# High Molecular Weight Deoxyribonucleic Acid Polymerase from Crown Gall Tumor Cells of Periwinkle (*Vinca rosea*)†

John M. Gardner‡ and Clarence I. Kado*

ABSTRACT: A high molecular weight (6 S) plant DNA polymerase from axenic *Vinca rosea* tissue culture cells has been purified 2200-fold and characterized. The enzyme has a molecular weight of 105 000 (±5000). Sodium dodecyl sulfate-acrylamide gel electrophoresis of the purified enzyme yields polypeptide subunits having molecular weights of 70 000 and 34 000. The purified enzyme has a pH optimum of 7.5; a cation requirement optimum of 6 mM $Mg^{2+}$ or 0.5 mM $Mn^{2+}$; an apparent requirement for $Zn^{2+}$; a $K_m$ of 1 μM for dTTP; and a 3.5-fold stimulation by 50 mM KCl. The enzyme is sensitive to *N*-ethylmaleimide (1 mM), heparin (0.1 μM), ethanol (5%), pyrophosphate (0.05 μM), and *o*-phenanthroline (0.1 mM) but is insensitive to rifamycin. Denatured DNA is found to be the best natural template, and only negligible activity can be demonstrated with the ribopolymer templates poly(dT)$_n$·poly(rA)$_n$ and p(dT)$_{10}$·poly(rA)$_n$. In addition to the polymerization reaction, the enzyme catalyzes a pyrophosphate exchange reaction. Antibody to calf thymus 6-8S DNA polymerase does not inhibit DNA polymerase from *Vinca rosea*, suggesting no antigenic relationships between the mammalian and plant enzymes.

There are numerous reports on DNA-dependent DNA polymerases from various microorganisms and mammalian sources. In comparison, there have been no detailed characterizations of purified DNA polymerase[1] from higher plant cells, although such activities have been described in various plant tissues (Dunham and Cherry, 1973; Keller et al., 1973; Srivastava and Grace, 1974; Stout and Arens, 1970; Tewari and Wildman, 1967). In view of the importance of DNA polymerases in plant growth and development, and in tumorigenesis, we report the purification and characterization of a high molecular weight 6S DNA polymerase from periwinkle (*Vinca rosea*) cells in axenic tissue culture.

## Materials and Methods

*Chemicals.* Calf thymus DNA was activated by the procedure of Ross et al. (1971). Five milligrams/ml of calf thymus DNA was incubated for 15 min at 37 °C with 0.2 mg/ml of pancreatic deoxyribonuclease in 0.01 M $MgCl_2$. Then NaCl was added to 1 M and the mixture was heated at 60 °C for 30 min. The DNA was extensively dialyzed against 0.01 M KCl and stored frozen. Calf thymus DNA was denatured by heating at 100 °C for 10 min, followed by quick freezing in a salt-ice bath. Cauliflower mosaic virus DNA (molecular weight of $4.4 \times 10^6$) was kindly provided by Steve Slack and Robert Shepherd, University of California, Davis. *Agrobacterium tumefaciens* DNA was purified as described previously (Kado et al., 1972).

Crystalline preparations of ribonuclease, ovalbumin, and trypsin were purchased from Worthington Biochemical Corporation. Bovine serum albumin (three times crystallized) was purchased from Pentex Corporation. Phosphorylase *a* and *Bacillus subtilis* α-amylase were purchased from Sigma. Actinomycin D was a gift of Merck, Sharp and Dohme. Rifamycin and rifamycin SV were purchased from Calbiochem. Deoxynucleoside triphosphates were purchased from Sigma, labeled deoxynucleoside triphosphates from Schwarz/Mann, and poly[d(A-T)$_n$], poly(dT)$_n$·poly(rA)$_n$, p(dT)$_{10}$·poly(rA)$_n$ from Miles Laboratories. Carrier-free $^{32}PP_i$, sodium salt, was purchased from New England Nuclear. Unless otherwise indicated, all chemicals were analytical reagent grade commercial preparations. *Escherichia coli* polymerase I was kindly provided by Arthur Kornberg, Stanford University, Calif. DEAE-purified γ-globulin active against calf thymus 6-8S DNA polymerase was very generously provided by Lucy Chang, University of Connecticut, Farmington. Normal rabbit γ-globulin was obtained from Antibodies Inc., Davis, Calif.

*Tissues.* Original clones of *V. rosea* tumor B6 tissue were kindly provided by Robert Manasse, Boyce Thompson Institute, New York, and clones of normal tissue were kindly donated by Armin Braun, Rockefeller University, New York. The normal tissues were cultured on agar media developed by Murashige and Skoog (1962) and by Wood and Braun (1962). All tumor lines were grown on either medium but without auxin and cytokinin. All cultures were grown at room temperature and harvested in log phase of growth (10-20 days).

*Buffers.* (1) TME: 50 mM Tris-Cl, pH 7.85 at 25 °C, 10 mM β-mercaptoethanol, 1 mM EDTA. (2) TMEG: TME plus 20% (v/v) glycerol. (3) TDEG: 50 mM Tris-Cl (pH 7.85), 1 mM EDTA, 1 mM dithiothreitol, and 40% glycerol.

*DNA Polymerase Assays.* DNA polymerase was assayed in 100 μl of the following reaction mixture unless otherwise stated: assay system A, 50 mM Tris-Cl (pH 7.85) at 25 °C: 15 mM $MgCl_2$ (or 0.5 mM $MnCl_2$), 1 mM dithiothreitol, 50 mM KCl, 0.1 mM each of dATP, dCTP, and dGTP, [*methyl*-$^3$H]dTTP (500-900 cpm/pmol), template (unless

† From the Department of Plant Pathology, University of California, Davis, California 95616. *Received September 18, 1975.* This investigation was supported by Grant No. CA-11526, awarded by the National Cancer Institute, DHEW.

‡ Recipient of National Institutes of Health Postdoctoral Fellowship 1F02CA54440-01. Present address: Department of Biology, American University, Beirut, Lebanon.

[1] The abbreviation DNA polymerase denotes DNA-dependent DNA nucleotidyltransferase (EC 2.7.7.7). Abbreviations used for nucleotide and polynucleotides are those of the IUPAC-IUB commission (1970). Abbreviations for homopolymers bearing the subscript *n* refer to chain lengths greater than 500. Definite subscript numbers refer to the chain length of chain nucleotides.

otherwise stated, calf thymus heat denatured DNA, 50 μg/ml), and enzyme protein. Assay system B: when RNA-dependent DNA polymerase was assayed, 0.5 mM $MnCl_2$ replaced $MgCl_2$ and from 0.006 to 0.06 OD unit of poly(dT)$_n$·poly(rA)$_n$ or p(dT)$_{10}$·poly(rA)$_n$ served as template. Incorporation was linear for at least 60 min at 37 °C and was proportional to enzyme protein input at the levels employed here (<40 μg/ml). However, crude extracts often showed some deviation from linearity between protein and activity. In all studies involving characterization of enzyme, 0.1–1.0 μg of phosphocellulose purified enzyme protein was used unless indicated otherwise. The reaction was run at 37 °C and an equal volume of cold 10% trichloroacetic acid containing 30 mM sodium pyrophosphate was added to stop the reaction. The mixture was filtered through glass fiber filters (Whatman type GF/C or Reeve Angel type RA), which were washed successively with 5% trichloroacetic acid plus 30 mM sodium pyrophosphate, 95% ethanol, and diethyl ether. Dried filters were counted in toluene based scintillation fluid in a Beckman spectrometer, Model LS-233.

The measurement of polymerase activity of crude extracts, except for minor modification, followed the procedure of Srivastava (1973). Tissue was ground in an ice-cold mortar with an equal volume of buffer (50 mM Tris-Cl, pH 7.85 at 25 °C, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10% glycerol, and 0.5 mM $Na_2EDTA$) and the extract was filtered through cheesecloth and Miracloth (California Biochemical Corp.). Any remaining unbroken cells were reextracted with 0.8 volume of buffer. The combined extracts were centrifuged at 4000*g* for 120 min and the pellet was washed twice in buffer (chromatin fraction). The supernatant was centrifuged at 20 000*g* for 10 min and the clear supernatant was used as soluble enzyme. One-half of the 20 000*g* supernatant was concentrated by slowly adding $(NH_4)_2SO_4$ to a final saturation of 80%, collecting the precipitate by centrifugation, and redissolving it in TMEG.

