JOURNAL OF BACTERIOLOGY, Feb., 1966
Vol. 91, No. 2

Printed in U.S.A.

Ribonucleic Acid and Protein Synthesis in *Rhizophlyctis rosea* Zoospores

HERBERT B. LÉJOHN[1] AND JAMES S. LOVETT
Department of Biological Sciences, Purdue University, Lafayette, Indiana

Received for publication 11 November 1965

ABSTRACT

LÉJOHN, HERBERT B. (Purdue University, Lafayette, Ind.), AND JAMES S. LOVETT. Ribonucleic acid and protein synthesis in *Rhizophlyctis rosea* zoospores. J. Bacteriol. **91**:709–717. 1966.—The uniflagellate zoospores of *Rhizophlyctis rosea* display active motility and a high endogenous respiratory metabolism, but neither growth nor net ribonucleic acid (RNA) or protein synthesis can be measured by ordinary procedures. Nevertheless, synthesis can be detected with isotopic precursors. Uracil-C^{14} is incorporated slowly into both the soluble and ribosomal RNA. Analysis of zoospore extracts (on diethylaminoethyl cellulose columns or sucrose gradients) after various periods of labeling suggested that most of the uracil incorporation represents slow synthesis of ribosomal precursor RNA and, ultimately, ribosomes. Actinomycin D caused an 80% inhibition of uracil incorporation. The most rapidly labeled RNA was susceptible to extensive degradation in cells treated with actinomycin, but the percentage of stable RNA increased with the time of incorporation before addition of the antibiotic. Neither the effects of actinomycin nor the results of chase experiments have established unequivocally the existence of turnover or the presence of a short-lived "messenger" fraction in motile spores. Both leucine and methionine were slowly incorporated into a spectrum of cellular proteins. The methyl group of C^{14}-methylmethionine also served as a methyl donor for the methylation of soluble RNA but not of ribosomal RNA. The observations that some of the newly synthesized RNA and protein occur in the intact 82*S* ribosomes and that actinomycin inhibits the low level of protein synthesis provide some indirect evidence for a very low rate of "messenger" synthesis and turnover in zoospores.

Rhizophlyctis rosea is a ubiquitous, easily isolated representative of the simple nonfilamentous aquatic fungi (17). The advantages of this and similar nonfilamentous organisms for experimental morphogenesis were discussed in a recent review (3). In brief, the mature, sac-like plant of *R. rosea* converts its entire multinucleate protoplast into a large number of small uninucleate, uniflagellate zoospores. These zoospores undergo a variable period of motility and then germinate to produce a new uninucleate plant. This tiny plant produces rhizoids and grows by enlargement of the coenocytic spherical thallus with concomitant nuclear division, eventually repeating the process of spore discharge, to complete one cell generation. The transition of the motile zoospore to a nonmotile growing plant, represented by germination, provides a very useful system for a study of intracellular control mechanisms and their role in differentiation.

The zoospores of *R. rosea* are exceedingly active and under conditions unsatisfactory for growth may retain their motility for many hours. They possess a very high metabolic rate, as reflected by an endogenous respiratory Q_{O_2} of 160 μliters of O_2 per mg (dry weight) per hr (LéJohn and Lovett, *unpublished data*), during the swimming phase, but do not show any evidence of growth. It thus seemed plausible that the zoospores might have an effective mechanism for repressing the major portion of their biosynthetic pathways, while retaining their catabolic, energy-yielding systems. It also seemed reasonable that the release of this repression could be intimately related to the triggering of morphological differentiation, i.e., germination with its

[1] Present address: Botany Department, Fourah Bay College, University College of Sierra Leone, Freetown, Sierra Leone, West Africa.

concomitant retraction of the flagellum, growth of rhizoids, and other changes. Except for some work with *Blastocladiella* (3), virtually nothing is known concerning the physiological activities of the flagellated zoospores in any of the numerous species of aquatic fungi. This paper reports a study of the ribonucleic acid (RNA) and protein metabolism in *R. rosea* spores, undertaken to gain some understanding of the function and synthetic capacity of these unusual cells.

MATERIALS AND METHODS

Organism. R. rosea strain 611B, obtained as a single spore isolate from tropical greenhouse soil at Purdue University (Smith, M.S. Thesis, Purdue Univ., Lafayette, Ind., 1962) was used for the experiments to be described. Strain 611B displays a minimal growth requirement for glucose (or other carbon source) and inorganic salts.

