and 'fluffy' layer, first centrifugation	2834	57	64
Pellet, first centrifugation	3524	15	13
Supernatant, second centrifugation	2967	0.8	0.9
Mitochondria	3606	14	12

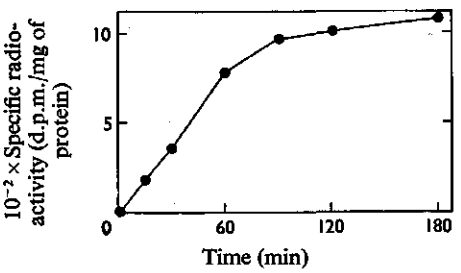

Fig. 3. *Cycloheximide-resistant incorporation of [^{14}C]leucine into mitochondrial protein as a function of time*

Animals were treated with 5μg of cycloheximide 10min before the injection of 0.1 μCi of [^{14}C]leucine. The flight-muscle mitochondria were isolated at the indicated times after the precursor injection. In control experiments (not shown here) it was established that a single dose of 5μg of cycloheximide per animal was sufficient to block cytoplasmic protein synthesis for all 3h.

bited the [^{14}C]leucine incorporation into the protein of the 100000*g* supernatant by more than 99.5%. In six experiments the percentage of [^{14}C]leucine incorporated into mitochondrial protein after injection of 5μg of cycloheximide per animal amounted to an average of 14.6±2.0 (S.D.). The time-course of precursor incorporation into mitochondrial protein resistant to the action of cycloheximide is given in Fig. 3. Here also a nearly linear incorporation rate during the first hour and a decline thereafter occurs. Table 3 shows that after a 30 min pretreatment with cycloheximide the [^{14}C]leucine incorporation into mitochondrial protein resembles the value obtained with a 10min period. Longer periods of pretreatment led to a decreased incorporation.

To establish the intramitochondrial origin of the

Table 3. *Effect of time of pretreatment with cycloheximide on [^{14}C]leucine incorporation into mitochondrial protein*

Beetles were treated with 5μg of cycloheximide at different times before the injection of 0.1μCi of [^{14}C]leucine. The flight-muscle mitochondria were isolated after a 30min labelling period.

Period of pretreatment (min)	Specific radioactivity (d.p.m./mg of protein)	
	Expt. a	Expt. b
10	387	374
30	403	348
60	298	272
120	187	141

protein labelled in the presence of cycloheximide, the effect of chloramphenicol was studied (Fig. 4). Administration of 150μg of chloramphenicol per animal blocked three-quarters of the cycloheximide-resistant incorporation into mitochondrial protein. To obtain this effect, it was necessary to extend the time-interval between antibiotic and precursor injection up to 30 min. At shorter time-intervals the inhibition by chloramphenicol was less pronounced (not shown). The possibility that the inhibitory effect of chloramphenicol was due to an interference with oxidative metabolism (Freeman, 1970) is not likely, because the antibiotic caused no inhibition of [^{14}C]-leucine incorporation into the 100000*g* supernatant protein. The specific radioactivities of the protein after administration of 0, 50, 100 and 150μg of chloramphenicol were in Expt. a 2903, 2822, 2932 and 3042d.p.m./mg of protein respectively and in Expt. b 3480, 2632, 3536 and 3366d.p.m./mg of protein. In these experiments beetles were treated with chloramphenicol 30min before the injection of 0.1μCi of

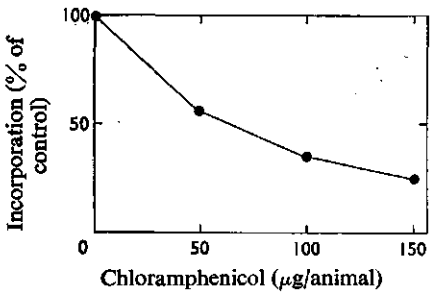

Fig. 4. *Effect of chloramphenicol on cycloheximide-resistant incorporation of* [^{14}C]*leucine into mitochondrial protein*

Beetles were treated with 5μg of cycloheximide and different amounts of chloramphenicol 30min before the injection of 0.1μCi of [^{14}C]leucine. After another 30min the flight-muscle mitochondria were isolated. The antibiotics were dissolved in ethanol. The 100% value amounted to 327d.p.m./mg of protein.

[^{14}C]leucine; after another 30min the thoraces were fractionated to yield the 100000*g* supernatant. Owing to the strong aerobic character of energy conservation in insect flight muscles (Sacktor, 1970), an interference by chloramphenicol on this level would probably also have led to a decreased synthesis of cytosol protein.

Effect of cycloheximide on leucine specific radioactivity

Experiments were undertaken in which a possible influence of cycloheximide on the specific radioactivity of free leucine in the flight muscles was examined. Animals were treated with 5μg of cycloheximide 10min before the injection of 0.1μCi of [^{14}C]leucine. Control animals received Ringer solution. The thoraces were isolated 30min after the injection of label and analysed for leucine specific radioactivity as described in the Materials and Methods section. Cycloheximide administration did not provoke a substantial change in leucine specific radioactivity. In Expt. a the specific radioactivities of the free leucine from control and cycloheximide-treated animals were 0.27 and 0.34μCi/μmol respectively and in Expt. b they were 0.31 and 0.39μCi/μmol. In both treated and control animals there was also some radioactivity associated with alanine, aspartic acid, glutamine, glutamic acid and proline. The specific radioactivities of these amino acids were, however, less than 1% of the corresponding leucine values.

Discussion

Although antibiotics have proved to be powerful tools in studying biogenesis of mitochondria, their use often introduces uncertainties. Our application of cycloheximide for the determination of extra- and intra-mitochondrial contributions to the synthesis of organelle protein therefore needs some comment.

The possibility exists that cycloheximide affects the intracellular leucine pool(s) and thereby the specific radioactivity of the precursor. Sebald *et al.* (1971) reported that in *Neurospora crassa* cells the size of the leucine pool increases eightfold after the addition of cycloheximide. They regard this accumulation as a consequence of the inhibitory action of the antibiotic on protein synthesis while the production of leucine still proceeds. The inability of insects to synthesize leucine and the use of starved animals makes a similar effect of cycloheximide in our experiments improbable. In this context we must also mention the action of cycloheximide on membrane transport. Ellis & MacDonald (1970) found in short-term experiments with discs of beetroot (*Beta vulgaris*) an inhibition of uptake of radioactive leucine. Under certain conditions Evans (1971) noticed in *Euglena gracilis* a rapid inhibitory effect of cycloheximide on the uptake of phenylalanine, glucose and 2,4-dinitrophenol. It is clear that if cycloheximide interferes with membrane transport in the flight muscles, the specific radioactivity of intracellular leucine may be affected. From the results presented in this paper, however, no fall in leucine specific radioactivity after cycloheximide poisoning is evident. The above-mentioned complications apparently do not occur with Colorado beetles.

Garber *et al.* (1973) reported that cycloheximide inhibits respiration and phosphorylation by rat and guinea-pig liver mitochondria oxidizing NAD-linked substrates. A similar observation was made with mitochondria from turnip roots (*Brassica napus*) by Wilson & Moore (1973), who found an inhibition of malate oxidation. Very high concentrations of the antibiotic, however, were required in these studies to obtain a marked effect. As up to 100μg of cycloheximide/ml did not affect amino acid incorporation *in vitro* by isolated Colorado-beetle mitochondria utilizing proline as oxidizable substrate (A. K. M. Bartelink, unpublished work), we consider it unlikely that an interference with energy-transfer mechanisms influenced the present experiments.

Another important problem is posed by the disturbing effect of cycloheximide on the co-operation between the two protein-synthesizing systems. This co-operation is best illustrated in the biogenesis of the enzyme complexes cytochrome oxidase and ATPase (Tzagoloff *et al.*, 1973). Blockage of cytoplasmic protein synthesis may have a secondary influence on the activity of the intrinsic mitochondrial system. Millis & Suyama (1972) treated whole cells of *Tetrahymena pyriformis* for different lengths of time with cycloheximide and then measured *in vitro* the capacity of the isolated mitochondria to incorporate [^{14}C]leucine into protein. It appeared

that even a 5min incubation period with the antibiotic led to a decreased incorporation activity of the isolated mitochondria. After the cells had been treated for 30min more than 50% of the activity was lost. On the other hand Schweyen & Kaudewitz (1970), working with *Saccharomyces cerevisiae*, observed no differences in cycloheximide-resistant labelling of mitochondrial protein *in vivo* when they extended the preincubation with the drug from 1 to 20min. Nor did we find any notable variance in [^{14}C]leucine incorporation after 10 and 30min of cycloheximide pretreatment. A decrease in the incorporation activity only took place when the antibiotic was administered 60min before injection of precursor (Table 3). This result, together with the nearly linear incorporation rate during the first hour (Fig. 3), suggests that under our normal experimental conditions (10min of pretreatment and a 30min labelling period) mitochondrial protein synthesis is not limited by a shortage of products from the cytoplasmic system.

In view of the preceding considerations it seems justifiable to conclude from the present results that in the flight muscles of 4-day-old beetles about 15% of the total mitochondrial protein is synthesized by the intrinsic system. Here it is assumed that the leucine content of the polypeptides formed by both translation systems is similar. Owing to the absence of experimental results, any possible influence by protein pools is not taken into account. Finally, lipoproteins extracted by the organic solvents used during the isolation of radioactive proteins are not included (see Burke & Beattie, 1973).

The above percentage is of similar magnitude to the values reported by others. Schweyen & Kaudewitz (1970) estimated with *Saccharomyces* cells grown in galactose that 8–9% of the mitochondrial protein is synthesized by the cycloheximide-resistant system. Their experiments were performed with a leucine-auxotrophic strain in a medium containing [^{14}C]leucine as the sole amino acid, thereby neatly avoiding the problem of amino acid pools. Using chloramphenicol and erythromycin Kellerman *et al.* (1971) found in glucose-repressed yeast cells a value of 5% for the intrinsic contribution and 13% in derepressed cells. After correcting for the increased leucine pool Sebald *et al.* (1971) calculated the value 8% for *Neurospora*. Hawley & Greenawalt (1970) arrived via other procedures at 15% for the same organism. When Sebald *et al.* (1969) simultaneously injected cycloheximide and ^{14}C-labelled amino acids into locusts (*Locusta migratoria*), they found that the radioactivity incorporated into the postmitochondrial-supernatant protein of the flight muscles was less than 3% of the control, the value for the mitochondrial protein being about 15%. In slices of rat cerebral cortex a similar percentage of the amino acid incorporation into mitochondrial protein was resistant to the cytoplasmic ribosomal inhibitor emetine and sensitive to chloramphenicol (Mahler *et al.*, 1971).

The described technique now makes it possible to estimate the relative contributions of the two protein-synthesizing systems to mitochondriogenesis at different stages during flight-muscle development and degeneration.

We are indebted to Dr. A. M. Kroon for critical discussions. Thanks are also due to Professor J. de Wilde for stimulating interest. The amino acid analyses were kindly performed by Dr. W. J. A. van Marrewijk and Mr. H. J. L. Ravenstein (Laboratory of Chemical Animal Physiology, State University of Utrecht, Utrecht, The Netherlands). Miss C. Peck corrected the English text. This work was supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

Borst, P. (1972) *Annu. Rev. Biochem.* **41**, 333–376
Burke, J. P. & Beattie, D. S. (1973) *Biochem. Biophys. Res. Commun.* **51**, 349–356
Burton, K. (1956) *Biochem. J.* **62**, 315–323
Chappell, J. B. & Hansford, R. G. (1969) in *Subcellular Components: Preparation and Fractionation* (Birnie, G. D. & Fox, S. M., eds.), pp. 43–56, Butterworths, London
de Kort, C. A. D. (1969) Ph.D. Thesis, University of Nijmegen
de Kort, C. A. D. & Bartelink, A. K. M. (1972) *Comp. Biochem. Physiol.* **43B**, 1009–1017
de Kort, C. A. D., Bartelink, A. K. M. & Schuurmans, R. R. (1973) *Insect Biochem.* **3**, 11–17
de Wilde, J. (1957) *Z. Pflanzenk. Pflanzenpathol. Pflanzenschutz* **64**, 589–593
de Wilde, J., Duintjer, C. S. & Mook, L. (1959) *J. Insect Physiol.* **3**, 75–85
de Zwaan, A. & van Marrewijk, W. J. A. (1973) *Comp. Biochem. Physiol.* **44B**, 429–439
Ellis, R. J. & MacDonald, I. R. (1970) *Plant Physiol.* **46**, 227–232
Ephrussi, B. & Beadle, G. W. (1936) *Amer. Natur.* **70**, 218–225
Evans, W. R. (1971) *J. Biol. Chem.* **246**, 6144–6151
Fiske, C. H. & SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
Freeman, K. B. (1970) *Can. J. Biochem.* **48**, 469–478
Garber, A. J., Jomain-Baum, M., Salganicoff, L., Farber, E. & Hanson, R. W. (1973) *J. Biol. Chem.* **248**, 1530–1535
Hawley, E. S. & Greenawalt, J. W. (1970) *J. Biol. Chem.* **245**, 3574–3583
Kellerman, G. M., Griffiths, D. E., Hansby, J. E., Lamb, A. J. & Linnane, A. W. (1971) in *Autonomy and Biogenesis of Mitochondria and Chloroplasts* (Boardman, N. K., Linnane, A. W. & Smillie, R. M., eds.), pp. 346–359, North-Holland Publishing Co., Amsterdam
Kleinow, W. & Neupert, W. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1205–1214
Kleinow, W., Sebald, W., Neupert, W. & Bücher, Th. (1971) in *Autonomy and Biogenesis of Mitochondria and Chloroplasts* (Boardman, N. K., Linnane, A. W. & Smillie, R. M., eds.), pp. 140–151, North-Holland Publishing Co., Amsterdam

Kroon, A. M., Agsteribbe, E. & de Vries, H. (1972) in *The Mechanism of Protein Synthesis and its Regulation* (Bosch, L., ed.), pp. 539–582, North-Holland Publishing Co., Amsterdam

Lennie, R. W., Gregory, D. W. & Birt, L. M. (1967) *J. Insect Physiol.* **13**, 1745–1756

Løvtrup, S. (1962) *Acta Biochim. Pol.* **9**, 411–424

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Madsen, N. P. (1969) *Anal. Biochem.* **29**, 542–544

Mahler, H. R., Jones, L. R. & Moore, W. J. (1971) *Biochem. Biophys. Res. Commun.* **42**, 384–389

Millis, A. J. T. & Suyama, Y. (1972) *J. Biol. Chem.* **247**, 4063–4073

Sacktor, B. (1970) *Advan. Insect Physiol.* **7**, 267–347

Schneider, W. C. (1957) *Methods Enzymol.* **3**, 680–684

Schooneveld, H. (1970) *Neth. J. Zool.* **20**, 151–237

Schweyen, R. & Kaudewitz, F. (1970) *Biochem. Biophys. Res. Commun.* **38**, 728–735

Sebald, W., Hofstötter, Th., Hacker, D. & Bücher, Th. (1969) *FEBS Lett.* **2**, 177–180

Sebald, W., Birkmayer, G. D., Schwab, A. J. & Weiss, H. (1971) in *Autonomy and Biogenesis of Mitochondria and Chloroplasts* (Boardman, N. K., Linnane, A. W. & Smillie, R. M., eds.), pp. 339–345, North-Holland Publishing Co., Amsterdam

Sherman, F. & Stewart, J. W. (1971) *Annu. Rev. Genet.* **5**, 257–296

Stegwee, D. (1964) *J. Insect Physiol.* **10**, 97–102

Tanguay, R. & Chaudhary, K. D. (1971) *Can. J. Biochem.* **49**, 357–367

Tzagoloff, A., Rubin, M. S. & Sierra, M. F. (1973) *Biochim. Biophys. Acta* **301**, 71–104

Wilson, S. B. & Moore, A. L. (1973) *Biochim. Biophys. Acta* **292**, 603–610

Biochem. J. (1973) **136**, 795–802
Printed in Great Britain

Synthesis of Mitochondrial Protein in the Flight Muscles of the Colorado Beetle

DIFFERENTIATION *IN VIVO* BETWEEN EXTRA- AND INTRA-MITOCHONDRIAL CONTRIBUTIONS TO THE AMINO ACID INCORPORATION INTO MITOCHONDRIAL PROTEIN

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By using cycloheximide, an inhibitor of cytoplasmic protein synthesis, conditions were investigated to estimate *in vivo* the extra- and intra-mitochondrial contributions to the synthesis of organelle protein in the flight muscles of Colorado beetles. With 4-day-old beetles about 15% of the [^{14}C]leucine incorporation into mitochondrial protein is resistant to the action of cycloheximide. The incorporation into cytosol protein is inhibited by more than 99.5% with cycloheximide. During the first hour after precursor administration the incorporation into mitochondrial protein proceeds, in both the presence and the absence of cycloheximide, at a more-or-less linear rate with time. The cycloheximide-resistant amino acid incorporation is sensitive to the inhibitor of mitochondrial protein synthesis, chloramphenicol. The uncertainties inherent in the use of cycloheximide were discussed in arriving at the conclusion that about 15% of the mitochondrial protein is formed inside the organelle.

In the past decade our knowledge of the mechanism of mitochondriogenesis has increased considerably. Mitochondria have been shown to contain genetic information and an intrinsic system for protein synthesis (for reviews see Borst, 1972; Kroon *et al.*, 1972). The properties of the mitochondrial DNA, RNA, polymerases, aminoacyl-tRNA synthetases, ribosomes and protein factors differ from those of the nuclear and cytoplasmic counterparts. The response of mitochondria to antibiotics exemplifies this. Mitochondrial protein synthesis is impaired by chloramphenicol and carbomycin, agents that also interfere with bacterial ribosome functioning but that do not have any effect on the cytoplasmic ribosomes of eukaryotes. Cycloheximide, on the contrary, inhibits protein synthesis by cytoplasmic ribosomes without affecting mitochondrial and bacterial ribosomes.

Besides the intrinsic genetic system there is also an extramitochondrial contribution to the biogenesis of mitochondria. The information content of mitochondrial DNA is far from sufficient to code for all mitochondrial proteins. Most are coded by nuclear genes, synthesized on cytoplasmic ribosomes and subsequently transferred to the mitochondria. With regard to cytochrome *c* this chain of events is now well documented (Sherman & Stewart, 1971).

Mitochondriogenesis thus comprises the assembly of proteins synthesized at different locations in the cell. Particularly with respect to the regulation of the two translation systems our knowledge is very incomplete. The series of studies we started on mitochondria in developing flight muscles of the Colorado beetle is associated with this.

The development of flight muscles in the Colorado beetle occurs mainly during the adult stage. This development involves a 50-fold increase in mitochondrial mass. As a result 14 days after the imaginal moult the mitochondria occupy about one-third of the muscle volume (de Kort, 1969). However, complete development only takes place if the beetles are reared at long photoperiods. When the animals are bred under short-day conditions, a treatment which induces diapause (de Wilde *et al.*, 1959), the development of the flight muscles and their mitochondria ceases soon after the moult. This arrest is followed by a complete degeneration (Stegwee, 1964; de Kort, 1969; de Kort & Bartelink, 1972). From experiments of de Kort (1969) it is evident that the photoperiodic effects on flight-muscle development are mediated by the endocrine system. These observations suggest that flight-muscle mitochondria of the Colorado beetle are an interesting object for studying mitochondriogenesis. The usefulness of insect flight-muscle mitochondria for such studies has been demonstrated (Kleinow *et al.*, 1971).

