Low Molecular Weight Deoxyribonucleic Acid Polymerase from Rabbit Bone Marrow†

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ABSTRACT: A deoxynucleotide polymerizing activity having a molecular weight of 40,000–50,000, estimated by gel filtration and sucrose gradient centrifugation, was purified from rabbit bone marrow. This polymerizing activity uses template information for selection of the monomer triphosphate polymerized and is therefore a *replicative* deoxynucleotidyl transferase (DNA polymerase). Cell fractionation experiments suggest that this low molecular weight enzyme is the only DNA polymerase present in purified nuclei. The purified enzyme has an alkaline pH optimum and requires a divalent cation, initiated template, and deoxynucleoside triphosphates complementary to the template for maximum activity. Nuclease activity was not detected in the purified enzyme. The reaction catalyzed by the low molecular weight polymerase proceeds for a single round of complementary synthesis, forming complete double-stranded polydeoxynucleotide products.

Two kinds of deoxynucleotide polymerizing enzymes have been isolated from mammalian systems: *terminal* deoxynucleotidyl transferase and *replicative* deoxynucleotidyl transferase (DNA polymerase). The terminal transferase from calf thymus glands was characterized by Kato *et al.* (1967) and purified to homogeneity (Chang and Bollum, 1971a). The terminal transferase is a low molecular weight enzyme (32,460), is found only in the thymus gland, and has a developmental cycle in the thymus during embryonic growth (Chang, 1971). The replicative DNA polymerase was first observed in 1957 (Bollum and Potter, 1957) and shown to be present in the cytoplasmic-soluble fraction of regenerating rat liver (Bollum and Potter, 1958). The replicative DNA polymerase was partially purified (Yoneda and Bollum, 1965) from calf thymus gland. A low molecular weight DNA polymerase (3.39 S) recently described (Chang and Bollum, 1971b) is present in both the cytoplasmic and nuclear fractions of mammalian tissue extracts. This enzyme is probably the same enzyme activity first described by Howk and Wang (1969) in rat liver nuclei, and subsequently in rat liver (Bellair, 1968), in tumor cells (Furlong and Gresham, 1971), and in HeLa cell nuclei (Weissbach *et al.*, 1971). We believe it to be the only form present in purified nuclei (Chang and Bollum, 1971b). Baril *et al.* (1971) also describe an enzyme from rat liver nuclei and free ribosomes that may be similar to the activity reported here.

This report is concerned with the intracellular distribution of DNA polymerase in rabbit bone marrow, the partial purification, and reaction properties of the low molecular weight DNA polymerase from rabbit bone marrow.

Methods and Materials

Monomers and Polymers. Deoxynucleoside triphosphates[1] (dNTP's), polydeoxynucleotides, and oligodeoxynucleotides were prepared as described earlier (Chang and Bollum, 1971a). Polydeoxynucleotide–oligodeoxynucleotide complexes were prepared by physical mixing of the appropriate materials followed by heating to 60° and slow cooling. The use of initiated[2] polydeoxynucleotide templates has been described in earlier work (*cf.* Bollum, 1964, 1967). Radioactive dNTP's were purchased from Schwarz/Mann or Amersham/Searle.

Activated calf thymus DNA (DNase I-treated DNA) used for routine enzyme assays was prepared as described by Aposhian and Kornberg (1962). DNase I nicked-exonuclease III-treated calf thymus DNA and exonuclease III-treated λ-DNA were gifts from Dr. M. Gefter, and fd-DNA was a gift from Dr. R. Benzinger. The human γ-globulin, bovine serum albumin, and sperm whale myoglobin used as molecular weight markers were purchased from Schwarz/Mann Biochemicals.

Biological Material. Rabbit bone marrow for cell fractionation was obtained fresh from the femur and tibia of 1- to 2-month-old New Zealand rabbits. Frozen rabbit bone marrow for enzyme purification was purchased from Pel-Freez Biologicals.

Enzyme Assays. The low molecular weight DNA polymerase activity is measured by the polymerization of radioactive dNTP into acid-insoluble form in the presence of activated calf thymus DNA or initiated homopolymer as template. The activated DNA reaction is carried out by incubating the enzyme protein at 35° in 0.05 M ammediol buffer (2-amino-2-methyl-1,3-propanediol) (pH 8.6), 200 μg/ml of activated calf thymus DNA, 1 mM 2-mercaptoethanol, 8 mM $MgCl_2$, 0.1

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[1] The abbreviations used for nucleotides and polynucleotides are those of the IUPAC–IUB commission (1970). Abbreviations for homopolymers bearing the subscript *n* refer to chain lengths greater than 500. Definite subscript numbers refer to the chain length of oligonucleotides. A bar over a definite subscript denotes average chain length, and a mixture of a finite range of oligonucleotides is designated, for example, by dC_{9-11}. Ammediol is used as a trivial name for 2-amino-2-methyl-1,3-propanediol, and KP_i is used to abbreviate potassium phosphate buffers.

[2] In this paper we use the term template to designate the polymer chain used for selection of complementary monomer triphosphates. The term initiator is used to designate the chain containing a free 3′-OH, physically complexed with the template. The initiator chain participates in chemical reaction with the monomer triphosphate forming phosphodiester bonds, while the template chain participates by physical interaction.

mM each of dGTP, dCTP, dATP, and [^{3}H]dTTP. The homopolymer reactions are generally carried out by incubating enzyme with 0.1 mM polymer nucleotide initiated with 5 to 10 μM oligodeoxynucleotide in 0.05 M ammediol buffer (pH 8.6) for $dC_n \cdot dG_{8-12}$, or 0.05 M Tris-Cl (pH 7.6) for $dA_n \cdot dT_{12}$ and $dT_n \cdot dA_{12}$, 1 mM 2-mercaptoethanol, 0.1 mM of the appropriate [^{3}H]dNTP's, and 0.5 mM $MnCl_2$. The specific activity of the [^{3}H]dNTP's used are from 10,000 to 70,000 cpm per nmole. Progress of the reaction is followed by measuring the amount of product formed as a function of time, on aliquots placed on glass fiber disks and worked up for acid-insoluble product as described previously (Chang and Bollum, 1971b). The amount of radioactive product formed is determined by scintillation counting of the disks in toluene containing 0.4% 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene. Reaction rate is expressed as nanomoles of deoxynucleotide incorporated per hour. One unit of enzyme is equal to 1 nmole of dNTP incorporated per hour and specific activity of the enzyme is expressed as enzyme units per mg of protein.

Routine enzyme assays on column effluents or gradient fractions were carried out by adding 50 μl of a reaction mixture to 10 μl of enzyme solution, and then incubating at 35° for 15 or 60 min. At the end of the incubation 50 μl of each reaction is processed on glass fiber disks for acid-insoluble radioactivity as described above.

Protein Determination. Protein is determined with the biuret reagent of Gornall *et al.* (1949) for crude extracts and by absorbance at 280 nm for partially purified fractions.

