The Preparation and Properties of Ribonucleic Acid Polymerase from Azotobacter vinelandii*

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(Received for publication, March 3, 1969)

SUMMARY

The DNA-dependent RNA polymerase (EC 2.7.7.6) of Azotobacter vinelandii has been obtained in a homogeneous protein of high and constant specific activity. At an ionic strength of 0.02 it exists as a species with \( s_{20,w} = 22.9 \) S and a molecular weight of 782,000 daltons, but when the salt concentration is raised to 0.4 M it dissociates reversibly to a 14 S form with a reduction in molecular weight to half. The purification was dependent on the recognition of the existence of a 14 S species, presumably a degradation product of the polymerase, and its removal by fractionation. This 14 S material is stable in low salt buffers and has a low and variable polymerase activity. The stability and behavior of the 14 S species has been examined by analytical sedimentation under a variety of conditions of pH and ionic strength; after aging; and in the presence of sulfhydryl compounds, Mg\(^{2+}\), and other reagents. The amino acid composition and free sulfhydryl groups have been determined.

DNA-dependent RNA polymerase (EC 2.7.7.6) has been purified from a number of bacterial species as well as from other sources. Investigations of the physical properties of the enzyme from Escherichia coli by Fuchs, Zillig, Hofschneider, and Preus (1) and Richardson (2) have identified the enzyme with a 21 to 23 S species in buffers of low ionic strength. This form of the enzyme was shown by Richardson (2) to dissociate reversibly into half-molecules having a sedimentation coefficient of 14 S in 0.5 M KCl. However, in recent work with E. coli polymerase there has been evidence for an enzymatically active 13 to 15 S protein that would not associate to form a 23 S species at low ionic strength (2-4). Partly as a result of this type of difficulty, no physical measurements have been made on preparations that are sufficiently free of minor components or sufficiently well defined with respect to the major 14 S and 23 S components to allow adequate characterization. Work in this laboratory has concentrated on the enzyme from Azotobacter vinelandii (5-7). In order to provide a physically and enzymatically characterized enzyme in quantities sufficient for studies of properties of the protein and of the transcription of \( \phi X174 \) replicative form and other DNA's we have redesigned the preparative procedure. The method described in the present study results in a homogeneous product of high and constant specific activity in good yield. The purified enzyme has been characterized by physical, analytical, and enzymatic methods, and the transitions in molecular weight with changes in ionic strength and pH have been studied and partially defined.

EXPERIMENTAL PROCEDURE

Materials—Chemicals were purchased from the following sources: labeled and unlabeled nucleoside triphosphates from Schwarz BioResearch, Inc., calf thymus DNA from Worthington; bovine plasma albumin from Armour and Company; putrescine, spermidine, dithiothreitol, and \( 5,5' \)-dithiobis(2-nitrobenzoic acid) from Calbiochem; \( \beta \)-chloromercuribenzoate, ammonium sulfate (special enzyme grade), and \( N \)-ethylmaleimide from Mann; and protamine sulfate (histone-free grade) from Sigma.

Nucleic acids were prepared as follows: A. vinelandii and Micrococcus luteus DNA essentially as described by Habich et al. (8); \( \phi X174 \) viral DNA by the method of Sinsheimer (9); \( \phi X174 \) replicative form by the method of Rush et al. (10) followed by a Pronase treatment and a phenol extraction; \( ^{14}C \)-Q\(^{+} \)RNA as described by Weissmann and Feix (11). MS2-RNA was made by Dr. Günter Feix and T2-DNA by Charles Gordon. A. vinelandii (ATCC 9104) cells were grown and harvested as described by Ochoa and Mif (12).

DEAE-cellulose with an exchange capacity of 0.97 meq per g was obtained from Schleicher and Schuell Company. It was washed successively with 0.1 M KOH, water, 0.2 M HCl, and water, suspended in the working buffer, packed without pressure into a column, and equilibrated with the same buffer. Hydroxyapatite with a capacity of 90 meq per g was obtained from the Clarkson Chemical Company (Williamsport, Pennsylvania) and the fine particles were removed by three cycles of heating and 5 min of settling before the material was packed into columns. Sephadex G-200 (Pharmacia) and Agarose beads, Bio-Gel A-1.5 m (Bio-Rad Laboratories, Richmond, California) were equilibrated with buffer and packed to give flow rates of...
10 to 15 ml per hour. Void volumes were determined with Dextran blue 2000 (Pharmacia).

**RNA Polymerase Activity**—The routine assay for polymerase was similar to that of Krakow and Ochoa (7). The reaction mixture in a volume of 0.25 ml contained 40 mM Tris-HCl buffer, pH 8; 10 mM MgSO₄; 24 mM putrescine; 12 mM spermidine, 2 mM 2-aminooethanol (mepacrine-2-ethanolamine) or 24 mM dithiothreitol; ATP, GTP, CTP, and UTP, each 1 mM; 64 μg of calf thymus DNA; and 10 to 30 μg of enzyme. ¹³C label was usually present in the UTP at a specific activity of 2 to 3 × 10⁵ cpm per μ mole. The mixture was incubated for 5 min at 37°C, chilled, and 2 ml of cold 6% trichloracetic acid were added. Bovine plasma albumin (500 μg) in 0.5 ml was added, and the acid-insoluble product was collected on Millipore filters (0.45 μ). washed four times with 6% trichloracetic acid, dried, and counted in a gas flow counter. A unit of enzyme is the amount that under these conditions will catalyze the incorporation of 1 nmole of UTP into acid-insoluble product per min at 37°C. The nucleoside triphosphates were used in this assay well below saturating concentrations. Specific activities were calculated on the basis of protein concentrations determined by the method of Lowry et al. (13). Because of the decrease in these concentrations the size of the unit used in this work is about 1.2 times larger than that of Krakow and Ochoa (7), i.e., our specific activities must be multiplied by 1.2 to be equivalent to theirs.

