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N.K. BOARDMAN

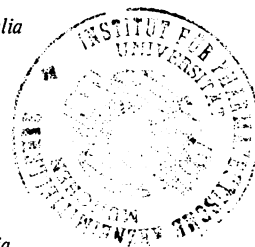*Division of Plant Industry, CSIRO, Canberra, Australia*

Anthony W. LINNANE

Department of Biochemistry, Monash University, Clayton, Victoria, Australia

and

Robert M. SMILLIE

Plant Physiology Unit, Division of Food Preservation, CSIRO,
and School of Biological Sciences, University of Sydney, N.S.W., Australia

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AMINO ACID INCORPORATION INTO MITOCHONDRIAL RIBOSOMES OF NEUROSPORA CRASSA WILD TYPE AND MI-1 MUTANT

W. NEUPERT, P. MASSINGER and A. PFALLER

*Institut für Physiologische Chemie und Physikalische Biochemie
der Universität München, Germany*

Up to the present time the best-characterized mitochondrial ribosomes are those from *Neurospora crassa*. As first shown by Küntzel and Noll [1] and by Rifkin et al. [2] they can be isolated in a rather pure state. They differ from cytoplasmic ribosomes in many respects; namely, in sedimentation constants, dissociation behaviour, electrophoretic and chromatographic patterns of their proteins, properties and composition of their RNAs and sensitivity to different inhibitors of protein synthesis [1–5,7,9].

In order to give an impression about the significance of these differences and to demonstrate the purity of the mitochondrial ribosomes used in the experiments to be described here, the two types of ribosomes will be compared with respect to their density gradient profiles and the properties of the ribosomal RNAs on gel-electrophoresis.

In fig. 1 sucrose density gradient profiles of mitochondrial and cytoplasmic ribosomes isolated from *Neurospora* wild-type cells (WT) in the late logarithmic growth phase are shown. With the cytoplasmic ribosomes a monosome peak and three distinct polysome peaks can be seen. No free subunits are present. The mitochondrial ribosomes also show the monosome peak, which moves slightly slower on the gradient, but they exhibit no clear polysome formation, and free subunits are present. The degree of dissociation into subunits of the mitochondrial ribosomes under our isolation procedure is dependent on the age of the *Neurospora* cells. The dissociation into subunits is greater the further they come into the stationary phase (cf. fig. 8). The S_{20} values of the cytoplasmic and mitochondrial ribosomes as determined by Küntzel and Noll [1] and Küntzel [3] are 77 S and 73 S for the monomers, respectively, and
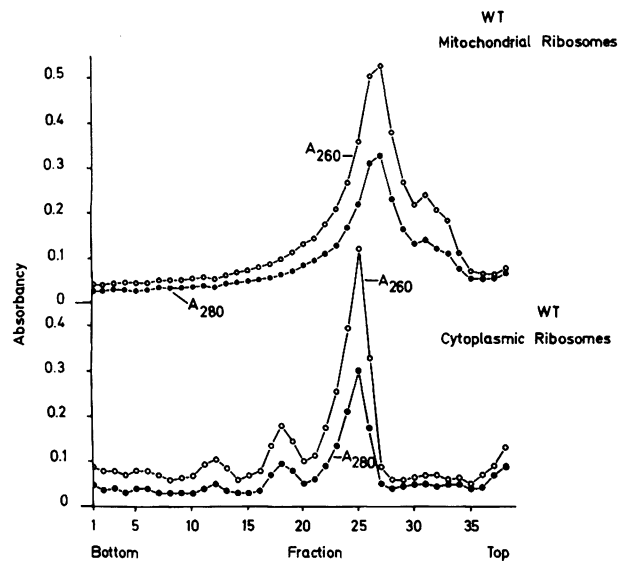
Fig. 1. Density gradient profiles of mitochondrial and cytoplasmic ribosomes from *Neurospora* wild type hyphae. Mitochondrial and cytoplasmic ribosomes were prepared from hyphae grown for 18 hr. The ribosomes were centrifuged for 2 hr at 41,000 rpm in the Spinco rotor SW 41 through a convex sucrose gradient according to Noll [24]. Fractions were collected and monitored at 260 and 280 mμ. For further details see [8].

57 S/37 S and 50 S/37 S for the subunits, respectively.

Küntzel and Noll [1] and Rifkin et al. [2] have also characterized the mitochondrial ribosomal RNA. The sedimentation coefficients vary, depending upon the salt concentration in the density gradient, from values similar to those of bacterial ribosomal RNA to those of cytoplasmic ribosomal RNA.

