Ligase Joining of Oligodeoxythymidylates†

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ABSTRACT: The joining by bacteriophage T_4 polynucleotide ligase of thymidylate oligomers in the presence of poly(dA) and poly(rA) has been investigated. Temperature-dependent curves were constructed for oligomers of different lengths and the curves were shown to be a sensitive tool for investigating associative effects of the oligomer·polymer complex. This oligomer·polymer complex minus the enzyme was unstable at the temperature for optimum joining. A method for extending the lengths of homooligomers with blocked partners is described.

Polynucleotide ligases are enzymes capable of repairing single-strand breaks ("nicks") in double-stranded polynucleotides. Their mechanism of action has been studied and elucidated by Olivera *et al.* (1968) and Harvey *et al.* (1971). These enzymes are now an important addition to the growing list used in the manipulation of nucleic acid chains. Thus, the polynucleotide ligase derived from *Escherichia coli* infected with bacteriophage T_4 has been used in the total synthesis of the gene for yeast tRNA (Agarwal *et al.*, 1970).

In preparation for similar applications in connection with the synthesis of a fragment of double-stranded DNA coding for an S-peptide analog of ribonuclease A (Heimer *et al.*, 1972; Poonian *et al.*, 1972), studies were conducted on the joining of thymidylate oligomers held together by poly(dA)[1] "splints." Gupta *et al.* (1968a) had established that the minimum length of oligomers joined was determined by the temperature at which the splint melted or separated from the chains to be joined. It was felt that a study extending these observations would be useful in determining associative properties of oligomers as well as in defining optimum conditions for joining.

Harvey *et al.* (1971) found that joining of thymidylate oligomers on a strand of poly(dA) proceeded as a function of temperature characteristic for the length of the oligomer. This observation has been extended in this report and the method is shown to be a sensitive tool for study of interaction of both ribo- and deoxyribonucleotide oligomers. The oligomer·poly(dA) association was shown to be reversible at the temperature at which the joining rate was optimal. This result demonstrates that the enzyme plays a role in the stabilization of the complex.

These studies have been used to derive a procedure for synthesizing thymidylate oligomers of defined length by block addition of chemically prepared oligomers. This method was used to prepare $(dT)_{18}$.

Experimental Section

Enzymes. Polynucleotide ligase was purified from T_4 r^+ infected *Escherichia coli* B. The *E. coli* B cells were grown in 100 l. of nutrient broth under forced aeration in a 75-gal. fermentor. When the cell density reached 1.2×10^9 cells/ml, 100 mg of L-tryptophan and T_4 r^+ phage at a multiplicity of two were added. The cell growth was continued for 45 min and the batch was cooled rapidly. The cells were removed by continuous centrifugation in a Sharples centrifuge. The T_4 infected cells (207 g net weight) were divided into 40-g batches and stored at −20°. Extraction and purification of the T_4 ligase was accomplished by the method of Weiss *et al.* (1968). Enzyme obtained from the phosphocellulose chromatography was concentrated to 2 ml with Carbowax 20,000 and 3 mg of purified bovine albumin (Pentex) added. The ligase was stored at −20° in 50% glycerol. The specific activity and yield were similar to those described in the literature. All ligase units are expressed in ATP-exchange assay units (Weiss *et al.*, 1968) and enzyme dilutions were made as described by these authors.

Polynucleotide ligase was assayed for endonuclease contamination as described by Weiss *et al.* (1968) except that purified lambda bacteriophage DNA was substituted for T_7 DNA. No endonucleolytic breakage (<1 break/strand) was found after incubation with 5 units of ligase for 1 hr. Phosphatase and ATPase contamination were assayed in the standard reaction mixture with either [5′-^{32}P]p(dT)$_9$ or [γ-^{32}P]ATP, but minus poly(dA). Less than 0.2 pmole of $^{32}P_i$ was released when 20 units of ligase were incubated with substrate for 1 hr.

Polynucleotide kinase was purified from T_4 r^+ infected *E. coli* B as described by Richardson (1965). The phosphocellulose fraction was stored at −20° in 50% glycerol. The purified enzyme contained no endonuclease, as determined by the above assay. Likewise, no phosphatase or ATPase was found when 20 units of kinase was incubated 1 hr with either 20 pmoles of [5′-^{32}P]p(dT)$_9$ or 100 pmoles of [γ-^{32}P]ATP in the standard kinase reaction mixture.

