STUDIES ON THE CYTOPLASMIC DNA POLYMERASES FROM THE INTESTINAL MUCOSA OF RAT

by

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ABSTRACT

Significant DNA polymerase activity has been found in cytoplasmic preparations from rat intestinal mucosa. The present work involves a partial purification and a study of the general properties of this cytoplasmic enzyme activity.

Crude cytoplasmic enzyme was prepared by high-speed centrifugation of the homogenate of washed mucosal scrapings. A strong inhibitor of DNA polymerase was sedimented by the high-speed centrifugation. The bulk of the enzyme activity was unadsorbed on DEAE-cellulose. However, a minor portion of the enzyme was adsorbed, and was eluted with 0.1 M KCl. When crude cytoplasmic enzyme was chromatographed by gel-filtration on Sephadex G-150, a single peak of DNA polymerase activity was detected. By the use of protein markers with known molecular parameters, the molecular weight of the DNA polymerase fraction was estimated to be 101,000.

The enzyme required the presence of a DNA template and Mg^{++} ions. Activity was only slightly enhanced by the addition of dithiothreitol. For maximum activity, the presence of all four deoxynucleoside triphosphates were required. Heat-denatured DNA was preferred as primer. The optimum pH for this enzymatic activity was found to be 7.2 in potassium phosphate buffer, and 8.0 in Tris-acetate buffer. Time course studies on the enzyme reaction indicated that the reaction was linear with respect to incubation time for at least 30 min. The DNA polymerase activity was stable up to 13 days under temperature conditions of 4°C to -20°C. Glycerol in 20% to

35% (v/v) concentrations was found to have both a stimulatory and a stabilizing effect on the enzyme activity. Ethylene glycol at 20% (v/v) concentration was also found to have a stimulatory effect on the enzyme activity. The enzyme was strongly inhibited in the presence of 0.10 M phosphate ions and activity was drastically reduced in phosphate ion concentrations of 0.20 M and above. The product of the DNA polymerase reaction could be destroyed by DNase, indicating that it was DNA in nature.

The purpose of the present work was to determine whether the DNA polymerase activity in the cytoplasmic preparation is actually of cytoplasmic origin, or whether it is due to nuclear contamination. The above results were compared with the results obtained by other workers on the nuclear DNA polymerases. The evidence seems to indicate that the cytoplasmic enzyme activity is not due to nuclear contamination. The nuclear preparation contained several DNA polymerases, while the cytoplasmic preparation contained a single major DNA polymerase activity. This cytoplasmic activity resembled one of the nuclear activities in many respects. The cytoplasmic preparation also contained a minor DNA polymerase activity which may be mitochondrial in origin.

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INTRODUCTION

Deoxyribonucleic acid (DNA) is believed to be the central storehouse of genetic information in most cells. This genetic information determines the biochemical specificity of the cell, and is passed intact from parent to progeny on cell division. The processes involved in DNA biosynthesis, especially those of self-duplication, are therefore of great interest and have been extensively studied.

On the basis of their structural model for complementary double-stranded DNA, Watson and Crick (1) proposed that each chain of the DNA duplex serves as template for the synthesis of a complementary chain, so that two replicas of the original double-stranded structure are produced. Meselson and Stahl (2), in their classical experiments with CsCl density gradient centrifugation of N^{15}/N^{14} hybrid E. coli DNA, demonstrated that this 'semi-conservative' type of replication actually takes place in vivo.

Bacterial DNA polymerases

The first isolation of an enzyme involved in DNA replication was from extracts of E. coli by A. Kornberg and his associates in 1960 (3). This enzyme converts deoxynucleoside polyphosphates into polymeric material, and was termed DNA polymerase (EC 2.7.7.7 Deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase). The overall reaction catalyzed may be described as follows:

$$\begin{matrix} n_1\ \text{dATP} \\ n_2\ \text{dGTP} \\ n_3\ \text{dCTP} \\ n_4\ \text{dTTP} \end{matrix} + \text{DNA} \xrightleftharpoons{Mg^{++}} \text{DNA} \begin{bmatrix} \text{dAMP} \\ \text{dGMP} \\ \text{dCMP} \\ \text{dTMP} \end{bmatrix}_{2n_1+2n_2} + 2(n_1+n_2)\ \text{PP}$$

DNA polymerase is now known to catalyze the addition of mononucleotide units to the 3'-hydroxyl terminus of a primer DNA chain (4). Synthesis proceeds in the direction of 5' to 3' (Fig. 1).

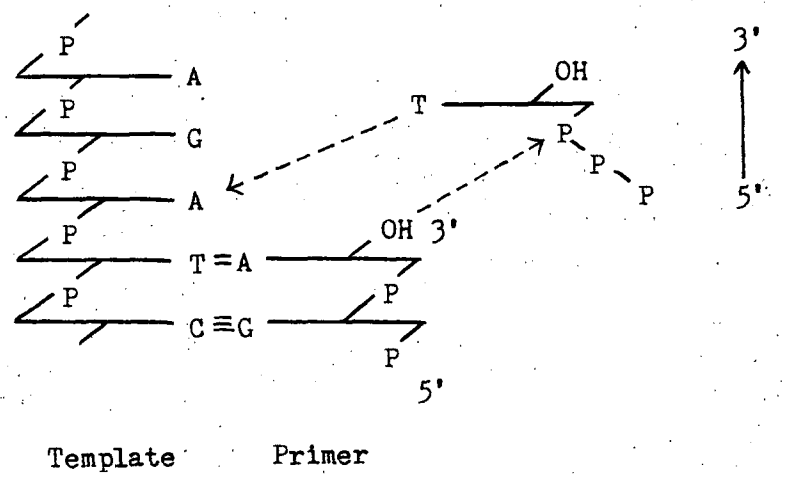


Fig. 1. Direction of chain growth catalyzed by DNA polymerase. (A, adenine; C, cytosine; G, guanine; and T, thymine.)

All four deoxynucleoside triphosphates are required; the diphosphates are not polymerized. The overall reaction is believed to be reversible, as incubation of DNA with high concentrations of pyrophosphate results in a partial depolymerization reaction. The reaction also requires the presence

of a divalent metal ion, usually magnesium, and of a DNA primer. The synthetic material resulting from the catalytic reaction is shown to be a DNA with physical characteristics largely resembling those of the primer.

Much work has been done on the DNA polymerase from E. coli, sometimes referred to as 'the Kornberg polymerase'. This is found to be a versatile enzyme with many catalytic properties. These include (4) : (a) the 5' → 3' growth of a DNA chain by the polymerization of nucleotides; (b) hydrolysis of a DNA chain in the 3' → 5' direction; (c) hydrolysis of a DNA chain in the 5' → 3' direction; (d) pyrophosphorolysis of a DNA chain from the 3' end; and (e) exchange of inorganic pyrophosphate with the terminal pyrophosphate group of a deoxyribonucleoside triphosphate.

Kornberg has presented a picture of several major sites within the active center of the enzyme (4). These sites specifically recognize and accommodate the template chain, the primer chain, the primer terminus, or an incoming triphosphate (Fig. 2). It was proposed that the triphosphate is bound adjacent to the 3'-terminus of the primer, and oriented so that it can form a base pair with the template. When a correct base pair is formed, a nucleophilic attack by the 3'-OH of the primer terminus on the innermost phosphate of the triphosphate takes place. Through movement of the entire chain relative to the enzyme, the newly added nucleotide is shifted to the primer terminus site and is then ready to attack another triphosphate and add the next

nucleotide. The specificity of DNA polymerase is probably based on its demand for one of the four Watson-Crick base pairs, all of which contain regions of identical dimensions and geometry and are symmetrical (4).

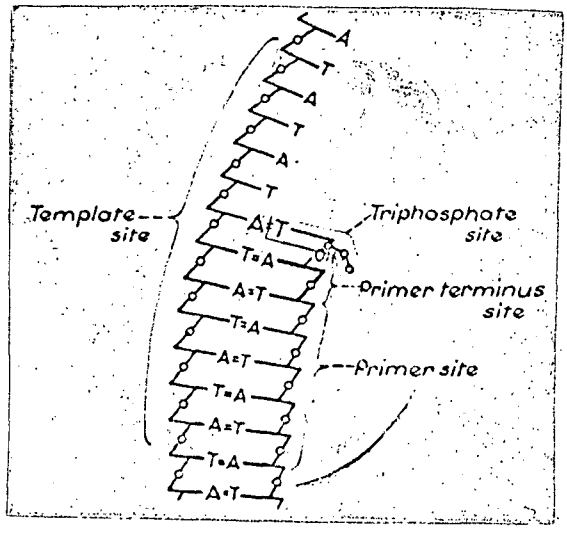


Fig. 2. Sites in the active center of DNA polymerase

Studies on DNA binding to DNA polymerase indicated that intact double-stranded DNA did not bind to the enzyme. Only nicked, denatured, or single-stranded DNA's were bound and replicated. This observation, together with the fact that DNA polymerization occurs only in the 5' → 3' direction, has caused some skepticism about the phusiological role of the Kornberg enzyme in replication. Various schemes have been proposed for the unidirectional replication of a duplex DNA chain. Replication is initiated by the introduction of a 'nick', a single break in one of the two DNA strands, at which DNA polymerase binds. Replication in the 5' → 3'

direction proceeds for some distance and then switches to the complementary strand as template to form a fork. The fork is then cleaved by an endonuclease. Repetition of this process results in small pieces of DNA near the replicating fork which may be linked up by a ligase. Okazaki and co-workers (5) have reported the isolation of small pieces of DNA at or near the nascent replicating region. This hypothesis is not entirely satisfactory as the replication is staggered, alternating from one strand to the other. Examination of dividing bacteria by autoradiography (6) or by gene duplication (7) have indicated that there is a simultaneous sequential replication of both strands.

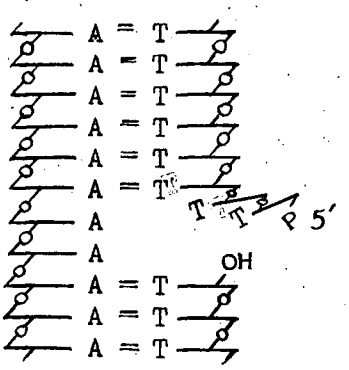


Fig. 3. Production of a 'nick' in DNA

The 5' → 3' nuclease activity associated with DNA polymerase readily removes non-base-paired segments, eg. thymine dimers, suggesting a repair function for the enzyme <u>in vivo</u>. An endonuclease recognises a disordered region in the DNA, and produces a nick to the 5' side (Fig. 3). Such dimer-specific endonucleases have been

identified and purified (8,9). DNA polymerase acts at the nick excising the damaged region, and simultaneously refilling the resulting gap. Closure with a ligase completes the repair process.

That E. coli DNA polymerase may function in repair in vivo is indicated by the UV sensitivity of mutants defective in the enzyme. DeLucia and Cairns (10) have isolated an amber mutant of E. coli containing less than 1% of the DNA polymerase activity of the parent strain. This mutant, called pol A, was discovered by assaying for the enzyme in extracts from a few thousand individual clones of mutagenized E. coli. It has essentially normal growth characteristics but is sensitive to ultraviolet irradiation and methylmethane sulfonate. Altogether six pol A mutants (pol 1 - 6) have now been isolated (11). This discovery of these mutants have strengthened the idea that E. coli DNA polymerase does not participate in chromosome replication.

Various attempts have been made to isolate from the Cairns mutant another enzyme which can replicate DNA. A membrane-bound enzyme is now believed to be involved in replication in pol A1. Knippers (12) has succeeded in solubilizing a DNA synthesizing enzyme activity from a crude cell-free membrane fraction. The enzyme has a molecular weight of between 60,000 and 90,000, and can synthesize DNA semi-conservatively for at least 90 minutes. Unlike the Kornberg DNA polymerase, this enzyme is strongly inhibited by mercuri-compounds and is resistant to an antiserum which inhibits the Kornberg enzyme.

Independently, T. Kornberg and M. Gefter (13) reported

the isolation from the Cairns mutant of an enzyme which could synthesize DNA *in vitro*. This enzyme, which they call DNA polymerase II, is apparently the same as Knipper's enzyme. It works best with double-stranded DNA as template. It polymerizes deoxynucleoside triphosphates in a 5' to 3' direction, and requires a free 3'-hydroxyl group. The enzyme is not inhibited by antisera against A. Kornberg's polymerase I.

Recent genetic experiments (14) indicate that polymerase I together with an excision function, can edit out pyrimidine dimers induced by UV irradiation. It appears that in *E. coli* there are two DNA repair mechanisms, only one of which involves polymerase I. The Cairns mutants, although lacking the repair function which involves polymerase I, can survive UV irradiation because the second repair mechanism is functional. Gross and co-workers (15) have also found that *E. coli* cells in which both repair mechanisms are inactivated are inviable.

That polymerase II is the enzyme responsible for DNA replication *in vivo* remains to be proven. Its discovery at least demonstrates, however, that there may be several enzymes in *E. coli*, all of which can polymerize DNA, although most of them are not involved in DNA duplication.

Mammalian DNA polymerases

A. Source

Shortly after the earlier reports on E. coli DNA polymerase, it became apparent that mammalian tissues also contain an enzyme that catalyzes a similar reaction. Such enzyme activities were reported by Davidson et al (16) from Ehrlich ascites tumor, by Bollum (17) from calf thymus gland, by Mantsavinos et al (18) from regenerating rat liver, and by Leung and Zbarsky from rat intestinal mucosa (19, 20). DNA polymerase activity has subsequently been discovered in all animal cells which have been examined (11, 21).