In fig. 2 a polyacrylamide gel electrophoretic run
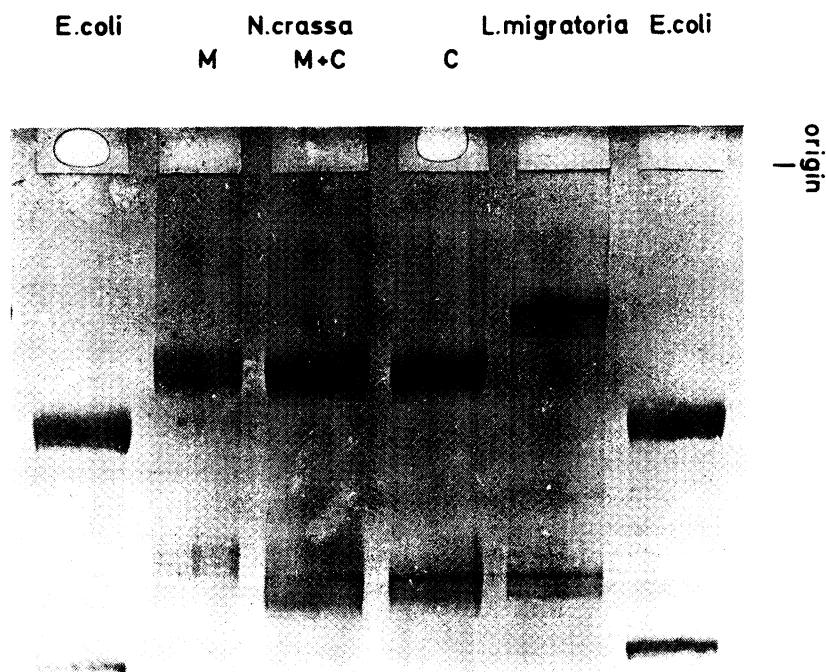
Fig. 2. Polyacrylamide gel electrophoresis of RNA samples. RNA was prepared from *E. coli* ribosomes, mitochondria (M) and 27,000 *g* supernatant (C) from *Neurospora* wild type hyphae, and from the cytoplasm of the flight muscle of *Locusta migratoria*. Electrophoresis on polyacrylamide gel (2.7% with 0.5% agarose) was carried out essentially as described by Dingman and Peacock [11,12]. The gels were stained with toluidine blue. For further details see [8].

of both types of RNA is presented. The large subunit RNAs of cytoplasmic and mitochondrial ribosomes have the same electrophoretic mobility, whereas the small subunit RNA of mitochondria has a lesser mobility than the cytoplasmic counterpart. To the same gel, RNA preparations from *E. coli* ribosomes and cytoplasmic ribosomes from locust flight muscle were applied as standards. According to Dingman and Peacock [13] and Loening [14], the electrophoretic mobility on polyacrylamide gel is a measure of the molecular weight of the ribosomal RNA species. The molecular weights estimated by this method are 1.28 and 0.67 million for cytoplasmic ribosomal RNA, and 1.28 and 0.72 million for mitochondrial ribosomal RNA. Mitochondrial and bacterial ribosomal RNA appear to be very dissimilar in size. A similar electrophoretic mobility for cytoplasmic and mitochondrial transfer RNA is obvious in pictures of the whole gel slab (cf. fig. 10). It should be noted that under our preparative conditions no 5 S RNA can be observed in mitochondrial ribosomes, in contrast to cytoplasmic and bacterial ribosomes.

Synthesis of peptide chains on mitochondrial ribosomes of Neurospora wild type

Are these mitochondrial ribosomes active in synthesizing peptide chains? In order to answer this question the following experiment was performed. Mitochondria were isolated from *Neurospora* hyphae and incubated with radioactive amino acids under

conditions which were determined to be optimal [6]. After 20 min incubation, the suspension was divided into two equal portions. One was immediately cooled to 0° and served as a control. To the other portion puromycin was added to a final concentration of 0.4 mM and incubation was continued for 7 min. This portion also was then cooled to 0°, and the ribosomes were isolated from both preparations in exactly the same way.

In table 1 the specific radioactivities of the different fractions of the isolation procedure of the ribosomes are shown. The control ribosomes have a very high specific radioactivity compared to the whole mitochondria, but only 7–10% of the total radioactivity incorporated is found in the ribosomal fraction. This percentage is dependent on the efficiency of incorporation and becomes higher when the rate of incorporation becomes lower. This observation indicates that, at least under certain conditions, the radioactive amino acids are incorporated into complete peptide chains which are released from the ribosomes and integrated into the insoluble membrane protein. There, as shown by Sebald et al. [6], they can be detected as definite bands by means of polyacrylamide gel electrophoresis.

In the preparation treated with puromycin, the specific radioactivity of the whole mitochondria is lower, probably because of loss of trichloroacetic acid

soluble peptides released from mitochondria. Similar results were obtained for rat liver mitochondria by Wheeldon and Lehninger [10]. Most interesting is the finding that the specific radioactivity of the ribosomes is very low compared to the control, indicating a substantial loss of radioactive amino acids from the ribosomes.

Fig. 3 represents sucrose density gradients of the ribosomes of these preparations. In the control ribosomes, radioactivity is found at the polysome region and at the monosome peak. The polysomes have the highest specific radioactivity. The radioactivity near the top of the gradient probably represents light mitochondrial particles not destroyed by the lysing agent, Triton X–100. In the preparation treated with puromycin, all of the radioactivity associated with poly- and monosomes has disappeared. This demonstrates that peptide chains are synthesized on the ribosomes, and that they can be released by puromycin, as is known for the bacterial system and for the cytoplasmic system of eucariotic cells.

To provide further proof that the label in the gradient below the monosome peak actually corresponds to peptide chains on polysomes, i.e., to demonstrate the existence of polysomes in mitochondria, another experiment was carried out. Isolated mitochondria were labelled with radioactive amino acids in the same way as described for the puromycin

Table 1

Specific radioactivities of mitochondrial fractions after incorporation of labelled amino acids into isolated Neurospora wild type mitochondria.

