"pure" methanol would act as a clean-burning fuel, having none of the potentially severe product quality problems associated with its use in motor gasoline, while the fuel oil or natural gas currently burned in these turbines could be diverted to other uses.

References and Notes

1. J. C. Gillis, J. B. Pangborn, J. G. Fore, "Synthetic fuels for automotive transportation," paper presented at the spring meeting of the Combustion Institute, Madison (March 1974).
2. P. Soedjanto and F. W. Schaffert, *Oil Gas J* (11 June 1973), p. 88.
3. T. B. Reed and R. M. Lerner, *Science* **182**, 1299 (1973); T. O. Wentworth, *Environ. Sci. Technol.* **7**, 1002 (1973); D. A. Howes, *J. Inst. Petrol. Technol.* **19**, 301 (1933); S. J. W. Pleeth, *Alcohol, A Fuel for Internal Combustion Engines* (Chapman and Hall, London, 1949).
4. E. S. Corner, "Octanes on the road—Leaded and unleaded," and W. E. Morris, "Engine-octane quality relationships—State of the art," papers presented at the 37th midyear meeting of the American Petroleum Institute, Division of Refining, New York (May 1972).
5. E. S. Corner and A. R. Cunningham, "Value of high octane number unleaded gasolines in the U.S.," paper presented at the American Chemical Society, Los Angeles (March 1971).
6. Committee for Air and Water Conservation of the American Petroleum Institute, *Use of Alcohol in Motor Gasoline—A Review* (Publ. No. 4082, American Petroleum Institute, Washington, D.C., 1971).
7. J. C. Ingamells and R. H. Lindquist, "Methanol as a motor fuel," in preparation.
8. P. J. Clark, "The effect of gasoline volatility on exhaust emissions," paper presented at the Society of Automotive Engineers' National Fuels and Lubricants Meeting, Tulsa (November 1972).
9. C. F. Taylor, *The Internal Combustion Engine in Theory and Practice* (MIT Press, Cambridge, Mass., 1965), p. 438.
10. B. Dimitriades and T. C. Wesson, *U.S. Bur. Mines Rep. Invest. No. 7527* (1968).
11. E. E. Wigg, R. J. Campion, W. L. Petersen, *Soc. Automotive Eng. Trans.* **81**, 923 (1972).
12. A. P. Altshuller, *J. Air Pollut. Control. Assoc.* **16**, 257 (1966).
13. Conversion factors for nonmetric units used in the text are: 1 mile per gallon equals 0.43 kilometer per liter; 1 pound per square inch equals 6.9×10^4 dynes per square centimeter; 1 gram per mile equals 0.62 gram per kilometer

DNA Ligase: Structure, Mechanism, and Function

The joining of DNA chains by DNA ligase is an essential component of DNA repair, replication, and recombination.

I. R. Lehman

DNA ligase is an enzyme that can join DNA chains to each other under certain very specific conditions. Although such a ligation activity had long been a feature of models for recombination between genes and for the repair of damage to DNA, the real impetus to search for a DNA joining enzyme stemmed from two experimental findings made in the early 1960's. The first was the discovery by Meselson and Weigle (*1*) and by Kellenberger, Zichichi, and Weigle (*2*) that genetic recombination can occur by the breakage and rejoining of DNA molecules; and the second was the observation by Young and Sinsheimer (*3*), and by Bode and Kaiser (*4*) that a large fraction of linear DNA from bacteriophage λ is rapidly converted to covalently closed duplex circles soon after it infects its host bacterium. The extent and vigor of the efforts to find a DNA joining activity is perhaps best conveyed by the independent and nearly simultaneous discovery in 1967 of DNA ligases in uninfected and bacteriophage-infected *Escherichia coli* in no less than five different laboratories (*5–9*).

Although there was a clear and implicit requirement for a DNA ligase in the repair of DNA and in recombination, yet another function became apparent with the report by Okazaki and his co-workers (*10*) that DNA may be replicated discontinuously as short segments which are subsequently joined into the continuous strands that make up the chromosome. As this model for DNA replication has gained acceptance, there has been a corresponding recognition of DNA ligase as an integral part of the cellular replication machinery.

After the discovery of ligases in uninfected and phage-infected *E. coli*, DNA joining activities were observed in a variety of eukaryotic tissues including rabbit bone marrow, spleen, and thymus (*11*), rat liver (*12*), and lily microsporocytes (*13*), so that their widespread distribution is by now well established. I will focus on two of these enzymes: that from *E. coli* and the one induced after infection of *E. coli* with bacteriophage T4. These two DNA ligases are the only ones now available in homogeneous form; they are also the most thoroughly investigated. Both catalyze the synthesis of phosphodiester bonds between directly adjacent 3′-hydroxyl and 5′-phosphoryl termini in duplex DNA. Phosphodiester bond synthesis catalyzed by the *E. coli* ligase is coupled to cleavage of the pyrophosphate bond of diphosphopyridine nucleotide (DPN), alternatively named nicotinamide adenine dinucleotide (NAD) (*14, 15*); the energy for phosphodiester bond synthesis by the bacteriophage T4–induced enzyme (as well as the eukaryotic ligases) is provided by the hydrolysis of the α,β-pyrophosphate bond of adenosine triphosphate (ATP) (Fig. 1) (*6, 8, 9, 11–13*).

In discussing the structure, mechanism, and function of DNA ligase, I will deal with (i) assay methods, (ii) physicochemical properties and substrate specificity, (iii) chemical mechanisms, (iv) functions in vivo, and (v) use of ligases as reagents in the construction of recombinant DNA molecules in vitro.

Assay Methods

DNA ligase activity can be measured in a variety of ways: the change in sedimentation coefficient after covalent closure of circles of phage λ DNA with two single-strand breaks (nicks) (*5*); covalent linkage of hydrogen-bonded dimers of λ DNA as measured by adsorption to hydroxyapatite after de-

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naturation (*15*); conversion of ^{32}P-labeled 5′-phosphoryl termini at nicks in duplex DNA to a form no longer susceptible to a phosphatase (*6*, *7*); covalent linkage of one polynucleotide to a second one immobilized in cellulose (*9*); and restoration of transforming activity to DNA that had been treated with deoxyribonuclease (*16*). A more recently developed assay measures the conversion of a copolymer of deoxyadenylate (dA) and deoxythymidylate (dT) to a covalently circular form lacking termini, which is then insensitive to exonuclease (*17*). Assays that measure the first step of the ligase reaction (see below) have also been described; the formation of enzyme-adenylate from DPN has been used for the *E. coli* enzyme (*18*), and the T4-induced ligase has been assayed by the exchange of ^{32}P-labeled inorganic pyrophosphate (PP_i) with ATP (*19*).

