Enzymatic Mechanisms of DNA Replication

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ABSTRACT DNA polymerases purified from several sources are characterized by replication of the 3′-hydroxy-terminated strand of a helical template. Failure to achieve simultaneous replication of the 5′-strand leads to aberrations in the synthesized DNA, described as nondenaturability and branching. Aberrations in synthesized DNA were not observed when (*a*) the 5′-strand was destroyed by a specific nuclease during the course of replication or (*b*) a single-stranded (circular) phage (M13) DNA served as template. Replication of a single-stranded, circular DNA produced a helical product, but the nature of initiation of a new strand by the circular template remains to be explained. Hypothetical mechanisms for simultaneous replication of the 5′-strand are presented as is the possibility that the tertiary structure of the DNA, as for example, a circular form of the helix, is of prime importance in in vivo replication.

Experiments during the past decade with in vivo and enzyme systems have convinced us that replication of DNA follows a semiconservative mechanism. It would also appear that replication of a linear chromosome starts at one end of the helix and proceeds sequentially, as proposed originally by Watson and Crick. What do we know about the molecular details of replication?

"Rules" of Polymerase Action

Studies on the DNA polymerases of *E. coli* (1), *B. subtilis* (2), *M. lysodeikticus* (3), phage-infected *E. coli* (4), and animal cells (5) warrant the following generalizations:

1. The unit assembled to make a new chain is a monomer, a single nucleotide; it is never a dinucleotide or oligonucleotide (Fig. 1).
2. The monomeric unit is a deoxyribonucleoside *5′-tri*phosphate.
3. Chain elongation occurs by nucleophilic attack of the 3′-hydroxyl end of the DNA chain on the nucleotidyl phosphorus with the elimination of inorganic pyrophosphate.
4. There is an absolute template requirement which directs which nucleotide is put into place; without such a template, no DNA synthesis occurs.

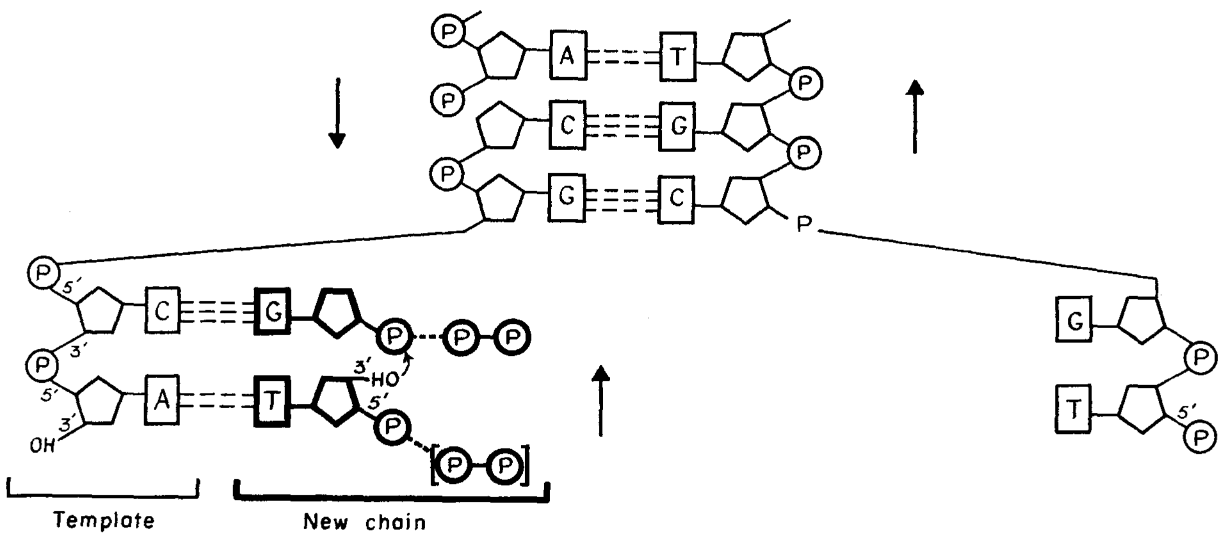

FIGURE 1. Mechanism of DNA replication by *E. coli* polymerase.

5. The assembly of these units, as dictated by the template, is determined by the Watson-Crick base-pairing of adenine with thymine and guanine with cytosine. This base-pairing mechanism is followed scrupulously without error. If errors do occur, they must be less frequent than one in a million.

6. The role of the template as the directing force in base-pairing is essential but probably not sufficient by itself to insure error-free replication. Emerging evidence suggests that recognition by the polymerase of a correct base pair is an important factor.

7. Finally, enzymatic replication of DNA proceeds rapidly with the production of chains containing several thousand base pairs; an estimate of the

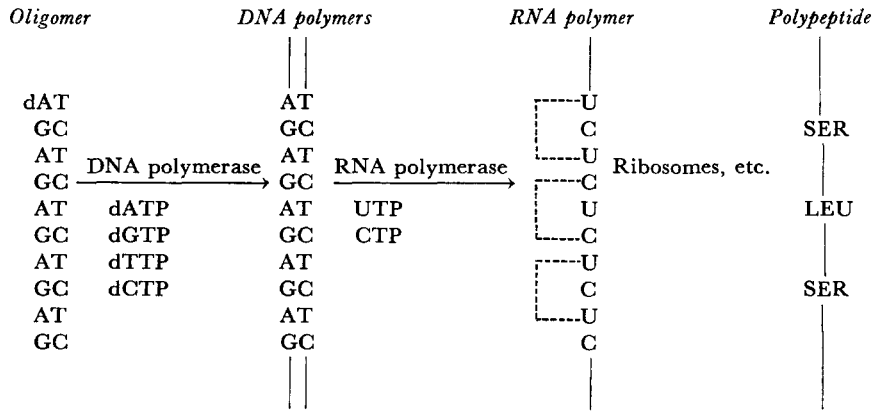

FIGURE 2. Oligomers to polypeptides (Khorana et al.).

minimal rate of polymerization is 1000 nucleotides per minute per molecule of enzyme.

We wish to cite two examples from recent work which illustrate that macromolecules synthesized by DNA polymerase (*a*) function in the synthesis of specific polypeptides and (*b*) possess physical and chemical characteristics that closely resemble those in DNA isolated from nature. We will then present evidence which illustrates the current limitations of the enzyme system in attempts to achieve the complete and orderly replication of a helical template.