Cell Fractionation. Rabbit bone marrow is mechanically removed from the femur and tibia and suspended in Swim's 67 media (Plagemann and Swim, 1966). A homogenous cell suspension is obtained by gently forcing the suspension through a 15-gauge needle with a syringe. The bone marrow cell suspension is then centrifuged in a clinical centrifuge to collect the cell pellet. The fatty top layer present after the low-speed centrifugation is resuspended in Swim's 67 media, and a second cell pellet is obtained by centrifugation. The two cell pellets are combined and washed three times with Swim's 67 media, and twice with 0.25 M sucrose containing 50 mM Tris-Cl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$ (TKM). The bone marrow cells are then suspended in four volumes of 0.25 M sucrose in TKM and homogenized with a Teflon pestle and a glass homogenizer. A portion of the homogenate is centrifuged at 7000 rpm to produce a crude supernatant fraction. The crude supernatant fraction is then centrifuged for 1 hr at 40,000 rpm in the No. 40 rotor in the Spinco centrifuge. The Spinco supernatant fraction is dialyzed against 0.1 M KP_i (pH 7.5) and the enzyme activities present in the soluble cytoplasmic fraction are analyzed on 5–20% sucrose gradients. A second portion of the homogenate is used to prepare nuclei by the step sucrose gradient described by Blobel and Potter (1966). The nuclear fraction obtained from the step gradient is washed once in 0.5% Triton X-100 in 0.25 M sucrose in TKM, twice in 0.25 M sucrose in TKM, and is then suspended in 0.2 M KP_i (pH 7.5) for extraction of enzyme activity. The 0.2 M KP_i (pH 7.5) suspension of the rabbit bone marrow nuclei is centrifuged for 2 hr at 40,000 rpm in the No. 40 rotor of the Spinco centrifuge and the resulting supernatant fraction is analyzed on 5–20% sucrose gradients.

Sucrose Gradient Centrifugation. Linear 5–20% (w/w) sucrose gradients are prepared as described by Britten and Roberts (1960). The gradients are made up in 0.1 M KP_i (pH 7.5) containing 1 mM 2-mercaptoethanol. The enzyme solution is loaded onto the gradient with a valve type sample applicator at 6000 rpm for 30 min in the SW 50L rotor in a Spinco L-2 centrifuge. Sedimentation is then carried out for 16 hr at 40,000 rpm at 5°. At the end of the centrifugation 24 equal volume fractions are collected from the top of each gradient by displacement from the bottom with 40% sucrose solution.

Column Chromatography. Whatman phosphocellulose (P-1 floc, purchased from H. Reeve Angel Company) was regenerated according to Yoneda and Bollum (1965). After regeneration and removal of fines it is packed into a 3.2 × 20 cm column, and equilibrated with 0.1 M KP_i (pH 7.5) in 1 mM 2-mercaptoethanol.

The Sephadex G-100 column (1.3 × 100 cm) was equilibrated with 0.1 M KP_i (pH 7.5) in 1 mM 2-mercaptoethanol.

Results

Cellular Distribution of DNA Polymerase Activities. Cytoplasmic-soluble fraction, and an 0.2 M KP_i (pH 7.5) extract of purified nuclei, obtained from the bone marrow of a 1-month-old rabbit, were analyzed on sucrose gradients as described in Methods and Materials. The distribution of DNA polymerase activities in the cytoplasmic and nuclear fractions are shown in Figure 1. The cytoplasmic fraction contains enzyme activities sedimenting at 3.39 S, and 8 S with a shoulder at 6 S while the nuclear extract contains only one molecular species, the 3.39S activity. Analysis carried out on 1 M NaCl extracts of purified nuclear fractions show identical activity profiles on sucrose gradients and total DNA polymerase activity comparable to that obtained by extraction with 0.2 M KP_i (pH 7.5). Since much less nuclear protein is extracted with 0.2 M KP_i, this extraction procedure was used throughout the work in this paper. The analysis shown here was done with bone marrow from a relatively young rabbit and a significant level of the 6–8S polymerase activity is seen. Bone marrow from older rabbits does not have as much of the 6–8S activity but does have about the same level of the 3.39S activity. About two-thirds of the total low molecular weight DNA polymerase in bone marrow cells from 2-month-old rabbits is present in the nuclear fraction. All of the 6–8S activity is in the cytoplasmic fraction.

Purification of the Low Molecular Weight DNA Polymerase from Rabbit Bone Marrow. Preliminary experiments (Chang and Bollum, 1971b) indicated that the nuclear and soluble 3.39S species behaved identically in properties and in fractionation. The following procedure was devised for total extracts.

CRUDE EXTRACT. Frozen rabbit bone marrow (195 g) is homogenized in a Waring blender in 635 ml of 0.2 M KP_i (pH 7.5) and the resulting homogenate is allowed to extract for 1 hr at 4° with stirring. The homogenate is then centrifuged at 7000*g* for 10 min and the residue is reextracted with 135 ml of 0.2 M KP_i. The pooled supernatant fractions are then centrifuged for 60 min at 105,000*g* in the Spinco centrifuge and the final supernatant fraction is designated fraction I. This fraction, 760 ml, contains 18.4 mg of protein per ml by biuret reaction.

PHOSPHOCELLULOSE COLUMN CHROMATOGRAPHY. Fraction I is diluted to 0.1 M KP_i (pH 7.5) with 1 mM mercaptoethanol and loaded onto a 3.2 × 20 cm phosphocellulose column previously equilibrated with the same buffer. The column is washed with 0.1 M KP_i (pH 7.5) and adsorbed proteins are eluted with a linear KP_i (pH 7.5) gradient from 0.1 to 0.4 M (750 ml each). The elution profile from the phosphocellulose column is shown on Figure 2. The active fractions from phosphocellulose are pooled and designated fraction II. This