Physicochemical Properties

Several of the physicochemical parameters that have been determined for the *E. coli* DNA ligase are given in Table 1. The molecular weight of the homogeneous enzyme as estimated by sedimentation equilibrium ultracentrifugation is 77,000. The agreement between this value and that found for the denatured and reduced form of the enzyme by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (74,000 ± 3,000) indicates that the *E. coli* ligase is composed of a single polypeptide chain (*20*). This finding is somewhat surprising in view of the relatively large molecular weight of the enzyme and the complexity of the reaction that it catalyzes. Earlier reports suggesting a subunit structure (*18*) are now clearly attributable to proteolysis of the enzyme during the long periods of dialysis required to demonstrate forms of lower molecular weight and with altered catalytic properties (*21*). The sedimentation coefficient ($s_{20,w}$) of the purified ligase determined by analytical sedimentation (3.9*S*) is lower than that expected for a spherical protein with a molecular weight of approximately 75,000, and hence suggests that the enzyme may have an asymmetric shape.

The number of DNA ligase molecules per bacterium can be estimated simply by comparing the specific activity of the pure enzyme with that observed in crude extracts. Wild-type *E. coli* growing in rich medium contains between 200 and 400 molecules of DNA ligase per cell, a number close to that calculated for DNA polymerase I (*22*). Inasmuch as DNA polymerase and ligase may act coordinately in the terminal steps of DNA replication (*23*), the near equivalence in the concentration of these two enzymes may be significant.

The T4 DNA ligase has recently been obtained in a physically homogeneous form, and although it has not yet been thoroughly characterized it too appears to consist of a single polypeptide chain, somewhat smaller than the *E. coli* enzyme (molecular weight between 63,000 and 68,000) (*24*).

Substrate Specificity

In addition to their different cofactor requirements, the *E. coli* and T4-induced enzymes have differing polynucleotide specificities: the T4 DNA ligase, but not the *E. coli* enzyme, can catalyze the joining of oligodeoxynucleotides or oligoribonucleotides in RNA-DNA hybrid duplexes (*25*) and can promote the end-to-end joining of two duplex DNA molecules with fully base-paired termini (*26*). However, the rates of these reactions are low relative to the rate of joining at single-strand breaks in duplex DNA and their significance in vivo remains to be determined. An RNA ligase activity distinct from DNA ligase has been found in extracts of T4-infected *E. coli*. This enzyme converts very short polyadenylate or polyinosinate chains (30 to 40 nucleotides) to a circular product in an ATP-dependent reaction. Duplex structures are not required and in fact appear to be somewhat inhibitory (*27*).

Phosphodiester Bond Synthesis in a Sequence of Three Partial Reactions

How does the cleavage of a pyrophosphate bond in DPN or ATP lead to the synthesis of a phosphodiester bond in DNA? It is now reasonably clear that this is accomplished in a sequence of three steps, involving two covalently linked intermediates (*28*) (Fig. 2). In the case of the *E. coli* ligase, the first step consists of a reaction of the enzyme itself with DPN to form ligase-adenylate and nicotinamide mononucleotide (NMN). Next, the adenylyl group is transferred from the enzyme to the DNA to generate a new pyrophosphate linkage, between the adenosine monophosphate (AMP) and the 5′-phosphoryl terminus at the nick. Finally, the 5′-phosphate is attacked by the apposing 3′-hydroxyl group to form a phosphodiester bond, and AMP is eliminated. The same sequence of reactions is catalyzed by the T4 DNA ligase, except that in the first step the enzyme reacts with ATP rather than with DPN, and PP_i rather than NMN is released.

Proof for this mechanism rests on three types of evidence: (i) isolation of intermediates, (ii) reversal of the reaction, and (iii) a steady state kinetic analysis.

Isolation of Covalent Intermediates

Ligase-adenylate is readily generated when the *E. coli* enzyme is incubated with DPN (*29*). With AMP-labeled DPN, approximately 1 mole of AMP is bound per mole of ligase, a value close to that obtained by difference spectroscopy of the free and

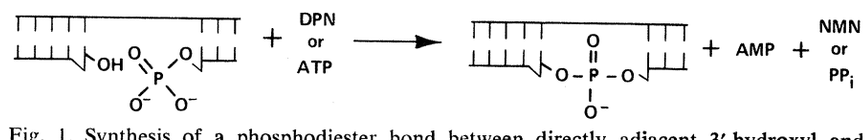

Fig. 1. Synthesis of a phosphodiester bond between directly adjacent 3′-hydroxyl and 5′-phosphoryl groups in duplex DNA by *E. coli* (DPN) and T4 (ATP) DNA ligases.

Table 1. *Escherichia coli* DNA ligase, a single polypeptide chain.

Measurement	Molecular weight
Polyacrylamide gel electrophoresis in sodium dodecyl sulfate	74,000 ± 3,000
Sedimentation equilibrium	77,000
Enzyme + DPN ⇄ enzyme-AMP + NMN	0.8 to 1.2 moles of AMP bound per 74,000*g* enzyme
Sedimentation velocity	$s_{20,w} = 3.9$

adenylylated enzyme (*20*) (Table 1). Once formed, the isolated ligase-adenylate promotes phosphodiester bond synthesis in nicked DNA without added DPN and, upon incubation with NMN, resynthesizes DPN (*29*). Ligase-adenylate is also formed by reaction of the T4 DNA ligase with ATP (*30*). The isolated enzyme-adenylate can then react with nicked DNA to generate a phosphodiester bond or, upon incubation with PP_i, it can regenerate ATP. These findings are all compatible with the participation of ligase-adenylate as an intermediate in phosphodiester bond synthesis.

In both *E. coli* ligase and T4 ligase-adenylates, the AMP moiety is linked to the ε-amino group of a single lysine residue of the enzyme through a phosphoamide bond (*31*) (Fig. 3). Hence, the first chemical step of the reaction catalyzed by these enzymes consists of the nucleophilic attack of the ε-amino group of the lysine on the adenylyl phosphorus of DPN or ATP. The *E. coli* and T4 ligases are, in fact, unique in their use of an ε-amino group as a nucleophile in enzyme-catalyzed nucleotidyl group transfer.

Identification of the phosphoamide linkage is based principally on the isolation of a compound that is indistinguishable by a variety of criteria from authentic ε-amino–linked lysine-AMP after alkaline hydrolysis or proteolytic degradation of ligase-adenylate. This assignment is fully consistent with the relative acid lability and alkaline stability of the isolated ligase-adenylate, and it is also compatible with its susceptibility to cleavage by acidic but not neutral hydroxylamine. These conditions distinguish a phosphoamide from other linkages, such as a phosphodiester or mixed anhydride.

