Multiple Forms of *Drosophila* Embryo DNA Polymerase: Evidence for Proteolytic Conversion

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ABSTRACT: The DNA polymerase in crude extracts of *Drosophila melanogaster* embryos sedimented at 9.0, 7.3, and 5.5 S on glycerol velocity gradients. The relative proportions of these enzymes depended on the method used to prepare the extract. Extracts of whole embryos contained the 7.3S and the 5.5S DNA polymerases and extracts of dechorionated embryos contained the 9.0S and 7.3S DNA polymerases. The proportion of the 5.5S DNA polymerase increased relative to the 7.3S DNA polymerase during storage of the extract of whole embryos. The protease inhibitor, phenylmethanesulfonyl fluoride, inhibited the formation of the 5.5S DNA polymerase, suggesting that it was proteolytically produced from the 7.3S DNA polymerase. This was demonstrated directly by converting the 7.3S DNA polymerase to the 5.5S DNA polymerase by treatment in vitro with trypsin. The degradation of the enzyme occurred without significant loss of DNA polymerase activity. It is further demonstrated that endogenous proteolysis reduced the chromatographic heterogeneity of the *Drosophila* DNA polymerase on diethylaminoethyl-Sephadex. When endogenous proteolysis was reduced, three forms of DNA polymerase were isolated by diethylaminoethylcellulose chromatography; two of these enzymes sedimented at 7.3 S and the third sedimented at 9.0 S. These results demonstrate the physical heterogeneity of the *Drosophila* DNA polymerase and suggest its similarity to vertebrate DNA polymerase-α.

Several lines of evidence suggest that DNA polymerase-α[1,2] is responsible for DNA replication in eukaryotic cells. First, it constitutes the majority of the total DNA polymerase recovered from crude extracts of growing vertebrate cells. Second, the amount of DNA polymerase-α in vivo and in cultured vertebrate cells increases with mitotic activity, whereas the amounts of polymerase-β and polymerase-γ do not. Regenerating tissues (Chang and Bollum, 1972; Baril et al., 1973) and embryo tissues (Ove et al., 1970; Margulies and Chargaff, 1973; Loeb, 1974) contain more DNA polymerase-α than do normal adult tissues; similarly, dividing tissue culture cells have more DNA polymerase-α than do quiescent cells (Chang et al., 1973; Spadari and Weissbach, 1974; Craig et al., 1975). Third, several eukaryotic microorganisms that have rapid growth rates, including *Saccharomyces cerevisiae* (Wintersberger, 1974), *Tetrahymena pyriformis* (Crerar and Pearlman, 1974), *Euglena gracilis* (McLennan and Keir, 1975a), and *Dictyostelium discoideum* (Loomis et al., 1976), lack DNA polymerase-β and contain only a DNA polymerase similar to vertebrate DNA polymerase-α.

These observations suggest that DNA polymerase-α is the replicative DNA polymerase, but do not prove it. A particular subcomponent of DNA polymerase-α or an, as yet, unidentified polymerase may function as the replicative polymerase. Proof of the functions of eukaryotic DNA polymerases may be accomplished only by a combination of genetic and biochemical techniques, as has proved invaluable to our understanding of DNA replication in bacteria. The isolation of mutants of *Escherichia coli* with reduced amounts of DNA polymerase I (De Lucia and Cairns, 1969) not only allowed the detection of the less abundant DNA polymerases II and III (Kornberg and Gefter, 1970, 1971), but also showed that the most abundant DNA polymerase (I) was not the primary replicative enzyme. Biochemical analysis of DNA polymerase I mutants is now being used to define the role of this enzyme (Lehman and Uyemura, 1976).

Of the complex eukaryotes, *Drosophila melanogaster* appears to be best suited for genetic and biochemical analysis of DNA polymerase functions. *Drosophila* embryos contain large amounts of DNA polymerase (Margulies and Chargaff, 1973; Loeb, 1974) and genetic analysis of this enzyme, with resolution similar to that in bacterial systems (Chovnick, 1966), may be possible. In preparation for genetic studies, it is important to purify and characterize the polymerases in *Drosophila* and to establish their relationship to the DNA polymerases of vertebrates (Weissbach, 1975). Karkas et al. (1975) have purified a DNA polymerase from *Drosophila* embryos which was ionically homogeneous and had a molecular weight of 87 000. These physical properties suggest that this DNA polymerase is unlike vertebrate DNA polymerase-α, -β or -γ (Weissbach, 1975). In this report, however, we show that the *Drosophila* DNA polymerase is considerably more heterogeneous than was reported earlier (Karkas et al., 1975) and is similar to vertebrate DNA polymerase-α. We show that proteolysis during enzyme preparation reduces the heterogeneity of the enzyme with respect to its sedimentation velocity and its chromatographic behavior. When endogenous proteolysis was uncontrolled, an ionically homogeneous, 5.5S DNA polymerase was obtained, as reported by Karkas et al. (1975). Using isolation conditions that reduce endogenous proteolysis, we have isolated ionically distinct forms of DNA

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[1] Abbreviations used are: DNA polymerase, deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7; LDH, lactate dehydrogenase, L-lactate:NAD oxidoreductase, EC 1.1.1.27; trypsin, EC 3.4.4.4; BSA, bovine serum albumin; DEAE, diethylaminoethyl; KPB, potassium phosphate buffer; EDTA, ethylenediaminetetraacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; DTT, dithiothreitol.

[2] See Weissbach (1975) for a review of the properties and nomenclature of vertebrate DNA polymerases.

polymerase that have the same sedimentation coefficients observed for the enzymes present in crude extracts, i.e., 7.3 and 9.0 S. We show that in vitro trypsin treatment of the 7.3S DNA polymerase produces a 5.5S DNA polymerase without reducing the polymerizing activity of the enzyme.

