complementary d(pPypPu) sequences. We verified in detail for d(pCpG) that a 2:1 complex is formed, and proposed a reaction mechanism similar to that advanced for actinomycin, namely, that the first-order step is a rearrangement of the 1:1 complex. A possible structural basis for the first order step is implied by the conclusion of Kreishman et al. (1971) that the complex of ethidium with single stranded poly(U) differs in geometry from the complex with double helix. By this interpretation the rearrangement step is conversion of the 1:1 complex from the single-stranded form to an intermediate able to accept a second dinucleotide and complete the miniature double helix.

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Studies on DNA α-Polymerase of Mouse Myeloma: Partial Purification and Comparison of Three Molecular Forms of the Enzyme†

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ABSTRACT: Activity of DNA α-polymerase in extracts from MOPC-104E was not associated with a single protein molecule, but with several molecular species that differed in isoelectric point. The three most abundant of these enzyme species were first separated from other DNA polymerases and then resolved from each other by repeated chromatography on diethylaminoethylcellulose columns. Next, with the use of glycerol gradient centrifugation and DNA-cellulose column chromatography, the three species were further purified to a state representing more than 5000-fold purification over the crude extract. These three highly purified enzyme species exhibited very similar catalytic properties, and the main activity of each species sedimented at the same rate (6–7S) in glycerol gradients containing 0.5 M KCl. Analysis of the polypeptide content of each species revealed that polypeptides of about 150 000 and 60 000 daltons cofractionated with the DNA polymerase activity. The multiple α-polymerase species did not appear to result from in vitro proteolytic cleavage, since multiple species were observed in extracts prepared under several different types of conditions, including the presence of the protease inhibitors, phenylmethanesulfonyl fluoride, or trasylol. The three species were recovered in about the same relative amounts from both the nuclear and cytoplasmic fractions of MOPC-104E, and it appeared that multiple species of α-polymerase were also present in extracts from fetal bovine liver.

The purification of DNA α-polymerase has been hampered both by the instability of the enzyme (Bollum et al., 1974) and by its molecular heterogeneity, which has now been observed in extracts from several mammalian cell types (Chang et al., 1973; Momparler et al., 1973; Ove et al., 1973; Holmes and Johnston, 1973; Yoshida et al., 1974; Holmes et al., 1974; Matsukage et al., 1974; Bollum, 1975; Hachmann and Lezius, 1975). Significant molecular heterogeneity, however, was not observed in some other mammalian tissues (Smith et al., 1975;

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Sedwick et al., 1975), suggesting that the heterogeneity may be tissue specific. The possible biological significance of the molecular heterogeneity and the relationship among the various molecular species of DNA α-polymerase are matters of current interest.

During our previous survey of DNA polymerases in the mouse myeloma MOPC-104E, we observed two forms of high-molecular-weight (>100 000 in 0.5 M KCl) enzyme that were *N*-ethylmaleimide sensitive and preferred activated DNA as template primer (Matsukage et al., 1974). These two enzymes, which could be separated by chromatography on a DEAE[1]-cellulose column, are α-polymerases according to the recently proposed nomenclature for mammalian cell DNA polymerases (Weissbach, 1975; Weissbach et al, 1975). The present study was undertaken to define the relationship between these two forms of DNA α-polymerase. First, the two relatively crude fractions of α-polymerase (designated D_2 and D_3) were isolated from MOPC-104E tumor by DEAE-cellulose chromatography as before (Matsukage et al., 1974). Further, ion-exchange chromatography then revealed that each fraction contained a mixture of at least three molecular species of α-polymerase. Based upon the chromatographic and catalytic properties tested, we concluded that the same species of DNA polymerase were represented in each of the two original crude fractions, D_2 and D_3. Therefore, in subsequent work, attention was focused on the relationship between the three molecular species in just one of the fractions, D_3.

In our initial work with myeloma DNA α-polymerase, we were unable to purify the enzyme activity more than 300-fold using conventional ion-exchange chromatographic procedures. However, we are now able to obtain enzyme in microgram quantities in a state representing a 5000-fold purification from the crude extract. This extent of purification is among the highest thus far claimed for an α-polymerase; it is achieved with the use of DNA-cellulose column chromatography, and by modifying procedures so that the enzyme is not frozen and thawed.

This improved purification procedure was exploited in order to study the relationship between the three separated species of α-polymerase mentioned previously. Each species was further purified by glycerol gradient centrifugation, then by DNA-cellulose and DEAE-cellulose column chromatography, and, finally, by polyacrylamide gel electrophoresis under nondissociating conditions. In this article, we report catalytic properties of three highly purified molecular species of α-polymerase and our initial attempts to understand their structure.

Experimental Procedures

Materials

Mouse myeloma MOPC-104E, in solid tumor, was grown as previously described (Wilson et al., 1974). The tumors, weighing about 2 g each, were stored whole in liquid nitrogen for about 1 month before use. For the subcellular fractionation experiments, fresh tumor that had not been frozen was used. Fetal calf liver was obtained from Roth Products, Inc., Gwynedd, Pa. Calf thymus DNA, from Sigma Chemical Co., was activated as a template primer for MOPC-104E α-polymerase by the method described by Schlabach et al. (1971). *Escherichia coli* DNA-dependent RNA polymerase, at greater than 90% purity, was a gift from Dr. P. Qasba, NIH. Trasylol (basic trypsin inhibitor or aprotinin), isolated from beef lung, was obtained from Sigma Chemical Co. Phenylmethanesulfonyl fluoride, from Sigma Chemical Co., was dissolved in 2-propanol at a concentration of 40 mM and this solution was diluted 20-fold in the final solution used in experiments. All other materials were as described previously (Matsukage et al., 1975) or were from standard commercial sources.

Methods

Assays for DNA Polymerase Activity. Two types of reaction conditions are described: assay no. 1 was used in the purification procedures and assay no. 2 was used in the examinations of catalytic activity. Unless specified otherwise, incubations were at 37 °C in silicone-treated soft glass tubes (10 × 75 mm). In assay no. 1, each reaction was incubated for 60 min in a final volume of 25 μl and contained 50 mM Tris-HCl buffer, pH 7.8, 40–50 mM KCl, 6 mM magnesium acetate, 1 mM DTT, 15% glycerol, 400 μg/ml of bovine plasma albumin, 200 μg/ml of activated calf thymus DNA, 0.5 mM each of dATP, dCTP, dGTP, and 0.1 mM [^{3}H]dTTP (500 cpm/pmol), and 1–5 μl of a diluted enzyme preparation. In assay no. 2, each reaction was incubated for 15 min in a final volume of 15 μl and contained 20 mM Tris-HCl buffer, pH 7.5, 50 mM KCl, 2 mM magnesium acetate, 1 mM DTT, 10% glycerol, 15 μg/ml of bovine plasma albumin, 5 μg/ml of activated calf thymus DNA, 15 μM each of dATP, dCTP, dGTP, and 15 μM [^{3}H]dTTP (11 500 cpm/pmol), and diluted enzyme preparation. After reactions were terminated by freezing, reaction mixtures were treated with a solution containing 10% Cl_3CCOOH at 4 °C and radioactive reaction products were measured by the method described by Matsukage et al. (1975). This procedure involved collection of cold acid-treated [^{3}H]DNA directly on nitrocellulose filters (Schrier and Wilson, 1975); it did not include the centrifugation step that was used previously in the collection of products from reactions containing poly(rA)·oligo(dT) as template primer (Matsukage et al., 1974; Wilson et al., 1974). The centrifugation step was omitted because, in reactions with activated DNA as template primer, most of the DNA product (90–95%) was not collected in the pellet and, therefore, was lost.