Synthetic DNA as a Template for a Functional Messenger

The capacity of a synthetic DNA to serve as a template for the transcription of a functional messenger RNA has been demonstrated by Khorana and his colleagues (6). They prepared, by organic chemical synthesis, the alternating copolymer $(AG)_5$—AGAGAGAGAG and its complementary copolymer $(TC)_5$—TCTCTCTCTC. Such a pair of alternating copolymers when incubated with DNA polymerase and the four triphosphates at the proper temperature under defined conditions serve as templates for DNA synthesis (Fig. 2). Macromolecules are produced which contain strands of thousands

of nucleotides, are many millions in molecular weight, and, as seen in the electron microscope, are several microns long. The extent of synthesis is governed by the amount of substrate supplied and can be as much as one hundred times greater than the amount of oligomeric templates added.

These macromolecules were then used as templates for transcription with RNA polymerase. By using only two triphosphates, UTP and CTP, only one strand of the DNA, the AG strand, was copied. The RNA polymer produced was the alternating copolymer, UC. This RNA polymer was then used as messenger in a ribosomal synthesizing system for the assembly of polypeptides. The remarkable things that Khorana et al. found were that of the twenty known amino acids only two, serine and leucine, were polymerized and, second, that the serine and leucine in the polypeptide were in a perfectly alternating sequence. This is exactly the result that would be predicted if the reading frame in the RNA were the triplets UCU and CUC and if these were read with regularity, such that UCU, the code word for serine, was followed by CUC, the code word for leucine. In comparable experiments in which transcription by RNA polymerase was carried out with ATP and GTP, the RNA produced was AG. In this case the translation into a polypeptide should result in an alternating sequence of arginine (for the AGA triplet) and glutamic acid (for the GAG triplet); the results of these experiments are being awaited.

A necessary control is the demonstration that the DNA polymer produced from the chemically synthesized oligomers is, in fact, a perfectly alternating copolymer of AG in one strand and TC in the other. A nearest neighbor frequency analysis of the DNA polymer synthesized with α-^{32}P–labeled dATP revealed that this nucleotide was positioned next to G exclusively; sequences of AA, AT, or AC were not detectable. Analogous determinations with α-^{32}P–labeled dGTP demonstrated that its nearest neighbor was invariably A and never G, T, or C; the alternating copolymeric nature of the TC polymer was established in the same way.

The infallibility of the enzyme in making these long copolymers is impressive to the chemist. There is something else that pleases the synthetic organic chemist. Minute quantities of an oligomer can be "immortalized" by DNA polymerase action. Starting with a few millimicromoles of the AG and TC oligomers, the macromolecular replicas produced by DNA polymerase can serve as templates for unlimited replication. Production of these polymers depends only on the supplies of commercially available triphosphate substrates. Thus, the synthetic creation of an organic chemist is, through proper selection by an enzyme system, preserved and multiplied.

Correct Restoration of Partially Single-Stranded DNA

The second example of replication by DNA polymerase to be cited is one in which extensive stretches of partially single-stranded macromolecules are

successfully restored (7). By the graded action of exonuclease III, as much as one-third of each strand of a helical DNA may be removed starting from each 3′-hydroxyl end (Fig. 3). With such a population of partially single-stranded DNA molecules, the remaining 3′-hydroxyl termini can serve as templates. Polymerase acts on such molecules to form a covalent attachment of a new strand to a 3′-hydroxyl terminus and proceeds to restore the portion of the molecule which had been removed by the exonuclease III.

The T7 phage DNA and *Bacillus subtilis* DNA molecules which have been repaired in this manner were examined by several techniques and appeared to be indistinguishable from the native DNA obtained from the phage or

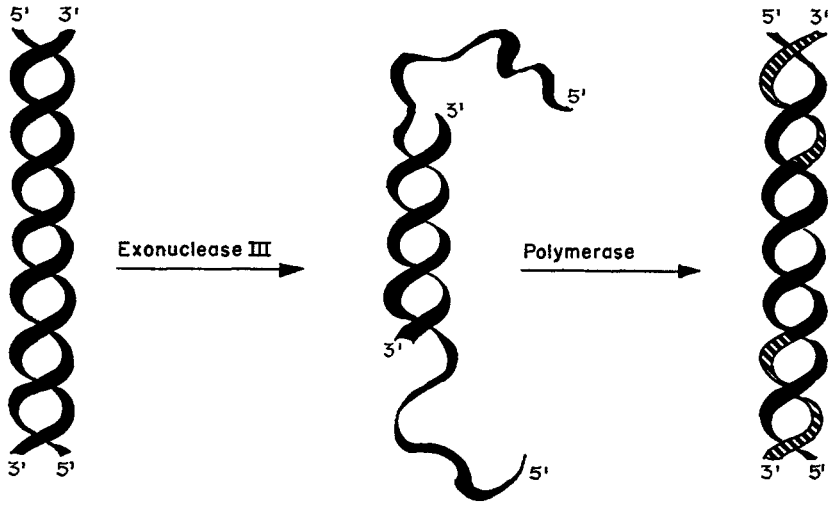

FIGURE 3. Postulated scheme for the polymerase repair of DNA partially degraded by exonuclease.

bacterium. One of the techniques used was electron microscopic measurements of the helical length of T7 DNA molecules. As seen in Fig. 4 *a*, the distribution of lengths is in the region of 11 μ. With degradation by exonuclease III to the extent of 17% from each end, the helical length distribution is close to 7 μ (Fig. 4 *b*). With further degradation to an extent of 29% from each end there is a shift in the mean helical length to 4 μ (Fig. 4 *c*), a value again consistent with what was anticipated. Upon exposure of the 29% degraded population to repair by the DNA polymerase system, there was a restoration of T7 molecules to a distribution quite close to the original (Fig. 4 *d*).