The present paper deals with experiments to work out conditions for determinations of extra- and intramitochondrial contributions *in vivo* to the synthesis of mitochondrial protein in the flight muscles. This was

accomplished by making use of the above mentioned differential sensitivity of cytoplasmic and mitochondrial ribosomes to cycloheximide.

Materials and Methods

Materials

Animals. Adults of the Colorado beetle (*Leptinotarsa decemlineata* Say) were obtained from the internal laboratory breeding stock. The beetles were reared at 25°C on fresh potato foliage as described by de Wilde (1957) and de Kort (1969). The daily length of the photophase amounted to 18h (long photoperiod). Unless otherwise stated the experiments were performed with 16h-starved beetles 4 days after adult emergence (the young adults emerge from the soil at the second day of the imaginal stage).

Chemicals. From Sigma Chemical Co., St. Louis, Mo., U.S.A., the following were purchased: cycloheximide, D(−)-*threo*-chloramphenicol, bovine serum albumin (fraction V powder), yeast RNA (type XI) and fish sperm DNA (type VI). L-[U-^{14}C]Leucine (331mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Nagarse was obtained from Teikoku Chemical Industry Co. Ltd., Higashi-Ku, Osaka, Japan, and succinate, cytochrome *c*, ATP and 4-nitrophenyl phosphate were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Oligomycin was a gift of Professor J. Dekker, Laboratory of Phytopathology, Agricultural University, Wageningen, The Netherlands. All other chemicals were of analytical-reagent grade. The solutions were prepared in deionized distilled water.

Methods

Injection of antibiotics and radioisotope. Antibiotics and L-[^{14}C]leucine, dissolved in Ringer solution (Ephrussi & Beadle, 1936), were injected abdominally as described by Schooneveld (1970), each beetle receiving 1μl of freshly prepared antibiotic solution and 0.1μCi of [^{14}C]leucine (331mCi/mmol) in a volume of 2μl. The 50μl Hamilton 705 SN micro-syringes (Micromesure N.V., The Hague, The Netherlands) used were fitted with a 31-gauge needle and operated by means of a Hamilton PB600-1 repeating dispenser. This device allows the delivery of microlitre quantities with a reproducibility of ±1%.

Preparation of subcellular fractions. At specified times after the injections the beetles, kept at 25°C, were decapitated and freed from elytra, wings, legs and abdomens. The thoraces of six beetles (three females and three males) treated identically were bisected and placed in 6ml of a preparation medium containing 0.25M-sucrose, 5mM-Tris–HCl buffer, pH7.4 at 20°C, 1mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate] and 10mM-L-[^{12}C]leucine. After the addition of 1.4mg of bacterial proteinase (Nagarse), dissolved in 1ml of preparation medium, the thoraces were briefly homogenized in a Dounce homogenizer (two strokes of the Teflon pestle). The suspension was incubated for 15min at 0°C, after which the homogenization was repeated. The homogenate was filtered through four layers of muslin and diluted with 10ml of preparation medium. The mitochondria were sedimented by centrifugation at 6000*g* for 5min in a Sorvall SS-1 centrifuge. After decantation the centrifuge tubes were cleaned with paper tissue and the 'fluffy' layer was removed by gentle agitation with a small volume of medium. The pellet was suspended in 8ml of preparation medium and re-centrifuged. The sedimented mitochondria were finally suspended in 1.2ml of preparation medium. The whole procedure, which is an adaptation of the method of Chappell & Hansford (1969), was performed at 0–4°C.

For the preparation of the 100000*g* supernatant the six thoraces were homogenized, with omission of Nagarse, in 7ml of preparation medium by four strokes of the Dounce homogenizer. The homogenate was passed through four layers of muslin and centrifuged for 5min at 6000*g*. The supernatant fluid was then centrifuged in a Christ 9530 rotor at 100000*g* during 60min. The resulting supernatant was carefully collected with the aid of a pipette. This preparation procedure was also carried out at 0–4°C.

Determination of radioactivity. Portions of the mitochondrial suspension or 100000*g* supernatant were added to equal volumes of cold 10% (w/v) trichloroacetic acid, containing 10mM-L-[^{12}C]leucine. After 10min the precipitates were recovered by centrifugation and then washed as described by Hawley & Greenawalt (1970). The final sediments were dissolved in 1M-NaOH. To 1.0ml of the solutions, 0.1ml of 11M-HCl and 10ml of toluene–Triton X-100 (2:1, v/v) liquid scintillant was added, the toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene (cf. Madsen, 1969). The samples were counted for radioactivity with a Nuclear–Chicago Mark I liquid-scintillation counter. The counting efficiency was 79–81%.

Determinations of enzyme activity. The activity of succinate dehydrogenase (EC 1.3.99.1) was determined spectrophotometrically as described previously (de Kort & Bartelink, 1972). ATPase* activity (EC 3.6.1.4) was measured in a medium containing 75mM-KCl, 50mM-sucrose, 50mM-Tris–acetate buffer, pH7.4, 1mM-EDTA, 6mM-$MgCl_2$ and 5mM-ATP. After a 15min incubation period the reaction was terminated by the addition of 10% (w/v) trichloroacetic acid. The amount of liberated phosphate was

* Abbreviation: ATPase, adenosine triphosphatase.

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AMINO-ACID INCORPORATION INTO PROTEIN BY ISOLATED FLIGHT MUSCLE MITOCHONDRIA FROM THE COLORADO BEETLE

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ABSTRACT

Mitochondria isolated from the flight muscles of Colorado beetles (*Leptinotarsa decemlineata*) incorporate [^{14}C]leucine into protein. Proline was found to be the most effective oxidizable substrate tested, in supporting the incorporation activity. Optimum [^{14}C]leucine incorporation also required appropriate concentrations of Mg^{2+}, phosphate, ADP, H^+ and a synthetic amino-acid mixture (*minus* leucine). The incorporation activity was inhibited by D-chloramphenicol, oxytetracycline, ethidium bromide, 2,4-dinitrophenol, and oligomycin. Erythromycin and cycloheximide had no effect. Evidence is presented that bacterial contamination of the mitochondrial preparations did not contribute to the results obtained.

ISOLATED mitochondria possess the intrinsic ability to incorporate amino acids into protein (for reviews *see* Beattie, 1971; Kroon *et al.*, 1972). Flight muscle mitochondria from insects constitute no exception in this respect. On account of the rapid proliferation and growth during adult development, these mitochondria are even very advantageous for studying mitochondrial protein synthesis *in vitro* (Bronsert and Neupert, 1966; Chan and Richardson, 1969; Williams and Birt, 1971a,b). Thus, as part of investigations on mitochondriogenesis in Colorado beetle flight muscles (de Kort and Bartelink, 1972; Bartelink and de Kort, 1973), we initiated incorporation studies *in vitro*.

In this paper experiments are described to define optimum conditions for amino-acid incorporation by isolated flight muscle mitochondria. In addition we tested the effects of various inhibitors on the observed incorporation activity and investigated whether the bacteria contaminating the mitochondrial preparations contributed significantly to the incorporation rate.

MATERIALS AND METHODS

ANIMALS

Colorado beetles (*Leptinotarsa decemlineata* Say) were reared under long-day conditions as described previously (Bartelink and de Kort, 1973). In the experiments beetles starved overnight were used 4 days after adult emergence. For 32 hr preceding the starvation period the animals received ampicillin-treated foliage (*cf.* Chan and Richardson, 1969): fresh potato shoots (100 g.) were allowed to absorb a solution of 100 mg. of ampicillin in water and were then offered to the beetles.

PREPARATION OF MITOCHONDRIA

Beetles were rinsed with water, wiped, and kept at 0 to 4° C. After 30 min the animals were

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cleaned with 96 per cent ethanol and then dissected with sterilized scissors and gloves. All further equipment was also sterile. Glassware, centrifuge tubes, instruments, etc. were autoclaved or dry heated. Solutions were passed through a membrane filter (pore size 0·2 μm.; Sartorius-Membranfilter GmbH, Göttingen, West Germany, and Gelman Instrument Company, Ann Arbor, Mich., U.S.A). The mitochondria were prepared from 30 or 60 thoraces according to the method of Chappell and Hansford (1969) as detailed previously (Bartelink and de Kort, 1973). Two modifications were made in the present experiments. The volumes were adjusted to the larger number of thoraces and the composition of the preparation medium was changed into: 0·25 M sucrose, 5 mM Tricine-KOH buffer (pH 7·4), and 1 mM ethyleneglycol-bis-(β-aminoethyl ether)*N,N'*-tetra-acetic acid (EGTA). The final mitochondrial pellet was resuspended in the preparation medium to give 8 to 12 mg. of protein per ml. and used immediately.

Determination of Amino-acid Incorporation

The incorporation activity of the isolated mitochondria was measured at 25° C in an incubation medium containing 0·04 mM L-[U-^{14}C]leucine (10 mCi per mmole), 50 mM sucrose, 50 mM Tricine-KOH buffer, 1 mM disodium-EDTA, 0·1 mM EGTA, 20 μg. ribonuclease per ml., and variable amounts of KCl, $MgCl_2$, potassium phosphate, ADP, oxidizable substrate, and amino-acids. The concentrations of these components, other additions, and the pH are indicated in the legends to Tables and Figs. With the exception of [^{14}C]leucine, the incubation medium was passed through a membrane filter before use. The final reaction volumes were 1·0 ml. and included 0·8 to 1·2 mg. of mitochondrial protein. All incubations were carried out with sterile 13-ml. round-bottom centrifuge tubes in a reciprocal shaking water-bath (290 oscillations per min). The reactions, initiated by the addition of [^{14}C]leucine, were terminated with 1 ml. of cold 10 per cent trichloroacetic acid containing 20 mM L-[^{12}C]leucine. The resulting precipitates were processed and assayed for radioactivity and protein as previously described (Bartelink and de Kort, 1973). Blanks were prepared by adding [^{14}C]leucine after trichloroacetic acid. To facilitate comparison with other studies the results were calculated as pmoles of leucine incorporated per mg. protein, using the specific radioactivity of the added [^{14}C]leucine. Dilution by endogenous amino-acid was not taken into account.

Determination of Bacterial Contamination

Dilutions of mitochondrial preparations were made into sterile 0·5 per cent NaCl and plated in duplicate on nutrient agar (0·5 per cent peptone, 0·5 per cent yeast extract, 0·5 per cent glucose, and 1·2 per cent agar; pH 7·0). After 48 hr. at 25° C the plates were evaluated. Unless otherwise stated the mitochondrial preparations contained less than 7×10^3 bacteria per mg. of protein.

Chemicals

Ampicillin (Penbritin) was obtained from Beecham (Ned.) N.V., Amsterdam, The Netherlands. Ribonuclease (E.C. 2.7.7.16), DL-α-glycerophosphate (disodium salt), non-radioactive L-amino-acids, oxytetracycline hydrochloride, and ethidium bromide were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. The amino-acid mixture (*minus* leucine) was prepared according to Roodyn *et al.* (1961), with a molar ratio for tyrosine of 1·6. Sodium pyruvate was from E. Merck, Darmstadt, W. Germany, and malic acid from Calbiochem, Los Angeles, Calif., U.S.A. Pyruvate kinase (E.C. 2.7.1.40) and sodium salts of phosphoenolpyruvate and ADP were obtained from C. F. Boehringer & Soehne, GmbH, Mannheim, W. Germany. ADP was assayed enzymatically with pyruvate kinase and lactate dehydrogenase (E.C. 1.1.1.27). 2,4-Dinitrophenol was a product of Eastman Kodak Co., Rochester, N.Y., U.S.A. Peptone was from Brocades, Amsterdam, The Netherlands, yeast extract (powder) from Oxoid Ltd., London, U.K., and Bacto-agar from Difco Laboratories, Detroit, Mich., U.S.A. The sources of other chemicals have been described previously (Bartelink and de Kort, 1973).

RESULTS

Bacterial Contamination

Axenic cultures of Colorado beetles could not be obtained because a proper artificial diet is not available at present. Thus, the starting material for the preparation of mito-

chondria, the thoraces, was inevitably contaminated with bacteria. The severance of the intestine during the dissection of the beetles is especially adverse. We, therefore, examined to what extent the bacteria contaminating the mitochondrial preparations contribute to the amino-acid incorporation into protein.

Mitochondrial preparations, isolated under non-sterile conditions from beetles reared on untreated potato foliage, contained in 6 experiments an average of 3×10^4 viable bacteria per mg. of protein (range: $1–6 \times 10^4$). The predominant organism was classified (Breed *et al.*, 1957) as *Streptococcus faecalis*. Two other bacteria, usually encountered, belong to the genera *Achromobacter* and *Pseudomonas*. The effects of extra additions of these bacteria to the incubation medium for amino-acid incorporation were tested and the results are shown in Table 1. It can be seen that at numbers exceeding 10^4 to 10^5 all three bacterial species enhanced the [^{14}C]leucine incorporation into protein and also to the same extent. The data show the necessity to reduce the number of bacteria contaminating the mitochondrial preparations. A combination of feeding the animals ampicillin-treated foliage and a sterile preparation procedure (*see* the Materials and

Table 1.—Effects of Extra Additions of Bacteria on [^{14}C]Leucine Incorporation into Protein by Mitochondrial Preparations from Flight Muscles

Species	Number of Added Bacteria per mg. Protein	[^{14}C]Leucine incorporated (pmoles per mg. protein)
Streptococcus faecalis	—	15·1
	$5{\cdot}0 \times 10^3$	15·7
	$5{\cdot}0 \times 10^4$	16·0
	$5{\cdot}0 \times 10^5$	45·4
	$5{\cdot}0 \times 10^6$	207·8
Achromobacter spec.	—	12·9
	$4{\cdot}2 \times 10^3$	13·2
	$4{\cdot}2 \times 10^4$	14·4
	$4{\cdot}2 \times 10^5$	29·9
	$4{\cdot}2 \times 10^6$	145·7
Pseudomonas spec.	—	12·9
	$2{\cdot}3 \times 10^3$	13·0
	$2{\cdot}3 \times 10^4$	13·7
	$2{\cdot}3 \times 10^5$	22·9
	$2{\cdot}3 \times 10^6$	111·7

Bacteria were grown aerobically at 25° C with reciprocal shaking in a liquid medium containing 0·5 per cent peptone, 0·5 per cent yeast extract, and 0·5 per cent glucose. Logarithmic phase cells were harvested by centrifuging and washed once with preparation medium (*see* the Materials and Methods section), using centrifugation conditions identical to those used during the preparation of mitochondria. The bacteria were resuspended in preparation medium, diluted, and then added to an incubation medium containing mitochondria, 20 mM KCl, 8·5 mM NaCl, 5 mM $MgCl_2$, 20 mM potassium phosphate, 2 mM ADP, 30 mM pyruvate, 30 mM malate, 50 μg. amino-acid mixture per ml., and the basic components listed in the Materials and Methods section. The final pH was 7·4, the incubation time 1 hr. The mitochondrial preparations were derived from beetles fed untreated foliage and were isolated under non-sterile conditions. The intrinsic contamination of these preparations and the number of added bacteria were determined by plating suitable dilutions on nutrient agar. The mitochondrial preparation contained in the experiment with *Streptococcus faecalis* $1{\cdot}9 \times 10^4$ bacteria per mg. protein; in the experiment with *Achromobacter* and *Pseudomonas* $2{\cdot}3 \times 10^4$ bacteria per mg. mitochondrial protein were present.

Methods section) was found to eliminate 80 to 90 per cent of the bacteria. In all further experiments the mitochondrial preparations contained less than 7×10^8 bacteria per mg. of protein, a level at which a significant contribution to the amino-acid incorporation is unlikely.

Optimum Conditions for [^{14}C]Leucine Incorporation into Mitochondrial Protein

The incubation medium, described by Kroon and de Vries (1971) for studying mitochondrial protein synthesis *in vitro*, was taken as the starting-point to define optimum conditions for amino-acid incorporation by isolated flight muscle mitochondria from Colorado beetles. Two modifications were made beforehand. NH_4Cl was omitted from the medium and initially 30 mM pyruvate + 10 mM malate were used as oxidizable substrate.

Varying the concentrations of KCl, $MgCl_2$, potassium phosphate, and ADP in the incubation medium revealed optima at 40 mM, 10 mM, 5 mM, and 2 mM respectively (not shown). The use of these optimum concentrations led only to a minor increase in the [^{14}C]leucine incorporation, at least when compared with the results obtained in testing the effects of various oxidizable substrates (Table 2). All substrates examined stimulated the incorporation activity but, to our surprise, pyruvate + malate was inferior to α-glycerophosphate and succinate. In measurements of respiration rates with Colorado beetle flight muscle mitochondria, the last two substrates yielded much lower values than pyruvate + malate (de Kort and Bartelink, 1972). Proline, which is very rapidly oxidized (de Kort *et al.* 1973), was by far the most effective substrate in supporting [^{14}C] leucine incorporation. The exogenous ATP-generating system used could not replace proline in this respect. The strongly stimulative effect of proline on [^{14}C]leucine incorporation is also shown in Fig. 1, where the dependence on the concentration is depicted. Maximum incorporation was obtained at 10 mM; a further increase in proline concentration was without effect. Fig. 2 illustrates that addition of more amino-acid mixture than the amount used in the former experiments (50 μg. per ml. medium) markedly raised the incorporation rate.

Table 2.—Effects of Oxidizable Substrates and an Exogenous ATP-generating System on [^{14}C]Leucine Incorporation into Protein by Isolated Mitochondria

Additions (final concentrations)	[^{14}C]Leucine incorporated (pmoles per mg. protein)
—	24
Pyruvate (30 mM) + malate (10 mM)	37
α-Glycerophosphate (30 mM)	62
Succinate (30 mM)	77
Proline (30 mM)	271
Phosphoenolpyruvate (5 mM) + pyruvate kinase (20 μg. per ml.)	56

The incubation medium contained 40 mM KCl, 10 mM $MgCl_2$, 5 mM potassium phosphate, 2 mM ADP, 50 μg. amino-acid mixture per ml. and the basic components listed in the Materials and Methods section. The final pH was 7·4, the incubation time 1 hr.

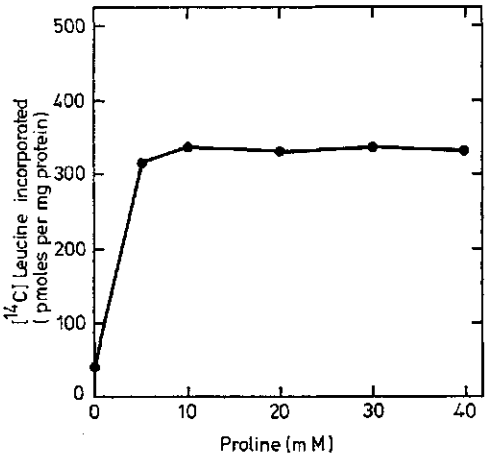

FIG. 1.—Effect of proline concentration on [^{14}C]leucine incorporation into protein by isolated mitochondria. For incubation conditions *see* the legend to Table II.