Fraction	Control		Puromycin incubated	
	Specific radioactivity cpm/mg protein	Total radioactivity cpm	Specific radioactivity cpm/mg protein	Total radioactivity cpm
Mitochondria after incubation	7,000	525,000	4,900	343,000
Mitochondrial lysate	7,210	392,000	4,960	238,000
Sediment of mitochondrial lysate 30 min, 35,000 g	7,910	13,050	3,650	6,930
Crude ribosomes	17,660	33,500	4,160	5,830
Supernatant after sedimentation of ribosomes	6,185	180,700	7,110	242,000

Isolated mitochondria were labelled for 20 min with 1-^{14}C-leucine, 1-^{14}C-isoleucine and 1-^{14}C-phenylalanine (0.1 μC/ml each) in a medium described by Sebald et al. [6]. For further details see text and ref. [8].
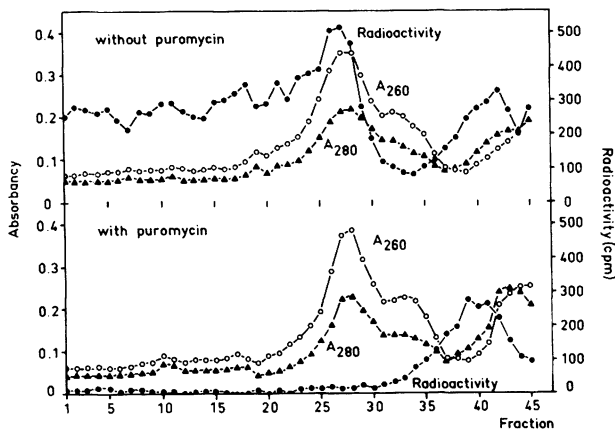
Fig. 3. Gradient profiles and incorporated radioactivity of ribosomes isolated from mitochondria labelled in vitro with and without successive incubation with puromycin. The crude ribosomal preparations of table 1 were used. For further experimental details see [8].
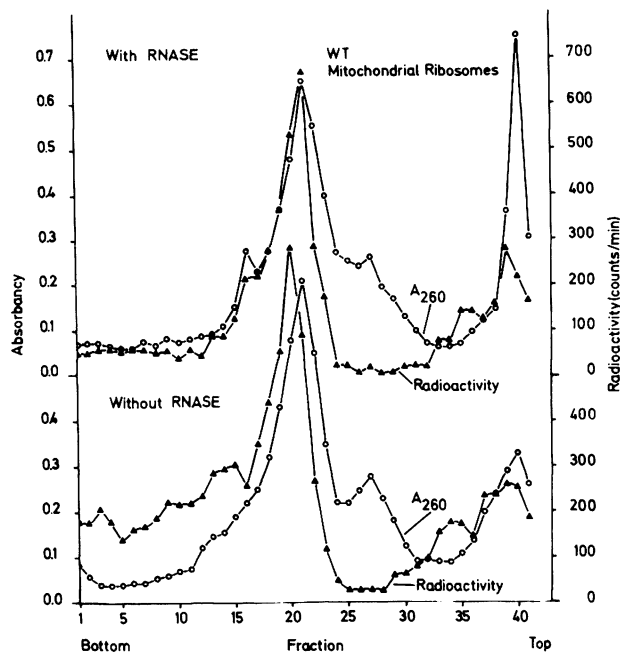
Fig. 4. Ribonuclease treatment of ribosomes isolated from Neurospora wild type mitochondria labelled in vitro with radioactive amino acids. Labelling of mitochondria, isolation of ribosomes and density gradient centrifugation were performed as described for table 1 and fig. 3. After isolation, one half of the ribosomes were incubated for 1 hr at 4°C with 1 μg/ml of pancreatic ribonuclease, essentially according to Rich et al. [18] prior to density gradient centrifugation.

experiment. After 20 min, the incubation was stopped by cooling and the ribosomes were isolated. These ribosomes were divided into two equal portions. One portion served as a control and was put on the gradient without further treatment. The second portion was incubated for 60 min at 4° with 1 μg/ml pancreatic ribonuclease essentially according to Rich et al. [18]. Both preparations were submitted to density gradient centrifugation, the result of which is demonstrated in fig. 4. When compared to the control, most of the radioactivity at the polysomes has disappeared in the ribonuclease experiment. The radioactivity removed from the polysome region cannot be found quantitatively at the monosome peak. The reason for this may be that under the conditions of the experiment part of the aminoacyl-transfer-RNA at the monosomes is degraded and removed.

It cannot be determined from our experiments whether peptide chains are synthesized only by polysomes, nor can it be determined how many of the polysomes present in the mitochondria, in vivo, might be converted to monosomes during the isolation.

One more interesting experimental observation was the inability of isolated mitochondria to incorporate amino acids into the structural ribosomal proteins. However, this provides no evidence that mitochondria are unable to synthesize their ribosomal proteins, since the protein-forming system as well as the system for the synthesis of the ribosomal ribonucleic acids may be damaged.

Biosynthesis of the mitochondrial ribosomal proteins

A more definite answer to the question of where the mitochondrial ribosomal proteins are synthesized can be expected from experiments in vivo with specific inhibitors of cytoplasmic and mitochondrial amino acid incorporation.

Table 2 represents experiments in which Neurospora cells were labelled with and without preincubation with cycloheximide and chloramphenicol, respectively, followed by a chase of unlabelled amino acids to make certain that the amino acids in the peptidyl-transfer-RNA on the ribosomes are not radioactively labelled. In the control experiment (first column) all fractions have a very similar specific radioactivity. In the experiment with cycloheximide

Table 2

Influence of inhibitors on the incorporation of labelled amino acids in vivo into fractions of *Neurospora* hyphae.

