A New Method for the Large-Scale Purification of Wheat Germ DNA-Dependent RNA Polymerase II†

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ABSTRACT: An improved method for the purification of the α-amanitin-sensitive deoxyribonucleic acid dependent ribonucleic acid polymerase [ribonucleosidetriphosphate:RNA-nucleotidyltransferase, EC 2.7.7.6] (RNA polymerase II or RNA polymerase B) from wheat germ is presented. The method involves homogenization of wheat germ in a buffer of moderate ionic strength, precipitation of RNA polymerase with Polymin P (a polyethylenimine), elution of RNA polymerase from the Polymin P precipitate, ammonium sulfate precipitation, and chromatography on DEAE-cellulose and phosphocellulose. RNA polymerase II is purified over 4000-fold with a 60% recovery, resulting in a yield of 25–30 mg of RNA polymerase from 1 kg of starting material.

Detailed studies of the chemical and physical properties of eukaryotic DNA-dependent RNA polymerases require that large amounts of enzyme be conveniently prepared in high purity. Eukaryotic nuclei contain multiple RNA polymerases with different functions. Thus RNA polymerase I, which is not inhibited by α-amanitin (Jacob et al., 1970), is localized in the nucleolus (Roeder and Rutter, 1970) and is responsible for the synthesis of ribosomal precursor RNA (Blatti et al., 1970). RNA polymerase II, which is inhibited by low levels of α-amanitin (Jacob et al., 1970), is nucleoplasmic (Roeder and Rutter, 1970) and synthesizes heterogeneous nuclear RNA (Blatti et al., 1970). RNA polymerase III, which is inhibited only by very high concentrations of α-amanitin, is believed to be responsible for synthesis of 5 S and precursor for tRNA (Weinmann and Roeder, 1974).

While RNA polymerases have been isolated and studied from a wide variety of eukaryotes (Chambon, 1974; Jacob, 1973), the low RNA polymerase content and limiting amounts and/or expense of the starting material prohibit large-scale purification of the RNA polymerases from many of these sources. Because RNA polymerase II is generally present in larger amounts and is more stable to purification than RNA polymerases I and III, it has been the most extensively studied. From calf thymus tissue, which has been reported to be the richest source of RNA polymerases (Chambon, 1974), milligram quantities of pure RNA polymerase II have been obtained. However, most of the purification procedures involve many steps resulting in low yields of enzyme (Gissinger and Chambon, 1972; Weaver et al., 1971). Often the procedures cannot be scaled up due to sonication (Gissinger and Chambon, 1972; Weaver et al., 1971) and preparative ultracentrifugation (Schmincke and Hausen, 1973) of large volumes.

Wheat germ is a convenient source for the purification of RNA polymerase. The material is inexpensive, easily stored, available in virtually unlimited quantities, ready for use with no preliminary tissue isolation, and is rich in the α-amanitin-sensitive RNA polymerase (Jendrisak and Becker, 1973). Although a method for the purification of this enzyme has been presented earlier (Jendrisak and Becker, 1974), the method described here is more efficient, results in higher yields, and is more suitable for scale up.

The new method is designed to avoid sonication, ultracentrifugation, and dialysis of large volumes. Fractionation of the crude extract with Polymin P (a method first introduced by Zillig et al. (1970) for the purification of *Escherichia coli* RNA polymerase) results in a substantial purification with high yields and complete removal of nucleic acids. The high purification achieved at the initial stages of this procedure allows more efficient use of subsequent column chromatographic steps, resulting in the complete purification of RNA polymerase from several kilograms of starting material in 2 days.

Since the enzymatic and physical properties of wheat germ RNA polymerase II have been shown to be similar, if not identical, to the analogous enzymes isolated from other eukaryotic sources (Jendrisak and Becker, 1973, 1974), any new information obtained from further studies of wheat germ RNA polymerase should be applicable to these other eukaryotic RNA polymerases. The availability of RNA polymerase in the large quantities obtained here should allow further studies and detailed analysis of the chemical, physical, and subunit properties of this enzyme.

Experimental Procedures

Materials. Raw wheat germ was obtained from VioBin Corporation, Monticello, Ill., and was stored in a cold room (4°C). Polymin P was kindly donated by Badische Anilin and Soda Fabrik, WHOZ Hauptlaboratorium B9, Hochschullieferungen, 6700 Ludwigshafen/Rhein, Germany, and was stored at room temperature. Ribonucleoside triphosphates (ATP, GTP, CTP, and UTP) were purchased from P-L Biochemicals and [5-^{3}H]UTP (26.9 Ci/mmol) was purchased from New England Nuclear. Calf thymus DNA was purchased from Worthington, bovine serum albumin from Miles (Pentex) and α-amanitin from Henley and Company, New York. Tris base and EDTA were purchased from Sigma, dithiothreitol and Miracloth from Calbiochem, $(NH_4)_2SO_4$ (enzyme grade), Brij 35, and Coomassie Brilliant Blue R-250 from Schwartz/Mann, and ethylene glycol (99+%) was from Aldrich. DEAE-cellulose

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(DE52) and phosphocellulose (P11) were purchased from Whatman. Electrophoresis reagents (acrylamide, methylenebisacrylamide, tetraethylmethylenediamine, and ammonium persulfate) were purchased from Bio-Rad. Sodium dodecyl sulfate was purchased from Matheson Coleman and Bell and was recrystallized as previously described (Burgess, 1969b).

