

AUTONOMY AND BIOGENESIS OF MITOCHONDRIA AND CHLOROPLASTS

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FORMATION OF MITOCHONDRIA OF LOCUSTA MIGRATORIA FLIGHT MUSCLE

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As you may remember, not long ago a well-known pioneer in chondriology called 'the tendency to erect the mitochondria to the dignity of an organelle or cell organ ... a blunder' [1]. Indeed, until the early sixties the multiplication of preexisting mitochondria was just one of several speculations concerning the cellular origin of mitochondria. Other mechanisms postulated by experienced investigators and carefully discussed in Rouiller's and Novikoff's reviews [2,3] were the synthesis *de novo* and the transformation of other cellular structures, such as the plasma membrane, the nuclear envelope, the endoplasmic reticulum, etc. [2,3]. With respect to multiplication, the splitting of mitochondria had been repeatedly observed [4]. The important question was whether mitochondria can grow [5], i.e., whether their mass could increase by a continuous process of insertion of specific material.

It was obvious that this problem could not be solved by morphological or biochemical investigations alone. Rather, a combination of both methods was required and that on a quantitative basis. In 1962 such studies were started, using different experimental approaches on quite different types of cells or tissues [6–8]. The current findings from our experiments with the wing depressors of the African migratory locust will be reported here. The wing depressors are a pair of strong muscle bundles, situated dorsal and longitudinal in the thorax of this orthopteran insect. They produce most of the work during flight [9].

In the days before and after the imaginal moult, the volume of chondriome in the flight muscle increases 25- to 30-fold during the formation of the muscle from a small precursor. This is mainly due to

an increase in volume of the single mitochondrion (fig. 1). Simultaneously, the content of inner membrane per unit of mitochondrial volume increases by a factor of 1.4. Therefore, the mature flight muscle contains about 40 times more mitochondrial membranes than its precursor at the beginning of the preimaginal interval.

In general, the enzyme activities of the cristae and of the matrix spaces increase by this same factor. During all phases of formation, the morphologically visible mitochondrial elements are enzymatically fully equipped [8,11]. This is established by the correlation of histiometric and biochemical results, and is demonstrated here for the final phase of the development of the flight muscle (table 1), the so-called duplication phase. In the beginning of this phase, around the fourth day after the imaginal moult, all histiometric and enzymatic parameters are present in proportions identical to those of the completely formed flight muscle. However, a doubling of all these parameters occurs. This is also illustrated by the cross-sections in fig. 2.

These findings present clear evidence of the ability of pre-existing mitochondria to grow. Moreover, in the beginning as well as at the end of this phase all cellular membranes of sarcolemma, sarcoplasmic reticulum and of nuclear and mitochondrial envelopes are clearly visible and separated from each other. Therefore, any growth by transformation of extra-mitochondrial structures is excluded.

After injection of an insect with labeled amino acids (fig. 3), the label can be found in all protein fractions in about equal proportions. However, the specific activity measured in the flight muscle declines markedly during the final phase. When mito-

