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The Fidelity of DNA Polymerase from Bacillus Licheniformis and Bacillus Stearothermophilus

Gerald R. McGowan

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THE FIDELITY OF DNA POLYMERASE FROM
BACILLUS LICHENIFORMIS
AND
BACILLUS STEAROTHERMOPHILUS

by
Gerald R. Mc Gowan

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment
of the
Degree of Master of Arts

Western Michigan University
Kalamazoo, Michigan
December 1972
ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. Jochanan Stenesh, without whose counsel, wisdom and patience this research and thesis could not have been completed.

Appreciation is also expressed to the Department of Chemistry for their financial support and to the faculty in the department for their instruction and encouragement.

Gerald Richard Mc Gowan
MASTERS THESIS M-4104

MC GOWAN, Gerald Richard
THE FIDELITY OF DNA POLYMERASE FROM
BACILLUS LICHENIFORMIS AND BACILLUS
STEAROTHERMOPHILUS.

Western Michigan University, M.A., 1972
Biochemistry

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INTRODUCTION

With the recognition of deoxyribonucleic acid (DNA) as the carrier of genetic information there developed the necessity of elucidating the nature of the self-replicating system which must provide for the transfer of this information from a cell to its progeny during cell division. During the mid 1950's it was noted that the incubation of labeled deoxynucleotides with a crude cell-free extract of *Escherichia coli* led to the incorporation of a small amount of radioactivity into the acid-insoluble DNA fraction, indicating the in vitro synthesis of DNA (1). The enzyme involved is known as DNA polymerase.

In 1958, Kornberg and co-workers reported the partial purification of DNA polymerase from *E. coli* and the characterization of its reaction (2,3). DNA polymerases have subsequently been isolated from numerous other sources including *Bacillus subtilis* (4), *Micrococcus luteus* (5,6), T-2 infected *E. coli* (7), T-4 infected *E. coli* (8) and recently in this laboratory from *B. licheniformis* and *B. stearothermophilus* (9).

While there are several nuclease activities associated with DNA polymerase (10), the primary reaction it catalyzes is the polymerization of deoxynucleoside triphosphates into a polynucleotide in the presence of a DNA template and with the concurrent release of pyrophosphate. The deoxynucleotides...
are joined via 3'-5' phosphodiester links. The equation for the reaction can be represented as (11):

\[
\begin{align*}
n \text{dGPPP} & \quad + \quad \text{DNA} \quad \xrightleftharpoons{\text{Mg}^{2+}} \quad \text{DNA} \quad + \quad 4n \text{PP}_i \\
n \text{dCPPP} & \\
n \text{dAPPP} & \\
n \text{dTPPP} &
\end{align*}
\]

The chain grows by addition to its 3' end and the reaction is known as proceeding in a 5' to 3' sense. The reaction involves the nucleophilic attack of the 3' hydroxyl group of the growing polynucleotide on the 5' phosphate group of the deoxynucleoside triphosphate with cleavage of inorganic pyrophosphate. The drawing below represents this proposed mechanism (11).
The above reaction is reversible; pyrophosphate-deoxynucleoside triphosphate exchange can occur and the reaction can be stopped by the addition of a large excess of pyrophosphate (10). \( \text{Mn}^{+2} \) can substitute for \( \text{Mg}^{+2} \) in the case of many organisms (4,5) and a limited reaction will take place in the absence of one or more of the deoxy-nucleoside triphosphates (3).

The efficiency of the reaction is highest with a partially denatured or "nicked" DNA template (4,7). Single-stranded DNA will also serve as an effective template. While double-stranded DNA requires a "nick", a free OH or phosphate terminal to bind DNA polymerase, single-stranded DNA will readily bind polymerase in the absence of any terminal groups, i.e. when it is in a closed circular form (10).

The template DNA determines the deoxynucleotide sequence in the newly synthesized DNA through the Watson-Crick type hydrogen bonding between the bases on the template DNA and the bases of the deoxynucleoside triphosphates. This was initially inferred from the similarity in base ratios of the template and the product for the DNA of a particular organism (12) and later from the similarity in nearest neighbor frequency analysis (NNFA) (11).

The final proof of the template directed synthesis of DNA was the in vitro synthesis of a biologically active DNA. This was accomplished in 1967 with the complete synthesis of infectious single-stranded circular phage \( \phi X 174 \) DNA (13).
The copying of a phage genome of about 5000 deoxynucleotides leading to the production of an infectious DNA requires that the replication must be extremely accurate. The frequency of occurrence of errors in replication was first studied by Trantner and co-workers in 1962 using E. coli DNA polymerase (14). The frequency of misincorporation of dGTP using poly dAT template at 37° was found to be less than 1/28,000-1/580,000 residues with a total polymer synthesis of 30-100%. The substitution of poly dA[c] as template increased the frequency of misincorporation to 1/2000-1/25,000 residues. The accuracy of DNA replication in vitro was concluded to be very high.

In a later study on the fidelity of DNA polymerase, Mc Carter et al. found a higher ratio of misincorporation (15). Using E. coli DNA polymerase at 37° the amount of dCTP and dGTP incorporated using poly dAT as a template was found to be about 1/500 residues. Attempts to study the incorporation of dATP and dTTP using the triple helix poly dG₂:dC were complicated by the de novo synthesis of poly dAT in the reaction mixture.

The de novo synthesis of poly dAT and poly dG:dC when the appropriate deoxynucleoside triphosphates are incubated with DNA polymerase in the absence of template was recognized early (11). The kinetics of this reaction follow a different time course than the normal replication of DNA, exhibiting a lag period of several hours. This apparent
lag period may be due to the inability of the assay techniques to measure the slow rate of the de novo synthesis. As the concentration of de novo product increases it serves as template for the production of more product and the reaction follows the regular course of synthesis in the presence of a template.

Hall and Lehman compared the fidelity of wild type *E. coli* DNA polymerase with DNA polymerase induced in *E. coli* by T-4 phage infection (8). Using poly dC as a template the misincorporation frequency of dTTP in place of dGTP at $30^\circ$ was found to be $1/420,000$ for the wild type and $1/120,000$ for the T-4 induced polymerase. For both enzymes, the T/G ratio was found to be independent of template concentration, pH (7.4 and 8.6), temperature ($30^\circ$ and $37^\circ$) and Mg$^{+2}$ concentration. The replacement of Mg$^{+2}$ by Mn$^{+2}$ however resulted in a 5-20 fold increase of the T/G ratio. The T/G ratio was found to be dependent on dGTP concentration. At a constant dTTP concentration the latter was incorporated competitively at low concentrations of dGTP and at a constant rate at high concentrations of dGTP. Using poly dAT as a template the misincorporation frequency of dCTP was found to be $1/10,000$ residues.

Bacteria are commonly divided into 3 groups according to the temperature at which they grow. Psychrophiles grow at low temperatures (below $25^\circ$), mesophiles grow at physiological temperatures ($25^\circ$-45$^\circ$) and thermophiles grow at
elevated temperatures (above 45°C).

