Characterization of a Novel Ribonucleotide-polymerizing Enzyme from a Fungus, *Histoplasma capsulatum* *

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Yeast-like cells of the pathogenic dimorphic fungus *Histoplasma capsulatum* have been found to contain a novel ribonucleotide-incorporating enzyme, which we have termed ribonucleotide polymerase. The enzyme, not detectable in the mycelial form of the fungus, is capable of incorporating any of the ribonucleoside triphosphates into short oligonucleotide chains. Ribonucleotide polymerase has an absolute requirement for Mn$^{2+}$ as the divalent cation; Mg$^{2+}$ cannot replace Mn$^{2+}$ at any concentration tested (up to 9.6 mM). DNA template is not required. Activity is inhibited by pyrophosphate but not by phosphate ions. Heparin and rifamycin AF/013 are strongly inhibitory, while actinomycin D and α-amanitin have no effect on the enzyme.

Ribonucleotide polymerase shows a rapid incorporation of ribonucleoside triphosphates into oligonucleotide product. The reaction reaches a plateau within 20 to 30 min, with little additional incorporation upon extended incubation. The cessation of incorporation is due to inhibition by the product which remains tightly bound to the enzyme.

Ribonucleotide polymerase has been purified by two glycerol gradient centrifugations and treatment with ribonuclease T1. The purified enzyme (estimated molecular weight of 900,000) yields a single band upon electrophoresis on polyacrylamide gels under nondenaturing conditions. Electrophoresis under denaturing conditions reveals two bands of protein with estimated molecular weights of 73,000 and 69,000 present in approximately 1:1 molar ratio.

Ribonucleotide polymerase makes the fungus an attractive model organism for the study of cell differentiation. During purification of RNA polymerases of *H. capsulatum* we have discovered a DNA-independent enzyme capable of utilizing any one of the four individual ribonucleoside triphosphates for the synthesis of oligonucleotides. The enzyme is found only in the yeast form of *H. capsulatum*. Here we describe the partial purification and properties of this enzyme which we have provisionally called ribonucleotide polymerase.

A preliminary account of this work has been presented.

**EXPERIMENTAL PROCEDURES**

Organism - *Histoplasma capsulatum*, Downs strain, mating type (+) was obtained from Dr. George Kobayashi, Washington University School of Medicine, St. Louis. The cells in the yeast or mycelial phase were cultivated as described previously (11, 14), except that 2% glucose, 1% yeast extract) at an initial density of 1.5 × 10⁶ cells/ml.

Biochemicals - The following materials were purchased from Sigma Chemical Co.: unlabeled ribonucleoside triphosphates, calf thymus DNA type I, yeast RNA and Escherichia coli tRNA, ribonuclease A, T1, and T2, deoxyribonuclease I, pronase, ovalbumin, catalase, actinomycin D, heparin, and α-amanitin. E. coli RNA

1. G. Boguslawski and W. A. Zehring (1976) Abstracts of the *Meeting of the American Society for Microbiology*, Atlantic City, New Jersey, May 3 to 7, 1976 Abstr. H 84, p. 110.
polymerase and homoribopolymers (polyU, polyC, polyG, and poly(A)) were purchased from Miles Laboratories. 3'-H-labeled nucleotides and [γ-32P]GTP were from Schwartz/Mann and Amersham/Scarse. Polyacrylamide gel reagents were obtained from Bio-Rad and DEAE-cellulose was from Roche. Destained thin layer chromatography plates were purchased from Brinkmann. Wheat germ ribosomal RNA was from Calbiochem, and β-galactosidase from Worthington. Rifampycin AF/013 was a gift from Dr. R. Weaver, University of Kansas.

Solubilization of RNA Polymerase and Ribonucleotide Polymerase-The enzymes from the yeast cells were extracted as described (11) with minor modifications. (The same procedure yielded mycelial extracts containing only traces of either enzyme.) The cells were disrupted with glass beads (0.45 mm diameter) and centrifuged at 9,000 × g to remove the beaded debris, and the supernatant was treated with solid ammonium sulfate. The precipitate was dissolved in a small volume of Buffer A (0.05 M Tris/HCl (pH 7.85), 25% glycerol (v/v), 0.6 mM EDTA, 0.5 mM dithiothreitol, and 5 mM MgCl2) and centrifuged for 60 min at 42,500 rpm in a Spinco type 50 rotor. The supernatant was passed through a Sephadex G-25 column equilibrated with Buffer A containing 0.025 M ammonium sulfate. The gradient was centrifuged for 56 h at 25,000 rpm in a Spinco SW 27 rotor. For analytical purposes, 1-ml samples were layered on 10-ml gradients and centrifuged for 15 h at 39,000 rpm in a Spinco SW 41 rotor. Thirty to thirty-eight fractions were collected from the bottom of each gradient. Fractions with the highest ribonucleotide polymerase and RNA polymerase activity were pooled and used in subsequent experiments.

