A Method for Monitoring Macromolecular Synthesis in Filamentous Fungi and its Use to Study Control of Nucleic Acid Synthesis in *Aspergillus nidulans*

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A method is described for measuring the syntheses of macromolecules in the filamentous fungus *Aspergillus nidulans*. It involves growth of mycelium on small filter papers in the presence of radioactively labelled L-leucine to monitor total protein synthesis, and hence growth, and following the incorporation of differently labelled D-uridine (to measure nucleic acid synthesis) or L-leucine (to measure protein synthesis) under various conditions such as starvation for a metabolite or the presence of an inhibitor. Comparison of $^3\text{H}/^{14}\text{C}$ or $^{14}\text{C}/^3\text{H}$ labelling ratios (depending on the labelling combination used) shows the effects of the treatments on nucleic acid or protein synthesis. The method allows large numbers of measurements to be made and enables economical use of radioisotopes. Results are presented to show that, under the labelling conditions used, uridine is incorporated mainly into stable RNA. The method is used to demonstrate probable stringent control of stable RNA synthesis in *A. nidulans* and that inhibitors of protein synthesis such as cycloheximide and anisomycin also inhibit stable RNA synthesis. In contrast, starvation for *meso*-inositol affects nucleic acid synthesis much more strongly than it affects protein synthesis.

INTRODUCTION

A number of years ago, we developed a method for monitoring the synthesis of nucleic acids and other macromolecules in the ascomycete *Aspergillus nidulans*. This method and certain of our results were described at several meetings (published only as abstracts: Arst & Scazzocchio, 1971, 1972*a, b*). As our research interests changed, we discontinued this series of experiments late in 1971. Nevertheless, even now full publication of the method and our results will be beneficial for at least two reasons. Firstly, the method deserves a wider availability. Although we used it mainly to look at control of nucleic acid synthesis in *A. nidulans*, it can be adapted for the study of the synthesis of other macromolecules and for use in other filamentous micro-organisms. Secondly, *A. nidulans* has proved to be one of the most suitable lower eukaryotes for a variety of biochemical and genetical investigations, including characterization of its RNA polymerases (Stunnenberg *et al.*, 1979), and interest in the organism continues to grow (see, for example, the series of papers in Smith & Pateman, 1977). Despite the passage of time, certain aspects of the methodology and the results remain novel and of potential use in further studies with this or other organisms.

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METHODS

Strains. Construction of *A. nidulans* strains for this work was by standard genetic techniques (Pontecorvo *et al.*, 1953; Clutterbuck, 1974). Genotypes of strains used for each experiment are given in the Table legends. All markers used are in standard use (Clutterbuck, 1974; Palmer & Cove, 1975). All strains carry the L-leucine auxotrophy *luA1* and the pyrimidine auxotrophy *pyrD23*. Use was also made of the adenine auxotrophy *adh23*, the L-arginine auxotrophy *argB2*, the choline auxotrophy *choA1*, the mutation leading to loss of choline-O-sulphatase *csuA6*, the L-histidine auxotrophy *hisC38*, the *meso*-inositol auxotrophy *inoB2*, the L-methionine auxotrophy *methG1*, the L-proline auxotrophy *proA6*, and the putrescine auxotrophy *puA2*. In addition, some strains carried *biA1* (biotin auxotrophy), *wA2* or *wA5* (white conidial colour), or *yA2* (yellow conidial colour).

Chemicals. L-[4,5-³H]Leucine, L-[U-¹⁴C]leucine, D-[5-³H]uridine, D-[6-³H]uridine and D-[2-¹⁴C]uridine were purchased from The Radiochemical Centre, Amersham. Cycloheximide, actinomycin D and emetine (hydrochloride) were purchased from Koch-Light, Calbiochem and Sigma, respectively. α -Amanitin was generously provided by Professor Th. Wieland (Heidelberg, F.R.G.), anisomycin by Pfizer (Sandwich, Kent) and rifampicin by Gruppo Lepetit Laboratories (Milan, Italy). All other chemicals were of reagent grade where possible.