**Other Enzyme Activities**—Exoribonuclease activity was measured by the conversion of ¹³C-Q₁₃-RNA into acid-soluble product. The 0.2-ml reaction mixture contained 40 mM Tris-HCl buffer, pH 8, 10 mM MgSO₄, 0.7 μg of ¹³C-Q₁₃-RNA, and 100 to 500 μg of protein in the fraction to be tested. The mixture was incubated for 1 hour at 37°C, and 10 μl of 1 M MgCl₂ and 50 μl of yeast RNA (5 mg per ml) were added. The solution was chilled and 0.5 ml of cold absolute ethanol was added. The radioactivity in the supernatant solution after centrifugation was measured. Endonuclease activities were detected by analytical zone sedimentation as described under “Results.”

DNA polymerase was assayed by the procedure of Richardson et al. (14) and polynucleotide phosphorylase by that of Ochoa and Mii (12).

**Ultracentrifugation**—Schlieren and zone velocity runs were carried out as previously described (10, 15). Routine schlieren velocity patterns for monitoring purification steps were obtained at 5° at 52,000 rpm after a 4-hour dialysis of the fraction against various concentrations of protein. For equilibrium sedimentation the methods of Yphantis (16) and Schachman and Edelstein (17) were used. The solvent density and viscosity were measured at the temperature of the centrifugation and at 20° where required for the calculation of results.

**Partial Specific Volume**—The partial specific volume was calculated from densities determined at two concentrations in equilibrium density gradients of the Linderstrom-Lang and Lanz type (18) at 20° as previously described (15). The protein concentration was determined by fringe count in a synthetic boundary at 20° in the ultracentrifuge. Samples of the same thoroughly dialyzed solutions were used for the two determinations. The observed refractive index difference between the buffer and the solution was converted to protein concentration by the use of 1.862 × 10⁻¹⁴ ml mg⁻¹ for the specific refractive increment of an average protein (19). Protein concentration determined by refractive index and by the method of Lowry et al. (13) agreed within the precision of the latter method.

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**RESULTS**

**Preparative Procedure**

In redesigning the preparative procedure, the effect of all precipitants was followed by analytical velocity sedimentation as well as enzyme assay in order to permit a continuous correlation of specific activity with sedimentation pattern. As the work progressed it became evident that several forms of the enzyme exist that have sedimentation coefficients of about 14 S but which differ in activity and other properties. Our subsequent effort was directed to purifying the species that at low ionic strength has a coefficient of 23 S. The initial steps were similar to those of Krakow and Ochoa (7) except that the conditions of the column separations on DEAE-cellulose and hydroxylapatite were modified. Unless otherwise indicated, all operations were carried out at 4°, and all buffers used in the preparative steps contained 1 mM EDTA and 2 mM mercaptoethanol in addition to the salt composition. The starting material was 800 to 900 g of fresh grown or frozen *A. vinelandii* cells. Table I contains a summary of the results of the purification procedure.

**Steps I, II, and III**—Steps I and II were identical with the description of Ochoa and Mii for the purification of polynucleotide phosphorylase (12). Step III was modified from that of Krakow and Ochoa (7) in the following ways. (a) Only histone-free gel of protamine sulfate was used. The first precipitation was carried out by adding protamine sulfate as a 1.5% solution to the protein solution at a concentration of about 30 mg per ml in a ratio of 1 g to 10 g of protein. The second precipitation was designed so that 85 to 90% of the polymerase activity was precipitated as determined by assay of the supernatant solution after dialysis. This was routinely accomplished by the dropwise addition of 400 ml of 1.5% protamine sulfate to the supernatant solution from the first precipitation for a preparation consisting of 15 g of protein at the beginning of Step III. The addition of more protamine than specified above in the second precipitation resulted in a loss of activity and conversion of 23 S component to more slowly sedimenting species. (b) The protamine sulfate pellet was washed and extracted by manual dispersion with a plunger of a plastic rod. The use of mechanical dispersing device resulted in a loss of activity. (c) The protamine sulfate eluted with 0.3 saturation with ammonium sulfate from the column fraction was discarded before precipitation of the polymerase fraction at 0.55 saturation. All centrifugations in these steps were carried out at 15,000 × g for 1 hour.