Bacterial alkaline phosphatase was obtained from Worthington Biochemical (BAPC) and diluted to 1 mg/ml after dialysis against 0.01 M Tris-HCl buffer (pH 8.0). Snake venom phosphodiesterase was from Worthington Biochemical.

Preparation of 5′-^{32}P-Labeled Oligomers. Thymidylate oligomers were prepared and characterized by the method of Khorana and Vizsolyi (1961). The oligomers were dephosphorylated, purified by DEAE-cellulose chromatography and labeled with ^{32}P in the 5′ position with polynucleotide kinase, as described by Harvey *et al.* (1971). [5′-^{32}P]Oligomers with four or more residues were chromatographed on DEAE-cellulose to remove the unreacted [γ-^{32}P]ATP as described by these authors. The labeled p(dT)$_3$ was separated by electrophoresis (Markham and Smith, 1952) in 0.05 M ammonium acetate buffer (pH 3.8) at a voltage of 1500 for 1.5 hr. Labeled p(dT)$_2$ was separated by descending paper chromatography

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[1] The abbreviations used are as specified in *Biochemistry* 9, 4022 (1970). We are greatly indebted to Dr. W. E. Cohn for his detailed suggestions.

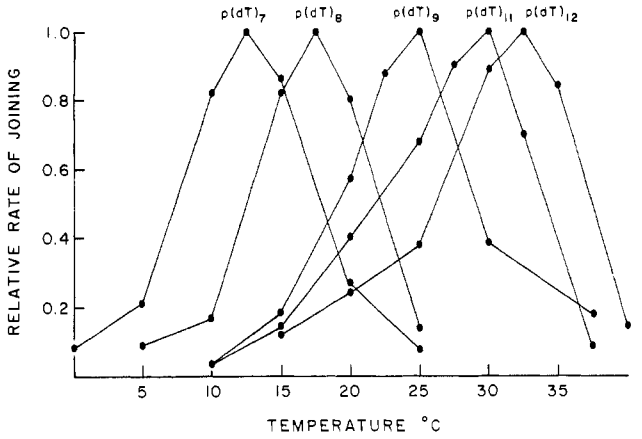

FIGURE 1: Relative joining rate of oligodeoxythymidylates with poly(dA) at different temperatures. Assay conditions are described in Methods.

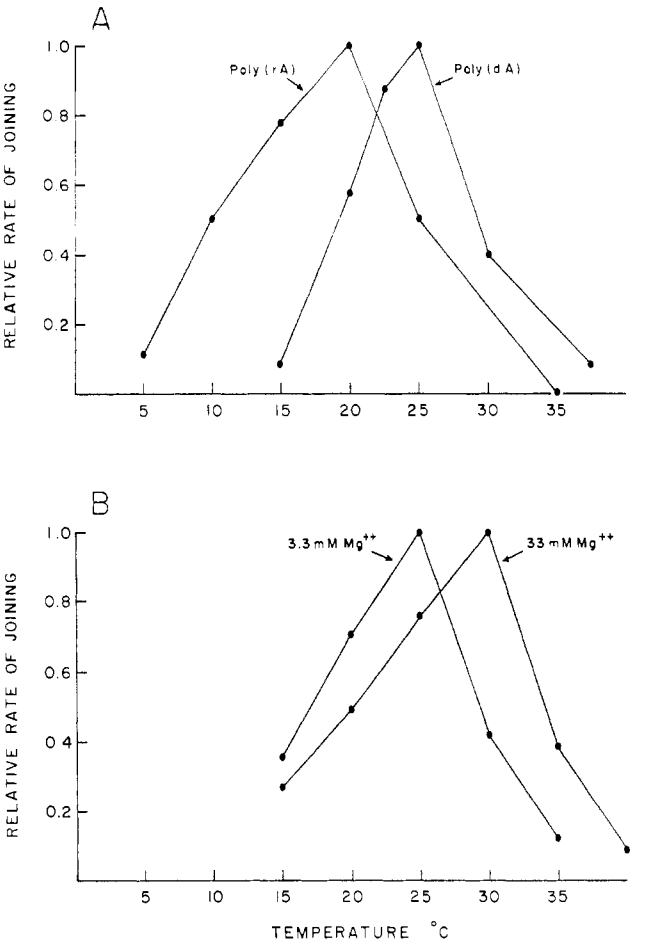

FIGURE 2: Dependence of temperature joining curves on magnesium concentration and "splint." Assay conditions are described in Methods.

using Whatman No. 1 and developing 20 hr with 1 M ammonium acetate (pH 7.0)–ethanol (1:1, v/v). Papers from either electrophoresis or paper chromatography were eluted with distilled water.

