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DNA Polymerase of Chicken Embryo: Purification and Properties*

(poly(rA)·poly(dT) template/9000-fold purified/Mn^{2+})

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ABSTRACT **The almost complete purification of the previously reported DNA polymerase of chicken embryo is described; in the final isolation step the enrichment of the enzyme, which comprises about 0.01% of the protein extractable from 10-day-old embryos, is more than 9000-fold. The enzyme is a basic protein (pI = 9.15), of molecular weight about 27,000. It exhibits no nuclease activity, requires Mn^{2+} ion for activity, and may contain Zn. It is very sensitive to oxidation.**

A recent orienting survey of the nucleic acid polymerases of the developing chicken embryo provided evidence of the presence of several enzymes (1). The outstanding role seemed to be played by a DNA polymerase, presumably of cytoplasmic origin, that reached its peak in 6- to 8-day-old embryos, was activated by Mn^{2+}, and appeared to prefer a DNA–RNA hybrid as template. We continue here the study of this enzyme, reporting its almost complete purification—a more than 9000-fold enrichment over the initial extract—and some of its properties.

MATERIALS AND METHODS

Materials. Unlabeled deoxyribo- and ribonucleoside triphosphates were supplied by Schwarz/Mann and Sigma, radioactive precursors by Schwarz/Mann and New England Nuclear. Sucrose came from Merck; Bio-Gel A-1.5 m and the acrylamide kit from Bio-Rad; nitrocellulose filters from Schleicher & Schuell; Sephadex G-100 and Sephadex CM-50 from Pharmacia; microgranular DEAE-cellulose DE52 and phosphocellulose P11 were Whatman products; *o*- and *m*-phenanthroline were kindly given us by Dr. A. S. Mildvan.

The hybrid template poly(rA)·poly(dT) was supplied by Miles Laboratories; it was prepared from poly(rA) having an s_{20} value of 7.9 and from poly(dT) with s_{20} of 2.6. Calf-thymus DNA was isolated in the usual manner (2); for denaturation, a 0.05% solution in 15 mM NaCl—1.5 mM sodium citrate (pH 7) was heated for 10 min at 100° and cooled rapidly. As a test substance for nuclease activity, DNA was rendered radioactive by the action of DNA polymerase I of *E. coli* (3) on denatured calf-thymus DNA in the presence of labeled precursors.

Buffers. The pH values stated were measured with the buffers at 25° before addition of KCl. The Tris buffers (0.05 M) had the following composition: pH 7.5, 6.35 g Tris·HCl and 1.19 g Tris base in 1 liter; pH 7.9, 4.88 g Tris·HCl and 2.3 g Tris base in 1 liter (compare p. 494 of ref. 4). The 2 M acetate buffer was prepared by dissolving 72 g of sodium acetate trihydrate and 93 g of glacial acetic acid in 1 liter; after 10-fold dilution, this buffer has pH 4.2 at 25° (compare p. 487 of ref. 4).

The other buffers used in this work carry the following designations: *extraction buffer*, 0.05 M Tris (pH 7.5)–0.35 M sucrose–0.025 M KCl–0.01 M $MgCl_2$; *buffer A*, 0.05 M Tris (pH 7.5)–0.35 M sucrose–0.3 M KCl–0.01 M $MgCl_2$; *buffer B*, 0.05 M potassium phosphate (pH 6.5)–0.2 M KCl–5 mM 2-mercaptoethanol; *buffer C*, same as *B*, but with 0.3 M KCl; *buffer D*, same as *B*, but with 0.4 M KCl; *buffer E*, same as *B*, but with 0.6 M KCl; *buffer F*, 0.05 M Tris (pH 7.9)–0.15 M KCl–5 mM 2-mercaptoethanol; *buffer G*, same as *F*, but with 0.25 M KCl.

Analytical Procedures. The molecular weight of the enzyme was estimated by sucrose density gradient centrifugation (5) and gel filtration (6). Fresh and stored preparations of the enzyme were subjected to polyacrylamide gel electrophoresis (7), the stored preparation also in the presence of sodium dodecyl sulfate (8). Its isoelectric point was measured by electrofocusing in an LKB 8101 apparatus with a pH gradient of pH 6–10. Protein was estimated either by spectroscopy (9) or by the biuret reaction (10).

