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Mode of Action of Thiolutin, an Inhibitor of Macromolecular Synthesis in *Saccharomyces cerevisiae*

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The sulfur-containing antibiotic thiolutin has been shown to be a potent, reversible inhibitor of the growth of *Saccharomyces cerevisiae*. Viability was unaffected over the concentration range of 4 to 100 μg/ml. At concentrations as low as 2 μg/ml, the drug inhibited ribonucleic acid (RNA) and protein synthesis in whole cells and spheroplasts. At these low concentrations, protein synthesis continued for a short period of time after RNA synthesis was completely stopped. With higher drug concentrations (greater than 20 μg/ml) protein synthesis was inhibited; concentrations of thiolutin up to 100 μg/ml did not affect translocation or peptide bond formation in cell-free protein-synthesizing systems from yeast. The effect of thiolutin on the activity of partially purified deoxyribonucleic acid-dependent RNA polymerases was examined, and the drug was found to be a potent inhibitor of RNA synthesis in vitro. Inhibition was greatest when the polymerase was preincubated with thiolutin. Several mechanisms are discussed to explain the multiple effects of thiolutin on *S. cerevisiae*. Since the action of the drug is easily reversed, thiolutin may prove to be of use in studies of various stages of yeast growth.

In bacteria, antibiotics have played useful roles in providing genetic markers for a number of macromolecules and have also been used in dissecting the roles of macromolecules in concerted reactions. The use of antibiotics in yeast has not been exploited to the same extent. This is due, in part, to the fact that many of the better-characterized antibiotics do not affect *Saccharomyces cerevisiae*, presumably because the yeast cell is impermeable to these compounds. Many antifungal compounds have been isolated during the process of screening for antimicrobial agents, and, although they may not be medically useful because of their toxicity properties, it is quite possible that some of these compounds may have biochemical applications. This report describes our initial studies on the mode of action of one such antifungal agent, thiolutin.

MATERIALS AND METHODS

Yeast strains, growth media, and preparation of Spheroplasts. The yeast strain used during the course of this work was *S. cerevisiae* Y166 (αtrp5 his4 MA1). Experiments with whole cells were carried out in YM-5 medium and those with spheroplasts were carried out in YM-5 medium containing 0.4 M magnesium sulfate (12). Solid media for strains was YEPD (1% yeast extract, 2% peptone, 2% glucose, and 2% agar).

Cell growth was followed by measurements of the optical density at 550 nm (OD_{550}) in a Bausch & Lomb Spectronic 20 colorimeter. An optical density of 0.32 corresponded to 10^7 cells/ml. Viability of cell cultures was determined by serial dilution and plating on YEPD medium; plates were scored after 48 h of incubation at 30 C.

The procedure of Hutchison and Hartwell (12) was used for the preparation of spheroplasts. Treatment with 1% glusulase in 1 M sorbitol for 1 to 2 h at 27 C was adequate to convert cells to spheroplasts, Metabolically active spheroplasts were obtained by harvesting spheroplasts after glusulase treatment and incubating them for 2 to 3 h at 27 C in YM-5 medium containing 0.4 M $MgSO_4$.

Incorporation of labeled precursors. The procedure described by Hartwell (9) was followed. Ribonucleic acid (RNA) and protein synthesis were monitored simultaneously by use of a mixture of ^3H-labeled uridine and ^{14}C-labeled leucine or recon-

stituted protein hydrolysate (Amersham-Searle). At various times, 1-ml samples were removed and added to 1 ml of ice-cold 10% trichloroacetic acid; the resulting precipitates were collected on glass-fiber circles (Whatman GF/C), washed several times with cold trichloroacetic acid, dried, and counted in a toluene-based scintillation fluid.

Preparation and analysis of polyribosomes. Metabolism in spheroplasts was stopped by the addition of cycloheximide (200 μg/ml) to a 5-ml culture. The culture was immediately cooled in ice, and spheroplasts were collected by centrifugation at 8,000 $\times$ *g* for 5 min in a Sorvall SS-34 rotor. The pellets were resuspended in 0.25 ml of ice-cold Tris-hydrochloride lysis buffer (10 mM tris[hydroxymethyl]-aminomethane, pH 7.4, 100 mM NaCl, and 30 mM $MgCl_2$), 0.5% sodium deoxycholate (DOC), and 0.75% Brij 58; they were immediately layered on top of a 5-ml 10 to 30% sucrose gradient in lysis buffer and centrifuged for 35 min at 35,000 rpm in a Spinco SW50.1 rotor. The gradients were analyzed at 254 nm in an Isco model D/UA-2 gradient analyzer. If the incorporation of labeled precursors into polyribosomes was to be measured, 33 fractions were collected and precipitated with ice-cold 15% trichloroacetic acid. The percentage of ribosomes present as polyribosomes was determined by planimetric tracing, or by weighing the areas under the peaks.

Analysis of radioactively labeled RNA. Spheroplast cultures (5 ml) were centrifuged for 2 min at 10,000 $\times$ *g*, and the pellets were lysed with 0.25 ml of a solution containing 100 mM NaCl, 50 mM sodium acetate, pH 5.4, 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate (SDS). The lysates were layered on a 5-ml 5 to 20% sucrose gradient made in the same buffer and were centrifuged for 2.5 h at 40,000 rpm in a Spinco SW50.1 rotor. The gradients were analyzed in an Isco gradient analyzer and 6-drop fractions were collected on 2.4-cm disks of filter paper (Whatman 3 MM). Fifty-eight fractions were collected from each 5-ml gradient. The disks were washed three times with ice-cold 10% trichloroacetic acid, once with alcohol-ether (1:1), and once with ether. The filters were air-dried and counted in a toluene-based scintillation fluid.

