Novikoff Hepatoma Deoxyribonucleic Acid Polymerase. Purification and Properties of a Homogeneous β Polymerase†

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ABSTRACT: Deoxyribonucleic acid polymerase-β (EC 2.7.7.7) from the Novikoff hepatoma has been purified over 200 000-fold (based on the increase in specific activity), by ammonium sulfate fractionation and chromatography on DEAE-Sephadex, phosphocellulose, hydroxylapatite, and DNA-cellulose. The enzyme is remarkably stable through all stages of purification until DNA-cellulose chromatography when it must be kept in buffers containing 0.5 M NaCl and 1 mg/ml bovine serum albumin for stability. The enzyme appears to be homogeneous as evidenced by a single stainable band when subjected to electrophoresis in polyacrylamide gels of different porosity. The stainable band corresponds to the DNA polymerase as determined by slicing sister gels and assaying for enzyme activity. The specific activity of the homogeneous preparation is about 60 000 units/mg. The enzyme lacks detectable exonuclease or endonuclease activity. It has a molecular weight of 32 000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In sucrose gradients, the molecular weight is estimated at 31 000. The isoelectric point of the hydroxylapatite fraction enzyme is 8.5. The Novikoff β-polymerase requires all four deoxyribonucleoside triphosphates, primer-template, and a divalent cation for maximal activity. The apparent K_m for total deoxyribonucleoside triphosphate is 7–8 μM and for DNA 125 μg/ml. Activated DNA, rendered 7% acid soluble by DNase I, is the preferred primer-template, although a number of synthetic polynucleotides can be efficiently utilized, particularly in the presence of Mn^{2+}. The Mg^{2+} optimum is 7 mM; the Mn^{2+} optimum is 1 mM. The pH optimum is 8.4 in Tris-HCl or 9.2 in glycine buffer. The β-polymerase is stimulated about twofold by NaCl or KCl at an optimum of 50–100 mM, and the enzyme maintains considerable activity at high ionic strengths. The DNA polymerase is inhibited by ethanol, acetone, and a variety of known polymerase inhibitors. Glycols stimulate the enzyme as does spermine or spermidine. Unlike most β-polymerases, the Novikoff enzyme is moderately sensitive to *N*-ethylmaleimide.

A low-molecular-weight DNA polymerase (EC 2.7.7.7), found in both nuclear and cytoplasmic extracts of a variety of mammalian cells, was first reported in 1971 by Chang and Bollum and by Weissbach et al. Low-molecular-weight (ca. 30 000–50 000) DNA polymerases are now recognized as a constant feature of all mammalian cells and have been designated as DNA polymerase-β to distinguish them from the higher molecular weight DNA polymerases now designated as DNA polymerase-α (see recent reviews by Loeb, 1974; Bollum, 1975; Weissbach, 1975). DNA polymerase-β has been purified to varying degrees from rabbit bone marrow (Chang and Bollum, 1971), HeLa cells (Weissbach et al., 1971; Spadari and Weissbach, 1974a), chick embryos (Stavrianopoulos et al., 1972; Brun et al., 1974), and rat liver (Baril et al., 1971; Berger et al., 1971). More recently Chang (1973a, 1973b) and Wang et al. (1974, 1975) have obtained homogeneous preparations in small yields from calf thymus chromatin and KB cells, respectively.

The role of DNA polymerase-β in DNA replication and/or repair is unknown. The levels of this enzyme appear to be relatively constant during the cell cycle (Chang et al., 1973; Spadari and Weissbach, 1974b; Craig et al., 1975; Chiu and Baril, 1975) or during liver regeneration (Chang and Bollum, 1972), while the levels of α-polymerase rise several fold. In order to investigate the in vivo role of β-polymerases in mammalian cells, it is essential that a highly purified (and preferably a homogeneous) DNA polymerase be used. We have chosen to work with the Novikoff hepatoma since it is one of the fastest growing mammalian cells, having an in vivo generation time of 12 h. Being an ascites tumor, the cells can easily be harvested and large yields of cells obtained. Novikoff cells can also be grown in culture, and can be experimentally manipulated both in vivo and in vitro.

In the present report we describe the purification and properties of a homogeneous DNA polymerase-β from the Novikoff tumor. We have used this enzyme to identify three accessory proteins which stimulate in vitro DNA synthesis by the polymerase (Stalker et al., 1974; Probst et al., 1975). A fourth stimulatory protein which binds to the β-polymerase has recently been found (Stalker et al., 1975; Stalker; Mosbaugh, Probst, and Meyer, manuscript in preparation).

Experimental Procedure

Materials

All chemicals were of analytical or reagent grade. Unlabeled deoxyribonucleoside triphosphates, ribonucleoside triphosphates, thymine, *p*-nitrophenyl phosphate, thymidine 5′-monophospho-*p*-nitrophenyl ester, thymidine 3′-monophospho-*p*-nitrophenyl ester, calf thymus DNA, neutral acriflavin, *o*-phenanthroline, cytochrome *c*, catalase (EC 1.11.1.6), lysozyme (EC 3.2.1.17), and yeast alcohol dehydrogenase (EC 1.1.1.1) were purchased from Sigma. Enzyme grade ammonium sulfate, bovine serum albumin, ovalbumin, actinomycin D, [^{3}H]UTP, [^{3}H]dATP, [^{3}H]dCTP, [^{3}H]dGTP, and [^{3}H]dTTP were obtained from Schwarz/Mann. New England Nuclear Corp. was a source of [^{3}H]thymine and [^{3}H]dTTP, and Gallard and Schlessinger a source of 5-aminoacridine. Ethidium bromide came from Calbiochem, and *N*-ethylmaleimide from Eastman Organic Chemicals. Pancreatic

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DNase I (EC 3.1.4.5), RNase A (EC 3.1.4.22), RNase T_1 (EC 3.1.4.8), and *E. coli* alkaline phosphatase (EC 3.1.3.1) were obtained from Worthington Biochemicals. Synthetic polynucleotides were a product of P-L Laboratories and Miles Laboratories. Fluorescamine (Fluram) was kindly donated by Hoffmann-La Roche, Nutley, N.J. K & K Laboratories supplied *m*-phenanthroline and *p*-hydroxymercuribenzoate. Hydroxylapatite and polyacrylamide gel reagents were obtained from Bio-Rad Laboratories, and phosphocellulose (P-1) from Reeve-Angel Co. Pharmacia was a source for DEAE[1]-Sephadex A-50. Ampholytes used in isoelectric focusing came from LKB. DNA-Cellulose was prepared according to Alberts and Herrick (1971).

