Saccharomyces cerevisiae mitochondria can synthesise FMN and FAD from externally added riboflavin and export them to the extramitochondrial phase

Maria Luigia Pallotta[a], Carmen Brizio[b], Alessandra Fratianni[a], Caterina De Virgilio[b], Maria Barile[b], Salvatore Passarella[a,*]

[a] *Dipartimento di Scienze Animali, Vegetali e dell'Ambiente, via F. De Sanctis, Università del Molise, 86100 Campobasso, Italy*
[b] *Dipartimento di Biochimica e Biologia Molecolare, Università di Bari, Bari, Italy*

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Abstract Evidence is given that mitochondria isolated from *Saccharomyces cerevisiae* can take up externally added riboflavin and synthesise from it both flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) probably due to the existence of the mitochondrial riboflavin kinase already reported and the novel mitochondria FAD synthetase. Moreover *Saccharomyces cerevisiae* mitochondria can export the newly synthesised flavin derivatives to the extramitochondrial phase. This has been proven to take place with 1:1 stoichiometry with riboflavin decrease outside mitochondria, thus showing that flavin traffic occurs across the mitochondrial membranes.

Key words: Mitochondrion; Riboflavin; Flavin adenine dinucleotide; Flavin mononucleotide; *Saccharomyces cerevisiae*

1. Introduction

One of the most outstanding problems in molecular biogenesis of mitochondria concerns the mechanisms by which vitamin and vitamin-derived cofactors enter mitochondria and, following a possible mitochondrial processing, reach mitochondrial compartments where they are involved in enzymatic catalysis.

As far as flavin cofactors are concerned, the existence of riboflavin kinase (EC 2.7.1.26) and flavin adenine dinucleotide (FAD) synthetase (EC 2.7.7.2) was shown about fifty years ago with both the enzymes studied in detail [1], however, today neither their cell localisation nor flavin transport has been exhaustively elucidated (see [2]). It is known that the majority of cell flavoproteins is located in the mitochondria, but a matter of debate is whether they contain enzymes designed to flavin metabolism. McCormick [2] showed that FAD and flavin mononucleotide (FMN) synthesis can occur in hepatocyte cytosol; however, we have reported that rat liver mitochondria (RLM) can synthesise FAD from FMN taken up in a carrier-mediated process [3]. Moreover, recently RLM were shown to contain their specific FAD pyrophosphatase (EC 3.6.1.18) and FMN phosphohydrolase (EC 3.1.3.2) [4]. At present, the knowledge of flavin metabolism and of its transport in mitochondria from other sources is rather poor. In particular, it was suggested that certain yeast mitochondria do not contain FAD synthetase, thus requiring a protein involved in FAD transport from the cytosol [5,6].

The pathway of riboflavin biosynthesis in microorganisms yields unphosphorylated riboflavin. For this reason, in an initial investigation *Saccharomyces cerevisiae* mitochondria (SCM) were added with riboflavin and measurements were made in order to find out whether they can synthesise flavin derivatives. In this work we show the capability of isolated SCM to synthesise FMN and FAD from externally added riboflavin, and to export them outside mitochondria, probably via the already reported riboflavin kinase, the novel FAD synthetase and the putative flavin translocator(s), respectively.

2. Materials and methods

All reagents and enzymes were from Sigma (St. Louis, MO, USA). Mitochondrial substrates were used as Tris salts at pH 7.0–7.3. Reagents used for HPLC were from J.T. Baker (Deventer, The Netherlands).

A strain (L220) of *Saccharomyces cerevisiae* (kindly supplied by C. Zambonelli, Diproval Bologna, Italy) was used. Cells were grown aerobically for 16 h at 28°C to a final A_{600} equal to 2 in a semisynthetic medium (1 l) containing 3 g bacto-yeast extract, 1 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $CaCl_2 2H_2O$, 0.5 g NaCl, 0.6 g $MgCl_2$, with 2% lactate as the carbon source and supplemented with 0.05% glucose. The pH of the medium was adjusted to 5.5 with NaOH.

Mitochondria were isolated according to [7] and mitochondrial protein measured as in [8].