Polypeptide synthesis in vitro. A method described elsewhere (A. Jimenez, B. Littlewood, and J. Davies, in D. Vazquez [ed.], Molecular mechanisms in antibiotic action on protein biosynthesis and membranes, Elsevier, in press) was modified as follows. The source of supernatant enzymes was the S-150 fraction precipitated with ammonium sulfate. The 40 to 70% precipitate was resuspended in a small volume of 20 mM Tris-hydrochloride (pH 7.4)-1 mM dithiothreitol (DTT) and dialyzed against the same buffer; small samples were stored at -30 C. Incubation mixtures (0.1 ml) for polyuridylic acid (poly U)-directed polyphenylalanine synthesis contained 50 mM Tris-hydrochloride, pH 7.7, 12.5 mM magnesium acetate, 80 mM KCl, 4 mM creatine phosphate, 40 μg of creatine phosphokinase/ml, 500 μg of yeast transfer RNA (tRNA)/ml, 1 mM DTT, 60 mCi of ^{14}C-phenylalanine/ml (specific activity, 33 mCi/mmol), 300 μg of poly U/ml, 16 A_{260} units of ribosomes/ml, and 2 mg of supernatant enzymes/ml. After 45 min of incubation at 30 C, reactions were stopped by the addition of 1 ml of 10% trichloroacetic acid. The precipitates were heated for 20 min at 90 C, cooled, collected on glass-fiber papers, and counted.

For endogenous messenger RNA (mRNA)-directed polypeptide synthesis, 400 ml of an active spheroplast culture was poured over frozen 1 M sorbitol and the thawed mixture was centrifuged for 15 min at 9,000 $\times$ *g*. The pellets were resuspended in cold lysis buffer (3 ml), and DOC (0.15%) and Brij (0.2%) were added. Lysis was assisted by three strokes in a Potter-Elvejhem homogenizer, and the lysate was centrifuged at 10,000 $\times$ *g* for 10 min to give an S-10 fraction. The S-10 fraction was purified by passage through a Sephadex G-50 column (previously equilibrated with elution buffer, 10 mM Tris-hydrochloride, pH 7.4, 10 mM $MgCl_2$, 40 mM KCl, and 5 mM DTT). The RNA-rich fraction (monitored at OD_{260}) was eluted with this buffer and stored in small portions in liquid nitrogen. Incubation mixtures (0.1 ml) contained 50 mM Tris-hydrochloride, pH 7.4, 10 mM $MgCl_2$, 80 mM KCl, 4 mM creatine phosphate, 40 μg of creatine phosphokinase/ml, 250 μg of yeast tRNA/ml, 1 mM DTT, 400 μM of the 20 amino acids, 5% (vol/vol) of ^{14}C-reconstituted algal hydrolysate (neutralized with KOH), and 30 μliters of purified S-10 fraction. Incubation was for 45 min at 25 C, and radioactivity incorporated into trichloroacetic acid precipitates was determined out as described above.

Preparation of RNA polymerases. RNA polymerases were prepared from *S. cerevisiae* A364A cells grown in YEPD medium as described in the accompanying paper. After breakage in a Buhler mill with glass beads and sonic treatment, the 180,000 $\times$ *g* supernatant fluid was fractionated on a column of diethylaminoethyl (DEAE)-cellulose with a gradient of increasing ammonium sulfate concentration (17). The assay consisted of incorporation of (*4-^{14}C*)-uridine triphosphate (UTP) into a polymer retained by DEAE paper during washing with 5% Na_2HPO_4 (2). Precipitation with 5% trichloroacetic acid at 0 C and filtration gave similar data. Unless otherwise stated, all assays were performed at 30 C and contained 0.2 mg of alkali-denatured salmon sperm deoxyribonucleic acid (DNA)/ml, 0.01 mM UTP, 0.2 mM adenosin, guanosine, and cytidine triphosphate (ATP, GTP, and CTP), 1.6 mM Mn^{2+}, and 0.05 μCi of ^{14}C-UTP in a final volume of 100 μliters. Counting efficiency was 45%, so that the incorporation of 50 counts/min was equivalent to incorporation of 1 pmol of UTP. Two peaks of activity, called I and II in order of their elution from DEAE-cellulose, were consistently recovered. Peak specific activities were usually about 5 and 50 nmol of UTP incorporated per mg of protein per h, respectively, and total enzyme II activity was three to eight times total enzyme I activity. Native salmon sperm DNA was about 80% as effective as denatured DNA for enzyme I, but only 10 to 15% as effective for enzyme II. Both enzymes had optimal activity in 1.5 to 2.5 mM Mn^{2+}, and were totally dependent on added template. ^{14}C-UTP incorporation required the presence of all four nucleotide triphosphates.

Chemicals. Thiolutin was generously provided by Nathan Belcher, Pfizer Inc., Groton, Conn. The drug was made up freshly in dimethyl sulfoxide (DMSO) before use. This solvent has been shown to have no effect on growth, viability, or macromolecular processes in cells or spheroplasts at concentrations up to 1% (vol/vol). Cycloheximide was the gift of G. B. Whitfield, Jr., The Upjohn Co., and cryptopleurine was provided by S. Földéak. Yeast tRNA was purchased from General Biochemicals Corp.; ^{3}H-uridine (26 Ci/mmol), ^{3}H-adenine (36 Ci-mmol), and (4-^{14}C)-UTP, from Amersham Searle; and ^{14}C-L-amino acid mixture, from New England Nuclear Corp.

RESULTS

Effect of thiolutin on cell growth and viability. Concentrations of antibiotic as low as 2 μg/ml inhibited the growth of *S. cerevisiae* in both rich and minimal media (Fig. 1). The drug appears to have a static effect, since incubation for 5 h with 100 μg of thiolutin/ml had no effect on cell viability; cell growth resumed promptly on removal of the drug.