Methods

Preparation of DNA Substrates. Calf thymus DNA, obtained from commercial sources, was further purified by phenol extraction, RNase treatment, and re-extraction with phenol. The product, spooled from ethanol, was dissolved in 0.015 M NaCl-0.0015 M sodium citrate, pH 7.0, at a concentration of 1-2 mg/ml.

Activated DNA was prepared by limited treatment of calf thymus DNA with DNase I as described by Loeb (1969), except that after incubation the mixture was heated to 70 °C for 15-30 min to inactivate the DNase. To generate a series of primer-templates activated to different degrees, the amount of enzyme and/or the time of digestion with DNase I was varied. For each batch, the amount of nucleotide rendered acid soluble was determined by the diphenylamine reaction (Burton, 1956) after precipitation of an aliquot with cold 0.5 M PCA.

Denatured DNA was prepared by heating in a boiling water bath for 10 min followed by rapid chilling in ice.

Radioactive DNA for nuclease assays was prepared by growing *Escherichia coli* strain $15T^-$ in a minimal medium supplemented with [^{3}H]thymine (5 μg/ml, 1 μCi/ml at a specific activity of 50 mCi/mmol). When the cells reached late log phase, they were harvested and the DNA was purified by standard phenol extraction techniques. Before use, the [^{3}H]-DNA was sheared by passing through a 26-gauge syringe needle.

Enzyme Assays. DNA polymerase assays were carried out in disposable 12 × 75 mm glass tubes in a final volume in 125 μl. Standard reaction mixtures contained 25 mM Tris-HCl[2] (pH 8.4), 5 mM 2-mercaptoethanol, 7 mM magnesium acetate, 0.5 mM EDTA, 50 mM NaCl, 0.015 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (specific activity 325 or 975 mCi/mmol), 15% (w/v) glycerol, 100 μg/ml activated DNA, and 0.01-0.3 unit of enzyme. Incubation was carried out in stoppered tubes for 60 min at 37 °C. After incubation, 100 μl samples of the reaction mixture were pipetted onto filter paper discs which were dropped into cold 10% Cl_3CCOOH. The discs were prepared for liquid scintillation counting as described previously (Meyer and Keller, 1972) and radioactivity was measured in a Packard liquid scintillation spectrometer using 0.4% 2,5-bis-2-(5-*tert*-butylbenzoxazolyl)thiophene (BBOT) in toluene as the scintillator at an efficiency of 16-20% (Meyer and Keller, 1972). In order to increase the detection of enzyme in column effluents or in sucrose gradient fractions, [^{3}H]dTTP was used undiluted at 0.3 or 0.9 μM (18 or 54 Ci/mmol). This is referred to as a "limited substrate assay". Changes from these standard conditions are noted in the legends to the tables and figures.

Exonuclease was assayed in Beckman Microfuge tubes containing 25 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 7 mM magnesium acetate, 100 μg/ml [^{3}H]DNA (746 cpm/pmol of nucleotide) and 15 μl of enzyme (0.1-0.3 unit) in a fluid volume of 125 μl. After 30 min at 37 °C, the tubes were chilled, 100 μl of a 1.0 mg/ml solution of calf thymus DNA was added as carrier, and the DNA was precipitated with 75 μl of 1.0 M PCA. After standing for 10 min, the tubes were centrifuged at top speed and samples of 150 μl of supernatant were removed for scintillation counting in Bray's solution (Bray, 1960). Exonuclease activity was measured with and without 2 mM ATP using native and denatured DNA, and at pH 5.0 with sodium acetate buffer substituted for Tris-HCl.

Endonuclease was measured by sucrose density centrifugation. Assays were carried out in a volume of 200 μl containing 20 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 7 mM magnesium acetate, and 50 μg/ml [^{3}H]DNA. After incubation, the samples were adjusted to 0.2 N NaOH and overlaid onto a 5-20% alkaline sucrose gradient. The samples were centrifuged for 8 h at 38 000 rpm in a Spinco SW 50L rotor. Fractions were collected from the bottom of the tube onto filter paper discs and prepared for scintillation counting. The sheared [^{3}H]DNA had a sedimentation value of 18 S when standardized against *E. coli* ribosomal RNA. A shift in this sedimentation value is indicative of endonuclease. Endonuclease assays were carried out with and without 2 mM ATP in the incubation mixture.

Acid and alkaline phosphatase, acid and alkaline phosphodiesterase I and phosphodiesterase II activities, and RNA polymerase were assayed as described previously (Meyer and Simpson, 1970; Meyer et al., 1972).

Polyacrylamide Gel Electrophoresis. Nondenaturing 5% polyacrylamide gels were prepared in 5 × 125 mm tubes to a height of about 80-90 mm and polymerized with ammonium persulfate. After polymerization, a 3% stacking gel of 10 mm was prepared on top of the running gel and polymerized with riboflavin by exposure to fluorescent light. In order to recover activity from the gels, the ammonium persulfate was removed by pre-running the gels for 2 h prior to adding the sample. Samples of 25-100 μl were made 50% in glycerol and 0.2% bromphenol blue (added as a tracking dye) and applied through the running buffer to the top of the gel. Electrophoresis was carried out in a Bio-Rad electrophoresis cell using 0.3% Tris-HCl and 1.44% glycine at pH 9.1 as the running buffer (Davis, 1964). Current was maintained at 2 mA per gel and electrophoresis carried out until the tracking dye had migrated to the end of the gel (usually 2-3 h). Gels were removed from the tubes, fixed in 7% acetic acid, stained for 2-6 h, with 0.1% Coomassie brilliant blue in 7% acetic acid, and destained by diffusion in 7% acetic acid. To recover activity, gels were run at 4 °C and immediately sliced after electrophoresis. Slices of 2 mm were extracted overnight in reaction buffer at 0-4 °C and then assayed for DNA polymerase activity.

