The program makes use of a sampling network for precipitation, organized jointly with the World Meteorological Organization. In addition to providing data as a basis for future studies, the work in progress is yielding immediate results in the field of hydrology of arid regions and in regard to problems of water supply and water contamination in developing countries.

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Active Center of DNA Polymerase

The operations are localized and arranged in multiple sites within a single area of the molecule.

Arthur Kornberg

DNA polymerases have now been isolated from a variety of bacterial and animal cells. These enzymes, including those produced specifically in response to virus infection, catalyze the addition of mononucleotide units to the 3′-hydroxyl terminus of a primer DNA chain. Synthesis therefore proceeds in the direction of 5′ to 3′ (Fig. 1) (*1*). There is an absolute requirement for a DNA template, and errors in copying the template are very infrequent. The synthesis of DNA proceeds rapidly, at rates near 1000 nucleotides per minute per molecule of enzyme, with the production of chains several million in molecular weight.

The polymerases are remarkable enzymes. A polymerase takes instructions as it goes along to build a chain according to specifications by a template. Bacterial DNA polymerase will make animal DNA and animal polymerase will make bacterial DNA. The DNA polymerase from *Escherichia coli* has additional catalytic properties. It may degrade DNA progressively from either end (5′ or 3′) of the chain by hydrolysis to produce deoxyribonucleoside monophosphates. Or it may degrade a chain by pyrophosphorolysis with inorganic pyrophosphate to produce deoxyribonucleoside triphosphates.

Until recently we understood little about how this enzyme works because we did not know enough about its physicochemical properties. We knew very little because our supplies of homogeneous enzyme were meager. Now with a simpler method for purification (*2*) and the invaluable use of the large-scale facilities of the New England Enzyme Center, we have had available 600 milligrams of homogeneous DNA polymerase obtained from 200 pounds (90 kilograms) of *E. coli*. The purpose of this article is twofold: (i) to assemble in a brief form the new physicochemical and functional observations concerning the pure enzyme; and (ii) to attempt an interpretation of these data in a model for the active center of the enzyme. This model is of course speculative, but it has helped us reconcile many hitherto confusing details and continues to suggest useful experiments.

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Physicochemical Properties

The molecular weight of the homogeneous polymerase, determined by sedimentation equilibrium, is 109,000 (*2*). This large molecular weight and the presence of both polymerase and multiple nuclease activities suggest a subunit structure. However, the molecular weight measured by sedimentation equilibrium under denaturing and reducing conditions (6*M* guanidine hydrochloride and 0.3*M* mercaptoethanol) was found to be the same as that of the native protein. Optical rotatory dispersion and velocity sedimentation studies showed that polymerase loses ordered structure in 6*M* guanidine hydrochloride, and would therefore be expected to be fully dissociated in this solvent.

More than 95 percent of the protein migrated as a single band on polyacrylamide-gel electrophoresis at *p*H 3.5, *p*H 8, and *p*H 11 (with or without 7*M* urea at *p*H 3.5 and *p*H 8). This result is most consistent with a structure composed of either a single polypeptide chain or of two or more identical subunits. However, the possibility of multiple, identical subunits is ruled out by the fact that polymerase contains, per 109,000 molecular weight, a single sulfhydryl group and a single disulfide group. The sulfhydryl group is probably not part of the active site

because it can be modified either with iodoacetate or mercuric ion to give derivatives with full polymerase and exonuclease activity (*3*). The reaction with mercuric ion will give either a polymerase monomer, with one mercury atom per protein molecule, or, in the presence of a molar excess of enzyme, a dimer, with two protein molecules linked through a mercury atom. The dimer also has full activity. (The reaction of polymerase with a single atom of ^{203}Hg provides a convenient way of incorporating a radioactive label of about 10,000 counts per minute per microgram of enzyme, wtihout affecting enzymatic activity. This label has served as a marker in DNA binding studies which will be described below.)

Amino acids (approximately 1000 per enzyme molecule) account for the dry weight. There is no evidence for any prosthetic group. The amino terminal residue, as determined by both the cyanate and the fluorodinitrobenzene procedures, is methionine.

Although these experiments indicate that DNA polymerase is a single polypeptide chain, the possibility still exists that there are subunits joined by nonpeptide linkages that resist disruption by guanidine hydrochloride–mercaptoethanol, or by urea, or by extremes of *p*H. The presence of blocked amino termini has also not been ruled out. There have been two recent reports of *E. coli* polymerase preparations with molecular weights in the range of 30,000 and 50,000, and these have been designated as possible subunits by Cavalieri (*4*) and by Lezius (*5*). However, their preparations are of relatively low specific activity and have not yet been fully characterized. If DNA polymerase is assumed to be roughly spherical, its diameter is calculated to be near 65 angstroms. The diameter of a DNA helix is about 20 angstroms.

Table 1. Influence of DNA structure on binding of DNA to DNA polymerase.