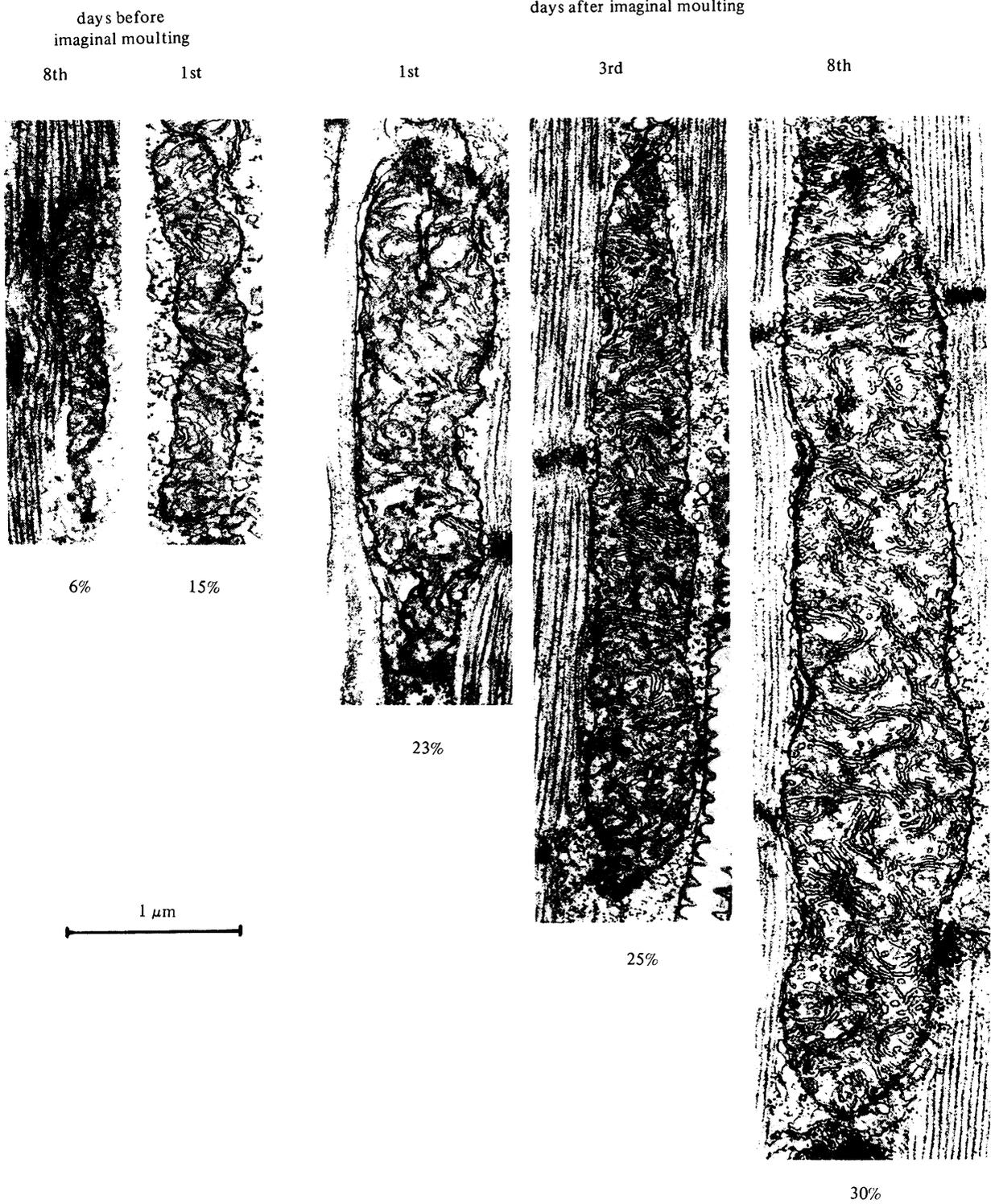


Fig. 1. The shape of mitochondria and percent of total volume during development of the flight muscle [11].

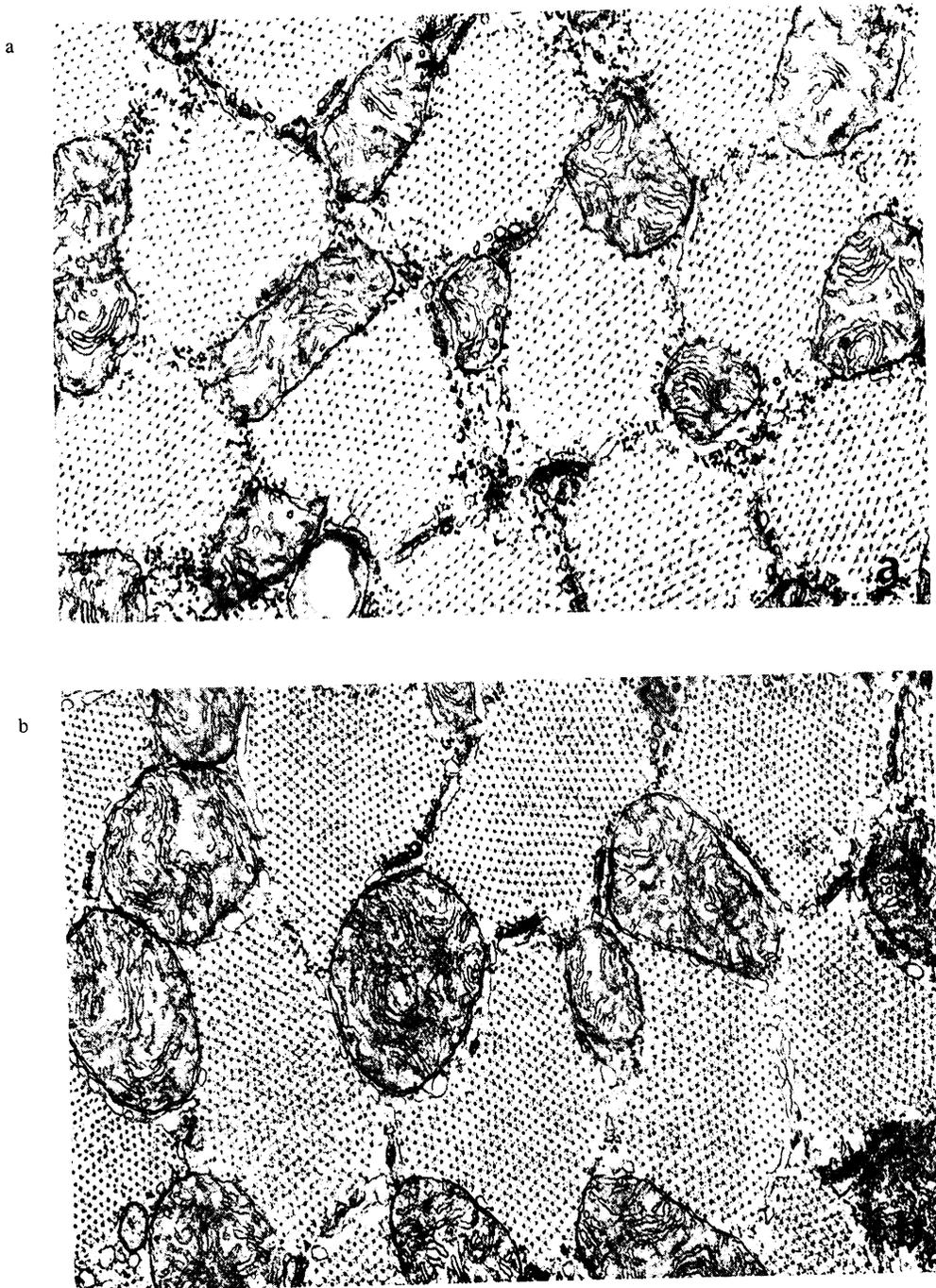


Fig. 2. Cross-sections ($\times 30,000$) of the dorsal longitudinal muscle at the beginning and the end of the duplication phase, at 4th day (a) and at 8th day (b) after imaginal moult [8].