B. Purification

Mammalian systems obviously offer a wider variety of tissues for study, with the possibilities of clinical as well as theoretical applications. However, the crude cell extracts or homogenates require much purification before use. Possible factors which might interfere with DNA polymerase studies include endogenous mononucleotides and nucleic acids, deoxyribonucleases (DNases), phosphodiesterases, and triphosphatases. The enzyme from calf thymus has been purified about fifty-fold with respect to protein in the crude extract (17) by acid precipitation, ammonium sulfate fractionation, and passage through DEAE-cellulose. It has also been freed from DNase and phosphodiesterase activity by chromatography on hydroxylapatite (22). In general, the techniques of ammonium sulfate fractionation, and chromatography on DEAE-

cellulose or hydroxylapatite are used (23, 24). However, extensive purification procedures are often not recommended as they may result in a highly purified but 'non-native' enzyme (21). Gel-filtration has been found to be a favorable fractionation technique since it does not require wide fluctuations of pH and ionic strength.

C. Requirements

Conditions for optimal DNA polymerase activity have been described for several mammalian systems. There is an absolute requirement for a divalent metal cation. Mg^{++} ions alone appear to give the optimum response, although partial replacement of Mg^{++} ions with Mn^{++} and Ca^{++} ions together has been observed (25).

DNA as primer or template is also required for the reaction. In most systems, denatured DNA is preferred over native DNA as primer (23, 26, 27), although in some cases (25, 28) the preference appears to be for native DNA. DNA activated by light treatment with DNase often produces high polymerase activity (28, 29, 30), indicating the ability of the enzyme to replicate at nicks.

Most animal cell DNA polymerases do not display a strict requirement for all four deoxynucleoside triphosphates (21). An exception is the Ehrlich ascites enzyme (29), which resembles the bacterial and phage polymerases in that synthesis drops to very low levels if one triphosphate is omitted. Cultured human KB cells (30) and sea urchin enzymes (28) can synthesize at 25 - 50% of the normal rate with only three triphosphates. The common explanation for these observations has been contamination

with terminal deoxynucleotidyltransferase. This appears not to be the case in the KB cell enzyme although the correct mechanism remains unknown (30).

D. Terminal transferase

Krakow et al (31, 32) were the first to report an enzyme in calf thymus nuclei which catalyzed the incorporation of a single deoxyribonucleoside triphosphate into terminal positions of DNA in the absence of the other three triphosphates. This enzyme is referred to as the 'terminal transferase', to distinguish it from the 'replicative' DNA polymerase. It requires heated DNA primer, Mg^{++}, and cysteine. The incorporation of mononucleotidyl residues is not stimulated, and is in fact inhibited, by the addition of the other three deoxyribonucleotides to the reaction mixture.

Using non-aqueous preparations of calf thymus nuclei and cytoplasm, Smith and Keir (33, 34) found that the nuclei and cytoplasm each contained both replicative and terminal transferases, the latter enzyme being about 1/27 as active as the former.

The terminal transferases from calf thymus have now been further purified by Bollum (35). The molecular weight calculated from equilibrium sedimentation is 32,600. In SDS-polyacrylamide gels the protein dissociates into two subunits. Enzyme activity is inhibited by low concentrations of metal chelating agents such as EDTA or o-phenanthroline, suggesting that the enzyme may

be a metalloprotein (36). Further studies indicate that the metal participates in the binding of oligodeoxynucleotide primer rather than in the binding of the triphosphate.

E. Distinct DNA polymerases

Numerous attempts have been made to isolate the DNA polymerase activities from animal cells. Animal cells infected with Shope fibroma virus have been found to contain a distinct polymerase activity. Infected rabbit kidney cells and rabbit tumors both contain two polymerase activities which can be distinguished by their behavior on phosphocellulose and reactivity with specific antibody (37, 38); the enzyme reacting with antibody is presumed to be induced by virus infection. A DNA polymerase activity associated with hepatomas has been found which differs from the polymerase of normal liver in its preference for denatured over native templates (39, 40, 41).

A DNA polymerase associated with mitochondria has been found, and has been purified from rat liver mitochondria (42, 43, 44) and yeast mitochondria (45). These mitochondrial polymerases have low specific activities, and differ from the nuclear enzyme with respect to chromatographic properties, Mg^{++} requirement, template preference, solubility, and catalytic parameters. The crude mitochondrial extracts are able to use both native and denatured DNA, but the highly purified mitochondrial polymerase freed from nuclease activity, preferred denatured rather than native DNA.

F. Intracellular location

DNA polymerases in mammalian tissues were originally found to be more readily obtained from soluble supernatant fractions after high-speed centrifugation of disrupted cells, than from nuclei and other intracellular particles (17, 24). This apparent cytoplasmic location of the enzyme was unexpected since DNA synthesis was believed to occur within the nucleus.

Using a technique involving only non-aqueous organic solvents, Keir et al (46) prepared nuclei and cytoplasm from regenerating rat liver; DNA polymerase was then extracted from these preparations with aqueous buffers. Much enzyme activity was found in the nuclear fraction and lower but appreciable amounts in the cytoplasm. Similar results were obtained from non-aqueous nuclei and cytoplasm from rabbit and calf-thymus.

Ca^{++} ions appear to be an important factor in the extraction of DNA polymerase in aqueous media; it is known that they are necessary for isolation of nuclei in a morphologically undamaged state (47). It was found that, when extracted with an aqueous medium containing 2mM Ca^{++} ions, DNA polymerase in rat thymus tissue was evenly distributed between the nuclear and the cytoplasmic fractions (48). Similar distribution of enzyme activity has been found in mouse embryo cells (49).

Loeb et al (50) have recently reported their findings in the early developing sea urchin embryos. Early development of sea urchin embryos is characterized by exponential cell division, accompanied by an exceptionally high level of DNA polymerase

activity *in vitro*. The majority of the polymerase activity was found in the cytoplasm of the egg. As the embryo developed, progressively more polymerase activity was found in the nuclear fraction with a concomitant loss of activity in the cytoplasm. By the time of hatching, 95% of the polymerase activity was recovered in the nuclei. The authors looked for, but could not find, evidence for selective synthesis of DNA polymerase. This indicated that the translocation of polymerase activity could not have resulted from either a breakdown in the cytoplasm and preferential synthesis of the polymerase in the nuclei, or an extreme rapid turnover in the cytoplasm with some transfer to the nuclei. They concluded then that there is a migration of a preformed enzyme from the cytoplasm into the nucleus.

Attempts to show differences between the nuclear and cytoplasmic DNA polymerases have been unsuccessful so far. Weissbach *et al* (51) recently reported the isolation of two separable DNA polymerase activities from the nucleus of HeLa cells, and only one DNA polymerase activity in the cytoplasm. The two nuclear enzymes differ in elution patterns on DEAE-cellulose and phospho-cellulose, molecular weight estimations, optimum Mg^{++} ion concentration, optimum pH, high salt concentration inhibition, and primer activation. One of the nuclear enzymes resembled the cytoplasmic enzyme in all these respects, although the actual relationship between the two enzyme activities remains to be established. The relationship between the two nuclear enzymes is also unclear; they may be distinct enzymes or they may

share some common structural features. An identical pattern of DNA polymerases has been found in the normal human lung diploid line WI-38, which also has two separable activities in the nucleus and only one detectable DNA polymerase activity in the cytoplasm.

The present work

The present investigation is a continuation of the work done by Leung and Zbarsky on rat intestinal mucosa (19, 20). The intestinal tissue was chosen for study because of its high mitotic rate and rapid replacement time for DNA, indicating the possibility of high DNA polymerase activity (52, 53).

Both a replicative and a terminal DNA nucleotidyl-transferase were detected in extracts of nuclei. The replicative enzyme could use either native or denatured DNA as primer, while the terminal enzyme preferred heat-denatured DNA primers. Results of chromatography on DEAE-cellulose and gel-filtration, and sucrose density gradient centrifugation indicated that the DNA polymerases were heterogeneous in nature, with molecular weights between 2.5×10^4 and 3×10^5.

In a study of the intracellular distribution of the DNA polymerases, the amount of enzyme activity was compared in nuclei isolated in the presence or absence of Ca^{++} ions, known to preserve nuclei in an undamaged state. The nuclei isolated in the presence of Ca^{++} ions retained a larger proportion of its protein and corresponding DNA polymerase activity than nuclei isolated in a medium without Ca^{++} ions (54). This indicated that there was some leakage of enzyme from the nucleus to the cytoplasm during extraction. However, when nuclear and cytoplasmic fractions were prepared in non-aqueous solvents from rapidly frozen and lyophilized tissue, enzyme activity was detected in both fractions, although the nuclear fraction

contained a higher total activity than the cytoplasmic fraction (54). These observations were in agreement with the findings in other mammalian systems in that DNA polymerase activity was present in both fractions (55).

The present work involves a partial purification and examination of the properties of the enzyme activity in the cytoplasmic fraction. The ultimate goal of the investigation is to determine whether the cytoplasmic enzyme activity is identical with that found in the nuclei, or whether it is a distinct enzyme. The following properties have been found for the cytoplasmic activity, and a comparison with the properties of the nuclear activity is now awaited.

The cytoplasmic DNA polymerase demonstrates an absolute requirement for Mg^{++} ions and DNA primer. Dithiothreitol appears to be dispensible. About 23% activity remained in the absence of three of the four nucleoside triphosphates. The optimum pH for the enzyme was found to be 7.2 in phosphate buffer, and 8.0 in Tris-acetate buffer. Heated DNA is always preferred to native DNA as primer. The product of the DNA polymerase reaction can be destroyed by DNase, indicating that it is DNA in nature. The enzyme appears fairly stable up to about two weeks. Enzyme activity is stimulated and stabilized when 20% to 35% glycerol or ethylene glycol is added to the enzyme preparation. The crude enzyme has been purified about 42-fold by Sephadex gel-filtration and about 5-fold by DEAE-cellulose chromatography, with the removal of about 78% of the contaminating DNase activity.

Further purification on phospho-cellulose has not been successful, however, possibly due to the strong inhibitory effect of phosphate ions present in the elution gradients applied.

MATERIALS AND METHODS

A. Preparation of crude enzyme extract

Generally four male Wistar rats, weighing about 185 to 200 gms each, were used per experiment. The rats were killed by a blow to the head and were immediately decapitated. The small intestine of each was rapidly removed and its contents flushed out with cold saline (0.9% NaCl). The entire length of the intestine was then everted as described by Perris (56). A 'sausage' was then made by tying up one end of the everted intestine, filling it with saline until the intestinal walls were fully extended, and tying up the other end of the intestine. The sausage was washed by a modification of the method of Perris (56). Swirling was done first in ice-cold saline, twice in Krebs-Ringer phosphate buffer, pH 7.4, containing 6% Dextran, and finally in saline again, for periods of $2\frac{1}{2}$ min in each solution. The washed intestine was slit open, and the mucosa was scraped off by stroking gently with the edge of a glass slide. The scrapings were then homogenized in 9 volumes of TKM buffer (0.05 M Tris, 0.025 M KCl, 0.005 M $MgCl_2.6\ H_2O$, adjusted to pH 7.4 with HCl) containing 0.32 M sucrose. Homogenization was done in a glass Potter-Elvehjem homogenizer with a Teflon-tipped pestle. The pestle was run electrically at a speed of 800 rpm, and five complete passes, each consisting of an upward and a downward stroke, were made. The homogenate was filtered through two layers of nylon, and the filtrate was centrifuged 10 min at 700 x g , 15 min

at 20,000 x g, and 1¼ hrs at 105,000 x g. The final supernatant was saved and stored at -20°C. In some experiments, as will be specified, glycerol was added to the final supernatant to a concentration of 20% or 35% before storage.

B. Column chromatography on DEAE-cellulose

About 20 gms of DEAE-cellulose (diethylaminoethyl-cellulose, Whatman DE 22) were washed by decantation first with 0.5 N HCl and then with 0.5 N NaOH. The slurry was then washed several times in TKM buffer. Fines were removed using the same buffer. The slurry was de-aerated under vacuum in a dessicator, and was packed under gravity at 4°C, into a 1.8 cm diameter column up to a height of 20 cm. The column was washed before use with TKM buffer containing 20% (v/v) ethylene glycol. Baril et al (57) have reported that ethylene glycol stabilized the activity of crude and purified rat liver DNA polymerases for at least 3 weeks at 4°C and at least 6 months at -20°C.

About 30 mg of protein were loaded onto the column, after which the column was washed with 100 mls of TKM buffer containing 1 mM dithiothreitol and 20% ethylene glycol. A stepwise gradient resulting from increasing concentrations of KCl was applied to the column. Fifty mls each of TKM buffer containing 1 mM dithiothreitol and 20% ethylene glycol and the following concentrations of KCl were used: 0.10 M, 0.25 M, and 0.50 M. Three ml fractions were collected by gravity. The gradient profile was followed by measuring the conductivity of each fraction collected. The

protein elution pattern was examined by measuring the absorbance of each fraction at 280 nm. The DNA polymerase activity of each fraction was determined by the usual enzyme assay.

In some experiments, a continuous gradient was used. A DEAE-cellulose column was washed and equilibrated with 0.005 M Tris-phosphate buffer, pH 8.0. The gradient consisted of 300 mls of 0.05 M phosphate buffer, pH 8.0, containing 1 M KCl and 2 mM dithiothreitol, into 300 mls of 0.005 M Tris-phosphate buffer, pH 8.0. Five ml fractions were collected by gravity and each fraction was examined in the manner already described.