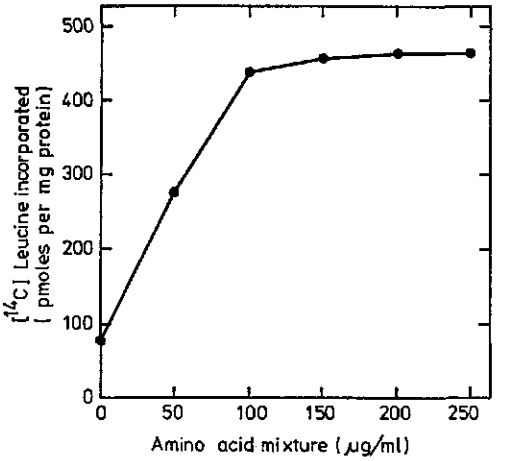

FIG. 2.—Effect of added amino-acid mixture on [^{14}C]leucine incorporation into protein by isolated mitochondria. Besides amino-acid mixture and the basic components listed in the Materials and Methods section, the incubation medium contained 40 mM KCl, 10 mM $MgCl_2$, 5 mM potassium phosphate, 2 mM ADP, and 30 mM proline. The final pH was 7·4, the incubation time 1 hr.

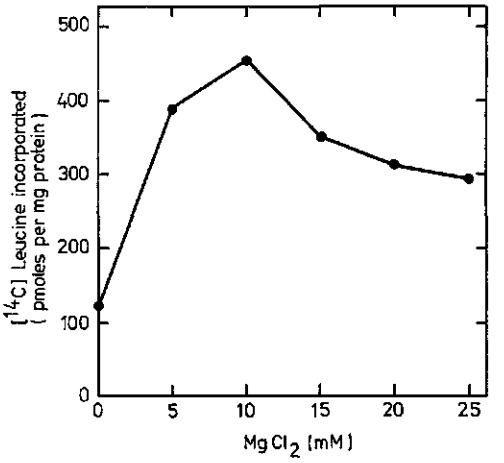

FIG. 3.—Effect of $MgCl_2$ concentration on [^{14}C]leucine incorporation into protein by isolated mitochondria. Besides $MgCl_2$ and the basic components listed in the Materials and Methods section, the incubation medium contained 40 mM KCl, 5 mM potassium phosphate, 2 mM ADP, 30 mM proline, and 200 μg. amino-acid mixture per ml. The final pH was 7·4, the incubation time 1 hr.

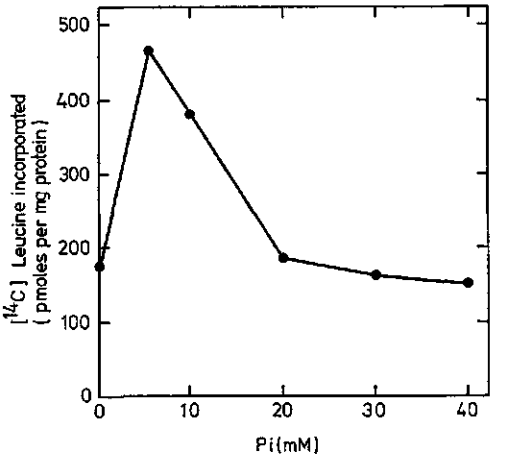

FIG. 4.—Effect of potassium phosphate concentration on [^{14}C]leucine incorporation into protein by isolated mitochondria. In addition to potassium phosphate and the basic components listed in the Materials and Methods section, the incubation medium contained 40 mM KCl, 10 mM $MgCl_2$, 2 mM ADP, 30 mM proline, and 200 μg. amino-acid mixture per ml. The final pH was 7·4, the incubation time 1 hr.

After this substantial improvement of the incubation medium by the use of proline and a higher concentration of the amino-acid mixture we felt it desirable to reinvestigate the dependence of [^{14}C]leucine incorporation on the concentrations of $MgCl_2$, potassium phosphate, and ADP. The results are shown in Figs. 3, 4 and 5. As may be seen, optimum incorporation was still obtained at 10 mM $MgCl_2$, 5 mM phosphate, and 2 mM ADP. The requirements for these components were, however, more pronounced than in the earlier trials.

The influence of pH on the amino-acid incorporation is illustrated in Fig. 6, the

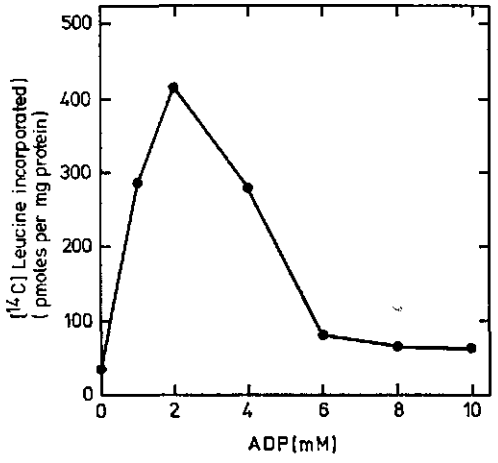

FIG. 5.—Effect of ADP concentration on [^{14}C]leucine incorporation into protein by isolated mitochondria. In addition to ADP and the basic components listed in the Materials and Methods section, 40 mM KCl, 10 mM $MgCl_2$, 5 mM potassium phosphate, 30 mM proline, and 200 μg. amino-acid mixture per ml. were present. The final pH was 7·4, the incubation time 1 hr.

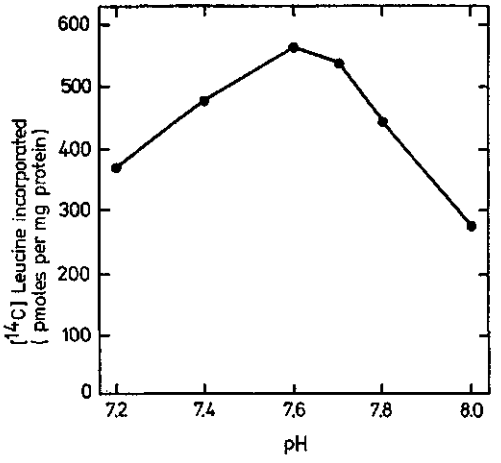

FIG. 6.—Effect of pH on [^{14}C] leucine incorporation into protein by isolated mitochondria. The incubation medium contained 40 mM KCl, 10 mM $MgCl_2$, 5 mM potassium phosphate, 2 mM ADP, 30 mM proline, 200 μg. amino-acid mixture per ml., and the basic components listed in the Materials and Methods section. Incubation time was 1 hr.

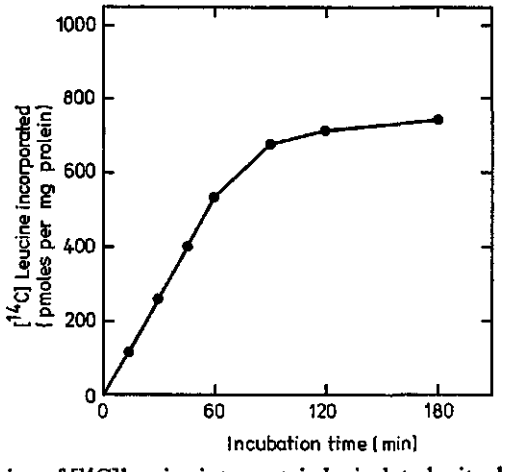

FIG. 7.—Incorporation of [^{14}C]leucine into protein by isolated mitochondria as a function of time. The incubation medium was as described in the legend to Fig. 6. The final pH was 7·6.

optimum pH being about 7·6.

Under the optimum conditions, as evaluated by the above experiments, the rate of precursor incorporation was linear with time during the first hour; thereafter a decline was observed (Fig. 7). Amino-acid incorporation was proportional to mitochondrial protein concentration in the range used in this study. Addition of 0·5 per cent bovine serum albumin to the incubation medium had no effect (not shown).

Effect of Inhibitors on [^{14}C]Leucine Incorporation into Mitochondrial Protein

In Table 3 the effects of various inhibitors on mitochondrial amino-acid incorporation are described. The incorporation activity was sensitive to chloramphenicol and oxytetracycline, two inhibitors of mitochondrial and bacterial protein synthesis (Borst and Grivell, 1971; Pestka, 1971; Kroon and Arendzen, 1972). The macrolide erythromycin (Borst and Grivell, 1971; Kroon and de Vries, 1971; Pestka, 1971; Kroon and Arendzen, 1972), on the other hand, had no effect. Resistance to the cytoribosomal inhibitor cycloheximide (Pestka, 1971; Bartelink and de Kort, 1973) was also observed. Interference with mitochondrial transcription by ethidium bromide (Kroon and de Vries, 1971) resulted in a strong inhibition. 2,4-Dinitropgenol and oligomycin, an uncoupler and inhibitor respectively of oxidative phosphorylation (Slater, 1967), both affected amino-acid incorporation.

Table 3.—Effects of Inhibitors on [^{14}C]Leucine Incorporation into Protein by Isolated Mitochondria

Additions (final concentrations)	[^{14}C]Leucine incorporated (percentage of control)	
D-Chloramphenicol (100 μg. per ml.)	16	(13–18)
Oxytetracycline (100 μg. per ml.)	27	(18–32)
Erythromycin (100 μg. per ml.)	98	(96–100)
Cycloheximide (100 μg. per ml.)	100	(96–103)
Ethidium bromide (2 μg. per ml.)	3	(1–5)
2,4-Dinitrophenol (0·1 mM)	12	(11–14)
Oligomycin (2 μg. per ml.)	5	(4–6)

The incubation medium contained 40 mM KCl, 10 mM $MgCl_2$, 5 mM potassium phosphate, 2 mM ADP, 30 mM proline, 200 μg. amino-acid mixture per ml., 1 per cent ethanol, and the basic components listed in the Materials and Methods section. The final pH was 7·6; the incubation time 30 min. The results shown are the averages of 3 experiments with the range in parentheses. The control values were 237, 262, and 278 pmoles per mg. protein.

DISCUSSION

The results reported in this paper indicate that isolated flight muscle mitochondria from Colorado beetles effect amino-acid incorporation into protein. A substantial contribution by contaminating bacteria to the measured incorporation rate is improbable. In short-term experiments, where the effects of extra additions of three different bacterial species were studied, we only noticed an increase in amino-acid incorporation at numbers exceeding 10^4 to 10^5 bacteria per mg. of mitochondrial protein (Table 1). Beattie *et al.* (1967a) showed that adding 10^4 bacteria to an incorporation system resulted in a slight labelling of protein (< 1 pmole per mg of protein per 30 min). Using *Streptococ-*

cus faecalis, Chan and Richardson (1969) needed 10^5 bacteria to enhance the incorporation. No differences in incorporation activities were observed by Kroon *et al.* (1968) between sterile mitochondria and preparations containing up to 10^5 bacteria per mg. of protein. In view of these data, amino-acid incorporation by mitochondrial preparations from flight muscles in which bacterial contamination was minimized to less than 7×10^3 bacteria has to be considered as an intrinsic activity of the mitochondria (cf. Krymkiewicz and Gonzàlez-Cadavid, 1970). The resistance of the incorporation activity to the macrolide antibiotic erythromycin (Table 3) supports this conclusion.

Flight muscle mitochondria from Colorado beetles oxidize proline at a higher rate than pyruvate, α-glycerophosphate, and Krebs cycle intermediates (de Kort and Bartelink, 1972; de Kort *et al.*, 1973). Amino-acid analysis of thorax extracts revealed that proline accounts for nearly half of the total content of free acidic and neutral amino-acids (de Kort *et al.*, 1973). The present results show that [^{14}C]leucine incorporation into mitochondrial protein is very effectively stimulated by proline (Table 2, Fig. 1). These observations point to a particular significance of the imino-acid for flight muscle metabolism in the Colorado beetle, as already discussed earlier (de Kort *et al.*, 1973). In the context of this study we further want to consider the following. Wheeldon and Lehninger (1966), working with rat liver mitochondria, likewise found a stimulation of [^{14}C] leucine incorporation by proline (*see also* Beattie *et al.*, 1967b; Hamberger *et al.*, 1969; Coote and Work, 1971). From their experiments the authors inferred that proline did not act by serving as an oxidizable substrate, but possibly via an effect on precursor uptake into mitochondria. Williams and Birt (1971b) reported that [^{14}C]leucine incorporation by flight muscle mitochondria from the blowfly *Lucilia cuprina* was enhanced by pyruvate, α-glycerophosphate, and to a lesser extent by proline. In contrast to Wheeldon and Lehninger they attributed the effect of proline in their system to a promotion of oxidative phosphorylation. Our experiments permit no definitive conclusion regarding the importance of both alternatives for the stimulation of [^{14}C]leucine incorporation by proline in the Colorado beetle system. However, the correlation between efficient phosphorylation (de Kort *et al.*, 1973) and a high incorporation rate in the presence of proline, together with the observed inhibition by 2,4-dinitrophenol and oligomycin (Table 3), suggest strongly that the phosphorylation process plays a significant role. It may be added also that stimulative effects of products of proline metabolism (Sacktor, 1970) cannot be excluded.

The requirements for Mg^{2+}, phosphate, and adenine nucleotide to achieve optimum amino-acid incorporation (Figs. 3–5) agree with the results of Burke and Beattie (1972). These workers found maximum incorporation by rat liver mitochondria at 10 mM Mg^{2+}, 5 mM phosphate, and 2 mM ATP (*see also* Wheeldon and Lehninger, 1966). Similarly Bygrave and Kaiser (1969), measuring [^{14}C]serine incorporation into the phospholipids of isolated flight muscle mitochondria from *Locusta migratoria*, needed in a substrate-supported system 10 mM Mg^{2+}, 5 mM phosphate, and 2 mM ADP for optimum incorporation rates. The optimized system, which Bronsert and Neupert (1966) had developed for the same object to study mitochondrial protein synthesis, included 3 mM Mg^{2+}, 4 mM phosphate, and 2 mM ATP. Greater disparity is found in the results which Williams and Birt (1971a, b) obtained with flight muscle mitochondria from *Lucilia*. Amino-acid incorporation into protein was maximum at 18 mM Mg^{2+}, 4 to 5 mM ADP or ATP, 20 mM phosphate in the presence of ADP and 10 mM phosphate with ATP. Insect systems are also not uniform with regard to optimum pH for

amino-acid incorporation. *Locusta* mitochondria show maximum incorporation activity at pH 6·7 to 6·8 (Bronsert and Neupert, 1966), flight muscle mitochondria from the moth *Manduca sexta* at pH 7·2 (Chan and Richardson, 1969), and Colorado beetle mitochondria at about 7·6 (Fig. 6). In the presence of an exogenous ATP-generating system the optimum pH value for *Lucilia* mitochondria was 7·4, with a substrate-supported system at pH 8·0 (Williams and Birt, 1971a).

Because of the large number of reactions involved in the process of amino-acid incorporation by isolated mitochondria, a study of this description cannot provide a rationalization for the observed optimum concentrations. The present study did result in a proper incubation medium for the further investigation of the factors controlling protein synthesis by Colorado beetle mitochondria. The incorporation rates recorded here are high because the mitochondria were isolated from beetles with rapidly developing flight muscles (de Kort, 1969). Mitochondria from full-grown flight muscles show similar incorporation rates as reported for rat liver (Bartelink, A. K. M., unpublished).

The observed inhibitory effects of chloramphenicol, oxytetracycline, ethidium bromide, 2,4-dinitrophenol, and oligomycin (Table 3) fit the results obtained with mitochondria from a variety of organisms (Beattie, 1971; Borst and Grivell, 1971; Kroon and de Vries, 1971; Kroon and Arendzen, 1972; Kroon *et al.*, 1972). As for erythromycin resistance the agreement only extends to intact animal mitochondria (Kroon and de Vries, 1971; Kroon and Arendzen, 1972; Towers *et al.*, 1972; Williams and Birt, 1972); mitochondria from yeast cells are sensitive to the macrolide (Lamb *et al.*, 1968). Amino-acid incorporation by mitochondrial preparations from flight muscles was refractory to ribonuclease and cycloheximide, indicating that microsomal contamination (Bartelink and de Kort, 1973) did not contribute to the activity observed. The above effects of inhibitors are also difficult to reconcile with the view that the observed labelling of protein merely reflects absorption of amino-acids (Hochberg *et al.*, 1972; *see also* Ibrahim *et al.*, 1973).

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REFERENCES

BARTELINK A. K. M. and DE KORT C. A. D. (1973) Synthesis of mitochondrial protein in the flight muscles of the Colorado beetle. Differentiation *in vivo* between extra- and intra-mitochondrial contributions to the amino acid incorporation into mitochondrial protein. *Biochem. J.* **136**, 795–802.

BEATTIE D. S. (1971) The synthesis of mitochondrial proteins. *Sub-Cell. Biochem.* **1**, 1–23.

BEATTIE D. S., BASFORD R. E. and KORITZ S. B. (1967a) Bacterial contamination and amino acid incorporation by isolated mitochondria. *J. biol. Chem.* **242**, 3366–3368.

BEATTIE D. S., BASFORD R. E., and KORITZ S. B. (1967b) The inner membrane as the site of the *in vitro* incorporation of L-[^{14}C]leucine into mitochondrial protein. *Biochemistry* **6**, 3099–3106.

BORST P. and GRIVELL L. A. (1971) Mitochondrial ribosomes. *FEBS Lett.* **13**, 73–88.

BREED R. S., MURRAY E. G. D., and SMITH N. R. (1957) *Bergey's Manual of Determinative Bacteriology*, 7th ed. Baltimore: Williams and Wilkins.

BRONSERT U. and NEUPERT W. (1966) Protein synthesis in locust-flight-muscle sarcosomes, in *Regulation of Metabolic Processes in Mitochondria* (ed. Tager, Papa, Quagliariello, and Slater) pp. 426–438. Amsterdam: Elsevier.

BURKE J. P. and BEATTIE D. S. (1972) Unpublished results; quoted from Ibrahim *et al.* (1973).

BYGRAVE F. L. and KAISER W. (1969) The magnesium-dependent incorporation of serine into the phospholipids of mitochondria isolated from the developing flight muscle of the African locust *Locusta migratoria*. *Eur. J. Biochem.* **8**, 16–22.

CHAN S. K. and RICHARDSON L. (1969) Biochemical studies on the developing thoracic muscles of the tobacco horn worm. I. Amino acid incorporation into mitochondria. *J. biol. Chem.* **244**, 1039–1042.

CHAPPELL J. B. and HANSFORD R. G. (1969) Preparation of mitochondria from animal tissues and yeasts, in *Subcellular Components: Preparation and Fractionation* (ed. Birnie and Fox) pp. 43–56. London: Butterworths.

COOTE J. L. and WORK T. S. (1971) Proteins coded by mitochondrial DNA of mammalian cells. *Eur. J. Biochem.* **23**, 564–574.

DE KORT C. A. D. (1969) Hormones and the structural and biochemical properties of the flight muscles in the Colorado beetle. *Ph. D. Thesis*, University of Nijmegen.