Buffers. Glass double-distilled water and the highest grade chemicals were used for all solutions. All buffers used in the purification procedure contained 0.05 *M* Tris-HCl (pH 7.9), 0.1 m*M* EDTA, and 1 m*M* dithiothreitol (TED).[1] Chromatography buffers in addition contained 25% (v/v) ethylene glycol (TEDG). These buffers were made from stock solutions, all Millipore filtered, of 2.0 *M* Tris-HCl (pH 7.9, 25°C) and 0.1 *M* Na_2EDTA (pH 8). Buffers containing $(NH_4)_2SO_4$ were freshly prepared from a Millipore-filtered, 2 *M* solution of $(NH_4)_2SO_4$, and solid dithiothreitol was added just before use of the buffer.

RNA Polymerase Assay. The standard RNA polymerase assay mixture contained in a final volume of 0.25 ml: 2.5 μmol of Tris-HCl (pH 7.9); 0.25 μmol of $MnCl_2$; 5 μmol of $MgCl_2$; 12.5 μmol of $(NH_4)_2SO_4$; 100 nmol each of GTP, CTP, and ATP; 1 μCi of [5-^{3}H]UTP diluted to a specific radioactivity of 1 μCi/0.1 nmol; 50 μg of heat-denatured calf thymus DNA; and 125 μg of bovine serum albumin.

Assays were incubated for 15 min at 25°C and the RNA was precipitated by adding 2 ml of 5% (w/v) ice-cold Cl_3CCOOH containing 25 m*M* $Na_4P_2O_7$. After 5 min at 0°C, the precipitates were collected on Whatman GF/C filters and were washed under suction with five 4-ml rinses of ice-cold 2% Cl_3CCOOH containing 10 m*M* $Na_4P_2O_7$ followed by 2 ml of 95% ethanol. After drying under a heat lamp, the filters were assayed for radioactivity by standard liquid scintillation counting.

One unit of RNA polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 10 pmol of labeled UMP into acid-precipitable form in 15 min under the above conditions. Specific activity is defined as units/mg.

Assays of Enzymatic Impurities. DNase. Tritiated *E. coli* DNA (10 μg; 3,000 dpm/μg) and RNA polymerase (10 μg) were incubated in 100 μl of nuclease assay solution (RNA polymerase assay mixture minus calf thymus DNA and ribonucleoside triphosphates) for 2 hr at 37°C. The mixture was chilled and after addition of 100 μl of 10% Cl_3CCOOH was centrifuged (10,000*g* for 10 min). The supernatant (100 μl) was assayed for radioactivity in 10 ml of Scintisol (Isolabs, Inc.) by liquid scintillation counting to determine how much of the DNA has been degraded and thus rendered Cl_3CCOOH soluble.

RNase. ^{14}C-labeled *E. coli* rRNA (10 μg; 80,000 dpm/μg) and RNA polymerase (10 μg) were incubated in 100 μl of nuclease assay solution for 2 hr at 37°C. The mixture was chilled, treated with 10% Cl_3CCOOH, and centrifuged, and the resulting supernatant was assayed for radioactivity as described above to determine how much of the RNA had been degraded and thus rendered Cl_3CCOOH soluble.

Protease. RNA polymerase in TEDG + 0.10 *M* $(NH_4)_2SO_4$ was incubated at 37°C for 24 hr. The sample was subjected to electrophoresis on dodecyl sulfate polyacrylamide gels as described below. After staining as described below, the gel was scanned at 550 nm on a Beckman Acta III recording spectrophotometer and compared with a scan of an identical amount of RNA polymerase which was not so incubated, in order to detect protein breakdown.

Column Chromatography. DEAE-cellulose was suspended in 10 volumes of TEDG + 0.5 *M* $(NH_4)_2SO_4$. After titrating to pH 7.9 and degassing, it was packed into a column at room temperature and equilibrated with 4 column volumes of TEDG + 0.15 *M* $(NH_4)_2SO_4$ at 0–4°C.

Phosphocellulose was prepared for use as previously described by Burgess (1969a) with the following modifications. The alkali- and acid-washed cake was resuspended in 5 volumes of TEDG + 0.5 *M* $(NH_4)_2SO_4$ and titrated to pH 7.9 with 10 *N* NaOH. The resulting slurry was degassed and packed into a column at room temperature. The column was then equilibrated with 5 column volumes of TEDG + 0.075 *M* $(NH_4)_2SO_4$ at 0–4°C.

Gel Electrophoresis. Dodecyl sulfate polyacrylamide gels were run as described by Laemmli (1970) with the modifications described in the accompanying paper (Burgess and Jendrisak, 1975). Gels were stained for at least 8 hr in 0.05% Coomassie Brilliant Blue R-250 in ethanol–acetic acid–water (5:1:5) at room temperature. They were diffusion-destained in 7.5% acetic acid at 50°C for 2 hr and then at room temperature until the background was clear.

Salt Determinations. Salt concentrations were made from conductivity measurements with a Radiometer/Copenhagen conductivity meter (CDM2e). Aliquots (100 μl) were diluted to 5 ml with glass double-distilled water at room temperature and the conductivity was determined. The salt concentrations in the samples were determined from a standard curve prepared with buffers of known salt concentrations.

Protein Determinations. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Because several buffer constituents and Polymin P strongly interfere with this assay, protein was precipitated from aliquots (10–1000 μl) of the various fractions with 10 ml of 10% ice-cold Cl_3CCOOH. After 10 min at 0°C the precipitates were collected by centrifugation (10,000*g* for 10 min), dissolved in 1 ml of 1 *N* NaOH, and diluted to 10 ml with water. Aliquots of the resulting solutions were used in the protein assays.

