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 intra-mitochondrial 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-¹⁴C]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-[¹⁴C]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 [¹⁴C]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-[¹²C]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 6000g 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 100000g 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 6000g. The supernatant fluid was then centrifuged in a Christ 9530 rotor at 100000g 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 100000g supernatant were added to equal volumes of cold 10% (w/v) trichloroacetic acid, containing 10mM-L-[¹²C]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-MgCl₂ 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-HClO₄ and washing the resulting precipitates as described by Tanguay & Chaudhary (1971). The final residues were extracted with 0.5M-HClO₄ 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 $\mu\text{g}/\text{mg}$ 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		

* 10-20 μg 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 1.4 μ g of DNA and 5 μ g of RNA per mg of protein (Lennie *et al.*, 1967). Tanguay & Chaudhary (1971) found 1.2 μ g 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* 4.6 μ g 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 100 000g supernatant protein are shown. After the administration of 1 μ g of this cytoplasmic ribosomal inhibitor per animal the radio-

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 0.1 μ Ci of [14 C]leucine (331 mCi/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 10 min before the injection of 0.1 μ Ci of [14 C]leucine per animal. After a 30 min labelling period the thoraces were fractionated as indicated in the Materials and Methods section. The 100% value for the mitochondrial fraction amounted to 3013 d.p.m./mg of protein; the control of the 100000g supernatant was 3402 d.p.m./mg of protein. ●, Mitochondrial protein; ○, 100000g supernatant protein.

activity incorporated into mitochondrial protein is decreased to about 13% of the control value. An increase in the amount of administered cycloheximide up to 5–10 μ g per animal does not lead to a further inhibition. These quantities of cycloheximide inhi-

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

Six beetles each received 0.1 μ Ci of [14 C]leucine. After 30 min 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 5 μ g of cycloheximide 10 min before the injection of 0.1 μ Ci 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 5 μ g of cycloheximide per animal was sufficient to block cytoplasmic protein synthesis for all 3 h.

bited the [14 C]leucine incorporation into the protein of the 100 000g supernatant by more than 99.5%. In six experiments the percentage of [14 C]leucine incorporated into mitochondrial protein after injection of 5 μ g 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 10 min 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 5 μ g of cycloheximide at different times before the injection of 0.1 μ Ci of [14 C]leucine. The flight-muscle mitochondria were isolated after a 30 min 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 150 μ g 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 100 000g supernatant protein. The specific radioactivities of the protein after administration of 0, 50, 100 and 150 μ g of chloramphenicol were in Expt. a 2903, 2822, 2932 and 3042 d.p.m./mg of protein respectively and in Expt. b 3480, 2632, 3536 and 3366 d.p.m./mg of protein. In these experiments beetles were treated with chloramphenicol 30 min before the injection of 0.1 μ Ci of

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

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

[^{14}C]leucine; after another 30min the thoraces were fractionated to yield the 100000g 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 $5\mu\text{g}$ of cycloheximide 10min before the injection of $0.1\mu\text{Ci}$ 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 $0.34\mu\text{Ci}/\mu\text{mol}$ respectively and in Expt. b they were 0.31 and $0.39\mu\text{Ci}/\mu\text{mol}$. 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. Sebalde *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 $100\mu\text{g}$ 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 de-repressed 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.

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