Branching and Nondenaturability in DNA Synthesized on a Helical Template

Up to this point we have cited examples which illustrate how the purified DNA polymerase can synthesize large stretches of DNA that are physically

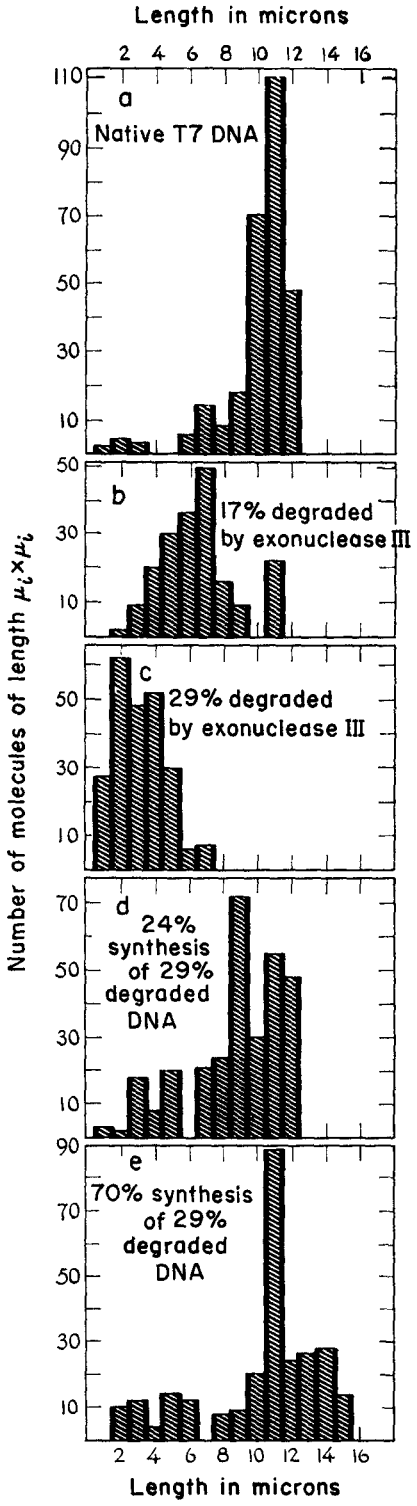

FIGURE 4. Length distributions obtained by electron microscopy.

indistinguishable from DNA isolated from nature (7) and, as illustrated by Khorana's studies (6), are functionally active. There is, however, a major problem. When native, *helical* DNA is provided as a template, and it is upon this that we wish to focus now, the new strands produced starting with helical templates have two properties which are distinct and unusual (8). One is an

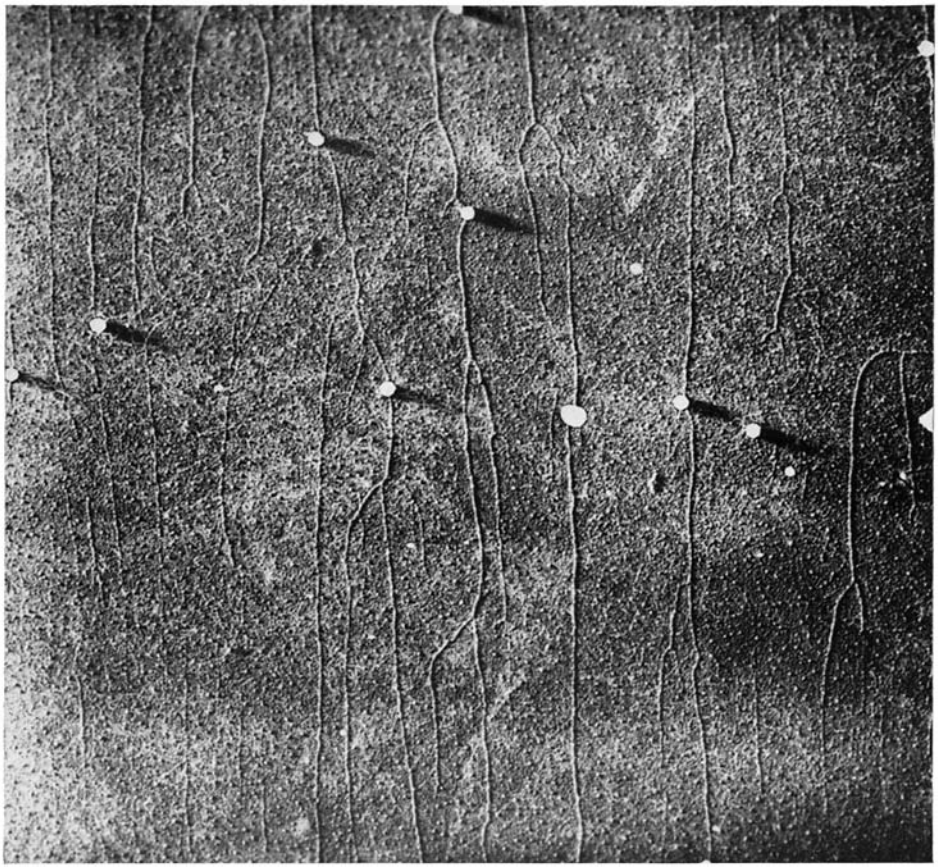

FIGURE 5. Electron micrographs of the product of DNA polymerase primed by a helical template.

insusceptibility to permanent denaturation. Upon exposure to denaturing conditions, the synthetic DNA strands separate and acquire a random-coil conformation but the collapse is not permanent. When the heat or alkali is removed, the enzymatically synthesized DNA strands, unlike those of native DNA, promptly resume a helical structure. This capacity of the DNA to snap back together into a helical form is referred to as nondenaturability. A simple assay for denaturability is the susceptibility of the DNA after a denaturing treatment to exonuclease I, an enzyme which degrades only single-stranded

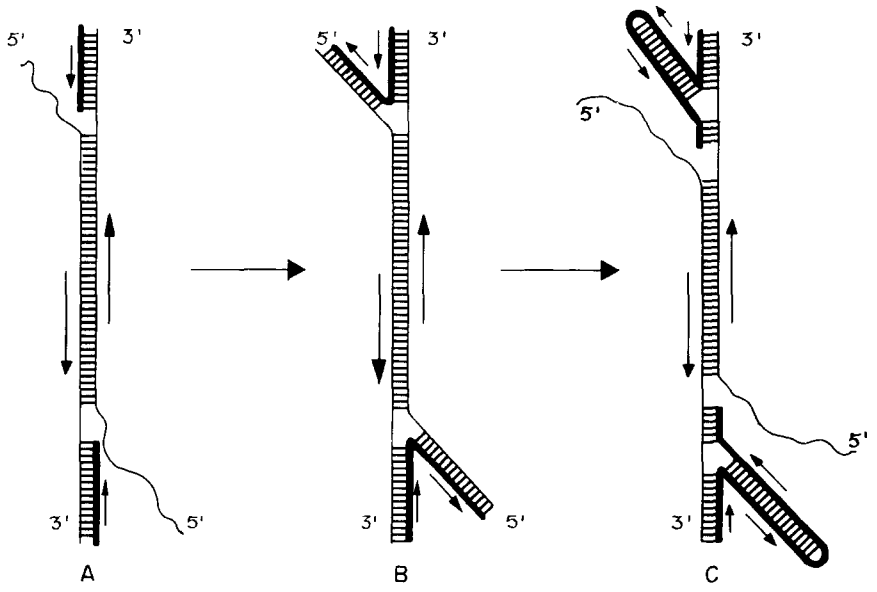

FIGURE 6. A hypothetical model to account for the nondenaturability of synthesized DNA.