Assay for Joining. The standard incubation mixture contained 66 mM Tris-HCl buffer (pH 7.6), 6.6 mM dithiothreitol, 3 μg of bovine plasma albumin, 10 nmoles of ATP, 2.38 nmoles (nucleotide phosphorus) of poly(dA), and 20 pmoles of [5′-^{32}P]thymidylate oligomer in a total volume of 0.3 ml. Joining rates were determined at an enzyme concentration that caused the joining of 0.2–2 pmoles of 5′-^{32}P-labeled oligomer in 20 min at the temperature used. After incubation, the reaction was placed in a 60° water bath and incubated 30 min with 5 μg of bacterial alkaline phosphatase. The reaction was stopped with 1 ml of 1 N HCl. After cooling, 0.2 ml of a solution containing 2 mM sodium pyrophosphate, 25 mM potassium phosphate buffer (pH 7.0), and 5 mg/ml of bovine plasma albumin was added, followed by 0.2 ml of a 20% Norit suspension (packed volume). The suspension was filtered through a Whatman GF/C glass fiber disk (2.4-cm diameter). The residue was washed three times with cold 0.01 N HCl. The wet Norit residue was placed in a vial with 10 ml of toluene-based scintillation fluid and the ^{32}P determined in a Packard scintillation spectrometer. The efficiency of the ^{32}P measurement was 80% under these conditions.

Large-Scale Preparation of $p(dT)_{18}$. The reaction mixture contained 66 mM Tris-HCl buffer, 6.6 mM dithiothreitol, 20 mg of bovine plasma albumin, 0.066 mM ATP, 200 nmoles of [5′-OH](dT)$_9$ chains, 30 A_{260} poly(dA), and 20 nmoles of [5′-^{32}P]p(dT)$_9$ chains (1×10^6 cpm) in a total volume of 30 ml. The reaction mixture was incubated for 4 hr at 25° with 2.5 units of T_4 ligase. The reaction was stopped by heating 3 min at 100° and separated by G-75 gel chromatography as described in Results.

Other Materials. The poly(dA) was obtained from Biopolymer, while poly(A) was from Miles Laboratories. Cold ATP was from Calbiochem or Pabst. All ATP was repurified by DEAE-cellulose chromatography using the same procedure and conditions as used to purify [5′-^{32}P]oligomers. The [γ-^{32}P]ATP was purchased from Amersham-Searle or New England Nuclear. Labeled ATP used contained less than 5% $^{32}P_i$. DEAE-cellulose was Whatman DE-23 and Sephadex G-75 was purchased from Pharmacia. When G-75 gel columns were to be used at 60°, the 0.5 M triethylammonium bicarbonate buffer (pH 7.6) was added to the gel and heated to 60° with occasional stirring. After degassing, the column was poured and 60° heated buffer used for development. The pH buffer after degassing at 60° was 8.3.

Results

Effect of Temperature on Joining. Figure 1 shows the relative joining rates of thymidylate oligomers in the presence of poly(dA). The temperature curves constructed by these experiments yielded a sharp optimum that was characteristic of the length of the strand. This optimum can discern a difference of one nucleotide in the oligomers that were joined. Thus, p(dT)$_{11}$ joined optimally at 30° while the oligomer p(dT)$_{12}$ was found to join most rapidly at 32.5°.

The heptamer of thymidylic acid was the shortest oligomer that yielded a temperature curve. Strands four, five, and six residues long could be joined consistently at 0° in ice, but joined only erratically at higher temperatures.