**Step IV**—DEAE-cellulose chromatography was performed on a column, 76 × 2.5 cm, equilibrated with 0.02 M potassium phosphate buffer, pH 7.3. Dialyzed Step III enzyme, in the amount and concentration indicated in Table I, was applied in three steps by a potassium phosphate buffer, pH 7. The buffer concentration was successively 0.02 M, 0.11 M, and 0.2 M plus 0.2 M KCl. The enzyme was precipitated from the fractions of the third peak at 0.55 saturation with ammonium sulfate and dialyzed against 0.02 M potassium phosphate, pH 6.9. The second peak contains DNA polymerase.

**Step V**—Hydroxylapatite chromatography was carried out on a column, 78 × 1.5-cm, equilibrated with 0.02 M potassium phosphate buffer, pH 6.9. The protein was eluted at a rate of 25 ml per hour with the same solutions used for Step IV except that the pH was 6.9. The enzyme was precipitated as before from the fractions of the third peak and dialyzed against 0.02 M potassium phosphate buffer, pH 6.9. The protein was eluted as a single peak with the same properties as the enzyme purified by DEAE-cellulose chromatography.
S. Lee-Huang and R. C. Warner

Purification of A. vinelandii RNA polymerase

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Ass. Aso (units)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield of units (%)</th>
<th>Purification (fold)</th>
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<td>27.5</td>
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<td>102.5</td>
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<td>18,800</td>
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<td>240</td>
<td>25.8</td>
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</table>

*In this preparation 850 g, wet weight, of A. vinelandii cells were used.

Phosphate, pH 7.3. The second peak contains polynucleotide phosphorylase.

Step VI—Agarose filtration was performed as described in Fig. 1. The small peak, A, consists predominately of nucleic acid and its position indicates approximately the void volume of the column. The enzyme is contained in Peak B. Peak C consists of inactive protein which sediments at a slower rate. The concordance of specific activity through the peak indicated by the coincidence of activity and absorbance in Fig. 1 was confirmed in several experiments by the results of the assay of individual fractions. The enzyme in these fractions was precipitated as more and stored at -20°C as a suspension containing 6 to 10 mg of protein per ml in 0.3 saturated ammonium sulfate. Under these conditions of storage a loss of activity of 10 to 15% in 6 months was observed. Samples were prepared for use by a 4-hour dialysis and storage at 4°C at a protein concentration of 3 to 6 mg per ml.

The specific activity of the enzyme purified in this manner was uniformly in the range of 220 to 240 units per mg. The ratio of absorbance, A₂₆₀/A₂₈₀, was 1.8 to 1.9. No increase in specific activity could be achieved by rechromatography on Agarose, DEAE-cellulose, hydroxylapatite, cellulose phosphate, or Sephadex G-200.

Variations on Preparative Method—During the development of the purification procedure more than 50 preparations have been made. The following points summarize our experience with variations on the final method. (a) The Agarose filtration shown in Fig. 1 was carried out in 0.02 M phosphate buffer. The 0.02 M Tris-HCl buffer containing 5 mM Mg²⁺, 2 mM EDTA, 1 mM dithiothreitol, pH 7.3, served equally well. (b) Attempts to perform gel filtration with either Sephadex or Agarose in the absence of 0.4 M KCl always resulted in low yields and the accumulation of low salt, 14 S material. (c) Disruption of the bacteria by grinding with alumina powder gave a slightly better yield than blending with glass beads, but the additional time and labor was too great to justify its regular use. (d) Attempts to replace protamine by streptomycin were unsuccessful. (e) A. vinelandii harvested at midlog growth gave a poorer yield of polymerase than the cells harvested as usual near the end of the phase.

Properties of Purified Polymerase

Sedimentation Velocity—The routine determination of the sedimentation pattern of fractions obtained during purification has been of great value in assessing the efficiency of individual steps and in correlating the physical state and enzymatic activity of the polymerase. The patterns of the Step VI enzyme are shown in Fig. 2. The product sediments as a single homogeneous component in buffers of low ionic strength with $s_{20,w} = 22.9 S$ (Fig. 2A). When the buffer is made 0.4 M KCl the enzyme is converted to a species with $s_{20,w} = 14.1 S$ (Fig. 2B). This transition is comparable to that observed by Richardson for the E. coli RNA polymerase (2). It is completely reversed when the KCl is removed by dialysis. The concentration dependence of the sedimentation coefficients under these two conditions of ionic strength is shown in Fig. 3. Similar changes were observed in 0.02 M potassium phosphate buffer at the same pH with and without KCl. A pattern in this buffer containing 0.2 M KCl is shown in Fig. 2C. The transition to the 14 S form is almost complete at this concentration of KCl. Specific activities lower than the maximum were invariably associated with the presence of a 14 S peak in the low salt buffer. This more slowly sedimenting component was observed regularly in earlier preparations in which Sephadex G-200 was used in place of Agarose in the final step. A pattern illustrating this is reproduced in Fig. 4A. Such preparations in 0.4 M KCl yield patterns indistinguishable from...
Fig. 2. Sedimentation patterns of purified RNA polymerase at 52,000 rpm and 5°. The phase plate angle was 60°. The pH values are those of the buffer at 25°. A, 0.02 M Tris-HCl, pH 7.3, 2 mM EDTA, 1 mM dithiothreitol, 5 mM MgSO4, 32 min. B, same buffer as A plus 0.4 M KCl, same preparation, 61 min. C, 0.02 M potassium phosphate, pH 7.3, 2 mM EDTA, 2 mM mercaptoethanol, 0.2 M KCl, 40 min. The direction of sedimentation is from right to left.