DNA to mononucleotides. The second unusual feature about the DNA is its appearance in the electron microscope. As seen in shadowed preparations (Fig. 5), the synthesized DNA forms long fibers, which in appearance and dimensions are exactly like those of native DNA except that the fibers are branched. A branch point seen at this level of resolution in the electron microscope represents several hundred base pairs and should not be taken to indicate simply a nucleotide branch on a polynucleotide chain.

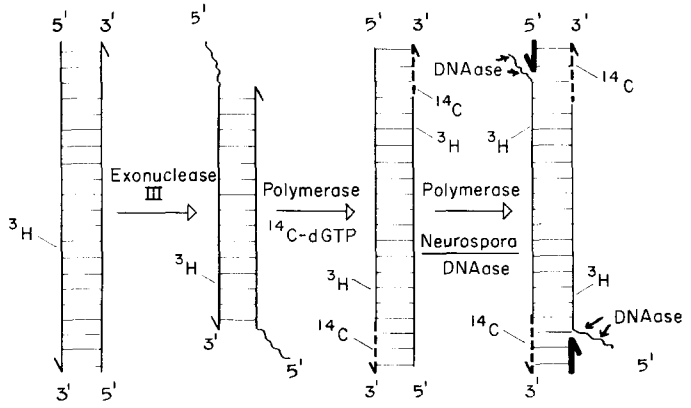

FIGURE 7. Scheme for replication of the ^{3}H-^{14}C–DNA in the presence of *Neurospora* nuclease.

A hypothetical model to explain the structure and development of synthetic DNA is shown in Fig. 6. The start of a new chain is along the 3′-strand at each end of the helix. However, the presence of the unreplicated 5′-sister strand at some point introduces a competitive element and becomes the template itself, as shown in Fig. 6. For reasons that are not known, the newly synthesized strand may in turn serve as a template, thus producing a hairpinlike loop; many such hairpins lead to a pleated structure. By virtue of having these closely matched complementary pairings within these hairpins, such a pleated

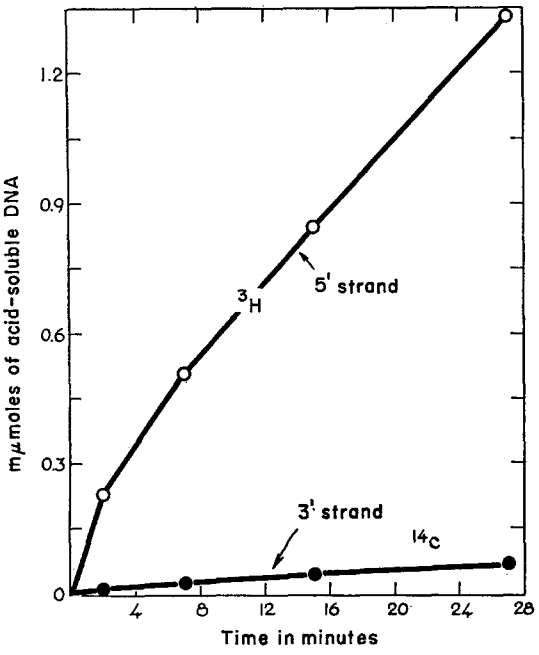

FIGURE 8. Preferential degradation of the 5′ strand during replication of helical DNA in the presence of *Neurospora* nuclease.

structure would be expected to reform after a denaturing treatment. This model has serious limitations but, at this time, it provides a better explanation of the available observations than a number of other plausible alternatives.

Removal of the 5′-Strand during Replication Permits Development of a Denaturable DNA

At this point, we would like to present two recent experiments that test this model. ^{3}H-labeled T7 DNA was first exposed to exonuclease III and then repaired by DNA polymerase with ^{14}C-labeled triphosphates. The repair was carefully controlled and exactly matched the predicted limit of full replacement of the DNA previously removed by exonuclease III. The product thus had ^{14}C at the end of each 3′-strand and ^{3}H at the end of each 5′-strand (Fig. 7). Replication was now carried out in the presence of a new enzyme

made available to us by Linn and Lehman (9). It is an endonuclease from *Neurospora* which splits *single strands specifically* to acid-soluble fragments. Replication of the doubly labeled DNA should lead to new strands developing along the 3′-strands forming helical complexes with them. Simultaneously, the unreplicated 5′-strands should be set free and in the presence of the *Neurospora* nuclease should be degraded promptly by the nuclease.

As shown in Fig. 8, the release of ^{14}C, representing the 3′-strands, was very small and represented only 5% of the new DNA synthesized. By contrast, the

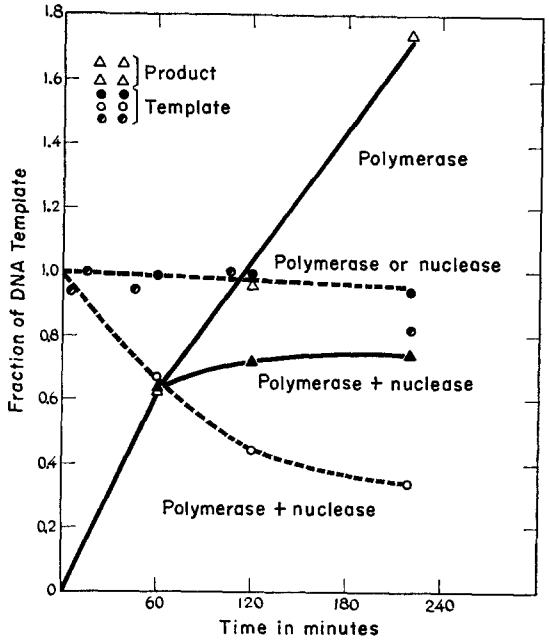

FIGURE 9. Replication of DNA and fate of template in presence of *Neurospora* nuclease.

release of ^{3}H, representing the 5′-strands, was extensive and of the order of magnitude anticipated from the extent of replication of the 3′-strand.