Assay for RNA Polymerase-Assays were performed in tubes (6 x 59 mm) in a final volume of 125 μl. The following components were usually premixed in each tube in a volume of 44 μl: 5 μM of Tris/HCl (pH 7.85), 0.2 μM of MnCl2, 0.065 μM each of unlabeled ATP, GTP, and CTP; 12.5 μg of native calf thymus DNA; the appropriate tube received 25 μl of Buffer B (0.06 M Tris/HCl (pH 7.85), 5% glycerol (v/v), 0.5 mM EDTA, and 0.5 mM dithiothreitol) which was made 0.12 M with ammonium sulfate. For preparative purposes, 2.5 to 3.0-ml samples of the dialyzed HSE fraction were then layered directly on top of 30-ml volumes of 5% Buffer B containing 0.12 M ammonium sulfate. The gradients were centrifuged for 15 h at 39,000 rpm in a Spinco SW 41 rotor. Thirty to thirty-eight fractions were collected from the bottom of each gradient. Fractions with the highest ribonucleotide polymerase and RNA polymerase activity were pooled and used in subsequent experiments.

**Polyacrylamide Gel Electrophoresis—** Electrophoresis under denaturing conditions was carried out on either 23 or 4% gels prepared according to procedures described by Maizel (17) in tubes (7.5 x 0.5 cm). Each tube contained 1.1 ml of separating gel (37.5% acrylamide, 0.1% bisacrylamide) and 0.3 ml of stacking gel (37.5% acrylamide, 0.1% bisacrylamide). After removal of excess ammonium persulfate, 0.3 ml of sample gel containing 2.5% total acrylamide and 1.0% bisacrylamide was layered on top of the separating gel. The ribonucleotides polymerase fraction was layered on top of the separating gel and allowed to polymerize in light for 60 min. The electrophoresis was then carried out at 2.5 mA/gel until the tracking dye reached the bottom of the gel. The gels were stained overnight for protein with 0.05% Coomassie brilliant blue and destained electrophoretically. Stained gels were scanned at 500 nm in an EC 910 densitometer (E-C Apparatus Corp.). Ribonucleotide polymerase activity in gels was measured as follows. After electrophoresis under denaturing conditions, a gel slice was excised and used as a source of radioactive protein. The gel slice was placed on Whatman DE81 paper, washed twice with water, dried, and placed on two filters. One of the filters was placed in a single scintillation vial and radioactivity was determined. In this manner, [3H]guanosine and [3H]GMP could be separated with ease (respectively Rf values: 0.4 to 0.5 and 0.7 to 0.8).

Ribonucleotide Polymerase Activity Reaction Products—A column of DEAE-cellulose (Whatman DE52) prepared according to procedures described as below was used for all four nucleotides triphosphates and the corresponding oligonucleotides in the manner described above (15).