(a) Cross-sections of fibrils are rounded and clearly distinct. The number of filaments seen on a cross section of a fibril amounts approximately to 200.

(b) Contrary to (a), this cross section shows a significant increase in the diameter of mitochondria, and their inner structure is more closely packed. The cross sections of fibrils are nearly unchanged, but the number of filaments therein has doubled to about 400 as compared with 200 in (a).

Table 1
Histiometric and enzymic data of the wing depressors at the beginning and the end of the final (duplication) phase of development^a

Days after imaginal moulting	2-4	8	Increase by factor of
Fresh weight $\times 10^3$ (g)	30	45	
Mitochondria			
Volume fraction occupied by chondriome ^b	25%	30%	
Membrane profile $\times 10^{-3}$ (cm/cm ²) ^c	450	560	
Membrane area (cm ²) ^d	3,600	8,000	2.2
α -Glycerolphosphate oxidase (U) ^e	1.05	2.27	2.2
Cytochrome <i>c</i> (nmoles) ^f	0.75	2.5	3.3
Condensing enzyme (U) ^g	2.40	5.0	2.1
Myofibrils			
Number per muscle fiber	ca 500	800-1,000	1.6-2
Myofilaments per fibril	210 \pm 20	430 \pm 20	2.1
Glycolytic enzymes			
Glyceraldehydephosphate dehydrogenase (U) ^h	4.40	10.8	2.5
α -Glycerolphosphate dehydrogenase (U) ⁱ	1.68	3.83	2.3
Aldolase (U) ^h	0.68	1.44	2.1

^a Average values per muscle pair. U denotes units of enzymic activity. Data from ref. [8].

^b From planimetry of E-M cross-sections.

^c Profile density in mitochondrial cross-sections.

^d Volume % $\times 10^{-2} \times$ membrane profile $\times 4/\pi \times$ fresh weight/1.2.

^e FAD dependent activity bound on inner membrane.

^f Protein associated to inner membrane.

^g Extractable matrix enzyme.

^h Extractable enzymes mainly located in the I-band regions of myofibrils (10).

ⁱ NAD dependent activity.

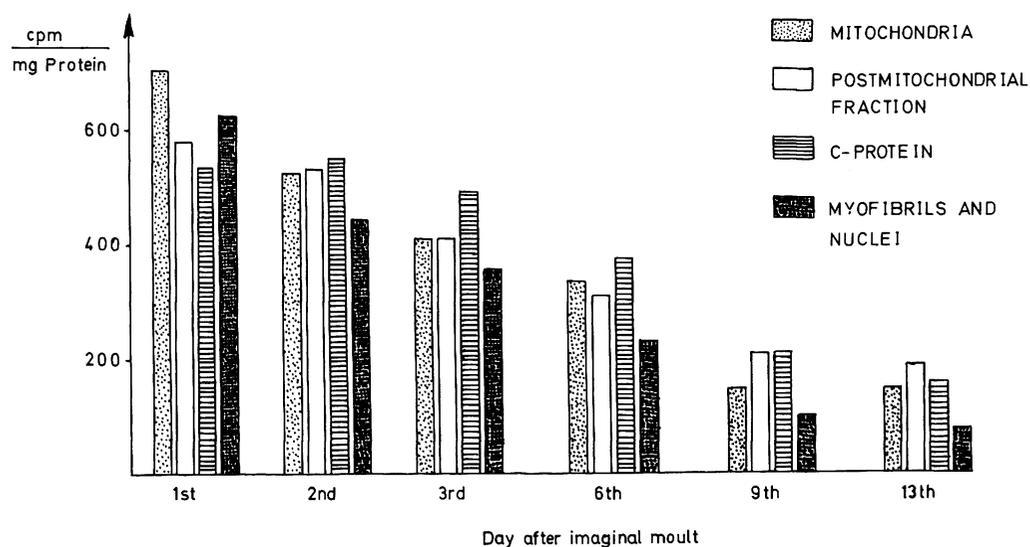


Fig. 3. Incorporation of ¹⁴C-isoleucine into protein fractions of the flight muscle at different stages of imaginal development. The specific activities are shown of total mitochondrial protein (first bar), the postmitochondrial fraction (second bar), cytosolic protein (third bar) and myofibrils (fourth bar) 24 hr after the injection of 0.1 μ C isoleucine per animal.

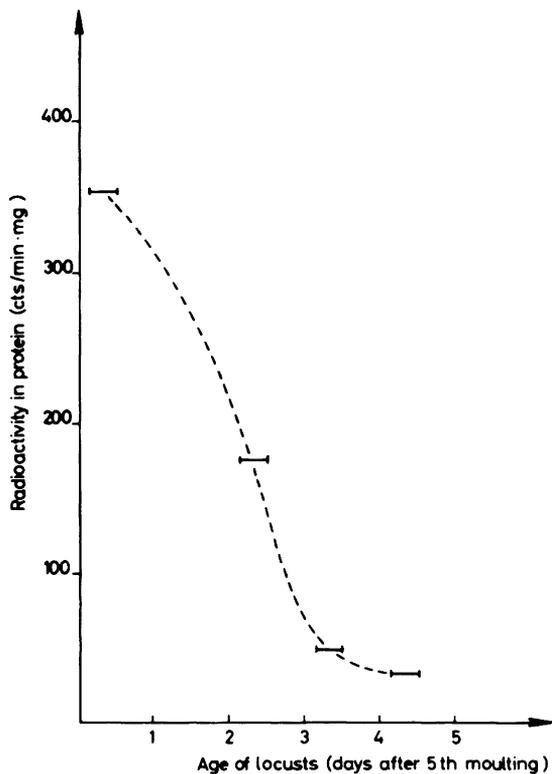


Fig. 4. Rate of incorporation of ^{14}C -isoleucine in vitro into mitochondria isolated at different stages of the developing flight muscle [12].