The second covalent intermediate, DNA-adenylate, does not normally accumulate in ligase reactions under steady state conditions. However, small amounts have been isolated after very brief incubations with DPN (0.5 minute) at 0°C in the presence of large amounts of the *E. coli* enzyme (*32*), and upon reversal of the reaction starting with DNA and AMP (see below). DNA-adenylate also accumulates in T4 ligase–catalyzed reactions at *p*H 5.6 and 0°C (*36*). When the isolated DNA-adenylate reacts with the unadenylylated form of the enzyme, a phosphodiester bond is synthesized and AMP is released.

Identification of DNA-adenylate as a DNA duplex in which AMP is linked through a pyrophosphate bond to the 5′-phosphate at a nick (see Fig. 2) rests on the isolation of the 5′ terminus as a trinucleotide containing 5′-AMP after exonuclease I digestion of denatured DNA-adenylate (*32, 33*). Since exonuclease I degrades single-stranded DNA sequentially from the 3′-hydroxyl end to produce 5′-mononucleotides and a dinucleotide derived from the 5′ terminus (*34*), linkage of the AMP to the DNA must be through a pyrophosphate bond to a 5′-phosphoryl terminus, rather than to a 3′-hydroxyl group through a phosphodiester bond.

Additional evidence for the structure of DNA-adenylate and its involvement in the ligase-catalyzed reaction has come from studies of a synthetic DNA-adenylate, namely, polydeoxythymidylate, in which the 5′-terminal phosphate is linked by a pyrophosphate bond to AMP, poly(dT)-adenylate (*32, 33, 35*). When multiple units of this poly(dT)-adenylate (chain length, 200 nucleotides) containing ^3H-labeled AMP and poly(dT) labeled with ^{32}P in its 5′-phosphoryl group are annealed to poly(dA) (chain length, 5000 nucleotides) and incubated with *E. coli* DNA ligase, there is a stoichiometric release of [^3H]AMP and incorporation of ^{32}P into a phosphodiester bond (Fig. 4). As might be anticipated, phosphodiester bond synthesis under these conditions is strongly inhibited by DPN, which converts the enzyme to the adenylylated form (*35*). Similar experiments performed with T4 DNA ligase have yielded essentially the same results (*33*).

Activation of the 5′-phosphoryl group by a pyrophosphate bond is a necessary but not sufficient condition for its re-

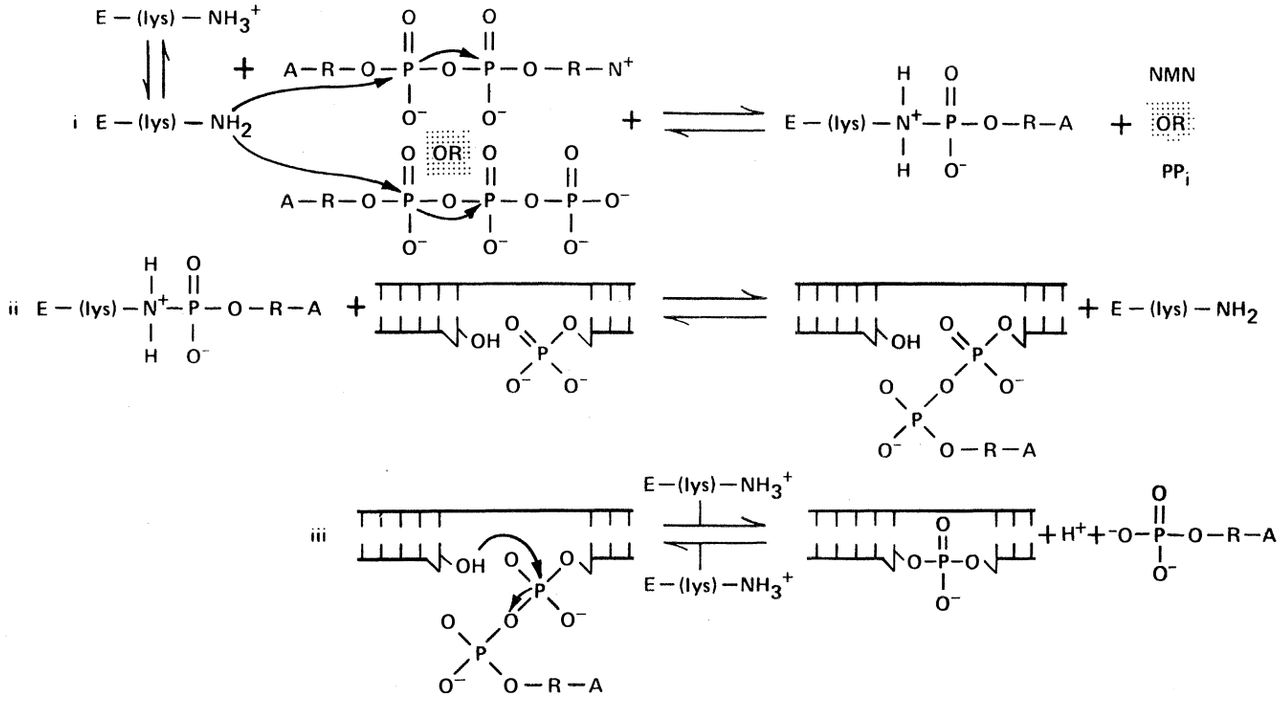

Fig. 2. Mechanism of DNA ligase reaction.

activity in ligase-catalyzed reactions. Thus, poly(dT) with a triphosphate rather than an adenylyl group at its 5′ terminus is inactive as a substrate for the *E. coli* enzyme (*32*). Similarly, when AMP is replaced by dAMP, the rate of the reaction catalyzed by the T4 DNA ligase falls by more than 90 percent; guanosine monophosphate (GMP) is completely inert (*33*).

Reversal of the Escherichia coli DNA Ligase Reaction

The reaction mechanism shown in Fig. 2 indicates that ligase should act as an AMP-dependent endonuclease in the reverse direction. Furthermore, if ligase can cleave phosphodiester bonds, as well as catalyze their synthesis, it could mediate a nicking and sealing activity similar to that postulated by Wang (*36*) to explain the activity of ω, an *E. coli* protein that removes negative superhelical turns from covalently closed duplex DNA circles. Consequently, either AMP-dependent endonuclease activity or superhelix relaxation should provide a measure of the reversibility of the ligase reaction (Fig. 5). As shown by the following experiments, the *E. coli* DNA ligase reaction is reversible, as judged by either of these criteria (*37*).