The hybrid poly(rA·dT)$_n$ was found by Riley *et al.* (1966) to be less stable than the corresponding homopolymer pair (dA·dT)$_n$. A comparison of the temperature curves of joining p(dT)$_9$ on poly(dA) and on poly(rA) is illustrated in Figure 2a. The optimum rate with the hybrid poly(rA)·p(dT)$_9$ was found to be 20° while poly(rA)·p(dT)$_9$ joined more rapidly at a higher temperature, 25°. Likewise, the stability of strand association is known to be effected by salt concentration.

TABLE I: Joining Rates at Optimum Temperature of Thymidylate Oligomers.[a]

Substrate	Temp (°C)	Rate (pmoles/Unit per 20 min)
$p(dT)_7$ + poly(dA)	12.5	10
$p(dT)_8$ + poly(dA)	17.5	244
$p(dT)_9$ + poly(dA)	25	425
$p(dT)_9$ + poly(rA)	20	2
$p(dT)_{11}$ + poly(dA)	30	1125
$p(dT)_{12}$ + poly(dA)	32.5	2010

[a] Rates were determined as described in Methods.

When $p(dT)_9$ was joined in the presence of poly(dA) at a concentration of 33 mM Mg^{2+}, the optimum was shifted from 25° to 30°. These results illustrate that T_4 ligase catalyzed joining is a sensitive tool for detecting associative effects.

Rates of Joining for Thymidylic Acid Oligomers. A comparison of the maximum joining rates of different strand lengths is given in Table I. The absolute joining rate increased 200-fold when the strand length was increased from 7 to 12 residues. This increased rate can not be explained by the Q_{10} of the enzyme which is <2 (Fareed and Richardson, 1967) but may be explained by the necessity of the 3′ and 5′ ends of two oligomers to be adjacent for joining to take place.

In agreement with Kleppe *et al.* (1970) and Fareed *et al.* (1971), substitution of an RNA strand for the "splint" also allows joining with T_4 polynucleotide ligase, but at a greatly decreased rate. Poly(rA)·$p(dT)_9$ joined at only 5% of poly(dA)·$p(dT)_9$ (Table I). Whether the decreased rate of $p(dT)_9$ joining on poly(rA) was caused by a difference in the type of complex formed with a DNA·RNA hybrid or whether the enzyme actually recognized the hybrid less efficiently is not known. Cassani and Bollum (1969) showed that $p(dT)_{10}$ forms a triple-stranded complex with poly(rA) and a double-stranded complex with poly(dA).

Oligomers with less than seven residues were found to join consistently at 0°. Table II illustrates that oligomers as short as the tetramer were joined. The extent of joining with the tetramer was 57%. Further experiments in which additional enzyme was added showed that the tetramer could be joined to the same extent as the nonamer (>85%). Reactions with dimer and trimer were found to join, but not consistently.

TABLE II: Joining Thymidylate Oligomers on Poly(dA) at 0°.[a]

Oligomer	Rate (pmoles/Unit per 30 min)	% Joined[b]
$p(dT)_4$	0.21	57
$p(dT)_5$	0.61	45
$p(dT)_6$	0.85	78
$p(dT)_7$	1.01	77
$p(dT)_9$	6.8	75
$p(dT)_{11}$	7.0	74

[a] Rates were determined as described in Methods. [b] Overnight incubation.

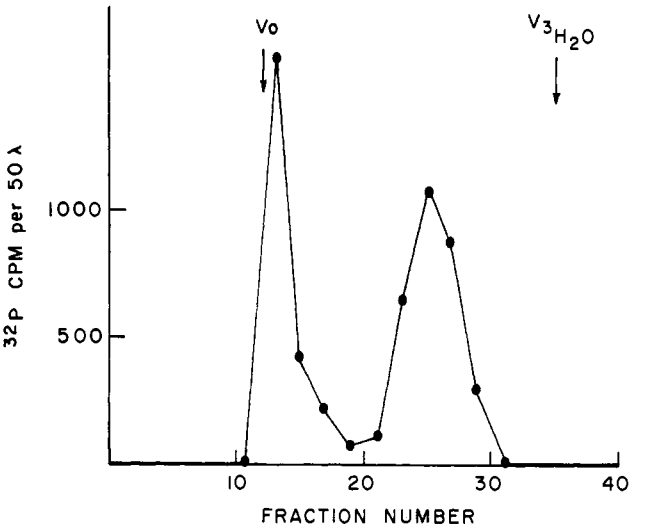

FIGURE 3: Separation of joining reaction by gel chromatography. A standard reaction mixture containing [5′-^{32}P]$p(dT)_9$ and poly(dA) was incubated 10 min with 5 units of T_4 ligase at 25°. The reaction mixture was layered on a G-75 gel column (0.9 × 23 cm) previously equilibrated with 0.5 M triethylammonium bicarbonate buffer (pH 7.6). The column was developed with the above buffer at 25° and 0.45-ml fractions were collected.