Preparation of Extracts. Unless indicated otherwise, extracts were prepared as follows. The frozen tumors were thawed at 25 °C and all subsequent procedures were carried out at 0–4 °C. The tumors were rinsed with solution A (50 mM Tris-HCl, pH 7.7, 1 mM DTT, 1 mM EDTA) containing 25 mM KCl, minced into small pieces, and then mixed with 4 volumes of solution A containing 500 mM KCl. The mixture was homogenized with three strokes of a motor-driven Teflon-glass homogenizer and then with three strokes of a hand-operated tight-fitting ground-glass homogenizer. The homogenate was sonicated for 5 min at 4 °C in a Raytheon ultrasonic oscillator (Model No. DF 101) and then centrifuged at 32 000*g* for 30 min. The supernatant fraction was dialyzed for 16 h against 20 volumes of solution A and fractionated with ammonium sulfate to obtain the precipitate that formed between 25 and 65% saturation. This precipitate was dissolved in solution A containing 25 mM KCl; the resulting solution was dialyzed and then used in experiments, as described in Figure

[1] Abbreviations for nucleotides and synthetic polynucleotides are according to the IUPAC-IUB Commission (1970). DNA polymerase, deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7; RNase H, ribonuclease H; DNase, deoxyribonuclease; DTT, dithiothreitol; MOPC-104E, a mineral oil plasmacytoma; KP, potassium phosphate buffer; DEAE-cellulose, diethylaminoethylcellulose; BPA, bovine plasma albumin; PC, phosphocellulose; ATP, CTP, GTP, adenosine, cytidine, and guanosine 5′-triphosphates; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

TABLE I: Summary of Purification of DNA α-Polymerase from MOPC-104E.

Fraction and Illustration	Protein (mg)[b]	DNA Polymerase Act. Units[c] (%)	Sp Act. (units/mg)	Fold Purification
Crude extract[a]	35100	214.9 (100)	0.006	1
30 000g supernatant	23260	247.5 (115)	0.01	1.7
1st DEAE-cellulose[d] (D3 fraction)	5100	136.0 (63)	0.026	4.3
Phosphocellulose[e] (D3P fraction)	1342	92.3 (43)	0.068	11.3
Hydroxylapatite[e] (D3PH fraction)	134	149.9 (70)	1.12	186.6
2d DEAE-cellulose[f] (D3PHD fractions)				
form A	7.3	31.4 (14.6)	4.3	717
form B	26.0	86.1 (40)	3.3	550
form C	9.2	39.5 (18.4)	4.3	717
3d DEAE-cellulose[g] (D3PHDD fractions)				
form A	2.8	4.6 (2.1)	1.7	275
form B	8.6	8.4 (8.6)	2.1	357
form C	2.5	10.7 (5)	4.3	717

[a] 530 g of MOPC-140E was used. [b] Determined by the method described by Lowry et al. (1951). [c] 1 unit = 1 μmol of dNMP incorporated/h using assay no. 1 described under Methods; these calculations were made with the assumption that dTMP incorporation was 25% of total dNMP incorporation. [d] See Figure 1. [e] Not shown. [f] See Figure 3. [g] See Figure 4a–c.

1. The fractionation scheme used in the purification is summarized in Table I.

Ion-Exchange Column Chromatography. Except as noted, all procedures were carried out of 0–4 °C according to the following specifications. The columns were adjusted so that they contained 1 ml of resin volume (bed volume) for each 10 mg of extract protein chromatographed. Before chromatography, enzyme extracts were dialyzed against the same buffer with which the column was equilibrated and any precipitate formed during the dialysis was removed by centrifugation at 10 000g for 10 min. After applying the extract, columns were washed with a volume of the equilibration buffer equal to five times the bed volume of the column. Each elution was performed in 8–9 h using a linear gradient of a total volume equal to seven times the bed volume of the column. Each fraction in the eluate contained about 2% of the total gradient volume.

DEAE-cellulose columns were loaded with extracts containing 20 mM KP, pH 7.5, 1 mM DTT, and 20% glycerol, and they were eluted with 20–400 mM KP gradients at pH 7.5 containing 1 mM DTT and 20% glycerol. Phosphocellulose columns were loaded with extracts containing 50 mM KP, pH 6.8, 1 mM DTT, and 20% glycerol, and they were eluted with 50–700 mM KP gradients at pH 6.8 containing 1 mM DTT and 20% glycerol. Hydroxyapatite columns were loaded with extracts containing 5 mM KP, pH 7.0, 500 mM KCl, 1 mM DTT, 1 mM EDTA, and 20% glycerol, and they were eluted with gradients of 5 mM KP (pH 7.0) to 100 mM KP (pH 6.4) in the same solution.

For concentrating and storing enzymes, fractions containing activity were pooled, as shown in the figures, and proteins were precipitated with ammonium sulfate at 65% saturation; the precipitates were dissolved in a minimal volume of 50 mM KP_i, pH 6.8, 1 mM DTT, and 50% glycerol, so that the protein concentration was 20–25 mg/ml, and the solutions were stored at −20 °C.

Isoelectric Focusing. Isoelectric focusing was performed by the method described by Godson (1970). Ampholine gradients between pH 3 and 10 were formed in 8.1 ml, 3–45% linear sucrose gradients containing 5 mM 2-mercaptoethanol, and 5% glycerol. Electrofocusing was for 5 h at 4 °C at an initial voltage of 500 V.

Glycerol Gradient Centrifugation. Samples in a volume of 250 μl were layered over linear 5-ml 10–30% glycerol gradients. Both the samples and the gradients contained 50 mM Tris-HCl, pH 7.7, 500 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 0.2% Tween 80.