**DEAE-cellulose and Thin Layer Chromatography of Ribonucleotide Polymerase Reaction Products—** A column of DEAE-cellulose (Whatman DE52) was prepared and used exactly as described by Colombo and Stone (16). A sample of ribonucleotide polymerase (300 μl) was incubated in a total volume of 375 μl with 1.6 mM MnCl2 and 6 μCi of [3H]UTP for 1 h at 23°. The reaction was terminated by the addition of 5 μl of RNase-free pronase solution (5 mg/ml), and the incubation was continued for additional 45 min. At that time, 175 μl of ice-cold chromatography buffer was added. The blockade of the radioactivity was removed with pronase, 0.004% RNase I (0.01 M Tris/HCl (pH 7.85), and 3 μM EDTA). The entire sample was mixed with oligonucleotide markers (wheat germ ribosomal RNA digested with ribonuclease A in 9.5 ml of ice-cold buffer, applied to a DEAE-cellulose column, and eluted with NaCl gradient as described (16). The absorbance at 260 nm and radioactivity were determined for each fraction. Material for thin layer chromatography was prepared as follows. A sample of ribonucleotide polymerase A (5000 μl) was incubated with 1.6 mM MnCl2 and 6 μCi of [3H]GTP in a total volume of 800 μl for 90 min at 23°. The reaction was terminated by the addition of 400 μl of ice cold 0.06 M Tris/HCl (pH 7.85), and the entire sample was loaded on a column (0.8 x 17 cm) of Sephadex G-50 equilibrated with 5% Buffer B. The ribonucleotide polymerase-product complex eluted in void volume, free of unreacted [3H]GTP and other low molecular weight contaminants. This material was treated with pronase (50 μg/ml) for 2.5 h at 37°, and then with RNase T1 (35,000 units/ml) and RNase T2 (74 μg/ml and 11 units/ml, respectively). The nucleolytic treatment was continued for 3 h at 37°. Following the hydrolysis, samples of 20 to 40 μl were analyzed with the guanosine and GMP standards were applied to EM cellulose (Celulose-P-25) plates (20 x 20 cm) and (15% acetic acid, 50% water) was used to elute the corresponding oligonucleotides in the manner described. The incorporated radioactivity was measured, and the corresponding oligonucleotides were pooled and used in subsequent experiments.

**Protein Determination—** Samples were precipitated with 10% trichloroacetic acid, pellets were dissolved in 0.1 N NaOH, and protein was determined by the Lowry procedure (18). Alternatively, a specific

2 The abbreviation used is: HSE, high speed enzyme.
Ribonucleotide Polymerase from H. capsulatum

Results

Solubilization and Glycerol Gradient Centrifugation—Solubilized extracts (HSE) were obtained from mycelial and yeast phase of Histoplasma capsulatum as described under "Experimental Procedures." Glycerol gradient centrifugation of the two extracts (Fig. 1) revealed that the yeast material contained high levels of both RNA polymerase and ribonucleotide polymerase activity, but the equivalent mycelial preparation showed only traces of either activity. The apparent lack of RNA polymerase activity in mycelia is probably due to the known presence in these cells of histin, an inhibitor of RNA polymerase (12, 14). Whether this also accounts for the absence of ribonucleotide polymerase activity is at present unknown.

As Fig. 1 shows, the yeast ribonucleotide polymerase activity can be detected in two regions of the gradient. The major peak, which we call ribonucleotide polymerase A, has a sedimentation constant of about 23 to 26 S (estimated molecular weight, 900,000). Most of our experiments employed this fraction. The small, light peak (ribonucleotide polymerase C, 6 to 8 S) appears consistently in all preparations. The properties of the light material are essentially the same as those of the heavy species, and it is possible that one is derived from the other (see "Discussion").

Purification Procedure—Ribonucleotide polymerase A was purified about 125-fold by two glycerol gradient centrifugations and treatment with RNase T1. The yield and purification at each step are summarized in Table I. The apparent recovery is relatively low. However, ribonucleotide polymerase C fractions were not included in the calculations, and only the fractions with the highest specific activity of ribonucleotide polymerase A were used at each step. Had all fractions been included, the recovery of activity after the first glycerol gradient would have been 82% (ribonucleotide polymerase A + C).

General Properties of Ribonucleotide Polymerase—In contrast to the RNA polymerase fraction which exhibited a nearly linear reaction rate with respect to enzyme concentration and time, ribonucleotide polymerase incorporated the bulk of the label within the initial 20 min of incubation. The rate and the extent of incorporation depended upon the amount of enzyme (Fig. 2), but the reaction exhibited a nonlinear response as increasing amounts of ribonucleotide polymerase were used. This cooperative effect may be analogous to a similar finding of Froehner and Bonner (20) with rat ascites RNA polymerase. However, no attempt has been made to search for a factor which could restore the linearity.

Ribonucleotide polymerase exhibited a broad optimum of ammonium sulfate concentration (0.02 to 0.06 M). The enzyme retained over 20% of control activity even at an (NH₄)₂SO₄ concentration of 0.3 M. Ribonucleotide polymerase also showed an absolute requirement for manganese ions (Fig. 3). The plateau of MnCl₂ concentration was reached at about 1.3 mM level. Magnesium ions could not substitute for manganese at any of the concentrations tested (up to 0.6 mM). This is a rather unusual property of ribonucleotide polymerase because most known nucleotide-polymerizing enzymes (RNA polymerase, polynucleotide phosphorylase, DNA polymerase) can utilize both metals, although with different efficiency and fidelity (5, 21-25).