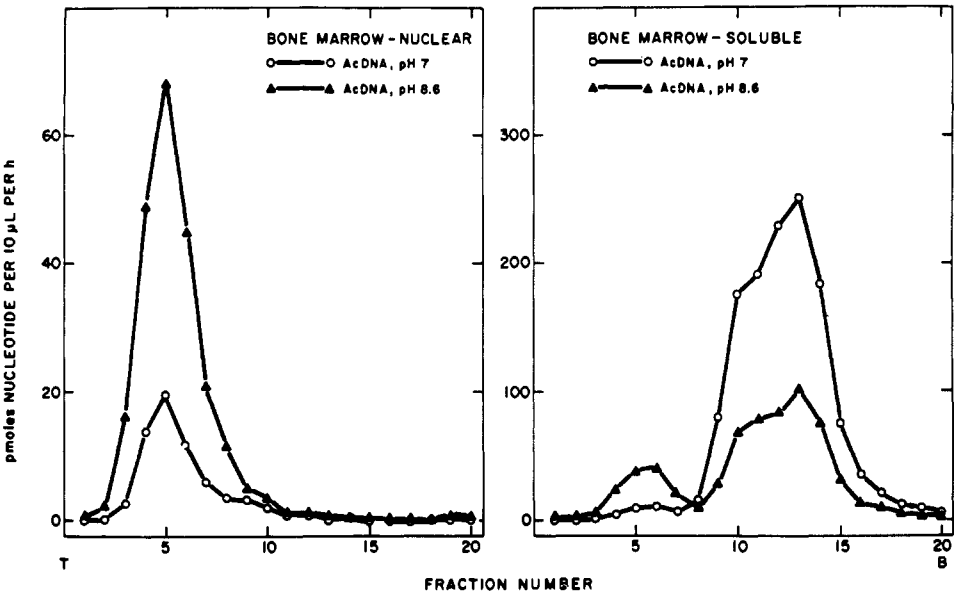

FIGURE 1: Sucrose gradient analysis of the nuclear and soluble fractions from rabbit bone marrow. Rabbit bone marrow nuclear and soluble extracts prepared by cell fractionation were analyzed for enzyme activities after separation on sucrose gradients as described in Methods and Materials. The direction of sedimentation is from left to right with T representing the top of the gradient and B representing the bottom of the gradient. The symbols, O-O, show results with activated DNA at pH 7.0, and ▲-▲ is the result obtained with activated DNA at pH 8.6.

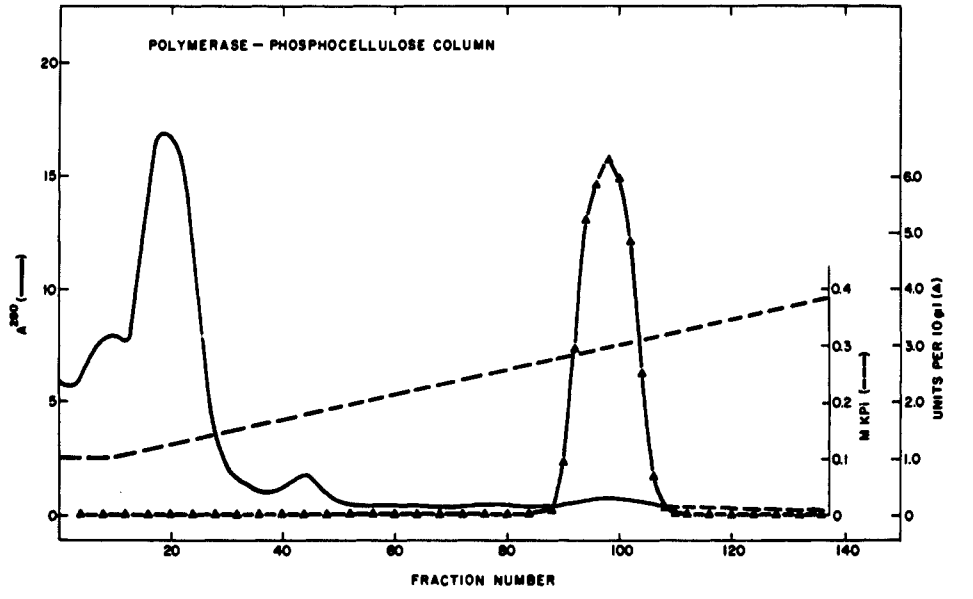

FIGURE 2: Phosphocellulose column chromatography of the low molecular weight DNA polymerase from rabbit bone marrow. See Methods and Materials and text for details. Enzyme activity in the column fractions was measured with $dC_n \cdot dG_{5\text{-}12}$ template at pH 8.6, and is shown as ▲-▲. The solid line is A_{280} and the dashed line is the phosphate gradient.

fraction, 161 ml, contains about 0.53 mg of protein per ml ($A_{280}/A_{260} = 1.2$) and is colorless.

GEL FILTRATION. Fraction II is precipitated by dialysis to a final ammonium sulfate concentration of 70% saturation. The ammonium sulfate precipitate is collected by centrifugation, redissolved in 0.1 M KP_i (pH 7.5), and one half of fraction II is loaded directly onto a 1.3 × 100 cm Sephadex G-100 column and eluted with 0.1 M KP_i (pH 7.5). The active fractions from the G-100 column are pooled and designated fraction III, which is the enzyme preparation used in most of the studies reported here. This fraction has about 0.61 mg of protein per ml ($A_{280}/A_{260} \simeq 1.5$, although the original fractions from G-100 had A_{280}/A_{260} greater than 1.7). A summary of the purification is shown in Table I. The purification is about 100-fold assayed on activated DNA template and 600-fold when $dC \cdot dG_{5\text{-}12}$ is used as template.

Contamination of the Low Molecular Weight Polymerase Preparation with Other Enzyme Activities. The specific exonuclease substrates used were [^{3}H]dA_4-dA_{300} and dA_{450}-[^{14}C]dA_{190} synthesized by use of appropriate initiators and triphosphates with terminal deoxynucleotidyl transferase from calf thymus gland. These substrates specifically measure 5′,3′-exonuclease and 3′,5′-exonuclease, respectively, and are used directly or as a 1:1 physical complex with dT_n. The level of exonuclease in the enzyme preparation was assayed by incubating 600 µg/ml of fraction III (ten times more concen-

TABLE I: Purification of Rabbit Bone Marrow DNA Polymerase.[a]

	Total Protein (mg)	Template: Activated DNA Total Units[b]	Activated DNA Spec Act.[c]	$dC_n \cdot dG_{5-12}$ Total Units	$dC_n \cdot dG_{5-12}$ Spec Act.
Fraction I (Crude)	13929	32355	2.3	30304	2.2
Fraction II (Phosphocellulose)	85	12186	143	25345	298
Fraction III (Sephadex G-100[d])	20	4074	204	26578	1326

[a] All assays were carried out at pH 8.6. From 195 g of frozen rabbit bone marrow. [b] 1 unit = 1 nmole of nucleotide incorporated per hr. [c] Spec Act. = units per mg of protein. [d] The concentrated protein solution from the phosphocellulose column was fractionated in two batches on Sephadex G-100.