Cultivation and harvesting. Cultures for production of zoospores were grown in 1-liter Roux bottles on medium A [glucose, 0.11 M; cellobiose, 0.0015 M; KH_2PO_4, 0.00114 M; K_2HPO_4, 0.00086 M, KNO_3, 0.02 M; $MgSO_4$, 0.002 M; $CaCl_2$, 0.001 M; agar (Difco), *p*H 6.8). Bottles were inoculated with dense suspensions of zoospores (3×10^5 cells) and incubated in the light for 4 to 5 days at 26 C. At that time the zoosporangia were mature, and the spores were harvested by flooding the surface of the cultures with sterile DS solution (a balanced salt mixture containing: 0.0005 M $MgCl_2$, 0.0005 M $CaCl_2$, 0.0005 M K_2HPO_4, 0.005 M KH_2PO_4, and 0.005 M NH_4NO_3 at *p*H 7.0) to induce discharge. The resulting suspensions were filtered through cotton to remove any plants, or debris, and the zoospores were concentrated by centrifugation at 4 C for 6 min at $4,500 \times g$. The pellet of spores was suspended in cold DS solution of double concentration and the suspension was centrifuged. The washed zoospores were then suspended in a small volume of medium C (KH_2PO_4, 0.00057 M; K_2HPO_4, 0.00043 M; KNO_3, 0.01 M; $MgSO_4$, 0.001 M; $CaCl_2$, 0.001 M) and stored on ice for a maximal period of 0.5 hr. Preliminary studies on the effect of storage on ice had shown no more than 3 to 8% difference in the total incorporation of labeled uracil during subsequent incubation at 24 C. The concentration of zoospores was determined by counting suitably diluted samples with a model B Coulter cell counter.

Standard incubation conditions. Experimental cultures were incubated with stirring in 100-ml Bellco spinner flasks mounted above a magnetic stirrer, and were maintained at a constant temperature of 24 C. Unless indicated otherwise, all incubations were carried out in medium C (of the composition outlined above) sterilized by filtration through Millipore filters. The number of zoospores used varied slightly, and the actual figures are given with each experiment. For purposes of comparison, 10^6 zoospores represent 50 μg of dry weight.

Homogenization and fractionation. Cells from incubation mixtures were centrifuged and resuspended twice (see above) in TM buffer [0.01 M tris(hydroxymethyl)aminomethane hydrochloride containing 0.01 M $MgCl_2$ at *p*H 7.4] to wash them free from the medium. The final pellet was taken up in a small volume of the same buffer, and the spores were disrupted by (i) three cycles of rapid freezing and thawing or (ii) passage through a French pressure cell at 12,000 psi. Homogenates were centrifuged at $10,000 \times g$ for 10 min at 4 C, and the pellet was discarded. The remaining cell-free extract was, in some experiments, used directly. In others, it was further fractionated by centrifugation in a Spinco model L preparative ultracentrifuge at $100,000 \times g$ for 2 or 5 hr. The resulting pellet or "ribosome" fraction was suspended in TM buffer. The upper phase will be referred to as the "soluble" supernatant fraction. All the analyses described in the experimental section were carried out with one or more of the above fractions.

For zone centrifugation analysis, sucrose gradients, buffered with 0.01 M TM, were prepared by the method of Britten and Roberts (2). The linear gradients ranged from 3 to 20% sucrose, and 0.2 to 0.5 ml of the material to be analyzed was carefully layered on the surface. The gradients were centrifuged at 4 C for 90 min at 37,000 rev/min in an SW39 swinging-bucket rotor of a Spinco model L preparative centrifuge. After centrifugation, the contents of the gradient tubes were collected in one-drop fractions. The fractions were diluted with 1 ml of TM buffer for measurement of optical density at 260 mμ, and samples were removed for assays of radioactivity.

The diethylaminoethyl (DEAE) cellulose (Cellex D, 0.91 meq/g; Calbiochem) used for the chromatography of cell-free extracts, ribosomes, and soluble RNA was washed three times with TM buffer before use. Columns (1.2×10 cm or 1.2×15 cm) were packed under nitrogen at 5 or 6 psi. Samples of RNA analyzed on such columns were restricted to 4 mg or less, and a fresh column was prepared for each run. Elution was at 4 C with linear NaCl gradients in the range of 0 to 1 M or 0.2 to 1.2 M in TM buffer. The flow rate was 0.25 ml/min.

RNA was isolated by extraction with an equal volume of buffer (TM)-saturated phenol and ethyl alcohol precipitation in the cold. After centrifugation, the RNA pellets were washed twice with cold 70% ethyl alcohol and three times with ether-ethyl alcohol (4:1, v/v), and were dried in vacuo. RNA nucleotides were isolated and separated chromatographically by the method of Katz and Comb (7).

Radioactivity measurements. The radioactivity of RNA samples was routinely determined in the following manner. The macromolecules were precipitated in 5% cold trichloroacetic acid containing an excess of the unlabeled compound used for incorporation. The samples were held on ice for 30 min, filtered on membrane filters (Schleicher and Schuell Co., Keene, N.H.; type B6), and washed with the same trichloroacetic solution. The filters were cemented to planchets, dried, and counted in a low-background, gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.; model C115 automatic low background system). The efficiency of the counter was 25%. When samples from sucrose gradients were used, the above procedure was modified by the addition of 2 mg of yeast RNA as carrier before precipitation. In some

cases, the samples were directly plated on planchets without filtration. Incorporation of C^{14}-amino acids into protein was estimated by the procedure of Neidhardt and Magasanik (16).