Ribonucleotide polymerase was completely inactivated by heating for 1 min at 63°. Incubation for 10 min at 45° prior to assay lowered the activity by 70% relative to 23° control. At 0°, the reaction exhibited a nearly linear reaction rate with respect to enzyme concentration and time, ribonucleotide polymerase incorporated the bulk of the label within the initial 20 min of incubation. The rate and the extent of incorporation depended upon the amount of enzyme (Fig. 2), but the reaction exhibited a nonlinear response as increasing amounts of ribonucleotide polymerase were used. This cooperative effect may be analogous to a similar finding of Froehner and Bonner (20) with rat ascites RNA polymerase. However, no attempt has been made to search for a factor which could restore the linearity.

TABLE I

| Fraction | Total activity | Total protein | Specific activity | Yield |
|----------|----------------|---------------|------------------|-------|
|          | units          | mg            | units/mg         | %     |
| Solubilized fraction | 7,935          | 345           | 23.0             | 100.0 |
| (HSE)    |                |               |                  |       |
| First glycerol gradient | 4,506          | 9.5           | 473.3            | 56.8  |
| Second glycerol gradient | 3,155          | 0.4           | 2,888.2          | 14.6  |

* Ribonucleotide polymerase A fraction from the first glycerol gradient (Fig. 1) was dialyzed against 0.12 M ammonium sulfate in 5% Buffer B. The dialysate was incubated at room temperature with RNase T1 (30,900 units/ml) for 30 min, and then centrifuged as described under "Experimental Procedures."
template, and thus the activity was not affected by actinomycin D. However, compounds which are known to interact with RNA polymerases directly, heparin and rifamycin AF/013, showed a considerable inhibitory effect. Phosphate ions at 0.64 mM concentration had no effect on ribonucleotide polymerase activity, but pyrophosphate was inhibitory. These results indicated that ribonucleotide polymerase used nucleoside triphosphate (UTP) in the polymerization reaction and that the incorporation was not due to the presence of a polynucleotide phosphorylase.

Utilization of Other Nucleoside Triphosphates by Ribonucleotide Polymerase—In addition to UTP, other nucleotides, CTP, GTP, and ATP, were tested in the ribonucleotide polymerase reaction. Table III shows that each of the nucleotides could be utilized by the enzyme and that the incorporation was additive. The apparent $K_m$ values were also determined using the Lineweaver-Burk method (26). Ribonucleotide polymerase showed preference for pyrimidine nucleotides ($K_m$ values: 18 $\mu$M for CTP and UTP, 75 $\mu$M for ATP and GTP). A similar preference has been shown for some RNA polymerases (21).

Ribonucleolytic Treatment of Ribonucleotide Polymerase—Preparations of ribonucleotide polymerase obtained by glycerol gradient centrifugation of HSE contained considerable amounts of RNA (over 20% as determined by the $A_{260}/A_{230}$ ratio). Since it was of interest to know whether a primer RNA is necessary for ribonucleotide polymerase activity, the pooled ribonucleotide polymerase A fractions were treated with ribonuclease A or T1 (see Fig. 4 and Table IV). After the treatment, the mixtures were recentrifuged on a 15 to 30% glycerol gradient to remove ribonucleases. The fractions containing most of ribonucleotide polymerase A activity were pooled and found to be essentially free of RNA ($A_{260}/A_{230}$ ratio less than 0.6). Nevertheless, a minute RNA fragment (not DNA, see below) must have been retained by the enzyme and used as a primer because no radioactivity was incorporated when $[\beta^32P]GTP$ was used as substrate. (A control experiment ascertained that no phosphatase or pyrophosphatase activity was present in the ribonucleotide polymerase preparation as such an activity could remove the $[\beta^32P]$phosphate label from the 5' terminus of the product.) We were unable to dissociate the primer from the enzyme by urea treatment or DEAE-cellulose chromatography. For this reason we could not demonstrate the dependence of the ribonucleotide polymerase A activity on the added RNA. Following RNase treatment there was a slight but discernible increase in sedimentation velocity of ribonucleotide polymerase, as if the removal of RNA increased the compactness of the protein. This is important with respect to the ribonucleotide polymerase product analysis (see below, 3 G. Boguslawski, unpublished observations.)
Ribonucleotide Polymerase from H. capsulatum