With progressive replication there should be a gradual replacement of the template DNA by the newly synthesized DNA. However, the total amount of synthesized DNA should approximate the input of template but never exceed it. At the same time there should be a progressive decrease in size of the helical molecules produced. The results did in fact conform to these predictions (Fig. 9). DNA synthesis, in the absence of the nuclease, continued linearly for a many-fold replication of the template but in its presence tapered sharply and approached a limiting value below one replication. There was also a concomitant elimination of the template DNA. The product, as judged by sedimentation analyses, was much smaller than the template, too small in fact for valid electron microscopic detection of branching. The denaturability of the product, unlike that of the usual synthetic product, was near that expected of

the original DNA. It would seem, therefore, that the problem in synthesizing new strands starting with helical DNA is the failure of the purified DNA polymerase to simultaneously replicate both strands of the DNA. We might imagine that the failure to copy the 5′-terminated strand leaves it loose and unattended and results in a product that is branched and nondenaturable.

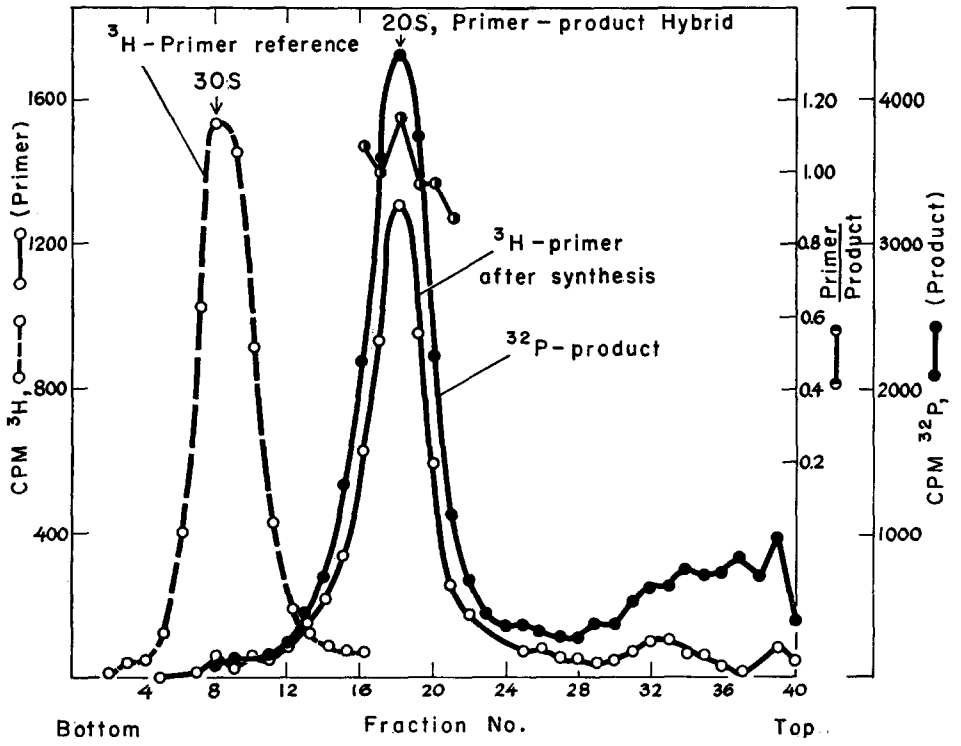

FIGURE 10. Sucrose density-gradient pattern of synthesized DNA in 0.5 M NaCl, 0.01 M Tris, pH 7.5.

Replication of Single-Stranded Circular DNA

Another experimental test of the hypothetical model (Fig. 6) involves the replication of a single-stranded, rather than a helical template. Experiments with preparations of denatured DNA had yielded results, which were only suggestive (8). We attribute these equivocal findings to the well known capacity of denatured DNA preparations to reanneal and reaggregate in ways that cannot be readily predicted or duplicated. This should not be the case with single-stranded DNA isolated as such from bacteriophages. The DNA's from phage M13 (10) and phage ØX174 (11) are examples of such molecules, which, incidentally, are also circular in form. With few, if any, stretches of complementary structure, these DNA's should show little tendency to ag-

gregate. If such single-stranded DNA's, despite the apparent absence of a 3′-hydroxyl at the end, were able to serve as a template for DNA polymerase, it should support the synthesis of a denaturable, unbranched helical product.

The circular DNA from phage M13 proved to be an excellent template for DNA polymerase. It was, in fact, as good on a molar basis as the linear helical molecules obtained from several T phages. A rapid rate of replication was apparent from the outset and was sustained. With ^{3}H-labeled M13 DNA as template and ^{32}P–deoxynucleoside triphosphates, the fate of the template and the size and shape of the product were determined by sucrose density-gradient centrifugation. In an experiment in which a full replication of the M13

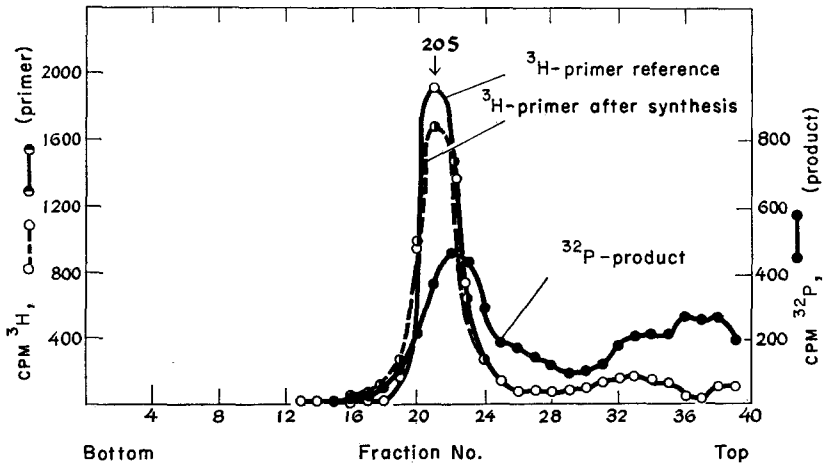

FIGURE 11. Sucrose density-gradient pattern of synthesized DNA in 0.9 M NaCl, 0.1 M NaOH.