Assay of Deoxyribonuclease Activity. Radioactive DNA was incubated with a large excess of DNA polymerase and tested at various time intervals for loss of radioactivity in the fraction precipitated by trichloroacetic acid. The composition of the assay mixture (volume, 0.65 ml) was: 0.05 M Tris·HCl (pH 8.3), 0.1 M KCl, 1 mM $MnCl_2$, 180 units of enzyme, 140 μg of DNA (270,000 cpm), 800 μg of bovine serum albumin, 2 mM dithiothreitol. The incubation extended to 15 hr at 35°, portions of 50 μl being removed at 3-hr intervals and tested for acid-precipitable radioactivity. Another portion of 10 μl was tested simultaneously for polymerase activity.

Assay of DNA Polymerase Activity and Definition of Unit. The composition of the assay mixture (0.13 ml) was: 0.05 M Tris·HCl (pH 8.3), 0.12 M KCl, 1 mM $MnCl_2$, 0.5–1 unit of enzyme, 5 μg of poly(rA)·poly(dT), 80 μg of bovine serum albumin, 20 nmol of labeled TTP, and 2 mM dithiothreitol. Incubation was for 20 min at 35°.

The polymerase unit is defined as the quantity of enzyme catalyzing the incorporation into an acid-insoluble product of 1 nmol of deoxyribonucleotide in 20 min at 35°.

* This is Paper II of the series: *Nucleic Acid Polymerases of the Developing Chicken Embryo.* For Paper I, see ref. 1.

TABLE 1. *Isolation of DNA polymerase from 10-day-old chicken embryos*

Step	Procedure	Volume (ml)	Total protein (mg)	Total activity (10^3 units)	Specific activity	Recovery (%)
I	Crude extract	8300	36,055	242	6.7	100
II	Precipitation at pH 4.2	2000	23,410	239	10.2	99
III	Fractionation with ammonium sulfate (pH 4.2)	300	1,684	208	123.5	86
IV	DEAE-cellulose column	770	1,173	186.8	159.2	77
V	Sephadex CM50 column (pH 6.5)	4	40.7	147.6	3,630	61
VI	Sephadex G-100 column	52	13.9	130.9	9,420	54
VII	Sephadex CM50 column (pH 7.9)	2.2	1.63	100.9	61,900	42

1000 Eggs were processed in this preparation. See *text* for definition of unit and experimental details. The specific activity is expressed as units per mg of protein.

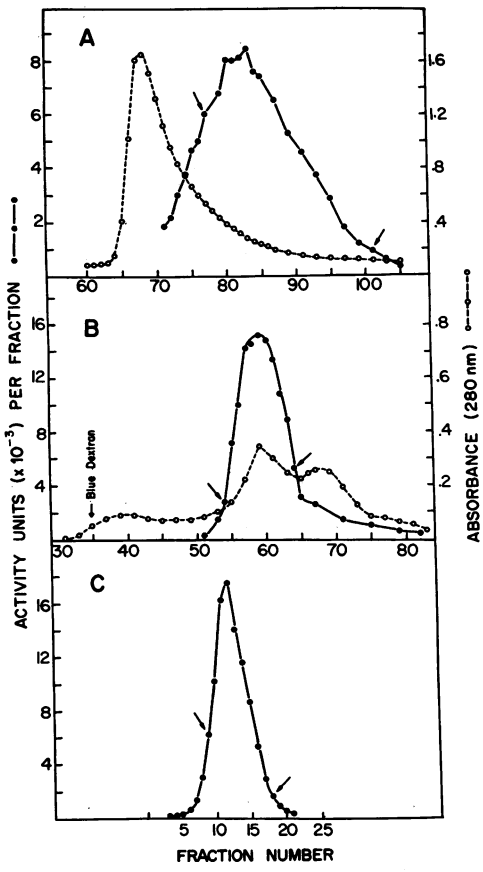

FIG. 1. Final steps of purification of DNA polymerase of chicken embryo. *A*: Step V, fractionation on Sephadex CM-50 at pH 6.5; 6-ml fractions were collected. Of the activity applied after Step IV, 90% was recovered; fractions between the two *arrows* (representing 90% of recovered activity) were used in the next step. *B*: Step VI, filtration through Sephadex G-100 at pH 6.5; 4.5-ml fractions were collected. Of the activity applied after Step V, 97% was recovered; the indicated fractions combined for use in the next step comprised 91% of recovered activity. *C*: Step VII, fractionation on Sephadex CM-50 at pH 7.9; 2.5-ml fractions were collected. Of the activity applied, 80% was recovered; the indicated fractions combined for final concentration comprised 97% of the recovered activity. See *text* and Table 1 for other details.