Effect of various additions on ribonucleotide polymerase activity

TABLE II

| Compound          | Concentration | Inhibition |
|-------------------|---------------|------------|
| Calf thymus DNA   | 50.0 μg/ml    | 2.8%       |
| Actinomycin D     | 80.0 μg/ml    | 10.4%      |
| Heparin           | 8.0 μg/ml     | 20.9%      |
|                  | 8.0 μg/ml     | 50.8%      |
|                  | 80.0 μg/ml    | 61.9%      |
| Rifamycin AF/013a | 0.8 μg/ml     | 0.0%       |
|                  | 8.0 μg/ml     | 26.0%      |
|                  | 80.0 μg/ml    | 84.3%      |
| α-Amanitin        | 400.0 μg/ml   | 0.0%       |
| Potassium phosphate | 0.64 mM | 1.6%       |
| Sodium pyrophosphate | 0.64 mM | 75.1%      |

a Rifamycin AF/013 was dissolved in dimethyl sulfoxide. The controls contained equivalent amounts of the solvent without the drug.

At this concentration RNA polymerase fraction (Fig. 1) was inhibited by 10.7 and 81.6% by phosphate and pyrophosphate, respectively.

Ribonuclease T1 was used here because it does not attack pyrimidine oligonucleotides, and therefore the assay for UMP incorporation could be performed in its presence. It can be seen that when RNA was not removed, ribonuclease T1 increased both the extent and the rate of the reaction. However, if

Utilization of ribonucleotide triphosphates by ribonucleotide polymerase

The incorporation was tested in the standard assay system containing 0.8 μg of partially purified ribonucleotide polymerase A/reaction. Individual nucleotides were present in the amounts indicated in the table (2 μCi each).

| Nucleotide input | Expected | Incorporation |
|------------------|----------|--------------|
|                  | pmol/reaction | pmol | % of expected |
| UTP (113.0)      | 1.29      | 1.15         | 91.1         |
| CTP (117.6)      | 0.67      | 0.62         | 92.5         |
| ATP (66.8)       | 1.22      | 1.23         | 71.9         |
| GTP (181.8)      | 1.40      | 1.22         | 87.1         |
| UTP + CTP (230.6)| 1.71      | 1.23         | 71.9         |
| UTP + ATP (199.8)| 1.29      | 1.15         | 91.1         |
| UTP + GTP (294.8)| 1.40      | 1.22         | 87.1         |
| ATP + GTP (268.6)| 1.29      | 1.15         | 91.1         |

a From the sum of individual incorporations.

b It was assumed that the joint incorporation was proportional to the molar fraction of each nucleotide present in the reaction mixture.

FIG. 4. The effect of ribonuclease T1 on the activity of ribonucleotide polymerase. Panel A, ribonucleotide polymerase A (1.55 μg of protein), from which RNA was not removed, was mixed with 1.0 μg of RNase T1 (952 units) in the total volume of 30 μl. The mixture was incubated for 5 min at room temperature and the reaction was started by the addition of the buffer, MnCl₂, and [³H]UTP (--;--). The controls without RNase T1 (--;--) were incubated with the equivalent volume of buffer. Panel B, ribonucleotide polymerase A (80 μg in 1 ml) was dialyzed against 5% Buffer B containing 0.12 M ammonium sulfate. The dialyzed material was incubated for 30 min with 100 μg of RNase A in the total volume of 1.1 ml at room temperature. After the treatment the mixture was layered on top of a 15 to 30% glycerol gradient (10 ml) and centrifuged as described under "Experimental Procedures." Fractions containing ribonucleotide polymerase A activity were pooled and tested for RNase T1 effect as described above, except that the amount of ribonucleotide polymerase protein was 0.39 μg, and the volume during RNase T1 treatment was 40 μl. The two top panels (--;--) show the ratios of activity in the presence and absence of RNase T1 for the respective ribonucleotide polymerase A preparations.
ribonucleotide polymerase was first freed of RNA, and then assayed in the presence of RNase T1, the nuclease had no influence on the activity. We interpret this as indicative of inhibition of UMP incorporation by endogeneous RNA. However, as Table IV shows, adding exogenous RNA to RNase A-treated ribonucleotide polymerase had little effect on the activity. The untreated ribonucleotide polymerase was somewhat inhibited by the addition of polyuridylic and polyguanylic acid. We have no explanation for this discrepancy, but it could be that the conformational change induced by the removal of RNA rendered the enzyme insensitive to added polyribonucleotides.

