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Nicoletta Beffagna, Sergio Cocucci, Maria Cecilia Cocucci, Erasmo Marrè

On the mechanism of the block of protein synthesis induced in Rhodotorula gracilis by nitrogen starvation

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Fisiologia vegetale. — *On the mechanism of the block of protein synthesis induced in* Rhodotorula gracilis *by nitrogen starvation* (*). Nota di NICOLETTA BEFFAGNA, SERGIO COCUCCI, MARIA CECILIA COCUCCI e ERASMO MARRÈ, presentata (**) dal Corrisp. E. MARRÈ.

RIASSUNTO. — In cellule di *Rhodotorula gracilis* in fase di crescita logaritmica il trasferimento in mezzo carente di fonte di azoto induce un arresto quasi immediato della sintesi netta di RNA seguito da una diminuzione e quindi un arresto della sintesi delle proteine. Esperienze di incorporazione nell'RNA di precursori marcati (ortofosfato ^{32}P acido orotico–6–^{14}C) suggeriscono che il blocco dell'aumento netto di RNA non dipende tanto da un blocco a livello trascrizione, quanto dalla rapida e precoce demolizione dell'RNA trascritto, prima che esso giunga alle sue forme definitive come RNA dei ribosomi o RNA messaggero funzionante.

Ammonia, as a source of nitrogen, is very efficiently taken up by *Rhodotorula gracilis* grown in a mineral minimal medium. When ammonia is the limiting factor, its exhaustion is shown by a sharp flattening of the growth curve. From this point on, biosynthesis of reserve lipids is enhanced, some cell division still goes on, but very little protein synthesis is observed [1].

In this paper some preliminary observations are reported on the apparently complex mechanism by which in this organism the deficiency of the nitrogen source in the medium leads to the inibition of protein synthesis.

EXPERIMENTAL

Cells were maintained on agar malt slants and transferred when wanted in a minimal mineral medium containing the required growth factors (thiamine and pantothenate), 5 % glucose as carbon source and 5 % ammonium sulphate as nitrogen source [2].

Incubation was then carried on at 30° C with eccentrical rotative agitation (140 rev/min). When still in the logaritmic growth phase, aliquots of the cells were collected by rapid filtration in the presence of decalite (0,2 % weight/volume) and resuspended in the same fresh medium containing or not ammonium sulphate. DNA , RNA, polyamines, aminoacids, were measured, and tests were performed for the capacity to incorporate nucleic acid and protein precursors.

(*) Laboratorio di Fisiologia Vegetale, Istituto di Scienze Botaniche, Università di Milano – Centro di Studio del C.N.R. per la Biologia Cellulare e Molecolare delle Piante. Milano (Italia).

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Total DNA, RNA and proteins were determined by the methods of Nieman and Poulsen [3]. Intact RNA was obtained by extracting cells grinded with alumina [4] using the SDS–phenol method [5]. Total free aminoacids (together with a comparatively small amount of polyamines) were determined by the ninhydrine reaction according to Moore and Stein [6] in a perchloric acid extract from cells washed several times to remove completely ammonium ion. In the labeled tracer studies the cells were supplemented with ^{32}P ortophosphate, carrier free, 2 μC/ml (final ortophosphate concentration 5.73×10^{-3} M) or with 6 ^{14}C orotic acid 0.04 μC/ml (final orotic acid concentration 5×10^{-4} M). Incorporation of radioactive compounds was measured by a Packard scintillation counter.

Results

As shown in fig. 1, during the first hour after the transfer of the cells into the nitrogen deficient medium the concentration of total aminoacids decreases to a value of ca. 55% of the initial. In the following period the

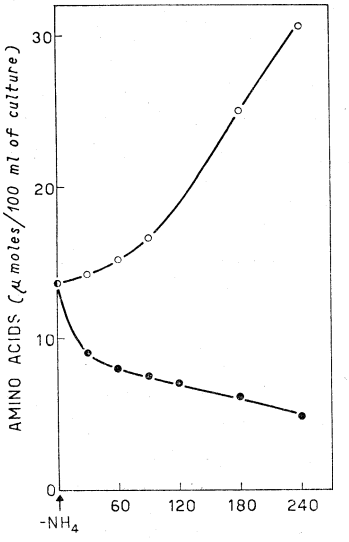

Fig. 1. – Effects of deficiency of the nitrogen source on the level of aminoacids in *Rhodotorula gracilis* cells incubated in a mineral minimal medium.
(○-○-○ control; ●-●-● minus ammonia).

rate of this decrease slows down consistently, so that at the end of the 4th hour an amount of aminoacids corresponding to ca. 40% of the initial is still present. No decrease, or even an increase of polyamines (namely spermidine) is observed during the same period.