Preparation of a 10% (v/v) Polymin P Stock. A 2-l. stock solution of 10% (v/v) Polymin P (pH 7.9) was prepared by dissolving 200 ml of Polymin P in 1700 ml of water. The solution was adjusted to pH 7.9 by adding concentrated HCl (approximately 77 ml) with stirring. This solution was diluted to 2000 ml with water and filtered through 1 layer of Miracloth (to remove slight particulate matter), resulting in a clear bluish solution. No changes in properties were observed after storage of this solution for several months at 0°C. Also, no differences in RNA polymerase precipitation behavior were observed for two different batches of Polymin P. Polymin P interferes strongly with the assay for protein but is efficiently removed by precipitating the protein as described in the previous paragraph.

Polymin P concentrations up to 1% do not appear to interfere with dodecyl sulfate polyacrylamide gel electrophoresis. Also, Polymin P is not precipitated by Cl_3CCOOH or $(NH_4)_2SO_4$ in any concentrations tested. A 1% solution has an absorbance of about 0.01 at 260 nm and no appreciable absorbance at 280 nm. We have found no difficulty in assaying for wheat germ RNA polymerase activity during

[1] Abbreviations used are: TEDG, 0.05 *M* Tris-HCl (pH 7.9), 0.1 m*M* EDTA, 1 m*M* dithiothreitol, and 25% (v/v) ethylene glycol; TED, TEDG minus ethylene glycol.

Table I: Summary of Purification of Wheat Germ RNA Polymerase II.[a]

No.	Fraction Description	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity (units/mg)	Fold Purification	Yield (%)	A_{280}/A_{260}
1	Crude extract	4150	191,000	12,000	0.063	1	100	0.60
2	Polymin P eluate	2040	6,830	13,200	1.9	31	110	1.6
3	$(NH_4)_2SO_4$ precipitate	150	1,480	10,200	6.9	110	85	1.6
4	DEAE-cellulose peak	60	92	9,000	98	1550	75	1.7
5	Phosphocellulose peak	23	30	7,560	250	4000	63	1.8

[a] From 1 kg of wheat germ.

Polymin P fractionation providing that small aliquots (5 μl) of protein are assayed in the manner described above.

Purification of RNA Polymerase II

Unless otherwise specified, all operations were carried out at 0–4°C, and all centrifugations were performed at 10,000*g* for 15 min in the Sorvall G-3 rotor (7,500 rpm).

Preparation of the Crude Extract. Wheat germ (1 kg) was ground for 1 min at full speed in a 1-gal Waring Blendor with 4 l. of TED + 0.075 *M* $(NH_4)_2SO_4$. After diluting with 1 l. of the same buffer the homogenate was centrifuged. The supernatant was filtered through 1 layer of Miracloth (to remove a lipid pellicle), resulting in approximately 4 l. of a turbid, yellow crude extract (fraction 1).

Polymin P Fractionation. RNA polymerase was precipitated from fraction 1 by addition of 0.075 volume (approximately 300 ml) of 10% Polymin P solution with rapid stirring. The resulting mixture was centrifuged and the clear amber supernatant solution discarded.

The Polymin P precipitate was washed by suspending it in 2 l. of TED + 0.075 *M* $(NH_4)_2SO_4$ and mixing in the 1-gal Waring Blendor for 2 min. Blending speed was adjusted with a rheostat such that it was sufficiently vigorous to completely suspend the precipitate but gentle enough to prevent foaming. The resulting suspension was centrifuged and the clear, yellow supernatant discarded. RNA polymerase was extracted from the resulting pellet by suspending it in 2 l. of TED + 0.20 *M* $(NH_4)_2SO_4$ and mixing in the blender as described above. The resulting suspension was centrifuged to give approximately 2 l. of a clear, yellow supernatant solution, containing the RNA polymerase (fraction 2).

$(NH_4)_2SO_4$ Precipitation. RNA polymerase was precipitated from fraction 2 by adding slowly with stirring, 20 g of solid $(NH_4)_2SO_4$ per 100 ml of solution. After all of the salt had dissolved, stirring was continued for an additional 10 min. The resulting mixture was centrifuged and the clear yellow supernatant discarded. The tightly packed pellet was dissolved in enough TEDG + 0.1% Brij 35 (about 150 ml) so that the final $(NH_4)_2SO_4$ concentration was 0.150 *M*, as determined by conductivity measurements. The resulting solution was cleared by centrifugation to yield a dark yellow solution (fraction 3).

DEAE-Cellulose Chromatography. Fraction 3 protein was applied to a DEAE-cellulose column equilibrated with TEDG + 0.15 *M* $(NH_4)_2SO_4$ (100 ml column volume/kg of starting material) at a flow rate of 100 ml/hr. After washing the column with 500 ml of TEDG + 0.15 *M* $(NH_4)_2SO_4$, RNA polymerase was eluted with 0.25 *M* $(NH_4)_2SO_4$ in TEDG. The fractions containing the bulk of the RNA polymerase activity were pooled to give fraction 4 (approximately 60 ml).