PREPARATION OF DNA POLYMERASE

Principles of fractionation

The final purification of the enzyme is achieved in seven steps, which are summarized in Table 1. Salt concentrations, pH values, and other preparatory details are critical and should be observed. The route of fractionation derived from several preliminary observations: (*a*) the enzyme can, in the range of pH 4.2–10, be kept for more than 10 hr without activity loss; (*b*) it is a basic protein; (*c*) it can be precipitated from the crude extract at pH 4.2 at a low salt concentration, but remains in solution at higher salt concentrations (0.8 M ammonium sulfate).

Step II, though not productive of great enrichment, is necessary because it permits a large part of the basic proteins, which otherwise would hinder the purification of the enzyme, to be removed; it also reduces the solution volumes considerably. Step III must be performed stepwise, as indicated later, since a portion of the inactive proteins precipitated below 18% $(NH_4)_2SO_4$ saturation go back into solution at a higher salt concentration. In Steps V and VII Sephadex CM-50 was used, because the enzyme loses 60–70% of its activity within 7 hr when adsorbed on phosphocellulose.

It should be noted that all subsequent operations were done at 4°.

Step I†. This is a modification of the previously described extraction procedure (1). A maximum of 500 eggs can be processed in 1 day. At one time, 125 eggs are opened on the side where the embryos are located; embryos are removed carefully (average wet weight, 2.5 g per embryo), placed in 1.2 liter of cold 0.35 M sucrose, collected on a plastic sieve, and again washed with the same sucrose volume. The

† Fertilized hen's eggs (White Leghorn), incubated for 10 days, were supplied by the Shamrock Poultry Farm, North Brunswick, N.J. Although the highest level of enzyme is found in 8-day embryos (1), the slightly older and much larger embryos are preferred owing to the greatly increased yield of enzyme. For reasons of economy, the glycyl-glycine buffer used before (1) was replaced by Tris·HCl buffer. Comminution of the tissue with a glass–Teflon grinder gives the best results: stirring of the tissue suspension in a Waring Blendor for 2 min at maximum speed reduced the enzyme activity by 50%.

embryos are distributed among 25 large test tubes, to each of which 36 ml of cold extraction buffer is added. The content of each tube is ground by means of five strokes at 1250 rpm in a 50-ml glass–Teflon grinder (clearance, 0.015–0.023 cm). The combined extracts are stirred for 20 min and centrifuged (15,000 × *g*, 1 hr). The supernatant fluid is stored overnight at 4° without the need of protection by a reducing agent.

Step II. The crude extract of 500 embryos is adjusted to 5 mM by the addition of 1 M 2-mercaptoethanol, 0.1 volume of 2 M acetate buffer is added, and the mixture is stirred for 40 min and centrifuged (10,000 × *g*, 20 min). The suspension of the sediment in 650 ml of 0.02 M Tris base + 5 mM 2-mercaptoethanol is blended in the grinder with three strokes, brought to pH 6.2–6.5 with about 20 ml of 1 M Tris base, and adjusted to 1 liter with 5 mM 2-mercaptoethanol. The solution is cloudy, the enzyme is completely dissolved.