Fig. 2 shows the behaviour of DNA, RNA and proteins during the first 3 hours of nitrogen starvation. It is seen that, while in the control medium protein level rapidly increases at a constant rate, in the N–deficient medium the rate of protein increase begins to slow down 30 minutes, and becomes close to nil 50–60 minutes after the beginning of the treatment.

Experiments of ^{14}C leucine incorporation show that the failure of the protein level increase corresponds to an almost complete block of protein

synthesis. A correlation appears clear between the rapid fall of aminoacid level and the progressive decrease of the rate of protein synthesis in the earlier period of N–deficiency, the almost complete inhibition of protein synthesis in the following period being paralleled by a much slower rate of decrease in aminoacids.

As shown in fig. 2, DNA synthesis is only slightly affected by nitrogen deficiency in the earlier 2 hours. This is in agreement with the finding that under these conditions the cells initiate cell division almost normally (by budding). The tendency of the newly formed buds, however, is to stop growing at an early stage, without separating from the mother cells.

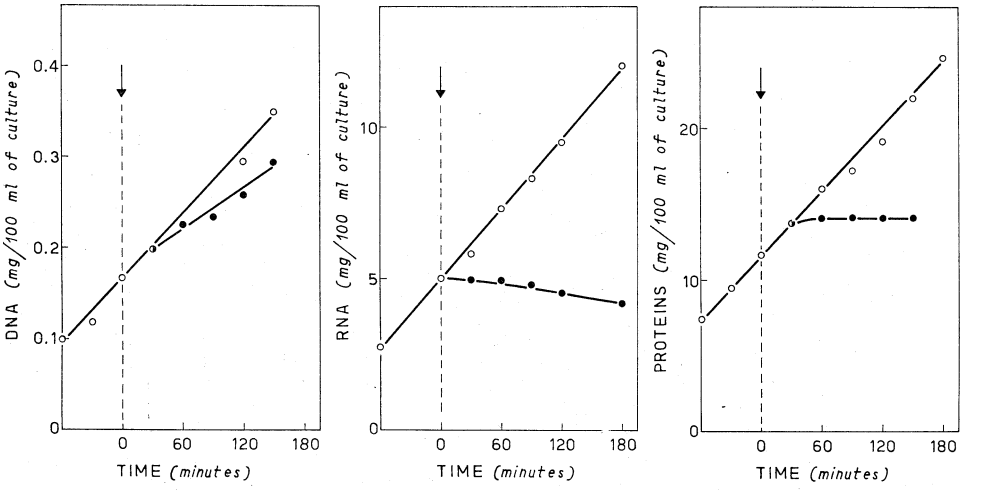

Fig. 2. – Effects of deficiency of the nitrogen source on the levels of DNA, RNA and proteins *in Rhodotorula gracilis* cells incubated in a minimal mineral medium. (○-○-○ control; ●-●-● minus ammonia).

In contrast with the behaviour of DNA, the RNA net synthesis (per ml. of culture) stops almost immediately after the transfer of the cells in the nitrogen deficient medium, and the RNA level begins to decrease slowly but steadily after 30 minutes.

Experiments of incorporation of labeled precursors such as ^{32}P orto-phosphate or 6 ^{14}C orotic acid into RNA show that in the nitrogen starved cells after 2 hours of incubation with the radioactive compound, practically no radioactivity is recovered in the ribosomes when these are isolated in a isocynetic sucrose gradient. In contrast, when short pulses (4 minutes) with phosphate or orotate are effected at 30 minutes as well as at 60 and at 90 minutes after the beginning of the treatment, the radioactivity recovered in the phenol extracted RNA fraction is almost equal to the one found for the controls grown in the presence of ammonium ions, fig. 3. Purification of the phenol extracted material by repeated washing and precipitation with ethanol and trichloroacetic acid eliminates only a minor fraction (ca. 20%) of the radio-

activity found in the preparation from the nitrogen deficient cells. Precipitation with sodium acetate according to Kirby (a procedure which eliminates DNA and soluble RNA) [7], leaves most of the radioactivity in the sediment, thus suggesting that the exchange of the terminal *t*–RNA nucleotides must play a minor role in the incorporation of radioactivity into RNA.