Fraction	Specific radioactivity (cpm/mg protein)		
	Control	Cycloheximide preincubated	Chloramphenicol preincubated
Mitochondrial lysate	25,010	2,380	13,280
Sediment of mitochondrial lysate 30 min, 30,000 g	27,700	1,460	10,830
Crude mitochondrial ribosomes	21,100	750	10,400
Cytoplasmic ribosomes	8,700	234	5,700
Supernatant of cytoplasmic ribosomes	17,700	95	11,810

Neurospora wild type hyphae grown for 18 hr were labelled for 20 min with $1\text{-}^{14}C$-leucine, $1\text{-}^{14}C$-isoleucine and $1\text{-}^{14}C$-phenyl-alanine (6.7 mμC/ml). Then a 20-min chase period with unlabelled amino acids (2 mM each) followed. The inhibitors (cyclo-heximide 100 μg/ml, chloramphenicol 4 mg/ml) were added 10 min prior to the addition of the radioactive amino acids. Separation of the crude mitochondrial ribosomes is shown in figs. 5–7 and ref. [9].

preincubation (second column) incorporation into cytoplasmic soluble proteins and into the cytoplasmic ribosomes is inhibited by 99%, while whole mito-chondria still show 10% of the incorporation of the control. However, incorporation into mitochondrial ribosomes is inhibited by 99%. In the experiment with chloramphenicol preincubation (third column), incorporation into all fractions is lower, if compared to the control. No distinct specific inhibition of any fraction can be observed. Similar experiments and results were described by Küntzel [4]. In the follow-ing figures sucrose density gradient profiles of the

mitochondrial ribosomes isolated in these experi-ments are presented.

In the control experiment (fig. 5) a heavy labelling of the monosome peak takes place; polysomes do not have a higher specific radioactivity. There is also no label at the subunits. Fig. 6 represents the mito-chondrial ribosomes from the experiment in which the *Neurospora* cells were preincubated with cyclo-heximide before adding the labelled amino acids. Virtually all radioactivity above the experimental limit of error has disappeared from the ribosomes. In fig. 7, representing the chloramphenicol experiment, the monosome peak is labelled to approximately the
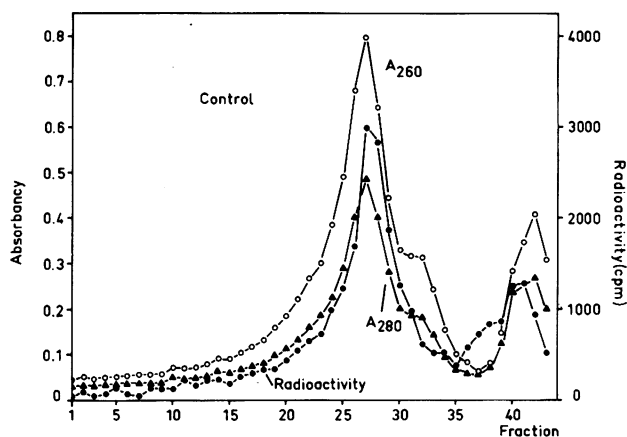

Fig. 5. Sucrose density gradient centrifugation of the crude ribosomal preparation from the control experiment in table 2.
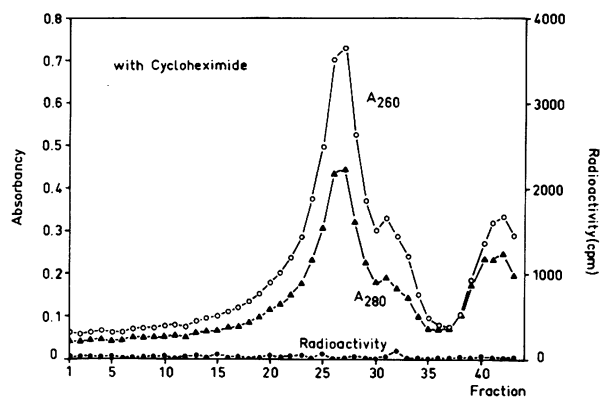

Fig. 6. Sucrose density gradient centrifugation of the crude ribosomal preparation from the cycloheximide preincubation experiment in table 2.
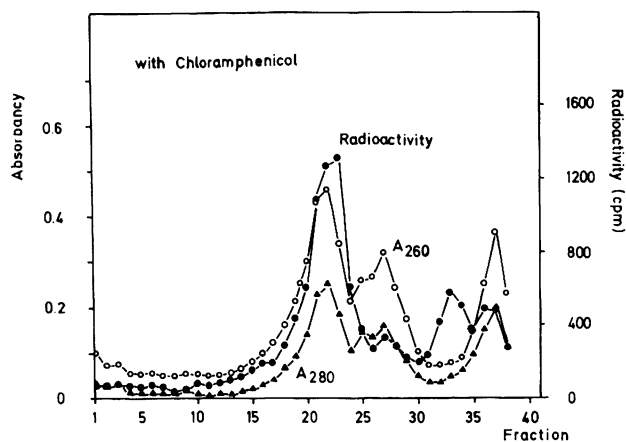
Fig. 7. Sucrose density gradient centrifugation of the crude ribosomal preparation from the chloramphenicol preincubation experiment in table 2.

same specific radioactivity as in the control experiment.