When superhelical closed-circular DNA from phage λ is incubated with ligase and AMP, two new forms appear: molecules with one single-strand break, and covalently closed circles that have lost their superhelical turns. These structures can be readily distinguished from each other and from supercoils by band sedimentation in 3*M* CsCl (Fig. 6), and both ligase and AMP are required for their formation. However, the rate of the reverse reaction is extremely low, and very large amounts of enzyme are required for its detection. With the use of [^{32}P]AMP it is further possible to demonstrate by the isolation of DNA-adenylate and ligase-adenylate that both are formed in the reverse reaction, thus providing additional evidence for these two intermediates in the reaction mechanism.

The ligase-catalyzed relaxation of superhelical DNA differs from the relaxation catalyzed by the ω protein in several respects, the most obvious being the absolute dependence of the ligase reaction on AMP. The two activities also differ in their specificity and in their kinetic properties. Ligase relaxes both positive and negative superhelices, while ω is inactive on circles with positive superhelical turns (*36*). Unlike ω, ligase removes all of the superhelical turns from a covalent circle with single-hit kinetics, implying that once the break is introduced, ligase frees one or both ends at the single-strand break for a time sufficient to allow complete unwinding to occur.

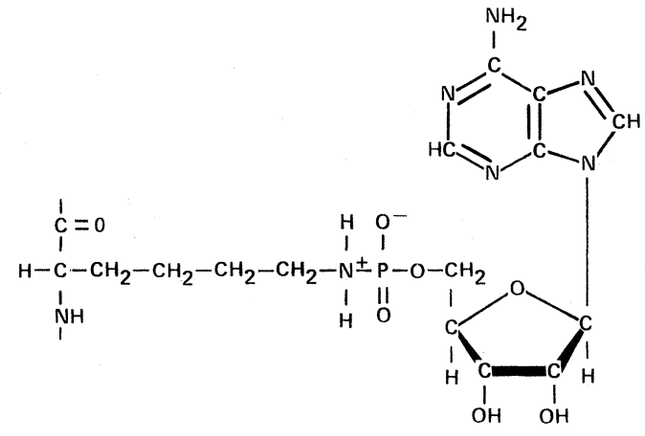

Fig. 3. Structure of ε-amino–linked lysine-AMP. The lysine is shown in peptide linkage.

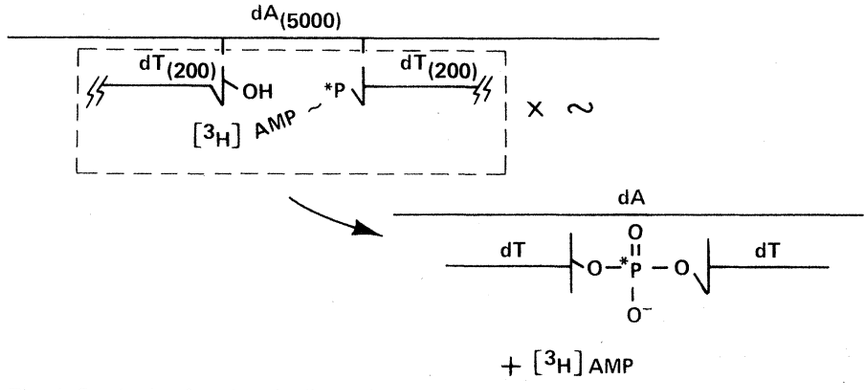

Fig. 4. Synthesis of a phosphodiester bond and release of AMP from poly(dT)-adenylate annealed to poly(dA)$_{5000}$, catalyzed by DNA ligase. The AMP is labeled with ^{3}H and the 5′-phosphoryl terminus of the poly(dT)$_{200}$ is labeled with ^{32}P (*P).

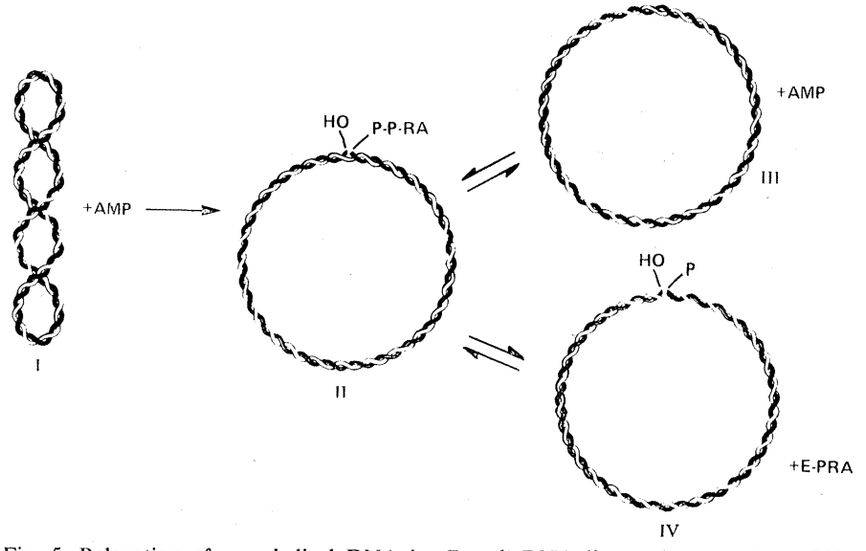

Fig. 5. Relaxation of superhelical DNA by *E. coli* DNA ligase. A superhelical DNA (I) is nicked in the presence of AMP to yield the adenylylated intermediate (II), which may be rejoined after relief of the superhelical twist (III) or hydrolyzed (IV).

Steady State Kinetic Analysis of the Ligase Reaction

The isolation of ligase-adenylate and DNA-adenylate as products of both the forward and reverse reactions implies that they are intermediates on the direct path of phosphodiester bond synthesis. However, proof of their direct participation requires that the rate constants for their formation and further reaction be equal to or greater than the rate of phosphodiester bond synthesis, that is, that they be kinetically significant intermediates.

Results of a steady state kinetic analysis of the overall joining reaction and two of the three partial reactions catalyzed by *E. coli* DNA ligase are given in Table 2. Joining was measured with the use of the homopolymer pair $(dA)_{5000} \cdot (dT)_{200}$ in which poly(dT) units approximately 200 residues in length are hydrogen-bonded to a long poly(dA) chain (5000 nucleotides).

