European J. Biochem. 10 (1969) 585–588

Puromycin Sensitivity of Ribosomal Label after Incorporation of ^{14}C-Labelled Amino Acids into Isolated Mitochondria from *Neurospora crassa*

W. Neupert, W. Sebald, A. J. Schwab, A. Pfaller, and Th. Bücher

Institut für Physiologische Chemie und Physikalische Biochemie der Universität München

(Received June 2, 1969)

Radioactive amino acids were incorporated into isolated mitochondria from *Neurospora crassa*. Then the mitochondrial ribosomes were isolated and submitted to density gradient centrifugation. A preferential labelling of polysomes was observed. However, when the mitochondrial suspension was treated with puromycin after amino acid incorporation, no radioactivity could be detected in either the monosomes or the polysomes. The conclusion is drawn that isolated mitochondria under these conditions do not incorporate significant amounts of amino acids into proteins of their ribosomes.

Mitochondrial ribosomes from *Neurospora crassa* have been isolated and characterized by Küntzel and Noll [1] and by Rifkin *et al.* [2]. Radioactivity has been found in these ribosomes after labelling of isolated mitochondria by incorporation of [^{14}C]-leucine [1]. However, no distinction was made between labelling of the proteins of the ribosomes and the peptide chains being actually synthesized. Puromycin, which is known to release the growing peptide chain from the ribosome, was used to examine this question.

MATERIALS AND METHODS

Hyphae of *Neurospora crassa* (wild type 74A) were grown for 18 h on a reciprocal shaker from an inoculum of 2×10^6 conidia/ml in 50 ml portions of Vogel's minimal medium supplemented with 2% sucrose. They were harvested by filtration.

Mitochondria were prepared by a slight modification of the method described by Luck [3]. Cells were mixed with 3 volumes (with respect to wet weight) of isolation fluid (0.44 M sucrose, 10 mM Tris, 2 mM EDTA, pH 7.2) and ground with sand (0.7 g per g of wet weight) for 8 min. The suspension, diluted with 6 additional volumes of isolation fluid, was passed through a filter cloth. The residue was ground again under the same conditions for 5 min. For isolation of mitochondria the combined filtrates were successively centrifuged in the following way: 10 min at $1500 \times g$, the resulting supernatant 30 min at $11000 \times g$, the resuspended pellet 10 min at $550 \times g$, the resulting supernatant 20 min at $17000 \times g$, and the resulting supernatant again 20 min at $17000 \times g$.

Unusual Abbreviation. A_{260} unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm when measured in a 1 cm path length cell.

Amino acids were incorporated into mitochondria as described by Sebald *et al.* [4], using 0.1 μC/ml L-[U-^{14}C]leucine (311 mC/mmole), L-[U-^{14}C]isoleucine (308 mC/mmole) and L-[U-^{14}C]phenylalanine (459 mCi/mmole) (The Radiochemical Centre, Amersham, England). Incubations were carried out in 1 ml samples with shaking for 20 min at 30°. Subsequently the samples were collected. The suspension of labelled mitochondria was divided into two equal portions. One portion served as a control. It was immediately cooled to 0°. To the other portion puromycin hydrochloride (Nutritional Biochemicals) was added to a final concentration of 0.4 mM. The incubation was continued for additional 7 min. This part of the suspension was then also cooled to 0°. From this point on control and puromycin treated mitochondria were handled in the same way. 3 volumes of isolation fluid containing 5 mM each of unlabelled L-leucine, L-isoleucine and L-phenylalanine were added. The mitochondria were centrifuged for 20 min at $17000 \times g$. The pellet was resuspended in isolation fluid containing amino acids. The suspension was centrifuged again for 20 min at $17000 \times g$.

For further purification mitochondria were resuspended in isolation fluid and centrifuged for 1 h at 25000 rev./min in the Spinco SW 25/2 rotor through a linear gradient of 25–68% sucrose in standard buffer (0.1 M NH_4Cl, 10 mM Tris, 10 mM $MgCl_2$, pH 7.6). The resulting dense mitochondrial band was collected and diluted with standard buffer to a sucrose concentration of 0.44 M.