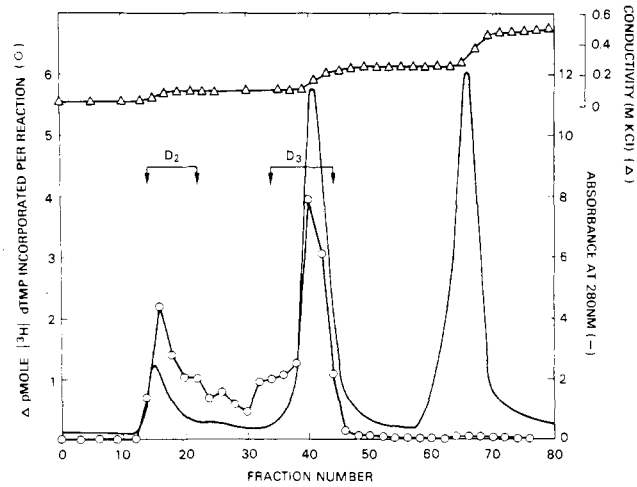

FIGURE 1: Preparative DEAE-cellulose column chromatography. After ammonium sulfate fractionation of the S30 extract, the dialyzed solution containing the enzyme activity and 2330 mg of protein was applied to a DEAE-cellulose column. The column was washed with 5 bed volumes of 25 mM KCl in solution A and then the stepwise elution was performed by changing the KCl concentration in the buffer to 100 mM, then to 250 mM, and, finally, to 500 mM. The flow rate was 40 ml/h, and each fraction contained 15 ml. DNA polymerase activity in 0.5 μl of fractions was measured by assay no. 1. The fractions D2 and D3, as designated in the figure, were pooled separately and stored as described under Methods. The DNA polymerase activity recovered in the D2 fraction and D3 fraction was 26 and 63%, respectively, of the original activity applied to the column.

Centrifugation was in nitrocellulose tubes at 50 000 rpm for 22 h at 2 °C in a Beckman SW 50.1 rotor. Fractions containing 150–200 μl were collected from the bottom of the tube and tested for refractive index and DNA polymerase activity by assay no. 1, using 0.1-μl portions. Bovine plasma albumin was analyzed in an accompanying tube and all sedimentation values reported represent migration distance relative to the migration of albumin; in making the calculations, it was assumed that the $s^0_{20,w}$ of the albumin was 4.3 and that the distance of migration and the $s^0_{20,w}$ were linearly related. Calculations were made this way in spite of the fact that such a linear relationship

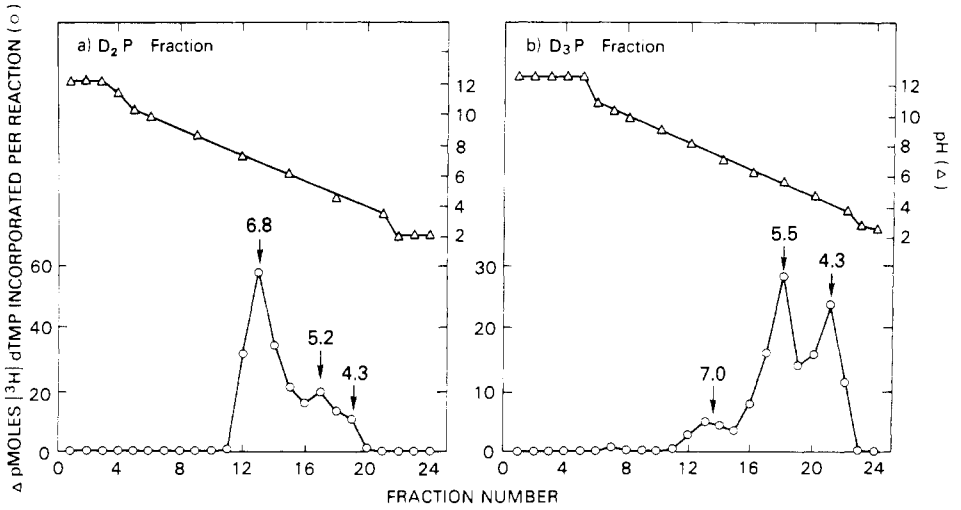

FIGURE 2: Analysis of DNA polymerase activities in the D2P and D3P fractions by isoelectric focusing. A 250-μl portion of each stocked fraction was mixed with one of the ampholine–sucrose solutions before the sucrose gradient was poured into the focusing device. The experiments were performed as described under Methods. DNA polymerase activity in 0.2-μl of fractions was measured by assay no. 1 without removal of the ampholines. During the focusing, no precipitation of protein was detected by visual inspection.

was not the case in the gradient system described above (see Figure 6d).

Polyacrylamide Gel Electrophoresis under Native and Denaturing Conditions. The preparation containing the A, B, and C species after the DNA-cellulose–DEAE-cellulose step was thawed and then further concentrated using a small DEAE-cellulose column and elution with 200 mM KP, pH 7.5, 20% glycerol, and 1 mM DTT. Each sample containing the concentrated enzyme was divided into two portions, and each portion was applied to a separate cylindrical 8% polyacrylamide gel (0.6 × 12 cm). After electrophoresis at 10 mA/gel for 10 h at 4 °C according to the method described by Brown (1969), one of the gels containing each enzyme species was stained with Coomassie brilliant blue (R-250) (Fairbanks et al., 1971) and the other gel was fractionated into 2-mm portions using a Gilson automatic gel fractionator. DNA polymerase was eluted from each fraction by mixing the gel fragments with 300 μl of 50 mM Tris-HCl buffer, pH 7.7, 0.1 mM EDTA, 1 mM DTT, 1 mg/ml of bovine plasma albumin, and 20% glycerol and allowing the mixtures to stand for 6 h at 0–1 °C. DNA polymerase was located by measuring activity in 1-μl portions of the eluate using assay no. 1.

For analysis of polypeptide content by sodium dodecyl sulfate–urea polyacrylamide gel electrophoresis (Dunker and Rueckert, 1969; Weber et al., 1972), a fraction from a native gel (described above) was mixed with 1 ml of 0.1% sodium dodecyl sulfate and allowed to stand for 3 h at 25 °C. The solution was removed and the gel fragments were reextracted with 0.5 ml of 0.1% sodium dodecyl sulfate. The pooled extracts, or a fraction from a glycerol gradient, were first dialyzed for 16 h against 10 mM NaP, pH 7.2, containing 0.1% sodium dodecyl sulfate at 25 °C, and then lyophilized. The residue was dissolved in 10 μl of H_2O, 1 μl of 20% 2-mercaptoethanol was added, and the solution was heated for 2 min at 100 °C. After cooling to 25 °C, a small amount (0.5–1 mg) of solid sucrose and tracking dye was added, and the solution was applied to a microscale 10% polyacrylamide gel that had been formed in the presence of 100 mM NaP, pH 7.2, 0.1% sodium dodecyl sulfate, and 5 M urea in a slab gel chamber (gel size, 15 × 15 cm and 0.1-cm thickness). After electrophoresis (9 h at 50 V) at 25 °C in the presence of 100 mM NaP, pH 7.2, and 0.1% sodium dodecyl sulfate, gels were stained and destained twice with 0.1% Coomassie brilliant blue (R-250) in 10% acetic acid, 25% and 10% 2-propanol, and 10% acetic acid, respectively. Marker proteins were the following: 0.5 μg of *E. coli* DNA-dependent RNA polymerase, which was composed of β', β, σ, and α subunits of assumed molecular weights 160 000, 150 000, 90 000, and 41 000, respectively; 0.1 μg of bovine plasma albumin (68 000 mol wt); 0.1 μg of α-chymotrypsinogen (23 700 mol wt); 0.2 μg of H-chain γ-globulin (55 000 mol wt); 0.1 μg of ovalbumin (45 000 mol wt); 0.2 μg of cytochrome *c* (12 500 mol wt).

