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Influence of monovalent cations on the activity of T4 DNA ligase in the presence of polyethylene glycol

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ABSTRACT

Monovalent cations such as Na^+ and K^+ inhibit the activity of T4 DNA ligase. However, the extent of inhibition varies with the terminal sequence of the duplex DNA used as substrate; in many cases, ligation of DNA is completely inhibited at 200 mM. The activity of the ligase is stimulated by raising the concentration of polyethylene glycol 6000 from 0 to 15% (w/v) when NaCl and KCl were both absent. Ligation was reduced as the concentration of NaCl or KCl was raised in a mixture containing 5 or 15% PEG 6000. With 10% PEG 6000, both cohesive- and blunt-end ligation of this ligase increased at high concentrations of salt (150-200 mM NaCl, or 200-250 mM KCl). Further, with 10% PEG 6000, inter- and intramolecular ligation occurred at low salt concentrations (0-100 mM NaCl, or 0-150 mM KCl); only linear oligomers were formed by intermolecular ligation at the high concentrations.

INTRODUCTION

T4 DNA ligase catalyzes cohesive- and blunt-end ligation (1). High concentrations (0.2 M) of monovalent cations such as Na^+ and K^+ inhibit these activities, because they decrease the enzyme affinity for the DNA substrate; thus, Km for the substrate increases (2). However, we have found to our surprise that the activity of this ligase was maximum at high concentrations of salt (150-200 mM NaCl, or 200-250 mM KCl) in the presence of 10% (w/v) polyethylene glycol 6000 (PEG 6000), which is a nonspecific polymer that stimulates the joining step of DNA ligases from *Escherichia coli*, rat liver, and cells infected with T4 phage, when NaCl and KCl are both absent (3). We here describe the influence of Na^+ and K^+ and of temperature on the activity of T4 DNA ligase when the concentration of PEG 6000 is changed. We also investigated the mechanism by which this polymer stimulates the ligase.

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MATERIALS AND METHODS

Materials

T4 DNA ligase was purified from E. coli 1100 lysogenic for NM989(λT4lig), in which the expression of the T4 DNA ligase gene is regulated by temperature (4, 5). The purified ligase was free from nucleases and contained no other polypeptides when examined by sodium dodecyl sulfate gel electrophoresis. The restriction endonucleases BamHI, EcoRI, EcoRV, HindIII, PstI, PvuII, SalI, and ScaI were purified at our laboratory. NruI restriction endonuclease was purchased from New England Biolabs. Both pBR322 DNA and λ DNA were prepared at our laboratory. pBR322 DNA was digested with one of the above endonucleases, and the digested DNA was isolated by phenol/chloroform extraction and ethanol precipitation. PEG 6000 was purchased from Wako Pure Chemical Industries, Ltd., Japan. A stock solution of 40% (w/v) PEG 6000 was made using distilled water. In this report, solutions of this polymer are expressed as (w/v) percent. ^{32}P-labeled PPi was purchased from New England Nuclear.

Methods

T4 DNA ligase was routinely assayed at 16°C for 30 min in polypropylene tubes in a mixture (20 μl) of 66 mM tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM ATP, 0.25 μg of linearized pBR322 DNA, and dilute T4 DNA ligase. The ligase was diluted in 50 mM tris-HCl (pH 7.6), 10 mM 2-mercaptoethanol, and 0.05% bovine serum albumin. One unit of T4 DNA ligase was defined as the amount needed to give more than 90% ligation of HindIII-digested λ DNA in 30 min in 20 μl of the same mixture as above except that 6 μg of the HindIII-digested λ DNA was substituted for the pBR322 DNA. After the reaction, 5 μl of a mixture of 1% sodium dodecyl sulfate, 0.02% bromophenol blue, and 50% glycerol was added and the samples were put on 0.7% agarose gels made in 40 mM tris-acetate (pH 7.8), 1 mM EDTA, and 1 μg/ml ethidium bromide. High levels of PEG 6000 and NaCl or KCl in the sample did not change the migration of DNA in the gels. The amounts of ligation products were measured on a densitometer (Gelman Sciences, Inc.)