Inhibition of macromolecular synthesis in whole cells. Thiolutin caused rapid inhibition of both protein and RNA synthesis in cells. A typical incorporation experiment is shown in Fig. 2; addition of thiolutin (4 μg/ml) immediately blocked the incorporation of ^{3}H-uridine into nucleic acids, and the incorporation of ^{14}C-amino acids into protein decayed to a minimum over a period of about 20 min. This can also be seen at lower concentrations of thiolutin, where the differential effect of the drug on protein and RNA syntheses is somewhat more apparent. Figure 3 shows that 45 min after the addition of thiolutin (1.5 μg/ml) uridine incorporation was reduced by 77% and protein synthesis by 55%. Similar results were found when incorporation into active yeast spheroplasts was studied. Inhibition of DNA synthesis was also observed, but this was not further analyzed.

The effect of thiolutin on the stability of RNA was determined by adding the drug after a short

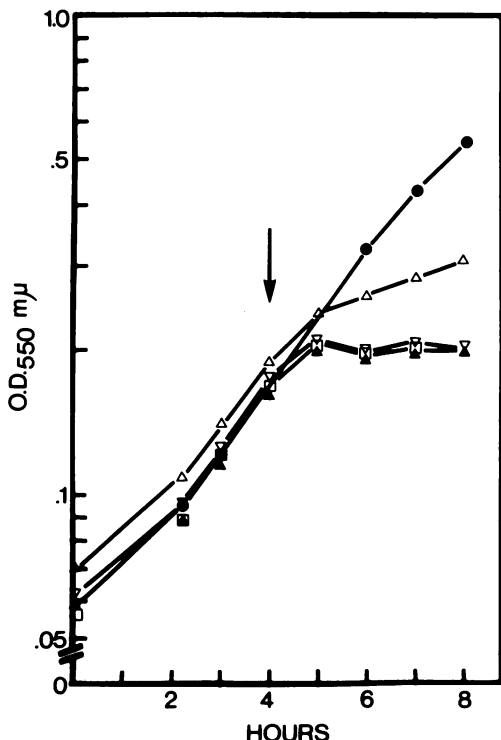

FIG. 1. *Effect of thiolutin on growth of S. cerevisiae. Cell growth in 5-ml incubations was monitored at OD_{550}. The arrow indicates the time of addition of the drug. Additions were as follows: 1% DMSO (●) and thiolutin at 2 μg/ml (△), 5 μg/ml (▽), 10 μg/ml (□), and 25 μg/ml (▲).*

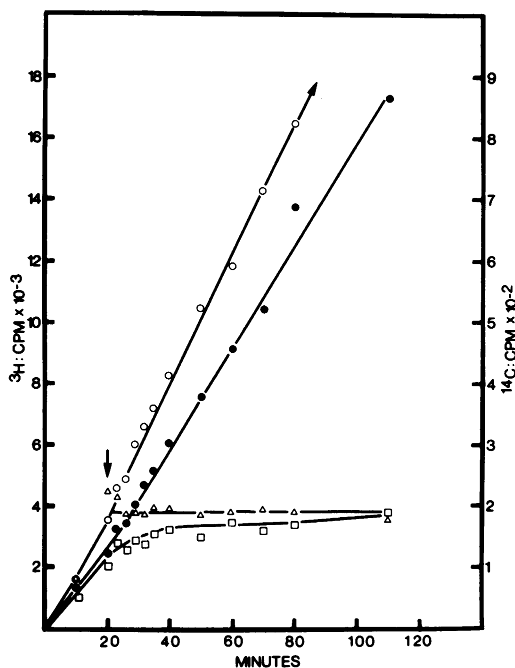

FIG. 2. *Effect of thiolutin on the incorporation of radioactively labeled uridine and amino acids into cells of S. cerevisiae. Exponentially growing cultures were divided into two parts, and ^{3}H-uridine (6.6 μCi/ml) and ^{14}C-amino acid mixture (0.66 μCi/ml) were added to each. Thiolutin or DMSO was added at 20 min, and samples (1 ml) were removed at the indicated times and precipitated with trichloroacetic acid (see Materials and Methods). Incorporation of ^{3}H-uridine in the absence of drug (○) and in the presence of thiolutin, 4 μg/ml (△). Amino acid incorporation in the absence (●) and in the presence (□) of drug.*

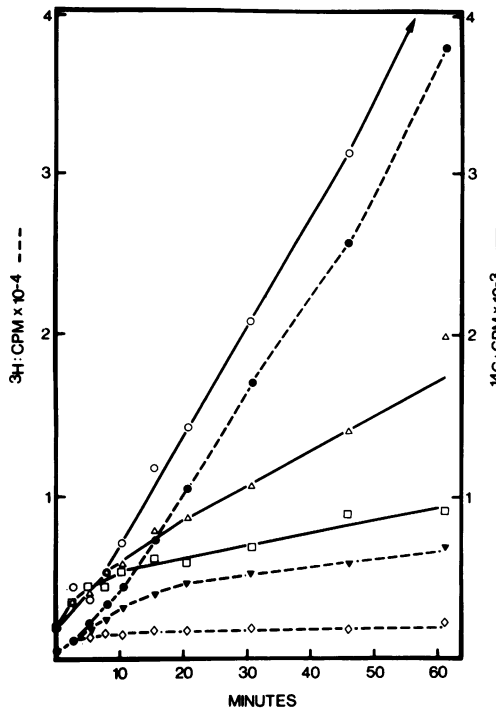

FIG. 3. *Effect of low concentrations of thiolutin on the incorporation of precursors of RNA and protein synthesis. Exponential cultures of S. cerevisiae in YM-5 received ³H-uridine (10 µCi/ml) and ¹⁴C-amino acids mixture (1 µCi/ml). After 2 min of incubation, additions were made as indicated, and samples were taken as described in Materials and Methods. ³H-uridine control (●), ³H-uridine in the presence of 1.5 µg of thiolutin/ml (□), ³H-uridine in the presence of 2.5 µg of thiolutin/ml (◇). ¹⁴C-amino acid control (O), ¹⁴C-amino acids in the presence of 1.5 µg of thiolutin/ml (△), ¹⁴C-amino acids in the presence of 2.5 µg of thiolutin/ml (▽).*