Measurement of nucleic acid synthesis. Suspensions of approximately 10^4 conidiospores ml⁻¹ were prepared in unsupplemented liquid minimal medium (Cove, 1966) containing 0.1 % (v/v) Tween 80. Sterile Whatman no. 1 filter papers (2.0 cm diam.) were briefly but thoroughly immersed in conidiospore suspensions and placed on solid minimal medium (Cove, 1966) containing 1 % (w/v) D-glucose as carbon source and supplemented with (final concentrations) 10 μ g biotin l⁻¹, 10 mM-NaNO₃ (as nitrogen source), 10 mM-D-uridine and 1 mM-L-leucine, either labelled with ³H in positions 4 and 5 at a final specific activity of 500 mCi mol⁻¹ (18.5 GBq mol⁻¹) or uniformly labelled with ¹⁴C at a final specific activity of 50 mCi mol⁻¹ (1.85 GBq mol⁻¹) or, in the experiment shown in Table 5, 12.5 mCi mol⁻¹ (462.5 MBq mol⁻¹). In addition, where appropriate to genotype, solid media were supplemented with (final concentrations) 100 μ M-adenine, 1 mM-L-arginine (hydrochloride), 10 μ M-choline (chloride), 250 μ M-L-histidine, 22 μ M-*meso*-inositol, 250 μ M-L-methionine, 1 mM-L-proline, or 8 μ M (limiting) or 20 mM (excess) putrescine (dihydrochloride). Filter papers were individually numbered with pencil before sterilization and the position of each was marked on the bottom of the Petri dish. Filter papers were handled with sterile forceps, and generally 12 were placed on a standard (90 mm diam.) Petri dish without touching each other. Growth then took place on the solid medium for 21 h at 37 °C. If starvation for a metabolite (adenine, L-arginine, choline, L-histidine, *meso*-inositol, L-methionine, L-proline or putrescine) was desired, it was invariably necessary to commence the starvation during the 21 h growth period on solid medium. In this case an appropriate number of plates with solid medium lacking the metabolite in question were poured before adding the metabolite and pouring the remainder of the plates. This ensured that the solid media were identical (particularly with regard to specific activity of isotope) except for the one metabolite. An appropriate number of filter papers was then transferred to the solid medium lacking the metabolite for the final 2 or 4 h (as indicated in the Tables or Table legends) of the 21 h period. The Petri dishes containing the solid media were incubated for an additional day at 37 °C to enable eventual contamination to be detected. As the positions of filter papers had been marked, a contaminated filter paper was easily identified (in any case, in our experiments considerably less than 0.1 % of filter papers were contaminated).

Once removed from solid medium, the mycelium-containing filter papers were washed by successive transfer through three 250 ml portions of unsupplemented liquid minimal medium at 37 °C (thus avoiding both osmotic and temperature shock) and thoroughly blotted. The filter papers were then placed, mycelium down (as they grew mainly on the side exposed to the air whilst on the solid media), in individual round receptacles (2.2 cm internal diam.) containing 800 μ l of liquid medium. These receptacles were made by heating about 1 cm lengths of sterile Pyrex tubing and inserting them severally into sterile heavy plastic Petri dishes such that a watertight seal was made. The liquid medium was a minimal medium (Cove, 1966) containing 1 % (w/v) D-glucose as carbon source and supplemented with (final concentrations) 10 μ g biotin l⁻¹, 10 mM-NaNO₃ (as nitrogen source), 1 mM-L-leucine with the same isotope label at the same final specific activity as in the solid medium, and 100 μ M-D-uridine, either labelled with ³H (when L-[¹⁴C]leucine was used) in the 5 or the 6 (as indicated) position at a final specific activity of 30 Ci mol⁻¹ (1.11 TBq mol⁻¹) or with ¹⁴C (when L-[³H]leucine was used) in the 2 position at a final specific activity of 2 Ci mol⁻¹ (74 GBq mol⁻¹). Other supplements at the concentrations as listed above for the solid medium and various inhibitors were included in the liquid medium as indicated in the Tables and Table legends. Except for the experiment shown in Table 6 where incubation times (at 37 °C) are indicated, filter papers were incubated at 37 °C for 2 h. They were then transferred to ice-cold 10 % (w/v) trichloroacetic acid and washed with more ice-cold 10 % trichloroacetic acid followed by ice-cold, distilled water on Hirsch funnels under vacuum. The filter papers

were then placed in scintillation vials and dried for 30 min at 90 °C. After cooling, 10 ml scintillant [4 g 2,5-diphenyloxazole and 40 mg 1,4-di-2-(5-phenyloxazolyl)benzene per litre toluene] was added, and vials were counted using the automatic setting for ^3H and ^{14}C double labelling in a Packard 3375 Tri-Carb liquid scintillation counter.

Measurement of protein synthesis. This was identical to the method for measurement of nucleic acid synthesis except that in the liquid medium the uridine was unlabelled whereas the L-leucine was labelled both with ^3H , in positions 4 and 5, at a specific activity of 500 mCi mol $^{-1}$ (18.5 GBq mol $^{-1}$), and with ^{14}C , uniformly distributed, at a final specific activity of 50 mCi mol $^{-1}$ (1.85 GBq mol $^{-1}$). To follow the effects of a treatment in parallel on nucleic acid and protein syntheses, all other supplements were added to the liquid medium which was then divided into aliquots to which either D-[2- ^{14}C]uridine or L-[U- ^{14}C]leucine was added.