ATP-PPi exchange activity was assayed at 37°C for 20 min in the mixture described elsewhere (6).

RESULTS

Influence of monovalent cations on DNA ligation

The influence of monovalent cations on the activity of T4 DNA ligase was measured when polymers such as PEG 6000 were not present. The DNA

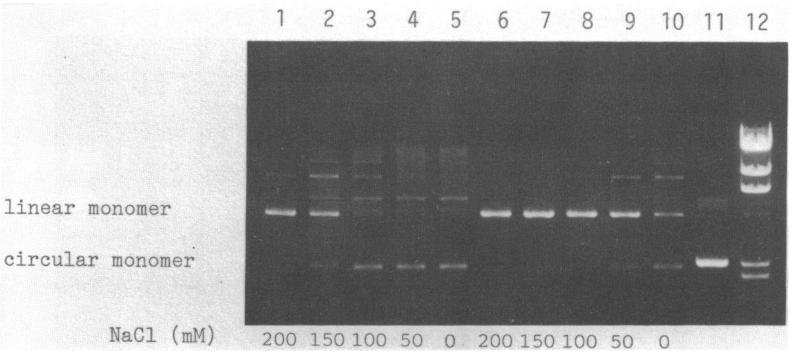

Figure 1. Comparison of cohesive-end ligation at HindIII and EcoRI sites. Assay mixtures were as in Methods, and contained HindIII-digested (Lanes 1-5) or EcoRI-digested pBR322 DNA (Lanes 6-10). NaCl was present at the concentrations indicated. T4 DNA ligase (0.35 u) was added. pBR322 DNA RFI, Lane 11; HindIII-digested λ DNA, Lane 12.

substrates we used to assay ligation were pBR322 DNA cut at a single site by EcoRI, BamHI, HindIII, PstI, or SalI, which generated cohesive-end termini, or by EcoRV, NruI, PvuII, or ScaI, which generated blunt-end termini. The extent of both cohesive- and blunt-end ligation decreased as the concentration of NaCl or KCl increased. Resistance to inhibition by monovalent cations varied with the terminal sequence of the DNA, and it was in the following order, from strong to weak:

Cohesive-end ligation	HindIII, PstI, EcoRI, BamHI, SalI
Blunt-end ligation	EcoRV, ScaI, PvuII, NruI

Figure 1 shows the differences in resistance at the HindIII and the EcoRI sites. At 100 mM NaCl, all of the linear pBR322 DNA substrate formed by HindIII digestion was ligated, but most of the same substrate formed by EcoRI digestion remained unligated (Fig. 1, Lanes 3 and 8). The extent of ligation in a mixture free from Na^+ or K^+ also varied with the terminal sequence of DNA, decreasing in the same order as above. All of the DNA substrates except for EcoRI-digested pBR322 DNA contain the same proportion of AT and GC pairs in their terminal sequences, so the extent of cohesive-end ligation probably depends not on the annealing efficiency of the terminal sites, but rather on the affinity of the enzyme for nicked regions in the duplex DNA.

Cohesive-end ligation in PEG 6000 solutions

Cohesive-end ligation was assayed with varying concentrations of PEG 6000 (5, 10, and 15%) and salt (NaCl or KCl). Figure 2 shows the effect of