(1 to 2 min) pulse of ³H-uridine. Over a range of 4 to 100 µg of thiolutin/ml for periods of up to 1 h, labeled RNA was stable and did not break down into trichloroacetic acid-soluble material. This is in marked contrast to the situation in bacteria, where inhibition of RNA synthesis results in a rapid decay of unstable RNA labeled by the short pulse (14). In HeLa cells, inhibition of RNA synthesis by actinomycin does not presage decay of synthesized RNA (19).

Effect of thiolutin on polyribosome metabolism in spheroplasts. The presence or absence of polyribosomes in spheroplasts can provide a useful indicator of the mode of action of a drug (5). Decay ("run-off") of polyribosomes can result from inhibition of RNA synthesis or from interference with initiation of protein synthesis. Inhibition of polyribosome activity (freezing) could result from inhibition of peptide bond formation, translocation, or a variety of other effects, such as starvation for an amino acid or a block in ATP production. The half-life of polyribosomes after inhibition of mRNA production has been used to determine mRNA stability, and studies with a yeast mutant have indicated that the half-life of mRNA in *S. cerevisiae* is 23 min at 36 C (13). When thiolutin (4 µg/ml) was added to an active culture of spheroplasts and the culture was examined periodically for polyribosome content, it was found that degradation of polyribosomes occurred, and after 60 min less than 20% of the ribosomes were present in polyribosomes (Fig. 4). The polyribosome "run-off" occurring after the addition of thiolutin is presumed due to the normal movement of ribosomes in protein synthesis, since it is completely blocked by the addition of inhibitors of protein synthesis in yeast, e.g., cycloheximide, anisomycin or cryptopleurine.

At low thiolutin concentrations (2 to 4 µg/ml), the half-life of polyribosome run-off in sphero-

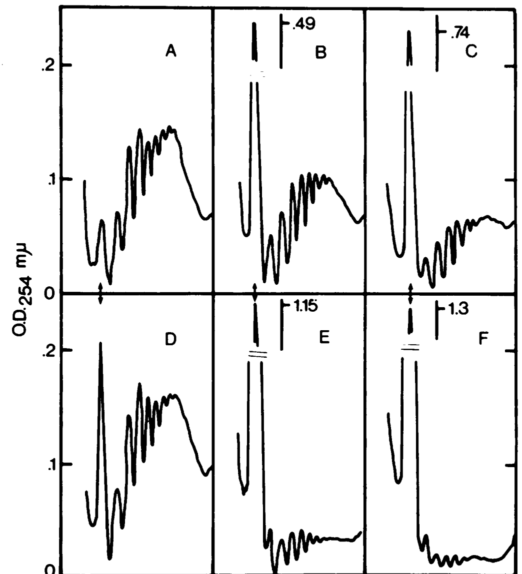

FIG. 4. *Effect of thiolutin on the stability of polyribosomes. Spheroplasts from S. cerevisiae in YM-5 medium containing 0.4 M MgSO₄ were treated with thiolutin (4 µg/ml) and incubated at 30 C. Samples (5 ml) were removed at various times, lysed, and analyzed on 10 to 30% sucrose density gradients as described in Materials and Methods. Panel A is zero time, no drug, and panel D is 60-min incubation, no drug. Panels B, C, E, and F indicate polyribosome profiles after incubation with drug for respectively 5, 15, 30, and 60 min. The arrows indicate the position of 80s monomers on the gradients.*

plasts of *S. cerevisiae* is 15 to 20 minutes at 24 C. This value is of the same order of magnitude as that found by Hutchison et al. (13) in a different yeast strain. The run-off of polyribosomes after addition of thiolutin is never complete, and the decay curves are biphasic (Fig. 5A). The half-life of polyribosome decay during the first 15 min is shorter than that at later times, which might indicate the existence of more than one population of polyribosomes or differential effects of the drug on RNA and protein synthesis. At concentrations of 10 μg/ml and higher, thiolutin partially inhibited the rapid phase of polyribosome run-off (Fig. 5B), and we assume that the drug begins to have secondary, nonspecific effects at these concentrations. Partial inhibition of polyribosome metabolism cannot be due to an effect on translocation since the drug has little or no effect on polypeptide synthesis in vitro.

Thiolutin has no effect on in vitro polypeptide synthesis. The effect of thiolutin on cell-free protein synthesis has been tested in two different systems. In one, amino acid incorporation was directed by endogenous mRNA in an S-10 extract prepared by lysis of active spheroplasts. As shown in Table 1, thiolutin at 100 μg/ml was only slightly inhibitory. In a more purified system, with the use of poly U-directed phenylalanine incorporation, the drug was equally ineffective. Since these two amino acid incorporation systems do not assay for all of the steps in protein synthesis (it is unlikely that normal initiation or termination occur), we can conclude only that thiolutin does not inhibit translocation or peptide bond formation, which is consistent with the inability of the drug to inhibit polyribosome run-off in sphereoplasts.

