Intracellular Protein Degradation in *Neurospora crassa*

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In exponentially growing cultures of *Neurospora crassa*, the basal rate of protein degradation increases as the constant of the rate of growth decreases, so that in slow growing cells (μ = 0.13) the rate of protein degradation is about 25% of the rate of protein accumulation. During glucose starvation and shift-down transition of growth, the rate of protein degradation is greatly enhanced, and a moderate reduction (about 30%) of the ATP level is observed. Treatment of glucose-starved cells with 2-deoxyglucose reduces the ATP content by 70% and blocks protein degradation. The addition of cycloheximide, given at the onset of glucose starvation, prevents the enhancement of protein degradation; instead cycloheximide is without effect if added when proteolysis has already started. At a supraoptimal temperature (42°C) the basal rate of protein degradation is not stimulated, contrary to the behavior observed in bacteria. Guanosine nucleotides, which appear to have a regulatory role for protein degradation in bacteria, are not found in *N. crassa.*

Protein degradation is one of the processes that contributes to the regulation of cell growth (1, 2). In bacteria it is regulated coordinately with RNA accumulation and other growth-related processes, such as synthesis of ribosomal protein, lipids, and nucleotides (3–5). Enhanced proteolysis and reduced RNA synthesis are observed in poor growth conditions and correlate with a large increase in the level of guanosine tetraphosphate (1, 5, 6). Changes in the overall rate of protein degradation contribute also in mammalian cells to the growth kinetics under a variety of normal and pathologic conditions (1, 7–11), whereas the mechanisms that control protein degradation in higher cells remain largely to be elucidated. Since most of the studies on this subject have been done either in bacterial or in mammalian cells, it seemed opportune to extend them to a lower eukaryote, such as *Neurospora crassa*, for which several investigations have already been carried out on the regulation of growth and macromolecular syntheses (12–15).

The studies presented in this paper aimed first to ascertain the relevance of protein degradation for the dynamics of growth in *Neurospora* cells in different conditions of exponential growth. Then the following aspects of the regulation of the enhanced protein degradation upon carbon starvation have been investigated: the ATP requirement, the effects of inhibitors of protein synthesis, and the occurrence of regulatory molecules.

### MATERIALS AND METHODS

**Organism and Growth Conditions**—The wild type strain 74 A (St. Lawrence) and, only for one set of experiments, the aclloidal strains 45 fl A, 1838 fl A, 291 col-3 A, 102 fr A, 88 sh A, 68 sp A, and 277 bis A of *N. crassa* were used. Seven-day-old conidia (2 × 10^10^/ml of final concentration; initial A_{450} about 0.02) were used to inoculate liquid Vogel's mineral medium (16) to which one of the following carbon sources has been added: glucose (2%, w/v), sodium acetate (40 mM), potassium acetate (2%, w/v), or ethanol (6%, v/v). Incubation was at 30°C (unless otherwise stated) in a Dubnoff water bath at 100 strokes/min, and growth was monitored as increase of absorbance at 450 nm (A_{450}), as previously indicated (17).

**Assay of Protein Degradation**—Proteins were labeled with L-[1-14C]leucine (New England Nuclear Corp.) during exponential growth. To a 200-ml culture (A_{450} about 0.15), radioactive leucine was added to a final concentration of 6 × 10^{-4} M (specific activity up to 50 Ci/mol). When practically all of the radioactivity had been incorporated into the material precipitable in hot trichloroacetic acid, after 30 min in glucose or after 45 min in the other media, [14C]leucine at 10 M final concentration was added.

This concentration was used as a higher (10^{-3} M) concentration of [14C]leucine, while not leading to an increase of the radioactivity released during the chase in mycelia exponentially growing on glucose, has an inhibitory effect (reversed by isoleucine) on growth of cultures growing on the other media (results not shown).