The joining reaction is specifically enhanced by low concentrations (1 m*M*) of NH_4^+. At saturating concentrations of NH_4^+ (5 to 10 m*M*) the true V_{max} (maximum velocity) for joining is increased as much as 20 times. Under these conditions the K_m (Michaelis constant) for DPN is 7 μM; the K_m for single-strand breaks is 0.04 to 0.06 μM, and the turnover number is 28 phosphodiester bonds synthesized per minute (*28*). In contrast to its profound activation of the *E. coli* enzyme, concentrations of NH_4^+ up to 10 m*M* have no effect on the activity of the T4-induced DNA ligase.

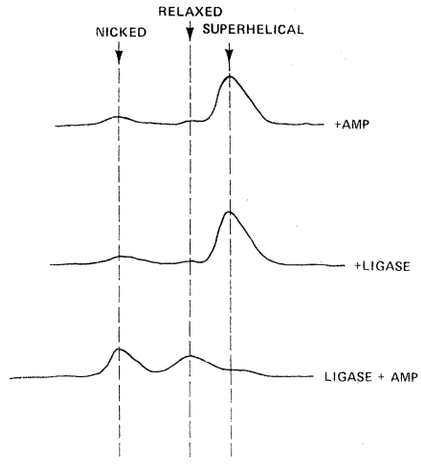

Fig. 6. AMP-dependent conversion of superhelical DNA from bacteriophage λ to relaxed and nicked circular forms by *Escherichia coli* DNA ligase as measured by band sedimentation of 3*M* CsCl (*37*).

The rate of enzyme-adenylate formation, as measured by exchange of the NMN moiety of [^{3}H]nicotinamide-labeled DPN with unlabeled NMN, is greater than the rate of DNA joining; hence, ligase-adenylate can be formed at a rate sufficient for it to be an intermediate in the overall reaction. The rate of this partial reaction is unaffected by NH_4^+, indicating that the activation occurs at a step subsequent to the formation of ligase-adenylate. Additional support for the direct participation of ligase-adenylate in phosphodiester bond synthesis comes from the finding that the joining reaction obeys ping-pong kinetics as seen in double-reciprocal plots of rate against substrate concentration (*28*). This result is most easily interpretable in terms of a series of partial reactions, with the intermediate formation of a complex or covalent compound of the enzyme with the group which is being transferred, in this case ligase-adenylate (*38*).

The ready reversibility of ligase-adenylate formation implies that a significant fraction of the free energy of the pyrophosphate bond of DPN is retained in the adenylylated intermediate. This is not unexpected since a high energy phosphoamide bond links the adenylyl moiety to the enzyme via the ε-amino group of a lysine residue. The equilibrium constant determined for the formation of ligase-adenylate under standard reaction conditions (*p*H 8 and 30°C) is 28. This value may not simply reflect the free energy difference between the pyrophosphate bond of DPN and the phosphoamide bond of ligase-adenylate, since a proton is probably removed from the protonated ε-amino group of the lysine residue prior to its nucleophilic attack on the adenylyl phosphorus of DPN (Fig. 2), and the disposition of this proton is not known. Since the intracellular concentration of DPN in *E. coli* is about 0.5 m*M* (*39*), an equilibrium constant of this magnitude suggests that virtually all of the ligase within the cell is in the adenylylated form.

Attempts at isolating the second of the three partial reactions, transfer of AMP from ligase-adenylate to DNA, and determining its rate constant have not been successful. Although DNA-adenylate does accumulate under abnormal conditions (0°C, *p*H 5.6) in T4 ligase catalyzed reactions (*33*), the kinetic parameters measured under these conditions cannot be extrapolated to joining reactions carried out at optimal *p*H and temperature.

A method of blocking the ligase reaction sequence at the stage of DNA-adenylate formation would be to use a nicked DNA lacking the 3′-hydroxyl group essential for synthesis of the phosphodiester bond. With such a DNA the reaction cannot proceed beyond activation of the 5′-phosphoryl terminus. However, when the homopolymer pair $(dA)_{5000} \cdot (dT)_{200}$ in which the $(dT)_{200}$ chains are terminated with a 3′-dideoxythymidylate residue

Table 2. Rate constants for reactions catalyzed by *E. coli* DNA ligase.

Reactions	NH_4^+	Turnover number (min^{-1})
Nicked DNA + DPN ⇄ joined DNA + AMP + NMN	−	1.4
	+	28
Enzyme + DPN ⇄ enzyme-adenylate + NMN	−	60
	+	60
DNA-adenylate ⇄ joined DNA + AMP	−	9.1
	+	10

Table 3. Phenotype of *E. coli* DNA ligase mutant *ligts7*. Ultraviolet and methyl methanesulfonate sensitivity were determined at 25°C. R, resistant; S, sensitive.

Strain	Viability		Ultraviolet sensitivity	Methyl methanesulfonate sensitivity	Enzyme-adenylate formed (pmole/mg)		DNA joining activity (unit/mg)	
	25°C	42°C			25°C	42°C	25°C	42°C
Wild type (*lig*$^+$)	+	+	R	R	1.4	1.2	1.6	1.4
Mutant (*ligts7*)	+	−	S	S	0.2	< 0.02	0.03	< 0.004

was tested, the rate of DNA-adenylate synthesis was extremely low, at least three orders of magnitude lower than the rate of joining $(dT)_{200}$ with a 3′-hydroxyl group. Thus, if DNA-adenylate is an obligatory intermediate in the DNA ligase reaction, it would appear that a 3′-hydroxyl group is essential for adenylylation of the adjacent 5′-phosphate at the single-strand break. At the present time, it is not clear whether the 3′-hydroxyl group is required merely for normal binding of the enzyme at the single-strand break, or whether it is essential for the catalysis itself.

The rate of release of AMP from a synthetic DNA-adenylate [poly$(dT)_{200}$-adenylate], and hence the rate of step (iii) (Fig. 2), is faster than the rate of the overall reaction when measured in the absence of NH_4^+ (Table 3), implicating DNA-adenylate directly in phosphodiester bond synthesis. However, in the presence of NH_4^+ (conditions optimal for the ligase reaction), the rate of formation of AMP from DNA-adenylate is significantly less than the rate of the overall reaction. A satisfactory explanation for this anomaly is not yet available. However, the rate constant for this partial reaction determined in the presence of NH_4^+ may be too low. When ligase is incubated with DNA-adenylate, the reaction can proceed in the forward direction to yield a phosphodiester bond and AMP, or it can go in the reverse direction to form ligase-adenylate, presumably in association with nicked DNA (Fig. 1). Free ligase-adenylate is unable to generate AMP from DNA-adenylate (*35*); thus, if NH_4^+ were to facilitate the dissociation of ligase-adenylate from DNA, it would cause a fraction of the enzyme to become catalytically inactive. In fact, when DNA-adenylate is incubated with ligase and NMN in the absence of NH_4^+, essentially all of the AMP released is recovered as free AMP. In its presence, however, a significant fraction (20 percent) is recovered as DPN. It would appear, then, that NH_4^+ does facilitate the dissociation of ligase-adenylate from DNA, and this dissociation may account for the anomalously low rate constant observed for AMP formation from DNA-adenylate in the presence of NH_4^+. Another explanation, as yet untested, is that the low rate constant may reflect a slow conformational change of the unadenylylated ligase required for binding to DNA-adenylate. Dissociation of ligase from