Effect of thiolutin on the synthesis and processing of stable RNA. In whole cells and spheroplasts, the addition of low concentrations of thiolutin leads to the rapid inhibition of uridine incorporation. This implies that the synthesis of all species of RNA is inhibited. Udem and Warner (20) have shown that, when yeast is treated with protein synthesis inhibitors, some RNA synthesis continues but the cells are blocked in the processing of the 37s ribosomal RNA (rRNA) precursor. The following experiment was carried out to determine

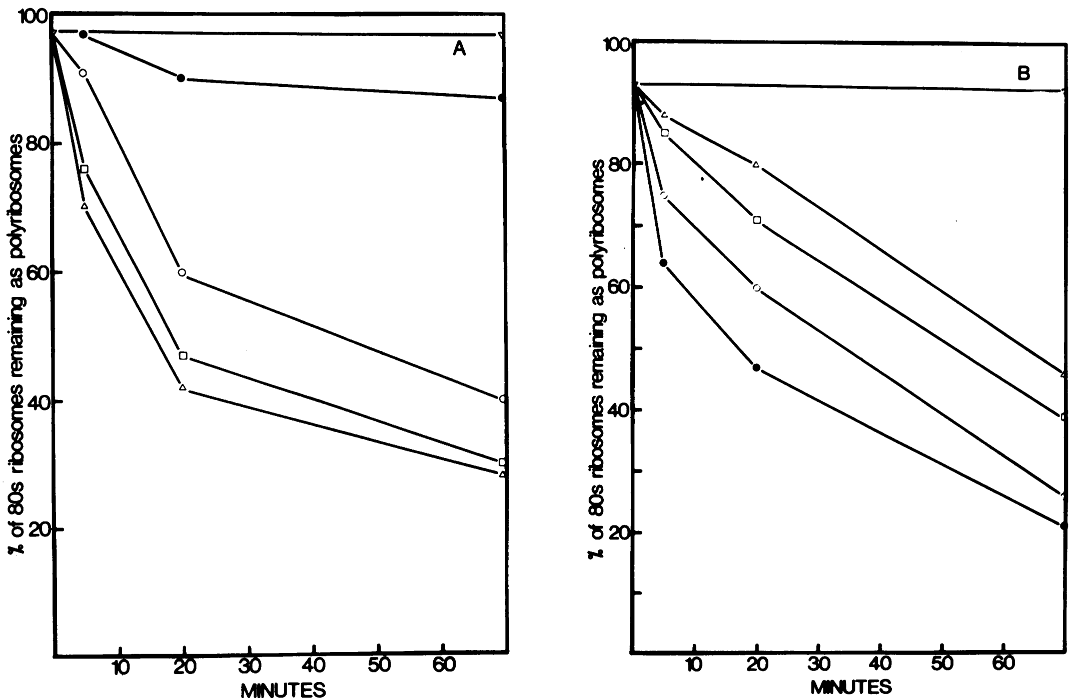

FIG. 5. *Effect of different thiolutin concentrations on polyribosome "run-off" in S. cerevisiae spheroplasts. Experimental details are as described in Fig. 4 and in Materials and Methods. Polyribosome patterns were determined at the indicated times after treatment with various concentrations of thiolutin. (A) Low concentrations of thiolutin: no drug (▽), 0.5 μg/ml (●), 1 μg/ml (○), 2 μg/ml (□), and 3 μg/ml (△). (B) High concentrations of thiolutin. No. drug (▽), 4 μg/ml (●), 10 μg/ml (○), 20 μg/ml (□), and 50 μg/ml (△).*

TABLE 1. *Effect of thiolutin on polypeptide synthesis in vitro*

System	Additions	Counts min	Percent inhibition
Endogenous incorporation	None	1,420	—
	DMSO, 2%	1,480	—
	Thiolutin,		
	10 μg/ml	1,480	—
	50 μg/ml	1,320	11
	100 μg/ml	1,170	21
	Anisomycin,		
	5 μg/ml	460	68
	Cycloheximide,		
	10 μg/ml	575	60
Poly U-directed	None	28,660	—
	DMSO, 2%	28,900	0
	Thiolutin,		
	10 μg/ml	28,120	0
	100 μg/ml	29,690	0
	Cycloheximide,		
	5 μg/ml	7,145	75

whether thiolutin also inhibits the processing of rRNA species in yeast. The stable RNA species (15*s* and 27*s*) were labeled by growing *S. cerevisiae* cells in the presence of ^{14}C-adenine, and the cells were converted to spheroplasts. The spheroplasts were incubated in YM-5 to restore metabolic activity and then were pulse-labeled for 2 min with ^{3}H-uridine; in this short period, the label would enter mRNA and the large-molecular-weight rRNA precursor. Portions of the pulse-labeled culture were treated in various ways (see Fig. 6), lysed, and analyzed to 5 to 20% sucrose gradients containing SDS. In this experiment, we have used cryptopleurine as the protein synthesis inhibitor; in the presence of this drug, further processing of the large precursor RNA is blocked. Identical results were obtained with cyclohexamide and anisomycin, and with thiolutin. Thiolutin inhibits the synthesis of all species of RNA and blocks the processing of precursor RNA.

Inhibition of yeast RNA polymerases by thiolutin in vitro. Since thiolutin was found to inhibit RNA synthesis in whole cells and spheroplasts of *S. cerevisiae*, we examined the sensitivity of an in vitro transcription system to this antibiotic, with the notion that this might represent the primary mode of action of the drug. The synthetic activity of RNA polymerases I and II from *S. cerevisiae* was found to be sensitive to low levels of the drug (Fig. 7). The degree of inhibition of the RNA polymerases depended on the assay procedure. Sensitivity was greatest if the enzyme was preincubated with thiolutin; enzymes I and II were 50% inhibited by 3 and 6 μg of thiolutin/ml, respectively (Fig. 7). In a number of experiments, it was found that preincubation of the drug with enzymes but not preincubation of drug and template enhances the inhibitory action of thiolutin on RNA synthesis; this would suggest that the site of action of thiolutin in inhibiting RNA synthesis is the enzyme and not the template. In all in vitro experiments, the presence or absence of a sulfhydryl reagent did not affect the inhibitory action of the drug.