**Table IV**

Effect of various polyribonucleotides on the activity of ribonucleotide polymerase

| Polynucleotide | Untreated ribonucleotide polymerase | Ribonuclease A-treated ribonucleotide polymerase |
|----------------|-----------------------------------|-----------------------------------------------|
|                | cpm | % control | cpm | % control |
| Poly(U)        | 7,262 | 100.0 | 12,148 | 100.0 |
| Poly(A)        | 4,379 | 60.3 | 11,679 | 96.1 |
| Poly(G)        | 6,155 | 84.6 | 11,825 | 97.3 |
| Poly(C)        | 4,197 | 57.8 | 11,205 | 92.2 |
| Yeast RNA      | 7,082 | 97.5 | 14,703 | 121.0 |

* Each polynucleotide was present at the concentration of 40 μg/ml.

**Fig. 5.** DEAE-cellulose chromatography of ribonucleotide polymerase reaction product. The chromatography was performed as described under "Experimental Procedures." Ribonucleotide polymerase A (35 μg) from the first glycerol gradient (Table I) was incubated in a total volume of 375 μl with 1.8 mM MnCl₂ and 6 μCi of [³H]UTP for 1 h at 23°C. The whole reaction mixture was then treated with pronase and loaded onto the column. The radioactivity (■) was eluted using a 0 to 0.25 M NaCl gradient (---), followed by 2 M NaCl. The first 40 fractions contained the bulk of unreacted [³H]UTP and the contaminating [³H]UDP and [³H]UMP. These fractions were omitted from the graph. The numbers next to each Aₒ₅₀₄ peak (O—O) show the length of the corresponding oligonucleotide markers.
Activity-The rapid cessation of UMP incorporation into oligo-
of L3H]UMP following the 90-min incubation period, whether
the tube contained ribonucleotide polymerase and the reaction
produced; thus apparent slower mobility on the column. These results also show that the primer
the presence of unlabeled RNA primer in the radioactive
added, and incubation was continued for 30
min and UMP incorporation was measured. A parallel reac-
tion then continued for an additional 30 min. The third tube
was incubated for 90 min, another 2 $\mu$Ci of [3H]UTP were
added, and incubation was continued for 30 min. The fourth
and UMP incorporation was measured. A parallel reac-
tion then continued for an additional 30 min. The third tube
was incubated for 90 min, another 2 $\mu$Ci of [3H]UTP were
added, and incubation was continued for 30 min. The fourth
tube contained ribonucleotide polymerase and the reaction
mixture without [3H]UTP. The labeled nucleotide was then
added at 90 min and incubation was allowed to proceed for 30
min. As shown in Table VI, there was very little incorporation of [3H]UMP following the 90-min incubation period, whether
or not new UTP was added (722 cpm and 407 cpm, respec-

tively). However, if the enzyme was first incubated for 90 min
without the substrate and then [3H]UTP was added, the
resulting incorporation was nearly 8.6 times higher (3488 cpm
versus 407 cpm). Therefore, the observed cessation of the incor-
poration in the standard assay was not due to substrate limita-
tion or enzyme inactivation on standing. Identical results were obtained when [3H]GTP was the initial substrate and [3H]UTP
was added at 90 min.4 In addition, when more enzyme was
added to the reaction which was completed at 90 min, there
was a burst of new oligonucleotide synthesis,5 again showing
that substrate availability was not limiting. Therefore, we
conclude that the termination of the reaction is due to product
inhibition. Supporting this notion is the finding that, when
the ribonucleotide polymerase reaction was allowed to proceed
to completion and then the mixture was centrifuged in gly-
cerol gradient, no enzyme activity could be recovered. This
interpretation is also consistent with the observed inability of
RNase A to hydrolyze all of the ribonucleotide polymerase

| Table VI |

| Assay number | Conditions of incubation | [3H]UMP incorporated | Net increase |
|--------------|--------------------------|----------------------|-------------|
| 1            | 90 min                   | 8704                 |             |
| 2            | 90 min + 30 min          | 9111                 | 407 (2-1)   |
| 3            | 90 min + 30 min + addi-
tional [3H]UTP             | 9,893                | 1120 (0-1)  |
|              | Total net incorporation due to the addition of new [3H]UTP | 722 (3-2) |
| 4            | 90 min without UTP + 30 min with 2 $\mu$Ci of UTP | 3488                 |             |