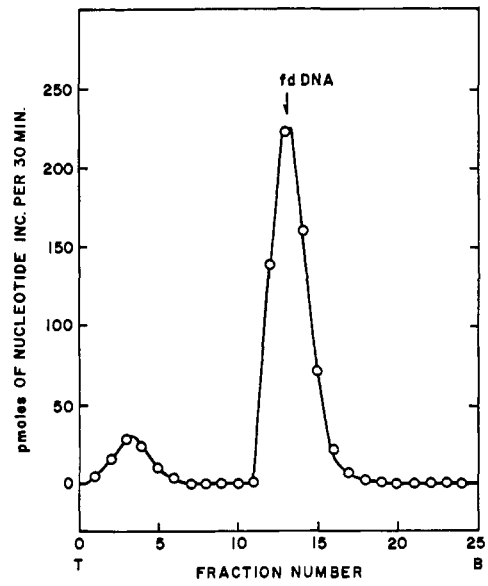

FIGURE 3: Binding of the low molecular weight DNA polymerase from rabbit bone marrow to fd-DNA on a sucrose gradient. The sucrose gradient was the same (5–20%) as described in Methods and Materials except it contained 0.1 M KCl in addition to 0.05 M KP_i, pH 7.5. Sample (0.25 ml) containing 2 A_{260} units of fd-DNA, and 200 $dC \cdot dG_{5-12}$ units of DNA polymerase in 0.05 M KP_i, 0.1 M KCl, and 1 mM mercaptoethanol was loaded onto the gradient with a disposable pipette. Centrifugation was carried out for 4 hr at 40,000 rpm in the SW 50 L rotor in the Spinco centrifuge. After centrifugation the gradient was fractionated as described in Methods and Materials, and A_{260} and DNA polymerase activity ($dC \cdot dG_{5-12}$ as template) was determined for each fraction. The arrow indicates the position of fd-DNA and the open circles (O-O) indicate levels of DNA polymerase activity. The direction of sedimentation is from left to right with T representing the top of the gradient and B the bottom.

trated than the protein concentration used for polymerase assays) with the single and double-stranded radioactive homopolymers noted above, in the presence and absence of ATP and in the presence of Mg^{2+} or Mn^{2+}. In no case is the release of radioactive acid-soluble material observed in 1 hr of incubation at 35°. The specific activity of the radioactive polymers used would easily detect either exonuclease activity at a level of 0.5% of the polymerizing activity. If a random endonucleolytic activity were present in important amounts, it would also be detected in this assay.

RNA polymerase activities, assayed according to Roeder and Rutter (1969), terminal deoxynucleotidyl transferase, and high molecular weight DNA polymerase activity (assayed on sucrose gradients and Sephadex G-100 columns) were not detected in fraction III.

Properties of the Partially Purified Rabbit Bone Marrow DNA Polymerase. MOLECULAR WEIGHT ESTIMATION. The low molecular weight DNA polymerase sediments at 3.39 S on 5–20% sucrose gradients. A molecular weight of about 45,000 is obtained for this activity using bovine serum albumin and lactate dehydrogenase (H_4-isozyme) as standards (Martin and Ames (1961)). A molecular weight determination on Sephadex G-100 using bovine serum albumin, sperm whale myoglobin, and human γ-globulin as standards gave an interpolated molecular weight of 48,000.

BINDING OF THE LOW MOLECULAR WEIGHT DNA POLYMERASE TO DNA. In contrast to the 6–8S DNA polymerases (Yoneda and Bollum, 1965), the 3.39S enzyme forms a stable complex with DNA and polydeoxynucleotides at reasonably high ionic strength. Figure 3 shows the binding of the low molecular weight DNA polymerase with fd-DNA on a 5–20% sucrose gradient containing 0.05 M KP_i (pH 7.5) and 0.1 M KCl. The bulk of the enzyme sediments with the fd-DNA. Preliminary results on the binding of the low molecular weight polymerase to homopolymers on sucrose gradients (not presented here) suggest tight binding of the enzyme with double-stranded polymers while some base specificity seems to be present in the interaction between enzyme and single-stranded polydeoxynucleotides. Under the ionic conditions described above the 3.39S enzyme sediments with poly(dT) on sucrose gradients but does not sediment with poly(dA). Since polydeoxynucleotides can interfere in the enzyme assay, quantitative results on the binding have not yet been obtained.

DIVALENT CATION REQUIREMENTS. The low molecular weight DNA polymerase requires the presence of a divalent cation for activity. When activated DNA is used as a template, Mg^{2+} is preferred with an optimum from 5 to 20 mM. Mn^{2+} is about 33% as effective with an optimum at about 0.3 to 0.6 mM. When $dA_n \cdot dT_{12}$ is used as template, Mn^{2+} is preferred with an optimum at 0.25 to 0.5 mM, and Mg^{2+} is only about 4% as effective at 3 to 8 mM. When initiated poly(dC) is used as template, Mn^{2+} at 0.4 to 0.7 mM is optimal and Mg^{2+} is about 12% as effective at 2 to 6 mM. These data are presented in Figure 4. The optimum cation concentrations reported here for the low molecular weight enzyme are dependent upon the dNTP and polymer concentration as might be anticipated. At higher concentration of the template and monomer a higher concentration of divalent cation is required.

pH OPTIMUM. While the high molecular weight DNA polymerase isolated from the soluble fractions of mammalian tissues in general has a neutral pH optimum, the low molecular weight DNA polymerase has an alkaline pH optimum. The pH optimum depends on the template used (Figure 5). The pH optimum of activated DNA and initiated poly(dC) reactions is 8.6, and the pH optimum of the initiated poly(dA) reaction is 7.6.

DEOXYNUCLEOSIDE TRIPHOSPHATE REQUIREMENTS. The low molecular weight DNA polymerase requires all four deoxy-