Thermophilic bacteria have been isolated from numerous sources (16). They have attracted considerable attention because they can grow at temperatures at which normal cellular components break down. Several theories have been advanced to account for thermophily. Gaughran noted differences in amount and saturation of lipids between mesophiles and thermophiles and postulated that lipids might have a protective role in thermophilic bacteria (17). Allen has proposed a kinetic theory where both synthesis and degradation rates are increased in thermophiles (16). A third theory proposes that the macromolecules and structures in thermophilic bacteria are unusually stable compared to those of mesophilic bacteria. Koffler reported on the increased heat stability of flagella from thermophiles (18). Numerous examples of unusual stability of enzymes from thermophiles have been reported (19).

In this laboratory, DNA isolated from thermophilic bacteria has been shown to have a higher \( \frac{(G+C)}{(A+T)} \) ratio and to have a correspondingly higher \( T_m \) value than DNA from mesophilic bacteria (20).

DNA polymerases isolated from \( B. \ licheniformis \), a mesophile and \( B. \ stearothermophilus \), a thermophile, were shown to have temperature optima of 45°C and 65°C respectively (9,21). In addition, the nearest neighbor frequency analysis for the product DNA varied for each enzyme as a function of
temperature. For a particular enzyme, the frequencies of nucleotide sequences were similar to those of the corresponding template when the reaction was carried out at the optimum temperature of the enzyme. At other temperatures, variations in NNFA were observed. The relative amount of G+C replicated increased at the higher temperatures, implying that either sections of DNA rich in G+C were preferentially replicated at higher temperatures or that the frequency of errors, substitution of G or C for A or T, increased as the temperature was increased.

The purpose of this investigation was to differentiate between these two possibilities, to establish unambiguously whether actual errors in replication occur and, if so, to determine the nature and frequency of these errors. It was hoped to determine whether the errors are temperature or organism dependent and whether specific or random nucleotide substitutions occur. At the same time the possibility would be explored that (G+C) may be replicated more rapidly at higher temperatures than (A+T). In order to carry out these experiments, DNA polymerases were isolated from B. licheniformis and B. stearothermophilus and used with the synthetic copolymers poly dAT and poly dG:dC.

There are several ways to measure the accuracy of DNA replication. The first is by comparing the base composition of the product and template DNA. This method was used originally to measure the accuracy of DNA replication (11,12).
The relative sensitivity of this technique is quite low. Since the base composition of DNA's from various sources is rather similar, and since the amounts of the 4 bases are about equal, only very gross abberations in base composition, on the order of 1%, can be detected.

A second way involves the use of nearest neighbor frequency analysis which provides a more sensitive method for comparing template and newly synthesized DNA (9,11). In this technique DNA is replicated using the 4 deoxynucleoside triphosphates, one of which is labeled with $^{32}\text{P}$ in the phosphate group attached to the 5' position on the deoxyribose. The product DNA is then subjected to enzymatic hydrolysis to yield 3' deoxynucleoside monophosphates. The $^{32}\text{P}$ label is thus passed from the 5' position of the original nucleotide to the 3' position of the adjacent nucleotide. By repeating the experiment four times, each time using one labeled deoxynucleoside triphosphate, the relative frequencies of the dinucleotide sequences in the DNA can be calculated.

While NNFA provides a more sensitive method for comparing template and product DNA than calculation of molar base ratios, complications can arise when the amount of product is smaller than the amount of template. Under conditions of limited synthesis only portions of the DNA template may be replicated and the relative frequencies of dinucleotide sequences may vary from the values of the template (11).
The NNFA approach was used previously in this laboratory to study the replication of several DNA's by DNA polymerases from \textit{B. licheniformis} and \textit{B. stearothermophilus} over a range of temperatures (21). The observed differences in dinucleotide sequences could be attributed to several sources including actual errors in copying the template or preferential replication of portions of the template, specifically those rich in (G+C).

A third way for measuring the accuracy of DNA replication involves the use of synthetic copolymers as templates. This allows for both an unambiguous demonstration of the occurrence of errors in replication, as well as an evaluation of the possibility that different portions of the template are replicated preferentially at different temperatures.

This approach was used in the present study. The replication of the synthetic copolymers poly dAT and poly dG:dC was investigated here. The former is a Watson-Crick type double helix consisting of two strands, each alternating in dA and dT. Poly dG:dC is also a Watson-Crick type double helix but consists of two strands, one being a homopolymer of dG and the other a homopolymer of dC. The structure of these two copolymers is shown below.

\begin{verbatim}
-C-C-C-C-C-C- \quad -A-T-A-T-A-T-

Poly dG:dC \quad \quad \quad Poly dAT
\end{verbatim}

If poly dAT is synthesized in a mixture containing
unlabeled dATP and dTTP plus labeled dGTP or dCTP, then any radioactivity incorporated into the newly synthesized copolymer must represent an error in incorporation. Likewise the incorporation of labeled dATP or dTTP in the presence of unlabeled dCTP and dGTP using a poly dG:dC template also indicates an error of replication. By varying the labeled and unlabeled deoxynucleotides it is possible to study the frequencies and the types of such errors.
MATERIALS AND METHODS

Organisms and Growth Conditions

The bacterial strains used in this experiment were a mesophile, *Bacillus licheniformis* (NRS 243) and a thermophile, *Bacillus stearothermophilus*.10

The bacteria were grown initially on slants containing 1% Trypticase (BBL), 0.2% yeast extract (Difco) and 2% Bactoagar (Difco). Stock cultures were grown on the same medium containing in addition 10 ppm manganese. Slants were incubated 12 hours at 37° for the mesophile and 10 hours at 55° for the thermophile. At the end of this period, 6 ml of sterile water was added and the contents of 3 slants, a total of 18 ml, were transferred to a seed flask containing 2 liters of sterile medium (1% Trypticase and 0.2% yeast extract). The seed flask was incubated at the appropriate temperature for 4 hours with constant aeration.

The contents of the seed flask were inoculated into a 25 liter fermentor (New Brunswick Scientific, Model MF-1285) containing 23 liters of sterile medium and 1 ml of silicone antifoam (Union Carbide Corp., SAG-471). The bacteria were grown to an absorbance of 1.5 at 540 nm for the thermophile (55°) and an absorbance of 2.5 for the mesophile (37°). This is slightly past the log phase of growth. The medium containing the cells was chilled and the cells harvested at 4° using a continuous-flow centrifuge (Sorvall...
RC-2 with KBS continuous-flow attachment) at 30,000 x g and a flow rate of 250 ml/minute.

The cells were resuspended in about 500 ml of buffer containing 0.01 M Tris (pH 7.4), 0.01 M magnesium acetate and 0.06 M NH₄Cl, and centrifuged at 11,700 x g for 20 minutes. The cells were then frozen in liquid nitrogen and stored at -20°. The yield of cells (wet weight) was about 80 g for *B. stearothermophilus* and 100 g for *B. licheniformis*.