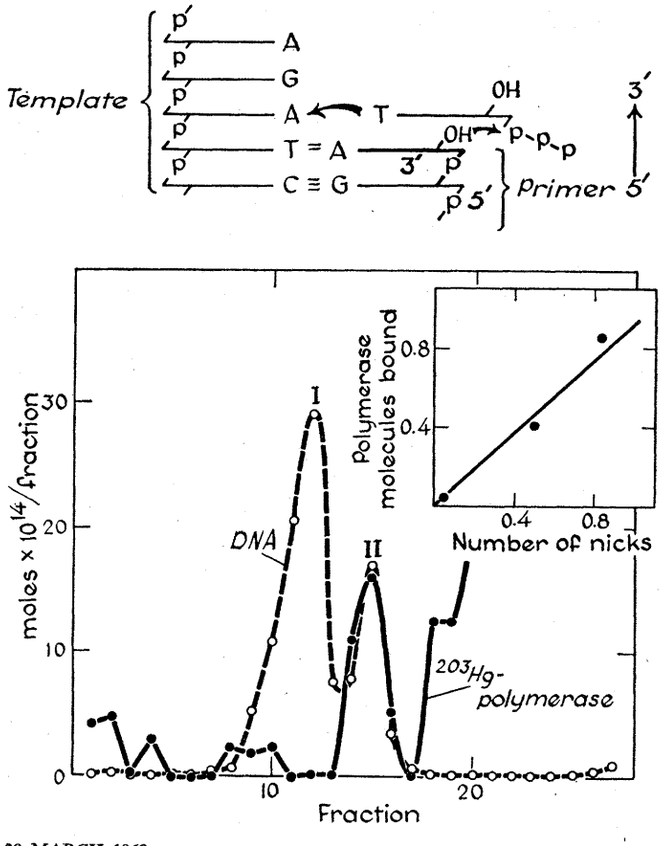
Conformation	Per DNA molecule: Nicks or ends	Per DNA molecule: Polymerase molecules bound
$d(AT)_{12}$ oligomer		
Hairpin	1	1
ØX174 DNA		
Circular, single strand	0	20
Closed circular, duplex	0	<0.1
Plasmid DNA		
Irreversibly denatured	0	21
3′-Hydroxyl nick	1	1
3′-Phosphate nick	5	6
T7 DNA		
Linear duplex	2.5	2.6
Single strand	2	240

DNA Binding Site

DNA binding to DNA polymerase (*6*) was studied with a variety of DNA structures (Fig. 2). The alternating copolymer of deoxyadenylate and deoxythymidylate (dAT) was partially digested by deoxyribonuclease. Oligomers were isolated from these digests either by gel filtration or by polyacrylamide-gel electrophoresis. Preparations were obtained with chain lengths of approximately 24 and 40 nucleotide residues [$d(AT)_{12}$ and $d(AT)_{20}$]; they were induced to assume "hairpin" conformations by melting and quick cooling at low ionic strength.

Binding was measured by sucrose density-gradient centrifugation of mixtures containing DNA labeled with ^{3}H or ^{32}P and polymerase labeled with ^{203}Hg. The mixtures were layered on top of the gradients, and the enzyme-DNA complexes were identified after sedimentation.

With excess enzyme, $d(AT)_{12}$ sedimented almost quantitatively with the enzyme, an indication of a very high binding affinity. The polymerase-dAT complex sedimented at 7.7*S*, compared to 6.1*S* for the free enzyme. With the dAT oligomer present in excess, all the enzyme sedimented as a complex



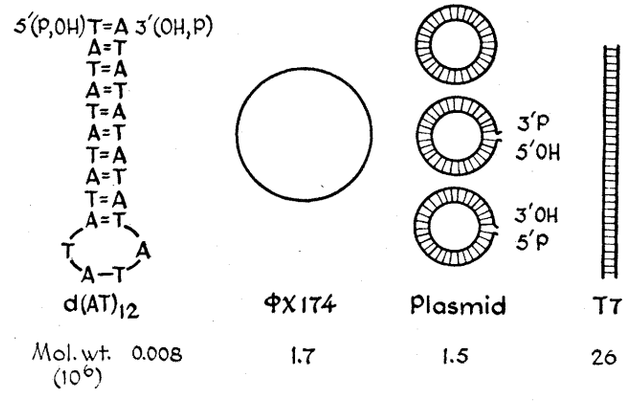


Fig. 1 (top left). Direction of chain growth catalyzed by DNA polymerase. Abbreviations are: A, adenine; C, cytosine; G, guanine; and T, thymine.

Fig. 2 (above). Various DNA structures used in studies of enzyme binding.

Fig. 3 (left). Binding of nicked plasmid DNA to the enzyme. Peak I contains intact, duplex, circular DNA in the supercoiled form; peak II contains the nicked form which is bound by polymerase. The inserted graph gives the number of polymerase molecules bound as a function of the number of nicks introduced by pancreatic deoxyribonuclease.

at 7.7*S* with an equimolar amount of dAT, an indication that DNA polymerase contains a single binding site for the dAT oligomer. Results identical to these were obtained with $d(AT)_{20}$. No difference was detected in the binding of oligomers terminated with a 3′-hydroxyl as compared with a 3′-phosphate group.

Binding of single-stranded circular ϕX174 DNA by polymerase resulted in about 20 enzyme molecules per molecule of DNA in the complex. A double-stranded, closed, circular plasmid DNA (*7*) was not bound at all by polymerase. However, when these duplex forms were denatured to make them single-stranded, they were bound by polymerase in proportion to their length and to the same extent as the single-stranded viral ϕX174 DNA.

We have introduced nicks into the plasmid DNA or the ϕX174 replicative form with pancreatic deoxyribonuclease. Such nicks have 3′-OH and 5′-P termini and are active points for replication (*8*). Nicks introduced with micrococcal nuclease produce 3′-P and 5′-OH termini and are not active points for replication; they inhibit replication (*9*). Regardless of the kind of nick, polymerase molecules formed complexes in numbers exactly equivalent to the number of nicks (Fig. 3, insert). The sedimentation pattern (Fig. 3) indicates that the polymerase molecules bound the nicked forms (II) and not the intact double circular forms of the plasmid (I).

The binding of DNA structures to polymerase is summarized in Table 1. There is no binding at all at helical regions. There is binding along single-stranded chains and to nicks and ends. In the case of the linear duplex DNA of bacteriophage T7, our preparation contained, on the average, one nick per two molecules; this explains why 2.6 molecules of the enzyme are bound per T7 DNA molecule.

Is binding of DNA at ends or nicks simply a consequence of fraying and single-strandedness in these regions, or is there more specific binding directed to the nucleotide termini at these points? We will assess this question in the formulation of a model for the active center of the enzyme.