Protein degradation was assayed by measuring at various times after the chase the radioactivity retained in the protein as hot trichloroacetic acid-precipitable material (12) or as the radioactivity released in the trichloroacetic acid-soluble fraction. The radioactivity released in the trichloroacetic acid-soluble fraction was assayed as follows: 1-ml aliquots of the culture were added to 1 ml of cold 20% trichloroacetic acid. After 30 min in ice, the suspension was centrifuged at 3500 rpm for 15 min in a MSE centrifuge at 4°C. An aliquot of the supernatant (0.5 ml) was mixed with 17 ml of aqueous scintillation fluid and counted in a Packard model 3320 liquid scintillation counter.

To determine the rate of protein degradation in glucose-starved conidial strains, a modified procedure was employed. After a 30-min pulse, the culture was filtered on a sterile Sartorius membrane (30 mm diameter), and the mycelia were quickly resuspended in fresh sterile glucose-free medium with 10^{-3} M [14C]leucine. Thereafter, the radioactivity released in the medium was assayed by filtering on Millipore HA filters (0.45 μm) at various times 2-ml aliquots of the culture and counting 0.5-ml aliquots of the filtrate.

**Determination of the Rate of Protein Synthesis**—A 200-ml culture was divided into 40-ml cultures that were pulse labeled with L-[1-14C]leucine (10^{-3} M final concentration, specific activity 5 Ci/mole) for 30 min. Duplicate 2-ml samples were withdrawn at various times, mixed with 2 ml of 20% trichloroacetic acid, and hydrolyzed at 90°C for 20 min; the hot trichloroacetic acid-precipitable material was filtered on Millipore HA filters (0.45 μm), washed four times with 5% trichloroacetic acid, dried, and counted (15).

**Determination of ATP, ADP, and AMP Levels**—[32P]Orthophosphate (carrier-free, Radiochemical Centre, Amersham, United Kingdom) was added to a 200-ml culture at the moment of inoculation of conidia. The phosphate concentration of the mineral medium had been lowered to 1 mM (17), and the specific activity of the [32P]-orthophosphate in the medium was about 4 Ci/mole and was controlled for each experiment as described previously (18). At specified times,

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aliquots of the culture (about 30 ml) were rapidly collected by filtration on Millipore HA filters. The mycelium was homogenized with 1 ml of 1 M formic acid and 50 mg of sea sand in refrigerated mortars, and the suspension was centrifuged at 20,000 x g for 10 min at 4°C. Twenty-five microliters of the clear supernatant were analyzed by bidimensional thin layer chromatography, together with commercial ATP, ADP, and AMP (Biochemia) as markers, on polyethyleneimine cellulose sheets (PEI CEL 300, Macherey Nagel Co., Duren, Federal Republic of Germany) according to Neuhard et al. (19). The nucleotides localized under an ultraviolet lamp were scraped from the sheets and counted in a Packard Tricarb liquid scintillation counter. AMP was also determined by monodimensional chromatography on polyethyleneimine cellulose using 35 μl of the same supernatant and developing with 1 N acetic acid for 4 cm and with 0.3 N LiCl for 12 to 15 cm, according to Randerath and Randerath (20).

Thin Layer Chromatography of Acid-soluble Guanine Nucleotides—Ten milliliters of culture (phosphate concentration 5 x 10^-4 M) (21) were labeled for at least one generation time with [32P]-orthophosphate (specific activity 100 Ci/mol). Thereafter, the mycelia were collected on Millipore HA filters and homogenized in refrigerated mortars with 50 mg of sea sand and 0.35 ml of 1 M formic acid, and the suspension was centrifuged at 20,000 x g for 10 min in a Sorvall refrigerated centrifuge. Twenty microliters of the clear supernatant corresponding to about 10^6 cpm were chromatographed on polyethyleneimine cellulose sheets, according to Gallant et al. (22), together with commercial GTP (Biochemia) as marker. The autoradiography (Kodak Regulix BB x-ray film) was exposed for 72 h. Alternatively, the cultures were labeled with [U-14C]guanosine (specific activity 100 Ci/mol), which was added to 20 ml of culture at a final concentration of 10^-4 M. After 30 min of labeling (this time was chosen because it yields the maximum endocellular soluble radioactivity), the culture was filtered, and nucleotides were extracted and chromatographed as described for the other 32P-labeled cultures, but the autoradiography was exposed for at least 14 days.