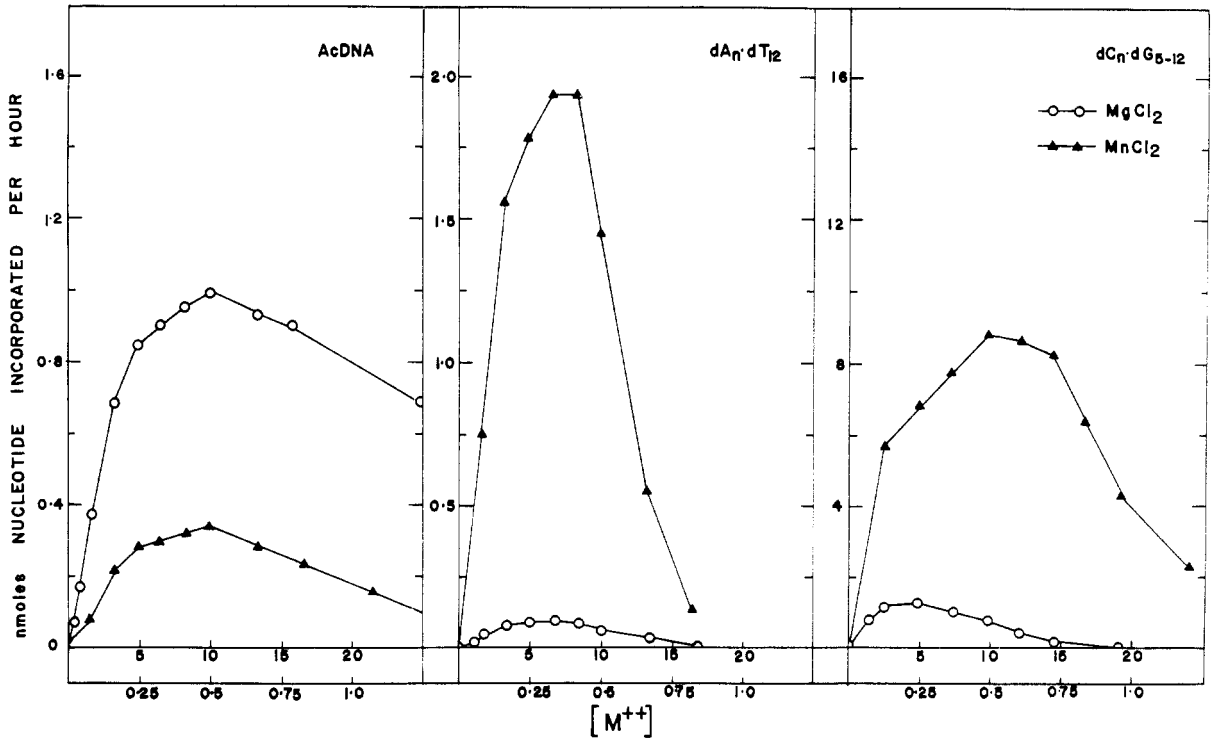

FIGURE 4: Divalent cation requirements for rabbit bone marrow low molecular weight DNA polymerase. Requirements for Mg^{2+} or Mn^{2+} were determined for the low molecular weight DNA polymerase from rabbit bone marrow using activated calf thymus DNA, $dA_n \cdot dT_{12}$, and $dC_n \cdot dG_{5-12}$ as templates in the presence of various levels of divalent cation. Rate of synthesis was determined as described in Materials and Methods. The lower abscissa is for Mn^{2+} and the upper is for Mg^{2+}.

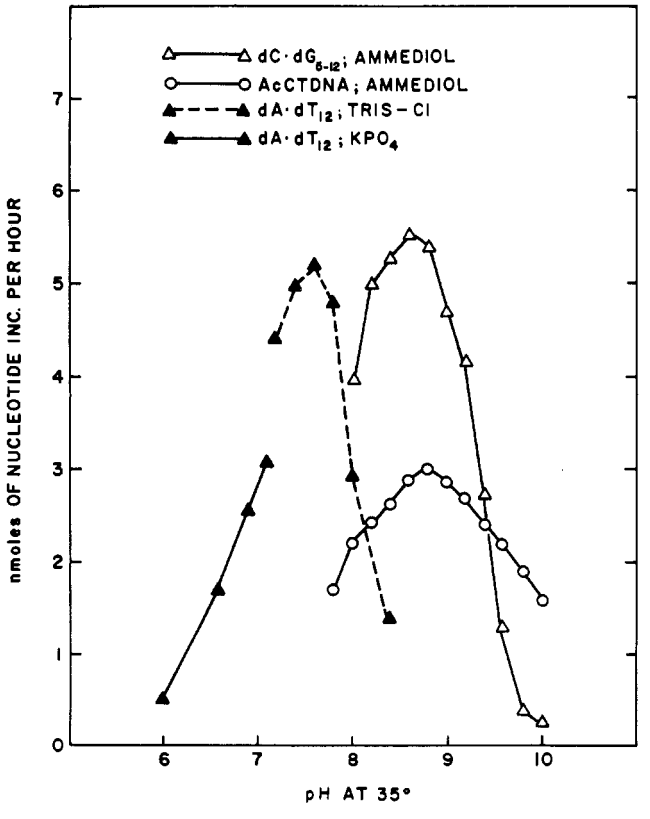

FIGURE 5: pH optimum of rabbit bone marrow DNA polymerase. The DNA polymerase reactions containing various templates were carried out in the presence of 20 mM KP_i, 50 mM Tris-Cl, or 50 mM ammediol buffers at various pH values. Reactions contained 6 μg of enzyme protein in 125 μl for $dC \cdot dG_{5-12}$ (△-△) and $dA \cdot dT_{12}$, (▲-▲) and 12 μg of enzyme protein in 125 μl for activated calf thymus DNA (O-O).

nucleoside triphosphates for maximum activity when activated DNA's are used as templates, as may be seen in Table II. When DNase I-treated DNA is used, a high level of activity is found in the absence of one to three triphosphates. This relaxed requirement for all four deoxynucleoside triphosphates is due at least in part to the nature of the activated DNA template. With DNase I-treated template only a small percentage of the template DNA (less than 1%) is replicated and the incorporation observed with less than a full set of dNTP's is probably due to the replication of many short regions of DNA template containing less than all four nucleotides. When the template DNA is nicked by DNase I and then treated with exonuclease III, a longer stretch of the DNA template may be available for replication and the requirement for all four triphosphates becomes more restrictive, as is shown in column 2 of Table II. With longer reaction times, the relative activity in the absence of three triphosphates is low compared to the complete system when both DNase I-treated and DNase I nicked, exonuclease III-treated DNA's are used as templates. The strict requirement for the presence of all four triphosphates can be best demonstrated by using exonuclease III-treated λ-DNA as template. Table II, column 3 shows that in the absence of one to three triphosphates, less than 5% of the activity of the complete system is obtained. The difference in the requirement for the presence of all four triphosphates for the different template DNA's used suggests that the nature of the DNA template is a major factor in determining the stringency of the triphosphate requirement.

BASE INCORPORATION RATIO. Since the nucleotide requirements on the usual DNA templates (such as DNase I-activated DNA) do not seem very stringent it is essential to confirm the fact that the low molecular weight DNA polymerase does use template information for replication. When activated calf thymus DNA is used as template, the enzyme incorporates

TABLE II: Deoxynucleoside Triphosphate Requirements for DNA Polymerase Reaction.[a]

Reaction Mixture	Template: DNase I-Treated CTDNA (pmoles/15 min)	DNase I Nicked Exonuclease III-Treated CTDNA (pmoles/10 min)	Exonuclease III-Treated λ-DNA (pmoles/15 min)
Complete	419 (100%)	119 (100%)	20.0 (100%)
−dCTP	199 (45%)	48 (40%)	
−dGTP	176 (42%)	55 (46%)	
−dATP	204 (49%)	49 (41%)	1.0 (5%)
−dCTP and dGTP	149 (36%)	33 (28%)	
−dATP and dCTP	144 (34%)	28 (24%)	
−dATP and dGTP	162 (39%)	36 (30%)	0.35 (1.8%)
−dCTP, dATP and dGTP	151 (36%)	21 (18%)	0.28 (1.4%)

[a] All reaction mixtures contain 18 μg of DNase I-treated calf thymus (CT) DNA or DNase I-nicked, exonuclease III-treated calf thymus DNA, or 9 μg of exonuclease III-treated λ-DNA, 50 mM ammediol buffer at pH 8.6, 8 mM $MgCl_2$, 1 mM mercaptoethanol, and 14 μg of enzyme protein in a final volume of 0.25 ml. The complete reaction mixture contains [^{3}H]dTTP, dCTP, dGTP and dATP, each at 0.1 mM.