Characterization of Joined Oligomers. The product from a $p(dT)_9$ joined ligase reaction was separated on a G-75 gel column (Figure 3). The ^{32}P-labeled peak near the void volume was characterized as shown in Table III; the $p(dT)_9$-joined product was >90% resistant to bacterial alkaline phosphatase. Heating the product at 100° before addition of phosphatase did not affect this resistance. This shows that the ^{32}P label was not masked by a pyrophosphate linkage as found in the DNA adenylate intermediate (Harvey *et al.*, 1971). Digestion of the joined thymidylate with venom phosphodiesterase unmasked the ^{32}P label which then could be removed with bacterial alkaline phosphatase. These results are consistent with a high molecular weight product with the label in a 3′–5′ linkage.

The molecular weight of the product was determined by removal of the 5′-^{32}P from the chain with bacterial alkaline phosphatase and separation from the remaining resistant

TABLE III: Characterization of Joined Product.

Oligomer Joined	% of ^{32}P Adsorbed on Norit after P'ase[a]	Heat + P'ase[b]	Venom + P'ase[c]
$p(dT)_9$	99	95	9
$p(dT)_4$	89	92	1

[a] Sensitivity to phosphatase was checked as described in Methods; P'ase = phosphatase. [b] Sample was boiled 15 min in 1 ml of 1 N HCl, then made 0.1 N with Tris, and the pH was adjusted to 8.0 with NaOH. Phosphatase sensitivity was assayed as described in Methods. [c] Sample was digested 30 min at 60° in a mixture (0.5 ml) containing 100 mM Tris-HCl (pH 8.9) buffer, 20 mM $MgCl_2$, 10 μg of venom phosphodiesterase, and 5 μg of phosphatase. Digested sample was assayed for Norit absorbable cpm as in Methods.

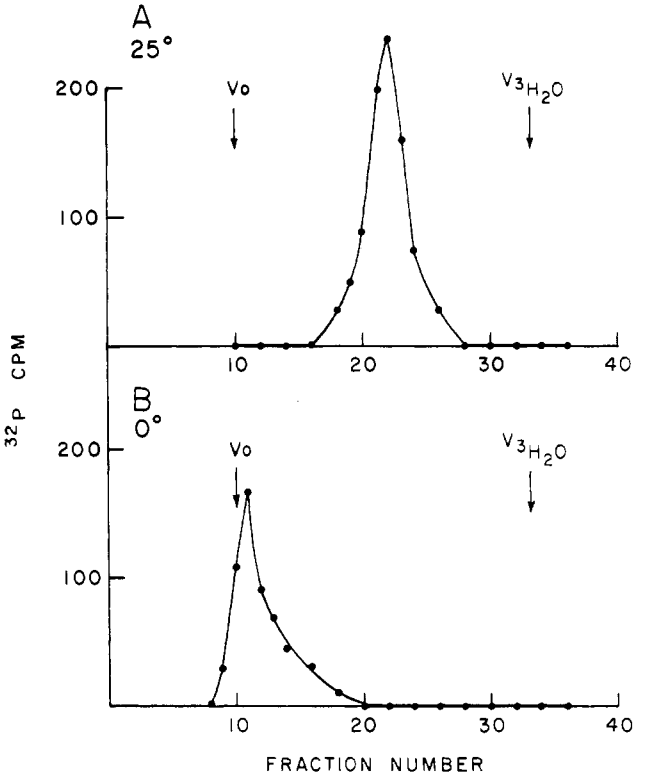

FIGURE 4: Determination of oligomer·polymer complex by gel chromatography. A standard reaction mixture containing [5′-^{32}P]p(dT)$_9$ and poly(dA) but minus ATP and enzyme was layered on a column (0.9 × 23 cm) equilibrated with 66 mM Tris-HCl buffer (pH 7.6) containing 6.6 mM $MgCl_2$. The column was developed with the same buffer and 0.45-ml fractions were collected.