chondria are isolated in different phases of the muscular development and are incubated with labeled amino acids in vitro under identical conditions, the incorporation rate is observed to decrease substantially during the first days after the imaginal moult. The incorporation rate into mitochondria isolated during the duplication phase is only 10% of that found on the first day after the imaginal moult (fig. 4).

Extrinsic and intrinsic contribution to formation of mitochondria

The observations presented in the preceding section indicate that the growth of the mitochondrial membrane is coordinated with the growth of other cellular constituents. It is harmonized with the formation of mitochondrial matrix enzymes as well as with the development of the extramitochondrial enzyme

pattern and extramitochondrial structures, such as the myofibrils. To understand the mechanism of this phenomenon we must discriminate experimentally between the extrinsic and intrinsic systems of mitochondrial membrane formation.

When isolated from the insect's flight muscle the mitochondria incorporate amino acids only into the water-insoluble fraction of the membrane proteins [12]. This is in complete agreement with the findings of Roodyn and his followers from their experiments with mammalian mitochondria incubated in vitro [13]. Furthermore, there is a reproducible separation of the insoluble membrane proteins into more than 20 bands by electrophoresis in phenol-formic acid on polyacrylamide gel. The band pattern obtained from flight muscle mitochondria is rather similar to those of mitochondrial membrane proteins from other sources (fig. 5). Only a few of these bands are labeled by incorporation of amino acids in vitro. Analogous to mitochondria of other species, the highest activity appears in band 4 [14,15].

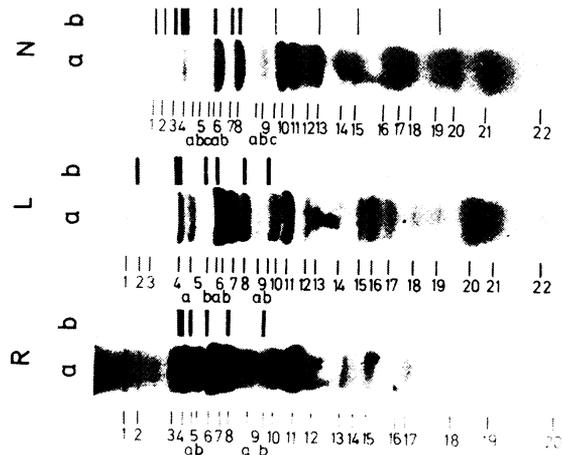


Fig. 5. Electrophoretic patterns after amido black staining (a) and in vitro labeling patterns (b) of insoluble mitochondrial proteins from *Neurospora* wild type (N), *Locusta* flight muscle (L), and rat liver (R). Mitochondria were extracted three times with phosphate buffer after sonification in order to remove soluble proteins. The resulting pellet was extracted with acetone, chloroform/methanol (2:1) and acetone and dissolved in phenol-formic acid-water (2:1:1, w/v/v). Electrophoresis was performed in 7.5% polyacrylamide plates, equilibrated with the same phenol-formic acid medium [14]. Radioactivity was determined after slicing the stained electropherograms (fig. 8).

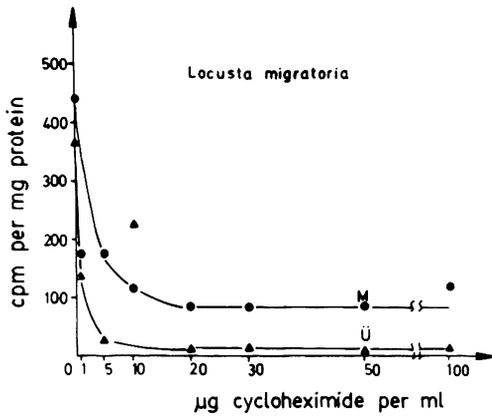


Fig. 6. Incorporation in vivo of ^{14}C -phenylalanine ($0.2\ \mu\text{C}$ per locust) into protein of whole mitochondria (M, ●---●) and $20,000g$ supernatant (U, ▲---▲) in the presence of different amounts of cycloheximide. The locusts were killed 30 min after the injection of the ^{14}C -amino acid [15].

It may be concluded from these findings that the contribution by the intrinsic system is restricted not only to the insoluble fraction of mitochondrial proteins, but even to a quantitatively minor part of this fraction.