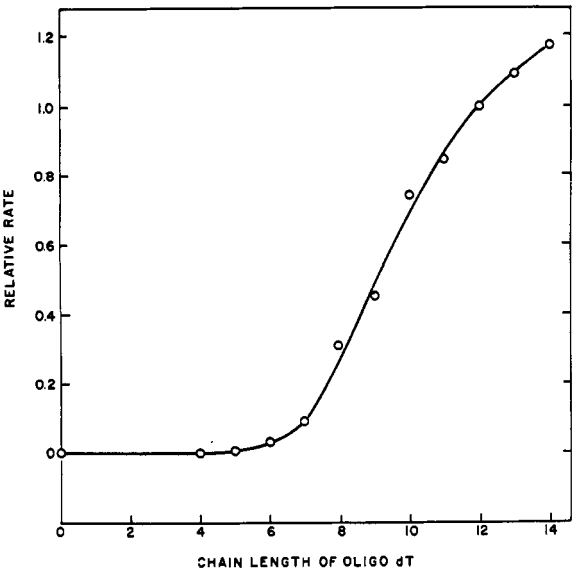

FIGURE 6: Effect of chain length of initiator on poly(dA) replication. Each reaction mixture (125 μl) contains 0.1 mM [^{3}H]dTTP, 0.1 mM dA_n (mM nucleotide), 0.05 M Tris-Cl (pH 7.6), 1 mM 2-mercaptoethanol, 0.5 mM $MnCl_2$, 8 μg of enzyme protein, and 4–5 μM oligodeoxynucleotide. All rates are expressed relative to the rate of the $dA_n \cdot dT_{12}$ reaction (3.16 nmoles of dT incorporated per reaction per 15 min).

approximately equal amounts of A and T, and equal amounts of G and C (Table III). The slightly high A + T to G + C ratio may be due to some bias in the available template in the activated DNA template as noted above. With more extensive replication of the template an A + T to G + C ratio of 1.29 is obtained, and this is more characteristic of calf thymus DNA. The replicative characteristic of low molecular weight DNA polymerase is best demonstrated by the use of initiated polydeoxynucleotides as templates. In the presence of all four dNTPs and initiated homopolymers as templates the enzyme copies the template with less than 1% error.

EFFECT OF CHAIN LENGTH OF THE OLIGODEOXYNUCLEOTIDE ON HOMOPOLYMER REPLICATION. The low molecular weight DNA polymerase requires initiated templates and for this reason native DNA, heat-denatured DNA (Chang and Bollum, 1971b), and single-stranded polydeoxynucleotides can not be used effectively as templates. The requirement of an initiator molecule for poly(dA) replication is shown in Figure 6. Oligodeoxythymidylate with chain length of eight is the minimum required for initiation. An increase in initial rate is observed with increase in chain length of the initiator. This effect is probably the result of the increased stability of oligonucleotide–polynucleotide interaction which produces an increased concentration of initiation sites (Cassani and Bollum, 1969).

EXTENSIVE REPLICATION BY LOW MOLECULAR WEIGHT DNA POLYMERASE. The time courses of the low molecular weight DNA polymerase catalyzed replication of several templates are shown in Figure 7. With activated DNA as template nonlinear kinetics is observed throughout the incubation and the reaction stops when all available single-stranded template regions are filled. When initiated homopolymers are used as templates in the presence of a twofold excess of dNTP the low molecular weight DNA polymerase catalyzes only one round of complementary synthesis.

The products of the polymerase reactions are fully double-stranded polymers. Figure 8 shows the product of poly(dA) replication analyzed by absorbancy *vs.* temperature profile and in a neutral cesium chloride gradient. The T_m of the product of $dA_n \cdot dT_{12}$ replication in 1 mM Tris-Cl (pH 8.0) and 15 mM NaCl is at 52°, corresponding to the T_m of $dA_n \cdot dT_n$ (Bollum, 1966). The radioactive product of $dA_n \cdot dT_{12}$ replication bands in the neutral cesium chloride gradient with radioactivity corresponding to the A_{260}, again indicating the full duplex structure of the $dA_n \cdot dT_n$ product. In this experiment, 115 nmoles of total nucleotide was applied to the gradient and all of this was recovered in the optical density band, which also contianed 49.1 nmoles of radioactive dT. The slight discrepancy in thymidylate residues is probably due to

TABLE III: Base Incorporation Ratios of DNA Polymerase.[a]

Template	[^{3}H]dNTP Incorporated (nmoles/hr): A	C	G	T
Ac CTDNA	0.42	0.25	0.28	0.41
$dT_n \cdot dA_{12}$	1.13	<0.01	<0.01	<0.01
$dA_n \cdot dT_{12}$	0.03	<0.01	<0.01	1.61
$dC_n \cdot dG_5$	0.03	0.02	6.98	0.02

[a] Reactions were carried out as described in Methods and Materials. All reaction mixtures contained all four deoxynucleoside triphosphates with only one radioactive deoxynucleoside triphosphate in each. Ac CTDNA is DNase I-treated calf thymus DNA.

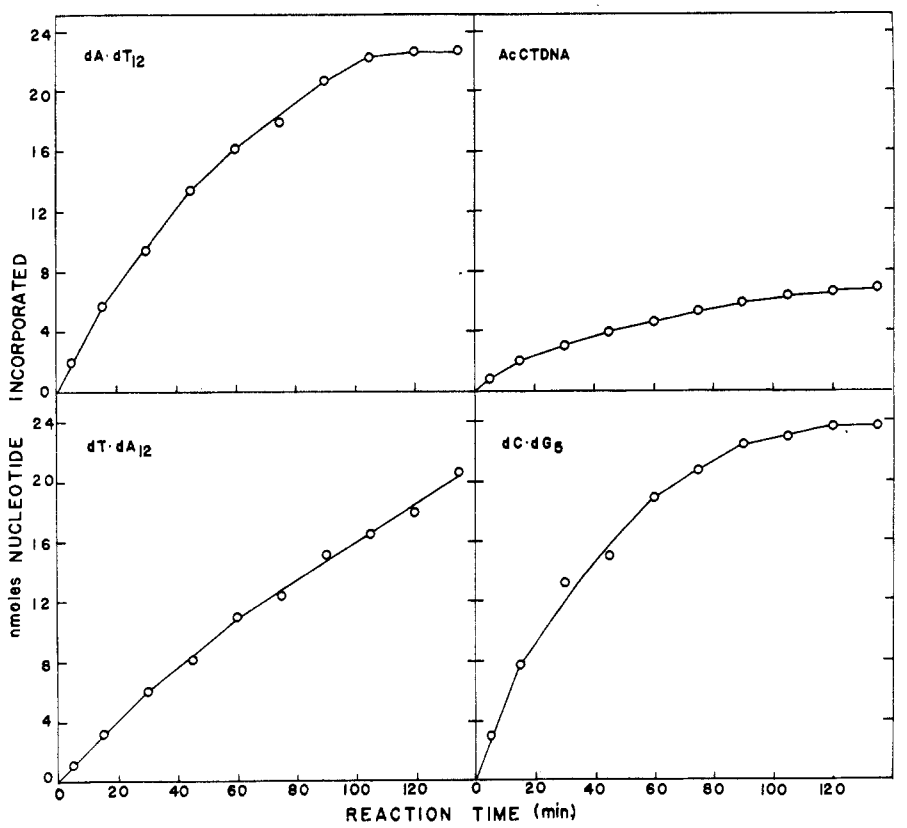

FIGURE 7: Extensive replication by the low molecular weight DNA polymerase. The synthesis of homopolymer complexes were carried out under the conditions described in Methods and Materials except that the concentration of the monomers was 0.2 mM (a twofold excess over template nucleotide concentration). A total of 25 nmoles of template nucleotide was present in each 0.25-ml reaction mixture. The reaction with activated calf thymus DNA as template was carried out as described in Methods and Materials.