RESULTS

Protein Degradation in Different Conditions of Exponential Growth—Protein degradation in N. crassa in four different conditions of exponential growth is shown in Fig. 1. The rates of protein degradation are very low in glucose minimal medium (μ = 0.51; μ = duplications/h) and in acetate medium (μ = 0.41). Instead, proteinolysis is substantial in media that support lower rates of exponential growth, such as glycerol (μ = 0.26) and ethanol (μ = 0.13). The kinetics of decay of labeled protein observed in glycerol indicated the presence of at least two components, a fast and a slow decaying one, whereas such biphasic kinetics data are not detectable with the other carbon sources.

The half-lives of protein calculated from the slopes of the best fitting straight lines on the semilogarithmic plot of Fig. 1 are 57 h in cells growing in glucose and in acetate and 24 h in cells growing in ethanol. Using the calculation developed for Escherichia coli (23), from the values of Fig. 1 we can estimate a half-life of 45 min for the fast decaying component in glycerol and about 70 h for the slow decaying one since the amount of rapidly decaying protein is about 4% of the total protein. It is interesting to recall at this point that glycerol kinase, a key enzyme for glycerol metabolism, has been shown in Neurospora to have a very high turnover rate (24). As for the cells growing on the other media it remains possible that the experimental approach used is not sensitive enough to detect the eventual presence of a very small percentage of rapidly turning over protein.

Protein Turnover during Nutritional Shifts—During a shift-down transition from glucose to glycerol (Fig. 2A), about 25% of the radioactive protein made during the exponential growth in glucose is degraded. The onset of proteolysis occurs immediately when glucose is exhausted and lasts for approximately 2.5 h (Fig. 2B), exactly in parallel with the block of rRNA accumulation (Fig. 2A) (12). By pulse labeling the protein at the beginning of the shiftdown transition, it is possible to measure the stability of newly made protein. As is shown in Fig. 2C, the proteins made during the diauxic lag are more stable than the old ones, and their turnover resembles that of the protein made by glycerol-grown cultures.
findings suggest that in some way the degradation process is a selective one and, furthermore, that it may facilitate the rearrangement of cellular structures that are quite different in the two nutritional conditions (25). On the contrary, during a shift-up transition from acetate to glucose, rRNA synthesis is enhanced (14), and no protein degradation is detectable (results not shown).

**Protein Degradation during Glucose Starvation**—When *N. crassa* mycelia are grown in low glucose (60 μg/ml of initial concentration), growth stops as soon as glucose is exhausted in the medium, with an immediate block of protein and RNA accumulations (results not shown), whereas at the same time protein degradation is largely enhanced (Fig. 3). In this condition we have determined protein degradation by measuring either the radioactivity retained in the hot trichloroacetic acid-precipitable fraction or the radioactivity released in the trichloroacetic acid-soluble fraction (Fig. 3, inset). The agreement between the two methods is good for the first 2 to 3 h and then the radioactivity found in the trichloroacetic acid-soluble fraction is less than that expected by its disappearance from hot trichloroacetic acid-precipitable material, probably due to a metabolic utilization of the released amino acids. From the data of Fig. 3, a degradation rate of about 12% (h⁻¹) can be calculated.

The addition of 3-0-methylglucose (100 μg/ml) or of α-methylglucoside (100 μg/ml) to glucose-starved cells does not significantly modify the rate of proteolysis, whereas the addition of 2-deoxyglucose (2 DG)¹ (100 μg/ml) almost completely blocks it (Fig. 4). In *N. crassa* α-methylglucoside and 3-O-methylglucose are substrates for the transport systems (26), but they are not utilized; 2-DG instead is taken up and phosphorylated by hexokinase (27). In our experimental conditions 2-DG neither supports growth or protein accumulation in glucose-starved cells for at least 3 h nor inhibits the growth rate of cells growing exponentially on glucose (200 μg/ml, initial concentration) (results not shown).