However, the question arises as to what extent the in vitro experiments on isolated mitochondria are representative of the situation in vivo. To answer this question, imagines of locusts were injected with cycloheximide in order to block the extrinsic (nucle-

ar-dependent) protein synthesis in vivo [15]. In the presence of cycloheximide, there is a residual amino acid incorporation into mitochondrial protein (fig. 6). The incorporation pattern shows negligible incorporation into the extractable proteins of the mitochondrial matrix (table 2), as well as into the extra-mitochondrial proteins (fig. 7b). Incorporation is observed in only a limited number of bands, the main peaks of which are similar to those found in the in vitro incorporation experiments (fig. 8). The pattern obtained in vivo in the presence of cycloheximide is distinctly sharper than that obtained in vitro. This

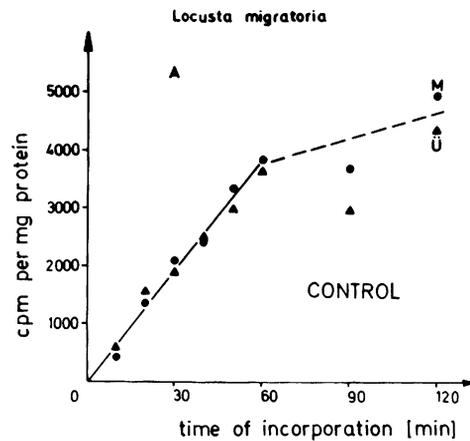


Fig. 7a. Time course of incorporation in vivo of ^{14}C -leucine plus ^{14}C -isoleucine plus ^{14}C -phenylalanine ($0.2\ \mu\text{C}$ each per locust) into protein of whole mitochondria (M, ●---●) and $20,000g$ supernatant (U, ▲---▲). Without cycloheximide.

Table 2

Specific radioactivities of submitochondrial fractions^a

Mitochondrial fractions	Mitochondrial labelling (cpm per mg protein)		
	in vivo ^b	in vivo with cycloheximide ^c	in vitro ^d
Insoluble protein	9,200	3,640	11,000
Soluble protein	8,300	540	960

^a See ref. [15].

^b 1 hr with $0.4\ \mu\text{C}$ leucine, $0.4\ \mu\text{C}$ isoleucine, and $0.4\ \mu\text{C}$ phenylalanine per locust.

^c 1 hr in the presence of $50\ \mu\text{g}$ cycloheximide per locust ($50\ \mu\text{g}$ cycloheximide was reinjected after 30 min) with $2\ \mu\text{C}$ leucine, $2\ \mu\text{C}$ isoleucine, and $2\ \mu\text{C}$ phenylalanine per locust.

^d 30 min with $1\ \mu\text{C}$ leucine, $1\ \mu\text{C}$ isoleucine, and $1\ \mu\text{C}$ phenylalanine per ml.

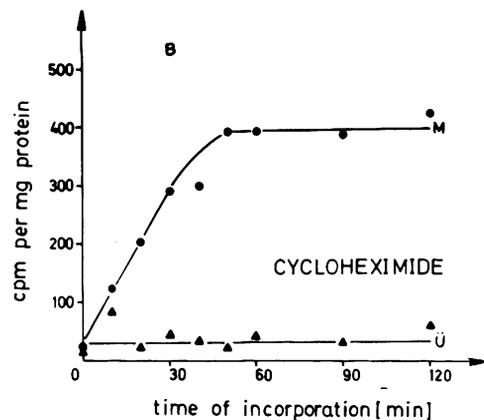


Fig. 7b. After the injection of $50\ \mu\text{g}$ cycloheximide per locust. $50\ \mu\text{g}$ cycloheximide was reinjected every 30 min [15].

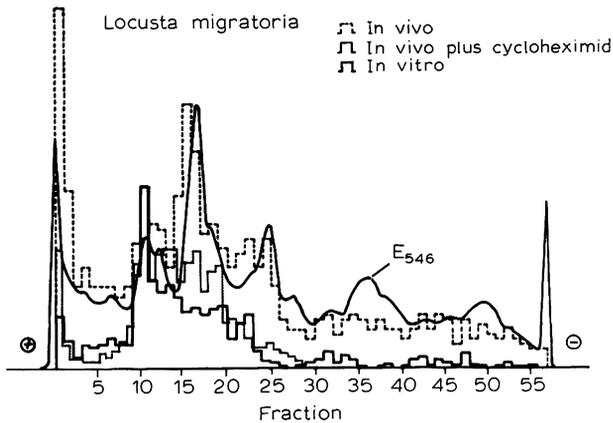


Fig. 8. Densitogram of amido black stained insoluble mitochondrial protein after gel electrophoresis (smooth line), and distribution of incorporated radioactivity over the electropherograms (edged lines). ---, in vivo (1 hr with 0.4 μ C leucine, 0.4 μ C isoleucine, and 0.4 μ C phenylalanine per locust); —, plus cycloheximide (1 hr in the presence of 50 μ g cycloheximide per locust, with 2 μ C leucine, 2 μ C isoleucine, and 2 μ C phenylalanine per locust. 50 μ g cycloheximide was reinjected after 30 min); in vitro (30 min with 1 μ C leucine, 1 μ C isoleucine, and 1 μ C phenylalanine per ml). Radioactivity in band 4 (fraction 11) was taken as equal [15].

suggests that in the in vivo experiment the number of incomplete peptide chains is less than in the in vitro experiment. In principal, however, the findings on incorporation in vitro and in vivo under action of cycloheximide are in excellent agreement. Hence, the conclusions drawn above are supported by two independent methods.

With respect to the coordination of the systems, it is remarkable, that the incorporation of amino acids by the intrinsic system continues to operate for at least 30 min after the extrinsic system is eliminated by cycloheximide (fig. 7b). This indicates a certain elasticity in the coupling of the two systems [16].