*Protein Determination.* Protein was determined by the method of Lowry et al. (1951).

*Nuclease Assay.* DNA exonuclease activity was assayed according to the procedure of Yoshida and Cavalieri (1971). The assay mixture (200 μl) included 50 mM Tris-Cl (pH 7.85), 1 mM dithiothreitol, 1 mM $MgCl_2$, and 10–20 μg of [$^{32}$P]DNA (3–7 × $10^4$ cpm/μg) from *Agrobacterium tumefaciens* strain 1D135 (ATCC 27912). To terminate the reaction, 0.5 mg of calf thymus DNA in 800 μl of 10% trichloroacetic acid was added and soluble acid-soluble radioactivity was counted by Cerenkov radiation. Background radioactivity from controls with no enzyme was subtracted from all values.

*Pyrophosphate Exchange Assay.* Exchange of $^{32}PP_i$ into deoxynucleoside triphosphates was assayed by a modification of the procedure of Chang and Bollum (1973). The assay mixture (400 μl) contained 50 mM Tris-Cl (pH 7.85), 50 mM KCl, 2 mM dithiothreitol, 6 mM $MgCl_2$, indicated concentrations of the four nucleoside triphosphates, 25 μg of calf thymus denatured DNA, and 0.2–1.0 mM sodium [$^{32}$P]pyrophosphate (specific activity from 31 cpm/mol to 124 cpm/pmol of pyrophosphate). After 60 min at 37 °C, 2 ml of 5% trichloroacetic acid containing 40 mM sodium pyrophosphate was added followed by 100 ml of 15% acid-washed charcoal. The charcoal was collected on glass filters. Filters were washed with 20 ml of trichloroacetic acid containing 40 mM $PP_i$ and 20 ml of $H_2O$, dried, and counted in toluene-based scintillation fluid. Parallel DNA polymerase assays were performed identically, except that $PP_i$ was unlabeled, dTTP labeled, and trichloroacetic acid precipitates were collected and washed as described for polymerase assays.

*Serological Reactions.* Polymerase preparations were treated with anti-calf thymus 6–8S DNA polymerase γ-globulin and assayed according to the procedure of Chang and Bollum (1972a). DNA polymerase (0.2–2.0 μg) in 0.05 M Tris-Cl (pH 7.85) and 20% glycerol was added to equal volumes of appropriate dilutions of γ-globulin (0.01–10 mg/ml) in 0.2 M NaCl and 0.05 M sodium phosphate (pH 7.5). The tubes were left at room temperature for 30 min, then transferred to 4 °C overnight. The assay components for DNA polymerase were added and incubated at 37 °C for 30 min.

*Sedimentation Velocity Centrifugation.* Linear sucrose density gradients (Martin and Ames, 1961) were made with either 5–20% sucrose or 7–16% sucrose in 0.05 M Tris-Cl (pH 7.85) or 0.1 M $KPO_4$ (pH 7.5) buffers containing 10% (v/v) ethylene glycol. Molecular weights were estimated using standard proteins: trypsin (24 000), ovalbumin (44 000), bovine serum albumin (68 000), *B. subtilis* α-amylase (96 000) and bovine γ-globulin (160 000). Equimolar amounts of $Zn^{2+}$ were added to the α-amylase preparations. Ethylene glycol was added to sucrose gradients to stabilize enzyme activity. This did not appreciably affect the semilogarithmic relationship between sedimentation and molecular weight.

*Gel Electrophoresis.* Acrylamide gels with sodium dodecyl sulfate were prepared and run according to the procedures of Weber et al. (1972). Molecular weight markers were as follows: phosphorylase *a* (96 000); bovine serum albumin (68 000); ovalbumin (44 000); trypsin (24 000). Gels were stained with Coomassie Blue, destained at 40–50 °C, and stored in 7.5% acetic acid–5% methanol. Gels were scanned in the Beckman Acta III spectrophotometer at 590 nm. Procedures for preparation of analytical acrylamide gels were as described by Gabriel (1971). In some cases, ethylene glycol (12% v/v, final concentration) was added to replace distilled water in the formula. Gels were run at 2 °C for 3–4 h at 2–3 mA/gel.

*pI Determination.* Isoelectric focusing was performed in a 100-ml capacity LKB 1800 focusing column with 1% ampholytes in 0–60% (v/v) glycerol gradients using pH ranges of 7–10 or 5–8. Length of runs were at least 96 h and power was maintained at 1 W or less. pH measurements were made immediately after fractions were collected. Fractions (20–40 μl) were assayed directly in the presence of 0.1 M Tris-Cl (pH 7.85) buffer.

*Column Chromatography.* DEAE-cellulose (Whatman DE-52 or Whatman DE-11) was washed with 0.5 N NaOH followed by water until neutral and then with 1.0 N HCl and water until neutral. Phosphocellulose (Whatman P-11) was washed with 1.0 N HCl followed by water until neutral and then with 0.5 NaOH and water until neutral. Both resins were then equilibrated with the appropriate buffer and DEAE-cellulose was degassed with nitrogen before use.

Soluble dialyzed enzyme extracts were adsorbed onto 2.5 × 15 cm columns of equilibrated DEAE-cellulose at a proportion of less than 30 mg of protein per g of resin. Each column was washed with 300 ml of TMEG and eluted with a 400-ml linear gradient from 0 to 0.4 M KCl in TMEG (pH 7.85) at a flow rate of approximately 1 ml/min. Fractions containing DNA polymerase activity were combined and dialyzed against TMEG containing 0.05 M KCl.

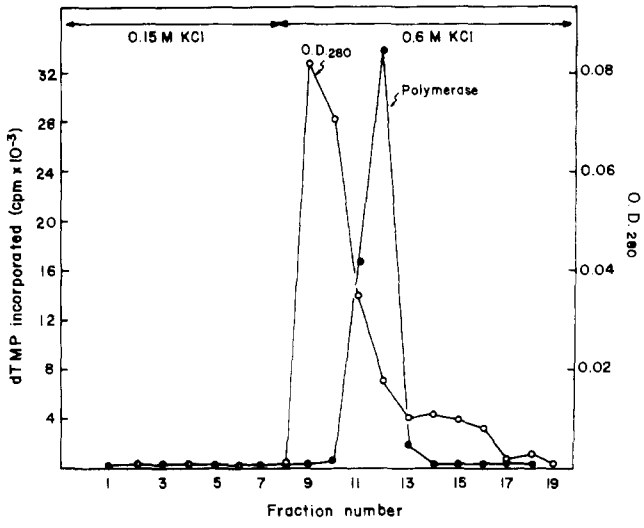


FIGURE 1: DNA-cellulose column elution profile of *V. rosea* DNA polymerase activity. A 2.5 × 8 cm column of DNA-cellulose was equilibrated with TMEG (see Materials and Methods) containing 100 μg/ml of bovine serum albumin and 0.15 M KCl. Enzyme from phosphocellulose column (0.62 mg of protein) was adsorbed on the column, and after a 500-ml wash with TMEG without bovine serum albumin, the KCl concentration was changed to 0.6 M. Fractions (5 ml) were collected and assayed with denatured DNA (system A). Most of the initial absorbance at 280 nM was due to DNA leakage (i.e., high 260/280 ratio) from the cellulose when the KCl concentration was raised to 0.6 M.

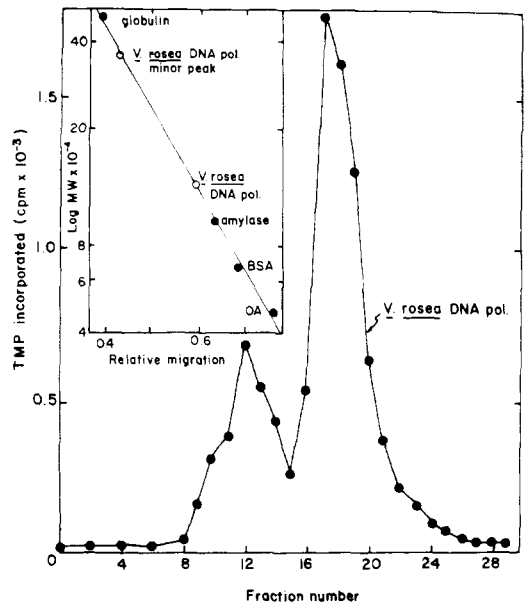


FIGURE 2: Sucrose density gradient profile of *V. rosea* 6S DNA polymerase and marker proteins (OA, ovalbumin; BSA, bovine serum albumin; amylase, *B. subtilis* α-amylase, and bovine γ-globulin). The enzyme was layered on a 5–20% (w/v) linear sucrose density gradient in 0.1 M $KPO_4$ (pH 7.5). The gradient was centrifuged in a SW50.1 rotor at 40 000 rpm for 16 h at 4 °C. Thirty 160-μl fractions were collected and 20-μl aliquots were assayed for DNA polymerase (system A) for 45 min at 37 °C.