Chemicals. Actinomycin D was generously provided by C. A. Stone of the Merck Institute for Therapeutic Research, West Point, Pa. The chloramphenicol was a generous gift from J. E. Gajewski of Parke-Davis & Co., Detroit, Mich. Puromycin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

Our prime interest was to study the physiology of zoospores *per se* under conditions as near to natural as possible. Glucose was omitted from the incubation medium of these experiments because preliminary unpublished work had shown that glucose had virtually no stimulatory effect on the very high endogenous respiratory metabolism and equally little influence on the synthesis of RNA and protein. The presence or absence of a carbon source also has no effect on the length of the motile period before zoospore germination. Under the conditions used in the experiments to be described, *R. rosea* zoospores remained actively motile for 4 hr or more. During this period, we were unable to detect an (net) increase in either RNA or protein per cell (i.e., by extraction and determination of whole-cell RNA and protein by conventional procedures). These results confirmed our earlier conclusions based on microscopic observation, but did not eliminate the possibility of slow synthesis or turnover in these fractions. To examine for the presence of these low rates in zoospores, we resorted to in vivo experiments using the appropriate isotopically labeled precursors for RNA and protein.

Incorporation of nucleic acid precursors by zoospores. In preliminary experiments, all four of the common RNA bases were found to be incorporated into the cold trichloroacetic acid-insoluble material of zoospores. The observation that all of the incorporated activity was released as free nucleotides by hydrolysis in 0.3 N KOH at 37 C for 16 hr established that the incorporation was due to RNA, rather than deoxyribonucleic acid (DNA), synthesis. The rates of incorporation were, however, very low in comparison with those of bacteria during exponential growth (13), or of the young growing plants of the water mold *Blastocladiella* (Lovett, *unpublished data*). Despite the relatively low rates, the specific RNA base, uracil, was incorporated significantly faster than the other bases. The higher rate and the relative specificity of uracil for RNA led us to choose uracil-C^{14} for all of our in vivo measurements of RNA synthesis. The

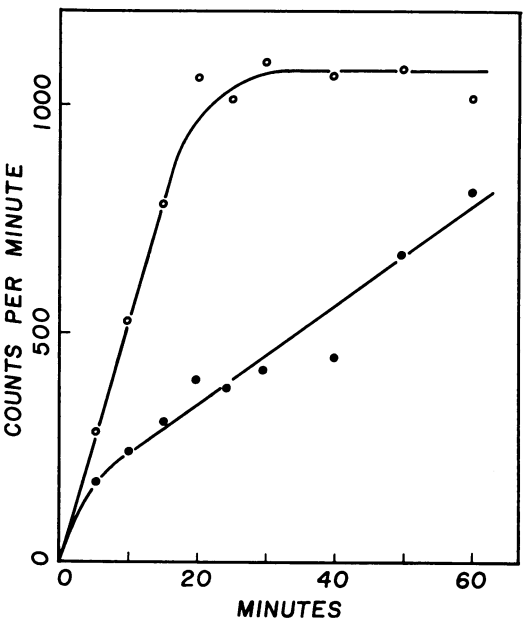

FIG. 1. *Uracil uptake and incorporation into RNA of zoospores. Duplicate 1-ml samples were removed from an incubation mixture containing 3.5 × 10^5 zoospores and 0.05 μc of uracil C^{14} per ml. One sample was filtered and washed rapidly with cold medium C plus uracil-C^{12} on a membrane filter for total uptake (○); the other was precipitated, washed in trichloroacetic acid, and filtered to measure RNA incorporation (●).*

data in Fig. 1 clearly show that there was little, if any, delay in the incorporation of uracil into RNA by pools of precursors. The same data also demonstrate the presence of a uracil pool(s) in the zoospores. At 30 min, when the extracellular uracil was exhausted, the radioactivity in whole cells was greater than 2.5 times that in RNA. However, after 30 min, RNA synthesis continued, with the result that at 60 min the radioactivity in the RNA fraction was approaching the total radioactivity. This increase could only have occurred at the expense of uracil-C^{14} previously taken up in the pools (13).

When the rates of uracil-C^{14} incorporation into "soluble" and ribosomal RNA were examined, it was found that its appearance was linear in the "soluble" fraction and nonlinear in the ribosomal fraction (Fig. 2). The difference in rates suggested to us that the soluble fraction might represent synthesis of precursors which were subsequently incorporated into intact ribosomal particles, via a process analogous to that described by McCarthy et al. (14) for *Escherichia coli*.

Because it was clearly important to know the type of RNA synthesized by motile spores, a more extensive fractionation of the cellular RNA

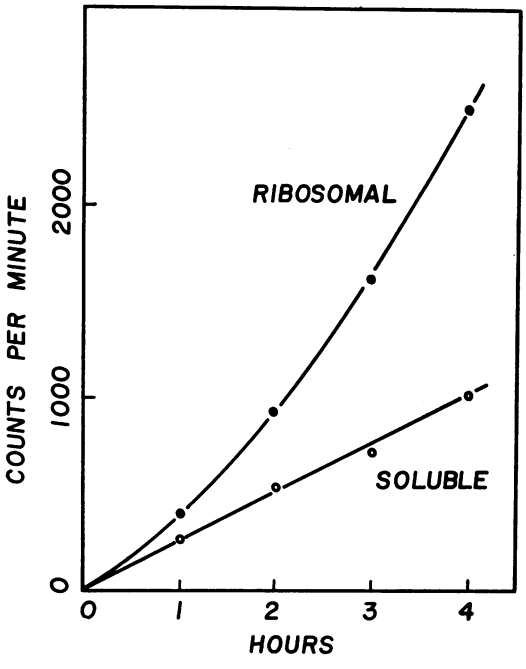

FIG. 2. *Time course of uracil entry into the "soluble" and ribosomal fractions of zoospores. At each time indicated, a 25-ml sample was removed from an incubation mixture containing 6.5 × 10⁵ spores and 0.2 μc (11.2 μc/mole) of uracil-2-C¹⁴ per ml in medium C. The cells were then homogenized, fractionated, and counted, as indicated in Materials and Methods.*

