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Inhibition of Yeast Ribonucleic Acid Polymerases by Thiolutin

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Received for publication 26 April 1973

Yeast ribonucleic acid (RNA) polymerase II, isolated after fractionation on diethylaminoethyl (DEAE)-cellulose (DE-52) or on DEAE-Sephadex (A-25), is 50% inhibited by 1.5 µg of α-amanitin. This inhibition is independent of the sequence of interaction of enzyme, template, nucleotides, and antibiotic and is expressed immediately on addition of α-amanitin to a preparation actively synthesizing RNA. Thus, α-amanitin’s primary effect is inhibition of elongation of preinitiated RNA sequences in this system, as in others. A single peak of α-amanitin-resistant RNA polymerase activity (I) was eluted before enzyme II on either column. On A-25 but not on DE-52, a third peak of activity (III) was eluted after enzyme II. This activity was also resistant to α-amanitin. Enzymes I, II, and III were 50% inhibited by 3, 4, and 3 µg of thiolutin per ml, respectively. The extent of inhibition was independent of the nature of the template (native or denatured salmon sperm deoxyribonucleic acid or poly(dA-dT)) or of the presence of 0.4 mM dithiothreitol, but this marked inhibition was only seen when enzymes were preincubated with thiolutin in the absence of template. Template protected the enzymes against thiolutin in the absence of nucleotides. Either the sensitive site on the polymerase is only accessible to thiolutin before interaction with template or thiolutin inhibits functional polymerase-template interaction but not elongation of preinitiated RNA chains.

Thiolutin, one of twelve metabolites produced by Streptomyces luteoreticuli (7), has been known as a potent inhibitor of growth of fungi and bacteria for some time (25). Its mode of action is unknown, but it is an effective and reversible inhibitor of growth of Saccharomyces cerevisiae and completely shuts down ribonucleic acid (RNA) and protein synthesis in this organism in vivo at 2 to 4 µg/ml (14a). In yeast spheroplasts, RNA synthesis stops immediately upon exposure to this concentration of thiolutin, and protein synthesis ceases within 20 min, with slower runoff of polysomes at a rate consistent with the estimated half-life of messenger RNA (mRNA) in yeast (14). In vitro protein synthesis, involving the endogenous polysomal messenger (when no reinitiation occurs) or poly U (where initiation is independent of normal mechanisms), is not inhibited by thiolutin. Thus, this antibiotic appears to have a primary effect on RNA synthesis with a possible secondary effect on the initiation of protein synthesis. These effects may be independent, or a consequence of interdependent coupling of mRNA synthesis and the initiation of mRNA translation. A direct effect of thiolutin on yeast polymerases I and II, isolated after diethylaminoethyl (DEAE)-cellulose (DE-52) chromatography, has been demonstrated in vitro (14a). When these enzymes were exposed to thiolutin before addition of template, they were 50% inhibited by concentrations of thiolutin similar to the minimal inhibitory concentrations in vivo or in spheroplasts. This paper describes the isolation of yeast RNA polymerases I and II by DE-52 chromatography, the isolation of a third RNA polymerase fraction (III) by DEAE-Sephadex (A-25) chromatography, and the interaction of these enzymes with thiolutin.

Metazoan nuclear RNA polymerases can routinely be separated into three or more fractions by DEAE-cellulose or DEAE-Sephadex chromatography (22). They are called I, II, and III (or A, B, and C) in order of elution. Enzyme II is recognized in vitro principally by its sensitivity to α-amanitin (15, 17), a property not shared by enzymes I and III. Thus, as additional peaks or subfractions are found (cf. 1, 28), they are called IIa, IIb, etc., if they are α-amanitin sensitive,
and Ia, Ib, etc., if they elute before II. In some cases, studies of the molecular size of subunit polypeptides indicate that one subtraction may be derived from another by (specific?) endopeptidase action (28), reminiscent of the modification of Bacillus subtilis RNA polymerase seen in enzyme purified from sporulating cells by similar conventional techniques (cf. 5, 16). Enzyme I is unique in location and probably in function since it predominates in isolated rat liver nucleoli and may thus be responsible for synthesis of ribosomal RNA (23). Enzymes II and III are found in the nucleoplasm and are therefore surmised to be responsible for mRNA synthesis. It has been suggested that enzyme III makes mitochondrial 5S and transfer RNA precursors (21). Enzyme III has a higher ionic strength optimum than enzyme I, but both transcribe native deoxyribonucleic acid (DNA) in vitro better than enzyme II does.

Yeast RNA polymerase was first studied by Frederick et al. (11), who probably fractionated only the relatively stable enzyme II activity (cf. 21). Separate peaks of RNA polymerase activity from yeast were first separated on DEAE-Sephadex by Roeder (22), and a number of recent reports over the past year have described similar fractionations (1, 6, 8, 9, 19, 20; J. Sebastian, et al., Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 162, 1972). The most complete data available describe the larger quantities of enzymes isolatable from whole yeast (1, 8, 20). It is difficult to exclude the possibility of contamination of such preparations by enzymes of mitochondrial or cytoplasmic origin, and this is, to a lesser extent, also true of preparations derived from yeast nuclei, since such nuclei are difficult to obtain and especially difficult to separate from other cellular components such as mitochondria and ribosomes. Luckily, the mitochondrial RNA polymerases of yeast appear to be either unstable to DEAE chromatography or relatively difficult to solubilize (24, 29). However, it is possible that nascent mitochondrial RNA polymerase, produced in the nucleus, may exist in soluble cytoplasmic form (cf. 2). It is also possible that the true mitochondrial polymerase is rifampicin sensitive (24), although this is disputed (3). A pattern of two or three activities is found by several laboratories and can probably be ascribed to the yeast nucleus.