Since several reports indicate that the ATP level and the adenylate energy charge are important factors in regulating protein degradation (1, 5, 28), we have measured the adenylate pools during glucose starvation with or without the addition of 2-DG (100 μg/ml). As shown in Fig. 5, the ATP level (expressed as nanomoles/A₅₀₅₀ unit of culture) slightly decreases during glucose starvation, whereas in the presence of 100 μg/ml of 2-DG it is much more reduced. However, also in this case there is not a complete depletion of the ATP pool, whose level stabilizes around 30% of the initial value. The energy charge decreases from a value of 0.80 to 0.84 to a value of 0.70 to 0.75 in glucose starvation, and the addition of 2-DG lowers the energy charge to values of 0.65, causing at the same time a partial depletion of the total adenylate pool (Table I).

Proteolysis in glucose-starved cells is immediately blocked, as shown in Fig 6A, by the readdition of glucose, which allows growth to start again. 2-DG also blocks protein degradation (Fig. 6B), of course without resumption of growth. Although the two responses are very similar, glucose and 2-DG appear to act with different mechanisms, as indicated by experiments done with cycloheximide (see below).

**Effects of Cycloheximide**—The addition of cycloheximide (CHI) (1 μg/ml) to *N. crassa* cultures severely inhibits the enhancement of protein degradation during glucose starvation. The effect of cycloheximide, added at the time of the glucose exhaustion, is shown in Fig. 7 as a marked inhibition of proteolysis. If CHI is added 15 min before glucose exhaustion, a complete inhibition is obtained; on the other hand, if

¹The abbreviations used are: 2-DG, 2-deoxyglucose; CHI, cycloheximide; ppGpp, 5'-diphosphate, 3'-diphosphate.

![Fig. 3. Protein degradation during glucose starvation.](http://www.jbc.org/)

![Fig. 4. Effect of α-methylglucoside, 3-O-methylglucose, and 2-deoxyglucose on protein degradation during glucose starvation.](http://www.jbc.org/)
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FIG. 5. ATP levels during glucose starvation: effect of 2-deoxyglucose. The cultures growing on limiting glucose (60 µg/ml) were labeled with [32P]orthophosphate (phosphate concentration 1 mM, specific activity about 4 Ci/mol) added at the moment of inoculation of conidia. At the specified times, 30-ml aliquots of the culture were rapidly collected on Millipore HA filters, and nucleotides were extracted with 1 ml of 1 M HCOOH as described under “Materials and Methods.” The ATP was separated chromatographically, and the levels were determined by measuring the 32P radioactivity incorporated in the ATP spots. At zero time glucose is exhausted: △ and O, without 2-deoxyglucose; □ and •, with 100 µg/ml of 2-deoxyglucose added at zero time.

TABLE I
Energy charges and total adenylate pool values in glucose-starved cells, with and without the addition of 100 µg/ml of 2-deoxyglucose

The values before the exhaustion of glucose were, respectively, 0.82 ± 0.035 for energy charges and 1.85 ± 0.07 nmol/Aeq unit of culture for the adenylate pool. The cultures growing in limiting glucose (60 µg/ml) were labeled with [32P]orthophosphate (phosphate concentration 1 mM, specific activity 4 Ci/mol) added at the moment of inoculation of conidia. At the specified times 30-ml aliquots of the culture were collected on Millipore HA filters, and nucleotides were extracted with 1 ml of 1 M formic acid as described under “Materials and Methods.” Adenine nucleotides were separated by means of two-dimensional chromatography on polyethyleneimine cellulose sheets according to Neuhard et al. (19) and Randerath and Randerath (20).

| Time after glucose exhaustion (min) | Energy charge | Adenylate pool |
|-----------------------------------|---------------|----------------|
| -Glucose +2-DG | -Glucose +2-DG |
| 20 | 0.77 | 0.65 | 1.86 | 0.88 |
| 30 | 0.71 | 0.66 | 1.76 | 0.81 |
| 60 | 0.65 | 0.53 | 1.89 | 0.99 |
| 75 | 0.73 | 0.63 | 1.44 | 0.90 |