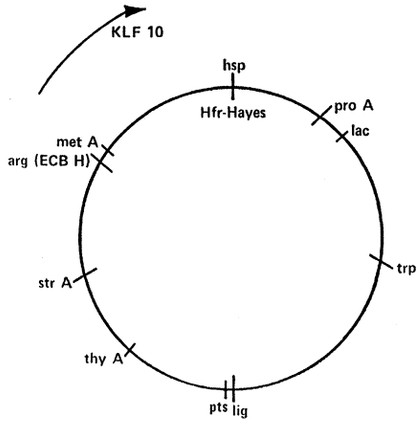

Fig. 7. Location of structural gene for DNA ligase on genetic map of *E. coli*. [Map drawn after Taylor and Trotter (*42*)]

DNA-adenylate may not normally occur before the latter is converted to products; consequently, experiments in which synthetic DNA-adenylate is incubated with the ligase may introduce a slow binding step that need not occur when the enzyme proceeds through its normal catalytic cycle.

Despite these uncertainties, the kinetic results taken as a whole are consistent with the mechanism for the *E. coli* DNA ligase reaction depicted in Fig. 2. The demonstration that both ligase-adenylate and DNA-adenylate accumulate during the reversal of the reaction offers additional support for this mechanism, particularly with respect to the involvement of DNA-adenylate.

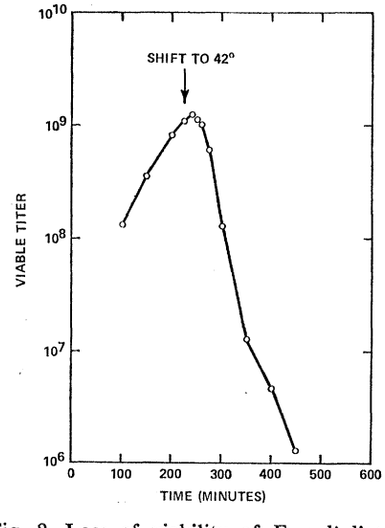

Fig. 8. Loss of viability of *E. coli ligts7* after shift from 25° to 42°C. The viable titer of *lig*$^+$ continues to increase exponentially after temperature shift. Viable titer was determined at 25°C (*43*).

Function of DNA Ligase in vivo

Much of what is currently known about the role that DNA ligase plays in vivo is based on the study of mutants in which the enzyme is defective. Since a ligation step has been invoked in almost all models of genetic recombination, in the repair of damage to DNA and in DNA replication, one might predict that a defective ligase would lead to aberrations in any or all of these processes.

Mutants in both T4 DNA ligase and *E. coli* DNA ligase have been described (*40, 41, 43, 44*). However, because of the uncertainty about the extent to which the *E. coli* ligase can substitute for the phage-induced enzyme when the latter is defective, more easily interpretable information on the role of DNA ligase in DNA replication and repair has come from an analysis of the *E. coli* mutants. Two mutations in the structural gene for ligase are known; both loci are at about 45 minutes on the genetic map of *E. coli* (*41, 42*) (Fig. 7). Although extracts of both mutants contain abnormally thermosensitive enzymes, one, *E. coli ligts7* (*41, 43, 44*), is a conditional lethal mutant that is inviable at 42°C, while the other, *lig4* (*45*), is not. Both grow normally at 30°C. This difference between the two is probably due to the amounts of residual ligase activity that persist at the elevated temperature. Whereas extracts of both mutants show less than 1 percent of the amount of joining activity at 42°C found in comparable wild-type extracts, the *ligts7* enzyme is defective even when assayed at 25°C. Moreover, in contrast to *lig4*, the *ligts7* ligase is abnormally thermosensitive even when measured by the formation of enzyme-adenylate, an assay that is able to detect a single turnover of the enzyme (Table 3) (*20*).

What, then, are the physiologic consequences of the *ligts7* mutation? The *ligts7* mutant as noted above, is unable to grow at 42°C, and in fact loses viability rapidly when shifted from a permissive (25°C) to a nonpermissive (42°C) temperature (Fig. 8). The *ligts7* mutant is also abnormally sensitive to the alkylating agent methyl methanesulfonate and to irradiation with ultraviolet light even at permissive temperatures (Fig. 9). It is therefore defective in its ability to repair damage to DNA caused by these agents.

As might be anticipated, the *ligts7* strain is defective in DNA synthesis

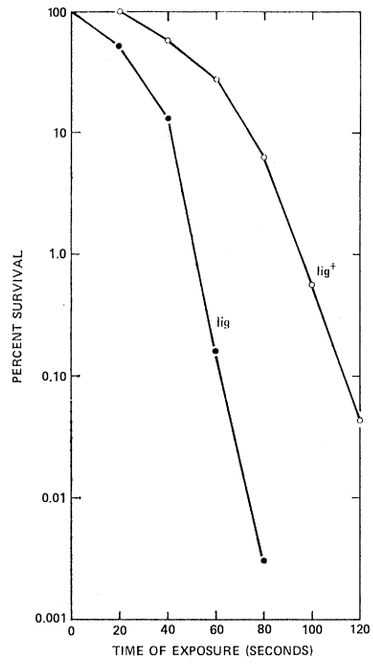

Fig. 9. Loss of viability of *E. coli ligts7* after irradiation with ultraviolet light at 25°C (*43*).

and accumulates 10*S* "Okazaki fragments" (*10*) to a strikingly greater extent than does the wild-type strain. Essentially all of the [^{3}H]thymidine that is incorporated into DNA by *E. coli ligts7* during a 10-second treatment (pulse) at 42°C has a sedimentation coefficient of about 10*S*. Under the same conditions, the *lig*+ strain incorporates [^{3}H]thymidine primarily into DNA with a sedimentation coefficient of 24*S* or greater (Fig. 10). When the 10-second pulse of *E. coli ligts7* at 42°C is followed by a 5-minute treatment with excess unlabeled thymidine at 25°C, most of the [^{3}H]thymidine incorporated during the pulse period appears in DNA with a median sedimentation coefficient of 30*S*, suggesting that the 10*S* material that accumulates in the mutant after the treatment with [^{3}H]thymidine at 42°C is a precursor of high molecular weight DNA. The *ligts7* mutant accumulates 10*S* fragments even at permissive temperatures, a result that is consistent with the greatly reduced ligase activity in extracts of the mutant at 25°C.