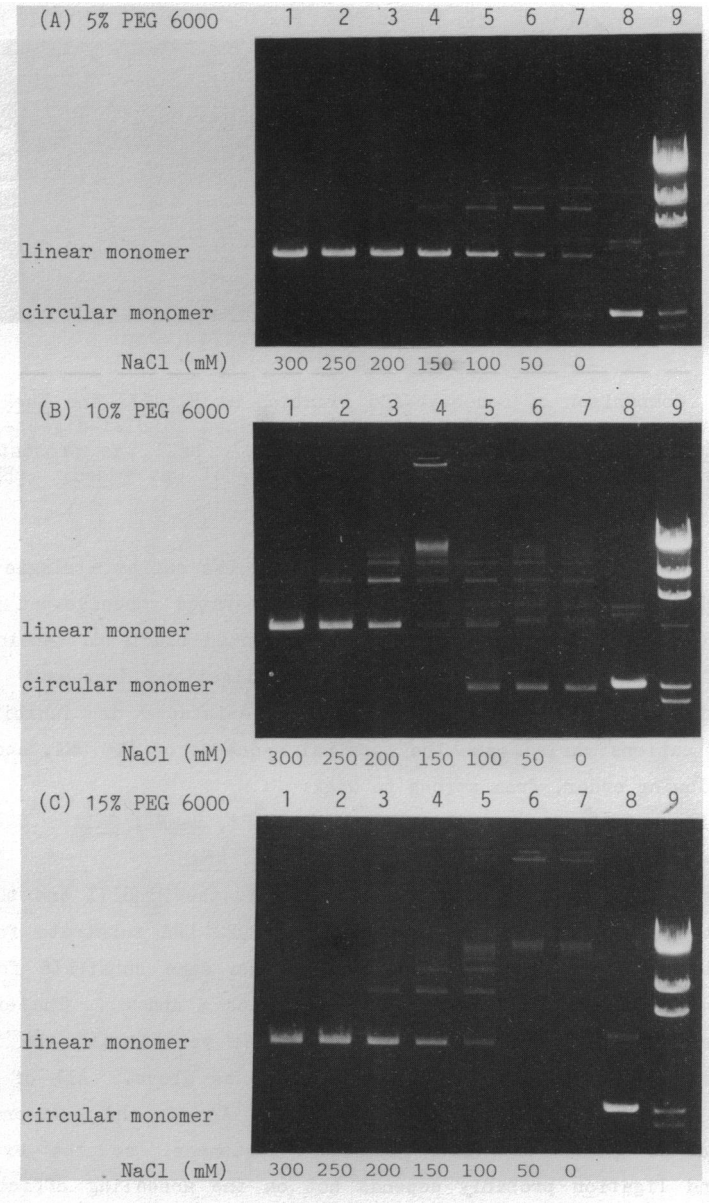

Figure 2. Effect of NaCl on cohesive-end ligation with 5 (A), 10 (B), or 15% (C) PEG 6000. Assay mixtures were as in Methods, and contained EcoRI-digested pBR322 DNA. PEG 6000 and NaCl were present at the concentrations indicated. T4 DNA ligase (0.35 u) was added. pBR322 DNA RFI, Lane 8; HindIII-digested λ DNA, Lane 9.

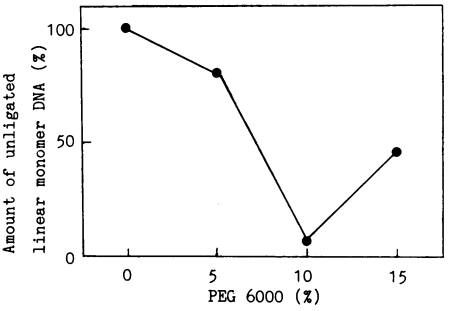

Figure 3. Unligated linear monomer DNA at 150 mM NaCl in 0, 5, 10, and 15% PEG 6000 solutions. Assay mixtures were as in Methods, and contained EcoRI-digested pBR322 DNA and 150 mM NaCl; 0.35 u of T4 DNA ligase was added. The amount of unligated linear monomer DNA was measured using a densitometer.