Mitochondrial ribosomes were prepared combining the methods described by Küntzel and Noll [1] and by Rifkin *et al.* [2]. Mitochondria were lysed by homogenisation for 5 min in 1% Triton X 100 in standard buffer. The clear lysate was centrifuged for

30 min at 30000 $\times g$. The supernatant was centrifuged for 3 h at 150000 $\times g$. The resulting yellowish pellet (crude mitochondrial ribosomes) was resuspended in standard buffer (100 A_{260} units/ml). The suspension was centrifuged for 2 h at 41000 rev./min in the Spinco SW 41 rotor through an isokinetic convex sucrose gradient as described by Noll [5]. (Volume 12 ml, starting concentration 15 %, reservoir concentration 30.5 %. Each beaker contained 10 A_{260} units.)

Generally the radioactivity of proteins was measured after precipitation and washing with trichloroacetic acid [6]. Crude ribosomes however, were resuspended in standard buffer, absorbed on a millipore filter and counted in a Packard liquid scintillation counter. Fractions of the isokinetic gradient were plated, dried and counted in a methane flow counter. The counting efficiency was 45 %. All values were corrected for quenching. Protein was measured by the Lowry method.

Ribosomal RNA was extracted from washed mitochondria by the method described by Rifkin *et al.* [2], without previous purification by sucrose gradient centrifugation. The RNA was precipitated with 2 volumes of ethanol, cooled to $-20°$, and then taken up in a solution containing 50 mM NaCl and 1 mM EDTA, pH 6.2 (25 A_{260} units/ml). Sucrose was added to a final concentration of 8 %. 20 µl of this solution were applied to gel electrophoresis using a slight modification of the method described by Peacock and Dingman [7,8]. The gel consisted of 2.7 % polyacrylamide with 0.5 % agarose. The buffer contained 10 mM NaCl, 90 mM Tris, 2.5 mM EDTA, and 90 mM boric acid, pH 8.3.

For preparation of cytoplasmic ribosomal RNA the hyphae were homogenized as described above using 1 % NaCl solution instead of isolation fluid. The homogenate was centrifuged for 30 min at 27000 $\times g$. The resulting supernatant was then treated further in the same way as the resuspended washed mitochondria.

Ribosomes from *Escherichia coli* were suspended in a buffer containing 10 mM Tris, 22 mM NH_4Cl, 10 mM magnesium acetate, 2.5 mM EDTA, and 10 mM mercaptoethanol, pH 7.3. The suspension was treated further as described above.

Cytoplasmic ribosomal RNA from the flight muscle of *Locusta migratoria* was prepared in our laboratory by Kleinow [8a].

RESULTS

To show the purity of the investigated mitochondrial preparation, a gel electrophoretic run of RNAs from various sources is shown in Fig. 1. It is seen that RNA extracted from the mitochondria (M)