The peak fractions from the DEAE-cellulose were dialyzed against 20 volumes of TMEG overnight and adsorbed on a 10 × 2.5 cm phosphocellulose column, previously equilibrated with TMEG containing 0.05 M KCl, at a ratio of less than 20 mg of protein/g of resin. The column was washed with 250 ml of TMEG containing 0.05 M KCl and eluted with a 400-ml linear gradient from 0.05 M KCl to 0.8 M KCl in TMEG (pH 7.85). The fractions containing polymerase activity were combined and dialyzed against TMEG and polymerase preparations were stored at −20 °C.

DNA-cellulose and DNA agarose-acrylamide were prepared according to Alberts and Herrick (1971) and Cavalieri and Carroll (1970), respectively. DNA was removed from tissue extracts by DEAE-chromatography or extraction with poly(ethylene glycol) (Alberts and Herrick, 1971). DNA polymerase and other DNA binding proteins were adsorbed onto a DNA-cellulose or DNA agarose-acrylamide column with TMEG containing 0.15 M KCl and washed with the same buffer until the optical density of the elute at 280 nm was <0.01. Both bacterial and plant DNA polymerases were then eluted from the column by raising the KCl concentration stepwise to 0.6 M and 3–5 ml fractions were collected. Active fractions were dialyzed against TMEG and stored at −20 °C.

## Results

### *Purification of DNA Polymerase*

All operations in the purification procedure were carried out at 4 °C

*Extraction.* The enzyme was purified solely from sterile *V. rosea* tissues grown on culture media. After ascertaining that the 6S enzyme was the major DNA polymerase activity in either normal, habituated, or crown gall tumor cell lines, we chose the latter as a source of highly purified 6S enzyme for the characterization studies herein since the tumor cells grow more rapidly on basic salts medium (Wood and Braun, 1962). Tissues were harvested and used immediately or stored frozen at −70 °C for later use. From 200 to 400 g of tissue was homogenized with an equal volume of TME in a motorized Potter-Elvehjem homogenizer. After filtering the extract through cheesecloth and Miracloth, the extract was made 0.8 M with KCl and 20% (v/v) with glycerol in TME. The extract was stirred for 8 h at 4 °C, then centrifuged at 20 000*g* for 20 min. In some cases, in order to reduce the volume of the high-salt extract, TME extracts were first centrifuged at 20 000*g* for 20 min and the pellet fraction was resuspended in 150–200 ml of TMEG plus 0.8 M KCl (followed by 8 h of stirring at 4 °C), whereas only 20% glycerol was added to the supernatant. The high-salt extracts were dialyzed for 10–12 h against TMEG and then were combined for the first chromatographic step.

Several extraction procedures were tested. Initially, Triton X-100 (0.1%) was included in TME when detection of RNA-dependent DNA polymerase was the main concern. High salt (0.8 M KCl) treatment of particulate matter was generally used in order that DNA polymerase adsorbed on membranes and chromatin would be extracted.

*DEAE Column.* When the crude enzyme preparation was adsorbed and eluted from DEAE-cellulose, only one peak of polymerase activity was observed. The unadsorbed material and material remaining on the column (eluted with 0.8 M KCl) were each checked for polymerase activity. Both portions were assayed, and also concentrated by dialysis against 80% ammonium sulfate and then assayed for polymerase activity. No polymerase activity was detected in either the unadsorbed or post-peak adsorbed material.

*Phosphocellulose Column.* Active fractions from the DEAE-cellulose were combined, dialyzed against TMEG overnight, adsorbed onto a 1.5 × 10 cm phosphocellulose

Table I: Purification of *Vinca rosea* 6S Polymerase from Sterile Tissue Cultures.

| Fraction | Total Protein (mg) | Activity[a] (units) | Specific Activity (units/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| I 40 000*g* crude supernatant[b] | 1237 | 74.2 | 0.06[c] | | 100 |
| II DEAE-cellulose | 94 | 58.2 | 0.62 | 10.3 | 78.4 |
| III Phosphocellulose | 2.37 | 62.5 | 26.4 | 440 | 84.2 |
| IV DNA-cellulose | 0.102 | 13.6 | 133 | 2217 | 18.3[d] |

[a] Assays were carried out as described in Materials and Methods with poly[d(A-T)$_n$] (1.0 $OD_{260}$ unit/ml) as template. Specific activity and concentration of dTTP were 1272 cpm/mol and 5 $\mu$M, respectively. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of dTTP into product in 30 min. [b] Enzyme extraction from 850 g of *V. rosea* tissue. [c] Incorporation was not perfectly linear with crude supernatants indicating the presence of possible inhibitors. [d] Dilute solutions of this enzyme fraction lost activity rapidly at −20 °C in 20% glycerol solutions (>50% in 7 days).

column, and eluted with a linear gradient from 0.05 to 0.8 M KCl in TMEG buffer. DNA polymerase eluted between 0.25 and 0.35 M KCl as a single relatively sharper peak than with DEAE-cellulose. Fractions before or after this peak showed no polymerase activity. The fractions possessing DNA polymerase activity also possessed nuclease activity, and these two enzyme activities cochromatographed on phosphocellulose. At least a 950-fold purification of polymerase activity was achieved when the peak fraction was assayed. Peak fractions were stored in 40% glycerol at −20 °C for 4–8 weeks and retained 60–80% activity during this time.

*Ammonium Sulfate Precipitation.* For electrophoresis studies, or other studies requiring concentrated enzyme, ammonium sulfate (30% saturation) was added to phosphocellulose peak fractions and the preparation was centrifuged. The supernatant was then dialyzed against 65% saturated ammonium sulfate at 0 °C for 12 h, the resulting precipitate was collected by centrifugation, and the pellet was frozen at −70 °C until use in assays and in electrophoretic analyses, or dissolved in 40% glycerol for storage at −20 °C.

*DNA-Cellulose Column.* Pooled peak fractions from phosphocellulose dialyzed against 0.5 M Tris-Cl (pH 7.85) containing 25% glycerol, 5 mM dithiothreitol, 1 mM EDTA, and 0.15 M KCl were placed on a 2.5 × 8 cm column of DNA-cellulose equilibrated with the same buffer including 100 $\mu$g/ml of bovine serum albumin. The column was then washed with 500 ml of the same buffer (without bovine serum albumin) and polymerase was eluted stepwise by raising the KCl concentration of the buffer to 0.6 M (Figure 1). The summary of polymerase purification through each column step (Table I) indicates that the greatest loss in polymerase recovery occurred at this step. The polymerase lost 50–80% of its activity when stored at −20 °C for 1 week under conditions of low protein concentration (10 $\mu$g/ml).

### *Physical Properties*

*Molecular Weight.* Purified *V. rosea* DNA polymerase sedimented slightly faster than the *B. subtilis* $\alpha$-amylase marker (mol wt 96 000). A molecular weight of 105 000 ± 5000 was estimated by velocity sedimentation analyses on both 5–20% and 7–16% linear sucrose density gradients (Figure 2) in 0.1 M potassium phosphate buffer (pH 7.5) employing protein standards of known molecular weights. Some gradients also contained 10% ethylene glycol which markedly stabilized enzyme activity. In some instances, a minor peak or shoulder around 8 S was observed as shown in Figure 2, suggesting an aggregation tendency similar to that of the 6–8 S mammalian polymerase rather than indicating any unique species of enzyme. A tentative nomenclature of 6 S for our high molecular weight enzyme will be used throughout this paper for convenience. An enzyme analogous to the 3S DNA polymerase of mammalian cells was not detected in our experiments with *V. rosea* callus cells; also we examined *Nicotiana tabacum* and found no 3 S enzyme despite the use of high salt to extract presumptive chromatin-bound 3 S enzyme.