Subcellular Fractionation. A 20% homogenate was prepared using 130 g of fresh MOPC-104E and solution F (10 mM Tris-HCl buffer, pH 7.5, 4 mM magnesium acetate, and 1 mM DTT) containing 0.25 M sucrose; the homogenization was with two strokes of a loose-fitting Teflon–glass homogenizer. After filtration through cheesecloth, the nuclear and cytoplasmic fractions were separated by centrifugation at 1100*g* for 10 min. The nuclear pellet was mixed with solution F containing 2 M sucrose, and then layered over this same solution in nitrocellulose centrifuge tubes. The tubes were centrifuged at 1 °C in a Beckman SW27 rotor for 90 min at 23 000 rpm. The resulting pellet of purified nuclei was resuspended in solution F containing 0.25 M sucrose and divided into two portions. One portion was allowed to stand at 0–1 °C while the other portion was adjusted to 0.25% in Triton X-100 (Blobel and Potter, 1966) and gently stirred for 2 min; both portions were then centrifuged at 1100*g* for 10 min. This process was repeated once and the final preparations of Triton X-100 treated nuclei and untreated nuclei were frozen and stored in liquid nitrogen until use.

DNA-cellulose Column Chromatography. Preparations to be chromatographed on DNA-cellulose were first applied to 1 × 20 cm Sephadex G25 (fine) columns that had been equilibrated with solution DC (10 mM Tris-HCl buffer, pH 8.1, 30% glycerol, 0.1 mM EDTA, and 1 mM DTT) containing 5 mM KCl. The fractions containing DNA polymerase activity were then applied directly to an activated DNA-cellulose column (Matsukage et al., 1975) with a bed volume of about 4 ml. The columns were washed first with 20 ml of solution DC containing 5 mM KCl and then successively with 20 ml of solution DC containing 60 mM, 250 mM, or 1 M KCl. Columns were run at a flow rate of about 15 ml/h and each fraction

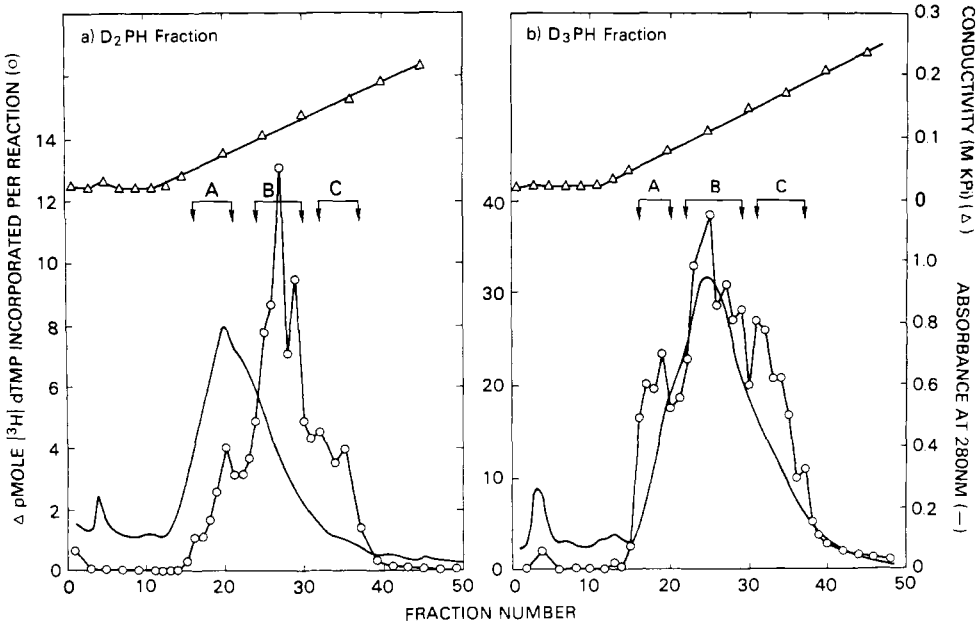

FIGURE 3: Preparative DEAE-cellulose column chromatography of the D2PH and D3PH fractions. In panel a, the D2PH fraction contained 44 mg of protein; in panel b, the D3PH fraction contained 134 mg of protein. The experiments were performed as described under Methods. The recoveries of activity were 60 and 61% in panels a and b, respectively.

contained about 1 ml. Protein was measured by reading absorbance at 280 nm and using the relationship of 1.5 A_{280}/mg of protein. DNA polymerase activity in 0.1-μl portions of fractions was measured using assay no. 1. Recovery of DNA polymerase activity ranged from 80–100%. The inclusion of 5 mM magnesium acetate in solution DC did not alter the chromatographic behavior of the enzyme tested (species B described under Results).

Results

Detection and Resolution of Multiple Species of DNA α-Polymerase. The purification to be described is summarized in Table I. After absorption onto DEAE-cellulose, DNA α-polymerase in an extract from a whole cell homogenate of MOPC-104E can be resolved into two fractions by stepwise elution with potassium chloride. An example of this is shown in Figure 1. DNA polymerase activity was measured with activated calf thymus DNA as template primer; it is attributed to α-polymerases both because β-polymerase(s) is not retained by the column and can be recovered from the flow through and because γ-polymerases are relatively inactive under the reaction condition used. The pooled fractions containing DNA polymerase activity that emerged from the column between 25 and 100 mM KCl (in buffer A) and between 100 and 250 mM KCl (in buffer A) are termed D2 and D3, respectively. DNA polymerase activity in these two fractions differed slightly, yet reproducibly in phosphocellulose column chromatography. The peak of activity with the D2 fraction emerged from the column at 155 mM KP, whereas the peak of activity with the D3 fraction was centered at about 140 mM KP. These profiles of DNA polymerase activity, although not shown, were similar to those obtained in a previous survey of MOPC-104E DNA polymerases (Matsukage et al., 1974).

DNA polymerase activities in the pooled fraction from the two phosphocellulose columns (D2P and D3P fractions) were analyzed by isoelectric focusing in ampholine containing pH–sucrose gradients. The results are shown in Figure 2. With both fractions, enzyme activity was distributed over a wide range of pH, although it appeared to be due primarily to species with isoelectric points of approximately 6.8–7.0, 5.2–5.5, and 4.3. The species with an isoelectric point of 6.8–7.0 was much more abundant in the D2P fraction than in the D3P fraction.

Separation of the Species of DNA Polymerase in Fractions D2P and D3P. Since both the D2P and D3P fractions of α-polymerase appeared to consist of several molecular species that differed in isoelectric point, we decided to use DEAE-cellulose column chromatography in the subsequent resolution of the species. However, before attempting this, the two fractions were chromatographed on hydroxyapatite columns in the presence of 500 mM KCl. In addition to achieving a 15- to 20-fold purification, this was done to remove any contaminating DNA β- or γ-polymerases, since both elute well before the peak of α-polymerase activity (Matsukage et al., 1974; 1975). The DNA polymerase activity in both the D2P and D3P fractions was eluted in a peak centered at about 40 mM KP.