RESULTS AND DISCUSSION

Salient features of the method for measuring macromolecular synthesis

In vivo measurement of many processes in a filamentous organism such as *Aspergillus nidulans* is more difficult than in unicellular organisms because changes in turbidity of the culture medium cannot be used effectively to measure growth. In compensation, however, many filamentous organisms can grow with attachment to a solid support such as a filter paper. This enables easy transfer from one growth medium to another (using sterile forceps) with intervening washing, if necessary. Although growth must commence on a solid medium so that it is confined to one side of the filter paper (the side exposed to the air), filter papers can then be transferred either to another solid medium or to a liquid medium. Growth can be monitored by including a radioactively labelled precursor of a macromolecule in all media. The use of an amino acid necessary for protein synthesis is an obvious choice because most *in vitro* measurements are reported relative to the amount of soluble protein in cell extracts. The use of strains auxotrophic for the amino acid increases labelling efficiency and ensures that labelling is an accurate measure of protein synthesis and therefore growth. L-Leucine was chosen because (1) a suitable auxotrophy, *luA1* (Clutterbuck, 1974), is available, (2) it is a frequent constituent of proteins, (3) it is catabolically rather inert (i.e. amongst the amino acids, it is one of the poorest nitrogen or carbon sources for *A. nidulans*), thus ensuring high specificity and efficiency of labelling and minimizing the danger of formation of volatile radioactive catabolites, and (4) it is commercially available labelled with ^3H or ^{14}C at reasonable cost.

The inclusion of a second, differently labelled compound in one or more subsequent media enables synthesis of another macromolecule to be followed relative to total protein synthesis/growth. For counting purposes, ^3H and ^{14}C form a convenient labelling combination, as used here. For these experiments, the second labelled compound was present in only the final medium (a liquid medium). Thus mycelia grown under identical conditions initially could be subjected to different treatments during which synthesis of a particular class of macromolecules relative to total protein synthesis/growth could be compared. The criteria for choosing the second labelled precursor are largely the same as those outlined above for the choice of L-leucine. To follow the effects of various treatments on protein synthesis, differently labelled L-leucine can be used (so that the final medium contains both ^3H - and ^{14}C -labelled L-leucine), and thus no second amino acid auxotrophy is necessary for labelling purposes. To monitor nucleic acid synthesis, labelled D-uridine was chosen, and these experiments utilized strains carrying the pyrimidine auxotrophy *pyrD23* (Palmer & Cove, 1975) in addition to *luA1*. Uridine is catabolically inert in *A. nidulans*, commercially available labelled with ^3H or ^{14}C , and supplements *pyr* auxotrophs of *A. nidulans* at lower concentrations than uracil. Recently, studies elsewhere have shown that both RNA and DNA can be labelled more efficiently *in vivo* in a *pyr* strain of *A. nidulans* than in a prototroph and that labelled uridine is incorporated more efficiently than labelled uracil (Zinchenko *et al.*, 1978). Labelled adenine can also be used for labelling nucleic acids very efficiently (Kessel & Rosenberger, 1968, and our unpublished results). However, adenine labelling

necessitates, in addition to an *ad* auxotrophy, a mutational block in purine catabolism which does not result in accumulation of a toxic intermediate of purine degradation (see Darlington & Scazzocchio, 1967). An *hxB* mutation, resulting in loss of the two hypoxanthine-oxidizing enzymes now designated purine hydroxylases I and II (Scazzocchio & Darlington, 1968; Scazzocchio *et al.*, 1973; Lewis *et al.*, 1978), meets these requirements. Care must also be taken to avoid adenine labelled such that the label will be retained during histidine biosynthesis (Orr & Rosenberger, 1976). [8-¹⁴C]- and [8-³H]adenine meet this requirement and are commercially available.

Unfortunately, DNA cannot be labelled specifically in *A. nidulans* because catabolism of thymine and thymidine (reviewed by O'Donovan & Neuhaud, 1970) results in labelling of RNA as well (Zinchenko *et al.*, 1978). Moreover, wild-type *A. nidulans* lacks thymidine kinase and therefore cannot incorporate exogenously supplied thymidine into DNA (Grivell & Jackson, 1968). Nevertheless, comparison of results obtained using D-[5-³H]uridine and D-[6-³H]uridine can be used to estimate the relative contribution of DNA synthesis to total nucleic acid synthesis (see below).

