

Incorporation *in vivo* of ^{14}C -Labelled Amino Acids into the Proteins of Mitochondrial Ribosomes from *Neurospora crassa* Sensitive to Cycloheximide and Insensitive to Chloramphenicol

W. NEUPERT, W. SEBALD, A. J. SCHWAB, P. MASSINGER, and TH. BÜCHER

Institut für Physiologische Chemie und Physikalische Chemie der Universität München

(Received June 2, 1969)

Radioactive amino acids were incorporated *in vivo* into *Neurospora crassa* cells, and the mitochondrial ribosomes were isolated. The incorporation of radioactivity into the proteins of these ribosomes was inhibited by cycloheximide, but not by chloramphenicol. It is therefore concluded that these proteins are synthesized on the cycloheximide sensitive and chloramphenicol insensitive cytoplasmic ribosomes.

Biogenesis of mitochondria involves the cooperation of two systems of protein synthesis, one extramitochondrial (extrinsic) and one mitochondrial (intrinsic). In order to investigate the contribution of one system, the other has to be separated. In the preceding paper we reported on the intrinsic incorporation of amino acids by mitochondrial ribosomes *in vitro* in isolated mitochondria [1]. In the present experiments, separation of the mitochondrial and extramitochondrial system was achieved *in vivo* by the use of specific inhibitors.

It has already been shown by Sebald *et al.* for the locust flight muscle [2] and for *Neurospora* [3] that in the presence of cycloheximide the cytoplasmic protein synthesis is inhibited to an extent of more than 99%, whereas the mitochondria under these *in vivo* conditions still incorporate amino acids into the same electrophoretic fractions of the insoluble protein as *in vitro*. Therefore, cycloheximide was used to study the contribution of the mitochondrial system of protein synthesis to the biogenesis of mitochondrial ribosomes.

In a second series of experiments, the effect of D-chloramphenicol on the incorporation *in vivo* of amino acids into mitochondrial ribosomal proteins was investigated. This antibiotic is known to inhibit protein synthesis in isolated mitochondria [4]. In concentrations up to the solubility limit (4 mg/ml) D-chloramphenicol does not suppress the growth of *Neurospora*. On the other hand hyphae grown under these conditions have mitochondria with an altered respiratory chain [5]. Therefore chloramphenicol is considered to be a specific inhibitor of the mitochondrial protein synthesis. Similar conclusions have been drawn for yeast [6].

MATERIALS AND METHODS

Hyphae of *Neurospora crassa* (wild type 74 A) were grown as described in the preceding paper [1]. Before harvesting, L-[U- ^{14}C]leucine (311 mC/mmole), L-[U- ^{14}C]isoleucine (308 mC/mmole), and L-[U- ^{14}C]phenylalanine (459 mC/mmole) (The Radiochemical Centre, Amersham, England) were added (6.7 nC/ml each) to the growth medium. After 20 min the three amino acids were added unlabelled, each in a final concentration of 2 mM. The chase was maintained for 20 min, then the hyphae were collected by filtration and washed with isolation medium [1] containing 5 mM of each of the three unlabelled amino acids. In the inhibitor experiments the hyphae were preincubated before addition of the radioactive amino acids for 10 min with 0.1 mg/ml cycloheximide or with 4 mg/ml D-chloramphenicol.

Cell fractionations and density gradient centrifugations were carried out as described in the preceding paper [1]. For preparation of cytoplasmic ribosomes, the postmitochondrial supernatant was centrifuged for 15 min at $27000 \times g$. The supernatant was centrifuged for 2 h at $80000 \times g$. The resulting pellet was resuspended in standard buffer [1] and centrifuged once more for 2 h at $80000 \times g$.