Polymerase activities in the pooled fractions from the two hydroxyapatite columns (D2PH and D3PH) did not behave as homogeneous species during chromatography on DEAE-cellulose columns when a relatively shallow gradient of KP was used for elution (Figure 3). Fractions from these DEAE-cellulose columns were pooled, as shown in Figure 3, and then each pool was rechromatographed two times on a separate DEAE-cellulose column. Chromatograms resulting from these two passes through DEAE-cellulose columns with the D3PH fractions are shown in Figure 4 and the partial purification of the species in the D3 fraction is summarized in Table I. Inspection of panels d, e, and f in Figure 4 revealed that discrete species of DNA polymerase that differed in behavior on DEAE-cellulose columns had been resolved from the mixture of species originally present in the D3PH fraction (see Figure 3, panel b). However, comparison of the specific activities (see Table I) after the steps shown in Figures 3 and 4a–c revealed that the three enzyme species were not purified by the rechromatography (Figure 4a–c) and that a portion of each activity had been lost.

In order to more clearly illustrate the distinct chromatographic behavior of the three resolved species of α-polymerase,

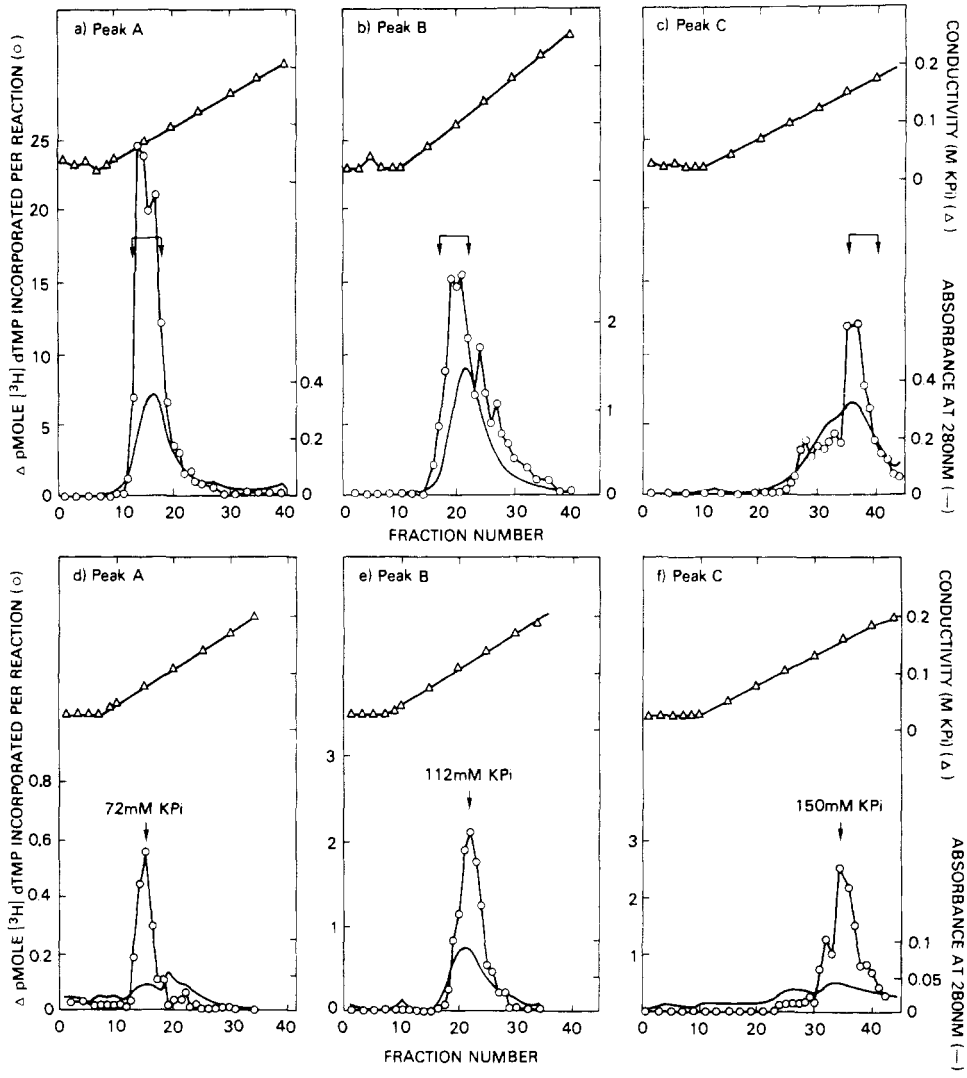

FIGURE 4: DEAE-cellulose column chromatography of the separated forms of DNA α-polymerase in the D3PH fraction. Preparative chromatography is shown in panels a–c, and fractions pooled, as shown in the figure, were used in the analytical chromatography shown in panels d–f, respectively. The experiments were performed as described under Methods, except that the volumes of the gradients were 45 times, instead of 7 times, the bed volumes of the columns. The amounts of protein chromatographed and the recoveries of activity were as follows: panel a, 7.3 and 47; panel b, 26 and 41; panel c, 9.2 and 14; panel d, 0.42 and 7; panel e, 1.3 and 19; panel f, 0.38 mg and 56%.

they were mixed and again subjected to chromatography on a DEAE-cellulose column. As seen in Figure 5, the forms were resolved in a manner expected from the behavior of each species when it was chromatographed alone (see Figure 4d–f).

In experiments not shown, virtually identical results were obtained when the D2PH fractions, A, B, and C (see Figure 3, panel a), were subjected to repeated chromatography on DEAE-cellulose. Therefore, from this result and the results of the isoelectric focusing, the two original fractions of α-polymerase (D2 and D3) appeared to be mixtures of the same enzyme forms. In the studies on the relationship between the enzyme species to be described below, fraction D3 was used.

Further Purification of the Species of DNA α-Polymerase. The enzyme species, A, B, and C, in the third DEAE-cellulose fractions shown in Table I were purified further. The first step was glycerol gradient centrifugation in the presence of 500 mM KCl and 0.2% Tween-80. The sedimentation behavior of each species was similar (Figure 6); the main peak was observed between 6.5 and 7 S and a shoulder was present at about 5 S. In experiments, not shown, using each of the species, lowering of the KCl concentration in the gradients to 250 mM, or less, resulted in aggregation.