Effects of Amino Acids—Since amino acids are the terminal products of proteolysis, it might be interesting to ascertain whether or not the addition of amino acids to glucose-starved cells blocks protein degradation, in a kind of negative feedback control. The addition of 1 mM casamino acids to glucose-starved cells gradually reduces protein degradation, and at the same time growth resumes (Fig. 10), whereas lower concentrations (100 µM) are without effect. Thus, the effect observed in the first instance is probably related to the recovery of growth and not to a direct regulatory effect of the exogenous amino acids on protein degradation. A process in which proteolysis has a significant physiological role is spore formation, both in fungi (29) and bacteria (30). The following experiments were performed to see whether or not there was an impairment of protein degradation in a number of aconidial mutants of Neurospora, which are able to produce conidia only in the

TABLE II
Effect of the addition at various times of 1 µg/ml of cycloheximide (CHI) on protein degradation in glucose-limiting cultures

Glucose is exhausted at zero time. The cultures growing on limiting glucose (60 µg/ml) were pulse labeled with L-[1-14C]leucine (6 × 10−4 M, specific activity 50 Ci/mol) before the glucose exhaustion. After 30 min [14C]leucine at a final concentration of 10−4 M was added. CHI (1 µg/ml of final concentration) was added at the specified times, and protein degradation was measured from the loss of radioactivity from hot trichloroacetic acid-precipitable material.

| Time of CHI addition (min) | Protein degradation initial rate (%) |
|---------------------------|-------------------------------------|
| -15                       | 2                                   |
| 0                         | 7                                   |
| +15                       | 12                                  |
growth in glucose at 42°C ($\mu = 0.37$), the basal rate of protein degradation is almost undetectable and even lower than that observed at 30°C. These results suggest that either Neurospora protein is fairly resistant to the processes of thermal denaturation or that the same proteases are thermolabile at 49°C. The latter suggestion does not hold, at least for the proteases that are active in glucose starvation. In fact, under this condition at 42°C the rate of protein degradation is largely enhanced over the rate measured at 30°C; it is, in fact, about 20% ($h^{-1}$).

A Search for Regulatory Signals—In E. coli a strict correlation between protein degradation and the net synthesis of ribosomal RNA (rRNA) is apparent since all of the conditions that block rRNA accumulation enhance protein degradation (1, 5, 6). Thus, it has been suggested that the same regulatory signals, for instance guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) as a negative signal or a guanosine nucleotide, called the "phantom spot" (22) as a positive signal, control both processes (1, 5, 6).

In N. crassa, ppGpp is not detectable under conditions that inhibit rRNA accumulation (21); thus, it seems interesting to extend the analysis to the phantom spot to find out whether or not it is present. For this purpose N. crassa cultures in exponential growth in glucose were labeled with [$^{32}$P]orthophosphate, and the acid-soluble nucleotides were separated by two-dimensional thin layer chromatography on polyethyleneimine cellulose sheets (22). After autoradiography a small spot (indicated as X) was observed near GTP, in a position close to that occupied by the phantom spot (Fig. 11A). This X spot is present also during shift-down; its level is quite constant with respect to that of the GTP (about 5% of the GTP counts are found in the X spot), and it is not absorbed by activated charcoal (Norit).

Furthermore, the X spot is not detectable after labeling the cultures with [U-$^{14}$C]guanosine (Fig. 11B), thus indicating that it is not a guanine derivative. The X spot observed after [$^{32}$P]orthophosphate labeling might, therefore, be a small polyphosphate (33) or a polyphosphorylated sugar.

Testing the Validity of the Measurement of Protein Degradation—The assay of the rate of the overall protein degradation performed with radioactive labeling techniques may be beset by two sources of error, i.e. the recycling of radioactive amino acids released from protein and a slow time course of

Fig. 8. Addition of cycloheximide to glucose-starved cells: lack of inhibitory effect on protein degradation. Growth and assay conditions are the same as for Fig. 3. Cycloheximide (1 $\mu$g/ml) has been added 60 min after the chase. Inset, [$^{14}$C]leucine incorporation into protein with 1 $\mu$g/ml of CHI (O) and without CHI addition (C). The cultures were pulse labeled with l-$[1-{^3}C]$leucine ($10^{-5}$ M final concentration, specific activity 5 Ci/mol) 10 min after CHI addition.