Molecular Weight Determination. Molecular weight was determined by centrifugation in 5-20% sucrose gradients (Martin and Ames, 1961) containing 20 mM Tris-HCl (pH

[1] Abbreviations used are: dNTP, unlabeled deoxyribonucleoside triphosphates; EDTA, ethylenediaminetetraacetate; NEM, *N*-ethylmaleimide; PCA, perchloric acid; PMEG buffer, 0.02 M potassium phosphate at pH 7.5, 0.005 M 2-mercaptoethanol, 0.001 M EDTA, and 10% (w/v) glycerol; PMG buffer, PMEG with EDTA omitted; Cl_3CCOOH, trichloroacetic acid; TMEG buffer, 0.02 M Tris-HCl (pH 8.0), 0.005 M 2-mercaptoethanol, 0.001 M EDTA, 10% (w/v) glycerol; Tris-HCl, tris(hydroxymethyl)aminomethane) hydrochloride; BBOT, 2,5-bis-2-(5-*tert*-butylbenzoxazolyl)thiophene.

[2] Buffers are routinely prepared as 1 M stock solutions and the pH adjustments made at 20 °C. Corrections for temperature and dilution coefficients have not been made.

TABLE I: Purification of Novikoff Hepatoma DNA Polymerase-β.[a]

Fraction	Protein (mg)	Total Units[b]	Sp Act. (Units/mg)	Purification (-fold)	Yield (%)
I. Cell extract	14 500	3 880	0.268	1.00	100
II. Ammonium sulfate	4 610	3 780	0.820	3.06	97.4
III. DEAE-Sephadex	8 866	9 820	11.3	42.2	253
IV. Phosphocellulose	81.2	4 390	54.1	202	113
V. Hydroxylapatite	1.34	3 150	2 350	8 770	81.2
VI. DNA-Cellulose	0.031	1 800	58 100[c]	217 000	46.4

[a] The reaction mixtures contained the following components in a final volume of 125 μl: 25 mM Tris-HCl, pH 8.4; 5 mM 2-mercaptoethanol; 7 mM magnesium acetate; 0.5 mM EDTA; 0.015 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (specific activity 975 mCi/mmol); 50 mM NaCl; 15% (w/v) glycerol; 250 μg/ml activated DNA; and 0.01–0.3 units of DNA polymerase fraction. Incubations were carried out for 1 h at 37 °C and acid-insoluble radioactivity was determined as described in Methods. When incorporation was not linear for 1 h, the data were extrapolated from a 30-min incubation. [b] A unit is defined as the incorporation of 1 nmol of total nucleotide into DNA per h at 37 °C. [c] With several different preparations, the specific activity varied from 32 000 to 62 000 units/mg.

8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 mg/ml bovine serum albumin, and 0.5 M NaCl. Samples of 200 μl were applied to the top of the gradient and centrifugation was carried out for 18 h at 35 000 rpm at 4 °C. Each gradient contained 1 μl of *E. coli* alkaline phosphatase (6.3 S, 80 000 molecular weight) as a marker.

An estimate of molecular weight was also made by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Weber and Osborn (1969). Standards included bovine serum albumin, *E. coli* alkaline phosphatase, phosphorylase *a*, ovalbumin, chymotrypsinogen, ribonuclease A, cytochrome *c*, catalase, lysozyme, and alcohol dehydrogenase.

Isoelectric Focusing. A 110-ml LKB Type 8101 isoelectric focusing column was used to determine the isoelectric point. Because of the instability of fraction VI, fraction V was used. Approximately 96 μg (5 ml) of fraction V protein was dialyzed for several hours into 1% glycine. A 3–50% sucrose gradient containing the sample and 2.5% ampholytes (pH 7–10) was poured into the focusing unit. The sample was focused for 36 h at 4 °C, and 2.3 ml fractions were collected. The pH of each fraction was read, and a small aliquot of each was assayed for DNA polymerase activity as described above.

Maintenance of the Tumor. The Novikoff hepatoma was maintained in Holtzman rats by intraperitoneal injection. The ascites fluid was harvested at 6–7 days postinjection and the cells pelleted by centrifugation at 12 000*g* for 10 min. The pellet was resuspended in 4 volumes of 0.01 M Tris-HCl at pH 7.0 and 0.002 M $MgCl_2$. After hemolysis of the erythrocytes, the tumor cells were collected by centrifugation, washed in Tris-Mg, and either frozen as a solid pellet at −20 °C or used immediately for enzyme purification.

Other Methods. Protein concentrations were determined by the Lowry procedure (Lowry et al., 1951), and by the fluorescamine technique (Böhlen et al., 1973). DNA was measured by the diphenylamine reaction (Burton, 1956).

Results

Purification of Novikoff DNA Polymerase-β. All steps were carried out at 0–4 °C. Generally 150 g (wet weight) of cells was conveniently processed in a single batch. A typical purification scheme is summarized in Table I.

Preparation of Novikoff Cell Extract. The cells were lysed by addition of two volumes of lysis buffer (0.02 M Tris-HCl (pH 7.0), 0.005 M 2-mercaptoethanol, 0.001 M EDTA, 0.2% Triton X-100, and 3.0 M NaCl). Extraction was carried out for 2 h with constant stirring and followed by centrifugation for 2 h in a Spinco 30 rotor at 70 000*g*. The red supernatant was dialyzed against TMEG–0.15 M NaCl buffer with two or more changes of buffer until the NaCl concentration equilibrated at 0.15 M. This usually required 24 h or longer, depending upon the amount of extract processed at one time. Precipitates formed during dialysis were removed by centrifugation at 12 000*g* for 10 min. The resulting extract constitutes fraction I.

Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to fraction I over a period of 30 min to bring the solution to 45% saturation. After stirring an additional 30 min, the precipitate was collected by centrifugation. The supernatant was brought to 75% saturation and the proteins precipitating at this concentration were collected, dissolved in TMEG–0.01 M NaCl, and dialyzed against this buffer overnight. This constitutes fraction II. The 75% ammonium sulfate soluble proteins were further fractionated for stimulatory proteins (Probst, 1974; Probst et al., 1975).

DEAE-Sephadex Chromatography. Fraction II was loaded onto a 4.9 $cm^2 \times 22$ cm column of DEAE-Sephadex A50 equilibrated with TMEG–0.01 M NaCl. The column was washed with 100 ml of starting buffer and the enzyme eluted with a 500-ml linear gradient of 0.01–0.30 M NaCl in TMEG buffer at a flow rate of approximately 40 ml/h. The DNA polymerase eluted at 0.12 M NaCl. Occasionally a 800-ml gradient of 0.01–0.50 M NaCl was used to recover DNA polymerase-α eluting at 0.33 M NaCl. Fraction II contains considerable quantities of Novikoff stimulatory factors II and III which elute on either side of the DNA polymerase at 0.09 and 0.15 M NaCl, respectively (Probst et al., 1975). These peaks were also pooled and saved for further purification. The combined DNA polymerase fractions were dialyzed overnight against PMEG buffer, and the dialyzed material represents fraction III.

Phosphocellulose Chromatography. Fraction III was loaded onto a 1.75 $cm^2 \times 24$ cm column of phosphocellulose equilibrated with PMEG. The column was washed with 100 ml of starting buffer and eluted with a 30-ml linear gradient of 0–0.45 M potassium phosphate in PMEG at a flow rate of about 35 ml/h. The enzyme eluted as a single peak at 0.22 M. The pooled fractions were dialyzed overnight against PMG buffer. The dialyzed material constitutes fraction IV.

Hydroxylapatite Chromatography. Fraction IV was loaded onto a 0.63 $cm^2 \times 8$ cm column of hydroxylapatite equilibrated with PMG buffer. The column was washed with 50 ml of PMG and eluted with a 300-ml linear gradient of 0–0.45 M potassium phosphate in PMG at a flow rate of about 25 ml/h. The enzyme eluted as a single peak at 0.15 M. The pooled fractions were dialyzed against TMEG overnight and bovine serum albumin was added to a final concentration of 1 mg/ml for

stability. This constitutes fraction V. Fraction V was usually frozen in liquid nitrogen for varying periods of time before continuing purification. Often two or more preparations were combined for the final purification step. In the experiment reported in Table I, a single preparation was used.

DNA-Cellulose Chromatography. Fraction V was loaded onto a 0.63 $cm^2 \times 5$ cm, single-stranded DNA-cellulose column equilibrated with TMEG plus 1 mg/ml bovine serum albumin. The column was washed with 40 ml of TMEG-bovine serum albumin and the enzyme eluted with a 200-ml linear gradient of 0.05–1.0 M NaCl in TMEG-bovine serum albumin at flow rate of about 30 ml/h. The polymerase eluted at 0.56 M NaCl. The pooled fractions were dialyzed for about 2 h against TMEG–0.5 M NaCl and stored in liquid nitrogen. This constitutes fraction VI.

In order to determine protein concentration and in preparation for gel electrophoresis, it was necessary to remove the bovine serum albumin. Therefore, a small hydroxylapatite column of 0.63 $cm^2 \times 1$ cm was poured as described above. A small aliquot (usually 1–2 ml) of fraction VI was applied to the column and the bovine serum albumin eluted by washing with 20 ml of 0.1 M potassium phosphate in 20% glycerol. The polymerase was then eluted with 2.0 ml of 0.3 M potassium phosphate containing 20% glycerol. Elution was by gravity flow and required about 30 min to complete. The entire fraction was used for protein determination by the fluorescamine reaction. Similarly prepared samples, used for polyacrylamide gel electrophoresis, showed this to be an effective method for removing bovine serum albumin.

The overall yield of enzyme was approximately 200 μg/kg of cells. The recovery of enzyme on the basis of activity was about 46% of the initial activity in the experiment reported in Table I. Accurate estimates of recovery are complicated by the presence of stimulating proteins in all but fraction VI (Probst et al., 1975; Stalker et al., 1975). The enzyme has been purified over 200 000-fold based upon increased specific activity over that of the initial extract. Homogeneous fraction VI DNA polymerase preparations had a specific activity ranging from 32 000 to 62 000 units/mg using DNase I activated DNA as primer-template.

Enzyme Stability. The enzyme is quite stable at 4 °C during all of the purification steps except the last, and, as a result, there is no need for rapid purification. After DNA-cellulose chromatography, however, the enzyme is very unstable in low ionic strength buffers but is stable in 0.5 M NaCl. The presence of glycerol in all fractions also promotes stability; bovine serum albumin is required for stability during DNA-cellulose chromatography and in fraction VI.

Under these conditions, the purified enzyme has been stored at −196 °C for 1 year without loss of activity. All fractions are stable to freeze–thawing. Whole cells may also be kept for several months at −20 °C before purifying the enzyme.

Evidence for Homogeneity. Electrophoresis of fraction VI DNA polymerase in nondenaturing polyacrylamide gels gave a single Coomassie-blue-staining band (Figure 1A). In sister gels which were sliced and assayed for DNA polymerase, the activity corresponded to the staining band (Figure 1B). When gels of different porosity were run, a single staining band was still found. Thus, if the preparation does have a contaminant, it must have the same size and charge as the DNA polymerase protein. In sodium dodecyl sulfate–polyacrylamide gels, a single polypeptide was observed (Figure 2).

Associated Enzyme Activities. Fraction VI was found to contain no other enzyme activity. Since nuclease is frequently associated with prokaryotic DNA polymerase (Kornberg and Kornberg, 1974), we examined the preparation for both exonuclease and endonuclease at acid and alkaline pH, with native and denatured DNA and in the presence and absence of ATP. No detectable nuclease activity was found.[3] The preparation also lacked RNA polymerase, and nonspecific phosphomonoesterase and phosphodiesterase activities.