DE KORT C. A. D. and BARTELINK A. K. M. (1972) Biochemical maturation of flight muscle mitochondria in the Colorado beetle, *Leptinotarsa decemlineata* Say. *Comp. Biochem. Physiol.* **43B**, 1009–1017.

DE KORT C. A. D., BARTELINK A. K. M., and SCHUURMANS R. R. (1973) The significance of L-proline for oxidative metabolism in the flight muscles of the Colorado beetle, *Leptinotarsa decemlineata*. *Insect Biochem.* **3**, 11–17.

HAMBERGER A., GREGSON N., and LEHNINGER A. L. (1969) The effect of acute exercise on amino acid incorporation into mitochondria of rabbit tissues. *Biochim. biophys. Acta* **186**, 373–383.

HOCHBERG A. C., STRATMAN F. W., ZAHLTEN R. N., and LARDY H. A. (1972) Artifacts in protein synthesis by mitochondria *in vitro*. *FEBS Lett.* **25**, 1–7.

IBRAHIM N. G., BURKE J. P., and BEATTIE D. S. (1973) Mitochondrial protein synthesis *in vitro* is not an artifact. *FEBS Lett.* **29**, 73–76.

KROON A. M., AGSTERIBBE E., and DE VRIES H. (1972) Protein synthesis in mitochondria and chloroplasts, in *The Mechanism of Protein Synthesis and its Regulation* (ed. Bosch) pp. 539–582. Amsterdam: North-Holland.

KROON A. M. and ARENDZEN A. J. (1972) The inhibition of mitochondrial biogenesis by antibiotics, in *Proc. 8th Meet. Fedn. Eur. Biochem. Socs., Amsterdam*, vol. 28, pp. 71–83. Amsterdam: North-Holland.

KROON A. M., BOTMAN M. J., and SACCONE C. (1968) The contribution of bacteria to protein and RNA synthesis in isolated mitochondria, in *Biochemical Aspects of the Biogenesis of Mitochondria* (ed. Slater, Tager, Papa, and Quagliariello) pp. 439–449. Bari, Italy: Adriatica Editrice.

KROON A. M. and DE VRIES H. (1971) Mitochondriogenesis in animal cells: studies with different inhibitors, in *Autonomy and Biogenesis of Mitochondria and Chloroplasts* (ed. Boardman, Linnane, and Smillie), pp. 318–327. Amsterdam: North-Holland.

KRYMKIEWICZ N. and GONZÀLEZ-CADAVID N. (1970) The significance of the incorporation of [^{14}C] leucine into different protein fractions by isolated ox heart mitochondria. *Biochem. J.* **116**, 269–276.

LAMB A. J., CLARK-WALKER G. D., and LINNANE A. W. (1968) The biogenesis of mitochondria. 4. The differentiation of mitochondrial and cytoplasmic protein synthesizing systems *in vitro* by antibiotics. *Biochim. biophys. Acta* **161**, 415–427.

PESTKA S. (1971) Inhibitors of ribosome function. *A. Rev. Microbiol.* **25**, 487–562.

ROODYN D. B., REIS P. J., and WORK T. S. (1961) Protein synthesis in mitochondria. Requirements for the incorporation of radioactive amino acids into mitochondrial protein. *Biochem. J.* **80**, 9–21.

SACKTOR B. (1970) Regulation of intermediary metabolism, with special reference to the control mechanisms in insect flight muscle, in *Adv. Insect Physiol.* **7**, 267–347.

SLATER E. C. (1967) Application of inhibitors and uncouplers for a study of oxidative phosphorylation, in *Methods in Enzymology* (ed. Estabrook and Pullman) **10**, 48–57. New York: Academic Press.

TOWERS N. R., DIXON H., KELLERMAN G. M., and LINNANE A. W. (1972) Biogenesis of mitochondria. 22. The sensitivity of rat liver mitochondria to antibiotics; a phylogenetic difference between a mammalian system and yeast. *Archs Biochem. Biophys.* **151**, 361–369.

WHEELDON L. W. and LEHNINGER A. L. (1966) Energy-linked synthesis and decay of membrane proteins in isolated rat liver mitochondria. *Biochemistry* **5**, 3533–3545.

WILLIAMS K. L. and BIRT L. M. (1971a) Incorporation *in vitro* of [^{14}C]leucine into the mitochondrial protein of *Lucilia cuprina*. 1. Basic requirements. *Eur. J. Biochem.* **22**, 87–95.

WILLIAMS K. L. and BIRT L. M. (1971b) Incorporation *in vitro* of [^{14}C]leucine into the mitochondrial protein of *Lucilia cuprina*. 2. Energy Requirements. *Eur. J. Biochem.* **22**, 96–103.

WILLIAMS K. L. and BIRT L. M. (1972) Sensitivity to erythromycin of mitochondrial protein synthesis in isolated flight muscle mitochondria of the blowfly *Lucilia*. *FEBS Lett.* **22**, 327–329.

Key Word Index: Colorado beetle, flight muscle mitochondria, mitochondrial protein synthesis.

1972; Hinds & Newburgh, 1972), RNA (Lennie *et al.*, 1967; Kleinow & Neupert, 1970) and ribosomes (Kleinow & Neupert, 1971; Kleinow *et al.*, 1971). Amino acid incorporation into protein catalyzed by flight-muscle mitochondria has been demonstrated both *in vitro* (Bronsert & Neupert, 1966; Chan & Richardson, 1969; Williams & Birt, 1971a, b; Williams, 1972) and *in vivo* (Sebald *et al.*, 1969; see also Polz & Kreil, 1970). The contribution of the mitochondrial genetic system to the biogenesis of flight-muscle mitochondria is poorly understood. To study this problem in the Colorado beetle, we developed some conditions for assay (Bartelink & de Kort, 1973; Bartelink *et al.*, 1974), which have been used in this research.

Materials and methods

Animals

Colorado beetles (*Leptinotarsa decemlineata* Say) were reared under long-day conditions (18 h light phase) at 25°C as described by Bartelink & de Kort (1973). Adult beetles were collected within 16 h after emergence and kept until the desired age (the young adults emerge from the soil at the second day of the imaginal stage).

Determinations of enzyme activity

Beetles were decapitated and elytra, wings, legs and abdomens were removed. The thoraces of six animals (three females and three males) were bisected and homogenized by hand with a Potter-Elvehjem (glass-Teflon) homogenizer (A. H. Thomas Co., Philadelphia, Pa., U.S.A.) in 6 ml of a medium containing 0.25 M sucrose, 5 mM Tris-HCl buffer (pH 7.4) and 1 mM disodium-EDTA. The resulting homogenate was filtered through one layer of cheese-cloth. The procedure was carried out at 0–4°C.

The cytochrome *c* oxidase activity (EC 1.9.3.1) in the filtered homogenates was assayed as described by Borst *et al.* (1967) and expressed as the first-order rate constant in min^{-1}. The excess of sodium dithionite, used to reduce cytochrome *c*, was removed by passage through a Sephadex G-50 column (40 cm high, 1.5 cm diameter), that had been previously equilibrated with 30 mM phosphate buffer (pH 7.4). The activities of succinate dehydrogenase (EC 1.3.99.1; measured as succinate: cytochrome *c* oxidoreductase) and soluble α-glycerophosphate dehydrogenase (EC 1.1.1.8) were assayed as previously described (de Kort, 1969). All enzyme assays were performed at 25°C.

Protein content of the homogenates was estimated on defatted trichloroacetic acid precipitates by the method of Lowry *et al.* (1951). Bovine serum albumin was used as the standard.

Determinations of cytochrome concentration

The thoraces of beetles, 30 or 60 according to age, were homogenized by the above procedure in 15 ml of a medium, that contained 1 mM EGTA [ethanedioxybis(ethylamine)tetra-acetate] instead of EDTA. The filtered homogenates were quickly frozen in liquid nitrogen and stored at −80°C until use.

Reduced *minus* oxidized difference absorption spectra of the homogenates were recorded at room temperature and at the temperature of liquid nitrogen with an Aminco-Chance dual-wavelength/split-beam spectrophotometer (American Instrument

Co., Inc., Silver Spring, Md., U.S.A.) operating in the split-beam mode. At room temperature, the recordings were carried out with a spectral band width of 1.5 nm, and at low temperature with a band width of 0.7 nm. The contents of the reference cuvette were oxidized by adding a few grains of potassium ferricyanide. Reduction of the measuring sample was accomplished with a small amount of sodium dithionite or by the addition of proline and KCN to final concentrations of 30 mM and 5 mM, respectively. The optical depth of the cuvettes used at room temperature was 1.0 cm. The aluminium cuvettes with Plexiglass windows for the low-temperature recordings had a light path of 0.3 cm and were filled as described by Chance & Schoener (1966).

The concentration of cytochrome aa_3 was calculated from the room-temperature spectrum, using an extinction coefficient of 24.0 $mM^{-1} \cdot cm^{-1}$ for the difference in absorbance between 604 and 625 nm (cf. van Gelder, 1966). For the calculation of concentrations of cytochrome *b* and *c*, the following procedure was adopted (cf. von Jagow *et al.*, 1973). The ratios of the absorbance of these cytochromes to cytochrome aa_3 absorbance were calculated from the spectrum recorded at the temperature of liquid nitrogen. The wavelength pairs used were 597–625 nm, 562–575 nm and 548–540 nm for cytochromes aa_3, *b* and *c*, respectively. After multiplication of the obtained ratios by the cytochrome aa_3 absorbance at room temperature, the cytochrome concentrations were calculated with the extinction coefficients 20.0 $mM^{-1} \cdot cm^{-1}$ cytochrome *b* (Estabrook & Holowinsky, 1961) and 17.8 $mM^{-1} \cdot cm^{-1}$ for cytochrome *c* (J. A. Berden, personal communication). To correct for the difference between cytochromes aa_3 and *b* in enhancement of absorbance at low temperature, the cytochrome *b* concentration was divided by a factor of 1.3 (Wilson, 1967).

Determinations of amino acid incorporation

In vivo. Beetles starved overnight were injected with 5 μg of cycloheximide in 1 μl of Ringer solution (Ephrussi & Beadle, 1936) as previously described (Bartelink & de Kort, 1973). Control animals received Ringer solution only. After 10 min, 0.1 μCi of L-[^{14}C]leucine (uniformly labelled, 331 mCi/mmol) was administered to each beetle in a volume of 2 μl. Mitochondrial fractions from the thoraces of six animals treated identically were isolated after a 30 min labelling period. The isolation procedure, the processing of the labelled protein and the measurements of radioactivity and protein concentration have been detailed previously (Bartelink & de Kort, 1973).

In vitro. Flight-muscle mitochondria were prepared from beetles starved overnight according to the method described earlier (Bartelink *et al.*, 1974). This method includes preparatory feeding of the beetles with ampicillin, and the use of sterile media and equipment to cut down bacterial contamination of the mitochondrial preparations to levels that contribute insignificantly to the incorporation activity. The mitochondria were incubated at 25°C with an optimized incorporation medium (Bartelink *et al.*, 1974) consisting of 50 mM sucrose, 50 mM Tricine-KOH buffer (pH 7.6), 1 mM disodium-EDTA, 0.1 mM EGTA, 40 mM KCl, 10 mM $MgCl_2$, 5 mM potassium phosphate (pH 7.6), 2 mM ADP, 30 mM L-proline, 20 μg/ml ribonuclease (EC 3.1.4.22), 200 μg/ml amino acid mixture without leucine (Roodyn *et al.*, 1961) and variable concentrations of L-[^{14}C]leucine (uniformly labelled, 10 mCi/mmol). The incubation time was 30 min. For further experimental details and the treatment of the radioactive protein, see Bartelink *et al.* (1974).

Chemicals

Sephadex G-50 (medium) was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden and digitonin (A-grade) from Calbiochem, San Diego, Calif., U.S.A. Dihydroxyacetone phosphate dimethylketal (dicyclohexylammonium salt) was obtained from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany, and converted to dihydroxyacetone phosphate as indicated by de Kort (1969). The sources of other chemicals have been described previously (Bartelink & de Kort, 1973; Bartelink *et al.*, 1974).

Results

Mitochondrial formation during flight-muscle development

Respiratory enzyme activities. Flight-muscle development in the Colorado beetle involves a tremendous increase in mitochondrial volume and membrane profile (de Kort, 1969). The changes in succinate dehydrogenase (measured as succinate: cytochrome *c* oxidoreductase) and cytochrome *c* oxidase activities accompanying this increase are illustrated in Fig. 1. At the moment of adult emergence, the flight muscles are still primordial and contain small mitochondria with few cristae (de Kort, 1969). The activities of the two mitochondrial inner-membrane enzymes were correspondingly low. During the first days after emergence, a rapid accumulation of succinate dehydrogenase and cytochrome *c* oxidase activity occurred, which was completed in the second week of adult life. At this stage the mitochondria contain many cristae and occupy about a third of the volume of the flight muscles (de Kort, 1969). The protein values (Fig.1_C) indicate that the specific activities of both enzymes have also increased over the experimental period.

Cytochrome concentrations. Dithionite-reduced *minus* ferricyanide-oxidized difference spectra of flight-muscle homogenates, which were recorded at room temperature, revealed the α-absorption bands of cytochromes aa_3, *b* and *c* as a peak or shoulder at 604, 562 and 551 nm, respectively (Fig. 2). Similar spectra taken at the temperature of liquid nitrogen showed a considerable intensification and sharpening of the absorption bands (Fig. 3, Trace A). This phenomenon and an accompanying blue shift of maxima are already known from the literature (e.g. Wilson, 1967) and produced a different picture. The α-region of the low-temperature spectra displayed at 597, 562 and 548 nm the maxima corresponding to cytochromes aa_3, *b* and *c*, respectively. In addition, a peak at 555 nm was observed. The nature of the absorbance at this position deserves some comment.

When reduction of the cytochromes was brought about with a physiological substrate (de Kort *et al.*, 1973) in the presence of KCN, the low-temperature spectrum showed no absorption band at 555 nm (Fig. 3, Trace B). Furthermore, the absorbance of the cytochrome *b* band was decreased. Estabrook & Sacktor (1958), studying the spectral properties of flight-muscle mitochondria from house flies (*Musca domestica*), also noted a dithionite-induceable band at 555 nm in their low-temperature spectra. The band was not elicited by ascorbate. Neither was the 555 nm band revealed by enzymic reduction of the respiratory pigments with α-glycerophosphate, in the presence or absence of a terminal inhibitor. But if the enzymic reduction was carried out in the presence of antimycin A, the spectra showed an absorption band at 562 nm, and the peak at 555 nm. These observations caused Estabrook & Sacktor to postulate an additional respiratory pigment,

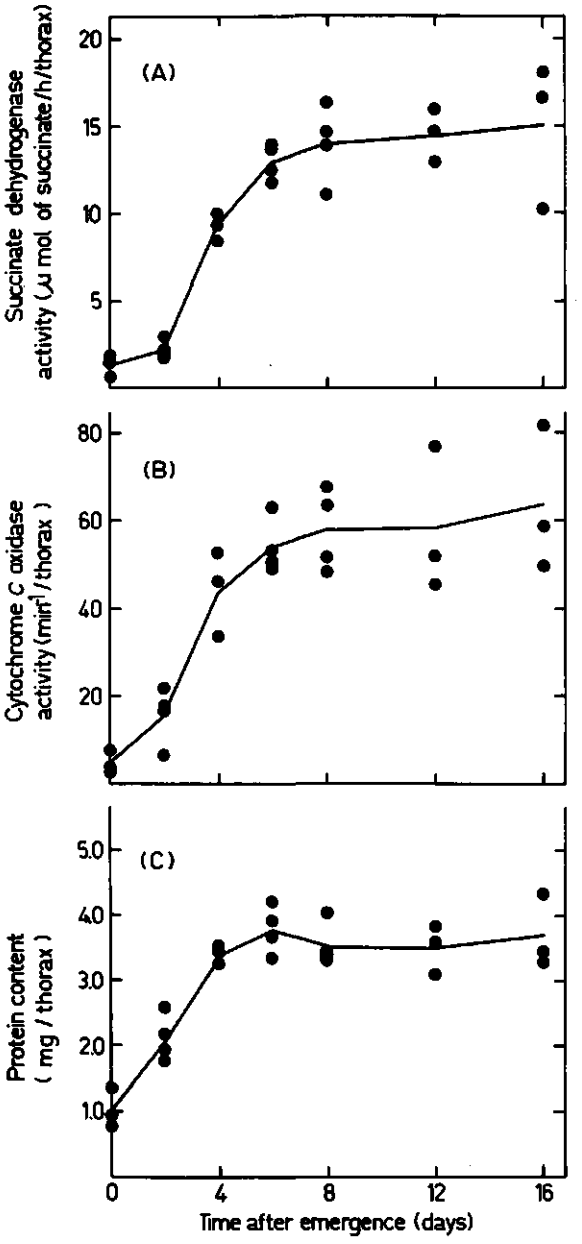

Fig. 1. Respiratory enzyme activities and total protein content of developing flight muscles. Homogenates of thoraces were prepared at the indicated stages and assayed for succinate dehydrogenase activity (A), cytochrome *c* oxidase activity (B) and protein content (C). For experimental details, see Materials and methods. At each stage 3–4 separate homogenates were assayed.

which was designated cytochrome 555. In view of recent spectrophotometric studies on the heterogeneity of cytochrome *b* (e.g. Sato *et al.*, 1971; Davis *et al.*, 1973) we tend to attribute the absorbance at 555 nm to the low-potential *b*-cytochrome, cytochrome b_T. The split α-band of this pigment[1] can be visualized in the difference spectrum between

1. There is also evidence that two different cytochromes are involved in the absorption structure (Wikström, 1973).

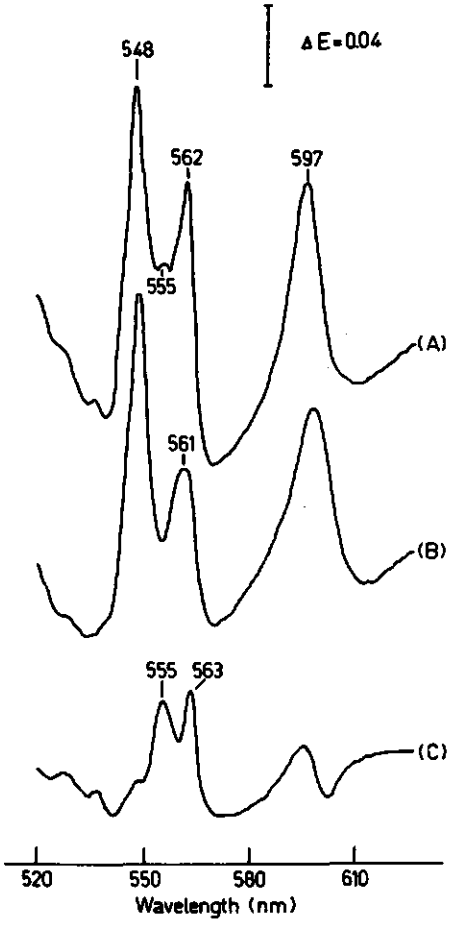

Fig. 2. Room-temperature difference spectrum of cytochromes in flight muscles. A homogenate was prepared from 30 thoraces of 16-day-old beetles and divided between two cuvettes. The contents of the measuring cuvette were reduced with dithionite; to the reference material ferricyanide was added. The spectrum was recorded as described under Materials and methods. The protein concentration was 7.9 mg/ml.