Fig. 3. Concentration dependence of the sedimentation coefficients of RNA polymerase. ○, sedimentation in 0.02 M Tris-HCl, 2 mM Mg2+, 1 mM EDTA, 2 mM mercaptoethanol, pH 7.3; •, same buffer plus 0.4 M KCl. The least squares lines for the upper and lower curves are given by \( s_{20,w} = 22.9 - 0.40c \) and \( s_{20,W} = 14.1 - 0.22c \), respectively, where \( c \) is the concentration in milligrams per ml.

that in Fig. 2B.

Larger quantities of the low salt 14 S component appeared (a) gradually on allowing Step VI enzyme to stand in solution; (b) more rapidly in the absence of a sulfhydryl compound; (c) in preparations made from Step II material that had been frozen for 8 years; and (d) after filtration on Sephadex G-200 in 0.4 M KCl. Several of these changes are also illustrated in Fig. 4. The enzymatic activity of the low salt 14 S component is extremely variable and no correlation between the relative areas of the 23 S and 14 S peaks and the specific activity of the preparations could be established. In some treatments of the type a and b in which a small amount of this component accumulated, the change in specific activity could be accounted for by assuming that it was inactive. In others a significant or high activity was indicated. An extreme example of this was a preparation of type c in which the specific activity was 140 but in which the 23 S component was less than 20% of the total. A limited

recovery of activity (15 to 81% increase) and reconversion (0, 25% increase) of the low salt 14 S component to 23 S has been observed by treatment of preparations of type b with dithiothreitol. Because of the lack of clearly defined criteria for distinguishing active from inactive low salt 14 S component, in particular in the presence of the 23 S species in the A. vinelandii polymerase, we have not studied the relationship among them in greater detail. It is clear, however, that in the case of the A. vinelandii polymerase, the sedimentation properties in buffers with an ionic strength of 0.2 greater cannot be used as criteria of purity.

Treatments a, b, and d produced, in addition to the low salt 14 S component, a series of more slowly sedimenting species without enzymatic activity that can be identified approximately 9 S, 6 S, and 3 S forms. Additional intermediate forms as well aggregates sedimenting faster than 23 S were sometimes observed.

Fig. 4. Sedimentation patterns of purified RNA polymerase at 52,000 rpm and 5°. The phase plate angle was 60°. The pH values are those of the buffer at 25°. A, Sephadex G-200 fraction of 0.02 M potassium phosphate, pH 7.3, 2 mM EDTA, 2 mM mercaptoethanol, 32 min. B, similar preparation stored for 3 months in the same buffer at 4° at a concentration of 6 mg per ml, 32 min. C, similar preparation dialyzed for 4 hours against the same buffer with the omission of mercaptoethanol. 32 min. D, similar preparation in 0.02 M Tris-HCl buffer, pH 7.9, to which p-mercuribenzoate had been added to provide a concentration of 4 mM excess of the dithiothreitol initially in the solution, 80 min. E, preparation similar to that shown in Fig. 2A but dialyzed against 0.02 M potassium phosphate, pH 7.3, 2 mM EDTA, 2 mM mercaptoethanol, 0.2 M guanidine hydrochloride, 32 min. F, preparation similar to that shown in Fig. 2A but dialyzed against the same buffer with the addition of 0.1% sodium dodecyl sulfate. G, preparation similar to that shown in A in 0.02 M Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM dithiothreitol. The direction of sedimentation is from right to left.
The 9 S, 6 S, and 3 S forms were also produced by treatment with protein-dissociating agents such as urea, guanidine, and sodium dodecyl sulfate. Although these species are reproducibly identifiable in sedimentation patterns, none has been isolated or well defined in the present study. Low concentrations of these agents produced partial dissociation as shown in Fig. 4E for 0.2 M guanidine hydrochloride. At higher concentrations patterns similar to that in Fig. 4E were obtained. These were invariably broad or skewed probably indicating the presence of more than one kind of subunit.

Similar patterns of dissociation were found at extremes of pH. The polymerase is insoluble in the pH range 4.5 to 5.5. Below pH 4.5 in formate or glycine buffers or above pH 10 in glycine buffers activity is lost and the protein exists chiefly in the form of 18 S and 6 S species. In the pH range 5.7 to 7, aggregation is favored as the pH is lowered and 23 S and more rapidly sedimenting components are formed even from the low salt 14 S species. The changes on exposure to pH 5.7 are largely reversible and are accompanied by only a small loss in specific activity.

The pH range between 7 and 8.5 is of more interest in relation to the enzymatic activity. In the early phases of this work potassium phosphate buffers containing EDTA were used. The patterns of purified polymerase in this buffer were similar to that in Fig. 4A although the use of Agarose rather than Sephadex resulted in relatively less 14 S component. A higher pH (8.1 at 0°C) in Tris-HCl buffer resulted in the pattern shown in Fig. 4C. A similar one was obtained in phosphate at this pH. This change was largely reversible and resulted in only a small increase in the low salt 14 S form on dialysis of the solution against phosphate buffer at pH 7.3. The change at this pH in Tris was entirely prevented by the addition of 5 mM Mg²⁺ to the buffer and the Tris-Mg²⁺ buffer used for Fig. 4A proved to provide the best conditions for the observation and stability of the 23 S form. Equally symmetrical peaks were obtained at pH 8.6 (3°C) in Tris-Mg²⁺ buffer, but at still higher pH values the 14 S component appeared.