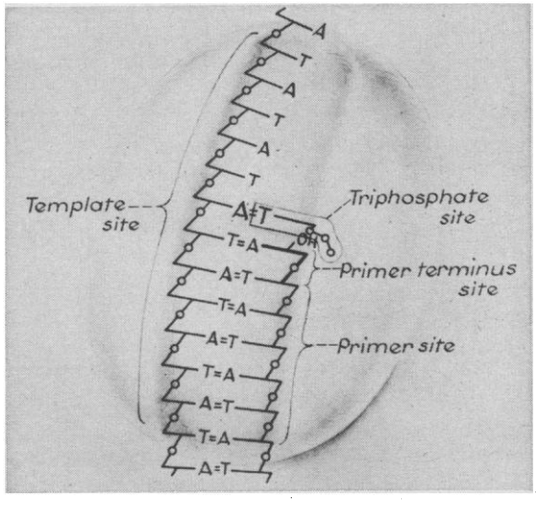
Deoxyribonucleoside Triphosphate Binding Site

Is one, or more than one, molecule of deoxyribonucleoside triphosphate bound to a polymerase molecule? This and related questions are crucial to understanding the nature of the active center and the mechanism of polymerase action.

Triphosphate binding was studied by equilibrium dialysis (*10*). Scatchard plots for triphosphate binding showed that there is one binding site for each triphosphate and that the dissociation constants for the enzyme-triphosphate complexes were 12, 33, 81, and 147 micromoles per liter for deoxyguanosine, deoxyadenosine, deoxythymidine, and deoxycytidine triphosphates, respectively. Although the four triphosphates differ in the affinity of their binding, the interpretation of these particular values is necessarily limited. DNA template and primer were not present, and their influence on binding, which is likely to be profound, has yet to be assessed.

Is there a separate site for each of the four triphosphates, or does the enzyme have a single site for which all four triphosphates compete? Equilibrium dialyses were run with each of the six possible combinations of two triphosphates, and each of the pair was labeled distinctively. These competition experiments established that there is a single binding site on the enzyme for which all four triphosphates compete. Further explorations (*10*) of the specificity of binding in this site emphasize the primary importance of the triphosphate moiety and the only secondary importance of the sugar and base components.

The effects of DNA template and primer on the binding of triphosphates are difficult to study experimentally. An active template and primer invariably promote polymerization or are degraded, and binding measurements are thus complicated. Among the questions to be answered are whether a template confers specificity on triphosphate binding and what influence the primer terminus exerts on the entry and orientation of the triphosphate in the site.



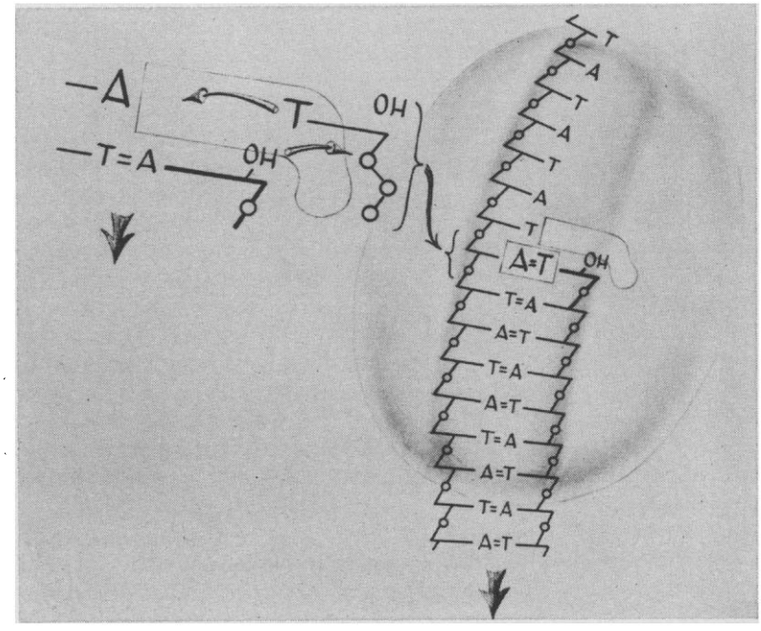


Fig. 4 (above). Sites in the active center of DNA polymerase. Fig. 5 (right). Mechanism of the polymerization step.

Multiple Functions of the Enzyme

At this point I would like to relate the results of the binding experiments to what we have learned about the catalytic properties of the enzyme in order to begin constructing a model of the many operations that may take place in the active center of the enzyme. These operations include: (i) the 5′ → 3′ growth of a DNA chain by the polymerization of nucleotides; (ii) hydrolysis of a DNA chain from the 3′-OH end (3′ → 5′ direction); (iii) hydrolysis of a DNA chain from the 5′ end (5′ → 3′ direction); (iv) pyrophosphorolysis of a DNA chain from the 3′ end; and (v) exchange of inorganic pyrophosphate (PP_i) with the terminal pyrophosphate group of a deoxyribonucleoside triphosphate.

We picture the active center of the enzyme as some specially adapted polypeptide surface that recognizes and accommodates several nucleotide structures (Fig. 4). We will present evidence that, within the active center, there are at least five major sites. (i) There is a site for a portion of the template chain. This area binds the chain where a base pair is formed and for a distance of several nucleotides on either side of it. The chain is oriented with a particular polarity. It seems likely that this is the site where circular, single-stranded DNA is bound, but we are uncertain whether this site recognizes an extended or a tightly stacked, helical conformation. (ii) There is a site for the growing chain, the primer. The primer is oriented with a polarity opposite that of the template. (iii) There is a site with special recognition for the 3′-OH group of the terminal nucleotide of the primer, the primer terminus. We shall discuss this region later as a site for the hydrolytic and pyrophosphorolytic cleavage of the 3′-OH–terminated primer chain (3′ → 5′ direction). (iv) There is a site for a triphosphate, and (v) there is an additional site, to be considered later, which provides for hydrolytic cleavage of the 5′-P–terminated chain (5′ → 3′ direction).

The Polymerization Step

When a linear duplex is partially degraded from each 3′-OH end, as for example by the action of certain exonucleases, these denuded portions are repaired with great facility by all DNA polymerases (*11*). How is this accomplished?