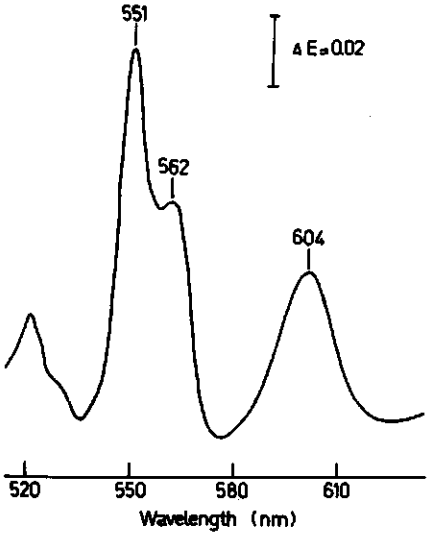

Fig. 3. Low-temperature difference spectra of cytochromes in flight muscles. Recordings were made with a homogenate prepared from 30 thoraces of 16-day-old beetles. For Trace A the measuring sample was reduced with dithionite and the reference material was supplemented with ferricyanide. The spectrum represented by Trace B was obtained after reduction of the measuring sample with L-proline (30 mM) in the presence of KCN (5 mM); the reference cuvette contained ferricyanide. Trace C shows the difference in absorption after reduction of the cytochromes by dithionite (measuring cuvette) and by L-proline in the presence of KCN (reference cuvette). The spectra were recorded at the temperature of liquid nitrogen as described under Materials and methods. The protein concentration was 6.7 mg/ml.

the dithionite-reduced state and the substrate-reduced state. A representative spectrograph of such a difference spectrum of flight-muscle homogenate is shown in Fig. 4 (Trace C). As seen a maximum at 563 nm is accompanied by the peak at 555 nm.

The cytochrome contents of flight-muscle homogenates prepared at various developmental stages are plotted in Fig. 4. Cytochrome aa_3 concentrations were estimated from difference spectra (dithionite-reduced *minus* ferricyanide-oxidized) taken at room temperature. For the evaluation of the contents of cytochromes *b* and *c* the corresponding low-temperature spectra were used, owing to the improved resolution of the absorption

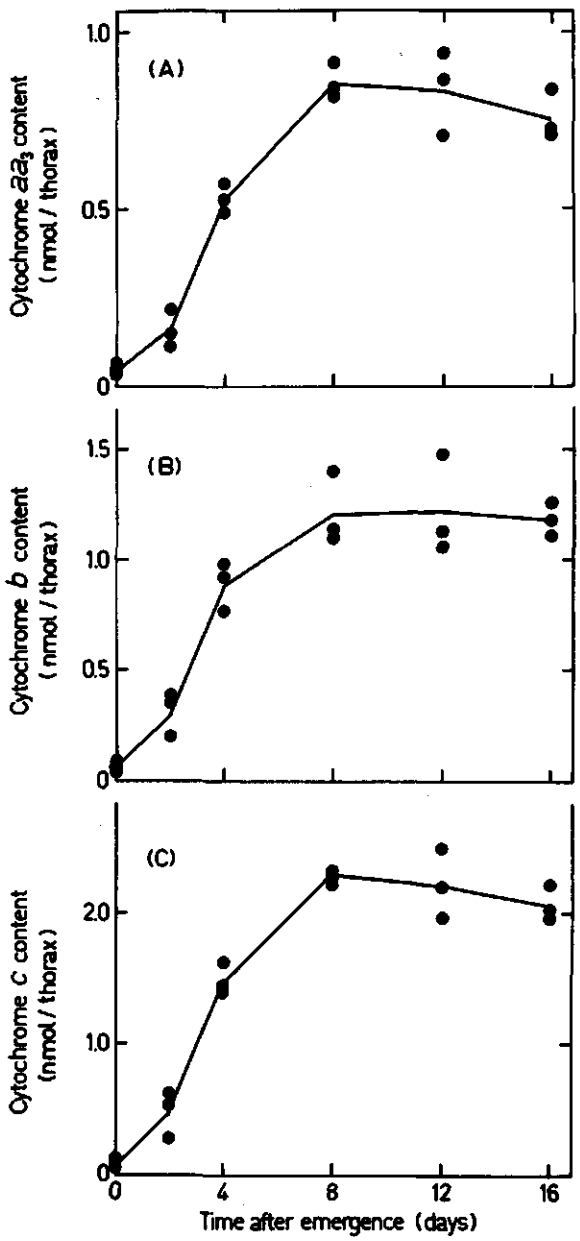

Fig. 4. Cytochrome contents of developing flight muscles. Homogenates of thoraces were prepared at the indicated stages. The concentrations of cytochrome aa_3 (A), cytochrome *b* (B) and cytochrome *c* (C) in the homogenates were calculated from difference absorption spectra as detailed in the text. At each stage three separate homogenates were analysed. The average protein content of these homogenates increased in a similar fashion as depicted in Fig. 1C from 0.93 mg of protein/thorax at day 0 to 3.2 mg/thorax at day 16.

bands. In order to assay 'total' cytochrome *b* content the absorbance was measured at the wavelength pair 562–575 nm. The cytochrome concentrations were calculated via the procedure detailed under Materials and methods with extinction coefficients for mammalian cytochromes. Mutual interference of the absorption bands was not taken into account (cf. von Jagow *et al.*, 1973). It is seen from Fig. 4 that the cytochrome contents of the flight muscles increased progressively during the first week after emergence and reached a maximum at day 8.

At no stage of development did we notice absorption indicative of cytochrome c_1 in the spectra of flight-muscle homogenates. Neither could Estabrook & Sacktor (1958) distinguish cytochrome c_1 absorption in low-temperature spectra of intact flight-muscle mitochondria from house flies (cf. Chance & Parsons, 1963; Herold & Borei, 1963). However, they did observe an absorption band at 551 nm with particles, which were depleted of cytochrome *c*. The pigment, designated by Estabrook & Sacktor as cytochrome 551, was reducible by dithionite, ascorbate and substrate. If this pigment, presumably cytochrome c_1, is also present in Colorado-beetle flight muscles, it contributes to the absorbance of the cytochrome *c* band. Thus, the cytochrome *c* contents presented in Fig. 4 are possibly overestimated.

Contribution of the intrinsic genetic system to mitochondrial formation

Effect of ethidium bromide on respiratory enzyme activities. The intramitochondrial contribution to the biosynthesis of the enzymes succinate dehydrogenase and cytochrome *c* oxidase was studied with ethidium bromide, which is an inhibitor of mitochondrial transcription (Kroon & de Vries, 1971; see also Bartelink *et al.*, 1974). The results are shown in Table 1. It can be seen that treatment of Colorado beetles with ethidium bromide inhibited the increase in cytochrome *c* oxidase activity in developing flight muscles. The elaboration of succinate dehydrogenase activity, however, was not depressed by the dye. Neither did the protein content and the activity of cytoplasmic

Table 1. Effect of ethidium bromide on enzyme activities and protein content of developing flight muscles[1].

	Control animals	Ethidium bromide-treated animals
Succinate dehydrogenase activity (μmol of succinate/h/thorax)	12.8 ± 1.0	13.0 ± 1.1
Cytochrome *c* oxidase activity (min^{-1}/thorax)	49.0 ± 6.5	22.2 ± 1.7
α-Glycerophosphate dehydrogenase activity (μmol of α-glycerophosphate/h/thorax)	46.4 ± 0.7	45.1 ± 2.2
Protein content (mg/thorax)	3.32 ± 0.22	3.05 ± 0.33

1. Beetles were injected two days after adult emergence with 10 μg ethidium bromide in 1 μl Ringer solution using previously described methods (Bartelink & de Kort, 1973). Control animals received Ringer solution only. The injections were repeated two days later. After another two days, the animals were killed. The values represent the averages (± S.E.M.) of five different experiments, except that α-glycerophosphate dehydrogenase activity was assayed in three experiments of the series.

α-glycerophosphate dehydrogenase in the treated animals differ from control values, which indicates that ethidium bromide had no influence on whole-cell protein synthesis.

Cycloheximide-resistant [^{14}C]*leucine incorporation into mitochondrial protein in vivo.* To obtain more quantitative data on the intramitochondrial contribution to the synthesis of organelle protein at different stages of flight-muscle development, we undertook pulse-label experiments *in vivo*. The incorporation of [^{14}C]leucine into mitochondrial protein was measured in the presence and absence of cycloheximide. This antibiotic blocks protein synthesis on cytoplasmic ribosomes, but has no direct effect on the mitochondrial protein-synthesizing system (Kroon *et al.*, 1972; see also Bartelink *et al.*, 1974). For 4-day-old beetles, a dose of 5 μg of cycloheximide per animal inhibits [^{14}C]leucine incorporation into the cytosol protein of flight muscles by more than 99.5% (Bartelink & de Kort, 1973). Under these conditions, however, a significant incorporation into mitochondrial protein was observed, which appeared to be age-dependent (Table 2). At the time of emergence, about 5% of the [^{14}C]leucine incorporation into mitochondrial protein was resistant to the action of cycloheximide. During the early phase of flight-muscle development, the percentage of cycloheximide-resistant incorporation rose to the value of 14, four days after emergence. With full-grown flight muscles the radioactivity incorporated in the presence of the antibiotic was again 5% of the control value. Through lack of data on the specific radioactivity of the leucine pool(s) at different stages of flight-muscle development, the magnitude of protein labelling cannot be compared vertically in Table 2. We did previously establish that cycloheximide has no substantial effect on the specific activity of free leucine in the flight muscles of 4-day-old beetles (Bartelink & de Kort, 1973). It was also shown that in these beetles in the presence and absence of cycloheximide the incorporation into mitochondrial protein proceeds, at an almost linear rate during the first hour after injection of 0.1 μCi of [^{14}C]leucine per animal.

Table 2. Effect of cycloheximide on [^{14}C]leucine incorporation into mitochondrial protein in developing flight muscles[1].

Time after emergence (days)	Number of experiments	[^{14}C]Leucine incorporated (d.p.m./mg of protein)[2]		Percentage of control[3]
		Control animals	Cycloheximide-treated animals	
0	3	5051	237	4.5 (2.5–5.9)
2	4	4361	420	9.7 (8.6–11.5)
4	7	3419	486	14.2 (11.5–16.4)
8	5	767	62	8.2 (6.5–9.0)
12	4	905	41	4.5 (3.6–5.0)
16	4	986	54	5.2 (3.2–7.0)

1. Beetles of the indicated ages were treated with 5 μg of cycloheximide in Ringer solution. Control animals received Ringer solution only. After 10 min 0.1 μCi of [^{14}C]leucine (331 mCi/mmol) was administered per beetle. The flight-muscle mitochondria were isolated after a 30 min labelling period. For further experimental details, see Materials and methods.
2. The values represent the average specific radioactivity.
3. The average percentage is given with the range in parentheses.

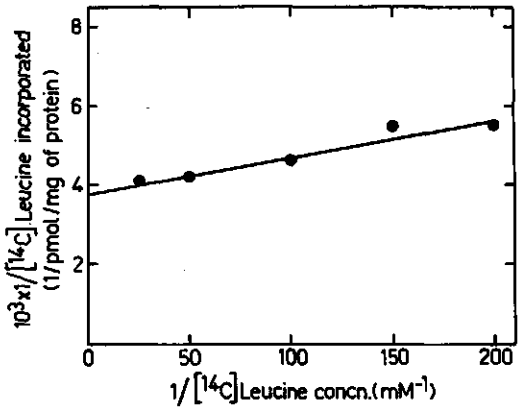

Fig. 5. Effect of concentration of added [^{14}C]leucine on incorporation into protein by isolated flight-muscle mitochondria. Mitochondria were isolated from the thoraces of 4-day-old beetles and incubated for 30 min with different concentrations of [^{14}C]leucine (10 mCi/mmol). For experimental details, see Materials and methods.

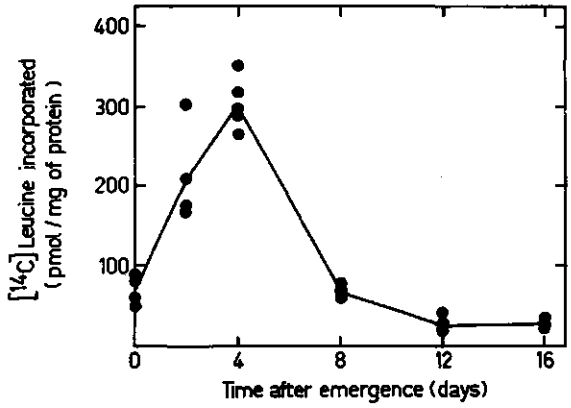

Fig. 6. [^{14}C]Leucine incorporation into protein by isolated mitochondria from developing flight muscles. Flight-muscle mitochondria were isolated at the indicated intervals and incubated for 30 min with 0.01 and 0.04 mM [^{14}C]leucine (10 mCi/mmol). The [^{14}C]leucine incorporation at infinite radioisotope concentration is given, as determined by extrapolation from reciprocal plots (cf. Fig. 5). Each point represents a separate mitochondrial preparation.

[^{14}C] *Leucine incorporation into mitochondrial protein in vitro.* Isolated flight-muscle mitochondria from Colorado beetles have the intrinsic ability to incorporate [^{14}C]leucine into protein (Bartelink *et al.*, 1974). To study the incorporation activity of the organelles at different developmental stages, we had to allow for possible variations in endogenous leucine content. Therefore we measured the [^{14}C]leucine incorporation into mitochondrial protein at various radioisotope concentrations (AB, 1968). Plotting the reciprocals of the amount of [^{14}C]leucine incorporated and the isotope concentration in the incubation medium resulted in a straight line (Fig. 5). The intersection with the ordinate represents the reciprocal of the amino acid incorporation at infinite isotope concentration. This value was next estimated at various stages during flight-muscle development. The results are shown in Fig. 6. Maximum [^{14}C]leucine incorporation into protein was found with mitochondria isolated from 4-day-old beetles. Both at emergence and after completion of muscular development, considerably less was incorporated.

Discussion

The development of flight musculature in the Colorado beetle is characterized by a rapid accumulation of respiratory enzymes. During this period succinate dehydrogenase activity was proportional to mitochondrial structure (de Kort, 1969). Thus, the results presented in Fig. 1_A depict mitochondrial growth in the developing Colorado-beetle flight muscles.

In their study on the development of the cytochrome system in the flight muscles of the honey bee (*Apis mellifera*), Herold & Borei (1963) distinguished three steps. Cytochrome aa_3 first started to increase in amount, at a second stage cytochrome *b* appeared and finally the formation of cytochrome *c* was initiated. Asynchronous development of the various cytochromes was also observed in yeasts during respiratory adaptation after anaerobiosis (Cartledge *et al.*, 1972; Chen & Charalampous, 1973) or glucose repression (Sherman & Stewart, 1971). In the flight muscles of the Colorado beetle, however, the elaboration of the cytochromes aa_3, *b* and *c* and the activities of succinate dehydrogenase and cytochrome *c* oxidase ran roughly parallel (Figs. 1, 4). It was previously shown that also the activities of malate dehydrogenase and citrate synthetase are directly proportional to succinate dehydrogenase activity during development (de Kort, 1969). Apparently, the enzymes of oxidative metabolism are formed synchronously in the flight-muscle mitochondria of the Colorado beetle. A parallel accumulation of mitochondrial enzymes has also been demonstrated during flight-muscle development in the locust *Locusta migratoria* (Bücher, 1965; see also Kistler & Weber, 1974).

The intrinsic contribution of the flight-muscle mitochondria to the synthesis of organelle protein was studied at different stages of development *in vivo* and *in vitro*. *In vivo*, the percentage of amino acid incorporation resistant to the action of cycloheximide was estimated. This cycloheximide-resistant incorporation is mediated by intramitochondrial ribosomes, since it is sensitive to chloramphenicol (Bartelink & de Kort, 1973), a specific inhibitor of mitochondrial protein synthesis. The results presented in Table 2 could therefore mean that the contribution of the mitochondria to the formation of their protein varies with developmental stage. This implies that the activity of the intramitochondrial translation system and the extrinsic synthesis of mitochondrial protein are not tightly coupled. It is also of interest that the time of maximum cycloheximide-resistant incorporation corresponded with the period of rapid mitochondrial development (Figs. 1,4). With chloramphenicol and erythromycin, Kellerman *et al.* (1971) estimated that in glucose-repressed yeast cells, 5% of the mitochondrial protein was synthesized by the intrinsic system, whereas after derepression a value of 13% was obtained. Evidently, mitochondrial development requires an enhanced contribution of the intrinsic protein-synthesizing system.

The interpretation of the observed differences in amino acid incorporation by isolated mitochondria (Fig. 6) in terms of variation in protein-synthesizing capacity is hampered by some uncertainties. Thus, for instance differences in incorporation values may reflect differences in cofactor requirements rather than changes in synthesizing capacity. However, the coincidence of the maximum in the incorporation activity with that in the percentage of cycloheximide-resistant incorporation *in vivo* strongly suggests that the values obtained in the experiments *in vitro* are of physiological significance. Changing rates of amino acid incorporation *in vitro* were also demonstrated with flight-muscle mitochondria of *Locusta* (Bronsert & Neupert, 1966), the moth *Manduca sexta* (Chan & Richardson, 1969) and the blowfly *Lucilia cuprina* (Williams, 1972). The incorporation in mitochondria from developing muscles was always substantially higher than after growth ceased. Similar observations were made with other systems. For instance, mitochondria isolated from logarithmic-phase cells of *Neurospora crassa* were more active in amino acid incorporation than mitochondria from lag-phase cells (Greenawalt *et al.*, 1972). With regenerating livers of partially hepatectomized rats, the highest incorporation activities were found if the mitochondria were isolated when the mitotic index was highest (Kroon, 1966). Changes in incorporation rates were observed also during normal liver develop-

ment. At the 19th foetal day, the incorporation rate of the isolated mitochondria was about three times the adult value (Hallman, 1971). In this context further the following observations are of importance. By electron microscopy André & Marinozzi (1965) found many more ribosome-like particles in the mitochondria of embryonic tissues of the rat than in the corresponding adult tissues. The intrinsic RNA content of mitochondria from regenerating rat liver was estimated by Kroon (1971) to be 3 μg per mg of protein, whereas for normal liver a value of 1 μg per mg of protein was found. Mitochondria from developing flight muscles of *Lucilia* also contained much more RNA per mg of protein than mature mitochondria (Lennie *et al.*, 1967). Therefore we may conclude that the higher incorporation activities of mitochondria from growing tissues indeed reflect a higher capacity for protein synthesis.