Materials and Methods

Chemicals. Deoxyribonucleotides were supplied by P. L. Biochemicals and [^{3}H]dTTP (49 Ci/mmol) was purchased from Amersham/Searle. Calf thymus DNA was purchased from Calbiochem and activated calf thymus DNA was prepared by the method of Aposhian and Kornberg (1962). LDH (from beef heart) was purchased from Worthington Biochemicals, and its activity was determined as described by Kornberg (1955). BSA was purchased from Sigma. Trypsin (TPCK treated) and soybean trypsin inhibitor were purchased from Worthington Biochemicals. $PhCH_2SO_2F$ was purchased from Sigma and a 0.1 M solution was prepared in 2-propanol. DEAE-Sephadex (A-50-120) was purchased from Sigma and was prepared for use as described by Karkas et al. (1975).

Buffer Solutions. All potassium phosphate buffers were prepared at pH 8.0 and contained 1 mM 2-mercaptoethanol and 10% (v/v) glycerol unless specified otherwise. These buffers are designated by their millimolar concentrations—e.g., 50 mM KPB denotes 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM 2-mercaptoethanol and 10% (v/v) glycerol.

Preparation of Ion-Exchange Resins. DEAE-cellulose (Sigma) (0.94 mequiv/g) was washed sequentially with 0.25 M NaOH plus 0.25 M NaCl, distilled water, 0.25 M HCl, and water. It was equilibrated to pH 8.0 with 25 mM potassium phosphate buffer and then with 25 mM KPB. The DEAE-cellulose resin was prepared for re-use by the same method. Phosphocellulose (Sigma) (0.99 mequiv/g) was prepared in the same manner except that it was first washed with HCl and then with NaOH plus NaCl. The phosphocellulose resin was equilibrated with 50 mM potassium phosphate (pH 8.0) and then with 50 mM KPB.

Drosophila Culture and Embryo Collection. Adult fruit flies (*Drosophila melanogaster,* Oregon R) were maintained as described by Kriegstein and Hogness (1974) except that the light and dark periods were 12 h. Embryos to be used for these experiments were collected for 16–17 h, starting 6 h after the light to dark transition.

Preparation of Embryos for Homogenization. Embryos were removed from agar collection plates by rinsing with a solution of 0.1% Triton X-100 and 0.7% NaCl and brushing with a camel-hair brush. The embryos were collected on a Nitex screen (HD-3-63) and dechorionated by incubation in 2.25% sodium hypochlorite for 2 min with occasional stirring. After dechorionation, the embryos were rinsed thoroughly with the Triton–NaCl solution. To remove remaining chorions, yeast, and fragments of flies, the dechorionated embryos were allowed to settle through the Triton–NaCl solution in a 1.5 × 70 cm tube. After the embryos settled, the supernatant was removed by aspiration. This settling procedure was repeated three to four times. The embryos were then rinsed with 50 mM KPB and drained thoroughly to give about 6×10^4 embryos/g.

Purification of DNA Polymerase. In several of the experiments reported here, we prepared enzyme fractions as described by Karkas et al. (1975). With the exception of the changes noted below, we attempted to reproduce their experimental conditions exactly. We were able to collect sufficient quantities of embryos in one collection period and we neither froze the embryos nor stored the stage I preparations before proceeding with the purification of the DNA polymerase. For some enzyme preparations, embryos were dechorionated and settled as described above before they were homogenized; in some cases, $PhCH_2SO_2F$ was included in the homogenization buffer. The enzyme fractions prepared by the methods of Karkas et al. (1975) are designated *stage* I, II, etc. as in that report. Briefly, the homogenate was centrifuged at 27 000*g* and the supernatant (stage I) was fractionated by ammonium sulfate precipitation to obtain the stage II preparation. The stage II preparation was further fractionated by DEAE-Sephadex chromatography (stage III).

We have developed another procedure for the partial purification of DNA polymerase from *Drosophila* embryos. Steps 1 through 4 are described below and a complete description of these techniques will be published elsewhere. The enzyme fractions prepared by this procedure are designated as *step* 1, 2, etc. to distinguish them from the enzyme fractions prepared by the methods of Karkas et al. (1975). For the experiments reported here, dechorionated embryos were suspended in five times their weight of 50 mM KPB and homogenized with 12 strokes of a Ten Broeck glass homogenizer. The homogenate was filtered through a Nitex screen (ASTM-400-37) to remove vitelline membranes, and the resultant preparation (step 1) was either used immediately or stored at −70 °C until needed. No loss of enzyme activity occurred during storage periods of up to 1 month. Frozen homogenates were thawed at 5 °C in batches of 200–225 mL (about 40 g of embryos) and centrifuged in a Spinco SW 27 rotor at 20 000*g* for 30 min at 5 °C. A lipid pellicle was removed and the supernatant fraction (step 2), which was carefully removed from the pelleted material, was applied to a 3 × 35 cm phosphocellulose column equilibrated with 50 mM KPB. After application of the sample, the column was washed with 50 mM KPB until all unadsorbed material was eluted (usually 1 L of 50 mM KPB was sufficient) and then the column was washed with 100 mM KPB (0.8–1.0 L) until all ultraviolet absorbing material was eluted. The DNA polymerase was eluted with 200 mM KPB (step 3). After elution from phosphocellulose, the enzyme preparation was applied to a 2.5 × 15 cm DEAE-cellulose column equilibrated with 25 mM KPB. After the sample percolated into the resin, the column was washed with 200 mM KPB. Fractions of 6 mL were collected and those containing DNA polymerase were combined (step 4). The removal of nucleic acids was indicated by the increase in the A_{280}/A_{260} quotient from about 1.4 to 1.8. The DNA polymerase we obtained in step 4 was approximately 30-fold purified relative to the enzyme in the crude homogenate and it had a specific activity of 850 nmol of total nucleotides incorporated per mg of protein per hour. Approximately 40% of the original DNA polymerase activity was recovered at this step. Different techniques, described in the appropriate figure legends, were used to further fractionate the DNA polymerase.