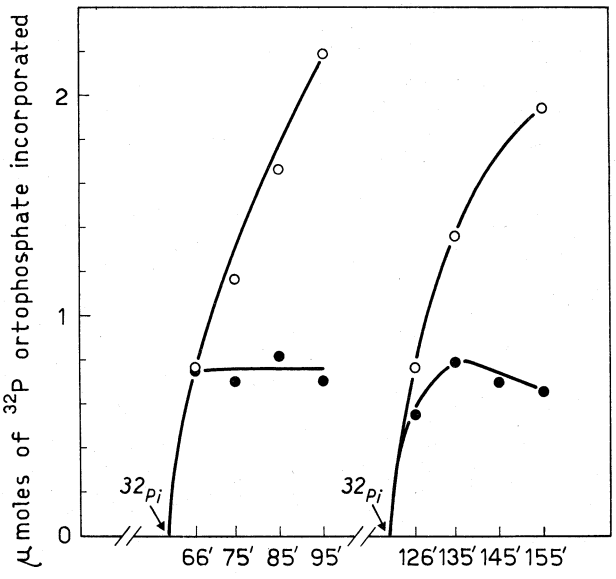

Fig. 3. – Effects of deficiency of the nitrogen source on the incorporation of ^{32}P ortophosphate in the RNA in cells of *Rhodotorula gracilis* incubated in a minimal mineral medium with (○-○-○) or without ammonia (●-●-●). The arrows indicate the time of addition of the ^{32}P ortophosphate (2 μC/ml; 5.73×10^{-3} M). The data are expressed as μmoles of ^{32}P ortophosphate incorporated in the RNA.

These results are interpreted as showing that even under the condiction of N–deficiency in the medium RNA synthesis still proceeds at a rate somewhat, but not very much, slower than in the control cells. This crucial point of the problem is presently under a more thorough investigation in this laboratory. More detailed data in this concern will be published elsewhere.

Discussion and conclusions

According to the results reported above, 15 minutes after the transfer of *Rhodotorula gracilis* cells in a nitrogen source-deficient medium the net RNA synthesis is already completely blocked. At the same moment the concentration of aminoacids has decreased by ca. 25%, and that of other important nitrogen compounds, such as polyamines are almost unchanged.

Protein synthesis after 15 minutes of treatment still proceeds almost unimpaired (thus indicating the presence of all factors required), its severe inhibition being observed only after a further 15 minutes delay. This behaviour

suggests that *a*) some sensitive and specific regulatory mechanism, rather than plain substrate deficiency, mediates the effects of nitrogen deficiency on the byosynthetic activities; *b*) this mechanism, in a very preliminary approximation, seems to originate in the aminoacid area and to invest as a primary target the metabolism of RNA.

On the other hand, the very early block of the net increase of RNA is not explained by the hypothesis of a block of RNA trascription, as short pulses with radioactive precursors show that RNA synthesis still proceeds at a rate only slightly lower than in the controls grown in the nitrogen rich medium. The simplest interpretation of these results seems that of a consistent acceleration of the breakdown of RNA. As practically no labelling of " heavy " RNA and of ribosomes is observed in long term (1 or 2 hours) labelling experiments, the breakdown of RNA should occur in the period between its synthesis and its processing to the final state in the cytoplasm.

For ribosomal RNA, one could theorise that some of precursors are particularly open to the attack of nucleases; in that case a block of the normal process of ribosome formation would leave it unprotected from the action of degradative enzymes. A similar model, implicating a very highly lable step between synthesis and stabilization in the cytoplasm, could also be valid for *m*–RNA.

The alternative hypotesis of an activation of RNA hydrolizing enzymes appears less likely, at least on the basis of the present data.

Further work is being carried on in this laboratory aimed at a better understanding of the characteristic and the fate of the RNA species being synthesized in *Rhodotorula gracilis* during the early phase of nitrogen deficiency, as well as the description of the general metabolic pattern characteristic of this condition.

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