*Gel Electrophoresis.* Purified enzyme from the peak fraction of the phosphocellulose column (ammonium sulfate precipitate) was subjected to electrophoresis in 7% polyacrylamide gels containing 12% polyethylene glycol at pH 4.3 and pH 7.3. A major stainable band coincident with polymerase activity was observed. Less than 5% of the polymerase activity was recovered from the pH 4.3 gels, over 50% from pH 7.3 gels, and no activity from gels containing sodium dodecyl sulfate. At pH 7.3 the enzyme protein constituting the major polypeptide band migrated less than 1 cm in a 7.5-cm gel. Activity was not recovered from gels that did not contain ethylene glycol. Electrophoresis at pH 8.3 and 9.3 resulted in aggregation or precipitation of the enzyme between the running and spacer gel. This aggregation behavior is identical with that observed for mammalian 6–8 S DNA polymerases (Haines et al., 1972; Brun et al., 1974). Upon further purification of the enzyme by stepwise elution on DNA-cellulose, a single major stainable band that was obtained in 7% gels (Figure 3) separated in dodecyl sulfate gels into two polypeptide bands with estimated molecular weights of 70 000 and 34 000 (Figure 4). No polypeptide band was observed in the 105 000 region.

*Isoelectric Point (pI).* The p*I* of the enzyme, determined by isoelectric focusing, was pH 6.8 in either pH 7–10 or pH 5–8 ampholytes. At least 95% of the enzyme activity was lost during the isoelectric focusing which may have been due to loss of enzyme (i.e., precipitation or aggregation) by conditions of low ionic strength, despite the presence of glycerol. The p*I* of the high molecular weight DNA polymerase from human KB cells is 5.6, in contrast to the p*I* of 9.2 for low molecular nuclear polymerase (Sedwick et al., 1972). High molecular weight polymerases have characteristically low isoelectric points (Loeb, 1974), and in this respect *V. rosea* 6S DNA polymerase is similar to the mammalian 6–8S enzymes.

### *Reaction Properties of 6S Polymerase*

*Template Preferences. V. rosea* polymerase, like other DNA polymerases, and in contrast to terminal deoxynu-

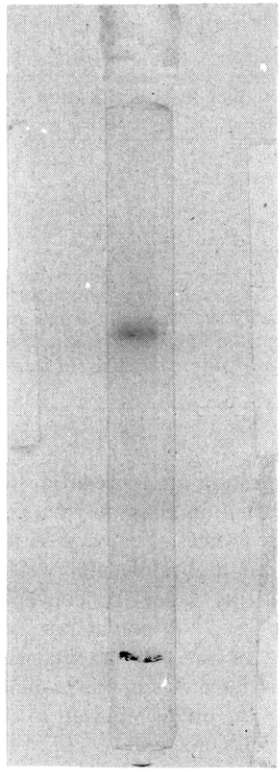

FIGURE 3: Polyacrylamide disc gel electrophoresis of *V. rosea* 6S DNA polymerase. Electrophoresis was performed in a 7.5% gel at pH 4.3, 2 °C, 2.5 *mA as described* by Gabriel (1971). After the methyl green tracking dye had migrated near the bottom toward the cathode (marked by the piece of copper wire as shown), the gel was removed and stained with Coomassie Blue.

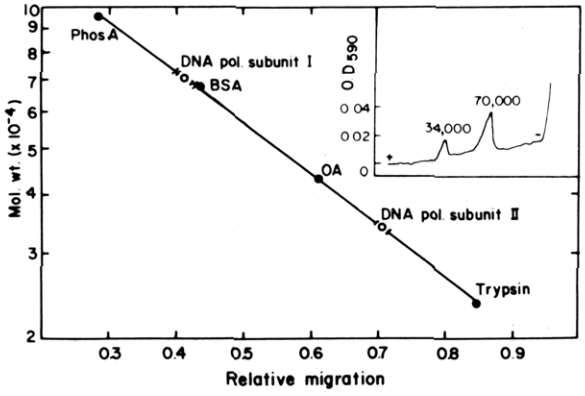


FIGURE 4: Separation of *V. rosea* DNA polymerase in acrylamide gel containing sodium dodecyl sulfate. Molecular weights were estimated *from semilog plot of migration distances with known standards:* phosphorylase A (phos A), bovine serum albumin (BSA), ovalbumin (OA), and trypsin. The molecular weight estimates of the disassociated enzyme is shown in the insert.

cleotidyl transferases, does not synthesize DNA in the absence of a template. The template preference of *V. rosea* polymerase is shown in Table II. The order of preference was denatured cauliflower mosaic virus DNA > denatured calf thymus DNA > poly[d(A-T)$_n$] > activated calf thymus DNA > native calf thymus DNA > (dT)$_{10}$·poly(rA)$_n$ and poly(dT)$_n$·poly(rA)$_n$. The optimum template concentration for denatured calf thymus DNA, which was used in most of the enzyme characterization studies was 50 μg/ml. The product of these reactions was destroyed completely by deoxyribonuclease but unaffected by ribonuclease.

Table II: Template Specificity of *Vinca rosea* 6S DNA Polymerase.[a]

| Template | Amount Added (μg) | Cpm Incorporated | dTMP Incorporation (pmol per μg of protein per h) |
|---|---|---|---|
| Native calf thymus DNA | 5.0 | 1 011 | 1.12 |
| Denatured calf thymus DNA | 5.0 | 12 225 | 13.5 |
| Activated calf thymus DNA | 1.0 | 2 590 | 2.78 |
| (−XTP) | | 81 | <0.1 |
| (−$Mg^{2+}$) | | 2 | <0.1 |
| (−Template) | | 20 | <0.1 |
| (−$K^+$) | | 835 | 0.8 |
| (10 mM *N*-ethylmaleimide) | | 737 | 0.8 |
| Poly[d(A-T)$_n$] | 0.01[b] | 7 680 | 8.5 |
| Poly(dT)$_n$·poly(rA)$_n$ | 0.01[b] | 26 | <0.1 |
| p(dT)$_{10}$·poly(rA)$_n$ | 0.01[b] | 90 | <0.1 |
| Native cauliflower mosaic[c] virus DNA | 2.5 | 2 084 | 2.3 |
| Denatured cauliflower mosaic virus DNA | 2.5 | 19 259 | 21.2 |
| *A. tumefaciens* B6 native DNA | 5.0 | 515 | 0.4 |
| *A. tumefaciens* B6 native denatured DNA | 5.0 | 3 765 | 4.15 |
| *V. rosea* native DNA | 5.0 | 258 | 0.3 |
| *V. rosea* denatured DNA | 5.0 | 3 395 | 3.7 |
| Yeast core RNA | 10.0 | 53 | <0.1 |

[a] Phosphocellulose purified enzyme (0.5 μg) was assayed for 30 min with the appropriate templates as described in Materials and Methods. Specific activity of dTTP was 910 cpm/pmol. [b] $OD_{260}$ units instead of μg. [c] This viral DNA contains homogeneous RNA segments (R. Hull and R. J. Shepherd, unpublished results) which could improve this DNA as a primer.

Table III: Deoxynucleoside Triphosphate Requirements for dTMP Incorporation by *Vinca rosea* 6S DNA Polymerase.[a]

| Nucleotide Additions | dTMP Incorporated per 30 min 37 °C (cpm) | % of Control |
|---|---|---|
| Control (dATP, dGTP, dCTP, dTTP) | 6911 | 100 |
| Control (minus dCTP, dGTP, and dATP) | 1229 | 18 |
| Control (minus dCTP and dGTP) | 2311 | 33 |
| Control (minus dGTP) | 4840 | 70 |

[a] Enzyme (0.5 μg) was incubated in the standard reaction mixture (system A) with denatured DNA unlabeled dATP, dGTP, and dCTP (0.1 mM each) and [$^3$H]dTTP (5 μM, 410 cpm/pmol) as indicated.

*Requirement for Deoxynucleoside Triphosphates.* When the polymerase reaction was run with only dTTP, incorporation into product was 18% of the control with dATP, dGTP, dCTP, and dTTP (Table III). These results indicate the relative absence of terminal deoxynucleotidyl transferase activity in the enzyme; partial synthesis in the absence of one or more deoxynucleoside triphosphates has been noted for other DNA polymerases (Loeb, 1974).