The triphosphate is bound adjacent to the 3′-OH group on the terminal nucleotide of the primer and oriented so that it can be brought into direct contact and form a base pair with the template (Fig. 5). When the 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. A plausible model, for reasons to be mentioned below, assumes that movement or translation of the chain relative to the enzyme is concurrent with diester bond formation. As the primer terminus loses its 3′-OH group during transformation into a diester bond, it is no longer held in the primer terminus site. Through movement of the entire chain, the old primer terminus is replaced by the newly added nucleotide, which has a terminal 3′-OH group and is therefore held in the primer terminus site. (The new primer terminus is now ready to attack another triphosphate and add the next nucleotide.) Inorganic pyrophosphate is displaced only as formation of the diester bond is being completed, and the chain movement is translating the newly added nucleotide into the primer terminus site.

The possibility has been raised that the interaction between template and triphosphate is not direct, but rather allosteric in nature. However, since there is only one triphosphate binding site, it is difficult to imagine this site assuming four conformations, each absolutely specific for one of the triphosphates.

The basis for specificity of DNA polymerase very likely is not in the recognition by the enzyme of an incoming triphosphate, but rather in its demand for one of the four base pairs. All of the Watson-Crick base pairs contain regions of identical dimensions and geometry and are symmetrical. When the correct base pair is within the active site, the enzyme may respond, possibly by a change in conformation, so that the subsequent catalytic steps can then proceed. If an

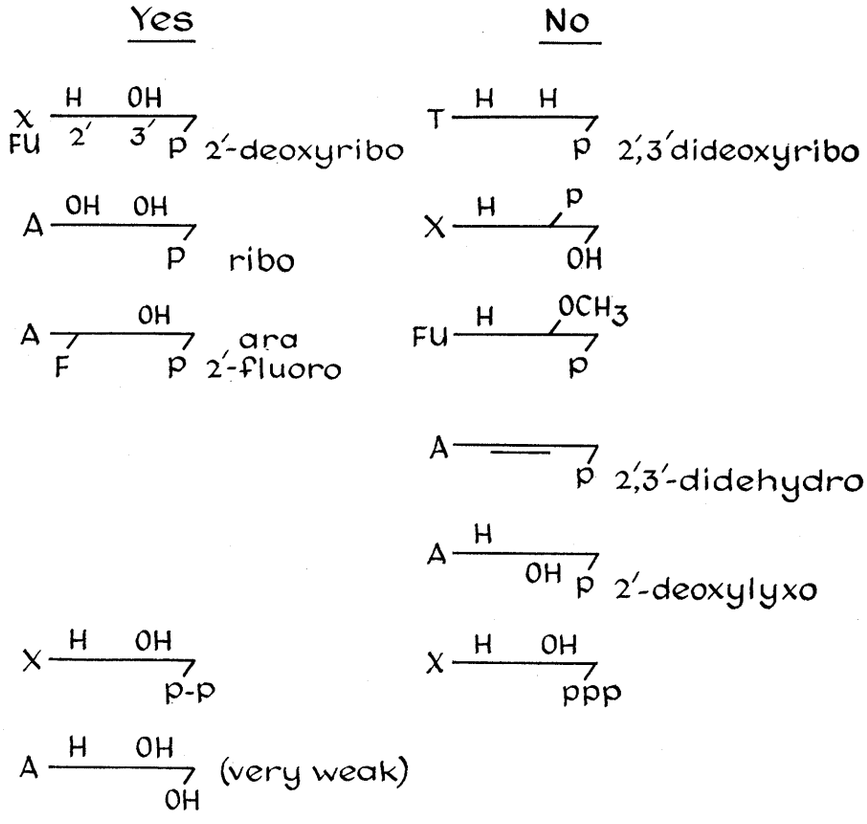
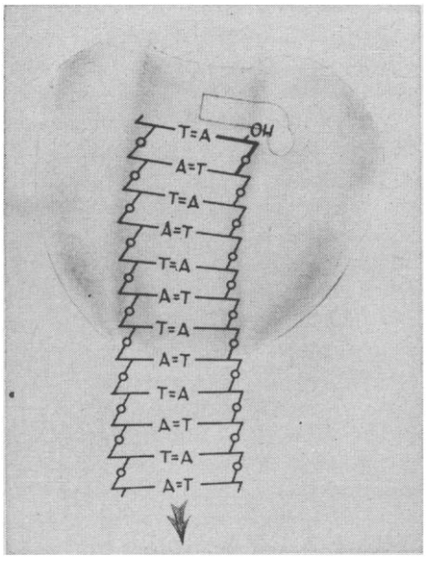


Fig. 6. Binding of monophosphates to DNA polymerase. Abbreviations are: X, the bases A, T, G, and C, as in the case of the 2′-deoxyribonucleoside 5′-monophosphate ($X\overset{H\ OH}{\underset{P}{\longrightarrow}}$); FU, fluorouracil; F, fluorine; ara, arabinosyl; and lyxo, lyxosyl.



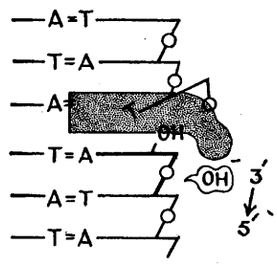


Fig. 7 (left). Binding of the end of a linear helix to the enzyme.

Fig. 8 (above). Binding of a nicked region of DNA to the enzyme.

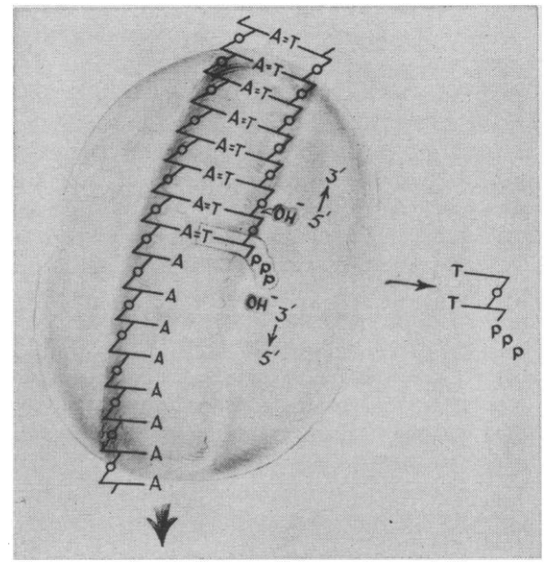


Fig. 9 (right). Scheme for 5′ → 3′ exonucleolytic degradation by the enzyme.

incorrect triphosphate were to bind to the enzyme, the correct base pair could not be formed, there would be no conformational change, and the triphosphate would be rejected.