Dezeele et al. (8, 9) obtained only two peaks of RNA polymerase activity from whole yeast on DEAE-cellulose. Brogt and Planta (6), starting with yeast nuclei, separated three peaks on DEAE-cellulose and Ponta et al. similarly obtained three peaks from whole yeast on DEAE-Sephadex (19), but only two peaks on DEAE-cellulose (20).

Adman et al. (1) found four peaks of RNA polymerase activity on DEAE-Sephadex chromatography of extracts of whole yeast cells. Two α-amanitin-resistant peaks (Ia, Ib) preceded the sensitive enzyme II peak. It is possible that Ia may be derived from Ib by proteolysis. Wintersberger (21) incorporates saturating phenylmethylsulfonyl fluoride (PMSF, an inhibitor of serine proteases) in all of his fractionation buffers, and does not see peak Ia. All workers agree that enzyme II is at least five times as efficient with denatured template as with native template, whereas the ratio for enzymes I and III is closer to unity. All fractions are resistant to rifampicin.

Enzyme II has been extensively purified by several groups (8, 9, 20; Sebastian et al., Fed. Proc. 32:646, 1973), and all find similar sodium dodecyl sulfate gel electrophoresis patterns. The major subunits have mobilities corresponding to molecular weights of about 185,000 and 135,000. Enzyme I has been purified to a similar extent by Wintersberger et al. (20) and Sebastian et al. (Fed. Proc. 32:646, 1973), and both find major subunits of about 175,000 and 145,000 daltons, clearly distinct from those of enzyme II.

More recently, DiMauro et al. (10) have reported the existence of a heat-stable protein "factor" in yeast which stimulates transcription from native templates by all four polymerase fractions, apparently without altering the template (e.g., by nicking it). This factor is not specific and also stimulates transcription by rat liver polymerases I and II and by Escherichia coli polymerase. It does not stimulate transcription of denatured templates. Sebastian et al. (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 159, 1973) report that enzyme I specific activity is inversely proportional to the division time in yeast, consistent with its probable role in ribosomal RNA synthesis.

My fractionation procedures and the properties of the enzymes I obtain most closely resemble those of Ponta et al. (20).

MATERIALS AND METHODS

DEAE-cellulose (Whatman DE-52) was obtained from Reeve-Angel, Clifton, N.J., and DEAE-Sephadex (A-25-120) was obtained from Sigma Chemicals, St. Louis, Mo. Phenylmethylsulfonyl fluoride was obtained from Calbiochem, Los Angeles, Calif. Poly(dA·dT) (copolymer of alternating dA and dT residues, $s_{20w} =$ 11) was obtained from Miles Research Laboratories, Elkhart, Ind. Salmon sperm
(type III) and calf thymus (type I) DNAs, triphosphates of adenosine, cytidine, uridine, and guanosine (ATP, CTP, UTP, and GTP, respectively), Tris(hydroxymethyl)aminomethane (Tris), and bovine serum albumin were obtained from Sigma Chemicals, St. Louis. Mo. Thiolutin was a gift from Julian Davies. Ammonium sulfate was obtained from Mann, Kansas, Ill. T2 phage DNA was a gift from I. K. Bendis. α-amanitin was a gift from T. Weiland.

Yeast strains and media. The Saccharomyces cerevisiae strain used in all experiments reported here was a derivative of the haploid strain A 364A of Hutchison and Hartwell (13) and, like its parent, was phenotypically ade-1 genotype, presumably through a second mutation in the adenine pathway. Similar data have been obtained by using ribonuclease “3, a mutant of strain Y226, which is deficient in ribonuclease activity (B. Littlewood, and J. Davies, Genetics 68:539, 1971). Both strains were grown at 30°C in YPD broth containing per liter, 10 g of yeast extract (Difco), 20 g of peptone (Difco), and 20 g of D-glucose (Dextrose, Difco). The glucose was separately sterilized as a 50% solution. Doubling time in exponential growth phase was 2 h.

S. cerevisiae A 364A was also grown at 30°C in a synthetic medium (13) whose components were filter-sterilized. They comprised, per liter, yeast nitrogen base (6.7 g; Difco), D-glucose (20 g), adenine (0.01 g) uracil (0.01 g), L-lysine (0.04 g), and L-tyrosine (0.04 g). Doubling time in exponential growth phase was 3.5 h.