Next in the purification, fractions from each gradient corresponding to the 5–9 S region were pooled and passed through a Sephadex G-25 column in order to adjust the KCl concentration in the buffer to 5 mM. The enzymes were then applied to separate DNA-cellulose columns. Under the conditions employed, the activity of each of the three species remained bound to the column in the presence of 60 mM KCl in solution DC. The enzyme activities were eluted by raising the KCl concentration in the solution to 250 mM. The three enzyme forms, A, B and C, were identical in their behavior during this DNA-cellulose chromatography; a chromatogram of the B form is shown in Figure 7. After this step, the enzyme activities per mg of protein, with activated calf thymus DNA as template primer, were 30, 26, and 34 μmol of dNMP incorporated/60 min for the A, B and C species, respectively. This represented about a tenfold purification of the activities in the DEAE-cellulose fractions shown in Table I and a 4000- to 5000-fold purification from the crude extract.

The final step in the purification was DEAE-cellulose

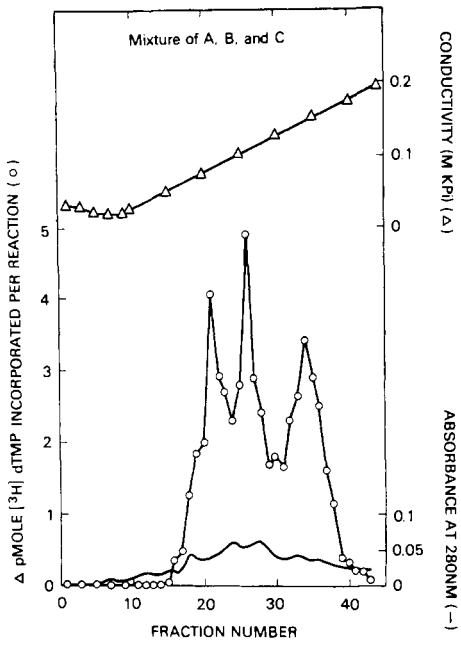

FIGURE 5: Analysis of an artificial mixture of the three species of α-polymerase by DEAE-cellulose column chromatography. Fractions containing the enzyme species A, B, and C were prepared as described in Figure 4a-c. A mixture containing 0.28 mg of protein of the A species, 0.43 mg of protein of the B species, and 0.38 mg of protein of the C species was prepared and then chromatographed as described under Methods. DNA polymerase activity in 0.2-μl fractions was measured by assay no. 1. The recoveries of activity were 38% for species A, 37% for species B and 74% for species C.

chromatography. The purpose of this step was to remove any contaminating DNA that may have eluted from the DNA-cellulose column and to concentrate the enzymes. Fractions containing the peak of enzyme activity from each of the DNA-cellulose columns were pooled, adjusted to 25 mM KCl in solution A by passage through a Sephadex G-25 column, and then each pooled fraction was applied to a separate DEAE-cellulose column (1 × 7 cm). After washing the columns with three bed volumes of solution A containing 25 mM KCl, enzymes were eluted by raising the KCl concentration to 250 mM. At this stage, we found that 50% of the activity of each species decayed in 3 h upon holding the enzyme at 0–1 °C in solutions containing 20% glycerol or upon one freeze-thaw cycle. Therefore, further purification was not practical and the fractions from these last DEAE-cellulose columns were used in characterizing the three enzyme species. The fractions were divided into small portions and stored in liquid N_2. The purity of these final preparations was examined by polyacrylamide gel electrophoresis under native conditions in an experiment to be described below (see Figure 10).

The Relationship between the Species of DNA α-Polymerase. Comparison of Catalytic Properties. The activities of the three species were essentially indistinguishable in their inhibition by increasing concentrations of potassium chloride, *N*-ethylmaleimide, phenylmethanesulfonyl fluoride, and a preincubation at 47 °C (Figure 8). The concentration curves of activated DNA, magnesium, manganese, and hydrogen ion were also similar for the three species (Figure 9), although close inspection of the graphs reveals that the B species was relatively less active at the lower concentrations of both divalent cations and relatively more active at the lower concentrations of hydrogen ion.

Comparison of Template Primer Preferences and Reaction Requirements. The template primer specificities of the three species of α-polymerase were compared using either Mg^{2+} or Mn^{2+} as the divalent cation, and homopolymer templates and primers (Table II). The species were very similar in their ability to use the various template-primer combinations under the conditions tested. All three species were relatively inactive with poly(rA)·oligo(dT) and were most active with the template-primer combination of poly(dC)·poly(dI) for the incorporation of dGMP. Detailed studies on the use of this and other template-primer combinations by the B species of DNA α-polymerase are described in a separate communication (Wilson et al., 1976, submitted for publicaton). In experiments not shown, the A, B, and C species of α-polymerase were similar in their apparent requirement for a primer molecule containing a free 3′-hydroxyl group. Incorporation was not observed with a deoxynucleoside 5′-diphosphate as substrate, and only very low levels of incorporation were observed with a ribonucleoside 5′-triphosphate or a deoxynucleoside triphosphate not complementary to the template.

Polypeptide Composition. In order to examine the polypeptide composition of the species of α-polymerase, two types of cofractionation experiments were performed. The results obtained with the B species are shown in Figure 10. Similar results were obtained with the A and C species.

In the first experiment, the final preparation of each species was applied to 8% polyacrylamide gels and electrophoresed under nondissociating conditions. Some of the gels were stained with Coomassie brilliant blue and other gels were sliced and incubated with a solution appropriate to extract the DNA

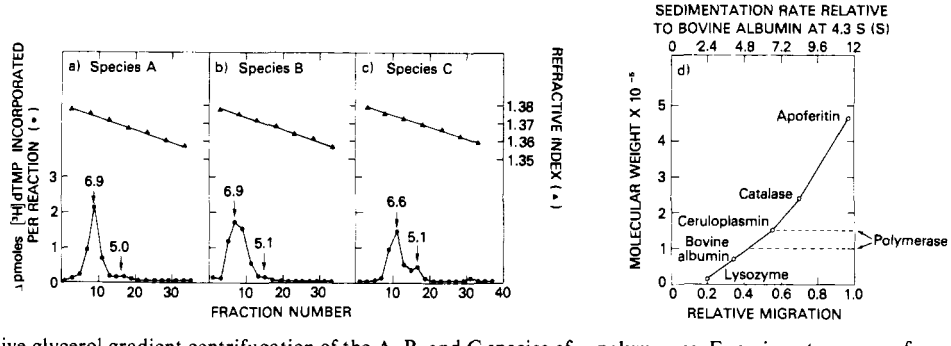

FIGURE 6: Preparative glycerol gradient centrifugation of the A, B, and C species of α-polymerase. Experiments were performed as described under Methods. DNA polymerase activity in 0.1 μl of fractions was measured by assay no. 1. The recoveries of activity were 25% for the A species, 30% for the B species, and 53% for the C species. The fractions from each gradient corresponding to the 5 to 9S region were pooled and stored in liquid N_2 before they were used in the next step in the purification. In panel d, the relationship between sedimentation rates of marker proteins and their molecular weights is shown.

TABLE II: Comparison of Template-Primer Specificity of the Species of MOPC-104E DNA α-Polymerase.