Primer Terminus Site

Evidence for the specificity of the primer terminus site comes from studies of the binding and functional behavior of several nucleotide analogs. Among the analogs studied are those that lack a 3′-OH group. Such an analog, if added to a chain, would prevent further chain growth (*12, 13*). Dideoxythymidine triphosphate is one example. It binds to the triphosphate site. One nucleotide is added per chain. The chain terminated with such a dideoxynucleotide is inert to further elongation; it is relatively inert to exonuclease action at the primer terminus end of the chain. In such a chain, as will be mentioned presently, attack by PP_i is inhibited. However, the chain can be degraded from the 5′ end (5′ → 3′ direction).

We interpret these results to mean that, without a 3′-OH group, the chain cannot bind properly in the primer site and is therefore not an effective substrate for exonuclease action. In keeping with this interpretation are the results of the following studies of the binding of monophosphate in what may prove to be the primer terminus site (*14*).

Each of the four common deoxyribonucleoside monophosphates binds to and competes for a single site on the enzyme. This site is, however, entirely distinct from the triphosphate site. Replacement of the 3′-OH group—by hydrogen as in dideoxythymidine monophosphate, by esterification with phosphate, or by *O*-methylation—prevents binding to this site. However, many other alterations in the nucleotide can be tolerated, provided that the 3′-OH group is in the "ribo" configuration (Fig. 6). For example, a certain arabinosyl nucleotide binds to the monophosphate site, whereas lyxosyl nucleotide does not.

In support of the interpretation that the 3′-OH monophosphates bind at the primer terminus site is the finding that these monophosphates inhibit hydrolysis of polynucleotides from the 3′ end of the chain. Other lines of evidence (*15*) have made it clear that there is only one primer terminus site serving for either polymerization or 3′ → 5′ hydrolysis.

Completion of a Linear or Circular Duplex

Picture a duplex in which template copying has been completed. What potential does such an intact, linear, double-stranded helix have for further replication? Consider a polymerase molecule which binds the end of such a linear duplex (Fig. 7). The primer strand is in its site with the 3′-OH group oriented in the primer terminus site; the strand is hydrogen-bonded to its complementary strand which is in the template site. But the template strand extends only as far as the primer terminus (Fig. 7). When a triphosphate enters the triphosphate site, there is no purine or pyrimidine base to serve as a template and thus no replication can take place. An intact linear duplex must therefore be inert.

Thus it appears that the replication of a linear duplex from its terminus, as pictured in the original Watson and Crick model, should not apply to DNA polymerase action in vitro, even for one of the strands. In recent studies (*16*) the DNA of phage T7 was prepared with care to avoid any internal breaks in this linear duplex and this DNA was essentially inert in supporting replication.

A special case of template copying is the replication of single-stranded circular DNA, such as the viral DNA of ϕX174 (*17, 18*). The circle provides no primer terminus and initiation of new strands by the enzyme does not take place readily (*18*). Therefore addition of an oligonucleotide which can anneal to the circle promotes replication by furnishing the necessary primer terminus (*19*). Copying then proceeds rapidly around the circle. The product is an incomplete circle. However, if a joining enzyme, called ligase, is present, the diester bond between the 3′-OH and 5′-P termini is made, and a fully covalent, double-stranded circle is produced (*20*). This synthetic molecule, as well as the double, circular molecules isolated from nature (Replicative Form I) do not bind to polymerase and are inert for replication.

Nicked Helix as the Functional Template Primer

Circular duplexes serve in vivo as chromosomes in bacteria (*E. coli* and *Bacillus subtilis*) and in viruses (polyoma), as replicative intermediates for other viruses (ϕX174, M13, and λ), and as bacterial episomes (*21*). Studies of ϕX174 replication indicate that a nicked form is the active replicative intermediate in vivo (*22*). Whereas the closed circular form is not replicated by DNA polymerase in vitro, the introduction by pancreatic deoxyribonuclease of one single-strand nick enables this DNA to bind to a polymerase molecule and converts the DNA to a favorable template and primer for replication (*16*). After replication has been initiated by introduction of a nick, the product, early or later in replication, is associated with the nicked form and is covalently linked to it (*16*).

As indicated earlier, an intact linear duplex, such as the DNA of phage T7, does not support replication. Upon the introduction of nicks by pancreatic deoxyribonuclease, the binding of these nicked duplexes to polymerase molecules and the appearance of productive sites for replication increase in direct proportion to the number of nicks introduced. In this instance, too, at least 90 percent of the DNA product is covalently attached to the primer (*16*).

How can the binding of a nicked region be visualized in the active center of the enzyme? The template and primer sites are filled. But the triphosphate site may not be vacant, and growth of a chain from the primer terminus is obstructed by the presence of the 5′-P–terminated strand, hydrogen-bonded to the template strand (Fig. 8). This dilemma might be resolved temporarily by the 3′ → 5′ exonuclease activity of the polymerase. Hydrolytic removal of the primer terminus nucleotide, accompanied by movement of the chain upward one nucleotide would open the triphosphate site for insertion and addition of a triphosphate. However, were this succession of events to take place nothing more would be achieved than the restoration of the original nicked region.

For progressive replication of the template to take place, the 5′-P–terminated strand must be displaced. Our evidence indicates that during the first phase of replication there is in fact a burst of hydrolysis of the template-primer, roughly matching the extent of replication (*23*). This hydrolysis is predominantly from the 5′ end of the DNA and entails degrading the chain from 5′ → 3′. The locus of this hydrolytic function appears to be distinct from that responsible for 3′ → 5′ hydrolysis.