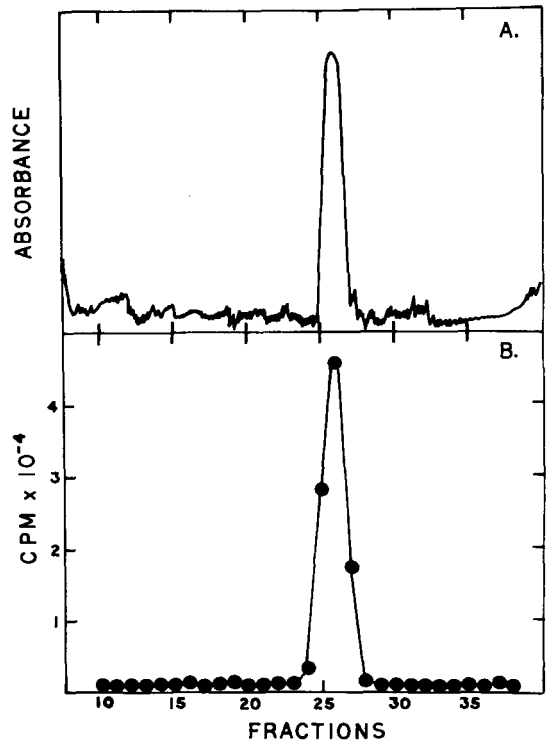

FIGURE 1: Polyacrylamide gel electrophoresis of Novikoff DNA polymerase-β. Approximately 9 μg of fraction VI, from which bovine serum albumin had been removed as described in Materials and Methods, was applied to the top of the gel and subjected to electrophoresis at 4 °C in Tris-glycine buffer (pH 9.1), at 2 mA per gel. Gel A was fixed in 7% acetic acid, stained with Coomassie brilliant blue, diffusion destained and scanned at 600 nm in a Gilford 2400 spectrophotometer. A sister gel (B) was sliced into 2-mm slices. Each slice was extracted overnight in 150 μl of reaction buffer at 4 °C and then assayed for activity using a limited substrate assay. Approximately 30% of the activity applied to the gel was recovered.

General Properties of the Enzyme. Molecular Weight. Molecular weight was estimated by two different methods. Sucrose gradient centrifugation (data not shown) gave an apparent molecular weight of 31 000, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2) gave 32 000. The molecular weight of the enzyme changes during purification and will be discussed in more detail in a future publication (Stalker, Mosbaugh, Probst, and Meyer, in preparation).

Isoelectric Point. Fraction V DNA polymerase was subjected to isoelectric focusing instead of fraction VI because of the instability of the latter in the absence of salt. The isoelectric pH was 8.5 (Figure 3) which is somewhat lower than the 9.5 found for the calf enzyme (Bollum, 1975), 9.15 for chick embryo (Stavrianopoulos et al., 1972), and 9.2 for human KB cells (Sedwick et al., 1972). An alkaline isoelectric point is typical of β-polymerases.

Reaction Requirements. Like all other DNA polymerases, the Novikoff DNA polymerase-β requires a divalent cation,

[3] The limit of detection of exonuclease activity for 0.2 unit of DNA polymerase was less than 0.01% of the polymerization rate.

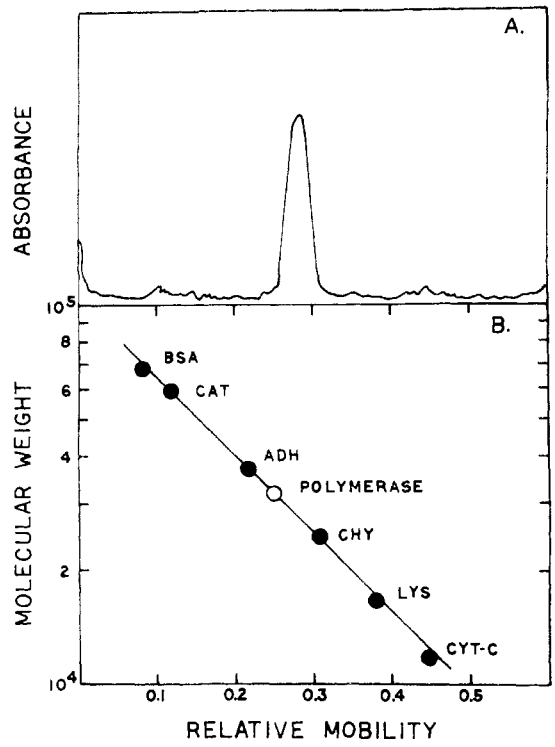

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of Novikoff DNA polymerase-β. Approximately 6 μg of fraction VI from which bovine serum albumin had been removed was subjected to electrophoresis in sodium dodecyl sulfate–polyacrylamide gels as described by Weber and Osborn (1969). Figure 3A is a scan of a Coomassie-brilliant-blue-stained gel. Figure 3B is a plot of the molecular weight vs. relative mobility as compared with standard proteins run under identical conditions. Abbreviations for this figure are: BSA, bovine serum albumin; CAT, catalase; ADH, yeast alcohol dehydrogenase; CHY, chymotrypsinogen A; LYS, lysozyme; CYT-C, cytochrome *c*.

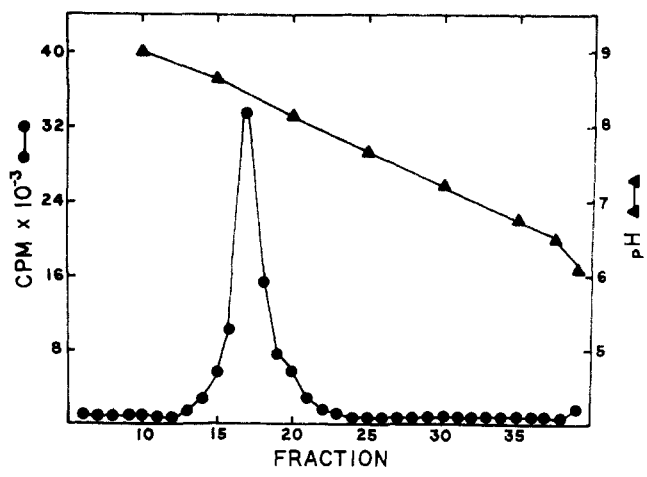

FIGURE 3: Isoelectric focusing profile for Novikoff DNA polymerase-β. Fraction V DNA polymerase (96 μg) in 1% glycine was focused for 36 h at 4 °C in an LKB Type 8101 isoelectric focusing column using 2.5% ampholytes (pH 7–10) in a 3–50% sucrose gradient. Each 2.3-ml fraction was measured for pH and for DNA polymerase activity with a limited substrate assay. All of the enzyme activity applied to the column was recovered.