Oxygen uptake by SCM was measured at 25°C by a Gilson oxygraph, in a medium (1.5 ml) containing 0.6 M mannitol, 20 mM HEPES-Tris (pH 7.4), 10 mM potassium phosphate, 2 mM $MgCl_2$, 1 mM EDTA and 5 mg/ml BSA. The intactness of the mitochondrial outer and inner membrane was checked as in [9]. Safranine response was monitored as in [10]. ATP synthesis and export was measured as in [11].

Flavin metabolism was investigated as follows: freshly isolated mitochondria (0.2 mg protein) were incubated for 1 min in 500 μl of standard medium (0.6 M mannitol, 50 mM Tris-HCl, pH 7, 1 mM $MgCl_2$), then the riboflavin was added. At the appropriate time the suspension was rapidly centrifuged for 2 min at 15000 rpm in a refrigerated microcentrifuge Heraeus, equipped with an Eppendorf rotor. Perchloric extracts of supernatant and pellet were obtained and neutralised as in [12]. In order to determine the amount of riboflavin, FAD and FMN, both in the supernatant and in the mitochondrial pellet, aliquots of the neutralised perchloric extracts were analysed by means of HPLC (Kontron Instrument HPLC system including a model 420 pump and a model 425 gradient former equipped with a data system 450 MT2) as previously reported [4], with a calibration made in each experiment.

The FAD appearance outside the mitochondria was assayed enzymatically as in [13] using a flavin adenine dinucleotide detecting system (FADDS) consisting of apo-D-aminoacid oxidase (0.14 mg), LDH (2.2 E.U.), NADH (180 μM), rotenone (2 μg), D-alanine (50 mM).

*Corresponding author. Fax: (39) (874) 417678.
E-mail: passarel@hpsrv.unimol.it

Abbreviations: FADDS, flavin adenine dinucleotide detecting system; RLM, rat liver mitochondria; SCM, *Saccharomyces cerevisiae* mitochondria

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FAD appearance was detected by photometrically measuring the holo-D-aminoacid oxidase (EC 1.4.3.3) activity. The latter was measured as a result of FAD binding to the partially inactive apo-D-aminoacid oxidase. Apoenzyme was obtained as previously described [3] and the rate of oxidation of the externally added D-alanine (50 mM) was expressed as the rate of NADH dependent reduction of pyruvate, which is the product of the D-aminoacid oxidase reaction. Both D-alanine concentration and lactate dehydrogenase activity were chosen in such a way that the measured reaction rate depends upon the FAD dependent D-aminoacid oxidase activity. The plots were obtained by means of Grafit software (Erithacus).

3. Results

Since both flavin derivative synthesis in mitochondria and flavin traffic across the mitochondrial membranes are expected to depend on the organelle energy state, in a series of preliminary experiments SCM were checked with respect to their functional features. The intactness of the mitochondrial membranes was evaluated by using the Triton X-100 test as in [9]: 85 and 95% integrity was found for the outer and the inner membrane, as revealed by measuring both cytochrome *c* oxidase and fumarase latency, respectively. SCM coupling and capability to synthesise ATP was also investigated. When added with 0.5 mM ADP, SCM show respiratory control indices 2.3 and 2.2, with NADH and 5 mM succinate used as a substrate, respectively. As shown by using the fluorimetric probe safranine as in [10], externally added succinate increased mitochondrial membrane potential. Finally, SCM was able to synthesise and export ATP from externally added ADP, with the rate of ADP/ATP exchange measured as in [11].

In order to gain some insight into SCM capability to synthesise FMN and FAD, 10 μM riboflavin was added to the organelles; the mitochondrial and extramitochondrial amounts of riboflavin, FMN and FAD were measured via HPLC and compared with those of parallel samples in which no riboflavin was added (Fig. 1). FMN and FAD contents in isolated SCM were 73 and 220 pmol/mg protein, respectively (Fig. 1a) (63 ± 23 and 243 ± 84 pmol/mg protein in 10 experiments), whereas the amount of riboflavin was not relevant. As a result of the addition of 10 μM riboflavin both FMN and FAD mitochondrial content increased by 120% and 330%, respectively; on the other hand riboflavin accumulation was found to occur up to a mM concentration (Fig. 1b). No appearance of FMN and FAD was revealed in the postmitochondrial supernatant both in the absence and in the presence of externally added riboflavin (Fig. 1a′,b′).