Reversibility of thiolutin inhibiton. Since incubation with thiolutin does not affect yeast cell viability, it was of interest to examine the timing of recovery of macromolecular synthesis after removal of the drug. Cultures of cells and spheroplasts were exposed to thiolutin (8 μg/ml) for 90 min, washed, and resuspended in medium containing radioactive precursors for RNA and protein synthesis, but lacking the drug. The cultures were then incubated and samples were taken at intervals to determine when RNA and protein synthesis were restored to normal. In Fig. 8 it can be seen that macromolecular synthesis was restored on removal of the drug and the rate of synthesis rapidly attained that of the uninhibited controls. Whole cells and spheroplasts behave differently in their recovery from thiolutin inhibition. In cells, protein synthesis recovers more rapidly than RNA synthesis, whereas in spheroplasts protein and RNA synthesis recover at approximately the same time. This difference may be only trivial, but it is known that spheroplasts tend to leak RNA (12), and it is possible that protein synthesis in spheroplasts is delayed as a consequence of leakage during thiolutin treatment. Not surprisingly, removal of thiolutin and restoration of macromolecular synthesis are accompanied by re-formation of polyribosomes (Fig. 9).

DISCUSSION

S. cerevisiae is a eukaryotic organism which is susceptible to facile genetic manipulation and biochemical study; it thus provides a good model system for studies of the synthesis and regulation of macromolecular synthesis in eukaryotes. The studies of Hartwell, McLaughlin, and Warner have provided much basic information on macromolecular synthesis in *S. cerevisiae;* a large number of temperature-sensitive mutants have been isolated (9), and various mutants have been characterized with blocks in cell division, protein, and RNA synthesis. The use of antibiotic inhibitors of protein synthesis, such as cycloheximide, has also been helpful in determining relationships between the synthesis of various macromolecules in yeast.

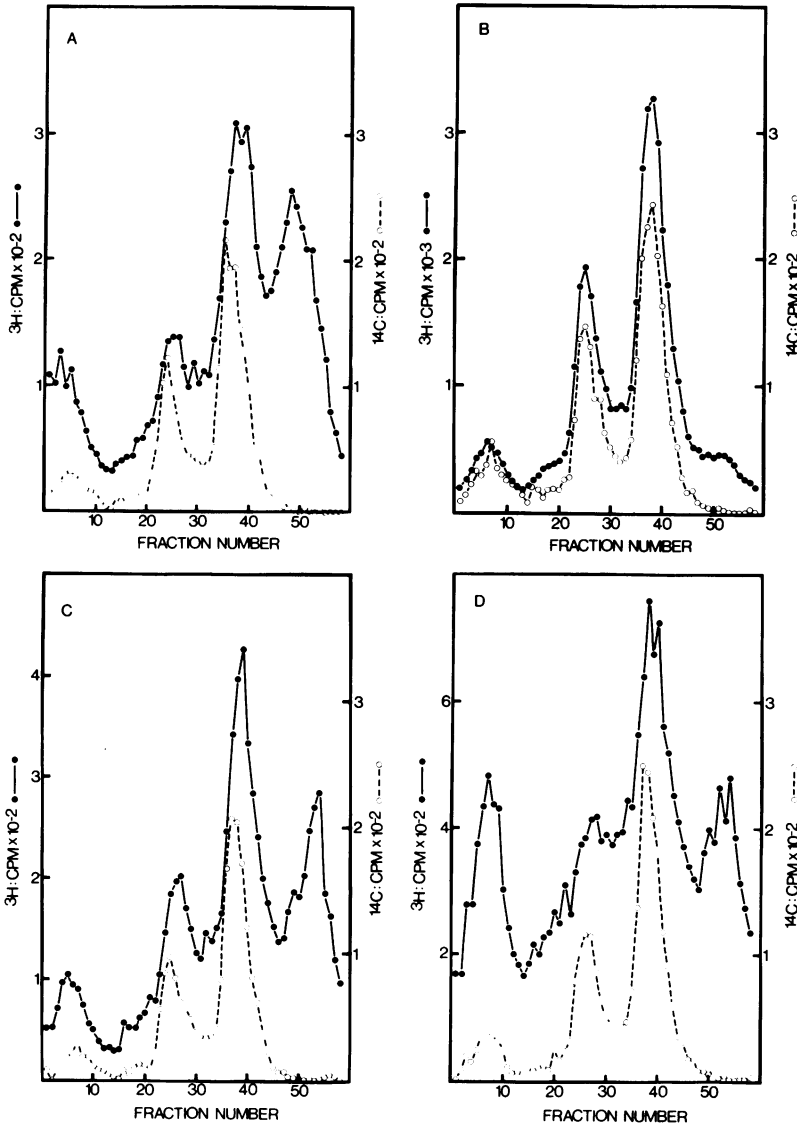

FIG. 6. Effect of thiolutin on the synthesis and processing of stable RNA species. Spheroplasts were prepared from S. cerevisiae cells which had been grown overnight in the presence of ^{14}*C-adenine (0.125 μCi/ml) to indicate steady-state labeling.* ^{3}*H-uridine (30 μCi/ml) was added to various 5-ml spheroplast cultures, which were incubated at 27 C. One culture (control) was poured over frozen 1.0 M sorbitol after 2 min of incubation. To the remaining cultures, unlabeled uridine (final concentration, 0.5 mg/ml) was added, and incubation was continued for 20 min, with additions as indicated. Spheroplasts were lysed, and gradients were collected and analyzed as described in Materials and Methods. (A) Control,* ^{3}*H-uridine for 2 min. (B)* ^{3}*H-uridine for 20 min. (C)* ^{3}*H-uridine for 2 min, thiolutin (10 μg/ml) and unlabeled uridine for 20 min. (D)* ^{3}*H-uridine for 2 min, cryptopleurine (60 μg/ml) and unlabeled uridine for 20 min.*