TABLE II: Reaction Requirements for Novikoff DNA Polymerase-β.[a]

Addition or Deletion	Act. (pmol of [^{3}H] dTMP)	% Control Act.
None (control)	62.7	100.0
− Mg^{2+}	0.50	0.8
− Mg^{2+}, + 1 mM Mn^{2+}	52.5	83.7
− Mg^{2+}, + 1 mM Ca^{2+}	0.01	0.1
− DNA	0.40	0.6
− dNTP	23.4	37.3
− DNA, + 10 μg/ml dAT	135.0	215
− DNA, + 10 μg/ml dAT, − dNTP	3.60	5.7

[a] Reaction mixtures contained the components described in Methods. Each assay tube contained 8 ng of fraction VI DNA polymerase. Changes from these conditions are noted in the table.

complementary deoxyribonucleoside triphosphates and a suitable primer–template for activity (Table II). Omission of DNA or Mg^{2+} results in less than 1% of control activity. Omission of the unlabeled deoxyribonucleoside triphosphates, however, still gives 37.3% maximal incorporation. The amount of incorporation varies with the DNA preparation used and probably reflects addition of one or two dTMPs to complementary ends. Similar results have been reported for other mammalian DNA polymerases (Sedwick et al., 1972; Chang, 1973a). When dAT is used in place of DNA and dNTPs omitted, the activity drops to less than 3% of that obtained with dNTPs present (Table II).

The enzyme is saturated with deoxyribonucleoside triphosphates at about 12 μM. The apparent K_m for dNTP is approximately 7–8 μM with DNA as primer–template.

The apparent K_m values for each substrate have not been measured individually and probably vary somewhat. In one experiment, the K_m for dATP using dAT as template–primer was found to be 3 μM.

Enzyme activity is proportional to protein concentration to approximately 0.3–0.4 unit per assay. Incorporation is proportional to time for usually an hour, although at some stages of purity incorporation levels off between 30 and 60 min. In the presence of stimulatory proteins, incorporation can be extended for several hours (Probst et al., 1975).

pH Optimum. The pH optimum of the enzyme, like other β-polymerases, is in the alkaline range. Maximal activity is obtained with Tris-HCl at pH 8.4 or 9.2 in glycine–NaOH; both buffers are equally effective. Phosphate buffer gives consistently lower activity than either Tris or glycine at all pHs tested up to 8.2.

Divalent Cation Requirement. A divalent cation is required for activity (Table II) and magnesium is preferred when DNA is used as the primer–template. The optimum concentration of Mg^{2+} extends from 5 to 10 mM. At 2 mM Mg^{2+}, 70% maximum velocity is obtained, and, at 15 mM Mg^{2+}, 62% is found. Manganese, when substituted for Mg^{2+}, is slightly less active with DNA template, but 1.5–5 times greater when synthetic polynucleotides are used (Table III). The optimum is fairly sharp at 1.0 mM Mn^{2+}; at 0.5 mM, 45% maximal velocity is obtained and at 2.0 mM 84%. When EDTA is omitted from the reaction tubes, the Mn^{2+} optimum drops to 0.5 mM. No synthesis of DNA occurred when calcium was used as a divalent cation from 0.1 to 25 mM.

Primer–Templates for the DNA Polymerase-β. The Novikoff β-polymerase has an absolute requirement for DNA (Table II). Unlike some bacterial DNA polymerases which are capable of de novo synthesis in the absence of primer–template (Kornberg and Kornberg, 1974), no incorporation was found after prolonged incubation in the absence of DNA. The best "natural" template is native DNA rendered approximately 7% acid soluble by pancreatic DNase I. Approximately sevenfold

TABLE III: Synthetic Polynucleotide Templates for Novikoff DNA Polymerase-β.[a]

Primer-Template		pmol of ^{3}H-Labeled Nucleotide Incorporated	
		Mg^{2+}	Mn^{2+}
Experiment I ([^{3}H]dTTP)			
Activated DNA		4.28	2.87
rA·dT_{12-18}	(20:1)	0.11	3.08
rA·dT	(1:1)	1.46	30.5
dA·dT_{12-18}	(20:1)	7.29	74.1
dA·dT	(1:1)	5.01	7.13
dAT		6.31	7.10
Experiment II ([^{3}H]dATP)			
Activated DNA		6.36	5.17
rA·dT_{12-18}	(20:1)	0.08	0.28
rA·dT	(1:1)	0.11	0.47
dA·dT_{12-18}	(20:1)	0.07	1.11
dA·dT	(1:1)	7.83	23.2
dAT		10.1	11.4

[a] Each assay tube contained, in a volume of 125 μl: 20 mM glycine buffer, pH 9.2; 7.5 mM magnesium acetate or 1.0 mM $MnCl_2$; 5 mM 2-mercaptoethanol; 0.5 mM EDTA; 100 mM NaCl; 15% (w/v) glycerol; 0.015 mM each of the complementary deoxyribonucleoside triphosphates with either [^{3}H]dTTP or [^{3}H]dATP as the labeled precursor; primer-template as indicated in the table at a concentration equivalent to 50 μM in deoxynucleotide phosphate and 1.0 ng of fraction VI DNA polymerase. Incubations were carried out at 30 °C for 1 h, and acid-insoluble radioactivity was determined as described in Methods.