DNA was attained, the DNA of the mixture was first analyzed in a gradient run at neutral pH (Fig. 10). Several things were evident. The product and template moved together with a calculated sedimentation coefficient of 20S as compared with a value of 30S for the original template. The slower sedimentation for a molecule presumed to be twice as heavy suggests a stiffened helical structure for the product-template complex. Based on the Studier plot (12) a 20S value for a helical molecule yields a molecular weight of 4×10^{6} for the hybrid as compared with 2×10^{6} calculated from a 30S value for a single-stranded DNA. It is also evident that virtually all of the template had been converted to a complex in this experiment. As seen from the ^{3}H and ^{32}P contained in the main peak, there are equimolar quantities of template and product in this complex.

Analysis in an alkaline sucrose gradient (Fig. 11) revealed the ^{3}H-DNA band sedimenting with exactly the same coefficient as the original circular M13 DNA. This indicates the absence of endonucleolytic scissions of the M13

template during the course of its replication. The ^{32}P band sedimented at a rate expected for a *linear* molecule the size of M13 DNA. Thus, the template appears to have been recovered as a single-stranded, circular molecule and the product strand, by virtue of this alkaline treatment, has been clearly separated from it. Relatively little ^{32}P was found in the area of the gradient where smaller fragments sediment.

Electron microscopic studies (Fig. 12) by Dr. Ross Inman show that the products are double-stranded helical structures with a contour length of 2 μ,

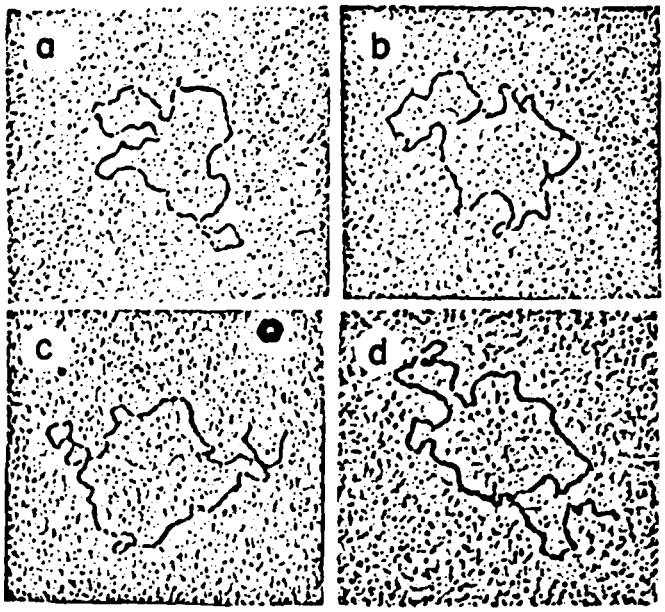

FIGURE 12. Electron micrographs of replicated M13 phage DNA (Inman).

a length corresponding to a molecular weight of 4×10^6. Single-stranded structures are not clearly visualized by this technique and when present are seen as puddles. As shown in *a* and *b* of Fig. 12, the molecules appear to be simple circles; in other instances (*c* and *d* of Fig. 12) there are branches attached to the circles. We attribute these branches to extensive synthesis beyond a full replication in which the complications associated with a helical template, cited earlier, lead to a pleated structure.

Under conditions in which synthesis was prevented from exceeding one round of replication, by either limiting the incubation time or conducting the reaction at 15°C, incomplete helical rings were seen (Fig. 13). The unreplicated, single-stranded regions appear as puddled aggregations of particulate matter.

The denaturability of the template and product strands of the helical

complex was determined by the usual technique of susceptibility of the DNA to exonuclease I after a denaturing treatment. The template remained resistant to exonuclease I, giving further proof that it had not been cleaved by endonucleolytic or shearing forces. The product DNA was largely denaturable (Fig. 14). However, when synthesis was extensive and exceeded a full replication, the DNA was nondenaturable (Fig. 14), and as seen in the electron micrographs (Fig. 12) showed branching.

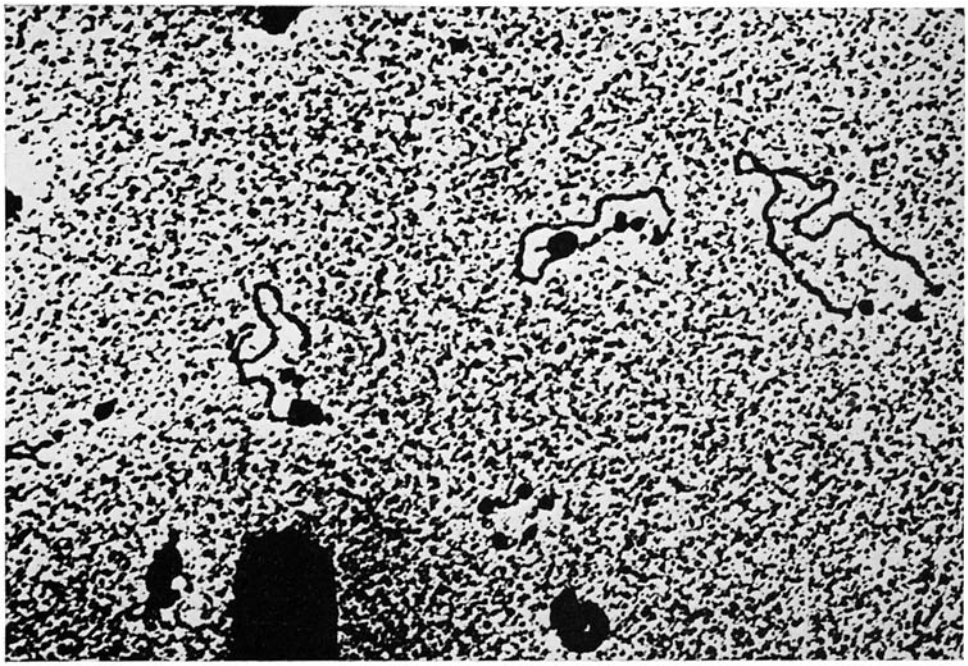

FIGURE 13. Electron micrographs of replicated M13 phage DNA (Inman).

Nature of Priming by M13 DNA

The foregoing results show that the M13 DNA is an excellent template. Similar results have been obtained with ØX174 DNA. Is M13 DNA a simple circle made up of phosphodiester bridges? If so, then what is the nature of the initiating reaction with such a circular template? Insusceptibility to exonuclease I implies an absence of a 3′-hydroxyl terminus. Dr. Charles Richardson has observed that polynucleotide kinase (13) fails to phosphorylate M13 DNA (14), even after phosphatase treatment, indicating the lack of a free 5′-hydroxyl (phosphoryl) terminus. On the other hand, such terminal points may exist but in a form unavailable to exonuclease I and polynucleotide kinase. Further investigations into the chemistry of M13 DNA and the related ØX174 DNA are necessary for a better grasp of their priming and template

activities. So too, are explorations of how the DNA polymerase itself might serve to orient the primary site of replication along the template.