The method enables economical use of radioisotopes. For example, 12 of the 2 cm diam. filter papers used here fit on to a standard (90 mm) plate of solid medium, and 800 μ l of liquid medium per filter paper is adequate for incubation. At the conclusion of experiments, extraction with ice-cold 10% (w/v) trichloroacetic acid removes both non-specifically adsorbed labelled compounds and pools of small molecules containing the labels. Comparison of ³H/¹⁴C or ¹⁴C/³H labelling ratios (depending on the labelling combination used) provides a relative measure of the effects of various treatments on macromolecular synthesis. Complete details of the protocol used here are given in Methods. The method should be adaptable to monitoring synthesis of macromolecules other than proteins and nucleic acids and possibly also to following synthesis of macromolecular structures such as membranes or the cell wall. This method has been used to monitor the effects of methylammonium on protein synthesis in wild-type and methylammonium-resistant mutants of *A. nidulans* (Arst & Page, 1973). A similar technique has been used for transport studies in *A. nidulans* (Arst & Page, 1973; Bailey & Arst, 1975; Arst & Scazzocchio, 1975; Scazzocchio & Arst, 1978). Filter papers have also been used as solid supports for cultures of *Trichoderma viride* (Gressel & Galun, 1967).

Examples of the effects of various treatments on nucleic acid synthesis

Results in Tables 1 to 4 illustrate how the method can be used to follow the effects of various treatments on nucleic acid synthesis. For example, purine starvation, using a strain having a mutation blocking the biosynthesis of all purines (Foley *et al.*, 1965), considerably reduced incorporation of labelled uridine (Table 1). It is possibly surprising that purine starvation did not altogether eliminate nucleic acid synthesis but this might be due to degradation of previously existing nucleic acid, which would make available sufficient purine nucleotides to support a low level of incorporation of the uridine label.

Results in Table 2 show that nucleic acid synthesis was inhibited by starvation for proline. Other results (not shown) using the same labelling combination showed that this was a general effect of amino acid starvation, as it was also elicited by deprivation of histidine, methionine or arginine. The efficiencies with which starvation for various amino acids inhibited nucleic acid synthesis appeared to differ widely, with histidine being the most efficient and methionine the least efficient in our experiments. These differences might reflect a variation in mechanisms of inhibition, but it seems more likely that they merely represent differences in the extents to which starvation was achieved. The efficiency of starvation will depend on pool sizes, extent of catabolism, and possibilities for synthesis by alternative pathways, and it is likely that comparable degrees of starvation for all amino acids cannot be achieved. In any case, it will be shown below that the labelled uridine was incorporated

Table 1. *Effect of adenine starvation on nucleic acid synthesis*

A strain of genotype *yA2 luA1 adh23 pyrD23* was labelled with L-[4,5- ^3H]leucine to measure growth. Nucleic acid synthesis was followed using D-[2- ^{14}C]uridine. $^{14}\text{C}/^3\text{H}$ ratios are the average of three determinations.

Duration of adenine starvation on solid medium (h)	Adenine concn in liquid medium (μM)	Nucleic acid synthesis	
		$^{14}\text{C}/^3\text{H}$	% of control
0	100	1.07	100
0	0	0.241	22.6
2	0	0.155	14.5
4	0	0.156	14.6

Table 2. *Effect of L-proline starvation on nucleic acid synthesis*

A strain of genotype *yA2 proA6 luA1 pyrD23* was labelled with L-[4,5- ^3H]leucine to measure growth. Nucleic acid synthesis was followed using D-[2- ^{14}C]uridine. $^{14}\text{C}/^3\text{H}$ ratios are the average of three determinations.

Duration of L-proline starvation on solid medium (h)	L-Proline concn in liquid medium (mM)	Nucleic acid synthesis	
		$^{14}\text{C}/^3\text{H}$	% of control
0	1	0.609	100
0	0	0.168	27.6
2	0	0.060	9.9
4	0	0.097	15.9

Table 3. *Effect of meso-inositol starvation on nucleic acid synthesis*

A strain of genotype *biA1 luA1 wA5 inoB2 pyrD23* was labelled with L-[4,5- ^3H]leucine to measure growth. Nucleic acid synthesis was followed using D-[2- ^{14}C]uridine. $^{14}\text{C}/^3\text{H}$ ratios are the average of three determinations.