Preparation of crude enzyme extract. Yeast cultures, growing exponentially at about one-third maximal growth yield (Klett, red filter = 120 in YPD, Klett = 70 in synthetic medium) were chilled by addition of ice and harvested at 4 C by centrifugation for 7 min at 3,000 × g. Unwashed cell pellet (20 g), breakage buffer (20 ml), and 80-ml glass beads (0.35 mm, RHO Scientific, Commack, N.Y.) were mixed at 0 C in the 100-ml cup of a Buhler mill (RHO Scientific, Commack, N.Y.), and the pH was adjusted to 7.9 at 0 C. Breakage buffer contained 20% glycerol, 100 mM Tris-hydrochloride, pH 7.9 at 4 C, 20 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 0.3 mM PMSF. The Buhler mill was cooled with circulating ethylene glycol at 0 C. After breakage for 8 min at maximum speed (final temperature of homogenate, 4 to 8 C), beads were removed by filtration through glass wool. The pH of the filtrate was adjusted to 7.9 at 0 C, and the filtrate was sonified for 30 s at one-half maximum power with a Branson model J17 V sonifier with a 0.5 in. (approximately 1.25 cm) step horn. The filtrate was then centrifuged for 1 h at 200,000 × g, and the pellet was resuspended in 10 ml of breakage buffer containing 0.4 M ammonium sulfate and sonified and centrifuged as before. The two supernatant fluids were combined (approximately 40 ml) and immediately frozen at −80°C or fractionated on DEAE-cellulose.

DEAE-cellulose and DEAE-Sephadex chro-

matography. Whatman DE-52 cellulose was washed at 25°C in 0.5 N HCl, water (three times), 0.5 N NaOH and water (three times), and then suspended at 0 C in 0.1 M Tris-hydrochloride (pH 7.9 at 4 C). After adjusting the pH of the suspension to 7.9, it was resuspended in TGME buffer (22) containing 50 mM Tris-hydrochloride, pH 7.9 at 4 C, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT, and 25% glycerol. The suspension was packed into a column of 100 ml bed volume and 30-cm height, and equili-

brated with TGME buffer (1 liter) at 4 C.

Crude yeast enzyme extract derived from 10 g (wet weight) of yeast was diluted with TGME buffer to 50 ml [approximately 0.04 M (NH₄)₂SO₄] and applied to the column at 0.5 ml/min. The column was then eluted at 0.75 ml/min with a 600-ml linear gradient of 0.05 to 0.6 M (NH₄)₂SO₄ in TGME buffer containing 0.05 mM PMSF. Fractions (7.5 ml) were collected and assayed for RNA polymerase activity and total protein content (18). Sephadex A-25 was hydrated overnight in water and then treated as above. The elution procedure was identical.

Assay for RNA polymerase activity. Samples (40 μl) of column fractions were incubated for 30 min at 30°C in a 100-μl assay containing 30 mM Tris-hydrochloride, pH 7.9 at 25°C, 1.6 mM MgCl₂, 0.8 mM K₂HPO₄, 0.1 mM EDTA, 0.4 mM DTT, 0.2 mM ATP, GTP, and CTP, 0.01 mM UTP containing 1.1 × 10⁻⁴ disintegrations per min of [3H]UTP (Amersham-Searle), 0.2 mg of bovine serum albumin per ml, and 0.2 mg of alkali-denatured salmon sperm DNA. The assay also contained 20 mM Tris-hydrochloride, 2 mM MgCl₂, 10% glycerol, and (NH₄)₂SO₄ derived from the 40-μlter samples of column frac-

tions. Tests of antibiotic sensitivity used 20 μlter of fractions with maximal polymerase activity. The extent of reaction with either enzyme was linearly dependent on the volume of peak fraction added over the range of 5 to 25 μlter per assay. After incubation, samples (90 μlter) of the assay mixtures were spotted on 2-cm squares of DEAE paper (Whatman DE-81). After 10 min at 25°C, these papers were washed at 25°C for 5 min, four times in 5% Na₂HPO₄, and twice in water (4). After rinsing in 95% ethanol and ether, the dry papers were counted in toluene scintillator (27) in a Nuclear Chicago Isocap 300 scintillation counter. Under these conditions, incorpo-

ration of 55 counts/min of [³H]CUTP corresponds to incorpo-

ration of 1 pmol of uridine. Specific activities are presented in units of micromoles of uridine incorpo-

rated per minute at 30°C per milligram of enzyme protein.

Templates. Native templates and poly(dA-dT) were dissolved at 2 mg/ml in 50 mM NaCl, 10 mM Tris-hydrochloride, pH 8, 0.1 mM EDTA, and stored at 2°C over CHCl₃. Salmon sperm DNA was dena-

tured by incubating for 5 min at 22°C in 0.1 M NaOH, followed by neutralization and storage as above. High-molecular-weight yeast DNA was a gift from M. Bhargava.

RESULTS

Enzyme fractionation on DEAE-cellulose.
The result of a typical DE-52 fractionation is shown in Fig. 1. Only two peaks of activity, called enzymes I and II in order of elution, were found, and only enzyme II was sensitive to α-amanitin, as reported by Dezelee et al. (9), Sebastian et al. (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 162, 1972), and Ponta et al. (20). No second peak of α-amanitin-resistant activity eluted after the α-amanitin-sensitive peak II. A small peak of activity, frequently seen on the tail of the enzyme II peak, was always α-amanitin sensitive (Fig. 1).