In a previous study (Bartelink & de Kort, 1973), we estimated the specific radioactivity of free leucine in the flight muscles after injection of 5 μg of cycloheximide and 0.1 μCi of the [^{14}C] labelled amino acid per animal. So we can compare the rate of cycloheximide-resistant amino acid incorporation *in vivo* with the incorporation activity of the isolated mitochondria *in vitro*. With an identical injection schedule as in the present experiments, the specific activity of the free leucine from 4-day-old beetles was found in three experiments to average 0.38 μCi/μmol. Assuming that this value equalled the specific activity of the actual precursor pool during the labelling period, we can calculate from the data of Table 2 that in 4-day-old beetles the rate of cycloheximide-resistant incorporation of leucine into mitochondrial protein averaged 576 pmol/30 min per mg of protein. Mitochondria isolated at the same stage of development showed *in vitro* about 50% of this activity (Fig. 6). Although the percentage is higher than previous estimates (Hawley & Greenawalt, 1970; Kroon *et al.*, 1972), it indicates that the incubation medium for the experiments *in vitro* is still suboptimal or that the mitochondria were damaged during isolation.

At the enzymic level, the intrinsic genetic system of the flight-muscle mitochondria appeared to contribute to the formation of cytochrome *c* oxidase (= cytochrome aa_3). Administration of ethidium bromide to young adults of the Colorado beetle inhibited the increase in cytochrome *c* oxidase activity. However, the accumulation of another mitochondrial inner-membrane enzyme, succinate dehydrogenase, was not affected (Table 1). Identical results were obtained for developing fat body of the cockroach *Blaberus discoidalis* (Keeley, 1972). Interference with cytochrome *c* oxidase formation by inhibitors of mitochondrial transcription or translation has also been demonstrated for non-insect systems, e.g. growing yeast cells (Clark-Walker & Linnane, 1967), regenerating rat liver (Kroon & de Vries, 1971) and rat intestinal epithelium (Gijzel *et al.*, 1972). These observations support recent evidence that part of the cytochrome *c* oxidase subunits are products of the mitochondrial protein-synthesizing system (Weiss *et al.*, 1972; Tzagoloff *et al.*, 1973).

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References

AB, G. (1968). Eiwitsynthese in een celvrij leversysteem. Ph. D. thesis, State University of Groningen.

André, J., & Marinozzi, V. (1965). Présence, dans les mitochondries, de particules ressemblant aux ribosomes. J. Microscopie 4, 615–626.

Bartelink, A. K. M., & de Kort, C. A. D. (1973). Synthesis of mitochondrial protein in the flight muscles of the Colorado beetle. Differentiation *in vivo* between extra- and intra-mitochondrial contributions to the amino acid incorporation into mitochondrial protein. Biochem. J. 136, 795–802.

Bartelink, A. K. M., de Kort, C. A. D. & Kortstee, G. J. J. (1974). Amino-acid incorporation into protein by isolated flight muscle mitochondria from the Colorado beetle. Insect Biochem. 4, 313–323.

Birt, L. M. (1971). Structural and enzymic development of blowfly mitochondria. In: Autonomy and Biogenesis of Mitochondria and Chloroplasts (N. K. Boardman, A. W. Linnane & R. M. Smillie, eds.), pp. 130–139. North-Holland Publishing Co., Amsterdam.

Borst, P., Ruttenberg, G. J. C. M., & Kroon, A. M. (1967). Mitochondrial DNA I. Preparation and properties of mitochondrial DNA from chick liver. Biochim. Biophys. Acta 149, 140–155.

Bronsert, U., & Neupert, W. (1966). Protein synthesis in locust-flight-muscle sarcosomes. In: Regulation of Metabolic Processes in Mitochondria (J. M. Tager, S. Papa, E. Quagliariello & E. C. Slater, eds.), pp. 426–438. Elsevier Publishing Co., Amsterdam.

Bücher, Th. (1965). Formation of the specific structural and enzymic pattern of the insect flight muscle. In: Aspects of Insect Biochemistry (T. W. Goodwin, ed.), pp. 15–28. Academic Press, London.

Cartledge, T. G., Lloyd, D., Erecińska, M., & Chance, B. (1972). The development of the respiratory chain of *Saccharomyces carlsbergensis* during respiratory adaptation. Biochem. J. 130, 739–747.

Chan, S. K., & Richardson, L. (1969). Biochemical studies on the developing thoracic muscles of the tobacco horn worm I. Amino acid incorporation into mitochondria. J. Biol. Chem. 244, 1039–1042.

Chance, B., & Parsons, D. F. (1963). Cytochrome function in relation to inner membrane structure of mitochondria. Science 142, 1176–1180.

Chance, B., & Schoener, B. (1966). High and low energy states of cytochromes I. In mitochondria. J. Biol. Chem. 241, 4567–4573.

Chen, W. L., & Charalampous, F. C. (1973). Kinetics of synthesis of cytochromes *a* and a_3 during respiratory adaptation of yeast. Biochim. Biophys. Acta 294, 329–342.

Clark-Walker, G. D., & Linnane, A. W. (1967). The biogenesis of mitochondria in *Saccharomyces cerevisiae*. A comparison between cytoplasmic respiratory-deficient mutant yeast and chloramphenicol-inhibited wild type cells. J. Cell Biol. 34, 1–14.

Davis, K. A., Hatefi, Y., Poff, K. L., & Butler, W. L. (1973). The *b*-type cytochromes of bovine heart mitochondria: Absorption spectra, enzymatic properties, and distribution in the electron transfer complexes. Biochim. Biophys. Acta 325, 341–356.

de Kort, C. A. D. (1969). Hormones and the structural and biochemical properties of the flight muscles in the Colorado beetle. Commun. Agricultural University Wageningen 69–2, 1–63.

de Kort, C. A. D., & Bartelink, A. K. M. (1972). Biochemical maturation of flight muscle mitochondria in the Colorado beetle, *Leptinotarsa decemlineata* Say. Comp. Biochem. Physiol. 43B, 1009–1017.

de Kort, C. A. D., Bartelink, A. K. M., & Schuurmans, R. R. (1973). The significance of L-proline for oxidative metabolism in the flight muscles of the Colorado beetle, *Leptinotarsa decemlineata*. Insect Biochem. 3, 11–17.

Ephrussi, B., & Beadle, G. W. (1936). A technique of transplantation for *Drosophila*. Amer. Natur. 70, 218–225.

Estabrook, R. W., & Holowinsky, A. (1961). Studies on the content and organization of the respiratory enzymes of mitochondria. J. Biophys. Biochem. Cytol. 9, 19–28.

Estabrook, R. W., & Sacktor, B. (1958). The respiratory metabolism of insect flight muscle. III. Low-temperature spectra of the cytochromes of flight muscle sarcosomes. Arch. Biochem. Biophys. 76, 532–545.

Greenawalt, J. W., Beck, D. P., & Hawley, E. S. (1972). Chemical and biochemical changes in mitochondria during morphogenetic development of *Neurospora crassa*. In: Biochemistry and Biophysics of Mitochondrial Membranes (G. F. Azzone, E. Carafoli, A. L. Lehninger, E. Quagliariello & N. Siliprandi, eds.), pp. 541–558. Academic Press, New York.

Gijzel, W. P., Strating, M., & Kroon, A. M. (1972). The biogenesis of mitochondria during proliferation and maturation of the intestinal epithelium of the rat. Effects of oxytetracycline. Cell Differentiation 1, 191–198.

Hallman, M. (1971). Changes in mitochondrial respiratory chain proteins during perinatal development. Evidence of the importance of environmental oxygen tension. Biochim. Biophys. Acta 253, 360–372.

Hawley, E. S., & Greenawalt, J. W. (1970). An assessment of *in vivo* mitochondrial protein synthesis in *Neurospora crassa*. J. Biol. Chem. 245, 3574–3583.

Herold, R. C., & Borei, H. (1963). Cytochrome changes during honey bee flight muscle development. Develop. Biol. 8, 67–79.

Hinds, T. R., & Newburgh, R. W. (1972). The DNA of the thoracic muscle of *Manduca sexta*. Biochim. Biophys. Acta 281, 514–521.

Keeley, L. L. (1972). Biogenesis of mitochondria: Neuroendocrine effects on the development of respiratory functions in fat body mitochondria of the cockroach, *Blaberus discoidalis*. Arch. Biochem. Biophys. 153, 8–15.

Keilin, D. (1925). On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants. Proc. Roy. Soc. London B98, 312–339.

Kellerman, G. M., Griffiths, D. E., Hansby, J. E., Lamb, A. J., & Linnane, A. W. (1971). The protein synthetic capacity of yeast mitochondria and the role of the mitochondrial genome in the economy of the cell. In: Autonomy and Biogenesis of Mitochondria and Chloroplasts (N. K. Boardman, A. W. Linnane & R. M. Smillie, eds.), pp. 346–359. North-Holland Publishing Co., Amsterdam.

Kistler, A., & Weber, R. (1974). Enzyme patterns in mitochondria of eggs, liver, and skeletal muscle during larval development of *Xenopus*. Develop. Biol. 37, 236–247.

Kleinow, W., & Neupert, W. (1970). RNA aus Mitochondrien des Thoraxmuskels von *Locusta migratoria*. Hoppe-Seyler's Z. Physiol. Chem. 351, 1205–1214.

Kleinow, W., & Neupert, W. (1971). The mitochondrial ribosome from *Locusta migratoria*: Dissociation into subunits. FEBS Lett. 15, 359–364.

Kleinow, W., Neupert, W., & Bücher, Th. (1971). Small sized ribosomes from mitochondria of *Locusta migratoria*. FEBS Lett. 12, 129–133.

Kroon, A. M. (1966). Protein synthesis in mitochondria. M. D. thesis, University of Amsterdam.

Kroon, A. M. (1971). Structure and function of mitochondrial nucleic acids. Chimia 25, 114–121.

Kroon, A. M., & de Vries, H. (1971). Mitochondriogenesis in animal cells: Studies with different inhibitors. In: Autonomy and Biogenesis of Mitochondria and Chloroplasts (N. K. Boardman, A. W. Linnane & R. M. Smillie, eds.), pp. 318–327. North-Holland Publishing Co., Amsterdam.

Kroon, A. M., Agsteribbe, E., & de Vries, H. (1972). Protein synthesis in mitochondria and chloroplasts. In: The Mechanism of Protein Synthesis and its Regulation (L. Bosch, ed.), pp. 539–582. North-Holland Publishing Co., Amsterdam.

Lennie, R. W., Gregory, D. W., & Birt, L. M. (1967). Changes in the nucleic acid content and structure of thoracic mitochondria during development of the blowfly, *Lucilia cuprina*. J. Insect Physiol. 13, 1745–1756.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Polz, G., and Kreil, G. (1970). Presence of *N*-formyl- and *N*-acetyl-methionine in the proteins of honey bee thorax. Biochem. Biophys. Res. Commun. 39, 516–521.

Roodyn, D. B., Reis, P. J., & Work, T. S. (1961). Protein synthesis in mitochondria. Requirements for the incorporation of radioactive amino acids into mitochondrial protein. Biochem. J. 80, 9–21.

Sacktor, B. (1970). Regulation of intermediary metabolism, with special reference to control mechanisms in insect flight muscle. Advan. Insect Physiol. 7, 267–347.

Sato, N., Wilson, D. F., & Chance, B. (1971). The spectral properties of the *b* cytochromes in intact mitochondria. Biochim. Biophys. Acta 253, 88–97.

Sebald, W., Hofstötter, Th., Hacker, D., & Bücher, Th. (1969). Incorporation of amino acids into mitochondrial protein of the flight muscle of *Locusta migratoria in vitro* and *in vivo* in the presence of cycloheximide. FEBS Lett. 2, 177–180.

Sherman, F., & Stewart, J. W. (1971). Genetics and biosynthesis of cytochrome *c*. Annu. Rev. Genet. 5, 257–296.

Tanguay, R., & Chaudhary, K. D. (1971). Studies on mitochondria. I. Mitochondrial DNA in relation to morphological development of sarcosomes in thoracic muscles of the African desert locust (*Schistocerca gregaria* Forsk). Can. J. Biochem. 49, 357–367.

Tanguay, R., & Chaudhary, K. D. (1972). Studies on mitochondria. II. Mitochondrial DNA of thoracic muscles of *Schistocerca gregaria*. J. Cell Biol. 54, 295–301.

Tzagoloff, A., Rubin, M. S., & Sierra, M. F. (1973). Biosynthesis of mitochondrial enzymes. Biochim. Biophys. Acta 301, 71–104.

van Bruggen, E. F. J., Runner, C. M., Borst, P., Ruttenberg, G. J. C. M., Kroon, A. M., & Schuurmans Stekhoven, F. M. A. H. (1968). Mitochondrial DNA III. Electron microscopy of DNA released from mitochondria by osmotic shock. Biochim. Biophys. Acta 161, 402–414.

van Gelder, B. F. (1966). On cytochrome *c* oxidase I. The extinction coefficients of cytochrome *a* and cytochrome a_3. Biochim. Biophys. Acta 118, 36–46.

von Jagow, G., Weiss, H., & Klingenberg, M. (1973). Comparison of the respiratory chain of *Neurospora crassa* wild type and the mi-mutants mi-1 and mi-3. Eur. J. Biochem. 33, 140–157.

Weiss, H., Lorenz, B., & Kleinow, W. (1972). Contribution of mitochondrial protein synthesis to the formation of cytochrome oxidase in *Locusta migratoria*. FEBS Lett. 25, 49–51.

Wikström, M. K. F. (1973). The different cytochrome *b* components in the respiratory chain of animal mitochondria and their role in electron transport and energy conservation. Biochim. Biophys. Acta 301, 155–193.

Williams, K. L. (1972). Protein synthesis during the metamorphosis of *Lucilia cuprina*. Ph. D. thesis, Australian National University, Canberra.

Williams, K. L., & Birt, L. M. (1971a). Incorporation *in vitro* of [^{14}C]leucine into the mitochondrial protein of *Lucilia cuprina*. 1. Basic requirements. Eur. J. Biochem. 22, 87–95.

Williams, K. L., & Birt, L. M. (1971b). Incorporation *in vitro* of [^{14}C]leucine into the mitochondrial protein of *Lucilia cuprina*. 2. Energy requirements. Eur. J. Biochem. 22, 96–103.

Wilson, D. F. (1967). Effect of temperature on the spectral properties of some ferrocytochromes. Arch. Biochem. Biophys. 121, 757–768.

...over. The haemo-
...ound to contain some juvenile hormone
...mergence, but the hormone level decreased rapidly

thereafter. No juvenile hormone was detectable at the entrance of diapause about two weeks after emergence. In active long-day animals, however, the titer increased until the fourth day and remained constant thereafter (DE WILDE *et al.*, 1968).

The state of the flight muscles and their mitochondria, therefore, is correlated with the titer of the juvenile hormone. Further investigation revealed that at the moment of adult emergence the flight muscles are still primordial and contain small mitochondria. In long-day animals, the flight muscles develop rapidly during the first few days after emergence and are fully formed by the second week. Flight-muscle development is initiated also in beetles exposed to a short photoperiod, but the process soon comes to a standstill and is then followed by degeneration (DE KORT, 1969; DE KORT & BARTELINK, 1972).

In a previous paper (BARTELINK *et al.*, 1974b) we described the elaboration of the cytochrome complement in the flight muscles of long-day animals. It appeared that the accumulation of cytochromes aa_3, *b*, and *c* proceeds roughly parallel and provides an index of mitochondrial development. Furthermore, our examination of the intrinsic protein-synthesizing system of the mitochondria revealed that maximum amino acid incorporation occurred during the period of rapid accumulation of cytochromes. The investigation reported here evaluates these biochemical parameters with beetles reared in a short-day environment.

MATERIALS AND METHODS

Animals

Colorado beetles (*Leptinotarsa decemlineata* Say) were bred at 25° C as previously described (BARTELINK & DE KORT, 1973). The experimental animals were reared *ab ovo* under a short-day régime with 10 h photophase. Adult beetles were collected within 16 h after emergence from the soil and kept until the desired age (the young adults emerge at the second day of the imaginal stage). About two weeks after emergence the animals leave the host-plant and re-enter the soil for diapause.

Topical application of juvenile hormone

Juvenile hormone, 100 μg dissolved in 1 μl of acetone, was applied on the dorsal part of the abdomen of each beetle by means of a micro-syringe and a Hamilton PB600-1 repeating dispenser (Micromesure N.V., The Hague, The Netherlands). Control animals received acetone only.

Determinations of cytochrome concentration

Homogenates of the thoraces from 30 or 60 beetles, depending on the age of the animals, were prepared as described by BARTELINK *et al.* (1974b). The concentrations of cytochromes aa_3, *b* and *c* in the homogenates were evaluated from dithionite-reduced *minus* ferricyanide-oxidized difference

spectra recorded at both room temperature and the temperature of liquid nitrogen. The spectrophotometric methods, spectra evaluation procedures and extinction coefficients have been described in detail elsewhere (BARTELINK *et al.*, 1974b).

Protein content of the homogenates was determined on defatted trichloroacetic acid precipitates according to the method of LOWRY *et al.* (1951) with bovine serum albumin as the standard.

Determination of amino acid incorporation

Flight-muscle mitochondria were isolated under semi-sterile conditions from 60 beetles starved overnight as reported previously (BARTELINK *et al.*, 1974a). The mitochondria were incubated for 30 min at 25° C with an optimized incorporation medium (KROON & DE VRIES, 1971; BARTELINK *et al.*, 1974a) containing 50 mM sucrose, 50 mM Tricine-KOH buffer (pH 7.6), 1 mM EDTA, 0.1 mM EGTA [ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetra-acetic acid], 40 mM KCl, 10 mM $MgCl_2$, 5 mM potassium phosphate, 2 mM ADP, 30 mM L-proline, 20 μg/ml ribonuclease (E.C. 2.7.7.16), 200 μg/ml amino acid mixture *minus* leucine (ROODYN *et al.*, 1961) and 0.01 or 0.04 mM L-[U-^{14}C]leucine (10 mCi/mmole). Additional incubation conditions, the treatment of the labelled protein and the determination of radioactivity and protein have been described earlier (BARTELINK *et al.*, 1974a).

Chemicals

Synthetic juvenile hormone was provided by Hoffmann-La Roche, Basle, Switzerland. The preparation (RO-08-9550) was a mixture of the geometrical isomers of methyl-10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate. The sources of the other chemicals have been given previously (BARTELINK *et al.*, 1974a).

RESULTS AND DISCUSSION

Cytochrome concentrations

The changes in cytochrome contents accompanying flight-muscle development and degeneration in beetles reared at a short photoperiod are depicted in fig. 1. For comparison the average concentration of cytochrome *b* in long-day animals has been included. At the moment of adult emergence, the flight muscles of the short-day beetles contained only small quantities of mitochondrial cytochromes. During the first week of the imaginal stage a distinct increase in the levels of cytochromes aa_3, *b* and c occurred. However, the cytochrome levels of short-day beetles did not, at any stage of development, approach the values previously measured in long-day beetles (BARTELINK *et al.*, 1974b). The level of the three cytochrome components reached a maximum during the second week and declined thereafter. This loss of cytochromes signifies the onset of degeneration. In deep diapause the thoraces were almost devoid of cytochromes.

By taking the cytochrome contents as a measure of mitochondrial development (see BARTELINK *et al.*, 1974b), it may be concluded that less mitochondrial mass is formed in short-day beetles, than in long-day animals. As reported earlier (DE KORT & BARTELINK, 1972), the flight-muscle mitochondria of pre-diapause beetles do show functionally normal characteristics. Respiratory and phosphorylating activities with various substrates were similar in mitochondria isolated from the flight muscles of both pre-diapause and long-day animals.