Template (160 μg/ml)	Primer (32 μg/ml)	Amount of dNMP Incorp by α-Polymerase Species (Δ pmol)[a]					
		A		B		C	
		Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
Activated DNA		25.2	7.2	50.8	21.2	10	4
Native DNA		0.8		0.8		0.3	
Denatured DNA		2		3.6		0.3	
Poly(dC)	Poly(dI)	62.2	41.8	120	54.6	39.1	22.3
Poly(dC)	$Poly(dG)_{12\text{-}18}$	7.6	4.5	17.7	8.8	1.7	0.4
Poly(dC)	Poly(rI)	7.3	2.0	7.2	3.4	2.3	2.1
Poly(dA)	Poly(dT)	1.7	2.9	5.2	12.7	1.6	2.4
Poly(dA)	$Poly(dT)_{12\text{-}18}$	0.7	2.6	2.8	9.9	0.4	1.2
Poly(dA)	Poly(rU)	0	0.5	0	0.7	0	0.1
Poly(rA)	$Poly(dT)_{12\text{-}18}$	0	0.4	0	0.5	0	0

[a] Reactions were according to assay no. 1 and contained the indicated template-primer combination, 0.1 mM dNTP base pair complementary to the template, and either 6 mM Mg^{2+} or 0.5 mM Mn^{2+} as indicated.

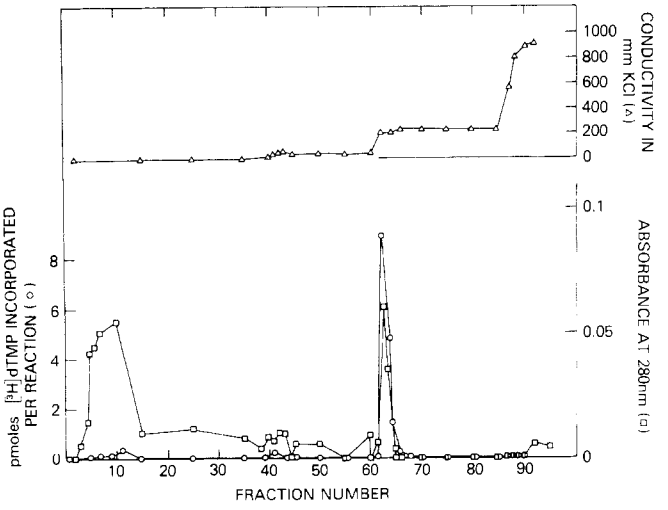

FIGURE 7: DNA-cellulose column chromatography of the B species of α-polymerase. A solution containing the pooled fractions corresponding to the 5 to 9S region from the glycerol gradient shown in Figure 9b was chromatographed as described under Methods.

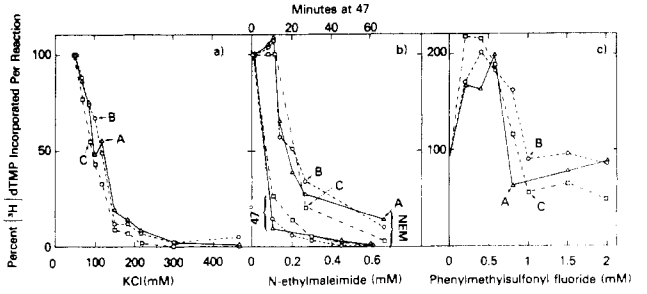

FIGURE 8: Comparison of the effects of KCl, preincubation at 47 °C, *N*-ethylmaleimide, and phenylmethanesulfonyl fluoride on the activity of the species of α-polymerase. Activities were measured using assay no. 2, as described under Methods and in the figure, except as follows. In the *N*-ethylmaleimide experiments, the dithiothreitol concentration was reduced to 0.2 mM. In the phenylmethanesulfonyl fluoride experiments, the dithiothreitol concentration was not changed from the usual 1 mM, and the 2-proponal concentration was 6.7% in all reactions; this concentration of 2-proponal had no effect upon the DNA polymerase activities. The 100% values (Δ pmol of [^{3}H]dTMP incorporated/reaction) for species A, B, and C, respectively, were as follows: in panel a, 1.2, 0.5, 2.2; in panel b *N*-ethylmaleimide curve, 2.2, 6.6, 0.1, and preincubation at 47 °C, 1.9, 6.3, 3.5; in panel c, 0.6, 3, 1.3.

polymerase activities. A stained gel and the corresponding DNA polymerase activity profile are shown in Figure 10, panel a. Note that the fraction containing the peak of polymerase activity corresponded with the most intensely stained region of the gel. The fractions containing polymerase activity were treated with sodium dodecyl sulfate and 2-mercaptoethanol at 100 °C, and polypeptides were analyzed by electrophoresis on slab gels of 10% polyacrylamide in 0.1% sodium dodecyl sulfate and 5 M urea. The fraction containing the peak of DNA polymerase activity (slice 4) contained three prominent bands that migrated at 157 000, 70 000, and 53 000 and six other bands ranging in apparent molecular weight from 118 000 to 40 000. Comparison of the various fractions indicated that the intensity of all of these bands, except the ones at 118 000 and 103 000, correlated with the amount of DNA polymerase activity.

In the second experiment, the final preparation of each species was centrifuged through a glycerol gradient and then the gradient fractions were tested for DNA polymerase activity and polypeptide content. As shown in Figure 10, panel b, the staining intensity of the bands at 150 000 and 60 000 mol wt correlated with the amount of polymerase activity. Such a correlation was not observed with the bands at 118 000, 103 000, or 70 000 mol wt; the lower molecular weight bands seen in panel a were not observed.

Different Extraction Conditions and the Molecular Heterogeneity. The effect of altering the extraction conditions on the molecular heterogeneity of α-polymerase was examined. Roughly, the same extent of heterogeneity was observed in the DEAE-cellulose column profiles when extracts were prepared under three different types of conditions: (1) the usual extraction conditions with 500 mM KCl and 50 mM Tris-HCl buffer at pH 7.5; (2) homogenization in 20 mM KP, pH 7.0, and preparation of an 100 000*g* supernatant fraction; (3) homogenization in 0.25 M sucrose, Tris-KCl-Mg^{2+} solution and preparation of an 100 000*g* supernatant. The heterogeneity observed with the 20 mM KP extraction was not changed by including either of the protease inhibitors, 2 mM phenylmethanesulfonyl fluoride or 500 KI units/ml of trasylol, during the extraction and DEAE-cellulose column chromatography.