*$K_m$ for dTTP.* Polymerase activity with poly[d(A-T)$_n$] copolymer as template was measured at dTTP concentrations ranging from 0.1 to 500 μM. The dATP concentration was maintained at 200 μM. The $K_m$ of the polymerase for dTTP as extrapolated from a Wolff plot is 1 μM (Figure 5) with poly[d(A-T)$_n$] copolymer as template which is consid-

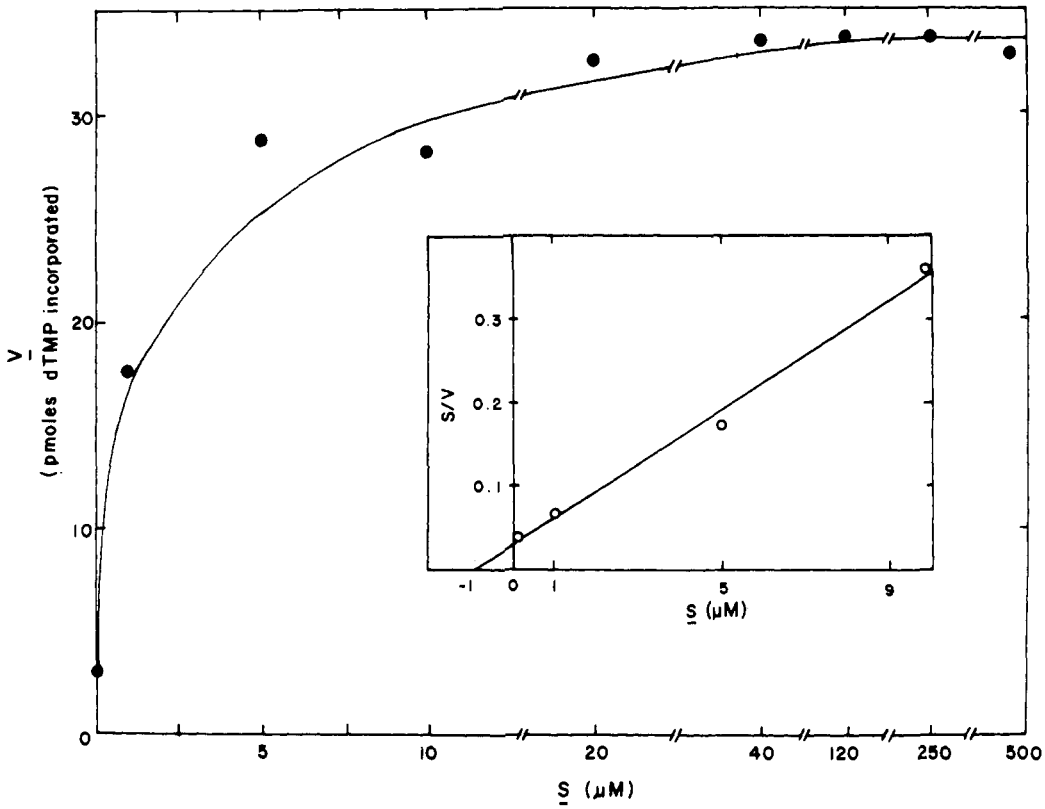


FIGURE 5: Michaelis and Wolff (insert) plots of *V. rosea* 6S DNA polymerase activity. Polymerase assays were performed with poly[d(A-T)$_n$] (4 $OD_{260}$ units/ml) as template (system A). The concentration of dATP was 400 μM. Specific radioactivity of dTTP was decreased from 62 × $10^3$ cpm/mol (100 nM) to 62 cpm/pmol (100 μM). Reaction volumes of assays with dTTP concentrations over 100 μM were increased to 200 and 400 μl to correct for the loss in specific radioactivity. $K_m$ for dTTP by extrapolation in the Wolff plot is 1 μM.

erably lower than $K_m$ values obtained for other DNA polymerases with the same copolymer template (Loeb, 1974). A Hill plot of the data indicated that there was only one binding site for dTTP ($n$ = 0.88).

*pH Optimum.* The rather sharp pH optimum was at pH 7.5 in both 0.1 M $KPO_4$ and 0.1 M Tris-tricine buffer. Tris-Cl buffer at 0.1 M was inhibitory. The optimum pH for 6-8S calf thymus DNA polymerase is 7.2 in contrast to the basic pH optimum of low molecular weight mammalian polymerase (Bollum et al., 1974).

*Cation Requirements Optima.* The $Mg^{2+}$ optimum was 6-15 mM, similar to that of 6-8S calf thymus polymerase (Bollum et al., 1974). The $Mn^{2+}$ optimum was 0.5 mM. The enzyme was at least 10 times as active with $MgCl_2$, than with $MnCl_2$, and combining optimal amounts of $Mg^{2+}$ and $Mn^{2+}$ completely inhibited enzyme activity.

$Zn^{2+}$ stimulated polymerase activity 34% when added in low concentration (50 μM) to reaction mixtures containing optimal amounts of $Mg^{2+}$. The inhibitory action of 1,10-phenanthroline indicates that $Zn^{2+}$ is associated with the purified enzyme (see inhibitors, below).

Monovalent cations such as $K^+$ and $NH_4^+$ are known to have a stimulatory effect on DNA polymerases (Keir, 1965). About 50 mM KCl stimulated 6S enzyme activity fourfold. The enzyme was not particularly sensitive to higher concentrations of $K^+$ (0.2 M KCl).

*Inhibitors and Competitive Effects of Nontemplates.* Table IV shows the results of the effects of various inhibitors of nucleic acid metabolism. The enzyme was sensitive to *N*-ethylmaleimide (60% inhibition at 1 mM; 90% inhibition at 10 mM); this is characteristic of mammalian high molecular weight polymerase in contrast to its comparatively weak inhibition of nuclear polymerases (Smith and Gallo, 1972). High levels of actinomycin D (200 μg/ml) inhibited activity by 90% but the inhibition was decreased by increasing the template concentrations; inhibition by this drug is known to be less efficient with denatured DNA as template (Reich and Goldberg, 1964). Actinomycin D was ineffective when poly[d(A-T)$_n$] was used as template. Rifamycin and rifamycin SV did not inhibit DNA polymerase activity.

Table IV: Effect of Various Inhibitors on *Vinca rosea* 6S DNA Polymerase.

| Addition[a] | Concn | Inhibition (%) |
|---|---|---|
| None | | 0 |
| Actinomycin | 100 μg/ml | 89 |
| *N*-Ethylmaleimide | 1 mM | 54 |
| Rifamycin SV | 200 mg/ml | 0 |
| Ethanol | 13% (v/v) | 88 |
| Heparin | 0.5 μM | 56 |
| *o*-Phenanthroline | 80 μM | 69 |

[a] Additions were made as indicated to the standard complete reaction mixture (system A) with 0.4 μg of enzyme and denatured DNA as template as stated in Materials and Methods.

In order to detect the presence of other possible polymerase species, heparin (a polyanionic carbohydrate) and ethanol, both potent inhibitors of high molecular weight mammalian DNA polymerase, but not low molecular weight nuclear polymerase (Lazarus and Kitron, 1973), were used. Both compounds inhibited *V. rosea* 6S polymerase at low

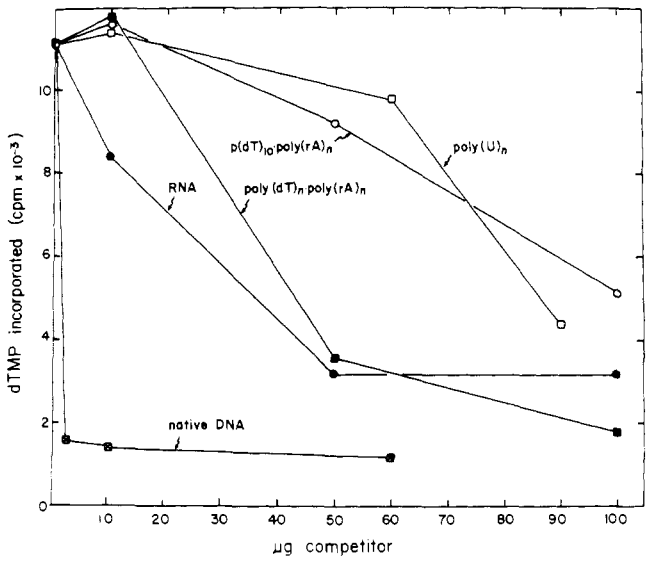


FIGURE 6: Inhibition of polymerization by competition of various nucleic acids with denatured calf thymus DNA as template. Native calf thymus DNA, RNA, poly(U)$_n$, poly(dT)$_n$·poly(rA)$_n$, and p(dT)$_{10}$·poly(rA)$_n$ were added to the reaction mixture (system A) with denatured calf thymus DNA (1 $\mu$g) as template for *V. rosea* 6S polymerase (0.4 $\mu$g) and incubated for 30 min at 37 °C.