The patterns of activity and protein concentration were reasonably reproducible and rechromatography of either peak of activity on the same column resulted in elution of a single peak of activity at the original elution volume. Different preparations had fairly constant peak-specific activities of enzyme II (1 to 2 mU per mg of protein) but had more variable peak-specific activities of enzyme I (0.07 to 0.3 mU per mg of protein). The routine breakage procedure involves sonic disruption first in low salt and then in high salt (see Materials and Methods). Fractionation of the two supernatant fluids separately showed that the high-salt extract contained about 30% of the enzyme I activity and 10% of the enzyme II activity. The procedure chosen reduces the volume of the diluted extract which is applied to the DEAE-
cellulose column, but similar results were obtained when the original breakage was performed in 0.4 M (NH₄)₂SO₄. Further purification has not been attempted.

**Enzyme fractionation on DEAE-Sephadex.** In contrast to the consistent recovery of only two peaks of RNA polymerase activity on DE-52, fractionation on A-25 gave three peaks, called I, II, and III in order of elution, with only enzyme II sensitive to α-Amanitin (Fig. 2). The pattern of total protein in the eluate resembled that seen on DE-52. Peak-specific activities of enzymes I, II, and III were 0.13, 0.9, and 0.33 mU per mg of protein, respectively.

**Properties of RNA polymerases.** Requirements: All enzymes are dependent on added template. Only enzyme I off, A-25 has significant endogenous activity (Table 1). At constant UTP-specific radioactivity but variable UTP concentration, the rate of incorporation catalyzed by enzyme II was half-maximal at about 1.5×10⁻⁴ M. The standard assay contains 1.10⁻³ M UTP.

Divalent metal ion optima: By using saturating (0.2 mg/ml) native or denatured salmon sperm DNA, optimal divalent metal ion concentrations were determined for RNA polymerase activity. Other components and procedures were as in the standard assay (see Materials and Methods, 20-μl enzyme sample).

All enzymes functioned best with Mn²⁺ (in the presence of 1 mM Mg²⁺ derived from the enzyme sample). The data for enzymes I and II (DE-52) and III (A-25) are given in Table 2. At optimal Mn²⁺ concentrations, the relative
Table 1. Requirements for RNA polymerase activity

<table>
<thead>
<tr>
<th>Mix</th>
<th>Enzyme I</th>
<th>Enzyme II</th>
<th>Enzyme III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100/100</td>
<td>100/100</td>
<td>100/100</td>
</tr>
<tr>
<td>Minus DNA</td>
<td>3/13</td>
<td>4/2</td>
<td>2</td>
</tr>
<tr>
<td>Minus CTP</td>
<td>19/22</td>
<td>20/34</td>
<td>35</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>20/11</td>
<td>15/16</td>
<td>20</td>
</tr>
<tr>
<td>Minus CTP and ATP</td>
<td>13/13</td>
<td>10/11</td>
<td>17</td>
</tr>
<tr>
<td>Counts per minute (complete)</td>
<td>1,800/1,180</td>
<td>7,700/5,450</td>
<td>1,750</td>
</tr>
</tbody>
</table>

*The complete assay contained 200 μg of denatured salmon sperm DNA per ml, 0.2 mM ATP, GTP, and CTP, 1.6 mM Mn++, and 40 μl of enzyme, as described in the standard assay. Data are presented as percentage of incorporation in the complete mix. The actual counts per minute incorporated (100%) are given in the bottom row.

Table 2. Optimal divalent metal ion concentrations

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metal</th>
<th>Template</th>
<th>Relative specific activity</th>
<th>Optimal concn (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mn²⁺</td>
<td>Native</td>
<td>85</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Mg²⁺</td>
<td>Native</td>
<td>30</td>
<td>5−8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>25</td>
<td>4−6</td>
</tr>
<tr>
<td>II</td>
<td>Mn²⁺</td>
<td>Native</td>
<td>16</td>
<td>2−10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Mg²⁺</td>
<td>Native</td>
<td>6</td>
<td>3−8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>10</td>
<td>3−8</td>
</tr>
<tr>
<td>III</td>
<td>Mn²⁺</td>
<td>Native</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>100</td>
<td>3−6</td>
</tr>
<tr>
<td></td>
<td>Mg²⁺</td>
<td>Native</td>
<td>10</td>
<td>5−10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured</td>
<td>14</td>
<td>5−10</td>
</tr>
</tbody>
</table>

*All assays contain 0.2 mg of native or denatured salmon sperm DNA per ml and 1 mM Mg²⁺ in addition to the above components.

specific activities on native versus denatured salmon sperm DNA with optimal concentrations of 75 to 200 μg/ml. However, at lower template concentration (10 to 20 μg/ml), patterns were somewhat different (Table 3). Enzymes I and III function best with their natural template at these concentrations, which appear to be saturating for both natural template and poly(dA-dT).

Kinetics of RNA synthesis: When enzyme II was incubated under the standard assay conditions (see Materials and Methods) and samples of the mixture were removed at intervals for determination of RNA synthesized, identical data were obtained by trichloroacetic acid precipitation followed by filtration and by the DEAE-paper procedure described in Materials and Methods. However, it was necessary to presoak the paper with 50 μl of 2 M acetic acid. When untreated DEAE paper was used, counts were higher in proportion to the time of incubation on paper before washing. Thus, some synthesis occurs after spotting an incubation mixture onto untreated DEAE paper. Acetic acid-presoaked DEAE paper was used for all kinetic experiments. Under these conditions, the kinetics of ¹⁴C-uridine incorporation by enzymes I and II were similar. At 22 C, curves were obtained indicating a decay of 50% in rate about every 20 min (Fig. 3). Addition of new enzymes (but not addition of new template) stimulated the reaction. Prolonged incubation at 30 C resulted in an eventual decay of recoverable radioactivity in the standard assay. The curves, therefore, probably represent both enzyme inactivation and product destruction by nuclease action.