Fig. 6. Sedimentation behavior of ribonucleotide polymerase reaction product on glycerol gradients. Ribonucleotide polymerase A (80 $\mu$g obtained as described in Fig. 1 was incubated in a total volume of 1125 $\mu$L with 1.5 mM MgCl$_2$ and 18 $\mu$Ci of [3H]UTP for 1 h at 23°C. The mixture was then distributed into three tubes (375 $\mu$L each). The first tube received 333 $\mu$L of 0.05 M Tris/HCl (pH 7.85). The mixture in the second tube was treated with 333 $\mu$L of RNase T1 (34,000 units). The third tube received 313 $\mu$L of buffer and 20 $\mu$L (100 $\mu$g) of pronase. The tubes were incubated at room temperature for 45 min, the volumes were adjusted to 1 ml with 0.05 M Tris/HCl (pH 7.85), and the samples were layered on 15 to 30% glycerol gradients. 7.85), and the samples were layered on 15 to 30% glycerol gradients.

4 J. S. Parr, unpublished observations.
Ribonucleotide Polymerase from *H. capsulatum*

**Polyacrylamide gel electrophoresis of ribonucleotide polymerase A.** Panel A, ribonucleotide polymerase (6.0 µg) obtained from the second glycerol gradient (Table I) was subjected to electrophoresis under nondenaturing conditions as described under "Experimental Procedures." After electrophoresis one gel was stained for product (Table V), as if it were tightly bound to the polymerase and thus protected from digestion.

**Binding of Oligonucleotides to Ribonucleotide Polymerase**—Further evidence for the interaction of ribonucleotide polymerase product with the enzyme was obtained when the ribonucleotide polymerase reaction mixture was centrifuged on a 15 to 30% glycerol gradient, and the distribution of radioactivity was studied (Fig. 6). When the ribonucleotide polymerase was incubated under standard reaction conditions the bulk of the label sedimented at exactly the same rate as the light enzyme species, ribonucleotide polymerase C (Fig. 11) and a consistently smaller, but significant, amount sedimented at the ribonucleotide polymerase A position. However, when incubation was carried out in the presence of RNase T1, the radioactivity was redistributed so that more product was found in the rapidly sedimenting fractions (note a one-fraction shift in sedimentation). Finally, when the material was treated with pronase, essentially all radioactivity remained at the top of the gradient. Therefore, it is likely that oligouridylate formed in the reaction binds tightly to ribonucleotide polymerase proteins unless some contaminating proteins exhibit the same sedimentation velocities as the two ribonucleotide species (also see "Discussion"). In addition, dialysis of ribonucleotide polymerase reaction mixture resulted in the removal of unreacted UTP but there was no loss of radioactivity in the oligonucleotide material. Had this material not been bound, it would have been removed by dialysis.

**Polyacrylamide Gel Electrophoresis**—When samples of ribonucleotide polymerase A were subjected to electrophoresis, under nondenaturing conditions, a single band appeared on staining with Coomassie blue (Fig. 7A). The band migrated relatively slowly in 3.85% acrylamide gel indicating, as expected, a high molecular weight protein. As shown in the figure, the position of the protein band coincided with the pattern of ribonucleotide polymerase activity in the parallel unstained gel, although the enzyme recovery was quite poor (less than 5% of the amount applied to the gel).

Electrophoresis under denaturing conditions revealed the presence of two prominent bands of protein (Fig. 7B). The molecular weights of these proteins were estimated to be 75,000 (slower band) and 69,000 (faster band). On the basis of densitometer scanning we conclude that the two components are present at about 1:1 molar ratio. However, these data are not sufficient to provide firm clues as to the possible subunit structure of ribonucleotide polymerase.