components was undertaken. The elution pattern obtained from a DEAE cellulose column charged with a whole-cell extract is given in Fig. 3. Figure 4 illustrates the elution profiles for 2 hr (Fig. 4a) and 4 hr (Fig. 4b) of incubation prior to extraction and fractionation of the cells. The distribution of the radioactivity in the nucleic acid region of the elution diagrams suggested that most of the newly synthesized RNA was either in ribosomes or closely associated with ribosomes. At 2 hr, the bulk of the activity was found to reside in the "soluble" RNA region, but by 4 hr a significant transfer of activity into the ribosomal region was obvious.

Density gradient centrifugations have also indicated a significant transfer of early-labeled RNA to ribosomes over a period of time. Figure 5 gives the results of an experiment where all of the material which sedimented during a 5 hr centrifugation, at 100,000 × *g*, was examined on sucrose gradients. The sedimentation of the zoospore ribosomes (82*S*) in sucrose gradients was calibrated with reference to the 30*S* and 50*S* particles of *E. coli*. For this experiment, spore

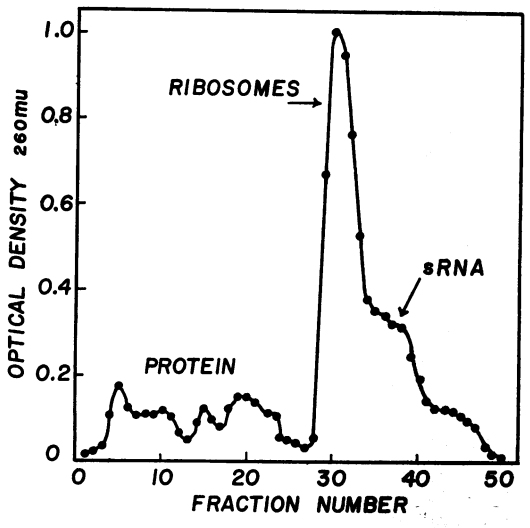

FIG. 3. *Separation of a whole-spore extract on a DEAE cellulose column. A linear gradient of 0 to 1 M NaCl in TM buffer was used with a salt gradient of 0.004 mole per ml. Fraction size, 3.4 ml.*

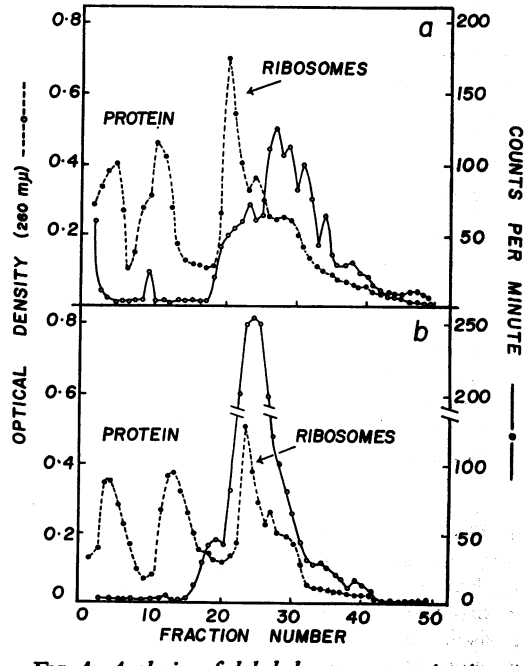

FIG. 4. *Analysis of labeled zoospore extracts on DEAE cellulose columns. Zoospores were incubated at a concentration of 7.5 × 10⁶ per milliliter with 0.2 μc/ml of uracil-2-C¹⁴ (specific activity, 1.5 μc/μmole) in medium C. Samples (25 ml) were removed, homogenized, and chromatographed after 2 hr (a) and 4 hr (b) of incubation. Chromatography carried out as in Fig. 3.*

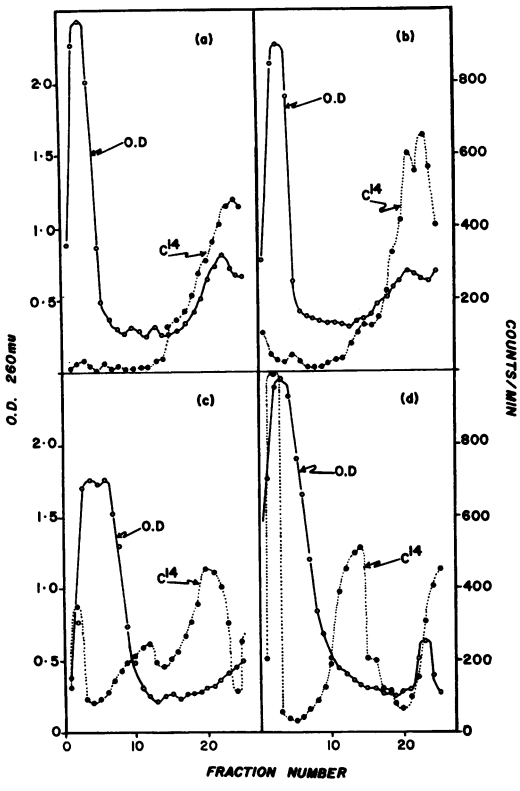

FIG. 5. *Density gradient analysis for time-dependent labeling of zoospore ribosomes. Ribosomal pellets, purified from low-speed cell supernatant fractions by two successive 5-hr centrifugations at 100,000 × g, were prepared and analyzed on sucrose gradients after exposure to uracil-2-C^{14} for (a) 15 min, (b) 30 min, (c) 60 min, and (d) 120 min.*