Inhibition of enzymes I and II by thiolutin and α-amanitin. Under the standard assay conditions (Materials and Methods), enzymes I and II are not particularly sensitive to thiolutin added at zero time to a complete assay mixture containing enzyme and template, whereas much greater sensitivity is seen when enzyme is

Table 3. Template efficiencies

<table>
<thead>
<tr>
<th>Template</th>
<th>Amt of template (μg/ml)</th>
<th>Relative sp act of enzyme (I, II, III) on</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE-52</td>
<td>A-25</td>
<td>I</td>
</tr>
<tr>
<td>d salmon sperm</td>
<td>20 100 100 100 100 100</td>
<td>100</td>
</tr>
<tr>
<td>n salmon sperm</td>
<td>20 64 18 70 29 83</td>
<td>100</td>
</tr>
<tr>
<td>n calf thymus</td>
<td>20 40 10 48 15 52</td>
<td>100</td>
</tr>
<tr>
<td>poly(dA-dT)</td>
<td>10-40 110 73 130 63 95</td>
<td>100</td>
</tr>
<tr>
<td>n yeast</td>
<td>10-20 160 50 350 70 160</td>
<td>100</td>
</tr>
<tr>
<td>n T2</td>
<td>40 26 8 24 12 28</td>
<td>100</td>
</tr>
</tbody>
</table>

*All assays contained 20 μl of enzyme fraction, 1 mM Mg²⁺, and 1.6 mM Mn++, and were performed under the standard assay conditions (see Materials and Methods).

*n, Native; d, denatured.
preincubated with thiolutin before the addition of template (14a). At 30 C in an assay mixture containing ATP but no DNA or other nucleotides, the kinetics of RNA synthesis by enzymes I and II in controls were independent of the time of addition of dimethyl sulfoxide (DMSO) (at -5 or 0 min, Fig. 4), whereas preincubation of enzyme in the absence of template with 40 \( \mu \)g of thiolutin per ml for 5 min at 30 C caused a 90 to 95% drop in the subsequent rate of RNA synthesis (Fig. 4). However, when enzyme and DNA were preincubated, addition of thiolutin at 0 min resulted in only a gradually increasing inhibition of RNA synthesis, and the same gradual imposition of inhibition was also seen when thiolutin was added after a period of 4 min of RNA synthesis (Fig. 4A). The addition of 10 \( \mu \)g of \( \alpha \)-amanitin per ml at +4 min to enzyme I had little effect, but the addition of 10 \( \mu \)g of \( \alpha \)-amanitin per ml to enzyme II at +4 min caused an immediate 90% inhibition of the rate of RNA synthesis (Fig. 4).

It is concluded that enzyme which has been preincubated with ATP and DNA, or enzyme which is in the process of active RNA synthesis, is insensitive to 40 \( \mu \)g of thiolutin per ml for several minutes at 30 C. Thiolutin is thus a poor inhibitor of elongation of preinitiated RNA sequences, in contrast to the effect of \( \alpha \)-amanitin on enzyme II.

The effects of thiolutin on the early kinetics of RNA synthesis were further studied by reducing the temperature to 22 C and taking more frequent samples. Enzyme III was also included in this experiment and the ATP was eliminated from the preincubation so that formation of any internucleotide bonds (cf. 26) should have been prevented. Again, the two controls containing only DMSO were almost indistinguishable (Fig. 5), and whereas preincubation of enzyme and thiolutin caused a 95% inhibition of the subsequent rate of RNA synthesis, preincubation of enzyme and DNA resulted in marked protection of the enzyme. It appears that enzyme is only immediately sensitive to thiolutin in the absence of template, even when no nucleotide triphosphates are present. When DNA and

![Graph](http://jb.asm.org/)

**Fig. 3.** Kinetics of RNA synthesis by DE-52 enzymes I and II at 22 C. Data from nine separate experiments are presented; open circles, enzyme I; filled circles, enzyme II. Standard assay conditions were used with 100 \( \mu \)g of denatured salmon sperm DNA per ml and 20 \( \mu \)l of enzyme fractions.

![Graph](http://jb.asm.org/)