Step III‡. The treatment with ammonium sulfate is performed in three stages: (*a*) precipitation below 18% saturation; (*b*) at 18–40%; (*c*) at 40–80%. (*a*) To 1 liter of Step II solution, 114 g of $(NH_4)_2SO_4$ is added with slow stirring for 15 min; then 0.1 volume of 2 M acetate buffer is added with faster stirring for 30 min, during which time a heavy protein precipitate appears that is removed by centrifugation (15,000 × *g*, 1 hr). The sediment, which contains the bulk of tissue protein, including the enzyme incorporating deoxynucleotides with poly(rI)·poly(rC) as template (Table 3 of ref. 1), retains only 5–10% of the DNA polymerase activity; it is discarded. (*b*) To the supernatant fluid, 133 g/liter of $(NH_4)_2SO_4$ are added slowly with stirring for 40 min. Centrifugation (15,000 × *g*, 1 hr) yields a supernatant fluid having most of the polymerase activity§. (*c*) The addition of 285 g/liter of $(NH_4)_2SO_4$ to the supernatant fluid of the preceding stage brought it to 80% saturation. The mixture was stirred for 3–4 hr and centrifuged (15,000 × *g*, 2 hr). The aqueous layer is decanted carefully from the precipitated protein, which is dissolved in 80–100 ml of Buffer *A;* the solution is clarified by centrifugation (100,000 × *g*, 2 hr), filtered through glass wool, and dialyzed overnight against 6 liters of Buffer *C*. An inactive precipitate is removed (15,000 × *g*, 30 min)¶.

The solution had an absorbance ratio (280/260 nm) of 0.72.

Step IV. The solutions resulting from the processing of 1000 embryos were combined and applied to a column (8 × 14.5 cm) of 725 ml (packed volume) of DEAE-cellulose that had been equilibrated overnight with Buffer *B*. The column was charged gradually with 40-ml portions of the enzyme solution, each diluted with 20 ml of 0.05 M phosphate buffer pH 6.5–5 mM 2-mercaptoethanol, at a flow rate of 200 ml/hr. Elution then was continued with Buffer *B* at the same rate; 25-ml fractions were collected. A large portion of the nucleic acids and about 30% of the protein are retained on the column, but the enzyme passes through. All protein-containing eluate fractions having an absorbance ratio (280/260 nm) higher than 1.1 are combined for the next step. The absorbance ratio of the pooled fractions is 1.2–1.3. After this step, the enzyme is no longer precipitable at pH 4.2.

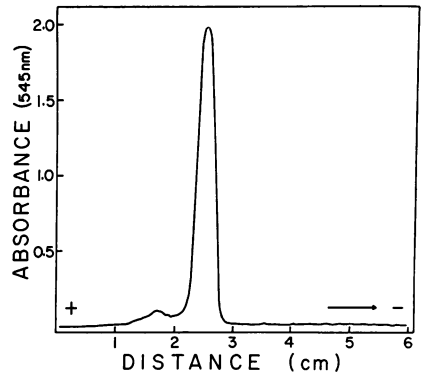

FIG. 2. Polyacrylamide gel electrophoresis of enzyme after step VII. To each tube (7.5% acrylamide, 6 × 60 mm) 8 μg of protein was applied. Electrophoresis was performed at 4 mA per gel and 5°. The gels were stained with Coomassie Brilliant Blue, destained electrophoretically, and scanned in a Gilford spectrophotometer. The areas under the peaks were used to estimate proportions.

Step V‖. The column (4 × 29 cm), equipped with a flow adaptor similar to the Pharmacia K50 columns, is packed with Sephadex CM50 and equilibrated with Buffer *B*. The enzyme solution is applied to the gel surface under a constant hydrostatic pressure of 60 cm, and the column is washed with 800 ml of Buffer *B;* much inactive protein is eluted. Elution is continued with Buffer *D*, while the outflow rate is reduced to 2/3 by means of a peristaltic pump. The elution profile is shown in Fig. 1*A*. All fractions exhibiting an at least 80-fold enrichment over Step I are combined and 611 g/liter of $(NH_4)_2SO_4$ is added; the mixture, after being kept for at least 8 hr at 4°, is centrifuged (15,000 × *g*, 1 hr). The sediment is dissolved in 3–4 ml of Buffer *B* and dialyzed overnight against 3 liters of the buffer.

Step VI. A column (2.5 × 93 cm) of Sephadex G-100 was equilibrated with Buffer *B* (outflow rate 20 ml/hr, hydrostatic pressure 50 cm); the enzyme was applied under the same conditions and eluted with Buffer *B* (Fig. 1*B*). The fractions showing a more than 550-fold purification compared with Step I (about 50 ml) were combined.

Step VII. A Sephadex CM-50 column (1.2 × 9 cm) was equilibrated with Buffer *B* and charged with the solution from

‡ The values for % $(NH_4)_2SO_4$ saturation at 25° are based on p. 76 of ref. 11.