Yeast RNA polymerase was first fractionated by Frederick et al., who probably isolated only the relatively stable enzyme II activity (8). More recently, by use of the procedure of Roeder and Rutter (17), multiple peaks of activity have been obtained, as first demonstrated by Roeder (2). Fractionation on DEAE-cellulose usually given only two peaks of activity, I and II, in that order. Only peak II is sensitive to α-amanitin (6, 16; D. J. Tipper, J. Bacteriol., in press; Sebastian, Bhargava, and Halvorson, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 162, 1972)

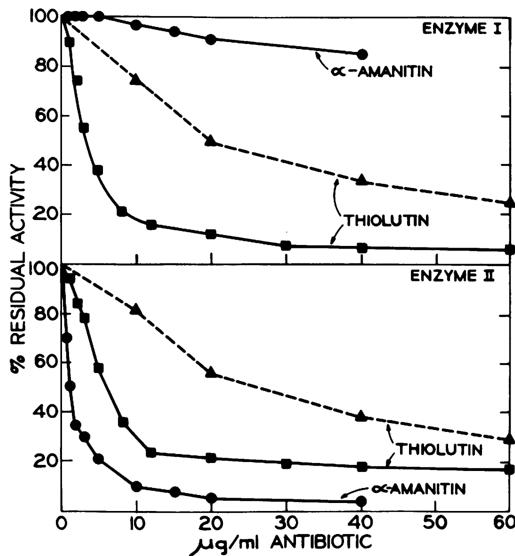

FIG. 7. *Inhibition of yeast RNA polymerases (I and II) by thiolutin. Enzyme (20 μliters of DEAE-cellulose column fraction) was incubated at 22 C in 50 mM Tris-hydrochloride buffer (pH 7.9 at 22 C), 1.6 mM* $MnCl_2$*, 0.4 mM DTT, 0.8 mM* KPO_4*, 0.2 mM EDTA, and dimethyl sulfoxide (2%) containing the indicated concentrations of thiolutin or α-amanitin. After 5 min, DNA template and nucleotide triphosphates (including 0.05 μCi of* ^{14}C*-UTP) were added (final volume of incubation, 100 μliters), and incubation was continued for 20 min at 30 C before assaying for amqunt of RNA synthesized. Thiolutin (■), α-amanitin (●). If thiolutin, DNA template, the other assay components, and enzyme were rapidly mixed in that order at 0 C and subsequently incubated for 20 min at 30 C, the inhibition of RNA synthesis by thiolutin was reduced (▲).*

though three peaks have been reported (3). On the other hand, fractionation on DEAE-Sephadex usually gives a third peak of activity (III) eluting after II. III, like I, is resistant to α-amanitin (1, 2, 15; Tipper, in press). Hall et al. (1) also found a fourth peak of activity eluting before I. The pattern of three RNA polymerase fractions, I, II, and III, with II being sensitive to α-amanitin, is common to most eukaryotic organisms investigated. In metazoans, only I is found in isolated nucleoli, and it is believed to be responsible for rRNA synthesis (2). It would be convenient if a good inhibitor of RNA synthesis were known for *S. cerevisiae;* actinomycin and rifamycin have been used extensively in studies of eukaryotes and prokaryotes, but neither of these inhibitors is effective against yeast. We therefore sought an inhibitor of RNA synthesis that would be effective in this organism, and this communication summarizes our studies on thiolutin, a sulfur-containing antibiotic, which is a potent antifungal agent (9). We have shown that the drug is effective against *S. cerevisiae;* it is static in its action and the inhibitory effect of the drug is easily reversible. The inhibitory effect of the drug on yeast RNA polymerases in vitro at relatively low concentrations would suggest that the primary site of action of thiolutin is at the level of transcription. However, this conclusion is not entirely in agreement with the results of experiments on inhibition of RNA and protein synthesis in cells and spheroplasts, since inhibition of protein synthesis occurs shortly after the cessation of RNA synthesis. These inconsistencies could be explained by any of the following possibilities.

(i) Thiolutin is primarily an inhibitor of energy metabolism in cells, which also binds to yeast RNA polymerases and inhibits their action in vitro. This possibility is difficult to prove or disprove. Thiolutin does not inhibit the incorporation of ^{14}C-labeled glucose into trichloroacetic acid-insoluble material in whole cells at concentrations when RNA synthesis is shut off (unpublished data), which may imply no effect of the drug on ATP production (10). In thiolutin-inhibited spheroplasts, polyribosomes

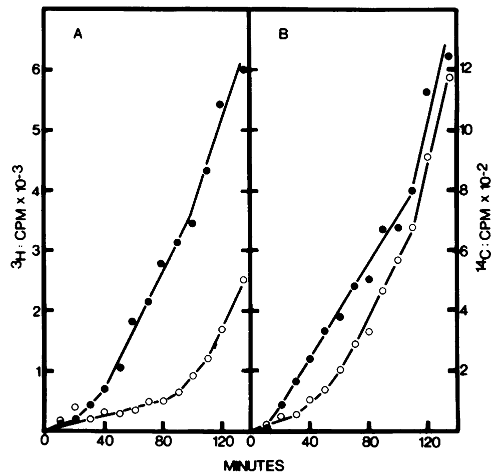

FIG. 8. *Recovery of macromolecular synthesis after removal of thiolutin. An exponential culture of S. cerevisiae was divided, and one half was treated with thiolutin (8 μg/ml) for 2 h at 27 C. Both batches of cells were washed three times with water; the pellets were resuspended in fresh YM-5 medium containing* ^3H*-uridine (6.6 μCi/ml) and* ^{14}C*-amino acid mix (0.66 μCi/ml) and were incubated at 27 C. Samples were taken for trichloroacetic acid-precipitable radioactivity (at the times indicated). (A) Incorporation of* ^3H*-uridine: control (●); thiolutin-treated (O). (B) Incorporation of* ^{14}C*-amino acids; control (●); thiolutin-treated (O).*

are active, and this activity is blocked by inhibitors of protein synthesis. Since protein synthesis cannot take place without a continued source of ATP, it is unlikely that thiolutin affects energy generation.