Fig. 9. Combined effect of cycloheximide and of the readdition of glucose or 2-deoxyglucose on protein degradation in glucose-starved cells. Growth and assay conditions are the same as for Fig. 3. A and C, growth as absorbance at 450 nm; B and D, radioactivity retained by the hot trichloroacetic acid-precipitable material. A and B, readdition of glucose; C and D, readdition of 2-deoxyglucose. The arrows indicate (a) pulse labeling, (b) chase, (c) addition of 1 $\mu$g/ml of CHI, and (d) addition of 100 $\mu$g/ml of glucose or 2-deoxyglucose.

presence of exogenous amino acids (31). Since growth starvation is required to start conidiation (31) and in the aconidial mutants exogenous amino acids have to be provided for the production of conidia, it might be thought that protein degradation may not be stimulated during glucose starvation in some aconidial mutants. Contrary to this prevision, all of the aconidial mutants tested (45 fl A, 1838 fl A, 291 col-3 A, 102 fr A, 88 sh A, 68 sp A, and 277 bis A) have been shown to be able to degrade intracellular protein during glucose starvation (results not shown).

Effect of Temperature—Temperatures higher than 38°C and lower than 20°C severely restrict growth of N. crassa mycelia growing exponentially in liquid medium in glucose (15). The experiments presented so far have been conducted at 30°C. We have previously reported that during exponential growth in glucose at 8°C, protein degradation is undetectable (15). Experiments conducted during this investigation indicate that there is not an enhancement of the basal protein degradation even at supraoptimal temperatures, contrary to the behavior reported for E. coli (32). In fact, during exponential growth in glucose at 42°C ($\mu = 0.37$), the basal rate of protein degradation is almost undetectable and even lower than that observed at 30°C. These results suggest that either Neurospora protein is fairly resistant to the processes of thermal denaturation or that the same proteases are thermolabile at 49°C. The latter suggestion does not hold, at least for the proteases that are active in glucose starvation. In fact, under this condition at 42°C the rate of protein degradation is largely enhanced over the rate measured at 30°C; it is, in fact, about 20% ($h^{-1}$).

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Fig. 10. Effect of the addition of casamino acids on protein degradation in glucose-starved cells. Growth and assay conditions are the same as for Fig. 3. Growth as absorbance at 450 nm; radioactivity retained by protein. The arrows indicate (a) pulse labeling, (b) chase, and (c) addition of 1 $\mu$mol concentration of casamino acids. The open symbols (O and C) indicate, respectively, $A_{400}$ nm and radioactivity in the control cultures (without addition of casamino acids).
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FIG. 11. Autoradiography of bidimensional chromatography of formic acid extracts of exponentially growing Neurospora cultures. A, a 10-ml culture was labeled for one generation time with $[^{32}P]$orthophosphate (100 Ci/mol). The mycelia were collected on a Millipore HA filter and homogenized with sand and 0.35 ml of 1 N formic acid. The suspension was centrifuged at 20,000 $\times g$ for 30 min and 20 $\mu$l of the clear supernatant were chromatographed on polyethyleneimine cellulose sheets, according to Gallant et al. (22), together with commercial GTP as ultraviolet marker. The autoradiography was exposed for 72 h. B, a 20-ml culture was labeled for 30 min with [U-14C]guanosine (final concentration 10$^{-4}$ M, specific activity 100 Ci/mol). Twenty microliters of the 1 N formic acid extract were chromatographed with commercial GTP. The autoradiography was exposed for 14 days.

The intracellular leucine content during exponential growth in glucose is quite small and it is largely increased by the addition of even fairly low concentrations (10$^{-5}$) of exogenous leucine (15), that appear to expand the vesicular pool. Under the experimental conditions used in this study, the chase with 10$^{-4}$ M leucine blocks completely and immediately the incorporation of [14C]leucine into protein (Fig. 12A), as expected if the specific activity of the nonorganellar cytoplasmic pool equilibrates very quickly with the external leucine. At the same time after the chase, the intracellular trichloroacetic acid-soluble radioactivity does not vary appreciably (Fig. 12B). These findings suggest that protein degradation, as other metabolic processes, utilizes the nonorganellar cytoplasmic pool, which is in active exchange with the external leucine, as indicated also by the results of Fig. 12A. The extent of isotope reutilization should, therefore, be minimized.