Possible Mechanisms for Replication of the 5'-Hydroxyl Strand

The burden of the foregoing results on replication by DNA polymerase is that synthesis of DNA on a helical template fails to copy both strands simultaneously from one end of the helix and, as a consequence, there are aberrations in the structure of the product. These results strengthen a conviction already strongly supported by recent studies of in vivo replication.

Genetic studies with dividing *B. subtilis* (15) and autoradiographic studies of Cairns with dividing *E. coli* (16) point to a linear, sequential replication of

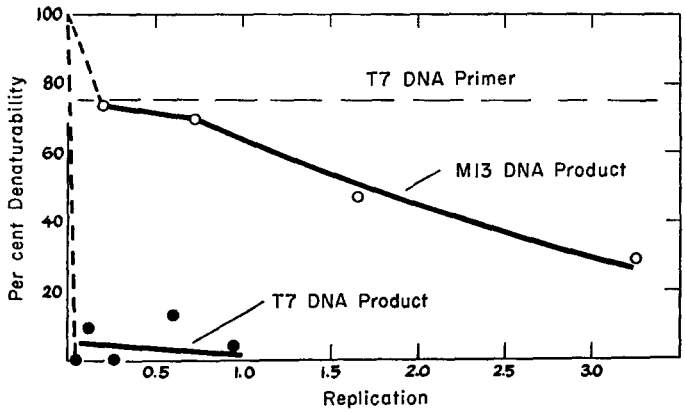

FIGURE 14. Denaturability of the product DNA as a function of replication.

chromosomes, starting from one point on the helix. The Cairns model assumes that the two-billion dalton DNA of *E. coli*, disposed as a circle, gives rise in replication to an expanding second circle. It seems likely that there is a mechanism in nature for the side-by-side simultaneous replication of both DNA strands.

The mechanism of synthesis by isolated DNA polymerases accounts for replication of the 3'-strand. The chain grows by having the initial insertion of a 5'-triphosphate whose hydroxyl group then attacks the next monomeric unit (Fig. 1). In this mechanism there is an attack by the terminal 3'-hydroxyl of the growing chain on the nucleotidyl phosphorus of the incoming monomeric unit. One might speculate about a related mechanism for replicating the complementary 5'-chain, one in which the triphosphate is put in place in the opposite, antipolar fashion (Fig. 15). The terminal triphosphate thus belongs to the growing chain, so that the added monomeric unit forms a diester bridge by having its 3'-hydroxyl group attack the nucleotidyl phosphorus of a triphosphate-ended terminus of the chain. It seems certain from