Distinctive Sites for 3′ → 5′ and 5′ → 3′ Hydrolysis

Klett, Cerami, and Reich (*24*) were the first to recognize that polymerase preparations contained an exonuclease activity which degraded from the 5′ end of a chain. Their conclusions were based on studies with a synthetic block polymer resistant to hydrolysis from the 3′ end of the chain. A similar discovery was made independently when we tried to explain how DNA with 3′-P termini (introduced by micrococcal nuclease) and presumably insensitive to 3′ → 5′ exonuclease action was nevertheless extensively degraded (*25*). It became clear that such a 3′-P–terminated chain is degraded exclusively from the 5′ end. The principal products of extensive hydrolysis proved to be mononucleotides and an oligonucleotide which bore the 3′-P terminus.

With the recognition of this new property of DNA polymerase, an interesting possibility was raised. If a DNA chain were initiated *de novo* by DNA polymerase, its starting 5′ terminal should, as in the case of RNA polymerase, be marked by the initiating triphosphate. Yet attempts to identify such a triphosphate initiation point have not succeeded. It seemed possible that the 5′ → 3′ exonuclease activity of polymerase might act also on a chain terminated in a 5′-triphosphate and would therefore have erased a terminal triphosphate group even if it had been present initially.

In order to test this possibility, a polydeoxythymidylate of about 300 residues was synthesized containing a ^{32}P-labeled triphosphate group at the 5′ terminus and ^{3}H in the thymidine residues (*26*). This polynucleotide was degraded by polymerase from the 3′ end but *not* from the 5′ end. However, when annealed to form a helix wtih a polydeoxyadenylate chain, it was degraded rapidly from both ends. After an incubation period limited to only 10 seconds, 90 percent of the ^{32}P was liberated from the polymer, whereas less than 5 percent of the ^{3}H was released. Most remarkably, the principal ^{32}P product proved to be not deoxythymidine triphosphate as expected, but instead a dinucleoside tetraphosphate (Fig. 9).

We interpret these results as follows. There is a hydrolytic site for progressive 5′ → 3′ release of mononucleotides from the 5′ end of a strand in a location just above the triphosphate site. When the chain is terminated in a triphosphate, the close resemblance of this terminus to a deoxyribonucleoside triphosphate directs its binding in the triphosphate site. As a consequence, the initial product is not a mononucleotide but rather a dinucleotide, the dinucleoside tetraphosphate. Subsequently as the chain moves downward one nucleotide at a time the products are principally mononucleotide residues.

The fact that the 5′ → 3′ degradation

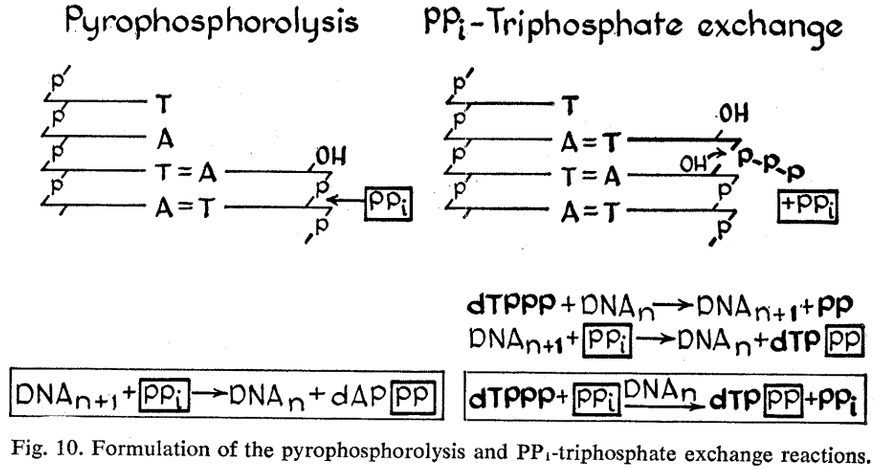


Fig. 10. Formulation of the pyrophosphorolysis and PP_i-triphosphate exchange reactions.

requires a helical structure indicates that this site cannot properly orient a single-stranded oligonucleotide chain. Because the site is occupied when such a chain is annealed to a complementary strand, we infer that the enzyme accommodates this complementary strand in the upper region of the template site.

The failure of the enzyme to degrade a 5′-terminated chain (from 5′ → 3′) when it is single stranded may help explain the stage in replication of helical DNA when synthesis proceeds without concomitant 5′ → 3′ hydrolysis. As the 3′-OH chain advances by growth along the template, the 5′ chain may be displaced from the active site for a stretch of several nucleotides and thus be rendered insusceptible to hydrolysis. The 5′ chain may be peeled back until some point when, for obscure reasons, it competes successfully for the template function by attracting the growing chain to switch templates (as in Fig. 13). Such a sequence of events would produce a covalent fork in the growing chain and has been suggested (*27*) as part of a mechanism to account for the branched structure and readily renaturable character of DNA synthesized on a helical template primer.

Pyrophosphorolysis and Pyrophosphate Exchange

Pyrophosphorolysis, the capacity of DNA polymerase to degrade DNA chains with PP_i, reaches a steady state when the accumulation of triphosphates supports synthesis at a rate that balances their removal (*15*). The enzyme also supports the exchange of PP_i into the β, γ groups of a triphosphate (*15*). This reaction can occur with only a single triphosphate present, but otherwise requires all the primer and template conditions demanded of replication (Fig. 10). All the evidence fails to suggest the formation of a nucleotidyl-enzyme intermediate.

The behavior of chains terminated with a dideoxynucleotide suggests a mechanism of pyrophosphorolysis and PP_i exchange (*12, 15*). Such chains are relatively insusceptible to degradation by nucleophilic attack of PP_i, just as they are to that of OH^- (nuclease). Furthermore, a triphosphate analog lacking a 3′-OH group supports little, if any, PP_i exchange. Inasmuch as such an analog can be added to a chain, it would seem that PP_i exchange with the triphosphate does not occur entirely in the triphosphate site. Rather, the lack of a 3′-OH group prevents binding of the transition state formed by attack of the primer terminus on the triphosphate, and the terminal nucleotide in this transition state cannot be displaced efficiently by PP_i or by OH^-.