C. Column chromatography on phosphocellulose

About 6 gms of phosphocellulose (Bio-Rad cation exchange cellulose, Cellex-P) were washed with glass-distilled water to remove fines. The slurry was then equilibrated with 0.05 M potassium phosphate buffer, pH 6.8, containing 1 mM dithiothreitol and 20% ethylene glycol, by washing six to seven times with the buffer. The slurry was de-aerated under vacuum in a dessicator, and was packed into a 1.2 cm diameter column up to a height of 18 cm. The column was washed with the same buffer before use.

About 5 mg of protein were loaded onto the column, after which the column was washed with 75 mls of 0.05 M potassium phosphate buffer, pH 6.8, containing 1 mM dithiothreitol and 20% ethylene glycol. A stepwise gradient resulting from increasing concentrations of potassium phosphate was applied onto the column. Fifty mls each of the following concentrations of potassium

phosphate buffer, pH 6.8, containing dithiothreitol and ethylene glycol were used: 0.1 M, 0.2 M, and 0.5 M. Three ml fractions were collected by gravity and each fraction was examined as previously described.

Another procedure has been used in which 17.5 mg of protein were allowed to stir gently at 4°C in a slurry of phosphocellulose previously washed and equilibrated in 0.05 M phosphate buffer, pH 6.8, containing 1 mM dithiothreitol and 20% ethylene glycol. The entire slurry, with the adsorbed protein, was packed into a 1.2 cm diameter column, and was washed with 60 mls of the buffer. A linear gradient resulting from an increasing concentration of phosphate was applied: one hundred mls of 0.50 M phosphate buffer, pH 6.8 was run into an equal volume of 0.05 M phosphate buffer, pH 6.8, both buffer systems containing 1 mM dithiothreitol and 20% ethylene glycol. The elution was followed by a final 60 mls of the 0.50 M phosphate buffer. Three ml fractions were collected, and each fraction was examined as previously described. Every third fraction was then dialyzed overnight at 4°C against TKM buffer containing 1 mM dithiothreitol and 20% ethylene glycol before being assayed for DNA polymerase activity.

D. Gel-filtration on Sephadex G-150

Forty gms of Sephadex G-150 (Pharmacia) were stirred into 2 l. of either TKM buffer or 0.1 M phosphate buffer, pH 7.2, and were allowed to swell, with occasional stirring, for 48 hrs at 4°C. The slurry was de-aerated under vacuum, and was poured

into a 100 cm column (2.5 cm diameter) according to the instructions from Pharmacia. The prepared column was washed by upward flow elution for 24 hrs with the original buffer containing 1 mM dithiothreitol and 20% ethylene glycol. Fractions were collected by upward flow elution, with a pressure head no greater than 30 cm.

Two mls each of a solution of Blue Dextran 2000 (Pharmacia) and 3 M NaCl were run into the column for the determination of void volume and total volume respectively. The column was calibrated with the standard proteins chymotrypsinogen A (5 mg), γ-globulin (5 mg), and bovine serum albumin (10 mg). In some experiments hemoglobin (4 mg) was used instead of bovine serum albumin.

To the calibrated column, a 2.0 ml sample of the crude enzyme preparation containing 10 to 42 mg protein was applied, followed by the buffer system used, containing 1 mM dithiothreitol and 20% ethylene glycol. Fractions of 2.0 or 3.0 mls were collected. The optical density at 280 nm and the DNA polymerase activity of each fraction was determined.

E. Enzyme assays

1. DNA polymerase assay

The activity of DNA polymerase was measured by the incorporation of a radioactively labeled deoxyribonucleoside triphosphate into an acid-insoluble product. The assay system used was similar to that described by Chiu and Sung (58).

The incubation mixture contained, in a total volume of 0.40 mls:

potassium phosphate buffer, pH 7.2	20 μmoles
$MgCl_2$	2 μmoles
dithiothreitol	2 μmoles
dATP, dCTP, dGTP, TTP	16 nmoles of each
[^{3}H]-TTP (Schwarz-Mann, 17 C/mmole)	115 pmoles (2 μC)
calf-thymus DNA (Armour Pharmaceutical Co.), heat-denatured	40 μg
enzyme preparation	0.1 to 0.3 mg protein

In some experiments, 20 μmoles of TKM buffer was used in place of the 20 μmoles of potassium phosphate buffer, pH 7.2. In the earlier experiments, only 0.5 μC of [^{3}H]-TTP was used per assay. The amount of [^{3}H]-TTP was later increased to 2 μC per incubation mixture, in order to increase the number of counts per minute observed. Heat-denatured DNA was prepared by heating the DNA solution at 100°C for 10 min and then cooling it in an ice-bath. In the preliminary experiments, the assay mixtures were incubated in a water-bath at 37°C for 60 min. It was later found that the reaction was linear with time for at least 30 min. The incubation time for the assay mixture was then cut back to 30 min.

After the tubes were incubated, they were rapidly cooled to 0°C in an ice-bath. To each tube, 1 mg of bovine serum albumin was added and mixed, followed by the addition of 2.5 ml of 10% trichloroacetic acid (TCA) to precipitate the DNA and protein.

After each tube was chilled for 10 min in ice, it was centrifuged for 5 min at top speed in a clinical centrifuge. The supernatant was decanted, and the pellet was washed by thoroughly resuspending it in 5.0 ml of 5% TCA. The suspension was re-centrifuged, and the washing procedure was repeated twice. The final pellet was dissolved in 0.2 ml of a 1 M solution of hyamine hydroxylate in ethanol, and was then mixed with 5.0 ml of scintillation solution containing 15 gm of 2,5-diphenylaxazole, 150 mg of 1,4-bis-(5-phenyloxazolyl-2)benzene, and 240 gm of naphthalene in 3 l. of a 1:1:1 solution of toluene, dioxane, and 95% ethanol. Radioactivity was then measured in a Packard Tri-Carb liquid scintillation spectrometer, model 314 AX. One unit of DNA polymerase activity was defined as the amount of enzyme required to convert 1 pmole of [^{3}H]-TTP into the acid-insoluble product in 30 min under the assay conditions described.

2. Terminal deoxyribonucleotidyltransferase assay

The procedure used was a modification of that reported by Krakow et al (31). The incubation mixture was identical with that for the DNA polymerase assay except for the omission of dATP, dCTP, and dGTP, and the addition of 2.5 μmoles of cysteine. The procedures of TCA precipitation, washing, and measuring of radioactivity were the same as those for the polymerase assay. One unit of terminal transferase activity was defined as the enzyme required to convert 1 pmole of [^{3}H]-TTP into the acid-insoluble product in 30 min under the assay conditions described.

3. DNase I assay

The diffusion slide assay developed by Jarvis and Lawrence (59) was used for the quantitative determination of DNase I activity. Concentrations of DNase down to 0.005 μg/ml could be measured with high reproducibility by this method.

A hot solution of agar (2% w/v) containing 2 mg/ml calf thymus DNA (Armour Pharmaceutical Co.) was mixed with an equal volume of hot 0.1 M Tris-HCl buffer, pH 7.8. $MnCl_2$ was added to the agar solution to a final concentration of 0.01 M. One ml of this hot mixture was spread on a microscope slide over an area of 2 in. by 1 in., which was outlined by means of cellulose tape. A hole 2.7 mm in diameter was bored with a thin steel tube, and 0.004 ml of the enzyme preparation was added to the well. The slides were placed in a plastic box containing moist blotting paper and incubated at 37°C for 20 hrs. The slides were then dipped in 1 N HCl for 15 secs, washed with water, and the diameter of each of the zones of clearing was read.

DNase I from bovine pancreas (Worthington Biochemical Corporation) containing 2,300 Kunitz units per mg was assayed by the method of Jarvis and Lawrence and the square of the radius of the zone of clearing was calculated. In the present work, DNase activities were assayed by the method of Jarvis and Lawrence and were then converted to Kunitz units. The assay method developed by Kunitz was based upon the increase in UV absorption at 260 nm observed during the course of depolymerization of DNA by DNase. One Kunitz unit is that activity which causes an increase in absor-

bancy of 0.001 per min per ml under the assay conditions at 25°C (67).

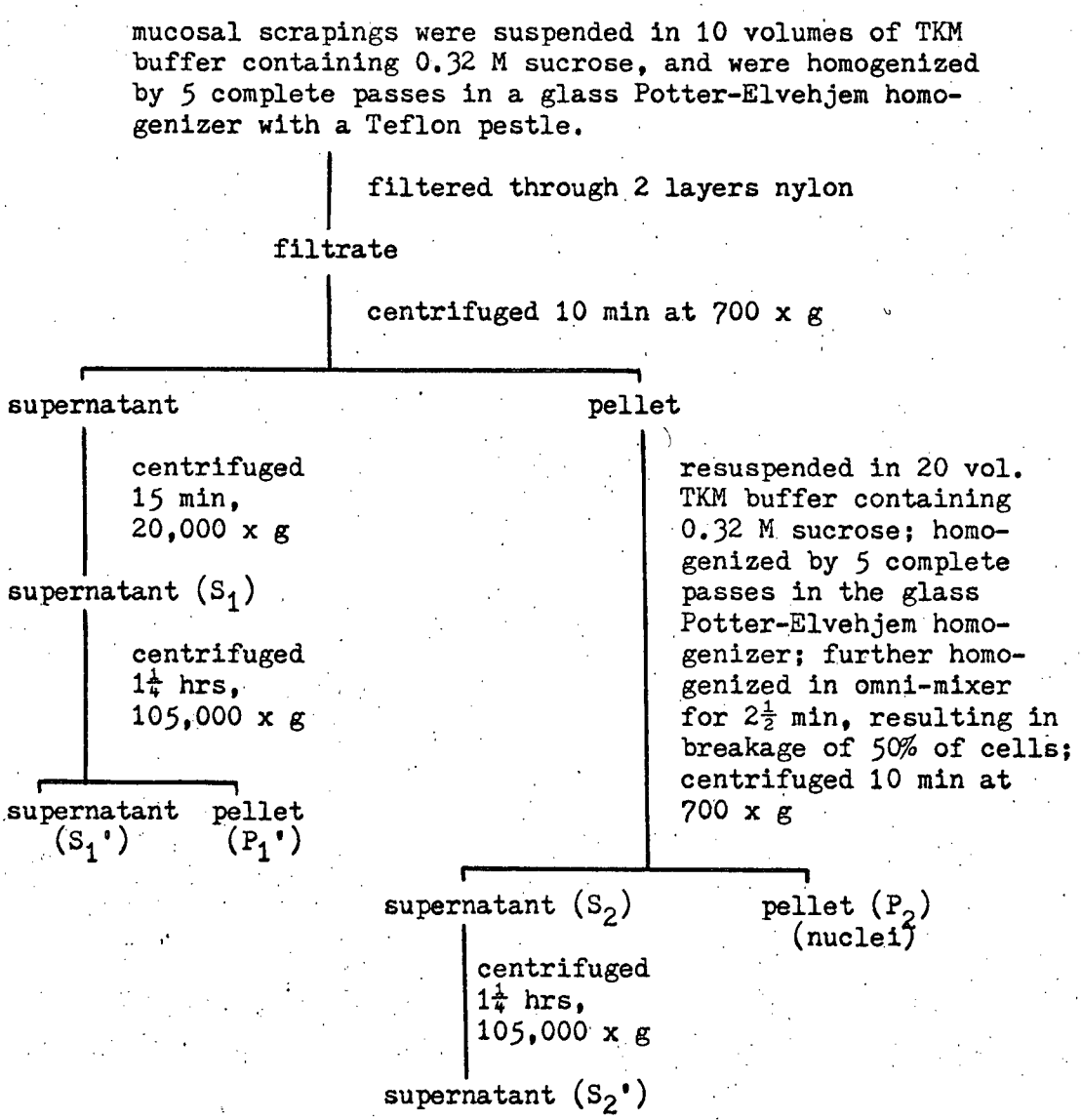
4. Protein determination

Protein was estimated according to the method of Lowry et al (60) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

A. Preparation of crude enzyme

In a search for a cytoplasmic, or extra-nuclear, DNA polymerase, the following study was made.



For studies of nuclear preparations, pellet P_2 could be further homogenized using a Servall Omni-mixer, and the soluble proteins could then be extracted.

As the present study involves the cytoplasmic preparations, the supernatants S_1, S_2, and S_2' were each assayed for DNA polymerase activity. The results are tabulated below.

Table I. DNA polymerase activity of various supernatant fractions from rat intestinal mucosal cells.

Enzyme preparation	Total activity (units x 10^3)	Total protein (mg)	Specific activity (units x 10^3 per mg protein)
S_1	858	149	5.8
S_1'	8286	132	62.8
S_2	710	320	2.2
S_2'	1588	190	8.4

Significant DNA polymerase activity was found in the supernatants S_1 and S_2. However, when S_1 and S_2 were centrifuged at high-speed, the total activities were increased approximately 10-fold and 2-fold respectively (S_1' and S_2'). Corresponding increases in specific activities were also observed. The supernatant S_1' was of particular interest because of the high total and specific activities associated with it. Other workers (61) have made similar observations also with rat intestinal mucosa. They have found that

on further centrifuging the S_1' fraction at 105,000 x g for 24 hrs, there was a further increase in the total DNA polymerase activity in the supernatant obtained. It appears then that S_1 may have contained a certain factor or factors which interfered with DNA polymerase activity. If this inhibitor was sedimentable by high-speed centrifugation, an increase in total DNA polymerase activity would be observed in the resulting supernatant S_1'. This possibility was strengthened by the results obtained from the following experiment.