To summarize, amino acid incorporation into the mitochondrial ribosomal proteins in vivo can be blocked by cycloheximide, a specific inhibitor of cytoplasmic protein synthesis [6,21,23], but not by chloramphenicol which specifically inhibits mitochondrial amino acid incorporation [15,20–22]. These results demonstrate that the synthesis of mitochondrial ribosomal proteins is dependent on the functioning of the cytoplasmic protein synthesis.

On the basis of the very strong degree of inhibition by cycloheximide the most probable explanation is that the vast majority, if not all, of the mitochondrial ribosomal proteins are synthesized by the cytoplasmic system. Of course, these experiments do not exclude the possibility that a minor part of these proteins, a few out of 40 or 50, are synthesized by the mitochondrial system. Our conclusions implicate a cooperation of mitochondrial and extramitochondrial systems in the biogenesis of mitochondrial ribosomes, since experimental evidence suggests that mitochondrial ribosomal RNA is coded for and synthesized within the mitochondria [25–28].

Other less probable interpretations of our results will be discussed in the following. (1) It could be that just one or a few protein components essential for the formation of the mitochondrial ribosome are synthesized by the cycloheximide-sensitive system. This explanation implies that the average time be-

tween the synthesis of these components and their integration into the mitochondrial ribosomes is much shorter than 10 min. In other words, only a small amount of these components would exist in the cell in a free state, not yet integrated into the mitochondrial ribosomes, so that this precursor protein pool is exhausted after the 10 min incubation with cycloheximide. (2) The mitochondrial ribosomal proteins could be all of mitochondrial origin, but a 'trigger' protein could be synthesized on the cytoplasmic ribosomes and then transferred into the mitochondria where it would regulate specifically the synthesis of the mitochondrial ribosomes or one of their components. For the same reason as stated above, this protein would have an extremely short half-life, i.e., it would be degraded or inactivated within less than 10 min. Such a high turnover for a protein seems improbable.

Mitochondrial ribosomes of Neurospora mi-1 (poky) mutant

We should like to turn now to experiments with the cytoplasmic mutant mi-1 (poky) of Neurospora crassa.

It is well known from the early work of Mitchell and Mitchell [16] that the mutant character of this slowly growing strain does not obey Mendelian genetics but is maternally inherited. It is also known from these authors that the hyphae possess mitochondria with an altered respiratory chain, with greatly decreased amounts of cytochromes a and b and an elevated amount of cytochrome c. The composition of the mitochondrial membrane, as shown by gel electrophoresis, is different from the wild type. Also the gel electrophoretic pattern of the mitochondrially labelled proteins is drastically changed as compared to the wild type [6]. The rate of amino acid incorporation into isolated mitochondria under optimal conditions amounts to about 20% of that of the wild type [6]. Reich and Luck [19] did not find a difference in the buoyant densities of poky and wild type mitochondrial DNA. Finally, the instability of the poky character should be mentioned, i.e., as the poky cells approach the stationary growth phase the more they aquire more of the cytochromes [17].

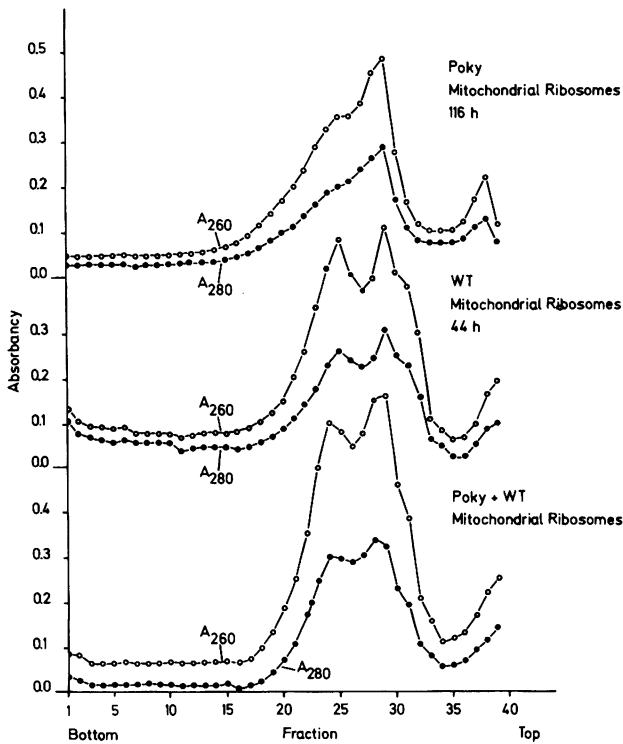Fig. 8 shows a comparison of sucrose density

Fig. 8. Density gradient profiles of mitochondrial ribosomes isolated from *Neurospora* mi-1 mutant (poky) and wild type (WT). Poky hyphae were grown for 116 hr, wild type hyphae for 44 hr. For experimental details of poky growth see [6]. In the mixture poky + WT, equal amounts of each type of ribosomes were applied.

gradient profiles of mitochondrial ribosomes from wild type and poky *Neurospora* hyphae. The wild type mitochondrial ribosomes were isolated from hyphae grown for 44 hr, i.e. from hyphae in the stationary growth phase. As mentioned above, a large part of the wild type mitochondrial ribosomes in the stationary growth phase are present as free subunits. One can distinguish the monosome peak, the large subunit peak and the small subunit present as a clear shoulder. Since the difference of the S values of the mitochondrial ribosomal subunits is relatively small as compared to the cytoplasmic ribosomes it is difficult to resolve the small subunit as a peak. In this context we refer to the excellent characterization of the mitochondrial ribosomal subunits by Küntzel [3].