Electron-microscopic investigation has shown that the degenerated flight muscles of diapausing beetles resemble the precursor musculature at adult emergence and contain only few small mitochondria (STEGWEE *et al.*, 1963; STEGWEE, 1964; DE KORT, 1969). This agrees with the extremely low cytochrome concentrations encountered during diapause (fig. 1). It should be pointed out that in the Colorado beetle the degeneration of the flight muscles and their mitochondria is reversible. After termination of diapause fully differentiated flight muscles and mitochondria were observed (STEGWEE *et al.*, 1963; STEGWEE, 1964; DE KORT, 1969).

In vitro [^{14}C]*leucine incorporation into mitochondrial protein*

The results above indicate that functionally normal mitochondria are formed in flight muscles of pre-diapause Colorado beetles, but to a lesser extent than in long-day animals. It was of interest to investigate, whether this is reflected in the protein-synthesizing activity of the isolated organelles. As mitochondria from other systems, flight-muscle mitochondria of the Colorado beetle have the capacity to incorporate amino acids into protein *in vitro* (BARTELINK *et al.*, 1974a). The incorporation activities measured with short-day beetles as a function of age are given in fig. 2. The dotted line again represents the average values estimated at long-day conditions (BARTELINK *et al.*, 1974b). A difficulty in this type of measurements arises from the presence of an endogenous amino acid pool in the mitochondria. Changes in pool size during flight-muscle development and degeneration will influence the magnitude of protein labelling. In a previous study (BARTELINK *et al.*, 1974b), this problem was approached by estimating the [^{14}C]leucine incorporation into mitochondrial protein at different radioisotope concentrations in the incubation medium. The plot of the reciprocal values of the amount of [^{14}C]leucine incorporated against the reciprocals of the isotope concentration (AB, 1968) gave a straight line. In the graph the intercept with the Y-axis equals the reciprocal value of the [^{14}C]leucine incorporation at infinite precursor concentration. Routinely, this value was determined by incubations at two concentrations of [^{14}C]leucine (0.01 and 0.04 mM).

Fig. 2 shows that except at day 8 the averages of the incorporation rates obtained with short-day beetles were somewhat lower than the rates measured during flight-muscle development in long-day animals.

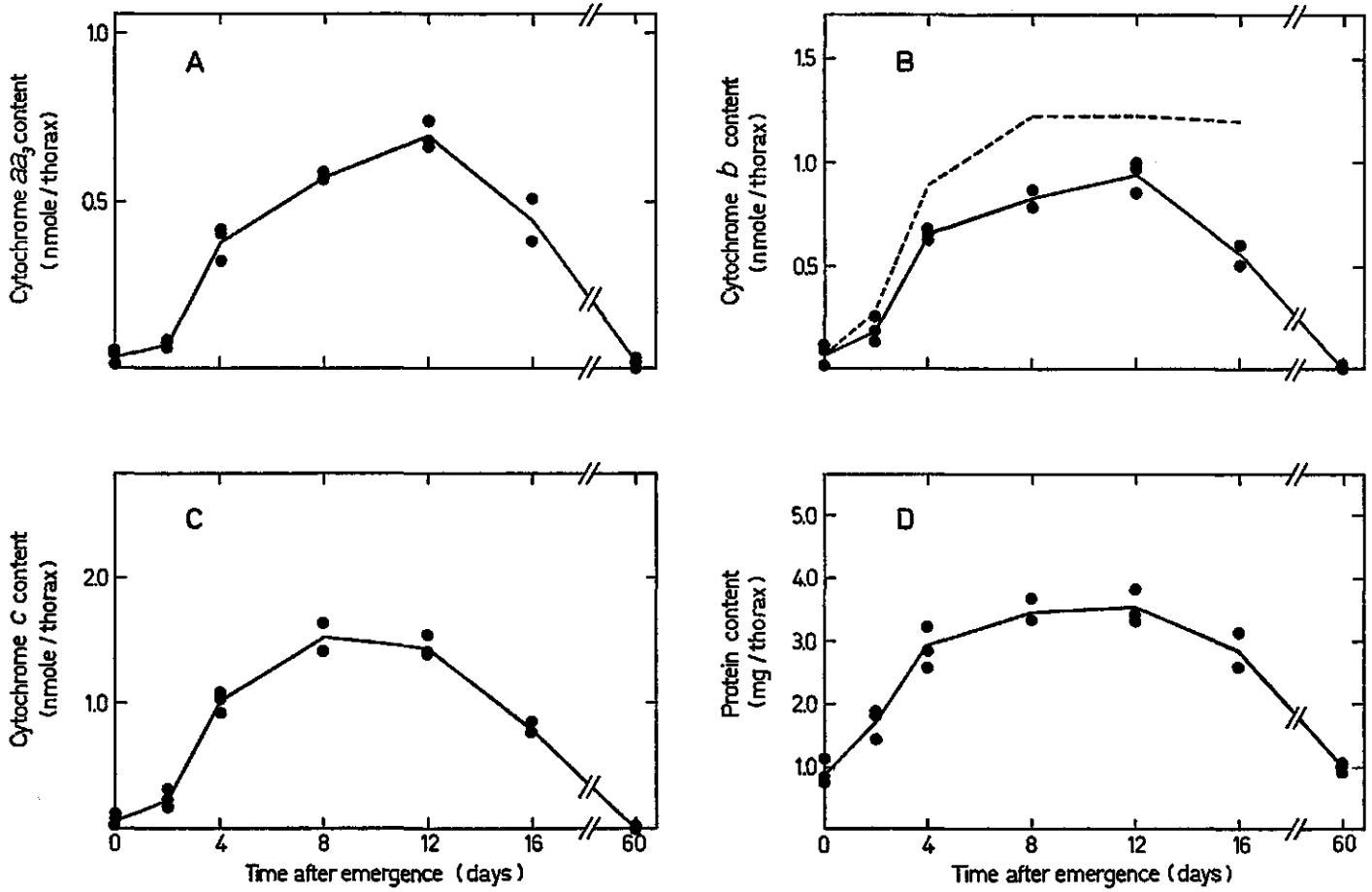

Fig. 1. Cytochrome concentrations and total protein content in thoraces of short-day beetles. Homogenates of thoraces were prepared at the indicated stages. The concentrations of cytochrome aa_3 (A), cytochrome b (B) and cytochrome c (C) were estimated from dithionite-reduced *minus* ferricyanide-oxidized difference spectra. The details of assay and the measurement of protein content (D) are described in the MATERIALS AND METHODS section. At each stage 2–3 separate homogenates were analysed. The dotted line represents the variation in cytochrome b content during flight-muscle development in long-day beetles (after BARTELINK *et al.*, 1974b).

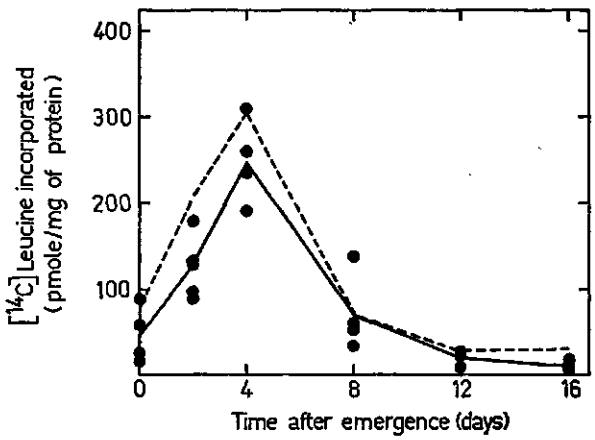

Fig. 2. [14C]Leucine incorporation into protein by isolated flight-muscle mitochondria from short-day beetles. Mitochondria were isolated at the indicated times and incubated for 30 min with 0.01 and 0.04 mM [14C]leucine (10 mCi/mmole). The [14C]leucine incorporation at infinite radioisotope concentration is given, as determined by extrapolation from reciprocal plots (see BARTELINK *et al.*, 1974b). Each point represents a separate mitochondrial preparation. The averages of the incorporation values measured with long-day beetles are connected by the dotted line (after BARTELINK *et al.*, 1974b).

Very low incorporation activities were found with mitochondria isolated from short-day beetles 16 days after emergence. Apparently, a condition of decreasing juvenile hormone titer is associated with reduced rates of mitochondrial protein synthesis. To establish this point more firmly the effect of the opposite treatment was explored.

Effect of juvenile hormone on [14C]leucine incorporation

Synthetic juvenile hormone was administered to 16-day-old beetles, reared at a short photoperiod. This treatment breaks diapause behaviour. Within a week after application of 100 μg of the hormone, SCHOONEVELD (1973) observed a reactivation of all animals. The response of the flight-muscle mitochondria is shown in table 1. Two days after hormone administration the level of [14C]leucine incorporation was three times greater than with mitochondria from control animals and, after four days the incorporation activity had increased to about six times the control value. No effect was found if the mitochondria were isolated immediately following hormone treatment.

Although the stimulation was clearly significant at day 4, the level of incorporation by no means reached the incorporation found during mitochondrial development in long-day beetles (see fig. 2, dotted line). Longer periods of exposure to juvenile hormone were not tested, because the treated animals leave the soil after the fourth day and start feeding. This might introduce undesirable side effects.

TABLE 1

Effect of juvenile hormone application on [^{14}C]leucine incorporation into protein by isolated flight-muscle mitochondria.

Time after hormone application (days)	[^{14}C]Leucine incorporated (pmole/mg of protein)	
	Control animals	Juvenile hormone-treated animals
0	5.9 ± 1.3	6.1 ± 1.2
2	4.7 ± 0.4	15.7 ± 2.6 [1])
4	5.0 ± 1.3	29.3 ± 5.9 [1])

To 16-day-old beetles, reared at a short photoperiod, 100 μg of juvenile hormone in acetone was applied. Control animals received acetone only. The beetles were used immediately or kept in plastic vials with moist sand. Each vial contained one female and one male. These animals rapidly entered the soil. Flight-muscle mitochondria were isolated and incubated for 30 min with 0.01 and 0.04 mM [^{14}C[leucine (40 mCi/mmole) as described in the MATERIALS AND METHODS section. The [^{14}C] leucine incorporation at infinite radioisotope concentration was determined from double reciprocal plots. The averages (± S.E.M.) of these values from five different experiments are given.

[1]) Significantly different from values of control animals ($p<0.01$), as determined by Student's t-test.

The above correlation between hormonal status and mitochondrial protein synthesis reminds of results described by MINKS (1967) with *Locusta migratoria*. Fat-body mitochondria isolated from allatectomized locusts were distinctly less active in amino acid incorporation than mitochondria from control animals. Analogous observations were made with mammalian systems. The incorporation activity measured with liver mitochondria from hypophysectomized rats was nearly half that of normal liver mitochondria. Treatment of the operated rats with growth hormone stimulated the incorporation rate significantly (MADDAIAH *et al.*, 1973). Furthermore, injection of growth hormone into normal mice enhanced the amino acid incorporation by the liver mitochondria *in vitro* (DELANEY & FENTON, 1967; DELANEY *et al.*, 1967). Castration of adult rats resulted in a decrease of prostate mitochondrial amino acid incorporation. Activity was restored by testosterone administration (PEGG & WILLIAMS-ASHMAN, 1968). KOTHS *et al.* (1972) reported similar effects of castration and testosterone on mouse-kidney mitochondria. Also relevant are the studies on the mode of action of thyroid hormones. Amino acid incorporation into protein by rat-hepatic mitochondria *in vitro* was depressed after thyroidectomy. Tri-iodothyronine or thyroxine administered to hypothyroid animals caused a stimulation of the incorporation activity (BRONK, 1963; ROODYN *et al.*, 1965; GORDON *et al.*, 1973; SATAV *et al.*, 1973).

The observations cited indicate that in several systems mitochondrial protein synthesis is affected by hormonal action. This raises the question

as to whether the hormone interacts directly upon the mitochondria. Our observation that juvenile hormone application did not produce an immediate stimulation of amino acid incorporation (table 1), does not preclude a primary hormonal effect on flight-muscle mitochondria. The lag time may be due to a slow penetration of the hormone through the cuticle. We therefore studied the influence of direct addition of juvenile hormone to the mitochondria *in vitro*.

Flight-muscle mitochondria were isolated from short-day beetles 16 days after emergence and incubated with the medium described under Materials and Methods. To each incubation tube juvenile hormone dissolved in 10 μl ethanol was added, giving final concentrations of 0, 0.002, 0.02, 0.2 and 2 μg of hormone per ml. After 10 min the incorporation of [^{14}C]leucine (0.04 mM; 40 mCi/mmole) was measured during a 30 min labelling period. The concentration of 0.02 μg hormone/ml corresponds, in the Galleria bioassay, with the juvenile hormone titer in the haemolymph of active long-day beetles (DE WILDE *et al.*, 1968; H. SCHOONEVELD, personal communication). In three experiments no effect of juvenile hormone whatsoever could be detected.

It is not probable that the lack of effect was due to the low water solubility of juvenile hormone. With a similar experimental design, CONGOTE *et al.* (1969) did find a stimulation of RNA polymerase activity in isolated fat-body nuclei from *Calliphora erythrocephala*. Hence, we conclude that juvenile hormone has no direct effect on *in vitro* protein synthesis by flight-muscle mitochondria. This is in contrast to the observations that thyroid hormones are able to stimulate *in vitro* amino acid incorporation by rat- and chick-liver mitochondria (KANDEMIR *et al.*, 1966; PRIMACK *et al.*, 1972; PRIMACK & TAPLEY, 1974). Similarly, cyclic AMP and dibutyryl cyclic AMP were found to increase protein synthesis in mitochondria isolated from rat-cerebral cortex and liver (BOSMANN, 1971). In both cases, however, rather high concentrations of the hormonal principle were used. Tri-iodothyronine over the range 0.001–10 μM failed to accelerate amino acid incorporation into protein by mitochondria from rat liver (ROODYN *et al.*, 1965). GORDON *et al.* (1973) presented evidence that the stimulation *in vitro* by thyroxine is mediated by other mechanisms than the increase observed under *in vivo* conditions. Thus, the physiological relevance of the effects *in vitro* is as yet questionable. It may be added that also direct supply of testosterone to prostate mitochondria from normal or androgen-deficient animals did not influence the incorporation activity (PEGG & WILLIAMS-ASHMAN, 1968).

On the basis of the present results we suggest that in the flight muscles of the Colorado beetle the mitochondrial response to juvenile hormone is indirect. Which mechanism is intercalated, remains unclear. From the biochemical point of view it is attractive to suppose that the nucleo-ergastoplasmic genetic system is involved. In endocrinological terms one can hypothesize about a role of neuro-endocrine centra (see KEELEY,

1972a, b; SCHOONEVELD, 1972). Further research will be needed to discriminate between these and other possibilities.

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REFERENCES

AB, G., Eiwitsynthese in een celvrij leversysteem. Ph. D. thesis, State University of Groningen (1968).

BARTELINK, A. K. M. & C. A. D. DE KORT, Synthesis of mitochondrial protein in the flight muscles of the Colorado beetle. Differentiation *in vivo* between extra- and intra-mitochondrial contributions to the amino acid incorporation into mitochondrial protein. Biochem. J. 136, 795–802 (1973).

———, ——— & G. J. J. KORTSTEE, Amino-acid incorporation into protein by isolated flight muscle mitochondria from the Colorado beetle. Insect Biochem., 4, 313–323 (1974a).

———, W. P. GIJZEL, C. A. D. DE KORT & A. M. KROON, Mitochondrial formation in the developing flight muscles of the Colorado beetle. Develop. Biol., in the press (1974b).

BOSMANN, H. B., The effects of adenosine 3′,5′-monophosphate, dibutyryl cyclic 3′,5′-AMP, and 5′-AMP on the synthesis of macromolecules by isolated mitochondria. Cytobios 4, 121–128 (1971).

BRONK, J. R., Thyroid hormones: Control of terminal oxidation. Science 141, 816–818 (1963).

CONGOTE, L. F., C. E. SEKERIS & P. KARLSON, On the mechanism of hormone action. XIII. Stimulating effects of ecdysone, juvenile hormone and ions on RNA synthesis in fat body cell nuclei from *Calliphora erythrocephala* isolated by a filtration technique. Exp. Cell Res. 56, 338–346 (1969).

DE KORT, C. A. D., Hormones and the structural and biochemical properties of the flight muscles in the Colorado beetle. Commun. Agricultural University Wageningen 69-2, 1–63 (1969).

——— & A. K. M. BARTELINK, Biochemical maturation of flight muscle mitochondria in the Colorado beetle, *Leptinotarsa decemlineata* Say. Comp. Biochem. Physiol. 43B, 1009–1017 (1972).

DELANEY, P. F. & P. F. FENTON, Role of growth hormone and dietary adaptation on incorporation of leucine into a mitochondrial protein. Proc. Soc. Exp. Biol. Med. 125, 832–834 (1967).

DELANEY, Jr., P. F., M. J. KEYES & T. I. SOULE, Growth hormone stimulation of protein synthesis in isolated mouse liver mitochondria. Endocrinology 80, 772–774 (1967).

De Wilde, J., The significance of the photoperiod for the occurrence of diapause in the adult *Leptinotarsa decemlineata* Say. Proc. First International Photobiological Congress (1955).

——— & J. A. de Boer, Physiology of diapause in the adult Colorado beetle. II. Diapause as a case of pseudo-allatectomy. J. Insect Physiol. 6, 152–161 (1961).

——— & D. Stegwee, Two major effects of the corpus allatum in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). Arch. Néerl. Zool. (Suppl.) 13, 277–289 (1958).

———, C. S. Duintjer & L. Mook, Physiology of diapause in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). I. The photoperiod as a controlling factor. J. Insect Physiol. 3, 75–85 (1959).

———, G. B. Staal, C. A. D. de Kort, A. de Loof & G. Baard, Juvenile hormone titer in the haemolymph as a function of photoperiodic treatment in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam 71C, 321–326 (1968).

Gordon, A., M. I. Surks & J. H. Oppenheimer, Thyroxine stimulation of amino acid incorporation into mitochondrial protein: Differences between *in vivo* and *in vitro* effects. Acta Endocrinol. 72, 684–696 (1973).

Kandemir, N., E. Eich, J. Alfano & R. L. Greif, Some effects of thyroxine on mitochondria from livers of newborn and partially hepatectomized rats. Endocrinology 78, 505–510 (1966).

Keeley, L. L., Biogenesis of mitochondria: Neuroendocrine effects on the development of respiratory functions in fat body mitochondria of the cockroach, *Blaberus discoidalis*. Arch. Biochem. Biophys. 153, 8–15 (1972a).

———, Neuroendocrine regulation of insect metabolism and the influence of nutrition. In: Insect and Mite Nutrition, (J. G. Rodriguez, ed.), pp. 541–554, North-Holland, Amsterdam (1972b).

Koths, K. E., W. Godchaux, L. H. Doeg & K. A. Doeg, The effect of testosterone on the synthesis of mouse kidney mitochondrial and microsomal proteins *in vivo* and *in vitro*. Endocrinology 91, 125–134 (1972).