Since a functional DNA ligase is essential for normal DNA replication and repair in *E. coli*, how can the normal growth and viability of the *lig4* mutant at temperatures where assays of crude extracts show 1 percent or less of normal joining activity be explained? The answer would appear to be that ligase is present in vast excess over what is normally required by the cell.

As was noted previously, an *E. coli* cell contains approximately 300 DNA ligase molecules (*20*). Given a turnover number of approximately 25 min^{-1} at 30°C (*28*), then 7500 single-strand breaks could be sealed per minute per cell. Since both strands of the *E. coli* chromosome are replicated discontinuously (*41, 43*), and given that the time to replicate the chromosome at 30°C is about 65 minutes (*46*) and that the average length of an "Okazaki fragment" is 1000 nucleotides (*23*), then approximately 200 sealing events per minute per cell should be sufficient to account for the joining of these intermediates in the discontinuous replication of DNA within a replication period. A few percent of the normal complement of ligase should therefore be sufficient to permit DNA replication to occur. Extracts of *ligts7* contain 1 to 3 percent as much DNA joining activity at permissive temperatures as does the wild-type parent assayed under similar conditions (*41, 43*). Since the mutant grows normally at permissive temperatures, it seems reasonable to conclude that the cell has a relatively limited requirement for ligase-catalyzed joining events and that this is well within the capabilities of the enzyme as determined in vitro.

Construction of Recombinant DNA Molecules in vitro

In addition to its indispensable function in the repair and replication of DNA in *E. coli* and almost certainly in other organisms, DNA ligase has proved to be an invaluable reagent in the construction of recombinant DNA molecules in vitro. A detailed account of the various instances in which ligase has been used in this way is beyond the scope of this article. However, two cases are cited that are particularly illustrative.

Inasmuch as the optimal substrate for DNA ligase is a duplex DNA with single-strand breaks, the first step in the construction of recombinant molecules is to provide cohesive termini for the two DNA duplexes to be joined. This has been accomplished by adding a single-stranded homopolymeric segment [for example, $(dA)_{100}$] to the 3′ end of one DNA strand and $(dT)_{100}$ to the other, by means of the enzyme terminal nucleotidyl transferase (*47*). The DNA's modified in this way are

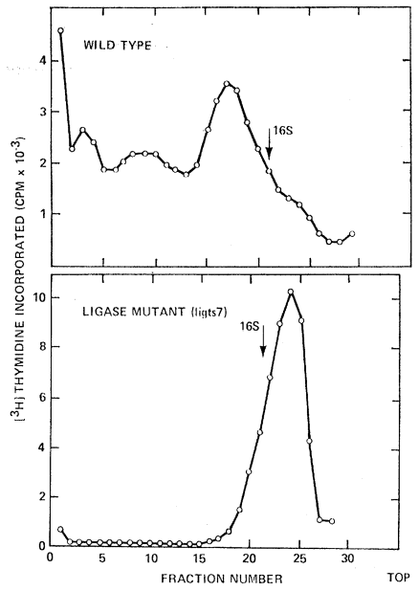

Fig. 10. Accumulation of "Okazaki fragments" by *E. coli ligts7* after 10-second treatment with [^{3}H]thymidine at 42°C (*43*).

first annealed to yield recombinant molecules joined by hydrogen bonds, and these structures are treated with *E. coli* DNA polymerase I or T4 DNA polymerase to fill in any gaps that may exist. The final closure is effected with DNA polymerase I or T4 DNA polymerase to fill in any gaps that may circles of SV40 virus DNA (*48*) and linear dimers of phage P22 DNA (*49*). It has also been used to form covalently closed circular SV40 DNA molecules into which the galactose operon of *E. coli* has been inserted (*48*).

A second, somewhat simpler procedure utilizes the restriction endonuclease specified by the drug resistance factor RTF-I, which cleaves unmodified DNA to produce staggered breaks, thus generating cohesive ends six residues long (*50*). This procedure, which permits direct ligation of the cleaved DNA molecules once they have been annealed, is simpler than that involving the addition of homopolymeric tails prior to ligase treatment. On the other hand, it does not possess the specificity of joining conferred by the directing homopolymers, and the yield of desired recombinant product is correspondingly diminished. This method has very recently been used successfully to insert restriction endonuclease–generated fragments of *Xenopus laevis* DNA, containing the genes for 18*S* and 28*S* ribosomal RNA into an autonomously replicating *E. coli* plasmid that carries the information for tetracycline resist-

ance. The recombinant DNA containing both *X. laevis* and plasmid DNA's replicates in stable fashion in *E. coli* and synthesizes RNA complementary to *X. laevis* ribosomal RNA (*51*).

Finally, as was noted previously, the T4-induced DNA ligase can promote directly the end-to-end joining of two duplex molecules with fully base-paired termini. In fact, linear dimers and small amounts of trimers have been generated on incubation of P22 DNA with T4 ligase (*52*).

Summary

DNA ligase of *E. coli* is a single polypeptide of molecular weight 75,000. The comparable T4-induced enzyme is somewhat smaller (63,000 to 68,000). Both enzymes catalyze the synthesis of phosphodiester bonds between adjacent 5′-phosphoryl and 3′-hydroxyl groups in nicked duplex DNA, coupled to the cleavage of the pyrophosphate bond of DPN (*E. coli*) or ATP (T4). Phosphodiester bond synthesis catalyzed by both enzymes occurs in a series of these discrete steps and involves the participation of two covalent intermediates (Fig. 1). A steady state kinetic analysis of the reaction-catalyzed *E. coli* ligase supports this mechanism, and further demonstrates that enzyme-adenylate and DNA-adenylate are kinetically significant intermediates on the direct path of phosphodiester bond synthesis.

A strain of *E. coli* with a mutation in the structural gene for DNA ligase which results in the synthesis of an abnormally thermolabile enzyme is inviable at 42°C. Although able to grow at 30°C, the mutant is still defective at this temperature in its ability to repair damage to its DNA caused by ultraviolet irradiation and by alkylating agents. At 42°C, all the newly replicated DNA is in the form of short 10*S* "Okazaki fragments," an indication that the reason for the mutant's failure to survive under these conditions is its inability to sustain the ligation step that is essential for the discontinuous synthesis of the *E. coli* chromosome. DNA ligase is therefore an essential enzyme required for normal DNA replication and repair in *E. coli*. Purified DNA ligases have proved to be useful reagents in the construction in vitro of recombinant DNA molecules.