the fact that the replication went to 91% completion, as measured by incorporation of the radioactive monomer, and the remaining 9% of the product may be derived from the nonradioactive oligodeoxynucleotide initiator.

TEMPLATE SPECIFICITY OF THE LOW MOLECULAR WEIGHT DNA POLYMERASE. The low molecular weight DNA polymerase requires initiated polynucleotides as templates. Table IV shows a comparison of the effectiveness of initiated polydeoxynucleotides and polyribonucleotides as templates for the low molecular weight DNA polymerase. Initiated dA_n, dU_n, dT_n, and dC_n are readily used as templates while initiated dI_n is not a good template. The inability of the enzyme to replicate initiated dI_n is probably due to instability of the polydeoxyinosinate and oligodeoxycytidylate complex, and the tendency for polydeoxyinosinate to form the $(dI_n)_2$ complex (Inman, 1964). The low molecular weight DNA polymerase utilizes initiated polyriboadenylate quite efficiently while the enzyme does not use rC_n, rI_n, nor rU_n as templates under the conditions tested. This result is in contrast to results obtained with the high molecular weight DNA polymerase isolated from calf thymus gland which uses initiated polyribonucleotide templates at less than 0.5% of the rate of comparable deoxynucleotide templates (L. M. S. Chang and F. J. Bollum, unpublished results).

Discussion

The low molecular weight DNA polymerase described in this paper is of interest primarily because of its intracellular localization. This enzyme is the only molecular species we have been able to detect in purified nuclei. It is not restricted to the nucleus, however, since it also occurs in the cytoplasmic fraction. Other investigators have described similar enzyme activities from rat liver chromatin (Howk and Wang, 1969), and rat liver nuclei and cytoplasmic particles (Baril *et al.*, 1971). We are of the opinion that all these activities are similar in enzymatic properties (*e.g.*, alkaline pH optimum) and molecular weight. Our current work has demonstrated the presence of a low molecular weight species of DNA polymerase in some 40 different mammalian tissues, including several lines of tissue culture cells. Cell fractionation studies demonstrating the low molecular weight polymerase to be the only DNA polymerase in nuclei have been carried out with rat liver, rat spleen, rat bone marrow, rabbit spleen, rabbit liver, calf thymus, human lymphocytes stimulated by phytohemagglutinin, mouse L-cells, HeLa cells, and Novikoff hepatoma cells. These studies, still in progress, demonstrate molecular forms of DNA polymerase in the cytoplasmic and nuclear fraction similar to that seen in rabbit bone marrow (Figure 1). The ratio of total low molecular weight DNA polymerase to total high molecular weight DNA polymerase varies between tissues. In general, fast-growing tissues (such as fetal calf tissues, regenerating rat liver, and tissue culture cells) contain a much higher level of the high molecular weight DNA polymerase, which is found only in the cytoplasmic fraction according to our analysis. The ratio of the low molecular weight DNA polymerase activity present in the cytoplasmic fraction to the activity present in the nuclear fraction also depends on the tissue studied, and may be partially related to the cytoplasmic to nuclear volume. All of

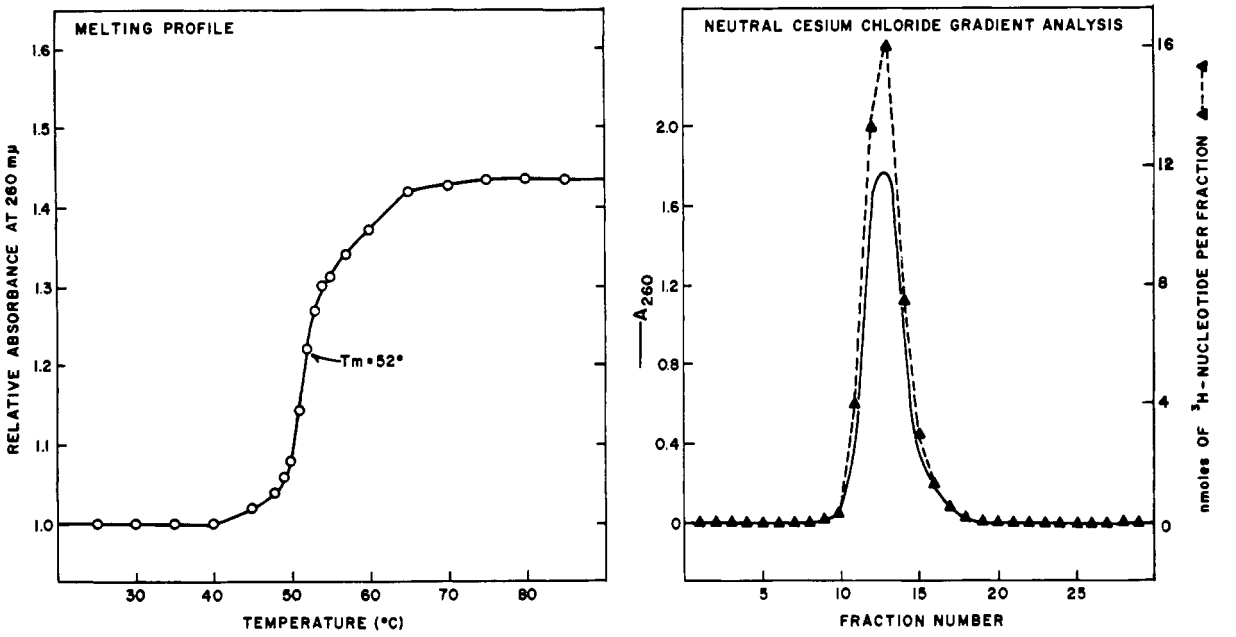

FIGURE 8: Analysis of the product of poly(dA) replication. The product of $dA_n \cdot dT_{12}$ reaction was isolated by gel filtration on a Sephadex G-50 column equilibrated with 1 mM Tris-Cl (pH 8.0) and 15 mM NaCl. The melting profile was obtained in the same buffer. The cesium chloride centrifugation of the product was carried out in the presence of 0.1 M Tris-Cl (pH 8.0) with an average density of cesium chloride of 1.6698. The centrifugation was carried out for 72 hr at 40,000 rpm at 25° in a SW 50 L rotor in the Spinco centrifuge. Fractions were collected from the bottom of the tube and A_{260} and acid-insoluble radioactivity was determined for each fraction.

these results indicate the common occurrence of multiple forms of DNA polymerase in mammalian tissues. A detailed analysis of the intracellular distribution of these molecular forms in a variety of tissues should be an aid in understanding the role of these enzymes in DNA metabolism.