RNA-pattern

A comparison of the results presented in the preceding section with those obtained from *Neurospora* communicated by Sebald and colleagues [17] reveals a striking similarity in amino acid incorporation of isolated mitochondria, as well as in the mode

of cooperation between the extrinsic and the intrinsic systems in vivo. Moreover, strictly comparable patterns and kinetics of labeling were found. These similarities in overall performance suggest corresponding similarities of composition and function of the intrinsic systems of both types of mitochondria. Whereas recently the characterisation of mitochondrial ribosomes in *Neurospora* has improved greatly [18,19], the results on animal tissues are less satisfactory. The current state of our investigations with *Locusta* is presented briefly in the following.

After phenolization of the flight muscle homogenate or of the pellet and the supernatant from the homogenate after centrifugation (fig. 9), 8 nucleic acid bands can be differentiated by electrophoresis on polyacrylamide gel after staining with toluidine blue.

One of the bands originating from the pellet fraction disappears upon treatment of the homogenate with DNase. It seems to be a low molecular weight DNA and is called 'DNA-s' (DNA small). The other seven bands are sensitive to RNase if the enzyme is applied to the purified nucleic acid preparation. Following centrifugation of the homogenate, a band corresponding to tRNAs was found in both the pellet and the supernatant. The tRNA band found in the pellet exhibits a greater average electrophoretic mobility than the band in the supernatant. In addition, there is a band of RNA that shows a position characteristic of 5S ribosomal RNA, corresponding to a molecular weight of 40,000. Under the Mg-free conditions described below, this RNA band appears mostly in the supernatant. It is not detectable in RNA preparations from mitochondria from which most of the adhering ribosomes of the extramitochondrial system have been removed.

A discrimination between cytosolic and mitochondrial contribution to the RNA patterns is difficult because under the conditions usually employed for the isolation of mitochondria from locust flight muscle the RNA bands are nearly entirely associated with the mitochondrial fraction. With the exception of 4S RNA, no nucleic acid can be extracted from the supernatant (fig. 10). Evidently under these conditions the extramitochondrial ribosomes are firmly attached to the mitochondria. Separation is made possible by homogenizing the tissue in a magnesium-free medium in the presence of high concentrations of EDTA [22]. As shown in fig. 10, increasing

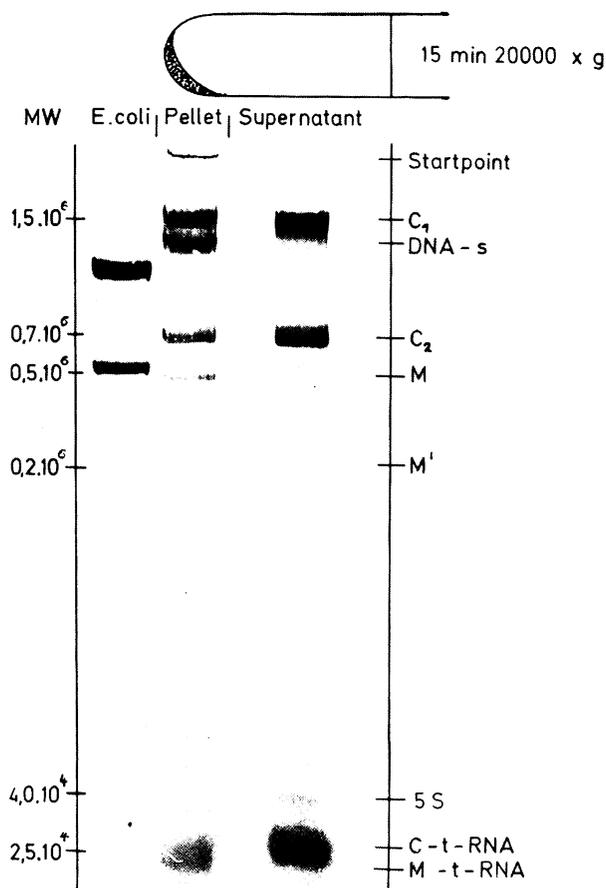


Fig. 9. Electropherograms of nucleic acids prepared from the precipitate and from the supernatant of a 15 min, 20,000 g centrifugation of a homogenate from thorax muscles of 7-day-old locusts. The homogenization was performed in 50 mM EDTA with addition of Subtilisin A.

