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

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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 Kuntzel and Noll [1] and by Rifkin et al. [2]. Radioactivity has been found in these ribosomes after labelling of isolated mitochondria by incorporation of $[^{14}\text{C}]$-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 $2 \times 10^6$ 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 $\mu$C/ml $\text{L-}[^{14}\text{C}]$leucine (311 mCi/mmole), $\text{L-}[^{14}\text{C}]$isoleucine (308 mCi/mmole) and $\text{L-}[^{14}\text{C}]$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$_4$Cl, 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 Kuntzel 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 × g. The supernatant was centrifuged for 3 h at 150000 × g. The resulting yellowish pellet (crude mitochondrial ribosomes) was resuspended in standard buffer (100 A260 units/ml). The suspension was centrifuged for 2 h at 41 000 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%o, reservoir concentration 30.5%o. Each beaker contained 10 A260 units.)

Generally the radioactivity of proteins was measured after precipitation and washing with trichloroacetic acid [el. Crude ribosomes however, were resuspended in standard buffer, absorbed on a millipore filter and counted in a Packard liquid scintillation 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 A260 units/ml). Sucrose was added to a final concentration of 8%o. 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%o polyacrylamide with 0.5%o 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%o NaCl solution instead of isolation fluid. The homogenate was centrifuged for 30 min at 27000 × 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 NH₄Cl, 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)
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 \(\mu\)C/ml each). Control mitochondria were withdrawn before further incubation with 0.4 mM puromycin for 7 min.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity of control</th>
<th>Radioactivity of puromycin incubated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>counts x min(^{-1}) x mg protein(^{-1})</td>
<td>counts/min</td>
</tr>
<tr>
<td>Mitochondria after incubation</td>
<td>7000</td>
<td>325,000</td>
</tr>
<tr>
<td>Mitochondrial lysate</td>
<td>7210</td>
<td>392,000</td>
</tr>
<tr>
<td>Sediment of mitochondrial lysate</td>
<td>7920</td>
<td>13,050</td>
</tr>
<tr>
<td>30 min, 35000 x g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude ribosomes</td>
<td>17,660</td>
<td>33,500</td>
</tr>
<tr>
<td>Supernatant after sedimentation of ribosomes</td>
<td>6185</td>
<td>18,070</td>
</tr>
</tbody>
</table>

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.

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REFERENCES

8a. Kleinow, W., unpublished results.

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