Duration of meso-inositol starvation on solid medium (h)	meso-Inositol concn in liquid medium (μM)	Nucleic acid synthesis	
		$^{14}\text{C}/^3\text{H}$	% of control
0	22	1.22	100
0	0	1.02	83.1
2	0	0.914	74.7
4	0	0.283	23.1

mainly into stable RNA, and therefore our results provide evidence for stringent control of stable RNA synthesis as has been demonstrated in the prokaryotes (reviewed by Edlin & Broda, 1968). Amongst the fungi, the stringent response in *Saccharomyces cerevisiae* has been demonstrated by a number of groups (Wehr & Parks, 1969; Roth & Dampier, 1972; Gross & Pogo, 1974; Wejksnora & Haber, 1974; Kudrna & Edlin, 1975; Oliver & McLaughlin, 1977; Shulman *et al.*, 1977; Warner & Gorenstein, 1978; Ray & Butow, 1979*a, b*) and has also been demonstrated in *Neurospora crassa* (Somberg *et al.*, 1966; Buckel & Böck, 1973).

Results in Table 3 show inhibition of uridine incorporation by meso-inositol starvation. We were unable to demonstrate any effect of starvation for another component of phospholipids, choline, using a *choA1* strain, auxotrophic for choline (results not shown). However, choline auxotrophies are not manifested during the early stages of growth (Arst, 1968; Markham & Bainbridge, 1979), and it is therefore possible that effective starvation for choline cannot be achieved during the relatively short period of the experiment.

Similarly there was no convincing effect of either putrescine starvation or excessive (20 mM)

Table 4. *Nucleic acid synthesis in the presence of cycloheximide or α -amanitin*

A strain of genotype *biA1 luA1 pyrD23* was labelled with L-[4,5- ^3H]leucine to measure growth. Nucleic acid synthesis was followed using D-[2- ^{14}C]uridine. $^{14}\text{C}/^3\text{H}$ ratios are the average of three determinations.

Inhibitor present in liquid medium	Inhibitor concn ($\mu\text{g ml}^{-1}$)	Nucleic acid synthesis	
		$^{14}\text{C}/^3\text{H}$	% of control
None	—	0.656	100
Cycloheximide	1	0.509	77.6
	10	0.196	29.9
	100	0.213	32.5
α -Amanitin	1.67	0.641	97.7
	16.7	0.656	100
	167	0.650	99.1

supplementation in a *pua2* strain, auxotrophic for putrescine (results not shown). However, later, it was shown that putrescine is required during germination of conidiospores of *A. nidulans* but not during subsequent hyphal growth (Stevens, 1975; Stevens *et al.*, 1976) so it is unlikely that, when measuring nucleic acid synthesis in mycelia, starvation for putrescine could have been effective. Winther & Stevens (1978) demonstrated a requirement for putrescine for both protein and nucleic acid syntheses but only by starving conidiospores from the time of inoculation. As the method described here requires sufficient mycelial growth to ensure adherence to the filter papers, it is doubtful whether it could be used in conjunction with putrescine starvation from the time of inoculation.

Results in Table 4 show that nucleic acid synthesis was inhibited by cycloheximide. Cycloheximide is an effective inhibitor of protein synthesis in *A. nidulans* (Cybis & Weglenski, 1972; Winther & Stevens, 1978) and there is presumptive evidence that the basis of its toxicity *in vivo* is due to its interaction with cytoplasmic ribosomes (Waldron & Roberts, 1974*a, b*). It is therefore likely that cycloheximide inhibition of nucleic acid synthesis is a consequence of its inhibition of protein synthesis. In contrast, neither α -amanitin at concentrations as high as $167 \mu\text{g ml}^{-1}$ (Table 4) nor actinomycin D at $67 \mu\text{g ml}^{-1}$ (results not shown) inhibited nucleic acid synthesis. Conidiospores of *N. crassa* are impermeable to these inhibitors (Tisdale & DeBusk, 1972) so the lack of inhibition might be a question of impermeability. However, Stunnenberg *et al.* (1979) have shown that even *in vitro* three RNA polymerases of *A. nidulans* are largely or completely insensitive to α -amanitin.

*Preliminary characterization of the nucleic acids whose synthesis is measured
by the method*

Results in Table 5 allow calculation of the contribution which DNA synthesis makes to total nucleic acid synthesis, as measured by the method under conditions of both normal growth and proline starvation. The details of the calculation are given in the Appendix, but the method is based on comparison of $^3\text{H}/^{14}\text{C}$ labelling ratios when parallel experiments are done using [5- ^3H]uridine and [6- ^3H]uridine at equal concentrations and specific activities. The ^3H in the 5 position of uridine is removed in the thymidylate synthetase-catalysed methylation of dUMP to form TMP. Hence (assuming the absence of further base modification), [6- ^3H]uridine labels all pyrimidine residues in both RNA and DNA whereas [5- ^3H]uridine labels pyrimidine residues of RNA but only cytosine residues of DNA. The actual calculation requires an estimate of the pyrimidine content of the RNA labelled in the experiment as well as of the guanine plus cytosine content of the DNA labelled in the experiment. We were then able to calculate that under proline-sufficient conditions DNA accounts for about 13 % of the total nucleic acid synthesis, whereas under proline-starvation the proportion of DNA rises to about 79 %. A cursory glance at Table 5 confirms the direction of this