Phosphocellulose Chromatography. Fraction 4 protein was precipitated by adding solid $(NH_4)_2SO_4$ to a final concentration of 50% saturation (30 g of $(NH_4)_2SO_4$/100 ml of fraction 4) and gently stirring for 1 hr. The precipitate was collected by centrifugation at 20,000*g* for 30 min and dissolved in enough TEDG (requires about 50 ml) so that the final $(NH_4)_2SO_4$ concentration was 0.075 *M*, as determined by conductivity measurements. The resulting solution was cleared by centrifugation at 20,000*g* for 30 min and then was applied at a flow rate of 50 ml/hr to a phosphocellulose column (25-ml column/kg of starting material) equilibrated with TEDG + 0.075 *M* $(NH_4)_2SO_4$. After washing the column with 50 ml of this same buffer, RNA polymerase was eluted with 0.15 *M* $(NH_4)_2SO_4$ in TEDG. The fractions containing the bulk of the RNA polymerase activity were pooled to give fraction 5 (approximately 25 ml).

Storage. Peak fractions from the phosphocellulose column were pooled and ethylene glycol was added to a final concentration of 50% (v/v). The resulting solution was stored at −20°C.

Results and Discussion

Purification Procedure

Preparation of the Crude Extract. Grinding the wheat germ for more than 1 min in the Waring Blendor is unnecessary because no additional RNA polymerase activity is extracted, although a slight additional amount of protein is extracted. Further blending also results in undesirable heating and foaming of the homogenate. The first centrifugation sediments the gross debris. This was pelleted hard (10,000*g*) in order to trap a minimal amount of solution, thus increasing yields. The force and duration of this centrifugation are also sufficient to sediment the chromatin and any RNA polymerase bound to it. It has been shown that wheat germ contains two RNA polymerase activities (Jendrisak and Becker, 1973). Since the RNA polymerase activity in the crude extract is almost completely (96%) inhibited by 0.4 μg/ml of α-amanitin, the α-amanitin-resistant RNA polymerase activity in wheat germ (RNA polymerase I) may for the most part be bound to the chromatin and thus was removed in the first centrifugation. Extraction of RNA polymerases from wheat germ by the sonication method of Roeder and Rutter (1970) solubilizes RNA polymerase I, but does not solubilize significantly more RNA

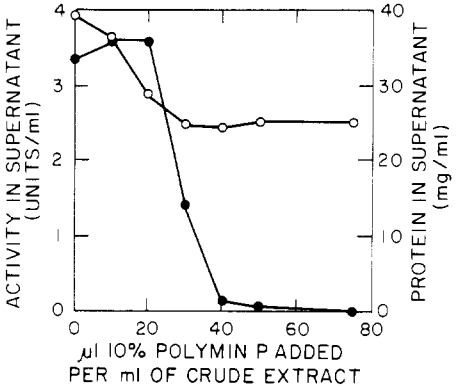


FIGURE 1: Precipitation of RNA polymerase and protein with Polymin P. The indicated volumes of 10% Polymin P were added to 1-ml aliquots of crude extract with thorough mixing. After centrifugation at 10,000*g* for 10 min (Sorvall SS-34 rotor; 10,000 rpm) 5-μl aliquots of the supernatants were assayed for RNA polymerase activity (●) and 20-μl aliquots were processed as described in Experimental Procedures to remove interfering substances prior to assaying for protein (○) by the method of Lowry et al. (1951).

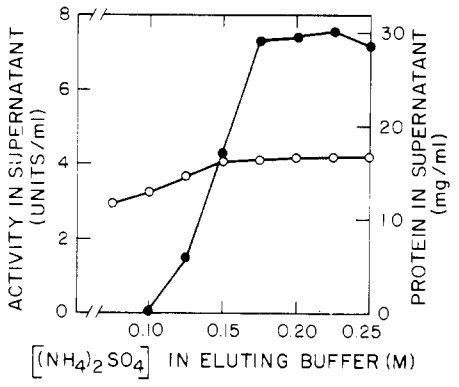


FIGURE 2: Elution of RNA polymerase and protein from the Polymin P precipitate with buffers of increasing salt concentrations. To 40 ml of crude extract was added 3.0 ml of 10% Polymin P with thorough mixing. The resulting mixture was divided into 5-ml aliquots and centrifuged at 10,000*g* for 10 min. The supernatants were discarded and the pellets were resuspended with the aid of a Dounce homogenizer in 2.5 ml of TED buffer containing the indicated concentrations of $(NH_4)_2SO_4$. After centrifugation at 10,000*g* for 10 min, 5-μl aliquots of the supernatants were assayed for RNA polymerase activity (●) and 20-μl aliquots were processed as described in Experimental Procedures prior to assaying for protein (○).

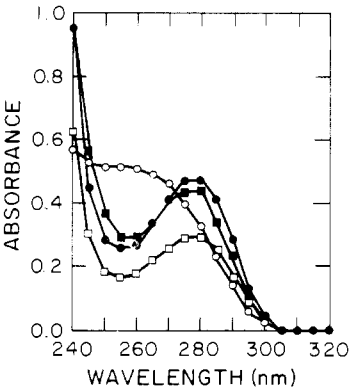


FIGURE 3: Ultraviolet absorption spectra of the crude extract and various fractions resulting from Polymin P precipitation: (a) crude extract (○); (b) 0.75% Polymin P supernatant (●); (c) 0.075 *M* $(NH_4)_2SO_4$ wash of the Polymin P precipitate (□); and (d) 0.20 *M* $(NH_4)_2SO_4$ eluate of the Polymin P precipitate (■). Samples were prepared for scanning by treating aliquots of the various fractions (a, 10 μl; b, 20 μl; c, 50 μl; d, 500 μl) with 10 ml of ice-cold 10% Cl_3CCOOH. After 10 min at 0°C, the precipitates were collected by centrifugation at 10,000*g* for 10 min and were dissolved in 2.5 ml of 1 *N* NaOH. The resulting solutions were neutralized with 2.5 ml of 1 *N* HCl and were buffered by adding 0.25 ml of 2.0 *M* Tris-HCl (pH 7.9). The solutions were scanned from 220 to 340 nm with a Beckman Acta III recording spectrophotometer in a 1-cm light path quartz cuvet against the appropriately constructed solvent blank. Scans were corrected for large particle and Rayleigh scatter by the extrapolation method of Leach and Scheraga (1960).