Na^+ using EcoRI-digested pBR322 DNA. Cohesive-end ligation was stimulated in the absence of salt by increasing the concentration of PEG 6000 from 0 to 15%. When PEG 6000 was not present, 32% of the DNA substrate was unligated; all was ligated in 15% PEG 6000 solution (Fig. 1, Lane 10, and Fig. 2C, Lane 7). In the presence of 5 or 15% PEG 6000, ligation was slower at the higher NaCl concentration; the absence of NaCl was optimal (Fig. 2A, C; Lane 7). Both inter- and intramolecular ligation took place in mixtures containing 5% PEG 6000 (Fig. 2A) as in mixtures with none (Fig. 1). In contrast, when there was 15% PEG 6000, all of the ligation products were linear oligomers from intermolecular ligation (Fig. 2C).

A phenomena that surprised us was observed in 10% PEG 6000 solutions. Here, intermolecular ligation was markedly stimulated at 150 mM NaCl; 94% of the linear monomer was converted to linear oligomers (Fig. 2B, Lane 4). The amount of unligated linear monomer DNA at 150 mM NaCl was least in 10% PEG 6000 solution among the different concentrations of PEG 6000 (Fig. 3). In 10% PEG 6000 solutions, only intermolecular ligation occurred at 150-300 mM NaCl; both inter- and intramolecular ligation occurred at 0-100 mM NaCl (Fig. 2B, Lanes 1-4 and Lanes 5-7, respectively). Identification of circular products from intramolecular ligation was based on their mobilities on a gel compared to standard materials and on their resistance to λ exonuclease (data not shown). This nuclease can degrade linear but not circular duplex DNA. Recutting of these products by EcoRI showed that the EcoRI site was actually joined by the ligase (Fig. 4). Similar phenomena occurred with K^+. The concentration of KCl at which intermolecular ligation

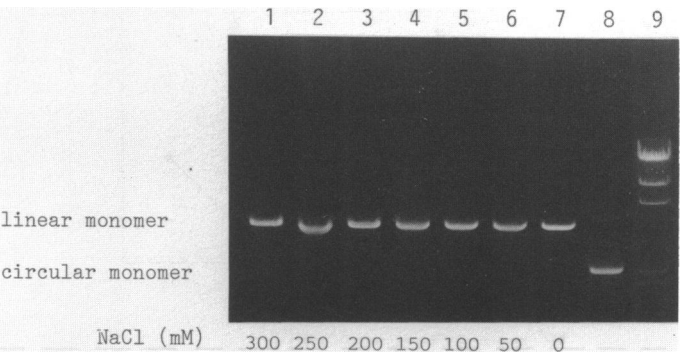

Figure 4. Recutting by EcoRI of products formed by ligation of EcoRI-digested pBR322 DNA in the presence of 10% PEG 6000 and 0-300 mM NaCl. T4 DNA ligase (0.35 u) was added to the mixtures described in Methods, with EcoRI-digested pBR322 DNA and 10% PEG 6000. NaCl was present at the concentrations indicated. Incubation was at 16°C for 30 min and then at 65°C for 5 min. After cooling at room temperature, 19 µl of water and EcoRI (84 u) were added and the mixtures were incubated at 37°C for 2 hr. pBR322 DNA RFI, Lane 8; HindIII-digested λ DNA, Lane 9.

was markedly stimulated was 200 mM (data not shown). These results were not specific to the EcoRI site. Using other cohesive-ended DNA as substrates gave similar results (data not shown). Table 1 shows variations in the optimal concentration of NaCl or KCl for cohesive-end ligation in 10% PEG 6000 solutions, for each cutting site.

Blunt-end ligation in PEG 6000 solutions

In mixtures containing 10% PEG 6000, blunt-end ligation was stimulated by higher concentrations of NaCl or KCl (Table 2). Figure 5 shows the effects of Na^+ on such ligation with ScaI-digested pBR322 DNA. Compared to cohesive-end ligation, the difference in the extent of blunt-end ligation at low and high concentrations of salt was much larger (Figs. 2B and 5). After incubation at 16°C for 30 min with 7.0 units of ligase in 10% PEG 6000

Table 1. Optimal salt concentration for cohesive-end ligation at different cutting sites in the presence of 10% PEG 6000.