RESULTS

In the Table and Fig. 1 the specific radioactivities of the cellular fractions from the successive steps of the preparation of mitochondrial ribosomes are shown. The control data listed in the Table and demonstrated in Fig. 1A were obtained from hyphae which had been grown from the same inoculum and were worked up in parallel (25 flasks each) to those

Table. Influence of inhibitors on the incorporation of ^{14}C -labelled amino acids *in vivo* into various fractions of *Neurospora crassa* hyphae

The labelling for 20 min with [^{14}C]leucine, [^{14}C]isoleucine, and [^{14}C]phenylalanine was followed by a 20 min chase period with unlabelled amino acids. The inhibitors (cycloheximide, 0.1 mg/ml, chloramphenicol, 4 mg/ml) were added 10 min prior to the addition of the radioactive amino acids

No.	Fraction	Specific radioactivity with		
		Control	Cycloheximide	Chloramphenicol
		counts \times min $^{-1}$ \times mg protein $^{-1}$		
1	Mitochondrial lysate	25010	2380	13280
2	Sediment of mitochondrial lysate, 30 min 30000 $\times g$	27700	1460	10830
3	Crude mitochondrial ribosomes ^a	21100	750	10400
4	Supernatant after sedimentation of mitochondrial ribosomes	23550	2800	12800
5	Cytoplasmic ribosomes	8700	234	5700
6	Supernatant of cytoplasmic ribosomes (cytosol)	17700	95	11810

^a The further separation of these fractions is presented in Fig. 1.

used in the cycloheximide experiment. The chloramphenicol experiment was made separately. It may be compared however to the same control.

In the control experiment (Table, first column) all mitochondrial fractions (1–4) exhibit a similar specific radioactivity. The specific radioactivity of the cell sap proteins (cytosol, fraction 6) is somewhat lower. The specific radioactivity of the cytoplasmic (extramitochondrial) ribosomes is about one half of that of the proteins in the supernatant. Probably this is not due to a lesser stability of the label during the chase. The specific radioactivity of the cytoplasmic ribosomes did not show significant changes during the chase period in preliminary experiments.

Under the action of cycloheximide (Table, second column) the incorporation into the cytoplasmic ribosomes (fraction 5) and the cell sap proteins (cytosol, fraction 6) is reduced to 2.5 and 0.5% as compared to the control. In contrast the incorporation into the whole mitochondrial protein (mitochondrial lysate, fraction 1) still amounts to about 10%. The larger part of this activity is found to be located in the Triton X-100 soluble fraction of the mitochondrial lysate (supernatant, fraction 4). Here the specific radioactivity amounts to 15% as compared to the control. The Triton soluble fraction includes the mitochondrial matrix and the mitochondrial membrane proteins in about equal proportions. Since it is known that the matrix proteins are not labelled by the mitochondrial system [4,5] the specific radioactivity of the mitochondrial membrane proteins can be estimated to about 30% as compared