polymease II activity. Therefore RNA polymerase I in wheat germ appears to be tightly bound to the chromatin (solubilizable only with sonication) while RNA polymerase II may be mainly soluble and extractable without sonication. We have not observed any indication of RNA polymerase III like activity in crude extracts (tested for by looking for an activity inhibited by high concentrations of α-amanitin), nor on DEAE columns developed by salt gradient elution. From 1 kg of wheat germ one can extract 200 g of protein (Table I) and 40 g of nucleic acid as judged by an A_{280}/A_{260} of 0.6 (Figure 3, scan a) for the crude extract.

Polymin P Fractionation. The optimal conditions for precipitating the RNA polymerase from the crude extract with Polymin P were determined from the curve in Figure 1. Addition of 10% Polymin P (pH 7.9) to a final concentration of approximately 0.75% precipitates all of the nucleic acid, since the A_{280}/A_{260} of the resulting supernatant after centrifugation is 1.6 (Figure 3, scan b). All of the RNA polymerase activity and about 30% of the protein in the crude extract are also precipitated (Figure 1). Polymin P, which is a polyethylenimine and very basic, is expected to precipitate acidic molecules including nucleic acids, nucleoproteins (chromatin and ribosomes), and acidic proteins (including RNA polymerase II). The optimal amount of Polymin P is not invariant since it depends on the salt concentration and the amount of nucleic acid and protein in the extract. Application of this method toward purification of RNA polymerase from other sources should include an analysis of the type carried out in Figure 1. Once optimal conditions are established, the method is extremely reproducible. The Polymin P precipitate is very dense and cleanly sediments under the moderate centrifugation conditions employed here yielding a firm pellet and a clear supernatant.

The optimal conditions for eluting RNA polymerase from the Polymin P precipitate were determined from the curve in Figure 2 which shows the elution of RNA polymerase and protein from the Polymin P precipitate as a function of various salt concentrations. By first washing the Polymin P precipitate with a low salt buffer (TED + 0.075 *M* $(NH_4)_2SO_4$), a large bulk of protein is removed from the still precipitated RNA polymerase activity (Figure 2). The nucleic acid also remains in the precipitate since the $A_{280}/A_{260} = 1.6$ for the resulting supernatant (Figure 3, scan c). The Polymin P precipitate has a chalky consistency and is easily suspended with the Waring Blendor as described in Experimental Procedures. Extracting the washed precipitate with high salt buffer (TEDG + 0.20 *M* $(NH_4)_2SO_4$) releases the RNA polymerase activity into the supernatant, following centrifugation, with a small amount of protein (Figure 2, Table I), and no nucleic acid ($A_{280}/A_{260} = 1.6$; Figure 3, scan d). It is suggested that the procedure be immediately continued through the $(NH_4)_2SO_4$ precipitation step since half of the RNA polymerase activity in fraction 2 is lost after 48 hr. Again, it should be stressed that application of this method for the purification of RNA polymerase

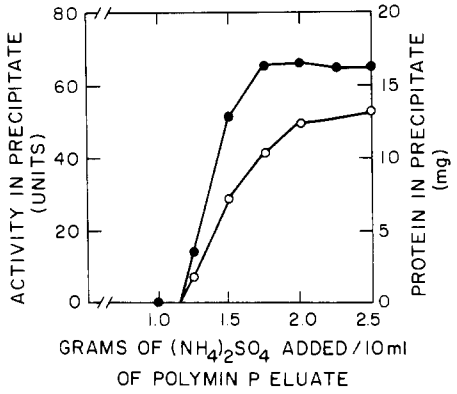


FIGURE 4: $(NH_4)_2SO_4$ precipitation of RNA polymerase and protein from the Polymin P eluate. The indicated amounts of solid $(NH_4)_2SO_4$ were added slowly with stirring to 10-ml aliquots of fraction 2. Stirring was continued 10 min after all of the salt had dissolved and the resulting mixtures were centrifuged at 10,000*g* for 10 min. The supernatants were discarded and the precipitates were dissolved in 1 ml of TEDG; 5-μl aliquots of the resulting solutions were assayed for RNA polymerase activity (●) and 20-μl aliquots were processed as described earlier prior to assaying for protein (○).