Pyrophosphorolysis is therefore taken to be a reversal of the polymerization step, including the concerted chain movement. Inorganic pyrophosphate exchange appears to be the result of a sequence of a polymerization step and a pyrophosphorolytic step repeated many times over (Fig. 10). Because the rate of PP_i exchange is considerably faster than that of pyrophosphorolysis (*15*), we suggest that the attack by PP_i occurs at a transition state short of completion of the polymerization step, and that this transition state is attained more readily from the direction of polymerization.

Insights from an Enzyme Modified by Acylation

Chemical alterations of DNA polymerase are beginning to provide important clues about its structure and function (*3*). Inasmuch as alkylation or Hg substitution of the single sulfhydryl group of DNA polymerase does not alter any of its activities, we assume that this group is relatively remote from the active center. However, acylation of the enzyme with *N*-carboxymethylisatoic anhydride results in a highly fluorescent derivative with markedly altered functional properties.

A derivative with 11 *N*-carboxymethylisatoyl groups had only 0.2 percent of the original polymerase activity but had 920 percent of the exonuclease activity when measured with DNA as primer or substrate at *p*H 7.4. Binding measurements and kinetic studies indicated that a major effect of this modification was a marked reduction in the affinity of deoxyribonucleoside triphosphate substrates for their site. Possibly, one or a few of the 61 lysine residues in the enzyme are located in this site, and their acylation had severely damaged the function of the site.

The remarkable increase in exonuclease activity implies that concomitant changes in the interaction of the enzyme with DNA are probably also involved. It remains to be determined whether one or both of the nuclease activities have been modified and how closer studies of the altered enzyme with various DNA's as substrates may elucidate the nature of the active center.

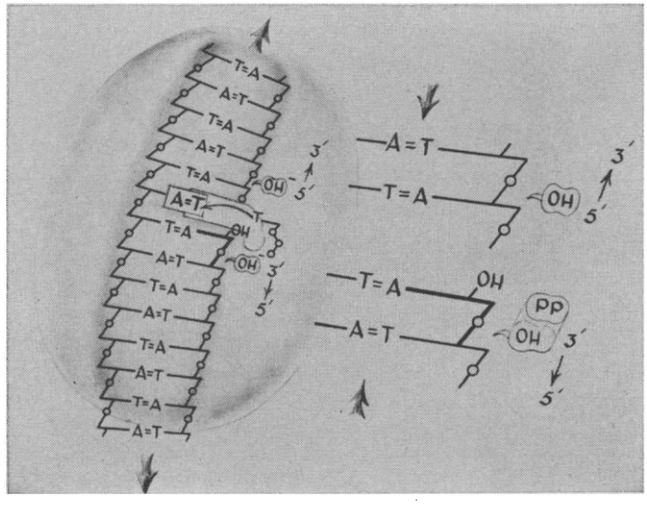


Fig. 11 (left). Model to account for the multiple functions of DNA polymerase within a single active center.

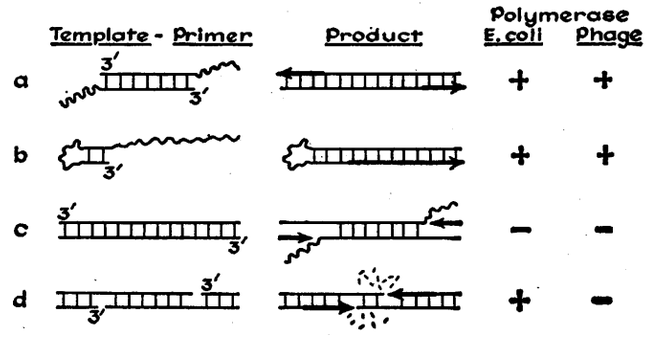


Fig. 12 (above). Template-primer requirements of the *E. coli* and phage-induced DNA polymerases.

Multiple Sites in One Active Center

A recapitulation of the several functional and physicochemical features of DNA polymerase is provided in a model proposed in Fig. 11. The template and primer functions in polymerization, the distinctive $3' \rightarrow 5'$ and $5' \rightarrow 3'$ exonuclease activities, and the various binding capacities of DNA are oriented in one active center. While alternative models have been considered (*28–31*), and some of these still have attractive features, the one we offer here goes farthest in explaining available data. A model by Beyersmann and Schramm (*28*), based exclusively on kinetic data, proposes an identical site for pyrophosphorolysis and hydrolysis and one active center for the degradative and polymerization functions of the enzyme. However, their model also proposes the pyrophosphorolysis of 3′-P–terminated chains and roles for specific inhibitors that are inconsistent with our formulation.

Yet, our model, even if proven correct in basic outline, is still far too simple to account for many of the enzyme's features. For example, the capacity of the enzyme to discriminate secondary structure both in polymerization and in nuclease activities will require a more striking three-dimensional recognition of template and primer strands within the active center. To accommodate the screwlike translation of an essentially helical structure through the active center would demand a surface adapted to one or more turns of the helical duplex. The important influences of specific metal cations, ionic strength, and temperature on the enzyme functions have not even been considered. Finally, it seems likely that enzymes and factors related to the cellular functions of DNA polymerase will interact with it, at or near the active center, in the regulation of these functions.