Assay of DNA Polymerase Activity. DNA polymerase activity was routinely assayed with activated calf thymus DNA. The reaction mixture contained 50 mM Tris-HCl (pH 8.5); 8 mM $MgCl_2$; 0.02% 2-mercaptoethanol; 0.1 mM each dATP, dGTP, and dCTP; 0.05 mM dTTP; 10 μCi/mL [^{3}H]dTTP; and 80 μg/mL activated calf thymus DNA in a final volume of 50 μL. Increasing the dTTP concentration above 0.05 mM did not increase the initial rate of the enzyme reaction. Enzyme activity was measured at 25 °C in our initial studies because *Drosophila* grow at 25 °C. In later studies, enzyme activity was measured at 37 °C, which increased the initial rate of [^{3}H]dTMP incorporation. The incorporation of

deoxynucleotides was stopped by adding 5 mL of ice-cold 2% perchloric acid. After incubation on ice for 15–30 min, samples were filtered onto Whatman GF/C filters and the filters were washed first with 2% perchloric acid, then with a solution of 10–20 mL of 1 N HCl and 0.1 M sodium pyrophosphate, and finally with 95% ethanol. The filters were then dried and the incorporation of [^{3}H]dTMP was determined by liquid scintillation spectrometry in Omnifluor (15.14 g/gal) (New England Nuclear) and toluene scintillant with 27% efficiency. One unit of enzyme activity is defined as 1 nmol of total nucleotide incorporated per hour. Total nucleotide incorporation was calculated by multiplying the amount of dTMP incorporated by 3.5 to account for the base composition of calf thymus DNA.

Protein Determination. The amount of protein was determined by the method of Lowry et al. (1951) in samples that were precipitated with 2.5% perchloric acid and redissolved in 1.0 N NaOH before analysis.

Results

Variation of Sedimentation Velocity of DNA Polymerase in Crude Extracts. The sedimentation velocity of the DNA polymerase in *Drosophila* embryos was affected by the method of preparation of the embryos prior to homogenization. Removing the chorions from the embryos had a marked effect on the size of the enzyme recovered in crude extracts prepared by the procedure of Karkas et al. (1975) (Figure 1). Most of the DNA polymerase in extracts from whole embryos sedimented at 7.3 S (Figure 1A) but the DNA polymerase in extracts of dechorionated embryos sedimented at either 7.3 or 9.0 S (Figure 1B). Furthermore, after 2 days at 4 °C, the preparation from whole embryos had lost about 50% of its original DNA polymerase activity and the remaining DNA polymerase sedimented at 7.3 and 5.5 S (Figure 1C). The DNA polymerase activity in extracts of dechorionated embryos was reduced by only 20% after 2 days at 4 °C and most of the enzyme sedimented at 7.3 S (Figure 1D). The amount of the 9.0S form was considerably reduced. These results suggest that the 5.5S enzyme may be a degradation or dissociation product of the 7.3S enzyme.

Two types of degradation could result in a decrease in the sedimentation velocity of the DNA polymerase: protease digestion of the enzyme or of proteins bound to the enzyme and nuclease digestion of associated nucleic acids. To test the first possibility, we examined the effect of the serine protease inhibitor, $PhCH_2SO_2F$, on the sedimentation velocity of the DNA polymerase in extracts prepared from whole embryos. $PhCH_2SO_2F$ inhibited the generation of the 5.5S enzyme in the stage I and II preparations: at both stages the enzyme sedimented at 7.3 S (Figure 2B,D). In the absence of $PhCH_2SO_2F$ both the 7.3S and 5.5S enzyme forms were observed in both the stage I and II preparations (Figure 2A,C). The ammonium sulfate precipitation procedure used to obtain the stage II preparation appeared to promote the formation of the 5.5S enzyme. This result suggests that proteolysis produces the 5.5S DNA polymerase.

Production of the 5.5S DNA polymerase by in Vitro Trypsin Treatment of the 7.3S DNA Polymerase. Although the results described above suggest that the 5.5S DNA polymerase is a proteolytic cleavage product of the 7.3S DNA polymerase, they do not exclude the possibility that nuclease digestion of contaminating nucleic acid also contributed to the reduction in sedimentation velocity of the DNA polymerase. This possibility would be excluded if the 5.5S DNA polymerase could be generated from the 7.3S DNA polymerase by treatment with a purified, nuclease-free protease, such as trypsin. The results presented in the preceding section suggest that it might be difficult to isolate either the 9.0S or the 7.3S DNA polymerase by the procedures of Karkas et al. (1975), because the amounts of these enzymes decreased during storage and during ammonium sulfate fractionation of the stage I preparation. Therefore, we used a new procedure (see Methods) to isolate the 7.3S DNA polymerase from the *Drosophila* embryos. The step 4 enzyme, described under Methods, was fractionated by ammonium sulfate precipitation and the resultant enzyme preparation contained a mixture of the 7.3S and 9.0S enzymes. Before testing the effect of trypsin treatment on the size of the DNA polymerase, we first determined its effect on the activity of these enzymes (Figure 3). Trypsin treatment for 40 min caused little loss in DNA polymerase activity until the ratio of the protein weights (enzyme fraction protein to trypsin) was less than 80 (Figure 3). At this or higher ratios, more than 90% of the DNA polymerase activity re-