DISCUSSION

The regulation of intracellular protein degradation in Neurospora, as in bacteria and in mammalian cells, appears to be part of a general control system that may be relevant to determine the dynamics of growth (2, 9) and allows the cells to adapt to a poor nutritional environment. The studies reported in this paper indicate the presence of similarities and differences in the control of protein degradation in Neurospora as compared to other well known systems.

In different conditions of exponential growth, the average rate of protein degradation in Neurospora increases by decreasing the rate of growth (Fig. 1), thus confirming the observation that slow growing cells have higher rates of pro-
tein degradation (1, 4). The rate of protein degradation be-
comes an increasing fraction of the rate of protein synthesis at lower growth rates. It can be calculated that the rate of protein degradation in cells growing in glucose, acetate, glyc-
erol, and ethanol media corresponds to 3%, 4%, 14%, and 25% of the respective rate of protein synthesis. Thus, in slow growing cells the considerable rate of protein degradation puts a heavy burden on growth metabolism since up to one-fourth of the protein synthetic activity is counteracted by protein degradation. This may be the reason for the presence of the "extra" rRNA (13, 40) observed in cells growing in ethanol.

The basal overall rate of protein degradation in cells grow-
ing exponentially on glucose is largely enhanced by carbon source starvation (Fig. 3). A slight decrease (about 30%) of the ATP level is observed during starvation (Fig. 5). A similar correlation is noted also during a shift-down transition of growth. The ATP level drops by 30% at the beginning of the diauxic lag (18), whereas protein degradation is largely stimu-
lated (Fig. 2). A second distinct effect of reduced ATP availability on protein degradation is evidenced by the treat-
ment with 2-deoxyglucose, which lowers the ATP level by 70% (Fig. 5) and greatly reduces the rate of protein degrada-
tion in glucose-starved cells (Fig. 4).

In *Neurospora* we would have, therefore, a situation similar to that described for *E. coli*, in which a moderate decrease of cellular ATP enhances the rate of protein degradation, whereas if the ATP level is almost depleted, protein degra-
dation is blocked (1, 5). The involvement of ATP in protein breakdown, which recalls to mind similar energy requirements of other catabolic processes such as glycolysis or fatty acids oxidation, has also been evidenced in *in vitro* systems of protein degradation (28). The energy requirement might perhaps explain the puzzling observation that the treatment with 2-deoxyglucose in the presence of cycloheximide does not block protein degradation (Fig. 9D), contrary to the effect of the same treatment without cycloheximide (Fig. 6B). It might be tentatively suggested that the treatment with cyclohexi-
mide spares ATP utilization, thus, keeping the ATP level, even in the presence of 2-deoxyglucose, at a value that allows protein degradation to occur.

As regards the regulatory function that a moderate decrease in the availability of cellular high energy phosphates may have to enhance the rate of protein degradation and the fall in RNA synthesis, it has been proposed that it may be mediated in *E. coli* by a large accumulation of guanosine ppGpp through a reduction of its rate of degradation (5).

We have shown that also in *Neurospora* an increased protein degradation (see "Results") is coordinated with a strong reduction of rRNA synthesis (12); however, the response is not mediated by ppGpp, which is not found in *Neurospora* (21). Nor did we detect in *Neurospora* another guanosine nucleotide, the so-called "phantom spot," which might be implicated in the regulation of stringent response in *E. coli* (22).

Moreover, when considering the control role that energy metabolism may have on protein degradation and rRNA synthesis in *Neurospora*, a preliminary question may be con-
sidered: whether it is the change of the ATP level or that of the rate of ATP production that triggers the regulatory re-
sponse. In fact, these two aspects of energy metabolism are not always correlated in *Neurospora* since there appears to be feedback processes that depress ATP utilization very quickly after ATP synthesis is inhibited (41).