extracts were prepared and examined after exposure to uracil-2-C^{14} over a period of 15 to 120 min. The bulk of the RNA labeled before 30 min was found in the region from 4*S* to 30*S*. After 1 hr of incubation, a detectable proportion of this label had been transferred to the ribosome peak, and by 2 hr a considerable increase was evident in this fraction, although the greatest activity was in the 50*S* region. These results were in substantial agreement with those obtained by DEAE-cellulose fractionation of whole-cell extracts. Both strongly suggest that much of the uracil incorporation in zoospores represents a slow synthesis of ribosomal subunits and the subsequent transformation of these to ribosomes.

An approach to the problem of RNA turnover was made by use of the antibiotic actinomycin D. This inhibitor of RNA polymerase (5, 6) has been used to demonstrate what was presumed to be the normal decay of messenger RNA in *Bacillus subtilis* (4, 10). Actinomycin at a concentration of 50 μg/ml caused a significant inhibition of the already low RNA synthesis in zoospores (Table 1). A concentration of 100 μg/ml resulted in a 79% inhibition of RNA synthesis, and this could not be increased significantly even at concentrations as high as 360 μg/ml. When 50 μg/ml of actinomycin D was added to a suspension of zoospores, after a 25-min exposure to uracil-C^{14}, RNA synthesis stopped immediately. The cessation of synthesis was followed rapidly by the loss of a large amount of the previously incorporated activity (Fig. 6a). The results given in Fig. 6b clearly show that the instability in the presence of actinomycin D decreased with time. After 1 min of synthesis, 90% of the labeled RNA was degraded in the presence of actinomycin, but after 20 min about 60% of the labeled RNA had become stable or resistant to the antibiotic. These results with actinomycin could be due, at least in part, to normal turnover; other possible interpretations will be evaluated in the Discussion.

Incorporation of protein precursors by zoospores. It was mentioned earlier that protein synthesis could not be detected when zoospores were analyzed during the first 4 hr for total protein by extraction and colorimetric assays. Nevertheless, as in the case of RNA, the incorporation of radioactive precursors could be demonstrated, albeit at low levels. This is clearly indicated by the data given in the first column of Table 2. During the initial 30 min of incubation, the inhibition of synthesis by actinomycin, chloramphenicol, and puromycin was 12, 28, and 21%, respectively. For the period between 30 and 120 min, the inhibition by all three antibiotics was essentially complete (94 to 99%).

Protein synthesis does, therefore, occur in motile zoospores and is sensitive to the RNA

TABLE 1. *Effect of actinomycin D on incorporation of uracil into zoospore RNA**

Actinomycin D (μg/ml)	Count/min incorporated	Per cent inhibition
0	380	0
50	142	63
100	80	79
200	81	79
360	68	82

* Assay tubes contained 10^6 zoospores and 0.2 μc of uracil-2-C^{14} (specific activity, 1.5 μc/μmole) per ml of medium C, in a final volume of 2.5 ml. They were incubated for 30 min at 24 C.