**Assay for exonucleolase in A. vinelandii RNA polymerase**

<table>
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<tr>
<th>Enzyme fraction</th>
<th>Relative RNase activity*</th>
<th>Specific RNase activity*</th>
<th>Specific polymerase activity</th>
<th>Ratio of activity of RNase to that of polymerase</th>
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<tr>
<td>(RNase)</td>
<td>1</td>
<td>66.6</td>
<td>0.227</td>
<td>5.8</td>
</tr>
<tr>
<td>Crude extract</td>
<td>0.333</td>
<td>1.32</td>
<td>1.04</td>
<td>1.51</td>
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<tr>
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<td>0.0865</td>
<td>4.8</td>
<td>0.0632</td>
<td>0.229</td>
</tr>
<tr>
<td>Step III</td>
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<td>8.4</td>
<td>58.2</td>
<td>0.144</td>
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<tr>
<td>DEAE-cellulose, Step IV</td>
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<td>8.4</td>
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<td>0.144</td>
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<tr>
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<td>120</td>
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<tr>
<td>Agarose, Step VI</td>
<td>0.0666</td>
<td>0.0066</td>
<td>240</td>
<td>0.00003</td>
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</table>

* Activity relative to that given by 15 μg of pancreatic RNase A in the 0.2 ml mixture.

**Guaranteed Activity**—Polyacrylamide gel electrophoresis was carried out at 25°C with 5 and 7.5% gels as described by Davis (20). The gels were stained in a 1% solution of naphthol blue black (Buffalo black) in 7% acetic acid and destained in 7%
acetic acid. The homogeneity of the purified (Step VI) polymerase is also established by the single band obtained under these conditions. Fig. 5 shows patterns obtained under two conditions at several stages of purification. Under the conditions chosen for Series A, the enzyme is in the 23 S form, and for Series B in the 14 S form.

Assay for Nuclease Contamination—The results of assay for exoribonuclease activity are given in Table II. This nuclease activity was concentrated, though not as much as polymerase, through the DEAE-cellulose step. However, it was effectively removed by hydroxylapatite and Agarose and was barely detectable in the final product.

Endonuclease activity was assayed by determining whether there was a change in the sedimentation properties of an intact nucleic acid after incubation with an enzyme fraction under the conditions of the standard assay for polymerase activity. The experiments were carried out by analytical zone sedimentation of the following nucleic acids on the indicated supporting electrolytes: (a) MS2 RNA on 33% ethylene glycol, 1% sodium dodecyl sulfate, 0.02 M Tris- HCl, pH 7.6,* (b) φX174 viral DNA on 0.9 M NaCl-0.1%, NaOH (c) φX174 replicative form DNA on neutral 3 M CsCl. The initial preparations of the first two showed six components with S20,W = 27 S and 19 S, respectively. That of φX replicative form was similar to those previously reported (21). In the case of the MS2 RNA, the incubation for 30 min at 37°C was terminated by the addition of sodium dodecyl sulfate, a concentration of 1%. Incubation mixtures containing D (30 min, 37°C) were chilled and layered without additions. Suzuki VI polymerase preparations and intermediate fractions were assayed in each case.

No degradation of MS2 RNA could be detected when a weight ratio of protein to RNA in the incubation mixture was 2.5. Slight tailing of the 27 S peak was evident at a ratio of Crude extract and Step II product at a ratio of 2.5 both gave patterns with maxima at 21 S and 27 S. For comparison puri- crestic RNase at a weight ratio of 2 x 10⁻⁵, equivalent to 0.01 μg per ml, degraded the RNA to a heterogeneous mixture with mean sedimentation coefficient of about 4 S. Higher concen- trations of RNase produced complete degradation.

In the assays for DNase activity with single strand φX DNA, the only change that was detected was the appearance of a shoulder on the slow side of the 19 S peak at weight ratios of 10 and 12. No change could be seen at a ratio of unity. This shift in sedimentation rate presumably corresponds to the change from circular DNA (19 S) in this solvent (22).

No change in the pattern of the double stranded replicative form could be detected at a weight ratio of 20. An increase of the ratio to 40 resulted in an increase of about 20% in circular nicked DNA (Form II, 17 S) and a corresponding decrease in a circular, superwound DNA (Form I, 21 S) (23). At a ratio of 100 a small percentage of linear DNA (14 S) was detected. In contrast, incubation with Step IV fraction at a ratio of 20 caused the disappearance of components sedimenting faster than about 8 S.

Assays were also made for DNA polymerase and polynucl- tide phosphorylase. Neither could be detected in the Step VI enzyme.