Kroon, A. M. & H. de Vries, Mitochondriogenesis in animals cells: Studies with different inhibitors. In: Autonomy and Biogenesis of Mitochondria and Chloroplasts (Boardman, N. K., A. W. Linnane & R. M. Smillie, Eds.), pp. 318–327, North-Holland Publishing Co., Amsterdam (1971).

Lowry, O. H., N. J. Rosebrough, A. L. Farr & R. J. Randall, Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951).

Maddaiah, V. T., R. K. Sharma, V. Balachandar, I. Rezvani, P. J. Collipp & S. Y. Chen, Effect of growth hormone on mitochondrial protein synthesis. J. Biol. Chem. 248, 4263–4268 (1973).

Minks, A. K., Biochemical aspects of juvenile hormone action in the adult *Locusta migratoria*. Arch. Néerl. Zool. 17, 175–258 (1967).

Pegg, A. E. & H. G. Williams-Ashman, Effects of androgens on incorporation of labeled amino acids into proteins by prostate mitochondria. Endocrinology 82, 603–610 (1968).

Primack, M. P. & D. F. Tapley, Stimulation of chick liver mitochondrial protein synthesis by thyroxine without mitochondrial swelling. Endocrinology 94, 871–874 (1974).

———, ——— & J. Buchanan, Thyroid hormone stimulation of mitochondrial protein synthesis supported by an ATP generating system. Endocrinology 91, 840–844 (1972).

Roodyn, D. B., P. J. Reis & T. S. Work, Protein synthesis in mitochondria. Requirements for the incorporation of radioactive amino acids into mitochondrial protein. Biochem. J. 80, 9–21 (1961).

———, K. B. Freeman & J. R. Tata, The stimulation by treatment *in vivo* with tri-iodothyronine of amino acid incorporation into protein by isolated rat-liver mitochondria. Biochem. J. 94, 628–641 (1965).

Satav, J. G., M. S. Rajwade, S. S. Katyare, M. S. Netrawali, P. Fatterpaker & A. Sreenivasan, The significance of promitochondrial structures in rat liver for mitochondrial biogenesis. Biochem. J. 134, 687–695 (1973).

Schooneveld, H., Effects of juvenile hormone on the endocrine system during break of diapause in the Colorado beetle. J. Endocrinol. 57, LV–LVI (1972).

———, Break of diapause by juvenile hormone in the Colorado beetle, and the role of the corpora allata during subsequent reproduction. Abstr. 7th Conf. Eur. Comp. Endocrinol. Budapest, p. 274 (1973).

Stegwee, D., Respiratory chain metabolism in the Colorado potato beetle. II. Respiration and oxidative phosphorylation in 'sarcosomes' from diapausing beetles. J. Insect Physiol. 10, 97–102 (1964).

———, E. C. Kimmel, J. A. de Boer & S. Henstra, Hormonal control of reversible degeneration of flight muscle in the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera). J. Cell Biol. 19, 519–527 (1963).

Final remarks

Results presented in Paper III (Table 2) indicate that during flight-muscle development in long-day beetles changes occur in the relative activities of the two protein-synthesizing systems concerned with mitochondrial formation. The same can be inferred from the measurements on short-day animals listed in the table of page 62. It is seen that the percentage of cycloheximide-resistant amino acid incorporation into mitochondrial protein increased during the first few days after emergence. This indicates a growing contribution of the intramitochondrial system to the synthesis of organelle protein (see Paper I). In the second half of the experimental period there was a decrease in the proportion of cycloheximide-resistant incorporation. The latter observation seems an answer to the question posed in the General introduction, as to which of the two translation systems becomes disrupted in short-day beetles. However, we must remember that also with long-day animals there are low percentages of cycloheximide-resistant incorporation during the second week of adult life (Paper III, Table 2). As a consequence, the present results do not provide a conclusive answer to the above question.

In his study on flight-muscle development in the Colorado beetle, de Kort (1969) used the level of succinate dehydrogenase activity as an index of the amount of mitochondrial mass. It appeared that mitochondrial formation in beetles reared under long-day conditions took 12 days. The results of Paper III, which were obtained with the same laboratory strain, indicate that at present development of flight-muscle mitochondria is completed within 8 days. The time courses of accumulation of cytochromes shown in Paper IV also suggest a more progressive formation of mitochondria in short-day beetles than can be inferred from the data of de Kort (1969). Hence, it seems that the laboratory strain has changed over the past six years. Slightly modified results as compared with earlier trials (de Wilde, 1955; de Wilde *et al.*, 1959), were also obtained in regard to the photoperiodic response and oviposition (de Kort, 1969; Schooneveld, 1970).

Thus in the present laboratory colonies the differences in mitochondrial development between long-day and short-day beetles are not pronounced. This lack of a distinct difference has prevented the analysis of the cause of arrested mitochondrial growth. However, the present study did characterize some variables which influence mitochondriogenesis in Colorado-beetle flight muscles. The functioning of the intramitochondrial system for protein synthesis requires the presence of several cofactors (Paper II) and is further dependent on the developmental and hormonal state of the animal (Papers III and IV). The extrinsic synthesis of mitochondrial proteins is also important for the activity of the intramitochondrial system. Extension of the time of pretreatment with cycloheximide up to 60 min and more led to a decreased amino acid incorporation into mitochondrial protein (Paper I, Table 3). This does not alter the fact that long-term changes can take place in the relative contributions of the two translation systems (see Paper III and the table on page 62).

Effect of cycloheximide on [^{14}C]leucine incorporation into mitochondrial protein in flight muscles of short-day beetles[1].

Time after emergence (days)	Number of experiments	[^{14}C]Leucine incorporated (d.p.m./mg of protein)[2]		Percentage of control[3]
		Control animals	Cycloheximide-treated animals	
0	3	5636	170	3.1 (1.2–6.6)
2	5	4601	443	9.6 (8.8–11.3)
4	5	4143	553	13.8 (11.2–15.6)
8	3	1184	125	10.0 (7.9–12.7)
12	5	700	35	5.5 (2.5–8.8)
16	4	1004	46	4.7 (3.5–5.2)

1. Colorado beetles were reared as described in Paper IV. At the indicated ages beetles were treated with 5 μg of cycloheximide in Ringer solution. Control animals received Ringer solution only. After 10 min 0.1 μCi of [^{14}C]leucine (331 mCi/mmol) was administered per beetle. The flight-muscle mitochondria were isolated after a 30 min labelling period. For further experimental details, see Paper III.
2. The values represent the average specific radioactivity.
3. The average percentage is given with the range in parentheses.

After addition of ethidium bromide or chloramphenicol to growing cultures of *Neurospora crassa* Barath & Küntzel (1972a, b) observed extra increased activities of mitochondrial RNA polymerase and methionyl-tRNA transformylase. Furthermore, the biosynthesis of mitochondrial elongation factors was stimulated. It was concluded that these proteins are coded for by the nuclear genome and synthesized on cytoplasmic ribosomes and that these processes are controlled by mitochondrial protein synthesis. To account for the control Barath & Küntzel postulated a repressor-like mitochondrial gene product(s) which binds to nuclear cistrons.

The repressor mechanism and the observed effect of the extrinsic synthesis of mitochondrial proteins on the activity of the intramitochondrial system (Paper 1, Table 3; see also Millis & Suyama, 1972) might explain the co-ordinated synthesis of mitochondrial proteins. At the onset of mitochondrial growth, a high contribution of cytoplasmic ribosomes will be necessary to produce, among others, the protein components of the mitochondrial genetic apparatus (see Ch'ih & Kalf, 1969; Brega & Baglioni, 1971; Barath & Küntzel, 1972a,b; Lizardi & Luck, 1972). In the next growth phase, the rate of intramitochondrial protein synthesis can increase (cf. Paper III). The mitochondrial translation products make possible the integration of cytoplasmically-synthesized proteins into the mitochondrial inner membrane (Schatz & Mason, 1974). In addition, the manufacture of these proteins will be lowered by repressor action. An excessive repression of the extrinsic synthesis of mitochondrial proteins is prevented because shortage of the proteins will lead to inhibition of the intramitochondrial system and thereby affect repressor production. This arrangement ensures the balanced synthesis and assembly of proteins during mitochondrial formation. After growth ceases, the activity of both translation systems will be decreased. In adult rat liver, the inner mitochondrial membrane turns over more slowly than matrix and outer membrane components (Brunner & Neupert, 1968; Beattie, 1969; Aschenbrenner *et al.*, 1970). Since the products of mitochondrial protein synthesis are found predominantly in the inner membrane (Beattie *et al.*, 1967; Bandlow, 1972; Werner & Neupert, 1972; Schatz & Mason, 1974), the intrinsic translation system may suffice with a lower relative contribution in fully-developed tissues (cf. Paper III, Table 2).

Further investigations are necessary to prove or dismiss the order of events and the nucleocytoplasmic-mitochondrial interactions outlined.

References

Aschenbrenner, V., Druyan, R., Albin, R. & Rabinowitz, M. (1970). Haem *a*, cytochrome *c* and total protein turnover in mitochondria from rat heart and liver. Biochem. J. 119, 157–160.

Bandlow, W. (1972). Membrane separation and biogenesis of the outer membrane of yeast mitochondria. Biochim. Biophys. Acta 282, 105–122.

Barath, Z. & Küntzel, H. (1972a). Induction of mitochondrial RNA polymerase in *Neurospora crassa*. Nature New Biol. 240, 195–197.

Barath, Z. & Küntzel, H. (1972b). Cooperation of mitochondrial and nuclear genes specifying the mitochondrial genetic apparatus in *Neurospora crassa*. Proc. Nat. Acad. Sci. USA 69, 1371–1374.

Beattie, D. S. (1969). The turnover of the protein components of the inner and outer membrane fractions of rat liver mitochondria. Biochem. Biophys. Res. Commun. 35, 721–727.

Beattie, D. S., Basford, R. E. & Koritz, S. B. (1967). The inner membrane as the site of the *in vitro* incorporation of L-[^{14}C]leucine into mitochondrial protein. Biochemistry 6, 3099–3106.

Brega, A. & Baglioni, C. (1971). A study of mitochondrial protein synthesis in intact HeLa cells. Eur. J. Biochem. 22, 415–422.

Brunner, G. & Neupert, W. (1968). Turnover of outer and inner membrane proteins of rat liver mitochondria. FEBS Lett. 1, 153–155.

Ch'ih, J. J. & Kalf, G. F. (1969). Studies on the biosynthesis of the DNA polymerase of rat liver mitochondria. Arch. Biochem. Biophys. 133, 38–45.

de Kort, C. A. D. (1969). Hormones and the structural and biochemical properties of the flight muscles in the Colorado beetle. Ph. D. Thesis, University of Nijmegen.

de Wilde, J. (1955). The significance of the photoperiod for the occurrence of diapause in the adult *Leptinotarsa decemlineata* Say. Proc. First Intern. Photobiol. Congr.

de Wilde, J., Duintjer, C. S. & Mook, L. (1959). Physiology of diapause in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). I. The photoperiod as a controlling factor. J. Insect Physiol. 3, 75–85.

Lizardi, P. M. & Luck, D. J..L. (1972). The intracellular site of synthesis of mitochondrial ribosomal proteins in *Neurospora crassa*. J. Cell Biol. 54, 56–74.

Millis, A. J. T. & Suyama, Y. (1972). Effects of chloramphenicol and cycloheximide on the biosynthesis of mitochondrial ribosomes in *Tetrahymena*. J. Biol. Chem. 247, 4063–4073.

Schatz, G. & Mason, T. L. (1974). The biosynthesis of mitochondrial proteins. Annu. Rev. Biochem. 43, 51–87.

Schooneveld, H. (1970). Structural aspects of neurosecretory and corpus allatum activity in the adult Colorado beetle, *Leptinotarsa decemlineata* Say, as a function of daylength. Neth. J. Zool. 20, 151–237.

Werner, S. & Neupert, W. (1972). Functional and biogenetical heterogeneity of the inner membrane of rat-liver mitochondria. Eur. J. Biochem. 25, 379–396.

Summary

This thesis deals with the biogenesis of flight-muscle mitochondria in the Colorado beetle (*Leptinotarsa decemlineata* Say).

In beetles, reared under conditions of long photoperiod, the activities of succinate dehydrogenase and cytochrome *c* oxidase and the contents of cytochromes aa_3, *b*, and *c* were found to increase roughly in parallel. This process starts around adult emergence and is completed after 8 days. The above enzymes provide an index of mitochondrial growth.

Particular attention has been paid to the relative contributions of the two genetic systems concerned in mitochondriogenesis. *In vivo*, the incorporation of [^{14}C]leucine was measured after administration of cycloheximide, a specific inhibitor of cytoplasmic protein synthesis. The incorporation into the cytosol protein of the flight muscles appeared to be inhibited by more than 99.5% with the antibiotic. However, a considerable amount of radioactivity was found in the protein of mitochondria. At the beginning of mitochondrial formation and in beetles with full-grown flight muscles the amino acid incorporation amounted to about 5% of the control value. During the period of rapid accumulation of mitochondrial enzymes, the percentage of cycloheximide-resistant incorporation increased to 14. This incorporation was sensitive to the inhibitor of mitochondrial protein synthesis, chloramphenicol.

The incorporation activity of the isolated mitochondria *in vitro* was also examined. Attempts were made to elaborate a suitable incubation medium. It appeared, that proline was the most effective respiratory substrate in promoting the incorporation of [^{14}C]leucine into protein. Optimum incorporation furthermore required appropriate concentrations of KCl, ADP, phosphate, Mg^{2+}- and H^{+}-ions, and a synthetic amino acid mixture. Subsequently, the incorporation activity was estimated at various times during mitochondrial formation. Maximum rates of amino acid incorporation were recorded in the phase of rapid enzyme accumulation. Apparently, formation of mitochondria involves an enhanced contribution of the intrinsic translation system.

While beetles reared at long photoperiods display locomotion and oviposition, treatment with a short-day régime induces diapause. In pre-diapause animals a development of the cytochrome system can be found after adult emergence, but the increase proceeds more slowly than in long-day beetles. This increase stops after one week and is then followed by a decline in cytochrome content. During deep diapause the cytochromes are hardly detectable in the flight muscles.

Incorporation experiments with pre-diapause animals *in vivo* yielded similar results to those obtained with beetles reared under long-day conditions. Firstly, there was an increase in the percentage of cycloheximide-resistant [^{14}C]leucine incorporation which later fell. Because of the similarities it is impossible to say which of the two protein-synthesizing systems is responsible for the stagnation in mitochondrial growth at short photoperiods.

The incorporation activities *in vitro* of flight-muscle mitochondria isolated from pre-diapause beetles were somewhat lower than those measured at corresponding times during normal flight-muscle development. Very low incorporation rates were recorded at the onset of diapause. Topical application of juvenile hormone not only broke diapause but also stimulated the incorporation activity of isolated mitochondria. No stimulation was found after direct addition of the hormone to the mitochondria *in vitro*. This indicates that the effect of juvenile hormone on amino acid incorporation is indirect.

Samenvatting

Dit proefschrift beschrijft een onderzoek naar de biogenese van vliegspiermitochondriën in de Coloradokever (*Leptinotarsa decemlineata* Say).

Bij kevers, die gekweekt waren bij een lange fotoperiode, werd de vorming van enkele mitochondriale enzymen tijdens de vliegspierontwikkeling bestudeerd. Het bleek, dat de activiteiten van het succinaatdehydrogenase en cytochroomoxydase en de concentraties van de cytochromen aa_3, *b* en *c* alle min of meer parallel toenamen. Dit proces begint op het moment, dat de kevers na het ontpoppen uit de grond komen en is 8 dagen later voltooid. De genoemde enzymen kunnen gelden als parameters voor de mitochondriale ontwikkeling.

Veel aandacht werd besteed aan de vraag, wat de bijdragen zijn van de twee genetische systemen die een rol spelen bij de mitochondriogenese. *In vivo* werd de incorporatie van [^{14}C]leucine gemeten na injectie van cycloheximide, een specifieke remstof van de cytoplasmatische eiwitsynthese. Terwijl de incorporatie in het cytosoleiwit van de vliegspieren voor meer dan 99,5% geremd was, werd een aanmerkelijke radioactiviteit in het eiwit van mitochondriën gevonden. Aan het begin van de mitochondriënontwikkeling en in volgroeide vliegspieren bedroeg de aminozuurincorporatie ongeveer 5% van de controlewaarde. Tijdens de periode, waarin een sterke toename van mitochondriale enzymen was waargenomen, steeg het percentage cycloheximide-resistente incorporatie tot 14. Vermeld dient te worden, dat deze incorporatie gevoelig was voor de remstof van de mitochondriale eiwitsynthese, chlooramfenicol.

Ook werd de incorporatie-activiteit van geïsoleerde mitochondriën *in vitro* onderzocht. Allereerst werd getracht een geschikt incubatiemedium samen te stellen. Hierbij bleek, dat van de geteste oxideerbare substraten, proline de incorporatie van [^{14}C]leucine in eiwit het meest effectief stimuleerde. Voor optimale incorporatie zijn tevens bepaalde concentraties KCl, ADP, fosfaat, Mg^{2+}- en H^{+}-ionen en een synthetisch aminozuurmengsel vereist. Vervolgens werd de incorporatie-activiteit op verschillende momenten tijdens de mitochondriënontwikkeling gemeten. Maximale aminozuurincorporatie vond plaats in de fase van snelle enzymvorming. Klaarblijkelijk gaat mitochondriale ontwikkeling gepaard met een verhoogde relatieve en absolute bijdrage van het intrinsieke translatiesysteem.

Vertonen de bij lange lichtperioden gekweekte kevers locomotie en ovipositie, behandeling met korte daglengten induceert, zoals bekend, diapause. Bij de prediapausedieren wordt na ontpoppen wel een ontwikkeling van het cytochroomsysteem gevonden. De toename verloopt echter minder snel en raakt na een week gestagneerd. Deze stagnatie wordt gevolgd door een daling van de cytochroomgehaltes. Tijdens diapause zijn in de inmiddels geatrofieerde vliegspieren de cytochromen nauwelijks aantoonbaar.

Incorporatie-experimenten met prediapausekevers *in vivo* gaven een overeenkomstig beeld te zien als bij dieren behandeld met lange fotoperioden. Eerst vond een toename

plaats van het percentage cycloheximide-resistente [^{14}C] leucine-incorporatie, later een daling. Als gevolg hiervan is geen conclusie mogelijk met betrekking tot de vraag, welke van de twee eiwitsynthetiserende systemen de stagnatie in de mitochondriale groei bij prediapausedieren veroorzaakt.

De incorporatie-activiteiten *in vitro* van vliegspiermitochondriën geïsoleerd uit prediapausekevers waren meestal enigszins lager dan op overeenkomstige tijdstippen in de normale vliegspierontwikkeling. Zeer lage incorporatiewaarden werden gemeten bij kevers, die op het punt stonden in diapause te gaan. Behandeling van deze kevers met juveniel hormoon leidde niet alleen tot een verbreking van de diapausereactie, maar ook tot een stimulatie van de incorporatie-activiteit van geïsoleerde mitochondriën. Deze stimulatie werd niet gevonden na directe toevoeging van het hormoon *in vitro*. Het effect van juveniel hormoon op de aminozuurincorporatie is dus waarschijnlijk secondair.

Curriculum vitae

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