Supernatant S_1 was prepared as before. S_1 was then centrifuged at 105,000 x g for 1¼ hrs, as before, and supernatant S_1' and pellet P_1' were obtained. P_1' was resuspended in TKM buffer containing 0.32 M sucrose. Protein determination of each of the fractions S_1, S_1', and P_1' indicated that 18% of the protein present in S_1 was pelleted, and that 82% remained in the supernatant S_1'. Each preparation was then assayed for DNA polymerase activity (Table II). S_1 contained a low enzyme activity. The pellet P_1' was found to contain a total activity very much lower than that of S_1. A large increase in both total and specific activities was again obtained in S_1'. When the pellet P_1' was recombined with the supernatant S_1', the DNA polymerase activity of the mixture was drastically reduced to a level not much higher than that of S_1 (Table II). These observations suggested that a strong inhibitor of DNA polymerase was present in the supernatant S_1, and was separated from the enzyme by sedimentation into P_1', thus allowing an increase in DNA polymerase activity detected in

S_1'. When the inhibitor was added back to the enzyme fraction, enzyme activity was immediately inhibited again.

Table II. DNA polymerase activities from recombination experiments with supernatant and pellet fractions from rat intestinal mucosal cells.

Enzyme preparation	Total activity (units x 10^3)	Total protein (mg)	Specific activity (units x 10^3 per mg protein)
S_1	2,343	93.1	25
S_1'	88,350	77.5	1140
P_1'	458	16.9	27
S_1' + P_1'	2,922		

It was possible that the observed inhibitory effect was due to interference by DNase I activity present in the preparations studied. DNase activity may have two effects on DNA polymerase assays. Firstly, DNase activity may produce nicks in the DNA primer strands, thus causing an activation of DNA polymerase activity. Secondly, DNase activity may cause the degradation of the newly-synthesized DNA product of the polymerase reaction, thus interfering with the DNA polymerase assay. Leung (54) had reported that crude homogenate preparations of rat intestinal mucosal cells contained a high DNase I activity. He found that when this crude homogenate was centrifuged at high-speed, there was a decrease in

the level of DNase I activity in the supernatant fraction. Leung also observed that fractions which contained a high DNase I activity generally showed a low DNA polymerase activity, indicating the possible interference of DNase I with DNA polymerase assays (54).

Each of the fractions S_1, S_1', and P_1' were therefore assayed for DNase I activity. The results are shown in Table III. Of the total original DNase I activity present in S_1, 77% was recovered in the supernatant S_1', and only 14% was sedimented with the pellet P_1'. These results do not fit in with the proposed sedimentation of the inhibitor with pellet P_1'. Moreover, as will be discussed in a later section, the levels of DNase I activity detected in any of the fractions were not sufficient to cause large effects on the DNA polymerase activities present (Table III). Thus DNase I does not appear to be the cause of the inhibitory effects observed in the DNA polymerase preparations.

Table III. DNase I activities in various supernatant and pellet fractions from rat intestinal mucosal cells.

Enzyme preparation	Total DNase I activity (Kunitz units)
S_1	6,646
S_1'	5,108
P_1'	923

Due to limitations of time, the nature of the inhibitory factor was not further investigated. In view of the high total and specific DNA polymerase activities obtainable with the S_1' supernatant, this preparation was chosen for further study. The routine procedure for preparing this fraction is described in the preceding section. This S_1' supernatant represents the first wash of presumably intact cells. Further experiments were carried out to determine whether this enzyme activity is of nuclear origin, or whether it has properties differing from the nuclear enzyme activity, indicating a possible cytoplasmic origin.

B. Partial purification of crude enzyme

Ammonium sulfate precipitation has been used by Bollum (17), by Shepherd and Keir (62), and by Furlong (63) to fractionate DNA polymerase from tissue extracts. This procedure has also been used by Leung (54) in the purification of the enzyme from the small intestinal mucosa of the rat. The DNA polymerase activity was recovered in the 60% ammonium sulfate fraction, but there was a loss of up to 60% of the original enzymatic activity. This result may have been due to the detrimental effects of high salt concentration on the structural conformation of the mammalian DNA polymerases as described by Keir (21). In view of these observations, ammomium sulfate fractionation was not used in the present work. The methods of purification used included DEAE-cellulose chromatography, phosphocellulose chromatography, and Sephadex gel-filtration.

1. Chromatography on DEAE-cellulose

The high-speed supernatant S_1', which will hereafter be referred to as the crude enzyme preparation, was chromatographed on a column of DEAE-cellulose. The column was eluted with a linear gradient in a Tris-HCl buffer system as described previously, and a typical elution profile obtained is shown in Fig. 4. A single protein peak containing 42% of the total protein was eluted before the gradient was applied. This protein peak, which was apparently not adsorbed to the anion-exchanger, contained about 92% of the total enzyme activity. The remaining 4% of the activity was obtained as a small peak occurring immediately after the start of the gradient. The large protein peak which was eluted with the gradient was completely devoid of enzyme activity.

Table IV. Chromatography of crude enzyme on DEAE-cellulose. Elution with Tris-HCl buffer system, with linear gradient of KCl.

	Protein (mg)	Total activity (units x 10^3)	Yield (%)	Specific activity of peak fraction (units x 10^3 per mg protein)	Purification
Applied in crude enzyme	48.0	12,696	100.0	282	1.0
Enzyme peak I	20.9	11,638	91.7	1,239	4.4
Enzyme peak II	0.5	529	4.2	817	2.9
Total	21.4	12,167	95.9		

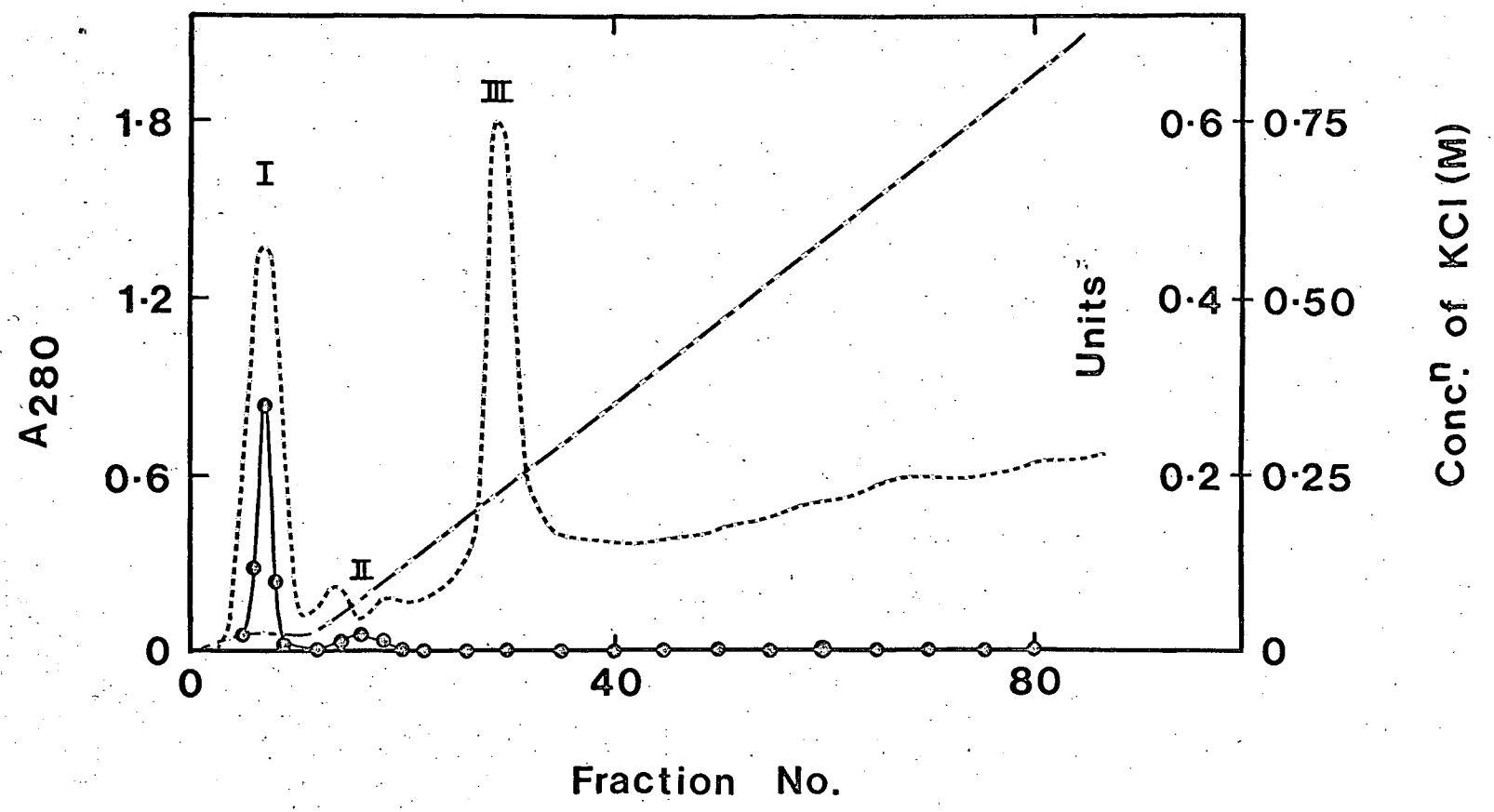


Fig. 4. Chromatography of crude DNA polymerase from rat intestinal mucosa on DEAE-cellulose. Elution with TKM buffer containing 1 mM dithiothreitol and 20% ethylene glycol, with linear gradient of KCl. Dotted line represents absorption at 280 nm; solid line represents units of DNA polymerase activity; alternate dots and dashes represents elution gradient of KCl.

Table IV refers to the enzyme peaks labeled in Fig. 4, and indicates that the purification of the enzyme activity in peak I is between 4- and 5-fold. These protein and activity profiles are consistently reproducible.

When the crude enzyme preparation was chromatographed on DEAE-cellulose using a stepwise gradient in a Tris-HCl buffer system, as described previously, a better protein separation was obtained. A large protein peak, with an overlapping smaller peak, was again obtained before the start of the gradient (Fig. 5).

Table V. Chromatography of crude enzyme on DEAE-cellulose. Elution with Tris-HCl buffer system, with stepwise gradient of KCl.

	Protein	Total activity (units x 10^3)	Yield (%)	Specific activity of peak fraction (units x 10^3 per mg protein)	Purification
Applied in crude crude enzyme	31.1	37,800	100.0	1,214	1.0
Enzyme peak I	14.0	30,186	79.9	3,367	2.8
Enzyme peak II	1.5	1,803	4.8	1,880	1.6
Total	15.5	31,989	84.7		

Several major or minor protein peaks were obtained with each stepwise increase in the gradient. About 5% of the enzyme activity was again detected at the start of the gradient. The other protein

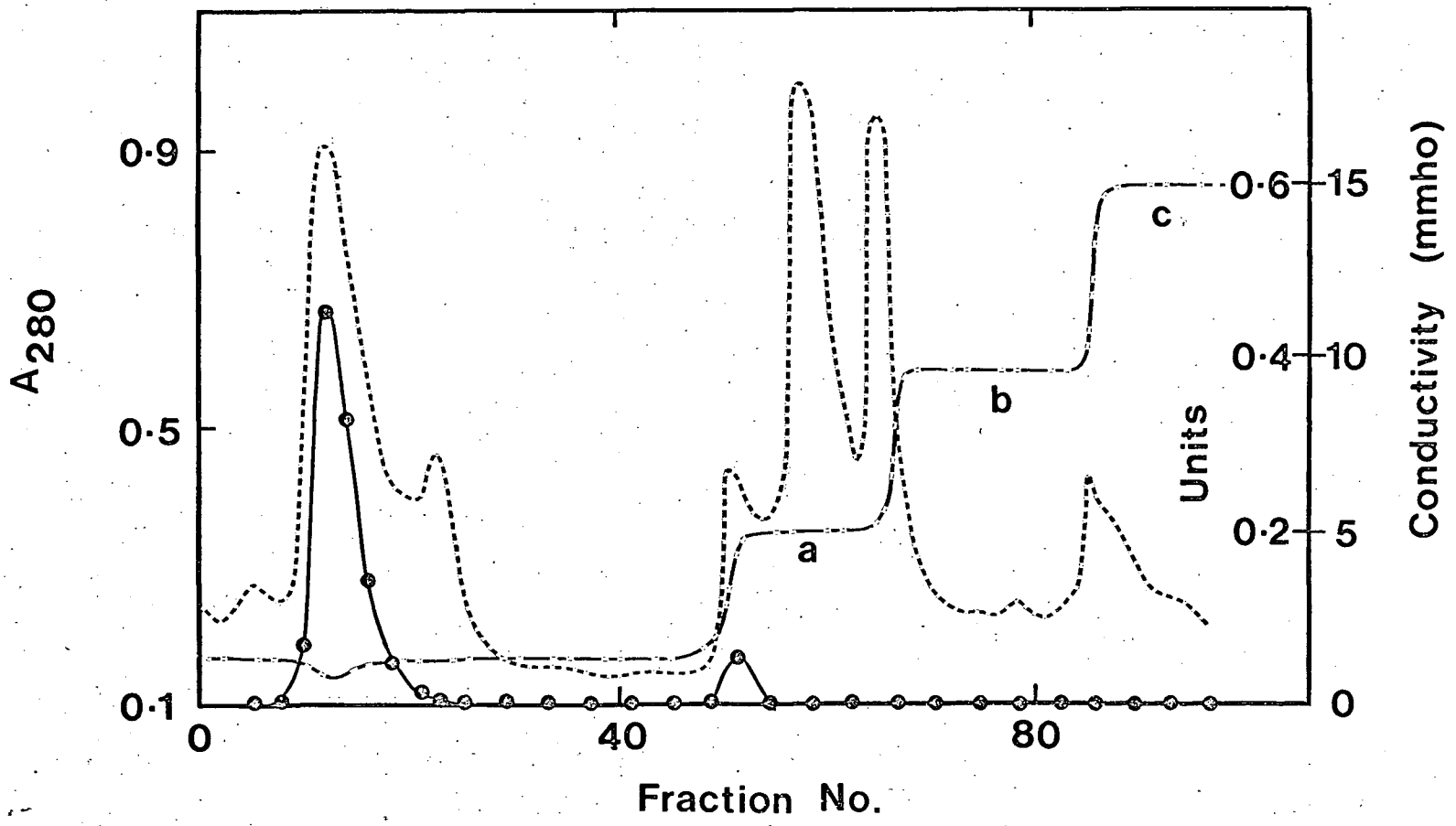


Fig. 5. Chromatography of crude DNA polymerase from rat intestinal mucosa on DEAE-cellulose. Elution with TKM buffer containing 1 mM dithiothreitol and 20% ethylene glycol, with stepwise gradient of KCl. Dotted line represents absorption at 280 nm; solid line represents units of DNA polymerase activity; alternate dots and dashes represents elution gradient of KCl (a: 0.10 M, b: 0.25 M, c: 0.50 M).

peaks eluted with the gradient were devoid of detectable enzyme activity. As indicated in Table V, the purification of DNA polymerase activity in peak I was about 3-fold.