Poky mitochondrial ribosomes isolated from the late logarithmic growth phase show a peak corresponding to the monosomes, and a stronger peak

corresponding to the large subunit. However, no shoulder corresponding to the free small subunit can be detected. Absence of the small subunit shoulder was observed in some 30 density gradients without an exception. When wild type and poky mitochondrial ribosomes are mixed in equal proportions and subjected to gradient centrifugation the small subunit shoulder can be seen clearly. These results strongly suggest that in the case of the poky mitochondrial ribosomes, the small subunits are not equivalent in number to the large subunits, and that only in the relatively small proportion of monosomes are small subunits present. In contrast, ribosomes from the cytoplasm of wild type and poky *Neurospora* do not differ. This is shown in fig. 9. Gradient profiles of both these types of cytoplasmic ribosomes and of a mixture of the two types are very similar.

This non-equivalence of large and small subunits in mitochondrial poky ribosome preparations should be reflected in the relation of large and small subunit ribosomal RNA. In fig. 10 polyacrylamide gel electrophoresis is shown in which RNA preparations from isolated whole wild type and poky mitochondria were run in parallel. Only a very small amount of small subunit RNA is present in poky mitochondria. As in the wild type, no 5 S RNA can be detected. This, and the observation that ribonuclease does not preferentially destroy the large subunit RNA when added to whole mitochondria, prove the mitochondrial localisation of the large subunit isolated from mitochondria. The small amount of small subunit RNA is presumably derived from the monosomes.

Are these poky ribosomes active in synthesizing peptide chains? The experiments reported by Sebald et al. [6] which demonstrate the ability of isolated poky mitochondria to incorporate radioactive amino acids, suggest that they are. However, as already mentioned, the electrophoretic pattern of the radioactively labelled bands is appreciably changed compared to that of the wild type. We have carried out the following experiment to check the function of mitochondrial ribosomes in whole poky cells. Two equal portions of poky hyphal suspensions were first incubated for 10 min with cycloheximide, then radioactive amino acids were added to each and the incubation continued for a further 10 min. One portion was then immediately harvested and cooled; the other was chased with approximately a 10,000-fold
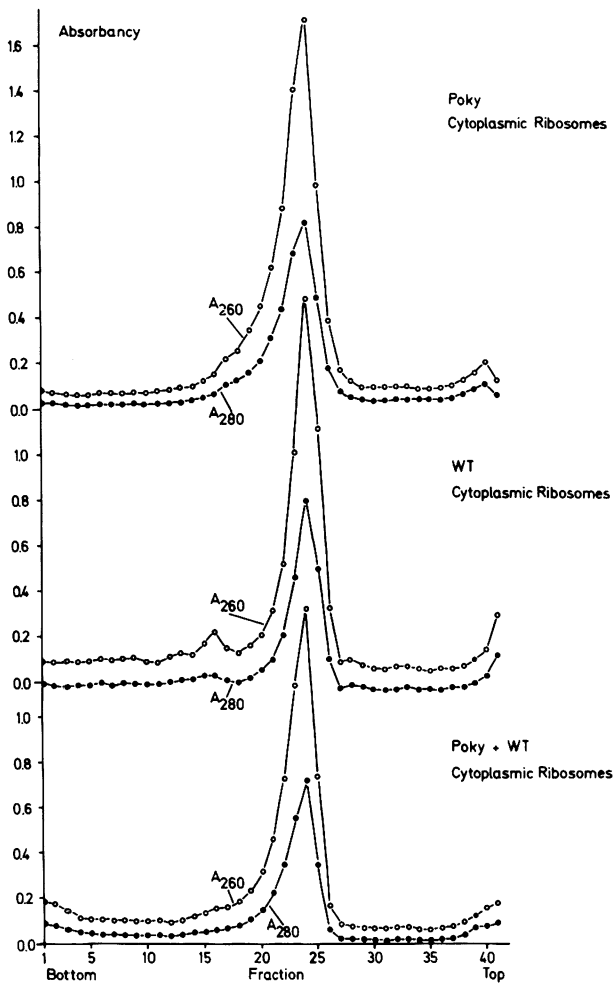
Fig. 9. Density gradient profiles of cytoplasmic ribosomes from Neurospora mi-1 mutant (poky) and wild type (WT). Poky hyphae were grown for 137 hr wild type hyphae for 47.5 hr. Other experimental conditions as described before.