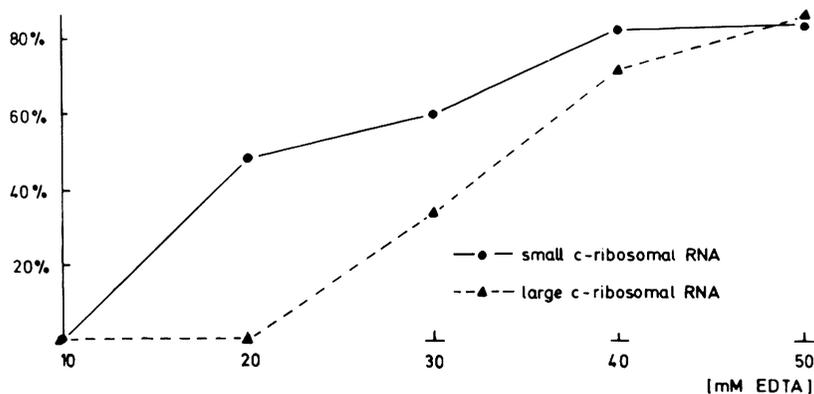


Fig. 10. Percentage of the cytosolic ribosomal RNA species appearing in the 15 min, 20,000 g supernatant at different concentrations of EDTA. Thorax muscles of 2- to 4-day-old locusts were homogenized, and the sediment and supernatant from centrifuging the homogenate 15 min at 20,000 g were examined. The homogenization medium was pH 7.2 and contained 0.3 M sucrose, 10 mM TRA and EDTA varying between 10 and 50 mM. Homogenization was accomplished by 10 strokes of a Potter homogenizer with Teflon pestle. Preparation of nucleic acids was performed by repetitive shaking with about one volume of 20% phenol:80% water and macaloid. In some cases the phenol:water was replaced by the mixture phenol:m-cresol:8-hydroxy-quinoline:water (500:70:0.5:55, w/w) following Kirby [20]. Lysis of mitochondria was accomplished by addition of 0.5 ml of 20% sodium dodecylsulphate to the first aliquot of phenol. Then the phenol was extracted by ether and the nucleic acids precipitated in the cold by two volumes of ethanol. Electrophoresis was performed according to Dingman and Peacock [21] in 2.7% acrylamide gel supported by 0.5% agarose, with Tris-borate buffer, pH 8.3, containing 2.5 mM EDTA. The relative amount of RNA corresponding to the electrophoresis band patterns was evaluated by densitographic means, from which the absolute amounts of the appropriate nucleic found in the 15 min, 20,000 g pellet and supernatant were calculated.

amounts of RNA species having molecular weights of $1,5 \times 10^6$ and of $0,7 \times 10^6$ appear in the supernatant by using increasing concentrations of EDTA. With this treatment, the smaller RNA is more easily removed from the sediment fraction than the larger one. This is similar to the action of EDTA in removing cytosolic ribosomes from liver rough endoplasmic reticulum as reported by Sabatini et al. [23]. The molecular weight of this RNA as determined from its mobility in gel electrophoresis [24,25] is in agreement with the molecular weight of cytosolic ribosomal RNA from other invertebrate animals as shown by Loening [26].

Upon treatment with 50 mM EDTA, about 10–15% of the c-ribosomal RNA remains in the mitochondrial fraction. This residue can be removed almost completely by gradient centrifugation of Subtilisin A treated homogenates (fig. 11). On the continuous gradient the flight muscle mitochondria separate into two fractions (fig. 11). The electrophoresis band patterns of the RNA isolated from these two fractions are almost identical. In addition to a prominent tRNA band with an electrophoretic mobility slightly different from that in the supernatant, there is another strong band corresponding to a molecular weight of about 500,000. Quantitatively – as deter-

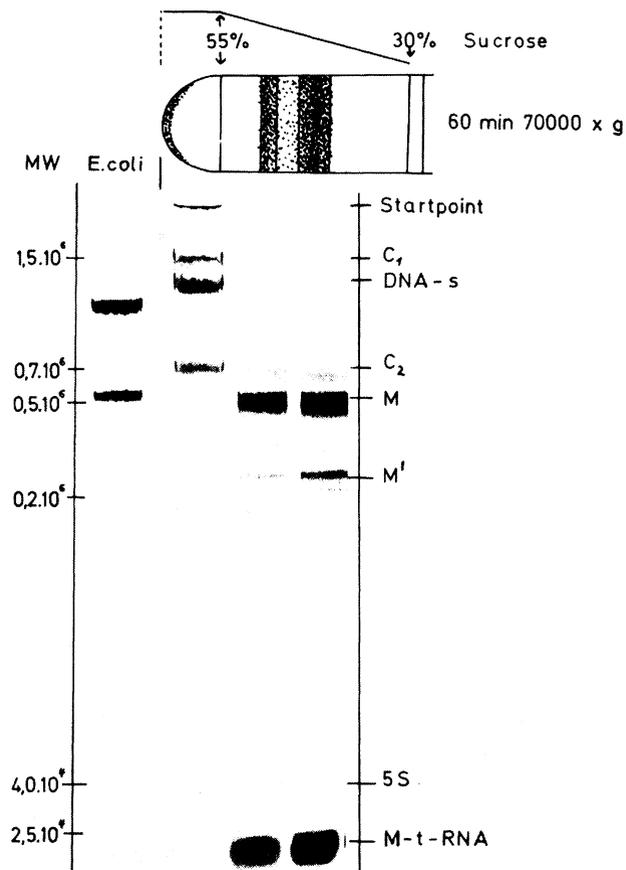


Fig. 11. Electropherograms of nucleic acids prepared from the mitochondrial fractions and from the pellet of a 60 min, 70,000 g gradient centrifugation. The gradient was 55–30% sucrose in 50 mM EDTA and 10 mM TRA, pH 7.2. The resuspended pellet from a 15 min, 20,000 g centrifugation of a thorax muscle homogenate from 7-day-old locusts, prepared in 50 mM EDTA with addition of Subtilisin A, was placed on top of the gradient. The relative amounts of RNA corresponding to the electrophoresis band patterns from the mitochondrial fractions shown, as determined by densitographic means, are approximately: C₁ 3%, C₂ 6%, M 35%, M' 16%, M-tRNA 37%.