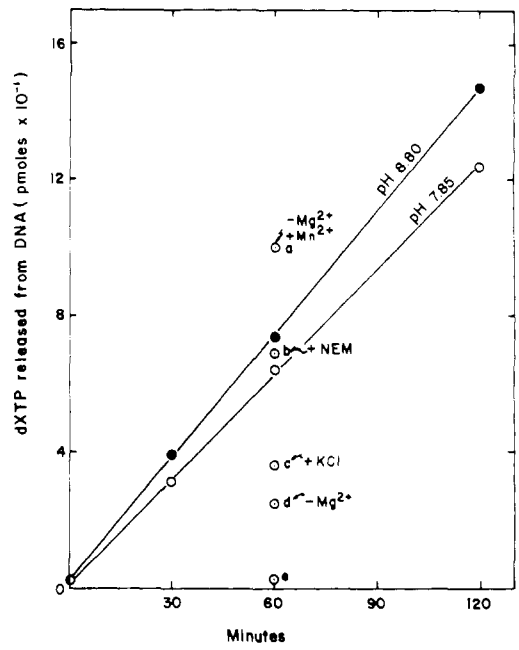


FIGURE 7: Time course and characteristics of nuclease activity associated with *V. rosea* 6S polymerase. Reactions were at pH 7.85 (○) or pH 8.80 (●) as described in Materials and Methods. Additions and deletions were as follows: (a) omission of $MgCl_2$ and addition of 0.5 mM $MnCl_2$; (b) plus *N*-ethylmaleimide, 1 mM; (c) plus KCl, 50 mM; (d) minus $MgCl_2$; (e) plus actinomycin D, 200 mg/ml.

concentrations and had similar effects on chromatin-bound DNA polymerase extracted with 0.8 M KCl. Heparin at 0.5 $\mu$M inhibited 6S polymerase and chromatin-bound polymerase 56 and 60%, respectively, and 13% ethanol inhibited these enzymes 88 and 92%, respectively. These results are compatible with the apparent absence of a chromatin-bound low molecular weight nuclear polymerase analogous to the 3S calf thymus enzyme.

Polynucleotide inhibition of polymerase can be a distinguishing characteristic for the enzyme's template preferences (Tuominen and Kenney, 1971). Various polynucleotides which are poor templates for the 6S enzyme competitively inhibited polymerase activity with denatured DNA as template. Figure 6 shows that native DNA competes out approximately 80% of the enzyme activity when it is at twofold higher concentration than the denatured DNA. Yeast core RNA and poly(dT)$_n$·poly(rA)$_n$ competed effectively at higher ratios of competitor to template; poly(U)$_n$ and p(dT)$_{10}$·poly(rA)$_n$ demonstrated even less competition. Poly(U)$_n$ competition at relatively low concentrations has been reported to be diagnostic for RNA-dependent DNA polymerases (Tuominen and Kenney, 1971). Poly(U)$_n$ inhibition was reversed by the addition of ribonuclease to the reaction mixture. It is apparent from these results that native DNA has high affinity for the enzyme despite being an ineffective template.

When 1,10-phenanthroline, a zinc chelator, was added to the reaction mixture at a concentration of 80 $\mu$M (in the presence of 6 mM $MgCl_2$), polymerase activity was inhibited 69% (Table IV). This indicates that $Zn^{2+}$ may be bound to the enzyme and is necessary for the 6S DNA polymerase, as has been shown with several other DNA polymerases (Loeb, 1974).

### *Associated Activities of 6S Polymerase*

*Nuclease Activity.* Nuclease activity was assayed according to Yoshida and Cavalieri (1971) with *A. tumefaciens* [$^{32}$P]DNA as substrate. This assay does not in itself distinguish between different nucleases. Nuclease activity was associated with the DNA polymerase eluted from the phosphocellulose column. Nuclease activity with native DNA was stimulated twofold by the presence of deoxynucleoside triphosphates, suggesting a possible synthesis-dependent activity. The nuclease was 50–60% more active with denatured DNA than with native DNA. The nuclease activity was favored by alkaline pH, required $Mn^{2+}$ or $Mg^{2+}$, and was inhibited 40% by 50 mM KCl and completely by actinomycin D (Figure 7). The specific activity on a total nucleotide basis is approximately 1/20th of the synthetic activity of the polymerase.

*Pyrophosphate Exchange.* Calf thymus 6–8S DNA polymerase catalyzes a pyrophosphate exchange into the triphosphate nucleotides, whereas the 3S nuclear polymerase is unable to do so (Chang and Bollum, 1973). Table V demonstrates that *V. rosea* 6S polymerase catalyzed pyrophosphate exchange (relative to polymerase activity) comparable to and greater than the exchange activities observed with *E. coli* polymerase I and *A. tumefaciens* polymerase, especially at lower $PP_i$ concentrations. Pyrophosphate was also a strong competitive inhibitor of the plant polymerase and the $K_i$ of $PP_i$ for the enzyme was approximately 60 $\mu$M.

### *Relationship to Mammalian Polymerase*

*Effect of Anti-Calf Thymus Polymerase $\gamma$-Globulin.* $\gamma$-Globulin specific for calf thymus 6–8S polymerase (Chang and Bollum, 1972a) was reacted with *V. rosea* 6S DNA polymerase as stated in Materials and Methods. The results indicated that there is no antigenic relationship between the high molecular weight polymerases from mammalian and plant sources, since as much as 10 mg/ml of purified anti-polymerase $\gamma$-globulin (a 1000-fold excess of purified globulin protein over polymerase protein and more than sufficient for complete inhibition calf thymus 6–8S polymerase) did not affect activity of the *V. rosea* 6S polymerase, as well as a range of lower antibody concentration (down to 40

Table V: Pyrophosphate Exchange.[a]

| Enzyme | $PP_i$ Concn | Exchange | Polymerization[b,c] | Polymerization: Exchange Ratio |
|---|---|---|---|---|
| *V. rosea* (6 S) | 1.0 | 53.5 | 30.7 | 0.574 |
| | 0.25 | 9.4 | 11.3 | 1.2 |
| *A. tumefaciens* | 1.0 | 92.0 | 285 | 3.1 |
| (6.3 S) | 0.25 | 29.1 | 339 | 11.5 |
| *E. coli* | 1.0 | 152 | 224 | 1.5 |
| (pol. I) | 0.25 | 17.9 | 197 | 11.0 |

[a] $PP_i$ exchange and DNA polymerase reactions were carried out under identical conditions as described in Materials and Methods except that [$^3$H]dTTP and unlabeled $PP_i$ were added to the polymerase assay. Reaction mixtures were incubated for 45 min at 35 °C. At 1 mM and 0.25 mM $PP_i$ concentrations, the respective specivic activities for $^{32}PP_i$ were 31 and 124 cpm/pmol and for [$^3$H]-dTTP they were 193 and 386 cpm/mol. Concentrations of each of the four nucleoside triphosphates were 25 and 6.25 $\mu$M at 1 mM and 0.25 mM $PP_i$, respectively. [b] Total nucleotide incorporation was obtained by multiplying dATP incorporated by 3.28 assuming percent GC of calf thymus DNA to be 39. [c] Inhibition of polymerization by $PP_i$ for *V. rosea*, *A. tumefaciens*, and *E. coli* polymerases was 70, 85, and 89%, respectively, at 1 mM $PP_i$ (0.1 mM total nucleotide), and 87, 80, and 89%, respectively, at 0.25 mM $PP_i$ (0.025 mM total nucleotide).

$\mu$g/ml of $\gamma$-globulin). Both 6–8S and 3.5S DNA polymerases from various mammalian sources are serologically related to calf thymus 6–8S polymerase (Chang and Bollum, 1972a).

## Discussion

We have purified a high molecular weight 6S plant DNA polymerase that comprises the major fraction of DNA polymerase in *V. rosea* normal and crown gall tumor callus cells. There are no distinctive differences between the 6S DNA polymerases of normal, habituated, and crown gall tumor callus cells (Gardner and Kado, in review). The 3.5S class of nuclear DNA polymerases, as found in some mammalian cells (Wang et al., 1974; Chang, 1973), was not detected in our preparations. *V. rosea* 6S DNA polymerase is serologically distinct from the calf thymus 6–8S DNA polymerase, but resembles the mammalian 6–8S polymerases in neutral pH optima, template preferences, elution behavior on DEAE-cellulose, and phosphocellulose, pyrophosphate exchange, isoelectric pH values below 7, sensitivities to inhibitors (*N*-ethylmaleimide, ethanol, heparin), requirements for $Mg^{2+}$ and endogenous $Zn^{2+}$, and sedimentation behavior in sucrose gradients.