**Fig. 4.** Effect of \( \alpha \)-amanitin and thiolutin on the kinetics of RNA synthesis by enzymes I and II. Enzymes were preincubated for 5 min at 30 C under the standard assay conditions (see Materials and Methods) except for the absence of UTP, CTP, and GTP, and, where indicated, of template (100 \( \mu \)g of denatured salmon sperm DNA per ml). At zero time, tubes were transferred to 0 C, nucleotides (including \( ^{14} \)C-UTP) and template (if required) were added, and incubation was continued at 30 C. Samples were removed at intervals and spotted on acidified DEAE paper for determination of RNA synthesized. The final thiolutin concentration was 40 \( \mu \)g/ml, in 2% DMSO. Controls contained only DMSO. Sequences of additions were as follows: DNA at -5 min, then DMSO and nucleotides at zero time (○); DMSO at -5 min, then DNA and nucleotides at zero time (○); DNA at -5 min, then thiolutin and nucleotides at zero time (△); thiolutin at -5 min, then DNA and nucleotides at zero time (-△-); DNA and nucleotides at zero time, then thiolutin at +4 min (arrow) (△); DNA, DMSO, and nucleotides at zero time, then \( \alpha \)-amanitin (final concentration, 10 \( \mu \)g/ml) at +4 min (○).
zyme, and template. By were that enzyme DNA at or enzyme and directly and already to time. Thus, 6). When thiolutin protected in denatured could, therefore, in incomplete mixtures, devoid of all nucleotides, were incubated for 10 min at 22 C. All missing components were added at zero time at 0 C, and incubation was continued at 22 C. RNA synthesized was determined at intervals. Sequences of additions were as follows: enzyme and DNA at -10 min, then DMSO and nucleotides at zero time ( ); enzyme and DMSO at -10 min, then DNA and nucleotides at zero time ( ); DNA and thiolutin at -10 min, then enzyme and nucleotides at zero time ( ); DNA and enzyme at -10 min, then thiolutin and nucleotides at zero time ( ); enzyme and thiolutin at -10 min, then DNA and nucleotides at zero time ( ).

Thiolutin were preincubated before addition of enzyme and nucleotides at zero time, the inhibition by thiolutin was barely enhanced. Clearly, thiolutin does not exert its effect by binding directly to template.

Kinetics of interaction of thiolutin, enzyme, and template. The data in Fig. 5 suggest that enzyme mixed at 0 C with template is already protected against the effect of thiolutin added immediately afterwards. When enzyme I or II was preincubated with native or denatured DNA at 22 C before simultaneous addition of nucleotides and thiolutin, the quantity of RNA synthesized was essentially independent of the time of preincubation with DNA (Fig. 6) and in each case corresponded to about 15% inhibition. When thiolutin and nucleotides were added shortly before DNA (-0.1 min), there was some increase in inhibition, and 4 min prior exposure to thiolutin resulted in 50 to 60% inhibition (Fig. 6). Thus, enzymes I and II are immediately protected against thiolutin by addition of native or denatured DNA.

The interaction of thiolutin and enzymes could, therefore, be effectively blocked by addition of template. Enzyme I or II was preincubated for varying times at 22 C in the presence of 20 µg of thiolutin per ml followed by addition of DNA and nucleotides at zero time. Synthesized RNA was assayed after incubation for an additional 30 min at 22 C (Fig. 7). Zero-time points are controls in which thiolutin was added immediately after DNA and before nucleotides, giving 17 and 14% inhibition of enzymes I and II, respectively. In the absence of template, 20 µg of thiolutin per ml caused 30 to 40% inhibition of both enzymes within 15 s, followed by a slower second phase resulting in 90% inhibition by 10 min. The inhibitory effect on enzyme I was more rapid, but the interaction of both enzymes with thiolutin at 22 C in the absence of template had a slow component. In the presence of template (100 µg of native or denatured salmon sperm DNA per ml), thiolutin at 20 µg/ml still inactivated enzyme II, but at a much slower rate, the half-life of activity being 14 min at 22 C with either template (see Fig. 7). This rate of decay is consistent with the data in Fig. 5.

In the absence of template, enzyme I activity
Nucleotides were and UTP DNA and rate C. 22 enzymes DMSO, decayed II native (20% at 22°C). vals salmon of added was also in the absence of ATP, decayed in template, whereas template, in the presence or absence of ATP, protected the activity somewhat (Fig. 8). In the absence of template, 20 µg of thiolutin per ml rapidly inactivated enzyme II at 30°C, whereas the half-life of enzyme activity in the presence of template, with or without ATP, was increased to 5 min (Fig. 8). This half-life is consistent with the decay of activity seen in Fig. 4A and shows that the presence of template merely retards the action of thiolutin at this concentration.

Effect of varying enzyme and template concentrations on the inhibitory effect of thiolutin. Enzyme II (20 µl) was preincubated for 5 min at 22 C with thiolutin (10 µg/ml in 1% DMSO) or 1% DMSO alone, followed by addition of nucleotides and varying amounts of denatured salmon sperm DNA at zero time. Polymerized RNA was assayed after incubation for an additional 30 min at 30 C (Fig. 9). The quantity of DNA synthesized, both in the presence and absence of thiolutin, was maximal at 80 µg of DNA per ml or greater. However, the extent of inhibition by thiolutin (average 54%) was independent of the concentration of DNA.

Enzyme II (10 to 40 uliters) was preincubated for 5 min at 22 C with thiolutin (10 µg/ml) or DMSO alone, followed by addition of nucleo-

![Graph](http://jb.asm.org/)

**Fig. 7.** Kinetics of interaction of thiolutin with enzymes I and II at 22 C. Enzyme I or II (20 uliters) was preincubated in the absence of nucleotides and DNA at 22 C. At zero time, nucleotides (including 14C-UTP) and DNA (final concentration, 100 µg of denatured salmon sperm DNA per ml) were added. RNA synthesis was assayed after 30 additional min at 22 C. DMSO alone (1% final concentration) or thiolutin (20 µg/ml in 1% DMSO, final concentration) was added to the incubations at the indicated intervals before zero time. “0 min” preincubation with thiolutin corresponds to incubations which received thiolutin 10 s after nucleotides and DNA. Enzyme I with DMSO, Δ; with thiolutin, ▲. Enzyme II with DMSO, O; with thiolutin, ●. Enzyme II (20 µl) was also preincubated in the absence of CTP, GTP, and UTP but in the presence of ATP and DNA (100 µg of denatured or native salmon sperm DNA per ml) and thiolutin (20 µg/ml) for the indicated times. Nucleotides were added at zero time, and RNA synthesis was assayed as before. Denatured DNA, □; native DNA, ■.