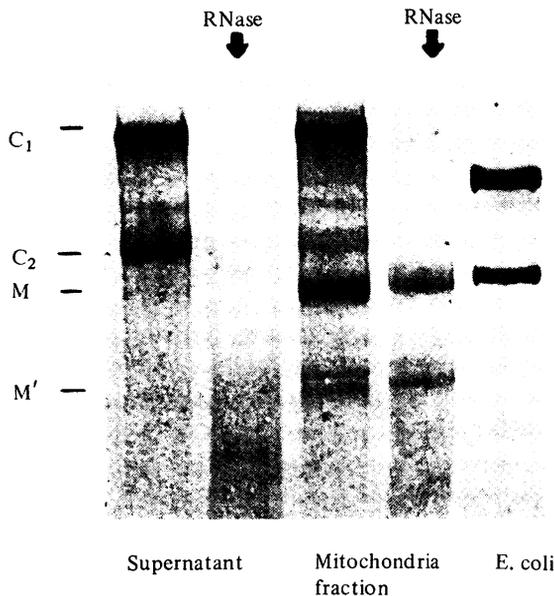


Fig. 12. Electropherograms of nucleic acids from the 15 min 20,000 g supernatant and from the mitochondrial fraction of an homogenate from thorax muscles of 2–4-day-old locusts, with and without treatment with beef pancreatic ribonuclease before phenolization. The tissue was homogenized in medium containing 50 mM EDTA, without addition of Subtilisin. The mitochondrial fraction was prepared by differential centrifugation. The resuspended pellet from 15 min, 20,000 g was centrifuged first for 10 min at 350 g and the resulting supernatant was further centrifuged 10 min at 10,000 g. The pellet from the last centrifugation was taken as the mitochondrial fraction.

mined by densitography of toluidine stain – this band amounts to up to 55% of the high molecular RNA. Besides this strong band, some minor bands, partly larger and partly smaller in molecular weight, are seen in varying proportions. Most prominent is one minor band appearing often as a double band and in a position corresponding to a molecular weight of 250,000.

Incubation with pancreas ribonuclease is another means by which the mitochondrial origin of the 500,000 and 250,000 MW bands can be demonstrated. It has been shown that ribonuclease added to suspensions of intact mitochondria, contrary to its action on cytosolic ribosomes, does not inhibit amino acid incorporation in mitochondria and leaves mitochondrial RNA unimpaired [13, 27–29]. Upon action of ribonuclease on flight muscle homogenates all

RNA bands disappear from the supernatant and also the corresponding bands in the mitochondrial fraction. The above described bands of mRNA remain with only slight diminution (fig. 12).

General remarks and conclusions

What conclusions may be drawn from the present finding? First, we must visualize, that the flight muscle is growing on an obligatorily aerobic basis, i.e. the energy required for the growth of the tissue, including that of its chondriome, is dependent upon the functional readiness of the chondriome itself. Interestingly enough, the same holds true for the growth of *Neurospora crassa* in the logarithmic phase [30]. In both tissues specific material is inserted into preexisting mitochondria in such a manner that growth in approximate constant proportions of constituents results. The preservation of constant proportions, useful in demonstrating mitochondrial growth, may be due to the special metabolic situation. In facultative aerobics and in general, the proportions of mitochondrial constituents will be subject to differentiations due to environmental circumstances and functional requirements.

Second, referring to the title of this symposium, the autonomy of mitochondria based on the results of the morphogenetic processes investigated is a rather restricted one, in that their formation is strictly coordinated with the formation of the other cellular constituents. Moreover, by far the greatest part of mitochondrial proteins is formed by the extrinsic system. Amino acid incorporation by the intrinsic system appears to be restricted to a few minor bands of the electrophoretic pattern of the insoluble mitochondrial protein fraction. The pattern of incorporation is strikingly similar to the one found in *Neurospora*. As in *Neurospora*, only a small degree of elasticity in the coupling between the extrinsic and intrinsic system could be demonstrated.

Third, the existence of RNA species in locust flight muscle mitochondria has been demonstrated. What are the functions of these RNAs? For that species, which has an electrophoretic mobility similar to cytoplasmic transfer RNA, a role as mitochondrial transfer RNA is probable. This conclusion is supported by other investigations on mammalian and *Neuro-*

spora mitochondrial tRNAs [31–34]. Considering the presence of mitochondrial tRNAs, the existence of mitochondrial ribosomes might be postulated, though recently, biosynthetic participation of tRNAs not linked to ribosomal function has been demonstrated in bacterial cell wall formation [35].

The molecular weight corresponding to the RNA-bands obtained upon gel electrophoresis are neither in that order of size observed for mammalian or insect cytoplasmic ribosomes nor in that observed for mitochondrial ribosomes from fungi, nor in that of bacterial ribosomes. In attributing the observed band pattern nevertheless to mitochondrial ribosomes, we must visualize mainly two possible explanations; either the observed bands represent the native ribosomal RNA chains or they contain specific breakdown products of these.

The first possibility would imply that the structure of locust mitochondrial ribosomes is different from that of all other types of ribosomes known at present. This difference in ribosomal structure, however, would be in contrast to the rather striking similarities in the mitochondrial amino acid incorporating systems. Therefore, attention must be drawn to the second possibility. As a matter of fact, the present standing of the problem resembles somewhat the discussion of length and continuity of bacterial ribosomal RNA occurring in the late fifties and early sixties. This matter has been reviewed by Spirin and Gavrilova [36]. Furthermore, the instability of chloroplast ribosomal RNA, as pointed out by Ingle et al. [37] seems to reflect a very similar situation.

Therefore in our case, we have to consider that at least one of the RNAs of the hypothetical large and small subunits is present as split pieces on the gel. One interpretation would be to correlate the prominent band M to the (unbroken) small subunit RNA, as well as to a major split piece of an unstable large subunit RNA.

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