The intracellular distribution of the low molecular weight DNA polymerase is rather unusual, being present (apparently free) in cytoplasm and bound to DNA in nuclei, particularly in view of the fact that the low molecular DNA polymerase binds tightly to isolated polydeoxynucleotides. Perhaps there are only a limited number of binding sites available in the chromatin, and the cytoplasmic component represents excess synthesis or a continuous level of synthesis of the low molecular weight DNA polymerase. Other possibilities, such as the need for additional proteins for interaction with DNA for initiation of DNA synthesis, are also open for exploration.

The low molecular weight DNA polymerase has been purified from the nuclear and soluble fractions of rabbit bone marrow and the activities have been demonstrated to be identical by chromatographic behavior, molecular weight, and reaction properties (Chang and Bollum, 1971b). Certain properties of the crude nuclear and soluble low molecular weight DNA polymerase did not always seem to be the same. For example, the pH optimum of the enzyme in the crude nuclear extract is lower than the pH optimum after the enzyme is partially purified. The change of pH optimum is probably due to the removal of a nuclease having an alkaline pH optimum. The presence of the nuclease may also increase the ability of the enzyme to use native DNA as template and decrease its apparent ability to use initiated homopolymer templates. When gradient fractions from soluble and nuclear extracts were tested for their ability to use homopolymer templates, the nuclear enzyme was inactive on $dT_n \cdot dA_{12}$. Further fractionation removed this apparent difference. These observations show that characterization of enzymes of this kind in crude extracts on the basis of template preferences and reaction properties is highly questionable. Additional properties, such as sedimentation rate or other physical properties are measurements necessary for comparative purposes.

In regard to mechanism, it appears that this 3.39S DNA polymerase will conform to that established with the calf thymus DNA polymerase (Bollum, 1964, 1967). The purified enzyme does not use native DNA, and denatured DNA is not much more effective. DNase I activated DNA is a good template and the enzyme probably works by filling up gaps in the double-helical molecules. Oligodeoxynucleotide initiated homopolymers are by far the best templates for this enzyme. This indicates that a short double-stranded region is sufficient to serve as the initiation point for chain growth. As indicated in Figure 7 the replication proceeds only to the extent of available template nucleotide. Preliminary characterization of the products of synthesis demonstrates that they have the same characteristics as the calf thymus DNA polymerase products (Bollum, 1967).

A point of current interest concerns the "reverse transcriptase" activity, that is, the ability to use ribopolymer templates to direct deoxynucleoside triphosphate polymerization. Table IV indicates that in certain cases, notably with rA_n, that ribopolymers are better templates than deoxypolymers. This does not hold true with rU_n or rC_n, and for these cases deoxytemplates seem to be much preferred. The high efficiency of ribotemplate utilization found for the low molecular weight DNA polymerase, particularly with rA_n, is not seen with calf thymus 6–8S polymerase.

The role of the low molecular weight enzyme in replication and/or repair is a difficult problem to approach since genetic techniques are not currently available for mammalian systems. Physiological experiments are possible, and we have examined the level of the low molecular weight DNA polymerase in regenerating rat liver. The enzyme activity increases only slightly as a result of the stimulus to regenerate. Generally

TABLE IV: Template Specificity of Rabbit Bone Marrow DNA Polymerase.[a]

Template	Monomer	Monomer Incorporated (pmoles per 30 min)
$rA_n \cdot dU_n$	dTTP	2193
$dA_n \cdot dU_n$	dTTP	1578
$rA_n \cdot dT_8$	dTTP	82
$dA_n \cdot dT_8$	dTTP	727
$rA_n \cdot dT_{12}$	dTTP	1376
$dA_n \cdot dT_{12}$	dTTP	4173
$rA_n \cdot dT_n$	dTTP	1282
$dA_n \cdot dT_n$	dTTP	188
$rU_n \cdot dA_{12}$	dATP	16
$dU_n \cdot dA_{12}$	dATP	920
$dT_n \cdot dA_{12}$	dATP	2087
$rU_n \cdot dA_n$	dATP	50
$dU_n \cdot dA_n$	dATP	209
$rI_n \cdot dC_n$	dCTP	66
$dI_n \cdot dC_n$	dCTP	27
$rI_n \cdot dC_{9-11}$	dCTP	10
$dI_n \cdot dC_{9-11}$	dCTP	20
$rC_n \cdot dG_{\bar{20}}$	dGTP	20
$dC_n \cdot dG_{\bar{20}}$	dGTP	1277
$rC_n \cdot dG_5$	dGTP	33
$dC_n \cdot dG_5$	dGTP	2934

[a] Each reaction mixture contained template nucleotide, 40 μM; complementary radioactive dNTP, 100 μM; $MnCl_2$, 250 μM; either Tris-Cl, 50 mM, pH 7.6 (for the A·U template systems) or ammediol, 50 mM, pH 8.6 (for the I·C and G·C systems); 2-mercaptoethanol, 1 mM, and 14 μg of fraction III protein in a final volume of 0.25 ml. The reactions mixtures were incubated at 35°.

speaking our results on a number of tissues show that the high molecular weight DNA polymerase is the major activity in rapidly growing systems. The level of low molecular weight polymerase seems to remain fairly constant. At the present time, major variations in the level of the low molecular weight DNA polymerase in a given tissue have not been found.

A most recent finding of interest is that all of the molecular weight forms of DNA polymerase we find in eukaryotic cells are inhibited by antibody directed against the high molecular weight DNA polymerase from calf thymus gland (Chang and Bollum, 1972).[3] This observation suggests common subunit or polypeptide sequence between various forms of DNA polymerases in the cell and between the DNA polymerases from different animals.

The isolation and purification of enzymes that polymerize deoxynucleotides is a subject of continuing interest in this laboratory. We believe that more than one protein is necessary for the complete replication process, as was clearly shown by the work on the multiplicity of replication defective mutations in bacteriophage T4 (Epstein *et al.*, 1963). It does seem likely to us, that the catalytic entities we are describing must be involved in DNA metabolism at the macromolecular stage because they use template information in the polymerization process. It seems clear to us that although the DNA polymerases are not necessarily the complete system for replication they do have a fairly good chance of being part of the replication complex. We imagine that other proteins, some similar in function to T_4-gene 32 protein (Alberts and Frey, 1970), must also be part of the replication complex. The resolution of this complex must come by analysis of the separate parts in eukaryotic systems.

[3] Manuscript submitted for publication.

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