Table 5. *Comparison of nucleic acid synthesis as monitored by [5-³H]uridine and [6-³H]uridine in cultures supplemented with or starved for L-proline*

A strain of genotype *yA2 proA6 luA1 pyrD23* was labelled with L-[U-¹⁴C]leucine to measure growth. Nucleic acid synthesis was followed using D-[5-³H]uridine or D-[6-³H]uridine at equal final specific activities. Non-supplemented cultures were starved for 2 h on solid medium lacking L-proline and then incubated in proline-free liquid medium. ³H/¹⁴C ratios are the average of three determinations.

Position of ³ H in uridine	Supplementation with L-proline (1 mM)	Nucleic acid synthesis	
		³ H/ ¹⁴ C	% of control
5	+	266	100
5	—	8.02	3.0
6	+	285	100
6	—	12.5	4.4

Table 6. *Effect of incubation time on cycloheximide inhibition of nucleic acid synthesis*

A strain of genotype *biA1 luA1 pyrD23* was labelled with L-[U-¹⁴C]leucine to measure growth. Nucleic acid synthesis was followed using D-[5-³H]uridine. Where indicated, cycloheximide was added to the liquid medium at a final concentration of 10 µg ml⁻¹. ³H/¹⁴C ratios are the average of three determinations.

Duration of incubation in liquid medium (min)	Nucleic acid synthesis		
	³ H/ ¹⁴ C		% inhibition by cycloheximide
	— Cycloheximide	+ Cycloheximide	
0	0.368	0.382	0
10	0.586	0.546	6.8
30	1.99	1.42	28.7
120	13.4	2.92	78.2

result because proline starvation has a slightly less drastic effect on the ³H/¹⁴C labelling ratio when the ³H is in the 6 position than when it is in the 5 position. Grossman *et al.* (1969) have reported preferential synthesis of mitochondrial DNA in *S. cerevisiae* when protein synthesis is prevented, and replication of nuclear DNA in the same organism, once initiated, can proceed in the presence of cycloheximide (Williamson, 1973; Golombek *et al.*, 1974).

Results in Table 6 show that the effect of cycloheximide on nucleic acid synthesis increased with the length of the uridine incorporation period. This indicates that the 2 h incorporation period used here monitors primarily stable nucleic acid synthesis. Thus we are looking mainly at the regulation of stable RNA synthesis.

A further point is to be noted from the comparison of Tables 5 and 6 with Tables 2 and 4, respectively. In each case, some of the results are derived from an experimental protocol which is virtually the same except that the labels have been reversed, so that ³H incorporation monitors nucleic acid synthesis and ¹⁴C incorporation measures growth for the experiments shown in Tables 5 and 6, whereas the reverse is true for the experiments shown in Tables 2 and 4. The substantial equivalence of results obtained with the two possible labelling configurations confirms the absence of labelling artefacts.

Examples of effects of combinations of treatments on nucleic acid synthesis

In the experiment shown in Table 7, proline starvation and cycloheximide (10 µg ml⁻¹) inhibited nucleic acid synthesis to about the same extent and the effects were neither additive nor cancelling.

Table 8 shows the results of an experiment in which proline starvation, cycloheximide and rifampicin in various combinations were used to inhibit nucleic acid synthesis. In contrast to the much more modest 11 to 15 % inhibition of nucleic acid synthesis by 500 µg

Table 7. *Effect of simultaneous proline starvation and cycloheximide treatment on nucleic acid synthesis*

A strain of genotype *yA2 proA6 luA1 pyrD23* was labelled with L-[U-¹⁴C]leucine to measure growth. Nucleic acid synthesis was followed using D-[5-³H]uridine. Non-supplemented cultures were starved for 2 h on solid medium lacking L-proline and then incubated in proline-free liquid medium. ³H/¹⁴C ratios are the average of ten determinations.

Supplementation with L-proline (1 mM)	Cycloheximide concn in liquid medium ($\mu\text{g ml}^{-1}$)	Nucleic acid synthesis	
		³ H/ ¹⁴ C	% of control
+	0	29.2	100
+	10	4.18	14.3
—	0	4.66	16.0
—	10	4.02	14.1

Table 8. *Effects of various combinations of proline starvation, cycloheximide treatment and rifampicin treatment on nucleic acid synthesis*

A strain of genotype *yA2 proA6 luA1 pyrD23* was labelled with L-[U-¹⁴C]leucine to measure growth. Nucleic acid synthesis was followed using D-[5-³H]uridine. Non-supplemented cultures were starved for 2 h on solid medium lacking L-proline and then incubated in proline-free liquid medium. ³H/¹⁴C ratios are the average of five determinations.