Site	NaCl (mM)	KCl (mM)
HindIII	200	200-250
PstI	200	300
EcoRI	150	200
BamHI	150-200	250
SalI	150	200

Table 2. Optimal salt concentration for blunt-end ligation at different cutting sites in the presence of 10% PEG 6000.

Site	NaCl (mM)	KCl (mM)
EcoRV	150-200	200-250
ScaI	150-200	200-250
PvuII	150-200	200
NruI	200	250

solution, 96% of the linear monomer DNA was converted to linear oligomers by intermolecular ligation with 150 mM NaCl (Fig. 5, Lane 5); at 0-100 mM NaCl, ligated DNA was hardly detected (Fig. 5, Lanes 6-8). ScaI-digested pBR322 DNA remained unligated even at 0 mM NaCl in the absence of PEG 6000 with 7.0 units of T4 DNA ligase. Blunt-end ligation was inhibited as the concentration of NaCl was raised in 5 or 15% PEG 6000 solutions. However, the activity was enhanced more than 1000-fold in the 15% PEG 6000 solution compared to activity without PEG 6000; ScaI-digested pBR322 DNA was almost completely converted to linear oligomers by intermolecular ligation even at 150 mM NaCl in 15% PEG 6000 solution with 0.35 units of T4 DNA ligase. Similar results were obtained with KCl (data not shown).

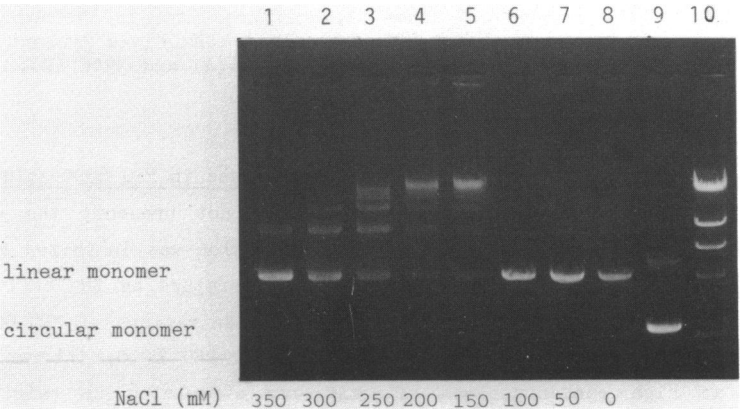

Figure 5. Effect of NaCl on blunt-end ligation in 10% PEG 6000. T4 DNA ligase (7.0 u) was added to the assay mixtures as in Methods, with ScaI-digested pBR322 DNA and 10% PEG 6000 added. NaCl was present at the concentrations indicated. pBR322 DNA RFI, Lane 9; HindIII-digested λ DNA, Lane 10.