Peak I from DEAE-cellulose chromatography of the crude enzyme was assayed for DNase I and terminal transferase activities, and the results were compared with those obtained with the crude enzyme preparation (Table VI).

Table VI. DNase and terminal transferase activities from crude and partially purified enzyme preparations.

	Total activity	
	DNase I (Kunitz units)	Terminal transferase (units x 10^3)
Crude enzyme	5,814	3,312
Peak I enzyme	1,261	1,944
Yield	21.7%	58.7%

When the crude enzyme preparation was chromatographed on DEAE-cellulose, the DNA polymerase peak collected in the peak of unadsorbed proteins contained only 21.7% of the total DNase activity applied onto the column. The terminal transferase activity in the DNA polymerase peak was 58.7% of that in the crude enzyme preparation applied.

DEAE-cellulose chromatography therefore provided a

simple and speedy procedure for partial purification of DNA polymerase. The bulk of the polymerase activity was washed through the column, leaving over 50% of the protein and about 78% of the DNase activity adsorbed onto the DEAE-cellulose. In the routine partial purification of the crude enzyme preparation, the S_1' supernatant was applied onto a DEAE-cellulose column and was washed through with buffer, no gradient being necessary. The optical density at 280 nm was determined for each fraction collected. Those fractions which constituted the first protein peak contained between 80% and 92% of the DNA polymerase activity, and these fractions were combined for further use.

In a study of rat liver DNA polymerases, Baril and co-workers (57) chromatographed the ammonium sulfate fractions of nuclei, mitochondria, ribosomes, and smooth membranes on DEAE-cellulose. The elution profiles of the enzymes from purified nuclei and ribosomes appeared quite similar. Neither enzyme was bound to DEAE-cellulose and all of the DNA polymerase activity appeared in the column wash. The column washes also contained some nuclease and terminal transferase activity. The DNA polymerases from both nuclei and ribosomes preferred native DNA as primer. Chromatography of the mitochondrial fractions produced multiple peaks of very low polymerase activity, most of which was eluted with 0.1 M and 0.25 M KCl. The elution pattern of the smooth membrane fraction was similar to that of the mitochondrial fraction, but the polymerase activity was 15 to 20 times higher than that in the latter. The elution profile obtained in the present work

indicates that the enzyme in the high-speed supernatant from rat intestinal mucosa was similar to those of the nuclear and ribosomal fractions from rat liver.

Previous studies have been made by Leung and Zbarsky (19, 20) on the soluble fractions extracted from the nuclei of rat intestinal mucosa. Chromatography of the nuclear extracts on DEAE-cellulose produced three peaks of DNA polymerase activity. The first enzyme activity was found to be associated with a peak of unadsorbed protein and nucleic acid material. The two other enzyme peaks were eluted with approximately 0.1 and 0.2 M KCl solutions. Re-chromatography of the first unadsorbed enzyme peak allowed the detection of a distinct peak of 'terminal' enzyme activity. In contrast to the three separable DNA polymerase activities found by Leung and Zbarsky (19, 20) in the nuclear preparations, the cytoplasmic preparations contained only a major and a minor enzyme activity peak. This major DNA polymerase peak from the cytoplasmic preparation was similar in chromatographic properties to one of the DNA polymerase peaks from the nuclear preparations, i.e., it was not adsorbed onto DEAE-cellulose. A similar pattern of distribution of DNA polymerases has been reported by Weissbach et al (51) in HeLa cells and in normal human lung diploid line WI-38, as has been discussed earlier. In each case, no separable nuclear DNA polymerase activities and only one cytoplasmic DNA polymerase activity were isolated. The two nuclear enzymes differed in chromatographic and other properties but the cytoplasmic enzyme resembled one of the nuclear enzymes in

all respects. The actual relationship between the three activities remains unclear.

2. Column chromatography on phosphocellulose

Since the DNA polymerase activity under study was not adsorbed onto the anion-exchanger DEAE-cellulose, it was hoped that it might be adsorbed onto a cation-exchanger, such as phosphocellulose.

Fractions from the enzyme peak (peak I) from the DEAE-cellulose column were combined and the solution was dialyzed overnight at 4°C against 0.05M phosphate buffer, pH 6.8, containing 1 mM dithiothreitol and 20% ethylene glycol. The dialysate was applied to a phosphocellulose column equilibrated with the same buffer. The column was eluted with a stepwise gradient as described in the preceding section. A sharp protein peak was obtained with each change in the gradient, giving a total of 4 peaks. Each peak was assayed for DNA polymerase, but no enzyme activity could be detected. The final protein peak, that eluted with the 0.5 M phosphate buffer, showed indications of a slight amount of activity. Fractions from this peak were therefore combined and the solution was dialyzed against TKM buffer containing 1 mM dithiothreitol and 20% ethylene glycol. The dialysate was concentrated by further dialysis against ice-cold sucrose, and was re-assayed. A low level of enzyme activity was present, but the total activity yield was less than 5%.

Another approach to phosphocellulose chromatography was

used. Fractions from the DNA polymerase peak (peakI) from the DEAE-cellulose column were combined and the solution was again dialyzed against TKM buffer containing 1 mM dithiothreitol and 20% ethylene glycol. The dialysate was added to a prepared slurry of phospho-cellulose and the mixture was stirred overnight at 4°C. The slurry with the adsorbed protein was packed into a column (1.2 cm diameter, 18 cm height) which was then eluted with a continuous gradient as previously described. The elution profile indicated that some protein did adhere to the column, but no sharp distinct peak was eluted. Every third fraction was dialyzed overnight against TKM buffer containing 1 mM dithiothreitol and 20% ethylene glycol, and was then assayed for DNA polymerase activity. No enzyme activity could be detected in any of the fractions.

The effect of phosphate ion concentration on DNA polymerase activity was later examined. It was found that DNA polymerase activity was drastically inhibited at phosphate concentrations of 0.20 M and above. At 0.10 M phosphate, the enzyme activity was reduced to 27% of that at 0.05 M phosphate. Since the phosphocellulose columns were run with gradients from 0.10 M to 0.50 M phosphate, it is not surprising that no enzyme activity could be detected in the fractions. Dialysis of the fractions against TKM buffer seems to be ineffective for the recovery of enzyme activity.

The ethylene glycol was added to the buffer systems to prevent deterioration of enzyme activity. It does not appear to have any detrimental effects on the DNA polymerase activities since DEAE-cellulose columns eluted in the presence of 20% ethylene glycol

allowed almost total recovery of enzyme activity.

In their work with rat liver DNA polymerases, Baril et al (57) re-chromatographed the enzyme peaks from DEAE-cellulose chromatography onto phosphocellulose columns. The columns were eluted by stepwise gradients of potassium phosphate buffer in concentrations of 0.1 to 0.5 M, containing 1 mM dithiothreitol and 20% ethylene glycol. The collected fractions were dialyzed overnight against TKM buffer containing 20% ethylene glycol before assaying. The polymerase activity from the nuclear and ribosomal fractions eluted at 0.5 M and that of the smooth membranes at 0.2 M phosphate concentration. In the present work, the slight enzyme activity detected at 0.5 M phosphate concentration again indicates a similarity to the nuclear and ribosomal enzymes from rat liver.

3. Gel-filtration on Sephadex G-150

Molecular-sieve chromatography, or 'gel-filtration' was used for the purification of DNA polymerase and for the estimation of its molecular weight. Sephadex G-150 columns were prepared and calibrated as described in the preceding section. A typical calibration profile is shown in Fig. 6.

When crude enzyme preparation was chromatographed on the column as previously described, elution with 0.1 M phosphate buffer gave the elution profile shown in Fig. 7. However, no DNA polymerase activity could be detected throughout the eluate. This result may be attributed to the presence of phosphate ions in 0.1 M concentration, which was later found to have a strong inhibitory effect on

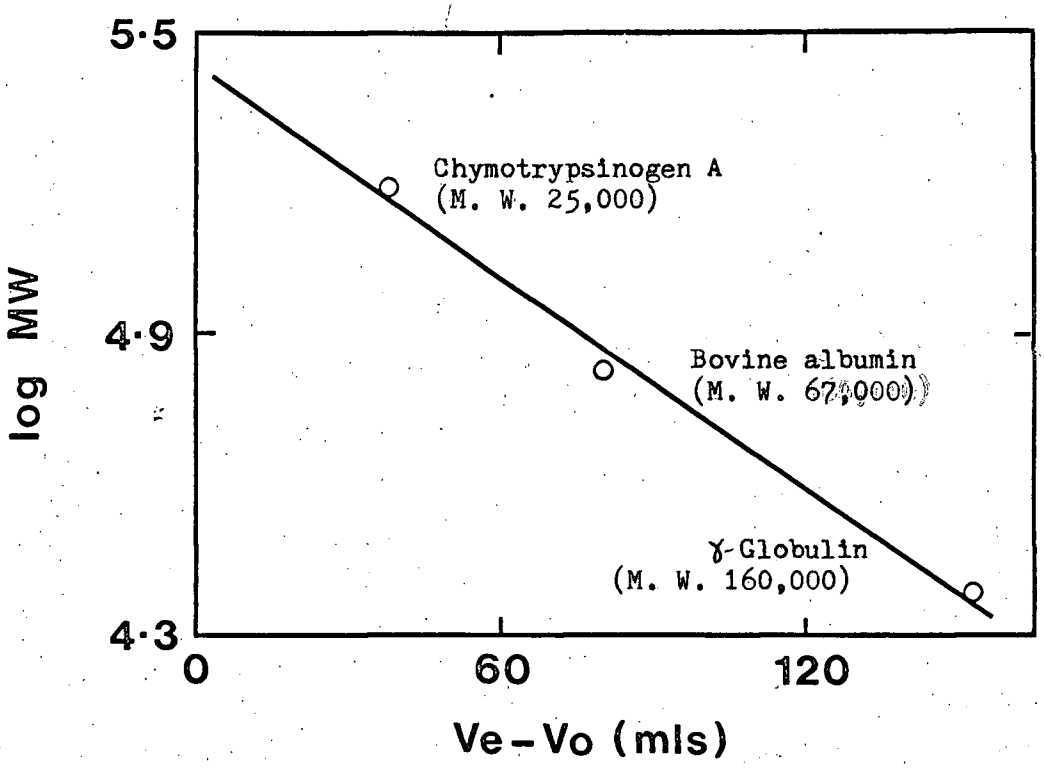


Fig. 6. Standard curve for the estimation of the molecular weights of protein sample on the basis of their elution volumes from gel-filtration on Sephadex G-150. (V_e = elution volume of sample; V_o = void volume of column.)