Fig. 10. Polyacrylamide gel electrophoresis of RNA samples from Neurospora mi-1 mutant and wild type. Abbreviations: PO, poky; WT, wild type; M, mitochondria; C, cytoplasm (27,000 g supernatant). The ages of hyphae from which RNA was isolated were: C (poky) 137 hr, C (WT) 44 hr, M (poky) 71.5 hr, M (WT) 47.5 hr. Preparation of RNA samples and conditions of electrophoresis as described for fig. 2.

excess of unlabelled amino acids for 20 min, then harvested. In figs. 11 and 12 density gradient centrifugations of the mitochondrial ribosomes from this experiment are shown. In the experiment without chase, a radioactivity peak is found at the monosomes and also some radioactivity at the polysomes. Also, an appreciable amount of radioactivity is located in the upper part of the gradient probably representing, as already pointed out for the wild type experiments, light mitochondrial particles. However, compared to the wild type gradients, the proportion of this frac-
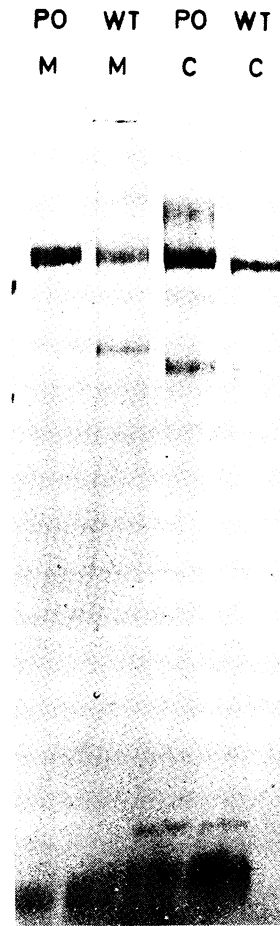
tion is much higher. The obvious explanation is the much lower labelling of the poky ribosomes. In the experiment with chase, the radioactivity in the polysome and monosome region has disappeared, whereas the label in the upper part of the gradient is still present.

These experiments suggest that poky ribosomes are also able to synthesize polypeptide chains, but they do not indicate whether or not these monosomes are 'normal'. Experimental evidence indicates that they are much more labile than those from the
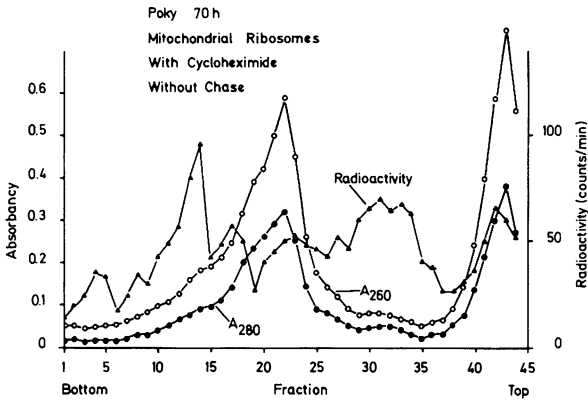
Fig. 11. In vivo labelling of mitochondrial ribosomes from Neurospora poky hyphae with radioactive amino acids after preincubation with cycloheximide. Poky cells grown for 70 hr were incubated first with cycloheximide (100 μg/ml) for 10 min, then 1-^{14}C-leucine, 1-^{14}C-isoleucine and 1-^{14}C-phenylalanine (12.5 mμC/ml each) were added and after 10 min the hyphae were harvested. Then mitochondrial ribosomes were isolated and subjected to density gradient centrifugation as described.
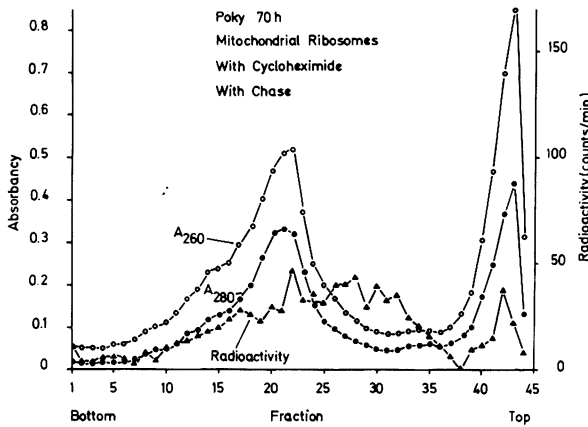
Fig. 12. In vivo labelling of mitochondrial ribosomes from Neurospora poky hyphae with radioactive amino acids after preincubation with cycloheximide followed by a chase with unlabelled amino acids. Poky cells were grown and labelled identically to those described in fig. 11. In this experiment after the incubation period with the ^{14}C-amino acids, unlabelled amino acids were added to a final concentration of 2 mM and incubation was continued further for 20 min. Then the hyphae were harvested and fractions were isolated in parallel to those of fig. 11.

wild type. Similar conclusions can be drawn from experiments in which puromycin was used to release newly synthesized peptide chains from poky ribosomes after incorporation of labelled amino acids into isolated poky mitochondria.