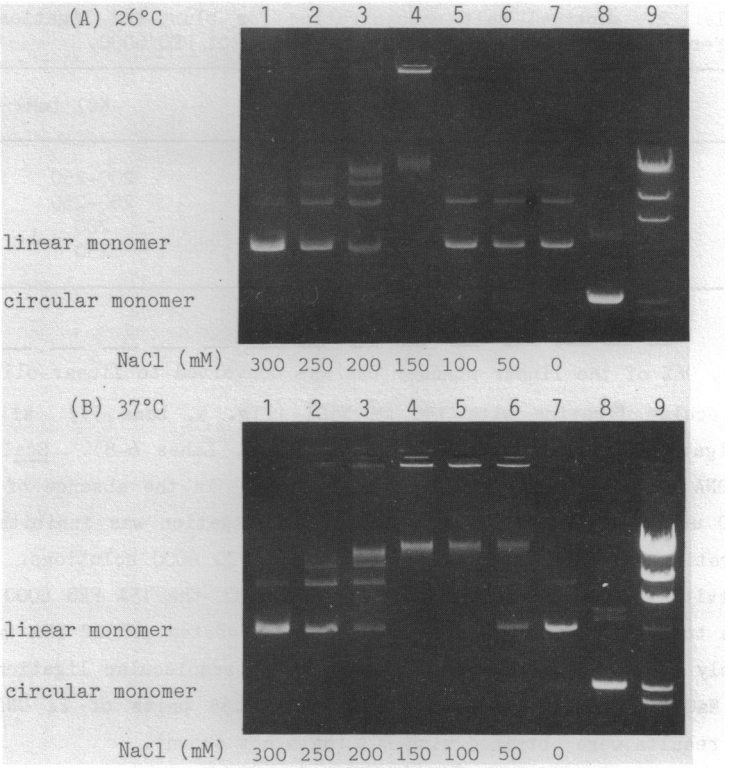

Figure 6. Effect of temperature on cohesive-end ligation in 10% PEG 6000. T4 DNA ligase (0.35 u) was added to mixtures as in Methods, with EcoRI-digested pBR322 DNA and 10% PEG 6000 added. NaCl was present at the concentrations indicated. Incubation was at 26°C (A) and 37°C (B). pBR322 DNA RFI, Lane 8; HindIII-digested λ DNA, Lane 9.

Influence of temperature on activity of T4 DNA ligase in PEG 6000 solutions

In the usual situation when PEG 6000 was not present, the optimal temperature for T4 DNA ligase was 10-16°C; ligation was inhibited at 26°C or more (data not shown). The influence of temperature on the activity of this ligase in a mixture containing different concentrations of PEG 6000 (5, 10, and 15%) was measured at 10-37°C. With 10% PEG 6000, intermolecular ligation at high concentrations of salt was stimulated by raising the temperature from 10 to 37°C; the salt concentrations at which such ligation was increased extended to lower concentrations at 37°C. Figure 6 shows the results at the EcoRI site at 26 and 37°C. The amount of unligated DNA substrate in the mixture without NaCl increased as the temperature was

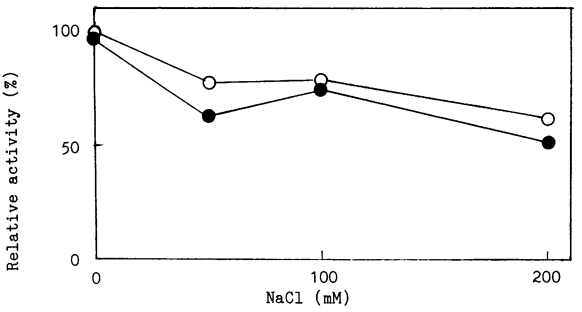

Figure 7. Influence of Na^+ on ATP-PPi exchange reaction in 10% PEG 6000 solutions. (○), PEG 6000-free; (●), 10% PEG 6000. The reaction was at 37°C with 12 u of T4 DNA ligase. Here, 100% activity is that in the mixture without PEG 6000 or NaCl.

raised from 10 to 37°C; only 15% of the substrate was ligated at 37°C (Fig. 6B, Lane 7). The opposite was true of mixtures containing 150-300 mM NaCl (Fig. 2B, Lanes 1-4; Fig. 6A, Lanes 1-4; and Fig. 6B, Lanes 1-4). The amount of unligated linear monomer DNA remaining in the mixture containing 150 mM NaCl at 10°C was 11% (data not shown), but this amount in the same mixture at 16, 26, and 37°C was 6, 0, and 0%, respectively (Figs. 2B, 6A, and 6B; Lane 4). Both inter- and intramolecular ligation occurred in the mixture containing 100 mM NaCl at 16 and 26°C, the amount of circular monomer decreasing at 26°C: 36% at 16°C and 5% at 26°C (Figs. 2B, 6A; Lane 5). However, at 37°C, all of the linear monomer DNA was converted to linear oligomers by intermolecular ligation (Fig. 6B, Lane 5). These results suggest that only intermolecular ligation is stimulated by raising the temperature of a 10% PEG 6000 solution. With 15% PEG 6000, the extent of ligation was stimulated by raising the temperature in all of the mixtures from 0 to 300 mM NaCl; 37°C was optimal. In contrast, with 5% PEG 6000, the extent of ligation decreased with increasing temperatures, whatever the NaCl concentration; 10°C was optimal. Similar results were obtained with K^+ and at other cohesive-end cutting sites (data not shown).