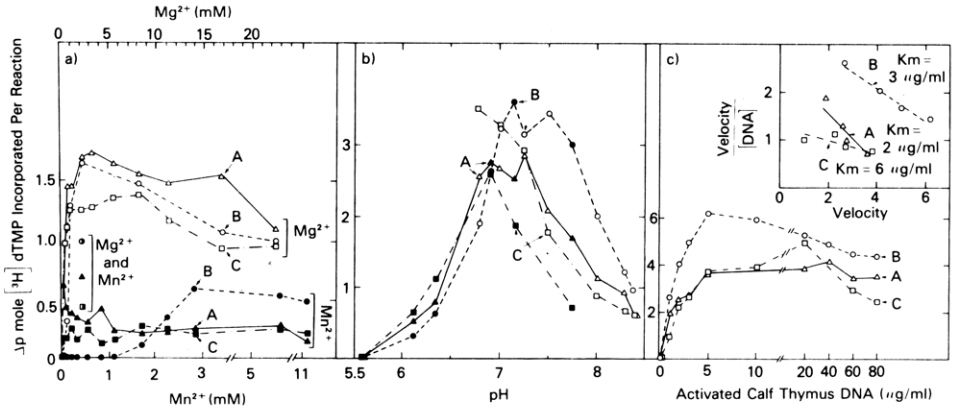

FIGURE 9: Comparison of the activities of the species of α-polymerase with activated DNA as template primer. Activities were measured using assay no. 2, as described under Methods and in the figure. In panel b, reaction mixture pH was determined using 0.4-ml reactions that were identical with the usual 0.015-ml reactions, except that the [^{3}H]dTTP was substituted with unlabeled dTTP. Results using reactions with potassium phosphate buffer are shown by closed symbols and with Tris-HCl buffer by open symbols.

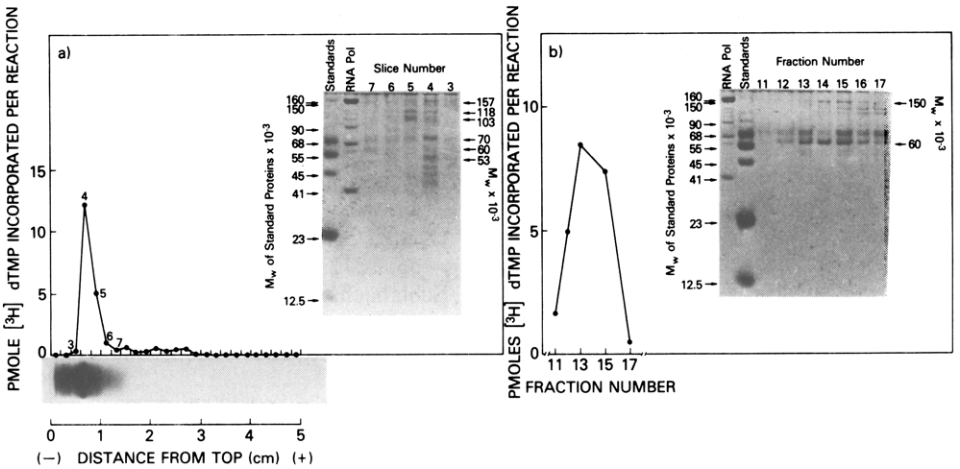

FIGURE 10: Comigration of DNA polymerase activity and polypeptides of the B species during polyacrylamide gel electrophoresis under native conditions (panel a) and glycerol gradient centrifugation (panel b). Experiments were performed as described in the text and under Methods, except that the centrifugation was at 40 000 rpm for 20 h and the glycerol gradient did not contain Tween-80. The recovery of DNA polymerase activity in panel a was 20% and in panel b 35%.

Extracts from Different Sources and the Molecular Heterogeneity. Nuclear and cytoplasmic subcellular fractions prepared from fresh MOPC-104E (see Methods) were analyzed for their content of DNA polymerase species that were identical in DEAE-cellulose chromatographic properties to the A, B, and C species described above. Of the total activity recovered in the two subcellular fractions, 85–90% was in the cytoplasmic fraction and this activity was distributed among the A, B, and C species in the ratio of approximately 1:3.5:0.4, respectively. The same species were recovered from the nuclear fractions in the ratio of approximately 1:3:1 for A, B, and C, respectively. Triton X-100 treatment of the nuclear fraction did not change either the amount of activity recovered or the distribution among the three species.

The occurrence of multiple species of α-polymerase was examined using a different tissue. Fetal bovine liver was homogenized and a 100 000*g* supernatant fraction was prepared according to the procedure described by Chang et al. (1973); this extract was chromatographed directly on a DEAE-cellulose column. Peaks of DNA polymerase activity emerged from the column in the same positions observed for the A, B, and C species of the MOPC-104E α-polymerase. These experiments show that the chromatographic heterogeneity observed in our work was not special to the MOPC-104E tumor or to the nuclear or cytoplasmic fractions of that tissue.

Discussion

The main points established in this study may be summarized as follows. (1) In MOPC-104E extracts, the chromatographic heterogeneity of DNA α-polymerase involved at least three species that were discrete and stable enough to be reproducibly chromatographed and extensively purified. This is important because it shows that the heterogeneity of the enzyme activity was due to a mixture of molecular species, rather than some artifact of the chromatographic analysis. (2) With both of the tissues and each of the extraction procedures tested, gross heterogeneity of α-polymerase was observed. This suggests that the heterogeneity may exist in vivo and was not solely the result of an extraction artifact, such as proteolysis. (3) The separated species of α-polymerase could be extensively purified by chromatography on DNA-cellulose columns and by avoiding freezing and thawing during the procedures. The extent of purification ultimately achieved and the final specific activity are among the highest reported. (4) The catalytic activities of the three highly purified molecular species from MOPC-104E were very similar, but not identical. In glycerol

gradients containing a high concentration of potassium chloride, no differences in sedimentation behavior of the three species were observed. The main portion of the activities sedimented at about 6.8 S and a small portion sedimented at about 5.1 S. These sedimentation rates, when compared with those of standard proteins in our system, suggest molecular weights of about 155 000 and 100 000, respectively. Thus, it appears that very similar enzyme active sites were contained within molecules of similar size, but quite different overall ionic change.

Analysis of the size distribution of the polypeptides that comigrated with the DNA polymerase activity provided evidence that each of the three enzyme species contained peptides of about 150 000 and 60 000 daltons. These experiments on polypeptide composition provide no insight into the mechanism of the molecular heterogeneity. However, it is interesting to note that these 150 000- and 60 000-dalton polypeptides are of comparable size to the polypeptides tentatively attributed to the calf thymus enzyme by Holmes et al. (1976) and also by Bollum et al. (1974). Our final preparations did not contain abundant 85 000- or 89 000-dalton polypeptides, as reported for preparations of human leukocyte and KB cell α-polymerase(s) by Smith et al. (1975) and by Sedwick et al. (1975), respectively.

Finally, as noted under Methods, we found that a procedure used earlier for isolating acid-treated reaction products led to incomplete recovery of the newly formed DNA. The loss occurred during a centrifugation step that had been included to reduce the background in reactions containing high concentrations (0.5 mM) of dNTP substrate. Such a loss was more pronounced in reactions that contained activated DNA as template primer and, thus, led to erroneous values for the amounts of DNA-directed DNA polymerase activity. With the stringent method (Schrier and Wilson, 1975) for product isolation used in the current study, our values for amounts of activity/g of tissue are higher than those we reported previously (Matsukage et al., 1974, 1975) and are in line with some values reported by others (Bollum, 1975).

Acknowledgments

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