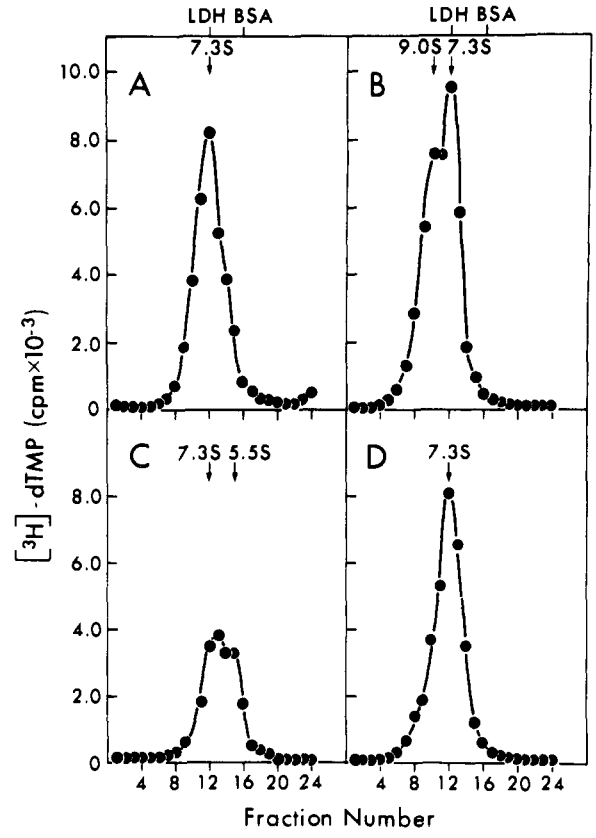

FIGURE 1: Effect of removal of chorions from embryos on the sedimentation velocity of DNA polymerase. Two batches (8 g each) of embryos, collected as described under Methods, were homogenized as described by Karkas et al. (1975) except that the embryos in one batch were dechorionated and settled before homogenization. After homogenization, stage I preparations were prepared as described by Karkas et al. (1975). A 2-mL portion of each stage I preparation was then dialyzed against the gradient buffer (50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, and 0.2 M $(NH_4)_2SO_4$). After a 12-h dialysis, 0.8 mL of each preparation was layered over 7–28% (v/v) glycerol gradients, which were formed over 0.6-mL cushions of 32% glycerol. Gradients were centrifuged for 28 h at 39 000 rpm and 2 °C in a Spinco SW 40 rotor and fractions were collected and the DNA polymerase activity of 10-μL portions of each fraction was determined. (A) Whole embryo extract; (B) dechorionated embryo extract. The remainder of each dialyzed preparation was kept at 4 °C for an additional 48 h and 0.8-mL portions were then centrifuged as above. (C) Whole embryo extract; (D) dechorionated embryo extract. LDH (7.3 S) was centrifuged in the gradients with the DNA polymerase preparations, and BSA (4.4 S) plus LDH were also centrifuged on a separate gradient.

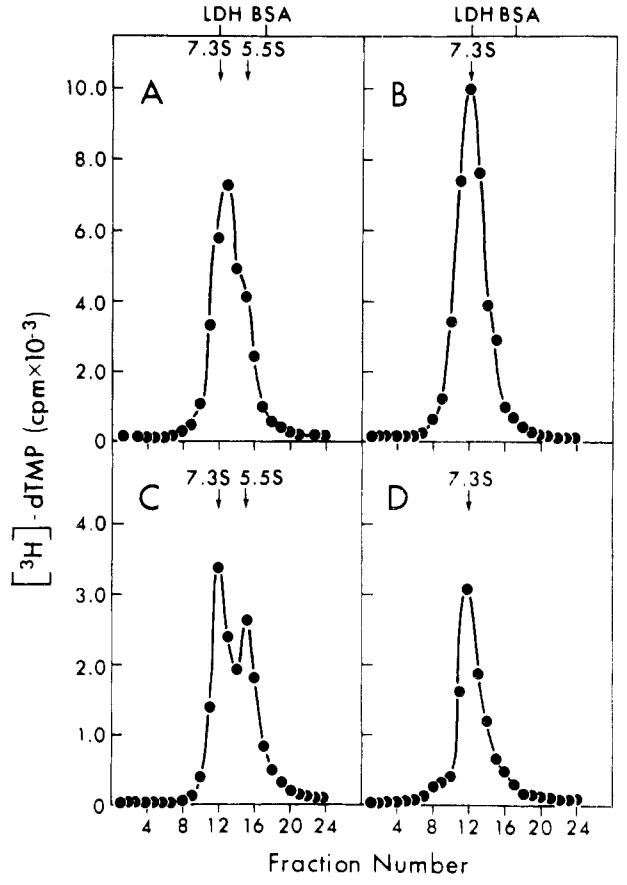

FIGURE 2: Effect of $PhCH_2SO_2F$ on the sedimentation velocity of DNA polymerase isolated from whole *Drosophila* embryos. Two 7-g batches of embryos were treated exactly as described by Karkas et al. (1975) except that $PhCH_2SO_2F$ (0.1 mM) was added to one of the suspensions of embryos. Stage I and II enzyme preparations, as described by Karkas et al. (1975), were made from each homogenate. Portions of each of the four preparations were dialyzed for 12 h in the gradient buffer described in the legend to Figure 1. After dialysis, 0.6 mL of each preparation was centrifuged on 7–28% (v/v) glycerol gradients as in Figure 1 and the DNA polymerase activity in 10-μL portions of each fraction was determined. (A) Stage I without $PhCH_2SO_2F$. (B) Stage I with $PhCH_2SO_2F$. (C) Stage II without $PhCH_2SO_2F$. (D) Stage II with $PhCH_2SO_2F$.