Isolation of DNA Polymerase

Preparation of crude extract

Cells, 15-70 g (wet weight), were thawed for 1 hour at room temperature and then suspended in buffer (30 ml/10 g of cells) consisting of 0.05 M glycylglycine (pH 7.0), 0.02 M EDTA and 0.002 M glutathione. The mixture was stirred for 1½ to 2½ hours at 4° to allow complete suspension of the cells. It was found, in the case of *B. stearothermophilus*, that this stirring after suspension of cells resulted in greater polymerase activity than when the cells were suspended in the buffer without prolonged stirring.

The cells were broken in a French Press (Aminco Model 5-596) at 18,000 psi. The mixture was centrifuged at 30,000 x g for 30 minutes, the pellet containing the cell debris was discarded and the supernatant was collected to give Fraction I (about 25 ml/10 g cells).
Phase partition

To each 100 ml of Fraction I were added 11.5 ml of 20% (w/w) Dextran T-500 (Pharmacia) and 32.2 ml of 30% (w/w) Polyethylene Glycol 6000 (Baker). To obtain a clean phase separation, the concentration of Dextran and polyethylene glycol in the final mixture must be 1.6 and 6.4% by weight, respectively (4).

Solid NaCl, 33.7 g for each 100 ml of Fraction I, was slowly added and the mixture was stirred for 2 hours at 4°C. Centrifugation of the mixture at 500 x g for 10 minutes separated it into two distinct layers. The upper layer (polyethylene glycol and protein) was poured off to give Fraction II. The centrifugation was repeated if necessary to insure adequate separation. The lower layer containing the nucleic acids was discarded.

If the proportions of Dextran and polyethylene glycol were not correct, the phase separation was not complete and centrifugation at 1000 x g for 20-30 minutes was necessary to separate the two layers. This could occur because the water content of the frozen cells varied, depending on their age and the absorbance at which they were harvested. With *B. licheniformis* this proved to be no problem but with *B. stearothermophilus*, harvested at a lower absorbance (1.5 instead of 2.5) a lower proportion of lysed cells was apparent. The resulting cell pellet was more compacted and
had a noticeably lower moisture content. This resulted in a higher actual cell concentration in the cell suspension and a greater solute concentration in Fraction I.

When Fraction I from \textit{B. stearothermophilus} was diluted with 20-50\% of water the phase partition proceeded normally. It was found, however, that the specific activity of the polymerase was the same whether or not the phase separation was complete. This was probably because the Dextran and nucleic acids which remained were removed in the first ammonium sulfate fractionation step. Consequently, Fraction I was not diluted in obtaining the enzyme fraction used in the actual experimental work.

Fraction II was dialyzed overnight (about 16 hours) against a buffer containing 0.3 M potassium phosphate (pH 7.4), 0.002 M EDTA and 0.01 M 2-mercaptoethanol using 5.7 liters for each 100 ml of Fraction II. The dialyzed solution, Fraction III, was straw-colored and normally clear.

If the phase partition in the previous step had been incomplete, the dialysis bag would contain two layers, a clear upper layer and a cloudy lower layer containing the remaining Dextran. In this case, only the clear upper layer was used as Fraction III.

\textbf{Ammonium sulfate fractionation}

Solid \((\text{NH}_4)_2\text{SO}_4\) (10 g/100 ml of Fraction III) was slowly
added to Fraction III and the mixture was stirred for 15 minutes. Centrifugation at 10,000 x g for 30 minutes yielded two layers. The lower layer, containing the enzyme, was siphoned off and designated as Fraction IV. The upper layer, containing the polyethylene glycol, was discarded.

Solid \((\text{NH}_4)_2\text{SO}_4\) \((10 \text{ g/100 ml of Fraction IV})\) was added to Fraction IV and the mixture was stirred and centrifuged as above. Again two layers were formed and the lower layer, containing the enzyme, was removed by siphoning to give Fraction V. The upper layer, containing polyethylene glycol, and any proteins which were pelleted during this step were discarded.

Solid \((\text{NH}_4)_2\text{SO}_4\) \((10 \text{ g/100 ml of Fraction V})\) was added to Fraction V and the mixture was stirred and centrifuged as above. The supernatant was poured off to give Fraction VI and the pellet discarded.

Solid \((\text{NH}_4)_2\text{SO}_4\) \((12.5 \text{ g/100 ml of Fraction VI})\) was added to Fraction VI and the mixture was stirred for 15 minutes. It was then centrifuged in 25 ml fractions at 10,000 x g for 30 minutes. The supernatant was discarded and the pellets, designated Fraction VII, were collected, transferred to screw-top culture tubes and stored at -20°.

**DNA-cellulose column chromatography**

The DNA-cellulose columns were prepared as described below. The columns were stored at 4° but used for
experiments at room temperature. The columns and buffers used for eluting the enzyme were allowed to equilibrate overnight at room temperature before use.

One pellet of Fraction VII (representing 25 ml of Fraction VI) was suspended in 6 ml of buffer containing 0.01 M potassium phosphate (pH 6.8), 0.1 M MgCl₂ and 20 % glycerol (v/v). This was the same buffer used in preparation of the column.

The suspension of Fraction VII was slowly run onto the column and allowed to equilibrate with the DNA-cellulose packing for 15 minutes. The column was then eluted with buffers of increasing ionic strength, starting with 25 ml of the buffer used to suspend Fraction VII, followed by 25 ml of buffer containing 0.01 M potassium phosphate (pH 6.8), 0.4 M NaCl and 20 % glycerol (v/v) and finally by 25 ml of buffer containing 0.01 M potassium phosphate (pH 6.8), 0.7 M NaCl and 20 % glycerol (v/v).

The bulk of enzyme activity was eluted with the first two 4 ml fractions of the final buffer (0.7 M NaCl) and was designated as Fraction VIII, while the majority of the protein passed unhindered through the column and was eluted with the first buffer. A typical elution profile for *B. licheniformis* is shown in Figure 1.

The specific activity of Fraction VIII was typically about 250-300 enzyme units/mg protein (9), representing about a 200-fold purification of Fraction VII.
Figure 1 DNA-Cellulose Chromatography (B. licheniformis)
recovery of activity from the column was about 200%. This large value is probably due to the presence of nuclease activity in Fraction VII and its absence in Fraction VIII (9).