**DISCUSSION**

**Relation to Known Ribonucleotide Polymerizing Enzymes**—There have recently been a number of reports describing the occurrence of enzymes catalyzing the synthesis of different polynucleotides in a variety of organisms (27-35). Our results indicate that the ribonucleotide polymerase activity isolated from yeast phase extracts of *H. capsulatum* differs from these enzymes in several respects. In contrast to the majority of polynucleotide polymerases, most of ribonucleotide polymerase activity is associated with a very rapidly sedimenting fraction of the yeast extract (HSE). Niessing and Sekeris (27) have shown that rat liver nuclei contain 30 S ribonucleoprotein particles capable of polymerizing UTP, CTP, GTP, and ATP to the corresponding ribohomopolymers. However, the polymers formed in their preparations, and in other systems as well (28, 31, 32), were of greater length than those made by the ribonucleotide polymerase, and the incorporation was linear for long periods of time as compared to only about 20 min for ribonucleotide polymerase. In addition, the incorporation was stimulated by the presence of RNA or other polynucleotides, indicating a primer requirement for the activity of those enzymes. We could not demonstrate polynucleotide stimulation in ribonucleotide polymerase system even after an extensive treatment of ribonucleotide polymerase with RNase A or T1, although the enzyme does seem to use an RNA primer (tightly bound). Moreover, the ribonucleolytic treatment of ribonucleotide polymerase did not seem to affect the integrity of the enzyme as evidenced by its sedimentation behavior and
catalytic properties. This again is in contrast to the behavior of the 30 S particles studied by Niessing and Sekeris (27), which lost activity upon exposure to RNase A.

Commercial preparations of nucleoside triphosphates usually contain 5 to 10% of contaminating diphosphates. Therefore, it is conceivable that the observed polymerizing activity was in fact due to a polynucleotide phosphorylase. The following evidence argues against this possibility. The enzyme was almost completely inactivated by rifampicin AF/013 (80 μg/ml, Table II), whereas polynucleotide phosphorylase from Micrococcus luteus is only slightly inhibited by 200 μg/ml (36). Furthermore, ribonucleotide polymerase was not inhibited by phosphate (Table II) but was sensitive to pyrophosphate. This indicated that nucleoside triphosphate was the substrate for polymerization. Polynucleotide phosphorylase in the presence or absence of a primer incorporates nucleotides for extended periods of time, although the unprimed synthesis takes place only after a prolonged lag (22). In either case, the resulting high molecular weight polymer differs dramatically from the small oligonucleotide produced by ribonucleotide polymerase in the rapid, and short lived, reaction. As shown in Table VI, the cessation of synthesis was not due to substrate limitation, but rather to product inhibition. This again indicates that polynucleotide phosphorylase is not the enzyme involved in the oligonucleotide formation of H. capsulatum because it is normally not inhibited by its own product (22).

Product Analysis and Structure of Ribonucleotide Polymerase – Perhaps the most puzzling feature of the ribonucleotide polymerase system is the distribution of the radioactive product of the enzyme through glycerol gradients (Fig. 6). Even though only the ribonucleotide polymerase A fraction was used in the experiment, the labeled material sedimented at positions at which ribonucleotide polymerase A and ribonucleotide polymerase C were found in parallel experiments. This is especially obvious after the treatment with RNase T1. Because of the product inhibition, no enzymatic activity could be detected under the radioactivity peaks. Nevertheless, the distribution of the label suggests that ribonucleotide polymerase A and ribonucleotide polymerase C may exist in a state of equilibrium, in which the catalytic form is most likely the ribonucleotide polymerase A, but in which the product binding ability is the property of both forms. The treatment with RNase T1 caused a shift in label binding from the ribonucleotide polymerase C region toward the almost even distribution. We assume therefore that the presence of endogenous RNA interferes with the binding (see also Fig. 4). This would explain why relatively little radioactivity was detected in the ribonucleotide polymerase A region before ribonucleolytic treatment. It would also suggest that ribonucleotide polymerase C form has either more binding sites per molecule or that these binding sites are not interfered with by the RNA present in the preparation.

Physiological Role of Ribonucleotide Polymerase – As is the case with almost all ribonucleotide-polymerizing activities described to date (except the true RNA polymerase), the role of ribonucleotide polymerase in the cellular metabolism remains unknown. Our results showed that the enzyme requires a primer for activity. It is conceivable, therefore, that ribonucleotide polymerase’s role in the cell is to add oligonucleotides onto the ends of RNA chains and thus to protect these RNAs from nucleolytic degradation. Supporting this idea is the report by Levy et al. (37) that polyadenylic and polyyguanylic acids are very effective inhibitors of a ribonuclease from Citrobacter.
Ribonucleotide Polymerase from H. capsulatum

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