Influence of Na^+ on ATP-PPi exchange in PEG 6000 solutions

The effect of Na^+ on the ATP-PPi exchange reaction in 10% PEG 6000 solutions was studied at 37°C. The first ligation reaction, Enzyme + ATP $\rightleftharpoons$ Enzyme-AMP complex + PPi, is reversible, so that T4 DNA ligase also catalyzes an exchange reaction between ^{32}P-labeled PPi and ATP that does not require the presence of DNA (7). This reaction was not stimulated by Na^+ in

the presence of 10% PEG 6000 (Fig. 7). The ATP-PPi exchange reaction was inhibited as the concentration of NaCl was raised even in 10% PEG 6000 solutions; the activity at 100 mM NaCl in 10% PEG 6000 solution was lower than that at 0 mM NaCl at 37°C. These results suggest that the first reaction of this ligase was not stimulated by PEG 6000, and that it was not stimulated in the mixtures containing high salt concentrations, in the presence of 10% PEG 6000.

DISCUSSION

T4 DNA ligase requires ATP as a cofactor and forms a phosphodiester linkage between 3'-OH and 5'-phosphate termini in duplex DNA (6). The reaction proceeds in three steps. (a) Transfer of the adenylyl group of ATP to an amino group of a lysine residue in the enzyme to form the ligase-AMP complex, with the elimination of PPi. (b) Transfer of the adenylyl group from the enzyme to the 5'-phosphoryl termini at the nick in DNA. (c) Phosphodiester formation by nucleophilic attack by the 3'-OH group at the nick on the 5'-phosphoryl group that has combined with the adenylyl group. The ligation of this ligase is stimulated by high concentrations of salt in 10% PEG 6000 solutions. This phenomenon is probably caused not by stimulation of the first reaction, but rather by the increased affinity of the enzyme for the DNA substrate, because the ATP-PPi exchange reaction is inhibited as the salt concentrations are raised in 10% PEG 6000 solutions. This affinity is enhanced not only by an increase in the effective concentration of the DNA termini by the presence of a volume excluder such as PEG 6000 (3), because there is some evidence that the presence of PEG 6000 causes changes in DNA, including an increase in its melting temperature (9). Changes in the concentrations of PEG 6000 and NaCl affect the location of nucleic acids and soluble proteins during phase separation (10). It seems that some changes in DNA are caused in 10% PEG 6000 solutions when there is a high concentration of salt and that these changes enhance the affinity of the enzyme for the DNA substrate. We also think that these changes in DNA are readily brought about in lower salt concentrations by raising the temperature. Our results suggest such a temperature effect; the concentrations of salt at which intermolecular ligation was stimulated extended to lower concentrations as the temperature was raised in 10% PEG 6000 solutions.

With 10% PEG 6000, the extent of ligation in the mixtures containing 100-200 mM NaCl was greatest at 37°C, close to the optimal temperature for

growth of E. coli. So, the presence of high concentrations of salt and PEG 6000 in solution may make the situation in vitro resemble that in vivo. Experimental stimulation of in-vitro DNA replication using polymers (11-13) supports this theory.

There are some restriction endonucleases such as SalI for which the optimal salt concentration is high (150-200 mM NaCl). When conventional methods are used, T4 DNA ligase cannot join the DNA fragments formed by digestion with such endonucleases under their optimal high salt concentrations, because it is necessary to replace the buffer of high salt concentration midway for one with a low salt concentration. The presence of 10% PEG 6000 makes such a maneuver unnecessary; with it the extent of ligation is increased at high concentrations of salt. Using 10% PEG 6000 may be helpful in making recombinant DNA.

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