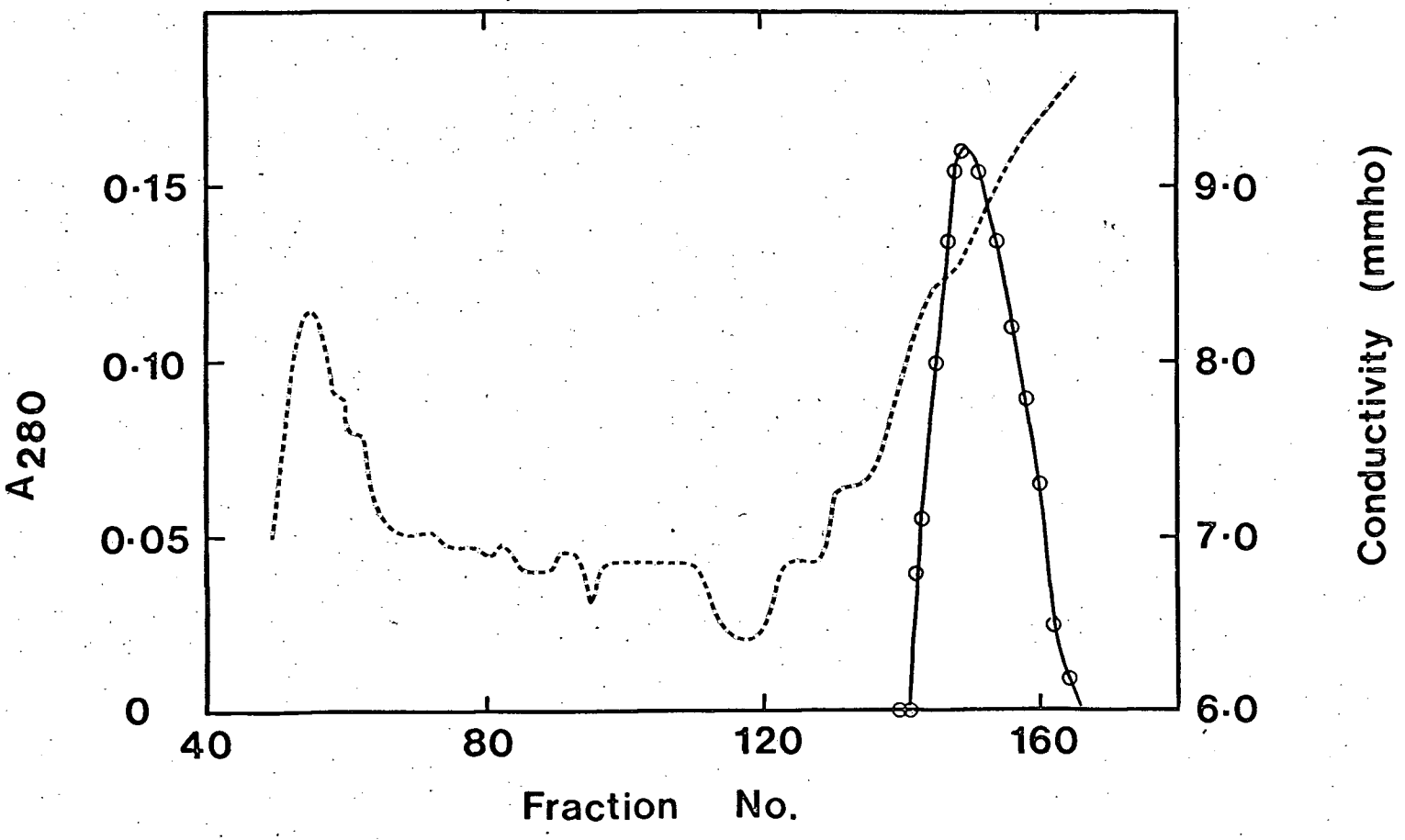


Fig. 7. Gel-filtration of crude DNA polymerase from rat intestinal mucosa on Sephadex G-150. Elution with 0.1 M phosphate buffer, pH 7.2, containing 1 mM dithiothreitol and 20% ethylene glysol. Dotted line represents absorption at 280 nm; solid line represents elution of NaCl, as measured by conductivity; The elution volume of NaCl was taken to be the void volume of the column.

the enzyme activity.

The experiment was therefore repeated in which the column was equilibrated and eluted with TKM buffer instead of phosphate buffer. In addition, the crude enzyme preparation was concentrated 4- to 5-fold against polyethylene glycol before loading onto the column. This concentration procedure led to a 55% decrease in specific activity. The elution profile obtained is shown in Fig. 8. A single DNA polymerase peak was observed (elution volume V_e 90.65 ml). The same column was calibrated using the markers -globulin (M.W. 160,000; V_e 78.75 mls), hemoglobin (M.W. 65,000; V_e 101.25 mls), and chymotrypsinogen-A (M.W. 25,000; V_e 111.25 mls). The DNA polymerase detected was thus estimated to have a molecular weight of 101,000. Previous investigators (19, 20) have obtained multiple peaks of DNA polymerase activity from Sephadex gel-filtration of nuclear preparations of rat intestinal mucosal extracts, and have obtained molecular weight values from 25,000 to 300,000. Bollum et al (64), using both a Sephadex G-100 and G-200 column, estimated the molecular sizes of the calf thymus enzymes to be 110,000 for the replicative and 37,000 for the terminal DNA nucleotidyl transferase.

The specific activity of the DNA polymerase in the peak fraction was 42-times greater than that of the original crude extract loaded onto the column (Table VII). The yield of total activity recovered from the column was over 12 times that loaded onto the column. The cause of this large increase in total activity is unclear. An obvious possibility is the removal of a strong inhibitory factor which differed in size from the DNA polymerase. As has

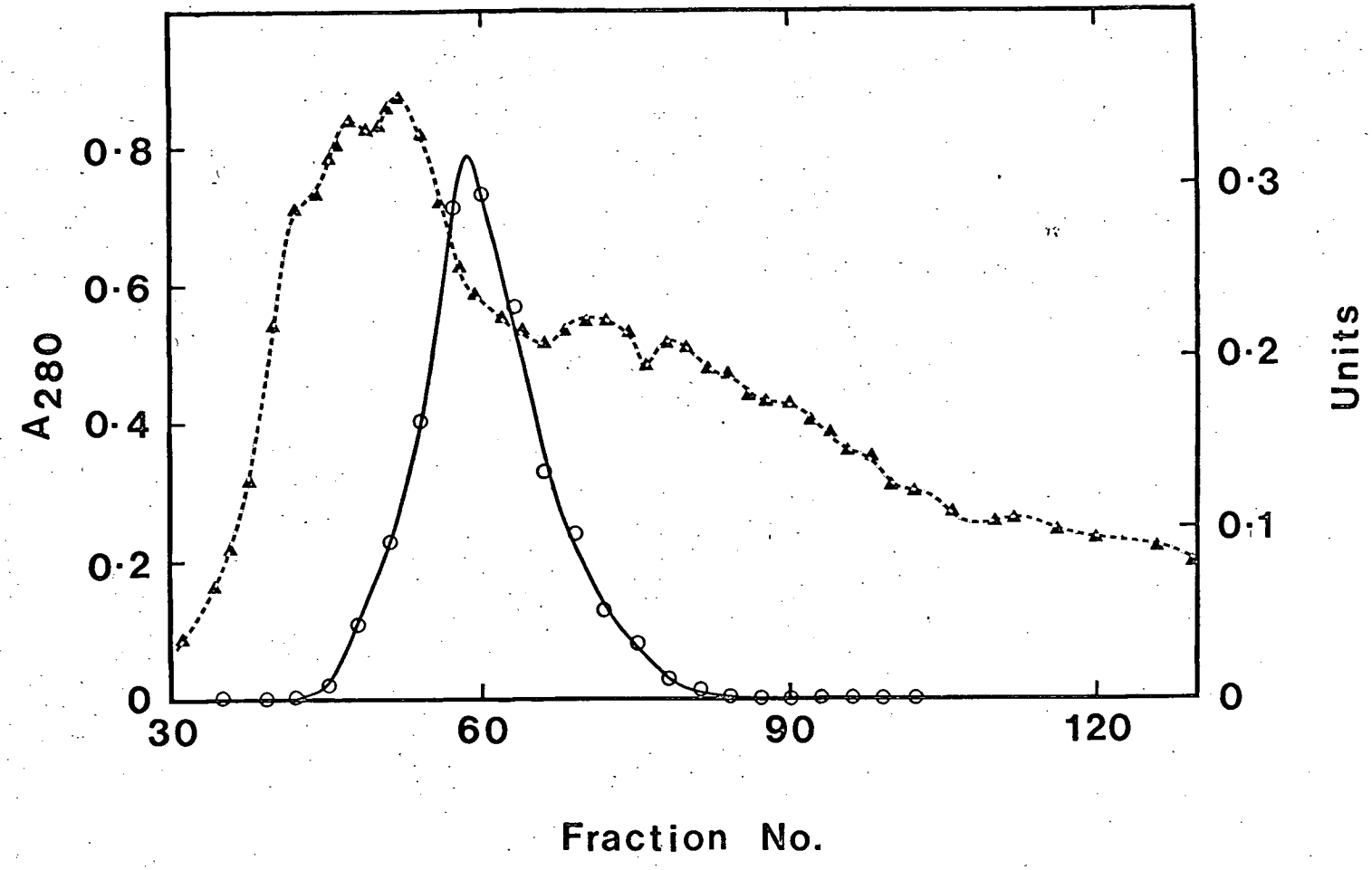


Fig. 8. Gel-filtration of crude DNA polymerase from rat intestinal mucosa on Sephadex G-150. Elution with TKM buffer containing 1 mM dithiothreitol and 20% ethylene glycol; Dotted line represents absorption at 280 nm; solid line represents units of DNA polymerase activity.

been described earlier, the crude enzyme homogenate did contain a strong inhibitory factor which was sedimentable by high-speed centrifugation. This indicated that the inhibitor differed in size from the DNA polymerase. It is possible that a portion of the inhibitory

Table VII. Gel-filtration of crude enzyme preparation on Sephadex G-150. Elution with TKM buffer system.

Enzyme fraction	Total DNA polymerase activity (units x 10^3)	Yield	Specific DNA polymerase activity (units x 10^3 per mg protein)	Purification
Crude enzyme applied	4,730		112	
Peak enzyme eluted	57,680	12-fold	4,719	42-fold

factor did not differ sufficiently from the polymerase to be pelleted, but did differ sufficiently to be separated from the polymerase by Sephadex gel-filtration. Removal of the strong inhibitor would result in a large increase in both total and specific DNA polymerase activities.

C. General properties of the enzyme

1. Requirements

The requirements for the enzymatic reaction catalyzed by DNA polymerase were studied. 'Partially purified' DNA polymerase,

hereafter referring to the enzyme peak (peak I) from DEAE-cellulose chromatography, was dialyzed against 0.05 M Tris-HCl buffer, pH 7.6,

Table VIII. Requirements for DNA polymerase activities from rat intestinal mucosal cells.

	Crude enzyme		Partially purified enzyme	
	units x 10^3	%activity	units x 10^3	%activity
Complete system	178	100.0	148	100.0
- DNA	15	8.3	0	0.0
- dATP, dCTP, dGTP	31	17.2	28	18.9
- dATP	57	32.1	38	25.7
- dCTP	86	48.1	59	39.9
- dCTP	70	39.2	49	33.1
- $MgCl_2$	15	8.3	8	5.4
- dithiothreitol	154	86.7	125	84.5
- enzyme	0	0.0	0	0.0
heated enzyme	1	0.6	-	-

containing 0.025 M KCl. The 'crude' DNA polymerase preparation, referring to the high-speed supernatant S_1' of the homogenate, was assayed without further purification. As shown in Table VIII, both enzyme activities required the presence of a DNA template and Mg^{++} ions. Activity was only slightly enhanced by the addition of dithiothreitol. Omission of one of the three unlabeled deoxynucleoside

triphosphates led to a reduction of the enzyme activity to between 25% to 48%, depending on the particular nucleotide omitted. With the omission of all three unlabeled triphosphates, the incorporation of [^{3}H]-TTP was less than 20% of that obtained in the complete system. Heating the enzyme at 100°C for 10 min resulted in drastic inactivation of the enzyme.

2. DNA primer preference

DNA polymerase activity with heat-denatured DNA and native DNA as primer was studied. Heat-denatured material was obtained by heating a solution of calf thymus DNA at 100°C for 10 min, and immediately cooling it to 0°C in an ice-bath. The DNA polymerase activities from the crude and the partially purified preparations were all found to be several-fold higher with heated rather than with native DNA as primer (Table IX). As has been discussed previously, DEAE-cellulose chromatography of the crude cytoplasmic extracts produced one large peak (I) containing 92% of the enzyme

Table IX. DNA polymerase activities with native or heat-denatured DNA as primer.

Enzyme preparation	Units activity x 10^3	
	Native DNA primer	Heat-denatured DNA primer
Crude enzyme	85	234
DEAE-cellulose peak I	726	2,571
DEAE-cellulose peak II	183	503

activity, and a small peak (II) containing 4% of the enzyme activity. These two peaks showed no differences in primer preference (Table IX). Heat-denatured DNA was therefore used in all subsequent assays.

This preference for denatured DNA as primer has also been reported by Bollum (55) in calf thymus DNA polymerase, and by Shepherd and Keir (62) in Landschutz ascites tumor cells. Leung and Zbarsky (19, 20) observed that nuclear DNA polymerase preparations from rat intestinal mucosa demonstrated variations in their preference for denatured or native DNA as primer, depending upon the enzymic fraction tested.

3. Effect of pH

The effect of pH on DNA polymerase activity was examined. Crude enzyme preparation was assayed in 0.05 M potassium phosphate buffers from pH 6.0 to 8.0, and also in 0.05 M Tris-acetate buffers from pH 5.0 to 9.5. The optimum pH values for the enzyme reaction was found to be 7.2 in the phosphate buffer, and 8.0 in the Tris-acetate buffer (Fig. 9).

Leung and Zbarsky (19) have reported pH optima of 7.4 in phosphate buffer, and 8.0 in Tris-HCl buffer for nuclear DNA polymerase in rat intestinal mucosa. Similar optimal pH values have been reported by Zimmerman in bacterial tissues (65) and by Mantsavinos in mammalian tissues (25) under similar conditions.

In the present work, potassium phosphate buffer, pH 7.2, to a concentration of 0.05 M, was used in the routine assay procedures.