Assay for DNA Polymerase

DNA polymerase was assayed by measuring the incorporation of $^3$H-labeled deoxynucleoside triphosphates into an acid insoluble product. The standard assay was carried out in a total volume of 300 ml in a 15 ml conical centrifuge tube. The incubation mixture contained the following:

Buffer, containing 0.2 M glycine (pH 9.0), 0.1 M MgCl$_2$----------------------------- 50 $\mu$l
Enzyme, Fraction VIII (0.5 units)--------- 50 $\mu$l
Polynucleotide, 6 mmoles or activated calf thymus DNA, 40 mmoles (nucleotide eq.)-- 50 $\mu$l
Deoxynucleoside triphosphates, as required, 10 mmoles each, one labeled with $^3$H (0.7-1.1 x $10^6$ total dpm)------------------ 100 $\mu$l
Water---------------------------------------- 50 $\mu$l

Total 300 $\mu$l

Activated calf thymus DNA (7) was initially used in the assay procedure but poly dAT was found to give higher activity and was used henceforth. When poly dAT was used as the template there was no appreciable difference in enzyme activity whether all four deoxynucleoside triphosphates were present or only dATP and dTTP. Likewise, when poly dG:dC was used as a template only dGTP and dCTP were necessary for full activity.
All assays contained one $^3$H-labeled deoxynucleoside triphosphate. This amounted to a total of 10 mmoles per incubation mixture and a total activity of $0.7-1.1 \times 10^6$ dpm. All of the results were normalized to a nominal activity of $1 \times 10^6$ dpm per incubation mixture by counting an aliquot of the labeled deoxynucleoside triphosphate solution, calculating the appropriate factor and multiplying the observed dpm by this factor.

The assay tubes were incubated for 30 minutes at the desired temperature: $37^\circ$, $45^\circ$, or $55^\circ$. At the end of the 30 minute period, the tubes were chilled in an ice bath at $0^\circ$ for 5 minutes. Calf thymus DNA (50 $\mu$l of an aqueous solution, 2 mg/ml) was added to each tube as a carrier followed by 1 ml of cold 1 M HClO$_4$. After an additional 5 minutes at $0^\circ$, 0.5 ml of cold saturated sodium pyrophosphate was added, followed by 5 ml of cold 1% trichloroacetic acid. The tubes were allowed to sit for at least 1 hour at $0^\circ$ before proceeding in order to insure complete precipitation of the DNA. The precipitate was collected by vacuum filtration on glass fiber filter paper (Whatman GF-C, 2.4 cm diameter, washed twice with 3 ml portions of saturated sodium pyrophosphate). The precipitate was washed on the paper 8 times with 5 ml portions of cold 1% trichloroacetic acid.

The filter papers were air dried, placed in scintillation vials and 10 ml of scintillation fluid was added (100 g of
naphthalene, 4 g PPO and 50 mg POPOP per liter of 1,4-dioxane). The vials were counted in a liquid scintillation counter (Nuclear Chicago Model Mark II) for 10 minutes or 10^6 counts. The counting efficiency was measured by the channels ratio method using ^3H quenched standards and the counts per minute (cpm) were converted to disintegrations per minute (dpm).

For the kinetic studies, a total reaction volume of 1.2 ml was used; the standard incubation mixture was increased by a factor of 4. Aliquots (100 µl) were removed at various intervals and pipetted directly into 1 ml of cold 1 M HClO₄. The carrier DNA was omitted and the tubes were treated as above. Although the total radioactivity of each assay was therefore reduced by a factor of three, the controls were correspondingly lowered so that the sensitivity remained about the same.

Preparation of DNA-Cellulose Columns

Cellex N-1 (Cal Biochem, 15 g) was suspended in 300 ml of 1 M HCl and stirred for 10 minutes. The Cellex was collected by vacuum filtration on Whatman No. 1 paper, washed with water and resuspended in 200 ml of 1 M HCl. After stirring and collecting as above, the Cellex was washed on the paper with water until the filtrate was neutral to litmus (about 1 liter of water) and air dried overnight.

The dry, acid washed, Cellex (3.5 g) was mixed with 25 ml
of calf thymus DNA (2 mg/ml in 1 mM NaCl) and the mixture was poured into two petri dishes. After drying overnight in a desiccator, the DNA-cellulose was scraped from the petri dishes, suspended in 80 ml of absolute ethanol, and stirred for 15 minutes. The suspension was placed in a 250 ml beaker under a low-pressure mercury lamp (Mineralight Model R-51) at a distance of 10 cm above the alcohol surface and irradiated for 15 minutes with gentle stirring. The activated DNA-cellulose was collected by vacuum filtration on Whatman No. 1 paper. It was washed three times by suspending it in 200 ml of 1 mM NaCl, stirring for 10 minutes and collecting by vacuum filtration. After the third washing, the filter paper with the collected DNA-cellulose was placed in a vacuum desiccator and dried overnight.

Activated DNA-cellulose (1 g) was suspended in 30 ml of buffer containing 0.01 M potassium phosphate (pH 6.8), 0.1 M MgCl₂ and 20% (v/v) glycerol, packed into a column (1 cm diameter) to a height of about 4.5 cm and washed with 50 ml of the same buffer.

The first time that the column was used, DNase (5 μl of an aqueous solution containing 5 μg) was mixed with Fraction VII prior to column chromatography. The solution was then run onto the column and allowed to react for 15 minutes before eluting the enzyme. On subsequent runs, the DNase was omitted from Fraction VII.

The above procedure must be carried out at room temperature to allow the DNase to efficiently "nick" the DNA bound
to the cellulose. If the DNase is omitted or if the column chromatography is carried out at low temperature, the majority (>60%) of the DNA polymerase activity will be eluted from the column with the rest of the proteins in the first two 4 ml fractions. When DNase activation is included the majority of the DNA polymerase activity (>50%) will be eluted with the first two 4 ml fractions of the final 0.7 M NaCl buffer when the column is used for the first time. During subsequent uses of the column, over 90% of the enzymatic activity will be eluted at that point.

Two columns were prepared, one for each bacterial preparation. The columns were regenerated by washing them after each run with 50 ml of the buffer used in packing the column, and were then stored at 4°C until the next run.

Protein Determination

Protein was determined by the method of Lowry. Sodium carbonate (2% Na₂CO₃) in 0.1 M NaOH, 1% CuSO₄·5 H₂O and 2% Na-K tartrate were mixed daily in proportions of 100:1:1. One ml of sample containing 10-100 μg of protein was mixed with 5 ml of the above solution. Phenol reagent (0.5 ml, 1 N) was added and after 10 minutes the absorbance was read vs. water at 750 nm on a Beckman DU spectrophotometer. Protein concentration was determined from a calibration curve using bovine serum albumin as a standard.
Reagents

Growth of organisms:

- Trypticase (BBL)
- Yeast extract (Difco)
- Bactoagar (Difco)
- Silicone anti-foam (Union Carbide SAG-471)
- Tris buffer (pH 7.4), 0.01 M containing 0.01 M magnesium acetate, 0.06 M NH₄Cl

Isolation of DNA polymerase:

- Glycylglycine buffer (pH 7.0), 0.05 M containing 0.002 M EDTA, 0.002 M glutathione
- Potassium phosphate buffer (pH 7.4), 0.3 M containing 0.002 M EDTA, 0.01 M 2-mercaptoethanol
- Potassium phosphate buffers (pH 6.8), 0.01 M containing 0.4 and 0.7 M NaCl, 20% glycerol (v/v)
- Potassium phosphate buffer (pH 6.8), 0.01 M containing 0.01 M MgCl₂, 20% glycerol (v/v)
- Dextran T-500 (Pharmacia), 20% (w/w)
- Polyethylene glycol 6000 (Baker), 30% (w/w)
- Ammonium sulfate (enzyme grade)
- NaCl, solid

Assay for DNA polymerase:

- Glycine buffer (pH 9.0), 0.2 M containing 0.1 M MgCl₂
- Pancreatic DNase (Worthington)
- Calf thymus DNA (Worthington)
Preparation of DNA-cellulose:

Cellex N-1 (Cal Biochem)

NaCl, 0.001 M

Calf thymus DNA (Worthington)

Pancreatic DNase (Worthington)

Protein determination:

Na$_2$CO$_3$, 2% in 1 M NaOH

CuSO$_4$.5H$_2$O, 1%

Na-K tartrate, 2%

Phenol reagent (Fisher)

Bovine Serum Albumin (Worthington)
RESULTS AND DISCUSSION

Replication of Copolymers in the Presence of 4 Deoxynucleoside Triphosphates

DNA polymerase from *B. licheniformis* and *B. stearothermophilus* was used to replicate poly dAT and poly dG:dC at 37, 45 and 55°C, respectively. These temperatures represent roughly the optimum temperatures of the two enzymes and an intermediate value. The incubation mixture contained all 4 deoxynucleoside triphosphates including one which was labeled with $^3$H. All the experiments were carried out in duplicate and the deviation from the mean of the duplicates was generally about 10% or less. The extent of replication varied from 60-110% for poly dAT and from 3-35% for poly dG:dC, depending on the temperature.

Zero time controls, where the reaction was stopped by the addition of HClO$_4$ before the addition of the enzyme, were also run. The controls averaged about 60 cpm compared to about 80,000 cpm for the product from the poly dAT reaction and 20,000 cpm for that from the poly dG:dC reaction. The cpm were converted to dpm and normalized as described in the previous section and the control values subtracted from the experimental values. The results are presented in Table 1.

The misincorporation of either dGTP* ($^3$H-dGTP) or dCTP* ($^3$H-dCTP), with the presence of poly dAT as template, is
<table>
<thead>
<tr>
<th>Table 1: Replication of Copolymers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organism</strong></td>
</tr>
<tr>
<td>Temperature</td>
</tr>
<tr>
<td>Deoxynucleotides</td>
</tr>
<tr>
<td>dATP*+dTTP*dGTP+dCTP</td>
</tr>
<tr>
<td>dATP+dTTP+dGTP*dCTP</td>
</tr>
<tr>
<td>dATP+dTTP+dGTP**+dCTP</td>
</tr>
<tr>
<td>% Incorporation **</td>
</tr>
<tr>
<td>dATP+dTTP+dGTP+dCTP*</td>
</tr>
<tr>
<td>% Incorporation **</td>
</tr>
<tr>
<td>Poly dG:dC Template</td>
</tr>
<tr>
<td>dGTP**+dCTP+dATP+dTTP</td>
</tr>
<tr>
<td>dGTP+dCTP**+dATP+dTTP</td>
</tr>
<tr>
<td>dGTP+dCTP+dATP**+dTTP</td>
</tr>
<tr>
<td>% Incorporation ***</td>
</tr>
<tr>
<td>dGTP+dCTP+dATP+dTTP*</td>
</tr>
<tr>
<td>% Incorporation ***</td>
</tr>
</tbody>
</table>

Values expressed as dpm (corrected for zero-time controls)
* Labeled with tritium (^3H)
** Based on total deoxynucleotide incorporation (dATP*+dTTP*)
*** Based on total deoxynucleotide incorporation (dGTP*+dCTP*)
seen to be remarkably constant, i.e. there was no apparent
temperature dependence. There was slightly less incor-
poration of dGTP* and significantly less incorporation of
dCTP* for the thermophilic system compared to the meso-
philic one. The ratio of dATP* (\(^3\text{H-dATP}\)) and dTTP* (\(^3\text{H-dTTP}\))
incorporated was close to unity in all cases. The incor-
poration of dATP* and dTTP* was essentially constant between
37-45° but decreased somewhat at 55°.

For the reaction using poly dG:dC as template several
observations can be made. First, the ratio of dGTP* to
dCTP* incorporated was not unity. Since the structure of
the copolymer consists of two homopolymer strands and since
it has been shown that DNA polymerase can replicate either
strand in vitro this fact is not surprising because the
rate for replicating the two different strands need not be
the same.

Second, the reaction was definitely temperature dependent.
The maximum and minimum total nucleotide incorporations
were 1.2 and 0.36 \(\mu\)moles for \textit{B. licheniformis} (at 37 and
55°) and 0.8 and 0.12 \(\mu\)moles for \textit{B. stearothermophilus}
(at 45 and 55°).

Third, the magnitude of the ratio of dGTP* to dCTP*
incorporated increased with temperature as shown below:

<table>
<thead>
<tr>
<th>Organism</th>
<th>\textit{B. licheniformis}</th>
<th>\textit{B. stearothermophilus}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37° 45° 55°</td>
<td>37° 45° 55°</td>
</tr>
<tr>
<td>dGTP*/dCTP*</td>
<td>7.6  5.6  21</td>
<td>16   23  30</td>
</tr>
</tbody>
</table>
It appears from this data that the *B. stearothermophilus* DNA polymerase is more selective than that of *B. licheniformis* since the dGTP*/dCTP* ratio is higher at all temperatures and also exhibits a smaller temperature dependence.

Fourth, the percent of misincorporation of dATP* and dTTP* into poly dG:dC was 5-50 times that of the misincorporation of dGTP* and dCTP* into poly dAT. At 55°C, this misincorporation was equal to, or greater than, the incorporation of dCTP* specified by the template.

**Replication of Copolymers in the Presence of 1, 2, or 3 Deoxynucleoside Triphosphates**

There are several mechanisms by which the incorporation of nucleotides not specified for in the template can occur during DNA synthesis. One mechanism involves the synthesis of a homopolynucleotide apart from, and not specified by, the template. Such de novo synthesis of poly dAT, poly dG, poly dC or other products has been described earlier (8,11). A second mechanism involves the attachment of single deoxynucleotides to the ends of existing DNA chains (22). In both of these cases there are no actual mistakes in replication but rather additional synthesis remote from the site of normal replication.

On the other hand a third mechanism, involving an actual replication error, would occur if an improper deoxynucleotide were incorporated in response to the template (substitution).
or if it were inserted as an additional nucleotide in a chain during synthesis (addition). These types of errors are, along with deletion errors, responsible for point mutations of DNA in vivo. A theory which would allow the accommodation of the non-complimentary bases within the double-stranded DNA helix has been proposed (23). This involves the formation of loops in one or both strands of the DNA to allow maximum pairing of adjacent nucleotides on either side of the non-complimentary bases. This theory does not require limitations as to the types of errors which may occur, i.e. the substitution of one purine or pyrimidine for another (transition) or the substitution of a purine for a pyrimidine or vice versa (transversion).