decayed slowly in 1% DMSO at 22 C, and enzyme II decayed more slowly still (Fig. 7). This rate of decay of enzyme II was accentuated at 30 C, although template, in the presence or absence of ATP, protected the activity somewhat (Fig. 8). In the absence of template, 20 µg of thiolutin per ml rapidly inactivated enzyme II at 30°C, whereas the half-life of enzyme activity in the presence of template, with or without ATP, was increased to 5 min (Fig. 8). This half-life is consistent with the decay of activity seen in Fig. 4A and shows that the presence of template merely retards the action of thiolutin at this concentration.

**Fig. 8.** Kinetics of interaction of thiolutin with enzyme II at 30 C. Enzyme II (29 µl) was preincubated at 30 C in the absence of nucleotides or DNA and in the presence of DMSO (1% final concentration, ▼) or thiolutin (20 µg/ml final concentration in 1% DMSO ▼). After the indicated times, DNA (100 µg/ml, final concentration of denatured salmon sperm DNA) and nucleotides (including 14C-UTP) were added. RNA synthesis was assayed after an additional 10 min at 30 C. In similar experiments, enzyme II was preincubated with DMSO (Δ) or thiolutin (▲) in the absence of nucleotides but in the presence of DNA, and enzyme II was also preincubated with DMSO (〇) or thiolutin (●) in the absence of CTP, GTP, and UTP but in the presence of DNA and ATP.

**Fig. 9.** Inhibition of enzyme II by thiolutin: effect of varying enzyme and DNA concentrations. Enzyme was preincubated for 5 min at 22 C with 10 µg of thiolutin per ml (〇) or with DMSO alone (●). DNA (denatured salmon sperm) and nucleotides were added at zero time, and synthesized RNA was assayed after a further 30 min at 30 C. The ratio of these data gives the percentage of inhibition by thiolutin (▼). A, Constant enzyme (20 µl) and variable DNA; B, constant DNA (200 µg/ml) and variable enzyme.
tides and DNA (200 μg of denatured salmon sperm DNA per ml) at zero time. Polymerized RNA was assayed after incubation for an additional 30 min at 30 C (Fig. 9). The quantity of RNA synthesized in the control was proportional to enzyme content up to 30 μlitters and the extent of inhibition with thiolutin was inversely proportional to enzyme concentration in this same range. In combination with the dependence of thiolutin inhibition on preincubation with enzyme rather than with DNA, this indicates that the primary effect of thiolutin in vitro is on the enzyme and not on the template. Similar data have been obtained with enzyme I.

Response of enzymes I, II, and III to different concentrations of thiolutin and α-amanitin. When enzyme I, II, or III was preincubated with template in the absence of nucleotides, subsequent addition of even 75 μg of thiolutin per ml failed to inhibit greater than 25% of the RNA synthesis over a period of 30 min at 22 C (Fig. 10). However, when enzyme I, II, or III was preincubated with thiolutin in the absence of template for 10 min at 22 C (a time period giving minimal loss of enzyme activity in controls lacking thiolutin; Fig. 7), marked inhibition of RNA synthesis occurred on subsequent addition of DNA and nucleotides (Fig. 10). Under these conditions, enzymes I, II, and III were 50% inhibited by 3, 4, and 3 μg of thiolutin per ml, respectively. When preincubation was only for 1 min at 22 C, 50% inhibition required 7, 7, and 6 μg/ml, respectively, perhaps not as large a difference as predicted from the data in Fig. 7, but consistent with a relatively slow interaction of thiolutin and enzyme. It is apparent that all three enzymes behaved indistinguishably towards thiolutin in these experiments. The RNA polymerase activity of enzyme II over 30 min at 22 C was independent of the presence of the 0.4 mM DTT in the assay mixture. The enzyme fraction itself (20 μlitters) would contribute 0.02 mM DTT derived from the column fractionation buffer, or rather the residual unoxidized fraction of it. Under these conditions, enzyme II, after a 10-min preincubation with thiolutin, was still 50% inhibited by 4 μg of thiolutin per ml.

By contrast to thiolutin, the inhibitory effect of α-amanitin was independent of mixing order or of preincubation of enzyme and template (Fig. 11). Enzyme II was 50% inhibited by 1.5 μg/ml, whereas enzymes I and III were only 20% and 25%, respectively, inhibited by 60 μg/ml. It is apparent that there was no contamination of this preparation of enzyme I with activity having α-amanitin sensitivity of enzyme II.

whereas about 15% of the activity of the pooled enzyme III used in this experiment was probably due to contaminating enzyme II (see Fig. 3). It is also possible that this enzyme III preparation is heterogeneous and contains a unique α-amanitin-sensitive component.