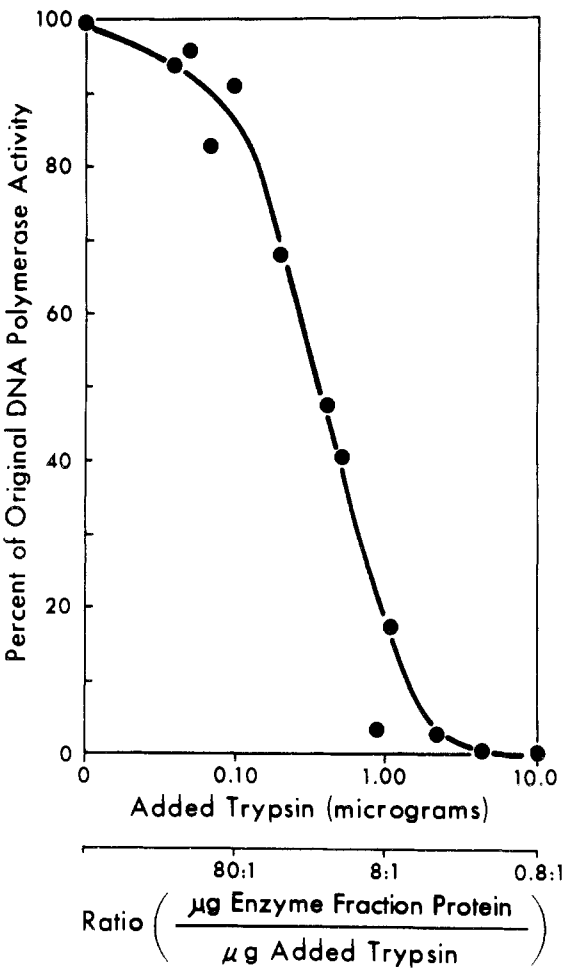

FIGURE 3: Effect of trypsin on DNA polymerase activity. DNA polymerase was prepared from 40 g of embryos through step 4 exactly as described under Methods except that $PhCH_2SO_2F$ (0.1 mM) was included in all buffer solutions. The step 4 preparation was fractionated by $(NH_4)_2SO_4$ precipitation. The protein which precipitated between 25 and 55% of saturation was collected by centrifugation at 20 000*g* for 30 min. The protein pellet was resuspended in and dialyzed against 200 mM KPB (minus glycerol). Portions of the dialysate (2 μL; 8 μg of protein) were incubated with the indicated amound of TPCK-treated trypsin. Reaction mixtures, containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, and 25% (v/v) glycerol in a total volume of 25 μL, were incubated for 40 min at 37 °C. After that incubation, 25 μL of DNA polymerase substrates was added, the incubation at 37 °C was continued for 15 min, and then the incorporation of [^{3}H]dTMP was determined. The total incorporation by the enzyme to which no trypsin was added was 345 pmol of dTMP.

mained after treatment and at lower ratios the activity was reduced, ultimately to undetectable amounts.

To test the effect of trypsin treatment on the size of the DNA polymerase, we first isolated only that portion of the DNA polymerase that sedimented at 7.3 S. When this 7.3S DNA polymerase was treated with a low concentration of trypsin (1 μg; protein weight ratio, 100:1), a mixture of two enzyme forms was produced: one form sedimented at 7.3 S and the other at 5.5 S (Figure 4B). No loss of DNA polymerase activity resulted from this treatment. Treatment with higher concentrations of trypsin (2 μg, ratio 50:1; or 5 μg, ratio 20:1) resulted in some loss of DNA polymerase activity (30% and 40%, respectively). At the higher trypsin concentrations, all of the DNA polymerase sedimented at 5.5 S and no enzyme cosedimented with LDH (7.3 S) (Figure 4C,D). Similar results were obtained by increasing the duration of trypsin treatment instead of the trypsin concentration. When the 7.3S DNA polymerase was treated with a mixture of trypsin plus soybean trypsin inhibitor, all of the DNA polymerase again cosedimented with LDH at 7.3 S (Figure 4A), as did both untreated DNA polymerase and DNA polymerase treated with only soybean trypsin inhibitor (data not shown).

When an identical 7.3S DNA polymerase preparation was incubated with pancreatic DNase plus Mg^{2+} and analyzed on glycerol velocity gradients (which separated the DNase and DNA polymerase), all of the original 7.3S DNA polymerase was recovered in the 7.3S form. Thus, protease digestion, but not nuclease digestion, was able to decrease the sedimentation velocity of the 7.3S DNA polymerase to 5.5 S.

Loss of Ionic Heterogeneity Caused by Endogenous Proteolysis. We investigated the effect of endogenous proteolysis on another physical property of the DNA polymerase: its affinity for anion-exchange DEAE-resins. We compared the DEAE-Sephadex elution profiles of two stage II preparations. One preparation was obtained exactly as described by Karkas et al. (1975) and the other was obtained by a modification of that procedure, using $PhCH_2SO_2F$ and dechorionated embryos (Figure 5). The DNA polymerase from untreated whole embryos eluted from DEAE-Sephadex in a narrow distribution between 50 and 150 mM $(NH_4)_2SO_4$ (Figure 5A), as was re-