^{32}P on DEAE paper (Gupta *et al.*, 1968b). The product from a p(dT)$_9$-joined reaction was some 400 nucleotides long while the product from the p(dT)$_4$ was ~100 residues.

Determination of Association by Gel Chromatography. The stability of oligomer association at the optimum temperature for joining was not known. A number of studies on the association of oligonucleotides and complementary polynucleotides have been made (Lipsett *et al.*, 1961; Rich, 1960; Cassani and Bollum, 1969). They established that the melting temperature of these complexes was concentration, as well as salt, dependent. Gel chromatography was used by Cassani and Bollum (1969) as a method for determining the presence of oligonucleotide·polynucleotide complexes.

When [5′-^{32}P]p(dT)$_9$ and poly(dA) were eluted from a G-75 gel column with 66 mM Tris-HCl buffer (pH 7.6) containing 6.6 mM $MgCl_2$, the labeled p(dT)$_9$ came through at the void volume with poly(dA) at 0° (Figure 4a). However, if the jacketed column was eluted at 25°, the temperature required for optimum joining of this length strand by T_4

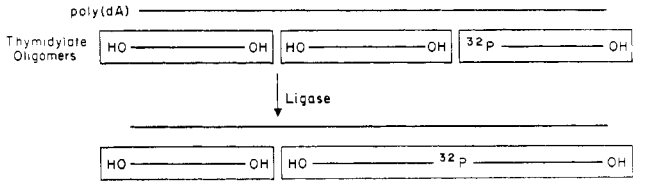

FIGURE 5: Scheme for joining [5′-^{32}P]p(dT)$_9$ in the presence of [5′-OH](dT)$_9$ and poly(dA).

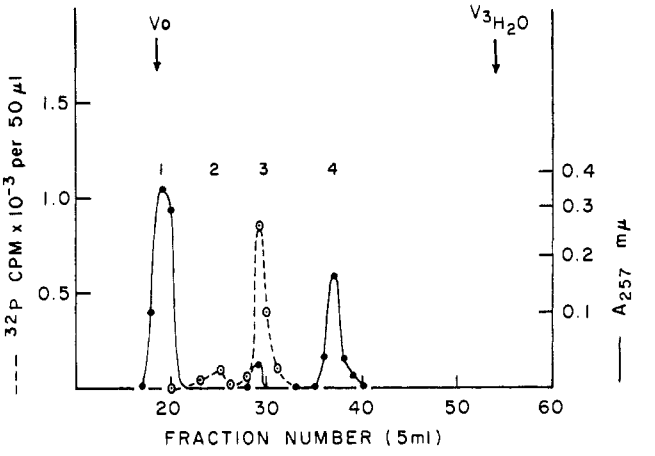

FIGURE 6: Separation of dimer by gel chromatography. The reaction mixture was concentrated to 3 ml and layered on a G-75 gel column (2 × 84 cm) that had been equilibrated with 0.5 M triethylammonium bicarbonate buffer (pH 8.3 after degassing). The column was developed at 60° with the same buffer and 5-ml fractions were collected.

ligase, the labeled oligomer was not complexed with poly(dA) but came through at the same volume as p(dT)$_9$ (Figure 4b). These results indicate that the oligomer does not form a stable complex in the absence of enzyme at the optimum joining temperature, but instead the polymer·oligomer complex appeared reversible.

The heptamer (p(dT)$_7$) was the shortest oligomer that formed a stable complex at 0° as revealed by gel filtration (not shown). This oligomer was also the shortest chain that gave a temperature dependent curve (Figure 1).

Preparation of a p(dT)$_9$ Dimer. The dimer of p(dT)$_9$ was made by adding a large quantity (10×) of HO(dT)$_9$ to the

TABLE IV: Characterization of Peak 3.