It has already been mentioned that several properties of poky cells and mitochondria change during the growth of a poky culture. Therefore, it seemed
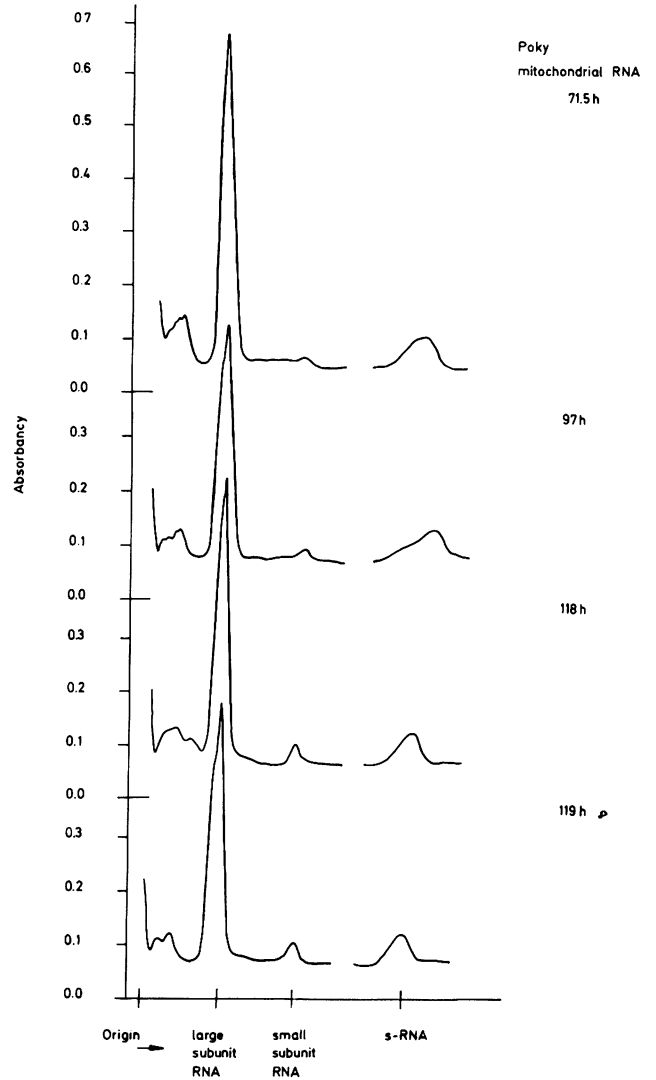
Fig. 13. Densitograms of polyacrylamide gel electrophoresis of RNA samples isolated from mitochondria of poky hyphae of different age (71.5 hr, 97 hr, 118 hr and 119 hr). Isolation of RNA and gel electrophoresis were performed as described for fig. 2, and the toluidine blue stained gel was submitted to densitometry at 546 mμ.
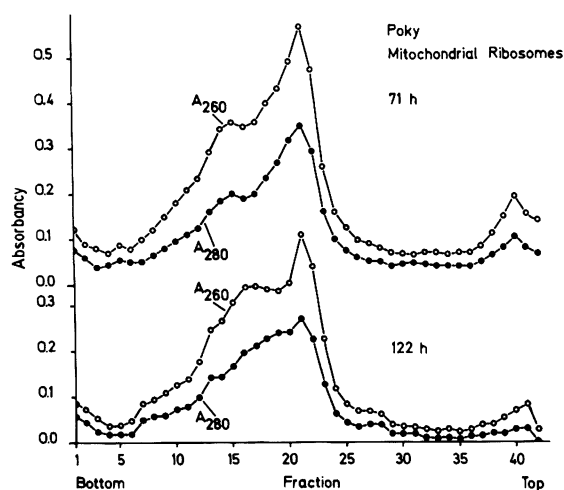
Fig. 14. Sucrose density gradient profiles of mitochondrial ribosomes isolated from *Neurospora* poky hyphae of different age (71 hr and 122 hr). Isolation and density gradient centrifugation were performed as described before.

worthwhile to determine whether the properties of the mitochondrial ribosomes might change also. In fig. 13 densitograms are shown of polyacrylamide gel electrophoreses of RNA preparations extracted from whole mitochondria isolated from poky cells of different ages. Hyphae were taken from 71.5, 97, 118 and 119 hr cultures. The first ones correspond to the early logarithmic phase, the last ones to the very late logarithmic phase. The proportion of small subunit RNA is relatively low throughout the whole period investigated. However, it can be clearly seen that it increases with the age of the hyphae. If this is true there should also be an increase of the proportion of the small subunit in density gradient profiles of ribosomal preparations, either as appearance of free small subunits or as an increase of the proportion of monosomes. This is illustrated in fig. 14. Ribosomes of the early and late logarithmic phase are shown. In both cases no small subunit shoulder can be seen. However, the shoulder corresponding to the monosomes is increased in the ribosome preparation from the late logarithmic growth phase. In order to explain the described changes of ribosomal composition in poky mitochondria, the following possibilities are considered: (1) the change in ribosomal composition is caused by a genetic change affecting the structure or the synthesis of one or several components of the mitochondrial ribosomes. (2) The genetic alteration

does not concern the information for structure or synthesis of a ribosomal component, and the change of the ribosome is a secondary effect. For instance, there could be an increased amount of endomitochondrial nuclease which specifically attacks and destroys the small subunit. Or conditions inside the poky mitochondria, such as alteration of membrane structure or the absence of normal messenger RNA, could favour the dissociation into subunits and the small subunit could be lost because of a peculiar instability. It is difficult to exclude these possibilities, but the reproducibility of the results and the dependence of ribosomal composition on the growth do not favour such an explanation. In this context it is interesting to note that the cytoplasmic mutant *mi-3*, which in many respects resembles *mi-1*, has large and small subunits in equal amounts [29]. Furthermore, in vivo pulse labelling experiments with radioactive amino acids of poky hyphae in the logarithmic phase show that the label is present almost entirely in the large subunit. We have to assume that either the newly synthesized small subunit is broken down immediately upon formation or the amount of small subunit synthesized is very small compared to that of the large subunit. Therefore the first explanation is preferred, namely that the small subunit is not formed because of a genetic defect in the mitochondrial DNA. Further speculations, such as whether the small subunit RNA is affected, should be based on further experimental data.

Acknowledgements

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