Sedimentation Equilibrium—The molecular weight of RN polymerase was determined at low and high ionic strength by the meniscus depletion method of Yphantis (16). At low ionic strength consistent results were obtained by adding 1% sucrose to the buffer to provide additional stabilization to the gradient. Plots are shown in Fig. 6 of the fringe displacement against r² of two initial concentrations of an enzyme preparation having sedimentation pattern comparable to that in Fig. 2A and for two initial concentrations in buffer containing 0.4 M KCl comparable to Fig. 2B. Since there is no significant change in slope with initial concentration in these and other experiments the values have been averaged to give 782,000 daltons for the molecular weight of the 23 S enzyme and 399,000 daltons for the 14 S form. Values close to this from other experiments, chiefly on preparations containing some 14 S component have not been averaged with the results of the experiment shown in Fig. 6. A value of 799,000 was obtained from an equilibrium experiment also shown in Fig. 6 observed with the automatic ultraviolet scanning system with an initial absorbance of 0.2 at 260 μm. The ultraviolet scanning was feasible only when dithiothreitol was used as the sulfhyd- reagent at a concentration of 1 mM or lower. Dissociation of half-molecules in 0.4 M KCl is indicated by these results. To conditions for sedimenting viral RNA in the presence of sodium dodecyl sulfate.

* We are indebted to Dr. Gunther Feix for establishing suitable

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**Fig. 6.** Plots of equilibrium centrifugation experiments for calculation of the molecular weight of RNA polymerase. Lines A and B, 0.2 and 0.5 mg of protein per ml in 0.02 M potassium phosphate, 0.4 M KCl, 2 mM mercaptoethanol, 2 mM EDTA, pH 7.5, 6000 rpm at 5°C, interference optics. Lines C and D, 0.2 and 0.5 mg per ml in the same buffer plus 1% sucrose but without KCl, 6000 rpm at 5°C, interference optics; Line E, initial absorbance at 280 μm, 0.2 in 0.02 M Tris-HCl, 2 mM Mg²⁺, 1 mM dithiothreitol, 1% sucrose, 6000 rpm at 5°C, absorption optics. The radius squared (r²) in the abscissa has been adjusted by an amount, δ, to permit plotting of all lines in the same figure. For Lines A, B, C, D, and E, δ is 42.6, 49.0, 41.4, 47.6, and 40.6, respectively. The displacement, Δy, in the ordinate is in centimeters and shown on the left for the interference runs and on the right for the absorption run. The straight lines were determined by the weighted least squares method of Yphantis (16).
consistent with the supposition that the two half-molecules are identical, but this has clearly not been proven.

In these calculations the partial specific volume has been taken as the apparent specific volume determined at protein concentrations of 2.8 and 4.2 mg per ml at 20°. The value of 0.74 ml per g in agreement with the value of 0.736 calculated from the amino acid analysis by the method of McMeekin and Marshall (26). The frictional coefficient, f/f0, calculated from the molecular weight and η0/η is 1.30 and 1.32 for the 28 S and 14 S forms, respectively.

Amino Acid Analysis—The amino acid composition of the polymerase is summarized in Table III. The analyses were performed as described by Spackman, Stein, and Moore (25) with the use of a Spinco amino acid analyzer equipped with an automatic integrator. In order to determine the cystine plus cysteine content samples were oxidized by the procedure of Hirs (26). The ratio of tryptophan to tyrosine was determined spectrophotometrically by the method of Benece and Schmid (27).

The table shows the amino acid composition relative to the histidine content. Insufficient material was available to put the analyses on an absolute weight basis by an analytical procedure.

### Table III

Amino acid composition of A. vinelandii RNA polymerase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Molar ratio relative to histidine</th>
<th>Average residues per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unoxidized</td>
<td>Oxidized</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>Asparagine</td>
<td>7.6</td>
<td>7.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.1</td>
<td>3.0</td>
</tr>
<tr>
<td>Serine</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Proline</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Valine</td>
<td>5.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Methionine sulfone</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

### Table IV

Sulfhydryl groups of A. vinelandii RNA polymerase

<table>
<thead>
<tr>
<th>Method</th>
<th>Sulfhydryl groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme</td>
<td>58</td>
</tr>
<tr>
<td>In 1 M urea</td>
<td>56</td>
</tr>
<tr>
<td>In 4 M guanidine hydrochloride</td>
<td>59</td>
</tr>
<tr>
<td>In 0.1% sodium dodecyl sulfate</td>
<td>56</td>
</tr>
<tr>
<td>Reaction with p-chloromercuribenzoate</td>
<td>61</td>
</tr>
<tr>
<td>Reaction with N-ethylmaleimide (native enzyme)</td>
<td>58</td>
</tr>
</tbody>
</table>

However, the actual recovery of total amino acid residue weight was about 90% of the weight of the initial air-dried protein samples in all cases. A tentative calculation of residues per molecule of 782,000 has therefore been made on the basis that the proportions of the amino acids recovered are the same as the proportions present in the initial sample.

### Sulfhydryl Groups—Titrations of the sulfhydryl groups were made by the procedures of Boyer (28), Ellman (29), and Roberts and Rouser (30). The results of all three methods are summarized in Table IV. They are in agreement and no significant difference in the number of groups available was found by titration with 5,5'-dithio-bis(2-nitrobenzoic acid) in the presence of the denaturing agents indicated in the table. A comparison of the titers with the estimate of 63 cysteic acid residues per molecule shown in Table III suggests that substantially all potential cystine plus cysteine is present as free sulfhydryl groups and that these are all freely accessible for titration by the reagents used.