Comparison of Various DNA Polymerases

Studies analogous to those described here for the *E. coli* enzyme have not yet been carried out on DNA polymerase from other sources. However, a comparison of the template-primer requirements of the *E. coli* enzyme with those of enzymes induced by infection with phages T2 (*8*), T4 (*32*), and T5 (*33*) is of considerable interest

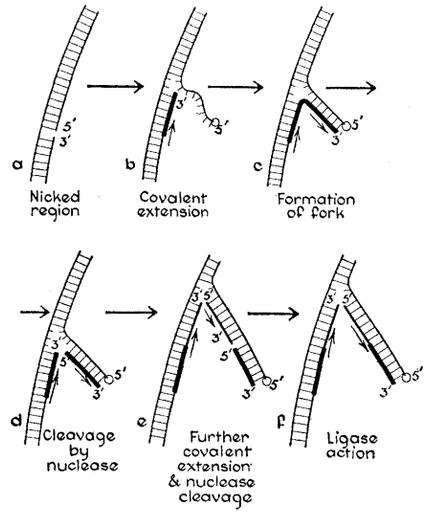


Fig. 13. Speculative scheme for unidirectional replication of a duplex chain.

(Fig. 12). Both the bacterial and the phage polymerases can utilize a double-stranded DNA helix, partially denuded from each 3′-OH end by exonuclease III (Fig. 12a). The helix is restored by replication to its original length by extention of the 3′ strands at each end. Similarly, both types of enzyme replicate single-stranded DNA. The latter, upon forming a looped structure with a short 3′-OH end, is converted by replication to a hairpin-like product (Fig. 12b).

The bacterial enzyme was regarded as different from the phage-induced polymerases in its apparent ability to utilize a linear segment of double-stranded helical DNA. This difference had been ascribed to the unique capacity of the *E. coli* enzyme to initiate a DNA strand *de novo* with the 3′-OH strand of the DNA duplex as template (Fig. 12c). It seems likely from the foregoing discussion that this earlier interpretation (*34*) was mistaken and that neither kind of polymerase can employ an intact linear duplex.

What seems clearer now is that a segment of duplex DNA which serves as template-primer for the *E. coli* enzyme does so by virtue of the nicked region that it contains and not by supporting new chain synthesis at its ends (Fig. 12d). It follows therefore that the phage-induced enzymes cannot exploit such nicked regions in the DNA. In support of this formulation are the long-standing observations that "activation" of duplex DNA by pancreatic deoxyribonuclease (that is, introduction of 3′-OH nicks) increases its template-primer capacity 10- to 20-fold for the *E. coli* enzyme while providing no significant improvement for the phage-induced enzymes (*8*).

How does one explain the inability of phage-induced enzymes to replicate at a nicked region of a duplex? Let us assume that the $5' \rightarrow 3'$ degradation by the $5' \rightarrow 3'$ nuclease function of the *E. coli* enzyme in the nicked region clears a path for the advancing synthetic chain, and that this is an essential step in the initiation of replication in vitro. Although the phage-induced polymerases are known to degrade polynucleotides from a $3' \rightarrow 5'$ direction, they may not possess the $5' \rightarrow 3'$ nuclease activity. Recent tests (*26*) show that there is, in fact, a striking absence of this $5' \rightarrow 3'$ activity in the phage T4-induced enzyme. Perhaps such a nuclease activity is present in the phage-infected cell, but has been eliminated on purification of the polymerase.

Inasmuch as the phage-induced DNA polymerase, as well as that from *E. coli*, has the $3' \rightarrow 5'$ nuclease activity, is there some physiological purpose attributable to this function? Again we can only conjecture. It appears that both nuclease activities are favored by some destabilization of the tight helical structure (*23*). Removal of DNA at or near disordered regions such as those produced by irradiation might then be performed by one of the nuclease activities. There is also the intriguing possibility that $3' \rightarrow 5'$ hydrolysis provides an opportunity for double-checking and editing to eliminate any newly polymerized nucleotide member of a poorly stacked or faulty base pair.

A Hypothetical Scheme for Replication Process in vivo

How might DNA polymerase serve a physiological role in replication? What follows is speculative. Bacterial chromosomes are duplex circular structures and, when intact, are inert in replication. Introduction of a nick, possibly at a specific site, starts replication. DNA polymerase binds at the nick and replication proceeds by covalent extension of the 3′-OH end (Fig. 13, a and b). The 5′ end may be degraded to some extent by $5' \rightarrow 3'$ nuclease action, or, if displaced, is freed from further attack. Fixation to some membrane site (*35, 36*) may facilitate displacement and preservation of the 5′ strand.

Replication 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 (Fig. 13, c and d). A repetition of this sequence leads to interruptions or small pieces of DNA near the replicating fork (Fig. 13e). Such pieces have been isolated by Okazaki and co-workers (*37*) at or near the nascent replicating region. These interruptions are sealed by ligase (Fig. 13f).

If this scheme were essentially correct, it would explain how one enzyme, DNA polymerase, replicating exclusively in a $5' \rightarrow 3'$ direction, would copy, almost simultaneously, the two maternal strands of opposite polarity. Examination of dividing bacteria at a gross level by autoradiography (*38*) or by gene duplication (*39*) makes it appear that there is a simultaneous, sequential replication of both strands. However, at the nucleotide level, as proposed in this scheme, the replicative action is staggered, alternating from one strand to the other.

Summary

DNA polymerase, a homogeneous protein of molecular weight 109,000, appears to be a single polypeptide chain. The enzyme contains one triphosphate substrate binding site and one site for binding a nicked region of duplex DNA. A model of the active center of the enzyme has been proposed (Fig. 11) in which there are distinctive sites for the template strand, the primer strand, the 3′-hydroxyl primer strand terminus, the triphosphate substate, and the 5′-phosphate–terminated strand beyond the nick (point of scission). The model attempts to account for the various synthetic and degradative functions within the closely related sites in the active center of the enzyme.

Initiation of replication is favored at a nicked region of a duplex. In the first phase of replication, extension of the 3′-hydroxyl primer strand appears to be related to the $5' \rightarrow 3'$ hydrolytic removal of the 5′-phosphate–terminated strand. The failure of phage-induced DNA polymerases to initiate replication at a nicked region may be due to the lack of a $5' \rightarrow 3'$ nuclease function in the phage enzyme. A speculative model for helix replication, in vivo (Fig. 13), suggests how DNA polymerase, in conjunction with endonuclease and ligase, may achieve the sequential and almost simultaneous replication of both strands of a helix.

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