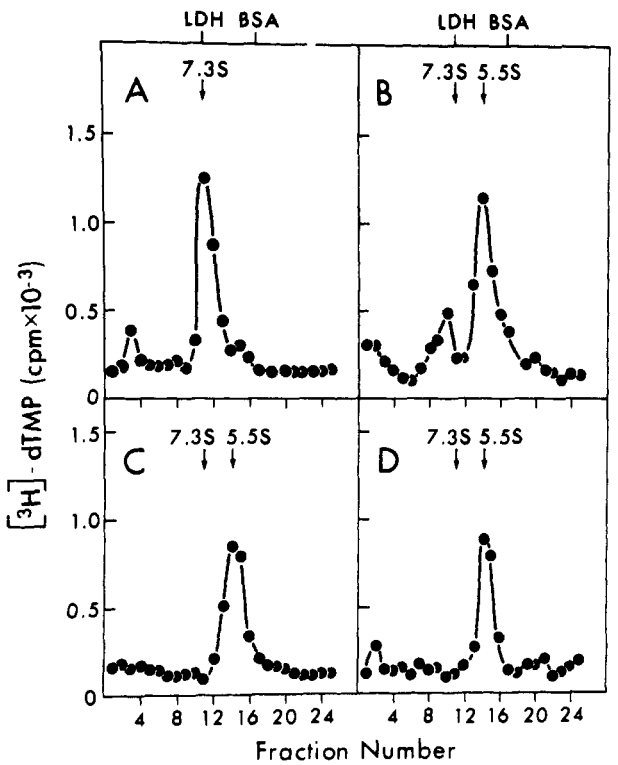

FIGURE 4: The effect of trypsin on the sedimentation velocity of the 7.3S DNA polymerase. The enzyme preparation described in the legend to Figure 5 was fractionated on 7–28% (v/v) glycerol gradients in 200 mM KPB. Gradients were centrifuged for 31 h at 39 000 rpm and 2 °C in a Spinco SW 40 rotor. LDH served as a sedimentation standard. Only the material that cosedimented with LDH (7.3 S) was combined and was then dialyzed against the trypsin digestion buffer: 50 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 0.1 mM EDTA, and 0.2 M $(NH_4)_2SO_4$. After dialysis, 250-μL portions (100 μg of protein) were incubated for 30 min at 37 °C with either (A) 5 μg of trypsin plus 10 μg of soybean trypsin inhibitor, (B) 1 μg of trypsin, (C) 2 μg of trypsin, or (D) 5 μg of trypsin. After a 30-min incubation, 10 μg of soybean trypsin inhibitor was added to B, C, and D, and the incubation was continued for 5 min. The samples were then layered over 7–28% (v/v) glycerol gradients prepared in the same buffer used for the trypsin digestion and formed over 0.6-mL cushions of 32% glycerol. Gradients were centrifuged for 35.5 h at 38 000 rpm and 2 °C in a Spinco SW 40 rotor. LDH (7.3 S) was centrifuged in all tubes and BSA (4.4 S) was centrifuged with LDH in a fifth tube; their sedimentation positions are indicated. DNA polymerase activity was determined using 10-μL portions of each fraction. Recovery of added DNA polymerase was 85–90% in each case.

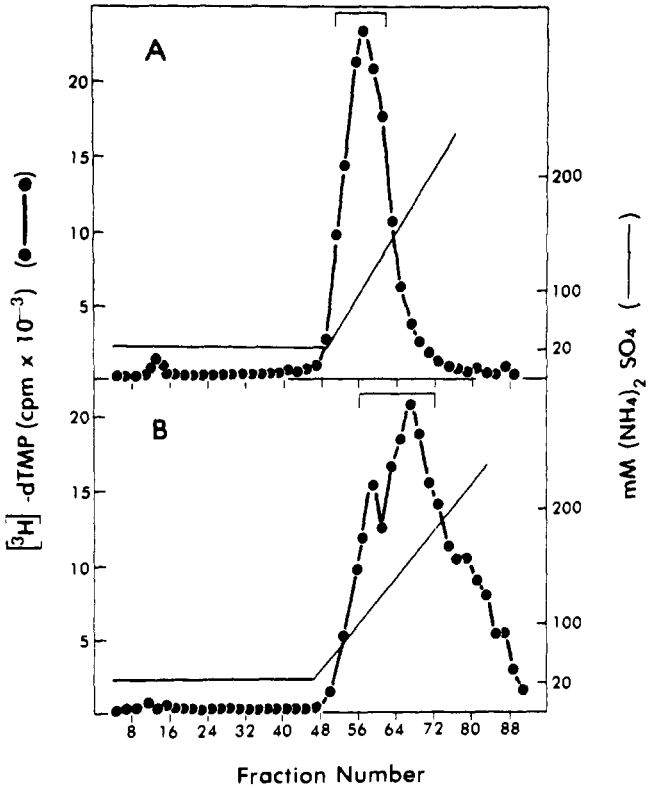

FIGURE 5: DEAE-Sephadex chromatography of DNA polymerase. (A) DNA polymerase was prepared exactly as described by Karkas et al. (1975) from 8 g of embryos. DEAE-Sephadex chromatography was performed on a 2.5 × 15 cm column with 100 mL of the stage II preparation (120 mg of protein). Five-milliliter fractions were collected and the DNA polymerase activity of 10-μL portions of every other fraction was determined. (B) DNA polymerase was prepared from 10 g of embryos which were dechorionated before homogenization. $PhCH_2SO_2F$ (0.5 mM) was added to the homogenization buffer and was also included in all other buffer solutions (at 0.1 mM). All other procedures were exactly as described by Karkas et al. (1975). The stage II preparation (80 mL, 160 mg of protein) was chromatographed and the fractions were analyzed as in A. The $(NH_4)_2SO_4$ concentration in every fifth fraction was determined using a CDM 3 conductivity meter (Radiometer/Copenhagen).

ported by Karaks et al. (1975). However, when the embryos were dechorionated prior to homogenization and $PhCH_2SO_2F$ was included in all buffer solutions, the DNA polymerase eluted in a heterogeneous distribution between 80 and 220 mM $(NH_4)_2SO_4$ (Figure 5B). These results indicated that endogenous proteolysis caused a reduction in the ionic heterogeneity of the DNA polymerase as well as a reduction in its size.