In order to ascertain whether FMN and FAD synthesis could occur outside SCM, caused by possible extramitochondrial contamination, in the same experiment 10 μM riboflavin plus ATP (i.e. the riboflavin kinase substrate pair) were added to the mitochondrial suspension. No change was found with respect to the results reported in Fig. 1, thus showing that the endogenous ATP does not limit flavin derivative synthesis and, more importantly, demonstrating that FMN synthesis cannot occur outside mitochondria. In order to confirm such a conclusion, in another experiment SCM were incu-

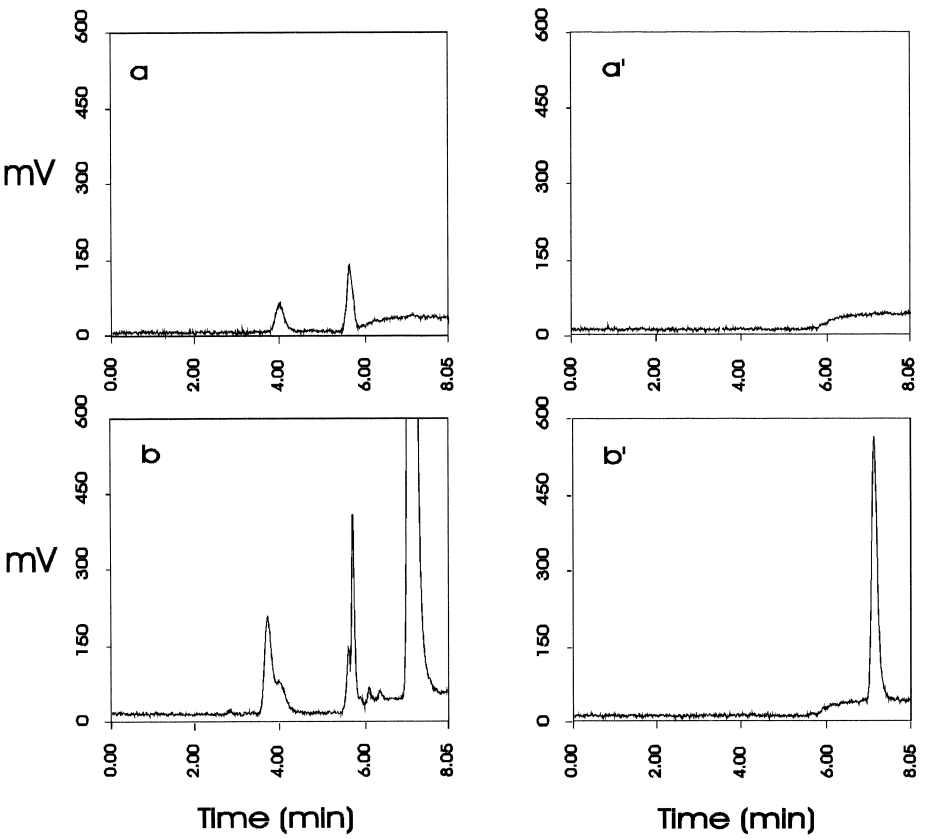

Fig. 1. HPLC evidence for FMN and FAD synthesis in SCM to which riboflavin was added. SCM (0.2 mg protein) were incubated at 19°C in 500 μl of the standard medium for 1 min, then 10 μM riboflavin (b,b′) or medium (a,a′) was added. After 90 s incubation time, the mitochondrial suspensions were rapidly centrifuged and neutralised perchloric extracts of mitochondrial pellets (a,b) and supernatants (a′,b′) were obtained. FAD, FMN and riboflavin were measured via HPLC in 5–30-μl extracts as reported in the Section 2.