The existence of a careful balance between the rate of syn-
thesis and that of utilization of ATP in *Neurospora* is supported by the observation that the intracellular ATP pool is very small as compared to the rate of ATP utilization for protein and RNA synthesis.³

³The ATP level in *Neurospora* cells growing exponentially in glucose at 30°C is 2.4 nmol/Arso unit of culture (18), i.e. 1.45 × 10¹⁵ molecules. The protein level per Arso unit of culture is about 140 μg, and a rate of protein synthesis (since protein degradation is negligible) of 4.1 × 10¹⁵ amino acid residues/Arso unit can be calculated (15), which corresponds to 16.4 × 10¹⁵ high energy phosphate bonds required for protein synthesis (minutes⁻¹/Arso unit). The RNA content per Arso unit of culture is about 35 μg, and the corresponding rate of synthesis is 4.7 × 10¹⁵ nucleotides (minutes⁻¹/Arso unit) (15). Since the rate of RNA synthesis is about 30% of the rate of total RNA synthesis in *Neurospora* cells in the indicated conditions of growth (42), it follows that 3.1 × 10¹⁵ high energy phosphate bonds (minutes⁻¹/Arso unit) are required for total RNA synthesis. Thus, considering these two major macromolecular syntheses, to sustain their rates of synthesis the unreplicated ATP pool would last only a few seconds. It follows that the ATP pool has to be in *Neurospora* a very fast turnover rate. Direct experiments in which the average intracellular ATP concentration has been determined after the addi-
tion of respiratory inhibitors (cyanide or azide) have demonstrated, in fact, that the ATP level decreases following a first order kinetics with a rate constant of 0.175 s⁻¹ (43).

In conclusion, the available findings suggest that in *Neu-
rospora* changes in nutritional conditions that bring about a decrease of energy production also provoke inhibition of rRNA synthesis and enhancement of protein degradation, presumably through the mediation of a metabolic signal(s) different from the one(s) that mediates similar responses in bacteria. The inverse correlation between rRNA synthesis and protein degradation is not absolute since there is at least one condition, the treatment with caffeine, that inhibits rRNA synthesis without stimulating protein degradation in *Neu-
rospora* (44). It is conceivable that the different response is related to a different mechanism of inhibition of rRNA syn-
thesis.

The enhancement of the rate of protein degradation upon carbon starvation is prevented by cycloheximide (Fig. 7 and Table I). Inhibitors of protein synthesis have been reported to reduce the rate of protein degradation in starved bacteria (1, 6, 45) and in resting mammalian cells (46, 47). Since in these cells protein degradation falls shortly after the addition of the protein synthesis inhibitor, it has been suggested by Hershko and co-workers (46, 47) that protein synthesis is necessary for the continuous synthesis of the short-lived protein required for the stimulation of protein breakdown during starvation. The experiments of Fig. 8 rule out the presence of such rapidly turning over protein in *Neurospora* since protein degradation continues at the same rate for at least 3 h after CHI is added after the onset of starvation. Similar results have been reported also in yeast (49).

In *E. coli* the inhibitors of protein synthesis cause a reduc-
tion of the ppGpp level and in this way appear to lower the rate of protein degradation (5, 6). The effects of CHI on protein degradation in *Neurospora* cannot be explained by a similar mechanism, with only the change of the metabolic signal involved. In fact, CHI prevents the onset of protein degrada-
tion with a clear time dependence (Table II), but does not affect protein breakdown once it is established (Fig. 8) or prevent the block of protein degradation by the readdition of glucose (Fig. 9). The simplest explanation seems, therefore, that the onset of protein degradation in starving *Neurospora* requires the synthesis of new, relatively stable protein, either proteolytic enzymes or regulatory polypeptides, in a different pattern of response than that of *E. coli*, in which the increased proteolysis seems to involve pre-existing proteases (6), but in a similar way as proposed for the regulation of protein degrada-
tion in sporulating yeast (49). On the other hand, the readdition of glucose (Figs. 6 and 9) and in a less pronounced way that of amino acids (Fig. 10) is able to quickly inactivate
the proteolytic system through a mechanism that is not inhibited by cycloheximide (Fig. 9B).

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