Even though proteolysis was reduced by dechorionating the embryos and by including $PhCH_2SO_2F$ in the buffer solutions, it may not be entirely eliminated by these precautions. Glycerol gradient analysis of the stage III enzyme preparations (pooled from DEAE-Sephadex chromatograms as indicated by the brackets in Figure 5A and B) showed that 75% of the activity in the untreated preparation sedimented at 5.5 S. In the preparation from dechorionated, $PhCH_2SO_2F$-treated embryos, the proportion of the 5.5S enzyme increased from the undetectable amounts (see Figure 2D) at stage II to approximately 25% of the total activity recovered after chromatography.

The ionic heterogeneity suggested by DEAE-Sephadex chromatography of the stage II preparation from dechorionated, $PhCH_2SO_2F$-treated embryos was also observed when the step 4 preparation (see Methods) was fractionated by DEAE-cellulose chromatography (Figure 6). In this fractionation procedure, three species of DNA polymerase (designated DEAE-I, eluted between 80 and 110 mM KPB; DEAE-II, eluted between 110 and 150 mM KPB; and DEAE-III, eluted between 180 and 210 mM KPB) were separated (Figure 6). Similar, if not identical, profiles were obtained repeatedly and the elution profile was unaffected by prior ammonium sulfate precipitation of the step 4 enzyme. Each of the three species resolved by the DEAE-cellulose chromatographic procedure eluted at its original salt concentration upon subsequent rechromatography, indicating that these enzyme fractions have distinct ionic properties.

When these enzymes were analyzed by glycerol gradient centrifugation, we observed that DEAE-I and DEAE-II both sedimented at 7.3 S and that DEAE-III sedimented at 9.0 S. Thus, the DNA polymerases obtained by these procedures were identical in size to those present in the crude extracts.

It is apparent from the DEAE-Sephadex chromatograms (Figure 5) that endogenous proteolysis reduced the affinity of the DNA polymerase for the anion-exchange resin. A similar result was obtained when the 7.3 S DNA polymerase was digested with trypsin. Recent results (to be reported elsewhere)

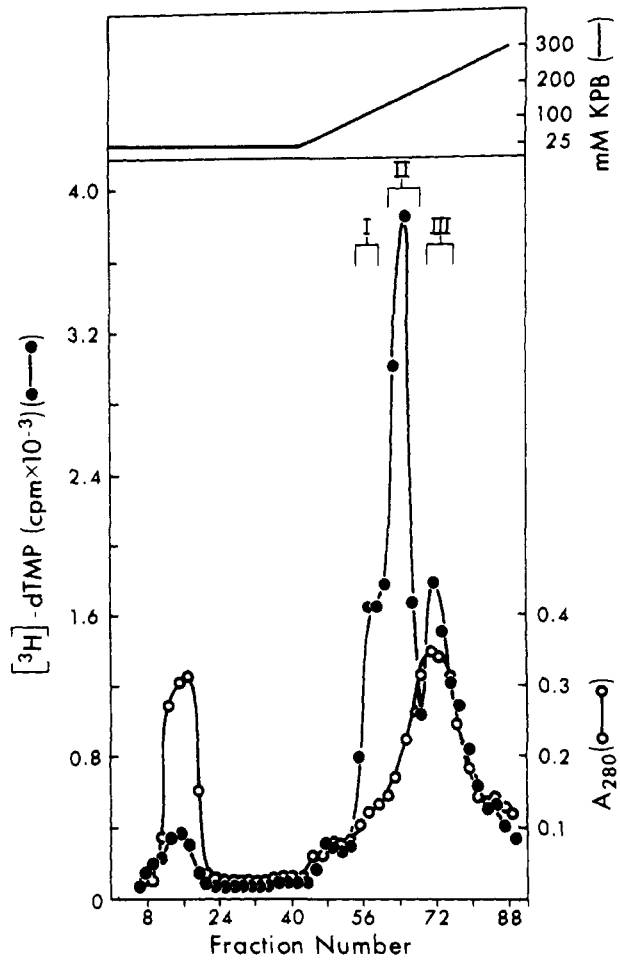

FIGURE 6: DEAE-cellulose chromatography of DNA polymerase. A step 4 enzyme preparation (see Methods) containing 56 mg of protein was dialyzed for 12 h against 25 mM KPB containing 20% (w/v) sucrose and then applied to a 2.5 × 15 cm DEAE-cellulose column which was equilibrated with 25 mM KPB. The column was eluted with 100 mL of 25 mM KPB and then a 260-mL gradient, from 25 to 300 mM KPB, was applied. Fractions of 6 mL were collected during the 25 mM KPB wash and 3.6-mL fractions were collected when the gradient was started. The DNA polymerase activity was determined by assaying 5-μL portions of every other fraction. Reaction mixtures were incubated at 25 °C for 20 min and the incorporation of [^{3}H]dTMP was then determined. The KPB concentration was determined in every fifth fraction using a CDM 3 conductivity meter (Radiometer/Copenhagen).

showed that the trypsinized 7.3S DNA polymerase (DEAE-II) did not adsorb to DEAE-cellulose in 25 mM KPB, the buffer which was used for enzyme adsorption as described in the legend to Figure 6. The 5.5S DNA polymerase which we prepared by the method of Karkas et al. (1975) also failed to adsorb to DEAE-cellulose in 25 mM KPB.

Discussion

One of the most striking characteristics of vertebrate DNA polymerase-α is its physical heterogeneity. This heterogeneity is easily detected by ion-exchange chromatography on DEAE-cellulose (Holmes et al., 1974) and by velocity sedimentation or gel-filtration chromatography (Holmes et al., 1974; Craig and Keir, 1975). The basis of the heterogeneity and the relationship of the resolved species is not clearly understood in most instances. However, recent evidence has shown that two species of calf thymus DNA polymerase-α resolved by DEAE-cellulose chromatography can be converted into a third form by mild urea treatment, indicating that they may be related (Holmes et al., 1975).