The effect of p-mercuribenzoate on the enzymatic reaction is shown in Fig. 7. The approximately linear relation between activity and fraction of the sulfhydryl groups titrated may indicate either that all sulfhydryl groups are equally necessary for activity or that certain critically required groups have the same reactivity with p-mercuribenzoate as the average sulfhydryl group.

### Characteristics of Enzymatic Reaction—The catalytic characteristics of RNA polymerase have been extensively described for the enzymes from E. coli (31-33) and M. luteus (34) and in previous studies of the A. vinelandii enzyme (7). They have all been re-examined with the use of the Step VI product. The results will not be reported in detail except where they differ from previous work. The divalent ion requirement is satisfied by Mg2+ for which K, = 11 mM and Vmax = 2.1 nmoles per min per unit of enzyme or by Mn2+ for which K, = 3.3 mM and Vmax = 3.1 nmoles per min per unit. In contrast to the results with E. coli and M. luteus polymerases (31, 32, 34), the maximum observed velocity before inhibition at high divalent ion concentration was about the same for Mg2+ [1.1 nmoles per min per unit at (Mg2+) = 15 mM] and for Mn2+ [1.2 nmoles per min per unit at (Mn2+) = 15 mM].
RNA Polymerase from *A. vinelandii*

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The pH of maximum activity was found to be 8.5 as shown in Fig. 8. This probably should not be considered to be an optimum because of the instability of the enzyme in this pH range. At the pH of the standard assay, 7.6 at 37°, the enzyme remains in the 23 S form in Tris-Mg²⁺ buffer as shown by centrifugation studies.

The effects of polyamines, ionic strength, and dithiothreitol on the time course of the reaction are shown in Fig. 9. The basic reaction mixture in this series of experiments has the optimum concentration of polyamines. Under these conditions the synthesis of product falls off only slowly over a 4-hour period and at the end of this time reaches a relative RNA synthesis equivalent to 3.3 times the amount of template present (Curve 1). The addition of KCl to a concentration of 0.2 M has little effect. When the polyamines are omitted, the formation of RNA levels off at a relative synthesis of 1.4 after about 2 hours. The addition of KCl restores the course essentially to that with polyamines or polyamines plus KCl. The effect of ionic strength in the absence of polyamines is thus essentially that reported by Fuchs et al. (35). Omission of sulfhydryl compound has the same effect as omission of polyamines. This is shown in Fig. 9 for an experiment with dithiothreitol. The relative RNA synthesis levels off at 1.1-fold after about 1 hour but was restored almost to the control value if dithiothreitol was added at this time. The addition of KCl in the absence of sulfhydryl compound depressed the synthesis further rather than increasing it as in the case of synthesis without polyamines. Omission of both dithiothreitol and polyamines resulted in still less synthesis which leveled off at relative values of 0.98 with, and 0.7 without, 0.2 M KCl.

The template activity of several native and denatured DNA's is shown in Table V under the conditions of the standard assay and in a more dilute reaction mixture without polyamines. The variation with type of template is similar to that observed with less purified polymerase (7, 36). The double stranded viral DNA's are effective templates, indicating that if a factor of the kind described by Burgess et al. (37) for the *E. coli* enzyme is necessary for transcription of these templates, it has not been removed from the *A. vinelandii* polymerase during purification. Additional experiments under both conditions show that 

![Graph](http://www.jbc.org/content/244/1/3800/F10.large.jpg)
in the fact that it can have greater activity than the 23 S form in
Varied from zero to 120. More striking differences are evident
in part from preparations that were mixtures with the 23 S form,
those of the 23 S species and the reversible dissociation product
treatment with dithiothreitol. The low salt 14 S species has not
Only a limited reversal to the 23 S form has been achieved by
However, it does not reassociate on reducing the ionic strength.
the presence of 0.4 M KCl this species cannot be distinguished
the product of the reversible dissociation of the 23 S species.
the recognition and removal of a low salt 14 S component. In
polyamines stimulate the incorporation with double stranded
templates, they reduce it when single stranded templates are
used. This result is similar to that reported by Karakow (36)
for the inhibition of template activity of synthetic polynucleotides.
Rifamycin—Because of recent reports of the binding of rifa-
mycin by RNA polymerase and the accompanying inhibition
(38), we have tested its effect on the sedimentation properties.
In the Tris-Mg+2 buffer with 20 µg of rifamycin and 7 mg of
enzyme per ml, no change in the sedimentation of the enzyme as
a 23 S component was found. Under these conditions the inhibi-
tion of enzymatic activity was complete.

**Table V**

<table>
<thead>
<tr>
<th>Template DNA</th>
<th>3H-UMP incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>mmoles/5 min</td>
</tr>
<tr>
<td>Calf thymus, native</td>
<td>36</td>
</tr>
<tr>
<td>Calf thymus, denatured</td>
<td>14^a</td>
</tr>
<tr>
<td>M. luteus, native</td>
<td>12</td>
</tr>
<tr>
<td>M. luteus, denatured</td>
<td>5^a</td>
</tr>
<tr>
<td>A. vinelandii, native</td>
<td>20</td>
</tr>
<tr>
<td>Tl</td>
<td>19</td>
</tr>
<tr>
<td>gX 174 replicative form</td>
<td>50</td>
</tr>
<tr>
<td>gX 174 viral DNA</td>
<td>9</td>
</tr>
</tbody>
</table>

^a Separate experiment; incorporation adjusted for the units of enzyme used.

