Evidence for the Existence of Membrane-bound Ribosomes in *Neurospora crassa*

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SUMMARY

There has been disagreement as to whether *Neurospora crassa* contains ribosomes bound to the endoplasmic reticulum. We have isolated a cellular fraction which by morphological and biochemical criteria appears to consist of membrane-bound ribosomes.

INTRODUCTION

There is increasing evidence that ribosomes exist in most eucaryotes either free in the cytoplasm or bound to the endoplasmic reticulum (Siekevitz & Palade, 1960; Blobel & Potter, 1967*a, b*). These two types of ribosomes appear to synthesize different types of proteins (Redman, 1968; Takagi & Ogata, 1968; Tanaka & Ogata, 1971). In rat liver, the bound ribosomes synthesize serum proteins and those intended for extracellular secretion (Redman, 1968; Takagi & Ogata, 1968), and the free ribosomes synthesize intracellular soluble proteins (Tanaka & Ogata, 1971). It has not been reported whether *Neurospora crassa*, an Ascomycete, contains membrane-bound ribosomes, although it is known that it does secrete extracellular enzymes (Chang & Trevithick, 1972; Drucker, 1972).

Several workers have made ultrastructural studies of *Neurospora crassa*. Weiss (1963) reported that the endoplasmic reticulum in conidia was almost undetectable. Although she noted an association of ribosomes with endoplasmic reticulum, she concluded that this association was non-specific as there was a large number of ribosomes free in the cytoplasm. Namboodiri (1966) also found the endoplasmic reticulum to be scantily distributed and discontinuous in conidia. This was not the case in hyphae, however. The mycelia contained well-defined segments of endoplasmic reticulum. No rough endoplasmic reticulum was observed, but this may have been because potassium permanganate, which is known to dissociate ribosomes from endoplasmic reticulum, was used as a fixative (Yotsuyanagi, 1962). Zalokar (1961), investigating centrifuged hyphal tips, found that the ribosomes were scattered throughout the cytoplasm, but noted no connexion of ribosomes with endoplasmic reticulum. However, with the endoplasmic reticulum were associated 'small granules, similar to ribosomes, but more irregular in size, more diffused in outline, and not as dark'. He made no study of ribonuclease-digested samples. Shatkin & Tatum (1959) found dense particles which were removed on treatment with ribonuclease and were associated with endoplasmic reticulum. Burnett (1968) states that although within the sheets of endoplasmic reticulum in *Neurospora* there are many granules which fall within the characteristic dimensions of ribosomes, as yet there has been no clear demonstration of 'rough' and 'smooth' endoplasmic reticulum and it is, indeed, unusual for granules to be attached to the membrane, even in cells of *Neurospora* engaged in active protein synthesis.

Thus it is still uncertain whether *Neurospora crassa* contains ribosomes bound to endoplasmic reticulum. We report here a biochemical and electron-micrographic study to investigate this.

METHODS

Organism. Wild-type *Neurospora crassa* wa-no. 1961 containing mainly the genetic background Em 5297a was used. The conidia were maintained on agar-plate cultures, containing 1.5 % sucrose, and Fries minimal medium (Beadle & Tatum, 1945). For the experiments outlined, the conidia were harvested by a vacuum technique, suspended in 20 ml of distilled water, and centrifuged at 650 *g* for 5 min. A uniform inoculum (equivalent to 0.1 ml of packed conidia) was inoculated into a 2 l flask containing 650 ml of Fries minimal medium (at 25 % of the recommended concentration) with 1.5 % sucrose as the carbon source, and was grown for 30 h at 30 °C in a gyratory shaker bath at 150 rev./min (New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.). The mycelia were harvested on a glass-fibre filter and were suspended in 20 ml of Medium A of Falvey & Staehelin (1970) containing 10 % sorbitol as an osmotic support, ruptured by sonic oscillation for 3 to 5 s, and homogenized with 20 strokes of a Ten Broeck homogenizer.

Isolation of free, bound and total ribosomal fractions. The procedure for the isolation of free, membrane-bound, and total ribosomal fractions is outlined in Fig 1. The homogenate was centrifuged at 13 500 *g* for 15 min in a Sorvall RC-2B centrifuge (SS 34 rotor, Sorvall Inc., Norwalk, Connecticut, U.S.A.), after which the post-mitochondrial supernatant was divided into two equal samples. Sample 1 was left untreated, sample 2 was treated with 4 % Triton-X-100 and 1 % sodium deoxycholate. The post-mitochondrial supernatants of both samples were layered on discontinuous gradients of 2.5 ml of 2.44 M-sorbitol over 2.5 ml of 3.76 M-sorbitol, and centrifuged in a Beckman L2-65B ultracentrifuge (Beckman Instruments Inc., Palo Alto, California, U.S.A.). In this procedure, the bound ribosomes of sample one banded throughout the 2.44 M layer, and the free ribosomes collected as a pellet at the bottom of the tube. The 2.44 M layer of sample one was isolated, suspended in three times its volume in buffer, and recentrifuged in an SW 40 rotor at 106 000 *g* for 35 min. The free ribosomes of sample one were rehomogenized in buffer and centrifuged in a Ti50 rotor (Beckman Instruments Inc.) at 165 000 *g* for 2 h. The total ribosomes of sample 2 collected as a pellet at the bottom of the tube. Both the pellet and the 2.44 M layer were assayed for RNA content by the orcinol method. The concentration of bound ribosomes was calculated in two ways: in sample 1, the free and membrane-bound RNA content was assayed directly, while in sample 2, the membrane-bound RNA content was calculated by subtracting the free RNA content obtained in sample 1 from the total RNA content obtained in sample 2.