The estimated molecular weight of the *V. rosea* DNA polymerase by rate sedimentation in sucrose gradients is 105 000 ± 5000; experiments with acrylamide gel electrophoresis containing sodium dodecyl sulfate (Figure 4) suggest that the enzyme is dissociated into two distinct subunits which combine 1:1 (molecular weights of 70 000 and 34 000) to form the 105 000 enzyme. The evidence is not conclusive because enzyme activity cannot be recovered from dodecyl sulfate gels and therefore enzyme activity has not been demonstrated with the two subunits resolved in dodecyl sulfate gels. Judging from various purification experiments in our laboratory, the specific activities of our enzyme preparations ranged from 364.7 to 925.1 units/mg of protein when calculated on a 1 h basis for the enzyme unit definition used in the papers cited below. Based on these values, our enzyme preparations are higher in specific activities than those of the cytoplasmic polymerases recently reported for calf thymus (Momparler et al., 1973), maize (Stout and Arens, 1970), and *Euglena gracilis* (Keller et al., 1973), comparable to those of chick embryo (Brun et al., 1974) and of rabbit bone marrow (Chang and Bollum, 1972b) and about onefold less than those of rat liver (Berger et al., 1971) and of human KB cells (Sedwick et al., 1972). Very high specific activities have been reported of the low molecular weight nuclear polymerase of calf thymus (Chang, 1973) and the polymerases of human blood lymphocytes (Smith and Gallo, 1972). Observations with *V. rosea* polymerase and mammalian 6–8S polymerase (Haines et al., 1972; Brun et al., 1974) suggest that these enzymes aggregate with themselves or perhaps with other proteins. However, the absence of protein at the 105 000 position in the dodecyl sulfate gel and the presence of two proteins with their combined molecular weights equal to 104 000 indicate that the enzyme may have a subunit composition. Definitive evidence awaits the purification of greater amounts of the plant enzyme. The 6–8S cytoplasmic polymerase from mammalian cells has not yet been purified to homogeneity, possibly due to its tendency to aggregate, and there is no definitive evidence concerning the possibility of subunit composition. Holmes et al. (1974) purified the high molecular weight polymerases from several mammalian tissues and obtained distinct multiple polymerase species with sedimentation coefficients ranging from 5 S to 8 S. However, their dodecyl sulfate gel electrophoretograms of these enzymes yielded a major protein band having a molecular weight of near 50 000. They suggest that the high molecular weight DNA polymerases are multiples of this subunit. The observation by Chang and Bollum (1972a) that 3.5S and 6–8S calf thymus polymerases are serologically related may also be significant in this respect.

Unlike many of the mammalian polymerases (Loeb, 1974), *V. rosea* DNA polymerase prefers denatured DNA to activated DNA. This template preference suggests the possibility that a nuclease is involved in replication, since 3′–5′ exonuclease is required to cleave the non-base-paired termini to expose the base-paired primer 3′ termini (i.e., single strands fold back for base pairing). Activated (deoxyribonuclease-gapped) DNA has many potential initiation sites without the requirement for exonuclease cleavage. Since the nuclease activity associated with the *V. rosea* polymerase has not been characterized as to its specificity or to its physical association with polymerase protein, experiments employing near homogeneous enzyme and specific terminally labeled polynucleotides are required. A 3′–5′ exonuclease is an integral component of *E. coli* polymerase I and is thought to serve a proofreading function (Brutlag and Kornberg, 1972).

Reversal of the DNA polymerase reaction should catalyze pyrophosphate exchange into deoxynucleoside triphosphate. The data in Table V demonstrate that *V. rosea* 6S DNA polymerase catalyzes pyrophosphate exchange to a greater extent than that of *E. coli* polymerase I and *A. tumefaciens* polymerase (6.5 S). It is interesting to note that calf thymus 6–8S polymerase catalyzes the exchange reaction, but the 3.5S nuclear polymerase does not (Chang and Bollum, 1973), thus demonstrating another similarity between *V. rosea* 6S polymerase and mammalian 6–8S polymerase. The competitive inhibition of *V. rosea* polymerase by $PP_i$ may be unrelated to $PP_i$ exchange activity, since

both 6–8S and 3.5S mammalian polymerases are inhibited by $PP_i$ (Chang and Bollum, 1973).

In an attempt to characterize the binding sites of polymerase for different templates, we have shown that templates which are unable to support significant polymerization with *V. rosea* 6S polymerase compete with the denatured DNA template and inhibit nucleotide incorporation (Figure 6). We interpret this inhibition as being a measure of the affinity of the enzyme for the template; this approach has previously been used with poly(U)$_n$ inhibition of potential RNA-dependent DNA polymerases (Tuominen and Kenney, 1971). Our polymerase activity with denatured DNA is inhibited nearly 80% when there was a twofold excess of native DNA. This may indicate that native DNA is a poor template for other reasons than its affinity for the enzyme; for example, free 3′-hydroxyl groups which are necessary for initiation may have little influence on the binding of template. On the other hand, the weak competition by poly(dT)$_n$·poly(rA)$_n$, poly(U)$_n$, RNA, and p(dT)$_{10}$·poly(rA)$_n$ suggests that affinity for the enzyme may be one of the limiting factors in their inability to act as templates.

The apparent $K_m$ of *V. rosea* polymerase for dTTP is 1 $\mu$M (Figure 5), at least seven times lower than the corresponding $K_m$'s reported for 6–8S and 3.5S mammalian polymerases (Loeb, 1974). Hill coefficients of less than one indicate that the enzyme has only one binding site for nucleotide substrate.

Previous literature on DNA polymerases in plant cells has not indicated any physical characteristics of these enzyme(s). Srivastava and Grace (1974) have recently observed DNA polymerase activity estimated to be 7 S from *N. tabacum* tissue cultures; however, this enzyme does not prefer denatured over native DNA whereas our 6S enzyme from *N. tabacum* and *V. rosea* tissue cultures prefers denatured DNA and is relatively inactive with native DNA. Srivastava and Grace (1974) also observed a low molecular weight polymerase; we have been unable to detect such an enzyme in our experiments. We have used the procedure described in Chang (1973) to extract chromatin associated enzyme and only obtained enzyme which was high molecular weight and sensitive to ethanol and heparin, reagents which can distinguish between the high and low molecular weight mammalian enzymes (Lazarus and Kitron, 1973; Chang, 1973). It is known, however, that the conditions under which cells grow can determine the proportion of 6–8S and 3.5S polymerase in mammalian cells (Chang et al., 1973), and that the DNA polymerase elution profiles and template preferences can change dramatically after washing plant tissues (Dunham and Cherry, 1973). It is also possible that small amounts of low molecular weight enzyme (<5%) may have gone undetected in our procedures. Such possibilities await further study.

The above characterization of the *V. rosea* 6S DNA polymerase will provide a basis for studies on DNA polymerizing enzymes in higher plant cells, in particular, DNA replication in defined tissue culture systems.

## Acknowledgments

We are very grateful to Lucy Chang for the gift of anti-polymerase $\gamma$-globulin and for many helpful suggestions in the preparations of the manuscript. Likewise, we thank Karl Drlica and Thomas Kornberg for their suggestions and criticisms. The excellent technical assistance of Patricia Okubara and Jesse Dutra is appreciated.

## References

Alberts, B., and Herrick, G. (1971), *Methods Enzymol. 21D,* 198.

Berger, H., Jr., Huang, R. C. C., and Irvin, J. L. (1971), *J. Biol. Chem. 246,* 7275.

Bollum, R. H., Chang, L. M. S., Tsiapalis, C. M., and Dorson, J. W. (1974), *Methods Enzymol. 29,* 70.

Brun, G., Rougeon, F., Lauber, M., and Chapeville, F. (1974), *Eur. J. Biochem. 41,* 241.

Brutlag, D., and Kornberg, A. (1972), *J. Biol. Chem. 247,* 241.

Cavalieri, L. F., and Carroll, E. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 807.