In order to decide whether the misincorporation which was observed (Table 1) was due to non-specific synthesis of the first two types of mechanisms or due to an actual replication error as described by the third mechanism, several experiments were set up.

The procedure was similar to that of the previous experiment except for the number of deoxynucleoside triphosphates present in the incubation mixture. The results are summarized in Tables 2 and 3.

In the reaction using poly dAT as template, the incorporation of dCTP* and dGTP* was generally significantly less when only two unlabeled deoxynucleoside triphosphates were present (Table 2), as in this experiment, compared to the
Table 2: Replication using Poly dAT Template

<table>
<thead>
<tr>
<th>Organism</th>
<th>B. licheniformis</th>
<th>B. stearothermophilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37°, 45°, 55°</td>
<td>37°, 45°, 55°</td>
</tr>
<tr>
<td>Deoxynucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dATP*+dTTP*</td>
<td>354,000, 338,000, 268,000</td>
<td>216,000, 366,000, 282,000</td>
</tr>
<tr>
<td>dATP+dTTP*</td>
<td>358,000, 279,000, 242,000</td>
<td>209,000, 319,000, 262,000</td>
</tr>
<tr>
<td>dATP+dTTP+dGTP*</td>
<td>760, 950, 440</td>
<td>950, 571, 464</td>
</tr>
<tr>
<td>% Incorporation**</td>
<td>0.11 %, 0.15 %, 0.09 %</td>
<td>0.22 %, 0.08 %, 0.08 %</td>
</tr>
<tr>
<td>dATP+dGTP*</td>
<td>175, 33, 126</td>
<td>215, 120, 17</td>
</tr>
<tr>
<td>dTTP+dGTP*</td>
<td>191, 99, 88</td>
<td>504, 37, 44</td>
</tr>
<tr>
<td>dGTP*</td>
<td>169, 280, 36</td>
<td>327, 28, 16</td>
</tr>
<tr>
<td>dATP+dTTP+dCTP*</td>
<td>529, 142, 69</td>
<td>336, 173, 39</td>
</tr>
<tr>
<td>% Incorporation**</td>
<td>0.07 %, 0.02 %, 0.01 %</td>
<td>0.08 %, 0.03 %, 0.01 %</td>
</tr>
<tr>
<td>dATP+dCTP*</td>
<td>107, 82, 201</td>
<td>257, 118, 21</td>
</tr>
<tr>
<td>dTTP+dCTP*</td>
<td>24, 35, 48</td>
<td>85, 124, ***</td>
</tr>
<tr>
<td>dCTP*</td>
<td>58, 63, 140</td>
<td>93, 99, -</td>
</tr>
</tbody>
</table>

Values expressed as dpm (corrected for zero-time controls)
* Labeled with tritium (3H)
** Based on total deoxynucleotide incorporation (dATP*+dTTP*)
*** Less than control value
Table 3: Replication using Poly dG:dC Template

<table>
<thead>
<tr>
<th>Organism</th>
<th>B. licheniformis</th>
<th>B. stearothermophilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37° 45° 55°</td>
<td>37° 45° 55°</td>
</tr>
<tr>
<td>Deoxynucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dGTP+dCTP*</td>
<td>8240 6110 560</td>
<td>2840 1310 110</td>
</tr>
<tr>
<td>dGTP*+dCTP</td>
<td>36,500 35,700 12,000</td>
<td>22,900 21,200 6540</td>
</tr>
<tr>
<td>dGTP+dCTP+dATP*</td>
<td>500 172 75</td>
<td>210 339 21</td>
</tr>
<tr>
<td>% Incorporation **</td>
<td>1.11 % 0.41 % 0.47 %</td>
<td>0.82 % 1.50 % 0.34 %</td>
</tr>
<tr>
<td>dGTP+dATP*</td>
<td>146 43 -***</td>
<td>372 122 8</td>
</tr>
<tr>
<td>dCTP+dATP*</td>
<td>19 - -</td>
<td>149 149 90</td>
</tr>
<tr>
<td>dATP*</td>
<td>- 25 -</td>
<td>- - 83</td>
</tr>
<tr>
<td>dGTP+dCTP+dTTP*</td>
<td>73 - -</td>
<td>98 148 42</td>
</tr>
<tr>
<td>% Incorporation **</td>
<td>0.16 % - -</td>
<td>0.38 % 0.66 % 0.63 %</td>
</tr>
<tr>
<td>dGTP+dTTP*</td>
<td>194 14 -</td>
<td>42 40 47</td>
</tr>
<tr>
<td>dCTP+dTTP*</td>
<td>105 38 10</td>
<td>119 39 88</td>
</tr>
<tr>
<td>dTTP*</td>
<td>96 44 -</td>
<td>9 69 103</td>
</tr>
</tbody>
</table>

Values expressed as dpm (corrected for zero-time controls)
* Labeled with tritium (^H)
** Based on total deoxynucleotide incorporation (dGTP*+dCTP*)
*** Less than control value
case when there were three unlabeled deoxynucleoside triphosphates present (see Table 1), as in the previous experiment. This is true of both organisms and at most temperatures. There is an apparent temperature effect with dCTP*; less misincorporation is apparent at higher temperatures than at low temperatures with both organisms. In the presence of three unlabeled deoxynucleoside triphosphates (Table 1) the misincorporation was essentially not affected by variations in temperature.

A similar statement can be made for the incorporation of dATP* and dTTP* in the reaction using poly dG:dC as template. In this case there was a similar temperature effect, i.e. there was less misincorporation at higher temperatures (Table 3) while the opposite trend was observed in the presence of three unlabeled deoxynucleoside triphosphates (Table 1).

With poly dAT as a template, dGTP* was incorporated more frequently than dCTP*. This is true for both organisms, at all temperatures and whether two or three unlabeled deoxynucleoside triphosphates were present (Table 1 and 2). The incorporation of dGTP* in the assays containing (dGTP*+dATP), (dGTP*+dTTP) and (dGTP*) was approximately equal and less than that of the assay containing (dGTP*+dATP+dTTP). This would suggest that the incorporation in the former three assays represents the de novo formation of poly dG; if dGTP* readily substituted for either dATP or dTTP then
either the \((dGTP^*+dATP)\) or the \((dGTP^*+dTTP)\) assay would be expected to show incorporation greater than that due to \((dGTP^*)\) alone. Since the de novo poly dG formation amounts to less than one fourth the total incorporation of dGTP* in the actively replicating system, this indicates clearly that dGTP* is actually misincorporated into the poly dAT product.