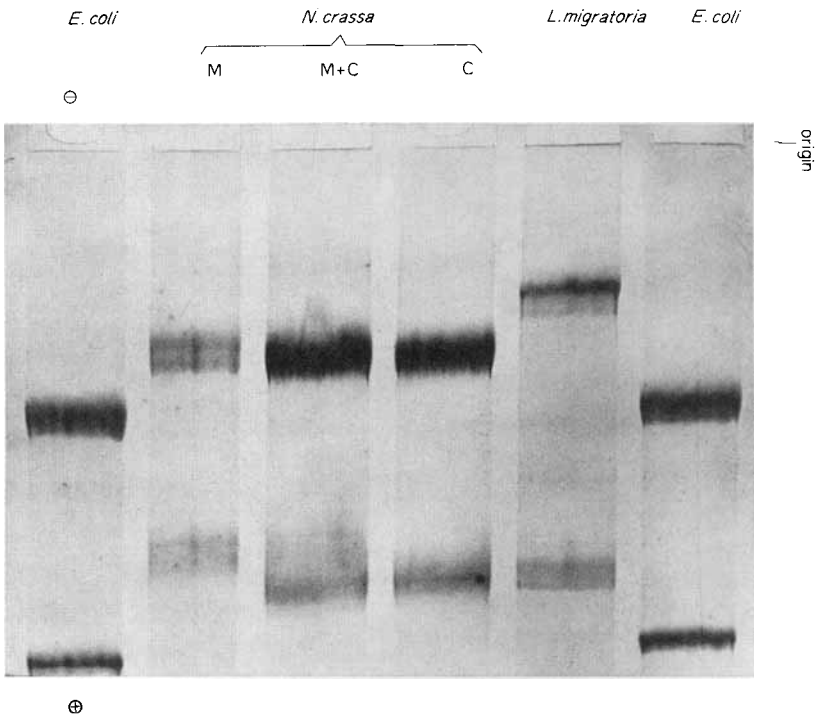

Fig. 1. *Gel electrophoresis of RNA samples.* RNA was prepared from *Escherichia coli* ribosomes (0.56 and 1.1), mitochondria (M) (0.72 and 1.28) and 27000 $\times g$ supernatant (C) (0.67 and 1.28) of *Neurospora crassa* and a mixture of both (M + C), and cytoplasm of the flight muscle of *Locusta migratoria* (0.67 and 1.55). Estimates of molecular weights in millions are given in brackets, using *E. coli* RNA as a standard [9]

Table. *Radioactivity incorporated into different fractions of mitochondria*
The mitochondria were labelled *in vitro* for 20 min with L-[^{14}C]leucine, L-[^{14}C]isoleucine and L-[^{14}C]phenylalanine (0.1 μC/ml each). Control mitochondria were withdrawn before further incubation with 0.4 mM puromycin for 7 min

Fraction	Radioactivity of control		Radioactivity of puromycin incubated samples	
	Specific	Total	Specific	Total
	counts × min^{-1} × mg protein^{-1}	counts/min	counts × min^{-1} × mg protein^{-1}	counts/min
Mitochondria after incubation	7000	525000	4900	343000
Mitochondrial lysate	7210	392000	4960	238000
Sediment of mitochondrial lysate 30 min, 35000 × *g*	7920	13050	3650	6930
Crude ribosomes	17660	33500	4160	5830
Supernatant after sedimentation of ribosomes	6185	180700	7110	242000

differs from RNA prepared from the postmitochondrial supernatant (C) with respect to the minor component. A mixture of both preparations (M + C) is also included. For comparison RNA from *Escherichia coli* ribosomes and from the cytoplasm of the flight muscle of *Locusta migratoria* were run in parallel. By this method, no contamination of mitochondria with cytoplasmic ribosomal RNA can be detected.

In the Table and in Fig. 2 the specific radioactivities of the mitochondrial fractions from the successive steps of the preparation of ribosomes are shown. In the control preparation (Table, left hand columns), the total radioactivity incorporated into the crude ribosomal fraction is less than 10% of the radioactivity of the mitochondrial lysate. The specific radioactivity of the ribosomes however, is higher by a factor of 2.5 than the one of the mitochondrial lysate.

Puromycin incubation (Table, right hand columns) for 7 min results in a decrease of specific radioactivity of the crude ribosomal fraction by a factor of about 4 as compared to the control fraction. An even greater decrease of the total radioactivity is observed. Also the total and specific radioactivities of whole mitochondria decrease. This may be at least partially explained by a loss of the smaller peptide chains released by the action of puromycin. These chains may possibly not be precipitated by trichloroacetic acid during the protein preparation procedure.

The results are more clear cut when the crude ribosomal fraction is further purified by density gradient centrifugation as shown in Fig. 2. In the control preparation (A) the largest part of the radioactivity is found in the polysome (fractions 1—23) and monosome (fractions 24—30) regions, the polysomes having a much higher specific radioactivity than the monosomes. By a preceding incubation with ribonuclease this label is shifted from the polysome region into the monosome peak. A smaller part of the radioactivity is found at the top of the gradient (fraction 40—45) in a region where the absorption at 280 nm is higher than at 260 nm. After incubation with puromycin (B) no radioactivity is associated with the monosomes and polysomes, and only the contaminating protein at the top of the gradient retains its radioactivity.

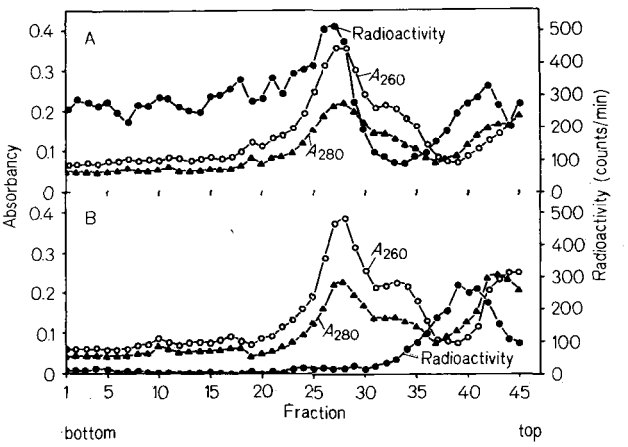

Fig. 2. *Gradient profiles and incorporated radioactivity of ribosomes isolated from mitochondria labelled* in vitro *with and without successive incubation with puromycin.* The crude ribosomal fractions of the Table were used