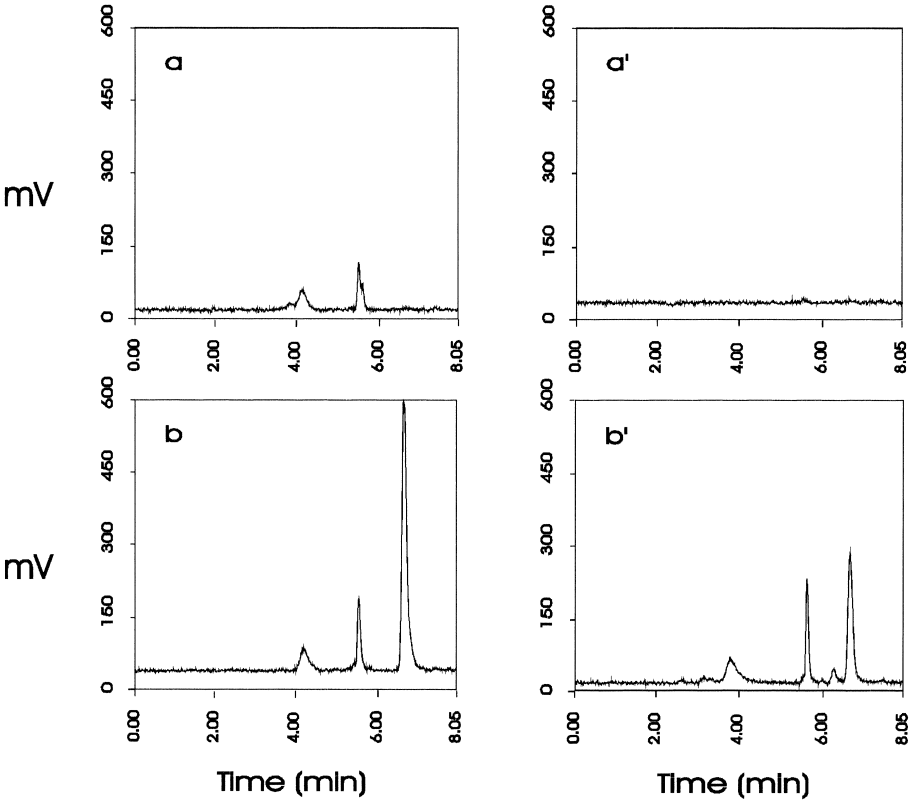

Fig. 2. HPLC evidence of flavin derivative export from SCM to which riboflavin was added. SCM (0.2 mg protein) were incubated in the same experimental conditions as in Fig. 1. After 1 min, 1 μM riboflavin (b,b′) or medium (a,a′) was added and 15 s later mitochondrial suspensions were rapidly centrifuged, neutralised perchloric extracts of mitochondrial pellets (a,c) and supernatants (a′,c′) were obtained. FAD, FMN and riboflavin were measured via HPLC in 60-μl extracts, as reported in Section 2.

bated in the absence of riboflavin under the same experimental conditions as in Fig. 1, then they were centrifuged and the supernatants added with either riboflavin or FMN. In both cases no flavin derivative synthesis was found to occur, thus definitively showing that both FMN and FAD synthesis must occur only in isolated mitochondria.

Riboflavin, FMN and FAD concentrations in yeast spheroplast cytosol were about 0.5, 4 and 8 μM, respectively, as calculated via HPLC measurement of the spheroplast neutralised perchloric extracts, by assuming both the spheroplast volume to be equal to 3 μl/mg protein and mitochondrial protein to be equal to 10% of spheroplast protein. Thus, in order to gain further insight into the mechanism of flavin derivative production due to SCM and to ascertain whether SCM can export newly synthesised flavin derivatives, 1 μM riboflavin was added to SCM and the content of the flavin compounds measured and compared with that of SCM without riboflavin addition (Fig. 2). No significant increase in the mitochondrial FMN and FAD content was found as compared to the control (Fig. 2a,b); surprisingly, FMN (300 pmol/mg protein) and more importantly, FAD (840 pmol/mg protein) were found in the extramitochondrial phase (see Fig. 2a′,b′). In SCM riboflavin accumulation up to 240 μM was also found as calculated by considering the mitochondria volume to be equal to 1 μl/mg protein.