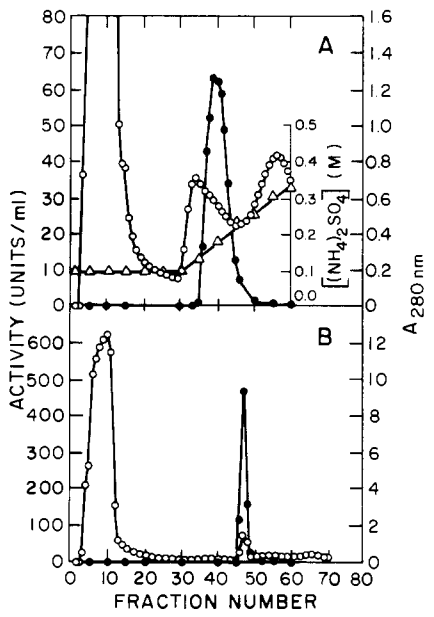


FIGURE 5: DEAE-cellulose chromatography of fraction 3 RNA polymerase. Fraction 3 protein (approximately 1.5 g) was applied to a 4.2 × 8 cm column of DEAE-cellulose and the RNA polymerase was eluted either with a salt gradient or by a salt step. For salt gradient elution (A), the sample was applied in TEDG + 0.1 *M* $(NH_4)_2SO_4$ and was followed by 250 ml of TEDG + 0.1 *M* $(NH_4)_2SO_4$ to wash out unbound protein. The RNA polymerase was then eluted with a linear gradient of $(NH_4)_2SO_4$ from 0.10 to 0.50 *M* in 1 l. of TEDG. Fractions of 20 ml were collected at a flow rate of 100 ml/hr. Tubes 33–50 contained the RNA polymerase activity eluting at between 0.15 and 0.25 *M* $(NH_4)_2SO_4$. Conditions for step elution of RNA polymerase from the DEAE-cellulose column (B) were as described in the text. Fractions of 15 ml were collected at a flow rate of 100 ml/hr. Tubes 1–45 contained the flow through material eluted with TEDG + 0.15 *M* $(NH_4)_2SO_4$. Tubes 46–49 contained the bulk of the RNA polymerase activity step eluted with TEDG + 0.25 *M* $(NH_4)_2SO_4$, which were pooled to give fraction 4. RNA polymerase activity (●); a_{280nm} (○); $(NH_4)_2SO_4$ concentration (△).

from other sources should include an analysis of the type carried out in Figures 1 and 2 in order to achieve the best results. RNA polymerase has been purified over 30-fold through this step and completely freed from nucleic acids with quantitative yield.

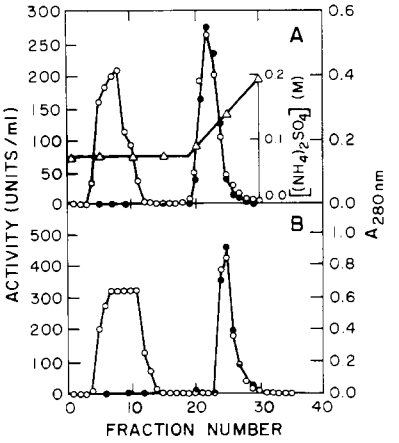


FIGURE 6: Phosphocellulose chromatography of fraction 4 RNA polymerase. Fraction 4 protein (90 mg) was applied to a column (1.8 × 10 cm) of phosphocellulose in TEDG + 0.075 *M* $(NH_4)_2SO_4$. The sample was followed by 50 ml of TEDG + 0.075 *M* $(NH_4)_2SO_4$ to wash out unbound protein. RNA polymerase was then eluted either by a salt gradient or by a salt step. For salt gradient elution (A), RNA polymerase was eluted with a linear gradient of $(NH_4)_2SO_4$ from 0.075 to 0.25 *M* in 100 ml of TEDG. Fractions of 7.5 ml were collected at a flow rate of 50 ml/hr. Tubes 21–25 contained the RNA polymerase activity eluting between 0.08 and 0.15 *M* $(NH_4)_2SO_4$. Conditions for step elution of RNA polymerase from the phosphocellulose column (B) were as described in the text. Fractions of 5 ml were collected at a flow rate of 50 ml/hr. Tubes 1–23 contained the flow through material eluted with TEDG + 0.075 *M* $(NH_4)_2SO_4$. Tubes 24–29 contained the bulk of the RNA polymerase activity step eluted with TEDG + 0.15 *M* $(NH_4)_2SO_4$, which were pooled to give fraction 5. RNA polymerase activity (●); A_{280nm} (○); $(NH_4)_2SO_4$ concentration (△).

$(NH_4)_2SO_4$ Precipitation. Optimal conditions for this step were determined from Figure 4. RNA polymerase was concentrated from about 2 l. (fraction 2) to about 150 ml (fraction 3) or roughly 13-fold by this step. Also achieved was a threefold purification with virtually quantitative recovery of RNA polymerase activity. The more concentrated protein solution allowed for more rapid DEAE-cellulose chromatography. Thus far, the purification has been performed with excellent yields in the absence of a stabilizing agent such as glycerol or ethylene glycol. This was probably due to the relatively high protein concentrations at all of these steps and the rapid and gentle method. Since large volumes are being dealt with in these initial steps, inclusion of large quantities of glycerol or ethylene glycol becomes expensive, as well as interfering with efficient centrifugation. After these first three steps, the RNA polymerase has been purified over 100-fold and concentrated over 25-fold with virtually quantitative yields. The $(NH_4)_2SO_4$ precipitation step is a convenient place to interrupt the procedure, if desired, since fraction 3 RNA polymerase activity is completely stable for several days at 0–4°C.