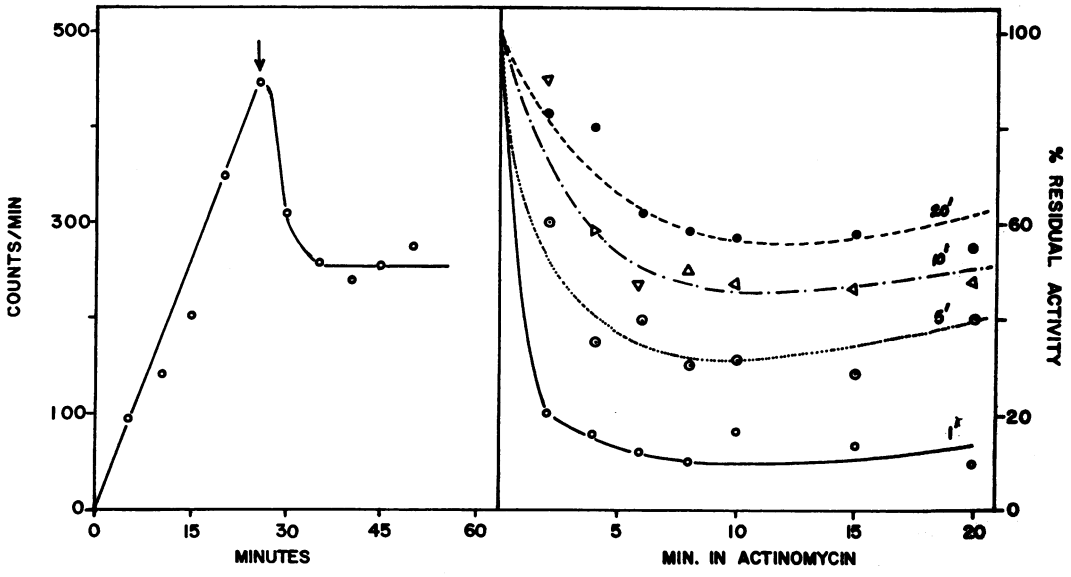

FIG. 6. *Effect of actinomycin D on newly labeled zoospore RNA. (a) The zoospores (10⁵ per milliliter) were incubated in medium C containing uracil-2-C¹⁴ (0.5 μc/ml; specific activity 1.5 μc/μmole) at 24 C, and 1-ml samples were removed for measuring RNA incorporation. At 25 min (arrow) actinomycin was added to give a final concentration of 50 μg/ml, and the sampling was continued. (b) Samples of zoospores were prelabeled in uracil-2-C¹⁴ for the times indicated in each curve. At that time 120 μg/ml of actinomycin D was added to each, and samples were removed for a subsequent 20-min interval. The activity at the time of actinomycin addition has been plotted as 100%, and all subsequent values as residual percentages of the original activity.*

TABLE 2. *Effect of inhibitors on leucine incorporation into zoospore proteins**

Time (min)	Count/min incorporated			
	Control	Actinomycin D, 120 μg/ml	Chloramphenicol, 100 μg/ml	Puromycin, 1,000 μg/ml
30	1,400	1,230	995	1,100
60	1,525	1,200	1,154	1,133
90	1,705	1,310	1,050	1,100
120	1,980	1,256	1,032	1,105
Total increase†	580	26	37	5

* Zoospores (2 × 10⁶ per milliliter) were preincubated for 60 min with or without antibiotics in medium C. Leucine-*1*-C¹⁴ (0.5 μc/ml; specific activity, 18.8 μc/μmole) was then added, and 1-ml samples were removed every 30 min for assay of incorporation into protein.

† Between 30 and 120 min.

inhibitor, actinomycin D (presumably indirectly), and the protein inhibitors, chloramphenicol and puromycin. The absolute rates were very low, and, as shown in Fig. 7a, leucine-*1*-C¹⁴ was incorporated in all protein fractions in a relatively nonspecific manner. A small shoulder of radioactivity appears under the peak in optical density for the ribosomes, but there is no conclusive evidence to suggest that any significant labeling of the structural protein of ribosomes occurred.

In contrast to the results obtained with leucine, methionine-methyl-C¹⁴ did lead to rather specific labeling of the ribosomal and soluble RNA (sRNA) fractions as well as of the soluble proteins (Fig. 7b). Table 3 reveals that the radioactivity in the sRNA region was the result of methylation of the sRNA bases with the methyl-C¹⁴ of the methionine. It is also obvious that the activity in the ribosomal fraction was not due to methylation of the ribosomal RNA. It could, of course, represent either structural protein of the ribosomes or newly synthesized protein remaining on them. It is certainly unlikely that the activity could represent a significant amount of attached sRNA, since this should have appeared in the phenol-extracted ribosomal RNA.

DISCUSSION

From the results presented above, it is obvious that the motile zoospores of *R. rosea* synthesize very little RNA or protein. Our interest in these low rates derives primarily from the unusual situation wherein macromolecule synthesis is repressed while energy metabolism proceeds at an active pace. In the absence of direct evidence, we

presume that this permits the swimming spore to serve economically its primary function in the life cycle, which is dispersal, a process requiring energy for motility but not for growth.

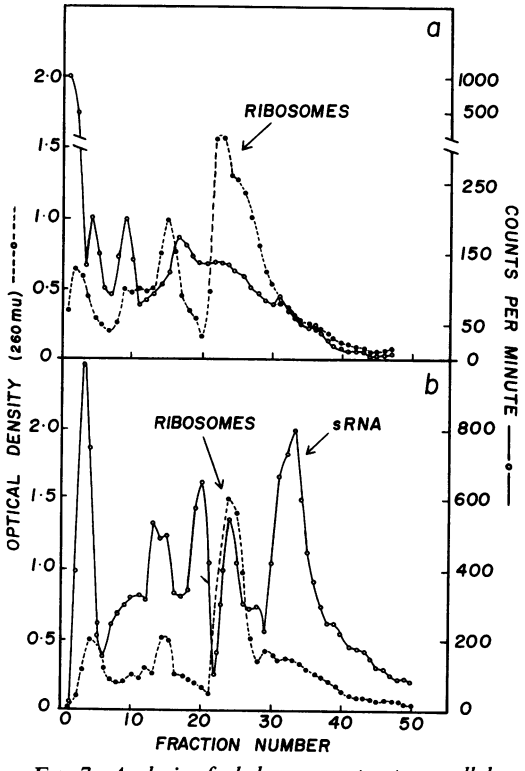

FIG. 7. *Analysis of whole spore extracts on cellulose after incorporation of amino acids. Zoospores were labeled for 4 hr with (a) leucine-1-C^{14} (3.75 × 10^6 spores and 0.5 μc/ml; specific activity 10 μc/μmole), and (b) methionine-methyl-C^{14} (4.13 × 10^6 spores and 0.5 μc/ml; specific activity 10 μc/μmole). Extracts prepared and analyzed as in Fig. 3. Fraction size, 3.5 to 3.7 ml.*