In order to further confirm FAD export from intact mitochondria and to check whether the exported FAD can still act as the prosthetic group of flavin enzymes, in another experiment FAD appearance outside mitochondria to which riboflavin (1 μM) was added, was revealed enzymatically, adding SCM with riboflavin in the presence of FADDS (Fig. 3). The monitored NADH oxidation in the presence of apo-D-amino-acid oxidase and D-alanine occurs essentially owing to the existence of rotenone insensitive yeast NADH dehydrogenase [14]; when 1 μM riboflavin, which per se has no effects on the FADDS, was added to SCM, an increase in NADH oxidation

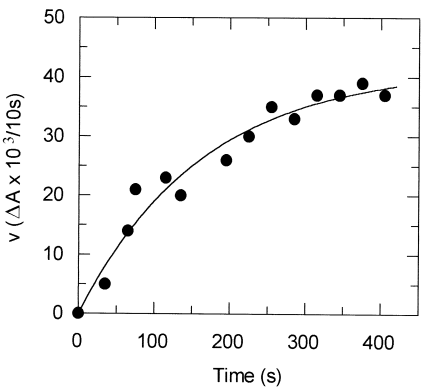

Fig. 3. Photomeric evidence of FAD export from SCM to which riboflavin was added. SCM (0.2 mg protein) were incubated at 19°C in the standard medium (800 μl). After 1 min FADDS was added and the rate of NADH oxidation (V_o) was measured as tangent at the experimental trace for 10-s intervals, every 20 s. When V_o was constant (about 1 min) riboflavin (1 μM) was added and the rate of NADH oxidation (V_r) measured as described above. In the ordinate $V=(V_r-V_o)$ is reported.

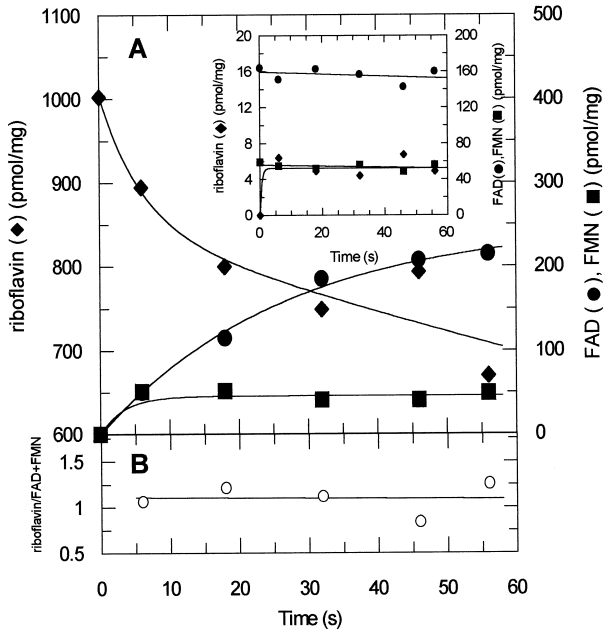

Fig. 4. Time course of flavin derivative export from SCM to which riboflavin was added. RLM (0.2 mg protein) were incubated at 2°C in 500 μl of standard medium for 1 min, then riboflavin (1 μM) was added. At appropriate points in time after riboflavin addition, the mitochondria were rapidly centrifuged and neutralised perchloric extracts of mitochondrial pellets and supernatants were obtained. The amount of FAD, FMN and riboflavin, measured via HPLC in 60 μl of supernatant (panel A) and pellet (see inset, panel A) extracts, was reported as a function of riboflavin incubation time. Panel B shows the ratio between the amount of riboflavin that disappeared in the supernatant plus that taken up by mitochondrial pellet and the amount of FMN plus FAD measured in the supernatant, as calculated at each riboflavin incubation time.

rate was measured, thus showing FAD appearance outside SCM. NADH oxidation rate was found to increase by time, reaching a constant value after 5 min after riboflavin addition (Fig. 3). FAD amount exported from mitochondria, as calculated by using a calibration curve, was found to be equal to about 190 pmol/mg, in fairly good agreement with measurement via HPLC under the same experimental conditions. Unfortunately, the rate of NADH oxidation appears to depend on the rate of the holoenzyme reconstitution, thus preventing any use of the FADDS in kinetic measurements.