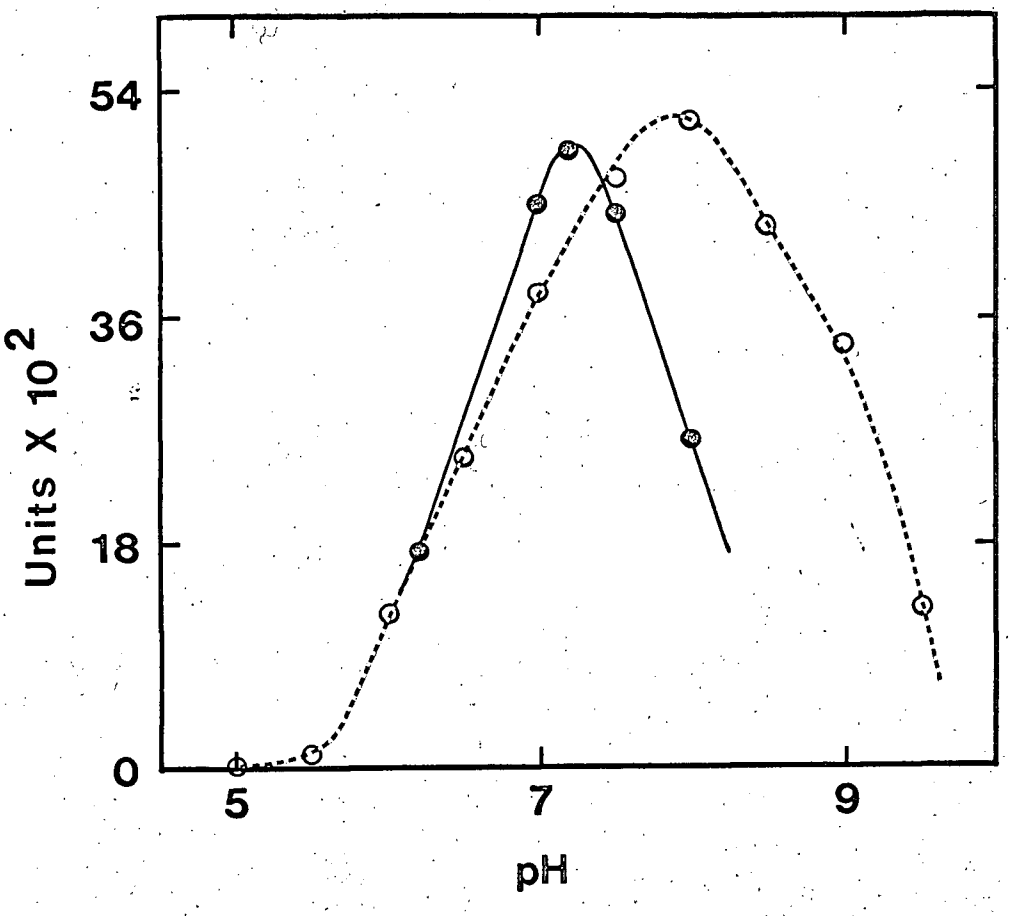


Fig. 9. The effect of pH on crude DNA polymerase from rat intestinal mucosa. Dotted line represents enzyme activity when assayed in 0.05 M Tris-acetate buffers from pH 5.0 to 9.5. Solid line represents enzyme activity when assayed in 0.05 M potassium phosphate buffers from pH 6.0 to 8.0.

4. Effect of varying amounts of radioactivity in assay

The effect of varying amounts of radioactivity on DNA polymerase was examined in an effort to determine the optimal proportions of unlabeled and tritiated TTP to be used in the assay mixtures.

The effect of varying the total concentration of TTP in the incubation mixture is shown in Fig. 10. Tritiated and unlabeled TTP were mixed to give a 1.8 mM solution of [^{3}H]-TTP containing 9 μC of radioactivity per ml. Varying amounts of this solution were used in the incubation mixtures for DNA polymerase assay. The specific radioactivity, i.e., the radioactivity per mole of TTP, was therefore constant for all incubation mixtures. The incorporation of [^{3}H]-TTP into DNA increased with increasing amounts of the [^{3}H]-TTP solution in the assay mixture, approaching a maximum level at about 81 nmoles [^{3}H]-TTP per assay (Fig. 10).

Another experiment was carried out in which the specific radioactivity in the assay mixture was varied, but the total amount of TTP present in each was kept constant. Each incubation mixture contained 16 nmoles TTP and varying amounts of radioactivity ranging from 0.2 to 2.0 μC. The incorporation of [^{3}H]-TTP into DNA increased linearly with increasing amounts of radioactivity (Fig. 11). No tapering off of the increase in activity was observed in the range studied.

In subsequent assays, 16 nmoles of a mixture of tritiated and unlabeled TTP, containing 2μC of radioactivity, were used per incubation mixture, as a suitably high level of DNA polymerase

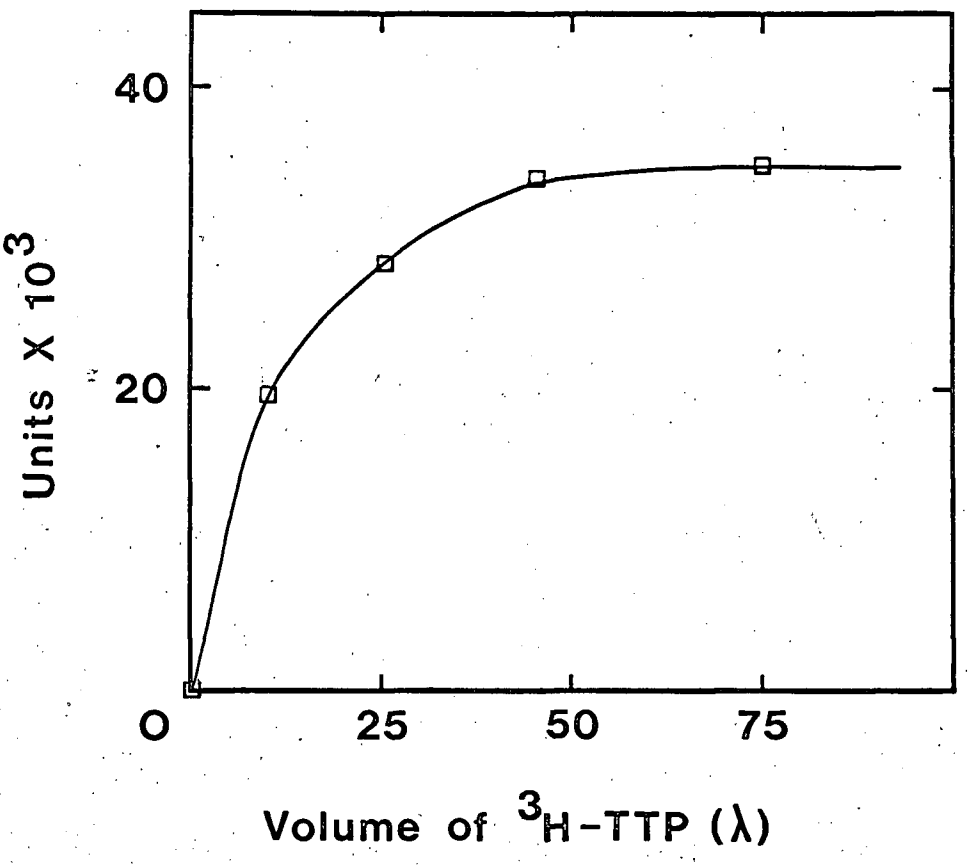


Fig. 10. The effect of varying the total concentration of TTP in the incubation mixture for the DNA polymerase assay, the specific radioactivity being kept constant. Unlabeled and tritiated TTP were omitted from the usual incubation mixture and were replaced with varying amounts of a 1.8 mM ^{3}H-TTP solution containing 9 μC per ml.

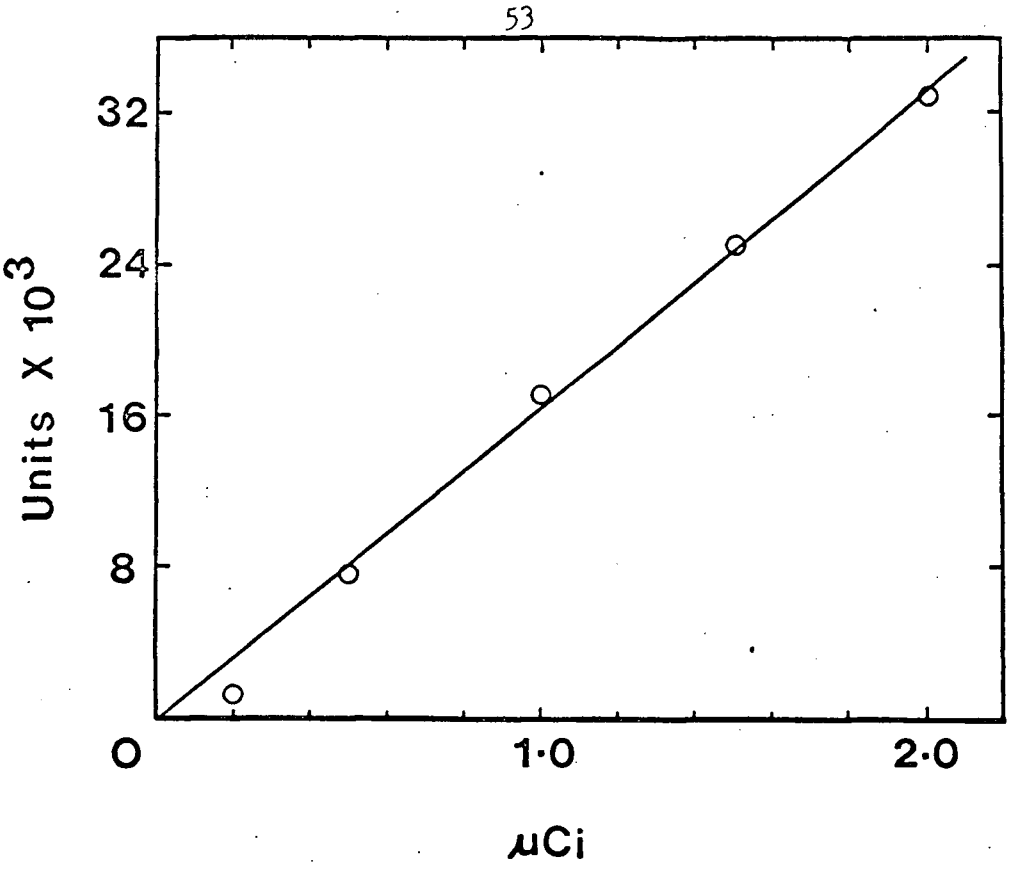


Fig. 11. The effect of varying the specific radioactivity in the incubation mixture for the DNA polymerase assay, the total TTP concentration being kept constant. Varying amounts of a ^{3}H-TTP solution of 10 μC/ml and 57 pmoles/μC were added to the incubation mixture. The amounts of ^{3}H-TTP present were negligible when compared with the 16 nmoles unlabeled TTP present in the incubation mixture.

activity could be obtained with these amounts.

5. Effect of glycerol and ethylene glycol

Glycerol is commonly used in the storage of enzyme preparations to reduce deterioration of enzyme activity. Therefore when crude DNA polymerase preparations were stored, glycerol was added to them in concentrations of 20% or 35%. The enzyme solutions containing glycerol did not solidify even at temperatures of -20°C.

Baril et al (57) have reported that ethylene glycol stabilized the activity of crude and purified rat liver DNA polymerases for at least 3 weeks at 4°C and at least 6 months at -20°C. In the present work, 20% ethylene glycol was therefore added to the buffer systems used in the elution of chromatographic columns.

The effects of glycerol and ethylene glycol on DNA polymerase activity were therefore investigated. Surprisingly, it was found that the presence of either glycerol or ethylene glycol in the enzyme preparations led to an activation of the enzyme activity observed (Table X). When 20% glycerol was added to an enzyme preparation immediately before assaying for DNA polymerase activity, a 1.5-fold increase in the incorporation of radioactivity was observed. Over 10-fold activation has been observed in certain preparations. The level of increase in activity was the same in 20% or 35% glycerol. An increased level of activity could be observed in enzyme preparations containing glycerol after at least 17 days of storage at -20°C. In the case of ethylene glycol, a 2-fold

Table X. Effect of glycerol and ethylene glycol on DNA polymerase activity.

Enzyme preparation	cpm	Control preparation	cpm
S_1'	3,364	S_1'	67
S_1' + 20% glycerol	5,177	water + 20% glycerol	79
S_1' + 20% ethylene glycol	6,537	water + 20% ethylene glycol	64

increase in DNA polymerase activity was observed when 20% ethylene glycol was added to the enzyme preparation immediately before assaying. Control samples were prepared in which enzyme was substituted by water, water containing 20% glycerol, and water containing 20% ethylene glycol. These preparations were assayed in exactly the same manner as the enzyme preparations. The results show no significant differences in the counts per minute of tritium incorporated. Similar stimulation of DNA polymerase activities in the presence of 5% glycerol have been observed by other workers in rat brain tissue (66). The cause of these increases in enzyme activity is unclear.

6. Time-course of the reaction

The time-course for the incorporation of [^{3}H]-TTP into DNA by the crude DNA polymerase extract was studied. The crude enzyme preparation was assayed both in the presence and in the absence of

35% glycerol. In both cases, the reaction was linear with respect to time for at least 30 min, as is shown in Fig. 12. A maximum level of activity was reached in both cases after an incubation time of about 60 min. The maximum level of activity reached by the enzyme containing 35% glycerol was much higher than that reached by the enzyme in the absence of glycerol.

In view of these results, an incubation period of 30 min was used in all subsequent assays for DNA polymerase activity.

7. Enzyme stability

The stability of the DNA polymerase activity upon storage under various temperature conditions was studied. Under condition A, a sample of crude enzyme preparation was stored at 4°C. Aliquots were withdrawn from it for each assay. Under condition B, a sample of crude enzyme preparation was stored frozen at -20°C. This sample was thawed and aliquots were withdrawn from it for each assay, after which the sample was re-frozen and stored for further use. Under condition C, several aliquots of crude enzyme preparation were stored frozen at -20°C. Fresh aliquots were thawed for use in each assay. Any remaining thawed enzyme preparation was discarded and was not re-frozen for further use. Enzyme preparations stored under each of the three conditions were assayed at 1, 6, and 13 days after extraction. The results are presented in Table XI.