DEAE-Cellulose Chromatography. This is a very efficient step for the further purification of the RNA polymerase (Figure 5B). It resulted in a 15-fold purification with 80% recovery. High recovery during chromatographic steps required the presence of high glycerol or ethylene glycol concentrations (25% v/v) in buffers. The latter agent was chosen because of its lower viscosity and density, which facilitated all manipulations and resulted in faster flow rates during column chromatography. We have chosen to step elute (Figure 5B) the RNA polymerase from the column rather than gradient eluting it (Figure 5A) since this resulted in higher recoveries. Although a slight purification was sacrificed by this method, the RNA polymerase eluted

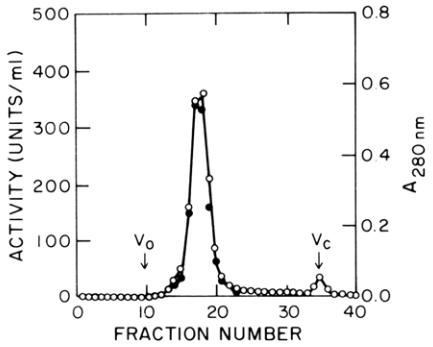
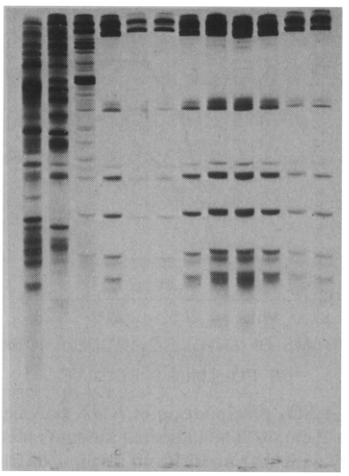


FIGURE 7: Bio-Gel A 1.5m chromatography of fraction 5 RNA polymerase. Fraction 5 protein was precipitated by adding solid $(NH_4)_2SO_4$ to a final concentration of 50% saturation (30 g of $(NH_4)_2SO_4$/100 ml of fraction 4) and gently stirring for 1 hr. The precipitate was collected by centrifugation at 20,000*g* for 30 min and dissolved in 5 ml of TEDG; 2.5 ml of the resulting solution (containing 12 mg of protein) was applied to a 2.6 × 28 cm column of Bio-Gel A 1.5m equilibrated with TEDG + 0.1 *M* $(NH_4)_2SO_4$. Fractions of 5 ml were collected at a flow rate of 10 ml/hr. The void volume (V_0) and column volume (V_c) were determined with Blue Dextran and $(NH_4)_2SO_4$, respectively, in a separate experiment. RNA polymerase activity (●); A_{280} (○).



a b c d e f g h i j k l

FIGURE 8: Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of RNA polymerase at various stages of purification. Gels containing 15% acrylamide were prepared and stained as described in the text: (a) fraction 2 protein from the Polymin P eluate, (b) fraction 3 protein from the $(NH_4)_2SO_4$ precipitate, (c) fraction 4 protein from the DEAE-cellulose column, (d) fraction 5 protein from the phosphocellulose column, (e–l) protein from 100-μl aliquots of fractions 14–21 from the Bio-Gel A 1.5m column profile of Figure 7.

at a high protein concentration which also appeared to contribute to its stability. Optimal conditions for step elution were determined from Figure 5A which shows the salt gradient elution profile. It can be seen that a single symmetrical peak of RNA polymerase activity eluted between 0.15 and 0.25 *M* $(NH_4)_2SO_4$. Brij 35 (a nonionic detergent) at a concentration of 0.1% in the applied sample improved the purification by preventing a slight aggregation of protein on the DEAE-cellulose column. The RNA polymerase through this step has been purified over 1500-fold with 75% yield of the initial activity (Table I). The A_{280}/A_{260} ratio of this fraction was 1.7.

Phosphocellulose Chromatography. This resulted in a 2.5-fold purification of RNA polymerase with approximately 80% recovery (Table I). We routinely step elute the RNA polymerase from this column (Figure 6B) since no further purification was achieved by salt gradient elution (Figure 6A). Step elution also simplified the chromatographic procedure and the RNA polymerase was eluted in a concentrated protein peak which usually resulted in higher yields. The A_{280}/A_{260} ratio of the purified RNA polymerase was 1.8.

Overall Purification, Yield, Recovery, and Stability

The overall purification of wheat germ RNA polymerase II from 1 kg of starting material is summarized in Table I. A final purification of 4000-fold relative to the crude extract was achieved. Each of the purification steps resulted in at least 80% recovery, leading to an overall recovery of approximately 65% of the initial activity in the crude extract and 30 mg of purified RNA polymerase. The purified RNA polymerase was stable for several months when stored in the manner described above.

RNA Polymerase Purity

The final purified RNA polymerase contained no detectable DNase, RNase, or protease activity when assayed as described in Experimental Procedures. Protein purity was estimated by gel filtration on Bio-Gel A 1.5m and dodecyl sulfate polyacrylamide gel electrophoresis. As shown in Figure 7, all of the protein from the phosphocellulose column coincided with the RNA polymerase activity when chromatographed on Bio-Gel A 1.5m. The small absorbance peak eluting with the salt contained no protein as determined in dodecyl sulfate polyacrylamide gels. The subunit pattern of the Bio-Gel A 1.5m column peak tube (Figure 8, gel i) was identical with that of the phosphocellulose fraction (Figure 8, gel d), indicating that no additional purification was achieved by gel filtration and that all of the polypeptides seen in fraction 5 protein are components of the purified enzyme.

The subunit pattern appears to be very complex for the wheat germ RNA polymerase. This complexity is seen in highly purified RNA polymerase preparations from several mammalian systems (Jacob, 1973; Chambon, 1974; Sklar et al., 1975). A detailed analysis of the subunit structure of wheat germ RNA polymerase II will be the subject of a future communication.

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References

Blatti, S. P., Ingles, C. J., Lindell, T. J., Morris, P. W., Weaver, R. F., Weinberg, F., and Rutter, W. J. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 649.

Burgess, R. R. (1969a), *J. Biol. Chem. 244,* 6160.

Burgess, R. R. (1969b), *J. Biol. Chem. 244,* 6168.