§ The sediment resulting from this centrifugation is taken up in 30–40 ml of Buffer *A* (three strokes in a glass-Teflon grinder) and again centrifuged (100,000 × *g*, 2 hr). If the resulting supernatant fluid contains more than 10% of the enzyme activity of Step II, it is dialyzed overnight against 2 liters of Buffer *C* and refractionated with $(NH_4)_2SO_4$ (0–40% and 40–80% saturation); the solution after dialysis against Buffer *C* is combined with the main fraction.

¶ The concentration of 0.3 M KCl is critical: at 0.2 M KCl, the precipitate may contain 40% of the enzyme activity. At this stage, the enzyme solution in the presence of 5 mM 2-mercaptoethanol is stable for at least 2 weeks at 4°. During the preceding steps, rapid activity loss is observed.

‖ The columns packed with Sephadex CM50 (Steps V and VII) must be tested with blue dextran about 3 hr after having been filled, and used within 5 hr, since otherwise the chromatography is disturbed by channeling.

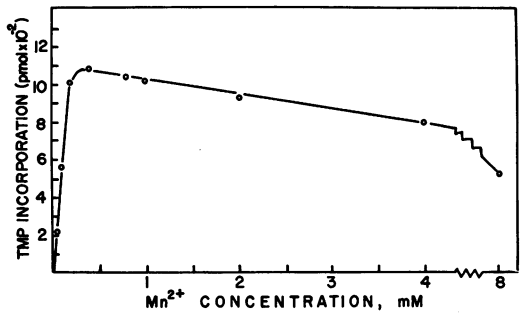

FIG. 3. Effect of concentration of Mn^{2+} ions. The standard assay conditions were used, with 25 ng of DNA polymerase and the indicated concentrations of metal ion.

Step VI. It was first washed with 10 ml of Buffer *B*, then treated with about 30 ml of Buffer *F*, at a flow rate of 10 ml/hr. At this point the column was equilibrated at pH 7.9. Washing with Buffer *F* was continued until the absorbance at 280 nm had dropped to 0.02–0.03 in the eluates. This step removes extraneous protein. The DNA polymerase then is eluted with Buffer *G*, at a flow rate of 5 ml/hr. At this stage the progress of elution, shown in Fig. 1*C*, could be followed only by measurements of enzymic activity. The enzyme was concentrated as follows. A column (1.2 × 5 cm) of Sephadex CM50 was equilibrated with Buffer *B* before completion of Step VII. Eluate portions of 2 ml each were diluted with 2 ml of 0.05 M phosphate buffer pH 6.5–5 mM 2-mercaptoethanol and were applied successively to the small column at an outflow rate of 15 ml/hr, followed by a wash with 10 ml of Buffer *B* and elution with Buffer *E*, at a rate of 5 ml/hr. The enzyme, now sufficiently concentrated to be measured at 280 nm, was collected in three 1.3-ml fractions. These were dialyzed in a dialysis tube (diameter, 0.625 cm; thickness, 0.005 cm) against 300 ml of a 1:1 mixture of glycerol and Buffer *C*** until the volume was reduced to 25% of the original. After the addition of 1 ml of the same mixture to the dialysis residue, the bag was closed and dialysis against the original mixture continued for 4 hr. The enzyme solution is stored at −20° under argon. As is seen in Table 1, the enrichment over the crude extract was 9240-fold††.

PROPERTIES OF DNA POLYMERASE AND CONCLUDING REMARKS

The enzyme is a basic protein, with an isoelectric point of pI = 9.15. Its molecular weight, estimated from gel filtration through a calibrated Sephadex G-100 column, is 27,000. Sucrose density gradient centrifugation yielded, on the assumption of a partial specific volume of 0.725, a value of 31,000. About 92% of the protein occurs in one main band upon gel electrophoresis of a fresh specimen, (Fig. 2). After storage of the enzyme (5 months at −20°), a second minor band appears. The same preparation, subjected to electrophoresis in the presence of sodium dodecyl sulfate, gave no evidence of the existence of subunits: the estimated molecular weight was 29,500.