The possibility that dGTP* is attached randomly to the end of an existing polynucleotide chain (22) can be ruled out on the following basis. If random attachment did occur then one would expect the amount of dGTP* incorporated in the presence of either dATP or dTTP to be one-half of that incorporated when only dGTP* is present, assuming equal probability of attachment. Furthermore, since an increase in temperature would tend to increase polynucleotide hydrolysis, thereby generating more chain ends, the incorporation of dGTP* would be expected to increase with temperature if it were merely attached to the end of an existing chain. However, no such trend can be observed.

The incorporation of dCTP* into poly dAT is temperature dependent, being maximal at \(37^\circ\) and decreasing to a barely detectable level of about 0.01 % at \(55^\circ\) for both organisms. In view of the arguments presented above, it appears that de novo formation of poly dC is smaller than that of poly dG and that dCTP* may substitute for dTTP preferentially, although the very low levels of incorporated radioactivity
do not allow a definitive statement.

Using poly dG:dC and dATP* it is apparent that de novo formation of poly dA did not occur to a measurable extent. Furthermore, dATP* is incorporated in the actively replicating system into the product and this appears to be a preferential substitution for dGTP. There appears to be a somewhat larger misincorporation of dATP* in the case of B. stearothermophilus.

The misincorporation of dTTP*occurs to a smaller extent than that of dATP* using poly dG:dC as template. It appears that the formation of poly dT may occur to a small extent, but no other conclusions may be drawn as to the type of incorporation error.

By comparing Tables 1 and 3 it becomes apparent that the incorporation of dATP* or dTTP* in the poly dG:dC directed reaction is much greater when three unlabeled deoxynucleoside triphosphates are present than when only two are present. This implies that poly dAT may be formed de novo when both dATP and dTTP are present. This is true with both organisms.

An attempt was made to show de novo synthesis directly. To this end, labeled deoxynucleoside triphosphates were incubated with the B. licheniformis enzyme in the absence of template. Incubation mixtures containing dATP*+dTTP, dGTP*+dCTP, and dCTP*+dGTP (at the usual concentrations) were incubated at 37, 45 and 55°. In all cases there was
no detectable incorporation of $^3$H above the control levels. This indicates that de novo synthesis requires the presence of a template for initiation of the reaction.

In summary we may say that the de novo formation of poly dG apparently does occur but not that of poly dA, poly dC or poly dT, at least not to a measurable extent. When both dATP and dTTP are present there apparently is de novo synthesis of poly dAT. There appears to be incorporation of improper deoxynucleotides into replicated DNA, especially dGTP in place of dATP and vice versa.

Tritium Exchange

The possibility existed that the incorporation of radioactivity during replication was due to an exchange reaction between the labeled improper deoxynucleoside triphosphate and the unlabeled proper deoxynucleoside triphosphate. For example, the reaction below may occur:

$$^3\text{H}\text{-dATP} + \text{dGTP} \leftrightarrow \text{dATP} + ^3\text{H}\text{-dGTP}$$

In this case, if $^3$H-dGTP were to be incorporated into the product of the poly dG:dC reaction, this would appear instead as an incorporation of $^3$H-dATP. In order to test for this possibility, $^3$H-dGTP was placed in the incubation mixture with dATP and dTTP, the buffer and poly dAT. It was kept at 40°C for periods of 3, 8 and 24 hours before the enzyme was added and the assay completed. The polymerase from *B. stearothermophilus* was used and the incubation
temperature was 55°. The incorporation was as follows:

<table>
<thead>
<tr>
<th>Time</th>
<th>3 hrs.</th>
<th>8 hrs.</th>
<th>24 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpm</td>
<td>830</td>
<td>396</td>
<td>793</td>
</tr>
</tbody>
</table>

It can be concluded from the data there was apparently no appreciable ³H exchange at 4° since the incorporation of ³H was independent of the time allowed for the exchange reaction to occur. It is unlikely that ³H exchange is an important factor since misincorporation decreased with increasing temperature (Tables 2 and 3) while ³H exchange would be expected to increase with temperature and lead to increased apparent misincorporation.

Kinetics of Polynucleotide Synthesis

The time course of the replication reactions of poly dAT and poly dG:dC was followed by removing aliquots from the assay mixture at time intervals and measuring the incorporation of ³H into the acid insoluble fraction. This was done for both organisms and at both 37 and 55°. The results are shown in Figures 2-5. It can be seen from Figure 2 that the B. licheniformis system in the presence of poly dAT reaches a maximum incorporation in 12 minutes at 37° and in 6 minutes at 55°. The incorporation level remains constant after that at 37° but declines to 60% of the maximum value at 55° by 30 minutes.

The decrease in incorporated ³H after reaching a maximum could be readily explained as a non-enzymatic hydrolysis.
Figure 2: Incorporation Kinetics-Poly dAT

*B. licheniformis

- dATP*, 37°
- dATP*, 55°
- dTTP*, 37°
- dTTP*, 55°

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Figure 3: Incorporation Kinetics-Poly dAT
B. stearothermophilus

- dATP*, 37°
- dTTP*, 37°
- dATP*, 55°
- dTTP*, 55°
Figure 4: Incorporation Kinetics – Poly dG:dC

E. licheniformis

- dGTP*, 37°
- dGTP*, 55°
- dCTP*, 37°
- No detectable dCTP* synthesis at 55°
Figure 5: Incorporation Kinetics—Poly dG:dC

*B. stearothermophilus*

- ○ dGTP*, 37°
- ▲ dCTP*, 37°
- □ dGTP*, 55°
- No detectable dCTP* synthesis at 55°
of the product. This would lower the incorporated radioactivity after synthesis had stopped and would be more rapid at the higher temperature. With the *B. stearothermophilus* system (Figure 3), the incorporation rises linearly for 30 minutes at 37° and for 20 minutes at 55°. Since these reactions were performed in a zero order portion of the substrate concentration curve (because generally the bulk of the substrate which underwent reaction did so in a linear fashion), this would suggest that the deviations from linearity were due to a change in a heat labile substance, such as the enzyme, substrate or template.

Figures 4 and 5, showing the kinetics of the poly dG:dC directed reaction, present similar patterns. In the case of the *B. licheniformis* system (Figure 4), a maximum is reached at 10-20 minutes at 37° for both dGTP* and dCTP* incorporation and the level remains relatively constant to 30 minutes. At 55° the incorporation of dGTP* reaches a maximum in 2 minutes and declines thereafter. There was no measurable dCTP* incorporation at 55°. The *B. stearothermophilus* system (Figure 5) shows a linear rise in dGTP* incorporation for 30 minutes at 37° and for about 12 minutes at 55°. Again there was no detectable dCTP* incorporation at 55°. The incorporation of dCTP* was slight but measurable at 37°.