Analytical methods. (i) RNA. RNA content was measured by a modified Mejbaum method (Merchant, Kahn & Murphy, 1964). Yeast RNA was used as a standard.

(ii) Electron-micrographic studies. Preparations were examined for simultaneous osmium-glutaraldehyde fixation, followed by post-fixation in uranyl acetate, as outlined by Hirsch & Federko (1968) and embedded in Epon resin. Preparations were examined for free ribosomes by staining with 5 % phosphotungstic acid (Horne, 1967).

(iii) Protein. Samples were hydrolysed in 5 % trichloroacetic acid and assayed by the biuret method (Layne, 1957).

(iv) Phospholipid. Phospholipid phosphorus was assayed by the method of Lindberg & Ernster (1956) after Folch extraction (chloroform:methanol, 2:1) and digestion (Denton & Randle, 1967).

Fig. 1. An isolation scheme for free, membrane-bound, and total ribosomes of *Neurospora crassa*.

Fig. 2. (a) Free ribosomes of *Neurospora crassa* stained with 5 % PTA (Horne, 1967). Membrane contamination was not evident. Bar marker = $0.1 \mu\text{m}$. (b) Membrane-bound ribosomes of *Neurospora crassa* fixed in osmium-glutaraldehyde and post-fixed in uranyl acetate (Hirsch & Federko, 1968). Serial thin sections were prepared. Bar marker = $0.1 \mu\text{m}$.

RESULTS AND DISCUSSION

At the time this work began, whether or not *Neurospora crassa* contained ribosomes bound to the endoplasmic reticulum was still in dispute. Although several workers had noticed an association of ribosomes with membranes (Burnett, 1968; Weiss, 1963; Shatkin & Tatum, 1959), whether or not these particles were specifically attached to or lying adjacent to the membrane was still in doubt, because of the large number of ribosomes lying free in

Table 1. *The RNA content of free, membrane-bound, and total ribosomal fractions*

The post-mitochondrial supernatant was divided into two equal fractions. Sample 1 was placed untreated on the modified gradient of Blobel & Potter (1967*a*). From this fraction was obtained the RNA content of the free and membrane-bound ribosomal fraction. Sample 2 was treated with 4% Triton-X-100 and 1% deoxycholate and placed on the modified gradient. From this fraction was obtained the RNA content of the total ribosomal fraction. The RNA content for the membrane-bound ribosomes for this fraction was obtained by subtracting the free ribosomal RNA concentration obtained in sample 1 from the total ribosomal fraction obtained in sample 2. RNA was determined by the orcinol method.

Experiment no.	Sample 1		Sample 2 Total ribosome fraction (mg RNA)	Total minus free ribosome fraction (mg RNA)	B/T* (%)
	Membrane- bound ribosome fraction (mg RNA)	Free + membrane- bound ribosome fraction (mg RNA)			
1	3.0	13.9	13.4	2.4	125
2	2.6	17.7	17.3	2.3	113
3	4.0	16.8	17.4	4.6	91
4	4.0	17.0	17.0	4.1	98

$$* \frac{B}{T} = \frac{\text{Bound ribosome fraction} \times 100}{\text{total minus free ribosome fraction}}; \quad \bar{x} = 107; \text{ s.e.} = \pm 8.$$

the cytoplasm and the fact that the ribosomes are irregularly spaced and infrequent along the membrane.

Electron-micrographic studies. Fig. 2 shows the electron micrographs of free and membrane-bound ribosomes. The dimensions of the particles of Fig. 2*b*, which are very close to or associated with the membrane, are in agreement with published dimensions of ribosomes (Spirin & Gavrilova, 1968). Similar photographs of bound ribosomes have been obtained in rat liver (Rolleston, 1972).

Biochemical studies. Table 1 gives the RNA concentration obtained for bound ribosomes from both sample 1 and sample 2. A calculation of the concentration of bound ribosomes gives the same value whether calculated directly from the RNA content of the free and bound ribosomal fractions (sample 1) or indirectly by subtracting the RNA content of the free ribosomal fraction from the RNA content of the total ribosomal fraction (sample 2), indicating that a cellular fraction contains RNA sediments at a density normally associated with bound endoplasmic reticulum.

The samples sedimenting throughout the 2.44 M layer were treated with 1% sodium deoxycholate to remove the ribosomes from the membrane. When this fraction was recentrifuged in the modified gradient of Blobel & Potter (1967*a*), this fraction collected as a pellet at the bottom of the tube and had an RNA/protein ratio similar to that found for free ribosomes (0.89).

In two separate experiments, when the 2.44 M layer normally associated with bound ribosomes was resuspended by homogenization and recentrifuged in the modified gradient of Blobel & Potter (1967*a*), 0% and 13% of the original RNA of this layer then appeared in the free ribosomal pellet. Similarly, when the free ribosomal fraction was added to the smooth membrane fraction and recentrifuged in the gradient previously outlined, the proportion of the original RNA of the free ribosomal fraction in the 2.44 M layer was 0% and 3%. This is therefore not a non-specific trapping of ribosomes within vesicles.

This fraction is also not an artifact of the isolation method, since samples which are

disrupted by grinding in acid-washed sea sand will sediment in a similar manner (Grinwich & Trevithick, 1973).

Both morphologically and biochemically, this material is similar to the rough endoplasmic reticulum found in plant and animal cells, although the ribosomes are not as abundant and are more irregularly spaced along the membrane. We conclude, therefore, that *Neurospora crassa* contains a cellular component that is similar in gross morphological structure and biochemical composition to the rough endoplasmic reticulum of higher plants and animals.

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