(ii) Thiolutin inhibits RNA synthesis and protein synthesis by a primary effect on protein synthesis (initiation or termination). It is known that RNA and protein syntheses in yeast are coupled in some way (18), and inhibition of protein synthesis causes a cessation of RNA synthesis. If initiation of protein synthesis was inhibited by thiolutin, it would be expected that polyribosomes would be unstable and would no longer be seen after incubation of spheroplasts for about 5 min. It is believed that the average time for the completion of polypeptide chains is 1 minute in yeast spheroplasts (11). In fact, 15 to 20 min after the addition of thiolutin, 50% of the ribosomes are still present in polyribosomes. This result is also not consistent with inhibition of the elongation or completion (termination) of polypeptide chains, since this would lead to "freezing" of the polyribosomes. However, thiolutin blocks the processing of the 37s rRNA precursor, which is typical of the mode of action of inhibitors of protein synthesis such as cycloheximide, anisomycin, or cryptopleurine.

(iii) Thiolutin inhibits RNA synthesis as its primary mode of action, but also has secondary effects on protein synthesis and other metabolic processes. Many antibiotics with a single mode of action at one concentration have multiple, secondary effects at higher concentrations. In vitro polypeptide synthesis experiments show that thiolutin does not inhibit translocation or peptide bond formation, even at high concentration (100 μg/ml). However, it is possible that some steps taking place in polypeptide synthesis in whole cells may not be assayed in these systems.

(iv) Thiolutin inhibits RNA synthesis as primary mode of action and, because of coupling between the two macromolecular processes, concomitantly causes inhibition of protein synthesis. This would imply that the control of RNA and protein synthesis in *S. cerevisiae* is coupled and stringent in both directions.

It has been established that RNA and protein synthesis in *S. cerevisiae* are coupled and that inhibition of protein synthesis by amino acid starvation, or with any one of several antibiotics (cycloheximide, anisomycin, or cryptoplurine), results in rapid cessation of RNA synthesis (8, 18). This may parallel the coupling between RNA and protein synthesis found in stringently controlled bacterial cells (7), but it is also possible that, in yeast, this inhibition is the result of failure to process the large rRNA precursor (20), an accumulation of which might lead to feedback inhibition of RNA synthesis. It is not inconceivable that RNA synthesis and protein synthesis in yeast are interdependent, and inhibition of RNA synthesis would lead to a rapid cessation of protein synthesis. At present, there is no evidence and no precedent for such a mechanism, and it is difficult to think of a resonable mechanism. We favor the notion that the primary effect of thiolutin may be on the transcription system, but secondary effects of the drug lead to inhibition of other processes (model iii). Thiolutin also inhibits DNA synthe-

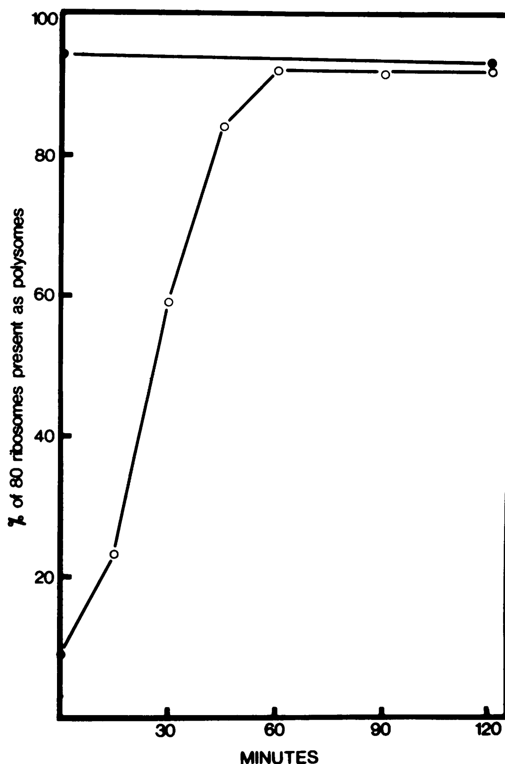

FIG. 9. *Re-formation of polyribosomes after removal of thiolutin inhibition. A spheroplast culture in YM-5 containing 0.4 M* $MgSO_4$ *was divided into two parts, and one half was treated with thiolutin (8* μ*g/ml); the two cultures were incubated at 27 C for 90 min. Samples (5 ml) were taken from each culture and chilled in ice in the presence of 200* μ*g/ml of cycloheximide. The remainders of the two cultures were centrifuged; the spheroplasts were washed by centrifugation and resuspended in fresh YM-5 medium containing 0.4 M* $MgSO_4$*. The two cultures were incubated at 27 C and, periodically, 5-ml samples were removed, lysed, and analyzed for polyribosome content as described in Materials and Methods.* (●) *Control, spheroplast culture not treated with thiolutin;* (O) *culture after removal of thiolutin.*

sis in *S. cerevisiae* (unpublished data); however, because of difficulties in monitoring DNA synthesis in yeast, these experiments are not conclusive. A direct demonstration of the site of action of thiolutin might come from studies of thiolutin-resistant mutants of *S. cerevisiae*. Unfortunately, to date, no stable thiolutin-resistant mutants have been obtained.

It is of interest that low concentrations (<10 μg/ml) of thiolutin are required to inhibit metabolism of whole cells or spheroplasts of *S. cerevisiae*, and similar low concentrations are sufficient to produce marked inhibition of the function of yeast RNA polymerases in vitro.

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