The incorporation rates, based on the initial linear portion of the curve are given in Table 4.
Table 4: Rates of $^3$H incorporation (dpm/min)**

**B. licheniformis**

<table>
<thead>
<tr>
<th>Deoxynucleotide</th>
<th>$37^\circ$</th>
<th>$55^\circ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGTP*</td>
<td>2000</td>
<td>80</td>
</tr>
<tr>
<td>dCTP*</td>
<td>120</td>
<td>--</td>
</tr>
<tr>
<td>dATP*</td>
<td>7250</td>
<td>21,000</td>
</tr>
<tr>
<td>dTTP*</td>
<td>5900</td>
<td>15,000</td>
</tr>
</tbody>
</table>

**B. stearothermophilus**

<table>
<thead>
<tr>
<th>Deoxynucleotide</th>
<th>$37^\circ$</th>
<th>$55^\circ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGTP*</td>
<td>80</td>
<td>50</td>
</tr>
<tr>
<td>dCTP*</td>
<td>2</td>
<td>--</td>
</tr>
<tr>
<td>dATP*</td>
<td>240</td>
<td>700</td>
</tr>
<tr>
<td>dTTP*</td>
<td>300</td>
<td>1100</td>
</tr>
</tbody>
</table>

** Rates are in dpm incorporated in acid-insoluble fraction per minute of incubation time.
It can be seen that the incorporation rate of dATP* and dTTP* at 55° is roughly three times that at 37°. This is true for both organisms. With dGTP* the situation is different, the incorporation rate at 37° being greater than that at 55°. For *E. licheniformis* this amounts to a factor of 25 and for *E. stearothermophilus* to a factor of 2.

Comparing the average rate of replication of dATP* and dTTP* with that of dGTP* we see that at 37° the ratio (dATP*/dGTP* or dTTP*/dGTP*) is about 3.2 for both organisms. At 55° the ratio has increased to 20 for the thermophile and 220 for the mesophile.

The replication of dATP and dTTP is so rapid that the replication of dGTP and possibly dCTP would be rate limiting for DNA synthesis in the presence of all 4 deoxynucleoside triphosphates. On this basis, and in view of the above rates of replication, one would expect that for the thermophile there would be a more rapid replication of portions of the template rich in (G+C) as the temperature is raised, as compared to the mesophile. This provides one explanation for the differences in NNFA observed in the earlier study (21).

Heat Lability of Incubation Components

The decrease in incorporation rate observed in the previous experiment (Figures 2-5) was interpreted as being due to the presence of one or more heat labile components.
in the incubation mixture. In order to determine which components are heat labile, various components of the incubation mixture were subjected to a pre-incubation of 30 minutes at 55°C before the assay was carried out for 30 minutes at 37°C. Poly dG:dC was used as the template with the enzymes from both organisms. The results are shown in Table 5.

It can be seen that pre-incubation of the enzyme alone results in complete loss of activity for *B. licheniformis* and 95% loss for *B. stearothermophilus*. This would indicate that DNA polymerase from the thermophile is more heat-stable than that of the mesophile. This agrees with findings made in the past concerning this and other thermophilic enzymes (19,21).

The incubation of the template causes a loss of about 60% of the activity, indicating partial but not complete denaturation and/or degradation. The incubation of the nucleoside triphosphates has little effect on the activity, indicating no hydrolysis of the phosphate groups occurs.

When the enzyme is incubated with the template there is complete loss of activity as expected. When the enzyme is incubated with dGTP* there is retention of 35% of the activity with the thermophile but none with the mesophile. Thus the substrate provides a protective action for the enzyme, at least in the case of *B. stearothermophilus*. This suggests that the deoxynucleoside triphosphate binds...
Table 5: Heat Lability of Incubation Components

<table>
<thead>
<tr>
<th>Label</th>
<th>Preincubation</th>
<th>B. licheniformis</th>
<th>B. stearothermophilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGTP*</td>
<td>None</td>
<td>50,000</td>
<td>4590</td>
</tr>
<tr>
<td>dGTP*</td>
<td>Enzyme</td>
<td>76</td>
<td>233</td>
</tr>
<tr>
<td>dGTP*</td>
<td>Template</td>
<td>19,000</td>
<td>1950</td>
</tr>
<tr>
<td>dGTP*</td>
<td>dGTP*</td>
<td>41,300</td>
<td>4110</td>
</tr>
<tr>
<td>dGTP*</td>
<td>Template + Enzyme</td>
<td>43</td>
<td>42</td>
</tr>
<tr>
<td>dGTP*</td>
<td>dGTP* + Enzyme</td>
<td>134</td>
<td>1580</td>
</tr>
<tr>
<td>dCTP*</td>
<td>None</td>
<td>9780</td>
<td>129</td>
</tr>
<tr>
<td>dCTP*</td>
<td>Enzyme</td>
<td>160</td>
<td>---***</td>
</tr>
<tr>
<td>dCTP*</td>
<td>Template</td>
<td>3480</td>
<td>7</td>
</tr>
<tr>
<td>dCTP*</td>
<td>dCTP*</td>
<td>9170</td>
<td>72</td>
</tr>
<tr>
<td>dCTP*</td>
<td>Template + Enzyme</td>
<td>51</td>
<td>--</td>
</tr>
<tr>
<td>dCTP*</td>
<td>dCTP* + Enzyme</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Values expressed as dpm

* Labeled with tritium (³H)
** Less than control value
to the enzyme in the absence of the template. With dCTP*
the levels of incorporation are too low to allow accurate
measurement and no statement can be made about its possible
protective role.
SUMMARY

DNA polymerase from *Bacillus licheniformis*, a mesophile, and *Bacillus stearothermophilus*, a thermophile, was used to study the misincorporation of deoxynucleoside triphosphates into the synthetic polynucleotides poly dAT and poly dG:dC as a function of temperature.

Misincorporation was apparent with both polynucleotides and both enzymes and in the presence of one, two or three unlabeled deoxynucleoside triphosphates. A study of the type of misincorporation showed that dGTP misincorporation into poly dAT occurred more often than any other type of misincorporation. There was de novo synthesis of poly dG and poly dAT which accounted for from 25 to 50% of the total misincorporation. Tritium exchange between deoxynucleoside triphosphates was shown to be of negligible importance.

A kinetics study showed that the replication of dGTP was rate limiting and that at 55°C the *B. stearothermophilus* system was relatively more efficient than the *B. licheniformis* system in replicating dGTP.

Both the enzyme and the template, particularly the former, were shown to be heat labile. The thermophilic enzyme could be partially stabilized by the addition of dGTP.
BIBLIOGRAPHY


7. Aposhian, H.V. and Kornberg, A., J. Biol. Chem., 237, 519 (1964)


VITA

The author was born on October 28, 1943 in Lawrence, Michigan. He was educated in the public school system in Lawrence. He attended Western Michigan University and received his B.A. in chemistry from the University of Hawaii in 1966. He attended Georgia Institute of Technology in 1966 and was employed by Chris Craft Corporation in Pompano Beach, Florida from 1967 to 1970. He enrolled in the Masters Degree program at Western Michigan University in 1970.