Supplementation with L-proline (1 mM)	Cycloheximide concn in liquid medium ($\mu\text{g ml}^{-1}$)	Rifampicin concn in liquid medium ($\mu\text{g ml}^{-1}$)	Nucleic acid synthesis	
			³ H/ ¹⁴ C	% of control
+	0	0	23.1	100
+	10	0	4.03	17.4
+	0	500	11.0	47.6
+	10	500	4.36	18.9
—	0	0	3.97	17.2
—	10	0	2.78	12.0
—	0	500	1.40	6.5
—	10	500	3.46	15.0

rifampicin ml^{-1} found in two previous experiments (results not shown), this same concentration of rifampicin inhibited nucleic acid synthesis by more than 50 % in this experiment. The reason(s) for this discrepancy is unknown. As a different strain was used for this experiment, it is conceivable that an unidentified genetic difference affecting, for example, cell permeability to rifampicin is responsible. It is worth noting that, in combination with amphotericin B (which presumably aids its penetration through membranes), rifampicin is a potent inhibitor of RNA synthesis and growth in *S. cerevisiae* (Medoff *et al.*, 1972; Battaner & Kumar, 1974) and in the yeast phase of *Histoplasma capsulatum* (Kobayashi *et al.*, 1972). In any case, rifampicin inhibition here appears additive in its effect with proline starvation but not with cycloheximide treatment. Moreover, cycloheximide treatment negates the additivity of rifampicin treatment with proline starvation.

Comparison of the effects of various treatments on nucleic acid and protein syntheses

Results in Table 9 show that proline starvation reduced nucleic acid and protein syntheses to rather similar extents, whereas results in Table 10 show that inositol starvation preferentially affected nucleic acid synthesis. The effects of inositol starvation are of particular interest because they lead to 'inositol-less death', a phenomenon which has been intensively studied in *N. crassa* and *S. cerevisiae* (Strauss, 1958; Shatkin & Tatum, 1961; Sullivan & DeBusk, 1973; Keith *et al.*, 1977; Henry *et al.*, 1977; Hanson & Brody, 1979). In *S. cerevisiae*, inositol starvation ultimately terminates macromolecular synthesis, and the results

Table 9. *Effects of L-proline starvation on protein and nucleic acid syntheses*

A strain of genotype *yA2 proA6 luA1 pyrD23* was labelled with L-[4,5-³H]leucine to measure growth. Nucleic acid synthesis was followed using D-[2-¹⁴C]uridine, and protein synthesis was followed using L-[U-¹⁴C]leucine. Non-supplemented cultures were starved for 2 h on solid medium lacking L-proline and then incubated in proline-free liquid medium. ¹⁴C/³H ratios are the average of four determinations.

Supplementation with L-proline (1 mM)	Protein synthesis		Nucleic acid synthesis	
	¹⁴ C/ ³ H	% of control	¹⁴ C/ ³ H	% of control
+	0.153	100	1.11	100
—	0.032	20.9	0.199	17.9

Table 10. *Effects of meso-inositol starvation on protein and nucleic acid syntheses*

A strain of genotype *biA1 luA1 wA5 inoB2 pyrD23* was labelled with L-[4,5-³H]leucine to measure growth. Nucleic acid synthesis was followed using D-[2-¹⁴C]uridine, and protein synthesis was followed using L-[U-¹⁴C]leucine. Non-supplemented cultures were starved for 4 h on solid medium lacking *meso*-inositol and then incubated in inositol-free liquid medium. ¹⁴C/³H ratios are the average of four determinations.

Supplementation with <i>meso</i> -inositol (22 µM)	Protein synthesis		Nucleic acid synthesis	
	¹⁴ C/ ³ H	% of control	¹⁴ C/ ³ H	% of control
+	0.196	100	1.17	100
—	0.176	89.8	0.412	35.3

Table 11. *Effects of anisomycin on protein and nucleic acid syntheses*

A strain of genotype *yA2 proA6 luA1 pyrD23* was labelled with L-[4,5-³H]leucine to measure growth. Nucleic acid synthesis was followed using D-[2-¹⁴C]uridine, and protein synthesis was followed using L-[U-¹⁴C]leucine. All media contained 1 mM-L-proline to supplement *proA6*. ¹⁴C/³H ratios are the average of four determinations.