The enzyme does not appear to exhibit any nuclease activity. In the assay described above, no degradation of radioactive DNA was observed even after an incubation period of 15 hr with large amounts of enzyme. The polymerase activity, however, dropped rapidly: it was 60% of the initial value after 3 hr, 25% after 6 hr, and the enzyme was inactive after 15 hr.

As can be seen in Table 2, the enzyme is very sensitive to dilution and oxidation. With the three additions listed, even a dilute solution (0.5 μg/ml) can be stored at 4° for 15 days with only a 20% loss of activity. Under assay conditions, i.e., in the presence of both template and precursors, the enzyme appears stabilized, but it is unstable if one of these two factors is omitted.

The enzyme exhibits an absolute requirement for Mn^{2+}, which cannot be replaced by Mg^{2+}: with Mg^{2+} only 3% of the activity is found. (See Fig. 3.) Changes in the KCl concentration have little effect. There is, however, evidence implicating a second metal, most probably Zn, whose presence has been demonstrated in the terminal deoxynucleotidyl transferase (12) and in DNA polymerases (13). We show, in Fig. 4, the inhibition of our enzyme by *o*-phenanthroline. It is, however, noteworthy that this inhibition can be observed only at low template concentrations. With template concentrations at the saturation level (5 μg), the enzyme is not inhibited, even after its preincubation with 1 mM *o*-phenanthroline.

The DNA polymerase described here amounts to about 0.01% of the protein found in the crude extract of chicken embryos. It is the predominant, but probably not the only, DNA polymerase present. We shall report later on the template specificity of the pure enzyme and also on the polymerases of adult tissue. In view of certain similarities in template preference between the enzyme isolated from chicken embryos and the DNA polymerases found repeatedly in RNA-containing tumor viruses, one could ask whether the enzyme discussed here derives from a virus. We are aware of the question, but lack an answer. It could be imagined that the

TABLE 2. *Sensitivity of pure DNA polymerase of chicken embryo (as % of maximum activity of Step VII enzyme in Table 1)*

Additions			Preincubation for 10 hr at 4°	Preincubation for stated periods (in min) at 35°		
Dithiothreitol	Bovine serum albumin	Argon		0	20	60
+	−	−	19	54	10	0
−	+	+	43	96	77	58
+	+	+	96	100	96	86

Each preincubation assay comprised, in a total volume of 0.05 ml, 0.025 μg of enzyme (Step VII in Table 1) in 0.02 M glycylglycine buffer of pH 7.5, with or without the additions indicated: 5 mM dithiothreitol, 80 μg of bovine serum albumin, or argon atmosphere. Nitrogen can replace argon, but it is less convenient. Subsequently, the enzyme was assayed under standard conditions.

** In subsequent experiments, it has proved advantageous at this point to substitute 5 mM dithiothreitol for 2-mercaptoethanol in Buffer *C*, since the stored enzyme retains its activity longer under these conditions.

†† If Step VI is omitted, so that the two fractionation runs through Sephadex CM50 at pH 6.5 and 7.9 (Steps V and VII) follow each other directly, a less homogeneous enzyme preparation (two minor bands in gel electrophoresis), with a 6000-fold enrichment over the crude extract, is obtained.

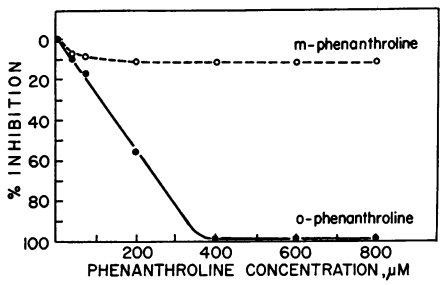

FIG. 4. Inhibition of DNA polymerase action by *o*-phenanthroline. The standard assay conditions were used, but with 1 μg of poly(rA)·poly(dT) and 12.5 ng of enzyme, together with the indicated concentrations of either *o*- or *m*-phenanthroline.

instruction for this enzyme is present in, or incorporated into, the genome of the organism, is expressed during embryonic development, but is partially or completely suppressed during further growth. It is to be hoped that such questions, which at the moment are part of the metaphysics of cancer, will eventually become answerable on a firm experimental basis. These studies are being continued. It will undoubtedly be of interest to extend them to other cellular systems engaged in differentiation.

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