Chang, L. M. S. (1973), *J. Biol. Chem. 248,* 3789.

Chang, L. M. S., and Bollum, F. J. (1972a), *Science 175,* 1116.

Chang, L. M. S., and Bollum, F. J. (1972b), *Biochemistry 11,* 1264.

Chang, L. M. S., and Bollum, F. J. (1973), *J. Biol. Chem. 248,* 3398.

Chang, L. M. S., Brown, M., and Bollum, F. J. (1973), *J. Mol. Biol. 74,* 1.

Dunham, V. L., and Cherry, J. H. (1973), *Biochem. Biophys. Res. Commun. 54,* 403.

Gabriel, O. (1971), *Methods Enzymol. 22,* 565.

Haines, M. E., Wickremasinghe, R. G., and Johnson, I. R. (1972), *Eur. J. Biochem. 31,* 119.

Holmes, A. M., Hesselwood, J. P., and Johnston, I. R. (1974), *Eur. J. Biochem. 43,* 487.

Kado, C. I., Heskett, M. G., and Langley, R. A. (1972), *Physiol. Plant Pathol. 2,* 47.

Keir, H. M. (1965), *Prog. Nucleic Acid Res. Mol. Biol. 4,* 81.

Keller, S. J., Biedenbach, S. A., and Meyer, R. R. (1973), *Biochem. Biophys. Res. Commun. 50,* 620.

Lazarus, L. H., and Kitron, N. (1973), *J. Mol. Biol. 81,* 529.

Loeb, L. L. (1974), *Enzymes, 3rd Ed. 10,* 173.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.

Momparler, R. L., Rossi, M., and Labitan, A. (1973), *J. Biol. Chem. 248,* 285.

Murashige, T., and Skoog, F. (1962), *Physiol. Plant. 15,* 473.

Reich, E., and Goldberg, I. H. (1964), *Prog. Nucleic Acid Res. Mol. Biol. 3,* 184.

Ross, J., Scolnick, E. M., Todaro, G. J., and Aaronson, S. A. (1971), *Nature (London), New Biol. 231,* 163.

Sedwick, W. D., Wang, T. S.-F., and Korn, D. (1972), *J. Biol. Chem. 247,* 5026.

Smith, R. G., and Gallo, R. C. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2879.

Srivastava, B. I. S. (1973), *Biochim. Biophys. Acta 299,* 17.

Srivastava, B. I. S., and Grace, J. T., Jr. (1974), *Life Sci. 14,* 1947.

Stout, E. R., and Arens, M. Q. (1970), *Biochim. Biophys. Acta 213,* 90.

Tewari, K. K., and Wildman, S. G. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 689.

Tuominen, F. W., and Kenney, F. T. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2198.

Wang, T. S.-F., Sedwick, W. D., and Korn, D. (1974), *J. Biol. Chem. 249,* 841.
Weber, K., Pringle, J. R., and Osborn, M. (1972), *Methods Enzymol. 26C,* 1.
Wood, H. N., and Braun, A. C. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 1776.
Yoshida, S., and Cavalieri, L. F. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 200.

# Nuclear Magnetic Resonance Studies of the Solution Conformation of Nucleoside Diphosphohexoses and Their Components†

Che-Hung Lee and Ramaswamy H. Sarma*

ABSTRACT: The solution conformations of UDPG, UDPGN, UDPGal, UDPM, UDPGluc, UDPGalc, ADPG, ADPM, GDPG, GDPM, and CDPG and their components Glu-1-P, Gal-1-P, Man-1-P, Gluc-1-P, Galc-1-P, ADP, GDP, UDP, and CDP are studied by high resolution fast Fourier transform nuclear magnetic resonance spectroscopy with iterative computer line shape simulation. The following results were observed. (1) The six-membered ring is in $^4C_1$ chair form with the C(5′)–C(6′) bond in gg ⇄ tg equilibrium for the derivatives of glucose and mannose and gt ⇄ tg for those of galactose. (2) No conformational preference can be detected for C(1′)–O(1′) bond in hexose-1′-P moiety. (3) Chemical shift dependencies for the pyranoid ring protons and their structural and conformational relations are: (a) axial proton is at higher field than equatorial; (b) the shielding effect of a gauche vicinal hydroxyl group is stronger than a trans vicinal; (c) the vicinity of a hydroxyl group located more than three bonds away tends to shift the proton downfield. (4) The conformation of the nucleoside 5′-diphosphate part is [anti, $^2E \rightleftarrows {}^3E$, g′g′ ⇄ g′t′, g″g″ ⇄ g″/t″], with slight variation of each conformation occurring for individual compounds. (5) No significant interactions are detected between the hexose and nucleoside parts in the nucleoside diphosphohexoses, and the hexose and nucleoside components display the same conformational preference as they become integrated to form nucleoside diphosphohexoses.

The derivatives of sugar are important components of biological systems and as such there has been intense effort in the past to unravel the interplay among constitutional, configurational, and conformational aspects of sugar chemistry with a hope of understanding how these molecules are assembled and transformed during cellular processes (Hall, 1964; Horton et al., 1973; Lemieux and Lineback, 1963; Stoddart, 1971). Studies of the aqueous solution conformational dynamics of several sugar derivatives which are also antileukemic agents (Evans and Sarma, 1975; Lee et al., 1975; Wood et al., 1973) have shown that one could advance a conformational rationale for their mechanism of action. In the present paper we attempt to delineate the aqueous solution conformation of known nucleoside diphosphohexoses such as UDPG,[1] UDPGN, UDPGluc, UDPGal, UDPGalac, UDPM, ADPG, ADPM, GDPG, GDPM, and CDPG and their monomeric components. They are cofactors in the biosyntheses of oligosaccharides, polysaccharides, glycoproteins, and glycolipids (Mahler and Cordes, 1971). The present study is undertaken with a hope that investigators in the above area will be able to relate their biological functions vis-a-vis their conformation.

## Materials and Methods

The various materials used in the present study are obtained from commercial sources. $^1H$ nuclear magnetic resonance (NMR) spectra of UDPG, UDPGN, UDPGal, UDPGluc, UDPGalc, UDPM, ADPG, ADPM, GDPG, GDPM, and CDPG (0.1 M, pH 8.0, 30 °C) were recorded at 100, 270, or 300 MHz in the Fourier transform mode. Details of the instrumentation are described elsewhere (Sarma et al., 1973a, Sarma and Mynott, 1972, 1973). $^{31}P$ NMR spectra of these compounds were recorded at 40.8 MHz to double check the $^1H$–$^{31}P$ couplings. Similar experiments for 5′-ADP, 5′-GDP, 5′-UDP, 5′-CDP, Glu-1-P, Gal-1-P, Man-1-P, Gluc-1-P, and Galc-1-P were conducted at pH 8.0 and 5.0 at which the phosphate group is a dianion and a monoanion, respectively. In order to detect the influence of the phosphate at the anomeric position of hexose, α-$CH_3$-glucose, β-$CH_3$-glucose, α-$CH_3$-galactose, β-$CH_3$-galactose, and α-$CH_3$-mannose were employed for comparison. Analyses of the spectra were carried out using a LAOCN III computer program. The data thus derived were used to obtain line shape simulation using a program developed in this laboratory. In line shape simulation, the

† From the Department of Chemistry, State University of New York at Albany, Albany, New York 12222. *Received September 8, 1975.* This research was supported by National Cancer Institute of the National Institutes of Health Grant CA12462 and National Science Foundation Grant B-28015-001. This research was also supported in part by National Institutes of Health Grant No. 1-P07-PR00798 from the Division of Research Resources,

[1] Abbreviations used are: UDPG, uridine diphosphoglucose; UDPGN, uridine diphospho-*N*-acetylglucosamine; UDPGluc, uridine diphosphoglucuronic acid; UDPGal, uridine diphosphogalactose; UDPGalc, uridine diphosphogalacturonic acid; UDPM, uridine diphosphomannose; ADPG, adenosine diphosphoglucose; ADPM, adenosine diphosphomannose; GDPG, guanosine diphosphoglucose; GDPM, guanosine diphosphomannose; CDPG, cytidine diphosphoglucose; Glu-1-P, α-glucose 1′-phosphate; Gal-1-P, α-galactose 1′-phosphate; Man-1-P, α-mannose 1′-phosphate; Gluc-1-P, α-glucuronic acid 1′-phosphate; Galc-1-P, α-galacturonic acid 1′-phosphate.