Anisomycin concn in liquid medium (µg ml ⁻¹)	Protein synthesis		Nucleic acid synthesis	
	¹⁴ C/ ³ H	% of control	¹⁴ C/ ³ H	% of control
0	0.157	100	0.973	100
100	0.112	71.3	0.752	77.3
333	0.013	8.1	0.404	41.5
1000	0.003	1.9	0.258	26.5
2000	0.002	1.4	0.239	24.5

Table 12. *Effects of emetine on protein and nucleic acid syntheses*

A strain of genotype *yA2 proA6 luA1 pyrD23* was labelled with L-[4,5-³H]leucine to measure growth. Nucleic acid synthesis was followed using D-[2-¹⁴C]uridine, and protein synthesis was followed using L-[U-¹⁴C]leucine. All media contained 1 mM-L-proline to supplement *proA6*. ¹⁴C/³H ratios are the average of four determinations.

Emetine concn in liquid medium (µg ml ⁻¹)	Protein synthesis		Nucleic acid synthesis	
	¹⁴ C/ ³ H	% of control	¹⁴ C/ ³ H	% of control
0	0.263	100	1.21	100
1	0.239	90.9	1.15	95.7
10	0.239	90.9	1.17	96.9
100	0.215	81.7	1.06	87.3
1000	0.201	76.4	0.949	78.7

suggest that RNA synthesis is affected slightly earlier than either protein or DNA synthesis (Henry *et al.*, 1977). Just as no effect of choline starvation on nucleic acid synthesis in a *choA1* strain could be detected (see above), neither was there any effect on protein synthesis (results not shown). In the case of protein synthesis, the experiment was performed using a strain which carried, in addition to *choA1*, *csuA6*, leading to loss of choline-*O*-sulphatase (Arst, 1971; Gravel, 1976), as well as using a *choA1 csuA⁺* strain. *csuA6* would have prevented release of choline from endogenous choline-*O*-sulphate, a fungal storage product (see Arst, 1971).

Results in Table 11 show that whilst preferentially affecting protein synthesis, anisomycin (see Grollman & Huang, 1973) strongly inhibited nucleic acid synthesis as well. Emetine, another inhibitor of eukaryotic protein synthesis (Grollman & Huang, 1973), was rather ineffective but inhibited protein and nucleic acid syntheses to similar extents (Table 12).

APPENDIX

The use of parallel experiments with [5-³H]uridine and [6-³H]uridine to estimate the contribution of DNA synthesis to total nucleic acid synthesis

Suppose that x nucleotides be incorporated into DNA containing m fraction of thymine (and hence $\frac{1}{2} - m$ fraction of cytosine) and y nucleotides be incorporated into RNA containing n fraction of pyrimidines. Then the proportion of nucleotides incorporated into DNA is given by $x/(x+y)$.

Let A be the amount of [5-³H]uridine incorporated per unit growth and B be the amount of [6-³H]uridine incorporated per unit growth. Then (assuming pyrimidine base modification in positions 5 and 6 is quantitatively negligible), if [5-³H]- and [6-³H]uridine are added in identical, parallel experiments at equal concentrations and specific activities and can be incorporated into nucleic acids with equal efficiencies (i.e. no isotope effects), they will have the same labelling constant k and

$$kB = ny + \frac{1}{2}x \quad (1)$$

$$kA = ny + (\frac{1}{2} - m)x \quad (2)$$

Subtracting (2) from (1):

$$k(B - A) = mx \quad (3)$$

$$\text{or} \quad x = k(B - A)/m \quad (4)$$

Substituting (4) in (1):

$$kB = ny + k(B - A)/2m \quad (5)$$

$$\text{or} \quad y = k[(2m - 1)B + A]/2mn \quad (6)$$

$$\text{Then} \quad x/(x+y) = 2n(B - A)/[(2m + 2n - 1)B - (2n - 1)A] \quad (7)$$

As cytoplasmic rRNA of *A. nidulans* contains approximately 50% pyrimidines (Verma *et al.*, 1971), a reasonable estimate for n would be $\frac{1}{2}$. Averaging the published values for the guanine plus cytosine content of *A. nidulans* DNA (Dutta *et al.*, 1967; Storck & Alexopoulos, 1970) gives approximately 50% so that m is about $\frac{1}{4}$. Thus for *A. nidulans*:

$$x/(x+y) = 2(B - A)/B \quad (8)$$

For the proline-sufficient cultures, $B = 285$ and $A = 266$ (Table 5), and therefore DNA accounts for approximately 13% of total nucleic acid synthesis. For the proline-starved cultures, $B = 12.5$ and $A = 8.02$, and therefore DNA accounts for approximately 79% of total nucleic acid synthesis.

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