**Discussion**

The purification of the RNA polymerase of *A. vinelandii* re-
ported here yields a homogeneous protein of high and constant
specific activity. This protein has been characterized as a 22.9
S species with a molecular weight of 782,000 daltons in dilute
buffers at neutral pH values. It exhibits a reversible conversion
of 0.4 ionic strength to 14 S half-molecules similar to that ob-
served for the *E. coli* polymerase (2). The attainment of constant
specific activity and centrifugal homogeneity was dependent on
the recognition and removal of a low salt 14 S component. In
the presence of 0.4 M KCl this species cannot be distinguished
from the product of the reversible dissociation of the 23 S species.
However, it does not reassociate on reducing the ionic strength.
Only a limited reversal to the 23 S form has been achieved by
treatment with dithiothreitol. The low salt 14 S species has not
thus far been characterized with respect to its physical behavior
except by the criterion of its stability at low ionic strength
(<0.05). It is evident that its catalytic properties differ from
those of the 23 S species and the reversible dissociation product
hereof. The specific activity in our standard assay, estimated
in part from preparations that were mixtures with the 23 S form,
varied from zero to 120. More striking differences are evident
in the fact that it can have greater activity than the 23 S form in
the unprimed synthesis of polynucleotides as described by Smith
et al. (39) and Krakow (40). Other characteristics are being
investigated. We consider that the low salt 14 S species is
probably a degradation product of the polymerase that accumu-
lates during the early stages of purification and which subse-
sequently can be formed from the 23 S species, more or less slowly
depending on the conditions. A similar species may be involved in
the reactions of the *E. coli* polymerase. Pettijohn and Kamiya
(3) found two forms of the enzyme, 21 S and 13 S, in different
fractions from a hydroxylapatite column. The 21 S form
was converted to 13 S at high ionic strength. However, it was clear
that the 13 S species obtained in this way had different properties
from the 13 S fraction eluted from hydroxyapatite. In addition,
they noted some shift to slower sedimentation on storage of the
21 S species. A similar observation was made by Richardson
(2). In a recently reported work, Lubin (4) was unable to re-
verse the dissociation of a 19 S species after exposure to 0.5 M
KCl although the dissociated product retained enzymatic activity.

The invariance of the specific activity of the Agarose fraction
after rechromatography under a variety of conditions is evidence
for its molecular and enzymatic homogeneity. More important,
the same specific activity of 220 to 240 nmoles per min per mg of
protein was attained in all preparations in which a single, sym-
metrical peak (Peak B, Fig. 1) from Agarose filtration was asso-
ciated with a single 23 S component (Fig. 2A). It is probable
that the specific activity reported here is greater than that
achieved in other preparations of polymerase from the same or
different sources. However, a precise comparison is difficult
because of variations in the conditions of assay used by different
workers. The possibility that 14 S material with variable en-
zymatic activity may be present could explain the low specific
activity of the enzyme used in a number of recent investigations.
Acrylamide gel electrophoresis of the Agarose fraction (Fig. 5)
shows a single component under two conditions and further shows
the removal of other species identifiable in the initial extract.
Recently reported electrophoresis patterns of the *A. vinelandii*
(41, 42) and *E. coli* (37) polymerases under similar conditions
show several bands that may be comparable to some of the faster
moving bands in Fig. 5, A and B, gels 2 and 3.

The relation of the reversible 23 S to 14 S transition to the
catalytic process has not been defined in the present study. In
our standard assay conditions at high polyamine concentration
the polymerase presumably exists in the 14 S form since the addi-
tion of 0.2 M KCl has little effect on the time course of the reaction
and the extent of net synthesis. This cannot be tested directly
by sedimentation under the same conditions of enzyme and
polyamine concentration. The assay conditions are arbitrary
and it does not follow that certain aspects of transcriptions may
not be determined at lower ionic strength by the existence of the
polymerase in the 23 S form.

**Acknowledgments**—We wish to acknowledge the counsel of
Professor Severo Ochoa throughout the course of this work. We
are indebted to Mr. Frank Zaboretsky for carrying out the ana-
litical centrifugation work; to Mr. Horace Lozina for his help
with the growth of *A. vinelandii* and preparation of Step I and II
fractions; to Mr. Henry (Chang Yuh) Lee for his technical assis-
tance in all phases of the work. Some initial work on revising
the purification procedure and on reaction of the polymerase with

---

* S. Lee-Huang and R. C. Warner, unpublished experiments.
p-mercuribenzoate was done by Dr. Kazuoki Kuratomi of the Institute of Public Health, Tokyo, when he was a visitor in this laboratory.

REFERENCES

The Preparation and Properties of Ribonucleic Acid Polymerase from *Azotobacter vinelandii*
Sylvia Lee-Huang and Robert C. Warner


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