DISCUSSION

Chromatography on DE-52 completely resolves two peaks of activity (I and II, Fig. 1)
which differ from each other in sensitivity to α-amanitin (Fig. 11) and in preference for denatured template. In particular, native high-molecular-weight yeast DNA was the best template for enzyme I, especially at lower DNA concentrations, whereas enzyme II was optimally active with high concentrations of denatured salmon sperm DNA (Table 3). These properties parallel those reported for similar fractions obtained by Dezeele et al. (8, 9), Ponta et al. (19, 20) and Adman et al. (1), except for the high efficiency of the natural yeast template. Another similarly prepared yeast DNA fraction had a much lower template efficiency. There was no sign of a third α-amanitin-resistant peak on numerous DE-52 fractionations; however, fractionation on A-25 gave such a peak (III) eluting after II. No early peak similar to the “Ia” of Adman et al. (1) was ever seen on either column. All fractions had similar divalent metal ion requirements (Table 2). Enzyme III is not distinguished clearly from enzyme I by any of the properties listed in this paper, and it remains possible that they are alternate aggregation forms. All fractions are relatively free of endogenous template and require all four ribonucleoside triphosphates for optimal activity (Table 1). RNA synthesis catalyzed by all fractions on native templates is at least 90% inhibited by 100 μg of Netropsin per ml, an intercalating agent with preference for AT-rich regions (cf. 28). At least 90% of the observed activity is thus DNA directed. The products have not been analyzed. All enzymes gave nonlinear kinetics over a 30- to 60-min incubation at 22 C (cf. Fig. 3), probably due to both enzyme inactivation (cf. Fig. 8) and product hydrolysis.

α-Amanitin, added to an incubation mix already synthesizing RNA at 30 C, has no effect on enzyme I but causes immediate cessation of enzyme II activity (Fig. 4). The sensitivity of enzyme II to α-amanitin is also independent of preincubation of enzyme and template (Fig. 11), and it can be concluded that α-amanitin inhibits elongation of preinitiated RNA chains catalyzed by enzyme I (cf. 15).

In marked contrast, thiolulin is an effective inhibitor of all three enzyme fractions and does not distinguish between them. The structure of thiolutin (7) is given in Fig. 12. The molecular weight is 230 and a 1 μg/ml solution is $4.3 \times 10^{-6}$ M. The structure apparently bears no more than a superficial resemblance to those of the nucleotide substrates of RNA polymerase. Possibly the disulfide bridge may be reduced in the presence of DTT; however, the presence of DTT is not required for activity. Other modifications of this structure, resulting in activation of thiolutin in vivo or in the crude enzyme fractions described in this paper, seem improbable but are not ruled out. An effect of thiolutin on template function is also improbable since the extent of inhibition is independent of the amount of template but is at least partially dependent on the enzyme content (Fig. 9). Also, the extent of inhibition is independent of the presence of native or denatured template or of preincubation of template and thiolutin (Fig. 7), whereas the extent of inhibition does depend on preincubation of enzyme and template. When incubated with any of the three enzymes in the absence of template at 22 C, thiolutin inactivates at least part of their activity rela-

![Fig. 12. The structure of thiolutin (7).](http://jb.asm.org/content/journal/jb/104/5/53123)
tively slowly (Fig. 7). Maximal inhibition of all three activities requires a 10-min preincubation of thiolutin and enzyme at 22 C. About twice as much thiolutin is required when the preincubation time is reduced to 1 min. When template is added before thiolutin, the interaction of thiolutin and enzyme is much slower and inhibition of RNA synthesis is complete only after 20 to 30 min at 30 C (Fig. 8). A similar time is required for inhibition of preinitiated RNA synthesis (Fig. 4). Thus, thiolutin does not inhibit elongation of preinitiated RNA chains, but may prevent the initiation of new chains by a slow inactivation of the fraction of enzyme which is not functionally interacting with template. Note that the protection of enzyme by template does not require the presence of nucleotides. Similarly, the rate of interaction of the E. coli RNA polymerase by rifampicin is 100 times slower in the presence than in the absence of template (12). In contrast, however, the E. coli enzyme is further protected by RNA chain initiation which ensues on addition of nucleotides (12, 26).

In conclusion, thiolutin inhibits in vitro RNA synthesis directed by all three fractions of yeast RNA polymerase, apparently by interacting with the enzymes. This implies the presence of a common critical binding site, and perhaps of a common subunit (or fragment of a subunit). Since enzymes I and II do not have high-molecular-weight subunits in common (20; Sebastian et al. Fed. Proc. 32:646, 1973), this would have to be a smaller subunit, unless the physically dissimilar larger subunits of enzyme I and II are derived from each other by proteolysis or by evolution, with conservation of the thiolutin-sensitive site. The immunological cross-reactivity of enzymes I and II (Sebastian et al. Fed. Proc. 32:646, 1973) is consistent with such an hypothesis. However, since thiolutin also inhibits the growth of a wide variety of bacterial species (25), it may also affect prokaryotic RNA polymerases and thus have a very broad specificity. This possibility is under investigation.

ACKNOWLEDGMENTS
This work was supported by National Science Foundation grant GB 18597.
I am greatly indebted to T. Wieland for the samples of a-amanitin, to J. E. Davies for the sample of thiolutin and for many useful discussions, and to Pamela Kesner for excellent technical assistance.

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