Polynucleotide Kinase[a]	
pmoles of Peak B	pmoles of ^{32}P Incorpd
30	29
60	52

Bacterial Alkaline Phosphatase Resistance[b]		
	pmoles	
Treatment	Supernatant	Norit
Phosphatase	3	59
Heat + phosphatase	3	59
Venom + phosphatase	55	5

[a] 5′-^{32}P incorporation by polynucleotide kinase was determined in a reaction mixture containing 66 mM Tris-HCl buffer (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 1 nmole of ATP containing approximately 44,000 cpm of [γ-^{32}P]ATP, and 0.5 unit of kinase in a volume of 0.05 ml. After incubation at 37° for 1 hr, 1 ml of 1 N HCl was added and heated 15 min at 100°. The solution was cooled and assayed for Norit adsorbable radioactivity as described in Methods. [b] Resistance to bacterial alkaline phosphatase was assayed as described in legend of Table III.

reaction mixture containing [5′-^{32}P]p(dT)$_9$ and poly(dA). Most of the labeled p(dT)$_9$ joined to HO(dT)$_9$ which possesses a 5′-OH in place of a phosphoryl group. Because polynucleotide ligase requires a 3′-OH adjacent to a 5′-phosphoryl, the joining stops after adding one oligomer (Figure 5).

The separation of a reaction mixture containing the dimer of p(dT)$_9$ is illustrated in Figure 6. The G-75 gel column was eluted at 60° to separate the joined thymidylate strands from the poly(dA). The poly(dA) was found at the void volume and was followed by two ^{32}P-containing peaks (peak 3 and 4). The second radioactive peak (peak 4) contained 13 nmoles of ^{32}P. When peak 3 was phosphorylated with polynucleotide kinase (Table IV), the amount of ^{32}P incorporated was equal to the pmoles of ^{32}P in the substrate. Further characterization of this peak showed that it was >95% resistant to bacterial alkaline phosphatase, but this resistance was lost following treatment with venom phosphodiesterase. After treatment with 1 N HCl at 100° for 15 min, the ^{32}P label remained resistant to bacterial alkaline phosphatase. These results are consistent with the fact that one [5′-^{32}P]p(dT)$_9$ chain was joined to one chain of HO(dT)$_9$ to form a dimer.

The second peak (peak 2) was not characterized but probably consisted of a trimer, *i.e.*, two [5′-^{32}P]p(dT)$_9$ chains joined to the 3′ end of HO(dT)$_9$. Peak 4 consisted of excess HO(pT)$_9$ as determined by elution volume and 280:260 ratio (0.72).

Discussion

Thymidylate oligomers as short as four units were found to join at 0°, and oligomers of seven or more residues joined in a temperature-dependent manner characteristic of their length. However, at the temperature at which the joining rate was maximal, the physical polymer·oligomer complex did not appear stable, as shown by gel filtration. It is possible that in the presence of ligase a stable complex is formed and that this enzyme·nucleic acid complex can "creep" or move randomly along the dA chain until the 5′-phosphoryl group of one chain is adjacent to the 3′-hydroxyl group of another chain. A similar mechanism was postulated by Olivera and Lehman (1968) for joining reactions with thymidylate oligomers 50–300 residues long associated with poly(dA). An alternate explanation would be that "creep" does not take place because of the low temperature and short strands used. Thus, joining can only take place at a temperature at which reversible binding of the oligomer to poly(dA) takes place. By either mechanism there should be an optimum temperature at which the enzyme·oligomer·poly(dA) complex is formed.

The minimum number of nucleotide residues necessary for joining under our conditions was four (Gupta *et al.* (1968b) also joined a tetramer, but their strands contained two G·C pairs). However, the reaction was extremely slow and it is doubtful that it would be practical to join segments of this size on a larger scale. Large amounts of nuclease-free ligase would be needed. In agreement with the above authors, we were unsuccessful in stabilizing the enzyme·nucleotide complex by raising the salt concentration without inactivating the enzyme. It is possible that an antibiotic that stabilizes the helix by intercalation could be used to raise the temperature and reaction rate. This possibility is being investigated on defined sequences with which there is no need for movement of the joining oligomers.

Polynucleotide ligase has been used for obtaining thymidylate chains with defined number of residues, thus extending the present method of chemical polymerization. By the procedure here described, it is possible to obtain chains of 10–24 residues by block addition. The excess 3′-OH chains and the poly(dA) may be recovered and reused. The procedure may be repeated to obtain thymidylate oligomers of any length.

Acknowledgments

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