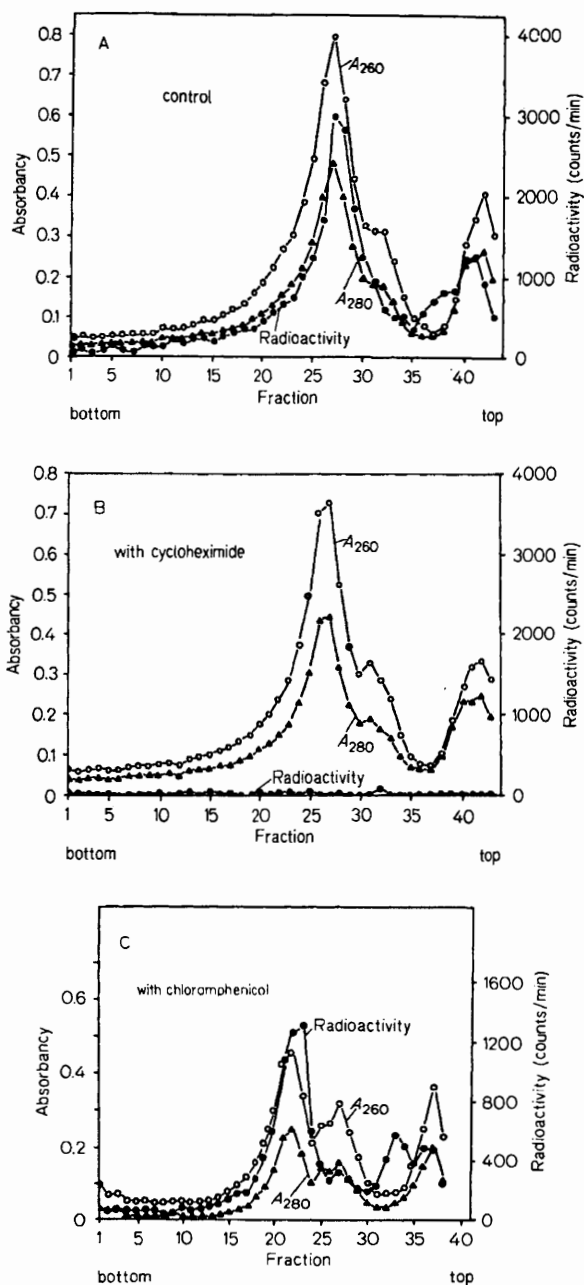


Fig. 1. Extension of the experiments presented in the Table. Gradient profiles of fractions 3. (A) from control hyphae, (B) from hyphae incubated in the presence of cycloheximide, (C) from hyphae incubated in the presence of chloramphenicol

to the control. Finally the crude ribosomal fraction of mitochondrial origin (fraction 3) entering the subsequent gradient centrifugation exhibits 3.5% of the controls specific radioactivity.

In the experiment with chloramphenicol, incorporation is almost uniformly lower in all fractions, as compared to the control experiment.

Fig.1 represents the density gradient profiles of the crude ribosomal fractions of the Table. In the control experiment (Fig. 1 A), a preferential labelling of the monosome peak appears. The subunit shoulder contains no radioactivity. On the top of the gradient a small part of the radioactivity remains. In the polysome region, where the growing peptide chains should appear [1], virtually no radioactivity is found. This shows the effectiveness of the 20 min chase with unlabelled amino acids.

With cycloheximide (Fig. 1 B), no significant radioactivity can be found in the mitochondrial monosome and polysome regions.

In contrast, with chloramphenicol (Fig. 1 C) the labelling profile is almost the same as in the control experiment.

DISCUSSION

In the experiments presented here cycloheximide blocks the cytoplasmic protein synthesis almost completely, whereas amino acids are still incorporated into mitochondrial membrane proteins.

The mitochondrial ribosomes are not labelled in this system under the action of cycloheximide.

Subsequent to the period of incorporation a chase with unlabelled amino acids, diluting the initially added radioactivity by a factor of 10^4 , has been applied. Therefore at the time of disruption of the fungi incomplete peptide chains can be assumed to be unlabelled. We therefore conclude that the radioactivity associated with the cytoplasmic and mitochondrial ribosomes represents to a large extent the labelling of the ribosomal proteins.

These results suggest that the proteins of mitochondrial ribosomes are synthesized by the extra-mitochondrial, cycloheximide sensitive system in *Neurospora crassa*. This is in agreement with the findings in the *in vitro* system [1] demonstrating the inability of isolated mitochondria to incorporate

amino acids into the structural proteins of their ribosomes. These conclusions are also supported by the experiments with chloramphenicol, a specific inhibitor of mitochondrial amino acid incorporation, which appears to be unable to block the synthesis of mitochondrial ribosomal proteins *in vivo*.

While this paper was in preparation, similar results and conclusions were published by Küntzel [7]. With respect to further speculations on the mechanisms of the biogenesis of mitochondrial ribosomes and the problems of translocation we may refer to this publication.

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 his support and help, and for the laboratory facilities put to their disposal for the cultivation of *Neurospora*. The authors wish also to thank H. G. Zachau for his critical revision of the manuscript.

REFERENCES

1. Neupert, W., Sebald, W., Schwab, A. J., Pfaller, A., and Bücher, Th., *European J. Biochem.* 10 (1969) 585.
2. Sebald, W., Hofstötter, Th., Hacker, and Bücher, Th., *FEBS Letters*, 2 (1969) 177.
3. Sebald, W., Schwab, A. J., and Hacker, D., *FEBS letters*, in press.
4. Sebald, W., Bücher, Th., Olbrich, B., and Kaudewitz, F., *FEBS Letters*, 1 (1968) 235.
5. Sebald, W., presented at the 20th Mosbach colloquium, Mosbach 1969.
6. Huang, M., Biggs, D. R., Clark-Walker, G. D., and Linnane, A. W., *Biochim. Biophys. Acta*, 114 (1966) 434.
7. Küntzel, H., *Nature*, 222 (1969) 142.

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