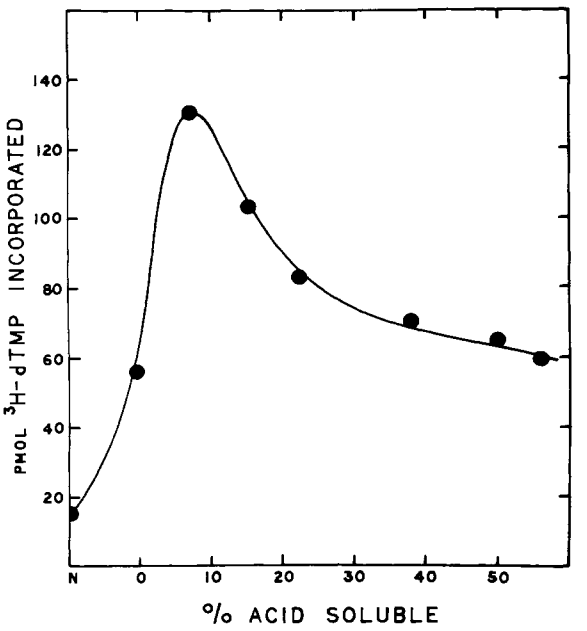

FIGURE 4: DNA polymerase activity as a function DNase I activation of primer-template. Samples of DNA were digested with pancreatic DNase I (Loeb, 1969) for varying periods of time at 37 °C and then heated to 70 °C for 15–30 min to inactivate the nuclease. Aliquots were precipitated with cold 0.5 M PCA and the amount rendered acid soluble was determined by the diphenylamine reaction. The efficiency of each sample to act as primer-template for the DNA polymerase was determined in a standard assay using 100 μg/ml of DNA and 12 ng of fraction VI enzyme. In the figure, N represents the activity of *untreated*, native calf thymus DNA, to distinguish it from the first point which represents a DNase-treated sample which did not render any of the DNA acid soluble.

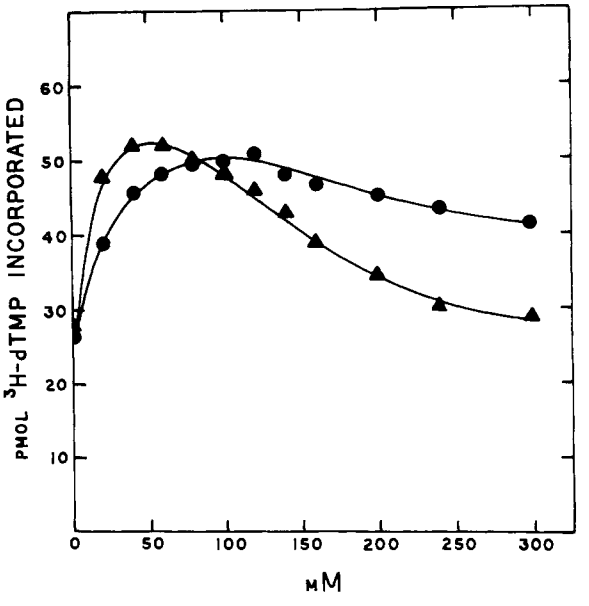

FIGURE 5: Effect of KCl and NaCl on Novikoff DNA polymerase-β. Standard assays were run as described in Methods, except the monovalent salt concentration was as given in the figure and 6 ng of fraction VI protein was used. (●) KCl; (▲) NaCl.

greater activity is obtained than with unactivated, native DNA (Figure 4). Further treatment with DNase I leads to less priming ability of this DNA. Heat denatured DNA is very inefficient, giving only 2–3% of the activity obtained with maximally activated DNA. Compared with other mammalian DNA polymerases, enzyme saturation with DNA occurs at fairly high concentration, about 250 μg/ml. The apparent K_m for DNA is about 125 μg/ml.

Synthetic polynucleotides are effective primer-templates for the Novikoff β-polymerase (Table III). Most adenine and thymine polymers can be used equally as well or several-fold better than activated DNA. The ability to use a particular template is dependent upon several factors including divalent cations, salt concentration, pH, and temperature of incubation. Manganese is consistently better than magnesium as a divalent cation with the synthetic polymers. While ribopolymers are efficiently used as templates, they are far less efficient as the primer strand (cf. [^{3}H]dTMP and [^{3}H]dAMP incorporation using rA·dT as template-primer in Table III). A more detailed investigation of the ability of this enzyme to use synthetic polymers is in progress.

The Novikoff β-polymerase is not able to incorporate a ribonucleotide when tested with [^{3}H]UTP in the presence of Mg^{2+} or Mn^{2+}.

Effect of Salt on the Polymerase. One of the distinguishing features of β-polymerases is their relative insensitivity to salt concentrations compared with α-polymerases which are strongly inhibited by salt (Weissbach, 1975). The Novikoff DNA polymerase-β is stimulated twofold at 50–100 mM concentration of NaCl or KCl (Figure 5). At higher concentrations (which almost totally inhibit α-polymerases), the β-polymerase activity drops off slowly, and at 300 mM is still higher than the activity measured in the absence of salt. There is a slight difference in behavior of the enzyme in NaCl compared with that in KCl (Figure 5) or other monovalent salts (data not shown).

Effect of Ethanol and Acetone. One of the interesting properties of the β-polymerase isolated from calf thymus is its relative insensitivity to acetone or alcohol (Chang, 1973a). To

see whether this is a general property of β-polymerases or unique to the calf enzyme, we measured the activity of the Novikoff enzyme in the presence of various concentrations of acetone or ethanol. Unlike the calf enzyme, inhibition was observed at all concentrations.

Effect of Glycols. Glycols provide a limited amount of stability to the enzyme and increase its activity approximately twofold. Sucrose and glycerol are effective over a wide range, but poly(ethylene glycol) shows a sharp optimum at 5%. For this reason, all buffers contained glycerol and all enzyme reactions were carried out in the presence of 15% (w/v) glycerol.

Effect of Spermine and Spermidine. Spermidine has been shown to stimulate prokaryotic DNA polymerases probably in a manner similar to DNA unwinding protein (Geider and Kornberg, 1974). In mammalian systems, spermidine has been reported to stimulate the partially purified β-polymerase of rat brain (Chiu and Sung, 1972). When tested with the Novikoff β-polymerase, spermidine increased the activity approximately twofold over a wide range of concentrations (up to 10 mM). In contrast, spermine showed stimulation at low concentration (optimum 2 mM) but was inhibitory above 3 mM.