The functional significance of the low synthetic rates in zoospores still remains to be clarified. The actual amount of RNA synthesized (as estimated by isotope incorporation) represents no more than 1 to 2% of the total cellular RNA, despite the long periods of incubation. From the distribution of RNA labeling with time, it is also apparent that much, if not all, of the RNA synthesis represents a limited production of ribosomal RNA. Turnover of RNA does not appear to represent a significant fraction of the total synthesis in these cells. However, in a situation where all the rates are very low, a small messenger-like fraction undergoing relatively rapid synthesis and turnover with efficient reutilization of the released nucleotides would probably go undetected.

The rapid degradation of newly labeled RNA in actinomycin-treated bacterial cells has been interpreted by Levinthal et al. (10) and Fan et al. (4) as normal turnover of a messenger fraction. They further reported that the 23*S* and 16*S* ribosomal RNA, synthesized before actinomycin was added, remained stable in the presence of the antibiotic. Our observation of an increased actinomycin stability with time was essentially similar to theirs obtained with *B. subtilis*. They did not, however, eliminate the possibility that some of the rapid turnover in the presence of actinomycin could be due to degradation of incomplete or unprotected molecules of ribosomal RNA. Acs, Reich, and Valanaju (1) concluded that there was an actinomycin-induced degradation of ribosomal RNA in a *B. subtilis* system where protein synthesis was inhibited by chloramphenicol. Also, the ribosomal RNA produced by a "relaxed" RNA control mutant of *E. coli*, in which protein synthesis was prevented by withholding a required amino acid, was reported to be rapidly degraded when RNA synthesis was stopped by 2,4-dinitrophenol (Nakada et al.,

TABLE 3. *Methylation of soluble RNA bases with methyl-labeled methionine**

Time (hr)	RNA sample	Count/min					
		Total in KOH hydrolysate	Nucleotides				Total recovered in nucleotides
			UMP	GMP	CMP	AMP	
2	Ribosomal	110	—	—	—	—	—
2	Soluble	750	100	106	114	108	428
4	Ribosomal	215	—	—	—	—	—
4	Soluble	850	130	154	136	132	552

* Spores (1.4 × 10^7) and 0.5 μc of methionine-methyl-C^{14} (specific activity, 9.95 μc/μmole) per ml were incubated in medium C at 24 C. At 2 and 4 hr, 20-ml samples were removed, and the soluble and ribosomal RNA was isolated. This was then hydrolyzed, and the nucleotides were separated and counted. UMP = uridine monophosphate; GMP = guanosine monophosphate; CMP = cytidine monophosphate; AMP = adenosine monophosphate.

15). Kennell (8) recently reported that precursors of the 50*S* ribosomal subunit of *B. megaterium* are degraded in the presence of actinomycin, which also inhibits incorporation into the 23*S* ribosomal RNA.

It seems reasonable to conclude from the above that both unstable ribosomal RNA precursors and messenger RNA (mRNA) may undergo degradation or turnover in the presence of actinomycin D, and in addition that the process is accelerated in the absence of protein synthesis. Our results with *Rhizophlyctis* zoospores are fully consistent with such an interpretation. The peak of high specific activity in the 50*S* region of Fig. 5d may well represent a precursor for the 57*S* subunit (LéJohn and Lovett, *unpublished data*) of zoospore ribosomes, and may be equivalent to the unstable precursor of the 50*S* particles of *B. megaterium* (8). If this is correct, the accumulation of this precursor could result from the depressed rate of protein synthesis and a concomitant lag in the rate at which these particles are stabilized in the form of the 57*S* ribosomal unit. The slow accumulation of precursors would, under this interpretation, reflect a greatly reduced rate in the later steps of ribosome assembly. The whole system is exceedingly slow and shows a much greater lag in the synthesis and assembly of ribosomal subunits than do growing *E. coli* cells (14). These unstabilized (accumulated) precursors might be expected to display a sensitivity to actinomycin in the absence of active protein synthesis, similar to the instability of the RNA particles in the "relaxed control" mutants of *E. coli* during a step down in growth [where 25% of the previously accumulated RNA is degraded without adding actinomycin (15)], or in normal bacteria when protein synthesis is inhibited by chloramphenicol. Certainly, in the absence of significant protein synthesis, an interpretation of the extensive actinomycin-induced degradation of the most rapidly labeled RNA in zoospores as "messenger" turnover seems unreasonable.

It seems clear that some ribosomal RNA is produced by zoospores, but we cannot be certain from these experiments whether mRNA is synthesized. That some may be made is suggested by (i) the appearance of RNA in complete 82*S* ribosomes, which would necessitate some concomitant protein synthesis (as observed in Table 2 and Fig. 7), and (ii) the inhibition of protein synthesis by actinomycin as well as by the protein inhibitors puromycin and chloramphenicol (Table 2). The latter could result from turnover of existing mRNA molecules and prevention of new synthesis by actinomycin, or from actual actinomycin-induced degradation of pre-existing mRNA. The relatively slower effect of actinomycin, compared with the protein inhibitors, provides some support for the existence of at least a little messenger turnover. Only the isolation of a fraction which displays messenger characteristics, e.g., by hybridization with DNA (12), can settle the question. This is a discouraging prospect in zoospores where the messenger fraction would, at best, represent a minute quantity of RNA with very low activity.