The DNA polymerase activities appeared to be stable for at least 13 days. An increase in enzyme activity was observed on day 6 under all three temperature conditions, the activity leveling

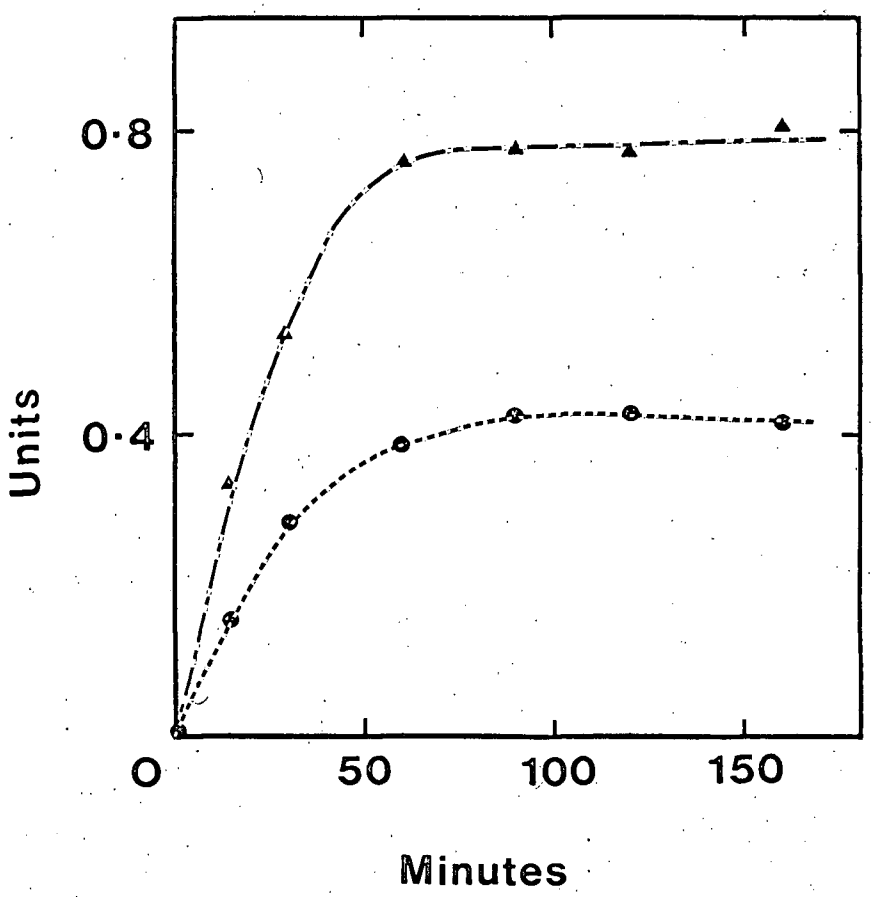


Fig. 12. Time-course studies on the DNA polymerase reaction. Circles represent the time-curve obtained when crude DNA polymerase was assayed in the absence of glycerol. Triangles represent the time-curve obtained when crude DNA polymerase was assayed in the presence of 35% (v/v) glycerol. Abscissa represents the incubation time in minutes.

Table XI. Stability of DNA polymerase under different temperature conditions. (See text).

Number of days after extraction	Units activity x 10^3 per ml enzyme		
	Condition A	Condition B	Condition C
1	60.0	60.0	60.0
6	97.0	94.5	93.5
13	67.0	106.5	80.0

off after 13 days, but remaining higher than the activity on day 1. Under condition B, where the enzyme preparation was frozen and thawed for use each time, the activity continued to increase even after 13 days. The observed increased in enzyme activity on storage were repeatable, and were found to occur as early as day 3.

A possible explanation for these results may be the degradation of an inhibitory factor present in the enzyme preparation. As has been discussed earlier, a strong inhibitor of DNA polymerase has been found to be present in the crude cell homogenate. The nature of the inhibitor is not known, but it was found to be sedimentable by high-speed centrifugation. It is probable that some of the inhibitor remained in the supernatant from the high-speed centrifugation, i.e., in the crude enzyme preparation. If this inhibitory factor is unstable and is degraded upon storage, an apparent increase in DNA polymerase activity would be observed.

In the present work, crude enzyme preparations were used

within one week after extraction.

8. Effect of phosphate ion concentration

An examination of the effect of phosphate ion concentration on DNA polymerase activity was prompted by the observation that elution of chromatographic columns with phosphate buffers always resulted in a total loss of enzyme activity in the eluate..

Table XII. Effect of phosphate ion concentration on DNA polymerase activity.

Phosphate ion concentration of incubation mixture	Units activity x 10^3 (average of two experiments)
0.05 M	972
0.10 M	262
0.15 M	116
0.20 M	13
0.25 M	2

It was suspected that phosphate ions might exert an inhibitory effect on the enzyme. Crude enzyme extract was assayed in the presence of increasing concentrations of phosphate ions, and the results are shown in Table XII.

It can be seen that enzyme activity was drastically inhibited at phosphate concentrations of 0.20 M and above. At

0.10 M phosphate, the enzyme activity was reduced to 27% of that at 0.05 M phosphate. This inhibitory effect of phosphate ions on DNA polymerase activity is believed to be the principal cause of the failure to detect enzyme activity in the eluates of phosphocellulose and Sephadex columns eluted with phosphate buffers at concentrations of 0.10 M or above.

9. Effect of DNase I

A study of the effect of DNase I on the DNA polymerase assay was made for two reasons. Firstly, Leung (54) had observed that fractions which contained a high DNase I activity generally showed a low DNA polymerase activity. He suggested the possible interference of DNase I with DNA polymerase assays. Secondly, the study was made in order to determine the nature of the product formed from the DNA polymerase reaction.

Crude enzyme preparation was measured in the presence or absence of added DNase I. The DNase I was added either at the beginning of the incubation period, i.e., at zero time, or after 30 min of incubation, after which the assay mixture was further incubated for 30 min. The effect of RNase on the DNA polymerase assay was also briefly studied. The results are summarized in Table XIII.

In the first case, crude enzyme was assayed in the absence of added DNase I, with an incubation time of 30 min. When 10 μg of DNase I was added to the assay mixture at zero time, and the mixture incubated for 30 min, a slight increase in DNA polymerase

Table XIII. Effect of DNase I and RNase on DNA polymerase assay.

Enzyme	RNase	DNase I (2,300 Kunitz units per mg)	Incubation time (min)	DNA polymerase activity cpm	DNA polymerase activity units x 10^3
S_1'	-	-	30	1,799	181
S_1'	-	10 μg, added at 0 time	30	1,912	194
S_1'	-	50 μg, added at 0 time	30	1,480	147
S_1'	-	50 μg, added after 30 min	60	580	52
S_1'	-	-	60	2,202	224
-	-	-	30	84	0
-	-	-	60	84	0
S_1'	10 μg, added at 0 time	-	30	2,061	209

activity was observed. This increase was probably due to nicking of the DNA primer by the added DNase I. When the level of DNase I added at zero time was increased to 50 μg and the assay mixture was incubated for 30 min, a 17% decrease in the DNA polymerase activity was observed. This indicated that the level of DNase I present was probable high enough to cause breakdown of the DNA product formed from the polymerase reaction.

In the next case, the DNA polymerase assay mixture was incubated for 30 min in the absence of DNase I. At this time, a significant level of [^{3}H]-TTP has presumably been incorporated

into the newly-formed DNA (see first case, 1799 cpm). Fifty μg of DNase I was added at this point and the assay mixture was further incubated for 30 min. The DNA polymerase activity observed was greatly reduced, indicating that a large portion of the pre-formed product of the polymerase reaction had been degraded by the DNase I. This clearly demonstrated that the product formed by the DNA polymerase reaction was DNA in nature. When the crude enzyme was incubated for 60 min without added DNase I, the DNA polymerase activity was slightly higher than that after 30 min of incubation, confirming that the loss in activity in the previous case was not due to the increase in incubation time. Blank samples containing no enzyme gave identical results for both 30 and 60 min incubation periods.

A brief study of the effect of RNase on DNA polymerase activity was made. Ten μg RNase was added to the assay mixture at zero time and the mixture was incubated for 30 min. The level of DNA polymerase activity obtained was slightly higher than that in the absence of RNase under similar conditions. This indicated that this level of RNase did not have any significant effect on the DNA polymerase activity.

SUMMARY

Previous studies have been made by Leung and Zbarsky (19, 20) on the nuclear DNA polymerases from the small intestinal mucosa of the rat. Significant DNA polymerase activity was also found in the cytoplasmic fractions even under non-aqueous separation conditions where leakage from the nuclei into the cytoplasm was minimized. The present work involves a partial purification and a study of the general properties of this cytoplasmic DNA polymerase activity. The experiments done and the observations made can be summarized as follows:

1. The crude cytoplasmic enzyme fraction studied was prepared by high-speed centrifugation of the homogenate of washed mucosal scrapings.

2. A factor which caused strong inhibition of DNA polymerase activity was sedimented by the high-speed centrifugation of the cell homogenate. The nature of this inhibitory factor remains unclear.

3. On chromatography of the crude cytoplasmic extract on DEAE-cellulose, over 96% of the DNA polymerase activity was found in the fractions representing unadsorbed material. Nevertheless, four-fold purification of the DNA polymerase was achieved because 78% of the contaminating DNase I activity was adsorbed onto the DEAE-cellulose.

4. A minor peak containing 4% of the total DNA polymerase activity was eluted when a gradient of KCl was applied to the DEAE-

cellulose column. This enzyme activity was similar to that in the major DNA polymerase peak in that both activities preferred denatured DNA as primer.

5. The unadsorbed protein peak containing the bulk of the DNA polymerase activity from the DEAE-cellulose column was re-chromatographed on a phosphocellulose column. No enzyme activity could be detected in the eluate. The absence of detectable DNA polymerase activity may have been due to the later discovered strong inhibitory effect of phosphate ions on the enzyme. However, no enzyme activity could be detected even after each fraction was dialyzed overnight against a Tris-HCl buffer system.

6. Crude cytoplasmic enzyme was also chromatographed by gel-filtration on Sephadex G-150 columns. Several protein peaks were eluted using a Tris-HCl buffer system, but only a single peak of DNA polymerase activity was detected. The specific enzyme activity was increased 42-fold and the total activity was increased 12-fold. The reason for this latter increase in total activity is unclear. The possible removal of certain inhibitory factors has been suggested.

7. By the use of protein markers with known molecular parameters, the molecular weight of the DNA polymerase fraction was estimated to be 101,000.

8. The activity of DNA polymerase was measured by the incorporation of [^{3}H]-TTP into an acid-insoluble product, under suitable assay conditions. The enzyme required the presence of a DNA template and Mg^{++} ions. Activity was only slightly enhanced by the addition of dithiothreitol. For maximum activity, the presence of all four

deoxynucleoside triphosphates were required. Only 25% to 48% of the enzyme activity remained when either of the three unlabeled deoxynucleoside triphosphates were omitted from the incubation mixture. When all three unlabeled triphosphates were omitted, the activity was less than 20% of that obtained in the complete system, indicating primarily replicative rather than terminal addition activity. Heat-denatured DNA was preferred as primer. The optimum pH for this enzymatic activity was found to be 7.2 in potassium phosphate buffer, and 8.0 in Tris-acetate buffer.

9. Time-course studies on the enzyme reaction indicated that the reaction was linear with respect to incubation time for at least 30 min.

10. The DNA polymerase activity was stable up to 13 days under temperature conditions of 4°C to -20°C.

11. Glycerol in 20% to 35% (v/v) concentrations was found to have both a stimulatory and a stabilizing effect on the enzyme activity.

12. Ethylene glycol at 20% (v/v) concentration was also found to have a stimulatory effect on the enzyme activity.

13. The enzyme was strongly inhibited in the presence of 0.10 M phosphate ions and activity was drastically reduced in phosphate ion concentrations of 0.20 M and above.

14. The product of the DNA polymerase reaction could be destroyed by DNase, indicating that it was DNA in nature.

CONCLUSION

The purpose of the present work was to determine whether the DNA polymerase activity in the cytoplasmic preparation is actually of cytoplasmic origin, or whether it is due to nuclear contamination. Work done by Leung and Zbarsky (19, 20) showed that the nuclear preparations contained several DNA polymerase activities with molecular weights ranging from 25,000 to 300,000. These enzyme activities demonstrated similar requirements and pH optima, but differed in chromatographic properties and DNA primer preferences. The present results indicate that the cytoplasmic preparation contained a single major DNA polymerase activity with a molecular weight of about 101,000. This activity resembled one of the nuclear activities in many respects, i.e., requirements, pH optima, chromatographic properties on DEAE-cellulose, DNA primer preference. As has been discussed earlier, Weissbach *et al* (51) have reported a similar pattern of DNA polymerases in HeLa cells and in normal human lung diploid line WI-38. In both these cases, two separable DNA polymerases were found in the nucleus and only a single activity was detected in the cytoplasm, the cytoplasmic enzyme resembling one of the nuclear enzymes in all respects tested.

The evidence indicates that the cytoplasmic enzyme activity is not due to nuclear contamination. Firstly, the amount of enzyme activity present in the cytoplasmic fraction is high compared with that in the nuclear fraction, and appears difficult to be accounted for by nuclear contamination alone. Secondly, the nuclear preparation

contained several distinct polymerase activities. Had there been nuclear contamination of the cytoplasm, one would expect to detect several polymerase activities in the cytoplasmic preparation, but only a single major activity was observed.

Results from other workers (61) studying mitochondrial DNA polymerases from rat intestinal mucosa suggest that the minor polymerase peak eluted with 0.1 M KCl on DEAE-cellulose may be of mitochondrial origin.

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