Effect of Inhibitors. Table IV summarizes the effects of adding known inhibitors of DNA polymerases to the reaction mixture. Of particular interst is the effect of sulfhydryl blocking agents. The Novikoff enzyme is inhibited by NEM.[4] Resistance to 10 mM NEM is a characteristic feature of β-polymerases (Bollum, 1975). However, the Novikoff enzyme shows 35% inhibition at this concentration and greater than 70% inhibition at 50 mM. The α-polymerase from Novikoff cells, like other α-polymerases, is far more sensitive to NEM than is the β enzyme (Mosbaugh and Meyer, unpublished observation). The β-polymerase is not inhibited by concentrations of *p*-hydroxymercuribenzoate up to 50 μM, and, in fact, shows a slight stimulation. Above this level, inhibition occurs. Other β-polymerases also show sensitivity to *p*-hydroxymercuribenzoate (Bollum, 1975). Actinomycin D, 5-aminoacridine, acriflavin, ethidium bromide, and *o*-phenanthroline all inhibit the enzyme to varying degrees (Table IV).

TABLE IV: Effect of Inhibitors on Novikoff DNA Polymerase-β.[a]

Addition	[^{3}H]dTMP Incorporated (pmol)	% Control Act.
None (control)	54.6	100.0
+ 50 μM *o*-phenanthroline	50.1	91.8
+ 150 μM *o*-phenanthroline	33.9	62.1
+ 50 μM *p*-hydroxymercuribenzoate	58.5	107.1
+ 75 μM *p*-hydroxymercuribenzoate	18.4	33.7
+ 4 mM *N*-ethylmaleimide	39.4	72.2
+ 10 mM *N*-ethylmaleimide	35.7	65.4
+ 50 mM *N*-ethylmaleimide	20.4	37.4
+ 10 μM actinomycin D	38.3	70.1
+ 50 μM actinomycin D	20.9	38.3
+ 5 μM 5-aminoacridine	40.6	74.4
+ 25 μM 5-aminoacridine	12.1	22.2
+ 10 μM acriflavin	49.3	90.3
+ 100 μM acriflavin	15.4	28.2
+ 10 μM ethidium bromide	49.1	89.9
+ 100 μM ethidium bromide	15.2	27.8

[a] Standard assay conditions were as given in Methods, except that inhibitors were added at the concentrations listed in the table. For *p*-hydroxymercuribenzoate and *N*-ethylmaleimide, DNA polymerase samples were dialyzed prior to the experiment in order to remove 2-mercaptoethanol and the assays were carried out in the absence of 2-mercaptoethanol. There was no loss of DNA polymerase activity by dialysis. Each assay tube contained 5 ng of fraction VI DNA polymerase.

Discussion

The Novikoff hepatoma DNA polymerase-β has been purified to apparent homogeneity and characterized. Like other β-polymerases, it has an alkaline pH optimum and an alkaline isoelectric point, is stimulated or unaffected by high salt concentrations, and has a low molecular weight. It has neither exonuclease nor endonuclease activity. It differs from other comparably pure mammalian β-polymerases by its sensitivity to acetone and alcohol, significantly lower p*I*, lower K_m for deoxyribonucleoside triphosphates, lower molecular weight, and particularly its sensitivity to NEM.

The properties of the Novikoff enzyme are quite similar to the homogeneous β-polymerase isolated from calf thymus gland (Chang, 1973a, 1973b), except for the sensitivity of the Novikoff enzyme to ethanol, acetone, and NEM. Our enzyme has a specific activity of roughly 60 000 units/mg which is about sevenfold greater than the homogeneous KB polymerase (Wang et al., 1974), but only one-third of that reported for the homogeneous calf thymus enzyme (Chang, 1973a); however, the inherent catalytic rate for our enzyme is difficult to determine accurately. The specific activity calculation for the final fraction depends upon accurate measurement of nanogram quantities of protein which is not an easy task. Inactivation of some enzyme molecules which co-purify with active DNA polymerase, a distinct possibility considering the relative instability of fraction VI, would lead to an underestimate of specific activity in the final fraction. The choice of assay conditions will also affect these calculations. For example, addition of 4 mM spermidine and increased levels of glycerol would give a specific activity of about 150 000 units/mg. Addition of stimulatory proteins for this enzyme (Probst et al., 1975; Stalker et al., 1975) could increase this value by at least an order of magnitude. Thus, our best estimate at present for the catalytic activity of the homogeneous 32 000-dalton polypeptide in the presence of 15% glycerol is about 60 000 units/mg.

The molecular weight of the homogeneous enzyme is estimated at 32 000, a value somewhat smaller than 43 000–45 000 for calf thymus and KB β-polymerases. Smaller β-polymerases have been described in chicken tissues (Stavrianopoulos et al., 1972; Brun et al., 1974). It is not certain that this represents the smallest catalytic unit in vivo. With *E. coli* DNA polymerase I, the enzyme can be proteolytically cleaved into a smaller, but still active polypeptide (Setlow et al., 1972). We cannot rule out the possibility that the Novikoff enzyme, as isolated, may be a product of limited proteolysis, and experiments designed to test this possibility are in progress.

[4] Since inhibition of a β-polymerase is rather unusual, we have repeated these experiments with several batches of freshly prepared NEM. Moreover, control tubes run simultaneously and containing NEM-sensitive polymerases, such as the α-polymerase from Novikoff cells and the sea urchin nuclear enzyme, showed the expected sensitivity while the NEM-resistant polymerase I of *Escherichia coli* was unaffected by the NEM.

The role of β-polymerases in DNA metabolism in vivo remains to be determined. It is likely that both replication and repair occur in a multienzyme complex consisting of DNA polymerase plus other accessory proteins. The availability of homogeneous Novikoff DNA polymerase we hope will be the first step in reconstituting such complexes in vitro.

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