The low rates of synthesis in zoospores may reflect the basal level required to maintain the integrity of the cell. The efficiency with which actinomycin inhibits protein synthesis encourages the concept that the rate of protein synthesis is directly regulated by the production of appropriate species of mRNA. This is in keeping with the steadily accumulating evidence to this effect from bacterial and mammalian systems. Even if this is the case, however, the mechanism(s) whereby the normal rates of RNA synthesis are repressed in the spore remains unknown, although it is apparent that such a mechanism(s) exists since spores do not germinate for hours even when placed in a normal growth medium.

Many questions remain concerning the actual levels of the enzymes, substrates, and cofactors necessary for the production of RNA and protein in zoospores. Still another important question concerns the presence or absence of a ribosome-containing nuclear cap in *R. rosea* spores. This type of compartmentalization of the spore ribosomes in a morphologically similar species, *Blastocladiella emersonii*, has presented the possibility of the control of protein synthesis by an isolation mechanism (11). The nuclear cap has been reported to be absent in *R. rosea* (9), but this was based upon light microscopic observations of the tiny spores (3 to 4 μ in diameter) in which the cap, if small, could be obscured by the numerous pigmented lipid granules. Since an unequivocal knowledge of its presence or absence is critical to our interpretation, the intracellular organization of zoospores at the ultrastructural level is presently under investigation. All of these questions must be answered before it will be possible to draw conclusions about the control mechanisms responsible for the repression of macromolecule synthesis in zoospores and the release of this repression at the time of germination.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI-04783 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. ACS, G., E. REICH, AND S. VALANAJU. 1963. RNA metabolism of *B. subtilis*. Effects of actinomycin. Biochim. Biophys. Acta 76:68–79.

2. BRITTEN, R. J., AND R. B. ROBERTS. 1960. High resolution density gradient sedimentation analysis. Science **131**:32–33.
3. CANTINO, E. C., AND J. S. LOVETT. 1964. Nonfilamentous aquatic fungi: Model systems for biochemical studies of morphological differentiation. Advan. Morphogenesis **3**:33–93.
4. FAN, D. P., A. HIGA, AND C. LEVINTHAL. 1964. Messenger RNA decay and protection. J. Mol. Biol. **8**:210–222.
5. GOLDBERG, I. H., M. RABINOWITZ, AND E. REICH. 1962. Basis of actinomycin action. I. DNA-binding and inhibition of RNA polymerase synthetic reaction by actinomycin. Proc. Natl. Acad. Sci. U.S. **48**:2094–2101.
6. HURWITZ, J., J. J. FIRTH, M. M. MALAMY, AND M. ALEXANDER. 1962. The inhibition of enzymatic synthesis of RNA and DNA by actinomycin D and proflavine. Proc. Natl. Acad. Sci. U.S. **48**:1222–1220.
7. KATZ, S., AND D. COMB. 1963. A new method for the determination of the base composition of RNA. J. Biol. Chem. **238**:3065–3067.
8. KENNELL, D. 1964. Persistence of messenger RNA activity in Bacillus megaterium treated with actinomycin. J. Mol. Biol. **9**:789–800.
9. KOCH, W. J. 1961. Studies of the motile cells of chytrids. III. Major types. Am. J. Botany **48**: 786–788.
10. LEVINTHAL, C., D. P. FAN, A. HIGA, AND R. A. ZIMMERMANN. 1963. The decay and protection of messenger RNA in bacteria. Cold Spring Harbor Symp. Quant. Biol. **28**:183–190.
11. LOVETT, J. S. 1963. Chemical and physical characterization of "nuclear caps" isolated from *Blastocladiella* zoospores. J. Bacteriol. **85**:1235–1246.
12. MCCARTHY, B. J., AND E. T. BOLTON. 1964. Interaction of complimentary RNA and DNA. J. Mol. Biol. **8**:184–200.
13. MCCARTHY, B. J., AND R. J. BRITTEN. 1962. The synthesis of ribosomes in *E. coli*. I. The incorporation of C^{14}-uracil into the metabolic pool and RNA. Biophys. J. **2**:35–47.
14. MCCARTHY, B. J., R. J. BRITTEN, AND R. B. ROBERTS. 1962. The synthesis of ribosomes in *E. coli*. III. Synthesis of ribosomal RNA. Biophys. J. **2**:57–82.
15. NAKADA, D., I. A. C. ANDERSON, AND B. MAGASANIK. 1964. Fate of the ribosomal RNA produced by a "relaxed" mutant of *Escherichia coli*. J. Mol. Biol. **9**:472–488.
16. NEIDHARDT, F. C., AND B. MAGASANIK. 1960. Studies on the role of ribonucleic acid in the growth of bacteria. Biochim. Biophys. Acta **42**:99–116.
17. SPARROW, F. K. 1960. Aquatic phycomycetes, 2nd ed. Univ. Michigan Press, Ann Arbor.