DISCUSSION

The absorbance profiles of the density gradients of mitochondrial ribosomes are essentially the same as those described by Küntzel and Noll [1]. Also the gel electrophoretic patterns of RNAs in Fig. 1 indicate that the mitochondria when isolated as described here do not contain appreciable amounts of contaminating cytoplasmic ribosomes. Consequently the results of the incorporation experiments are characteristic for the mitochondrial system.

After incorporation of radioactive amino acids into isolated mitochondria, the mitochondrial ribosomes have a higher specific radioactivity than the whole mitochondria, especially in the polysome

region. The ribosomal label can be removed by incubating the mitochondria with puromycin. It is therefore concluded that mitochondrial ribosomes incorporate amino acids into polypeptide chains. At least part of the ribosomes are active as polysomes. This confirms and extends recent experiments of Küntzel [10]. He demonstrated a poly U dependent activity of amino acid incorporation with mitochondrial ribosomes isolated from *Neurospora crassa*.

After release of the polypeptide chains from the mitochondrial ribosomes, no radioactivity is left in the ribosomal proteins. It can be concluded that isolated mitochondria do not synthesize significant amounts of the proteins of their ribosomes. It might be still assumed that ribosomal precursor proteins or peptides are synthesized by the mitochondrial system which are not integrated into the ribosomal proteins under these *in vitro* conditions. In preliminary gel electrophoretic studies (phenol—acetic acid system) no correlations have been found between the bands of the ribosomal proteins and the bands of insoluble mitochondrial proteins labelled by incorporation of radioactive amino acids into isolated mitochondria. Therefore the hypothetical precursor protein if existent at all must be quite different from the completed and integrated ribosomal proteins.

Less than 10% of the total incorporated radioactivity is found in incomplete peptide chains, in the experiments described here. Under non-optimal incorporation conditions however, these relationships are different. In an experiment in which incorporation into whole mitochondria was lower by a factor of 12.5 as compared to the experiment of the Table the specific radioactivity of the crude ribosomes was higher by a factor of 7.5 than the one of whole mitochondria. As much as 25% of the total radioactivity incorporated was found in the crude ribosomes in this case.

This work was supported by the Deutsche Forschungsgemeinschaft („Schwerpunktprogramm Biochemie der Morphogenese"). The authors wish to express their most sincere appreciation to F. Kaudewitz of the Institut für Genetik der Universität München for the laboratory facilities for cultivation of *Neurospora* and for his continuous support. The ribosomes of *E. coli* were generously supplied by W. Zillig of the Max-Planck-Institut für Biochemie, München. The authors thank also H. G. Zachau for his critical revision of the manuscript.

REFERENCES

1. Küntzel, H., and Noll, H., *Nature*, 215 (1967) 1340.
2. Rifkin, M. R., Wood, D. D., and Luck, D. J. L., *Proc. Natl. Acad. Sci U. S.* 58 (1967) 1025.
3. Luck, D. J. L., *J. Cell Biol.* 16 (1963) 483.
4. Sebald, W., Bücher, Th., Olbrich, B., and Kaudewitz, F., *FEBS Letters*, 1 (1968) 235.
5. Noll, H., *Nature*, 215 (1967) 360.
6. Neupert, W., Brdiczka, D., and Bücher, Th., *Biochem. Biophys. Res. Commun.* 27 (1967) 488.
7. Peacock, A. C., and Dingman, C. W., *Biochemistry*, 6 (1967) 1818.
8. Dingman, C. W., and Peacock, A. C., *Biochemistry*, 7 (1968) 659.

8a. Kleinow, W., unpublished results.

9. Dingman, C. W., and Peacock, A. C., *Biochemistry*, 7 (1968) 668.
10. Küntzel, H., *Nature*, 222 (1969) 142.

W. Neupert, W. Sebald, A. J. Schwab, A. Pfaller, and Th. Bücher
Institut für Physiologische Chemie und Physikalische Biochemie der Universität
BRD-8 München 15, Goethestraße 33, Germany