References and Notes

1. M. Meselson and J. J. Weigle, *Proc. Natl. Acad. Sci. U.S.A.* **47**, 857 (1961).
2. G. M. Kellenberger, M. L. Zichichi, J. J. Weigle, *ibid.*, p. 869.
3. E. T. Young and R. L. Sinsheimer, *J. Mol. Biol.* **10**, 562 (1964).
4. V. C. Bode and A. D. Kaiser, *ibid.* **14**, 399 (1965).
5. M. Gellert, *Proc. Natl. Acad. Sci. U.S.A.* **57**, 148 (1967).
6. B. Weiss and C. C. Richardson, *ibid.*, p. 1021.
7. B. M. Olivera and I. R. Lehman, *ibid.*, p. 1426.
8. M. L. Gefter, A. Becker, J. Hurwitz, *ibid.* **58**, 240 (1967).
9. N. R. Cozzarelli, N. E. Melechen, T. M. Jovin, A. Kornberg, *Biochem. Biophys. Res. Commun.* **28**, 578 (1967).
10. R. Okazaki, T. Okazaki, K. Sakabe, K. Sugimoto, A. Sugino, *Proc. Natl. Acad. Sci. U.S.A.* **59**, 598 (1968).
11. T. Lindahl and G. M. Edelman, *ibid.* **61**, 680 (1968).
12. K. Tsukada and M. Ichimura, *Biochem. Biophys. Res. Commun.* **42**, 1156 (1971).
13. S. H. Howell and H. Stern, *J. Mol. Biol.* **55**, 357 (1971).
14. B. M. Olivera and I. R. Lehman, *Proc. Natl. Acad. Sci. U.S.A.* **57**, 1700 (1967).
15. S. B. Zimmerman, J. W. Little, C. K. Oshinsky, M. Gellert, *ibid.*, p. 1841.
16. E. K. F. Bautz, *Biochem. Biophys. Res. Commun.* **28**, 641 (1967).
17. P. Modrich and I. R. Lehman, *J. Biol. Chem.* **245**, 3626 (1970).
18. S. B. Zimmerman and C. K. Oshinsky, *ibid.* **244**, 4689 (1969).
19. B. Weiss, A. Jacquemin-Sablon, T. R. Live, G. C. Fareed, C. C. Richardson, *ibid.* **243**, 4543 (1968).
20. P. Modrich, Y. Anraku, I. R. Lehman, *ibid.* **248**, 7495 (1973).
21. P. Modrich and I. R. Lehman, *Fed. Proc.* **31**, 441 (1972).
22. C. C. Richardson, C. L. Schildkraut, H. V. Aposhian, A. Kornberg, *J. Biol. Chem.* **239**, 222 (1964).
23. R. Okazaki, M. Arisawa, A. Sugino, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2954 (1971).
24. A. Panet, J. H. Van de Sande, P. C. Loewen, H. G. Khorana, A. J. Raae, J. R. Lillehaug, K. Kleppe, *Biochemistry* **12**, 5045 (1973).
25. B. M. Olivera and I. R. Lehman, *J. Mol. Biol.* **36**, 261 (1968); K. Kleppe, J. H. Van de Sande, H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 68 (1970); G. C. Fareed, E. M. Wilt, C. C. Richardson, *J. Biol. Chem.* **246**, 925 (1971).
26. V. Sgaramella, J. H. Van de Sande, H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1468 (1970).
27. R. Silber, V. G. Malathi, J. Hurwitz, *ibid.* **69**, 3009 (1972).
28. P. Modrich and I. R. Lehman, *J. Biol. Chem.* **248**, 7502 (1973).
29. J. W. Little, S. B. Zimmerman, C. K. Oshinsky, M. Gellert, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 2004 (1967); B. M. Olivera, Z. W. Hall, Y. Anraku, J. R. Chien, I. R. Lehman, *Cold Spring Harbor Symp. Quant. Biol.* **33**, 27 (1968).
30. B. Weiss, A. Thompson, C. C. Richardson, *J. Biol. Chem.* **243**, 4556 (1968).
31. R. I. Gumport and I. R. Lehman, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2559 (1971).
32. B. M. Olivera, Z. W. Hall, I. R. Lehman, *ibid.* **61**, 237 (1968).
33. C. L. Harvey, T. F. Gabriel, E. M. Wilt, C. C. Richardson, *J. Biol. Chem.* **246**, 4523 (1971).
34. I. R. Lehman, *ibid.* **235**, 1479 (1960).
35. Z. W. Hall and I. R. Lehman, *ibid.* **244**, 43 (1969).
→ J. C. Wang, *J. Mol. Biol.* **55**, 523 (1971).
37. P. Modrich, I. R. Lehman, J. C. Wang, *J. Biol. Chem.* **247**, 6370 (1972).
38. W. P. Jencks, *Catalysis in Chemistry and Enzymology* (McGraw-Hill, New York, 1969), pp. 56–64.
39. J. London and M. Knight, *J. Gen. Microbiol.* **44**, 241 (1966).
40. G. C. Fareed and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 665 (1967).
→ M. M. Gottesman, M. Hicks, M. Gellert, *J. Mol. Biol.* **77**, 531 (1973).
42. A. L. Taylor and C. D. Trotter, *Bacteriol. Rev.* **31**, 332 (1967).
43. E. B. Konrad, P. Modrich, I. R. Lehman, *J. Mol. Biol.* **77**, 519 (1973).
44. C. Pauling and L. Hamm, *Proc. Natl. Acad. Sci. U.S.A.* **60**, 1495 (1968).
45. M. Gellert and M. L. Bullock, *ibid.* **67**, 1580 (1970).
46. R. E. Bird and K. G. Lark, *J. Mol. Biol.* **49**, 343 (1970).
47. K. Kato, J. M. Gonçalves, G. E. Houts, F. J. Bollum, *J. Biol. Chem.* **242**, 2780 (1967).
48. D. A. Jackson, R. H. Symons, P. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2904 (1972).
49. P. E. Lobban and A. D. Kaiser, *J. Mol. Biol.* **78**, 453 (1973).
50. J. E. Mertz and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3370 (1972); J. Hedgpeth, H. M. Goodman, H. W. Boyer, *ibid.*, p. 3448.
51. J. F. Morrow, S. N. Cohen, A. C. Y. Chang, H. W. Boyer, H. M. Goodman, R. B. Helling, *ibid.* **71**, 1743 (1974).
52. V. Sgaramella, *ibid.* **69**, 3389 (1972).
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