Burgess, R. R., and Jendrisak, J. J. (1975), *Biochemistry,* preceding paper in this issue.

Chambon, P. (1974), *Enzymes, 3rd Ed. 10,* 261.

Gissinger, F., and Chambon, P. (1972), *Eur. J. Biochem. 28,* 277.

Jacob, S. T. (1973), *Prog. Nucl. Acid Res. Mol. Biol. 13,* 93.

Jacob, S. T., Sajdel, E. M., and Munro, H. N. (1970), *Biochem. Biophys. Res. Commun. 38,* 765.

Jendrisak, J. J., and Becker, W. M. (1973), *Biochim. Bio-*

phys. Acta 319, 48.
Jendrisak, J. J., and Becker, W. M. (1974), *Biochem. J. 139,* 771.
Laemmli, U. K. (1970), *Nature (London) 227,* 680.
Leach, S. J., and Scheraga, H. A. (1960), *J. Am. Chem. Soc. 82,* 4790.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Roeder, R. G., and Rutter, W. J. (1970), *Nature (London) 224,* 234.
Schmincke, C. D., and Hausen, P. (1973), *Z. Naturforsch. Teil C 28,* 610.
Sklar, V. E. F., Schwartz, L. B., and Roeder, R. G. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 348.
Weaver, R. F., Blatti, S. P., and Rutter, W. J. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2994.
Weinmann, R., and Roeder, R. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790.
Zillig, W., Zechel, K., and Halbwachs, H. (1970), *Hoppe-Seyler's Z. Physiol. Chem. 351,* 221.

A Fluorometric Study of DNA-Bound Benzo[*a*]pyrene†

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ABSTRACT: Comparisons were made among the fluorescence spectra of DNA-bound benzo[*a*]pyrenes which were produced in vivo and in vitro. DNA from mouse skin treated with benzo[*a*]pyrene had a maximum emission beyond 400 nm, which was clearly distinguished from that of DNA-bound benzo[*a*]pyrene 4,5-oxide. The emission spectra from mouse skin were classified into two groups, type I and type II. The former was similar to the spectrum of benzo[*a*]pyrene, although the two maxima were shifted to longer wavelengths (410 and 435 nm). Type II was characterized by a broad peak around 430 nm. Type I and type II were obtained from different fractions of hydroxylapatite chromatography, but type I was changed into type II during storage. This suggests that type II is a modified product of type I. The emission spectra of both groups also were detected in in vitro activating systems, including photoirradiation, iodine treatment, and hydrogen peroxide treatment. Treatment of *Escherichia coli* with benzo[*a*]pyrene during culture produced only fluorescence of type I. Although the relationship between types I and II remains to be established, both types of fluorescence evidently indicate that the conjugated ring structure of the parent compound, benzo[*a*]pyrene, is preserved intact in DNA-bound benzo[*a*]pyrene. Several lines of evidence suggest that the proximate (active) form is an unidentified hydroxylated product including an oxy radical, but a cation radical cannot be completely excluded.

Polynuclear aromatic hydrocarbons represented by benzo[*a*]pyrene (B[*a*]P)[1] are important carcinogens in our environment. However, the active forms of their metabolites are still obscure in spite of many investigations. The candidates for the proximate (active) form of aromatic hydrocarbons are cation radicals, carbonium ions, epoxides, and oxy radicals. Cation radicals have never been detected among metabolites but in an in vitro model system they are able to be bound to nucleic acids (Hoffmann et al., 1970). Carbonium ions have been discussed only from a theoretical point of view (Dipple et al., 1968; Jeftic and Adams, 1970). K-Region epoxides were recently confirmed as metabolites of several aromatic hydrocarbons (Grover et al., 1971, 1972) and it was discovered that the 6-oxy radical was important in the metabolism of B[*a*]P (Nagata et al., 1968).

There are a number of activating model systems which include one or two active forms. Iodine produces cation radicals, while hydrogen peroxide, especially in combination with ferrous chloride, results in hydroxylation at several sites (Ioki, Kodama and Nagata, to be published), and photoirradiation forms the 6-oxy radical in the case of B[*a*]P (Inomata and Nagata, 1972). A model system which includes epoxide formation is not known at present, but organic synthesis of K-region and non-K-region epoxides can substitute for it (Sims, 1971, 1972; Waterfall and Sims, 1972; Dansette and Jerina, 1974). These in vitro model systems are superior to the enzymatic system using microsome preparation, because they simplify the analysis of the mechanism of activation and they provide marker materials for the analysis of carcinogen-bound macromolecules in vivo.

For characterizing the nature of the conjugates, the chromatographic analysis of radioactive hydrolysates is a useful tool (Baird and Brookes, 1973); however, more direct information on molecular structure is provided by the fluorometric method. In this paper the authors wish to describe the characteristic fluorescences of DNA-bound B[*a*]P which were obtained in vivo as well as in vitro.

Materials and Methods

B[*a*]P was purchased from the Nutritional Biochemical Corp. and purified by chromatography over an alumina column. 6-Hydroxy-B[*a*]P was prepared according to the method of Fieser and Hershberg (1938). B[*a*]P 4,5-oxide was a generous gift from Professor Okamoto of The University of Tokyo. [^{3}H]B[*a*]P (3.0 Ci/mmol, Radiochemical Center) was used for acid hydrolysis of B[*a*]P-bound DNA.

Highly polymerized calf-thymus DNA from Sigma

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[1] Abbreviation used is: B[*a*]P, benzo[*a*]pyrene.