The high-molecular-weight DNA polymerases of lower eukaryotes are also heterogeneous. In *Saccharomyces cerevisiae* (Wintersberger and Wintersberger, 1970) and *Euglena gracilis* (McLennan and Keir, 1975a) two enzyme forms were resolved by DEAE-cellulose chromatography. The DNA polymerases from yeast sedimented at about 7.5 S (Wintersberger and Wintersberger, 1970) and the DNA polymerases from *Euglena* sedimented at 8.7 and 10.3 S (McLennan and Keir, 1975a). The *Euglena* enzymes sedimented more slowly after prolonged storage, perhaps as a result of dissociation of subunits (McLennan and Keir, 1975b).

The results presented here demonstrate that the *Drosophila* DNA polymerase is also heterogeneous in size and ionic composition. However, endogenous proteolysis during purification can produce an enzyme that is smaller (5.5 S) than those present in the initial extracts (7.3 and 9.0 S) and that elutes homogeneously from DEAE-Sephadex. When proteolysis was reduced by dechorionating the embryos and by using $PhCH_2SO_2F$, the enzyme sedimented at 7.3 S and eluted heterogeneously from anion-exchange DEAE-resins.

The source of the endogenous proteolytic activity is not certain. The proteolytic effect is reduced by the dechorionation procedure, indicating that the protease may be associated with the chorion membrane or with contaminants, such as yeast or bacteria, which are trapped by the chorion. However, extracts of the live yeast paste (used as fly food) did not alter the sedimentation velocity of the DNA polymerase. Present evidence suggests that the protease may be a stage-specific *Drosophila* enzyme because we observe primarily the 9.0S DNA polymerase in extracts of cleavage-stage embryos.

Our preparative procedures allowed the isolation of 7.3S and 9.0S DNA polymerases. These enzymes were purified to the same extent (30–40-fold) as the stage III enzyme described here and by Karkas et al. (1975), which sedimented at 5.5 S. Thus, the rapid sedimentation of the 7.3S and 9.0S enzymes could not be attributed to their being more highly contaminated.

To prove that the enzymes we prepared are related to the 5.5S enzyme, we have demonstrated that in vitro trypsin treatment of the 7.3S enzyme produces a 5.5S enzyme without reducing the polymerizing activity. This type of observation is not without precedent: *Escherichia coli* DNA polymerase I can be split by protease treatments into two fragments, one fragment contains the polymerase and the 3′ → 5′ exonuclease activities and one contains the 5′ → 3′ exonuclease activity (Brutlag et al., 1969; Klenow and Henningsen, 1970). Although we have identified the polymerase fragment of the *Drosophila* DNA polymerase, the functions and physical properties of the other fragment have not been determined.

The results of this work clearly demonstrate that the *Drosophila* DNA polymerase is physically heterogeneous. The chromatographic and size heterogeneity of the enzyme indicates it may be more similar to vertebrate DNA polymerase-α than was suggested by earlier findings (Karkas et al., 1975). The enzyme activities of the 7.3S and 9.0S DNA polymerases (to be reported elsewhere) also indicate that they are closely related to DNA polymerase-α. Although the 5.5S DNA polymerase can be proteolytically produced from the 7.3S DNA polymerase, the relationship of the 9.0S DNA polymerase to these two enzymes remains to be established.

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^{1}H Nuclear Magnetic Resonance Studies of Transfer RNA: The Methyl and Methylene Resonances of Baker's Yeast Phenylalanine Transfer RNA and Its Fragments†

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ABSTRACT: The methyl and methylene resonances of intact Baker's yeast $tRNA^{Phe}$ and its four key fragments in D_2O solution with Mg^{2+} have been measured by both 220- and 360-MHz spectrometers in a temperature range of from 16 to 98.5 °C. Totally, 12 methyl and 2 methylene resonances in $tRNA^{Phe}$ can be unambiguously assigned at high temperatures. Therefore, the profile of chemical shifts vs. temperature of each resonance can be measured. Four conclusions can be reached by this study based on chemical shift and line width: (i) the anticodon loop protrudes from the molecule and does not associate with any parts of the molecule; (ii) the presence of Mg^{2+} greatly stabilizes the native form, not only because the T_m is higher than that without Mg^{2+} (Kan, L. S., et al. (1974), *Biochem. Biophys. Res. Commun. 59,* 22) but also because the transition profile is more narrow; (iii) the residues D's, m^2_2G, T, m^1A, and perhaps m^7G, are involved in the tertiary structures of native tRNA; (iv) the T residue may have two different conformations, probably with respect to the mode of stacking to G_{53} in the native state. Based on the refined atomic coordinates of $tRNA^{Phe}$ in orthorhombic crystal and on the recent advances in the distance dependence of the ring-current magnetic field effects (Giessner-Prettre, C., et al.(1976), *Biopolymers 15,* 2277), a computed shielding effect ($\Delta\delta$) for these high field resonances was made. The computed $\Delta\delta$'s were compared with the observed $\Delta\delta$'s and the comparison indicates that the conformation of yeast $tRNA^{Phe}$ in aqueous solution is closely similar but not identical with that found in the crystal, especially in the TψC and D regions.

Recently, the structure of yeast phenylalanine transfer ribonucleic acid ($tRNA^{Phe}$) in crystalline state has been clearly elucidated by x-ray diffraction studies (Kim et al., 1974; Ladner et al., 1975a). Furthermore, the three-dimensional coordinates of all atoms (except hydrogen) in this tRNA molecule have been reported by several laboratories (Quigley

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