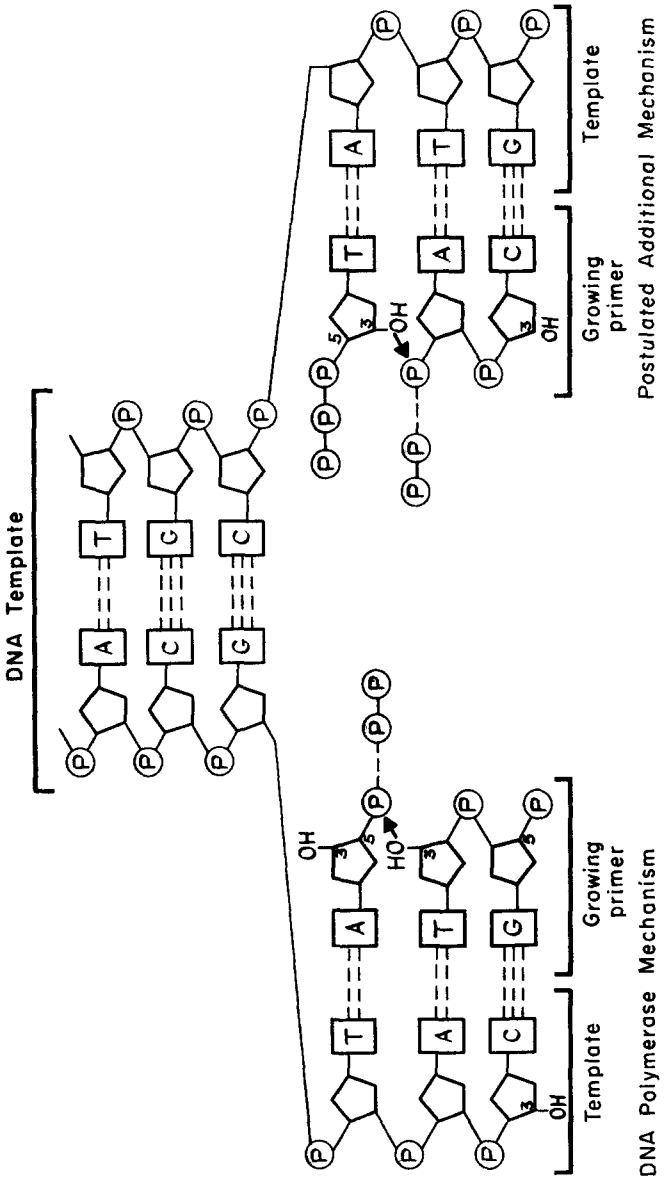

FIGURE 15. *Mechanisms of DNA replication.*

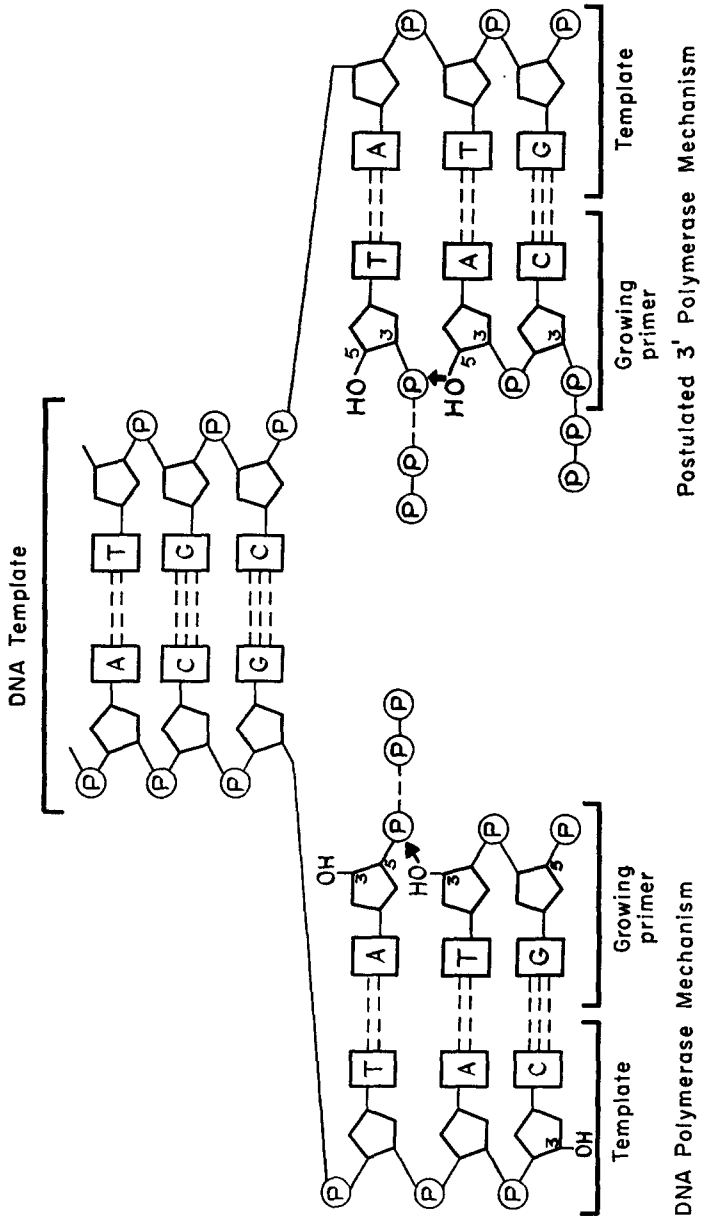

FIGURE 16. Mechanisms of DNA replication.

our recent work that this type of reaction is not carried out by the DNA polymerases that have been purified. However, this is not to say that a polymerase of this kind does not exist in nature. Whereas search in crude cell extracts has thus far failed to provide evidence for such a mechanism, the noise level of the assays devised to detect this type of polymerase has been relatively high and this mechanism should therefore not be dismissed.

Another plausible mechanism is one in which the monomeric units are 3′-triphosphates rather than 5′-triphosphates (Fig. 16). This type of mechanism was never considered seriously because the *de novo* pathways of nucleotide biosynthesis yield the 5′-mono- and triphosphates. However, if the cell were able to synthesize 3′-triphosphates, a mechanism involving their use in DNA synthesis would become attractive. Starting with a 5′-ended chain as template, the first triphosphate would be inserted much as postulated for the insertion of the initial triphosphate by DNA polymerase along the 3′-strand (Fig. 16). However, because the 5′-hydroxyl of the first unit is free, it would be able to attack the nucleotidyl phosphorus of the incoming 3′-triphosphate monomer, thus providing a growing end which would in each case be a 5′-hydroxyl group. Whereas the unit attacked by the standard DNA polymerase is a 5′-triphosphate, it would in this case be a 3′-triphosphate. Work in recent months has established that there are enzymes in extracts of microorganisms which convert deoxynucleoside 3′-monophosphates to the corresponding 3′-triphosphates (17). The natural origin of these 3′-monophosphates has not been determined nor has there been any success in recent intensive efforts by Dr. John Josse with extremely radioactive 3′-triphosphates to detect even traces of polymerase activity in extracts of *E. coli*, *B. subtilis*, or *M. lysodeikticus*.

Still another alternative mechanism is one which regards the physiological template as a circular, rather than a linear, molecule. In addition to Cairns' autoradiographic studies which propose a circular chromosome as the active replicative form, there are the strong indications in the work of Bode and Kaiser (18) and of Young and Sinsheimer (19) of the importance of a circular form of phage λ-DNA in replication. More recently, Thomas (20) has presented evidence that the T-even phages may be made to form circles in vitro. The natural occurrence of circular DNA in viruses has been mentioned in the case of M13 and ØX174 and also includes the animal viruses: polyoma (21), rabbit papilloma, and human wart.

How is a circular helical DNA replicated? According to Cairns, replication starts at some point on the circle and each strand is copied forming a second circle. For a second circle to be formed, each of the strands of the parental circle must unwind. To avoid a hopeless snarl, Cairns postulates a swivel joint, the chemical nature of which is a mystery. Another possibility, premature for discussion at this time, is suggested as a result of studies by Lucas with a polymerase induced by T4 phage infection of *E. coli* (22). The polymerase is

physically distinct from *E. coli* polymerase and easily separated from it. (de Waard et al. (23) have also shown that mutants of T4 which lack the gene for this polymerase cannot make phage DNA, indicating that this phage-induced polymerase is essential for the replication of phage DNA.) Current in vitro studies have led us to speculate that the invading helical DNA of the T4 phage which is linear upon injection becomes looped within the cell and supports semiconservative replication by the phage polymerases.

Whereas the foregoing discussion dwelt on the possibility that a circular DNA may be the form which is active in replication, the point to be stressed is the general importance of tertiary structure in the DNA rather than any one particular type of tertiary structure. For example, the finding by Ganesan and Lederberg (24) that the growing region of DNA in *B. subtilis* is associated with the cell membrane suggests that a particular complex of DNA with a certain cell constituent may be a critical factor in replication.

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Discussion

Dr. C. Coutsogeorgopoulos: I just wanted to make a comment to say that Dr. Josse is probably not the only one who fails to detect polymerizing activity from 3′-deoxyribonucleoside triphosphates in *E. coli*. We have synthesized chemically all four 3′-deoxyribonucleoside triphosphates, and we also failed to detect any activity in *E.*

coli extracts. Possibly for the same reason, others share this disappointment with us. As for the existence of 3′-kinases, which is relevant, we have already reported on the enzymic formation of thymidine-3′-triphosphate in extracts of regenerating rat liver (Coutsogeorgopoulos, C., Hacker, B., and Montsavinos, R., *Biochem. and Biophysic. Res. Commun.*, 1965, **20**, 129), and Canellakis has made a similar observation using extracts of *B. subtilis* (*Proc. Nat. Acad. Sc.*, 1965, **53**, 184).

Dr. Kornberg: Curious kind of sharing of disappointment. Is there anyone who has some success to announce? Are there any other comments?