In the light of the reported results, which require both riboflavin uptake into SCM and FMN/FAD export from SCM, an initial investigation was carried out in order to find out how SCM can regulate flavin traffic across the mitochondrial membranes. First the time course of the investigated processes was examined. In order to achieve this, 1 μM riboflavin was added to SCM at 2°C and the content of the flavin components was measured as a function of riboflavin incubation time, both in the mitochondria and in the supernatants (Fig. 4). The amounts of mitochondrial FMN and FAD were found to remain rather constant in time; in contrast riboflavin accumulated immediately in the matrix up to 6 μM (see inset), with no further significant change in the investigated times. Riboflavin content was found to decrease in the supernatant with a rate equal to 25 pmol/s/mg protein; on the other hand, in agreement with Fig. 2, FMN and FAD were found to appear outside mitochondria. In a few seconds 50 pmol/mg protein FMN were measured in the extramitochondrial phase, which remained constant within 1 min; on the other hand the amount of FAD was found to increase up to 200 pmol/mg protein at a rate equal to 9 pmol/s/mg protein. Interestingly, the ratio between the amount of riboflavin that disappeared in the supernatant, plus that taken up by SCM and FMN plus the amount of FAD that appeared in the extramitochondrial phase was found to be in fairly good agreement with a 1:1 stoichiometry (Fig. 4, panel B). This experiment further shows that SCM can synthesise and export FMN and, to a higher extent, FAD from the mitochondrial matrix.

4. Discussion

This paper shows a novel feature of isolated SCM, i.e. the capability to synthesise FAD from externally added riboflavin. This has been assumed to occur via riboflavin kinase and FAD synthetase which catalyse FMN synthesis from riboflavin and ATP and FAD from FMN and ATP [5,6], respectively. Indeed, this paper confirms the existence of the mitochondrial riboflavin kinase and gives the first evidence in favour of the existence of the mitochondrial FAD synthetase. We show that both FMN and FAD synthesis occur in the mitochondria, in fact no flavin derivative is revealed both in SCM supernatant, to which riboflavin and ATP were previously added, and in the supernatant to which riboflavin was added after mitochondria sedimentation. These results excluded that FAD synthesis was due to any extramitochondrial contamination.

At present the localisation of mitochondrial enzymes, which synthesise FMN and FAD, is not clear. In fact, in agreement with [5,6], we have found riboflavin kinase activity in the matrix fraction; however, no FAD synthesis was found as a result of FMN and ATP addition in the same fraction, thus suggesting either the occurrence of this reaction in the other mitochondrial compartment or, more probably, FAD synthetase inactivation in the purified matrix fraction.

Whatever flavin derivative synthesis occurs, riboflavin uptake into SCM is required; the mechanism by which this occurs is, at present, unknown. Indeed, since no FMN and FAD efflux takes place as a result of the addition of 10 μM riboflavin to SCM, the possibility of the existence of the riboflavin uniporter and/or the riboflavin diffusion across the mitochondrial membrane should be taken into consideration. Unfortunately, a detailed kinetic study of riboflavin uptake is prevented by its rapid conversion into FMN, which is slowly transformed into FAD. Research aimed at investigating riboflavin uptake into SCM is presently in progress.

In this paper the initial proposal of the existence of a protein involved in flavin transport in SCM [5,6] has been confirmed. At present the nature of the flavin carrier must be a matter of speculation; indeed the 1:1 stoichiometry reported in Fig. 4 could account for the existence of the nucleotide/riboflavin antiporter; however, the possibility of the existence of a high affinity uniporter for the newly synthesised flavin nucleotides cannot be ruled out.

Thus, whatever the transport mechanism is, flavin traffic across the mitochondrial membrane is suggested to involve both riboflavin uptake and nucleotide export mediated by either the nucleotide/riboflavin antiporter or by two independent uniporters. The same translocator(s) would also be able to account for FAD movement across the mitochondrial membranes, as measured in experiments in which FAD concentra-

tion (60 μM) was very high with respect to the physiological concentration [6]. In this paper the riboflavin concentration used was similar to that measured in yeast spheroplasts, thus preventing other artificial phenomena which might impair FAD efflux. In this regard we explain the lack of both FMN and FAD appearance outside mitochondria to which 10 μM riboflavin was added with the inhibition of FMN and FAD transport due to riboflavin accumulated in the matrix.

The question as to whether SCM could provide both FMN and FAD for other cell compartments per se or together with other extramitochondrial enzymes [5,6] remains to be established. However, in the light of cytosolic riboflavin, FMN and FAD concentrations, as calculated by measuring both spheroplast and mitochondrial content via HPLC, we are forced to suggest that mitochondria play a major role in regulating the flavin pool in yeast.

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